**SEMESTER VI**

**17BTU611A MOLECULAR DIAGNOSTICS PRACTICAL 3H - 1C**

**Total hours/week: L:0 T:0 P:3 Marks: Internal: 40 External: 60 Total: 100**

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**Practical**

1. Perform/demonstrate RFLP, RAPD and analysis
2. Kirby-Bauyer method (disc-diffusion method) to study antibiotic sensitivity of a bacterial culture
3. A kit-basd detection of a microbial infection (Widal test)
4. Study of Electron micrographs (any four).
5. Perform any one immuno diagnostic test ( Typhoid, Malaria, Dengue)

**References**


1. Willey, J.M., Sherwood, L.M., & Woolverton, C.J. (2008). *Prescott, Harley and Klein's Microbiology* (7th ed.). McGraw Hill Higher Education.
2. Goering, R., Dockrell, H., Zuckerman, M., & Wakelin, D. (2007). *Mims' Medical Microbiology* (4th ed.). Elsevier.
3. Ananthanarayan, R., & Paniker, C.K.J. (2005). *Textbook of Microbiology* (7th ed.). University Press Publication.
4. Brooks, G.F., Carroll, K.C., Butel, J.S., & Morse, S.A. (2007). Jawetz, Melnick and *Adelberg's Medical Microbiology* (24th ed.). McGraw Hill Publication.
5. Joklik, W.K., Willett, H.P., & Amos, D.B. (1995). *Zinsser Microbiology* (19th ed.). Appleton-Centuary-Crofts publication.


*(Wherever wet lab experiments are not possible the principles and concepts can be demonstrated through any other material or medium including videos/virtual labs etc.)*

# MOLECULAR DIAGNOSTICS PRACTICAL

**(Course code: 17BTU611A)**

**Practical Manual**

## EXP No. 1 Restriction Fragment Length Polymorphism (RFLP)

**Aim:**

To learn the process of DNA fingerprinting following Restriction Fragment Length Polymorphism (RFLP) method by restriction digestion of DNA and analysis of the digested fragments on agarose gel.

**Introduction:**

Restriction fragment length polymorphism (RFLP) method in molecular biology was evolved for detecting variation attheDNAsequencelevelofvariousbiologicalsamples.Theprincipleofthismethod is based upon the comparison of restriction enzyme cleavage profiles following the existence of a polymorphism in a DNA sequence related to other sequence. In RFLP, DNA of individuals to be comparedis digested with one or more restriction enzymes and the resulting fragments are separated according to molecular size using gel electrophoresis along with a molecular weight marker. Through this approach two individuals can present different restriction profiles.

**Principle:**

Restriction fragment length polymorphism (RFLP) analysis is extensively used in molecular biology for detecting variation at the DNA sequence level. The principle of this analysis is to compare restriction digestion profilesofDNA samples isolated fromdifferent individuals. RFLP functions as a molecular marker as it is specific to a single clone/restriction enzyme combination. Most RFLP markers are co-dominant and highly locus-specific. In molecular biology, restriction fragment length polymorphism, or RFLP is a technique that exploits variations in homologous DNA sequences. It refers to a difference between samples of homologous DNA molecules that come from differing locations of restriction enzyme sites, and to a related laboratory technique by which these segments can be illustrated.

RFLP is a difference in homologous DNA sequences that can be detected by the presence of fragments of different lengths after digestion of the DNA samples in question with specific restriction endonucleases. The basic technique for detecting RFLPs involves fragmenting a sample of DNA by a restriction enzyme,which can recognizeand digest DNA wherever a specific short sequence occurs, in a process known as restriction digestion. The resulting DNA fragments are then separated by length through a process known as agarose gel electrophoresis. Molecular markers are used to estimate the fragment size. RFLP is specific to a single clone/restriction enzyme combination and it occurs when the length of a detected fragment varies between individuals.

RFLP analysis was the first DNA profiling technique for genetic fingerprinting, genome mapping, localization of genes for genetic disorders, determination of risk for disease, and paternity testing. Presence and absence of fragments resulting from changes in recognition sites are used for identificationofspeciesor populations.

**Procedure:**

1. Before starting the experiment, crush ice and place the vials containing DNA samples, restriction enzymes and assay buffers onto it.
2. In this experiment three reference DNA samples and the test sample are digested simultaneously with two restriction enzymes *Eco*RI and*Pst*I.

3. Set up four separate reaction mixtures as follows:

| | |
|---|---|
| DNA sample | – 15.0 µl |
| 10X Assay Buffer | – 3.0 µl |
| Molecular Biology Grade Water | – 10.0µl |
| *Eco*RI | –1.0 µl |
| *Pst*I | – 1.0 µl |
| **Total** | **30µl** |

4. After preparing the four reaction tubes, mix the components by gentle pipetting and tapping.
5. Incubate the tubes at $37^{O}$C for 2-3 hours.
6. After incubation, immediately add 5 µl of 6X Dye to each tube.
7. Run the samples on agarose gel as given below:

## EXP No. 1 Random Amplification of Polymorphic DNA (RAPD)

**Aim:**

To understand the concept of DNA fingerprinting by Random Amplification of Polymorphic DNA (RAPD) in context of bacterial strain identification.

