Lecture 30

Surface Plasmon Resonance- Principles and Assays-II

Welcome to MOOC codes, on applications of Interactomics using Genomics and Proteomics, technologies, in the previous lecture, we discussed about SPR technology, especially biacore platform to perform surface Plasmon resonance based experiment. Dr. Uma Sinha Datta an application scientist and a trainer, from G healthcare, gave you the basic idea for, doing these experiments. Today she is going to continue, where it was stopped in the last lecture. And she is also going to provide you, a hands-on

session, on vehicle technology. So, let's have Dr. Uma Sinha Datta for today lecture. So, I pointed out one thing to you, when you're directly immobilizing you're like,

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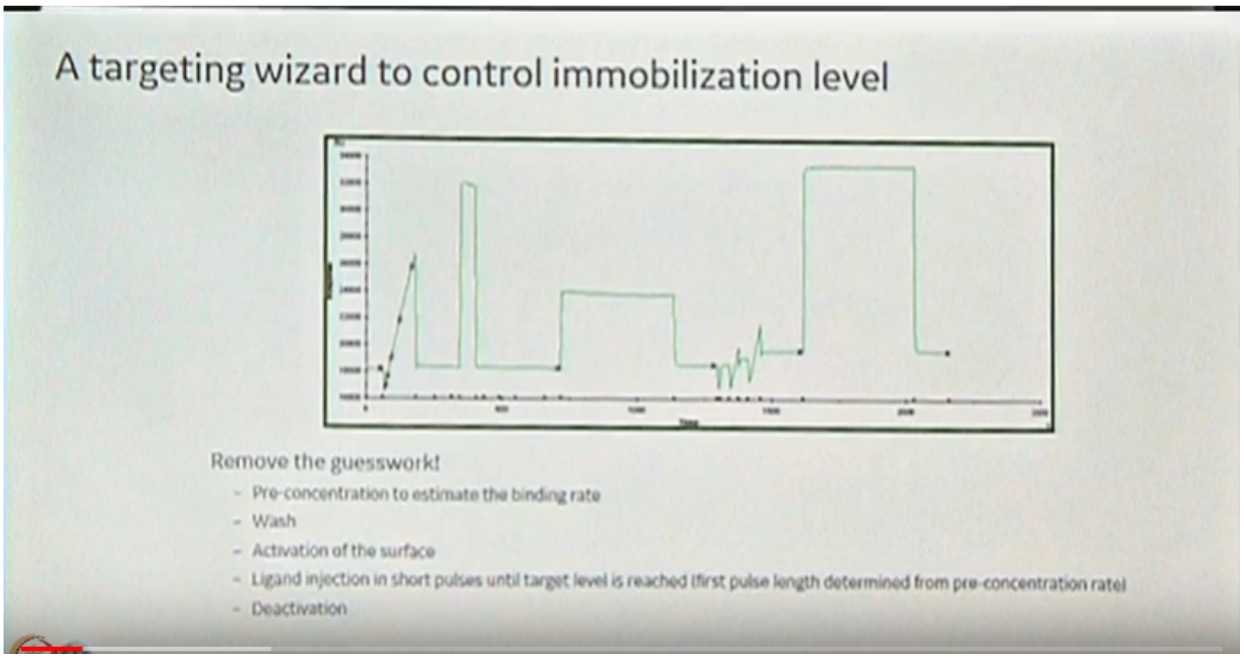

in its covalently, linked. Right? And once you immobilize, it its immobilized for you good, you cannot use the chip to immobilize anything else you, you use the same ligand, to do your experiments in that, but there is a smart way to actually save, your or use your chips more judiciously you know. So, when you are actually immobilizing, it and you are playing with the AR use how much you know immobilize and you don't know how much you would like to immobilize or you are attaining some part of it, say we decided to immobilize, 600 our use but how would you go and immobilize if you then you have to actually, look at your contact time, how do you attend that 600are used. Right? So, there is a visit that we have which, we call it as this control wizard and when you set up your immobilization, you can say aim for immobilization. Okay? Rather than putting a contact time for immobilization and when you do so, what happens is your system actually pulses small volumes, there on a microlitre or so, on the surface and does a pre concentration for you. And with the rise in the RU it calculates, whatever you have aimed to attend, around 600 are use, it'll calculate and let you know in, in, in the first six or five to six pulses it will let you know whether it is attainable or not, if it thinks that it's going to attend, then it proceeds to the EDC NHS step, before that it does a quick an Irish wash, wash to remove all the electrostatic ally bound you know ligands. And then you do an EDC NHS and then you pulse your ligand, based on the information that you had collected and then finally you do the ethanolamine step. In case you know it decides, that the concentration that you have supplied, is not enough to attend your 600 or use whether it's more or high, it's going to abort your cycle post here. So, your chip is actually intact in this case. Okay? And this is a very useful tool to use, when we are because the chips come really, expensive, there is another one which is not added here, which is the C m7 typically used for the, small molecules. Okay? A little bit more detail

on the chips. So, you, you know the basic, structure of the chip, the chips are actually on the glass surface on which there is a gold coating, then there is a linker molecule.

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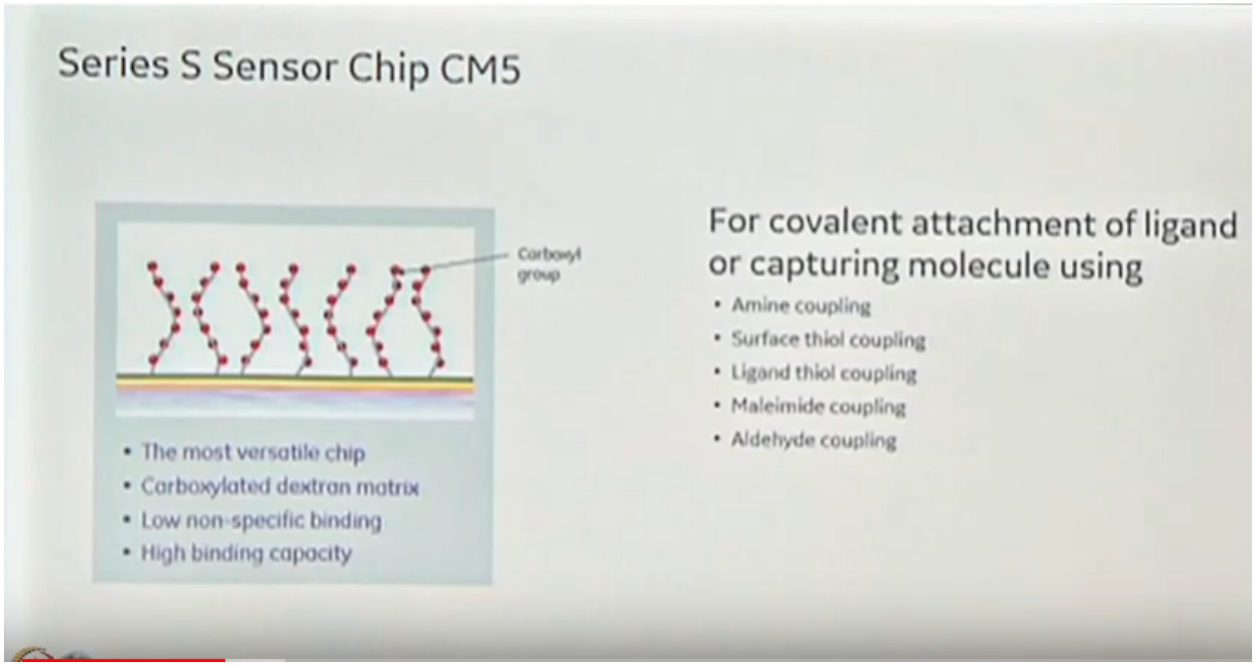

Where the dextran molecules are stuck, to it and the dextran molecules have car boxy methyl groups and in these carboxyl methyl groups, is where your covalent linking happens. Okay? The cn5 chip is, is actually our most vers versatility, you know any most of your, you know even now when I do my, first experiment in via core with a particular system, I would straightaway go with cm5, if it doesn't work I then go and choose, a different chip. Because it is it's been proved to be very, very versatile to work for most proteins. Okay? And it can support almost all kinds of you know, covalent chemistry there, now as light variation of the,

