LECTURE-28

Label-free Proteomics

Handout

PREAMBLE

Over the last two decades detection techniques in proteomics have experienced phenomenal achievements with introduction of different ultra-sensitive detection techniques, which can selectively detect target analytes from even complex biological samples. Additionally, quite a few detection techniques are proficient for multiplexed detection, which is very useful for high-throughput proteomics applications, particularly in protein/antibody microarrays. There are two major detection approaches used in protein microarrays; label-based and label-free. In label-free detection approaches, inherent properties of the query molecules like mass and dielectric propertyare measured. This eliminates any interference due to the presence of tagging molecules. Recently, label-free detection methods are gaining recognition due to their easy operation procedure, real-time detection, exclusion of the need of secondary reactants and prolonged labeling practice (Yu et al., 2006; Ray et al., 2010). This lecture will provide an introduction and working principle of different label-free detection techniques, which are commonly applied in proteomics.

PROF. SANJEEVA SRIVASTAVA
DEPARTMENT OF BIOSCIENCES & BIOENGINEERING
IIT BOMBAY, MUMBAI, INDIA

OUTLINE OF LECTURE

I. Different label- free detection techniques commonly used in proteomics

(a) SPR and related techniques

(b) Ellipsometry-based techniques

(c) Interference-based techniques

(d) Scanning Kelvin nanoprobe and atomic force microscope

(e) Microcantilever

II. Application of label-free techniques in proteomics

(a) Study of biomolecular interactions

(b) Detection of disease biomarkers

III. Challenges

IV. Conclusions

Label-based detection techniques

With rapid advancements in gel-free proteomics techniques, particularly protein microarrays, the need for improved detection systems has been imperative. Label-based detection systems have taken rapid strides to satisfy this demand with significant improvements in sensitivity, multiplexing capability and reproducibility.

Illustration: Chemiluminescence

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.

Illustration: Fluorescence-based detection

The array surface is functionalized with probe antibody molecules specific for the target antigen of interest. The target antigens are 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.

Illustration: Chromogenic detection

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 has been achieved by this detection technique.

Illustration: Radioactive labeling

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.

I. DIFFERENT LABEL-FREE DETECTION TECHNIQUES COMMONLY USED IN PROTEOMICS

Development of reliable, sensitive and high-throughput label-free detection techniques has become imperative for proteomic studies due to drawbacks associated with label-based technologies. Label-free detection methods, which monitor inherent properties of the query molecule, promise to simplify bioassays (Fig 1).

BOX-1: Overview of label-free techniques

Label-free detection: Label-free detection techniques monitor inherent properties of the query molecules such as mass, optical and dielectric properties. Unlike label-based detection methods, these techniques avoid any tagging of the query molecules thereby preventing changes in structure and function. They do not involve laborious procedures but have their own pitfalls such as sensitivity and specificity issues.

Surface plasmon resonance-based techniques

i) **Surface plasmon resonance (SPR):** Detects any change in refractive index of material at the interface between metal surface and the ambient medium.

ii) **Surface plasmon resonance imaging (SPRi):** Image reflected by polarized light at fixed angle detected.

iii) **Nanohole array:** Light transmission of specific wavelength enhanced by coupling of surface plasmons on both sides of metal surface with periodic nanoholes.

Ellipsometry-based techniques

i) **Ellipsometry:** Change in polarization state of reflected light arising due to changes in dielectric property or refractive index of surface material measured.

ii) **Oblique incidence reflectivity difference (OI-RD):** Variation of ellipsometry that monitors harmonics of modulated photocurrents under nulling conditions.

Interference-based techniques: Interferometry is based on the principle of transformation of phase differences of wave fronts into readily recordable intensity fluctuations known as interference fringes. The various detection strategies that make use of this principle include:

Spectral reflectance imaging biosensor (SRIB): Changes in optical index due to capture of molecules on the array surface detected using optical wave interference.

ii) **Biological compact disc (BioCD):** Local interferometry i.e. transformation of phase differences of wave fronts into observable interference fringes, used for detection of protein capture.

iii) **Arrayed imaging reflectometry (AIR):** Destructive interference of polarized light reflected from silicon substrate captured and used for detection.

Electrochemical impedance spectroscopy (EIS) -aptamer array: Aptamers are short single-stranded oligonucleotides that are capable of binding to a wide range of target biomolecules. EIS combined with aptamer arrays can offer a highly sensitive label-free detection technique.

Atomic force microscopy (AFM): Vertical or horizontal deflections of cantilever measured by high-resolution scanning probe microscope, thereby providing significant information about surface features.

Enthalpy array: Thermodynamics and kinetics of molecular interactions measured in small sample volumes without any need for immobilization or labelling of reactants.

Scanning Kelvin nanoprobe (SKN): A non-contact technique that does not require specialised vacuum or fluid cell; SKN detects regional variations in surface potential across the substrate of interest caused due to molecular interactions.

Microcantilever: These are thin, silicon-based, gold-coated surfaces that hang from a solid support. Bending of cantilever due to surface adsorption is detected either electrically by metal oxide semiconductor field effect transistors or optically by changes in angle of reflection.

