Proteomics: Principles and Techniques

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Lecture No. # 35
Label-Free Techniques: SPR and SPRi

Welcome to the proteomics course. In today's lecture, we will talk about Label-free techniques such as SPR and SPRi. Detection techniques are often classified as label-based and label-free techniques. Most of the biological applications have employed label detection such as fluorescent chemiluminescent and radioactive labeling these labeling strategies have synthetic challenges multiple label issues and may exhibit interference with the binding side. Therefore, development of sensitive reliable high-throughput label free detection techniques are now attracting significant attention the label-free detection techniques monitor the biomolecular interactions and simplify the bio assays by eliminating the for secondary reagents required in label-based detection techniques. Moreover, they can provide the quantitative information on the binding kinetics surface Plasmon resonance SPR surface Plasmon resonance imaging SPRi are most commonly used label-free techniques which we will discuss today.

The commonly SPR measurements are collected in one of the three modes the scanning angle SPR, SPR wavelength shift and SPR imaging the surface Plasmon resonance or SPR is a popular surface analysis method, which is used to detect changes in the refractive index or thickness of an adsorbed layer on or near an SPR active surface with high sensitivity. Commonly the SPR measurements are collected for SPR imaging for various high-throughput applications the SPR imaging provides an expanded and collimated polarized laser beam, which travels through the prism and reflects from metal dielectric interface the reflected light intensity from the illuminated area is monitored by the CCD camera which produces image in SPRi.

SPRi is the potential alternative to the label-based detection techniques and it offers nearly comparable sensitivity to single color as well as dual color labeling methods, but despite the promises of SPR and SPRi. There is a still need for further improvement for various high-throughput biological application.

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So, in today's lecture we will talk about detection techniques, the label-based and label-free. We will try to provide you a comparative vision of both label-based and label-free detection techniques then we will discuss about some of the promising label-free techniques such as SPR and SPRi. So, let us first talk about detection techniques there are several conventional label-based detection techniques, which employ fluorescence chemiluminescence and radioactive isotopes they are in practice, but researchers are now exploring the methods for label-free analysis to eliminate the interference due to the tagging molecules and reduce the complexity of assays.

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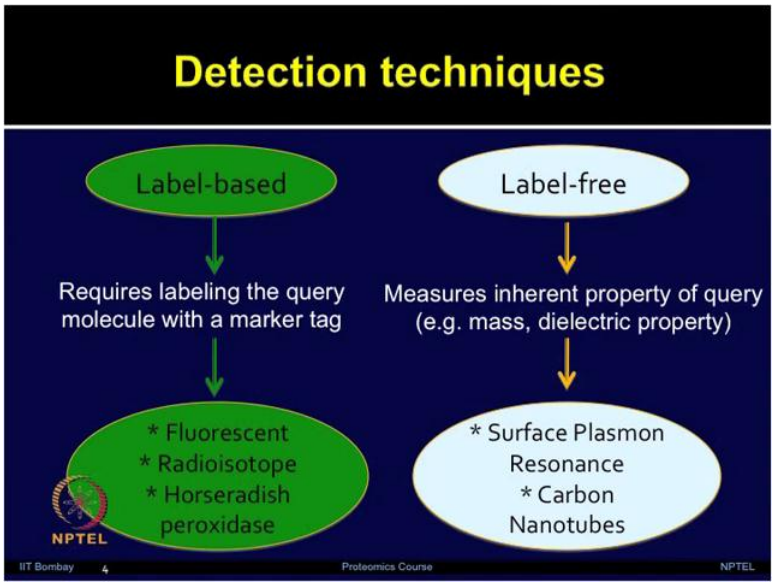

There are two different type of Detection techniques broadly we can classify them as Label-based and Label-free. The label-based method requires labeling the query molecule with a marker tag this tag could be Fluorescent based Radioisotopes or HRP based Horseradish peroxidase. Whereas, the label-free methods they measure the inherent property of the query the mass dielectric properties etcetera. There are various label-free techniques, which are emerging some of the most promising one includes SPR SPRi and carbon Nanotube and different type of nanotechnique based biosensors.

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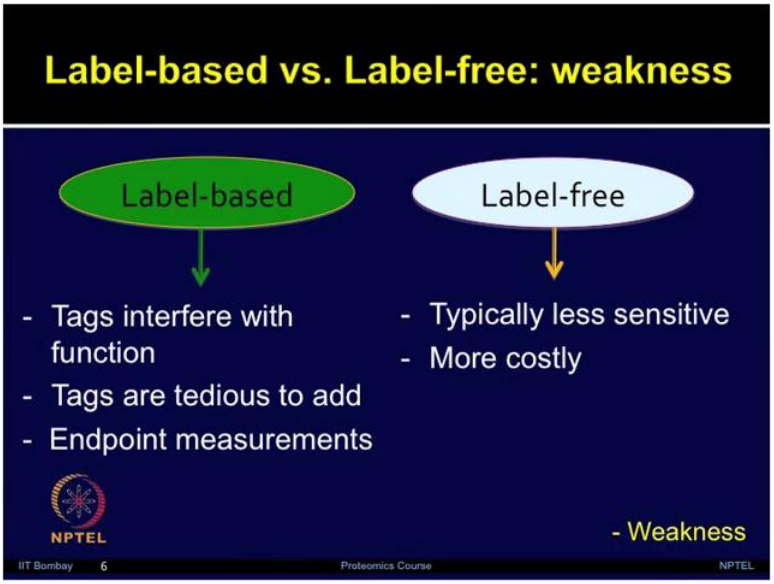

So, let us look at the strengths and weakness of label-based and label-free methods label-based methods they are most commonly used in most of the laboratories worldwide. So, most of the reagents are very commonly available. So, as the instrumentation the label-free methods; however, are mostly new. So, the instrumentation is still very costly it is not. So, easily available, but this method avoids adding any tag-related issues.

So, that is an advantage over the label-based method moreover it provides measurement in real-time and also provides the information for the kinetics which is not exactly possible by the most of label-based methods. Now let us look at the comparative weakness of both Label-based and Label-free methods the Label-based methods since they are employing some tags that tag itself can interfere with the protein function.

Now, these tags are not always easy to add to the query molecules and finally, these label-based methods they provide end point measurement. You have to perform assay

whole day and then at end of day you can learn whether your assay had worked or did not worked whereas, the label-free techniques gives you an advantage of providing the data in real-time. So, that is one of the another advantage of label-free over label-based methods, but comparing the label-free they are typically less sensitive with the label-based methods and they are most costly as I mentioned the instrumentations are not so easily available.

So, now let us focus on the Label-free measurements. The label-free techniques rely on measurement of inherent properties of the query molecules such as mass and dielectric property and it allows the direct real-time detection of biomolecules in high-throughput manner, which eliminates the need for addition of secondary reactant which is a case for label-based detection techniques.

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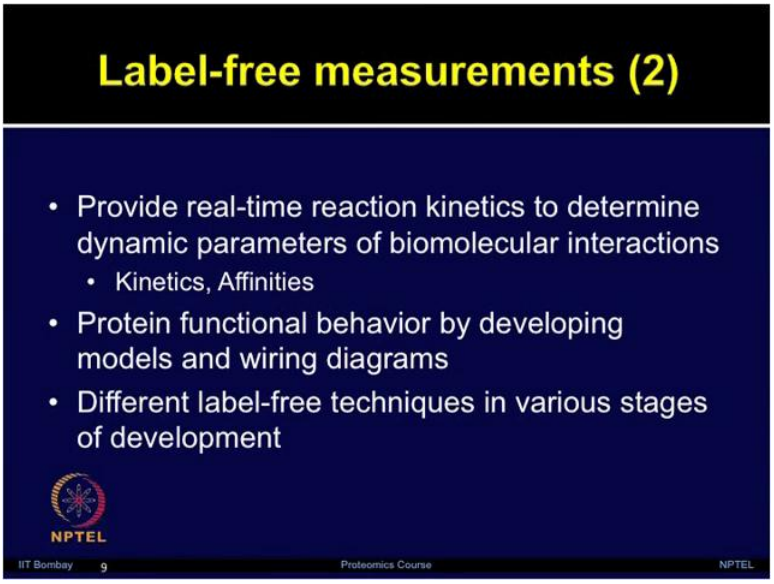

So, Label-free measurements they rely on the property of query itself such as mass and dielectric properties they avoid modification of interactors. So, since there is no label added separately there is no effect from the conjugated fluorescent labels or radioactive material the label-free measurements have advantage over the label-based techniques as we discussed and one of the most striking advantage here is, that they can provide real-time reaction kinetics which can determine the dynamic properties of biomolecular interactions. Such as kinetics and affinity the protein function behavior can also be studied by developing various type of models and wiring diagrams.