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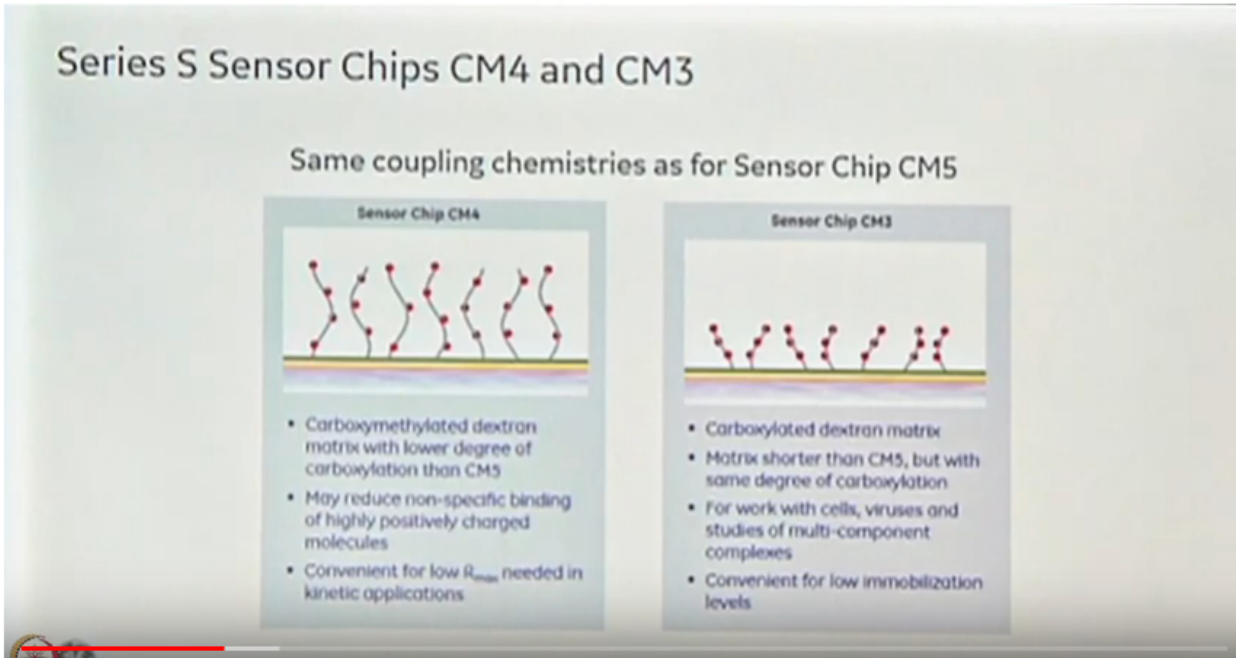

CM 5 is actuallycm3 and cm4. Okay? So, they are exactly the same, it's just that you know the length in one case the length of the carboxymay dextran, is the same as CM 5, but the car boxy methylation, the intent the number of car boxy methyl degree, of carboxylation out here, is less than same, in CM 5 whereas in CM 3 the degree of carboxylation, is the same, but the length of the dextran is actually, smaller. Okay? So, this is used, mostly to you know if you want to have work in a lower max region, you know if you have a cm 5 sometimes you know you it's sometimes difficult to attend, to our max, whereas in this case it's much easier, to attend the lower our max. The smaller size, of Dexter on here, helps to you know do your asses in larger molecules, like for example you ‘reworking with viruses or cells, where the size of your cell is quite big and you know, it moves away from the surface. So, the surface Plasmon resonance, will happen quite far away, from the surface and where you’re the SPR phenomenon reduces, as you go far away from the surface. So, that's where you actually, user smaller branch. So, that you can keep your proteins or your cells closer, to your surface, c1 I think we discussed about that which is the dextran is all gone, it's only the car boxy methyl groups which are stuck from the surface in cases where your dextran is causing no specificity, you can use this kind of chip. This is your streptavidin; this is streptavidin chip, for your biotinylated protein. But remember, this protein the streptavidin, biotin binding, is extremely high carry, I think it's in the range of 10 to the power minus 12 or so, which is actually very, very close to being a covalent linkage. So, once a streptavidin and biotin links, you can never,

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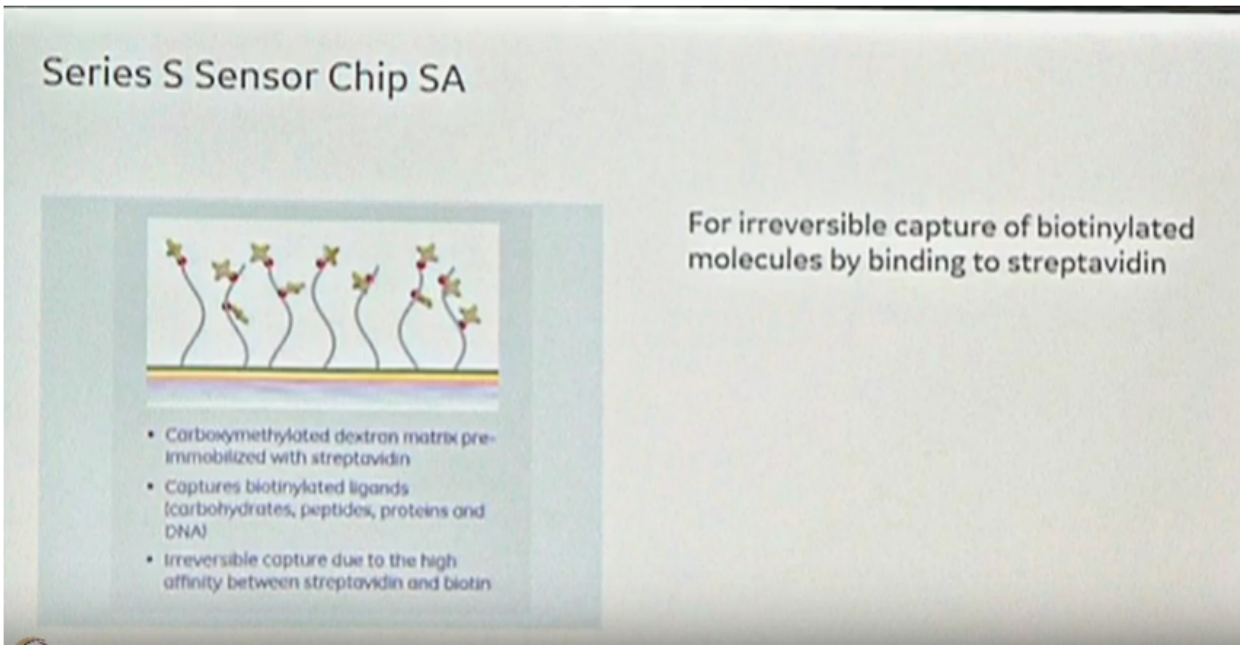

you know open it up. So, you have to be a little careful, when you're using this chip, because once bound, it is good to go like a covalent, chemistry.

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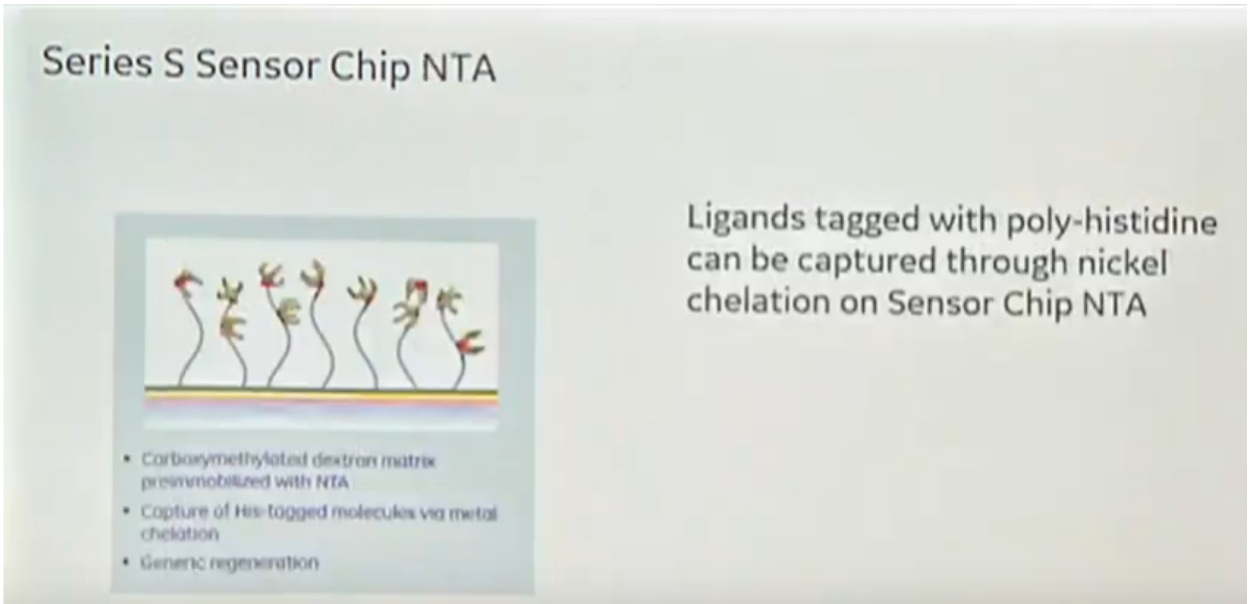

NTA this is for the histidine tag, this is reusable you can recharge your chip all the time, with the regular you know chemicals, that are available for entire columns.