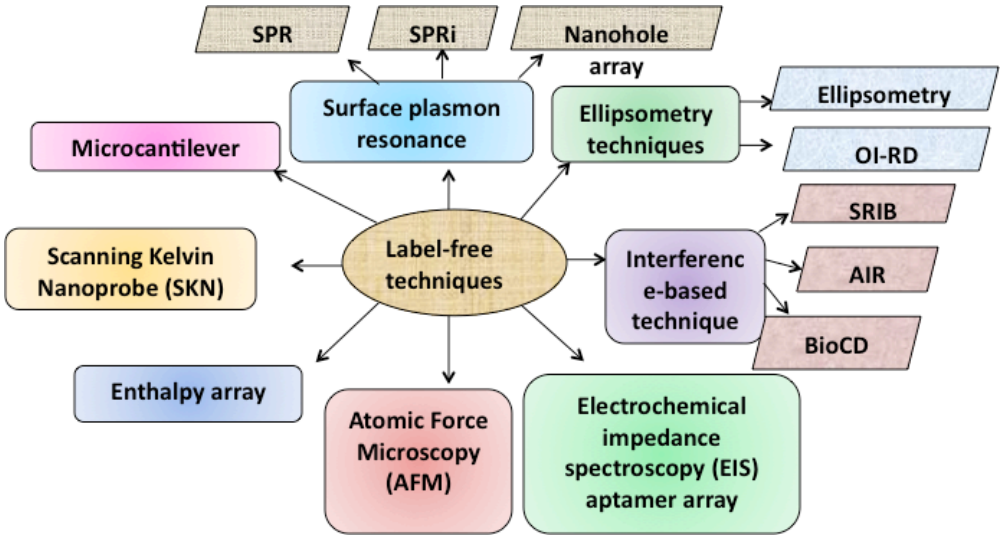

Fig 1. An overview of label-free techniques

1A. SPR and related techniques

Surface plasmon resonance (SPR) is a label-free technique that measures variations in refractive index of the dielectric layer adjoining to the sensor surface as a result of the adsorption or desorption of molecules (Fig1). SPR provides real-time measurements of alteration in refractive index in the locality of a surface (Shankaran et al., 2007). The variation in reflection intensity with respect to incident angle before and after binding of the target molecule is shown as sensorgram.

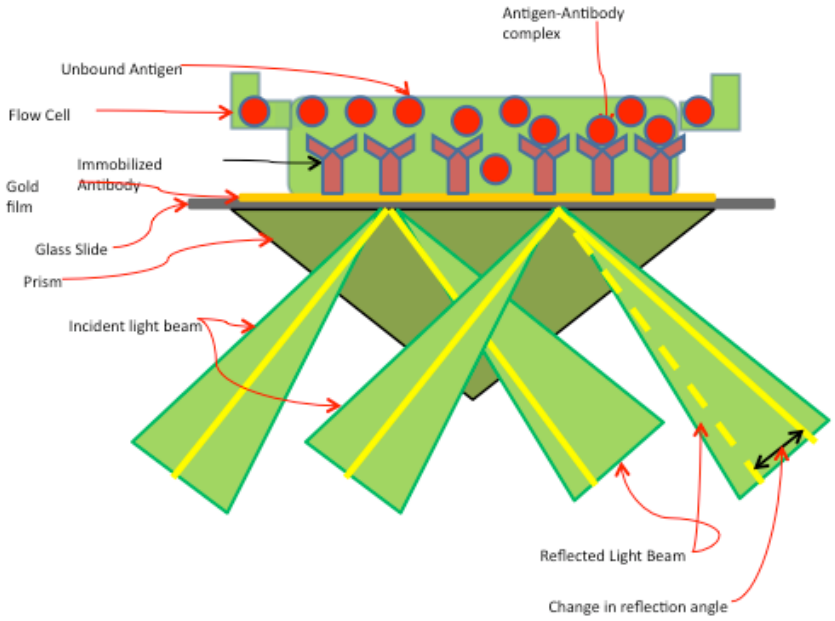

Fig 2. Variations in the refractive index of the medium directly in contact with sensor surface is measured in SPR.

In SPR imaging (SPRi) a spatially resolved measuring device is introduced in SPR set-up (Ladd et al., 2009). SPR and SPRi are suitable for instantaneous label-free analysis of several biomolecular interactions in a quick and HT style. SPR-based biosensors

capable of detecting very minute amounts of target analytes with high selectivity are promising for discovery of disease biomarkers.

Apart from SPR and SPRi, nanohole arrays are considered as a advantageous label-free approach for biosensing, since they require plain optical alignment and simple miniaturization, and offer high accuracy, robustness, increased fluorescent signal, multiplexing and collinear optical detection (Ji et al., 2008; Lesuffleur et al., 2008). If prearranged arrays of nanoscale holes are designed in a metal film, unusual optical transmission characteristics at resonant wavelengths are observed. Surface plasmons (SPs) are excited on both sides of metal surface. It increases the light transmission for a specific wavelength and makes nanohole arrays a prospective surface-based biosensor (Fig 3).

