Proteomics: Principles and Techniques
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Lecture No. # 39
Detection System: Diffraction-based Biosensors

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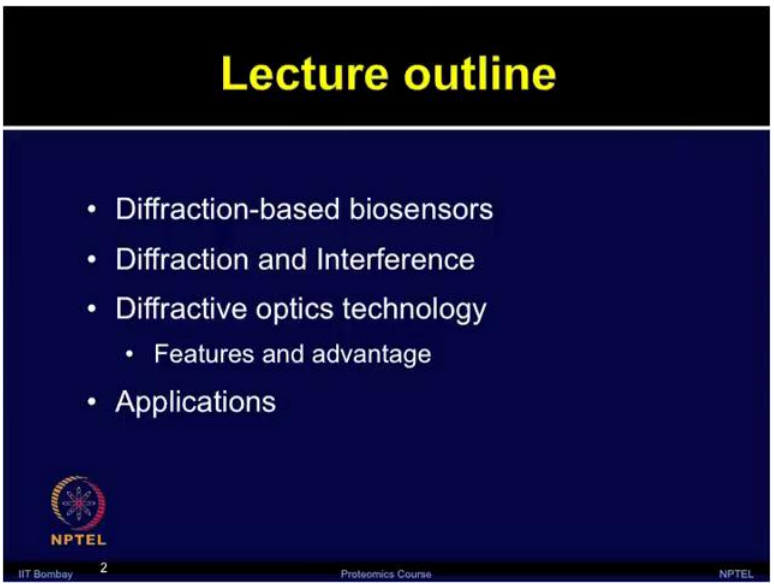

Welcome to the proteomics course. Today, I have guest with me Professor Cynthia Goh she is a professor in department of chemistry, at University of Toronto. She is also director of optical Sciences University of Toronto. So, we have been discussing about label free methods for a measurement of different type of bio molecule interactions; especially protein-protein interaction, and we have discussed different type of methods which are currently being used including surface Plasmon resonance based optical sensors.

Now, today with doctor Cynthia Goh, we will discuss about diffraction based biosensors, which her lab is actively working on and during discussion she will also show some examples of how these types of diffraction based biosensors can be used for measurement of protein-protein interaction; and it can also be applied for different type of diagnosis and point of care diagnostics. With that I would like to welcome Professor Cynthia Goh and we will talk more on the how to measure protein interactions welcome, Miss Cynthia.

Thank you, it is please to be here. So, I should know bio-sensing is about measurement of interactions between two biomolecules, and I will be discussing an unusual approach which actually surprising that it has not been used before but it was invented in my lab which is to examine the interaction between two sets of molecules using the principles of diffraction. So, let me just take you back to what you may remember from either your basic physics course or perhaps you may have met diffraction in the context of x-ray diffraction on crystals if you look at the hand side.

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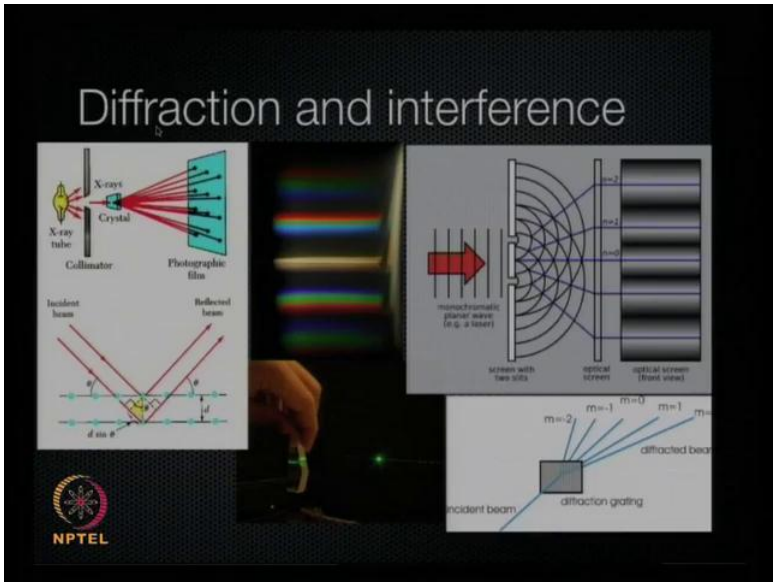

There I have two slates if you been watching waves of water passing through two slates you see theirs is diffraction this with a wavelets that are formed that resolves in an interference pattern that has light and dark spots. So, in the middle part of this slide we see a beam of light passing through a grating a diffraction grating and it shows the main beam and the lot of little beams, that is generated and the pattern of this diffraction image the image of the diffraction depends on the pattern of the grating.

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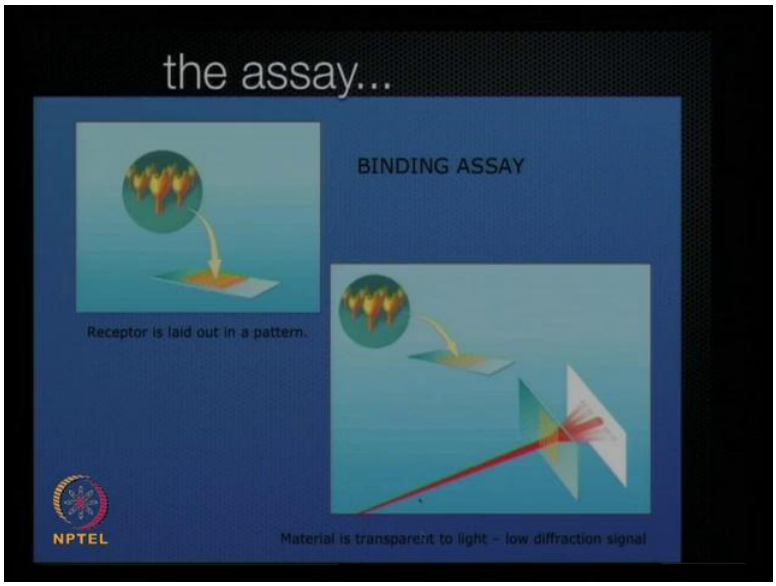

So, let me show how we can use diffraction the principles of diffraction to actually measured interaction between molecules. So, let me take a piece of glass slide here and put a coating just one molecular layer take of coating that is in a pattern. So, this is a grating lines made up of bio molecules that are spaced approximately micro and micro and a half of part. As such that when light shines through that grating which is very faint this going to be a little bit of diffraction not much in fact you could barely see it in this in this cartoon drawing. However, if binding were to take place so that, this molecule now has a complementary partner binding to it is so, you can imagine this yellow one protein one with green one, protein two what you say is that the grating becomes more pronounced and so, when light shines through that grating you going to get a much brighter spot. So, again let us just do this in a different representation I will take you to a different slide where… So, Cynthia it means?

You are actually measuring how much material is there and to begin with if we have small material one nanometer size and then if you are adding more material to it then the change in the diffraction that is being measured? That is correct so, effectively we have a surface first like a piece of glass where light will go through and when this just imagine writing with your pencil lines on the piece of glass if you shine light you can get a diffraction except the lines that were writing is one molecule thick which is one protein layer thick. And so, the diffraction is very fat so, let me illustrate that in this little cartoon. And so, the diffraction is very fat so, let me illustrate that in this little cartoon.

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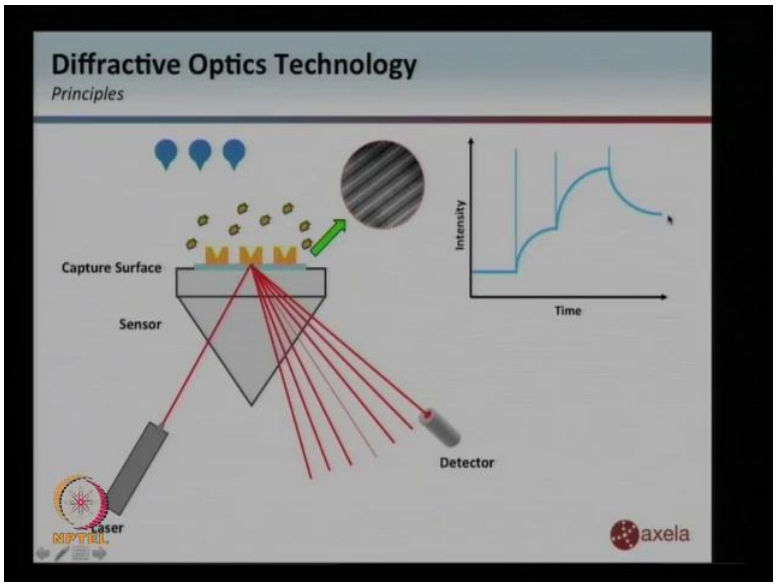

So, the sensors surface is a piece of glass it has lines as you can see in this inset and when I shine light through it this going to be a very faint signal. Now, suppose I introduce molecules that bind on this line the signal gets darker and it is represented in the hand side here by the intensity of lights. So, effectively if I have a detector in one location you can see that the signal increases with time as binding takes place.

Now, forward to introduce a second molecule that binds to that first one again the signal gets darker so, my detector then has an intensity increase with time. A good thing in a multiplexing is possible in that way? We can talk about multiplexing and a different one this one is basically, different one this is one molecule binding to another molecule you can also bind the second one. So, you can imagine an antigen and an antibody and secondary antibody.

You can also play games if you into trying to measure relative strengths of interaction you can imagine trying to imagine whether you can displace this antibody with another thing. So here, something comes in another molecule and if the binding is stronger it may actually detach the previous one and that will be indicated by a change in signal in this case a decreasing signal. So, I think very similar to what we have talk in the previous class on the surface Plasmon resonance methods I think same way we have the base line here and we are measuring the time versus intensity on x and y axis. That is right. And then, we will have an on rate we will have an off rate.

That is right. Depending on the interaction how it is strong or weak one can actually compute the values for measuring the on rate off rate and the connected of it. That is right. So, it is very similar to surface Plasmon and in fact lots of the principles were similar in that it depends on the index of refraction difference. The main difference here is that in surface Plasmon you are only a looking at the main beam. In this case because we are putting things in a pattern then you are going to have a diffracted beam and we are looking specifically at the diffracted beam and there are advantages of doing that is so, why would we actually want to measure this way instead of just directly through surface Plasmon? Well, you can actually imagine in one area if you are doing surface Plasmon you can only put one molecule in that area that is correct.

And therefore, I think also like now there are some new or methods there people are trying to four plates or at least at least that is why now you have different areas and you can put things down on different areas. Correct. So, in the case of diffraction you noted there is a one to one correspondence between you are your grating look like and what your diffraction image looks like so, even just if I have a grating facing one way versus another way. I will have this grating will have dots in this direction this grating will have dots in this direction.

