

Lecture 31

Light Microscopy

Introduction: Light microscopy is the simplest form of microscopy. It has tools that are used to observe the small organisms or object and even macromolecules. It has wide variety of microscopic tools for studying the biomolecules and biological processes.. It includes all forms of microscopic methods that use electromagnetic radiation to achieve magnification.

Instrumentation of a typical light microscope-The typical diagram of a light microscope is given in the Figure 31.1. The light is produced by a lamp (with tungsten filament) as source and light rays are focused on the specimen by the condenser. The specimen is kept on the stage and fixed by clipped present on the side. The light diffracted by the sample is then collected by the objective lens (objective lens varies from 10x-100x magnification) and additional magnification is achieved by the eyepiece (usually gives additional 10x magnification). Hence, if you observe a sample with 40x objective lens, microscope is actually magnifying the object by 400x (40x from objective and 10x from the eye piece, $40 \times 10 = 400x$).

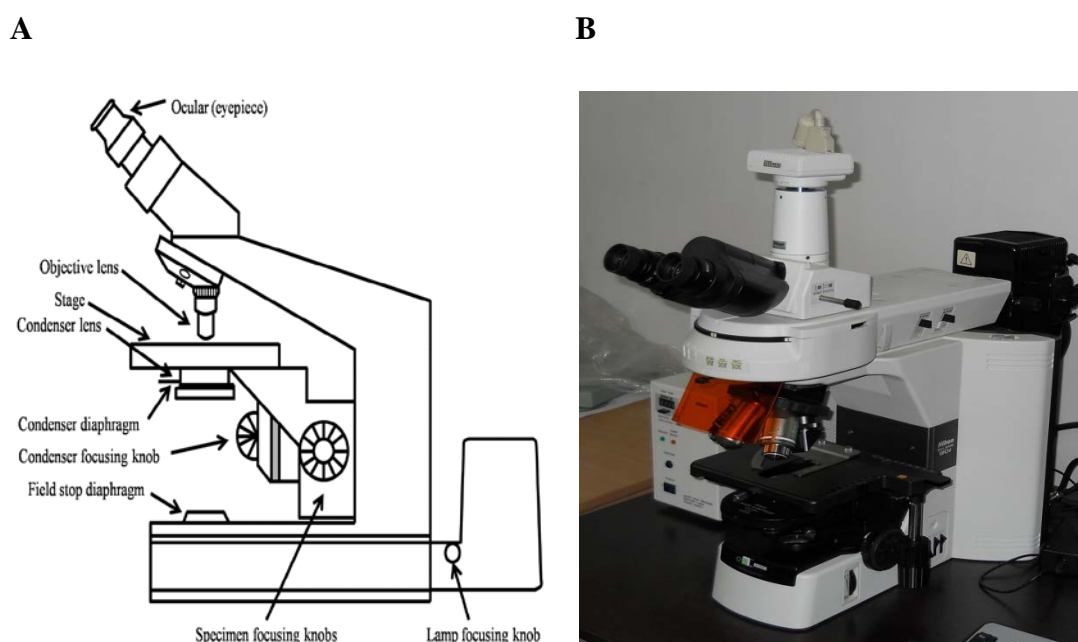


Figure 31.1: Instrumentation of a typical light (binocular) microscope with its different components. (A) Schematic Diagram and (B) Actual microscope

Light microscopes come in two designs: upright and inverted (Figure 31.2).

Upright microscope: In an upright microscope, the objective turret is usually fixed and the image is focused by moving the sample stage up and down.

Inverted microscope: In an inverted microscope, the sample stage is fixed and objective turret is moved up and down to focus the final image.

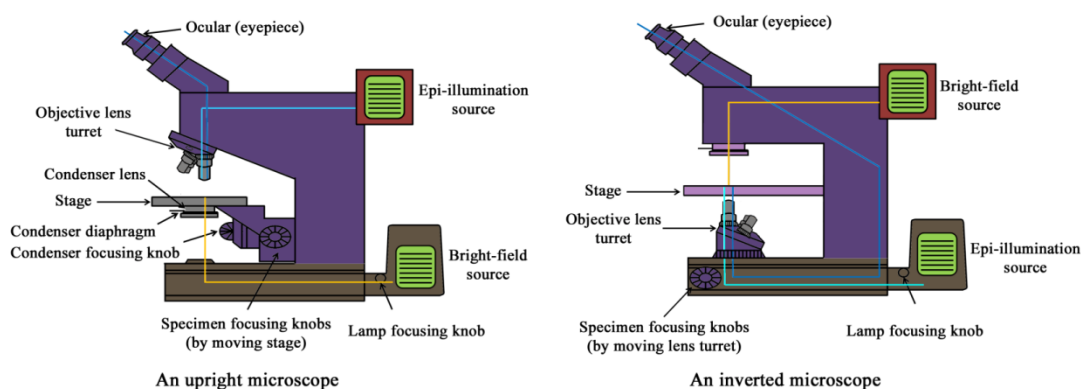


Figure 31.2 Designs of upright (A) and inverted (B) microscopes

Lab Experiment 30.1: Calculate the IC_{50} of chloroquine against malaria parasite in an in-vitro microscopic schizonticidal assay.

Background Information: Light microscope can be visualized the object in two different modes (bright field/dark field) and both of these modes give different information of an object. Both of these modes are extensively been used to perform multiple task in the biological research.

Bright-field microscopy: In a bright-field microscope, both diffracted (diffracted by the specimen) and undiffracted (light that transmits through the sample undeviated) lights are collected by the objective lens (Figure 30.3). The image of the specimen is therefore generated against a bright background, hence the name bright-field microscopy. Most biological samples are intrinsically transparent to the light resulting in poor contrast. To increase the contrast of the image, the specimens are therefore generally stained with the dyes.

Dark-field microscopy : Dark-field microscopy increases the contrast of the image by eliminating the undiffracted light. If there is no specimen in the optics path, no light is collected by the objective lens. Presence of specimen results in the diffraction

of light; the objective lens collects the diffracted light generating a bright image against a dark background.

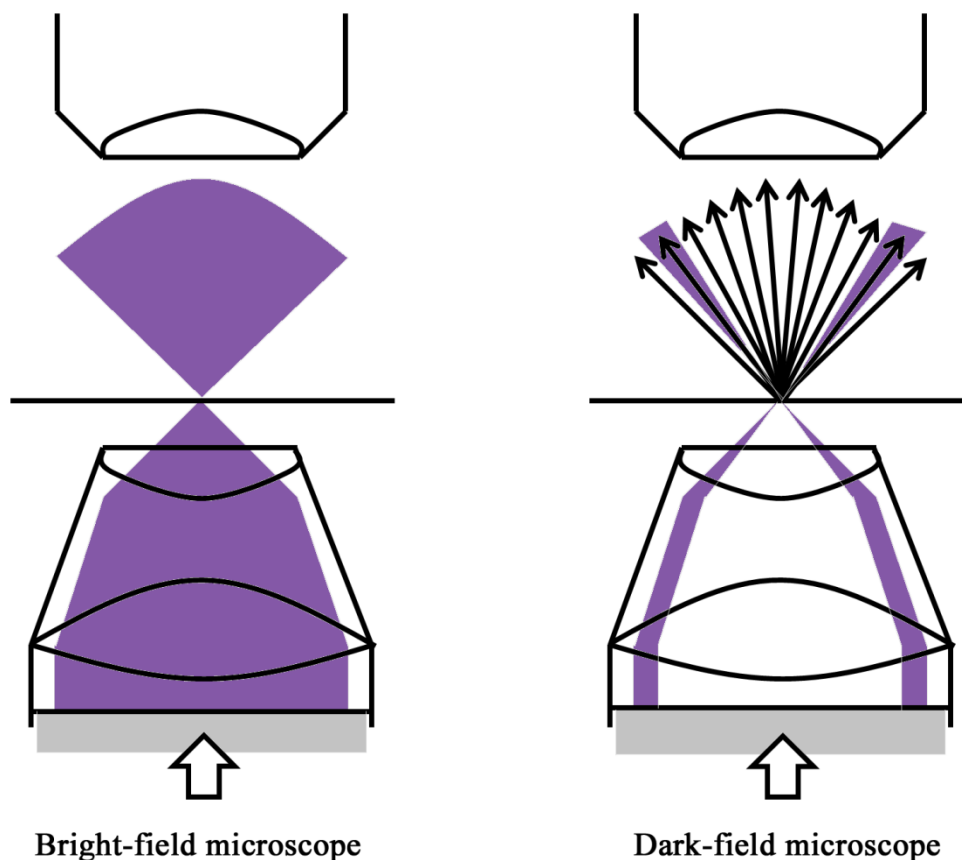


Figure 30.3: Optical diagrams of bright-field and dark-field microscopes

Material and Instruments:

1. RPMI 1640 cell culture media
2. Albumax-II
3. 0.22 μ m membrane filter
4. Filtration Unit
5. Autoclave
6. Vacuum Pump
7. Upright microscope

Procedure:

1. Culture of malaria parasite: malaria parasites are cultured by candle-jar method of Treger and Jensen. For detail procedure, student can follow it from the http://www.mr4.org/Portals/3/Pdfs/ProtocolBook/Methods_in_malaria_research.pdf.

The screening of candidate drug molecules can be performed against malaria parasite by multiple ways: **1. ^3H -Hypoxanthine uptake assay:** This is a radioactive assay to monitor the growth of the parasite. Malaria parasite synthesizes nucleic acid along with the growth of the parasite and DNA content of the culture is proportional

to the parasite load. During the nucleic acid synthesis, parasite takes up the hypoxanthine and it gets incorporated into the parasite DNA. Hypoxanthine is radioactive and the amount of radioactive associated can be used to assess the parasite.

2. SYBR Green I based Drug Sensitivity Assay: This is a fluorescence based assay to monitor the growth of the parasite. SYBR Green I is a nuclear stain used to visualize the parasite DNA. The amount of fluorescence is proportional to the nuclear content and the parasite number in the culture.

3. Microscopic schizonticidal assay: This is the conventional light microscopy based assay to screen compounds for antimalarial activity. During the intra-erythrocytic life-cycle, malaria parasite undergoes different stages, such as ring, trophozoite and schizont (Figure 30.4). During the life-cycle, it has ring stage (0-10hrs), trophozoite (11-32hrs) and schizont (32-40hrs) and then merozoites are produced to invade new RBCs to initiate another cycle. In the microscopy based assay, ring stage parasite containing RBCs are incubated with the test compound and then the parasite growth is monitored and number of schizonts are counted after 48hrs (Figure 30.5). Hence, this assay tests the effect of compounds on progression of the life-cycle of parasite and it is believed that the compounds inhibiting development of ring into the schizont stage may have potential to inhibit the growth of the parasite. With few modifications, the assay can be used to test the parasitostatic and parasicidal potential of the compounds. The complete details of the assay are as follows:

A. Synchronization of malaria parasite: This is the first step where parasite culture (mixture of stages of malaria parasite) is synchronized to the ring parasite containing RBCs. It has the following steps:

1. Take 4ml of a culture of >5% parasitemia.
2. Centrifuge the parasite culture at 720g for pellet down.
3. re-suspend the parasite pellet with 4ml of 5% sorbitol (in distilled water) and incubate for 10 min at room temperature. Mix and Shake it 2-3 times.
4. Centrifuge the culture at 720g and wash it 3 times with media and bring the parasite to the 5% hematocrit.
5. Repeat the step 1-4 after culturing the parasite after one cycle (approximately 48 hrs).
6. calculate the parasitemia after giemsa staining. The calculation of parasitemia is discussed later.

