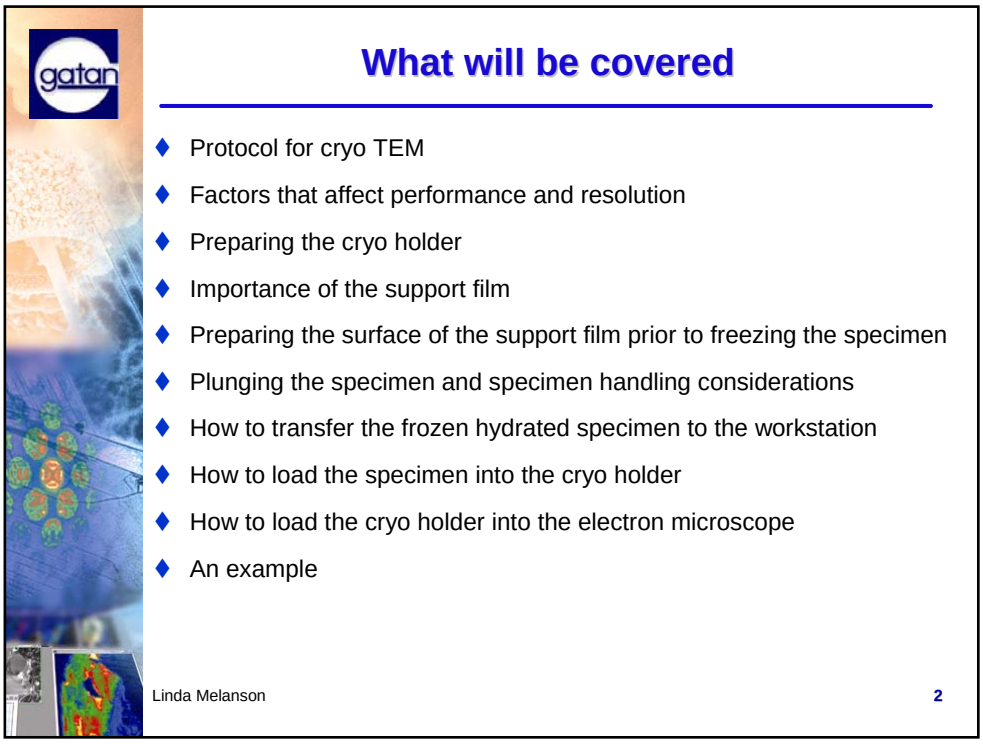
Cryo TEM workshop
Baylor College of Medicine
October 17-19, 2006


gatan
What will be covered
Protocol for cryo TEM
Factors that affect performance and resolution
Preparing the cryo holder
Importance of the support film
Preparing the surface of the support film prior to freezing the specimen
Plunging the specimen and specimen handling considerations
How to transfer the frozen hydrated specimen to the workstation
How to load the specimen into the cryo holder
How to load the cryo holder into the electron microscope
An example
Linda Melanson
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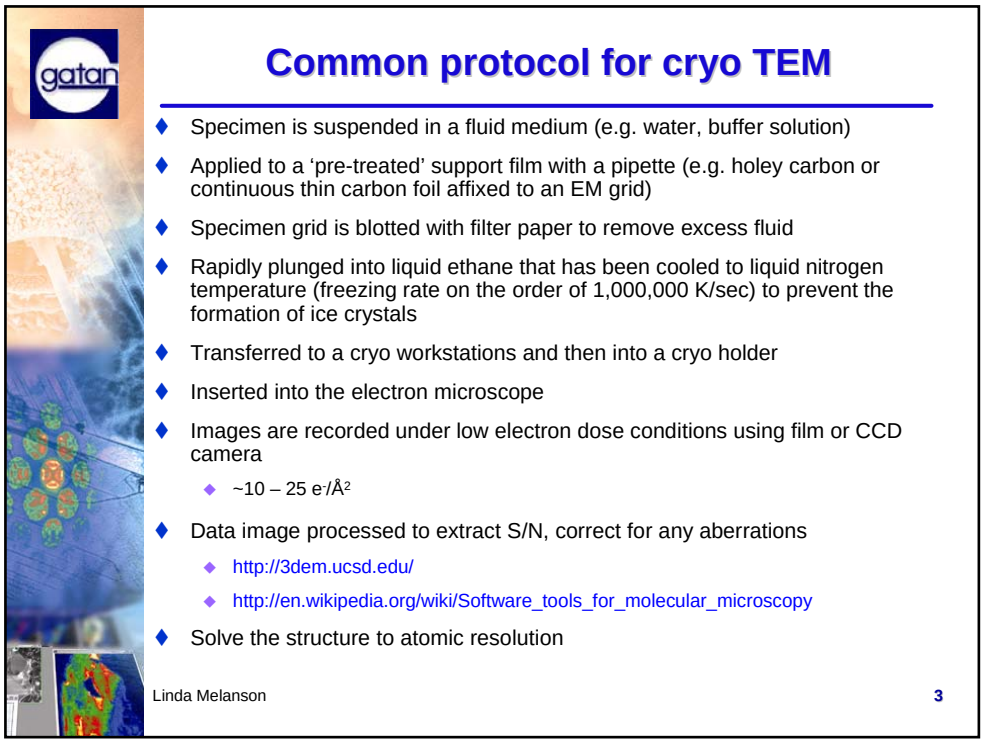


There are many different ways to prepare a specimen for viewing via cryo methodologies. This talk will only look at methods that center around specimens that are suspended in an aqueous medium and that can be blotted and plunge frozen in liquid ethane and then viewed, using low electron dose techniques, on a TEM.

- Specimen is suspended in a fluid medium (e.g. water, buffer solution)
- Applied to a 'pre-treated' support film with a pipette (e.g. holey carbon or continuous thin carbon foil affixed to an EM grid)
- Specimen grid is blotted with filter paper to remove excess fluid
- Rapidly plunged into liquid ethane that has been cooled to liquid nitrogen temperature (freezing rate on the order of 1,000,000 K/sec) to prevent the formation of ice crystals
- Transferred to a cryo workstations and then into a cryo holder
- Inserted into the electron microscope
- Images are recorded under low electron dose conditions using film or CCD camera
  - ~10 – 25 e-/Å2
- Data image processed to extract S/N, correct for any aberrations
  - http://3dem.ucsd.edu/
  - http://en.wikipedia.org/wiki/Software_tools_for_molecular_microscopy
- Solve the structure to atomic resolution

All steps involved in the production of cryo EM data are not particularly straightforward and most often are not 100% reproducible for any given specimen.

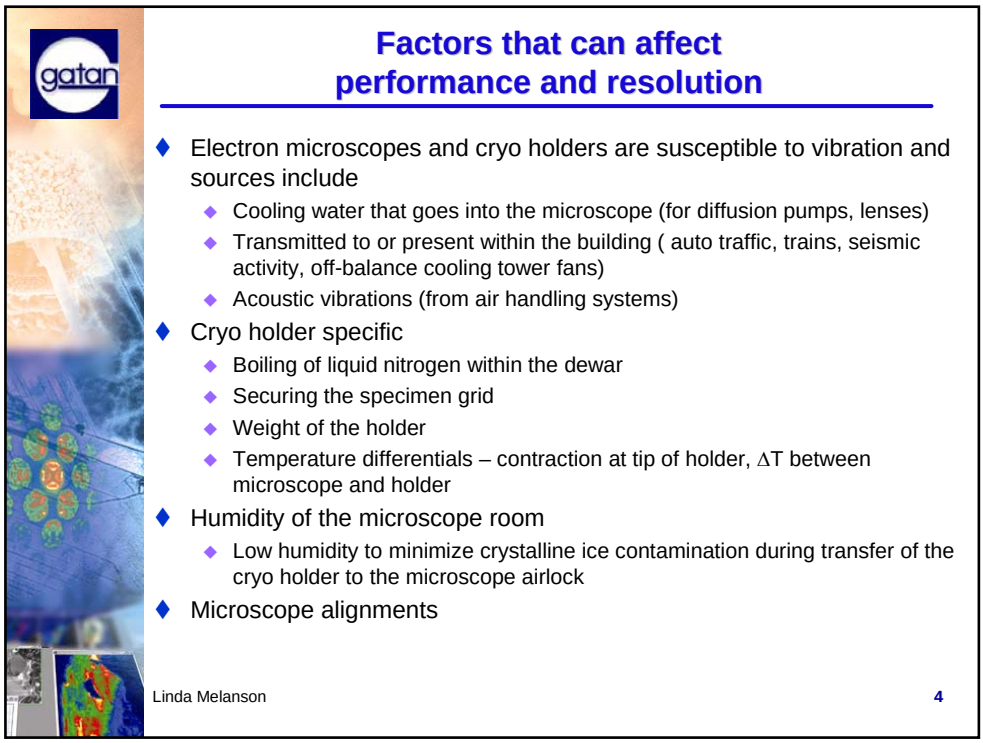
For all of the steps involved in the process of preparing a frozen hydrated specimen (biological or otherwise) there are many caveats that must be taken into account to try to insure that the results obtained are an accurate representation of the native state of the specimen being studied.

