Lecture 22
An overview of label-free technologies-I

Welcome to MOOC course, on applications of Interactomics, using Genomics and Proteomics technologies. In the next two lectures, I will focus upon, the label-free detection platforms and their applications, in today's lecture, I will walk you through, various label-free, detection techniques available and their advantages, over the label based, detection platforms, to study the molecular interactions and their kinetics. There's always a debate, whether we want very accurate quantification, using labels or

labels are going to affect, the properties of our molecule and we should use, the innate properties and have the label-free quantification. So we'll have these discussions, in this lecture, further we will discuss about, a few label free platforms, in detail and their interpretation of difference programs obtained. So, let us continue, with my lecture, which I delivered in this workshop. Let me start with the outline, what I'm going to talk? A couple of things which you have been hearing already the feel of Interactomics.

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Outline

- Interactomics
- Detection Platforms
- An Overview of Label-free Techniques
- Latest Popular Label-free Techniques
- Applications of Label-free Techniques

I will not take much time on that. But then, I'll move to comparing the detection platforms, which is because you are, hearing a lot of lecture some Josh, on protein microarrays, you've heard many of these speakers on rivets feel arrays and peptide arrays, all of those require, some level of you know, label based, detection system and study you also started getting exposed to some of the label free platforms. so brief comparison, of those two type of reduction platforms, some of the overview of the label free technologies, there are many in fact, you know, you just got some exposure of SPR a steady, surface Plasmon resonance. But, they're already know, many emerging technologies, which are already in have it started making some impact, but many of them are still in the testing phase, some of the latest popular label free technology platforms, as you go along in the course and then some of them are brief overview of applications, I will not have too many cases studies. But, I will at least give you the flavor of what all things can be done. Because, our intention with this course is, that meaning of you can start planning your experiment and think about, you know how broadly these technologies could be used for everybody. Because, they're not biased for any kind of sample, they're not biased for any specific project, depending on your objectives; I think you can very much modulate; these things for your own research. So, I'm sure by now, you are convinced that you know, these kinds of technologies.

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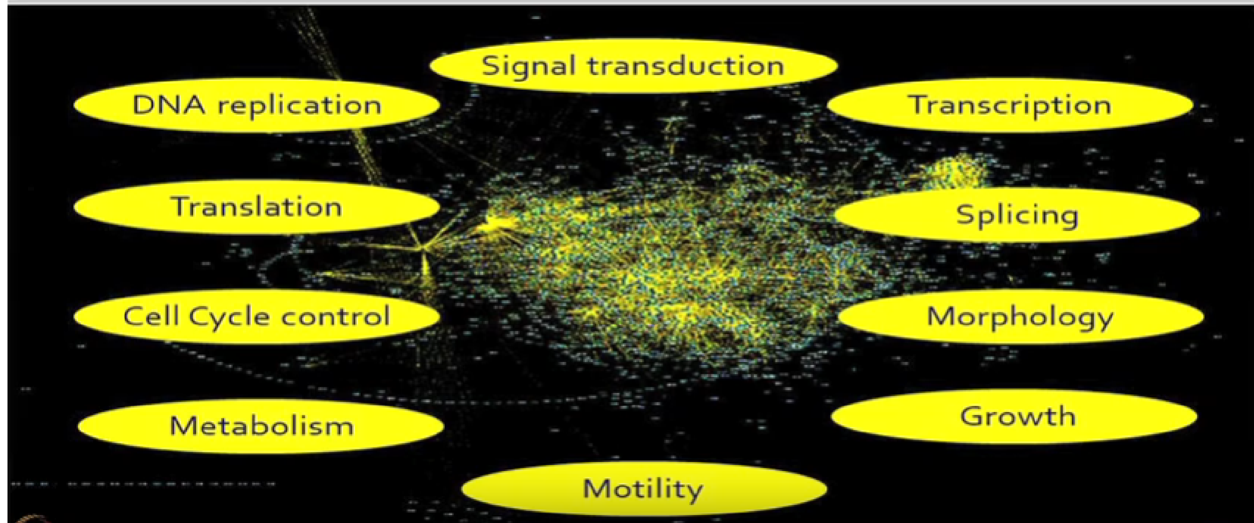

Are very useful to study the entire Interactome, which is involved in many of the important, activities whether you talk about, signal transduction, to splicing, morphology growth ,metabolism, translation, DNA replication, these are all pretty relevant, for any kind of physiological processes, which we want to study.