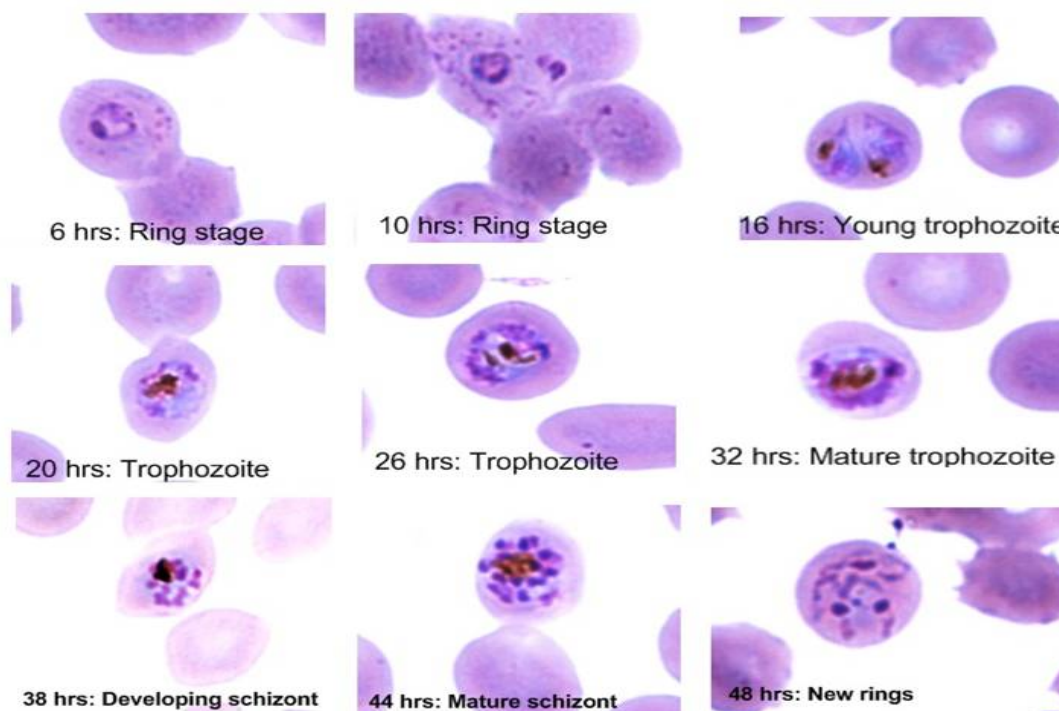


Figure 30.4: Different stages during intraerythrocytic life-cycle of malaria parasite.

B. Preparation of compound solution: The test compound can be dissolved in the organic solvent at a concentration of 5mg/ml. It is recommended to use DMSO as solvent has no significant effect on parasite growth.

C. Setup of the assay: Parasite culture synchronized at ring stage by D-sorbitol treatment brought to the 1% parasitemia with 3% hematocrit. In a total volume of 100µl, 50µl parasite culture is mixed with the various concentration of test compound (0, 1.5, 3.0, 6.25, 12.5, 25, 50µg/ml) in 25µl and remaining complete media. Chloroquine can be added as “**positive control**” and solvent as “**negative control**”. Incubate the compounds for 48hrs. Monitor the appearance of hemolysis or any such effect. If appeared, stop the assay and screen the compounds using other assay.

D. Monitoring the growth of parasite: After 48hrs, After exposure, smears were made. Parasitemia has been determined after JSB staining (Fields’ stain) using oil immersion objective.

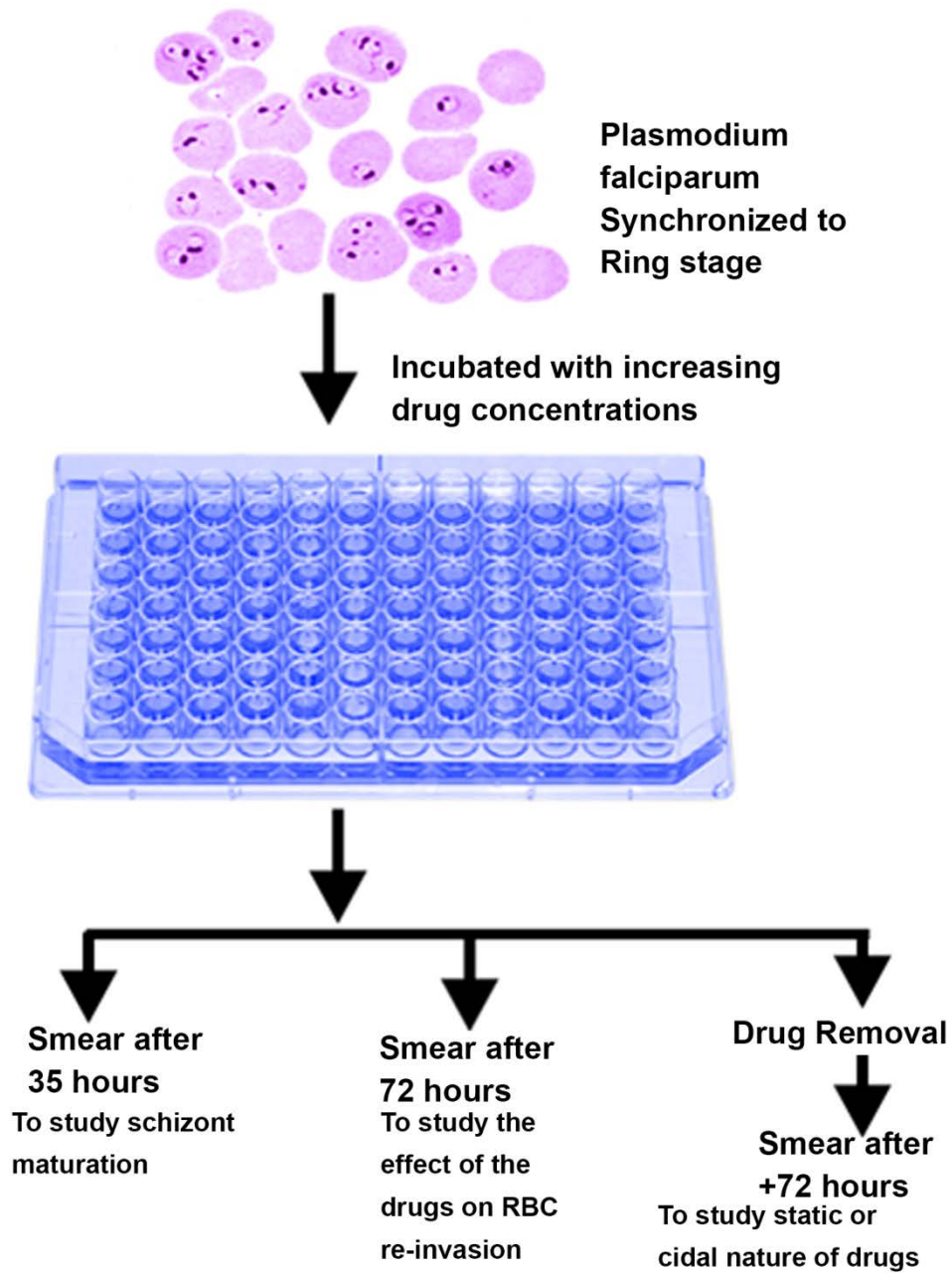


Figure 30.5: Over-view of the microscopy based antimalarial assay.

E. Observation: A typical observation of the drug treated parasite is given in the Figure 30.6.

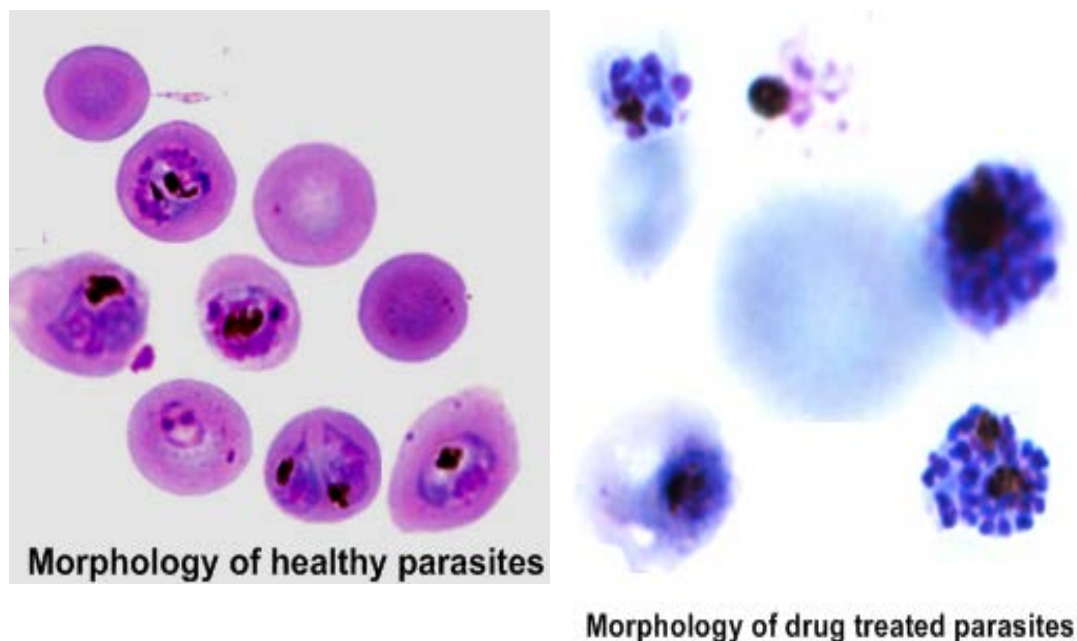


Figure 30.6: Differences in the cellular morphology of healthy parasites vs. drug treated parasites

F. Results and calculation of IC_{50} : The number of schizont containing RBCs were counted against each concentration. The schizont inhibition data from the in vitro in vitro schizont inhibition assays of the above compounds were fed into a specially pre-programmed excel sheet HN-NonLin available freely from (www.malaia.farch.net) . To determine the nature of action (parasitotatic/parasitocidal), in 100 μ l volume, 3 % haematocrit with 1 % parasites were exposed to trial compounds for 48 hours. After 48 hours, parasites were washed twice with complete media and again incubated for 48 hour in drug free media. Then smears were made and parasitemia has been determined microscopically.

Lecture 32

Microscopy-II

Lab Experiment 31.2 : Study the structural changes in the proliferative cells such as macrophages.

Background Information: A phase contrast microscope provides very high contrast as compared to the bright-field and dark-field microscopic methods. The image in a phase contrast microscope is generated from both diffracted and undiffracted lights as shown in Figure 32.1. Like dark-field microscopy, the specimen is illuminated by the light coming from a ring, called a condenser annulus. The diffracted and the undiffracted lights are separated in space allowing selective manipulation of their phases and intensities. The diffracted as well as the undiffracted light is collected by the objective lens. A phase plate is placed at the back side of the objective lens that increases the phase of the undiffracted light by $\frac{\lambda}{4}$ and decreases that of diffracted light by $\frac{\lambda}{4}$ as shown in Figure 32.1. A total phase difference of $\frac{\lambda}{2}$ is therefore obtained between the diffracted and the undiffracted light beams before they are focused on the image plane. As the intensity of the undiffracted light is very high, it is selectively reduced to ~30% of the initial intensity by a semi-transparent metallic film on the phase plate. Two waves that have $\frac{\lambda}{2}$ phase difference interfere destructively thereby diminishing the light intensity. Any phase change caused by the specimen is therefore converted into an amplitude signal by a phase contrast microscope thereby increasing the contrast.

