Interactomics: Protein Arrays and Label-Free Biosensors.
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Lecture-16.
Kinetic Analysis of Protein-Protein Interaction Using BLI

Welcome to MOOC intractomics course. Kinetic analysis of antibodies and other proteins is critical to characterization of molecules. In today's lecture, we will use bio-layer interferometry BLI technique to perform a kinetics interaction study between mouse monoclonal antibody and protein A. Here, protein A will be immobilized on a matrix at the tip of a fiber optic sensor. The binding between the immobilized ligand and the analyte, for example, the mouse monoclonal antibody will produce a change in optical thickness at the tip and resulting in a wavelength shift proportional to binding.

The data acquision and data analysis will be demonstrated followed by determination of interaction kinetics between the analyte and the ligand. So, let us start the lab experimental session now.

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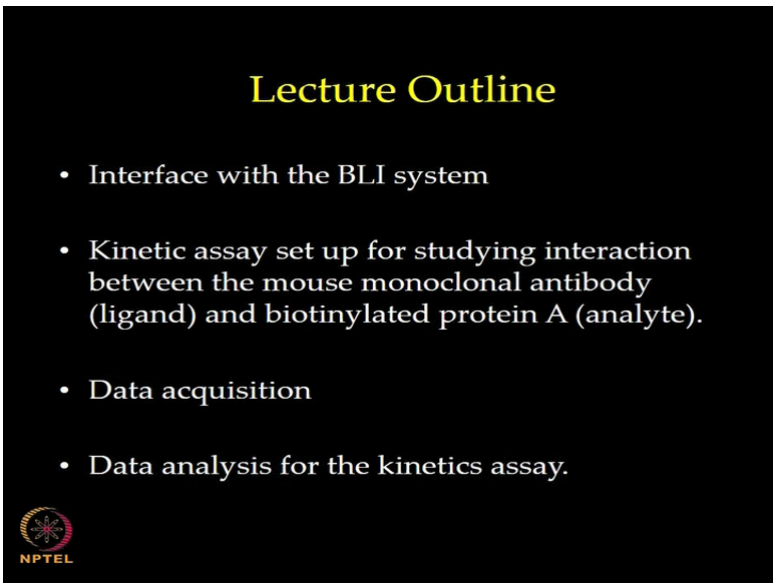

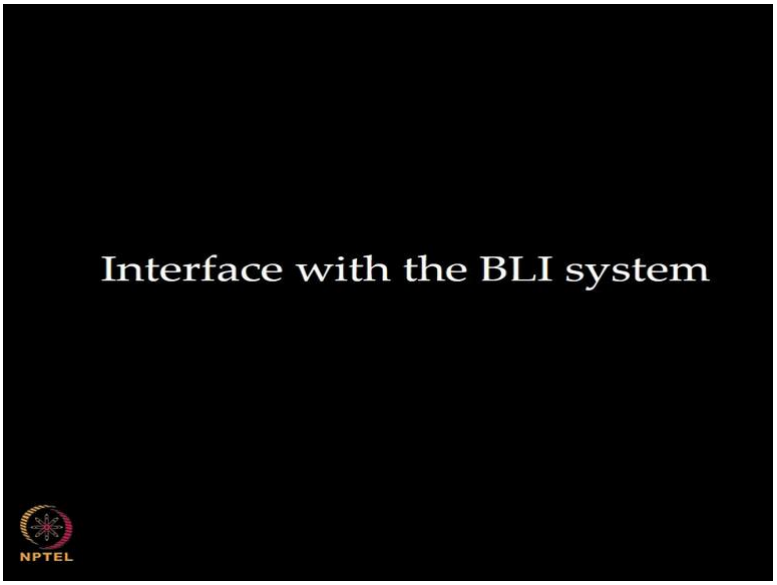

Uhh myself Shushilendra Vaidya, applications specialist for the ForteBio Pall Corporation, just now in the the last talk Dr. Shenglan Cao spoke about the bio-layer interferometry and the applications. Now, I am going to demonstrate how this bio-layer interferometry works. Now, I am going to demonstrate the kinetic studies of the monoclonal antibodies that is the mouse monoclonal antibody to the protein A ligand.

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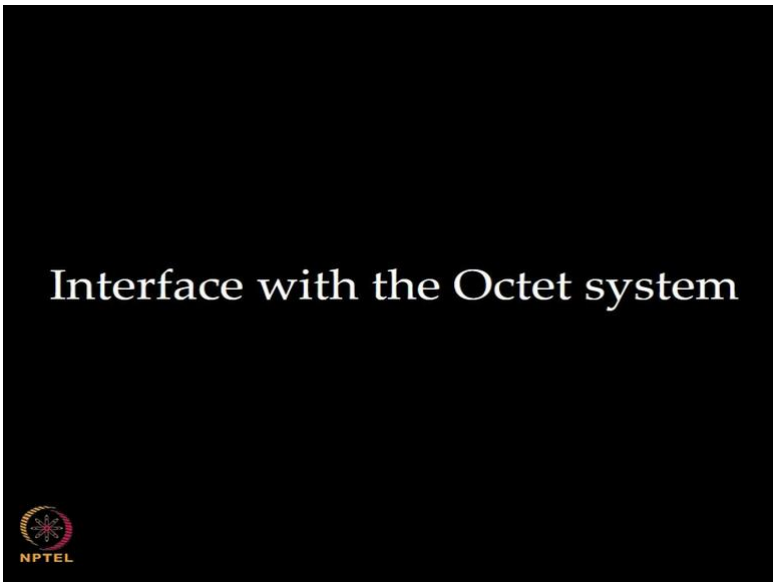


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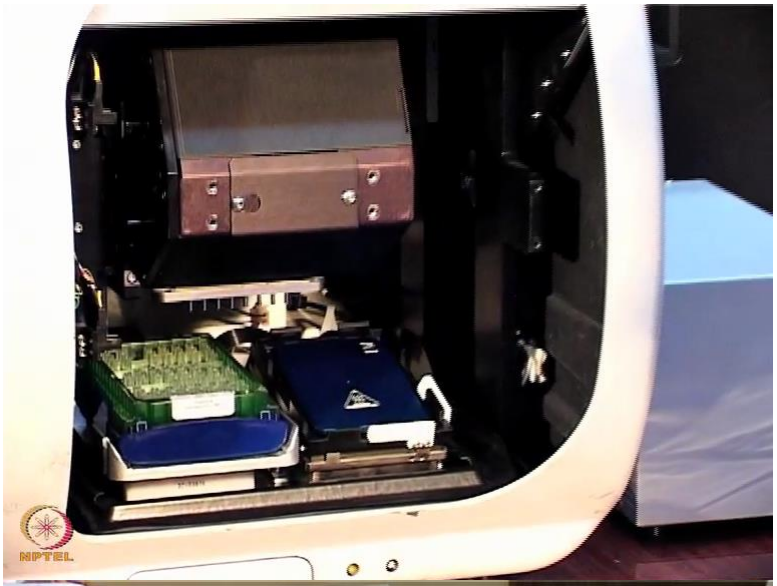
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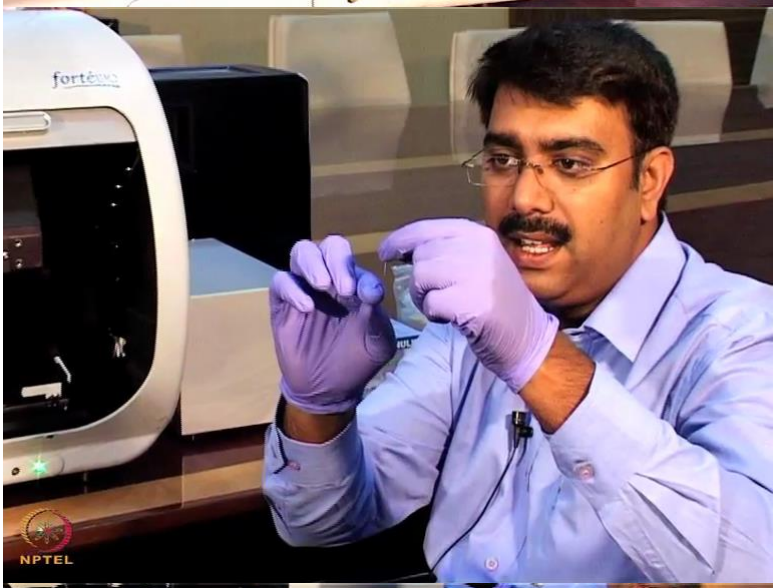
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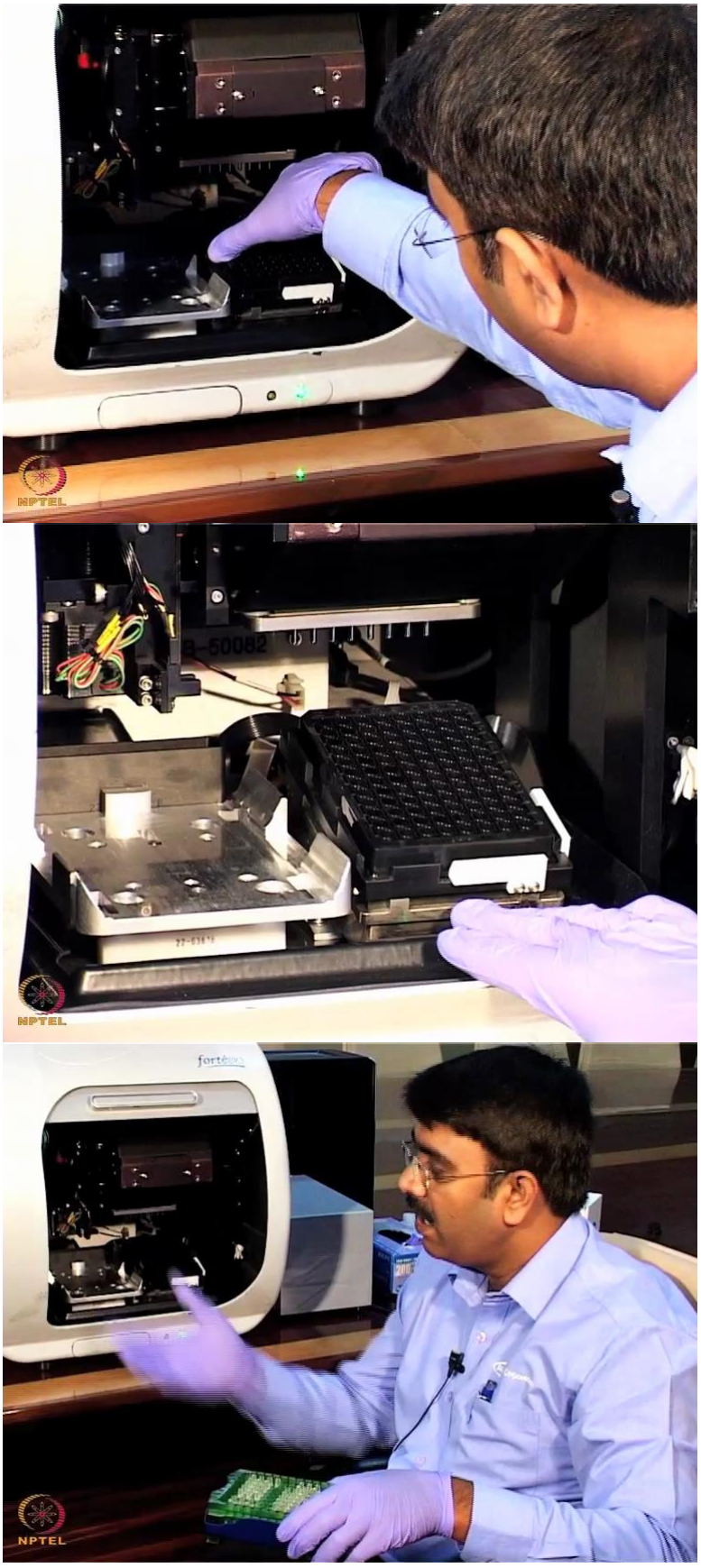

