

# In Vitro Reconstitution of Eukaryotic Ion Channels Using Droplet Interface Bilayers

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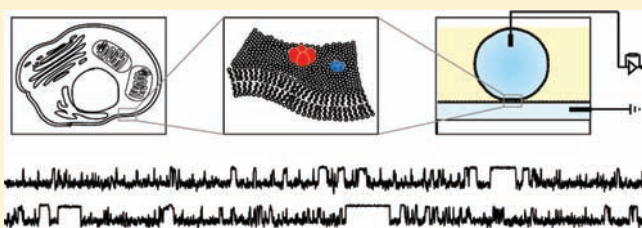
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 Supporting Information

**ABSTRACT:** The ability to routinely study eukaryotic ion channels in a synthetic lipid environment would have a major impact on our understanding of how different lipids influence ion channel function. Here, we describe a straightforward, detergent-free method for the in vitro reconstitution of eukaryotic ion channels and ionotropic receptors into droplet interface bilayers and measure their electrical activity at both the macroscopic and single-channel level. We explore the general applicability of this method by reconstitution of channels from a wide range of sources including recombinant cell lines and native tissues, as well as preparations that are difficult to study by conventional methods including erythrocytes and mitochondria.



## INTRODUCTION

Ion channels are a highly divergent and structurally complex class of proteins that play key roles in cellular signaling.<sup>1</sup> Much of our understanding of ion channels has arisen from the patch clamp's ability to record the ionic flux through a single channel.<sup>2</sup> However, patch clamping is not without its drawbacks, and the need to apply a glass micropipet to a single cell requires time, patience, and a highly trained operator. This restricts the throughput possible with conventional patch clamping and limits the size of cell that can be routinely patched. Higher throughput chip-based patch clamp methods are possible, but they typically sacrifice seal quality and sensitivity in return for parallelization.<sup>3</sup>

It is also now clear that the lipid environment plays a major role in determining eukaryotic ion channel function,<sup>4</sup> yet most patch-clamp methods do not allow easy manipulation of this experimental parameter. Alternative methods that allow manipulation of the lipid environment typically employ reconstitution of detergent-solubilized or vesicle-reconstituted proteins into synthetic bilayers.<sup>5–7</sup> Both throughput and bilayer stability are still a problem for many of these approaches. These problems are also compounded by the fact that detergent purification and reconstitution of eukaryotic ion channels is by no means trivial.<sup>5,8</sup> Synthetic bilayers do however present a trade off: control of bilayer composition typically comes at the expense of working with ion channels sufficiently robust to survive purification in detergent and reconstitution into lipid vesicles.

We recently developed a new method for creating synthetic lipid bilayers in which an aqueous droplet and an agarose surface are brought into contact within an oil/lipid solution:<sup>9–12</sup> A monolayer forms at both oil–agarose and oil–droplet interfaces,

and when the droplet and agarose surface are brought into contact, a bilayer is created. By placing electrodes in the agarose surface and droplet, gigaohm seal recordings from single ion channels present in the bilayer can be made.<sup>12</sup> Here, we report a method to reconstitute eukaryotic membrane proteins into these droplet interface bilayers. We have obtained rapid and efficient reconstitution into the bilayer in all cases attempted.

## RESULTS

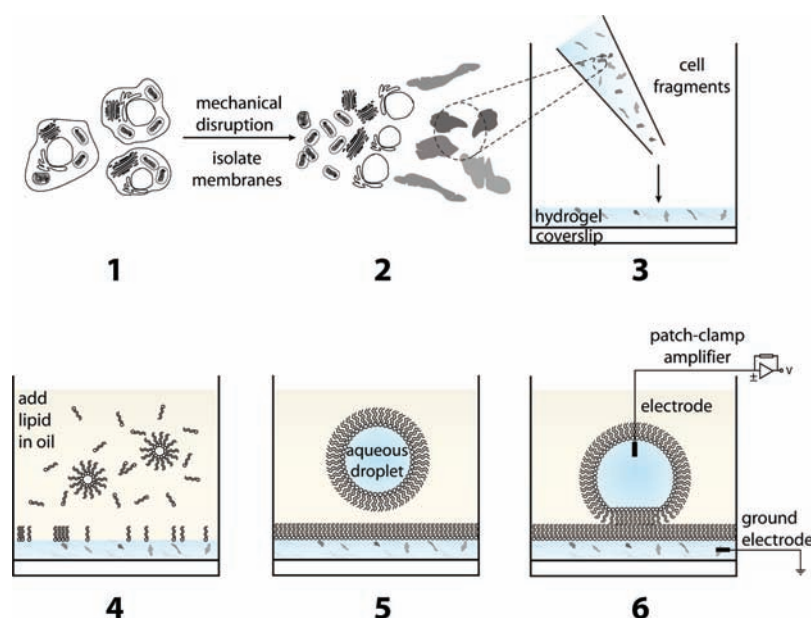
The basic steps required for reconstitution of eukaryotic ion channels into droplet bilayers are depicted in Figure 1: (1) Eukaryotic cells are ruptured; (2) Cell membranes are isolated and diluted; (3) Membrane samples are added to an agarose layer deposited on a glass coverslip; (4) A mixture of phospholipid in hexadecane is applied, and a monolayer self-assembles at the agarose–oil interface; (5) A nanoliter aqueous droplet is introduced, which also acquires a second lipid monolayer; (6) A bilayer forms when both monolayers are brought into contact. Upon bilayer formation, ion channels are immediately present in the bilayer. Bilayer formation and reconstitution methods are described in detail in the Supporting Information.