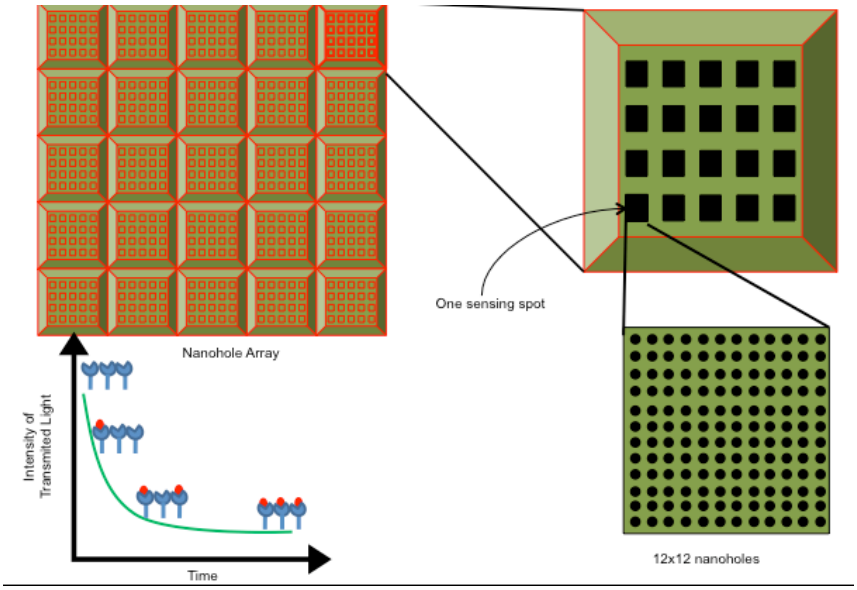

Fig 3. Nanohole arrays are label-free approach for biosensing, which require plain optical alignment and simple miniaturization, and offer high accuracy, robustness, increased fluorescent signal, multiplexing and collinear optical detection. If prearranged

arrays of nanoscale holes are designed in a metal film, unusual optical transmission characteristics at resonant wavelengths are observed.

(b) Ellipsometry-based techniques

Ellipsometry-based label-free detection methods measure the polarization state of the reflected light, which is changed when dielectric property or refractive index of the sample surface is altered (Fig 4). In imaging ellipsometry microscopy the CCD camera are coupled with ellipsometer (Jin et al., 2004). If microfluidic system is coupled with imaging ellipsometry multiple advantages such as high automation, less sample consumption, fast detection and HT assays with superior sensitivity can be achieved. Ellipsometry-based techniques are useful for studying kinetics of biomolecular interactions, hormonal activity, detection of microorganisms, and quantification of competitive adsorption of protein.

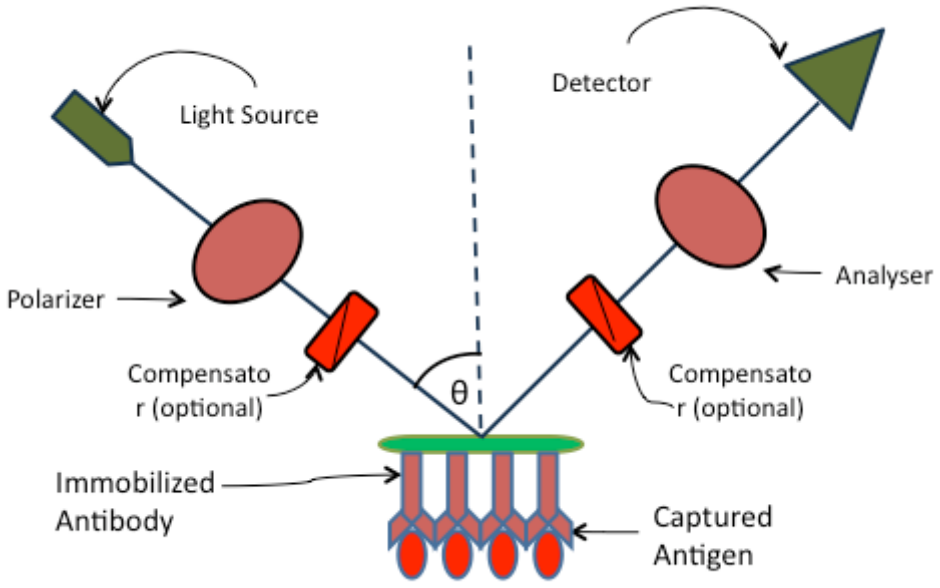

Fig 4. Basic principle behind ellipsometry. A photodetector is used to monitor the intensity of the reflected light.

Another form of ellipsometry; oblique incidence reflectivity difference (OI-RD), in which the harmonics of modulated photocurrents are measured under appropriate nulling conditions, is used as a label-free detection platform in proteomics (Zhu et al., 2007; Fei et al., 2008). Variation in thickness and/or dielectric response as a result of biomolecular interactions generates a detectable OI-RD signal.

(c) Interference-based techniques

Interference-based techniques detect optical phase difference as a result of biomolecular mass addition (Fig 5). There are quite a few potential interferometric techniques such as spectral reflectance imaging biosensor (SRIB), dual-channel biosensor, SPR interferometry, on chip interferometric backscatter detection, porous silicon-based optical interferometric biosensor, biological compact disc (BioCD) and spinning disc interferometry, which are very promising for label-free detection of biomolecules (Ray et al., 2010). Biochemical and functional analysis of proteins are also possible using interference-based label-free detection methods (Table 1) (Gao et al., 2006; Ozkumur et al., 2008).

