Proteomics: Principles and Techniques
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Lecture No. # 27
Microarray workflow: Label-based Detection Techniques

Welcome to the proteomics course. In today's lecture we will continue our discussion on microarrays workflow, we will talked about label based detection techniques. Most of the microarray applications to date have used label based detection techniques due to their several advantages. Such as ease of use, common availability of reagents and simple instrumentations which are required for detection of these label based systems. Conventionally, the label based techniques such as fluorescence labeling, radioactivity, chemiluminescence they were used. But now several new techniques such as quantum dots, gold Nanoparticles, dye doped Nanoparticles. As well as different type of (( )) based labels are currently employed for various microarray applications. In proteomics application there is need to detect a very dynamic range of proteins those which are present in the low abundance, as well as those which are present in the high abundance. It is very easy to detect high abundant proteins, but detection of very low abundant proteins requires very sensitive detection platforms.

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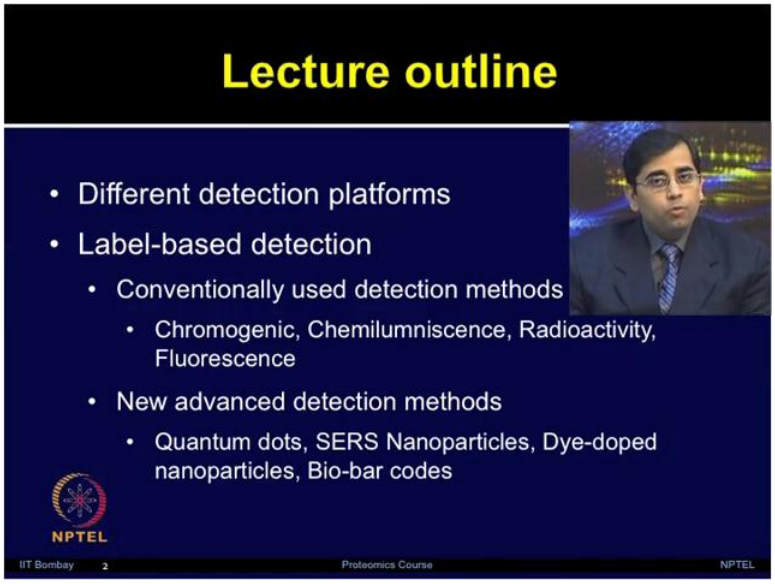

So, in today's lecture we will talk about different type of detection platforms. We will focus more on label based detection techniques. Although for detection there are various type of label free approach is also available, but those will be discussed separately. So, label based detection, the several conventional used label based systems such as chemiluminescence, chromeogenic based detections, radioactivity and fluorescence which is most commonly and widely used. Now, there are several new advanced detection techniques are also emerging, which includes quantum dots, SERS based Nanoparticles, and dye doped Nanoparticles, bio bar codes. As well as many other Nano technique based detection systems.

The label based platform for microarrays is very commonly used. For microarray applications, detection systems have now improved significantly as compared to what are the available from last a few years ago. The sensing technologies they aim to improve the sensitivity, the limit of detection, the dynamic range as well as they try to provide multiplexing capability and high resolution. So, all the new latest sensing techniques they aim for achieving all of these advantages. So, broadly we can group these detection platforms which are now improvised for microarray applications in label based and label free detection systems.

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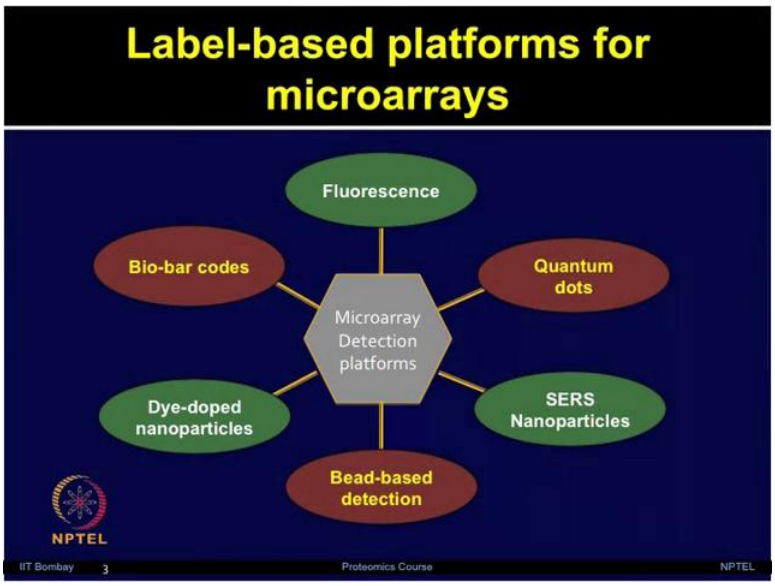

The label based systems, we will discuss fluorescence based methods, quantum dots, SERS Nanoparticles, bead based detection systems, and dye doped Nanoparticles as well

as bio bar codes. These are just a few names there are many other detection system which are also available. Although today we will not discuss about the label free platforms, but just for your information not only label based, but there is an increasing trend now, to also apply the label free platforms for microarray based applications. On one hand the label based systems use tags for detection of the signals. The tags could be fluorescent dyes, radiolabels. Whereas, the label free system they try to avoid any labels present in the query molecules. They depend on the inherent properties of the query, it is mass, dielectric properties, the optical properties etcetera.

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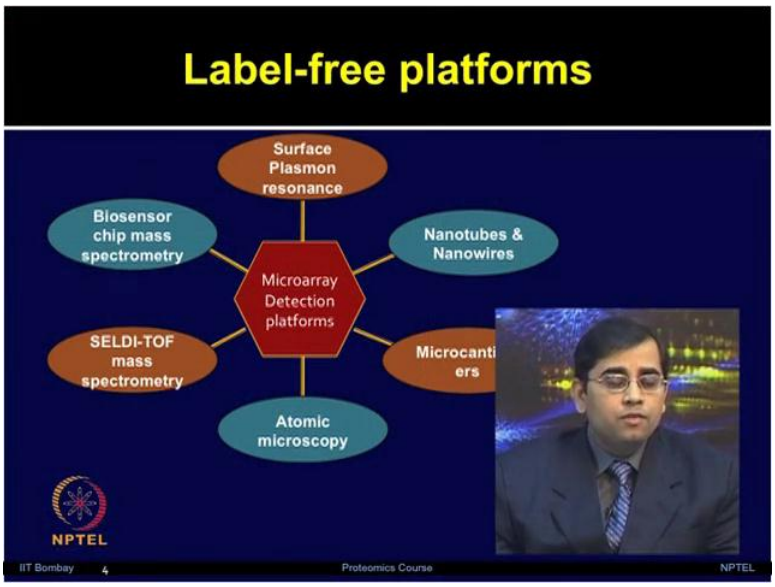

The main commonly and recently more advanced systems which have emerged for label free system, include the surface Plasmon resonance, nanotubes and nanowires, microcantilever based systems, atomic force microscopy, cell rate of mass spectrometry base systems and biosensor chip based m s technologies. Again, we will discuss label free techniques as well as several Nano techniques in more details in the next lectures.

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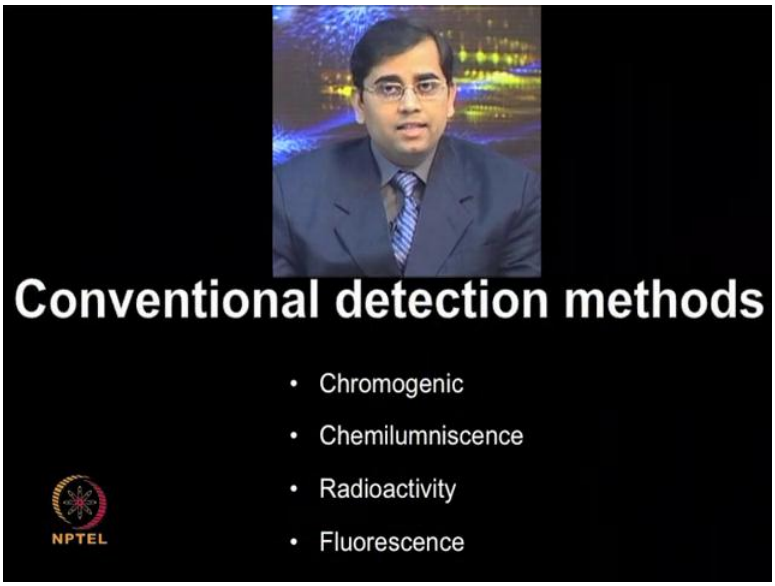

So, let us start discussing about conventional detection techniques for label based detection system. The most conventional one available are chromogenic, chemiluminescence, radioactivity and the fluorescence based detection systems. Let us discuss each one of these in some more detail and then we move on to the some more recently emerged advanced platform, which has used for label based detection system. Chromogenic detection, the antigen antibody interactions can easily be detected by use of chromogenic reactions. The chromogenic substrate is a molecule which is catalyzed by the enzyme linked to the antibody to provide a colored product which can be easily detected.

