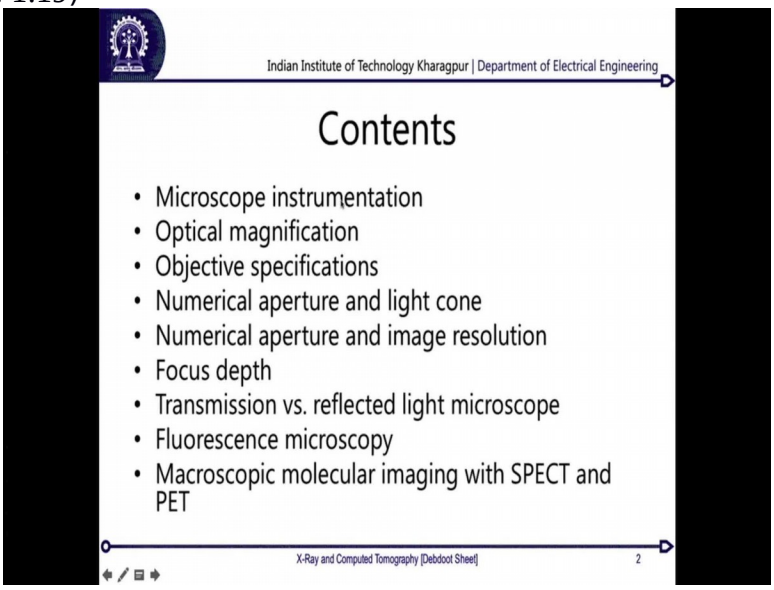
Course on Introduction to Medical Imaging and Analysis Softwares
Professor Debdoot Sheet
Department of Electrical Engineering
Indian Institute of Technology Kharagpur
Module 01
Lecture 05: Optical Microscopy and Molecular Imaging

Welcome to today's topic which is on Optical Microscopy and Molecular Imaging. So this is basically the last module which we are going to cover on all the modalities over here so we have already covered down modules on CT, MR, ultrasound which are majority of macro imaging modalities where you look into larger parts of your body and the whole body in as a whole in total. Now in microscopy is where you will be looking into very specific part of your body and now this is more of aim of at a portion of looking into just say one of the cells one single cell or a cluster of cells within your body which was not possible with any of the other modalities over there.

And from there enter into a very interesting phenomenon called as molecular imaging and this molecular imaging is where initially we will do it for molecular imaging for microscopy and from there we will move on to molecular imaging on the mass scale as well.

(Refer Slide Time: 1:19)

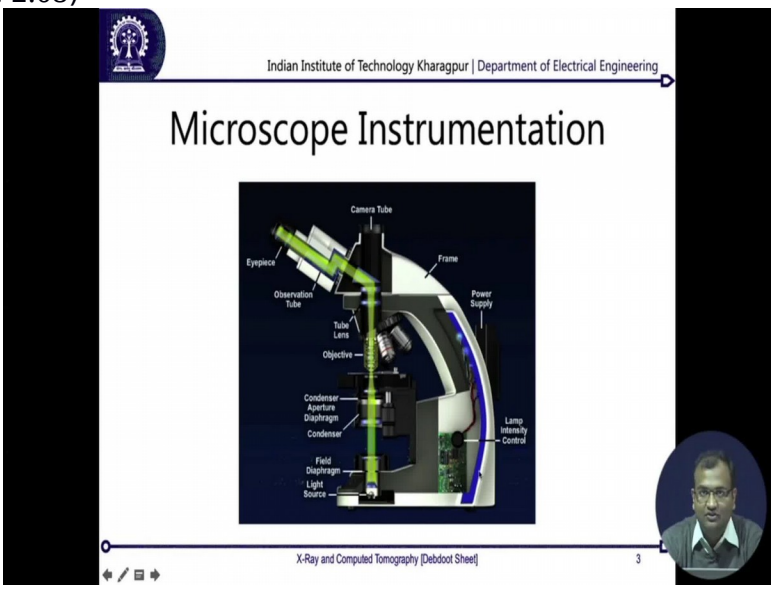


So this lecture is arranged something like this so I would be introducing you to you the microscope instrumentation and then the concepts of optical magnifications, what is an objective

and what are specifications of an objective, the relation between numerical aperture and light cone and the image resolution and what we mean by depth of focus.

From there to two different modes of microscopy which is transmission and reflected light microscopy. Although reflected light microscopy is not something which is very traditionally used for medical applications, it is used more of for material science applications but we do have one specific mode of operation called as florescence microscopy in which reflected light microscopy is the basic principle and that is where you get introduced to molecular imaging for the first time and from there I would enter into molecular imaging on the microscopic scale with SPECT and PET.

(Refer Slide Time: 2:08)



So this is what a microscope looks like, you might have seen them in your high school as well or some other experiments in some point of time. Now what it has is this part is basically a very sturdy body thing and you have some objectives over here which is an assembly of lenses tact one beyond the other and you have a few lenses over here which are called as cube lenses which are again for the purpose of magnification, there is a small prism over here which basically divides the light on to this part which is which goes into your eyepiece where you put your eyes and look into and the other part of light comes out over here which is called as a camera tube, you can put a digital camera over here, something like your web cam and just acquire those digital images over there.

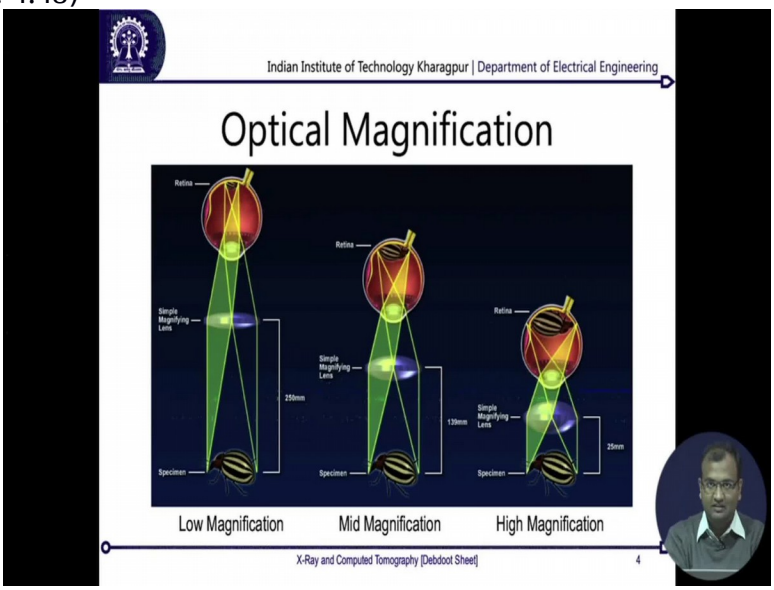
Now the light comes down from this part which is called as a light source so if you were in you are looking at your high school microscopes then you had some sort of a small mirror concave mirror and you had to take somewhere near the window and do your experiments over there. In more of these microscopes since we do not make them move them a lot and carry them along where the sun moves so we have a light box in which you just have one simple lamp and a diffuser placed over there and then this whole light passes through this assembly.

So from there we can control out the total width of the light which is coming out using this field diaphragm and aperture control diaphragms over here together such that we have a very narrow focused cone of light passing down through the lens, okay. So this is the standard diagram of any kind of a microscope so be it from a very cheap microscope you use in your high school to a very high end microscope which we have in the demonstrator on using a microscope for imaging and image acquisition.

So all of them have the same sort of a path connector diagram. Now when you have these objectives which are placed on top of the object placed on a slide then this kind of a microscope is called as an upright microscope. In other kind of a microscope which we will see in the demonstration as well these set of objectives are placed over here which is below and the light box is on top so that is just say vertically flipped configuration of this microscope so you have to look at the eyepiece which is present over here as well.

So that kind of a microscope where the objective is below the sample, that is called as an inverted microscope, just it is an inversion of the optical design of this one, that is the only difference between them otherwise the rest of the imaging and physics remains the same.

(Refer Slide Time: 4:48)



Now from there let us enter into what this microscope does. Now I will try to again review the concept of optical magnification. Now say that you had an object over here and through a lens you were looking at it. Now if the object is quite far off from the focal plane so you would from the focal length of this lens then you would see a very smaller version of it casted on your image over here and that is a very small one.

Now, if I keep on changing the distance between my eye and the lens as well as the distance between the lens and the object over there I would eventually come down to a mid-level magnification where I have almost the same size casted over here and if they are very close to each other then I would see that the total image which is casted over here is much wider and that is what is called as optical magnification in any way. So the total image which is cast on my retina or which is cast on the censor is much bigger than what it is in actual practice then it is so.

And microscope gets its name from the perspective that it can magnify objects in the order of micro which is in microns, so 10 power of minus 6 of a centimeter is what gets magnified to in order which is visible with naked eye, so in the order of centimeters is what is visible to naked eye. So if I magnify something in the order of 10 power minus 6 to a order of 10 power minus 2, then it makes it visible to the human eye in a very direct sense.

So that is what optical magnification means in its direct way and a microscope just does this part of it. Now it is not just optical that you have, you have other microscopes like electron microscope and atomic force microscopes which are very different from it but whenever in terms of medical image analysis and digital pathology we speak about optical microscopy then it just means that there are lenses and it is a lens based microscope and I am trying to magnify micron level appearances to something which is visible with naked eye.

(Refer Slide Time: 6:53)

Now one important thing which plays this major role in this magnification is called as the objective. Now what this does is this is basically a set of collection of lenses which will help you in magnifying, so instead of using one single lens which will make it really big and thicker and you will no more have a thin lens phenomenon, you can put cascade of lenses and pack them together. So this is one cylinder in which there are multiple lenses which are packed together and such that you get a very high magnification.

Now if you look at it, typically you would have a so I we do not have any so relation with Zeiss as such, it was just one of these designs I took and more of from the reason that we would be demonstrating on one of the microscope which has this sort of kind of kind of lens over there so it makes it easier for us to go through it. So one first part which you would see over there is the magnification, so this is a 63 times magnification one. So whatever comes over here is 63 times magnified when you look through this one.

So this is the primary condition of objective so you have objectives starting from 4x to 10x to 20x to 40x, 63x, 100x reaches a standard configuration. Now this is the first part which you will have to keep in mind, the second one is that what is the focal correction? So this is infinity corrected which means that whatever you see over here is a parallel beam and it would appear as if the object is placed at infinity and you are seeing that object over there which is quite important otherwise if this looks into a particular point that means that along this optical tarot over there or wherever we have the tube lenses than those tube lenses will also be have to place replaced exactly at the focus point.

Now if you are moving this objective up and down in order to look into your focus points, then your tube lenses will also have to move and it becomes a very complicated optical arrangement to solve. That is why we generally prefer to have a focal correction at infinity but there are some microscopes and some lenses which have certain limitations which they do not offer and they do make it very implicitly mention over here as well. Now the other part is this is a NEOFLUAR lens and what that means is that generally if you are looking down so think of this one.

Let us say you have a prism and you have a white beam of light going down over there so you will have a spectral slicing across the prism from with your total. Now, a lens is basically two different prisms with their bases together, okay. So obviously there is going to be some diffraction and now every point which is on focus that we not say there is a white point in focus on the other side but that white point will no more appear as white whereas there will be a rainbow like pattern which comes down over there.

Now we do not want to have that one because we want colors preserved and everything, otherwise this will create some sort of optical distortion. Now in order to get rid of that what they do is we put down certain chemical coatings on top of the lens such that for different wavelengths there are different refractive indices such that all of them convert at the same point and a white spot is again seen as a white spot you do not have optical abrasions.

So NEOFLUAR is a particular grade of such lens which specifies as to what is the amount of optical abrasion which is present over in that lens. And these are something which are of very good quality otherwise it is so a standard one is just a plan lens from where you will be getting down a fluar lens from there you get a apochromatic lens and and different varieties as well. So

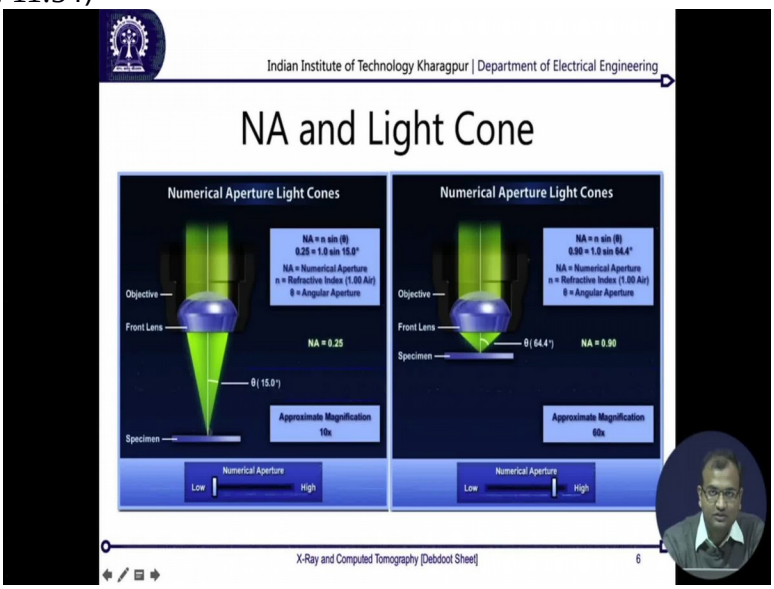
the other part is whenever you have a very high magnification lens over there you cannot just put it.

So it has to be put very close to the object. So as we were looking into that earlier slide where for higher magnification the lens is actually very close to the object. Now if it is very close it will just go and touch the surface of the object. So over there in order to avoid some wear and tear because of the object and the glass rubbing against each other we basically put down some kind of a lubricant and generally this can be something like a glycerin or oil or water.

And now the other point is that since you have some lubricant and everything paced over there, so this can leak inside the lens as well and that can create other kind of optical distortions, you do not want them to happen so there are certain sealing jackets and mechanical design constraints. So that are also mentioned over here, so this lens it points down as W so it is water immersion lens so you can only use it with water, so please do not use it with oil or anything and you need to be very cautious in the designs over there because this are specified and you need to use them only with those specified fluids for emersion over there.

So there are air emersion lenses now which are to be operated in air, you cannot use those lenses with water otherwise water would leak in and that would distort the whole optical arrangement and the durability of the lens as such.

(Refer Slide Time: 11:54)



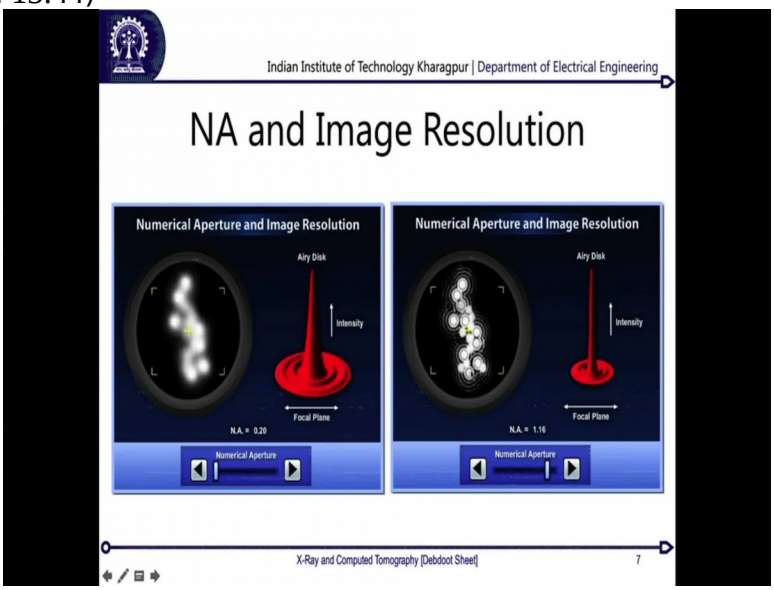
So once we are done with this one, now another interesting fact which is observed is the effect of numerical aperture and light cone. Now what happens is something like this that if your object is very far off and from the lens then you have a very small cone being projected over there and as the cone is small your numerical aperture which is dependent on n times sine theta and n is the refractive index of the material through which it is passing.

So in this part it is just air, so refractive index is 1. So your numerical aperture is again low because your theta over here is low. Now, as you keep on moving this object close to it your numerical aperture is also going to be high because your theta is going to be high. Now you need to keep something in mind that the numerical aperture or the angle of this cone should be matched with the angle of the cone of the objective as well.

So your object because if you have a lower numerical aperture of this lens over there and you are putting at a higher numerical aperture over here then you will basically have a less amount of light passing down over there, you cannot collect effective amount of light. And now, if you have a smaller numerical aperture smaller angle of the light being casted on the lens and you have a lens with a wider numerical aperture than you are getting going to get a lot of stray reflections coming down from the sides which is just going to be noise.

So the quality of image actually depends a lot on matching the numerical aperture to the light cone of the object which is being done and you generally from a user's perspective you do not need to be worried too much about that because these are matched down by the manufacturers themselves over there. And for each lens it is pre calibrated where you put the objective and where you put the sample and what should be the numerical aperture for that objective as well but if you are changing down objectives so if you want to replace one of your native objectives with a new objective, then do keep in mind and recalibrate the whole system, this is one thing to be kept in mind.

(Refer Slide Time: 13:44)



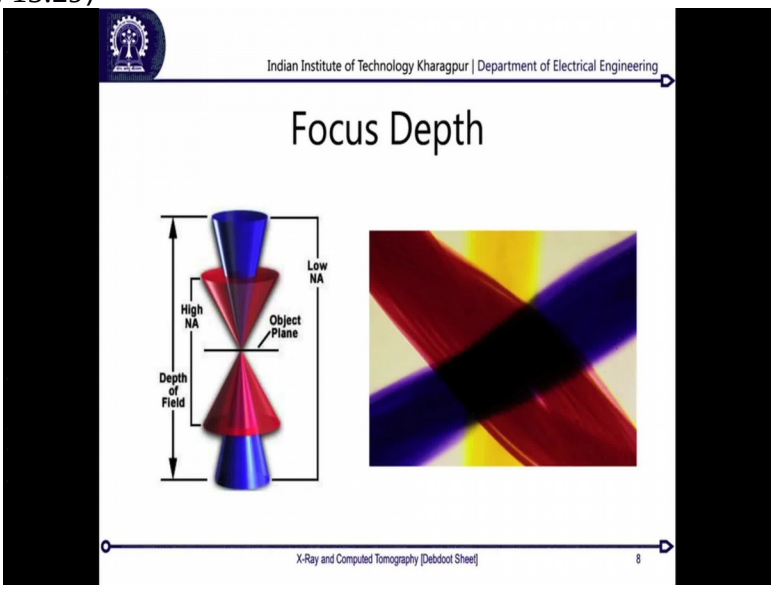
The other part is now that we have looked into that numerical aperture and I said what will mismatch cause over there does this have some relation with image resolution as well? It should in some way, right? Now what I do is a very simple experiment over there. So assume that there are small beads placed down over there and each of them is appearing in white and now if you are using a very lower numerical aperture what would happen is you will not be able to discriminate between the beads in a very good way.

So what is the structure and resolution you are trying to look and what is the numerical aperture we will have to choose is very selective of that. At a lower numerical aperture you get a much lower resolution, so discriminability is not that good. At a higher numerical aperture you will get a much higher resolution, you get a good amount of discriminability, the down side is something called as an airy disk effect.

And what this means is that since you have a whole series of lenses coming down over there. So they will introduce some sort of an aberration over there eventually. Now cumulatively they start producing if it is a spherical object then it will start producing some sort of rings around over there, and these are again depended on those frequency effects which are over here because you do not an infinity projecting lens, you are just taking a small part of the light over there. So you are truncating down all the high frequency light or the rest of the incident rays which are needed to reconstruct this image perfectly.

Now because of that at a higher numerical aperture you will start tend to tending to have these kind of ringing artifacts over there although you have a much higher resolution, so these are what come down into some advanced concepts on microscopy and how to get rid of them is what we will study in the case study part over there as well.

(Refer Slide Time: 15:29)



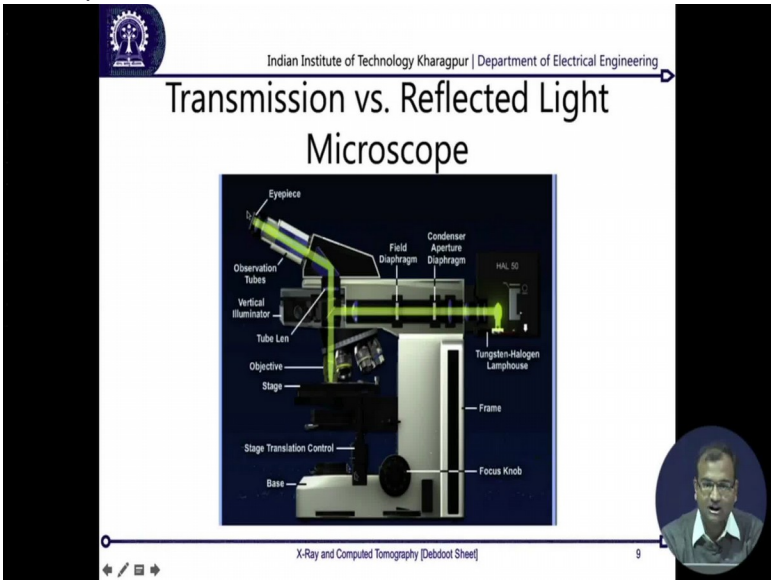
Now another interesting fact is called as focus depth. So what it does is as I said from my earlier example that you have some sort of an aberration always being created because your lens is not a actual planar lens and it is made out say assume two different prisms and they will be causing down spectral distortions over there. Now for that reason what happens is that if you have a very high numerical aperture your depth of focus actually becomes very lower, whereas if you have a lower numerical aperture your depth of focus is larger.