This is the Pall ForteBio octet (())(2:17) instruments if we look at inside this is we call it as a optical head box. The optical head box consist of spectrophotometer as well as the channels

or we call it as a manifold this is 8 channel manifold. So, it can take up the 8 sensors. So, here we have look at this is the sensor compartment, this is we call it as a sample compartment.

Now, you can see this, this is we call it as a sensor holder or sensor manifold. So, here we can put it the sensor in this fashion and then we can put it this as a sample. So, this is the 96 well plate format we can view, analyze 96 sample in one go. If you look at this, this is works on the principle of the depend rate as well as the bio-layer interfero metry. You know about the principle behind the bio-layer interferometry in the last talk, here it consist of white light as if you look at here, this is called as a white light, can able to see here.

This is the light emitting diode; we are passing the light emitting diode, the light pass through the sensor. If you look at this, this is the sensor. It is actually made up of the glass it consists of fiber optic inside and at the end at the holder the part this is made up of the plastic. So, inside there is a fiber optic at the tip of the sensor we are putting a ligand of our interest.

So, we are passing a white light, light gets reflected back. It is reflection based phenomenon. One light which is coming from the internal optical layer another light which is coming from the ligand end. So, when there is an interaction between the two binding partner, say suppose I am coating with a kind of a matrix, it is a protein A, when you dip into the the sensor into the corresponding binding partner, example, like monoclonal antibody, when human IgG starts binding to the protein A matrix.

As you know that, it is concentration dependent as the more molecule starts binding to the protein A matrix then we can able to see there is a change in the spectrum or I mean to say, it is like a change in the wavelength.

So, the change in the wavelength is nothing but the as the concentration increases, change in the wavelength, it is corresponds to the optical thickness. The more the optical thickness as we can see as change in the wavelength. It indicates that the more the molecules are binding. So, from this we can able to like determine the concentration. This instrument even we can useful for the quantification purpose. Say suppose in case of industry or any a protein if you want to do a quantification uh directly we can take known concentrations of the standards, we can generate a standard curve and from we can bale to determine the unknown concentrations.

Uhh apart from that the major useful of this instrument is to study the binding kinetics where we will determine the rate constants like on rate, off rate as well as the affinity constants. I

am going to demonstrate you kinetics interaction of the mouse monoclonal antibody to the protein A ligand. Here, I am using biotinylated protein A as a ligand at the concentration I am using a 10 microgram per ml. 10 microgram per ml I am putting into the 6 wells and mouse monoclonal antibody I am using as a analyte here, I am taking the starting concentration at a 25 microgram per ml I am going to do a serial dilution with the two-fold from 25 to 12.5 then serially I will go for a two-fold concentration around 6 data points.

I am this is the plate mapped here, this is the buffer the first column and the second column I am going to put the by 10 microgram per ml concentration of a biotinylated protein A and the third column I am going to a buffer. It will be useful as a for a unbound biotinylated protein A get washed off here. And also, this well I am going to useful for the dissociation purpose, and the last well, I am going to put the analyte that is a 25 microgram per ml serially diluted in a all these 6 wells.

This is the sample plate 96 well plate, I am going to place in the sample compartment here like this, there is a clicks on, it will come, firmlyit fits into the sample compartment. Here, if the sample compartment if you look at it is work on depend rate the BLI technology works on the depend rate method, here we do not have a like a any microfluidic devices.

So, to assist for a binding we have a like shaker, that shaker assists in the binding. the shakers works from 150 RPM to 1500 RPM and also, the sample plate have a like there is a temperature we can work from the ambient room temperature like 23 plus 4 degree to the 40 degree centigrade. So, here I am going to place the sensor compartment. So, we can from, we pick up the sensors from the either column one, column two or column three like anyway, we can make a program according to the in the instrument, we are placing the sensor rack like this.

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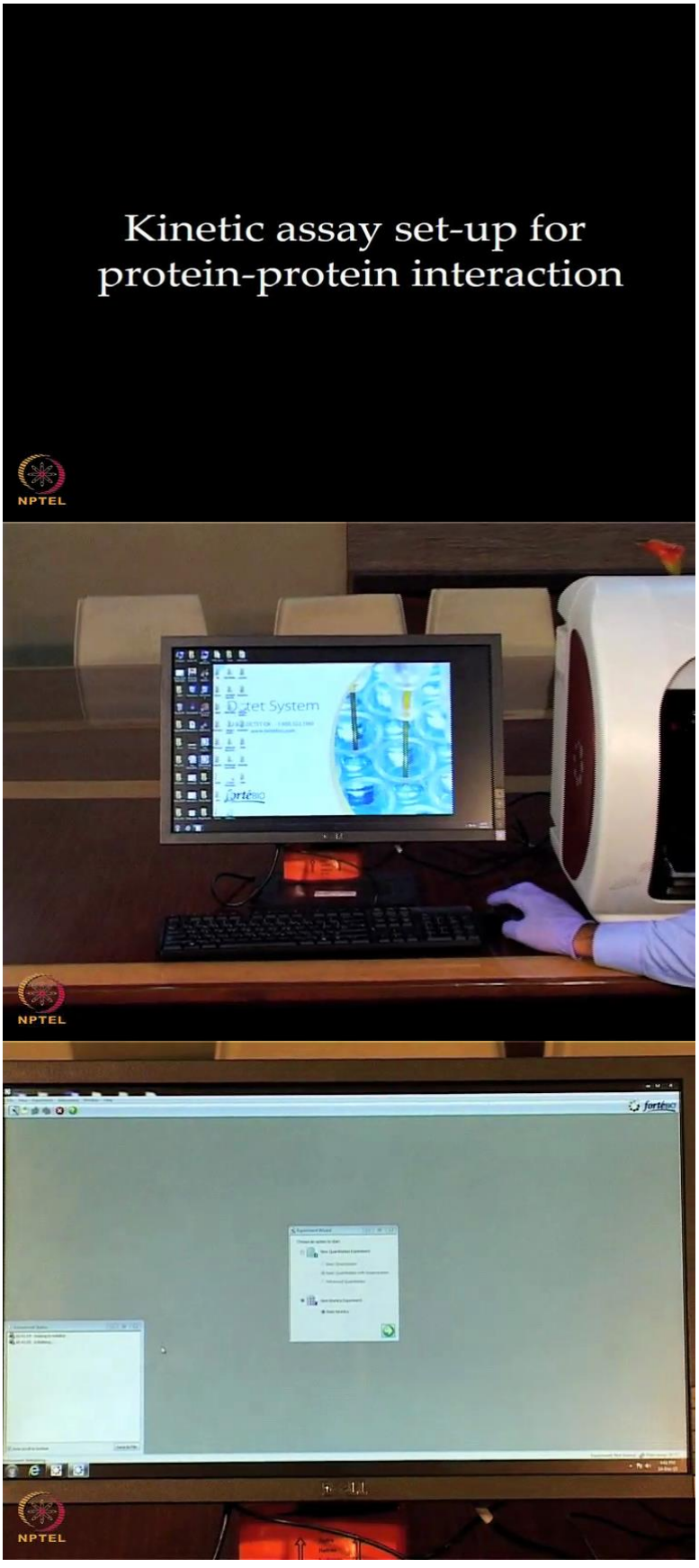