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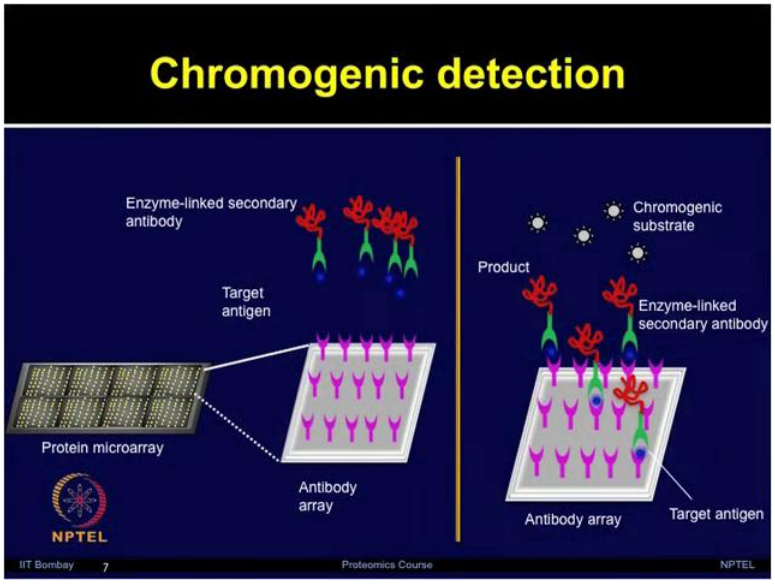

In chromogenic detection an enzyme that can give a colored reaction upon the addition of suitable substrate, which we just discussed. And then that molecule can be linked to secondary antibody. This acts as a probe by binding to a different epitope on antigen from the primary antibody bound to the microarray surface. Binding of substrate molecules result in to the colored product formation which can be easily detected and quantified by means of a microarray scanner. Let us look at the chromogenic detection system in the following animation to understand the mechanism in more detail Chromogenic detection.

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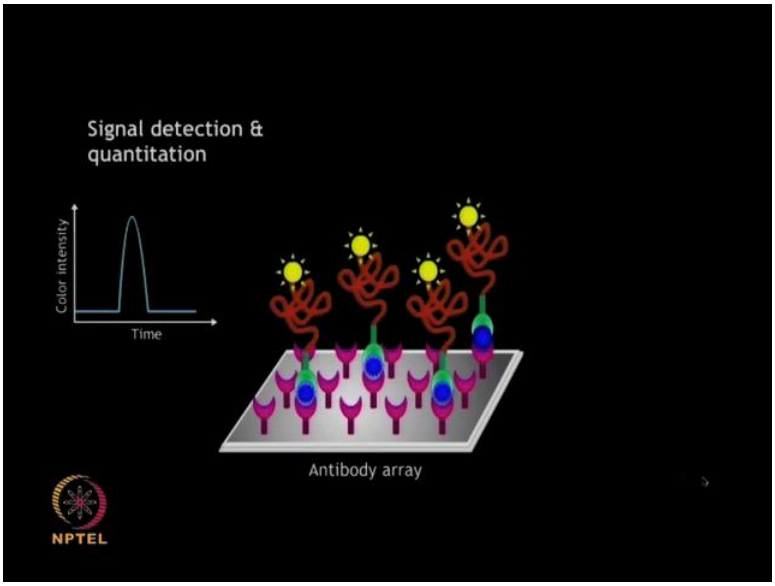

The antigen antibody binding interaction can easily be detected by a means of chromogenic reactions. An enzyme that can give a colored reaction upon addition of suitable substrate molecules is linked to the secondary antibody. This acts as a probe, by binding to a different epitope on the same antigen from that of the primary antibody bound to the array surface. Binding of the substrate molecule results in the colored product being formed, which is easily detected and quantified by a means of an microarray scanner.

Let us now move on to chemiluminescence based detection system. The emission of light as a result of a chemical reaction is referred to as chemiluminescence. And this phenomenon can be used very effectively to detect the molecules of interest. For example, luminal that is used to detect trace quantities of food gives out light when it comes in contact with iron of the hemoglobin molecule. Similar chemical interactions between the target analyte and the probe molecule can be used for detection of binding interactions for microarrays. The horseradish peroxidase h r p linked antibodies are most commonly and widely used to catalyze the reaction of chemiluminescent substrate molecules.

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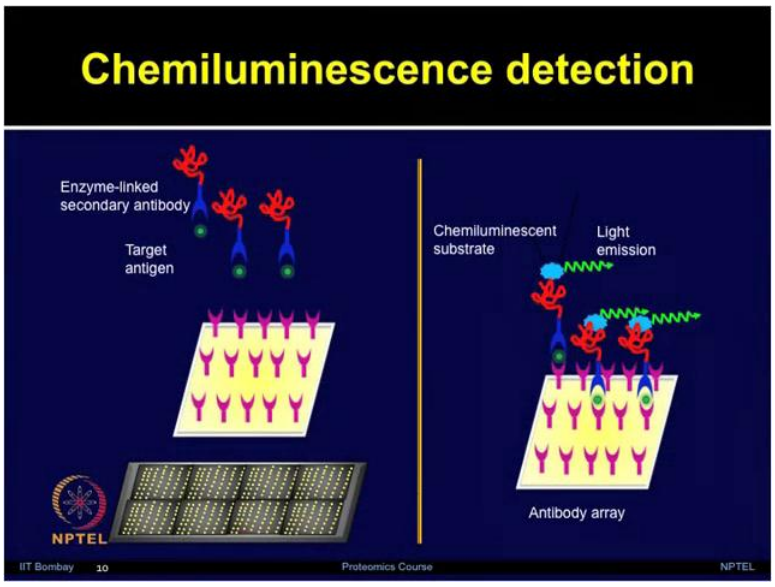

In the chemiluminescence detection system, the antigen of interest binds to the corresponding antibodies which are coated on the microarray surface. The microarrays can then be probed by an enzyme linked secondary antibody which recognizes a different

epitope on same antigen. Now the excess antibody which is unbound can be washed off. And chemiluminescence substrate is added which reacts with the enzyme and emits the light signal. Let us watch this animation on chemiluminescence detection system to understand it is concepts and mechanism.

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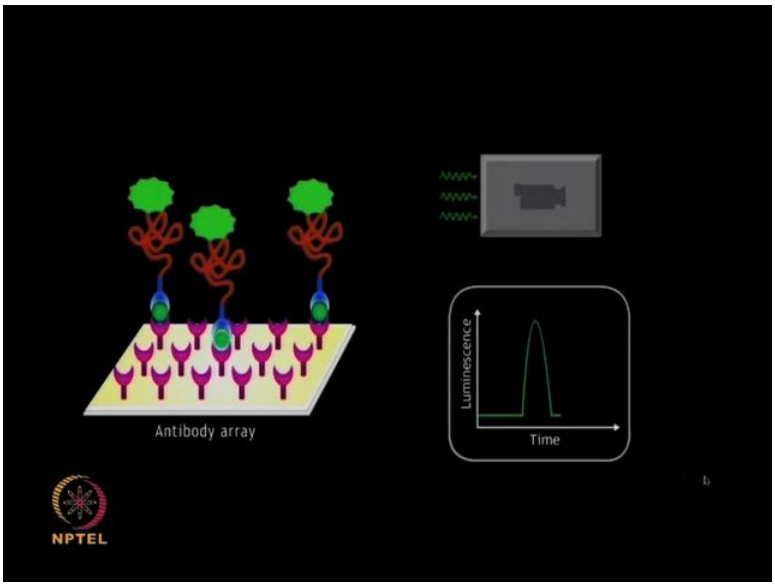

The antigen of interest binds to the corresponding antibodies which are coated on the microarray surface. The arrays then probed by an enzyme linked secondary antibody that is capable of recognizing a different epitope on the same antigen. The excess unbound antibody is washed off, and the chemiluminescence substrate is then added which reacts with enzyme and emits the light. This is detected by a means of CCD camera and a plot is obtained as shown on the right hand side. A graph is plotted between time and luminescence.

Let us now move on to radioactivity labeling. Radioactivity is a process by which certain element is spontaneously emits energy in the form of waves or particle by disintegrating the unstable atomic nuclei into a more stable form. These radiations can be detected by auto radiography or Geiger counter. The various applications were radioactivity labeling have been employed. Antigen antibody, protein protein, protein DNA and protein RNA interactions on the microarray surface can be studied by using the radio labeled query protein on antigen that gives out radiation on binding to the corresponding target molecule.