Isolating the specimen is generally the most difficult part of the procedure. But, once isolated, macromolecules in solution may not be exactly as they appears within an intact cell so this creates a question as to whether or not what is viewed is the true native state of the specimen.

The forces that the specimen is subjected to in a fluid suspension when it is being transferred via a pipette to the surface of the grid are many and may affect the native structure. The specimen can be subject to local charge changes, dipoles, van der Waals interactions and hydrodynamic effects. For very long, filamentous specimens in particular or even for isolated single particles, velocity gradients may cause the specimen to orient in the direction of the stream of the of the fluid as it is deposited onto the surface of the substrate.

It is known from the work of (Peters and Fromherz, 1975; Quinn and Dawson, 1969; Steinemann and Laeuger, 1971) that when proteins adsorb onto a surface their enzymatic activity is reduced and the proteins suffer denaturation.

Kellenberger and Kistler, 1979 paper discusses damage that occurs to biological specimens when they are mounted for TEM studies.



Side entry cryo transfer holders

- Unlike room temperature holders, cryo holders have
  - Liquid nitrogen dewar to maintain specimen at near liquid nitrogen temperature
  - Protective shutter mechanism to protect frozen hydrated specimen during transfer
  - Specialized work station to load the specimen at liquid nitrogen temperature
  - Temperature controller to monitor or adjust the temperature
  - http://www.gatan.com/


Gatan 626 cryo transfer holder


Gatan UK CT3500 cryo transfer holder

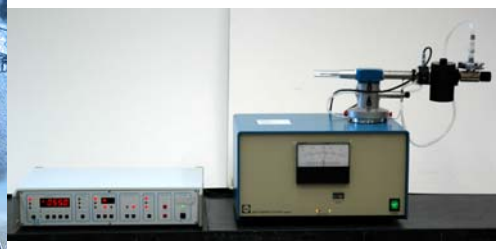
Preparing the cryo transfer holder for use

- Sorb material located (internally) at the base of the dewar
  - Different materials can be used
    - Gatan 626 uses Zeolite
    - CT3500 uses activated carbon
- Acts as a passive cryo pump
  - Prolongs the dewar vacuum hold time
  - Allows dewar to maintain low, stable temperature
- The dewar vacuum degrades over time and with use

Pumping the holder

- Dewar is inserted into one of the pumping ports for the turbo pumping station
- Electrical connection between the dewar electronics and the temperature controller
- Valves on turbo pumping station and dewar are opened once bake-out temperature is achieved
- Bake-out 4 hours or over night to re-establish a good vacuum and long hold time
  - Gatan 626 baked at ~ 90 to100 °C
  - Gatan UK CT3500 baked at 55 °C

Gatan 626 cryo transfer holder, 655 turbo pumping station and SmartSet 900 Cold Stage Controller



Gatan UK CT3500 cryo transfer holder, ITC temperature controller and 655 turbo pumping station with pump-out adapter

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Importance of the support film

- Jacques Dubochet (1982) - *"Lacking precise knowledge about the surfaces on which he mounts the sample, the microscopist uses indicators which are rough but of great practical importance. These are in particular the hydrophilic or hydrophobic nature of the surface and the way charged molecules adsorb to the film."**
- Robert Glaeser (January 15, 1990) – *"Every carbon film is an individual; no two ever seem alike."***
- Same can be said of holey carbon specimen supports.

**Dubochet, J., Groom, M. and Mueller-Neuteboom, S. (1982), Mounting of macromolecules for electron microscopy with particular reference to surface phenomena and treatment of support films by glow discharge. Advances in optical and electron microscopy, Barrer, R. and Cosslett, V. E. (eds.), Academic Press, London, New York. 107-135.*

*** Robert M. Glaeser, Professor Emeritus of Biochemistry and Molecular Biology, Department of Molecular and Cell Biology, University of California, Berkeley, CA. Personal Communication.*

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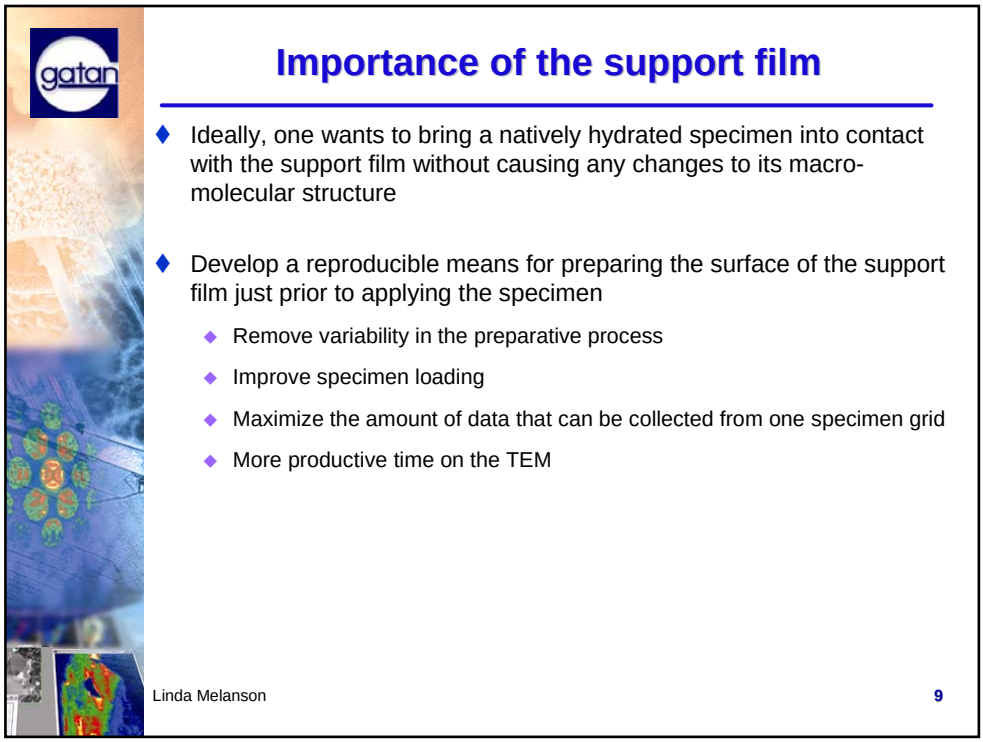
Quotes from Dubochet and Glaeser point out the variability of dealing with carbon support films, be they holey or continuous.

Surface Chemist: Schmidt, 1975 commented on lack of understanding adsorption and reaction of complex molecules such as hydrocarbons as remaining an unknown partially based on the experimental techniques available during that time: Dubochet comments w.r.t. Schmidt's statement w.r.t. the adsorption of hydrocarbon molecules w.r.t. electron microscopy: "lacking precise knowledge about the surfaces on which he mounts the sample, the microscopist uses indicators which are rough but of great practical importance. These are in particular the hydrophilic or hydrophobic nature of the surface and the way charged molecules adsorb to the film."