Now if what this would allow is that in this depth of focus wherever be your object placed you will always get a image which is in focus. So now the total say span over which you have or the tolerance on which you can do it for a lower numerical aperture is much larger than the total tolerable limit which you can have for a higher numerical aperture. And for this reason what you would see when you are at a higher magnification you have to be very cautious in tuning down your focus point for that object.

Whereas if you are in a lower magnification you do not have that problem. So when we later on in the lecture when I will be demonstrating out with an actual practical microscope I will would be showing these and telling you about how the problems will occur in them as well. So as a simple example say the if you want to look at the effect, so this is just a bunch of three different thread stands over here you would see that the red thread is now in focus whereas the yellow thread does not appear in focus and this blue thread also does not appear in focus because they are somewhere much above this focal zone over there.

So now this does allow us another interesting thing that you can basically move up and down and sort of create a tomography projection of the microscopic objects as well by looking into the in focus planes. But that is not something which we are going to discuss in this lecture, that is in the case studies in the last week when we are going to look into a microscopy in much more details.

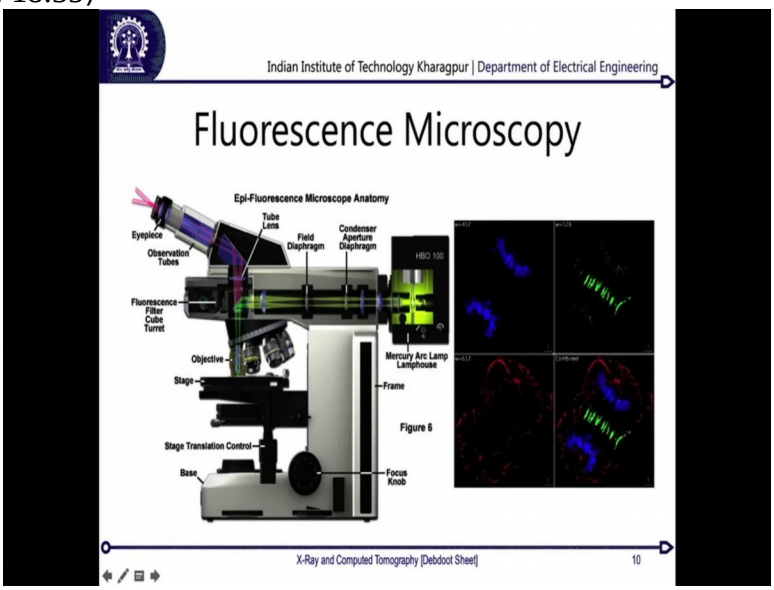
(Refer Slide Time: 17:32)



So these where some of the aspects you need to keep in mind and with that I move on to another variant of a microscope which is called as a reflected microscope. So in transmission light microscope what we had seen is that there was light box over here, you could see the light going from here to your objective. In reflected microscope it is interesting that there is a light box which is placed some here over here. Light comes down through your objective piece over here.

Now what happens is that say you have an object and this is opaque, so if you have a light coming down from here it will never pass and I cannot see anything. Now for that if I want to do I need to have the light from top and then look through it, so the only pipeline through which I can send this light is basically my objective but again I have to be cautious that the same light should not go into my eyepieces over there, otherwise if the same light goes into my eyepiece then I am going to get blinded and I will not be able to see what is around this object. So there is a splitter over here which does the purpose of that one so that I can use the same objective for illumination as well as for looking back at the reflected light.

(Refer Slide Time: 18:35)



Now, with this kind of a microscope the practical application is actually in florescence imaging. So here what happens is that so this is a initial introduction to molecular imaging so here what we do is we have some sort of chemical compounds which are called as chloroforce and they are certain compounds which will bind onto certain specific kind of proteins on my cells. And now say I want to look into mitochondria, I want to look into certain sort of DNA structures and

strands over here and certain kind of protein structures then I can actually have a different color representation coming for each of them.

And then based on what wavelength I am exciting which is getting selected from the filter banks present over here, then it will have created a different excitation. So in florescence what we do is typically we have a different excitation emission spectra ratio over there and we do imaging at individual time. So for one particular frequency I take down this blue excitations coming, for the second one I take the green excitation and for the other one I take the red florescence protein excitations.

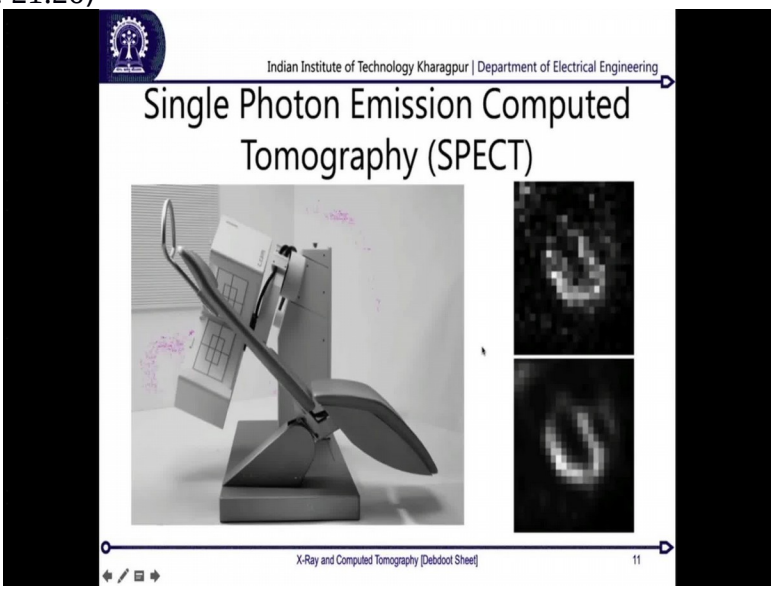
Now using all of these three together we can resynthesize an image which is basically an addition of all the three color planes in these three images. So these images are not necessarily which appear in color because typically we put down a monochromatic sensor over here to have a much higher photon yield over there, so they are again pseudo colored and back retraced onto producing my tri color or multi-color florescence images.

So this is a molecular image of a splitting cell so you would be seeing down the whole splitting phenomenon the main nucleus over here and the splitting in the DNA present over here. So you can although these are not used typically for standard medical diagnostic purposes, but we do make use of florescence imaging a lot for research purposes and for understanding biology of certain disease processes in total.

These are much more advanced microscopy than you would be using in a day to day clinical diagnosis platform. Now that we have known about the use of these kind of molecular markers for in florescence microscope one question would definitely strike us as can we use for macro imaging as well? I mean I just want to look into so from the CT to T1, T2 and everything we had seen that from CT to x rays to MR's I can look into the structure of the body.

Now I want to look at what function is a particular structure doing or say different tissue layers within a particular organ if they are behaving in a different functional way and I just want to look at it. So in order to do that you can again use molecular markers with this are a bit different because they are not optical emitting one but they say they are responsive to x rays or gamma rays and we can find out what object is behaving in what way.

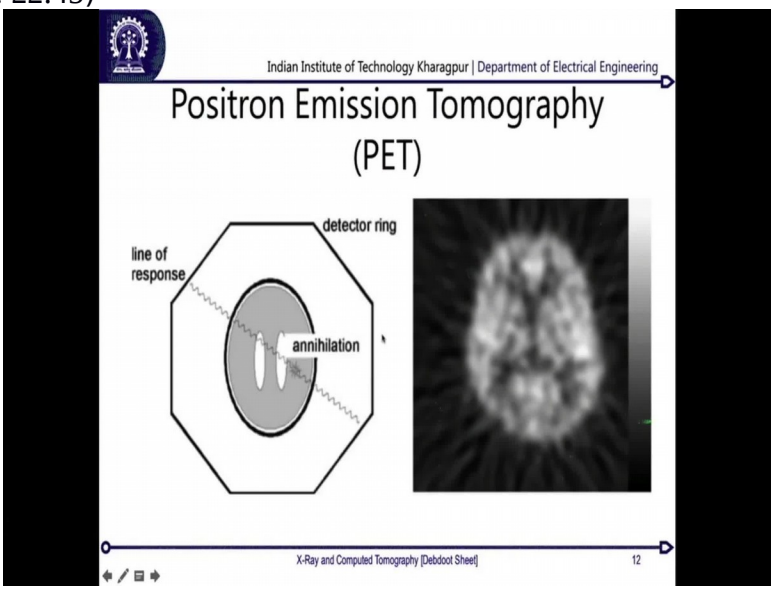
(Refer Slide Time: 21:26)



So one of the first one is called as Single Photon Emission Computed Tomography or a SPECT system which is very much useful in order to look into gamma photon generation from radio nuclides. So what we do is we basically put down a radio isotrop along with glucose and just give it to the patient there and now if certain organ has more amount of glucose consumption than other organs over there, so I would see more of radio nuclide traces coming down from there.

But given the fact that these radio nuclei traces, they are just pass phenomena which occurs only once, so you need a very good kind of a detector over there coming down which can trace down one single photon which is coming down, one single gamma photon. And that is why this kind of a tomographic system is called as a SPECT system. Now the resolution generally offered is much lower. So there is a raw resolution, there is just a corrected resolution over there and that is why you always have a structural scan system either a structural CT or a structural MR along with a SPECT. And these two images are registered together in order to come down to a diagnosis about the by both knowing the structure and the function along that structure because these molecular imaging they do not give you a very high structural resolution as such.

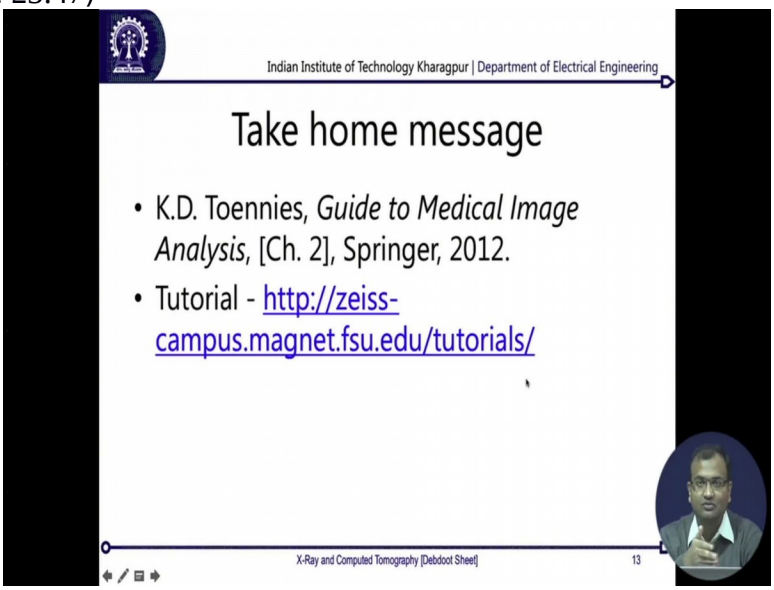
(Refer Slide Time: 22:45)



So the other one is called as a positron emission tomography and what this does is whenever there is a positron emitted you would see a annihilation created and a pair of gamma photons created over there. So this means that I always need to have a pair of detectors which are 180 degree apart and if these are rotating then I will be sensing down a pair of incidences. So this actually helps in reducing a lot of noise coming down so noise will be independent on each detector.

But if it is a pair wise production and compensated with the travel time, then I can always predict down in space exactly from where my proton was emitted over there and my positron was emitted and that is the concept of a positron emission tomography. This is also used in couple with a CT or an MR because the general resolution offered for this one is much lower and you would need a couple one in order to get a much higher structural resolution as well. Since so these are to just give you a basic introduction and an idea of what different modalities are.

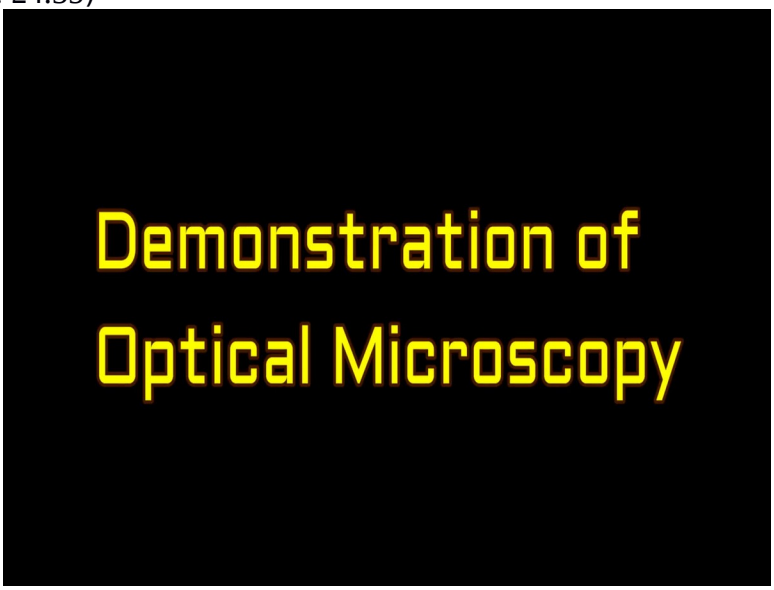
(Refer Slide Time: 23:47)

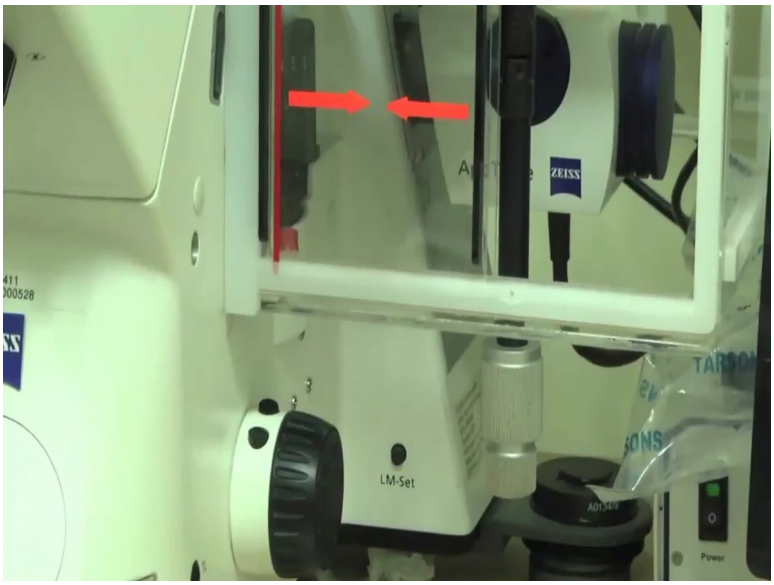


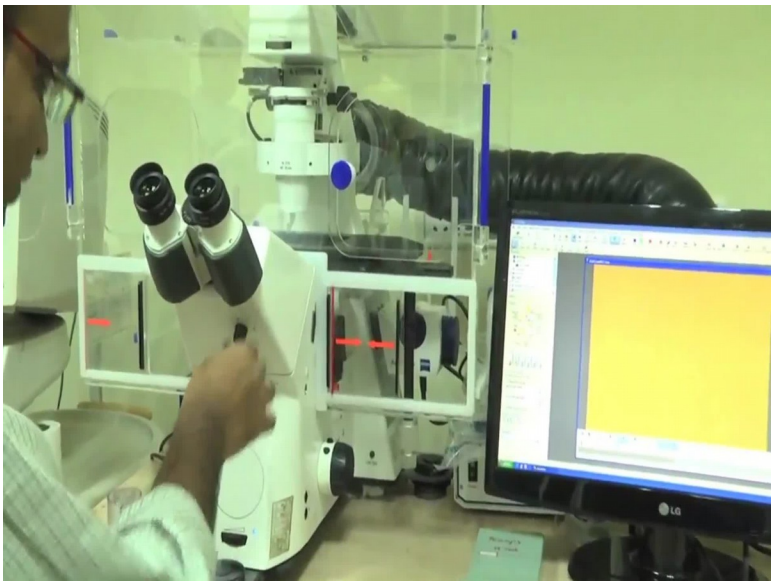
You can read more about these modalities which we have discussed on microscopy and molecular imaging in Toennies Guide to Medical Imaging on the chapter 2 on digital imaging techniques and for microscopy you can have this wonderful tutorial from Zeiss campus magnet at FSU and they have very interactive tutorials which are flashed based and you can just move through them and change different configurations of a microscope and see what happens to the light beam or the image formation processed together.

So with that we come an end to the first week of lectures on getting introduced. In the second week we are going to start with more of basic introduction to analytical tools which are useful for medical image analysis and eventually we built up on top of that, so with that thank you.

(Refer Slide Time: 24:35)



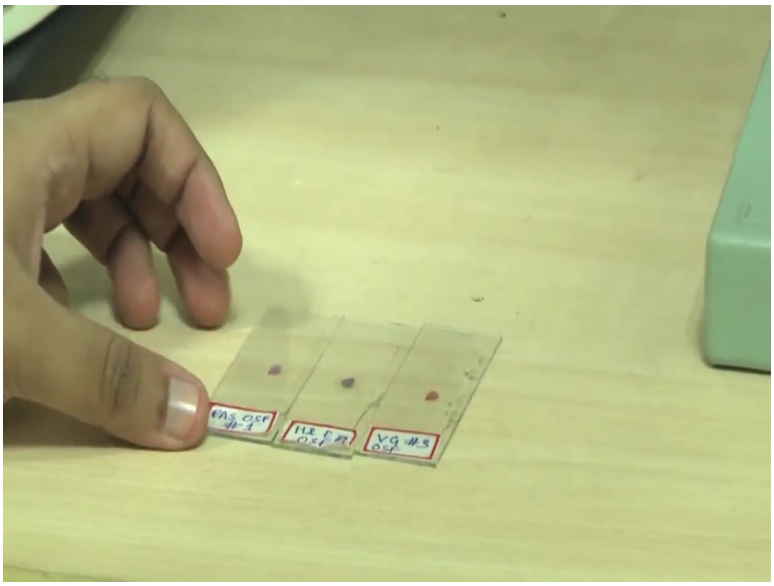

ZEISS
LM-Set
Power

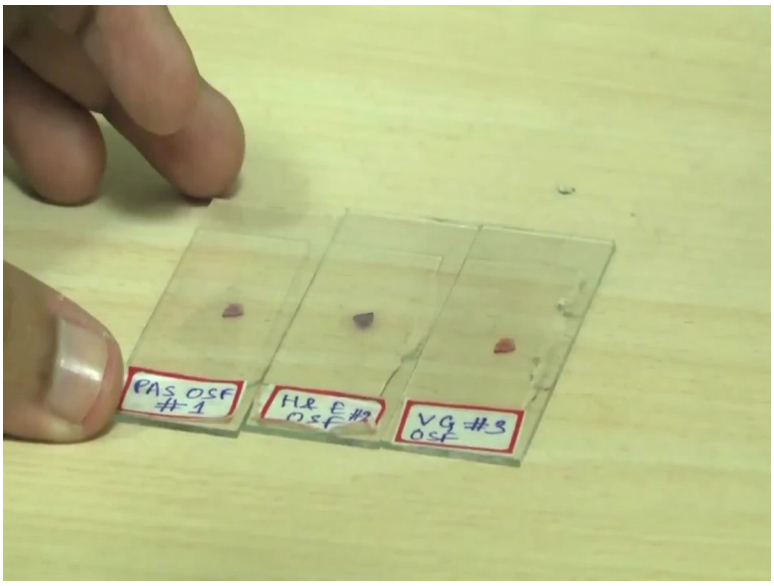

LG

Welcome to this demonstration on optical microscopy and although this is a very high end microscope which is for the purpose of showing how you would be acquiring digital images in perspective of taking them for digital pathology applications but in general any standard microscope would have basically an illumination over here, so since this is we are operating at a bright fold and at bright field and a transmission mode microscopy so you have your illumination this is from where the light source comes down and somewhere over here you would be seeing down the objective over there.

And now since and from there the rest of the optical assembly is housed inside over here, this is my control nob for changing my illumination which I can increase or decrease and here is my eyepiece from which I can actually look into so generally if I do not want to look onto the screen side of it I would be looking through this particular piece and I can see everything magnified on the present on my slide over there.