To test the ubiquity of our method, we studied a number of representative ligand- and voltage-gated ion channels. We also explored a range of different sample conditions, ranging from purified prokaryotic proteins to mammalian tissue samples.

**Electrical Recording of Ion Channels from Recombinant Overexpressing Cells.** We first examined a number of

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**Figure 1.** Reconstitution method for incorporating eukaryotic ion channels into droplet bilayers. (1 and 2) Cells are mechanically disrupted and membranes isolated. (3) Cell membrane fragments are deposited onto an agarose-coated coverslip or incorporated into the agarose. (4) A lipid in oil solution is added and a lipid monolayer forms at the interface. (5) An aqueous droplet with a monolayer is added into the oil. (6) Both monolayers come together to form a lipid bilayer; reconstitution of ion channels occurs during this process. An electrode is introduced into the droplet to measure ion flux.

overexpressed ion channels (Figure 2a–c). By changing the degree of dilution of membrane fragments (Figure 1 Step 2) we can control the number of ion channels in the bilayer, to achieve either macroscopic, or single-channel current recording. Typically, we diluted membrane fragments around  $1:10^4$  (v/v) for macroscopic recordings, and  $1:10^6$  for single-channel recording.

Although this manuscript deals primarily with eukaryotic channels, our first experiments were with isolated membranes from a porin-free *E. coli* strain overexpressing the archetypal bacterial potassium channel, KcsA. Membrane preparations were first diluted into isotonic buffer before being incorporated into the agarose support. The presence of  $50 \mu\text{M Ba}^{2+}$  on both sides of the bilayer resulted in a 93% block in current<sup>13</sup> (Figure 2a). Upon further dilution of the membrane preparation, we observed single channels with low open probability<sup>14</sup> and a conductance of  $62 \pm 2.6 \text{ pS}$  at  $140 \text{ mM KCl}$  (Figure 2a). The  $+200 \text{ mV}$  chord conductance of KcsA under similar conditions in a planar lipid bilayer is approximately  $66 \text{ pS}$ .<sup>15</sup> Any discrepancy is likely due to the limitations in our estimation of channel conductance from an individual single-channel recording at a single voltage. Control experiments using *E. coli* cells without transformation showed no ionic current.

