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## Preparation of Sample Support Films in Transmission Electron Microscopy using a Support Floatation Block --Manuscript Draft--

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**TITLE:**

Preparation of Sample Support Films in Transmission Electron Microscopy using a Support Floatation Block

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**KEYWORDS:**

TEM; grid preparation; support film; amorphous carbon; graphene; graphene oxide; floatation block.

**SUMMARY:**

Sample preparation for cryo-electron microscopy (cryo-EM) is a significant bottleneck in the structure determination workflow of this method. Here, we provide detailed methods for using an easy-to-use, three-dimensionally printed block for the preparation of support films to stabilize samples for transmission EM studies.

**ABSTRACT:**

Structure determination by cryo-electron microscopy (cryo-EM) has rapidly grown in the last decade; however, sample preparation remains a significant bottleneck. Macromolecular samples are ideally imaged directly from random orientations in a thin layer of vitreous ice. However, many samples are refractory to this, and protein denaturation at the air-water interface is a common problem. To overcome such issues, support films—including amorphous carbon, graphene, and graphene oxide—can be applied to the grid to provide a surface which samples can populate, reducing the probability of particles experiencing the deleterious effects of the air-water interface. The application of these delicate supports to grids, however, requires careful handling to prevent breakage, airborne contamination, or extensive washing and cleaning steps. A recent report describes the development of an easy-to-use floatation block that facilitates wetted transfer of support films directly to the sample. Use of the block minimizes the number of manual handling steps required, preserving the physical integrity of the support film, and the time over which hydrophobic contamination can accrue, ensuring that a thin film of ice can still be generated. This paper provides step-by-step protocols for the preparation of carbon, graphene, and graphene oxide supports for EM studies.

## INTRODUCTION:

Over the last decade, breakthroughs, principally in detector technology, but also in other technical fields, have facilitated a succession of substantial increases in the resolution at which biologically relevant systems can be imaged by transmission electron microscopy (TEM)<sup>1,2</sup>. Despite the fact that cryo-EM already allows the resolution of high-resolution structures from as little as 50 µg of protein through single-particle analysis (SPA), cryo-EM sample and grid preparation remain major bottlenecks<sup>3–5</sup>. SPA samples consist of macromolecules distributed approximately randomly within a layer of vitreous ice. The ice must be as thin as possible to maximize the contrast difference between the particles and the solvent. Biological macromolecules are more stable (*i.e.*, less likely to lose their native structure) in thicker ice, because they remain better solvated. Moreover, particles are often found to be much better distributed over the field of view in ice much thicker than the particle size<sup>6</sup> and frequently may not be found within holes in the carbon films at all.

Additionally, thicker layers of ice decrease the probability of molecules being close to the air-water interface due to the high surface-to-volume ratio, and it has been estimated that using standard plunge-freezing methods for cryo-EM studies results in the adsorption of ~90% of particles to the air-water interface<sup>7</sup>. Thicker ice results in undesirably high background due to increased scattering events within the solvent and concomitant attenuation of the signal<sup>6,7</sup>. It is therefore necessary to achieve as thin a layer of vitreous ice as possible; ideally, the layer would be only slightly thicker than the particle. The challenge for the researcher, which must be overcome for every different sample applied to a grid, is to prepare specimens thin enough for high-contrast imaging whilst maintaining the structural integrity of the particles within their sample. Protein adsorption to the air-water interface is accompanied by several, usually deleterious, effects.

First, binding of proteins to this hydrophobic interface often induces denaturation of the protein, which proceeds rapidly and is typically irreversible<sup>8,9</sup>. A study conducted using yeast fatty-acid synthase showed that up to 90% of adsorbed particles are denatured<sup>10</sup>. Second, evidence from a study comparing the orientation distribution of 80S ribosome datasets collected either on amorphous carbon<sup>11</sup> or without support<sup>12</sup> showed that the air-water interface can cause severe preferential orientation compromising 3D reconstruction of the volume<sup>13</sup>. Methods to reduce particle interaction with the air-water interface include supplementation of the freezing buffer with surfactants (such as detergents), the use of support films, affinity-capture or scaffolding of substrates, and accelerated plunging times. The use of surfactants is associated with its own problems, as some protein samples may behave non-ideally in their presence, whilst affinity-capturing and scaffolding substrates generally require engineering bespoke grid surfaces and capture strategies. Finally, although there is a lot of research on the development of rapid-plunging devices<sup>14–16</sup>, these require apparatus that is generally not widely available.

Although the standard TEM grid for biological cryo-EM already features a perforated amorphous carbon foil<sup>17</sup>, there are a number of protocols available for the generation of additional support films and their transfer to TEM grids. The use of these films is a long-established method for sample stabilization<sup>18</sup>. Amorphous carbon supports are generated by evaporation and deposition

on crystalline mica sheets<sup>19</sup>, from which the layers can be floated onto grids, with the utility of floatation supports as useful tools established in prior reports<sup>20</sup>. Graphene oxide flakes, typically prepared using a modified version of the Hummers method<sup>21</sup>, have been used as a preferable support structure to amorphous carbon for their decreased background signal as well as the ability to immobilize and stabilize macromolecules<sup>22</sup>. More recently, there has been a resurging interest in the use of graphene as a TEM support film due to its mechanical stability, high conductivity, extremely low contribution to background noise<sup>23</sup>, as well as the emergence of reproducible methods for generating macroscopically large areas of monolayer graphene<sup>24</sup> and transferring it to TEM grids<sup>25</sup>. When compared to amorphous carbon, which undergoes beam-induced motions similarly to, or worse, than ice lacking a support film<sup>11,12,17</sup>, graphene showed a significant reduction in beam-induced motion of cryo-EM images<sup>12</sup>.

