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1 **TITLE:**
2 **Sample Preparation Using a Lipid Monolayer Method for Electron Crystallographic Studies**
3

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18 **SUMMARY:**
19 Lipid monolayers have been used as a foundation for forming two-dimensional (2D) protein
20 crystals for structural studies for decades. They are stable at the air-water interface and can serve
21 as a thin supporting material for electron imaging. Here we present the proven steps on
22 preparing lipid monolayers for biological studies.
23

24 **ABSTRACT:**
25 Electron crystallography is a powerful tool for high-resolution structure determination.
26 Macromolecules such as soluble or membrane proteins can be grown into highly ordered two-
27 dimensional (2D) crystals under favorable conditions. The quality of the grown 2D crystals is
28 crucial to the resolution of the final reconstruction via 2D image processing. Over the years, lipid
29 monolayers have been used as a supporting layer to foster the 2D crystallization of peripheral
30 membrane proteins as well as soluble proteins. This method can also be applied to 2D
31 crystallization of integral membrane proteins but requires more extensive empirical investigation
32 to determine detergent and dialysis conditions to promote partitioning to the monolayer. A lipid
33 monolayer forms at the air-water interface such that the polar lipid head groups remain hydrated
34 in the aqueous phase and the non-polar, acyl chains, tails partition into the air, breaking the
35 surface tension and flattening the water surface. The charged nature or distinctive chemical
36 moieties of the head groups provide affinity for proteins in solution, promoting binding for 2D
37 array formation. A newly formed monolayer with the 2D array can be readily transfer into an
38 electron microscope (EM) on a carbon-coated copper grid used to lift and support the crystalline
39 array. In this work, we describe a lipid monolayer methodology for cryogenic electron
40 microscopic (cryo-EM) imaging.
41

42 **INTRODUCTION:**
43 Electron diffraction through 2D crystals or helical arrays of proteins can achieve sub-nanometer
44 resolutions in favorable cases[1–3]. Of particular interest are reconstituted 2D membrane protein

45 arrays or crystals in their near-native environments[1]. Because a crystal acts as a signal amplifier
46 enhancing the intensities of the structural factors at specific spatial frequencies, electron
47 crystallography allows probing a target with a smaller size at high resolutions, such as small
48 molecules, than those for single-particle cryo-EM. The electron beam can be diffracted by an
49 ordered 2D array of proteins, generating a diffraction pattern or a lattice image depending on
50 where the image plane is recorded on the detector[4]. The diffracted intensities can then be
51 extracted and processed to reconstruct a 2D projection structure of the crystal. Electrons have a
52 larger scattering cross-section than X-rays and its scattering mostly follows the Rutherford model
53 based on the Coulomb interaction between the electrons and the charged atoms in the
54 molecule[5]. The thicknesses of 2D membrane crystals are usually less than 100 nm, suitable for
55 electron transmission without dynamical scattering occurring within specimen[6]. Electron
56 crystallographic studies have been shown to be a powerful tool to probe high-resolution
57 structural information of membrane proteins and lipid-protein interactions[7–17].
58
59 A lipid monolayer is one single lipid layer composed of phospholipids densely packed at an air-
60 water interface[6], which can assist the 2D array formation for soluble proteins or peripheral
61 membrane proteins[18]. Depending on the density of the lipids and their lateral pressure, the lipid
62 molecules can form an ordered 2D array on the air-water interface with their acyl chains
63 extended to the air and hydrophilic headgroups exposed in the aqueous solution[1,6,19]. The lipid
64 headgroup can interact with proteins via electrostatic interaction or can be modified to provide
65 an affinity tag to bind a specific protein domain. For example, the DOGS-NTA-Ni (1,2-dioleoyl-*sn*-
66 glycero-3-[(N-(5-amino-1-carboxypentyl)iminodiacetic acid)succinyl]$^{2-}$ Ni^{2+}) is often used in
67 forming a lipid monolayer to bind the proteins with a poly-histidine tag[20–22]. Also, the cholera
68 toxin B can bind a particular pentasaccharide of ganglioside GM1 in a lipid monolayer for
69 structural studies[23,24]. By anchoring the proteins on the lipid headgroups, the lipid monolayer can
70 assist the formation of the 2D arrays that are thin for high-resolution electron crystallographic
71 studies. The lipid monolayer technique has been used in electron crystallography for structural
72 studies of proteins, such as streptavidin[2,25], annexin V[26], cholera toxin[27], *E. coli* gyrase B subunit[28],
73 *E. coli* RNA polymerase[25,29,30], carboxysome shell proteins[31] and the capsid proteins of the HIV-
74 1[32] and Moloney murine leukemia virus[33]. Due to the stability and chemical property of the lipid
75 monolayer, different applications for sample preparation have been explored for cryo-EM
76 imaging[34]. However, optimization will be needed for protein array formation.
77
78 Here, we provide extensive details of the general preparation of lipid monolayers for cryo-EM
79 imaging and some considerations that could affect the quality of the formed monolayers.
80
81 **PROTOCOL:**
82
83 **1.   Teflon block preparation**
84
85 1.1.   Prepare the Teflon block from chemical-resistant PTFE (polytetrafluoroethylene) resin.
86 Make holes on the block using a general drill followed by the dimensions labeled in **Figure 1**.
87
88 **2.   Monolayer lipid preparation**

