LECTURE-22

Antigen and Antibody Microarrays

Handout

PREAMBLE

All the biological processes are mediated by the macromolecules in the cell and the most dynamic and versatile of these are the proteins. Proteins are extensively involved in every cell process, including receptors, hormones, enzymes, metabolites, etc. Thus, for a complete understanding of the cellular processes it is important to understand how the proteins interact with each other and other macromolecules. Traditional approaches such as yeast two-hybrid systems, immunoprecipitation and immunofluorescence, etc have been in use for studying protein interactions. However, very limited number of proteins can be studies at a time, making these approaches very time-consuming.

For obtaining large amount of data at the same time, high-throughput techniques have been developed recently. One of the high-throughput techniques used for studying protein-protein interaction is microarrays. Thousands of proteins can be screened simultaneously, thus combining many experiments in a single one. There are many ways in which the antibodies can be used to bind the antigen. Also, there are various methods for detection of the antibodies. Thus, depending on the type of experiment antigen or antibody microarrays can be used for detection of the antigen-antibody reaction.

OUTLINE OF THE LECTURE

I. Microarrays

II. Antigen antibody binding

III. Antigen-Antibody reactions used in microarrays

- i. Direct labeling
- ii. Sandwich labeling
- iii. Reverse-phase microarrays

IV. Label-based techniques for detection

- i. Fluorescence
- ii. Single walled carbon nanotubes
- iii. Gold nanoparticles
- iv. Quantum Dots
- v. Dye-doped silica nanoparticles
- vi. Magnetic micro-particle probe

V. Applications

VII. Advantages and challenges

VIII. Conclusions

BOX-1. TERMINOLOGY

Protein microarrays - a concept that evolved from DNA microarrays, provides a valuable platform for high-throughput analysis of thousands of proteins simultaneously.

Protein purification – Since the target protein is expressed along with other proteins native to the host system, it is essential to purify the desired protein prior to printing on to the array surface. For this reason, the gene of interest is often fused with a convenient tag sequence such as His6 that will facilitate the purification process.

Array functionalization - The microarray surface must be suitably derivatized with a chemical reagent that can react with the groups present on the protein surface in order to firmly immobilize them on the microarray. Functionalization is often done with silane derivatives as these react easily with the groups present on the protein. Aldehyde groups react with amine groups present on the protein to form Schiff's base linkages, which hold the protein firmly in place.

SWNTs: Single-walled carbon nanotubes – these are SERS based method for detection of antigen-antibody reactions

SERS: Surface enhanced Raman Scattering – used for enhancing the electrical properties around the surface of the particle, which can be detected using Raman scattering.

I. MICROARRAYS

Microarrays are platforms on which a large number of proteins can be studies at the same time. The setup includes a solid support like a glass slide on which proteins are spotted. The microarray slides are usually treated with amines, aldehyde or epoxy in order to facilitate the immobilization of the protein molecules. The sample is then incubated with a probe, which is linked to a label. The probe matches to the appropriate target in the sample and label can be used to detect the spot. This approach enables simultaneous screening of hundreds of proteins on a single chip. Thus, it is a high-throughput method for screening large number of targets at the same time. There are many types of microarrays - depending on the nature of the proteins being captured on the array and different types of detection platform – label-based and label-free (Fig 1 and 2).

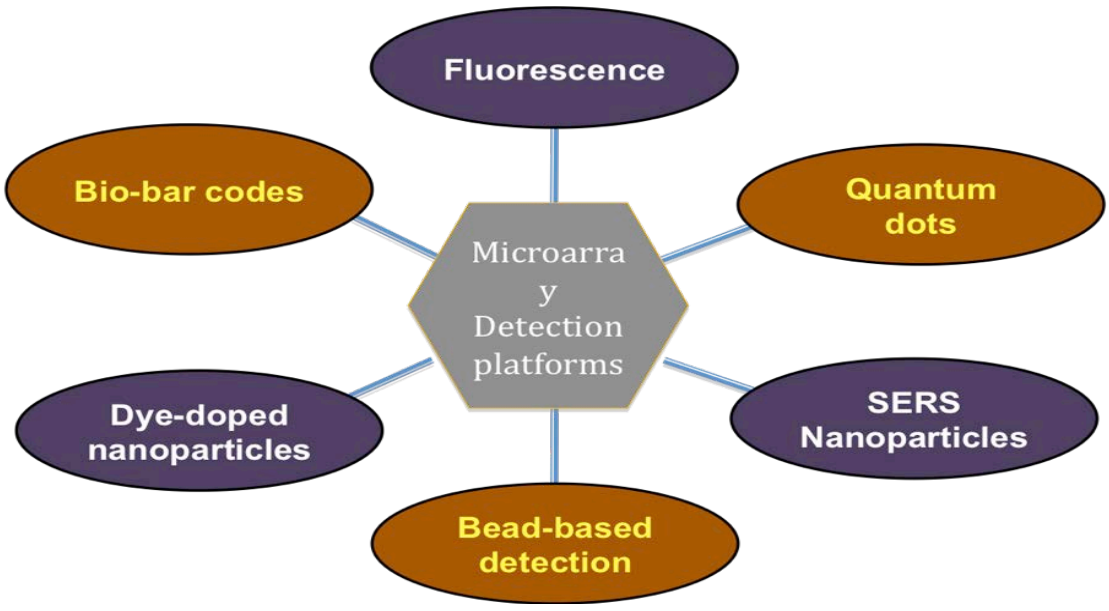


Fig 1. Label-based platform for microarrays.