And then, you can identify whether molecule a is binding to this one versus this one so, you can multiplex very easily and that is one advantage, from the technical perspective we can actually choose that kind of pattern to enhance the signal and for create a better sensor in many cases. But from if you are trying to look into the diagnostic area in the future one advantage of getting diffraction pattern is that if molecule b it is not bind in but just drops somewhere accidently we will call that nonspecific binding.

In surface Plasmon you will measure that because if attaches to the surface. In diffraction experiment if you do not drop in a linear on a grating pattern, then you would not get a signal. So, it means like you are able to increase the specificity here and much more controlled manner as compare. It is correct. To what one can do? And you In SPR another method that is you can reduce what is called the postpositive.

Postpositive, where you get a signal that is not really meant to be a signal. Sure, I think that is big advantage because then you are talking about diagnostics and you are looking at the very specific signal. That is, I think giving a false signal actually is very

diplomatic. For diagnostic that is very important, but even for experiments in your lab. You of course, do not definitely, want to have you no big error bus because, some proteins just like falling out of your solution. Exactly, so, let me show you just implementation wise how simple this can be said this was the technique was invented in my lab and here is the example of substrate where we have patterned the bio molecules

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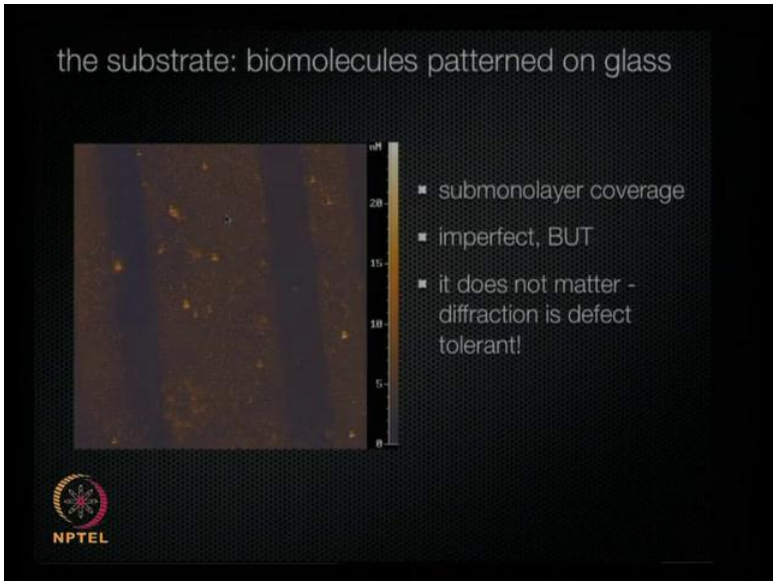

This protein on the piece of glass and it is sub monolayer coverage. It is very small various parts amount of protein in there, and it is imperfect as you can see not even at all but it is does not matter, because diffraction is defect tolerant. What do you mean by defect tolerant? Because, if there is some defect are we going to have different type of diffraction pattern or that can be compensated because of the nature of diffraction.

Once the nature of guides it is such that it will pick out the repeats that is of the order of it is wavelength are higher and so, if you look at this picture this is an atomic force microscope image. So, it is the little dots in their proteins and you can see that this area where there is parts coverage an areas, where there is more of them, you can see lot of clamps. But as first slide is concerned it does not matter, because these clamps are nonrepeating and if they are not periodic it does not show or process a signal.

And so, it ignores all these see this is probably a protein that just or may be this is a piece of junk and it is ignores that completely. So, I think some error can be tolerated in that way where the defects are not going to. That is right. And so, from the point of view if

building a device and building instrument it does not. It does not have to cost much, because you can you do not have to make things perfect, making things perfect is very expensive. But a still I had at the end, we can get the perfect signal well I think that is what matters.

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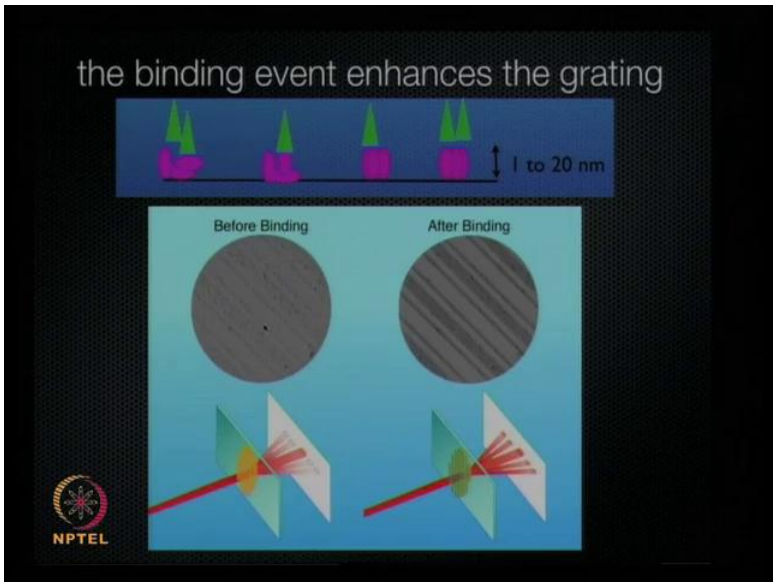

That is right. So, again let me just show you different vision or we have here the grating made up of individual protein molecules, and then binding takes place on it more binding takes place and what happens is here is this surface is before binding, and you can see that if the coverage is not very strong after binding the coverage is strong, and you get much a bigger signal intensity of signal. That is right.

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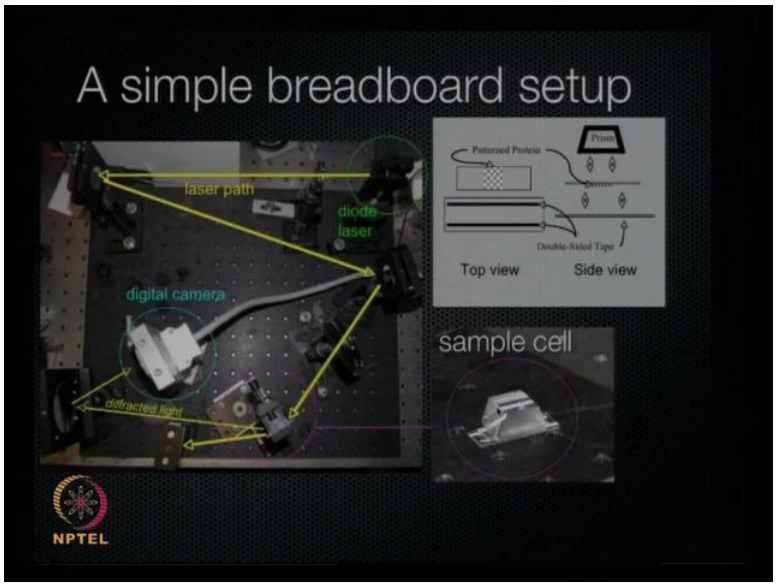

So, you can quantify that and here is some implementation of how simple it can be in our lab. Really, it has three components the light source which is in this case a laser pointer is a 3 mill watt red laser and the detector here is this is a see most detector, but it could be a webcam it could be what is call the photodiode, which is the very expensive piece and here is the sample cell and let me enhance that. So, it seems you have a prototype earlier to begin with? Well, this is how it built it in the lab, because you know you take a piece and put in together. This is where the actual interaction takes place and let me just does that schematically. So, at the bottom here a two pieces of glass slide separated by a double sided tape that makes a channel that is about of 50 microns that is a thickness of the tape and on one of the glass slides you put down the pattern of the proteins and it is out here. So, that you can then flow your analyte your medium with the analyte in between and on the other side we put in a prism, the prism helps to guide the light so that where under what is called total internal reflection. So, the light does not go all the way through it just comes to surface and actually detects the binding on this upper substrate so, it is a very simple. So, many of a prism then you have the matching fluid for refractive in that correction then you got the slides which contain the protein.

That is right. And then that the light beam then that is Initial interaction and with this simple assembly you can actually measure down to nanograms per milliliter a label free. So, this other components in this set up or simply mirror to make it a little bit more compact that is very neat concept, I think then one can achieve various applications on it.

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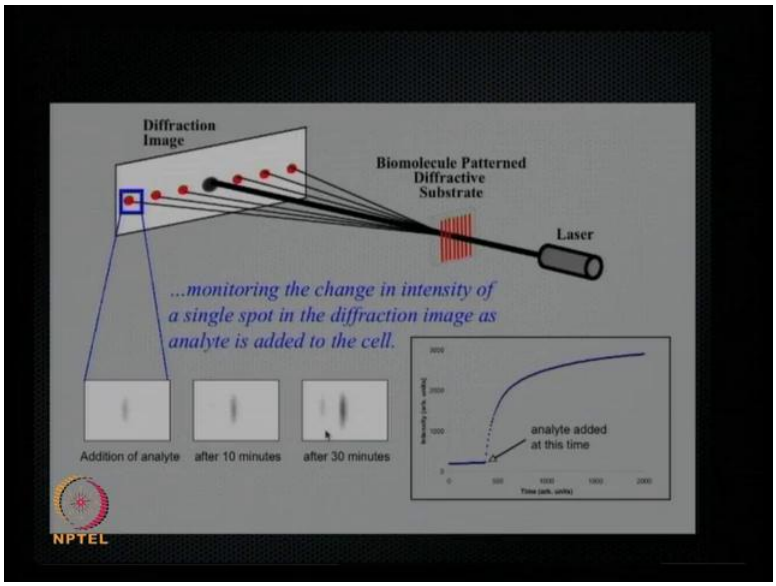

That is right. So, in our assessor in our first implementation it is diode laser a laser pointer and a webcam in these are actually pictures captured from webcam. So, actually monitor the change in intensity in one spot of the diffraction image up an addition of analyte. So, after few minutes its gets darker and then gets darker even there.

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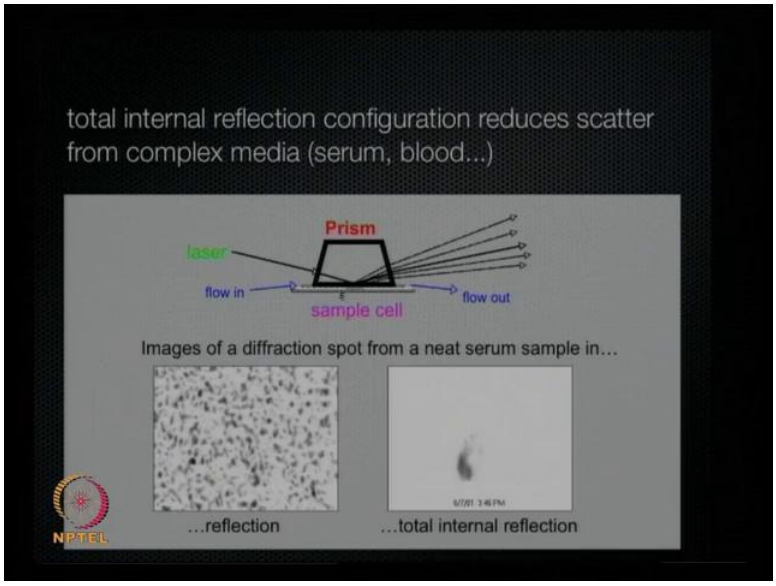

The role of the prism is to make sure that the light beam does not actually get scattered by whatever assent solution. So, here is a picture what the diffractions part would be if the if there were no prism if it is not under total internal reflection with total internal

reflection. We get it a lot cleaner and that means you can actually use a fluid like blood over that something equivalently murky. So, many times I think the intention is look for some biomarker or some other diagnostics so, measuring blood or serum?