**Introduction:**

Random Amplified Polymorphic DNA (RAPD) is a method of producing a biochemical fingerprint of a particular species. Relationships between species may be determined by comparing their unique fingerprint information. RAPD-PCR is means of creating a biochemical fingerprint of an organism and is used to analyze the genetic diversity of an individual by using random primers. In this method random primer sequences are added each to an individual sample of DNA which is then subjected to PCR. The resulting amplified DNA markers are random polymorphic segments with varying band sizes which can be analysed after performing gel electrophoresis.

**Principle:**

Genetic analysis of organisms at the molecular level is a very important and widely practiced scientific tool. Several techniques have been developed to identify each individual or type of individual in a species unambiguously. One important PCR-based genetic analysis is random amplified polymorphic DNA analysis (RAPD). RAPD uses small (10 nucleotide length), nonspecific primers to amplify seemingly random regions of genomic DNA. If primers with arbitrary sequences (random primers) are used for PCR amplification, DNA segments to be amplified will be selected at random which will thus provide a truly random sample of DNA markers. In RAPD no knowledge of the DNA sequence for the targeted gene is required, as the primers will bind somewhere in the sequence, but it is not certain exactly where. The principle is that, the single, short oligonucleotide primer, which binds to many different loci, is used to amplify random sequences from a complex DNA template. This means that the amplified fragment generated by PCR depends on the length and size of both the primer and the target genome. The assumption is made that a given DNA sequence (complementary to that of the primer) will occur in the genome, on opposite DNA strands, in opposite orientation within a distance that is readily amplifiable by PCR.

**Procedure:**

1) **Preparation of master mix for PCR**
   Prepare a PCR master mix by adding the following reagents:

| Sr. No. | Ingredients for PCR | Volume in |
|---|---|---|
| 1 | Molecular Biology Grade Water | 75 µl |
| 2 | 10X Assay Buffer | 10 µl |
| 3 | 2.5mM dNTP each | 7.5 µl |
| 4 | Random Primer | 5 µl |
| 5 | Taq DNA Polymerase | 2.5 µl |

2) Tap the tubefor 1 – 2 secondsto mixthe contents thoroughly.

3) Aliquot 20µl of the above PCR master mix to each of the five different PCR tubes (placed on ice) and label them as 1, 2, 3, 4 and 5.

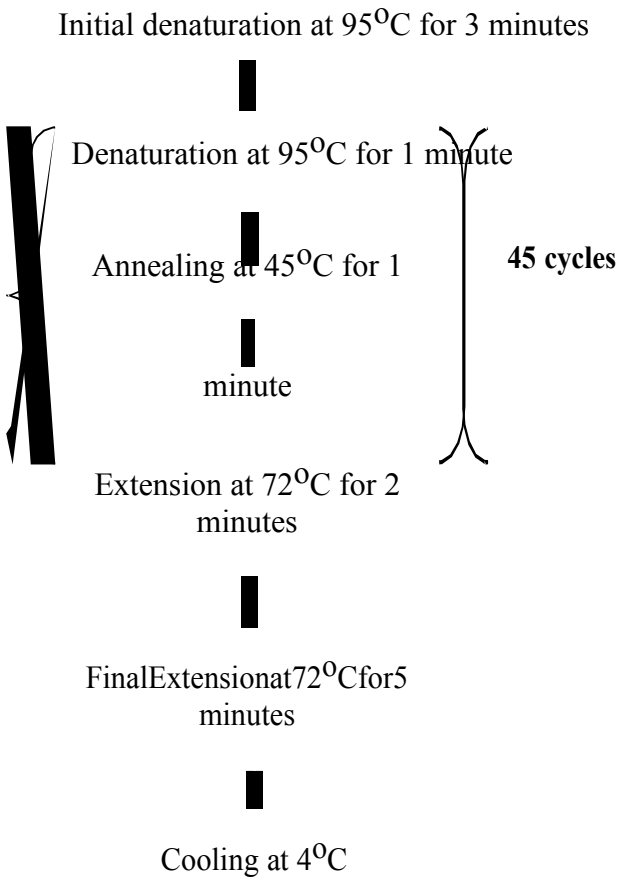
4) Add 1 µl genomic DNA of *E. coli* K12, *E.coli* 0103:K:h8, *Bacillus cereus*, *Bacillus subtilis* and Test Genomic DNA to the tubes 1, 2, 3, 4 and 5 respectively. Mix the contents gently.

5) Add 25 µl of mineral oil to the PCR tubes to avoid evaporation of the contents.