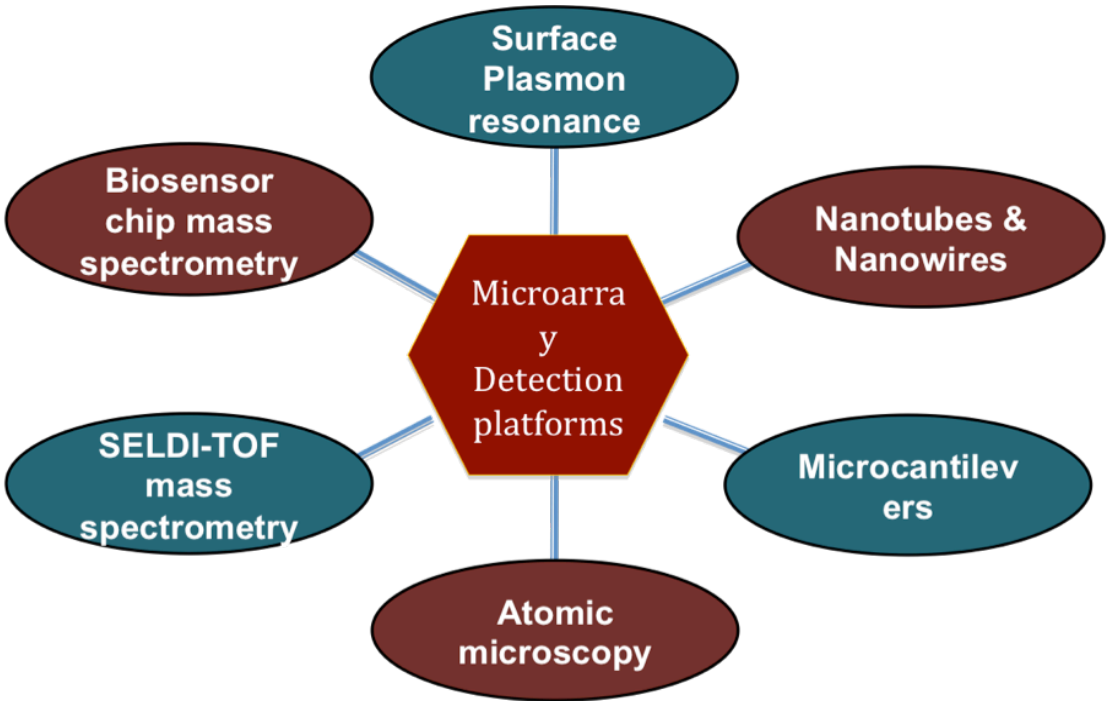


Fig 2. Label-free platform for microarray-based analysis.

Illustration 1: Need for protein microarrays

Functional analysis of proteins is a time consuming process that requires many steps. Analysis of a single protein at a time would be a tedious and laborious process. Analysis of several protein samples will undoubtedly take long time if they are run one at a time. Protein microarrays successfully overcome this hurdle by allowing analysis of several samples simultaneously.

Illustration 2: Protein expression & purification

The gene coding for the protein of interest is expressed in a suitable heterologous host system such as E. coli by means of expression vectors like plasmids. The host cell machinery is used for transcription and translation, which results in a mixture of proteins consisting of the target protein along with other host proteins.

Since the protein of interest is expressed along with other proteins native to the host, it is essential to purify the target protein before it can be used for microarray applications. This can be done by chromatographic procedures to obtain the pure target protein. Protein purity is tested on SDS-PAGE gels. Tags like His6 are often fused with the protein of interest to facilitate the purification process due to its specific affinity towards nickel.

Illustration 3: Array functionalization & printing

Commonly used array surfaces include glass, gold, nitrocellulose and hydrogels. While glass slides are easy to handle, available at low cost and can be used with existing scanning equipment, they show relatively low surface absorption and need to be derivatized with more reactive groups. They however continue to be used more extensively than the other array surfaces, which are more expensive. Comparative features for commonly used array surfaces are demonstrated in the table.

The array surface is functionalized with a suitable chemical reagent that will react with groups present on the protein surface. Aldehyde and silane derivatizations are commonly used as they interact well with amino groups present on the protein surface resulting in firm capture of the protein.

The protein solution is printed onto the array surface in extremely small volumes by means of a robotic printing device that has small pins attached to it for this purpose. The slides are kept for a suitable duration following the printing step to allow capture of the protein on to the array surface. The unreacted sites are then quenched by a blocking solution such as BSA, which also prevents any non-specific protein binding in subsequent steps.

There are two types of protein arrays that are commonly used. In forward phase arrays, the analyte of interest such as an antibody or aptamer is bound to the array surface and then probed by the test lysate that may contain the antigen of interest. In reverse phase arrays, however, the test cellular lysate is immobilized on the array surface and then probed using detection antibodies specific to the target of interest.

Illustration 4: Protein detection & analysis

In the direct labeling detection technique, all the target proteins are labeled with a fluorescent or radioactive tag that facilitates easy detection upon binding to the immobilized capture antibody on the array surface. In the sandwich assay, however, a fluorescent-tagged secondary antibody that recognizes a different epitope on the target antigen binds to it and is detected by means of the fluorescence.

The protein microarray is then scanned in a microarray scanner that allows detection of the fluorescently labeled proteins or antibodies. The output from this scanner is then received by software after which the data can be analyzed.

Protein microarrays have found wide applications for discovery and functional proteomic studies. They allow rapid analysis of thousands of proteins at the same time.

II. ANTIGEN-ANTIBODY BINDING

Antigen-antibody reaction is one of the most specific interactions in biochemistry. The antibodies have a very high affinity for the corresponding antigens and the commonly occurring reaction is very strong. The antibodies are very diverse and they can be easily produced for almost any protein. The antigen and its antibody are held together by non-covalent bonds such as hydrogen bond, Van der Waal's force etc., thus making the reaction reversible. Hence, the antigen-antibody reaction is used to detect the proteins in various techniques like Western Blot, ELISA, Microarrays etc.