That is right. Becomes very important and actually measuring that is very challenging because of that issue this had and that is where I think correction for this type of scattering. That is right. But even in your doing your experiments like if you using cell lysate, then cell lysate has different kinds of particles and will actually scatter light. That is right. Definitely so, we have lot of complex sample let us not always the clean purified protein which one has to look for direct application, so in this case you do not have to purify your sample before you actually do the experiment. That is right.

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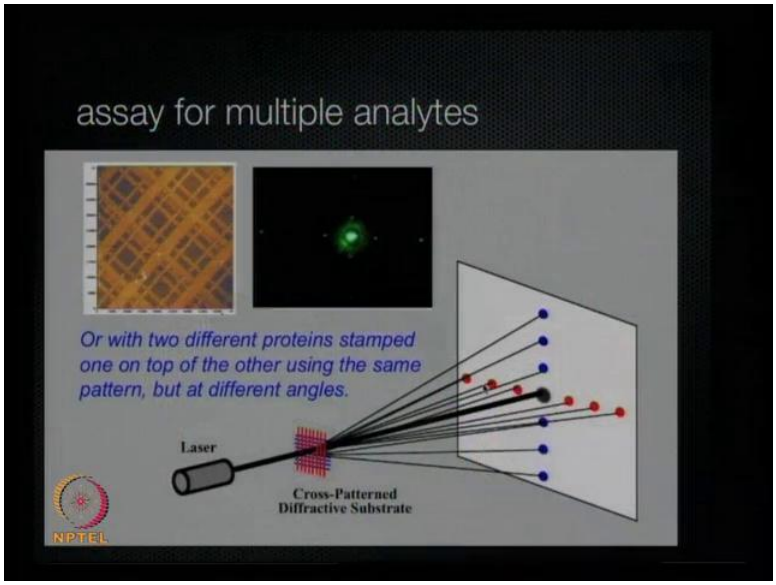

And if you have we said before about multiple analyze so, in this case you can have protein one, in one direction and protein two as the other grating and you end up with two grating patterns and here is a webcam image of what it would look like. So, this part here will be due to protein one, whereas this part perpendicular this part is for perpendicular will be due to protein two. Correct. And so, if you introduce your a medium if this part lights up you know there is binding to protein one if this part light off this biding due to protein two. Sure so, you can examine multiply analytes that way so, to show specificity here is our example now, we have names for the analytes.

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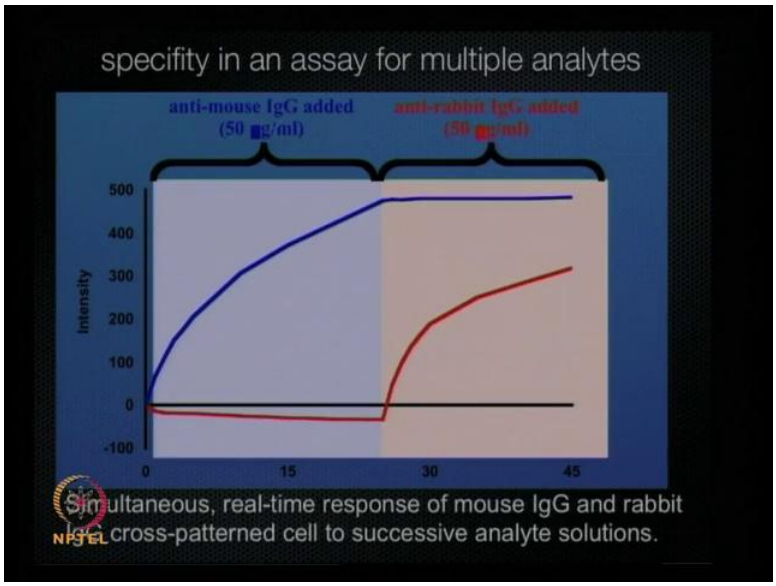

This is mouse IgG on one analyte; the other one is rabbit IgG so, when we introduce anti mouse IgG you can see the increasing signal in one of the spots, but not in the other spot. The red versus, the blue, and then at this point we introduce anti rabbit IgG in which case one of this spots the second spot increase, but the first one just remain constant.

So, actually one need to show that specificity of the assay and I think you test it out you have probably mobilized different type of proteins including one from rabbit one from mouse. That is righ and now, when you looking at the how much the specific signals are then only anti mouse is binding on the feature where you will have the mouse IgG. That is correct. And now, were we have the anti rabbit IgG, it is only binding with the Rabbit IgG. That is right. That is and so, yes and this is showing it with two so now, you can actually imagine generalizing with more. That is right. And it only is a question of how many you want pattern into the little substrate that you have that there is important point here is that you are able to measure the signal simultaneously for all the features.

That is right. So, that actually you can compare those visibly while the experiments are going on so. It just gives little bit more room for even errors one can correct for the errors one can try to change the concentration of antibodies or different analytes and one can have different room I think that is one of the other major advantage of having the label free systems where user can have the visible feel of the experiment how its progressing.

So, label free detection is how you would actually do it best if you trying to measure kinetics example because, you have the actual signal not adulterated by a secondary reaction but in this case having multiple analytes is actually very good in building in controls because you can imagine one of your spot is always controlled. In fact, we do that with in my lab when we were doing measurement. I think controls are very important is, I think that is where it is good to have these features.

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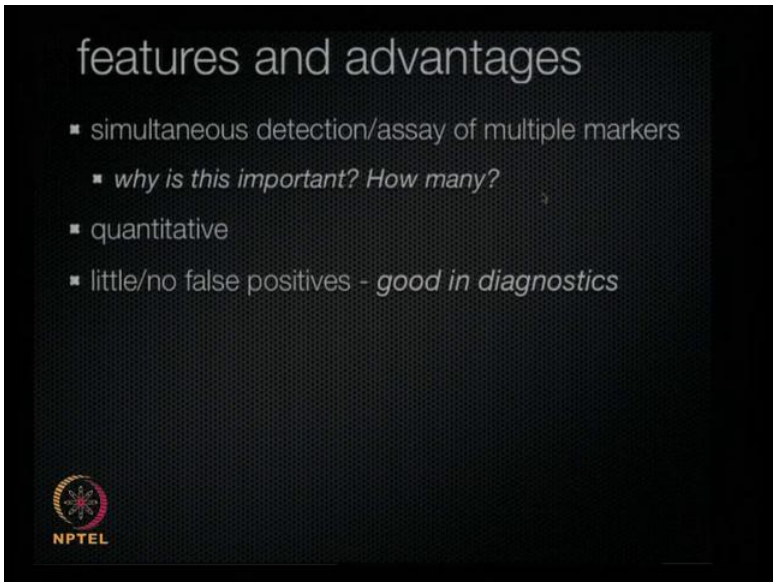

So, just to summaries the features and advantages we actually talk about being able to detect more than one at a time simultaneously. Because, the question is you know why you would want that and how many is a good number and that really depends on what project you are you are engaged in. But usually like based on your experience in the field for diagnostic purpose what do you feel like what will a good number be in terms of how many one to measure simultaneously?

Well, I think it is a question of cost now rate the more things you put down the more expensive it becomes so, in an in any disease you are indentifying how many markers do you want or if you want multiple diseases how many of them are likely took at same time. So, I would say it should be less than ten because you know chances you are not going be sick with ten more than ten different things and in a complex in this like cardiovascular a diseases probably there is four or five relevant markers that one would like to detect.

That is correct. So, one at actually take a call like a in terms of what they are actually trying to measure and I think having as a good marker is always good but having too many is also not good because controlling them and actually keeping them functional for long time again. That is all the cost for the measurement and everything that is comes in the picture. Yes, so I would say some more between four and a dozen is probably, typical number.

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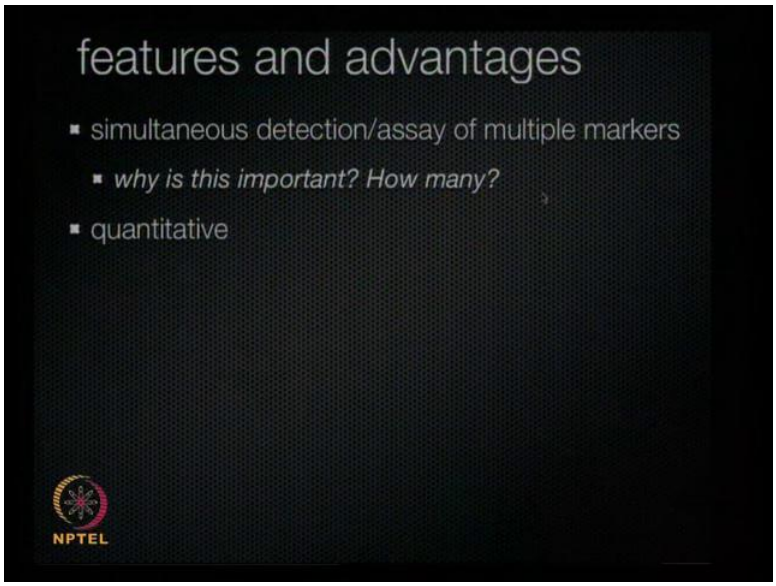

So, the approaches also quantitative because of course, the intensity of the signal is proportional to the amount of the material that is come down and it is you know of course, you have to run calibration curves to get those standards going. As we have mention this earlier that there is a little postpositive because, if things do not fall down on a grating then it is just not going be measured and that information is real time again that is that is characteristics of all label free techniques it is real time measurement of the actual interaction. And therefore, you can extract from kinetic information binding information. In our case. the sample volume that is needed is very small.

