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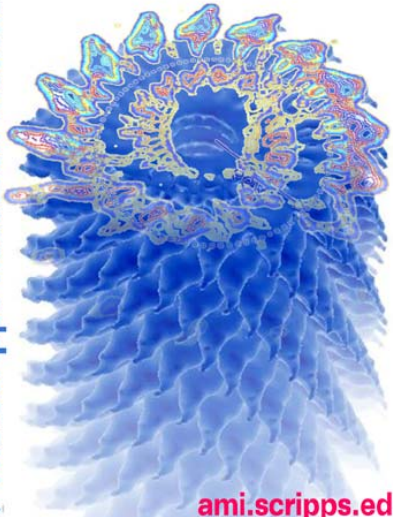
Fax: (858) 784-9090

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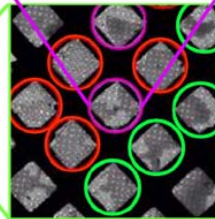
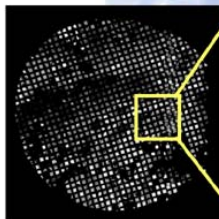
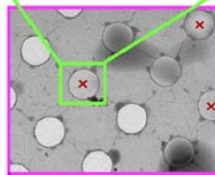
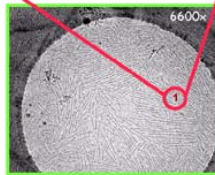
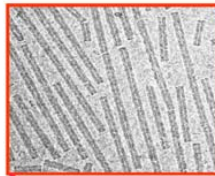
AMi Automated Molecular Imaging Group

LEGION

The Scripps Research Institute



[ami.scripps.edu](http://ami.scripps.edu)



## Automation in Molecular Microscopy

Clint Potter  
Bridget Carragher



## Step 1 Sample Requirements



0.6 or 1.6ml Eppendorf Tubes

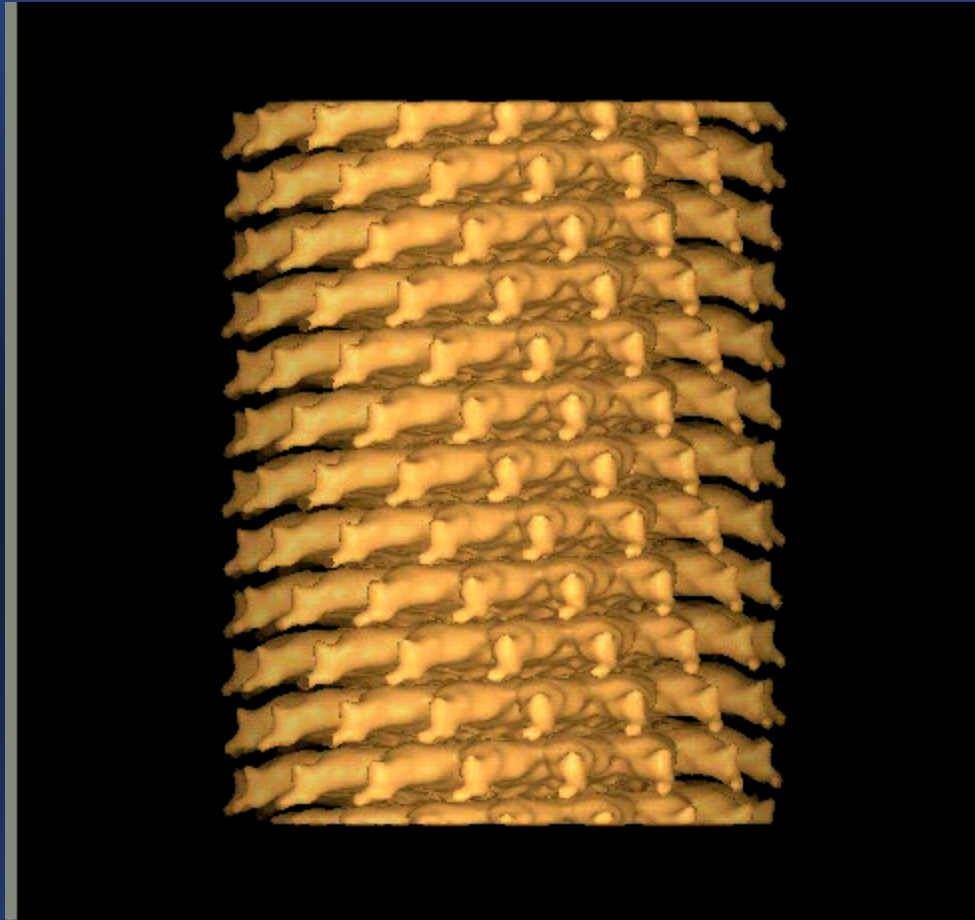
- Protein concentration at or above 1 mg/ml.
- Buffer and salt concentration below 100mM.
- Biological pH range.
- No glycerol. If not possible, can it be dialyzed out and how long is the protein stable?
- Low concentration of sugar(s), less than 50mM.
- Low concentration of detergent(s).
- 50-100  $\mu$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

