NATIONAL PROGRAMME ON TECHNOLOGY ENHANCED LEARNING (NPTEL)

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Applications of Interactomics using Genomics and Proteomics technologies

Course Introduction by
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MOOC-NPTEL

Applications of Interactomics using Genomics and Proteomics Technologies

Lecture-28
Biomolecular interactions analytics using MicroScale Thermophoresis

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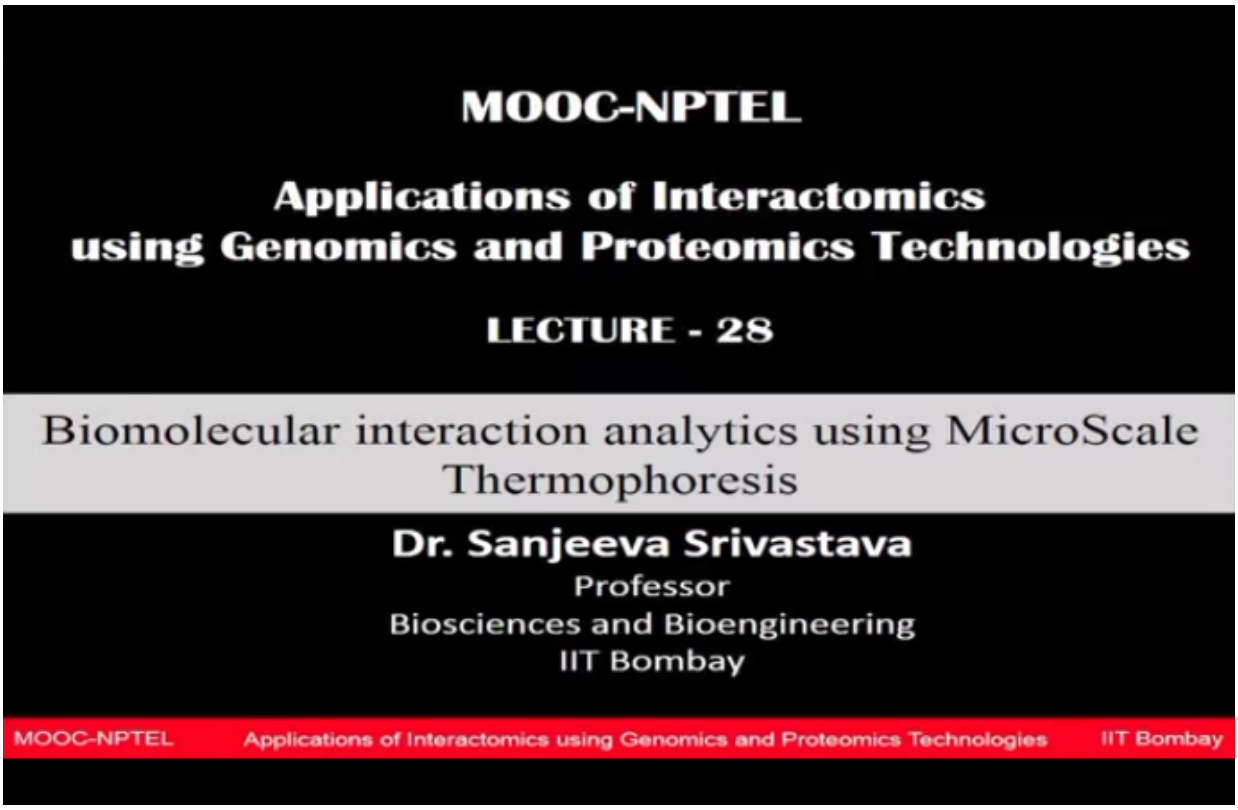

Dr. Sanjeeva Srivastava:- Welcome to MOOC course on Applications of Interactomics using Genomics and Proteomics Technologies. There are advent of new technologies which are really trying to provide high-throughput solution and much more reproducible and reliable results.

One way of doing high-throughput assays is to tag the proteins of interest with different type of tags or reagents like fluorescent tags or radioisotopes or chemiluminescence tags, and then trying to monitor the signals obtained from them. However if you are adding a chemical moiety on your proteins of interest it may provide some multi fact results, it may altered the protein binding or the protein functions as well.

So there is lot of emphasis about how to do the protein studies in the label-free manner. And there is advent of new technologies which are trying to achieve the protein-protein interaction, protein other bio-molecular interactions studies by using label free platforms.

In this slide today we have invited a guest Dr. Shiv, who is head of Business Operations at Nano Temper technologies to talk to you about a latest technology MST which is Microscale thermophoresis for quantifying the binding events and studying the bio-molecular interactions.

Dr. Sheev will also provide you a demonstration of how to use MST technologies for biophysical assays. Towards the end one of the colleagues and application scientist will explain the basic principles of Tycho which is used to check the protein quality and purity in a very, very short time, so in this manner you have a good understanding about how to first test your proteins of interest, quantify them to check their quantification and also their purity level, and then further study how they can bind with another protein of interest or a drug molecule of interest, so let's have this lecture with Dr. Sheev.

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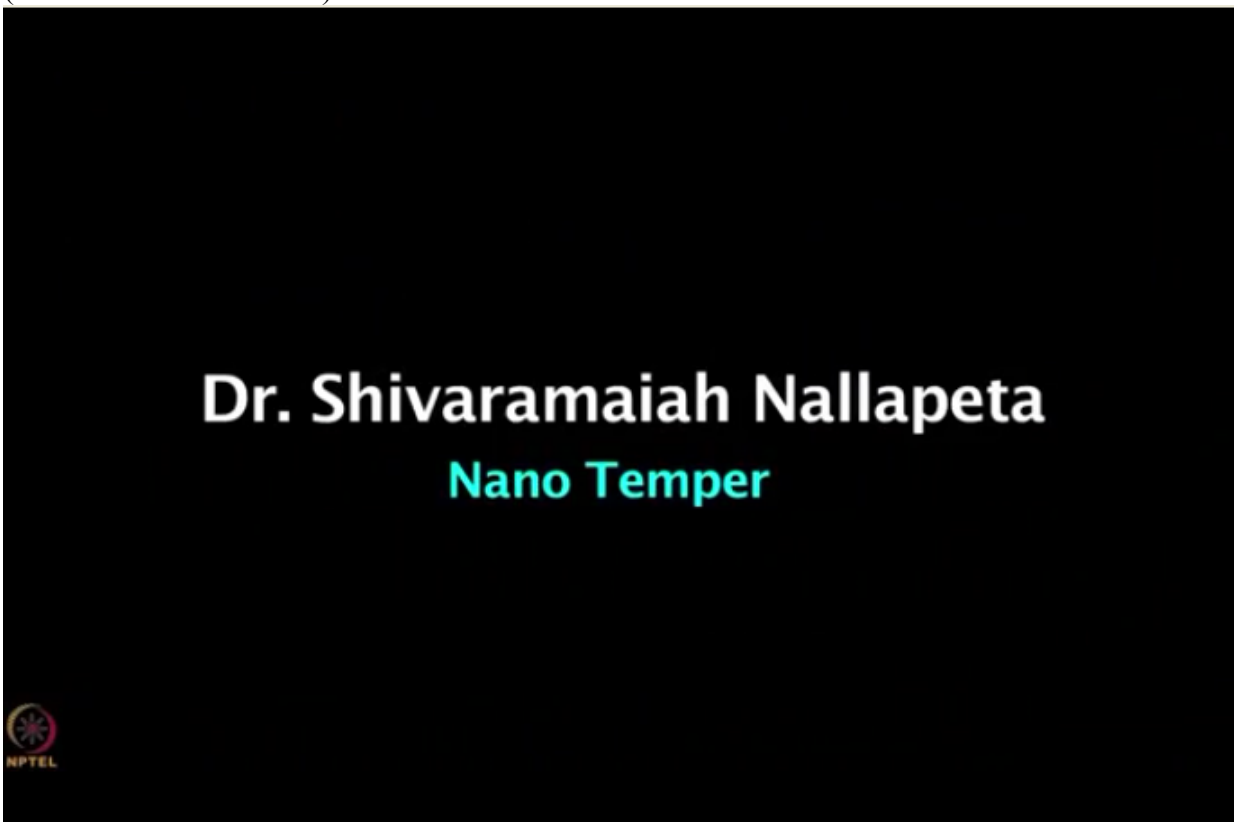

Dr. Shivaramaiah Nallapeta:- I'll quickly start my presentation without wasting much time, but before that I'd like to know like are you doing any biophysical assays for the interaction studies protein-protein, protein small molecule, if you are working on those lines for sure this talk should be very interesting to you, so I am sure like you are working on those lines, okay, okay, great, okay.

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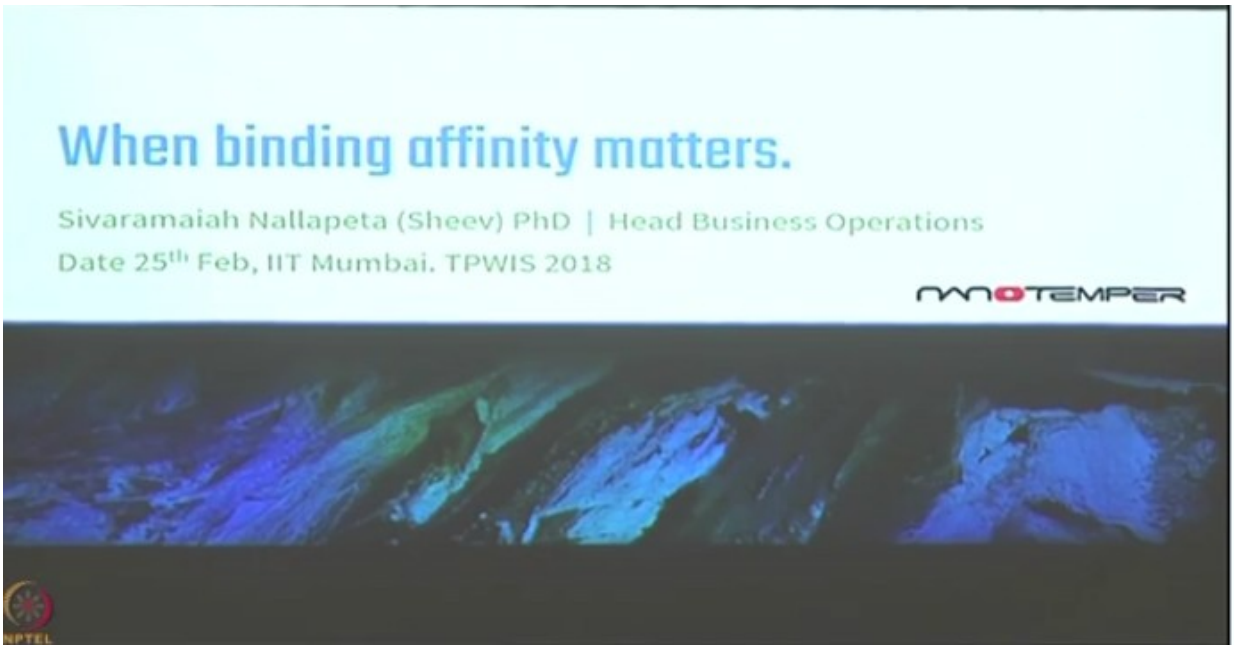

So this is something like you know, a biophysical technique that determines the binding affinities, so as I said like you know this is a technique about a binding affinities, how you determine the binding affinity between two biomolecules, it can be anything protein-protein, protein small molecule, protein ligand like anything you can do in the binding affinity.

So but before that I would like to quickly introduce myself as myself Shivaramaiah Nallapeta, you can call me Sheev, I'm currently taking care of Nano Temper business in India, we are based in Bangalore, and my other two colleagues Ajay application specialist and my other colleague Ram is also here with us.

So before going into details of the technology I would like to give a quick introduction on how our company has formed, which would be very interesting.
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The technology what I'm talking about is thermophoresis, so with thermophoresis have you ever heard of it before? Yeah we say that like you know whenever there is a heat the molecules will move from hotter region to the colder region, but it will be a director moment, so this principle was discovered by this two gentlemen 150 years ago, but there was no any application, it was only a physical principle, but there was no any application,
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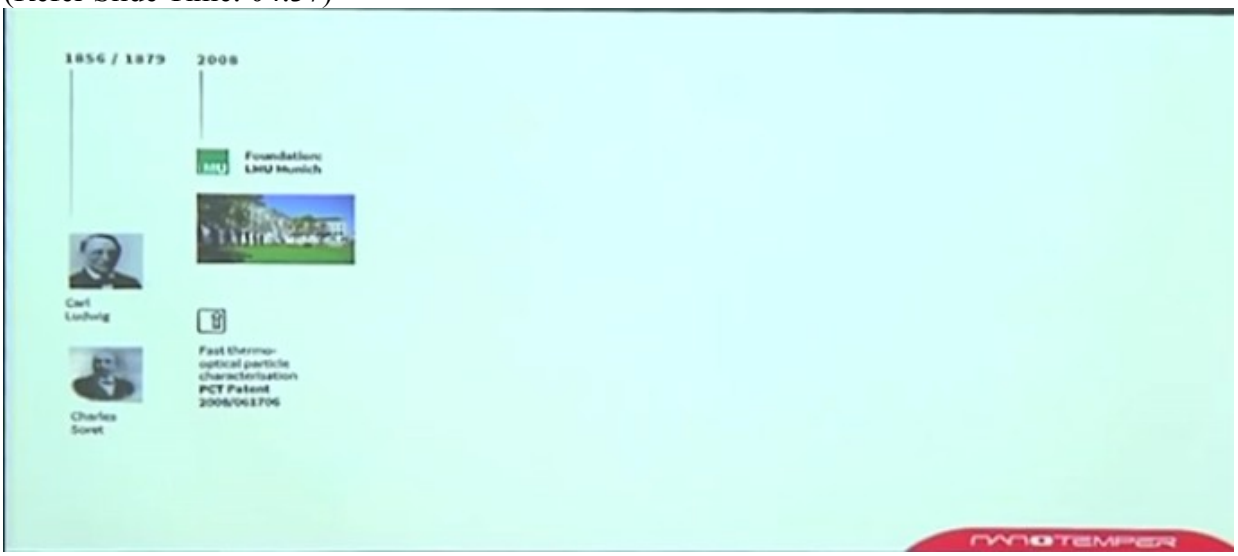

until the two CEO's of our company who filed a patent in 2008, they said that the thermophoresis can be established at a microscale level which can be used to determine the binding affinities, so from there the company has started and as I said like the first instrument which was launched in 2010, which is in front of you they said that using this thermophoresis principle you can determine the binding affinities.

Later on different versions came into the picture which I'm really not interested, but other than this we have a one more technology which measures the proteins stability like you know you
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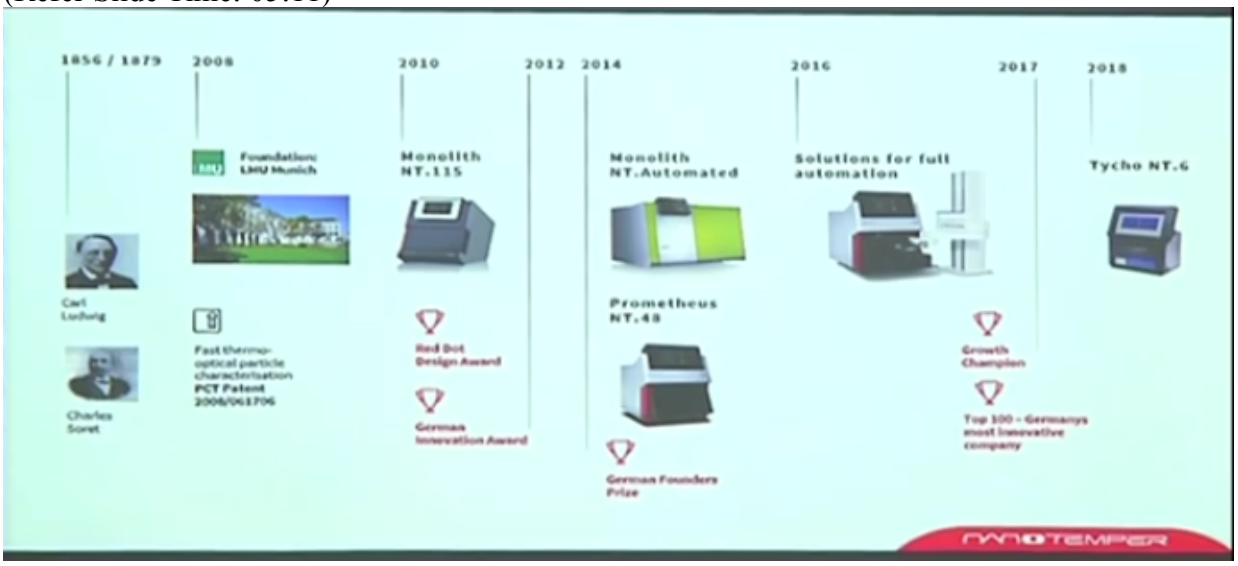

just wanted to know till at what temperature your protein is stable, like what you call that TM, melting temperature of your protein, so this are the two technologies what company has, and as

I said like this two instruments are very handy just beside me on that side and this side, maybe during this talk is over we'll also have a quick demonstration and we will see how easy it is to run a experiment without any optimization, so this are the two CEO's of our company as I said
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like they are 31 year old who has actually filed a patent and started this company in 2010, and now we are successfully spread across the globe and as you can see a coffee machine in between them, we say that our machines are very simple, they are like a very robust like a coffee machine like you know, it's a vending machine like we want to do an assay everything you just prepare and keep it and 15 minutes of time everything is ready on your table, we'll see it as well.

