Journal of Visualized Experiments

Lipid bilayer experiments with Contact Bubble Bilayers for Patch-clampers
--Manuscript Draft--

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Molecular Physiology & Biophysics

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September 24, 2018

Dr. Nandita Singh
Senior Science Editor
Journal of Visualized Experiments

Dear Dr. Singh:

Please find enclosed our manuscript entitled, "Lipid bilayer experiments with Contact Bubble Bilayers for Patch-clampers" by Masayuki Iwamoto and Shigetoshi Oiki, which we are submitting for publication in the Journal of Visualized Experiments.

We would like to thank the Editor for handling our manuscript and are grateful for the constructive comments provided. We have responded to all points raised by the reviewers and revised the manuscript accordingly. We have uploaded a pdf file in which all revised sentences are highlighted.

We believe that the manuscript satisfies the requirements of the journal.

Yours sincerely,

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1   **TITLE:**
2   Lipid Bilayer Experiments with Contact Bubble Bilayers for Patch-Clampers
3
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16 **KEYWORDS:**
17 Lipid Bilayer, Patch Clamp, Water-In-Oil Droplet, Monolayer, Ion Channel, Electrophysiology,
18 Surface Chemistry
19
20 **SUMMARY:**
21 Here, we present a protocol for the formation of lipid bilayers using a contact bubble bilayer
22 method. A water bubble is blown into an organic solvent, whereby a monolayer is formed at the
23 water-oil interface. Two pipettes are manipulated to dock the bubbles to form a bilayer.
24
25 **ABSTRACT:**
26 Lipid bilayers provide a unique experimental platform for functional studies of ion channels,
27 allowing the examination of channel–membrane interactions under various membrane lipid
28 compositions. Among them, the droplet interface bilayer has gained popularity; however, the
29 large membrane size hinders the recording of low electrical background noise. We have
30 established a contact bubble bilayer (CBB) method that combines the benefits of planar lipid
31 bilayer and patch-clamp methods, such as the ability to vary the lipid composition and to
32 manipulate the bilayer mechanics, respectively. Using the setup for conventional patch-clamp
33 experiments, CBB-based experiments can be readily performed. In brief, an electrolyte solution
34 in a glass pipette is blown into an organic solvent phase (hexadecane), and the pipette pressure
35 is maintained to obtain a stable bubble size. The bubble is spontaneously lined with a lipid
36 monolayer (pure lipids or mixed lipids), which is provided from liposomes in the bubbles. Next,
37 the two monolayer-lined bubbles (~50 μm in diameter) at the tip of the glass pipettes are docked
38 for bilayer formation. Introduction of channel-reconstituted liposomes into the bubble leads to
39 the incorporation of channels in the bilayer, allowing for single-channel current recording with a
40 signal-to-noise ratio comparable to that of patch-clamp recordings. CBBs with an asymmetric
41 lipid composition are readily formed. The CBB is renewed repeatedly by blowing out the previous
42 bubbles and forming new ones. Various chemical and physical perturbations (*e.g.*, membrane
43 perfusion and bilayer tension) can be imposed on the CBBs. Herein, we present the basic
44 procedure for CBB formation.

45

INTRODUCTION:
For ion channels, the cell membrane is not simply a supporting material but a partner for generating the ion flux. Functionally, the membrane is an electrical insulator in which ion channels are embedded, and all cell membranes are imparted with a resting membrane potential. In previous studies, an arbitrary membrane potential was imposed from an external circuit by which electrical current through the channels was measured. This quantitative evaluation of the ion flux at different membrane potentials revealed the molecular properties of these channels, such as their ion-selective permeation and gating functions[1,2]. The membrane platform for functional studies of ion channels is either the cell membrane or the lipid bilayer membrane. Historically, single-channel electrical current recordings were first performed in lipid bilayers[3,4], and the relevant techniques were developed for cell membranes, such as the patch-clamp method (**Figure 1A**)[5,6]. Since then, these two techniques have evolved separately for different purposes (**Figure 1**)[7,8].

Membrane lipids and bilayer membranes are currently the focus of research for their roles in supporting the structure and function of channel proteins. Therefore, the ready availability of methods to vary the lipid composition in bilayers is in high demand. Lipid bilayer formation methods such as the planar lipid bilayer (PLB)[8–11], water-in-oil droplet bilayer[12], and droplet interface bilayer (DIB)[13–19] techniques (**Figure 1**) are common choices, providing an opportunity for examining the channel function under varying lipid compositions[20]. Although the DIB is technically much easier to produce than the conventional PLB, the large size of the DIB has created a disincentive for patch-clampers to apply it for studying single-channel current recordings with usual-sized conductance (<100 pS).

To circumvent the background noise, the bilayer area must be minimized. This issue recalls the repetitions of history in developing electrophysiological techniques for lipid bilayers (**Figure 1**). In the early days, a small-sized bilayer (1–30 μm in diameter) was formed at the tip of a pipette (tip-dip method; **Figure 1C**)[21–23], rather than using a free-standing bilayer (~100 μm in diameter) on a hydrophobic septum in a chamber (**Figure 1B**). The tip-dip method allowed for electrical measurements with much lower background noise[24]. Our experiences with the PLB[25, 26], tip-dip[22,23,27], and patch-clamp[28–31] methods led us to a novel idea of forming lipid bilayers by using the principles of the water-in-oil bilayer. We have referred to this as the contact bubble bilayer (CBB) method[20,32]. In this method, rather than hanging the water droplets in an oil phase (**Figure 1D**), a water bubble is blown from a glass pipette (with tip diameter of approximately 30 μm) into the oil phase (**Figure 1E and 2**), where the bubble is maintained by applying a steady pressure. A monolayer forms spontaneously at the water–oil interface at the surface of the bubble. Then, two bubbles are docked through the manipulation of two glass pipettes, and the bilayer is formed as the two monolayers approach each other, yielding an equilibrium bilayer area. The size of the bubble is controlled by the intra-bubble pressure (holding pressure), and likewise the bilayer size. An average diameter of 50 μm is frequently used. Although the volume of the bubble is small (<100 pL), it is connected to the larger volume of the pipette solution that is in the microliter range, constituting the bulk electrolyte phase.

89   There are many benefits to use the CBB method (**Table 1**). As a lipid bilayer formation technique,
90   membranes of various lipid compositions can be produced, and asymmetric membranes are
91   more readily formed[32] than are those by the conventional folding method[33]. The bilayer can be
92   mechanically manipulated, unlike the conventional PLB that can only be bent with a hydrostatic
93   pressure difference[34,35]. By changing the holding pressure, the bubbles either expand or shrink,
94   leading to increased or decreased membrane tension[32]. The bilayer is mechanically detachable
95   into monolayers, similar to the freeze-fracture technique[36,37] of membranes in morphological
96   studies, but with the CBB, a maneuver allows for repeated detach and attach cycles[32]. The small
97   volume of the electrolyte solution within the bubble allows efficient fusion of channel-
98   reconstituted liposomes into the bilayer, and the probability of getting channel recordings is
99   much higher than with the conventional PLB technique. The small bubble volume also allows
100   rapid perfusion (within ~20 ms) once another injection pipette is inserted into either of the
101   bubbles. Unlike the patch-clamp method, a broken CBB-based membrane is re-formed
102   immediately and repeatedly, and pipettes can be used several times a day. By integrating benefits
103   of the patch-clamp and PLB methods, the CBB provides a versatile platform to vary the
104   physicochemical conditions of the membrane, allowing for unprecedented studies of channel–
105   membrane interactions.

106

107   Before presenting a detailed protocol of the CBB formation process, the physicochemical
108   background of the bilayer formation is presented first, which will be helpful for patch-clampers
109   to resolve experimental difficulties relating to the membrane formation that are encountered.

110

111   CBB experiments impart lessons of surface chemistry science[38]. The CBB is similar to a soap
112   bubble blown from a straw into the air, where likewise, a water bubble is blown into an organic
113   solvent. One will notice that a water bubble is hardly inflated when membrane lipids are not
114   included in either the water bubble or the organic solvent. In the absence of amphipathic lipids,
115   the surface tension at a water–oil interface is high, and the intra-bubble pressure for blowing a
116   bubble will be high. This is a realization of the Laplace equation ($\Delta P = 2\gamma/R$, where ΔP is the intra-
117   bubble pressure, γ is the surface tension, and R is the bubble radius). When the concentration of
118   lipids in either the organic phase or the electrolyte solution is high, the density of lipids in the
119   monolayer increases, as dictated by the Gibbs adsorption isotherm ($-d\gamma = \Gamma_i\,d\mu_i$, where Γ_i is the
120   surface excess of compound i, and μ_i is the chemical potential of component i)[39], leading to a
121   lower surface tension and ease of bubble formation. In the CBB, the bilayer can be observed from
122   a tangential angle (**Figure 2**), and the contact angle between the monolayer and bilayer is
123   measurable. This angle represents an equilibrium between the surface tensions of the monolayer
124   and bilayer (Young equation: $\gamma_{bi} = \gamma_{mo}\cos(\theta)$, where γ_{bi} is the bilayer tension, γ_{mo} is the monolayer
125   tension, and θ is the contact angle). The changes in the contact angle indicate changes in the
126   bilayer tension, given that the monolayer tension is evaluated from changes in the contact angle
127   as a function of the membrane potential (Young-Lippmann equation: $\gamma_{mo} = C_m\,V^2/4\,(\cos(\theta_0)$ -
128   $\cos(\theta_v))$, where C_m is the membrane capacitance, V is the membrane potential, and θ_0 and θ_v are
129   the contact angles at 0 and V mV, respectively)[40–42]. When two bubbles are close enough, they
130   approach each other spontaneously. This is due to the van der Waals force, and we can visually
131   observe this dynamic process in CBB formation.

132

133 A CBB system consists of distinct phases: namely, a bulk oil phase, water bubbles coated with a
134 monolayer, and a contacting bilayer (**Figure 3**). These are reminiscent of the multiple phases
135 observed in a PLB, such as a solvent-containing torus around the bilayer phase and a thin organic
136 phase sandwiched by two monolayers[43,44]. In the CBB, the monolayer phase is continuous with
137 the bilayer leaflet, and lipid molecules readily diffuse between the monolayer and the leaflet. The
138 monolayer phase covers most of the bubble surface, constituting the major phase that serves as
139 a lipid reservoir. Because the hydrophobic tail of lipids in the monolayer extends outward to the
140 bulk oil phase, the bilayer interior or the hydrophobic core opens to the bulk oil phase. Thus, a
141 hydrophobic substance injected into the oil phase close to the bilayer is able to readily access the
142 bilayer interior. This is the membrane perfusion technique we had developed recently[45], by which
143 the lipid composition in the bilayer is changed rapidly (within a second) during single-channel
144 current recordings. We found that the cholesterol content in the bilayer could be reversibly
145 controlled by switching the cholesterol perfusion on and off[45]. In the event that the concentration
146 of the relevant substance in the monolayer and bilayer differs, the concentration gradient of the
147 relevant substance is immediately dissolved through diffusion, which is known as the Marangoni
148 effect[46,47]. On the other hand, flip-flops across the monolayers are slow[48–50].
149
150 Using the CBB method, the bilayer is formed under versatile physicochemical conditions, such as
151 an electrolyte pH as low as 1 [51], a salt (K^+, Na^+, *etc.*) concentration up to 3 M, a membrane
152 potential as high as ±400 mV, and a system temperature of up to 60 °C.
153
154 There are several options for formation of the CBB and incorporation of channel molecules
155 therein. For formation of the monolayer at the water–oil interface, lipids are added either in an
156 organic solvent (lipid-out method; **Figure 4A, 4C**) or in a bubble as liposomes (lipid-in method;
157 **Figure 4B, 4D**). Notably, the lipid-in method allows for the formation of asymmetric
158 membranes[15,32]. Channel molecules soluble in aqueous solution (*e.g.*, channel-forming peptides)
159 are directly added into the bubble (**Figure 4A, B**)[52,53], whereas channel proteins are reconstituted
160 into liposomes, which are then added into the bubble (**Figure 4C, D**). Herein, the formation of
161 CBBs by the lipid-in method for either a channel peptide (polytheonamide B (pTB); **Figure 4A**) or
162 a protein (KcsA potassium channel, **Figure 4C**) is shown.
163
164 **PROTOCOL:**
165
166 **1   Prepare Liposomes**
167
168 1.1   Disperse phospholipids (*e.g.*, 10 mg in powder) in chloroform at a desired concentration
169 (*e.g.*, 10 mg/mL).
170
171 1.2   Evaporate chloroform.
172
173 1.2.1   Place the phospholipid solution in a round-bottom flask and set it on a rotary evaporator
174 (see **Table of Materials**) connected to a N_2 gas cylinder. Rotate the flask under N_2 flow at room
175 temperature until a thin phospholipid film appears (after ~30 min).
176

