

Santi M. Mandal · Debarati Paul

Automation and Basic Techniques in Medical Microbiology



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*The book is dedicated to
God
for His provision during the toughest Covid
times
and
our beloved parents and family
who have been constant sources of
motivation.*

Preface

In this handbook every endeavour has been made to make a comprehensive one-volume reference text and practical book which is of value to practising microbiologists throughout their undergraduate, postgraduate, and subsequent careers.

During the preparation of the text the interest of students has been taken into consideration such that they get an overview of safety guidelines while working in the laboratory before embarking on actual experiments and handling various instruments. Students have been instructed about how they should follow the protocols and write lab manuals with precise documentation. Code of conduct in a microbiology laboratory set up has been stated to make them aware of their roles and responsibilities while performing various experiments. There is a summary of hazards that may be encountered in the microbiology laboratory, intending at acquainting the students with some essential elements needed to maintain a safe working practice, and to advise strongly on the importance of consulting with senior members of the laboratory or the appointed safety officer, and of consulting comprehensive specialist texts which should be readily accessible to laboratory users. In particular the advice of safety officers should be sought, since by virtue of their office they have available the latest information.

Hazards associated with particular chemicals and procedures are noted in the text as far as information is available.

Classical knowledge has been presented along with the updates in the field with application of microbiology as the focal point. Sample preparation and interpretation of data thus obtained have been introduced making this handbook exceptional amongst existing books of microbiology.

The book provides a bridge between the theory books provided by current standard undergraduate textbooks and the wider specialist fields of research literature. Furthermore, it is our intention to reflect on research developments in the area of medical microbiology, since we consider that the teaching of concepts must closely integrate mechanistic theory with the strategy and methodology of experimentation and data thereof.

The use of robotics and automation in medical biology has been described in the first chapter, stating its history of origin and advancements and implications in modern microbiology. The chapter describes the history and types of different

auto analysers used in medical microbiology labs, along with their uses and limitations. The differences between semi-automated and fully automated systems have been highlighted along with their numerous applications.

Manual and automated characterization of multi-antibiotic-resistant (MAR) bacteria have been described in the next section. The types of resistance shown by microbes have been described along with the necessity and implications of antibiotic susceptibility testing. Qualitative tests such as the disc diffusion method by Kirby-Bauer; quantitative tests including minimum inhibitory concentration (MIC); and automated tests for resistance, e.g. turbidimetric detection and molecular techniques namely multiplex PCR and RAPD, have been detailed. The use of other processes for the detection of MARs has been described (Padlock PCR and microarray analysis, quantitative real-time PCR), and these may be applicable to all microbial strains irrespective of pathogenesis.

Currently whole genome sequencing for detection of pathogens and for study of microbiota of healthy and unhealthy people has led to several important diagnoses about disease-causing agents and has immensely assisted in patient care. As compared to other techniques of clinical microbiology that give partial information, such as PCR-based techniques discussed previously, the WGS-based techniques are faster and have the potential to generate wholesome datasets. Few WGS methods have been discussed and their applications, and bottlenecks have been discussed.

The next section includes the modern advanced techniques used for studying microbial and other cells. The principles of spectrometry, spectroscopic methods, and the interpretation of spectra are now treated together in Chaps. 5 and 6. The most significant addition to this chapter is a more detailed coverage, with examples of spectra from different types of spectroscopy which is considered alongside with usage of laboratory equipment. The assistance of the company representatives of very many manufacturers of chemicals and of laboratory, spectroscopic, and chromatographic equipment has been invaluable.

The use of MALDI-TOF as an aid for bacterial identification and identification of toxins produced by microbes that can in turn be used to characterize pathogenic bacteria has been discussed in detail in the sixth chapter. Description of applications and limitations of this technique have been included to apprise readers of the latest techniques in this upcoming field.

The following section describes a very popular and important technique used for immune assays for the detection of various microbial strains based on antigen and antibody interactions. The various types of ELISA (enzyme-linked immunosorbent assay) techniques have been vividly described, and a protocol has been suggested for students to perform this assay. The advanced forms of this assay have also been described, e.g. radioimmunoassay (RIA) and automated ELISA. The significance, advantages, and limitations have also been detailed for getting an overall perspective of the topic.

Isolation of normal microbiota from human body and microbial identification using various basic and automated techniques have been described with methodology and applications. The progress made in this area in this current era of modern technologies has been focussed on.

Immunotechniques apart from ELISA have been described that will help the reader to understand the very basics and importance of such techniques in clinical microbiology.

The advances in microscopy chapter would then encompass advances in this technique from the oldest to the latest technology and from the simplest designs to the intricate modern engineering and progress in electronics and other fields. The chapter also encompasses sample preparation for electron microscopy which will help the reader to carry out experiments, after getting a wholesome knowledge of the instrumentation and their applicability.

We are indebted to the referees who made valuable comments on the manuscript which undoubtedly helped to improve the final text. The authors are grateful to their colleagues in academia, industry, and government who have made useful suggestions for improving this handbook, both as a book and as a reference source. The authors are especially thankful to all the contributors, whose combined expertise has made this unique textbook possible.

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1.1 Introduction

The potential risks associated with any microbiology laboratory are from handling infectious agents in addition to other hazards posed by use of certain chemicals and/or radioactive elements. Many cases have been documented stating conditions where lab personnel acquired diseases at work, especially due to continuous or prolonged exposure to harmful agents. Therefore, lab practices should be streamlined, and every worker should be educated to understand and follow safety guidelines, to stay protected from potential occupational risks. Potentially harmful microbes may get isolated from environmental materials that might require the use of BSL2 or above containment practices. Immunocompromised should carefully consider such guidelines and refrain from enrolling in such courses.

Safety in the microbiology laboratory is therefore of utmost interest and the primary concern.

Three main techniques for safe containment of microbes are as follows:

1. Good laboratory practices
2. Use of safety equipment
3. Suitable design of facility

In this chapter we shall review the suitable or good laboratory practices to be followed to work safely. The points have been enumerated below but not prioritized. All the safety rules are therefore equally important.

1. Laboratory access should be restricted, and doors may be locked when the lab is not in use or unattended.
2. One should wash hands with disinfectants after entering the lab and again before leaving.

3. It should be ensured that whosoever enters the laboratory is clear about potential hazard associated. Before starting any lab work, relevant SDS and MSDS should be reviewed.
4. One should be familiar with the safety equipment located in the lab (e.g., first aid kit, eye-wash station, sinks, shower, fire extinguisher, biological safety cabinet, emergency gas valve).
5. At the entrance always post an Emergency Information sign for the concerned department. This should include names and contact of the lab supervisor(s) or any other responsible person.
6. A lab should be clean, neat and clutter free. One should clean their assigned bench-top area with a disinfectant solution before starting lab work and after finishing.
7. Backpacks, copies/books, purses, bottles and coats should be placed in the designated areas and in the aisle. Free movement should not be restricted due to these.
8. Food or drinks is not permitted in the laboratory, for personal safety and non-transmittance of potential pathogens outside the lab. Mouth pipetting should be discouraged.
9. Labels should be clearly written and placed.
10. Only appropriately dressed people should gain entry to the lab. The dress should include the following:
 - Fast knee-length lab coats are needed, and these should not be worn outside the laboratory.
 - The lab coat should not have holes, tears, contamination, and debris in pockets. If any of these are found, notify lab supervisor/staff and obtain a new lab. Only use lab coats that are free from the above deficiencies.
 - Gloves should be used when necessary and during the handling of infectious agents or in a biological safety cabinet. They should be removed and disposed before leaving. Like lab coats, inspect gloves for holes/tears or imperfections. Hands must be washed after removing gloves.
 - Avoid loose fitting clothing in the lab. Appropriate shoes should be worn, and never wear shorts or sandals in the laboratory.
 - Loose hair, clothing, dangling jewelry, etc. should be avoided.
 - Hands should be away from one's face/eyes and mouth while working with microorganisms or chemicals. This includes not applying cosmetics, not adjusting contact lenses, and not biting your finger nails.
11. If accidentally any chemical enters the eyes, splashing with cold water immediately is recommended, but do not rub your eyes.
12. Special care should be taken while using open flames or heated surfaces. Never leave any heating source (hot plate/Bunsen burner) unattended. The containers of flammable liquids, e.g. alcohol, acetone, etc., should be stored away from heaters/flames.
13. Any contaminated material and instrument must be appropriately decontaminated and labelled or tagged before being taken away from lab for disposal or servicing.

14. The use of syringes, needles or sharp objects should be strictly limited. Precautions should be taken while handling sharps, needles and syringes in order to avoid auto-inoculation or generating aerosols. Inoculating loops must be flame-sterilized in a Bunsen burner before laying them down.
15. Secure the caps on reagent bottles and bacterial cultures soon after use. Petri plates should not be opened in the lab outside laminar flow.
16. All microorganisms pose the risk of being potential pathogens, and they should not be taken out of the laboratory.
17. Kindly dispose off broken glass in the “broken glass” container.
18. Each one should report spills and/or accidents to the instructor as soon as possible.
19. Similarly, all injuries should also be reported immediately to the instructor, even if they are small.
20. Small spills should be cleaned up with care, but seek help from experts or instructor for clearing large spills.

1.2 Basic Record and Lab Note Book

Lab record book is for daily use in the lab and also for planning experiments/schedules, notes, rough works, memoirs, calculations, etc. Recording experiments should be systematic and regular. All experimental details and readings taken in the lab should be written legibly in this notebook, along with date. Since it is a compilation of entire experiments performed by a student or researcher, it must be neatly maintained for future reference by oneself or others.

The following tips should be kept in mind while writing in a lab record book:

1. Title or aim of the experiment should be documented along with the date of performance.
2. A list of various requirements for performing the experiment should be noted.
3. Summarization of the theory/principle behind the experiment should be stated in short.
4. All the observations and calculations should be noted.
5. One should use only indelible ink; pencil and ink pens should be discouraged.
6. Sticky notes should not be used for taking down reading or noting observations.
7. The result section should have the final result in accordance to the aim of the experiment. It should be organized in the form of statistically valid tables and figures and should be discussed logically. All raw data, consisting of gel photographs, graphs, autoradiographs, printouts, etc. should be included.
8. The results obtained should be interpreted in accordance with the principle of the experiment.
9. Scribbling or random cutting should be avoided.
10. Any suggestions from the protocol done, any refinements required, etc. may be noted too.

1.3 Laboratory Safety Equipment

1. Biological safety cabinet

A biological safety cabinet (BSC) is the first barrier for infectious microbes. It is characterized by laminar airflow where air moves at a uniform velocity through high-efficiency particulate air (HEPA) filters. BSCs are meant for safety from microbes but cannot be used as a fume hood. The HEPA filters only trap particulates of sizes about 0.3 μm . Any other contaminant can pass through.

2. Safety shower and eye-wash station

This facility is important to reduce dangers leading to workplace hazard due to accidental spills or spreading of hazardous elements, e.g. chemicals or even culture broth containing microbes. These are used in emergency situations.

3. Fire extinguisher

The best fire extinguisher for a microbiology lab is the one that can control A, B and C fires. The unreactive, dry chemical of the fire extinguisher douses the fire and prevents the burning of oxygen, thereby extinguishing the fire, and also re-ignition is prevented.

4. First aid kit

Injuries due to broken glassware: Wound should be immediately washed to remove pieces of glass. Mercurochrome ointment may be applied to the wound, which should be dressed with gauze. No glass piece should be pulled out forcefully; in such cases medical help should be sought.

Alkali/acid burns: After washing thoroughly the affected part of the skin may be lightly sponged with cotton wool containing dilute sodium carbonate solution (5% w/v) and vinegar, in case of acid and alkali, respectively.

Acid/alkali in eye: Water should be sprayed from a wash bottle into the eyes.

Swallowing acid: Immediately gargling should be done with water to rinse any acid out. If the lips or tongue get acid burn, after rinsing with water, one may rinse with 2% sodium bicarbonate solution.

5. Gas valve (for emergency use)

Electrical circuits must be protected and earthed. Emergency gas valve should be shut off for fire safety.

1.4 Biosafety Levels and Practices

Microbes have been classified on the basis of their pathogenicity, and accordingly practices and lab facilities, or associated safety equipment, have been classified into four biosafety levels (BSLs) (<http://www.cdc.gov/OD/ohs/biosfty/bmbl4/bmbl4toc.htm>). BSLs are selected to provide users with a description of the minimum containment required for handling different microorganisms safely in a laboratory setting and reduce or eliminate exposure to potentially hazardous agents.

BSL type	Agents	Practice
1	Non-pathogenic (<i>Agrobacterium radiobacter</i> , <i>Aspergillus niger</i> , <i>Bacillus thuringiensis</i> , <i>Escherichia coli</i> strain K12, <i>Lactobacillus acidophilus</i> , <i>Micrococcus luteus</i> , <i>Neurospora crassa</i> , <i>Pseudomonas fluorescens</i> , <i>Serratia marcescens</i>)	Common microbiological practices; lowest safety level; special containment devices like the biosafety cabinets are not required for BSL-1
2	Associated with disease, moderate potential hazards to the laboratory personnel, the environment, and the agent. Pathogenic strains of <i>E. coli</i> , <i>Staphylococcus</i> , <i>Salmonella</i> , <i>Plasmodium falciparum</i> , <i>Toxoplasma</i> and herpes simplex viruses	BSL-2 laboratories like BSL-1 laboratories are not necessarily separated from the general traffic patterns in the building. However, access into the laboratory is limited, while BSL-2 experiments are in progress
3	Work is performed with agents that may cause severe or potentially lethal disease through inhalation or aerosol formation, to the personnel, and may even contaminate the environment. The pathogens that require BSL-3 laboratories include HIV, H1N1 flu, <i>Yersinia pestis</i> , <i>Mycobacterium tuberculosis</i> , SARS, rabies virus, West Nile virus, rickets, etc.	Individual protection gears like personal protective equipment, coats, gloves and respiratory protection should be worn while entering the laboratories and then removed before leaving. The air flowing in the laboratory shouldn't be recirculated to any area of the laboratory and should be HEPA-filtered prior to being discharged to the outside
4	Highest level that is employed while working with dangerous infectious agents that present a high individual as well as environmental risk in the form of life-threatening disease, aerosol transmission or unknown risk of transmission. Pathogens include the risk group IV organisms like Ebola virus, SARS-CoV-2, Central European encephalitis virus, haemorrhagic viruses, etc.	The requirements of BSL-4 laboratories are extensive with specific laboratory design, training procedures and highly protective equipment and personal gears BSL-4 laboratories are also termed the maximum containment laboratories as they have secondary barriers to prevent hazardous materials from escaping into the environment

Adapted Sapkota, weblink: <https://microbenotes.com/biosafety-levels/#requirements>

2.1 Introduction

Automation means the use/introduction of automatic machines or equipment in a manufacturing or work process, e.g. automated plate streakers and specimen processors including complete solution for laboratory-based automation. Automation has been applied in clinical haematology and chemistry-related arenas of diagnostic labs, but in the case of microbiological labs, this privilege has not been commonly observed. However, in few areas of clinical microbiology, e.g. identification of microbes, blood culturing systems with automated continuous monitoring and also anti-microbial resistance/susceptibility tests are being routinely used for microbiological labs, mainly for processing specimens or culturing them. The below listed reasons limit the application of automation to microbiology labs.

- Microbiology as a subject is highly complex as compared to haematology or biochemical analysis labs that use urine/blood samples and difficult to automate, and the lab space, on the other hand, is too small for automation.
- In such labs specimen includes urine samples, blood, catheter tips, sterile body fluids, tissues, other specimen from prosthetic devices and also specimen from the respiratory tract most commonly. After collection from homes or collection centres, these specimens are transported to the facility via numerous techniques including various swab collecting tools; sterilized container for urine/stool/tissues, aspirates and/or prosthetic residues; etc.

2.2 Applications of Automation

Automation means the use of various robotic control systems for operating any work tools/equipment or lab process with minimal or reduced human intervention.

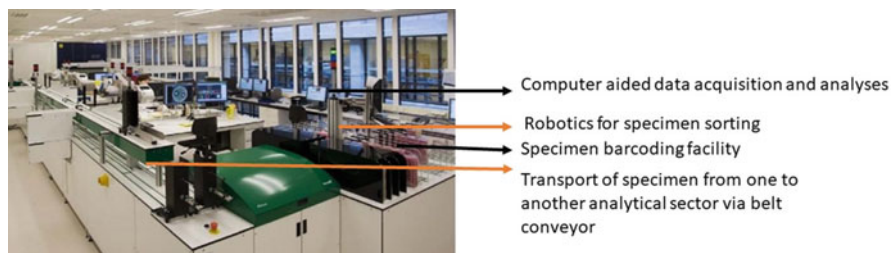


Fig. 2.1 A fully automated clinical microbiology lab, i.e. TLA (total lab automation) system containing conveyor/track system to link the various modules to combine them and create the fully automated system. Modules are mainly the specimen processing and streaking units. Figure adapted from Kiestra TLA system (Bourbeau and Ledebøer 2013) and labelled for understanding

Automation and robotics are in use for specimen processing, plate reading, organism identification, antibiotic sensitivity profiling, hormone level determination, haematological analysis, etc. Though costly, the automated systems are faster, more accurate and efficient, than manual existing processes (Fig. 2.1).

These automated labs perform test for several types of specimen, including:

- Urine
- Tissue(s)
- Blood
- Swab from throat
- Respiratory specimen

Application of Automation

Automation has a vast array of applications; the most common have been listed below:

1. Inoculating culture plates, followed by detecting microbial colonies on culture media, antimicrobial susceptibility tests and molecular detection through extraction of nucleic acids for detection from samples.
2. Molecular diagnostics is evolving continuously, and so is the whole laboratory-based industry. PCR or RT-PCR is the most popular technique playing important roles.
3. Rapidly improvising test methods lead to newer techniques for identification of pathogens that depend upon their molecular specificity, to predict their susceptibility and resistance towards the existing treatment methods. They also mediate the development of new treatments (antibiotics) that are specific to the genetic makeup of pathogens. Therefore, developing treatments having broad spectrum is of no use, because these otherwise give way to “superbugs”.

2.3 Advantages and Disadvantages

2.3.1 Advantages of Using Auto-analysers

In medical labs the overall test quantities have been on a rise by 10–15% annually due to high percentage of aging population, because of increased life expectancy, testing innovations, infection control policies and determination and characterization of drug-resistant microbes.

- Due to this increasing need, it is now mandatory to work round the clock, and a 24/7 work culture is becoming inevitable. In this scenario, only automation can shorten the physical contact time and turn-around time. Hence the importance of automation has been realized and propagated worldwide (Novak and Marlowe 2013).
- The crisis of skilled manpower may be ameliorated because automated labs have the potential to perform with the efficiency of 50–100 human beings.
- Quality issues can be minimized. The need for better testing units or facilities and improved techniques for testing samples has been realized off late, and there is demand for efficient tests not only catering to high numbers but also increased range and accuracy. New assays based on molecular analysis, e.g. using biomarker-based analytical assays for diagnosing infection causing diseases, is being popularized (Leo et al. 2020).
- Precision: Advancements in the area of improvised instrument or platform development have been able to promote the automation of traditional microbiological methods, thereby making them more precise.
 - Accurate diagnosis possible. No manual errors.
 - Less time-consuming. Rapid assay detection of pathogens in the case of infectious disease.
 - Classical pathological laboratory or testing centres need more time, although the automation will be able to decrease complexity and decrease the time taken for analysis.

2.3.2 Disadvantages of Automation

- Human intelligence and decision-making in the microbiology laboratory cannot be replaced by any machine. The ongoing prejudice is that man can think and machines can't although machines perform faster than man.
- Any machine is incapable of making critical decisions and basic skills targeted towards microbiological experimentations.
- The human eye can observe and predict about microbial growth on culture plates more efficiently than machines.
- On the other hand, a machine can be programmed, and chances of mistakes are less as compared to humans.

- Automation has proven to be cost ineffective and therefore not preferred previously.
- Specimen sizes or sample volume used for microbiology is relatively smaller as compared to biochemical analyses; hence automation is not preferred.
- Set-ups for microbiological experiments are comparatively smaller in size and so unsuitable for automation. Mostly, automation is preferred in bigger laboratories. In smaller labs, automation facility may be underutilized.

2.4 Types of Auto-analysers

Auto-analysers have been classified into two types based upon mode of operation:

1. Semi-automated: Few initial steps are manual like sample preparation and reagent addition; the remaining are done by robotic systems fitted in the analyser (reading absorbance, analysis of results, diagnosis, printing of results, etc). Partly automated clinical analysers are user-friendly tools for diagnostic use and clinical applications.

Examples: PCR reactions, e.g. multiplexing PCR to detect serogroups/serotypes of *Streptococcus pneumoniae*, PCR for microbial typing, pulse field gel electrophoresis (PFGE) for analysing methicillin-resistant *Staphylococcus aureus*, Zika virus, etc.

2. Automated: A fully automated device/instrument is designed to detect and analyse various chemicals or other analytes in several biological samples quickly and require minimal human assistance, in a high-throughput mode. These analyses help in the diagnosis of diseases and measure the indicators of good health or well-being. All the steps right from the sample preparation, dilution, application, addition of reagents, reading the results, documentation and interpretation are fully automated.

Examples:

- a. Automated urine analysis via flow cytometry
- b. Identification of fungal strains via sequencing of ITS2 region
- c. Automated fluorescent capillary electrophoresis system
- d. Automated immunoassays

When a single solitary test is performed using one analytical channel or “line”, it is known as a “single-channel analysis”, and similarly, a “multiple-channel parallel analysis” is performed for obtaining data from more than one channel or “line”, wherein every line is dedicated for one test, but analysis of many lines occurs simultaneously. For random-access analyses, the specimen is tested in random sequence, that is, in and out with respect to each other, depending on the availability of containers, needed for the specific reaction. This is done with no adherence to any existing accession/serial number, even when specific samples, e.g. stats, are given priority over others. The assays may be termed as end-point tests, where the reaction

gets completed after fixed time or may have many or series of data points that are recorded at specified intervals of time (continuously monitored test).

The auto-analyser system with continuous flow was used for analysis in batches, which stood completely revolutionized by using other systems (positive displacement) that utilized pipettes of various volumes/fixed volumes for processing the sample or for dispensing of reagents. A particular step for washing was introduced in between steps to avoid contamination of sample/reagent carryover. Mixing of samples with reagents was done via forced dispensing, stirring (magnetic/mechanical using rods/mixers based on piezo-electricity). Reaction temperature is regulated using water bath, heating block or compartments with heated air, also called air-bath. Mostly the discrete analysers utilize separate cuvettes of glass or plastic; the flow cell may be the same throughout. Glass cuvettes are normally permanent, whereas the plastic ones may be disposed off after every use or may be replaced after a fixed number of tests. The detectors also vary in their make with a variety of lamps (tungsten, quartz, mercury, xenon, lasers, halogen, etc.). Another important component is the monochromator, which uses prisms, interference filters or diffraction grating to produce a single beam. The photodiode arrays (PDA) allow the use of a large spectra of wavelengths, and so analytical signals are typically detected using this PDA.

2.5 History of Auto-analysers

The earliest evidence of application of automation was when the auto-analyser using continuous flow analysis was introduced and a Robot Chemist then automated all traditionally manual steps for analysis. Successively more generations of detached or individual analysers were used for increasing the analytical speed. Such a system thus paved the way of testing elevated numbers of patient specimen and also facilitated a more elaborate assay menu (Fung 1992). These automated systems were bifurcated subsequently, where a number of analysers were designed to perform regular clinical tests dedicated to chemistry and immunoassay (Fung 1992). Many integrated systems were developed, greatly improving the analytical phase of clinical testing. Afterwards, automation was implied to carry out pre-analytical procedures, e.g. identifying samples, sorting, centrifuging and performing post-analytical procedures, i.e. by appropriately storing the specimen storage and also archiving them. Various phases for tests were finally combined in a process called “total laboratory automation” (TLA) in which every individual analysis sections that may be involved becomes physically connected via a tracking system, where samples travel via this processing starting from the beginning to the very end. A powerful and very evolved analytical technique is liquid chromatography coupled to mass spectrometry/mass spectrometry (LC-MS/MS). Such automated techniques are being gradually incorporated into modern TLA systems.

Another important tool of automation is bioinformatics, exemplified by middleware that forms an interface between an analysis software and the hospital's information system (HIS) and/or lab information system (LIS). This software

includes control of the overall operation of a TLA configuration and combines analytical results with patient demographic information to provide additional clinically useful information. It is a given that automation will continue to evolve in the clinical laboratory, limited only by the imagination and ingenuity of laboratory scientists.

Dubosq colorimeter was previously used for comparing colour to distinguish and quantitate creatinine in urine, using Folin's reagent. This method applied in the year 1904 then led to a fresh era for application of automation for the study of clinical chemistry. Colorimetric methods were adapted to a photometer in the 1960s, and this manual method was slowly adapted for the auto-analyser based on continuous flow; this caused displacement of individual analysers. Modern technology includes colorimetry/spectrophotometry, nephelometry/immunonephelometry, turbidimetry/immunoturbidimetry, atomic absorption, flame emission photometry, potentiometry (ion specific electrodes or ISE) and a variety of chromatographic techniques, including gas chromatography (GC), GC coupled to mass spectrometry (GC-MS), high-performance liquid chromatography (HPLC) and LC coupled to tandem mass spectrometry/mass spectrometry (LC-MS/MS), plus various ligand assay methodologies, including fluorescence polarisation immunoassay (FPIA), enzyme immunoassay (EIA), radioimmunoassay (RIA) and chemiluminescence immunoassay (CMIA).

To meet the need of fully automated system for measuring calcium, urea, glucose, etc., in 1956, Leonard Skeggs conceived the very first practical "auto-analyser", for meeting some specified requirements of a clinical chemistry laboratory. The machines were capable of performing analyses of blood samples, where the manual inference was not required by the technician. Skeggs introduced many modules for automating different steps of any reaction, and Technicon Corporation, Tarrytown, NY, USA, manufactured them. In the same year, Skeggs along with Technicon Company filed for a commercially available method. Technicon single and multi-channel continuous flow auto-analysers came into being. The initial model of an auto-analyser was made up of dialysis membranes for obtaining protein-free filtrate. Other tools used are tygon tubing, air bubbles to separate patient samples and "scrub" tubing, mixing coils, a flow-through cuvette with photometric monitoring and a strip-chart recorder. The designs that evolved afterwards lured the users with simultaneous analysis for above 20 specimens where about 150 samples per hour were running out. Some multi-channel systems are non-selective batch analysers. These multi channel systems may be utilized by learners even though they were used on every sample whether all tests were requested or not. Continuous flow analysis has the potential to change the image of biochemical tests such that the analysis duration is converted from hours (or days) to just minutes, leaving the clinician/technician free for developing further sub-specialty tests related to molecular diagnostics, toxicology and endocrinology. Example of such system is the auto-analyser SMAC (sequential multiple analyser with computer) developed in 1974 and came with a built-in computer.

In the 1970s, apparently, the auto-analysers led to the ample use of batch analysers, many of which measured only 1 analyte but could test up to 100 samples in continuous mode, whereas, in the early 1980s, the photodiode array for spectrophotometers with grating monochromators allowed a sample to be tested simultaneously for multiple analytes, each test detecting an analytical signal at

different wavelengths. The advantages of the auto-analyser include less labour, fast analysis and the use of batch panels. The disadvantage came in the form of pursuing “defensive medication” given to counteract threats posed by malpractising lawsuit. Another method of analysis was popularized in 1959, after the wave of continuous flow analysis. This was a product from the Robot Chemist where discrete analysis was utilized along with traditionally used cuvettes clubbed with use of automatic mixing and pipetting and also automating all manual activities that were otherwise practised by a clinical microbiologist. However, the Robot Chemist did not succeed finally since it became complicated and also impractical and soon doused off in 1969. The good part was that this effort then promoted manufacturing of automation equipment, and so the discrete analysis model was developed; that ultima could now completely replace the auto-analyser and also the earliest continuous flow analysis.

Yet another new automation approach was introduced in 1968 that used the concept of centrifugal analysis, which was developed by Norman Anderson. Centrifugal analysis is a single test conducted at any given time. Batch analysis is performed in which tests are conducted in sequence, discretely and parallelly. Centrifugal analysis makes use of a spinning rotor to mix, equilibrate, transport and measure. The sample or reagents can be pipetted into specific compartments of rotors where they get mixed during the spinning of the rotor. The action causes them to flow into a reaction chamber, where the test is finally performed. These centrifugal analyser models succeeded within 2 years in clinical laboratories.

Automation was reinforced and supported during the 1950s with the introduction of a “kit”-based technique which contained packaged reagents for assay, along with step-wise instructions for use, by Sigma Chemical Company (St. Louis, MO). This advent was considered as very novel during this era. Such automated analysers were seen as a big boon for clinical chemistry; however, the invention and application of reagent “kits” for analysis made the automation more successful and practical. This period of automation got promoted further after the auto-analyser was introduced. In 1976, an edition of Tietz (Fundamentals of Clinical Chemistry) included a text that detailed 13 automated analytical systems. Here the DuPont ACA, a revolutionary and unique analyser having long life, was introduced.

Spectrophotometry or colorimetry (older terminology) is used for various photometric techniques: fluorescence polarization, nephelometry, chemiluminescence, fluorescence and electro-chemiluminescence. The random-access analysers, also called discrete analysers, selectively perform only specified assays ordered on each sample. During the 1990s, batch analysers gained popularity, such as centrifugal and auto-analysers which were capable of performing one assay on several samples simultaneously. However, batch analysers are still important, and discrete analysers may be utilized in batch mode along with conducting one or more tests using random-access mode.

Any centralized laboratory can include various modern instruments, namely, electrophoresis, GC-MS, LC-MS/MS, colorimetry/spectrophotometry, potentiometry and immunoassay (using various methodologies). However, few traditional instruments may also be included in the facility, e.g. radioimmunoassay, atomic absorption and flame photometry. This may be for reference purposes.

2.6 Laboratory Automation and Total Laboratory Automation

Two approaches are there for automation: stand-alone or task-targeted technique and total laboratory automation (TLA). TLA may be recognized due to the presence of a tracking system connecting different parts or components. Stand-alone technique can also provide nearly all benefits of TLA, without including the expenses of running a track. Using stand-alone automation technique, the sample transport can be performed by laboratory technicians, who are also sometimes referred to as the “sneaker network”. The option of stand-alone, however, offers limited components at lower cost and therefore can serve as a good choice for labs having less floor space (McAdam 2018).

The advent of robotics along with informatics promoted the development of TLA set-ups. TLA comprised of facilities for pre-analysis, analysis and post-analysis operations. The automated systems lacking any of the above components are considered as sub-total facilities. Dr. Masahide Sasaki of Kochi Medical School, Japan, during the early 1980s, made the first attempt to discover TLA. He used the conveyor system, having robots for loading or unloading specimen and analysers/instruments, clubbed to a control software. Later in the 1990s, other companies started to offer automation, but these systems were expensive and had limited functionality and generally fell somewhat short of TLA.

2.7 Types and Applications of Auto-analysers in Microbiology

2.7.1 Microbiological Specimen Processor

In the past, few large laboratories utilized instruments for processing of, e.g. spread-plating urine samples by InocuLAB (Dynacon, Canada), but it had limitations in speed and functionality. The plus point is that InocuLAB plated the specimens in a more reproducible manner as compared to manual plating via technicians.

In the present scenario, the specimen processors are capable of automation for processing various types of liquid specimens, e.g. Innova specimen processor.

This instrument is made up of 5 sample holding drawers that can hold about 40–200 containers. About 6 input stacks having a capacity to hold 45 plates/stack (meaning 270 plates in total) are present. Each stack holds either all same type of agar plates or various types of agar plates (including bi-plates). This specimen processor has a complete library of classical streaking methods, and the streaked ones get ejected and placed in another stack or output carousel. Reusable 1, 10 and 30 µl nichrome loops are used, and there is no requirement of disposable supplies making it environment friendly.

Some processors may possess a further capacity to store 30 plates in a stack, meaning 150 plates in total. Here a disposable pipette and disposable applicator are required for each specimen and each plate, respectively. A unique radial-comb streak pattern is used on the plates, and unlike the previous one, no other streaking pattern options are seen. The maximum capacity is 180 plates/h. The walk-away specimen

processor utilizes a specimen loading and unloading conveyor belt having pallets of various sizes for differentially sized sample tubes. A universal de-capper is incorporated that can decap/recap differently sized tubes even without the need for manual adjustment.

2.7.2 Routine Biochemistry Analysers

These are based upon a special flow technique named “continuous flow analysis (CFA)”, invented in 1957 by Leonard Skeggs, PhD, and first made by the Technicon Corporation.

Such machines can process majority of the specimen that go to any hospital or an associated private lab. Now due to automation facilities, the time required for analysis and testing has decreased from hours to minutes in several cases. The history of discrete sample analysis for the clinical laboratory began with the introduction of the “Robot Chemist” invented by Hans Baruch and introduced commercially in 1959.

The first applications were for clinical (medical) analysis. The auto-analyser profoundly changed the character of biochemical analytical labs by permitting prominent elevation in sample numbers that may be processed. The design based on separating a continuously flowing stream with air bubbles largely reduced slow, clumsy and error-prone manual methods of analysis.

The categories of biochemical tests usually performed are to test enzyme/hormone levels (indicators of malfunctioning of organs, e.g. thyroid, liver, etc.), ionic levels (e.g. Na^+ and K^+) or other micro-/macro-biochemical components (e.g. creatinine, glucose, serum albumin).

Simple ions are often measured with ion-selective electrodes, which let one type of ion through and measure voltage differences. Activities and rates of enzyme-catalysed reactions can be measured by the rate they change one coloured chemical substance to another. In these tests for enzymes, the results are given as an activity, not as a concentration of the enzyme. Other tests use colorimetric changes to determine the concentration of the chemical in question. Turbidity may also be measured especially in the case of antigen-antibody reactions.

2.7.3 Immunology-Based Analysers

Antibodies may be used by few analysers for detecting antigenic agents via immunoassays and also other reactions that are based on the application of antibody-antigen reactions. If the titres or concentration of such compounds are too low, a measurable complex formation does not occur. Thus specialised methods are then warranted. Therefore, recently such automated immuno-haematology has been developed that is also called transfusion medicine.

2.7.4 Haematology Analysers

These are used for performing erythrocyte sedimentation rates (ESRs), complete blood count or coagulation test.

2.7.5 Cell Counter

These counters can take blood samples and classify or quantify the various types of blood cells by using optical and electrical methods. Electrical analysis is conducted by passing a dilute blood solution through an aperture, across which the electrical current is made to pass. Passage blood cells through an electric current causes the flow between cathode and anode to change (the Coulter principle). A specific reagent causes lysis of RBCs (red blood cells) selectively, thereby leaving the platelets and white cells (WBCs) intact. Thereafter this solution is passed via a second detector, which can now count RBCs, platelets and WBCs. The platelet counts are easily distinguished from the WBC count and calculated by determining the smaller impedance spikes produced by platelets because of their small sizes.

Optical detection can be applied for obtaining differential counts from WBCs of different types. Like before, a dilute cellular suspension may be passed through flow cells, such that cells of particular type are passed one at a time, via a capillary tube, and a laser beam is shone on them. Properties of transmittance, reflection and scattering from each cell can then be analysed using a dedicated software. The software represents the data numerically and gives the exact count and distribution of different cell populations.

Applications

1. The morphological information of leukocyte population may be utilized for marking the cellular abnormalities relating to diseases or acting as trigger of some diseases.
2. The counts of reticulocyte can be obtained via some analysers, instead of manually counting using traditional time-consuming means. Several automated reticulocyte counts make use of a supravital dye, e.g. methylene blue for staining RBCs.
3. Analysers having modular slide maker can generate blood films of a fixed quality which is stained and then reviewed by a specialist or medical lab professional.

2.7.6 Coagulometer(s)

Coagulometer is an automated coagulation machine to determine the blood clotting ability. This may be determined via assays, e.g. lupus anticoagulant screens, partial thromboplastin times, D dimer assays, prothrombin times (and the calculated INRs commonly used for therapeutic evaluation) and factor assays. For functioning these machines need blood samples which must have been collected in tubes containing

anticoagulants, e.g. sodium citrate. The anticoagulation caused by sodium citrate is reversible, for which this method is popularly used. Various substances may be added to blood plasma for triggering blot clot formation. This reaction may be monitored by using spectrophotometric means, i.e. by measuring the absorbance of a particular light by the sample, and the change in the absorbance pattern is also determined.

2.7.7 Additional Instrument for Haematology-Based Methods

Automatic erythrocyte sedimentation rate (ESR) readers have to perform in compliance to “Procedures for the Erythrocyte Sedimentation Rate Test: H02-A5” and also to “ICSH review of the measurement of the erythrocyte sedimentation rate”. These two documents indicate Westergren as the reference method. Diluted blood samples (in sod citrate) are passed via 200 mm pipettes having bore size of 2.55 mm. After being in vertical position for 30–60 min, RBCs are allowed to fall through, and optical reader determines how far the RBCs travel after fall.

2.7.8 Other Miscellaneous Analysers

Some of the tests require separate analysers for few tests, and some need it for one single test. The other tests may be performed less frequently as compared to others, since they may be expensive and take lot of time. However, due to the shortage of skilled or trained clinical labour technicians, manufacturers have tried to innovate newer and better automated systems to conduct the rare but important tests. Such analysers include instruments that can be used for performing the following:

- Measurement of osmolarity and osmolality
- Labelling of DNA and its detection
- Moving samples to different platforms within the lab by aliquoting
- Measuring glycosylated haemoglobin, i.e. haemoglobin A1C

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Manual and Automated Characterization of Multi-antibiotic-Resistant (MAR) Bacteria

3

3.1 Introduction

Antibiotics were defined as biochemical compounds that are produced by microbes to deter or stop the growth of other microorganisms but act in a very selective way against various types of organisms. Currently, chemical laboratories around the world are able to synthesize compounds that mimic the biochemical structures of naturally occurring ones and also may possess chemically modified structures of bio-synthetic compounds.

To be used as a drug, an antibiotic should be safe for the host and also compatible with the microbiota existing in the host while acting against the infecting organism. It should be stable in toxic form and not be metabolized in the body, as long as the growth of infecting organism is not arrested.

Microbes inhibit two types or modes for resisting the effects of antibiotics: (1) intrinsic and (2) acquired. If the microbe shows resistance to an antibiotic even before it has been exposed to an antibiotic, then this phenomenon is intrinsic resistance. Acquired resistance towards an antibiotic is demonstrated by a microbial species which was previously susceptible to it but later gained tolerance. Resistance to antibiotics may be acquired from other co-existing microbe, via conjugation, transformation or transduction and also via mutations and natural selection of mutants. Whatever be the means, an unpredicted acquisition of tolerance towards a specific drug may pose impedance while treating infections.

Co-resistance and cross resistance mechanisms have paved the way to multi-drug resistance (to several different antibiotics at the same time) in microbes resulting in colossal snag in health-care, much to the annoyance and anxiety to doctors and care-givers. There are evidences stating that the huge amount of antibiotics and disinfectants used in clinics, dairy farms and pesticides, used in agricultural lands, promote multi-drug resistance, making disease-causing agents stronger and fitter for survival (Romero et al. 2017; Kurenbach et al. 2018).

3.2 Types of Antibiotic Sensitivity Tests

Microbes showing reduced susceptibility or complete resistance to any particular drug should not be prescribed for treatment, thereby necessitating the need for “antibiotic susceptibility” testing, which tests the suitability of a particular drug for treating a patient. Different methods are employed by clinical microbiologists to test the sensitivity/resistance of microorganisms to a range of drugs/antibiotics. The methods have been broadly classified and listed below along with examples:

1. Qualitative tests: Disc diffusion method, e.g. Kirby-Bauer Disc Method, etc.
2. Quantitative tests: Minimum inhibitory concentration (MIC) in broth or on agar plates
3. Automated tests for resistance: Turbidimetric detection, VITEK (takes ~4–15 h); fluorimetric detection, AutoSCAN; walkaway (takes ~3.5–7 h); video image detection, ALADIN (takes ~18–24 h)
4. Molecular techniques: multiplex PCR, RAPF, AFLP, etc.

Two procedures for the same have been described below. Kirby-Bauer disc method may be applied for determining the particular antibiotic that serves as the most effective antimicrobial agent against any particular pathogen. The second method of determining MIC may be utilized for determining lowest concentrations of any drug required for destroying microbial pathogens during infection.

One qualitative and one quantitative procedure for testing the resistance/susceptibility of microbes to one or multiple drugs at one time has been described in this section. The first one (the Kirby-Bauer disc method) is used to determine which antibiotic is the most effective against a certain pathogen. The second (MIC) is used to determine the lowest concentration that is needed to kill the pathogen at the site of infection.

3.2.1 Kirby-Bauer Disc Diffusion Method

This method is also known as (1) agar diffusion method or (2) disk diffusion method because it utilizes a filtering disc that is impregnated by any antibiotic that is generally layered on the surface of the agar plate which has been inoculated with the test organism. The drug/biochemical compound slowly diffuses into the agar, depending on its chemical nature. This creates a gradient of concentrations of that particular drug being highest near the disc and lowest at the periphery of the agar plate. This gradient in concentration has an inverse proportion to the square of the distance of diffusion. Clear areas surrounding the disk form due to the action of the chemical in the disc and are called zones of inhibition (ZOIs). At some particular distance from each disk towards the periphery of the plate, the concentration of drug is so low that it does not inhibit microbial growth. Growth inhibition zones/circles are formed surrounding the filter disc, and the diameter of the zone indicates the effectiveness of the antibiotic in question. The diameter of the ZOI is measured with

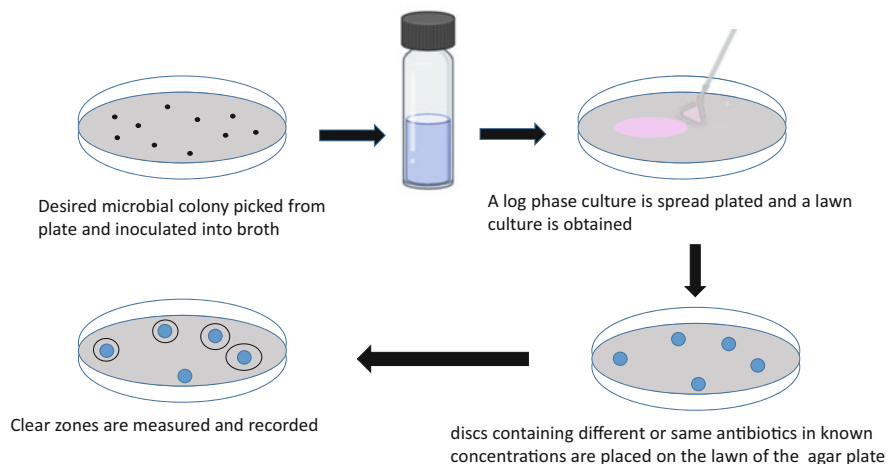


Fig. 3.1 Showing the protocol for antimicrobial assay using the Kirby-Bauer disc diffusion method

a ruler, and the results are used to construct the antibiogram. In the case of testing for multi-drug resistance, several antibiotic discs may be placed on the lawn (Fig. 3.1).

Points to be remembered here are the following:

Size of the zone may depend on the density and viscosity of the agar medium, which consequently affects the rate of diffusion of the antibiotic and the spread of the drug thereof. Therefore, chemo-therapeutic agents are not chosen simply on the basis of the drug producing the widest ZOI. Sensitivity of the organism to the antibiotic is however determined by this assay, and the disk diffusion method therefore represents a simple qualitative procedure for screening substances to determine the level of antimicrobial activity.

Material required: BHI-agar plate, overnight grown microbial cultures (5 ml), forceps, 95% ethanol, sterile swabs/spreader or glass beads, antimicrobial discs

Strict aseptic conditions should be maintained at all times.

Equipment: Incubator, laminar flow

Methodology:

1. Obtain a swab/spreader and dip it into the microbial broth culture.
2. Use the spreader to spread the culture on the plate uniformly to obtain a lawn. Label the plate. Allow the plates to dry for 2–5 min.
3. Draw lines on the back of the plate to divide it into required number of sections.
4. Lift the pair of forceps from alcohol, and pass through a Bunsen burner, until alcohol burns off, and then use the sterile one for picking up one antibiotic disc from the dispenser, aseptically and placing on each agar plate. These discs may be containing antibiotics, e.g. gentamicin, tetracycline, penicillin G, chloramphenicol, ampicillin and erythromycin.

5. Replace lids of plates, invert them, label the plates, and incubate them for 2 days at 37 °C.
6. Measure the diameters of the zones of inhibition (ZOI) formed due to clearance of susceptible microbes.
7. Zone diameter for the individual antibiotics can be classified as resistant, intermediate or susceptible. For this the interpretative table mentioned below may be referred ([https://bio.libretexts.org/Demos%2C_Techniques%2C_and_Experiments/Microbiology_Labs_I/09%3A_Kirby-Bauer_\(Antibiotic_Sensitivity\)](https://bio.libretexts.org/Demos%2C_Techniques%2C_and_Experiments/Microbiology_Labs_I/09%3A_Kirby-Bauer_(Antibiotic_Sensitivity))).
8. See Note 1 for details and precautions to be taken while interpreting the results.

Note 1

- Zone of inhibition may be measured in terms of its diameter, using rulers, sliding calipers or a particular template, to the nearest whole millimeter.
- The measuring device is held on the back of the Petri plate, which is illuminated with reflected light, if supplemented medium is used.
- The zone formed on blood-containing agar media is measured at the agar surface.
- Ending points of reading and recording data are determined visually.
- Minuscule colonies or inappropriate growth in colonies that are barely visible should be ignored.
- Conspicuously large microbial colonies that form within the inhibition zone should represent resistant variants.
- A mixed inoculum may necessitate re-identification and re-test.
- In the case of double zones, one should measure the innermost zone.

Precautions

Points to remember about:

1. Antimicrobial discs
 - a. Discs should not be used after the expiry date.
 - b. Discs should not be stored in freezer.
 - c. FDA clearance on products should be verified.
 - d. Relocation of any disc after it touched agar surface on plate should be avoided.
2. Incubation of plates
 - a. Inversion of plates for incubation keeping agar side up is the correct method.
 - b. Incubation at 35 °C is recommended for non-fastidious bacteria, for 16–18 h.

3.2.2 The Minimum Inhibitory Concentration (MIC) Method

The disk diffusion (Kirby-Bauer) method for in vitro susceptibility testing gave a good indication of whether any bacterium is susceptible, resistant or intermediate to a particular antibiotic using the size of the growth-free zone. However, Kirby-Bauer testing could not indicate the exact concentrations of the antibiotic that is needed for achieving a reliable result valid therapeutically. The minimum inhibitory concentration (MIC) determination technique is a quantitative tool for precise determination of the antibiotic concentration(s) required to deter/stall microbial growth. It also helps to determine the class of antibiotic that is best in inhibiting growth of pathogen, giving vital knowledge and guidance to an appropriate choice of the antibiotic to improve chances of cure and act against drug resistance (Reller et al. 2009).

The minimum inhibitory concentration (MIC) is defined as the lowest amount (in $\mu\text{g/mL}$) of an antibiotic required to inhibit the growth of a given microbe.

Although it is widely useful, MICs are not performed in certain cases, especially under the following circumstances:

- The growth requirements of special organisms require different conditions that are not included in the protocol for MIC testing.
- Unavailability of certain drugs/antibiotics commercially.
- The drug is reported as ineffective via clinical results against the organism, regardless of the in vitro results.

How Are MICs Used?

Comparing the MICs of different antibiotics on different microbes is not a simple task as it is based not alone on the numerical value but on how far the MIC is from the breakpoint (*dilution of drug at which microbe starts showing resistance*), the actual spot affected by infection and associated considerations, e.g. species, age, patient health, etc. (Coyle 2005). The plausible side effect of the medicine, cost, dose frequency and route of administration are also notable parameters and important factors.

Note 2: Points to Remember

- MIC value obtained for a specific antibiotic CANNOT be comparable to the MIC number obtained for any other antibiotic.
- The MIC value should be the basis for choosing an antibiotic, for treating any infection.
- Other parameters to consider for choosing the optimal antibiotic are the ease of use, safety and cost.

Material required: BHI-agar plate, overnight grown microbial cultures (5 ml), forceps, 95% ethanol, microtitre plate (24 well).

Strict aseptic conditions should be maintained at all times.

Equipment: Incubator, laminar flow

Procedure for Broth Microdilution (MIC) Testing

The steps described for disc diffusion methodology, i.e. selecting suitable test colonies, preparation and standardization of the inoculum, are similar to those used for MIC determination.

1. Isolated colonies from an agar plate having freshly growing culture (18–24 h old). The plate should have non-selective medium, e.g. blood agar, for preparation of inoculum.
2. Prepare the inoculum using colonies from agar plate and suspending in a broth medium and incubated under optimal conditions till it reaches the turbidity of 0.5 McFarland standard such that the culture has $\sim 1-2 \times 10^8$ CFU/mL. Alternately, a direct colony-culturing method can be employed for fastidious organisms, e.g. *N. gonorrhoeae*, *Haemophilus* spp., streptococci, etc. This may also be used to determine the anti-bacterial potential of methicillin or oxacillin, against staphylococci.
3. Purity of the inoculum should be checked, and the inoculum should be well suspended to avoid wrong results for misinterpretations.
4. To prevent the panels of microtitre plate from dehydration during incubation, place a plastic seal to lock the plate, or alternately the panel may be enclosed within a clear plastic bag.
5. Non-pathogenic, slow-growing bacteria may be incubated at 35 °C, for 16–20 h; however, more than four panels should never be stacked.
6. Label the wells A1 to A6 and B1 to B6 for antibiotic 1 (T), while wells C1 to C6 and D1 to D6 are for antibiotic 2 (G). Well numbers B6 and D6 should be maintained as untreated negative controls having no antibiotic. Add 0.5 ml broth (tryptic soy broth) by maintaining sterility, to all wells except A1 and C1.
7. Add 0.5 ml antibiotic 1 (T) to wells A1 and A2 and 0.5 ml antibiotic 2 (G) to wells C1 and C2, in aseptic manner. A twofold serial dilution of antibiotics is prepared and added to well numbers 3–6 in A and C lanes and wells 1–5 in lanes B and D of microtitre plate. Every time when mixing is done, a new tip should be used.
8. About 0.5 ml from diluted (100-fold) culture suspension should be added to each well as indicated in Table 3.1.
9. Incubation to be done at optimal conditions for about 2 days and the special incubation conditions should be referred to.
10. Calculation of antibiotic concentrations should be done using per ml volume. Ending point of this assay would be considered as the highest value of dilution at which the antibiotic is still inhibitory towards the particular bacteria. Resistant results can be reported when detected.

Specific incubation cases: Normally within 16–20 h, most anti-microbial agents can produce results against non-fastidious bacteria. However, in some cases, to determine antimicrobial resistance to few microbicidal reagents, this procedure

Table 3.1 Showing antibiotic dilution for calculation of MICs. Antibiotic used is tetracycline (Antibiotic A1)

Antibiotic well	Broth (ml)	Antibiotic (100 µg/ml)	Diluted culture	Antibiotic conc.
A1	0	0.5	0.5	50 (µg/ml)
A2	0.5	0.5	0.5	
A3	0.5	0.5 from A2	0.5	
A4	0.5	0.5 from A3	0.5	
A5	0.5	0.5 from A4	0.5	
A6	0.5	0.5 from A5	0.5	
B1	0.5	0.5 from A6	0.5	
B2	0.5	0.5 from B1	0.5	
B3	0.5	0.5 from B2	0.5	
B4	0.5	0.5 from B3	0.5	
B5	0.5	0.5 from B4 (discard 0.5ml)	0.5	
B6	0.5	0.0	0.5	

Adapted from “Antibiotic sensitivity testing” Lab Manual, by Dr. Eby Bassiri, Dept. of Biology, University of Pennsylvania, USA

must be altered, e.g. in the case of staphylococci, incubation of vancomycin and oxacillin should be considered for 24 h. For enterococci incubation in vancomycin, gentamicin (synergy test) and streptomycin (synergy test) should be done in high levels for 24 h. In case negative results are obtained against streptomycin after incubating for 24 hours, reincubation is necessary for another 24 h, prior to documenting and reporting of results (a total of 48 h).

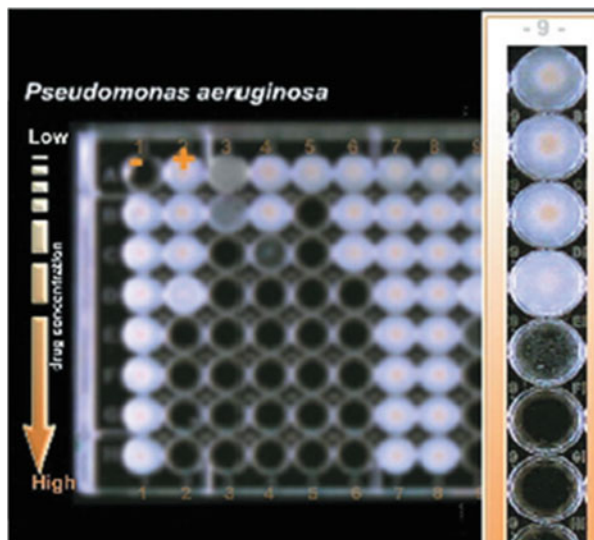
Reading MIC Panels

1. Check the positive and negative control well for growth and clarity, respectively.
2. MIC endpoints considered should be the highest dilution of microbicidal agents that can abolish the organism, as read visually by one's unaided eye.
3. In cases of sulphonamides, trimethoprim and trimethoprim-sulfamethoxazole, end point considered should be the concentration showing >80% reduction in growth compared to positive control. Bacteriostatic agents, which do not kill the organism but only arrest its growth, may exhibit very blurred colony or small buttons.