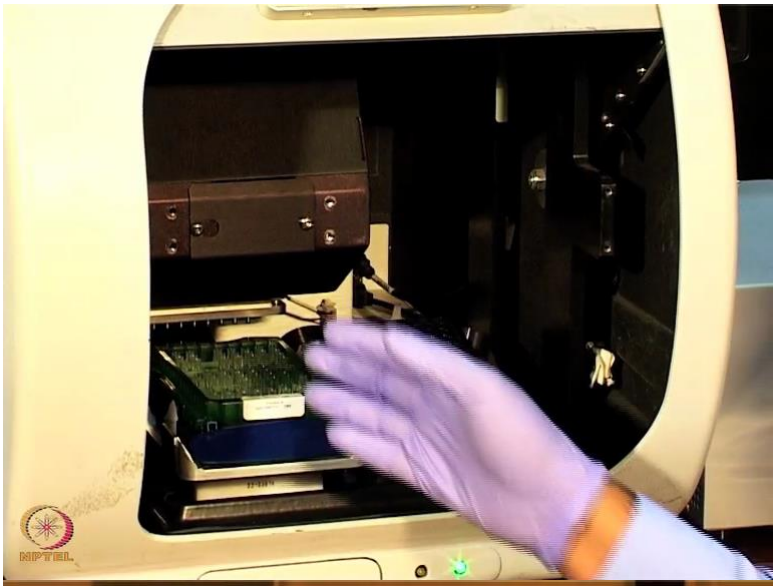

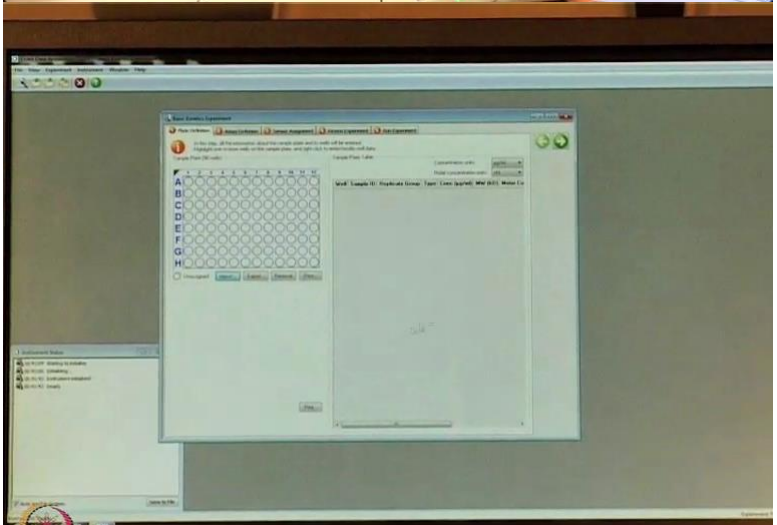

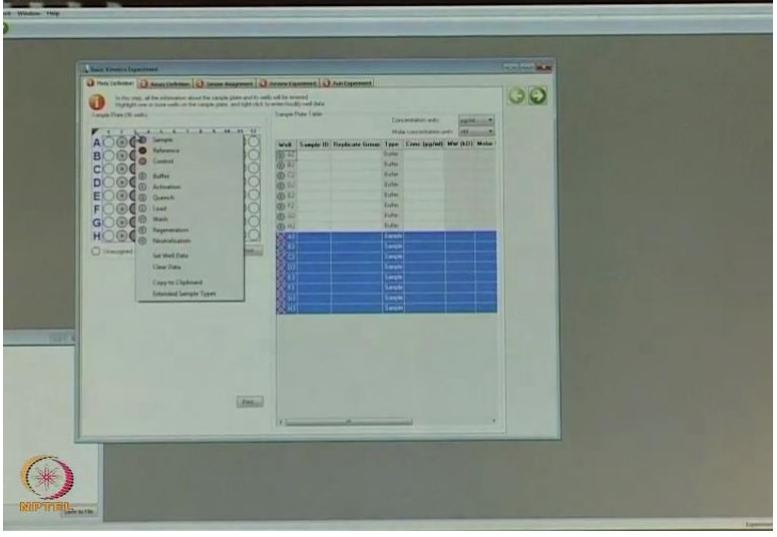

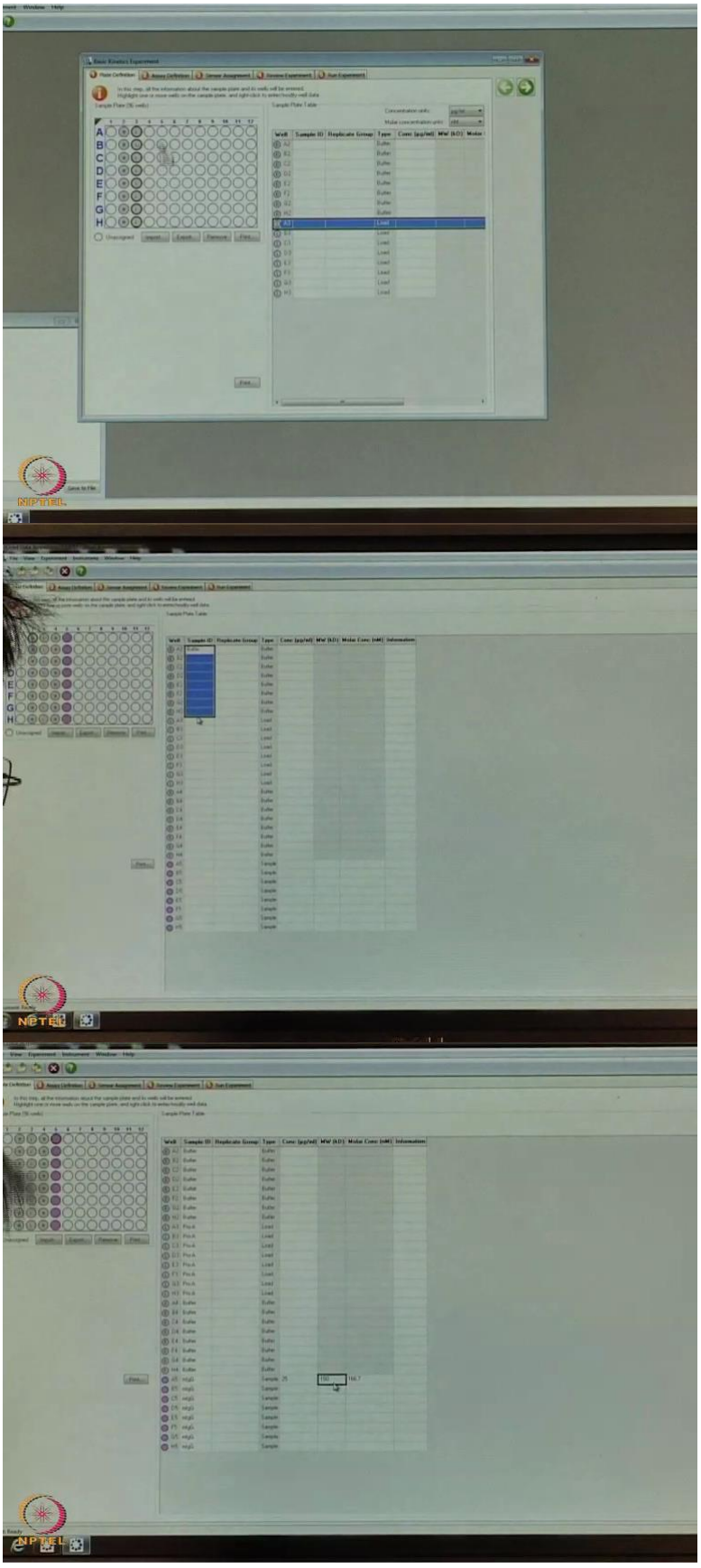

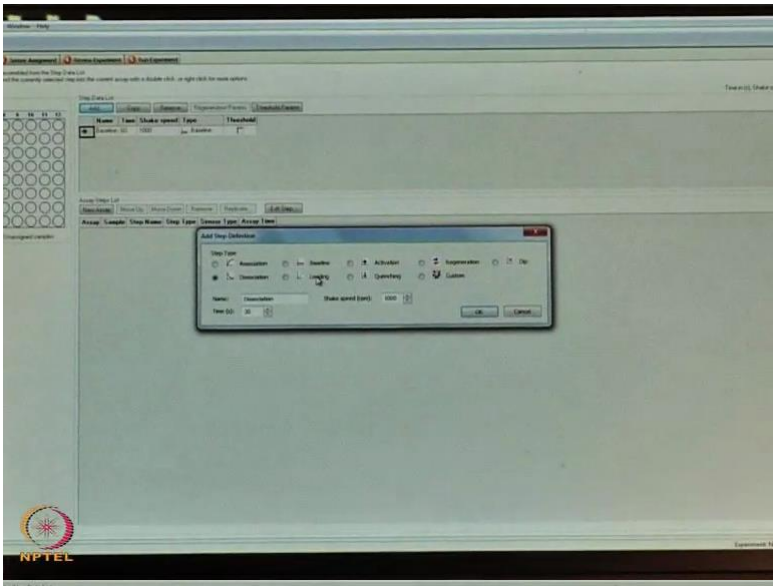
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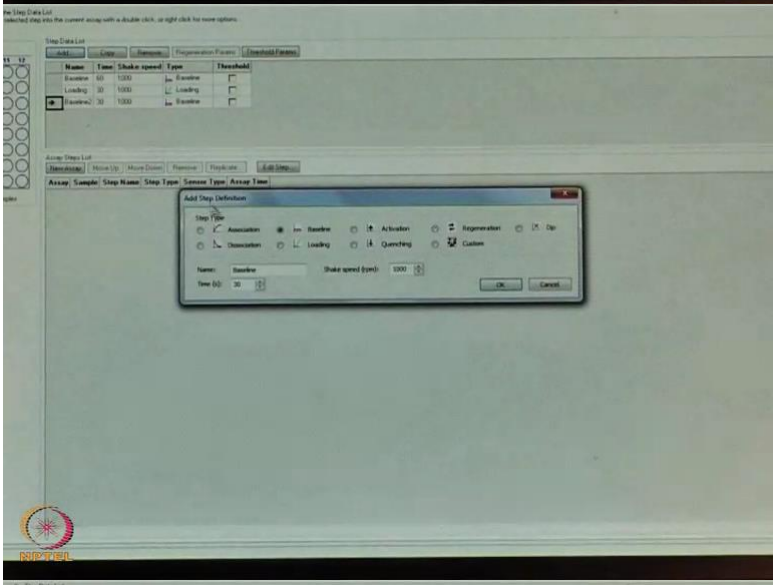
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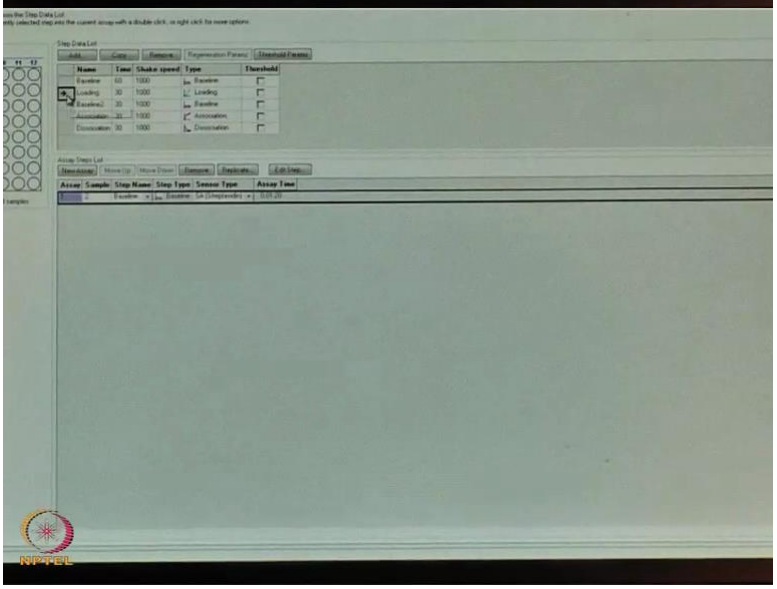
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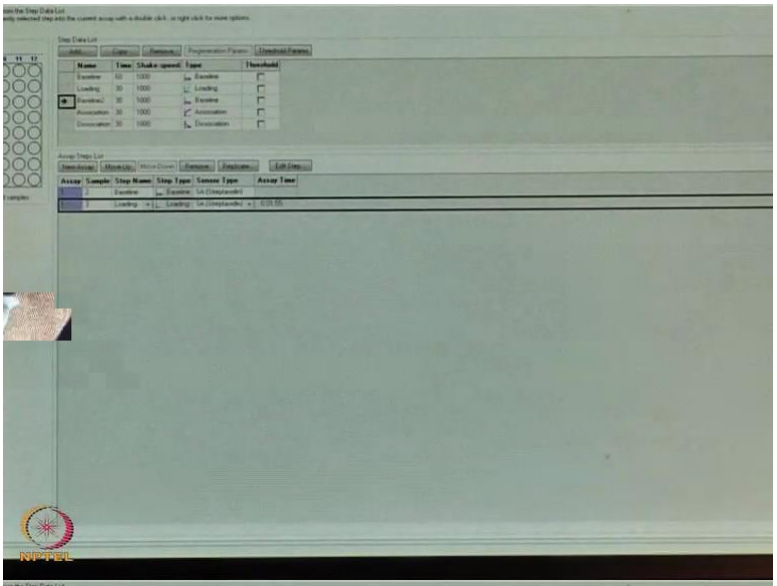