So with this quick introduction of the company as I said like you know it started in a small garage in 2008 and we slowly spread across the world and we have more than 700 instruments across the world and the best part of this company is like you know you see from 2010 to 2017 we got 1700 publications in all reputed generals, so that is how we had been connecting more to the scientist, it's completely a technology driven company, and it has been estimated that 15 million experiments are done using our technologies till date, so I think this is more than introduction of the company what I can give, and now I'm just coming to the technology, as I said it is a thermophoresis, we have established at a microscale, at a very small temperature gradient we have demonstrated this thermophoresis, this is called the microscale thermophoresis in a short form you call it as an a MST, so but before going into the details I just want to elaborate more on the basic principle what is thermophoresis? We all know what is electrophoresis, so in the,
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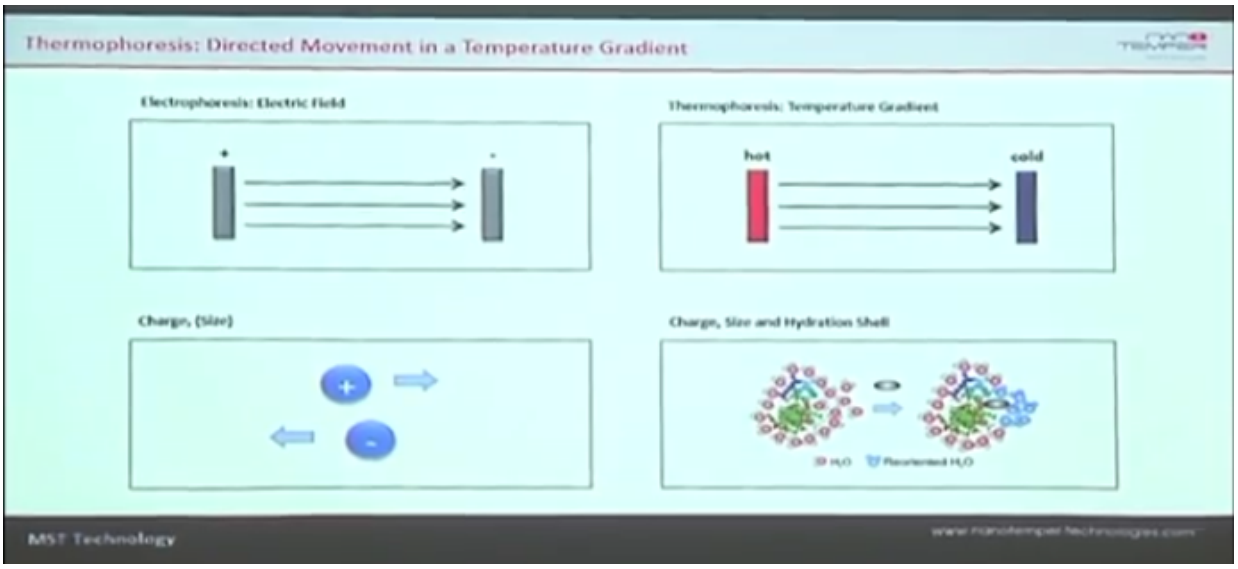

like in the electric field the ions or molecules will move based on their sizes, if there is any gradient in between them like acrylamide or like any kind of a gel.

So similarly thermophoresis is the moment of the molecules in the temperature gradient, thermo means temperature, phoresis moment, the moment of the molecules in the temperature gradient is called as a thermophoresis, but this thermophoresis is sensitive to for 3 things, size, charge, and the hydration shell, so as an electrophoresis you are only seeing the size right, but here this movement is depending on three important things, size, charge and the hydration shell, so if any of these things changes due in the molecular interaction the thermophoresis behavior changes, so that is the basic principle what we have been using it, maybe I'll go more into the detail, size, charge and the hydration shell, so normally when a protein-protein binds the size changes, when a protein DNA binds the charge changes, and when a protein small molecule binds the charge never changes or size never changes, but still one water molecule is displaced upon the binding, it all depends how it is binding and all this stuff, so this technique has become a very sensitive for all this three parameters and there will be a change in the thermophoresis behavior when there is any change in this parameters.

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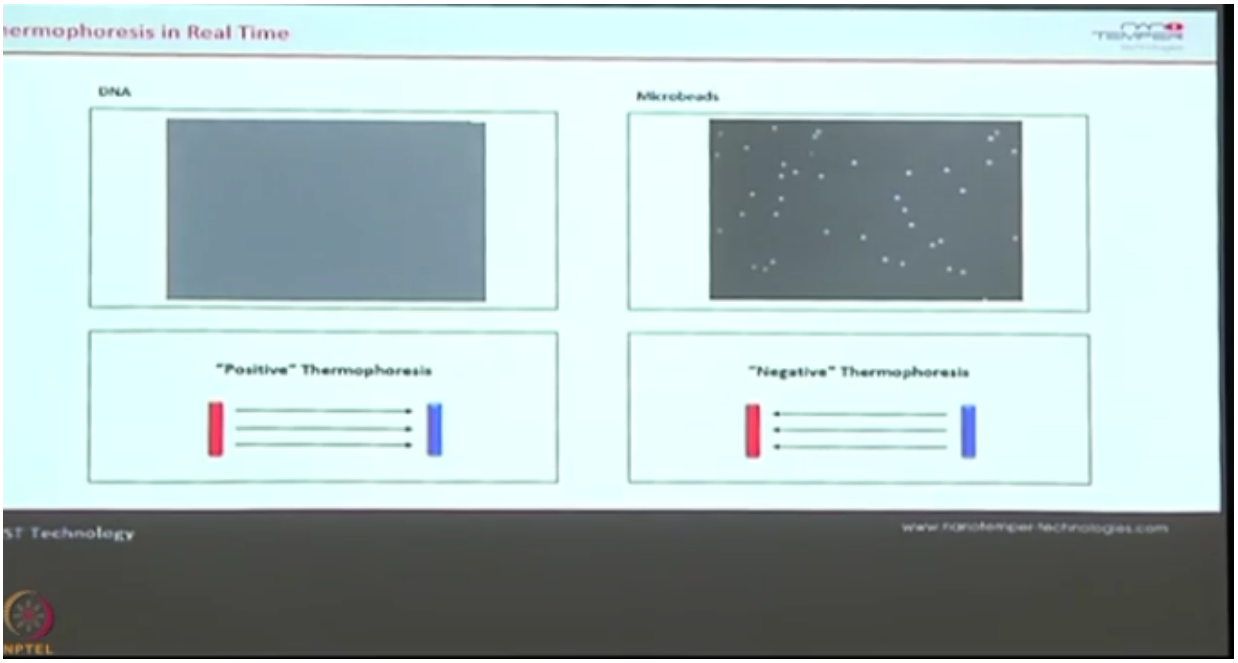

Yeah, so generally as I said like you know thermophoresis, the moment you know you put a heat all the molecules move away from the heat that is the general principle, as you can see here this is just a cover slip slide which has been quoted with LMU, the moment you are shining with a laser all the fluorescent molecules are moving away, so that's a reason why you were able to see the LMU, this is a Munich University, so that is called the positive thermophoresis, the molecules moving away from the heat which is a general property is called the positive thermophoresis.

Is similarly there will be like you know the vice-versa affect what you call it as a negative thermophoresis, sorry, in the negative thermophoresis what happens is the molecule move towards the heat, so it is like example glass beads when you shine some laser or when you heat them all the molecules move towards the heat that is called the negative thermophoresis, so in principle when you are doing a thermophoresis experiment you always see these two kind of a phenomenon like they move towards the heat or they move away from the heat, but in our principle you can measure both their movement, how we measure maybe I can articulate the next slide.

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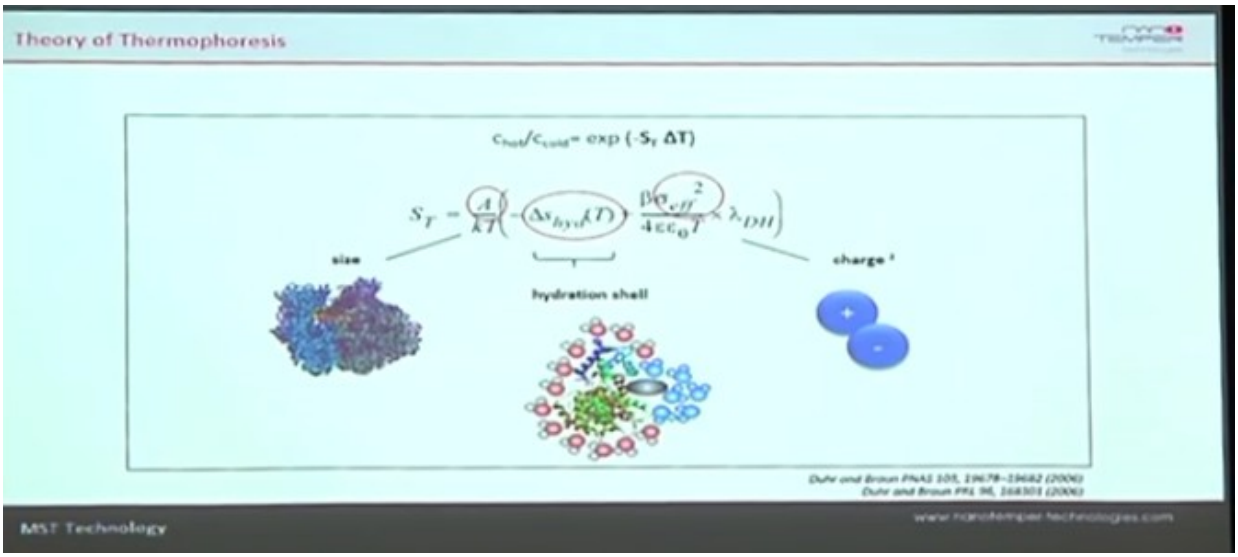

So if you look into the first slide, the two gentlemen who has proposed the theory of thermophoresis they state that the study state concentration of the molecules before an heating, before an heating or directly dependent upon the three parameters that is size, charge and the hydration shell, so as I said like you know before heating whenever you are shining some heat, the movement of the molecules that has gone towards the hotter region and the molecules in the colder region their ratio is always dependent upon these three parameters that is what the law of thermophoresis says, and all our experiments have been highly validated and it follows this principle.

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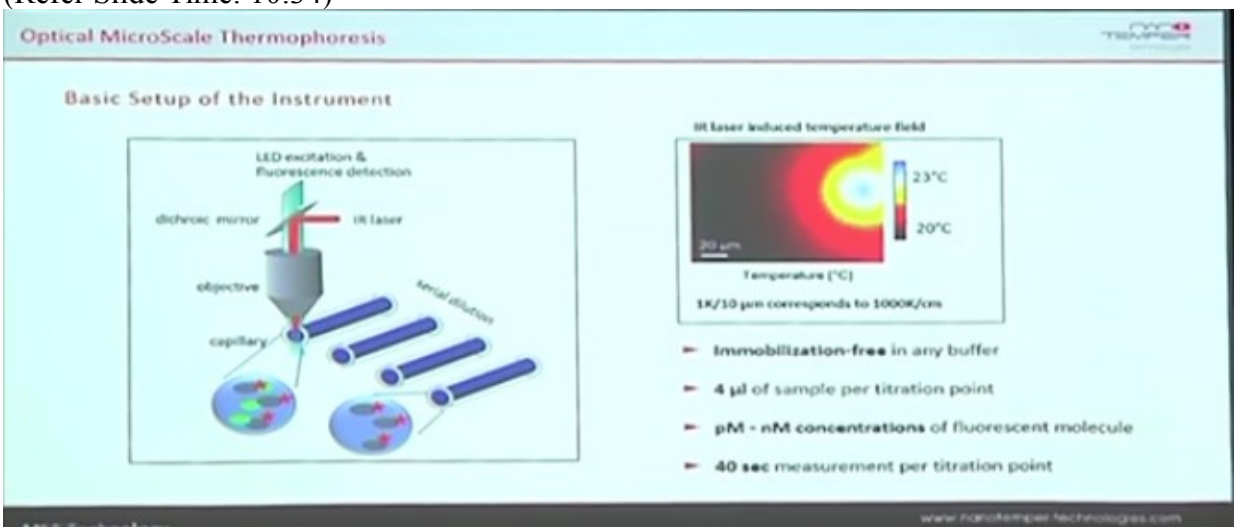

So with this basic principle of thermophoresis we built up an instrument which has two important things one is a laser, which creates a temperature for thermophoresis, and here one of the binding partner is fluorescently labeled, so just to see the fluorescence you have fluorescent detection unit, and the biggest advantage in this technique is you are not immobilizing anything as in other techniques, so you directly determine the binding affinity in the buffer, and you require only 4 to 5 microliters of the sample and the time, so normally like you do a dilution series up to like 12 to 16 dilutions, and every dilution take 40 seconds to scan, so in 12 to 15 minutes of time you are able to determine the time being affinity without any compatibility with

the buffers, there is no any cross torque, there is no any like you know buffer interferences in your assay, there is a biggest advantage of this technique and many people ask the temperature or creating how much is the temperature we create, it's a very steep gradient as you can see in the slide 20 to 23 degrees, so that temperature doesn't affect any of your molecules, so in that way what we do is you fix one of your partner and titrate the second one of a different dilutions, and irradiate with the laser and you see how the biomolecules move away upon the interaction and when they are not interacting, so that difference is will help us to determine the binding affinity.

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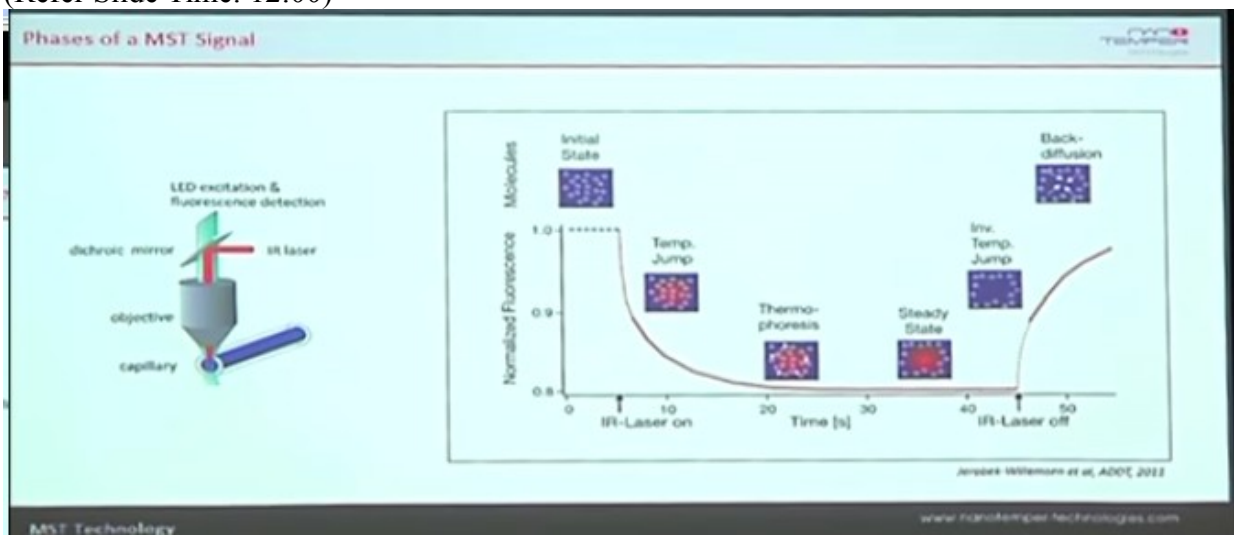

So let's see more in detail in the next slide, see if you look into the typical signal what is actually happening, as I said like you're fixing one of the partner and the second partner you are titrating it right, so on every concentration, on every dilution whatever you are taking it, you are radiating the laser, so as you can see here for the first 5 seconds all the molecules are uniformly distributed, this is generally what is in principle it is there, the moment you put the laser on, IR laser on what is happening is all the molecules are moving away from the heat, and it states that within 30 seconds they attain the study state, so for 30 seconds I'm putting the laser on, okay, so what is happening is all the molecules are moving away from the heat and within 30 seconds they attain the study state as you can see here, and after that I'm putting the laser off, since there is fluorophore again there will be a, like you know regearing of the fluorescence, so again your signal will shoot up, right, fine.

So now I have a sample where one of the binding partner and a different concentration of a ligand is mixed and that has been taken the small glass capillary, and on every glass capillary I'm taking this thermophoresis reading, first 5 seconds it is very uniformly distributed after 5 seconds you are putting the laser till 30 seconds we assume that it attains the study state and after that you are putting the laser off, so in this way for all the different dilutions whatever I'm taking it I'll collect the thermophoresis signal, right.

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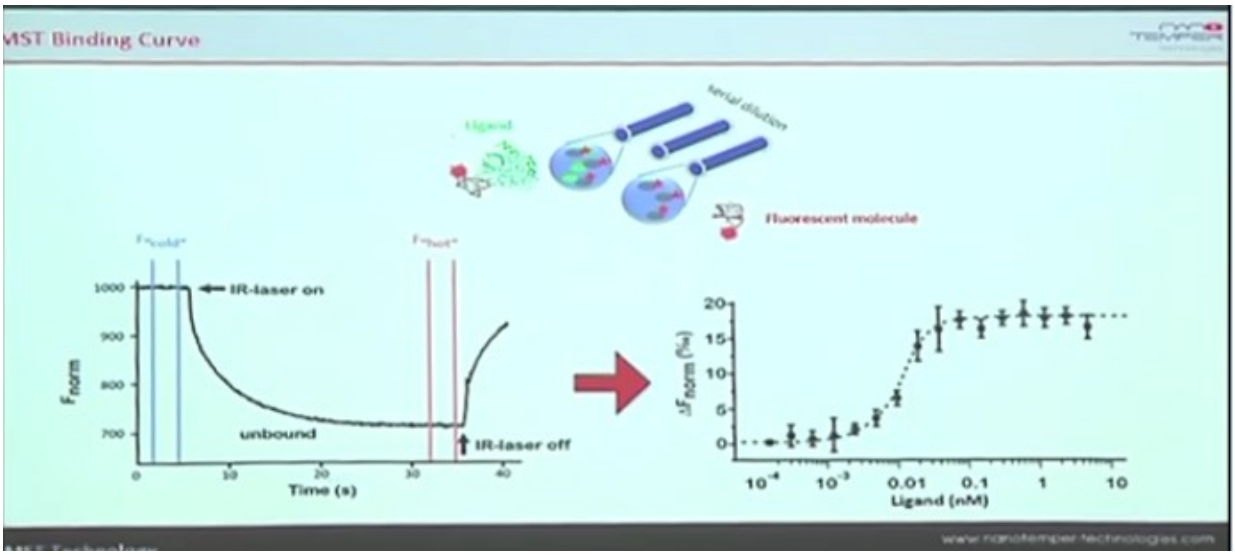

So this is how typically as I said like you know in the top you can see and each and every glass capillary I have a partner along with the ligand which is like you know a different concentration, so now every of the glass capillary is going for the thermophoresis measurement you see here, so there is a change in the movement.

Can you tell why this change is happening? Yeah upon binding it is the size or a charge is changing, so that way the moment is also changing. If the moment is not changing that means that there is no any binding at all, then you have a signals all are overlapping one over the other as the clutter, but here as you will see here, as and how there is a binding the different concentration of the ligand is coming the moment is changing, right, so this is how we track the difference between the unbound state and the bound state, the unbound is where it is not binding, and on the bound state is the where the biomolecule is actually interacting.

So in this way we are actually capturing the small differences in the thermophoretic behavior to determine the binding affinity. So once you determine all the 16 thermophoretic curves for all the different ligand concentrations you just go to the dose fit curve as you can see here, this is the baseline where typically there is no binding and here there is a saturation, and the midpoint is generally as a KD is what we call it as a binding affinity, so where your molecules are half bound and half unbound is call your KD.

