Proteomics: Principles and Techniques

Prof. Sanjeeva Srivastava
Department of Biosciences and Bioengineering
Indian Institute of Technology, Bombay

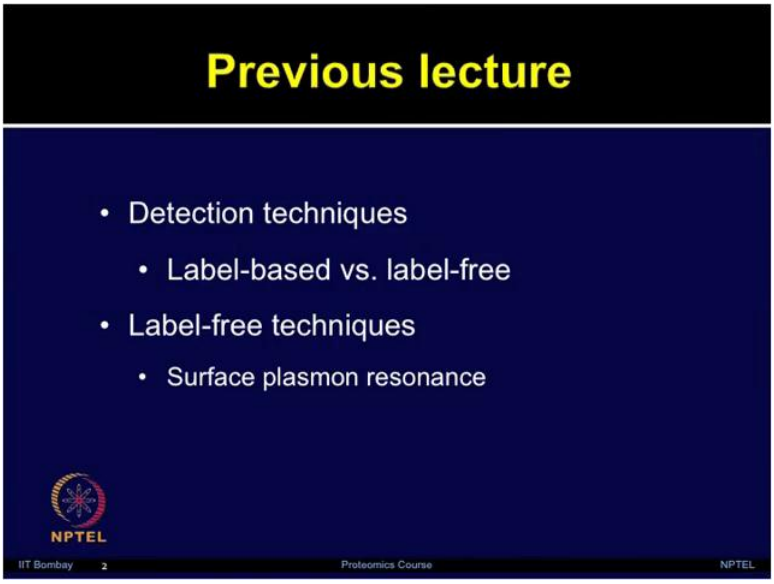
Lecture No. # 36
Label-free techniques: SPRi, Ellipsometry, Interference

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

So the label-free techniques they relay on the measurement of some inherent properties of the query molecules such as mass, dielectric properties, and it allows for the direct real-time detection of bimolecular protein interactions as well as various other applications in high throughput manner by eliminating the requirements of secondary reactants various labels. Among the different immerging label-free techniques - surface Plasmon resonance SPR and SPR imaging SPRi, they are most promising candidates for various diagnostic applications as well as studying the biomolecular interactions.

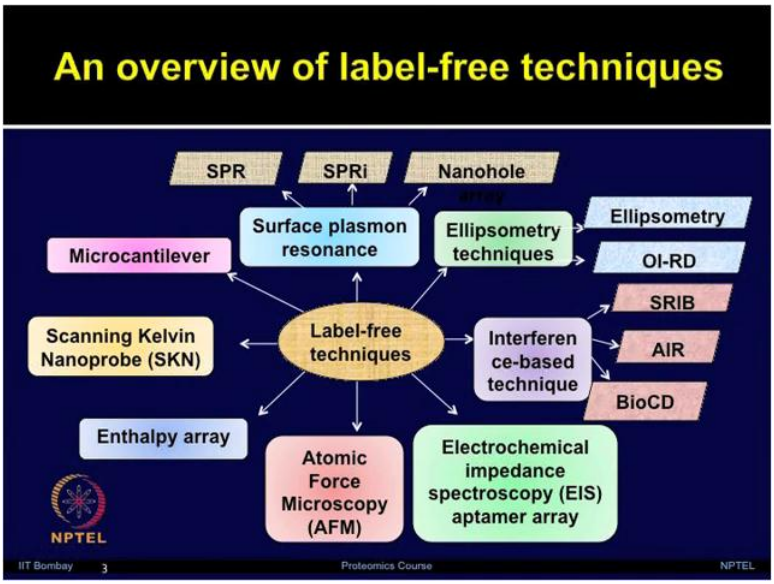
We studied the SPR or surface Plasmon resonance in more detail in the previous lecture, but other than SPR based techniques, there are various nano techniques, ellipsometry and interferometric based detection techniques which have also emerged in the label-free techniques. SPR although is most widely available and used the label-free detection platform, but SPR imaging has several high throughput potential and that is why it is immerging as an alternative to SPR which allows the simultaneously analysis of multiple biomolecular interactions in a high throughput manner.

(Refer Slide Time: 02:50)



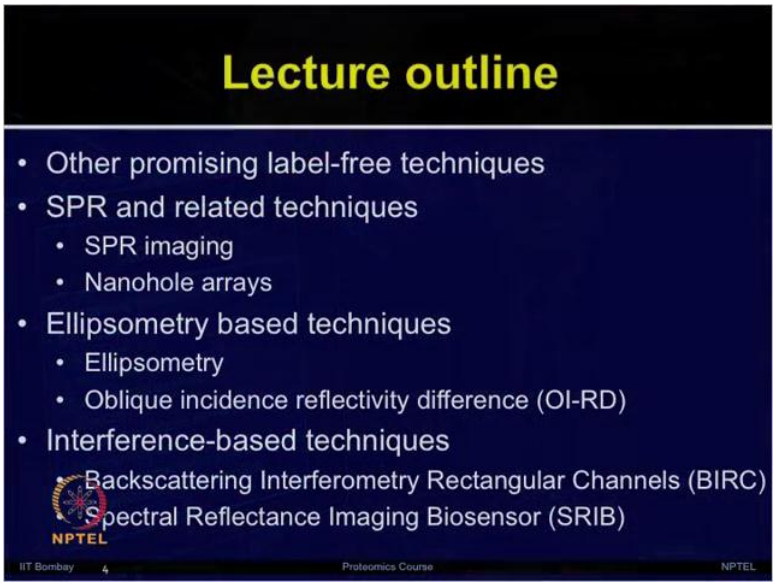
So today, we will discuss from our previous lectures which were more on the label-based and label-free comparison. Today we will focus on the label-free detection techniques and we will talk more onto the SPR imaging; different type of ellipsometry and interferometry based techniques. So, in the last lecture if you remember, we talked about comparison of detection based methods such as label-based and label-free methods, and different type of label-free techniques where we discussed in more detail about surface Plasmon resonance.

(Refer Slide Time: 03:31)



Just to refresh you about various label-free techniques which were discussed briefly in the last lecture; this overview slides give you various available options for studying the different applications in using different label-free techniques such as SPR based platforms like SPR, SPR imaging 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, we have arrayed imaging reflectometry or AIR then, we have bioCD; then they are options such as electrochemical impedance spectroscopy aptamer arrays, atomic force microscopy or AFM, enthalpy arrays, scanning Kelvin nanoprobes or SKN and microcantilever. This is a detailed list, but not the complete list; there are still many other emerging label-free techniques which are available there which was not possible to include everything in this overview slide.

(Refer Slide Time: 04:57)



Today, we will focus from our previous discussions on SPR and related techniques, we will now cover the SPR imaging and nanohole arrays then, we will move on to ellipsometry based techniques; we will talk about what ellipsometry is and then followed by OI-RD or oblique incidence reflectivity difference. We will then talk about interference-based techniques such as back scattering interferometry, rectangular channels or BIRC and spectral reflectance imaging biosensor or SRIB.

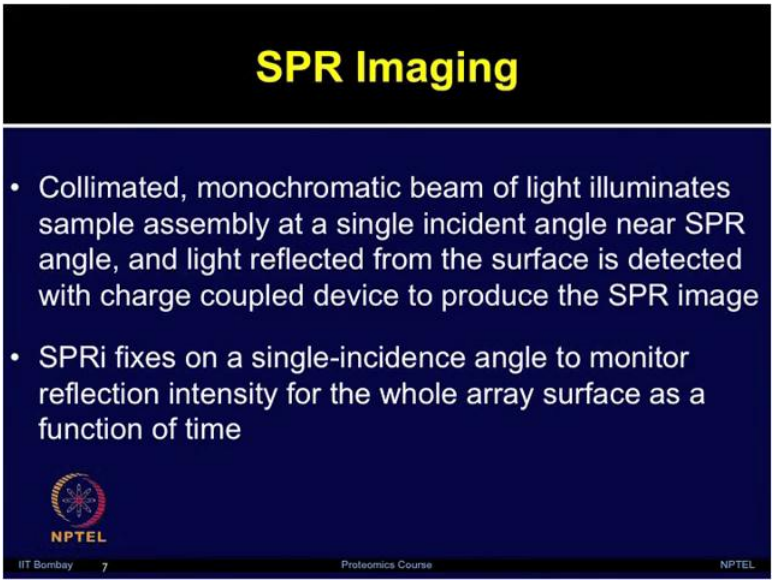
(Refer Slide Time: 05:36)

So, let us first start with the SPR and related techniques. We have discussed about surface Plasmon resonance.

(Refer Slide Time: 05:45)

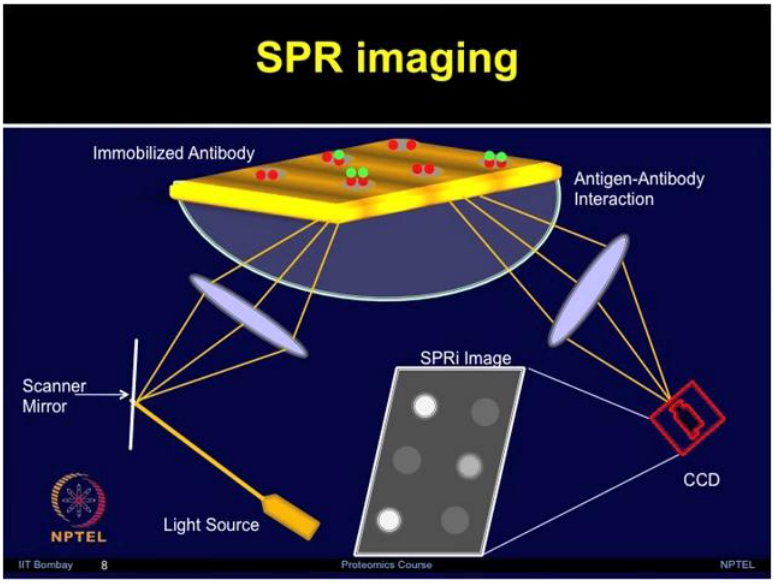
Now today, we will talk about surface Plasmon resonance imaging or SPRi. So, SPRi the entire biochip surface is illuminated at a time with a broad beam of monochromatic polarized light, and the reflected time is captured from each spot by a CCD camera simultaneously. This CCD camera continuously monitors the changes which are occurring on the surface and provides the real-time kinetic data in high throughput manner.