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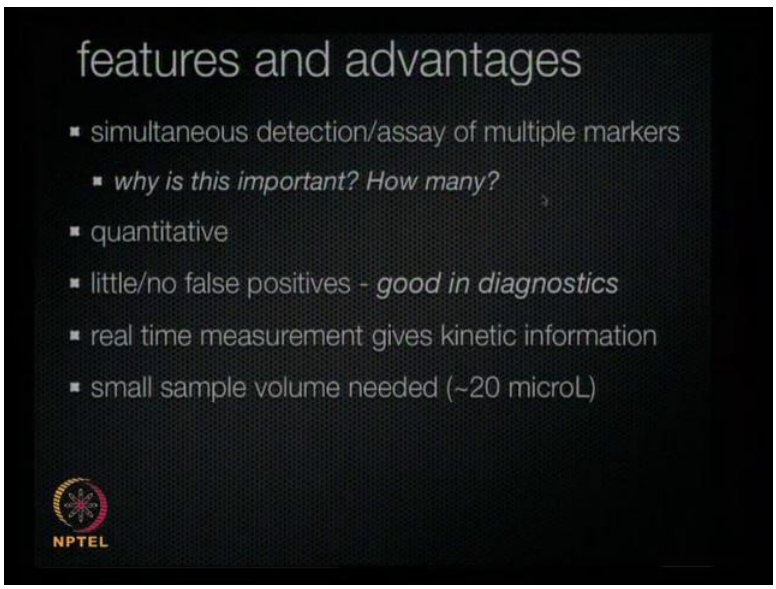

It is really you know all depends on how good at that little sample cell is, as you have seen double sided tape we can get it down to 20 micro liters and that is like a small droplet. Like I say it is a very important because, if you are talking about clinical sample and measuring the things in the clinical settings I think it is very important how low we can go, that is even in your experiments in the lab of course, Right proteins are very expensive. So, if you have then smaller the rates for more experiment. You can do for cheap. Definitely or it is always better to do the in the small volume what it is possible.

Now, the sensitivity people ask me how sensitive can this get well, if you notice that it is all about measuring that grating. So, the more pronounced the grating the bigger your signal and therefore, if you want to work with low concentration it depends on how big your molecules are the bigger the molecules that better your signal going be but also the stronger rate the binding the better your signal is going to be at low concentrations. So, there is no direct answer to that is but in sometimes it would be comparable to SPR because it is based on a similar principle which is just an index of refraction change at that at that interface it is label free now.

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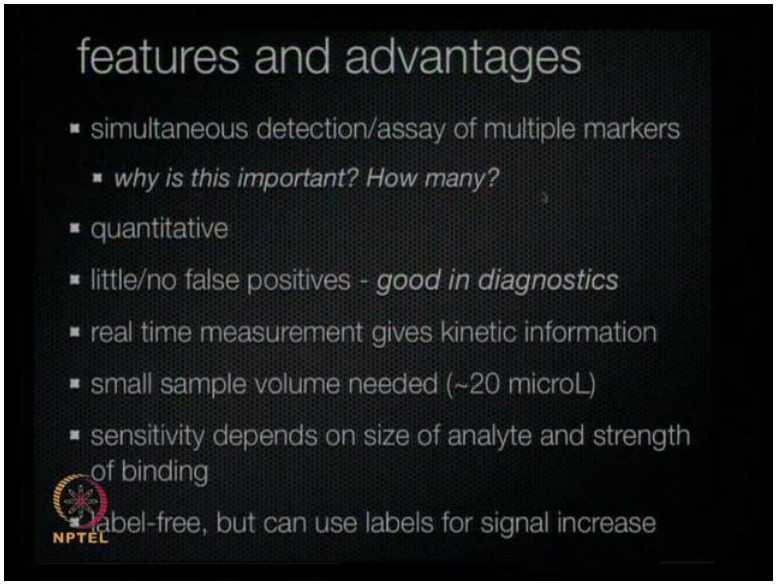

But actually if you want to get better sensitivity you can also add labels. So, you can actually start with protein one or an antigen you put in an antibody and that should binding if you if that signal is too weak you could put in a secondary to identify your antibody. That will be I think very important because if you are not able to detect the

signal at very low level then obviously, then you have to have some mechanism to bring the signal up and for statistic. That is right.

You can amplify or perhaps the other way of doing it to that the signal may below and you need any instrument you need good photo diode to do that. But then you want to sometimes amplify it. So, that it is not; obviously, to your eye and what you can do is you can add your secondary.

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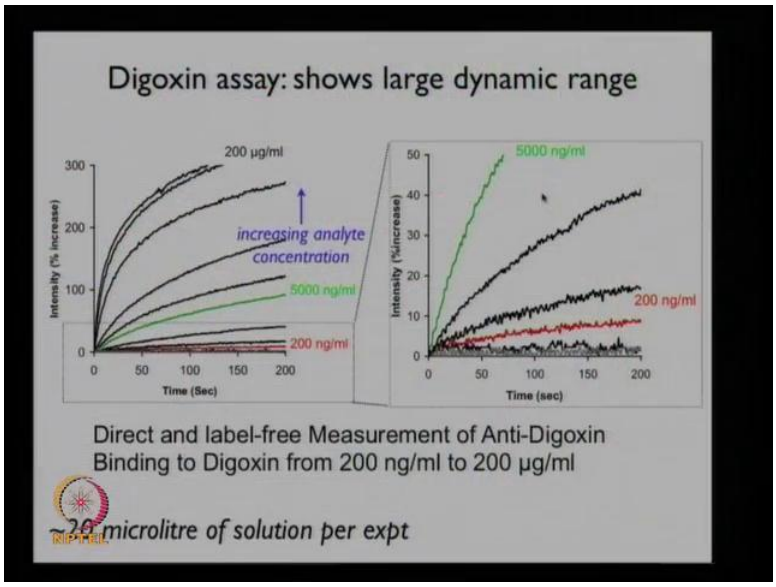

Here I just show an example here is an assay this resolves from an experiment in this set up that I showed you earlier that little photo type. So, basically looking at the intensity light, as a function of time has we introduce anti digoxin to bind to digoxin which has been immobilized on the glass surface. So, you can see that is when the analyses, when the anti dioxin is 200 micrograms per ml you get the upper mouse curve.

I should decrease the analyte concentration you get the lower and lower curves until down to 200 nanograms per ml and here on the hand side we blow it up so, that you can see 200 nanograms per ml still pretty reasonable I can still believe that still signal above noise but there is a noise here. So, we can go from 200 micrograms per ml to 200 nanograms per ml label free in 20 micro liters of solution in this experiment.

I think that is quite good, but probably we can tweak it around to enhance signal. Well this is still of course, this is label free. It also is in that little breadboard set up I showed

you earlier so, I do remember, not optimize in any way. In fact, some of this experiments we have done earlier just on a web come with manual capture of the intensity. So, now this many ways of pushing it higher, but one way of doing so, it is by putting in a label I know this is a label free course. But you can see what the label would do to you so, I think broadly we are discussing about different type of addition system I think it will fit.

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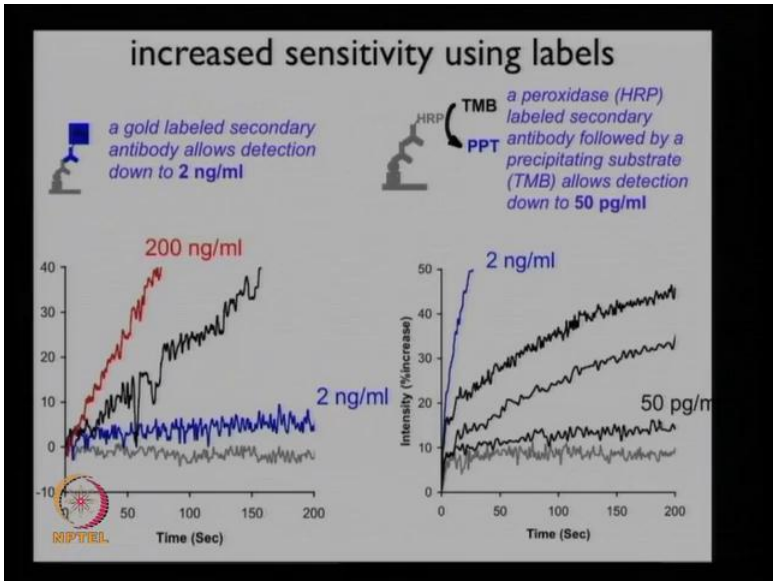

So, you remember that we have the 200 nanograms per ml that I was in the previous slide. But now I am going to introduce a secondary, here is a secondary antibody and in this case a label with a little piece of gold actually, we found a later we do not need that gold label. But anyway for this graph, that was had a gold label in there and we can bring it down 200 nanogram per ml to 2 nanograms per ml. That is fine.

And now, this is now the noise of the detector so, we are invited to go better than that we have to do a little bit more of our signal averaging. And we can do a different type of labeling which is this is a precipitation assay. So, here is our initial antigen and then you put in the antibody digoxin and then you put in the anti the secondary which has horseradish peroxidase in it, the horseradish peroxidase, then x on a substrate TMB to form of precipitate, if you do that we can actually go down to 50 pictograms per ml.

You achieve much higher sensitivity by from 2 nanograms here is 2 nanograms per ml here and now 2 nanograms per ml is huge and we go down to 50 Pico grams per ml. I think one can actually even tweak it further. That is right.

But you are starting, now to 5 kinetics, because if you are very low of course, you now this binding is taking a very long period, because it takes some time for them to find each other. So but anyway just in this.

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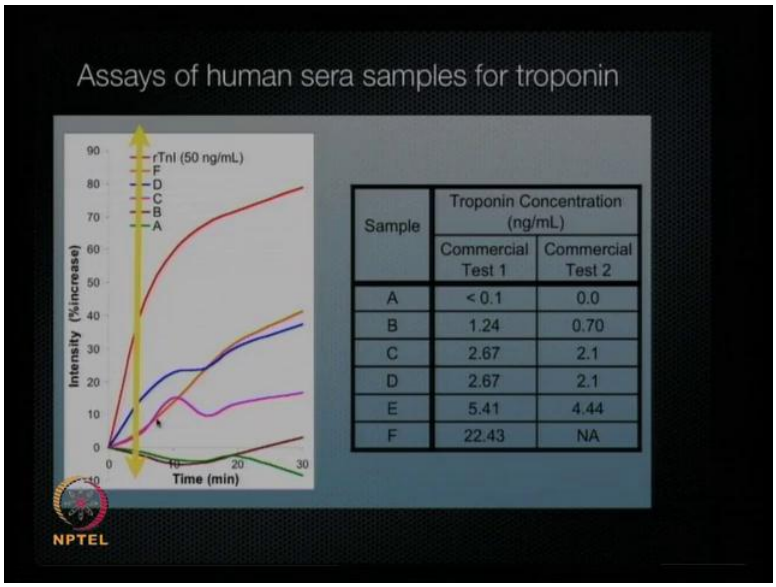

Sample	Troponin Concentration (ng/mL)	
	Commercial Test 1	Commercial Test 2
A	< 0.1	0.0
B	1.24	0.70
C	2.67	2.1
D	2.67	2.1
E	5.41	4.44
F	22.43	NA

But this could be useful I think one we are talking about very weak protein-protein interactions or different type of analytes, which are very low in abundance. So, that becomes the one issue about assay development what we have here is a tool that allow should to measure the signal. Now, you can configure your assays. So, that you introduce in this case we will introduce them linearly. But in some cases you can actually premix a cocktail and then let it bind together and that make sometimes work better.

