Basics of Microscopy with Visualization of Mammalian Cells

&

Immunolabelling and Visualization of Marker Proteins Inside Cells

Aim

Visualization of mammalian cells (stained and unstained) using phase contrast and confocal microscope.

Introduction

Enormous advancement has been made in the field of microscopy over the past nine decades. There are different types of microscopes available now for different applications. The reason for the continuous development in microscopy is the increasing need to observe finer features on sample surfaces i.e., high-resolution images. Other demands on microscopic techniques have been reproducibility of results, ease of sample preparation, less or no damage during investigation of samples, especially biological tissue samples, ease of handling and lower maintenance cost. The term 'light microscopy' is used to indicate microscopic examination of a sample by shining a light radiation on it. Light can interact with tissue or cell samples in different ways viz., part of it is simply transmitted through the sample, part of the light is reflected by certain regions in the sample while some part may get scattered, diffracted or refracted. Also certain samples can absorb light of a particular energy and emit light of lower energy in a process known as fluorescence. A microscope can be built based on any of these interactions of light and sample, which can be converted into an image.

Terms and Definitions

Depth of Field- the distance along the optical axis throughout which the object can be located and yet be imaged with satisfactory clarity. This is used in stereo microscopy.

Field-Of-View- the visible area through the eyepiece when the microscope is in focus.

Magnification- the enlargement of an object through the lens system. This is determined by multiplying the magnifying power of the objective by the eyepiece.

Magnifying Power- It is the number of times the image is seen through the microscope is larger than the item appears to the unaided eye.

Numerical Aperture- (N.A.) a term representative of the angle included by a cone of light accepted by the objective of a microscope. The higher the N.A., the greater the resolving power.

$$\mathbf{N.A. = n \times \sin \Theta}$$

n = refractive index of the medium

Θ is the maximal half-angle of the cone of light that can enter or exit the lens

Resolution- a measure of the ability of a lens to image closely spaced objects so they are recognized as separate objects.

Resolving power- the capacity of any optical system to distinguish and separate details in a specimen.

$$\text{Resolving power (R.P.)} = \frac{\text{Wavelength of light in nm}}{2 \times \text{Numerical aperture of objective lens}}$$

Working Distance- the distance between the cover glass or object and the tip of the objective.

Parts of Microscope

- **Mechanical parts**

These are secondary but are necessary for working of a microscope. A 'Base', which is horseshoe shaped supports the entire framework for all parts. From the base, a 'Pillar' arises. At the top of the pillar through an 'Inclination Joint' arm or limb is attached. At the top of the pillar, a stage with a central circular opening called 'Stage aperture' is fixed, with a stage clip to fix the microscopic slide. Beneath the stage, there is one stage called 'sub stage' which carries the condenser. At the top of the arm, a hollow cylindrical tube of standard diameter is attached in-line with the stage aperture, called 'body tube'. The body tube moves up and down by two separate arrangements called 'coarse adjustment' worked with pinion head and 'fine adjustment' worked with micrometer head. At the bottom of the body tube an arrangement called 'revolving nose-piece' is present for screwing different objectives. At the top of the body tube eye- piece is fixed.

- **Optical parts**

It includes mirror, condenser, objective and ocular lenses. All the optical parts should be kept in perfect optical axis.

a. **Objectives**: Usually 3 types of magnifying lenses (i) Low power objective (10x) (ii) High dry objective (45x) and (iii) Oil immersion objective (100x)

b. **Eye-piece**: Mostly have standard dimensions and made with different power lenses. (5x, 10x, 15x, 20x). A compound microscope with a single eyepiece is said to be monocular, and one with two eyepieces is said to be binocular.

c. **Condenser**: Condenses the light waves into a pencil shaped cone thereby preventing the escape of light waves. Also raising or lowering the condenser can control light intensity. To the condenser, iris diaphragm is attached which helps in regulating the light.

d. **Mirror**: It is mounted on a frame and attached to the pillar in a manner that it can be focused in three different directions. The mirror is made of a lens with one plane surface and another concave surface. Plane surface is used, when the microscope is with a condenser.