(Refer Slide Time: 06:21)



So, SPR imaging technique involves collimated monochromatic beam of light which illuminates the sample assembly at a single incident angle near the SPR angle, and the light reflected from the surface is detected by using CCD device charge coupled device to produce the SPR image. Now, the SPR imaging it fixes on a single incidence angle to monitor the reflection intensity for the whole array surface as a function of time.

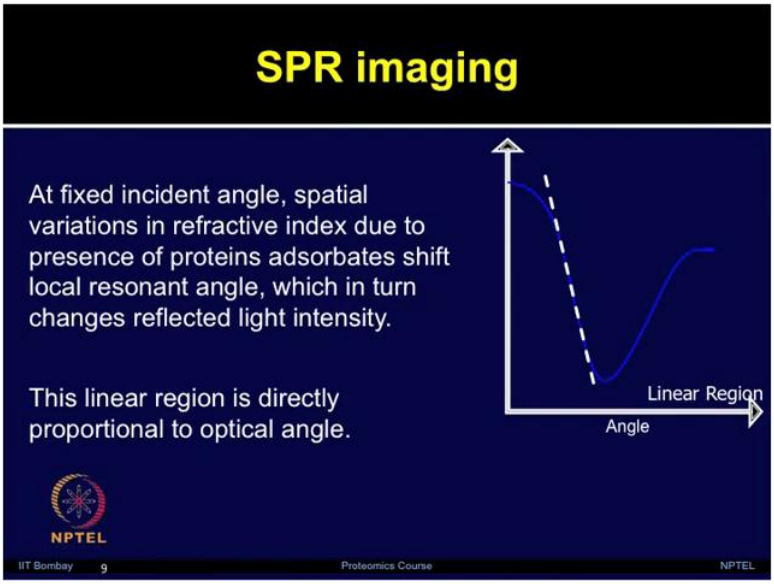
(Refer Slide Time: 06:58)



This is a set up of SPR imaging. The configuration shows that light from a collimated polychromatic source passes through a polarizer, and impinges on a prism or gold interface

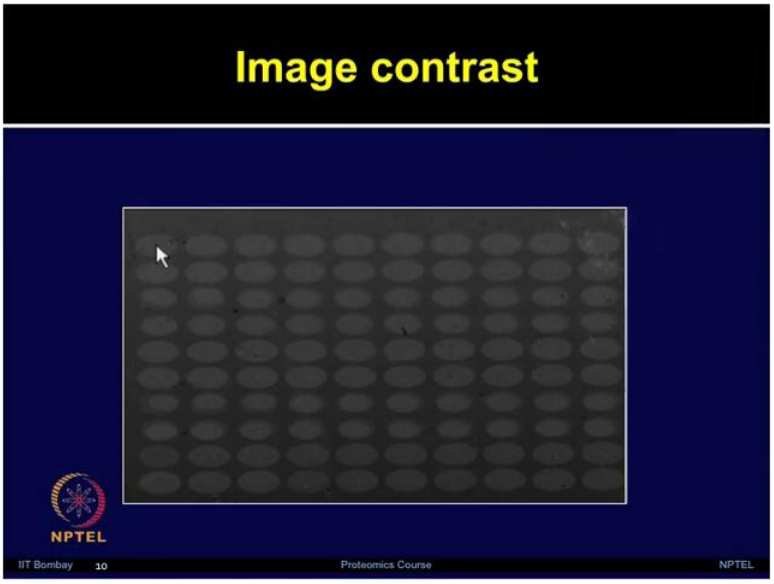
at a specific angle of incidence which you can see here the gold surface, the prism and the light source. Now, the reflected light passes through the narrow band interference filter, and it deducted with the CCD camera as you can see in this PPT here.

(Refer Slide Time: 07:33)



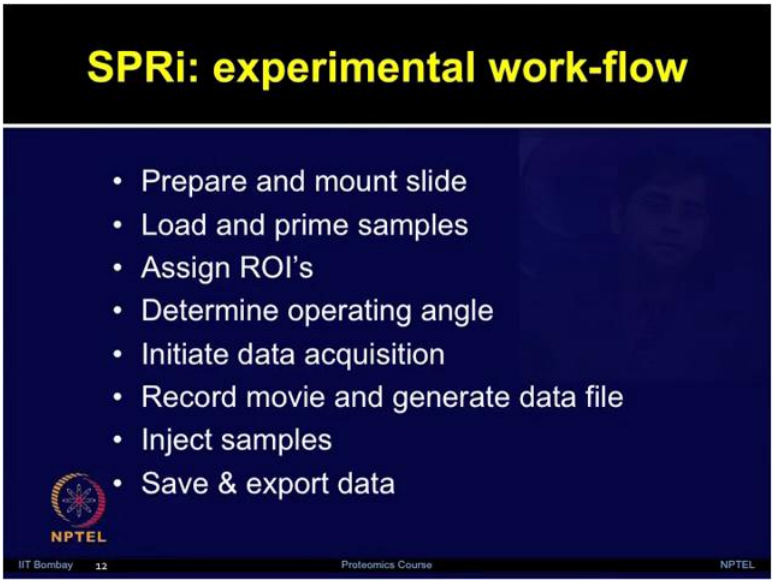
So at a fixed incident angle, the spatial variations in refractive index due to the presence of protein or other biomolecules which are printed on the surface those adsorbates shift local resonant angle which in turn changes the reflected light intensity. So, this linear region as you can see in this slide here, this is directly proportional to the optical angle. The linear region of SPR curve quantitatively correlates the changes in reflected light intensity with amount of material present on the surface.

(Refer Slide Time: 08:16)



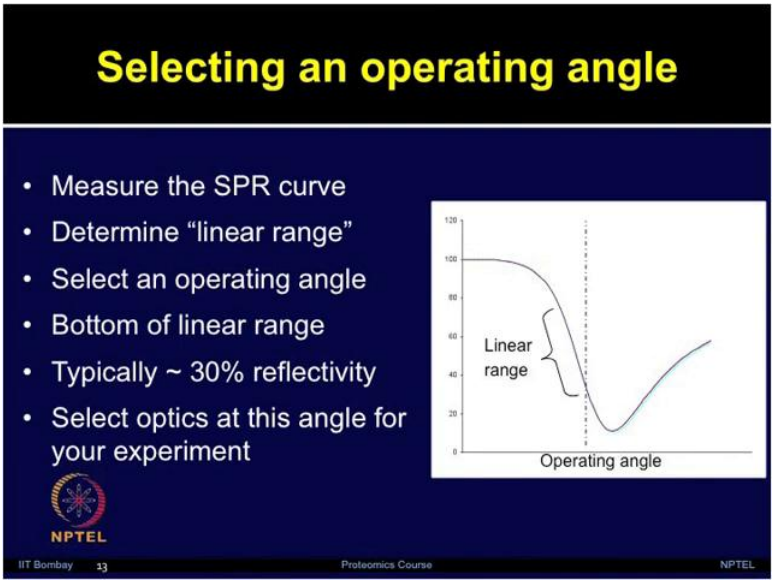
Now from SPR image as the term says SPRi, an image is generated and as you can see in this slide here, the image contrast is shown. So in SPRi, the collimated monochromatic beam of light is used to illuminate the sample assembly at single incident angle near the SPR angle and the light reflected from the surface can be detected by charge coupled device camera which produces this SPR image. As you can see here, each of this circle shows a spot printed on the surface of gold. Now, let us talk about the experimental workflow involved in SPR imaging 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 SPR imaging experiments.

(Refer Slide Time: 09:20)



So, let us go through some of these steps in more detail. So, various steps involve preparation and mounting the slide on the prism when you are starting the SPR imaging experiment; load and prime the samples; assign the region of interest; determine the operating angle; initiate the data acquisition; record movie and generate data file; inject the sample and then finally, you can save and export the data.