(Refer Slide Time: 25:44)

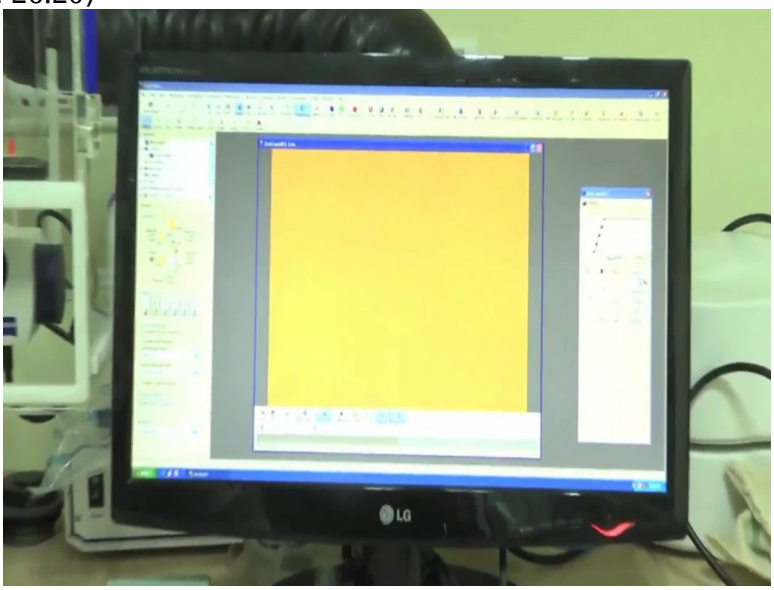


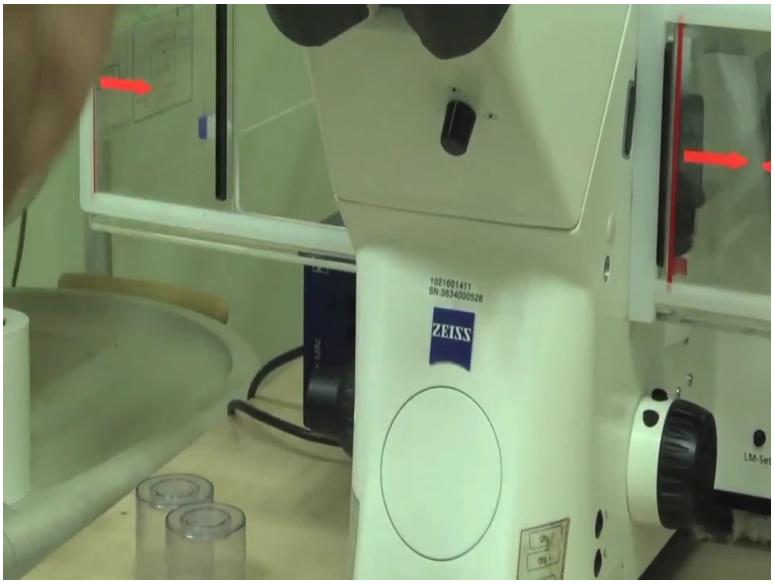


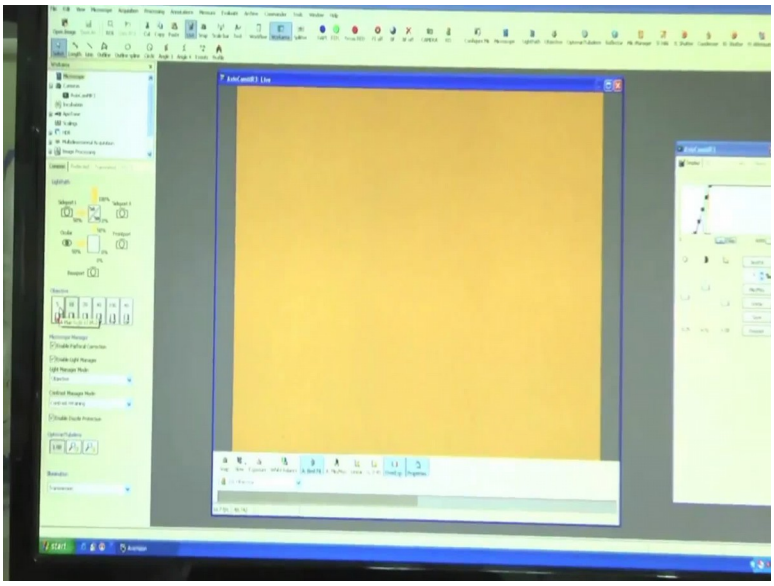
So I would be demonstrating basically with three different slides today and one of them is hematoxylin and eosin stain H and E stains slide, the other one is PAS Periodic acid–Schiff staining and the other one is (())(25:59) staining over there. So these three slides are what I would be showing down through this microscope (())(26:05) and they are basically the same tissue block subsequent serial sections neighboring sections which are stained down by independent individual slides.

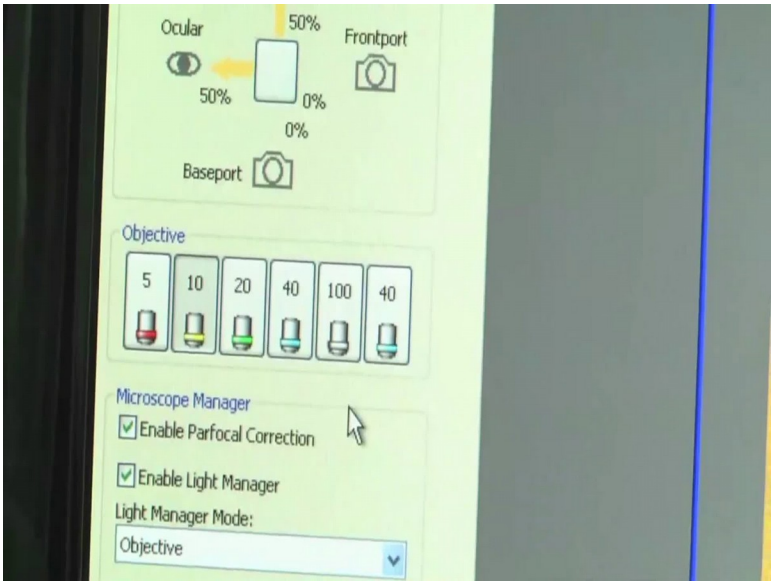
Now as per our microscope we had done on the lecture theory over there so we have a set of beam splitters.

(Refer Slide Time: 26:20)








Ocular
50%
Frontport
50%
0%
0%
Baseport
Objective
5
10
20
40
100
40
Microscope Manager
Enable Parfocal Correction
Enable Light Manager
Light Manager Mode:
Objective

So over here since this is a research level one it allows you to show whether you want to turn all the like the like 50-50 part into this objective over here and you want to turn the rest of the 50 part into your side port which has the camera mounted. So on this microscope you would be seeing there is a camera this is the digital camera in blue which you see.

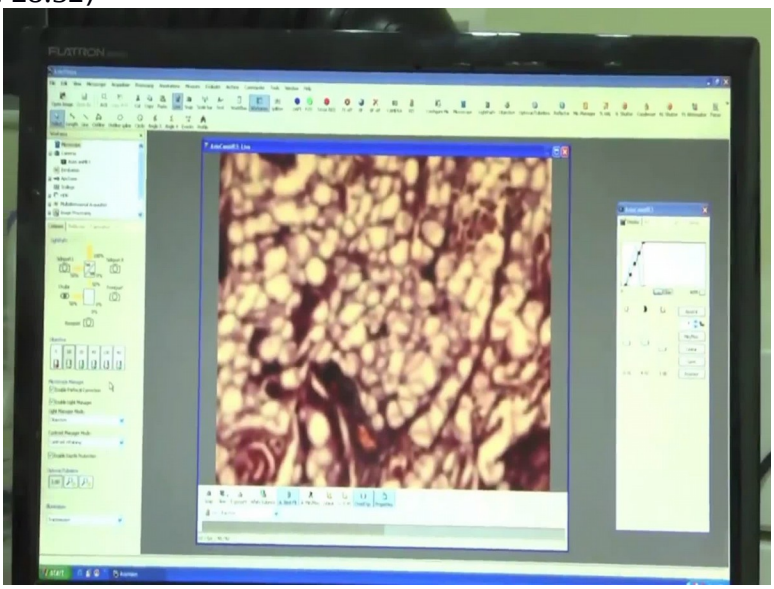
So in this particular configuration I can see both on my objective over here on the eyepieces and I can see whatever is coming from the objective on the camera and on my digital screen over there. Now moving back on to the digital screen where I have all of my controls, so initially we are using just a 10x objective over here for our magnification. We can change over to a 20x magnification, 5x, 40x as and when we require them. There is also an 100x one and there is a 40x with oil emersion also available.

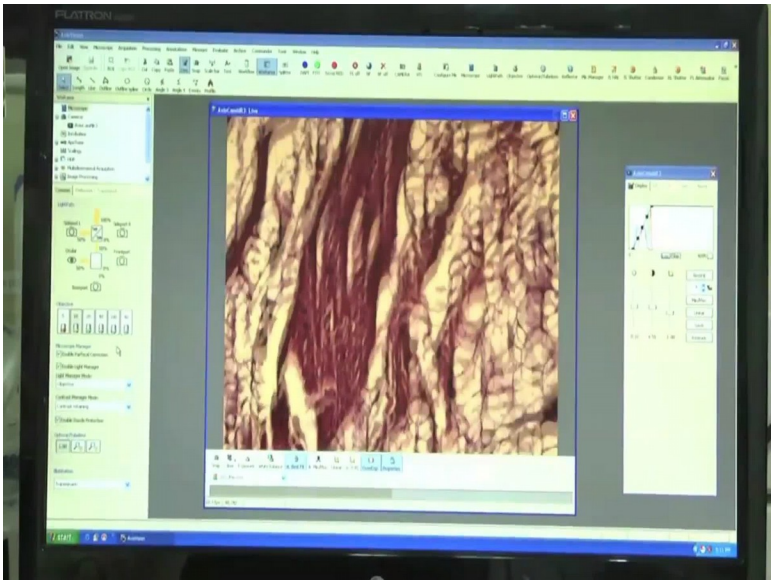
So in total this one has 6 different objectives which are available. So without taking much more time explaining them because they are not what is objective of our whole class over here, so I am taking initially this hematoxylin eosin stains slide over here and I am just going to put it over there. Now remember that since the objective is from below this is a inverted microscope this kind of an objective one the where you have the objective on top and you are seeing that is called as upright microscope.

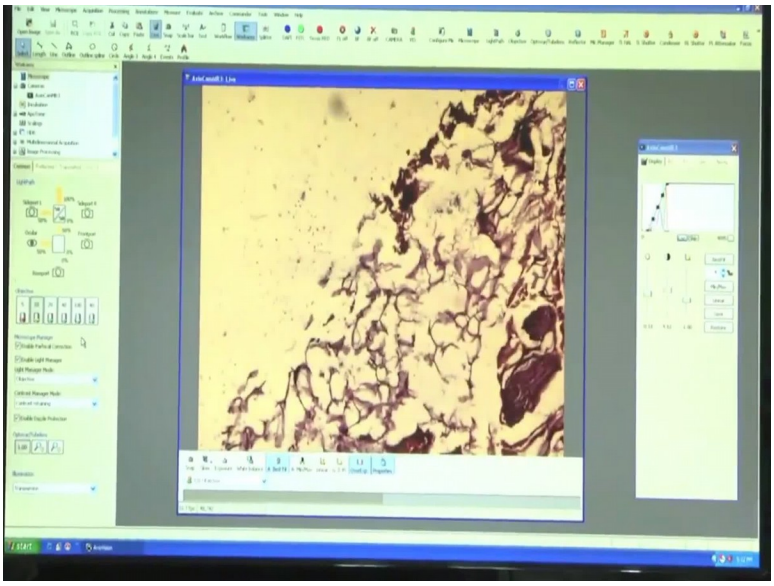
So since it is inverted microscope and if you look on the slide you will see a small segregation over here which is my cover slip which is used to prevent down oxidation over here so basically

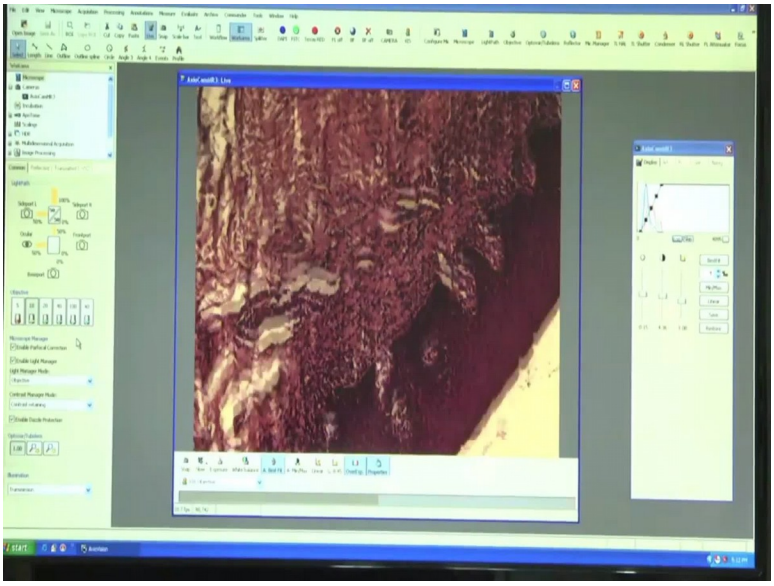
there is a slide the tissue section which is stain and then I put down this cover slip on top of it to prevent oxidation. Now this layer this side so there are two so this is one surface of the slide, this is other surface of the slide. I am supposed to put this surface which has the tissue section over there closer to the objective lenses, so that is why I am going to mount it this way on over this microscope.

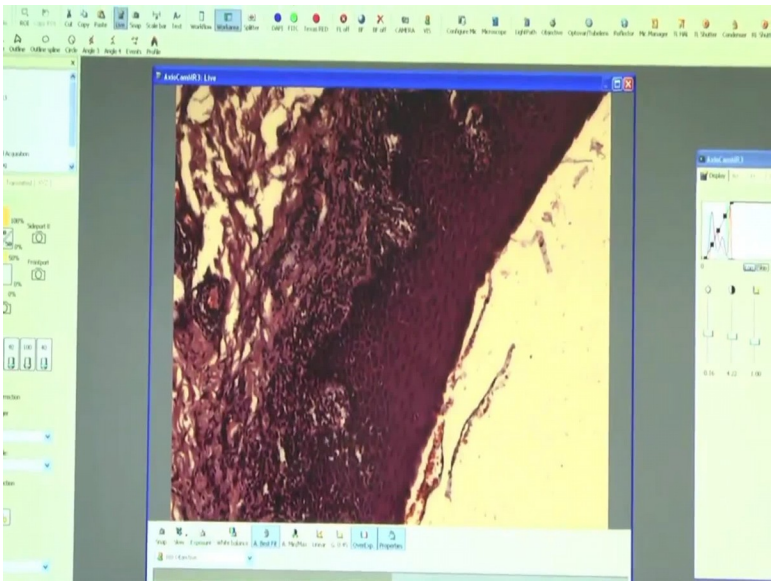
(Refer Slide Time: 28:32)











So the moment I mount it you can see something's on the screen and now the next thing I will have to do is basically do a focusing control over there and try to focus on what I am able to see on the screen. So as you see I mean to a untrained eye this would basically appear as if a garbled and what I am doing is I have a stage position control nob over here which is present over here. This can do my xy direction moments as well as my up and down movements.

So this is what I see now. Now this is a section of the biopsy of oral tissue, so you would be seeing down your epithelium and then the basement membrane somewhere over here and since this is oral (())(29:22) and fibrosis so just below the mucosa you do have a lot of fibrous cells and fibrous tissues over there. Now I mean that is just part of the pathology which we are going to discuss.

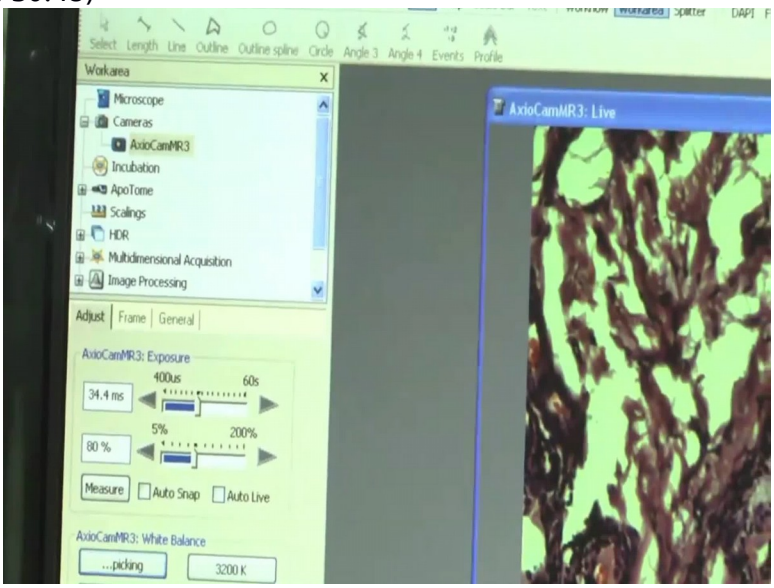
Now for the control side of it since this is again a standard camera so as I keep on changing my illumination obviously I am going to see changes over there and now I have my over exposure indicator on. So these regions, these particular pixels over there are already getting saturated and over exposed, okay. So given that I am at a particular exposure level the first thing I would be doing is setting it down to a exposure compensation and after that the best one is to do a auto white balancing.

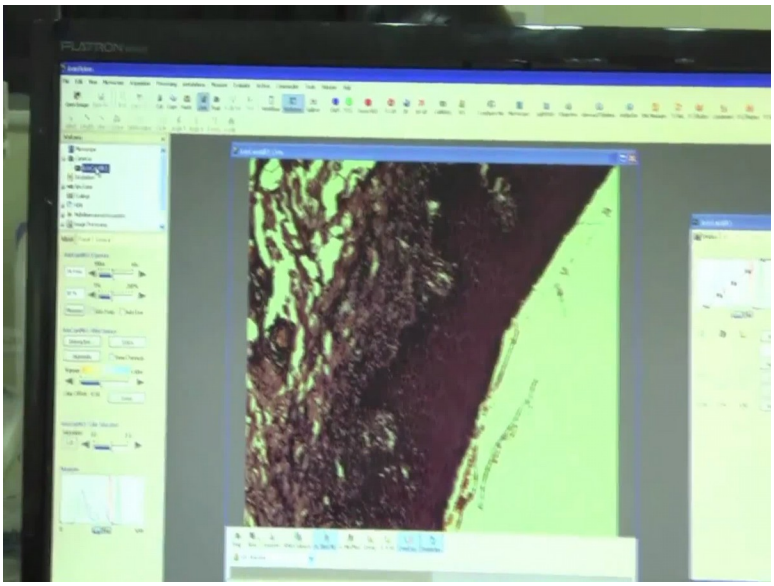
This is what it comes on auto white balancing but the cardinal rule always for white balancing is that whatever however you whatever you see with your eyes the same color is to be reproduced

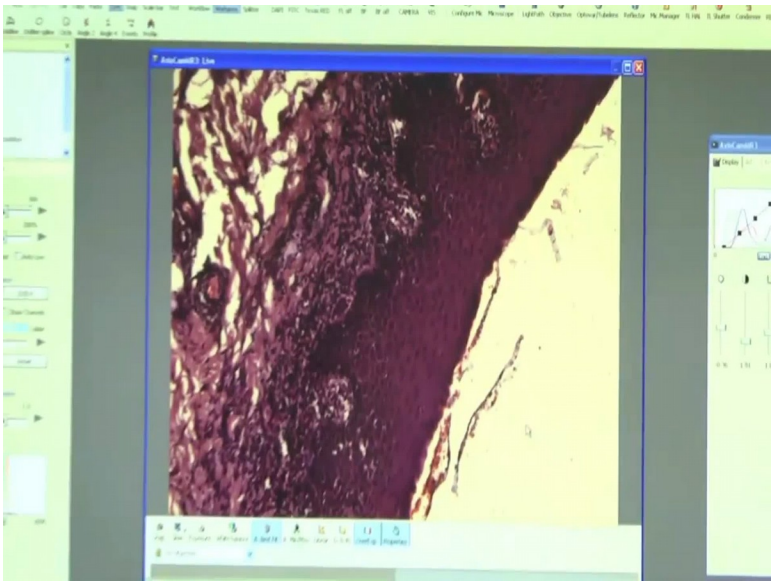
on the screen as well and again taking into account the non-linearity in mapping down colors by the monitor itself, we have to invert that together.

So together it should be something like what I am seeing on these eyepieces since for a purpose we cannot fit a camera on this eyepiece to show you well so I am try to basically match down that color with one, so the best practice is that you basically look over here you know what is the color of the tissue and the color of this so color of the tissue and this over here. So this part is supposed to be a bit more yellowish in nature and that is what not coming down over here.