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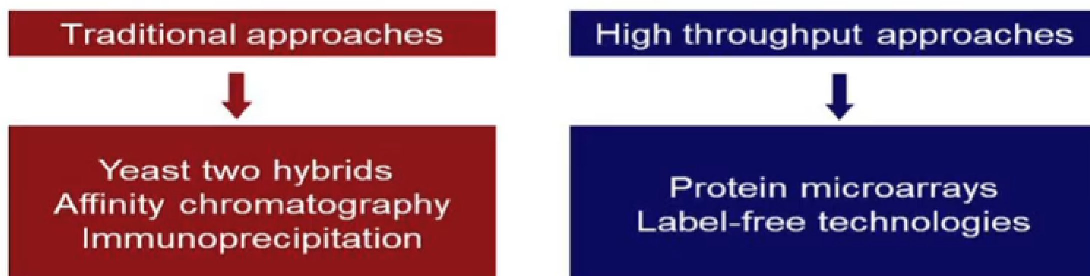

So therefore, many type of protein interaction methods have come forward, the traditional approaches includes is to hybrid and different type of IP methods. and the latest technologies, you are now happy and convinced that, you know protein microarrays, are emerging as the one of the solid platforms ,including cell free expression based arrays, which includes NAPPA arrays and then, we have couple of label free platforms, biosensors, which are equally crucial as well.

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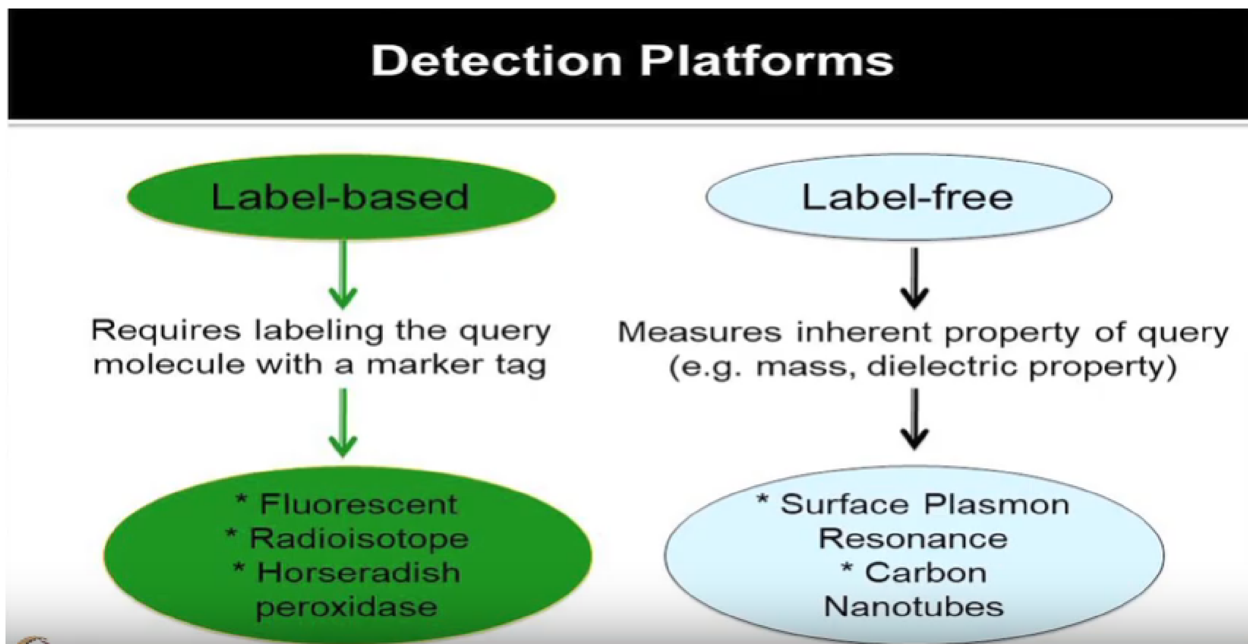

So let's talk about, detection platforms and their comparisons. So, you want to detect the signal after doing the SI. Right? And that's where, whatever you have hypothesized, at the end only looking at the dots, are looking at the curve, you know, that your experimental work or not. so therefore, you need to have some robust detection platforms and to do that, either you are using some sort of label, to follow your experiment and then, you are trying to measure those labels, either with the fluorescence based methods or Chemiluminescence based methods, something, even radioactivity businesses, can be very powerful. So, you require some sort of labels, which could be used as a marker, to follow your experiments or you can also think about label free approach, because whenever you are labeling a protein, irrespective of what chemistry you use, you are adding something from outside on a given molecule and that is going to definitely, in some way affect, the overall structure, overall binding and it may result into certain artifacts.

So, for many kind of you know, critical drug discovery platforms, it is, you know, it becomes more apparent that you probably do not want to change, the narrative structure of the molecules and you want

to study them, in their own you know, natural environment, as much as possible. Without adding any sort of extra label on top of them, as it is at many type of label free platforms have emerged, which essentially aims, to look for, the properties of the molecule itself. if two molecules are binding ,what kind of mass is getting changed? What kinds of dielectric properties are getting changed? do we see some sort of percentage effectively change, because of the binding intensity can we see some sort of interference change, many of the physical principles, are being used to look at the molecules and molecular interactions and can we measure those properties. So, as I mentioned the label based, the red ox could be many of these, fluorescent, radioisotope, HRP based systems, for label free you got little exposure of SPR, even many of the nanotechnology based platforms, like Carbon Nanotubes, etc, also equally powerful and they have been used, for doing this kind of measurement as well.