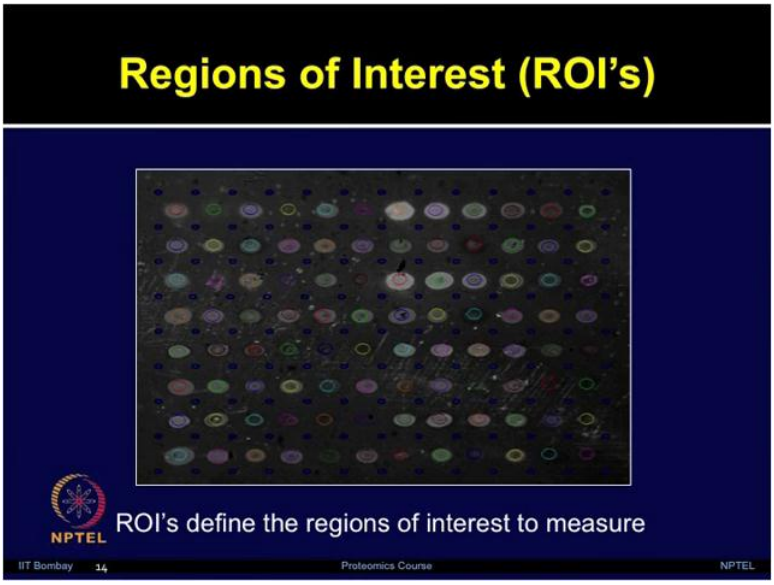
(Refer Slide Time: 09:55)



Now in SPR imaging, the first most crucial step is selection of an operating angle. So here as you can see in the slide, the SPR imaging device it measures the SPR curve and

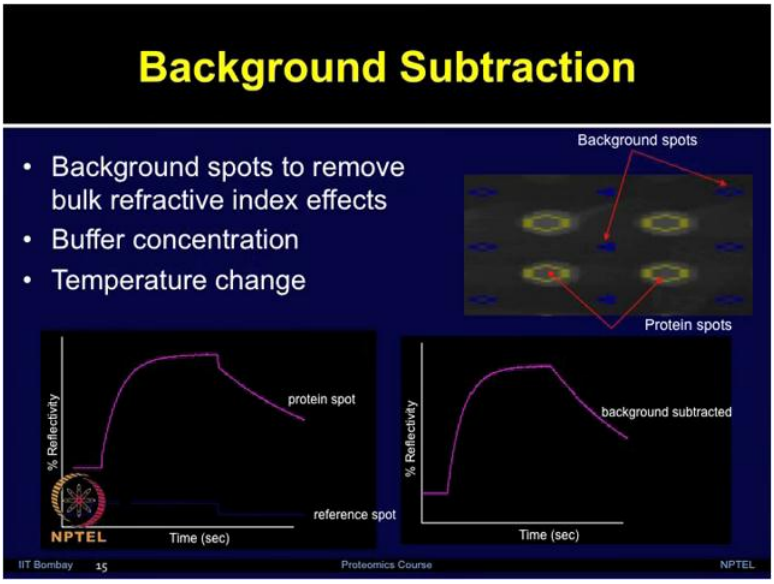
determines the linear region as you can see here in this region. Now, first of all one need to stand the whole optics to range of angles and determine the linear range. Once you select an operating angle which is usually the bottom of the linear range. There are also some rough estimate that typically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

(Refer Slide Time: 10:59)



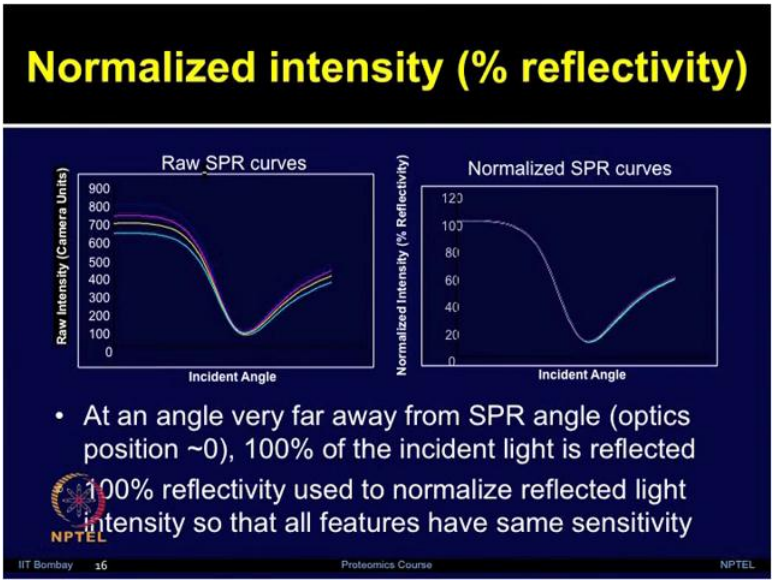
Now, this image shows that SPR image generated from the CCD camera and now software adds up some spots on the background surface. This is image taken from proteomic processor instrument SPR imaging device. Each of the ROI or regions of interest can be defined as the region of interest for the measurement.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

(Refer Slide Time: 11:45)



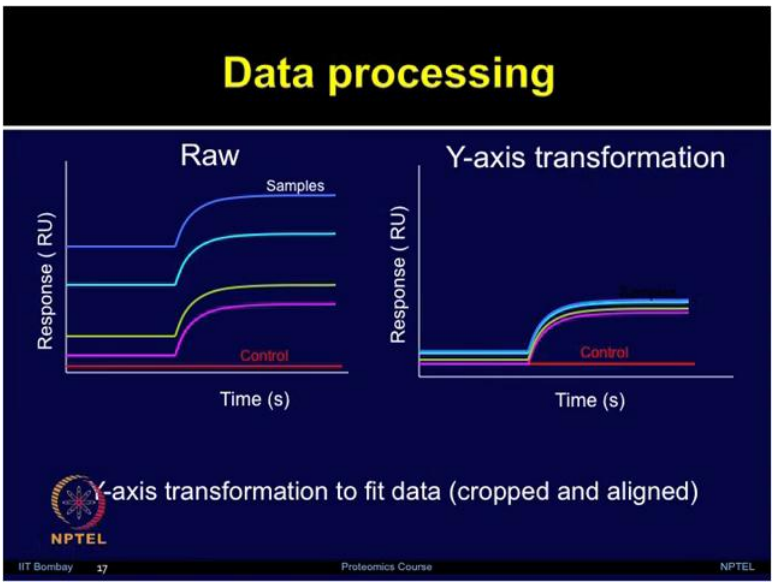
So, background spots to remove the bulk reflective index effects as it is more visible in this side. These spot is the spot of interest and there are four software generated spots, the reference spots and the bottom panel is showing a graph where you can see the pink one, the protein spot signal and the blue one is the reference spot signal. So, X axis is for the time in the second scale and Y axis percentage reflectivity. The right hand side graph shows the background subtraction. So, if you subtract the reference spot generated from the software with the protein signal then, the background subtracted image can be generated.

(Refer Slide Time: 12:34)



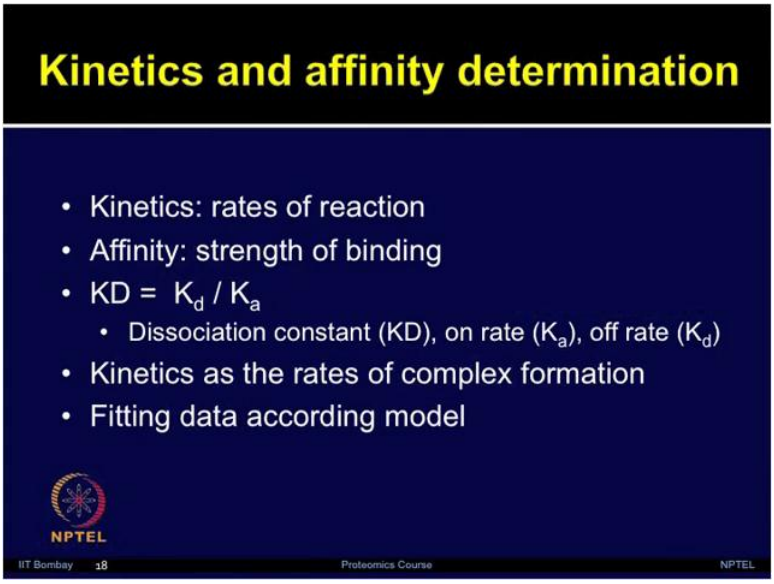
Now, next important aspect is the normalized intensity or percentage reflectivity. So, left image is showing is raw SPR curves and the right image is showing the normalized SPR curves. Certain angle which is very far away from the SPR angle, the optics position will be at zero, 100 percent of the incident light is reflected. Now, the 100 percent reflectivity can be used for the normalization; it can be used to normalize the reflected light intensity so that all the features have similar intensity.

(Refer Slide Time: 13:13)



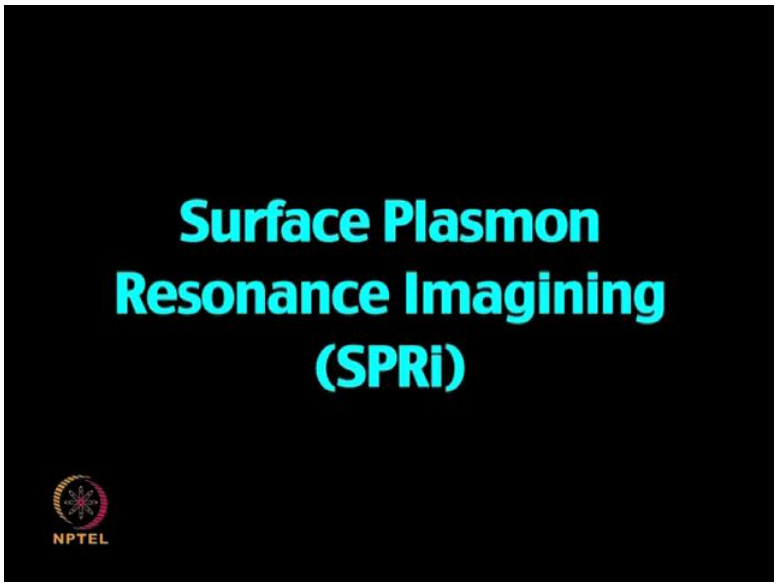
Now, data processing involves multiple steps. We have also discussed few consideration for data processing and analysis in the previous lecture when we talked about SPR. Similar concepts can be applied here; also as I have shown in this slide. The y axis transformation is important because, you have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

(Refer Slide Time: 14:10)



