Analytical Technologies in Biotechnology

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Module - 1
Microscopy
Lecture - 4
Differential interference contrast and polarization microscopy

In this lecture, we are going to discuss two light microscopy techniques, which uses plane polarised light. These are differential interference contrast microscopy and polarisation microscopy, each of that we are going to discuss in detail. Now, if you could recall from the last lecture, we discussed about phase contrast microscopy. Now, in phase contrast microscopy if you could recall, the face differences have been translated into intensity differences, and that is how contrast was created for image formation.

Now, in this technique also here in differential interference contrast microscopy, the face differences will be converted to intensity differences, but with a difference. If you could recall in phase contrast microscopy there were diffraction hallows surrounding the specimen which blurred the area near the edges actually, and you are not able to see things clearly. But in differential interference contrast microscopy that problem has been solved.

So, this particular technique like microscopy technique is also called Nomarski in interference contrast microscopy and who's invented by its name comes from George Nomarski in mid nineteen fifties. Now, this technique is used to enhance the contrast in unstrained transparent samples, the images formed without bright diffraction hallows as was the case in phase microscopy. Now, in transmitted light differential inter interference contrast I call it d i c are for convenience. The two Nomarski modified bolasterone prism are inserted, now one prism and then again I will call this prism as d i c prism. So, one pair prism is inserted before the condenser and the second after the objective lens.

Now, these bolasterone prism are type of prism which are made of two layers of crystalline substances such as quartz, which is due to variation in refractive index depending on the polarisation of light it splits the light according to their polarisation.

So, these are the ones which are used in polarisation microscopy in place of face ring or in place of phase annular aperture.

So, here are it is quite different from phase contrast microscopy, though the basic principle of conversion of face differences into the intensity differences is same. Now, let us first understand this and let us go to the screen and see how this works actually. Now, here in d i c microscopy technique like I said, there are two prisms are inserted. So, apart from other lenses like condenser lens, objective lens, the additional insertions are analyser, polariser and two prisms actually.

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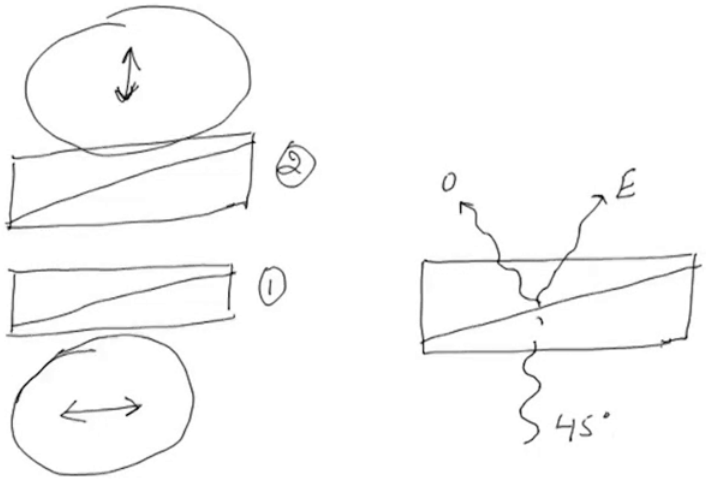

Let us see how this works out and to understand this if I say, if this is one of the analysers which stars and this is oriented it is axis is oriented in east-west direction. Now, above this will be a condenser and above this will be a prism that is the modified Wollaston prism. This prism is number 1 prism or we can say first d i c prism before condenser lens. Now, second is a general schematic I am giving you, I will give detailed optics later on and above this will be another prism, now this another prism like I am not saying like I said whole thing here.

Another prism which is number 2 d i c prison and it is after the objective lens, it is placed after the objective lens. Above that you will have another polariser which we call analyser actually and which has the axis oriented in different, like here analyser was in east-west direction, but this is in north south direction. So, what you have is this is the

arrangement, now when you have analyser and polariser at ninety degrees or perpendicularly oriented to each other, they are called crossed actually. Because any light which passes from polariser will not pass through analyser.

So, now let us see how the d i c microscopy works, now in this particular one the first prism that is the prism just above the condenser lens. Now, here first thing is the light has to be polarised, so if I say most of the light general light is unpolarised that is it is vibrating in different directions, when it is being propagating in one direction. Now, all these vibrations if they are there then is called unpolarised or non-polarised, but when you allow only one direction or you allow the light through polariser which becomes, which is vibrating in simple one and at one angle then it is called plane polarised light.

