PROTOCOLS

Preparation of a 0.75% uranyl formate solution

Attention: uranyl formate is radioactive, toxic and light-sensitive

- Weigh out 37.5 mg of uranyl formate into a small beaker
- Add 5 ml of boiling deionized water and stir for 5 min in the dark
- Add drops of 5 M NaOH until the stain solution becomes slightly darker yellow (too much NaOH will precipitate the stain) and stir for another 5 min in the dark
- Filter the solution with a 0.2 µm syringe filter into a Falcon tube wrapped with aluminum foil and add deionized water to a final volume of 5 ml

Conventional negative staining protocol

- Place two 50 µl drops of deionized water and two 50 µl drops of uranyl formate stain on a piece of parafilm
- Apply 2.5 µl of sample to a glow-discharged EM grid covered with a continuous carbon film and let the sample adsorb for 30 sec
- Blot the grid from the side with a piece of filter paper, briefly touch the first drop of water with the grid, blot with filter paper, briefly touch the second drop of water, blot with filter paper, briefly touch the first drop of uranyl formate, blot with filter paper, touch the second drop of uranyl formate for 20 sec, and blot with filter paper (avoid complete drying of the grid in between the drops)
- Completely dry the grid by vacuum aspiration touching only the rim of the grid

The particles on the carbon film should be well separated (to allow for their extraction into individual images for computational processing) but not too sparse (to avoid having to collect too many images). The particle concentration on the grid is best adjusted by dilution of the sample solution. It is also possible to vary the time for glow discharging and for sample adsorption.

Useful modifications to the protocol

- For sensitive specimens, distilled water can be replaced by buffer solution for the washing steps
- The number of washing drops can be increased to remove detergent or omitted to induce immediate fixation of the specimen
- Glycerol or glucose can be added to the sample solution or the staining solution to minimize specimen flattening (only recommended for calculating 3D reconstructions)

Carbon sandwich technique

- Place two 50 µl drops of deionized water and one 50 µl drop of uranyl formate stain on a piece of parafilm
- Float thin piece of carbon in a small container of uranyl formate stain
- Apply 2.5 µl of sample to a glow-discharged EM grid covered with a continuous carbon film and let the sample adsorb for 30 sec
- Blot the grid from the side with a piece of filter paper, briefly touch the first drop of water with the grid, blot with filter paper, briefly touch the second drop of water, blot with filter paper, briefly touch the first drop of uranyl formate, blot with filter paper, touch the second drop of uranyl formate for 20 sec
- With the sample side facing up, plunge the grid into the container holding the uranyl formate with the floating piece of carbon. Position the grid under the carbon and then lift the grid out of the container picking up the floating piece of carbon in the process. Gently blot the grid from the side using filter paper

Useful modifications to the protocol

- Uranyl formate can be substituted with any other stain
- Glycerol or glucose can be added to the sample solution and the grid can be frozen in liquid nitrogen to minimize specimen flattening

Cryo-negative stain using uranyl formate (Walz lab method adapted from Golas *et al.*)

- Float thin piece of carbon in a small container of deionized water. Pick up piece of carbon with a holey carbon grid (QUANTIFOIL, Germany). The thin piece of carbon should rest on the carbon side of the holey grid. Allow the grid to air dry overnight or for several hours. Do not blot the grid after picking up the carbon
- Sample should contain 5-10% glycerol or sucrose
- Glow discharge the thin layer of carbon supported by the holey carbon film
- Place one 50 µl drop of deionized water and one 50 µl drop of uranyl formate stain on a piece of parafilm
- Float thin piece of carbon in a small container of uranyl formate stain
- Apply 5.0 µl of sample to a glow discharged holey carbon grid prepared as described above and let the sample adsorb for 30 sec
- Blot the grid from the side with a piece of filter paper, briefly touch the drop of water with the grid, blot with filter paper and then touch the drop of uranyl formate for 30 sec
- With the sample side facing up, plunge the grid into the container holding the uranyl formate with the floating piece of carbon. Position the grid under the carbon and then lift the grid out of the container picking up the floating piece of carbon in the process. Dry for 20-30 sec and then carefully blot the grid from the side using filter paper. Dry at room temperature for approximately 2 minutes and then freeze the sample in liquid nitrogen

Cryo-negative staining (according to Adrian *et al.*)

- Place a 100 µl droplet of 16% ammonium molybdate (pH range 7.0 to 8.0) on a piece of parafilm
- Apply 4.0 µl of sample to a holey carbon film (QUANTIFOIL, Germany) that has not been glow discharged. Adsorb for 30 sec
- Float the grid in the 16% ammonium molybdate droplet (sample side facing droplet) for 60 sec
- Blot with filter paper, air dry 1-3 sec, and plunge into liquid ethane

Both the length of blotting and the amount of time the sample is allowed to dry before plunge freezing is critical for obtaining the optimal thickness of vitrified ice. These times must be experimentally determined.