However, while hydrophilized graphene protected fatty acid synthase from air-water interfacial denaturation, the authors of this study noted that the graphene became contaminated during specimen preparation, likely due to a combination of atmospheric hydrocarbon contamination and from the reagent used to hydrophilize the grids<sup>10</sup>. Indeed, despite many of the superior qualities of graphene, its widespread use is still hindered by the derivatization required to decrease its hydrophobicity<sup>12</sup>, which ultimately is chemically difficult and requires specialist equipment. This paper reports protocols for the preparation of amorphous carbon, graphene oxide, and graphene sample supports using a three dimensionally (3D) printed sample floatation block<sup>27</sup> to directly transfer support films from the substrates on which they were generated to TEM grids (**Figure 1**). A key advantage of using such a device is the wetted transfer of films, minimizing hydrophobic contamination of the supports and consequently the need for further treatment, and reducing the number of potentially damaging manual handling steps. These approaches are inexpensive to implement and therefore widely accessible and applicable for cryo-EM studies where sample supports are necessary.

## PROTOCOL:

### 1. General preparation of TEM grids presupport transfer

1.1. Using a pair of clean, fine tweezers, lift and submerge TEM grids sequentially in double-distilled water (ddH<sub>2</sub>O) or ultrapure water for 10–15 s, followed by ethyl acetate, for 10–15 s.

NOTE: Here, negative-action, oblique-tip tweezers were used.

1.2. Place the tweezers, with grid still in grip, to one side to air-dry for ~5 min.

1.3. Plasma-clean the grids to strip the surface of any contaminants accrued through the air or washing steps.

NOTE: Here, plasma-cleaning was done for 10–15 s in air with a radiofrequency power of 25 W.

### 2. General preparation of reagent solutions

2.1. Uranyl acetate (UAc) solution (2% w/v)

2.1.1. Wrap a 50 mL tube in foil fill with 50 mL of ultrapure water, and add 1 g of UAc powder.

NOTE: UAc is light-sensitive and precipitates over time when exposed. As UAc is radioactive and toxic, maintain a high level of cleanliness. With the most serious hazard arising from inhalation or ingestion, extra care should be taken to prevent any possibility of inhaling fine particles. Gloves must always be worn when handling or weighing out the uranium salts. Masks and goggles highly recommended. Uranium salts must be disposed of according to the legal requirements set out for radioactive hazards within the state.

2.1.2. Leave the solution stirring for 1 h to allow for all the UAc to dissolve. Store at 4°C.

2.1.3. Before use, filter 1 mL of the stain solution into a small vial using 0.22 µm filter to remove any remaining acetate crystals.

2.2. Graphene oxide (GrOx) suspension

2.2.1. Pipette 2.5 µL of GrOx into a 1.5 mL tube (1% final concentration). Pipette 2.5 µL of 10% (w/v) *n*-dodecyl β-D-maltoside (DDM) detergent into the GrOx, and gently mix (0.1% (w/v) final concentration).

2.2.2. Add 245 µL of ultrapure water to the GrOx-DDM mix, and immediately vortex vigorously for 5 min. Use GrOx suspension within 1 h of preparation, vortex vigorously for at least 1 min before immediate use.

2.3. Iron(III) chloride (FeCl<sub>3</sub>) solution (10% w/v)

2.3.1. Carefully weigh 5 g of FeCl<sub>3</sub> in a weighing boat. Transfer to a 100 mL measuring cylinder containing 35 mL of ddH<sub>2</sub>O and a magnetic stir bar.

2.3.2. Place on a magnetic stirring plate, and dissolve FeCl<sub>3</sub>, adding ddH<sub>2</sub>O to a final volume of 50 mL. Filter the FeCl<sub>3</sub> solution through a 0.8 µm syringe filter into a clean bottle for storage.

NOTE: FeCl<sub>3</sub> is corrosive and an irritant; wash hands and other exposed areas with mild soap and water before eating, drinking or smoking and when leaving work. Provide good ventilation in process area to prevent formation of vapor. Do not breathe mist, vapors, spray. Gloves must always be worn when handling or weighing out the salt. Masks and goggles highly recommended whenever in use.

3. Buffer exchange for carbon support films on mica to prepare negatively stained samples using the support floatation block

177 3.1. Wash and plasma-clean TEM grids.  
178

179 NOTE: Here, 300 mesh holey-carbon copper grids were used as outlined above in section 1.  
180

181 3.2. Pipette 10–12  $\mu\text{L}$  of sample into the buffer exchange well (with the small channels) of the  
182 floatation block and 10–12  $\mu\text{L}$  of 2% UAc solution (see section 2.1) for negative staining into the  
183 adjacent non-buffer exchange well.  
184

185 NOTE: The well has a volume of 10  $\mu\text{L}$ ; however, adjust the sample volume so that a convex  
186 meniscus is formed at the surface of the liquid to allow proper film floatation. A low volume of  
187 sample may cause film breakage.  
188

189 3.3. Carefully cut two small pieces of mica with predeposited carbon film on top. Ensure that  
190 the mica fragments are wide enough to fit into the well (3.4 mm width) and longer than the well  
191 length (3.45 mm), such that the fragment will sit on the well while carbon is floating, and there  
192 is enough space to handle the fragment with the tweezers.  
193

