Membrane Proteins

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Quantification of Membrane Protein Inhibition by Optical Ion Flux in a Droplet Interface Bilayer Array**

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Despite the importance of membrane proteins as drug targets the discovery of new compounds is hampered by a lack of high-throughput methods for their study. Membrane proteins remain such challenging targets primarily because of the difficulty of measuring a functional response from a folded protein in a membrane environment.

Ion channels are a major class of membrane protein targets important in a wide range of diseases, including those of the central nervous[1] and cardiovascular systems.[2] High-throughput measurements of ion channel function are also important to fulfil the regulatory requirements for cardiotoxicity screening.[3] Whilst high-throughput methods exist for the measurement of ionic flux,[4] scaling such measurements is challenging,[4] as parallelization of electrical measurements requires increasingly complex electronics. Optical methods using fluorescent detection in response to ion flux provide an alternative means for measuring electrical activity.[5] Since data is collected simply by imaging, these methods are arguably more scalable than techniques based on conventional electrophysiology.

One route to parallelization of ion flux measurements is the generation of arrays of synthetic bilayers.[6] The ability to precisely control the constituents of such an experimental system provides significant advantages in reproducibility, scalability and sensitivity over existing methods.

Whilst there are many previous reports of lipid bilayer[7] or liposome[8] arrays, which have been the subject of recent reviews,[6] those that report the ability to measure protein function are limited: The successful parallel measurements of pore-forming protein in individual artificial bilayers by electrical measurement have been reported.[9–12] However, scale-up of the methodology is limited. The potential for optical approaches to provide improved costs and throughput scalability has led to much recent interest. However, to date optical methods have failed to produce quantitative measurements of protein function in bilayer arrays. Fluorescence techniques have been used for imaging of bilayer arrays themselves[7a,b,d] or for the detection of the presence of membrane protein through fluorescent antibody[7h] or ligand binding.[7g,i,13] Flux measurements of fluorogenic species,[14] have been performed, but the ability to quantify changes in the protein function in the presence of interacting species has yet to be realized.

A promising approach for scalable, arrayed, measurements of membrane protein function is the parallel optical quantification of ion flux in an array of droplet interface bilayers (DIBs).[15] DIBs exploit the self-assembly of a lipid monolayer at the interface of aqueous droplets submerged in immiscible oil. Upon contacting the monolayers of the droplets the droplet interfaces spontaneously form a bilayer. DIBs afford high stability and show high resistance (GΩ) electrical seals.[16] Sequential electrical measurements have previously been done on multiple DIBs for the detection of a pore forming protein[17] and the screening of potassium channel blockers,[18] but in such configurations throughput is limited by the scalability of standard electrophysiology techniques. DIB formation between a single droplet and a planar hydrogel support allows easy optical interrogation of the bilayer.[19] In this scalable, planar, configuration, it should be possible to make parallel optical measurement of multiple lipid bilayers simultaneously.

Here, we have built a DIB array in which multiple bilayers formed between droplets and a hydrogel surface are imaged synchronously using wide-field total internal reflection fluorescence (TIRF) microscopy (Figure 1). To investigate the suitability of this method to assay membrane protein functions, we detect the insertion of the pore forming toxin, α-hemolysin (αHL) from S. aureus. αHL is perhaps the archetypical model protein pore for such ion flux measurements.[10,12,20] Importantly, we also show the ability of the array to measure protein function by quantifying the dose-dependent response of α-hemolysin to the pore-blocker, γ-cyclodextrin, reporting for the first time quantitative measurements of membrane protein function made from an optically addressable lipid bilayer array.

αHL forms heptameric membrane-spanning protein pores through which ions passively diffuse.[21] We put the calcium-sensitive fluorescent dye, Fluo-8, in the droplet (0.25 mM Fluo-8, 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 333 μM ethylenediaminetetraacetic acid (EDTA), 2 M KCl, ±300 nM αHL, pH 7), and $CaCl_2$ in the agarose (1 M $CaCl_2$, 10 mM HEPES, pH 7.0). Following αHL insertion, Ca^{2+} diffusion through the pore yields a fluorescent response proportional to the concentration of Ca^{2+} cations in the droplet.