Now, this plane polarised light when it enters, if I say this is a plane polarised light and it is entering the modified wollaston or d i c prism number 1. Now, when it enters the prism number 1 it gets splitted into two separate rays actually, now one is called ordinary ray, which is denoted by E and another is called extra ordinary. Now, these two rays they are specially separated or we call in this term sheared, sheared means they are adjacently they are moving in the light path or an optical path. Now, these two rays here which are sheared, they are like if this ray is oriented its axis of rotation is 45 degree than these are, two of these rays are perpendicularly oriented which could be 0 degree and 90 degree, so they are perpendicularly oriented here.

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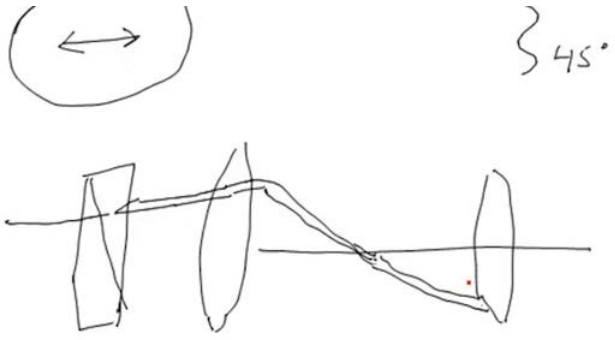

Now, if we go further, if I would like to show this on in a different way it could be like supposing there is a prism here and there is a condenser lens which will be focusing the light. Than the light comes in here and then light is divided into two and the adjacent light goes in here, which will enter the condenser and then will be focused onto the specimen plane.

Then finally, it will be passing through the objective lens which will again be sheared only and then finally, it will be combined by the next one which is the wollaston prism number 2. Now, let us see how this is combined by your prison number second. So, what is done is in here is like I said the face differences are converted into intensity differences.

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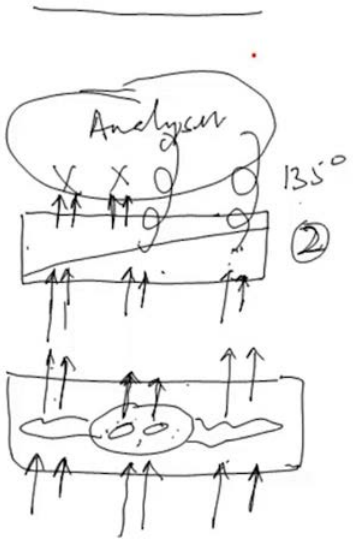

So, you have number 1 prism and you have the light coming in here, there be different lights will be coming in here and this light will have two. So, it has to pass through first the specimen and then the prism number 2, now before it passes through specimen it is both are perpendicularly oriented. Now, when they pass through, so this is prism I will show you this later, but hear what you have is, if you have a specimen in here and specimen will contain a lot of things, it might contain like cell contains like I have shown you earlier the cell will contain different things here.

What you have is in this particular one as the light, which is adjacently moving around, the light which is passing directly will not be changed so much in face, but this light

which is passing here will be changed in face actually. So, what you have is you have two lights passing here which are perpendicularly polarised, when they pass through the specimen or other parts of the specimen, they will be differentially, they will be retarded.

This light which is differentially retarded will be these two lights will be combined by wollaston prism number 2 and they will be like one could be combined and another could be blocked actually at this particular, if I say this is analyser. So, some of them which are perpendicular to the axis of analyser will be stopped and one which is parallel to the analyser, this I will explain it further, it is polarised 135 degree. So, what is happening? Let us little bit repeat it to make it clear.

So, light from the first prism, number 1 prism which goes through and this light passes through the specimen, now this light was single and here it is sheared actually. So, all the parts of this prism this is sheared this passes through condenser lens focused onto the specimen, this specimen might contain different things. So, light is retarded differentially here and these two lights are then combined as they passes through the specimen and through objective into the condenser number into the d i c prism number 2, they are combined and they are now polarised at 135 degree.

So, first it was 45 degree polarisation when they came out of the polariser, then as they move through condenser and specimen they are still sheared, they are spatially displaced, they are not combining, they are not allowed to interfere. The sheer distance is around 0.2 micrometre or less, as you decreased the sheer distance also you increase the resolution, but that one has to set accordingly. Now, once it passes through, then like I said there would be differentially absorption of due to the thickness and refractive index. This light which goes will be combined by prism number 2 and where it could be combined into elliptically polarised light at 135 degree.