III. Antigen-Antibody reactions used in microarrays

Antibodies are used in microarrays for capturing the target proteins or for reading the signal from the experiment. The antigen-antibody interaction can be used on an array in multiple ways:

i. Direct labeling

For a direct labeling procedure, the microarray surface is immobilized with the antibodies. Upon incubation with the sample, the target antigens bind to the appropriate antibody on the microarray (Fig 3).

Merits

- Only single antibody required
- High reproducibility
- Highly sensitive for abundant proteins

- Multiple sample assay

Demerits

- Less sensitivity for low abundance proteins
- Chemically modified sample
- Cross reactivity

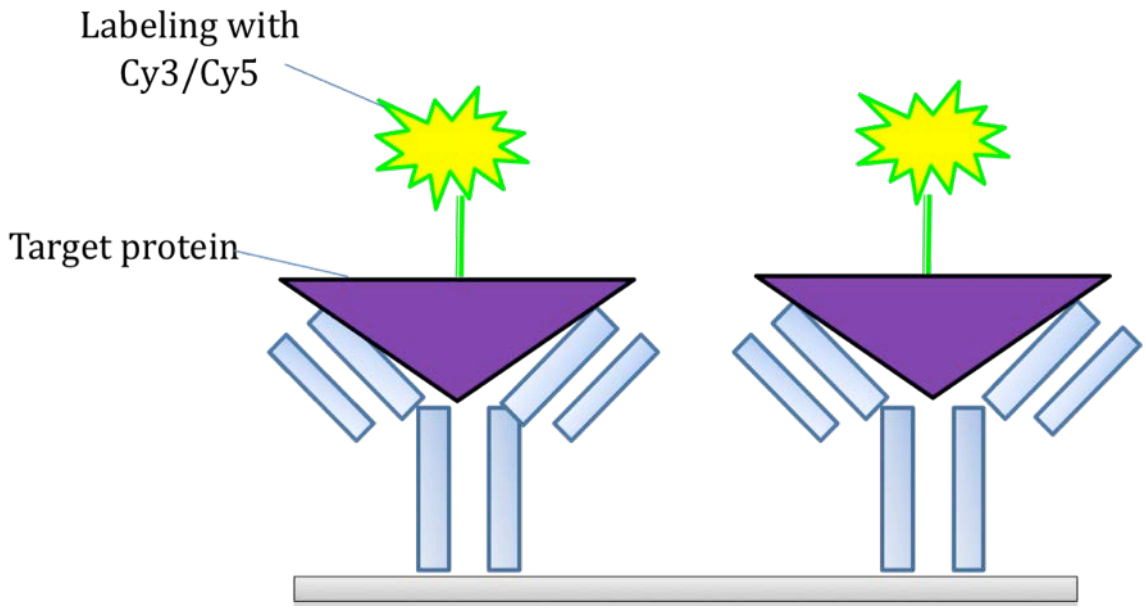


Fig 3. Direct labeling detection for microarrays.

ii. Sandwich Assay

In Sandwich assay, the antibodies are spotted on the microarray. The array is then incubated with the sample and the target antigen bind to the appropriate antibody on the array. A second antibody is then introduced which binds to the antigen, sandwiching the antigen in between two different antibodies (Fig 4 and 5).

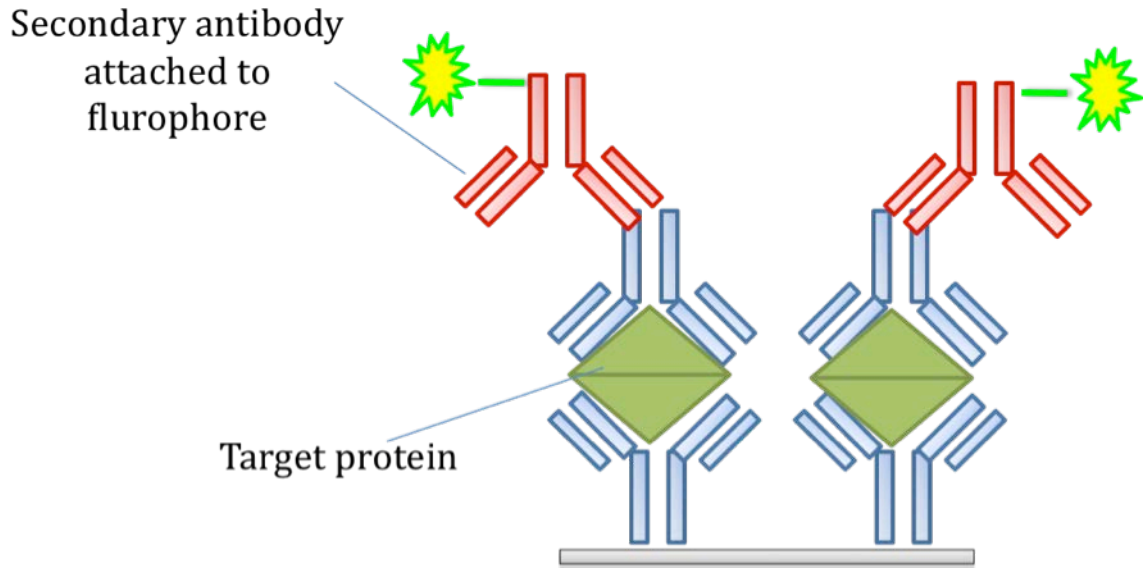


Fig 4. Indirect labeling detection for microarrays.