(Refer Slide Time: 30:48)

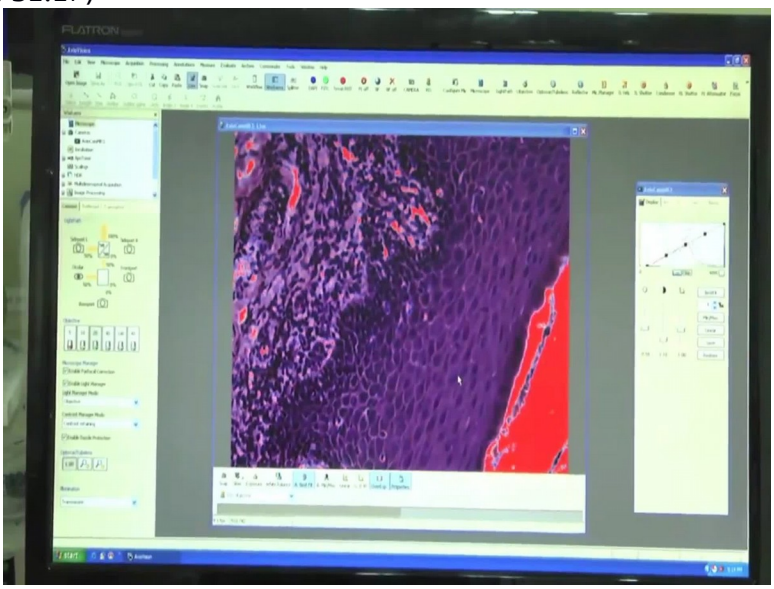


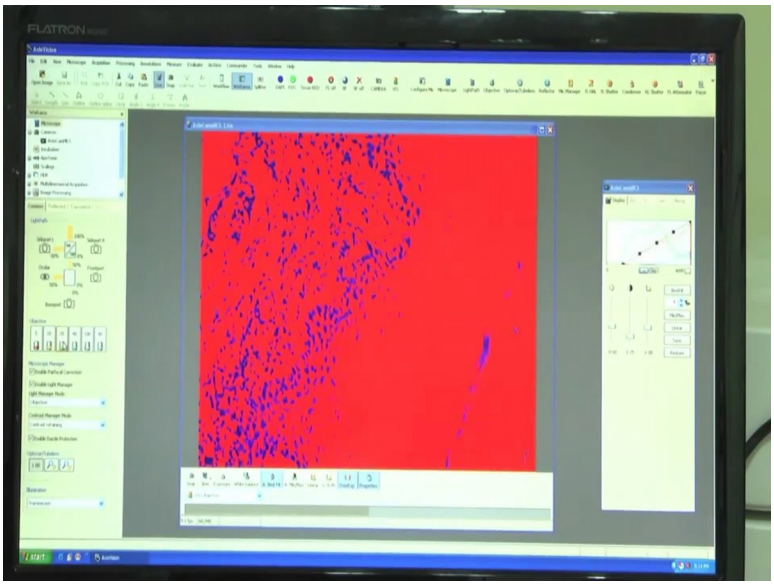


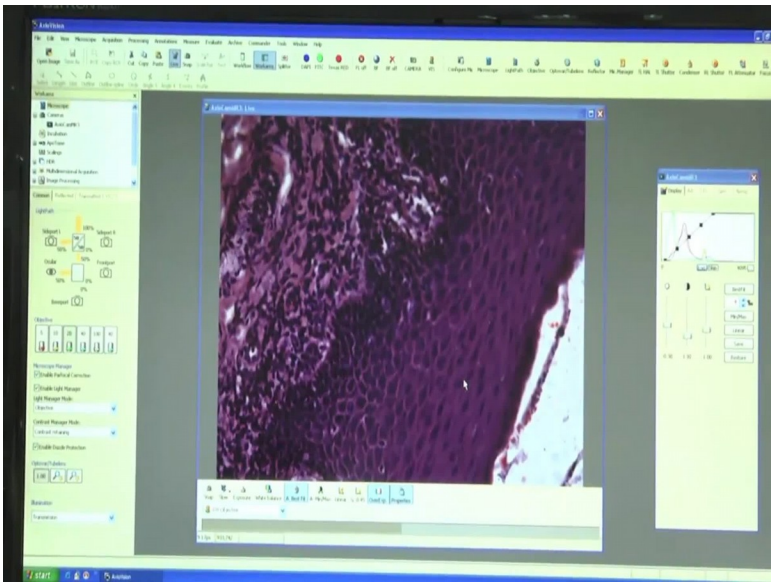


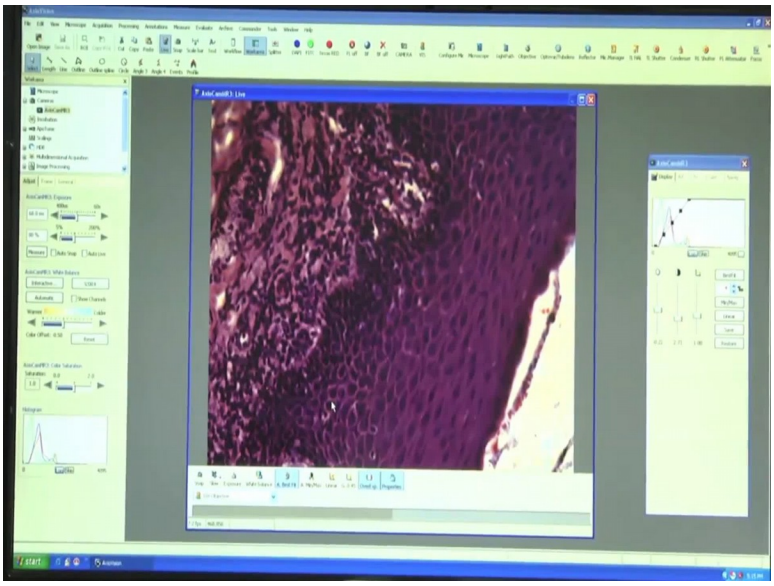
So what we will do is we go down to the advanced settings on the Axio cam controller over here which is the camera controller and start a interactive white balancing by picking up a location say I pick up this particular location. And now I see that since this part was supposed to match down with my color, this actually does a good decent job of color compensation over there and is a very important step whenever you are doing the edle pathology because otherwise color shifts will introduce major croma shifts and your algorithms might not work, so that is one part of it.

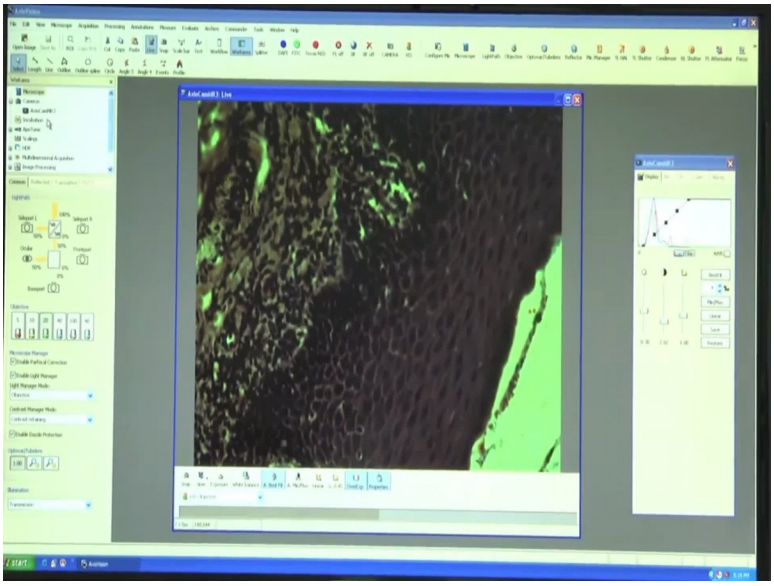
(Refer Slide Time: 31:17)

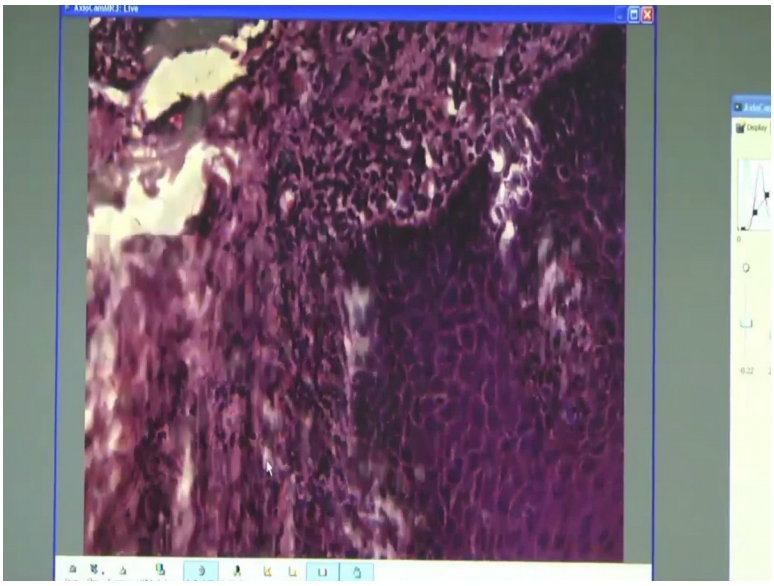








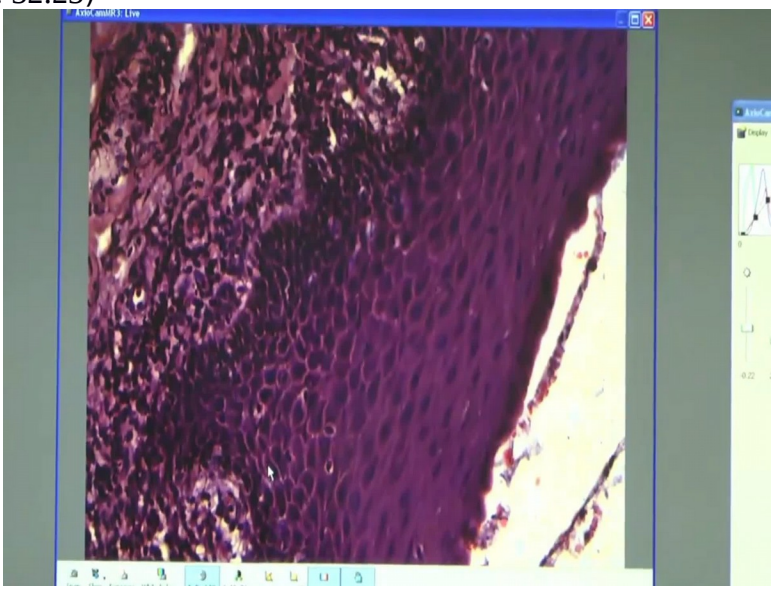




Now coming to the major parts that since you have different magnifications and what happens over here so this is at a particular magnification, now I change this one to 20x over here so it moves and then puts me on a 20x magnification. Obviously I see there is a lot of part which got over exposed and that is because in particular 20x magnification you have a much smaller area which is being seen and then the total amount of light to be compensated matched on with a numerical aperture has to be appropriately done.

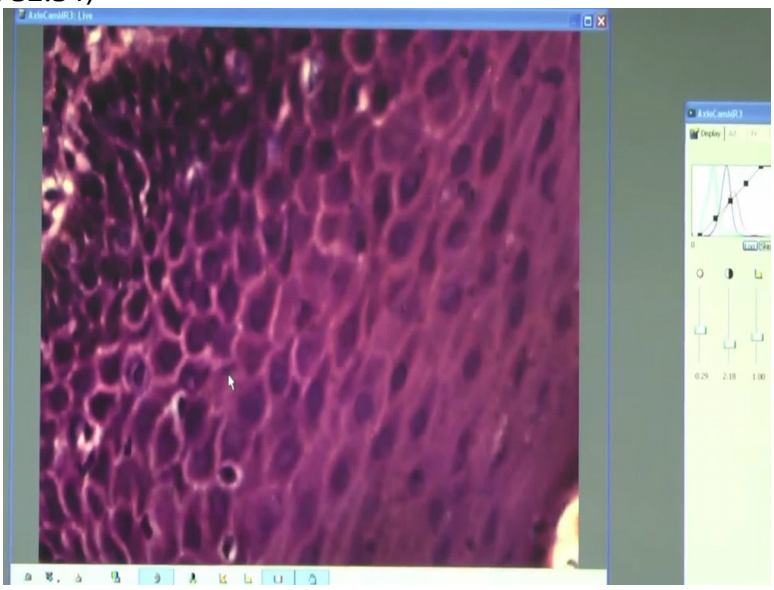
So basically I am going to pull down my intensity levels over there, then do another level of exposure compensation and a white balancing and again that I see this change coming down I will go into my camera settings over there, do an interactive one and balance it out and now I have it, perfect. So I we do see that some part of it is again out of focus so I need to focus it very carefully, so this is where I get most of my objects now in focus, okay now if I change this is how it would be translating and you can see.

(Refer Slide Time: 32:23)



Now at this particular magnification of 20x you can rather make out all of these individual nuclei for the first time which was not so evident in 10x magnification.

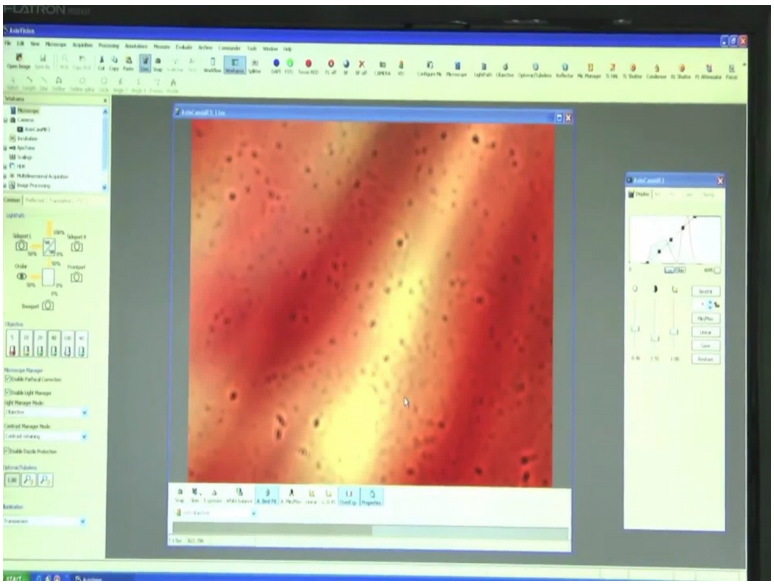
(Refer Slide Time: 32:34)



So from there I move onto say 40x on my air mount one over there and I have a much higher magnification but I just need to focus on my objects so. Now that we are at a 40x objective magnification you can that the cells and the nuclei so the cellular membrane over here as well as the nucleus is quite distinctly visible which was not there in lower magnification. So this are the

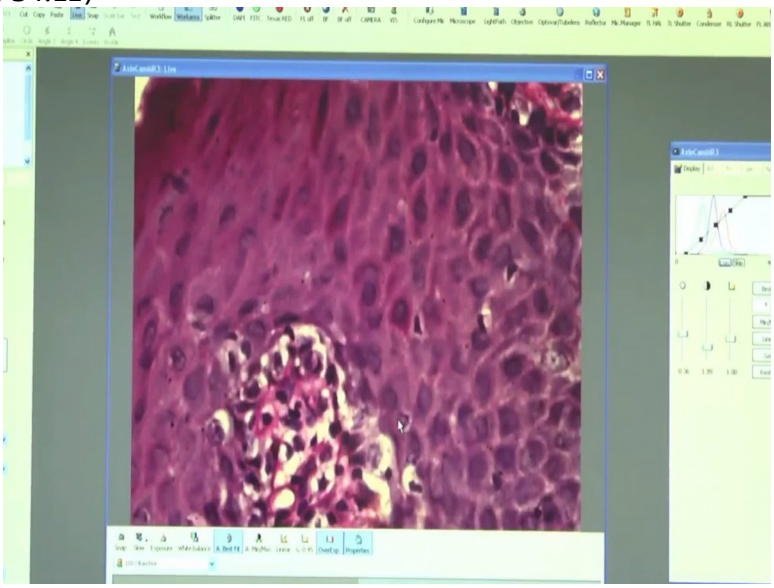
different effects different magnifications have and what we use over there for individual purposes.

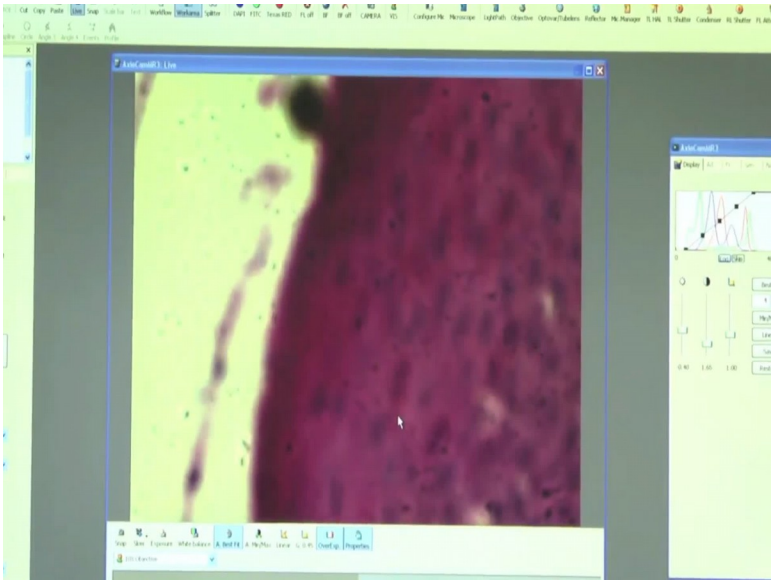
(Refer Slide Time: 33:14)

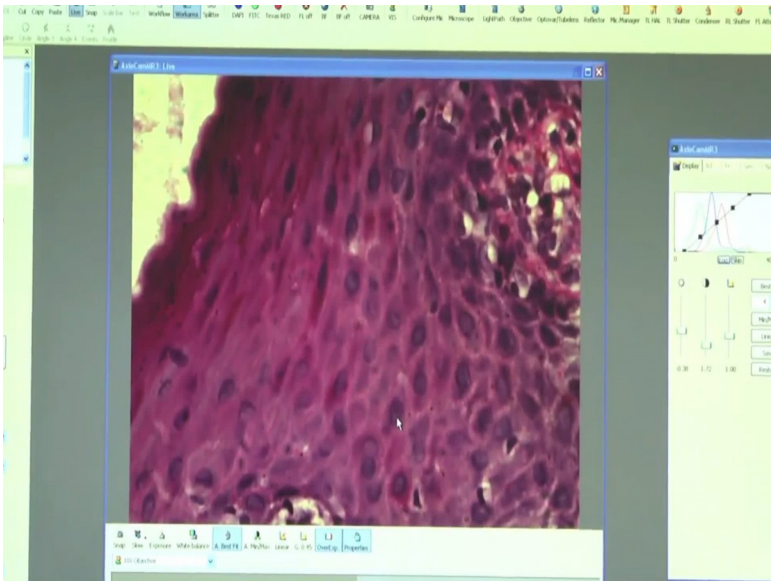


So this was about an H and E stain slide. Now I am going to change this one and put down another slide which is called as Van Gieson staining or VG. So let us position it over there at 40x I again try to put them onto yeah. So this is my now you have your epithelium region again present over there, so for a different stain you have a different way. One thing you see is this is actually reddish in color for Van Gieson stain, you do not have the bluish staining component present over there at all.

(Refer Slide Time: 34:11)







So I can change this one and let us put down a PAS staining Periodic acid–Schiff (())(34:12) agent stain over here. So I have placed it. Now I am going to just center it appropriately and somewhere I am close to it, I do a fine focus control and yes now I a at over here, okay.

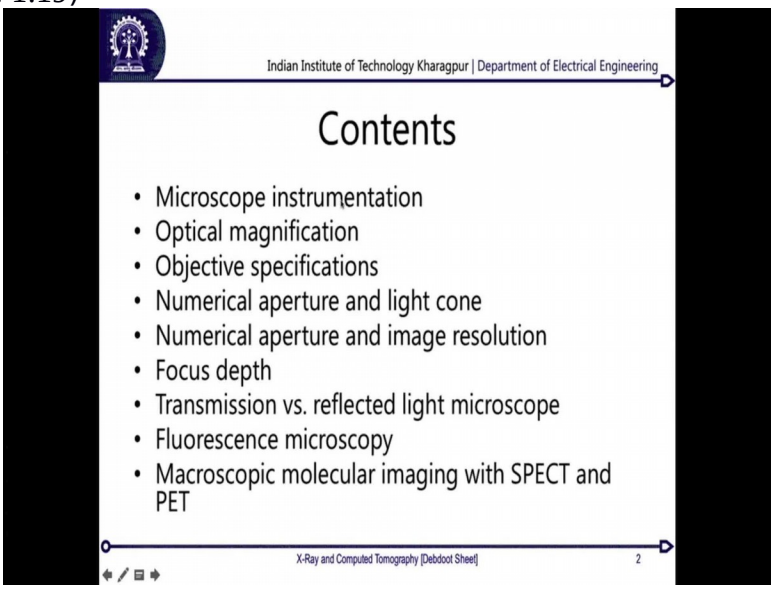
So this was the effect of different stains and some common magnifications over there, you have many more mode supported on it, it can do florescence microscopy, it can do dark field microscopy with phase contrast as well but our whole subject is related only to digital pathology which is again with bright field microscopy only and this is one of those examples of how to use microscopes for digital pathology. So the details have been covered over here. We have a much more detailed case study on digital pathology problem as well somewhere in the fourth week upcoming forth week over there, so with that thanks.