Now, light which is like this light, if I say it is blocked or this light is blocked here, which is perpendicularly oriented to analyser, then this will form the background. So, there will be two kinds of interference, constructive interference and destructive interference, so one which is direct light will be blocked, because analyser is set perpendicular to the polariser. The one which is polarised after combination will are in the plane of polarisation matches with axis of analyser, they are parallel to the axis of analyser and they will be passed and they will form the image on the image plane.

So, this particular one here plane polarised light as it is passing, that is combined light only passes and gives various interference patterns to give image which could be dark or bright as mostly it has a three-dimensional quality, because it gives shadow on one side. Let us return to our discussion, so let us go through and understand this like I have shown you on the screen that how a plane polarised light is separated. So, let us go through the path of the light in this particular technique.

Now, what is happening? First thing is a polarised light is generated at 45 degree; this polarised light is generated by a polarised. Then the polarised light enters the first d i c prism, where it is separated into two beams polarised perpendicular to each other by first Nomarski modified wollaston prism or d i c prism as we call it. Now, both are especially displaced or we call it sheared at the sample plane and therefore are not able to combine to cause interference.

Now, sheer distance is less than the resolving inability of objective, like unlike microscopy it is 0.2 micrometre. Now, two rays separated by the sheer will be focused by the condenser onto the specimen plane. Now, the specimen plane as the sheared light and they are remember these two rays of light will pass through adjacent areas and they will be differentially retarded due to the thickness and the refractive index of specimens components and so they will be retarded accordingly. Now, what will happen? This will cause a change in the faces of two rays, so they are little offset here relative to each other, they are offset from beginning actually there is one extraordinary day another is ordinary day.

Now, once this face differences introduced, now they are travelling with particular phase difference from each other. So, what you get is this parallel beams enters the objective and they are focused for on the second d i c prism. Now, what does d i c prism does? Second one its function is to recombine the two raise at a defined distance from the prism. The second polariser or we call it analyser above the prism will facilitate the interference of two parallel beams by bringing them in the same plane and access.

Remember like I told you that the finally combined beam is 135 degree and it is passed because it is parallel to the plane of polariser or axis of polariser sorry analyser, I should say it is analyser that is a second polariser, but it is analyser after it is being placed at the

second place. So, this will not allowed in the direct light to pass through which will form the background and this phenomenon is called extinction also.

As you see the images in d i c microscopy the one side of an object appear bright, while other side appears darker in parting us to do three-dimensional appearance to the specimen. So, this is one very important technique for various applications. Now, let us have a look at the complete optics of like we were explaining this, but let us have a look at the complete optics of this d i c microscopy.

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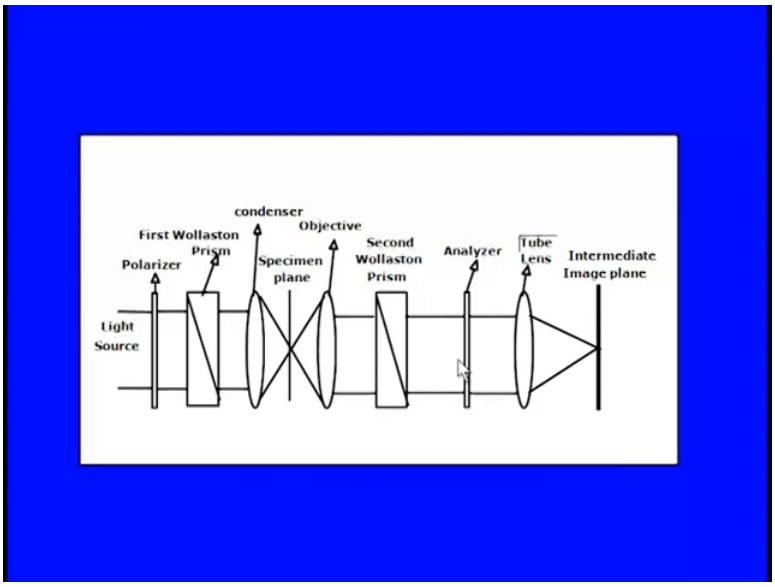

As we have explained we will just go through here and will try to understand. The light source which is unpolarised pass through polariser, which is now a plane polarised light at incidence angle of 45 degree. First wollaston prism here it is not shown, but this is a sheared like that is two rays adjacent to each other very close to each other, but they cannot interfere. So, they pass through the condenser lens, as they pass through the condenser lens, they passed through a specimen plane where they are differentially retarded because of thickness and refractive indices differences.