So typically by just extracting the data from the thermophoresis curve we are fitting the dose fit curve to get a KD value, so this is the basic principle what it is underline in the instrument, just by the thermophoretic behavior changes from the bound state to the unbound state you are able to determine the KD, since this technique is very sensitive for size, charge and the hydration shell we always get a differences in the thermophoretic behavior which will help you determine the binding affinity. The biggest advantage is you are taking a smaller volumes and you have no interferences with the buffer and there is no any limitation with the sizes, so this is the basic principle I'm sure like you know if you have any questions you can stop me here, the rest are all like you know how they examples like you know how protein-protein interacts have some good data I want to show it to you, so this is the basic principle how this instrument works, and how we did in the binding affinities.

So with this you see a wide variety of samples from ionic interactions to the liposome interactions, nanodisc, any kind of interactions can be studied in this instrument,
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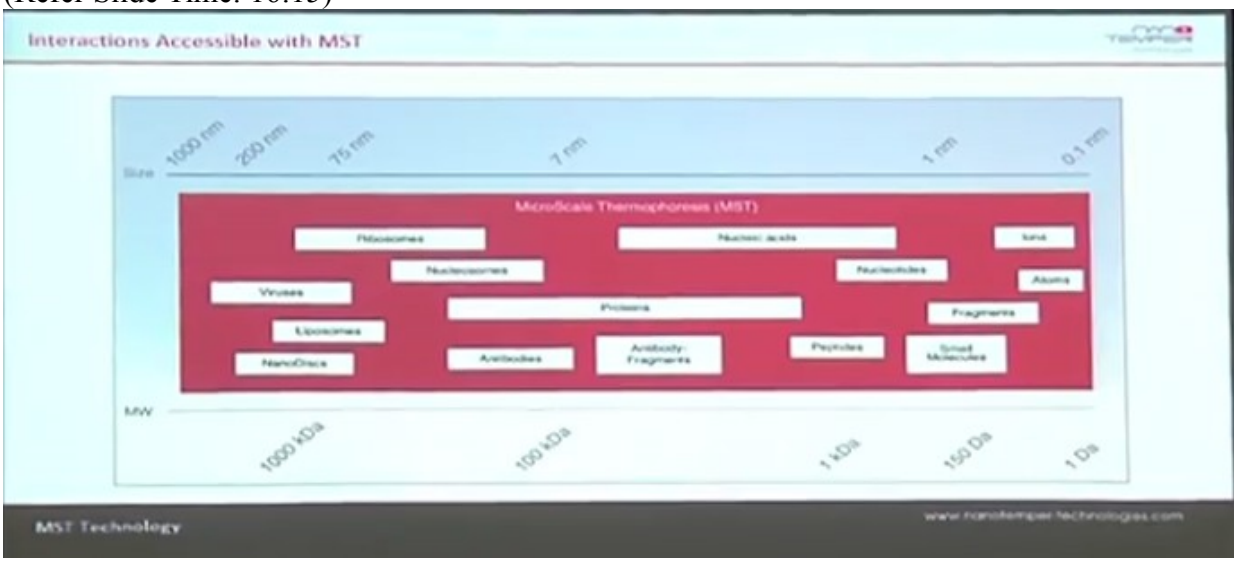

so there is no any limitation of size here, there is no any limitation of any of the properties of the molecule here, so anything even like ionic interactions you can imagine there, ions are almost in a daltons, and even if you know liposomes or ribosomal complexes they are in megadaltons, even if there is an interaction between these, these things still the techniques work very nicely.

As I said like you know we got 1700 publications in the last 5 years we have got all the supporting data with all different kind of interactions we have, so if you are working on a specific like protein-protein, protein small molecule we can still discuss, I can help you out like you know how we can do the assay another stuff, so this is the main use free of this technique without any limitation of the sizes you can work with the wide variety of the samples to get the binding affinity.

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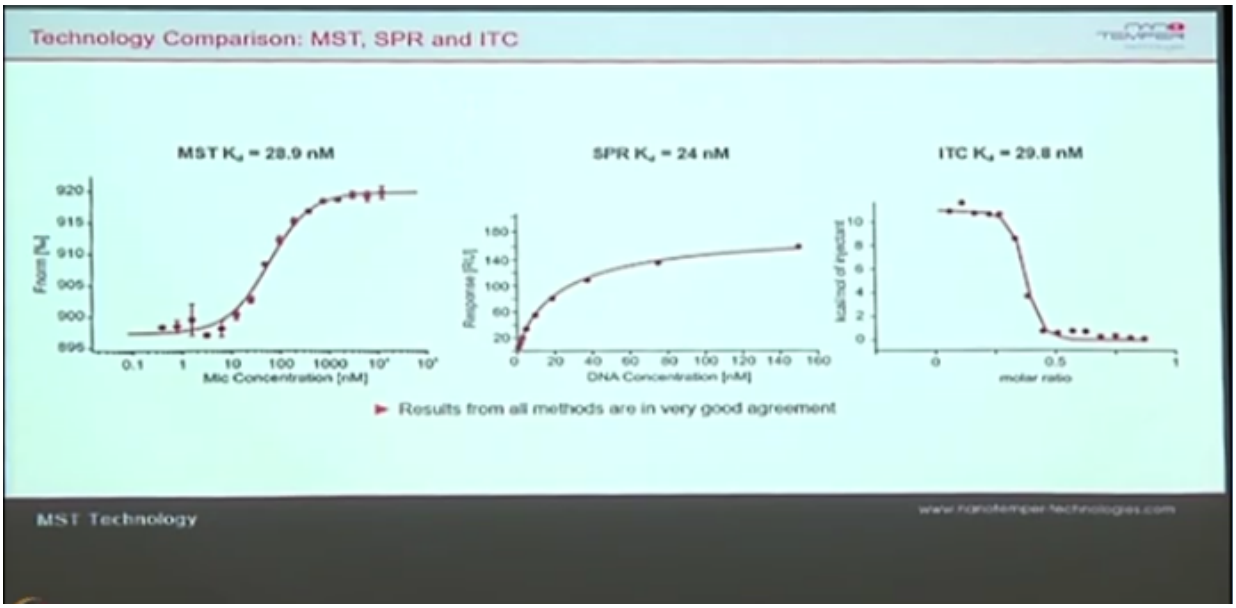

So typically this technique is comparable to the SPR, have you ever heard of a SPR? I think many people might be using the SPR as well, so this technique is a very much comparable to the SPR and ITC, isothermal calorimetry, so in the chemistry people will be just measuring the heat change, heat exchange driven the binding affinity.

So the biggest advantage over all this techniques is as I said like you know we did a protein DNA interactions and you see in all the three techniques you are almost getting the same KD, we got 29 nanomolar, SPR 24 and ITC got 30 nanomolar, so whatever the binding affinity determining the MST is very similar to the ITC and SPR assays, but what is the biggest advantage?

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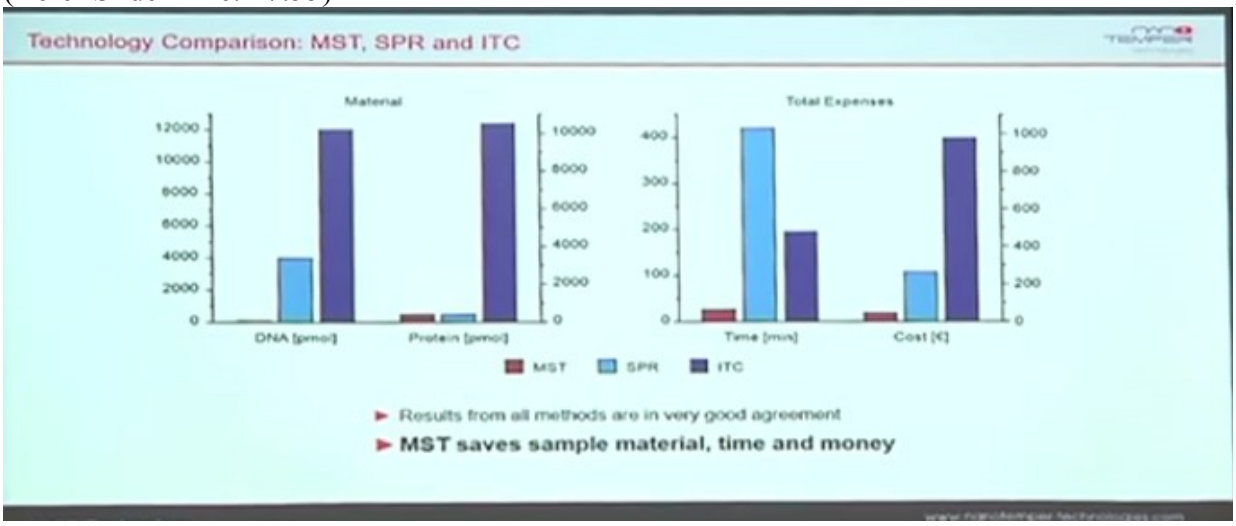

So as we all are familiar with the SPR you always have a chip, you immobilize one of your protein and pump your different buffers and ligands and it involves a lot of method development and method optimization and of course the cost for every assay.

And in the ITC we know like you know you have a huge pump and then you need to fill your pump and then start putting it up, so they are higher volumes of the sample is required, so if you look into the biggest advantage of the MST is you don't require the higher volume of the sample we look with the always the lower concentration of the sample, and the assay is done in 10 minutes of time with no cost, the glass capillaries are only the consumables what we use for doing the assay and it will cost you 5 euros per assay, so at the end of the assay if you look on like to perform one KD measurement you require 5 euros, so that is the biggest advantage, the smaller volumes, time and the overall cost of the experiment, so this has been the highly like you know without with all the three experiments as you can see here the cost you know, it's almost like $1/10^{th}$ is what we generally do it for an SPR, another assay experiments.

So maybe people working with SPR still might have some questions you can still feel free to ask me, so as I said like you know we do a labeling, one of the binding partner so we recently came up with the kit where you can label you're his-tag, so the sixth history in position you can label your protein,

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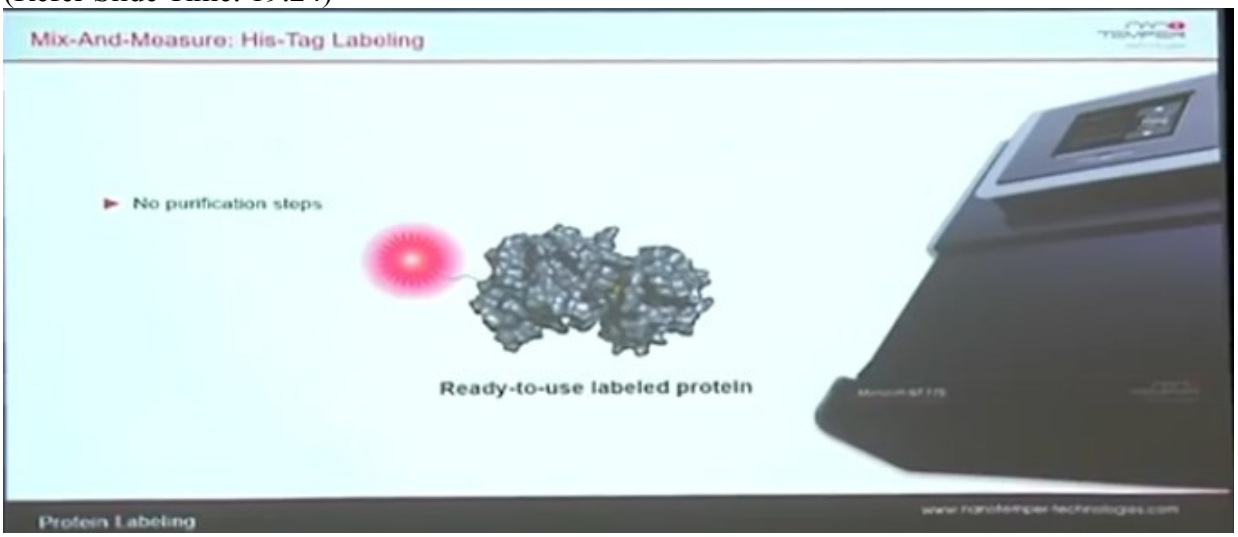

so in such a way what is the biggest advantage is like you know you need not purify your protein, if your protein is in the cell lysate and it is already his-tagged, just simple add the dye and do the titrations, so in that way even in the cell lysates if you want to have a specific interaction studies this can be successfully done.

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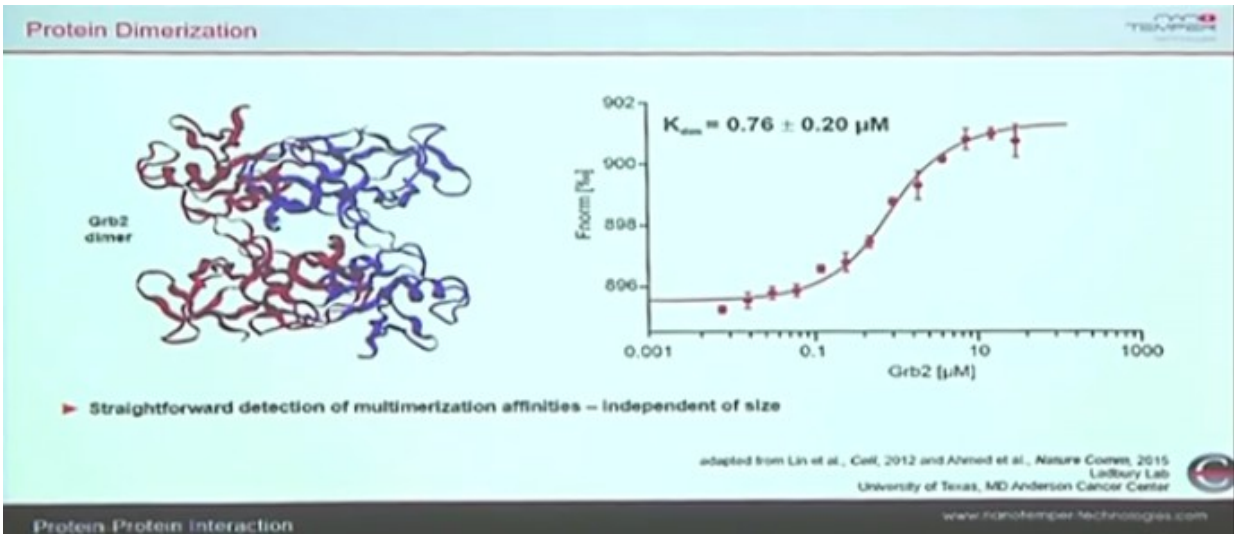

So now I'll just take it to some examples of like you know how this application has been used in a different variety of interactions, one is the dimerization, so when you are talking about the protein-protein interaction the same protein when it interacts it becomes a dimer, so it's extremely difficult in other techniques like maybe in SPR or ITC, when the same protein you immobilizing and same protein you are running through you get a lot of artifacts, so but here labeling one of the protein and titrate in the same protein you can determine the dimerization KD as well.

As you can see here you can clearly see a clean fit of KD coming up here, so even the protein dimerizations and protein dimers can be easily studied using this technology, and other most interactions people are interested is protein nucleic acids,

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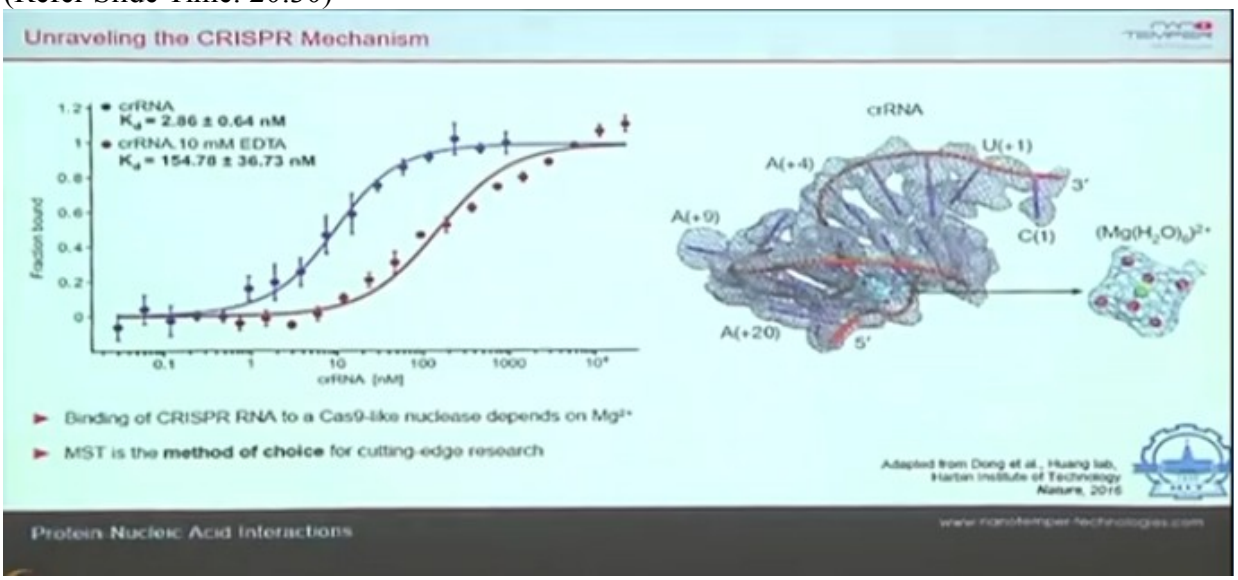

so I'm sure you are well aware of this Chris Packer Mechanism, so here the RNA to the nucleus protein has been studied and this paper hit the nature and here in this paper they quoted that this is only biophysical technique that has been to determine the binding affinities, so even the cutting edge technologies also MST has become much handy tool to determine the binding affinity, so maybe traditionally people does EMSA,

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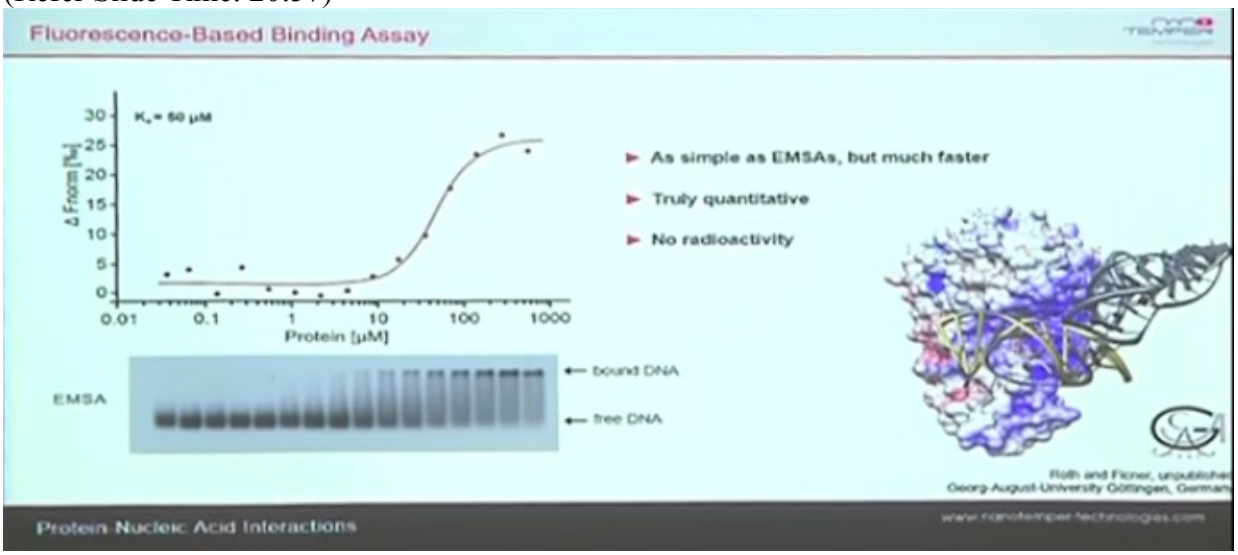

so have anybody done EMSA here? Great, so you know like what is a pain of an EMSA there and you always get a qualitative, you never get a quantitative information there, so in the same studies whichever done with the EMSA we tried to label the DNA like a sai 5 or sai 6 or whatever you have the dye and then titrate your other things and you are able to determine the KD which is very much similar.