194 NOTE: To handle the carbon, use flat negative-action long-tip tweezers. When cutting the mica  
195 fragments, cut using single movements to maintain the integrity of the carbon film.  
196

197 3.4. Immerse the mica into the well with an approximate angle of  $45^\circ$  until the mica sits on  
198 the ramp of the well and a layer of carbon is observed at the surface of the liquid sample.  
199

200 3.5. After the initial incubation on sample (typically 20 s to 20 min depending on the sample  
201 adherence; optimize this period based on experimental needs), withdraw the mica sheet very  
202 slowly to recover the carbon film and minimize residual viscous sample retention.  
203

204 3.6. Carefully blot the mica by tapping the lower surface (non-carbon side) with filter paper to  
205 remove excess liquid, and subsequently exchange the carbon bearing the sample into negative  
206 stain by application to the opposing well (*i.e.*, immerse the mica as in step 3.4) containing the 2%  
207 UAc solution.  
208

209 NOTE: A carbon layer should be observed floating on top of the stain solution at this point.  
210

211 3.7. Recover the floating carbon layer with the holey carbon-covered side of a washed and  
212 plasma-cleaned EM grid. Leave the grids to air-dry until imaging on a TEM. Ideally, cover the grids  
213 during the drying process to avoid airborne contamination.  
214

215 4. Application of the support floatation block to prepare graphene oxide-coated TEM grids  
216

217 4.1. Wash and plasma-clean TEM grids using 300 mesh holey-carbon copper grids as outlined  
218 above (section 1).  
219

220 4.2. Pipette 10–12  $\mu\text{L}$  of GrOx suspension (see section 2.2) into the 4 non-buffer exchange

wells along the floatation block. Pipette 10–12  $\mu\text{L}$  ddH<sub>2</sub>O or ultrapure water into the remaining 4 buffer exchange wells of the block.

NOTE: This volume of water should be sufficient to form a slight convex meniscus rising above the height of the block.

4.3. Drop 4 grids gently onto the GrOx suspension of each well for 1 min, ensuring that the holey carbon-covered side makes contact with the solution. After 1 min, recover each grid carefully by sliding the tweezers into the tweezer groove of each non-buffer exchange well.

4.4. Very gently and briefly touch the copper, non-carbon-covered side of each grid to the ddH<sub>2</sub>O in the adjacent well. Then, carefully and gently hold the grid, water droplet-side down, against a piece of filter paper.

NOTE: Blotting off the water will draw the GrOx suspension through the grid by capillary action. It is crucial to avoid submerging the grid in the ddH<sub>2</sub>O, so contact should be very brief. When the grid is lifted, a droplet of water should hold to the underside of the grid. Take care not to move the grid on the filter paper as this could upset the settling of the GrOx flakes.

4.5. Leave the grids in the tweezers to air-dry until preparation with sample. Ideally, cover the grids during the drying process to avoid airborne contamination.

## 5. Application of the support floatation block for the preparation of samples on monolayer-graphene films

5.1. Wash the TEM grids as outlined above (section 1), but omitting plasma-cleaning.

NOTE: Here, 300 mesh holey-carbon gold grids were used, but other non-copper grids or copper alloy grids are also practicable.

5.2. To deposit grids with graphene, directly transfer from graphene grown on copper (Cu-graphene) substrates to cryo-EM grids, as described previously<sup>25</sup>.

5.2.1. Place four washed grids on top of a Cu-graphene sheet (10 mm  $\times$  10 mm) deposited onto a glass slide, and cover each grid with a drop of isopropanol (5–10  $\mu\text{L}$ ) to allow intimate contact between the monolayer graphene and the grid.

NOTE: Make sure to place the holey carbon-covered side of the grids in contact with the graphene sheet.

5.2.2. When the isopropanol is completely evaporated (typically 2 h), float the Cu-graphene sheet with grids onto 10% (w/v) FeCl<sub>3</sub> solution (see section 2.3) in a glass Petri dish, and leave to etch at room temperature overnight. Cover the dish to avoid airborne contamination.

NOTE: After etching is complete, only the graphene monolayer will remain floating on the  $\text{FeCl}_3$  solution—this should be visible by eye with suitable lighting.

5.2.3. Use a loop with diameter larger than the TEM grid size to fish the grids floating on the graphene monolayer, and carefully transfer to a glass Petri dish containing ddH<sub>2</sub>O to wash.

NOTE: Be extremely cautious when fishing the grids to avoid hitting the walls of the Petri dish, which may cause graphene film breakage or bending.

5.2.4. Wash two more times in water by fishing grids and transferring to a clean Petri dish containing ddH<sub>2</sub>O to remove all residual  $\text{FeCl}_3$ . Finally, transfer grids into a Petri dish containing sample buffer until sample preparation and plunge-freezing.

NOTE: The graphene-covered side of the grids must be kept wetted at all times to avoid their exposure to airborne contaminants.

5.3. Pipette the sample (10–12  $\mu\text{L}$ ) into a non-buffer exchange well of the floatation block. When the sample is ready in the block, pick a graphene-coated grid from the buffer solution using a pair of clean tweezers, and place onto the surface of the sample-containing well.

5.4. After an appropriate incubation period (1–5 min depending on the sample; optimize according to experimental needs), pick the grid with a pair of clean freezing tweezers, and proceed with blotting and vitrification.