The objective focuses them onto the second wollaston prism which combines these adjacent rays into one, where plane polarised light which is at 135 degree and is elliptically polarised, which is allowed to pass through the analyser and images formed at the intermediate image plane. The light which is direct which is perpendicular to the axis of analyser will not be allowed to pass and form the background and this phenomenon is

also known as extinction. So, this is about the optics of d i c microscopy and like I said it could be used for examining the lifestyles and lot of other, it can be used in medicine, it could be used in geology and a lot of other nonbiological samples can also be examined on d i c.

So, and then it is devoid of any diffraction hallows like in phase contrast, this is one of the most useful technique in the light microscopy with a lot of applications, so this was about d i c microscopy. Now, let us move onto the second technique which we are going to discuss in here which is polarisation microscopy. Now, polarisation microscopy also utilises a plane polarised light, but what is the main principle behind this microscopy, let us understand this.

Now, I think all of you know that certain structures in biological samples have a particular pattern, like these consists of either elongated particles in parallel arrays or they are arranged in stacked disks, embedded in the medium with a different refractive index from that of the individual structure particles. When there are arranged in this particular fashion they exhibit form birefringence, that is the structures will pass plane polarised light only if the plane of the polarisation is parallel to the particles and this shall be true even of the particles themselves alone are not intrinsically birefringence.

So, these are birefringent objects, which could be a non biological source also like crystals or this is like particular arrangements of these particles individual particles allows them to become birefringent and this phenomenon is used in polarisation microscopy. Form birefringence is easily observed in cellular systems using the polarisation microscopy. Now, before we go any further I would like to little bit explain what is a parallel array and what does I mean by the perpendicular arrays actually here or a perpendicularly stacked disk? So, let us see what does that mean actually?

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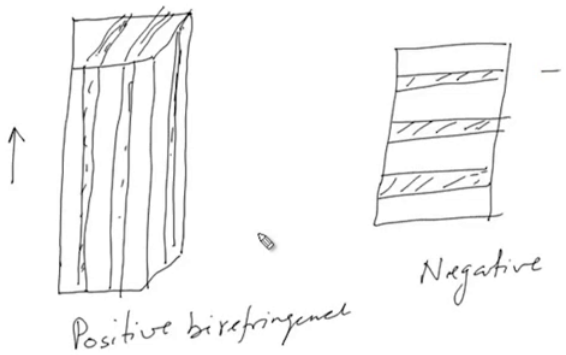

Now, the arrangements are that if you have this particular kind of petrol here and if your things arranged like I would say these particles are arranged in a fashion like this. You could observe them, these are we will call this is parallel arrays actually and this is called positive birefringence. Now, vector which is may be a plane polarised light will pass through this because plane of polarisation is same as the arrangement of these fibres that is parallel to the arrangement of these fibres. Now, again another arrangement could be stacked disks which could be differently arranged, rather than parallel they are arranged in that stacked on top of each other.

So, this could be another arrangement which could be present in here and this is called Negative birefringence and here your vector will be in this direction, which will be passing through the negative birefringence. So, you have two kinds of arrangements positive or parallel arrays, which shows Positive birefringence and the negative birefringence which shows stacked disks which shows negative birefringence, so let us return to our discussion.

So, what we have is that like I said this particular technique is of great utility, because it can be analytically used to determine orientations also, not only that in certain cases it is the only property to visualise the structure. For example, if particles cannot be stained for certain reason or the specific refractive increment is not too large to generate phase differences which are large enough to be visualised by phase contrast or d i c

microscopy, then you would like to use the polarisation microscopy. If you take examples like this thylakoid arrangement in chloroplast has been seen before in polarisation microscopy before it was seen clearly in the electron microscopy.

