Analytical Technologies in Biotechnology

Dr. Ashwani K. Sharma
Department of Biotechnology
Indian Institute of Technology, Roorkee

Module - 1
Microscopy
Lecture - 5
Fluorescence and confocal microscopy

In this lecture, we are going to discuss about two light microscopy techniques, which utilize the phenomenon of fluorescence, the fluorescence microscopy and confocal microscopy, the extension of fluorescence optics. Now, fluorescence microscopy is a technique that utilizes phenomenon of fluorescence to generate an image. Now, it is an extraordinarily sensitive method for detecting minute amount of material in a specimen as for any fluorescent material. The total intensity of fluorescence is proportional to the intensity of incidence light.

Now, here in this technique, a sample is illuminated with light of a specific wavelength, we call it exciting wavelength or excitation wavelength, which is absorbed by the fluorophore or the fluorescent compound, and which causes fluorescence that is the light emitted by the fluorescence or emission wavelength, we call it fluorescence emission wavelength is that a different and longer wavelength. Then the illumination and mostly it is in visible region. This fluorescence emitted wavelength is detected through objective lens. Finally, it generates an image, it could be captured on a digital device or it could be absorbed.

Now, the illumination light is separated from weaker emitted fluorescence, through use of optical filters. This is because as we know that the excitation wavelength is a particular characteristic of a compound. So, you have to eliminate other wavelengths, which may not be useful and optical filters are utilized, that is an addition in optics here, we will be dealing with the optics later on.

Now, one filter is placed between light source and condenser. What is the function of this? This particular filter allows only excitation wavelength almost near monochromatic, and not any other wavelength in the fluorescence spectrum to be transmitted through the condenser lens. So, only excitation wavelength will pass through the specimen stage. The second filter optical filter will be transmitting only fluorescence wavelength, that is

emission wavelength. It will not allow excitation wavelength to pass through, and it is placed between objective and eye.

So, what you have is 2 filters, which have different filtration or which filters the wavelength, different wavelengths. One allows the excitation wavelength. Another does not allow excitation wavelength, but rather allows emission wavelength of fluorescence wavelength to pass through. Now, in absence of fluorescence or in absence of a fluorescent object, the field is dark. In case of a fluorescent sample, the contrast is very strong with respect to background. It got excellent images of the specimen.

Now, most fluorescence microscopes used in life sciences are epifluorescence microscopes. They will show you how the optics differs here. Now, this epifluorescence microscopes means the excitation and observation of the fluorescence are from above the specimen that is from above the specimen. The main components of fluorescent microscope are as follows like we have already discussed different components and some are common components.

There are certain additions here like we have seen in dic or polarization microscopy. Likewise here, light source, which is with excitation filter and the dichroic. So, the main components of fluorescence microscope are a light source, which is little different like we discussed the excitation filter that is first optical filter. The dichroic mirror is also called a beam splitter objective lens emission filter and a detector.

Let us see each of them. Now, light source used in fluorescence microscopy are xenon arc lamp or mercury vapor lamp with excitation filters. Also, lasers could be utilized. Lasers are mostly used for complex fluorescence microscopy technique like confocal microscopy, which we are going to discuss later on.

Now, in fluorescence microscopy, halogen lamps are not used as they cannot provide intense near monochromatic illumination. So, to get near monochromatic illumination, you have to avoid halogen lamps. Non-fluorescing slides; cover slips and lenses are required for the analysis of weak fluorescence because if the slides cover slips itself fluoresced, then it will be very hard to analyze the weak fluorescence. So, you have to use to see that those things are avoided.

