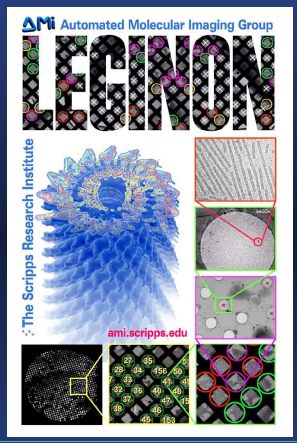


Automation I: Data Collection

Clint Potter

NRAMM CryoCourse 11/2005

THE SCRIPPS RESEARCH INSTITUTE

Automated Molecular Imaging Group:

THE SCRIPPS RESEARCH INSTITUTE

Step 1 Sample Requirements

0.6 or 1.6ml Eppendorf Tubes

FedEx Express 10:20 AM Delivery

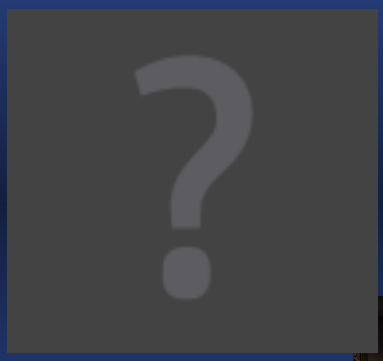
- Clean Preparation
- Protein concentration at or above 1 mg/ml.
- Buffer and salt concentration below 100mM.
- Biological pH range.
- No glycerol. If not possible, can it be dialyzed out and how long is the protein stable?
- Low concentration of sugar(s), less than 50mM.
- Low concentration of detergent(s).
- 50-100 µl of sample + 1-5 mls of dilution buffer.

Step 2

GO

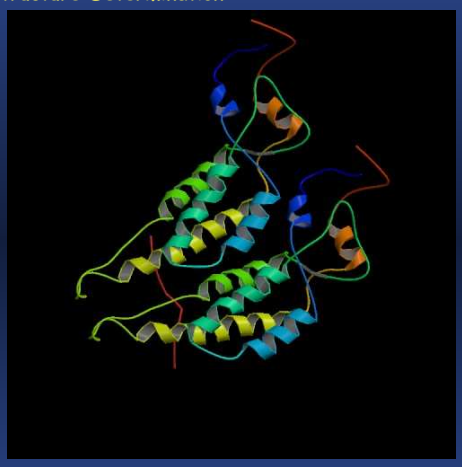
10:30 AM

Step 3 Examine Map



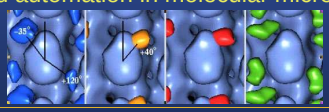
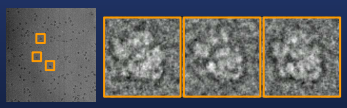
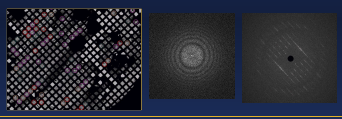
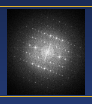
1:10 PM

Step 4 Structure Determination

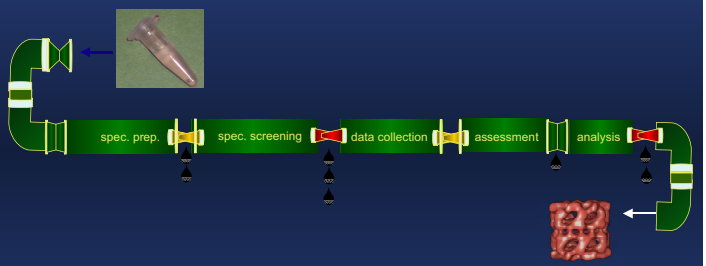


4:50 PM

Why do we need automation in molecular microscopy?