Course on Introduction to Medical Imaging and Analysis Softwares
Professor Debdoot Sheet
Department of Electrical Engineering
Indian Institute of Technology Kharagpur
Module 01
Lecture 05: Optical Microscopy and Molecular Imaging

welcome to today's topic which is on Optical Microscopy and Molecular Imaging. So this is basically the last module which we are going to cover on all the modalities over here so we have already covered down modules on CT, MR, ultrasound which are majority of macro imaging modalities where you look into larger parts of your body and the whole body in as a whole in total. Now in microscopy is where you will be looking into very specific part of your body and now this is more of aim of at a portion of looking into just say one of the cells one single cell or a cluster of cells within your body which was not possible with any of the other modalities over there.

And from there enter into a very interesting phenomenon called as molecular imaging and this molecular imaging is where initially we will do it for molecular imaging for microscopy and from there we will move on to molecular imaging on the mass scale as well.

(Refer Slide Time: 1:19)

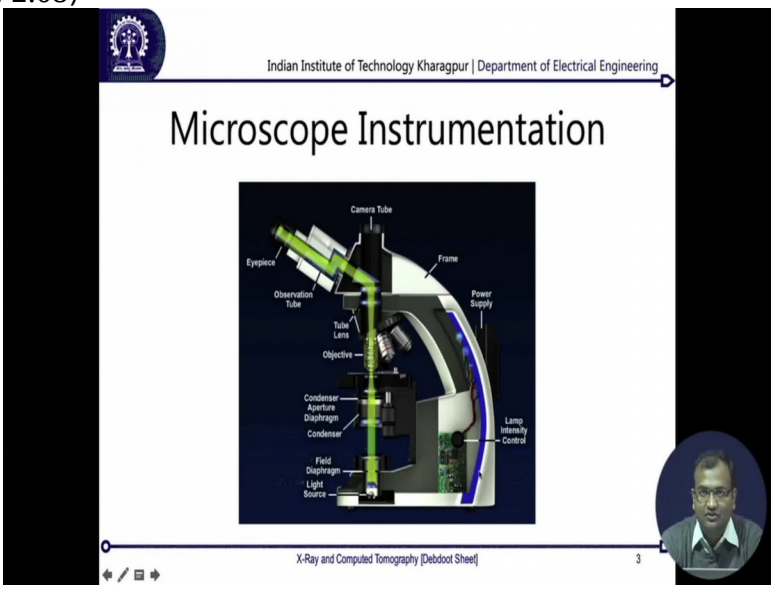


So this lecture is arranged something like this so I would be introducing you to you the microscope instrumentation and then the concepts of optical magnifications, what is an objective

and what are specifications of an objective, the relation between numerical aperture and light cone and the image resolution and what we mean by depth of focus.

From there to two different modes of microscopy which is transmission and reflected light microscopy. Although reflected light microscopy is not something which is very traditionally used for medical applications, it is used more of for material science applications but we do have one specific mode of operation called as florescence microscopy in which reflected light microscopy is the basic principle and that is where you get introduced to molecular imaging for the first time and from there I would enter into molecular imaging on the microscopic scale with SPECT and PET.

(Refer Slide Time: 2:08)



So this is what a microscope looks like, you might have seen them in your high school as well or some other experiments in some point of time. Now what it has is this part is basically a very sturdy body thing and you have some objectives over here which is an assembly of lenses tact one beyond the other and you have a few lenses over here which are called as cube lenses which are again for the purpose of magnification, there is a small prism over here which basically divides the light on to this part which is which goes into your eyepiece where you put your eyes and look into and the other part of light comes out over here which is called as a camera tube, you can put a digital camera over here, something like your web cam and just acquire those digital images over there.

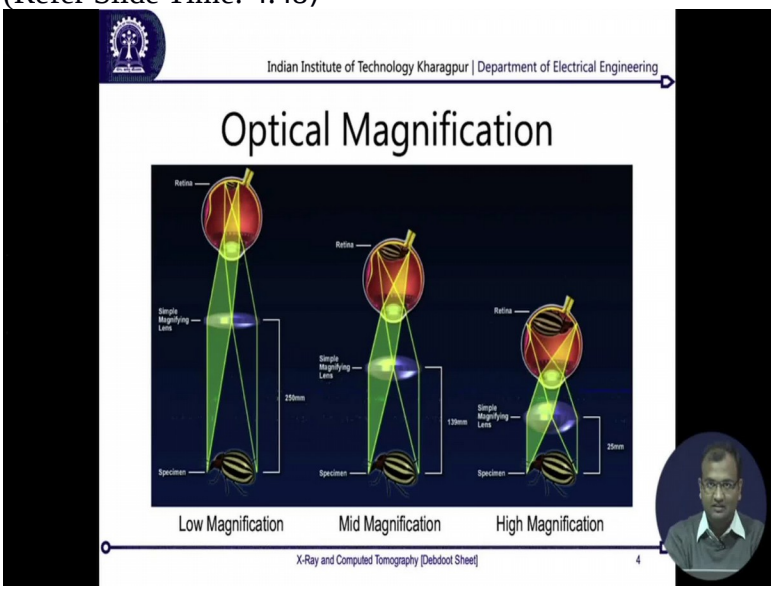
Now the light comes down from this part which is called as a light source so if you were in you are looking at your high school microscopes then you had some sort of a small mirror concave mirror and you had to take somewhere near the window and do your experiments over there. In more of these microscopes since we do not make them move them a lot and carry them along where the sun moves so we have a light box in which you just have one simple lamp and a diffuser placed over there and then this whole light passes through this assembly.

So from there we can control out the total width of the light which is coming out using this field diaphragm and aperture control diaphragms over here together such that we have a very narrow focused cone of light passing down through the lens, okay. So this is the standard diagram of any kind of a microscope so be it from a very cheap microscope you use in your high school to a very high end microscope which we have in the demonstrator on using a microscope for imaging and image acquisition.

So all of them have the same sort of a path connector diagram. Now when you have these objectives which are placed on top of the object placed on a slide then this kind of a microscope is called as an upright microscope. In other kind of a microscope which we will see in the demonstration as well these set of objectives are placed over here which is below and the light box is on top so that is just say vertically flipped configuration of this microscope so you have to look at the eyepiece which is present over here as well.

So that kind of a microscope where the objective is below the sample, that is called as an inverted microscope, just it is an inversion of the optical design of this one, that is the only difference between them otherwise the rest of the imaging and physics remains the same.

(Refer Slide Time: 4:48)



Now from there let us enter into what this microscope does. Now I will try to again review the concept of optical magnification. Now say that you had an object over here and through a lens you were looking at it. Now if the object is quite far off from the focal plane so you would from the focal length of this lens then you would see a very smaller version of it casted on your image over here and that is a very small one.

Now, if I keep on changing the distance between my eye and the lens as well as the distance between the lens and the object over there I would eventually come down to a mid-level magnification where I have almost the same size casted over here and if they are very close to each other then I would see that the total image which is casted over here is much wider and that is what is called as optical magnification in any way. So the total image which is cast on my retina or which is cast on the censor is much bigger than what it is in actual practice then it is so.

And microscope gets its name from the perspective that it can magnify objects in the order of micro which is in microns, so 10 power of minus 6 of a centimeter is what gets magnified to in order which is visible with naked eye, so in the order of centimeters is what is visible to naked eye. So if I magnify something in the order of 10 power minus 6 to a order of 10 power minus 2, then it makes it visible to the human eye in a very direct sense.

So that is what optical magnification means in its direct way and a microscope just does this part of it. Now it is not just optical that you have, you have other microscopes like electron microscope and atomic force microscopes which are very different from it but whenever in terms of medical image analysis and digital pathology we speak about optical microscopy then it just means that there are lenses and it is a lens based microscope and I am trying to magnify micron level appearances to something which is visible with naked eye.

(Refer Slide Time: 6:53)

Now one important thing which plays this major role in this magnification is called as the objective. Now what this does is this is basically a set of collection of lenses which will help you in magnifying, so instead of using one single lens which will make it really big and thicker and you will no more have a thin lens phenomenon, you can put cascade of lenses and pack them together. So this is one cylinder in which there are multiple lenses which are packed together and such that you get a very high magnification.

Now if you look at it, typically you would have a so I we do not have any so relation with Zeiss as such, it was just one of these designs I took and more of from the reason that we would be demonstrating on one of the microscope which has this sort of kind of kind of lens over there so it makes it easier for us to go through it. So one first part which you would see over there is the magnification, so this is a 63 times magnification one. So whatever comes over here is 63 times magnified when you look through this one.

So this is the primary condition of objective so you have objectives starting from 4x to 10x to 20x to 40x, 63x, 100x reaches a standard configuration. Now this is the first part which you will have to keep in mind, the second one is that what is the focal correction? So this is infinity corrected which means that whatever you see over here is a parallel beam and it would appear as if the object is placed at infinity and you are seeing that object over there which is quite important otherwise if this looks into a particular point that means that along this optical tarot over there or wherever we have the tube lenses than those tube lenses will also be have to place replaced exactly at the focus point.

Now if you are moving this objective up and down in order to look into your focus points, then your tube lenses will also have to move and it becomes a very complicated optical arrangement to solve. That is why we generally prefer to have a focal correction at infinity but there are some microscopes and some lenses which have certain limitations which they do not offer and they do make it very implicitly mention over here as well. Now the other part is this is a NEOFLUAR lens and what that means is that generally if you are looking down so think of this one.

Let us say you have a prism and you have a white beam of light going down over there so you will have a spectral slicing across the prism from with your total. Now, a lens is basically two different prisms with their bases together, okay. So obviously there is going to be some diffraction and now every point which is on focus that we not say there is a white point in focus on the other side but that white point will no more appear as white whereas there will be a rainbow like pattern which comes down over there.

Now we do not want to have that one because we want colors preserved and everything, otherwise this will create some sort of optical distortion. Now in order to get rid of that what they do is we put down certain chemical coatings on top of the lens such that for different wavelengths there are different refractive indices such that all of them convert at the same point and a white spot is again seen as a white spot you do not have optical abrasions.

So NEOFLUAR is a particular grade of such lens which specifies as to what is the amount of optical abrasion which is present over in that lens. And these are something which are of very good quality otherwise it is so a standard one is just a plan lens from where you will be getting down a fluar lens from there you get a apochromatic lens and and different varieties as well. So

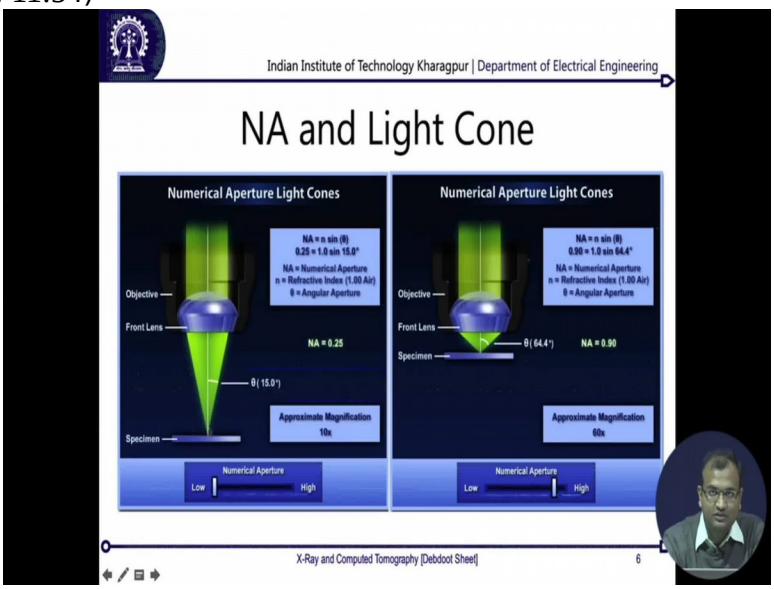
the other part is whenever you have a very high magnification lens over there you cannot just put it.

So it has to be put very close to the object. So as we were looking into that earlier slide where for higher magnification the lens is actually very close to the object. Now if it is very close it will just go and touch the surface of the object. So over there in order to avoid some wear and tear because of the object and the glass rubbing against each other we basically put down some kind of a lubricant and generally this can be something like a glycerin or oil or water.

And now the other point is that since you have some lubricant and everything paced over there, so this can leak inside the lens as well and that can create other kind of optical distortions, you do not want them to happen so there are certain sealing jackets and mechanical design constraints. So that are also mentioned over here, so this lens it points down as W so it is water immersion lens so you can only use it with water, so please do not use it with oil or anything and you need to be very cautious in the designs over there because this are specified and you need to use them only with those specified fluids for emersion over there.

So there are air emersion lenses now which are to be operated in air, you cannot use those lenses with water otherwise water would leak in and that would distort the whole optical arrangement and the durability of the lens as such.

(Refer Slide Time: 11:54)



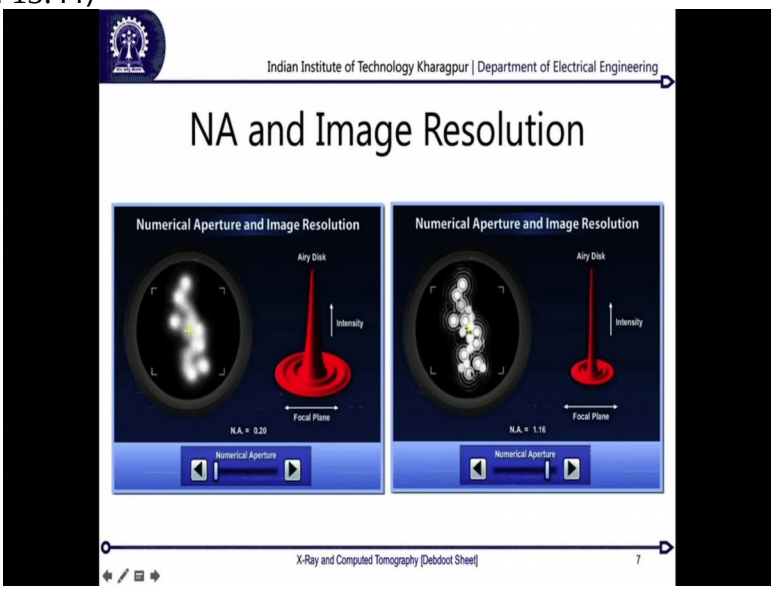
So once we are done with this one, now another interesting fact which is observed is the effect of numerical aperture and light cone. Now what happens is something like this that if your object is very far off and from the lens then you have a very small cone being projected over there and as the cone is small your numerical aperture which is dependent on n times sine theta and n is the refractive index of the material through which it is passing.

So in this part it is just air, so refractive index is 1. So your numerical aperture is again low because your theta over here is low. Now, as you keep on moving this object close to it your numerical aperture is also going to be high because your theta is going to be high. Now you need to keep something in mind that the numerical aperture or the angle of this cone should be matched with the angle of the cone of the objective as well.

So your object because if you have a lower numerical aperture of this lens over there and you are putting at a higher numerical aperture over here then you will basically have a less amount of light passing down over there, you cannot collect effective amount of light. And now, if you have a smaller numerical aperture smaller angle of the light being casted on the lens and you have a lens with a wider numerical aperture than you are getting going to get a lot of stray reflections coming down from the sides which is just going to be noise.

So the quality of image actually depends a lot on matching the numerical aperture to the light cone of the object which is being done and you generally from a user's perspective you do not need to be worried too much about that because these are matched down by the manufacturers themselves over there. And for each lens it is pre calibrated where you put the objective and where you put the sample and what should be the numerical aperture for that objective as well but if you are changing down objectives so if you want to replace one of your native objectives with a new objective, then do keep in mind and recalibrate the whole system, this is one thing to be kept in mind.

(Refer Slide Time: 13:44)



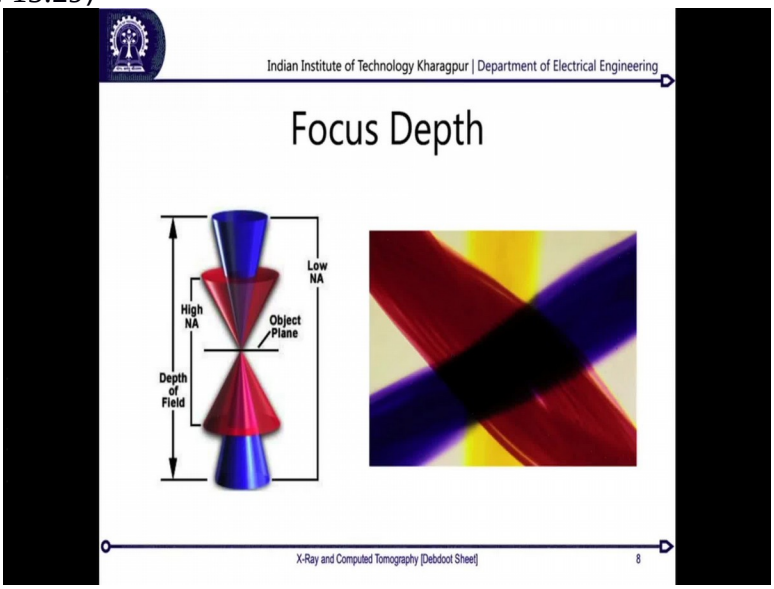
The other part is now that we have looked into that numerical aperture and I said what will mismatch cause over there does this have some relation with image resolution as well? It should in some way, right? Now what I do is a very simple experiment over there. So assume that there are small beads placed down over there and each of them is appearing in white and now if you are using a very lower numerical aperture what would happen is you will not be able to discriminate between the beads in a very good way.

So what is the structure and resolution you are trying to look and what is the numerical aperture we will have to choose is very selective of that. At a lower numerical aperture you get a much lower resolution, so discriminability is not that good. At a higher numerical aperture you will get a much higher resolution, you get a good amount of discriminability, the down side is something called as an airy disk effect.

And what this means is that since you have a whole series of lenses coming down over there. So they will introduce some sort of an aberration over there eventually. Now cumulatively they start producing if it is a spherical object then it will start producing some sort of rings around over there, and these are again depended on those frequency effects which are over here because you do not an infinity projecting lens, you are just taking a small part of the light over there. So you are truncating down all the high frequency light or the rest of the incident rays which are needed to reconstruct this image perfectly.

Now because of that at a higher numerical aperture you will start tend to tending to have these kind of ringing artifacts over there although you have a much higher resolution, so these are what come down into some advanced concepts on microscopy and how to get rid of them is what we will study in the case study part over there as well.

(Refer Slide Time: 15:29)



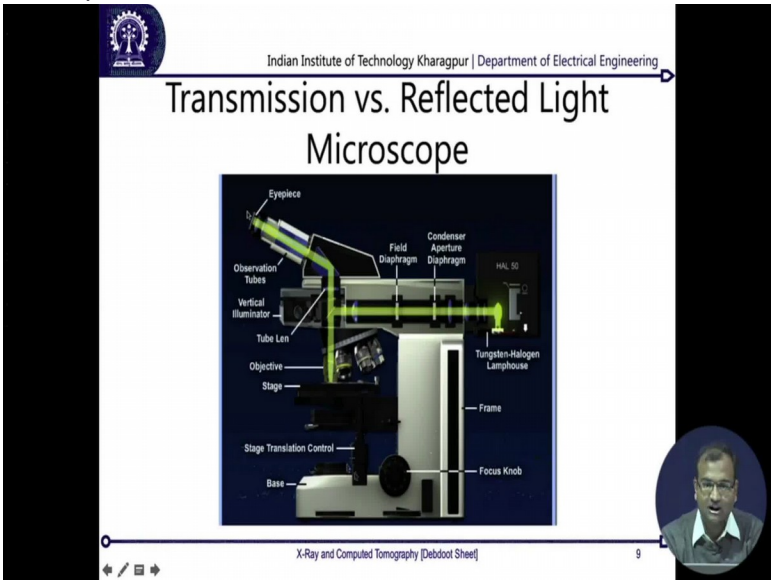
Now another interesting fact is called as focus depth. So what it does is as I said from my earlier example that you have some sort of an aberration always being created because your lens is not a actual planar lens and it is made out say assume two different prisms and they will be causing down spectral distortions over there. Now for that reason what happens is that if you have a very high numerical aperture your depth of focus actually becomes very lower, whereas if you have a lower numerical aperture your depth of focus is larger.

