Optical Spectroscopy and Microscopy
Prof. Balaji Jayaprakash
Centre for Neuroscience
Indian Institute of Science – Bangalore

Lecture – 54
Fundamentals of Optical Measurements and Instrumentation

Hello and welcome to this lecture series and optical spectroscopy and microscopy. So far what we have seen is we have seen in parts I mean part by part different components of a light microscope. We talked about the light source, we talked about the lenses, we talked about the detectors; we talked about the filters, and how do we choose different filters to be able to optimize our correction for the fluorescence and so on and so forth.

In this part of the course or this part of the lecture what we will be concentrating is we in fact saw in little bits the detectors themselves; we would see the detector separately. But the point is we will; in this part of the course in the lecture what we will concentrate is the associated electronics and how are we actually going to synchronize all of these different components such that in a laser scanning system we optimize the light detection.

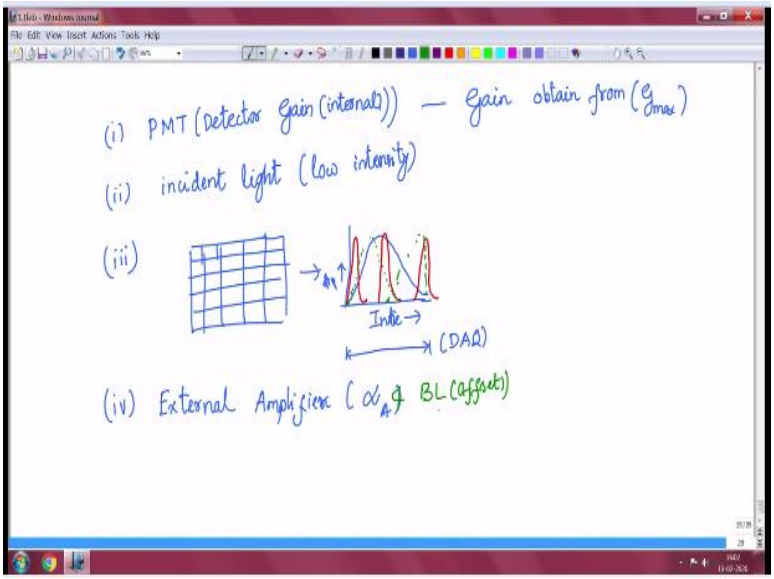
And what are the things that we need to watch out for to get an optimal image purely in terms of detection perspective, not necessarily in terms of the spectroscopy that; what do you mean by that is that not in terms of optimizing the filters; not in terms of choosing the fluorophore so on and so forth that is been dealt with separately. But now here given a microscope given of the sample now I am going to go ahead and acquire an image.

Now I need to know what parameters do I need to look for, how do I go about obtaining an image that would be a better or the best possible representation of my sample that I have with me, all right. So, so this would require a step by step checks that we need to tick check as we go along and understanding why are we ticking this. Clearly these are something some of these things are discussed in the course before so I will not go into them in detail.

But I would briefly mention in which context we discussed. And there will be some aspects that I need to discuss with you which is not discussed before. So let us assume that we have a sample

and we are going to try to take a image which is basically a representation of that image using a laser scanning system. Now how do I go about doing this? So please note I am talking about laser scanning system in general which means it is true for both confocal as well as multiphoton. So let us go ahead and look at what are the boxes that we need to take.

(Refer Slide Time: 03:16)



Now the first; first and foremost, when you are having a sample the thing that you want to optimize is the step number 1 is you want to look at the photomultiplier tube or I would call this as detection system so detected gained internal, okay. So we will call this as an internal; basically this is in very, very many sense it is actually the gain that we obtained from the cascading effects of the photomultiplier tube.

So we have described this in the class right in the beginning or once in between and perhaps later in a much more detailed manner. But this is again that is coming from the internals of the PMT itself right the grids the; each of this electrons coming and then generating secondary electrons and each of these secondary electrons in turn producing more secondary electrons so on and so forth that gain we are talking about.

We do not want to be too much where any change in the intensity; so the whole idea here is to match the dynamic range of your sample to the detection system right, okay. So you do not want to be too high wherein the brighter objects are totally saturated you lose the differentiation at the

brighter range but at the same time you are able to see the smallest mean as close to the dark signal as possible, alright.