So with a smaller volume of the sample you are quantitatively determine the KD over this, all this EMSA and radioactive labeling everything is all been like you know overlooked when you do these kind of assays here, so other example is a protein ionic contractions, these are always have a very special interactions, as I said like you know when a protein and ion interacts there is no size at all, there is no any size making any difference, the ion is a very small in size and your protein is a very big, so that time the size doesn't change at all, but still when a protein goes and binds there is a displacement of 1 water molecule at least outside the protein or anything, so even with this small change we are able to determine the binding affinity, this is the biggest advantage as you can see this is 1 calcium binding protein which has been done.

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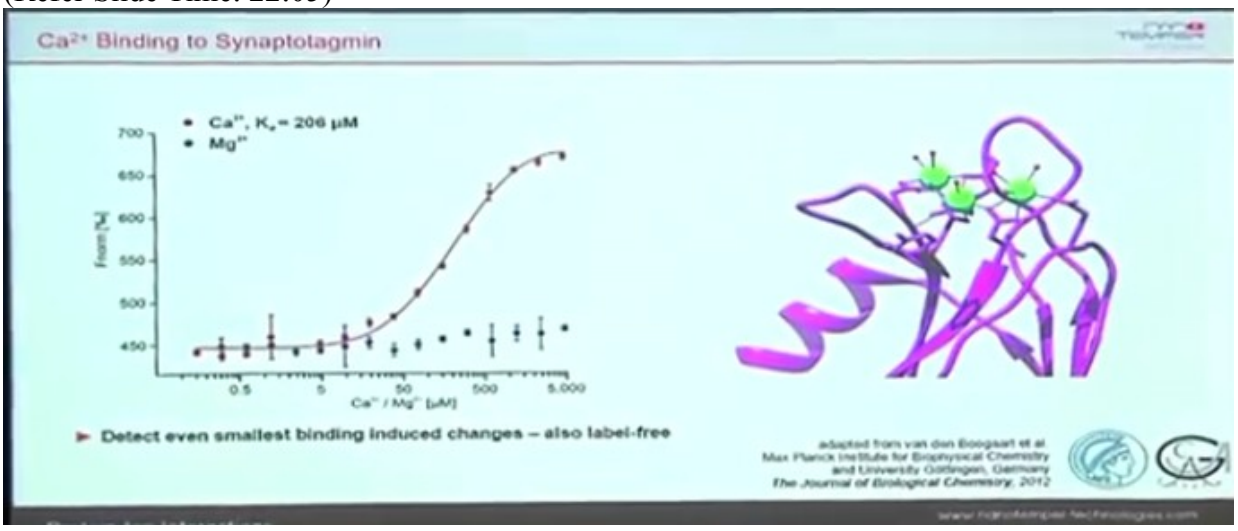

And when the same experiment was done in the presence of the magnesium there was no binding as a positive and negative control this experiment has been done, and as you can see here this is from the Max Planck Institute, the person who has developed the ITC who is like you know known to be the father of ITC, so always when you are working with the nanomolar, picomolar interactions and a very small size molecules, this addresses very nicely over any other techniques.

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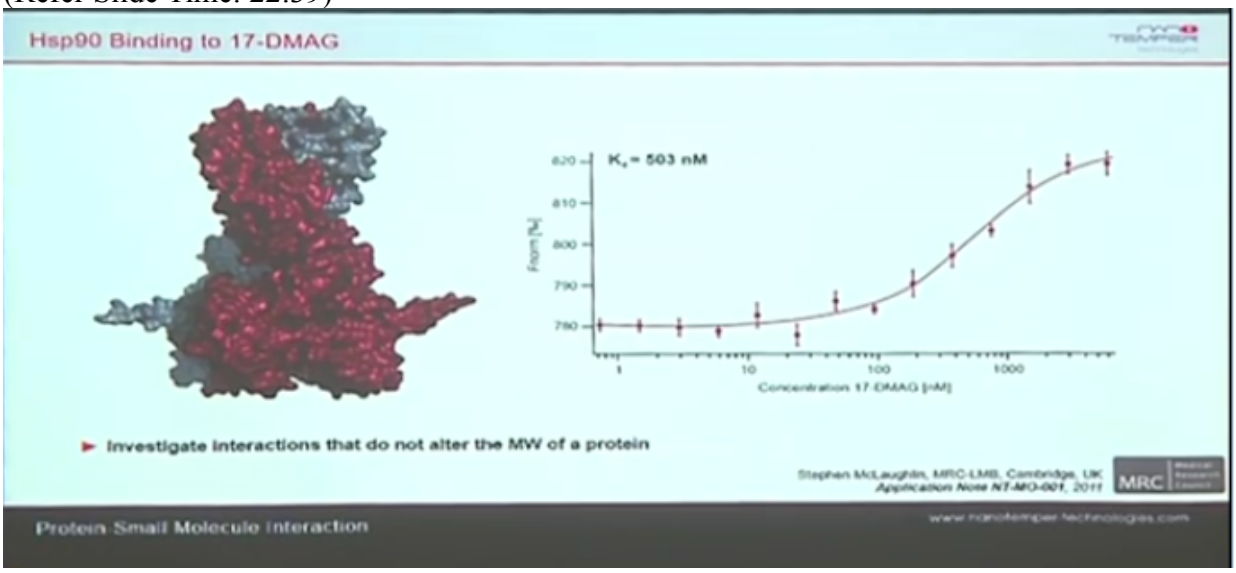

So again this is like you know a protein small molecule interaction, here you have a ribosomal protein complex in the red colour which is like you know few megadaltons, and a small molecule which is about like you know a 120 daltons, the cancer drug, so when it binds the size is absolutely not like you know making any difference, like if you know imagine like megadalton protein and 120 dalton small molecule interacts, the size doesn't change at all, but in principle if you look into this structural information like you know the water molecule is actually being displaced which we call it as the hydration shell because of which we are determining the thermophoresis change and the binding affinity.

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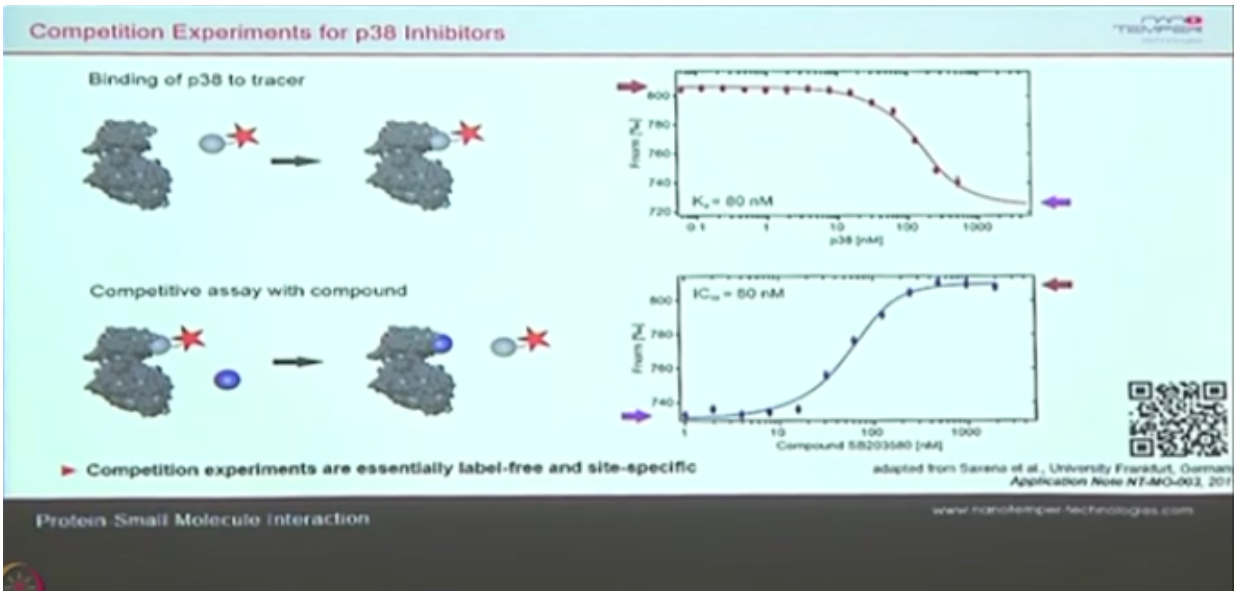

So and other experiments many people who are working with enzymes, they might be more interested with the like this kind of what you call that? Inhibitor studies, inhibitor screenings, so generally as you know like you know you do a binding with one specific, what you call this? The tracer molecule, and once you know the KD of it and again you titrate the second one, so wherever it displaces then you'd always know the binding like competitive assay experiments can also be easily studied, we have a well robust softwares to support, all this kind of studies at the end of the day,

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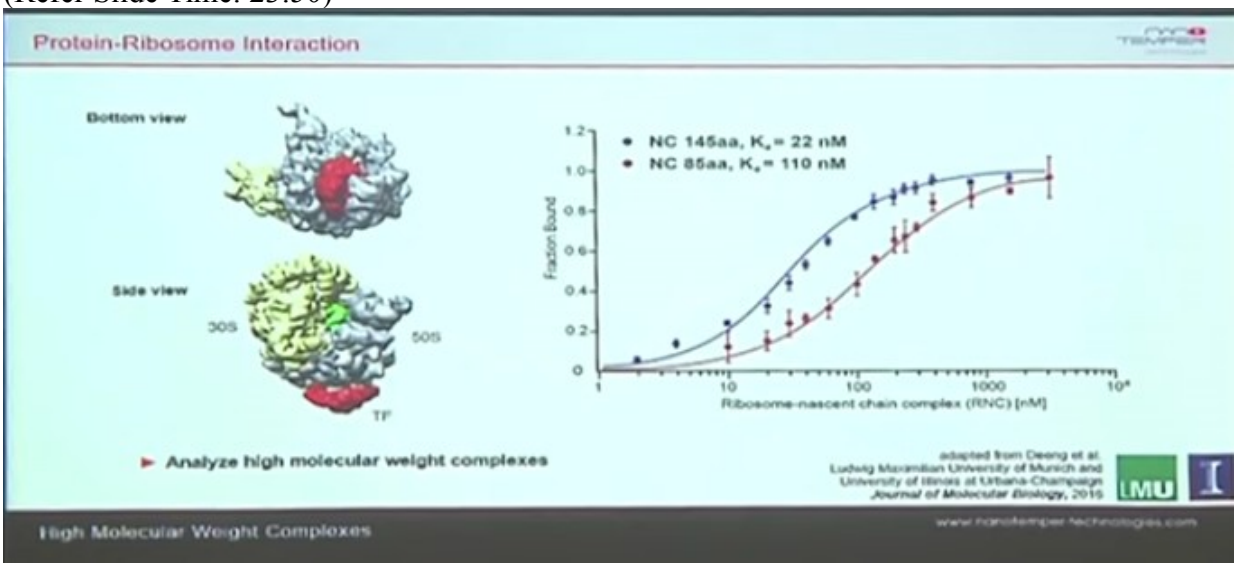

so this is again a same example with the high molecular weight protein complexes as I said like you know few megadalton proteins when in fact with the proteins still you are able to determine the binding affinities very easily.

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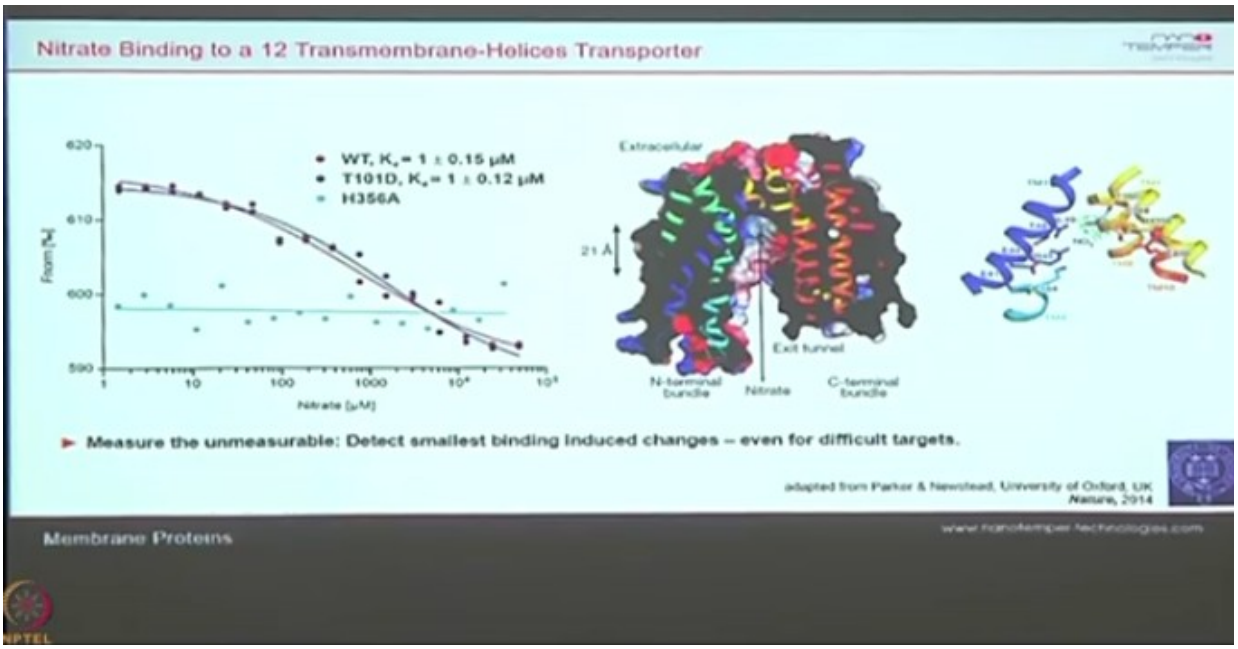
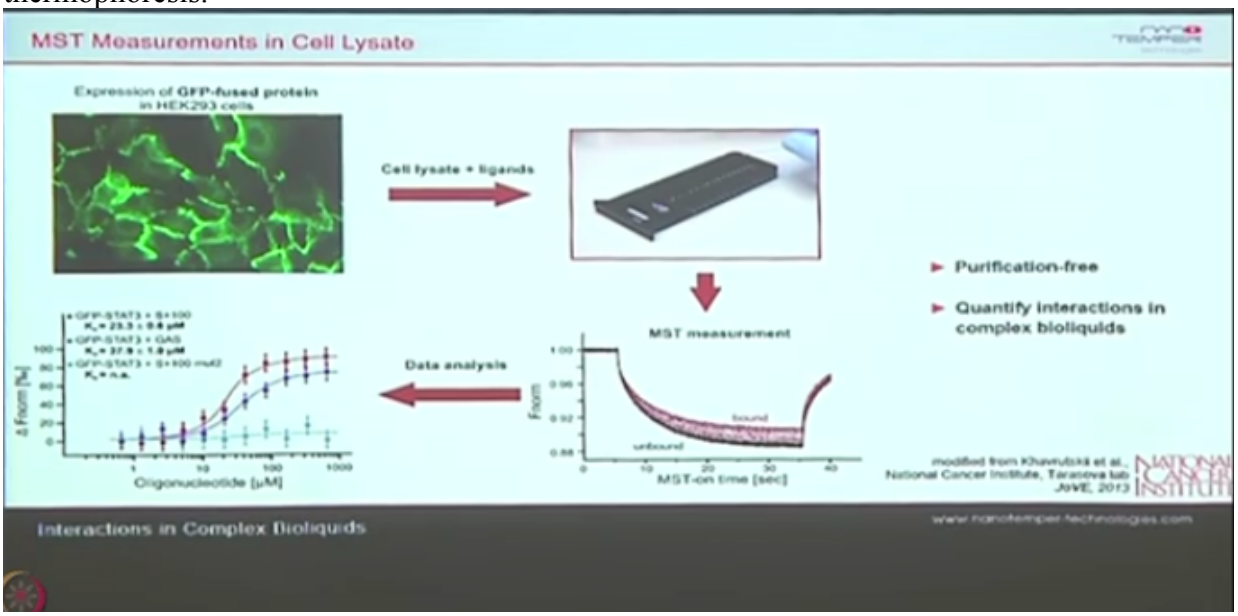


And the other challenging thing is like if anybody is working on the membrane proteins, hope you know what is a pain of like making the membrane proteins more stable, it requires a detergence, so any other techniques like you know when you have a detergents you always have an a buffer incompatibility, for suppose if I have an STS, if I have some any like a foaming agents in your things, then obviously you get lot of artifacts, so here in this technique as I said like we are using only 3 to 4 microliters of the sample that thing can be nullified, so even membrane proteins can successfully be studied here, as you can see the paper again hit the nature where the membrane bromeprotein has been expressed with the GFP and that particular protein has been studied for the microscale thermophoresis, so in this way any variety of sample, it need not be protein-protein, any kind of molecular interactions, protein-carbohydrate, protein-ion, anything, anything between them can be studied using the microscale thermophoresis.



