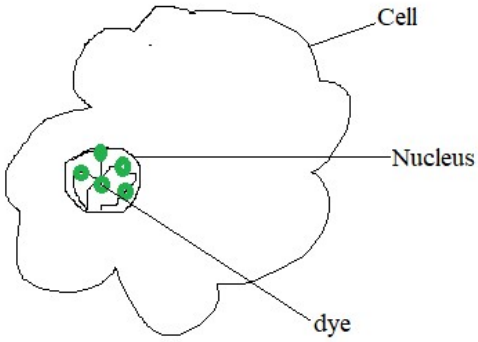
Introduction of Fluorescence imaging

(CHM409 Physical Methods in Chemistry, Course teacher: Dr. Biplab Kumar Kuila)

Fluorescence imaging is the visualization of fluorescent dye or its spatial distribution in material or sample especially biological samples. The fluorescence imaging technique is widely used in molecular biology, biochemistry laboratories for a variety of experimental, analytical, and quality control applications. Commonly used techniques, including total nucleic acid and protein quantification, Western, Northern and Southern blotting, PCR product analysis, and DNA sequencing, can all benefit from the application of fluorescence based methods for detection like fluorescence imaging.



Let consider a cell whose nucleus is labeled or strained with green fluorescent dye so that these dye molecules reside only on the nucleus of the cell. The dye can absorb blue light and emit green light which can be measured. By looking at the image inside the cell, we can find the green coloration due to its green emission and can say where exactly the dye molecules are present insight the cell as well as the position of the nucleus because the dye molecules only bind to the nuclueus. Fluorescence microscope is used to produce such images. Fluorescence microscopy works by tagging or labeling various cellular or sub-cellular structures by molecules called fluorophores which display fluorescence. For e.g. a fluorophore called DAPI (4′,6-diamidino-2-phenylindole) binds to DNA (as shown in Fig. 2. 1). So it has been used to tag the nucleus which contains DNA. Similarly other cellular components can be labeled by choosing an appropriate fluorophore that will bind to it, or a protein bound to a fluorophore which will bind to the desired location. The basic type or most simplified fluorescence microscope is widefield fluorescence microscope. Later, fluorescence microscope is further modified to confocal and two photon fluorescence microscopy depending on the use and limitation of each type of microscope. The basic principle of fluorescence microscope is described in Fig. 2.2. Fluorescence microscope

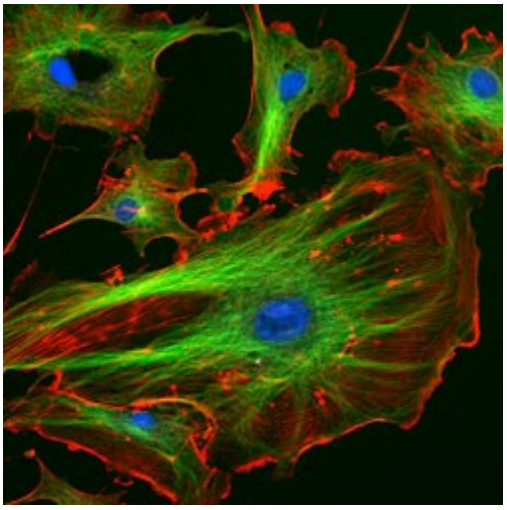


Fig. 2. 1 Endothelial cells observed under fluorescence microscope. Nuclei are labeled blue with a fluorphore called DAPI which binds to DNA. Microtubules are labeled green by an antibody bound to a green fluorphore called FITC(Fluorescein isothiocyanate) and actin filaments are labeled red with a fluorphore called TRITC(Tetramethylrhodamine-isothiocyanate).

