

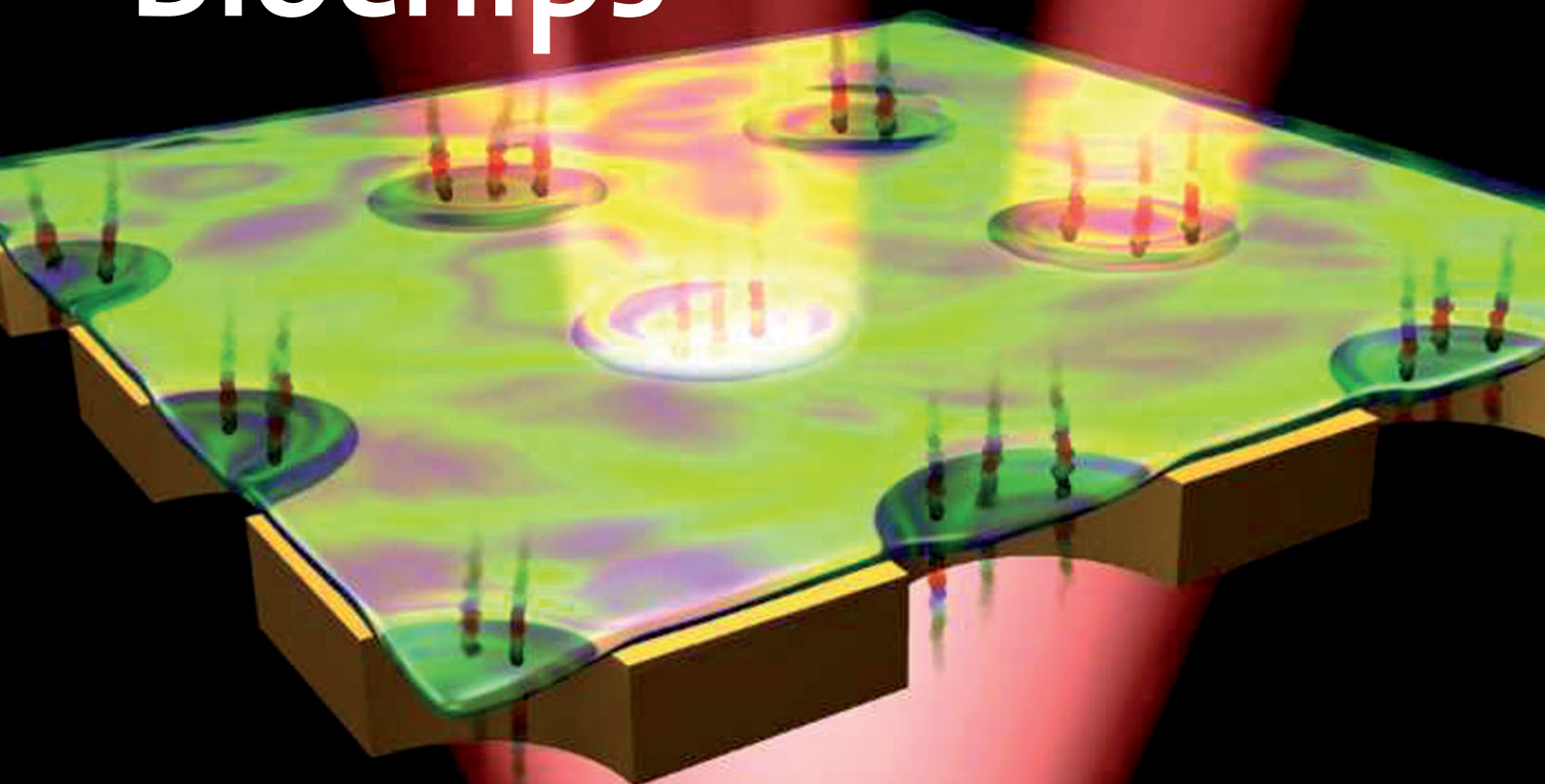
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Review

Surface plasmon resonance for high-throughput ligand screening of membrane-bound proteins

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Technologies based on surface plasmon resonance (SPR) have allowed rapid, label-free characterization of protein-protein and protein-small molecule interactions. SPR has become the gold standard in industrial and academic settings, in which the interaction between a pair of soluble binding partners is characterized in detail or a library of molecules is screened for binding against a single soluble protein. In spite of these successes, SPR is only beginning to be adapted to the needs of membrane-bound proteins which are difficult to study *in situ* but represent promising targets for drug and biomarker development. Existing technologies, such as BIAcore™, have been adapted for membrane protein analysis by building supported lipid layers or capturing lipid vesicles on existing chips. Newer technologies, still in development, will allow membrane proteins to be presented in native or near-native formats. These include SPR nanopore arrays, in which lipid bilayers containing membrane proteins stably span small pores that are addressable from both sides of the bilayer. Here, we discuss current SPR instrumentation and the potential for SPR nanopore arrays to enable quantitative, high-throughput screening of G protein coupled receptor ligands and applications in basic cellular biology.

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1 Introduction

Technological advances facilitate scientific breakthroughs by providing previously inaccessible data and accelerating the pace of scientific discovery. In particular, modern biology has been transformed by the ability to describe biological phenomena in quantitative physical terms, a development pro-

vided by innovations such as surface plasmon resonance (SPR) to measure protein-ligand binding kinetics. Similarly, assay miniaturization has allowed development of high-throughput screening (HTS) programs, from developments as simple as increasing the density of assay plates from 96 to 1536 wells to integrated lab-on-a-chip devices. Combined into a single device, SPR and HTS could allow rapid quantitative analysis of, for instance, thousands of small molecule ligands binding a cell surface receptor to identify agonists meeting specific criteria.

While many ligand screening programs rely on equilibrium binding as a first level of analysis, sub-

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Abbreviations: ELISA, enzyme linked immuno-sorbent assay; EOT, extraordinary optical transmission; GPCR, G protein-coupled receptor; LSPR, localized SPR; MHC, major histocompatibility complex; pMHC, peptide-MHC complex; SLB, supported lipid bilayer; SPR, surface plasmon resonance; SUV, small unilamellar vesicle; TCR, T cell receptor

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sequent characterization of “hits” includes detailed characterization of binding kinetics and selectivity for the receptor of interest. The current standard for characterization of binding partners is quantitation of association and dissociation rate constants by SPR, most successfully characterized by BIAcore™. This technology typically works by tethering one binding partner to a microfluidic chip constructed from a thin gold film on a glass support. To measure equilibrium and binding kinetics, a solution containing ligand flows across the surface. As ligand binds the immobilized partner, the mass of material bound to the surface increases. This change is detected as a change in the angle of polarized light reflected from the bottom surface of the chip (see Fig. 1). SPR has been extensively used in industry and academia for antibody engineering [1] and drug screening programs, as well as to understand basic mechanisms of molecular recognition [2]. Binding kinetics are important to quantify since small differences can provide a rationale for selecting lead molecules during development and binding kinetics will impact both the dosing and potency of a molecule *in vivo*. Mechanistically, kinetic analysis of site-directed variants provides insight into the mechanism and dynamics of binding [3].

However, membrane-bound proteins, which require a lipid bilayer for native function, present a series of challenges for currently available SPR technologies. Membrane-bound proteins are an important class of molecules for several reasons – almost half of the 100 best-selling drugs on the market are targeted to membrane-bound proteins

[4]. These proteins represent the interface between a cell and its surroundings, mediating responses to growth factors and immune cells and representing potential diagnostic and therapeutic targets. While 30% of genes in the human genome are predicted to encode for membrane proteins, these molecules remain poorly characterized, largely due to difficulties in purifying protein for analysis. As an example, the structure of only the second G protein-coupled receptor (GPCR) was solved in 2007 after enormous effort [5–8].