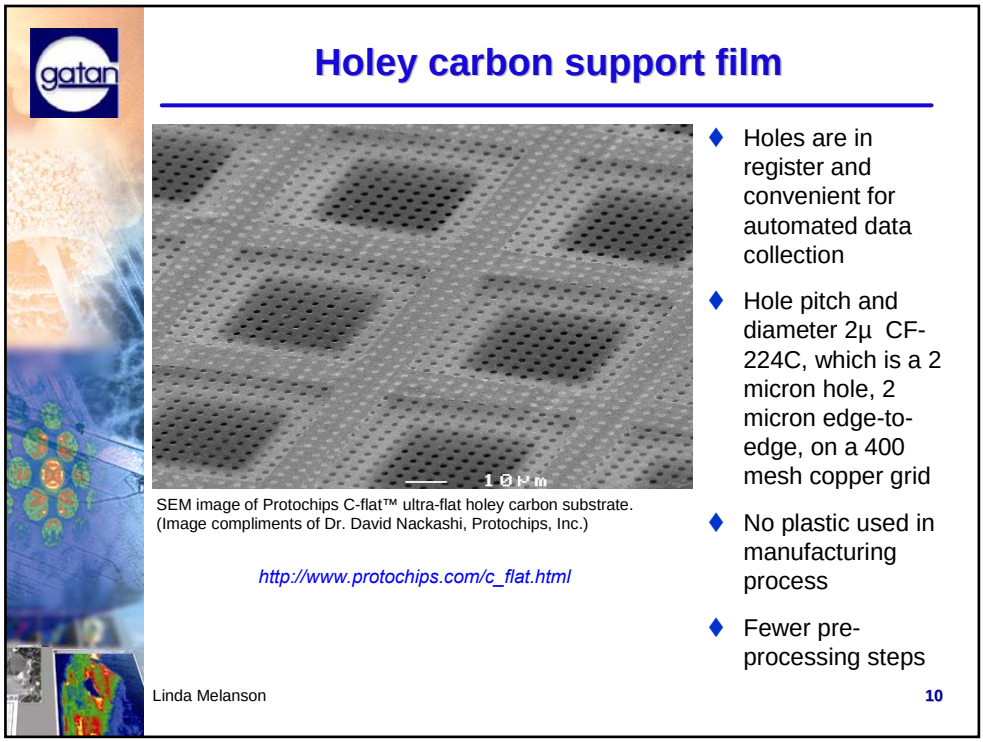


- Ideally, want to bring a natively hydrated specimen into contact with the support film without causing any changes to its macro-molecular structure -- no flattening artifacts – this is one reason why getting the specimen to locate within the holes of a holey carbon film is important. Another important parameter is to get the ice thin enough without compressing the specimen due to surface forces of the ice layer being too thin. Ice that is too thin over the hole can cause the specimen to migrate toward the edges of the hole – not always a bad thing because it is good to have some of the carbon in the imaging area to draw off the charge during the exposure but, if the specimen is too crowded it is sometimes difficult to parse individual particles.

- Develop a reproducible means for preparing the surface of the support film just prior to applying the specimen to improve specimen loading

    - Improve specimen loading
        - many times the specimen in solution is at such a low concentration that 'loosing' the specimen to the surface of the carbon as opposed to having it locate in the holes of the holey carbon support film wastes too much of the specimen which decreases the amount of useable data that can be recorded
    - Want a method to reduce or eliminate variability between one carbon support and the next
        - no more individuals

- More productive time on the TEM
    - Running and electron microscopy facility is very expensive so it is more cost effective to have the highest efficiency w.r.t. data collection on the TEM



Many different types of support films used in cryo TEM:

- Lacey films
    - used when minimization of the carbon surface is most important w.r.t. viewing the specimen
    - Variable distribution of hole size and number per grid
    - Difficult to focus off-axis in low dose mode if there is not some carbon available for this purpose
- Home made holey carbon films
    - have more carbon surface area and also variable distribution of hole size and number
    - Depending upon the method used to make the film, the holes in the film can be round or variable in shape but for both methods the size is always variable
- Above methods involve a multi-step, non-reproducible method for making the supports
    - involves casting plastic film onto a glass slide and exposing the slide to either a high humidity environment to create the holes or incorporating low concentration of glycerol in the plastic solution prior to casting the film onto the surface of the glass slide
    - the plastic is floated off onto the surface of a water bath containing the EM grids which are submerged below the surface
    - the plastic is brought into contact with the grids by lowering the water surface to affix the plastic layer to the grid surface
    - further steps involve coating the plastic layer with carbon in a carbon evaporator
    - removing the plastic by submerging the grids into a solvent

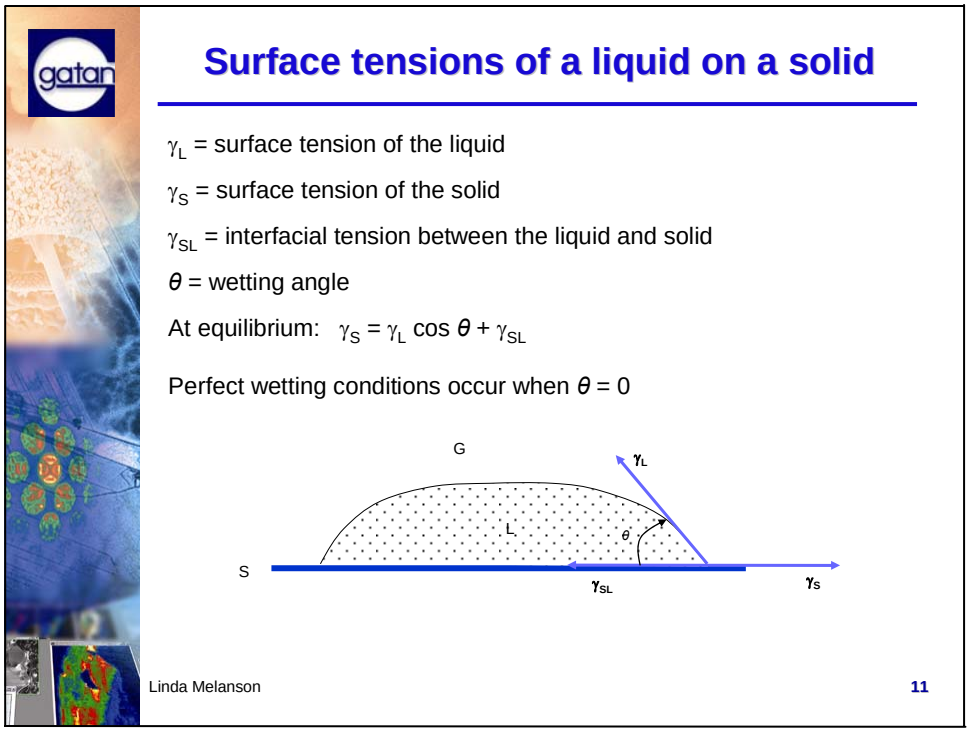
- Lots of people still use this method but it is time consuming to make the holey supports and there are many steps to get to the finished product.

- In addition, if you want 'in register' holes (to facilitate automated microscopy) this is not possible with the homemade methods available.

Commercially available holey carbon foils:

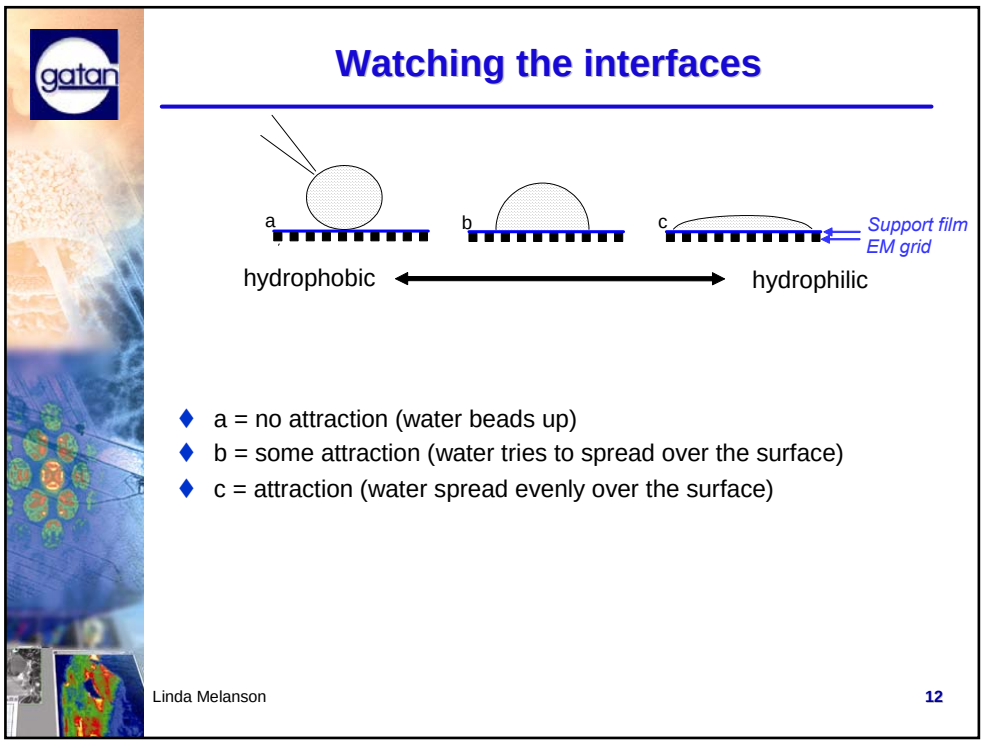
- Quantifoils and C-flats have 'in register' holes and can be purchased directly from supplier or distributors. Somewhat expensive: range of $4-8/grid

- Quantifoils still involve extra steps to make them useable for cryo TEM specimen application
    - most people soak these grids in solvent/vapor upon receipt to remove any residual plastic that was used in the fabrication process
    - This action of soaking the grids in solvent vapor causes the hole size to vary sometimes (depending upon how long you soak the grids)
    - many users follow this 'in-house' cleaning procedure by evaporating an additional layer of very thin carbon on the 'top' side (side away from the grid bars) and then render that new carbon layer hydrophilic using available methods (glow discharge using a vacuum evaporator or instrument designed specifically for plasma/etch)
- One advantage of C-flat support films is that holes are in register, there are no plastic layers used in the manufacturing process so one does not have to soak them in solvent before use.
    - More of an 'out of the box' solution.
    - Product just released for sale mid-2006.
- Commercially purchased support films are expensive but so are the man-hours involved in producing the home-made version
- In the end, one has to decide which support film is right for the specimen under investigation.