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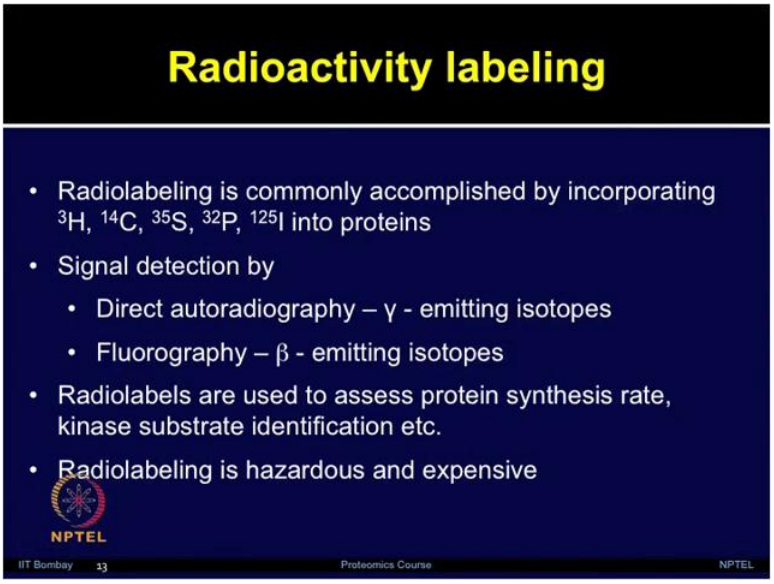

In radioactivity detection, various types of radio labeling molecules are used. These commonly used radio labels include 3 hydrogen, 14 carbon, 35 sulphur, 32 phosphorus, 125 iodine as well as many other radio nucleotides into the proteins. The signal detection it can be performed by the direct autoradiography, which emits the gamma emission or by the fluorography which emits the beta emission. The radio labels have been used for many applications and microarrays including studying about tiny substrates identification which is most commonly used. The radio isotopes have also been used to access the protein synthesis rate. As well as there are many other applications.

Although these are very robust system, but the radio labeling is hazardous as well as expensive. Due to the hazardous nature of these radio labels this technique people use only for very essential applications. However, the sensitivity as well as various type of applications you need to the radio labeling still are providing the unique opportunity for radiolabels to be used for microarray based applications. So, let me show you this animation on radioactivity based detection system to give you a better understanding of how to use the radio labels for microarray based detection system.

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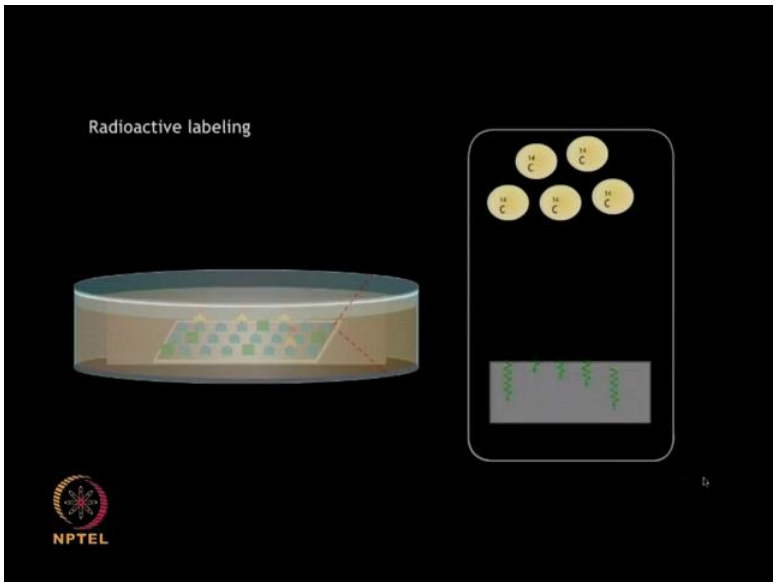

The array surface is coated with the protein mixture containing the target protein of interest. A suitable radio labeled query protein that can specifically interact with the protein of interest is used to probe the array surface. Once binding has occurred the axis unbound query protein is washed off the surface. The washed off array surface is then developed in an autoradiography solution beta emissions from the radioactive carbon atoms of the query protein strike the photographic film on which the final images then developed.

Let us now move on to fluorescence labeling. Fluorescence is a phenomenon by which a substance absorbs radiation of one wavelength and emits another, usually the longer wavelength and that is known as fluorescence. The fluorescent labels, they can be used to tag the probe molecules which bund to the analyte of interest on microarray surface. The excess fluorescent labeled can be washed off from the microarray surface. And, the fluorescence from the binding interactions can be used to identify as well quantify the target molecules.

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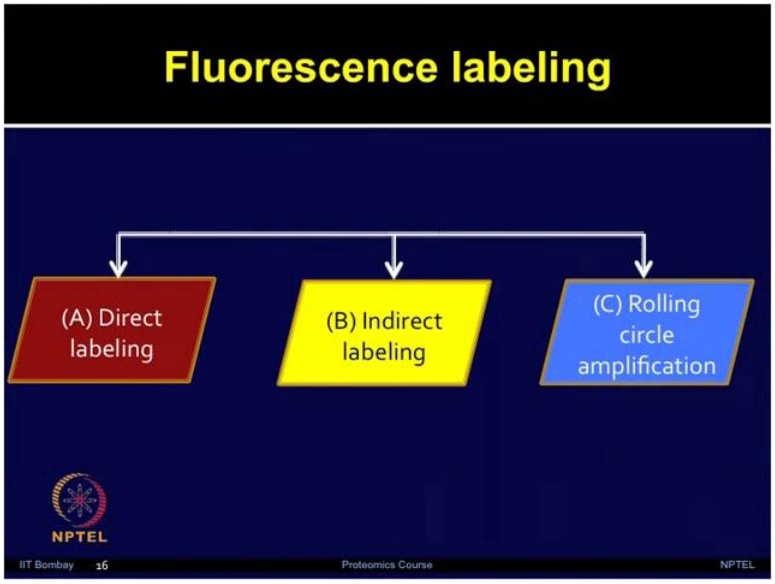

The different type of fluorescence labeling methods have been employed for protein microarray base applications which includes direct labeling, indirect labeling as well as rolling circle amplification or RCA base method. I will talk to each one of these a little bit more detail.

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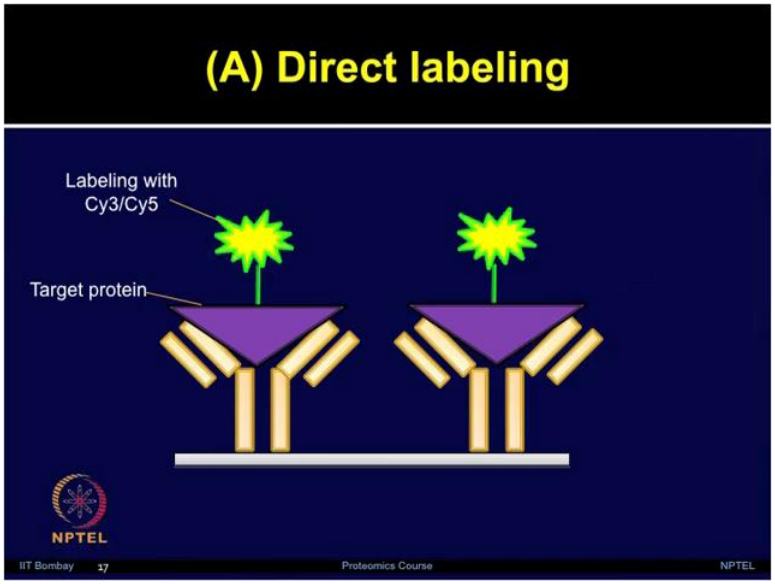

Let us first talk about direct labeling method. In direct labeling, the target protein is labeled directly with a fluorophore commonly used ones are Cy 3 or Cy 5, which is captured by the immobilized antibody on microarray surface. The direct labeling, it

allows the co incubation of reference sample or the controlled sample with an analyte of interest. So, that both containing different tags Cy 3 and Cy 5, that can facilitate the internal normalization.

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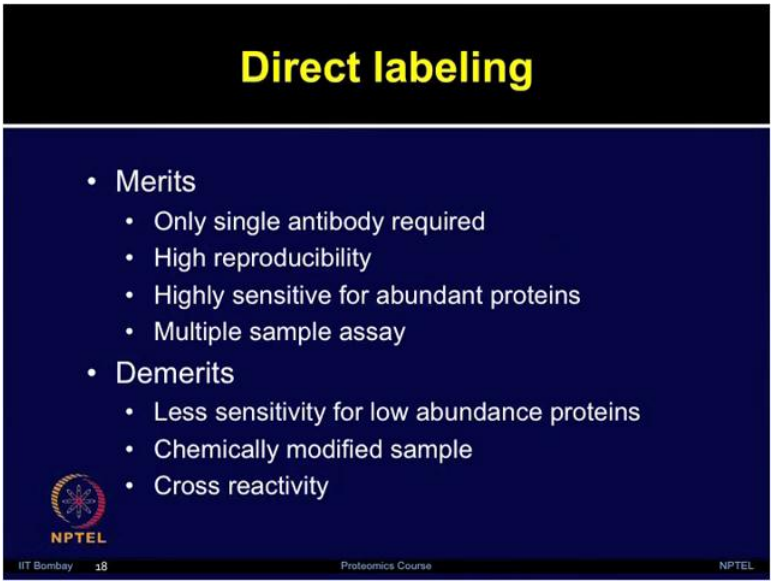

The direct labeling method has many advantages. It requires only a single captured antibody. It has the capacity for multiplexed detection of hundreds of analytes as well as it offers accurate and reproducibility which is required for abundant proteins. However, the direct labeling method has few disadvantages or demerits. It is less sensitive for very low abundant proteins. It is the chemically modified samples are used and there is some chance of cross reactivity.