There are several types of optical microscopes. Each of them has its own advantages and choice of samples as tabulated below:

Type of light microscope	Construction/ Working principle	Specimens		Remark
Upright microscope	Specimen placed below the objective lens	Tissue slice (Histology work)		High resolution but short working distance
Inverted microscope	Objective lens positioned beneath the specimen	Live cells in transparent tissue culture flasks or well plates		Lower resolution but long working distance; can be integrated with flow cells
Bright Field microscope	Based on transmission of light through samples; background appears bright and specimen features appear dark	**Stain**	**Inform-ation**	Unstained cells cannot be seen; immuno-histochemistry can be done
		Eosin	Cytopla-smic proteins	
		Hematoxylin	Cell nucleus	
		Alizarin red	Calcific deposits on implants	
		Ver Hoeff's stain	Elastin	
Dark Field microscope	Oblique illumination of the specimen; based on scattering of light by the dark and features as bright spots	Specimens with small particulates		
Fluorescence microscope	Monitors light emission from fluorophore-tagged lipids, enzymes, membrane components and antibodies	Fluorophore-tagged specimens		
Confocal laser scanning microscope	A special tool for fluorescence, prevents blurring of images by placing a pin hole at the confocal image plan	Location of proteins, lipids and cellular components		
Polarization based microscope	Measures changes in polarization of light caused by ordered arrangements in a sample	Collagen, muscle sarcomeres, Remnant synthetic polymers in explanted tissues		Cannot be performed in tissue culture plastic wares since they themselves cause polarization changes

We have used phase contrast and confocal microscopes for our experiment; basic principles of them have been discussed in the following sections.

Phase Contrast Microscope

Phase contrast microscope has a special type of condenser, objective and a special magnifier. Light passing from one material into another of slightly different refractive index will undergo a change in the phase. This change in the phase of the light wills in-turn increase the contrast. A system of rings in the condenser and objective separate the diffracted rays from the specimen. These diffracted rays from the specimen and the undiffracted rays combine and the phase difference is converted into difference in light intensity. In phase-contrast microscopy, unstained/living organisms can be examined. As a result, the internal contrast of various parts of a specimen against its surroundings is increased. It has a resolving power of 0.1 – 0.2 μm.