To enable rapid, quantitative screening of ligands binding GPCRs and identification of membrane-bound immune and tumor-associated biomarkers, these ligand-receptor interactions must be probed in lipid bilayers that resemble their native membrane environment. To interface with existing SPR instrumentation, membrane proteins can be immobilized as detergent “solubilized” protein, deposited in supported lipid bilayers or trapped in vesicles that are subsequently captured. Newer SPR-based technologies offer the potential to analyze membrane proteins in completely native environments. One option is a periodic metallic nanopore array supporting free-standing lipid bilayers on a gold film (see Fig. 1d). In this format, membrane proteins would be presented in a lipid bilayer that mimics the natural biological membrane to allow functional studies and label-free kinetic measurements. This review focuses on the applications of existing and emerging SPR technologies for ligand screening programs, biomarker discovery for cancer and basic cellular biology. Alternative options do exist for label-free kinetic

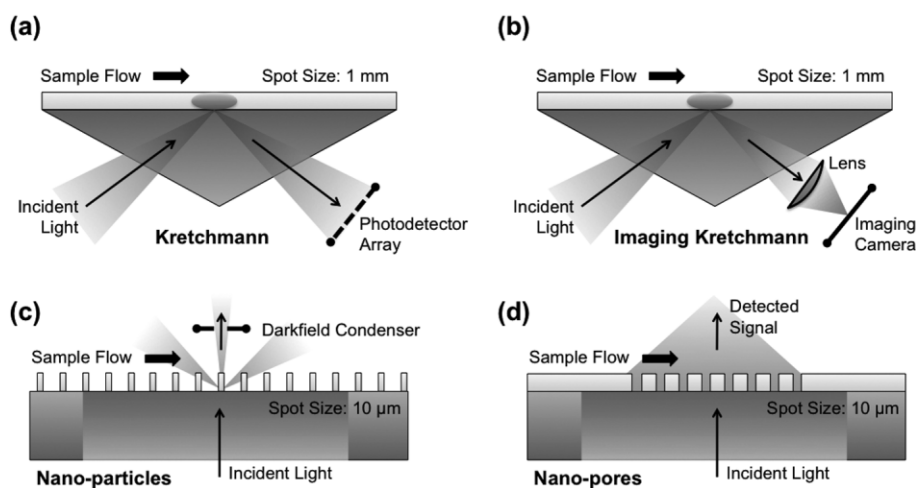


Figure 1. Comparison of SPR technologies. (a) The standard BIAcore™ measurements with a prism-based Kretschmann setup have a large sensing spot size. (b) SPR imaging uses a similar setup, but with imaging optics for the detectors. (c) Nanoparticle arrays use a dark-field condenser for collecting the signal. (d) Nanopore arrays have a high spatial resolution and can easily be made highly multiplexed.

biosensing, such as a quartz crystal microbalance, nanomechanical resonators [9], nanowire sensors [10] and high-Q optical microcavities [11], but these are beyond the scope of this review.

2 Membrane proteins and lipid bilayers

Lipid membranes are responsible for compartmentalizing the many functions and components of a cell, including the cell itself (see Fig. 2). However, to replicate and interact with its surroundings, cells need to transport molecules across membranes, detect and respond to external molecules and to interact with other cells. These processes are particularly complex in the eukaryotic cell, and are mediated by a host of peripheral, integral and transmembrane proteins. The lipid bilayers themselves are a complex mosaic of different lipids, with cholesterol, sphingolipids, lipoproteins and membrane proteins forming “lipid rafts,” membrane microdomains serving to transiently compartmentalize membrane functions such as formation of the immune synapse [12].

A major challenge to the biochemical study of membrane proteins in general, and seven-transmembrane GPCRs in particular, has been the lack of robust recombinant expression systems resulting in purification of large (milligram) quantities of pure, functional material (for review, see [13]). In fact, despite intense efforts, only two GPCR crystal structures have been solved, that of the highly expressed native bovine rhodopsin [14] and the recombinant human beta-2 adrenoceptor [8]. Challenges include low-level endogenous expression, poorly understood folding and stability pathways, host cell toxicity and the need to solubilize these integral membrane proteins with detergents or lipids. However, advances are being made using various expression hosts and fusion proteins, with bacterial systems able to produce 0.5–2 mg/L of cannabinoid and bradykinin receptors [15], respectively, and the yeast *Saccharomyces cerevisiae* producing ~4 mg/L adenosine A_{2A} receptor [16].

3 Experimental approaches for analysis of membrane proteins

3.1 Soluble membrane proteins

Membrane proteins are frequently studied using a variety of “soluble” formats because of the ease of experimentation. In the simplest case, proteins tethered to the membrane *via* a single pass alpha helix or lipid-linked anchor are simply produced as truncated extracellular variants. Because the functional domain folds independently of the anchor, truncation usually results in a properly folded soluble variant of the original membrane protein, which faithfully reproduces many protein functions. Truncation has been widely used, especially for analysis of immune recognition proteins with low expression levels and weak binding affinities, such as the T cell receptor (TCR) and major histocompatibility complex (MHC) proteins [17, 18], which limits analysis on the cell membrane. For multi-pass transmembrane proteins such as GPCRs, which have significant hydrophobic domains and altered tertiary structures and binding affinities in the absence of a lipid bilayer, two options are available. Surfactant screening can identify a detergent whose presence allows the protein to be purified from the cell membrane while retaining function [19]. Alternatively, the hydrophobic surface residues usually in contact with the lipid tails of the membrane can be altered to hydrophobic residues to generate a completely solubilized variant, an approach which has resulted in crystallization of the pentameric transmembrane protein phospholamban [20]. While successful, there is a valid concern that the amino acid changes necessary for solubility may modify the protein's function and compromise interactions with accessory proteins.

3.2 Cell capture technologies

When recombinant soluble expression is not an option, or when membrane proteins need to be

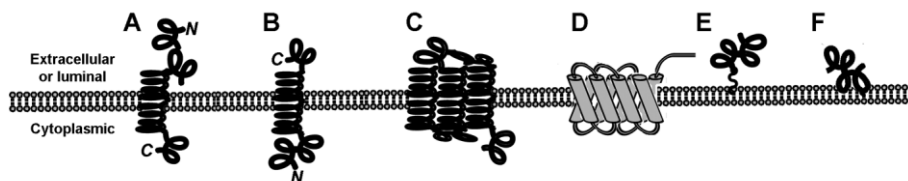


Figure 2. Membrane protein topology. (A) Type I integral membrane protein with an alpha helical transmembrane domain and a cytoplasmic C terminus; (B) Type II integral membrane protein with an extracellular C terminus; (C) Type III and IV multi-pass transmembrane proteins (including GPCRs); (D) a beta-barrel protein, such as the eight stranded, anti-parallel bacterial outer membrane protein OmpA; (E) a lipid- or GPI-linked peripheral membrane protein; and (F) a peripheral membrane protein with an alpha helix lying in the plane of the lipid bilayer.

studied *in situ*, binding of soluble ligands can be used to measure the binding affinity and approximate the number of receptors on the cell surface. Typically labeled with fluorescent or radioactive probes, the soluble ligand is incubated with cells prior to analysis by low-throughput methods such as flow cytometry or ELISA. This approach is widely used for its ease, but is unable to deconvolute complexity – for instance, if co-receptors are involved in binding and influence the binding kinetics, this information is lumped into a single equilibrium binding constant. In an effort to access the same information with high-throughput and multiplexing capabilities, ligand capture has been extended to array formats, in which the soluble binding partner is immobilized in a feature on the array and cells specifically binding the ligand are quantified under equilibrium binding conditions.

