IMPROVING AUTOMATION FOR CRYO-EM SPECIMEN PREPARATION

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Cryo-Electron Microscopy (cryo-EM) was started in the 1960-70's and perfected in the early 1980's [1]. It is a method in which 2-5 uL of a biological sample is placed on a grid with a holey carbon film and blotted with filter paper, then quickly plunged into liquid ethane. The buffer is frozen into amorphous ice, and the biological sample can be visualized using TEM while keeping the grid at cryogenic temperatures. A 50-100 nm layer of ice is ideal for studying proteins and 2-D crystals. For the most part, obtaining a layer of ice this thin has been controlled by the use of filter paper and the manipulations of the experimenter. For a novice this process is irreproducible and frustrating. The increasing interest and number of facilities that collect cryo-EM data is pushing the need for semi-automated sample preparation techniques that yield reproducible results.

The commercial availability of automated blotting devices (Vitrobot, FEI Co.) has aided sample preparation. The Vitrobot can control blot and plunge times as well as the humidity and temperature in the blotting chamber, which will increase the reproducibility of ice thickness. The majority of the time the ice created on the grid is a wedge that stretches across the grid. This is acceptable and desired because the optimal ice thickness is located somewhere on the grid but the characteristics of the ice layer is dependant on the types of grids one uses. For our automated data collection requirements, we use a commercially available holey carbon film with a regular spaced array of holes (2µm in diameter) spaced 4 µm ((Quantifoil [2]) grids: Qfoil R2/4, Cu/Rh, 400M). Our experience has been that these grids are hydrophobic and have contaminants on the support film that interferes with 'thin ice' formation on the grid. There are several techniques used to overcome this: evaporating a fresh coat of carbon, pre-irradiation (using an e- beam), washing in organic solvents, and glow discharging. These treatments may not always work and are sometimes sample or buffer dependent.

We present a more reliable and efficient process of preparing cryo-EM grids using a plasma cleaner (Fischione Instruments, fig 1), Vitrobot, and Quantifoil grids. In our procedure the grids are 'plasma cleaned' and then the sample is frozen within the hour on the Vitrobot.

Plasma cleaners have been widely used by the materials science community for specimen preparation. The theory is that free electrons are accelerated to high velocities by an oscillating electromagnetic field that excites gas atoms and creates the plasma. A mixture of 25% oxygen and 75% argon is then used to optimize the cleaning. The plasma process creates dissociated oxygen, which combines with carbonaceous material to produce H2O, CO, and CO2 thus eliminating all contamination. We use the plasma cleaner for 30s to clean the Quantifoil grids

Figures 2-4 show cryo-EM images prepared using this method, Samples were prepared form TMV (in ddH2O), GroEl (in 100 mM Hepes, 10 mM Mg-Acetate, 10 mM K-acetate, and 2mM DTT, pH 7.5), and PBCV-1 (in 50 mM Tris, pH 7.0). Each sample has a different buffer and particle size and shape.

The process has proven reliable enough that Rachel Banez a work-study undergraduate student with no prior experience in EM has been able to reliably reproduce good cryo-EM grids. Our group continues to

have success freezing samples provided by other labs. There is no learning curve associated with the procedure, and most of the steps are computer or robot controlled.

References

[1] Dubochet, J. et al. Cryo-electron microscopy of vitrified specimens. Q. Rev. Biophys. 21, 129–228 (1988).
[2] Quantifoil Micro Tools GMBH, Winzerlaer Str 2A, Jena, 00745 Germany .
[3] This research was conducted at the National Resource for Automated Molecular Microscopy which is supported by the National Institutes of Health though the National Center for Research Resources' P41 program (RR17573).