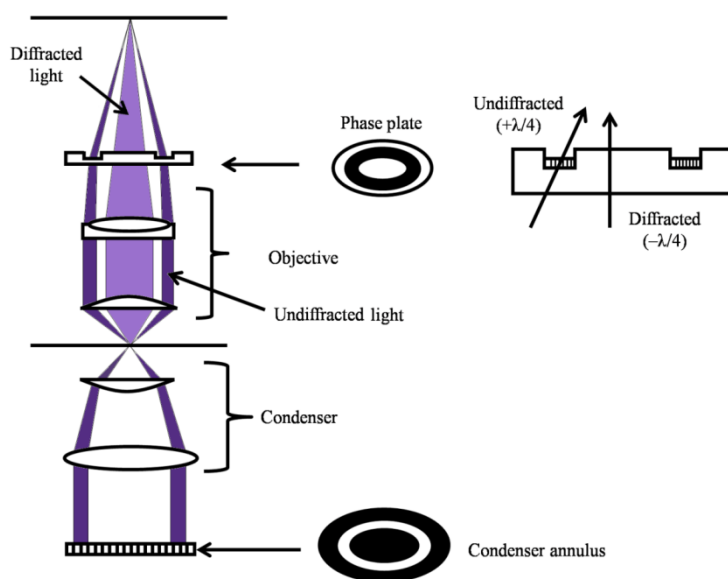


Figure 32.1 Optical diagram of a phase contrast microscope

Material and Instrument:

Glass Slides

Cover Slip

Inverted microscope with phase plate.

Observation: Take out the cells from the cell culture plate by trypsinization or by 0.5% EDTA in PBS. Place a small amount of cells on the glass slide and cover them with cover slip. Observe the cells under the 40x objective using inverted microscope with phase plate. A typical observation of healthy and abnormal macrophage is given in the Figure 32.2.

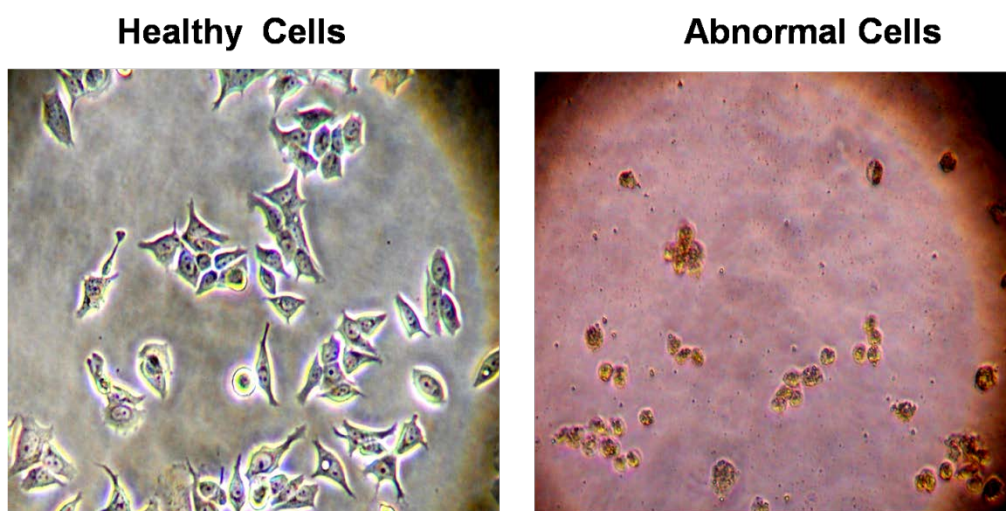


Figure 32.2 Observation of macrophage in the inverted phase microscope phase.

Conclusions: In a Typical observation, healthy cells will diffract the light rays and as a result cell membrane, nucleus and cytosol can be observed. Where as in the case of disease or damaged cells will show condensed nuclear content, several cell bodies or apoptotic bodies and scrambled membrane. The contrast of cytosol will not be very clear in the cells exposed to the cyto-toxic compounds.

Lab Experiment 31.3 : Determine the number of viable cell present in the cell culture using Trypan Blue exclusion method.

Material and Equipment

- 1. Glass Slides**
- 2. Cover Slip**
- 3. Inverted microscope with phase plate.**

4. Hemocytometer

Trypan Blue solution.

Protocol: Remove the cells from the cell culture plate by trypsinization or by 0.5% EDTA in PBS. Plate a small amount of cells on the glass slide and cover them with cover slip. Mix 50ml of cell suspension with the 50ml of trypan blue solution (0.4%) and fill the hemocytometer chamber. Observe the cells under the 20x objective using inverted microscope with phase plate. Trypan blue is a charged dye and viable cells exclude this dye to the presence of membrane potential where as dead cells (in the absence of membrane potential) accumulates the dye in the cytosol (Figure 32.3). Hence, viable cells appear colorless where as dead cells appear blue or dark colored. The hemocytometer is placed on the microscope stage and the cell suspension is counted. There is a "V" or notch at either end through which cell suspension is loaded into the hemocytometer. The cells are counted in the chambers and that gives the number of cells. In addition, blue colored cells can be counted to know the number of dead cells.

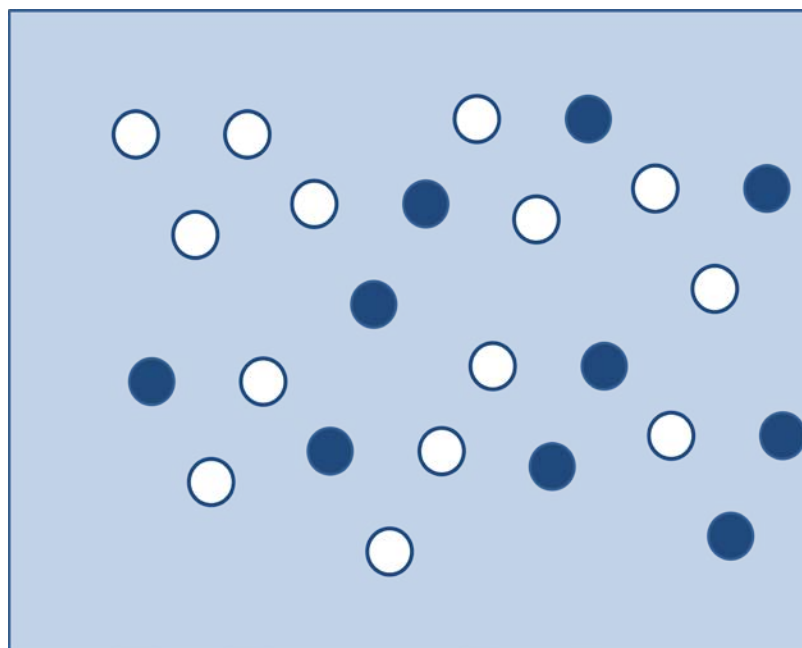
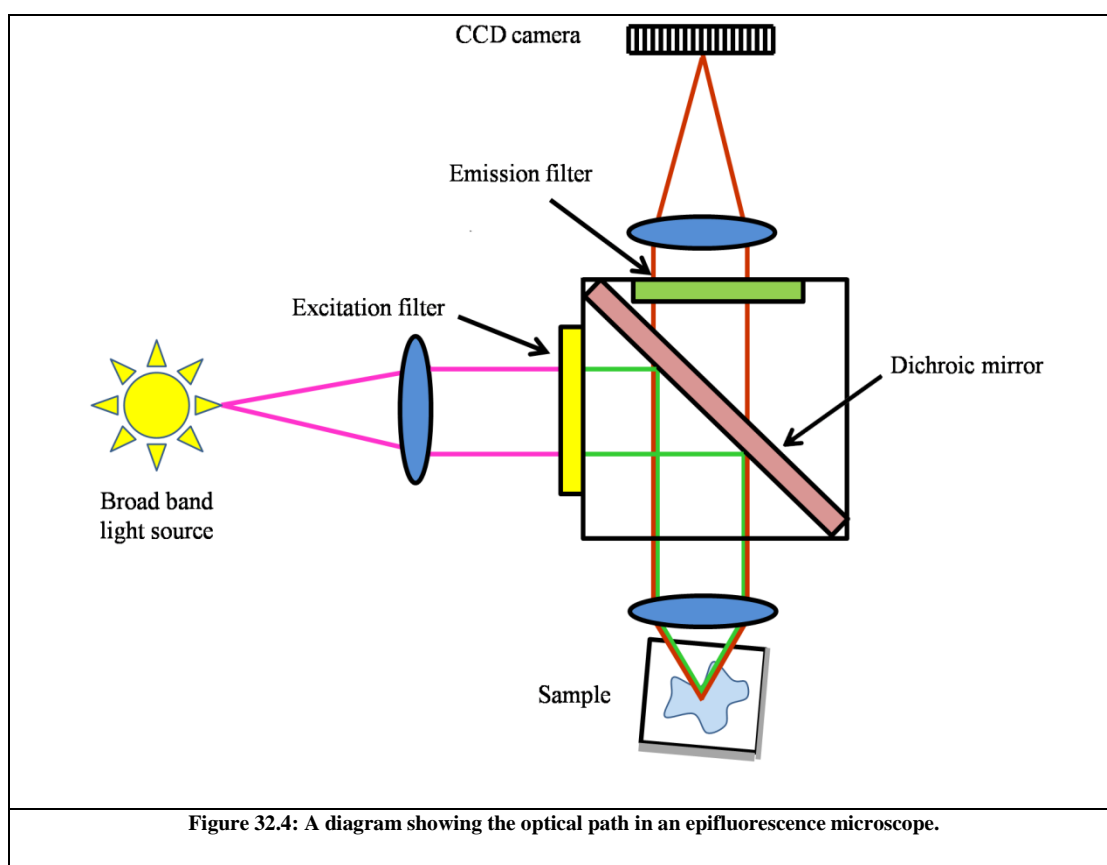
A**B**

Figure 32.3 Observation of cell suspension after trypan blue staining. (A) Viable cells appears colorless where as dead cells takesup dye and appear dark blue. (B) Hemocytometer

Lab Experiment 32.1 : Immuno-localization

Background Information: Unlike the other types of light microscopy that need special optics to enhance the contrast, fluorescence in visible region of electromagnetic radiation is directly detected. The cellular features, however, can be studied using extrinsic fluorescent probes that can go inside the cell and bind to the intracellular molecules with high specificity. The fluorescence emission of the dyes used in biological microscopy span the entire visible region of the electromagnetic spectrum. Optical diagram of an epifluorescence microscope is given in the Figure 32.4. In an epifluorescence microscope, the illumination of the specimen as well as the collection of the fluorescence light is achieved by a single lens. This has become possible due to the incorporation of dichroic mirror in the optics. A dichroic mirror is largely reflective for the light below a threshold wavelength and transmissive for the light above that wavelength.