This approach has been used most extensively with antibody arrays, in which a panel of monoclonal or recombinant antibodies specific for different membrane proteins are immobilized in discrete features on the array surface. A report by Borrebaeck and coworkers [21] used 20 recombinant single-chain antibodies recognizing different cell-surface receptors to detect corresponding cells in mixed cell populations, representing a semi-quantitative technology for rapid profiling of the plasma membrane. Similar immobilized antibody arrays have been used for phenotype characterization of leukemic, stem and blood cells and have also been combined with planar wave-guide detection systems [22]. Immobilized peptide-MHC (pMHC) complexes have created arrays for T cell capture to characterize cellular immune responses to cancer and vaccination [23–25]. While these arrays are readily adapted to high-throughput analysis, their reliance on equilibrium-based measurements limits the quality of the information. For instance, two anti-HIV antibodies binding the same protein with similar K_d of ~35 nM achieved equilibrium behavior with very different binding mechanisms, as the on-rates differed by fivefold while the off-rates differed by sixfold [26].

3.3 Supported lipid bilayers

A compromise between the completely native environment of the cell membrane and the ready analysis of a soluble protein is a supported lipid bilayer (SLB), in which membrane proteins and lipids are immobilized on a solid support (see Fig. 3). In this format, membrane proteins are analyzed in native or near-native environments with the practical appeal of easy preparation, stability, patterning and availability of compatible surface

characterization techniques. First exploited to study the requirements for T cell activation [27] and the interaction of cholera toxin with the cell surface ganglioside G_{M1} , SLBs can be formed by vesicle fusion, microcontact printing or direct deposition of lipids onto a solid surface to achieve protein-lipid ratios between 1:500 and 1:5000 for large transmembrane proteins (see supporting material for more information). The key advantages are that the solid support confers excellent mechanical stability while the lipids retain their fluid nature, and the system is compatible with many surface characterization techniques. Supported lipid membranes on silicon or SiO_2 -based substrates have been successfully used as a model systems for investigating natural cell membranes in pioneering work by several groups [28–31]. Detection can be achieved by a number of optical techniques, including fluorescence, SPR and plasmon waveguide analysis.

A thin layer of water (1–2 nm) beneath the lipid layer acts as a lubricant to allow lateral and rotational mobility. However, there is still evidence of friction between the lipids and the solid support, as lipid diffusion coefficients in a supported bilayer are more than two times slower than in a free-floating bilayer under identical conditions [32]. Mobility of transmembrane proteins is ever further reduced, due to drag of the external loops against the surface and incorporation of native membrane proteins with large intracellular domains is impossible [33]. The common solution is to lift the bilayer away from the solid support by some type of spacer molecule, such as a polymer cushion [34], a hydrogel [30] or a DNA tether [35]. However, it has been challenging to form stable lipid bilayers on planar noble metal films (gold or silver) without extensive surface modifications [4]. The problem is compounded by the intrinsic roughness of as-deposited metal films, which interferes with lipid membrane formation and reduces transmembrane protein lateral mobility and function. While the use of a polymer cushion or a hydrogel layer [36] (see Fig. 3) can heal these surface defects, the addition of a passivation layer can sharply degrade the SPR detection sensitivity. Tethered lipid bilayers can partially overcome this challenge by lifting the membrane a few nanometers above the substrate [37], it requires difficult chemistry, and the membrane is only accessible from above, making this technique not readily applicable to natural cell membranes.

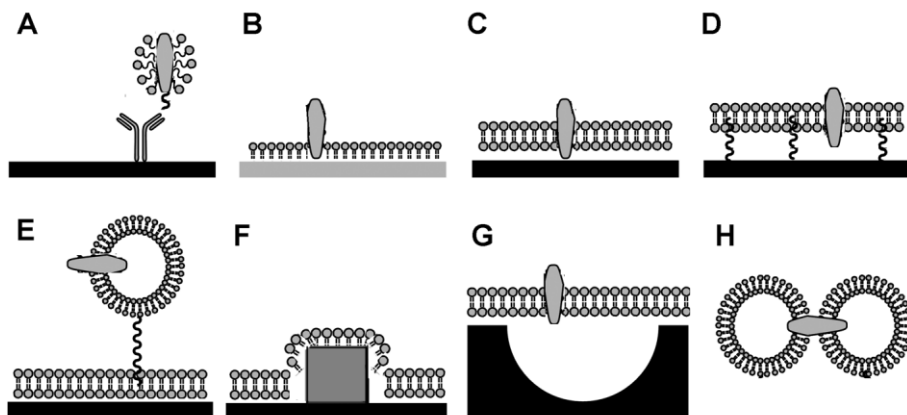


Figure 3. Membrane protein immobilization for *in vitro* analysis. (A) Capture of detergent-solubilized membrane proteins by a C-terminal peptide tag and an immobilized antibody; or formation of lipid layers, including (B) lipid monolayers self-assembled on hydrophobic surfaces, including the BIAcore HPA chip; (C) lipid bilayers formed on hydrophilic surfaces; (D) tethered or polymer-cushioned lipid bilayers reduce frictional drag of membrane proteins along the solid surface; (E) capture of vesicles by single-stranded DNA tethers, anti-LPS antibodies or the L1 BIAcore chip; (F) LSPR signal from nanocrystals; (G) suspended lipid bilayers over nanopores to allow access to both sides of the lipid bilayer; and (H) droplet interface bilayers [42].

3.4 Suspended lipid bilayers

An even more physiological environment for analysis would be a free-standing or suspended lipid bilayer, allowing the membrane to be addressable from both sides of the bilayer. Early efforts to create suspended lipid bilayers over micron-sized pores (so-called “black lipids” because of their black appearance) were limited by the poor stability of the suspension. Recent developments in nano-fabrication have allowed the metal substrate to be machined to include nanometer-sized pores (~30 nm to micrometers). Lipid bilayers deposited by vesicle fusion, Langmuir-Blodgett or detergent dialysis techniques (similar to the methods described for SLBs above, see [38] for review) span these pores, which can be characterized by atomic force microscopy (AFM) indentation force as a measure of elasticity [39, 40] or electrochemical impedance spectroscopy. These experiments have revealed that the bilayers respond to stress by local bending rather than lateral tension. Danelon *et al.* [41] were able to spread native membranes across silicon nitride films containing apertures of 50–600 nm in diameter and total surface areas of coverage of 100 μm^2 . Remarkably, not only did this approach allow access to both sides of the membrane, but it preserved the native orientation of the membrane proteins.

4 SPR instrumentation

For a variety of applications, including membrane protein ligand screening, biomarker discovery and cellular signaling, it is critical to measure and quantify binding rates and affinities, and not only

the mere presence of binding events easily obtainable with basic fluorescence imaging. SPR techniques enable such real-time, label-free quantification of molecular binding kinetics and affinities [43–47] and are currently the gold standard for quantifying the binding kinetics of molecules. In these techniques, capture molecules immobilized on a thin gold film are immersed in a liquid solution containing analytes, and surface plasmon waves probe the molecular activity on the surface (see Fig. 1).