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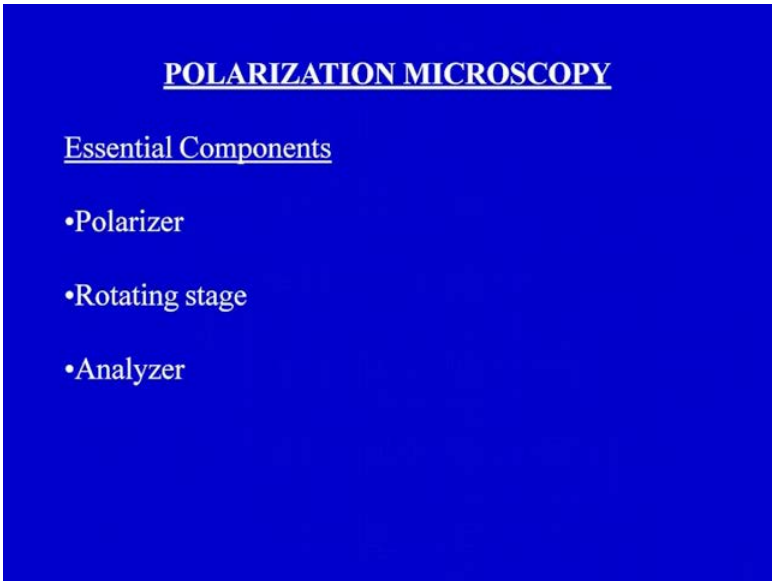

Now, let us move onto what are the essential components of the polarisation microscopy or polarised light microscopy we also call it. Now, the specific components of polarisation microscopy are, one is like we said different polariser and analyser, like have shown you the d i c microscopy there will be to polariser and analyser. Polariser is placed between the light source and condenser and the analyser is placed between objective and eyepiece and we will see how in the optics will see how it works out, then there could be other like components.

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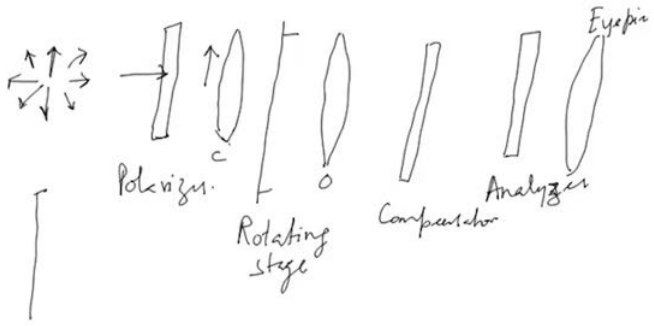

Let us see on the screen how does it work out, so what you have is first thing is you have Unpolarised light actually which is vibrating in different directions, which could be many directions it could be vibrating in. This unpolarised light will be converted to the polarised light here. Now, this polarised light is formed by the polariser like I said what you have to have is polariser, the essential components we were talking about first thing is you have to have a polariser.

So, polariser will be placed if there is a polariser placed in here or I could say I can make it here and polariser which will generate a polarised light. Then there is there has to be analyser which will placed between the objective and eyepiece. So, now what is going to happen? This plane polarised light, the plane of the polarisation axis of polariser and analyser are crossed, that is as I explained in d i c microscopy they are at oriented at 90 degree each other. So, any light passes to polariser will not be able to pass through analyser.

So, it means that that since there 90 degrees oriented, the field or the image field will look dark here. What are the other components? Will come to this how the image formation takes place, but let us see what are the other components which are important here? So, you will have a condenser lens and I will show you this complete optics clearly, you have condenser lens and then you have a specimen play. Now, this has to be

remembered that this stats another very important part of the polarisation microscopy is the rotating state, which is can be rotated at 360 degree.

Why do you require a rotating stage which could rotate at 360 degree? The rotating stage allows us to rotate the sample and so that it could be a oriented at different angles to the polariser and analyser and therefore we could see the image in the image plane and will explain it further as I go along. Your condenser lens and then you will have a objective lens, this will be condenser lens, this will be objective lens both has to be strain free as you are including a lot of things in here, so these are strain free objective and condenser lens.

Now, light will pass through analyser and many times you might have a compensator put in here, which is will compensator will see the activity of function of compensator we will discussed later on. Then there be eyepiece which is eyed ocular lens, now this eyepiece has a particular property that it is which fitted with a crossed wire radicles of graticules is to mark the centre of field of view.

So, these are few different important parts of the polarisation microscopy and which are little different like I said strain free objective and condenser lens, the polariser, analyser, special eyepiece with crossed wire fitted radicals of graticules, these are important parts of particular polarisation microscopy. Now, what happens actually in the polarisation microscopy and how this path of the light is followed, let us see that particular thing.