Disc diffusion technique and broth dilution method are most commonly used protocols for determining antimicrobial susceptibility (Fig. 3.2), *in vitro*. These easy and inexpensive methods suffer from some disadvantages, e.g. time consumption, requirement of skillful labour and dependency on the investigator (Driscoll et al. 2012; Hombach et al. 2013). Therefore, many studies indicated the feasibility of using polymerase chain reaction (PCR)-based analysis to detect pathogens in the form a diagnostic tool. This is due to the fact that PCR is very sensitive and can enable high-throughput reads and provide quick results (Gao

Fig. 3.2 Showing the results of MIC using *P. aeruginosa* as the test microbe to show its susceptibility to various antibiotics along with positive and negative controls.

Adapted from *Manual of Antimicrobial Susceptibility Testing* (Coyle 2005)



et al. 2012). In addition, the technique of PCR may be employed to identify genes of resistance.

3.2.3 RAPD PCR Analysis

RAPD PCR (random amplification of polymorphic DNA PCR) is a quick, simple, cost-effective and popular typing method that does not need skilled or trained hands or DNA sequence information with respect to the targeted organism. RAPD typing assays have previously been successfully applied for epidemiology studies of a larger number of bacterial species (Hassan et al. 2015; Ben-Hamouda et al. 2003). RAPD assay often gives non-reproducible, but distinctive, arrays of amplicons when template genomic DNA is using short (10–25 base) oligonucleotide primers of random sequence. This approach has been applied to detect genomic diversity amongst plants (Wolff and Peters-Van Rijn 1993), animals (Rothuizen and Van Wolferen 1994), parasites (Murphy and Pellec 1994) and microbial organisms (Makino et al. 1994; Maslow et al. 1993). The most lucrative feature of this assay is that several arbitrary primer sets can be used, and still prerequisite of DNA sequence for the target organism is not needed. Therefore, application wise various sets of primers can be used.

RAPD PCR finds use in various streams of biology, e.g. ecology, plant tissue culture, genetics, etc. It can be given to students as an introduction to PCR and also for hands-on practice, as well as to answer their queries on the assay. Microbes that are isolated from environmental resources or clinical specimens may be typed on basis of specific gene(s) in comparison to a known strain. The effect of differences in the amplification pattern of various strains after performing RAPD PCR may be

evaluated prior to undertaking this study or analysis. Various other microbes, namely, yeast, fungi, viruses and parasites, may also be subjected to RAPD PCR to identify them or study their population genetics. Effects of physical or chemical mutagenic agents on various bacteria may be determined via RAPD PCR. This analytical tool may also be employed for initially evaluating unknown bacteria, before performing other biochemical tests that re-confirm identity.

Preparing Samples for RAPD

Material required:

Procedure: Boiling Cells (BC) Method

1. Desired bacteria were grown in a broth, until log phase.
2. Cells were spun down (4000 g; 2 min), rinsed in 0.85% NaCl and spun down again.
3. The pellet was re-suspended in TE buffer (1.0 mL) and boiled for about 15 min. TE buffer contained Tris-HCl 10 mM and EDTA 1.0 mM (pH 8.0).
4. Serially dilute the sample with Tris-HCl (10 mM, pH 8.0), and 2.0 µL of the diluted template may be used for RAPD PCR reactions.

RAPD Analysis

Procedure

1. RAPD PCR should be carried out in a volume of 15 µL, having 2.5 ng template DNA or 2.0 µl dilute boiled and denatured culture; 0.4 mM primer; 200 µM each of dGTP, dCTP dTTP and dATP; 5 mM MgCl₂; and finally 1.5 U Taq DNA polymerase in 20 mM Tris-HCl, pH 8.4 having 50 mM KCl. (Concentrations vary as per PCR kit.)
2. The thermal cycling was done starting with 4 min of denaturation at 92 °C, followed by 40 cycles of denaturation for 1 min at 92 °C, 2 min primer annealing at 37 °C, 3 min extension at 72 °C and a final extension for 3 min at 72 °C. Extension temperatures depend on primer's specification.
3. RAPD PCR can be done using low annealing temperatures as compared to other PCRs. Lowering of annealing temperatures is needed for non-specific binding of primers to the target sequence. This results in good numbers of amplification product formation.
4. These RAPD primers are short (about 10 bases long), and thus they are expected to bind after every 4¹⁰ bases, i.e. 1,048,576 bases.
5. *Qiagen Operon* has random 10 base long primers about 1200 in count having a G/C content of 60–70% and lacking any self complementarity. The kit is user-friendly and affordable. Several primers and experiments should be planned with RAPD PCR to determine a pattern quality so that it can serve as a desirable fingerprint that is generated by various randomly picked PCR primers meant for RAPD.
6. After PCR, RAPD PCR products are analysed by electrophoresis on 1% agarose gels. Each gel should include DNA ladder (a band-size marker). Gels may be

placed for 15 min in a 0.5 ug/ml EtBr solution, and the GelDoc should be used for digital photo documentation.

7. Data is analysed by comparing the band patterns within a gel and across gels, for known bacteria having band patterns and also for unknown species.

Precaution

1. DNA should be extracted without contaminating (Note 3).
2. Time and conditions of electrophoresis for all gels should be exactly the same.
3. Staining should be uniform and thus post staining the gels is advised.
4. Misidentification of bacteria after data analysis may be due to mislabelling of PCRs or gel electrophoresis lanes.

Note 3: Contaminating DNA effect RAPD—Since RAPD primers do not target specific sequences, contaminating DNA could be amplified and either add to or completely obscure the pattern of bands from the target genome. This underscores the importance of using isolated bacterial colonies, negative controls that exclude template DNA and the care that must be taken in performing RAPD PCR.

ERIC PCR

A popular technique is genotyping using “repetitive DNA elements” in a PCR-based method. This is seen in the use of ERIC (enterobacterial repetitive intergenic consensus) sequences common to Gram-negative enteric bacteria (Syrmis et al. 2004). ERIC stretches have a good potential for studying about the evolution of interspersed repeat sequences in bacteria, since these stretches are long and have more information to aid comparative microbial analyses and also these exist in a large number of target organisms (Wilson and Sharp 2006). The ERIC sequence-based PCR genotyping (ERIC-PCR) technique has reportedly aided in the study of *P. aeruginosa* epidemiology in Brazil and Australia (Stehling et al. 2010; Kidd et al. 2011). Rapid and discriminating methods were used for sub-typing to determine the strains involved in nosocomial outbreaks. Studies were designed for understanding gene diversity of multi-drug-resistant (MDR) *P. aeruginosa* strain isolates taken from wound and burn infection via ERIC PCR. To determine genetic relations of MDR strains, dynamics of transmission of infection can be mapped throughout the region under study. It also gives an understanding of epidemiology of drug-resistant strains.

Material required: PCR kit, DNA extraction kit (or as required), ethidium bromide (EtBr) stain

Instrument: Thermocycler, horizontal agarose electrophoresis machine, GelDoc

Protocols

1. Extraction of DNA: DNA can be extracted from bacterial colonies by boiling method as has been detailed earlier in the previous section.
2. ERIC PCR fingerprinting

- a. PCR amplification should be performed on extracted DNA by using primers designed on the basis of the spacer sequences of 16S and 23S ribosomal DNAs (Jensen et al. 1993; Tosin et al. 2003). Normally, various primer sets for ERIC PCR can be obtained by literature search and validation by experimentation.
- b. PCR mixture with a final volume of 50 μ l should be prepared containing 10X PCR buffer (10 μ l), 50 mM $MgCl_2$, 2.5 mM dNTP mix, 10 μ M of each primer, 1U *Taq* DNA polymerase and 50 ng template DNA.
- c. The amplification parameters should be initial denaturation at 94 for 3 min, followed by 35 cycles of PCR consisting denaturation at 94 for 1min; annealing at optimal temperature, depending on the primers, for 7 min; and extension at 72 for 2 min and a final extension at 72 °C for 5 min. The time of extension may be varied to get bands of proper size.
- d. The PCR products can be separated using electrophoresis using 1.5% agarose gel which is stained using 0.5 μ g/ μ l ethidium bromide (Qiagen, Germany) and can be viewed under UV radiation of a gel documentation system. A mixture of fragments from HindIII digested lambda DNA and HaeIII digested ϕ X174 DNA may be used to mark molecular masses. TBE buffer might be tried for better results as compared to TAE (Note 4).
- e. The size of every band should be determined using softwares, e.g. Stratascan 7000 densitometry with one-dimensional analysis software where the data is coded as 0 (negative) or 1 (positive). A hierarchical cluster analysis should be performed for the construction of a dendrogram via SPSS program for Windows, Release 6.0 (SPSS Inc., Chicago, IL). Finally, different typing techniques have different distinguishing ability and may be calculated by employing Hunter and Gaston's method (Hunter and Gaston 1988).

Note 4:

TBE (Tris-borate-EDTA) buffer can conduct electric current better than TAE (Tris-acetate EDTA) buffer and therefore is less susceptible to over-heating. Therefore, TBE may be used for long electrophoretic runs.

Applications of ERIC PCR to Study Multi-drug-Resistant (MDR) Pathogenic Strains

Jena et al. (2017) tried to understand the underlying clonal relation amongst MDR *E. coli* isolates that produce β -lactamase enzymes using enterobacterial repetitive intergenic consensus (ERIC) polymerase chain reaction (PCR). Their dendrogram results indicated about 14 unique clusters of ERIC (CL-1–CL-14) included within 41 *E. coli* isolates, revealing the diverse gene-based diversity between them. Similarly, Khosravi et al. (2016) also analysed the diversity of genes in MDR *P. aeruginosa* strains that were isolated from wound and burn infections in Ahvaz, Iran, via ERIC PCR.

3.2.4 Multiplex PCR

The major challenge concerning molecular diagnostic tests for pathogen characterization is the huge number of different genetic targets. In addition to these enzymes, more than 500 acquirable antibiotic resistance genes conferring resistance to other antibiotic classes are also disseminated amongst pathogens, and thousands of “single nucleotide polymorphisms” (SNPs) contributing to resistance towards antibiotics or housekeeping genes alter the drug susceptibility and, thus, have to be characterized as well (Zankari et al. 2012). Therefore, it becomes evident that sophisticated diagnostic methods are necessary to detect all these gene targets using ideally only a single test. Although cultivation-based characterization methods are dominant in clinical laboratories today, the long time period to start a potentially life-saving antibiotic treatment optimization is problematic, especially in the light of emerging multi-drug-resistant pathogens (Barisic et al. 2016). DNA-based multiplex detection methods have the potential to characterize the pathogens directly from the patient samples and provide results within a few hours upon sampling.

Many multiplexing PCR-based methods used for detecting genes for antibiotic resistance in *S. aureus* (Strommenger et al. 2003), *E. coli* (Sianglum et al. 2009), streptococci (Malhotra-Kumar et al. 2005), etc. have been developed proving the potentials for this assay for analysis of multi-drug resistance in pathogenic strains.

Material required: PCR kit, DNA extraction kit (or as required), ethidium bromide stain

Instrument: Thermocycler, horizontal agarose electrophoresis machine, GelDoc

Protocols

DNA extraction: DNA extracted from colonies of all isolates by simple boiling method as described earlier in the previous section. Alternately, use of “DNeasy Blood & Tissue Kit (QIAGEN®)”, which is suitable for such bacterial samples, is recommended. The DNA concentration and purity should be assessed using Nanodrop 2000 (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Multiplex PCR: Various drug resistance gene(s) are selected—resistance to aminoglycoside (*strA*, *strB*), sulphonamide (*sulI*, *sulII*), tetracycline (*tetA*, *tetB*, *tetK*, *tetM*, *tet*), macrolide and lincosamide (*msrA*, *ermA*, *ermB*, *ermC*, *mefA/E*), etc.—and primers are designed/searched for amplifying these. The primers should have annealing temperatures that vary by 5 °C. Primer3 software may be used for designing desired primers and checked for self-dimers, dimers and hairpin structures.

Multiplex PCR conditions: The PCR mixture (total volume 10 µl) should consist of 5 µl of PCR Master Mix (PCR kit/see components as in ERIC-PCR), 1.4 µl of primer mix (0.1 µl of each primer [10 pmol]), 3.1 µl PCR-grade water and 0.5 µl template DNA (20 ng/µl). PCR can be done under the given conditions: initial denaturation for 1 min at 95 °C, denaturation for 30 s at 95 °C for 35 cycles, primer annealing for 30 s at optimum temperature and elongation for 30 s at 72 °C, followed by a final elongation for 7 min at 72 °C.

Reaction products should be detected using a 2% agarose gel with ethidium bromide. All PCR products should be sequenced, and data should be compared to corresponding sequences in the GenBank using the BLAST algorithm available at the National Center for Biotechnology Information website (www.ncbi.nlm.nih.gov).

Challenges: Multiplexing PCR methods can include amplification of target sequence with generic primers containing species-specific sequence or multiple sets of primers. These techniques are considered as great advancements over classical assays based on PCR, but they too suffer from limitations. To target conserved genome sequences can limit analyses to any specific group of microbes that are known taxonomically. Combination of many primer sets in an assay might pose a technical challenge. Recently, microarray technique coupled to universal amplification has been accepted as an unbiased approach for detecting pathogens (Vora et al. 2004).

3.2.5 Padlock PCR and Microarray Analysis

Padlock probes (PLPs) are oligonucleotides, having ends complementary to adjacent target sites. After hybridizing with target sequence, both the ends come in contact that allows circularization of PLP via ligation. PLPs facilitate specific recognition of targets, followed by its amplification and then detection via microarray. The PLPs serve as a combination of pathogen-specific recognition by molecular tools, with PCR. This combination results in increase in sensitivity of the reaction and can also result in multiplex PCR reactions, such that the wide range of organisms can be targeted at the same time. PLPs are ~100 bases long oligonucleotides that contain the sequences complementary to target regions which lie both at the 5' and 3' ends (Fig. 3.3). Such regions can identify sequences adjacent to target DNA, and also in between the regions, the universal primer sites are placed along with unique sequence identifiers, which are also known as "ZipCode" (Nilsson et al. 1994). After hybridization the ends of the probe come together in adjacent positions and may be ligated enzymatically. Ligated and circularized molecule with both segments at the ends can correctly recognize the target sequence. Non-circularized probes can be removed by treating with exonucleases, and the circular ones should be amplified via universal primers. Thereafter, amplified products can be determined via the universally complementary ZipCode (cZipCode) microarray system (Shoemaker et al. 1996). PLPs are highly specific and have good capability for multiplexing for genotyping assays (Hardenbol et al. 2003). They have been proven to be effective for multiplexed diagnosis. The amplification uses universal non-degenerate primers, and so some bias is expected, but in this method uniform sized amplicons are obtained. Additionally, the universal microarray platforms provide lower degree of random hybridization, thereby allowing a better spectrum of detection of pathogens. Moreover, the probes and target sequences present on the array are independent, to render this assay flexible and include additional target pathogens (Marianna et al. 2005).

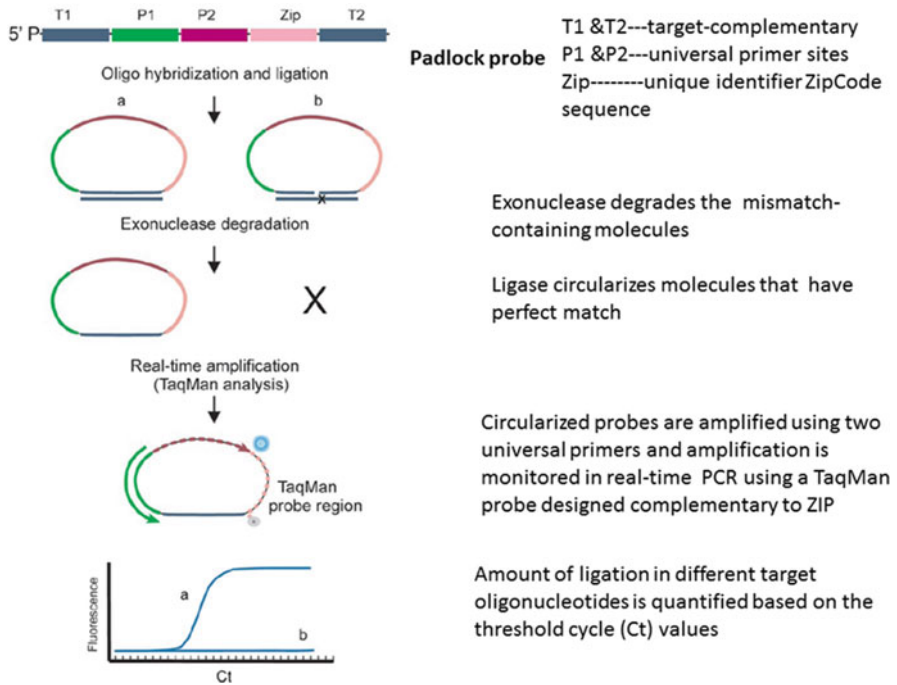


Fig. 3.3 Showing the working of pad lock probes for genotyping assays as they allow multiplex diagnosis

3.2.6 Real-Time PCR for Quantitative Data

RT-PCR is also known as quantitative PCR (qPCR) which monitors the amplification target DNA or PCR product in real time and is detected by using fluorescence technology. The “real time” denotes that the PCR can monitor the progress of the amplification when the process is going on and in contrast to the conventional PCR method where analysis is done only at the end of the process. Several models of real-time PCR are seen, and each one shares few common characteristics: a common platform of thermal cycler linked to any excitation source (mostly tungsten or laser lamps), camera with ability to detect fluorescence and for data processor system like computer and software. Various fluorescence dyes are used in RT-PCR. Two most commonly used RT-PCR methods use SYBR Green and TaqMan probes. The two methods where PCR products are detected are the following:

- Non-specific fluorescent dye that can bind to double-stranded DNA in PCR. As the DNA product increases during each PCR cycle, the intensity of fluorescence increases.
- Sequence-specific DNA probes that permit detection only after the hybridized probe is bound to its complementary sequence.

SYBR Green: This dye on binding with the minor groove of DNA non-specifically emits prominent fluorescent signal, and as the dye intercalates with dsDNA, the green signal increases. It provides information about each cycle of amplification as well as about the melting temperature.

TaqMan probe: TaqMan probes also known as hydrolysis probes are specifically designed to increase the specificity of quantitative PCR and was first reported by Kary Mullis in 1991. It bears a reporter dye, a fluorophore (e.g. 6-carboxyfluorescein or tetrachlorofluorescein) attached with covalent linkages to 5' ends of an oligonucleotide probe, and at the 3' end, a quencher (e.g. tetramethylrhodamine) is bound. As the fluorescence is emitted by the fluorophore, the quencher molecule quenches when it becomes excited by the cyclers' light source via Forster resonance energy transfer (FRET). The probe is designed in such a way that it can anneal within a DNA region amplified by a specific set of primers.

Molecular beacons: These are short singled-stranded nucleic acid sequences (30–50 bases) with unique sequence having indirect repeat regions on both ends, to create a structure like stem-loop such that the 5' and 3' ends lie in close proximity. Like TaqMan at one end of the probe, they have a fluorochrome, and at other end this fluorescence is suppressed by a quencher molecule. However, molecular beacons don't get degraded during PCR as in the case of TaqMan probes; rather they remain intact for functioning and can bind to target(s) during each PCR cycle and produce a fluorescence that is detected and measured.

Clinical microbiology requires rapid characterization of pathogen samples for faster treatment of infection in patients to contain any outbreak or spread of infectious elements. These above methods are very appropriate for monitoring and checking infections especially caused by MAR strains. However, for identifying and characterizing new infectious agents, a complete genome sequencing facility is required. With the advent of and a sharp fall in costs of WGS (whole-genome sequencing) techniques, this method has become favourable for clinical microbiology.

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Rapid Microbial Genome Sequencing Techniques and Applications

4

4.1 Introduction

Antimicrobial susceptibility tests performed via established methods for MIC (minimum inhibitory concentration), e.g. determining zones of clearance or concentration at which bacterial growth is inhibited, are now considered as preliminary data, and they suffer from drawbacks due to a limited number of antimicrobials that can be simultaneously get tested and is not cost-effective when extended to a larger pool of antimicrobials. Sequence-based techniques also suffer from errors due to DNA contamination while generating fingerprints. However, they are now becoming more prevalent due to their homogenous protocols that may be applied throughout a region or even countries.

Ultra-high-throughput sequencing (UHTS) has helped research in many scientific fields, especially in clinical microbiology, where pathogens have small genomes. When tools and techniques for analysing sequence data become automated and get correlated to databases that contain associated meta-data, then WGS (whole-genome sequencing) might be considered as a common and most effective tool for larger clinical microbiology labs. Continued reduction in costs and time for nucleic acid sequencing has made it a lucrative strategy for both researchers and diagnostic labs.

Here the major techniques and applications of UHTS have been discussed to apprise the students about the need and importance of UHTS. They include (1) identifying target sequences of DNA and the antigens for developing rapid tools for effective diagnosis, (2) identifying strains precisely during epidemics for typing and monitoring the pathogenic agent during epidemic or pandemic outbreaks, and (3) determining the properties of particular strains, like virulence factor or antibiotic resistance. In addition, recent developments in comparative metagenomics and single-cell sequencing offer the prospect of a better understanding of complex microbial communities at the global and individual levels, providing a new perspective for understanding host-pathogen interactions. Being a high-resolution tool,

high-throughput sequencing will increasingly influence diagnostics, epidemiology, risk management and patient care.

By using UHTS the number of steps involved for complete microbial identification and characterization of pathogens or antigen gets reduced, and thereby the time taken to get results decreases. To obtain the whole-genome sequence (WGS) of any microbial strain, a pure culture may be used for DNA extraction. Also, WGS data may be determined from a clinical sample (blood/urine) or even from a single bacterium obtained in a given sample, and the data may be generated as qualitative or quantitative. Genome finishing—the costliest and time-consuming step—is often not necessary. WGS data may be used directly for development of diagnostic tests, e.g. multiplex PCR, qPCR, etc.

The cost of bench-top sequencers is about US\$125,000 on an average, and they have the capability of sequencing many small genomes per day where every sample reasonably costs about US\$150. The increasing number of sequencers and the databases for genome analysis have gained importance for surveying antibiotics/drug-resistant microbes for containing them (Didelot et al. 2012; Peacock 2014).

As compared to other techniques of clinical microbiology that give partial information, such as PCR-based techniques discussed previously, the WGS-based techniques are faster and have the potential to generate wholesome datasets for thorough analysis (Fig. 4.1) and accurate characterization.

The WGS process may be divided into some significant steps, which are outlined as follows:

- a. Bacterial/microbial isolation, DNA extraction, quality assessment of DNA
- b. Preparation of a library and determining its quality (Fig. 4.2)
- c. Sequencing via various tools
- d. Bioinformatic analyses (Fig. 4.4)

The most common methods used nowadays are pyrosequencing (454), Illumina technologies (Illumina, Inc., San Diego) that may be available as MiniSeq or NextSeq or HiSeq and SOLiD sequencing. The other sequencing platforms are Oxford Nanopore and Thermo Fisher ion Torrent.

4.2 WGS Techniques

The 454 Genome Sequencer was the first sequencer for commercialization, and it was used profusely for metagenomics and also de novo sequencing owing to long reads (up to 750–800 bp) provided by the technique and was comparable to automated Sanger sequences (Bertelli and Greub 2013). Homopolymer tails and repeat sequences were a problem with this technique, leading to further research for novel and better techniques. This was followed by “sequencing by synthesis” from Solexa that also gained popularity amongst the researchers due to the applications in resequencing and RNA sequencing, at lower cost/base, although the reads (36–150 bp) were shorter in this case. Then evolved the SOLiD (sequencing by

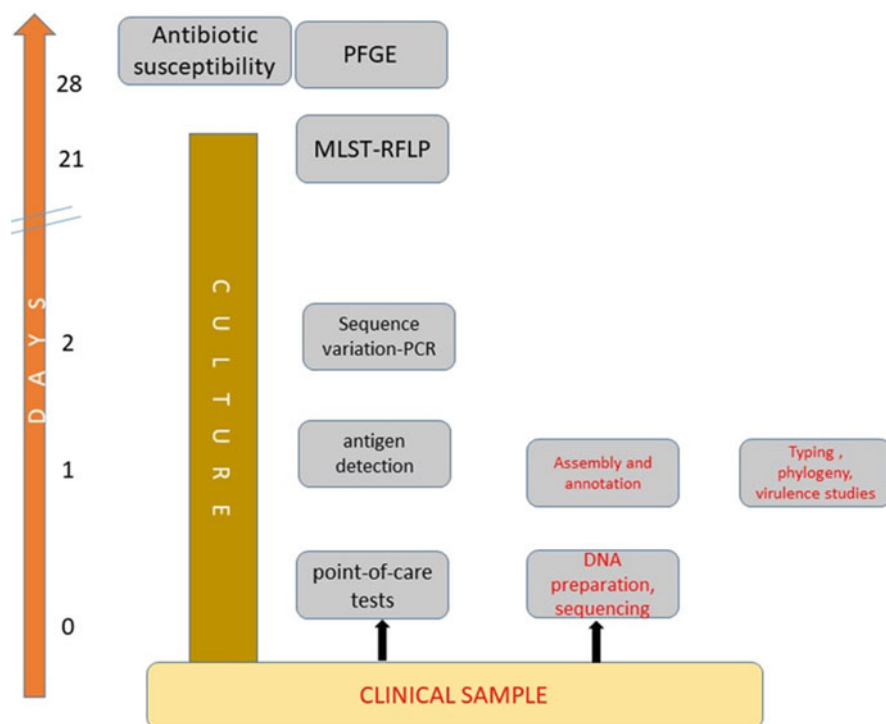


Fig. 4.1 Showing the time required (in days) for various methods used in clinical microbiology

oligonucleotide ligation and identification) sequencing system, which was based on ligation of oligonucleotide probes and the special feature being that each base was read twice, leading to decrease in errors and accuracy for SNP detection. The method was insensitive to homopolymer errors and was effectively used in resequencing and epigenomics/transcriptomics studies. Later, Heliscope was developed but was not as popular with users.

Both 454 and Solexa offer the facility for paired-read reads that assist in the assembly of draft genomes. Here samples may easily be pooled during a single run because they are tagged individually with 6–8 bp long sequences incorporated in the adaptors for barcoding purpose. Illumina sequencing also enables multiplexing (in a single run) and reading about 100 small genomic samples at the same time, which is cost-effective and high throughput (Fig. 4.2).

Third-generation sequencing technologies are the most commonly used technique for clinical purposes, especially for RNA sequencing. Ion Torrent technology launched by Life Science is based on the release and sensing of a hydronium ion during the incorporation of a new base in the DNA string. The advantage is therefore the time saved, and the precision of the advanced material of ion sensors makes this lucrative and efficient for high-throughput studies. The time of obtaining sequence data has therefore been decreased to hours instead of days and weeks. A

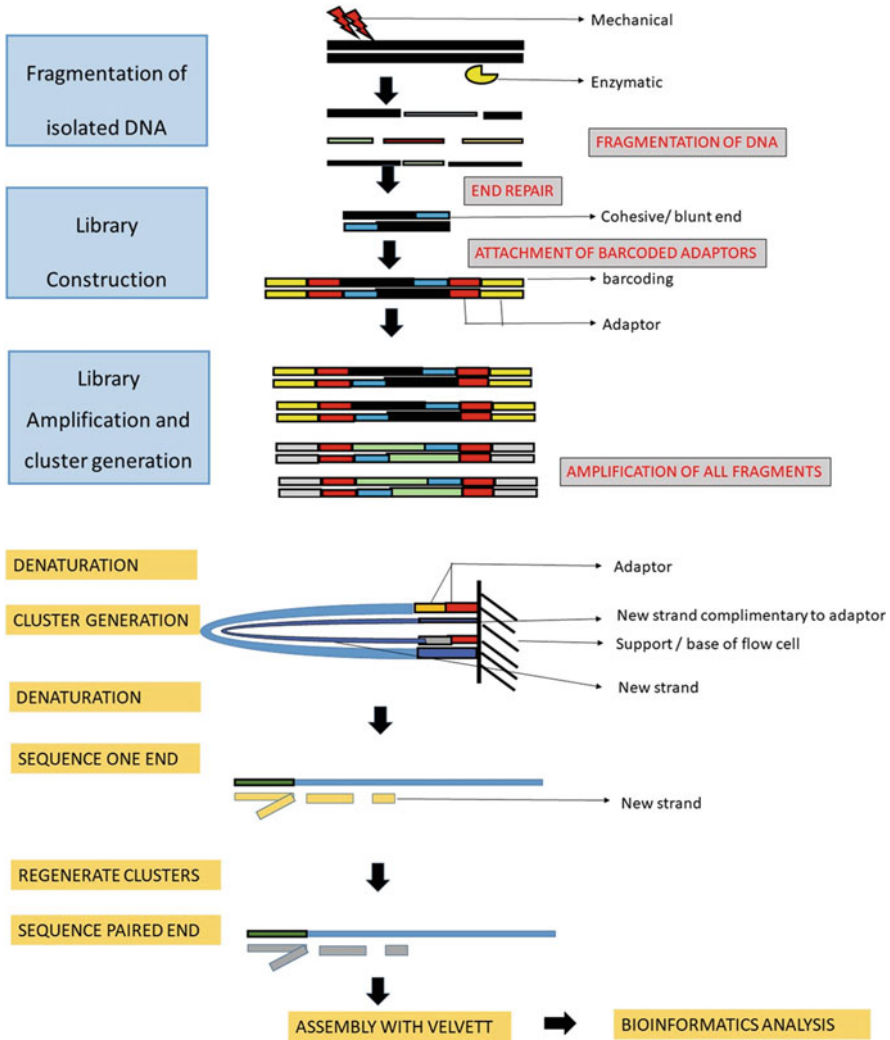


Fig. 4.2 Diagrammatic representation of steps showing paired end Illumina sequencing

biology-cum-nanomaterial-based system, Nanopore, was based on direct sequencing of RNA or single-stranded DNA by sensing the ionic current when the individual bases pass through a pore and get excised by an exonuclease, positioned near the pore. Long reads may be obtained and facilitate assembly and mapping of repeat regions for elucidating sequences of heterochromatin-rich regions of DNA (Margulies et al. 2005; Bennett 2004; Pandey et al. 2008; Steinmann et al. 2011; Rothberg et al. 2011; Eid et al. 2009; Stoddart et al. 2009).

4.3 Data Analysis

With the advent of the second-generation and third-generation sequencers, the journey from plate to paper (Fig. 4.3) has become relatively simple, less manual, time-saving and cost-effective. There are still several challenges related to sequences that are generated by the UHTS, especially related to sequence length, sequencing errors and repeat regions or SNPs. The techniques, e.g. 454 and Ion Torrent, are prone to unrealistic insertions or deletions while reading long homopolymeric stretches. Solexa results show substitution errors or errors during first PCR amplification step that results in biased sequence representation. The results are therefore low-coverage sequences in some sequences. To analyse and assemble such data, robust software has to be developed, and these are evolving each day to minimize error rates and yield data to 99.9% accuracy (Fig. 4.4).

Sequence of human genomes is possible using the current generation of sequencers, and for clinical practices in a diagnostic lab, the batch sizes of several samples have to be reduced and maintained at a minimum. The sample preparation process for WGS has been simplified, to reduce the time to just few hours with the aid of highly trained technical staff. However, the techniques chosen should yield reads that are sufficiently long for detecting sequence-linked mutations that lead to drug resistance or other phenotypes (Gega and Kozal 2011).

WGS has now become efficient in sequencing DNA extracted from even single colonies without or even DNA pre-amplification step that leads to errors and loss of time. After a single colony is picked up for anti-microbial resistance test, the remainder on the plate may be utilized for extracting DNA, followed by overnight sequencing. This will enable the results of both experiments, i.e. phenotypic resistance/susceptibility along with whole-genome typing may be available within 48h of sample collection or sample arrival. The platforms, namely, 454, Ion Torrent and Illumina MiSeq/HiSeq, have met some of the above targets, but research is still ongoing in this area for developing fool-proof techniques. Kim et al. (2017) proposed a new poly-dimethylsulfoxide (PDMS) microfluidic circuit technique

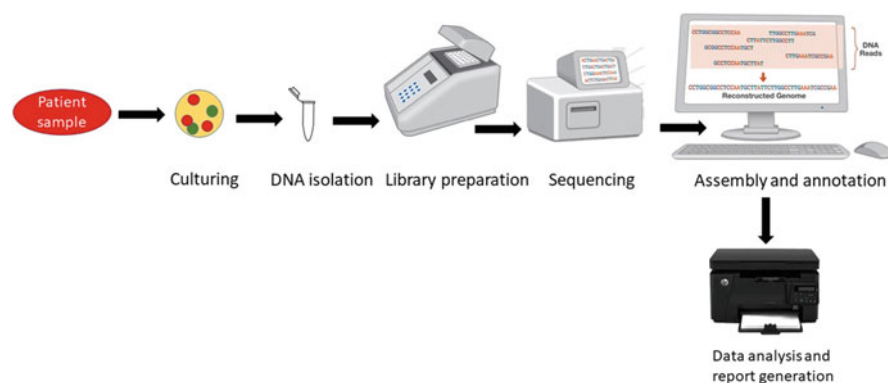


Fig. 4.3 Showing sample preparation and analysis (from plate to paper)

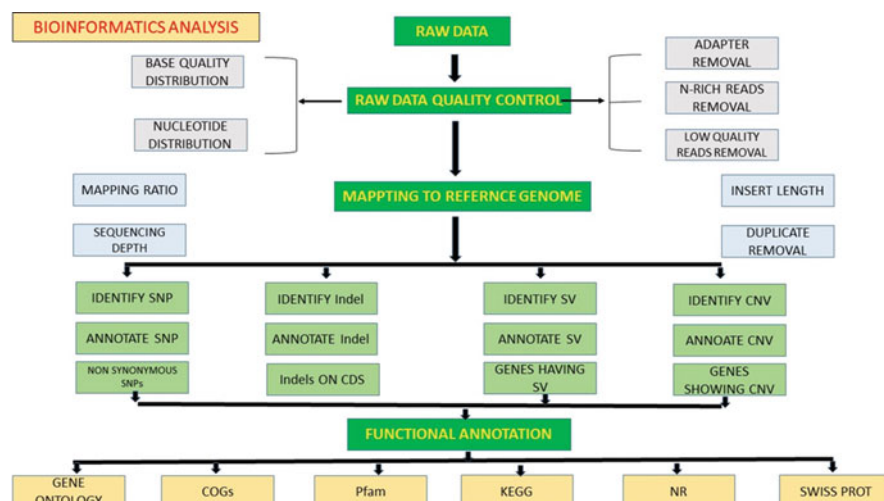


Fig. 4.4 Showing bioinformatics analyses of the WGS sequences. *SV* structural variations, *CNV* copy number variations, *CDS* core data services, *COGs* clusters of orthologous groups of proteins, *SNP* single-nucleotide polymorphism, *Indel* insertion and deletion, *Pfam* protein families database, *KEGG* Kyoto encyclopedia of genes and genomes, *NR* non-redundant

integrating all major steps in a sample preparation, thereby making significant advances in the type and amount of input material needed and efficacy of result or dataset generation while not compromising on data quality. A new micro-architecture was innovated to prepare DNA samples from clinical isolates and environmental isolates. Further evaluation is needed to assess their utility for routine clinical practice as opposed to their roles as tools for research (Loman et al. 2012). With regard to the ease of sample preparation, Oxford Nanopore promises to be very interesting for pathogen WGS (Eisenstein 2012).

Protocol for WGS (adapted from Gautam et al. 2019)

1. Extraction of bacterial genomic DNA
2. Quantification of extracted DNA and quality check
3. Tagmentation and PCR amplification of whole genomic DNA
4. Cleaning the PCR product
5. Library normalization
6. Preparation of pooled normalized library for MiSeq
7. Bioinformatical analysis

The steps mentioned should be performed using kits specified by the company that provides sequencing facility

WGS has resulted in opening a wide range of diagnostic tools that may be offered by using only one machine, and it has the capability to replace multiple numbers of different methods, for diagnosing pathogens and diseases, allowing economical patient care. This is noteworthy that analysing any genome using WGS is a single experiment, but any error may lead to several assays going wrong. WGS analyses the entire HIV genome in a single step, as opposed to seven runs using Sanger sequencing, thereby eliminating complications due to errors in partial sequences. This may apply to other typing tools, e.g. MIRU-VNTR typing done for *Mycobacterium tuberculosis* complex (Köser et al. 2012).

Regional sequencing hubs are needed to be developed in several centres across the world having a strict quality control process associated with all protocols and should have technical expertise needed for handling WGS. There should also be universal application of methods and analysis tools, but they also should be open for operating on alternative models and decentralization with respect to changes in the technology.

Description of steps for WGS

1. DNA extraction and quality assessment: During extraction of DNA, all care should be taken such that no foreign isolates or reagents like EDTA get into the extracted DNA. Any kit may be used for this, and a lab having established isolation protocols may be used too. DNA quantity and purity should be determined using fluorometric and spectrophotometric methods. DNA may also be extracted using fully automated robotic workstations, e.g. MagNA Pure96.
2. Kits recommended by the sequencing platform should be used, for example, Nextera DNA Flex and XT Library using the associated guide. The protocols may also be found at the website (www.illumina.com). The Illumina software also offers quality check parameters for every sequencing run which are explained by their software guide, and they also offer free guidance and training for users. The protocols can be also found at <https://www.cdc.gov/pulsenet/pathogens/wgs.html>.
3. Quality control (QC) guarantees accuracy of sequencing results, where the raw reads of poor quality lead to errors in prediction of AMR-related mutations/genes. Errors might also come from contamination of DNA due to error-prone data handling. The important parameters for QC are the number of reads generated, average read length, size of assembly, coverage and depth, number of contigs and shortest contig length needed for covering 50% genome (N50).
4. Bioinformatics analyses related to pathogenesis:
 - a. *Gene prediction for AMR-related genes/mutations*: A number of web-based tools, e.g. ResFinder, may be used for this purpose.
 - b. *Analysis and sub-type determination*: Bioinformatics analysis, e.g. determination of species, using pre-validated pipelines or through the CGE database (Center for Genomic Epidemiology). Various parameters like MLST (multi-locus sequence type), cgMLST (core genome multi-locus sequence typing), virulence factor identification, serotype determination and

phylogenetic predictions of SNP (single nucleotide polymorphism) may be useful for clinical isolates, especially pathogens.

It has been seen that SNP thresholds should not be determined for understanding the source of microbial origin. The populations of bacteria or other microbes are subject to evolutionary factors that can alter genomes. For these reasons SNP counts should be combined with a phylogenetic analyses and bootstrapping for correct analyses (Pightling et al. 2018). It is important to note that different populations of bacteria contain different levels of genetic diversity that investigators must consider when interpreting WGS results. Evolution due to natural selection, genetic drift, population bottlenecks, etc. results in SNPs driving this diversity. Also, isolation and culture conditions (such as multiple passages) can result in the accumulation of small numbers of additional SNPs (Allard et al. 2012).

Generally, the WGS datasets from any population, e.g. clinical, environmental, etc., may be first submitted to NCBI's Sequence Read Archive under the BioProject, and the automated pipeline aggregates these isolates into clusters based on mutations or differences in the gene sequences. These are visible via construction of phylogenetic trees.

The well-known application of WGS is GenomeTrakr network (GT) which has been developed by international collaboration of government and academic laboratories including FDA (US Food and Drug Administration), US National Center for Biotechnology Information (NCBI), USDA (US Department of Agriculture), Centres for Disease Control and Prevention, state health departments and international bodies. The bacterial species, e.g. *Listeria monocytogenes*, *Escherichia coli* and *Salmonella enterica*, are important for food and clinical microbiology labs, and study of such genomes leads to the identification of potential causes of illness. The data is publicly available at NCBI in BioProjects associated to GT.

NCBI has several tools to explore and analyse sequence information, and they are the preliminary analytical tools to support WGS studies.

First using GT database, the genome sequences are compared without editing or removing any taxa. This helps in evaluating all relationships that exist between a query sequence and the closely related ones existing in database. The Genomic Epidemiology Ontology project also necessitates global standardization of contextual information (metadata) associated with WGS data.

Presence of a few SNPs indicates that the isolates have a close relationship phylogenetically, thereby increasing the chances of isolates arising from a single source. However, presence of many SNPs (100 or above) indicates isolates did not originate from identical population and are distantly related (Pightling et al. 2018). Therefore, despite genome sequences being nearly identical, the populations are deemed distantly related. Such approaches may lead to problems in the interpretation of WGS results.

For analysing paraphyletic relation among isolates, WGS data of entire GT for a particular genome, e.g. *Salmonella*, may be compared to query isolates from

environmental or clinical or food samples. Epidemiological or traceback evidence is required to prove such relationships predicted using phylogenetic analyses.

It is also possible that isolates that arose from the same source by WGS analyses are not linked by a common population (Pightling et al. 2018). The tree topologies show that the environmental and clinical isolates form distinct sub-groups, instead of being interspersed, suggesting that there is no link. Therefore, traceback evidence is very important as proof, where SNP data analysis fails to give correct prediction.

There are a number of other limitations that affect WGS analyses, and presumption should not be the basis of any outcome. The analysis is totally dependent on genome sequence databases which may be biased or limited to a category of people that sought the help of clinicians for their sickness, who in turn suggested genome sequencing. The control group of normal or healthy individuals are not screened for the isolates, which may have been present without causing disease. Therefore, the GT database lacks a complete pool of genetically diverse sequences of the same organism in question.

Diagnosis can be designed for germline disorders using WGS, but that too has challenges. Clinical tests on WGS analysis depend on two factors, i.e. reporting the variant types and also the loci on the genome sequences that should be interrogated, and this sequence depends on the type of variant to be detected. However, there is a confusion whether the tests should be based only on the classes of variants or depend on the specific phenotype alone. In this case it is advisable to evaluate clinical presentation showing wide ranges of genetic etiology, with respect to those which are associated with fewer sets of genetic events. However, with the advancement of protocols and tests, WGS analyses certainly hold a promise for easy and rapid detection of disorders (Marshall et al. 2020).

4.4 Applications

1. The recent outbreak of the virulent *E. coli* O104:H4 and SARS-CoV2 is an excellent illustration of the speed of data acquisition and major outcomes that can be obtained by WGS.
2. WGS is an ultimate typing methodology used for epidemiology, instead of older techniques, e.g. restriction fragment length polymorphism (RFLP), pulsed-field gel electrophoresis (PFGE) or multilocus sequence typing (MLST) to type bacterial strains. It helps in the identification of single nucleotide genomic changes between two isolates. Due to the onset of cost-effective high-throughput sequencing tools, routine epidemic-based investigations utilise WGS. In 2001, anthrax letter attack in the USA led to the first attempt for sequencing any whole genome of a pathogenic organism that could lead to an epidemic. The complete genomic sequence was obtained by Sanger sequencing for this anthrax-causing agent, *Bacillus anthracis*. It was then compared to other genotypes, and it was clearly determined that the variants sent in letters were not environment strains.

3. The spread of the leprosy agent, *Mycobacterium leprae*, due to migration of man and trading was investigated by using Sanger and Illumina sequencing.
4. The resistome of bacterial strains *A. baumannii* and *Burkholderia dolosa* was investigated through comparison of genome sequences.
5. Profiling microbial communities obtained from particular environments involved cloning of 16S rRNA and Sanger sequencing followed by phylogenetic analyses. Currently, because of the high coverage rate, high-throughput sequencing techniques have prevented the cloning requirements and are still being able to provide sensitive data to study rare DNA (Hugenholtz et al. 1998; Huse et al. 2008; Tringe and Hugenholtz 2008).
6. WGS was used to analyse the methicillin-resistant *Staphylococcus aureus* (MRSA) reported from Rosie Hospital. This study demonstrated the cost-effective WGS had application for controlling the spread of an antimicrobial infection.
7. Usually, drug resistance/susceptibility can be tested quicker than WGS. However, for cultures of *M. tuberculosis* complex (MTBC), the causative agent of tuberculosis, slow growth rate leads to extended time for complete susceptibility testing, which can result in few weeks. However, via WGS the variations in genome arising due to point mutations or small deletions/insertions lead to drug resistance and make it ideal for genotypic tests.
8. Detailed analysis of the microbiome in human, for understanding diversity/microbial load of healthy and unhealthy people, may now become an important area for routine patient management.
9. Several of the greatly correlated strains of *Salmonella montevideo* often lead to faulty investigations during epidemics, because PFGE (pulsed-field gel electrophoresis) is insensitive to distinguish the strain involved in an outbreak from other genetically related strains but are not associated with the same epidemic (Allard et al. 2012). These unrelated strains often retain a common PFGE pattern despite their origin or sporadic nature. In such a case, accuracy is needed while subtyping and clustering isolates that resulted from any food that caused infection or an outbreak. It is also important to get this data for traceback to a specific source of food or drink or the environment. NGS contributes to solutions in such cases for proper genetic analysis of pathogens because it gives high resolution to distinguish between isolates that have undergone micro-evolutionary genetic changes. Such changes are often seen in food-related outbreaks, and these need to be differentiated from similar environmental isolates or even clinical isolates. Thus NGS is now a tool for molecular epidemiological studies that can validate ancestry and help in traceback of very similar isolates that are difficult to distinguish otherwise.

4.5 Challenges

1. A fool-proof data analysis pipeline for the rapidly transforming genome databases into understandable and decipherable data for clinically significant knowledge is still a challenge. This information requires adept workforce and but an automatized process to allow computational analysis of genomes. Primarily, global or national servers may be preferred for storing information of pathogens, their resistance profiling and other symptomatic/asymptomatic details. This may be developed for bioterrorism causing pathogens especially. Therefore, this issue poses a challenge for creating world-wide resources, because they would be capable of providing analyses of millions of genomes that are sequenced annually. The associated meta-data may also be openly provided to the entire scientist community for better analyses or understanding the implications. An effort of this dimension should be upheld by governments and reinforced collaboration between biologists, clinicians, scientists, industry and bioinformaticians, such that barriers are surpassed. Vital information may be shared amongst nations on real-time basis, and the scientific community should not hide it from the public in view of its relevance and importance to the people. Also, harmony in lab procedures used for data analysis would facilitate methodical comparison and unlimited exchange of analytical insights or results that are obtained from various laboratories.
2. Isolation of microbial strains may be laborious at times and also labour intensive, especially in the case of unculturable strains and also intra-cellular strains or slow-growers. Automation and a development of various chromogenic selective or differential culture media may facilitate strain isolation in such cases.
3. Culturing approaches are of insignificant value if samples are taken from any patient who went through antibiotics treatment, because of the loss in the actual diversity of bacterial strains in the sampling. Also many unculturable bacteria are present that we cannot decipher through culturing.

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Spectroscopy: Principle, Types and Microbiological Applications

5

5.1 Introduction

Spectroscopy is a study of the absorption and emission of light and other radiation by substances related to the wavelength dependence of electromagnetic radiation. More recently, the definition has been extended to include the study of interactions between particles such as electrons, protons and ions and interactions with other particles as a function of impact energy. Spectroscopy refers to the decomposition of light from an object into its constituent colours (i.e. energy). Astronomers can infer the physical properties of an object (temperature, mass, luminosity, composition, etc.) by decomposing and analysing the light of the object. This can be used to determine the structure and functional groups of organic compounds.

Spectroscopy is the analysis of the interaction between a substance and any part of the electromagnetic spectrum. Traditionally, spectroscopy has included the visible spectrum, but X-ray, gamma ray and UV spectroscopy are also valuable analytical techniques. Spectroscopy can involve any interaction between light and matter, including absorption, luminescence and scattering. The data obtained from spectroscopy is usually represented as a spectrum, which is a plot of measurement coefficients as a function of frequency or represents the wavelength. Emission and absorption spectra are common examples. This is a technique for measuring the interaction between molecules and electromagnetic radiation. The energy of light is used to move electrons from the ground state to the excited state. Measuring the absorption of light as a function of its frequency or wavelength gives a spectrum. Molecules with delocalized aromatic electrons often absorb light in the near-ultraviolet range (150–400 nm) or visible range (400–800 nm). Absorption spectroscopy is usually performed on molecules dissolved in a clear solvent such as aqueous buffer. Absorption spectroscopy is ideal for quantitative measurements because the absorption of solutes is linearly dependent on their concentration. The absorption wavelength and intensity of a molecule depend not only on its chemical properties but also on the molecular environment of its chromophore. Therefore,

absorption spectroscopy is a great way to track ligand binding reactions, enzyme catalysis and protein-nucleic acid conformational changes. Spectroscopic measurements are extremely sensitive and non-destructive and require a small amount of material for analysis.

5.2 General Types of Spectra

Continuous and discrete spectra are the two types of spectra. The light in a continuous spectrum is made up of a wide, consistent range of colours (energies). Only magnificent or dim lines at very distinct and clearly described colours are visible with discrete spectra (energies). Emission spectra are discrete spectra with bright lines, while absorption spectra are discrete spectra with dark lines.

5.2.1 Continuous Spectra

Thick gases or solid objects that radiate their heat away by creating light provide continuous spectra. These solid objects emit light over a wide range of wavelengths, giving the impression of a smooth and continuous range.

5.2.2 Discrete Spectra

Discrete spectra are the discernible after-effect of the physics of atoms. There are two sorts of discrete spectra:

1. Emission spectra (bright line spectra)
2. Absorption spectra (dark line spectra).

5.2.2.1 Emission Line Spectra

Unlike a continuous spectrum source, which can have any energy it requires, the electron mists that surround particle nuclei have definite energies that are controlled by quantum mechanics. With few exceptions, every element on the periodic table has its own set of potential energy levels, which are distinct and distinguishable. Atoms will also tend to settle at the lowest energy level possible (known as the ground state). This means that an excited atom in a higher energy level will have to “dump” some energy. A particle’s vitality is “dumped” by it generating a wave of light with the appropriate energy. A hydrogen atom drops from the second to the first energy level in the diagram below, emitting a wave of light with energy equal to the difference in energy between levels 2 and 1. We observe a brilliant line at that exact wavelength because this energy correlates to a particular colour or wavelength of the light (Fig. 5.1).

Photons with modest energies and long wavelengths, such as radio waves, are created by slight changes in energy in an atom (Fig. 5.2). Large energy changes in an

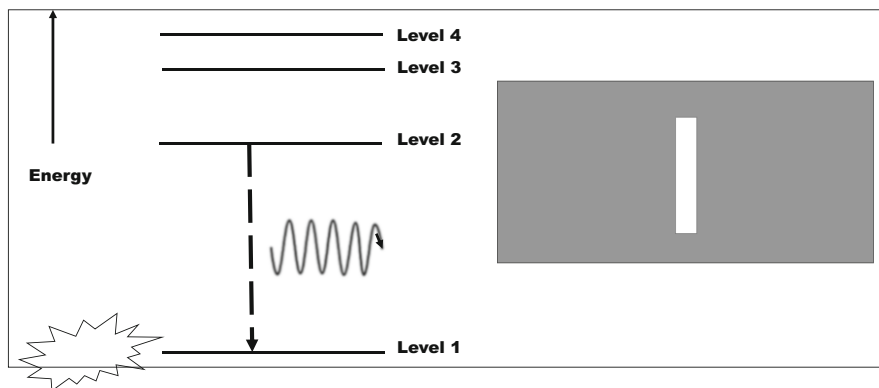


Fig. 5.1 An excited hydrogen atom relaxes from level 2 to level 1, yielding a photon, energy corresponding to a specific wavelength and colour results in a bright emission line

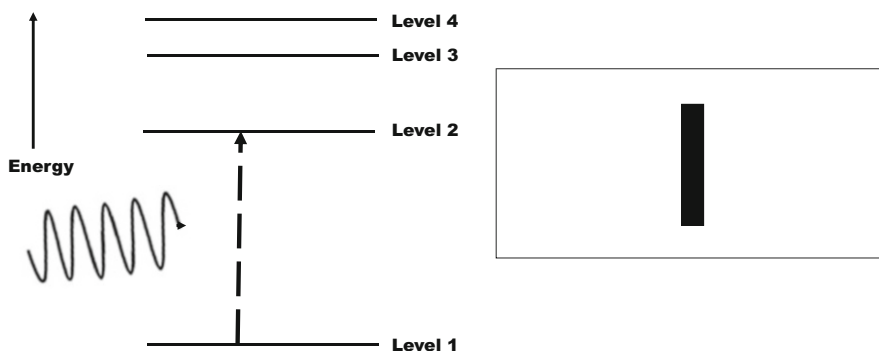


Fig. 5.2 Different energy levels and photon absorption by an atom cause transition of electrons from ground state to the excited state as illustrated in this diagram from level 1 to higher level (level 2)

atom result in the emission of high-energy, short-wavelength photons (UV, X-rays and gamma rays).