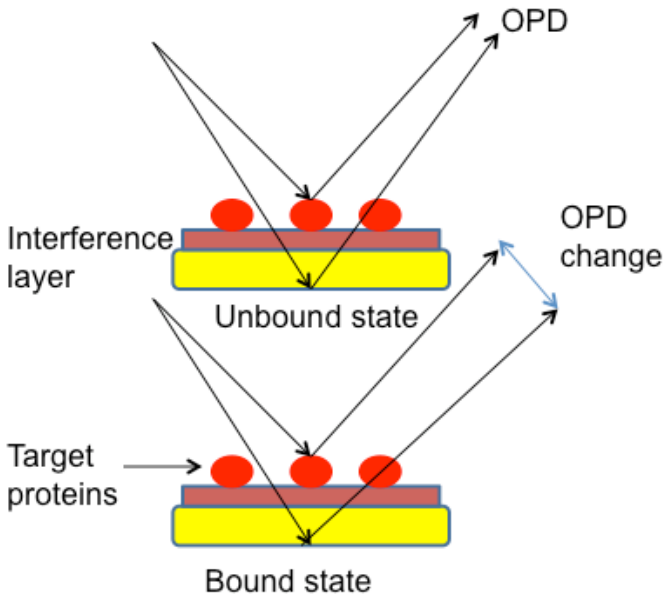

Fig 5. Interferometric techniques measure the phase differences of the wave fronts and convert it into observable visible intensity fluctuation known as Interference fringes.

Illustration: Spectral reflectance imaging biosensor (SRIB)

A SiO_2 coated Si surface is functionalized with the biomolecule of interest. The magnitude of total reflected light at a particular wavelength depends entirely on the OPD between the top surface and the SiO_2-Si interface. Binding of the target to the immobilized biomolecule further increases the OPD and is seen as a shift in the spectral reflectivity. SRIB therefore serves as a useful tool for HT, real-time detection of biomolecular interactions.

Spectral reflectance imaging biosensor (SRIB) is the most promising interference-based label-free detection method, which monitors alterations in the optical index due to the capture of biological material on the sensor surface (Ozkumur et al., 2008). SRIB directly monitors primary molecular binding interactions with high sensitivity. Back-scattering interferometry (BSI) is another promising platform for studying label-free molecular interactions within very small amount of samples (Bornhop et al., 2007). It can quantify a wide dynamic range (Kd spanning six decades) of molecular interactions in free solution and very compatible for multiplexing.

(d) Scanning Kelvin nanoprobe

The Kelvin probe force microscope (KPFM) measures local changes in surface potential across a substrate (generally gold) (Fig 6). KPFM is very promising for label-free biomolecular label-free detection and offers a number of advantages. It is a non-contact technique; therefore specialized vacuum or fluid cell is not required. High-speed screening is possible with KPFM while maintaining the signal fidelity. Another significant aspect of KPFM technology is its ability to analyse high-density arrays (Sinensky et al., 2007). This label-free detection technique also has ability to reduce noise by decreasing the non-specific binding of biomolecules.

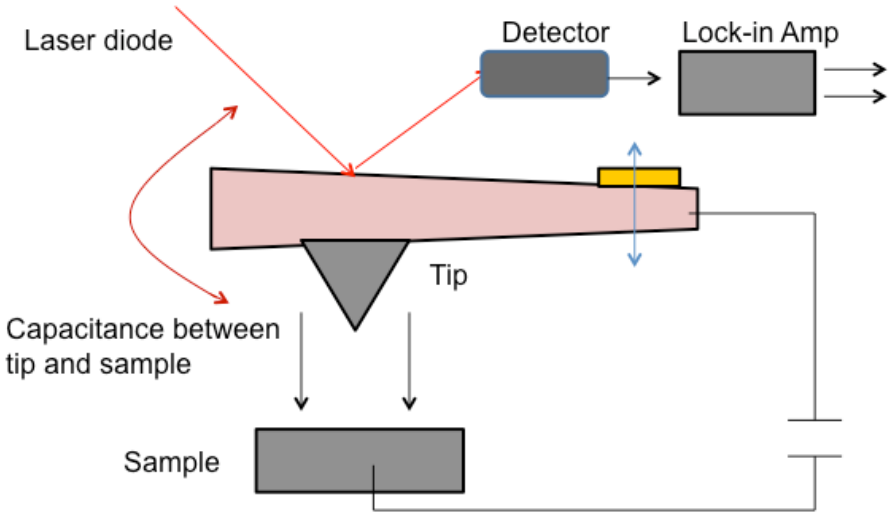

Fig 6. Working principle of Scanning Kelvin nanoprobe. Variations in work function and surface potential occurred due to molecular interactions are measured using SKN.

(e) Microcantilever

Microcantilevers are silicon-based, gold-coated, thin (1 mm) surfaces, horizontally attached to a solid support (Braun et al., 2009). Binding of biomolecules on the cantilevers bends them and level of bending is measured optically or electrically for label-free detection (Fig 7). Analysis of thermodynamics of protein–protein and other biomolecular interactions, detection of cancer markers, antigen–antibody interactions can be performed using microcantilever-based label-free sensors (Table 1) (Ray et al., 2010).