needs a light source which may be tungsten lamp, mercury arc lamp or laser where laser is better option due to its high energy. The specimen needs to excite the sample with a light of particular wavelength (here blue light). The excitation filter is used to filter only the blue light from the initial light which consists of lights of different colors or wavelengths. The blue light will be further reflected by dichromatic mirror and passed through objective lens which focuses the excitation light on the specimen sample. Once the sample which contain fluorophore, will be excited by the excitation light and emit radiation of higher wavelength green light (let say). This green light will pass through objective lens in opposite direction and encounter the dichromatic mirror. The dichromatic mirror is a special type of mirror that it will only allow certain wave length of light to pass and reflect other wavelength of light. In this case the dichromatic mirror is designed in such a way that it will only allow passing green color light but reflect blue color. After passing through dichoric mirror, the green light will encounter the emission filter. The emission filter will allow only green light to pass and arrange all the emitted lights from different focal planes of the sample. The green light after passing through the emission filter and ocular it will be detected by the detector. As the detector only detects the green light, we can quantify the amount of green light emitted and also the fluorophore present in the sample. It also

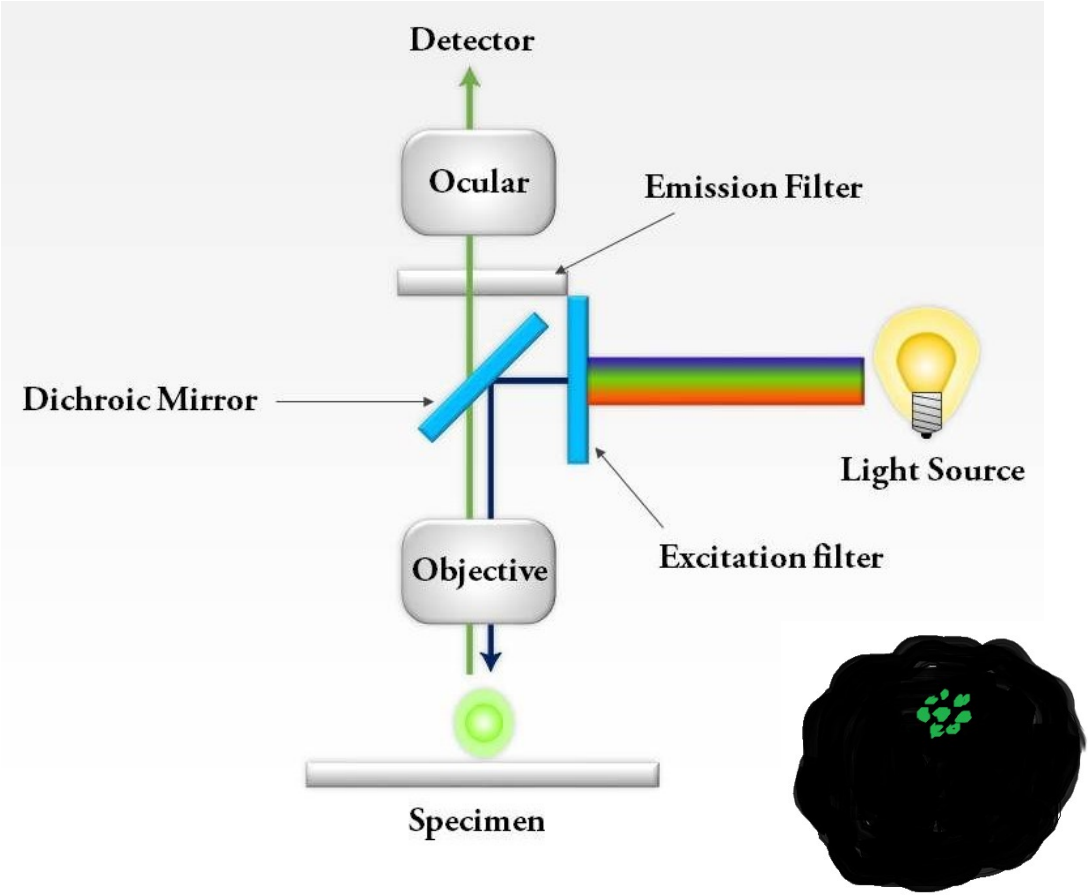


Fig. 2.2 basic principle of fluorescence microscope

give us good contrast of the sample, i.e. in a cell, only the positions where the green fluorophore binds and labeled will show green coloration and other position will be dark (Fig. 2.2).