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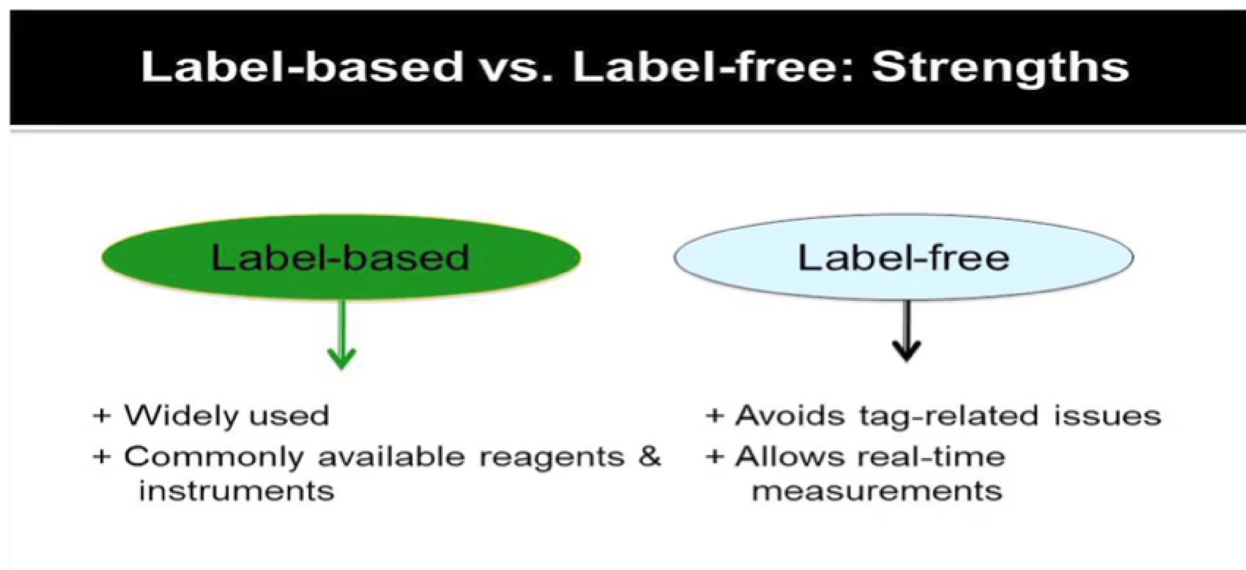

Label based, one of the major at is, that these technologies are available, the kind off you know, every lab, every Center will have those scanners. So, that you have easy readouts, you can definitely do the scanning, I'm sure in any given building you will have some of these, instruments available, Regents are easily available, even if you don't have a sharp, you don't have sight 3 or any of the dice, I'm sure you can borrow from somebody in the neighboring lab and you can do the experiments. But, you cannot do these kind of experiments, with the label-free kind of approaches, when the technologies are very specific, however the label-free platforms are avoiding the tag rated, issues which you observe in case of label based and most importantly, if you are actually monitoring, the bio-molecular interactions in the real time manner. and that's pretty powerful, because all the protein micro technology are great, but it's like you know takes whole day time, to do the experiment sometimes, when you no longer, if you are doing a blocking at all and at the end of the day, then only you are relying on the scanner to show you, that you know, your spots are lighting up or not.

Or you see some sort of you know huge background, you see you know, your controls did not work out and you really you know hugely disappointed, but you had literally no control, on has the experiment progressed whole day, in this case here if binding is not working, you will you know, after universe just for 20 minutes time stop it and now you will bring the solution, change the concentration, change the temperature, do different pH scouting, so you have many ways of planning, to modulate the experiment, you're not going to just wait for something to, happen 10 hour time and then you say, that is happen or not happen. More importantly, that you know in case of microarray or other kind of label based platforms, you are only measuring the signal at the end, which gives you an idea it happened or did not happen. So, you have a positive signal or a negative signal. So, an interaction happened or a biomarker is present, you can just say qualitatively, it is showing you signal. And of course, you know, that you have many different measures, of having different controls, which could be used to do the condition as well, but in this case when you are talking about some of the label-free platforms, you have even ability to say that you know yes, binding is happening. But, what can be on rate? What can we offer eight? What can be overall decision constant for these? And how my kinetics is actually getting changed. So, these are the added information on top of, what you only obtained in the microarray-based or any kind of label based platform. So, that is added advantage of the label free technologies.

