

## LECTURE-23

### Reverse-Phase Protein Microarrays

#### Handout

#### PREAMBLE

Proteomics is study of protein properties such as expression levels, post-translational modifications and their interactions with other molecules to understand various biological processes. Since proteins are the actual effector molecules that carry out majority of the functions in the cell and most drug targets are present at protein level, therefore, proteomic studies are extremely important for better understanding of physiology of living systems. Protein microarray platform comprised of thousands of discrete proteins and allow multiplex proteins analysis in a single experiment and is a promising tool to understand complex biological processes. Recent studies have shown its wide applications in basic and clinical research where sample size is a limitation. In reverse-phase protein microarrays (RPPAs), a set of proteins or entire cellular proteomic repertoire from different samples to be assessed are printed on slide and probed with a single antibody; in contrast to traditional protein array, where probes are immobilized on chip and incubated with the samples to be analyzed. It requires less volume of antibody, shows high-degree of sensitivity and uniformity of results. In this lecture principle and workflow of RPPA, as well as how this high-throughput technique can be used to study signaling networks, biomarker discovery and post-translational modifications will be discussed.

## **OUTLINE OF LECTURE**

I. Reverse-phase protein microarrays (RPPAs)

II. Principle

III. Procedure

IV. Applications

V. Advantages and challenges

VI. Conclusions

## **BOX 1. Terminology**

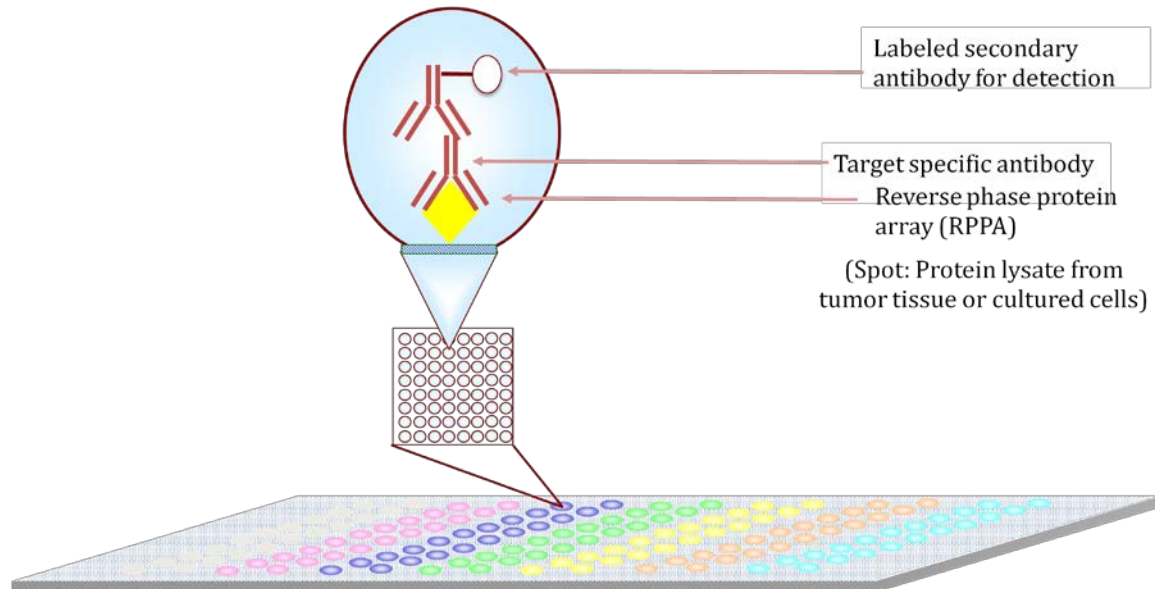
- **Laser capture microdissection (LCM):** LCM is a technique used for the isolation of cells of interest from tissues, cells or organisms.
- **Fine-needle aspiration cytology (FNAC):** FNAC is technique that involves a hollow needle, which is used to collect samples from stained lumps or masses that are superficial.
- **Reverse Phase Protein Arrays (RPPA):** A set of discrete proteins or entire cellular proteomic repertoire from biological sample to be assessed is printed on a glass slide and array is probed with an antibody.

### I. Reverse-phase protein microarrays

The concept of RPPA was developed by Paweletz et al. (Paweletz CP et al 2001). The RPPA is an antibody-based array, where set of discrete proteins or entire cellular proteomic repertoire from biological sample to be assessed is printed on a glass slide. The microarray is then probed with a single antibody in contrast to traditional protein array where a specific probe or bait protein is immobilized on chip and incubated with a complex sample to be analyzed. RPPA uses small amount of sample, which remains a limitation with clinical samples, therefore RPPA provides a useful method to assay such samples. It has been used to determine protein expression level in diseased samples, to study post-translational modifications and for profiling of signaling networks or validation of targets in cell line and diseased samples (Spurrier B et al 2008).