Merits

- Higher specificity
- Very sensitive

Demerits

- Cross reactivity

• Multiplexed analysis not possible

• High cost

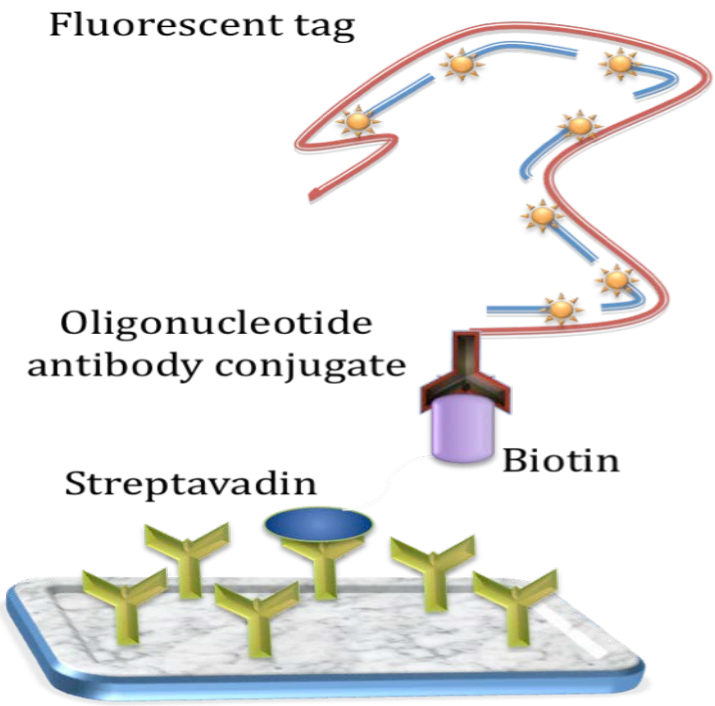


Fig 5. Rolling Circle Amplification for microarrays.

Merits

• High sensitivity

• Reproducibility

• Broad dynamic range

• Multi-color detection

• Detection of low-abundance proteins

Demerits

- Critical validation procedures
- Higher variations due to different incubation times
- Decrease in robustness

ii. Reverse-phase microarrays

In reverse-phase microarrays, instead of the antibody, the antigen is immobilized on to the microarray plate. It is then incubated with a solution of antibodies and appropriate antibody binds to the specific antigen.

IV. Label-Based Technologies For Detection Of Antigen-Antibody Microarrays

Once the microarray assay is performed for the antigen-antibody interaction, one needs to use appropriate detection method for detecting the interaction. There are various techniques available for the detection of antigen-antibody binding and most of them involve a different secondary antibody. Few of these antibody-based detection techniques are listed below:

i. Fluorescence based techniques

In Fluorescence based techniques, direct or indirect labeling is done. In direct labeling, the target antigen is labeled with a fluorescent dye, which then binds to the capture antibody on the microarray surface. In indirect labeling or sandwich technique the unlabeled target antigen binds to the capture antibody on the microarray surface. A second antibody that is fluorescently tagged is introduced, which then binds the target antigen.

ii. Single-walled carbon nanotubes (SWNTs)

SWNTs fall under the Surface-enhanced Raman Scattering (SERS) type of detection methods. In SERS detection methods, the Raman dye is coated to the particle, along with the antibody. The SWNTs have electrical and spectroscopic properties, which have a characteristic Raman signature. These SWNTs are coupled with Raman-labeled antibodies, which make the detection of the target possible (Fig 6).

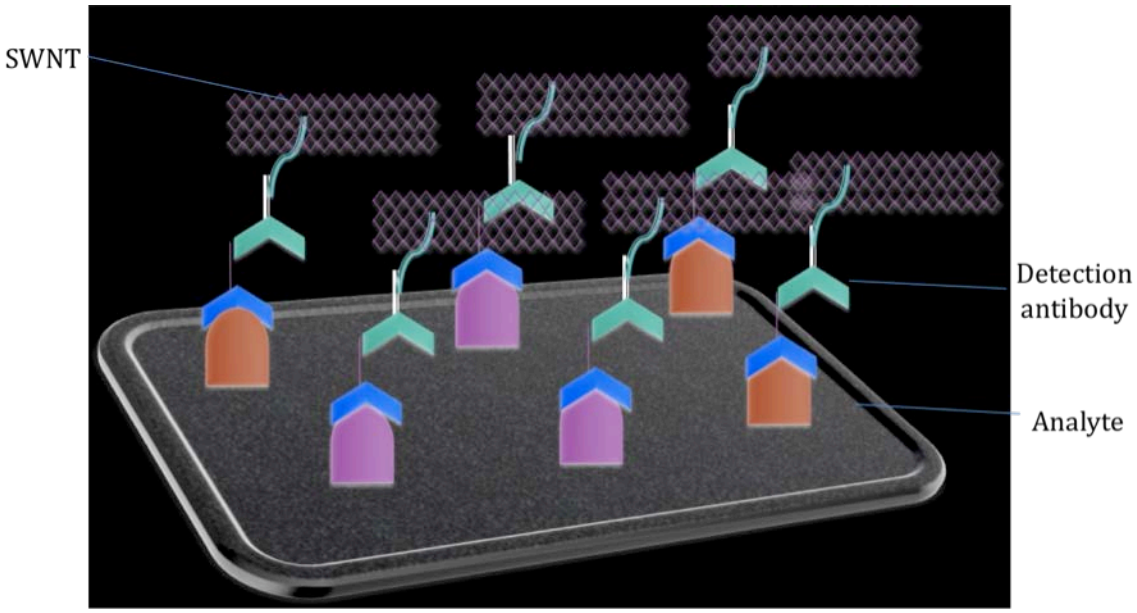


Fig 6. Single walled carbon nanotube (SWNT) for microarray detection.