6) Place the tube in a thermocycler block and set the program to get DNA amplification.

**PCR Amplification Cycle:**

Carry out the amplification in a thermocycler for 45 cycles using the following reaction conditions.




Following PCR amplification, add 2 µl of 6X Gel loading buffer to each of the PCR tubes and mix thoroughly. Let standfor sometime forthetwo layerstoseparate(ifmineraloilisused) and performagarosegel electrophoresis as described below.

## EXP No. 2 Kirby-Bauyer method

Aim:

To determine the sensitivity of bacteria against various antibiotics.

Introduction:

Antibiotic sensitivity describes the susceptibility of bacteria to various antibiotics. Clinical microbiologists have a major role to play in prescribing antibiotics for either treatment or prophylaxis of infection. The antibiotics for therapy are selected after performing Antibiotic Susceptibility Test (AST). It is often done by the Kirby-Bauer method in which antibiotic impregnated discs are used to test the susceptibility of any bacterial strain to a specific antibiotic.

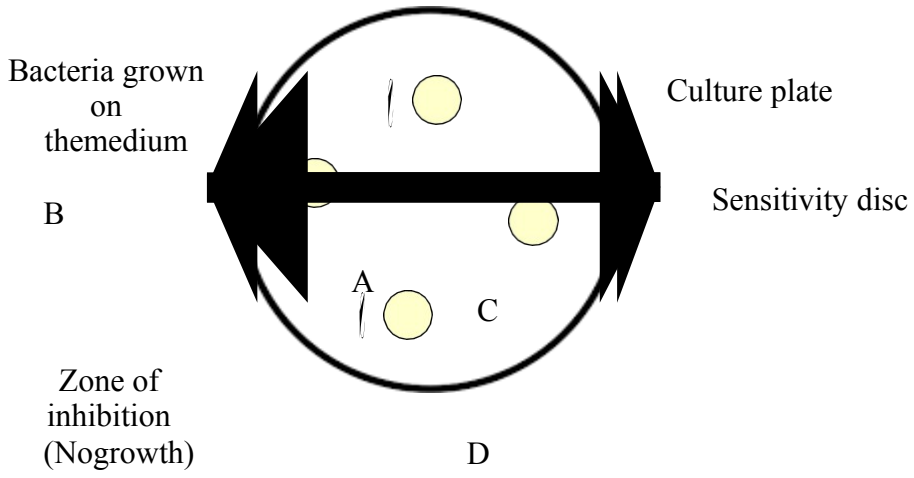



**Fig 1: Sensitivity of bacteria to antibiotics B and D but not to A and C**

Principle:

Antibiotic Sensitivity Teaching Kit allows classification of bacterial strains as susceptible, resistant, or intermediate to various antimicrobial agents. In this method, the antibiotic impregnated discs are placed on the Mueller Hinton Agar plates on which the bacterial culture is spread. As the antibiotic impregnated disc comes in contact with the moist agar surface, water is absorbed in the disc paper and the antibiotic diffuses out in the surrounding medium. As the distance from the disc increases, there is a logarithmic reduction in the antibiotic concentration which creates a gradient of drug concentration in the agar medium surrounding each disc. Though the diffusion of drug occurs, the bacteria that are inoculated on the agar surface are not inhibited bythe concentration of antimicrobial agents but continue to multiplyuntil a lawnof growth is visible. No growth occurs in the areas where the concentration of drug is inhibitory thus forming a zone of inhibition. Thus when an organism is sensitive to any antibiotic, a clear zone appears around that specific disc where the growth has been inhibited (zone of inhibition) whereas if an organism is resistantnoclearzoneof inhibition appears.

**Sensitive (S):** An organism is called 'sensitive' to a drug when the infection caused by it is likely to respond to the treatment with that specific drug at the recommended dosage.

**Intermediately sensitive (I):** It is applicable to organisms that are moderately sensitive to an antibiotic that can be used for treatment at a higher dosage and as a result leads to uncertain therapeutic effect.

**Resistant (R):**Anorganismiscalled'resistant'to adrugwhentheorganismdoesnotrespond toagiven drug irrespective of the dosage.
Thediameterofthezoneofinhibitionsurroundingtheantibioticdiscismeasuredtodetermine whetherthe microorganism issensitiveorresistant toaparticular antibiotic. Thezonesize dependson:

1. The rate of diffusion of the antibiotic through agar
2. The concentration of the antibiotic present in the disc
3. The degree of sensitivity of the microorganism
4. The growth rate of the bacterium

Thus,byperformingASTclinicianscanselectthemostappropriateantibioticfortreatment. Alsovarious microbial strains can be studied for their susceptibility to various antibiotics.

**Procedure:**

**Day 1: Revival of Strains**

1. Open the vials containing cultures (*E.coli, S.aureus, P.aerruginosa*) and resuspend the pellet individually with 0.25 ml of LB broth.
2. Pick up a loopful of culture and streak onto MH agar plate.
3. Incubate overnight at$37^0$C.