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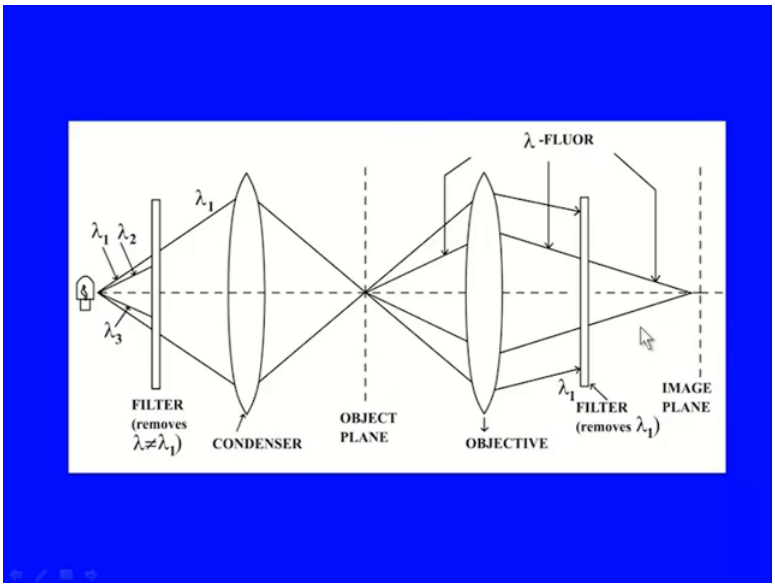

Now, let us get into a try to understand the optics of a fluorescence microscope. This is a very simple schematic of the optics of fluorescence microscope. Now, here if you see a light source as we have discussed is different from halogen lamp. It could be xenon arc lamp or mercury vapor lamp. Here, the light as it comes as near monochromatic or so what you have in optical filter here attached, this is the first optical filter. It says it will allow only a one wavelength only. It will not allow anything. So, it will only allow one kind of monochromatic wavelength to pass through.

Now, this is then passed onto the condenser. The condenser focuses the wavelength here onto the specimen plane or object plane. Now, this object has to be fluorescent that is it should have a fluorescence substance, which could be excited by this wavelength. So, it could emit fluorescence. Now, as we go along, if you can see that, after the objective lens, another optical filter has been placed.

This optical filter is not allowing the excitation wavelength, but rather it is allowing only emission wavelength to pass through. So, this optical filter is different form optical filter number 1. Finally, you get image in the image plane that is a very simple schematic, but like I said the epifluorescence microscopes are mostly used rather than a simple schematic.

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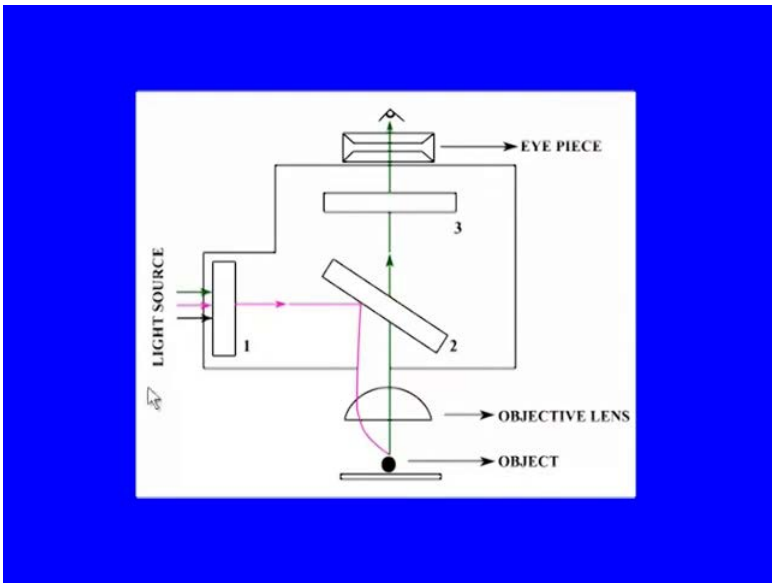

So, here is the schematic of epifluorescence microscope. So, what happens here? You have a light source. This light source in confocal. It will be laser. As we will see now, there is an optical filter here at number 1 position, which allows a particular wavelength. The first filter allows blue light though it is shown here in different color, but interests to be wavelength; between 450 and 490 nanometer light is allowed. It will be different things. Here, we are showing a particular element, which is now at this number 2 is being a splitter or dichroic mirror. It is a beam splitting mirror reflects the light below 510 nanometers. It reflects the light below 510 nanometers; but allows the light above 510 nanometers to go through.