## REPRESENTATIVE RESULTS:

TEM grids prepared with amorphous carbon supports are typically covered across the entire grid surface. Although breakage of the carbon film occurs in some instances along with some ruffling (**Figure 2A**), a large number of grid squares are pristine and thus widely applicable for negative staining purposes. The major factor affecting the integrity of the support is the carbon thickness, which is determined during carbon evaporation. Similarly, with this GrOx protocol, good coverage is routinely achieved across the entire grid (**Figure 2B**). A single application of GrOx suspension for 1 min is sufficient to ensure few areas with multiple layers, which are easy to see due to flake edges. GrOx grids can be prepared quickly from raw materials and are highly protective of the sample.

However, flake edges, incomplete coverage, and ruffling are more frequently visible with GrOx grids than for the other techniques because of the nature of the GrOx flakes. Although the integrity of the graphene support film, like the amorphous carbon, depends on the deposition process, areas that are well-covered display the characteristic diffraction pattern of single-layer graphene. Importantly, by keeping graphene support films wetted, samples can be recovered from the floatation block after an incubation period and data collected in a manner amenable for single-particle analysis. This method does not require any other treatment of the graphene for wetting, thereby removing the requirement for expensive equipment to render graphene hydrophilic, and it is best to prepare support films shortly prior to sample preparation and grid



freezing (**Figure 2C**).

#### FIGURE AND TABLE LEGENDS:

**Figure 1: Sample floatation block design and application during support film preparation.** (A) Schematic of top, well, and side views of the floatation block including measurements of the shape, depth, and incline. The groove for tweezer tips to rest, as well as channels to insert needles, are indicated. (B) Amorphous carbon layers can easily be floated onto the surface of buffer contained within the wells of the floatation block using the ramp, *i.e.*, during the preparation of negatively stained TEM grids. (C) The width of the wells is suited to accommodate one TEM grid, whilst the tweezer grooves reduce the need to release and pick up grids unnecessarily during preparation steps, but offer a defined path to recover grids without risk of bending if grids are released. Images in **B** are modified from <sup>26</sup>. Abbreviation: TEM = transmission electron microscopy.

**Figure 2: Typical examples of sample support films prepared using the floatation block.** Grid square (left) and image (right) views are shown for (A) amorphous carbon, (B) graphene oxide, and (C) graphene support films prepared using the floatation block. The amorphous carbon support was used in the preparation of 70S ribosomes for negative staining, whereas the graphene oxide and graphene supports were used in the preparation of 70S ribosomes for cryo-EM. Images in **A** and **C** are modified from <sup>26</sup>. Scale bar for **A** grid square = 10  $\mu\text{m}$ ; scale bars for **B** and **C** grid squares = 5  $\mu\text{m}$ ; scale bars for **A–C** image views = 50 nm. Abbreviation: cryo-EM = cryo-electron microscopy.

#### DISCUSSION:

This paper presents protocols for handling of both amorphous carbon and graphene films for cryo-EM sample preparation using a sample floatation block<sup>27</sup>. An STL file for the support block is freely available from the public Thingiverse repository [[www.thingiverse.com/thing:3440684](http://www.thingiverse.com/thing:3440684)], and can be 3D-printed with any suitable stereolithography printer from a suitable resin. The use of carbon films covering a TEM grid usually involves the carbon floatation onto the sample<sup>28</sup>. This approach to preparing negative stain grids minimizes air exposure during support handling, thus reducing contamination and protein denaturation. The preparation of grids using floating carbon in small wells is advantageous to floating a larger surface area, *i.e.*, in a water bath or Petri dish, in which case mechanical shearing of the carbon occurs much more readily.

UAc may be difficult to purchase due to current health and safety regulations at the time of publication. Many other commonly used, non-radioactive, negative staining reagents are available, and protocols for their preparation have been described previously<sup>29</sup>. Although alternative stains have not been used with this support floatation block, it is not likely that there would be any differences in these protocols besides the optimization of incubation time with sample (step 3.5), which is already inherently sample-dependent. The key step in this GrOx support preparation protocol is step 4.4, highlighted by the note to prevent the water and GrOx solution from making contact around the grid edge. Inappropriate mixing of the water and GrOx solutions prevents unidirectional settling of the GrOx flakes by capillary action. Having GrOx flakes on both sides of the carbon foil results in thick layers, thus negating the advantages of

using GrOx as a near-single layer support, as well as trapping water between the flakes, which causes contamination of useable areas with additional layers of ice. Graphene oxide support preparation is relatively easy to achieve using droplets of solution on flexible polyolefin film.

However, when performed in that way, it is easier to accidentally contaminate the copper side of the grid by mishandling errors; the use of the floatation block reduces the likelihood of this eventuality. Finally, this paper presents a protocol to prepare graphene-covered grids that avoids any kind of graphene pretreatment to render it hydrophilic, thus reducing its cost and increasing its accessibility. Maintaining a wetted film throughout specimen preparation and applying the sample *in situ* in the block just before freezing is sufficient to allow the generation of suitable ice layers for cryo-EM with a homogeneous sample distribution. Overall, the protocols presented here minimize sample contact with the air-water interface, therefore reducing sample denaturation and support contamination. For the three support films used in these approaches, homogeneous sample distributions could be achieved across the grids along with imaging of intact, well-preserved single particles.

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#### DISCLOSURES:

The authors are not aware of any conflicts of interest with respect to this work.