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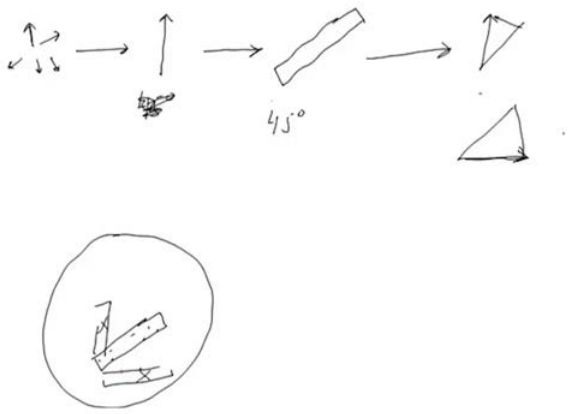

So, like I said what happens is that you have a non-polarised light, which is through a polariser becomes a polarised light at 45 degree or particular incidence angle actually. So, this is as per the axis of the polariser you will get a particular plane polarised light, now this plane polarised light has to pass through the specimen through condenser actually. So, polarised light like if I say my sample is oriented in this direction and I will just not mention this here for just for now, now this is oriented at a particular angle, your sample is oriented at a particular angle.

Now, remember when the polariser and analyser are crossed then there is a dark field shown here, because light will not pass through the analyser. Likewise if the plane of polarisation or the arrangement of the particles either in a parallel arrays or a stacked disks as per their arrangement. If their oriented parallel to the either analyser or polariser light will not pass through and there will not be any image seen here. But when they are oriented at some other angle, other than the parallel or perpendicular to the analyser or parallel to the analyser or polariser they will not be any light, but if any other angle is there then there will be certain light will pass through analyser.

Now, this light will be maximal at 45 degree angle say it is oriented, if their arrangement is oriented at 45 degree to either analyser polariser, the maximum brightness will be shown here. So, what you get? So, when polariser there is an angle here which is different, than the plane polarised light will be divided or be broken into two components. One will be, we can show here this will be one component will be like parallel and another will be perpendicular.

So, another component will get is this one and one component will get this, so this parallel component which is perpendicular to the analyser or through the polariser will pass through analyser, so what will happen? Through analyser you will be able to pass this particular component, that is which is parallel to the axis of analyser. Now, so what is going to happen? If I say this is the rotating stage and like I said, this is the one at 45 degree oriented its arrangement to analyser and polariser you will see the maximum brightness, through a bright crystal, bright image will be shown or seen of this particular material.

Now, if I rotate it like, so why rotating stage is required? As you rotated, if is it is in this direction or if it is in this direction they will not be seen, no image will be seen. Because

like I said they are again either they are aligned to polariser or their aligned to analyser and light will not pass to through. So, I hope you have been able to understand that how the path of light is followed and at an angle which is different from the axis of polariser or analyser. Some light can pass through with a maximum brightness at 45 degree, so when you rotate the rotating stage you will encounter, bright, less bright and dark and bright less bright likewise as you rotate.

As the angle changes the brightness of the image is also changed and that is how you can see the image in this particular way. So, this was to explain to you how this whole thing works. Let us return to our discussion and understand whole thing. So, most of the time the light which falls onto the specimen is parallel light to avoid the internal reflections of refractions, so that there is no problem in the image formation. Now, like I explained there could be two kinds things, there two kinds of objects one showing positive birefringence and another showing the negative birefringence.

Now, this will be depending on how their arrangement is, as I have shown earlier there are parallel arrays and positive arrays. Now, how do you distinguish between the two kinds, let us understand this. So, what we have seen is the light when it passes through either positive birefringence or the negative birefringence. It is has been observed that positive birefringence will be faster and the negative birefringence will be slower. Now, parallel direction has a lower index of refraction and allows light to travel faster than the perpendicular direction, alright?

So, how does that work out? If I say like I have shown you, now before I show this like let me explain this. What is done is a compensator is introduced in between and compensator and I will show you in this optics. The compensator we know it could be a simple crystal birefringent crystals of mica or gypsum or other things and we know the slow and fast direction of this crystal. So, if you align it with the fast direction of the object, then that is parallel, then it will be showing much higher brightness, when you align with the perpendicular direction then it will the brightness will lower term.