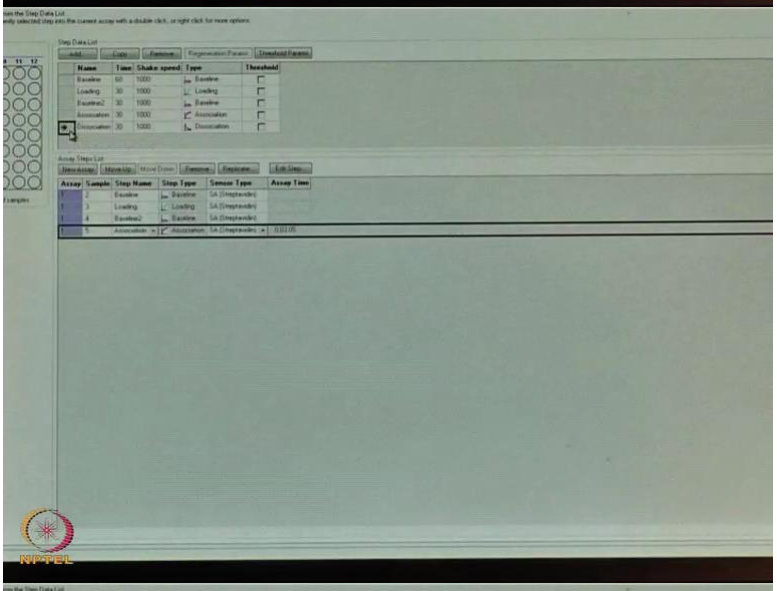

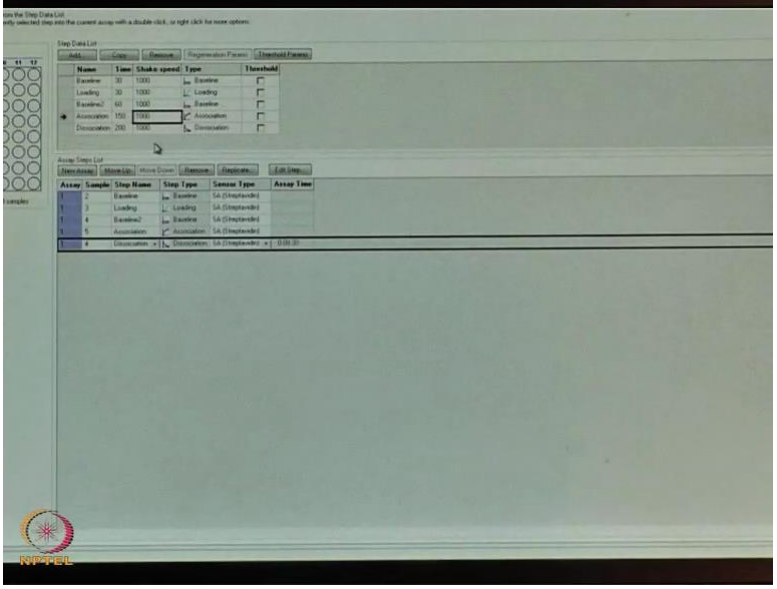

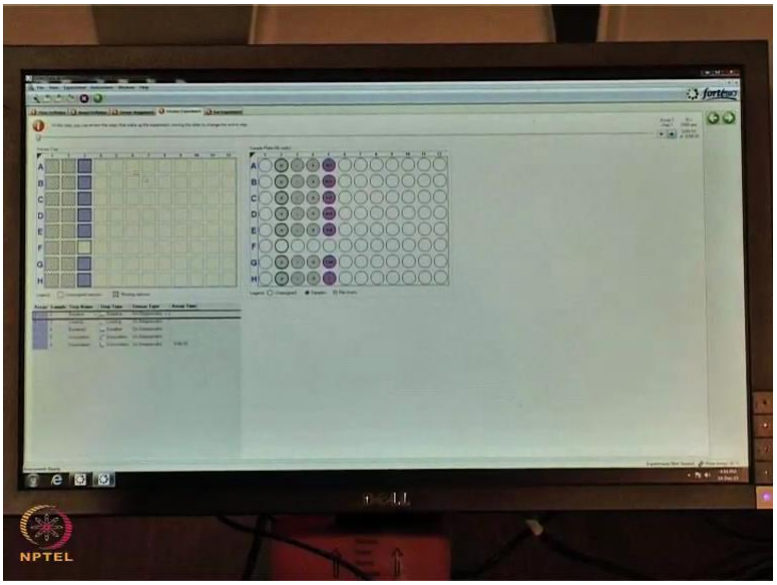

If you look at this in the software, there is a we have in ForteBio platform the BLI technology, we have a acquisition software as well as the data analysis software. Now, I will show you the acquisition software what are all the features it have. If I double click on this, instruments starts nationalizing. If you look at it is moves in a XYZ direction itself it initializes. Okay, now we can see that there is initialization process is going on, it takes a roughly around 30 seconds of time.

So, we can see in the software what the event is happening on the the dialog box opened. Now, it is showing that the instrument status, it is ready. So, now we can start the experiments, when there is a acquisition software opened we can see there is a experimental wizard here. So, here in the wizard we can see there is a two major experiments whether you want to do a kinetics experiments or the quantitation experiments.

Now, we are going to perform the kinetics experiments, I will go here clicking on thethis green arrow mark. So, if you look at this, there is a page get opened here if you see on your left hand side there is a 96 well plate design. This is what we call it as a plate design we have to mention in the plate design what things we had put at it. So, I had mentioned we had put a like ligand biotinylated ligand, buffers as well as the the two-fold dilutions of the different two-fold dilutions concentration of the mouse monoclonal antibody.

Now, we have to mention where all these things we had put into the 96 well plates. So, I had put in the 96 well plate here, this column I put it as a buffer I will right click it here, right click I am showing this as a buffer. Here, second column I had put a biotinylated ligand that

we call it as a load. Load is nothing but we are performing the biotinylated protein A immobilization step we are performing on the streptavidin sensor.

Now, the third column I had put here this one as a buffer once again unbound material get washed off here. Now, we have to put analyte we call it as a sample. So, these are the sample ideas we have to mention what we had put it here, we can put it this here just like a buffer. So, what I will do is just I will take I will copy it then buffer and once again here, I had put a once again there is a buffer here I put a protein A as a ligand. Here, as I mentioned the buffer we can use any kind of buffer.

Now, I in this experiment I am using a PBS buffer having a additive like BSA, it is a 0.1 percent BSA as well as it having a like a surfactant like a 0.02 percent of the tween 20. So, this composition in a PBS at the pH 7.2. So, I am here I am mentioning the mouse monoclonal antibody I will use M small g capital G MIgG. Okay.

I am using a 25 microgram per ml and the molecular weight of the mouse monoclonal antibody is 150 kilo Dalton, is automatically calculates it is the 166.7 nanomolar. Okay, just I will copy this one, what I will do I will select this, search well data, then it is a 25 divided by 2 then automatically last one I am put it as a zero concentration. These are the concentrations 25, 12.5, 6.25, 3.125, 1.56, and 0.7 corresponding nanomolar concentrations.

Now, we have in the there is one more step we call it as assay definition. So, these are the some of the steps I am going to add here. There is a baseline by default baseline just acquires the the buffer. Now, we are going next step we call it as a loading. Loading is nothing but we are immobilizing a 10 microgram per ml protein A on to the streptavidin sensor. So, I am going to add here then next step I am performing baseline and I will say it as a ok.

Then our instruments says that already there is one more first already we have a baseline. There is a exist, it is saying do you need one more? Yes, I need one more, I will say ok, then I will add one more step, we call it as a association steps we call ok, then second we call it as a dissociation step ok.

Now, we have to tell instrument what is the first step? This is the first step, this line I am going here I am double click on that. This is the first step we call it as a baseline step, the second step we call it as a immobilization protein A immobilize biotinylated protein A immobilization on the streptavidin sensor, I will double click on this. Then the third step, this base line once again unbound material gets washed off here, just I am clicking on this.

Next step is the association step. This is the sample, this is the mouse monoclonal antibodies, two-fold diluted 6 data points then the dissociation step I am performing same buffer that is the column four. Okay. These are the stages what type of sensors we are using I am using a streptavidin sensor.

Now, we have to save for instrument how much time each step has to be acquired. the first step I require a around the 30 seconds and the second step, it is require around once again the 30 seconds, third step it is good to have 60 seconds, association I will perform for around 150 seconds and the dissociation I will perform for around 200 seconds. This is the for this experiment, these are the conditions we already pre-optimized.

So, for this it is well established I am performing and here, I am performing the shake speed at the 100 RPM. If you look at the total time, it is roughly around the 9 minutes and now, we have to say in the sensor compartment from where the sensor has to be pick up.

Now, I had put up the sensors somewhere here at the third column in the sensor compartment. If you look at to the next step review experiment. In the review, it is like that, your sensor is picking from the third column then the first step will be your baseline, second step here is loading, third step your baseline and the fourth step is your association, then the fifth step your baseline. .

So, run experiment then where exactly you want to store the data, here I am going to store the data in the desktop, I have a IIT demo, I am creating this folder ok. Whenever you are performing the experiment sensors has to be hydrate or prior to the experiment, at least 10 minutes is require for the hydrations.

So, suppose your sensors are not hydrated we can say in the software delay 600 seconds. 600 seconds is nothing but a 10 minutes after the hydration, the sensors the instruments starts acquisition in second option is like that during the the hydration whether your sample has to be in the shaking conditions because the instruments have a shaker, it will during the affectively mix. In some cases, it is requires, some cases not you know that shaking sometimes enhances the aggregation.

So, we can uncheck this and also, I think a prior to the experiment we already started hydration of the sensors. Now, sensors got hydrate you can directly uncheck this we can go for the experiment and sample plate temperature, it is at the 30 degree so, already it is stabilized plate we can now, go for the experiment. Here if you look at the, I open the door

for the demonstration purpose in all the cases we have to close this door because stray lights affects the interactions.