As I said this is one example where the cell lysate, like when you are working with the biological fluids like maybe a serum, cell lysates, you have a thousands of protein, so you can even bypass by doing them a, not doing any purification, if your protein of interest is always with the histac you are the dye which is very specifically binds to the histac and then titrate the other partner, so in this way even for business markers many people working with the proteomics are also using the MST as a complementary tool to screen the different samples for the analysis here, so this is again just the example like how we get the data here, so as I said like you know any one of the binding partner should be labeled, it can be A or it can be B, there is no a mandate that you know you should label only a protein, you can label anything, you can label a small molecule, you can label a protein,
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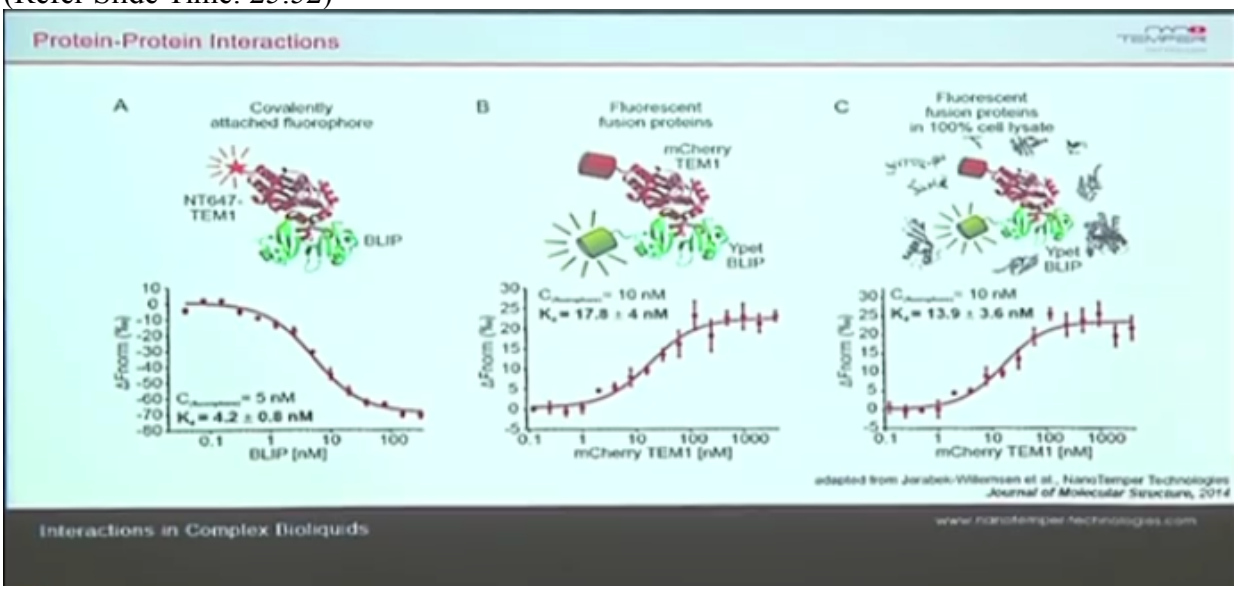

any partner can be labeled, but the binding affinity doesn't change at all, so that is the main USP of this, so this has some examples what I want to tell for a variety of interactions, how this techniques work so successfully, so any questions or any other things you wanted me to know or I shall be glad to address.

So now just as I said you are aware of the principle how we does it, and you know what variety of applications can be done on this, now I'll just quickly take to this software like you know how this instrument like how it can be operated and all this things, maybe in a nutshell I just want to tell this software is an artificial intelligence driven, so that means that the moment you set up the assay,
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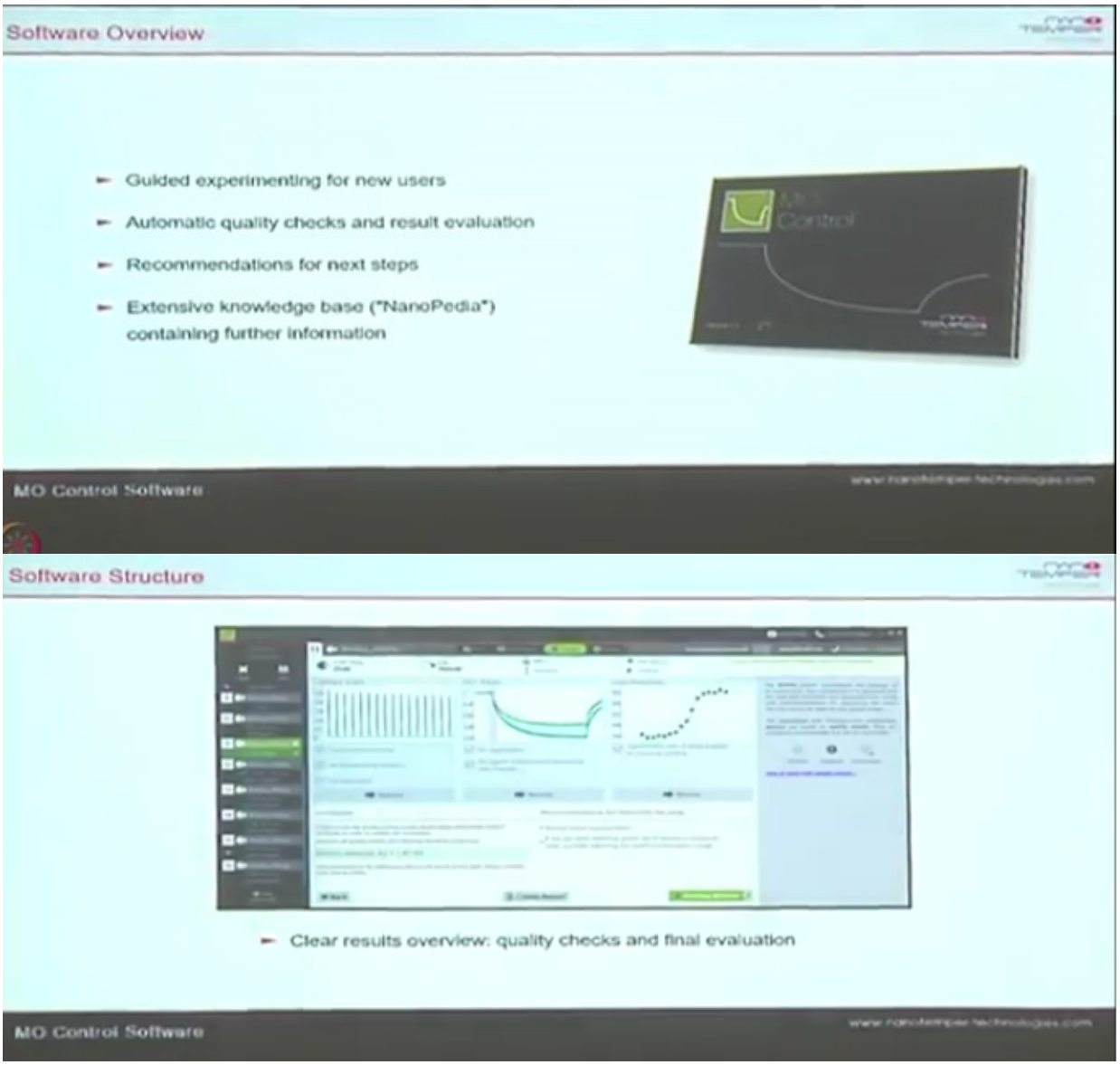

it runs on its own and once the analysis is over the software automatically assesses whether this data is good enough or not, is there something like you know still you are not getting a proper results or because of any reasons it will automatically give you a suggestions, okay, if this is not a binding fit then it will tell you, you please change the buffer, you change the PH or you change your like you know the protein concentrations, so like this automatically software does the assessment, so in a way it is very much easier, of course like you know as a user we always know technically what is happening with the software by looking to the data we can always assess, but still software will help you to make a judgment on your analysis, and you always get all the data in a single page as you'll see here, this is a clean binding fit, so and these are all the thermophoretic phrases of all your dilutions what you have made it.

So for suppose like you know your samples are making an aggregates, so for suppose you know upon particular concentration when it is interacting with the ligand it becomes an aggregates, so then aggregates means then the sample will be nonhomogeneous, the thermophoresis behavior also will be vary like random, the curve likes you know looks very, very random, I'll just show

how it looks like, see, sorry, see, whenever there is an aggregation, see you see here the curve is completely like you know a wavy,
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so like this qualitatively we can always judge the aggregation of your sample also, so in other experiments when your sample is aggregating, it is already in contacts with the instrument, it spoils the next run, it spoils the next things, so the biggest advantage here is you are using a different independent capillaries for every dilution, so if one capillary is having an aggregate you can just discard them, and you know at what concentration your protein is actually aggregating, so in this way qualitatively you can judge the aggregation in your samples by using this kind of a technique.

So maybe all this things I don't want to bore you much because as I said like it's more for users who are actually doing it,
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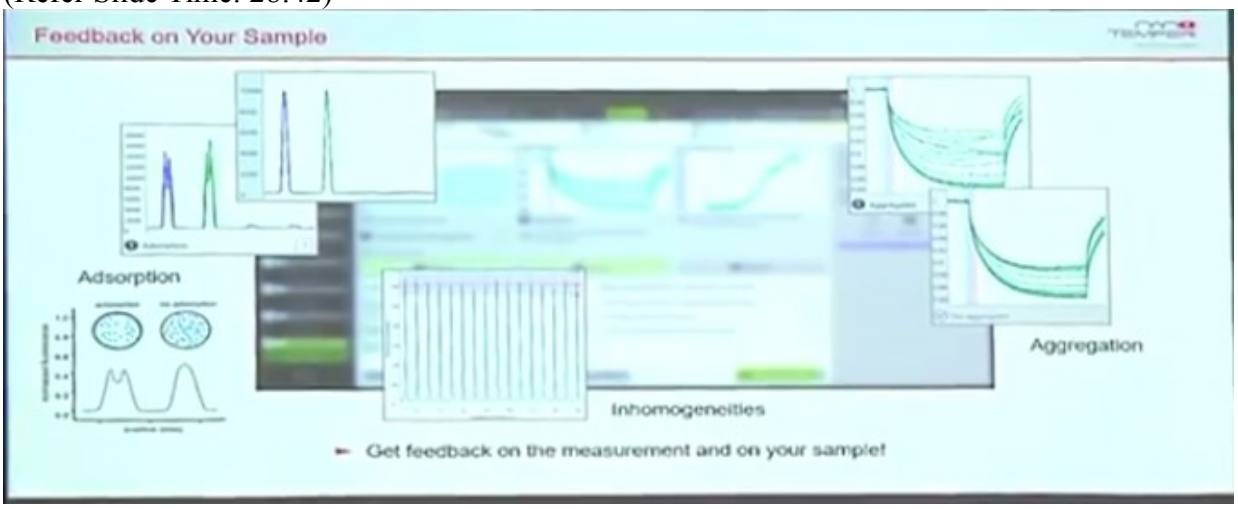

in principle the software is completely a artificial intelligence driven, it gives you a suggestions at the end of the day like how you need to move on.

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So this is the, I just want to summarize quickly, we are already associated with the top premium institutes across the globe and I'm very glad that in India we are associated with the IIT Hyderabad, RCB, Novozymes, and many other customers who are using this technology.

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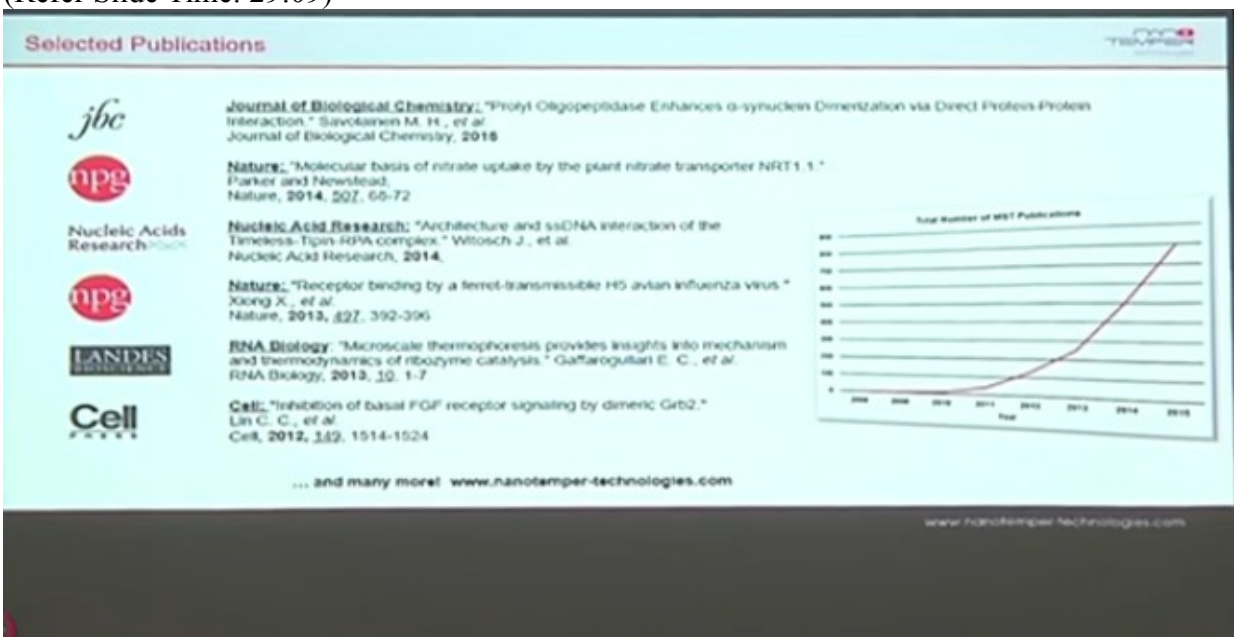

And as I said like you know in all kind of generals you can find the MST papers like in nature we have more than 15 papers, science, all the best generals have acknowledged this is as a biophysical tool that actually be very useful tool in measuring the binding affinities,
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so he has some customers who had been using a lot of SPR, ITC experiments and now made a shift to the techniques and found how easy it is, like if you are doing any screening, like anyway you are doing a drug screening you have a small molecules, libraries, hundreds or thousands of molecules, so I am sure like you know other techniques will be a nightmare, so here since you are using a 10 to 12 minutes of time you can always screen them very quickly.

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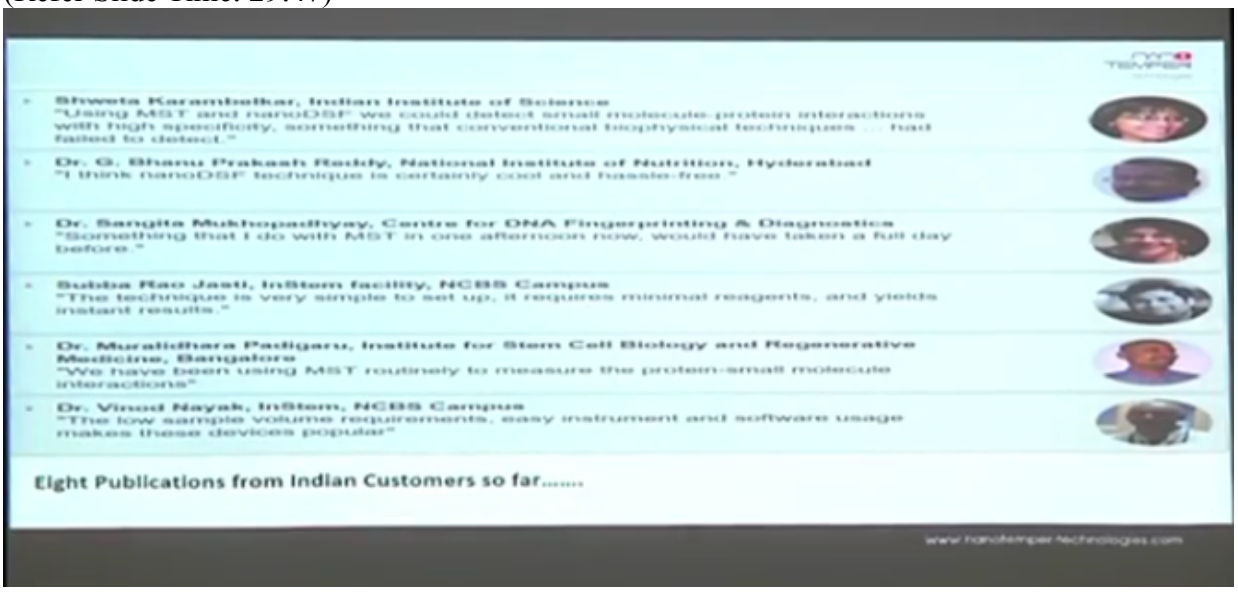

And these are our some customers who had used it and gave a very good feedback and we have more than 10 publications from Indian customers in the last 24 months.

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The final slide is always what I say, as I said in the beginning it is a company of scientist, so along with the, it's not just telling about the instrument, it is all about giving a good support, so you're always bundle with a good scientist to support you and develop the assays on your own, so that's all I want to tell, since we already are going to give a quick demo, maybe like you know we can just quickly share some questions you have or if you are working on any other things, what are the challengers you are facing I shall be very glad to help you out.

What kind of samples you do on SPR basically at the moment? ITC, okay, so for sure like you know as I said like now we have a small positive control kit, it is a DNA aptamer at a small molecule, ATP basically ADP and the DNA aptamer, so anybody is interested they can just come in front and then just do it because as I said like this doesn't required any much training, much thing to understand, as I said the basic principle is sophomores, and how the every dilution there is a difference is what we need understand, and rest everything is done by the software automatically.

See that's why I'm saying in principle anything is possible, alkaloid can you tell me the example, curcumin, so we recently I think as I said like 15 minutes of the time is a measurement time so maybe people can come front and this volunteer or you can sit there and see however you wish, so we have a control where we are just going to load in to the capillaries and do the like a MST assay, and we will go to this one, the stability 1, so there also the things are ready, it takes only 3 minutes to measure the stability of your protein that is the biggest advantage, so maybe she will give a small talk on few slides, what is the basic principle and what is the applications.

So anybody working with the protein can use this instrument for quickly checking like you know the functionality of your protein, whether your protein is really functional or not, okay, so this are the glass capillaries what we use for the assay, you can see this as a simple glass capillaries which can accommodate maximum of 5 to 6 microliters, you just dip into the solution, so normally you take a PCR steps and then like you know fix your partner A, and titrate the ligand, like then only like 12 to 16 dilutions is what we prepare, and all the 12 to 16 dilutions, each dilution you take into the glass capillary, so on this glass capillary we'll go for the measurement.

So and here you have, already like you know we already mixed that DNA aptamer and ATP here, so what I'm just going to do is now you know, I'm just dipping the glass capillary and then loading it, so it's all like once you load it within 10 minutes of time you can do it, so normally you know proteins are very like you know unstable at a room temperature, so the DNA has been used, so DNA is very stable here, so for the generally for demos I prefer to do with the DNA because it's most stable you can carry anyway what you wanted, yeah, so this is the tray which holds your glass capillaries, so it's like label 1 to 16, so like whatever you are taking it you just try to put into this and then do it.

And we have a small magnetic strip 2RS to glass capillaries, it's like you know when you are loading it they might not fell down, so it's just a magnetic strip to arrest the glass capillaries when you are loading it, that's all.

So maybe I'll take two more minutes to load, and after this we can just start that, yeah, yeah, somebody want to load they can come and load, you can load it no problem, as a protein purification like columns or anything like that, so how you maintain the quality, how you check the quality? That is nothing but the stability or the functionality, so we have recently launched this instrument only not even a one month old is this, so we can quickly check the protein quality, how, what is the basic principle of this and what kind of applications, she will try to summarize and give it in this 15 minutes of time, and after that that instrument also you can give a demo, it takes in principle only 3 minutes to run one like run 6 samples, we got some controls, we can quickly run that also, Swathi please go ahead.