The microscope has a high power lamp source, usually a mercury or xenon arc lamp. An excitation filter transmits the band of the excitation radiation. The excitation radiation is reflected by the dichroic mirror towards the condenser/objective lens that

focuses the light on the specimen. Light emitted by the fluorescent molecules (higher wavelength due to Stokes shift) is collected by the same lens and is transmitted by the dichroic mirror towards the ocular lens. Immunofluorescence, that makes use of the very high specificity of antibodies towards their targets, is a very useful method for studying cellular markers and organelles. Immunofluorescence microscopic analysis of cell surface markers is straightforward wherein the cells are treated with the fluorescently labeled antibodies and studied under microscope.

Materials and Instruments

1. Methanol

2. Acetone

3. PBS (1X)

4. 1% Triton X-100

5. BSA (Fat free, acetylated): Prepare 5% BSA solution in PBS and filter with the 0.45mm filter to remove particulate matter.

6. Primary antibody (anti-protein): An antibody can be developed against protein (antigen of interest) in rabbit or mice.

7. Secondary antibody: An antibody coupled with fluorescent marker (such as FITC) and directed against mouse IgG.

8. Epi-fluorescence microscope

Procedure

1. Fixation: This is the first steps and it is required for two purpose. (1) Stopping biological activity and (2) it stops the relative movement of cellular components and intracellular macromolecules. In addition, it reduces the damage to the cellular system and morphology. Fix the biological sample with Methanol: Acetone (7:3) mixture at -20⁰C for 15 min. Hydrate the sample with 1X PBS.

2. Permeabilization: Cell membrane is non-permeable to the charged as well as macromolecules. Only small molecule or hydrophobic dyes can pass through the membrane and reach to the inner compartments of the cell. Hence, cellular membrane needs to make porous by partially removing lipids from them. This process is known as permeabilization. Cells are permealized with 1% Triton X -100 for 15 min at room temperature.

3. Blocking: The intracellular spaces contains several antigenic sites and these need to block to reduce non-specific binding of the primary antibody. The cells are incubated with 5% BSA in 1X PBS for 15 min at room temperature. This step will allow masking of non-specific antigenic sites.

4. Primary Staining: Incubate the sample with primary antibody (1:50 in 2% BSA) for overnight at 4⁰C or 1hrs at 37⁰C. The primary staining at low temperature reduces the background signal and give good staining for sample where as staining at room temperature gives more amount of non-specific signal.

5. Washing : The primary antibody needs to wash to reduce the background signal. Sample is washed with 2% BSA prepared in PBS.

6. Seconadry Staining: Incubate the sample with secondary antibody (1:500 in 2% BSA) for overnight at 4⁰C or 1hrs at 37⁰C.

7. Washing: The secondary antibody needs to wash to reduce the background signal. Sample is washed with 2% BSA prepared in PBS.

8. Mounting: The sample is sensitive to the loss of water and needs to preserve in a mounting media containing glycerol. In addition, fluorescence signal is sensitive to the high laser beam and it require protection by adding antifading agent. In a typical mounting media, glycerol containing PPD is used to mount fluorescent sample.

9. Observation and visualization: Sample is fixed on the microscope stage and then observe under bright light to check the cells morphology by turning focusing knob. If the sample's morphology is good then it can observe under fluorescence channel (Figure 32.5).

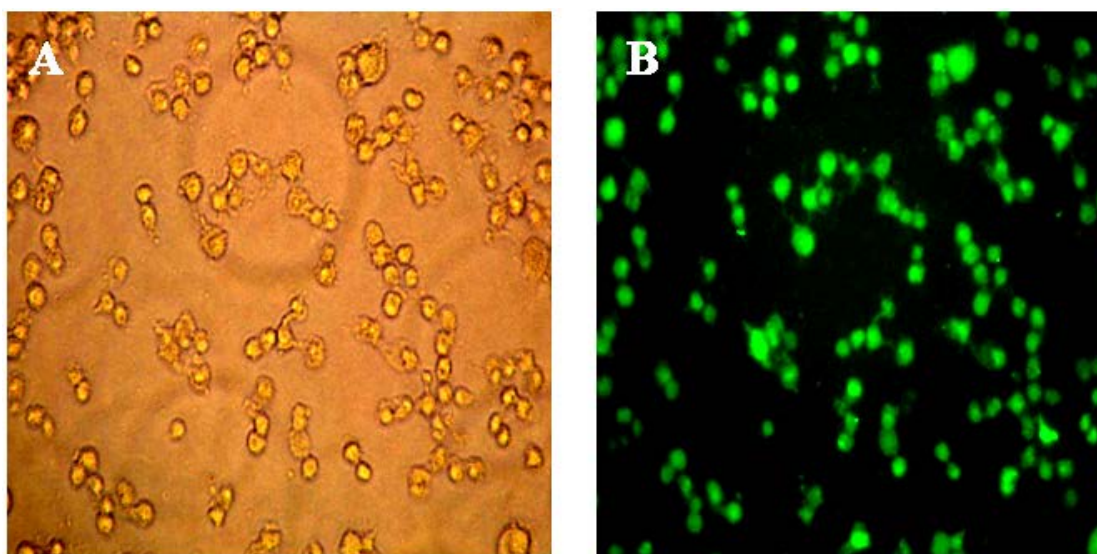


Figure 32.5: Bright-field (A) and epifluorescence (B) images of Cos-7 cells expressing GFP.

Lecture 33

Microscopy (Part-III)

Lab Experiment 33.1: Determine the Phagocytosis of beads in J774A.1 macrophage cells.

Material and equipments

1. Methanol
2. Acetone
3. PBS (1X)
4. 1% Triton X-100
5. **BSA (Fat free, acetylated):** Prepare 5% BSA solution in PBS and filter with the 0.45mm filter to remove particulate matter.
6. **Primary antibody (anti-protein):** An antibody can be developed against protein (antigen of interest) in rabbit or mice.
7. **Secondary antibody:** An antibody coupled with fluorescent marker (such as FITC) and directed against mouse IgG.
8. **mounting medium.**
9. **1 μ m Latex Beads**
10. **Filipin:** Prepare 5mg/ml stock solution of filipin in 100% alcohol. The working solution is 50 μ g/ml in PBS.
11. **Glass slides**
12. **Cover Glasses:** 12mm circular cover glasses available from the vendor are polished and are not suitable for cell attachment. Cover glasses are washed with alcohol and allow the cover glasses to air dry. Keep the cover glasses in a 50ml glass beaker and wrap with the aluminium foil. Autoclave the cover glasses to avoid contamination during phagocytosis experiment.
13. **Forcep:** Autoclave the forcep to avoid contamination during phagocytosis experiment.
14. **Epi-fluorescence microscope**

Procedure:

1. J774A.1 cells are cultured in the DMEM media containing 10% FBS and 1% antibiotics cocktails (pencillin/streptomycin sulphate).
2. Remove the cells from the cell culture plate by trypsinization or by 0.5% EDTA in PBS.
3. Plate 10,000 cells on 12mm cover glasses and incubate it in the 24 well dish with 0.5ml DMEM media containing FBS and antibiotic cocktail.
4. Incubate cells over night at 37⁰C and 5% CO₂ and it will allow the cells to attach to the cover glasses.

5. Wash the cells with DMEM without FBS media.
6. Prepare a suspension of latex beads (10^6 beads/ml) in DMEM without FBS media.
7. Remove media and add beads suspension to the well and centrifuge the 24 well dish at 1000rpm for 1mins at 4°C .
8. Incubate the plate for 1hrs at 37°C and 5% CO_2 .
9. Wash the well with 1ml DMEM without FBS media to remove uninternalized beads.
10. Fix the biological sample with Methanol: Acetone (7:3) mixture at -20°C for 15 min. Hydrate the sample with 1X PBS.
11. Stain the cells with filipin ($50\mu\text{g/ml}$) for 1hrs at 37°C in dark.
12. Keep one drop ($\sim 20\mu\text{l}$) of mounting medium (glycerol mounting media containing antifading agent) on the glass slide and keep the cover glass on it. Firm the cover glass by making a thick rim by nail polish.

Observation: Observe the cells in the bright field and look for the beads on the cells. Observe the cells in the fluorescence microscope with UV filter.

Results : A typical phagocytosis of bead will represent by the appearance of beads in the phase and the same bead will be circled by fluorescence (Figure 33.1).

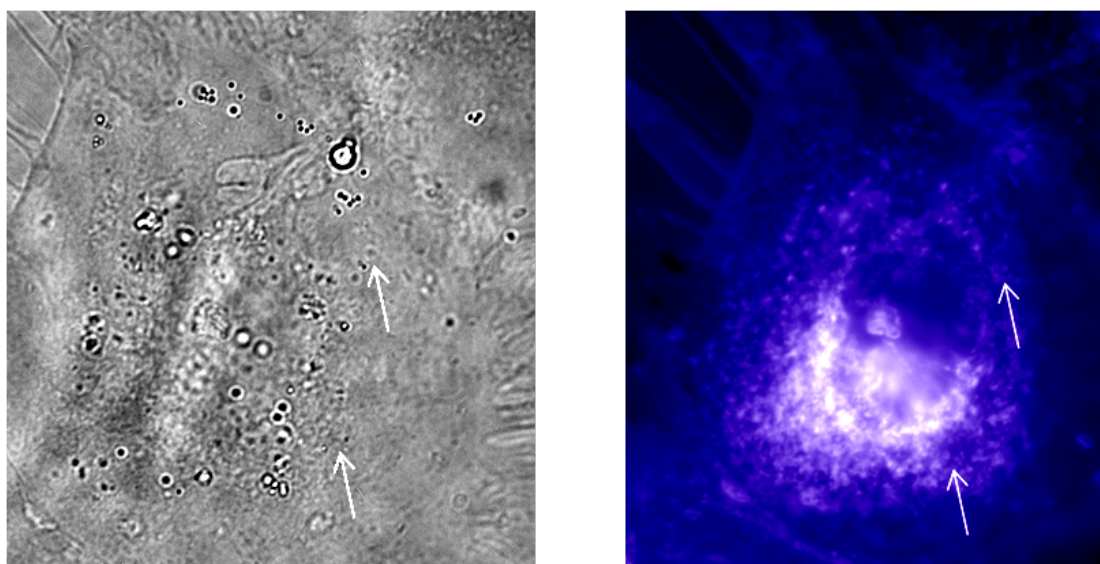


Figure 33.1 Observation of macrophages fed with latex beads after staining with filipin. Arrow indicates the position of phagocytosed beads.

Lab Experiment 33.2: Study the interaction between phagosome and lysosome in J774A.1 macrophage cells.

Material and Instrument

1. Methanol

2. Acetone

3. PBS (1X)

4. 1% Triton X-100

5. BSA (Fat free, acetylated): Prepare 5% BSA solution in PBS and filter with the 0.45mm filter to remove particulate matter.

6. Primary antibody (anti-protein): An antibody can be developed against protein (antigen of interest) in rabbit or mice.