**Day 2: Antibiotic Sensitivity Test**

1. Pick up a single colonyofeach strain(*E. coli, S. aureus and P. aeruginosa*) from the MH agar plate and inoculate into three sterile test tubes respectively containing 1 ml of

sterile saline. Mix the tubes thoroughly.

2. Take a sterile cotton swab and dip it into the *E. coli* labeled test tube.
3. Spread the cotton swab containing the *E.coli* culture evenly onto the *E.coli* labeled Mueller Hinton Agar plate.
4. Similarly, follow steps 2 and 3 for *S. aureus* and *P. aeruginosa.*
5. Allow the plates to dry for 5 minutes.
6. Using applicator place the antibiotic discs onto the surface of the *E. coli* Mueller Hinton Agar plateas shown in figure2.
7. Similarly follow steps 9 and 10 for *S. aureus* and *P. aeruginosa.*
8. Incubate all the plates at $37^{o}$C for 24 hours

**Table 2: Measurement of zone of inhibition**

| Sr. No. | Antibiotics | Zone of inhibition ( in mm) *E. coli* | | |
|---|---|---|---|---|
| | | | ***S. aureus*** | ***P. aeruginosa*** |
| 1 | Chloramphenicol | | | |
| 2 | Gentamycin | | | |
| 3 | Kanamycin | | | |
| 4 | Tetracycline | | | |
| 5 | Vancomycin | | | |

Compare the results obtained with that of the standard to determine the susceptibility of each organism to each of the five antibiotics and record them as follows:
S- Sensitive
I- Intermediate
R- Resistant

**Table 3: Susceptibility of each organism**

| Sr. No. | Antibiotics | Organism *E.coli* | | |
|---|---|---|---|---|
| | | | ***S. aureus*** | ***P. aeruginosa*** |
| 1 | Chloramphenicol | | | |
| 2 | Gentamycin | | | |
| 3 | Kanamycin | | | |
| 4 | Tetracycline | | | |
| 5 | Vancomycin | | | |

## EXP No. 3 Widal Test

Aim:

To detect the presence of *Salmonella* genus which causes enteric or Typhoid Fever by using qualitative slide agglutination test.

Introduction:

Widal test is a serological method to diagnose enteric fever or typhoid which is caused by the infection with pathogenic microorganisms like *Salmonella typhi*,*Salmonella paratyphi* A, B and C. This method of diagnostic testisbaseduponavisibleagglutinationreactioneitherinatesttubeor on a slide between antibodies of patient serum and antigens specifically prepared from *Salmonella sp*.

Principle:

Enteric fever or typhoid is a life threatening disease which usually occurs due to the infection of

pathogenic microorganisms, e.g. *Salmonella typhi*, *Salmonella paratyphi* A, B and C. These microorganisms are transmitted to human body through food and drinks contaminated with fecal matter. Early diagnosis and treatment for this fever are essential to avoid serious clinical complications. During the course of infection antibodies are produced against Salmonella antigens. Widal test, a serological method for the detection of *Salmonella sp*., was developed by F Widal in 1896. During this test a visible agglutination is formed dueto the reaction in a test tube or on a slide between antibodies present in the infected person's blood sample and specific antigens of *S. typhi* and *S. paratyphi*. For the slide agglutination test, stained *Salmonella* antigens are used to detect the presence of specific agglutinin in the patient's serum. The slide agglutinationtestisusedas a primary screeningprocedure.

Theorganismscausing entericfeverpossessestwomajorantigensnamely somaticantigen,Oand a flagellar antigen, H along with another surface antigen, Vi. During infection antibodies are produced in patient's sera against *Salmonella typhi* O and H and *Salmonella paratyphi* AH and BH antigens. During infection antibodies are produced in patient's sera against these antigens. Antigens specifically prepared from this organism are used in the agglutination test to detect the presence of antibodies in patients' sera which are elucidated in response to infection by these bacteria. There are some agglutinins that are produced in the patient's serum during the fever period, which react with somatic antigen O of *Salmonella typhi*, A or B of *Salmonella paratyphi* and then with flagellar antigen H which is common in most of the *Salmonella* species. In this test four specific antigen suspensions are used e.g. *Salmonella typhi (* H antigen), *Salmonella typhi* (O antigen), *Salmonella paratyphi* - A and *Salmonella paratyphi* - B. If agglutination occurs with O antigen then it is considered positive for*Salmonella typhi*. Ifagglutinationoccurs inAorBantigenthenitisconfirmedaspositivefor*Salmonella paratyphi*.

**O Antigen**: This is a somatic antigen and is present on the outer membrane of the cell. Its specificity is determined by the nature of the repeating units in the outer O-polysaccharide chain. Somatic antigens are heat stable, alcohol resistant and forms compact and granular clumps whenmixedwithOantisera.