So, the light is reflected and focused onto the object through objective lens. This could be you can say condenser as well as objective. Then as it is focused onto the object, the excitation object is excited and emits a longer wavelength or fluorescence. That fluorescent wavelength passes through the dichroic mirror without any problem. Then it passes through this filter, which is the second barrier filter and allows only a specific green fluorescent emission between 520 and 560 nanometer to pass through. So, unwanted signals are cut in here. Then you can either observe it through eye piece or it could be recorded.

So, this is typical optics of epifluorescence microscope where like I said observation and emission is recorded from above actually. So, optics of epifluorescence microscope is

different from a simple schematic diagram, which I have shown you earlier. So, let us move on to understand this whole thing. Now, because very few cell components, the problem with fluorescence is that most of the cell components or biological samples are not fluorescent. For example, there could be like in proteins, certain amino acids could be fluorescent or certain other like pigments could be fluorescent, but not many of the biological samples are fluorescent.

Now, because very few cell components are fluorescent here and even lesser can be excited by shorter wavelengths, you have to use extrinsic fluorophores or you have to attach dyes or other fluorophores. They have to be bound to the particular cell component in order to visualize those cell components. So, this requires a specimen preparation here, so that you can attach, you can bind those extrinsic fluorophores and you can observe the fluorescence. Now, four very useful dyes, which are utilized for fluorescent staining are one is rhodamine and taxes red, which emit red light and when excited with green and yellow light. So, rhodamine and taxes red emits red light.

There are others like cite three emits orange light and fluorescence emits green light when excited with blue light. Now, most fluorescent dyes emit visible light. Some like cite five or cite seven could be emitting infrared light also. We are not going into that. Now, there could be lot of other flours, which are used like for example, acridine orange, and cuna green.

They are used for lot of analysis of DNA samples or RNA samples or we can say nucleic acids. There could be other techniques also like in immunofluorescence microscopy; specific proteins or other molecules can be revealed in the specimen by chemically coupling these dyes to purified antibodies a specific for a particular macromolecule. So, antibodies bind to this.

Since, there is a dye attached to them, the fluorescence could lead to the localization or viewing of those particular macromolecules or a structures. So, this is immunofluorescence. As you are utilizing the antibodies in this case, fluorescence microscopy can also be applied to life cells. It could be you can stain the cells or life cells directly could be seen through fluorescent microscopy.

For example, a purified acting may be chemically linked to a fluorescent dye. It could be microinjected into cultured cells. The endogenous cellular and injected TED active

monomer copolymerizes into long acting fibers. You can observe those arrangements in vivo or as they are going on technique is very useful to study individual microtubule within cells. So, even you can utilize it for life cells. Fluorescence microscopy has been used for determination of intracellular concentration of say for example, calcium and H plus ions.

Now, this is done. For example, for calcium ions, the fluorescence properties of a dye called furan two have been used. What it does is it facilitates the measurement of concentration of free calcium in cytosol. The fluorescence of furan two at one particular wavelength is enhanced when calcium is bound and fluorescence is proportional to calcium at another wavelength.

The fluorescence of this dye is seen whether or not calcium is bound or not. By examining cells continuously at two wavelengths, you can determine, quantify the concentration of the cytosolic calcium. Likewise, there could be other dyes, which could be used for other ions like H plus or to determine ph. So, there could be lot of different ways fluorescence could be used to in fluorescence microscopy.

Another very important technique has been developed to detect specific proteins within living cells as they are going locating them as a function of time. Now, what is done is there is a green fluorescent protein. This is a natural fluorescent protein and found in jelly fish and the bioluminescence of these organisms. It radiates a green fluorescence. It is due to the green fluorescent protein. It is a 238 amino acid protein containing corin thyrocin and glycine residues whose cytosines have spontaneously reacted with another to form fluorescent chromophore.

Now, by recombinant DNA technique, the protein can be introduced into the living cultured cells either fused or otherwise. The expression of protein fused with other protein could be utilized for location of a protein in vivo. So, this is a very good technique to detect these specific proteins within living cells with the help of a green fluorescent protein. Let us discuss some of the important applications of this particular technique.

