LECTURE-27

Microarray workflow: Label-based detection techniques

TRANSCRIPT

Welcome to the proteomics course. In today's lecture we will continue our discussion on microarray workflow, we will talk about label-based detection technique. Most of the microarray application so far have used label-based detection techniques due to several advantages such as ease of use, common availability of reagents and simple instrumentation which are required for label-based methods. Conventionally the label-based techniques such as fluorescence labeling, radioactivity, chemiluminescence were used but now several new techniques such as quantum dots, gold nanoparticles, dye-doped nanoparticles as well as different types of Raman-based labels are currently employed for various microarray applications. In proteomics applications there is a 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 the detection of very low abundant proteins requires very sensitive detection platforms.

So in today's lecture we will talk about different type of detection platforms. We will focus more on label-based detection technique. Although for detection there are various types of label-free approaches also available but those will be discussed separately. There are several conventional label-based systems such as chemiluminiscence, chromogenic based detection, radioactivity and fluorescence which is most commonly and widely used. Several new advanced techniques are emerging which include quantum dots, SERS based nanoparticles, dye-doped nanoparticles, biobar codes as well as many other nanotechnique 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 last few years. The sensing technologies aim to improve the sensitivity, limit of detection,

dynamic range as well as they try to provide multiplexing capability and high resolution. So broadly we can group these detection platforms which are now provided for microarray applications in label-based and label-free detection systems. In label-based systems we will discuss fluorescence based methods, quantum dots, SERS nanopartcles, bead-based detections systems, dye-dope nanoparticles and bio-bar codes. These are just few names.

There are many other detection systems which are available. Although today we will not discuss label-free platforms but just for your information not only label-based but there is an increasing trend now to apply label-free detection for microarray applications. On one hand the label-based systems use tags for the detection of the signals. The tags could be fluorescent dyes, radiolabels whereas the label-free system try to avoid any labels present in the query molecules. They dependent on the inherent properties of the query, its mass, dielectric property, optical property, etc. There are many recent advanced systems which have emerged for label-free system which include the surface plasmon resonance nanotubes and nanowires, micro-cantilever based systems, atomic force microscopy, SELDI-TOF mass spectrometry based methods and biosensor chip based MS technologies. We will discuss label-free techniques as well as several nanotechniques in detail in next lectures.

So let's discussing about conventional label-based detection systems. The most conventional methods available are chromogenic, chemiluminiscence, radioactivity and fluorescence based detection systems. Let's discuss each of these in detail and then we can move on to recently developed advanced platforms which are used for label-based detection system.

The antigen-antibody interactions can easily be detected by chromogenic reactions. A chromogenic substrate is a molecule which is catalyzed by the enzyme linked to the antibody to provide a coloured product which can be easily detected.

In chromogenic detection an enzyme that can give a coloured reaction upon the addition of suitable substrate which we just discussed and that molecule can be linked

to secondary antibody. This acts as a probe by binding to different epitope on antigen from a primary antibody bound to the microarray surface. Binding of substrate molecules result in to the coloured product formation which can be easily detected and quantified by the means of a microarray scanner.

Let's look at the chromogenic detection system in the following animation to understand the mechanism in more detail.

Animation: Antigen-antibody binding interaction can easily be detected by means of chromogenic reactions. An enzyme that can give a colored reaction upon addition of suitable substrate molecule is linked to the secondary antibody. This acts as a probe by binding to a different epitope on the 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 means of an array scanner. Sensitivity down to femtomolar levels have been achieved by this detection technique.

Let's move on to chemiluminiscence based detection system. The emission of light as a result of chemical reaction is referred to as chemiluminiscence and this phenomenon can be used very effectively to detect molecules of interest. For example, luminol is used to detect trace quantities of food. It gives out light when it comes in contact with iron of haemoglobin molecule. Similar chemical interactions between the target analyte and the probe molecule can be used for detection binding interactions for microarrays. The horse radish peroxidase (HRP) linked antibodies are most commonly used to catalyze the reaction of chemiluminsecent substrate molecules.