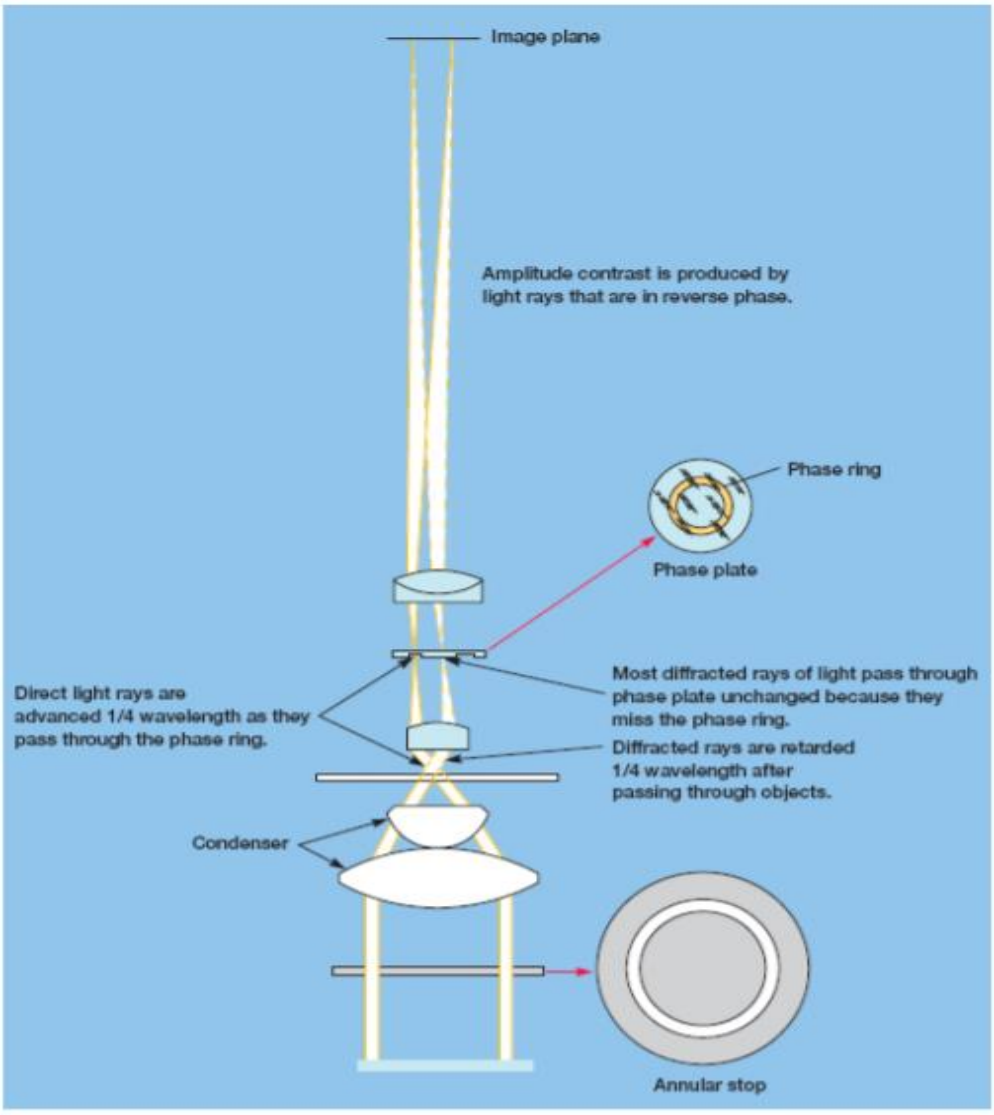

Figure 1: Basics of phase contrast microscopy. To the left: Arrangement of the ring-shaped mask below the objective and of the phase-ring within the objective to the right: The path of light rays within a phase-contrast microscope. 1. Ring-shaped mask, 2. Condenser, 3. Specimen, 4. Objective, 5. Phase plate, 6. Focal plane of the objective. The wave character of the light is indicated by the change of light and dark areas.

Confocal Laser Scanning Microscope

Light sources used in confocal microscopes are lasers. The microscope works in epi-illumination mode. The laser beam is spread by a diverging lens so as to fill the back aperture of the objective lens which functions as condenser as well. The expanded laser light is reflected by the dichroic mirror on the objective that focuses the light as an intense diffraction-limited spot on the sample. The fluorescence from the illuminated spot is collected by the objective and sent to the eyepiece/camera/detector through a pinhole aperture. The fluorescence light emitted by the illuminated sample is focused as the confocal point at the pinhole. Any light coming from below or above the focal plane is blocked by the pinhole plate. As the fluorescence is detected from a diffraction limited spot, the focused laser spot is scanned over the sample in a raster fashion collecting light from the entire focal plane. As the position of the illuminating spot changes, the pinhole moves so as to be confocal with the illuminated spot of the same focal plane. The light that passes through the pinhole is filtered by the emission filter before it reaches the detector.