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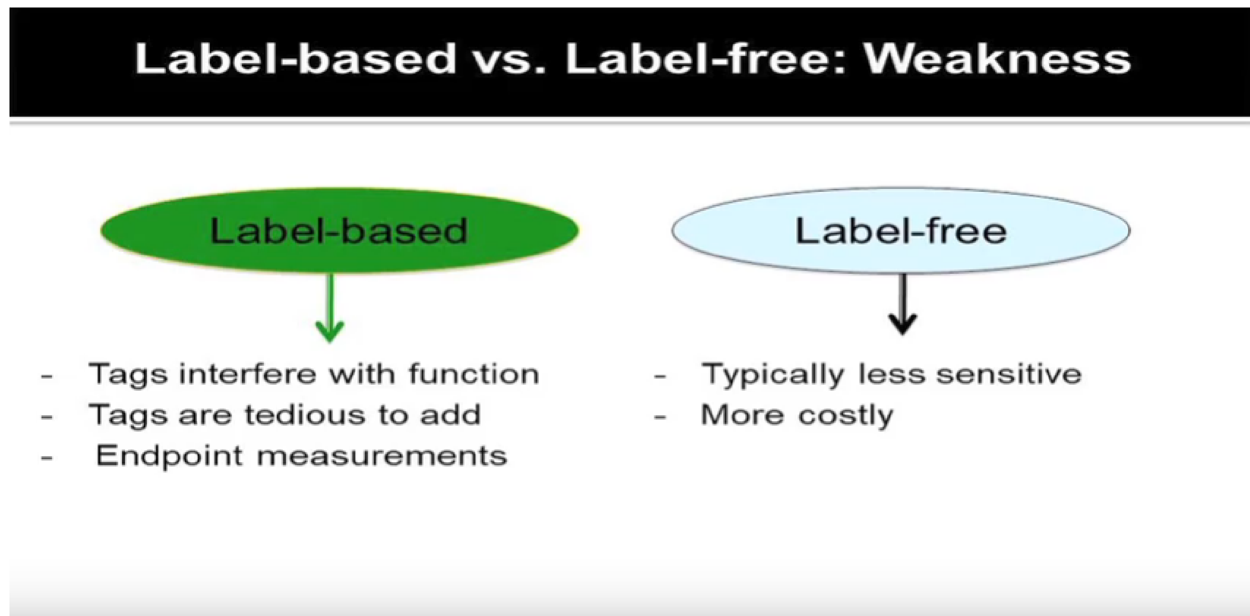

So, label based because of the tax, you know, you there might interfere with a function; you may have certain kind of issues, which can give you artifacts. And this is endpoint measurement. Because, you are not having control, on how the SE progresses. label-free technology having lot of advantages, but there is still very specialized, many of them are not available in many labs, you will not find the those biosensors available everywhere, they are sometime more costly and again you still have to ensure that, all the signal

which you measure is coming because of the actual binding and not because of the bulk effect, not because of something else which is happening and you are seeing in art factual response ,so many of the things that is still under the kind of, you know, more in fancy more early developmental stage, which needs to be tested out well.

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So now, just to give you a feel that you know, we are covering, two or three level free technologies in this course. But, there are many which are actually under development. and there are lot of physics, people who are actually know contributing towards this way of making many type of biosensors, where they are involving different type of physical principles, how they could be used to measure, molecular interactions.

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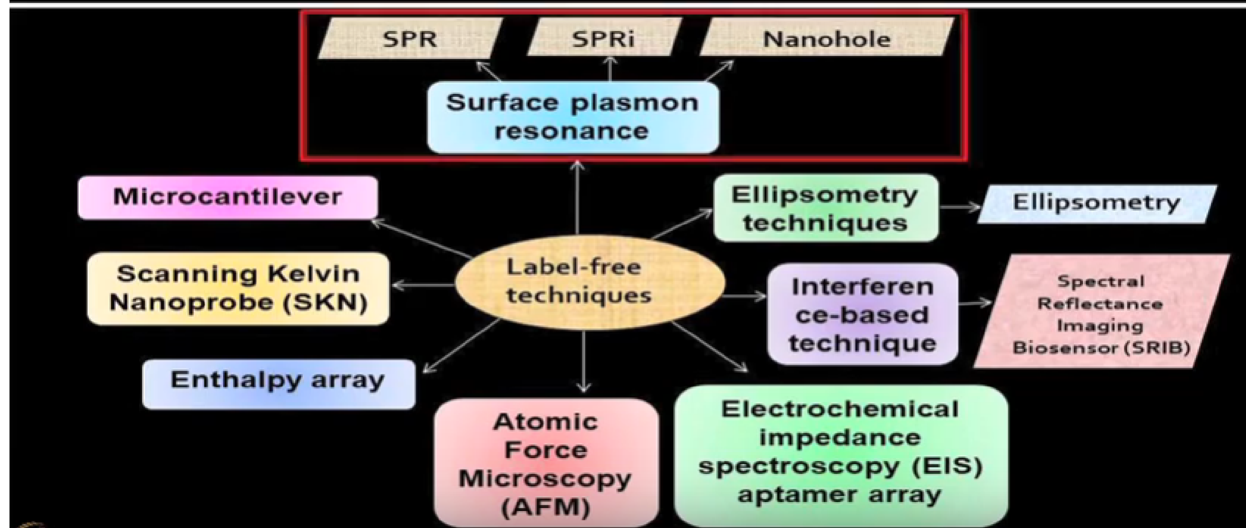

So, you know, so many I'm sure you know, you can find out SPR just one among them, one of the label field platform, which can give rise to multiple type of even technology platforms, including surface Plasmon resonance, which we talked briefly, surface Plasmon resonance imaging based platform, SPRI or Nanohole arrays, all these three depends on one of the principle, which is SPR then there are many platforms, which are emerging based on the ellipse symmetry. We have interference based methods, we have electrochemical impedance spectroscopy, atomic force microscopy, enthalpy erase, scanning Kelvin Nanoprobes, micro cantilever, so I'm sure, you appreciate there are many technologies which are under development right now. They're all looking at different type of physical principles, which could be used for, looking at the bio-molecular interactions in the label-free manner.