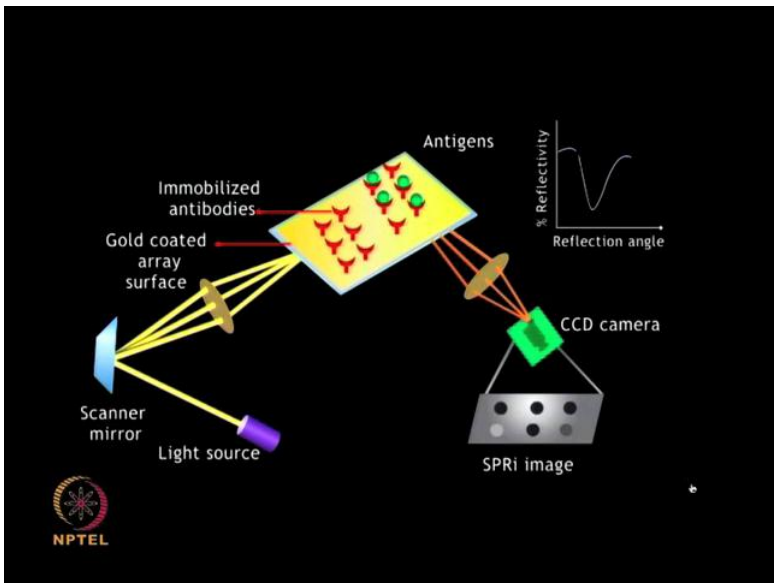
By using SPR and SPR imaging, the kinetics and affinity can be determined. Kinetics represents the rate of reaction; affinity is the strength of the binding; KD, K capital D or dissociation constant is represented by K d; K small d which is showing the off rate divided by K small a which is on rate. So, kinetics as the rate of complex formation and the fitting data can be performed according to the models. So as we have discussed, the SPR imaging 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, after discussing some of the basic concepts involved in SPR imaging,

(Refer Slide Time: 15:18)



Let us watch this animation for further understanding of SPR imaging concepts. Surface Plasmon resonance imaging or SPRi.

(Refer Slide Time: 15:25)



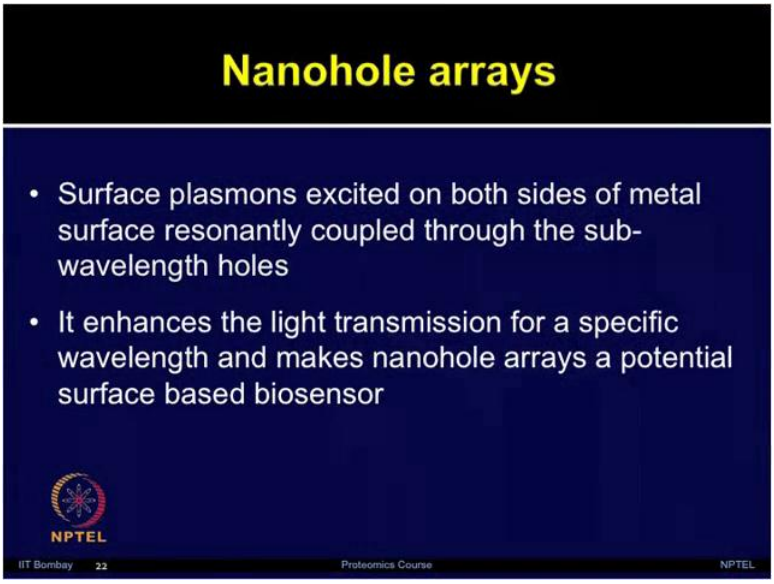
In this technique, a gold coated glass array surface is used for immobilization of antibodies complementary to the target protein of interest. A broad beam monochromatic polarized light originating from a suitable light source is used to illuminate the entire biochip surface with the help of mirrors placed at suitable angles that will reflect the light on to the surface. The reflected light from each spot on the array surface is captured by a CCD camera and

used to generate the SPRi image. Binding of target antigen with an antibody can be detected in real-time due to changes in the 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.

(Refer Slide Time: 17:07)

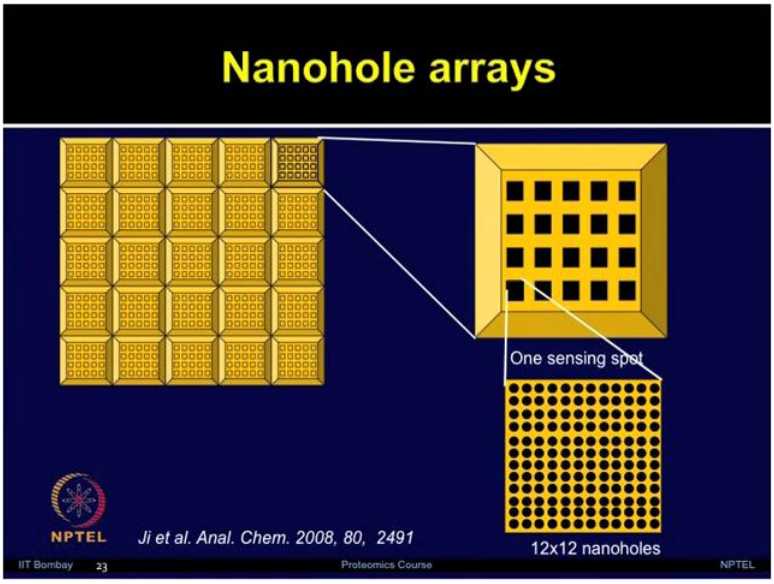
So, after discussing the SPR imaging animation, now let us talk about few applications of SPR imaging. The SPRi has been used for various applications such as protein-protein interactions, DNA DNA and DNA protein interactions. It has been widely used for direct, multiplexed detection of unlabeled low molecular weight protein biomarkers and protein expression profiling experiments. There various studies which have demonstrated that SPR imaging can be used for relative and absolute protein expression as well as different type of drug discovery applications. Now, let us talk about another technique which is also based on the SPR principle which is nanohole arrays. So, nanohole array technique utilizes the label-free detection of biological binding events in very high throughput manner with temporal and special resolution.

(Refer Slide Time: 18:17)



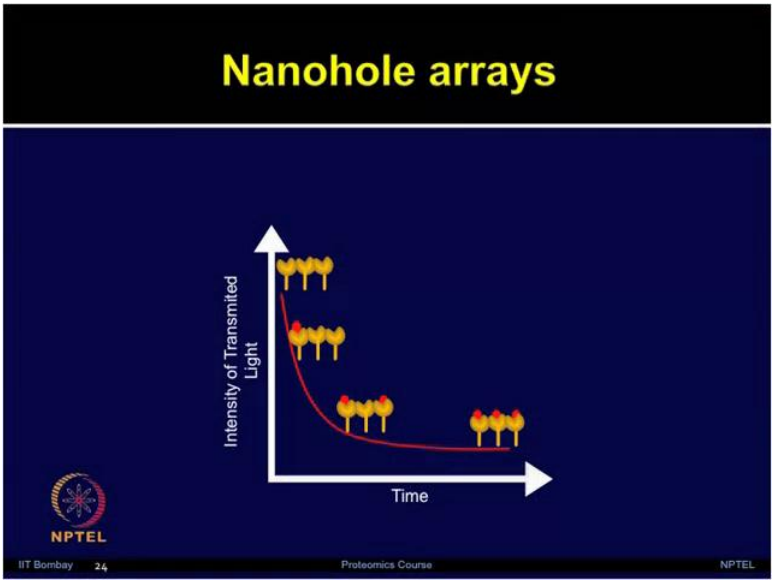
So, nanohole arrays the surface Plasmon exited on the both sides of metal surface which are resonantly coupled through the sub wavelength holes which enhance the light transmission for a very specific wavelength and makes nanohole arrays a potential surface based biosensor. There is some unusual optical transmission characteristics at the resonant wavelength which are shown by the (( )) arrays which I will show you in the next image for the nanohole scale on the metal film. There is some recent studies which have shown that an integrated nanohole array with extraordinary optical transmission or EOT intensity can achieve above 10 times more sensitivity as compared to the prism based SPR configurations.

(Refer Slide Time: 19:22)



So, let us look at this configuration of nanohole arrays. So, where a gold coated chip of nanohole array shown here, now one sensing is spot of nanohole array is around 150 nanometers; that is the diameter of the one spot and now one sensing spot is again expanded, and you can see there are 12 by 12 matrix of the nanoholes. So the periodicity of each nanohole is in the nanometer range depending on the transmission light intensity.

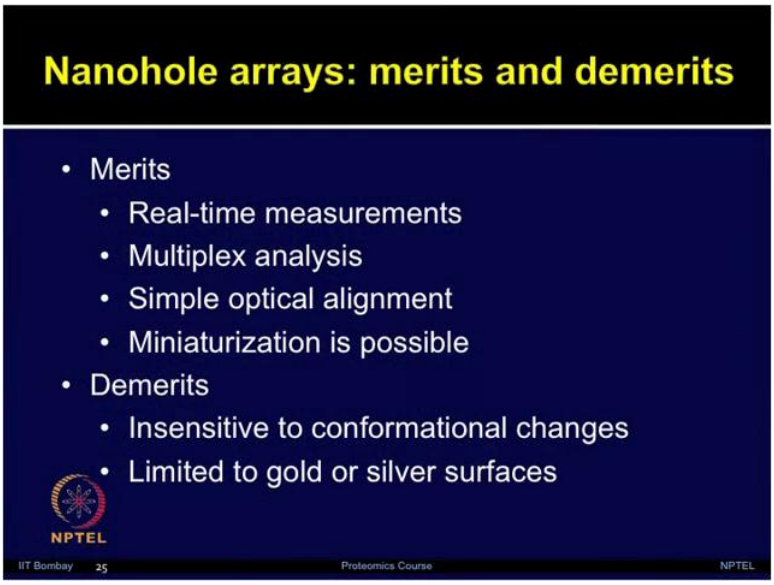
(Refer Slide Time: 19:56)



Now, in this graph which is intensity of the transmitted light versus time scale; as analyte concentration increases on the nanohole arrays, the transmission intensity of the light

decreases exponentially. So, nanohole arrays are promising for studying the binding kinetics of protein-protein interactions and there also compatible for the micro array format.