Multiple conformational states		lots of experiments
Low SNR Need averaging		lots of data
Systematic evaluation and improvement of techniques		lots of luck
Screening trials	 	lots of patience
Challenging technique		lots of skill

Automated Pipeline for Molecular Microscopy

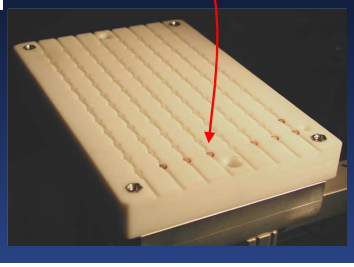


Adapted from a slide courtesy of: Peter Kuhn, Scripps-PARC Institute for Advanced Biomedical Sciences, TSRI

Specimen Screening :

Crystallization Screening Trial

Screen: 6 lipids x 8 conc., x 4 time pts = 192 grids



Robotic screening

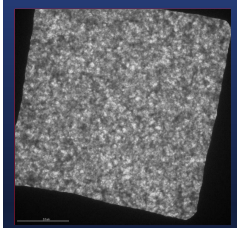
Stain not ice!

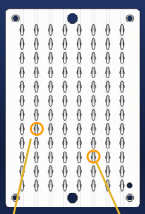
Throughput goal: ~100 grids per day

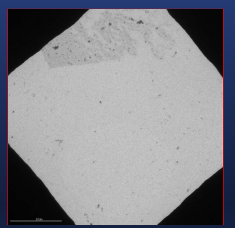
Potter, et al. JSB, 2004

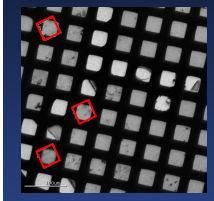
2D Crystallization Screening Trials

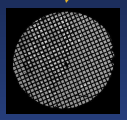
Screen: 6 lipids x 8 conc., x 4 time pts= 192 grids

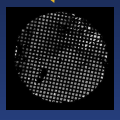


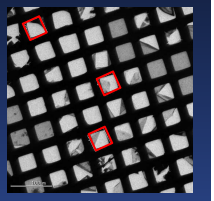






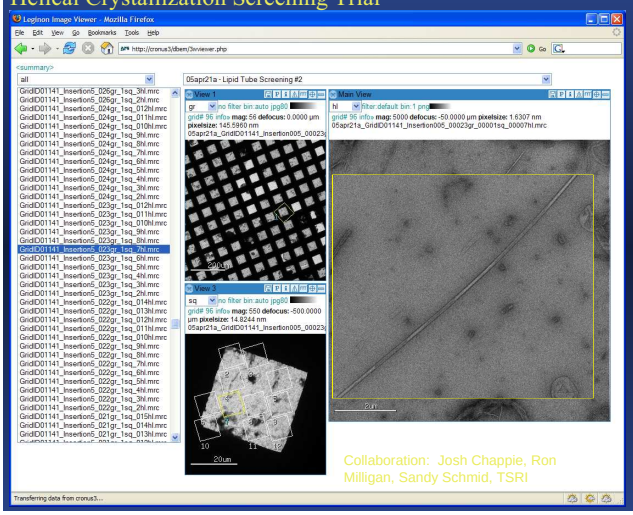






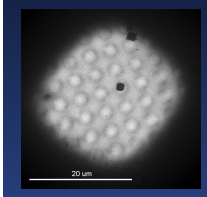
Collaboration: Holly Heaslet and David Stout, TSRI

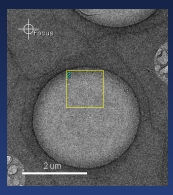
Helical Crystallization Screening Trial



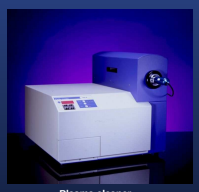
Collaboration: Josh Chappie, Ron Milligan, Sandy Schmid, TSRI