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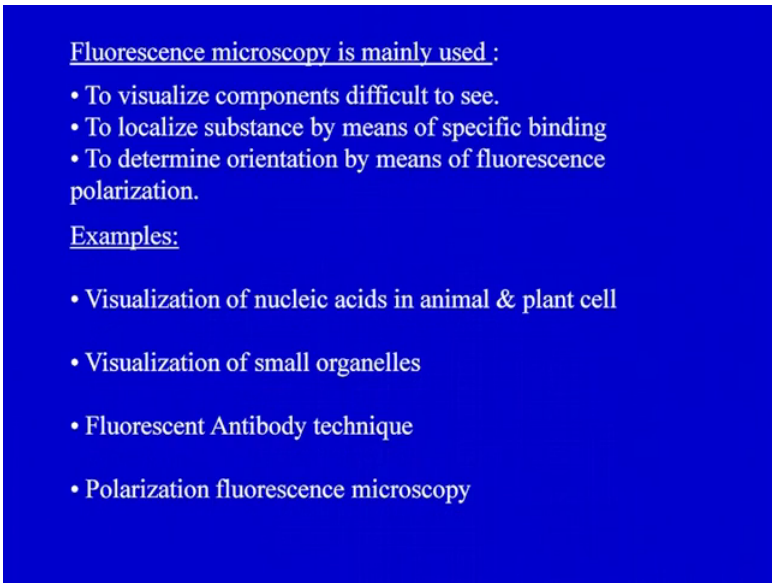

Now, what for fluorescence microscopy is mainly used, one is to visualize components, which are difficult to see. Then it is used to localize substances by means of a specific binding and to determine orientation by means of fluorescence polarization. We have already discussed about polarization. If you know, we will discuss this, how fluorescence polarization could be utilized.

So, when many components, which are in very low quantity, can be visualized by fluorescence microscopy, if you tag it with fluorescent compound, a particular macromolecule could be located and followed by observing its fluorescence as a function of time. Let us take some examples of application of fluorescence microscopy. One is visualization of nucleic acids in animal and plant cells.

Now, acridine orange, one of the dyes binds to both DNA and RNA, but gives a green fluorescence with DNA. If dye concentration is high, it will give an orange fluorescence with RNA. Now, eukaryotic cells will exhibit a bright green nucleus and pale green cytoplus at low concentration of dye. But, at high concentration, the cytoplus will also become orange. That is RNA will be also showing the fluorescence.

So, during mitosis, chromosomes glow bright green and could be observed in living cells for their morphological characteristics. Likewise a cell, which could be infected with DNA virus, can be detected. So, you can detect the DNA of virus as the green spots in

cytoplus rather than nucleus. So, this is a clear indication that DNA virus has infected a particular cell.

Another could be visualization of small organelles, for example, the yeast nucleus was hard to see because of its small size, but with the help of acridine orange, the DNA or the nucleus could be localized or nucleus could be seen actually here. Since, the yeast has the large RNA to DNA ratio; that is why, it was very hard to see the nucleus. It will take time actually, but with this particular technique, it was able to, scientists were able to do that.

Another application could be through fluorescent antibody technique. Like I told you earlier, fluorescent dyes covalently coupled to antibody can be used to localize the specific substances in a sample. Now, cell components, viral antigens, cistones and many other substances for that matter could be localized and individual cells or tissues could be followed.

Also, the proteins, actins and myosin in muscle fibers were distinguished by fluorescent labeled anti-actins and anti-myosin antibodies. So, like immunofluorescence could be a very useful for application of in various areas. Another important application could be by utilizing polarization fluorescence microscopy. That is you would like to know the orientation, the angular polarization of the fluorescence with respect to DNA helix axis could be, if you know the plane of the polarization of a fluorescent dye.

For example, if you know the plane of polarization of acridine orange, which intercalates between DNA biospheres, then you will be able to know the orientation of DNA helix as well. This has been used to find orientation of DNA molecule with respect to chromosomes actually here. So, this is one very good technique. Polarization fluorescence could be a very good technique to know the orientation of a macromolecule in a particular complex structure actually.