The successful insertion of functional αHL into the bilayer was confirmed by electrical recording (Figure 2c). Figure 2

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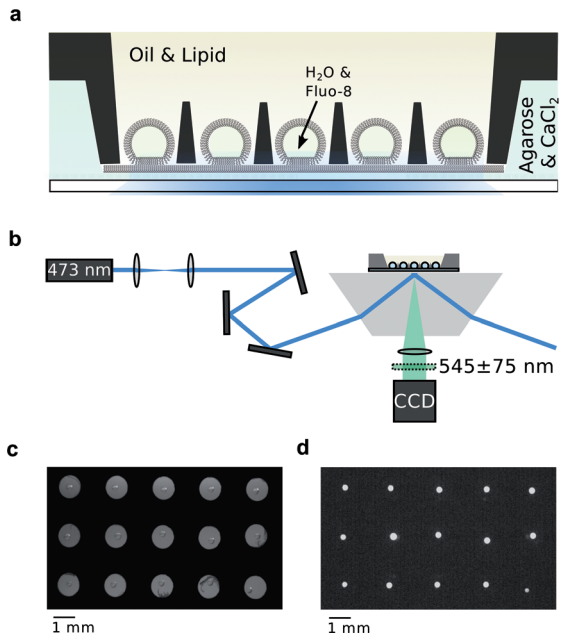

Figure 1. a) Cross-section of the droplet interface bilayer array and b) wide-field TIRF illumination of the array. c) White-light and d) fluorescence images of a 15 bilayer droplet interface bilayer array. Droplets contain a calcium-sensitive fluorescent dye (Fluo-8, 250 μM) and fluoresce following diffusion of Ca^{2+}, present in the agarose substrate ($CaCl_2$ 1 M), across the bilayer through α-hemolysin protein pores.

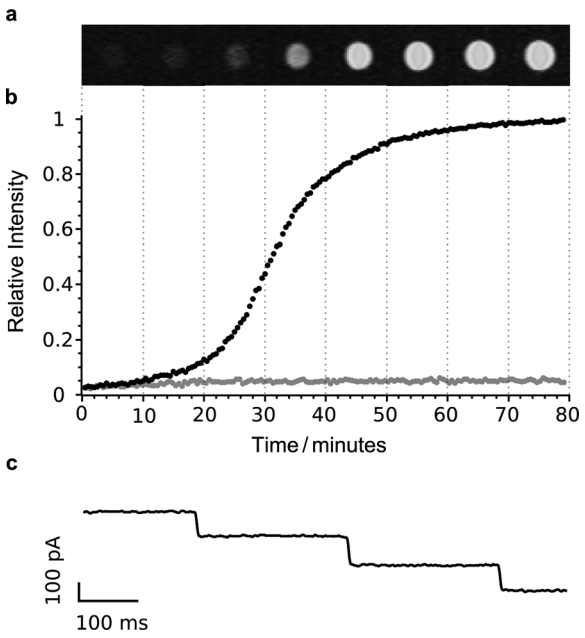

Figure 2. The time-course of fluorescence from a single droplet interface bilayer containing α-hemolysin. Ca^{2+} diffusion through the α-hemolysin elicits a fluorescence response from the calcium-sensitive dye, Fluo-8, present in the droplet. a) The image sequence shows the droplet at intervals of 10 min. b) Plot depicting the mean pixel intensity of the bilayer area normalized to the maximal fluorescence response observed upon saturation of Fluo-8 (black circles) together with the fluorescence response of an otherwise identical non-α-hemolysin containing droplet (grey circles). c) Electrical recording of a DIB from within the array. The stepwise increase in negative current under an applied potential of −100 mV confirms the functional insertion of α-hemolysin in the bilayer.

shows a typical fluorescent intensity time-course for a single droplet containing αHL from within the array. An increase in fluorescence is observed as Ca^{2+} diffuses through the αHL pores present in the bilayer. The initial lag phase is a consequence of chelation of Ca^{2+} by EDTA present in the droplet to minimize initial background fluorescence. The later plateau occurs as the Fluo-8 dye response is saturated by calcium. αHL-free droplets elicited no increase in fluorescent response over the same time period.