So, it is actually not just protein that we can actually look for these are some data on a Troponin this set up protein antibody assay. The clinically relevant level is around here above this line above two and you can see this is the signal. This is indicative of the noise of the system that is why there it goes in that signal. But the interesting thing here is the point of view of clinical diagnostics just ten minutes here.

So, in less than 5 minutes you already have a difference between higher levels clinically relevant levels and clinically you know absence of troponin. If you no very short time, I think you are able to measure the signal that is with high intensity. That is so, imagine that this is a marker for cardiovascular disease marker for stroke then you can know within few minutes that it is there or not.

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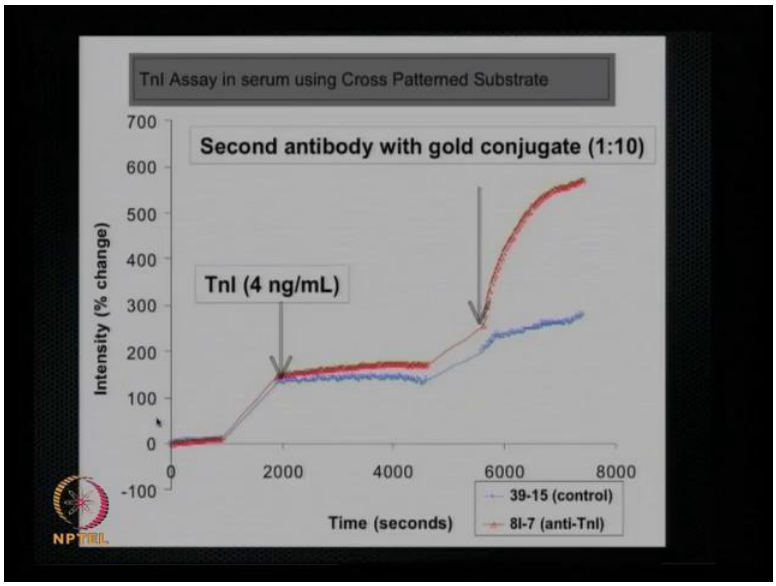

Here is an example for how you can actually assay for couple of antibodies for TB.

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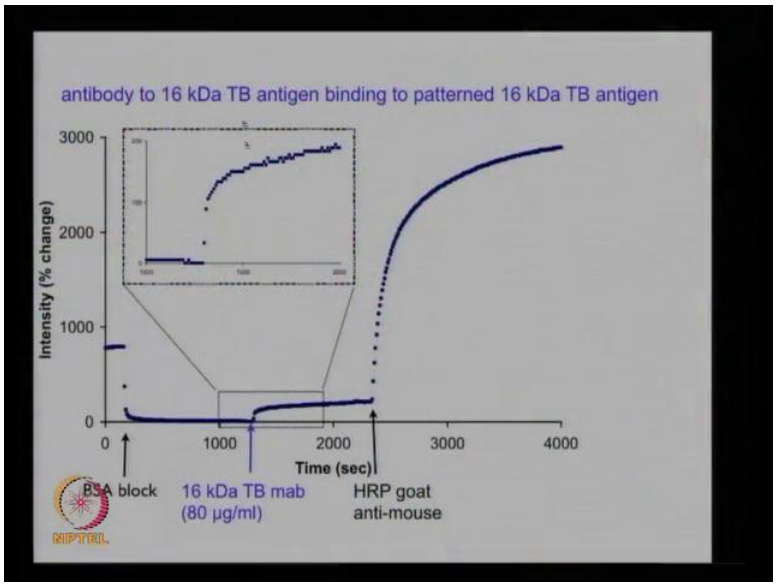

Here is looking at the 16 kilodalton so, this we are just going to show you in this case an example of two markers a two antibody antigen binding for TB. Again it is looking two different spots simultaneously happening and the introduction of antibody to the 16 kilodalton TB antigen, again you start with the experiment here and so how do you correct the baseline in beginning because I see it is beginning from 800 or something?

Yes so, there is a baseline sorry about that, there is a base line here because there is a diffraction pattern we put down this 16 kilodalton antigen in on the substrate.

And so, when you shine light and there is going be a baseline and to actually reduce nonspecific binding we put in a BSA block and so basically, here that is to show that your medium will not have all the proteins sticking to the line. So, you introduce BSA block. Suppose you do the blocking of the surface and then support the interaction.

So, here this signal drops to 0 because of the blocking because the BSA sticks in between in the lines and then at this point we introduce the 16 kilodalton antibody for TB that will bind to the antigen and you can see there is a little blip in here. Now, let me expand this area you can see the expansion here that blip is actually real because, the signal to noise is good enough.

But in case of basically want something that is much more obvious or something that you can see, because at this point when you have the precipitation you can actually see this signal already, but you have introduce HRP goat anti mouse and the TMB substrate here and you can see the enhancement in signal and see this is actually a very big intensity change you can look at it this is a 3000 media. So, it is 3000 percent intensity change. Now, that is a think sort of clear less no answers that is yes so, if you want to create a diagnostic just say or no now well here I can see yes or no.

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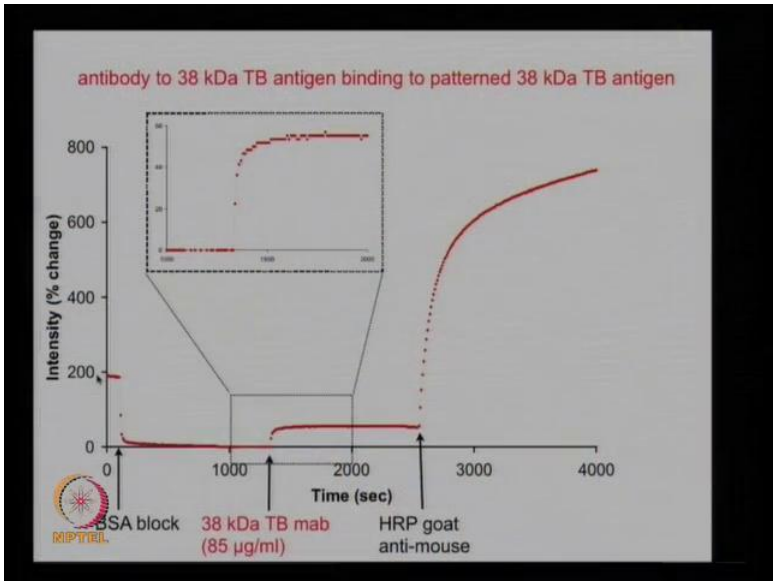

Yes, that is one of this spots now, if you look at this second spot which is which is filled with the 38 kilodalton TB antigen. You can do the same experiment and then we will put them together that this two parts together at this point we introduce the 16 kilodalton TB antibody and one of the spots get more intense the other one is did not.

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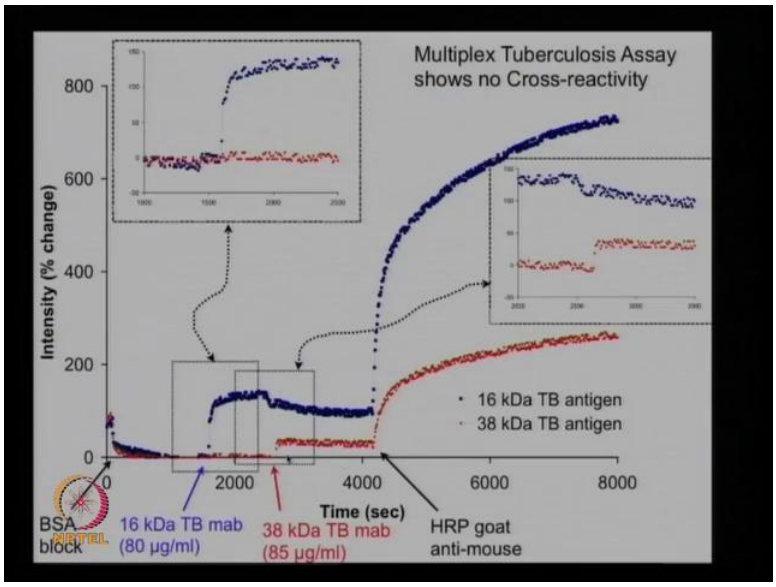

And then at this point, we introduce the 38 kilodalton and then you get the other spot got more intense and then we the other we amplify them both and both spots now. Blue 1 is 16 kDa and 38 kDa is the red one and It is able to measure simultaneously both that is at this signal and if we see the signal both then I think we know that person is actually positive for that.

Well we particularly. You have for TB is a very high thing to detect. But at least now you know the you have two signs that it is there and you can imagine adding because, there is other types of our potential markers per TB that we can just add to that and so, you get more and more sure that actually something is happening. It is more sort of I think a proof of concept which you are showing but I think it can be applicable depending on a context. That is right. Yes. Even can move the make the assay more robust with the introduction of type of proteins and more mass.

Yes, well now, the other way to do it also it is now, you can imagine making the second spot be a blank and that way you can make sure that the signal is you know in reference to a blank and you know that you do not have force on positives in there.