Now if what this would allow is that in this depth of focus wherever be your object placed you will always get a image which is in focus. So now the total say span over which you have or the tolerance on which you can do it for a lower numerical aperture is much larger than the total tolerable limit which you can have for a higher numerical aperture. And for this reason what you would see when you are at a higher magnification you have to be very cautious in tuning down your focus point for that object.

Whereas if you are in a lower magnification you do not have that problem. So when we later on in the lecture when I will be demonstrating out with an actual practical microscope I will would be showing these and telling you about how the problems will occur in them as well. So as a simple example say the if you want to look at the effect, so this is just a bunch of three different thread stands over here you would see that the red thread is now in focus whereas the yellow thread does not appear in focus and this blue thread also does not appear in focus because they are somewhere much above this focal zone over there.

So now this does allow us another interesting thing that you can basically move up and down and sort of create a tomography projection of the microscopic objects as well by looking into the in focus planes. But that is not something which we are going to discuss in this lecture, that is in the case studies in the last week when we are going to look into a microscopy in much more details.

(Refer Slide Time: 17:32)

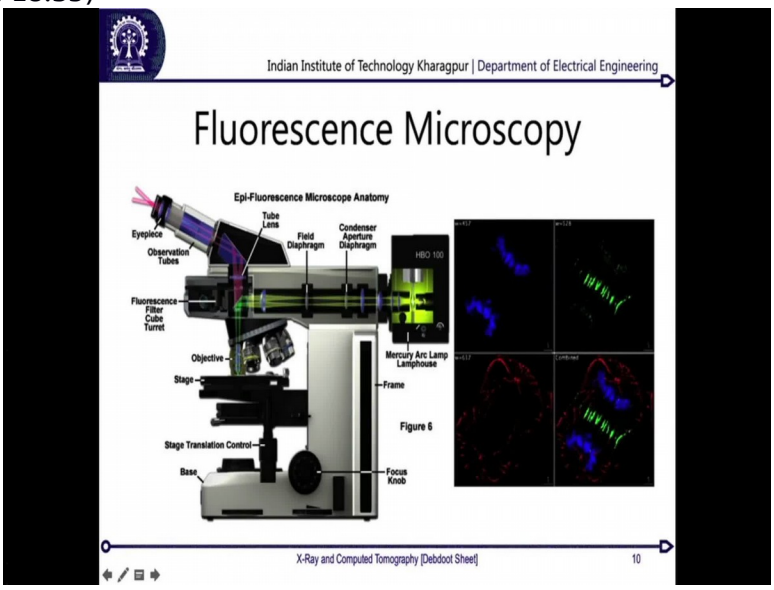


So these where some of the aspects you need to keep in mind and with that I move on to another variant of a microscope which is called as a reflected microscope. So in transmission light microscope what we had seen is that there was light box over here, you could see the light going from here to your objective. In reflected microscope it is interesting that there is a light box which is placed some here over here. Light comes down through your objective piece over here.

Now what happens is that say you have an object and this is opaque, so if you have a light coming down from here it will never pass and I cannot see anything. Now for that if I want to do I need to have the light from top and then look through it, so the only pipeline through which I can send this light is basically my objective but again I have to be cautious that the same light should not go into my eyepieces over there, otherwise if the same light goes into my eyepiece then I am going to get blinded and I will not be able to see what is around this object.

So there is a splitter over here which does the purpose of that one so that I can use the same objective for illumination as well as for looking back at the reflected light.

(Refer Slide Time: 18:35)



Now, with this kind of a microscope the practical application is actually in florescence imaging. So here what happens is that so this is a initial introduction to molecular imaging so here what we do is we have some sort of chemical compounds which are called as chloroforce and they are certain compounds which will bind onto certain specific kind of proteins on my cells. And now say I want to look into mitochondria, I want to look into certain sort of DNA structures and strands over here and certain kind of protein structures then I can actually have a different color representation coming for each of them.

And then based on what wavelength I am exciting which is getting selected from the filter banks present over here, then it will have created a different excitation. So in florescence what we do is

typically we have a different excitation emission spectra ratio over there and we do imaging at individual time. So for one particular frequency I take down this blue excitations coming, for the second one I take the green excitation and for the other one I take the red florescence protein excitations.

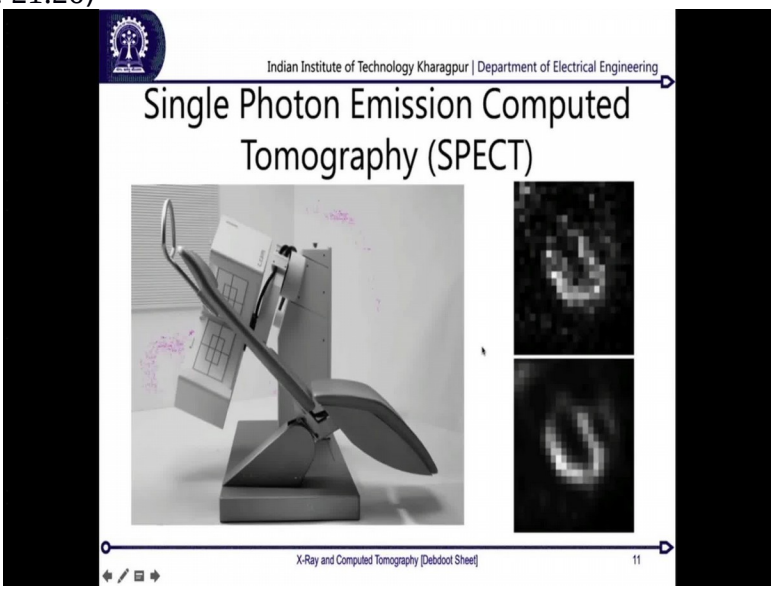
Now using all of these three together we can resynthesize an image which is basically an addition of all the three color planes in these three images. So these images are not necessarily which appear in color because typically we put down a monochromatic sensor over here to have a much higher photon yield over there, so they are again pseudo colored and back retraced onto producing my tri color or multi-color florescence images.

So this is a molecular image of a splitting cell so you would be seeing down the whole splitting phenomenon the main nucleus over here and the splitting in the DNA present over here. So you can although these are not used typically for standard medical diagnostic purposes, but we do make use of florescence imaging a lot for research purposes and for understanding biology of certain disease processes in total.

These are much more advanced microscopy than you would be using in a day to day clinical diagnosis platform. Now that we have known about the use of these kind of molecular markers for in florescence microscope one question would definitely strike us as can we use for macro imaging as well? I mean I just want to look into so from the CT to T1, T2 and everything we had seen that from CT to x rays to MR's I can look into the structure of the body.

Now I want to look at what function is a particular structure doing or say different tissue layers within a particular organ if they are behaving in a different functional way and I just want to look at it. So in order to do that you can again use molecular markers with this are a bit different because they are not optical emitting one but they say they are responsive to x rays or gamma rays and we can find out what object is behaving in what way.

(Refer Slide Time: 21:26)

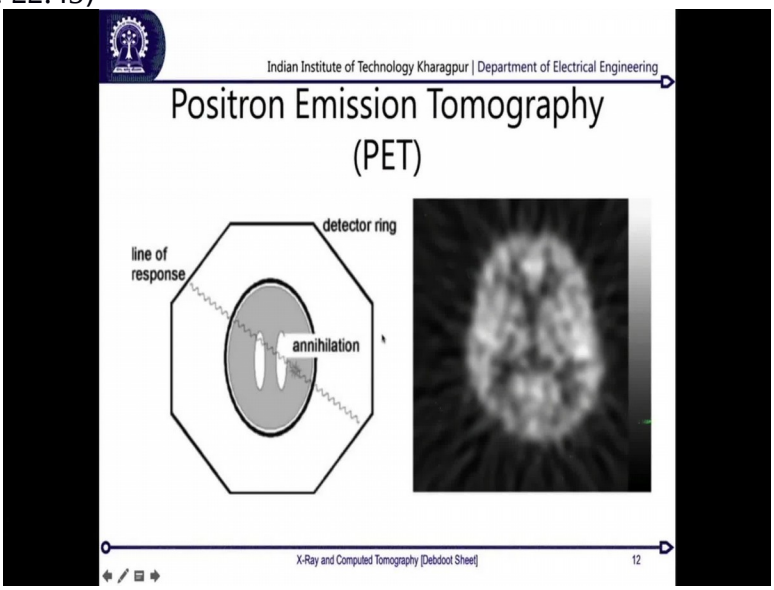


So one of the first one is called as Single Photon Emission Computed Tomography or a SPECT system which is very much useful in order to look into gamma photon generation from radio nuclides. So what we do is we basically put down a radio isotrop along with glucose and just give it to the patient there and now if certain organ has more amount of glucose consumption than other organs over there, so I would see more of radio nuclide traces coming down from there.

But given the fact that these radio nuclei traces, they are just pass phenomena which occurs only once, so you need a very good kind of a detector over there coming down which can trace down one single photon which is coming down, one single gamma photon. And that is why this kind of a tomographic system is called as a SPECT system. Now the resolution generally offered is much lower. So there is a raw resolution, there is just a corrected resolution over there and that is why you always have a structural scan system either a structural CT or a structural MR along with a SPECT.

And these two images are registered together in order to come down to a diagnosis about the by both knowing the structure and the function along that structure because these molecular imaging they do not give you a very high structural resolution as such.

(Refer Slide Time: 22:45)

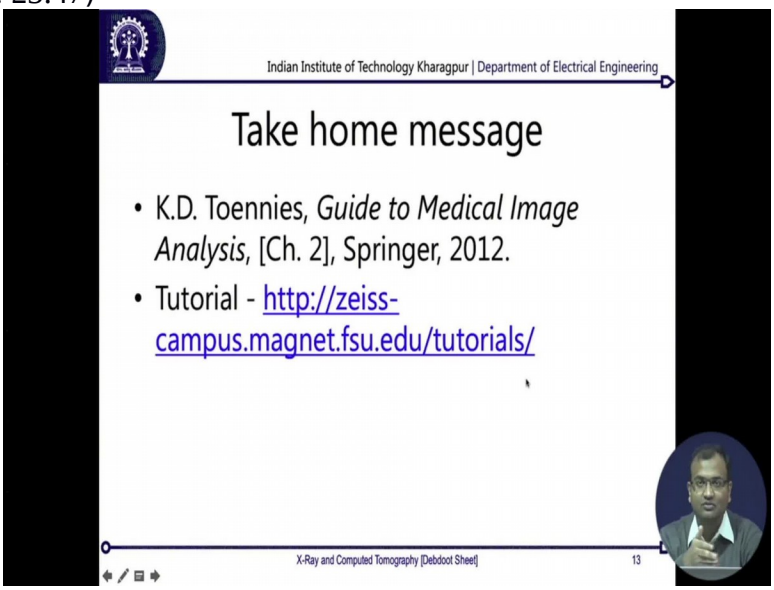


So the other one is called as a positron emission tomography and what this does is whenever there is a positron emitted you would see a annihilation created and a pair of gamma photons created over there. So this means that I always need to have a pair of detectors which are 180 degree apart and if these are rotating then I will be sensing down a pair of incidences. So this actually helps in reducing a lot of noise coming down so noise will be independent on each detector.

But if it is a pair wise production and compensated with the travel time, then I can always predict down in space exactly from where my proton was emitted over there and my positron was emitted and that is the concept of a positron emission tomography. This is also used in couple with a CT or an MR because the general resolution offered for this one is much lower and you would need a couple one in order to get a much higher structural resolution as well.

Since so these are to just give you a basic introduction and an idea of what different modalities are.

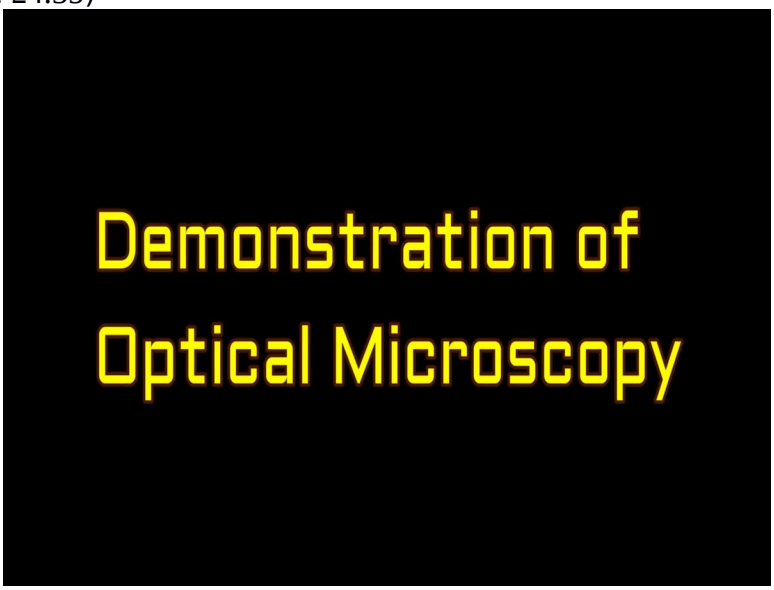
(Refer Slide Time: 23:47)

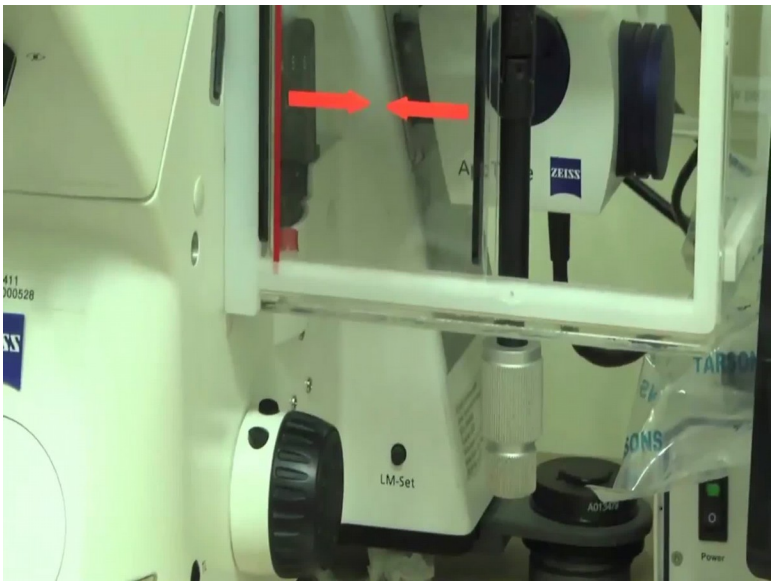


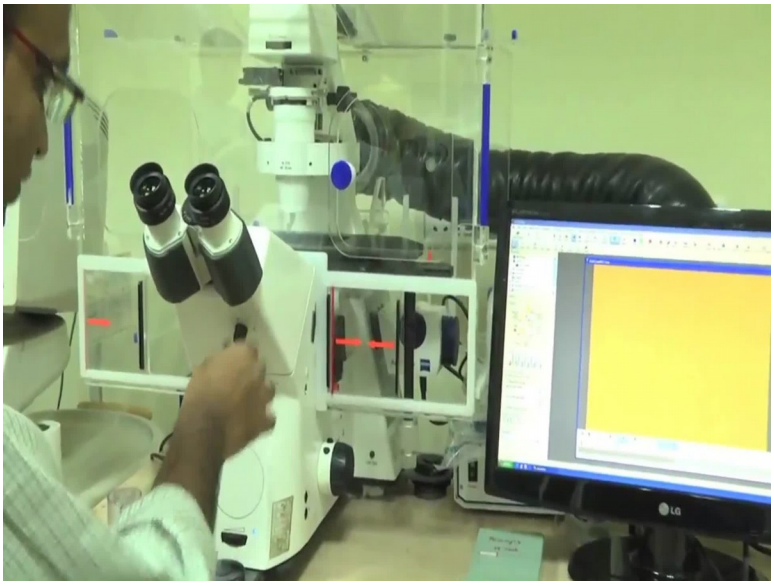
You can read more about these modalities which we have discussed on microscopy and molecular imaging in Toennies Guide to Medical Imaging on the chapter 2 on digital imaging techniques and for microscopy you can have this wonderful tutorial from Zeiss campus magnet at FSU and they have very interactive tutorials which are flashed based and you can just move through them and change different configurations of a microscope and see what happens to the light beam or the image formation processed together.

So with that we come an end to the first week of lectures on getting introduced. In the second week we are going to start with more of basic introduction to analytical tools which are useful for medical image analysis and eventually we built up on top of that, so with that thank you.

(Refer Slide Time: 24:35)



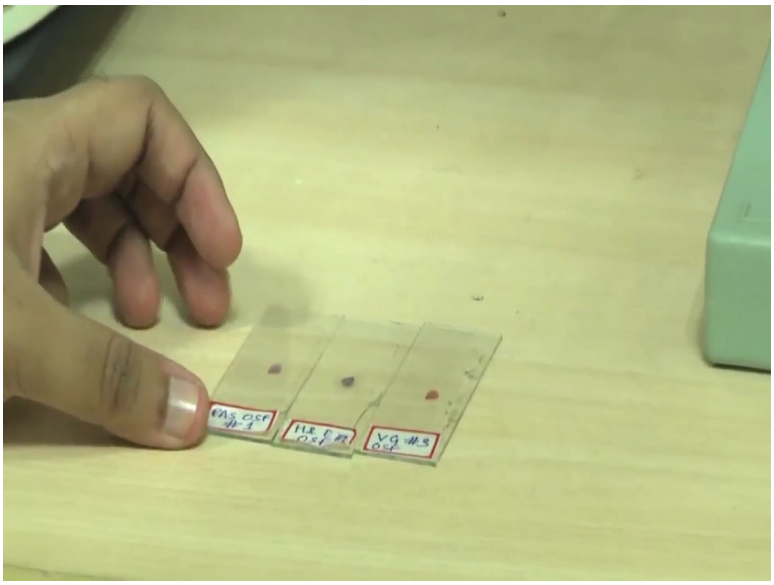


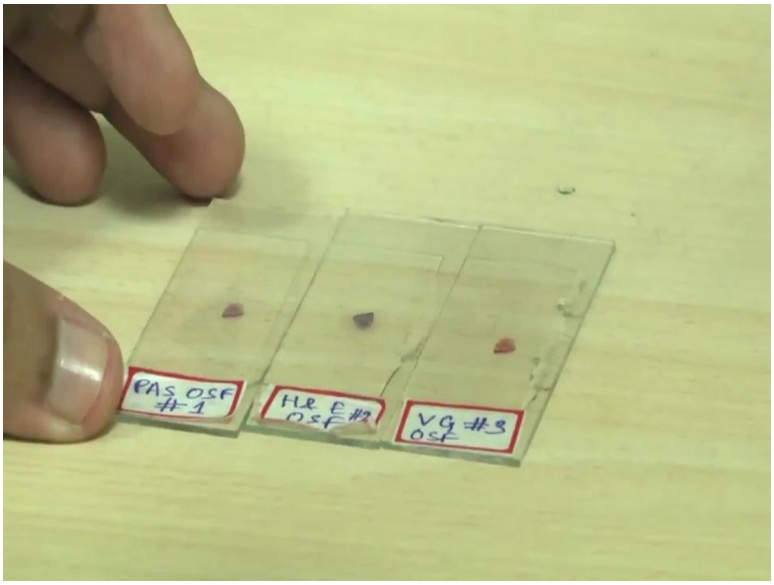


Welcome to this demonstration on optical microscopy and although this is a very high end microscope which is for the purpose of showing how you would be acquiring digital images in perspective of taking them for digital pathology applications but in general any standard microscope would have basically an illumination over here, so since this is we are operating at a bright fold and at bright field and a transmission mode microscopy so you have your illumination this is from where the light source comes down and somewhere over here you would be seeing down the objective over there.

And now since and from there the rest of the optical assembly is housed inside over here, this is my control nob for changing my illumination which I can increase or decrease and here is my eyepiece from which I can actually look into so generally if I do not want to look onto the screen side of it I would be looking through this particular piece and I can see everything magnified on the present on my slide over there.