177 1.2.2 Place the open flask into a desiccator that is connected to a vacuum pump. Using the
178 vacuum pump, aspirate the inside of the desiccator for several hours to remove the chloroform
179 thoroughly.
180
181 1.3 Add an appropriate volume of an electrolyte solution to the flask and suspend the
182 phospholipid to obtain a 2 mg/mL phospholipid suspension.
183
184 1.4 Sonicate the suspension for several tens of seconds using a bath sonicator (see **Table of**
185 **Materials**) to obtain a multilayered vesicle (MLV) suspension.
186
187 1.5 For preparation of proteoliposomes that contain ion channel proteins, add a protein
188 solution (with the proteins solubilized using appropriate detergents; 2% volume is the maximum)
189 to the MLV suspension and sonicate for several seconds using the bath sonicator.
190
191 **2 Prepare Large-Bore Glass Pipettes**
192
193 2.1 Set a glass capillary to a pipette puller and fabricate micropipettes with a fine tapered tip
194 through two-step pulling.
195
196 2.2 Set the micropipette on a microforge and contact the tip of the micropipette to a platinum
197 filament at the tapered portion with a diameter of 30 to 50 μm.
198
199 2.3 Heat the filament briefly (5 s) and immediately turn it off.
200
201 NOTE: This manipulation forms a crack at the heating point, whereby the tip of the micropipette
202 is cut off, leaving a wide bore with a diameter of 30 to 50 μm.
203
204 **3 Treat the Surface of Glass Slide with a Shallow Concave Well (Siliconization for a Water-**
205 **Repellent Finish)**
206
207 3.1 Clean the surface of the glass slide with a shallow well with distilled water and ethanol.
208
209 3.2 Apply an appropriate volume (*e.g.*, 100 μL) of a siliconizing reagent (water-repellent) onto
210 the hole slide glass.
211
212 3.3 Dry the reagent completely in the air.
213
214 3.4 Place the glass slide on the stage of an inverted microscope.
215
216 **4 Form the CBB and Perform Electrophysiological Measurement**
217
218 4.1 Add 100 μL of hexadecane into the shallow well of the siliconized hole slide glass.
219

220   NOTE: For the lipid-out method, phospholipids are dispersed in hexadecane (20 mg/mL)
221   beforehand.

223   4.2   Fill the electrolyte solution up to half the length of the micropipette, using a tuberculin
224   syringe.

226   4.3   Set the micropipette onto the micropipette holder with a pressure port, allowing the
227   Ag/AgCl wire electrode to soak into the pipette electrolyte solution.

229   4.4   Connect one of the micropipette holders to the head stage of a patch-clamp amplifier and
230   the other one to the electrical ground.

232   4.5   Connect a microinjector to the pressure port of the micropipette holder.

234   4.6   Set the micropipette to an appropriate position above the stage of an inverted
235   microscope by manipulating the micromanipulator.

237   4.7   Adjust the electrode offset potential.

239   4.7.1   Place 1 µL of the same electrolyte solution used to fill the micropipette on the flat surface
240   around the shallow well of the hole slide glass, creating an electrolyte dome.

242   4.7.2   Soak the tip of both micropipettes into the electrolyte dome by manipulating the
243   micromanipulator.

245   4.7.3   Adjust the electrode offset potential of the patch-clamp amplifier.

247   4.7.4   Confirm the correct offset at the end of experiments by breaking the CBB via application
248   of a high membrane potential (electrical breakdown; using Zap on the amplifier), causing the two
249   bubbles to be fused into one (bubble fusion).

251   4.7.5   Correct the liquid junction potential[54] in the cases where asymmetric electrolyte
252   solutions are used, such that the calculated value is added to the applied membrane potential
253   for the true membrane potential.

255   NOTE: The liquid junction potential is calculated using the program JPCalc[55].

257   4.8   Draw the liposome solution from the tip.

259   NOTE: When water-soluble channels are examined using the lipid-out method, suctioning of the
260   liposome solution is not necessary.

262   4.8.1   Place 1 µL of liposome solution on the flat surface around the shallow well of the hole
263   slide glass (liposome-containing dome).

4.8.2   Manipulate the micromanipulator and insert the tip of the micropipette into the liposome-containing dome.

4.8.3   Aspirate the liposome-containing solution by lowering the pressure inside the micropipette holder using the microinjector.

4.8.4   Repeat the procedure for the other pipette.

4.9   Manipulate the micromanipulator and dip the tip of the micropipette into the hexadecane in the shallow well.

4.10   Blow a water bubble slowly by increasing the pressure until the bubble reaches the desired size (*e.g.*, 50 µm in diameter) and maintain the same pressure thereafter.

4.11   Discard the bubbles by passing the tip through the oil–air interface if it is hard to keep the size of the bubbles stable.

4.12   Repeat steps 4.8 to 4.9 until stable bubbles are formed.

4.13   Manipulate the bubbles to allow contact between them (**Figure 5**).

NOTE: Sometimes, the bubbles approach each other spontaneously to form the CBB. In other cases, the bubbles are close but do not contact each other. In this case, push the bubbles each other mechanically.

4.14   Fine-tune the pressure to maintain the bubble size, because the size may gradually change even at the constant intra-bubble pressure.

4.15   Set the membrane potential to the appropriate value using the patch-clamp amplifier and wait for the channel current to emerge (**Figure 6**).

5   Measure Bilayer Capacitance

5.1   Measure the bilayer electrical capacitance (C_{el}) by applying a ramp potential.

NOTE: When the rate of the voltage change in the ramp command is 10 mV/10 ms (or 1 V/s) followed by −10 mV/10 ms, the amplitude of the current jump upon changes in the slope corresponds to the read of the membrane's capacitance value (*e.g.*, 100 pA → 100 pF).

5.2   Evaluate the bilayer area of two bubbles stacked one on the other and focus the microscope at the bilayer level to view the edge of the bilayer (**Figure 6**).

NOTE: The bilayer shape is mostly circular, and the area is calculated from the radius.

308

309 5.3   Calculate the specific membrane capacitance (C_{sp}) by dividing the electrical capacitance
310 by the bilayer area ($C_{sp} = C_{el}/A$).

311

312 5.4   Calculate the bilayer thickness (thickness of the hydrophobic core) using $D_c = (\varepsilon_r \varepsilon_0)/C_{sp}$
313 (where ε_r and ε_0 represent the permittivity of the hydrophobic region of the bilayer and the
314 permittivity of a vacuum, respectively).

315

316 **REPRESENTATIVE RESULTS:**
317 A typical CBB had a diameter of 50 µm (**Figure 5, 6**) and the specific membrane capacitance in
318 hexadecane was 0.65 µF/cm^2. The bubble size was arbitrarily controlled by the intra-bubble
319 pressure. When small bubbles are necessary for low-noise recordings, the tip diameter should be
320 correspondingly small. For example, for a bubble size of 50 µm in diameter, the tip diameter
321 should be 30 µm.

322

323 Once the CBB had formed, the channel molecules either in aqueous solution or in the liposome
324 were spontaneously inserted into the bilayer within a span of a few to dozens of minutes.
325 Insertion of the channels was confirmed by the stepwise increase of current amplitude (**Figure 7**)
326 under the applied membrane potential. The smaller membrane area (<1,000 µm^2) relative to that
327 in the conventional PLB and DIB systems substantially improved the electrical signal-to-noise
328 ratio.

329

330 Current recordings could be continued until the membrane was disrupted and the two bubbles
331 merged. New bubbles were blown and the CBB formed immediately and repeatedly. The pipette
332 could be used repeatedly within a day.

333

334 **Duty cycle of attach and detach**. A bilayer formed by the CBB method can be disintegrated into
335 two monolayers. Detach-attach of the CBB can be repeated by manipulating the two bubbles
336 (duty cycle of detach-attach). This process was monitored by the appearance of the channel
337 current of pTB, a peptide channel from a marine sponge, as it was readily inserted into the lipid
338 bilayer to form a monovalent cation-selective pore[52,56]. The pTB channel current emerged
339 immediately after attaching the two monolayers, and the current became larger as the area of
340 contact increased (**Figure 8**). The amplitude of the current was synchronized with the detach-
341 attach manipulation of the CBB.

342

343 **Single-channel measurements of the KcsA channel**. The KcsA potassium channel is pH sensitive,
344 being activated by acidic intracellular pH[57]. Thus, the electrolyte solution was set to be
345 asymmetric[58–60]. In step 4.2 of CBB formation, the pipette solution of the left side was set at pH
346 4, whereas that of the right side was set at pH 7.5[25]. In step 4.7.1, a proteoliposome suspension,
347 rather than liposome suspension, was placed onto the slide glass for aspiration into the left
348 pipette. Accordingly, the KcsA channel was oriented in the membrane with its cytoplasmic
349 domain facing the left bubble. Liposomes with the protein:lipid weight ratio of 1:2000 are
350 suitable for single-channel current recording, and those with a 1:10 ratio are for macroscopic
351 current recording.

352
353 **Asymmetric membrane**. An asymmetric lipid bilayer can be formed using different liposome
354 suspensions for each bubble (lipid-in)[15,32]. The orientation of the KcsA channel in the membrane
355 was regulated by setting an asymmetric solution pH (pH 4_{in}/pH 7_{out}), leading to its cytoplasmic
356 side being toward the left side. Accordingly, the left side was assigned as "in," whereas the right
357 side was assigned as "out." To examine lipid dependence on the gating of the KcsA channel, four
358 types of CBB (including an asymmetric CBB) were used; namely, PG_{in}/PG_{out}, PG_{in}/PC_{out}, PC_{in}/PG_{out},
359 and PC_{in}/PC_{out} (PG: phosphatidylglycerol) (**Figure 9**). The KcsA channel exhibited high open
360 probability (>90%) only in the PG_{in}/PG_{out} and PG_{in}/PC_{out} membranes. The KcsA channel requires
361 the existence of anionic phospholipids in the inner leaflet of the membrane for a high open
362 probability[26].
363
364 **TABLES AND FIGURE LEGENDS:**
365 **Figure 1: Lipid bilayer and patch-clamp methods.** Various methods have been developed for
366 forming the lipid bilayer. (A) Patch-clamp method. (B) Conventional planar lipid bilayer method,
367 which provides a free-standing, mostly vertical, bilayer on a small hole. (C) Tip-dip method. A
368 monolayer at the air–water interface is positioned on the tip of a glass electrode. Various
369 modifications have been developed. (D) Droplet interface bilayer method. (E) Contact bubble
370 bilayer method.
371
372 **Figure 2: Contact bubble bilayer formation.** As a bubble is blown into an oil phase, lipid
373 molecules transfer spontaneously to the water–oil interface. Different types of lipids as
374 liposomes are included in each bubble (lipid-in), and monolayers are formed exclusively by the
375 relevant lipids. Docking two monolayers generates an asymmetric bilayer membrane.
376
377 **Figure 3: Distinct phases in the contact bubble bilayer, and the dynamics of lipids therein**. Lipids
378 lining the bubble constitute a phase where they belong to either a monolayer or a bilayer. Flip-
379 flop of lipids across the bilayer is infrequent, and the asymmetric membrane is retained for a long
380 time.
381
382 **Figure 4: Delivering lipids with the lipid-out or lipid-in method.** Lipids are added either in an
383 organic solvent (lipid-out; A and C) or in aqueous solution as liposomes (lipid-in; B and D). In the
384 lipid-in method, an asymmetric membrane is formed. Channel-forming substances that are
385 soluble in aqueous solution (blue), such as peptides, are added into one of the bubbles and
386 spontaneously inserted into the bilayer. In the lipid-in method, a part of the channels is inserted
387 into liposomes, which are then fused to the bilayer. Channel proteins (red) are reconstituted into
388 liposomes in either the lipid-out or lipid-in method, which are then added into one of the bubbles
389 and spontaneously fused to the bilayer.
390
391 **Figure 5: Formation and microscopic image of the contact bubble bilayer.** The bilayer is
392 observed from a tangential direction.
393

394 **Figure 6: Observation of the bilayer for measuring the bilayer area.** Two bubbles are stacked
395 one on the other through pipette manipulation, and the microscope is focused at the level of the
396 contact bubble bilayer.
397
398 **Figure 7: Stepwise insertion of the KcsA channel into the contact bubble bilayer membrane.**
399 The E71A mutant of the KcsA channel was used to show the immediate detection of the inserted
400 channels.
401
402 **Figure 8: Detach-attach of monolayers and responses of channels.** A peptide channel,
403 polytheonamide B, was added into one of the bubbles, which was then spontaneously
404 incorporated into the membrane. When detached, channels in the membrane withdrew but
405 were retained at the membrane leaflet of the channel-added side. Upon attachment, the
406 channels were inserted into the membrane.
407
408 **Figure 9: Formation of an asymmetric membrane and channel activity dependent on the**
409 **composition of the leaflet.** The KcsA channel is regulated by acidic lipids, such as PG, in the inner
410 leaflet of the membrane.
411
412 **Table 1: Characteristics of various lipid bilayer membranes.**
413
414 **DISCUSSION:**
415 The CBB method of lipid bilayer formation is based on the principle of a water-in-oil droplet lined
416 by a monolayer[20]. Technically, the procedures for forming CBBs are easy, especially for patch-
417 clamp researchers, who are proficient in manipulating glass micropipettes. The
418 electrophysiological setup for the patch clamp is readily used in the CBB when two pipette
419 manipulators with microinjectors are available. On the other hand, because the CBB is a
420 successor of the conventional PLB, for which a large amount of physicochemical knowledge has
421 been accumulated[8,61], this background as well as knowledge of surface chemistry[38] is helpful for
422 operating and manipulating CBBs. The CBB serves a versatile platform for studying channel–
423 membrane interplays. By using the CBB method, the bilayer can be modulated in terms of
424 chemical composition and physical manipulation. In the CBB, lipids lining the bubble can diffuse
425 freely across the border of the monolayer phase and leaflet of the bilayer, which enables various
426 experimental procedures. Various physical and chemical modifications to the bilayer are now
427 made possible, and we have developed other techniques such as the bilayer attach-detach and
428 the membrane perfusion methods[45]. We have extended CBBs for *in vitro* channel protein
429 synthesis, where channel synthesis was performed in the bubble, and newly synthesized channel
430 proteins were subjected to spontaneous transfer to the bilayer under application of a membrane
431 potential where nascent channel functions of the KcsA channel was traced from the time of
432 initiating the transcription/translation of KcsA DNA[62].
433
434 Among the steps of the protocol, maintaining the bubble size is critical. The bubbles may slowly
435 swell or shrink spontaneously because lipid transfer to the interface is slow and the monolayer
436 tension is prone to change. In particular, the first bubble inflated just after suction of the
437 liposome solution (steps 4.8.3 and 4.10) is hard to maintain because the monolayer tension is

