Cryo Preparation at NRAMM

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Overview of the freezing procedures at NRAMM

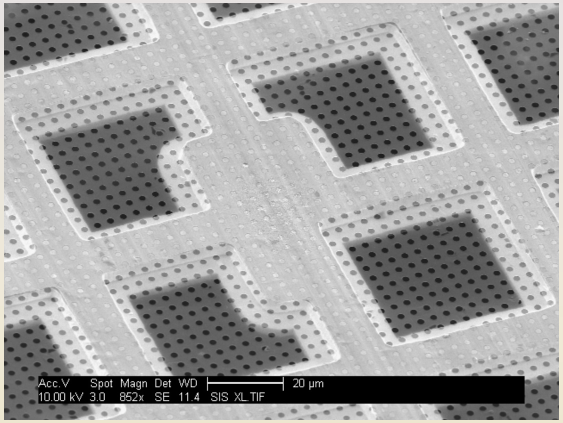
- First I’ll go over some background of cryo-EM
- Then the principles of freezing
- Steps we follow at NRAMM to freeze grids
- Problems we have encountered and how we have solved them
- Making freezing easier
- Some examples of bad and good ice we have imaged
- Future experiments and developments we are pursuing

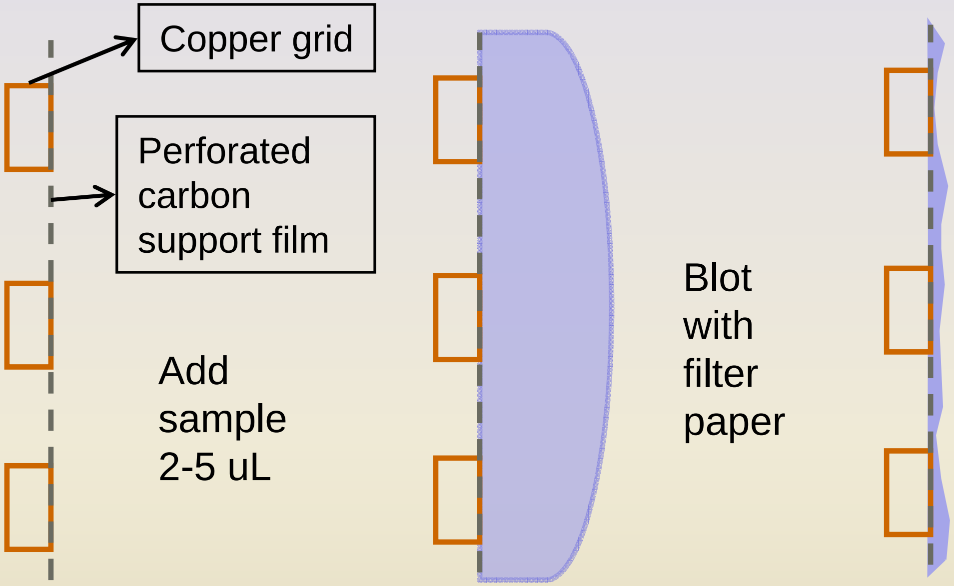
Background and Literature for Cryo-EM

- Cryo-EM was described by Taylor, K.A. & Glaeser, R.M. (1974) Electron Diffraction of frozen, hydrated protein crystals. *Science* 106, 1036-37.
- Since then many papers have been written on techniques and principles about sample preparation (many of the authors are in this room!).
- One of the most in depths reviews is Dubochet J, Adrian M, Chang JJ, Homo JC, Lepault J, McDowall AW, Schultz P. Cryo-electron microscopy of vitrified specimens. *Q Rev Biophys.* 1988 May;21(2):129–228.
- Now (2007) the principles are the same and we have better equipment and supplies.

Principle of cryo freezing

- The basic procedure of freezing is to remove enough liquid (sample) from a carbon coated grid with filter paper to leave a micro-environment of liquid behind to keep the sample hydrated (can be 10nm to 300nm).





Copper grid

Perforated carbon support film

Add sample 2-5 uL

Blot with filter paper

Immediately plunge into liquid ethane

- Then it is rapidly plunged into liquid ethane.
- The grid with vitreous ice is then removed from the ethane and place into LN2 and then placed on a cryo-stage and then imaged in a TEM.

Several things that can affect the freezing process

- The filter paper removes too much or too little liquid.
- The support film on the grid is hydrophobic or the sample acts hydrophobic or the film breaks.
- The micro-environment is good but evaporates before freezing.
- The ethane is not cold enough or has solidified.
- The transfer from the ethane to the LN2 bends the grid, is too slow, or picks up contamination.
- The cleanliness of the LN2 and ethane are important.

Cleanliness of gases and LN2 is important

- **The LN2 used at Scripps is**
  - **Specification**

    Nitrogen ≥ 99.998%
    Oxygen ≤ 10ppm
    Water ≤ 4ppm
    Dew Point ≤ -90°F

Meets or exceeds purity requirements of Mil PRF-27401E, A-A-59503A, CGA G-10.1 Grade L

- **The Ethane is**
  - **Specification**

    Ethane > 99.0%*
    Oxygen < 200 ppm
    Nitrogen < 800 ppm
    Methane < 2000 ppm
    Ethylene < 75 ppm
    C4+ < 150 ppm
    Carbon Dioxide < 30 ppm
    Moisture (H2O) < 20 ppm

* Liquid Phase

Grid choice

Grids are chosen mainly by the sample and how they react when frozen.

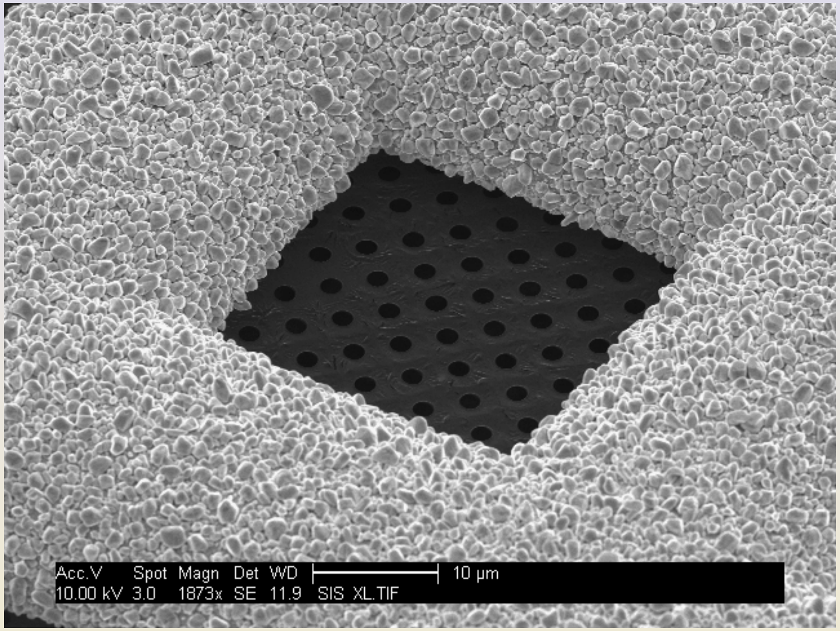
- 1st step is to do a quick screen in negative stain.

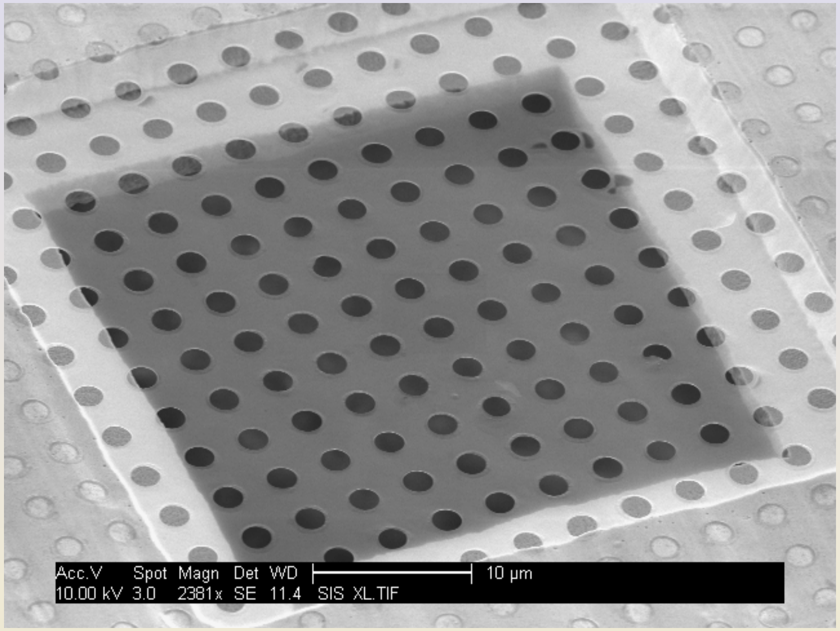
- 2nd we determine a good concentration for using holey grids. ~10x more concentrated than negative stain.

- We start with C-flats with 2 micron holes and 2 micron spacing, 400 mesh copper grids. The 2 micron spacing gives plenty of room on the carbon to focus and the hole size is large enough to fit small single particles, vesicles, and tubes. The smaller mesh size helps to keep the carbon from breaking.

- The orientation of the grid and carbon is also very important.