(Refer Slide Time: 25:44)

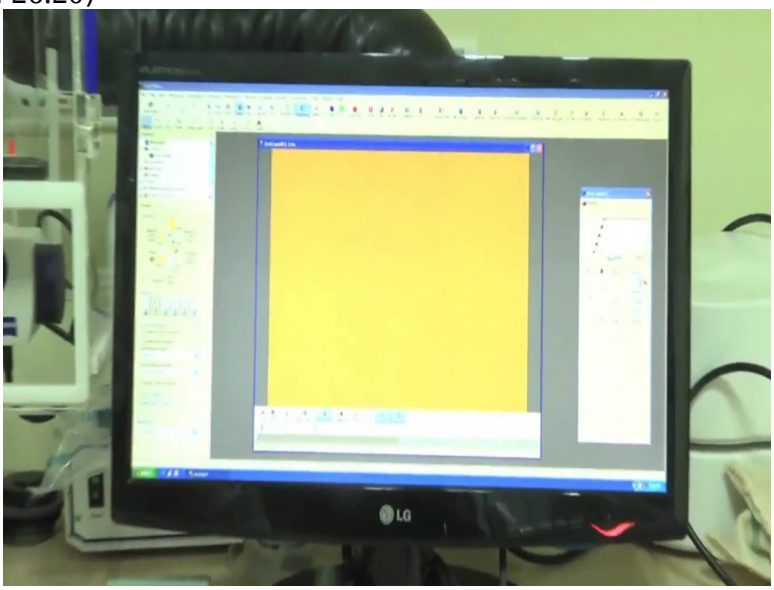


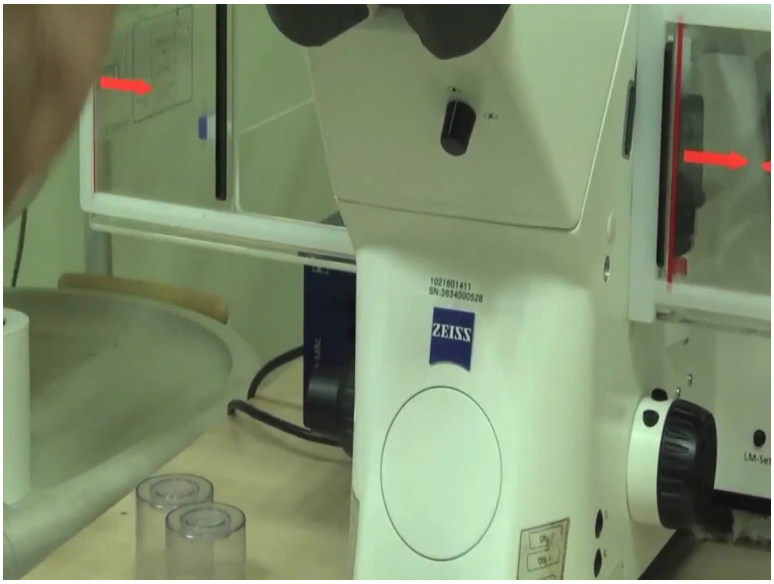


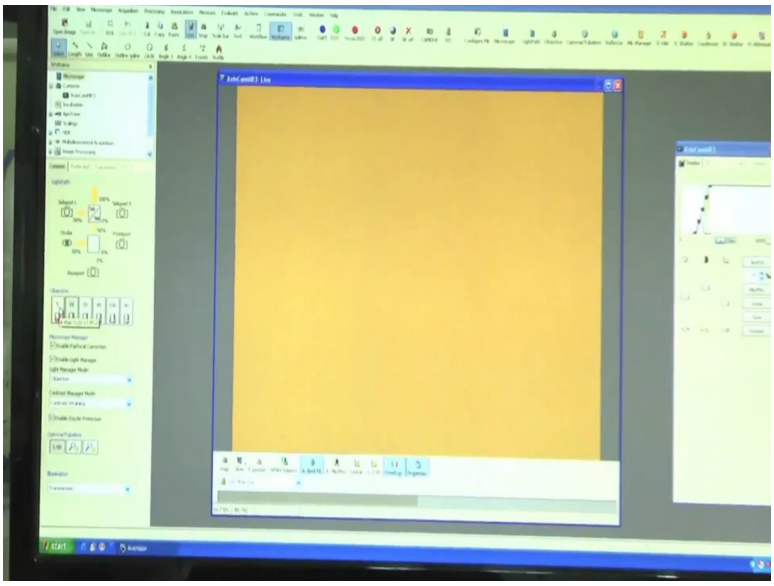
So I would be demonstrating basically with three different slides today and one of them is hematoxylin and eosin stain H and E stains slide, the other one is PAS Periodic acid–Schiff staining and the other one is (())(25:59) staining over there. So these three slides are what I would be showing down through this microscope (())(26:05) and they are basically the same tissue block subsequent serial sections neighboring sections which are stained down by independent individual slides.

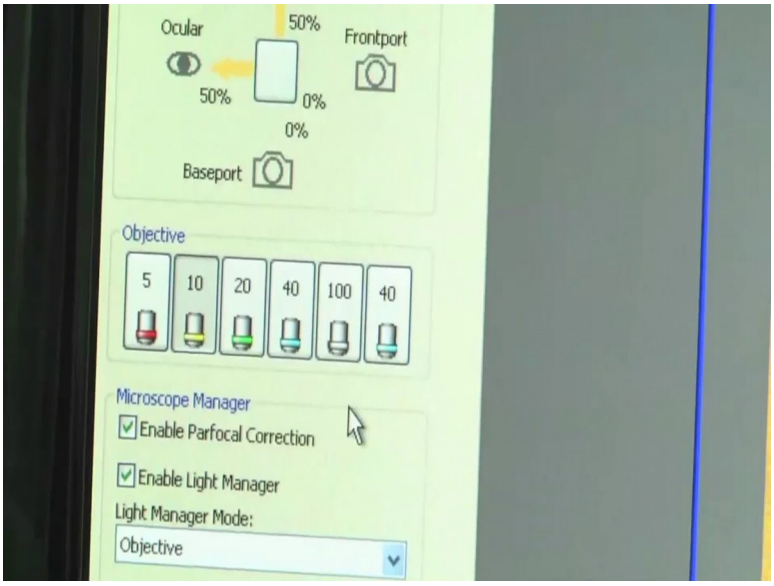
Now as per our microscope we had done on the lecture theory over there so we have a set of beam splitters.

(Refer Slide Time: 26:20)








Ocular
50%
Frontport
50%
0%
0%
Baseport
Objective
5
10
20
40
100
40
Microscope Manager
Enable Parfocal Correction
Enable Light Manager
Light Manager Mode:
Objective

So over here since this is a research level one it allows you to show whether you want to turn all the like the like 50-50 part into this objective over here and you want to turn the rest of the 50 part into your side port which has the camera mounted. So on this microscope you would be seeing there is a camera this is the digital camera in blue which you see.

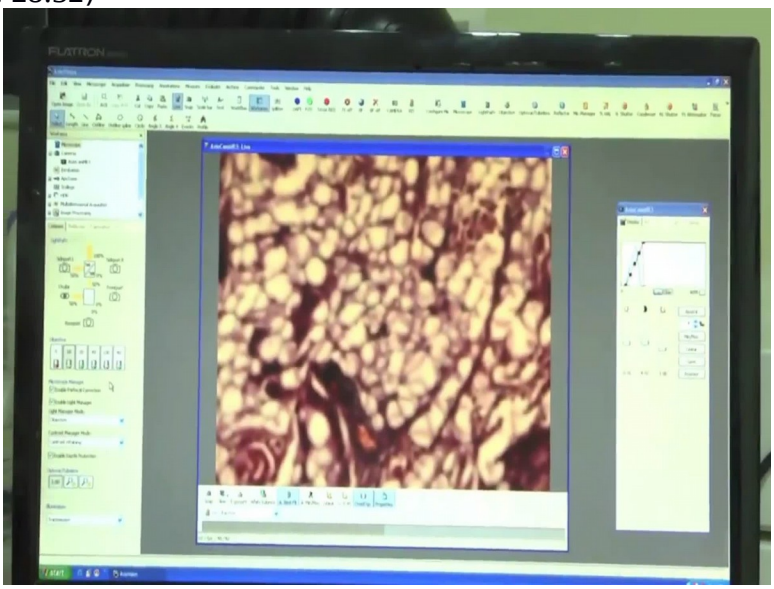
So in this particular configuration I can see both on my objective over here on the eyepieces and I can see whatever is coming from the objective on the camera and on my digital screen over there. Now moving back on to the digital screen where I have all of my controls, so initially we are using just a 10x objective over here for our magnification. We can change over to a 20x magnification, 5x, 40x as and when we require them. There is also an 100x one and there is a 40x with oil emersion also available.

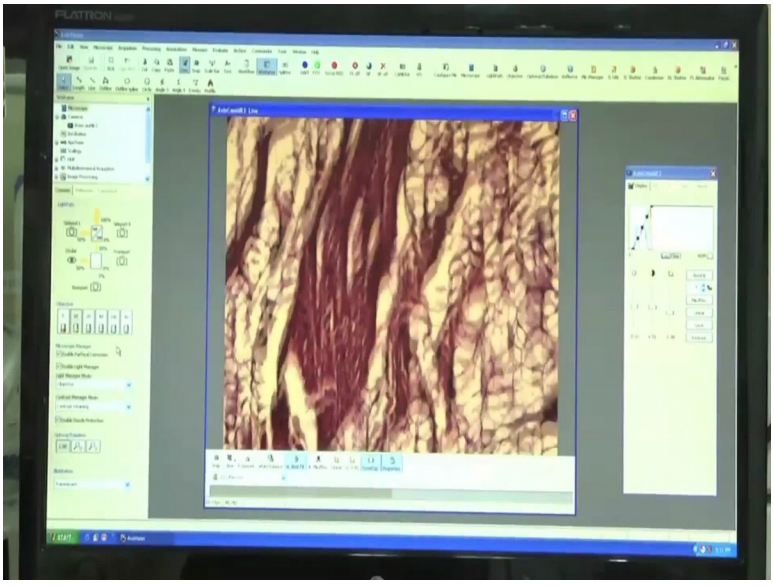
So in total this one has 6 different objectives which are available. So without taking much more time explaining them because they are not what is objective of our whole class over here, so I am taking initially this hematoxylin eosin stains slide over here and I am just going to put it over there. Now remember that since the objective is from below this is a inverted microscope this kind of an objective one the where you have the objective on top and you are seeing that is called as upright microscope.

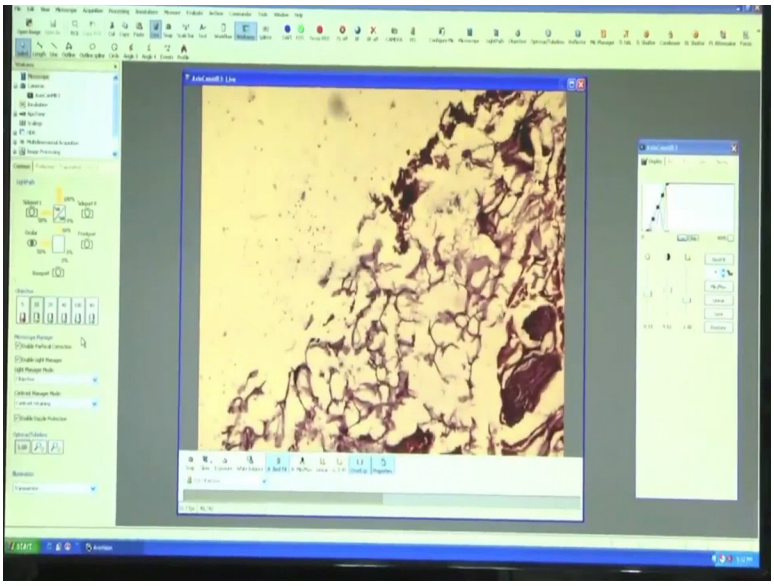
So since it is inverted microscope and if you look on the slide you will see a small segregation over here which is my cover slip which is used to prevent down oxidation over here so basically

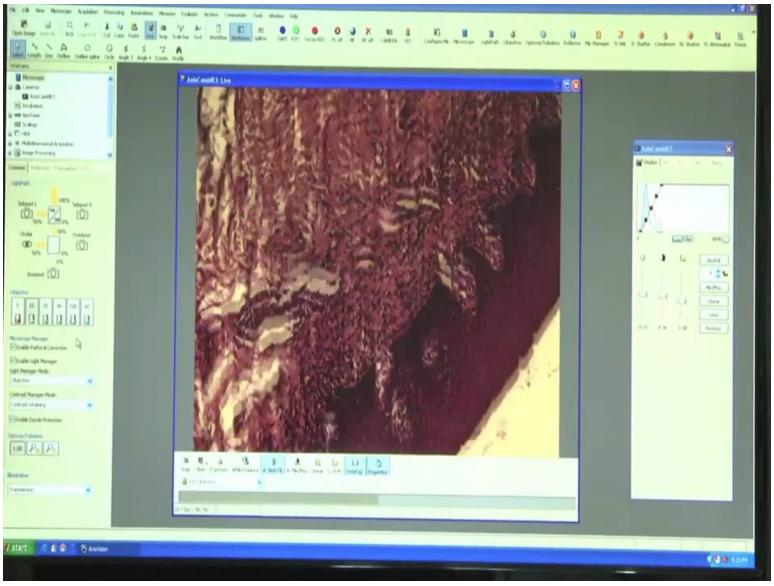
there is a slide the tissue section which is stain and then I put down this cover slip on top of it to prevent oxidation. Now this layer this side so there are two so this is one surface of the slide, this is other surface of the slide. I am supposed to put this surface which has the tissue section over there closer to the objective lenses, so that is why I am going to mount it this way on over this microscope.

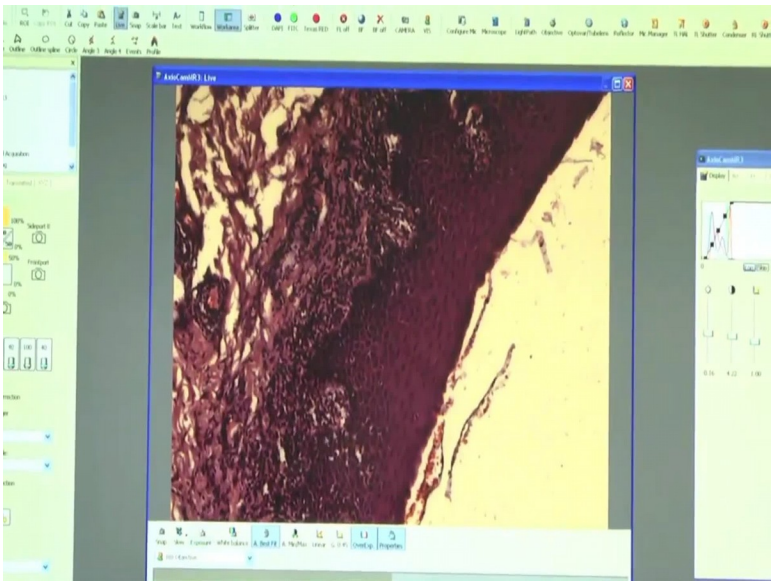
(Refer Slide Time: 28:32)











So the moment I mount it you can see something's on the screen and now the next thing I will have to do is basically do a focusing control over there and try to focus on what I am able to see on the screen. So as you see I mean to a untrained eye this would basically appear as if a garbled and what I am doing is I have a stage position control nob over here which is present over here. This can do my xy direction moments as well as my up and down movements.

So this is what I see now. Now this is a section of the biopsy of oral tissue, so you would be seeing down your epithelium and then the basement membrane somewhere over here and since this is oral (())(29:22) and fibrosis so just below the mucosa you do have a lot of fibrous cells and fibrous tissues over there. Now I mean that is just part of the pathology which we are going to discuss.

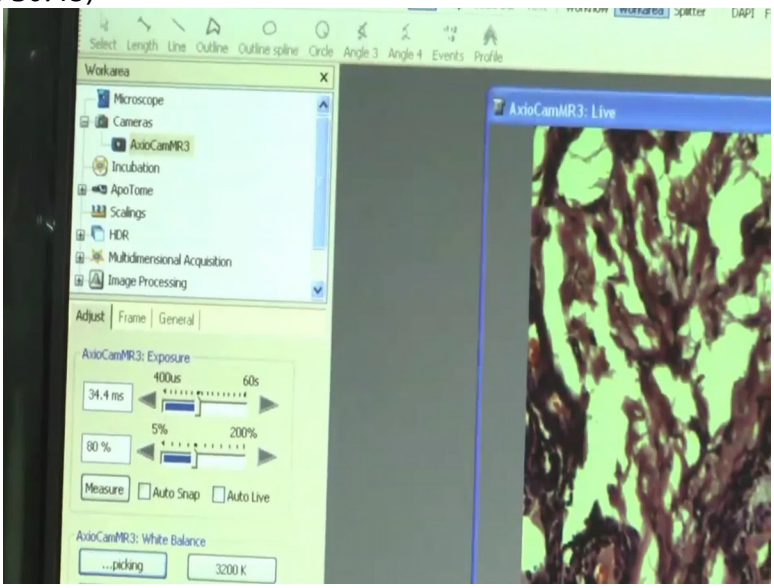
Now for the control side of it since this is again a standard camera so as I keep on changing my illumination obviously I am going to see changes over there and now I have my over exposure indicator on. So these regions, these particular pixels over there are already getting saturated and over exposed, okay. So given that I am at a particular exposure level the first thing I would be doing is setting it down to a exposure compensation and after that the best one is to do a auto white balancing.

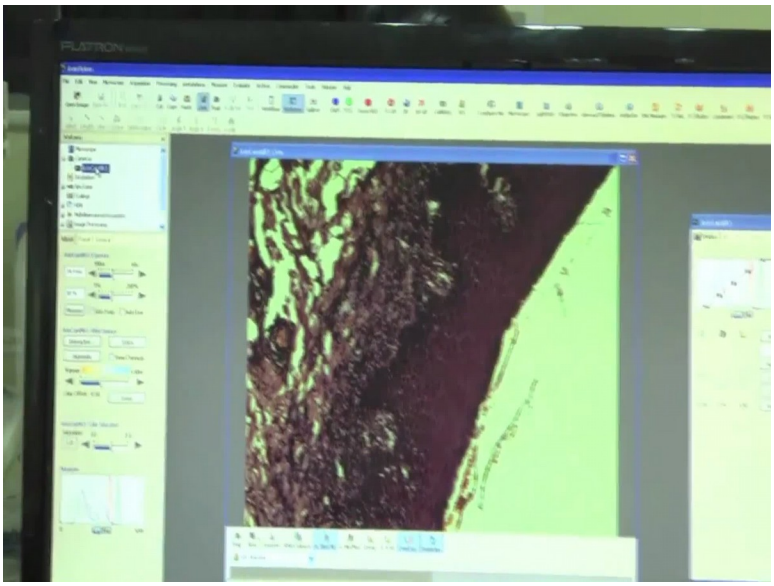
This is what it comes on auto white balancing but the cardinal rule always for white balancing is that whatever however you whatever you see with your eyes the same color is to be reproduced

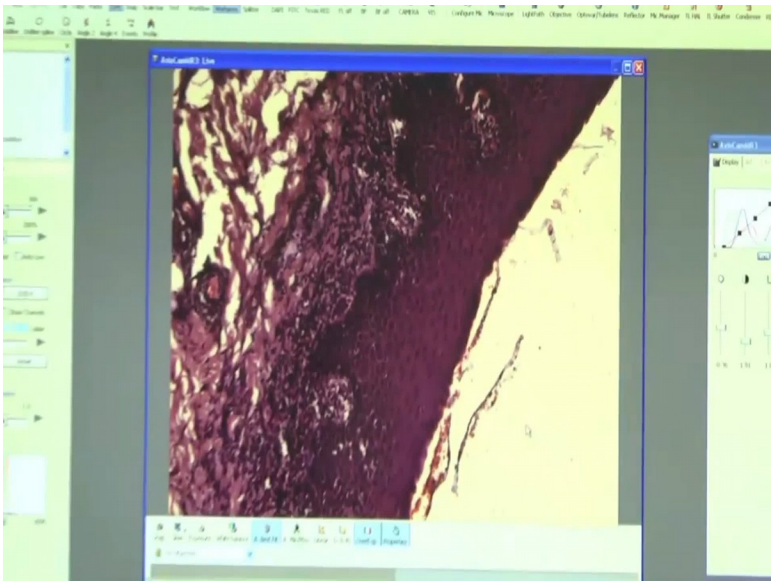
on the screen as well and again taking into account the non-linearity in mapping down colors by the monitor itself, we have to invert that together.

So together it should be something like what I am seeing on these eyepieces since for a purpose we cannot fit a camera on this eyepiece to show you well so I am try to basically match down that color with one, so the best practice is that you basically look over here you know what is the color of the tissue and the color of this so color of the tissue and this over here. So this part is supposed to be a bit more yellowish in nature and that is what not coming down over here.