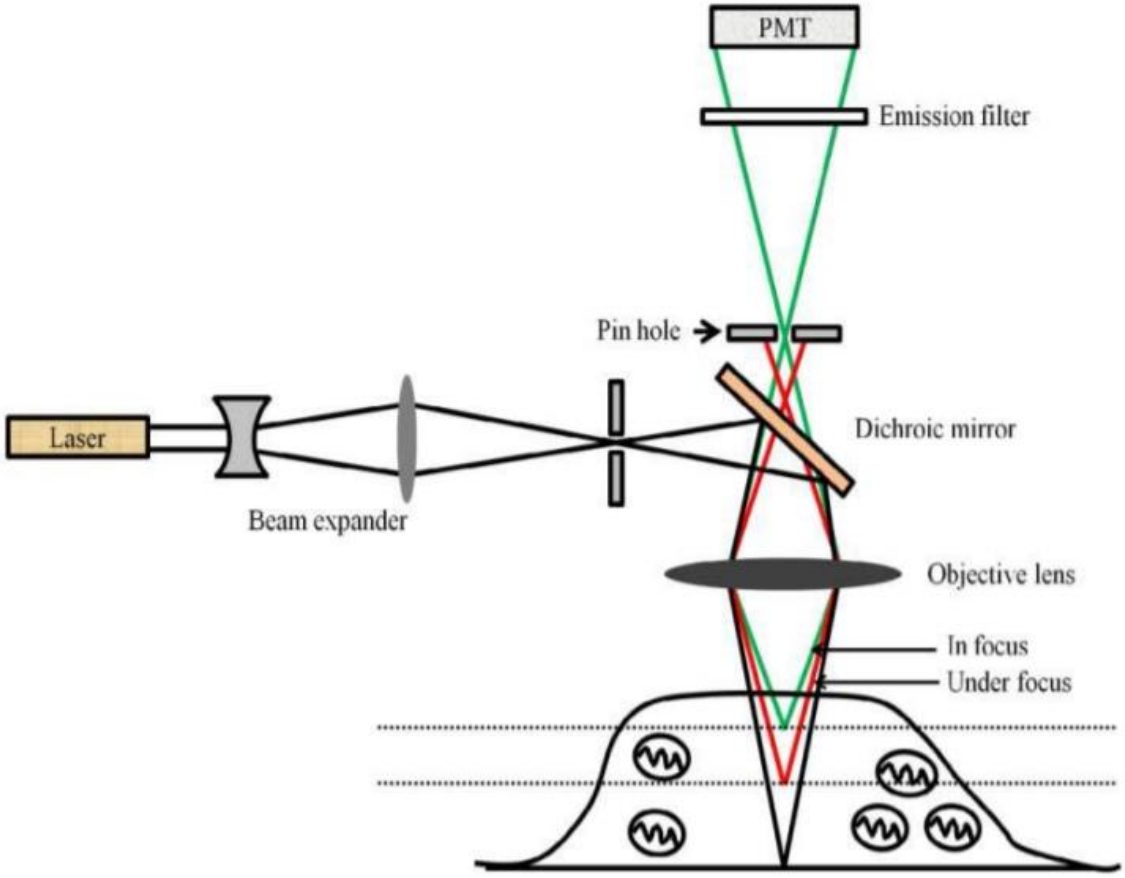

***Figure 2*:** Optical diagram of a confocal laser scanning microscope

Immunolabeling

So, Immunolabeling is a biochemical process that enables the detection and localization of an antigen to a particular site within a cell (immunocytochemistry), tissue, or organ (immunohistochemistry). These antigens can be visualized using a combination of antigen-specific antibody as well as a means of detection, called a tag, that is covalently linked to the antibody. The antibodies with fluorescent dyes can react with their antigen within a cell and allow visualization of the distribution of proteins, glycans, and small biological and non-biological molecules. There are two complex steps in the manufacture of antibody for immunolabeling. The first (direct method) as you can see is by producing an antibody that binds specifically to the antigen of interest. Now here the antibody is fused to a Fluorophore. Second, an indirect method employs a primary antibody that is antigen-specific and a secondary antibody fused to a fluorophore that specifically binds the primary antibody.

Sample Preparation

For Unstained cells

Seed the cells in tissue culture dishes/flasks. Keep the tissue culture dishes/flasks in incubator (37˚C, 5% CO_2) for 24 hours for proliferation. Observe the cells under microscope.

For Immuno-stained/immunolabelled cells

1. Coat coverslips with polyethylineimine or poly-L-lysine for 1 hr at room temperature or use coated tissue culture dishes.

2. Rinse coverslips well with sterile H_2O (three times 5 min each).

3. Allow coverslips to dry completely and sterilize them under UV light for at least 4 hr.

4. Grow cells on glass coverslips/ tissue culture dishes or prepare cytospin or smear preparation.

5. Rinse briefly in phosphate-buffered saline (PBS).

- **Fixation**

1. Fix the samples either in ice-cold methanol, acetone (1-10 min) or in 3-4% paraformaldehyde in PBS, pH 7.4 for 15 mins-60 mins at room temperature.

2. Wash the samples twice with ice cold/room temperature PBST (phosphate-buffered saline with Tween 20).

- **Permeabilization**

If the target protein is localized intracellularly, it is very important to permeabilize the cells.

1. Incubate the samples for 10 mins-15 mins with PBS containing 0.25% Triton X-100 (or 100 µM Digitonin or 0.5% Saponin). Triton X-100 is the most popular detergent for improving the penetration of the antibody. However, it is not appropriate for membrane-associated antigens since it destroys membranes.

2. Wash cells in PBST three times for 5 min.

- **Blocking and Immunostaining**

1. Incubate cells with 1% BSA in PBST for 30 min to block unspecific binding of the antibodies (alternative blocking solutions are 1% gelatin or 10% serum from the species from which the secondary antibody was raised in).

2. Incubate cells in the diluted primary antibody in 1% BSA in PBST in a humidified chamber for 1 hr at room temperature or overnight at 4°C.