Once you have obtained the kinetic values from these experiments which is not possible from the label-based methods. So, there are different type of label-free techniques which are currently in various stages of development.

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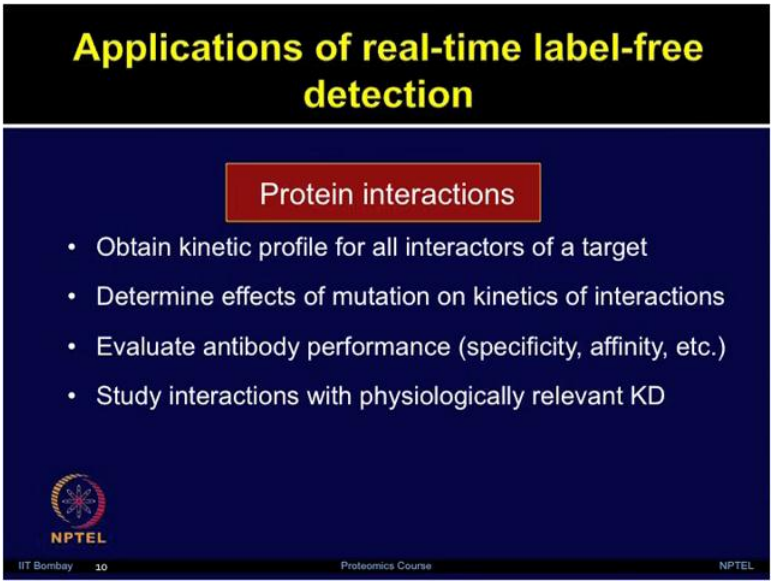

There are various Applications of label-free detection techniques which can measure the biomolecular interactions in real-time first most important application is protein interactions.

So, these label-free techniques can obtain the kinetic profile for all the interactions of a target. If you want to study the effect of mutation then these methods can be used to determine the effect of mutation on kinetics of protein interactions different type of label-free methods, such as SPR have been used for evaluation of antibody performance detection of specificity affinity etcetera they are also used for studying the interactions with physiologically relevant K D or dissociation constant.

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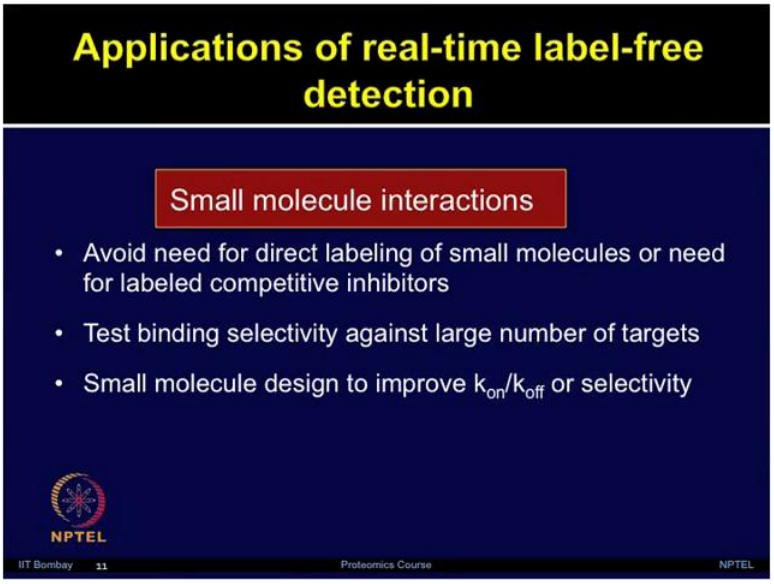

Other applications include studying about a small molecular interactions. Since there is no need for labeling these small molecules, it can provide a very good binding results the test binding selectivity against large number of targets are used by various label-free methods the small molecule designing is also evaluated by these type of label-free techniques which can improve the k on k off the on rate or off rate ratio or selectivity.

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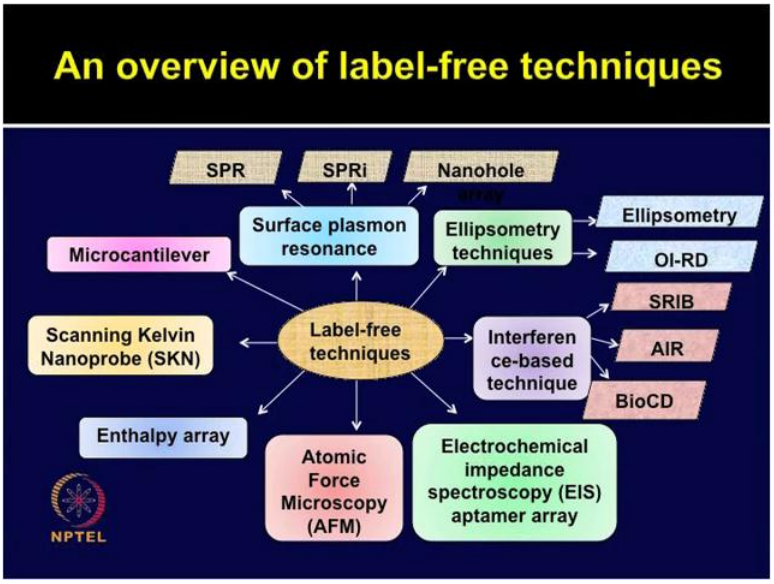

So, there are many label-free techniques let us have a look on the overview of these label-free techniques. So, many label-free techniques are emerging rapidly as potential

complement with the labeling methods, as shown in PPT's these methods include Surface plasmon resonance, Ellipsometry, interference based techniques, Electrochemical impedance, spectroscopy, Atomic force microscopy, Enthalpy arrays, Scanning Kelvin Nanoprobe, Microcantilever etcetera. So, let us have a look on an overview of label-free techniques in following animation.

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Label-free techniques, SPR and SPRi animation for Overview of Label-free techniques. An overview of label-free techniques the label-free detection techniques monitor inherent properties of the query molecules such as mass, optical and dielectric properties. Unlike the label-based detection methods these techniques avoid any tagging of the query molecules and thereby preventing changes in the structure and function they do not involve laborious procedure, but have their own pitfalls such as sensitivity and specific issues.

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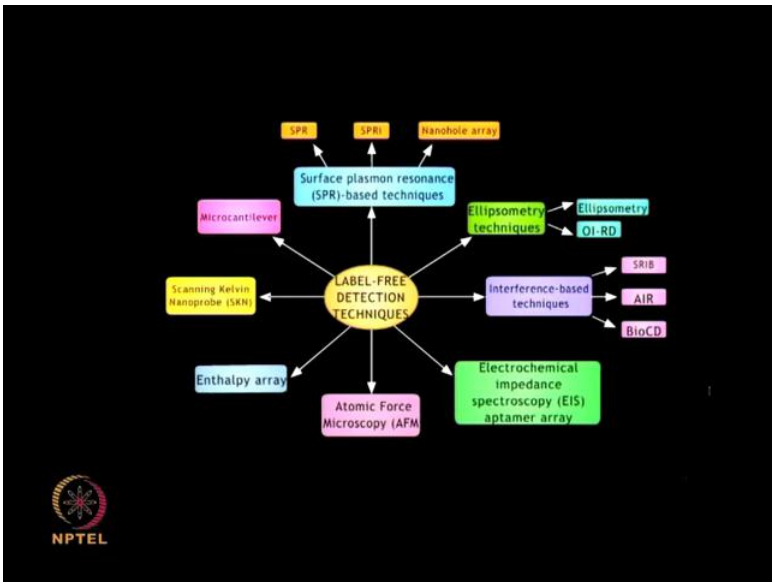

So, let us first talk about Surface plasmon resonance based techniques. First is SPR surface Plasmon resonance which detects any change in refractive index of material at the interface between metal surface and the ambient medium. Second SPRi or surface Plasmon resonance imaging. Image reflected by the polarized light at fixed angles are detected. Third Nanohole arrays the light transmission of specific wavelength enhanced by coupling of surface plasmons on both sides of metal surface with periodic Nanoholes Ellipsometry based techniques. First Ellipsometry the change in polarization state of reflected light arising due to changes in dielectric property or refractive index of surface material measured. Second OI-RD or oblique incidence reflectivity difference.

