

# NPTTEL VIDEO COURSE – PROTEOMICS

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### HANDOUT

### LECTURE-27

### MICROARRAY WORKFLOW: LABEL-BASED DETECTION TECHNIQUES

#### **Slide 1:**

In today's lecture, we will continue our discussion on microarray workflow, we will talk about label-based detection techniques. Most of the microarray applications 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 large 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 highly abundant proteins but the detection of very low abundance proteins requires very sensitive detection platforms.

#### **Slide 2:**

Lecture outline:

- 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 chemiluminescence, chromogenic based detection, radioactivity and fluorescence which are most commonly used.

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- Several new advanced techniques are emerging which include quantum dots, SERS based nanoparticles, dye-doped nanoparticles, bio-bar codes as well as many other nanotechnology based detection systems.

### **Slide 3:**

Label-based platforms for microarrays are 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 nanoparticles, bead-based detection systems, dye-doped nanoparticles and bio-bar codes.

### **Slide 4:**

There are many other detection systems which are available. Both label based and label-free techniques are available, with an increasing trend towards the latter being seen. This lecture will however, only discuss label-based platforms. The label-based systems use tags for the detection of the signals. The tags could be fluorescent dyes, radiolabels whereas the label-free system tries to avoid any labels present in the query molecules. They are 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 systems 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.

**Slide 5:**

**Conventional label-based detection systems.**

- The most conventional methods available are chromogenic, chemiluminescence, 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.

**Slide 6:**

**Chromogenic Detection**

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.

**Slide 7:**

In chromogenic detection, an enzyme that can give a colored 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.

**Slide 8:**

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

**Animation 1:**

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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.

### **Slide 9:**

#### **Chemiluminescence-based detection systems.**

- The emission of light as a result of chemical reaction is referred to as chemiluminescence 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 chemiluminescent substrate molecules.

### **Slide 10:**

- In the chemiluminescence detection system, the antigen of interest binds to the corresponding antibody which is coated on the microarray surface.
- Microarrays can then be probed by an enzyme linked secondary antibody which recognizes a different epitope on same antigen.

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- Now the excess antibody which is unbound can be washed off and the chemiluminescence substrate is added which reacts with the enzyme and emits the light signal.

### **Slide 11:**

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

### **Animation 2:**

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.

### **Slide 12:**

#### **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 Geiger counter.
- 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.

### **Slide 13:**

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- In radioactivity detection, various types of radioactive molecules are used. These commonly used radio-labels include  $^3\text{H}$ ,  $^{14}\text{C}$ ,  $^{35}\text{S}$ ,  $^{32}\text{P}$ ,  $^{125}\text{I}$  as well as many other radio-nucleotides into the proteins.
- The signal detection can be performed by direct autoradiography which emits the  $\gamma$ -emission or by fluorography which emits the  $\beta$ -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 provide a unique opportunity for radio-labels to be used for microarray based applications.

### Slide 14:

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.

**Slide 15:**

**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 binds 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.

**Slide 16:**

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).

**Slide 17:**

Direct labeling method:

- In direct labeling the target protein is labeled directly with a fluorophore. Commonly used fluorophores include Cy3 or Cy5. Fluorophore is captured by immobilized antibody on the microarray surface.
- Direct labeling allows the co-incubation of reference sample or control sample with an analyte of interest, so that both containing different tags Cy3 and Cy5 can facilitate the internal normalization.

**Slide 18:**

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- 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.

### Slide 19:

The indirect labeling:

- In this 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.

### Slide 20:

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.



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### Slide 21:

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 color 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.

### Slide 22:

- The advantages of RCA method as compared to direct or indirect fluorescence labeling includes higher sensitivity, reproducibility, broad dynamic range of detection, multicolour detection and detection of low abundance proteins.
- The demerits include the requirement for 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.

### Slide 23:

Following is a generic scheme of fluorescence detection, depicted by animation.

For protein microarray detection, various types of dyes such as fluorescein, rhodamine, nitrobenzoxazole, acrydines and cyanines are most commonly used. Factors which

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govern the choice of fluorophore depends on sample type, substrates, light emission spectra, various types of characteristics and number of target proteins which one wants to study.

### **Animation 4:**

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.

### **Slide 24:**

So far we have discussed various conventional detection methods employed in various types of microarrays, including protein microarray.

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.

### **Slide 25:**

#### **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

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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.

### **Slide 26:**

#### **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.

### **Slide 27:**

Raman dye labeled gold nanoprobe Compared with fluorescence based detection methods:

- Merits: High sensitivity, flexibility due to non-overlapping probes, sharp scattering peaks and cost effectiveness of the assay.
- Demerits include the complexity in synthesis of nanoparticles and the lack of uniformity.

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### Slide 28:

- The macromolecular single walled nanotubes (SWNTs) functionalized with specific Raman dye labeled antibodies are 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.

### Slide 29:

- 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.

### Slide 30:

#### 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 the amount scattered 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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### Slide 31:

- 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.

### Slide 32:

- GNPs offer several advantages. They have 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, including a lack of biocompatibility and increased 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.

### Slide 33:

#### 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.

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### Slide 34:

- 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.

### Slide 35:

- 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.

### Slide 36:

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

### Animation 5:

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

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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.

### **Slide 37:**

#### **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.

### **Slide 38:**

- 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.

### **Slide 39:**

- The dye-doped silica nanoparticles have been applied in a variety of areas. 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.

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- The demerits include the complex synthesis process.

### Slide 40:

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

### Slide 41:

#### **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 are dehybridized and then sequenced to identify the protein of interest. The nanoparticle based bio-barcode has increased the detection limits down to attomolar range. The liberated oligonucleotide barcodes can be identified on microarray surface by scannometric detection as well as by using conventional fluorophores.

### Slide 42:

- 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 factors for performing nanoparticle based bio-barcode assays. And in fact, the same is also true for many applications in proteomics which require antibodies.



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### **Slide 43:**

So to summarize all the various type of detection techniques which we discussed, advancements 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 applied to high throughput assays. 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 capability and reproducibility.

Amongst label-based methods, although fluorescence-based methods remain one of the most commonly used methods for various microarray applications, there is an increasing trend to try out new labels so that one can achieve an ideal system which can be applied for microarrays and also provide a system with high specificity, sensitivity and large dynamic range.

### **Slide 44:**

So in summary, for successful proteomics studies, the emerging protein microarray platforms should be coupled with sensitive, rapid and robust detection systems. Today we have discussed label-based detection systems. 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 throughout this lecture, use a tag or a query molecule. Conventionally people have used fluorescent dyes or radioisotopes. Fluorescence based methods remain in wide use, because of the availability of the scanners and readers in the labs throughout the world. But recently the nano-based system such as quantum dots, GNPs, Raman dye based systems, SERS-based dyes, silica

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nanoparticles 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 applications in our next lecture and then we will talk about label-free detection systems.

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