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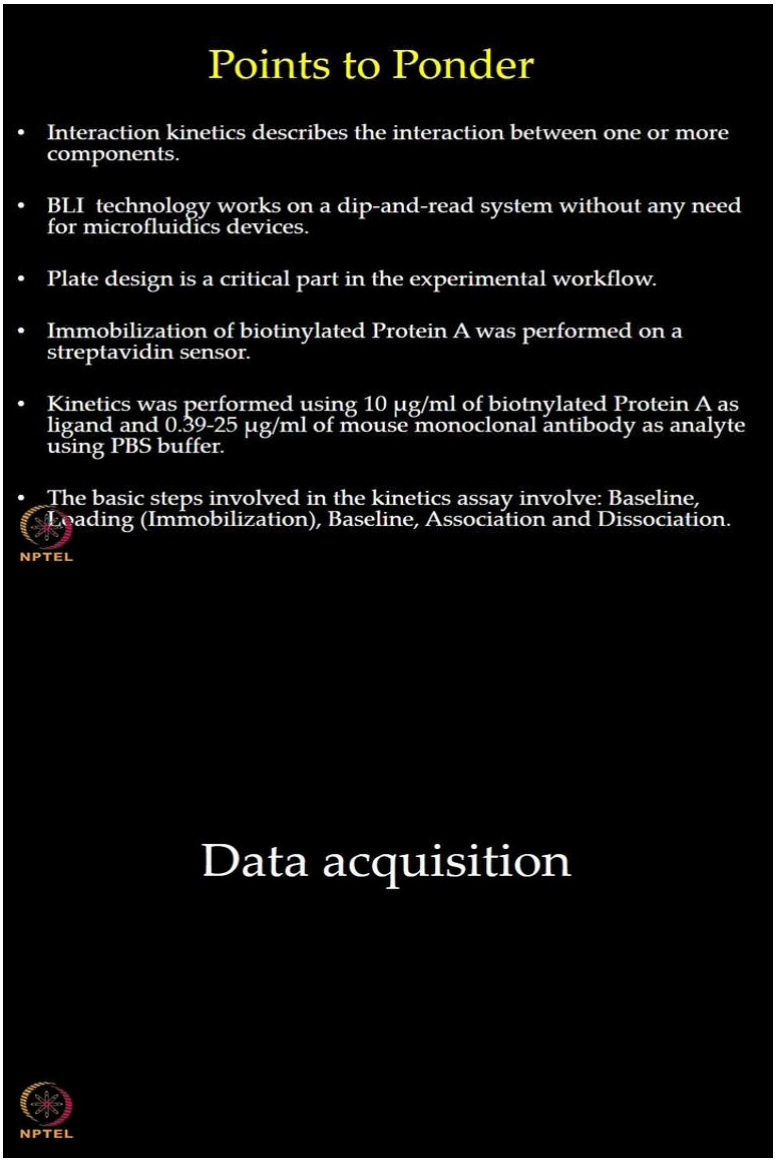

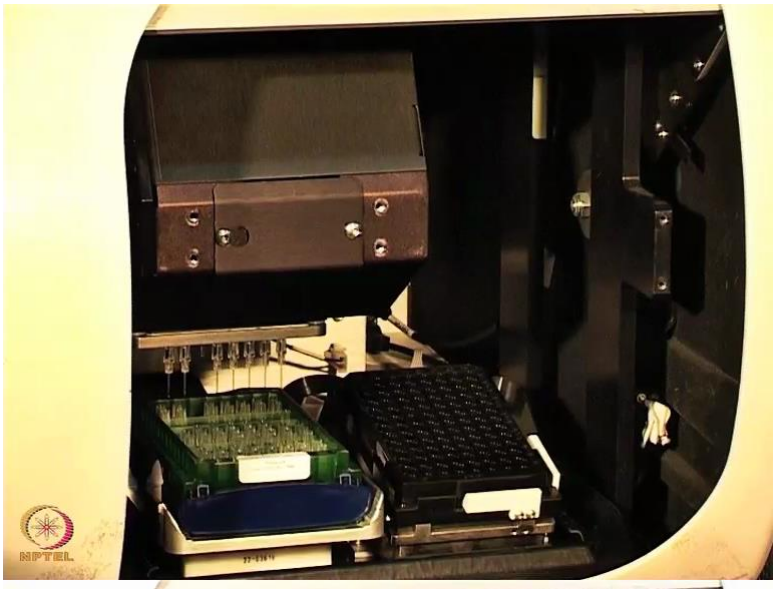

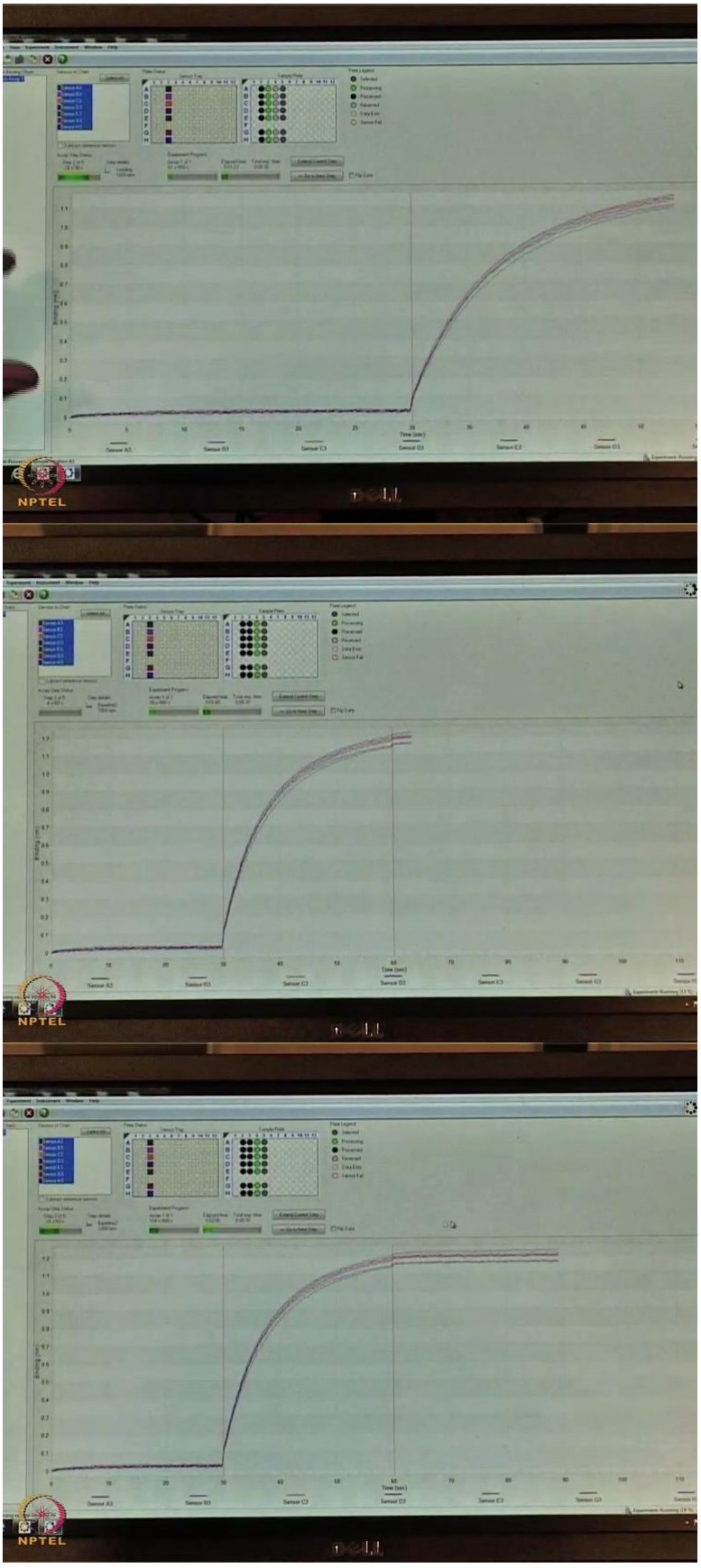
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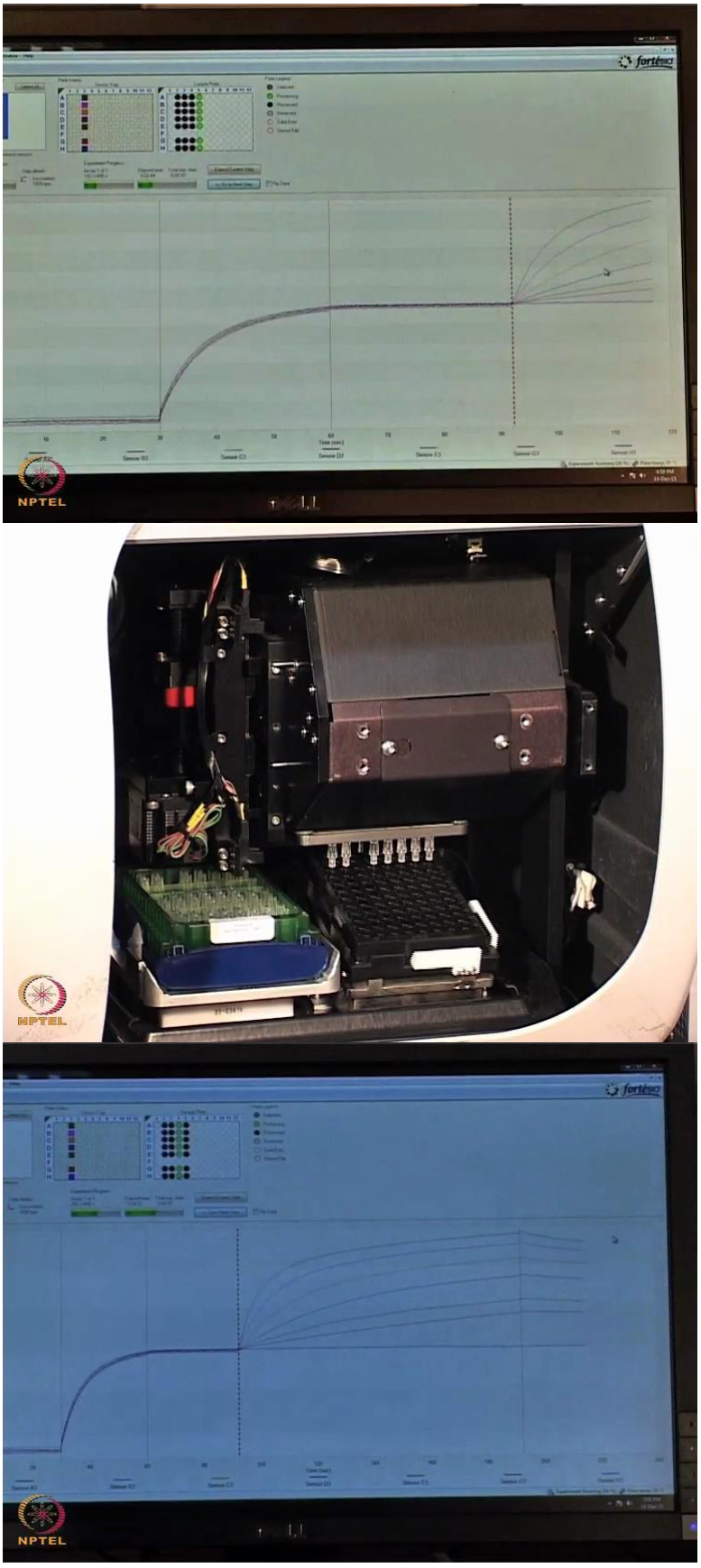

Now, for the demonstration purpose I am keep opening the door I will say go. Sensor is picking from here, from the sensor rack, it is now it is go into the sample plates. Sample plate

if you look at now, there is a shaker which is a working at the 1000 RPM. So, you can able to see there is as when it dips into the sample compartment you see there is a signal. So, now this signal we are acquiring for the 30 seconds. There is a first step we called as a baseline.