Shiny side compared to dull/darker side of grid

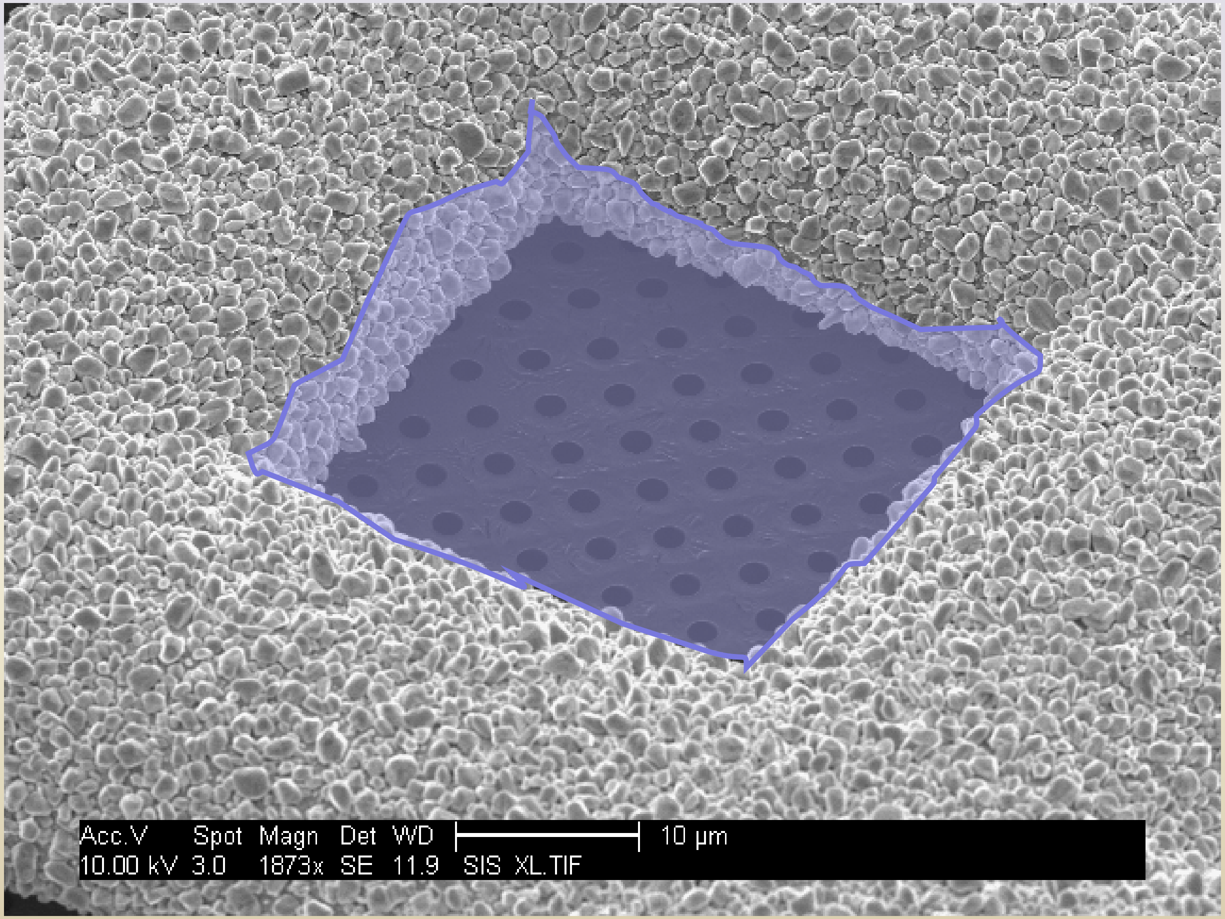




The left image is the shiny/bright side of a grid. The right image is the dull/darker side and should have the carbon film.

Note: It is sometimes hard to explain the different shades of copper to new users. If the sample is placed on the wrong side it will pool in the pocket of the copper and carbon.

Adding sample to wrong side, or sample diffuses to other side of grid.



When the sample is on this side of the grid, it will most likely result in thick ice. Unless it is blotted for a long time 5-8 seconds.

Plasma Cleaning theory

In a non-equilibrium, high-frequency plasma, free electrons are accelerated to high velocities by an oscillating electromagnetic field that excites gas atoms and creates the plasma.
To optimize cleaning, a mixture of 25% oxygen and 75% argon is generally recommended. An oxygen plasma is highly effective in removing hydrocarbon contamination. The plasma process creates disassociated oxygen which combines chemically with the carbonaceous material on the specimen and specimen holder. This reaction chemically reduces the organic contamination to H2O, CO, and CO2 that are evacuated by the vacuum system. Complete cleaning of highly-contaminated specimens can occur in 2 minutes or less.[1]

1. *Fischione 1020 plasma cleaner manual*

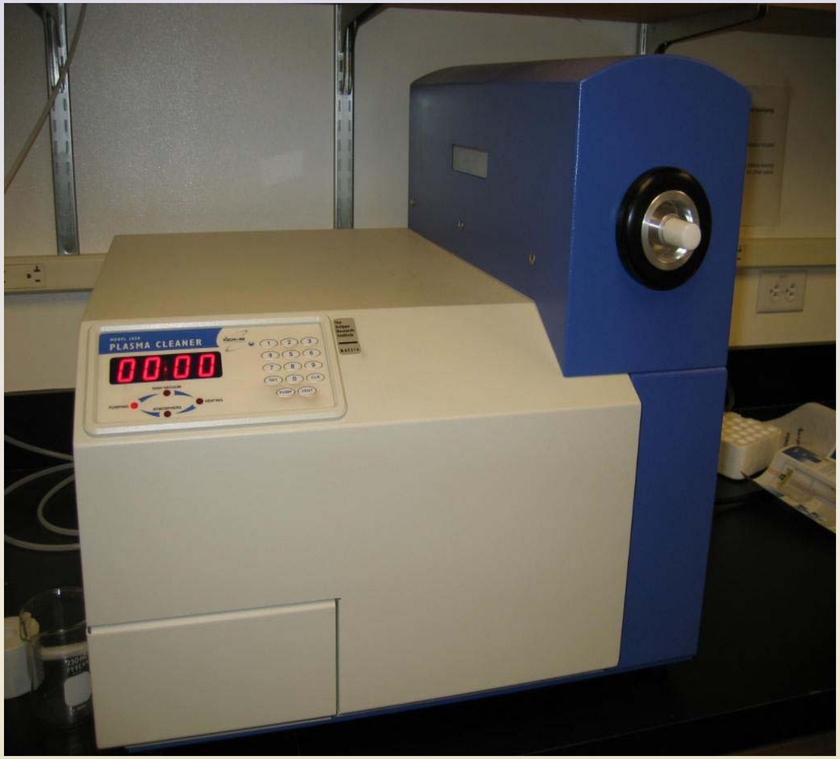
Glow Discharging

The glow discharging concept is the same as plasma cleaning except that it doesn’t use specific gases. It uses air and organic solvents (amyl amine) to create a hydrophilic surface and + or - charges on the support film.

The purpose for glow-discharging is to make the carbon support film hydrophilic and add a charge that will cause proteinaceous material to adhere to the carbon film.

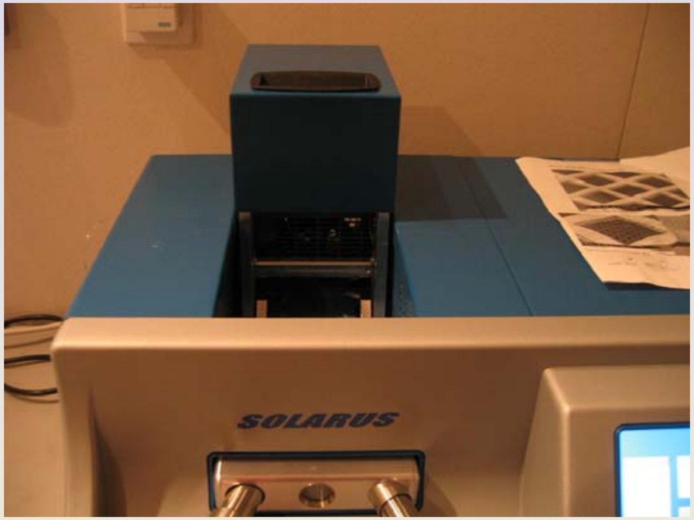
The glow discharger starts by evacuating the a chamber to ~100-200 mTorr of air. Then a high voltage is applied between a cathode and anode, this causes the air molecules to become ionized and form a plasma cloud. The plasma then reacts with the grids and makes the carbon film hydrophilic and negatively charged.

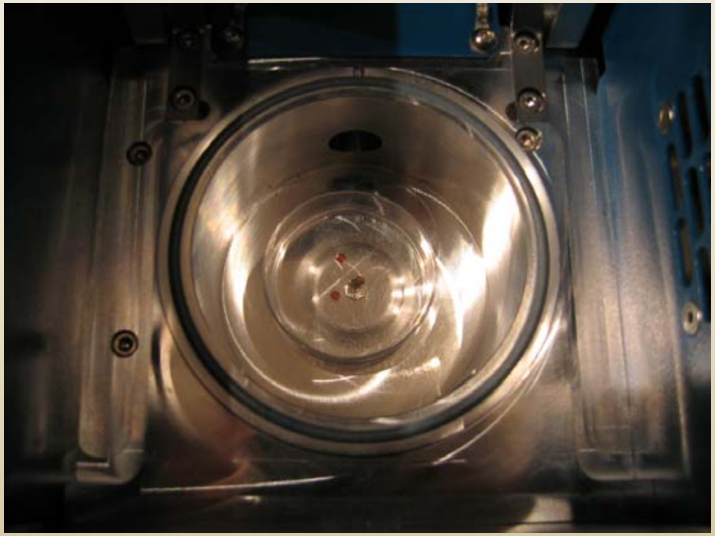
Plasma cleaning with the Fischione



- Fischione plasma cleaner with 5 grid holder. Clean time is 10-20 seconds with dampening shield. Uses 25% Oxygen/75% Argon.
- The shield dampens the effects of the Oxygen plasma and reduces the amount of carbon removed from the grids. Needed for thin carbon grids, not needed for plastic coated grids or thick carbon.

