LECTURE-36

LABEL-FREE TECHNIQUES: SPRi, ELIPSOMETRY, INTERFERENCE

TRANSCRIPT

Welcome to the proteomics course. In today's lecture we will talk about label free techniques, which is in continuation with our previous lecture. Today we will focus on SPRi, Ellipsometry, and Interference based techniques. So as discussed in the last lecture there are several conventional label-based techniques which are in practice however now researchers are exploring new methods for real time label-free analysis so that they can get rid of interference from the tagging molecules and also it can reduce complexity and assay time.

So the label-free techniques they rely on the measurement of the some inherent properties of the query molecules such as mass dielectric properties and it allows for the direct real time biomolecular protein interactions as well as various other applications in high throughput manner by eliminating the requirements of secondary reactants/ various labels.

Among the different emerging label-free techniques, SPR and SPRi are most promising candidates for various diagnostic applications as well as studying biomolecular interactions. We studied the SPR in more detail in the previous lecture. But other than SPR based techniques there are various nanotechniques, Ellipsometry and interference based detection techniques which have emerge in the label-free techniques. SPR although is most widely available and used the label-free detection platform but SPRi has several high throughput potential and that is why it is emerging as an alternative to SPR which allows simultaneous analysis of multiple biomolecular interactions in a high throughput manner.

So today we will discuss from our previous lectures which were more based on label-based and label-free comparison. Today we will focus on label-free techniques and we will talk more about SPRi, Ellipsometry and Interferometry based detection techniques

which have also emerged in the label-free techniques. So, in the last lecture we talked about comparison of label-based and label-free methods and different type of label-free techniques.

Just to refresh you about various label-free techniques, which we discussed about SPR which we briefly discussed in the last lecture, this overview slide give you various available options for studying the different application using label-free techniques such as SPR based platforms like SPR, SPRi or nanohole arrays, Ellipsometry based techniques such as Ellipsometry and oblique incidence- reflectivity difference or OI-RD, then we have various interference based techniques like spectral reflectance imaging biosensors, arrayed imaging reflectometry or AIR, we have BioCDs. Then there are options such as electrochemical impedance spectroscopy (EIS) aptamer arrays, atomic force microscopy (AFM), enthalpy arrays, scanning Kelvin nanoprobe (SKN) and microcantilever. This is a detailed list but not the complete list there are still many other emerging label-free techniques.

Today we will focus on SPRi and nanohole arrays then we will move on ellipsometry based techniques. So let's first talk about SPR and related techniques, we have discussed about SPR. Today we will talk about SPRi. So in SPRi the entire biochips are freezed is illuminated at a time with a broad beam of monochromatic polarised light and the reflected time is captured from each spot by a CCD camera simultaneously. This CCD camera continuously monitors the changes, which are occurring on the surface and provides real time kinetic data in high throughput manner.

So SPRi techniques involve collimated, monochromatic beam of light illuminates sample assembly at a single incident angle near SPR angle, and light reflected from the surface is detected with charge coupled device to produce the SPRi.

SPRi fixes on a single-incidence angle to monitor reflection intensity for the whole array surface as a function of time.

This is a set up of SPRi. The configuration shows that light from a collimated polychromatic source passes through a polariser and impinges on prism or gold interface at a specific angle of incidence. Which you can see here the gold surface the prism and the light source. Now the reflected light passes through the narrow band interference filter and is detected by CCD camera as you can see in slide.

So at a fixed incident angle, the spatial variations in refractive index due to the presence of protein or other biomolecules which are printed on the surface those adsorbates the shift local resonant angle, which in turn changes reflected light intensity.

So this linear region shown in graph is directly proportional to optical angle. The linear region of SPR curve quantatively correlates the changes in refractive light intensity with amount of material present on surface.

Now SPRi as the term says an image is generated and as you can see in the slide the image contrast is shown. So in SPRi collimated monochromatic beam of light illuminates sample assembly at a single incident angle near SPR angle, and light reflected from the surface is detected with charge coupled device which produce the SPRi. As you can see here each of these circles show a spot printed on the surface of gold.