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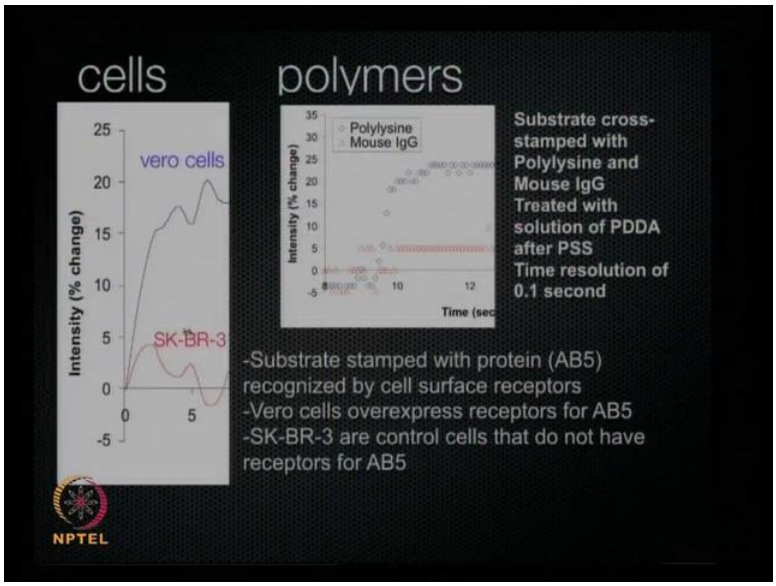

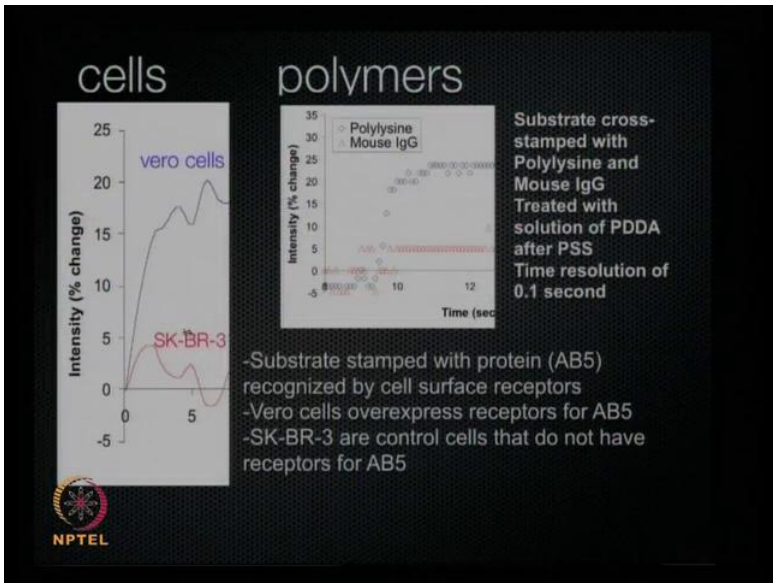

We can we can skip the other one is that other than proteins, but just to show you that you can measure look at cells you can look at polymers, because actually the measurement of interaction is you know, it is general you can measure the Interaction between the two types of molecules.

Yes.

Second now, you got the prototype and probably you can discuss that how you took the prototype and actually took it at the commercial scale because, ultimately need a lot of applications to develop.

Yes right.

Well, you know I have showed you what it look like in my lab. So, you know and I can teach you how to make one of those but unless you want to be an expert in optics then you do not really want to have to build one every time you do a measurement.

So, I mean I could have just keep cracking out data but that is not the point we want to be able to create an instrument that will be useful to other people and if we are going for medical diagnostics. It is important that the instrument is functional in and useful for others and this is the role of commercialization. So, at that point I actually around January 2002 or so, basically I have decided that we have to commercialize the device in order to get it to be useful by others, and this is important that the thing that I believe in that for science to be able to benefit society it has to be turned into a product that others can actually use.

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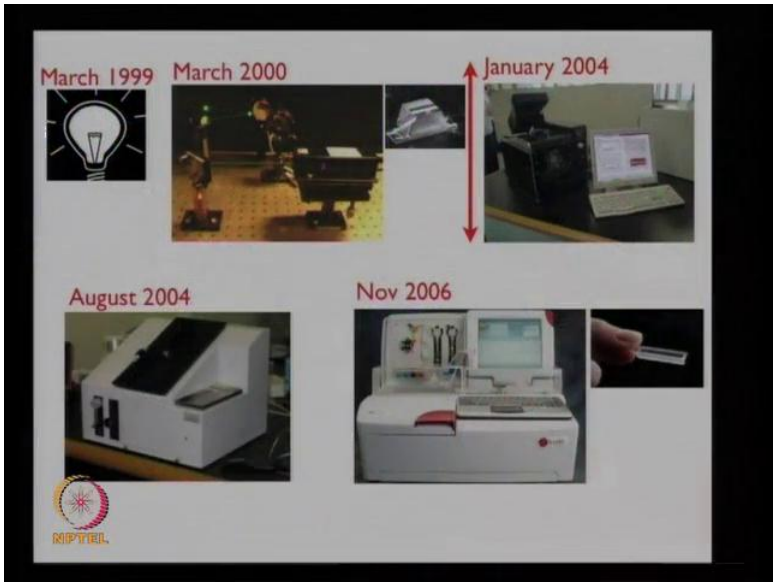

And so, this the path and the way this is how it was in my lab and up to now, we still have this instruments in my lab we still work on the bench top and, but what we did is we translated it into a series of steps So, that the current device that went out in the market late 2006 early 2007 is an instrument that other people can use and that little piece of double sided sticky tape and you know it is not really going to be good for you to just have to make that yourself. Now, a little piece of plastic that is actually a lot

cheaper took a lot to get there but it is now much more efficient and cheaper. That is right.

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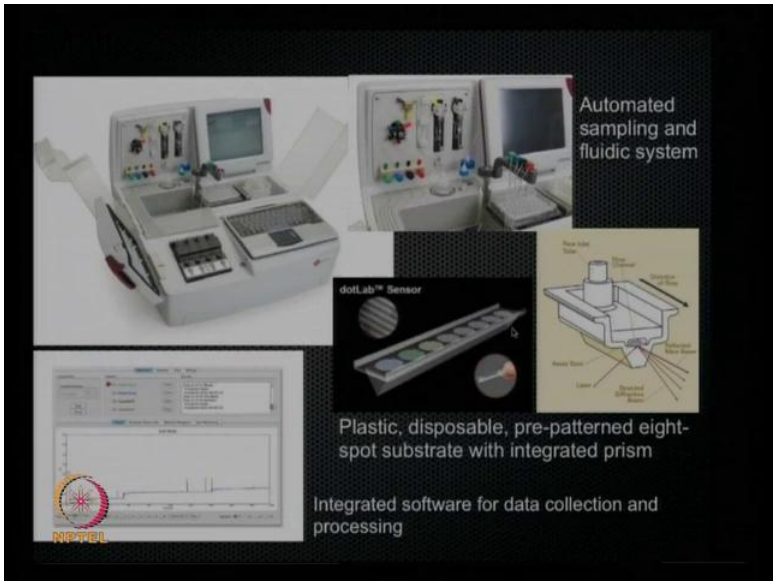

So, here is the instrument it has it is own computer as there is a pumping system inside their software and it has built a little sample cell that has eight spots in it that you can put down different proteins or different snippets of DNA or whatever bimolecular you are trying to a safer. So, now you are providing multiplexing capabilities for multiplexing?

Yes, it has multiplexing instead of on top of each other it is now in eight difference spots and effectively in the instrument the laser beam runs through back and forth into this and there is a channel, there is an insertion chord, the injection port, where you inject the analytes and it is just goes to the little channel which is about 20 microlitres or less. Yes.

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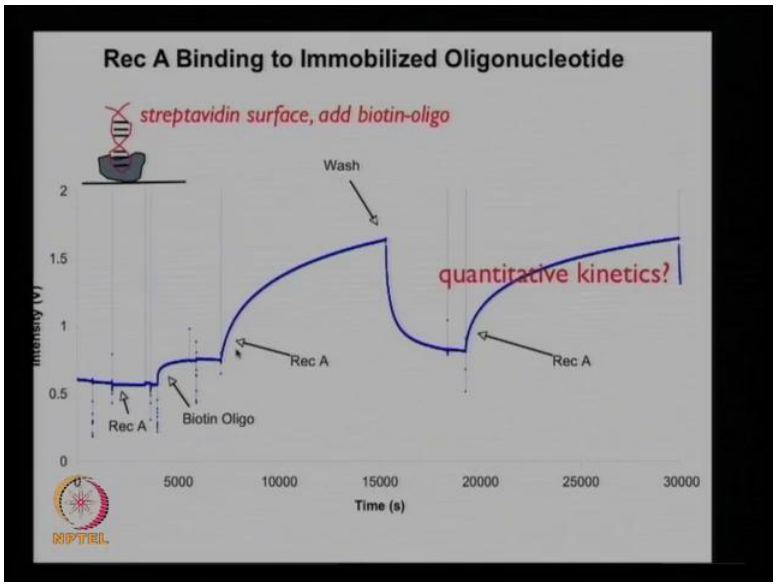

But it has a pumping system so, you can actually control the flow rates and so on which is becomes important when you are doing kinetics studies. So, here is an example of study on the binding of a protein to a DNA. So, in this case this is the substrate with put down streptavidin in a pattern and we basically, take that substrate and here is the initial the signal from that substrate. What is the y x is here are you relative?

This is the time versus intensity; this is just intensity in arbitrary units at this point. Yes. So, Rec A is the protein that binds to the DNA and that is what we wanted here to see is the kinetics of binding and an unbinding. So, we start with the substrate that has only streptavidin and when you introduce Rec A to the sample cell nothing happens. Rec A it does not bind so, at this point here we introduce a biotinilated oligo and so, that binds to the strptavidin and you get a little blip and signal. When you introduce Rec A at this point you get big Increase in signal and where we want to unbind Rec A. So, we have introduce just the buffer to you know flush out the Rec A and the signal goes down that is because in the interaction between Rec A and DNA is a lot weaker than antibody antigen interaction and you can repeat the experiment etcetera so, and you can actually, analyze these curves to measure the binding and the unbinding kinetics of Rec A and DNA. This will be similar to what we are talking about in SPR like in terms of on rate and off rate?

That is right. I think similar… Yes. So, this is an on rate and off rate of Rec A on to DNA. Now, mine you know similar of both in here and SPR one has to actually we have been put together the correct model in order to extract the correct the kinetics. Definitely, Yes. So, I think the software's play the role over there like how you best fit the model that is and extract the data for. Yes.

So, you know I think and I know that the SPR systems come with the associated software now, if you are actually trying to study a specific system it may or may not be the model for your system and in that case actually you write down the equations and do your own fitting of the data so, that is in order to extract real quantitative kinetics.

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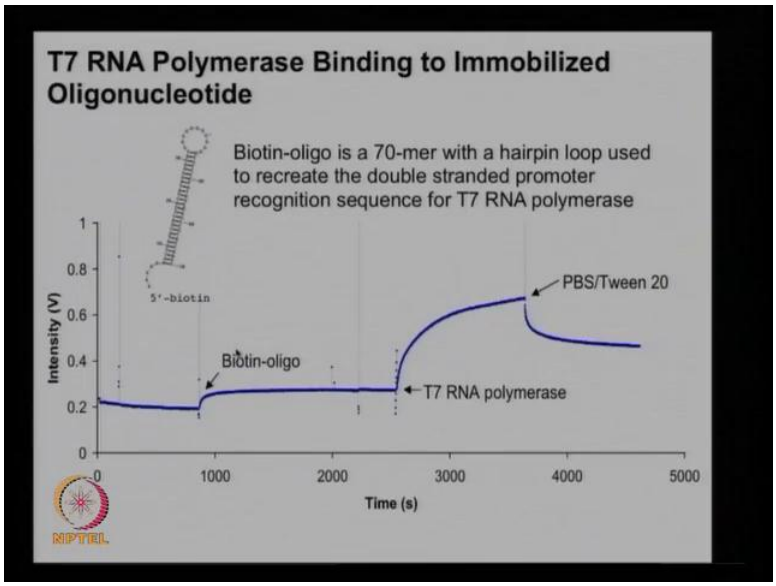

So in this case, as long as we are reasonably at the certain range of concentration the intensity is linear with concentration and therefore, you can actually model the kinetics nicely. It is just a, this is the similar experiment and this is under binding of polymerase RNA polymerase to their immobilized oligo.