7. Secondary antibody: An antibody coupled with fluorescent marker (such as FITC) and directed against mouse IgG.

8. Epi-fluorescence microscope

9. 1µm Latex Beads

10. Filipin: Prepare 5mg/ml stock solution of filipin in 100% alcohol. The working solution is 50µg/ml in PBS.

11. Rhodamine Dextran.

Procedure:

A. Identification of phagosome:

1. J774A.1 cells are cultured in the DMEM media containing 10% FBS and 1% antibiotics cocktails (penicillin/streptomycin sulphate).
2. Remove the cells from the cell culture plate by trypsinization or by 0.5% EDTA in PBS.
3. Plate 10,000 cells on 12mm cover glasses and incubate it in the 24 well dish with 0.5ml DMEM media containing FBS and antibiotic cocktail.
4. Incubate cells over night at 37⁰C and 5% CO₂ and it will allow the cells to attach to the cover glasses.
5. Wash the cells with DMEM without FBS media.
6. Prepare a suspension of latex beads (10⁶ beads/ml) in DMEM without FBS media.
7. Remove media and add beads suspension to the well and centrifuge the 24 well dish at 1000rpm for 1mins at 4⁰C.
8. Incubate the plate for 1hrs at 37⁰C and 5% CO₂.
9. Wash the well with 1ml DMEM without FBS media to remove unentrained beads.

10. Fix the biological sample with Methanol: Acetone (7:3) mixture at -20°C for 15 min. Hydrate the sample with 1X PBS.

11. Stain the cells with filipin ($50\mu\text{g/ml}$) for 1hrs at 37°C in dark.

12. Keep one drop ($\sim 20\mu\text{l}$) of mounting medium (glycerol mounting media containing antifading agent) on the glass slide and keep the cover glass on it. Firm the cover glass by making a thick rim by nail polish.

B. Labeling of Lysosome:

1. Plate cells on cover glasses in 24 well plate.

2. Grow them with $100\mu\text{g}$ rhodamine dextran O/N in DMEM + 10% FBS+1% antibiotics cocktails.

3. Wash the cells with PBS and chase for 1 Hrs in media without rhodamine dextran.

C. Fusion assay:

1. Add $10\mu\text{g/ml}$ $1\mu\text{M}$ latex/IgG beads in 0.5ml media and spin at 1000G for 2 min.

2. Incubate for another 5 min in 37°C in water bath.

3. Remove the beads and wash them two time with PBS at 37°C .

4. Media is removed and fixed with 4% paraformaldehyde.

5. Slide were visualized in fluorescence microcope.

D. Observation: Observe the cells in the bright field and look for the beads on the cells. Observe the cells in the fluorescence microscope with UV filter. If the bead has blue fluorescence, then the cells can be visualized through red channel.

E. Analysis: A typical phagocytosis of bead will represent by the appearance of beads in the phase and the same bead will be circled by blue fluorescence from filipin (Figure 33.2). If the bead has blue fluorescence ring, and it further encircled by red ring indicates interaction of lysosome and phagosome (Figure 33.2).

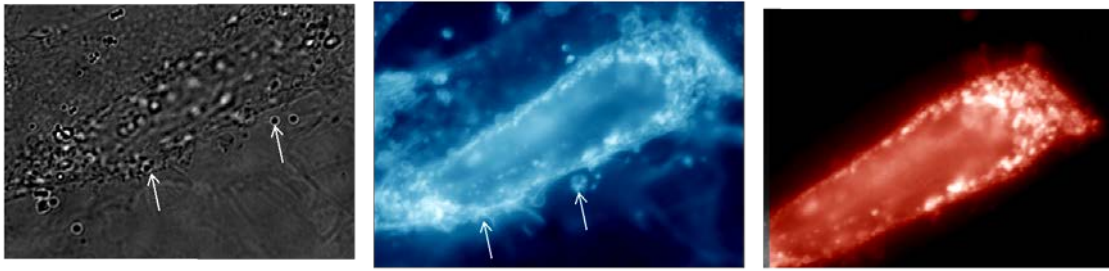


Figure 33.2 : Phagosome-lysosome interaction by fluorescence microscope. Arrow indicates the position of phagosome fused with lysosome.

Lecture 34

Electron Microscopy-I

Lab experiment 34.1: Preparation and analysis of the E.coli bacterium cells in scanning electron microscope.

Background Information: There are two basic models of the electron microscopes: *Scanning electron microscopes (SEM)* and *transmission electron microscopes (TEM)*. In a SEM, the secondary electrons produced by the specimen are detected to generate an image that contains topological features of the specimen. The image in a TEM, on the other hand, is generated by the electrons that have transmitted through a thin specimen. Let us see how these two microscopes work and what kind of information they can provide:

Scanning electron microscope: Figure 34.1 shows a simplified schematic diagram of a SEM. The electrons produced by the electron gun are guided and focused by the magnetic lenses on the specimen.

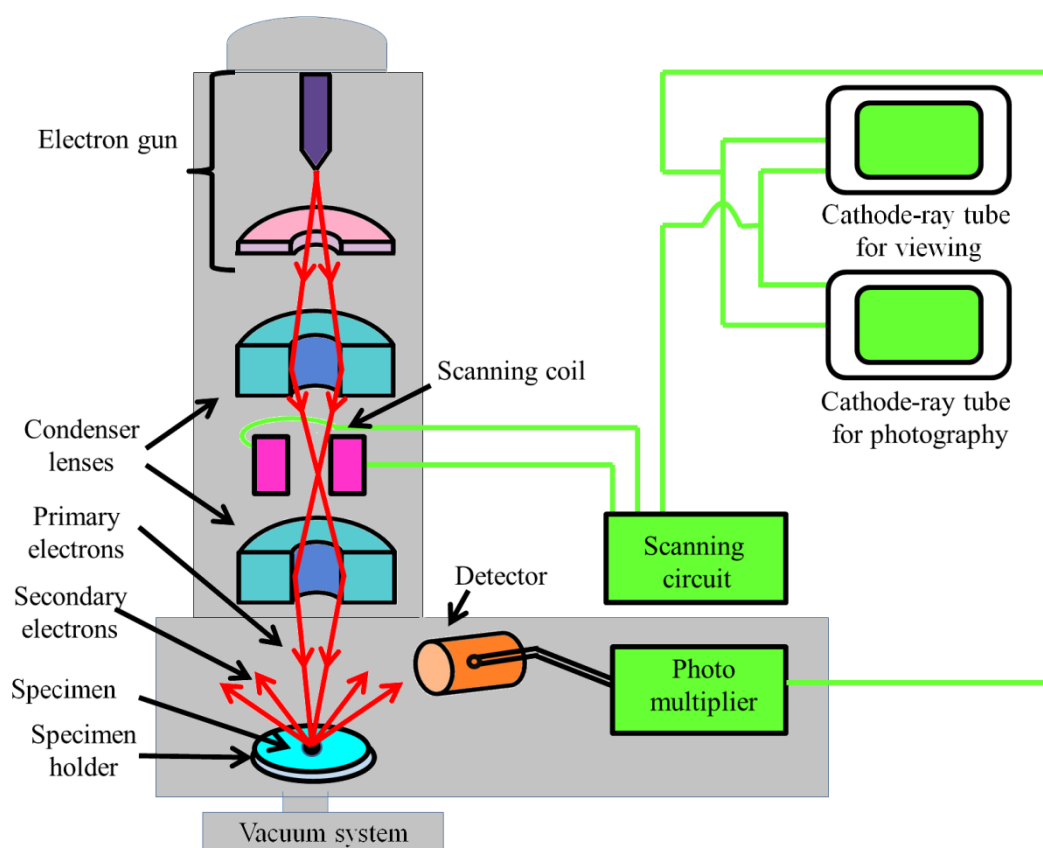


Figure 34.1 A simplified schematic diagram of a scanning electron microscope.

The focused beam of electrons is then scanned across the surface in a raster fashion. This scanning is achieved by moving the electron beam across the specimen surface by using deflection/scanning coils. The number of secondary electrons produced by the specimen at each scanned point are plotted to give a two dimensional image. In principle, any of the signals generated at the specimen surface can be detected. Most electron microscopes have the detectors for the secondary electrons and the backscattered electrons. As backscattered electrons come from a significant depth within the sample, they do not provide much information about the specimen topology. However, backscattered electrons can provide useful information about the composition of the sample; materials with higher atomic number produce brighter images.

Material and Equipment:

1. Ethanol
2. foil
3. E.Coli culture
4. Gold Sputter
5. Scanning Electron microscope
6. Dessicator

Step 1 Sample preparation: *The cells or non-biological material for SEM analysis needs to place on a small piece of aluminium foil.*

Step 2 Fixation: *Biological samples are fragile and fixation of biological sample is required for two purpose. (1) Stopping biological activity and (2) it stops the relative movement of cellular components and intracellular macromolecules. Sample is fixed with 2-5% glutaraldehyde and 1% osmium tetroxide in 50mM sodium cacodylate pH 7.4 for overnight at 4°C.*

Step 3 Dehydration: Biological samples are fragile and contains large amount of water. Water present in the biological sample diffract electron rays and may increase the background signal. Following osmium fixation, water is chemically extracted from the specimen using a graded series of ethanol.

It is performed in the following steps:

- Sample is incubated with 50% ethanol for 30mins.

- Sample is incubated with 70% ethanol for 30mins.
- Sample is incubated with 80% ethanol for 30mins.
- Sample is incubated with 90% ethanol for 30mins.
- Sample is incubated with 95% ethanol for 30mins.
- Sample is incubated with 100% ethanol for 30mins.
- Sample is incubated with anhydrous ethanol for 30mins.

Step 4 Drying: During dehydration, sample needs to prevent air drying. Once the dried material is removed, it needs to be stored in a desiccated environment until viewing.

Step 5: Specimen Coating: The specimen needs to be coated with the conductive material to view with scanning electron microscope. In the sputter, a discharge is formed between anode and cathode using a suitable gas (argon). The bombardment of gas ions on cathode material (usually gold) erode the target material and deposit it on the specimen (Figure 34.2). The target used in the sputter consists of 60% gold and 40% palladium. The uniform deposition of sputtered material (gold) form even coating on the surface of specimen and make the specimen conductive to perform scanning electron microscope.

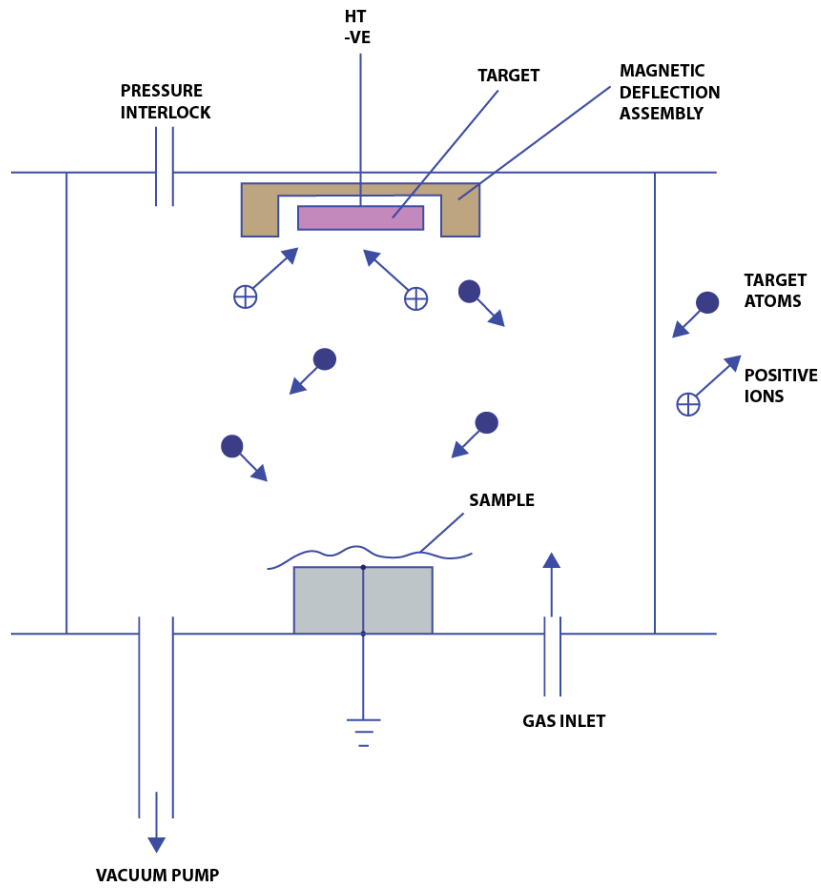


Figure 34.2 Mechanism of Sputter to coat specimen. [Kaushik redraw]

Observation: A typical Scanning electron microscopic image of E.coli Bacterium is given in the Figure 34.3.