## II. Principle

Reverse phase protein microarrays employ two existing technologies; (i) laser capture micro dissection (LCM), where stained tissue slide is placed under a microscope and the tissue is visualized in real-time manner, and (ii) microarray fabrication (Paweletz CP et al 2001). In RPPA whole protein lysate either from histopathologically relevant cell populations from diseased tissue procured by LCM or fine-needle aspiration cytology (FNAC), from cultured cells, serum, body fluid or lumps and masses is immobilized on coated slides. Signal intensity depends on amount of analyte protein present in individual spot thus a range of serial dilutions of each cell lysate are printed/immobilized on slide to make sure that analyte of interest remains within the linear range of detection and this dilution series is used to estimate expression of a protein. Then it is probed with highly specific antibody followed by detection with a secondary antibody labeled usually with fluorescent dyes like Cy3 or Cy5. Binding affinity of primary antibody and saturation effect is another important parameter to be taken care of; therefore highly specific antibody is used in analysis.



**Fig. 1 Reverse phase protein array (RPPA)** This illustration represents assay of RPPA. Proteins from tumor or cultured cells are immobilized onto the glass slide. The antibodies, in the solution that is used for evaluation, bind to the corresponding proteins on the microarray slide surface. This interaction can be detected by using a labeled secondary antibody, which has affinity to the primary antibody.

## III. Procedure

- Tissue sample collection/ cell culture sample/ serum/ other body fluid
- Laser capture micro-dissection (LCM) for tissue
- Protein extraction/protein lysates
- Serial dilution of protein lysates
- Printing on slide
- Incubation with specific antibody
- Detection
  - ✓ Fluorescence,
  - ✓ Enzyme-activated colorimetry
  - ✓ Chemiluminescence
- Analysis of reverse phase protein array data

### IV. Applications

The major applications of RPPAs include biomarker discovery and signaling pathway profiling. Srivastava et al (Srivastava M et al 2006) successfully used RPPA platform for validation of plausible biomarker for cystic fibrosis. An antibody microarray platform which had 507-features was used at first to identify and quantify low abundance signaling proteins in serum from cystic fibrosis patients, and several candidates were identified. Further, using RPPA platform three of the antibodies, prostaglandin E2 Synthase (p23), glucocorticoid receptor and Caspase 4 were quantified and recognized as serum proteomic signature for cystic fibrosis.

Similarly, RPPA platform is also useful to profile proteins involved in disease progression. RPPA was introduced by Paweletz et al. where whole protein lysates from histopathologically relevant cell populations from human tissue procured by LCM were immobilized on nitrocellulose coated slides. Various stages of microscopic progressing cancer lesions were captured within individual patients on array and each patient set was arrayed in several dilutions (Paweletz CP et al 2001). Cancer progression was found to be related to increased phosphorylation of Akt, suppression of apoptosis signaling, and decreased ERK phosphorylation.

RPPAs can also be used for quantitative profiling of disease associated proteins or molecular networks profiling (Wulfschlegel JD et al 2003; Grubb RL et al 2003; Sheehan KM et al 2005), studying therapeutic responses (Carey MS et al 2010), validation of mass spectrometry discovered candidate biomarkers by RPPA (VanMeter AJ et al 2008; Mueller C et al 2010) and personalized medicine (Mueller C et al 2010).



### V. Advantages and challenges

There are several advantages of using RPPAs for detecting protein-protein interaction. One can perform multiplex analysis where in multiple analytes could be evaluated simultaneously from relatively smaller numbers of cells than required by other techniques. RPPAs can be used for studying post-translational modifications (PTMs), such as phosphorylation and de-phosphorylation mediated by protein kinases. These PTMs are mediated by protein kinases which are critical in transduction networks. The assays performed using RPPAs provide high sensitivity in comparison to western blot (detection capabilities of 50 fg/l, or 1,000 to 5,000 molecules per spot (Liotta et al. 2003; Paweletz et al. 2001; Geho D et al. 2005). Apart from high sensitivity, RPPAs also provide high reproducibility and robustness as it does not require any direct labelling of the sample (Sheehan KM et al 2005). Problems associated with antigen retrieval while using other types of microarrays can be avoided by using RPPA, since RPPAs can use denatured lysates.

Despite having distinct advantages, RPPAs have certain drawbacks, which need to be addressed. Availability and specificity of primary antibodies are one of the challenges of RPPAs, which limits the use of this technique. Other problems associated with RPPAs are sample degradation, preservation and proper data normalization.

### **VI. Conclusions**

The RPPAs provides a high-throughput proteomic platform with high sensitivity. With continuous advancement in technique its wide applications has been reported in various fields including clinical research, as discussed in this lecture (Charboneau L et al 2002; Mueller C et al 2010). Although there are several challenges to be taken care of but this promising approach may be helpful for biomarker discovery & validation, and personalized medicine.

### VII. REFERENCES

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