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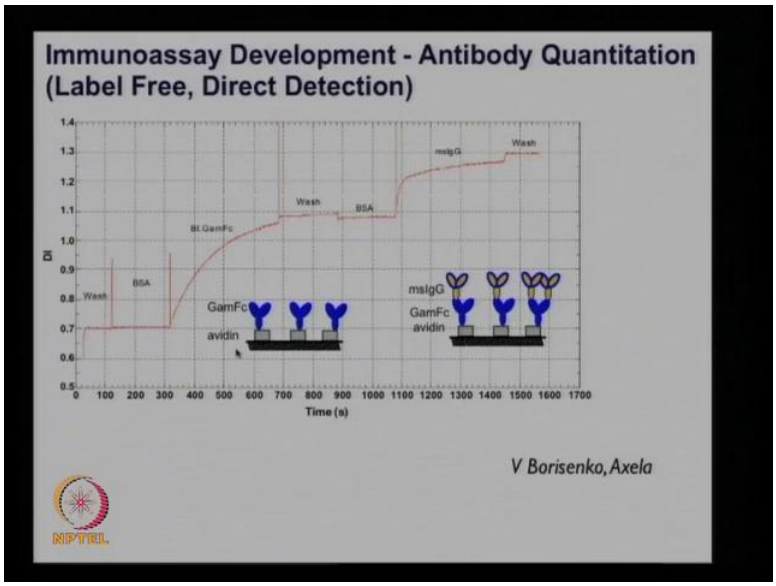

For sure the kind of different level of applications one can achieve not only it has the strong antibody and protein that also protein-protein, or DNA protein or polymers or cells. That is right. I think these cells are actually very easy, because cells are big reins. So, you know as long in fact the challenge with the cells we have to redesign the lines, because the lines in well then the lines originally were about one point microns. But you know cells are bigger than that and. So, we have to redo the whole thing with much bigger line so, the different setting has to be in line.

But it is the same principle and In fact, it actually a very simple to detect cells. Yes. So, this is just an again this example is antibody immunoassays so, you can play games on that. So, what Avid in and what you have written IDG? Yes. So, this one is just antibody quantitation and showing a slightly different surface avid in with a gam fc in air it is not different from the other one so, as passed by.

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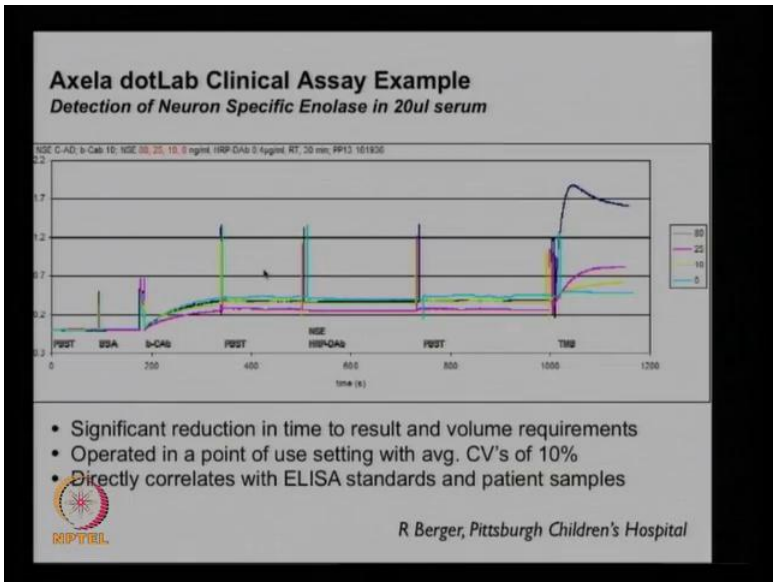

Here may be some more interesting example and I think this one of the early clinical example using now, the instrument as I said now to fact that there is an instrument that somebody else can use that means, people can actually configured assay and can play around with how you going to get it to work best for measuring whatever it is you want to measure. That is right. So, this is the detection of neuron specific enolase, which is basically associated with traumatic brain injury and in this case.

So, this kind of a marker which indicates traumatic injury? That is correct. Yes. And I am not an expert on the subject but doctor Berger was using this primarily to look at markers imbibes. When your baby sub shaken baby syndrome they actually have issues about it whether a baby has been shaken and in this case being able to use only 20 micro liters it is very important, because you cannot get too much blood from babies.

And so, here is an example what they are actually have you know it is an effectively the same as we have been discussing before, you put down even protein that identifies another protein and sure not it works. I mean as a physical chemise I feel like well, if it works for one it has work for another it is a question of how strong the signal is which is dependent on how big the sample. But criteria will be like in terms of the level of the protein in the different patients. That is right. Yes, What is the significant level?

So, in designing biodiagnostic instruments one has to always ask you know if I am detecting for disease x what is the relevant concentration to detect. Whether I am able to

capture the dynamic range and… That is right. Well, or rather it is even important to have dynamic range. So, if you are actually looking for a marker that is you knows present in you know in micromolar quantities that is another challenge you can use anything that you have.

Definitely, But also then there's no sense building an instrument. That can go down to nanomolar if you all detecting it is micromolar. Yes. Now, for many if you are interest is primarily in terms of kinetics then you do not really run kinetics at low concentration and so, you know you do not have to give do an instrument that can do a nanomolar if you are trying to actually do kinetics so, you usually run that at micromolar.

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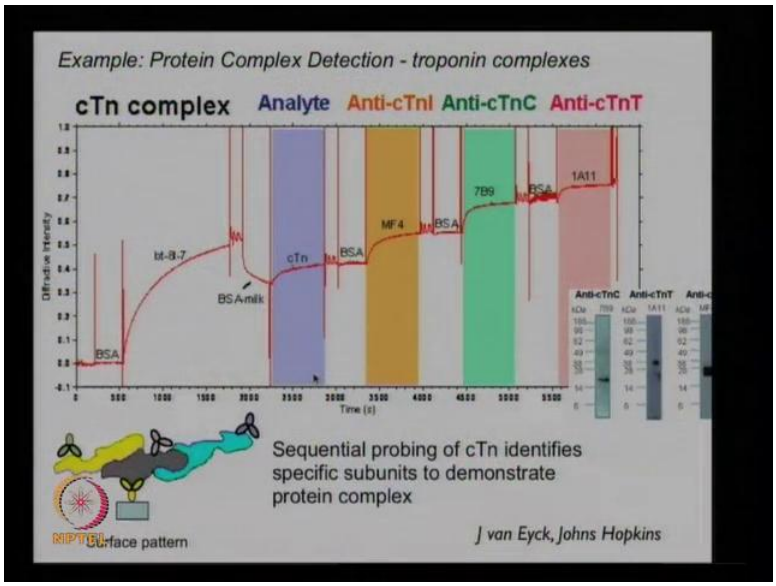

So, here is an example of troponin detections so, the interesting thing here is being able to detect a complex system. So, in this case and this is the work of professor J Van Eyck, Johns Hopkins again using the instrumental ready to commercial instrument in the surface you put down the antibody untied troponin.

In this case, troponin is a complex that has three parts and you can identify whether all three parts are present or in what amount are the three parts present by putting now, an antibody for each of the parts of troponin in measuring various parts. So, it is almost a multiplex assay except for its all on the same protein.

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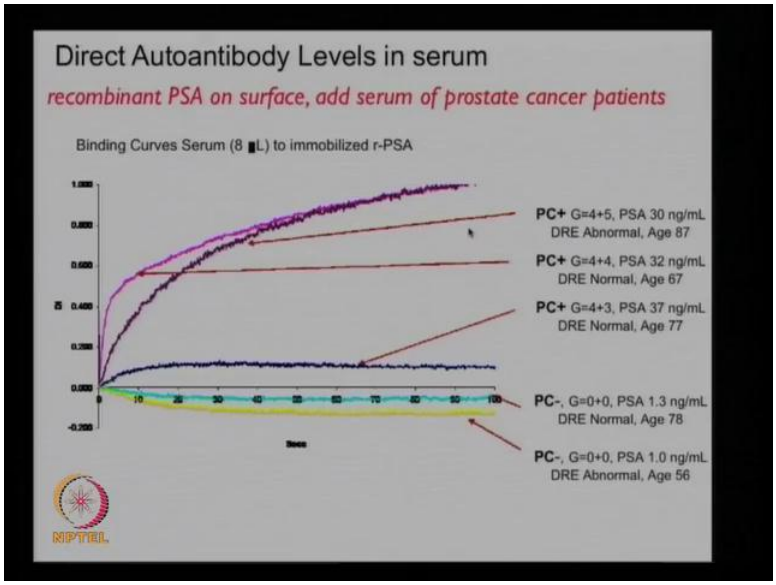

And again so, at each point here is the introduction of another thing so, the introduction of the troponin and the introduction of all the different antibodies to the different parts organs. The way they we have configured instrument is actually in order to introduce a different solution there is a little gas bubble and so, this gas bubble marks where you introduce. So, it is a time zero if you are doing a kinetic measurement you need to know exactly what time zero is when things were introduced.

I think in this case since, I think they know the biology of it well. So, probably measuring different components will be easy because, I think they were able to generate anti cTnl and anti Tlc and TnT fragments and then specific antibodies for it I think it is pretty neat experiment where we are actually measure the complex protein.

That is right. But also it is individual components with their specifications. Yes and so, you are being able to and then configuring you assay you can actually do this placements and take them away. You see them and this can be done in how long is to say. So, this is 5000 seconds but it does not have to be even 5000 seconds if you are not doing kinetics you can cut this thing shorter. So, does not have to be that long the comparable technique is to actually do a western blot you know how long western blot stay, a whole day in fact more, and a lot work, and a lot of material. So, that was actually a very nice way you can actually do experiments faster.