The variation of Ellipsometry that monitors harmonics of modulated photocurrents underlying conditions. Interference based techniques the interferometry is based on the principle of transformation of phase difference of wave fronts into readily recordable intensity fluctuations known as interference fringes. The various detection strategies that make use of this principle include SR-IB AIR and bio CD first SR-IB spectral reflectance imaging biosensors. The changes in optical index due to capture of the molecules on the array surface detected using optical wave interference. AIR a rate imaging refractometry the destructive interference of polarized light reflected from silicon substrate captured and used for detection. Third, Bio C D biological compact disc the local interferometry means, the transformation of phase difference of wave fronts into observable interference fringes used for detection of protein capture electrochemical impedance spectroscopy

aptamer array. Aptamers are short single stranded oligonucleotides that are capable of binding to a wide range of target biomolecules. Electro chemical impedance spectrometry aptamer array. Aptamers are short single stranded oligonucleotides that are capable of binding to a wide range of target biomolecules. EIS combined with aptamer arrays can offer a highly sensitive label-free detection technique.

Atomic force microscopy A F M, the vertical or horizontal deflections of cantilever measured by high resolution scanning probe microscope there by providing the significant information about surface features. Enthalpy array thermodynamics and kinetics of molecular interactions measured in a small sample volumes, without any need for immobilization or labeling of reactants scanning Kelvin probe. S K N a non contact technique that does not require a specialized vacuum or fluid cell. S K N detects regional variations in surface potential across the substrate of interest caused due to molecular interactions. Microcantilivers these are thin silicon based gold coated surfaces that hang from a solid support, bending of cantilever due to surface adsorption is detected either electrically by metal oxide semiconductor field. In fact, transistors or optically by changes in angle of reflection.

The success of sensing technologies are mostly determined by their sensitivity resolution and detection limit dynamic range real time monitoring multiplexing and high through put capability wide SPRead applicability and data handling are several important factors. I hope by looking at the overview of the label free techniques in the animation you got glimpse of various type of methods currently available in label free detection methods.

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So, now let us talk about one of the very promising label free technique Surface Plasmon Resonance or SPR the optical methods based on Surface Plasmon resonance and evanescent waves are promising methods to provide kinetic resolution of binding reaction in label free manner in Surface Plasmon resonance spectroscopy base sensors are popular, because of their simple instrumentation and high sensitivity. The SPR based bio sensors are in great demand, because they can provide label free real time detection of various bio molecular interactions SPR has been used for various applications in biology including the drug discovery rapid diagnosis and security applications.

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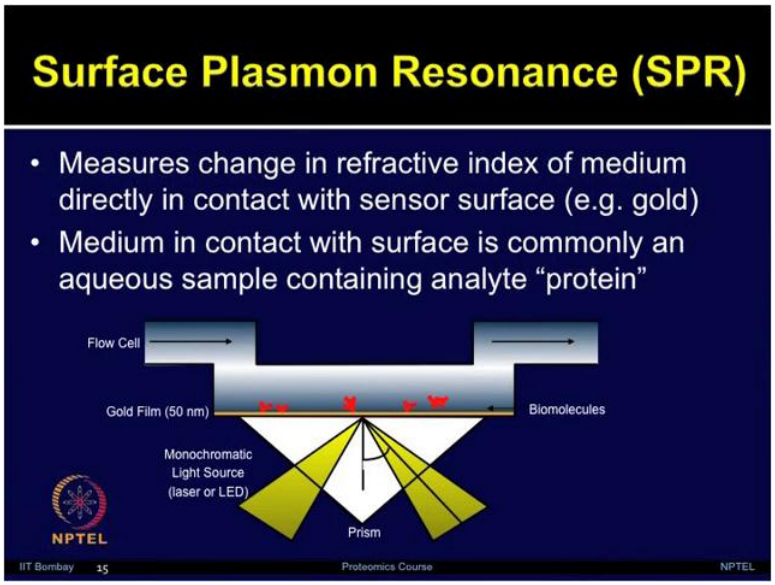

So, let us discuss what is SPR. SPR is the surface sensitive spectroscopic method, which measures the changes in refractive index of medium directly in contact with the sensor surface. Commonly employed metal is gold as you can see in this PPT in this slide, it is shown that you have a prism a light source the antibodies are immobilized on the gold surface you have a flow cell from which you can add the target proteins for which you want to study the interaction. So, medium in contact with the surface is commonly an aqueous sample containing analyte protein the test proteins such as antibodies or purified proteins can be immobilized on the gold surface.

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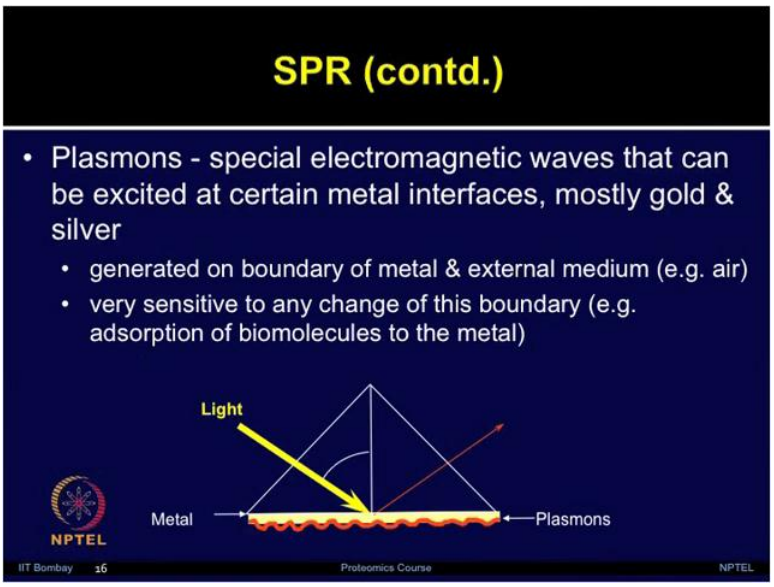

So, first we discuss about surface Plasmon's these are special electromagnetic waves that can be excited at certain metal interfaces mostly gold and silver are used for this purpose. The surface Plasmon's are electromagnetic wave that propagate parallel to the metal or dielectric surface from that interface the surface Plasmon's are created when the light energy from polarized incident photons coupled in the oscillation mode of free electron density metal at the which is present at the metal film. Now from this gold surface this Plasmon's are generated on boundary of metal and external medium .Usually air these are very sensitive to any change of the boundary with their adsorption of the bio molecule to the metal in the schematic diagram, you can see the working interface of an SPR spectrometer and how these surface Plasmon's are generated