To show the ability of the DIB array to detect the presence of a particular protein relative to other droplets in the array, we then generated a droplet bilayer array containing Fluo-8 and either αHL, no protein, or 0.1 mM $CaCl_2$. Following bilayer formation αHL was identified by the increase in fluorescent response caused by calcium diffusion though the protein pores (Figure 3). Protein-free droplets displayed a constant low-level background fluorescence, and calcium containing positive controls showed a constant moderate fluorescence.

γ-Cyclodextrin is a cyclic oligosaccharide which can bind reversibly within the lumen of the αHL pore with micromolar affinity.[22] Such binding results in the narrowing of the pore and a partial restriction of the ion flux across the membrane.[16,22] Consequently, it has been used with αHL as a model system for ligand binding and pore block.[23] Here, we use γ-cyclodextrin to characterize the dose-response relationship for the partial block of αHL.

γ-Cyclodextrin was incorporated into the agarose at a range of concentrations (0–77 mM) with purified heptameric αHL incorporated into the bilayer from the droplet (see the

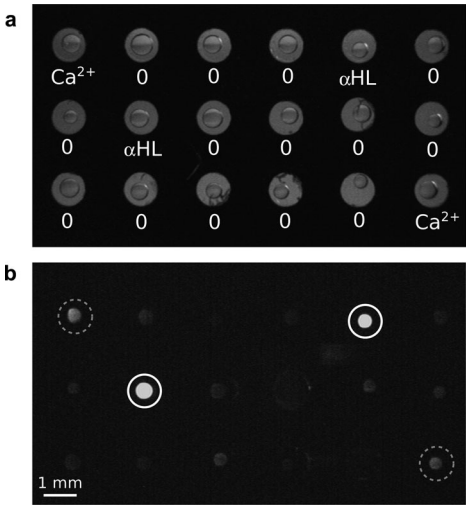

Figure 3. The white-light and fluorescent images of a DIB array identify the presence of α-hemolysin. a) White-light image of a droplet array at experiment start and b) fluorescent imaging of the DIB array ($t = 60$ min). The identification of α-hemolysin (αHL) containing droplets (solid white circles) in a mixed population bilayer array is easily made by the increase in fluorescence response upon the passive diffusion of Ca^{2+} across the bilayer through the functional protein pores. Droplets containing no protein (0) and those preincubated with 0.1 mM of $CaCl_2$ (Ca^{2+}) show no increase in fluorescence response following the bilayer formation.

Supporting Information). The rate of fluorescence increase from a droplet is proportional to the rate of Ca^{2+} flux through the αHL pores in the bilayer. Ionic flux is reduced when γ-cyclodextrin binds within the pore and hence, the rate of Ca^{2+} flux into the droplets decreases as the γ-cyclodextrin concentration is increased. (Figure 4a). The extent of the γ-cyclodextrin block was determined from the maximum rate of fluorescence intensity increase. Comparison of this rate at each γ-cyclodextrin concentration enabled the calculation of a dose-response curve for the blocking of αHL by γ-cyclodextrin (Figure 4b). A K_d of 340 μM is measured. This compares favorably to a previously reported K_d of 70 μM from the electrical measurement of ion flux under an applied potential in 1M KCl and 10 mM sodium phosphate at pH 7.[22] Cyclodextrin block of αHL is known to vary with conditions of pH,[23] applied potential,[23] and ionic strength[23] which may account for the small difference in our measurement and previously reported values.