438   lowered by the liposomes accumulated at the tip of the pipette. Discarding the initial bubbles
439   and subsequent formation of the bubbles provide the stable CBB. Visual tracing of the bubble
440   size and fine tuning of the pressure are necessary.
441
442   There are experimental limitations to the CBB method. Although the CBB is designed for
443   electrophysiological measurements, the electrical resistance of the bilayer for certain lipid
444   compositions may not be high enough (*e.g.*, 100 GΩ) for single-channel recordings. For example,
445   dioleoyl phosphatidylcholine is frequently used for liposomes, but it forms electrically leaky CBBs
446   at 25 °C[63]. CBBs cannot even be formed with lipid species whose phase transition temperature is
447   above the recording temperature. Indeed, CBB formation was difficult with dipalmitoyl
448   phosphatidylcholine at room temperature, but raising the temperature above T_m circumvented
449   the problem[64]. In these experiments, the temperature was controlled by using a transparent heat
450   plate (see **Table of Materials**) beneath the slide glass[62]. The phase transition temperature of the
451   frequently used lipid diphytanoyl phosphatidylcholine is below 0 °C [65], and CBBs are readily
452   formed[32,45,51] at a broad temperature range.
453
454   Phospholipids of the water–oil interface are provided from either the lipid-in or lipid-out method,
455   where the transfer rate is relatively slower in the lipid-out than in the lipid-in method[63]. In
456   addition, for reasons unknown, the electrical resistance of the bilayer membrane is relatively
457   smaller with the lipid-out method than with the lipid-in method.
458
459   In the protocol (step 4.8), a solution of liposomes and channel-reconstituted liposomes (1 µL) is
460   loaded from the tip of the pipette (steps 4.9 and 4.10), and the rest of the pipette contains the
461   previously filled electrolyte solution. This is merely for conserving materials, such as lipids and
462   channel molecules. Consequently, the liposomes diffuse gradually toward the upper pipette
463   solution, and after a while, the lipid concentration at the tip of the pipette becomes insufficient
464   to form lipid monolayers. In this case, fresh liposome solution should be aspirated from the tip
465   (step 4.8). This temporal concentration change can be circumvented when the whole pipette is
466   filled with the solution containing lipids and channel molecules, whereas liposomes gradually
467   settle down into the bubbles. In this case, bubbles are renewed.
468
469   Electrophysiologically, the CBB size is larger than that of the patch clamp, thus yielding a larger
470   membrane capacitance. However, the series resistance (the electrical resistance in a series of
471   membrane resistance) is much lower (~100 kΩ) than that for the patch clamp (pipette resistance
472   > 1 MΩ), which accelerates the speed of the voltage clamp and attenuates the background noise
473   and series resistance errors in the voltage-clamped membrane potentials[66–68]. The background
474   noise is a function of the membrane capacitance and the series resistance, where a low series
475   resistance complements a high membrane capacitance, resulting in low background noise[9].
476
477   As a rule of lipid bilayer experiments, detergents should not be used to wash glassware. Even a
478   trace amount of detergent perturbs the integrity of the bilayer. Organic solvents such as
479   chloroform/methanol and ethanol should be used for such cleaning purposes instead.
480

481 Overall, the CBB integrates the benefits of both the patch clamp (*e.g.*, the mechanical
482 manipulation of the membrane) and the PLB (*e.g.*, the ability to modify the lipid composition of
483 the membrane). Various types of channel-forming substances and channel proteins have been
484 studied[52,69–71]. The development of this method is opportune since increasingly more researchers
485 are focusing on channel–membrane interplay, and the CBB provides a versatile platform for
486 various experiments. Further experimental developments in the CBB method are expected.
487
495
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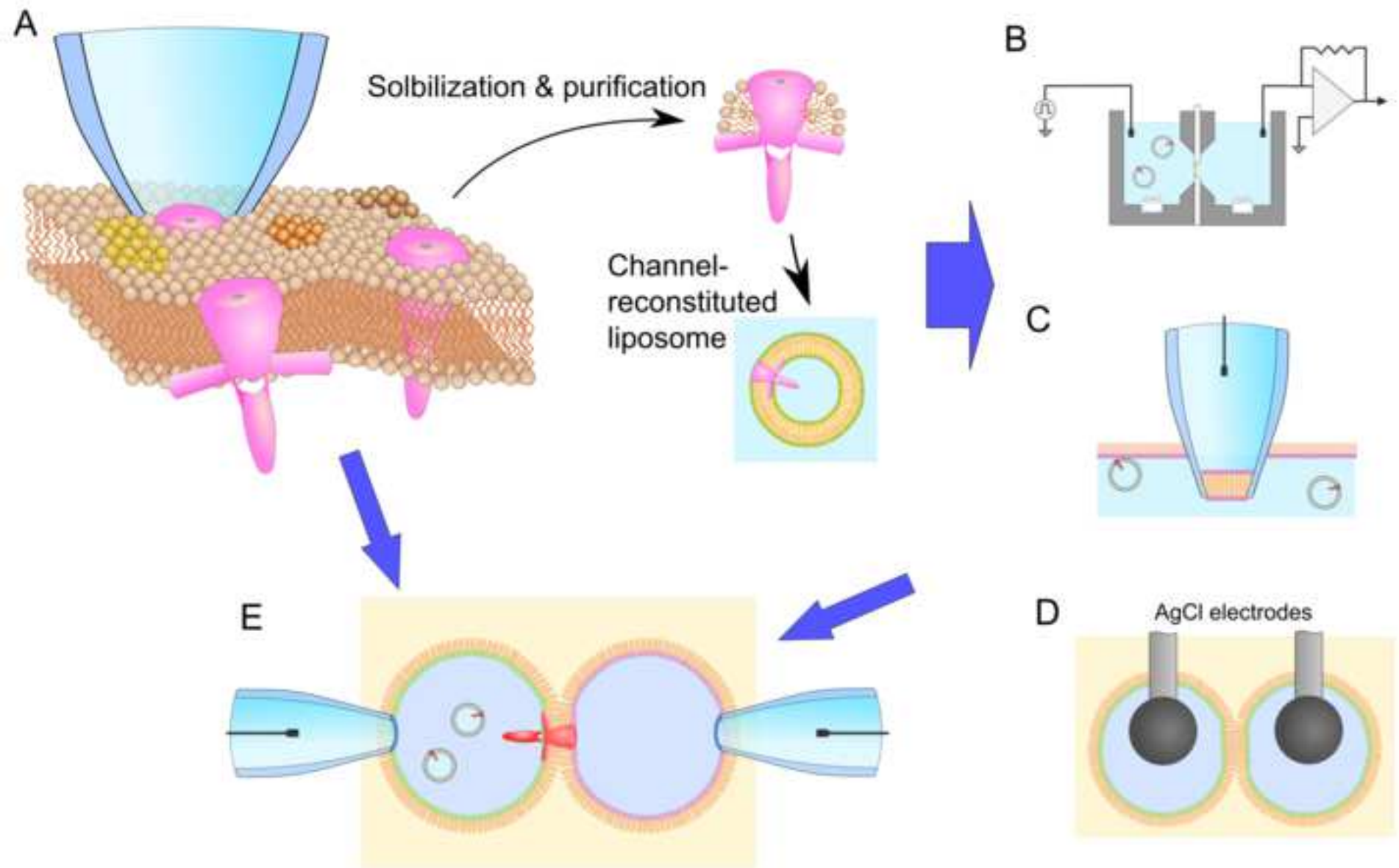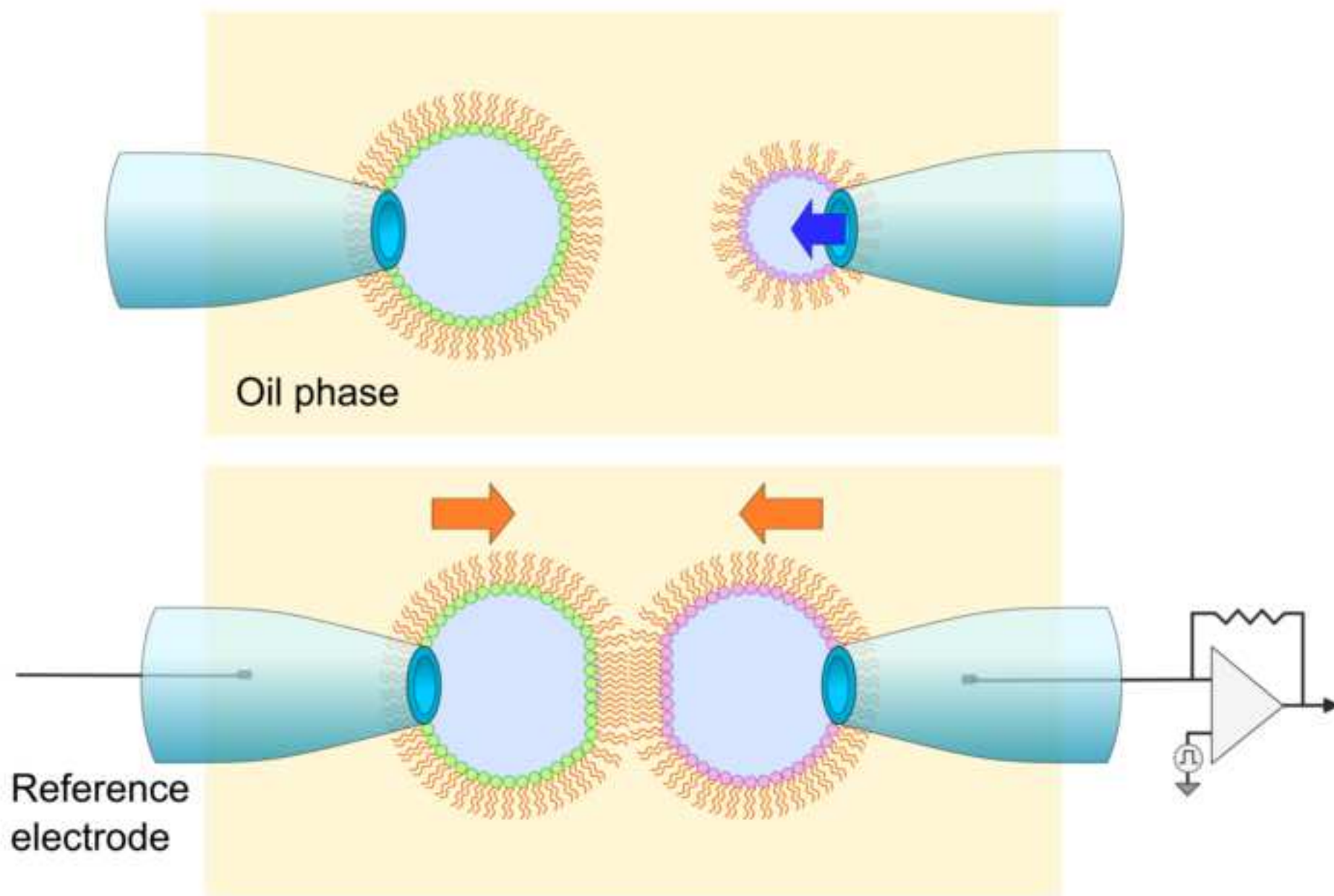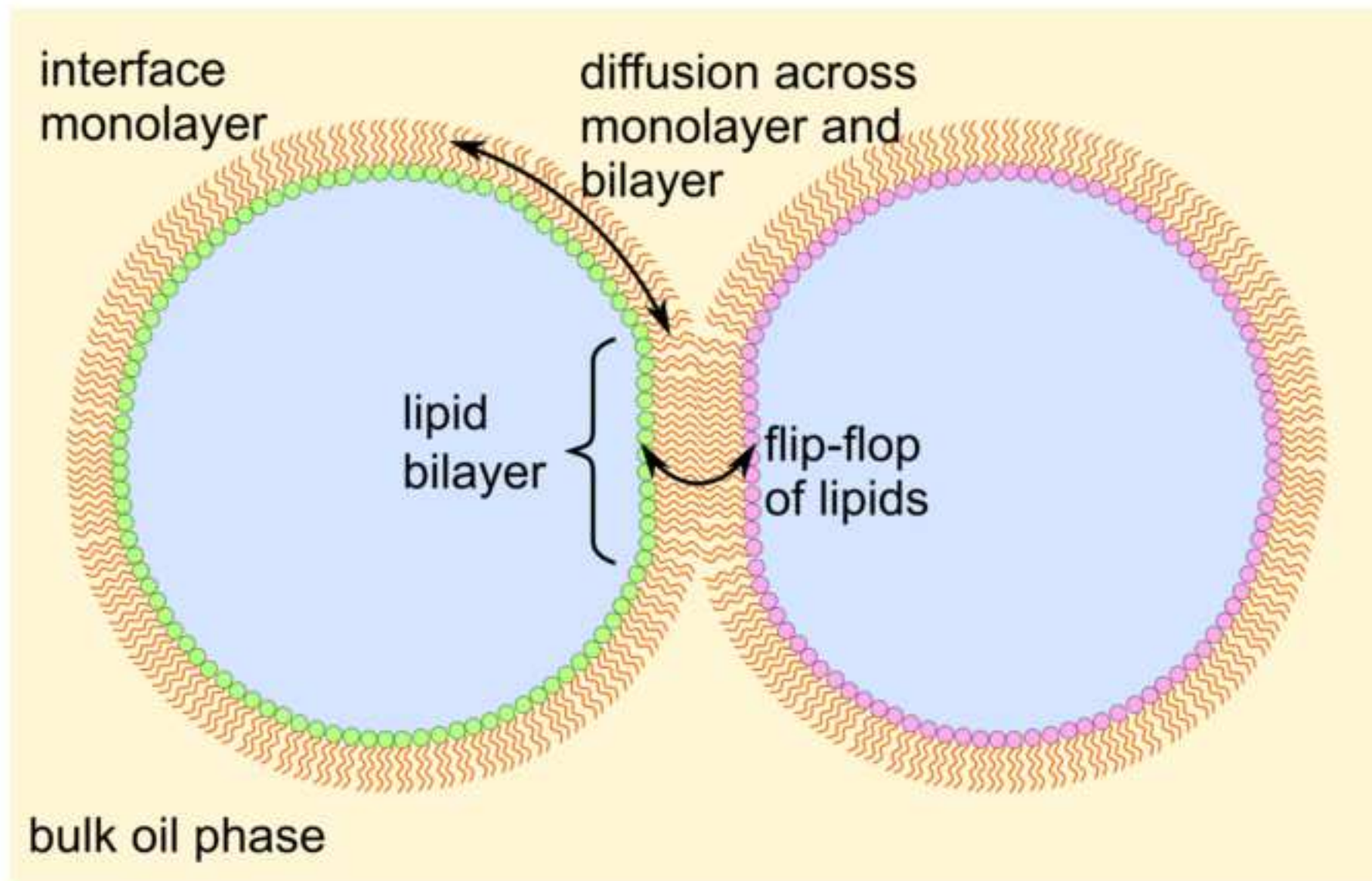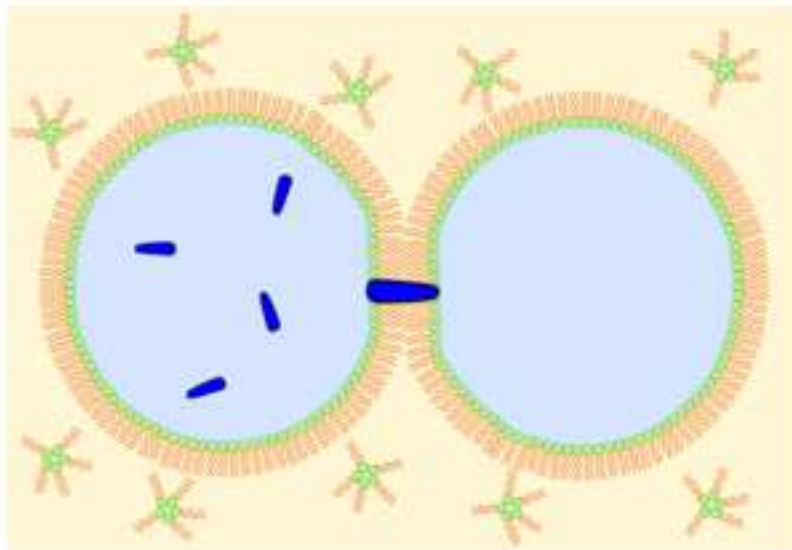675