In the chemiluminscence detection system the antigen of interest binds to the corresponding antibody which are coated on the microarray surface. Microarrays can then be probed by an enzyme linked secondary antibody which recognizes different epitope on same antigen. Now the excess antibody which is unbound can be washed

off and the chemiluminiscence substrate is added which reacts with the enzyme and emits the light signal.

Let' watch this animation on chemiluminiscence detection system to understand its concepts and mechanisms.

Animation - The antigen of interest binds to the corresponding antibodies coated on the array surface. The array is 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 chemiluminescent substrate is then added which reacts with the enzyme and emits light. This is detected by means of a CCD camera and a plot is obtained.

Let's move on to radioactivity labeling. Radioactivity is a process by which certain elements spontaneously emit energy in the form of waves or particle by disintegrating the unstable atomic nuclei in to a more stable form. These radiations can be detected by autoradiography or Geigercounter. There are various applications where radioactivity labeling has 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.

In radioactivity detection various types of radioactive molecules are used. These commonly used radio-labels include 3H, 14C, 35S, 32P, 125I as well as many other radio-nucleotides into the proteins. The signal detection can be performed by direct autoradiography which emits the γ-emission or by fluorography which emits the β-emission. The radio-labels have been used for many applications on microarrays including studies on kinase substrate identification. The radio-labels have also been used to assess the protein synthesis rate as well as there are many other applications. Although this is a very robust system, the radio-labeling is hazardous as well as expensive. Due to the hazardous nature of these radio-labels these technique is used

only for very essential studies. However, the sensitivity and various types of applications unique to the radio-labeling still are providing the unique opportunity for radio-labels to be used for microarray based applications.

Let me show you this animation on radioactivity detection system to give you a better understanding of how to use the radio-labels for microarray based detection system.

Animation 3: 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, excess unbound query protein is washed off the surface.

The washed 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 image is then developed.

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

Different types of fluorescent labeling methods have been employed for protein microarray based application which includes direct labeling, indirect labeling and rolling circle amplification (RCA). I will talk about these in detail.

Let's first talk about direct labeling method. In direct labeling the target protein is labeled directly with a flourophore. Commonly used flourophores include Cy3 or Cy5. Flourophore is captured by immobilized antibody on the microarray surface. Direct labeling allows the co-incubation of reference sample or control sample with an anlyte of

interest, so that both containing different tags Cy3 and Cy5 can facilitate the internal normalization.

The direct labeling method has many advantages. It requires only single capture antibody. It has capacity of multiplexed detection of hundreds of analytes as well it offers accuracy and reproducibility which is required for abundant proteins. However the direct labeling method has few disadvantages. It is less sensitive for low abundance proteins. Chemically modified samples are used and there is some chance of cross reactivity.

Let's now discuss the indirect labeling. In indirect labeling method the unlabeled target proteins are captured by antibodies which are immobilized on microarray surface. Detection is carried out by secondary antibody which is attached to a flourophore molecule. The indirect labeling method offers higher sensitivity due to the binding of two target antibodies at different epitope to the analyte of interest and high sensitivity because of no background labeling. The analyte can also be captured with one analyte specific reagent and detected with second antibody specific to a different epitope in sandwich immunoassay based method. The sandwich immunoassay method is shown in this slide.

Indirect labeling method has various merits. It offers high sensitivity and specificity but it has few demerits. For example, the use of sandwich assay for multiplex detection is usually limited to few targets like 30-40 targets due to lack of specific antibodies for all the purified antigen targets. It can also lead to cross-reactivity. The multiplex analysis is not possible and high cost is also required. So these are the demerits of indirect labeling methods.

Let's now talk about rolling circle amplification or RCA method. RCA is very effective method for on-chip signal amplification to improve the detection limits in microarray experiments. In RCA the captured antibody is printed on the microarray and it binds to the analyte of interest. After that it can be detected by a biotin-labeled secondary antibody. This is then detected by oligonucleotide linked anti-biotin antibody as shown

in this slide. The two colour RCA method has also been used for detection of various labeled proteins from serum samples that are captured on antibody microarrays. RCA method produces 30-fold higher fluorescence as compared to direct or indirect fluorescence labeling approaches. The advantages of RCA method as compared to direct or indirect flourescence labeling include higher sensitivity, reproducibility, broad dynamic range of detection, multicolour detection and detection of low abundance proteins. The demerits include the critical validation procedures, higher variations due to inherent incubation timings which are different for these assays as well as decrease in the robustness of the assays.