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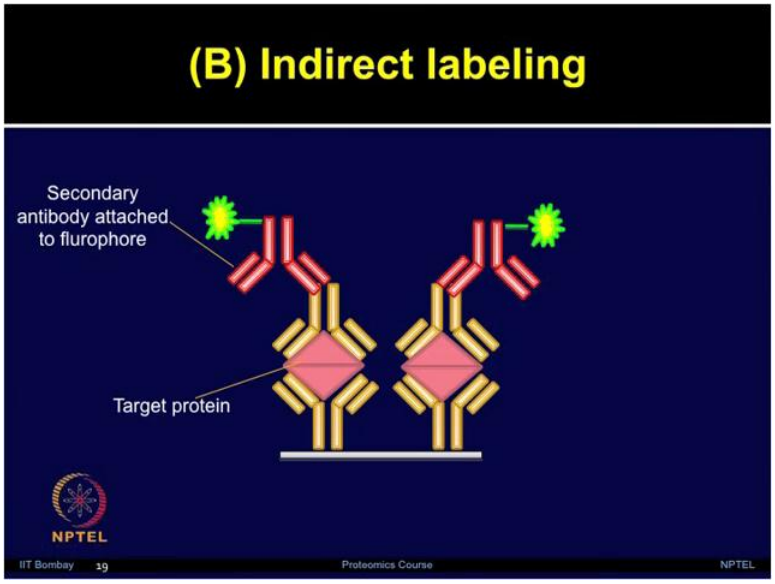

Let us now discuss the indirect labeling. In indirect method, the unlabeled target proteins are captured by the antibodies which are immobilized on microarray surface. Detection is carried out by secondary antibody which is attached to a fluorophore molecule. The indirect labeling method offers higher specificity due to the binding of two target antibodies at different epitopes to the analyte of interest and high sensitivity because of low background labeling. The analyte can also be detected by capture with one analyte specific reagent and detected with second antibody specific to a different epitopes in sandwich immunoassay based method. Sandwich immunoassay fashion is shown in this slide.

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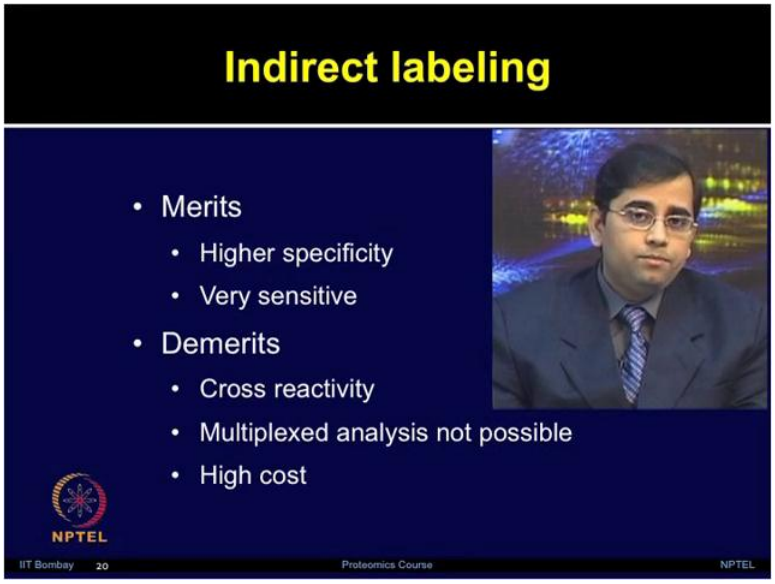

Indirect labeling method, it has various merits. It offers high sensitivity, high specificity, but it has few demerits. For example, the use of sandwich assay for multiplex detection is usually limited to few targets like thirty to fifty targets, due to lack of specific antibodies for all the purified antigen targets. It can also lead to the cross reactivity. The multiplexing analysis is not possible and high cost is also required. So, some of these are the demerits or limitation of indirect labeling methods.

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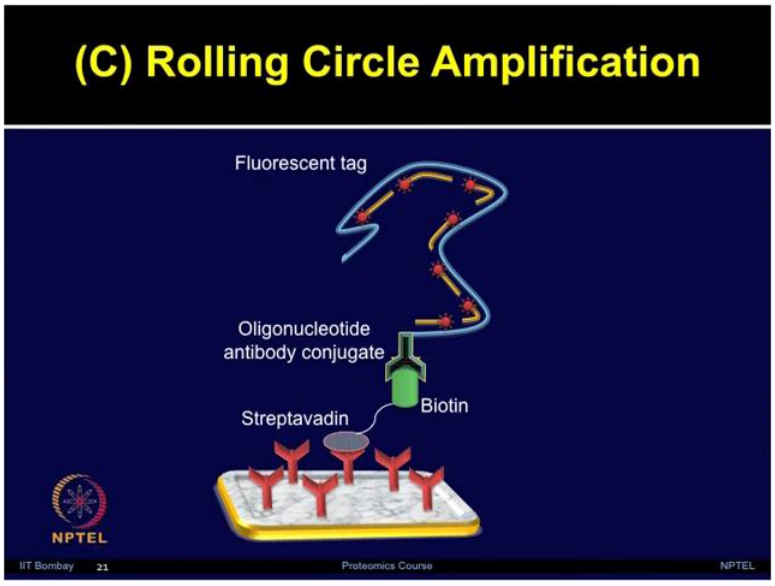

Let us now talk about rolling circle amplification or RCA method. RCA is very effective approach for on chip signal amplification to improve the detection limits in microarray experiments. In RCA, the capture antibodies are printed on the array binds to the analyte of interest. After which it can be detected by a biotin labeled secondary antibody. This is then detected by oligonucleotide linked and anti biotin antibody as shown in the slide. The two color rolling circle amplification method has also been used for detection of various labeled proteins, from the serum samples that are captured on antibody microarrays. This two colored RCA method produce thirty four higher fluorescence as compared to the direct or indirect labeling approaches.

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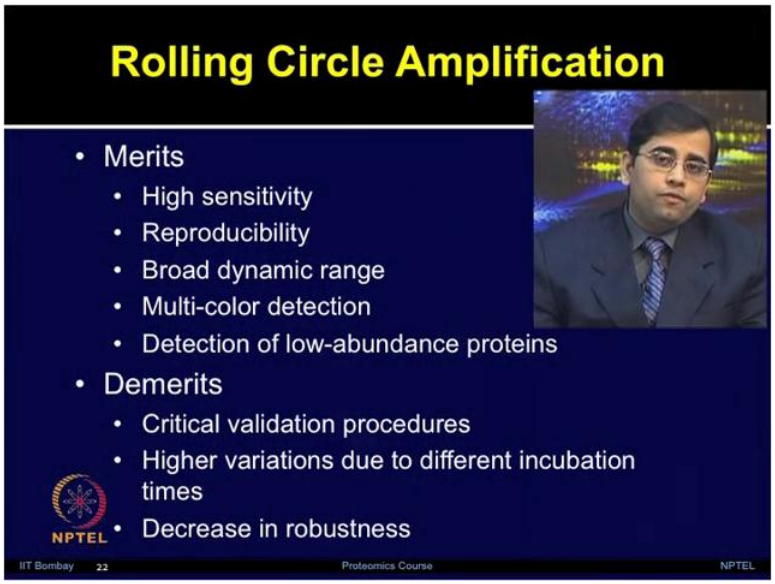

The advantages of rolling circle amplification method as compared to the direct and indirect fluorescence labeling include higher sensitivity, reproducibility, the broad dynamic range of the detection, multi color detection as well as detection of low abundance proteins. It's demerit includes the critical validation procedures, higher variation due to the inherent incubation timings which are different for the, these assays as well as decrease in the robustness of assay. So, I will show you a generic scheme of fluorescence detection by using this animation.

For microarrays especially the protein microarray detection various type of dyes. Such as rhodamine, nitrobenzoxadiazole, acridness as well as cyanines are most commonly used. Factors which govern the choice of fluorophore molecules that depends on sample types,

substrate, light emission spectra, the various types of characteristics as well as the number of target proteins which one wants to study. So, let me show you this animation for your better understanding.