Specimen Preparation



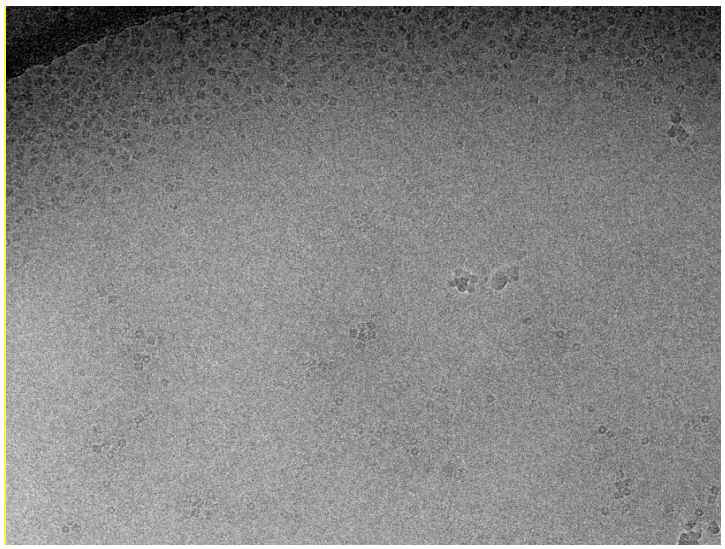


http://cimbio.scripps.edu/misc/documentation/protocol/freeze.php



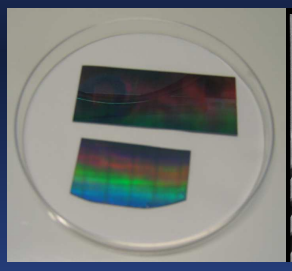
Plasma cleaner

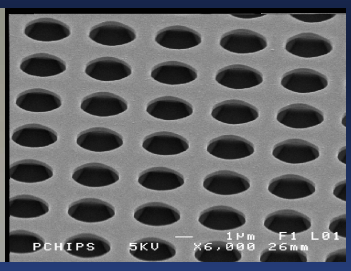
Vitrobot



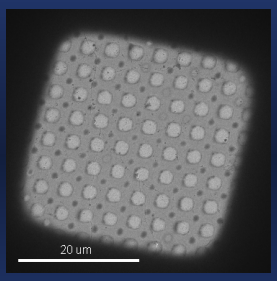
New technique for making an array of holey carbon film

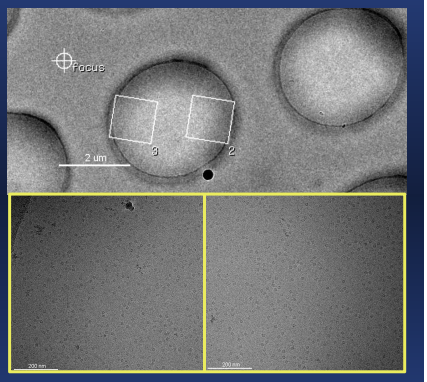
- Use a silicon-nitride template with regularly spaced wells. Developed by the Protochips Co. We are currently working with a template similar to the R2/4 from Quantifoil, but many different arrays and holes can me made.
- Make a carbon replica of the template using Victawet as a releasing agent.