A surface plasmon (SP) wave is a rippling motion of the conduction electrons of a metal (typically gold), right at the interface between the metal and a sample solution. As an SP wave propagates along a gold-liquid interface, its wavelength changes when it encounters a thin layer of biomolecules bound to the gold film. By monitoring the changing behavior of the SP waves in real time, affinity and binding kinetics between capture molecules immobilized on the gold surface and target molecules in the liquid can be obtained. Since SPs are coupled to free electrons, for a given energy they have a larger momentum than free-space electromagnetic waves, necessitating various geometries to increase the momentum of the exciting light, such as the use of an optical prism or grating. In BIAcore™, a convergent light cone illuminates the detection spot (~1.6-mm spot size) on a gold film *via* prism coupling in total internal reflection mode. The angular distribution of the reflected light is measured by a photodiode array in real time, scanning for a steep drop in intensity that indicates the resonant excitation of SPs. As molecules bind to the surface of the gold, the resonance angle changes. This gives a local refractive index sensi-

tivity of $\Delta n/n \sim 10^{-6}$. In various formats, this technique has found wide application in pharmaceutical development (small molecules and proteins) and basic research, and has also been successfully commercialized [48].

In contrast to radioactive or fluorescence labeling methods, label-free SPR kinetic assays provide several unique advantages: (i) ligand-analyte binding kinetics can be probed without the costly and time-consuming labeling process that can also interfere with the binding interactions; (ii) binding kinetics and affinities can be measured directly, as opposed to only the mere presence of binding events; and (iii) a wide range of molecular interactions – especially low-affinity interactions that require a large amount of antibodies for saturation – can be characterized with less reagent consumption than other equilibrium measurement techniques.

5 SPR technology for membrane proteins: State of the art and challenges

While the SPR technique has been successfully commercialized by several companies, most notably BIAcore™ (GE Healthcare), its main function has been measuring the average affinity between known pairs of purified proteins immobilized over a large area ($\sim 1 \text{ mm}^2$) of the gold surface. For many membrane protein applications, a new class of SPR technology is needed that is capable of directly measuring antibody-antigen interactions occurring on a cell membrane at a much higher spatial resolution than BIAcore™ can offer, and with a consequent increase in multiplexing capabilities. Furthermore, a solid gold film as the sensing surface does not provide a natural environment to study cell-surface antigens that are positioned within a lipid bilayer, as the procedures of isolating and immobilizing membrane proteins often adversely affects their function.

For high-throughput, functional studies of transmembrane protein binding kinetics using real-time label-free SPR techniques, the following key challenges must be addressed: throughput, imaging resolution, and maintenance of biological function of transmembrane proteins on a gold film. In its current implementation, BIAcore™ is a low-throughput instrument that can measure binding kinetics from only four channels with an associated cost of \$300K. For membrane protein microarray applications, it is necessary to simultaneously measure kinetics from thousands of sample spots. SPR microscopy (sometimes called SPR imaging) based on a similar setup is one such technique. An-

other type of high-throughput SPR instrument utilizes a diffraction grating instead of a prism, to convert incident light into SP waves. The FlexCHIP system uses this mechanism to measure binding kinetics from 400 sample spots, but at a reduced sensitivity compared to BIAcore™. Both approaches, however, suffer from low imaging resolution and limited field-of-view because the image plane is tilted at a sizable angle to the sample surface, creating significant optical aberrations and prohibiting the use of high-resolution imaging optics. Finally, while BIAcore™ is good at measuring kinetics between capture ligands immobilized on the gold surface and target molecules in solution, it cannot easily be applied to functional studies of membrane proteins because the gold surface of the sensor chip may perturb the biological activity of these proteins, as illustrated in Fig. 2.

5.1 Conventional prism-based SPR platforms

Two specialized chips, the HPA and L1, have been developed to facilitate membrane protein analysis on BIAcore™ systems. The hydrophobic association analysis, or HPA chip includes a monolayer of long-chain alkanethiol groups covalently attached to the gold surface. When injected over the surface, small unilamellar vesicles containing membrane proteins rupture and fuse to form a supported lipid monolayer on the surface of the chip. This chip has been used, for instance, to analyze recombinant antibodies binding LPS molecules and to demonstrate bacterial species selectivity [49]. In contrast, the L1 chip presents a surface coated with carboxymethyl dextran with terminal alkane groups to capture liposomes containing integral membrane proteins in a lipid bilayer. The exact form of the captured lipid membranes is not precisely known but appears to be dominated by captured liposomes rather than a lipid bilayer [50]. This chip was developed specifically to allow identification of orphan GPCR ligands by coupled SPR-mass spectrometry (MS) analysis, in which a library of potential ligands is injected, molecules binding non-specifically are washed away, and binding ligands are eluted and recovered for identification by MS. A direct comparison of the two chips analyzed coagulation factor VIII binding to synthetic membranes containing phosphatidyl choline and varying amounts of phosphatidyl serine (4–25%). In this study, the L1 chip provided superior sensitivity, most likely due to the presence of more binding sites due to the capture of vesicles *versus* planar bilayers. Apart from the different immobilization techniques, the chips are used in a similar manner

as the standard CM5 chip, with cycles of binding and regeneration.

Early reports demonstrated that functional light-mediated activation of rhodopsin and the subsequent dissociation of G proteins could be monitored by plasmon-waveguide resonance systems in tethered lipid bilayers [51, 52] and used to measure GPCR-G protein affinities in the presence of agonists and antagonists [53]. Standard CM5 chips have been used to study binding of detergent-solubilized neurotensin receptor-1 GPCR to immobilized peptide ligand [54], while the L1 chip has been used to capture GPCR-containing micelles in a proof-of-concept experiment with transducin [55]. Myszka's group has used two complementary approaches to study GPCRs on BIAcore chips. In the first, the CXCR4 GPCR (a co-receptor for the gp120 protein during HIV invasion of T cells) was expressed with a C-terminal peptide tag and purified in the presence of detergent. This solubilized CXCR4 was then immobilized *via* an antibody specific for the peptide tag. Second, to more closely mimic the receptor's native environment, CXCR4 was immobilized from crude supernatant *via* the 1D4 tag-specific antibody on a chip with alkanes, followed by reconstitution of the lipid bilayer around the receptor [56].

6 Next generation SPR instrumentation based on nanostructured materials

Despite the success of BIAcore™ for ligand screening and pharmaceutical research, it is clear from the previous sections that there is a critical need for a new generation of SPR technology capable of high-throughput microarray sensing, detecting small ligands with higher sensitivity, and integrating transmembrane proteins. Emerging SPR technologies based on patterned nanostructures, such as noble metal nanoparticles and nanostructured metal films, provide new design freedoms, enhanced detection sensitivity, and the unique geometry to address some of these challenges. These nanostructured SPR sensors can be divided into two categories: (i) nanoparticle-based sensors utilizing localized surface plasmon resonance (LSPR) [57] and (ii) the inverse structure utilizing nanopore arrays in a thin metal film [58]. While both systems harness collective oscillation of conduction electrons, the LSPR in nanoparticles and the propagating SPR wave in a metal film perforated with nanopores exhibit very different characters.

A particularly desirable feature of these patterned metal nanostructures is their ability to di-

rectly convert incident light into SPR, obviating the need for a bulky coupling prism used in the BIAcore™ system. On the curved surface of a metal nanoparticle, light can directly couple into an LSPR that has the symmetry of a time-varying dipole. For more detail, see "Online Supporting Material: Nanoparticle-based LSPR biosensors". Similarly, a subwavelength hole patterned in a metal film can also efficiently couple incident light into SP waves. The elimination of a prism can considerably simplify the optical design, assembly and alignment of an SPR imaging system that is required for high-throughput imaging.

Furthermore, compared with an unpatterned gold or silver film, metal nanoparticles or nanopores can resonantly amplify the intensity of incident light by up to 10 000 times. Such strong field enhancement was shown to significantly increase the Raman scattering cross-section of surface-adsorbed molecules by as much as 10^{12} , thereby facilitating the identification of the bound molecules *via* surface-enhanced Raman scattering (SERS) [59, 60]. While label-free SPR technology can measure the affinity of binding partners, it does not reveal a chemical signature for the bound molecules. By coupling SPR and SERS measurements in nanostructured metals, it will be possible to identify "hits" in a high-throughput SPR binding screen and then capture the vibrational signature of the bound target molecule using SERS, which will be an important step forward for biomarker discovery.