Some of the common fluorophores used in microscopy are 4',6-diamidino-2-phenylindole, (DAPI, blue emission), Fluorescein Isothiocyanate (FITC, green emission), Cy3 (Green), Cy5 (Red), Rhodamine (Red). There are also synthetic proteins called fluorescence proteins which can be used as fluorophores. Some biomolecules show intrinsic fluorescence. These are called endogenous fluorophores, e.g. Nicotinamide adenine dinucleotide (NADH). These fluorophores produce autofluorescence which may sometimes interfere with fluorescence imaging using other fluorophores. Endogenous fluorophores are limited and therefore don't offer a great deal of flexibility. So fluorophores external to the biological system, called exogenous fluorphores are used to label different structures. In some cases the fluorphores may directly bind to the various

structures, e.g. DAPI to AT rich segments of DNA. In other cases the fluorophore is first attached (conjugated) to a protein or biomolecule which can then attach to a desired structure. For e.g. an antibody can be conjugated with a fluorophore and inserted into a cell to find a cancer cell. The brightness of a fluorophore is controlled by a factor called quantum yield. It refers to the fraction of fluorescently emitted photons to the incident photons. Fluorophores with high quantum yield will produce brighter images.

Advantages of Fluorescence microscope:

(i) High contrast imaging: Fluorescence microscope provide high contrast image for example which part of the cell contain fluorophore or which part does not contain fluorophore.

(ii) **High specificity:** As the fluorophores are tagged or bound to the particular cellular component, it has high specificity

(iii) **Quantitative imaging:** It can provide information of the fluorophores in the cell in a quantitative manner.

(iv) **Live cell imaging:** Most importantly, we can actually image the live cell. We can tag fluorophore with the cells which are growing or dividing and then take snap shots in various formation using various types of dyes in different time and super impose all the snaps together. We get information about the real changes that are going on in the cell during its different formation and time.

Disadvantage:

Photobleaching: Fluorophores suffer from a degradation process called photobleaching. Photobleaching refers to the reduction of intensity of fluorescent emission from a fluorophore upon continuous illumination. As shown in fig. 2.3, that the intensity of the fluorescence from the Hella cells strained with FITC-conjugated phalloidin decreases after 30 minutes constant illumination due to photobleaching. There are several mechanisms for photobleaching. For instance, the excited state may undergo some photochemical reactions which inhibit fluorescence of the reacted products. The fluorophore may get photooxidized leading to decrease in intensity of fluorescent emission over time. Photobleaching causes problems in long-term fluorescence imaging. Currently some fluorophores with very low photobleaching are available, for e.g.,

Alexa Fluor dyes. It is also possible to have inorganic nanoparticles, called Quantum Dots, which exhibit fluorescence but do not suffer from photobleaching