Figure 1

A

Solbilization & purification

Channel-
reconstituted
liposome

B

C

D

AgCl electrodes

E

Figure 2

Oil phase

Reference electrode

Figure 3

interface monolayer

diffusion across monolayer and bilayer

lipid bilayer

flip-flop of lipids

bulk oil phase

Figure 4

Figure 5

50 μm

Figure 6

Figure 7

Oil

← Bilayer

Objective lens

Bilayer

Figure 8

Figure 9

PG

PC

PG$_{in}$/PG$_{out}$

10 pA

1 s

PG$_{in}$/PC$_{out}$

PC$_{in}$/PG$_{out}$

PC$_{in}$/PC$_{out}$

Table 1

Success rate[1]
Immediate reformability[2]
Asymmetric membrane
Solution exchange
S/N ratio[3]
Membrane deformation
Mechanical manipulation
Membrane perfusion
Lipid compositions

[1]The success rate of forming stable lipid bilayer per trial.

[2]Formation of a new membrane quickly after break down of a membrane. Also, membrane is formed repeatedly.

[3]The signal-to-noise (S/N) ratio represents the electrical background noise primary generated from the membrane capacitance.

[4]Concave or convex membrane can be formed by applying different inner pressure between two bubbles.

[5]Only increasing the membrane tension.

[6]Phospholipids that can form giant unilamellar vesicles are applicable, but the giga-seal may not be attained.

Conventional PLB	Liposome patch
high	low
yes	no
yes	no
slow	very fast
low	high
no	no
no	yes[5]
no	no
diverse	limited[6]

DIB	CBB
very high	very high
intermediate	yes
yes	yes
slow	fast
low	high
no	yes[4]
yes	yes
yes	yes
diverse	diverse

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
Azolectin (L-α-Phosphatidylcholine, Type IV-S)	Sigma-Aldrich	P3644	
A/D Converter	Molecular Divices	Digidata1550A	
Ag/AgCl electrode	Warner Instruments	64-1317	
Bath Sonicator	Branson	M1800H-J	
Camera	Hamamatsu Photonics	C11440-10C	
Glass Capillary	Harvard Apparatus	30-0062	
Hepes	Dojindo	342-01375	
Hole Slideglass	Matsunami Glass	S339929	
Inverted Microscope	Olympus	IX73	
Isolation Table	Herz	TDI-86LA(Y)2	
Micro Injenctor	Narishige	IM-11-2	
Micro Manipulator	Narishige	EMM	
Microforge	Narishige	MF-830	
Micropipette holder			
n-Hexadecane	Nacalai	07819-32	
Patch-Clamp Amplifier	HEKA	EPC800	
Pipette Puller	Sutter Instrument Co.	P-87	
POPC (1-palmitoyl-2-oleoyl-glycero-3-phosphocholine)	Avanti Polar Lipids	850457	

POPE (1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine)	Avanti Polar Lipids	850757
POPG (1-palmitoyl-2-oleoyl-sn-glycero-3-phospho-(1'-rac-glycerol))	Avanti Polar Lipids	840457
Potassium Chloride	Nacalai	28514-75
Rotary Evapolator	Iwaki	REN-1000
Succinic Acid	Nacalai	32402-05
Vacuum Pump	Buchi	V-100

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Dear Editor,

We would like to thank the Editor for handling our manuscript and are grateful for the constructive comments provided. We have responded to all points raised by the reviewers and revised the manuscript accordingly. We have uploaded a pdf file in which all revised sentences are highlighted.

Responses to Reviewer #1:

Thank you very much for the constructive comments. We have added a statement regarding the experimental condition, as far as possible. Also, we added suggestions on how to form a stable lipid bilayer.

Minor Concerns:
Since lipids and lipid bilayers and their stability is significantly effected by the temperature, pH, salt and buffer conditions, I will recommend authors to elaborate on the information regarding their conditions in the paper. What are the limitations of forming successful and high efficiency contact bubble bilayers in a variety of electrolytes (such as NaCl, KCl), and pH conditions. If the authors have experience with modulating these conditions or having a condition, which performs the best for bilayer stability, they should definitely include it in this manuscript. This will be highly useful for researchers adapting this protocol in their labs.

The limitations of the CBB method have been added and are summarized in the Discussion section.

Responses to Reviewer #2:

We appreciate the valuable comments on our manuscript. The awkward expressions were corrected according to the suggestions of a native English speaker.

-The term "patch-clampers" is overly colloquial English for me. I would replace these words with a different terminology in all places where the term appears.

We agree that the term "patch-clampers" is colloquial English, and we had discussed this term with a native English speaker doing patch clamp experiments. The term is one that we all understand. The alternative would be to refer to such people as "specialists in patch-clamp recording techniques" or "users of patch-clamp recording techniques." These phrases are awkward and cumbersome. Also, there is no concise alternative. So generally speaking, we would prefer to retain "patch-clamper."

-In the abstract, the word "manipulability" is awkward. I would choose a different wording.

We have replaced "bilayer mechanical manipulability" with "to manipulate bilayer mechanics" in the text.

-In the short abstract (and a few other places), there is a choice to be made between past tense and present tense ("was blown" and "was formed" versus "is blown" and "is formed"). There are other places where the past tense and present tense are mixed. Please try to be consistent.

We have unified the tenses in the text.

-In line 101 the word "impat" is incorrect. Perhaps "impart" was intended.

-Insert "the" at the end of line 140 to give: "We found that the cholesterol content . . . "

We have inserted "the" at the indicated position.

-In line 141 change "switch" to "switching" and delete "of".

We have revised the phrase as suggested.

-In line 355, change "is" to "are" to agree with "procedures." The remainder of the Discussion has multiple places where there is awkward use of the English language. Please edit the entire manuscript for minor improvements in the grammar and the flow of the language. Notably, in line 391, see "glass wears" ?? There are other examples, too numerous, that need to be checked.

Thank you for this indication. The entire manuscript has been copy edited by a professional English-language editor.

Responses to Reviewer #3:

Thank you very much for

1. Line 115: Please elaborate what you mean by changes in surface tension? Changes in the bilayer tension or changes in the monolayer tension? Presumably both, but how do you sort this out?

Thank you for pointing out this important aspect. We have revised the relevant part as follows:

"The changes in the contact angle indicate changes in the bilayer tension, given that the monolayer tension is evaluated from changes in the contact angle as a function of the membrane potential (Young-Lippmann equation: $\gamma_{mo} = C_m V2/4 (\cos(\theta_0) - \cos(\theta_v))$, where C_m is the membrane capacitance, V is the membrane potential, and θ_0 and θ_v are the contact angles at 0 and V mV, respectively)."

2. Line 119: At first reading, I did not understand what was meant by "multiple phases." Multiple components is likely to be a better term.

We have revised the indicated part as follows:

"A CBB system consists of distinct phases; namely, a bulk oil phase, water bubbles coated with a monolayer, and a contacting bilayer."

3. Line 125: Is it necessary to introduce the Marangoni effect? It is likely to confuse, unless you make use of this later in the presentation.

The "Marangoni effect" is a concept known among surface chemists and admittedly might be unfamiliar to other readers. However, this phenomenon is an essential one that must be kept in mind for the CBB experiment. In the revised version, we have explained the Marangoni effect more clearly. Also, the reference #47 was replaced.

4. Lines 127 - 130: This needs to be clarified; the transition temperature for DOPC is close to -20 C, which should allow for stable bilayers.

We have moved the indicated part to the Discussion, revised as follows:

"For example, dioleoylphosphatidylcholine is frequently used for liposomes, but it forms electrically leaky CBBs at 25 °C[62]. CBBs cannot even be formed with lipid species whose phase transition temperature is above the recording temperature. Indeed, CBB formation was difficult with dipalmitoylphosphatidylcholine at room temperature, but raising the temperature above T_m circumvented the problem[63]."

5. Line 183, and elsewhere: The term "hole slide glass" is unknown to me, and I did not see it illustrated in any of the figures.

We have illustrated the hole slide glass in the text: hole slide glass (slide glass with a shallow concave well)

6. Line 193: This is in part redundant with what is stated on line 188.
7. Line 201: Again some redundancy.

We have revised the indicated parts.

8. Lines 213-214: Please change the tense to be present tense.

We have revised the sentence using the present tense.