So, as we have seen in fluorescence microscopy that you could utilize it for detecting very small amount of components, it is a very sensitive method. It gives a very good contrast. It can be used for both life cells as well as for like stem cells and life cells. You can detect small amount of material. You can follow it like we have seen through immunofluorescence or gfp binding and other techniques could be utilized for that purpose.

So, you have lot of applications of confocal microscopy. The important part in optics of fluorescence microscopy, important part in fluorescence microscopy is that in optics of microscopy is that you have to use optical filters. So, that particular excitation wavelength and emission wavelength could be separated. You can detect the material most of the or you can say modern fluorescence microscopes are epifluorescence microscopes, where the observation is from above and excitation is also from above. So, this was about fluorescence microscopy.

Let us discuss another technique, which is an extension of fluorescence optics. It is confocal microscopy. Now, confocal microscopy also utilizes the fluorescent optics like I said here. But, there are certain differences between confocal microscopy and the fluorescence microscopy. In confocal microscopy, you can even use thick cells, no thick tissues or what we say tissues and thick specimens. This could give you a three dimensional image, where the relative positions of different components can be visualized or could be observed.

Now, here in confocal microscopy is usually used with lasers like we will discuss here in optics. Now, instead of illuminating whole specimen at once as in conventional fluorescence microscopy, we have seen confocal microscope uses point illumination that is you will illuminate a particular point in the specimen. A pinhole in optical conjugate plane, in front of the detector is placed to eliminate out of focus signals. So, what you have is the excitation wavelength, which is coming out of one pinhole is in same focal depth as the pinhole in front of the detector. So, they are called confocal and another part which is in focus, same focal depth is the point on specimen.

So, almost three points are here and the same focus. Now, the optical system focuses the spot of light on a particular focal plane in the sample. Now, supposing if you would like to make a three dimensional image of certain object or sample say a tissue, and then you have to make lot of optical sections. You have to make three sections of that particular one. You have to take the images of each section. Later on, you have to combine those sections. Each of the images are from different optical section are to build a three dimensional image.

Here, in confocal microscope, you can do that in the same tissue without sectioning. What you have to do is that you have to rather focus the light at different points like you

are restoring it over or you are scanning the whole specimen in a rectangular fashion at various depths. Now, this particular technique requires bright source of illumination that focuses on a particular focal plane on the specimen.

We will come to the optics in a little while, but the fluorescence emitted from the sample is collected and brought at an image suitable like detector. So, what is done here? If you see the technique enables the least construction of three dimensional images by scanning the sample over a regular raster, which we call rectangular pattern. Parallel scanning lights technique allows a high optical resolution and contrast as compared to conventional techniques.

Also, thick samples could be scanned at different depths to obtain images and these all the optical images, like I told you like optical sections. You are not really making a section. It could be combined to reveal relative positions of components in three dimensions. Let us see the optics of a schematic of optics of confocal microscope.

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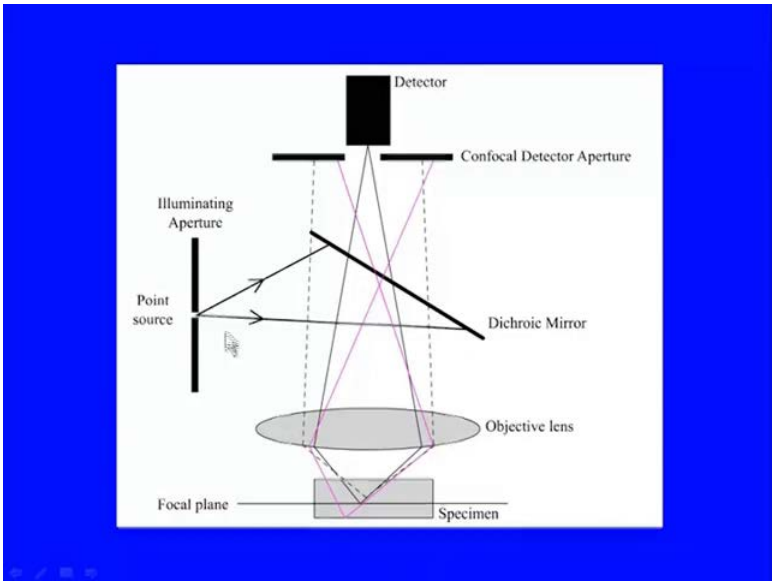