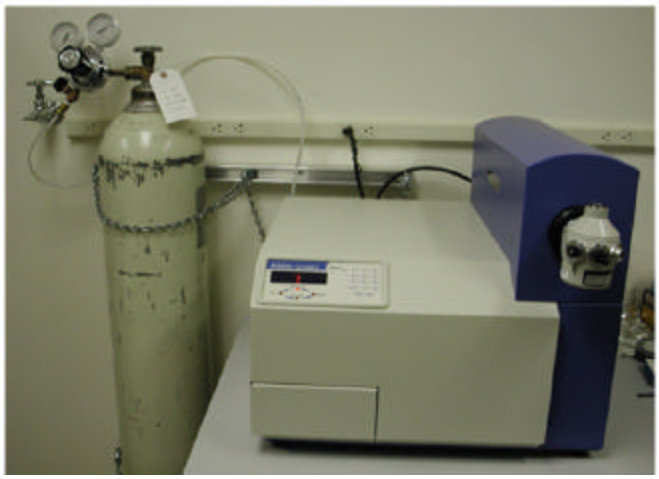

Fig 1. Model 1020 Plasma Cleaner

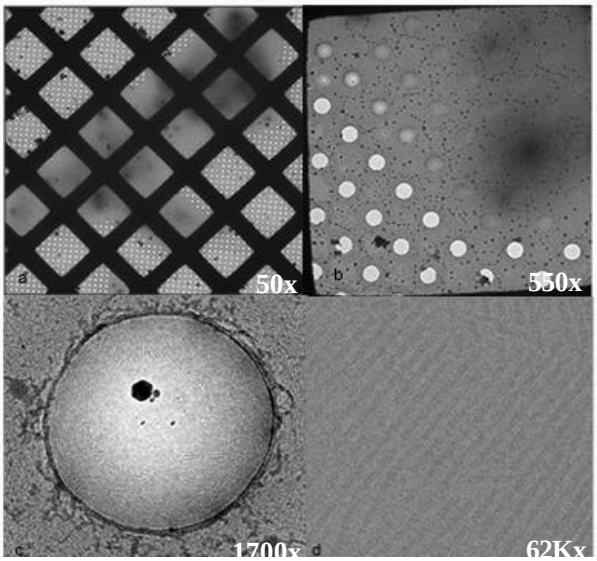

Fig 2. Tobacco mosaic virus (TMV) preserved in vitreous ice showing distribution of ice at various magnifications.

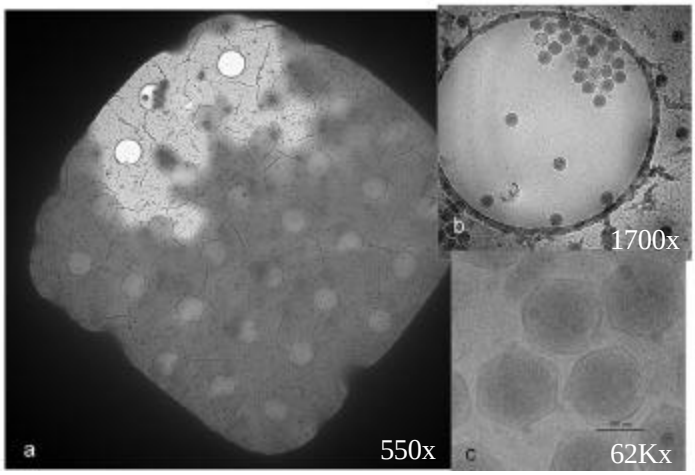

Fig 3. Paramecium bursaria chlorella virus type-1 (PBCV-1)

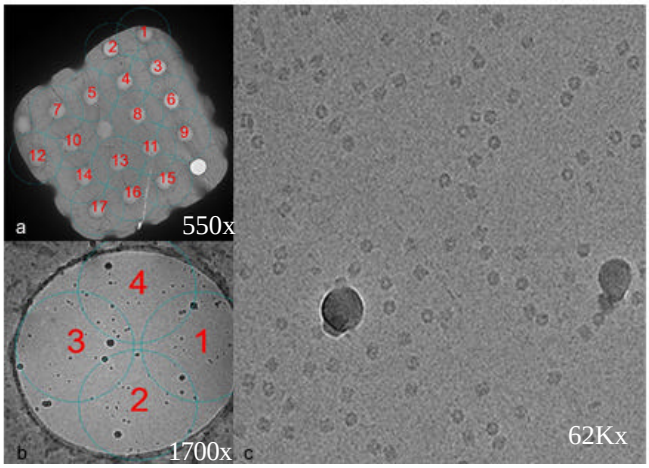

Fig 4. Escherichia coli chaperonin GroEL.