9. Lines 226 - 231: This was unclear to me. Please revise.

We have moved the indicated part to the Discussion, revised as follows:

"In our protocol (step 4.8), a solution of liposomes and channel-reconstituted liposomes (1 µL) is loaded from the tip of the pipette (steps 4.9 and 4.10), and the rest of the pipette contains the previously filled electrolyte solution. This is merely for conserving materials, such as lipids and channel molecules. Consequently, the liposomes diffuse gradually toward the upper pipette solution, and after a while, the lipid concentration at the tip of the pipette becomes insufficient to form lipid monolayers. In this case, fresh liposome solution should be aspirated from the tip (step 4.8)."

10. Lines 234 - 235: This probably should moved to be right after lines 127 - 130.

We have moved the relevant part to the Discussion.

11. Lines 263 - 264: It is not clear to me what the authors refer to. It would be good to refer to Figure 5, which I assume shows what the authors have in mind.

We have added a new figure (Figure 7) to illustrate the indicated point.

12. Line 355: Please change "is" to "are."

This has been corrected.

13. Line 360: Suggest deleting "and unprecedented."
14. Line 365: Same comment.

These have been deleted.

15. Table 1: It would be preferable if Rows 1 "Success Rate" and 5 "S/N ration" had numbers assigned

to "low," "high" and "very high."

In this table, we intended to show the rough characterization of and comparison between the techniques. Each item was expressed qualitatively because they varied substantially in each experiment and channels used.

Responses to the editorial comments:

We have made the corrections throughout the text according to the editor's comments.

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
2. Please provide an email address for each author.
3. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

This has been corrected (Lines 15–18).

4. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

This has been corrected (Lines 95–98).

5. Please spell out each abbreviation the first time it is used.

This has been corrected.

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This has been corrected.

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, dashes, or indentations.
8. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

This has been corrected (Lines 154–258).

9. Lines 147-154, 226-235, etc.: The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please move the discussion about the protocol to the Discussion.

This has been moved to Lines 389–392.

10. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:
Line 157: Listing an approximate volume to prepare would be helpful.

Line 160: What temperature is set during rotary evaporation and how long does it take to form a thin phospholipid film?

Line 175: Please specify the settings of the pipette puller.

It is generally difficult to show the settings since the platinum heating sheet varies substantially, and so too the settings.

Line 179: Heat for how long?

Line 185: What is considered to be an appropriate volume?

All the points, except for Line 175, were corrected.

11. Step 5 is difficult to follow. Please try to combine this specific protocol with previous steps. For instance, indicate the modifications made for KcsA potassium channel in steps 2 and 7.

Step 1.5 was included in Lines 170–172.

12. Please include single-line spaces between all paragraphs, headings, steps, etc.

This has been corrected.

13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

This has been corrected.

16. Discussion: Please also discuss critical steps within the protocol.

We have added sentences for the critical steps (Lines 378–384)

17. For in-text references, the corresponding reference numbers should appear as superscripts after the appropriate statement(s) in the text (before punctuation but after closed parenthesis). The references should be numbered in order of appearance.

This has been corrected.

18. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. See the example below:

Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

19. References: Please do not abbreviate journal titles.

This has been corrected.

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1 Lipid Bilayer Experiments with Contact Bubble Bilayers for Patch-~~Clamper~~Clampers

2

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12

13 **KEYWORDS:** Lipid bilayer, patch-clamp, water-in-oil droplet, monolayer, ion channel,

14 electrophysiology, surface chemistry.

15

16 **SHORT ABSTRACT:** *(10 words minimum, 50 words maximum)50*

17 ~~Contact~~Herein, we present a protocol for the formation of lipid bilayers using a contact bubble

18 bilayer ~~is a water-in-oil droplet bilayer, which can be mechanically manipulated via glass~~

19 ~~pipette. From a patch pipette (tip diameter = 30 μm), a~~method. A water bubble ~~was~~is blown

20 from a patch pipette into an organic solvent, whereby a monolayer ~~was~~is formed at the water–

21 oil interface~~, subjected~~. Two pipettes are manipulated to ~~be docked~~ dock the bubbles to form a

22 bilayer.

23

24 **LONG ABSTRACT:** *(150 words minimum, 300 words maximum) 250,*

25 Lipid ~~bilayer experiments~~bilayers provide a unique experimental platform for functional studies

26 of ion channels, allowing the examination of channel--membrane interactions under various

27 membrane lipid compositions. Among them, ~~water-in-oil droplet bilayers, such as the~~ the

28 droplet interface bilayer ~~(DIB), have~~has gained popularity; ~~but a~~however, the large membrane

29 size hinders the ~~recordings~~recording of low electrical background noise ~~that the patch-clampers~~

30 ~~anticipate.~~. We have established ~~the~~a contact bubble bilayer (CBB) method~~, which~~ that

31 combines the benefits of planar lipid bilayer and patch-clamp methods, such as the ability to

32 vary the lipid ~~compositions~~composition and to manipulate the bilayer ~~mechanical~~

33 ~~manipulability~~mechanics, respectively. ~~With an experimental~~Using the setup for conventional

34 patch-~~clamping~~clamp experiments, CBB-based experiments can be readily performed.

35 ~~Electrolyte~~In brief, an electrolyte solution in a glass pipette ~~was~~is blown into an organic solvent

36 phase (hexadecane), and ~~a bubble was~~the pipette pressure is maintained to obtain a stable

37 bubble size. The bubble is spontaneously lined with a lipid monolayer (pure lipids or mixed

38 lipids~~) through the transfer of lipids from either the electrolyte solution or the organic solvent.~~),

39 which is provided from liposomes in the bubbles. Next, two monolayer-lined bubbles (~50 μm

40 in diameter) at the tip of the glass pipettes ~~were docked for bilayer formation. Thereafter,~~

41 ~~pipette pressure was maintained~~are docked for ~~a stable bubble size. By applying a high~~

42 ~~membrane potential (~1000 mV), a membrane was broken and the two bubbles were fused,~~

43 ~~whereby the electrode offset potential is readily adjusted. The CBB is renewed by blowing out a~~

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44 ~~fused bubble and forming new bubbles.~~<u>bilayer formation.</u> Introduction of channel-
45 reconstituted liposomes ~~in a~~<u>into the</u> bubble ~~led~~<u>leads</u> to the incorporation of ~~the~~ channels in the
46 bilayer, allowing for single-channel current ~~recordings~~<u>recording</u> with a signal-to-noise ratio
47 comparable to ~~patch-clamp recordings.~~<u>that of patch-clamp recordings. CBBs with an</u>
48 <u>asymmetric lipid composition are readily formed. The CBB is renewed repeatedly by blowing</u>
49 <u>out the previous bubbles and forming new ones.</u> Various chemical and physical perturbations
50 ~~can be applied~~ (e.g., membrane perfusion and bilayer tension) <u>can be imposed</u> on ~~CBB and~~
51 ~~here~~<u>the CBBs. Herein,</u> we present the basic procedure for CBB formation.
52
53
54 **INTRODUCTION:** *~~(150 words minimum, 1500 words maximum)~~*
55 ~~Ion channels carryout their function in the membrane, without which the channel function that~~
56 ~~is required for generating ion flux is never attained. Functionally, the membrane is an electric~~
57 ~~insulator in which ion channels are embedded, and all the cell membranes are imparted with the~~
58 ~~resting membrane potential. In experiments, an arbitrary membrane potential is imposed from~~
59 ~~an external circuit by which electric current through channels is measured. Measurements of ion~~
60 ~~flux at different membrane potentials constitute quantitative studies of channel molecular~~
61 ~~properties, such as ion permeation and gating (1, 2). The membrane platform is served by either~~
62 ~~the cell membrane or lipid bilayer membrane. Historically, single channel electric current~~
63 ~~recordings were first performed in lipid bilayers (3, 4), and the relevant techniques were~~
64 ~~developed for cell membrane, such as the patch-clamp (Fig. 1E)(5, 6). Since then these two~~
65 ~~techniques have been evolved separately for different purposes (Fig. 1)(7, 8).~~
66 ~~Membrane lipids and bilayer membranes are currently in focus for their roles in supporting~~
67 ~~structure and function of channel proteins, and the readily availability of methods to vary the~~
68 ~~lipid compositions in bilayers is in demand. The lipid bilayer methods such as planar lipid bilayer~~
69 ~~(PLB)(8–11) and a water-in-oil droplet bilayer (12), including droplet interface bilayer (DIB)(13–~~
70 ~~19) (Fig. 1), are methods of choice, providing an opportunity for examining channel function~~
71 ~~under varying lipid compositions (20). Even though DIB is technically much easier than the~~
72 ~~conventional planar lipid bilayer, the large size of the bilayer has created a disincentive for patch-~~
73 ~~clampers to apply it for studying single channel current recordings having usual sized~~
74 ~~conductance (< 100 pS).~~
75 ~~To circumvent the background noise, the bilayer area must be minimized. This issue recalls~~
76 ~~to the repetitions of history for developing electrophysiological techniques (Fig. 1). In early days,~~
77 ~~a small size bilayer (1 – 30 μm in diameter) was formed at the tip of a pipette (tip-dip method;~~
78 ~~Fig. 1C)(21–23), rather than using a free-standing bilayer (~100 μm in diameter) on a hydrophobic~~
79 ~~septum in a chamber (Fig. 1B). The tip-dip method allowed electric measurements with much~~
80 ~~lower background noise (24). Our experiences with the planar lipid bilayer (25, 26), tip-dip (22,~~
81 ~~23, 27) and patch-clamp (28–31) methods led us to an idea of a novel method for forming a lipid~~
82 ~~bilayer using the principles of water-in-oil bilayer. This is referred to as the contact bubble bilayer~~
83 ~~(CBB) method (20, 32). Rather than hanging the water droplets in an oil phase (Fig. 1D), a water~~
84 ~~bubble was blown from a glass pipette (the tip diameter of approximately 30 μm) into the oil~~
85 ~~phase (Fig. 1F and 2), where the bubble was maintained by applying steady pressure. A~~
86 ~~monolayer was formed at the water-oil interface at the surface of the bubble. Two bubbles were~~

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87 docked through manipulation with two glass pipettes, and the bilayer was spontaneously formed
88 as the two monolayers approached each other, yielding an equilibrium bilayer area. The size of
89 the bubble is controlled by the intra-bubble pressure (holding pressure), and so is the bilayer size.
90 The average size of 50 μm in diameter is frequently used. The volume of the bubble is small (<
91 100 pL), but it is connected to the pipette solution, whose volume is in μL range.
92 There are many benefits to usin the CBB (Table 1). As a lipid bilayer method, membranes of
93 various lipid compositions can be formed and asymmetric membranes are more readily formed
94 (32) than by the conventional folding method (33). The bilayer is able to be mechanically
95 manipulated, which cannot be performed in the conventional planar lipid bilayer, except for
96 bending the bilayer with a hydrostatic pressure difference (34, 35). By changing the holding
97 pressure, the bubbles either expand or shrink, leading to increased or decreased membrane
98 tension. The bilayer is mechanically detachable into monolayers, similar to the freeze-fracture
99 technique (36, 37) of membranes in morphological studies, but this unprecedented maneuver
100 allows for repeated detach and attach cycles (32). A small volume of the electrolyte solution
101 within the bubble allows efficient fusion of channel-reconstituted liposomes to the bilayer, and
102 the probability of getting channel recordings is much higher than with the conventional planar
103 lipid bilayer technique. The small bubble volume also allows rapid perfusion within ~20 ms once
104 another injection pipette is inserted into either of the bubbles. Unlike the patch-clamp, a broken
105 membrane is reformed immediately and repeatedly, and pipettes can be repeatedly used several
106 times a day.
107 Before presenting a detailed protocol for the formation of the CBBFor ion channels, the cell
108 membrane is not simply a supporting material but a partner for generating the ion flux.
109 Functionally, the membrane is an electrical insulator in which ion channels are embedded, and
110 all cell membranes are imparted with a resting membrane potential. In previous studies, an
111 arbitrary membrane potential was imposed from an external circuit by which electrical current
112 through the channels was measured. This quantitative evaluation of the ion flux at different
113 membrane potentials revealed the molecular properties of these channels, such as their ion-
114 selective permeation and gating functions[1, 2]. The membrane platform for functional studies of
115 ion channels is either the cell membrane or lipid bilayer membrane. Historically, single-channel
116 electrical current recordings were first performed in lipid bilayers[3, 4], and the relevant
117 techniques were developed for cell membranes, such as the patch-clamp method (Figure 1A)[5,
118 6]. Since then, these two techniques have evolved separately for different purposes (Figure 1)[7, 8].
119 Membrane lipids and bilayer membranes are currently the focus of research for their roles
120 in supporting the structure and function of channel proteins. Therefore, the ready availability of
121 methods to vary the lipid composition in bilayers is in high demand. Lipid bilayer formation
122 methods such as the planar lipid bilayer (PLB)[8–11], water-in-oil droplet bilayer[12], and droplet
123 interface bilayer (DIB)[13–19] techniques (Figure 1) are common choices, providing an opportunity
124 for examining channel function under varying lipid compositions[20]. Although the DIB is
125 technically much easier to produce than the conventional PLB, the large size of the DIB has
126 created a disincentive for patch-clampers to apply it for studying single-channel current
127 recordings with usual-sized conductance (<100 pS).
128 To circumvent the background noise, the bilayer area must be minimized. This issue recalls
129 the repetitions of history in developing electrophysiological techniques for lipid bilayers (Figure