6.1 Nanopore SPR sensors

While the LSPR of metal nanoparticles give them strong plasmon resonance effects, the inverse structure, *i.e.*, nanopores in a metal film, also exhibits unique SPR characteristics because of the extraordinary optical transmission (EOT) effect [61]. An obvious distinction between a nanopore array patterned in a continuous metal film and an array of disconnected nanoparticles is that the former can support propagating SPR (used in BIAcore™), whereas the latter can only sustain the short-range LSPR.

A single subwavelength nanopore milled through a thin gold film will transmit very little incident light. It can, however, convert the incident light into an SP wave, acting as a local source for SP waves, like a stone tossed into a pond will generate surface waves from a single point. When many of these nanopores, or SP sources, are arranged in a periodic array (Fig. 4), at certain resonance wavelengths (Fig. 4b), the SP waves constructively interfere and intensify, efficiently "funneling" their en-

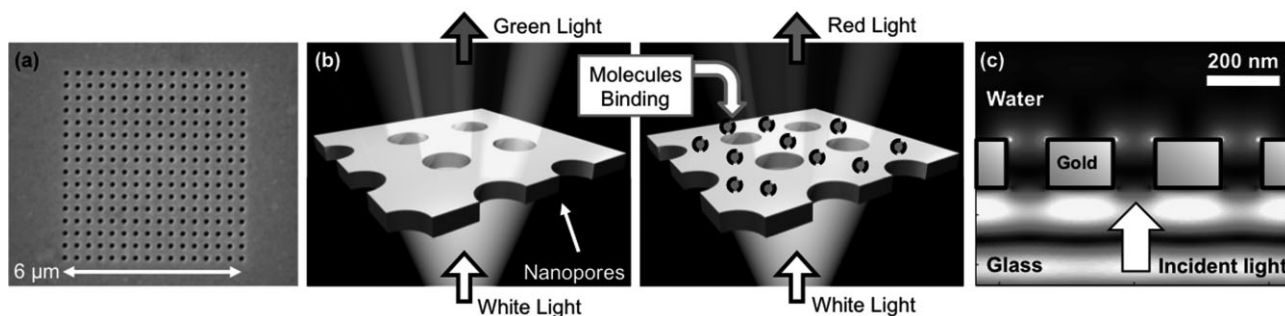


Figure 4. (a) A periodic array of nanopores milled through a thin gold film. (b) At resonant wavelengths, the incident light is efficiently transmitted, giving a sharp transmission peak that is easily monitored. As molecules bind, the peak shifts, modulating the transmission. (c) Side view: Computer simulation of light transmission (“funneling”) through a nanopore array. Intense optical energy is observed, confined within ~100 nm from the gold surface. Molecular binding on or near the gold surface sharply modulates this field distribution, and the optical transmission process, providing the basis for measuring binding events.

ergy through the tiny nanopores. Molecules on the gold surface sharply modulate the resonance wavelength (Fig. 4b) and this funneling process. On the other side of the gold film, these funneled SP waves are then re-converted into light, which freely propagates away. Overall, the optical transmission is far more efficient than one would expect considering only the openings created by the tiny nanopores. By continuously measuring the transmitted light, molecular binding events, binding rates and affinities can be monitored as a function of time. Thus, each nanopore array behaves as a single SPR biosensor.

Several groups have demonstrated the potential of nanopore arrays for label-free SPR biosensing. Following the discovery of the EOT effect, Brolo *et al.* [62] first demonstrated a proof-of-concept using periodic nanopore arrays in a gold film for biochemical sensing. There, using a broadband light source and a spectrometer, they reported a 4-nm shift of the EOT transmission peaks after the immobilization of a molecular monolayer on the gold surface. Using a tunable IR laser source (1520–1570 nm), Tetz *et al.* [63] demonstrated refractive index sensing and estimated the sensitivity of periodic nanopore arrays to be close to 10^{-6} , comparable to BIAcore™. Larson and coworkers

[64–67] demonstrated the potential of the nanopore platform for highly multiplexed analysis of ligand interactions. Furthermore, recent advances in the fabrication of large-area nanopore arrays in a metal film [68, 69] show promise to push this technology further toward next generation SPR biosensing that is more sensitive, miniaturized, has the ability to multiplex and uses very small amounts of sample. In our group, we recently reported using shape-enhanced periodic nanopore arrays in a microfluidic flow cell for real-time measurements of molecular binding with a 50% improvement in sensitivity [70]. The shape-enhancement came from producing sharp apexes by overlapping two circular nanopores. For multiplex, microarray applications, Lesuffleur *et al.* [71, 72] used periodic nanopore arrays with laser illumination and an imaging camera, which was also incorporated with multiple microfluidic channels as shown in Fig. 5.

Later, Lindquist, *et al.* [73] demonstrated sub-micrometer-resolution nanopore-based SPR imaging with enhanced sensitivity and sensor-to-sensor isolation (Fig. S1). Recent work by Ferreira *et al.* [74] has even shown that each nanopore on a glass substrate can detect attomolar concentrations of proteins using in-hole SPR effects. Detailed reviews of the physics and applications of nanopore

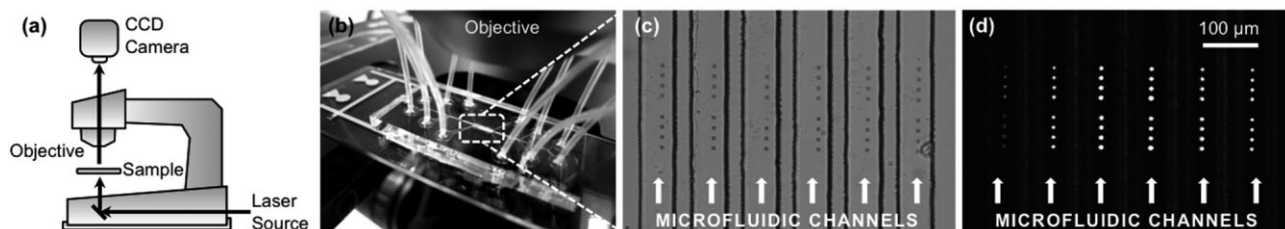


Figure 5. Real-time SPR sensing platform based on periodic nanopore arrays. (a) With a standard microscope, CCD camera, and laser, a (b) microfluidic chip with (c) multiple parallel channels is (d) illuminated from below and imaged. Each bright spot is a single nanopore array, whose brightness changes with molecular binding events [72].

arrays in metal films can be found in a review article by Gordon *et al.* [75]. Using a random array of nanopores, it is possible to perform LSPR detection, since there is no longer any long-range order to support travelling SP and pore-to-pore interference effects, as in EOT. Dahlin *et al.* [76] utilized this technique for membrane sensing. However, the ability to harness propagating SPs in a continuous gold film can increase the possible probing range (beyond the 10–30 nm mentioned previously) as well as the tunability of the structure. The periodic nanopore structure, therefore, is uniquely suited for SPR biosensing, achieving high sensitivities, seamless integration with inexpensive optics in a transmission-mode setup, and high-resolution, highly multiplexed detection. The nanopore array is also distinctive in its geometry, which may be suitable for investigating transmembrane proteins.