Merits

- High sensitivity
- Multiplexed detection
- Minimum background signal
- Resistance to photobleaching

Demerits

- Metal impurities interfere with activity
- Insoluble in biological buffers
- Difficult to determine degree of purity

iii. Gold nanoparticles

The Gold nanoparticles work on the principle of Surface Plasmon Resonance. The gold nanoparticles are attached to the antibody. Upon binding of the antibody to the antigen, a change in the emission spectra of the gold nanoparticles is observed which allows the detection of the interaction.

Merits

- Improved optical property
- Superior quantum efficiency
- Compatible with wide range of wavelengths
- Resistance to photo bleaching

Demerits

- High cost
- Cytotoxicity
- Non-uniform size and shape of NPs

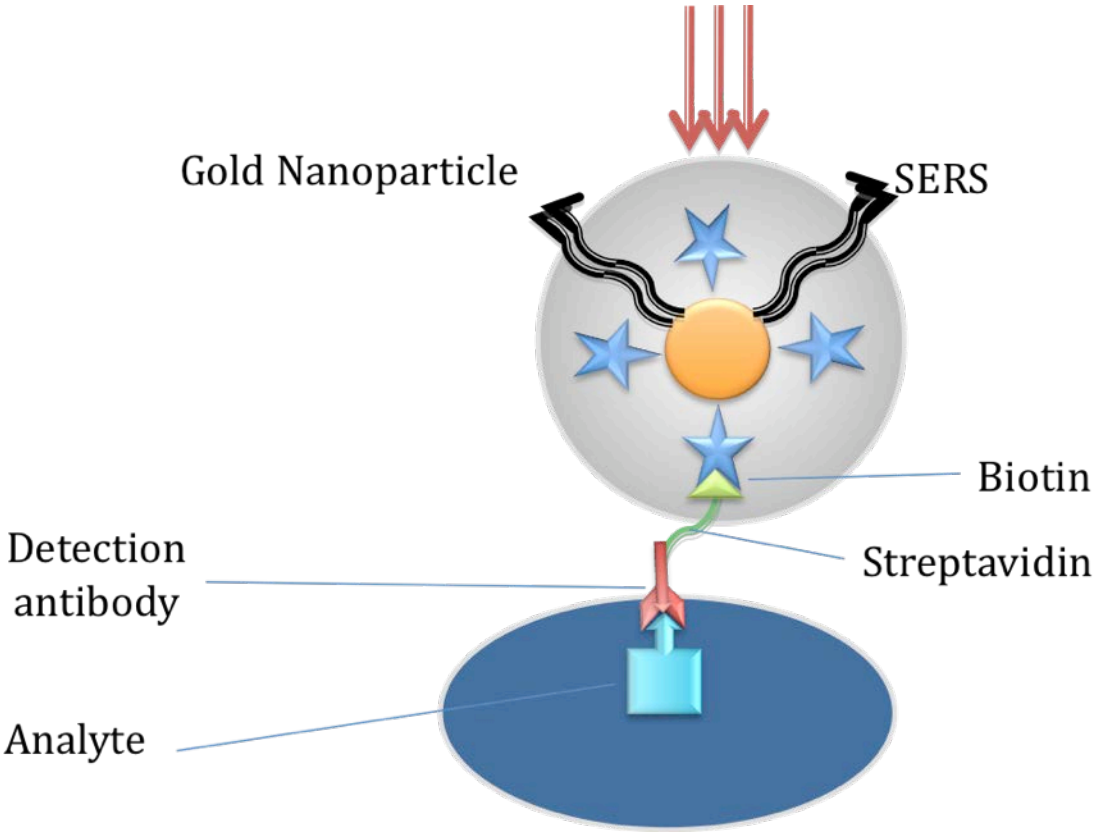


Fig 7. Gold nanoparticles for microarray detection.

iv. Quantum Dots

Quantum Dots are crystals which have a semiconductor fluorescent core coated with another semiconductor shell. When a light of higher frequency falls on the quantum dot, it leads to excitation. As the quantum dot returns to the lower energy level, the energy is released, which can be read as emission spectra (Fig 8).

Merits

- Brighter fluorescence
- Excellent photostability

• Multicolor fluorescent excitation

• Greater quantum yield

Demerits

• Toxicity

• Unknown mechanism

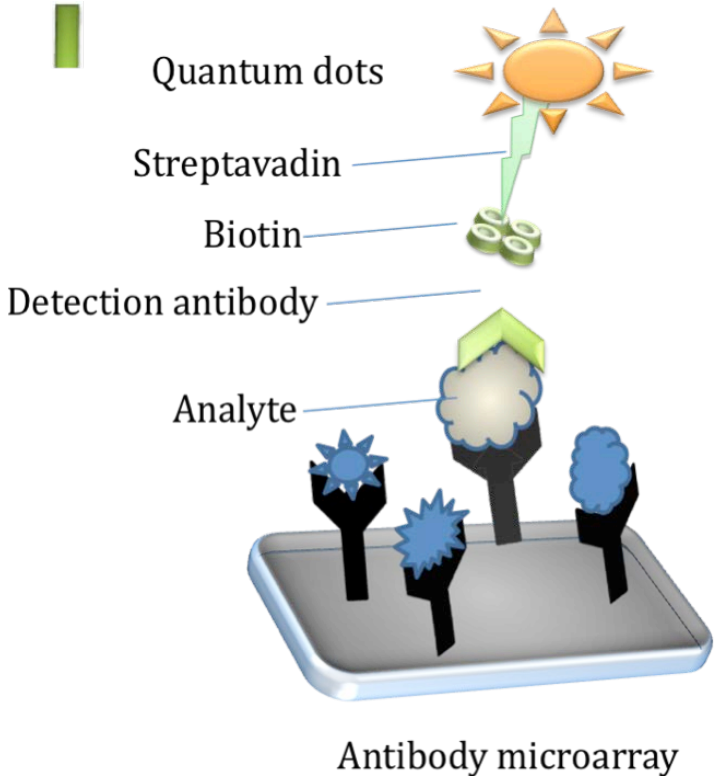


Fig 8. Quantum dots for microarray detection.

v. Dye-doped Silica nanoparticles

The dye-doped silica NPs consists of a silica matrix in which fluorescent molecules are packed. It can be tagged to various targets including antibodies and the interaction can be detected using fluorescence (Fig 9).