5.2.2.2 Absorption Line Spectra

What would happen, on the other side, if we sought to reverse this process? With that example, what would happen if we returned this unique photon to a ground-state-atom? The atom may absorb that “specially energetic” photon and become stimulated, causing it to move from the ground state to a higher energy level. If a star with a “continuous” spectrum shines on an atom, the wavelengths corresponding to conceivable energy changes within that atom will be absorbed and hence not visible to an observer. As a result, a dark line absorption spectrum, as illustrated below, is formed:

5.3 Principle of Spectroscopy

Spectroscopy is a broad scientific approach, with approaches differing depending on the species studied (atomic or molecular spectroscopy), the region of the electromagnetic spectrum studied and the sort of radiation-matter interaction seen (such as emission, absorption or diffraction). Nonetheless, the underlying premise shared by all approaches is the application of an electromagnetic radiation beam to a targeted sample in order to see how it responds to such a stimulus. A spectrum is often represented as a plot of such reactions as a function of light wavelength. A spectrum may be produced by any energy of light (from low-energy radio waves to high-energy gamma rays). The general goals of spectroscopy are to understand how light interacts with matter and how that information can be used to quantitatively explore specific samples; however, spectroscopy should also be viewed as a set of tools that can be used in conjunction to understand the different devices and solve complex chemical problems.

5.4 Optical Instruments in Spectroscopy

A spectroscopic study can be performed using a variety of instruments, but even the most basic ones require an energy source (usually a laser, but a radiation or ion source can be used) and a device to measure the change in the energy source after interaction with the material. The light normally travels from the entrance slit via the lens to the prism, where it is dispersed. The radiation emerging from the exit slit is perceived by the eyes as a spectral line that is an image of the entry slit. Finally, the size of the prism determines the resolution, which is proportional to the length of the prism's base. The instrument is referred to as a spectrograph when the exit slit is substituted by a photographic plate detector. Other types of detectors, known as spectrometers or spectrophotometers, are more useful and are usually specific electrical devices that record the strength of radiation falling on it as a function of wavelength.

In spectroscopic techniques, the operational region of the source is traditionally utilized to name the technique. If a UV source is utilised, for example, the technique is known as ultraviolet spectroscopy. Other techniques, such as infrared, fluorescence and atomic spectroscopy, are named using the same approach. Photons interact with a sample when a beam of electromagnetic energy passes past it. They can be absorbed, reflected or refracted, amongst other things. The electrons and chemical bonds in a sample are affected by absorbed radiation. In some circumstances, absorbed radiation causes lower-energy photons to be emitted. Spectroscopy investigates how incoming radiation affects a sample. The emitted and absorbed spectra can be utilized to learn more about a substance. There are many distinct forms of spectroscopy since the interaction relies on the wavelength of light.

5.5 Is Spectroscopy Different from Spectrometry?

In practice, the terms “spectroscopy” and “spectrometry” are used interchangeably (with the exception of mass spectrometry); however they do not signify the same thing. The term spectroscopy is derived from the Latin word *specere*, which means “to gaze at”, and the Greek word *skopia*, which means “to see”. The term spectrometry is derived from the Greek word *metria*, which means “to measure”. Spectroscopy is the non-destructive study of the electromagnetic radiation produced by a system or the interaction between the system and light. The measuring of electromagnetic radiation in order to gain information about a system is known as spectrometry.

In other terms, spectrometry is a technique for examining spectra. Mass spectrometry, Rutherford scattering spectrometry, ion mobility spectrometry and neutron triple axis spectrometry are all examples of spectrometry. Spectrometry produces spectra that aren't always intensity versus frequency or wavelength. A mass spectrometry spectrum, for example, shows intensity versus particle mass. Another frequent word is spectrography, which pertains to experimental spectroscopic procedures. Both spectroscopy and spectrograph are concerned with the strength of radiation versus wavelength and intensity. Spectrometers, spectrophotometers, spectral analysers and spectrographs are examples of spectrum-measuring instruments.

5.6 Uses of Spectroscopy

The nature of substances in a sample can be determined via spectroscopy. It is used to analyse the purity of goods and to monitor the development of chemical operations. It may also be used to determine the impact of electromagnetic radiation on a sample. This can be used to determine the intensity or length of exposure to the radiation source in various instances.

5.7 Types of Spectroscopy

5.7.1 Ultraviolet and Visible Spectroscopy

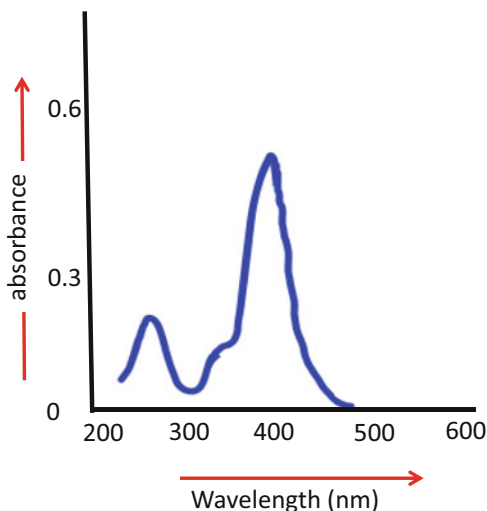
5.7.1.1 Background

The colour of various compounds is an obvious distinction between them. As a result, quinone is yellow, chlorophyll is green, the 2,4-dinitrophenylhydrazone derivatives of aldehydes and ketones range in colour from brilliant yellow to deep red, and aspirin is colourless. In this situation, the human eye acts as a spectrometer, evaluating light reflected off a stable's floor or travelling through a liquid. Although humans perceive sunshine (or white light) to be uniform or homogenous in colour, it is made up of a wide variety of radiation wavelengths from the ultraviolet (UV), visible and infrared (IR) portions of the spectrum.



Fig. 5.3 Colours of visible light ranges from 400 to 800 nm with variable wavelength are separated by passing sunlight through prism, which bends light in different ranges consistent with wavelength

Fig. 5.4 A typical graph obtained in UV-visible spectrophotometric studies, plotted between absorbance and wavelength of UV and visible region



The visible element's aspect colours may be separated by passing sunlight through a prism, which bends the light in distinct ranges corresponding with the wavelength. Electromagnetic radiation, including visible light, is often treated as a wave phenomenon, with a wavelength or frequency used to define it. The distance between neighbouring peaks (or troughs) is referred to as the wavelength, and it can be measured in metres, centimetres, or nanometres (10^{-9} m). Frequency is the number of wave cycles that move past a certain location in time and is generally expressed in cycles per second or hertz (Hz). The visible wavelength ranges from 400 to 800 nm. Red has the longest visible wavelength, while violet has the shortest (Fig. 5.3). Figure 5.4 depicts a typical graph generated in UV-visible experiments.

These approaches are perhaps the most extensively employed for analytic work in these areas of the electromagnetic spectrum. The chemical substructures that interact with electromagnetic radiation are referred to as chromophores. Peptide linkages, specific amino acid side chains (mainly tryptophan and tyrosine) and particular prosthetic groups and coenzymes are the sorts of proteins that are significant in UV-vis spectroscopy (e.g. porphyrin groups present in haem). Colorimetric tests necessitate the plotting of a calibration curve (concentration against absorbance), which should be linear as long as the Beer-Lambert law is followed.

Using this, the absorbance of unknowns is measured, and their concentration may be estimated from the plot's linear section. Qualitative analysis may be used to identify certain classes of substances in pure samples as well as biological mixtures. This sort of spectroscopy is most typically employed for directly or indirectly quantifying biological substances using colorimetric tests. Proteins may be measured directly in many circumstances by employing their inherent chromophores, tyrosine and tryptophan. Scanning from 500 to 210 nm is used to get protein spectra. The area between 500 and 300 nm contains useful information on the existence of any prosthetic groups or coenzymes.

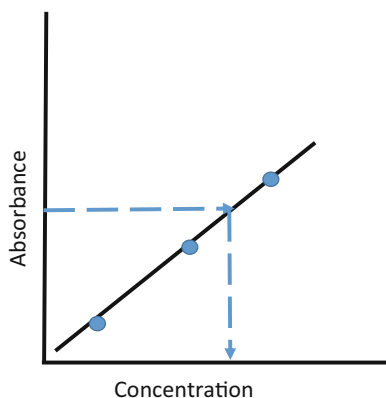
5.7.1.2 Principle

The Beer-Lambert law states that the concentration of a substance in solution is directly proportional to the “absorbance”, A , of the solution (Fig. 5.5).

$$\text{Absorbance } A = \text{constant} \times \text{concentration} \times \text{cell length}$$

The law only applies to monochromatic light, or light with a single wavelength or limited band of wavelengths, and only if the physical or chemical state of the material does not vary with concentration. When monochromatic light flows through a homogeneous solution in a cell, the intensity of the emitted radiation is determined by the solution's depth and concentration.

Fig. 5.5 Graphical representation of Beer-Lambert law stating the direct relation between absorbance and concentration of the substance in the solution, the greater the absorbance, the greater the concentration of the substance in the solution



5.7.1.3 Applications of UV-Vis Spectroscopy

Spectroscopy is a scientific measuring technique used to analyse matter by observing its interactions with various components of the electromagnetic spectrum. It can measure light by splitting it down into its component colours using a prism and studying the resulting spectrum. Later, the notion was broadened to cover any possible interaction with radiative energy as a function of frequency or wavelength. The consequence of such an encounter allows researchers to derive analytical information about the matter's atomic or molecular structure. A wide range of spectroscopic methods may be used in almost every field of scientific inquiry, from environmental studies and biological sciences to space exploration.

5.7.1.3.1 Spectroscopy in Environmental Analysis

Environmental scientists have utilized visible and ultraviolet spectroscopic tools for many years. Colorimetric assays that explore various water parameters (such as acidity) are now accessible in easy kit form, utilizing visual colour matching or portable colorimeters. Metals in water or solid samples can be determined using emission spectroscopy or atomic absorption in the visible and ultraviolet ranges. These methods need immersing the analyte in solution before proceeding with the analysis. Certain solid or semisolid materials, however, can be examined directly using atomic absorption spectrometry (by using electrothermal atomization). The emergence of long-range infrared sensors that can determine the concentration of particular substances in the air has made infrared spectroscopy a crucial addition to the environmental analysts' arsenal. Ultraviolet long-path approaches are also becoming more popular, albeit not as frequently as infrared spectroscopy. X-ray techniques (such as X-ray fluorescence) have been used to assess the atomic composition of solid materials, and they have also been used to determine metal concentrations in airborne particulate matter and soil samples. In some environments, microwave region spectroscopy and magnetic resonance spectroscopy have been used.

5.7.1.3.2 UV-Vis Spectroscopy for Water Analysis and Environmental Applications

Water analysis is the most essential topic of discussion now even in our environmentally conscious society. Water pollution is, in fact, of critical importance to environmental safety. UV-vis spectroscopy is not only a low-cost method of water analysis, but it also offers a wide range of applications in the field of environmental technology. When compared to other spectrophotometric methods, UV-vis spectroscopy is more versatile and flexible than many scientists previously imagined.

5.7.1.3.3 Spectrophotometric Analysis of Bacterial Water Contaminants

Bacteria are one of the most serious concerns when it comes to water contamination. Standard bacterial checking out is effective, but it is time-consuming, taking about 2 days to complete an examination. However, recent UV-vis spectroscopy developments already use light absorption measurements to accurately determine the concentration level of bacteria in water samples. This method is fast, portable and

cost-effective, making it the preferred analytical instruments for determining water safety.

The Environmental Protection Agency (EPA) is constantly on the lookout for innovative technologies to monitor pollutants in drinking water. Despite the fact that it was previously disregarded, new developments in UV-vis technology now provide more information and data regarding water quality and safety. Spectrophotometers that measure light in the UV and near-infrared ranges are built for mobility and durability, making them ideal for environmental research in this area.

5.7.1.3.4 Spectrophotometers for Chlorine and Flouride Quantification

Those that routinely store water add chlorine for disinfection purposes. Because this procedure leaves a tiny amount of residual chlorine in the water, it should be closely monitored to ensure that it is safe for human consumption. There are two main types of residual chlorine: “free chlorine” and “combine chlorine” residuals. UV-vis spectroscopy is a useful tool for distinguishing between the two. This is significant because free residual chlorine is commonly thought to become a more effective disinfectant and may be utilized more effectively while being detected in drinking water.

Flouride is another prevalent ingredient to our drinking water. UV-vis spectroscopy may also be used to measure amounts of this element for both protection and efficacy, and it’s a simple, non-adverse, chemical-free and effective way to detect it. Environmental professionals can be alerted to excessive quantities of these compounds in our drinking water if precise quantification and data are available.

5.7.1.3.5 UV-Vis Spectroscopy for Geological Studies Linked to Water Contamination

UV-vis spectroscopy is being used by geologists in the field of environmental study. A distinctive reflectance signature of minerals and chemicals found in nature may be created using spectral statistics. This data may then be used to map those geological components and offer estimates of possible contamination patterns in rivers, lakes, streams and oceans. UV-vis spectroscopy is used by geologists to examine the environmental impact of mining on our natural water supplies. The use of UV-vis spectroscopy may identify trace amounts of infection, and harmful acid-producing mine drainage can be discovered instantly, allowing for rapid implementation.

5.7.1.3.6 Other Applications

Spectroscopy is also used in astronomy to collect information on a particular celestial object’s composition, density, temperature and other major physical processes. Scientists may utilize spectroscopy to measure the relative velocities of supernovae and galaxies by detecting red shift (recession speed). Raman spectroscopy may reveal a particular analyte’s vibrational spectrum (often known as its “fingerprint”), allowing for easy identification and interpretation. It might be used in everything from archaeology to current nanotechnology.

5.7.2 Infrared Spectroscopy

5.7.2.1 Introduction

Infrared spectroscopy is the study of how infrared light interacts with molecules. The intensity of radiation absorbed, emitted or reflected can be used to do the analysis. Transitions between vibrational and rotational energy levels manifest in the form of bands, also known as IR spectral bands, in the infrared absorption spectra. The absorption plotted against wave number displays descending peaks in the spectrum (ν). From $12,500\text{ cm}^{-1}$ to 100 cm^{-1} , the IR range is between the visible and microwave realms. It is separated into three regions from the standpoint of application: near-infrared (overtone), mid-infrared (normal vibrational transitions) and far-infrared (normal vibrations of weak and heavy atom bonds) regions (Table 5.1). IR spectroscopy can provide information about a molecule's functional group, composition and interactions with other compounds (Sharma 2008).

Energy is absorbed by the molecule when the frequency of IR radiation matches the natural vibrational frequency of the molecules. The absorbed energy raises the amplitude of the bonds' vibrational motion. Every link needs a specific quantity of energy to vibrate (Table 5.2).

Table 5.1 Different regions in IR radiation band along with wave number

S no	Spectral region	Wave number (ν)
1	Near-infrared region (NIR)	$12,500\text{--}4000\text{ cm}^{-1}$
2	Mid-infrared region (mid IR, MIR)	$4000\text{--}400\text{ cm}^{-1}$
3	Far-infrared region (FIR)	$400\text{--}10\text{ cm}^{-1}$

Table 5.2 Important IR stretching frequencies

Type of bond	Wave number (cm^{-1})
$\text{C}\equiv\text{N}$	2260–2220
$\text{C}\equiv\text{C}$	2260–2100
$\text{C}=\text{C}$	1680–1600
$\text{C}=\text{N}$	1650–1550
C_6H_6	~ 1600 and $\sim 1500\text{--}1430$
$\text{C}=\text{O}$	1780–1650
$\text{C}-\text{O}$	1250–1050
$\text{C}-\text{N}$	1230–1020
$\text{O}-\text{H}$ (alcohol)	3650–3200
$\text{O}-\text{H}$ (carboxylic acid)	3300–2500
$\text{N}-\text{H}$	3500–3300
$\text{C}-\text{H}$	3300–2700

5.7.2.1.1 Molecular Vibrations and Vibrational Frequency

5.7.2.1.1.1 Vibration of Diatomic Molecules

The vibration of diatomic molecules causes the compression and extension of the covalent bonds. The vibrating diatomic molecule can be considered as a simple harmonic oscillator whose oscillation frequency can be given as

$$\omega_{\text{osc}} = 1/2\pi\sqrt{(k/\mu)}$$

where μ is the reduced mass given as $m_x \cdot m_y / (m_x + m_y)$ where m_x and m_y are the mass of atom x and y and K is the force constant. The value of K varies from one bond to another. Like the force constant for triple bonds, it is greater than that of single bonds. Stronger bonds have larger force constant and vibrate at higher frequencies. Bonds between heavier atoms vibrate at lower frequency than those between lighter atoms. Hybridization affects force constant, $sp > sp^2 > sp^3$. Resonance also affects the strength and length of the bond and thus the force constant.

When we convert this to wave number, $\nu = 1/2\pi c\sqrt{(k/\mu)} \text{ cm}^{-1}$.

The vibrational energy for a simple harmonic oscillator can be calculated as.

$$E\nu = [\nu + 1/2] h\omega_{\text{osc}} \quad (\nu = 0, 1, 2 \dots)$$

ν is known as vibrational quantum number.

5.7.2.1.1.2 Vibrational Transitions

There are various types of vibrational transitions observed in a molecule in the presence of IR radiations:

1. Fundamental (normal): The vibrational quantum number changes by 1 ($\Delta\nu = 1$). The probability of this change is high and thus has a high value of ϵ .
2. Overtones: The change in the vibrational quantum number is greater than 1 ($\Delta\nu = 2, 3 \dots$). The probability of their occurrence is lower and thus has lower ϵ value.
3. Combination: A simultaneous change in two or more vibrational quantum number is observed (example in a polyatomic molecule).
4. Difference bands: When two interacting bands diverge, a new IR active frequency emerges.

Fermi resonance is the linked resonance that occurs when a fundamental vibration couples with an overtone or combination band, for example, carbonyl compounds.

5.7.2.1.1.3 Types of Vibrations (Sharma 2007)

There are mainly two types of vibrations observed:

1. Stretching: The length of the chemical bond (inter-nuclear distance) changes. They are comprised of symmetric and asymmetric stretching vibration.

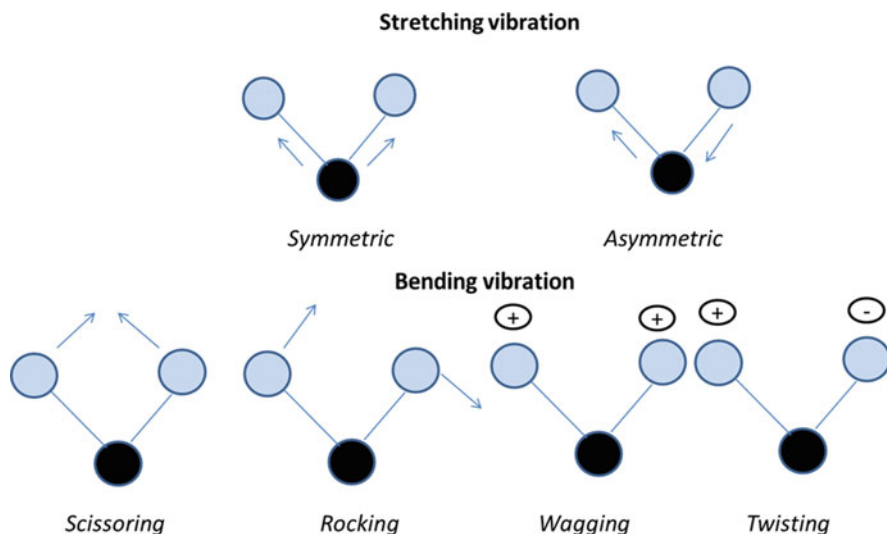


Fig. 5.6 Illustrated the stretching and bending vibrations of triatomic non-linear and multi-atom molecules, symmetric and asymmetric are the types of stretching vibrations and scissoring, rocking, wagging and twisting are the types of bending vibrations

2. **Bending:** On vibration the angle between the bonds changes. These vibrations maybe symmetric or anti-symmetric or vibrations in plane and out of plane.

The stretching and bending vibration in a triatomic non-linear molecule and a multi-atom molecule is as shown in Fig. 5.6.

Bending vibrations require less energy and occur at longer wavelength than stretching vibration.

Only certain molecules give an IR spectrum or are IR active. Molecules which contain polar bonds, i.e. molecules composed of atoms of different elements, are IR active, e.g. HCl, NO₂, H₂O, CO₂, etc. Pure elements in their molecular or crystal state are IR inactive, e.g. O₃, N₂, C, graphite, Si, etc.

The square of the change in dipole moment that happens during molecular vibration is proportional to the IR signal detected in spectra.

Based on their relative strengths in the IR spectrum, the IR bands can be classed as strong (*s*), medium (*m*) or weak (*w*). The size of the dipole moment associated with it determines the strength of the bands. Strongly polar bonds, such as C=O, create strong bands; medium polar bonds, also known as asymmetric bonds, produce medium bands; and weakly polar links, also known as symmetric bonds, produce weak or non-observable bands.

The bands that are formed might be small or wide. For alcohols and carboxylic acids, O-H produces a wide band, and the broadening of the band is generally caused due to rotational coupling.

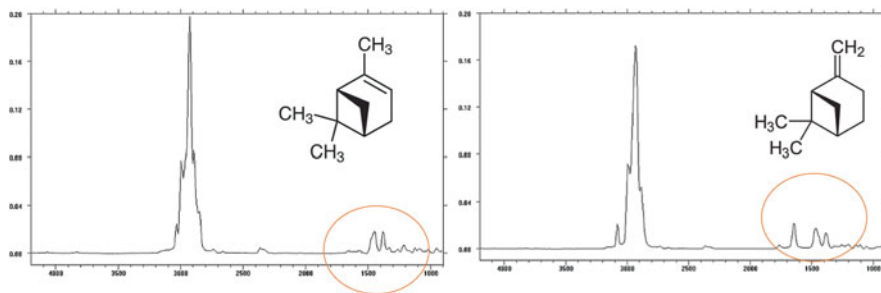


Fig. 5.7 Variation in spectra of two similar molecules in fingerprint region, containing bending vibrations which characterize the molecule

The MIR region comprises of bands of the fundamental vibrational frequency. The number of vibrations of a molecule having N atoms is $3N-6$ for non-linear molecules and $3N-5$ for linear molecules. If two or more vibrations have the same frequency, the absorption bands are stronger and are called as degenerate vibrations. Multiple polar bonds give strong signals like ($C=O$), while low polarity bonds give weak signals ($C-C$). Non-polar bonds give no signal at all.

MIR is divided into two sub-regions:

- The region with characteristic vibrations of functional groups in the range $4000-1250\text{ cm}^{-1}$ which generally corresponds to stretching vibration.
- The fingerprint region ($1250-400\text{ cm}^{-1}$) which contains bending vibrations; thus the spectrum in this region characterizes the molecule as a whole (Fig. 5.7).

5.7.2.2 Instrumentation

There are various types of instruments used for recording an IR spectrum like grating dispersive, filter dispersive and Fourier transform spectrometers. They may be simple instruments with a filter or monochromator or based on an interferometer. The main components of the instrument being used for IR studies are radiation source, measuring cell, wavelength selector and detector. The spectrophotometer maybe single beam or double beam (Fig. 5.8).

5.7.2.2.1 Source

The source used for near IR is a tungsten lamp, while for mid-IR region is an electrically heated silicon carbide rod (1100°C) called as globar or lasers. Nichrome wire, Nernst glower or Opperman can also be used. Nichrome wire is a simple coil which may or may not be supported on a ceramic rod. Nernst glower is a mixture of rare earth oxides heated by electric current. The Opperman is a ceramic rod filled with rare earth oxides with a metal wire located at the centre.

5.7.2.2.2 Sample Types and Preparation

The cells for measuring the transmission are generally made of $\text{NaCl/KBr/CaF}_2/\text{ZnSe/AgCl}$ (for MIR), CsBr/polyethylene (for FIR) and glass/quartz (for NIR) (Table 5.3).

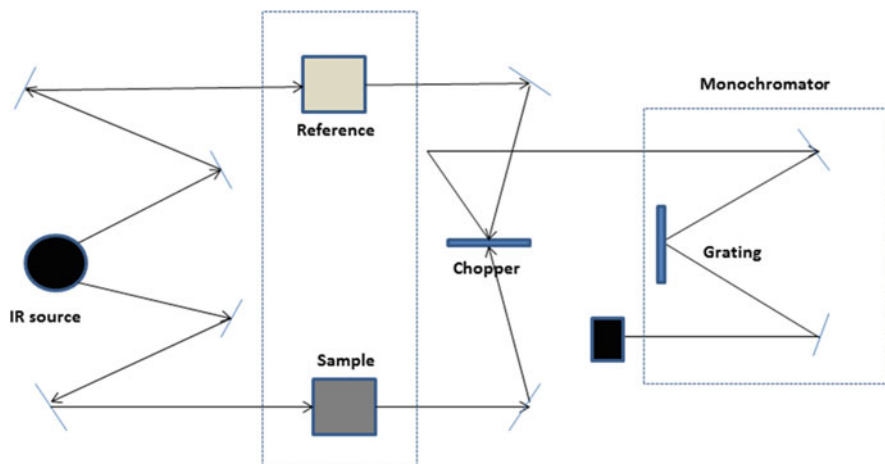


Fig. 5.8 Line diagram of IR spectrophotometer (double beam), consisting of IR source, reference, sample, chopper, detector, etc.

Table 5.3 Some IR-transmitting materials

Window material	Frequency
NaCl	40,000–625 cm^{-1}
KBr	40,000–400 cm^{-1}
AgCl	25,000–435 cm^{-1}
AgBr	20,000–286 cm^{-1}
CaF ₂	6670–1110 cm^{-1}
BaF ₂	50,000–870 cm^{-1}
CsBr	10,000–270 cm^{-1}
ZnSe	10,000–55 cm^{-1}
Polyethylene	625–33 cm^{-1}

If the sample is in the gaseous form, the cell is first evacuated and then filled with the sample. They are directly scanned in a 10-cm-path length cell. In the case of trace analysis, 1.5–120 m cells are used. For liquid samples solvents like CCl_4 , CHCl_3 and CS_2 can be used. The sample thickness is usually 0.001–0.05 mm which gives a transmittance of 15–70%. Solid samples are mixed with KBr and set in the form of pellets or dispersed in paraffin oil to form Nujol mull.

For polymers, resins and amorphous solids, the sample is dissolved in a solvent, and a drop of it is placed in a window and sealed. As the solvent evaporates, a thin film is left behind which can be scanned directly.

The detectors used are generally based on the change in temperature, influenced by the heat capacity of the detector element and independent of the wavelength of incident radiation or photosensitive detectors.

5.7.2.2.3 Various Types of Detectors Used

- (a) Pyroelectric triglycine sulphate detector which works at normal temperature.
- (b) Photoconductive detector (HgTe/CdTe) which works at a temperature of $-196\text{ }^{\circ}\text{C}$ (liquid nitrogen) and has high sensitivity and fast response. They are generally used in MIR or FIR.
- (c) Germanium bolometers work at the temperature of $-271.7\text{ }^{\circ}\text{C}$ (liquid helium) and are used in FIR.
- (d) Thermocouple contains two dissimilar metals like bismuth and antimony which produce a small voltage proportional to the temperature of the junction.
- (e) Thermistors are made of sintered oxides of manganese, cobalt and nickel and have high coefficient of resistance.
- (f) Golay pneumatic detector uses the pneumatic expansion of gas as the measuring device. A metallic cylinder is used as the chamber which, if filled with xenon, expands when radiation falls on it.

Recorder (Electronics and data handling).

5.7.2.3 FTIR (Fourier Transform IR Spectrometers)

The Michelson interferometer is used for this. The source's beam is split into two parts, one of which is reflected to a fixed mirror and the other to a moveable one. The reflected beams recombine in the splitter, resulting in wave interference.

When polychromatic light passes through the interferometer and the sample, an interferogram is produced, which is subsequently Fourier transformed into an IR spectrum.

5.7.2.4 Advantages of FTIR

Only one moving part is mounted on a frictionless air bearing.

Slits and filters are not required.

Frequency is more accurate.

32 scans per minute; thus time can be saved.

5.7.2.5 Applications of IR Spectroscopy

1. Identification of functional groupings and clarification of structure
2. Studying the progress of a reaction
3. Detection of impurities
4. In food industry
 - (a) Determination of trans-unsaturated fatty acids in fats: Peak is obtained at $960\text{--}970\text{ cm}^{-1}$ (Mossoba et al. 2007).
 - (b) Determination of main components of food (Fig. 5.9) (Sun 2009).
5. In Medicine
 - (a) Tumour diagnosis: The change in functional groups of molecules from the tissues or cells in the MIR region as compared to the normal cells can serve as a tool using FTIR to detect cancer (Simonova and Karamancheva 2014).
 - (b) Analysis of stone samples which occur recurrently help to understand the further prevention of stone formation (Bhattacharyya et al. 2014).

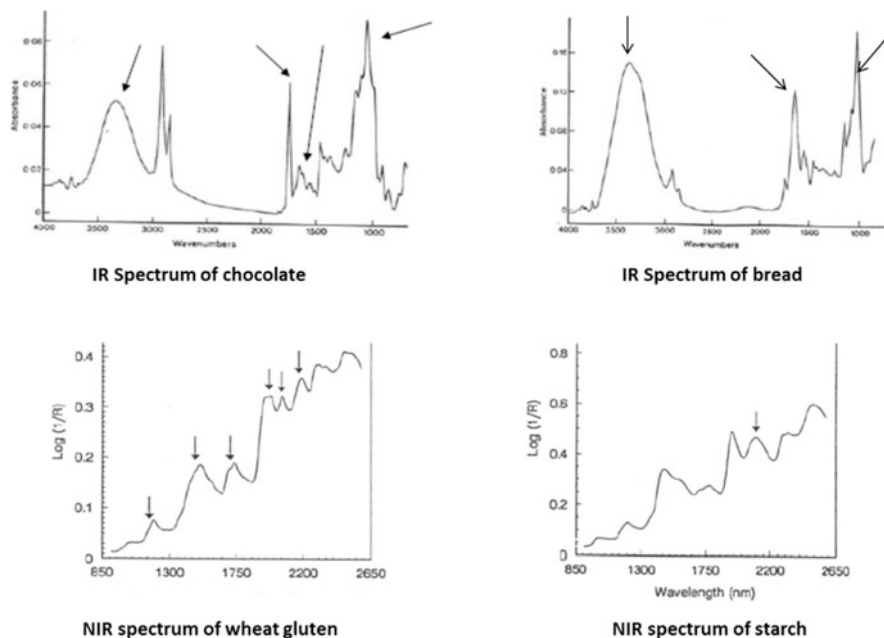


Fig. 5.9 IR spectra of some food products determine the main components of the food products

6. Environmental monitoring: Air pollutants can be monitored using FT-IR. Gases like CO₂, SO₂ and HF are toxic and are emitted in large quantities and need periodic monitoring (Pandita 2013).
7. Monitoring bioremediation process: Can be used to identify and isolate bacterial strains which can remediate hydrocarbon soils.

5.7.3 Mass Spectrometry

High-energy electrons ionize sample molecules. Electrostatic acceleration and magnetic field perturbation are used to determine the mass to charge ratio of these ions, yielding an exact molecular weight. The structure of the molecular ion may be connected to ion fragmentation patterns.

5.7.3.1 The Mass Spectrometer

A mass spectrometer transforms individual molecules to ions so that they may be moved around and manipulated by external electric and magnetic fields in order to assess their properties. A mass spectrometer is made up of three parts:

1. *The Ion Source*: It ionizes a small sample, usually to cations by loss of an electron.
2. *The Mass Analyzer*: In mass analyser, the ions are sorted and separated according to their mass and charge.

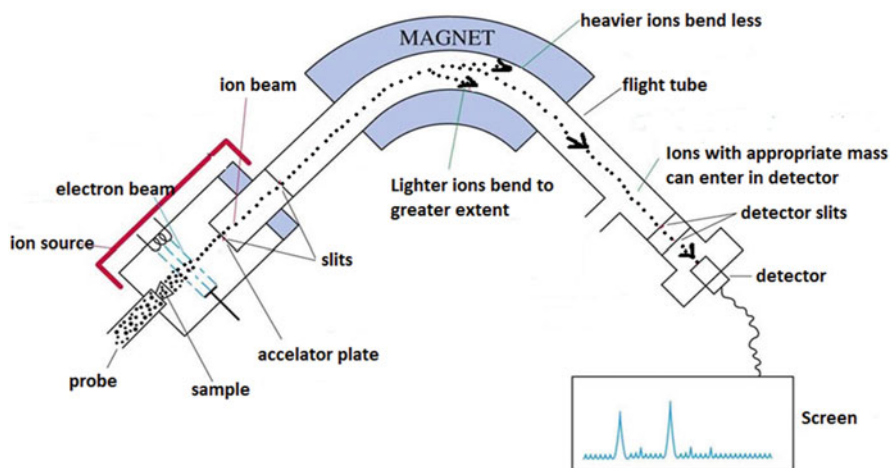


Fig. 5.10 Schematic representation of mass spectrometer, represents the process of the spectrometer; lighter ions reach the detector first and gets detected

3. *The Detector:* The separated ions are then measured, and the results displayed on a chart by detector.

Because ions are very reactive and have a short lifetime, they must be formed and manipulated in a vacuum. The atmospheric pressure is around 760 torr (mm of mercury). Ions can be handled at pressures ranging from 10^{-5} to 10^{-8} torr (much less than a billionth of surroundings). Ionization is accomplished with a high-intensity electron beam, and ion separation is accomplished by accelerating and concentrating the ions in a beam, which is then bent by an external magnetic field. The ions are then electrically detected, with the resulting data being recorded and examined on a laptop. Figure 5.10 depicts a mass spectrometer that works in this manner. The ion supply is the spectrometer's beating heart. The pattern's molecules (black dots) are attacked by electrons (light blue lines) emitted from a heated filament. An (electron impact) source is what this is called. Gases and unstable liquid samples from a reservoir are allowed to flow into the ion source (as proven). Solids and beverages that aren't dangerous can be offered all at once. Cations that have been formed by electron bombardment (red dots) are repelled by a charged repeller plate (anions are interested in it) and multiplied towards different electrodes, having slits via which the ions bypass as a beam.

Several of the ions break down into smaller cations and neutral particles. The ion beam is deflected in an arc whose radius is inversely proportionate to the mass of each ion by a perpendicular magnetic field. Ions that are lighter are deflected more than heavier ions.

5.7.3.2 The Nature of Mass Spectra

The mass spectrum is often shown as a vertical bar graph, with each bar representing an ion with a certain mass-to-charge ratio (m/z) and the length of the bar indicating the ion's relative abundance. The abundance of the most severe ion is 100, and it is referred to as the base peak. Because the majority of ions generated in a mass spectrometer have a single charge, the m/z value is equal to the mass. Modern mass spectrometers can easily identify (resolve) ions that vary by employing the most effective single atomic mass unit (amu) and so provide perfectly exact readings for a compound's molecular mass.

If the pattern is a single pure molecule, the molecular ion is often regarded the best-mass ion in the spectrum, while decrease-mass ions are fragments from the molecular ion.

5.7.3.3 The Working Principle of a Mass Spectrometer

In a traditional mass spectrometer, the substance to be studied is there at first, but it must be ionized in order to pass through the spectrometer with sufficient energy. As a result, electrons assault the sample, causing it to ionize. Table 5.4 depicts several ionization processes.

Depending on the type of sample and its characteristics, this ionized beam is now passed through a sequence of electric or magnetic fields. The ions are deflected by the field in such a way that ions with the same mass to signal ratio take the same path to the detector.

These deflected and charged ions are now incident on a detector that can discriminate between the charged particles that fall on it. We can identify the atoms or molecules that make up the sample by comparing their masses to known masses or looking for a distinctive fragmentation pattern in the mass spectrum created by the charged ions.

Mass spectrometry is an analytical method that may be used to detect unknown chemicals inside a material, quantify the amount of a known substance present in a

Table 5.4 Mass spectrometry methods

Ionization method	Typical analytes	Sample introduction	Mass range
Electron impact (EI)	Relatively small volatile	GC or liquid/solid probe	To 1000 Daltons
Chemical ionization (CI)	Relatively small volatile	GC or liquid/solid probe	To 1000 Daltons
Electrospray (ESI)	Peptides, proteins, non-volatile	Liquid chromatography or syringe	To 200,000 Daltons
Fast atom bombardment (FAB)	Carbohydrates, organometallics, peptides, non-volatile	Sample mixed in viscous matrix	To 6000 Daltons
Matrix-assisted laser desorption (MALDI)	Peptides, proteins, nucleotides	Sample mixed in solid matrix	To 500,000 Daltons

sample and clarify the chemical characteristics or structure of various molecules, amongst other things. The impact of ionizing energy on molecules is studied using mass spectrometry, which depends on chemical interactions in the gas phase to provide data. To conduct the measurement, a mass spectrometer uses electron ionization from the material to create numerous ions. Fragmentation occurs in each molecular ion. The mass spectrometer then separates each of these ions based on their unique mass-to-charge ratio, and the amount of each ion type is measured.

5.7.3.4 Applications of Mass Spectrometry

5.7.3.4.1 Analysis of Biomolecules

Mass spectrometry is quickly becoming a vital tool for studying biomolecules. Electrophoretic, chromatographic and ultracentrifugation technologies were the only analytical techniques that offered equivalent information until the 1970s. Because the results were based on criteria other than molecular weight, they were not absolute. As a result, the only way to determine a macromolecule's actual molecular weight was to calculate it using its chemical structure. Desorption ionisation technologies based on the emission of pre-existing ions, such as plasma desorption (PD), rapid atom bombardment (FAB) and laser desorption (LD), enabled mass spectrometry to be used to analyse complex biomolecules.

5.7.3.4.2 Analysis of Glycans

Oligosaccharides are molecules made up of multiple monosaccharides bound together by glycosidic linkages. The full structure of oligosaccharides is more difficult to determine than that of proteins or oligonucleotides. Because of the isomeric nature of monosaccharides and their ability to create linear or branched oligosaccharides, it necessitates the identification of additional components. An oligosaccharide's structure is determined not only by its monosaccharide sequence and branching pattern but also by the isomer location and anomeric configuration of each of its glycosidic linkages. Glycobiology research entails a thorough examination of the structure, biosynthesis and biology of sugars and saccharides. In the fields of glycomics and glycobiology, mass spectrometry (MS) is developing as a key tool.

5.7.3.4.3 Analysis of Lipids

Lipids are made up of many molecular groups that are soluble in organic solvents. Lipidomics is a subset of metabolomics that involves the extensive investigation and global characterization of the structure and function of lipids (the lipidome) inside a biological system, both spatially and temporally.

Many innovative mass-spectrometry-based lipid analysis techniques have been developed. Electrospray ionization (ESI) sources and triple quadrupole analysers are the most used lipidomics methods. The molecular weight, elemental composition, branching location and type of substituents in the lipid structure may all be determined using mass spectrometry.

5.7.3.4.4 Analysis of Proteins and Peptides

Proteins and peptides are linear polymers made up of 20 amino acid combinations connected together by peptide bonds. Proteins go through a number of post-translational alterations that broaden the scope of their activity.

Proteomics is the study of a biological system's whole protein composition, including co- and post-translationally modified proteins as well as alternatively spliced variations. Almost all proteomics investigations currently use mass spectrometry as a critical tool. It enables for the exact measurement of peptide molecular masses as well as sequences. This data may be utilized for protein identification, de novo sequencing and post-translational modification detection.

5.7.3.4.5 Analysis of Oligonucleotides

Oligonucleotides (DNA or RNA) are nucleotide linear polymers. A nitrogenous base, a ribose sugar and a phosphate group make up these molecules. Oligonucleotides can undergo a variety of natural covalent modifications, such as those seen in tRNA and rRNA, as well as artificial covalent modifications caused by interactions with foreign chemicals. In order to identify these alterations and determine their structure as well as their location in the oligonucleotide, mass spectrometry is used. It not only enables for the calculation of oligonucleotide molecular weights but also for the indirect or direct detection of their sequences.

5.7.4 Nuclear Magnetic Resonance (NMR) Spectroscopy

This technique helps to determine the structure of molecules and ions and their quantization based on transition between energy levels of atomic levels under certain conditions. The radiations involved are in the radiofrequency region. Only nuclei which have a spin show NMR signal. The nuclei of certain isotopes have an intrinsic spinning motion around the axis which generates a magnetic moment along the axis of spin.

In the presence of an external magnetic field, the spin can align with or against the field (Fig. 5.11). The spinning motion of the nucleus is quantized and has a spin quantum number. Addition of energy leads to flipping of nucleus, and nuclei shed excess energy by the process of relaxation.

A nucleus will be magnetic if it has a spin quantum number with a magnitude of $(h/2\pi) \sqrt{I(I+1)}$.

Those nuclei which have $I = 1/2$ act like charged spinning spheres and give a spectra, Eg. ^1H , ^{13}C , ^9F , etc. (Table 5.5).

Nuclei with $I = 3/2$ (^7Li , ^{11}B) and $I = 2$ (^2H , ^{14}N) are important but behave as non-spherical rotating bodies and show line broadening in NMR.

Many organic molecules contain only C, H and O. ^{12}C and ^{16}O do not have nuclear magnetic moment, and thus only proton spectra are observed. ^{13}C has very low abundance (1.1%) and thus is not observed in NMR.

Nuclei which have even A and Z and have $I = 0$ do not show NMR (Kemp 1991).

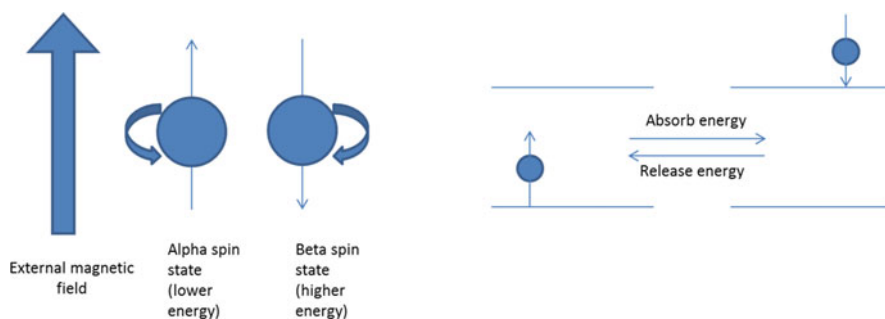


Fig. 5.11 Change in energy level of a spinning molecule in the external magnetic field. Alpha spin state and beta spin state are two energy levels in external magnetic field, align parallel and opposite to the direction of magnetic field

Table 5.5 Details of molecules showing NMR spectroscopy

Mass number (proton+neutron)	Atomic number (no of protons) Z	No of neutrons	Spin quantum number (I)	Examples
Even	Even	Even	0	^{12}C , ^{16}O , ^{32}S
Odd	Odd	Even	$\frac{1}{2}$, $\frac{3}{2}$	^1H , ^{13}F , ^{31}P , ^{11}B , ^{79}Br
Odd	Even	Odd	$\frac{1}{2}$, $\frac{3}{2}$	^{13}C , ^{127}I
Even	Odd	Odd	1	^2H , ^{14}N

The spinning nucleus has a magnetic moment which can have two orientations ($2I + 1$) with respect to the external magnetic field. The transitions between the energy levels by the application of magnetic field occur by the selection rule $[\Delta m = \pm 1]$.

If the applied magnetic field is kept constant and if radiofrequency is increased, an alternate magnetic field is created. When this equals the resonance frequency, energy is absorbed by the spinning nucleus, which flips the spin direction such that its magnetic moment processes against the applied field. There is a linear relationship between the applied magnetic field and the resonance frequency.

The strength of the signal is approximately equal to the square of the magnetic field applied.

Two types of relaxation processes occur: spin-lattice or longitudinal relaxation occurs when nuclei with higher energy transfer their energy to the other nuclei to increase their translational or rotational energy. The other is the spin-spin relaxation in which nuclei exchange spin with neighbouring nuclei by interaction of their magnetic moment and although no energy is lost, the nuclei lose their phase coherence.

5.7.4.1 NMR Spectrum

It is the plot of the intensity of the NMR signals versus the magnetic field (frequency) in reference to TMS. The intensity is measured by the integration of the area under the triangle (Fig. 5.12).

Interpreting the ^1H NMR spectrum:

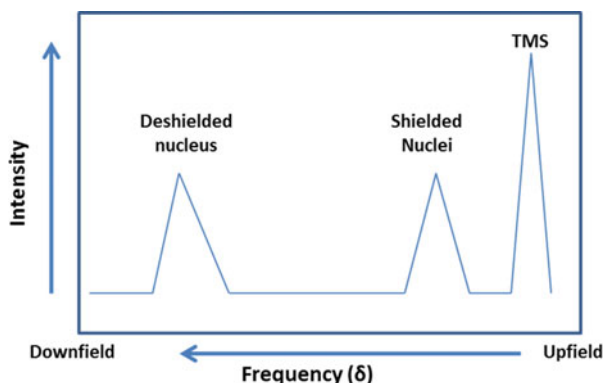
1. Number of signals: Chemically equivalent protons experience different magnetic environments, which give separate signal in NMR spectrum.
2. Position of signals: The position in the horizontal scale at which the signal occurs is called a chemical shift.
3. Relative intensity of signals: The area under the signals corresponds to the number of proton responsible for the signal.
4. Splitting of signals: The interaction between the neighbouring protons produces different flip energies.

5.7.4.2 NMR Spectrometers

The NMR spectrometers contain the following basic units as shown in Fig. 5.13 (Kalsi 2005):

1. A powerful magnet
The magnet must have high field strength which is homogeneous and stable. The stronger the magnetic field, the better the separation of absorption lines. Multiplet overlapping decreases with increasing field strength, and sensitivity also increases. For a homogenous magnetic field, two large pieces of homogenous metal are used. Permanent magnets are cheaper but electromagnets are better for changing the field strength.
2. Two or more radiofrequency channels (to stabilize the magnetic field and to provide irradiating energy)
3. A probe with coils for coupling the sample with the radiofrequency field. This is the sensing element and inserted between the magnetic poles by an adjustable probe holder. The sample holder is a thin-walled glass tube of 5 mm diameter and

Fig. 5.12 A typical NMR spectrum, plot of intensity of NMR signal versus magnetic field frequency showing shielding and deshielding with the reference TMS



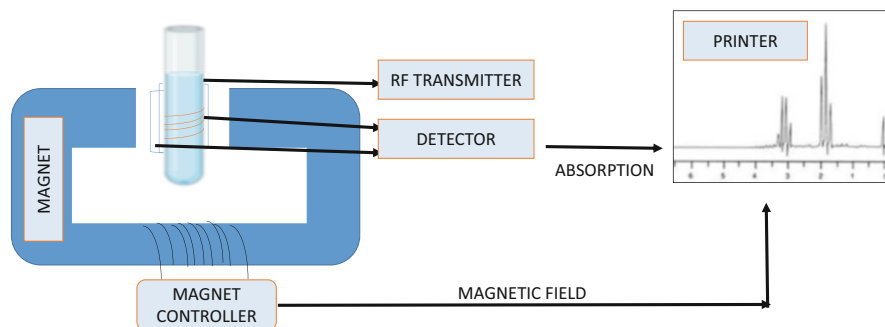


Fig. 5.13 Instrumentation used in NMR studies consisting of (a) powerful magnet, (b) radiofrequency channels, (c) probes with coils, (d) detector, (e) sweep generator, (f) recorder

rotated at 1200–2400 rpm. A few micro- to milligrams of the sample is dissolved in a solvent like CCl_4 , CS_2 , deuterated chloroform, benzene or acetone. The solvent should not contain hydrogen. Single as well as dual coil probes can be used. Tetramethylsilane (TMS) is generally used as the reference standard.

4. **Detector:** The radiofrequency signal produced by resonating nuclei is detected by a coil surrounding the sample. The electrical signal is amplified before recording.
5. **Sweep generator:** It is a set of Helmholtz coils placed parallel to the magnet which allows alteration of the applied field over a small range. By varying the direct current, the effective field can be changed.
6. **Recorder:** The signal detected by the detector is then amplified and fed to a strip chart recorder. The spectrum contains a series of peaks that correspond to different field strengths where each field corresponds to a set of protons. Thus, an NMR spectrum is a plot of detector signal against magnetic field at constant oscillator frequency.

The commonly used solvents are CS_2 , $(\text{CCl}_3)_2\text{C}=\text{O}$ (hexachloroacetone), CCl_4 , CDCl_3 , C_6H_6 , d-DMSO and D_2O . The choice of the solvent depends on the solubility of the compound being studied.

A Fourier transform NMR spectrometer is similar to the one described above except that it has a computer-controlled pulse generator and digital computer for signal processing and presentation of spectra.

A nucleus in different chemical environments experiences different shielding from the applied field depending on the distribution of electronic charge in its surroundings. The greater the field introduced by the circulating electrons shielding the nucleus, the higher the applied field to achieve resonance. Shielding effect depends on hybridization and electronegativity of the groups attached to the atom to which the nucleus belongs. TMS, the reference standard, gives a sharp signal due to 12 equivalent protons and is taken as 0.0 in the chemical shift scale. It is a liquid and miscible in most solvents. It is volatile and easy to remove. TMS is inert and does not react with samples.

Chemical shift: The nucleus is surrounded by electrons which have charge as well as spin which shields the electrons from the external magnetic field known as diamagnetic shielding. Chemical shift is expressed as δ which is a dimensionless quantity.

$$\delta \text{ (in ppm)} = (\nu_{\text{sample}} - \nu_{\text{reference}}) \times 10^6 / \text{spectrometer frequency}$$

Factors affecting chemical shift:

1. Intramolecular factors

- (a) **Inductive effect:** As the electron density surrounding a proton increases, so does the induced magnetic field, requiring a larger applied field to overcome the shielding effect. De-shielding occurs when electronegative atoms or groups remove electron density from surrounding the proton, causing the peak to shift downfield.
- (b) **Van der waal's deshielding:** The protons in bulky molecules may occupy sterically hindered positions which will repel the electron cloud.
- (c) **Anisotropic effect:** In a spherical electron density (s orbital), the induced magnetic field will be uniform and is known as isotropic. In non-spherical electron density (π electron cloud), the induced field will be non-uniform known as anisotropic.

2. Intermolecular factors

- (a) **Hydrogen bonding:** During hydrogen bonding, the hydrogen atom shares its electrons with two electronegative atoms and thus is deshielded and comes into resonance at low field. In this way inter- and intra-molecular hydrogen bonding can be distinguished as in the case of intramolecular hydrogen bonding, there is no shift in the peak with increase in concentration.
- (b) **Temperature:** Most signals are not affected except $-\text{OH}$, $-\text{NH}$ and $-\text{SH}$ which resonate at higher field as temperature increases.
- (c) **Solvent:** The solvent used in NMR must preferably not have hydrogen atoms (Table 5.6). Use of benzene can cause a shift in the peak (Table 5.7).

Spin-Spin Coupling

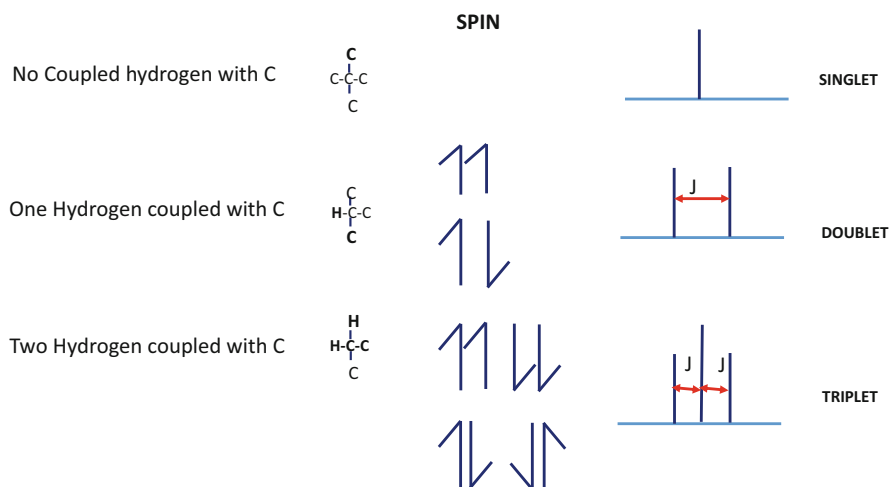
Nuclei in proximity with each other interact to cause splitting of resonance lines called as spin-spin coupling or J coupling. J, which is equal to the spacing of multiplets in Hertz, denotes the coupling strength. $2n + 1$, where n is the number of nuclei involved, gives the number of lines in a multiplet. The overall intensity of a multiplet is proportional to the number of nuclei involved, and the relative intensity of the relative peaks follows the binomial expansion coefficient. Example, one neighbouring proton splits the peak into a doublet (1:1), two produce triplet (1:2:1), and three a quartet (1:3:3:1) (Fig. 5.14). The magnitude of J is independent of field strength. When a proton is coupled to a nucleus which has quadruple moment, spin-lattice relaxation comes into play, and the spin-spin interaction with the proton is decoupled.