6.2 Novel SPR sensing scheme based on suspended lipid membrane over nanopores

Toward membrane protein sensing applications, we note that nanopore arrays provide a unique geometry, since a thin lipid bilayer can be suspended over the nanopores while maintaining mechanical stability and being surrounded by a buffer on both sides (Fig. 6). Membrane proteins can thereby be seamlessly integrated with the SPR sensing capability of periodic nanopore arrays, maintaining their functionality in an environment that more closely mimics their natural state. Furthermore, membrane proteins integrated in the free-standing lipid bilayer can be easily accessed from both sides, making this approach more attractive for studying membrane protein interactions than planar lipid bilayers supported on a flat substrate. Each lipid monolayer will join at the nanopore area to form bilayers spanning the nanopore. While the formation of pore-spanning lipid bilayers was previously studied by several groups using AFM or impedance measurements [38, 39, 77, 78], the unique abil-

ity of metallic nanopore arrays that can concurrently act as a mechanical support for lipid membrane as well as an ultra-sensitive SPR biosensor has not been realized.

Most existing work on making periodic nanopore arrays relied on milling metallic holes through a metal film deposited on a glass substrate. This process results in dead-ended nanopores, suitable for substrate-supported lipid membranes (Fig. 6a). A few groups have demonstrated processing schemes for making free-standing nanopores, suitable for flow-through SPR sensing [79] or for lipid membrane sensing [80] (Fig. 7). The process typically begins with backside etching of a silicon wafer covered with a thin nitride film. A thin gold film is then deposited on the free-standing nitride membrane, through which the nanopores are then milled. Microfluidic channels can then access both the top and the bottom openings of the nanopores, allowing both sides of a suspended lipid membrane to be in contact with a buffer solution (or with two different buffers, as is the case for the inside and outside of a cell).

We believe the scheme proposed in Figs. 6 and 7 provides a new platform for studying transmembrane proteins such as GPCRs and ion channels. Small nanopores perforated through a thin gold layer are ideally suited to provide mechanical support, since smaller diameter free-standing membranes are more stable than larger diameter membranes, and for detection of molecular binding events, since the gold film sustains SPR effects. Importantly, such a set-up would allow transmembrane proteins to be presented in native or near-native environments and allow interrogation from both sides of the membrane. This will be particularly important for fundamental studies of signal transduction, in which ligand binding on the extracellular side of the membrane triggers association and dissociation of multiple membrane proteins on the internal or cytoplasmic side of the membrane, such as occurs during GPCR activation.

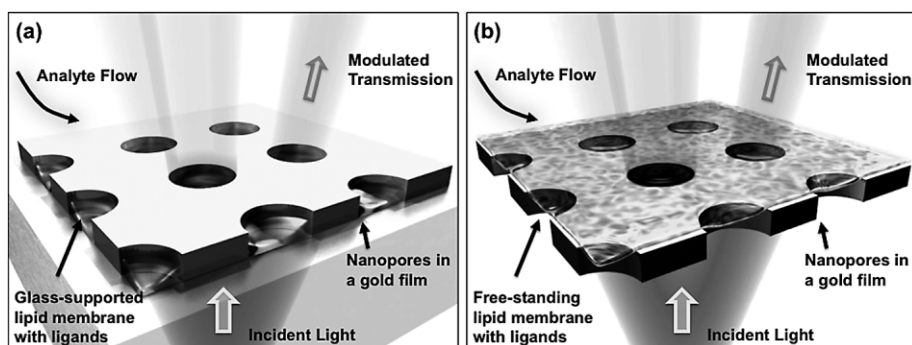


Figure 6. Proposed nanopore sensing schemes. (a) A cell membrane is reconstituted on a glass substrate and is surrounded by a thin gold film sidewall inside each nanopore. Ligands binding to transmembrane proteins can drastically modulate light transmission through the nanopores, enabling label-free SPR measurements. (b) A cell membrane that is free-standing and surrounded with a buffer solution on both sides.

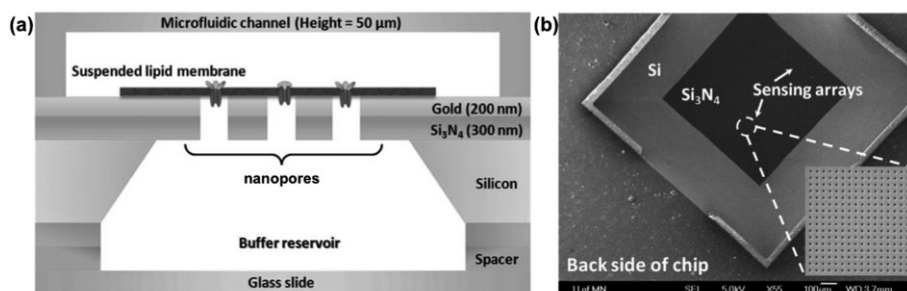


Figure 7. Schematic of the nanopore platform made on a suspended nitride membrane. (A) The ability to fill both sides of the membrane with a buffer and access them makes this geometry a unique platform to study transmembrane proteins. (B) SEM image of a free-standing silicon nitride membrane on a silicon wafer (flipped for imaging). Adapted from [80].

7 Applications of SPR to membrane proteins

7.1 GPCR ligand screening

GPCRs are the largest family of membrane proteins in human genome and while sequence homology across the family is low, all exhibit a seven-transmembrane α -helical topology. The majority of hormones and neurotransmitters communicate extracellular information to cells via GPCRs, and drugs acting on GPCRs can impact a broad spectrum of diseases. While endogenous ligands have been proposed for several hundred GPCRs, there remain over 100 “orphan” GPCRs for which ligands have yet to be identified and likely represent opportunities for new drug development. Moreover, even for those GPCRs for which suitable pharmacologically active drugs have been identified, modified ligands with greater binding specificity, affinity and selectivity for a given GPCR could represent an improved drug. Importantly, it has been observed that the ligand equilibrium dissociation constant (K_d) scales with biological responses, such that a partial agonist is less avid than a full agonist. These dissociation constants range from 0.2 to 3000 nM for the small molecule and peptide ligands (0.5–8 kDa) [81], falling precisely within the normal detection window of an SPR instrument. Sensitive, high-throughput activity screens are currently used to identify novel and more potent molecules from large chemical libraries, although with the recent advances in recombinant GPCR expression and structural characterization [82], structure-based drug design is becoming an increasingly attractive approach.

Currently available assays to assess GPCR ligand binding affinity and specificity fall into two main categories: those that use radio-labeled ligands to measure binding to cells overexpressing a specific GPCR [81], and those that use complex cell-based assays to indirectly measure downstream events of the signal transduction cascade (e.g., intracellular cAMP or intracellular Ca^{2+} con-

centrations, see Fig. 8 [83]). A major limitation of the simple cellular receptor binding assay is the uncertainty in the GPCR concentration [84] and that overexpressed GPCRs may exhibit constitutive activation. Molecular assays, such as those employing SPR imaging, that directly report ligand binding kinetics and G-protein activation could bridge the gap between simple binding assays and the complexity of cell-based systems. An ideal GPCR screen should be simple, non-radioactive, with a high signal to noise ratio, contain minimal reagent additions and be amenable to automation [85].