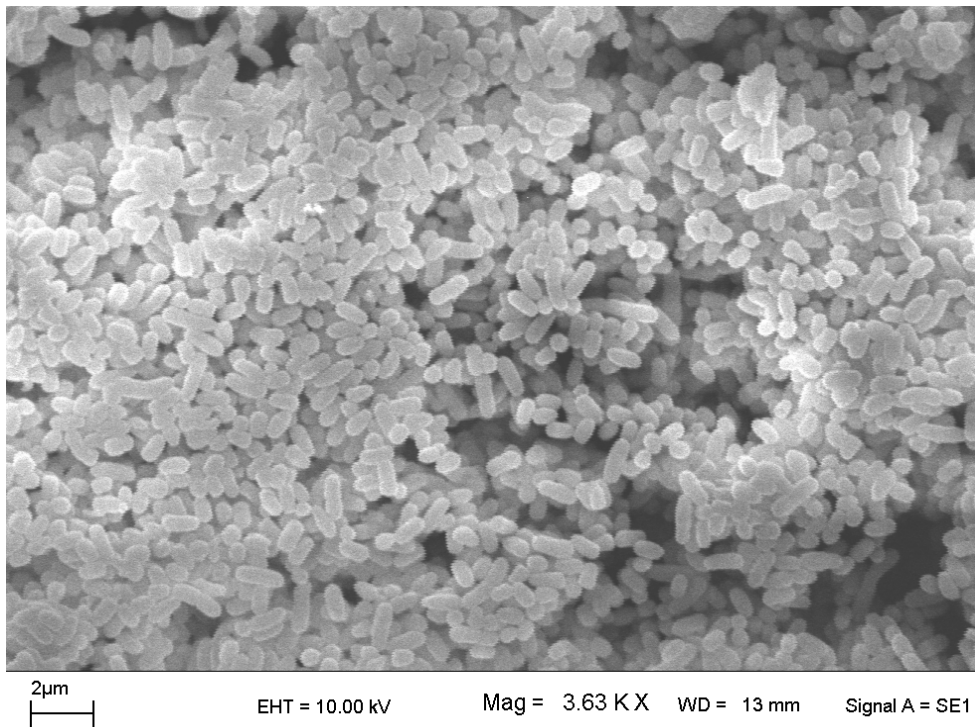


Figure 34.3 SEM image of *E.Coli* bacterium.

Lecture 35

Electron Microscopy-II

Lab Experiment 35.1 : Localize the protein inside the macrophage using transmission electron microscope.

Background Information: In the transmission electron microscope; the electrons were focused on a thin specimen and the electrons transmitted through the specimen were detected. Figure 35.1 shows a simplified optical diagram comparing a light microscope with a transmission electron microscope.

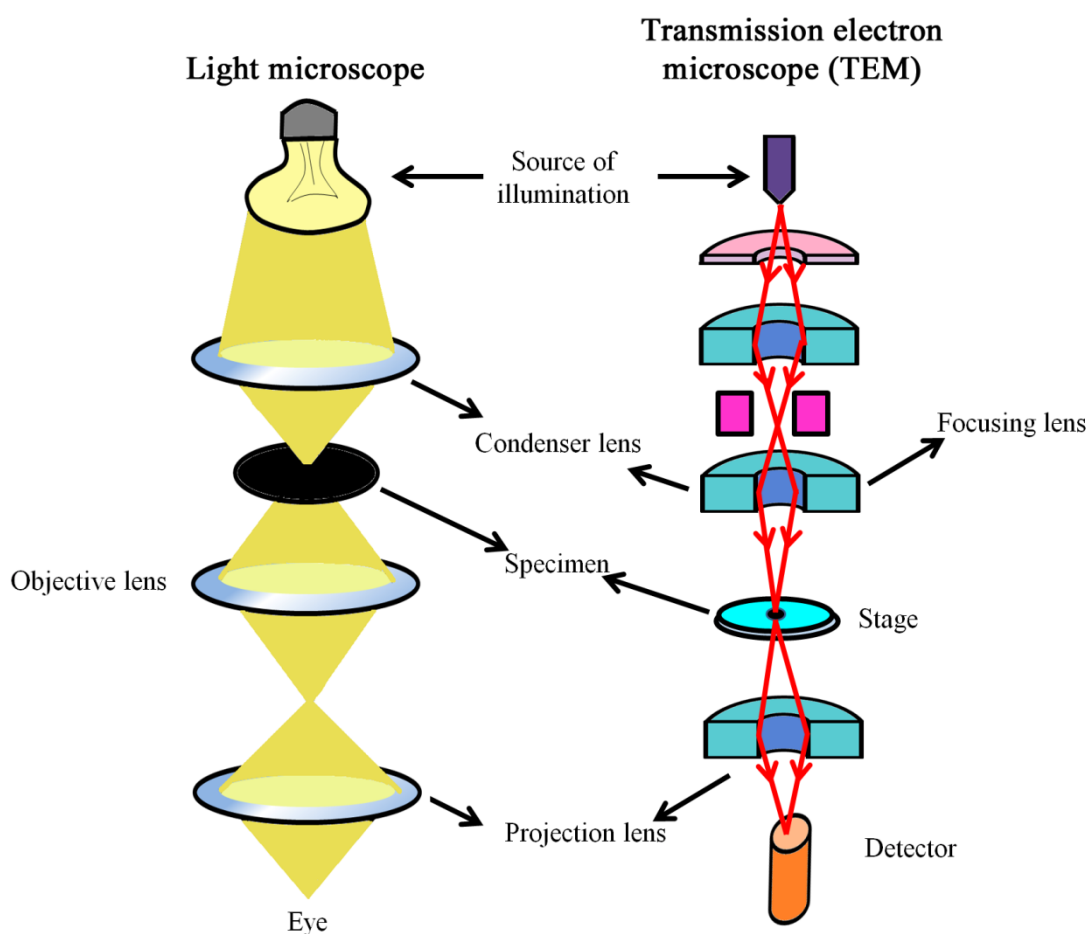


Figure 35.1: A simplified comparison of optics in a light microscope with that in a TEM.

Transmission electron microscopes usually have thermionic emission guns and electrons are accelerated anywhere between 40 – 200 kV potential. However, TEM with >1000 kV acceleration potentials have been developed for obtaining higher resolutions. Owing to their brightness and very fine electron beams, field emission guns are becoming more popular as the electron guns.

Material and Equipments

1. Paraformaldehyde
2. Glutaraldehyde
3. PBS (1X)
4. 1% Tween-20
5. BSA (Fat free, acetylated): Prepare 2% BSA solution in PBS and filter with the 0.45mm filter to remove particulate matter.
6. Primary antibody (anti-protein): An antibody can be developed against protein (antigen of interest) in rabbit or mice.
7. Secondary antibody: An antibody coupled with gold particle and directed against mouse IgG.
8. Uranyl acetate
9. Osmium tetroxide
10. Propylene oxide
11. Ethanol
12. Epon resin
13. LR white resin
14. Microtome
15. Transmission Electron microscope.

Procedure:

(A) **Fixation** : The samples for TEM are fixed by two different ways, (1) immersion or (2) perfusion. Fixation time, concentration of fixative agents depends on tissue thickness. It is performed in following steps:

1. Sample is incubated with 2% paraformaldehyde/2.5% glutaraldehyde in 50mM sodium cacodylate pH 7.4 for overnight at 4⁰C.
2. Post fixation, samples are incubated with 2% osmium tetroxide in 50mM sodium cacodylate pH 7.4 for overnight at 4⁰C.

(B) **Dehydration**: Biological samples are fragile and contains large amount of water. Water present in the biological sample diffract electron rays and may increase the background signal. Following osmium fixation, water is chemically extracted from the specimen using a graded series of ethanol.

It is performed in the following steps:

- Sample is incubated with 35% ethanol for 15 mins.
- Remove the solution and sample is incubated with 50% ethanol for 15 mins.
- Remove the solution and sample is incubated with 70% ethanol for 30mins.
- Remove the solution and sample is incubated with 95% ethanol for 15 mins.
- Remove the solution and sample is incubated with 100% ethanol for 15 mins.
- Remove the solution and sample is incubated with 100% ethanol for additional 30mins.
- Remove the solution and sample is incubated with propylene oxide for 15 mins.
- Remove the solution and sample is incubated with propylene oxide for additional 15 mins.

(C) Embedding : A thin (~10nm-100nm) section of the sample so that electron can pass through it to form an image. Biological samples are fragile and can not be processed to cut thin section. Hence, sample needs to be embedded into a solid matrix to cut the sections. There are two different matrix used to embedded the sample for sectioning purposes:

(i) Epon embedding

- Remove the solution and sample is incubated with propylene oxide: Epon resin (1:1) over night.
- Remove the solution and sample is incubated with fresh Epon 812 resin for additional 1-5 hrs.
- Dispense few μ l fresh epon 812 resin in polyethylene capsules and specimen is transferred into it.
- Place capsule at 60⁰C in hot oven for 48hrs.

(ii) LR white embedding

- Dehydrate the specimen in ethanol as described.

- Incubate tissue or cells for 30 min in mixture of Ethanol and LR white resin (1:1).
- Subsequently, incubate tissue or cells for 30 min in mixture of Ethanol and LR white resin (2:1).
- Incubate tissue or cells in LR white resin for 1hr. repeat this step 3 times.
- Finally leave the tissue or cells for overnight in 100% LR white resin.
- Fill the capsule with LR white containing tissue/cell and seal the capsule to allow polymerization of resin in 50⁰C oven overnight.

(D) Sectioning: Initially, thick sections are cut with the help of razor blade to reach the tissue in the block. Afterwards, block is mounted on the microtome and ultra thin (10-100nm) sections are cut and floated onto the water placed within the boat. Cut sections are stacked on each other in a definite pattern known as “**ribbon**”. These ribbons are collected on electron microscope grid.

(E) Staining to visualize cell structure: Incubate the grid in uranyl acetate for 2hrs and subsequently in lead citrate for additional 2hr. Uranyl acetate staining will allow to observe the cellular structure and to study the changes in the cellular or sub-cellular morphology.