89

90 NOTE: Estimated operating time: 30- 45 minutes

91

92 2.1.   Lipid stock preparation

93

94 2.1.1. Prepare a 0.01 mg/mL lipid mixture in 9:1 (v/v) chloroform/methanol using 8.91 mL of
95 chloroform, 0.99 mL of methanol and 0.01 mL of 10 mg/mL lipids.

96

97 2.2.   Teflon plate preparation

98

99 2.2.1. Sonicate a Teflon plate in a methanol bath for 5 minutes.

100

101 2.2.2. Wash with hot water for 15 minutes.

102

103 2.2.3. Rinse with distilled water.

104

105 2.2.4. Dry the Teflon plate in a desiccator for 30 minutes and keep it under vacuum when not in
106 use (**Figure 1**).

107

108 **3.   Formation of lipid monolayer on buffer reservoir**

109

110 NOTE: Estimated operating time: 1.5 hours

111

112 3.1.   Freshly prepare a lipid mixture of 0.01 mg/mL in a 9:1 mixture of chloroform/methanol
113 on the day of the experiment.

114

115 3.2.   Place a Type-1 filter paper in a Petri dish and arrange the Teflon block on top of the filter
116 paper.

117

118 3.3.   Fill the wells of the Teflon plate with 60 µL of the buffer.

119

120 3.4.   Carefully add lipid mixture on top of the buffer surface drop by drop (ideally 1 µL per drop)
121 using a Hamilton syringe.

122

123 3.5.   Wet the filter paper with distilled water to keep humidity in the Petri dish.

124

125 3.6.   Incubate at room temperature for 60 minutes. The chloroform should evaporate, and a
126 monolayer of lipid on the surface of the buffer should be formed.

127

128 **4.   Application of an EM grid on a lipid monolayer**

129

130 NOTE: Estimated operating time: 1.5 hours

131

132   4.1.   Gently place a holey carbon-coated EM grid without glow-discharging, carbon side down,
133   onto the top of each buffer reservoir.

134

135   4.2.   Carefully inject proteins into the side injection well. Use final protein concentrations in
136   the well around 1 µM of protein as a starting point. Pre-optimize the protein concentration
137   before the experiment.

138

139   4.3.   Incubate for 60 minutes at room temperature.

140

141   4.4.   Gently inject approximately 20-30 µL of buffer into the side injection port. This will raise
142   the grid above the surface of the Teflon block.

143

144   4.5.   Immediately pick up the grid with a tweezer and lift it vertically off the droplet.

145

146   4.6.   Prepare monolayer samples for negative staining or cryo-TEM imaging (**Figure 2**). If the
147   proteins form a 2D array, the image power spectrum or Fourier periodogram will show diffraction
148   intensities at the spatial frequencies based on the crystal symmetry. If no diffraction spots are
149   shown in the power spectrum, the proteins are unlikely to form a 2D crystal.

150

151   4.7.   Check the coverage of the lipid monolayer on the EM grid using a transmission electron
152   microscope (TEM). A lower-magnified electron image is helpful to identify the morphology of the
153   covered monolayer. Usually, the image contrast of the monolayer-covered area will be slightly
154   lower than that of the uncovered areas. In addition, the computed 2D image power spectrum
155   will help identify the location of the 2D crystalline.