Now let's talk about SPRi experimental work flow involved in SPRi experiment. The SPRi fixes on a single incidence angle and monitors the reflection intensity for the whole array as a function of time. There are various steps which are involved in performing SPRi experiment.

So let's go through some of these experiments in more detail. So various steps involves preparation of sample and mounting of slide on the prism when you are starting SPRi experiment.

Load and prime the samples, Assign the region of interest (ROI's), Determine the operating angle, Initiate the data acquisition, Record movie and generate data file, Inject

samples and then save and export, Now in SPRi the first most crucial step is selection of an operating angle.

So here as you can see in the slide the SPRi device it measure the SPR curve and determines the linear region. Now first of all one need to scan the whole optics through range of angles and determine the linear range. Once you select an operating unit which is usually the bottom of the linear range. There are also some rough estimate that typically around 30% reflectivity can be taken as bottom part which represents linear region so linear region starts there and that can be selected as operating angle. So select the optics at this angle for your entire experiment so that all the spots printed on the chip surface can be measured simultaneously.

Now this image shows SPR image generated by CCD camera and now software adds up some spots on the background surface. This is image taken from proteomic processor instrument SPR device. Each of the ROI's or regions of interest can be defined as region of interest for measurement. So you want to subtract the background and software generate some spots on the background which are automatically generated so surrounding each spot four background spots are generated which can be used for background subtraction with the protein or biomolecules which is printed.

Background spots to remove bulk refractive index effects as it is more visible here in this slide. These are the spots of interest and there are four software generated spots the reference spots and bottom panel is showing a graph where you can see the pink one the protein spot signal and blue one is the reference spot signal. X–axis represents time in second scale and Y-axis show % reflectivity. The right hand side graph shows background subtraction so you subtract the reference spots generated by software with the protein signal then the background-subtracted image can be generated.

Now next important aspect is normalized intensity or percentage reflectivity so left image is showing you raw SPR curve and the right image is showing normalized SPR curve. So at an angle very far away from SPR angle (optics position ~0), 100% of the

incident light is reflected. Now the 100% reflectivity used to normalize reflected light intensity so that all features have same sensitivity.

Data processing involves multiple steps we have already discussed few conditions for data processing and analysis in the previous lecture when we talked about SPR. Similar concepts can be applied here. Also I have shown in this slide the Y-axis transformation is important because you have printed many features on the surface and now you want to bring the signals from the same scale so Y-transformation is important to fit the data which is already cropped and aligned. So the left panel image is showing raw graph generated, Y-axis is showing response unit (RU) and X-axis is showing is showing time in seconds. Now Y-axis transformation is showing that all the spots which were showing sensogram from different levels are now with the same baseline.

By using SPR and SPRi the kinetics and affinity can be determined. Kinetics represents the rate of reaction, affinity is strength of binding. KD or Dissociation constant is represented by Kd/Ka , where Kd is off rate and Ka is on rate. So kinetics as the rate of complex formation and the fitting data can be performed according to the model.

So as we have discussed, the SPRi combines the advantages of SPR- the kinetic and affinity analysis with high throughput capabilities. It is convenient, sensitive and offers high throughput label-free measurement of biomolecular interaction. Now we will discuss some of the very basic concepts involved in SPRi. Let's watch this animation for further understanding.

A gold-coated glass array surface is used for immobilization of antibodies complimentary to the target protein of interest. A broad beam, monochromatic, polarized light originating from a suitable light source is used to illuminate the entire biochip surface with the help of mirrors placed at suitable angles that will reflect the light onto the surface. Reflected light from each spot on the array surface is captured by means of a CCD camera and used to generate the SPRi image.Binding of target antigen to the antibody is detected in real-time due to changes in intensity of reflected light from every spot on the array surface. Multiple biomolecular interactions can be studied

simultaneously in a high throughput manner and changes occurring on the array surface can provide kinetic data about the interactions.