#### REFERENCES:

1. Frank, J. Advances in the field of single-particle cryo-electron microscopy over the last decade. *Nature Protocols*. **12** (2), 209–212 (2017).
2. Lyumkis, D. Challenges and opportunities in cryo-EM single-particle analysis. *Journal of Biological Chemistry*. **294** (13), 5181–5197 (2019).
3. Elmlund, D., Elmlund, H. Cryogenic electron microscopy and single-particle analysis. *Annual Review of Biochemistry*. **84** (1), 499–517 (2015).
4. Thompson, R. F., Walker, M., Siebert, C. A., Muench, S. P., Ranson, N. A. An introduction to sample preparation and imaging by cryo-electron microscopy for structural biology. *Methods*. **100**, 3–15 (2016).
5. Arnold, S. A. et al. Miniaturizing EM sample preparation: opportunities, challenges, and “Visual Proteomics.” *Proteomics*. **18** (5–6), 1700176 (2018).
6. Wu, S., Armache, J. P., Cheng, Y. Single-particle cryo-EM data acquisition by using direct electron detection camera. *Microscopy*. **65** (1), 35–41 (2016).
7. Noble, A. J. et al. Routine single particle CryoEM sample and grid characterization by tomography. *eLife*. **7**, e34257 (2018).
8. Grimm, R., Typke, D., Bärmann, M., Baumeister, W. Determination of the inelastic mean free path in ice by examination of tilted vesicles and automated most probable loss imaging.

397 *Ultramicroscopy*. **63** (3–4), 169–179 (1996).

398 9. Glaeser, R. M. Proteins, interfaces, and cryo-EM grids. *Current Opinion in Colloid and*  
399 *Interface Science*. **34**, 1–8 (2018).

400 10. D’Imprima, E., Floris, D., Joppe, M., Sánchez, R., Grininger, M., Kühlbrandt, W. Protein  
401 denaturation at the water-air interface and how to prevent it. *eLife*. **8**, e400432 (2019).

402 11. Bai, X. C., Fernandez, I. S., McMullan, G., Scheres, S. H. W. Ribosome structures to near-  
403 atomic resolution from thirty thousand cryo-EM particles. *eLife*. **2013** (2), e00461 (2013).

404 12. Russo, C. J., Passmore, L. A. Controlling protein adsorption on graphene for cryo-EM using  
405 low-energy hydrogen plasmas. *Nature Methods*. **11** (6), 649–652 (2014).

406 13. Naydenova, K., Russo, C. J. Measuring the effects of particle orientation to improve the  
407 efficiency of electron cryomicroscopy. *Nature Communications*. **8** (1), 8–12 (2017).

408 14. Jain, T., Sheehan, P., Crum, J., Carragher, B., Potter, C. S. Spotiton: A prototype for an  
409 integrated inkjet dispense and vitrification system for cryo-TEM. *Journal of Structural Biology*.  
410 **179** (1), 68–75 (2012).

411 15. Razinkov, I. et al. A new method for vitrifying samples for cryoEM. *Journal of Structural*  
412 *Biology*. **195** (2), 190–198 (2016).

413 16. Feng, X. et al. A fast and effective microfluidic spraying-plunging method for high-  
414 resolution single-particle cryo-EM. *Structure*. **25** (4), 663–670.e3 (2017).

415 17. Ermantraut, E., Wohlfart, K., Tichelaar, W. Perforated support foils with pre-defined hole  
416 size, shape and arrangement. *Ultramicroscopy*. **74** (1–2), 75–81 (1998).

417 18. Adrian, M., Dubochet, J., Lepault, J., McDowell, A. W. Cryo-electron microscopy of viruses.  
418 *Nature*. **308** (5954), 32–36 (1984).

419 19. Fujiyoshi, Y. The structural study of membrane proteins by electron crystallography.  
420 *Advances in Biophysics*. **35**, 25–80 (1998).

421 20. Hummers, W. S., Offeman, R. E. Preparation of graphitic oxide. *Journal of the American*  
422 *Chemical Society*. **80** (6), 1339 (1958).

423 21. Pantelic, R. S., Meyer, J. C., Kaiser, U., Baumeister, W., Plitzko, J. M. Graphene oxide: A  
424 substrate for optimizing preparations of frozen-hydrated samples. *Journal of Structural Biology*.  
425 **170** (1), 152–156 (2010).

426 22. Pantelic, R. S. et al. Graphene: Substrate preparation and introduction. *Journal of*  
427 *Structural Biology*. **174** (1), 234–238 (2011).

428 23. Li, X. et al. Large-area synthesis of high-quality and uniform graphene films on copper  
429 foils. *Science*. **324** (5932), 1312–1314 (2009).

430 24. Regan, W. et al. A direct transfer of layer-area graphene. *Applied Physics Letters*. **96** (11),  
431 2008–2011 (2010).