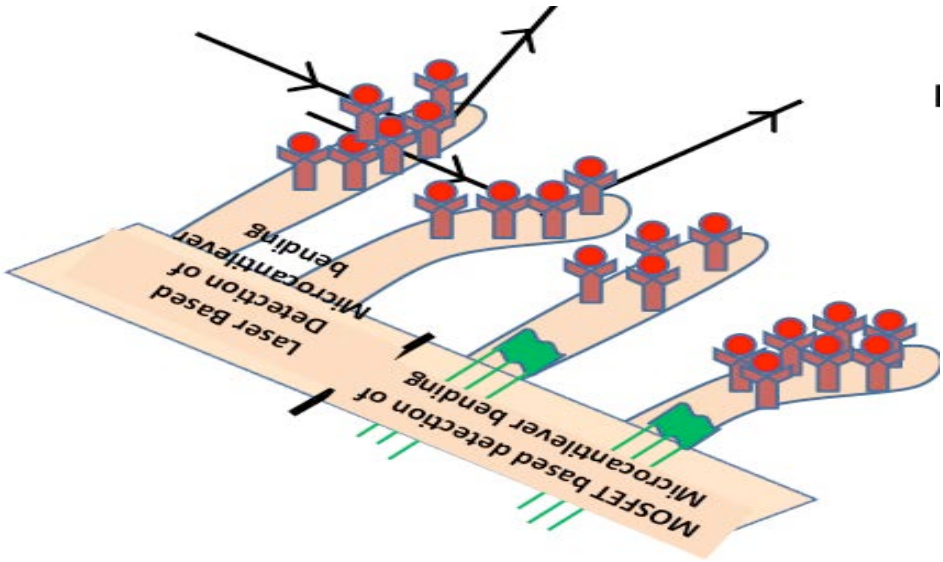

Fig 7. Working principle of microcantilever. The interaction of query molecules with immobilized target molecules leads to bending of microcantilevers and changes the resonant frequency.

II. APPLICATION OF LABEL- FREE TECHNIQUES IN PROTEOMICS

Quite a few label-free approaches, like SPR, SPRi, interference-based techniques, microcantilever etc. are considered as impending platforms for studying biomolecular interactions and detection of disease biomarkers (Table 1).

Table 1: Application of label- free techniques in proteomics

Techniques	Principle	Applications	Merits	Demerits	Sensitivity & Resolution	†Through-put	Used for protein microarrays
1. Surface plasmon resonance (SPR) and related techniques							
(i) Surface plasmon resonance (SPR)	Measures changes in the refractive index of the medium directly in contact with sensor surface	• Studying association or dissociation kinetics • Drug discovery (Shankaran et al. 2007) • Rapid diagnosis of cancer patients (Campagnolo et al. 2004) • Antigen-antibody interactions in protein microarrays (Hiep et al. 2007)	1. Real-time measurements 2. Multiplex analysis 3. Sensitive to conformational change 4. Quantitative and qualitative	Restricted to gold/silver surfaces	10 ng/mL for casein by LSPR (Hiep et al. 2007) High [B]	++ (**) 400 protein–protein interactions (Wassaf et al 2006)	((Wassaf et al 2006, (Hiep et al. 2007)
(ii) Surface plasmon resonance imaging (SPRi)	Captures an image reflected by polarized light at a fixed angle, and simultaneously detects many biomolecular interactions	• Used for DNA-protein interaction [(Zhu et al. 2001) • Protein-glycan interactions (Yuk et al. 2006) • Disease marker detection and protein expression profiling (Lausted et al. 2008)	1-4 as above+ 5. Suitable for HT	1. Restricted to gold/silver surfaces 2. Requires sophisticated instrumentation	[nM-zM range] 0.5pM (Lokate et al. 2007) 64.8 zeptomole [Best achievable sensitivity] (Beusink et al.	+++(*) 792 features on microarray (Lausted et al 2008)	(Lee et al. 2006, Yuk et al. 2006, Lokate et al. 2007, Huang et al. 2006, Dong et al. 2008, Huk et al. 2009, Aing et al 2005, Ladd et al.2009,

		• Antigen-antibody interactions on microarrays (Xinglong et al. 2005)			2008) Very High [A]		Lausted et al 2008)
(iii) Nanohole array	Periodic nanoholes couple incident photons into surface plasmons (SPs). SPs of both the side couple through periodic nanoholes to enhance light transmission	• Binding kinetics measurement (Lesuffluer et al. 2008) • Protein-protein interaction (Ji et al. 2008)	1,2,4,5 as above+ 6. Simple optical alignment 7. Unlike SPR, bulky prism is not required 8. Use of high numerical aperture is possible 9. Miniaturization is possible	1. Insensitive to conformational changes 2. Restricted to gold/silver surfaces	333 nm/RIU (Leebeeck et al. 2007)● $9.4x10^{-8}$ RIU (Ji et al. 2008) ●● 80 nM (Eftekhair et al. 2009) High [A]	+++(**) 20,164 sensors can be fabricated (Ji et al. 2008)	(Ji et al. 2008, Lesuffluer et al. 2008, Lindquist et al 2009)
2.Ellipsometry	Measures change in polarization state of the incident light which depends on the dielectric properties and refractive index of thin film	• Real time and end point measurement of biomolecular interactions (Qi et al. 2005) • Hormone detection & cancer marker test (Jin et al. 2004) • Clinical diagnosis and narcotics detection (Klenkar et al. 2008) • Affinity determination (Chamritski et al. 2007) • Intrinsic pathway of coagulation (Elwing et al. 1998)	1,2,4 as above+ 10. Not restricted to gold/silver 11. Cheaper than SPR based biosensors 12. Simple instrumentation 13. Large field of view for simultaneous monitoring of the entire microarray	1. Less sensitive than SPRi 2. Insensitive to conformational changes	1 ng/mL (Jin et al. 2004, Qi et al. 2009) 10 pg/mm^2 (Westphal et al. 2002) High [B]	+ 12 protein spots [Wang *et al.,*2006]	(Wang *et al.,*2006; Chamritski 2007; Qi *et al.,*2005; Jin *et al.,*2004)
3. Oblique incidence reflectivity difference	Based on polarization modulated nulling	• Real-time and end point analysis of antigen-antibody	All the merits of ellispometry +5+	Insensitive to conformational	10 pm thickness change. Sensitivity	**+++**(*) 2760 spots (Fei *et al.,*	(Zhu *et al.,*2007; Fei *et al.,* 2008;