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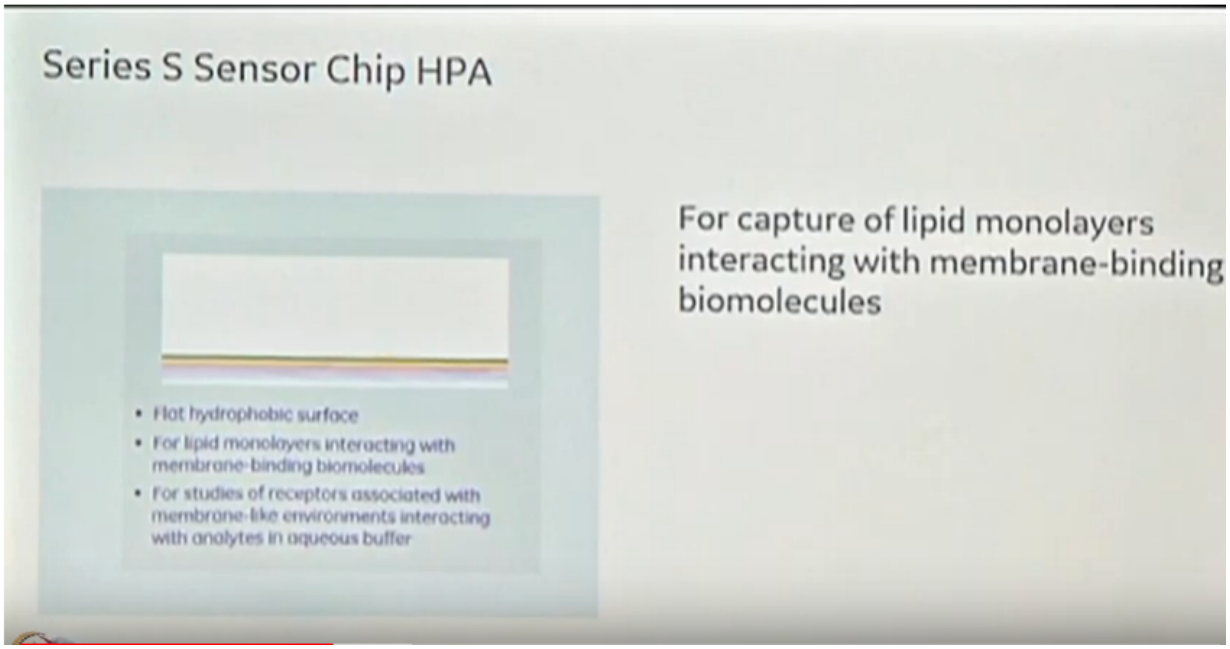

HPA this is for hydrophobic interactions, you can go to your hydrophobic proteins on here it's a flat hydrophobic thing. This is to make in this case you can only make a mono layer, of hydrophobic layer.

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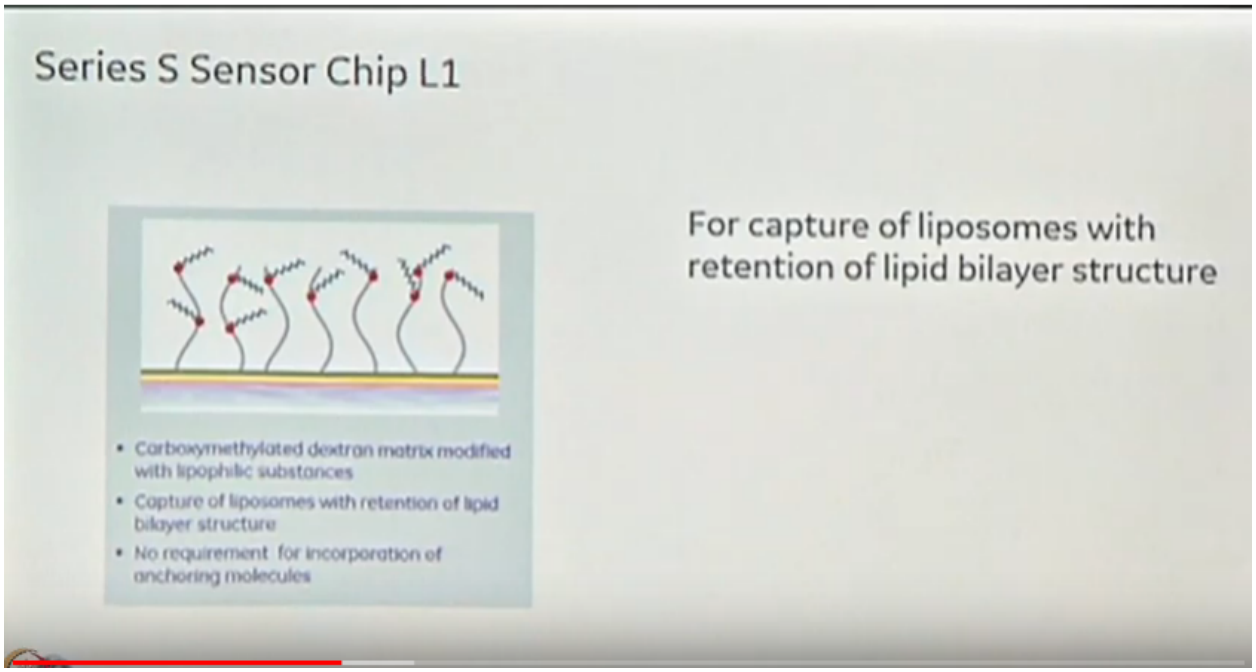

Whereas here you can make a lipid bilayer for using the liposomes if you have liposomes the mixture you can, make a lipid bilayer kind of a structure, on this thing and then have the protein studies done ,on them.

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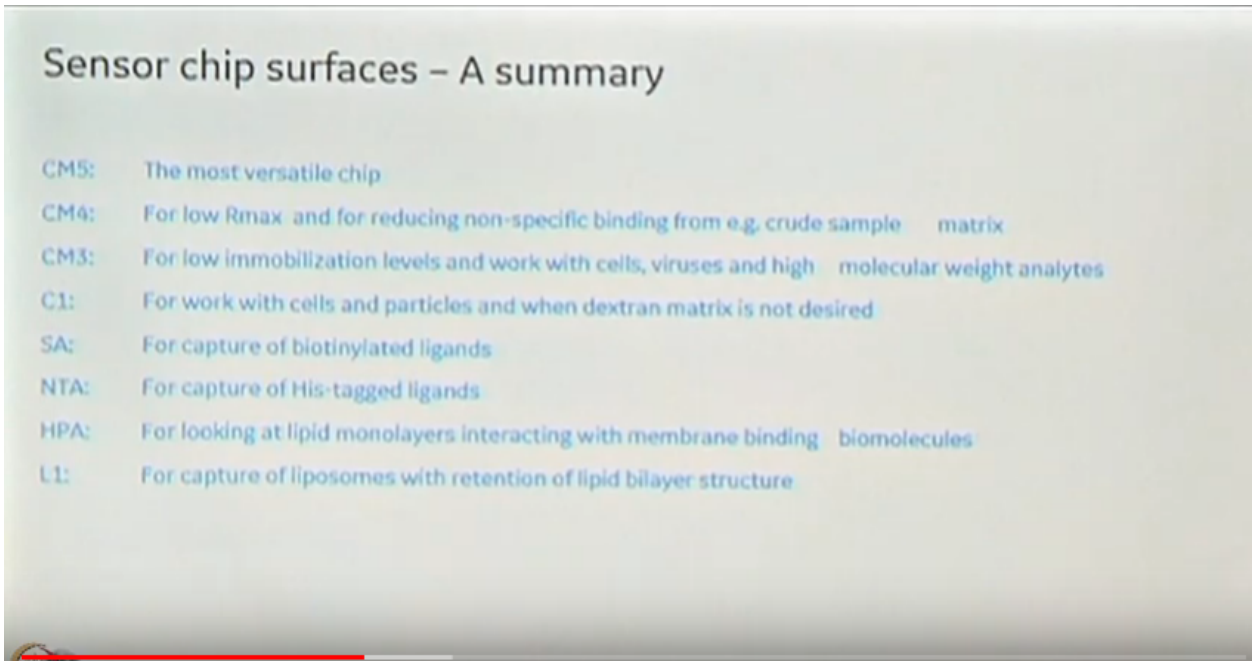

So, that's the summary of all the chips, I'm not going to go through this, anymore because we have talked, about the various kinds of chips. Now unless you have any questions I can answer them.