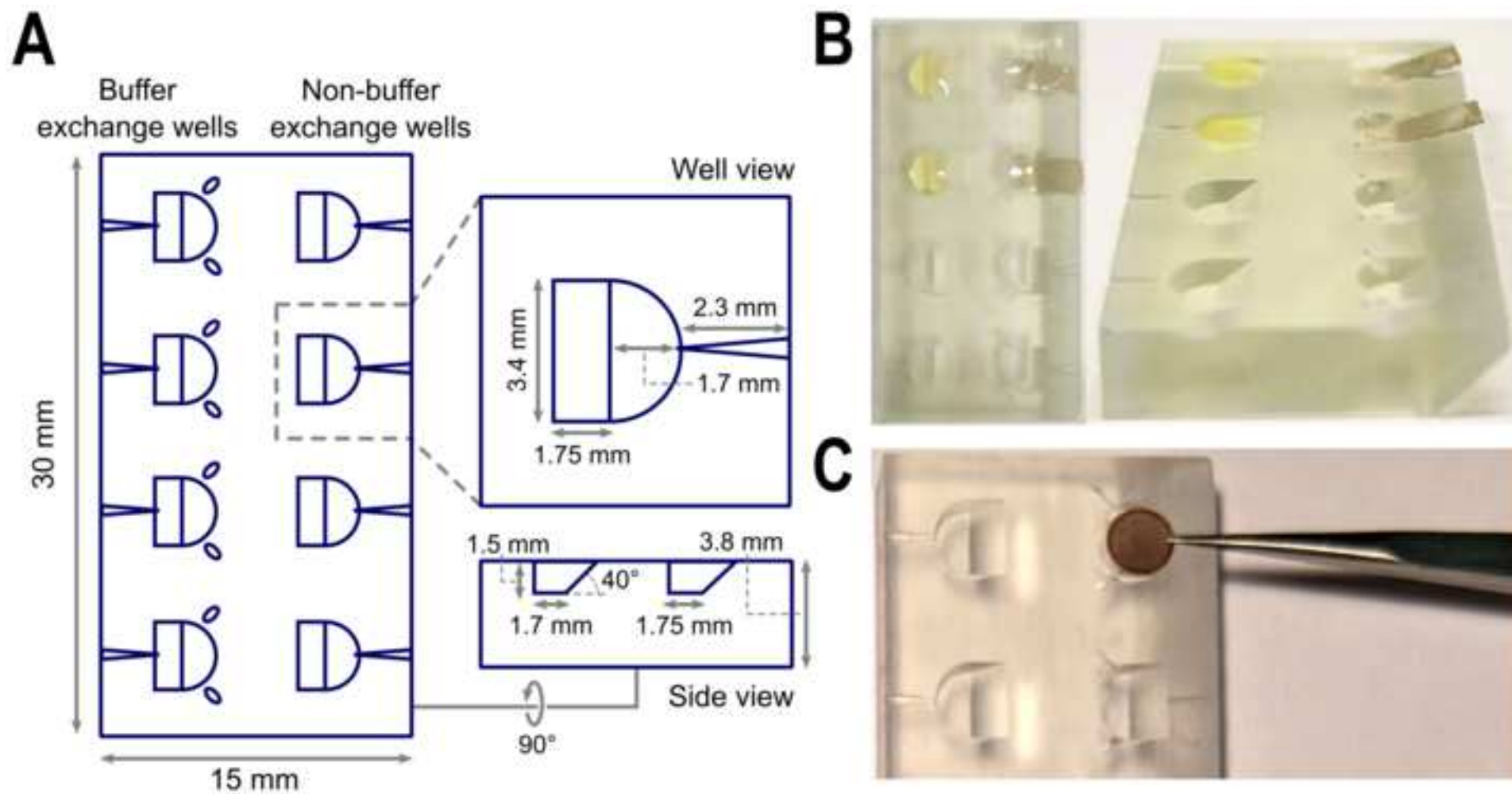
432 25. Brilot, A. F. et al. Beam-induced motion of vitrified specimen on holey carbon film. *Journal*  
433 *of Structural Biology*. **177** (3), 630–637 (2012).

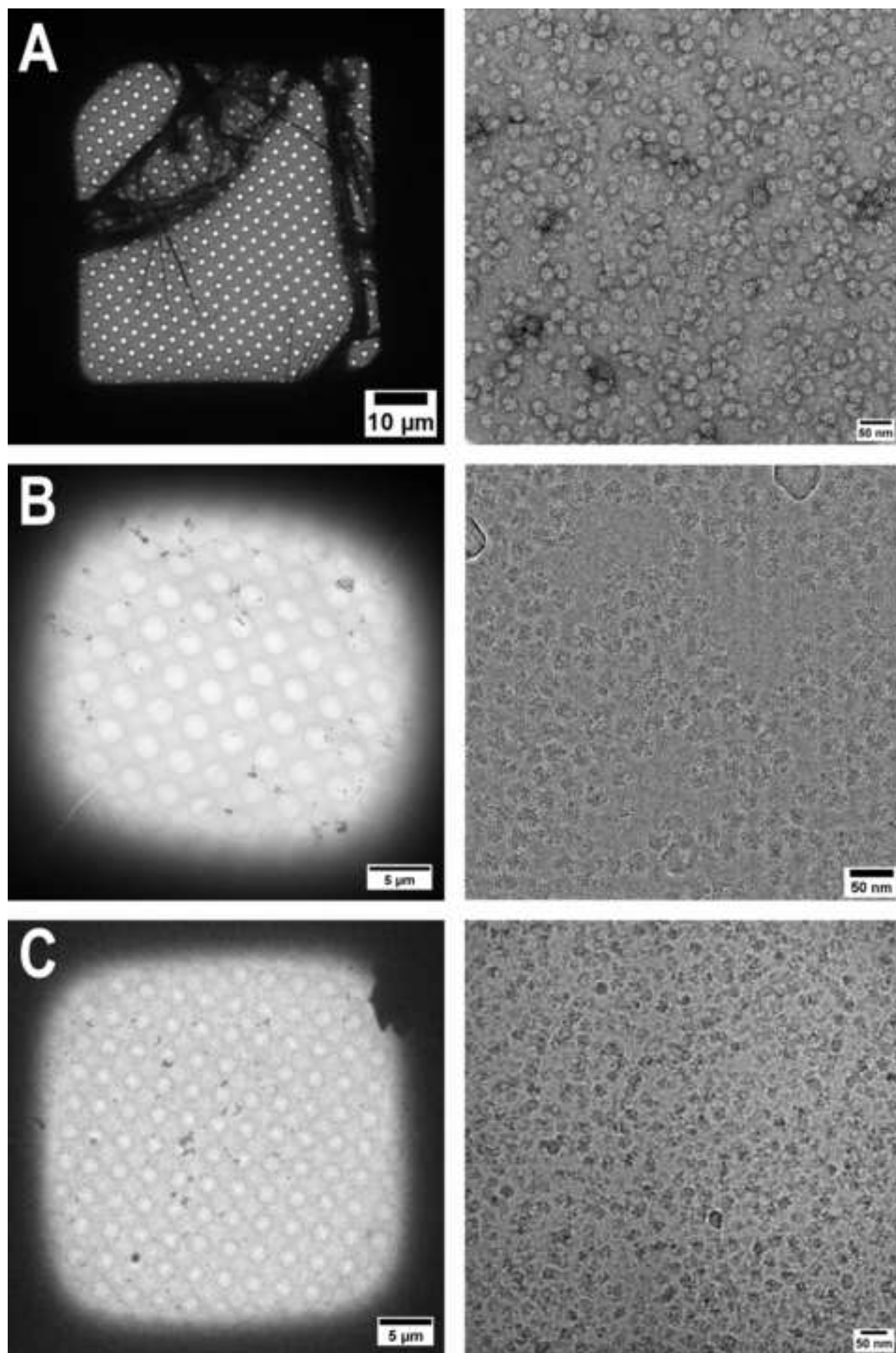
434 26. de Martín Garrido, N. et al. Direct transfer of electron microscopy samples to wetted  
435 carbon and graphene films via a support floatation block. *Journal of Structural Biology*. **213** (1),  
436 107677 (2021).