(F) Immunogold labeling:

- Block the grid sample with 5% BSA for 45 mins at room temp.
- Incubate the grid in the primary antibody for 1hr at room temperature or overnight at 4⁰C in humified petridish.
[it is advisable to centrifuge primary antibody on full speed for 5mins to remove aggregated antibod].
- Dip the grid in PBS drop 3 times.
- Dip the grid in PBS containing tween 20 drop for 6times.
- Incubate the grid in the secondary antibody (antibody coupled to 10nm gold nanoparticle) for 1hr at room temperature or overnight at 4⁰C in humified petridish.
[it is advisable to centrifuge secondary antibody on full speed for 5mins to remove aggregated antibod].
- Dip the grid in drop of PBS containing tween-20 for 6 times.

- Dip the grid in PBS drop for 3times.
- Finally stain the grid again with uranyl acetate for 1min and lead acetate for 30sec.

Observation: A typical TEM image is given in the Figure 35.2. As you can observe that cell shows black dots which indicate the presence of secondary antibody.

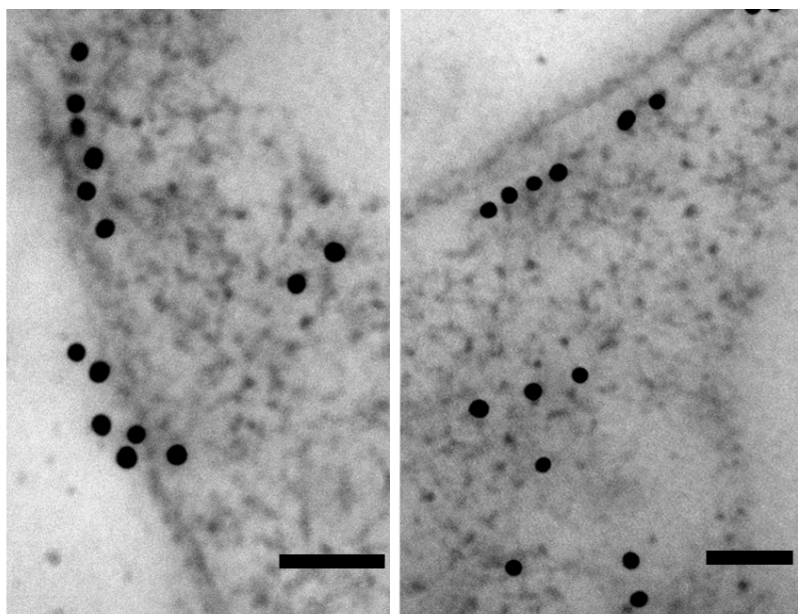


Figure 35.1: A typical image of eukaryotic cell observed with TEM. Gold particle, 10 nm; Bar, 50 nm

Laboratory experiment 35.2: Study the co-localization of two proteins in eukaryotic cell using TEM.

Material and equipment:

- All material and equipment related to the TEM experiment related to experiment 35.1.
- Primary antibody against protein-x generated in rabbit.
- Primary antibody against protein-x generated in mouse.
- Secondary antibody against rabbit coupled to 5nm gold particle.
- Secondary antibody against mouse coupled to 15nm gold particle.

Observation: A typical TEM image is given in the Figure 35.3. As you can observe that cell shows black dots with two different diameters (5 or 15nm) which indicate the presence of two proteins in the eukaryotic cell.

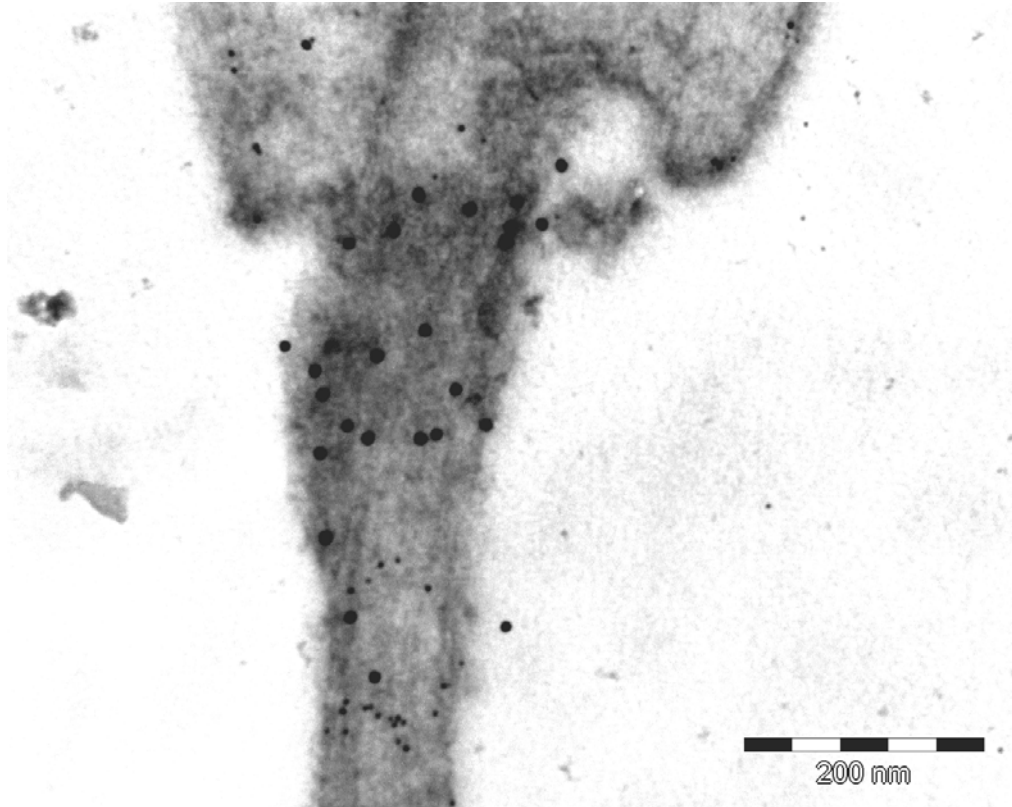


Figure 35.3: A typical image showing localization of two proteins in eukaryotic cell observed with TEM. Small gold particle, 5 nm and large particle 15nm; Bar, 200 nm

Lecture 36

Atomic Force Microscopy

Aim:

To study the nanotubes formed by diphenylalanine using atomic force microscopy

Introduction:

Atomic force microscopy belongs to the class of microscopic methods together known as scanning probe microscopy (SPM). The working principle of scanning probe microscopes is very different from conventional optical microscopes. An SPM scans the surface of the sample using a very fine pointed probe measuring one or more of the sample properties at each point. Atomic force microscope (AFM) is a scanning probe microscope that measures the force between the probe and the specimen.

An AFM has a cantilever (a cantilever is a beam fixed at only one end) that has a finely pointed probe, also referred to as the AFM tip, at its free end. The other end is anchored to a piezoelectric displacement actuator (Figure 36.1).

Attachment to the piezoelectric material allows precise positioning of the cantilever with respect to the specimen. For imaging, the probe is brought in close proximity to the specimen surface. Interaction (attractive or repulsive) between the probe and the specimen imposes a bending moment on the cantilever. Responding to this moment, the cantilever deflects towards or away from the specimen. The deflection of cantilever is detected using a laser beam that is focused on the cantilever, just above the probe. The back surface of the cantilever is highly reflective, the reflected beam is focused on a split photodiode (Figure 36.1). The cantilever is scanned across the specimen surface in a raster pattern. Any deflection in the cantilever as a result of sample interaction causes displacement in the laser spot on the photodiode; this displacement signal (difference in response in the upper and lower sectors of the split diode) is used to calculate the deflection in the cantilever.

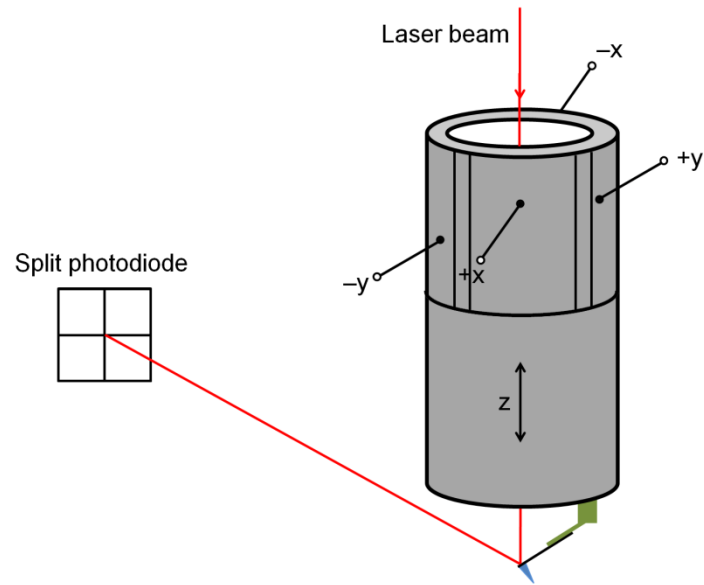


Figure 36.1: Diagrammatic representation of a cantilever attached to a piezoelectric tube. The laser beam falls on the back of the cantilever and gets reflected to hit the split photodiode detector.

Modes of AFM

Figure 36.2 shows the Lennard-Jones potential for a pair of atoms.

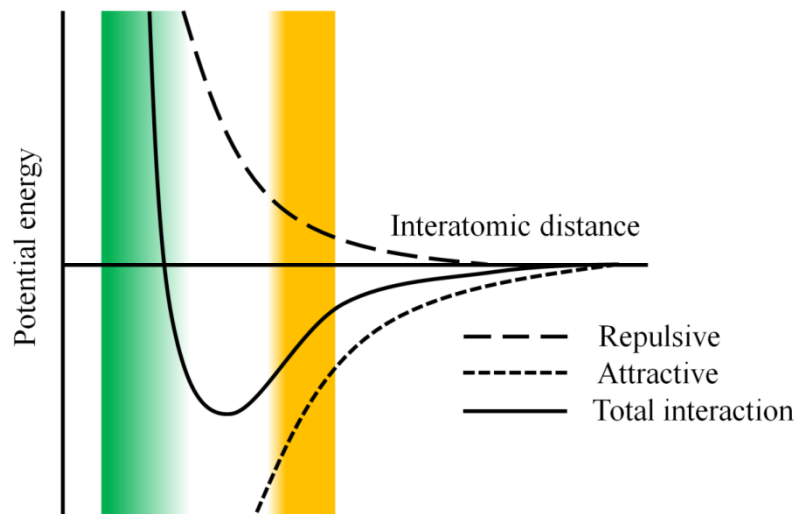


Figure 36.2: Lennard-Jones potential energy curve for two atoms

An AFM experiment can be performed in either attractive or repulsive regime of the Lennard-Jones potential. Depending on the working regime of the Lennard Jones potential, AFM imaging methods are divided into three basic modes:

Contact mode AFM: In contact mode AFM, the probe is brought in contact with the specimen surface; the interaction between the probe and the specimen, therefore is repulsive. As the tip is in contact with the sample, the frictional forces are very high during scanning. Contact mode imaging, therefore, may not be suitable for soft samples including biological samples.

Non-contact mode AFM: In non-contact mode AFM, a cantilever with very high spring constant is oscillated close to its resonance frequency. The probe does not contact the specimen and interacts with it through long range surface interactions. The forces between the probe and the specimen are very small, of the order of piconewtons. This mode, therefore, is well-suited for soft samples; resolution, however, is compromised.