Now let's talk about few applications of SPRi. The SPRi has been used for various applications such as protein-protein interactions, DNA-DNA and protein-DNA interactions. It has been widely for used direct, multiplexed detection of unlabelled low molecular weight protein biomarkers and protein expression profiling experiments. There are various studies which have demonstrated SPRi can be used for relative and absolute protein expression as well as different type of drug discovery applications.

Now let's talk about other technique which is also based on SPR principle. It is called nanohole array. So nanohole array technique utilizes the label-free detection of biological binding events in very high throughput manner with temporal and spatial resolution.

Nanohole arrays - The surface plasmons are excited on both sides of metal surface resonantly coupled through the sub-wavelength holes which enhances the light transmission for a specific wavelength and makes nanohole arrays a potential surface based biosensor.

There are some unusual optical transmission characteristics at the resonant wavelength which are shown by the ordered arrays which I will show you in the next image for the nanohole scale on the metal film. There are some recent studies which have shown that an integrated nanohole array with extraordinary optical transmission or EOT intensity can achieve ten times more sensitivity as compared to the prism based SPR configuration.

So let's look at configuration of nanohole arrays-A gold coated chip of nanohole is shown here. One sensing spot of nanohole array is 150 nm (diameter of one spot). One sensing spot is again expanded and you can see there is 12x12 matrix of nanoholes. So the periodicity of each nanohole is in the nm range depending on transmission light intensity.

Now in this graph which is intensity of transmitted light versus time scale. As analyte concentration increases on the nanohole arrays, the transmission intensity decreases exponentially. So nanohole arrays are promising for studying binding kinetics of protein-protein interaction and they are also compatible for microarrays format.

There are many advantages of using nanohole arrays. They provide information in the real time which has multiplexing capability. The optical alignment is very simple and miniaturization is very much possible with this type of platform. One can get rid of bulky prism which is the case of traditional SPR which is not required here. And also the sensitivity is much higher in comparison to traditional SPR. But there are few demerits as well, it is insensitive for the conformational changes and it is again limited to gold or silver surfaces.

Now let's discuss few applications of nanohole assays. So the simple optical alignment, easy miniaturization, very small foot print, very high sensitivity, multiplexing capability and collinear optical detection makes nanohole arrays a very useful platform for various applications. Nanohole arrays are promising for high throughput protein microarray based applications. They have been used for binding kinetics measurements, biomarker screening as well as studying protein-protein interactions.

After discussing SPR and SPR based techniques lets now discuss Ellipsometry-based techniques. Just to give you word of caution here that we cannot cover all the label-free techniques in very much detail. There are many promising techniques which we have to miss out here but there are some which I am going to give you glimpse so you aware of various label-free techniques which are available and again reference can be used for further study.

Ellipsometry is based on the polarization state of the reflected light which is altered due to the changes in dielectric property or the reflective index of the sample surface. Ellipsometry is based on the polarisation state of reflected light which is altered due to the changes in dielectric property or the reflective index of the sample surface. The

imaging Ellipsometry that combines ellipsometer, microscopy and CCD camera, it can measure the total protein content which is printed on solid surface.

This is configuration of Ellipsometry-based label-free technique. A monochromatic laser light which is linearly polarised by the polarizer is passed through a compensator and it produces elliptically polarised light. This light is reflected from a sample surface and again becomes linearly polarized, which is detected by the analyzer filter. This reflected light intensity is monitored with photo-detector.

Ellipsometry has many merits. The instrumentation is very simple unlike the SPR based instrumentation. It is not restricted to the gold or silver. It is cheaper than previous discussed SPR based techniques. It provides high throughput capability. It can provide simultaneous measurement of entire microarray. Demerits- It is less sensitive than SPR and SPR-based techniques. It is insensitive for the conformational changes.