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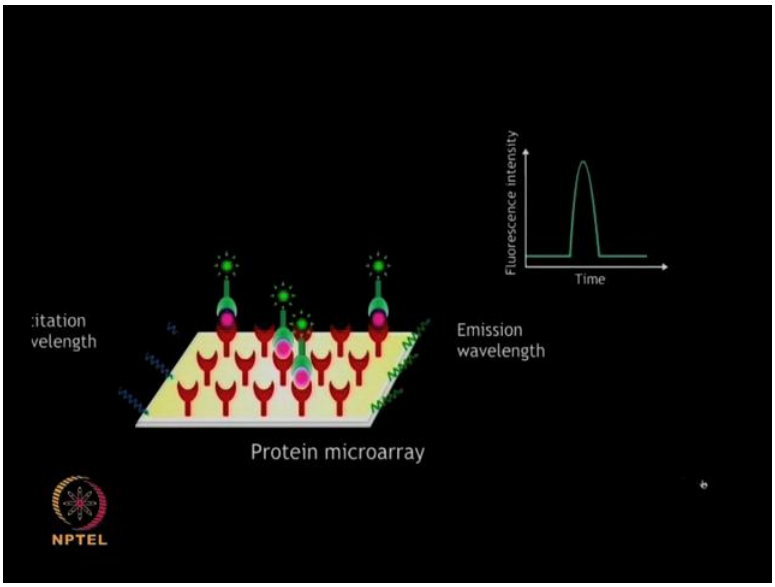

The array surface is functionalized with probe antibody molecules, specific for the target antigen of interest. The target antigens get bound to their primary antibodies on the array surface. Detection is carried out by means of fluorescent labeled secondary antibodies. The excess unbound secondary antibody is washed off and the fluorescence measured by exciting the array with light of suitable wavelength. The resulting emission is measured using microarray scanner and can be used to quantify the corresponding antigen antibody interaction.

As shown on the right hand side in the graph, the time versus fluorescence intensity is plotted and one can look at the peak to measure this fluorescence intensity. Sensitivity of less than one nanogram are achievable by using these fluorescence dyes. So, far we have discussed various conventional our detection methods employed for protein microarrays as well as overall in the microarray field.

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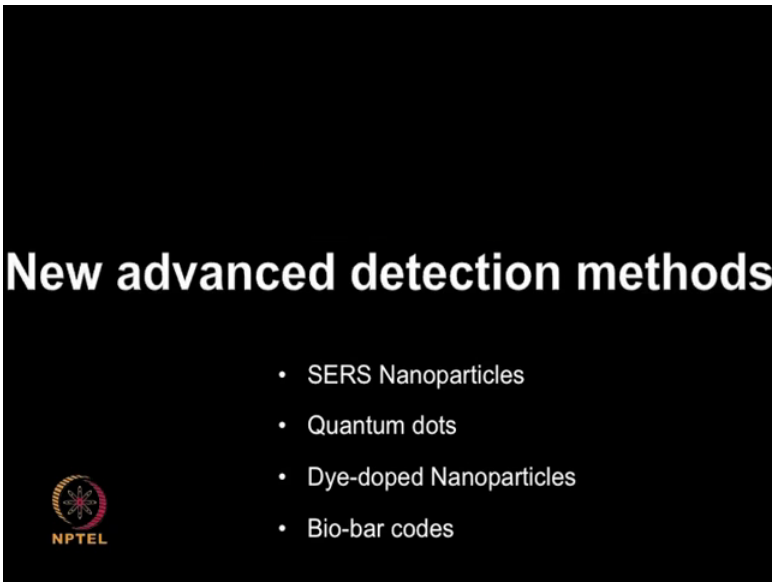

Now let us move on to the new advanced detection methods. We will talk about few methods in more detail such as SERS based Nanoparticles, quantum dots, dye doped Nanoparticles and bio bar codes. Let us first start with surface enhanced Raman scattering or SERS based methods. Light which is incident on an atom or molecule it is scattered back with the same energy and same wavelength that is a generic phenomenon. However, the Raman Effect prevails in a small fraction of photons where energy of scattered photon is different from the incident photons.

Therefore, the improved optical properties are obtained because of enhanced electromagnetic field at the surface of the particle which is detected by using spectroscopic techniques, such as SERS or surface enhanced Raman scattering. In surface enhancing agents include gold and silver. As well as functionalization with target molecules which enhance the sensitivity of Raman spectroscopy.

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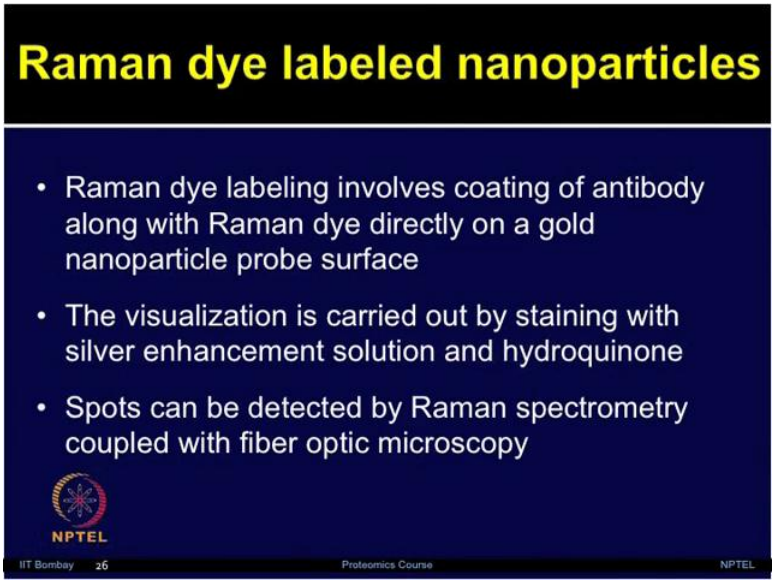

Let us now look at how the system has been employed for detection methods for microarray surface. Let us talk about Raman dye labeled Nanoparticles. The Raman dye labeling involves the coating of antibodies on the array surface, usually the surfaces gold. By using the Raman dye directly on the gold surface which are the Nanoparticles probe. The visualization can be carried out by staining with a silver enhancement solution as well as hydroquinone. The spots can finally be detected by using Raman spectrometry coupled with fiber optic microscopy. As compared to the fluorescence based detection methods the Raman dye labeled gold nanoprobes offer several advantages.

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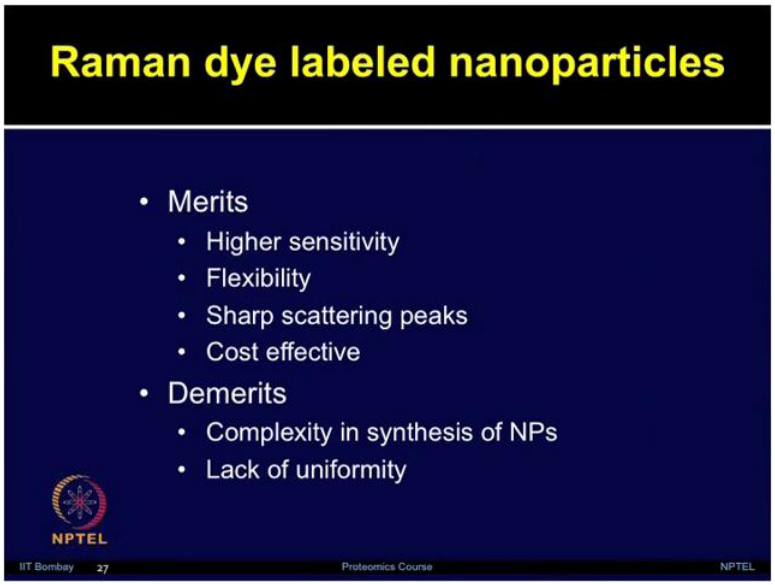

Which includes high sensitivity, the flexibility due to the non overlapping probes very sharp scattering peaks, as well as the cost effectiveness of the assay. However, there are certain demerits of using Raman dye labeled Nanoparticles which includes the complexity in synthesis of these Nanoparticles, as well as the lack of uniformity.