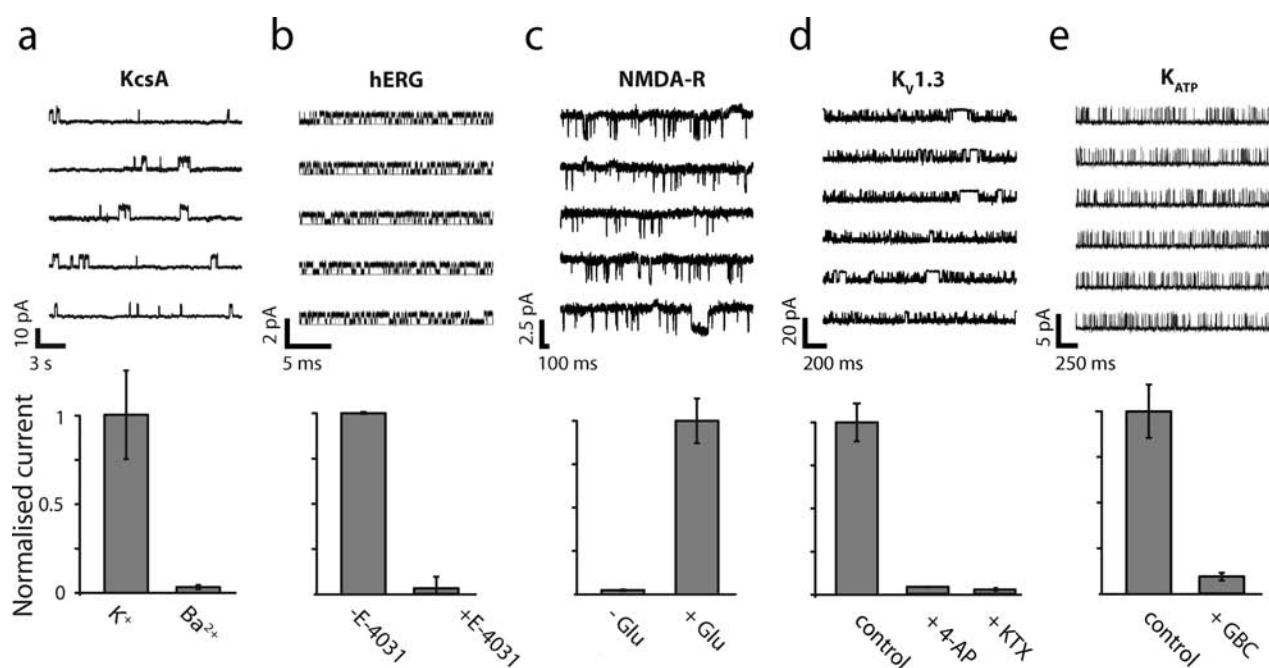
We then measured ionic flux across membranes derived from cells overexpressing the voltage-gated potassium channel hERG (Kv11.1), crucial to repolarization during the cardiac action potential.<sup>16</sup> Growth arrested HEK293 cells overexpressing hERG were mechanically disrupted by sonication, and membranes isolated by centrifugation and washed. Finally, membranes were resuspended into buffer ( $350 \text{ mM KCl}$ ,  $10 \text{ mM Hepes}$ ,  $\text{pH } 7.5$ ) and diluted before incorporation into the agarose layer. We obtained a voltage-dependent macroscopic current response, and ion current was blocked (97% Figure 2b) by  $1 \text{ mM}$  of the hERG blocker, E-4031.<sup>17</sup> Upon further dilution of the cell membrane preparation, we were also able to record single channel traces with an average conductance of  $8 \pm 0.21 \text{ pS}$  ( $350 \text{ mM KCl}$ )

similar to previous reported values at high ionic strength<sup>18</sup> (Figure 2b). No single channel events were observed in the presence of blocker.

We also studied the ligand-gated ionotropic *N*-methyl-D-aspartate (NMDA) receptor. Mouse fibroblast (Ltk–) cells expressing recombinant human NMDA receptor<sup>19</sup> were washed in isotonic buffer before mechanical disruption, isolation of the membranes, and dilution ( $140 \text{ mM NaCl}$ ,  $10 \text{ mM Hepes}$ ,  $\text{pH } 7.5$ ). Membranes were then incorporated into the agarose layer before bilayer formation. The presence of glutamate ( $10 \mu\text{M}$ ) and glycine ( $10 \mu\text{M}$ ) showed a 98% increase in ionic flux consistent with activation of the receptor<sup>20</sup> (Figure 2c). When the cell preparation was further diluted, we observed single channels that exhibited slow opening and closing events typical of the NMDA receptor. Observed conductance levels with  $47.3 \pm 3.2 \text{ pS}$  and a subconductance level of  $36.2 \pm 3.55 \text{ pS}$  correspond to values previously reported in the literature.<sup>21</sup>

**Electrical Recording of Endogenous Ion Channels.** We recorded single channel currents and the voltage dependence of macroscopic current from endogenously expressed voltage-gated potassium channels in a primary lymphocyte culture (Figure 2d,e). Lymphocyte potassium channels are important for signaling during immune response and regulate membrane potential and calcium signaling of T cells.<sup>22</sup> A suspension of SupT1 cells was lysed by hypo-osmotic shock, and membranes were isolated by centrifugation and washed in isotonic buffer. Membranes were then diluted before being deposited onto an agarose-coated coverslip.