So, while we cannot talk everything right now, but at least some of them I will, I will talk, I will give you some examples of those, but idea here is that you know, many times when a physical scientist work on these kind of biosensors, their objective is to only show, that two molecules are interacting and by looking at interference or conductance change or you know, reflectance change, you can measure a binding. and then, they stop over there because they would have taken, very standard protein six, you know they will take BSA, anti BSA or they will take you know, if you till there are five more interesting proteins to test out they, will take those, highly abundant protein and the robust antibodies, to test out those assists and this thing's are usually not good. Because, when you want to try out your actual experiment, which is depending on many, time low abundant proteins and antibodies are not so great for them, then I think you know, your assay don't work as robust, as you would have seen the you know, proof-of-concept level. So, many of these technologies are still under proof-of-concept level, which needs to show, that what could be done at the actual medical sample level, therefore what all I have shown here, not everything has reached to biological lab and not everything is actually holding up, to the biological experiment. Because, when it's got samples, they have lot of low abundant proteins, you will not have that as strong and relies to test out, alright.

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Label-free Measurements

- Provide real-time reaction kinetics to determine dynamic parameters of biomolecular interactions
  - Kinetics, Affinities
- Protein functional behavior by developing models and wiring diagrams
- Different label-free techniques in various stages of development

So, all the label-free measurements, a respective of which platform we talk, they're all measuring the bio-molecular properties and it's pretty much kind of, without changing anything on top of them. And it's adding the information, which is providing you, either kinetics information or you are looking at the affinities, these are added information watchman could obtain, along with you know, just yes/no answer which you can obtain from other binding spirits. you know more importantly, the new area which are emerging, which is essentially systems Banerjee area, the intention is can be generate lot of these values, which is on rate of, rate for different type of binding, interactions which are happening and now develop those other model and wiring diagrams, which could be used to predict many unknowns, because how many actual experiment can you do, for so many no proteins.

So let's say, you know for a given protein, vial type condition and there are many mutant forms, which you want to test, their affinity certain you know, with a drug or with other, other molecule. Now, you can build it safe from 50 of their mutants, you can develop some of the values, which can give you the some models. And now, can those models be used, to predict the behavior of non-mutant. So, that kind of stuff is going to really happen, if we have the available information or at each level, of on rate of binding, off rate of binding, what is the KD value? And then, you build the models and there's a lot of systems bellow just, have a started working in this area and are going to contribute, much more heavily towards these directions. So, different type of label-free techniques, as I mentioned are in various developmental phases, of course way a core is, pretty leading in that way. Because, you have already over know 20 plus years, of the technologies in field available, but many technologies you know, which are still just emerging including ,some of the bio layer Interferometry based methods, have really come off very forward, fast even a masti technologies are emerging, so those could be good platforms to be used.

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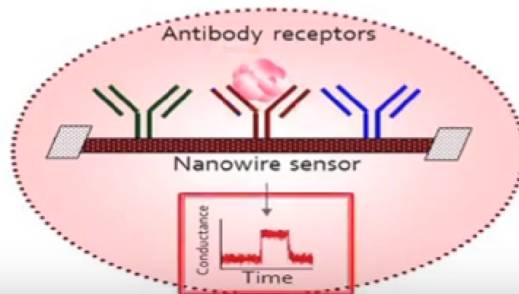
Carbon Nanotubes (CNTs) & Nanowires

- CNTs are hollow cylindrical graphite sheets
  - Exhibit high level of chemical stability and mechanical strength
  - Unique and well-defined electrical, mechanical properties
- Most promising candidates for development of nanoscale biosensors



Zheng et al. Nat. Biotechnol. 23, 1294–1301 (2005)

Change in conductance when protein binds specifically to its receptor on nanowire

I'm just showing you, very briefly, some of the principles involved of a various type of label free technologies. One of them is showing you here, know these kind of nano wires, Nanowires sensors. On which antibodies are immobilized, if a protein binds specifically to this empty body, then you will see a change in the conductance happening and this is recorded here, over the time period. simple technology, simple concept, can have so much utility clinical voice, imagine that you know, when a lot of Start's issues were happening, different type of viruses people are getting affected, that team you know immediately on lot of you know, metal detectors on the airport's, these type of platforms were used, to detect that whether patients or the individuals who are coming, are they carrying these kind of antigens, possible and then they were quarantined and all kind of these things are happening on the Air Force, so just you know, immediately this papers were published, lot of you know, applications are started emerging out of them. simple concept you are measuring, antibody antigen interactions, looking at the conductance change and if that can give you some idea, that you know, some individuals are carrying, those antigens, which are going to give rise to certain viral disease, then probably you can do, much more specific tests on those.