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130 1). In the early days, a small-sized bilayer (1–30 μm in diameter) was formed at the tip of a
131 pipette (tip-dip method; Figure 1C)[21–23], rather than using a free-standing bilayer (~100 μm in
132 diameter) on a hydrophobic septum in a chamber (Figure 1B). The tip-dip method allowed for
133 electrical measurements with much lower background noise[24]. Our experiences with the PLB[25,]
134 [26], tip-dip[22, 23, 27], and patch-clamp[28–31] methods led us to a novel idea of forming lipid bilayers
135 by using the principles of the water-in-oil bilayer. We have referred to this as the contact
136 bubble bilayer (CBB) method[20, 32]. In this method, rather than hanging the water droplets in an
137 oil phase (Figure 1D), a water bubble is blown from a glass pipette (with tip diameter of
138 approximately 30 μm) into the oil phase (Figure 1E and 2), where the bubble is maintained by
139 applying a steady pressure. A monolayer forms spontaneously at the water–oil interface at the
140 surface of the bubble. Then, two bubbles are docked through the manipulation of two glass
141 pipettes, and the bilayer is formed as the two monolayers approach each other, yielding an
142 equilibrium bilayer area. The size of the bubble is controlled by the intra-bubble pressure
143 (holding pressure), and likewise the bilayer size. An average diameter of 50 μm is frequently
144 used. Although the volume of the bubble is small (<100 pL), it is connected to the larger volume
145 of the pipette solution that is in the microliter range, constituting the bulk electrolyte phase.
146   There are many benefits to use the CBB method (**Table 1**). As a lipid bilayer formation
147 technique, membranes of various lipid compositions can be produced, and asymmetric
148 membranes are more readily formed[32] than are those by the conventional folding method[33].
149 The bilayer can be mechanically manipulated, unlike the conventional PLB that can only be bent
150 with a hydrostatic pressure difference[34, 35]. By changing the holding pressure, the bubbles either
151 expand or shrink, leading to increased or decreased membrane tension[32]. The bilayer is
152 mechanically detachable into monolayers, similar to the freeze-fracture technique[36, 37] of
153 membranes in morphological studies, but with the CBB, a maneuver allows for repeated detach
154 and attach cycles[32]. The small volume of the electrolyte solution within the bubble allows
155 efficient fusion of channel-reconstituted liposomes into the bilayer, and the probability of
156 getting channel recordings is much higher than with the conventional PLB technique. The small
157 bubble volume also allows rapid perfusion (within ~20 ms) once another injection pipette is
158 inserted into either of the bubbles. Unlike the patch-clamp method, a broken CBB-based
159 membrane is re-formed immediately and repeatedly, and pipettes can be used several times a
160 day. By integrating benefits of the patch-clamp and PLB methods, the CBB provides a versatile
161 platform to vary the physicochemical conditions of the membrane, allowing for unprecedented
162 studies of channel–membrane interactions.
163   Before presenting a detailed protocol of the CBB formation process, the physicochemical
164 background of the bilayer formation in CBB is presented first, which iswill be helpful for patch-
165 clampers to solve issues whenresolve experimental difficulties relating to membrane formation
166 that are encountered.
167
168 **The Sense of surface chemistry in the CBB:** CBB experiments impart lessons of surface
169 chemistry science[38]. The CBB is similar to a soap bubble blown from a straw into the air, where
170 likewise, a water bubble is blown into an organic solvent. One will notice that a water bubble is
171 hardly inflated when membrane lipids are not included in either the water bubble or the
172 organic solvent. In the absence of amphipathic lipids, the surface tension at a water–oil

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interface is high, and the intra-bubble pressure for blowing a bubble will be high. This is a realization of the Laplace equation ($\Delta P = 2\gamma/R$, where ΔP is the intra-bubble pressure, γ is the surface tension, and R is the bubble radius). When the concentration of lipids in either the organic phase or the electrolyte solution is high, the density of lipids in the monolayer increases, as dictated by the Gibbs adsorption isotherm ($-d\gamma = \Gamma_i \, d\mu_i$, where Γ_i is the surface excess of compound i, and μ_i is the chemical potential of component i)[39], leading to a lower surface tension and ease of bubble formation. In the CBB, the bilayer can be observed from a tangential angle (Figure 2), and the contact angle between the monolayer and bilayer is measurable. This angle represents an equilibrium between the surface tensions of the monolayer and bilayer (Young equation: $\gamma_{bi} = \gamma_{mo}\cos(\theta)$, where γ_{bi} is the bilayer tension, γ_{mo} is the monolayer tension, and θ is the contact angle). The changes in the contact angle indicate changes in the bilayer tension, given that the monolayer tension is evaluated from changes in the contact angle as a function of the membrane potential (Young-Lippmann equation: $\gamma_{mo} = C_m V^2/4 \, (\cos(\theta_0) - \cos(\theta_v))$, where C_m is the membrane capacitance, V is the membrane potential, and θ_0 and θ_v are the contact angles at 0 and V mV, respectively)[40–42]. When two bubbles are close enough, they approach each other spontaneously. This is due to van der Waals force, and we can visually observe this dynamic process in CBB formation.

A CBB system consists of distinct phases; namely, a bulk oil phase, water bubbles coated with a monolayer, and a contacting bilayer (Figure 3). These are reminiscent of the multiple phases observed in a PLB, such as a solvent-containing torus around the bilayer phase and a thin organic phase sandwiched by two monolayers[43, 44]. In the CBB, the monolayer phase is continuous with the bilayer leaflet, and lipid molecules readily diffuse between the monolayer and the leaflet. The monolayer phase covers most of the bubble surface, constituting the major phase that serves as a lipid reservoir. Because the hydrophobic tail of lipids in the monolayer extends outward to the bulk oil phase, the bilayer interior or the hydrophobic core opens to the bulk oil phase. Thus, a hydrophobic substance injected into the oil phase close to the bilayer is able to readily access the bilayer interior. This is the membrane perfusion technique we had developed recently[45], by which the lipid composition in the bilayer is changed rapidly (within a second) during single-channel current recordings. We found that the cholesterol content in the bilayer could be reversibly controlled by switching the cholesterol perfusion on and off[45]. In the event that the concentration of the relevant substance in the monolayer and bilayer differs, the concentration gradient of the relevant substance is immediately dissolved through diffusion, which is known as the Marangoni effect[46, 47]. On the other hand, flip-flops across the monolayers are slow[48–50].

Using the CBB method, the bilayer is formed under versatile physicochemical conditions, such as an electrolyte pH as low as 1 [51], a salt (K^+, Na^+, etc.) concentration up to 3 M, a membrane potential as high as $\pm$400 mV, and a system temperature of up to 60 °C. There are several options for formation of the CBB and incorporation of channel molecules therein. For formation of the monolayer at the water–oil interface, lipids are added either in an organic solvent (lipid-out method; Figure 4A,C) or in a bubble as liposomes (lipid-in method; Figure 4B,D). Notably, the lipid-in method allows for the formation of asymmetric membranes[15, 32]. Channel molecules soluble in aqueous solution (e.g., channel-forming peptides) The Sense of surface chemistry in the CBB: CBB experiments impat lessons of surface

216 chemistry science (38). CBB is similar to a soap bubble blown from a straw into the air. A water
217 bubble is blown into an organic solvent, and one will notice that a water bubble is hardly
218 formed when membrane lipids are not included in either the water bubble or the organic
219 solvent. In the absence of amphipathic lipids, surface tension at a water-oil interface is high,
220 and the intra-bubble pressure for blowing a bubble will be high. This is a realization of the
221 Laplace equation ($\Delta P = 2\,\gamma/R$; ΔP: intra-bubble pressure, γ: surface tension, R: the bubble
222 radius). When the concentration of lipids in either the organic phase or the electrolyte solution
223 is high, the density of lipids in the monolayer becomes higher as dictated by the Gibbs
224 absorption isotherm ($d\gamma = \Gamma_i\,d\mu_i$; Γ_i: the surface excess of compound i, μ_i: the chemical potential
225 of component i) (39), leading to lower surface tension and ease of bubble formation. In CBB,
226 bilayer is observed from a tangential angle, and the contact angle between monolayer and
227 bilayer is measurable. This angle represents equilibrium of surface tension of monolayer and
228 bilayer (Young equation; $\gamma_{bi} = \gamma_{mo}\cos(\theta)$; γ_{bi}: the bilayer tension, γ_{mo}: the monolayer tension, θ:
229 the contact angle), and the changes in the contact angle indicate changes in surface tension
230 (40–42). When two bubbles are close enough, they are approach each other spontaneously.
231 This is due to van der Waals force, and we are visually observing this dynamic process in CBB
232 formation.
233   CBB provides unique phases (Fig. 3), which is reminiscent of multiple phases observed in a
234 planar lipid bilayer, such as a torus around the bilayer phase (43, 44). In CBB, a monolayer phase
235 is continuous with a leaflet of the bilayer, and lipid molecules readily diffuse between the
236 monolayer and the leaflet. A monolayer phase covers most of a bubble surface, constituting a
237 major phase serving as a lipid reservoir. When a concentration gradient of a lipid constituents
238 exists between a monolayer and a leaflet, the relevant molecule diffuses rapidly down the
239 gradient, which is known as the Marangoni effect (45, 46). On the other hand, flip-flops across
240 the monolayers are slow (47–49).
241   For CBB composed of certain lipid species, the electrical resistance of CBB is low and cannot
242 be used for single-channel current recordings. Even the bilayer cannot be formed. This is mostly
243 because of the physical feature of constituent lipids, whose phase transition temperature is
244 above the recording temperature. For example, dioleoylphosphatidylcholine (DOPC) is frequently
245 used for liposomes, but the electrical resistance is low at 25 °C (50). CBB formation was difficult
246 with dipalmitoylphospatidylcholine (DPPC) at room temperature but was possible above the
247 phase transition temperature (51). The frequently used lipid diphytanoylphospatidylcholine
248 (DϕPC) has its phase transition temperature below 0 °C (52), and CBB is readily formed (32, 53,
249 54) at a broad temperature range.
250   As the hydrophobic tail of lipids extends outwardsare directly added into the bubble
251 (Figure 4A,B)[52, 53], whereas channel proteins are reconstituted into liposomes, which are then
252 added into the bubble (Figure 4C,D). Herein, the formation of CBBs by the lipid-in method for
253 either a channel peptide (polytheonamide B (pTB); Figure 4A) or a protein (KcsA potassium
254 channel, Figure 4C) is shown.
255   to the bulk oil phase, the bilayer interior or the hydrophobic core opens to the bulk oil phase.
256 Thus, a hydrophobic substance injected into the oil phase close to the bilayer is able to readily
257 access the bilayer interior. This is the membrane perfusion we recently developed (53), by which
258 the lipid composition in the bilayer is changed rapidly, within a second, during single-channel

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current recordings. We found that cholesterol content in the bilayer can be reversibly controlled by switch on and off of the cholesterol perfusion.

PROTOCOL:

1   For formation of the monolayer at the water-oil interface, lipids are added either in an organic solvent (lipid-out) or in a bubble as liposomes (lipid-in) (Fig. 4). In either case, lipids transfer to the water-oil interface, while the transfer rate is relatively slow in lipid-out that in the lipid-in method (50). In additon, the electrical resistance of the bilayer membrane is relatively smaller in lipid-out than that with the lipid-in method for reasons unknown. Notably, the lipid-in method yields asymmetric membranes (15, 32).Prepare liposomes

Channel molecules soluble in aqueous solution (e.g., channel-forming peptides) can be directly added in a bubble (55, 56), and channel proteins are reconstituted into liposomes, which are then added in a bubble.

1   Preparation of liposomes

1)1.1   Phospholipids in chloroform: Disperse phospholipids (e.g., 10 mg in powder) in chloroform at an appropriate a desired concentration (e.g., 10 mg/mlmL).

2)1.2   Chloroform evaporation: Evaporate chloroform

i)1.2.1 Place the phospholipid solution in a round-bottom flask and set it on a rotary evaporator (see table of materials) connected withto a N₂ gas cylinder. Rotate the flask under N₂ flow at room temperature until a thin phospholipid film appears. (after ~30 min).

ii)1.2.2 Place the open flask ininto a desiccator, which that is connected to a vacuum pump. AspirateUsing the vacuum pump, aspirate the inside of the desiccator using the vacuum pump for several hours to remove the chloroform thoroughly.

3)1.3   Phospholipid suspensionSuspend the phospholipid: Add an appropriate volume of an electrolyte solution to the flask and suspend the phospholipid to obtain a 2 mg/mlmL phospholipid suspension.

4)1.4   Sonicate the suspension for several tens of seconds using a bath sonicator (see table of materials) to obtain a multilayered vesicle (MLV liposome) suspension.

5)1.5   For preparation of proteoliposomes that contain ion channel proteins, add less than 2% volume of a protein solution (with the proteins are solubilized using appropriate detergents; 2% volume is the maximum) to the MLV liposome suspension and sonicate for several seconds using the bath sonicator.