Let's now discuss few Ellipsometry based applications- Ellipsometry has been successfully applied for many applications- such as biomolecular interactions, hormonal activity, cell factor and its receptor binding, diagnosis for Hepatitis B, kinetic measurements of multi-protein interaction processes and quantification of competitive adsorption of protein. The combination of imaging Ellipsometry with micro-fluidic system can provide many advantages which are not possible to obtain from individual techniques and this configuration, the combined imaging ellipsometry and microfluidics was applied for real time measurement of binding kinetics of SARS virus.

Let's talk about OI-RD. So OI-RD is a form of Ellipsometry in which, harmonics of modulated photocurrents are measured under the suitable nulling conditions. The changes in the thickness or dielectric response due to a reaction, such as protein binding, can provide a detectable signal and that is how the reactions are monitored.

This is the configuration of OI-RD. Now in this image PD denoting the photodiode detector and A is analyzer, OBJ is objective lens, PS is here phase shifter, PEM is photo-elastic modulator. The scanning OI-RD microscope, the X-scan is performed by

moving sample holding stage and Y-scan by a combination of rotating mirror and theta lens. The sample is coated on the glass slide, which is directly in contact with fluidic system.

The OI-RD platform is highly sensitive than imaging ellipsometry. It provides rapid detection system, real time measurement as well as high throughput affinity detection. Its demerits involve insensitivity to conformational changes. OI-RD has been used for various applications. OI-RD microscope is applied for real time monitoring of antigen-antibody interactions, nucleic acid hybridizations and protein-small ligand binding reactions. Its various applications makes it a good choice for label-free detection of proteins on microarrays.

So we have seen that label-based techniques are widely used for microarrays but just to avoid the issues due to tag there is increasing trend now to couple the microarrays with label-free platforms and that's why we are looking at different type of emerging label-free techniques such as SPRi, Ellipsometry, OI-RD based methods which are trying to take power of both microarray and label-free detection system to provide high throughput data without interference of a tag.

Now let's move on to interference-based techniques, we have already discussed about SPR and imaging based techniques, ellipsometry based techniques and now we will talk about interference-based techniques. So let's first talk about interferometry. Interference based detection techniques are powerful tools biochemical and functional analysis of proteins. It principle is "transformation of phase differences of wave front into observable intensity fluctuations known as interference fringes". There are many promising interferometry techniques have emerged which include spectral reflectance imaging biosensor (SRIB), dual-channel biosensor, SPR interferometry, on chip interferometry backscatter detection, biological compact disc (BioCD).

So let's look at interferometry principle in detail here-In interferometry, the phase differences of wave fronts are transformed into observable intensity fluctuations known as interference fringes. The interferometry techniques relate the optical phase to

biomolecular layer density on the surface. The signal is created by additional phase shift or optical path length (OPD), which is caused by the adsorbate biolayer. So the biomolecules printed on this surface, the left hand panel is showing the unbound state, the right hand panel is showing the bound state. The optical path length difference which is caused due to the adsorbate which is the biomolecule printed on the surface is giving you the change in OPD length and that is measured here.

So one of the interferometry technique is BIRC, let's briefly look at it. This is a highly sensitive interferometry performed within rectangular channels of micrometer size which are formed in inexpensive PDMS [poly (dimethylsiloxane)]. Here is configuration of BIRC, the micrometer sized rectangular channels are moulded within the PDMS chips and this interferometry principle is applied here for measurement of interference due to the molecules printed on surface.

BIRC has been applied for many applications. It is highly sensitive and enables study of reversible and irreversible protein interactions. The solute quantification and quantification of irreversible streptavidin-avidin binding, this type of application have been demonstrated using BIRC technique. This technique facilitates the label-free studies within the very small pico-litre volume and overall, this technique has potential to quantify binding affinities in high throughput manner.

So similar to other label-free techniques which we have discussed today. BIRC also provide real time measurements, it is applicable for reversible and irreversible for protein-protein interactions. This technique is very cost effective however it has some demerits such as use of PDMS chip, surface preparations are multistep and this is complex process which is time consuming.