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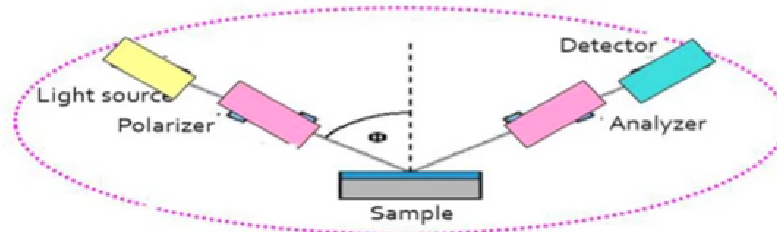
Ellipsometry

- Based on polarization state of reflected light
  - altered due to changes in dielectric property or refractive index of sample surface
- Imaging ellipsometry combines ellipsometer, microscopy & CCD camera – measures protein content on solid surface



Laser light, linearly polarized by polarizer produces elliptically polarized light. Light reflected from sample surface, detected by analyzer.

So, another platform which is you know, which has shown some promise, because of the low cost and that is Ellipsometry based platforms, where you are measuring how much polarization change can see, as a result of binding. and you know, again it's very a simple setup with, involves a microscopy and CCD camera and it idle emails lately protein contents, which is not unlike the other platforms, which are requiring lot more costly setup, this platform is very cost effective method, all that has not shown as many biological application, as we have seen, from other platforms but this was a robust thing to it to show, that in cost-effective manner you can do label free technologies.

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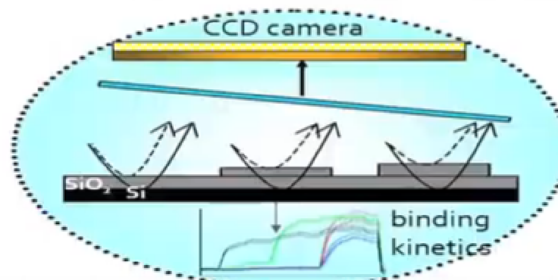
Spectral Reflectance Imaging Biosensor (SRIB)

- SRIB, a label-free approach based on interference
  - Changes in optical index as a result of capture of biological material on surface detected using optical wave interference
  - Directly monitors molecular binding interactions



Interference of light reflected from SiO_2 surface. Increase in optical path length differences caused by biomolecular binding is measured

One more thing, which you know, we have seen some good promise of this paper, based on a spectral reflectance imaging biosensor or SRI B. where you have you know different layers of silicon and silicon dioxide and by varying the low concentration of these, if you have now the protein printed in different amount, you can see the change in the binding happening and this particular, concept will again no published in PNAS, which shows how beautifully, just looking at the change in the interference ,you can see that, how much material is printed in different quantity? And can be measured, in the label free manner.

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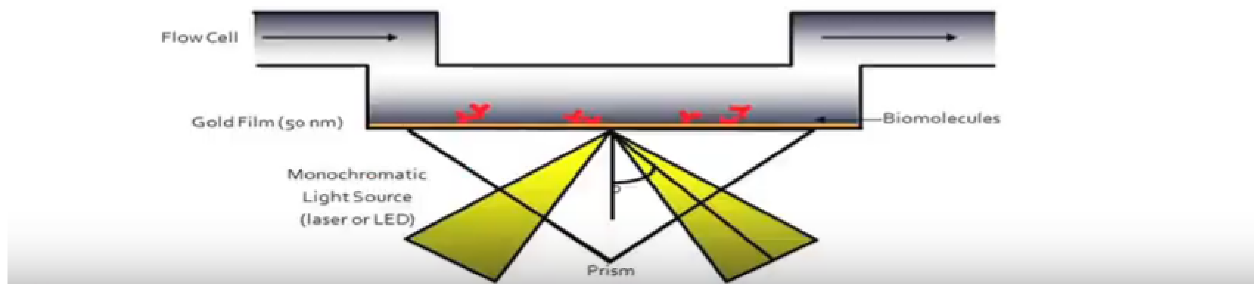

Then comes the SPR technology, which is surfaced on resonance, which you are exposed already on a prism and on a gold slide, you have certain antibodies printed for example, some protein molecules are flowing here and as you see, the binding is happening, by changing the percentage of activity, you can measure those particular things in the real-time manner.

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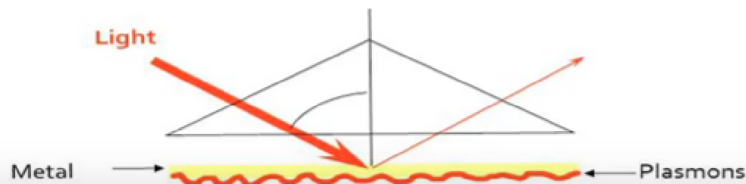
What are Plasmons?