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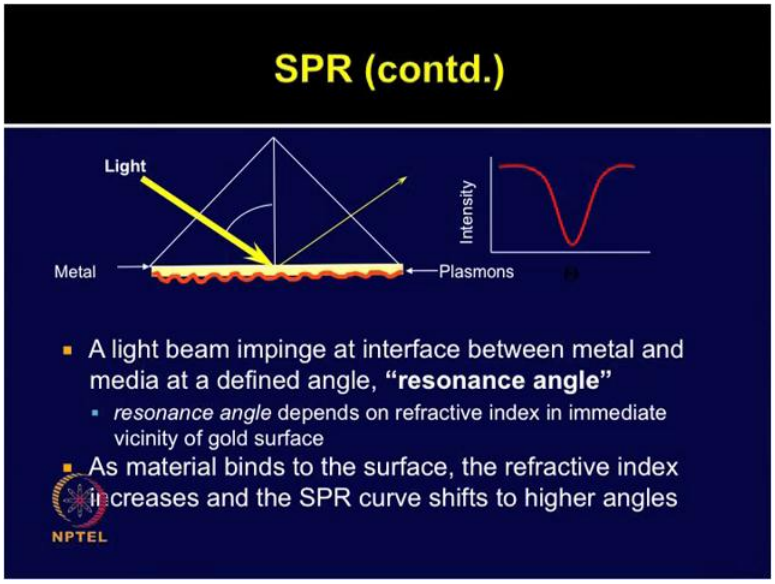

Now I will continue the working principle of SPR. So, a light beam which impinges at the interface between metal and the media that is defined as a resonance angle the resonance angle it depends upon the refractive index which is in immediate contact with the gold surface. When metal binds to the surface the refractive index increases and the SPR curve shifts towards the higher angle. So, the changes in angle of reflection of light caused due to the binding of probe to the immobilized protein is measured for the characterization of bio molecular interactions in real time

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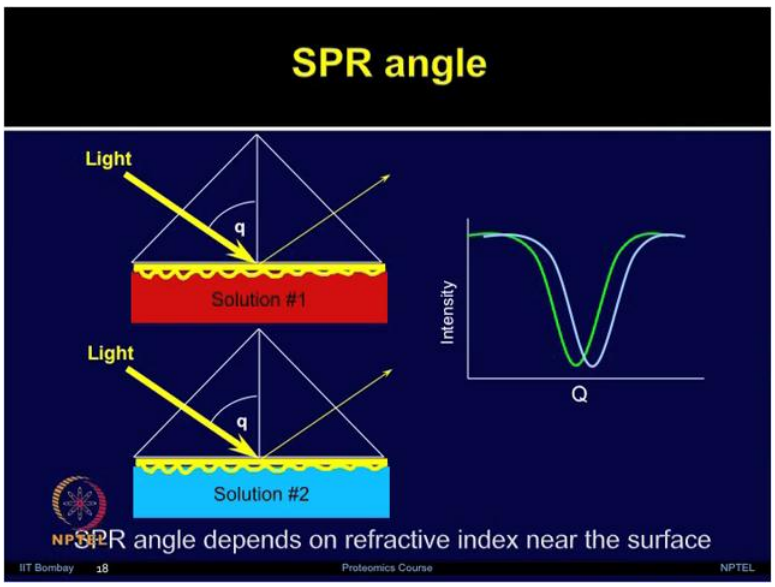

Now in this slide, I have shown you the SPR angle how it depends upon the refractive index near the surface by changing different type of solution, different SPR angles are generated. So, the angle at which the minimum intensity of the reflected light is obtained is known as the SPR angle which is directly related to the amount of biomolecules binding on the gold surface.

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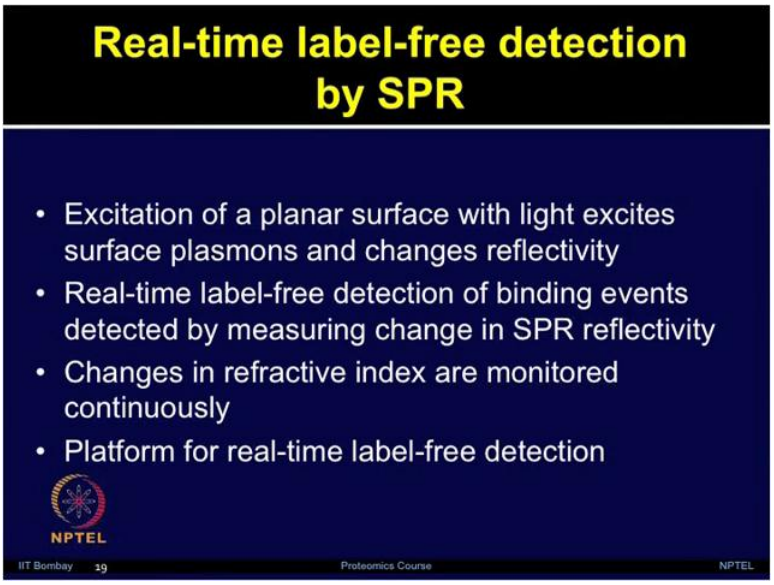

Now, let us discuss about the real time label free detection by using SPR. So, when excitation of a planar surface with light excite the surface Plasmon's and changes the reflectivity as we have seen in the last slide the real time label free detection of binding events can be detected by measurement of change in SPR reflectivity. The changes in this reflective index are continuously monitored to obtain the kinetic data in the real time. Let us now talk about SPR sensorgrams the sensorgrams describe the changes in SPR signal versus time as molecules bind and disassociates from the sensor surface the resulting change in resonance signal creates sensorgrams.

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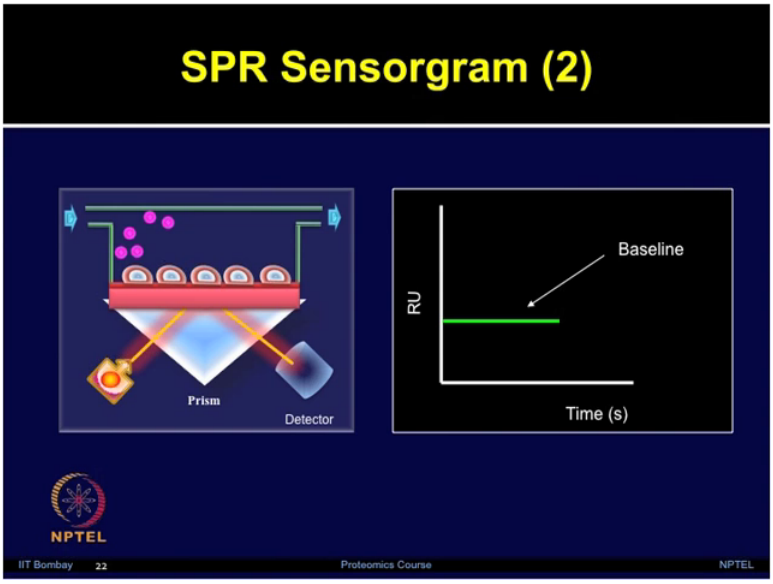

Let us look at the various steps involved in SPR sensorgrams as you can see in the slide, initially you have a prism a light source a gold slide and your proteins or antibodies are immobilized on the surface. Initially the running buffer is added on to SPR chip containing printed antibodies or proteins and the base line is straight. Now the base line remains same until the query molecule or test molecule is added in the medium. So, if you want to study about biomolecular interactions. Initially, you have to wash the surface with just running buffer and then following by add your query molecule in same running buffer. So, base line remains straight until these query molecules start interacting with the molecules already printed on the chip surface.

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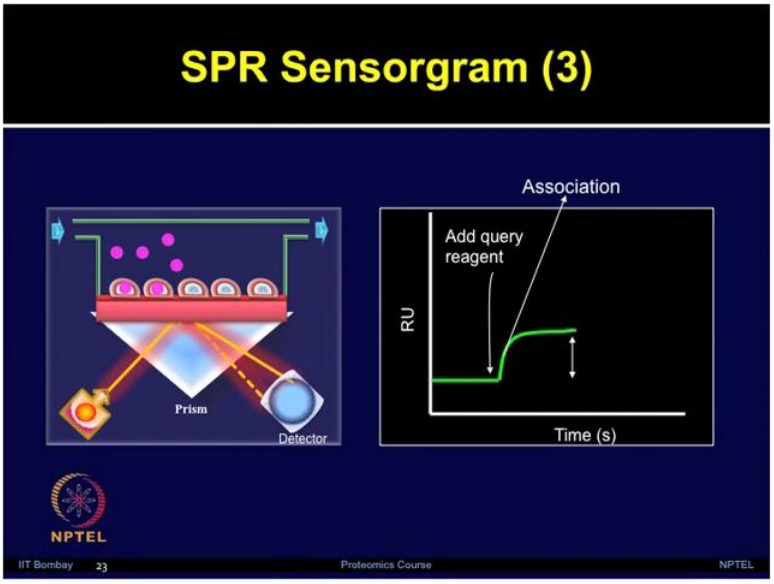

So, in the SPR sensorgrams when query molecule is added in flow cell the interacting antigen binds to the antibody and this association can be same in SPR sensorgrams which is known as association rates or k on, which is denoted by k small a or k on. So, in this image you can see some of the query molecules have started binding to the proteins immobilized on the chip surface.