2.2   Preparation of Prepare large-bore glass pipettes

1)2.1   Set a glass capillary (see table of materials) to a pipette puller (see table of materials) and fabricate two micropipettes with a fine tapered tip through two-step pulling.

2)2.2   Set the micropipette on a microforge (see table of materials) and contact the tip of the micropipette to a platinum filament at the tapered portion with a diameter of 30 to 50 μm.

300 3)2.3 Heat the filament for a momentbriefly (5 s) and immediately turn it off. This
301 manipulation forms a crack at the heating point and, whereby the tip of the micropipette is cut
302 off, leaving a wide bore with a diameter of 30 to 50 μm.
303
304 3.3   Surface treatmentTreat the surface of hole slide glass (slide glass with a shallow concave
305 well; see table of materials; siliconization for a water-repellent finish)
306 1)3.1 Clean the surface of athe hole slide glass with distilled water and EtOHethanol.
307 2)3.2 Apply an appropriate volume (e.g., 100 μL) of a siliconizing reagent (water-repellent,
308 e.g., Sigmacote) on to; see table of materials) onto the hole slide glass.
309   3.3 Use afterDry the reagent dries completely.
310   3.4 The hole slide glass is placed on the stage of the inverted microscope.
311
312 4   Formation of the CBB and electrophysiological measurement
313 3.3   Equipment: micropipette holders (x 2): The micropipette holder should be equipped
314 with a pressure port. An Ag/AgCl wire electrode was used. in the air.
315 3.4   Place the siliconized hole slide glass on the stage of an inverted microscope (see tables
316 of materials).
317
318 4   Form the CBB and addperform electrophysiological measurement
319 1)4.1 Add 100 μlL of hexadecane into the holeshallow well of the siliconized hole slide glass.
320 Note: For the lipid-out method, phospholipids are dispersed in hexadecane (20 mg/mL)
321 beforehand.
322 2)4.2 Fill the electrolyte solution up to half the length of the glass pipettemicropipette, using a
323 tuberculin syringe.
324 3)4.3 Set the micropipette toonto the micropipette holder, which is mounted on with a micro
325 manipulator, and pressure port (see table of materials), allowing the Ag/AgCl wire electrode
326 (see table of materials) to soak the Ag/AgCl wire electrode into the pipette electrolyte solution.
327 4)4.4 Connect one of the micropipette holders to the head stage of a patch-clamp amplifier
328 and another(see table of materials) and the other one to the electrical ground.
329 5)4.5 Connect a micro injectormicroinjector (see table of materials) to the pressure port of
330 the micropipette holder.
331 6)4.6 Set the micropipette to an appropriate position above the stage of an inverted
332 microscope by manipulating the micro manipulator.micromanipulator (see tables of materials).
333 4.7   SuctionAdjust the electrode offset potential.
334 4.7.1 Place 1 μL of the same electrolyte solution used to fill the micropipette on the flat
335 surface around the shallow well of the hole slide glass, creating an electrolyte dome.
336 4.7.2 Soak the tip of both micropipettes into the electrolyte dome by manipulating the
337 micromanipulator.
338 4.7.3 Adjust the electrode offset potential of the patch-clamp amplifier.
339 4.7.4 Confirm the correct offset at the end of experiments by breaking the CBB via application
340 of a high membrane potential (electrical breakdown; using Zap on the amplifier), causing the
341 two bubbles to be fused into one (bubble fusion).

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342 4.7.5   Correct the liquid junction potential[54] in the case where asymmetric electrolyte
343 solutions are used, such that the calculated value is added to the applied membrane potential
344 for the true membrane potential.
345 Note: The liquid junction potential is calculated using the program JPCalc[55].
346 ~~7)~~4.8   Draw liposome solution from the tip
347 ~~Place 1 µl droplet of~~Note: When water-soluble channels are examined using the lipid-out
348 method, suctioning of the liposome solution is not necessary.
349 ~~i)~~4.8.1 Place 1 µL of liposome solution on the flat surface around the ~~hole~~shallow well of the
350 hole slide glass (liposome-containing dome).
351 ~~ii)~~4.8.2 Manipulate the ~~micro manipulator~~micromanipulator and insert the tip of the
352 micropipette into the ~~droplet~~liposome-containing dome.
353 ~~iii)~~4.8.3   Aspirate the ~~droplet~~liposome-containing solution by lowering the pressure inside
354 the micropipette holder using the ~~micro injector~~microinjector.
355 ~~iv)~~4.8.4   Repeat the procedure for the other pipette.
356 ~~8)~~4.9   Manipulate the ~~micro manipulator~~micromanipulator and dip the tip of the micropipette
357 into the hexadecane ~~on~~in the ~~hole~~shallow well.
358 ~~9)~~4.10 ~~A~~Blow a water bubble ~~was~~ slowly ~~blown~~ by increasing the pressure until the bubble
359 ~~reached a~~reaches the desired size (e.g., 50 µm in diameter~~),~~) and maintain the same pressure
360 ~~was maintained~~thereafter (maintaining pressure).
361 ~~10)~~4.11   ~~If it is hard to keep the size of the bubbles stable, discard~~Discard the bubbles by
362 passing the tip through the oil~~–~~—air interface if it is hard to keep the size of the bubbles stable.
363 ~~11)~~4.12   Repeat steps 4.8 to 4.9 until stable bubbles are formed.
364 4.13   ~~Manipulators are used~~Manipulate the bubbles to allow contact between ~~bubbles.~~them
365 (Figure 5).
366 ~~12)~~Note: Sometimes, the bubbles approach each other spontaneously ~~approach~~to form the
367 CBB. In other cases, the bubbles are close but do not contact each other. In this case, push the
368 bubbles each other mechanically.
369 ~~13)~~4.14   ~~The bubble size should be maintained by fine-tuning~~Fine-tune the pressure to
370 maintain the bubble size, because the size may gradually change even at the constant intra-
371 bubble pressure.
372 ~~14)~~4.15   Set the membrane potential to the appropriate value using the patch-clamp
373 amplifier and wait for the channel current to emerge~~.~~ (Figure 6).
374
375 ~~In our study, a solution of liposomes and channel-reconstituted liposomes was drawn from~~
376 ~~the tip into the pipette electrolyte solution (step 9 and 10). This is merely for conserving~~
377 ~~materials such as lipids and channel molecules. Consequently, the sample and lipid~~
378 ~~concentration gradually decrease because of diffusion towards the upper pipette solution, and~~
379 ~~after a while, monolayers cannot be formed. In this case, fresh lipid solution should be~~
380 ~~aspirated from the tip (step 7). This temporal concentration change can be circumvented when~~
381 ~~the whole pipette is filled with the solution containing lipids and channel molecules, whereas~~
382 ~~liposomes gradually settled down into the bubbles.~~Measure~~In this case, bubbles are renewed.~~
383 ~~When the phase transition temperature (Tₘ) is high, CBB must be performed at above the Tₘ,~~
384 ~~and the temperature is controlled using a transparent heat plate beneath the slide glass (57)~~

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385
386 ~~5   KcsA potassium channel specific protocol (58–60)~~
387   ~~1) Asymmetric electrolyte solution: The channel is activated only when the cytoplasmic~~
388   ~~side is acidic (61). Accordingly, in step 2 in the CBB formation, 'functional' orientation of~~
389   ~~the KcsA channel in the membrane was controlled by setting the pH of the two pipette~~
390   ~~solutions asymmetric (i.e., pH4 and 7) (25).~~
391   ~~2) In step 7 for the CBB formation, proteoliposome suspension rather than liposome~~
392   ~~suspension is placed on the glass vessel for aspiration into pipettes.~~
393   ~~3) Concentration of KcsA: In the case of the KcsA potassium channel, use of liposomes with~~
394   ~~the protein/lipid weight ratio of 1/2000 is suitable for single-channel current recording,~~
395   ~~and that of 1/10, for macroscopic current recording.~~
396
397 ~~6   Adjustment of the electrode offset potential~~
398 ~~Setting the zero offset potential is prerequisite for the electrophysiological measurements.~~
399   ~~1) Upon aspirating the liposome solution, two electrodes are soaked in a same liposome~~
400   ~~dome. Now, two AgCl electrodes are connected via a electrolyte solution of the dome,~~
401   ~~and the electrode offset potential can be adjusted.~~
402   ~~2) At the end of experiments, CBB is broken by applying high membrane potential~~
403   ~~(electrical breakdown; using Zap in the Axopatch amplifier) and the two bubbles are~~
404   ~~fused into one (bubble fusion) to confirm the correct offset.~~
405   ~~3) In case of asymmetric solutions, the liquid junction potential (62) should be corrected~~
406   ~~using the program JPCalc (63).~~
407
408 ~~7.~~5   ~~Measurement of~~ bilayer capacitance
409 ~~1)~~5.1 ~~The~~Measure the bilayer ~~electric~~electrical capacitance ~~is measured~~(C_{el}) by applying a
410 ramp potential.
411 Note: When the rate of the voltage change in the ramp command is 10 mV/10 ms (or 1
412 V/~~sec),~~s) followed by −10 mV/10 ms, the ~~measured current~~ amplitude of the current jump upon
413 changes in the slope corresponds to the ~~jump in current upon the application of ramp potential.~~
414 read of the membrane's capacitance value (e.g., 100 pA $\rightarrow$ 100 pF).
415 ~~2)~~5.2 ~~To evaluate~~Evaluate the bilayer area~~,~~ of two bubbles ~~are~~ stacked ~~next to each~~one on the
416 other, and focus the microscope ~~is focused~~ at the bilayer level. ~~The~~ to view the edge of the
417 bilayer ~~is visually discernible.~~ (Figure 6).
418 ~~Specific membrane capacitance can be measured from the electric~~Note: The bilayer shape is
419 mostly circular, and the area is calculated from the radius.
420 ~~3)~~5.3 Calculate the specific membrane capacitance ~~and the bilayer area as~~ (C_{sp} ~~= Cel/A. The~~
421 ~~specific membrane~~) by dividing the electrical capacitance ~~in hexadecane is 0.65 μF/cm².~~by the
422 bilayer area ($C_{sp} = C_{el}/A$).
423 ~~4)~~5.4 ~~Bilayer~~Calculate the bilayer thickness (thickness of the hydrophobic core) ~~is evaluated~~
424 ~~as~~using $D_c = (\varepsilon_r \varepsilon_0)/C_{sp}$~~,~~ (where ε_r and ε_0 represent the permittivity of the hydrophobic region of
425 the bilayer and the permittivity of a vacuum, respectively~~.~~).
426
427 ~~8   Asymmetric membrane~~

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428 ~~An asymmetric membrane can be formed, when different types of lipids are added in each~~
429 ~~bubble, in the form of liposomes (lipid-in) (15, 64).~~
430
431
432 **REPRESENTATIVE RESULTS:**
433
434 A typical CBB ~~has~~had a diameter of 50 μm (~~Fig.~~Figure 5,6) and the specific membrane
435 capacitance in hexadecane was 0.65 μF/cm². The bubble size ~~is~~was arbitrarily controlled by the
436 intra-bubble pressure. ~~The size of the CBB is governed by the bubble size because the bilayer is~~
437 ~~spontaneously formed as two bubbles approach each other.~~ When small bubbles are necessary
438 for low-noise recordings, the tip diameter should be correspondingly small. For example, for a
439 bubble size of 50 μm in diameter, the tip diameter should be 30 μm.
440   Once the CBB ~~is~~had formed, the channel molecules either in aqueous solution or in the
441 liposome ~~are~~were spontaneously inserted into the ~~CBB~~bilayer within a span of a few ~~minutes~~to
442 dozens of minutes. Insertion of the channels ~~can be~~was confirmed by ~~a~~the stepwise increase of
443 current amplitude (~~Fig. 6~~Figure 7) under the applied membrane potential. The smaller
444 membrane area (<-1000 μm²) relative to that in the conventional PLB and DIB ~~system~~systems
445 substantially ~~improves~~improved the electrical signal-to-noise ratio.
446   Current recordings ~~can~~could be continued until the membrane ~~is~~was disrupted and the two
447 bubbles ~~are~~ merged. New bubbles ~~can be~~were blown and the CBB ~~is~~formed immediately and
448 repeatedly. The pipette ~~can~~could be used repeatedly ~~used, and the new bubbles readily form~~
449 ~~another CBB~~within a day.
450
451 ~~**The duty cycle of attach and detach:** Similar to freeze fracture procedure for morphological~~
452 ~~studies usingf the electron microscope, a bilayer formed by the CBB method can be disintegrated~~
453 ~~into two monolayers. Detach-attach of the CBB can be repeated by manipulating the two bubbles~~
454 ~~(duty cycle of detach-attach). Such a process is also monitored by the appearance of the channel~~
455 ~~current of polytheonamide B (pTB), a peptide channel from a marine sponge, as it is readily~~
456 ~~inserted into the lipid bilayer to form a monovalent cation-selective pore (55, 65). The pTB~~
457 ~~channel current emerged immediately after attaching the two monolayers and current became~~
458 ~~larger as the area of contact increased (Fig. 7). The amplitude of the current was synchronized~~
459 ~~with the detach-attach manipulation of the CBB.~~
460
461 **Duty cycle of attach and detach:**
462 A bilayer formed by the CBB method can be disintegrated into two monolayers. Detach-attach
463 of the CBB can be repeated by manipulating the two bubbles (duty cycle of detach-attach). This
464 process was monitored by the appearance of the channel current of pTB, a peptide channel
465 from a marine sponge, as it was readily inserted into the lipid bilayer to form a monovalent
466 cation-selective pore[52, 56]. The pTB channel current emerged immediately after attaching the
467 two monolayers, and the current became larger as the area of contact increased (Figure 8). The
468 amplitude of the current was synchronized with the detach-attach manipulation of the CBB.
469
470 **Single-channel measurements of the KcsA channel:**