Now, if you can see here, there is illuminating aperture through which the lasers are pointed at the dichroic mirror. That is the beam splitter as we have seen earlier. Now, here lasers are required because it requires high intensity and focus. So, these lasers are, and then through objective lens are focused onto the specimen. Now, difference is here is rather than focusing or illuminating the whole specimen, it is focused onto a particular point in the specimen.

There it goes like distance in rectangular fashion at different depths. You have lot of images generated. Now, here like when it is at a particular focal depth, a focal point, then light, which or fluorescence, is emitted from here. As it goes through objective lens, it is allowed to pass through detector like I earlier, we have already discussed about this that it will allow the particular wavelengths to pass through. So, one in focus with the detector, the point source and the point at the specimen, the light emitted from there or fluorescence emitted from there will pass through.

This particular aperture, which is confocal detector aperture and the out of focus lights, will not be allowed to pass through. So, what you get is at a time, you get an image, which is in focus. Then to change the focus like to raster or to go to different depths, you will change the focus of both these points in here. Subsequently, the focal point on the specimen will also change. You will collect lot of optical images, which could be combined to give a three dimensional image.

So, this is a very simple schematic of confocal microscope. It is like epifluorescence microscope with difference that laser is being used. These 2 points are in focus with each other that is they are confocal. So, this was about confocal microscope and confocal microscope as we have seen are you can say, could be utilized for thicker specimens. It could be utilized to generate three dimensional images. Now, before let us before we end this section, let us also discuss about little bit about specimen preparation for light microscopy.

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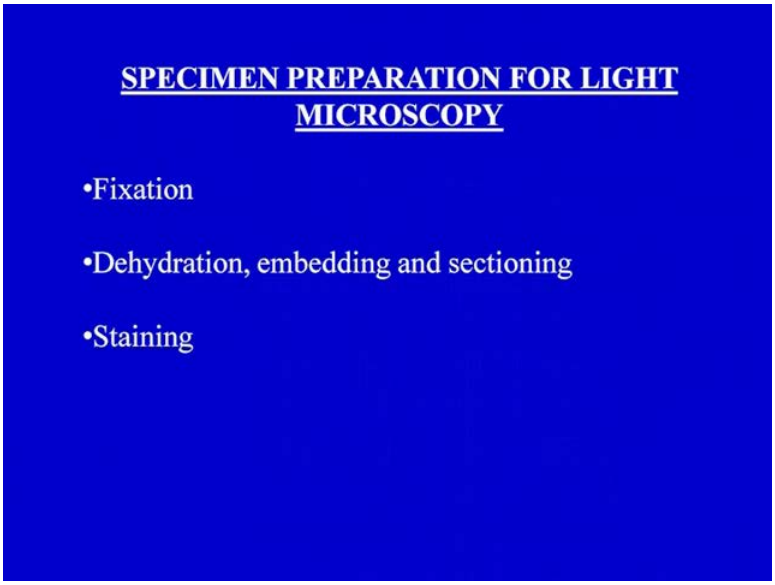

Now, if you could remember, we have told, I have told you that there could be two kinds of samples can be used here. One is you can use stain cells, but staining kills the cell. Lot of processing is required where many times the internal architecture could be disturbed, but you try to retain that architecture through different ways or different processing steps. We will discuss here. Another was life cells where you can utilize the cells as they are. You have thin layer of cells or a single cell could be utilized, could be used as a specimen to see in vivo phenomenon or activities as they are going on.