- **Plasmons** - special electromagnetic waves that can be excited at certain metal interfaces, mostly gold & silver
  - generated on boundary of metal & external medium (e.g. air)
  - very sensitive to any change of this boundary (e.g. adsorption of biomolecules to the metal)



And what you are actually measuring, these kind of plasmids. which are on the interface of the gold surface, when these inner electrons are being generated and those you are measuring throughout your, you know, reaction and by changing the solution, then you are seeing, what is the SPR angle being changed, from one to other condition and those SPR angle is being used to monitor, your entire binding reactions.

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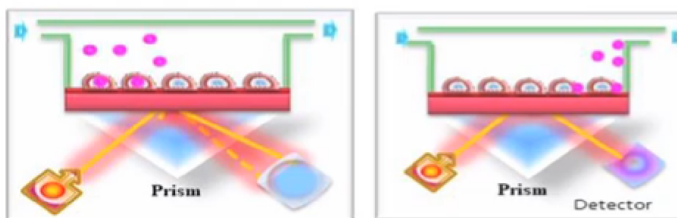
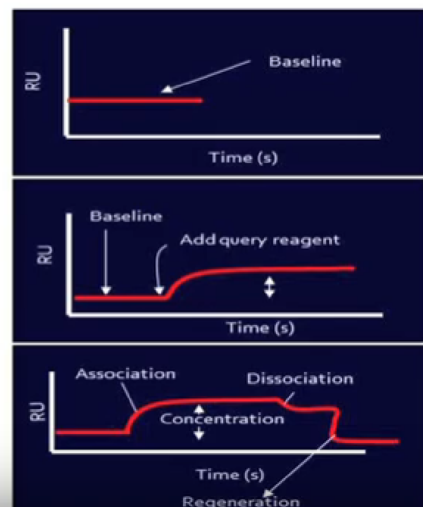
SPR Sensorgram

- Changes in SPR signal versus time





And that you measure in, in the terminology, which is used as a speed sensor gram. these sensor grams could be used to monitor your entire binding activities, for the reactions, for example, initially you were you are just you know, having the molecules coming in and start to bind, with the antibody for example, you can start seeing there is an initial baseline there, which is stable, as the reactions are happening, there is some binding happening, you can see a non rate happening, association of the molecules and then, after sometime they will you know get saturated, you're still injecting your buffer and then ideally they should start washing off and then, he will see you know, off rate going in and now, the you know you are doing these experiments on the noble metals, which are very costly, essentially gold you are using ,so you want to regenerate, you want to reuse the same slide, again and again, at this couple of times. so then, you are trying to do regeneration and you are using some mild assets, to chop off your this binding of molecules, without disturbing your you know, printed molecules and then this chip can be used to after, you know ,again you have to wash off in the buffer couple of rounds and the same chip could be used again for next round of interactions. So, in this way, you know you can reduce the cost of the experiment, otherwise the gold slide and all these chips, which we are buying are way too costly.

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Kinetics and Affinity Determination

- Kinetics: Rates of reaction
- Affinity: Strength of binding
- KD = Kd / Ka
  - Dissociation constant (KD), on rate (Ka), off rate (Kd)
- Kinetics as the rates of complex formation
- Fitting data according to model

So, but what in its offering you, of course it's offering you, much more powerful information as we talked, you are getting the kinetics or the rate of reaction, how you you know you can monitor in the label-free and the real-time manner, you are looking at, both on rate and off rate of these, reactions to happen, unlike the label based methods, where you are just talking, a binding is happening or not happening, here you are saying, that okay binding is happening. But, probably the one rate was very fast or offer it was we know very slow, so those kind of information you can obtain. And many of these information's having lot of utility in the Pharma sector. and that's where you know, even we see that you know, more than academic labs, the you know, via code and SPR and many of the label, fill technologies are much heavily used by the Pharma sector, who are discovery and drug designing and testing kind of you know experiments, they have lot more utility for no using these platforms because, it's just having

binding information is not sufficient, how the binding is happening, on rate and offer differences are much more, crucial to to know, over there.

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SPR: Advantages

- Label-free
  - No need for tedious or expensive labeling protocols
  - Avoid potential labeling artifacts
- Direct
  - Measures binding of the actual analyte
- Real-time
  - Allows user to watch the experiment as it happens
  - Not an “end point assay”
- Measure binding kinetics and affinity (k_a, k_d, K_D)

So, advantage of SPR. I sure you are all convinced that you know, you have lot of promise here, you are not doing any sort of extra thing, on your molecules, which you want to test for interaction, so there is no, labeling which you are applying. you are directly measuring ,the bio-molecular interactions using the physical properties and then, you have full control on, how the reactions are progressing and as a result, you can actually modulate the conditions to make it work. many we are trying out you know, different type of concentrations of different antibodies, than we are doing the first initial screening or you know, you can use different type of flow rates to even try out, so many things you can play with it, in that in the initial round of experiments, to find out what can be the best condition for your binding, to be observed. and now, once you finalize those condition, then you can apply the same on your, full experiment with many of the molecules of interest, alright. So, I think we have talked, that you know there is lot of merit for using these things, but of course there are limitations, as well.