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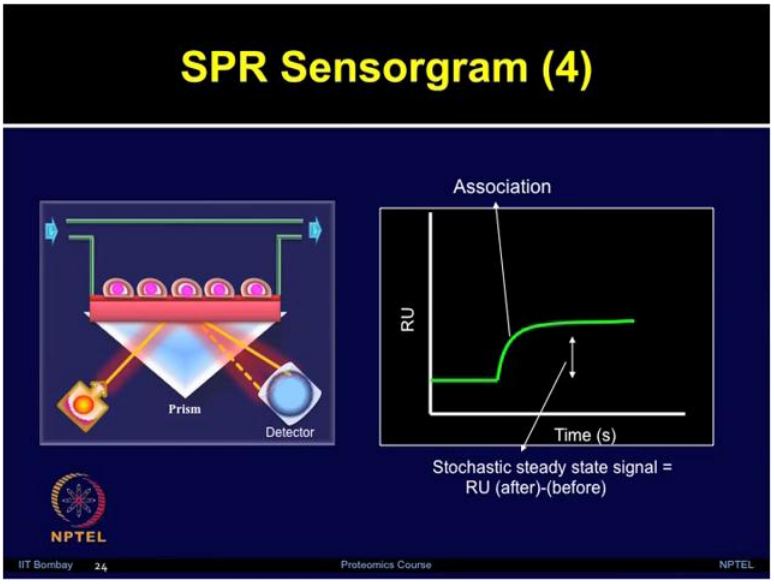

Now, after some time when the binding is reaching to a saturation level almost all the query molecules have occupied binding with the proteins immobilized in the chip then

they reach to a state known stochastic steady state which is representative of response unit signal after and before.

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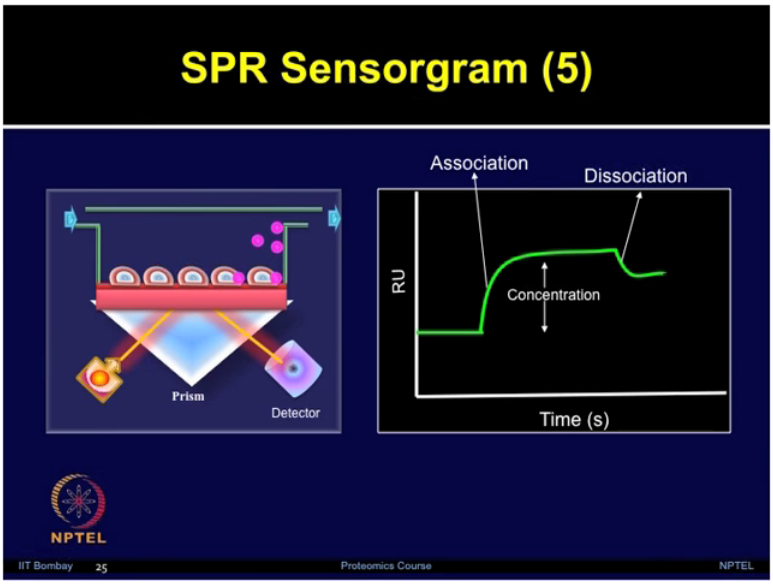

Now in this p r sensorgrams, when running buffer is further injected the bound proteins are dissociated, which is known as Dissociation rate k of or k small d as shown here. Initially, you can obtain a straight base line followed by Association and then dissociation which you can see in the right panel of the graph. The left panel is showing the proteins are being dissociated from the bound molecules.

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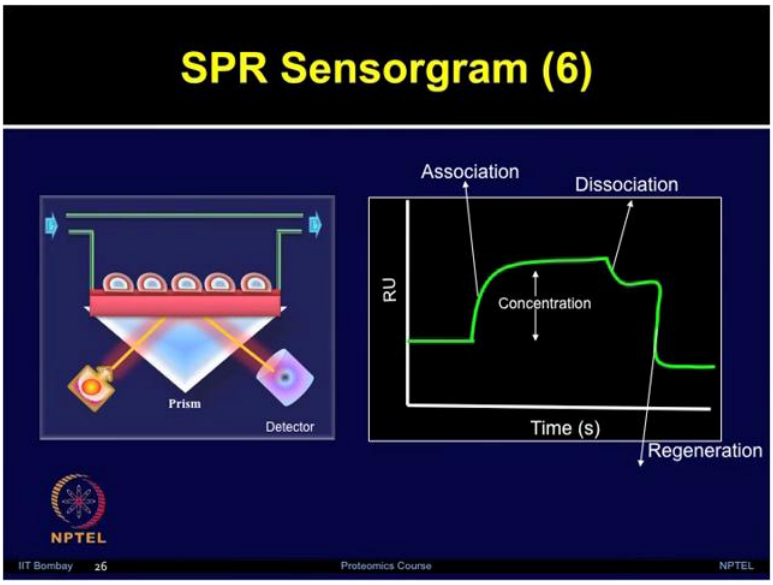

So, after this experiment in completed the SPR run is finished the same chip can be reused for further experiments, but one needs to apply a mild assay treatment and further washing with the running buffer and this process of making use of the same chip for further experiment is known as regeneration.

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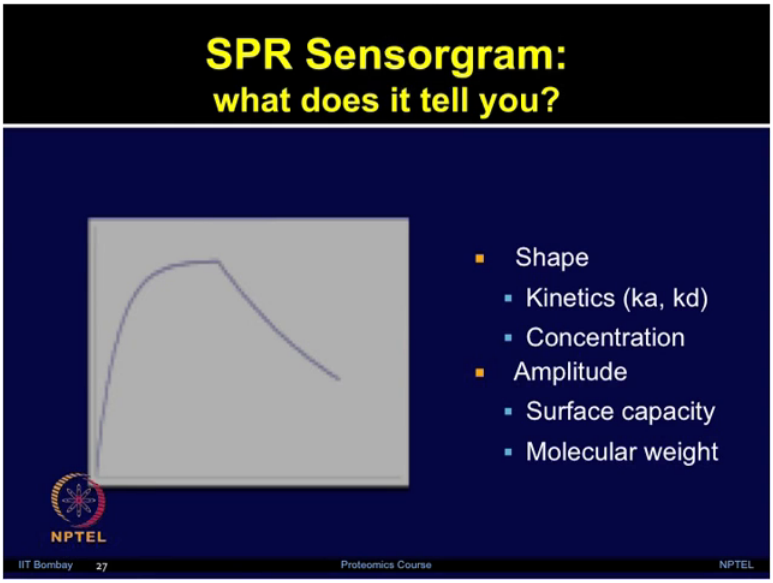

So, what these SPR sensorgrams can tell us. These curves the shape and amplitude of binding measurements can be used to determine the overall concentration and the kinetics of interaction. The analysis of SPR sensorgrams can answer many questions, how much by looking at the active surface? How fast with bulk concentration? How strong gives the kinetic information? How specific the affinity specificity. So, the shape and the amplitude provides various information by looking at these SPR sensorgram curves.