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471 The KcsA potassium channel is pH sensitive, being activated by acidic intracellular pH[57]. Thus,
472 the electrolyte solution was set to be asymmetric[58–60]. In step 4.2 of CBB formation, the pipette
473 solution of the left side was set at pH 4, whereas that of the right side was set at pH 7.5[25]. In
474 step 4.7.1, a proteoliposome suspension, rather than liposome suspension, was placed onto the
475 slide glass for aspiration into the left pipette. Accordingly, the KcsA channel was oriented in the
476 membrane with its cytoplasmic domain facing the left bubble. Liposomes with the protein/lipid
477 weight ratio of 1/2000 are suitable for single-channel current recording, and those with a 1/10
478 ratio are for macroscopic current recording.
479
480 **Asymmetric membrane:** ~~Asymmetric~~
481 An asymmetric lipid bilayer can be formed using different liposome ~~suspension~~suspensions for
482 each bubble~~.~~ (lipid-in)[15, 32]. The orientation of the KcsA channel in the membrane was regulated
483 by setting an asymmetric solution pH (pH 4_{in}/pH 7_{out}), leading to its cytoplasmic side being
484 toward the left side. Accordingly, the left side was assigned as "in," whereas the right side was
485 assigned as "out." To examine lipid- dependence on the gating of the KcsA channel, four types
486 of CBB (including an asymmetric CBB) were used~~, i.e.,~~; namely, PG_{in}/PG_{out}, PG_{in}/PC_{out}, PC_{in}/PG_{out},
487 and PC_{in}/PC_{out} (~~Fig. 8). Here, the pH of the two bubbles was set asymmetrically as~~
488 ~~pH4in/pH7out.~~PG: phosphatidylglycerol) (Figure 9). The KcsA channel exhibited high open
489 probability (>-90%) only in the PG_{in}/PG_{out} and PG_{in}/PC_{out} ~~membrane~~membranes. The KcsA
490 channel requires the existence of anionic phospholipids in the inner leaflet of the membrane
491 for a high open probability ~~(26).~~[26].
492
493
494 **Tables and Figures:**
495 **Figure 1:** Lipid bilayer and patch-clamp methods. Various methods have been developed for
496 forming the lipid bilayer. (A) Patch-clamp method. (B) Conventional planar lipid bilayer method,
497 which provides a free-standing, mostly vertical, bilayer on a small hole. (C) Tip-dip method. A
498 monolayer at the air–water interface is positioned on the tip of a glass electrode. Various
499 modifications have been developed. (D) Droplet interface bilayer method. (E) Contact bubble
500 bilayer method.
501
502 **Figure 2:** Contact bubble bilayer formation. As a bubble is blown into an oil phase, lipid
503 molecules transfer spontaneously to the water–oil interface. Different types of lipids as
504 liposomes are included in each bubble (lipid-in), and monolayers are formed exclusively by the
505 relevant lipids. Docking two monolayers generates an asymmetric bilayer membrane.
506
507 **Figure 3:** Distinct phases in the contact bubble bilayer, and the dynamics of lipids therein. Lipids
508 lining the bubble constitute a phase where they belong to either a monolayer or a bilayer. Flip-
509 flop of lipids across the bilayer is infrequent, and the asymmetric membrane is retained for a
510 long time.
511
512 **Figure 4:** Delivering lipids with the lipid-out or lipid-in method. Lipids are added either in an
513 organic solvent (lipid-out; A and C) or in aqueous solution as liposomes (lipid-in; B and D). In the

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lipid-in method, an asymmetric membrane is formed. Channel-forming substances that are soluble in aqueous solution (blue), such as peptides, are added into one of the bubbles and spontaneously inserted into the bilayer. In the lipid-in method, a part of the channels is inserted into liposomes, which are then fused to the bilayer. Channel proteins (red) are reconstituted into liposomes in either the lipid-out or lipid-in method, which are then added into one of the bubbles and spontaneously fused to the bilayer.

Figure 5: Formation and microscopic image of the contact bubble bilayer. The bilayer is observed from a tangential direction.

Figure 6: Observation of the bilayer for measuring the bilayer area. Two bubbles are stacked one on the other through pipette manipulation, and the microscope is focused at the level of the contact bubble bilayer.

Figure 7: Stepwise insertion of the KcsA channel into the contact bubble bilayer membrane. The E71A mutant of the KcsA channel was used to show the immediate detection of the inserted channels.

Figure 8: Detach-attach of monolayers and responses of channels. A peptide channel, polytheonamide B, was added into one of the bubbles, which was then spontaneously incorporated into the membrane. When detached, channels in the membrane withdrew but were retained at the membrane leaflet of the channel-added side. Upon attachment, the channels were inserted into the membrane.

Figure 9: Formation of an asymmetric membrane and channel activity dependent on the composition of the leaflet. The KcsA channel is regulated by acidic lipids, such as PG, in the inner leaflet of the membrane.

DISCUSSION: *(3-6 paragraphs)*
The CBB method of lipid bilayer formation is based on the principle of a water-in-oil droplet lined by a monolayer[20]. Technically, the procedures for forming CBBs are easy, especially for patch-clamp researchers, who are proficient in manipulating glass micropipettes. The electrophysiological setup for the patch clamp is readily used in the CBB when two pipette manipulators with microinjectors are available. On the other hand, because the CBB is a successor of the conventional PLB, for which a large amount of physicochemical knowledge has been accumulated[8, 61], this background as well as knowledge of surface chemistry[38] is helpful for operating and manipulating CBBs. The CBB serves a versatile platform for studying channel–membrane interplays. By using the CBB method, the bilayer can be modulated in terms of chemical composition and physical manipulation. In the CBB, lipids lining the bubble can diffuse freely across the border of the monolayer phase and leaflet of the bilayer, which enables various experimental procedures. Various physical and chemical modifications to the bilayer are now made possible, and we have developed other techniques such as the bilayer attach-detach

and the membrane perfusion methods[45]. We have extended CBBs for *in vitro* channel protein synthesis, where channel synthesis was performed in the bubble, and newly synthesized channel proteins were subjected to spontaneous transfer to the bilayer under application of a membrane potential where nascent channel functions of the KcsA channel was traced from the time of initiating the transcription/translation of KcsA DNA[62].

Among the steps of the protocol, maintaining the bubble size is critical. The bubbles may slowly swell or shrink spontaneously because lipid transfer to the interface is slow and the monolayer tension is prone to change. In particular, the first bubble inflated just after suction of the liposome solution (steps 4.8.3 and 4.10) is hard to maintain because the monolayer tension is lowered by the liposomes accumulated at the tip of the pipette. Discarding the initial bubbles and subsequent formation of the bubbles provide the stable CBB. Visual tracing of the bubble size and fine tuning of the pressure are necessary.

There are experimental limitations to the CBB method. Although the CBB is designed for electrophysiological measurements, the electrical resistance of the bilayer for certain lipid compositions may not be high enough (e.g., 100 GΩ) for single-channel recordings. For example, dioleoylphosphatidylcholine is frequently used for liposomes, but it forms electrically leaky CBBs at 25 °C[63]. CBBs cannot even be formed with lipid species whose phase transition temperature is above the recording temperature. Indeed, CBB formation was difficult with dipalmitoylphosphatidylcholine at room temperature, but raising the temperature above T_m circumvented the problem[64]. In these experiments, the temperature was controlled by using a transparent heat plate (see table of materials) beneath the slide glass[62]. The phase transition temperature of the frequently used lipid diphytanoylphosphatidylcholine is below 0 °C [65], and CBBs are readily formed[32, 45, 51] at a broad temperature range.

Phospholipids of the water–oil interface are provided from either the lipid-in or lipid-out method, where the transfer rate is relatively slower in the lipid-out than in the lipid-in method[63]. In addition, for reasons unknown, the electrical resistance of the bilayer membrane is relatively smaller with the lipid-out method than with the lipid-in method.

In our protocol (step 4.8), a solution of liposomes and channel-reconstituted liposomes (1 μL) is loaded from the tip of the pipette (steps 4.9 and 4.10), and the rest of the pipette contains the previously filled electrolyte solution. This is merely for conserving materials, such as lipids and channel molecules. Consequently, the liposomes diffuse gradually toward the upper pipette solution, and after a while, the lipid concentration at the tip of the pipette becomes insufficient to form lipid monolayers. In this case, fresh liposome solution should be aspirated from the tip (step 4.8). This temporal concentration change can be circumvented when the whole pipette is filled with the solution containing lipids and channel molecules, whereas liposomes gradually settle down into the bubbles. In this case, bubbles are renewed.

The CBB is a method to form a lipid bilayer based on the principle of water-in-oil bilayer (20). Technically, procedures for forming CBB is easy, especially for patch-clampers. Electrophysiological setup for the patch-clamp is readily used in the CBB when two pipette manipulators with microinjectors are available. On the other hand, CBB is a successor of the conventional planar lipid bilayer, for which a large amount of physicochemical knowledge has been accumulated, and this background, as well as knowledge of surface chemistry, are a prerequisite for operating and manipulating CBB. The CBB serves a versatile and unprecedented

600 platform for studying channel-membrane interplays. By using the CBB method, bilayer can be
601 modulated in terms of chemical composition and physical manipulation. In CBB, lipids lining a
602 bubble are freely diffusible across the border of the monolayer phase and leaflet of the bilayer,
603 which enables various experimental procedures. Various applications are now made possible and
604 we have developed unprecedented techniques such as the attach-detach and the membrane
605 perfusion. We extended CBB for *in vitro* channel protein synthesis (*in bulla* synthesis), in which
606 channel synthesis was performed in the bubble, and newly synthesized channel proteins are
607 subjected to spontaneous transfer to the bilayer where nascent channel functional of the KcsA
608 channel was traced from the time of initiating the transcription/translation of KcsA DNA (57).
609 There are experimental limitations in the CBB method. CBB is designed for
610 electrophysiological measurements, but the bilayer resistance should be as high as 100 GΩ. This
611 high resistance membrane cannot be attained for lipid molecules having T_m above experimental
612 temperature. Raising temperature above the T_m circumvents the problem. Electrophysiologically,
613 the bilayer size is larger than that of the patch-clamp, yielding larger membrane capacitance in
614 the CBB method. However, the series resistance (the electric resistance in series of the
615 membrane resistance) is much lower (~100 kΩ) than that for the patch-clamp (the pipette
616 resistance > 1 MΩ), which accelerates the speed of voltage clamp and attenuates the background
617 noise and the series resistance errors in the voltage-clamped membrane potentials (66–68). The
618 background noise is a function of the membrane capacitance and the series resistance, and a low
619 series resistance complements high membrane capacitance, resulting in low background noise
620 (9).
621 Overall, CBB integrates the benefits of patch-clamp, such as the mechanical manipulability
622 of the membrane, and those of planar lipid bilayer, such as the ability to modify lipid composition
623 of the membrane. Various types of channel-forming substances and channel proteins have been
624 studied (55, 56, 69, 70). Development of this method is opportune since more and more
625 researchers are focusing on channel-membrane interplay, and the CBB provides a versatile
626 platform for various experiments. Further experimental developments are expected in CBB.
627
628 **Trouble shooting**Electrophysiologically, the CBB size is larger than that of the patch clamp,
629 thus yielding a larger membrane capacitance. However, the series resistance (the electrical
630 resistance in a series of membrane resistance) is much lower (~100 kΩ) than that for the patch
631 clamp (pipette resistance > 1 MΩ), which accelerates the speed of the voltage clamp and
632 attenuates the background noise and series resistance errors in the voltage-clamped
633 membrane potentials[66–68]. The background noise is a function of the membrane capacitance
634 and the series resistance, where a low series resistance complements a high membrane
635 capacitance, resulting in low background noise[9].
636 Overall, the CBB integrates the benefits of both the patch clamp (e.g., the mechanical
637 manipulation of the membrane) and the PLB (e.g., the ability to modify the lipid composition of
638 the membrane). Various types of channel-forming substances and channel proteins have been
639 studied[52, 69–71]. The development of this method is opportune since increasingly more
640 researchers are focusing on channel–membrane interplay, and the CBB provides a versatile
641 platform for various experiments. Further experimental developments in the CBB method are
642 expected.

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Troubleshooting: As a general rule of lipid bilayer experiments, detergents should not be used in the washing of glass wears~~to wash glassware~~. Even a trace amount of detergent perturbs the integrity of the bilayer. Organic solvents such as chloroform/methanol and ethanol should be used for such cleaning purposes instead.

DISCLOSURES:
The authors have no conflict of interest to disclose.

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