LECTURE-35

LABEL-FREE TECHNIQUES- SPR AND SPRi

TRANSCRIPT

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-based detection such as fluorescent, chemi-luminescent and radioactive labeling. These labeling strategies have synthetic challenges, multiple labeling issues and may exhibit interference with binding site. Therefore, developments of sensitive, reliable, high throughput label-free techniques are now attracting significant attention.

The label free detection techniques monitor bio-molecular interaction and simplify the bioassays by eliminating the need for secondary reagents which are required in label-free detection techniques. Moreover they can provide quantitative information on binding kinetics. SPR and SPRi are both 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 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 SPR active surface with high sensitivity. Commonly the SPR measurements are collected for SPR imaging for various high throughputs applications.

 The SPR imaging provides an expanded and collimated polarized laser-beam which travels through the prism and reflects from metal-dye electric interface. The reflected light intensity from the illuminated area is monitored by CCD camera which produces an image in SPRi. SPRi is a potential alternative to the label-based detection techniques and it offers nearly comparable sensitivity to single color as well as dual color labeling

method. But despite the promises of SPR and SPRi, there is still need for further improvements for various high throughput biological applications.

So in today's lecture, we will talk about detection techniques, the label-based and label-free. We will try to provide you comparative vision of label-based and label-free techniques. Then we will discuss about some of the promising label-free techniques such as SPR and SPRi.

So let's first talk about detection techniques - There are several label-based conventional techniques, which employ fluorescence, chemi-luminescence and radioactive isotopes. They are in practice but researchers are now exploring the methods for label-free analysis to eliminate the interference due to tagging molecule and reduce the complexity of assays.

There are broadly two types 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, these marker can be fluorescent-based, radioisotope-based and horseradish peroxidase-based whereas label-free techniques measure inherent property of query example - mass, dielectric property etc.

There are various label-free techniques are emerging some of the most promising includes, SPR, SPRi and carbon nanotubes and different type of nano-technique based biosensors.

Let's look at strengths and weaknesses of label-based and label-free methods. Label-based methods are most commonly used in most of the laboratories worldwide so most of the reagents and instruments are very commonly available. However, the label-free methods are mostly new so the instrumentation is very costly. It is not so easily available but this method avoids tag-related issues so that is an advantage over tag-based assays. Moreover it provides measurement in real time and also provides the information for the kinetics which is not exactly possible by the most of the label-based methods.

Now let's look at the comparative weakness of label-based and label-free methods. The label-based methods since they are employing some tags, the tag itself can interfere with the function. Now these are not always easy to add to the query molecule and finally these label-based methods, they provide endpoint measurements. You have to perform assay whole day and then at the end of the day you can learn that your assay had worked or not worked whereas label-free techniques gives you an advantage of providing the data in real time. But comparing the label-free, they are typically less sensitive with the label-based methods and they are more costly as I mentioned instrumentations are not easily available.

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

Label-free measurements rely on inherent property of query itself such as mass and dielectric property etc. they avoid modification of interactors so since there is no label added separately there is no effect from conjugated fluorescent labels or radioactive material.

The label free measurements have advantage over label-based techniques as we discussed and one of the most striking advantage here is they can provide real time reaction kinetics to determine dynamic parameters of biomolecular interactions such as kinetics and affinity. The protein function behavior can also be studied by developing models and wiring diagrams once you have obtained kinetic values from these experiments which is not possible by label-based methods. So there are different label-free techniques which are in various stages of development.

There are various applications of label-free detection techniques which can measure the biomolecular interactions in real time. Most important function is protein interactions. These techniques can obtain kinetic profile for all interactions of a target or you can

study 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 affinity, specificity etc. they are also used for studying interactions with physiologically relevant KD or dissociation constant.

Other applications include studying about small molecule interactions. Since there is no need for labeling the small molecules it can provide very good binding result. They test binding selectivity against large number of targets are used by various label-free methods. The small molecules' designing is also evaluated by these type of label-free techniques which can improve Kon/Koff or selectivity.

So there are many label-free techniques-let's have a look on the overview of these techniques. Many label-free techniques are emerging rapidly as potential complement to the labeling methods. As shown in slide these methods include SPR, ellipsometry, interference-based techniques, electrochemical impedance spectroscopy (EIS), atomic force microscopy, enthalpy arrays, scanning Kelvin nanoprobe (SKN), microcantilever etc. So let's have a look on overview of label-free detection techniques in following animation.

Label-free detection techniques monitor inherent properties of the query molecules such as mass, optical and dielectric properties. Unlike label-based detection methods, these techniques avoid any tagging of the query molecules thereby preventing changes in structure and function. They do not involve laborious procedures but have their own pitfalls such as sensitivity and specificity issues.

Surface plasmon resonance-based techniques. Surface plasmon resonance (SPR): Detects any change in refractive index of material at the interface between metal surface and the ambient medium. Surface plasmon resonance imaging (SPRi): Image reflected by polarized light at fixed angle detected. Nanohole array: Light transmission of specific wavelength enhanced by coupling of surface plasmons on both sides of metal surface with periodic nanoholes.