SPR imaging has the potential to address the limitations of current GPCR ligand screening methods, although the sensitivity and throughput remain inadequate for screening of large chemical libraries. Three distinct SPR approaches include: (i) Immobilization of GPCR in lipid bilayers, with ligand-binding dose-response curves monitored by G-protein α unit dissociation and the consequent decrease in SPR signal [52] (for review, see [86]). Because the $\text{G}\alpha$ subunit is relatively large

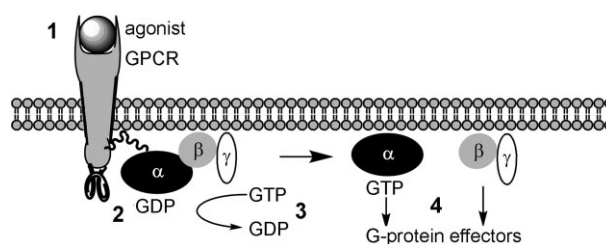


Figure 8. GPCR-G protein coupled activation. In step (1), the agonist-GPCR interaction promotes a series of conformational changes favoring GPCR interactions with G proteins. In step (2), formation of an agonist-GPCR-G protein trimolecular complex induces G protein conformational changes resulting in (3) the exchange of the α subunit bound GDP for GTP. Step (4), the activated G protein dissociates to form a GTP-bound α subunit and a $\beta\gamma$ complex. The dissociated G proteins then regulate the activity of a number of intracellular effector proteins, resulting in changes in cAMP or calcium levels and regulation of signal transduction pathways. These activities stop when the GTP is hydrolyzed to GDP and the $\alpha\beta\gamma$ G protein complex reforms. (Adapted from [83].)

(~45 kDa), the SPR signal is more sensitive than that resulting from direct monitoring of small molecular weight ligand binding (~0.5–8 kDa); (ii) antibody capture or immobilization of detergent-solubilized GPCR, followed by SPR imaging of ligand binding [56, 87, 88]; and (iii) immobilization of biotinylated ligand, followed by capture of the high molecular weight, detergent-solubilized GPCR [54]. The most appealing approach for development of high-throughput screens is immobilization of the GPCR in a suspended lipid bilayer across a nanopore. In this way, ligand can be added to one side of the bilayer with the G protein attached to the other side. GPCR activation could then be sensitively and directly monitored by α subunit dissociation, without the complications associated with monitoring downstream functional effects, such as changes in cAMP levels.

7.2 Biomarker discovery with membrane-bound antigens

Both cancer and autoimmune diseases induce autoantibodies – cancer due to expression of protein variants or dysregulation of key proteins that can be recognized by the humoral immune system, aberrant recognition of self-antigens in autoimmune disease. When considered alone as diagnostic tools, most autoantibodies show poor sensitivity and/or specificity for their associated diseases. While it is usually difficult to identify a single biomarker for which the presence of specific antibodies is diagnostic of disease presence and severity, there is evidence that autoantibody binding patterns can indicate disease pathology and severity years before the onset of clinical symptoms [89]. For instance, there is some evidence that the occurrence of autoantigens to specific antigens in lung cancer may have prognostic relevance, and tumor regression has been demonstrated in some patients with small cell lung carcinoma and autoantibodies to onconeural antigens [90]. Similarly, antibodies binding a set of 18 signaling proteins has been identified that can distinguish Alzheimer's from control patients with 90% accuracy [91].

Sensitive, high-throughput, equilibrium-based technologies have been developed for analysis of antibodies binding soluble proteins. These include antigen arrays to detect serum autoantibody responses to soluble antigens immobilized on a microarray [92]. These arrays simultaneously detect femtomolar concentrations of antibodies recognizing up to 230 antigens while utilizing very small volumes of patient samples [93]. Other emerging technologies include multiplexed assays using fluorescent microspheres, a technology developed by

Luminex and which has been licensed by three companies for lupus characterization [94]. Autoantibody binding patterns to soluble antigens have been primarily characterized because these are readily accessible with the current technology. However, autoantibodies also recognize intact cells and thus membrane proteins. For instance, antigen microarrays have identified unique patterns of antibodies binding lipids in patient samples which predict disease pathology in Alzheimers and multiple sclerosis [95]. Analysis of autoantibody binding to membrane protein antigens *via* a high-throughput nanopore SPR array would further enhance the power of this approach by monitoring not just patterns of binding but also the kinetics, as clinical relevance may correspond to high- or low-affinity autoantibodies.

7.3 Autoantibody-based therapeutics binding membrane proteins

Not only do the binding patterns of autoantibodies have diagnostic potential, some of these autoantibodies are mechanistically involved in repair of disease and may ameliorate disease when administered therapeutically. IgM antibodies binding asialo-G_{M1} glycolipids have been successfully characterized using SPR and G_{M1}-containing liposomes [96]. Approved antibody-based therapeutics are used to treat cancer, inflammatory diseases, transplantation recipients, infections and cardiovascular disease, and have a high rate of approval as drugs compared to small molecule based drugs [97].

By isolating mAbs from humans with monoclonal gammopathy, a condition in which the individual carries the mAb in high concentration for long periods of time, and focusing only on those individuals free of antibody-based disease, candidate mAbs can be isolated that have already been tested for long-term, high-dose, toxicity in at least one human [98, 99]. Human mAbs from serum were selected based on cell surface binding and then assayed for efficacy in models of disease [100–102]. Mouse and human mAbs have been isolated that promote CNS protection and repair, bind specifically to surface plasma membrane antigens, activate intracellular signals that promote neuron or glial cell survival [103], and cross the blood-brain barrier to accumulate within injured regions of the CNS [101]. This process results in candidate mAbs with proven *in vivo* efficacy and a degree of toxicology data, but without an identified antigen or mechanism of action. To transition to clinical trials, data regarding the antigen and mechanism of protection would greatly increase the probability of a molecule's regulatory approval.

Some of the target antigens bound by reparative mouse mAbs are known, and all antigens are lipids or carbohydrates [104]. Our data suggest that the reparative IgMs, which have a total of ten antigen binding sites, do not bind to a single membrane molecule, but to a membrane micro-domain complex composed of multiple antigens. If this native membrane complex is disrupted, IgM binding to the target cell is lost. Cell and tissue specificity of the reparative IgMs is maintained only when bound to intact plasma membranes. When candidate antigens are presented in isolated form, such as an ELISA, the IgMs often bind nonspecifically. Therefore, a new antigen screening technology is required to study these difficult, but critical, lipid and carbohydrate molecules of the plasma membrane antigens in their native state to preserve appropriate antibody binding kinetics. It has not been possible to use a commercial BIAcore™ system to model the complex interactions of these natural autoantibodies with cell surface antigens because studying individual proteins, which the BIAcore™ does well, does not allow the study of a multiple antigen complex. The combination of SPR with suspended lipid bilayers spanning nanopores has the potential to identify the individual components of the membrane complex recognized by the IgMs. One example of a therapeutic antibody is an IgM autoantigen whose binding to white matter in the CNS promotes remyelination in *in vitro* and *in vivo* models of multiple sclerosis (see Fig. 9). SPR analysis would facilitate the study of reconstituted myelin membranes isolated from glycolipid knock-out mice and allow the introduction of candidate antigens back into these membranes for IgM binding studies.

7.4 Basic membrane biology

The union of SPR and SLB technologies will illuminate many fundamental issues in biology, from the basic physics of lipid membranes to membrane biogenesis and the molecular details of cellular interactions [106]. For instance, the fine details of HIV fusion with cell membranes [107], bacterial outer membrane protein transport, assembly and insertion into the outer membrane [106], membrane protein diffusion [108, 109] and formation of lipid raft structures [110] are all important questions that are beginning to be quantitatively addressed with SPR monitoring of SLBs.