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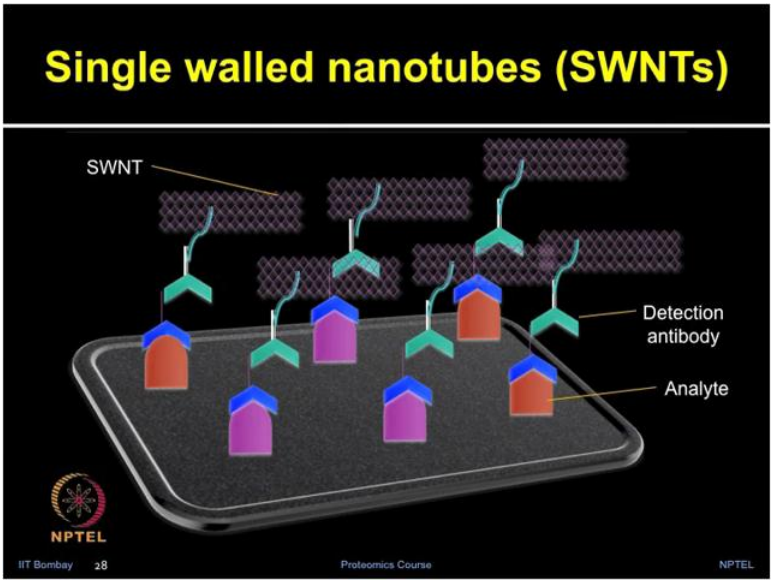

The macromolecular single walled nanotubes functionalized, with specific Raman labeled antibodies are used for multiplexed detection of target proteins bound on the microarray slide, which is gold coated. On in this slide you can see the single walled nanotubes functionalized with Raman labeled antibodies, which are used for multiplex detection of target proteins, which are bound on the gold coated microarray surface. The single walled nanotubes they offer several advantages. Such as high sensitivity, the multiplex detection capability of proteins, minimum background signal due to the sharp scattering peaks, as well as high signal to noise ratio. They also offer resistance to the photo bleaching.

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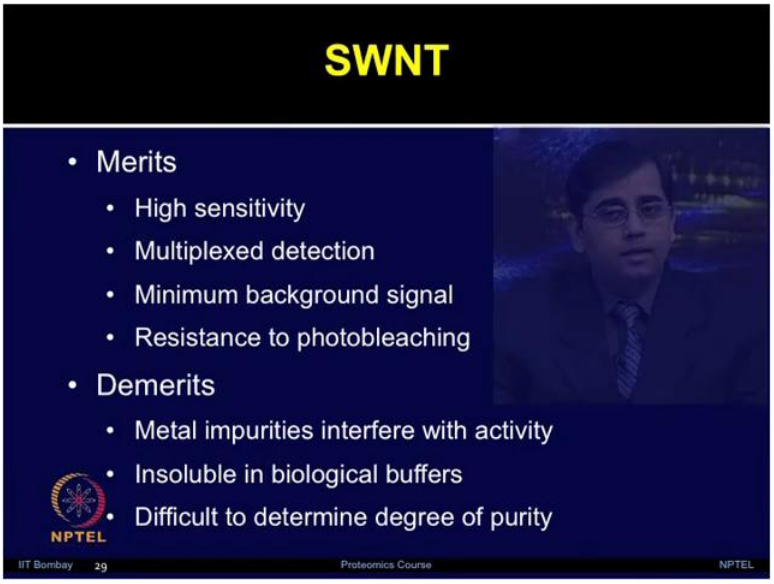

Now SWNTs also have several limitations. Such as the metal impurities during the preparation of these a nanotubes that can interfere with the activity. They are insoluble in the biological buffers and there is difficulty to determine how pure your preparation is. So, the degree of purity is also one of the limitations. Let us now discuss on gold Nanoparticles or GNPs. The excitation of coherent electron oscillations that exist on interface of two materials is known as surface plasma resonance which forms the basis for use of gold Nanoparticles as detection system. The proportion of light absorption to scattering depends on size of the Nanoparticle. The large Nanoparticles can be used for biological imaging due to the need for high scattering cross section.

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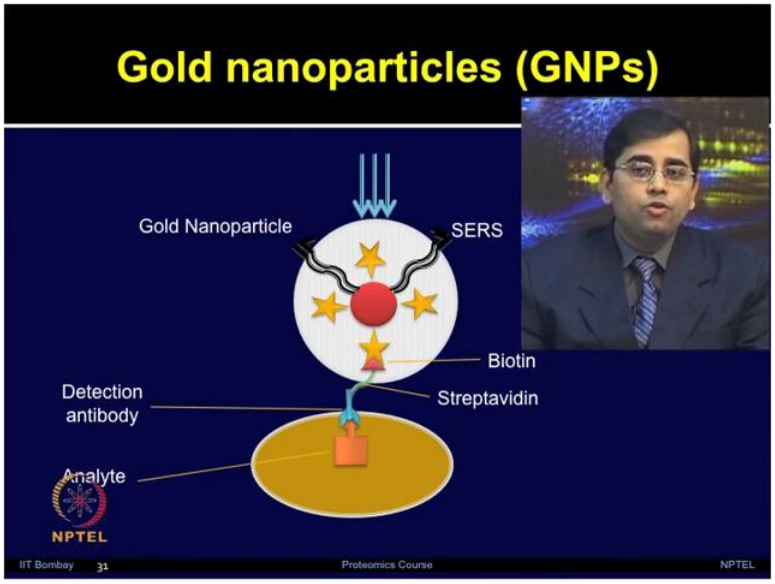

So, the gold Nanoparticles labeled with a suitable capture molecule exhibit change in the emission spectrum of scattered light, upon binding to the analyte of interest, from a protein mixture due to a specific biomolecular interactions. As shown in this slide, the change in the emission spectrum of scattered light directed upon binding of the gold Nanoparticles which is conjugated with antibody to the analyte of interest. Gold Nanoparticles offer several advantages. It has been used for various applications for sensitive detection of standard proteins.

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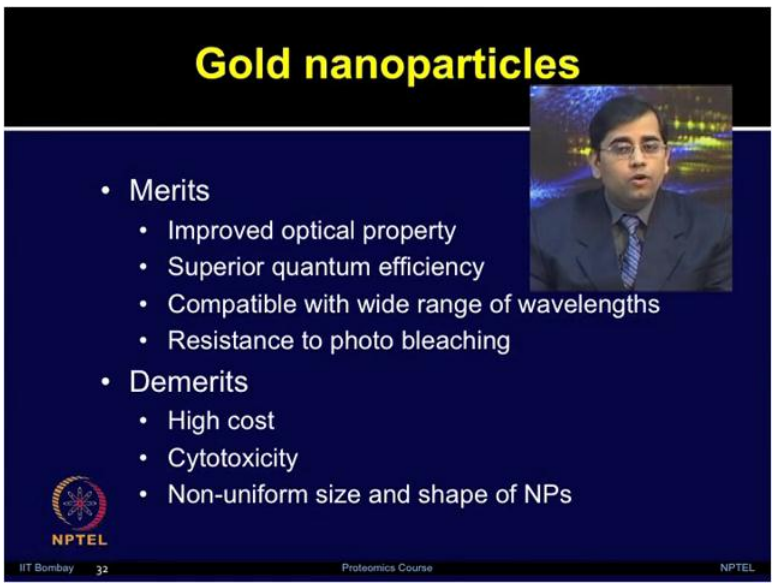

They provide optical property, superior quantum efficiency, show compatibility with the wide range of wave lengths and chemical stability against photo bleaching. However, the certain limitations of using gold Nanoparticles which are also similar to some of the other Nano technique based methods, such as the biocompatibility and low cellular toxicity. The systematic cytotoxicity studies should be performed if you want to use these gold Nano particles for variety of applications including protein microarrays. So, high cost cytotoxicity, the non uniform size and shape of the Nanoparticles, these are some of the limitations of using gold Nanoparticles as sensitive detection platform for microarray based applications.

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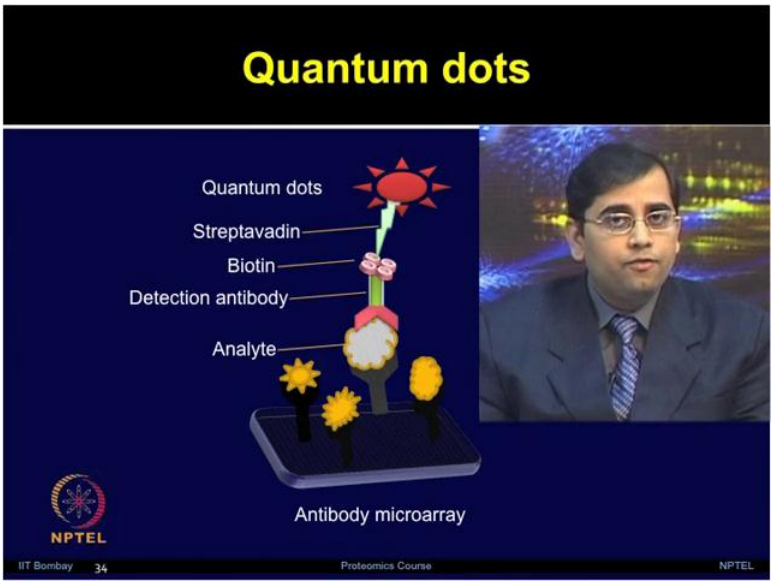

Let us now move on to quantum dots. The quantum dots are nanometer sized crystal, composed of semiconductor fluorescent core coated with another semiconductor cell having large spectral band gap, which is a stable light scattering or emitting properties. In quantum dots the formation of excitons takes place when light of higher energy than that of the band gap of composing semiconductor is incident on the quantum dots. When these excitons return to their lower energy level, emission of a narrow symmetric energy band takes place. As shown in this slide here, the change in the optical properties because of the formation of excitons upon binding of quantum dot conjugated antibody, to the target analyte can be used as a method for detection in the microarray based systems.