Most of the microscopic techniques, which we have discussed other than bright field microscopy, they utilize, and they could be unstained samples. But, still you have to prepare the samples, which could be unstained. They should be like you have to prepare sections of the specimen to visualize that is thin sections of the specimen has to be made for visualizing. Of course, you can use simple thin layer of cells or life cells could be utilized, but still preparation of sample is required in lot of cases.

So, we have in bright field microscopy, certainly you have to stain the cells because you will not be able to see the specimen without staining. So, let us see how the specimen preparation for light microscopy is done and very brief we are going to discuss this here. Now, specimen could be whole amount or that could be section. Now, many times specimen could be opaque.

Now, if there is an opaque object, then light will not pass through it. You will not be able to observe anything. So, opaque objects needs to be made translucent by substituting water with alcohol and immersing object in solvents such as toluene or xylene in which they become clear. So, that has to be done if there is opaque object. Now, the specimen preparation steps involved in specimen preparation are fixation dehydration embedding sectioning.

Finally, staining is required. So, let us see each of these steps. Now, fixation, the specimens are generally fixed with solutions containing alcohol or formaldehyde or acetic acid. Now, what is the role of this? What these compounds do is they denature most proteins and other macromolecules and formaldehyde cross links also amino groups to adjacent molecules.

Now, when this cross linking occurs, it stabilizes the protein. Protein nucleic acid interactions eventually render molecules insoluble and stable for further procedures. So,

what you have done is you have tried that the internal arrangement is not disturbed. Somehow, you have tried that the internal arrangement through cross linking could be preserved here.

Once fixation has been done, then dehydration has to be done. In dehydration, you have to remove the water. So, after fixation, the tissue is dehydrated by series of transfers through alcohol water solutions. Now, the alcohol water solutions to pure alcohol and finally, a solvent like xylene. So, what happens? You have removed water. Now, it is like you have completely dehydrated the sample.

Once you have dehydrated, the next step involves hardening of the sample to make sections by placing the sample in warm liquid paraffin or a plastic. So, what is done is in a tube or a plastic tube, warm paraffin is taken and cells. Your sample is put in there. Now, as they cool down, the warm liquid paraffin will solidify. So, the sample will solidify. So, it is like this process is called embedding. So, after embedding, once you have embedded the samples in, then piece of specimen will be mounted on the arm of a microtome. What is a microtome? Microtome is utilized for sectioning thin sections.

To obtain thin sections of the specimen, I will show you how it works actually. The arms move up and down and over a metal or glass blade cutting sections of very thin sections of a few microns, which is micrometer. Around 1 to 10 micrometer thick sections is cut. Now, alternatively there could be another method where you do not want to process, do so much of processing, could be fusing the samples, and then sectioning them, but you require special instrumentation for that to be used in a cold condition. So, we are not going into that, but that is also very useful technique.

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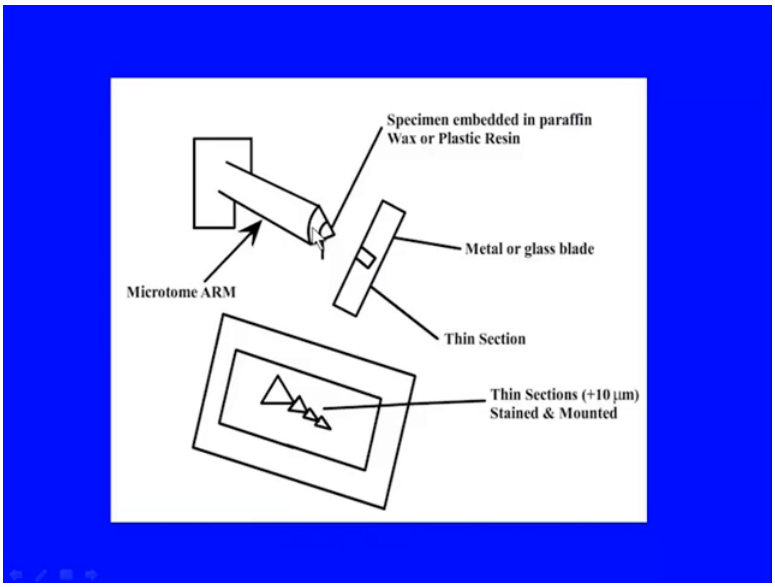

Now, this figure shows in here a microtome. So, microtome has this. This is a microtome arm. This microtome arm, if you can see here is specimen, which is embedded specimen paraffin or wax, which is hardened is put in here. This microtome arm is moved up and down rapidly. It moves through this glass blade or metal blade. There is a trough, water trough here where the sections fall. These are sections. Like I said, there would be around, what you call 1 to 10 micrometer sections here.