Now, it is going to a next column. It is consist of a biotinylated protein A at 10 microgram per ml. now, we can able to see, there is a rise in the signal. This is what we call it as a immobilization step, the biotinylated protein A is binding to the the streptavidin sensor. Now, that is we are acquiring for around roughly a 30 seconds time and it is around in a less than 20 seconds we can able to see it is reaching to the 1 nanometer the binding we are measuring in terms of the nanometer here. This is a good enough loading for to get the interaction studies.

Now, the moving to the next column where there is a buffer PBS buffer unbound material get washed off and if this steps is required for the to get stabilized baseline. The baseline is now stabilized we can go from here we can say ok. It is automatically goes to the next step 30 seconds. Now, the sensors are moving to the association step. Now, here there is a biotinylated program A interaction to your mouse monoclonal antibody. it is like a two-fold different concentrations if you look at this, the graphs this is how the curves are two-fold dilutions, this is the highest concentrations, this is the second fold, this is third folds, this is fourth fold, one at the bottom I had put the zero concentration.

So, in a software we have a function like that we can extend the current step as well as if you want like it is already like saturated, we can go the next step. I will say go to next step and the second says is good. The sensors went back to the buffer well where exactly if there is a interactions happening if it is a loosely bound kind of interactions just get come out from the sensor, if it is a very strong then we can able to see there is a still kind of a straight line here. So, if it is a straight line then it indicates that, it is very strong binding. Once the dissociation step perform the sensors once again rerack back to the sensor tray and the instrument the robotic arm mode to its original position.

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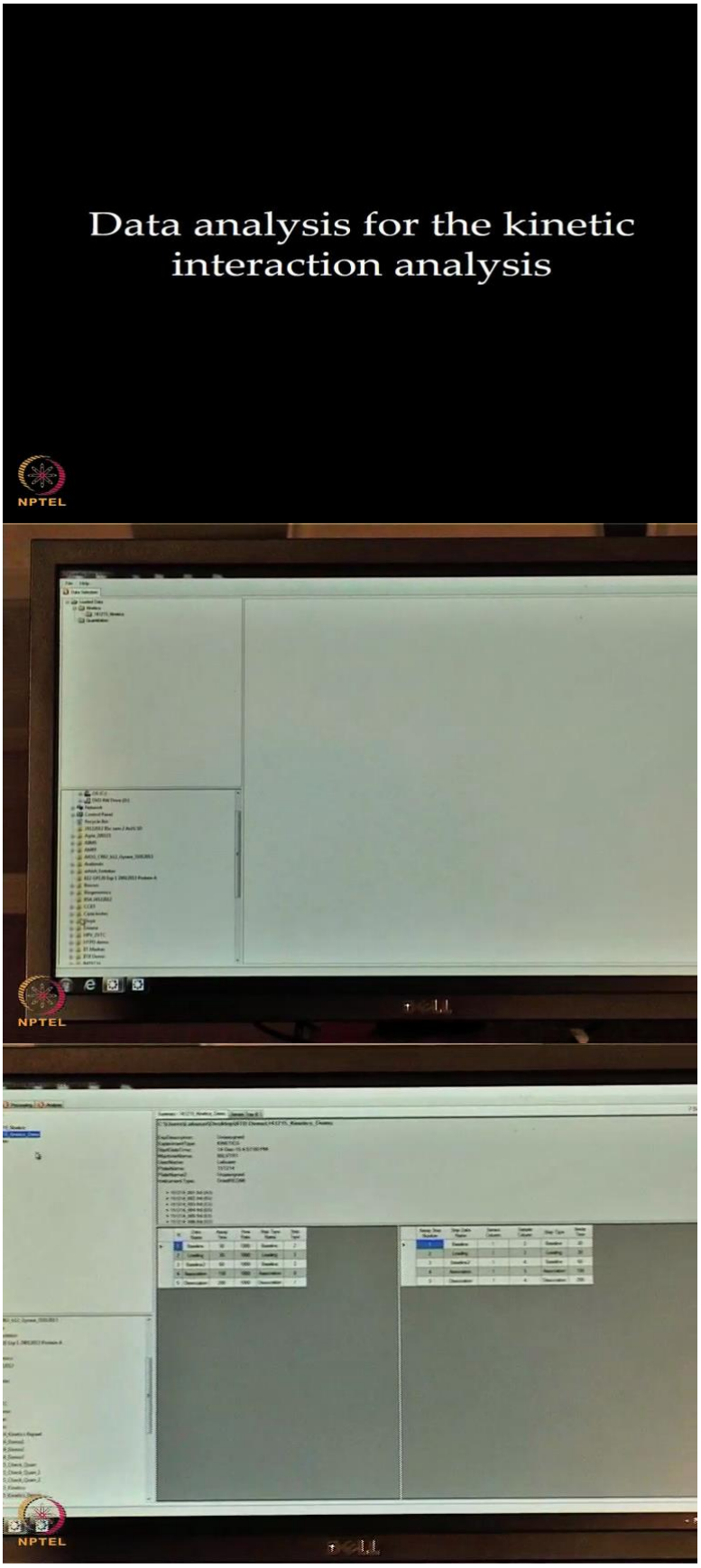