Merits

- Biocompatible
- High sensitivity
- Minimal aggregation & dye leakage
- Photostability
- High capacity

Demerits

- Complex synthesis process

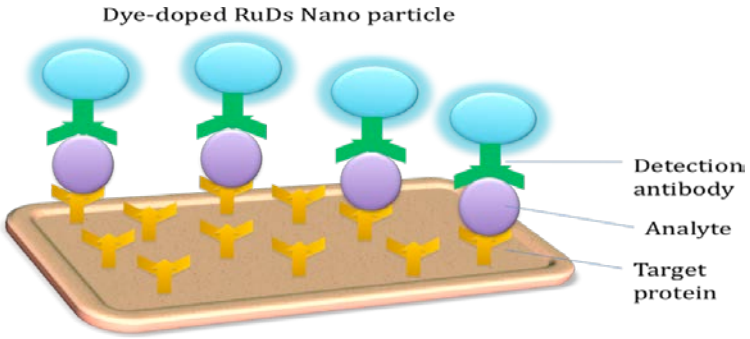


Fig 9. Dye doped silica nanoparticles for microarray detection.

vi. Magnetic nano-particle probe bio-barcodes

In magnetic nano-particle probe bio-barcodes, the nanoparticle probes are coated with DNA unique to the protein of interest. The magnetic microparticle probes or MMPs consisting antibodies for the target analytes, are captured by corresponding antibodies. This complex is then separated in presence of magnetic field, dehybridization of oligonuclelotides and sequenced for the identification of protein of interest. The identification of the liberated oligonucleotide barcodes can be done on the microarray surface by using either scannometric detection or conventional flourophores (Fig 10).

Merits

- High sensitivity
- Less detection time
- Easy adaptability to multiple targets

Demerits

- Can be used only with known antibodies

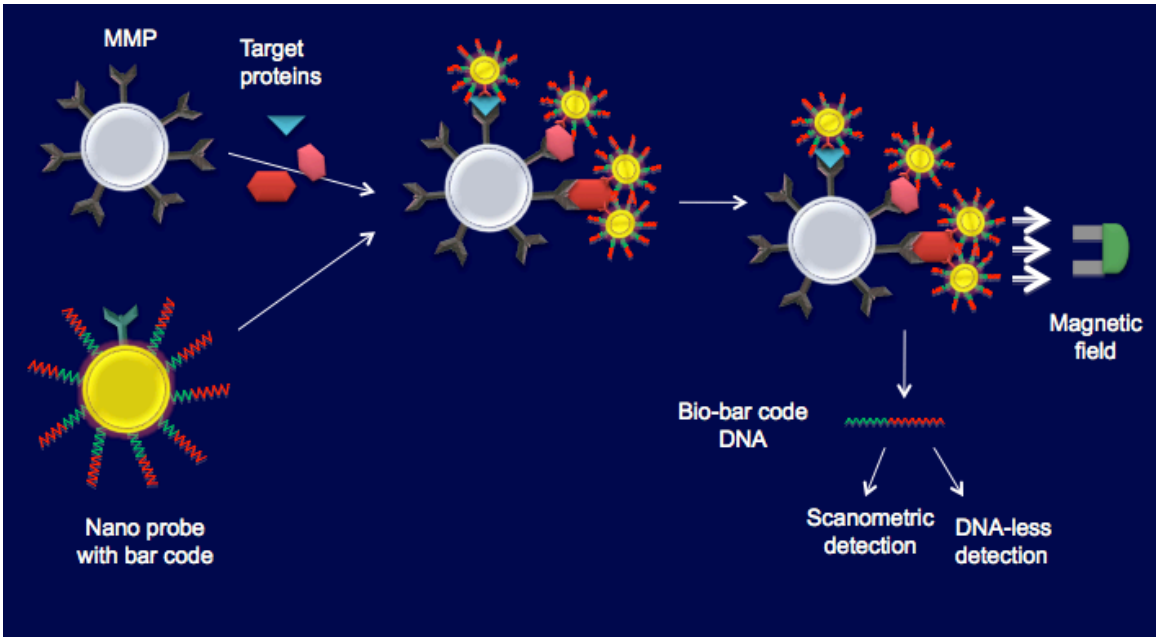


Fig 10. Magnetic nano-particle probe bio-barcodes for microarray detection.

APPLICATIONS

The antigen and antibody microarrays have been shown to be useful in various applications such as:

- Biomarker discovery
- Autoantibody detection
- Protein-protein interaction studies
- Cytokine detection
- Discovery of small molecule inhibitors for signal transduction
- Protein screening
- Antibody screening

Illustration 5: Application-Antigen-antibody interactions

Ref: Haab B, Dunham M, Brown P: Protein microarrays for highly parallel detection and quantitation of specific proteins and antibodies in complex solutions. Genome Biol 2001, 2 (2).

Haab et al. (2001) printed six arrays of 114 different antibodies on to poly-L-lysine coated glass slides using a robotic arrayer. These were used to analyze interactions in six unique antigen mixtures ranging in concentration from 1.6 μg/mL to 1.6 ng/mL. The antigens were tagged with Cy3 and Cy5 fluorescent labels.