437 27. Valentine, R. C., Shapiro, B. M., Stadtman, E. R. Regulation of glutamine synthetase. XII.  
438 Electron microscopy of the enzyme from *Escherichia coli*. *Biochemistry*. **7** (6), 2143–2152 (1968).

439 28. Scarff, C. A., Fuller, M. J. G., Thompson, R. F., Iadaza, M. G. Variations on negative stain  
440 electron microscopy methods: Tools for tackling challenging systems. *Journal of Visualized*

441     *Experiments.* (132), 57199 (2018).  
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<b>Name of Material/ Equipment</b>	<b>Company</b>	<b>Catalog Number</b>
Basic Plasma Cleaner (230 V)	Harrick Plasma	PDC-32G-2
Dumont tweezers N5A INOX.	Dumont Swissmade	0302-N5A-PO
Dumont tweezers NGG INOX.	Dumont Swissmade	0102-NGG-PO
Ehtylacetate	Sigma-Aldrich	270989-250ML
Fishing Loops 10 µL	VWR	612-9353
Graphene Oxide 2 mg/mL	Sigma-Aldrich	763705-25ML
Iron (III) chloride	Sigma-Aldrich	31232-250MG
Mica Sheets 75 mm x 25 mm x 0.15 mm	Agar Scientific	AGG250-1
Monolayer Graphene on Cu	Graphenea	N/A
n-dodecyl β-D-maltoside (DDM)	GLYCON Biochemicals GmbH	D97002-C
Quantifoil R1.2/1.3 300 mesh copper grids	Enzo Life Sciences	JBS-X-101-Cu300
Quantifoil R2/1 300 mesh copper grids	Enzo Life Sciences	JBS-X-102-Cu300
Quantifoil R2/1 300 mesh gold grids	Electron Microscopy Sciences	Q350AR1
Scissors	Agar Scientific	AGT577
Uranyl Acetate	TAAB Laboratories Equipment	U001
Vitrobot Mark IV	FEI	N/A
Whatman filter paper 55 mm	GE Healthcare Life Sciences	1441-055
Whatman filter paper 70 mm	GE Healthcare Life Sciences	1441-070

## Comments/Description

We usually coat mica with a target carbon film thickness of 2 nm  
10 mm x 10 mm, pack of 4



10<sup>th</sup> February 2021

**Christopher HS Aylett**

BA MA MSci PhD  
Sir Henry Dale Fellow

To the Editorial Committee: Journal of Visualised Experiments

**Re: de Martin Garrido, *et al.* – Preparation of Sample Support Films in Transmission Electron Microscopy using a Support Floatation Block**

Dear Sir / Madam,

We thank the reviewers and the editor for their comments and recommendations on our manuscript.

We have edited and reformatted our manuscript as requested. Please find below our responses in-line with the editor's and reviewers' comments.

We hope that the amendments are satisfactory and that our manuscript can be considered ready for publication, however we look forward to receiving further comments as necessary.

Yours faithfully,



Christopher H S Aylett

## Comments from Editor:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have revised and proofread the manuscript as requested. We have made several adjustments in line with below comments from both the editor and reviewers and have tracked all changes as requested.

2. Please consider revising the title to “Preparation of Sample Support Films in Transmission Electron Microscopy using a Support Floatation Block”

We have revised the title and changed it according to the editor’s recommendation:

“Preparation of Sample Support Films in Transmission Electron Microscopy using a Support Floatation Block”.

3. Please provide an institutional email address for each author.

An email address for each author has been added as requested by the editor.

4. Please define all abbreviations before use (EM, ddH<sub>2</sub>O).

We have revised the manuscript and defined all abbreviations before use as the editor asks.