156
157   **REPRESENTATIVE RESULTS:**
158   A lipid monolayer deposited on the EM grid can be visualized under a transmission electron
159   microscope (TEM) without staining. The monolayer presence can be recognized by the contrast
160   difference from the area without any specimen in the beam path. Areas that have lipid monolayer
161   coverage have lower local contrast than the ones with no coverage, since the electron beam
162   through the empty holes has no scattering and shows a brighter illumination (**Figure 3**).

163

164   To screen the conditions for 2D monolayer crystallization, investigate the image of the specimen
165   using negative-stain EM or cryo-EM imaging. Whether the monolayer covers the holes could be
166   observed by a stronger edge contrast when the stage is tilted. If a crystal is formed, the image
167   power spectrum or periodogram will show the Fourier or diffraction spots. Crystals in small sizes
168   (< 200 nm) and randomly oriented may show high-intensity rings in the power spectrum. If the
169   crystals are not grown, the power spectrum will not show Fourier spots.

170
171   **FIGURE AND TABLE LEGENDS:**
172   **Figure 1. Teflon block used for lipid monolayer formation**. A Teflon block is shown in top (above)
173   and cross-section (below) views. It contains 12 wells arranged in six rows and two columns. The
174   main buffer well labeled A, shown in darker blue, in the middle is 4 mm in diameter and 5 mm

175   deep with 60 µL sample volumes. The monolayer is formed on top of the buffer reservoir in the
176   main well and proteins are injected through the side port labeled B shown in light blue.

177

178   **Figure 2. Schematic presentation of the development of 2D crystal on lipid monolayer.** The
179   buffer reservoir is first filled with the buffer, followed by the lipid deposited on top of the well.
180   The carbon side of an EM grid is placed on the lipid monolayer, which will interact with the
181   hydrophobic tails. First, proteins are injected through the side injection well B (step 1). Over time,
182   the proteins are arranged into a 2D crystal lattice and interact with the lipid head groups (step
183   2). To pick up the EM grid for further analysis, a tweezer is used to lift the grid off the buffer
184   reservoir vertically (step 3).

185

186   **Figure 3**. **Example of the lipid monolayer coverage on an EM grid.** A monolayer- and holey
187   carbon-coated EM grid without staining was imaged using a Philips CM12 transmission electron
188   microscope at room temperature. The orange arrow indicates the area that has no lipid
189   monolayer coverage, and the blue arrows indicate the full coverage of the lipid monolayer in the
190   grid hole. Scale bar is 0.5 µm.

191

192   **DISCUSSION:**
193   A lipid monolayer is a powerful tool that facilitates the growth of large 2D crystals for structural
194   studies of biological macromolecules. To successfully prepare an intact lipid monolayer at the air-
195   water interface, it is strongly recommended that the lipids are prepared freshly on the day of the
196   experiment, because oxidization of the lipid acyl chain could lead to packing disruption in the
197   monolayer and adversely affect the resulting crystal formation. Purchased lipids in powder form
198   should be dissolved using a mixture of chloroform:methanol solvent, which is commonly used to
199   dissolve phospholipids[35]. The chloroform:methanol solvent can be premade and stored in a
200   sealed glass vial. While the dissolved lipids are applied at the air-water interface, wait until the
201   chloroform:methanol solvent completely evaporates from the buffer. Proper waiting time for the
202   formation of lipid monolayer and the formation of 2D crystals is essential in this experiment.
203   Typically, the formation of a lipid monolayer and 2D arrays can take place within approximately
204   30 to 60 minutes of incubation time[18].

205

206   When a lipid monolayer is properly formed, ensure that the 2D crystal array formation can be
207   promoted by the lipid effectively. The buffer composition should be optimized to stabilize the
208   establishment of 2D arrays, especially when DOGS-NTA-Ni lipid is used[18,36]. The success of the
209   lipid monolayer crystallization for this lipid may be affected if there are high concentrations of
210   EDTA (ethylenediaminetetraacetate), DTT (1,4-dithiothreitol), or various ions in the buffer. The
211   soluble chelator EDTA can disrupt the lipid-metal ion-protein complex[36]. One example of this is
212   the streptavidin protein interaction with iminodiacetate-Cu (II) lipid; the addition of EDTA
213   removes Cu (II) and interrupts the lipid-metal ion-protein complex[20]. Hence, the sample may
214   need to be diluted or buffer exchanged to remove EDTA, allowing a stronger binding of protein
215   and promoting crystal formation. The optimization of the buffer needs to be done before it is
216   added to the buffer reservoir in the Teflon block.