(Refer Slide Time: 20:23)

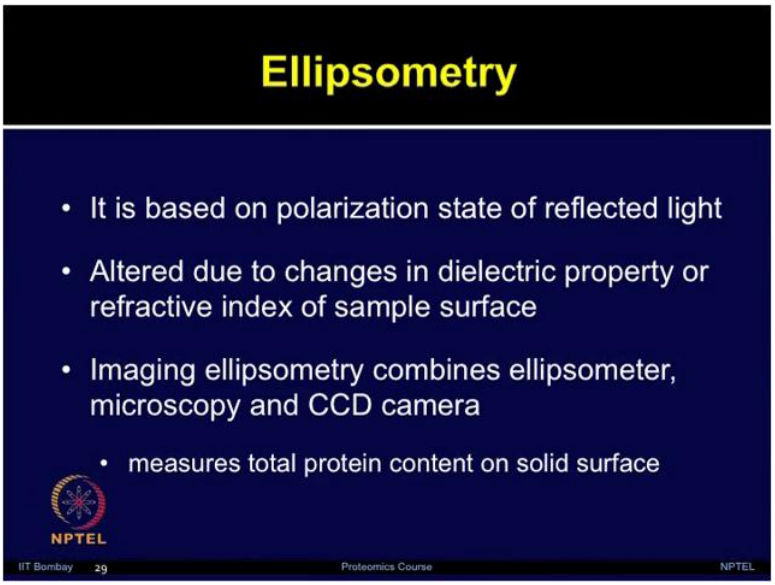


There many advantages of using nanohole arrays; they can provide information in the real-time which as multiplexing capability. The optical alignment is very simple and miniaturization is very much possible by using this type of platform. One can get rid of bulky prism which is the case for traditional SPR which is not required here, and also the sensitivity is much higher as compared to the traditional configuration of SPR. But there are few demerits as well. It is insensitive for the conformational changes; it is again limited to the gold or silver metals surfaces. Now, let us discuss few of the nanohole array based applications. So the simple optical alignment; it is easy miniaturization; very small footprint, very high sensitivity, multiplexing capability and collinear optical detection makes the 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 measurement, biomarker screening as well as studying protein-protein interactions.

(Refer Slide Time: 21:54)

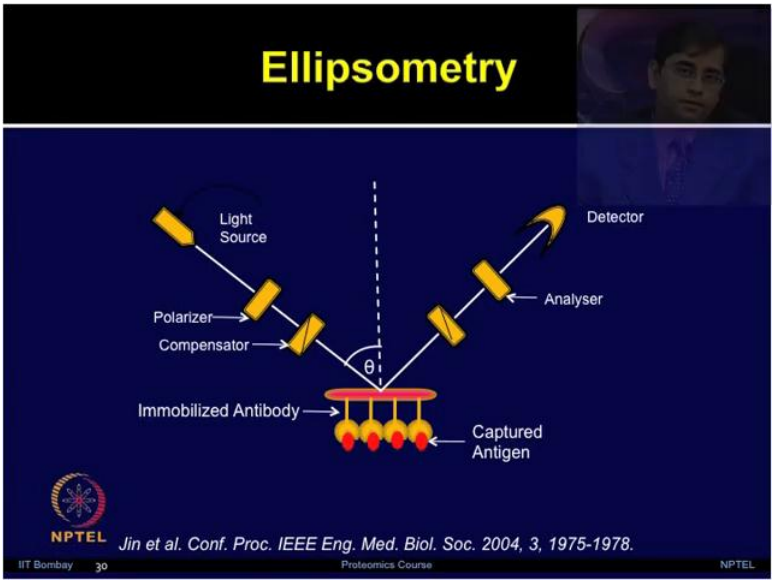
After discussing SPR and SPR based techniques, now let us move on to ellipsometry based techniques. So, just to give you word of caution here that, we cannot cover all the label-free techniques in very much details; there are many promising techniques which we have to miss out here, but there are some which I am going to give you glimpse so that you are aware about various type of label-free techniques which are available and again some of the references can be used for further studies. So, ellipsometry is based on the polarization state of the reflected light which is altered due to the changes in dielectric property or the refractive index of the sample surface.

(Refer Slide Time: 22:38)



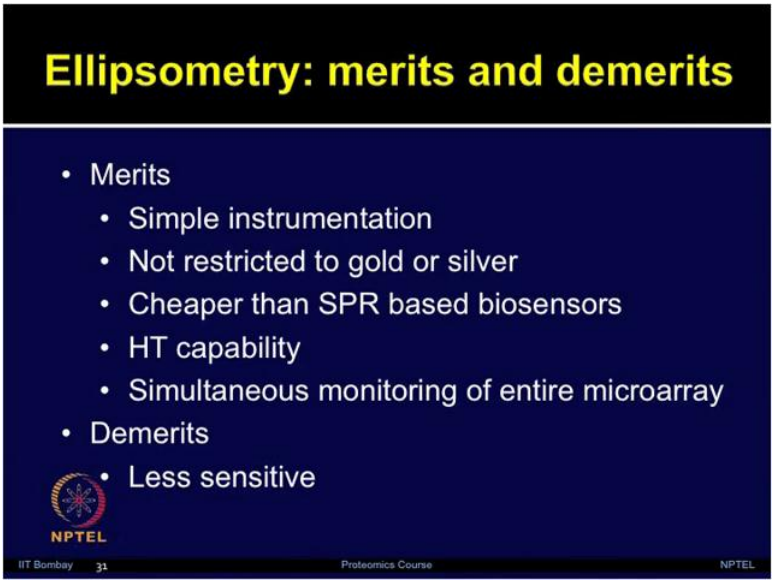
Ellipsometry is based on the polarization state of reflected light which is altered due to the changes in dielectric property or refractive index of the sample surface. The imaging ellipsometry that combines ellipsometer, microscopy as well as CCD camera, imaging ellipsometry can measure the total protein content which are printed on the solid surface.

(Refer Slide Time: 23:10)



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

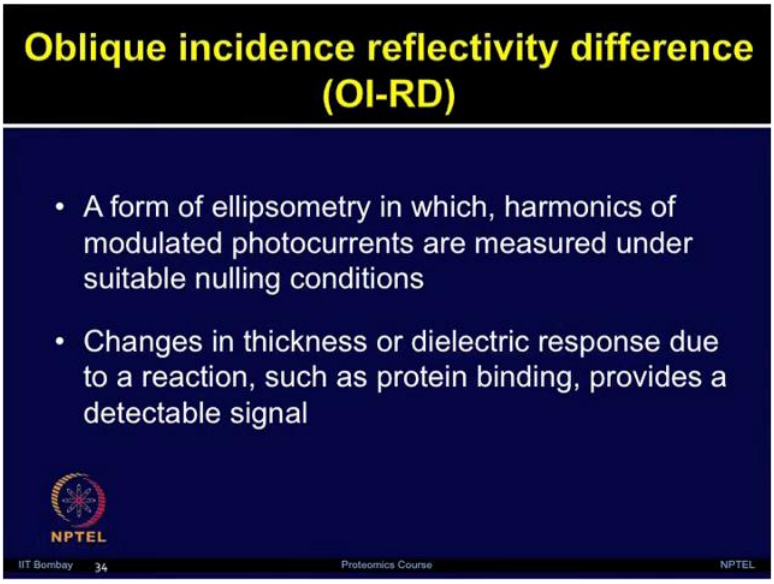
(Refer Slide Time: 23:46)



The ellipsometry technique has many merits; the instrumentation is very simple unlike the SPR based instrumentations. It is not restricted to the specific metals such as gold or silver; it is much cheaper than the previously discuss techniques based on SPR configurations. It also provide the high throughput capability, and it can provide the simultaneous measurement of chip surface which is applicable for the microarray base applications. It demerits; it is less sensitive than SPR, SPR imaging; it is insensitive for the conformational changes.

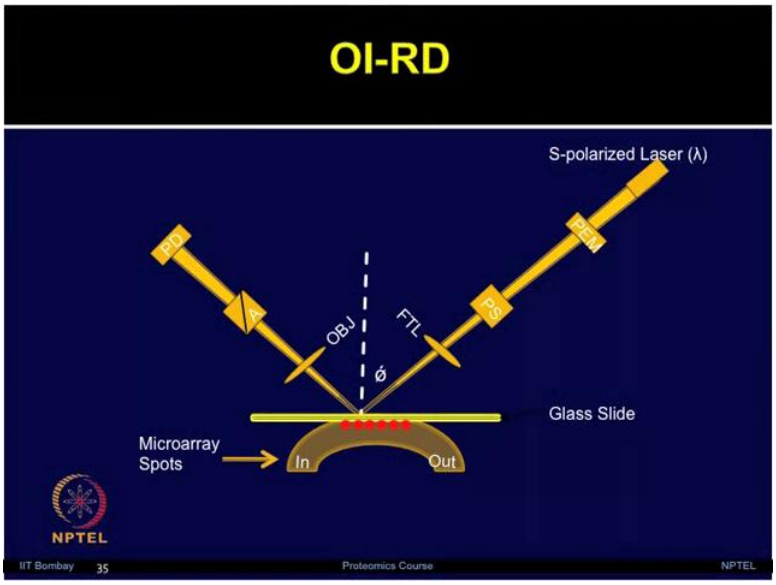
Now, let us discuss few of the ellipsometry based applications. So, ellipsometry has been successfully applied for many application such as biomolecular interactions, hormonal activity, cell factor and it is a sector binding, diagnosis for hepatitis b, kinetic measurements of multi protein interaction processes and quantification of competitive absorption of protein. The combination of imaging ellipsometry with microfluidic system can provide many advantages which is not possible to obtain from the individual these instruments, and this configuration the combined imaging ellipsometry and microfluidics was applied for real-time measurement of binding kinetics of sars virus Now, let us talk about oblique incidence reflectivity difference or OI-RD which is based on polarization modulated nulling ellipsometry.