So I will show you a generic scheme of fluorescence detection by using this animation. For protein microarray detection various types of dyes such as flourosin, rhodamine, nitrobenzoxide dizol, acrydines and cyanines are most commonly used. Factors which govern the choice of flourophore depends on sample type, substrates, light emission spectra, various types of characteristics and number of target proteins which one wants to study. So let me show you this animation for your better understanding.

Animation - 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. 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 a microarray scanner and can be used to quantify the corresponding antigen-antibody interaction. Sensitivities of less than 1 ng are achievable by these fluorescent dyes.

So far we have discussed various conventional detection methods employed for protein microarrays as well as overall in the microarray field.

Now let's move on to 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's first start with surface enhanced Raman scattering (SERS) based methods. Light which is incident on an atom or molecule is scattered back with same energy and wavelength. It 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 electro-magnetic field at the surface of the particle which is detected by using spectroscopic methods such as SERS. The surface enhancing agents include gold and silver as well as functionalization with target molecules which enhance the sensitivity of Raman spectroscopy. Let's now look at how this system has been employed for the detection methods for microarrays.

Let's talk about Raman dye labeled nanoparticles. The Raman dye labeling involves the coating of antibodies on the array surface, like gold, by using the Raman dye directly on the gold surface which are the nanoparticle probes. The visualization can be carried out by staining with silver enhancement solution as well as hydroquinone. The spots can be detected by Raman spectrometry coupled with fiber optic microscopy.

As compared to the fluorescence based detection methods, the Raman dye labeled gold nanoprobes offer several advantages which include high sensitivity, flexibility due to non-overlapping probes, sharp scattering peaks and cost effectivenss of the assay. How ever there are certain demerits of using Raman dye labeled nanoparticles which include the complexity in synthesis of nanoparticles and the lack of uniformity.

The macromolecular single walled nanotubes (SWNTs) functionalized with specific Raman dye labeled antibodies are used for used for multiplexed detection target proteins bound on the microarray slide which is gold coated. In this slide you can see SWNT functionalized with Raman dye labeled antibodies which are used for multiplex detection of target proteins which are bound on the gold coated microarray surface.

The SWNTs offer several advantages such as high sensitivity, the multiplex detection capability of proteins, minimum background signal due to sharp scattering peaks as well as high signal-to-noise ratio. They also offer resistance to photobleaching. SWNTs have several limitations such as metal impurities during the preparation of these nanotubes that can interfere with the activity. They are insoluble in biological buffers and there is a difficulty in determining how pure your preparation is. So the degree of purity is also one of the limitations.

Let's discuss gold nanoparticles (GNPs). The excitation of coherent electron oscillations that exist on interphase of two materials is known as surface plasmon resonance (SPR) which forms the basis for the use of GNPs as detection system. The proportion of light absorption to a 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.

So the GNPs labeled with a suitable captured molecule exhibit change in the emission spectrum of scattered light upon binding to the analyte of interest from a protein mixture due to specific biomolecular interactions. As shown in this slide the change in the emission spectrum of scattered light directed upon binding of gold nanoparticle which is conjugated with antibody to the analyte of interest.

GNPs offer several advantages. It has been used for several applications for sensitive detection of standard proteins. They provide improved optical property, superior quantum efficiency, show compatibility with wide range of wavelengths and chemical stability against photobleaching. However, there certain limitations of using gold nanoparticles which are similar to some other nanotechnique based methods such as the biocompatibility and low cellular toxicity. The systematic cytotoxicity studies should be performed if you want to use these GNPs for variety of applications including protein microarrays. So the high cost, cytotoxicity and non-uniform size and the shape of the nanoparticles are some of the limitations of using GNPs as sensitive detection platform for microarray detections.

Let's now move on to quantum dots. The quantum dots are nanometer sized crystals composed of semi-conductor fluorescence core coated with another semi-conductor shell having large spectral band gap which is stable light scattering or emitting properties.

In quantum dots the formation of excitons take place when light of higher energy than that of the band gap of composing semi-conductor is incident on the quantum dots. When these excitons return to their lower energy level, emission of narrow symmetric energy 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 of microarray based methods.