Table 5.6 Chemical shift of reference standard relative to TMS

Solvent	Relative δ value	Solvent	Relative δ value
Water	-5.1	Chloroform	-7.25
Acetone	-2.09	Cyclohexane	-1.49
Benzene (Int)	-7.27	Benzene (ext)	-6.73
Dioxane	-3.57	Me ₄ Si	-

Table 5.7 Shift in absorption position of benzene protons

Substituent	Position			Substituent	Position		
	Ortho	Meta	Para		Ortho	Meta	Para
-COOH, -COOR	-0.8	-0.15	-0.2	-SO ₃ H, -SO ₂ NH ₂	-0.4	-0.1	-0.1
-CH ₃	+0.15	+0.1	+0.1	-CN	-0.3	-0.3	-0.3
-COR	-0.6	-0.3	-0.3	-CONH ₂	-0.5	-0.2	-0.2
-OR	+0.2	+0.2	+0.2	-OH	+0.4	+0.4	+0.4
-NH ₂	+0.8	+0.15	+0.4	-N(CH ₃) ₂	+0.5	+0.2	+0.5
-Cl, -Br	0	0	0	-I	-0.3	+0.2	+0.1
-OCOR	-0.2	+0.1	+0.2	-NO ₂	-0.1	-0.3	-0.4

**Fig. 5.14** Spin-spin coupling, depicting one neighbouring proton splits into two peaks (doublet) and three peaks (triplet)

J spin-spin coupling constants depends on the following:

- Distance between coupling partners and intervening bonds.
- Dihedral angle between the coupling partners for vicinal protons.
- Coupling constant is largest when dihedral angle is 180° and least when the angle is 90°.
- In freely rotating bonds, average J values are obtained.

5.7.4.3 Applications of NMR

1. Structural (chemical) elucidation
 - Natural product chemistry (Jackman and Zechmeister 1965)
 - Synthetic organic chemistry
 - Analytical tool of choice of synthetic chemists
 - Used in conjunction with MS and IR
2. Study of dynamic processes
 - Reaction kinetics (Kim et al. 1999)
 - Study of equilibrium (chemical or structural)
3. Structural (three-dimensional) studies
 - Proteins, protein-ligand complexes (Wishart 2005)
 - DNA, RNA, protein-DNA complexes
 - Polysaccharides
4. Metabolomics (Yoon et al. 2013)
5. Drug design (Diercks et al. 2001; Roberts 2000)
 - Structure-activity relationships by NMR
6. Medicine (Moudgil et al. 1985)

5.8 Applications of Spectroscopy in Microbiology

1. Study of bacterial growth

Light passes through a particular sample in the spectrophotometer, after passing through a wavelength selector. A reference sample, called “blank”, is imperative to measure first. Normally blank contains a solvent that was used to prepare the test sample, for example, a buffer can be the blank if it was used to prepare the test sample. The autoclaved culturing medium will be a reference for bacterial cultures. The OD of the blank may be subtracted manually or by the instrument itself to obtain the exact absorbance value of analytes.
2. Purity check of nucleic acids extracted from microbes

Chances of protein contamination may be revealed by taking the ratio of absorbances at 260 nm and 280 nm in nucleic acid samples. Pure DNA shows 1.8 as ratio of 260/280, and in the case of pure RNA, the ratio is 2.0, because uracil in RNA gives a higher 260/280 value. Proteins give higher absorbance at 280 nm, and so protein contamination in nucleic acid samples decreases the ratio. Protein-contaminated samples have a lower 260/280 ratio due to a greater absorbance at 280 nm.
3. Fluorescence correlation spectroscopy (FCS) applications in medical microbiology

This technique works on the principle that the size of a fluorescing molecule is correlated to specific diffusion velocity, such that a bigger molecule shows slower diffusion through any specific spherical volume. Fluorescence spectroscopy has been used for polyphasic taxonomy for pseudomonads, as it provided more information than nucleic acid-based homology grouping. FCS provided better resolution of strains that were difficult to resolve using PCR. Fluorescence

spectroscopy has potential as a novel diagnostic tool for detecting viruses and viral infections of cells. FCS was successfully applied to viruses belonging to the cystovirus family which infect pseudomonad cells and to study rhinovirus receptor interaction in human.

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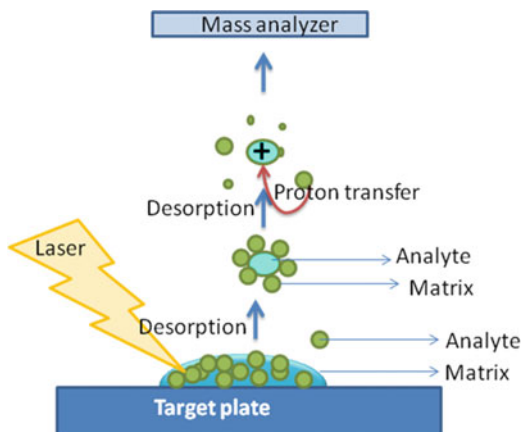
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6.1 Introduction

Mass spectrometry has been used for several decades to characterize molecules and their proper identification. In 1975, Anhalt and Fenselau first proposed that matrix-assisted laser desorption/ionization-time of flight mass spectrometry (MALDI TOF MS) could be used to characterize bacteria. He observed unique mass spectra from bacterial extracts of different genera and/or different species that were helpful to recognize the individual bacteria. In the 1980s, the development of desorption/ionization technologies such as plasma desorption (PD), laser desorption (LD) and fast atom bombardment (FAB) enabled the generation of molecular biomarker ions from microorganisms, enabling bacterial profiling. In the early stages of bacterial characterization, only low molecular weight biomarker molecules such as bacterial lipids are analysed because the process used to ionize biomolecules is too energetic to avoid unpredictable degradation of the analyte (Heller et al. 1988). In the late 1980s, with the development of soft ionization technologies such as MALDI and electrospray ionization (ESI), Tanaka and Fen succeeded in analysing large biomolecules such as intact proteins. Several groups (Cain et al. 1994) have confirmed that MALDI-TOF can be used for protein profiling after whole cell extraction and purification. Holland et al. (1996) reported that MALDI-TOF was able to obtain the first spectral fingerprint from whole bacterial cells without pretreatment prior to MS analysis. This approach was subsequently used by several research teams to identify bacteria at the genus and species level (Mandal et al. 2007). The success of this technique for the rapid identification of fungi and yeasts as well as bacteria has been demonstrated since the late 1990s by the remarkable exponential growth of publications on the detection of bacteria using MALDI-TOF MS.

Analysis of cell proteomes is a unique method that occupies an intermediate position in relation to the dichotomy of phenotypic genotypes. The increasing use of DNA fingerprinting methods over the last two decades emphasizes their applicability and usefulness for the identification and classification of microorganisms. In recent

Fig. 6.1 Schematic representation on the basics of MALDI-TOF mass spectrometry



years, automation of proteomics technology has led to several applications in microbiology such as strain typing of microorganisms and epidemiological studies, identification of microorganisms inhabiting specific ecosystems, detection of aquatic and food pathogens including the pathogen causing blood infection, etc. The following outlines the current status and new applications of mass spectrometry (MS) for microbial identification and the usefulness of this technique for diagnosing diseases caused by bacteria, viruses and fungi (Fig. 6.2).

MALDI has additional advantage over other analytical techniques that the sample is mixed with the chemical matrix to generate ions, which requires less preliminary analysis. The MALDI-time of flight (TOF) MS combines two technologies as a MALDI source and TOF mass (Fig. 6.1). Since its inception, this tool has revolutionized the way microorganisms are identified in clinical microbiology laboratories because it is a fast, inexpensive, efficient and high-throughput system. One of the main benefits of using MALDI-TOF MS is that it saves time because bacterial identification can be performed in less than an hour instead of 48–72 h. This time is important for patients with underlying autoimmune disease, immunocompromised persons or infections with multi-drug-resistant bacterial pathogens.

6.2 MALDI: Sample Preparation and Analysis

Prior to MALDI-TOF MS analysis or data acquisition, a fresh bacterial isolate was inoculated into and cultured at 28 °C for 24 h. Mass spectra can be obtained according to standard direct transfer protocols (commonly referred to as whole cell or intact cell measurements). In short, approximately 0.1 mg of cellular material can be transferred directly from a single bacterial colony (if possible) or colony smear to the MALDI target point. After drying at laboratory temperature, the sample spots can be covered with 1 µL of matrix solution (10 mg/ml α -cyano-4-hydroxycinnamic acid in 50 µL acetonitrile and 2.5% trifluoroacetic acid). Each measurement should be performed three times (technical replica). MS analysis can be performed on any

machine, such as the Autoflex MALDITOF mass spectrometer (Bruker Daltonics, Germany), using the Flex Control 3.4 software (Bruker Daltonics, Germany). Calibration was performed using the Bacterial Test Standard (Bruker Daltonics, Germany). Here is a step-by-step protocol to follow for proteome analysis of microbial cells.

6.2.1 Sample Preparation

1. Collect cells, wash and add lysate buffer, containing chemicals (e.g. SDS) to break the plasma membrane and also inhibitors of protease enzyme to prevent degradation of proteins.
2. Homogenize cells and incubate with the lysis buffer, and then centrifuge to precipitate the cell debris from lysate which would contain the proteins.
3. Sometimes separation steps are needed to distinguish between proteins having different physical and chemical characters, for which two-dimensional gel electrophoresis (2DE) may be used. Stain and extract proteins from the gel.
4. Analyse the stained proteins separately, to obtain better assessment of proteome. However, the main error in analysis results due to unequal precipitation of various peptides, and horizontal, vertical or diagonal shifts are observed in two-dimensional gels (2D), thereby aligning all gels to one reference gel which is important.

6.2.2 Protein Digestion

1. Digest proteins into short fragments either chemically or enzymatically for ease of identification of protein/peptides. Complex structures of protein are broken by digestion, and hence resulting peptides are tractable and amenable to MS analysis, because of reduced size. Trypsin is often used for cleaving the COOH of LYS and ARG (except if either is followed by PRO) and a chemical CNBr (cyanogen cleaves COOH end of MET). Enzymatic digestion may be performed on gel or in lysate, but gel is difficult to separate after digestion leading to missed cleavages or missed peptides and skewed results upon database searches.
2. MS spectra may be measured using Flex Control software (or any other depending on the equipment used) in accordance to a standard method for microbial identification. Specifically, the set-up values in linear positive mode are adjusted, e.g. ion source 1 voltage, 20 kV; ion source 2 voltage, 19 kV; lens voltage, 6.5 kV; mass range, 2–20 kDa; the final spectrum will be the cumulation of 10 spectra, each obtained by 200 laser shots on random target spot positions. With regard to the functioning of MALDI-TOF MS, by which +1 ions are predominantly generated and detected, Da may be used as a unit of m/z throughout the study.

6.2.3 MALDI/MS Analysis

1. Prepare samples for MALDI/MS analysis by coating with matrix (solution of an energy-absorbent, organic compound). The matrix crystallizes on drying, therefore the captured sample that co-crystallizes. The laser beam ionizes the sample in the matrix and produces protonated ions from the analyte in the sample. The protonated ions are then accelerated to a fixed potential, and the ions are separated from each other due to the mass-to-charge ratio (m/z). Charged objects are then detected and measured by various types of mass spectrometers, such as quadrupole mass spectrometers, ion trap analysers and time-of-flight (TOF) analysers. TOF mass spectrometers are mainly used for microbiological applications. The MALDI-TOF analysis measures the m/z ratio of ions by determining the time it takes to travel the length of the flight tube. Some TOF analysers include an ion mirror at the near end of the flight tube. It is used to reflect ions from the flight tube to the detector. Therefore, ion mirrors not only increase the length of the flight tube but also compensate for small energy differences between ions (Yates 1998). Based on the TOF information, a characteristic spectrum of the analyte in the sample is generated. This is called peptide mass fingerprinting (PMF). PMF or intact protein mass profiling is the output that is used to compare with the predicted mass values obtained from MALDI analysis. Companies have developed specific software such as MALDI Biotyper, a novel approach for the rapid identification of microorganism using MALDI-TOF MS. Other than Biotyper, common search programs used in PMF are MASCOT, PROFOUND, MSFIT and ALDENTE. For protein identification also four to five peptide mass matches are adequate to identify the correct protein hit (Fig. 6.2).

6.3 Uses of MALDI-TOF

MALDI-TOF has made a shift from research to clinical for identification of microorganisms from patients and highly acceptable to detect the pathogens in infection. Microbial colonies cultured from patient samples are placed on a MALDI “target” plate, treated with formic acid, embedded in a matrix, and the laser is directed at the target plate to initiate sampling. The detected biomolecules are not identified from the resulting spectrum. Rather, unique patterns created by microbial proteins and other biomolecules are matched against known microbial spectra from databases, such as fingerprints matched to identify an individual. Each spectrum contains peaks that are unique to a particular genus, species and strain, and the MALDI-TOF device uses an algorithm to generate a confidence-level value for that identification (Fig. 6.3). Many studies have shown the accuracy of MALDI-TOF MS in the identification of a wide range of bacteria, including Gram-positive cocci and bacilli, fermentable and non-fermentable, gram-negative bacilli and anaerobic bacteria (Croxatto et al. 2012; van Veen et al. 2010). In general, more than 90% of all isolates are identified at the species level, 98% are identified at the genus level, and

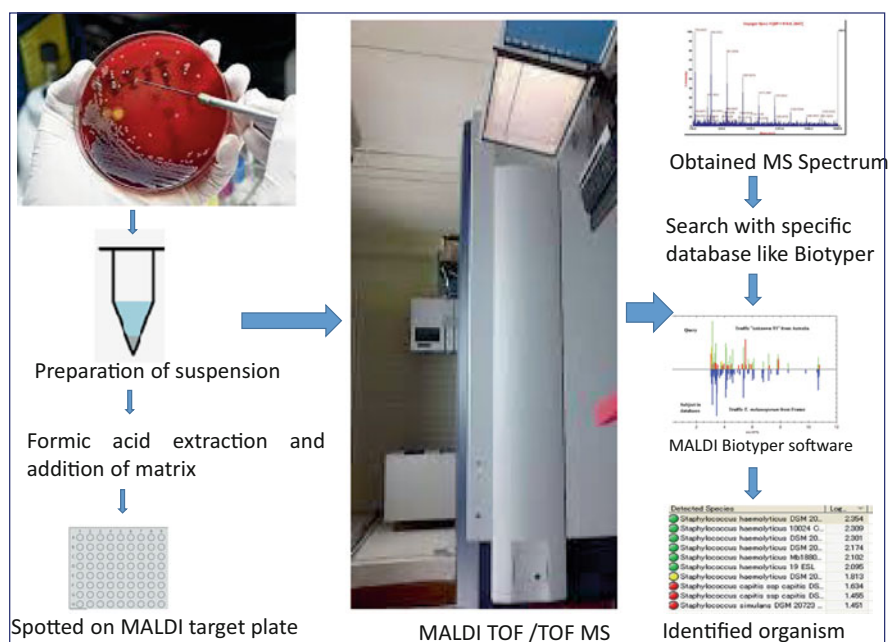


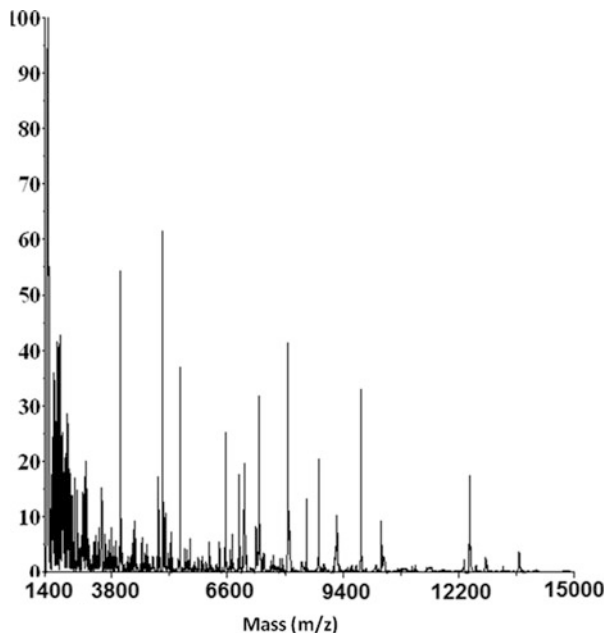
Fig. 6.2 Schematic workflow of MALDI techniques for rapid bacterial identification

less than 1% are misidentified. The most common reason an isolate is not identified is because it is not included in the database. Misidentifications are most commonly observed with taxonomically related bacteria, such as *Shigella* with *Escherichia* and *Streptococcus pneumoniae* with *S. mitis*. As experience is gained with MALDI-TOF MS, it is expected that the database will be expanded to resolve many of the current inadequate identifications (e.g. no identification, genus-level identification), and algorithms for potential misidentification will be developed (e.g. rapid bile solubility test to confirm a *S. pneumoniae* identification).

6.4 MALDI-TOF MS-Based Antimicrobial Susceptibility Testing

Recently, the multi-antibiotic-resistant bacteria are of great threat to human health due to the overuse of conventional antibiotics and their inappropriate use. Most of the conventional antibiotics are not working against antibiotic-resistant bacteria (ARB) that delayed to start the appropriate treatment. Antimicrobial susceptibility testing (AST) of ARB bacteria is very much urgent with minimum time to prescribe appropriate drugs. The conventional AST-based methods are reliable for the detection of bacterial growth but are very slow. Therefore, an urgent necessity has been created to search for new method that can perform AST quickly. MALDI-TOF MS is

Fig. 6.3 A representative MALDI MS image obtained from whole cell *Rhizobium* useful for rapid bacterial identification using Biotyper

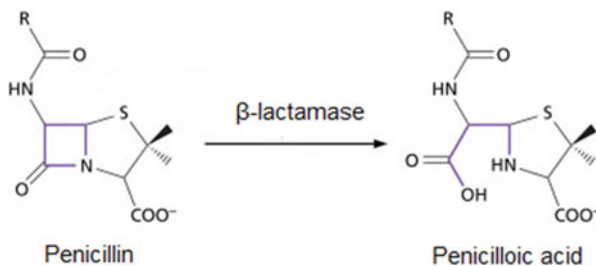


increasing their attention due to its rapidity, accuracy and simplicity including promising results in detecting antimicrobial resistance. The modification of antibiotic is a very common phenomenon in antibiotic resistance which is induced by bacterial enzymes and easy to detect by MALDI-TOF MS. It is very much well known that bacterial metabolism is directly related to antibiotic resistance. This metabolic profiling approach has been used in mass spectrometry technology to determine the antibiotic resistance profile by comparing microbial metabolic processes in the presence or absence of antimicrobials very fast. MALDI-TOF MS-based microbial identification is regularly used in clinical laboratories and it has the potential to expedite analysis of AST. Several methods have been designed to analyse AST using MALDI-TOF MS which are described below:

6.4.1 Detection of Antibiotic Degradation

β -Lactam antibiotics are the most widely used antibiotics which kill the bacteria by inhibiting the bacterial cell wall biosynthesis (Lovering et al. 2012). The most important mechanism of resistance to β -lactam antibiotics is the production of β -lactamases that hydrolyse the β -lactam ring (Fisher et al. 2005) as shown in Scheme 6.1. These lactamase enzymes catalyse the hydrolysis of amide bond in β -lactam ring corresponding to the addition of a water molecule. Therefore, this reaction leads to an 18 Da mass change, which can be easily detected by MALDI-TOF MS (Sparbier et al. 2012). It has been evidenced that MALDI-TOF MS can

Scheme 6.1 Hydrolysis of penicillin to penicilloic acid in the presence of the enzyme β -lactamase



detect the carbapenemase that hydrolyses carbapenem and inactivates the antibiotic (Hrabák et al. 2012).

This enables to make a very quick decision and speeds up the selection of antibiotics that is put into practice of infection control measures. However, sometimes for few cases, it was observed the very low enzymatic activity or delayed enzyme expression that could lead to a false-negative result. This problem can be overcome by increasing the incubation time at a maximum of 4 h and nullify the false-negative result or mistakes (Chong et al. 2015).

6.4.2 Identification of Biomarker for Detecting Antibiotic-Resistant Strains

MALDI-TOF MS-based WC (whole cell)—WCMS—is used for biomarker determination in antibiotic-resistant strains. One of the important studies summarizes that methicillin-resistant *Staphylococcus aureus* (MRSA) is possible to identify using WCMS concept. MRSA is an important clinical pathogen burden to public health and hospital hygiene in most countries throughout the world. Josten et al. have shown a signal at 2415 m/z is corresponding to small peptide PSM-*mec* encoded on three SCCmec cassettes, recognized as the biomarker of MRSA (Josten et al. 2014). The specificity of ion, 2415 m/z, is too high ($\geq 98\%$), which indicates that this ion is a specific biomarker for MRSA strain. Similarly, *cfiA* is encoded by a class B metallo- β -lactamase, which also confirms the biomarker of carbapenem-resistant *Bacteroides* strains (Nagy et al. 2011).

6.4.3 Phenotypic Antibiotic Resistance Analysis of Bacterial Strains

MALDI-TOF MS is very useful to analyse the whole cell proteome of bacteria. It is possible to determine the type of antimicrobial resistance by the analysis of microbial proteome changes after antibiotic treatment (Fenyvesi et al. 2014). A rapid and phenotypic AST approach, the MALDI Biotyper antibiotic susceptibility test rapid assay (MBT-ASTRA), is developed (Lange et al. 2014). Clinical isolates of *Klebsiella pneumoniae* and *Klebsiella oxytoca* were tested successfully and determined the resistance pattern against meropenem. This method achieved a sensitivity of 97.3%

and a specificity of 93.5%. Further, MBT-ASTRA approach is used for bacterial cultures and determined the antibiotic resistance profile successfully (Jung et al. 2016). In brief, bacterial isolates were incubated in minimal or nutrient broth medium with and without antibiotics directly on a disposable MALDI-TOF MS target followed by 18 h of incubation; however high accuracy is also achieved within 4–5 h of incubation for *K. pneumonia* and *P. aeruginosa*. In this way, samples are easy to handle, easy to perform, useful for high-throughput screening and considerably a rapid technique for clinical applications.

6.5 Advantages and Limitations

MALDI-TOF MS-based bacterial identification depends on the nature of microbial proteins which are normally well conserved from species to species. Thus, it provides a more reliable means of discriminating one species from another. But following the traditional techniques, it is difficult to discriminate among species that are phenotypically, biochemically or even genetically similar.

MALDI-TOF MS has proved its effectiveness to show the more accurate nutritionally variant streptococci (NVS), including *Granulicatella* and *Abiotrophia* species.

The speedy process with MALDI-TOF MS-based identification and antibiotic susceptibility result quickly guides the further treatment.

Bacteria can be identified directly from the blood culture bottle within an hour.

Anaerobic bacteria and *Saccharomyces* species identification is one the important benefits of MALDI-TOF MS.

This is a potential time and cost-saving technique as the primary method of identification in the clinical laboratory.

Therefore, the beginning of MALDI-TOF MS into clinical laboratory would be more beneficial for timely and accurately identifying the microorganisms that will improve the diagnosis procedure as well as reduce time to start appropriate therapy.

6.6 Challenges

Determination of few targetted resistance mechanisms in resistant strains is the main inherent limitation of MALDI-TOF MS-based AST approach.

The technique cannot distinguish between co-related species due to similarity of organisms, e.g. *E. coli* and *Shigella*. The cause may be that these are one species, against the suggestion of taxonomists. Despite of these problems, discriminating organisms using MALDI-TOF MS is possible. In clinical cases differentiation to species level is important and necessitates supplementary tests. In the future, adding proteomics analyses to MALDI-TOF MS system can improvise its discriminatory power to identify particular strains or serotypes.

Incorrect identification may also be due to a lack of satisfactory or abundant spectral information in databases. This leads to incorrect identification at species

level, or sometimes strains are not identified, as has been stated in the case of *Trichophyton* species.

In conclusion, MALDI-TOF MS has a great potential in microbial identification and taxonomic studies if supported by other complementary studies, and it is a rapid and efficient technique for clinical laboratories because it can identify and discriminate between strains and serotypes of microbes. Application of this technique has resulted in more accurate and timely identification of microbes, thus subsequently improving diagnosis and also by reducing time to suitable treatment. Since the techniques are improvising over the years, we hope that soon the limitations will be resolved, and with the help of this platform, clinical microbiology will see positive transformation.

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Enzyme-Linked Immunosorbent Assay (ELISA)

7

7.1 Introduction

An enzyme-linked immunosorbent assay (ELISA) is generally used to detect and measure antibodies in the blood. Antibodies are the specific proteins that are produced by the body in response to harmful substances called antigens. This technique is independently developed by Engvall (1972). ELISA is highly specific (>99%) as well as highly sensitive (>99%); therefore this is routinely used for diagnosis of infectious diseases like HIV, Lyme disease, rotavirus, syphilis, toxoplasmosis, Zika virus, chickenpox and shingles and pernicious anaemia, including squamous cell carcinoma, and pregnancy test (Leng et al. 2008). ELISA is a qualitatively or quantitatively assay of either antibody or antigen. The most important point is the use of enzyme that attaches to the antibody along with colourless substrate for generation of chromogenic substrate. In general, few specific enzymes have been used in ELISA, such as beta-galactosidase (*E. coli*), alkaline phosphatase (calf intestine), horseradish peroxidase and urease (jack bean).

There are several types of ELISA (Fig. 7.1) that have been routinely used in clinical diagnosis which are described below with their schematic steps of preparation protocol:

7.2 Indirect ELISA

- Indirect ELISA involves the binding of target antigen to the first antibody, that is then detected by the second antibody (Ab₂).
- This is used for detection of viral antigen, i.e. HIV.
- Antibody qualitatively and quantitatively is determined by indirect ELISA.

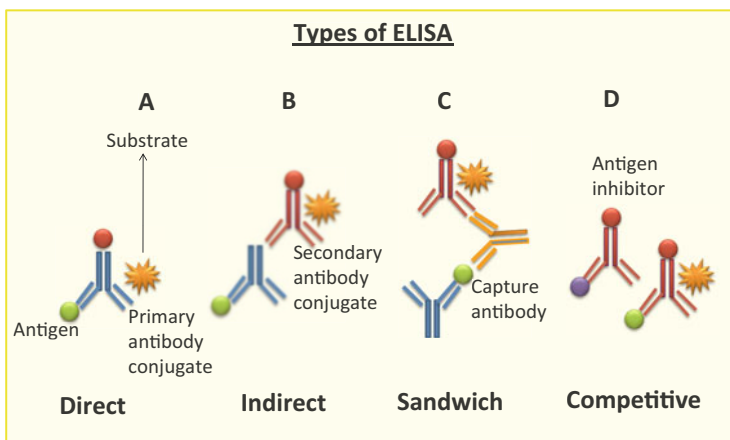


Fig. 7.1 Showing the schematic representation of various types of ELISA commonly performed

7.2.1 Steps of Indirect ELISA

- Collection of primary antibody containing the sample (i.e. serum).
- Processed (i.e. centrifugation) sample or direct sample is added to an antigen-coated well during reaction at optimum incubate condition.
- Unbound antibodies are washed out by buffer.
- Primary antibody-specific enzyme-linked secondary antibodies were added.
- After completion of reaction, specific colour should be produced.
- During this reaction the intensity of colour developed is proportional to the antigen and may be determined by a spectrophotometer.

7.3 Direct or Sandwich ELISA

- Sandwich ELISA can be helpful for the detection of antigen.
- During this technique the antibody is immobilized on a microtitre well.
- Sandwich ELISA is of two types such as double antibody sandwich ELISA and triple antibody sandwich ELISA.

7.3.1 Steps of Double Antibody Sandwich (DAS) ELISA

- DAS ELISA is the widely used immunochemical technique for diagnostic purpose.
- It is rapid but reliable and sophisticated technique.
- Specific antigen is immobilized to a solid surface by a primary antibody and detected with marker enzyme-linked second antibody.

- Commonly usable marker enzymes are horseradish peroxidase and alkaline phosphatase.
- In some cases the enzyme is replaced by radioactive label, and that is called immunoradiometric assay (IRMA).
- This technique is commonly used for agriculture and horticulture purpose.
- Specific antibodies are immobilized to a solid surface of microtiter well.
- Antigen-containing sample is added to the antibody-coated well.
- After washing, second enzyme-linked antibody is added.
- Another washing is step is performed to eliminate the unbound antibodies.
- Finally the amount of colour is measured by a spectrophotometer.

7.3.2 Steps of Triple Antibody Sandwich (TAS) ELISA

- TAS is also called indirect ELISA.
- Hepatitis B virus (HBV) antibodies are used as a diagnostic test for this disease.
- ELISA plate is coated with antibody.
- HBV coat protein is added to the antibody-containing well (live virus is not used as antigen due to being infectious; instead of this synthetic HBV coat protein has been used).
- After incubation wash out unbound antigen by a buffer.
- Antibody-containing sample (patient serum) is added.
- Anti-human antibody conjugated to an enzyme that is added to the well for detection of disease.

7.4 Competitive ELISA

- It is primarily used for assay of small molecules such as hormone where a single epitope is present on the antigen.
- The principle is based on competition between natural antigen (hormone) to be tested for and an enzyme-conjugated form of the antigen which is the detection agent. The test sample and a specific amount of enzyme-conjugated antigen are mixed together and placed into the coated well of a microtitre plate. The antigen and conjugated form of it compete for the available space on the coating antibody layer. The more natural antigen present, the more it will displace (compete out) the conjugated form leading to a reduction in enzyme bound to the plate.
- This type of ELISA is routinely used for testing blood sample for thyroxine.
- Competitive ELISA is used for this as an accurate measure of the circulating level of hormone can be made from a standard curve of known dilutions.

7.5 Radioimmunoassay (RIA)

- Radioimmunoassay (RIA) is the most sensitive technique for detection of the concentration of antigen or antibody in a sample, which is based upon competitive binding between unlabelled and radioisotope-labelled antigen for its specific antibody (Goldsmith 1975).
- Yalow and Berson (1960) developed the concept about RIA for the assessment of insulin in animal plasma.
- The principle of RIA involves competitive binding of unlabelled antigen and radiolabelled antigen to a high affinity antibody.

7.5.1 Steps of RIA

- A specific amount of antibody is attached to the bottom portion of the tube.
- A selective amount of radiolabelled antigen is added to the tube.
- The amount of radiolabelled antigen is typically high enough to saturate all of the antibody molecules attached to the bottom of the tube.
- The tube can be counted by gamma counter chamber to obtain a counts per minute (CPM).

7.6 Automated ELISA

Automated ELISA is used in places where unattended operation is required in low-, medium- or high-throughput laboratories. The workstations for automated ELISA may be bulky or expensive but provide accurate data and involve very less chances of contamination that results from handling errors. The workstations are generally easy to set up and maintain, and most assays are performed in 96-well microplates. The example of such a workstation is the Crocodile Mini-workstation which has five functions in one compact unit. It can do all the jobs required in ELISA, i.e. to wash, dispense, shake, incubate and also record absorbance at required wavelengths (<https://www.berthold.com/en/bioanalytic/products/automated-elisa-systems/>).

Most large clinics/hospitals use automated ELISA on regular basis, and depending on the workload, they use either low-, medium- or high-throughput machines. These machines can process a single plate daily, 2–3 plates per day or up to 16 plates every day, respectively. Due to the various advantages of automated ELISA, it is widely used in research, clinics and other set-ups. The operator can be free after setting up the automated workstation and attend to other jobs, and the accuracy of incubation time, washing, etc. is unquestioned and does not vary from batch to batch that otherwise causes errors. This also decreases the requirement for trained manpower.

It may be therefore concluded that ELISA is a widely used technique for microbiology and clinical set-ups and is very improvised for reproducibility, reliability, process economy and increasing throughput. Several other techniques are

based on the same principles with differing modes of detection which are evolving, e.g. chemiluminescence immunoassays (CLIA), for further improvement and benefit for end users.

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Isolation of Normal Microbiota from the Human Body and Microbial Identification

8

8.1 Introduction

The microbes constituting the routine microflora of a human body are mostly harmless, but few of them are potential pathogens or opportunistic microbes. These latter microbes result in disorders or diseases under specific favourable circumstances. Therefore, we should learn about the prevailing normal microbiota found in various parts of the human body. This will lead to understanding various microbes residing in/on specific body parts and also provide deep insights of plausible infections resulting from injury to these sites. In order to help a clinician with knowledge of the existing microorganisms, sources, infectivity, significance, etc., such studies are warranted. Also consequences of over-/under-growth of such indigenous microbes aid in diagnosis, treatment and post-treatment care. Therefore, such awareness is required for developing interest to decipher the roles played by indigenous microflora in stimulation of the immune responses from host, to get protection from pathogens, which otherwise cause sickness.

For clinical microbiology, it is important to accurately identify the microbial isolates since treatment of diseases relates to successful identification of both microbe and disease. In this case not only indigenous but also food-borne, air-borne and water-borne microbes need proper and authenticated identification. Again, during outbreaks and epidemics, accurate identification of causative pathogen helps in containing the epidemic and suggesting suitable strategies for treating and preventing the pathogens. This is the case of all cases related to cholera, plague and the recent COVID-19 outbreak.

Identification and characterization of pathogenic microbes and their serotypes play a significant role in clinical and pharmaceutical processes for which there is a limited spectrum of distinguishing tests that are appropriate for the problem under study (Stackebrandt 1989). Any microorganism is characterized by several factors such as its origin, product, niche/environment and metabolic pathways. The microbial load or variety of microbes present in the manufacturing environment in pharma

Table 8.1 Various techniques for identification and classification of microbes, as recommended by European Pharmacopeia

Method	Example	Chapter name
Phenotypic	Staining and biochemical tests (manual/kit)	This chapter
	Fatty acid profiles	This chapter
	FTIR spectroscopy	Spectroscopy for microbial identification
	Microscopy	Advances in Microscopy
	Immunological tests	Immunotechnology
Genotypic	PCR and real-time PCR	Manual and automated characterization of multi-antibiotic-resistant (MAR) bacteria
	Sequencing 16S or 23S or ITR regions of rDNA	Rapid microbial genome sequencing techniques and applications
	Whole-genome sequence	Rapid microbial genome sequencing techniques and applications

companies, and also in raw materials (e.g. water), in-process materials, finished product, should be determined accurately. Classical methods for microbial identification included staining techniques and culturing on different media and differential tests (discussed in Sect. 8.2). In the 1970s improved methods were developed via the onset of API (analytical profile index) test strip that consisted of miniaturized biochemical substrates inside a series of ampules (PDA 2000). Thereafter, by the 1990s, several new phenotypic tests were available for microbiological laboratories for identification of microbes in quality control processes. These techniques were affordable, fast and more accurate than the current existing techniques but lacked the expected speed and desirable accuracy. So in the 2000s, several genotype-based techniques were introduced that were designed for regular sample processing in pharmaceutical or microbiological laboratories in various set-ups. The genotypic-phenotypic-based differentiation techniques are now being used for identifying the microorganisms in question. Genotypic method of classification relies on the hereditary information that is represented by the genetic makeup, and phenotypic methods were based on an organism's properties, such as optimal environment (pH, temperature, salt content, etc.), morphology, reactivity to chemicals, staining, microscopic visualization, habit, etc. Phenotypic methods assist in identification of microbes to genus level and sometimes even up to species level, but confirmatory tests require genetic-level tests. Biochemical or physiological tests form the second level of identification, after phenotypic tests. They may be primarily growth-dependent methods, and identification requires the isolation of a pure culture. For genotypic techniques, a pure culture of growth is not required, and these differences have been comprehended in Table 8.1.

This chapter will primarily focus on the techniques of isolating pure cultures of microbes from various body parts, e.g. throat, rectal area and skin, to acquaint the readers with the normal microbial diversity of the human body and be able to understand and perform basic techniques for culturing microbes, identification and

characterization, via basic and automated methods and also computer-aided technique.

8.2 Collection of Samples from Various Parts of the Body

Throat swab may be taken after a sterile tongue depressor has been applied to prevent interference by the tongue.

Method The specific area to be swabbed is around the tonsillar area (touching pharyngeal regions around tonsils, not touching the palate/roof of one's mouth) using a sterile cotton swab and has been demonstrated in Fig. 8.1.

- a. Each student takes swab from one's own throat or from their partner.
- b. The swab should be rolled back and forth near the edges of blood agar plate.
- c. A sterile loop may be used to streak from this region so that single colonies can be obtained by quadrant streaking.
- d. It is mandatory to label the plate with the body part (throat), date, time, name of person and type of agar.
- e. Plates are to be incubated at 35–37 °C for up to 72 h, as required.
- f. A number of well-isolated colonies should be selected, and Gram staining should be done as well as catalase test (precaution: RBCs have catalase; so with the colonies, agar should not be picked up as it has RBCs).
- g. Table 8.2 gives a summary of colonies expected on agar plates and their result of Gram staining.

Skin culture may be taken using a moistened swab by rolling on an exposed part of the skin.

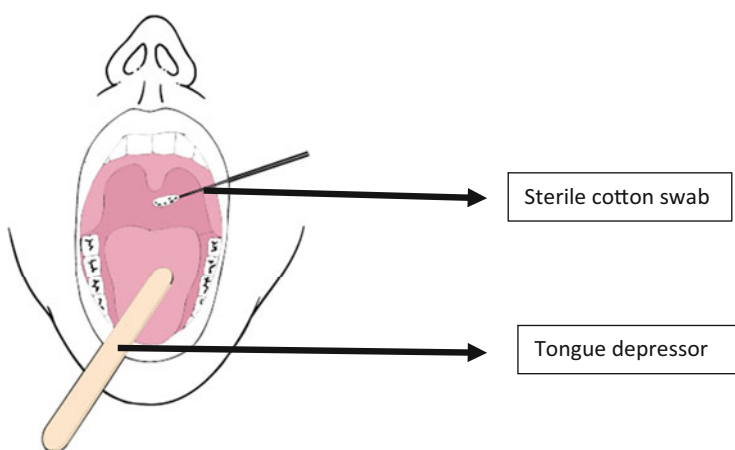


Fig. 8.1 Sample collection from tongue using cotton swab

Table 8.2 The variety of expected microbes isolated from various body parts of human

Plate	Appearance on plate	Microorganism	Staining/microscopic view
Blood agar	Dome-like colonies surrounded by greenish zone	<i>Streptococcus viridans</i>	Gram positive, cocci and chains
	Similar but has depressed centre	<i>Streptococcus pneumoniae</i>	Gram positive, cocci, pairs and chains
	Raised colonies surrounded by clear zone	<i>Streptococcus pyogenes</i>	Gram positive, cocci and chains
	Large, round, tan to yellowish colony surrounded with clear zone	<i>Neisseria</i> spp.	Gram positive, cocci in clusters
	Vary with species; some look dry, wrinkled	<i>Corynebacterium</i>	Gram-positive rods, club shaped, barred, bipolar (pleomorphic)
Tryptic soy agar	Large, round, white, glistening colony	<i>Staphylococcus epidermidis</i>	Gram-positive cocci in cluster
	Large, round, glistening, generally yellow	<i>Staphylococcus aureus</i>	Gram-positive cocci in cluster
	Large, round, moist colony	Yeasts	Large budding cells
	Round or irregular; surface dull becoming		Gram-positive rods, single and chains
	Large, round, yellow, glistening colony	<i>Micrococcus luteus</i>	Gram-positive cocci
Eosin methylene blue agar	Colonies are 2–3 mm in diameter and have greenish metallic sheen in reflected light, dark or even black centre in transmitted light	<i>Escherichia coli</i>	Gram-negative rods
	No metallic sheen, grey-brown centres by transmitted light; colonies are 4–6 mm in diameter, raised and mucoid, tending to become confluent	<i>Enterobacter aerogenes</i>	
	Colourless colonies	<i>Proteus vulgaris</i>	
	Irregular colourless colonies	<i>Pseudomonas</i>	

Method

- Each learner can prepare a swab by moistening with 0.85% saline (extra liquid to be squeezed and discarded) and rolling it on his/her arms.
- A TSA plate should be streaked in this case, instead of blood agar (as in the case of throat swab).
- Labelling should be done according to the method stated above and incubated, in inverted position at room temperature for overnight or up to 72 h.
- The colonies should be stained by Gram stain.
- General results have been tabulated in Table 8.2. The observations obtained may be matched with the table for identification.

For obtaining a rectal culture, the swab has to be obtained in a sealed tube and taken to rest room.

Method

- a. A sterile cotton swab kept in sealed packet or tube and an EMB plate should be taken to the rest room.
- b. Culture from rectum may be obtained by insertion of a swab (1/4:1/2 inch) into the rectum.
- c. About 1/3 of the EMB plate may be streaked with the swab in the rest room.
- d. The swab should not be flushed down.
- e. It should be placed properly, sealed, contained and discarded with safety measures.
- f. In the laboratory, a sterile loop should be used to streak the culture, and plate may then be incubated as mentioned in the case of throat swab.
- g. The common rectal isolates may be differentiated using biochemical tests (Sect. 8.3), although prevalent stains are *Escherichia coli* and *Enterobacter aerogenes* (see Table 8.2).

8.3 Biochemical Tests for Identification of Bacteria

The biochemical or physiological characters of a microbe are useful for developing methods to classify and identify them. Analysis of the functional, nutritional or metabolic abilities of an isolate can aid in the development of specific approaches to determine the taxonomical position and identification of the organism. Few microbes have special features that help us in identification and classification, e.g. storing certain compounds in the form of granules or lipid bodies within their cell. The examples are certain non-fluorescent strains of *Pseudomonas* that store poly- β -hydroxybutyrate (PHB) or some oleaginous microorganisms that store oil globules or produce toxins or show resistance to some antibiotics, which can be used for biochemical identification. However, other techniques are based upon metabolic or biochemical pathways present in the microbe, such as ability to consume certain sugars and ability to live in presence of oxygen or produce enzymes, e.g. catalases, proteases, etc. These help in identifying the microorganisms in small laboratories and are usually undertaken in a small number of test tubes and one after the other. Nowadays, the advent of latest techniques, e.g. Biolog's system (developed by Biolog Inc.), depends on series of biochemical tests that are almost performed in synchronization and may be analysed using computer-aided software. Biolog's system can identify and classify various microbes depending on the capability of metabolizing specific chemicals/biochemical compounds and also on some of their physiological nature, including oxygen tolerance, temperature, pH, salinity and chemical sensitivity. It uses all major classes of biochemical tests for its analysis. Identification is based on manual or semi- or completely automated equipment.

In this section such unique feature-based techniques will be discussed, and also semi-automated and automated techniques would be described, such as carbon utilization and metabolite production.

8.3.1 Carbohydrate Fermentation

Carbohydrate fermentation by microbes leads to production of acids and gases that may be detected to understand the capability of any microbe for utilization of sugars. Each microbe uses different carbohydrates in a different way and up to varied extent depending mainly on their metabolome or secretome. During fermentation, sugars and sugar alcohols undergo anaerobic breakdown, and organic acids are produced. If the medium contains an indicator for determining alkali or acid, the anaerobically produced acid changes the colour of the indicator, confirming the presence of an acid. The pH indicator used is phenol red which is red at neutral pH and yellow in slightly acidic pH that is formed due to weak acids. If hydrogen or carbon dioxide is emitted, a Durhams tube may be placed in the fermentation broth, and the bubbling of gas may be visualized.

Requirement: Test tubes, phenol red, sugar (for fermentation)

Method

- Inoculate the culture into appropriately labelled fermentation medium using an inoculation loop.
- Different sugars, e.g. glucose, sucrose and lactose, was used for preparing broths.
- For every tube inoculated, an uninoculated control should be maintained.
- Incubation should be done at 37 °C for 24 h.

Observation

Gas bubble formation and change in medium's colour indicated fermentation.

Result

8.3.2 Indole Production Test

Tryptophanase enzyme found in microbes enables them to oxidize tryptophan (amino acid) and leads to formation of indole, ammonia and pyruvic acid. In the medium, tryptophan is added that may be assimilated by the microbes, and indole is produced, which is detected by Kovac's reagent (cherry red layers are produced due to *p*-dimethyl amino-benzaldehyde). Positive colonies therefore give cherry red layers indicating that the test is negative.

Ehrlich's reagent is an alternate reagent used for classifying the members of *Enterobacteriaceae* family (Gram-negative gut microbes) and is more sensitive to Kovac's reagent.

Method

A bacterial broth culture is supplemented with tryptone water and incubated for 1 or 2 days at 37 °C. The experiment may be conducted in tubes.

To about 10 ml of peptone water inoculated with bacterial culture, 1 ml Kovar's reagent may be added. An uninoculated control set should be maintained.

Tube should be gently shaken after 10–15-min intervals and a pink top layer appears. Absence of pink colour is negative test. The examples for indole-positive bacteria are *E. coli* and *Proteus vulgaris*, whereas Gram-negative bacteria, e.g. *Klebsiella* sp., *Proteus mirabilis*, etc., give a negative reaction.

8.3.3 Methyl Red Test

Enteric microorganisms can oxidize sugars like glucose for energy production, and the end products of this process will vary depending on the specific enzymatic pathway present in the bacteria. For the test, a pH indicator like methyl red is used to detect the production of acidic organic compounds that result in decreasing the pH. The test can differentiate *Enterobacter aerogenes* from *Escherichia coli* (both coliform bacteria that cause food-/water-borne diseases), and so this test helps to understand the quality of food, water, etc.

At the end of incubation period, *E. coli* produces acidic pH of about 4, but *Enterobacter aerogenes* converts the acid into non-acid alcoholic products (2,3-butanediol and acetyl methyl carbinol), using intracellular enzymes, and pH of 6 is maintained. At pH level 4, the indicator methyl red turns red throughout the tube, indicating *E. coli* in the sample. At pH 6, the indicator gives yellow colouration indicating the presence of *Enterobacter aerogenes*.

Requirements

MR broth, bacterial culture in log phase, inoculating loop

Method

Bacterial culture should be inoculated in tubes containing MR broth using an inoculating loop, and an uninoculated tube should be kept as control. Tubes should be incubated for 24–48 h at 37 °C.

Following incubation, five drops of MR indicator may be added to the tubes and mixed properly, and the development should be observed.

Stable red colour indicates the presence of *E. coli*, *Proteus* sp. and *Salmonella* sp.; red colour turning to yellow indicates *Klebsiella* and *Enterobacter* sp.

8.3.4 Voges-Proskauer Test

Microbes that can ferment sugars and produce neutral/non-acidic organic compounds, e.g. acetyl methyl carbinol or 2,3-butylene glycol, will give positive test with Barrett's reagent. 2,3-Butanediol being a neutral end-product resulting from

glucose fermentation does not change the media pH, and MR test gives yellow colour. Hence VP test is done to confirm. VP reagent consists of a mixture of 40% KOH (potassium hydroxide) solution and alcoholic α -naphthol (α -naphthol is Barritt's reagent A and KOH is B). The end product which is acetyl methyl carbinol should be oxidized to a di-acetyl compound. The VP test is given by the intermediate product, i.e. acetoin formed just before butanediol in the pathway. Acetoin reacts with VP reagent (α -naphthol + KOH) to give pinkish red complex (rose colour) upon exposure to oxygen, which indicates positive test.

Method

Using a sterile technique, the experimental organism may be inoculated into VP broth by means of a sterile loop, and an uninoculated tube should be kept as control. All the tubes should be incubated for about 24–48 h at 37 °C. After completing incubation time, around 3 ml Barritt's reagent A and about 1 ml Barritt's reagent B may be added to all tubes and also in control.

After shaking the tubes gently for ~30 s, the caps should be taken off to allow oxygen into the tubes.

The reaction may be completed in 15–30 min, and crimson red colour in tubes should be observed.

A positive test is given by *Klebsiella* sp., *Enterobacter*, etc. whereas negative test is given by *E. coli* and *Proteus* sp.

8.3.5 Citrate Utilization

Citrate utilization test is commonly employed as part of a group of tests, the IMViC (indole, methyl red, VP and citrate) tests, that distinguish between members of the *Enterobacteriaceae* family based on their metabolic by-products. This test is done by inoculating microbes in a synthetic medium, e.g. Simmons citrate agar (solid) or Koser's citrate medium (liquid), which has sodium citrate as the only carbon and energy source.

Citrate utilization test can differentiate amongst enteric bacteria based on their citrate utilizing capability as a sole carbon source. Assimilation of exogenous citric acid occurs due to the action of permeases (citrate transport proteins). This citrate is lysed by enzyme citrate lyase to oxaloacetate and acetate. The oxaloacetate is then metabolized to pyruvate and CO₂. The CO₂ released subsequently reacts with water and sodium ions of the medium producing alkaline sodium carbonate. The shift in pH turns the bromothymol blue indicator in the medium from green to Prussian blue above pH 7.6. When the medium is acidic (about pH 6.8 or below), green colour is obtained or blue colour in alkaline pH. The appearance of blue colour indicates positive reaction.

Requirements

Bacterial broth in log phase, Simmons citrate agar slants, inoculation loop

Method

Bacterial culture in log phase may be streaked on the slants of Simmons citrate agar, and slants were incubated at temperature of 37°C for about 24–48 h. The uninoculated sample was used as a negative control.

Blue colour of the medium indicates the presence of citrate utilizing *Klebsiella* or *Enterobacter* sp.

8.3.6 Urease Test

The enzyme urease is detected when microbes are cultivated on urea broth medium, where phenol red is the pH indicator. Urea is enzymatically cleaved to form ammonia and carbon dioxide as products. This test is also called CLO test (*Campylobacter*-like organism test) and reaction for the presence of urease.

Method

Bacterial cultures, slants of Christensen's urea agar, inoculation loop

Procedure

The test sample should be inoculated into the slant using a sterile loop, and an uninoculated tube is used as control. Slants should be incubated for 24 h at 37 °C, and pink colour may be detected.

8.3.7 Catalase Test

Catalase is produced by several microbes living in oxygen-rich environment to break down H_2O_2 and other toxic forms of oxygenated metabolites. So this phenomenon helps microbes to neutralize the effects of hydrogen peroxide and protects them. Anaerobes generally lack the catalase enzyme. Catalase mediates the breakdown of hydrogen peroxide H_2O_2 into oxygen and water. Catalase-positive bacteria can give oxygen bubbles if inoculated in hydrogen peroxide solution, in small quantities. Strict aerobes and facultative anaerobes may be catalase positive having the ability to carry out respiration by using O_2 as a terminal electron acceptor.

This test is used for distinguishing amongst Gram-positive cocci, e.g. *Staphylococcus* and members of the *Enterococcus* and *Streptococcus* (catalase negative). It can also differentiate between *Bacillus* species and aero-tolerant *Clostridium* strains (negative). Identification of *Mycobacterium tuberculosis* and *Enterobacteriaceae* (catalase positive) may be done.

Requirement

A 24-h-old bacterial culture, glass slide, Petri dish, 3% H_2O_2 , applicator sticks

Method

A microbial colony may be transferred to a clean and dry glass slide via inoculation loop to which a drop of 3% H_2O_2 is placed and mixed properly to spread the culture. Bubbling of oxygen (almost immediately) indicates positive test. Scattered or no bubbles indicates negative test.

Notes

- Slide should be disposed off in the biohazard glass disposal container.
- Metal loop or needle should not be used in the presence of H_2O_2 . It might corrode the metal and show a false-positive result.
- If colonies are picked from blood agar plates, all agar containing RBC should be scraped off as blood cells are catalase positive and give positive test.

8.3.8 Coagulase Test

Staphylococcus aureus can secrete coagulase that clots plasma into a gel or can agglutinate cocci cells. This test can differentiate between *S. aureus* and other strains of *Staphylococci*. Two types of coagulases are produced by *S. aureus*, free coagulase and bound coagulase. While free coagulase is an enzyme that is secreted extracellularly, the bound form of coagulase (protein) is associated with cell membrane. Free coagulase can be detected via tube coagulase test, and the bound coagulase enzyme may be detected via slide coagulase test. Slide coagulase test can also be used for screening of *S. aureus* isolates followed by tube coagulase for confirmation. There are seven types of free coagulase, but only one bound coagulase form exists. Also, free coagulase generally seems to be heat labile as compared to bound coagulase.

Coagulase Slide Test The bound coagulase is also called clumping factor. It cross-links α and β chain of fibrinogen in plasma to form fibrin clot that deposits on the cell wall. As a result, individual coccus sticks to each other and clumping is observed.

Coagulase Tube Test Free coagulase enzyme produced by *S. aureus* reacts with CRF (coagulase reacting factor) in the plasma forming a complex called thrombin. This in turn transforms fibrinogen to fibrin protein and clot formation is observed.

Requirement

Clean and dry glass slides, test tubes, EDTA anticoagulant of human plasma, culture, sterile inoculating loop

Method

Slide Coagulase Test: Dense suspensions of *Staphylococci* culture are spotted on the two ends of a slide and labelled as “test” and as “control”, respectively. The control is maintained to rule out possibilities of false-positive results due to auto-

agglutination. A drop of citrated plasma is put on the “test” and mixed properly. Clumping of the cocci (agglutination) that occurs almost immediately, within 5–10 s, is considered to be a positive reaction. Some strains of *S. aureus* may not produce bound coagulase, and such strains must be identified by tube coagulase test.

Coagulase Tube Test

Three test tubes labelled as “test”, “positive control” and “negative control” are taken and filled with 0.5 ml of rabbit plasma (10 times diluted). The “test” should have about 100 µl of log phase grown culture of the given microbe, “positive control” should contain 100 µl of overnight grown broth culture of a strain that gives positive coagulase test, and negative control may have water or 100 µl of overnight grown broth culture, of any known coagulase-negative strain. After incubating the tubes at 37 °C for 4 h, observations may be recorded. In the “positive” tube, gelling of plasma is observed even after inversion of the tube, whereas in the “negative” tube, this phenomenon does not occur. The test sample is then observed for a positive or negative reaction. Usually coagulase is secreted by *Staphylococcus aureus*, whereas *E. coli* is coagulase negative.