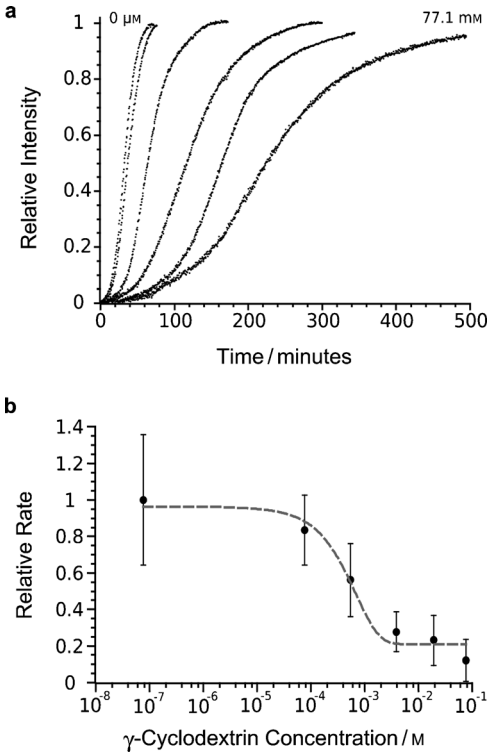

Figure 4. Ca^{2+} flux through α-hemolysin is restricted by the pore-blocker, γ-cyclodextrin. a) Fluorescence time course for single droplets at a range of γ-cyclodextrin concentrations (0, 0.0771, 0.540, 3.85, 19.3, and 77.1 mM). b) The dose-dependent response of α-hemolysin is calculated from the maximal gradient of the time-course fluorescence response for droplet bilayers ($n > 15$, error bars represent the standard deviation) at each γ-cyclodextrin concentration. The rate of fluorescence intensity increase is normalized relative to the absence of γ-cyclodextrin. A K_d of 340 μM is measured.

Droplet interface bilayers address several key challenges in using artificial bilayers for the study of incorporated membrane proteins: 1) It is straightforward to measure a response from hydrogel-supported DIBs, whilst maintaining hydration of the bilayer and the free diffusion of integrated membrane components.[19] 2) Droplets provide complete segregation of the *cis*- and *trans*-sides of each bilayer, and allow each bilayer to be individually addressed. 3) Critically, optical imaging of DIB arrays is readily scalable for parallel measurements. Whilst optical imaging of multiple lipid bilayers has previously been reported[7b,c,d,g] and the detection of membrane proteins in such arrays has been made,[7h,i,13] functional measurements have largely been restricted to electrical detection[9,10,12,20] and to a maximum of four protein-containing bilayers.[12] Here we present a proof-of-principle optical measurement of membrane protein function in an array of 18 bilayers, enabling calculation of the concentration-dependent block of αHL protein pores by γ-cyclodextrin. In contrast to previous electrical measurements, this optical response is readily scalable to larger arrays.

To make comparative measurements of ion flux on the array, it is important to control the droplet volume, as both calcium concentration and bilayer area are sensitive to variations in volume between droplets. The fluorescent response depends on the concentration of Ca^{2+} in the droplet, and this concentration obviously depends on the droplet volume. Hence, variation in volume alters the absolute magnitude of fluorescent response. Variation in the bilayer area will affect the insertion of pores, and consequently the resulting ion flux. Additionally, in these experiments, the droplet volume also defines the quantity of protein available for insertion into the bilayer. For the quantitative measurements reported here, droplets of reproducible volume (9.2 nL ± 1.2 nL (standard deviation), bilayer area 38200 μm² ± 4500 μm² (standard deviation)) were prepared by piezo nanoinjection. Droplets can also be produced over a wide range of diameters using multiphase microfluidics.[24] This may also afford additional advantages for monolayer assembly resulting from accelerated and reproducible mass transfer at the droplet interface, achieved by chaotic advection.[25] It is also likely that improved control over the conditions under which the lipid monolayer assembles at the droplet interface will further improve the reproducibility of protein insertion. Due to the limitations imposed by the conditions required to optimize the reproducible insertion of αHL in these experiments, conditions were chosen that achieved maximal fluorescence response in less than one hour. However, the rate could also be increased to achieve maximal measurement of Fluo-8 saturation in less than five minutes. The opportunity for reduction in the droplet volume provides a convenient mechanism for increasing detection sensitivity and time resolution of concentration-dependent ion flux assays, enabling access to low conductance channels and increasing array density.