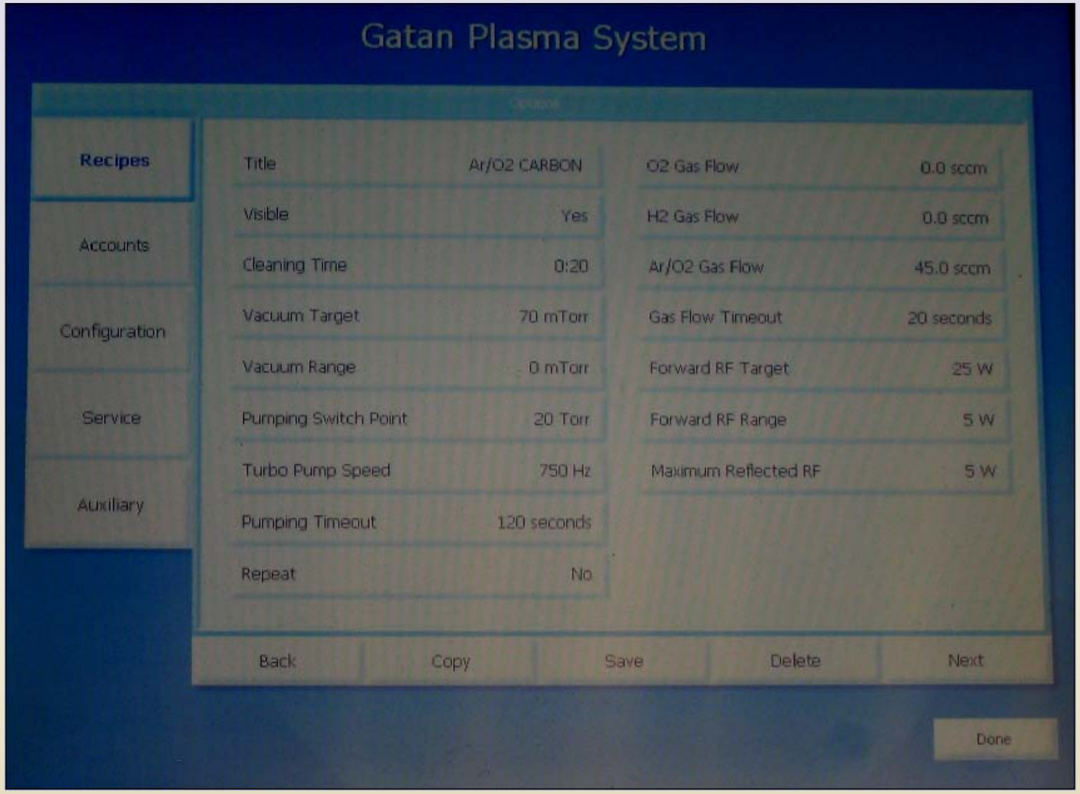
Gatan Solarus Plasma cleaning





Use 25% Oxygen/75% Argon, Power : 25 Watts,
Clean time 5-10 seconds for carbon film,

Solarus Features

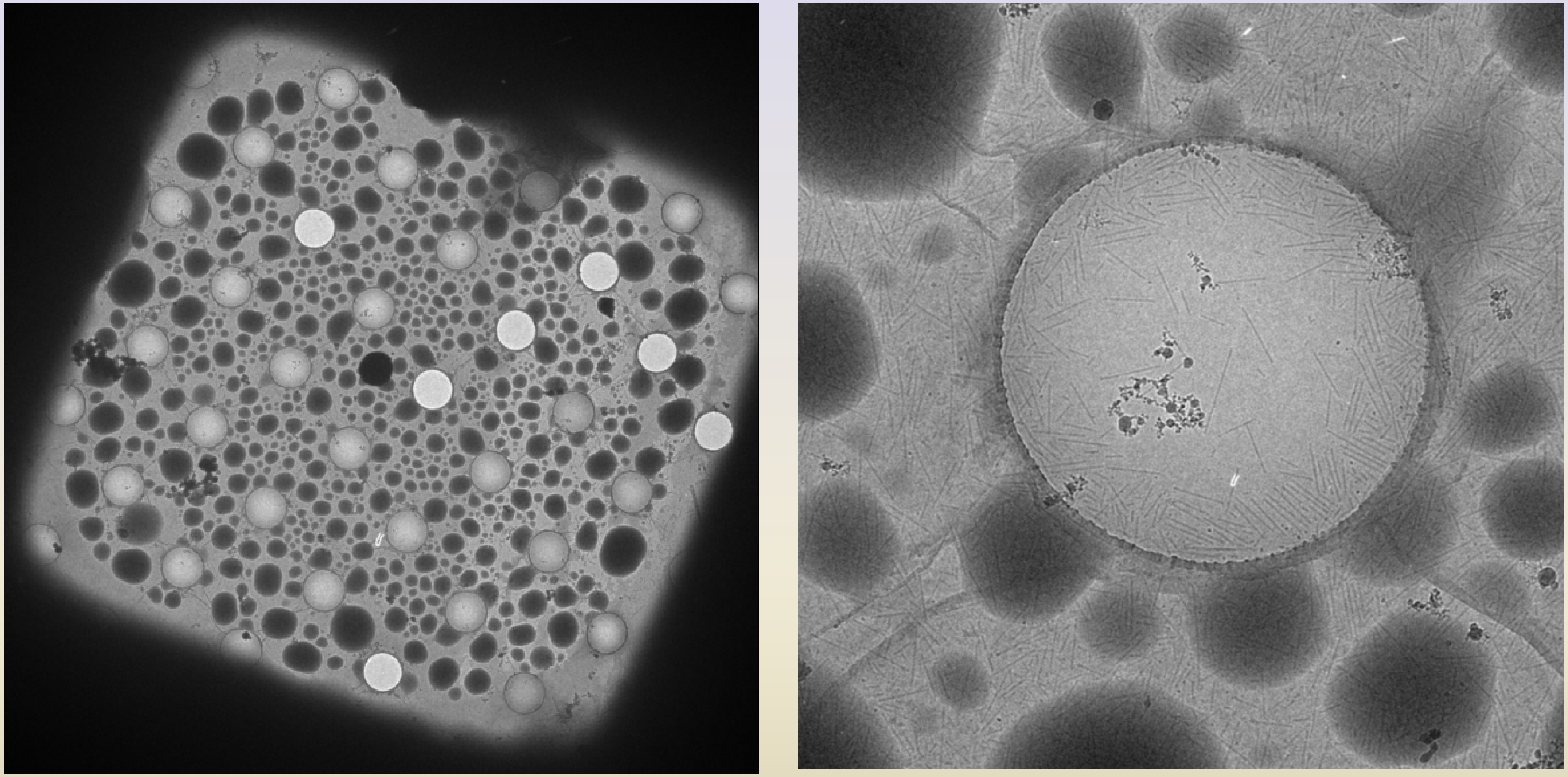


Title	Ar/O2 CARBON
Visible	Yes
Cleaning Time	0:20
Vacuum Target	70 mTorr
Vacuum Range	0 mTorr
Pumping Switch Point	20 Torr
Turbo Pump Speed	750 Hz
Pumping Timeout	120 seconds
Repeat	No
O2 Gas Flow	0.0 sccm
H2 Gas Flow	0.0 sccm
Ar/O2 Gas Flow	45.0 sccm
Gas Flow Timeout	20 seconds
Forward RF Target	25 W
Forward RF Range	5 W
Maximum Reflected RF	5 W

Solarus features:

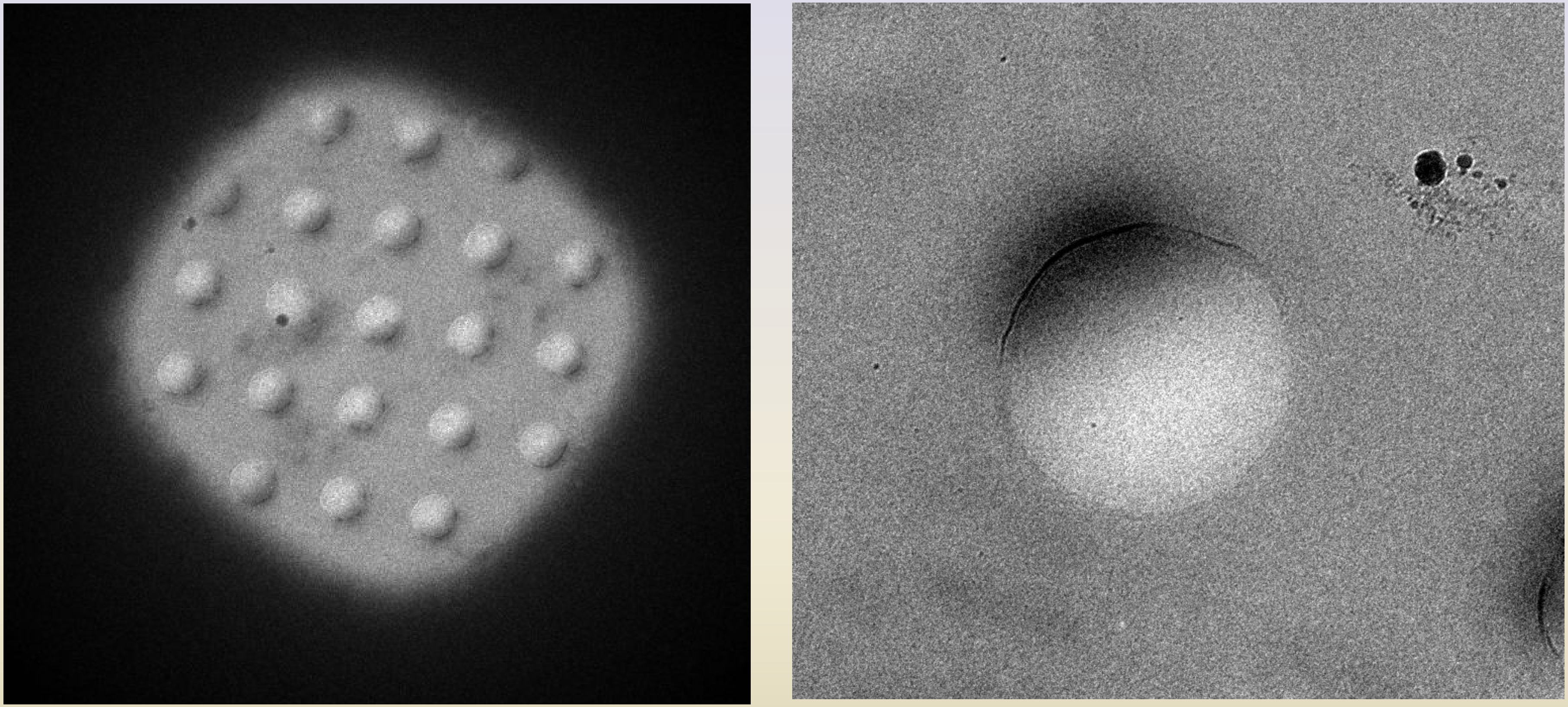
- Quick pump down and vent times.
- Various gases can be used: Oxygen, Hydrogen, O_2/Ar.
- The gas flow and power settings can be adjusted.
- Multiple recipes can be stored.
- Also used to prepare grids for negative staining.

Hydrophobic carbon



Common result for us when we use the glow-discharger.

Hydrophilic Grid



Results when we use plasma cleaning, either Fischione or Gatan.

Freezing Environment is important

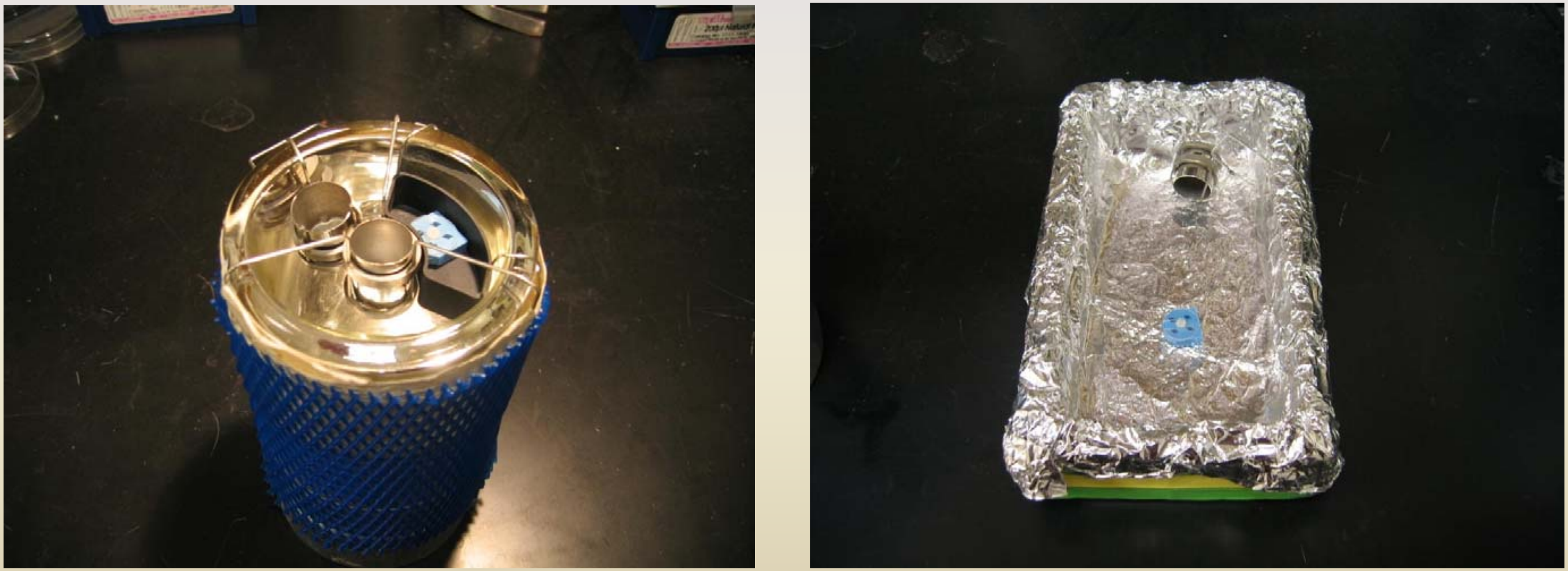
- The ideal environment would be a blotting chamber that has high humidity and low temperature to minimize evaporation, and keep samples at a stable temperature.

- A cryo chamber that has a low humidity and at room temperature.

- Good freezing can be accomplished if these requirements are not met, but precautions must be made to minimize contamination will cause problems.

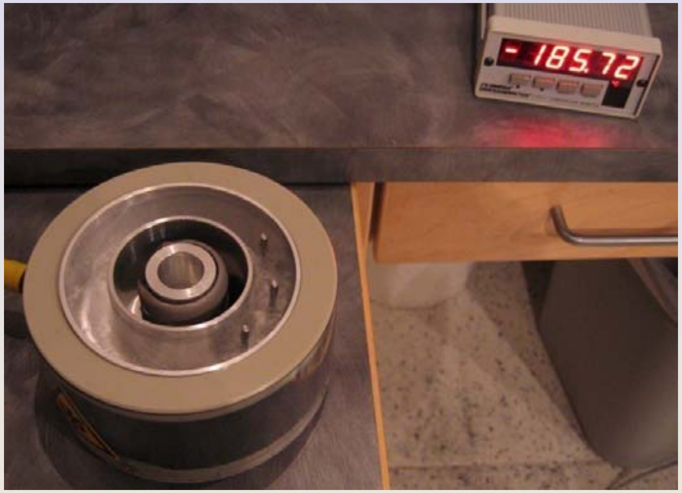
Manual Freezing

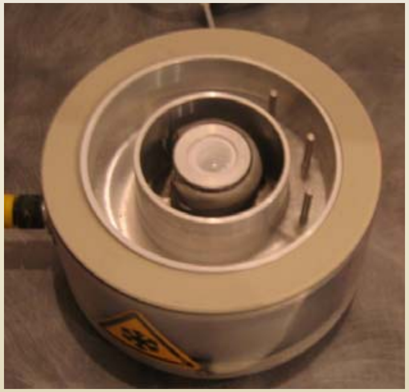
- Keeping the ambient humidity low around the ethane and LN2 will keep contamination (water crystals) at a minimum.
- When freezing by hand it is a good idea to keep the grids and/or boxes in a different environment than the cooling LN2.
- This can be done by making a small container (see pic)



The Vitrobot can help to separate the blotting & cryogen environment.

Vitrobot freezing (old version)

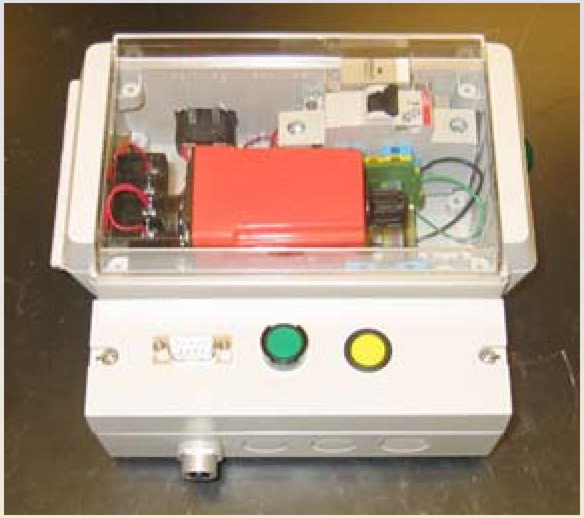




The old ethane container has been redone to accommodate square boxes and a temp sensor, heater, and controller has been added.

The Vitrobot has been in use in our lab for 4 years. We have made modifications to the ethane container and have moved it to a low humidity environment.