So, likewise knowing the slow and fast directions we are able to know the orientation of about sample. This is a very useful technique to know how the particular thing, even you do not know the details of that, but you will be able to tell that how it is arranged by simple particular technique. Now, let us see go to the optics of the polarisation

microscopy in little detail. We have tried to understand whole thing let us summarise everything in here.

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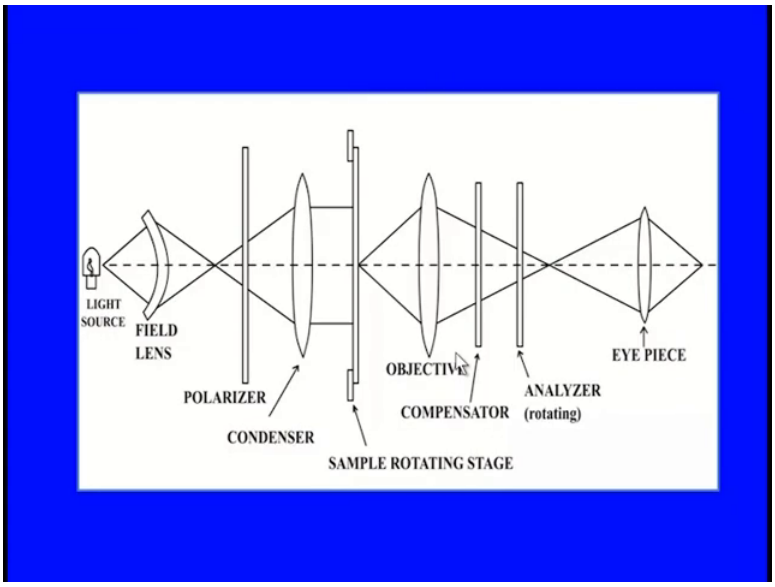
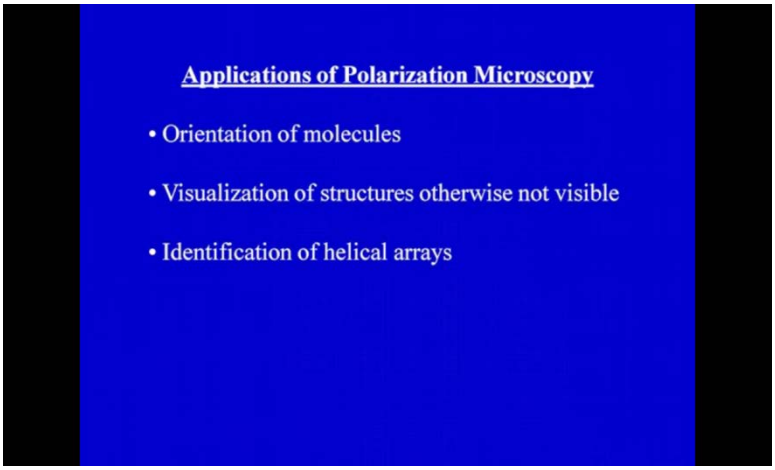


So, what you have is you have a light source, now light source like I said give unpolarised light and then you have a polariser through which plane polarised light will come through. Now, this plane polarised light is focused in parallel rays by the condenser, it is focused parallel onto the specimen. Now, this is specimen plane is a rotating stage, like I said you need to rotating stage to see relative brightness in darkness. As per the alignment of the plane of your arrangement of the particles in the structure and the plane of polarisation, they will show brightness as they will allow the light to pass through analyser or it will not allow the light pass through analyser or partly light is allowed to pass through analyser.

Now, objective will focus the race from the specimen onto the analyser and analyser will allow certain lights like we have discussed earlier to pass through. Eyepiece has cross wire with ratical like I said and you can focus the image at the centre of the field. Then image is formed, which will be bright at 45 degree most bright and you can rotate the stage to see different variations in the brightness of the object. So, this was about the polarised optics of the polarisation microscopy. Now, let us see what are the uses of polarisation microscopy?