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SPR: Limitations

- SPR detection relies on mass changes
- SPR detection decreases exponentially with distance from surface
- Estimated detection limit ~200 nm
- Limited to choice of metal which results in SPR

Because, everything what you are relying, looking at the mass changes and sometimes this could also come from the bulk effect, that is may not be the actual binding happening from the molecules. So, where you are immobilizing the molecules, depending on the size of those, detection also you know, going to get less or more sensitive, because exponentially as you know, the distance is further from the chip surface, you are going to see the loss in the signal. and the detection limit estimated is around 200 nanometers, in this case is, limited to the choice of you know metal which you are using, because that is the gold mantle, which is useful for doing these, kind of experiments.

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SPR: Limitations (2)

- Sample must be homogeneous
- Sample preparation and probe attachment to metal surface can be difficult
- Non-specific interactions also results into SPR signal
- Need to ensure specific signal
- Avoid bulk effects
- Refractive index is temperature dependent

Your samples has to be really clean, it has to be homogeneous, you have to do the you know proper removal of all the gas etc, just so that you know, bubble is blocking ,your columns there is nothing is getting clogged, as your you know flow rate is changing for measuring the binding. So, sample preparation and these you know probe attachments, could be difficult, but I am sure - these are some of the generic thing, which is applied for any kind of you know, the basis in which you have to follow, you have to ensure that, you know, your washing and your all kind of cleanup is steps your well performed, otherwise you will have many nonspecific interaction which may result, to ensure that you are getting a specific signal. it's good idea, to have lot of positive controls and you can vary the concentrations of those and see you know, tell what is smallest, lowest, concentration you can still measure those signals and those can be used to follow your various, you know, not everything you can do on one chip, if you have to test many of your molecules. so then, those kind of you know, one flow say let's say, I'm sure you are aware of the different, flow cells involved in the way a core experiment. For example.
So one flow cell you can just block it, which can be used for, you know, your QC for many experiments and then those could be and you can vary the other flow cell, for testing your unknowns, so therefore you will have a still full control even as you are going along, the chip has not gone bad and your you know, the molecules are for positive controls are behaving same way. So, of course you know, many of these things in the field of label free technologies, you have to avoid ensure that, the binding what you are seeing is actually real binding ,it's not artifacts, it's not and sometime you will see a reaction is happening on the temperature dependent manner, so you have to also you don't play with those, I think fortunately not some of these instrument and technologies, have not to give you the range from four degrees to know, you can vary till 37 to 50 degrees even ,to try your different conditions. of course you know, we are in this course about, high-throughput platforms, intention is I'm sure you know, with Josh talk you are pretty much, you know convinced that, if you can have the chip which can screen 10,000 proteins, of course you know you are going to talk very quality late, high quality data you are reducing your time, you are reducing the cost, everything and so much variation which comes with a by doing you know, to and experiments on if you had 1,000 protein, on one chip or sis 10,000 on one chip. Right? same thing is applicable here, as well on the SPR base platforms, where if you are doing only 4, maximum interactions or 4 maximum testing of the you know binding, on the wire co-trainer platform, what says if you can do thousands, or on a different platform. and that's where the concept of SPR imaging, became very powerful, with hope that can we build the platform ,fish can do the high throughput interaction studies, using the same principle, what we have used for SPR?

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Points to Ponder

- Advantages of label-free detection platforms when compared to label-based detection systems
- Various label-free detection platforms available and their principles
- Utility of Surface Plasmon Resonance (SPR) in understanding the kinetics of biomolecular interactions and their affinities
- Analysis of the sensogram and basic steps involved in performing interaction studies using SPR

So I hope, after attending my today's lecture, you are now able to appreciate, the utility of using label free, detection techniques, to define the bio-molecular interactions and analyzing, their binding and kinetics. Although there are many label free, detection platforms currently available. But, most of them are is still under infancy and they have shown the utility, only at the proof-of-concept level, it means, most of these new technologies and bio sensor platforms, they have shown the experimental evidences, that these principles were, only with the known pair of robust antigen and antibody based detection. however when you have to test out, the actual biological samples and the bio-molecules, which are in the physiological context, they are very very in spatial amount, very low concentration ,then we need to think about, how to use technology platforms robustly, in this light one of the technology based on the SPR and the Bayer Core Technology, have over the period ,from long time showed its utility, also a new advancement in the area bio layer Interferometry technology, has also shown a lot of promise, nevertheless SPR is one of the most acceptable platform to study, the bio-molecular interactions, whose principles and data analysis was explained in this lecture. I hope now by looking at a sensor or ground, looking at these binding curves, you can now make sense of what has happened in this particular experiment, especially for the interaction candidates. We'll continue, our discussions on latest advancement in this area and also how to integrate, different technology platforms with existing label-free biosensors, in the next lecture. See you then. Thank you.