Swathi:- So have you ever asked to yourself that whatever protein you are purifying, whether it's pure or not,
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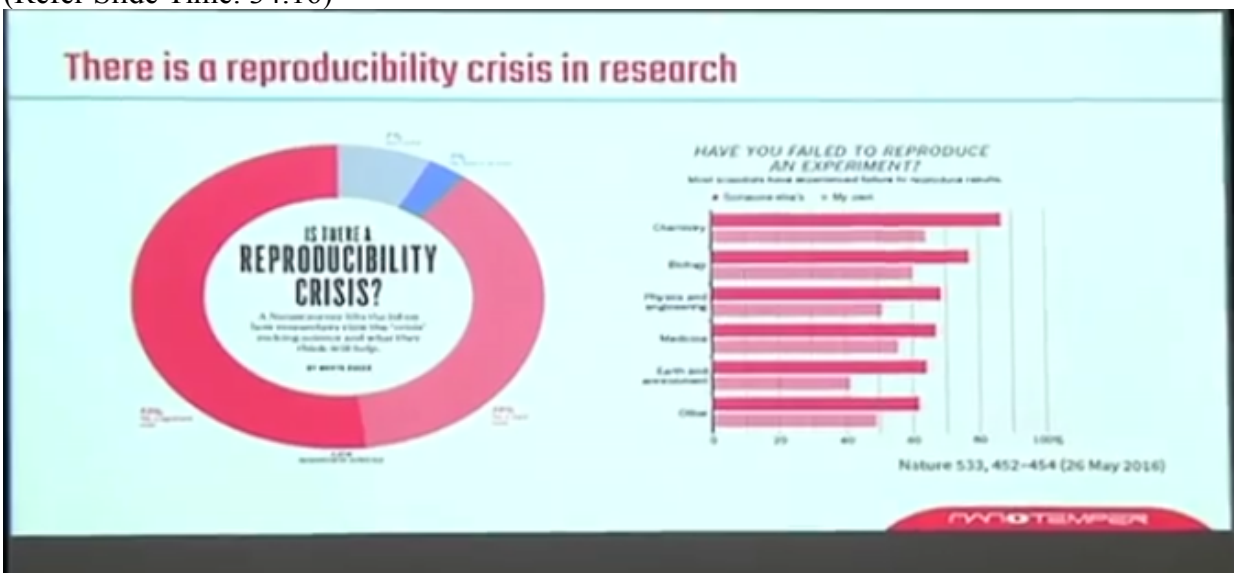

I mean whether that can be used for your future assay experiments or not? So that's a very big question that always comes in the mind of any researcher, so before going for any assay what you do is you run a column, you do a SDS page, or you do spectrophotometric analysis.

So these are like very started gold methods, we are going for a spectrophotometry or electrophoresis or a column chromatography, but still you are not aware whether your protein is a functional protein or not,

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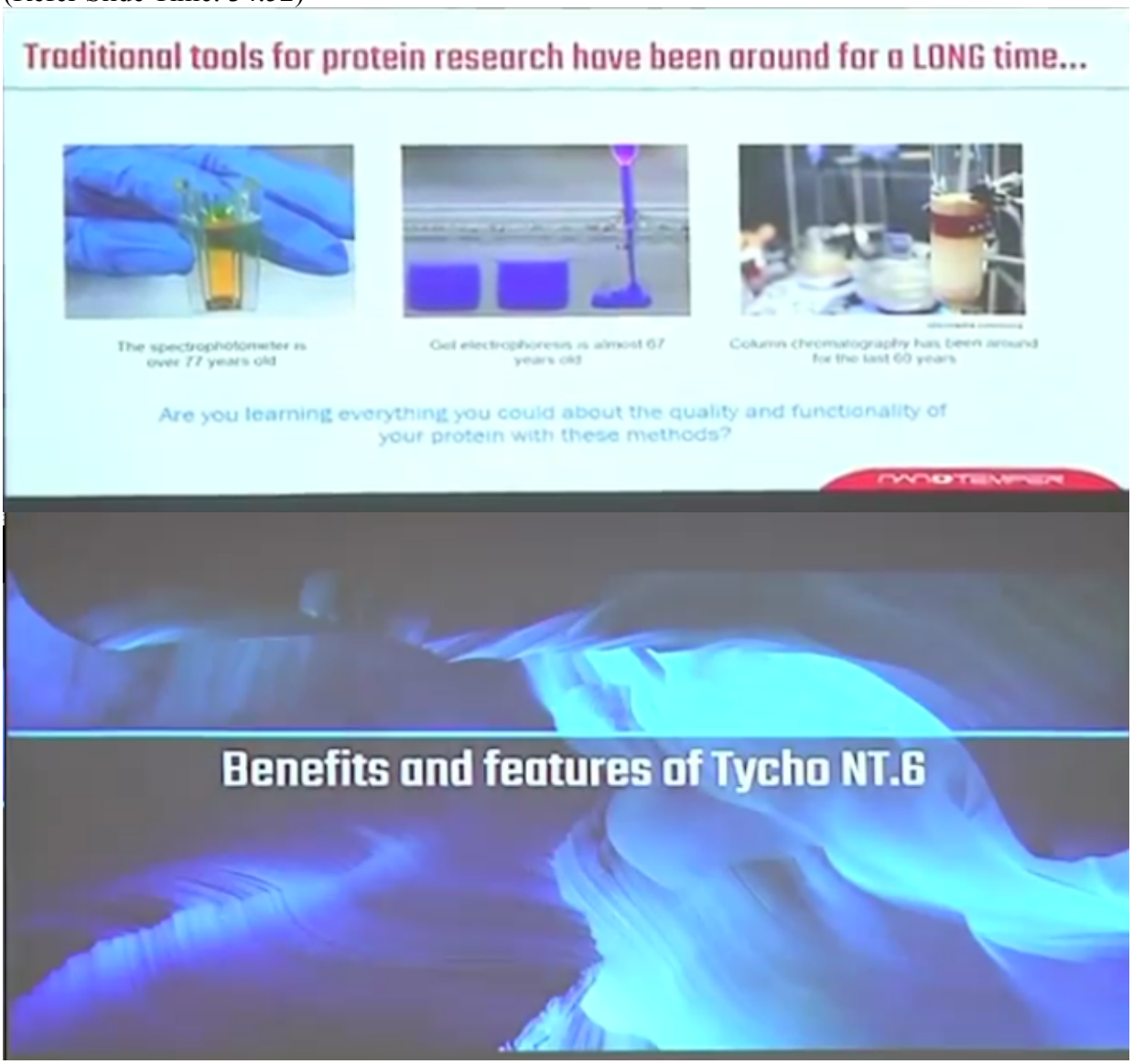

so we have this newly launched product which is known as Tycho, and in one go it takes 6 samples and you can actually check the quality of the protein, so it will come to know whether the protein what you have, you know, purified is functional or not.

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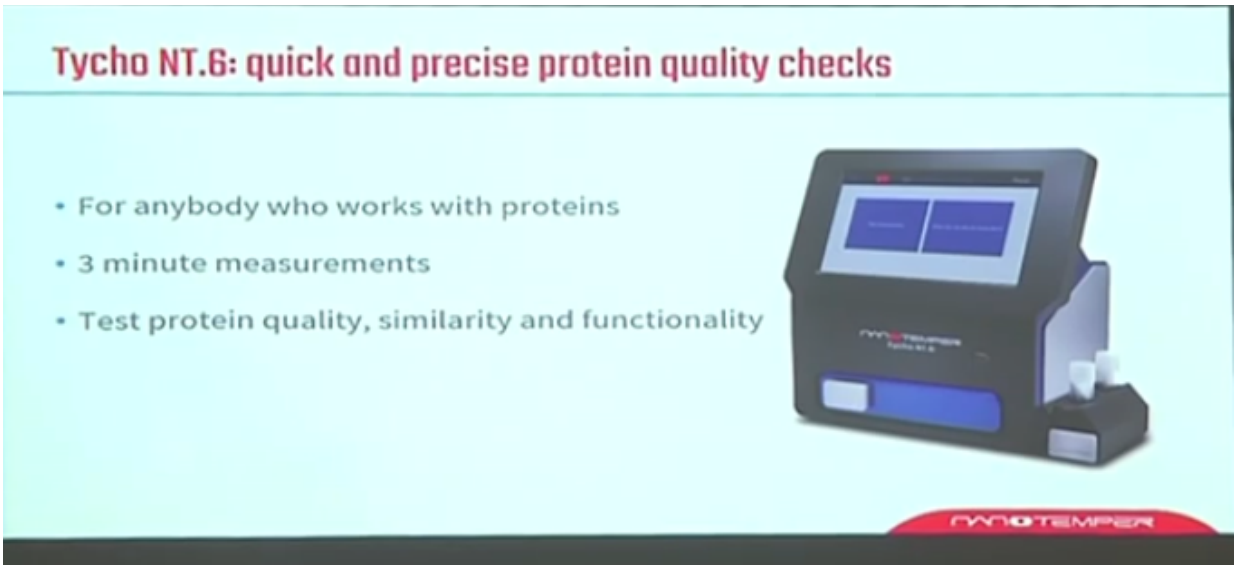

So what's the basic principle of this machine is that it can work with any kind of protein, whether its antibody or whether it's a normal protein, membrane protein, receptor protein, any kind of protein and it give a 3 minute measurement, so within 3 minutes you are actually checking your quality and the purity of the protein, so it's a label free measurement, like here one of the partner is labeled,

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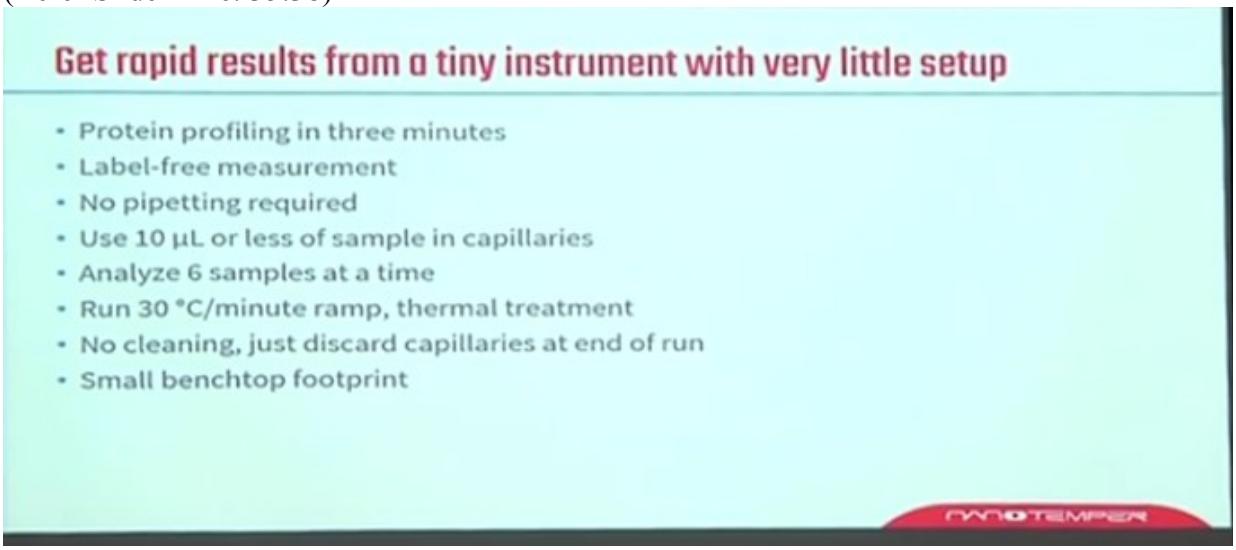

we are not labeling here, it's the intrinsic protein fluorescence that we are checking which will give you a label free, what you call inflection temperature which is very equivalent to the melting temperature.

And you are similarly using capillaries and the amount of all, the volume that you are using is less than 10 microliter, and in one go almost 6 samples you can analyze, then the run-rate here is little fast because you are analyzing, you know, in 3 minutes, so the run-rate here is 30 degree per minute, and as of our all the instruments are maintenance free, there is no cleaning, you are just putting the capillaries in the instrument, taking off once the run is complete.

So what are the basic benefits here is that it's a faster measurement technique and it checks the purification and the, I mean it checks at the purification and the characterization levels,
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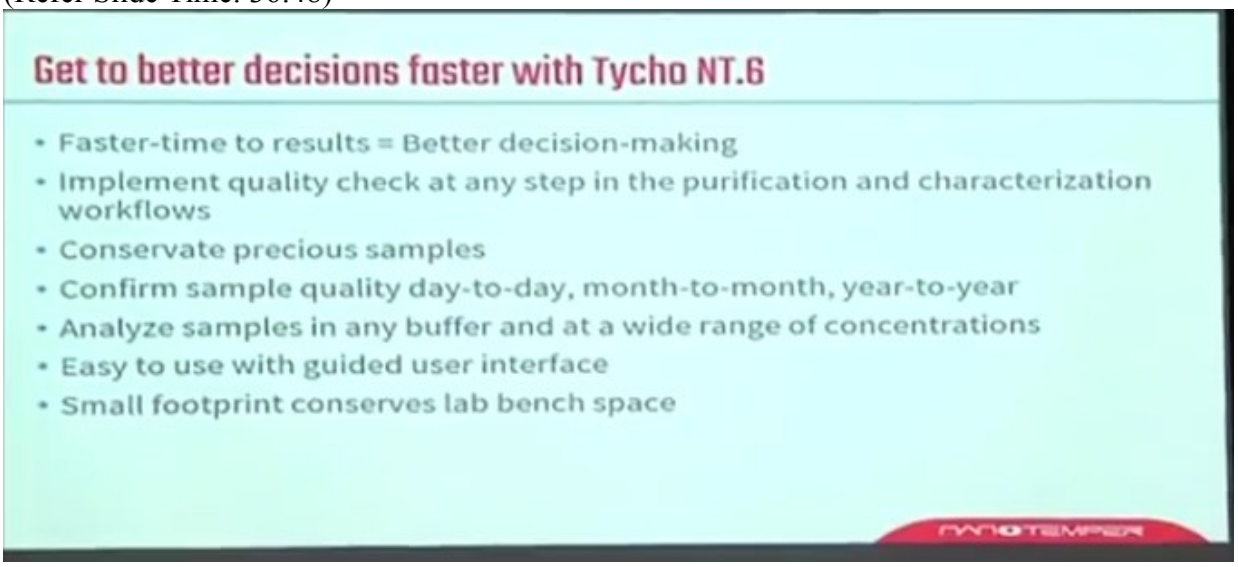

so if you have like batch to batch samples or like one day old samples or ten day old samples or like very old samples and you have a free storing the samples, all that samples when you take and check one by one, you can check the actual purity, whether it has denature or not, like that you can always check batch wise.

Then you can analyze the samples in a wide range, that is from 5 microgram per ML to 250mg per ML, and as you can see it's a very small instrument you can hand carry the instrument here and there, so it's a very easy to you know work with, so when you see the characterization workflow where all the Tycho can be used,
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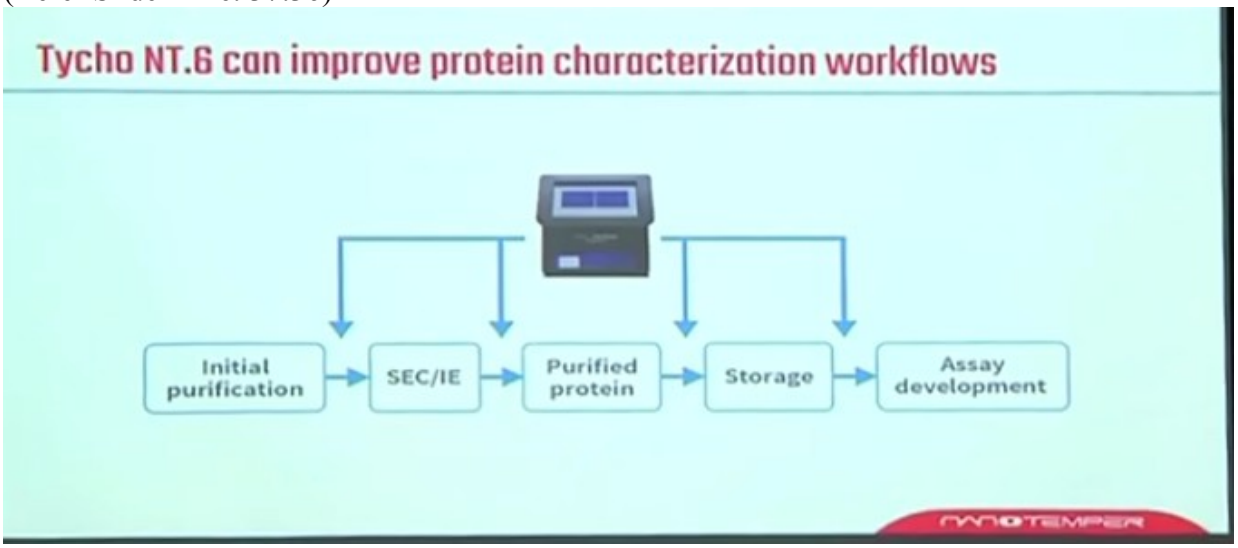

so when you go for the initial purification, after that when you get the protein you can check the purity at this level, after chromatography I mean an exchange chromatography or column purification you can again check the purity of your protein, once the protein is purified then you

can again, you have to store it at different conditions, so at which buffer, at which PH you want to store it, you can always check that and then store it in that conditions.

Once you have stored the proteins, suppose you have kept it for 10 days or 20 days after that you want to again check the purity, because for assay you need to have a pure protein, if it's not pure then definitely like suppose you are going for SPR, one chip you can't waste if your protein is not pure, so before the assay development you always have to check the protein, how the protein is, if it's totally denatured and you are doing an assay, definitely you losing your time as well as the chip what you are using for a SPR, so all these are the steps where you can actually check your protein quality.