In contrast to our experiments with overexpressing channels, we cannot make any assumptions as to the identity of an endogenously expressed channel; however, potassium channels from this cell type are almost exclusively  $\text{K}_v1.3$ .<sup>23</sup> We observed voltage-gated behavior typical for  $\text{K}_v1.3$ ,<sup>24</sup> with increased open probability at higher voltages (Figure 2d). The identity of  $\text{K}_v1.3$  was confirmed using a nonspecific  $\text{K}^+$ -channel blocker<sup>25</sup> ( $5 \text{ mM}$  4-aminopyridine (4-AP), 96% block) and a specific  $\text{K}_v$ -channel



**Figure 2.** Single Channel Recordings (SCR, upper panels) and macroscopic ion currents (lower panels) from (a–c) overexpressed and (d and e) endogenous ion channels. Potentials are applied to the droplet electrode, with identical solution conditions on both sides of the bilayer. Errors bars are standard deviation from 5 experiments. All SCR are from a single contiguous trace. (a) SCR of KcsA expressed in the porin-free *Escherichia coli* strain PC2889 (+50 mV, 150 mM KCl, 10 mM Succinic acid, pH 4 on both sides of the bilayer). Macroscopic currents are in the absence or presence of 150  $\mu$ M Ba<sup>2+</sup>. (b) SCR of a hERG channel expressed in HEK293 cells (+100 mV, 350 mM KCl, 10 mM HEPES, pH 7.5). Bar chart shows macroscopic currents in the absence or presence of 1 mM E-4031. (c) SCR of the NMDA receptor overexpressed in 3T3 cells (+50 mV, pH 7.5, 140 mM NaCl, 10 mM HEPES in the droplet and 140 mM NaCl, 10 mM HEPES, 10  $\mu$ M glycine, 10  $\mu$ M glutamate). Macroscopic currents are in the absence or presence of glutamate. (d) SCR of a K<sup>+</sup> channel from lymphocyte membranes (150 mM KCl, 10 mM HEPES, pH 7.5, +25 mV). Bar chart shows macroscopic currents. In the presence of blocker, ion flux is reduced by 96% using 4-aminopyridine (5 mM, 4-AP) and 99.4% using Kaliotoxin (50  $\mu$ M KTX) compared the absence of KTX ('control'). (e) SCR of a K<sub>ATP</sub> channel from Min6 cells (140 mM KCl, 10 mM HEPES, pH 7.5, +50 mV). (f) Macroscopic currents are in the presence or absence of glibenclamide (112  $\mu$ M) (GBC).

blocker<sup>26</sup> (50  $\mu$ M Kaliotoxin, 99% block) (Figure 3b). From this pharmacological profile and the current voltage behavior (Supporting Information), we identify this channel as K<sub>v</sub>1.3.

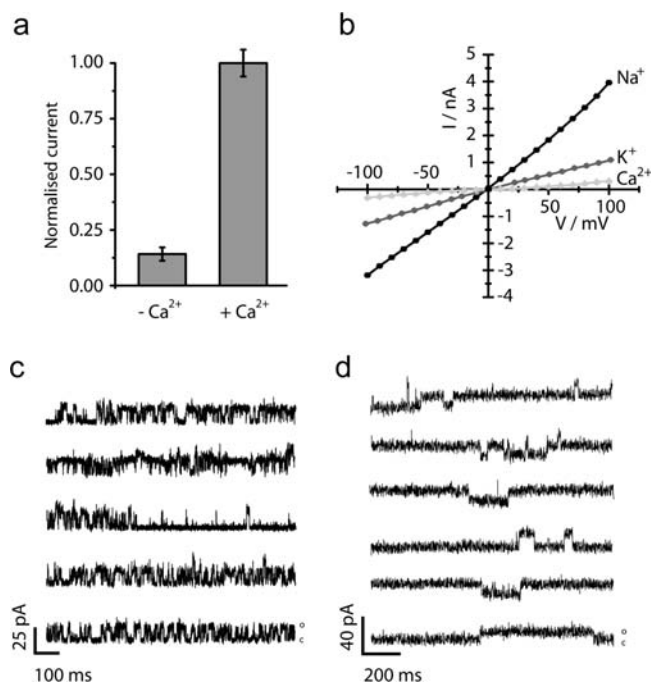
We also measured currents across membranes derived from a mouse insulinoma cell line (Min6) endogenously expressing the K<sub>ATP</sub> potassium channel<sup>27</sup> thought to play a key role in insulin secretion. Macroscopic currents were blocked (91%; Figure 2f) by glibenclamide, a specific K<sub>ATP</sub> channel blocker.<sup>28</sup> Further dilution of the cell preparation revealed single channels (Figure 2e) with a conductance of  $50 \pm 3.1$  pS similar to previous observations.<sup>29</sup>

**Electrical Recording of Ion Channels from Erythrocytes and Mitochondria.** One additional advantage of our method is the measurement of ion currents from membranes that would be extremely difficult to access using conventional patch clamping. To demonstrate this, we examined both erythrocyte and mitochondrial membranes.