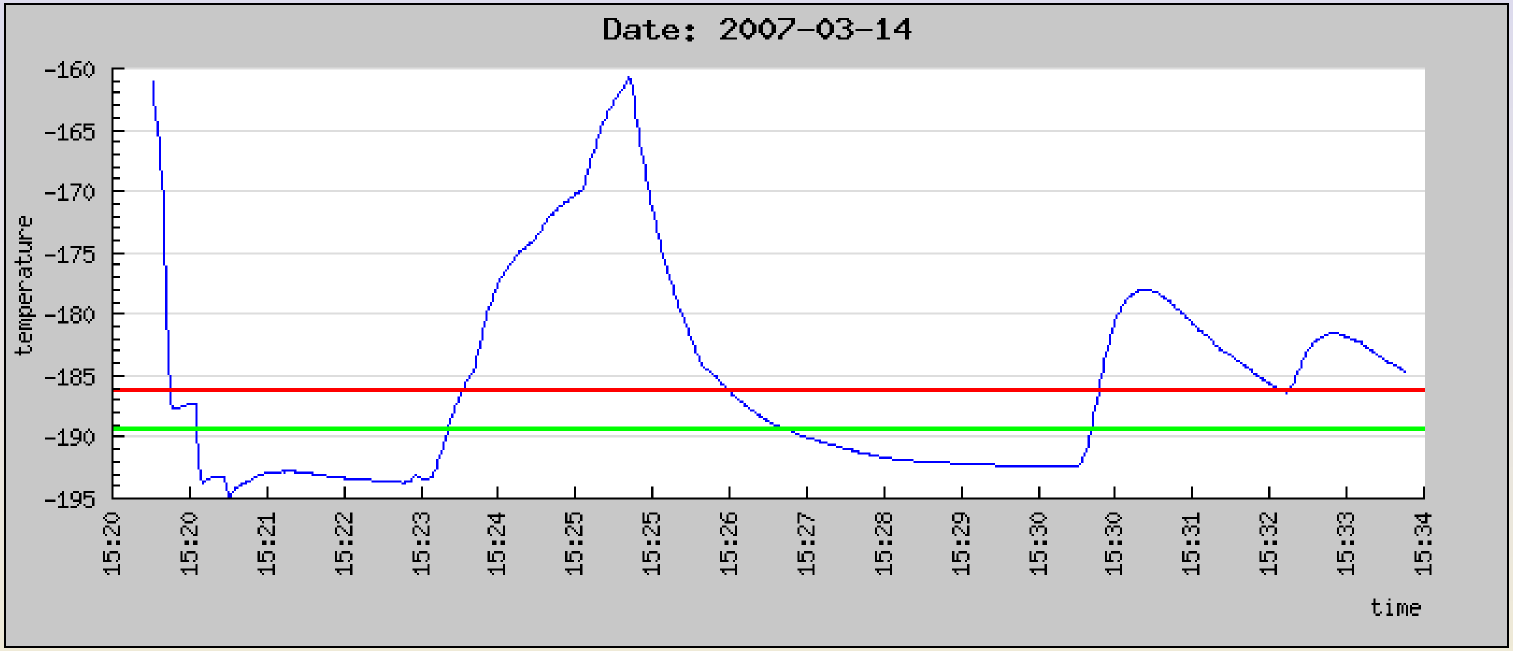
Ethane Heater schematic

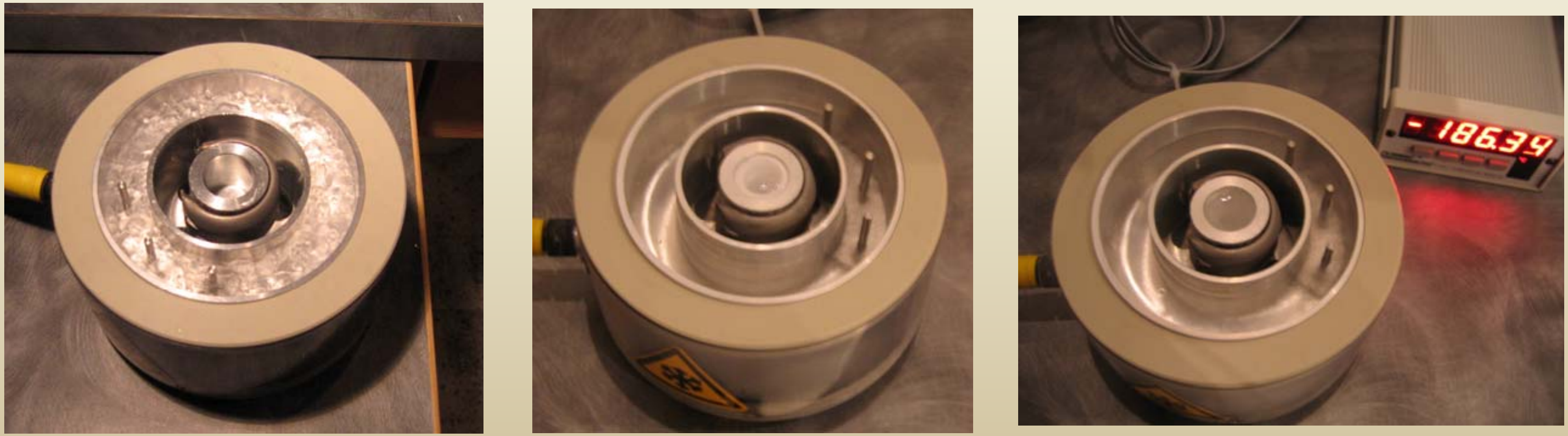


Ethane Temperature °C		Dewer Temperature °C	
Initial	Final	Initial	Final
-178.0	-174.7	-186.2	-182.2
-179.2	-177.2	-186.2	-182.0
-179.4	-177.1	-186.2	-182.2
-179.7	-177.0	-186.1	-182.4
-179.7	-176.9	-186.1	-182.3
-179.7	-177.0	-186.1	-182.3

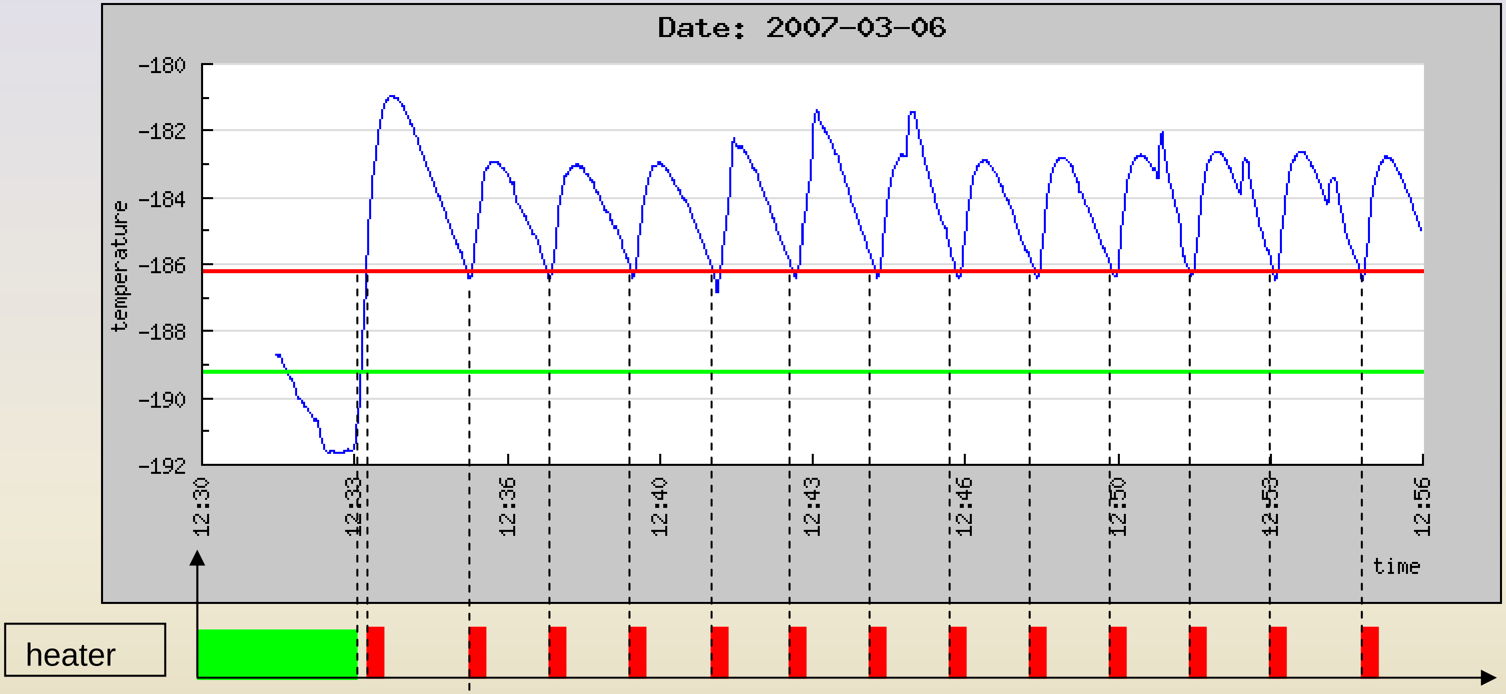
Denis Fellman designed and assembled the heater. The heater has a cryo temperature sensor in the wall of the ethane container. The heating device is placed around the ethane cup and the controller is attached to the container by an industrial wire rated for high temp and humidity. We have been using this heater since December of 2006. The chart shows there is a temperature difference between the ethane and container.