(OI-RD)	ellipsometry	interaction • DNA-DNA hybridization and protein-small ligand binding reactions (Piciu et al. 2006)	14. Higher sensitivity than imaging ellipsometry	changes	comparable to SPRi (Lesuffleur et al. 2008) Very High [A]	2008;)	Landry *et al.*,2007; . Landry *et al.,* 2008)
4.Interference based techniques:							
(i) Spectral reflectance imaging biosensor (SRIB)	Detection of optical phase difference due to biomolecular mass accumulation.	• Dynamic measurements of protein-protein interactions (Leebeeck et al. 2007)	5+ 15. Cost effective 16. Fast determination of binding kinetics	1. Suitable for only smooth layered substrates 2. Non-specific binding	19 ng/mL (Leebeck et al. 2007) High [B]	**++**(**) 200 spots [O¨zkumur et al.,2008]	(O¨zkumur et al.,2008)
(ii) Dual-channel biological compact disc (BioCD)	Simultaneous interferometry and fluorescence detection	• Detection of mass and fluorescence signals from protein (Eftekhari et al. 2009)	5+17 Extremely fast 18. Specific and non-specific bindings can be differentiated	Expensive and complex	30-70 pg/mL (Eftekhari et al. 2009) High [A]	**+++**(*) 6800 spots (Wang et al.,2008)	(Wang et al.,2008)
(iii) Arrayed imaging reflectometry (AIR)	Measures small-localized changes in optical thickness of a thin film	• Detection of human proteins in cellular lysate and serum (Asinovski et al. 2008) • Biomolecular binding (Elwing et al.1998) • Protein spot homogeneity evaluation (Asinovski et al. 2008)	6+15+17	Sensitivity	250 pg/mL, (Asinovski et al. 2008) High [A]	+ 4 spots (Mace *et al.,* 2006)	(Mace *et al.,* 2006; Mace *et al.,* 2006)
5. Scanning kelvin nanoprobe (SKN)	Measures alteration in work function and surface potential due to molecular interactions	• Antigen–antibody interactions (Landry (Asinovski et al. 2007) • DNA structure analysis (Asinovski et al.	17+ 19. Non-contact	Unsuitable for very complex samples	< 50 nanomolar (Fei et al. 2008) High [B]	+ (**) 36 spots (Cheran *et al,*,2004; Thompson *et al,*,2005)	Sinensky *et al,*,2007; Cheran *et al,*,2004; Thompson *et al,*,2005; Saoud *et*

		2007) • • Isoelectric point determination (Fei et al. 2008)					*al.*,2008
6. Atomic force microscope (AFM)	High resolution scanning probe microscope detects vertical and horizontal deflection of cantilever	• Pathogen detection Nikitin et al. 2000) • Protein interaction (Glaser et al. 2007)	20. Detection under physiologically relevant conditions 21.High specificity	1. Imaging in aqueous solutions is very difficult 2. Image artifacts	Picoliter volume (Glaser et al. 2007) High [B]	+ (**) 12 protein spots (Lee *et al.*,2006)	Lynch *et al.*,2003; Huff *et al.*,2004; Soultani-Vigneron *et al.*,2005; Lee *et al.*,2006
7. Enthalpy array	Arrays of nanocalorimeters, Measures heat generation of the reaction	• Enzyme kinetics (Km, Kcat) and inhibitor constants (Ki) can be determined (Chen et al. 2005) • Biomolecular interactions enzymatic turnover and mitochondrial respiration determination (Joo et al. 2009)	10+ 22. Immobilization of biomolecules not required 23. Very rapid, small sample volumes required 24. Can be used for complex samples (i.e. serum)	1. False positives when two reacting solutions have different pH or ionic strength 2. Complex instrumentation 3. Real-time analysis not possible 4. Not sensitive to conformational change	µM-nM range, [(Joo et al. 2009) Moderate [C]	**++**(**) 384 samples (Recht *et al.*,2008;)	Torres *et al.*,2004; Recht *et al.*,2008; Recht *et al.*,2009
8. Microcantilevers	The binding of query molecules to the immobilized target molecules causes bending of microcantilever and change the resonant	• Investigating thermodynamics of biomolecular interactions (Zhu et al. 2001) • Detecting conformational changes • Determining mass of	1,4,10,15	False positives with complex sample (i.e. serum)	0.2 ng/mL (Beusink (Joo et al. 2008) High [B]	**++**(**) 80-120 reaction wells (Yue *et al.*, 2008)	(Yue *et al.*, 2008; Huber *et al.*,2006; Backman *et al.*,2005)

	frequency	single virus or bacterium and measurement of cell growth on cantilever surface (Zhu et al. 2000)					