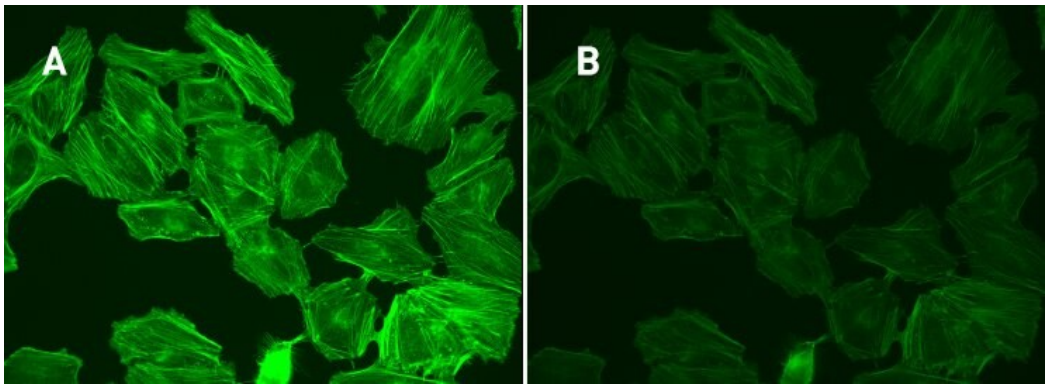


Figure 2.3. *HeLa cells were fixed and labeled with FITC-conjugated phalloidin. Coverslips were mounted in 50% glycerol (in PBS). Panel (A) shows the initial intensity of the fluorophore, while panel (B) shows the photobleaching that occurs after 36 seconds of constant illumination.*

Confocal Fluorescence microscope: Confocal fluorescence microscopy is an imaging technique that enables increased optical resolution as compared to conventional "wide-field" epifluorescence microscopy. The basic principle of confocal fluorescence microsope is same as the normal fluorescence microscope as discussed earlier. In case of confocal microscopy, laser is usually taken as light source. The light beam used to excite a fluorophore is focused by the objective lens of a microscope and converges at a "focal point" where it is maximally focused. Beyond the focal point the light again diverges. The entering and exiting beams may be imagined as a pair of cones touching at the focal point as shown figure 2.4 (left side). The phenomenon of diffraction imposes a limit on how tightly a beam of light can be focused. The beam actually focuses to a spot of finite size. Mainly two factors determine the size of the focal spot: (a) the wavelength of the light, and (b) the light-gathering ability of the objective lens, which is characterized by its numerical aperture (NA). The focal "spot" extends not only in the *x-y* plane, but also in the *z* direction, and is in reality a focal volume. The dimensions of this focal volume define the maximum resolution achievable by optical imaging. Although the number of photons is greatest within the focal volume, the conical light paths above and below the focus also contain a lower density of photons. Any fluorophore in the light path can thus be excited. In

conventional (wide-field) epifluorescence microscopy, emission from fluorophores above and below the focal plane contribute out-of-focus fluorescence (a "hazy background"), which reduces image resolution and contrast, as depicted in Figure 2.4, with the red cube representing

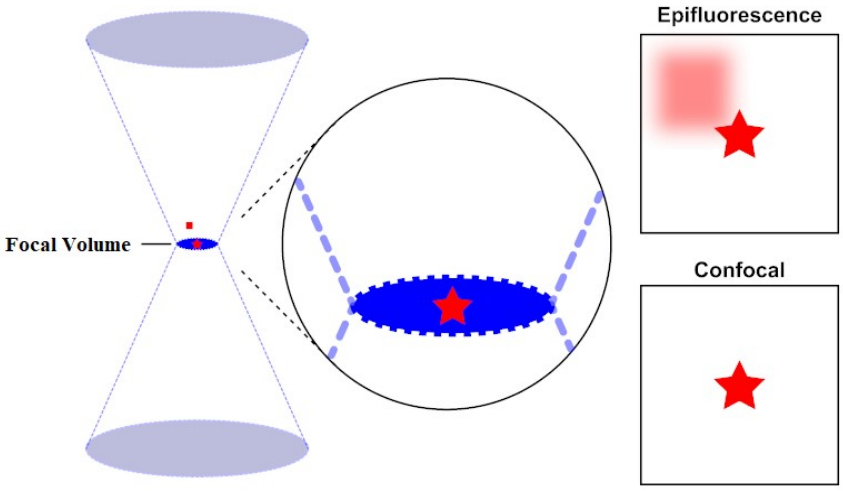


Figure 2.4. Optical resolution of epifluorescence versus confocal microscopy.