Because of their size, erythrocytes represent one of the smallest and most difficult cells accessible by patch clamp. After isolation of erythrocytes from whole blood, we lysed cells by hypo-osmotic shock, and resuspended membranes in isotonic buffer. By conducting experiments with or without Ca<sup>2+</sup> (5 mM), we observed a Ca<sup>2+</sup> induced K<sup>+</sup> flux which increased 7-fold in the presence of calcium, consistent with currents derived from the hSK4 (KCNN4) channel (Figure 3a). The hSK4 (or Gardos) K<sup>+</sup> ion channel is the most important calcium-activated cation channel in the membranes of erythrocytes.<sup>30</sup>

We also compared the membranes of red blood cells reconstituted from both healthy individuals and those suffering from sickle cell disease. Sickling is known to occur via a change in the cation permeability of erythrocyte cell membranes and leads to dehydration of the cell;<sup>31</sup> however, the molecular basis of this initial step is still the subject of debate.<sup>32,33</sup> In this work, we incorporated erythrocyte membranes into the agarose. Membranes from both sickled and healthy erythrocytes exhibit similar Ca<sup>2+</sup> induced K<sup>+</sup> fluxes. However, in contrast to healthy erythrocytes, reconstituted membranes from sickle cells were highly conductive for Na<sup>+</sup>, K<sup>+</sup>, and Ca<sup>2+</sup> ions, with an essentially Ohmic response for each ion. Healthy erythrocytes were not conductive for any of these ions in isolation (Figure 3b). Upon further dilution of the sample preparation (1:20), we also obtained single channel recordings from membranes derived from sickle cells that were absent in membranes from healthy individuals (Figure 3c). Clearly, this experiment alone cannot attribute this channel activity to a specific channel.

To provide a further example of the ease with which small cells or organelles can be studied using this method, we also reconstituted mitochondrial membranes isolated from porcine liver. Organelles are very difficult to investigate with conventional patch clamping.<sup>2</sup> After mechanical disruption of isolated mitochondria, we separated and washed the membranes in isotonic buffer before reconstitution into a droplet bilayer. We were able to resolve both single channel and macroscopic currents using either potassium or calcium salts (Figure 3d). We did not observe



**Figure 3.** Electrical recordings from primary cells and organelles (a) Normalized macroscopic currents from red blood cell membranes, recorded in the absence or presence of 5 mM Ca<sup>2+</sup> (150 mM KCl, Hepes, pH 7, +50 mV). Error bars are determined as the standard deviation from 5 experiments. (b) Macroscopic current recording of reconstituted sickle cell membranes testing the conductance of different cations (Na<sup>+</sup>, K<sup>+</sup>, Ca<sup>2+</sup>). Sickle cell membranes are conductive whereas control membranes from healthy individuals show no conductance from a single type of ion. (c) Single channel recordings of a sample of red blood cells with the HbSS genotype, conducting sodium (140 mM NaCl, 10 mM Hepes, pH 7.4, +125 mV). (d) Single channel recordings of a potassium conductive channel in mitochondrial membranes (150 mM KCl, 10 mM Hepes, pH 7.5, +50 mV).

any voltage dependence, which suggests this channel is not VDAC;<sup>34</sup> however, we have not attempted to identify the specific nature of this channel.

## DISCUSSION

We have shown successful reconstitution from a wide variety of ion channel types and sample conditions. These experiments required no specialist equipment beyond a sensitive current amplifier, and membrane protein reconstitution was reliable and reproducible in all cases.

We have not provided a quantitative comparison of reconstitution efficiency in droplet bilayers as compared to, for example, vesicle fusion to a planar lipid bilayer. This is primarily due to the difficulties, at least in our hands, of obtaining reliable reconstitution in these other systems, even after careful optimization of conditions.<sup>35</sup> For samples that gave essentially no reconstitution via vesicle fusion, we see immediate and efficient incorporation of channels using our method.