Mathematical description of surface tension of a liquid on a solid substrate can be described. When $\theta = 0$ perfect wetting conditions can be achieved.

From Jacques Dubochet's book chapter: **Dubochet, J., Groom, M. and Mueller-Neuteboom, S. (1982), Mounting of macromolecules for electron microscopy with particular reference to surface phenomena and treatment of support films by glow discharge. Advances in optical and electron microscopy, Barrer, R. and Cosslett, V. E. (eds.), Academic Press, London, New York. 107-135.*



Phenomena related to interfaces:

Most electron microscopists do not calculate the level of hydrophilicity but rather observe the fluid/solid interface to see if the fluid is spreading evenly onto this surface prior to plunging the specimen into liquid ethane.

Image shows qualitative view of a small drop (maybe 3-5 µl) of specimen solution placed onto the surface of a holey carbon support film that is affixed to an EM grid.

Top diagrams:

- Image 'a' shows what happens to the small drop when the surface is very hydrophobic
- Image 'b' shows an intermediary state where the small drop tries so spread out over some of the area
- Image 'c' shows what happens when the surface of the support film is rendered hydrophilic – the drop spreads out over the entire surface

Glow discharge summary*

Atmosphere	Surface	Charge
Air	Hydrophilic	Negative
Air	Hydrophilic	Positive (With subsequent Magnesium Acetate Treatment)
Alkylamine	Hydrophobic	Positive
Methanol	Hydrophobic	Negative

**Dubochet, J., Groom, M. and Mueller-Neuteboom, S. (1982), Mounting of macromolecules for electron microscopy with particular reference to surface phenomena and treatment of support films by glow discharge. Advances in optical and electron microscopy, Barrer, R. and Cosslett, V. E. (eds.), Academic Press, London, New York. 107-135.*

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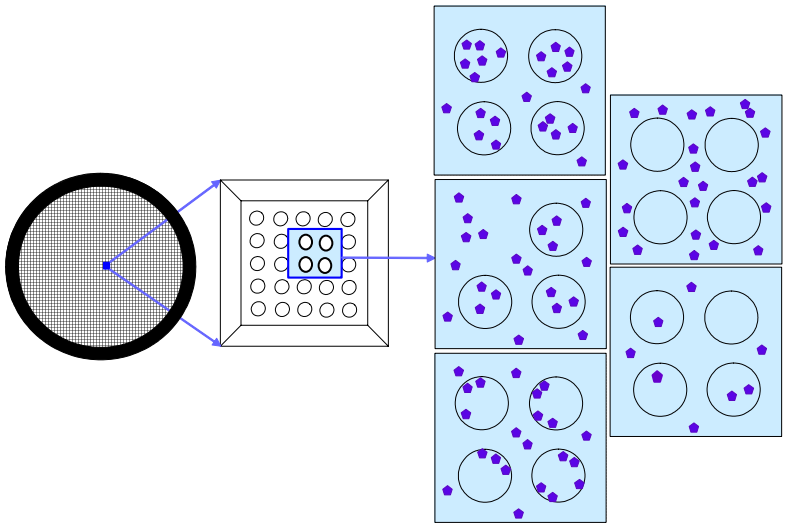
Specimen partitioning



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Specimen partitioning for the frozen hydrated grid:
Left to right:

Representation of a frozen hydrated specimen grid. The highlighted blue grid square is magnified to show the array of holes in the support film that is mounted to the surface of the grid. This array is typical of what one would find for a holey carbon specimen support. The five images to the

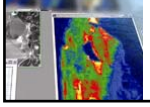
right depict an area from the previous (middle) image showing four holes from one area of the specimen support and show some of the possible ways that the specimen in solution partitions once on the support film depending upon how hydrophobic or hydrophilic the support film is as well as the charge that can be assigned to the specimen which will also depend upon the amount of dissolved solutes in the supporting fluid medium (buffer, water, etc.)

1. Shows specimen locating in the holes of the film with a few on the carbon support. This is usually what one is trying to achieve – even distribution of the specimen over the holes embedded in a thin, uniform layer of vitreous (non-crystalline) ice in order to maximize the amount of data that can be collected from one specimen grid.
2. Shows approximately equal distribution over the holes and the surrounding carbon support. Not as optimal as in #1 but so long as the specimen is embedded in thin layer of vitreous ice a lot of data can still be collected from this grid.
3. Shows the specimen locating at the interface of the hole and the liquid in which it is suspended – usually this is not optimal because it could indicate that the drying of the grid during blotting caused the middle of the hole to become thinner than that near the edge of the hole forcing the particles to the edge – also, the particles could be attracted by other forces to the edge of the hole. This is not always the best condition and it depends on the sample being studied as to whether or not it is advantageous to have the sample crowd at the solid/liquid interface at the edge of the hole. Some time the particles pack so tightly that it is difficult to parse them for image processing.
4. Shows an example whereby the specimen adsorbs to the surface of the support film and does not enter the holes – this is a non-optimal condition for freezing the specimen. Usually not an optimal condition. The higher resolution scenario is to have the particles suspended over the holes in a thin layer of ice with either no background carbon or (as some people may use) a very thin layer of carbon for CTF correction purposes.
   http://www.maxsidorov.com/ctfexplorer/webhelp/background.htm and
   http://www.aber.ac.uk/~ecmwww/journal/smi/pdf/smi97-11.pdf#search=%22contrast%20transfer%20function%22
5. Shows and example where the specimen is not partitioning properly due to incorrect surface charge for the grid or low concentration specimen. One way to overcome 'low loading' is to saturate the surface with several droplets of the specimen suspension, allowing it to concentrate through minimal evaporation at 100% humidity over several minutes and then blot and plunge.

The question is always – what to do I have to do to get the sample to evenly distribute in the holes in a uniform layer of vitreous ice

Optimizing surface charge for carbon support films

- Evaporated carbon is hydrophobic and can be made more hydrophobic by heating for 1 hour at 80 °C
- Most specimens like a hydrophilic surface
  - Treating the surface of the grid with an organic solvent
  - Adding chemical to the specimen suspension to improve the spreading characteristics
  - Ionization systems
    - UV light
    - Glow discharging using a vacuum evaporator
    - Plasma cleaners to produce controlled glow discharge



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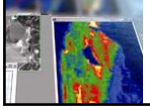
Optimizing surface charge for carbon support films

- UV light source
  - Low pressure Hg light source
    - generates ozone which is decomposed to produce activated oxygen
    - organic molecules on surface of the support film can be cracked and oxidized by the UV light and the activated oxygen generated by the light
    - Cleans organic contaminants from the surface of the support film making it more hydrophilic
  - Surface becomes hydrophilic, negatively charged
  - Works at atmospheric pressure
  - Not good for removing thick contaminant layer because not as strong as using plasma at low vacuum
  - Results are often variable for specimen substrates



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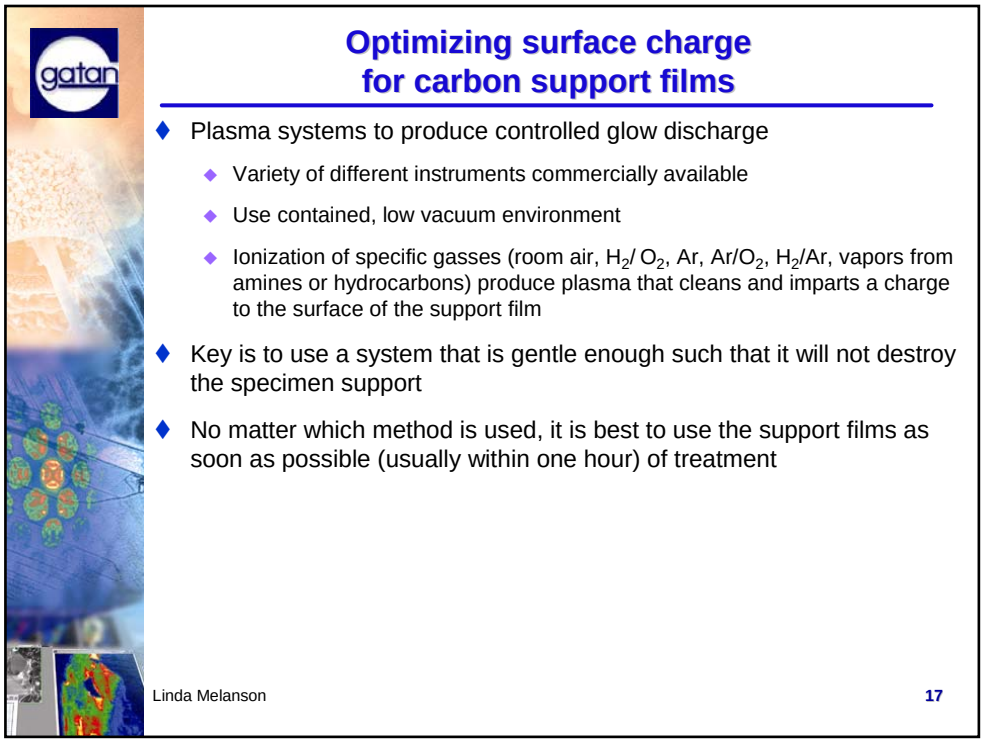
http://www.crystec.com/senteche.htm