(Refer Slide Time: 25:36)



So, OI-RD is a form of ellipsometry which we just discussed in which the harmonics of modulated photocurrents are measured under the suitable nulling conditions. The change in the thickness or dielectric properties due to a reaction such as protein binding can provide a detectable signal and that is how the reactions are monitored.

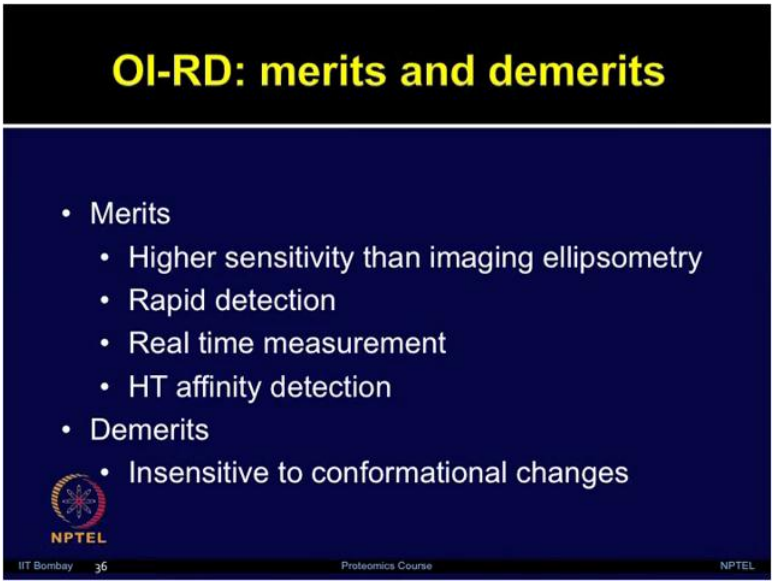
(Refer Slide Time: 26:00)



This is the configuration of OI-RD. Now in this image, PD detecting the denoting the photodiode detector; A is analyser; OBJ objective lens; we have PS here phase shifter; and PEM which is photoelastic modulator. The scanning OI-RD microscope the access can is

perform by moving the 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 the contact with fluidic system.

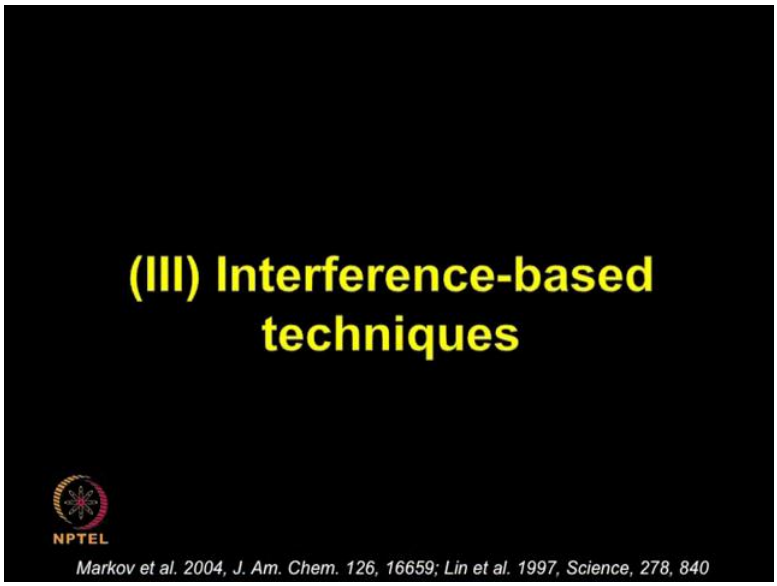
(Refer Slide Time: 26:42)



The OI-RD platform is highly sensitive as compared to the imaging ellipsometry base system. It provides ability for rapid detection, real-time measurement as well as high throughput affinity determination. Its demerit involves insensitive for the 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. There are various application of ORID which makes it a good choice for label-free detection of proteins on microarrays.

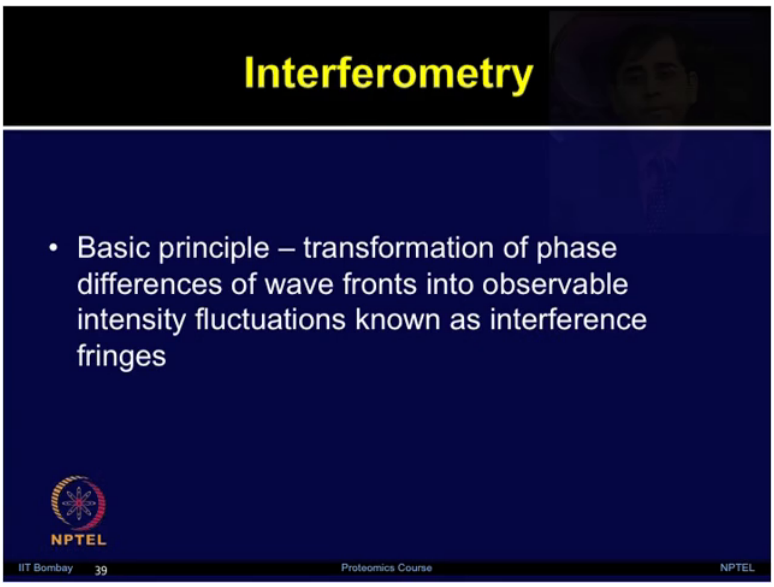
So, we have seen that label-based detection techniques are widely used for or microarrays, but just to avoid the issue due to the tag. There is increasing trend now to couple the microarrays with label-free platforms and that is why, we are now looking at different type of emerging label-free techniques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

(Refer Slide Time: 28:08)



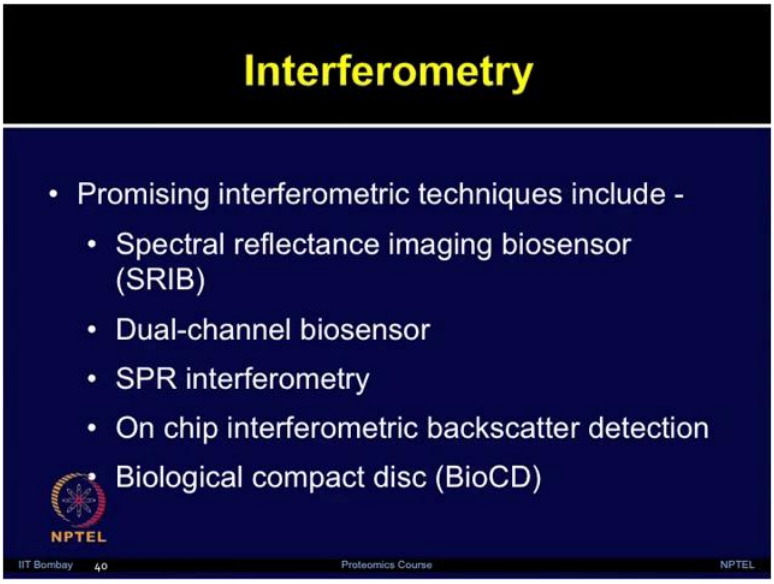
So, now let us move on to third label-free techniques which is interference based techniques, we have already discussed today about SPR and imaging based techniques, ellipsometry based techniques. Now, we will talk about interference based techniques.

(Refer Slide Time: 28:25)



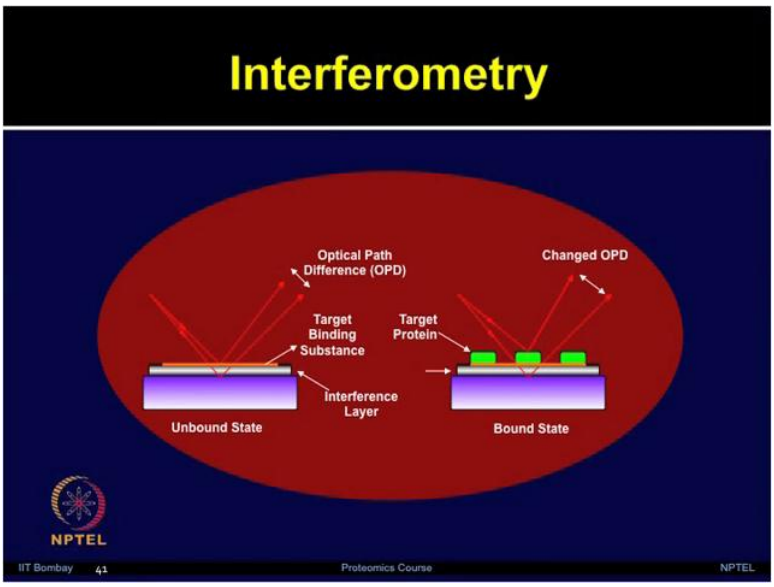
So, let us first talk about interferometry. So the interference based detection techniques are powerful tool for biochemical and functional analysis of proteins. Its basic principle is the transformation of phase difference of wave front of wave front into observable intensity fluctuations which is known as intensity fringes.

(Refer Slide Time: 28:50)



There are many promising interferometry techniques have emerged which includes spectral reflectance imaging biosensor or SRIB, dual channel biosensor, SPR interferometry, on chip interferometric backscatter detection and biological compact disc or BioCD.