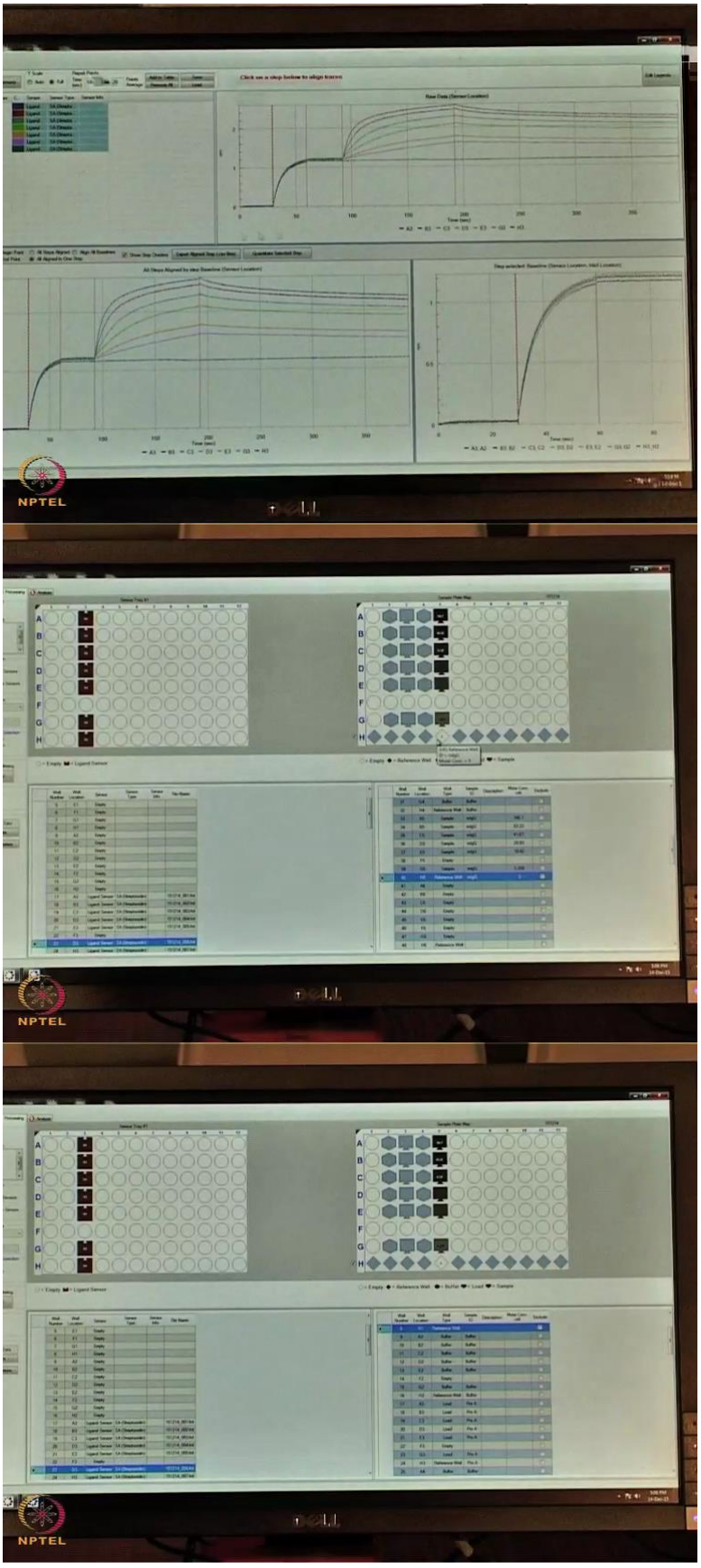
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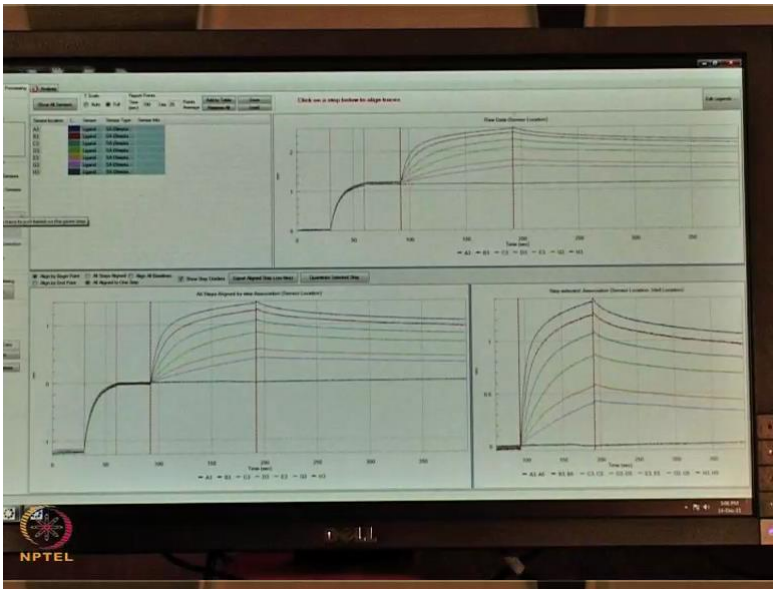
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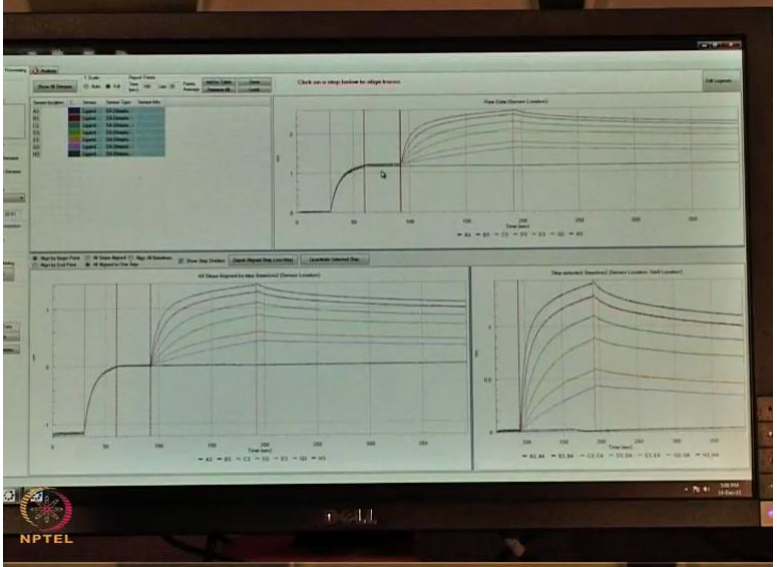
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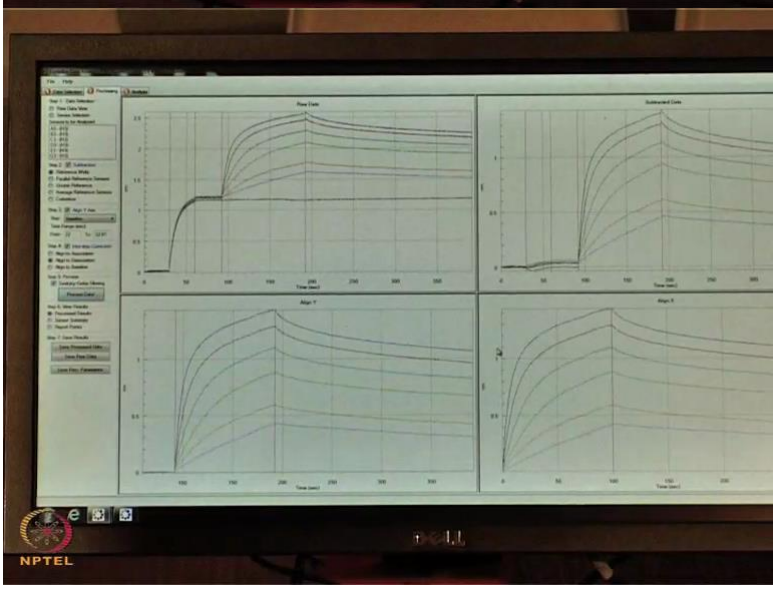
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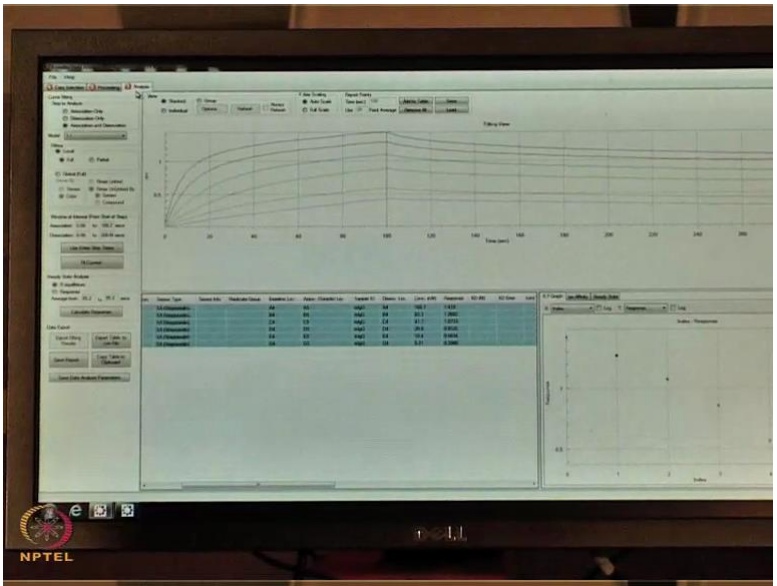
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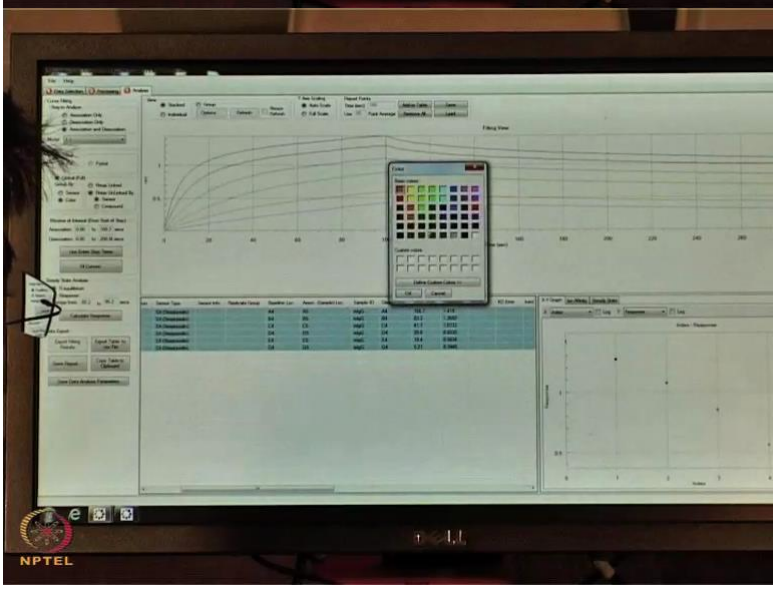
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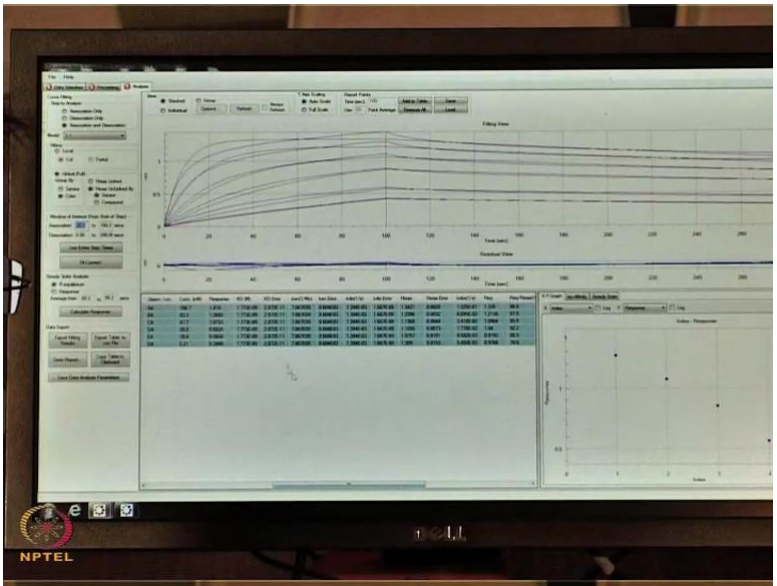