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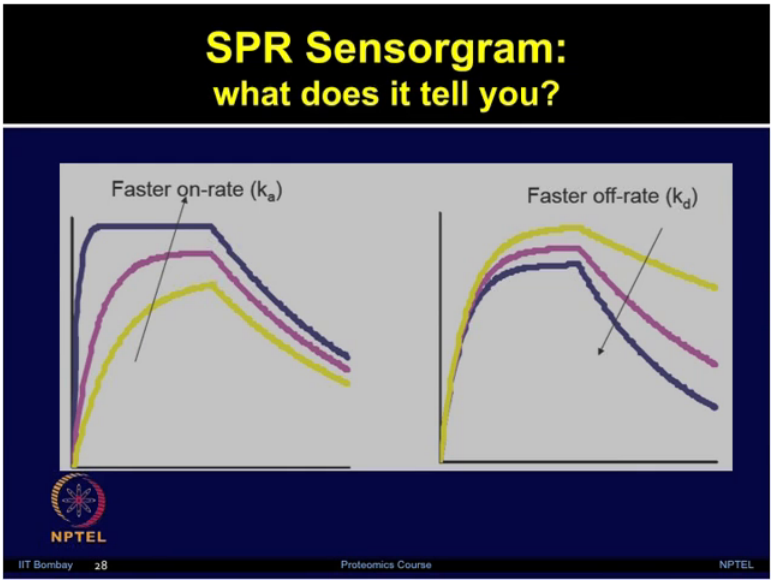

As shown here, in this slide the on rate and off rate these information whether it is slow or fast and comparison of various analytes can be made by looking at the SPR sensorgrams.

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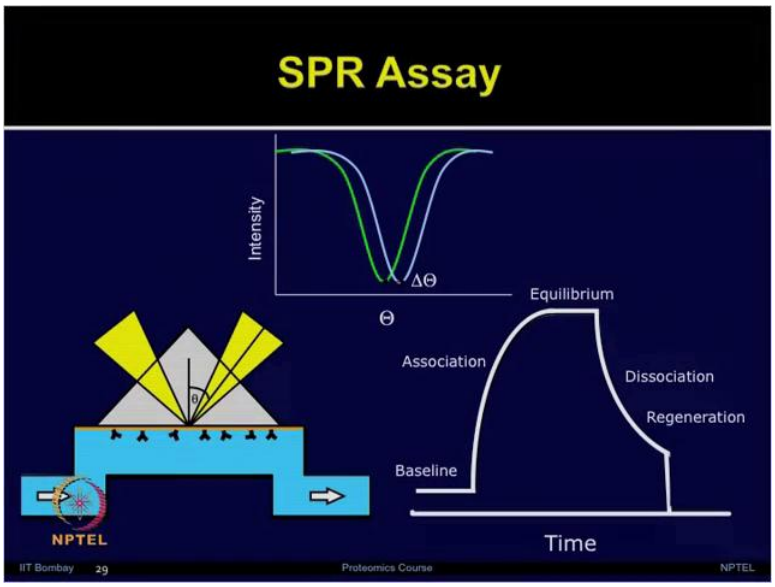

So, overall the SPR assay means tracking the SPR angle to measure binding events. Now, by looking at some basic concepts and details of SPR sensorgrams, I think it should be very clear that how SPR angle is used to measure these binding interactions. As shown here, initially you can obtain a base line once event happened association can

be seen after sometime, if it reaches to a equilibrium or steady state then by further washing with buffer, it can be dissociated shown as a dissociation followed by same chip can be reused for regeneration. So, I will show you the Surface Plasmon Resonance Animation after discussion of some of the basic concepts. I think this animation will help you to further clarify the optics and various concept involved in Surface Plasmon Resonance.

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Surface Plasmon Resonance, SPR is a highly sensitive a spectroscopic tool, that is increasingly being used for label free detection studies, test proteins such as antibodies are immobilized onto the gold coated glass array surface incident light is striking the surface is constantly reflected at a particular angle in this state.

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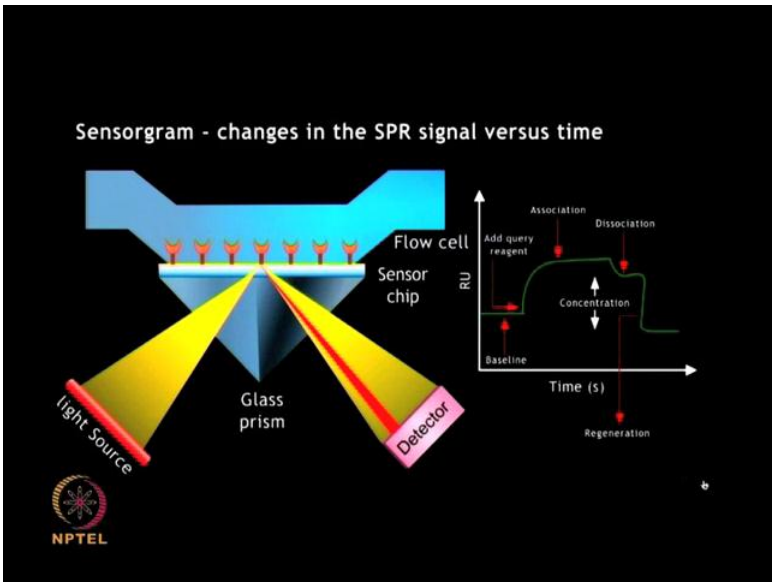

So, let us watch this animation where gold film on top of glass slide then there is a prism the test antibodies are immobilized on a gold surface. The incident light strike the surface is constantly reflected at a particular angle. In SPR experiment, the unlabeled free antigens or other query proteins enter via the flow cell and move towards the immobilized antibodies or other test proteins.

Initially, there is no change in reflected light the binding of antigen to antibody immediately brings about a change in the angle of reflection of light due to changes in the refractive index of the medium. These changes can be continuously monitored to characterize biomolecular interactions in real time the SPR angle or the angle at which minimum intensity of reflected light is obtained is indicative of the amount of biomolecule binding to the surface. The graph shown on the right side represents change in reflection intensity before and after the antigen binding.

SPR sensorgrams, a sensorgram describes the changes in SPR signal versus time. Initially, the running buffer is added onto SPR chip. So, in animation the prism light source gold slide immobilized antibodies are shown. So, when the running buffers is added onto the SPR chip containing printed antibodies. Initially the base line is straight when query molecule is added in flow cell the interacting antigen binds to the antibody and association can be seen in SPR sensorgram.

Now, the graph is showing association or on rate k a after some time the binding reaches

to a saturation level known as stochastic steady state. When running buffer is further, added the bound proteins are dissociated which can be seen as the dissociation rate in the graph. Which is represented by off rate or k off. After the SPR run is finish same chip can be reused by applying a mild assay treatment and further washing steps by a process known as regeneration

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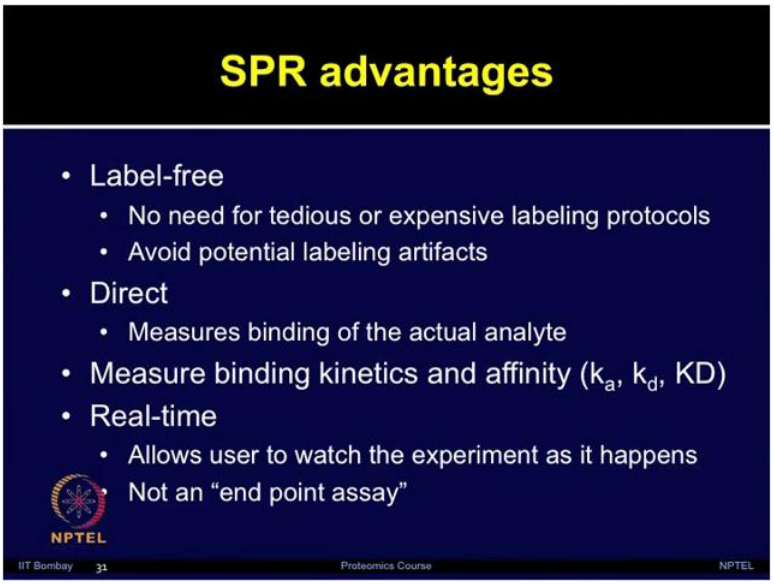

There are many advantages of using SPR first of all it is a label free method. So, there is no need for addition of t d s tax or labeling methods. It avoids the artifacts due to the labeling it is direct method, because it provides the measurement of binding of actual analyte. It gives information in the real time and allows user to watch the experiment and change the experiment as or when they want to make some changes in the exclamatory conditions in the real time. So, this is not an end point assay unlike the label based detection method and finally, and most importantly it gives you the measure of binding kinetics and affinity. So, it gives you k a k d and K D on rate, off rate and disassociation constant.