So in such a case we would like to attain the maximum possible gain in the PMT, so you would go and make sure that it is safe to operate and then really go at the highest gain possible. Now there are ways to characterize this gain so this is totally dependent on the device and the associated electronics powering up the device and the internal voltage and etcetera. So in a lab you could actually do a small little experiment.

We will not go into that in this lecture but you would; we would perhaps if time permits we will see in later in the course or we would go into any of the PMT catalogs you would be able to arrive at this tests and then perform this experiment where you actually vary the PMT gain and measure the signal-to-noise ratio and it turns out that beyond a certain gain internal gain it is not of much help. So you want to be as close to that as possible.

The reason why you still have a higher gain is for a completely different reason so; but right now we are going to up reach that gain and then we I would right now call that as call I would call it as g max, okay. So you have your sample you turn down your; put the sample focused with your eye make sure that this is the region that you have; want to be imaging.

And then now you have; go set the PMT to g max make sure these safe lights are not going to; I mean unnecessary lights are not going through and all that. Then at this instant what you need to do is that you should start letting the incident light beam, okay instant light in starting from lowest intensity. The goal here is that I just want to be able to use the minimum amount of intensity that is sufficient to capture the entire dynamic range entire contrast right here in my sample and with as less as less noise as possible.

Higher the intensity the photo damage and nonlinear effects will kick in so we would like to not have that, right. So we would start increasing the intensity from the lowest possible value. At that point how do I know is that sufficient; I mean if this, a good image or not so that definition is

provided to you by very simple measure. So once you have an image, so what you are actually looking at is basically; remember this image is a set of pixels.

Each of them representing some point in space in your; where your sample is located. So you are actually looking asking; what you should ask for is you ask; you should ask for a histogram of the pixel intensity. So the intensity is here or in the in this axis while the number of pixels and the y-axis. So what you would like to see is that this histogram is spread out like that. Now where the Mac; the range of the intensity, okay.

What determines this range, we will come to that in a minute, okay. So this range is the maximum dynamic range of your digital data acquisition card, okay. So make sure that you are looking at that entire range so it typically it is; if it is an 8-bit it is 0 to 255; 12-bit 0 to 495 so on so forth. So just make sure that you are looking at the entire range. When you are looking at the range you would like to get a histogram that is like this blue line and not this or this or this.

You actually would like to spread it and be in the middle so that you know that you are capturing the entire thing. Now how do we do this? In case if you have a histogram that is coming out like this then what do we exact; what do we do as a next step? First thing is that immediately you realize that the PMT the; since you have actually maxed out and the gain from the PMT you really do not have any multiplicative gain from the photo detection process itself that is no that is not available to you so the only way you; now you can do is that you have to use the external amplifier gain.

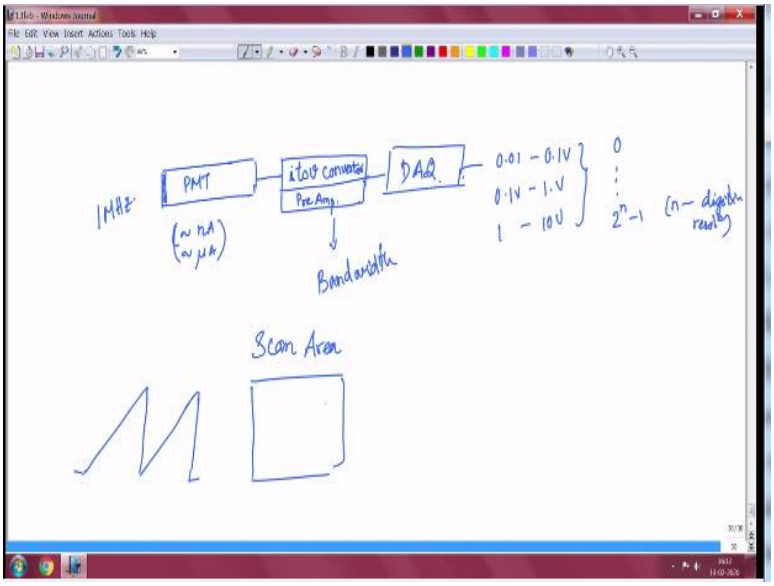
And only in this circumstance you actually want to utilize this otherwise it is unnecessary and then you are going to add and unnecessary noise to the system. So at this place you can actually use the external amplifier gain, so we will call this amplifier gain as alpha A. So as you increase the amplifier gain what you will see is that there will be a slight shift in the most probable value but most noticeably you would see the widening of this histogram, alright.