Ethane heater





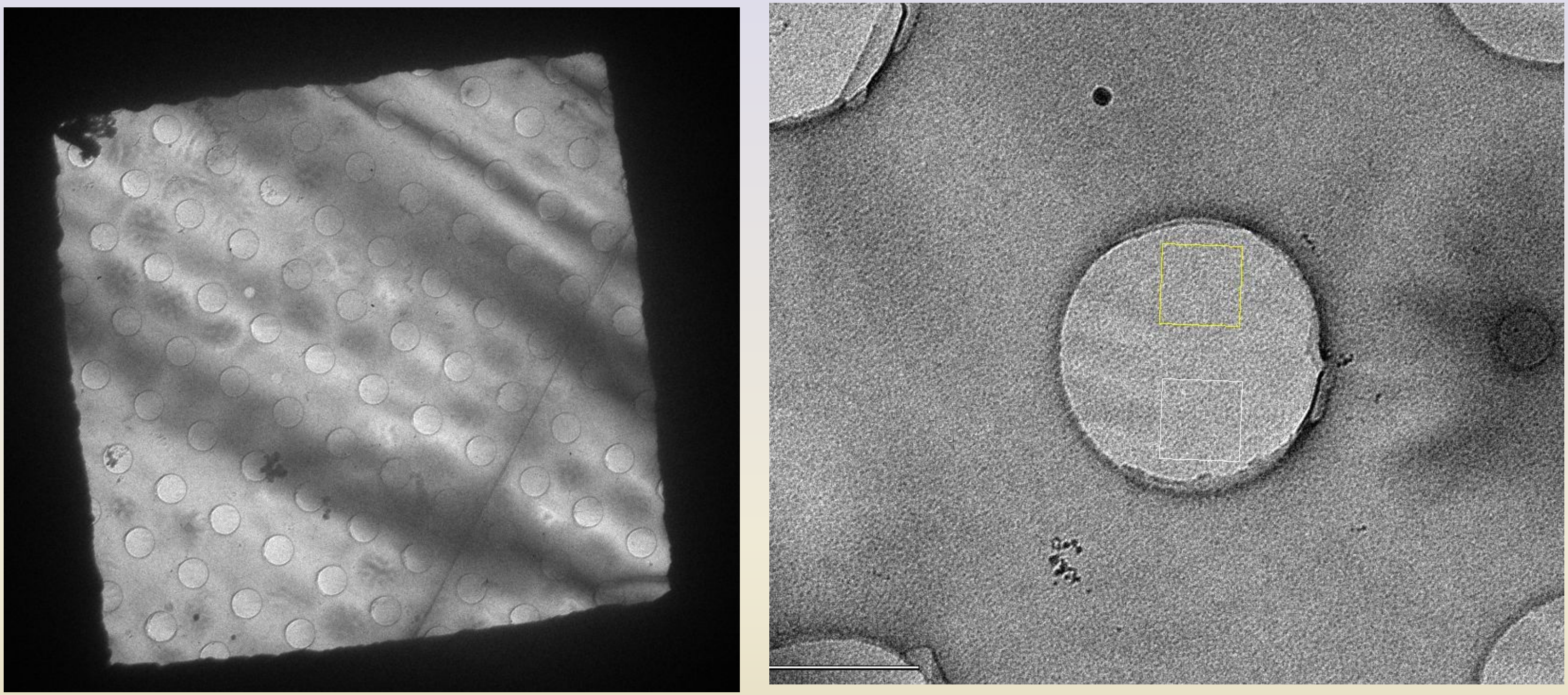
Ethane heater temperature curve



The temperature variation during a normal blotting session on the Vitrobot. The spikes that occur on the top of the curves are when the container is raised up to blotting position and comes in contact to the bottom of the humidity chamber.

Vitrobot freezing Mark III version (not the Mark IV)

Freezing with continuous carbon



Freezing with continuous carbon requires that the grids be plasma cleaned and the blotting be 2-3 seconds longer. If the blotting is over 10 seconds, then using less volume helps to lower the blot times.

We want to also thank Michael Radermacher and Teresa Ruiz for supplying us with thin carbon on mica.

Image of ‘good’ ice melting

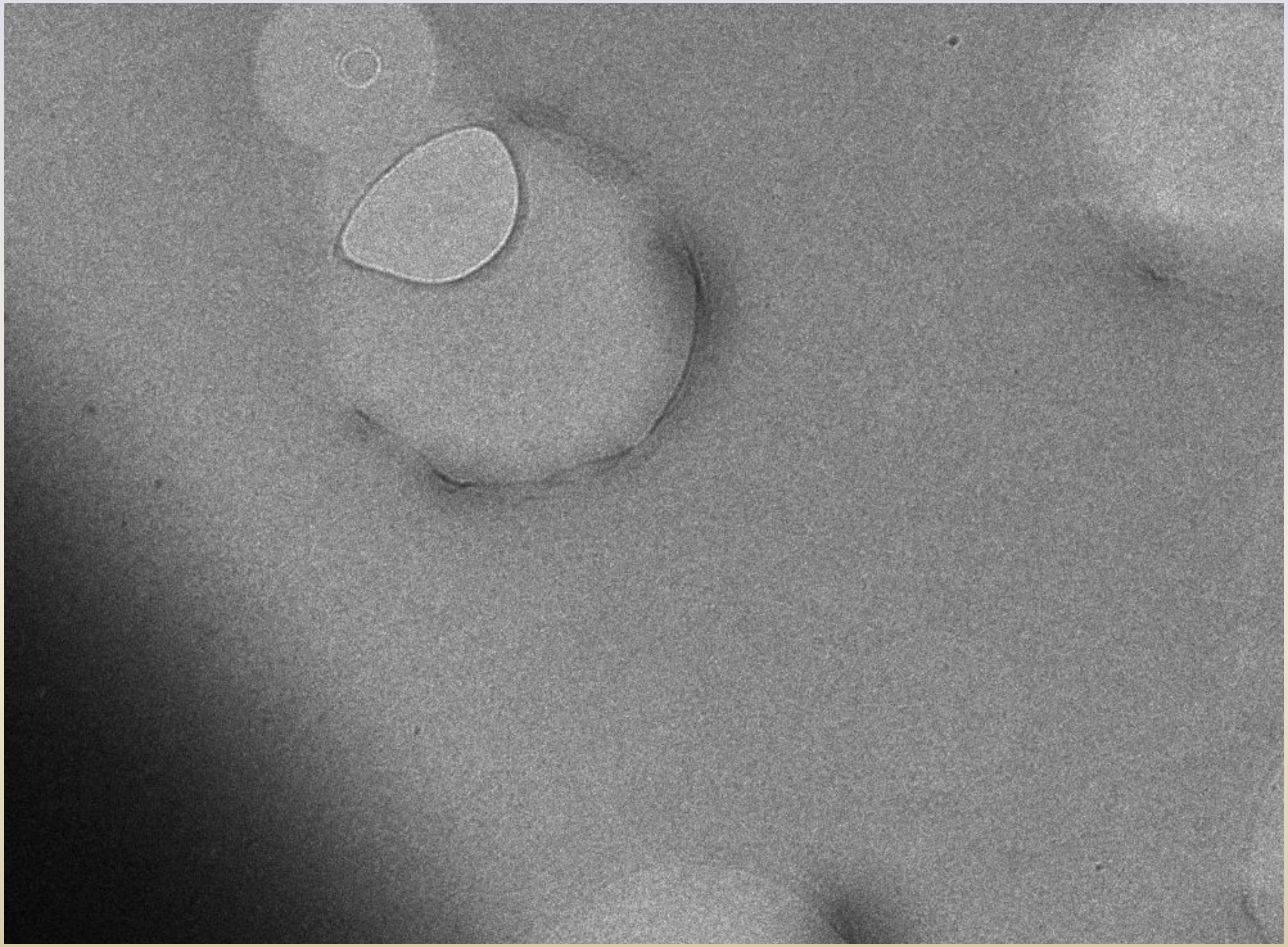
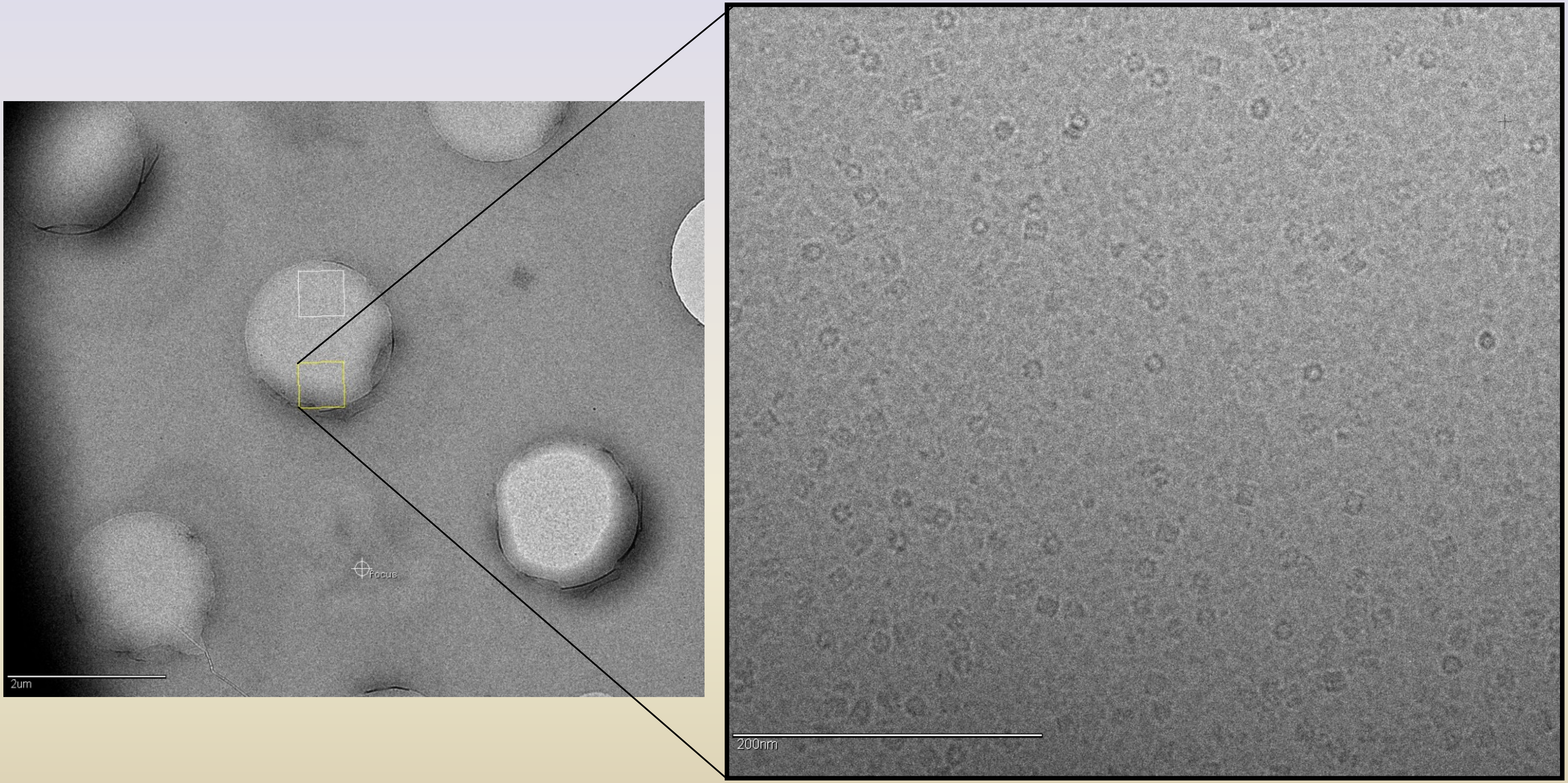
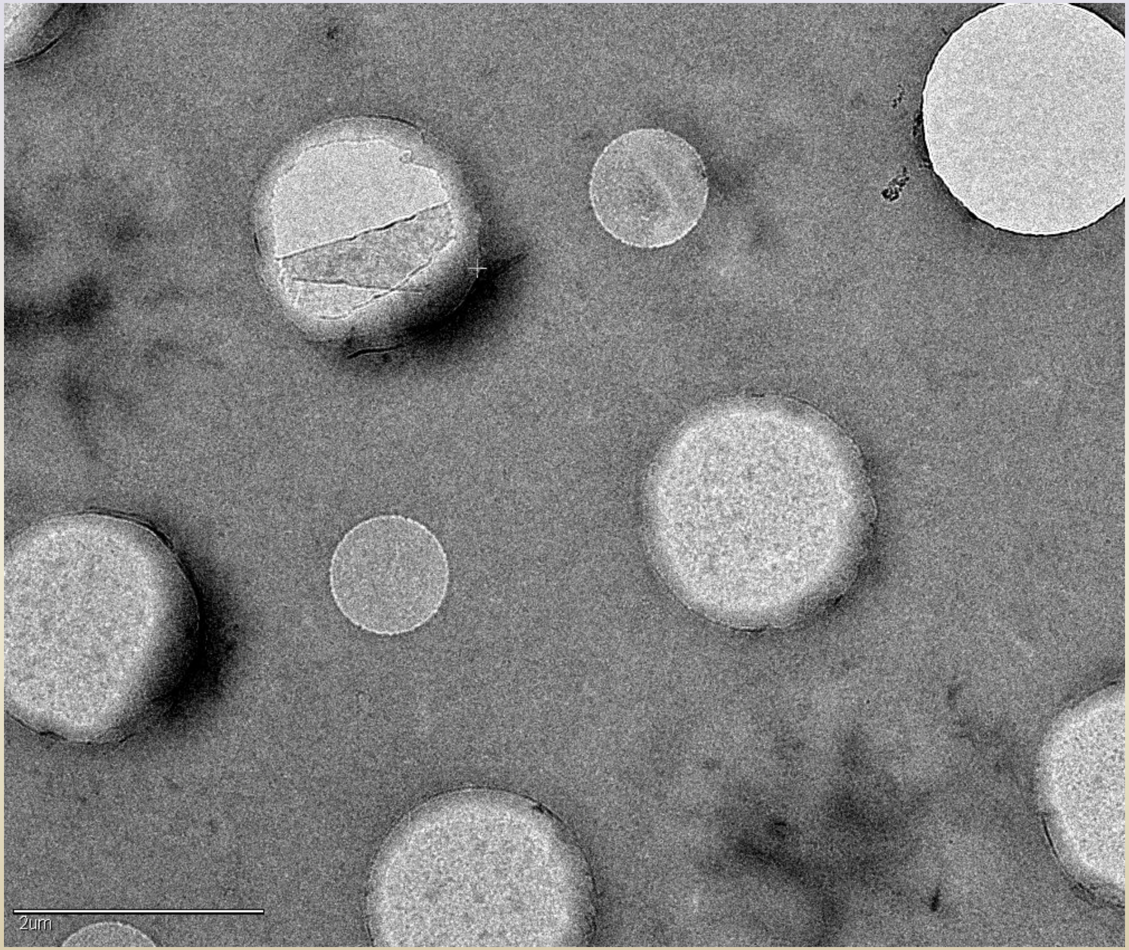