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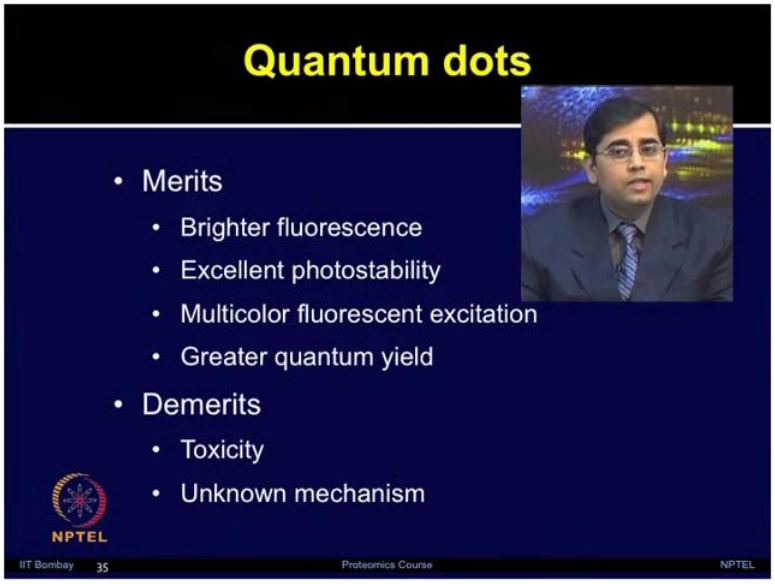

The key advantages of quantum dots compare to the organic dyes include it is brighter fluorescence, excellent photo stability, multicolor fluorescent excitation and greater quantum yield. However, despite a several benefits and applications for variety of biological samples, it is demerits include toxicity. So, the quantum dots have shown various applications which we will discuss during the course in some more time.

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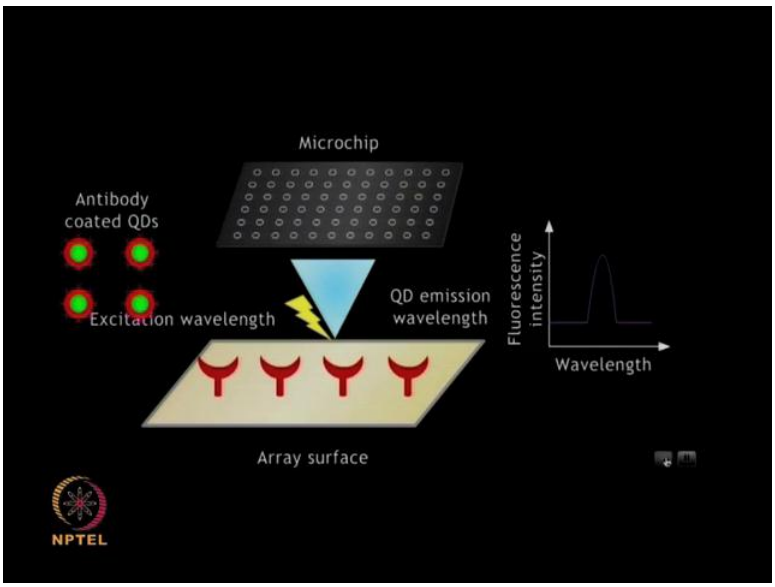

Let us now look at this animation for the quantum dots, to understand it is functioning in a better way. Changes in the emission wave length upon binding to the antibody

conjugated quantum dots are recorded by the micro chip and use for detection of various biomolecules. Quantum dots are capable of detecting molecules down to femto molar levels and provide significant advantages, over conventionally used organic fluorophores. In this interaction we will see how quantum dots work.

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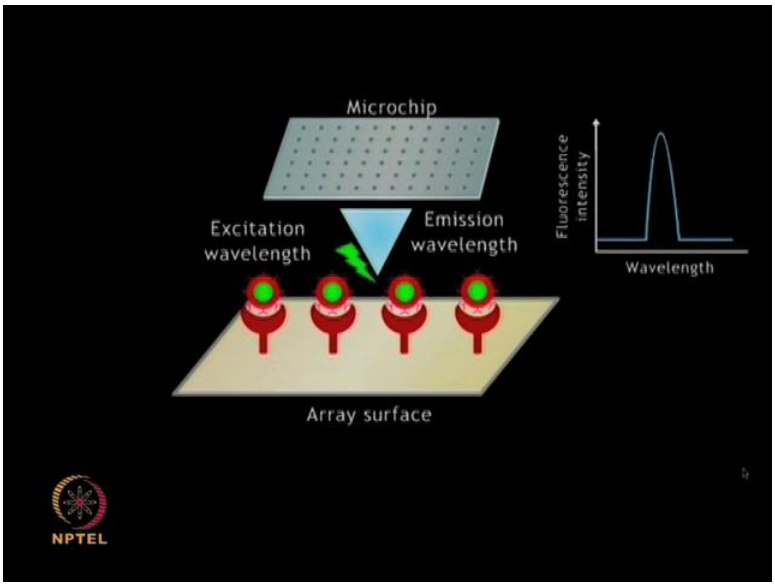

The inorganic fluorophores known as quantum dots, has been develop that can conjugate with several biomolecules and be used for protein microarrays signal detection. They are made up of semiconductor devices which form excitons upon absorption of light. There is emission of a narrow energy band when these excitons are return to their lower energy level. Let us click on these quantum dots to view how they work. So, as you can see upon binding of the target protein to the antibody. Now these changes are plotted on the wave length versus fluorescence intensity graph. So, these quantum dots can detect molecules with very high sensitivity in femto molar range.

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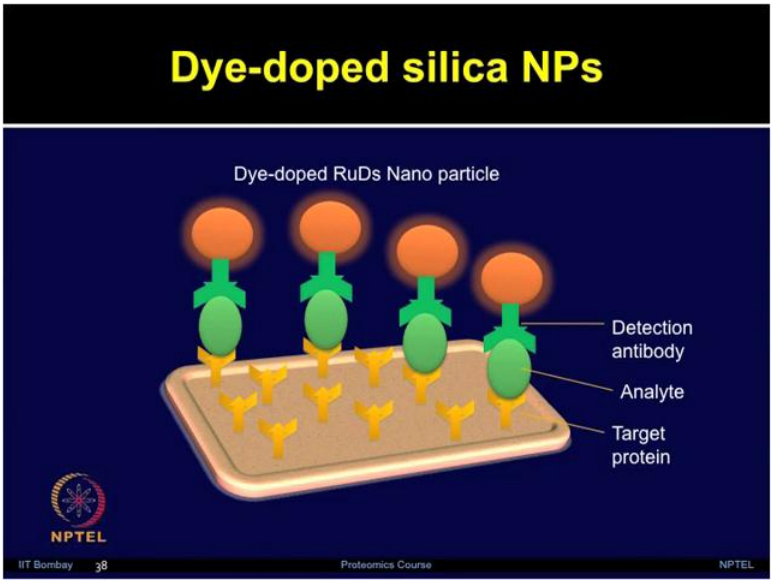

Let us now us a discussing about dye doped silica Nanoparticles. The silica based Nanomaterials which have a large quantity of fluorescent dye, packed inside the silica matrix, have the ability to selectively tag a wide variety of biologically important targets. Such as cancer cells, bacteria as well as many other biological samples. As shown in this slide here the silica based Nanoparticles, which have large amount of fluorescent dye packed inside them, can be use for selectively labeling of the protein molecules for detection of biomolecular interactions.

The dye doped silica Nanoparticles, it is application have been used for variety of biological problems. So, the application of various functionalized silica Nanoparticles have been demonstrated in diver speed, such as biomolecular discovery, drug delivery, and multiplex signaling in bioanalysis.

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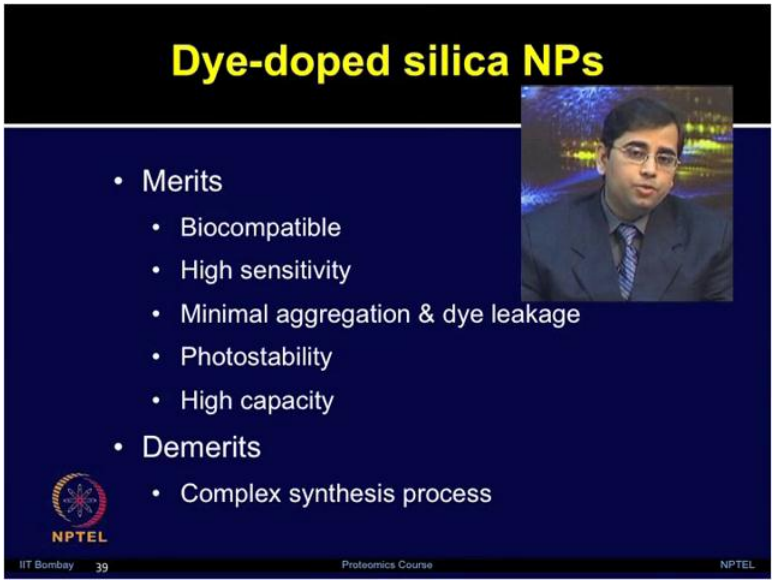

It is various merits include biocompatibility, high sensitivity, minimal aggregation and dye leakage, photo stability as well as high capacity. The demerits of dye doped silica Nanoparticles include the complex synthesis process. Let us now discuss about Nanoparticles based biobarcodes. The narrow particle probes in code with DNA unique to the protein of interest and suitable antibodies capture the magnetic microprticle probes or MMPs having antibodies for the target analyte. Thereby, sandwiching the target protein as you can see in this slide.