217

218   To preserve the high-resolution structural detail of the 2D crystal, maintaining the specimen
219   flatness is critical. However, due to surface tension, differential heat capacities between the
220   specimen and support or human intervention, it is not trivial to maintain the flatness of the 2D
221   crystal during the sample transfer. When the EM grid is lifted from the buffer reservoir, one must
222   operate it with as little disturbance as possible to leave the monolayer interface intact. Any
223   drastic rotation or movement of the grid may fracture the monolayer interface. This also applies
224   for sample freezing steps, where the grid with crystals must be handled with care and rapidly
225   frozen to avoid ice crystals, which can damage the newly formed 2D protein crystals.
226
227   One may use a lacey carbon-coated grid to support monolayers in different sizes, depending on
228   the hole sizes on the lacey carbon film. The flatness of the monolayer interface may be affected
229   by various hole sizes on the lacey carbon film, which may help screen a flat monolayer with the
230   diffraction intensities in isotropic directions.
231
232   There are four common methods used to grow and develop 2D crystals for structural studies:
233   dialysis, dilution, hydrophobic adsorption and lipid monolayer[37–39]. The dialysis method removes
234   the detergents that are used to solubilize membrane proteins in the early step of purification.
235   The 2D crystals are grown by reconstituting detergent-solubilized membrane proteins into lipid
236   bilayers under favorable conditions[35]. For the reconstitution to take place, detergents need to be
237   removed by dialysis. Therefore, the dialysis method helps to drive the formation of 2D crystals[40].
238   However, the dialysis method is usually time-consuming, especially for detergents with a small
239   critical micelle concentration (CMC). The formation of 2D arrays can also be achieved via a
240   controlled dilution. This method provides a set ratio for the protein–lipid–detergent mixture to
241   induce crystal formation if the detergent concentration drops below its CMC. This could be
242   facilitated by a specific dilution machine to carry out the work starting with a higher initial protein
243   concentration[41]. In addition, hydrophobic adsorption prompts the growth of 2D crystals by using
244   polystyrene beads (e.g., Bio-beads) to remove the low CMC detergents[42]. If the protein of interest
245   is solubilized with high CMC detergents, this method is not ideal. In contrast, the lipid monolayer
246   method is versatile and provides non-convoluted steps for 2D crystallization[43]. As discussed here,
247   it requires a simple apparatus and some materials to conduct the experiments. With our method,
248   the total time needed for the experiment can be shortened to 4 hours[17]. Even though pre-
249   optimization of certain parameters, such as protein concentration and buffer components, may
250   still be required beforehand, it is possible to maximize one's efficiency in routine lab work.
251
252   For crystallizing integral membrane proteins using a lipid monolayer, one should test whether
253   the detergent concentration for solubilizing protein and lipids disturbs the monolayer interface.
254   Generally, it is suggested to use the detergent concentration lower than the CMC [44]. In cases that
255   the monolayer interface is sensitive to the detergent existence, an alternative approach can be
256   to form the lipid monolayer using fluoro-lipids, which are hardly solubilized by detergent and
257   expand the applicability of integral membrane protein crystallization using the monolayer
258   method [45].
259
260   Although the lipid monolayer method is simple and less costly, the reproducibility of the
261   monolayer preparation depends on various factors, such as ambient humidity and temperature.