- The 185nm UV line decomposes oxygen molecules and synthesizes ozone O_3. The 245nm UV line decomposes ozone and produces high energy O* (activated oxygen).
- Radicals such as *OH, COO*, CO* and *COOH are formed with an increased hydrophilic nature.

- Organic molecules can be cracked and oxidized by UV light and active oxygen generated by UV light. CO_2 and H_2O are formed, which desorb from the surface.
- The surface will be cleaned of organic contaminants and becomes hydrophilic.
- Results in the surface of the support film being cleaned of any adsorbed hydrocarbon contaminants

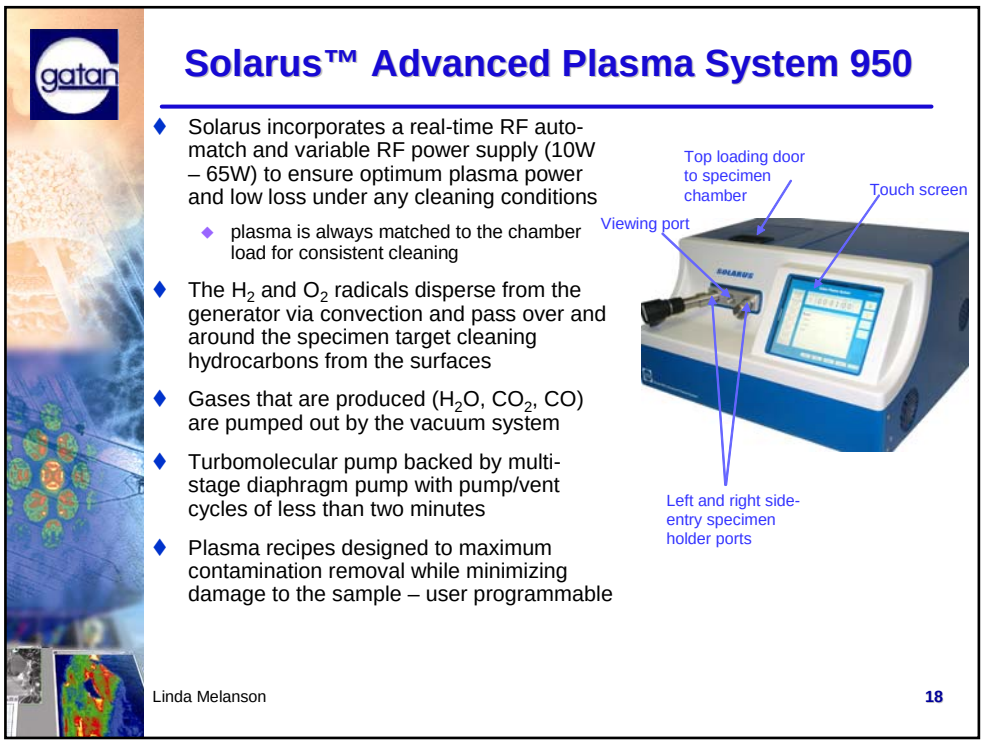


- Plasma systems to produce controlled glow discharge
  - Variety of different instruments commercially available
  - Use contained, low vacuum environment
  - Ionization of specific gasses (room air, H_2 O_2, Ar, Ar/O_2, vapors from amines or hydrocarbons) produce plasma that cleans and imparts a charge to the surface of the support film
    - atmospheric air, hydrophilic, negatively charged surface*
    - Atmospheric air, hydrophilic, positively charged surface with post treatment using Magnesium*
    - Alkylamine atmosphere, hydrophobic with positive charge (~3 µl of the solution on a piece of filter paper is placed in the chamber prior to the glow discharge.* Dangerous to work with (should not breathe the vapors) and exhaust from process should be vented to a fume hood.)
    - Methanol (hydrocarbon) atmosphere, hydrophobic with negative charge.* (~3 µl of the solution on a piece of filter paper is placed in the chamber prior to the glow discharge.)

**Dubochet, J., Groom, M. and Mueller-Neuteboom, S. (1982), Mounting of macromolecules for electron microscopy with particular reference to surface phenomena and treatment of support films by glow discharge. Advances in optical and electron microscopy, Barrer, R. and Cosslett, V. E. (eds.), Academic Press, London, New York. 107-135.*

Key is to use a system that will not destroy the specimen support (i.e. will not break cause breakage of the support so that there is no longer any holey carbon foil bridging the holes of the grid)

No matter which method is used, it is best to use the support films as soon as possible (usually within one hour) of treatment



Features of the Gatan Solarus 950 and why it may be the best choice for plasma cleaning:

- Solarus incorporates a real-time RF auto-match and variable RF power supply (10W – 65W) to ensure optimum plasma power and low loss under any cleaning conditions
    - plasma is always matched to the chamber load for consistent cleaning
- The H_2 and O_2 radicals disperse from the generator via convection and pass over and around the specimen target cleaning hydrocarbons from the surfaces
- Gases that are produced (H_2O, CO_2, CO) are pumped out by the vacuum system
- Turbomolecular pump backed by multi-stage diaphragm pump with pump/vent cycles of less than two minutes
- Plasma recipes designed to maximum contamination removal while minimizing damage to the sample – user programmable

Image of the Solarus showing front viewing port, left/right hand ports for inserting and cleaning specimen rods, the touch screen operating panel and the access door on the upper left that can be opened to insert larger samples or up to ~50 TEM grids for pre-treatment (cleaning and rending surface of a holey carbon foil hydrophilic)

Solarus™ Advanced Plasma System 950

- Exclusive H_2/O_2 gas chemistry (Patent Pending)
  - This unique chemistry provides superior cleaning with less sputter damage of all samples including holey carbon films
  - 40 - 50% cooler than cleaning with traditional Ar/O_2 (75%:25%) mixture
  - Internal mass flow controllers accurately control flow rate of the chosen gas mixture
  - Fast! 1 minute of cleaning with H_2/O_2 reduce/prevent buildup of contamination
  - Excellent for cleaning fragile holey carbon support films without damage
- Multi-language support includes operational instructions in English, German, French, Chinese, Korean, and Japanese
- One touch operation for consistent results for every user

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Simple to operate

- Switch on Mains
- Load sample (or holders) for cleaning
- Chose appropriate recipe
- Press start

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Simple one touch operation

- Switch on Mains
- Load sample (or holders) for cleaning
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Evacuation of the chamber to low vacuum

- Two stage variable speed diaphragm pump (electronic speed control) backs a 70 liter/sec turbo molecular drag pump
- 50 second pump-down

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Evacuation of the chamber to low vacuum

- Two stage variable speed diaphragm pump (electronic speed control) backs a 70 liter/sec turbo molecular drag pump
- Pump-down for Solarus is 50 seconds vs. 3 minutes for other commercially available units

Introduction of process gases

- Gases are introduced into the chamber via precision mass flow controllers
- Repeatable cleaning results achieved by accurate gauging of the gas levels by the MFCs