**Vi antigens**: This is a virulence antigen which is a capsular polysaccharide that overlays the O antigen. This capsule is not necessary for infection butit increases the infectivity by making it

less detectable by the body's immune system. It is heat labile and can be detected using Vi antisera. Vi antigencaninterferewithOantigen testing.

**H Antigens**: This is a heat labile flagellar antigen which is inactivated both by boiling and alcohol. H antigens rapidly form fluffy clumps when treated with the corresponding antisera. H antigen inducesrapidformationof corresponding antibodies as it is strongly immunogenic.

**Procedure:**

1. Before starting the experiment, bring allreagents to roomtemperature and mix well.
2. MarkthecirclesofslidesasPC(Positivecontrol), NC(Negativecontrol),O,H,AH,BHas perantigen solutions used for testing (as shown in figure 2).
3. Add a drop (25 µl) of positive control into the circle marked as PC of given glass slide.
4. Then add 25 µlof negative control into the reaction circle marked as NC.
5. Add 25 µl of test sample into each reaction circle labeled as O, H, AH, BH according to given antigen solution.
6. Add25 µlof Antigensolutionof*Salmonella typhi* 'H'into PCandNC circle each. Mix wellusingnew mixing stick for each circle.
7. To circles labeled as O, H, AH, BH in which test samples have been added, add antigen solutions of *Salmonella typhi* 'O', *Salmonella typhi* 'H', *Salmonella paratyphi* 'AH' and *Salmonella paratyphi* 'BH', respectively.
8. Mix the content of each reaction circle uniformly with separate mixing stick.
9. Rockthe glass slide gently(approximately for one minute) and observe for agglutination.

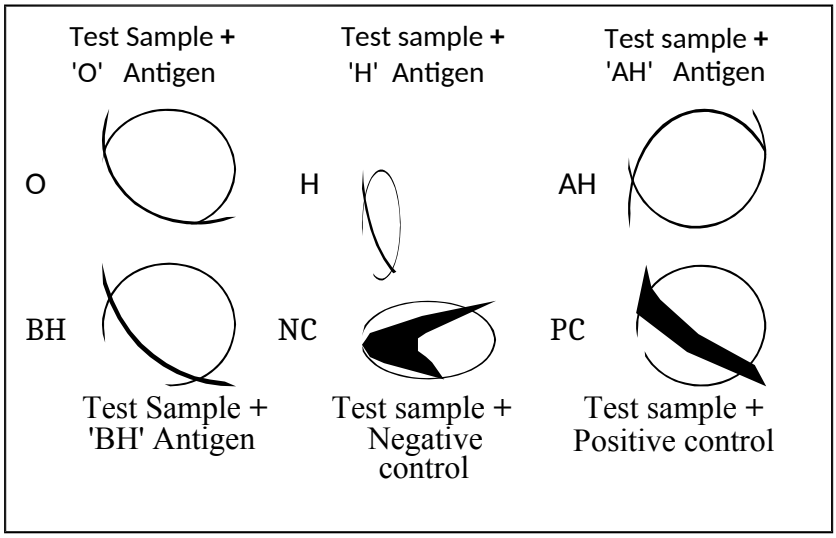



**Fig 2: Diagrammatic representation of Widal Slide test**

## EXP No. 4 Electron microscope

Electron microscope constructed by Ernst Ruska in 1933

An **electron microscope** is a microscope that uses a beam of accelerated electrons as a source of illumination. As the wavelength of an electron can be up to 100,000 times shorter than that of visible light photons, electron microscopes have a higher resolving power than light microscopes and can reveal the structure of smaller objects. A scanning transmission electron microscope has achieved better work than 50 pm resolution in annular dark-field imaging mode[1] and magnifications of up to about 10,000,000× whereas most light microscopes are limited by diffraction to about 200 nm resolution and useful magnifications below 2000×.

Electron microscopes use shaped magnetic fields to form electron optical lens systems that are analogous to the glass lenses of an optical light microscope.

Electron microscopes are used to investigate the ultrastructure of a wide range of biological and inorganic specimens including microorganisms, cells, large molecules, biopsy samples, metals, and crystals. Industrially, electron microscopes are often used for quality control and failure analysis. Modern electron microscopes produce electron micrographs using specialized digital cameras and frame grabbers to capture the images.

In 1926 Hans Busch developed the electromagnetic lens.

According to Dennis Gabor, the physicist Leó Szilárd tried in 1928 to convince him to build an electron microscope, for which he had filed a patent. The first prototype electron microscope, capable of four-hundred-power magnification, was developed in 1931 by the physicist Ernst Ruska and the electrical engineer Max Knoll. The apparatus was the first practical demonstration of the principles of electron microscopy. In May of the same year, Reinhold Rudenberg, the scientific director of Siemens-Schuckertwerke, obtained a patent for an electron microscope. In 1932, Ernst Lubcke of Siemens & Halske built and obtained images from a prototype electron microscope, applying the concepts described in Rudenberg's patent.