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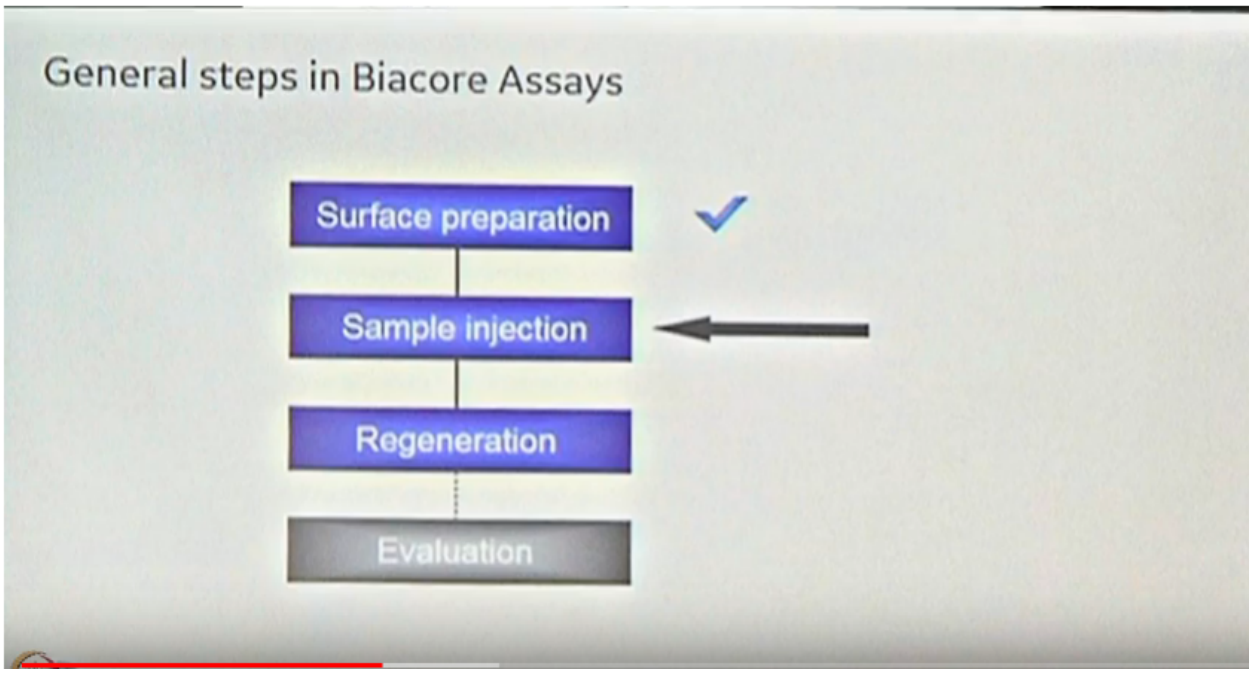

So, that was all about the surface preparation, you know what which one should, you use what is your ligand, you know the P is that is related important, you know how you can optimally, use your chips, by not wasting them. Right? And we also learnt about the R max, in watch which R max you are going to work, things like that. So, the next one is sample injection, this is rather a simple thing, it's just that you know, you are passing your analyte, over the ligand that you have prepared, now if you remember we talked about, that SPR is actually a refractive index, measure. Right? The change in mass causes, a refractive index change which you are reading and so, difference in concentration or in your buffer would also actually, change your response unit that you're reading. Right? So, what we call it is actually,

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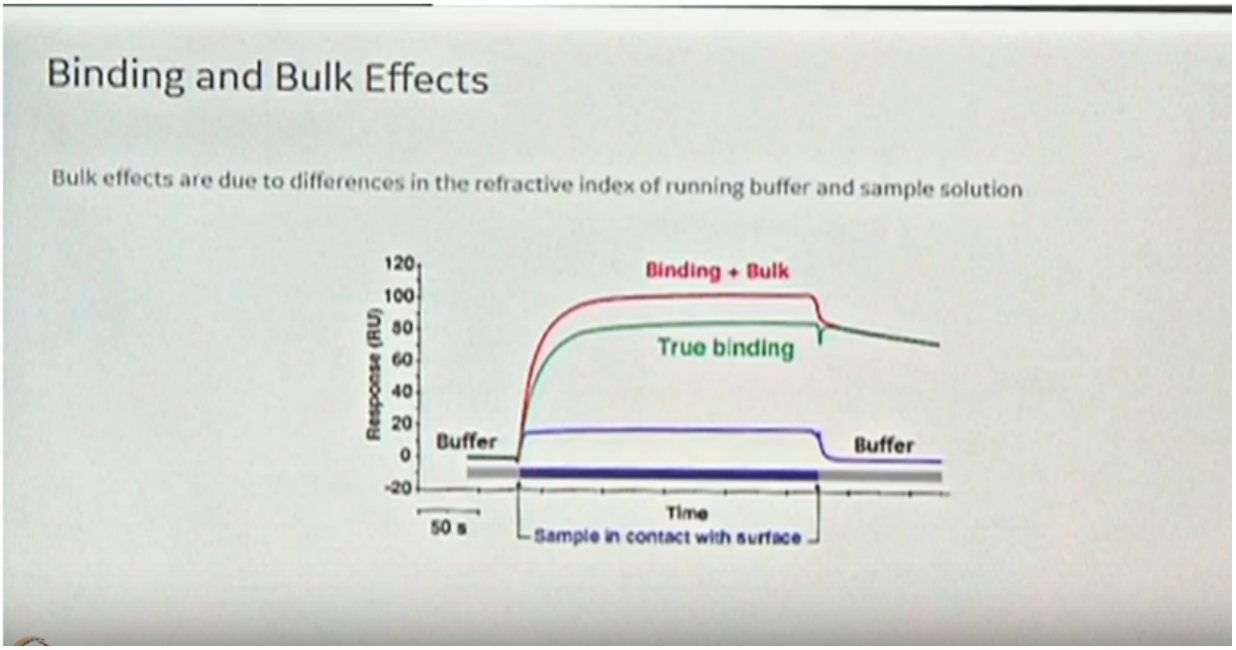

a bulk effect, you know when you are passing your analyte, your analyte is in a particular buffer, the buffer also gives you some response unit. Okay? So, if you look at this graph, your the blue bar is just the buffer, running without anything. Okay? So, it has a teeny bit of response unit, the red one is actually, your but you know binding as well as your bulk, you know. So, the you 'relooking at a response unit, which you have received, after binding as well as you're looking at the bulk if you subtract, these two you actually get your true binding. So, all SPR experiments should have zero concentration, which is subtracted. And the buffer needs to be exactly the same so, whatever is the running buffer or the zero buffers has to be used to make sample. Right?

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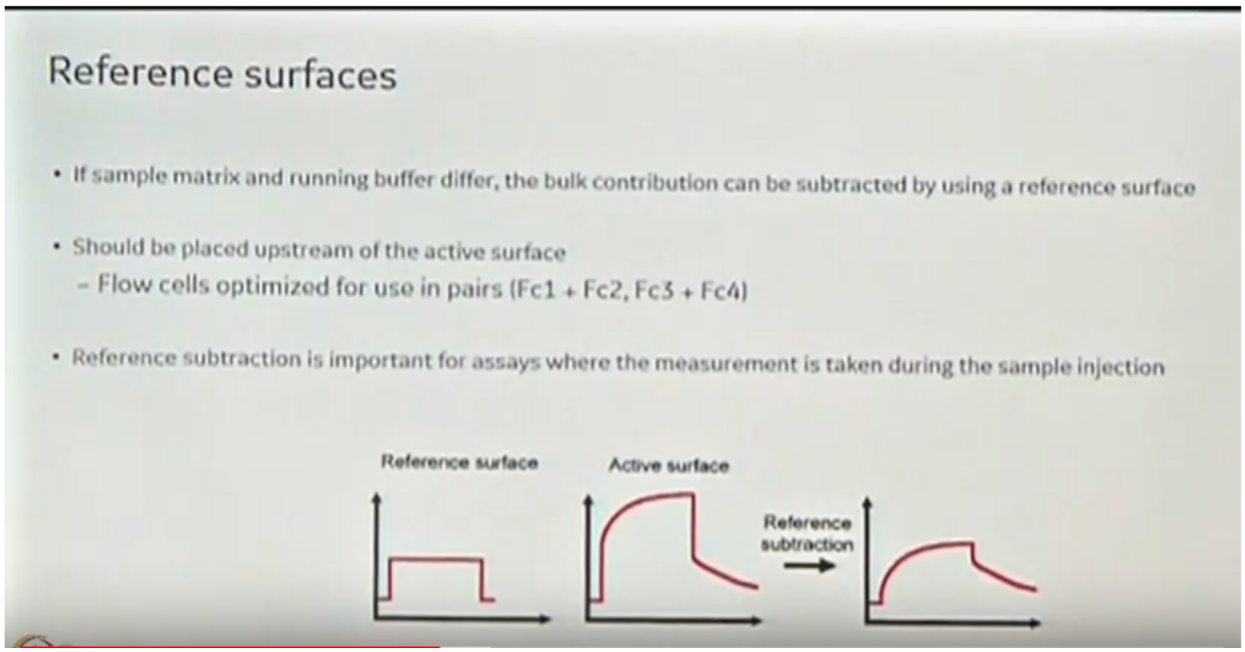