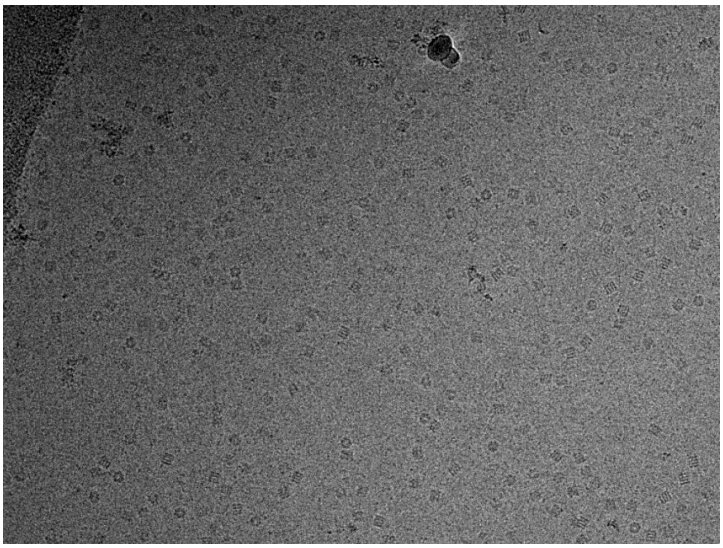




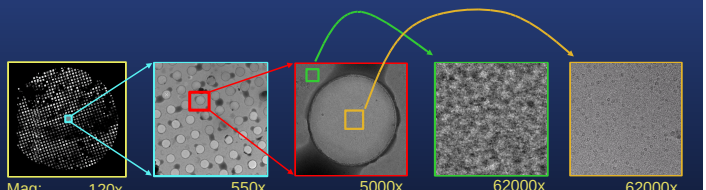
Vitreous ice across JAHC's







Automated image acquisition: A mutiscale targeting problem

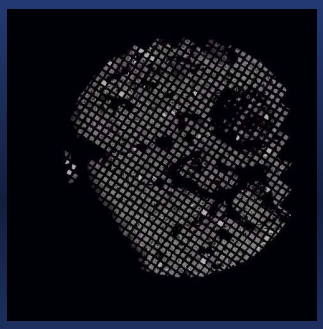


Mag:	120x	550x	5000x	62000x	62000x
Dose:	~0e⁻/Å²	~0.003e⁻/Å²	~0.3e⁻/Å²		~10e⁻/Å²

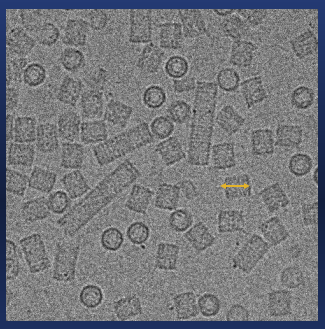
Grid	Grid square	Hole	High mag image
2mm x 2mm	~500 squares/grid 20μm x 20μm	~50 holes / square 2μm x 2μm	4K CCD: ~6 / hole 0.5 μm x 0.5 μm

~25,000 holes / grid
~150,000 high magnification target areas / grid

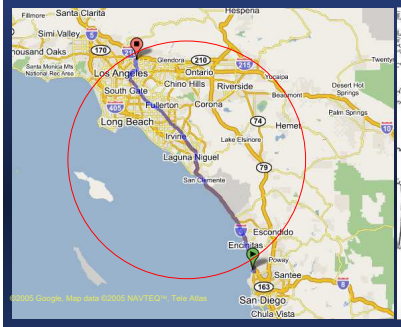
Total dose allowed: ~10 e⁻/Å²



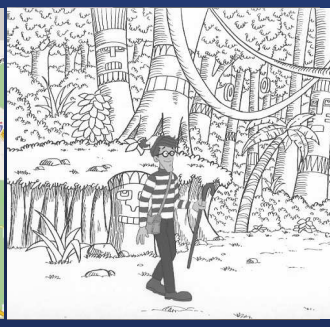
grid: ~2 mm



particle : ~20 nm



Southern California: ~200 km



Waldo (~2m)
(1m)

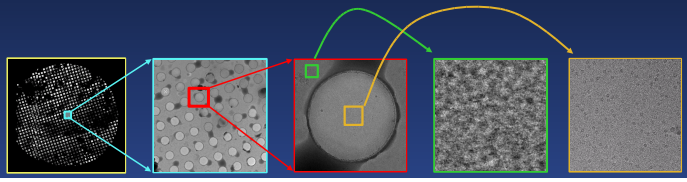
Accurate targeting across a magnification range of ~600 requires accurate and stable calibrations, precisely defined relationships between preset magnification settings, and well defined targeting parameters.

Calibrations:

- Magnification/Pixel Size
- Dose rate
- Goniometer
- Image/beam tilts and shifts
- Focus

Presets:

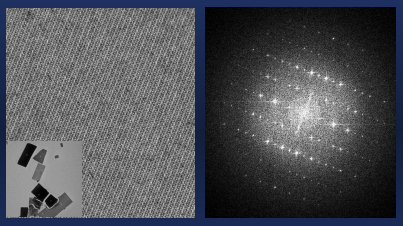
- Atlas
- Square
- Hole
- Focus
- Image



Calibrations: Magnification/Pixel Size @ Specimen

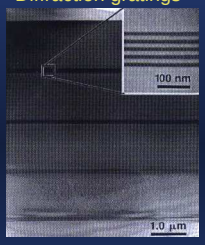
Calibration standards:

Crystals (catalase / TMV)



Wrigley, N. G. The lattice spacing of crystalline catalase as an internal standard of length in electron microscopy. *J Ultrastruct Res* 24, 454-64 (1968).