3. Decant the solution and wash the cells three times in PBST, 5 min each wash.

4. Incubate cells with the secondary antibody in 1% BSA in PBST for 1 hr at room temperature in the dark.

5. Decant the secondary antibody solution and wash three times with PBST for 5 min each in the dark.

Observation

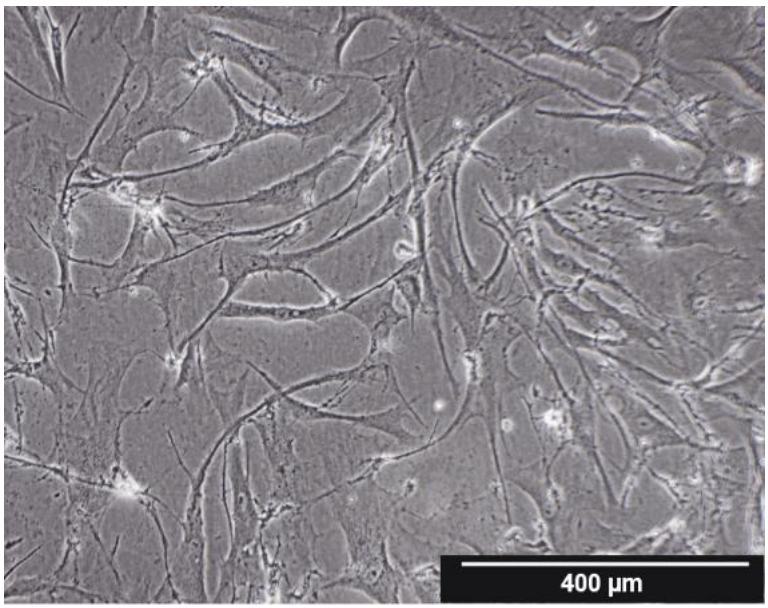

Figure 3: Image of unstained human dermal fibroblasts (HDFs) using phase contrast microscopy.

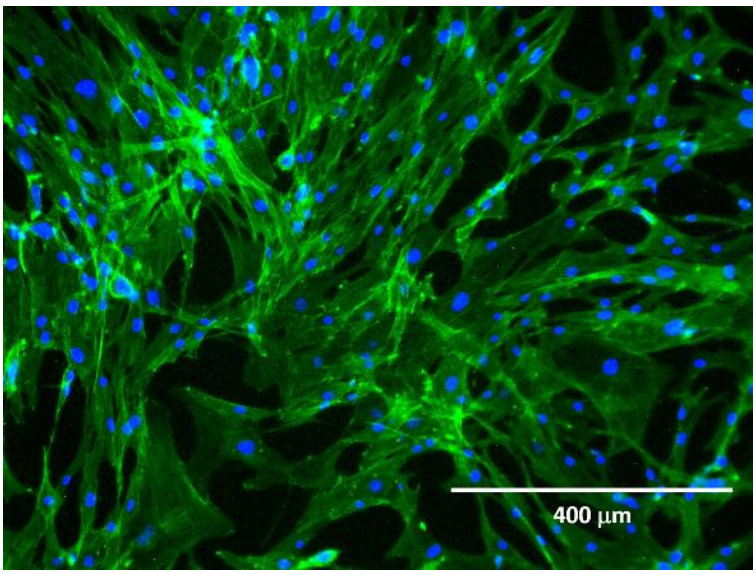

Figure 4: Fluorescence image of immunolabelled HDFs using fluorescence microscopy.

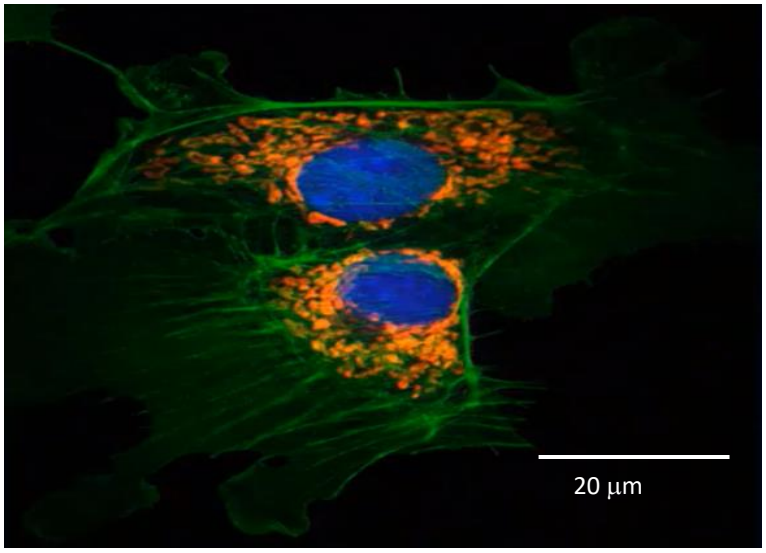

Figure 5: Fluorescence image of immunolabelled fibroblasts using confocal microscopy.