And then you use a reference surface, this is to remove no specificity of your analyte, with the surface. Right? When you're passing, your analyte over the ligand somebody asked, me I think from within you that how do you make sure that it is binding to the ligand and not to the surface. Right? So, you have another blank surface, which is just before, before your active surface and there you actually see, if there is any nonspecific binding, that is happening with your extra and you subtract, that which we call it as a reference surface. So, when your analyte passes to the reference surface, you see a certain pattern, of response unit, this is your active surface and when you subtract here this is actually, your you know the real binding minus the nonspecific binding, in some cases if you have too much, non specificity, it might interfere with your binding, in those cases you are supposed to use other, additives like detergents or there is something called an,' NSB', reducer which is nothing but free of car boxy methyl groups which are mixed with your analyte, to you know reduce the non specificity. Okay? There are three different ways to, produce or make your reference surface, there are various ways you can have a reference surface, the first one is unmodified you don't have to do anything. Okay? In most cases that works, but if you're not happy, with that unmodified you can also treat it exactly how you immobilize. So, you activate and deactivate without passing, the ligand so, you're treating the surface exactly, the same way that you have treated your active surface. Okay? So, the first one is unmodified, the other one is activated and deactivated and the third variety can be you can have, a dummy ligand a knockout ligand, which is knocked, out of binding, that is also possible. Okay? So, if you have it if you have the luxury to have that, you can also have that another way of doing the surface, certain some sample considerations, not I mean very I mean they're very critical, but also very logical, I mean you know the sample has to be homogeneous you cannot have particulate matter or you know they will create spikes dirty sensor grams, eventually difficult, to evaluate right the quantity of analyte also has to be extremely, you know it's very important particularly, in certain applications, like kinetics the concentration, needs to be determined very accurately.
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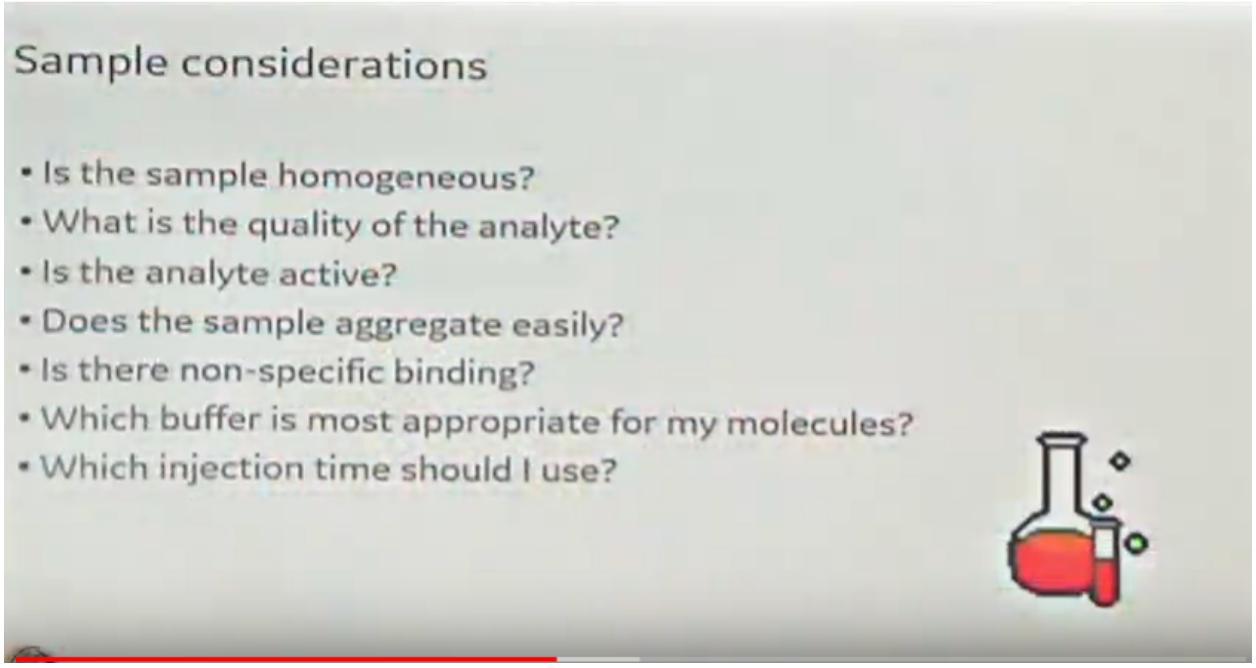

Wrong concentrations will land up giving you, you know different values, you need to make sure your analytes are active, they are free of aggregates, if it is aggregated then you get higher response, than usual which is not. Right? Okay? And buffers, that's again what buffers to use you know that's also very, very important, because sometimes you know your interact, ants may require certain metals you know, for binding to happen, that is something that you need to find out from literature's whether you want to add some additives, to inhale you know promote your binding. So, these are some things that need to be considered and also injection, time we spoke about how much of time does it take, to run a full cycle. Right? And it's a very generalized question because it depends, on your association and the dissociation that we understood. Right? So, if it's a very quick associate ER and a dissociate ER you would obviously need a smaller time. And if you have a very slow Association and dissociation obviously the time needs to be increased, buffer requirements the buffers, that go in should be point to two micron filter they're of course available, to buy from G but it's not a big deal, I mean you can make it yourself as long as your chemicals, that you are buying are on good quality and just pass them to through a point to micron, do make sure you have P 20, which is a detergent in the.

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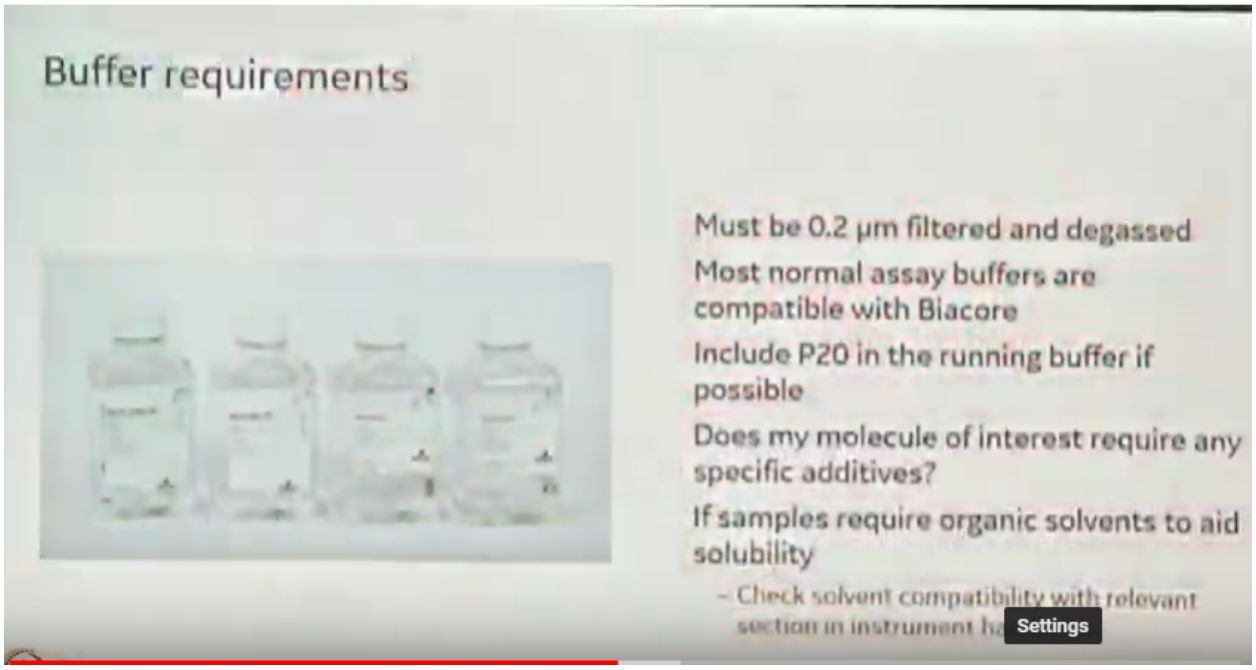

And it's a non ionic detergent, because you don't want, any ionic detergent running over the surface, that can foil your things, it is important to have a detergent to remove the stickiness, because the proteins are quite sticky and they stick to your flow cell and you know for the maintenance, of your system, it's you know important, also in some cases where you do not see binding you might, want to remove the p20 and do your experiments and see if it is working. But make sure after that you run through a good, you know maintenance cycle. Okay?