In the following year, 1933, Ruska built the first electron microscope that exceeded the resolution attainable with an optical (light) microscope. Four years later, in 1937, Siemens financed the work of Ernst Ruska and Bodo von Borries, and employed Helmut Ruska, Ernst's brother, to develop applications for the microscope, especially with biological specimens. Also in
1937, Manfred von Ardenne pioneered the scanning electron microscope. Siemens produced the first commercial electron microscope in 1938. The first North American electron microscope was constructed in 1938, at the University of Toronto, by Eli Franklin Burton and students Cecil Hall, James Hillier, and Albert Prebus. Siemens produced a transmission electron microscope (TEM) in 1939. Although current transmission electron microscopes are capable of two million-power magnification, as scientific instruments, they remain based upon Ruska's prototype.

### Transmission electron microscope (TEM)

The original form of the electron microscope, the transmission electron microscope (TEM), uses a high voltage electron beam to illuminate the specimen and create an image. The electron beam is produced by an electron gun, commonly fitted with a tungsten filament cathode as the electron source. The electron beam is accelerated by an anode typically at +100 keV (40 to 400 keV) with respect to the cathode, focused by electrostatic and electromagnetic lenses, and transmitted through the specimen that is in part transparent to electrons and in part scatters them out of the beam. When it

emerges from the specimen, the electron beam carries information about the structure of the specimen that is magnified by the objective lens system of the microscope. The spatial variation in

this information (the "image") may be viewed by projecting the magnified electron image onto a fluorescent viewing screen coated with a phosphor or scintillator material such as zinc sulfide.

Alternatively, the image can be photographically recorded by exposing a photographic film or plate directly to the electron beam, or a high-resolution phosphor may be coupled by means of a lens optical system or a fibre optic light-guide to the sensor of a digital camera. The image detected by the digital camera may be displayed on a monitor or computer.

The resolution of TEMs is limited primarily by spherical aberration, but a new generation of hardware correctors can reduce spherical aberration to increase the resolution in high-resolution transmission electron microscopy (HRTEM) to below 0.5 angstrom (50 picometres), [1] enabling magnifications above 50 million times. The ability of HRTEM to determine the positions of atoms within materials is useful for nano-technologies research and development.

Transmission electron microscopes are often used in electron diffraction mode. The advantages of electron diffraction over X-ray crystallography are that the specimen need not be a single crystal or even a polycrystalline powder, and also that the Fourier transform reconstruction of the object's magnified structure occurs physically and thus avoids the need for solving the phase problem faced by the X-ray crystallographers after obtaining their X-ray diffraction patterns.

One major disadvantage of the transmission electron microscope is the need for extremely thin sections of the specimens, typically about 100 nanometers. Creating these thin sections for biological and materials specimens is technically very challenging. Semiconductor thin sections can be made using a focused ion beam. Biological tissue specimens are chemically fixed, dehydrated and embedded in a polymer resin to stabilize them sufficiently to allow ultrathin sectioning. Sections of biological specimens, organic polymers, and similar materials may require staining with heavy atom labels in order to achieve the required image contrast.

**Serial-section electron microscopy (ssEM)**

One application of TEM is serial-section electron microscopy (ssEM), for example in analyzing the connectivity in volumetric samples of brain tissue by imaging many thin sections in sequence.

**Scanning electron microscope (SEM)**

The SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). When the electron beam interacts with the specimen, it loses energy by a variety of mechanisms. The lost energy is converted into alternative forms such as heat, emission of low-energy secondary electrons and high-energy backscattered electrons, light emission (cathodo luminescence) or X-ray emission, all of which provide signals carrying information about the properties of the specimen surface, such as its topography and composition. The image displayed by an SEM maps the varying intensity of any of these signals into the image in a position corresponding to the position of the beam on the specimen when the signal was generated. In the SEM image of an ant shown below and to the right, the image was constructed from signals produced by a secondary electron detector, the normal or conventional imaging mode in most SEMs.

**Reflection electron microscope (REM)**

In the **reflection electron microscope** (REM) as in the TEM, an electron beam is incident on a surface but instead of using the transmission (TEM) or secondary electrons (SEM), the reflected

beam of elastically scattered electrons is detected. This technique is typically coupled with reflection high energy electron diffraction (RHEED) and *reflection high-energy loss spectroscopy (RHELS)*. Another variation is spin-polarized low-energy electron microscopy (SPLEEM), which is used for looking at the microstructure of magnetic domains.