Ellipsometry-based techniques - Ellipsometry: Change in polarization state of reflected light arising due to changes in dielectric property or refractive index of surface material measured. Oblique incidence reflectivity difference (OI-RD): Variation of ellipsometry that monitors harmonics of modulated photocurrents under nulling conditions.

Interference-based techniques - Interferometry is based on the principle of transformation of phase differences of wave fronts into readily recordable intensity fluctuations known as interference fringes. The various detection strategies that make use of this principle include: Spectral reflectance imaging biosensor (SRIB): Changes in optical index due to capture of molecules on the array surface detected using optical wave interference. Biological compact disc (BioCD): Local interferometry i.e. transformation of phase differences of wave fronts into observable interference fringes, used for detection of protein capture. Arrayed imaging reflectometry (AIR): Destructive interference of polarized light reflected from silicon substrate captured and used for detection.

Electrochemical impedance spectroscopy (EIS)-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 (AFM): Vertical or horizontal deflections of cantilever measured by high-resolution scanning probe microscope, thereby providing significant information about surface features.

Enthalpy array: Thermodynamics and kinetics of molecular interactions measured in small sample volumes without any need for immobilization or labelling of reactants.

Scanning Kelvin Nanoprobe (SKN): A non-contact technique that does not require specialized vacuum or fluid cell. SKN detects regional variations in surface potential across the substrate of interest caused due to molecular interactions.

Microcantilever: 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 effect transistors or optically by changes in angle of reflection.

The success of sensing technologies is mostly determined by their sensitivity, resolution and detection limit. Dynamic range, real time monitoring, multiplexing and high throughput capability, wide spread applicability and data handling are several important factors. I hope by looking at the overview of label-free techniques in animation. You got a glimpse of various types of methods currently available in label-free detection methods.

So let's now talk about one of the very promising label-free technique- Surface Plasmon Resonance (SPR) - the optical methods based on surface plasmon resonance and Evanscent waves are promising methods to provide kinetic resolution of binding reactions in label-free manner. The SPR spectroscopy biosensors are popular because of their simple instrumentation and high sensitivity. The SPR based biosensors are in great demand because they can provide label-free real time detection of various biomolecular interactions. SPR has been used for various applications in biology including the drug discovery, rapid diagnosis and security applications.

So let's discuss what is SPR? SPR is the surface sensitive spectroscopic method which measures the changes in refractive index of the medium directly in contact with the sensor surface commonly employed metal is gold. As you can see in this slide, it is shown that you have a prism, light source, the antibodies 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 interactions. So medium in contact with the surface is commonly an aqueous sample containing analyte "protein". The test protein such as antibodies or purified proteins can be immobilized on the gold surface.

Surface plasmons- these are special electro-magnetic waves that can be excited at certain metal interfaces, mostly gold and silver are used for this purpose. The surface

plasmons are electro-magnetic waves that propagate parallel to the metal or dielectric surface. From that interface surface, the plasmons are created when the light energy from polarized incident photons is coupled in the oscillation mode of free electron density which is present at the metal film. Now from these gold surfaces, these plasmons are generated on the boundary of metal and external medium usually air. These are very sensitive to any change of this boundary whether adsorption of biomolecules to the metal. In the schematic diagram you can see the working interface of SPR spectrometer and how these surface plasmons are generated.

I will continue to discuss working principle of SPR. So a light beam which impinges between metal and media at the defined angle, it is defined as "resonance angle". The resonance angle depends upon refractive index in immediate vicinity of gold surface. When metal binds to the surface, the refractive index increases and the SPR curve shifts towards the higher angles so the changes in angle of reflection of light caused due to the binding of probe to the immobilized proteins is measured for the characterization of biomolecular interaction in real time.

Now in this slide I have shown you the SPR angle- how it depends on refractive index near the surface by changing different type of solutions, the 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.

Now let's discuss about real time label-free detection by SPR. So when excitation of a planar surface with light excites surface plasmons and changes reflectivity as we have seen in the last slide. The real time label-free detection of binding events can be detected by measuring change in SPR reflectivity. The changes in this refractive index are continuously monitored to obtain the kinetic data in real time.

Let's now talk about SPR sensograms. The sensograms describe the changes in SPR signal versus time. As molecule binds and dissociates from the sensor surface the resulting change in resonance signal creates sensogram.

Let's look at various steps involved in SPR sensograms. As you can see in the slide initially you have a prism, a light source, a gold slide and your protein 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.

The baseline remains straight until the query molecule or the test molecule is added in the medium. So if you want to study about a biomolecular interaction, initially you will have to wash the surface with running buffer and then following add your query molecule in same running buffer so baseline remains straight until these query molecules start interacting with the molecules already printed on the chip surface.

So in the SPR sensograms when query molecule is added in flow cell the interacting antigen binds to the antibody and this association can be seen in SPR sensograms which is known as association rate or Kon or Ka. In this slide you can see some of the query molecules have started binding to the proteins immobilized on the chip surface.