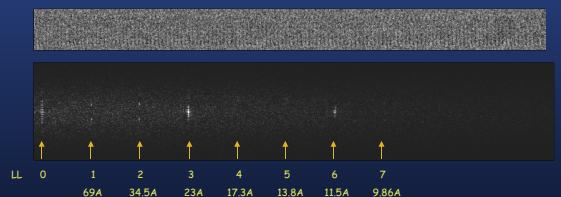
Diffraction gratings



Mag*I*Cal Standard

* Good catalase crystal prep protocol at http://www.itg.uiuc.edu under tech reports.

Example of calculating pixel size from known diffraction spot:



Given: TMV LL3 (23A) diffraction is 295 pixels from LL0. Filament has a length of 3072 pixels.

-> Pixel size at specimen= 23Å (295/3072)= 2.21Å

Microscope Nominal Magnification Setting: 62,000X

Camera pixel size is 24µm.

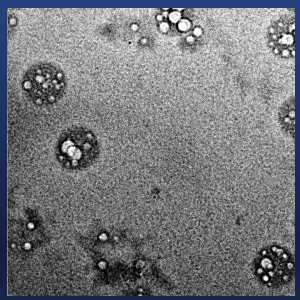
-> Measured magnification is 109,000X (24µm/2.21Å) Additional post magnification of ~1.7X due to camera extension flange.

Calibrations: Dose -> Beam Intensity

measured at the specimen
dose in $e^-/Å^2$
dose rate in $e^-/Å^2/sec$
1 amp = $6.25x10^{18}$ e^-/s

Analytical Holder w/ Faraday Cup

Screen Current

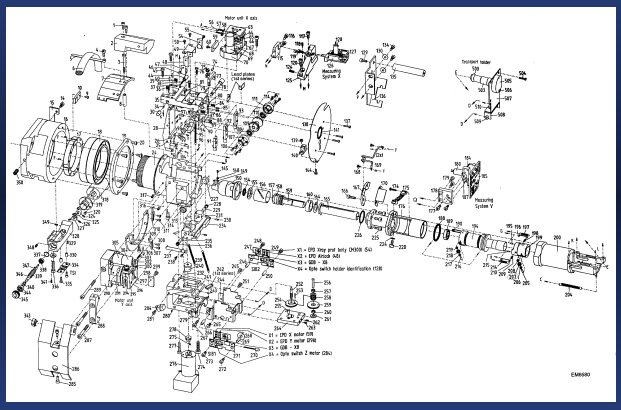


Measure dose rate from screen current:
Dose rate ($e^-/Å^2/s$) = (K* B) / A

K = $6.25x10^{18}$ e^-/s
B = beam current (amps)
A = Area on screen (m^2)
B = C * (measured screen current)
C = correction factor (1.04 on our T1)

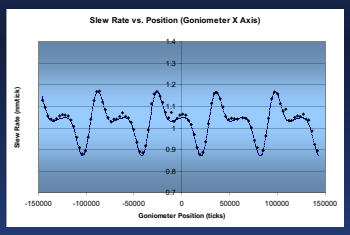
Dose measurements are critical!!

FEI Compustage

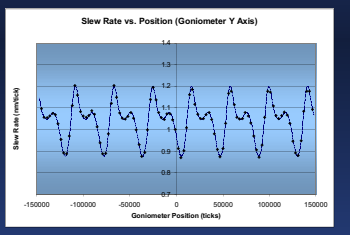


Characterization of Goniometer Slew Rate

- Measured piecewise slew rate (nm/tick) over range of goniometer (CompuStage) using image cross correlation.
- Results: 18% periodic variation over range of goniometer.
- Slew rate for X and Y axis a function of position:



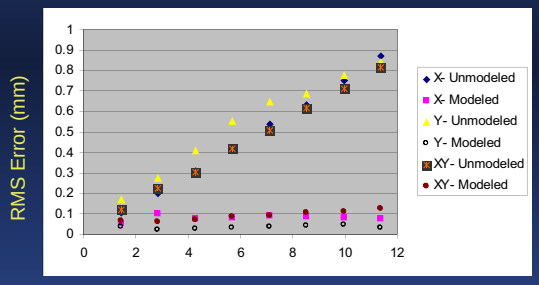
X axis (period = 61.9 mm)



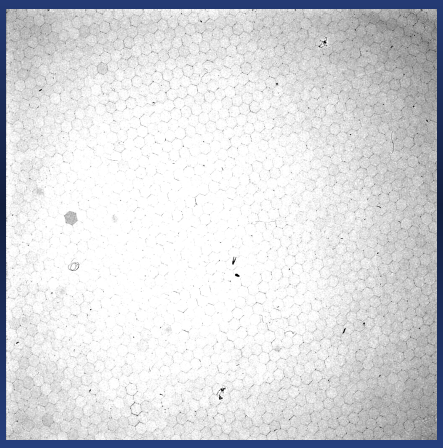
Y axis (period =41.6 mm)

Goniometer Modeling: Results

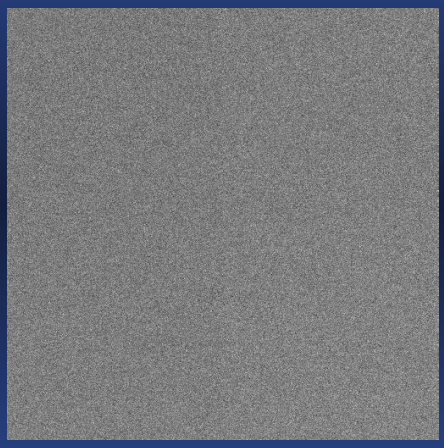
- 2 CompuStages characterized with similar results.
- Slew rate for X and Y modeled with Fourier Series
- Validation: RMS error as a function of distance moved over range of goniometer:
- Pulokas et al., J. of Struct. Biology, 128, p.250-256 (2000)



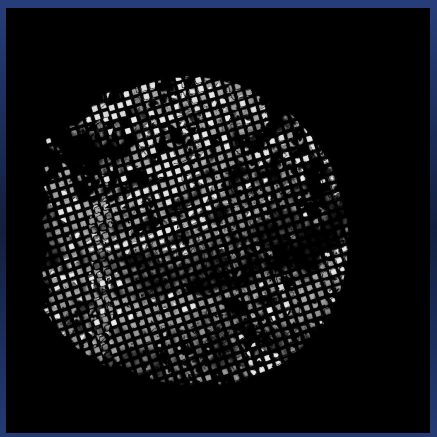
Calibrations: Gain Normalization and Flat Fielding of CCD camera



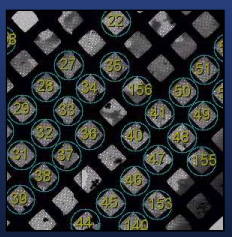
Calibrations: Gain Normalization and Flat Fielding of CCD camera

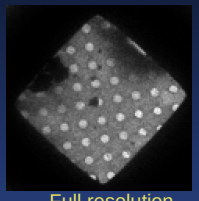


1. Constructing a grid atlas (~60x) and targeting "good" squares



Atlas typically constructed of a mosaic of 25 images (180nm / pixel)