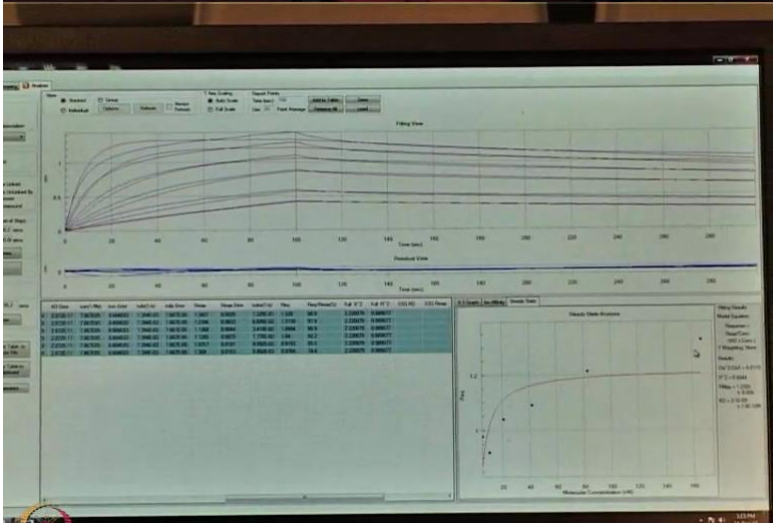

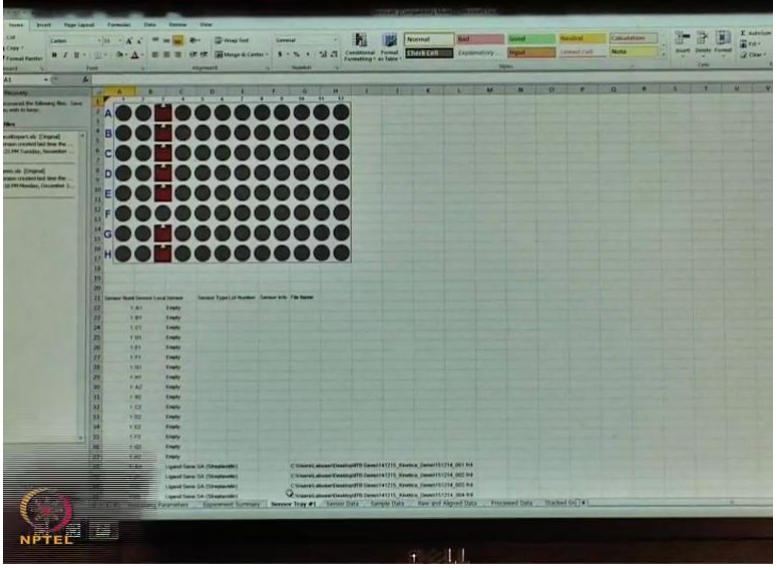

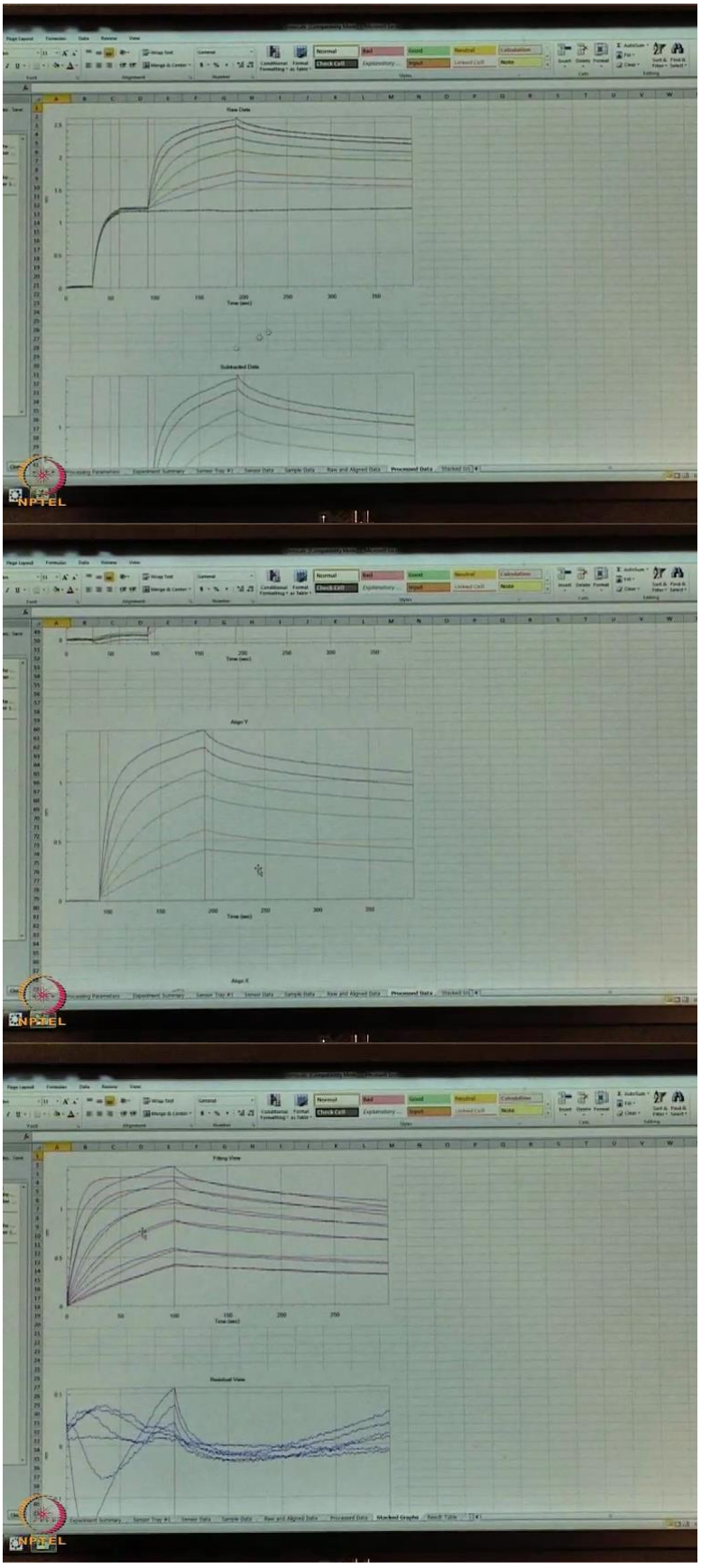

So, now acquisition is done. Now, we have to go for the data analysis. There is a data analysis software I am going to double click on the data analysis software. In the bottom of

this where exactly you have data so, IITB demo, I will go with this kinetics demo I have folder here, this I am double click on this if you see that there is a page got opened. I will go to processing here, in the processing tab we will see this is the raw data.

So, what we are doing is we subtracting, I had like one of the column I had put a zero concentration. In this column I will change, change to the reference well, this is what have zero concentration because some of the artifacts from the buffer we can subtract from the data. So, that is why I am going for the reference subtractions.

On the left hand side, there is a here we have icon subtraction we have different types the subtraction systems. So, I am using here one of this just only a zero concentration I am using for the subtraction. So, reference well I am going to for the in the raw data I will go that, I will select this association step then there is a Y align y-axis, I am going to do a baseline. This is the baseline step for this particular baseline I am going to align.

Now, I have to go for the process data. So, if you look at now, the data what we got. This is what the process data. Now, we have to go for the analysis. Analysis there are the 6 data points we have here, in the analysis part we have association only, dissociation only, association and the dissociation.

For to calculate the rate constants we have to go always choose the association and the dissociation. We have a different binding models like one is to one, two is to one for heterogeneous ligand mast transport affect if there is a any mass transport involve in the interaction studies we have a bivalent analyte one is to two binding model, depletion studies yes. So, local in the fitting model apart from the what kind of model we have a like another kind of a fitting, it is the local fitting as well as global fitting.

The most of the times when you are performing for the interactional studies we always go with global fitting. Global fitting with respect to color and the Rmax unlinked by a sensor. To perform global fitting we have to select all the samples change to a one color. So, I will change to a blue color. Now, we can see this now, is a one is to one binding model, I have to try use entire such step systems I would fit the curve.

So, now fitting is okay and the KD it is showing that around a 17 nanomolar. Easily we can able to see the good fit through the with respect to the Chi square values as well as the r square values. If you look at in this table, we have like a what is the Kd? The KD errors, Kon with the Kon errors and if you look at the the good fits, it is always when in case of the

kinetics the good fit a comes, it is a 0.5 and 0.95 above and also Chi square should be a less than 3.

So, from this indicating that it is behaves one is to one binding model, it is it is supported by a your r square as well as the Chi square stastical parameters. Also, we have a graphs like xy graph, iso-affinity graphs as well as a steady state. Because in this experiments, we not reached the steady states, we not able to like a calculate the steady states. But still software can pickup and the KD value, it is determining, it is around a 2.1 nanomolar and also, here, also if you look at this is around roughly a 1.7 nanomolar.

Uhh It is almost like a similar very near to those values, yes it is a good fit and we can export this the into a printed versions like a making a report. Here we have to say save report then then say export we get like a kind of excel sheet what the parameters I had used for that experimental summary, the sensor tray where exactly you had put a sensors, the sensor data what sensor we had use in sample data, how your sample plate design is, raw data you can see the raw data, aligned datas as well as the process data how this we had subtracted using a reference sensor subtraction method, the graphs the fitted graphs, the residual view the experimental view, the blue ones which as experimental red ones which are the theoretical.

In the results tables what is the (())(24:23) sensor locations sample id, the concentrations response what we got, what is the KD, KD errors. So, all entire details we can get it into the report. Now, we successfully demonstrated the protein-protein interactions here with a protein A with a mouse monoclonal antibodies we used and we got a very good data with one is to one binding model with a affinity is a around the 1.7 nanomolar range. So, with this I am concluding my demonstrations.

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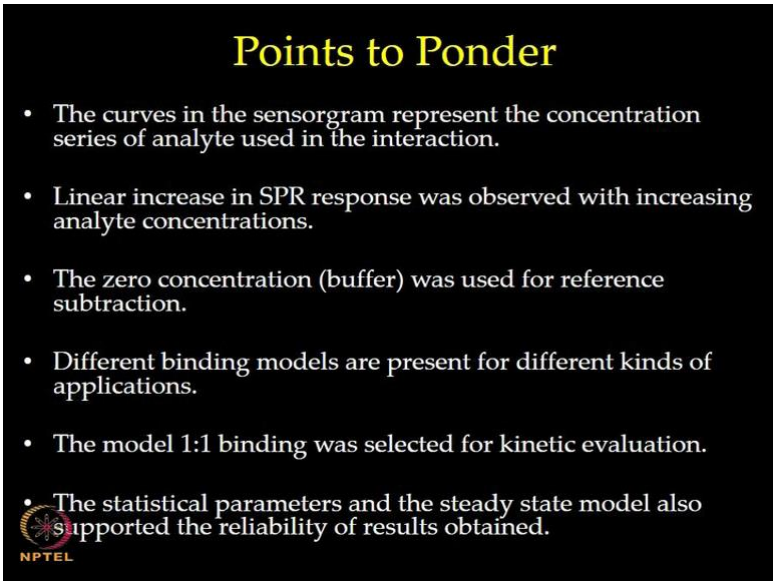

So, today we have witnessed the application of BLI to measure interaction between protein A and mouse monoclonal antibody. As demonstrated, the system monitored association of the analyte with the immobilized ligand and dissociation after moving the sensor to solution without the binding partner. The changes in interference patterns were quantified and used to determine the kinetic rate of binding and dissociation. Thank you.

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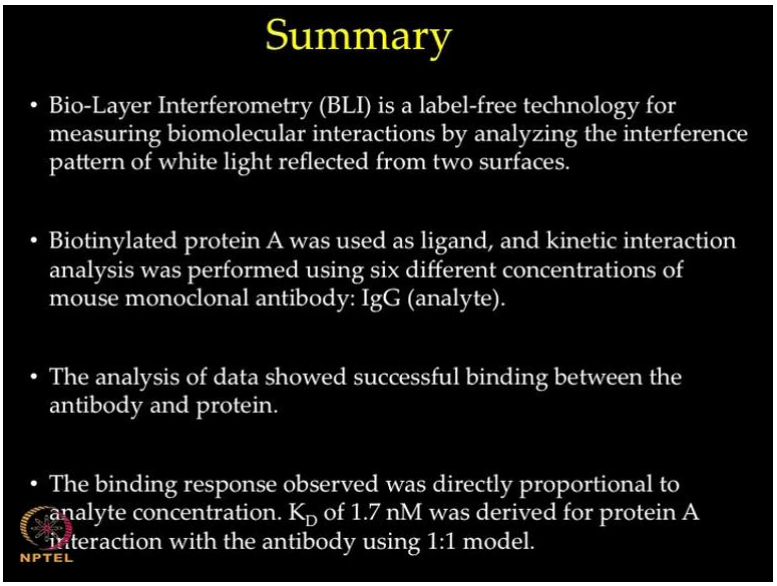

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