8.3.9 Lactophenol Cotton Blue

Lactophenol cotton blue (LPCB) staining technique (Fig. 8.2b) is used in identifying fungi and yeast. In LPCB staining, cotton blue stains the chitin present in the cell wall of fungi and yeast and imparts a dark blue shade to the fungal cells, which are prominently visible against light blue background. The cytoplasm of cells also takes a light blue colour stain. The phenol acts as fungicidal agent, and the lactic acid component is a preserving agent that does not allow the dead cells to disintegrate. Various mycelium can be visualized to give distinct morphology and conidium structure.

Requirement

LBCB stain, fungal culture, needle or inoculation loop

Method

For staining fungal mycelium with lactophenol cotton blue stain, a freshly grown culture from the periphery of agar plates may be used. One or two drops of LPCB stain should be put on a clean dry slide, and then a very small amount of fungal culture should be picked up from the plate using a sterile needle and teased and spread in the stain. Coverslip should be placed carefully to avoid any air bubble, and the excess LPCB stain should be soaked by a piece of tissue paper before observing the slide under a microscope with objective lens at 10× and then 45×.

The structure of the mycelium and conidia helps in the identification of fungal sample.

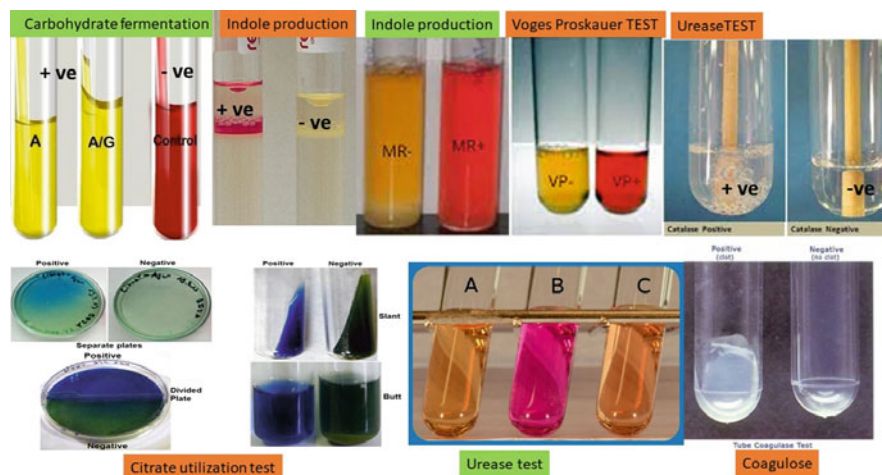
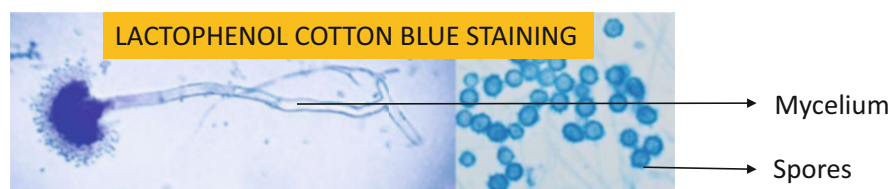
a**b**

Fig. 8.2 (a) Various biochemical tests performed for bacterial identification. (b) Lactophenol cotton blue stained fungal mycelium and spores

8.4 Rapid Multitest Systems

The above techniques involved in determining microbial characteristics, identification and classification eventually, require a significant amount of chemicals, media, culture plates, test tubes and, above all, labour plus time. Also manual or mechanical errors may be introduced at different steps such that even with sufficient controls, errors cannot be totally evaded. In order to reduce the above factors, several quick multi-testing protocols have been developed for identifying medically important microbial species.

McDonnell Douglas Corporation launched a separate program for transfer of basic microbial load monitoring technology to clinical application, in 1973. The result was development of a system called the AutoMicrobic System (AMS), which totally evaded the traditional techniques and procedures used for identification and detection in microbiology. The system used the concepts underlying traditional processes to design and improvise techniques involved in identifying specific

organisms or a class of microbes, in the presence of similar competing organisms, when directly sampled out from clinical samples. This novel approach was made possible via evolution in the area of selective growth media that were specially designed to a sealed micro-environment (micro-cuvette), using minimal volumes of selective broths (about 30 μ l of broth). After inoculating the clinical sample or strain in microcuvettes, they are incubated within an automated equipment fitted with an optical system to monitor all changes in the transmission of light via the microcuvettes. When all prerequisites have been completed or understood and applied, the mini-computer can display the results plus provide a printed copy after about 12–13 h of incubation.

Another recent and more evolved system is of the API 20E System. This system is a miniaturized but standardized version of traditional biochemical tests used for identifying *Enterobacteriaceae* and also other Gram-negative gut bacteria. Around 127 taxa may be identified through this system which is nearly a “ready to use” micro-tube system with the ability to perform 22 standard biochemical tests, on a pure bacterial specimen picked up from a primary isolation media. The methodology is very user-friendly and easy to perform. There is a strip with 20 chambers, each having a micro-tube and also a depression called cupule. Dehydrated substrates are placed in the micro-tubes which can be rehydrated when a bacterial saline suspension is added to them. To mimic anaerobic systems, sterilized mineral oil may be added to the micro-tubes. The strip containing these micro-tubes is incubated at 35 °C to 37 °C for 18–24 h, to allow the bacterium to utilize or metabolize the substrates. All reactions are colour change ones, and the strip is therefore read by noting colour changes after the various indicator systems have been affected by the metabolites or added reagents (Fig. 8.3). All unknown samples are provided with a seven-digit PIN (profile index number) after consulting with guidelines of API 20E

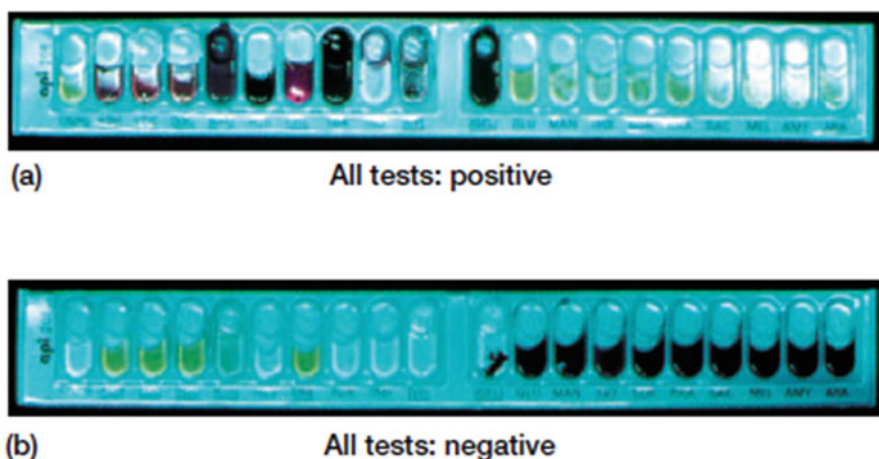


Fig. 8.3 Microbial identification using the API 20E Manual Biochemical System. (a) Positive result is shown in these tubes. (b) The results indicate a negative result in each tube. Adapted from Harley-Prescott (2002) Laboratory Exercises in Microbiology, Fifth Edition, p 209

Profile Index Booklet or the API 20E Profile Recognition System. These charts help in identifying the unknown specimen.

8.4.1 Automated Validation of Every Result (VITEK) System for Microbial Identification

In the last 30 years, various automated systems have been developed to facilitate identification of microbes and also for carrying out antimicrobial susceptibility tests (AST) after interpreting the results on the automated system. These tests are performed on a micro-dilution tray, and after overnight incubation they are spectrophotometrically analysed, and data is recorded (Stager and Davis 1992). In the 1970s, the VITEK platform was found for automated identification and AST analysis which evolved further to the present form VITEK 2 system, and this can automatically perform all steps involved in identification and AST. The initial manual step required is preparation of a primary inoculum and standardization of process (Funke et al. 1998). This new system (Fig. 8.4) allows kinetic analyses by recording data from every test after 15-min interval. A system developed by combining an optical system with multi-channel photometer and fluorimeter is “API 20 Strep” for members of the *Streptococcaceae* family. Other tests developed include serological typing of the streptococci with beta-haemolytic ability, coagulase test with *Staphylococcus aureus*, optochin susceptibility test for *S. pneumonia* and yellow pigment formation plus motility test for enterococci.

Bacterial diseases commonly caused by *Streptococcus agalactiae*, *Enterococcus* spp., *Streptococcus pneumonia*, *Staphylococcus* spp., etc., which are resistant to multiple drugs, need thorough investigation through identification and serotyping. Therefore the VITEK 2 platform (version of software, VT2-R01.02) was tested for identification and AST of Gram-positive cocci (Woodford et al. 1995; Murray



VITEK[®] 2 Compact (bioMérieux) is an automated system for extended identification database, enabling the detection of varied and broad range of microbes, through an optimized workflow. The bar-coded cards endorsing complete reliability and traceability minimizes the risks of transcription error. In this system, every process is automated and so set up and response are easy and time efficient. About 2 to 18 hours are sufficient in most cases. The VITEK[®] 2 Compact software is efficient, and productive, requiring less technical support from trained personnel. The efficiency relies on an advanced colorimetry technique where the latest generation of VITEK test cards, containing 64 wells for accuracy are read. Every 15 minutes three wavelengths are used for reading the system and the data generated thus is analysed and results are generated, ensuring high accuracy.

Fig. 8.4 VITEK 2 Compact system with cards (a, b) and the instrument. This system allows kinetic analyses by recording data for every test after intervals of 15 min. The optical system records turbidity, fluorescence and colorimetric signal by combining multi-channel photometer and fluorimeter readings

1990), using a rapid and accurate system of identification along with evaluation of MIC of certain antimicrobial chemicals, since these pathogens are becoming alarmingly drug resistant.

The total time needed for identifying the strains varies. Ligozzi et al. (2002) reported that the VITEK 2 system was able to identify all *S. pneumoniae* and *S. agalactiae* isolates within 2 h. They also reported that about 98% *S. aureus* strains and about 71% CNS (coagulase-negative staphylococci) could be identified in approximately 3 h. For identifying the other isolates, e.g. in the same study performed using VITEK 2 system, the researchers achieved equal accuracy rates during the identification of *Staphylococci*. Very few taxa had low degrees of discrimination during identification of strains. For such strains, alternate routes of identification were used, e.g. one strain of *S. agalactiae* could not be easily differentiated from a very similar strain of *Streptococcus acidominimus*. Therefore, this discrepancy had to be resolved by using immunological tests. Garcia-Garrote et al. (2000) tested and documented the validity of the VITEK 2 platform as 76.3% and 98.3 in the identification of *E. faecium* and *E. faecalis*, respectively; in that study the latter species was mostly misidentified as *E. casseliflavus*-*E. gallinarum*. For detecting oxacillin-tolerant staphylococci using VITEK 2 system, high accuracy was recorded (Ligozzi et al. 2002). The VITEK 2 system also correctly distinguished all the enterococci which showed vancomycin resistance. Supplementary tests were needed for the accurate identification of phenotypes or genotypes. Therefore, the VITEK 2 system is an easy-to-use system that provides a rapid (4–15 h) and accurate means for identification and AST of most commonly isolated species of Gram-positive cocci.

8.4.2 Biolog: Phenotype Microarrays

To complement the traditional methods by proteomics, transcriptomics and genomics-based analyses, “phenotype microarrays” have been developed. They offer the opportunity to scientists and clinicians for correlating and confirming the microbial systems analyses with modelling, to get a broad and quantitative assessment of the microbial physiology and metabolism to various substrates and environmental conditions. “Biolog” phenotypic assays can achieve these analyses by coupling tetrazolium dyes to minimal media containing defined nutrients, so that the impact of different sources of nitrogen, carbon, phosphorous, sulphur, etc. can be understood and also quantified. The sources of redox reaction sources that induce the electron transport chain can also be determined. Biolog’s assays are highly reproducible and sensitive for distinguishing between the related isolates, even if they are quite similar metabolically. It helps in understanding metabolic differences and modelling their physiological behaviour via flux balancing analyses. The various components/reagents and protocols of Biolog’s phenotype microarray system have been described hereby, in particular for identification of microbes and to characterize them and understand their metabolic capabilities (Shea et al. 2012; <https://www.biolog.com/support/bibliography/>).

From Genomics/Proteomics to Phenotype: Biolog's Phenotype MicroArrays

Nucleic acid-based microarrays and latest proteomics-based techniques enable scientists to determine exact genes or proteins that may be co-regulated and also find out if changes in their cellular levels correlated with any specific incidence such as a disease state or a particular agent. These certainly do not ensure that such changes are significant for cells, and confirmation has to be done through other assays. Phenotype MicroArray technology is a complementary technique to provide the necessary information at cellular level. These MicroArrays have been pre-configured sets of phenotype-related tests organized on micro-plate panels. In the panel, each well of the array is inoculated with a standardized cell suspension to test a different phenotype, which allows the simultaneous tests of several phenotypes within a single experiment.

Phenotype MicroArrays use the patented redox-based technology from Biolog, which uses cellular respiration coupled to NADH production, as the reporter universally. If we find a strongly “positive” phenotype in a particular well, it means the cells show respiration actively that reduces a tetrazolium indicator dye and give a deep colour. Less intense colour development or absence of colour indicates that cells are respiring weakly or not being able to respire under given conditions, which therefore means reaction is weakly positive or totally negative. This assay based on redox reaction can provide for the amplification process and also allow quantifying the specific traits from various phenotypes very precisely. Incubating and data compilation of phenotypic trait can be performed by the OmniLog instrument, automatically.

For comparing two cell lines phenotypically, data for the first is recorded using a reddish tracing dye, and for the other, a green dye is used for tracing. Graphs are then overlaid using bioinformatics-based tools for detecting the differences between the two cell types. The overlapping area get coloured by yellow, and subsequently differences between them get highlighted with green or red (Fig. 8.5).

Kinetic Data Capture and Analysis

The OmniLog PM software consists of a suite of algorithms which can work in synchronized manner with the Phenotype MicroArray panel and OmniLog PM platform for automating the incubation of about 50 micro-plates, under fixed temperature controlled by users and having a comprehensive collection of assay data (from colorimeter) versus time. Such programs facilitate the displaying of kinetic data obtained from PM panels that were recorded by OmniLog PM system. The

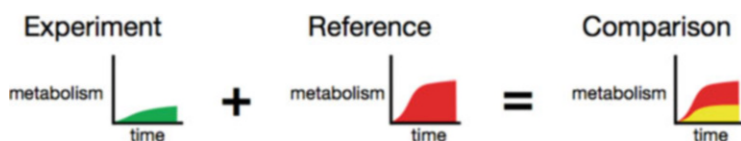


Fig. 8.5 Comparison of experimental and reference graphs to understand the phenotypic similarities or differences between cell lines/types

programs also systematically store the data and analyse it along with exporting it in various raw and processed formats, for displaying in presentations and reports (<https://www.biolog.com/products-portfolio-overview/phenotype-microarrays-for-microbial-cells/>).

Functions of the Software

- Driving the OmniLog PM system
- Guiding of loading and plate-reading or reading PM panels
- Creating kinetic data files for every plate/PM panel for subsequent application to other program modules

Kinetic analysis includes assembling plates or data files of PM panel into lists, displaying kinetic plot of the listed data and facilitating creation of figures with the kinetic plot data in the form of bmp or jpeg files.

Parametric analysis extracts the data list available from kinetic analysis/file management module to calculate the parameters from the obtained datasets and facilitates the comparison of various data lists. A report can be then generated with specified highlight on PM phenotypes showing significant variation for a specific or selected kinetic parameter, used for analysis. It helps to identify the substrates included in all PM panels and creates a link between various metabolic substrates, and the PM panels therefore get linked to the KEGG database. The data may be exported to the original form of OmniLog kinetic data, and also the data parameter (s) used for any bioinformatics and statistical analyses may also be exported for records.

8.4.3 Electromigration Techniques

Currently, electromigration-based techniques have attracted a lot of interest for identification of microorganisms (Table 8.3 excel). This technique is relying on migration of some charged ions/molecules when a homogeneous electric field is applied. Due to differences in electrophoretic mobility, test molecules are separated, and the migration is directly proportional to their charges (Buszewski and Kłodzinska 2016). The most common electromigration techniques are GE (gel electrophoresis), two-dimensional GE, capillary zone electrophoresis (CZE), capillary isotachopheresis and CIEF (Petr and Maier 2012). The surface of the cell has biopolymers, e.g. phospholipids, polysaccharides, receptors, membrane proteins and other small molecules, which result from metabolism or are produced due to cell signalling molecules (Buszewski and Kłodzinska 2016). The components have many functional groups which dissociate and thereby affect the ionic interactions between the double-layered cell wall creating a z-potential on the cell's surface (Crispo et al. 2016). Thus, because of their charge and size, microbes can be considered as charge-containing biologically active colloidal entity (i.e., biocolloids). Every species possesses unique spectroscopic fingerprints, e.g. UV spectra that are recorded by a diode array detector. Under some specified condition

Table 8.3 Indication of type of culture, based on sugar utilization abilities

Sugar	Fermentation result	Microorganism
Glucose	Acid production only (colour change)	<i>Staphylococcus aureus</i>
	Acid plus gas formation	<i>E. coli</i> , <i>Klebsiella</i>
	No fermentation	<i>Acinetobacter</i>
Lactose	Acid production	<i>S. aureus</i>
	Acid production and gas formation	<i>E. coli</i> , <i>Klebsiella</i>
	No fermentation	<i>S. typhi</i> <i>S. paratyphi</i> <i>Pseudomonas</i> sp.
Sucrose	Acid production	<i>S. aureus</i>
	Acid production and gas formation	<i>E. coli</i> , <i>Klebsiella</i>
	No fermentation	<i>S. typhi</i> <i>S. paratyphi</i> <i>Pseudomonas</i> sp.

during analysis, cells can move along the electric field with varying velocities. Because of such properties, identification and characterization of microbes become facilitated via their electro-migration properties (Crispo et al. 2016). Microbes (tabasco moscati virus and *Lactobacillus casei*) were first time analysed by Hjerten et al. (1987) using CE (capillary electrophoresis). Later, Ebersole and McCormick (1993) could suitably separate *S. pyogenes*, *S. agalactiae*, *S. pneumoniae* and *Enterococcus faecalis* using CZE.

The configuration and type of surfaces play a determining role in identifications via electro-mobility tests. At various time points during the growth of organisms, their surface properties change, thereby changing the charges on the surface and the z-potential consequently. Certain factors imposed by the environment also lead to such changes (Buszewski and Kłodzinska 2016; Petr and Maier 2012; Crispo et al. 2016). Growth of microbes may be monitored continuously by determining the z-potentials. Soni et al. (2008) measured z-potentials of bacteria during various stages and differing growth parameters, by conducting CE-based experiments, and they showed that in the presence of a nutrient-rich medium, z-potential showed greater absolute value in comparison to the nutrient-devoid or starved/dead cell minimal media. Researchers used CZE to separate three strains of *Saccharomyces cerevisiae* electrophoretically which showed varying growth phases. After regular time intervals, during cultivation of organisms, the electrophoresis profile for the three strains showed two distinct peaks. Such studies indicated that employing CE to identify various yeast strains from fermented grape, and also to assess their viability in a fermentation medium, was successful. Such information can be implied to identify and therefore control bacterial or yeast growth in food material. The major shortcoming of the separation via electrophoresis is when microorganisms try to interact with each other or with internal surface of the capillary causing agglomeration and adhesion, respectively. Adhesion occurs due to positive charges present on microbial surfaces that readily interact with silanol groups of the capillary tube.

A dominant influence on the aggregation of cells and, thus, on the behaviour of microorganisms during electrophoretic separation does have a z-potential value. Researchers showed that (Kłodzinska et al. 2010) in electropherograms, the shapes of peaks and numbers depend upon the z-potential values. Results showed that absolute values of z-potential more than 30 mV indicate the presence of stable clusters during the electrophoresis. Gram-positive and Gram-negative bacteria have different z-potentials because of their cell wall differences and are easily differentiated using this technique.

For controlling unnecessary cellular adsorption onto capillary walls, several procedures have been innovated. Adding polyethylene oxide (PEO) in the buffer strategically changed the interior surface of capillaries. Szeliga et al. (2011a, b) identified both Gram-positive coccus-shaped bacteria and rod-shaped Gram-negative bacteria using this method and achieved a specificity and sensitivity of 100% and 88.1%, respectively. In another approach the fused capillary wall after chemically modifying using various monomers, e.g. divinylbenzene trimethylchlorosilane, acrylamide, etc., was successful where the coating prevented cellular adsorption onto capillary surfaces. This coating also suppressed the phenomenon of electro-osmotic flow, such that the cells moved along capillaries using electrophoretic mobility. Buszewski and Kłodzinska (2008) reported that microbes thriving in the GI (gastrointestinal) tract can be identified very rapidly after making various modifications in the interior capillary wall. Horka et al. (2012) identified methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA) from blood samples by employing CZE on capillaries of trimethoxysilane-modified fused silica (FS) that were etched with super-critical water. In about 2 mL human blood sample, around 200 MRSA cells were detected and provided enough datasets for determining effective therapy using antibiotic. Also the sample volume was quite less, and still it was sufficient for rapid screening of clinical specimens. A very quick technique to screen and identify *E. coli* in biological samples using CZE was reported by Szeliga et al. (2011a, b) where nearly 30 infected wounds or ulcer samples were collected to compare the results with conventional techniques. In about 30 min, the *E. coli* that infected surgical wound could be identified at 86.7% and 85% sensitivity and specificity, respectively. A triple-plug injection protocol in conjunction with CE was developed to identify microbes in urine specimen (Song et al. 2013), and the innovation was capable of detection of bacteria in about 10 min time using CE. The CE chromatogram showed some peaks due to urine components, e.g. protein/lipids and cell from blood, but this did not have detrimental effect on the retention time of peaks representing bacteria, and the assay also indicated quantitation of different bacteria, from peak area of the individual peaks in the chromatogram. Therefore, such types of an electrophoresis-based techniques may be employed for quantitative detection of microbial load. Similar studies were carried out for the quantitative detection of microbes in natural waters (Oukacine et al. 2011), by developing a system for repeated pre-concentrated electrophoresis method to analyse bacteria. Isotachophoresis was coupled to a hydrodynamic electro-kinetic injection simultaneously for quantitation of *Enterobacter cloacae*. Electromigration-based methods were appropriately

employed for microbial identification especially to *Candida* sp., which are phenotypically very similar and indistinguishable (the strains which cause bloodstream infection (BSI) frequently). The very first and accurately reproducible technique developed for separating and identifying *Candida parapsilosis*, *C. metapsilosis* and *C. orthopsilosis* was based on capillary isoelectric focusing followed by CE (Horka et al. 2012). This technique was also applicable to biofilm forming species. Horka et al. (2012) employed about five distinct analytical protocols to characterize *Monilinia* spp., namely, CZE first; then CIEF, followed by gel IEF and complemented by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE); and lastly MALDI-TOF mass spectrometry, and found that both CZE and CIEF techniques may be employed for rapid, effective and economical discrimination between morphologically alike species; however the protocol cannot be employed for analysing different strains belonging to one single species. SDS-PAGE and gel-based IEF techniques could also differentiate between the various phenotypically similar *Monilinia* spp., also analyse various strains belonging to the same species. MALDI-TOF MS also may be deemed as a suitable method to differentiate between morphologically similar *Monilinia* spp. very fast, efficiently and with accuracy *Monilinia*, up to strain level. However, the host origin of the spores of *Monilinia* spp. played an important role in analysis via MALDI-TOF MS, and according to a suggestion by Horka et al. (2012), the issue may be resolved by simultaneous data collection jointly with CIEF or CZE techniques.

8.4.4 MIDI Sherlock System for FAME Analysis

There are three important criteria for identifying bacteria in pharmaceutical Quality Control set-up, namely, biochemical tests, fatty acid profile analysis and DNA sequence data. We have discussed several biochemical tests that are manually or automatically performed to facilitate the identification process. The MIDI Sherlock System is designed to identify all aerobic bacteria that are included in its library by using a standard technique for sample preparation (Fig. 8.6a–c), and thus biochemical tests and Gram staining are not required at all. All environmental strains of bacteria should be cultured on a common medium, e.g. TSA (tryptic soy agar) at temperature 28 °C for about 24 h. Bacteria should be harvested from a plate having quadrant streaking, which is closest to log phase growth and provides adequate number of cells for further analysis. Species that respond discretely to fatty acid methyl ester (FAME) analysis include *Bacillus* sp., *Pseudomonas* sp., Gram-positive cocci and rods (such as *Corynebacterium* sp.), *Acinetobacter* sp. and some very unusual organisms ubiquitously found in pharmaceutical labs. The Sherlock System does not require high technical proficiency for operating the machine, and also the cost of consumable is lower than \$3.00 per sample. The most important advantage is that it is a high-throughput system with about 200 samples getting analysed per day. This plus points make the Sherlock System a routinely used technique for microbial quality control. The MIDI Sherlock System has been validated by the National

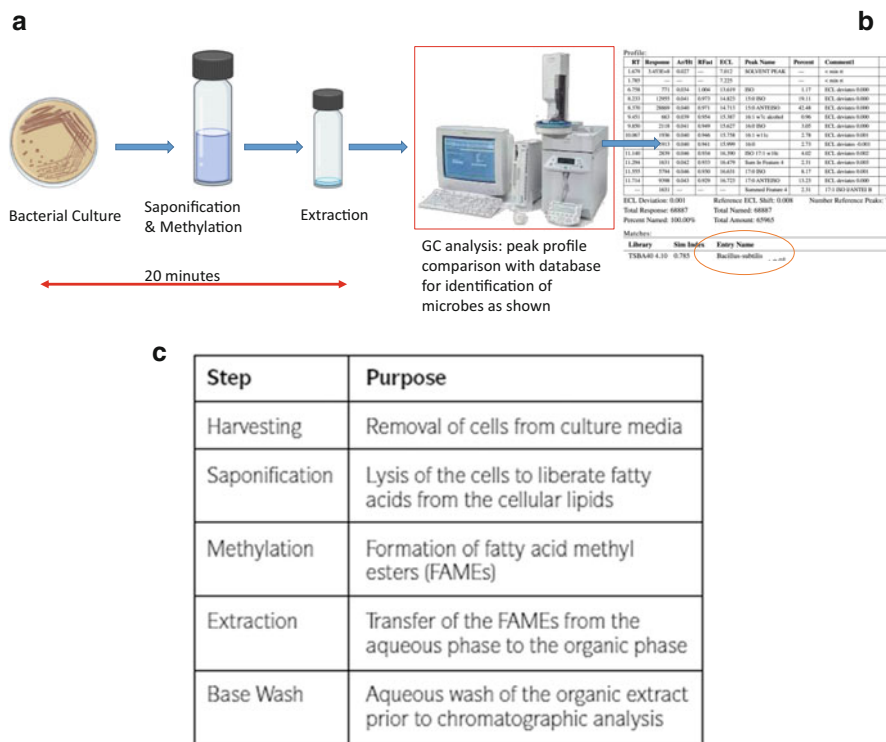


Fig. 8.6 (a, b) The MIDI system and printout from MIDI showing the similarity index of identified organism. (c) The steps for using MIDI for identification of microbes. This has been adapted from the MIDI Sherlock Microbial identification protocol (Kunitzky et al.)

Institute for Occupational Safety and Health (NIOSH) because of its rapid and accurate identification ability for aerobic bacteria (Pendergrass 1998).

Over 300 fatty acids or related components exist in bacteria, and these compounds are extremely informative to project out both genus level differences (qualitative mainly) and species level differences (commonly quantitative). When metabolic pathways for synthesis of fatty acids are deciphered, many other relationships can also get decoded. Thus from 16:0 fatty acid when 16:1 molecule forms, then it requires the desaturase enzyme activity which is direct “mole for mole” conversion. After this step when a bacterial cell gets physiologically more developed, there is a shift from 16:1 to 17:0 cyclopropane molecule, and this reaction is also “a mole for a mole” basis conversion. Such kind of information may suggest that using cells for an actively proliferating stage can minimize critical differences between the microbial cells. Therefore, the process uses about 24-h-old growing culture only from a plate which has quadrant streaking of the culture, and it is known that quadrant streaking of plate actually can reduce such differences that lead to problems. In addition to this standardization, covariance matrix is also used by the Sherlock software for minimizing impacts of such changes.

A controlled temperature for growth and the use of a commercial and standard medium also contribute to reproducibility of results as the medium affects the fatty acid profile. The branched iso- and anteiso-fatty acid chains are commonly found in various Gram-positive bacterial strains, whereas Gram-negative bacteria predominately have fatty acid molecules with straight chains. Presence of LPS (lipopolysaccharide) amongst Gram-negative bacteria increases the hydroxy fatty acids in such genera, and so, the presence of 10:0 3-OH, 12:0 3-OH, and also 14:0 3OH molecules indicates the nature of the organism as Gram negative or positive; absence of LPS layer including peaks of hydroxy fatty acid molecules is indicative of Gram-positive bacteria. This technique therefore allows us to embark upon this FAMES analysis without staining techniques, and like electrophoretic mobility separation-based techniques, FAMES analysis also is independent of presence or absence of plasmid and deletion or insertion of plasmids, in the bacterial cells as seen in several pseudomonads. This is an important conclusion because bacteria keep exchanging plasmids and extrachromosomal DNA from the exterior environment, and if such changes caused alterations in the fatty acid profile, MIDI Sherlock System would fail. Mutagenic treatment with ultraviolet light (causes frame-shift mutagenesis) or ethyl methanesulphonate/nitrosoguanidine (causes point mutagenesis) can kill 99.999% of microbial cells and also alter strains to make them auxotrophic mutants, but these too did not seem to affect fatty acid profiles, when the growth rate of the culture stayed quite normal. This indicates that lipid profile/composition has high degree of conservation at the genetic level, and so significant changes only occur after a very long time gap.

Method

It is necessary to culture the bacteria for analysis by Sherlock System, and then fatty acids should be extracted from the cells using a standardized protocol which starts with saponification with diluted sodium hydroxide and methanol solution, and then saponified fats are derivatized with mixture of dilute hydrochloric acid and methanol for generating methyl esters of the fatty acids (FAMES). These FAMES should then be extracted and transferred to an organic solvent such that the organic phase containing fatty acids can be analysed by GC (Fig. 8.6c).

The Sherlock System presents its results based upon a Similarity Index (SI) which is a number to express the closeness or relatedness of FAME profile of an unknown strain with known strains. The known strain has the entire fatty acid composition compiled as a library for identification of unknown strains according to matches. This "SI" does not indicate "probability" or a percentage; instead it expresses the relative closeness or distances of unknown strain (from the specimen) from a population average (see Fig. 8.6b). Exact matches show SI equal to 1.000 and fatty acids differ the least from the known profile. However, the more the variance from the known strain's profile, the SI decreases, and SI of 0.500 or greater and separated by 0.100 from the first and second hits in the library may be considered as a proper library comparison. If SI falls between values 0.300 and 0.500 plus it stands properly separated from the second hit (above 0.100), then this is considered as a good match, indicating atypical strain. But values less than 0.300 suggest that

microbe may not be included in the library, although the software still indicates closely related species, and such a data might become useful in case a new species is discovered.

The Sherlock library comprises of several different organisms listed by strain, i.e. *Bacillus anthracis* has more than 70 strains alone. So the combined Sherlock library has nearly 2000 species, with 700 aerobic environmental bacteria, 620 anaerobic ones and 200 yeast species.

FAME analysis may be based on nearly a universal feature compiled from all bacteria that possess a unique complementation of cell's fatty acids, and currently the library is expanding to upgrade the system's applicability. MIDI has established an extensive networking platform and has collaborated with researchers all over the world who share their analytical results whenever they encounter novel species or organisms. The data is subsequently employed for strengthening the existing library by adding new information about completely new microbes for which information is lacking. The MIDI Sherlock Microbial Identification System is completely automated comprising of gas chromatography system for identification of more than 2000 species of microorganisms. This is only based upon the unique composition of cell membrane fatty acid profiles. The Sherlock System is accurate, reliable high throughput and inexpensive. It also facilitates data acquisition system complying with the 21 CFR Part 11 data export for analysis of trend. The Agilent GC hardware is also reliable and a fully automatic system, and also its ability of tracking microbes is very unique. Since subjective tests for identification are not required, the system of naming is reproducible and objective. The latest version is capable of incorporating data from DNA sequencing, such that "polyphasic" (genotypic (DNA sequence) combined to phenotypic data) taxonomical analysis can be done.

8.5 Computer-Aided Gene Analysis for Identification of Microbes

The classical method to identify and classify any microbe is based on staining and morphology, biochemical activities and rapid multitext systems, as explained above. With the onset of the new millennium, gene sequencing coupled to bioinformatics support from various organisms is offering newer and quicker means of identification. "Bioinformatics" implies to the field of science that is devoted towards computational study and management of biological data collection, storage, analysis and dissemination. This field has been innovated upon by expertise from the information technology sector and projects dealing with gene sequencing. Database involving DNA, RNA or proteins along with analytical tools required to manage the data are easily available for students and scientists, free of charges on the worldwide web.

8.5.1 Ribosomal RNA Gene Sequencing

It is presently being used for study of diversity amongst various prokaryotes and other microbes or even eukaryotes. Thereafter the phylogenetic relationship amongst

them is analysed. Studies using rRNA have several advantages. All organisms have ribosomal RNA, and these have evolved very slightly over generations and are therefore comparable. In case the sequences were very dissimilar, there would be no comparison possible. Also culturing is not needed for rDNA sequencing. Apart from this, there are some particular sequences that are peculiar to individual species and are called signature sequences or markers, and these too can be used for identification and classification, especially in phylogenetic analysis.

Logically usefulness of rDNA may be contributed by the enormous rate of evolutionary changes found in the various regions of rDNA (can be amongst some species and also within the genes of same species). Also, there are several copies of rDNA sequences per genome, and there exists a specific pattern for combined/concerted evolution occurring amongst the repeated copies. Such features may facilitate analysing of rDNA directly via sequencing of RNA or DNA that is possible either after cloning it or via amplification and also using restriction digestion technique. All the constraints that have been imposed due to rRNA's secondary structure and evolution should be taken into consideration for doing phylogenetic analysis; however, such limitations may be resolved, and though they tend to impede analysis, the usefulness of rDNA/rRNA cannot be questioned (Hillis and Dixon 1991).

Gene sequences of over 16,000 organisms, both prokaryotes and eukaryotes, have been submitted to one or the other public database. Upon submitting new sequences to any database management system, it responds within seconds and gives the most likely homologous (similar base sequence) gene sequence of any species. It may or may not be exact match, and the amount of similarity within the sequences is also compared and reflected (Fig. 8.5). Database for submitting 16S rRNA of bacteria/archaea and 18S/23S and ITS region sequences are submitted to Ribosomal Database Project (<https://rdp.cme.msu.edu/html/>).

It provides ribosome-related data services to the scientific community, such as online data analysis, rRNA-derived phylogenetic trees and aligned and annotated rRNA sequences. Other important websites that contain the link to sequencing databases and computational hubs for sequencing data analyses include the following:

- The Institute for Genomic Research (<http://www.tigr.org/>)
- Genomics: A Global Resource (<http://genomics.phrma.org/>)
- European Bioinformatics Institute (www.ebi.ac.uk/)
- GenBank (<http://www.ncbi.nlm.nih.gov/>). The GenBank is maintained by the National Center of Biotechnology Information (NCBI).

8.5.2 Phylogenetic Analysis

Phylogenetics is the area of bioinformatics that applies a statistical model utilizing the gene sequences to determine evolution and for suitable reconstruction of evolutionary history various taxa. This reconstruction of evolutionary relationships

between taxa is known as phylogeny of the taxa (O'Halloran 2014). In order to establish phylogenetic relationships, theoretical knowledge is needed for selecting one or several sequences or molecular markers. In doing so, nuclear ribosomal DNA (rDNA) and mitochondrial DNA (mtDNA) that illustrate the general characteristics and phylogenetic applications have been most frequently used for phylogenetic studies. Highly conserved regions of molecular markers and genetic sequences are also used for determining phylogeny to elucidate deep branches of evolution. Hypervariable genetic markers or sequences may be useful to elucidate a phylogenetic relationship based on the recently diverging branches of the phylogenetic tree. Varied selection forces lead to evolution of different molecular markers with differing extents, and therefore few species are more evolved than the others. It is therefore necessary to select suitable genetic markers with for depiction of true evolutionary relations over a varied spectrum of taxa through plotting of phylograms.

The mitochondrial DNA or mtDNA present in all multicellular animals is usually a circular DNA molecule, but sometimes it consists of linear DNA molecules about one to two (Bridge et al. 1992). The sizes range from 14 to 17 kb; however, some are about 40 kb in molecular weight. However, genetic variation is little even if sizes vary due to the variations in noncoding DNA of the mtDNA (Stanton et al. 1994). As compared to nuclear rDNA, designing of universal primers for amplification of specified mtDNA sequences is more difficult, because of its increased variability. Therefore, very few mitochondrial gene(s), e.g. 12S rDNA, Cytb, COI and ND1, are used for phylogenetic analysis. It may be noted that mtDNA has been evolving at a faster rate as compared to nuclear genome. The 12S rDNA sequence is more conserved than the nuclear SSU rDNA, which was used for depicting phylogeny at even higher levels where phyla or subphyla are concerned. In comparison to 12S rDNA, 16S rDNA sequences are more variable and are commonly employed for phylogeny-based analysis where family- or genus-level study is required (Kambhampati et al. 1996).

Here a step-wise pipeline has been described to infer the phylogeny derived from a given set of nucleic acid sequences. It gives detailed steps for interrogation of database by using the Basic Local Alignment Search Tool (BLAST) tool via a web-based interface. This is the first step for obtaining the list of homologous or similar sequences to any query sequence provided by the user. The same type of data may be obtained by researchers employing a web interface, e.g. Phylota (<http://www.phylota.net/>). The BLAST tool compares nucleotide or amino acid sequences against the databases of nucleic acids or proteins to obtain "hits" resembling query sequences. The BLAST algorithm was designed by Stephen Altschul et al. (1990) at NIH (the National Institutes of Health). The server of BLAST comprises of various programs that have been listed and explained here:

1. *Nucleotide-nucleotide BLAST (blastn)*: *blastn* program can run a DNA sequence as input, and the most similar sequences are returned as hit from the database using specifications given by the user (e.g. for a specific organism).
2. *Protein-protein BLAST (blastp)*: User fills an amino acid sequence in the box for unknown sequence or may fill in the accession number if the sequence is an

already submitted one. The tool returns most homologous protein sequence that is present in the protein database using the specifications.

3. *Position-specific iterative (PSI) BLAST*: Any protein sequence is used as input, and the tool returns all closely related proteins, and now a conserved profile is generated through this tool. In the next step, another query gets generated that uses only conserved “motif” for interrogating protein databases. This step therefore returns a large set of proteins from which a new set of conserved “sequences/motifs” get extracted, and then these are used to interrogate a protein database until an even larger set of proteins are returned. This creates another profile and this process is subsequently repeated. Identification of sequences which are more divergent gets selected by the repeated steps.
4. *Nucleotide six-frame translation protein (blastx)*: When any nucleotide sequence is used as input, the tool converts it into a predicted/theoretical six-frame translational products (includes positive and negative strands) using a protein database.
5. *Nucleotide six-frame translation-nucleotide six-frame translation (tblastx)*: This tool uses any DNA sequence as the input and can translate it into conceptually six-frame translation products and compares it against six-frame translations of nucleotide sequence databases, subsequently.
6. *Protein-nucleotide six-frame translation (tblastn)*: This tool needs an amino acid sequence as an input for comparing it against all the six translational frames of the nucleotide database.

Method

1. Click on this link to visit the BLAST¹ web server at the National Center for Biotechnology Information (NCBI)—<http://blast.ncbi.nlm.nih.gov/Blast.cgi>.
2. Input a FASTA formatted text sequence into the query box.
3. Click the appropriate BLAST program and relevant database or individual species of interest to use in the search, and then click “BLAST”. *Note*: FASTA formatted sequence begins with a description line indicated by a “>” sign. The description must follow immediately after the “>” sign; the sequence (i.e. nucleotides or amino acids) follows the description on the next line. The output from the BLAST search is viewed as HTML, plain text, XML or hit tables (text or csv) with the default set to HTML.

8.5.3 Generating Multiple Sequence Alignments

1. Links to access common used Multiple Sequence Alignment (MSA) programs may be used, such as the following:
 - (a) ClustalW <http://www.clustal.org/>
 - (b) Kalign <http://msa.sbc.su.se/cgi-bin/msa.cgi>
 - (c) MAFFT <http://mafft.cbrc.jp/alignment/software/>

¹Basic local alignment search tool (BLAST): online interface.

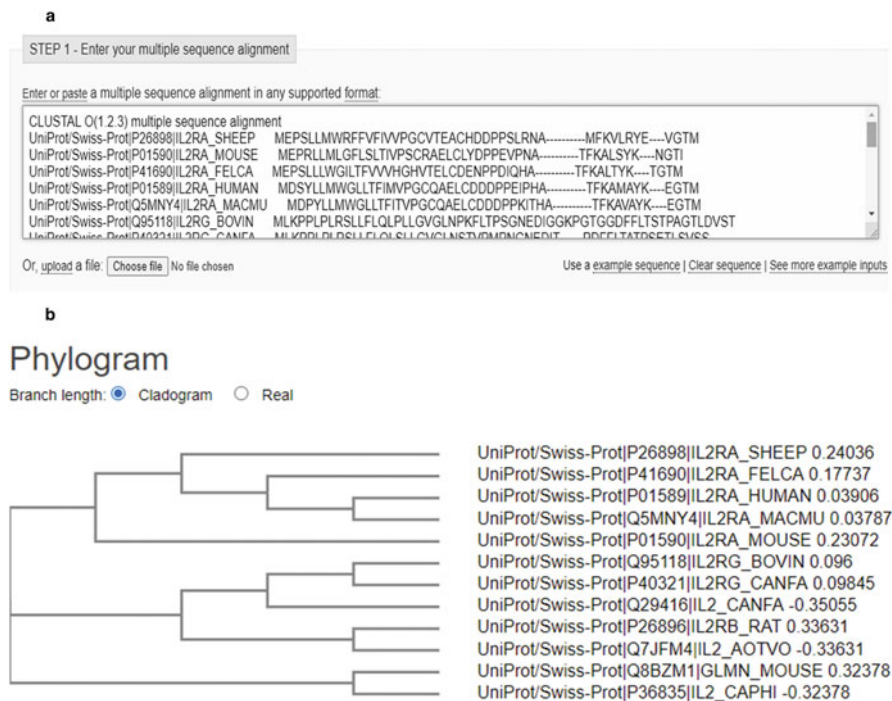


Fig. 8.7 (a) The snapshot of input sequences for simple phylogeny tool. (b) A phylogram constructed using simple phylogeny tool

- (d) MUSCLE <http://www.drive5.com/muscle/> T-offee <http://www.tcoffee.org/Projects/tcoffee/>
 - (e) PROBCONS <http://toolkit.tuebingen.mpg.de/probcons>
2. Clustal MSA may be downloaded by clicking this link <http://www.clustal.org/clustal2/> to use a command line version (i.e. ClustalW) or a graphical version (i.e. ClustalX). An appropriate executable (i.e. win, Linux, Mac OS X) may be used.
 3. Sequence data should be uploaded as FASTA formatted sequences in text (*.txt) format, and click align.

Phylogenetic Tree

1. The downloaded alignment file should be uploaded in the webtool: Simple Phylogeny (EBI) https://www.ebi.ac.uk/Tools/phylogeny/simple_phylogeny/#sequence (Fig. 8.7)
2. Set the phylogeny options, e.g. tree format, distance correction and clustering method, and then submit the job.
3. The phylogram appears as seen below (constructed in neighbour joining clustering method) (Fig. 8.7b).

After sequence alignment using Clustal Omega, subsequent phylogenetic tree may be constructed using other programmes, e.g. Maximum Likelihood (ML) of Molecular Evolutionary Genetics Analysis (MEGA) and Bayesian Inference via MrBayes (<https://www.jove.com/t/56684/using-phylogenetic-analysis-to-investigate-eukaryotic-gene-origin>).

8.6 Conclusion

Rapid, inexpensive, effective, reliable and reproducible techniques are required for screening and identifying microbes and need a lot of focus by modern scientists, in order to meet challenges by rapidly growing population and clinical/industry requirements. Replacement of phenotype-based methods, with modern methods, such as Biolog/VITEK, or electromigration techniques not only improves the time consumed but also offers high-throughput options at a lower price per sample. These solutions therefore should be considered for prevailing challenges in the field of clinical diagnostics and food/pharma/biopharma/cosmetics industries. However, these methods are mostly for bacteria and fungi. For viruses, molecular biology-based methods and immune techniques are more valuable.

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Microarrays and Its Application in Medical Microbiology

9

9.1 Introduction

Microarray technology has emerged as a method for high-throughput gene expression analysis, required for a wide range of research goals and objectives (Kozian and Kirschbaum 1999). In the years 1995 and 1996, the term “microarray” was adopted for describing the technique due to its miniaturized form and published by the research team of Pat Brown from Stanford University and soon adopted by others from the year 2001 onwards, and several publications were seen on applications of microarray technology.

This technology makes use of a number of existing techniques and may be seen as a combined effort of evolving gene expression and nucleic acid hybridization studies, along with advances in fluorescence microscopy (Baldwin et al. 1999). These techniques are also combined with strategies of adsorbing or imprinting biomolecules to solid surfaces and robotics for preparing miniature versions representing complete genes/genomes, proteins or proteomes of organisms. A robust computational software is also mandatory for analysis of data obtained from capturing signals coming off microarray or microchips. Classically analysis of gene expression may be achieved by using northern hybridization, reverse transcriptase-PCR or nuclease protection assays; however, these are limited to a set of genes at a time. They are also not applicable for high-throughput studies. A filter-based gene immobilization technique combined to reversed probe-based detection can facilitate the expression of a large number of genes at the same time in one experiment. Due to this improvisation of being able to evaluate thousands of genes together, cost decreases, and comparison between many samples concomitantly becomes possible. These advantages have made microarrays popular which are being evolved continuously for even better results and dependability. However, there are certain disadvantages, for example, the membrane has a comparatively large degree of autofluorescence, limiting its use and preventing multiplexing of different fluorescent probes. Use of microscopic slides was also used for immobilizing chromosomes

for gene expression studies (Watson et al. 1998). Glass surface immobilization and photolithography technique were combined for depositing nucleic acids onto a rigid glass support, and a process of “contact printing” was evolved.

Despite having a very small size, large amounts of data can be generated using this technology. Replicate arrays are used for analysing samples for authenticity, and a computer-based data analysis is mandatory to handle the huge amount of data collected. The special software used for analysis of microarrays uses the raw data from slide images and then normalizes it, so as to avoid any experimental discrepancies caused due to over- or under-representation of sequences in the array. The final data is derived in a form such that it has a conclusive biological meaning and serves to speculate gene expression or compare relative gene expressions.

9.2 Basic Principle

All microarray techniques are underplayed by a few common strategies:

1. Targets or immobilized nucleic acids/peptide sequences
2. Probes or labelled moieties that hybridize with the target
3. A robust detection system

The “target” is the immobilized biomolecule (unique nucleic acid/peptide). The sequence is spotted on a miniaturized surface and deposited on slides/wafers of glass, using many different methods of spotting. The size of spots varies from each other but is usually lesser than 200 μm in diameter. The miniature “chip” thus contained about 10,000 genes spotted in a specific pattern or array, in only about few centimetre square of area.

The “probe” is mRNA for gene expression studies, or DNA derived from genome study has to be labelled using fluorescence, and these molecules are termed as probe. The different probes are labelled with a different dye, and over 10,000 different probes may be used to interrogate 100,000 different immobilized target sequences. Therefore, such probes have to be “unique” allowing no overlap that can cause errors in analysis of gene expression. The cyanine dyes C3 and C5 (fluorescent) are used for labelling generally, because fluorescent labels allow multiplexing, are not harmful as radioactive probes and ensure accuracy in quantitative analyses and multiplexing. Microarray has labelled fragments in the probe which are unique, and hybridization of probes and complementary targets is allowed to form hybrids. This means that the probe and target are both single stranded and have to be accessible for forming duplexes. More than one probe labelled with a different dye can be used simultaneously, and the fluorescent signals from each spot are detected by cameras, and these are converted to data via certain software which gives the relative density or abundance of specific sequences in each sample being compared.

The “detection” system and “data analysis” are a very critical part of using microarray technique. The scanners contain laser that emits light usually in a

wavelength that can excite particular fluorescent dyes, called “labels”. A confocal microscope attached to the detector system is used for recording the light emitted from the spots, and thereby the hybridization signals are recorded. The intensity of the emitted light reflects the strength of hybridization and is therefore an indicator of gene expression and level of complementarity between the target and probe.

9.3 Immobilization Strategies Used for Preparing Microarrays

A plethora of strategies exist for preparing the immobilized target DNA for microarrays, e.g. the nucleic acids may be extracted directly on the microarray, cDNA may be used, and oligonucleotides or short DNA fragments may be generated and printed using robotics. The use of the strategies depends on the biological query to be resolved, and a huge number of selection techniques make the process flexible and user friendly.

The various types of microarrays have been described below:

1. **Oligonucleotide based:** Oligonucleotides are 50–70 dNTP long and may be spotted using contact- or non-contact-based methods of printing. The microarrays can be prepared by a researcher in the lab by using microarray spotter, and thus customized microarrays are easily obtained. The nucleotide sequence of the oligo should be known. Genome sequencing has greatly assisted this technique; however, the accuracy of sequences is mandatory for proper analysis; errors can lead to partial or no hybridization of probe. The different parts of same gene may be spotted on the array, and so various forms of the same gene may be discriminated from each other. These are easily available commercially and save time and effort.
2. **cDNA or fragments of DNA:** amplified exons, expressed sequence tags (ESTs) or cDNA can be used for microarray. Usually the cDNA clones are obtained from subtraction libraries where the highly expressed DNA sequences have been removed and cDNA clones may not be completely sequenced to be used for microarray (DeRisi et al. 1996). Also by knowing specific areas on cDNA, they can be used as unique probes, which do not hybridize with non-complementary DNA, leading to lesser chances of error. The PCR fragments, which are prepared by using specific primers, should be purified to remove extra dNTPs, enzyme or other buffer salts that may otherwise lead to the interference in microarray detection and analysis. For this any column or kit-based protocols may be used. Labelling of such fragments should be accurate, and contamination with other nucleic acid should be strictly prevented. Targets should never have repetitive sequences. The optimal length of DNA targets is between 300 and about 800 dNTPs and may be easily spotted or immobilized on microarray wafers/slides, and increasing length also means that the concentration required for high number of target molecules is more and amount of target molecules should be more than that of probe for a good analysis. Else the signals will show saturation if the probe is more than the target. Single-stranded DNA may not be required;

using denaturing agents might be used for creating single-stranded DNA fragments.

3. **Control targets:** Microarray analysis should have proper controls because variations in signals occur between different slides and also between the replica slides containing same target sequences but spotted onto different locations on the slide. Various factors influence hybridization, the most important being the labelling method and number of transcripts, conditions and printing methods. Adequate amounts of exogenous DNA sequences may be spiked, and some housekeeping genes may be used as targets because their expression is not expected to differ in different treatments. The oligonucleotide targets allow using mismatched dNTPs in sequences as control. The comparison of signal strengths from these controls and experimental sequence can validate the accuracy and reliability of this technique.

9.4 Manufacture of the Different Components of Microarrays

9.4.1 Oligonucleotide Synthesis

Targets can be prepared on the slides directly, or they can be deposited on the slide after purification via kits. The slides are the solid supports that are capable of binding to DNA or RNA. Oligos are attached to chemically altered linkers containing protective groups that can be photochemically deleted, upon attachment to the solid surfaces. Targets are synthesized at different positions of the supports by masking and unmasking the support in a step-wise manner and exposing the activated areas to nucleosides that bind to the suitable positions only (ref. 35 Chap. 3). The arrays have an area of about 1–2 cm square and contain 4 lakhs of “features” that in turn have oligos with same nucleotide sequences and are fixed by 3′ ends on the surface of the array. Multiple arrays may be simultaneously synthesized on large surfaces for automating the synthesis process and are later divided into small single arrays for use in labs.

For depositing targets, kit-purified sequences in solutions are used in nano- or micro-litres for forming covalent bonds with the surface, for example, amino groups of oligos get linked to aldehyde groups on surfaces. Imidazole can be used to derivatize the 5′ phosphate groups, or bi-functional cross-linking groups may also be used where aminated nucleotides are linked to aminated surfaces.

Spot sizes should be small and separated from each other equidistantly. Usually they are deposited in a grid. Slight movement by mistake while printing slides may disrupt the pattern. The spots should also be of uniform shapes with same amounts of target sequences, necessitating proper calibration of printing pens. The environmental contaminations should be prevented; else background signal may be a problem. Humidity also should be controlled such that concentration of spots is not altered. Higher humidity might cause smears and lower moisture can cause dryness. So optimization between 10 and 70% is required.