So now let's discuss about SRIB. It is a label-free approach which is based on interference. The changes in optical index as a result of capture of biological material on surface can be detected using optical wave interference. SRIB monitors molecular binding interactions directly.

So in this slide the principle of SRIB is shown. The interference of light reflected from the silicon dioxide surface increase in the optical path-length differences are caused by this biomolecular binding which measured by SRIB.

So similar to some of the other recently discussed label-free techniques, SRIB is also very useful but recently introduced which has been applied to study dynamic protein-protein interaction. This technique has also shown very promising application for microarrays so it can be coupled with the protein microarray for high throughput applications.

The SRIB's are cost effective, they are used for fast determination of binding kinetics, they can be easily implemented and provide high throughput data for biomolecular interaction studies. It's demerits include it is suitable for only smooth layered substrates and also there are some non-specific binding which has to be improved with advancement in this technique.

Let's see this animation for further understanding of its principle. In SRIB technique, a silicon dioxide coated silicon surface is functionalized with the biomolecule of interest. The magnitude of total reflected light at a particular wavelength depends entirely on the optical path length difference or OPD between the top surface and the silicon dioxide-silicon interface. Binding of the target to the immobilized biomolecule further increases the optical path length difference and is seen as a shift in the spectral reflectivity. SRIB therefore serves as a useful tool for high throughput, real-time detection of biomolecular interactions.

After discussing many label-free techniques, in this slide I am just giving you an overview of several sensitive label-free techniques which are currently promising for microarray application. Some of these we have discussed in detail, it was not possible to discuss all of these in detail and also there are some nanotechniques based label-free technique which we will discuss in subsequent lecture but what I have shown in this slide is that some of these label-free techniques are very promising for high throughput protein microarrays or DNA microarrays based applications. So the panel A on the top-

that is based on Raman labels based on multicolour 12 C (12 carbon) and 13 C (13 carbon) single bond nanotubes tags for multiplex protein detection. The panel B is SPR which measure changes in the refractive index along with a sensorgram which is shown in the bottom of same image. The real time multiple binding events by monitoring the intensity of EOT or extraordinary optical transmission through the nanoholes sensing arrays are shown in panel C. The panel D describes SRIB which we just talked in last slide based on the light reflected from Silicon dioxide surface. Panel E shows a planer wave guide array system for fluorescence imaging where all the molecules are excited simultaneously with a laser light and only surface confined fluorescence labels are selectively excited for emission. This is not a label-free technique but this is very sensitive technique applicable for high throughput protein microarray platform. Panel F shows nanowire sensor arrays where protein binds specifically to its receptor on the nanowire and produces conductance change.

So this just gives you an overview of various platforms which are currently in progress for coupling with the protein microarrays and DNA microarrays so that one can utilize potential of both the label-free detection techniques as well microarray capabilities. So other than techniques which we talked today, there are many nanotechniques which are very promising analytical tools for disease diagnosis, cellular detection, screening of potential drugs as well as environmental monitoring. By using wide variety of nanomaterials numerous label-free detection techniques have been introduced for protein microarray based techniques which we will discuss in the next lecture. So in summary in today's lecture we talked about SPR and SPR based techniques such as SPR imaging and nanohole arrays. We also talked about ellipsometry based technique such as OI-RD and imaging ellipsometry. We then talked about interference based detection techniques such as BIRC and SRIB.

It just shows you that there are many new label-free techniques which are emerging for many applications. Some of these are in very early stage it is very tough to say which one is better than the other just because these techniques are evolving they are in field

of optimization and most of the applications and studies have only shown prove of concept but not biological meaningful application.

Only SPR has been used for long enough and we have discussed it in much more detail. There are many label-free techniques which are emerging and promising, we have tried to cover, at least principle of the technique as well advantages and disadvantages of each of these techniques along with it configurations. We will continue discussion on some of the latest technologies especially nanotechniques based proteomics in the following lecture. Thank you!