**Scanning transmission electron microscope (STEM)**

The STEM rasters a focused incident probe across a specimen that (as with the TEM) has been thinned to facilitate detection of electrons scattered *through* the specimen. The high resolution of the TEM is thus possible in STEM. The focusing action (and aberrations) occur before the electrons hit

the specimen in the STEM, but afterward in the TEM. The STEMs use of SEM-like beam rastering simplifies annular dark-field imaging, and other analytical techniques, but also means that image data is acquired in serial rather than in parallel fashion. Often TEM can be equipped with the scanning option and then it can function both as TEM and STEM.

**Scanning tunneling microscopy (STM)**

In STM, a conductive tip held at a voltage is brought near a surface, and a profile can be obtained based on the tunneling probability of an electron from the tip to the sample since it is a function of distance.

In some configurations information about several specimen properties is gathered per pixel, usually by the use of multiple detectors. In SEM, the attributes of topography and material contrast can be obtained by a pair of backscattered electron detectors and such attributes can be superimposed in a single color image by assigning a different primary color to each attribute. Similarly, a combination of backscattered and secondary electron signals can be assigned to different colors and superimposed on a single color micrograph displaying simultaneously the properties of the specimen.

Some types of detectors used in SEM have analytical capabilities, and can provide several items of data at each pixel. Examples are the Energy-dispersive X-ray spectroscopy (EDS) detectors used in elemental analysis and Cathodoluminescence microscope (CL) systems that analyse the intensity and spectrum of electron-induced luminescence in (for example) geological specimens. In SEM systems using these detectors, it is common to color code the signals and superimpose them in a single color image, so that differences in the distribution of the various components of the specimen can be seen clearly and compared. Optionally, the standard secondary electron image can be merged with the one or more compositional channels, so that the specimen's structure and composition can be compared. Such images can be made while maintaining the full integrity of the original signal, which is not modified in any way.

The technique required varies depending on the specimen and the analysis required:

- *Chemical fixation* – for biological specimens aims to stabilize the specimen's mobile macromolecular structure by chemical crosslinking of proteins with aldehydes such as formaldehyde and glutaraldehyde, and lipids with osmium tetroxide.
- *Negative stain* – suspensions containing nanoparticles or fine biological material (such as viruses and bacteria) are briefly mixed with a dilute solution of an electron-opaque solution such as ammonium molybdate, uranyl acetate (or formate), or phosphotungstic acid. This mixture is applied to a suitably coated EM grid, blotted, then allowed to dry. Viewing of this preparation in the TEM should be carried out without delay for best results. The method is important in microbiology for fast but crude morphological identification, but can also be used as the basis for high-resolution 3D

reconstruction using EM tomography methodology when carbon films are used for support. Negative staining is also used for observation of nanoparticles.

- *Cryofixation* – freezing a specimen so rapidly, in liquid ethane that the water forms vitreous (non- crystalline) ice. This preserves the specimen in a snapshot of its solution state. An entire field called cryo-electron microscopy has branched from this technique. With the development of cryo- electron microscopy of vitreous sections (CEMOVIS), it is now possible to observe samples from virtually any biological specimen close to its native state.[*citation needed*]
- *Dehydration* – or replacement of water with organic solvents such as ethanol or acetone, followed by critical point drying or infiltration with embedding resins. Also freeze drying.
- *Embedding, biological specimens* – after dehydration, tissue for observation in the transmission electron microscope is embedded so it can be sectioned ready for viewing. To do this the tissue is passed through a 'transition solvent' such as propylene oxide (epoxypropane) or acetone and then infiltrated with an epoxy resin such as Araldite, Epon, or Durcupan; tissues may also be embedded
- directly in water-miscible acrylic resin. After the resin has been polymerized (hardened) the sample is thin sectioned (ultrathin sections) and stained – it is then ready for viewing.
- *Embedding, materials* – after embedding in resin, the specimen is usually ground and polished to a mirror-like finish using ultra-fine abrasives. The polishing process must be performed carefully to minimize scratches and other polishing artifacts that reduce image quality.
- *Metal shadowing* – Metal (e.g. platinum) is evaporated from an overhead electrode and applied to the surface of a biological sample at an angle. The surface topography results in variations in the thickness of the metal that are seen as variations in brightness and contrast in the electron microscope image.
- *Replication* – A surface shadowed with metal (e.g. platinum, or a mixture of carbon and platinum) at an angle is coated with pure carbon evaporated from carbon electrodes at right angles to the surface. This is followed by removal of the specimen material (e.g. in an acid bath, using enzymes or by mechanical separation[21]) to produce a surface replica that records the surface ultrastructure and can be examined using transmission electron microscopy.
- *Sectioning* – produces thin slices of the specimen, semitransparent to electrons. These can be cut on an ultramicrotome with a glass or diamond knife to produce ultra-thin sections about 60–90 nm thick. Disposable glass knives are also used because they can be made in the lab and are much cheaper.
- *Staining* – uses heavy metals such as lead, uranium or tungsten to scatter imaging electrons and thus give contrast between different structures, since many (especially biological) materials are nearly "transparent" to electrons (weak phase objects). In biology, specimens can be stained "en bloc" before embedding and also later after sectioning. Typically thin sections are stained for several minutes with an aqueous or alcoholic solution of uranyl acetate followed by aqueous lead citrate.
- *Freeze-fracture or freeze-etch* – a preparation method particularly useful for examining lipid membranes and their incorporated proteins in "face on" view. The fresh tissue or cell suspension is frozen rapidly (cryofixation), then fractured by breaking or by using a microtome while maintained at liquid nitrogen temperature. The cold fractured surface (sometimes "etched" by increasing the
- temperature to about −100 °C for several minutes to let some ice sublime) is then shadowed with evaporated platinum or gold at an average angle of 45° in a high vacuum evaporator. The second coat of carbon, evaporated perpendicular to the average surface plane is often performed to improve the stability of the replica coating. The specimen is returned to room temperature and pressure, then