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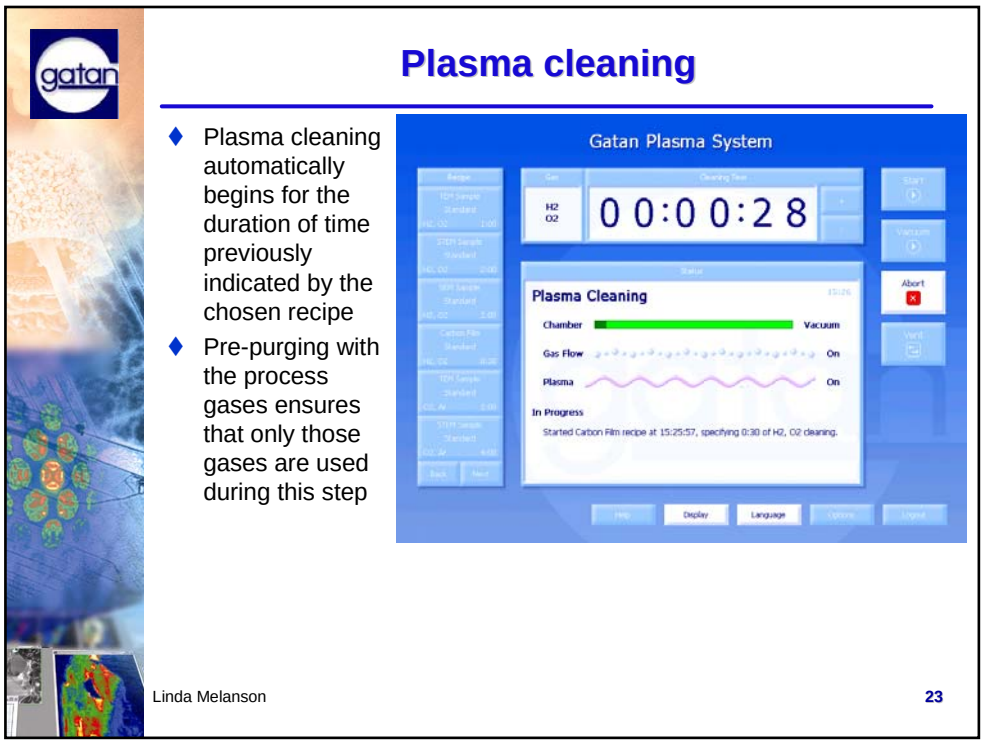
Introduction of process gases

- Gases are introduced into the chamber via precision mass flow controllers
- Repeatable cleaning results achieved by accurate gauging of the gas levels by the MFCs (mass flow controllers)
    - Cleaning rates 30 seconds to 120 seconds vs. 2 minutes to 10 minutes for other commercial units

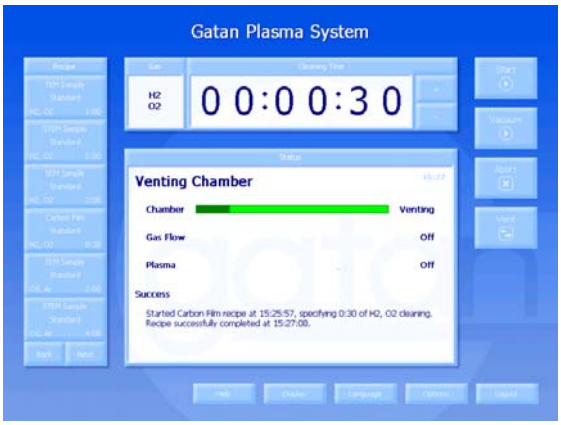


Plasma cleaning

- Plasma cleaning automatically begins for the duration of time previously indicated by the chosen recipe
- Cleaning rates of 30 to 120 seconds can be used
- Pre-purging with the process gases ensures that only those gases are used during this step

Venting the chamber

- When the plasma has completed its cycle, press 'Vent' to retrieve your grids
- 5 seconds to vent system
- H_2O_2 grid prep recipe takes about ~3 minutes start to finish



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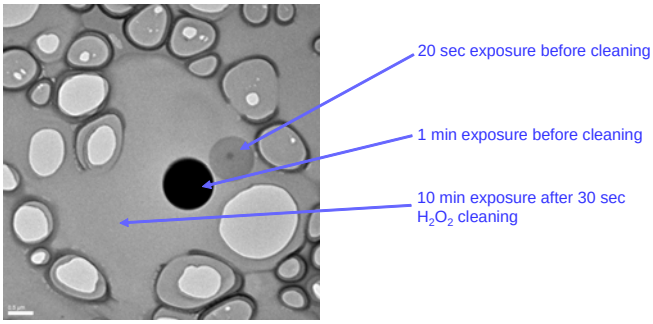
Cleaning power of Solarus™

- Example of a holey carbon film that has been contaminated with 1 drop of oil in 50 ml of acetone



Courtesy of Mr. Richard Mitro, Gatan, Inc.

http://www.gatan.com/knowhow13/index.htm

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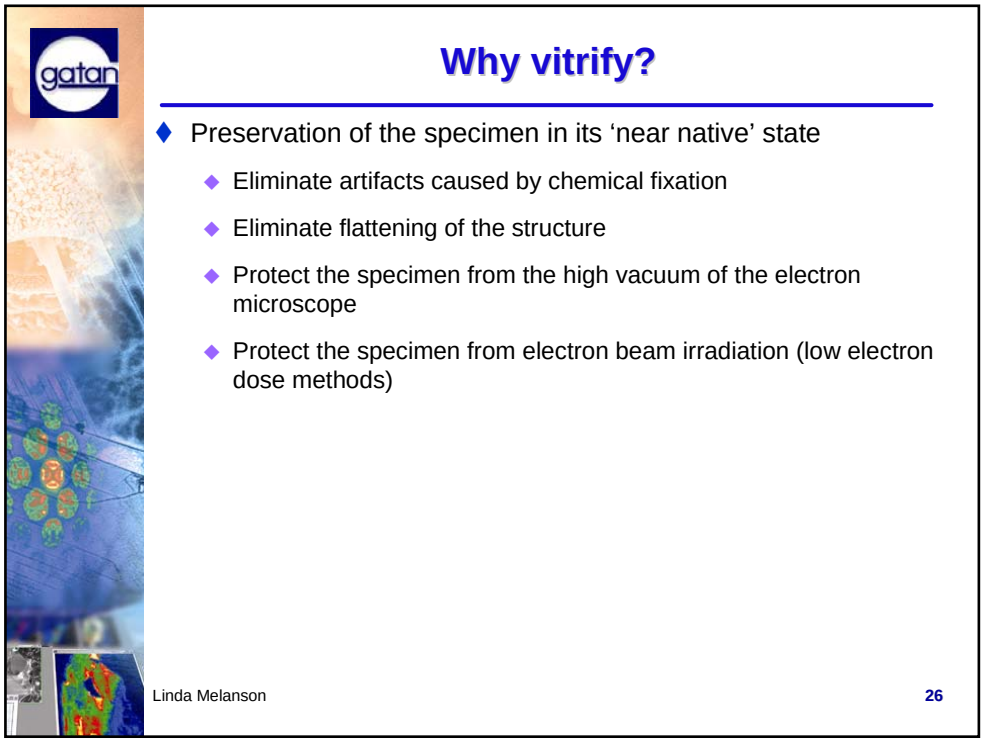
Cleaning power of Solarus:

- Example shows a homemade holey carbon film that has been contaminated with 1 drop of oil dispersed in 50 ml of acetone.
- Image shows 20 second and 1 minute exposures by the electron beam prior to treatment with the Solarus.

- After cleaning the holey carbon support in the Solarus for 30 seconds using H_2O_2 gas chemistry, all of the hydrocarbon contamination has been removed and thus, when the electron beam interacts with the surface of the support, there is no residual burn mark from the beam (no residual ash from the incineration of the now removed oil suspension.)

For more on the Solarus (or information on other products manufactured by Gatan) visit the www.gatan.com and click on ***'KnowHow'***



Freezing the specimen

- Three main steps
  - Apply a quantity (3-5 µl) of the aqueous suspension that contains your specimen to the (holey) carbon support film
  - Blot the specimen to produce a thin layer of the aqueous suspension (~100nm)
  - Rapidly plunge the specimen grid into liquid ethane that has been cooled to near liquid nitrogen temperature

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Plunging protocol - supplies

- Plasma cleaned specimen supports
  - Holey supports, continuous carbon supports
  - Use within one hour of plasma cleaning
- Cryo grid boxes
- Fine and coarse tweezers
  - Plunger comes with tweezers
  - Coarse tweezers for manipulating bulk items (grid boxes)
- Liquid nitrogen and liquid nitrogen dewars
  - 240 liter supply
  - 35 liter storage
  - 4, 5 or 10 liter transfer with pour spouts
- High purity grade ethane
- Nitrogen gas (if plunger utilizes a pneumatic supply)
- Appropriate regulators, tubing, fittings, securing mechanisms for gas cylinders
- Small styrofoam box lined with aluminum foil

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Have all of these supplies ready before you start plunging your specimens.