Image of buffer drying out over hole



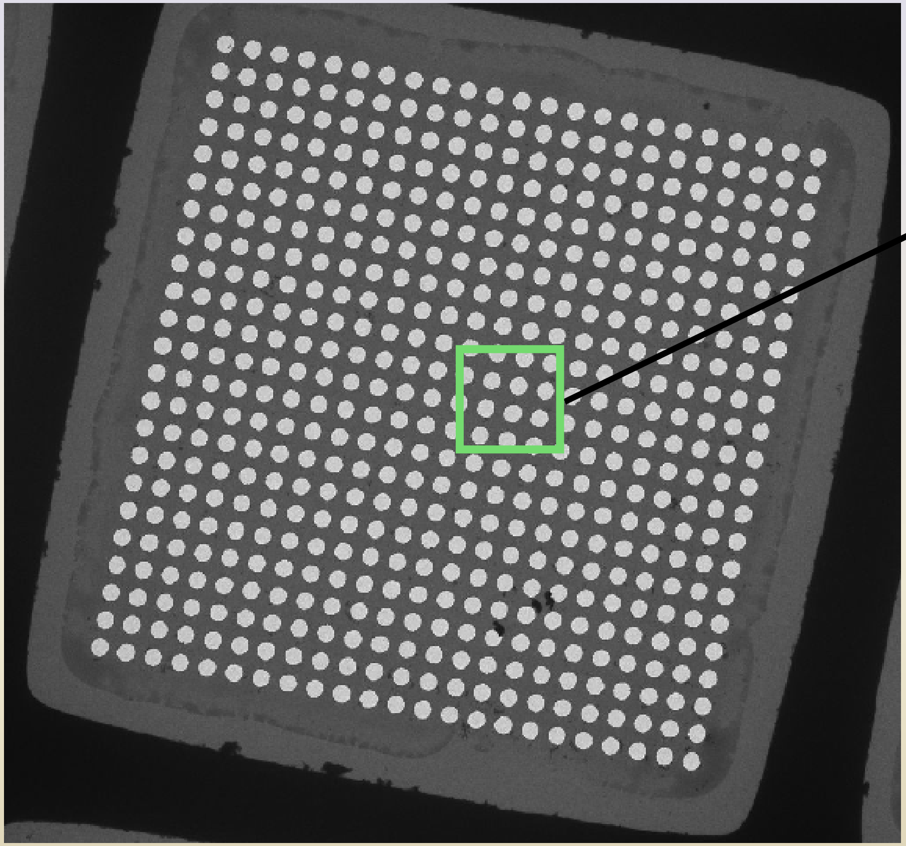
Sample images of eutectic ice.



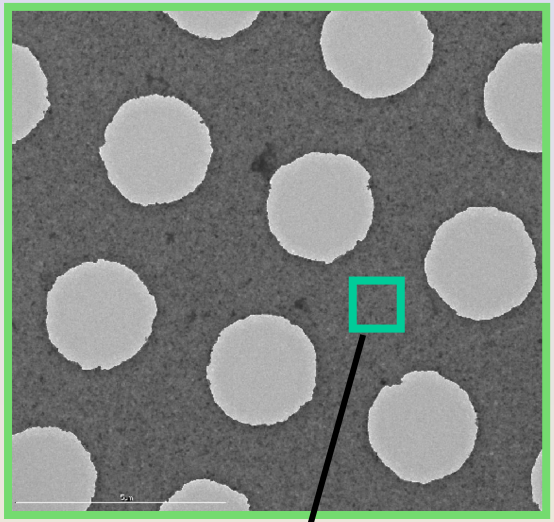
Future experiments and projects

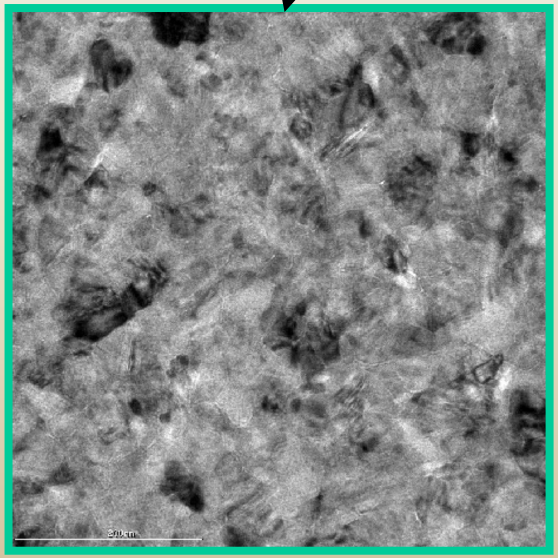
We are also testing new support films made by Protochips.