Each spot should have distinct target sequences, and nucleic acid from one spot should not be allowed to enter into another.

In some cases non-contact deposition may be adopted, e.g. piezoelectric and syringe solenoid-based methods. Contact deposition uses pens of varied designs, and they are dipped onto target nucleoside solution and then contacted with solid surfaces, requiring more than one pen for completion of printing and attaining high throughput. Contact deposition ultimately requires less target sequences and also results in smaller spots.

Examples of contact-based spotter is the Lucidea array spotter from Amersham Biosciences. It monitors and controls humidity and temperature while spotting and has an integrated control software to integrate all aspects of spotter operation. The thickness and uniform distribution of surface coatings result in better quality of results and accurate prediction. The standard solid surface varies from manufacturer to manufacturer and should be well analysed before printing, so that background fluorescence is minimal. The surfaces have different chemistries to enable strong binding of nucleic acids, and also the surfaces determine the density of spots per unit area of the solid support. Common surface chemistries include adding amino-, aldehyde, amino-silane, poly-lysine, etc. groups.

Fluorescent light from a probe gets scattered in several directions while using glass slides. But if a reflecting surface is overlayed on slides, then scattering is decreased, and all the scattered light gets directed towards a detector, and signal strength gets improved. Aluminium surfaces over the glass can help in this controlled reflection. SiO₂ spacer laid on the top of this Al layer further improves the signal. Lucidea reflective slides enhance Cy5 signals, and Cy3 signals are already enhanced when they chemically bind to molecules.

Few factors that aid in microarray preparation are as follows:

1. Clean environment with controlled humidity, temperature and airflow.
2. Powder-containing gloves should never be used, and touching with bare hands should be avoided.
3. Low background signals from slides may be permitted, but never high.
4. Purity and concentration of target samples should be verified.
5. Shelf-life should be taken into account while using slides, and they should be stored away from light under desiccation.

9.5 Properties of Fluorescence and Fluorophores

Fluorescence is the property of molecules where they absorb photons of a particular (usually shorter) wavelength and emit light at another (longer) wavelength, and such molecules are called fluorochromes or fluorophores. The excited state of the molecule is just a fraction of a second long, and the energy absorbed may be dissipated in the form of vibrational and rotational energy causing heat energy to be released. Once the lowest vibrational state is reached, the electrons fall to ground state from excited state, thus emitting photons of lower energy and larger wavelength. The

difference between the maximum and minimum wavelength is called Stokes shift. In conditions where intense light is used, the fluor molecule might get “photobleached”, i.e. the ability to have fluorescent properties. CyDye fluors are resistant to photobleaching and therefore are suitable for many machines having high laser powers. Good separation between emitted spectra is important for sensitivity of detection, and the background remains low. Multiplexing is made possible by using fluorochromes having pairs of excitation and detection wavelengths that are different, and so many different wavelengths can get detected from the same sample at the same time. Hence we can use Cy3 and Cy5 together as there is minimal overlap between their spectra. The fluorescent dyes are also less harmful as compared to radioactivity and have a better shelf-life and stability. However, if two or more fluorochromes are in proximity, the excitation energy is dissipated, and there are decreased signals. This phenomenon is called “quenching” and is a disadvantage when samples are overly labelled or too much sample is used.

The cyanine dyes or CyDye family was used primarily in photography for film sensitization and thereafter in molecular biological experiments. The fluors have emission spectra in visible light and are soluble due to presence of sulfonate groups and do not form aggregates due to the presence of negatively charged groups, and thus “quenching” is minimal. Cy3 spectra and Cy5 spectra are easily resolved because they are excited by two different lasers at 532 nm and 633 nm, respectively, and specific filters are used to collect only the emitted part of the light from either dyes, having minimal overlap. But the pH and DMSO may hamper their efficiency, although minimally. Temperature elevations do not deter the performance of these dyes, and so they are used for many applications in molecular biology. The dyes, however, should not be exposed to light and should be stored in amber-coloured ampules or wrapped in Al foil. Single-use aliquots should be prepared to avoid freeze and thaw. If illumination is very intense, fluorochrome molecules remain continuously in an excited state, and this is known as “saturation”.

9.6 Measuring Fluorescence

The fluorescent signals should be able to give high resolution in a short span of time and should allow differential expression of genes by using two dyes at least at a time. During detection of fluorescent, the low detection threshold should be set to determine the signals from low abundance or weaker signals which are rare but important. The more abundant gene expression signals should be also picked up by using a wide range of detection. The intensity of the signals depends on the intensity of the light used for excitation, but the concentration of the fluorochrome governs the brightness of spots.

Measuring the intensity of fluorescence is possible by using photosensitive devices, e.g. photomultiplier tubes (PMT) which amplify signals; the light intensity is calculated in “relative fluorescence unit” (RFU).

9.7 Labelling Samples for Analysis of Gene Expressions

The gene expression is usually analysed in terms of identity of particular genes and abundance in the total transcriptome (total mRNA content of the microbe). For this, the transcriptome has to be labelled or tagged with fluorescent dyes and allowed to hybridize with microarray. Wherever positive hybridization occurs, signals are detected and then analysed. mRNA can be present in just few copy numbers per cell to thousands, and they change in abundance with changing environmental conditions of the cell. However, major genes are expressed at constant intermediate levels. The size of transcripts also varies and the average length falls between 1.5 kb and 1.7 kb. This diversity should be represented by microarray gene analysis techniques and is achieved via efficient labelling.

A few factors should be optimized for labelling probes, e.g. length of probes, amounts and label density.

The length of the labelled sequences is an important parameter governing the strength of hybridization where above 100 dNTPs hybridize strongly to target sequences but probes with 200–500 dNTPs are considered to be optimal for withstanding harsh washing and stringent temperature conditions during hybridization. Longer than 500 nucleotides, hybridization of probe and target is again inefficient. Concentration of probe used is also critical, because signals have to be above the minimal detectable limit. If higher amounts of mRNA are used for probing, then the differences in labelling of probes using different methods will not cause difference in detectable signals. In case amplification is required to label the dNTPs, then it should still represent the original expression levels of the different population of mRNAs present originally. Too less or too high fluorescent signals are not properly detected, and hence optimization is required. Excessive label causes quenching, and low density labels do not give sufficient signal strength. Gene expression studies require relative variations in numbers of target genes in many samples. So differences in signals actually indicate the variations in the numbers of the mRNA sequences in question. Sometimes, the sequence of nucleotides governs the amount of label associating with a probe. For example, highly adenine-rich mRNA sequences can be labelled using CyDye dCTP. But label density should not be considered as the most important factor for achieving high signal. The T_m (melting temperature) of highly labelled molecules may be less than unlabelled counterparts, and so excessive label might bring down the signals obtained upon hybridization.

9.8 Labelling Strategies

Regardless of the type of primer, amount should always exceed the actual target sites so that it does not become a limiting factor. So concentration of each probe/primer should suffice for covering the entire range of gene expression. The section describes the various primers and strategies used for labelling.

All labelling strategies start with RNA population. mRNA constitutes only about 2% of the total cellular RNA, and this fraction has to be labelled. In eukaryotes, the poly-A tail facilitates use of poly dT primers, complementary to the tails, for isolation of the mRNA fraction. However, additional purification steps for mRNA samples give high noise signals. Therefore, total RNA may be used for decreasing this problem.

However, for cDNA synthesis polydT primers are used, and if target sequences are derived from 5' end of long cDNAs, probes labelled using oligodT priming may hybridize, leading to wrong results. This is because only one strand is synthesized, complementary to a strand that had 3' polyA tail.

To counteract this problem, random priming may be done for cDNA synthesis such that a mixture of oligos have short sequences as primers and these have transcripts from all parts of the mRNAs. This method is compatible for mRNA only; otherwise it will anneal to other RNAs also and give non-specific results. The yield of cDNA is highest when both oligo dT and random hexamer primers are used together and also is considered most suitable as it can copy various parts of the genes that can hybridize with targets. Using specific primers for targeting specifies sequences may also be a good option but can be expensive, and so the use is limited.

9.8.1 Labelling Bacterial Transcripts

mRNA of bacteria don't have polyA tailing and are not easy. Conversion to cDNA would help in removing the other RNA species. Random priming may be used, and stringent conditions should be used for hybridization so that high amounts of labelling and consequent saturated signals do not deter proper analysis. Target-specific primers or short primers capable of targeting several genes at a time may be used too. This strategy helps in noise reduction and facilitates specific binding.

9.9 Labelling Samples for Gene Expression Microarray

Molecular biology-based protocols have been used widely for labelling purpose, although using more than one enzyme changes the sequence depending on the property of enzymes used. Here, chemical methodologies have an upperhand, as the sequence is copied without changes and covalent linking of label is possible. Fluorescent dyes may be incorporated using chemical or enzymatic methodologies, and these give bright signals and may be multiplexed. Labelling might be achieved by priming cDNA synthesis (as discussed before) with total RNA as template. The ratio of fluorescent dNTPs and the other non-labelled dNTPs is an important factor to estimate the amount of label incorporated in cDNA. This has to be optimized with respect to dyes and also enzyme. The polymerases do not synthesize a long strand when labelled dNTPs are used. mRNA synthesis gets discontinued when a stretch of labelled dNTPs are incorporated consecutively. If the labelled nucleotide

concentration decreases, the cDNA will increase in amount, but signal strength will decrease due to lower density of label. Therefore optimization requires planning and effort.

Post-labelling strategies of cDNA are implied in many cases, e.g. Amersham Biosciences has discovered CyScribe Kit for the first synthesis of modified cDNA with amine group incorporation, followed by chemically treating with a derivative of CyDye. This forms covalent bonds between the cDNA and fluorescent dyes, and the non-labelled dNTPs are neutralized using hydroxylamine. No further purification steps are needed. The benefit of this strategy is that cDNA yield is high and equal degree of labelling achieved with the cyanine fluorescent dyes, e.g. Cy3 or Cy5. Affinity chromatography, suitable for binding the amine groups, can be used for purification of the cDNA from the reverse transcriptase and buffer in the first step; alternately, ethanolic precipitation of cDNAs may be done for concentration and purification of the cDNA molecules prior to CyDye labelling. The presence of moisture in the lab can, however, destroy the CyDye-labelled cDNA, and therefore, aliquoting, desiccating and storing are important, and the lab should not have excessive moisture.

RNA polymerases may be used for amplifying RNA. mRNA may be converted to cDNA that has a promoter for RNA polymerase, introduced by attaching promoter via a modified oligo-dT primer. After 2000 times amplification, labelling may be done. In another technique, labelling and amplification may be done simultaneously. Other strategies include the use of hapten-labelled ribonucleotide, e.g. biotin-labelled ribonucleotide.

The RNA is fragmented before using in microarray, since the secondary structure of RNA does not allow it to hybridize to target sequences. The fragments are of varying lengths from 50 to 200 bases.

Probes should be characterized before use, e.g. the level of CyDye and nucleic acid in the probe, purity, relative fluorescence, distribution of dNTPs, etc. This can be achieved via spectrophotometry, thin layer chromatography, polyacrylamide gel electrophoresis and radioactive spiking of probe.

9.10 Calculating Label Density in Probe

The density is calculated as the amount of CyDye present in a given amount of nucleic acid and expressed as pmol of dye per microgram of nucleic acid. This may also be defined in terms of number of molecules of CyDye in 100 dNTPs.

Labelling density = $\text{pmol CyDye in labelled sample} / (\mu\text{g nucleic acid in labelled sample})$

Therefore, 1 μg of cDNA has about $1 \times 10^{-6} / 330 = 3030$ pmol nucleotides.

So, label density of 100 pmol/ μg is equivalent to 100 pmol/3030 pmol of nucleotides = 3.3 CyDye nucleotides per 100 nucleotides = 3030 pmol of nucleotides.

Recovery percentage of purified labelled nucleic acids can be done using:

$$\text{Recovery}\% = \frac{(\text{cpm after}) \times (\text{volume recovered}) \times 100\%}{(\text{cpm before}) \times (\text{incorporation}\%/100) \times (\text{volume purified})}$$

9.11 Steps for Microarray Hybridization

Microarray technique is similar to Southern blotting in many ways; however, it differs in that the microarray technique is performed in reverse order, where target sequence is attached first onto any solid support, e.g. slide, instead of a nylon or nitrocellulose membrane in Southern hybridization.

There are majorly three underling processes and have been explained in the following section for a good understanding, before describing the protocol (Fig. 9.1).

- Attachment
- Hybridization
- Stringent washings

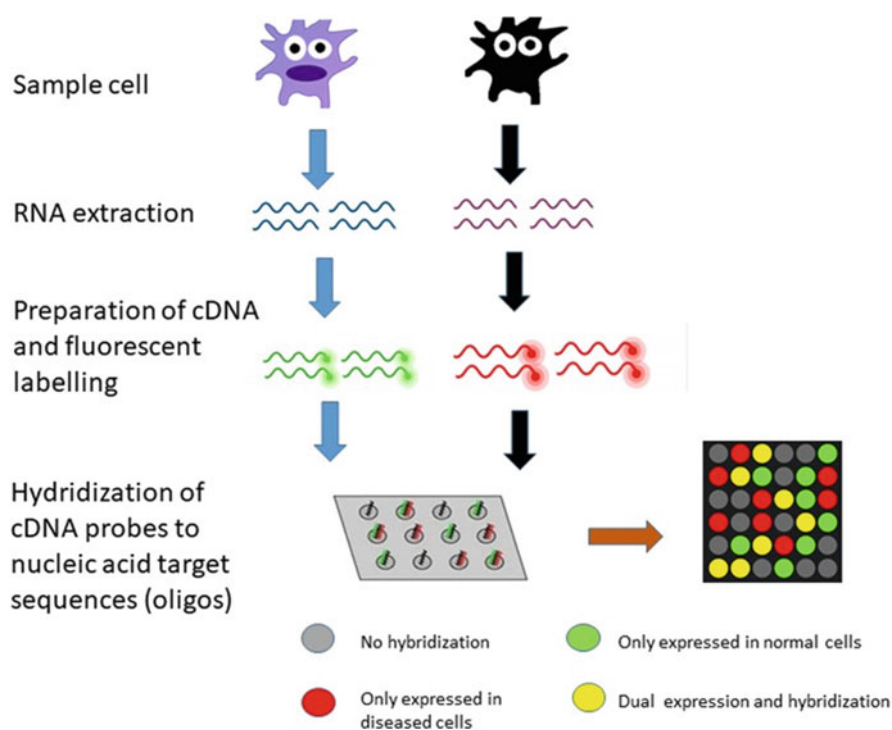


Fig. 9.1 Diagrammatic representation of steps involved in microarray hybridization method to determine differences between a normal and diseased or treated cell

“Prehybridization” steps are followed before the probe is added. This involved incubation of slide spotted with target in buffer, such that wrongly adhered target is removed, to avoid loosening of poorly adhered targets into the hybridization buffer which then combine with probe in solution and give signals. This step also ensures that the target is in single-stranded form. Before the probe is added, non-specific hybridization is prevented by adding blocking agents (e.g. poly-dA oligo) that hybridizes to poly-dT tails or salmon sperm or yeast tRNA to stop unspecific probe hybridization. The solution may be then heated for denaturing the ds DNA (if any) to maintain only ss sequences for good hybridization.

In the next step, hybridization is performed via different methods as suitable for the different slides. The “coverslip” method allows hybridization of probe with slide under the coverslip. This method does not need any special instrument but has the disadvantage of drying out of samples especially at the edges. However, a humid chamber for the process is helpful in this. The hybridization buffer should maintain the pH and maintain a low surface tension for easy flow of buffer under the coverslip. The buffer also contains reagents to speed up the hybridization. Formamide may be used to decrease the T_m (melting temperature) of probe to facilitate hybridization at lower temperatures, so that dryness is prevented. The buffer has to be free flowing so that the probe finds its complementary target easily by flowing freely under the coverslip. However, in case there are many target spots in a particular spot, the probe stays there and gets concentrated there, and spots further away would get limited access to the probe. This results in probe depletion which finally lowers the sensitivity of microarray. Target saturation is another problem faced during microarray hybridization by coverslip method, where in cases of high abundance genes, target and probe genes are similar in concentration. Use of hybridization chamber and suitable buffers and pre-hybridization steps are important for ameliorating the problems encountered due to target saturation and probe depletion.

Stringency washing is done after hybridization for removing attached and loosely attached probes. Buffers such as SSC/SDS solutions at variable concentrations are used. The primary washing is done at temperature for hybridization and has high salt concentrations (about 1 to $2 \times \text{SSC}/0.1\% \text{ SDS}$), to remove the unwanted buffering components during hybridization. Then secondary washing having lower salt content (around $0.1 \times \text{SSC}/0.1\% \text{ SDS}$) removes loose probe molecules and remaining salt from slides. Warmed buffer solutions are effective, but over-heating buffer using microwave or use of low salt concentrations in the buffer results in stripping away the probe signals. Stringent washing treatment prevents false signalling and reduces background noise that is emitted from smears or unwanted spots. Washing should be done in darkness and should be dried immediately and stored in a desiccator. If background noise is high, the slides may be again washed under stringent buffers, and the steps may be repeated.

9.12 Different Slide Types for Microarray

Depending on the slide type, the right protocol for hybridization should be used. However, the manufacturer's protocol has to be referred to while using microarrays. Three basic types of microarrays exist as shown in Table 9.1: (1) spotted arrays on glass, (2) self-assembled arrays and (3) in situ synthesized arrays (Bumgarner 2013).

9.13 Comparing Automated and Manual Hybridization (Table 9.2)

Table 9.1 Different types of microarrays with their specific features and application

Type	Description	Reference
Spotted arrays	Multiple or single “pen” is used for spotting; it is dipped into the solution containing DNA/RNA of our interest and deposited on glass slide coated with agents, e.g. polylysine, or a chemically reactive surface	DeRisi et al. (1996)
Self-assembled arrays	Applying beads containing set of oligos to a surface having pits which are of same size as beads. After array is constructed, a series of hybridizations are done to determine which probe is at which place in the array which is unique	Ferguson et al. (2000), Steemers et al. (2000)
In situ synthesized arrays	These are produced using inkjet oligo synthesis method or by photolithography (e.g. affymetrix)	Dalma-Weiszhausz et al. (2006)

Table 9.2 Comparison of manual and automated hybridization

Function	Manual hybridization under coverslip in hybridization chamber	Automated hybridization using Lucidea Slide Pro
Intensity of signal	Variable and less reproducible results	Consistent Cy3/Cy5 signal ratio; more reliable results
Mixing of probe	Localized probe depletion may occur	Better mixing
Re-using probe	Possible with decreased efficiency	Probe is effective after second wash also because Cy3/Cy5 signal ratio was unchanged; background decreased after multiple re-use
Duration of hybridization	12–18 h	3–6 h

9.14 Imaging for Microarray System

This is also a very critical feature of using microarray system. The various types of scanners, light sources, detection systems and signal amplification affect the overall success of the technique, and therefore appropriate instrumentation should be procured for specific types of assays for desirable results (Fig. 9.2).

Mechanism for Incident Light and Collection of Emitted Light

9.15 Optical System for Imaging in Microarray

Light sources are critical for fluorescence-based imaging in microarray. Light sources may be of two types: wide-area light source or broad wavelength light source, e.g. xenon/ultraviolet arc lamps, used for spectrometers and other camera-based imaging, and sources of light giving discrete wavelength light or line sources, e.g. laser, that directs the beam to the sample through filters. Light collection system includes lens, mirror, filters, etc., for proper wavelength selection and transmission. Light filtration system allows the collection of selected wavelengths of interest and, in systems that are fitted with more than one detector, usually has additional beam splitting filters to separate the light emitted by the sample and direct it along distinct paths to the corresponding detectors.

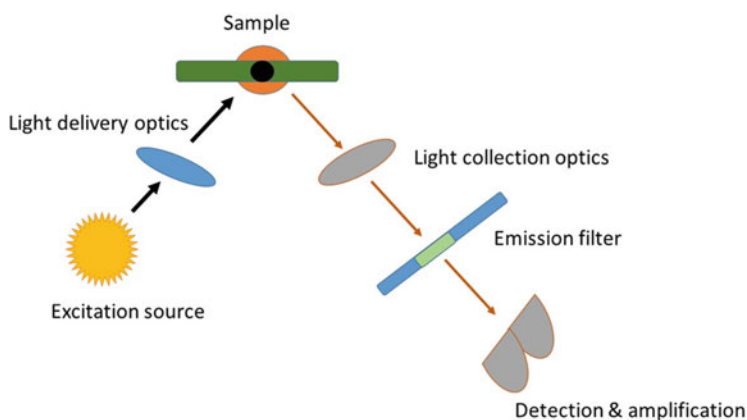


Fig. 9.2 The key elements: excitation sources, lighting and signal collection, filtration of light emitted from slide and detection plus amplification of signals to understandable digital signals

9.16 Detector System, Amplifier System and Digital Resolution for Imaging in Microarray

Photomultiplier tubes or char-coupled devices have been commonly used for quantitation and detection of light emitted by samples, where the light energy is converted to electrical signals and so the photons emitted become directly related to current, which is then amplified and finally changed to a digital signal (means the analog signal is converted to numbers that represent intensity). The digital resolution is the number of intensity levels that may be discretely discerned by the system, and the output is given as 8 bit, 12 bit and 16 bit digital files. If two signals have nearly equal intensities, but are emitted by different dyes, then they should be discriminated with least degree of error, and the ability to do so is called resolution. Intensity levels may be distributed in a linear manner, giving same spacing for all levels of intensity, or non-linearly, wherein lower intensities of signals are divided into more levels and high-end signals are divided into relatively lesser number of levels. The second system maintains digitalization error as constant across dynamic signal ranges.

9.17 Scanners and Excitation Light System for Microarray

Mostly laser is employed for exciting the fluorochromes because they emit a monochromatic, collimated light and is a coherent light source with narrow width of beam which results in good sensitivity and resolution. Lasers may be argon ion lasers, helium neon lasers, neodymium:yttrium aluminium garnet (Nd:YAG) laser or diode laser. Other sources of light may be LEDs (light-emitting diodes), which are less expensive and smaller in size and weight, but the power output is lower and the beam is not as narrow and thus less sensitivity is achieved. They can emit light in the visible range above 430 nm. Due to the power and collimated light emitted by lasers, there are negligible losses during excitation process, which is not the case with LEDs.

The light is focused as a point, although the target spreads over a large space, which is two dimensional. For effectivity the beam of light has to be swiftly crossing the target spread in two-dimensional area. In order to achieve this, two methods are employed to spread the point beam (source of excitation), firstly by using a galvanometer-based small and rapidly vibrating mirror to deflect the incident light and the other by moving head scanners which use a mechanism such that the power density of the beam is the same at every point of the target.

9.18 Data Analysis in Microarray

These systems are still evolving, and efforts are being made to set up annotation standards to create public databases for results of microarray analyses (Brazma et al. 2001; Lee et al. 2000). The analysis involves few main steps, i.e. image analysis, control assessment and data normalization followed by visualization and data



Fig. 9.3 The importance of controls to validate microarray data. *CC* calibration control, *RC* ratio control, *NC* negative control and *UC* utility control, respectively

clustering. Image analysis makes use of a dedicated software for quantitating the fluorescent intensity of every spot, by a process known as spotfinding. The controls are carefully examined and the data is normalized to calculate mean ratios. The data is finally represented in a graphical form, and clustering is also done for deriving meaningful analysis and visualization.

The amount of data obtained from microarray experiments is generally vast and high throughput, but the quality of data may not always be sufficiently of good quality. Three types of values for quality are important and should be taken into consideration during data collection.

The first important parameter is to know whether good, regularly shaped spots have been quantitated; secondly; control datasets are needed to ensure efficient and accurate hybridization; thirdly, replicate data is needed to ensure statistically valid data collection.

The scanner obtains data in the form of pixels that are obtained from the spots and the background. So a good software is required to locate the proper spots by a process “gridding”, where spotfinding is facilitated by designating a spot on the grid which is a properly sized spot with equidistant spacing from other circles and properly arranged. All spots may not be of the same intensity and the software does not take that into account. Example of a software is the Array Vision.

The information or data collected from spots is measured as micron units or as pixel, and after the grid definition, the scanned spots are aligned with it by taking their image. This avoids tedious manual alignment. The manual method first divides the grid into subgrids, and visually alignment is done by adjustment of circle position. In semi-automated ones, a software finds the spots with the help of the user, and in fully automated ones, softwares analyse many images, and no human intervention is required in this process. The background signal is subtracted from the actual signal; background is determined by a user-defined area outside the positioned grid, whereas actual spot signals are obtained from within the grid. If spots are closely spaced, the background correction is not effective.

Controls used are of three types (Fig. 9.3): (a) Housekeeping gene is used as control, e.g. GAPDH (glyceraldehyde 3-phosphate dehydrogenase), to ensure hybridization and may also be used for normalization, although under experimental conditions, expression of these genes may vary, necessitating the use of another such gene. (b) Control are use to minimize mechanical errors, e.g. the pen to pen or slide surface variations caused during spotting and coating of slides. Maintaining positive controls spotted in replicates can help to limit such variations. In the Lucidea

Universal Scorecard, there are 23 control genes (intergenic sequences from yeast) that serve as control for validation and normalization of data. (c) Negative controls which cannot hybridize with the labelled probe and therefore should not generate signals are used to ensure that the stringency washings are effective enough and non-specific hybridizations have been delimited. Signals from negative controls make the data inaccurate and less dependable.

9.19 Normalization of Data for Correcting Experimental Variation Between Slides

While comparing one or more slides, often Cy3 and Cy5 signals vary, because mRNA levels used in labelling reactions differ or due to detection system's efficacy. In such cases normalizing the data is important. In linear normalization, a normalization factor is obtained from signals of housekeeping genes or from spikes added to labels, and this factor is used as a conversion factor. Thus all signals or data is expressed in terms of ratio between signals obtained from experimental spots and control. The methods therefore work by assuming that the normalization factor remains constant over the whole range of signals obtained.

Slide 1	Slide 2
Housekeeping gives Cy3/Cy5 ratio = 2	Housekeeping gives Cy3:Cy5 ratio = 3
Gene of interest (A) gives Cy3/Cy5 ratio = 5	Gene of interest (A) gives Cy3/Cy5 ratio = 7.5
After normalization	
Gene A has normalized signal = $5/2 = 2.5$	Gene A has normalized signal = $7.5/3 = 2.5$

Non-linear normalization is used when Cy3/Cy5 ratio has more low Cy5 levels but is different in the case where Cy5 levels are higher, showing that the two cases need a different normalization factor. Non-linear methods of calculating the normalization factor give more accurate results after normalization, using software packages.

The control data is analysed post-normalization of microarray data. Signal/noise ratios are calculated for negative controls and should be lower than 3, where signal/noise ratio is calculated as (average signal/background noise signal)/standard deviation in signals of background. In case spikes have been added to Cy3 and Cy 5 in different amounts, then theoretically valid ratios are compared to actual experimentally derived ratios, to get normalization factor. Statistical validation of data is required and is achieved only by repeating multiple times or using replicates on same slide. Log ratios are obtained such that any ratio which is less than one has negative values. The coefficient of variation (CV) is calculated by dividing standard deviation of the ratios, by mean ratio. The data points showing high CV values are rejected, as they result from low signals. If noise or SNR values are below 3, the spots are also rejected.

9.20 Visualizing of Data and Clustering

After normalization, the microarray data is imported into various softwares for visualizing the data and analysing it (Fig. 9.4). Any significant differences in gene expression levels can be judged by the softwares, and through helpful features like data integration, pattern recognition or query resolution systems, the data is visualized by user. The software groups or clusters the similar gene expression responses, thereby indicating at pathways which may be linked or over-expressed and so on. One such software is Spotfire Decision Site (Amersham Biosciences), used for functional genomics studies. It adds the core features of Spotfire Decision Site software with tools specialized to interrogate and extract data from the spotted microarray slides.

Softwares should be easy to handle such that user can access the data, and the visual representations should be sharable and publishable. The calculations, cluster analysis, etc. should be clear and may have signature profiles.

The aid of information management tools is also required for microarray-based experimentation.

Microarray workflow systems can do the following tasks:

- a. Prepare array.
- b. Prepare samples and label probes.
- c. Hybridize array and scan it.
- d. Image analysing.

The Sierra Microarray Lab Workflow system (MA-LWS) from Amersham Biosciences and Axion Propel high-throughput workflow from ThermoFisher Scientific are for handling microarray experiments in laboratories that mainly involve such work.

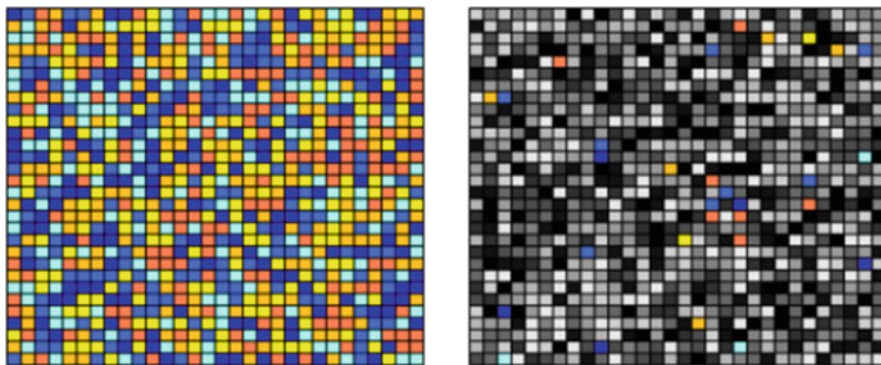


Fig. 9.4 Visualization of microarray slides using softwares

9.21 Troubleshooting During Microarray-Based Experiments

System should be optimized and all components of microarray should be tested together. The target sequences which are very similar to real samples and probes should be used for optimizing the system. Such experiments should incorporate controls. Controls are needed because they help to economize resources, and problems are identified easily by step-wise analysis, and also the validity of the results are ensured. Extra sets of controls may be used while changing a batch of reagents or setting up new protocols. RNA extracted from various cell cultures or specimens contains slightly varying amount of some mRNAs, and so pooled RNA extracted from several specimens should be used for optimization of microarray system. The yellow experiment is therefore recommended. It is an efficient tool for optimizing the microarray system. Any particular RNA sample is labelled with Cy3 and Cy5 fluors and allowed to hybridize with microarray slide such that equal concentration of signals of both colours is obtained. All the hybridized spots should appear yellow.

The numerical data obtained from the yellow experiment is plotted as a scatter plot and is very informative. Since differential expression of genes is not expected, the normalized Cy3 signals are plotted versus the normalized Cy5 signal, and the plot should be a straight line. These yellow hybridization experiments provide a lot of information for standardizing a microarray system, but cost wise it is economical as only one species of RNA sample is needed. Two microarray experiments never yield the same result even if they are replicates as every step contributes to such variations. This can lead to masking the actual differential gene expression and give false-positive (is level of expected variation is unknown) or false-negative result. However, replication is the key for identification and quantification of differential gene expression in a microarray experiment.

The general problems experienced during setting up microarray experiments have been tabulated, and probable causes and remedies have been suggested (Table 9.3).

9.22 Applications of Microarrays

1. Study of gene expressions using real-time PCR or northern hybridization applies to small numbers of genes at one time. Microarray systems allow analysis of thousands of genes with replicates and controls at one point of time and in few hours, when the protocols are standardized.
2. Microarrays promote genomics studies by identifying new genes or analysis of SNPs and aid in genome annotation and mapping. This technique can predict splice variants and can also aid in sequencing for identification of genetic variants. Diseases are sometimes related to occurrence of sequence variations, and microarrays aid in such studies (Alizadeh et al. 2001; Meltzer 2001).
3. Microarray technology aids in drug discovery in various steps (Van Berkum and Holstege 2001; Meltzer 2001; Lockhardt and Winzeler 2000). The method helps in elucidation of metabolic pathways by understanding simultaneously

Table 9.3 The troubleshooting of various problems encountered during microarray experiments (adapted from Amersham Microarray handbook)

Problem	Cause	Remedy
No hybridization	Target conc low or not clean	Purified and quantified target should be used
	Target not retained on slide or no mRNA in sample	Buffer and protocols should be reviewed, and slides may be changed or RNA may be extracted again
	No labelling or faulty labelling	Checking hybridization reaction beforehand is needed; reagents to be checked; controls should be used
	Loss of probe during purification	Check protocols beforehand
	Failed scanner; fluorescent dyes exposed to light beforehand; target genes poorly expressed in specimen; mechanical error	Check before use in final experiment; protect fluors from light and control with housekeeping genes and positive controls should be used; replicates should be used to detect variations from handling/mechanical error
Low signals from fluors	Unequal amounts of 2 dyes were used; loss of probe; free fluors in probe; poor quality RNA or contaminated RNA sample	Purification steps for probe and target and quantification of RNA in preparation is mandatory. DNase should be used to clean RNA preparations and purified further to remove enzyme
	Hybridization has high background signals	Slides of different batch may be tried, and buffers and slide surface should be checked
	Laser source errors and detection sensitivity	Check settings
Unbalanced Cy3/Cy5	High density of label used or too much of one probe in reaction leading to quenching	Use appropriate volumes and use labelled nucleotide sparingly; controls should be used
	High background in one coloured label; normalization not adequate; too much variation in signals	Use a different method; optimized and standardized protocol should be followed
Background has speckles	CyDye-labelled nucleotides remain in probe	Optimization of probe purification needed
Deformed spots	Tiny or different sized spots	Controlled humidity, test printing pen, spotting buffer, use slides of different batch

co-expressing genes and also determining protein targets of drug treatment, for further understanding the action of drugs (Jain 2000; Gray et al. 1998).

- Specialized techniques using microarray technology, e.g. CHIP-chip (chromatin immunoprecipitation on microarray chip), can be used for study of gene expression and transcriptomics (Gade and Kalvakolanu 2012). The binding of transcription factors or any other DNA-bound proteins (antigens) may be detected in vivo in fixed cells using formalin and extracting DNA bound to proteins. Antibodies

are used to target the protein antigen bound on the chromatin, and thus the exact sequence where the protein remains bound to the DNA segment can be determined. The sheared nucleic acid segments bound to protein antigen are purified via immunoprecipitation reaction, and the DNA fragments bound to proteins are using a microarray chip, which has spots representing the entire genomic DNA (Zheng et al. 2007).

5. Pharmacogenomics is an upcoming area of research, and microarrays have a special role in this frontier area of biotechnology (Jain 2000). The DNA chips have now found a way to personalized medicine and have integrated disease diagnosis and therapeutics and genome information.
6. Genotyping for detection of single-nucleotide polymorphism (SNP) can be done using Affymetrix allele discriminating platform where PCR-based approach is used and SNPs that lie between restriction sites less than 1kb apart are detected. The technique allows users to understand a lot of complex biological systems, but the data obtained requires analytical methods and also allow data exchange. For this, Function Genomics Data Society (D.org) or the Microarray Gene Expression Data Society has created the standards for describing experiments and exchange of microarray data via Minimum Information About a Microarray Experiment (MIAME). Such efforts have motivated databases for microarray data (Brazma et al. 2001).

9.23 Limitations of Microarray Technique

Microarrays are devices for measuring relative concentrations of many different DNA or RNA sequences, but the technique has some limitations. The signals give the concentration of labelled probe that can bind to a specific target, but signal level may not be linearly proportional to the concentration of probe, especially because array gets saturated and at very low concentration no binding happens. So the signal is linear over a limited concentration range.

The other problem is that for mammalian genomes, arrays are almost impossible to construct, because closely related sequences of DNA/RNA bind to the same probe, due to multigene families. It is difficult to design arrays that can detect every exon in genes which are a part of multigene families.

Genes that are unannotated cannot be represented on the array, and also non-coding RNAs that are expressed will never get detected. Some genes are annotated with alignment with reference sequences existing in databases, and those might have sequences that are unique and can't be recognized by microarray technique.

9.24 Conclusion and Future Direction

The advantages of microarrays especially for gene expression studies are immense, and high-throughput data can be obtained. However, the cost of sequencing has decreased rapidly paving the way for regular sequencing strategies being applied to most analytical procedures, which now have limited the importance of microarrays. Also sequencing is a direct method, and microarrays are indirect, thereby making sequencing the choicest method above microarrays.

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10.1 Introduction

Immunology is the study of the body's own defence against any type of infections, although we are surrounded by various types of microorganisms that cause disease. We are getting sick very rarely, whereas all the time the protection against infection comes from our defence molecules which are called immunity. The origin of immunology began in 1796 when Edward Jenner, an English physician, took the pus of the pustules of cowpox-infected person, and inoculated into an 8-year-old boy through an incision in the arm. He demonstrated that the child developed immunity to smallpox. Over the past few decades, there have been numerous developments in the present perceptive of the immune system and its role in protecting the body from any type of infections (Varadé et al. 2021).

Immunotechniques performed belong to the category of enzyme immunoassays. These are used routinely to diagnose infectious agents such as viruses, bacteria, fungi and other substances in the blood. Antigen is the main object of substance to be measured and immobilized on a solid phase. Principally, an antigen is a substance that can trigger an immune response that leads to the formation of antibodies and highly specific. These reorganization, detection and analysis of antibody and antigen reactions using different types of bioassay or analytical measurements are focused on different immune techniques. Immunoprecipitation is a technique of purification of a specific protein from a complex mixture using an antibody. Immunotechniques are based on selective binding, reversible and non-covalent, of antigens, with antibodies; therefore, it is generally used to detect or quantify antigens or antibodies (Warrington et al. 2011).

10.1.1 Monoclonal Antibodies: Purification and Concentrate

10.1.1.1 Principle

Affinity chromatography separates proteins on the basis of a reversible interaction between a protein and group of proteins and a specific ligand coupled to a chromatographic matrix. The technique can be used to separate active biomolecules from denatured or functionally distinct biomolecules, separate low concentrations of pure material from large volumes of raw samples, and remove specific or non-specific impurities.

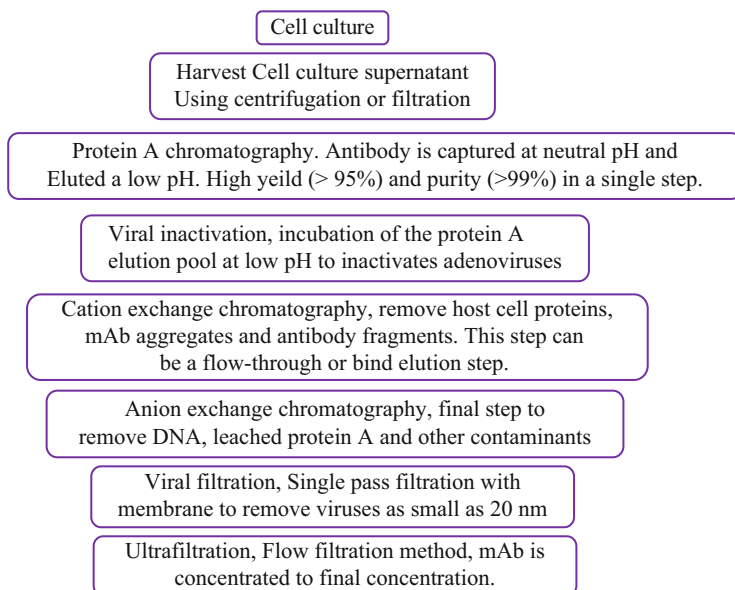
Biological interaction between ligand and target molecules can be a result of hydrophobic or electrostatic interaction or van der Waals force (Reverberi and Reverberi 2007). To elute the bound molecules from the affinity matrix, the interaction can be reversed, either specifically using a competitive ligand or alteration of ionic strength or polarity by changing the pH. The binding ligand must retain its specific affinity for binding to the target molecule, and the ligand and the target molecule can be removed, so the target molecule can be removed in active form after washing away the unbound substance. The bond between them must be reversible. Changes in the elution buffer conditions reverse the interaction between the target molecule and the ligand, allowing the target molecule to elute from the column. Changes in pH alter the ionization potential of the charged groups of the ligand and/or bound protein. This change can directly affect the binding site, reduce their affinity or cause indirect changes in affinity. The most common way to elute the binding material is to gradually lower the pH. The chemical stability of the matrix, ligand and target protein determines the pH limit that can be used.

Immunoglobulin G (IgG) is a type of antibody that accounts for about 75% of human serum antibodies. IgG is the most common type of antibody in the bloodstream. Each IgG molecule has two antigen binding sites. The Fc region of IgG bears a highly conserved N-glycosylation site.

Protein A is derived from a strain of *Staphylococcus aureus* and contains five regions that bind to the Fc region of IgG. Protein A binds to Sepharose as an affinity ligand, so these regions are free to bind. One molecule of bound protein A can bind to at least two IgG molecules, depending on the starting species of the immunoglobulin. Dynamic binding capacity depends on factors such as binding strength and flow velocity at some point in sample application. Peristaltic pumps may be used to maintain a constant flow rate of 0.5–1 mL/min during sample application. The basis for purification of IgG, IgG fragments and subclasses is the high affinity of protein A for the Fc region of IgG-type polyclonal and monoclonal antibodies. Protein A-Sepharose is a complex where protein A is immobilized on Sepharose by the CNBr. Protein A-Sepharose complex is very helpful in isolating immune complexes.

10.1.1.2 Method

Purification of monoclonal antibody depends on several steps such as preparation and collection of cell culture supernatant, preparation of protein A-Sepharose



Scheme 10.1 Schematic diagram of monoclonal antibody purification and concentration

column and loading of sample, washing of column and elution of bound antibody from the column (Scheme 10.1).

Suspension hybridoma cells grown in culture were transferred into 50 mL centrifuge tubes and centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was removed from the centrifuge tubes and kept in the bottle, whereas cell pellet was discarded. The bottle containing the culture supernatant was stored at −20 °C or −70 °C until purification.

Further, protein A-Sepharose column was prepared and cell culture supernatant was loaded. In brief, 0.5 g of protein A-Sepharose was weighed and swollen in 5 mL of PBS. The swollen protein A-Sepharose was put in an empty column, and the outlet was connected with a peristaltic pump. The flow rate was adjusted to 0.5–1 mL/min. The column was washed with 10–15 mL of PBS (1×) for equilibration with the buffer, and one must be careful that the column should not be dry any time. The cell culture supernatant was applied at the top of the column and then allowed to flow slowly. The flow-through was collected in a separate tube. The flow-through material was re-applied and passed through the column; again the flow-through was collected and stored at −20 °C.

The column is repeatedly washed with 1× PBS (phosphate buffered saline, pH 7.4). In brief, PBS 10–20 mL was applied at the top of the column and allowed to flow through the column. When no pink colour was visible in the column matrix, the flow-through was collected in 10 Eppendorf tubes. The optical density of each fraction was checked in a UV spectrophotometer at 280 nm wavelength of light. If

the optical density of the collected washing fraction reaches zero, PBS application was stopped at the top of the column, and the outlet of the column is closed.

Elution of bound antibody from the column can be done using Tris (pH 9.0) buffer followed by elution buffer (50 mM glycine/0.15 M NaCl, pH 8.5). The elution buffer was applied at the top of the protein A-Sepharose column containing bound antibody and relaxed antibody. The antibody was collected in the tubes containing Tris buffer.

The tubes were mixed and OD was checked at 280 nm wavelength against blank Tris buffer. The tubes containing the elution buffer were pooled and stored at 4 °C. After the elution of the antibody, the protein A-Sepharose column was washed with 10 mL PBS, pH 7.4, and stored in the same buffer containing 0.02% sodium azide at 4 °C.

The obtained OD values at 280 nm were converted into protein concentration after calculation following standard curve with BSA. Fractions with higher protein concentration are found to be IgG fraction.

10.1.2 Concentrate the Purified Antibody

Purified monoclonal antibodies are typically concentrated using a filter membrane. Centrifugation is a very useful and common technique to separate target molecules from a heterogeneous mixture. The gravity of matter is the central principle of centrifugation. Here, more dense particles fall out from the solution quickly, and the less dense particles fall later. Centrifugation helps separate particles that do not separate naturally. Centrifugal filter is another important tool for separation. This filter helps separate particles by size and density in a single saturation motion. It is also commonly used to separate RNA and DNA molecules, unsoltered proteins and molecules by size or remove impurities from liquids. Centrifugal filters work on the same principle as standard filters. Particles larger than the pore size cannot pass through and are concentrated together. The advantage of centrifugal filters is that they separate faster due to the centrifugal force, which is much larger than gravity. The particles collect on the membrane to form a cake-like structure, and the rest of the mixture flow through the filter. The cake can be dried by centrifugation, while the liquid passes through the membrane. Centricon's centrifugal filter device enables rapid and efficient concentration and desalting of polymer solutions by ultrafiltration using a cellulose membrane developed for use in centrifuges with fixed angle rotors. It can provide up to 80× sample enrichment while minimizing solute loss due to adsorption.

Concentration is achieved by ultrafiltration of the sample solution through an anisotropic membrane. Centrifugal force sends the solvent and low molecular weight solute through the membrane into the filtrate vial. The retained macrosolute is on the membrane of the sample reservoir. When the sample volume decreases, the concentration of retained solute increases. Transfer the sample to a holding vial by placing the vial on top of the sample reservoir for recovery. This minimizes adsorption losses on the membrane and reservoir walls. Filtration stops when the solvent comes out.

Filtration stops when the solvent meniscus reaches the outer edge of the solvent bottom channel.

The centricon was washed and filled with PBS and centrifuged at 5000–6000 rpm for 10–15 min. Further, the centricon was taken out and emptied. It was filled with the purified monoclonal antibodies (IgG) and centrifuged at 6000 rpm. It was centrifuged till most of the solvent passed through the membrane, and about 500 μL of it remained above the membrane. The retained solvent along with the concentrated protein was collected in an Eppendorf tube. One microlitre the concentrated protein was taken in micropipette, and the optical density was checked in NanoDrop instrument or Bradford method was followed to determine the concentration of IgG. The purified monoclonal antibody was taken as a sample on centrifugation; it was observed that the solvent had passed through the membrane and protein with molecular weight larger than the cut-off value of the membrane was retained on the membrane.

10.1.3 Analysis and Quality Assurance

The purified antibody is necessary to check the purity and quality measurement. In general, the purified antibody checked the purity with SDS-PAGE (sodium dodecyl sulphate polyacrylamide gel electrophoresis). SDS-PAGE is an analytical method for separating charged molecules in a mixture according to the molecular weight in the electric field. Use SDS to give equal charge-to-mass ratios to protein molecules. Therefore, SDS-PAGE is an electrophoresis method that allows protein separation by mass.

SDS-PAGE is a discontinuous electrophoresis system. The medium is a polyacrylamide-based discontinuous gel. About one SDS molecule can bind two amino acids. SDS acts as a surfactant, covering the protein's intrinsic charge which is conferring them uniform and equal charge-to-mass ratio. The intrinsic charge of the protein is negligible compared to the SDS charge, and the positive charge is significantly reduced in the basic pH range of the separation gel. When a constant electric field is applied, proteins move to the anode at different rates depending on their mass, allowing accurate mass separation of proteins.

This electrophoresis system uses two gel systems with different concentrations. Gel stacking and separation differ in pore size, ionic strength and pH value. The pH of the stacking gel is 6.8, while the pH of the separation gel is 8.8. The most commonly used electrolyte is the tris-glycine buffer system containing SDS. At neutral pH, glycine forms a predominantly zwitterionic form, and at high pH, glycine loses its positive charge and becomes predominantly anionic. In stacking gels, small negatively charged chloride ions move in front of the protein, and slightly larger zwitterionic glycine move behind the protein (as initial training ions). In the separation gel, both ions move in front of the protein. The pH gradient between the stacking gel buffer and the separation gel buffer provides a stacking effect at the boundary between the stacking gel and the separation gel. This is because the glycine ion loses

its positive charge as the pH value increases. The bands of various proteins are narrowed, and the stacking effect is sharpened.

For gel solutions, acrylamide is mixed as a gel-forming agent, and methylbisacrylamide is mixed as a cross-linking agent to stack or separate gel buffer, water and SDS. With the addition of a TEMED catalyst and radical initiator, ammonium persulfate (APS) provides the free radicals required for the polymerization of acrylamide and bisacrylamide. TEMED catalyses ammonium persulfate to form free radicals, and TEMED promotes the polymerization of acrylamide and bisacrylamide.

Glycerine is usually added to increase the density to prevent the protein sample from diffusing into the electrode buffer. To observe the mobility of the protein sample (purified antibody), add a bromophenol blue dye or any tracer dye to the sample. These colourants can move faster than all macromolecules as long as the dye moves to the edge of the gel until electrophoresis stops.

SDS is generally added in excess amount to the sample, and the mixture is heated at 95 °C for 5 min. Heating disrupts the secondary and tertiary structure of the protein by disrupting hydrogen bonds and extending the molecules. Disulphide bridges can be cleared by reduction. For this purpose, reducing thiols such as *p*-mercaptoethanol, dithiothreitol (DTT) or dithioerythriol (DTE) are added to the sample buffer.

10.1.4 Preparation of Separation Gel

The glass plates were assembled after placing two spacers in between two clean glass plates. The sandwich was locked to the casting stand. Further, the separating gel solution was prepared by mixing of 30% acrylamide/0.8% bis-acrylamide, Tris-Cl/SDS (pH 8.8), 10% ammonium persulfate and 10 µL of TEMED, and optimum water was poured. The mixture was stirred gently and used immediately. The final concentration of the separating gel varies from 8 to 18%. The top of the gel was slowly coated with a layer of 70% ethanol or Milli-Q water. The gel is allowed to polymerize for 30–60 min at room temperature. A layer of water or alcohol has been poured from the top of the plates. Stacking gels were prepared in a 50 mL conical flask by mixing 30% acrylamide/0.8% bisacrylamide, Tris-Cl/SDS (pH 6.8), 10% ammonium persulfate and 5 µL TEMED, and optimal water was poured. The mixture is gently stirred and loaded immediately. The final concentration of the stacking gel was 5%. The stacking gel solution is slowly poured into the centre of the sandwich panel along one edge of one of the spacers until the height of the solution in the sandwich panel is 1 cm above the top of the plate, followed by a sizing insert a comb. The stacked gel solution is allowed to polymerize for 30–45 min at room temperature.

10.1.5 Preparation of Protein Sample and Loading

Approximately 15 μL of the protein (antibody) sample was mixed with 5 μL of the reductive dye in an Eppendorf tube, and 5 μL of the unreduced dye was mixed with 15 μL of the protein sample in another tube. They were heated for 3–5 min at 100 °C. Protein molecular weight standards were prepared by mixing 5 μL of standard with 1 μL of reducing and non-reducing dyes, subsequently heating as above. The gel sandwich is attached to the upper and lower spacers in the electrophoresis device by means of clamps and screws. The upper and lower buffer chambers were filled with $1\times$ electrophoresis buffer. The Teflon comb is carefully removed without tearing the edges of the polyacrylamide well. Protein samples and standards are carefully loaded. The power supply has been connected to the cell and is starting to work. After the next dye, bromophenol blue, nearly reached the bottom of the separating gel, the power was cut off. The spacer is removed from both compartments, and the glass plates are removed with the gel sandwich attached from the unit, and the sandwich is placed on a sheet of blotting paper. One of the pads is carefully removed and the glass is opened, revealing the gel. The gel is carefully removed from the bottom plate.

10.1.6 Staining and Destaining of the Gel

The gel was placed in a plastic or glass tray, and approximately 2550 mL of the dye solution was poured and gently shaken in a slow rotating platform for a minimum of 20 min at room temperature. To make destain, the gel was removed and placed in a tray containing 500 mL of decolourization solution (50 mL of methanol with 50 mL of glacial acetic acid and 400 mL of H_2O) and rotated for approximately 30 min to completely destain.

10.1.7 Quality Assurance

The bands in the protein molecular weight marker will be sharp. The bands of the purified sample should be much darker than that of a sample that may have a higher protein concentration because IgG is purified on a laboratory scale. In the case of non-reducing gels, bands were observed near 150 kDa for standard IgG, laboratory purified IgG and IgG samples in the same position. This indicates that the sample eluted from the protein A-Sepharose column contains IgG molecules. The presence of bands other than those mentioned above indicates that impurities are present in the eluted sample. In the case of reductive dyes, the standard IgG bands will be pale and stained, indicating degradation of the IgG molecules. Both purified and standard antibodies will display cut-off bands near 50 kDa and 25 kDa, respectively, for the heavy and light chains in the antibody. The other band may be a reduced form of the impurity present because the band corresponding to the impurity in the non-reducing

PAGE is not present in the reducing PAGE. Therefore, the eluted protein must be identified as IgG.

10.2 Immuno-electrophoresis

Immuno-electrophoresis refers to deposition on agar by the action of an electric field. It is a process that combines immunodiffusion and electrophoresis. When an electric current is applied to the gel-covered lateral layer, the antigen mixture placed in the well is separated into individual antigenic components according to charge and size. After electrophoresis, the isolated antigen reacts with a specific antisera arranged parallel to the electrophoretic migration, and diffusion occurs. The presence of antisera migrates to antigenic components and forms separate sedimentation lines within 18–24 h, each representing a reaction between an individual protein and an antibody (Csako 2019).