Plunging protocol - supplies

- Transfer pipettes and tips (Pipetman 10, 100, 200, 1000 or equivalent)
- Eppendorf tubes for dilutions
- Extra buffer, deionized water
- Conical tubes for storing the frozen hydrated specimens in their grid boxes
- Cryo gloves
- Grid box transfer tools
- Small screw driver
- Tape and marking pens
- String
- Razor blades
- Scissor
- Nitrile gloves
- MSDS for any of the gases or cryogens that you will be using

Plungers for freezing the specimen

- Cryo Plunge devices
  - Homemade models
    - Advantage
      - inexpensive to build
      - portable
    - Disadvantages
      - Most incorporate manual blotting which can be inconsistent from one user to the next
      - No real environmental (humidity, temperature) controls
      - No safety controls
      - Variability in cryo preparations may lead to unproductive cryo sessions on the microscope

Advantages offered by commercial plunger

- Several commercial models now available (Gatan, FEI, EMS, Leica)
  - Features that offer numerous advantages over homemade plungers
    - Provide automatic, programmable blotting
    - Humidity and temperature control
    - Safety interlocks to protect the user
    - protection for splashing liquid ethane during plunging process
    - Some can be modified for different freezing methods
    - More consistent results even for novice users
  - Disadvantage is that they can be quite expensive

Plunging protocol

- Prepare the chamber for required temperature and humidity
- Prepare the blot pads
- Cool the workstation
- Condense the ethane
- Attach the tweezers/grid to the plunging post and set plunging post to the 'ready' condition
- Pipette specimen solution onto surface of the grid
- Initiate the blotting/plunging cycle
- Remove the frozen hydrated grid to the storage grid box
- Transfer cryo grid box to pre-cooled workstation for the cryo holder for immediate viewing or transfer grid box to a storage dewar and view at a later date

Helpful hints

- Safety first!
  - Protect your eyes and hands from ethane and liquid nitrogen
  - No open flames near the ethane
  - Vent ethane to an explosion proof hood
    - Liquid cryogens expand in excess of 700X at temperatures above their boiling point so no enclosed vessels (boiling point of ethane is -88.6 °C)
- Practice all the procedures at RT (without cryogens) until you are confident with every step
- Work quickly but carefully and become accustomed to the order by which each step should be carried out
- To avoid devitrification of the grid, always pre-cool any tools that will come into contact with the frozen hydrated grid or the grid box and never lift the grid out of the protected (cold) gaseous environment of the workstation during transfer to the grid box
- Clean the inside of the cryo holder dewar with compressed air and never insert something that might scratch the inner surface of the dewar as this will cause continual bubbling of the liquid nitrogen which can lead to vibration in the image

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Liquid cryogens expand in excess of 700X at temperatures above their boiling point so no enclosed vessels (boiling point of ethane is -88.6 °C) and no sources of ignition in the area where the plunging will take place.

Cryo holder and workstation

Gatan 626 70° cryo transfer holder, workstation and cover, blue cryo grid box, clip ring tool, fine tweezers, cryo gloves (plug for the workstation is inserted.)

Workstation at RT showing transfer grid box and tip of the cryo transfer holder.

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Close-up of the Gatan 626 70° cryo transfer holder and workstation.

Cooling the workstation with liquid nitrogen

Conical tube with cryo grid boxes in transfer dewar filled with liquid nitrogen.

Pre-cool the workstation. Insert the plug into the support cylinder that extends from the workstation if the holder is not already inserted.

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Transferring the grid box from a conical tube that is often used for storing the cryo grid boxes with the frozen hydrated specimen grids. Keep everything cold including all tools that will contact the grid box during transfer. In the following images the lid for the workstation is purposefully left off so that the images could show what the users is doing with in the station but, in normal use, it is best to work through the holes in the cover to prevent condensation of ones breath onto the surface of the grid.

Insert the cryo holder

Using pre-cooled large tweezers, quickly transfer the cryo grid box containing the frozen hydrated grids to the liquid nitrogen in the workstation.

Remove the plug and insert the cryo holder into the workstation.

Take care not to damage the tip of the holder on insertion.

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Once the specimen grid box is in the workstation, load the holder by removing the black plug (which is held in place via an o-ring seal – so remove it by just pull the plug straight out from the arm of the workstation.)

Fill the dewar

Avoid spilling the liquid nitrogen by using the trapped funnel that comes with the workstation.

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Quickly fill the liquid nitrogen dewar of the holder to get it to temperature. The tip will cool down immediately once it is inserted into the cool environment of the workstation. There are many

ways to accomplish this step. Cooling the holder down in the high vacuum of the microscope column and then transferring the cryo holder to the pre-cooled workstation is method that is commonly used in many laboratories.

For the purpose of observing what is happening within the workstation chamber, the workstation cover has been removed for the following images. Normally, one would work with the cover in place, working through the two opening in the cover in order to protect the specimen from condensation of atmospheric vapor (and subsequently the formation of frost) onto the surface of the grid.

The 70° cryo transfer holder has a special tool to remove the clip ring that holds the specimen grid in place. Insert the tool over the clip ring and rotate the knob at the end of the tool. This action will cause the metal blades located at the opposite end of the tool to engage the clip ring. Once firmly attached, tilt the tool sideways to disengage the clip ring. It is sometimes helpful to view the action of these blades under a stereo microscope to have a close-up view of how they work.

Carefully remove the previously loaded grid from the tip of the holder and discard.

Carefully remove the previously loaded grid from the tip of the holder and discard. It is important to remove the ‘old’ grid from the workstation prior to loading the new grid to eliminate the possibility of accidentally carrying it over into the airlock of the electron microscope. This can sometimes cause a vacuum leak in the airlock of the microscope.

Insert the frozen hydrated grid and secure the clip ring

Keep the grid close to the metal surface of the workstation insert during the loading process.

Press the clip ring securely to lock the grid in place.

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- Keep the grid close to the metal surface of the workstation insert during the loading process.
- Press the clip ring securely to lock the grid in place.
- The higher up from the metal surface that you raise the grid, the greater the chance that it will warm up and devitrify the ice layer.
-

Make sure the clip ring is attached

Pre-cool the opposite end of the clip ring tool and then press it gently against the clip ring to ensure that the clip ring is firmly seated in place.

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- Pre-cool the opposite end of the clip ring tool and then press it gently against the clip ring to ensure that the clip ring is firmly seated in place.

Move the cryo holder to the microscope console

Close the shutter to protect the grid and transfer the workstation over to the console of the microscope.

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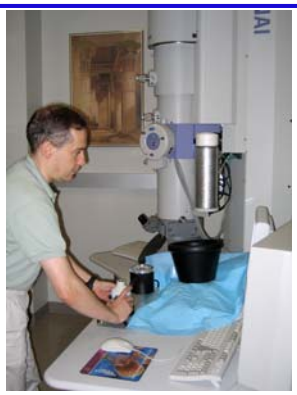
- Close the shutter to protect the grid and transfer the workstation over to the console of the microscope. You want to minimize the transfer of the grid in room air even though it is protected by the cryo shutter so it is best to move the entire workstation with holder and grid to the microscope console.

Preparing to insert the cryo holder into the airlock

Top of the anti-contaminator. You may want to protect the surface of the microscope.



Pre-tilt the microscope stage to minimize loss of nitrogen as the holder is inserted into the airlock. Wait for any vacuum sequence to finish before inserting the holder.