Now, these sections could be mounted. They could be observed under microscope if staining is not required directly or they could be mounted after staining. So, this is how the microtome is utilized for sectioning or making optical sections of the specimen. Remember as we will discuss later on electron microscopy, there will be ultra microtome. It will be similar, but much thinner sections are required in electron microscopy that we will discuss later on.

Now, supposing if staining has to be done, then what are different methods? What are different dyes to be are utilized for staining? Many chemical stains or we say dyes are available for staining procedures. Now, these chemical stains will bind 2 molecules with specific features. They could be specifically or differentially binding to different molecules, so that you can differentiate them. For example, haematoxylin binds 2 basic amino acids like lysine and arginine on many different kinds of proteins, whereas using binds to acidic molecules like nucleic acids aspartate or glutamix cysteines.

So, these are aspartic and glutamix cysteines are acidic in nature, whereas lysine and arginine are basic in nature. So, one binds to basic residues. Another binds to acidic residues. Now, because of this differential binding properties, these dyes stain various cells to or in components of the cells differentially so that you can distinguish them visually. There are many other dyes utilized. They could be like 2 other common dyes.

Benzedrine is utilized, which binds 2 hemi containing proteins. There is fusion, which binds 2 nucleic acids or DNA. It could be used in different stains. Other dyes could be malachite green, Sudan black, coomassie blue. They have a specific specificity for particular components, which could be sub cellular components of macromolecules. I think all of you must have heard about gram stain, which has been developed by Christian gram. It has been used for differentiating 2 large groups of bacteria, which is gram positive bacteria, which stains and gram negative bacteria, which does not stain. So, there are whole lot of different kinds of staining dyes are available, which bind specifically.

There could also be cytochemical staining. In cytochemical staining, it could be employed for detecting an enzyme in cell sections of an enzyme. Enzyme catalysis is a reaction that produces a colored visible precipitate from a colorless precursor. So, cytochemical staining could be utilized to visualize a certain sample actually. Likewise, antibodies could be utilized. So, lot of different methods is available for staining the samples for bright field microscopy and other microscopy techniques.

So, this completes our section on light microscopy. In this section, we have discussed all different light microscopy techniques right from bright field microscopy to dark field microscopy, phase contrast microscopy, differential interference contrast microscopy, fluorescence microscopy, polarization microscopy, confocal microscopy. These techniques, they have different applications, but mainly they are utilized for viewing different kinds of samples. You have seen that dark field microscopy gives you limited resolution, but gives you excellent contrast.

Phase contrast microscopy is a valuable technique for visualizing lot of different cell components, sub cellular organelles, macromolecular assemblies and so on. It is widely used technique for even recording or even visualizing the phenomenon as it is going on

in real time in the cell. Like for example, the movement of mitochondria or other different phenomenon.

Differential interference contrast microscopy is where you could get rid of those diffraction hallows. As we have seen in phase contrast, a pseudo three dimensional quality could be obtained. In polarization microscopy, certain amount, certain structures, which are arranged in a particular fashion, which could be a parallel bundles or step sticks could be utilized for giving you certain patterns through polarizer and analyzer. You can visualize lot of different images, which are very hard to see with other microscopy techniques.

Likewise fluorescence microscopy utilizes and confocal microscopy utilizes the fluorescence phenomenon. In confocal microscopy, even you can use thick sections or tissues. Three dimensional views could be obtained by combining lot of different optical section images from lot of different optical sections and a very useful technique for different application. So, I hope you have been able to understand the different microscopy techniques, light microscopy techniques and would benefit from it.

Thank you.