Now as the histogram widens and the most probable value starts to shift you might reach a position where it is something like this so because; so when you see asymmetric; skewed

histogram like this either in this end or on the other end. This means you are saturating you are reaching the max of the DAQ card so you need to do something to bring this back. So at this point what you want to do is you want; I mean you have to play with the gain as well as the baseline or else sometimes called as an offset.

So depending on your; depending on your system that you are using one of these; I mean it could be called any of that but by adjusting these two you want to make ensure that your histogram is really in the middle. Sometimes despite your maximum utilization of this amplifier gain and the baseline offset you may not be able to reach it in the; such a case, only in such a case you start going; go about increasing the incident intensity of the light. So once you have reached the maximum levels only under such circumstance you actually go about doing that. So now what is happening here is that.

(Refer Slide Time: 12:55)



Let us look at the little bit of an electronic that goes in here. So we have a PMT and then the PMT signal for the multiplier tube signal has an interfacing. If you are actually using an analog PMT you will have a current to voltage converter, all right or i to v converter. Typically, this is an integral part of a preamp so; which will allow you to amplify that; it will allow you not only to convert the current into the voltage but also will allow you to amplify that voltage such that it is like a buffer, right.

So, such that the range that you see here, right. The amount of current that comes out from the photomultiplier tube is anywhere between; depending on the kind of the PM to use it can be anywhere between nanoampere and the best of them are about microamperes. This is for photon counts of about one megahertz or so, okay. So any more than that you are actually starting to it eat out the photocathode that is present on the PMT, so that is not advisable.

So in this range; so now it is; the DAQs that are available that are finally going to associate a numeric value to this current that I am going to measure this current at the speed where you; where the speed of the galvanometric; at which you are operating galvanometric scanner, right. This speed matching is super critical. Now well this is because you are ability to localize the photon intensity in space comes from the synchronization of the galvo operated signal with that of the PMT output digitization.

So if; for some reason you are; if you are; digitization is slow then you would see streaks basically the light originating from the previous space extends to the next space that is; that will appear like a streak; the images will have long horizontal streaks that is a very good example that; assuming that you are going a raster scan with the horizontal axis being fast, that is a very good example; indication that your speed of either the digitization or the amplifier electronics, the bandwidth of the amplifier electronics.

Basically this is an integrator, right the i to v and then preamplifier. It is a basically axial, I can integrate in circuit and that bandwidth; one of them is slow. It is not; it is not as fast as your galvo so either you have to increase that bandwidth our slow down your galvanometric mirrors. On the other hand, if you have to digitize faster than the galvanometric mirrors, you will see that you would be missing; mean you will be many of the photons sensitivity will be very, very low.

Because your; I have not still moved from that space but you, you sample that and then you are taking one of them; it is not a sample and the whole so; it is not integrating over this entire time you are integrating over very short time. So you are missing out a lot of photons from that. So your sensitivity for that time; the amount of time that you spend would be very low. So you; it is

super critical to match this and the indications are if it is too noisy then you have to increase your galvano speed.

And if it is streaky then you have to reduce your galvanometric speed. Ideally, the speckle size is a very good indication and you should see the speckle; the balance between the speckle counts and the speckle size tells you, you are in an optimal range of your detection system, alright. So now assuming that you have done that now when you; take that current out from the preamp you the, the preamp would place this voltage such that it is in the range where the DAQ can start operating; digital data acquisition card can acquire the; or can digitize this to its entire dynamic range, right.