Once the antigen-antibody binding reaction was complete, excess unbound antigens were washed off using phosphate buffered saline and water at room temperature.

Once the excess antigens were washed off, the bound antigen-antibody interactions were detected by means of a microarray scanner at wavelengths of 532 nm and 635 nm. The authors found that such microarrays of antibodies could detect their corresponding antigens at concentrations as low as 1 ng/mL.

In a complimentary experiment, the authors generated six antigen arrays having 116 different antigens, which they probed with Cy3 or Cy5 labeled antibodies of varying concentrations.

The antigen-antibody binding reaction was allowed to go to completion and excess unbound antibody was washed away using PBS and water at room temperature.

The microarray slides were scanned at 532 nm and 635 nm. It was found that these antigen arrays allowed detection and quantitation of antibodies down to absolute concentrations of 100 pg/mL. These detection limits can further be improved by using high affinity and purity antibodies, thereby showing great promise for high throughput and sensitive clinical applications.

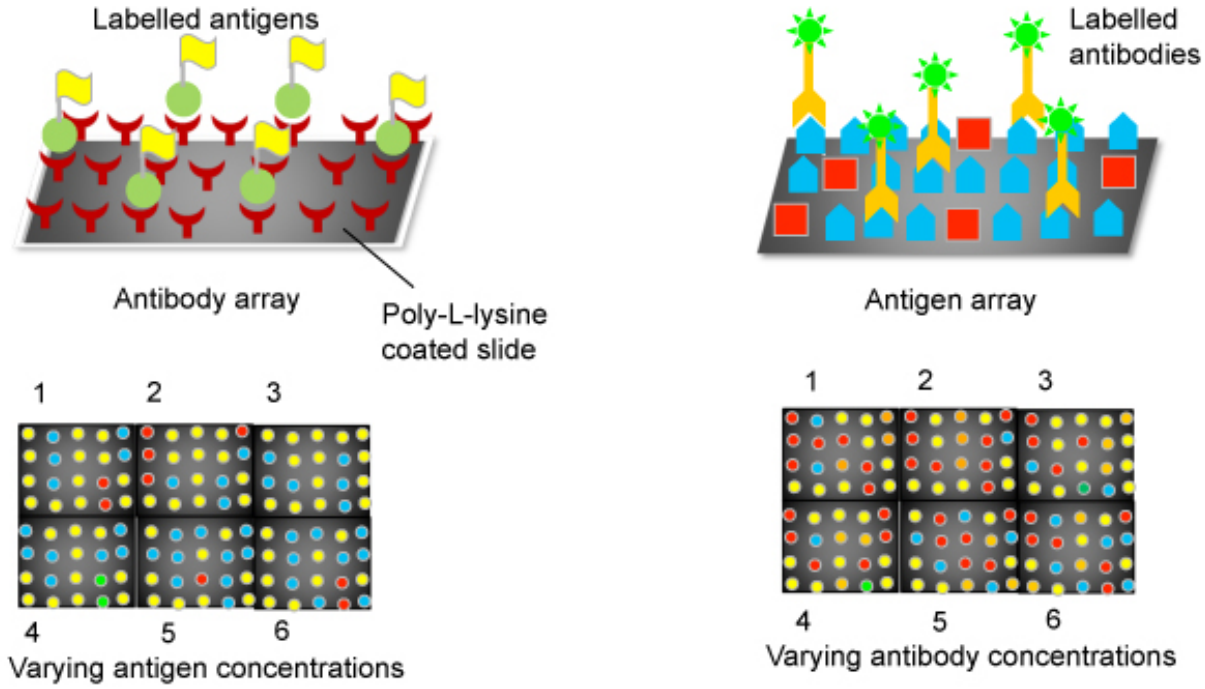


Illustration 6: Application-Biomarker detection

Ref: Steller, S. et al. Bacterial protein microarrays for identification of new potential diagnostic markers for Neisseria meningitidis infections. Proteomics 2005, 5, 2048-2055.

Steller et al. (2005) amplified and subcloned 102 genes from N. meningitidis for expression in E. coli. Clones were grown for 16h at 37^{o}C in antibiotic containing medium, following which protein expression was induced by addition of isopropyl-□-D-thiogalactoside.

The cells were harvested four hours after induction and their proteins purified based on

specific Ni-NTA binding followed by analysis on SDS-PAGE. The 67 purified proteins obtained were then printed on nitrocellulose coated glass slides using a robotic arrayer.

The array was probed with sera from 20 convalescent patients by incubating it overnight at 4°C. The array was then washed with PBS and detection carried out by means of Cy5 labeled secondary antibody. Excess detection antibody was washed off and the array then dried and scanned. Authors detected 47 immunogenic proteins, one of which showed response in 11 of the patients. Protein microarrays have been successfully used for detection of several other disease biomarkers like cancer, autoimmune disorders as well as for diseases like Q-fever and other viral infections.