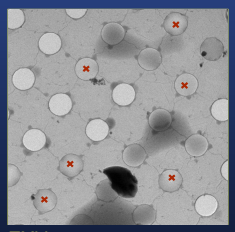




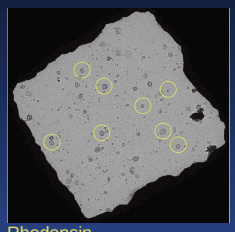
Full resolution

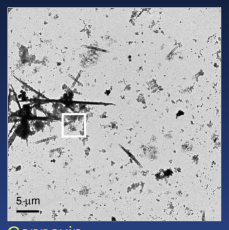
Dose accumulated: ~0 $e^-/Å^2$

2. Finding targets at low magnification (~600x)


TMV


RNA PolII

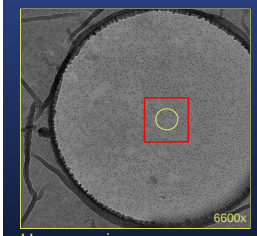

Rhodopsin



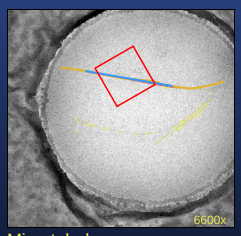
Connexin

Dose: ~0.003 e⁻/Å²

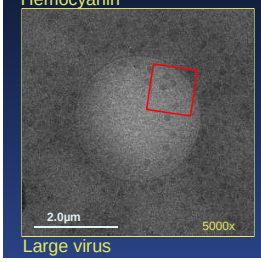
3. Finding targets at intermediate magnification (~6000x)



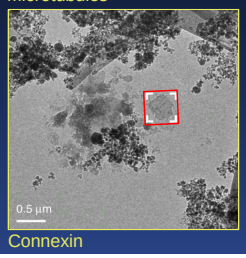
Hemocyanin



Microtubules



Large virus

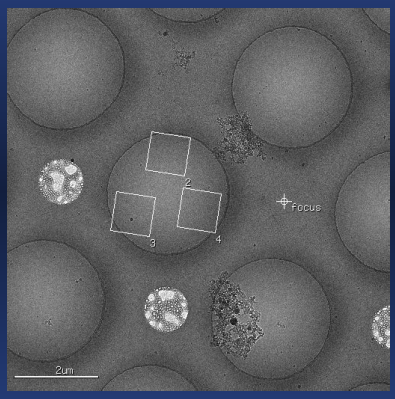


Connexin

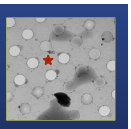
Area of acquired image at ~60,000x on 4K CCD (~2 Å/pixel)

Dose: ~0.3 e⁻/Å²

4. Low dose drift check and focus



Drift check



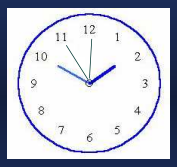


Image1(time1)

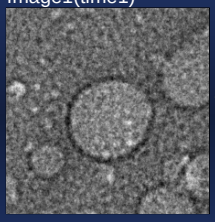


Image2(time2)

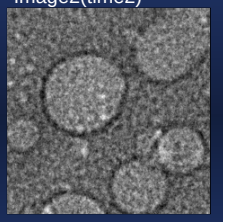
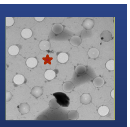


Image displacement

Image displacement(time) α (drift)

Focus and astigmatism measure



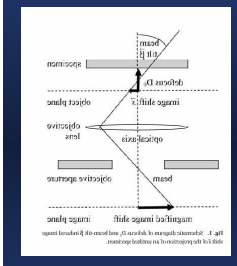


Image1(tilt1)

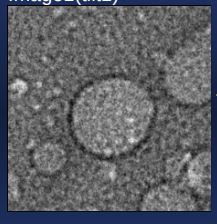


Image2(tilt2)

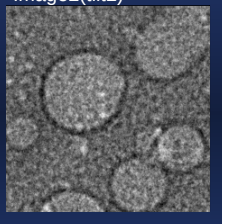


Image displacement

Ziese et al, J. Microsc. 211, 179 (2003)
Koster and de Ruijter, Ultramicroscopy, 40, 89 (1992)

Image displacement(beam tilt) α (defocus)

Drift measurement using cross correlation

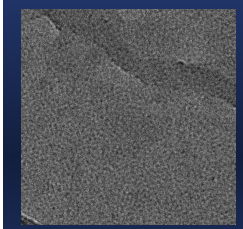


Image1

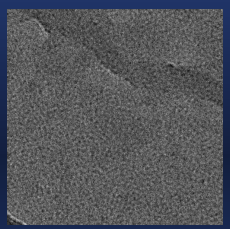


Image2


CC

Typical drift tolerance: < 2 Å/s w/ 0.5 s exposure

Autofocus Technique:

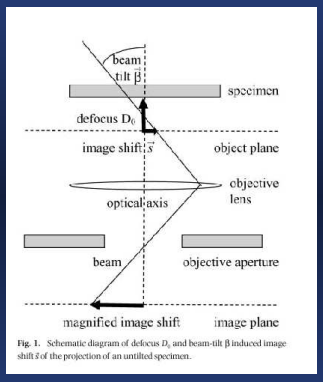


Fig. 1. Schematic diagram of defocus D_0 and beam-tilt β induced image shift δ of the projection of an untilted specimen.