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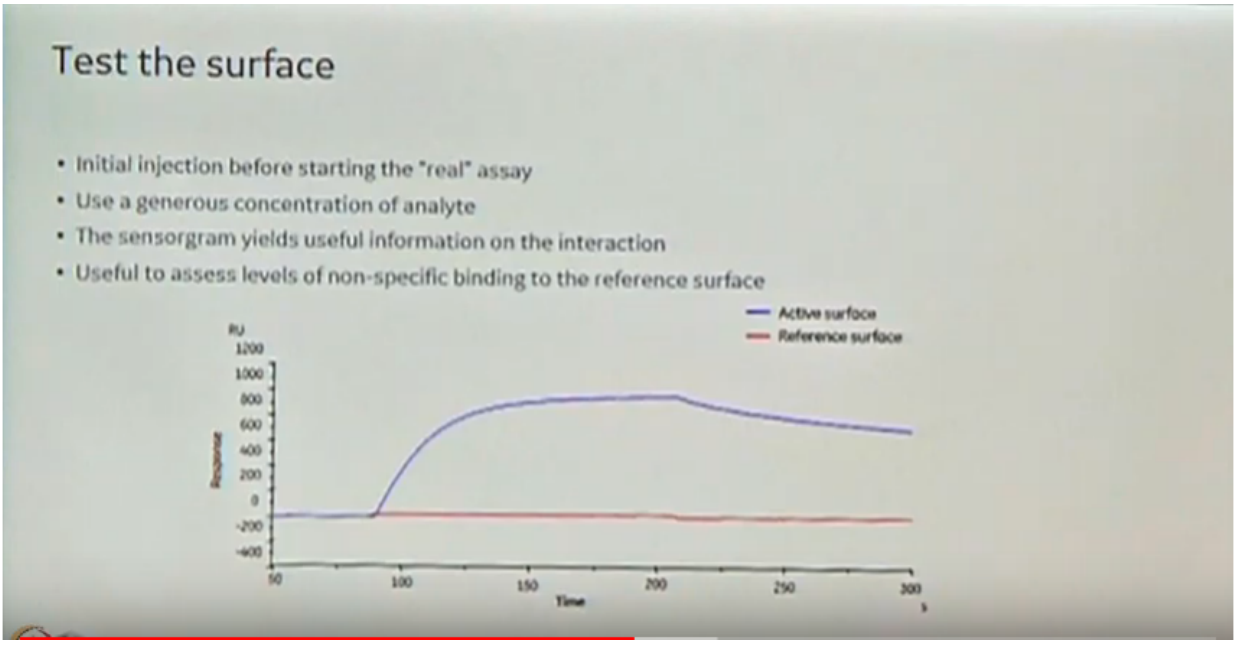

p20 is a detergent, it's a non ionic detergent, sorry I don't have the full name so, once you have your immobilization ready, you pass your sample exactly that the experiment that you did today in the lab, you know you did a zero concentration, usual low concentration and you need a high concentration, you need to test the surface exactly that way, because the first thing is you don't want to use huge amount of analyte which is which could be very expensive, for you to get it actually to set up. So, much of run and then you realize your life you know Ligon, was not proper all your surface was not right, Right? So, you test the surface, once before you set up big kind of experiment. So, you run two different concentrations, low and a high first you get to see the shape of the curve, which gives you a lot of information like we discussed, you know the shape of the curve you get to know and you get to see, that exactly what you were expecting, you have got that the other thing is you see a dose dependency. Right? That a low concentration higher concentration you see a dose dependency, the third point is you have also calculated your R max and based on that you have had immobilized, your ligand and with that are you getting your R max are you getting your ru or not. So, these are very, very important things to keep in mind when you are setting up, your asses because if you don't if something is actually not falling in place it's, it's time to check, right there rather than you know setting up the whole thing and going. Okay? So, that was all about the sample injection, you know the last one is your regeneration. Right? After your association and association, before you go to the second cycle, you actually find out the regeneration which is stripping, removing all your analyte from the bound ligand. Okay?

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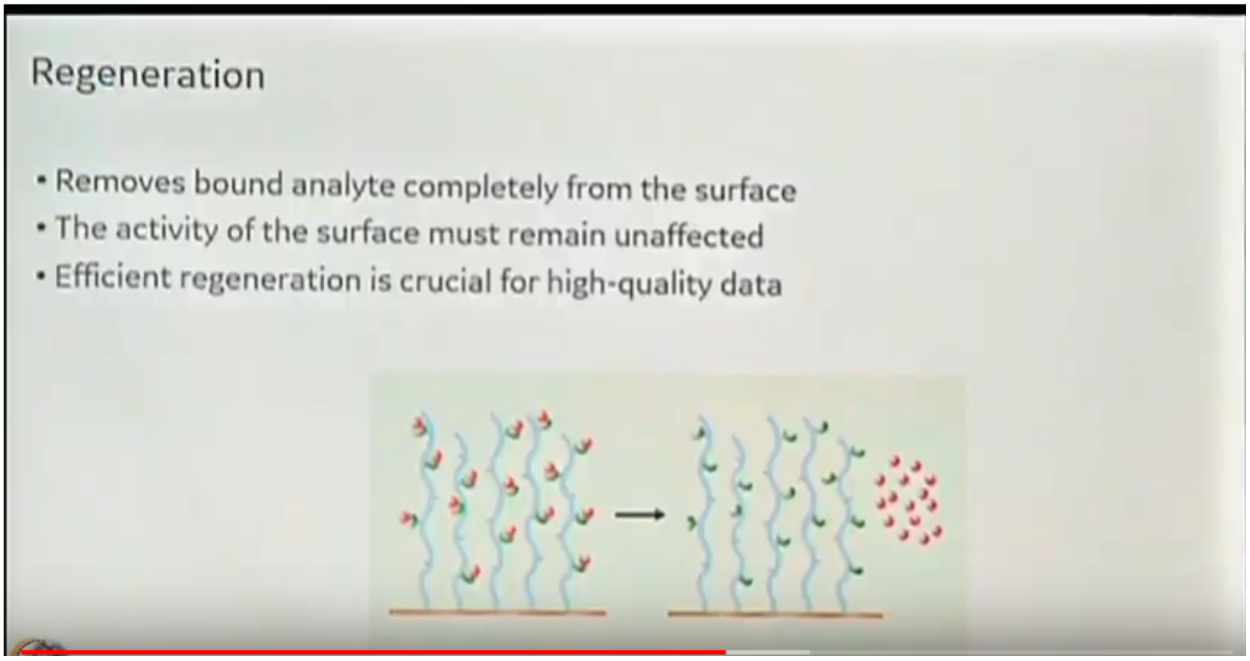

I think you saw this it's just removing, all your bound analyte from the ligand.

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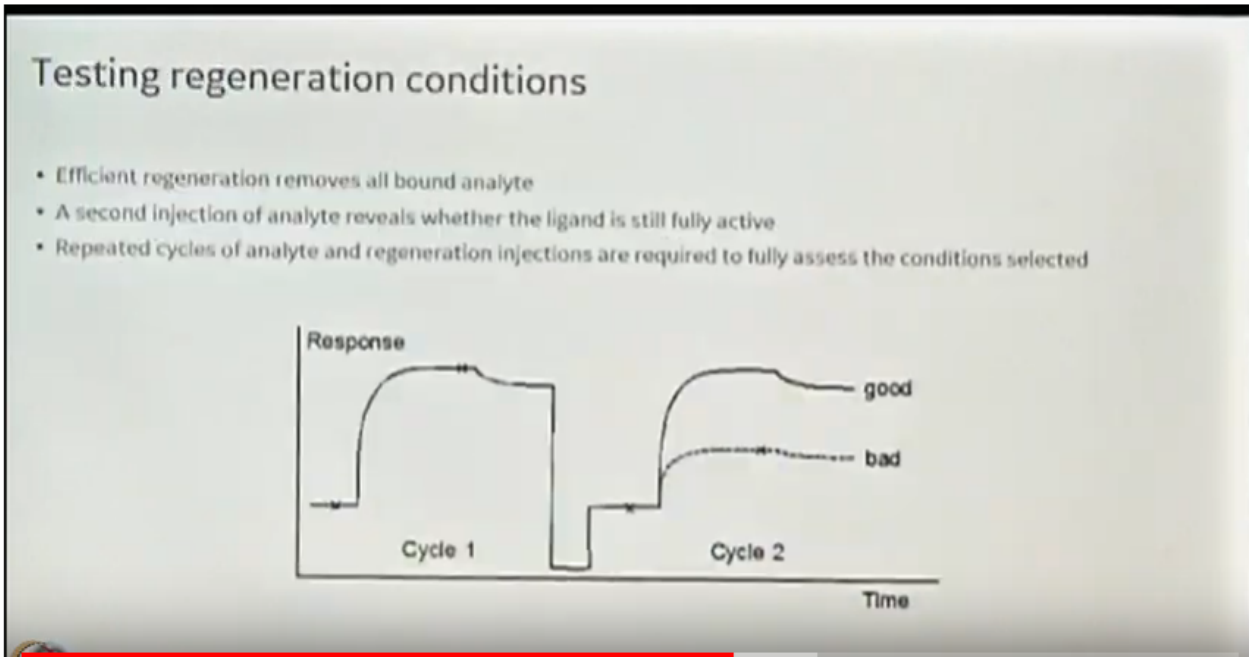

So, there's a way to check, whether your regeneration conditions are fine or not, Okay? So, so if you may have taken the same 50millimolar glycine, pH to 0.5. Right? And you have done a regeneration and you see so, you run the first cycle you get your curve. Right? And you do a regeneration with your desired, regeneration condition, then you run exactly the same cycle, again the second time, if your regeneration cycle is good, then you're supposed to get the same response unit, because you're using the same concentration, of analyte. Okay? If you do not if it goes down, then it is not optimal, but at this point you do not know whether, it is with this one cycle, you do not know whether it is it was harsh or it was mild. Right? You just know that it was not optimal; your regeneration was not fine.