Sensitivity scale

[A] Very high: atto-femtogram/mL [10^{-18}-10^{-15}gram/mL]

[B] High: pico-nanogram/mL [10^{-12}-10^{-9}gram/mL]

[C] Moderate: microgram/mL [10^{-6}gram/mL]

† Throughput (maximum number of sample spots analyzed simultaneously)

+++ High (capable to monitor thousands of biomolecular interactions simultaneously)

++ Moderate (capable to monitor about hundred biomolecular interactions simultaneously)

+ Low (capable to monitor less than hundred biomolecular interactions simultaneously)

HT applications demonstrated/Proof-of-concept

(*) HT applications already demonstrated using this technique

(**) Proof of concept for HT but actual demonstration of HT application is not yet done

- Amount of protein used in the microchannel during incubation 17.5 μg
- ●● Corresponds to 200µl of 290nM GST (~34.9 X 10^{12} molecule)

III. CHALLENGES

Regardless of rapid advancement in field of label-free proteomics achieved with introduction of new and versatile technologies, label-free detection approaches have several limitations as well. Both label-free and label-based detection methods have their own advantages and limitations (Ray et al., 2010; Chandra et al., 2011). Although, label-free detections techniques are very promising and potential candidates for real-time measurements of low-abundance analytes and protein-protein interactions, issues regarding sensitivity and specificity remain to be explored further. Additionally, costly fabrication techniques, morphological anomalies of sample spots and insufficient knowledge regarding the exact working principles of the label-free biosensors often restrict their use for practical clinical applications. Label-free measurements have capabilities of detection of low-abundance analytes and protein-protein interactions; but further improvement of specificity and sensitivity is required when complex body fluids have to be analyzed rather than simple buffer solutions commonly used in most of the proof-of-principle experiments. For making label-free sensors popular in routine clinical applications, cost-effective fabrication techniques are required to be developed, and mechanism of working principle of label-free detection approaches need to be explored further.

IV. CONCLUSIONS

Label-free detection techniques are definitely attractive for the large-scale, real-time analysis of protein–protein and other biomolecular interactions and measurement of concentrations of multiple target molecules in HT manner. Such extremely sensitive, fast, label-free detection approaches are useful in various applied fields including pharmaceutical analysis, screening of potential drug molecules, cellular detection, characterization of biomolecules, detection of disease markers and environmental monitoring. Coupling of microarrays and label-free techniques is emerging rapidly and found to be highly effective in detection of extremely low-abundance analytes in buffer solutions. Nonetheless, sensitivity and specificity frequently become the prime limitation for label-free detection methods when very complex biological samples are concerned. Hitherto, label-free detection approaches found to be efficient in analysis of antigen–antibody interactions, but it will be useful in actual bedside applications in clinics, if they can detect multiple protein–protein interactions simultaneously with similar efficacy. Considering the present scenario, it can be concluded that label-free proteomics is still at a premature stage of development and has shown promises mainly for targeted detection of known protein parkers; however, it has not contributed effectively in discovery of new markers which can be directly translated in clinics. It is anticipated that with efforts from different research groups world-wide, the field of label-free proteomics will turn into more robust, sensitive, fast, cost-effective and overcome existing limitations.

REFERENCES FOR FURTHER READING

- Ray S, Mehta G, Srivastava S. Label-free detection techniques for protein microarrays: prospects, merits and challenges. Proteomics 2010; 10:731–48.
- Yu, X., Xu, D., Cheng, Q., Label-free detection methods for protein microarrays. Proteomics 2006, 6, 5493–5503.
- Shankaran, D. R., Gobi, K. V., Miura, N., Recent advancements in surface plasmon resonance immunosensors for detection of small molecules of biomedical, food and environmental interest. Sens. Actuat. B 2007, 121, 158–177.
- Ladd, J., Taylor, A. D., Piliarik, M., Homola, J., Jiang, S., Label-free detection of cancer biomarker candidates using surface plasmon resonance imaging. Anal. Bioanal. Chem. 2009, 393, 1157–1163.
- Jin, G., Zhao, Z. Y., Wang, Z. H., Meng, Y. H. et al., The development of biosensor with imaging ellipsometry. Conf. Proc. IEEE Eng. Med. Biol. Soc. 2004, 3, 1975–1978.
- Gao, T., Lu, J., Rothberg, L. J., Biomolecular sensing using near-null single wavelength arrayed imaging reflectometry. Anal. Chem. 2006, 78, 6622–6627.
- Ozkumur, E., Needham, J. W., Bergstein, D. A., Gonzalez, R. et al., Label-free and dynamic detection of biomolecular interactions for high-throughput microarray applications. Proc. Natl. Acad. Sci. USA 2008, 105, 7988–7992.
- Sinensky, A., Belcher, A., Label-free and high resolution protein/DNA nanoarray analysis using Kelvin probe force microscopy. Nat. Nanotechnol. 2007, 2, 653–659.