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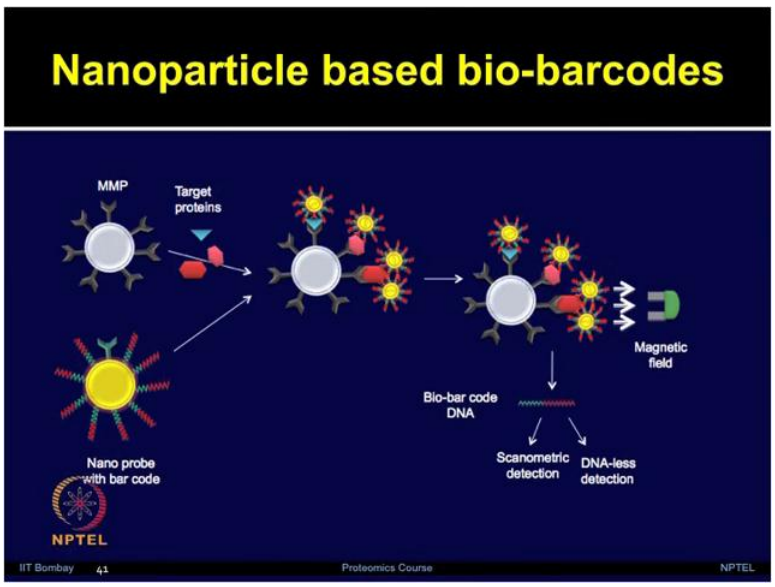

So, these are magnetically separated the oligonucleotide dehybridized and then sequenced, to identify the protein of interest. The Nanoparticles based biobarcodes; they have increased the deduction limits down to auto molar range. The librated oligonucleotide bar codes can be identified on microarray surface by scanometric detection, as well as by using conventional fluorophores.

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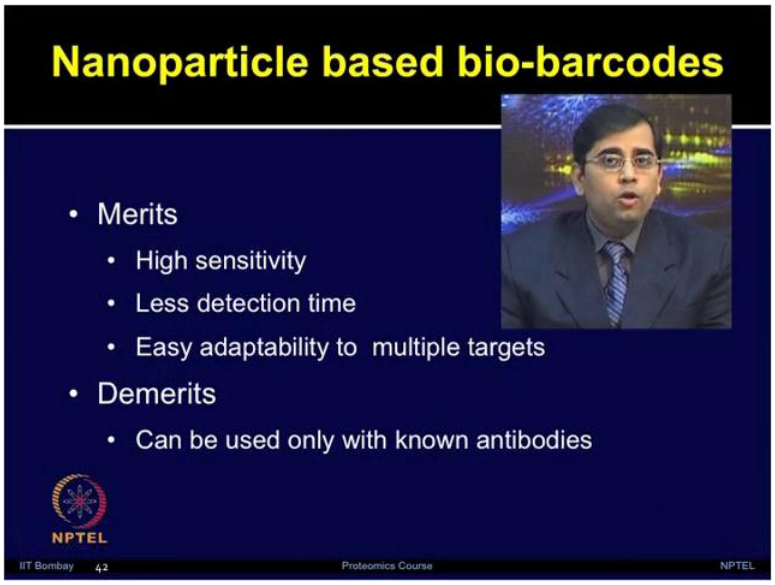

The Nanoparticles based biobarcodes offer various advantages. It is merit include high sensitivity, less detection time, as well as it can be easily adapted to the multiple protein targets. Now, it is demerit includes that it can be used only for the known antibodies. So, number of antibodies as well as good quality antibodies is one of the limiting factors for performing the Nanoparticles based biobarcodes assays. And, in fact the same is also true for many applications in proteomics which require antibodies.

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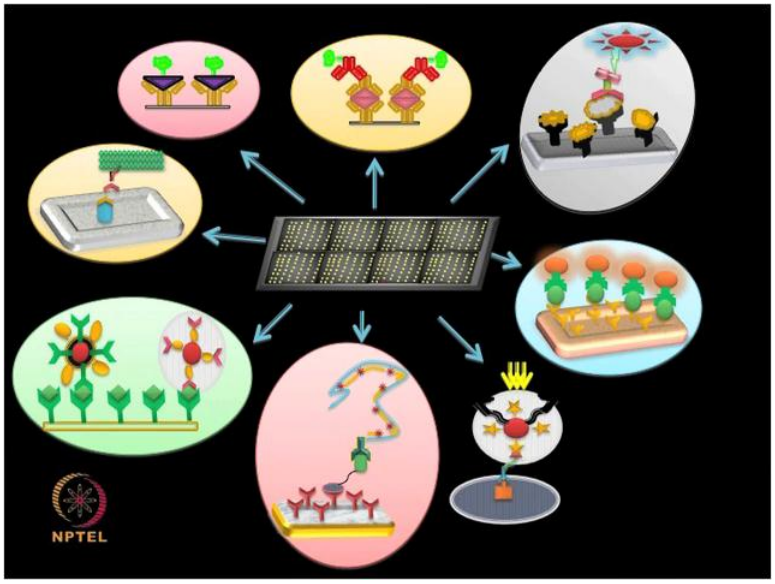

So, to summaries all the various type of detection techniques which we discussed. They advancement in the microarray technologies have led to the devolvement of sensitive and reliable detection systems. The different label based detection techniques which have been employed to study high throughput ways, thousands of protein as well as their interactions and function by using protein microarray platforms. These various novel detection techniques which we have discussed today have facilitated sensitive specific high throughput as well as rapid analysis for many proteomics based applications.

The label based detection systems have taken rapid strike to satisfy the demand of proteomic applications that significant improvement in sensitivity, multiplexing capability and reproducibility. So, we have discussed variety of label based methods. Although fluorescence based methods is still remain one of the very commonly used platform for various microarray application. But there is increase in trend to try out new labels. So that one can achieve an ideal system, which can be applied for microarrays and also provide good detection system with high specificity, sensitivity and large dynamic range.

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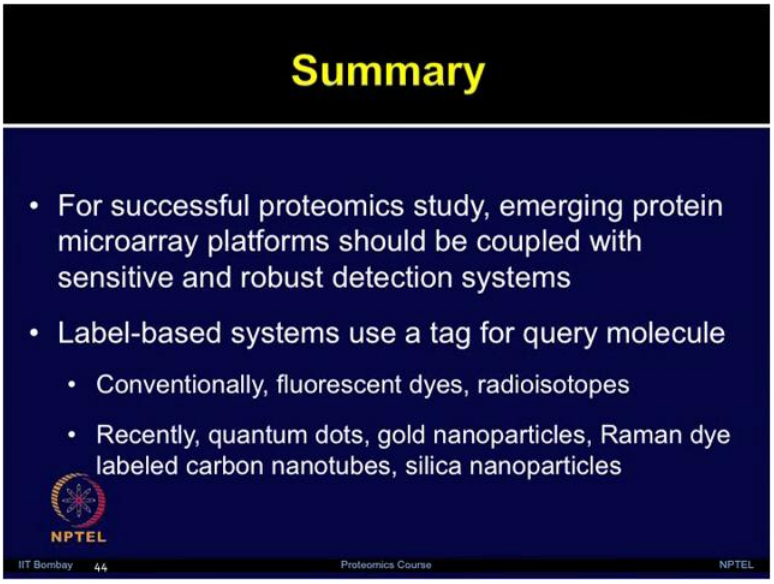

So, in summary for the successful proteomics study the emerging protein microarray platforms should be coupled with sensitive and rapid, very robust detection systems. Today we have discussed, on label based detection system. We will also discuss about label free as well as different type of Nanotechnique based detection system in our continuous lectures in the same flow of microarrays. The label base system as I discussed throughout, they use a tag or query molecule. Conventionally people have used florescent dyes or radio isotopes.

And still the fluorophores, fluorescence based methods is most widely used because of availability of the scanners, because of availability of the radius which are available in the laps throughout the world. But recently the Nano technique base system such as quantum dots, gold Nanoparticles, Raman dry base systems, cells based or dyes, silica Nanoparticles. They have started integrating with the microarray platform. So, we will continue our discussion about detection techniques which are employed for protein microarrays as well as other proteomics application in our next lecture. And then we will talk about label fee detection system. Thank you.