Now I have few applications where it will tell you, where all Tycho can be used, now in this when you see what is the basic principle that is applied here,
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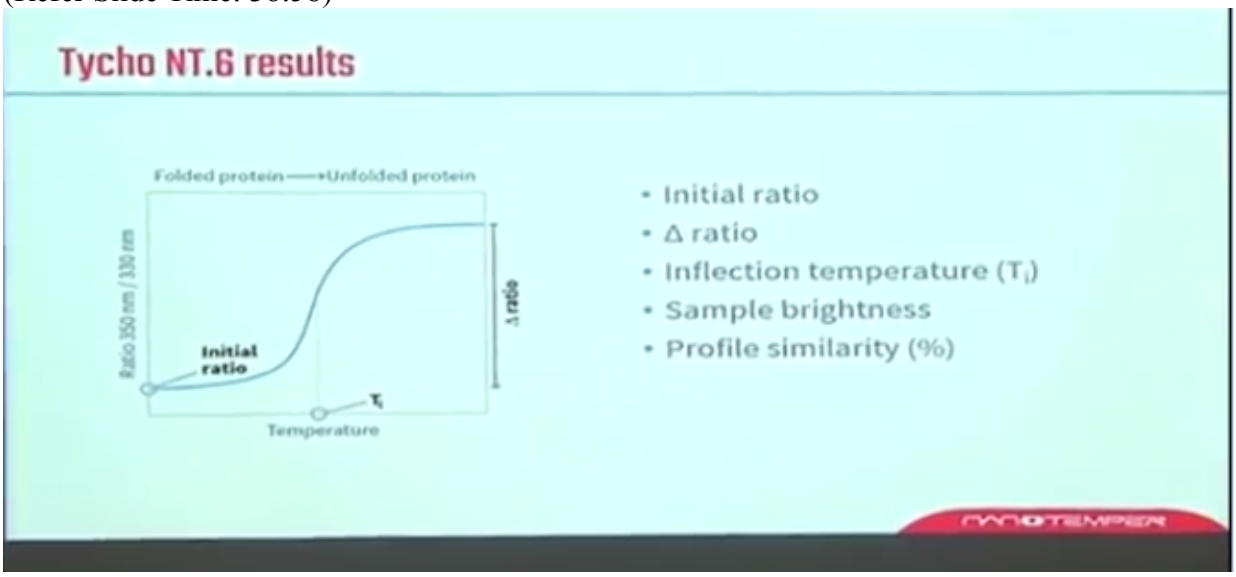

is that it is checking the intrinsic tryptophan fluorescence, so protein from a folded to a unfolded state it will give you a particular TI value, so the temperature, the results that a Tycho is going to give is the initial ratio, the initial ratio is the ratio where you know, it will give you the purity of the protein, I mean whether its denatured or not.

And delta ratio is the ratio which is from the folded to the unfolded region, like that when the protein folds, when the protein unfolds you will get a TI value as well as this sample brightness, so sample brightness here will give you a relative concentration of your protein molecule, it will not give you the actual concentration, but a relative concentration that TI this much amount of protein is present.

And then protein profile similarity is that if you have a reference sample of your protein, that can be taken as a standard protein sample and then when you are going for batch to batch or like if you are doing a free storing, at that time you can always take that as a reference sample and crosscheck the other, batch to base or you can free stored ones, and then check how much it has denatured or how much it has changed.

Now here is one example where you have compared it with batch to batch you know screenings, so here you can see the protein which is a pure protein and a protein that has been kept in -80, it's almost showing the similar profile, but when it is used after one week, but kept it 4 degree centigrade the profile has changed, so you can see the shift has gone up, that means the protein has slightly unfolded, so that means you can definitely go for assay but still you can check the quality of the protein how it has valued.

Now here you can check the buffer screening as well as the PH stability measurements, so you know like basically for SPR you need low PH,
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so if you have, if you can, if you have a standardize form of how to do it you can check at PH4, it's you know, it's totally denatured and then at PH5 which is like a basic PH for SPR measurements, the curve is almost good, I mean when you compare it with different buffers you can always check which PH you can use, which buffer you can use.

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Now here you can also check the thermal shift assay, I mean a protein is there and if you want to check whether the protein can bind with the particular ligand or not, so when you singly when you run the protein without the ligand you will see a particular profile and then with the, in the presence of a ligand the shift will change, so this shift can either be a positive shift or a negative shift, if it is a positive shift that means it's a, it's a true binder, if it's a negative shift that means it's not a true binder.

We can actually go for a yes or no that it whether the shift is happening or not, I mean whether the interaction is possible or not, with this you can go for further assays like if you are going for SPR or if you are going for ITC you can say that oh okay this protein, with this ligand it's good, because there is the thermal shift, because the TI's are shifting here.

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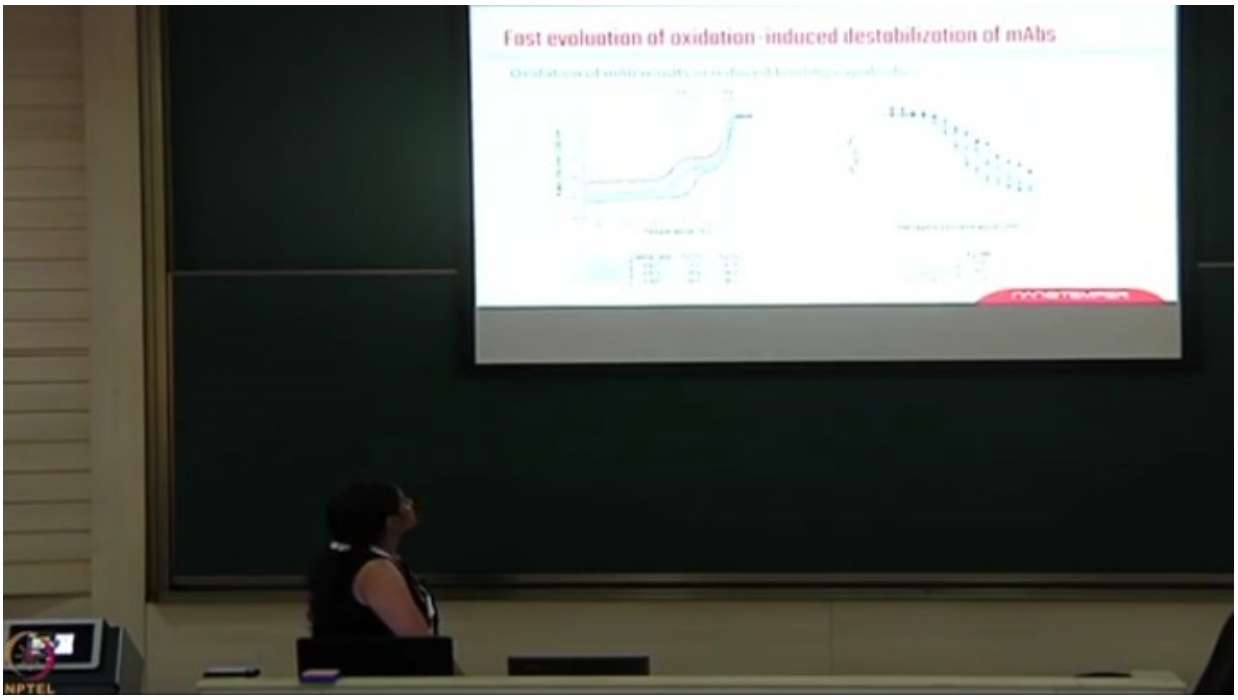

Now I have few examples which will show you the data, and you know this is the example in which the monoclonal antibodies was checked at different oxidation levels, so you can see at the native state it has given a different profile and then a 3 hour oxidation with hydrogen peroxide it has given a different profile and a 18 hour oxidation it's giving a different profile. So you can see there is a TI shift in all the 3 samples, and when you had gone for the MST assay the same thing you can see, the shift is I mean the KD value is changing with respect to oxidation.

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Now if you want to go for a biosensor assay like the SPR, so here you have to check the PH values, definitely which PH is best for the SPR, so you can always screen different PH ranges and check which buffer is best, so like here kinase is taken as a standard and at different PH it's tested, so you can see at PH7 and PH4 and PH4.5 it's just giving a planar line, that means it's already denatured, okay, but the other in PH7 and PH5.5 it's giving a curve which is showing the stability of the protein.

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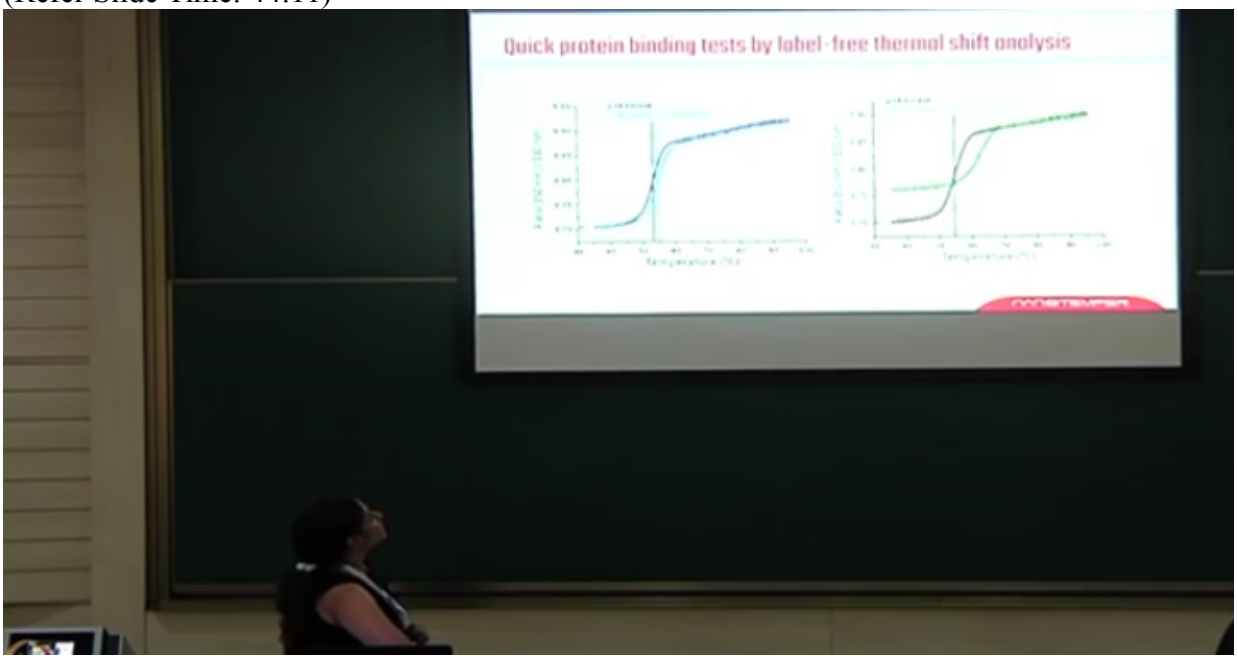

Now this is a thermal shift assay that we have done where we have taken a kinase, and then the kinase is treated with a ligand molecule, you can see the shift in the assay, you can see protein alone was giving a shift at 50, 55, and once its added with the ligand the shift has gone to 62, so that is how you can go check the thermal assays.

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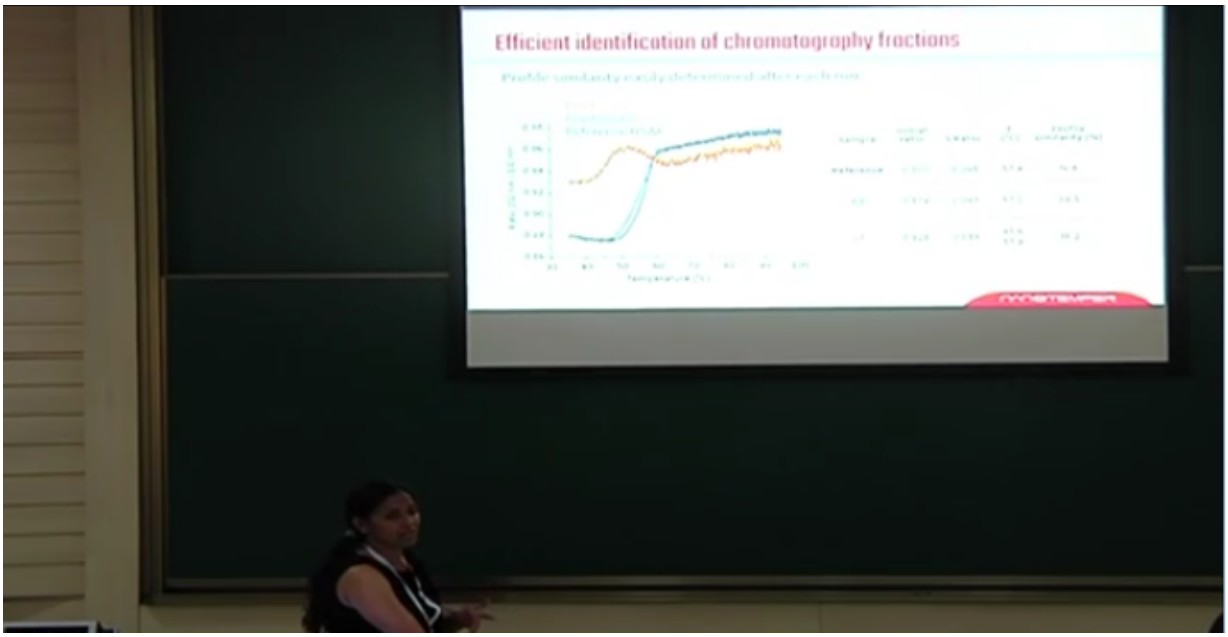

Now this is once the chromatography is run, I mean you get lot of fragments, so you check all the fragments and see which is your best, which is the best fragment where your protein is there, so this will give you a protein profile that this fragment or the first fragment or the second fragment is having the best protein, but if you have a standard reference along with it you can always validate that the protein is actually the pure protein.

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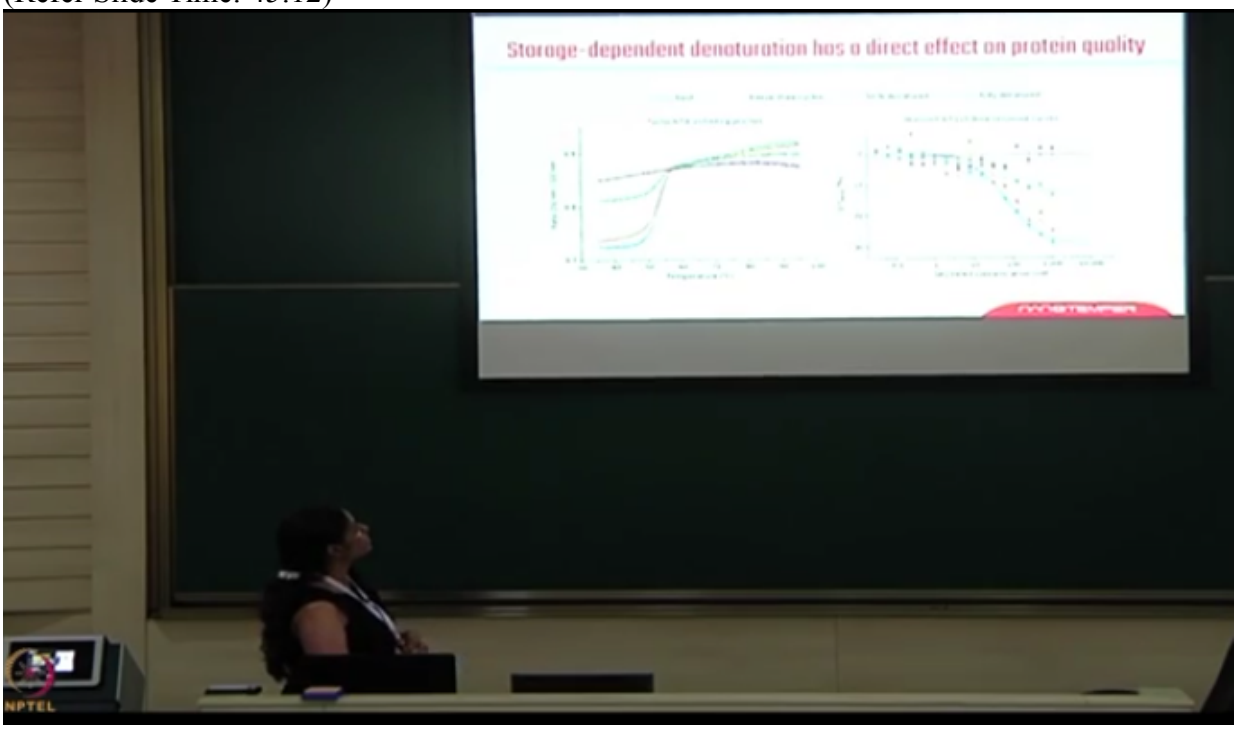

And here is the another example that we have conducted where you have taken fresh samples, free store cycle samples and fully denatured and partially denatured samples, so you can see how the trend has changed when you are checking the unfolding profiles, so the fully denatured

sample is giving you a very planar profile whereas the partially denatured is giving a different profile compared to the free store and the fresh samples.

So the same is analyzed with the MST, so you can see the fully denatured sample is you know there is no KD value to it, but the other samples you can get a KD value, but the KD value will vary, compared to the fresh sample.
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So in all if you see it's a very quick tool to determine the purity as well as the you know stability and functionality of the protein, and when you want to characterize some kinases and receptor or some membrane proteins or transmembrane proteins like that, you can always check the purity of this proteins, and like it will give you a yes or no, or signal when you want to do a thermal stability assays, or you want to go for SPR assay or Cryo EMS assay or NMR studies, so before that you want to check whether your protein is stable or not, whether your protein is pure or not, so yes or no will always, this machine will always help you with the yes or no decisions.

So I can give you a small demo with one sample that is the BSA that is 1mg per ML I have taken, so I can just give you because it's a 3 minute run that will be there, and we can check the profile how it goes.

Unidentified Speaker: Do you have any sample of this lot of reference proteins whether they change this?

Swathi: Yes.

Unidentified Speaker: Yeah, so you know many sample proteins that reference proteins that are _47:11_?

Swathi: As such reference proteins we don't have, but in yeah, but like if we do for you, yes, so like if we have a reference protein we can always you know, if you give us a for sample, if you have a standard protein we can always correlate with that.

Dr. Shivaramaiah Nallapeta:- If we say positive shift that goes the right side, and they usually like other side _47:41_, yeah, that in principle no, or something –taken all this.

Swathi: Because in few of the demos we have seen this kind of shifts where there is a positive shift as well as a negative shift, so positive shift, yeah, yeah, yeah, so the positive shift will always say that yes it's a binder, but the negative shift is destabilizing your protein and you can see the profile when it's goes above 0.9 or something, whether it's fully denatured or not.

Unidentified Speaker: (00:48:25 to 00:48:41) somewhat it will be down only the result comeback to the original.

Swathi: Exactly, that's the main purpose,

Dr. Shivaramaiah Nallapeta:- As long as then this question it's fine, if your protein is unfolded with 30, 40% then obviously like when you are buffering to that protein which is a 90%, therefore suppose you are immobilize your protein and you are changing lot many purpose and you are trying to teach everything and if it's not working, the problem is your protein is really not like you know a good say actually, it will checkpoint always be taken care off.