- Braun, T., Ghatkesar, M. K., Backmann, N., Grange, W. et al., Quantitative time-resolvedmeasurement ofmembrane
- protein–ligand interactions using microcantilever array sensors. Nat. Nanotechnol. 2009, 4, 179–185.
- Chandra H, Reddy PJ, Srivastava S. Protein microarrays and novel detection platforms. Expert Rev Proteomics 2011;8:61–79.
- Yuk, J. S., Kim, H. S., Jung, J. W., Jung, S. H. et al., Analysis of protein interactions on protein arrays by a novel spectral surface plasmon resonance imaging. Biosens. Bioelectron. 2006, 21, 1521–1528.
- Zhu, H., Klemic, J. F., Chang, S., Bertone, P. et al., Analysis of yeast protein kinases using protein chips. Nat. Genet. 2000, 26, 283–289.
- Hiep, H. M., Endo, T., Kerman, K., Chikae, M. et al., A localized surface plasmon resonance based immunosensor for the detection of casein in milk. Sci. Technol. Adv. Mat. 2007, 8, 331–338.
- Zhu, X., Landry, J. P., Sun, Y. S., Gregg, J. P. et al., Oblique-incidence reflectivity difference microscope for label-free high-throughput detection of biochemical reactions in a microarray format. Appl. Opt. 2007, 46, 1890–1895.
- Landry, J. P., Sun, Y. S., Lam, K. S., Zhu, X. D., High-throughput endpoint and real-time detection of biochemical reactions in microarrays using label-free oblique-incidence reflectivity difference microscopes. Lasers and Electro-Optics. Optical Society of America 2007, DOI: 10.1109/CLEO.2007.4453099

- Mace, C. R., Yadav, A. R., Miller, B. L., Investigation of nonnucleophilic additives for the reduction of morphological
- anomalies in protein arrays. Langmuir 2008, 24, 12754–12757.
- Thompson, M., Cheran, L., Zhang, M., Chacko, M. et al., Label-free detection of nucleic acid and protein microarrays by scanning Kelvin nanoprobe. Biosens. Bioelectron. 2005, 20, 1471–1481.
- Mace, C. R., Striemer, C. c., Miller, B. L., Detection of human proteins using arrayed imaging reflectometry. Biosens. Bioelectron. 2008, 24, 334–337.
- Wu, G., Datar, R. H., Hansen, K. M., Thundat, T. et al., Bioassay of prostate-specific antigen (PSA) using microcantilevers. Nat. Biotechnol. 2001, 19, 856–860.
- Capobianco, J. A., Shih, W. Y., Yuan, Q. A., Adams, G. P. et al., Label-free, all-electrical, in situ human epidermal growth receptor 2 detection. Rev. Sci. Instrum. 2008, 79, 076101.
- Suwansa-ard, S., Kanatharana, P., Asawatreratanakul, P., Wongkittisuksa, B. et al., Comparison of surface Plasmon resonance and capacitive immunosensors for cancer antigen 125 detection in human serum samples. Biosens. Bioelectron. 2009, 24, 3436–3441.
- Vaisocherová H, Faca VM, Taylor AD, et al. Comparative study of SPR and ELISA methods based on analysis of CD166/ALCAM levels in cancer and control human sera. Biosens Bioelectron 2009;24:2143–8.

- Masson, J. F., Battaglia, T. M., Khairallah, P., Beaudoin, S. et al., Quantitative measurement of cardiac markers in undiluted serum. Anal. Chem. 2007, 79, 612–619.
- Ji, J., O'Connell, J. G., Carter, D. J., Larson, D. N., High-throughputnanohole array based system to monitor multiple binding events in real time. Anal. Chem. 2008, 80, 2491–2498.
- Lesuffleur, A., Im, H., Lindquist, N. C., Lim, K. S., Oh, S. H., Laser-illuminated nanohole arrays for multiplex plasmonic microarray sensing. Opt. Expr 2008, 16, 219–224.
- Fei, Y. Y., Landry, J. P., Sun, Y. S., Zhu, X. D. et al., A novel high-throughput scanning microscope for label-free detection of protein and small-molecule chemical microarrays.
- Rev. Sci. Instrum. 2008, 79, 013708–1–013708–7.
- Bornhop, D. J., Latham, J. C., Kussrow, A., Markov, D. A. et al., Free-solution, label-free molecular interactions studied by back-scattering interferometry. Science 2007, 317, 1732–1736.
- Chamritski, I., Clarkson, M., Franklin, J., Li, S. W., Real-time detection of antigen–antibody reactions by imaging ellip- sometry. Aust. J. Chem. 2007, 60, 667–671.
- Campagnolo, C., Meyers, K. J., Ryan, T., Atkinson, R. C. et al., Real-time, label-free monitoring of tumor antigen and serum antibody interactions Christine. J. Biochem. Biophys. Methods 2004, 61, 283–298.

- Wassaf, D., Kuang, G., Kopacz, K., Wu, Q. L. et al., High- throughput affinity ranking of antibodies using surface plasmon resonance microarrays. Anal. Biochem. 2006, 351, 241–253.