We have not yet characterized the physical mechanism underlying the success of our reconstitution method. However, we speculate that the most likely route is via the incorporation of cell membrane fragments containing native protein *during* bilayer formation. This may help explain why our reconstitution method is more successful than most previous methods.<sup>35,36</sup> For example, fusion of

proteoliposomes relies on reconstitution *after* rather than *during* bilayer formation. Our suggested mechanism is supported by the observation that addition of membrane fragments into the droplet after bilayer formation does not result in channel reconstitution. It is therefore perhaps this different method of channel incorporation, combined with the enhanced local concentration due to the close proximity of the agarose support to the lipid bilayer that is the most likely reason for our improved reconstitution efficiency.

Our current experiments required compounds to be present on both sides of the bilayer in order to achieve complete block. This, combined with the observed symmetric macroscopic current–voltage responses, suggests that there is little preferential channel orientation. Like other bilayer reconstitution methods, channel block from one side of the bilayer, or the preparation of proteoliposomes with a specific channel alignment is needed to measure a single orientation. In these experiments, compounds were added to either agarose or droplet before bilayer formation. Our previous work describes how droplet translation can be used to modulate the composition on the agarose side of the bilayer.<sup>12</sup> It is also easy to change droplet composition via fusion of a second droplet, microfluidic access, or nanoinjection; however, we are yet to publish this work. One further limitation of our current method is the use of synthetic lipids in addition to the native lipids present when reconstituting channels from cell extracts. In this case, we presume we have a mix of synthetic and endogenous lipids. In this work, we have made no attempt to characterize either the lipid composition, or any potential subtle variation in channel function due to the different lipid environment within these droplet bilayers. Although we detect no gross change in function, clearly, in the future, it would be advantageous to more carefully control and characterize the lipid composition in these cases.

Reconstitution from cell membrane fragments does not exclude endogenous channels from the membrane, and as with other synthetic lipid bilayer methods, interference from other channels will become a problem for macroscopic current recording of channels present at very low levels of endogenous expression. For these cases, we must rely on single-channel recording and dilution of the membrane preparation. This method can be used to study a single pure ion channel in the absence of known accessory components; however, to achieve this, we must rely on either prior purification, or dilution in a pure known lipid composition to the point where we can be confident about the composition of the lipid bilayer.

A key feature of this method is the incorporation of ion channels directly from cell membranes into a synthetic lipid bilayer without the need for detergent purification. Detergent-free reconstitution protocols have been reported since the 1970s;<sup>35–38</sup> however, we believe that reconstitution into droplet bilayers is considerably easier and more reliable than these previous methods, and that the data presented here supports our opinion. Previous approaches are reportedly unreliable<sup>5,7</sup> and require considerable sample preparation; the lack of recent work in this area is perhaps another indicator of the difficulties in achieving efficient protein incorporation using prior methods. The relative ease with which both prokaryotic and eukaryotic channels are incorporated into the bilayer suggests that for many experiments our method may be a useful alternative to patch clamping. Alternative approaches have, for example, exploited the use of a glass probe to directly introduce membrane proteins from a bacterial colony;<sup>39</sup> however, such methods have only been successful in reconstituting prokaryotic proteins.<sup>40</sup>

The reconstitution of ion channels from erythrocytes and mitochondria also suggests that this method should find application in the study of other small cells and cellular compartments that are currently inaccessible to all but the most skilled patch clampers. At present, smaller objects can only be patched through nanoscopic control of the patch pipet,<sup>41</sup> by the use of giant proteoliposomes,<sup>42</sup> or by studying atypically large variants of the membrane component.<sup>43</sup> In our case, recording of single channels can be achieved with relatively straightforward sample preparation and without the need for microscopic positioning of a patch pipet.

Ion channels remain an under-exploited target class for therapeutic drugs, largely due to the lack of techniques capable of high-throughput interrogation of channel function.<sup>3</sup> As multiple droplet bilayers are simple to produce,<sup>44</sup> this method has significant potential as a tool for the high-throughput screening of both overexpressed and endogenous ion channels. The low quantities of material required for this reconstitution method (less than one cell per measurement), electrical control, and access to both sides of the bilayer are particular advantages of this technique.

This work has focused on the reconstitution and measurement of ion channels, primarily because the ionic flux provides a very convenient measurement of protein function. We have previously shown that high-sensitivity optical measurements of such lipid bilayers can also be made.<sup>10,11</sup> Thus, we believe that it will be simple to extend this method to interrogate other classes of membrane protein where an optical probe of protein function exists, including receptor kinases, G protein-coupled receptors, and transporters.

## ■ ASSOCIATED CONTENT

**S Supporting Information.** Additional description of the reconstitution method, and further electrical characterization of ion channels present in this manuscript are included as Supporting Information. This information is available free of charge via the Internet at <http://pubs.acs.org/>.

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