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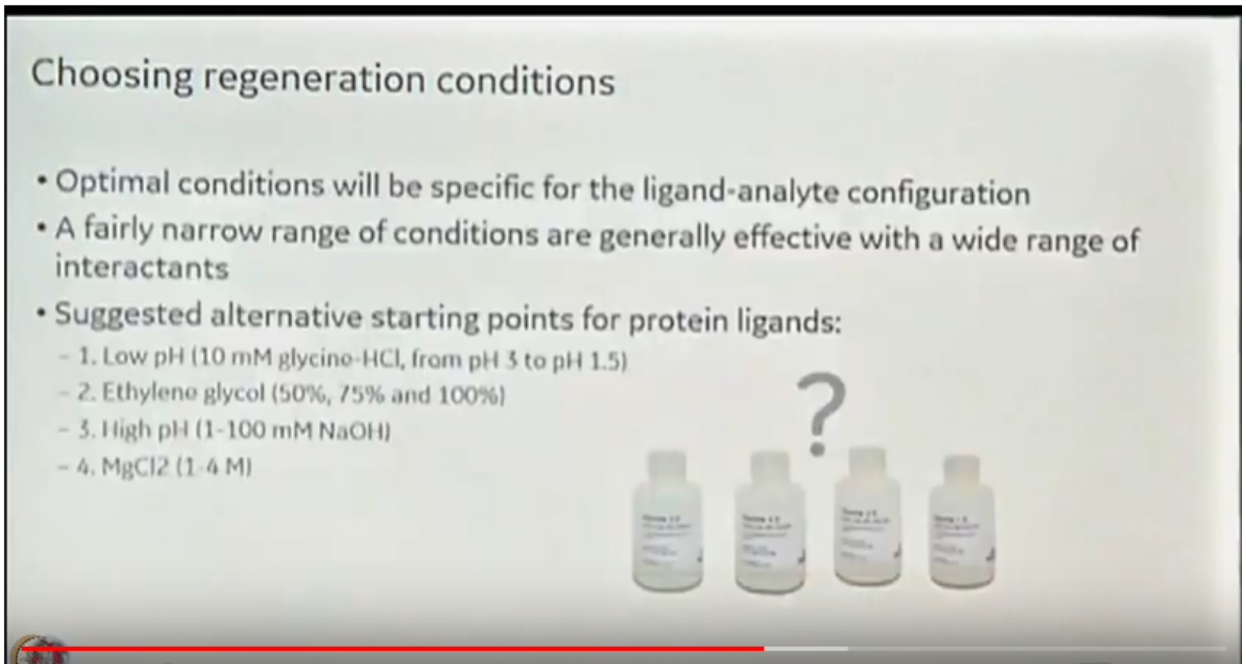

So, you need to do couple, more things actually so, before that let's look at some of the regeneration buffers that we supply but again, these are very generic buffers you can have them yourself ,the regeneration can be low pH, ranging from pH 3.5 to 1.5it can be salt sodium, sodium chloride, it can be detergents, ethylene glycol. Okay? So, various things typically similar things that you use in downstream processing also so, some guidance to find out, whether it was the regeneration, you know how to find out the ideal condition and there is again a scouting wizard that that you can use to check, you know which is your ideal pH condition. Right? So, what you do is a minimum run five cycles, of your, you know use the same analyte concentration, the same page and you run five cycles of it. Okay? So, what you're seeing out here is the analyte binding response and at the same time you need to see your baseline. Okay? How your baseline, is if you do not if your baseline, is increasing that is some definitely something is stuck on the surface. Right? If your baseline is either stable or going down that means it is too harsh. Right? So, let's look at this situation, whereas this one is 5.5 so you have run five cycles, with its three two point five two and one point five. Okay?

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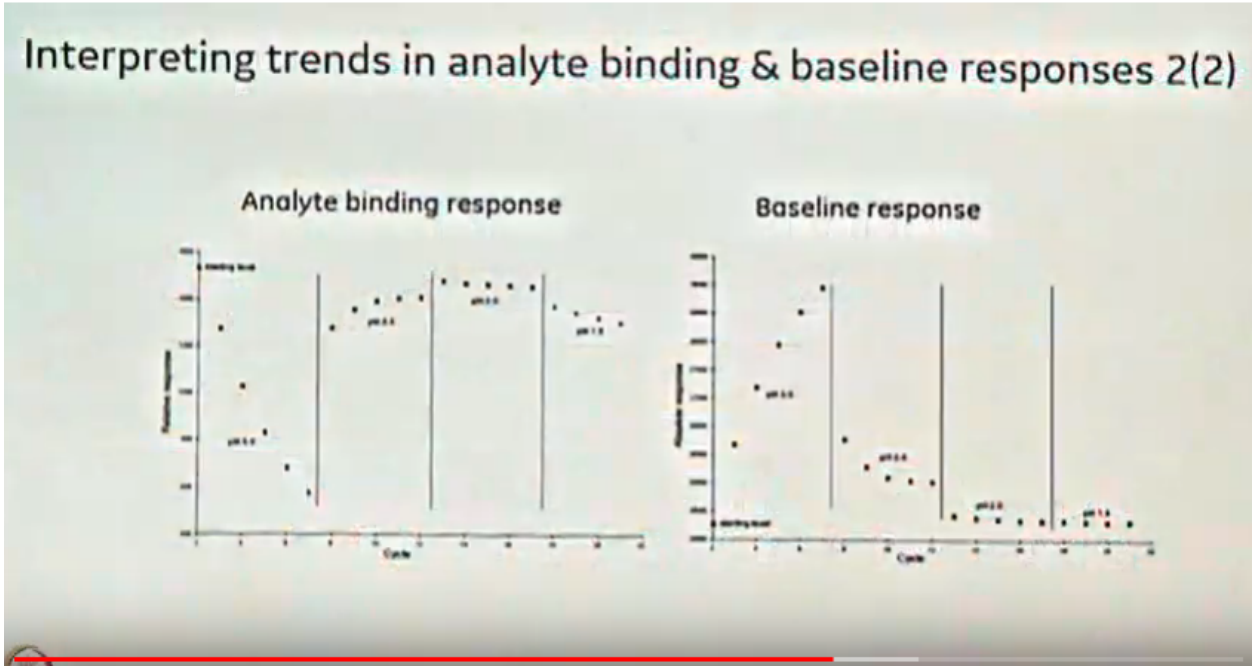

Three two point five two and one point five so, these are the red regeneration that done and you're looking at and an analyte binding here and the baseline response here. If you see at three what is happening? The analyte binding is dropping. Okay? And your baseline is increasing, it's a clear case, of what it's clear it's too mild. Right? Because your baseline is increasing it is truly it is definitely, it's very mild you're not removing all your ligand, as a result your baseline is increasing. Right? And this is dropping because your ligand is not available to bind in the next cycle. Okay? And when you go to 2.5, you see a slide here there is a slight increase and then here there's a slight decrease this is probably, slightly are not explainable because you know in earlier because it was running just after this so, some spillover from three pH 3 is still there. so, not yet you don't know the conditions, yet but if you look at pH2, both are stable kind of like a actually ideal place where you are keeping both the analyte binding as well as your baseline perfect. Right? So, meaning that you are stripping out, everything and each after each regeneration you're binding the same amount, but if you look at 1.5 your baseline is stable, but your binding is dropping. Right? So, when it's too harsh not necessarily, it's going to strip out your ligand so your baseline typically, remains stable but, you're binding goes down. So, that's your harsh condition so, this is again a very good great to find out, your, you know regeneration condition.