7.4.1 TCR-pMHC interactions

Antibodies are currently one of the most rapidly growing classes of therapeutic molecules [111, 112], able to treat solid and circulating tumors and limit inflammation associated with autoimmune reactions by virtue of specific, high-affinity ligand recognition [113]. In contrast, the exclusively membrane-bound cellular immune system, which plays a central role in defense against cancer and viral infections and the pathology of autoimmune diseases such as diabetes, is much less well understood [114–116]. T cell discrimination between self *versus* non-self occurs based on the tri-partite binding kinetics between a TCR and peptide antigens presented by MHC proteins on a cell. Proteins produced intracellularly or ingested from the external milieu are proteolyzed into short peptides approximately nine amino acids residues long and complexed with MHC proteins. After trafficking of the pMHC complex to the cell membrane, the composite surface is surveyed by $\alpha\beta$ TCRs (for review,

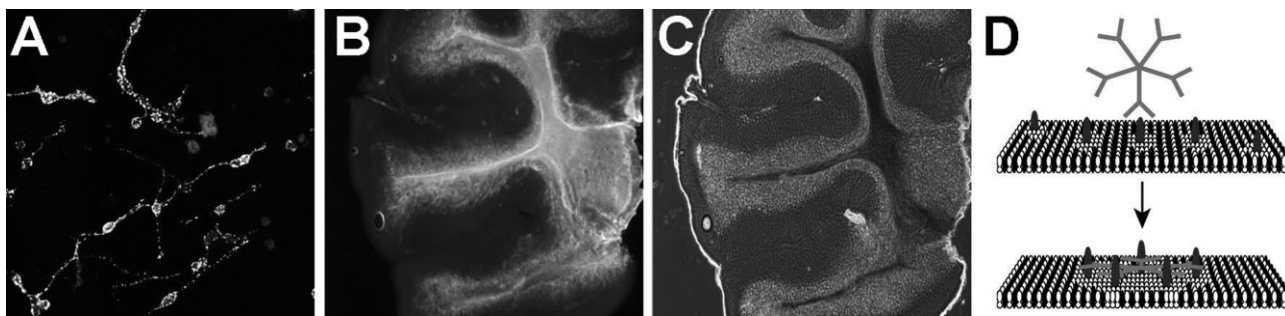


Figure 9. Application of antigen identification in reconstituted membrane binding screening assays. (A) A human IgM that binds to the surface of neurons (green label) promotes neurite extension from rat cerebellar granule cells when presented as a substrate. This IgM was identified by screening for biological properties; whereas the antigens recognized are unknown, preliminary evidence suggests the antigens are lipids [105]. (B, C) A recombinant human IgM that promotes repair in models of demyelination such as multiple sclerosis binds specifically to the central white matter in an unfixed slice of mouse cerebellum; immunocytochemistry (B), whole tissue visualized by phase contrast (C). This IgM binding specificity is maintained only on live tissue, and is lost using frozen or fixed specimens, suggesting intact cell membrane is critical for IgM binding. (D) Proposed model of reparative IgM binding, which could be tested by SPR on supported membranes. The pentameric IgM binds to the surface of a target cell; antibody multivalency initiates clustering of plasma membrane molecules and activation of signaling which can lead to cellular responses such as proliferation, differentiation, or increased resistance to apoptosis.

see [117]). The outcome of a productive TCR-pMHC binding interaction depends on the subclass of T cell involved, but can include induction or repression of immune responses or lysis of the pMHC-bearing cell, with direct relevance to vaccine design and anti-cancer therapeutics.

Key issues in TCR-pMHC recognition include: (i) identification of immunodominant peptides bound by both the MHC and TCR; (ii) identification of TCRs binding known pMHC complexes; and (iii) characterization of the TCR-pMHC binding kinetics resulting in T cell activation or deactivation. Currently there is a paucity of methods for characterizing TCR molecules and peptides associated with disease; in general these approaches study the interaction indirectly using whole cells or quantitatively using artificial soluble pMHC variants. Recombinant systems for production of soluble variants of TCRs and pMHCs have been developed [17, 18, 118, 119], but still require significant effort, including identification of solubilizing mutations for each unique receptor studied. The resulting soluble pMHC tetramers have been immobilized on arrays and used to capture T cells to identify activating peptides and characterize T cell responses to a peptide vaccine against melanoma [23–25, 120, 121]. SLB technology was first developed to study the TCR-pMHC binding interaction under more physiological conditions [27]. pMHC molecules were purified from antigen-presenting cells and immobilized on a solid support, and T cells allowed to bind, which was monitored by fluorescence. This technology has been extended in conjunction with modern photolithographic techniques and multi-parameter fluorescent protein labeling to visualize the coordinated movement of TCR-pMHC and costimulatory molecules during formation of the “immune synapse,” a pre-requisite for T cell activation [122–125]. The ability to monitor TCR-pMHC interactions both quantitatively and in the context of a native lipid membrane would represent a major advance.

8 Conclusions

For two driving industrial biological needs, ligand screening and biomarker discovery with membrane proteins, as well as fundamental research in membrane biology, currently available quantitative screening technologies such as BIAcore™ have limitations. The systems have been retrofitted to accommodate the unique needs of membrane proteins, but still suffer, for instance, from the poorly defined form of the lipid bilayer coupled to L1 BIAcore chips™. The problem is compounded for

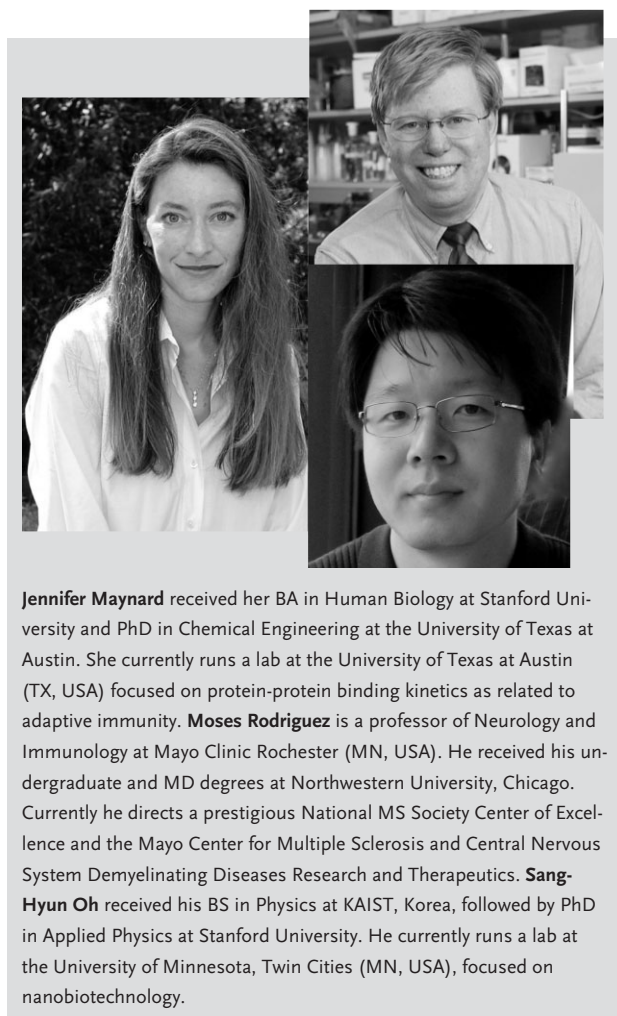
transmembrane proteins such as GPCR because proteins in direct contact with a solid substrate (in particular the gold substrate in BIAcore™) often lose their functionality or denature. The nanopore-based dynamic sensing architecture in development by several groups has the unique potential to overcome this challenge. Each nanopore sits on a glass substrate and forms a tiny well in which to confine supported lipid membranes, while the surrounding gold film provides SPR effects to dynamically measure the binding kinetics of molecules onto the membrane. Furthermore, this nanopore geometry offers the intriguing possibility of suspending lipid bilayers over metallic nanopore arrays to mimic the structure of natural biological membranes as proposed here. This new platform is beginning to be used, and we anticipate a number of breakthroughs in biological research.

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