So typically the DAQs I mean that we get; the in the sensitive region can operate between 0.01 to easily 0.1 volts or in some, 1-volt dynamic range, so 1-volt dynamic range. So when you say I mean by actually choosing different voltage ranges at which you want to operate your DAQ; I mean there will be different ranges let us say one is a; let us say if it were the DAQ what allows you to go in decades then things can be as similar to something like 1 to 10 volts or something similar to this, so then why not I mean why to choose one particular range why not have the entire 0.1 to 10 volt I mean why you have to subdivide this.

The reason is very simple which is, the entire the DAQ is going to associate a number anywhere between 0 and 2 n - 1 value, right where n is the number of bits or the digitization resolution. So you would like to maximize it so that small change in intensities; intensity across that is happening across the space you will be able to better represent or better detect. So that is the whole goal that is the entire goal of this operation.

So you want to match the bandwidth to that of the scan; scanning speed; we will talk about the scanning speed in a little bit and then you also want to actually the frequency of digitization should be such that it is matched with the scanning speed as well as the intensity range or digitization should be optimal. So if you know that the variation in intensity of your sample is in the nanoampere range and it is the output of the; i to v is going to be only between at the max could be at 0.1 volts, so you are better off to choose this.

So it is; this is critical, if you want to measure changes in a sensitive manner, okay. It is a sensitive to the changes the; increase the sensitivity of the delta that you want to measure you need to actually do this; this to; to better capture the contrast you actually need to match this intensity. On the other hand, there is also the notion; there is this; the; we are doing this entire thing so that we not only capture the intensity variation but we want to capture this intensity variation across space in the highest resolution possible.

Now that means we have all; we have previously seen the; what is about (()) (21:52) criterion and spatial frequency and how we have to match it. But what it also means is that it tells you, given an amplitude or given a field of view in the sample how frequently; I mean how fast can I actually keep; can it go, alright. So if you have a larger scan area; so now; then the galvanometer needs to oscillate with a longer amplitude to generate voltage pulse.

I mean you will be generating a voltage pulse such that the amplitude of the voltage that oscillates is going to be at larger range. So now remember, this voltage itself is digitally generated from your data acquisition card so there is a slew rate in the rate at which you can actually generate these voltages. So you; and that need to be even; that need to be; you need to be able to accommodate that such that your ability to scan at a certain speed matches with that of the ability to digitize the PMT voltage that is coming out.

So this matching is also critical, so that we do not attribute I mean to minimize the discrepancy or the distortions that are introduced by non-uniform scanning of the galvo mirrors and during the image formation. So assuming that you have one can do that; this is a simple easy detection. What we need to do is that you need to put a spatial grid a calibration grid that has repeated patterns of square or line and you, you have to just measure the reflection.

So that reflection get in our reflection image so that you optimize this scan amplitude and the scan speed. So you can always make any amplitude that you want but then the point is that, that is going to come at the cost of how fast can you actually go. So given that optimization what we; next you; what next you want to do is to understand what it means to reduce the scan amplitude.

Reduction and increasing increase of the scan amplitude we have seen previously amounts to zooming in and zooming out of the sample region simply because you are actually taking the same space and putting it in a denser or a rarer pixel grid. So, so naturally affecting the spatial frequency at which you are actually sampling. So you need to optimize when you are optimizing the scan amplitude you need to keep that in mind.

Alter the scan speed along with that because when you alter the scan speed you fixate on the scan speed then the amount of photons that a fluorophore that can produce is limited, right you cannot expect the fluorophore to generate arbitrary amount of photons. Clearly we know we have; at this point you would have solved some of these problems in the assignments and we know what is the max? How to estimate the maximum rate of fluorescent photons for a given molecule?

How we can estimate that and taking that into account so assuming that you are actually measuring or obtaining these images with that high sensitivity of single molecules then are close to that then what we what it is allows you to do is that it sets the speed of scanning; once you have set the speed of scanning you need to match that with the bandwidths and the acquisition rates of the DAQ.

At that place then we have to go and modulate the gain of the PMT. Then what the image that you get would possibly the best image that you could get from your setup. And remember the histogram readout and so forth and so on. I hope you were able to gain enough information or the information that you actually wanted from both the theoretical as well as the experimental point of view and we would have lab sessions and then hopefully that will enhance your understanding and the real-world use of the methodologies that you have learned. Thank you.