The key advantages of quantum dots compared to the organic dyes include its brighter fluorescence, excellent photostability, multicolour fluorescent excitation and greater quantum yield. However, despite its several benefits and applications of variety of biological samples its demerits include toxicity. So the quantum dots have shown various applications which we will discuss during the course some other time.

Let's now look at this animation about quantum dots to understand its functioning in a better way.

Changes in the emission wavelength upon binding to the antibody conjugated quantum dots are recorded by the microchip and used for detection of various biomolecules. Quantum dots are capable of detecting molecules down to femtomolar levels and provide significant advantages over conventionally used organic flourophores. In this interaction we will see how quantum dots work. The inorganic flourophores known as quantum dots have been developed that can conjugate with several biomolecules and been used for protein microarrays signal detection. They are made up of semi-conductor devices which form excitons upon absorption of light. There is an emission of narrow energy band when these excitons are returned to their lower energy levels.

Let's click on the quantum dots to view how they work. As you can see upon binding of the target protein to the antibody, these changes are plotted on wavelength versus wavelength intensity graph. These quantum dots can detect molecules with very high sensitivity in femtomolar range.

Let's now discuss dye-doped silica nanoparticles. The silica based nanomaterial 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 quantity of fluorescent dye packed inside the silica matrix can be used for selectively labeling of the protein molecules for the detection of biomolecular interactions.

The dye-doped silica nanoparticles' applications have been used for a variety of problems. So the application of various functionalized silica nanoparticles have been demonstrated in diversified fields such as biomarker discovery, drug delivery, multiplex signaling in biomolecules. Its various merits include biocompatibility, high sensitivity, minimal aggregation and dye leakage, photostability and high capacity. The demerits dye-doped silica nanoparticles include the complex synthesis process.

Let's now discuss about nanoparticle based bio-barcodes.

The nanoparticle probes encode with DNA unique to the protein of interest and suitable antibodies capture the magnetic microparticle probes or MMPs having antibodies for the target analytes thereby sandwiching the target protein as you can see in the slide. So these are magnetically separated, oligonucleotides dehybridized and then sequenced to identify the protein of interest. The nanoparticel base bio-barcode have increased the detection limits down to attomolar range. The liberated oligonucleotide barcodescan be identified on microarray surface by scannometric detection as well as by using conventional flourophores.

The nanoparticles based bio-barcodes offer various advantages. The merits include high sensitivity, less detection time and it can be easily adapted to multiple protein targets. the demerit of this method is that it can only be used with known antibodies. So number of antibodies as well as good quality antibodies is one of the limiting factor for performing the nanoparticle based bio-barcode assays. And infact the same is also true for many applications in proteomics which require antibodies.

So to summarize all the various type of detection techniques which we discussed, the advancement in the microarray technology have led to the development of sensitive and reliable detection systems. There are different label-based detection techniques which have been employed to study high throughput ways, thousands of proteins as well as their interactions with proteins and their functions by using protein microarray platform. 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 strides to satisfy the demands of proteomic applications with significant improvement in sensitivity, multiplexing capabitlity and reproducibility. So we have discussed variety of label-based methods, although fluorescence based method is one of the most commonly used methods for various microarray applications but there is an increasing trend to try out new labels so that one can achieve ideal system which can be applied for microarrays and also provide good protection system with high specificity, sensitivity and large dynamic range.

So in summary for the successful proteomics study, the emerging protein microarray platformsshould be coupled with sensitive, rapid and robust detection systems. Today we have discussed the label-based detection system. We will also discuss about label-free as well as different types of detection systems in the next lectures. The label-based systems, as discussed through-out this lecture, use a tag or a query molecule. Conventionally people have used fluorescent dyes or radioisotopes. And still the fluorescence based method is widely used, because of the availability of the scanners and readers in the labs through out the world. But recently the nanobased system such

as quantum dots, GNPs, Raman dye based systems, SERS based dyes, silica nanoparticles have started integrating with the microarray platform. So we will continue our discuss about detection techniques which are employed for protein microarrays as well as other proteomics applications in our next lecture and then we will talk about label-free detection system. Thank you!