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Simultaneous Measurement of Ionic Current and Fluorescence from Single Protein Pores

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We recently developed a new method for creating artificial lipid bilayers, using nanoliter water droplets immersed in a lipid/oil solution,[1−4] and here we adapt it to achieve simultaneous fluorescence and current measurements of stochastic blocking of individual staphylococcal alpha-hemolysin (αHL) pores.

The ability to simultaneously monitor both the ionic current and fluorescence from membrane channels and pores has the potential to link structural changes with function in such proteins.[5−11] Moreover, by making these measurements at the single molecule level important subpopulations and intermediates might be resolved that would otherwise be overlooked.[12] However, demonstrating the detection of correlated current and fluorescence from a single molecule remains a serious challenge.[13−19] Evidence of correlated electrical and fluorescence measurements from single channels has been demonstrated using live cells,[13,15,16] but similar experiments using artificial bilayers have been less convincing.[17−19] Artificial bilayers, such as black lipid membranes (BLMs), have one important advantage over *in vivo* approaches: the ability to control the bilayer composition and the constituents of the system. However, integrating a BLM into a configuration suitable for microscopy is difficult. Although pioneering work by Harms et al.[18] and Borisenko et al.[19] showed some evidence of simultaneous measurements from single dye-labeled gramicidin channels using pipet-patched bilayers[18] and painted BLMs,[19] in our opinion the collective evidence for simultaneous measurements using *in vitro* systems remains unconvincing. This is largely due to the great difficulty of carrying out such experiments. Crucially, a method capable of imaging the entire bilayer is needed, so that each fluorescence change can be associated with a change in the electrical current carried by an individual channel.

In our system a 50 nL aqueous droplet is pipetted onto an agarose substrate immersed in a lipid/oil solution (5 mM 1,2-diphytanoyl-*sn*-glycero-3-phosphocholine in hexadecane). Both the droplet and agarose become coated with a self-assembled lipid monolayer, and when the two are brought into contact, a bilayer spontaneously forms between them (Figure 1a). These droplet hydrogel bilayers (DHBs) form with high efficiency and are extremely stable.[3,4] We have shown previously that DHBs are suitable for both single-molecule fluorescence imaging[4] and single-channel recording (SCR).[3] By using a suitably thin hydrogel, it is possible to image the bilayer using total internal reflection fluorescence (TIRF) microscopy, and by inserting Ag/AgCl electrodes into both droplet and hydrogel we can record ionic currents using a patch-clamp amplifier. We can control the bilayer size by varying the droplet position relative to the substrate with the inserted micromanipulator-mounted electrode, making it easy to image the entire bilayer. We provide a complete description of this method as Supporting Information.

Optical patch clamping[13] has previously used fluorescence detection of ion flux as an alternative means of measuring the electrical activity of ion channels, and here we adapt it to measure

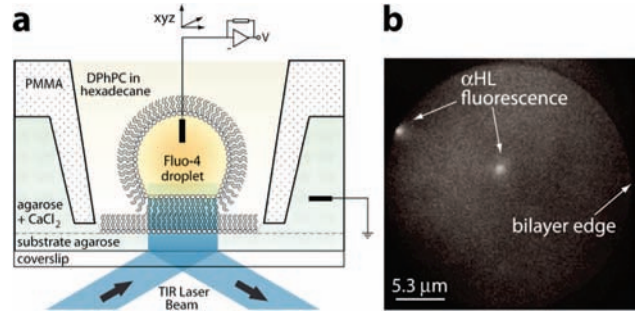

Figure 1. (a) Bilayers are formed by placing an aqueous droplet (∼50 nL) on an agarose substrate immersed in a lipid/oil solution. A 100 μm Ag/AgCl electrode in the droplet, and a ground electrode in the agarose permits electrical recording. TIRF microscopy allows fluorescent molecules to be visualized. (b) Fluorescence image of the entire bilayer containing two αHL pores. αHL heptamer in the droplet (∼10 pg/μL) spontaneously inserts into the bilayer. Ca^{2+} is included in the agarose, and the Ca^{2+} indicator dye Fluo-4 is included in the droplet. Ca^{2+} flux through the pores is monitored using the fluorescence from the Ca^{2+} indicator dye Fluo-4, and current due to the overall ion flux is recorded at the same time.

Ca^{2+} flux through single pores of αHL, thereby evaluating DHBs for simultaneous measurements. We use αHL as a well characterized model pore and exploit its ability to bind molecular adapters to fluorescently and electrically detect stochastic blocking events.[20,21] To fluorescently image ion current through the αHL pores, $CaCl_2$ (750 mM) was incorporated into the hydrogel, and the bilayer-impermeant Fluo-4 dye (25 μM) was included in the droplets (Figure 1a). EGTA (50 μM) was also present in the droplet to chelate residual Ca^{2+}. A low level background fluorescence was observed, which permitted clear visualization of the bilayer (Figure 1b).

Purified αHL heptamer included in the droplet (∼10 pg/μL) inserts spontaneously into the bilayer.[3,21] When αHL pores were present, fluorescent spots were observed under negative applied potential (Figure 1b) as a result of Ca^{2+} being driven through the pores into the droplet. The proteins were observed to diffuse in two dimensions, and under an applied square-wave potential the fluorescent spots corresponding to the pores blinked synchronously with the driving potential (Supplementary Figure 2, Supplementary Movie 1).