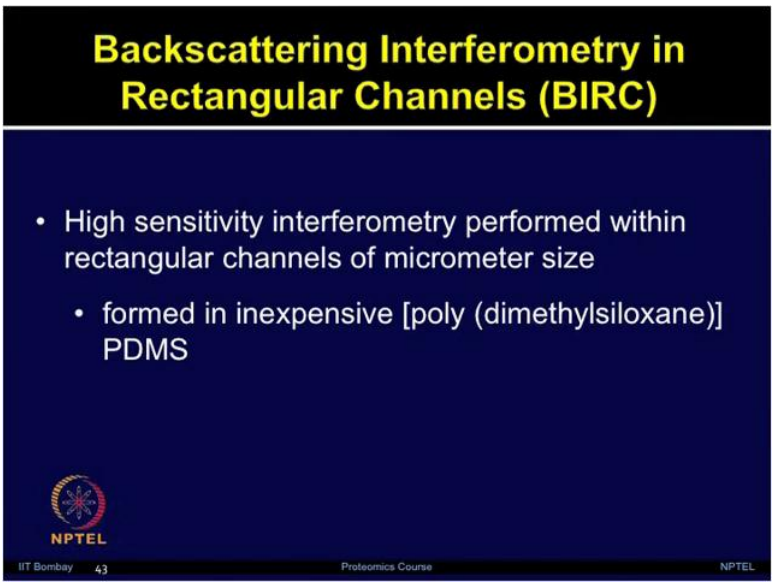
(Refer Slide Time: 29:15)



So, let us look at this interferometry principle in detail here. In interferometry, the phase difference of wave fronts are transformed into observable intensity fluctuations called as interference fringes. The interferometric techniques relate the optical phase to biomolecular layer density on the surface. The signal is created by an additional phase shift or optical

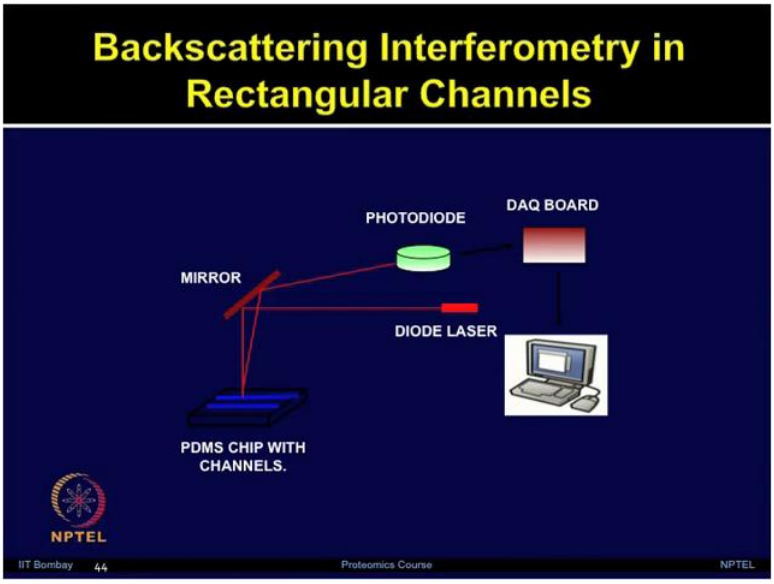
path length difference OPD which is cause by the adsorbate biolayer. So the biomolecules are printed on these surface; the left hand panel showing the unbound state; the right hand showing the bound state. The optical path length difference which is caused due to the adsorbate which is biomolecule printed on the chip surface is giving you the change in that OPD length, and that is measured here with the interferometry principle. (( )) of the interferometry technique is BIRC or backscattering interferometry in rectangular channels.

(Refer Slide Time: 30:24)



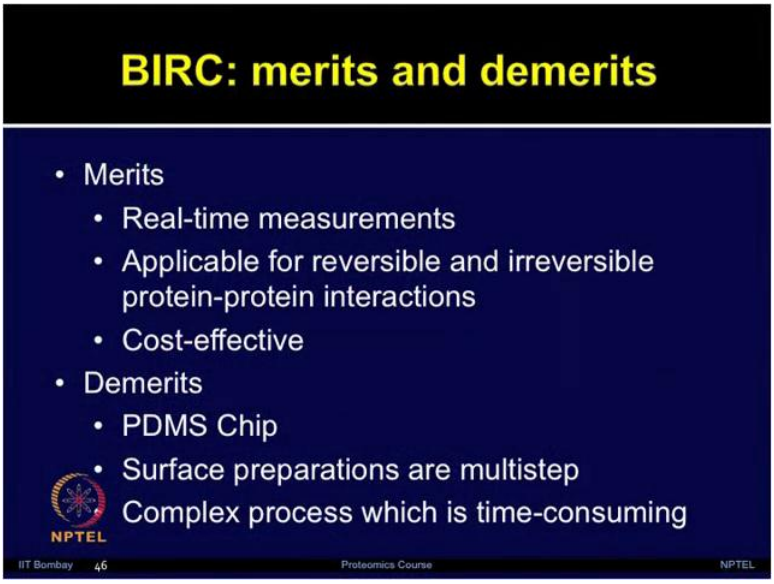
Let us briefly look at this technique. This is highly sensitive interferometry technique which is perform within the rectangular channels of micrometer size which are formed in the inexpensive PDMS or poly dimethylsiloxane.

(Refer Slide Time: 30:40)



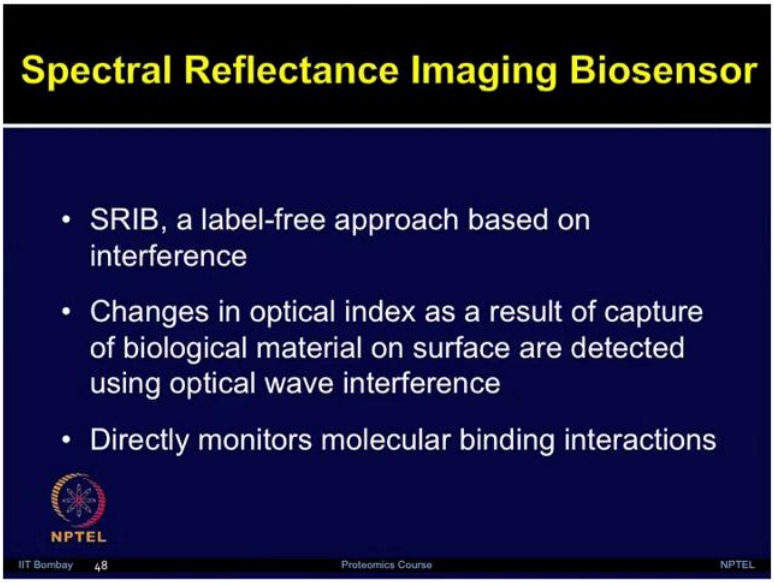
Here is the configuration of backscattering interferometry in rectangular channels or BIRC. The micrometer sized rectangular channels are moulded within the PDMS or poly dimethylsiloxane chips, and this interferometry principle is applied here for measurement of interference due to the biomolecules printed on these 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 biotin binding; these type of applications have been demonstrated by using BIRC technique; this technique facilitates the label-free studies within the very small peculator volume, and overall this technique has potential to quantify binding affinities in high throughput manner.

(Refer Slide Time: 31:49)



So, similar to other label-free techniques which we have discussed today BIRC also provides real-time measurement. It is applicable for reversible and irreversible protein-protein interactions. This technique is very cost effective. However, there are some demerits such as use of the PDMS chip, the surface preparations involves multiple steps and this is complex step, complex process which is time consuming.

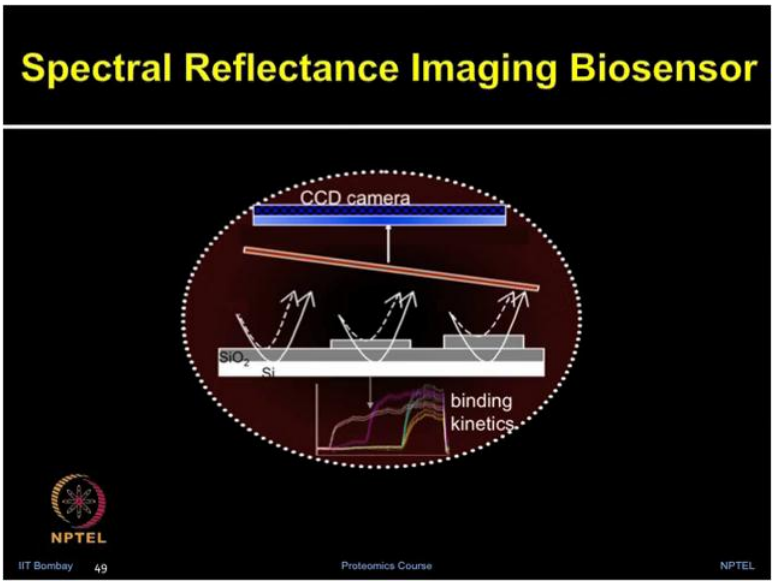
(Refer Slide Time: 32:34)



So, now let us discuss about spectral reflectance imaging biosensor or SRIB. SRIB is a label-free approach which is also based on interference. The changes in optical index as a

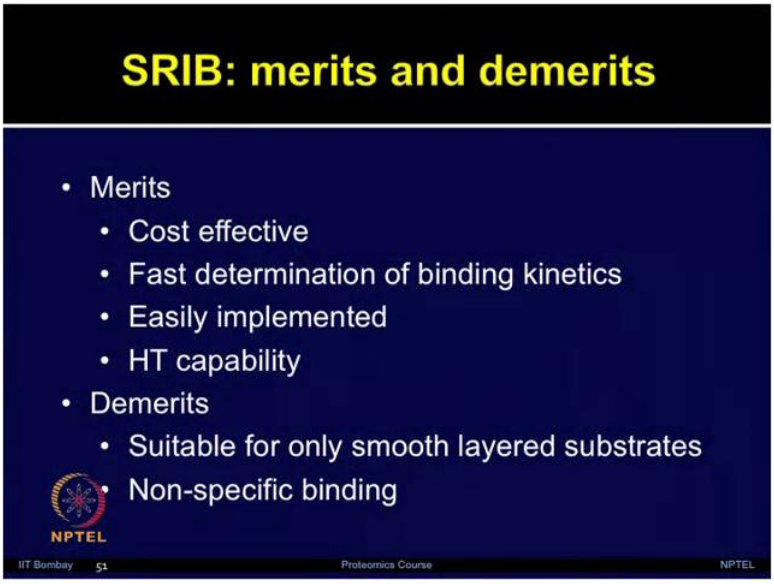
result of capture of the biological material on the surface can be detected by using optical wave interference. SRIB monitors the molecular binding interactions directly.