New semiconductor grids: Protochips Inc.

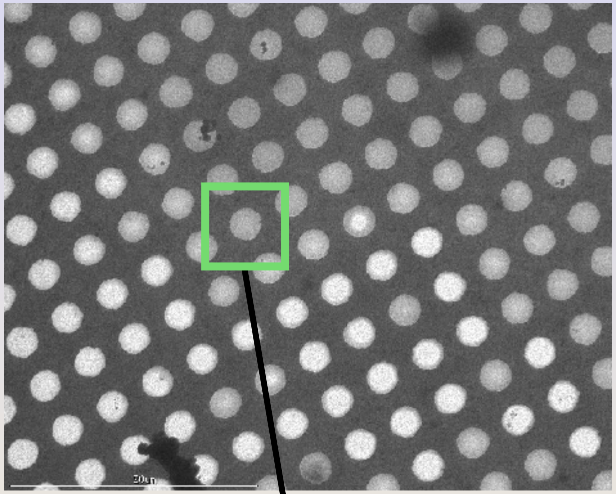


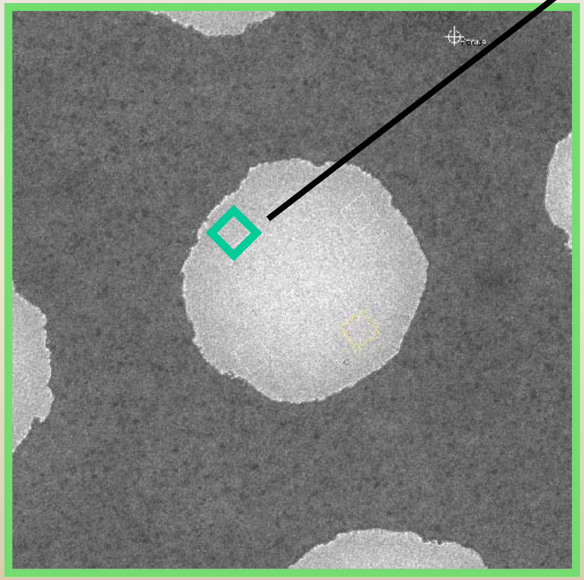
200 mesh grid,
2 micron holes with ~2 micron spacing.

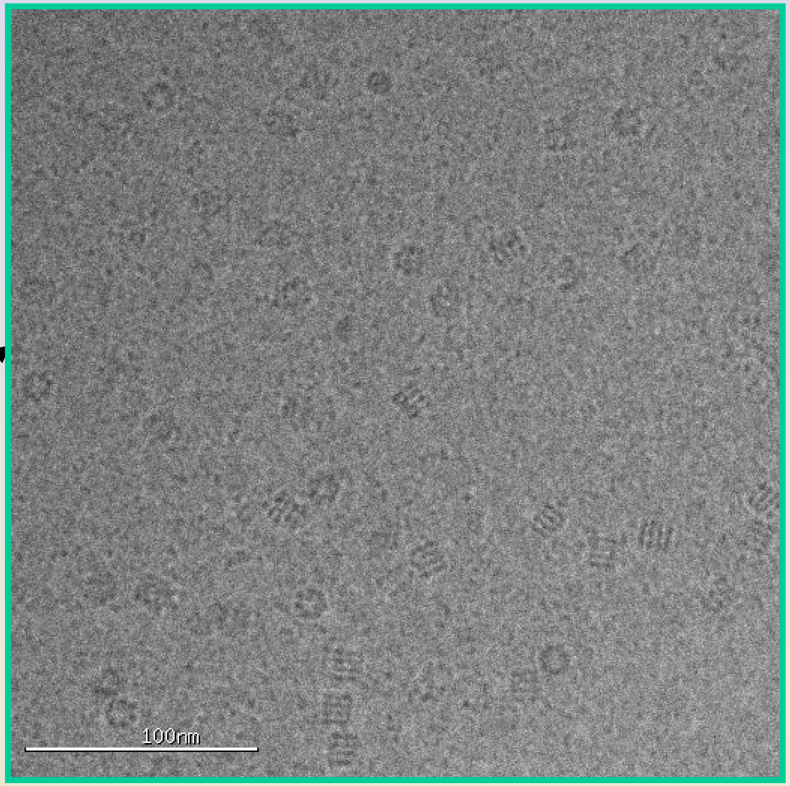




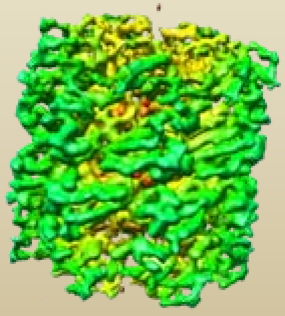
GroEL in vitreous ice prepared over semi-conductor grids





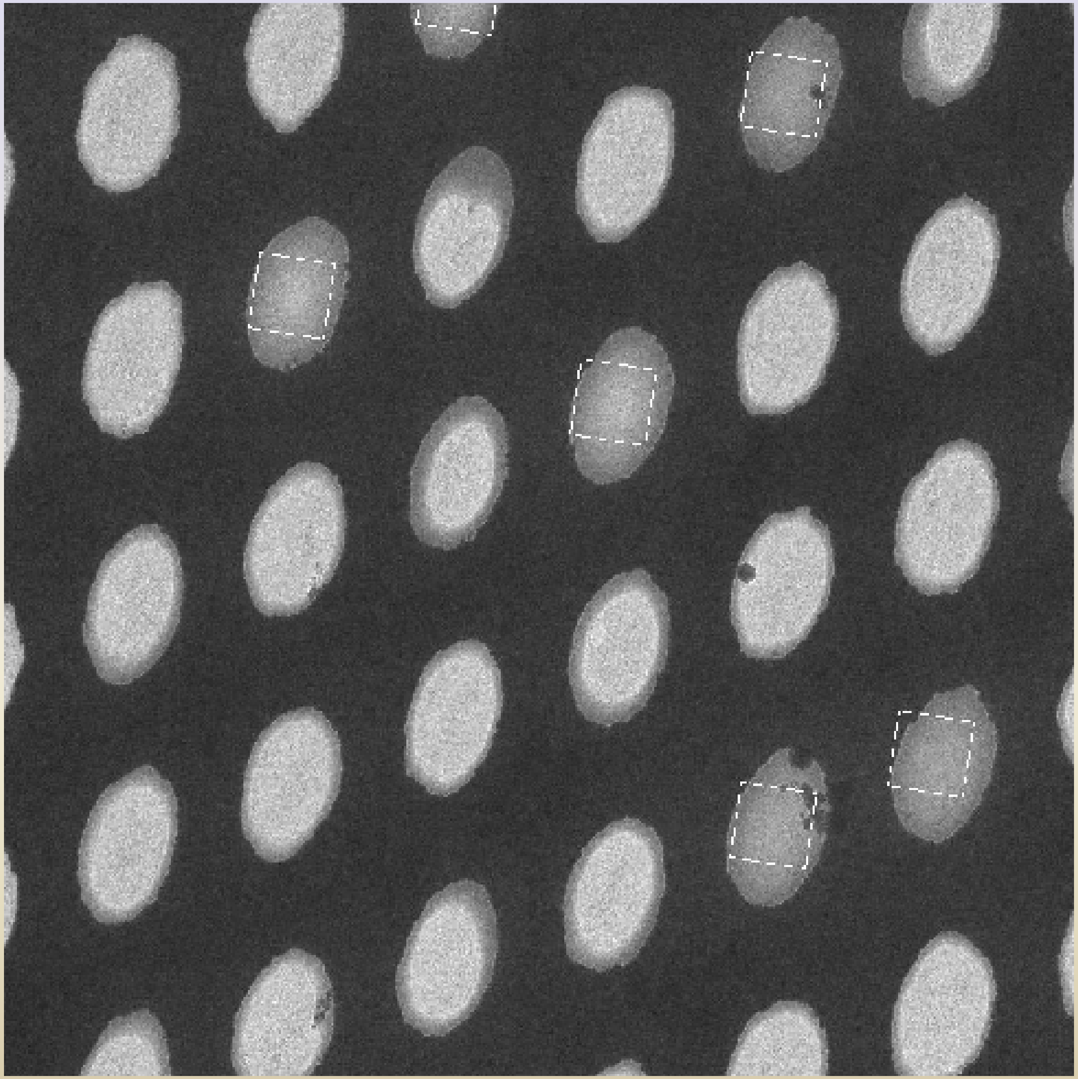


~1,000 images (mag 100,000x) acquired using Leginon;
50,000 particles selected



7.5Å resolution
38714 particles used

Tilt pairs acquired over semi-conductor grids show minimal charging compared to carbon grids



Resisitivity of amorphous carbon vs Protochips semi-conductor material

	Room Temperature	77K
Amorphous Carbon*	$1x10^{8}$ ohm-cm	$\sim 1x10^{16}$ ohm-cm
Protochips Material	$3x10^{-2}$ ohm-cm	10^{1-2} ohm-cm *** ***
Reduction in resistivity	10^{8-9}	10^{15-16}

*** Shimazaka and Miyake, Phys Rev Letters, 61, 994 (1988).**

*** * Estimated**

Ethane heater movie