Counter-current immuno-electrophoresis is a technique in which antigen and antibody migrate in opposite directions, forming a visible white precipitate in the area between the wells. Since the movement of antigen and antibody through agarose gel is not simple double immune diffusion, but due to applied voltage, it is also called tension-increased double immune diffusion. In this method, antigen and antibody are placed in parallel wells and moved towards each other under the action of an electric field. At some point, an equivalence region appears between the wells, and the antigen-antibody complex precipitates as a visible white line. This qualitative method is much faster and more sensitive than the double diffusion method and is used to rapidly identify antisera for antibodies to a specific antigen. Immunoprecipitation occurs when antigen molecules (negatively charged in alkaline buffer) at the level of the cathode (cathode) migrate within an electric field, causing a pair of antibodies (positively charged) to migrate back from the anode (Emmett and Crowle 1982).

10.2.1 Protocol

The glass slides will be cleaned gently with soap water, wiped with tissue paper and made grease free by wiping with alcohol. The slide was marked with a marker that will be towards the negative electrode during the electrophoresis and placed in the smooth surface of the table. A total of 500 mL of $1\times$ TAE buffer was prepared by dilution of $50\times$ TAE buffer with water. In a 1000 mL conical flask, 0.75 g of agarose was weighed, and 500 mL of $1\times$ TAE buffer was added and boiled in microwave oven to dissolve the agarose completely with intermittent swirling which will make 1.5% agarose gel. The agarose solution was cooled to approximately 50–60 °C and poured slowly (required amount app. 3 mL) and carefully on the top of each glass slide. The agarose is allowed to form solid gel for about 30 min, and then two wells were made about 1.5 cm apart by punching through the agarose gel using a gel puncher. The inner gel piece was removed from the well carefully. The slides are

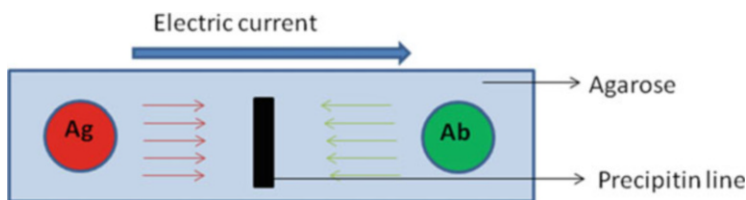


Fig. 10.1 Counter-current immunoelectrophoresis

placed on a horizontal electrophoresis apparatus, and the buffer ($1\times$ TAE) was poured in both sides of buffer chamber sufficient enough just to touch the slides. Paper wicks were used to connect both sides of glass slides with buffer chamber. Fifty microlitre of BSA antigen will be added into the well placed towards negative electrode, and 50 μ L of anti-BSA polyclonal antibody is added in the well towards the positive electrode. Electrophoresis is performed by connecting to the power supply until precipitin line was observed between the wells (Fig. 10.1). The precipitin line between the antigen and corresponding antiserum is to be observed. A faint white precipitin line will be observed in between the two wells. The precipitin line indicates that the antigen and the antibody have undergone cross-linking and formed antibody-antigen complex.

10.3 Western Blotting

Western blotting is a widely used method to detect and analyse proteins based on their ability to bind specific antibodies. Western blotting is performed quickly using simple equipment and inexpensive reagents. It is very useful and is commonly used in molecular biology laboratories. The specificity of antigen and antibody responses allows the identification of target proteins in small complex protein mixtures. In Western blot, a protein sample is electrophoresed by SDS-PAGE electrophoresis and electrophoresis on polyvinylidene difluoride (PVDF) or nitrocellulose membranes. The delivered protein is detected using specific antibodies labelled with primary and secondary enzymes. Antibodies bind to specific amino acid sequences known as epitopes. Because the amino acid sequence varies from protein to protein, an antibody can recognize a specific protein in a group or among several groups. Thus, one protein can be identified in cell lysates containing thousands of different proteins, and the amount can be quantified by Western blotting. First, proteins separate from each other according to their size. Second, the antibody is used to detect the protein of interest. Finally, the antibody-protein complex is observed using a substrate that reacts with the enzyme (Kurien and Scofield 2015).

Western blotting is the transfer of proteins from an SDS-PAGE gel to a solid support membrane. There are two types of blotting machines used to transfer proteins to a solid medium, wet transfer (reservoir blotting) or semi-dry transfer. Semi-dry systems require less soaking time than staple systems, and the buffer volume required for semi-dry systems is less than those required for tank systems.

The reservoir system has high delivery efficiency and uniform delivery of high molecular weight proteins. Although more expensive, the PVDF membrane is stronger and has a higher protein absorption capacity, making it suitable for Coomassie bright blue staining and detection and rescan of specific substrates for alkaline phosphorylation. Different buffers are used for delivery depending on the molecular weight and properties of the target protein. Methanol in transfer buffer stabilizes the gel and prevents gel swelling. When delivering high molecular weight proteins, lowering the methanol concentration slightly swells the gel to facilitate protein delivery (Jensen 2012).

10.3.1 Required Material

Vertical gel electrophoresis apparatus and power supply, the reagents for preparing and running SDS-PAGE, staining and destaining solutions, PVDF (polyvinyl difluoride) membrane, protein sample, bovine serum albumin (BSA), electroblotting apparatus, Whatman paper (3 mm), transfer buffer (25 mM Tris/192 mM glycine/20% methanol, pH 8.3), casein, antibody, PBS (pH 7.4), protein A-HRP conjugate, hydrogen peroxide, DAB (3,3-diaminobenzidine).

10.3.2 Protocol

At first, the separation of proteins in SDS-PAGE is necessary. With a 10-well comb, 12% SDS polyacrylamide gel was prepared. Different protein samples were prepared, and it was mixed with loading dye boiled for 5 min, cooled on ice and loaded onto the gel. The gel is run at 50 V till the sample enters into the separating gel, and then, the voltage is increased to 200 V such that the loading dye migrates 1 cm from the bottom of the gel. Following electrophoresis, the glass plates were removed from the electrophoresis apparatus using a spacer. The same gel is to be run in two sets, and one set is process for staining with Coomassie brilliant blue and destaining consequently.

The separated proteins were transferred from gel to the membrane. Another half of the gel was transferred into a tray containing about 100 mL of transfer buffer and shaken for 15–30 min. Two pieces of Whatman filter paper were cut and saturated by soaking in transfer buffer. The fibre pads were also saturated in transfer buffer, and the buffer tank was half filled with transfer buffer. The gel holder was opened, and the pre-wetted fibre pad was placed on top of the opened panel of the holder. A piece of saturated Whatman filter paper was placed on top of the pad, and the pre-equilibrated gel was placed on top of it. A pre-wetted PVDF membrane was placed on the top of the gel, and air bubbles (if any) were removed by rolling a glass pipette gently over the membrane. A saturated filter pad was placed on the top of the filter paper that was placed on top of the pVDF membrane, and the holder was closed. The gel holder containing gel sandwich was placed in the transfer tank so that the gel side should be present on the cathode side of the tank and the membrane side

on the anode side of the tank. The buffer tank was filled with transfer buffer, and the lid was placed, and the unit was plugged into the power supply. The apparatus was seen at 30 V (100 mA) overnight preferably at 4 °C. Following transfer turning the power supply switched off, the lid was removed, and gel cassette is disassembled, the membrane was taken out and stored in between two sheets of Whatman paper at 4 °C.

10.3.3 Blocking of Membrane

The PVDF membrane was taken out from 4 °C and placed in a plastic tray containing 50–100 mL of PBS supplemented with 3% casein and was incubated for 1 h at room temperature to block the sites in the membrane non-occupied by the transferred proteins. After 1 h of incubation, the membrane was washed thrice with 100 mL of 1 × PBS containing 0.15% Tween 20.

10.3.4 Binding of Primary Antibody

Antibody (monoclonal or polyclonal) of the target protein is added to 20 mL of PBS (containing 1% BSA), diluted 250 times and incubated at room temperature for 45 min. After incubation, the membrane was washed thrice with 100 mL of PBS containing 0.15% Tween 20.

10.3.5 Binding of Secondary Antibody

Protein A-HRP conjugate was diluted in PBS, and the membrane was incubated with this solution at room temperature for 45 min. After incubation, the membrane was washed thrice with 100 mL of PBS containing 0.15% Tween 20.

To detect immunoreactive proteins, a solution must be added next to this substrate. The substrate concentrate was diluted in buffer and added to the solution in the required amount. The membrane was immersed in the chromogenic solution and incubated at room temperature with gentle agitation until colour development was complete. Instead of immunoreactive proteins, a light brown colour appears. Wash the membrane with water for 10 min with gentle stirring to stop colour development, air-dry Whatman paper, and store in the dark.

The proteins are separated successfully based on their size using SDS-PAGE. The part of the gel which was stained with Coomassie blue had a well-resolved molecular weight marker. The band corresponding to the target protein can be seen at desired molecular weight (Fig. 10.2).

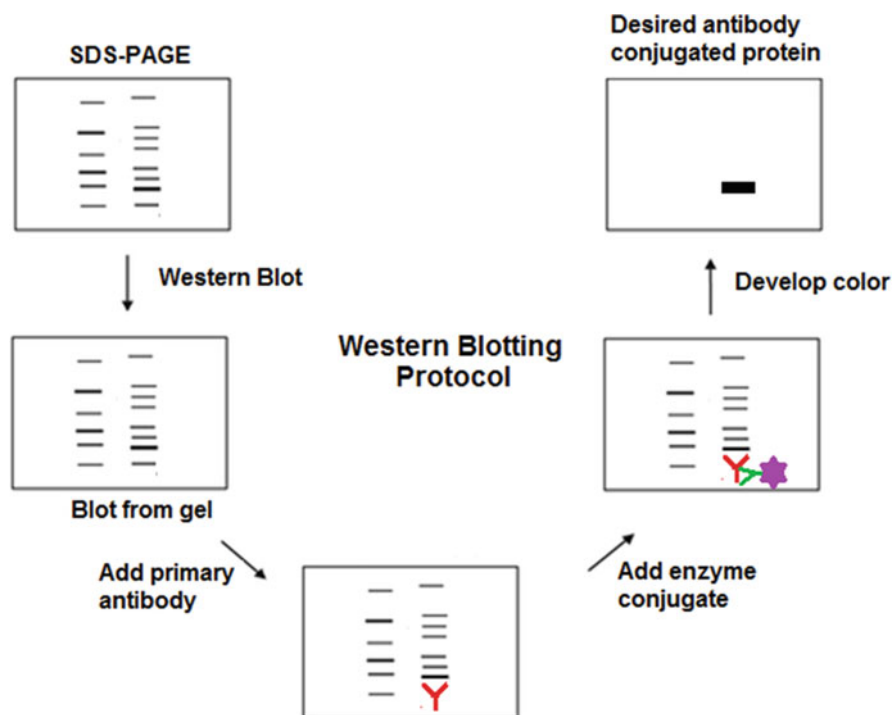


Fig. 10.2 Schematic representation of Western blotting to identify the target protein

10.4 Determination of Cell Number

The percentage of viable cells in a cell suspension can be estimated using a variety of methods. The simplest and most widely used method is the dye exclusion method. In the dye exclusion method, viable cells exclude dye, but dead cells do not. Trypan blue is the most commonly used. The trypan blue dye exclusion assay is used to determine the number of viable or dead cells in a cell suspension. Trypan buoys are large, negatively charged molecules. The trypan blue dye exclusion assay is based on the principle that these cells have intact cell membranes that exclude this dye, but dead cells do not. This is a simple and inexpensive method that is a good indicator of membrane integrity, and dead cells turn blue within 1 s of exposure to the dye. Cell counting is usually done with a haemocytometer. Therefore, a calculation error of 5–10% can be considered. Calculation errors are due to poor cell dispersion, loss of cells during cell dispersion, incorrect dilution of the cell suspension, improper chamber filling and the presence of air bubbles in the chamber (Crowley et al. 2016).

10.4.1 Required Material

Haemocytometer, coverslip, 70% ethanol, tissue paper, trypan blue solution, cell suspension, Eppendorf tip, tube and pipettes.

10.4.2 Method

About 100 μL cell suspension was taken into a 1.5 mL Eppendorf tube, and an equal volume of trypan blue solution (100 μL) was added to it and mixed properly. The haemocytometer was cleaned properly with detergent; also the cover slip should be cleaned with 70% ethanol and dried. The coverslip is put at the top of the haemocytometer, and 10 μL of cells were applied and mixed with trypan blue in between coverslip and haemocytometer. The cells are observed under the inverted microscope. The total number of cells should be counted in four squares present at four corners in the haemocytometer. The total number of cells as well as live (unstained) and dead (stained) cells should be calculated per mL of cell suspension as follows.

The area of each square of haemocytometer is $1\text{ mm} \times 1\text{ mm} = 1\text{ mm}^2$

Volume of each square = $1\text{ mm}^2 \times 0.1\text{ mm} = 0.1\text{ mm}^3$

Number of cells present in one square (0.1 mm^3)

Cells present in right lower (RL)-A number of cells; right upper (RU)-B number of cells; left lower (LL)-C number of cells and left upper (LU)-D number of cells.

So, average number of cells— $A + B + C + D/4 = \text{let } X$

X cells are present in 0.1 mm^3

In 1 mm^3 , $1000 \times$ cells are present.

In 1 cm^3 , no of cells present = $1000 \times \text{cells}/\text{mm}^3 \times (10\text{ mm}/1\text{ cm})^3 \times \text{dilution factor} \times 2$.

10.5 Immunofluorescence Assay

Immunofluorescence (IF) is an incredible method for getting knowledge in cellular structures and internal organization of the cell, using microscopic or spectroscopic instruments. Specific or target protein is easy to determine for their appearance, location and interaction with other biomolecules. An immunofluorescence analysis depends on some basic principles where specific antibodies bind to the target protein. Fluorescent colours are coupled to these immune complexes in order to visualize the target protein.

Antigens and antibodies have their own specificity to each other. When linked/labelled with an appropriate fluorescent dye, it is easy to recognize cells carrying the surface receptor of interest or to identify specific cell types. This extraneous fluorescence can be detected with fluorometer (fluorimeter or UV/fluorescence microscope). The antigens and antibodies have an inborn property of explicitness for

each other. When connected/labelled with a reasonable fluorescence dye, it is easy to recognize the cells bearing surface receptor of interest or detecting specific cell type. This sort of extraneous fluorescence can be distinguished with a fluorescence measuring device (fluorimeter or a UV/fluorescence microscope).

Sometimes the fluorescence molecule (propidium iodide binds to DNA in dead cells; Evans blue attach to non-fluorescence cells) might have the option to bind with intracellular organelle. On the other hand, some cell organelles showed some inherent fluorescence without any addition of dye, and this stain is called as counterstain (Odell and Cook 2013).

10.5.1 Principle

In immunofluorescence assays (IFA), specific monoclonal or polyclonal antibodies are conjugated to fluorescent dyes (fluorochromes/fluorophores), which can be visualized using a fluorescence microscope, fluorometer, fluorescence scanner or waft cytometer. Fluorophores are excited thru manner of way of slight at a specific wavelength and launch the greater strength through emitting mild at another, longer wavelength. Fluorescence microscopes or plate-readers used for the detection consist of filters specific for every emission wavelength. Conventional fluorophores like fluorescein isothiocyanate (FITC) emit mild at 550 nm wavelength. The absorbance of strength thru manner of way of the fluorescence moiety occurs in plenty much less than 10–15 s. The excited molecules then fall back to lower energy levels, resulting in loss of energy in the form of heat and photon longer wavelength, as compared to incident rays. This happens in a very short period of time (less than 10–8 s). The released energy is detected with the detectors. The fluorescence may be observed through an UV/fluorescence microscope. Dyes emitting at longer wavelengths (red) are more appropriate because several biological materials can emit green light after exposing to UV rays (autofluorescence). The fluorescent cells are visible in dark to facilitate best detection as well as to minimize the effect of drift radiations that might negatively impact the results.

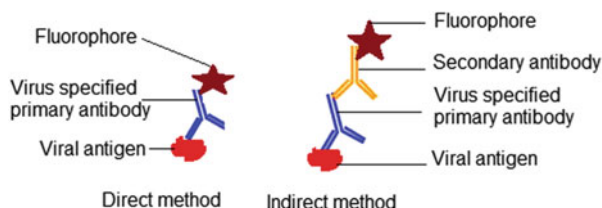
Excitation—Emission = ΔE Or $h\nu$ excitation – $h\nu$ emission = $\Delta h\nu$; thus emission < excitation. The strength related to the emitted rays is usually much less than the strength associated with the excitation rays due to the fact the excited molecules generally tend to lose their strength ($\Delta h\nu$) via interaction with the impurities (salt ions, mobile components, particulate matter, etc.) in the solution or tissue.

10.5.2 Immunofluorescence Technique

Two types of immunofluorescence assays are (1) direct fluorescent antibody (IFA) test and (2) indirect fluorescent antibody (IFA) test.

The fluorophore is visualized under a fluorescent microscope (e.g. epifluorescence and confocal microscopes). It is very easy to distinguish

Fig. 10.3 Schematic representation of immunofluorescence technique



among IF techniques relying on whether or not the fluorophore is conjugated to the primary or the secondary antibody: direct IF makes use of a single antibody directed towards the target of interest. The primary antibody is at once conjugated to a fluorophore. The signal received in direct techniques may also appear susceptible, whilst in comparison to indirect techniques, signal amplification furnished with the aid of the usage of secondary antibodies does now no longer occur.

Indirect IF uses two antibodies. The primary antibody is not conjugated, and a fluorophore-conjugated secondary antibody to the primary antibody is used for detection (Fig. 10.3). Due to the additional complexity of the indirect strategy, the correct secondary antibody may also be selected. This is especially true for multiplex experiments that require multiple secondary antibodies, each targeting a specific species and conjugated to a different dye. Several secondary antibodies bind to the primary antibody, enhancing the signal. Since the fluorophore is already conjugated to the primary antibody, the direct method minimizes cross-species cross-reactivity. Secondary antibodies may also further cross-react with non-target species. The use of pre-adsorbed secondary antibodies can reduce cross-reactivity. Direct and indirect methods are not limited to immunofluorescence. They are also applicable to a variety of methods based on the use of fluorophore-binding antibodies, including flow cytometry, ELISA, Western blotting and immunohistochemistry. Despite indirect methods, finding low-protein proteins can sometimes be difficult. Biotinylated antibodies provide an additional layer to enhance signal amplification.

10.5.3 Labelling of Antibodies with Fluorochromes

Antibodies can be sorted by binding directly to fluorochromes. For example, detection of CD4⁺Th (T helper cells) in the human blood can be achieved using a defined anti-human CD4⁺ antibody against fluorescein. Therefore, the detection of target cells using specific fluorochrome antibodies is called direct fluorescence. Alternatively, an anti-human CD4⁺ antibody (no fluorochrome molecule) can be used in combination with a fluorescently labelled anti-human antibody (e.g. a secondary antibody, a rabbit anti-human IgG fluorescein antibody). This technique is called indirect fluorescence. For indirect methods, fluorochrome-labelled secondary reagents are suitable. Direct labelling may be an optional technique that does not require these conjugates or specifically requires immediate labelling.

10.5.4 Detection of Fluorochrome-Labelled Reagent

A fluorescence microscope is required to detect a reagent labelled with a fluorochrome. For the low fluorescence that occurs when staining cells, the microscope must be adjusted for epifluorescence, where excitation radiation is delivered through the objective lens to the bottom of the sample. Absorbing radiation of the appropriate wavelength raises the fluorescent electrons to a higher energy level. When these electrons return to their ground state, light of a characteristic wavelength is emitted. This emitted light bureaucracy the fluorescent image seen inside the microscope. Individual fluorochromes have discrete and feature excitation and emission spectra. Filters are used to make certain that the specimen is irradiated with light at the perfect wavelength for excitation. By putting a second set of filters inside the viewing light direction that transmit light of the wavelength emitted with the aid of using the fluorochrome, images are formed only by the emitted light and form a high-resolution image in dark background. Another extension of immunofluorescence technique is a fluorescence-activated cell sorting (FACS) that is able to detect, count or sort diverse types of cells on the premise in their size (30–100 micrometer size), viability (the usage of propidium iodide that binds DNA of useless cells), granularity and unique surface receptors. Thus FACS is used for multi-parametric analyses for detection, counting and sorting of specific type of cells of interest. The FACS is used for quantitative detection and evaluation of T helper cells (Th cells; CD4⁺) and cytotoxic T cell (Tc cells; CD8⁺) to display the reputation of AIDS patients and to take a look at the effectiveness of the anti-AIDS therapy.

10.5.5 Selection of Fluorochrome

The desire for fluorochromes is mainly limited by commercially available filter kits for microscopy (Table 10.1). Most filter kits are best suited to contain rhodamine or fluorescein. Texas magenta/red can be used with a rhodamine filter kit, but these filters no longer match their emission spectra exactly. The increased availability of phycobiliproteins, which could theoretically be about 50-fold brighter, will have a significant impact on the design of immunofluorescence experiments over the next

Table 10.1 Properties of fluorochromes used in cell staining

Fluorochrome	Excitation wavelength (nm)	Emission wavelength (nm)	Colour
DAPI	365	>420	Blue
Fluorescein	495	525	Green
Hoechst 33258	360	470	Blue
R-Phycocyanin	555, 618	634	Red
B-Phycoerythrin	545, 565	575	Orange, red
R-Phycoerythrin	480, 545, 565	578	Orange, red
Rhodamine	552	570	Red
Texas red	596	620	Red

few years, as various filter sets become available. Fluorescein emits a faint yellow colour that is correctly detected using the human eye and a maximum filter. However, fluorescein is prone to discolouration of images quickly, and bleach retardants containing DABCO or *o*-phenylenediamine should be delivered to the editing environment. Rhodamine gives a purple/red colour and no longer carries the risk of fading like fluorescein, but the rhodamine conjugate is more hydrophobic and therefore provides a better background than fluorescein. Texas red additionally emits a strong red/purple light, whose emission spectrum is different from that of fluorescein than that of rhodamine. If dual labelling is used, fluorescein can be mixed with Texas red and rhodamine.

10.5.6 Materials

Milli-Q or purified deionized water. Phosphate buffered saline (PBS) (10×), Triton X-100, bovine serum albumin (BSA), 1 L graduated cylinder or glass beaker, NaCl, KCl, Na₂HPO₄, and KH₂PO₄.

10.5.7 Blocking Buffer

Buffers have been used to block free sites of proteins or membranes. BSA containing blocking buffer is a good choice due to its high affinity to proteins. The blocking buffer is more powerful when it improves the sensitivity of an assay with the aid of using interference and enhancing the signal-to-noise ratio. Required material of blocking buffer are as follows: 1× PBS, 0.3% Triton X-100, 25 mL. Add 42.50 mL water to 100 mL conical tube. Measure 5 mL of 10× PBS and 2.5 mL of 5% normal serum, and mix well. During stirring, add 75 µL of Triton X-100.

10.5.8 Dilution Buffer

Seventy-two millilitre water to 100 mL conical tube. Eight millilitre 10× PBS and add 0.5 g BSA, and mix well. While stirring, add 240 µL Triton X-100.

10.5.9 Fixative Solution

Four percent formaldehyde in PBS. To prepare 1 L of 4% formaldehyde, take 800 mL of 1× PBS in a beaker, and start stirring over a heat plate to approximately 60 °C. The solution does not boil, and one should be careful in this stage. Further, 40 g of paraformaldehyde powder is added to the heated PBS solution. The powder will dissolve slowly and raise the pH by adding 1 M NaOH dropwise. After few minutes the solution turns to a clear solution, and stop adding NaOH. When the paraformaldehyde is dissolved completely, the solution should be cooled and

filtered, and volume should be adjusted with the solution to 1 L with $1\times$ PBS. Adjust pH at approximately 6.9–7.0. The solution can be aliquot and stored at 2–8 °C.

10.5.10 Immunostaining

Most required reagents: Streptavidin; 0.2 M sodium phosphate buffer, pH 7.0; 0.1 M sodium phosphate buffer, pH 7.0; biotin, streptavidin (0.1%). For 1 mL, 1 mg of streptavidin is mixed with 1 mL of 0.1 M sodium phosphate buffer, pH 7.0; dilution of primary antibody 1:100; dilution of polyclonal biotinylated secondary antibody 1:100; dilution of fluorophore on conjugated streptavidin 1:100; glutaraldehyde; and extension and Gold Antifade reagent with DAPI.

10.5.11 Immunofluorescence Staining Method

At first, block specimen in 200 μ L blocking buffer for 15–20 min, and add 200 μ L streptavidin (0.1%) to the section for 20 min to block the endogenous biotin. Wash the sample repeatedly with PBS ($1\times$) for 5 min each and minimum two times. Next to that, 1:100 dilutions of primary antibodies are added in antibody dilution buffer. Wash thrice in 100 mL wash buffer for 5 min each, and 1:100 dilution of polyclonal biotinylated secondary antibodies is added immediately; incubate the sample with 200 μ L of secondary antibody dilutions, and incubate at 4 °C for overnight, or incubate at room temperature for 1 h. Again, wash gently with PBS wash buffer. Now, 200 μ L of 1:100 dilution of fluorophore-conjugated streptavidin which is prepared in antibody dilution buffer is added and incubated at room temperature in dark for 30 min. Wash three times with 100 mL PBS $1\times$. Add 200 μ L of 1% glutaraldehyde buffer for 25 min to fix the antibody-streptavidin conjugate, and immediately wash gently with PBS. Samples are placed on glass slides and coverslips with 200 μ L of ProLong Gold Antifade Reagent containing DAPI that is observed at the appropriate excitation wavelength of the fluorophore used.

10.5.12 Uses

- Bacterial, viral and fungal infections can be detected in nasopharyngeal samples using direct fluorescent antibodies (DFA), which are also commonly used to detect respiratory syncytial respiratory virus (RSV), influenza A and B and adenovirus.
- Immunofluorescence is a successful technique which is routinely used to identify a number of subpopulations of lymphocytes, such as CD4⁺ and CD8⁺ T cell.
- The technique is also suitable for detecting Ag-Ab complexes in autoimmune disease; organelles in tissues and hormone localization and cellular products in situ are also easily detected.

- The most important application is the localization of antigens in tissue sections or in subcellular compartments.
- Bio-imaging of tissue samples. Rapid prognosis of the infections is now viable withinside the point of care (POC).

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11.1 Introduction

Microscope is the only instrument that enables us to visualize any organism or cellular structure because we cannot see such structures with our naked eye or unaided eye. Microscopy is the technical field of using microscopes for visualizing specimen that fall outside the resolution range of the human eye and for elucidation of fine structures or cellular view of a given sample (Johnson 2018).

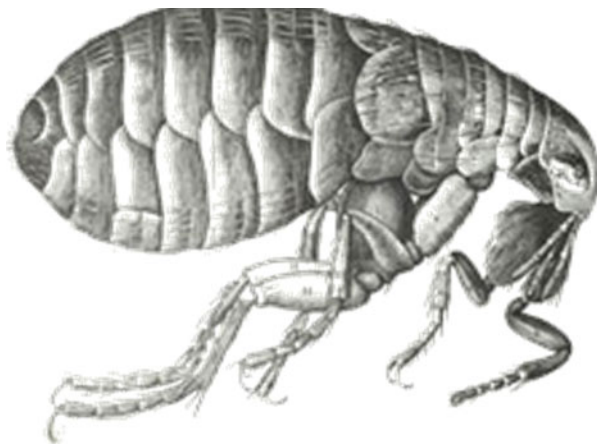
It is not quite certain who invented the first microscope. Man always wanted to look at minute details of objects and describe the intrinsic details of various organisms, living or non-living. Glass was invented in the first century AD, leading Romans to experiment with various types/shapes of this new material when they realized that if it was thicker in the middle and thin on the edges, it can cause an object held near it to appear bigger. They coined the term “lens” taken from lentil (beans in Latin) due to the peculiar shape. However, only towards the end of the thirteenth century the spectacle producers started preparing lens that could be used as spectacles. The early simple “microscopes” which were actually modified forms of the magnifying glass having only one power were used for viewing tiny insects, e.g. fleas, hence the name “flea glasses” (Fig. 11.1).

Probably, the spectacle makers in Middleburg, Netherlands, invented the first compound microscope plus the refraction telescope, between 1590 and 1610. The first well-known users and consequently “Father of Microscopy”, van Leeuwenhoek, used a painstakingly grounded single lens microscope to count threads in woven cloth. Thereafter he made small lenses with great curvatures such that his microscopes were able to magnify up to $270\times$! These microscopes were so difficult to focus that instead of changing specimens, Leeuwenhoek preferred to build a new microscope for each of the specimen. However, he was able to view bacteria, yeast, blood cells and many tiny animals swimming about in a drop of water. His efforts for and contribution gave us the first modern microscopy. In a letter to the Royal Society of London in 1676, Leeuwenhoek described his first



Fig. 11.1 A simple microscope constructed by Antonie van Leeuwenhoek and the first compound microscope constructed by Janssen. (Source: <http://www.visioneng.com/resources/history-of-the-microscope#sthash.B3yOq0Fb.dpuf> and ‘The microscope and Its Revelations’)

Fig. 11.2 Plate showing the drawing of a flea as seen under the microscope. (Adapted from *Micrographia*, by Robert Hooke, 1665)



observations of bacteria and protozoa in water, and this discovery made him the first person to detect bacteria under a microscope. Initially, Leeuwenhoek was not at all willing to share his discoveries (419 microscopes) to various parts of the world; it was only near the time of his death his daughter revealed about his invention to the rest of the world and donated his invented instrument to the Royal Society. Thereafter this magnificent discovery surfaced for the world (Egerton 2006).

Simultaneously, about the year 1590, the Dutch made a very important discovery where Zaccharias Janssen, with his father Hans, arranged many lenses inside a metal tube. In doing so, objects kept near the tube got enormously enlarged, as compared to a simple magnifying glass. They invented the compound microscope (which is a microscope that uses two or more lenses) although their early compound microscopes were of poor quality and were not suitable to visualize bacteria.

By the middle seventeenth century, Robert Hooke, from England, worked with microscopes to improve their capabilities by altering designs, and he was eventually known as the “English Father of Microscopy”. While studying a sample of cork, Hooke saw small structures that looked like rooms in monasteries called “cells” (*Micrographia*). Hooke also initiated the use of lowest level configuration of three lens that is employed for microscopy even today (Fig. 11.2).

Fig. 11.3 Model of a “microscope” that was designed by Ernst Abbe. (Adapted from <http://www.visioneng.com/resources/history-of-the-microscope#sthash.B3yOq0Fb.dpuf>)



Until 1830, no significant development was noticed in this field. Ernst Abbe (1870s) discovered the laws of physics which governs how light is collected by the objective and he could maximize this collection with the use of oil and water immersion lens. Maximal resolution Abbe achieved was approximately ten times more than what Leeuwenhoek attained more than 100 years ago. Such a resolution of 200 nm or 0.2 μm is a physical barrier that is imposed due to light (Fig. 11.3).

Later on, Joseph Jackson Lister discovered the design of the first improvised version of a compound microscope which took the final form or shape of a modern compound microscope after a number of improvements. In fact, the invention of microscope offered a better view, understanding and knowledge of microbial world. To understand the mechanism of compound microscope, one should understand the physical properties of light, functioning of eye and principles of magnification and resolution. All these concepts work collectively in the evolution of compound microscope. In the middle of the nineteenth century, huge steps were taken for improving good quality instrument, e.g. microscope, and that is when the type of microscope that we use today emerged. German companies, such as Zeiss and American company that was started by Charles Spencer, started to produce fine instruments using lenses.

Important discoveries leading to the invention of the modern microscope have been tabulated in Table 11.1.

Table 11.1 History of discovery of the microscope

Year	Discoverer	Achievements
1590	<i>Hans and Zacharias Janssen</i>	Spectacle producers from Denmark made the initial models of compound microscope which magnified objects about 3–9× times
1625	<i>Johannes Faber</i>	Framed the name microscope which had 3 parts <i>micron</i> , <i>scopos</i> and <i>skopein</i> meaning “small”, “aim” and “to look at”
1665	<i>Robert Hooke</i>	Published the book <i>Micrographia</i> . Coined the term “cells”
1670	<i>Antonie van Leeuwenhoek</i>	Inventor of modern microscope. He then described his discovery in more than 100 letters written to the Royal Society of England. The lenses made by him magnified objects up to 270 diameters
1674	<i>George Ravenscroft</i>	Improved the glass designs of Antonio Neri (1612)
1685	<i>Cherubin d’Orleans</i>	Invention of binocular type telescope (<i>tele menas</i> “far” and <i>skopein means</i> “to see or look at”)
1744	<i>John Cuff</i>	Metal-based microscope was made by him
1872	<i>Ernst Abbe</i>	Theory of microscopy was published by him, and his formula for calculating resolution that was based on light theory of waves became popular
1888	<i>Carl Zeiss</i>	Sold the design of apochromatic objective of microscope
1953	<i>The Carl Zeiss Company</i>	Sale of the first operative modern microscope

Adapted from http://web.szote.u-szeged.hu/expsur/hefop/angol/microsurgery/microsurgery_history.htm

11.2 Light Microscopy

The light microscope employs visible light to detect small objects and is probably the most well-known and well-used research tool in learning basic biology. Although a beginner thinks that the challenge of viewing small objects is only related to sufficient magnification, the actual challenges are faced to acquire the correct contrast or find a suitable focal plane and obtain best resolution followed by finally identification of the objects under study after visualization. Optical or light microscope uses the principle of passing visible light through a sample and studying the transmitted or reflected light from the sample via a one or many lenses such that a magnified image is obtained and it can be and the image thereby can be viewed by the eye or the image can be obtained on photographic plates or virtually captured by digital means. Single lens system having attached lenses and imaging equipment, a suitable lighting system and stage for holding the sample and support are the main components of a basic light microscope. Recently, digital microscope that uses a CCD camera for focusing the desired sample/exhibit is used popularly. In this case the eyepieces become unnecessary, because the image appears on a screen.

Understanding the principles of light complements understanding of microscopy. When light falls on different media, its properties change. The medium allows light

to pass through or intercepts it. Principles of microscopy are thus steered by such properties of the intercepting medium and incident light.

11.2.1 Physical Properties of Light

Light is constituted of electromagnetic radiation that has electric and magnetic fields placed orthogonally, and these propagate together traversing space and many types of media. Light shows various properties, including image formation when used for studying samples by using microscopes, and this image may be determined using the wave theory of light. Some other properties, however, are appropriately understood by making the assumption which states that light is composed of material particles (photons), such that rays of light incident from distant stars are bent due to strong gravitational attraction of the earth. Various types of interaction take place when light interacts and passes through a medium, such as air, water or any object. Some common interactions are discussed in the following paragraph.

11.2.2 Reflection

When light rays are incident on any object and bounce back to its originating medium, this is called reflection, e.g. rays of light falling within the green range of the electromagnetic spectrum (sun light) are reflected off the surface of leaves and impart green colour to the leaves. Mirrors can reflect all or specific wavelength (polychroic mirrors) and are used to deflect light, to various image-forming devices inside the microscope.

11.2.3 Transmission

Transmission refers to the passing of light through an object. We can see through an object because light can pass through it but not through a rock as light cannot pass through it. Transparent substances have 100% transmission. Microorganisms are visible to us as transmission is allowed through it. Lenses can be framed using specialized glass and coating for maximizing light transmission of specified wavelengths, e.g. visible, IR or UV. The fluorescence filters may be designed for specifically absorbing or transmitting of definite wavelengths of light.

11.2.4 Absorption

Absorption occurs if light is absorbed by an object. Here rays of light do not pass through any object; neither they get bounced back from any solid object but are completely taken up by it. For example, when sunlight interacts with leaves, most of it is absorbed, but only light of green range is reflected off. Some of this absorbed

energy gets utilized during photosynthesis and is employed by plants for the preparation of food.

11.2.5 Refraction

After the rays of light pass from a medium to another, they change their directions. This bending of light is known as refraction, and the angle through which it bends is called the angle of refraction. One has often observed how the underwater portion of a straw dipped into a tumbler containing water seems to appear bent. But when it is then removed from water, it again appears normal and straight like it was initially. The bent look is due to light rays deviating from its own path or bending when it travels to air medium from water medium causing alteration in speed across the water interface.

Various interactions of light with an object are as follows:

- (a) Reflection: Light may be reflected back from an object.
- (b) Transmission: Light is transmitted through the object directly.
- (c) Absorption: Light may be absorbed by the object.
- (d) Refraction: Light rays may be bent/refracted when passing through the object.

11.2.6 Diffraction

When light rays pass via small openings like that of a slit, hole or narrow gap, the light rays seem to get bent around the slit, and this phenomenon is diffraction. Light rays incident on a tiny circular aperture, from any point source, do not create an image looking like a bright dot; a diffused circular disc (Airy disc) that is surrounded by fainter circular concentric rings is observed instead. These concentric rings can be produced because of interference of many diffracted light rays. Similar effect is seen while getting an image by using an objective lens where light is incident from any point source. Airy disc has a size greater than the point source of light always, which makes it impossible to get the desired maximal resolution. The degree of bending becomes directly proportional to its wavelength, and hence the red light can be bent to a greater degree than the blue light. This is the reason why resolution is better when blue light is used over red light. Bending is inversely proportional to the pinhole's size or diameter, and the size of the object where bending of light is more for small objects compared to larger ones. Due to the small size of one's pupil (eye), it has less diffraction and less resolving power. When the pupil becomes even smaller in bright sunlight, poorer resolution is observed as compared to dimmer light. However, in this case the contrast is poor and so resolution is affected adversely. Diffraction becomes a major factor limiting the effectiveness of image formation while performing light microscopy.

11.2.7 The Human Eye

The eye is an optical device with the ability of focusing the images of objects onto a light-sensitive surface called retina. The focusing elements are the cornea and the lens. The portion of light entering into the eyes is controlled by the iris, a muscular diaphragm that regulates the diameter of the pupil. Retina is composed of two types of cells. Cones response mainly to light at 555 nm (green, yellow) and are responsible for daylight (phototropic) and colour vision. Rods are about 1000 fold sensitive compared to cones and are most sensitive at 507 nm (blue-green light) during night vision and do not provide colour. The resolution of the eye is limited by the diffraction of light passing via the small pupil aperture and the size of the photoreceptors. Nyquist sample collection theorem states that for a periodic signal needing resolution, it must be sampled at twice its frequency. So it seems that two points must be resolved by the human eye: their images must (Airy discs) fall on two nonadjacent receptors; otherwise it is not possible to determine whether there are one or more objects. Photoreceptor cell has a size of around 2 or 3 μm . Between two non-adjacent photoreceptors, the distance falls within the range of Nyquist criteria for sampling and also matches the photoreceptor's size and resolving power.

11.2.8 Polarization

Natural light is not polarized because it is composed of waves that vibrate in all possible direction, while laser light is strongly polarized as vibration is unidirectional. A polarizer filters light rays by allowing specific waves or wave components in a particular plane to pass through it. Two polarizers oriented at right angles will not let any light pass. Polarization is used for contrast enhancing, in microscopy (e.g. polarization microscopy, DIC).

11.2.9 Fluorescence

Fluorescent molecules can absorb light rays of specified wavelengths, and they emit the energy absorbed, in the form of light having a larger wave length, i.e. lower energy. Fluorescence is a phenomenon which results because of interactions of light waves and electronic shells of certain molecules. An incident wave of light has an electric field that momentarily displaces electrons to higher energy states and can be determined using the formula $E = h\nu$. However, after a nanosecond, it goes back to the normal energy level, and it releases the difference of energy between the initial and final energy levels, in the form of another radiation of certain wavelength, lower than the incident light. Some fraction of this energy gets dissipated during this process, and hence, the emitted light has lower energy (larger wavelength).

Phosphorescence happens by a similar model, but here the time duration between incidence and emission of light may be much larger (seconds as compared to nanoseconds).

11.2.10 Important Concepts in Microscopy

Optical microscopes use light rays as probe for obtaining structural information about a small object. Contrary to common belief, magnification is not the main objective of microscopy because high degrees of magnification may be achieved by obtaining a photo image of a sample and then enlarging it. However, after a certain point, no further special information would be achieved with this method. Primarily the task of microscopes is resolving structural details of objects under study, and then magnification becomes the next step, for making the details of the exhibit visible to us. Thus the main aim of a microscope is to provide a well-resolved and magnified image of an object to the human eye.

The basic criteria of light microscopy depend on the following few parameters.

Resolution: Visualizing small details of any object with clarity and without overlap is called resolution. It is the ability to separately visualize spots distinctly, rather than to see objects. The ability to see small objects is mainly limited to contrast which will be discussed later. Resolution is basically dependent upon the objective's numerical aperture and the condenser lens and also the specific wavelengths of incident light. If the wavelength of light used to view any object is not large enough to pass between two points, they will appear as one. The primary requirement is to get light of very short wavelength to fit between two objects or two points, so that we can see it distinctively. Various studies have shown that cell structures less than one half of the specifically selected wavelength will not be visible to the human eyes. Visible light has an average wavelength of 550 nm. So by using visible light, objects separated by more than 220 nm can only be resolved. Thus microscope that uses UV light for illumination can generate more details of cellular structure than ordinary light.

The resolution of a given lens is equivalent to the minimum distance between two distinguishable objects, and it is denoted by D . The smaller the D value, the better the resolution. It is also called limit of resolution. Three parameters govern D ; they are the refractive index (n), of the medium present in between the objective lens and specimen; angular aperture (α) or half the angle of light cone that enters the objective lens after passing through the specimen; and finally the wave length, λ , of the incident light.

$$\text{Limit of resolution or } D = 0.61\lambda/n \sin \alpha$$

The half range of the cone of light that enters the objective lens from specimen is the angular aperture (α), and it is one of the parameters for determining a microscope's resolution, where the larger the value, the better the resolution. Here NA equals $n \sin \alpha$, where NA is the numerical aperture, n is refractive index of any medium and appears printed on objectives lenses and α is the resolution. The angular aperture α is dependent on objective lens width and distance from the specimen. If objective lens is moved closer to a specimen, the angle (α) increases, and also $\sin \alpha$ value; and so D 's value gets reduced resulting in better resolution. Increase in α

means that a larger amount of light emitted or deflected by a specimen enters objective lenses, so by increasing refractive index, the value of D may be reduced; resolution would be increased. For example, air has refractive index ($n = 1$), whereas immersion oil has refractive index ($n = 1.5$), so if we replace air by immersion oil between specimen and the objective lens, the bending of the direction of light emanating from specimen will be more, and α will increase, thus better resolution.

Finally, the shorter the wavelength of the light of incident radiation, the lesser the value of D . All α , n and λ can be varied up to a particular value, so the maximum resolution achieved by light microscopy by applying wavelengths in visible range is about $0.2\ \mu\text{m}$ ($200\ \text{nm}$). No matter how many times the image is magnified, the resolving power could never be less than $\approx 0.2\ \mu\text{m}$ (by applying maximum limit of all parameters).

Maximum angular aperture for the best objective lens is 70° ($\sin 70^\circ = 0.94$), the shortest wave length $\lambda = 450\ \text{nm}$ (blue) and an immersion oil ($n = 1.5$) above the sample; applying all these values, we get

$$\text{Limit of resolution } D = 0.61 \times 450\ \text{nm} / 1.5 \times 0.94 = 194\ \text{nm} \approx 0.2\ \mu\text{m}$$

Magnifying small objects is necessary for research in the area of biology, although the finer details of cells and of sub-cellular compartments need imaging systems capable of providing data having spatial information considering small distances.

11.2.11 Contrast

Contrast is the change in colour or intensity of two objects or between the background and object. For the eye, a certain degree of contrast becomes necessary for separating two objects. For light microscopes, if samples are transparent, then some contrast has to be enhanced to be able to view the details of the specimen. Contrast is usually enhanced by applying specific stains to stain or alternately to change the transmission of light from cellular constituents or the specimen.

11.2.12 Magnification

The total magnification seen in any light microscope is calculated by multiplying the magnification power of objective lens by using an ocular lens (the lens nearest to your eye). Compound microscopes may be available having six interchangeable objective lenses with different powers of magnification, whereas the magnification of an ocular lens is somewhere increased or decreased by ten fold. The common ones are listed below. The resolving power is determined solely by objective lenses.

Scanning $3\times \times 10 = 30\times$ magnification.

Low power $10\times \times 10 = 100\times$ magnification.

High “dry” $40\times \times 10 = 400\times$ magnification.

Oil immersion $100\times \times 10 = 1000\times$ magnification.

In optical microscope, 500–1000 times magnification with respect to the numerical aperture of the objective lens is desirable. In low magnification details of the objective are not visualized by the human eye or recorded by the imaging instrument (e.g. eye or CCD camera). Optimal magnification is achieved if microscope's resolution can be matched with the imaging device's resolution.

11.2.13 Sensitivity

The signals from the microscope may be several times lesser compared to that obtained from distant stars. Therefore, a highly sensitive device is needed for imaging, and so photodetectors are used to capture microscope signals. However because there can be just few photons, therefore, a noisy data can be obtained from imaging devices in such cases.

11.2.14 Simple Theory of Microscopy

Image formation by a lens can be explained in terms of either geometric or physical optics. Geometric optics explains focus and aberration; however, physical optics is essential to understand why images are not perfectly sharp and how contrast is achieved. The two rules of geometric optics describe that (a) light travels in a straight path and (b) the path bends (refracts) at an interface between two transparent media. An ideal convex lens with two spherical surfaces does not have the same curvature radius. So it has two focal points. If two light rays are emanated from any given point of an object (such that one is parallel to lens axis and the other passes via focal point), enter into the lens and emerge out via another side, they form an image at the point of intersection of the emerging rays.

Actually real lenses fail to bring all rays from a given point of an object to unique focus. This is called aberration. The most easily understood aberration is chromatic aberration. We know that white light consists of many wavelengths having different refractive index and the position of the focal point is dependent on various wavelengths. Hence, image formed by white light will be fuzzy as it is the superposition of a large number of images, not all of which can simultaneously be in focus. Chromatic aberration is easy to rectify. One way is by using a lens system consisting of several types of glasses where the relation between refractive index (n) and wavelength λ is balanced to make n independent of λ ; such a lens system is called achromatic. The way to reduce aberration is the use of monochromatic rays or cut-off filters to reduce the range of incoming light rays. The principle of correction is to nullify one aberration produced by one lens by an opposite aberration of the same degree by another lens. Thus one positive and one negative lens in combination produce aberrations in opposite senses, and hence they nullify each other. It is

advised to use a filter even when a colour correction system of lens is used because colour correction is never perfect. The other aberration found even with monochromatic light is spherical aberration, a point imaging aberration. This arises because all rays from a single point on the optical axis of the lens are refracted at different degrees by different parts of the lens and therefore do not come to the focus at the same point on the axis, and ultimately the image is softened in the image plane. The other point imaging aberration is called coma; a comet-shaped blurred area is produced due to differences in the surface of the lens. By appropriate lens correction, these aberrations are easily corrected. A lens so corrected is called aplanatic lens. The other aberrations are not that important except that where highest resolution is required. Objective lenses of microscopes are achromatic commonly, and when imaging is done using green light, best results are obtained. The green filter can make achromatic objective more effective by narrowing down the bandwidth of incident light, and the system becomes very efficient most routine use. However, for using white light and for obtaining high-resolution images, achromatic lens is not suitable. This is primarily due to the planes of blue and red light not being focused in the plane as that of green light. Colour photomicrography is performed for obtaining best resolution with clarity of image using fully corrected apochromatic lenses as objective lens. For a lesser degree of correction, one can use fluorite lens, but these are still better in performance as compared to achromatic lens, but cannot compete the apochromats. Fluorite lens is suitable for performing fluorescence microscopy due to higher transmission of short wavelengths of light. High degrees of corrections contribute to making these objective lenses expensive as compared to other lens. The price for apochromatic objectives may range in between 3000 to 10,000 dollars. In many microscopes, ocular lens have been designed for working with objective lens procured from the same manufacturing company. The manufacturers introduce some specific spatial and colour correction on both the ocular and objective lenses, and they should therefore be procured as a pair. Therefore, mixing of brands with different lenses will actually cause inferior image formation. The image obtained via oculars is actually a virtual image, which is viewed in a corrected form when viewed by the eye. This is, however, not suitable for producing a photographic image or video via microscopes. A projection lens is required for photographic or video-based microscopy, in order to generate corrected real images. Several modern microscopes can provide a completely corrected image via objective lens, such that many concerns about matching of glass components sold by the same manufacturer become obviated. However, mixing different parts of any microscope made by one manufacturer with that of another may still result in an undesirable image quality.

11.2.15 Metric Units Used in Microscopy

Microscope is meant to visualize objects, normally not visible to the human eye. As the micro-organisms are tiny, the units used to measure it should be different from the macroscopic world. Some commonly used units in microscopic domain are summarized in Table 11.2.

Table 11.2 Units of length that are commonly used

Unit	In English	In metric terms (m)
Meter (m)	3.28 ft.	1
Centimetre (cm)	0.39 in.	10^{-2}
Millimetre (mm)	0.039 in.	10^{-3}
Micrometre (μm)	0.000039 in.	10^{-6}
Nanometre (nm)	0.000000039 in.	10^{-9}
Angstrom (\AA)	0.0000000039 in.	10^{-10}

Micrometre (μm) formerly known as micron (μ) is expressed as 10^{-6} m, and nanometre formerly called m μ is expressed as 10^{-9} m. The third unit, Angstrom (\AA) used in old literature, is equivalent to 10^{-10} m.

11.2.16 Light Microscopes

11.2.16.1 The Compound Light Microscope

Light microscopy refers to any kind of microscope which uses visible light to illuminate an object. Over the years, several types of light microscopes have been developed; each adopted certain kind of improvement to achieve a better view of the specimen. The modern compound microscope which uses more than one lens is a descendant of Hooke's microscope.

Illumination: An important factor for the production of a desirable image using a light microscope is provision of adequate amount of light to the specimen or to the object plane. It is important to obtain a bright light around objects, and also for optimum image generation, uniform light should be provided across the field of view. Light enters into the microscope from a source called illuminator. Generally, a tungsten filament lamp is used in light microscope except fluorescence microscope. Light then passes through a reflector and collector lens for maximum collection, then through a diaphragm or stop for defining the illuminated area and again through filters, e.g. colour balancing filters, heat filters, neutral density filters and diffusers required for achieving suitable light intensity and attenuation. For right illumination of an object, it must satisfy two criterions: firstly the incident light must fill the numerical aperture of the objective lens, and secondly the illumination should be uniform across a specimen.

To illuminate any specimen, another lens system may be employed, and this is known as the condenser. Any source of light of small dimension is employed which focuses a very small area of the specimen which is achieved by using a set lens mounted in a single unit called a condenser. Basically it is an objective operated with the light path reversed. Condenser provides two types of illumination, critical and Kohler illumination.

In critical illumination, the condenser is adjusted with respect to the object so that the spot of illumination is as small as possible. This is done by using a diffusing

screen in front of the light source to avoid the imaging of the structure of the light filament on the object.

Kohler illumination is more intense and precisely controlled. Here an accessory lens, called a field lens, is placed between the light source and the condenser so that it forms an image at the focal plane of the condenser.

Light from the condenser then passes through the specimen, which is kept on the specimen stage. A large flattened lens placed beneath the object is the front element of the condenser. The object is placed on a movable rack to facilitate focusing of the beam of light that comes through the object and maximizes the intensity and controls the uniformity of illumination. Specimen stage can be motorized and if required can be oriented in the desired direction. Light after passing through specimen passes through the objective lens, collects light from the specimen and accounts for the actual resolution and magnification of the microscope. In modern system, the objective does not form image in the microscope tube but rather projects an image at infinity. A tube lens must be used to form the primary image at infinity so that it does not form an image inside the microscope tube. The NA or numerical aperture defines the ability of an objective to gather light, and that in turn determines its resolution. The numerical aperture is normally increased by decreasing the focal length because the diameter decreases with increasing magnification. Immersion media (water, glycol, oil) are used to increase the numerical aperture of high-performance objectives. By using oil whose refractive index is the same as that of glass coverslip above the object and that of the front lens element, all surfaces between the object and lens are eliminated except when the object touches the coverslip. The front lens element of an oil immersion objective is usually greater than a hemisphere so that the object can be considered within spherical lens.

The focusing mechanism of the specimen consists of a coarse adjustment and a fine adjustment knob. Coarse adjustment knob facilitates the proper localization of the specimen by changing the distance between the specimen and objective lens rapidly and conveniently. Fine adjustment knob changes the distance very slowly and imparts a sharp focus of the specimen. Compound microscopes may have a maximum of six objective lenses that are interchangeable and have different powers of magnification. The microscopes are designed such that in slight changes of microscopic instrument, the specimen will remain more or less in the same focus. Such microscopes are said to be par-focal. It increases the efficiency of microscopes and prevents major damage to both the slides and also objective lenses. Thus the objective lens magnifies the image and corrects various aberrations. But to achieve higher magnification, the focal length of lens should be very small, and the eye needs to be placed virtually on the opposite surface of the lens. To avoid the problems of making lens of very short focal length and to provide a reasonable distance from the lens to eye, a secondary lens system called an eyepiece is introduced. The ocular lens or eyepiece can provide additional magnification of 5 to 20 \times , and it focuses image into the retina of the eye with small detailed description.

11.2.16.2 Inverted Microscope

An inverted microscope was developed for analysing metals such as steel and iron, prior to World War II. Its action is opposite than a normal light microscope. Afterwards advances were made for biological research, and then scientists began to employ these models of inverted microscope to study living cells. The condenser and the light source are placed atop the stage, which points down. Therefore the objectives are placed below the stage which points upward. The part in common with standard light microscope is that a specimen is placed on the stage and the binocular tube lies in a standard position allowing a conventional angle for viewing. For these reasons, one can visualize the exhibit placed over the stage, after it has been covered with a cover glass.