Intermittent mode or tapping mode AFM: A stiff cantilever is oscillated close to its resonance frequency, at a probe-specimen separation that allows a small part of oscillation lie in the repulsive regime of the Lennard-Jones potential. The probe-specimen interaction therefore varies from long-range attraction to weak repulsion. The tip intermittently touches the sample while scanning. Interaction of the probe with the sample surface causes changes in the amplitude and the phase of oscillation. This mode of imaging allows imaging with very high resolution and has become the method of choice for scanning the soft biological samples.

In this experiment, we shall be using intermittent mode of imaging to study the ordered superstructures formed by a self-assembling peptide. In intermittent contact mode of imaging, user can define an amplitude set-point. This amplitude can be maintained using a feedback mechanism that moves the cantilever up or down to maintain the user defined vibrational amplitude. The cantilever displacement directly corresponds to the height of the specimen. In constant amplitude mode, the oscillating cantilever scans the sample, moving up/down to maintain the defined amplitude. A plot of cantilever displacement against the specimen coordinates generates the topographic image of the specimen surface.

Resolution

Atomic force microscopes can provide resolutions comparable to or even better than those obtained with electron microscopes. As images are not obtained using light or particles (such as electrons), resolution of AFM does not depend on any wavelength. The resolution of an AFM is determined by the diameter and the geometry of the probe. The influence the probe dimensions on the resolution is diagrammatically represented in figure 36.3.

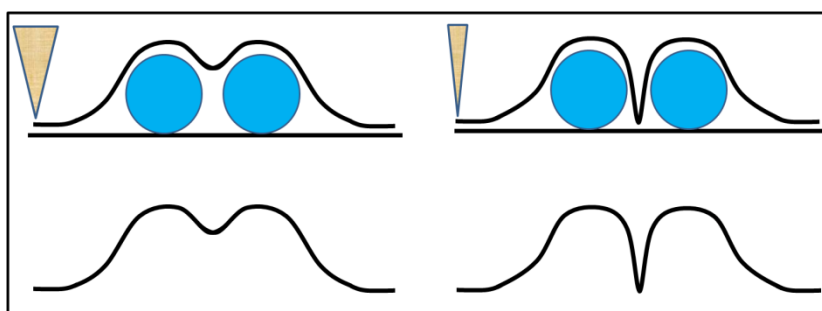


Figure 36.3 Effect of tip dimensions on the lateral resolution of an AFM.

It is evident that the resolution in the X-Y plane is poor and strongly depends on the probe dimensions. Resolution in Z-dimension (height), on the other hand, is very high; resolutions of ~ 0.2 nm or better are routinely achieved using high-resolution tips.

Materials

Equipments:

1. An atomic force microscope
2. Weighing balance

Reagents:

1. The peptide, diphenylalanine ($\text{NH}_2\text{-Phe-Phe-COOH}$)
 - a. Diphenylalanine ($\text{NH}_2\text{-Phe-Phe-COOH}$) is a dipeptide that readily assembles into highly ordered nanotubes in aqueous solutions.
2. 1,1,1,3,3,3 hexafluoro-2-propanol (HFIP)

Other materials:

1. Pipettes
2. Pipette tips

3. 1.5 ml microfuge tubes
4. V1 grade mica sheet

Procedure:

Preparing diphenylalanine nanotubes

1. Weigh 5mg of diphenylalanine peptide and dissolve it in 50 μ l HFIP. This gives a peptide concentration of 100 mg/ml.
2. Dilute the peptide into distilled water to a final concentration of 2 mg/ml.
3. Allow the solution to age for one day at room temperature. This results in the self-assembly of the peptide to give tubular assemblies that can be visually observed.

AFM sample preparation

4. Take a small (~ 1 cm² area) V1 quality mica sheet.
5. Place the mica piece on a solid support and peel off the upper mica surface using a sticky tapeto obtain a smooth surface.
6. Deposit ~ 0.1 - 1 μ g of the peptide on the mica surface.
 - a. Take 2 μ l of the clear part of the diphenylalanine solution in a microfuge tube and add 198 μ l distilled water.
 - b. Take 50 μ l (theoretical peptide amount ~ 1 μ g) of the peptide solution and deposit on the freshly cleaved mica surface.
7. Remove the excess fluid from the mica surface after one minute. This can be done by carefully touching lint-free tissue paper to the edge of mica.
8. Air-dry the mica.

AFM imaging

We shall be discussing the steps that are to be followed for performing the intermittent mode (also known as AC mode) imaging on the AFM from Agilent Technologies (Figure 36.4).



Figure 36.4 An atomic force microscope (Agilent Technologies)

Some of the important components and accessories that we shall be referring to in the procedure for carrying out the imaging are shown in figure 36.5.



Figure 36.5 Components and accessories of an atomic force microscope from Agilent Technologies

9. Switch ON the AFM instrument and the computer as instructed by the manufacturer.
10. Take out the ‘Scanner’ and place it on the cantilever mounting block (Figure 36.6 A).
11. Fix the specified ‘Nose cone’ into the scanner (Figure 36.6B).
12. Carefully hold the AC mode specified cantilever chip using the tweezers (Figure 36.6C, 36.6D).
13. Lift the clip of the Nose cone by pressing it against the beak pusher (Figure 36.6E).
14. Mount the cantilever chip into the assembly, carefully placing it into the groove provided for the chip (Figure 36.6E).
15. Orient the chip carefully such that the free cantilever tip hangs over the center of the nose cone.
16. Release the pressure on the beak pusher, allowing the clip to hold the cantilever (Figure 36.6F).
17. Place the scanner into the center of the microscope head’s base plate ensuring that it fits in the slot perfectly (Figure 36.6G).
18. Fix the scanner into position by tightening the locking screws (Figure 36.6H).

19. Plug the scanner's connectors on the microscope head.



Figure 36.6 Steps showing fixing the different components of the AFM. The steps are discussed in the text

20. Switch ON the laser.
21. Place a small piece of white paper under the scanner.
22. Use the laser tilting screws and the detector alignment screws to obtain a clear red spot on the paper.
23. Move the laser spot in the direction perpendicular to the cantilever until diffraction of the laser beam is seen on the paper and the Lucite block (Figure

36.7A). Lucite block acts as a screen for viewing safety; the laser beam reflected from the cantilever is projected on the Lucite block. The diffraction of laser beam is suggestive of the beam hitting one of the cantilever legs (Figure 36.7A).

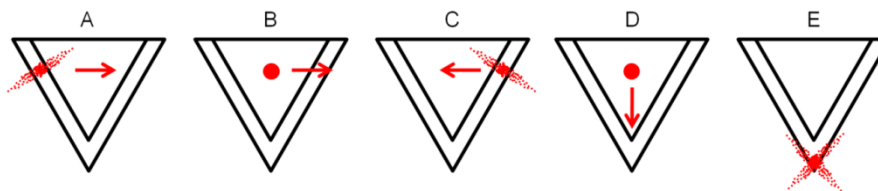


Figure 36.7 Steps showing focusing of the laser on the cantilever just opposite to the probe.

24. Continue moving the spot in the same direction. As we continue moving, the spot should reappear on the paper (Figure 36.7B) and again give diffraction (Figure 36.7C).
25. Bring back the laser spot between two legs and move it towards the tip of the cantilever (Figure 36.7D).
26. Focusing on the cantilever is suggested by almost completely obscured spot on the paper screen.
27. The spot on the Lucite block should look like an 'X' (Figure 36.7E). This happens when the laser beam is right on the tip of the cantilever.
28. Insert the Photodiode detector into the detector groove by sliding it into the groove (Figure 36.6I).
29. Plug the detector's connector into the correct slot present on the head base plate.
30. Check the 'Amplitude', 'Deflection', and 'LFM' values in the instrument controller. The deflection value should be zero or close to zero (Preferably within ± 0.7).
31. Fix the mica on the metallic disc provided with the instrument using a double-adhesive tape (Figure 36.6J).
32. Fix the circular 'Sample plate' on the sample stage holder and place the metallic disc having mica piece at the centre of the plate. The disk is held in place by a magnet present in the sample plate (Figure 36.6J).
33. Place, without tilting, the sample plate into the screws present beneath the base of the microscope's head base plate (Figure 36.6K, 36.6L). A magnet present on the sample plate secures its position.

34. Bring the sample close to the cantilever tip using coarse adjustment screw.
35. Start the software, PicoScan.
36. Go to the 'Preferences' and set the 'Scanner type'. An AFM can have multiple scanners; select the appropriate option. In this experiment, we shall be using large scanner compatible with the Agilent AFM system.
37. Go to the 'Mode' and select AC–AFM.
38. A dialogue box appears that shows that the AC mode controller is online *i.e.* connected.
39. Go to the SPS tab and select the 'AC Mode' frequency plot.
40. Provide the frequency range for oscillation. It is better to cover the entire frequency range specified by the manufacturer for a particular cantilever slot.
41. Click Sweep/Connect button present in the 'AC Mode Control' dialogue box.
42. A graph between amplitude and the frequency is generated.
43. Select the frequency slightly lower than the resonance frequency.
44. Go to 'View' menu.
 - a. In the 'Buffer Assignment' window, add a buffer by clicking the + button. Buffer means the type of data required, such as topography, amplitude, etc.
 - b. In the 'Servo Control' window, set the Force Setpoint to 0 V and set the 'Integral Gain' and 'Proportional Gain' to 0.6 and 0.3, respectively.
 - c. In the 'Scan and Approach Control' window, set the 'Stop' at 0.9 V and set the motor speed for probe approach and withdraw.
45. Go to 'File' menu in the main toolbar and select 'Live Scan'.
46. Click the 'Approach' button in the 'Scan and Approach Control' window.

When the instrument reaches the set point, a dialogue box prompting 'Setpoint Reached: Servo Active' is displayed.
47. Click OK and go to the Scan tab and set the following:
 - a. Scan size (area)
 - b. Scan speed (number of lines/sec)
 - c. Direction of scan (Up/Down/Toggle)
 - d. Number of scans (Single/Multiple)
48. Go to the 'Advanced Scan' tab and set the 'Datapoints per line' (dpi).
49. Click Start to begin the scan (**Note 1**).
50. Save the obtained image as diphenylalanine.STP file (**Note 2**).

51. Go to the 'Withdraw' tab and click on 'Withdraw' to withdraw the probe.
52. Exit the 'PicoScan' software.
53. Remove the sample stage and unmount the mica sheet from the metallic disc.
54. Switch off the instrument as per manufacturer's instructions.

Results and analysis:

1. Start the 'PicoScan' software.
2. Open the recorded image, the diphenylalanine.STP file.
3. In the 'Data rendering' window, go to the 3D scale tab and adjust the Z-scale to see the sample features.
4. In the same window (Data rendering), go to the 'Optimizing' tab and flatten the image to obtain a flat background. This results in a clearer image on a flatter background.
5. A number of data processing options are present in the software. It is highly recommended to go through the software manual to realize its full potential.

Notes:

1. While the imaging is underway, any one of the image modes like Raw, Derivative, Flattened, and Tilted (in the 'Optimizing' tab under 'Data rendering' window) can be selected to obtain the image as per requirement. It is, however, recommended to obtain a raw image as any other processing can be done on the recorded image.
2. If the imaging is not good or the desired features are not obtained, it is recommended to scan a different area on the sample. This can be done without withdrawing the probe and giving an offset for the scanning area.