And when you are dealing with purification like you know in all the partners you need not go to the STC and then check with that, so quickly you can always check it, when you are storing it then you go, since basically like every check point to know what is the particular of protein what are you working with, and if you left in a, you don't have any direct method to like you know measure this kind of quantification, to the quantification was _49:40_ and you do all the purification, you run this STC, but you know that the protein you worked with is really straight, it's functional or not, so many more to the biopsy's, and it's your protein is not function there is no point in that you know saying about assay and spending lot of time, and since it's like you know label free, you don't require anything like you know, you just dip it and then take the measurement, so that will be the quickest way to check the protein quality.

Swetha: And many times when you take the proteins out, I mean you keep it in eyes but still you're taking in out, in out, so you know how whether the protein is stable or not, so this is like a quick tool.

Dr. Shivaramaiah Nallapeta:- It's also show that percentage unfolding right just for after 10 days or after one month, what is a percentage according to say, I'm going to choose 30% is fine, when it goes above 50% then show like you know everything changes, there is any 2 protein what you are actually working on, but the background we never know what is that will happen here when you are taking from -20 or -80, you protein might be small, but you are not sure but still you are going for the biopsy, but you want to know then you think that there is something is

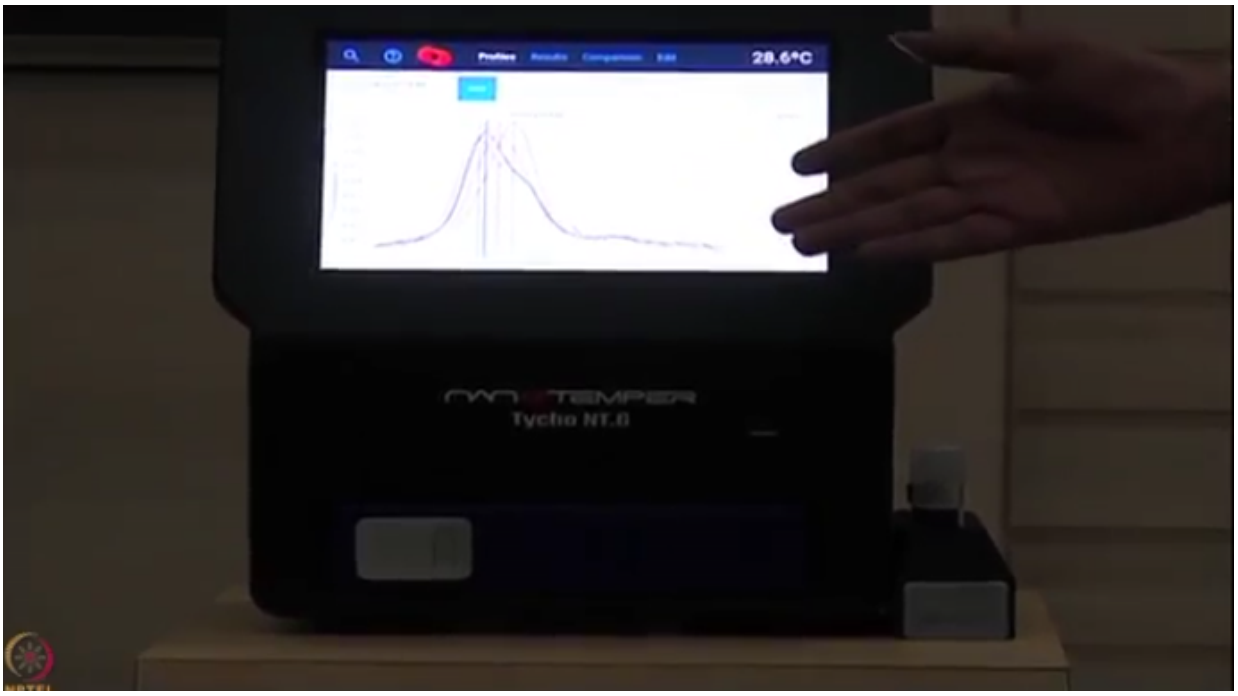
wrong, so we doesn't know the protein itself has doing right now unfolded while doing _50:54_.

Unidentified Speaker: _51:00_

Dr. Shivaramaiah Nallapeta:- See our basic principle here is, see maybe you see a protein the tryptophan is actually bury inside, right, all the tryptophan is buried inside the protein, and then now we are keeping that the tryptophan gets exposed, once your tryptophan gets exposed in order to say, so when you complete all your tryptophan is exposed the protein is lost his you know the antibody or that you know unfolding is happening, so that is the basic principle what I'm taking in, and with protein 30 minutes for hydrate, from 30 to 90 we are reaching to 3 minutes in between, just giving the sample and once the all the tryptophan is exposed you can sample, then we are getting a like you know the transition like TR, inflection point, so that's the basic principle.

But we have other version of instrument where you can get it through TI's maybe that pressure like you know 1 TI of many, all that 2 TI always control the _51:57_ and you can get the TI values also, this is very similar to DSC, but this only starting with any, but it was any mass spectrometer like you know you're working with the intact proteins, when you're using a LCA mass like the protein is all way there, I'm sure you don't get it any, you just go and because nano LC is something like that, so here you can definitely say micro proteins, sorry, it's going to that LCMS, so like this as a repeat check point at the different experiments like NMR or _52:27_ any kind of LC you can always use this as a, with whom to know the protein quality, that's the main purpose of this, and cost effective and also that 3 minutes like you know it's hardly matters like your 6 samples you are giving the data instruments, just you are reading a _52:46_, fine, so that's all, anything else?

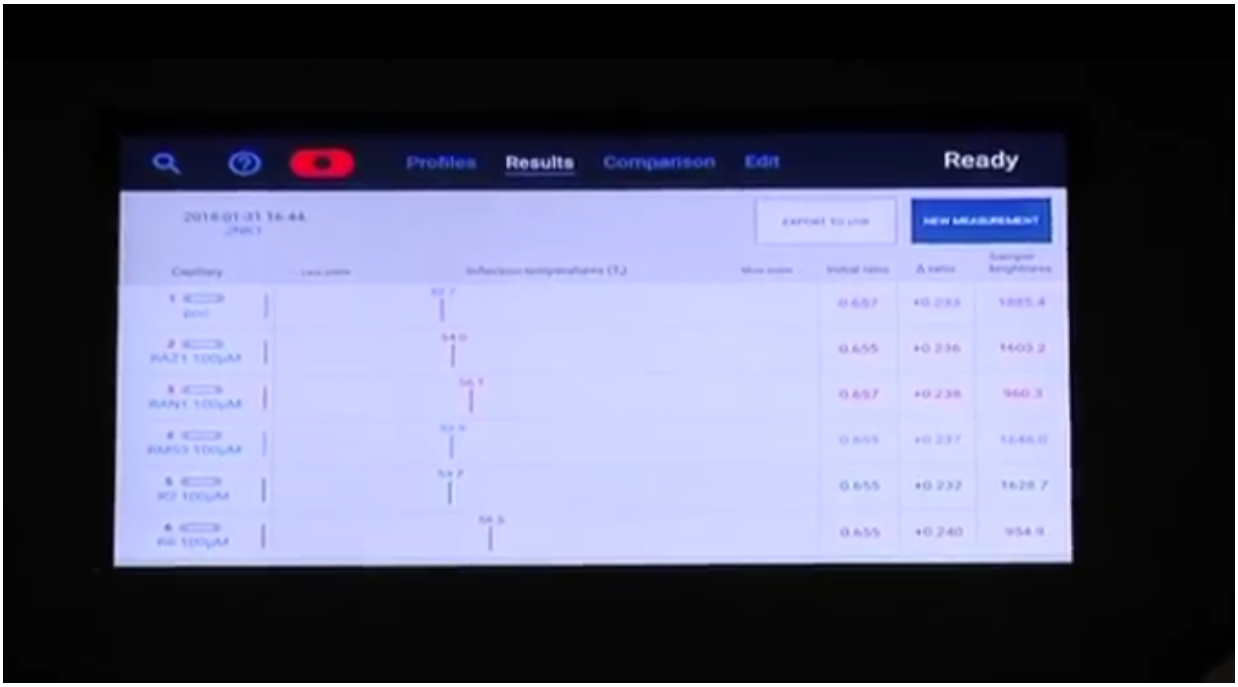
3 minutes as I said she will load one sample quickly, by the time she comes here the time unit from there, Swathi just load one sample is fine. Just mentioning that previous data to clear just get understanding because we have only one BSA, so like you know you can clearly see there is a change in the TM's, like you know TI points like whenever they are doing the interaction,
(Refer Slide Time: 53:26)



generally when the obvious stabilizer complex, so there will be a shoot in the TM's, so typically you always get the curves with a clear TM's, I mean you just see the lasers and this like you know, you always get the temperatures at least they got unfolded and it's clearly showing you shift of somewhere 3, somewhere 4 degrees, so like this you can always see the differences in the TM's, so that is the biggest advantage.

And other thing you are seeing the stability of your protein, so always I know like many times same protein over someone or something like that, so you can always compare like you know what is a profile similarity,

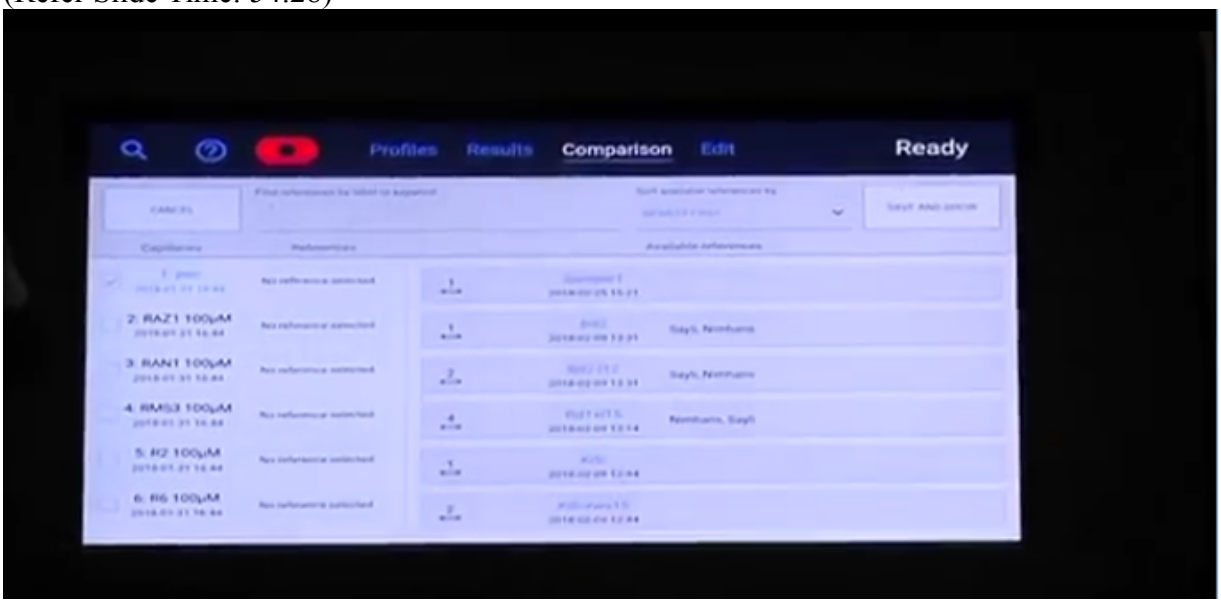
(Refer Slide Time: 54:00)



what is the percentage loss I'm doing here, so this kind of all things you can always, once more to measure the proteins temporary.

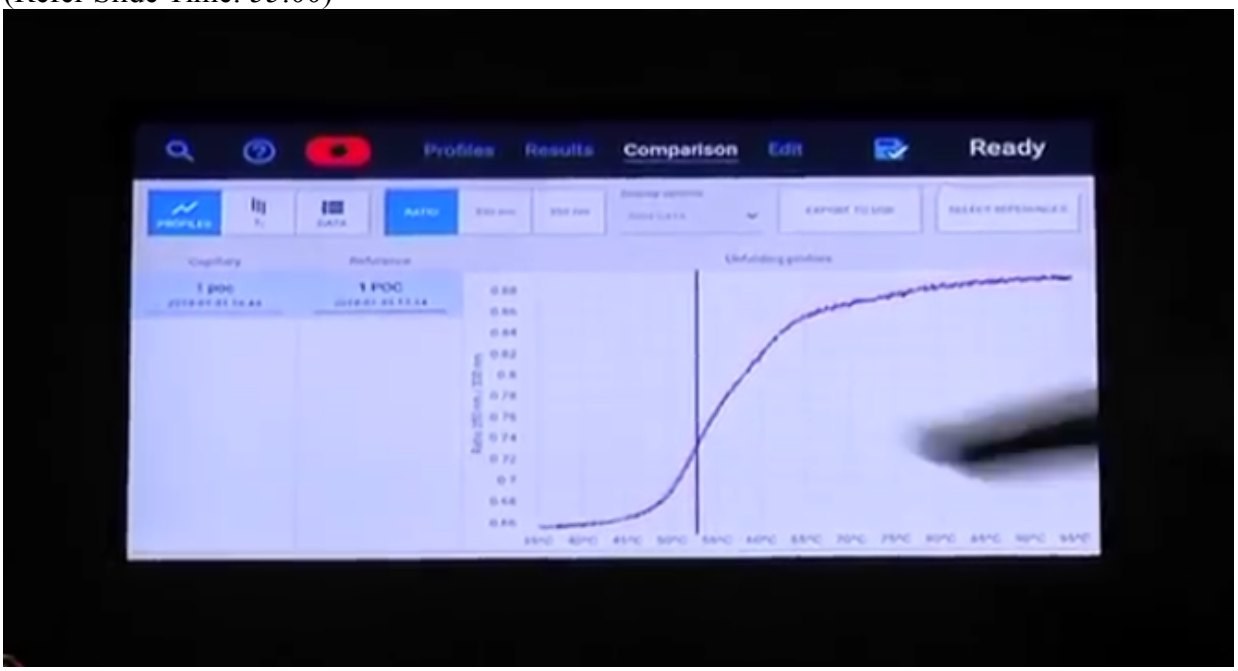
So you always get to know the data ratio, I'll tell you the percentage unfolding, the sample brightness, not exactly at quantitative to parameter, it is kind of ability, like you know if I'm taking one samples standard, again is that one what is the brightness what you have it, like you always give the ability quantitation for that also.

(Refer Slide Time: 54:28)



See it's very easy like you know the settling point where you can always assess the protein quality for, before going to any biopsy it can be SPR, it can be like any ITC, anything like that, so you can always quickly know the quality of your protein, whether it would be really good or not, and if it is good then compatibility with your references how will actually got some, this is

the biggest advantage what you have here. Because one sample is not make sense to show you all the data, so we are showing previous sample, so this is how typically you are getting it here, (Refer Slide Time: 55:00)



software automatically calculation then show you this is the TM.

Unidentified Speaker: _55:01_

Dr. Shivaramaiah Nallapeta:- That thing all depends like you know, when you do the actually unfolding, so in tryptophan might be exposed, some tryptophan are like already inside, so in principle you get always a very non-specific data, now it's a biophysical always, what we recommend protein is recommended, but yes many of our customers having two proteins they have tried it, so always they know like you know one protein TM they know, the other one whatever it was getting to the concluding.

It's always like RND can affect, sorry, yeah absolute, absolute, I think you can always see the multiple of the same protein, you can have that you know same protein have multiple domains which are like you know exposed in different temperatures, then particular maps if you see to get the fab region and we have seen region in different, different times, so like this even sure same protein multiple teams and you still get the direct input. So this is what we can do, so this is how you get the difficult results in _56:08_, so any questions, I'm sure like you know here proteomics means everybody will be doing some kind of purification, mass spectrometry all this kind of things, we should be very glad if you are interested we can evaluate and we can still give more insides in this technology.

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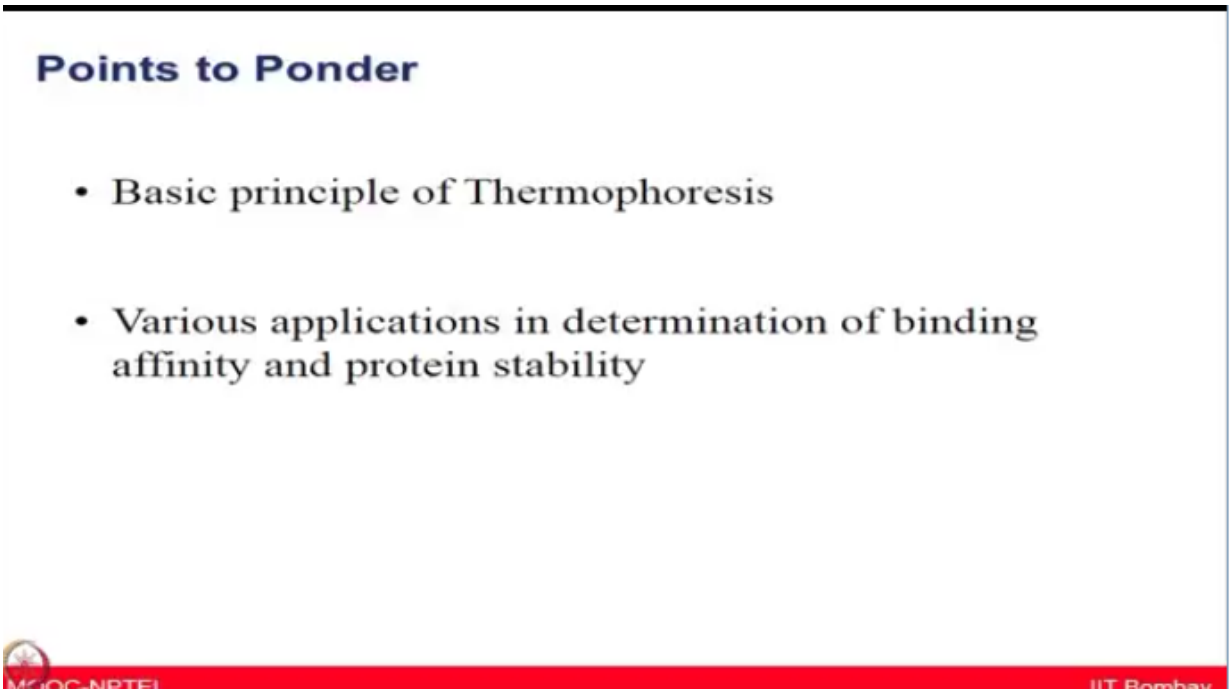

Dr. Sanjeeva Srivastava:- The advent of new technologies both label based and label free have now started offering us new inside for lot of new functions for the proteins of interest for which there was no function defined earlier, in this slide there is advent of new technologies which aims to do these experiments without taking the protein of interest and also provide the binding and the kinetics information.

In this slide there are new developments, new technologies are coming forward, and today we'll try to provide you interaction with one of the leading companies and their application scientist who talk to you about MST technologies and also the quality control assays where done using Tycho technology. I hope these provide you some insight about how to do your experiments for the proteins to further characterize using latest label free biosensors. Thank you.

Prof. Sridhar Iyer

NPTEL Principal Investigator
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