Determine defocus by measuring the image shift that is given by the cross correlation of two images acquired with different beam tilts.

$S = D_0 \cdot \tan(B)$

Reference:

Koster and de Ruijter, Ultramicroscopy, 40, 89-107 (1992)

Ziese et al, J. Microscopy 211, 179-185 (2003)

Autofocus accurate to within ~150nm

Defocus = 2.1 +/- 0.13 µm

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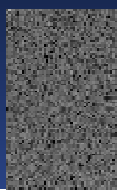
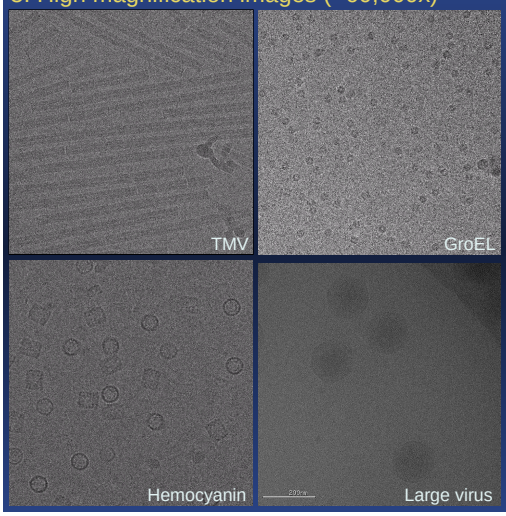


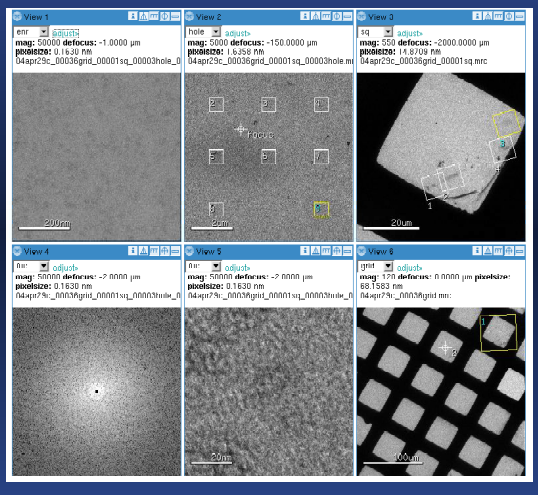
5. High magnification images (~60,000x)

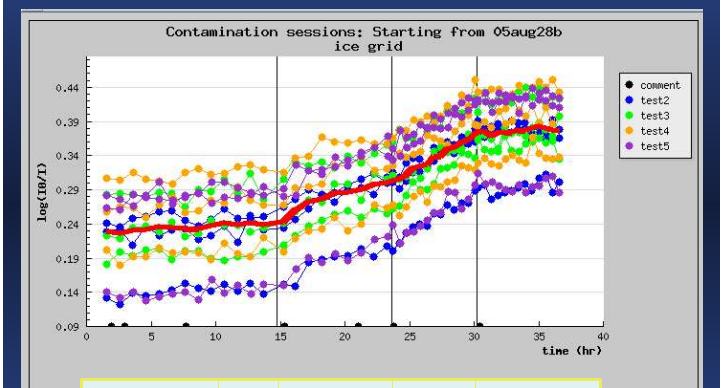


Acquired as defocus pairs or sequences

Total dose: ~ 10 e/A^2

Leginon Observer Interface (LOI)





Film	-	+	+	-
Aperture	+	+	-	+

Leginon Database: Images and Acquisition Parameters
- Multi-scale: Keeps track of relationships between scales.