(Refer Slide Time: 30:48)

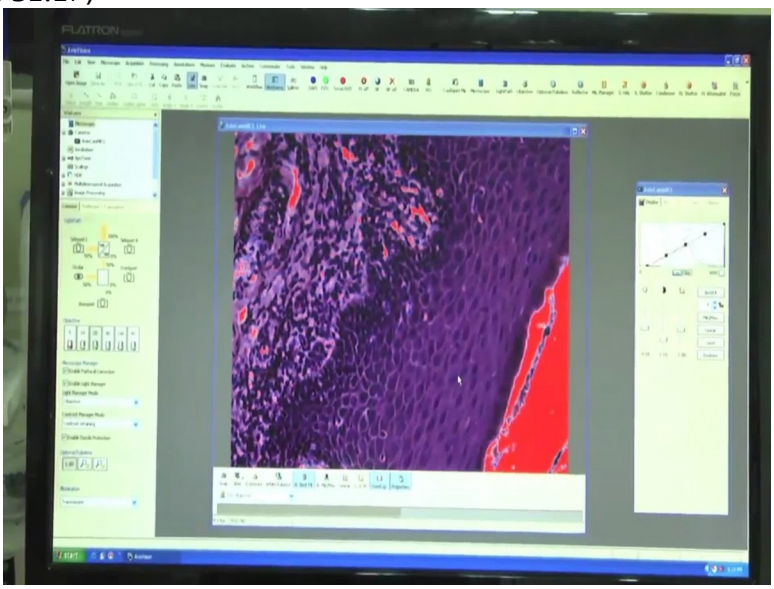


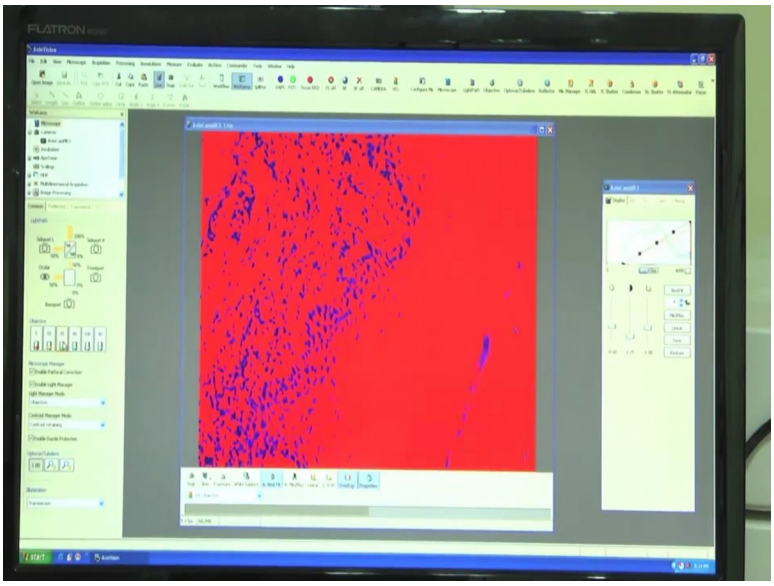


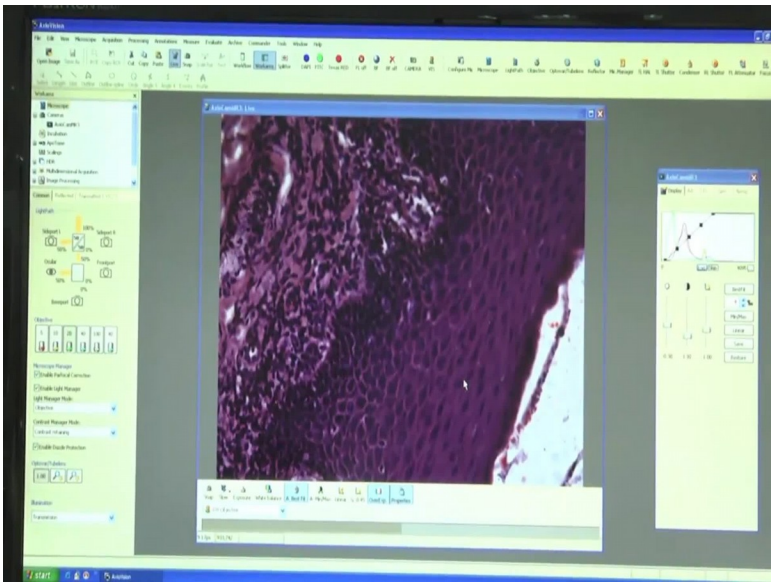


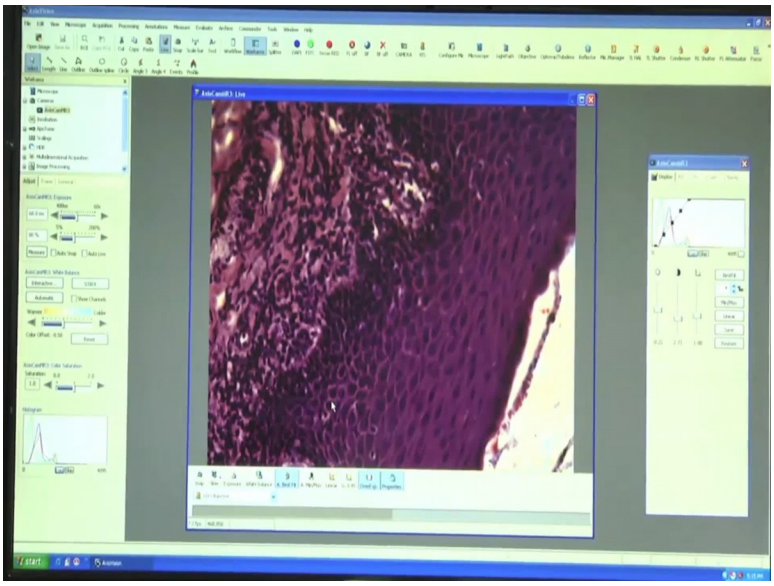
So what we will do is we go down to the advanced settings on the Axio cam controller over here which is the camera controller and start a interactive white balancing by picking up a location say I pick up this particular location. And now I see that since this part was supposed to match down with my color, this actually does a good decent job of color compensation over there and is a very important step whenever you are doing the edle pathology because otherwise color shifts will introduce major croma shifts and your algorithms might not work, so that is one part of it.

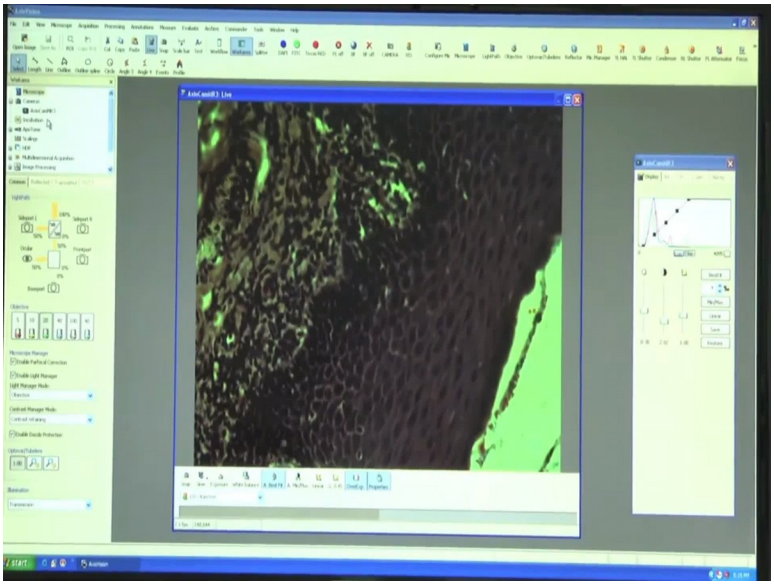
(Refer Slide Time: 31:17)

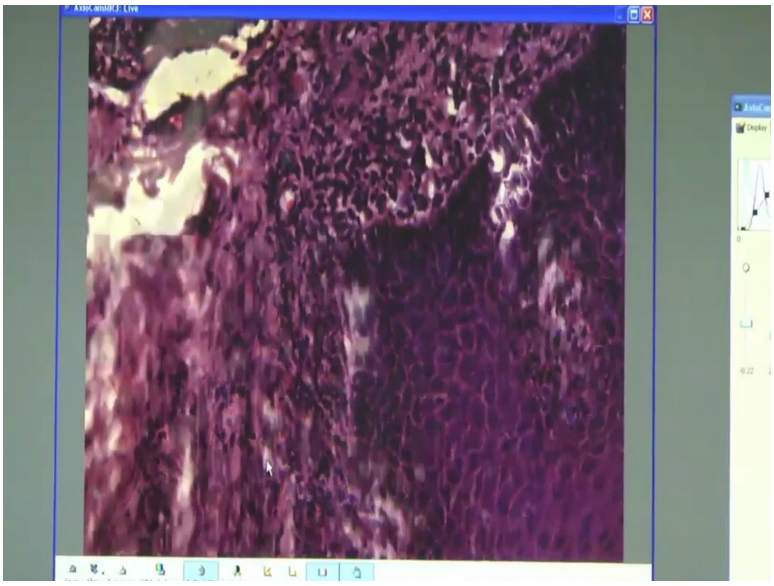








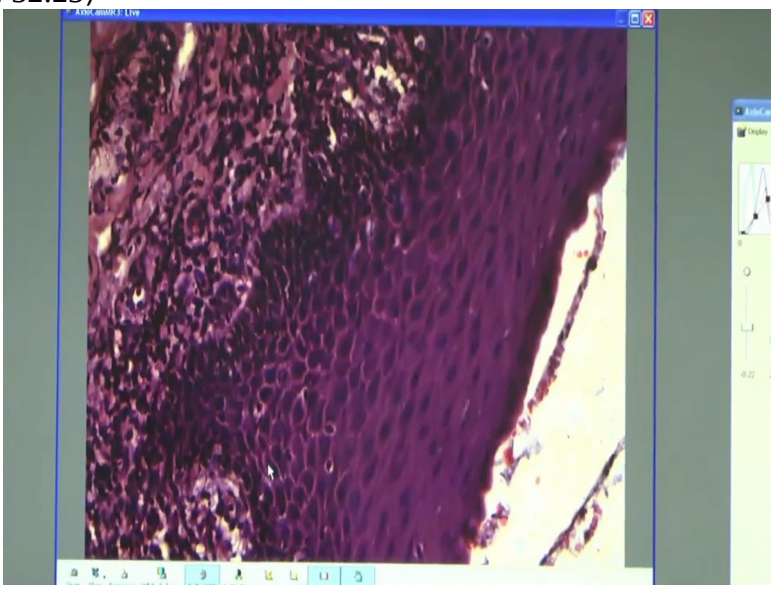




Now coming to the major parts that since you have different magnifications and what happens over here so this is at a particular magnification, now I change this one to 20x over here so it moves and then puts me on a 20x magnification. Obviously I see there is a lot of part which got over exposed and that is because in particular 20x magnification you have a much smaller area which is being seen and then the total amount of light to be compensated matched on with a numerical aperture has to be appropriately done.

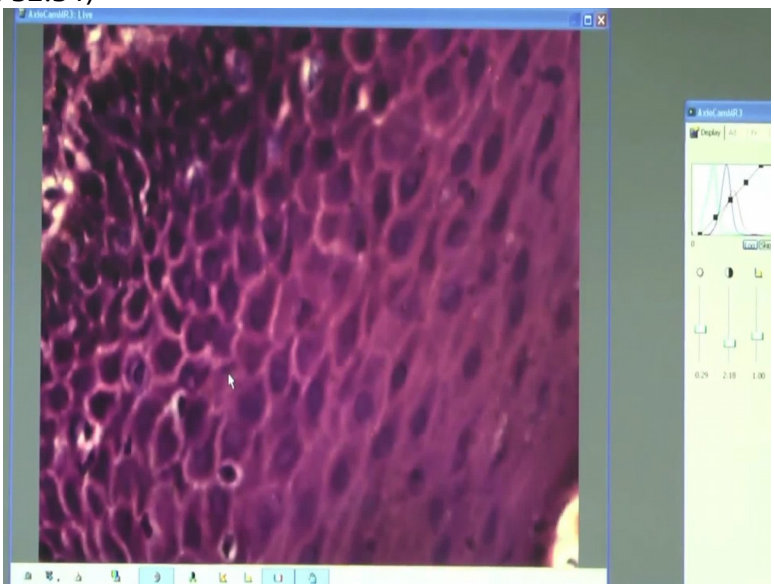
So basically I am going to pull down my intensity levels over there, then do another level of exposure compensation and a white balancing and again that I see this change coming down I will go into my camera settings over there, do an interactive one and balance it out and now I have it, perfect. So I we do see that some part of it is again out of focus so I need to focus it very carefully, so this is where I get most of my objects now in focus, okay now if I change this is how it would be translating and you can see.

(Refer Slide Time: 32:23)



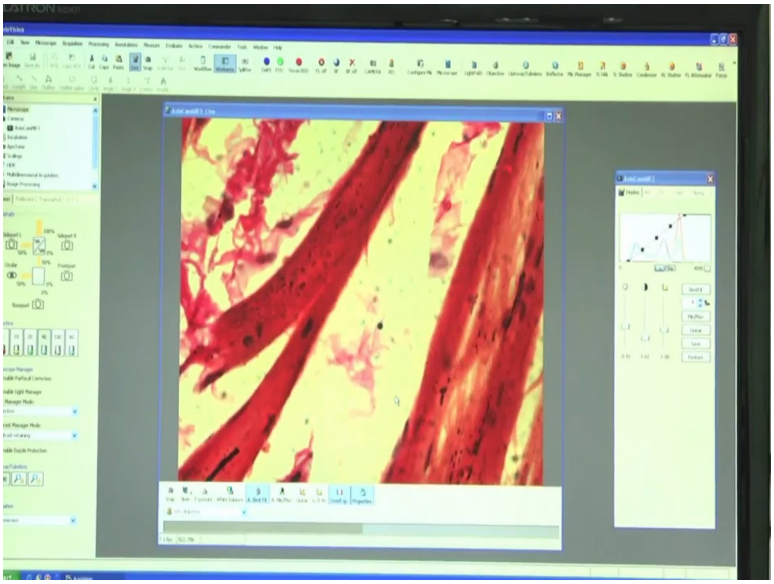
Now at this particular magnification of 20x you can rather make out all of these individual nuclei for the first time which was not so evident in 10x magnification.

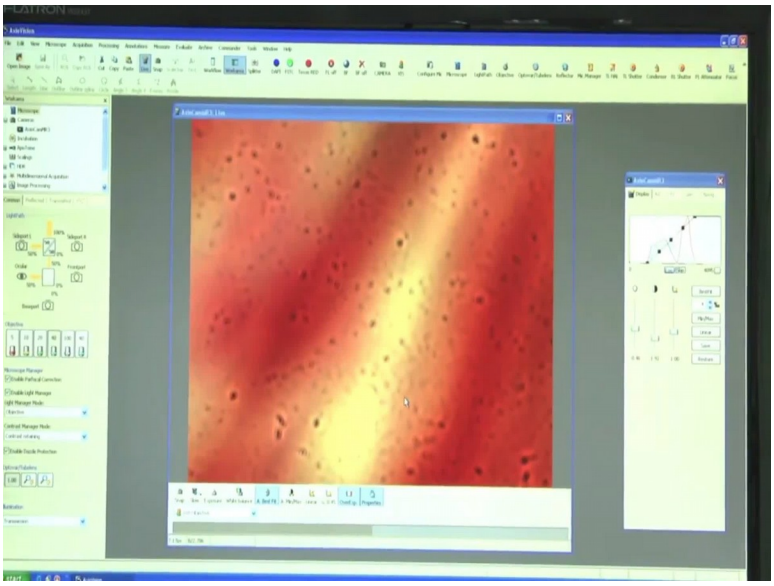
(Refer Slide Time: 32:34)



So from there I move onto say 40x on my air mount one over there and I have a much higher magnification but I just need to focus on my objects so. Now that we are at a 40x objective magnification you can that the cells and the nuclei so the cellular membrane over here as well as the nucleus is quite distinctly visible which was not there in lower magnification. So this are the different effects different magnifications have and what we use over there for individual purposes.

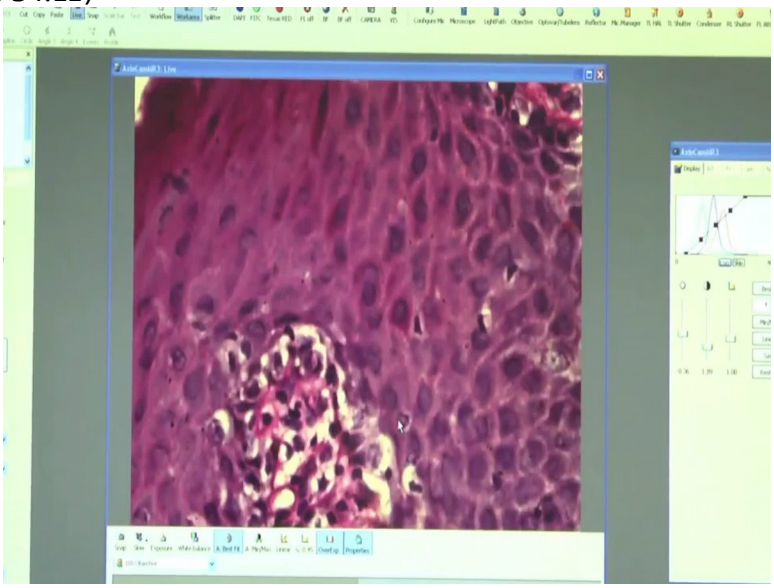
(Refer Slide Time: 33:14)

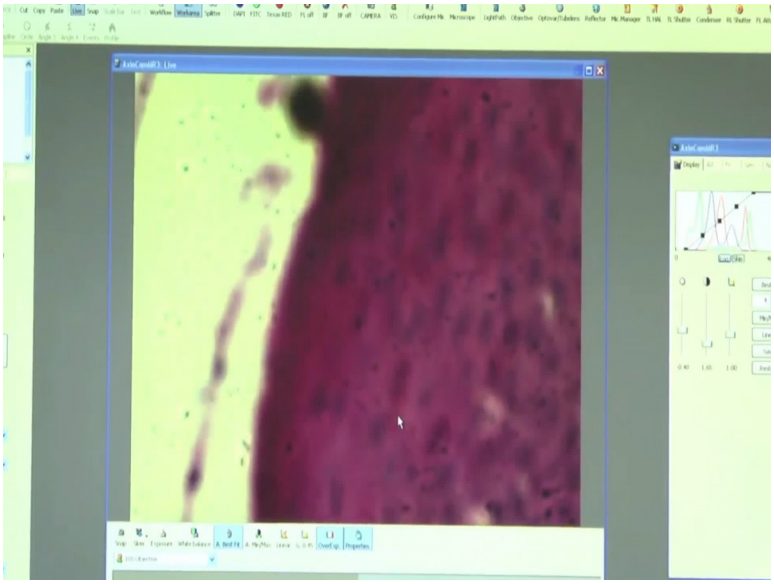


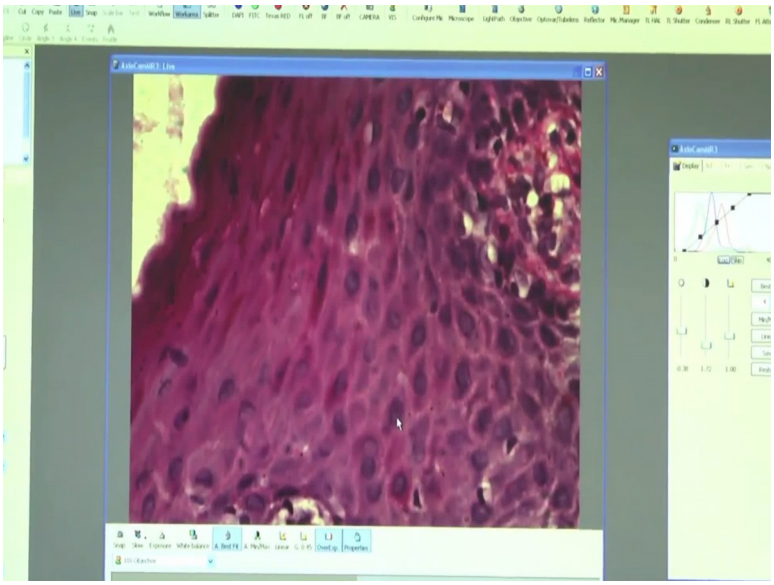


So this was about an H and E stain slide. Now I am going to change this one and put down another slide which is called as Van Gieson staining or VG. So let us position it over there at 40x I again try to put them onto yeah. So this is my now you have your epithelium region again present over there, so for a different stain you have a different way. One thing you see is this is actually reddish in color for Van Gieson stain, you do not have the bluish staining component present over there at all.

(Refer Slide Time: 34:11)







So I can change this one and let us put down a PAS staining Periodic acid–Schiff (())(34:12) agent stain over here. So I have placed it. Now I am going to just center it appropriately and somewhere I am close to it, I do a fine focus control and yes now I a at over here, okay.

So this was the effect of different stains and some common magnifications over there, you have many more mode supported on it, it can do florescence microscopy, it can do dark field microscopy with phase contrast as well but our whole subject is related only to digital pathology which is again with bright field microscopy only and this is one of those examples of how to use microscopes for digital pathology. So the details have been covered over here. We have a much more detailed case study on digital pathology problem as well somewhere in the fourth week upcoming forth week over there, so with that thanks.