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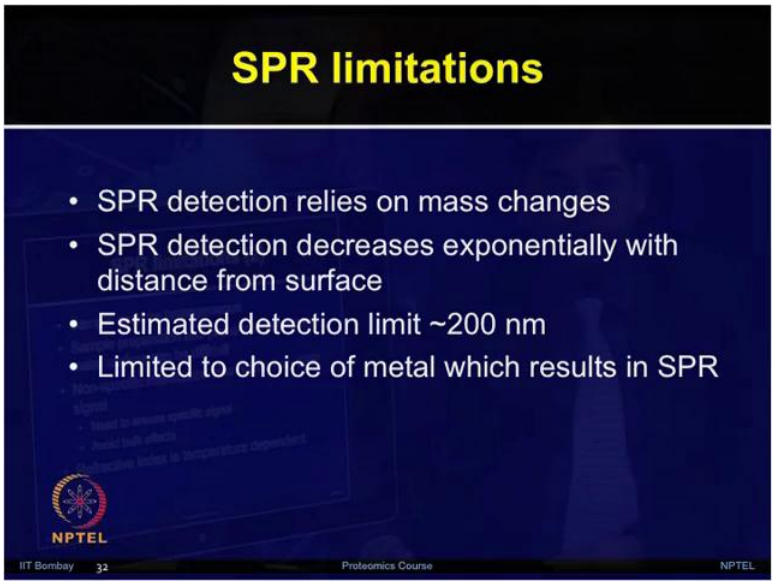

But SPR also has certain Limitations. The SPR detection it relies on mass changes the detection decreases exponentially with distance from the surface. It is estimated that approximate detection limit is around 200 nanometers it is also limited to the choice of metal which can result into the surface plasmon's such as gold and silver.

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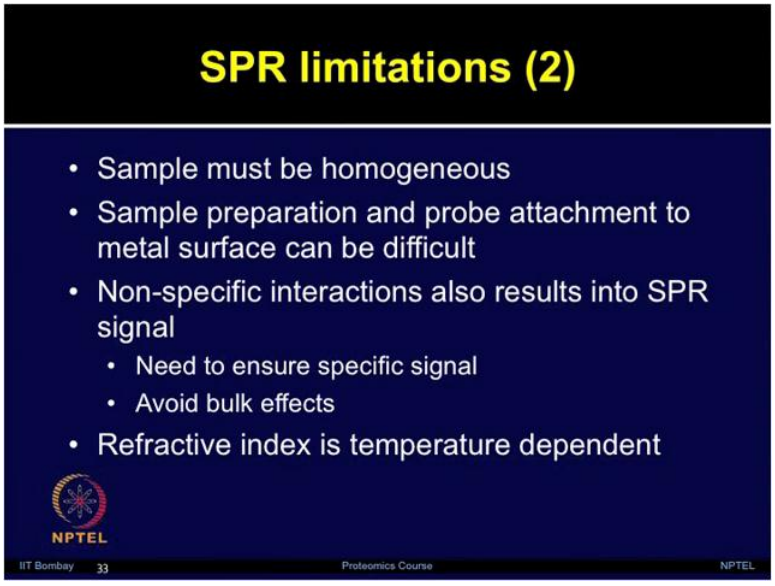

Now, you cannot use very viscous or all type of samples for SPR analysis. The samples has to must be homogeneous the sample preparation has to be very meticulous and how the probe can be attached to the metal surface that requires lot of optimization and

different type of surface chemistry.

So, that is not very straight forward. Often non specific interactions can also result into the false SPR signal. So, there is need to ensure that we can obtain a specific signals from these experiment, often the bulk effects can also be seen which has to be avoided. Now refractive index is temperature dependent so, these are some of the limitations of using surface Plasmon resonance, but due to the advantages mentioned it is still one of the very promising label free detection technique.

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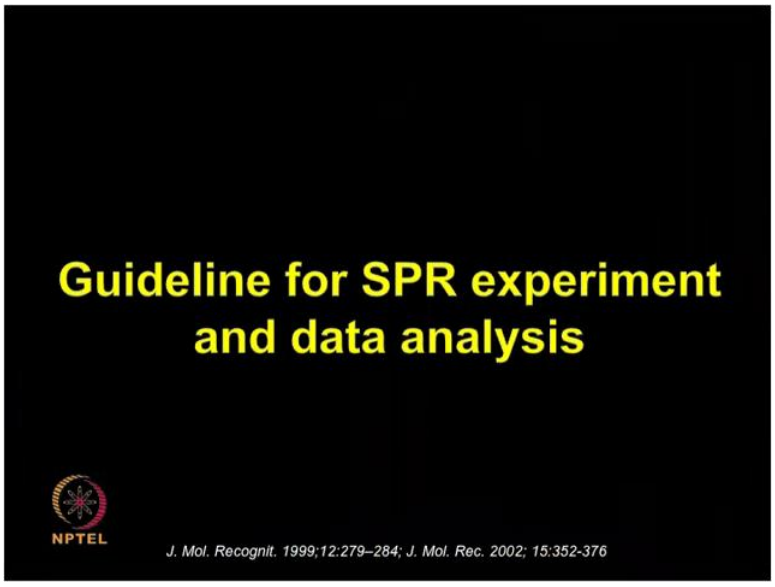

Let us now, discuss the guidelines for performing SPR experiment and data analysis. So, performing a good SPR experiment and accurate interpretation of binding reactions from these bio sensors are always very challenging David mischa from university of Utah in u s a has provided very detailed guidelines as shown in some of these references for bio sensor analysis.

So, performing good SPR experiment data collection and processing can eliminate many artifacts and provide good quality data. I will briefly describe some of the guidelines which can be used for SPR experiment and data analysis, but for detailed description and more information you should read these references.

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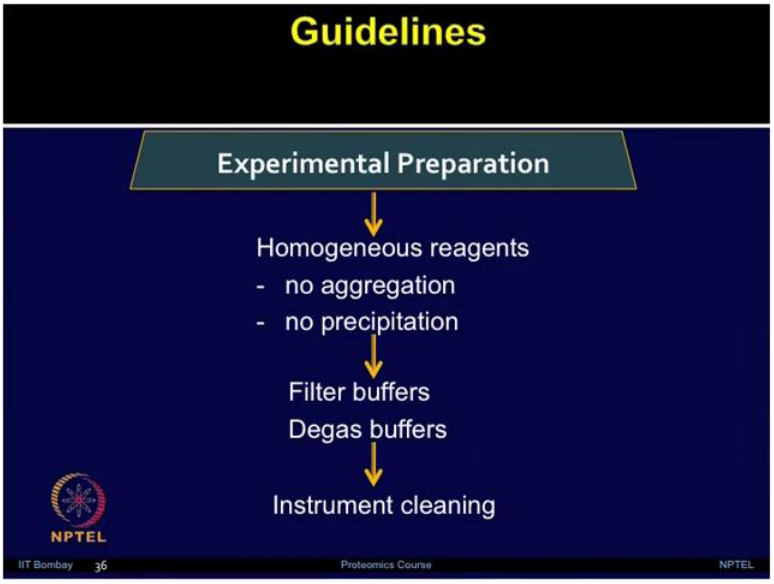

So, first of all, experimental preparation that is very important, if you are starting with quality reagents that is going to ensure the success of further high quality data. So, homogeneous reagents there has to be no aggregation no precipitation one need to ensure during preparation of all the protein samples as well as buffers. Filter the buffers degas it, because during the run a small bubble can totally screw up the whole experiment. Instrument cleaning is very important you have to keep the instrument always clean, because any dust particle or any type of interference can give you artifact results. So, the analyte and ligand should be monomeric in solution and it should form one to one complex for a data to fit simple biomolecular reaction model. Therefore, your good quality reagents are going to determine the good quality output data.