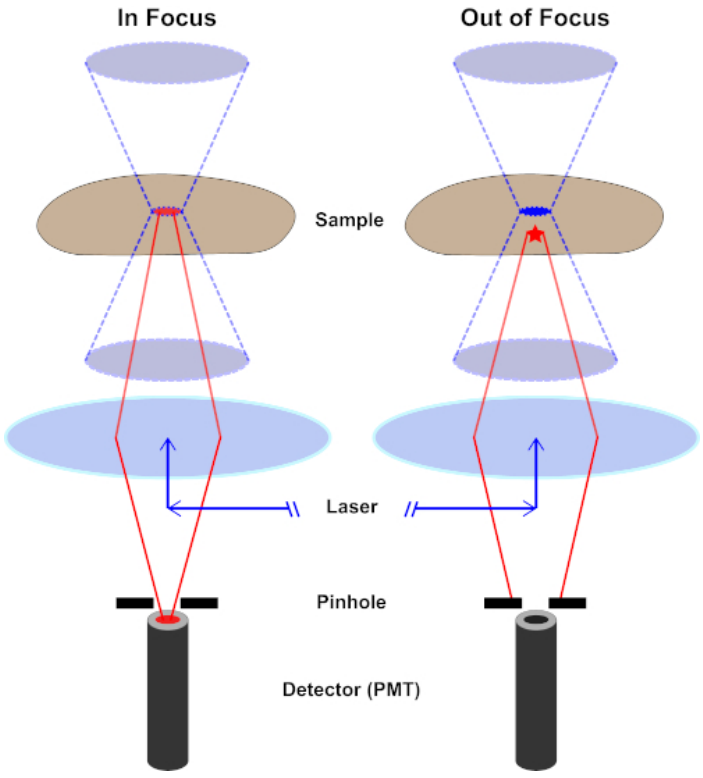


Figure 2.5 Pinhole effect in confocal microscopy.

fluorophore emission above the focal plane (red star) that results in out-of-focus fluorescence (top right). This problem is avoided in confocal microscopy, due to the utilization of a pinhole. (Figure 2.5, bottom right). As depicted in Figure 2.5, the pinhole allows emissions originating from the focal place to reach the detector (left), while blocking the out-of-focus fluorescence (right) from reaching the detector, thus improving both resolution and contrast.

A detail schematic of the confocal microscope is shown in fig. 2.6. Here laser is used as the energy source to excite the molecules. First laser will pass through the pin hole and excitation filter and encounter to the dichromatic mirror. Later it will pass through objective lens and heating different specimen focal planes. But only one focus point will be focused properly due to the presence of pin hole which will only allow passing emission light coming from that focal point to the detector. So the region or space which belongs to the focal point will be only clearly visible and any other region above or below of that focal point will not be visible. The emitted light will pass through the dichromatic mirror and finally through barrier filter/emission filter which will prevent any further scattering. Then it will pass through pinhole aperture and detected by the detector and converted to image.