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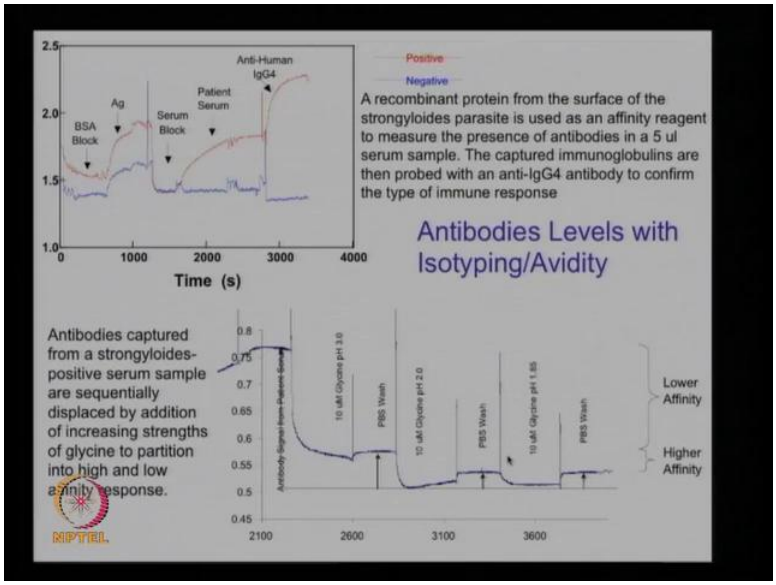

You know this various applications that different groups have studied and this is about detection in a PSA and some interesting work have we done on antibodies of isotyping and avidity experiments. So, I thought that once the instrument is commercial I do not even know what these things are anymore. Obviously I mean, it is just a measuring interaction by molecules. But and one can use the same principle.

But you can use the same instrument but now you can configure your assay to get you the number you want because for example, if I told you that we will mess large molecules well, how do you start doing small molecules? Well, you have to be more clever at it you might have to do a displacement assay. Yes. It is still or it is labor free but you are going to have to do a displacement to compare the strands of interaction. In this case you need to look at the isotyping and avidity it is about the displacement where there. Let us see for all technology. Yes. But you see I personally could not have done all this wonderful applications. Sure. So, again I go back to you know the result benefit society by sharing your science and it is not just about publishing it but actually creating a device that facilitates other studies which can give rise to multiple application that and hopefully, get it to be more useful quickly I mean if you can have a diagnostics that are useful quickly that is great. That is right Yes.

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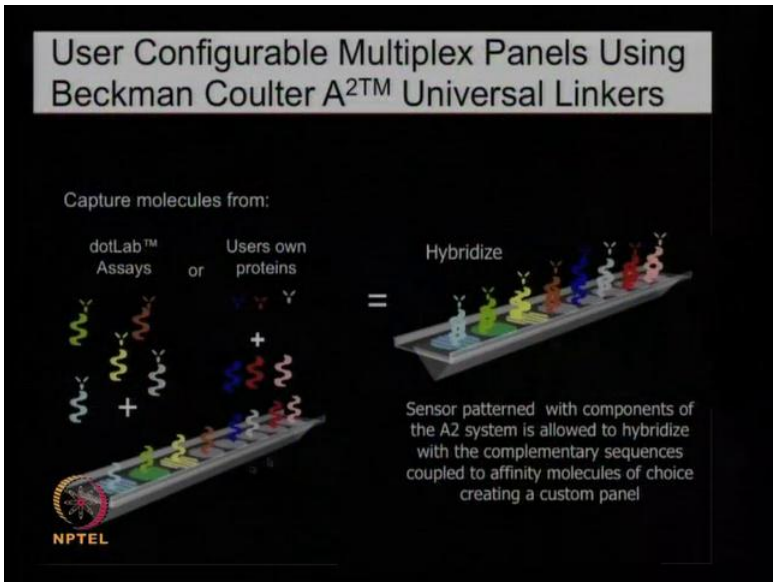

So, let me just show you how we are actually did multiplex a kind of glossed over a cedar eight parts in there but put eight different proteins on a piece of a glass slide is in a pattern is actually nontrivial. Yes. And you know you can do it by physically doing them one by one but that becomes very, very expensive in prone to errors. That is right.

So, one approach that we have actually done together with in collaboration with the excella the company is to use what is called Beckman Coulter. A squared linkers which are short snippets of DNA is that they actually have created in order to bind to proteins but they are distinct enough so, that each snippet is on one location. So, for example, when I bind this dark blue single stranded oligo to the protein it was specifically bind to this site.

So, it will hybridize it is specific location so, it makes it a lot easier to you know you have to react your protein with this DNA but that chemistry is known and then you just inject them all and they go to the locations and I think you can achieve more interactions simultaneously and you can measure eight different things at the same time and that is the whole idea.

So, the instrument works and hopefully other people will start developing more now, there is the main people using it or in the diagnostics area trying to actually develop ways of detecting certain analysis but it can be used for research to actually look at the

binding kinetics. Yes, I think. Now since, we have developed this device. So, now, one has to think is like where is the next. What? So, what is the?

Well of course, the dream is point of carried diagnostics from any it may basically to be able to actually get yourself diagnosed without to having to have you know lot's and lot's of byles of blood extracted sent to the clinic sent to some laboratory and to take by. So, if you going to have diagnostics in the doctor's office you can know quickly that is a dream and there is a lot of issues in it and it is not scientific it is a question really of business and policy and so on. So, that is the goal for the company.

In terms of detection systems obviously, you are looking at defection base system here and you are aware like the optical base system and different type of other platforms are available. How do you foresee the kind of like they based on the user requirement? How the field is going to progress in terms of what has to really rely, one type of only principle and device, based on it one need have a combination of it or depending on the type of samples one can select these types of things. So, what is like since you are working in this area from longtime, what will be your perspective like where the field is progressing and what do you think like is there any specific way one can just select a platform and move forward on it or depending our kind of application one has to see what actually?

Now, I do not think there one answer to everything because different devices have different strengths for example, what is the weakness for something like diffractive optic? Remember, I have told there has to be repeats. So therefore, we cannot take one single molecule so, what is the best sensitivity you can achieve well, I do not know the answer to that, but it certainly multiples of molecules. Right.

So, if you are trying to go very low numbers that is not a way to go. So, it works best probably its main advantage is in detection of multiplexing that needs multiplexing particularly there is the things that needs a wide dynamic range. So for example, if you looking at cardiac marker some are present at nanograms some are present at micrograms it is very hard to find the technique that will expand a very wide dynamic range but there are other advantages of different techniques and what has to consider also the ease of use the cost the time needed etcetera. So, if you are trying to do early detection of cancer you willing to pay premium because it is you know very important whereas, if you are trying

to do a surveillance of malaria you not willing to pay premium because, after all what are you going to do. So, there is a lot of considerations not just technology When you are looking at kind of an application. Yes, this is why and I think sometimes, when people talk about diagnostics they really do not think about the part past to markets and In fact, different countries of the world or may be even different towns with in a country may have different diagnostics that may suit them because of the local situations, local requirements. Yes, I think one has to actually customize their devices based on their needs for the local market and the regulations locally. And that is what is the kind of awareness is that people might be having to address. So, sometimes we sign to tend to concentrate on signal and sensitivity.

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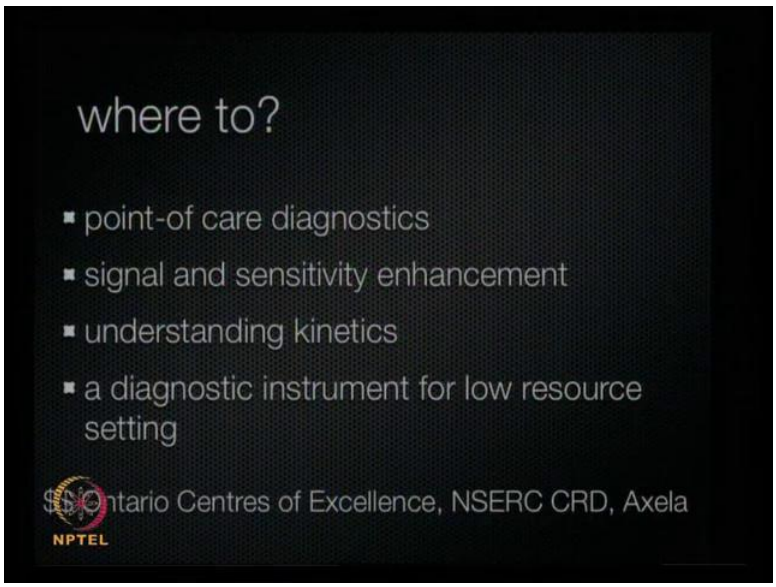

Which I start put it down and we know we want to have better signal higher sensitivity we of course, want more accurate diagnostics but then again you know just how accurate do you need it you know basically, you know usually when it is when you are ninety percent chance of having a disease probably you get treatment for the disease.

But from the basic science perspective we would like to understand kinetics of it better this is that there are models there are software where you can press a button and outputs an answer and some of them shift as well is that really what is happening because, once you start to deal with confined media very small volumes and you start having other issues special if you are the other things and solutions that you have to take into account.

Do you foresee your diffraction base senses in terms of integrating it with more nano elements some nanomaterial or with the Plasmon's or integrating different components for having the better applications. Yes. Well, this is so that is what when a way in improving sensitivity. So right now, we built this to be as cheap and as simple as possible I thought did the three elements are a light source, a sample cell and a detector.

The rest are you know the things that you can remove but it is there nuts nice for research if you want to increase the signal then you can put in plasmonic surface actually there is two approaches that I am working it with colleagues. One is using plasmonics by having gold either on the surface or as particles or on the lines and the other one is to actually do elements that are critical to actually have confined which is called black surface waves. Again they will make the device more expansive because its more complex and probably because, it is more complex it is less robust, but if you can get two orders more sensitivity then you have the chance of looking at early stage cancer. So, this all plus and minus.

So, I think their application is to look for the very low abundant molecule at very early stage I think that is what probably we need to have leads the integration. Well, that is what everybody seems to be after that now my main interest is to actually look at it to reengineer everything for low resource static. Yes. Just how cheap can we get it is so, that we can actually do say dengue surveillance where you do not want to spend too much because really again what after all what would you do if somebody is sick. At the same time you need to know whether there is going to be a there is a breakout happening at the remote village.

So that means, I need to be able to do very low cost thousands of experiments quickly in a device that is robust enough to probably be powered by the sun So, that is what I am after. So, thank you very much Cynthia, I think as you learned from her lecture the science part of it how one has to think about the even simple physics principle and then build devices from it which can be applicable for various type of applications and also she is being an entrepreneur.

She always has an insight of making the devices of a low cost which can have the better implication for various type of market and that is where I think during our entire discussion you have been hearing like not only in terms of making the device in its

application but also how well it can be applicable for the different type of consumer different type of people for different type of point of devices.

So, I would like to thank again in the Cynthia for sharing your work with us and giving us a very good insight about different type of detection system including the diffraction bases, and how one can play with the different type of molecules over there and have either label free or label based detection for better sensitivity thank you very much.

Well and thank you.