5. Line 43: Please consider removing the reference in parenthesis.

The reference in line 43 (Abstract) has been removed as asked.

6. JoVE cannot publish manuscripts containing commercial language. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials: e.g., Dumont N5A, Falcon, Quantifoil R1.2/1.3, etc. We must maintain our scientific integrity and prevent the subsequent video from becoming a commercial advertisement.

We have removed all instances of commercial language from the manuscript (lines 130, 144, 163, 190, 206, 233, 265, 311, and 398).

7. Please include a one line space between each protocol step and highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

We have added a one line space between each protocol, reformatted the Protocols section to be left-aligned, and highlighted the sections of the protocol text that we would like to include in the protocol section of the video.

8. Please remove the embedded figure(s) from the manuscript. All figures should be uploaded separately to your Editorial Manager account. Each figure must be accompanied by a title and a description after the Representative Results of the manuscript text.

The Figures have been removed from the main text and the title and figure legends added after the Representative Results section of the manuscript text.

9. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

We have taken information and images from Figures 1, 2 and 4 from our previous publication "de Martín Garrido, N. et al. Direct transfer of electron microscopy samples to wetted carbon and graphene films via a support floatation block. Journal of Structural Biology. 213 (1), 107677, doi: 10.1016/j.jsb.2020.107677 (2021)." We have used Figure 1A and 1B to reproduce the schematic of the sample floatation block in the current Figure 1A, and images were adapted from Figure 4B for the current Figure 1B. The negatively stained sample image was adapted from Figure 4H in the current Figure 2A. The graphene images were adapted from Figure 2I and 2K in the current Figure 2C. This was published under a Creative Commons Attribution 4.0 International license (<https://creativecommons.org/licenses/by/4.0/>), and reprinting is therefore explicitly allowed under the licence. Could the editor please clarify whether or not this is satisfactory - we are happy to provide alternative images if necessary. We have amended Figure legend 1 (lines 363-364) accordingly with:

"Images in B are taken from de Martín Garrido et al., 2020 under a Creative Commons (BY 4.0) license."

And Figure legend 2 (lines 374-375) with:

“Images in A and C are taken from de Martín Garrido et al., 2020 under a Creative Commons (BY 4.0) license.”

We hope that the provided document and changes are satisfactory to the editor.

## Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript describes a new method for the preparation of various types of carbon support on quantifoil grids in order to avoid denaturation of proteins on the air water interface, a very common problem for cryo-TEM nowadays.

We would like to thank the reviewer for their careful consideration of our manuscript, and are glad they have no major concerns.

Minor Concerns:

Two references mentioned in the text are not in the reference list. Valentine 1968 and De Martin Garrido et al 2020.

The two references have been added to the reference list as the reviewer requests.

I remember a method described in 2003 by Koning et al. Ultramicroscopy (not me by the way), where a double layer of carbon was applied onto the grid from a Teflon well. It might be worth mentioning this paper in the introduction.

We thank the reviewer for bringing this paper to our attention. We have reviewed this publication and amended our Introduction as follows (lines 96-97):

“... from which the layers can be floated onto grids, with the utility of floatation supports as useful tools established in prior reports<sup>20</sup>”.

We therefore provide the new reference (20):

“Koning, R.I., Oostergetel, G.T., Brisson, A. Preparation of flat carbon support films. Ultramicroscopy. 94 (3–4), 183–191, doi: 10.1016/S0304-3991(02)00263-2 (2003).”

Uranyl acetate is scarcely available and difficult to purchase at the moment due to health and safety regulations unfortunately. may be good to give an alternative to those who can not get access to it.

We appreciate the reviewer's point and we therefore have modified our Discussion section accordingly. Lines 388-394 now read:

“We note that UAc may be difficult to purchase due to current health and safety regulations at the time of publication, however many other commonly used, non-radioactive, negative staining reagents are available and protocols for their preparation have been described previously<sup>28</sup>. Although we have not used alternative stains with our support floatation block, we do not envisage any differences required for our protocols besides optimisation of incubation time with sample (Step 3.5), which is already inherently sample-dependent.”

We also provide a new reference (28) in the Discussion and Reference List:

“Scarff, C.A., Fuller, M.J.G., Thompson, R.F., Iadanza, M.G. Variations on negative stain electron microscopy methods: Tools for tackling challenging systems. *Journal of Visualized Experiments*. 2018 (132), 57199, doi: 10.3791/57199 (2018).”

... which details alternative negative staining reagents and protocols for their preparation and use.

Reviewer #2:

Manuscript Summary:

The manuscript introduces a method to direct transfer of the graphene oxide, graphene and carbon support films to the sample and consequently deposit it on TEM grid for EM investigations. The main advantage of this method is lowering the risk of contamination during the sample and grid preparation.

Major Concerns:

There is no major concern.

We would like to thank the reviewer for their careful consideration of our manuscript, and are glad they have no major concerns.

Minor Concerns:

Is what you stir in section 2.1.2 the same UAc containing Falcon you filled in 2.1.1? If yes why would you expose it for 10 min and prevent the exposure to light for the next 60 min of stirring?

We appreciate that the reviewer noticed this contraindication in our protocol, and we have now changed it to read correctly as follows (lines 144-154):

1.1.1. Wrap a 50 mL tube in foil (UAc is light-sensitive and precipitates over time when exposed), fill with 50 mL of ultrapure water and add 1 g of UAc powder.

1.1.2. Leave the solution stirring for 1 hr to allow for all the UAc to dissolve.