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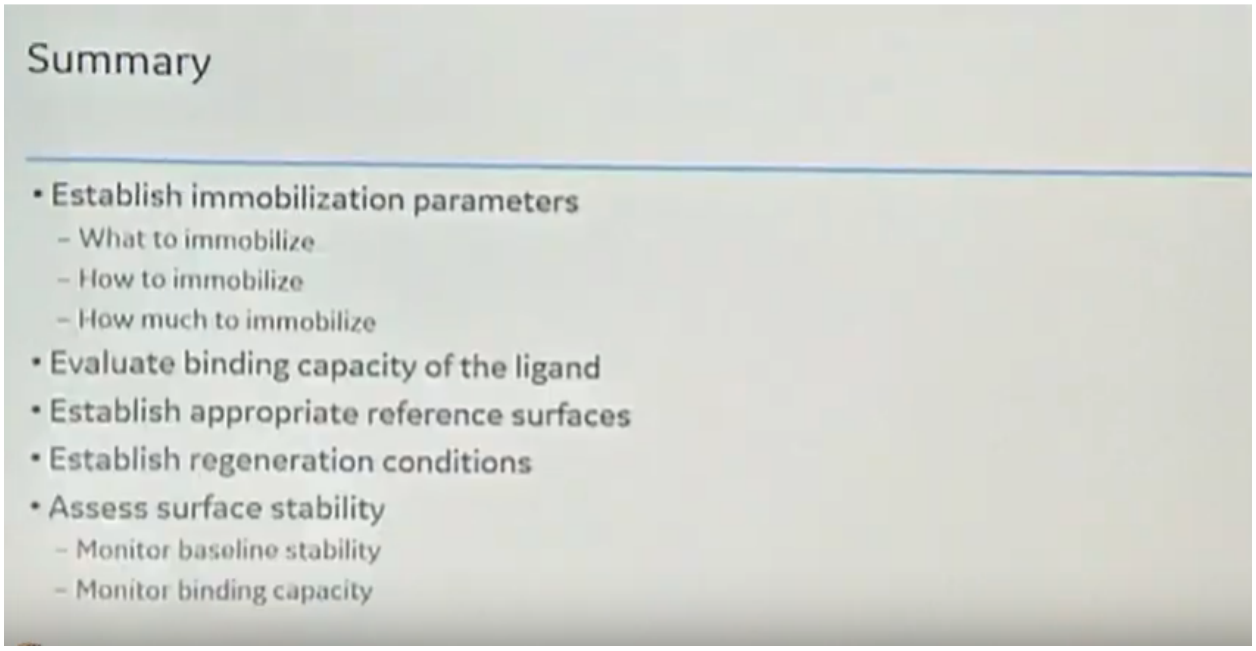

I think that's you know the end of the talk. So, we talked about some you know ideas, to you know how do you do your immobilization, what are the things that you need to take care before you run your samples, your regeneration conditions, I think it'll give you a good start when you start with your BIA caresses. Thank you. If you have any more questions, I'm happy to answer yes sir yeah, yes you can do it but in separate runs. Right? Together yes, absolutely you can do those. So, there are ways that you can do, you know multiple whether, you know one binding is affecting the other binding those kind of studies, can definitely be done absolutely. So, you do one after another. So, once you have your ligand you either mix, to sell you two things and then see the binding what is your response unit and then do the bindings in single, analytes and then check, what is the binding if the binding has increased with mixing, these kind of things can also be done. So, what, what other methods do you use for KD, either the cell based method or the valve or maybe Eliza you can also use to find out the KD. Right? So, you know finding out the KD is not the crux out here, the KD is the affinity value there are other techniques, to find out the affinity value what you and based on the KD or based on the affinity value, if you are actually rating your molecules you might be limited in the knowledge that you have, because two molecules can have the same KD. But they might have different Association and dissociation constants, because KD is actually the ratio between, the Association and the dissociation constant. So, you would choose SPR, when you are actually wanting to look at, the detail of how the molecule is really behaving, you know whether it is a fast associate or a slow dissociate or things like that. So, that is the key her yes, yes. so, you know in typically, when you look at the papers that is published, you typically do it at room temperature. So, whenever you report a KD value, you mention the temperature, at room temperature 25 degrees, 22 degrees, things like that. Okay? Now if you are say for example you do not see a binding, at a temperature room temperature, you might lower it a little bit you know 10degrees, 12 degrees, 15 degrees, oh you know to, to check, whether it is still binding or not in that cases ,you can actually do it a lot of people want to find out their KD values at 37 thinking, that it is more close to the physiological pH to look, at it in that case also you can choose a different temperature yeah. So, the degassing actually removes, all the suspended bubbles or air which is inside, the liquid and you know if you don't remove them they tend to

create, spikes in the response, you know and, and you do not get a very neat, it does I am probably, a little but I don't but it still maintains, well I don't think. So, because you know you, your samples are kept at a particular temperature. Right? Your analysis is happening at a particular temperature, which you are actually setting. So, I mean if even if you are if you claim that you know it changes the temperature, then it is still set at the temperature where you 'resetting and you know you get the, at least the binding and that yeah because, well K, on and K off, are the property of the interact, ants you don't change them typically unless you're changing the temperature they don't change. Okay? So, you can only changes you know increase the temperature, to increase your association. Okay? The rate of association you do need push it a little higher but it is it is the property of the interact ants. So, you know you don't change them, by anything.

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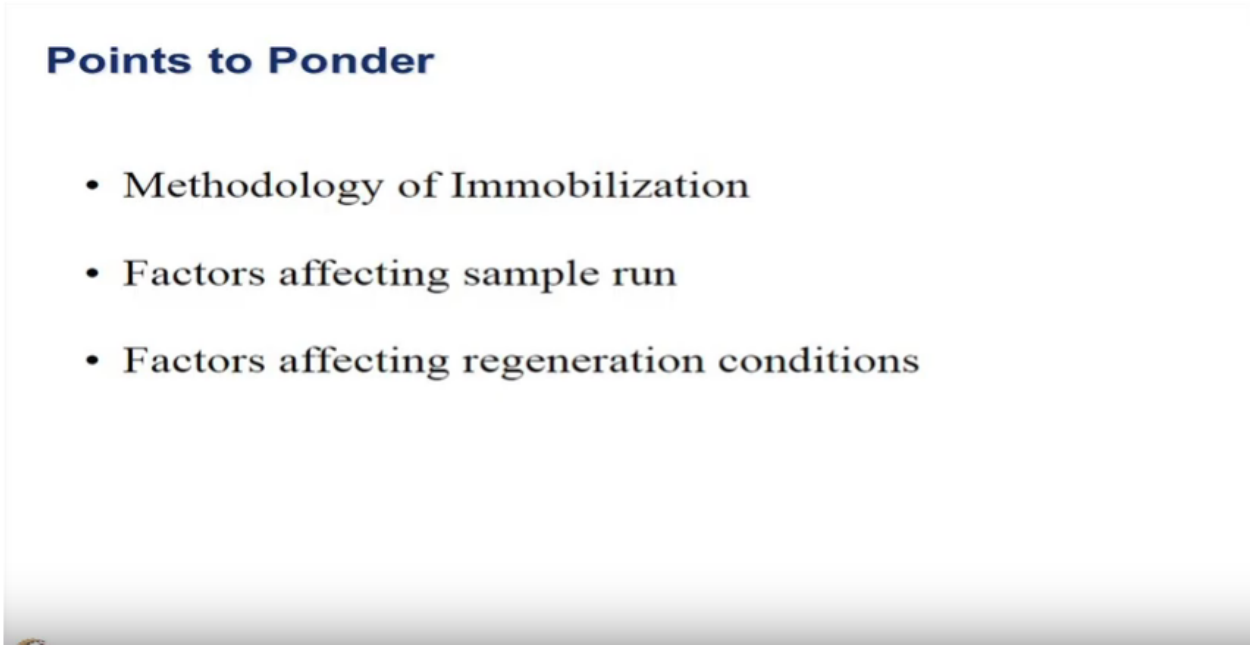

I'm sure by now you're familiar, with the SPR based experimental workflows, you areal. So, pretty confident about the very basic processes, involved in doing these experiments, till the data processing and data analysis, please understand that if you are not very clear about, experimental design and if you are not really clear about data interpretation, you may end up with a lot of false positives and artifacts, in label-free biosensors one of the major, concerns have been how to ensure that a binding which is seen, is it coming from two bio molecules or is it not a specific binding, is it a bulk effect just coming from the buffer itself or some other artifacts which are present on the there's gold chips. And that's where your understanding of these experiments and Integrity's where a Croma talked, to you today, because very crucial to distinguish, delineate that what is the right binding and the data obtained, from that the sensor gram, what says, what could be an artifact, which comes from the bulk effect, some of these concepts,

will be taken further again, in the next lecture, where we are going to have a scientist, coming from whatever moral center, Atrac, who's going to talk to you about applications of SPR technology in biologically relevant problems. Thank you.