The main advantage of inverted microscope is that it allows one to observe microorganisms in a big container kept under natural or least stressed configuration. Because of its configuration, an entire culture or large sample may be placed in a relatively large container, e.g. a Petri dish, and viewed under the microscope under non-stringent conditions. Such a sample may sustain life over a much longer period. The cover of the container slows evaporation greatly while at the same time allowing some gas exchange. The use of large quantity of water helps to maintain optimal temperature; especially if the specimen is stored inside a room, it will acquire room temperature. With high power objectives, it is possible to correct differences in thickness in specimen. It is useful to see pond life under a coverslip facing the objectives. The condenser also allows for an unusually long working distances.

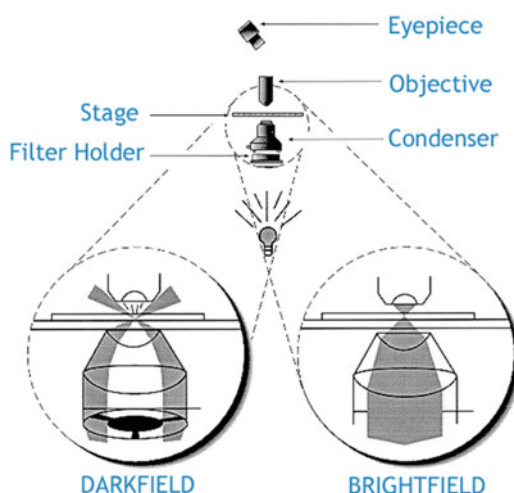
A traditional light microscope warrants that the exhibit should be kept over a glass slide, covered with a coverslip. This usually means an artificial environment with a change of temperature and oxygen content of the sample. Also, the sample will dry out unless repeatedly replenished with water. Such changes lay stress on microbes and affect their metabolism, or movements may also kill them after sometime. The major disadvantages are cost and limitation in magnification, as it is more complex and sophisticated and so more expensive; increase in magnification by oil immersion objective is not available here.

Before the World War II, Olympus marketed the microscopes meant for metallurgical purposes having horizontally placed optical axes and also dry plates for use as photosensitive objects. During photography these were not user friendly. Olympus later introduced a model for inverted microscope for study of metals and for research application to observe metal surface observations or composition and research on ceramics, plastics, etc. Olympus introduced the ME standard inverted microscope in 1956 and then the high-performance PMG in 1964, which also had an internal photographic connected with light metre, followed by the PME in 1967 (Fig. 11.4).



Fig. 11.4 The four models of inverted microscopes developed by Olympus. The PMF being the most ancient (before World War II) and the GX series being the most modern (2001 onwards) (<http://www.olympus-global.com/en/corc/history/story/micro/headstand/>)

Fig. 11.5 The relationship between dark field and light field microscopy. (Illustration provided courtesy of Washington State University and adapted from https://www.microscopeworld.com/t-darkfield_microscopy.aspx)



11.3 Dark Field Microscope

Dark field microscopy is simple to operate and an inexpensive device with good contrast enhancement technique. It is very suitable for specimen having refractive indices that are different from the suspending medium, but are still not clearly visible due to the lack of stain or colour. In dark field microscopes, the specimen is seen as a bright object suspended on a dark background, i.e. the medium. In a light microscope, light coming through a condenser is directly transmitted through a specimen (Macnab 1976). Under a bright field of illumination, contrast relative to the background decreases, and most of the live samples cannot be stained as they get distorted by staining. In such a situation, where contrast is needed to visualize a specimen, a dark field illumination (Fig. 11.5) is used where an opaque disc containing a transparent annulus is inserted just below or within the condenser

lens, so that the object plane is illuminated by a hollow cone of light passing through the object. If this hollow cone is sufficiently large and does interact with the object positioned in an object plane, it will diffract light, and some of the diffracted light at a particular angle will enter the objective lens. Even though the intensity of the diffracted light is low (it is illuminated with annulus of light not with direct condensed light), a bright image will be produced against a black background.

In order to perform dark field microscopy, a dark field filter (“patch stop”) should be placed into the given holder for the filter, in the condenser. The filter thus prevents all beams of light radiated by the lamp from entering the objective directly, thus making the background appear darker. The specimen may be illuminated from its sides and cause them to scatter all the light that then enters the objective. In this way the specimen shall appear bright, whereas the background remains dark.

Dark field image may be obtained by two methods:

- (a) Use of special condensers (dark field): This method may be the best but also the most expensive solution.
- (b) Use of a filter (dark field/“patch stop”): This filter is placed into the condenser in a filter holder. A stop-out patch can be cut out from a tin or a cardboard with a knife or scissors and is therefore inexpensive.

Advantages of dark field imaging:

- Technically simple methodology and may be used on live transparent specimen that otherwise has to get killed and stained. To view motile microbes, this will be applicable.
- Images appear relatively better and are more impressive, visually.
- Dark field microscope can facilitate the viewing of objects that fall below the microscope’s resolving power, since these objects appear as lighter spots on a darker background. However, the shape is not visible in this case.
- Dark field microscope may be handy for counting of small particles that is otherwise not possible via bright field microscopes, and so its high contrast is useful even though it can elucidate minimal details of specimens.

Limitations of dark field microscopes:

- A dark field microscope may give faulty results due to presence of dirt.
- When the refractive indices of any transparent specimen is very close to its surrounding, then in this case the light will not get scattered into the objective; instead it will pass through the specimen and can’t be viewed.
- Illumination has to be optimized for correct viewing of specimen. Also optimizing is needed for proper resolution.
- The filter-based one patch stop usually is sufficing for studies requiring low magnification; however, when magnification is higher, image quality is poor. Experimentation is needed using various sizes of patch stops for different objectives.

11.4 Phase Contrast Microscopy

Frits Zernike, a Dutch physicist, invented phase contrast microscope in the 1930s, but could not commercialize it, due to the outbreak of World War II (1935–1945). Finally, the invention of phase contrast microscope was awarded Nobel Prize in physics in the year 1953. Zernike discovered the direct light path can be speeded up; a destructive interference pattern may be introduced in the viewed image. These patterns make the details of the image look dark when viewed against a lighter background. Zernike discovered a system of rings that are located in objective lens and also in condenser system, to create interference patterns. After proper alignment, light rays emitted by the illuminator reach the eye about $1/2$ wavelength out of phase, causing the images, thus created to get more enhanced and making better visualization of the specimen possible. This microscope requires specialized phase condenser and phase objective lenses. Each phase setting numbered as phase 1, phase 2 and phase 3 is matched with each condenser and objective lens. When parallel light falls in a perpendicular direction on a transparent disc in a transparent medium, the disc remains invisible because the intensity of the light passing through the disc and medium is the same. But if the refractive indices of the disc and medium are different, the light transmitted through it differs in phase because of the difference in velocity. (Velocity of light wave is slower in denser medium). Still it cannot be distinguished because neither our eye nor photographic film can distinguish this difference of phases; hence the disc is invisible.

Let us consider the situation when light passes through a point of transparent disc and falls on the objective. This light consists of undiffracted or direct beam, might be called undeviated light. However, under the same situation when light is diffracted at various angles, it is called deviated light. It differs in phase by one-quarter wavelength from the undeviated light, and the phase of the combined ray will be the combination of the phases of its component rays. The light passing through the medium is also broken down into deviated and undeviated portions.

In phase contrast microscopy, the phase difference is converted into an intensity difference by following a simple method. Here an annular diaphragm is placed in the focal plane below the condenser so that light from the source comes in circular annular form and illuminates the object. Suppose there is no specimen; then light will pass through as unobstructed and is focused on the objective lens forming a tiny ring of light on the eyepiece side of the objective; an image of the annulus is formed. At this point a phase plate is introduced to produce phase difference. The phase plate consists of either annular groove of one-quarter wavelength in depth or an additional layer of one-quarter wavelength. The size of phase plate is exactly the same as that of the image of the condenser annulus. Thus all light passing through condenser annulus will be deviated by one-quarter of wavelength.

Phase contrast microscopy uses the differences in the refractive index of cellular structures. Light that passes through the dense part of the cell is held up relative to the light passing through the less denser part, for example, cytoplasm. Phase is only useful on specimens that are colourless and transparent and usually difficult to distinguish from their surroundings. Such specimens are known as “phase objects”

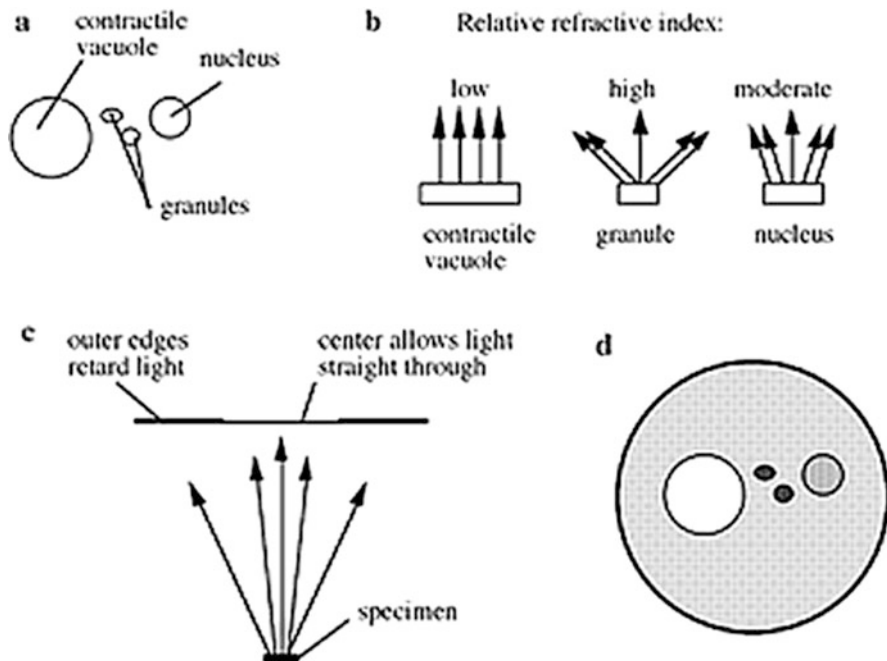


Fig. 11.6 (a) Organelles having different refractive indices (RI) are hardly distinguishable in bright field microscopy; (b) objects with higher RI cause light to bend to higher degrees; (c) a phase plate has been kept in the path of incident light for phase contrast. Light that is not refracted to detectable degree can travel via the plate from the centre and will not be retarded. Light that is refracted to higher degree can pass through the plate, but far from its centre, and is thus retarded by one-quarter wavelength; (d) the field of the microscope has darker background such that the organelles are visualized in sharp contrast (here the cell's cytoplasm shows higher refractive index as compared to the contractile vacuole). (Adapted from <http://www.ruf.rice.edu/~bioslabs/methods/microscopy/phase.html>)

such as cell parts in protozoans, bacteria, sperm tails and other types of unstained cells. Phase contrast microscopy is widely used to visualize live cells growing in tissue culture, cell organelle preparations, for lysis, etc.

When an object is placed, incident light interacts with two components of an object, specimen and medium. Now these two components have different refractive index and hence phase difference. When this phase difference is superimposed with the phase difference created by the phase plate, there is a loss of destructive interference, and the object will appear brighter (Fig. 11.6a–d). When wave peak of light superimposes with peak of another, there is reinforcement or brightness, but when the wave peak of one light source coincides with the wave trough from another, it causes interference or relative darkness. The darkness created by an object depends on its particular refractive index and thickness. In this way invisible objects can be made visible, but the disadvantage of this microscopy is its alignment; the condenser annulus must be concentric and focused on the phase plate. Any

sample which does not absorb light can be observed on phase contrast microscopy. For light absorbing object, difference in light intensity is observed, and it is added up to the phase effect. It results in reduced contrast and poor image quality.

Phase contrast microscopy is most useful in visualizing the organelles of living cells. The diagnosis of hematologic disease can be resolved by phase contrast microscopy by identifying and characterizing the cells of the bone marrow and blood. Often the demarcation of normal from pathological forms of the cells depends upon subtle changes in cell morphology (Wolffe and Van der Velden 1987). Phase contrast microscopy can offer a relatively easy visualization to study living cells, and its use in many laboratories has been promoted as a good tool for diagnosis of hematologic diseases. A membrane can be seen clearly as dark line with bright halo on either side, and due to this, unit membrane is not easily distinguished from double membranes. Small objects like bacteria are difficult to see in detail. By placing bacteria in a medium whose refractive index equals to that of cell wall or cytoplasm (e.g. concentrated solutions of bovine serum albumin), internal details become (nuclear bodies) relatively easy to see.

11.5 Differential Interference Contrast Microscopy (DIC)

Live cells or specimen that are transparent or unstained may be difficult to view using the traditional techniques of bright field microscopy where full aperture is used and so there is low resolution of the microscope objective. Frits Zernike first conceptualized the “phase contrast”, in the 1930s, for viewing challenging exhibits; however, the method suffered due to halo artifacts and was then restricted to sample preparations which were extremely thin. Interference microscopy resembles phase contrast microscopy in principle and more sensitive because instead of one, two separate beams are transmitted through the specimen by using a special prism. In other words, DIC offers good contrast when unstained specimen is used, using the difference in refractive indices of various specimen constituents. Along with phase contrast microscopy, the DIC can transform the shifting of phase in light, that is, induced RI of specimen, and converts it to a detectable amplitude.

DIC generates an image that looks as if the specimen is casting a shadow to one side. This shadow principally represents a difference in refractive index and thickness of a specimen rather than topography. A special type of prism is used to split light rays into two components. One beam passes through the specimen, while the other passes through the medium in the absence of the specimen. When these two beams are recombined, any change that has occurred in the phase of the specimen beam causes it to interfere with the control beam. The magnitude of phase changes can even be quantified and related to the dry weight of cellular components. DIC microscopes are in general interferometers such that they can exploit the phase difference between the specimen and reference in terms of emanated light and induced light, to result in contrasting light ray and a reference ray. In interferometry, like in the case of DIC, a single ray of light splits into two rays, where one traverses the specimen and other one interacts with the background. The rays thereafter

combine again on the image plane, such that wave interference is seen. Contrast of the image is modified due to alteration of phases between the rays of the specimen and reference rays. In DIC microscopy, the sample's reference and rays get created when the rays traverse through, and its phase is distorted by the object. Unlike phase contrast, DIC can convert the gradient of specimen's optical path length to differences in amplitude and may be seen as improvement in contrast for the resulting image.

The basic **DIC** system was first proposed in 1955 by Francis Smith, and it is conceptualized as a polarized microscope working with polarized light. It has two Wollaston prisms, in front side of the condenser on the focal plane and the other placed at the rear focal plane of the objective. After several years, Georges Nomarski, French physicist from Poland, altered the traditional configuration of the Wollaston prism to insert very thin optical components which are actually situated far away from the aperture of conjugate planes.

A polarized light is used for Nomarski-modified Wollaston prism which can be separated into two rays polarized at 90° to each other, the ordinary and extraordinary rays. The Nomarski modification brings the rays to meet at the focal point outside the body of the prism. This step facilitates a higher level of flexibility while setting up the microscope and so determines if the prism can be focused actively. Then these two rays are focused by the condenser lens for passage through the sample. The two rays in question are then focused in a manner that they now penetrate through adjacent two points virtually placed in the sample, about $0.2\ \mu\text{m}$ apart. Through this technique, the samples are suitably lighted using two sources of coherent light, one having 0° and another having polarization at 90° . However, they are not completely aligned, and one of them is slightly offset with respect to the second. These rays pass via adjacent but different sites on the sample. The specimen is not uniform throughout, and so the optical path lengths across the sample are different. When the wave of light traverses an optically denser material or part of the specimen, there is a change in the phase of one ray with respect to another due to the delay caused. Passage of several pairs of rays through the adjacent pairs of spots in the sample helps in image formation. If each of these is scrutinized individually, they would form bright field images, which are somewhat offset from one another. Then light rays travel through the objective lens and get focused on the modified Wollaston prism. The two rays are recombined into a single polarized plane at 135° . The joint action of rays results in interference, darkening or brightening of the image at that particular point as determined by the optical path difference. The prism can overlay the images from the two bright fields so that their polarization is aligned and they can interfere.

DIC has strong advantages for examining both unstained and live samples, e.g. unicellular water-borne microbes or smears from tissue culture. The clarity and resolution obtained in DIC are much superior than the traditional optical microscopic techniques (Fig. 11.7).

The primary problem of DIC is that it requires a transparent sample having refractive indices that coincide with the surrounding medium. For thick samples DIC is not suitable for thick biological samples, e.g. pigmented cells, tissue sections,

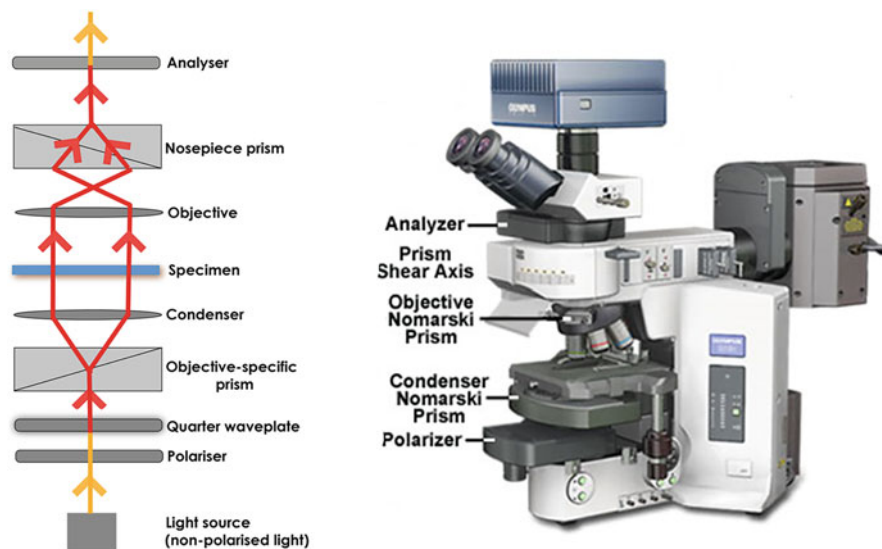


Fig. 11.7 The standard DIC configuration needed for a transmitted light microscope which is coupled to fluorescent light

etc. DIC is unsuitable for many non-biological materials too due to its principle of polarization of light since many physical non-biological specimen get affected (Obara et al. 2013).

However, even though there are limitations, the image quality is outstanding and free of artifacts. Analysis of images from DIC should take the orientation of Wollaston prisms into account because the parallel features will not be visualized. This problem is easily taken care of by rotation of the sample to observe the changes arising in the image.

11.6 Fluorescence Microscopy

Fluorescence can be defined as the ability of a substance to absorb light rays of short wavelength, e.g. ultraviolet rays, and emit rays of a longer wavelength, e.g. in visible range. The light excites the fluorescent chromatophore present in the sample, and longer wavelength of light is emitted. Fluorescence microscope generates magnified images of objects; however visualization is based upon the light that is emanated from the fluorescent species, and not the original source of light that was incident on the sample to cause excitation of the fluorescent molecules. Here the focus of illumination must fall to a greater extent on an object than is required in conventional microscope using a simple condenser lens system. The total intensity of the

fluorescence is proportional to the intensity of the incident light. In order to generate enough excitation light intensity to furnish secondary fluorescence emission capable of detection, powerful light sources like mercury or xenon arc lamps are used.

Absorption of light followed by re-radiation by specimen which are inorganic or organic in nature is due to well-known phenomena of light, commonly referred to **fluorescence** or **phosphorescence**. During the phenomenon of fluorescence, light is emitted and appears almost as soon as the specimen gets excited with a certain wavelength of light. The incident light causes excitation of specimen, and the excitation light gets absorbed, and a light of a lower wavelength is emitted. There is relatively small time interval between emission and absorption of photons that can be limited within microseconds in duration. If emissions persist for a long duration even after the excitation wavelength is not existing, then the phenomenon is referred to phosphorescence (Spring and Davidson 2021). In 1852, Sir George G. Stokes (British scientist) first described fluorescence as a phenomenon where upon excitation of specimen using UV light, a mineral called fluorspar could emit red light, and this led to his coining the term fluorescence. As observed by Stokes, the fluorescent rays emitted were always longer than the incident wavelength of light that caused excitation of flour. In the nineteenth century, several researchers showed that several specimens (such as chlorophyll, crystals, butter minerals, resins, vitamins crude drugs and also inorganic compounds) could show the phenomenon of fluorescence upon being irradiated with UV light.

Several samples/materials can show the property of autofluorescence (even if fluorophores are not attached to them externally) if they are excited by UV rays or other wavelengths of light. This phenomenon is very significant and has been thoroughly researched upon during the study of subjects, e.g. botany, semiconductors and petrology. However, the study of animal tissues and pathogens is often complicated with either extremely faint or bright, non-specific autofluorescence (also termed **fluorophores**). In such a case or if a sample does not fluoresce naturally, then it is stained with one group of fluorescent dye called fluorochromes (also termed **fluorophores**). Fluorochromes are colouring agents or stains that may get attached to the visible or invisible components of the target and show the phenomenon of fluoresce. They are highly specific in attachment with targets and show an immense quantum yield (i.e. number of photon absorbed divided by photons emitted) and may be excited only by specified wavelength of incident light, whereby they emit light having a defined intensity. In about 1930 application of fluorochromes was started for biological studies in order to stain tissue components, bacteria and other pathogens. When microorganisms, stained with a fluorochrome, are examined under a fluorescent microscope with an ultraviolet or near ultraviolet light source, they appear as luminescent, bright object against a dark background. Many stains developed for their fluorescent properties were found to be highly specific, and they in turn promoted further evolution of the fluorescence microscope. A wide range of synthetic fluorophores having different profiles of intensity upon excitation and emission are currently in the market, and these are prorogating the applications of fluorescence microscopy in wider aspects along with the earlier known biological applications (Gitai 2009).

For excitation of the specimen using a specific wavelength, through specified excitation filters, a light coming from a source having multiple wavelengths is allowed to pass, but the filter moves, allowing only a desired frequency to pass through. Radiation gets reflected by the dichroic mirror in downwards direction which is then focused onto the sample through the objective lens. Fluorescent moieties of samples are excited for emitting rays at specific but longer wavelengths. The objective lens focuses the light emitted by the sample, and majorly this light traverses the dichroic mirror and moves upwards and is not reflected. To block residual light transmission or reflection, a barrier filter is placed, and it does not correspond to the fluorescent substance.

The major problem however is the detecting fluorescence or speculating fluorescence from the incident light with a fluorescence microscope. The problem is taken care of by using optical filters. The most commonly used procedure utilizes two filters:

1. A filter which is placed between the light source and the condenser and allows only the desired wavelength (e.g. 488 nm) to pass through barring any other wavelength of the fluorescence spectrum.
2. A filter of barrier type placed between the objective and eye, transmitting only fluorescence wavelength (518 nm) but not the exciting light.

So in the absence of an object, no light reveals the eye, and the field is black, but if an object is placed, it will contrast strongly with the background. To analyse weak fluorescence, it is necessary to use microscope slides, coverslips and lens elements made of non-fluorescing glass because any of these can lighten the background significantly.

Most of the cell components fluoresce very poorly even under UV illumination. Exceptions are molecules like tryptophan, tyrosine and riboflavin very commonly found in the cell. So extrinsic fluors, which bind to a particular cell type, are used to increase absorption of light. Common fluors are rhodamine and Texas red, which emit red light; cytochrome 3 which emits orange light; acridine orange and fluorescein, which emit green light; etc. These dyes have very low nonspecific affinity to biologically active molecules; however, these might be attached to antibodies, using chemical means, such that antibodies specifically targeted to a desired macromolecule appear distinct. Fluorescence microscopy is used mainly to see complicated object or to know orientation of an object. For example, acridine orange binds with both DNA and RNA but gives green fluorescence with DNA in low concentration of the dye; at higher concentration of the dye, it gives orange fluorescence with RNA.

Hence, at low concentration of the dye, eukaryotic cells show bright nucleus and pale green cytoplasm, but at higher concentration, the cytoplasm becomes orange. This typical fluorescence is observed even with cells infected with DNA virus. The yeast nucleus is very small, difficult to detect. Yeast has a very large RNA/DNA ratio, so when it is stained with acridine orange, at high concentration, a small nucleus with bright green colour and the cytoplasm with an orange glow are observed.

11.6.1 Fluorescent Antibody Technique or Immunofluorescence

Fluorescent antibody technique is used to identify any foreign body or antigen in the body. Here, at first specific antibodies are produced by injecting a particular antigen to an animal. After a sufficient time, the antibodies are collected from the serum of the animal. Fluorescence dye is then chemically combined with that particular antibody. These fluorescent tagged antibodies are then added to a microscopic slide containing an unknown bacterium. If the unknown bacterium is the same bacterium that was injected into the animal, the fluorescent tagged antibodies bind to antigen on the surface of the bacterium, and the antigen fluoresce, when illuminated with ultraviolet light.

This technique can detect bacteria and other pathogenic microorganisms, even within cell tissues and clinical specimen. It is a very sensitive technique and can identify microorganism in minutes.

Fluorescence tagged antibodies can also be generated against proteins and various cell fractions. These antibodies are then subjected to cell section or inside the cell by making it permeable by acid treatment or treatment with acetone which helps in localization of the protein, for example, viral antigen, cell membrane components, histones and many other substances can be detected by this method.

Auramine-O gives yellow glow with *Mycobacterium tuberculosis*. Fluorescein isothiocyanate produces apple green colour with *Bacillus anthracis*, causative agent of anthrax.

11.6.2 Identification of Chromosome

Quinacrine, a fluorescent dye, binds very efficiently by reacting with free amino groups of nucleotides in DNA or with chromosomes. When it is added to a cell chromosomes, it not only gives fluorescence but also shows a pattern of bright and dark bands in sequence. By observing a particular pattern, one can characterize a chromosome and can find out if there is any morphological defect or abnormalities. High absorption is observed with adenine, thymine and to the region where histones are preferentially bound.

11.6.2.1 Fluorescence In Situ Hybridization (FISH)

This technique combines the visualization of any specific gene or gene segment on the chromosomes directly by using suitable probes under a fluorescence microscope (cytogenetics). This technique is very powerful for rapid detection of some chromosomal abnormalities that otherwise remain undetectable or cannot be characterized using traditional cytogenetics-based methods. The technique has put forth advanced research and diagnostics related to haematologic problems and tumours and may also be employed for the detection of genetic abnormalities, e.g. aneuploidy, deletions, gene fusions and loss of chromosomal regions, and for monitoring the progress of aberrations. Thus it finds applications in prenatal and postnatal testing.

FISH can also be applied to such research applications as gene mapping and for cytogenetics used for cancer-related diagnosis.

Spectral karyotyping (SKY) includes a coloured, multi-chromosomal staining assay with 24 colours, and it facilitates visualizing of human chromosomes all together in a single experiment. This technique of SKY for detecting complex chromosomal rearrangements, and identification of the origin of marker chromosomes or of extra-chromosomal entities, confers the technique with high sensitivity, and so it serves as an invaluable tool to identify chromosomal aberrations (Hu et al. 2014).

Apart from mammalian systems, it can be used for plant systems as well although it is more challenging due to the presence of cell wall and cytoplasmic debris. FISH was first used to study plant chromosomes by Lee et al. (1996). A variety of probe-labelling schemes are now available for simultaneous detection of two or more sequences in the same nucleus. When the genomic DNA (including entire nuclear DNA of a plant species) is employed to probe another chromosomal DNA in hybridization experiments, the technique is referred to as GISH (genomic *in situ* hybridization).

The various applications of FISH techniques using fluorochrome include the following:

1. Viewing multigenic families, e.g. 5S, 8S-26S and 18S-5 ribosomal RNA genes for their location on chromosome.
2. Physical map of ribosomal genes, transposable DNA and microsatellite on sugar beet chromosome.
3. GISH facilitates the characterization of the chromosomes and the genome of hybrid plants, recombinant species and allopolyploid breeding lines. The inheritance patterns of hybrid or polyploid species may be elucidated using southern hybridization or *in situ* hybridizations.
4. Multicolour FISH (mFISH) for screening total genomic DNA using probes is another promising technique used for simultaneously distinguishing genomes that exist in natural conditions or artificial amphidiploids. It uses various fluorescence dyes for representing a range of coloured probes, all at the same time. Again, this method is a robust tool to investigate genomic homology between two polyploid species and their diploid progenitor.

11.7 Polarization Microscopy

Polarized light instead of visible light is used for illumination in polarization microscopy. Polarized light consists of light waves having same direction of vibration, or it is linearly polarized. A polarizer generates a very ordered light, in a privileged plane from the natural light source.

When two filters arranged crossed across the path of the beam at 90° angle to one another, they do not let any light to pass through. The first filter filters out extra vibration such that the next filter does not let the same pass through it. The first one is

known as the polarizer, and the second one is the analyser; since the second filter is oriented in the wrong plane, none of the light normally is expected to pass through the second filter, and so no image would be seen with the microscope.

However, some material exhibit a property called birefringence, which allows the rotation of plane polarized light. Birefringent structures present in cells can rotate plane-polarized light in a typical manner such that it can then pass through the second filter and hence create an image. Birefringence (multiple RI or refractive indices) is a phenomenon that starts with alignment of molecules or atoms in specific planes of the object. Such moieties (atoms/molecules) show strong interaction with incident light beams that impinge upon them from a particular direction. But the interaction is weak or poor when light beams from another direction impinge on them from a different direction. Birefringence is most commonly exhibited by highly ordered structure. The major components of a polarization microscope are a polarizer, situated between the light source and condenser. This ensures that the specimen is illuminated by linearly polarized light via the condenser. A rotating stage, which holds a sample and an analyser, is situated between the objective and eyepiece. The analyser can be set so that its axis is perpendicular to the polarizer axis and a compensator. The analyser is arranged at an angle of 90° to the polarizer. When polarizer and analyser are situated in crossed direction and either there is no object or an isotropic object, in the object plane, the image appears uniformly dark. Isotropic objects have only one refractive index and have no restriction on the vibration direction of light passing through them, whereas an anisotropic object has optical properties which vary with the orientation of incident light and with the crystallographic axis. It demonstrates a range of refractive indices depending both on the propagation direction of light through the substances and on the vibrational plane coordinate. Such specimens are also called birefringent materials if a substance consists of elongated particles in a parallel array, or stacked layers stuck in a medium whose refractive index is different from that of the medium also exhibit birefringences. A birefringent object present on the sample stage, at an angle other than 0° or 90° with respect to the plane of polarization, will resolve incident light into two components: one parallel and one perpendicular to the plane of the analyser. Hence some light will pass through the analyser, and the object will appear bright against a dark background. If the object is rotated, then it will no longer be parallel, and the object will appear dark. It will have maximum brightness at an angle of 45° , but the main problem is to differentiate between positive and negative birefringent structure. This can be identified by the position of the compensator, producing minimum and maximum brightness. The sign of birefringence tells how the components are oriented.

Studies on sperm cell and muscle cell show that they contain molecules parallel to the direction of fibre. Chloroplast and rod cells of retina have a stacked disc structure. A positively birefringent fibre looks dark for all rotations, but if the fibre is helical, it appears alternatively dark and light. This way helical structure can be identified. The advantage of polarizing microscope lies in its ease of sample preparation and relatively short time required to get detailed information about molecular architecture. Cell structure, which is not visible by common microscopy, could be easily

seen in a polarization microscope, for example, mitotic spindle and the orientation of molecules in the boundary region between plant cells undergoing cell division (Abrahamsson et al. 2015).

11.8 Confocal Microscopy

Fluorescence is emitted through the entire depth of the specimen, observed under fluorescence microscope, but the viewer can only focus the objective lens on a single plane at any point of time. As a result, light emitted from the regions of the specimen above and below the focal plane causes a blurring of the visible image. Because of this limitation, fluorescence microscopy has traditionally been used on cells that are well flattened, such as cultured cells attached to the surface of a culture dish. However, a modified version called confocal scanning microscope overcomes this problem by employing a laser beam to illuminate a single plane of the specimen at a time. Thus it eliminates the blur, normally caused by fluorescence emitted from adjacent focal planes (Jang et al. 2018).

Marvin Minsky first employed the basic principle of confocal imaging in confocal microscopes, and he got this instrument patented in the year 1957. An optical confocal scanning microscope is expensive and can illuminate any object using a narrow beam via a series of points, eliminating photooxidation-related issues and thereby allowing the visualization of objects over extended duration with low signal losses and at higher resolution (Fig. 11.8). A small aperture placed along a narrow beam facilitates field depth and eliminates information that are out of focus, effectively during image formation (Costa et al. 2018).

The action of this microscope involves the use of a bright spot of light that can scan across the object in a line from either top to bottom or sideways; like the incident light which falls in a point by point fashion, the image too gets generated likewise.



Fig. 11.8 A confocal microscope LSM 700 for high sensitivity and performance (www.zeiss.com/lsm)

The formation of an image gets translated in the form of intensity of each spot adjusted via a photomultiplier tube in the X–Y raster. The intensity data can be digitized for subsequently storing in a computer. Visualizing and manipulating the images then become facilitated through a software package for image processing. The resolution is determined via spot sizes of the laser light used for excitation and ranges between 0.12 and 0.15 μm for any ideal specimen, even when we have the best objective lens that is available. These microscopes have a pinhole in the diaphragm placed in the optical path in a special way close to the positioning of a photomultiplier tube. The pinhole is situated at a plane from where the incident light and the part of “in-focus” converge at a particle point.

Light rays travelling from the object plane do not converge with the light above or below the focused image, in the optical path that leads to the pinhole. This special design causes darkening of spots out of focus and so it is not detectable. Consequently, all the image information that is out of focus gets removed, and the confocal image is from a comparatively thicker object. Since these confocal images obtained are then stored in a computer, they can get overlaid to generate 3D images which are actually reconstruction of the various images captured. The image processing is done via software, and they facilitate the rotation and inversion of the images for best recreation, very conveniently. This leads to 3D aspects of cell structures. For these functions, a robust and fast processing computer is required. The image formed may be over 1000 KB, and the storage space should be sufficient in the computer; else just a few images would fill the hard disks.

This technique is used for obtaining images (3D) of whole cells or cell organelles. Beside this, it is used for evaluating the cell's physiology via monitoring of distribution and concentration of molecules, e.g. ATP and calcimines. The field of biomedical research, majorly confocal microscopy, is applied for imaging living cells or fixed tissues that carry fluorescent labels or probes. Several types of fluorescent probes have been developed, specifically stains for cellular organelles and structure, for example, dyes that label nucleus, Golgi apparatus, endoplasmic reticulum, mitochondria, etc. (Abrahamsson et al. 2015).

11.9 Electron Microscopy

11.9.1 Introduction

Ernst August Friedrich Ruska, German electrical engineer, was awarded half of the Nobel Prize (Physics) for the invention of electron microscope in the year 1966. In the early 1920s, it was established that electrons have the properties of waves and its wavelength is about 100,000 times shorter than those of light, so it could be focused in an object the way light is focused. An extremely high magnification could be achieved with greater detail than a conventional microscope does. In 1931 he built the first electron lens, an electromagnet, consisting of coil of wire through which current flows producing a magnetic field. Various functions of electron microscope, such as brightness, focus and magnification, are achieved by altering the level of

electric current. The control of this current must be very precise, and it must remain stable once set.

By using several such lenses in a series, he invented the first electron microscope in 1933. It was the first primitive electron microscope, not fit for practical purpose. The very first working model of the electron microscope was designed in 1938, by Eli Franklin Burton and his students, at the University of Toronto (Canada). Modern electron microscope can magnify an object up to two million times, but under optimal conditions, a resolution of 0.10 nm can be obtained with transmission electron microscopes about 500 times better than the best resolution of light microscope.

11.9.2 Transmission Electron Microscope (TEM)

The source of illumination in transmission electron microscope (TEM) is a high-voltage electron beam transmitting through a specimen. The transmitted radiation forms an image, which is not visible to the human eye. As the image is formed by the transmission of electron through the specimen, it is called transmission electron microscope.

The electron beam is produced by a cathode, made of tungsten or lanthanum hexaboride. The beam is accelerated towards the anode. The potential of the anode is normally 40–100 KV greater than that of the filament (cathode). The electron gun is made up of both the anode and the filament. Through a minute orifice present in the anode, electrons can pass through at high speed. Since electrons get deflected slightly, the beam is also slightly divergent towards the edge of the orifice. The divergent beam is made convergent by passing it through a condenser electromagnetic lens. The electron beam then passes through a very thin cross section of the specimen. Some of the electrons are scattered by the atoms of the specimen, but the specimen absorbs the majority of it. The pattern of this loss of electron generates the image pattern. The objective lens, adjusted at the focus of the specimen, refocuses the beam to produce an image. The image is then magnified by a series of lenses. In general for high magnification, lenses of short focal length are needed, and the focal length decreases as the magnetic field increases, which is achieved by increasing the current in the magnetic wires. Finally the projector lens forms the image on a fluorescent screen or a photograph plate. Some areas in the specimen scatters electron more or do not allow the light to pass through it, appear as dark regions and are referred to as electron opaque. Conversely molecules with less electron scattering ability appear as lighter areas. The final image thus depends on electron scattering abilities of various structures. The electron scattering ability depends on the atomic number: the higher the atomic number, the greater the ability to scatter electrons. Biological molecules are mostly composed of elements like C, H, O, N, S and P and have relatively low atomic number and hence more or less same scattering power. In order to increase the contrast between different parts, staining is done with high molecular weight compounds of osmium, uranium and lead. The

electron opaque stains form complexes with biological molecules and thereby enhance their visibility.

TEM has very high resolution, and it is extremely valuable for examining different layers of specimen (Curry et al. 2006). But also it has certain disadvantages as the penetration power of electron is very weak; it can pass only a very short distance through air, so the internal chamber must be kept in vacuum. The thickness of the specimen should not be more than a few hundredth nanometres. It causes technical difficulties in preparing samples. Moreover, the specimen has no three-dimensional aspect. Treatment with heavy metal stains and high vacuum kills the specimen and causes some shrinkage and distortion; sometimes it happens to such an extent that it appears as an additional structure in a prepared cell.

11.9.3 Scanning Electron Microscope

Scanning electron microscope overcomes most of the disadvantages of TEM and provides a three-dimensional view of the specimen, problem of sectioning of specimen was minimized, and it also allows the investigator to view the surfaces of the un-sectioned specimens (Allgeier et al. 2018).

In SEM the finely focused beam of electrons produced by electron gun is called primary electron beam. It passes through electromagnetic condenser lenses and is projected on the surface of the specimen and scan the surface rapidly. The electron get knocked out from the surface of the specimen by the primary electron beam, and then secondary electrons are generated, and these get transmitted to the electron collector in the form of a signal. This signal is then amplified and used to produce an image on a viewing screen or photographic plate. The number of secondary electron produced by any point on the specimen is dependent upon the electron beam's angle with the surface. TEM resolution is much better than SEM; however SEM images depend on surface-linked activities instead of transmission. It can capture images of high-volume samples and the depth of viewing is high.

11.9.4 Scanning Tunneling Microscope (STM)

In 1980, Gerd Binnig and Heinrich Rohrer invented the first of scanning tunneling electron microscope. They got Nobel Prize in 1986 for this invention. Though scanning tunneling microscope (STM) and atomic force are considered a type of electron microscope, actually it is a type of scanning probe microscopy and is non-optical in nature.

An atomically sharp probe (the tip) made of platinum and iridium is moved over the surface of the specimen material under study. The probe acts just like a fingertip, which can feel the bumps while reading Braille. A certain voltage is applied between the probe and the surface, and depending on its magnitude, electrons will jump from the tip of the probe to the surface of the specimen just like a tunnel (or vice versa depending on the polarity). This process results in the generation of a weak electric

current. The stronger the current, the closer the top atoms of the surface to the probe. Moving the probe across in straight line reveals the highs and lows of individual molecules or atoms in a surface. Thus the size of this current is exponentially dependent on the distance between the probe and surface. Actually STM employs principles of quantum mechanics to determine the height of a surface. STM is very useful in the study of live specimen such as virus-infected cells exploding and releasing newly from viruses. Besides this it first showed as individual fibrin molecules coming together to form a blood clot.

11.9.5 Atomic Force Microscope

This type of microscope has a micro-scale cantilever bearing a sharpened tip (probe) at one end which primarily is used for scanning the surface of a specimen. The cantilever is made of silicon or silicon nitride with a tip radius of curvature in the order of nanometres. When the tip is brought into the proximity of a sample surface, force is generated on the sample and tip, thus leading to the deflection of the cantilever as per Hooke's law. This deflection is classically determined by using reflection from a laser spot at the top of the cantilever into some photodiodes lying in an array. But a detecting system may be bulky and expensive. If tips are scanned at constant heights, the risk of collision with surfaces remains and can cause damage. So in many instances a feedback mechanism is used for adjustment of the distance from the sample to the tip such that constant forces are maintained between them. In general cases, samples are mounted onto piezoelectric tubes, which move the sample up and down for maintenance of constant force along x and y directions, for scanning the sample. The resulting map represents the topography of the sample.

AFM can be operational using many modes. Generally, the possible modes can be divided as static modes or dynamic modes of various types. In static mode operation, the static tip deflection is used as a feedback signal. Cantilevers having lower stiffness are preferred for boosting the deflection signal because measuring a static signal is susceptible to noise and drifting. However, close to the surface of the sample, attractive force is quite strong, causing the tip to snap in the surface. AFM is performed in static mode when the repulsive force is employed; overall, hence it is also referred to as contact mode. Here a constant force is maintained between the surface and tip, while scanning is done by maintaining constant deflection.

While working in dynamic mode, cantilever is constantly under oscillation at or around its resonance frequency. The amplitude of oscillation phase and the frequency of resonance can be altered by interaction forces between the sample and the tip. Such changes in oscillation compared to external references of oscillation and such references also provide knowledge about the characteristics of the sample. Operations at dynamic mode can mean modulating the frequency and also commonly the amplitude. During frequency modulation, or amplitude modulation, changes in the oscillation frequency and amplitude can give information on topography or characteristics of the sample.

The AFM has an edge over the performance of the scanning electron microscope. This technique has the ability to generate images of materials ranging from 1 nm to around 100 nm. AFM facilitates the generation of a real 3D profile of the surface. Samples viewed under the AFM may not need other exclusive treatments although working on electron microscopes requires a vacuum for operating, and this makes the technique expensive. AFM works well in all optimal environment, be it air a liquid. This makes the technique applicable to living organisms and also macromolecules from biological systems. The main disadvantage of AFM compared with the scanning electron microscope is the image size. Here the image size is small compared to SEM.

11.9.6 Sample Preparation for Light Microscope

Preparation of specimen from microbial culture for light microscope involves fixation, staining and mounting, while in histological techniques, the study of either animal or plants tissue is concerned primarily with preparation of thin slices of tissue, which must be properly stained with suitable dyes to identify its components in an explicit manner. The study of tissue under microscope consists of the following steps: fixation sectioning, staining and mounting.

Fixation mainly is of two types, wet mount and smears.

11.9.6.1 Wet Mount Method

The microbes present as suspension in a medium are mounted upon a microscope slide, and then a thick syrupy solution of carboxymethyl cellulose is added into it to slow down the fast-moving organisms so that they can be studied under light microscope. A special method of wet mount is the hanging drop technique which is used in combination to dark field illumination. A drop of culture is placed on a coverslip, ringed with petroleum jelly, and then the coverslip is inverted and mounted on the depressed spot of a depression slide. The petroleum jelly is used for sealing the culture drop, which does not fall and also prevents evaporation.

The second type, smear, involves the spread of a drop of microbial culture onto the surface of a glass slide and viewing under microscope, but this technique kills the microorganism while fixing it onto the slide. After the preparation of smear, it is air-dried completely and quickly and passed three to four times through an open flame. The process is called heat fixation.

Complete drying before heat fixation is very much required; otherwise the organisms will be destroyed by the boiling medium.

The second step, staining, is required to incorporate a contrast between the microorganisms and their background. A stain or dye is a chemical that can bind to a cellular structure and induces colours. In microbiology the most commonly used dyes are cationic or basic dye, which includes methylene blue, crystal violet, safranin, malachite green, etc. The cell membrane of most bacteria has negatively charged surfaces and thus reacts with the positively charged basic dyes. Anionic or

acidic dyes include eosine, picric acid, etc. Negative stains are attracted to any positively charged cell materials.

From chemical composition point of view, stains used in microbiology are of two basic types, simple stains and differential stains. Simple stain consists of a single dye which reveals basic cell shape and arrangements. Methylene blue, crystal violet and carbol fuchsin are few examples of this type. A differential stain contains two or more dyes which distinguish two kinds of microorganisms or two different parts of organisms. Common differential stains are Ziehl-Neelsen acid fast stain and the Schaeffer Fulton spore stain. In this connection it is necessary to have an idea about Gram staining. Microorganisms may be categorized into four major classes based on staining. The Gram-positive bacteria show a blue black coloured cell when stained with crystal violet stain; Gram-negative ones do not retain this stain and so get pink stained by the counter-stain safranin. The other two classes have organisms that are non-reactive to Gram stain which do not stain or stain poorly and Gram-variable organism, which stain unevenly. This observation helps in distinguishing groups with radical difference in taxonomic character. The most widely used stain in Gram staining is differential stain. Here first the Gram's crystal violet stain is taken up by the bacterial cells; the mordant, i.e. iodine, is then added, which helps to retain the stain in certain cells. It is then rinsed with alcohol 95% to remove stain from that cell which is not strongly bound to the dye. Subsequently it is stained with counter-stain safranin.

11.9.7 Histological Techniques

Fixation, an act that arrests the movement and further changes in the tissue, is the first step in histological techniques. Fixation is required to stop denaturation of enzymes, cell membrane, proteins and all organelles. It makes tissues hard so that thin slices may be cut from it. It also stops fungal deterioration of tissue that occurs after death. Some common fixatives are formaldehyde (10% solution), ethanol, picric acid and its combinations, mercuric chloride and its combinations, potassium dichromate, osmium tetroxide, glacial acetic acid, etc. The second step is section cutting, where a section tissue must be embedded with paraffin wax which fills the interstitial spaces in tissues in a very firm way, so that sectioning is possible without creating damage to the tissue components. Paraffin wax is most widely used for impregnation because of its easy handling and easy staining as well as low cost. A number of additives are used with ordinary paraffin wax to improve its consistency and alter its melting point. Few such additives, with different degrees of success, include bay berry wax, beeswax, stearic acid, rubber and diethylene glycol, distearate, etc.

Sections of tissues are cut on the machine called microtome. It is a machine designed to cut thin slices of tissues. The most important feature of microtome is a feedback mechanism that forwards the tissue for a prefixed distance (in microns) followed by cutting stroke.

Several types of microtome are available, for example, Cambridge rocking microtome, base sledge microtome, freezing microtome, rotary microtome, ultra-microtome, sliding microtome, etc.

The next step in this process is staining and mounting. But before staining, the wax, which is insoluble in water, should be removed by heating over a flame to melt and then placing it in a suitable solvent like benzene, xylene, etc. After total removal of wax, the slide is placed in absolute alcohol with a cover to prevent contamination with atmospheric water vapor for dehydration. The section is then left in water for sufficient time (5–10 min) to remove alcohol.

The process of staining starts after the total de-waxing and hydrated sections. There are several procedures for staining, double (containing two stains), triple (three stains), and polychrome (more than three) procedures. The effectiveness of the staining process depends on dye-tissue or reagent-tissue affinity. In many cases we find that the majority of stains have a very low affinity and very slow rate of solubility in the processing fluids and the mounting media. The staining condition selected must favour maximum selective affinity. For example, haematoxylin must be applied in a neutral or acidic solution to keep proteins with positive charge and are thus prone to be stained by haematoxylin. Haematoxylin is a powdered dye obtained from small leguminous plant as haematoxyl. Haematoxyl on oxidation produces haematoxylin. Haematoxylin stains nuclei and cytoplasm in different shades.

Eosine, another dye, stains cytoplasm and connective tissues in different shades. And the mixture of two eosine-haematoxylin combinations is used as a double stain. An example of triple stain is Mallory's triple stain. Commonly used mounting media is Canada balsam, a natural product, which acts as a natural plasticizer. Nowadays Canada balsam has been replaced by DPX, a commercially available mounting media, for its low cost, sufficient consistency (required for slide storage) and ease of work. It also does not fade the stain for long time.

Although staining and microscopy offer valuable information about microorganisms, it is insufficient for identifying of samples. A large number of microbial species appear very identical when viewed under the microscope, but actually they are different. A limited number of basic shapes and arrangements are known, whereas thousands of kinds are available in nature, so for identification genetic characteristic and biochemical knowledge are required. Some stains are used to detect a particular type of microorganism, for example, an acid-fast stain (Ziehl-Neelsen) is used to detect tuberculosis and leprosy causing organisms of genus *Mycobacterium*. Here the dye combination used is carbol fuchsin and Loeffler's methylene blue. Bacteria which contain lipid component in their cell wall will retain red colour even after the use of decolourizer. Bacteria that are not acid-fast lose the red colour and capture blue colour from Loeffler's methylene blue. Mounting media is the same as that of the tissue dying technique.

11.9.8 Sample Preparation for Electron Microscope

The primary requirement for sample preparation is that the sample should be very thin; otherwise beam of electron will not be able to pass through it. The maximum permissible thickness is approximately 50–100 μm . Virus, fibrils or macromolecules can be observed clearly, but for cells, in the range of 1–50 μm in thickness, it is necessary to follow embedding, sectioning and staining steps just like light microscopy. For this a sample support is needed, which must be very thin with uniform thickness without any particular structure. This type of support is called grid, made up of rigid copper or platinum mesh with an opening of about 75 μm on every side and overlaid with a deposit of support film which is a thin electron transparent film. Electron scattering power of this film must be low and uniform. Formation of image depends on the differential scattering of the incident electrons by molecules in the preparation; when the sample is not stained, the incident electron passes through a cell or tissue sample uniformly, and it appears with uniform brightness. Thus staining techniques are required to increase contrast and to reveal location and distribution of specific materials. Generally, salts of heavy metals like molybdenum, tungsten, uranium, lead or osmium tetroxide vapours are used for staining. Staining is depositing metal on the specimen to increase its electron density. This can be done by chemical reactions or by forming metal complex with some cellular components in the sample. Thus the sample now appears dark on the micrograph.

11.9.9 Sample Preparation of TEM and SEM

The tissue is cut into small cubes and plunged into a fixing solution, for example, glutaraldehyde, osmium tetroxide that cross-links and immunoglobulin proteins. The sample is then dehydrated by placing it successively in increasing alcohol- or acetone-containing solutions. Finally they are immersed in plastic embedding media (suspension) and dried in hot air oven. On heating, the solution polymerizes into a hard plastic block, which is cut into hard plastic block, followed by very fine sections (0.1 μm) with an ultra-microtome (instrument having a diamond blade indicates their weight or thickness; heavy ones sink and are not selected. The sections are then picked up on grids of Cu coated with carbon or other material. Transmission electron microscope usually provides two-dimensional view of a cell interior. However a three-dimensional view could be obtained by taking the photograph of the same specimen at two slightly different angles and then viewing the two photographs through a spectrocope. Although cutting sections is the most common method for preparing materials for transmission electron microscopy, other techniques have also been developed for particular purposes. For example, shape and surface structure of a cell or organelles can be examined by simply positive or negative staining techniques.

Macromolecules or isolated particulate matter may become visible by metal shadow casting process. Here the specimen is overlaid upon a mica sheet and vacuum-dried in a vacuum evaporator. The specimen is then sprayed with gold or

platinum that is in vapour phase. This can be achieved by electrically heating the heavy metal filament (platinum or gold), till the metal evaporates. Spraying is done across the specimen at an angle, such that the metal accumulates on one side and a shadow region is created which lacks the metal on another side. It creates a three-dimensional view of the specimen. The biological material is then dissolved by acid, leaving the metal replica of its surface which can be viewed by transmission electron microscope. Variation in angle and thickness of the deposited metal allow image formation since the incident electron meeting the metal will reflect in various directions rather than passing through the specimen. As the metal is deposited at one side of the sample, that area appears dark, whereas the other side appears light.

The SEM can be used to view un-sectioned tissues or cells. The sample is fixed and dried before coating it with a thin film of some heavy metal, for example, platinum, in a uniform manner, platinum. The solvent is evaporated in vacuum.. The sample is then scanned with a beam of electron which produces secondary electrons. The resolving power of SEM becomes limited by the metal coating thickness which is about 10 nm. This is well below the thickness of the transmission instrument.

11.9.10 Cryoelectron Microscopy

Sometimes, the macromolecules get nonfunctional and denatured due to removal of water. Cryoelectron microscopy technique however examines unfixed, hydrated and unstained specimen under the TEM directly. Using this technique, sample is dissolved in the form of aqueous suspension and is applied to the grid like a very thin film, frozen in liquid nitrogen and then mounted in this state using a special technique. Thereafter this sample is visualized under the TEM. Due to the persistence of low temperatures during this process, evaporation of water even in vacuum is resisted, and so the sample is studied in its hydrated native state without shadowing or fixing it. Using a computer-aided average calculation involving hundreds of images of the particle, a three-dimensional model can be generated.

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