- the extremely fragile "pre-shadowed" metal replica of the fracture surface is released from the underlying biological material by careful chemical digestion with acids, hypochlorite solution

or SDS detergent. The still-floating replica is thoroughly washed free from residual chemicals, carefully fished up on fine grids, dried then viewed in the TEM.

- *Freeze-fracture replica immunogold labeling (FRIL)* – the freeze-fracture method has been modified to allow the identification of the components of the fracture face by immunogold labeling. Instead of removing all the underlying tissue of the thawed replica as the final step before viewing in the microscope the tissue thickness is minimized during or after the fracture process. The thin layer of tissue remains bound to the metal replica so it can be immunogold labeled with antibodies to the structures of choice. The thin layer of the original specimen on the replica with gold attached allows the identification of structures in the fracture plane. There are also related methods which label the surface of etched cells and other replica labeling variations.
- *Ion beam milling* – thins samples until they are transparent to electrons by firing ions (typically argon) at the surface from an angle and sputtering material from the surface. A subclass of this is focused ion beam milling, where gallium ions are used to produce an electron transparent membrane in a specific region of the sample, for example through a device within a microprocessor. Ion beam milling may also be used for cross-section polishing prior to SEM analysis of materials that are difficult to prepare using mechanical polishing.
- *Conductive coating* – an ultrathin coating of electrically conducting material, deposited either by high vacuum evaporation or by low vacuum sputter coating of the sample. This is done to prevent the accumulation of static electric fields at the specimen due to the electron irradiation required during imaging. The coating materials include gold, gold/palladium, platinum, tungsten, graphite, etc.
- *Earthing* – to avoid electrical charge accumulation on a conductively coated sample, it is usually electrically connected to the metal sample holder. Often an electrically conductive adhesive is used for this purpose.

Scanning electron microscopes operating in conventional high-vacuum mode usually image conductive specimens; therefore non-conductive materials require conductive coating (gold/palladium alloy, carbon, osmium, etc.). The low-voltage mode of modern microscopes makes possible the observation of non-conductive specimens without coating. Non-conductive materials can be imaged also by a variable pressure (or environmental) scanning electron microscope.

Small, stable specimens such as carbon nanotubes, diatom frustules and small mineral crystals (asbestos fibres, for example) require no special treatment before being examined in the electron microscope. Samples of hydrated materials, including almost all biological specimens have to be prepared in various ways to stabilize them, reduce their thickness (ultrathin sectioning) and increase their electron optical contrast (staining). These processes may result in *artifacts*, but these can usually be identified by comparing the results obtained by using radically different specimen preparation methods. Since the 1980s, analysis of cryofixed, vitrified specimens has also become increasingly used by scientists, further confirming the validity of this technique.

## EXP No. 5 Malaria Parasite

### Malarial Parasite-Kit

Malarial Parasite-Kit is used for observation of malarial parasite in thick blood films.

**Composition**

| | |
|---|---|
| Methylene blue | 1.300 gm |
| Potassium phosphate | 6.250 gm |
| Disodium hydrogen phosphate | 5.000 gm |
| Fresh distilled water | 550.000 ml |

Field's Stain B(S009)

**Ingredients**

| | |
|---|---|
| Eosin | 1.300 gm |
| Disodium hydrogen phosphate | 5.000 gm |
| Potassium dihydrogen phosphate | 6.250 gm |
| Distilled water | 500.000 ml |

Formula adjusted, standardized to suit performance parameters

### Principle and Interpretation

Field Stains contain methylene blue and eosin. These basic and acidic dyes induce multiple colours when applied to cells. The fixative, methanol does not allow any further change in slide. The basic component of white cells (cytoplasm) is stained by acidic dye and they are described as eosinophilic or acidophilic. The acidic component (nucleus with nuclei acid) takes blue to purple shades of the basic dye and are called basophilic. The neutral component of the cells are stained by both the dyes. This staining method is used for screening thick films of malarial parasites.