Cyclodextrins such as heptakis(2,3,6-tri-*O*-methyl)-β-cyclodextrin (TRIMEB) act as noncovalent blockers that lodge inside the β barrel of αHL.[21] They also block other β-barrel pore-forming toxins and have recently shown promise in treating anthrax infection.[22] We detected the stochastic binding of TRIMEB to αHL (Figure 2 and Supplementary Movie 2). TRIMEB binding restricts the ion flux through a pore, which is observed as a reversible stepwise change in the single-channel current. As TRIMEB binds preferentially from the β-barrel side of αHL, we incorporated protein in the agarose substrate during the preparation of the DHB device (Figure 2a). In

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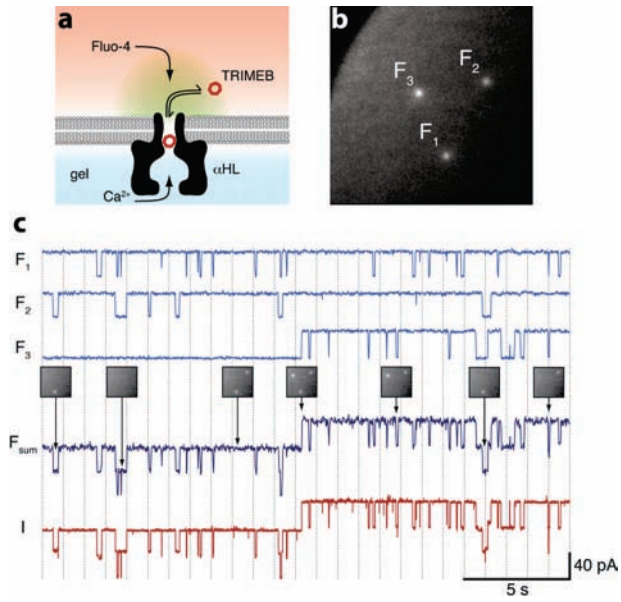

Figure 2. Simultaneous fluorescence and electrical detection of stochastic cyclodextrin binding to αHL pores. (a) αHL pores were inserted from the hydrogel interface. TRIMEB binding inside the β barrel of αHL produces a stepwise change in ion current. (b) An image from supplementary movie 2 showing Fluo-4 fluorescence from the three αHL pores in the bilayer (−50 mV; 10 mM HEPES pH 7.0, 1.5 M KCl, 25 μM Fluo-4, 50 μM EGTA and 10 μM TRIMEB in droplet; 10 mM HEPES pH 7.0, 750 mM $CaCl_2$ in hydrogel). (c) The time-dependent fluorescence from each pore (F_1, F_2, F_3), the summed fluorescence (F_{SUM}), and the measured ionic current across the bilayer (I). The individual fluorescence traces show stepwise changes resulting from TRIMEB blocks. F_3 shows a pore insertion event occurring halfway through the trace, followed by TRIMEB blocking events. The summed fluorescence for all three spots (F_{SUM}) correlates with the measured electrical current (I) through all three αHL pores. Individual frames from the movie show stochastic blocking events of individual pores. The electrical trace is inverted to aid comparison.

this configuration, αHL diffusion was not observed, presumably because the cap domain of αHL is sufficiently large to be restricted by the agarose gel. Data were typically collected from a DHB in which several αHL proteins were detected (three in the case shown; Figure 2b). TRIMEB binding to αHL pores could be observed both in the single-channel recording and by fluorescence (Figure 2c). The fluorescence associated with each pore switched between two levels in a stepwise manner, which we attribute to a reduction in the Ca^{2+} flux when TRIMEB binds within the pore. Both the kinetics of binding ($k_{on} \sim 10^5$ M^{-1} s^{-1}) and the extent of block (95%) of ionic current by TRIMEB were consistent with those observed for other cyclodextrins in conventional BLMs.[21]

Strikingly, the step changes in fluorescence were mirrored in the electrical trace measured concurrently (Figure 2c and Supplementary Movie 2), and the sum of the three fluorescence traces displayed an identical sequence of TRIMEB blocking steps to that recorded electrically over the entire bilayer. This shows that no binding events are missed in the fluorescence recordings. The correspondence between the electrical and fluorescence traces demonstrates the simultaneous detection of correlated ionic current and fluorescence from all of the channels present in the bilayer. To our knowledge, this is the first straightforward demonstration of simultaneous measurements from single channels.

The present approach is not subject to many of the problems inherent in previous simultaneous measurements: (1) DHBs are easy to create and very stable,[3,4] which is in stark contrast to alternative *in vitro* approaches based on artificial bilayers.[17−19] (2) No events are missed as the entire bilayer can be imaged. (3) The agarose support provides an optically flat substrate that avoids many of the imaging problems associated with unsupported bilayers. (4) The ability to implement TIRF imaging gives a low fluorescence background and sufficient sensitivity for single-molecule imaging.

With this system we clearly show that we can simultaneously detect synchronized fluorescence and electrical events from multiple αHL pores in a bilayer, and with correlated fluorescence measurements we can unambiguously assign the electrical events to specific channels. This method would be directly applicable to the screening of inhibitors of other pore-forming toxins, such as the anthrax protective antigen,[22] or the study of Ca^{2+} channels.[13,15,16] We have previously shown that single-molecule fluorescence imaging is also possible in DHBs[4] and that a wide variety of membrane proteins can be incorporated,[1] so a next step must be the application of the technique to a labeled membrane protein, where the fluorescence signal yields additional information. For example, we believe it would be straightforward to apply the method to studies of ligand binding, voltage-sensitive conformational changes in ion channels, and the assembly of multimeric channels.

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Supporting Information Available: Experimental details, movies for the figures, and additional analysis of the Ca^{2+}/Fluo-4 system.This material is available free of charge via the Internet at http://pubs.acs.org.

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