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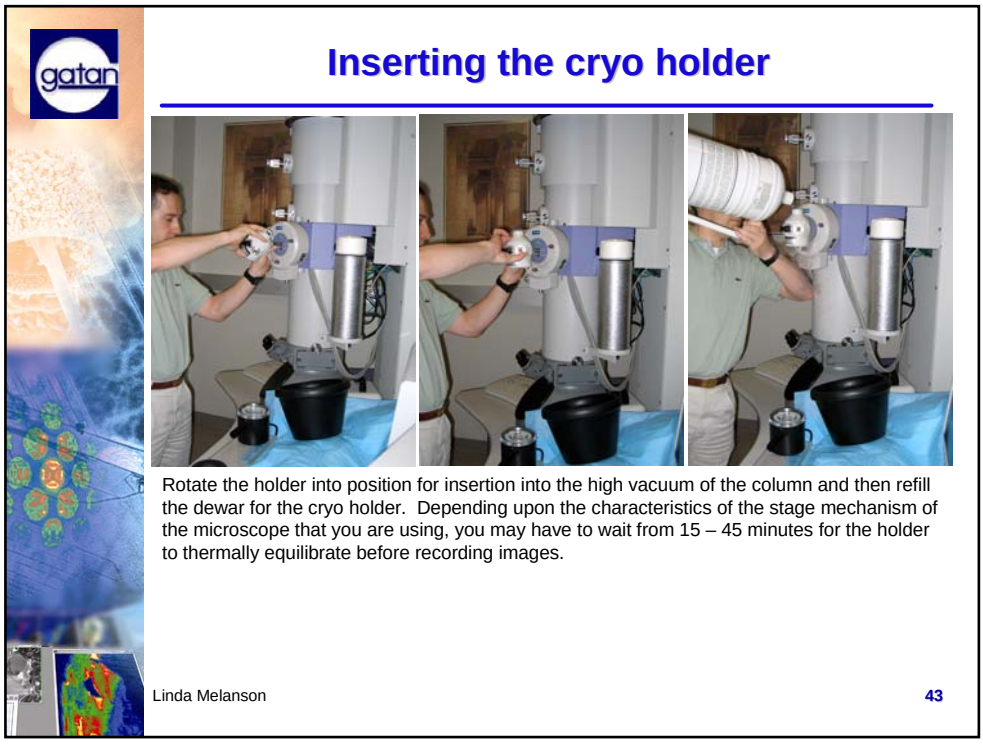
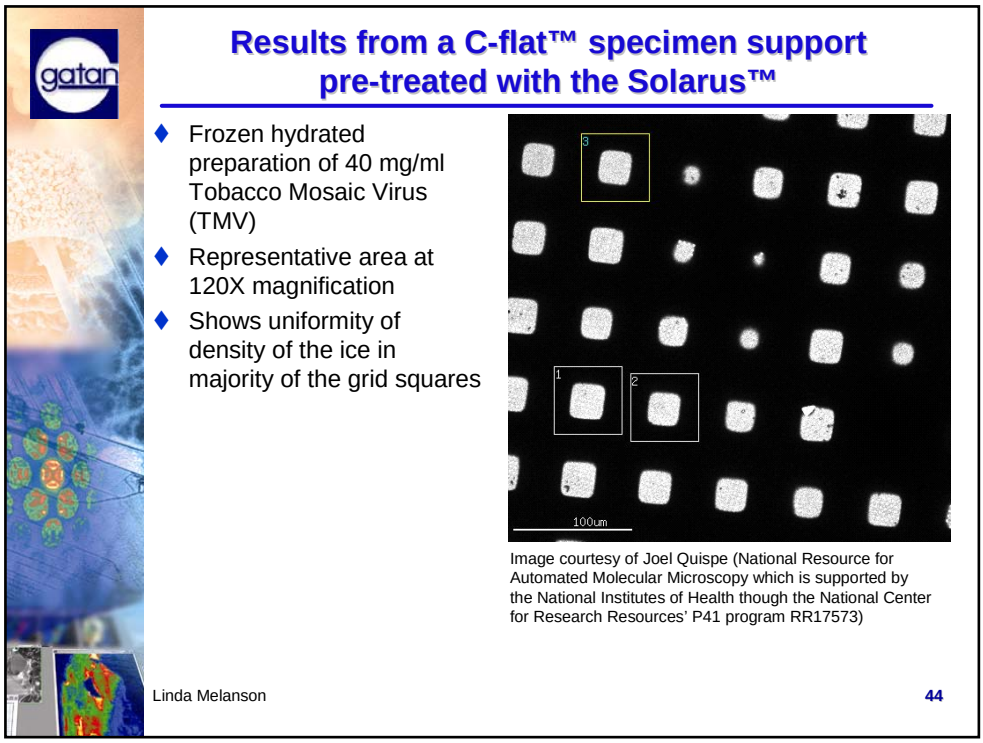
- Top off the anti-contaminator. You may want to protect the surface of the microscope.

- Pre-tilt the microscope stage to minimize loss of nitrogen as the holder is inserted into the airlock. Wait for any vacuum sequence to finish before inserting the holder. Consult with the microscope manufacturer to get explicit instructions for loading specimen holders into the airlock.

- Make sure the anti-contaminator on the microscope is topped off. Make sure the valve to the electron gun is closed (especially if it is a FEG) and pre-tilt the stage of the microscope to accommodate loading the holder such that a minimum amount of nitrogen will spill out and then insert the holder into the high vacuum of the optical column.

- 

- Following manufacturer's directions for your particular electron microscope, insert the holder into the airlock. Rotate the holder into position for insertion into the high vacuum of the column and then refill the dewar for the cryo holder. Depending upon the characteristics of the stage mechanism of the microscope that you are using, you may have to wait from 15 – 45 minutes for the holder to thermally equilibrate before recording images.



This image is the first in a series of images showing a grid pre-treated with the Solarus to make it hydrophilic and then plunge frozen in liquid ethane and viewed on an electron microscope under low electron dose conditions.

Imaging conditions:

- Tobacco Mosaic Virus (TMV) at 40mg/ml was used as the biological test sample
- The acquisition parameters were:
  - Tecnai F-20(FEG), operated at 120kV
  - Gatan 4kx4k CCD, 50,000x magnification (2.26 Å/pixel on CCD)
  - Gatan 626-cryo-stage temperature in scope –178 C
  - Leginon automated data acquisition, and Protochips, Inc. C-flat holey carbon specimen support. ~15e-/ $Å^2$ dose per image, -0.8 to -2 micron defocus
  - 36 high mag pairs, extracted 511 TMV filaments, imaged 67 holes, and ran Leginon for 2 hours 47minutes.
  - The FFTs of the extracted TMV showed the same if not better diffraction from previous data collections.

Suitable ice density

- Enlargement of previous image
- Blue arrows indicate examples of suitable ice density
- Note that almost every hole for this grid square is suitable for recording images

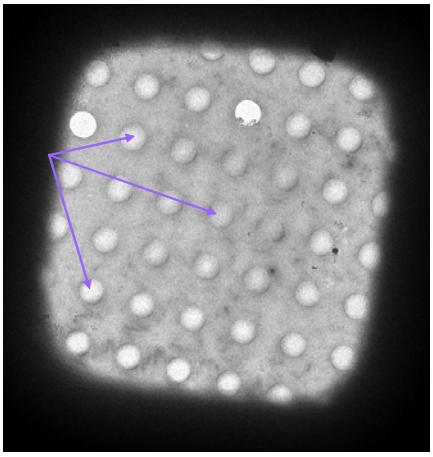


Image courtesy of Joel Quispe (National Resource for Automated Molecular Microscopy which is supported by the National Institutes of Health though the National Center for Research Resources' P41 program RR17573)
Image magnification 800X.

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Look for loading characteristics with each hole

- Enlargement of previous image
- The TMV can be seen at the edges of the holes and on the carbon
- Boxed areas were recorded at higher magnification
- Focus indicates area on the grid where focus and astigmatism corrections were made.

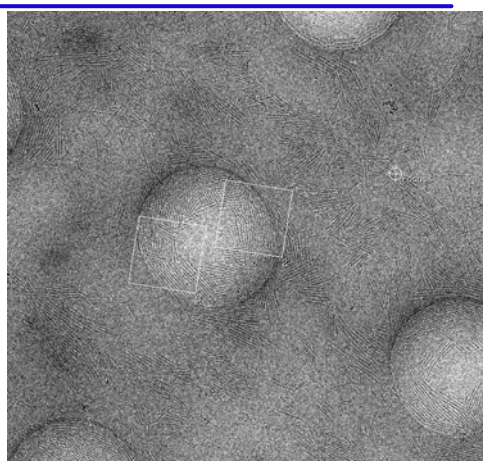

Image courtesy of Joel Quispe (National Resource for Automated Molecular Microscopy which is supported by the National Institutes of Health though the National Center for Research Resources' P41 program RR17573)
Magnification 5000X

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- The loading on this grid is very high (within and outside of the holes) because the sample concentration is 40 mg/ml. The ice is consistently thin and the resolution of individual particles of the Tobacco Mosaic Virus showed layer line diffraction to 11.5 Å

Frozen hydrated TMV

- One of the boxed areas from the previous image
- Edge of one hole in the support film showing the loading and distribution for the frozen hydrated TMV specimen
- 50,000x with a 2.0 µ defocus

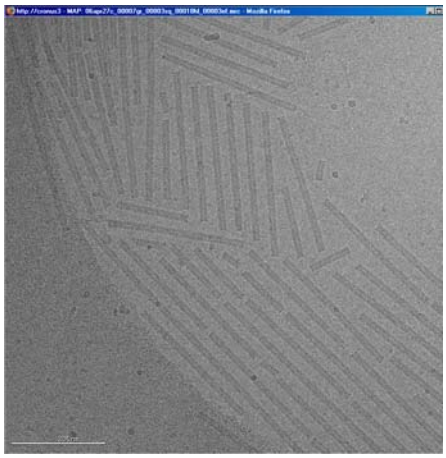

Image courtesy of Joel Quispe (National Resource for Automated Molecular Microscopy which is supported by the National Institutes of Health though the National Center for Research Resources' P41 program RR17573)

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The Solarus is a faster method for preparing the grids and makes the carbon substrate very hydrophilic, which is very important for thin ice.

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