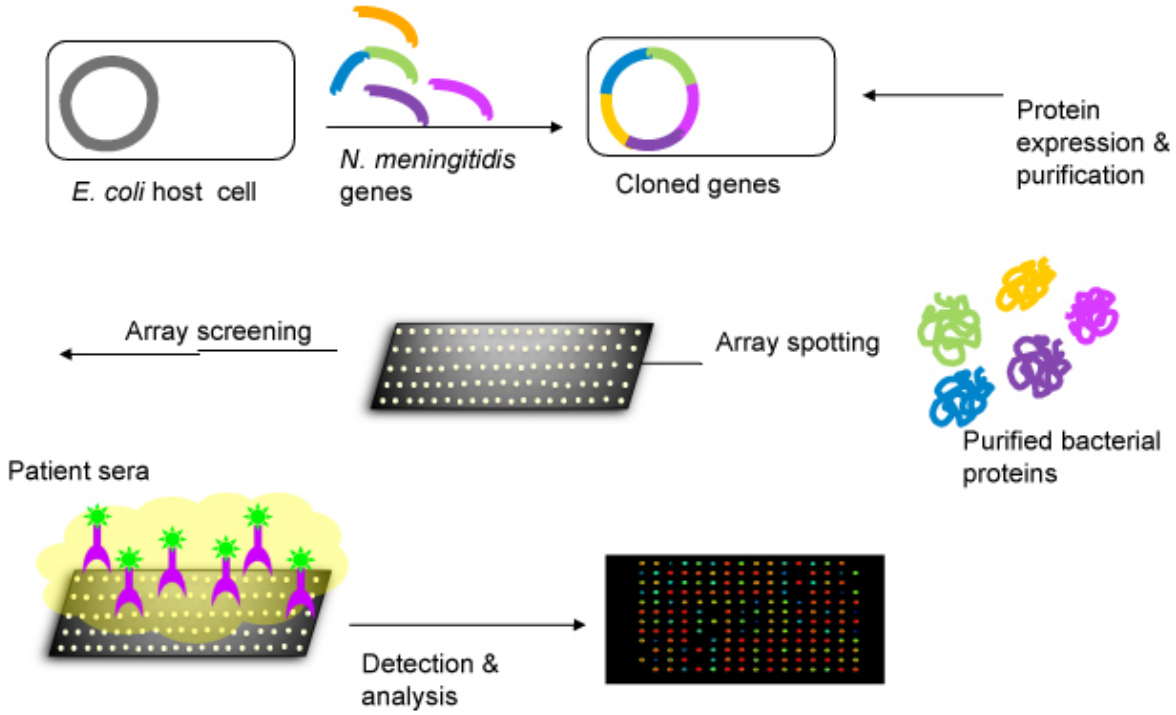


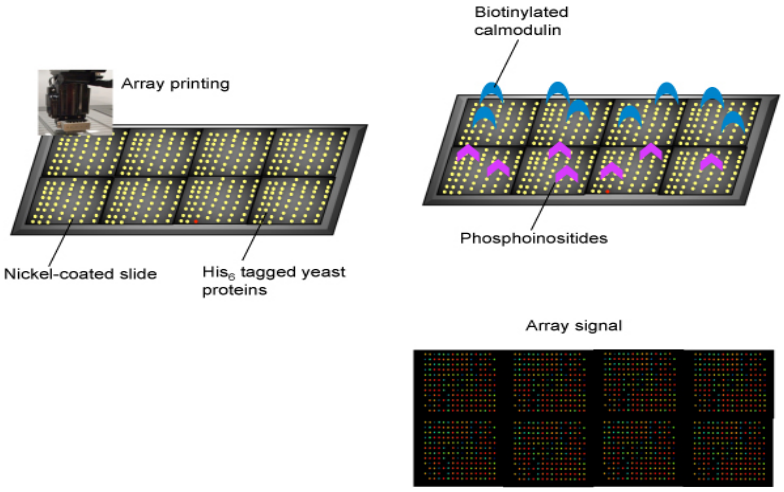
Illustration 7: Application-Protein interaction

Ref: Zhu, H. et al. Global analysis of protein activities using proteome chips. Science 2001, 293:2101-2105.

Zhu et al. (2001) generated a yeast whole proteome array by expressing 5800 purified proteins on a single nickel coated slide. The chips were probed with anti-GST antibodies to determine the reproducibility of protein immobilization. More than 93.5% of protein samples were found to give significant signals and over 90% contained 10 to 950 fg of protein.

To understand the potential applications of such whole proteome arrays, the authors screened the immobilized proteins for protein-protein and protein-lipid interactions. They used biotinylated calmodulin in presence of calcium and phosphoinositide liposomes, respectively. Detection was carried out using Cy3 labeled streptavidin.

6 known calmodulin targets and 33 potential partners were identified with 14 of these proteins possessing a consensus sequence. The PI liposomes were able to identify 150 protein targets, of which 45 were found to be membrane associated, predicted to have membrane spanning regions. This study testified the tremendous potential of whole proteome arrays in identifying new protein targets.



ADVANTAGES AND CHALLENGES

TECHNIQUE	ADVANTAGES	CHALLENGES
Fluorescence based techniques	• Single antibody required • High reproducibility • Excellent for abundant proteins • Multiple samples can be assay	• Sensitivity low for abundant proteins • Cross-reactivity concerns
Single walled carbon nanotubes	• High sensitivity • Multiplexed detection possible • Very less background signal • Photobleach resistant	• Metal contamination may interfere with activity • Biological buffer insoluble • Hard to determine purity
Gold nanoparticles	• Good optical properties • Improved quantum efficiency • Large wavelength range	• Expensive • May be toxic to cells • Variations in shape and size of particles

	• Photobleach resistant	
Quantum Dots	• Photostable • High fluorescent • Multicolor excitation possible	• Toxic • Mechanism not yet understood
Dye-doped silica nanoparticles	• High sensitivity • Biocompatible • Less dye leakage from particles • Particles do not aggregate • Photostable	• Difficult to synthesize
Bio-barcodes	• High sensitivity • Less detection time • Easily adapted to multiple protein targets	• Can only be used with known antibodies

CONCLUSIONS

Antigen-antibody interactions are highly specific and very strong. Apart from the simplicity of production of antibodies, the antibodies themselves can act as antigens. This helps in easy detection using a secondary antibody such as fluorescent antibodies in case of microarrays. New SERS based tags for antibodies are currently the latest method for detection. It is highly sensitive method for detection of low-abundance proteins upto femto and even atto molar levels.

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