Although this proof-of-principle demonstration is on the bacterial toxin, αHL, it has recently been shown that a wide variety of prokaryotic and eukaryotic membrane proteins can be incorporated into DIBs.[26] This, in combination with recently emerging strategies for reducing the challenges associated with membrane protein purification[27] will increase the future utility of the reported technique. In the measurements reported here the greatest source of error in the quantification of protein function is the uniformity of insertion of the membrane protein into the bilayer (Support-

ing Information). This gives rise to a significant standard deviation in the measured rates of ion flux into the droplets. However, reproducibility is sufficiently high to enable effective droplet-to-droplet comparisons for quantitative measurements of the inhibition of protein function by the blocking species, γ-cyclodextrin. Two-color fluorescent imaging, reporting on the concentration of labeled protein within each bilayer, would enable correction for insertion variability for further improved precision. It is likely that further development of reconstitution strategies will enable further optimization. Protein localization by agarose patterning,[28] stamping,[7g,29] or dip-pen lithography[30] could be employed to control protein concentration on the hydrogel support, or self-assembled nanoscaffolds[31] would also enable tethering of membrane protein in the vicinity of the resulting bilayer at defined concentrations. Despite these challenges, the reported work here, is, to our knowledge, the first report of optical quantification of membrane protein function in a parallel lipid bilayer array, and represents a significant advance.

The ability to quantify protein function by optical detection should be readily transferrable to other assay protocols including indicators for ions or small molecules, pH, and charge changes. Assays using fluorescent antibody binding, labeled ligand binding or displacement could also be used to study nontransporting membrane components, such as GPCRs[32] or kinases.[33] We have previously shown that DIBs can be used to make electrical measurements on single ion channels in the bilayer[26] and combined single channel recording with optical measurements.[34] Development of the DIB array to allow electrical access to selected droplets would enable rapid high-throughput optical screening followed by "gold standard" electrical measurements on promising candidate compounds. Voltage-gated ion channels could be measured by adapting a DIB array to include either contact or non-contact electrodes to apply a potential across each bilayer. A chemical potential may be used, as in this demonstration, where ionic asymmetry gives rise to a potential of −32 mV.[35] Such potentials may be controlled by the light-activated release of caged ions, enabling chemical modulation of an applied potential. Ligand-activated proteins might be accessed by perfusion of the droplet or hydrogel support, or by nanoinjection into the droplet.

In conclusion, droplet interface bilayer arrays provide a robust, scalable method for the optical screening of ion flux across a lipid bilayer, enabling quantification of membrane protein function.

Experimental Section

Details of device construction are provided in the Supporting Information. Briefly, an array of wells was machined from polyoxymethylene resin to spatially separate each droplet. DIBs were formed within each well of the device on an agarose-coated glass coverslip. A lipid monolayer was formed on the agarose support by spin-coating a lipid in decane solution onto the coverslip prior to device assembly and immersion in hexadecane. Aqueous droplets were first incubated in hexadecane and lipid solution, allowing self-assembly of a lipid monolayer around the droplet, before addition to the wells of the device. Expanded, collimated, 473 nm laser light was totally internally reflected from the glass–agarose interface through a dove prism, at a power density of 0.67 mW cm^{-2}, enabling fluorescence measurements at the bilayer itself. Images were captured using a CDD below the bilayer array.

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