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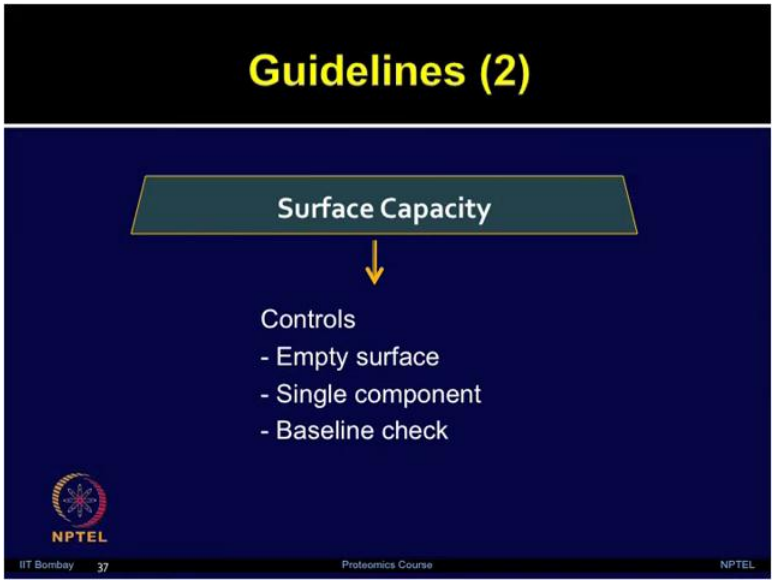

Surface Capacity is another consideration, how your control should be printed. How you should keep the surface empty the single component baseline checks, all of these are important point to consider. So, initially the running buffer should be injected at the beginning of starting any experiment and the base line should be completely flat as you have seen in the SPR sensorgrams as well. A sensor surface capacity is low that can help to minimize issue such as mass transport aggregation and a steric hindrance etcetera.

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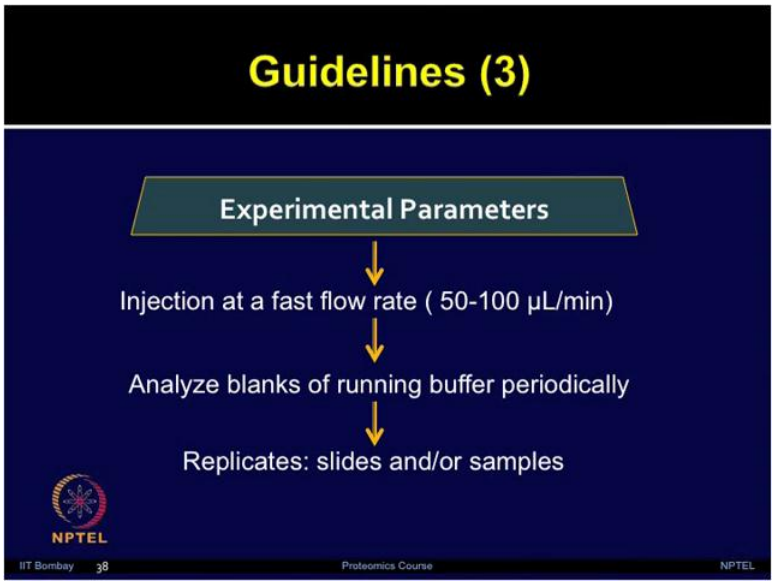

There are various Experimental Parameters one need to keep track of for example, how

slow or fast the injection rate should be ideally the fast flow rates can minimize mass transport type of artifacts analyze blanks of running buffer periodically. So, that you are confident that your chip is clean and the baseline is stable it is always important to reproduce these experiment perform various replicates of same experiment on independent slides as well as on independent samples

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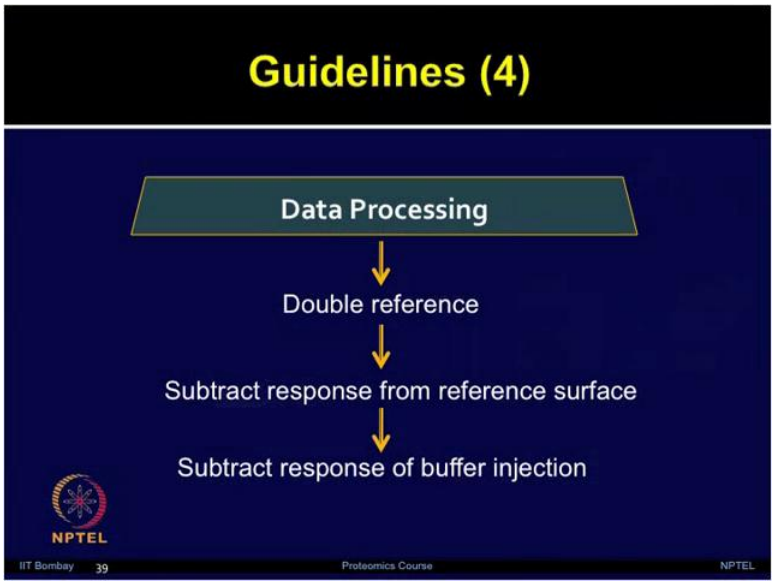

Data processing that is very important one often need to do double referencing, especially if you are talking about SPR imaging type of data subtract the response from the reference surface and subtract response of buffer injection. Subtracting the reference surface data from reaction surface can reduce the issues which are related to the refractive index changes. The double referencing is the blank injection response which is used to remove such artifacts.

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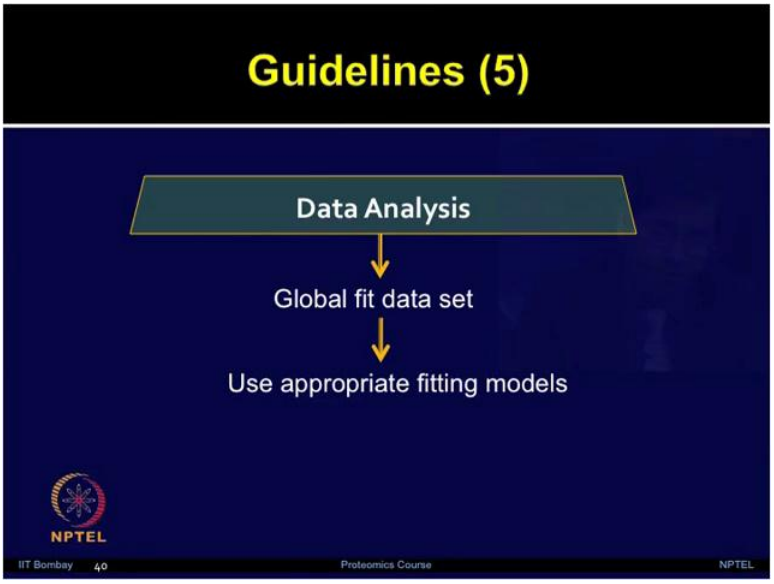

Now, how to fit the data the global fitting modals or selection of appropriate fitting modals are always very critical one issue which people often encounter is avidity issue. So, the experiment must be designed accurately if binding data has to be correlated with interaction model one need to avoid the avidity issues. So, what is the avidity issue? So, for a study, if you are talking about antigen or antibody interaction, if antibody is for example, used as a query in solution it can bind with 2 antigens and it will result into high apparent affinity which is not going to give you one to one modal. Therefore, for such a studies, such as antigen antibody interactions the antibodies should be printed on the chip surface and antigen should be floated to study the biomolecular interaction.

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So, in summary in today's lecture, we talked about label free detection techniques which monitor biomolecular interactions and simplify the bio assays for various applications by eliminating the need for secondary reagents. Moreover, they provide quantities information for the binding kinetics. We discussed about its strength and weakness of label based and label free techniques. Then we started discussing about some of the promising label free techniques such as surface plasmon resonance. We will continue our lecture on surface plasmon resonance and surface plasmon resonance imaging as well as other label free techniques in following lecture. Thank you.