Now after some time when the binding reaching to a saturation level almost all the query molecules have occupied binding with the proteins immobilized on the chip. Then they reach to a state known as stochastic steady state, which is representative of response unit signal after and before.

Now in the SPR sensograms when running buffer is further injected the bound proteins are dissociated which is known as dissociation rate, Koff or Kd. As shown here initially you can obtain a straight baseline followed by association and then dissociation, which you can see in the right panel of the graph. The left panel is showing proteins are being dissociated from bound molecules.

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

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

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

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 sensograms, 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 baseline, when binding event happen association can be seen, after some time it reaches to a steady state then by further washing with buffer it can be dissociated shown as dissociation followed by same chip can reused for regeneration. So I will show you SPR animation of a discussion of some basic concepts.

SPR is a highly sensitive 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 striking the surface is constantly reflected at a particular angle in this state.

In this animation, gold film on top of glass slide, then there is a prism, the test antibodies are immobilized on the gold surface. The incident light strike the surface is consistently reflected at a particular angle. In SPR experiment the unlabelled free antigen or other query proteins enter via the flow and move towards the immobilized antibodies or other test proteins. Initially, there is no change in reflected light upon entering into the system.

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 i.e. 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 antigen binding.

Sesnorgram describes the changes in SPR signal versus time. Initially the running buffer is added onto SPR chip containing printed antibodies and the baseline 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, known as association rate, Kon or on rate. After sometime the binding reaches to a saturation level known as Stochastic steady state. When running buffer is further injected the bound proteins are dissociated, which is known as Dissociation rate, off rate or Koff. After the SPR run is finished the same chip can be re-used by applying a mild acid treatment and further washing steps by a process known as 'regeneration'.

There are many advantages of using SPR. First of all, it is label-free method so there is no need of addition of tedious tags or labeling methods. It avoids the artifacts due to the labeling. It is a direct method because it provides measurement of binding of actual analyte. It gives information in the real time and allows the user to watch the experiment as it happens and change the experiment as and when they want to make some changes in the experimental conditions in real time. So this is not an end point assay unlike label-based detection methods. And finally and most importantly it gives you measure of binding kinetics and affinity (Ka, Kd, KD).

SPR also has certain limitations. SPR detection relies on mass changes and it decreases exponentially with distance from surface. It is estimated that approximate detection limit is around 200 nm. It is also limited to the choice of metal which results in surface plasmons such as gold and silver.

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

type of surface chemistry, it is not very straightforward. Often non-specific interaction can also result in the false SPR signal so there is need to ensure that we can obtain specific signal from these experiments. Often, the bulk effect can also be seen which has to be avoided. Now, refractive index is temperature dependent. So these are some of limitations using SPR but due to the advantages mentioned it is still one of the very promising label-free detection technique.

Let's 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 biosensors are always very challenging. David Myska from University of Utah in USA has provided very detailed guidelines as shown in some of these references for biosensor 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.

First of all, experimental preparation, that is very important if you are starting with quality a reagent that is going to ensure the success of further high quality data. So homogenous reagents there has to be no aggregation, no precipitation one need to ensure during preparation of all protein samples as well as buffers. Filter the buffer; degas it because during the run a small bubble can 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 data to fit into simple reaction model. Therefore, good quality reagents going to determine good quality output data.

Surface capacity is another consideration. How your control should be printed, how you should keep the surface empty, the single component, and the baseline checks all these are important point to consider. So initially running buffer should be injected at the

beginning of any experiment and the baseline should be completely flat as you have seen in SPR sensograms as well. The sensors surface capacity is low that can help to minimize issues such as mass transport, aggregation and steric hindrance etc.

There are various experimental parameters, one need to keep track of. For example how fast or slow the injection rate should be. Ideally, the fast flow rate 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's always important to reproduce this experiment. Perform various replicates of same experiment on independent slides and/or independent samples.

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

Now how to fit the datamthe global fitting models or selection of appropriate fitting models are always very critical. One issue, which people normally encounter, is avidity issue. So the experiment must be designed accurately. If binding data has to be correlated with interaction model, one needs to avoid avidity issues.

So what is the avidity issue? For a study if you are talking about antigen and antibody interaction if antibody is used as a query in solution, it can bind with two antigens and it will result into high apparent affinity which is not going to give you one to one model. Therefore for such studies such as antigen-antibody interactions the antibodies should be printed on the chip surface and antigen should be floated to study biomolecular interaction.

So in summary, in today's lecture we talked about label-free detection techniques, which monitor biomolecular interactions and simplify the bioassays for various

applications by eliminating the need for secondary reagents. Moreover they provide quantitative information for the binding kinetics. We discussed about strength and weakness of label-based and label-free techniques. Then we started discussing about some of the promising label-free techniques such as SPR. We will continue our lecture on SPR and SPRi as well as other label-free techniques in following lecture. Thank you.