Now, polarisation microscopy gives detailed information about molecular architecture and in some studies of living cells this is the only applicable method, because the more precise and sophisticated techniques require are either bright sample or large volumes. Let us get into some of the examples here and see how it could be applied. (Refer Slide Time 38:07)



Now, the polarisation microscopy could be utilised for knowing the orientation of molecule, like I was discussing with you. Now, birefringence itself indicates there is a structure containing oriented molecules, and this birefringence measurements gives you indication of that how they are oriented. For example, this like a muscle cells have been seen to be parallely oriented by polarisation microscopy, likewise thylakoid in granum and the wrought cells on the retina has been found to be oriented in a stacked disk fashion. So, you will be able to know a lot of like these molecules which ever shows the birefringence, you will be able to know the orientation and with the help of the compensator this could all easily be facilitated.

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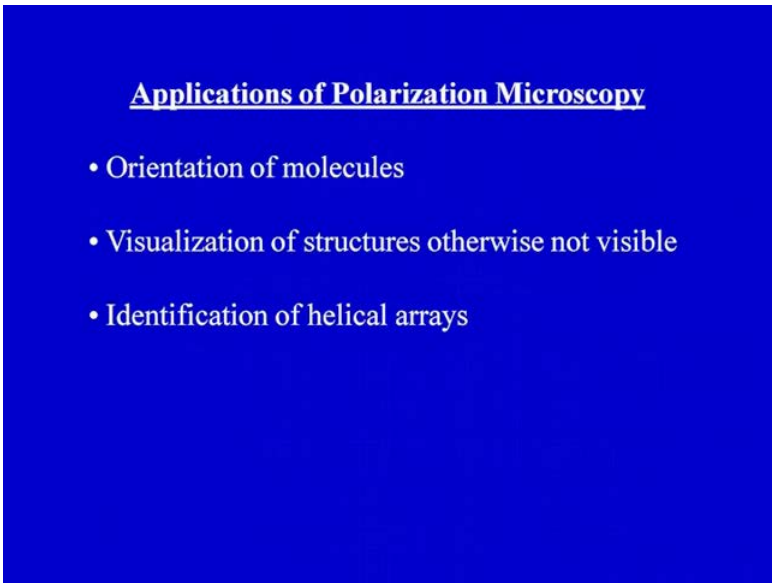

Then visualisation of the structures, like I said there are a lot of structures which are not being visible by other techniques. For example, mitotic spindles that are not visible by either bright field or phase contrast microscopy and this could be easily seen in polarisation microscopy. So, this one makes easier for to see as things are going on in mitotic spindle. Then you can identify the helical arrays. For example, if positively birefringent fibres are object or observed looking down fibre axis, then they we be like seen as a dark thing.

But if there are helical arrays, then light and dark patterns corresponding to the helical regions will be seen and so you will be able to recognise that the helical structure is present there. Likewise there could be a lot of different applications, like you can know the (( )) studies you can use a polarisation microscopy. You can use the polarisation microscopy to know the orientation of a macromolecule in a particular structure and there are whole lot of different applications in field of biology as well as non biology. So, this completes the section on this particular technique.

So, today what we have done is we have discussed about two important light microscopy techniques using clean polarised light. One was d i c or differential interference contrast microscopy, where the two modified prisms which are Wollaston prism were utilised and which used the principle of face conversion of face differences into intensity differences, where the light was plane polar polarised light was sheered. When it passes through

adjacent area of specimen it is differentially retarded and face differences are introduced. This technique was gives you much higher picture quality as compared to phase contrast microscopy, because it gives you bright and dark shadows on the same image and gives you a pseudo-three-dimensional quality.

In polarisation microscopy, like I said it also utilises plane polarised light, but it has certain other components also. Now, here the important part is that you can only observe those components or samples which are birefringent, owing to the arrangement of individual particles in particular fashion, that could be parallel arrays or stacked disk. So, when polariser and analyser are crossed and no object is present that to see the dark field, but when an object is present at a certain angle. If it is aligned with either polariser or analyser you will not see anything, but if it is aligned at certain angle to both of them and light would pass through analyser.

At 45 degree you will see the maximum brightness and you will able to observe different birefringent objects and also you will be able to calculate or you will be able to do the orientation studies. So, it could be both qualitative and quantitative kind of technique and this is very useful technique, where you could see like at least if you are not able to see it with some other technique. You can see the particular structures in this technique, like for example we have taken mitotic spindle.

So, here you have to have a objective condenser and eyepieces which are little different from other optics and this is one of the most useful techniques in the light microscopy. So, we complete these two techniques which used plane polarised light. In the next lecture we are going to discuss about other two techniques which utilises Fluorosis phenomena.

Thank you.