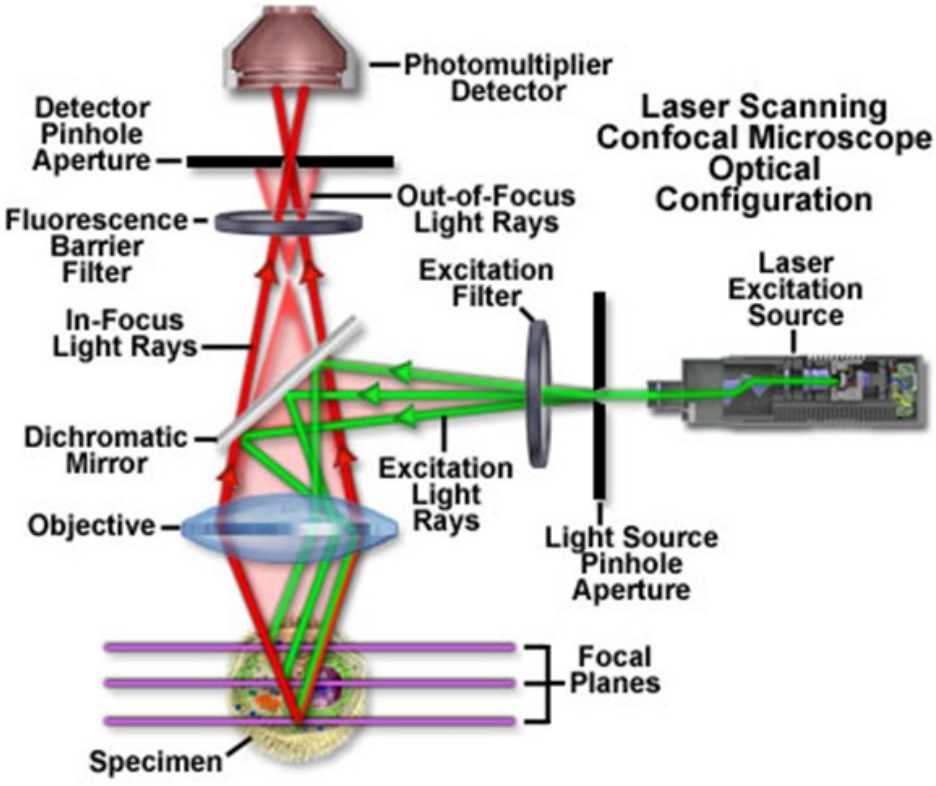


Fig. 2.6 Basic principle of confocal fluorescence microscope.

In case of confocal microscope one only gets image of a small area or point of a cell at a particular time. But, we need to take snap of a complete cell. Then the cell is divided into small microgrids. Microscope will scan a particular point in the cell at a particular time and in each case it will take a snap of that particular point. It will provide image of all the points and combine all these snaps into an image of a complete cell with the help of a CPU.

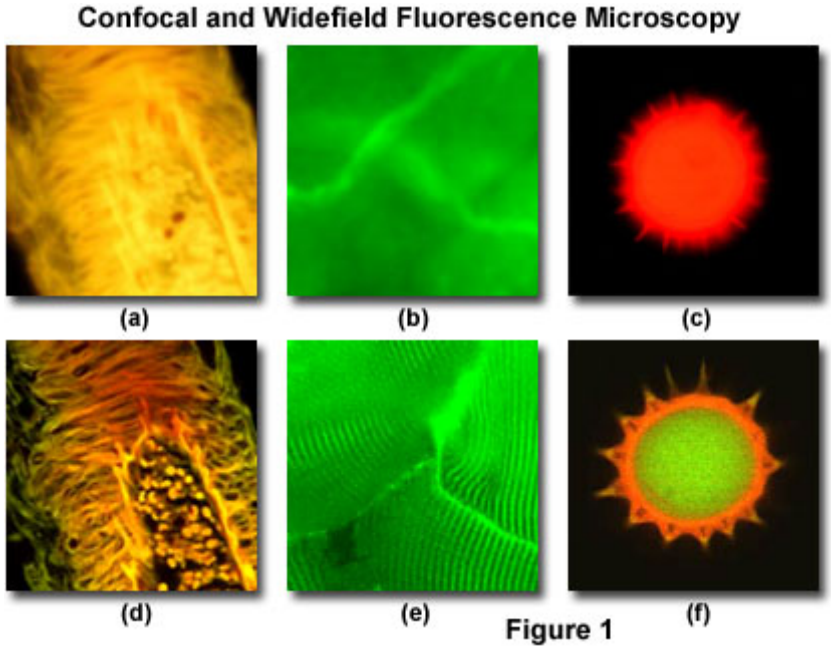


Figure 1

Figure 2.7 showing a series of images that compare in traditional widefield and laser scanning confocal fluorescence microscopy. The upper (a, b, c) images are widefield fluorescence microscope images where as the lower (d, e, f) are their corresponding laser confocal fluorescence microscope images respectively. The figure clearly shows that widefield images become more clearly visible when observed through confocal microscope.