262 The success of the sample transfer for cryo-EM imaging relies on careful specimen handling. In
263 some cases, it may be challenging to obtain high-resolution results by a novice or in the beginning
264 phase of a new research project.
265
266 In conclusion, the lipid monolayer method offers an opportunity for studying protein structures
267 due to its simplicity. It may provide an alternative approach to facilitate the process of 2D crystal
268 formation.
269
270 **ACKNOWLEDGMENTS:**

273
274 **DISCLOSURES:**
275 The authors have no conflict of interest to declare.
276
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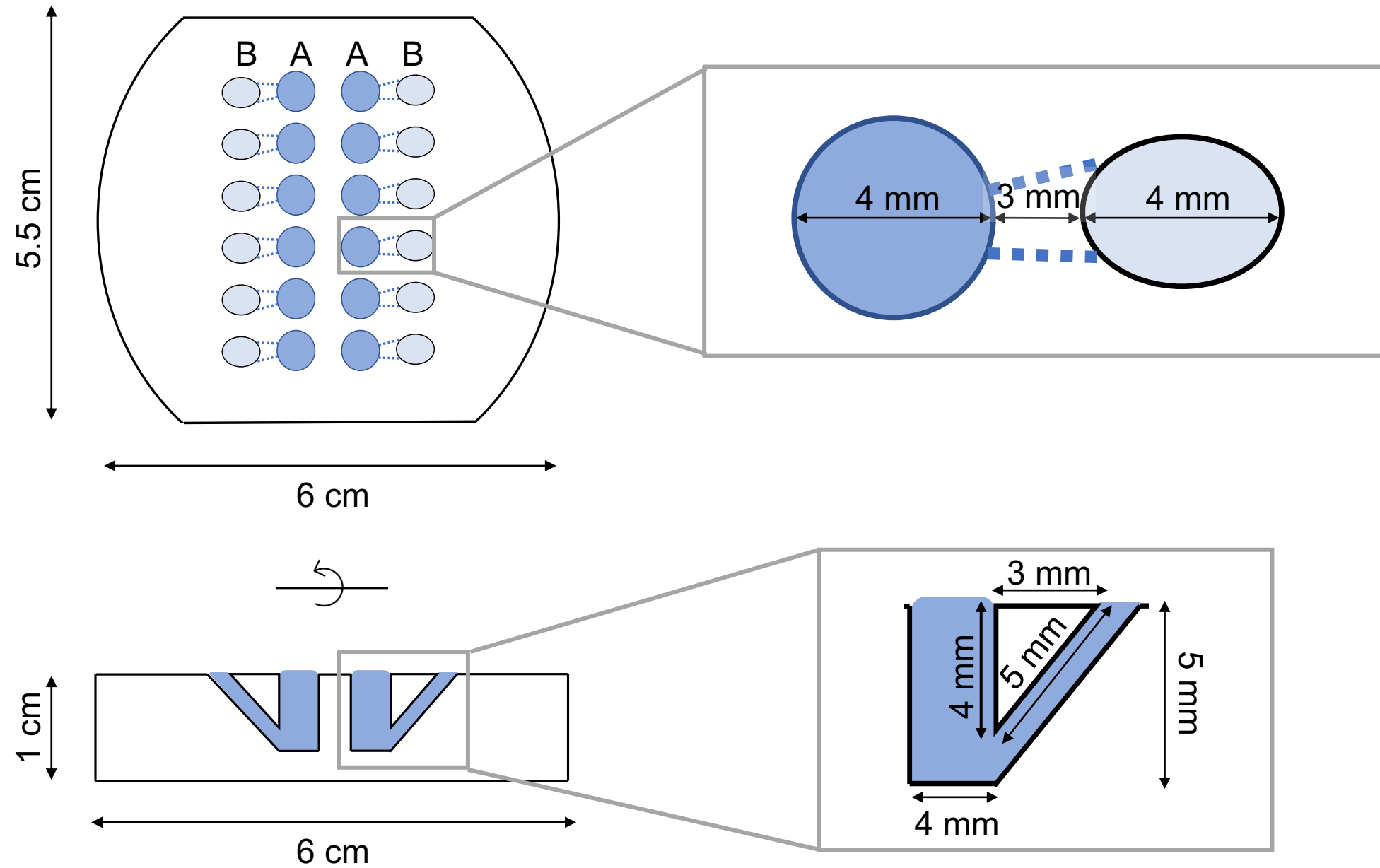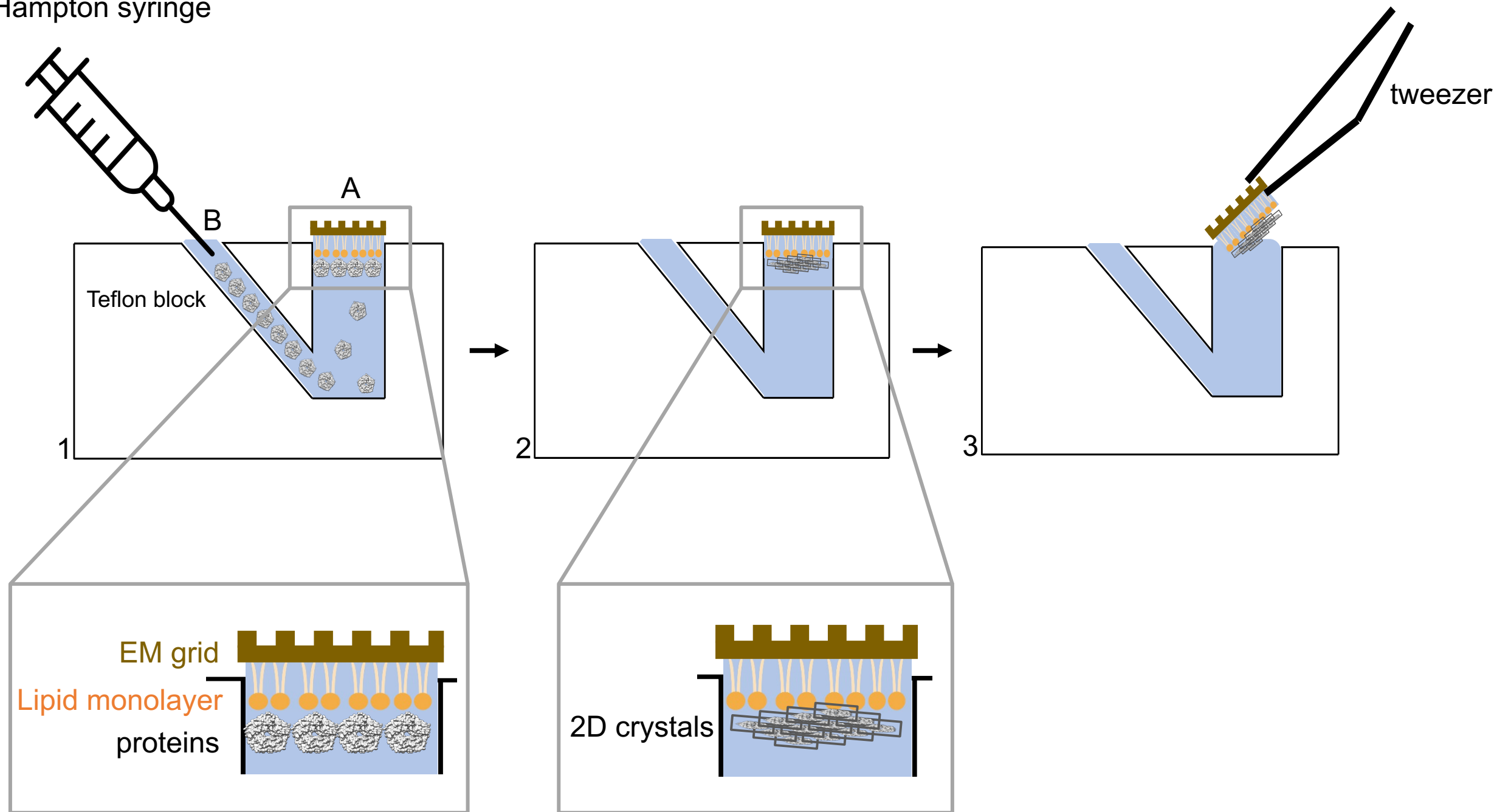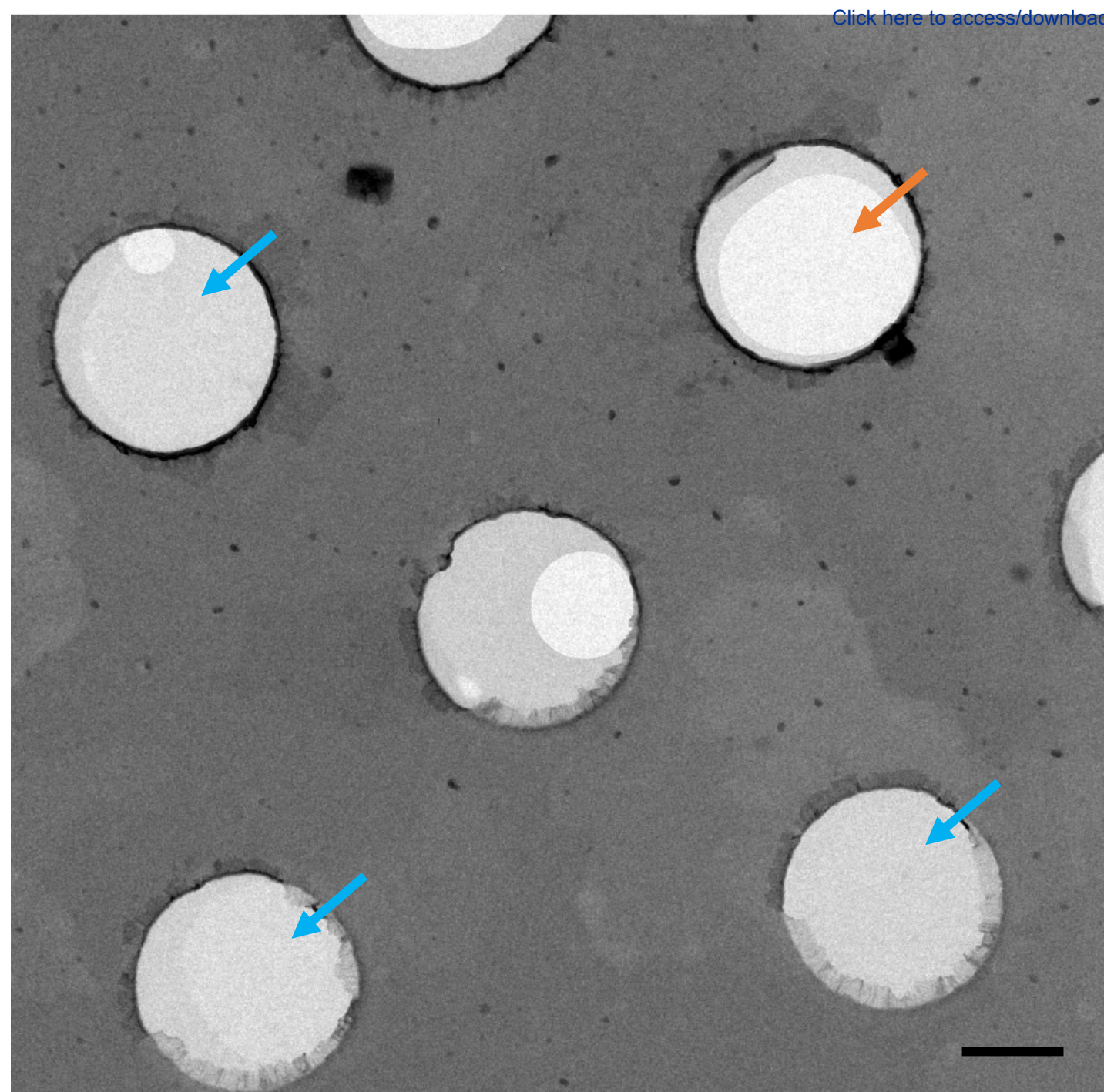
Figure 1

Figure 1

Figure 2

Figure 2

Figure 3

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Table of Materials

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Dear receiving editors,

On behalf of all the co-authors, I want to thank you sincerely for handling and revising our manuscript. Enclosed is the revised version of our manuscript JoVE63015, entitled "*Sample Preparation Using Lipid Monolayer Method for Electron Crystallographic Studies.*" We have followed editorial comments as best as possible to improve our manuscript. The changes in the revised manuscript were tracked. Please find the attached revised manuscript and video and figures in high resolutions in PDF format.

We hope that with these changes, our manuscript is suitable for publication in the *Journal of Visualized Experiments*. Please do not hesitate to let us know if you need any more materials.

Thank you very much for your consideration.

Sincerely,

Po-Lin Chiu
Assistant Professor of Molecular Sciences