(Refer Slide Time: 32:59)



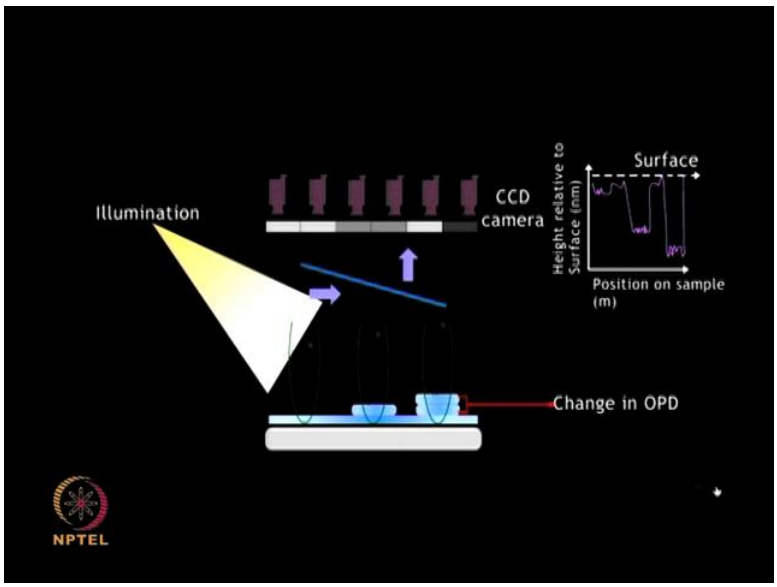
So in this slide, the principle of SRIB is shown. The interference of light reflected from the silicon dioxide surface; increase in the optical path length differences are cause by this biomolecular binding which is measured by spectral reflectance imaging biosensor. So, similar to some of the recently discussed label-free techniques, SRIB is also very useful, but recently introduce technique which have been applied for dynamic measurement of protein-protein interaction. The technique has also shown very good promising application for protein microarrays. So it is possible that, it can be coupled with the protein microarray for the high throughput applications.

(Refer Slide Time: 33:53)



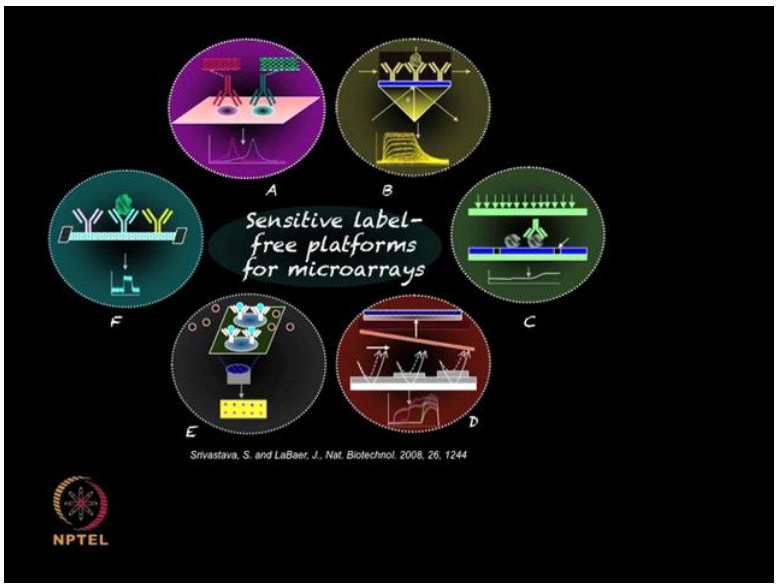
Spectral reflecting imaging biosensor; they are cost effective; they can fast determine the binding kinetics; they can be easily implemented and provide high throughput platform for biomolecular interaction studies. It demerits includes; it is suitable for only smooth layered substrates, and also there are nonspecific binding which has to be improved with the advancement in this technology. Now, after discussion on some of the basics of spectral reflectance imaging biosensor or SRIB, let see this animation for further understanding of its working principle.

(Refer Slide Time: 34:42)



Spectral reflectance imaging biosensor or SRIB. In SRIB technique, a silicon dioxide coated silicon surface is functionalize with biomolecules of interest. The magnitude of total reflected light at a particular wave length depends entirely on the optical path difference or OPD between the top surface and the silicon dioxide silicon interface. As you can see here, in this animation when the biomolecules are printed at different depth, that difference in the optical path differences measured here on the silicon dioxide and silicon surface. Binding of the target to the immobilized biomolecule further increases the optical path difference, and it is shown as a shift in the spectral reflectivity. This spectral reflectance imaging biosensor or SRIB therefore serves as a useful tool for high throughput real-time detection of biomolecular interactions.

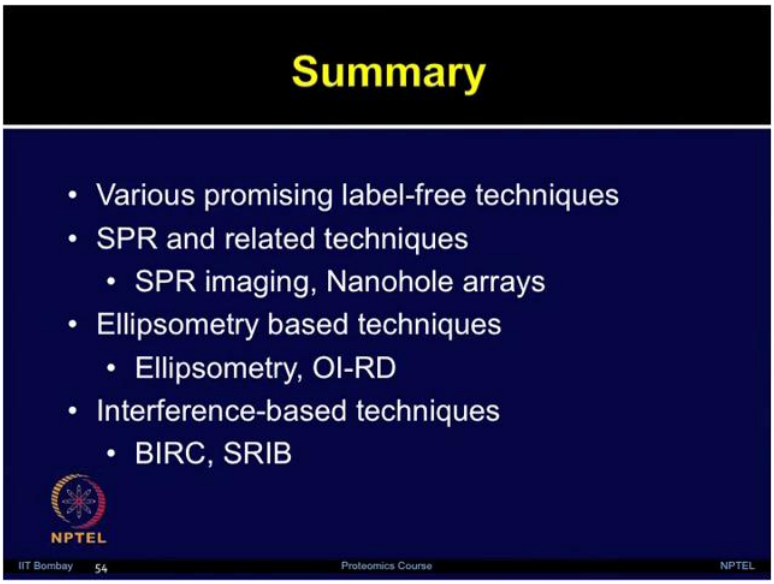
(Refer Slide Time: 36:06)



After discussing many label-free techniques in this slide, I 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 this in detail and also there are some nanotechnique based label-free technique which we will discuss in the subsequent lecture. But what I have shown in this slide is that some of these label-free techniques are very very promising for a high throughput protein microarrays DNA microarray based applications. So, the panel A in the top that is based on the Raman labels based on multicolor 12 carbon and 13 carbon single walled nanotube tags for multiplex protein detection. The panel B is surface Plasmon resonance which measures changes in the reflective index along with sensorgram which is shown in the bottom of the

same image in the panel B. The real-time multiple binding events by monitoring the intensity of EOT or extraordinary optical transmission through the nanoholes and single rays are shown in panel C. The panel D describe the spectral reflectance imaging biosensor which we just talked in the last slide based on the interference of light reflected from the silicon dioxide surface. Panel E shows a planer wave guide array system for fluorescence imaging, where all the molecules are excited simultaneously with the laser light and only surface confined fluorescence labels are selectively excited for a mission. This is not a label-free technique, but this is a highly sensitive technique applicable for the high throughput protein microarray platform. Then panel F shows the nano wire sensor arrays where protein binds specifically to its a sceptre on the nano wire and produces the conductance change which is shown in this figure in panel F. So, this is just given an overview of various platforms which are currently in progress for coupling with the protein microarrays or DNA microarrays so that one can utilize the potential of both the label-free detection detection techniques as well as microarray capabilities.

(Refer Slide Time: 38:54)



So, another technique which we talked today, there are many nano techniques which are very promising analytical tools for disease diagnosis, cellular detection, screening of potential drugs as well as environment monitoring. By using wide variety of nano materials, numerous label-free detection techniques have been introduced for protein microarray based applications 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 detection techniques such as OI-RD and imaging ellipsometry. We then talked about interference based detection techniques such as BIRC and SRIB or spectral reflectance imaging biosensors. It just shows you that, there are many a new techniques, 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. Then the phase optimization and most of the applications or studies have actually shown only the proof of concept, but not actually the biological meaningful application.

So, only SPR has been used form long time, and we have discussed that in much more detail. There are many techniques, label-free techniques which are emerging, which are very promising and we have try to cover that at least the principle of the techniques as well as some of the advantages, and disadvantage of each of these techniques along with its configurations. We will continue our discussion on some of the latest technologies especially the nano technique based proteomics in the following lecture. Thank you.