## Step 2

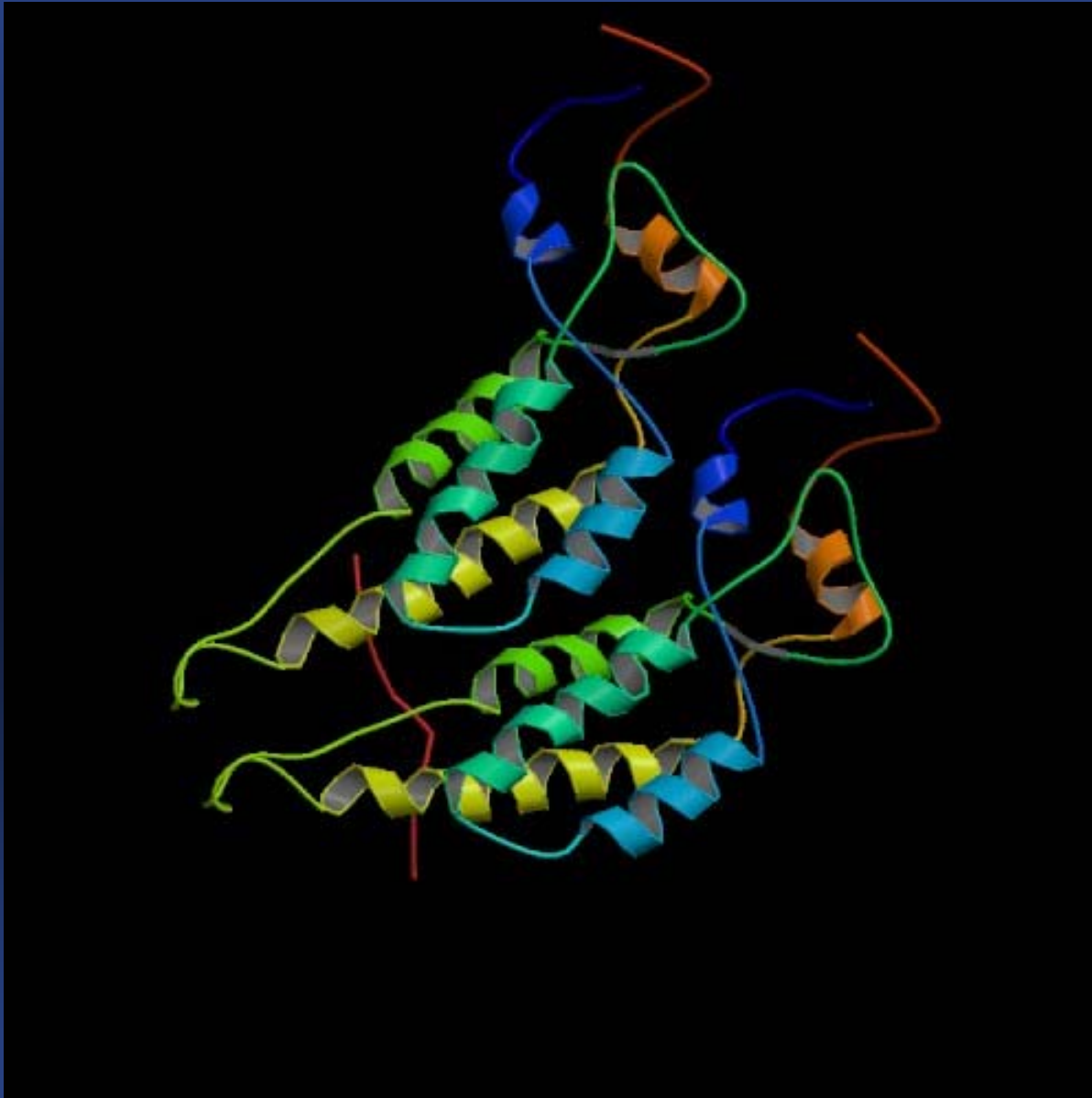


**GO**

## Step 3 Examine Map



## Step 4 Atomic Model



Where can we automate?

Specimen preparation

Specimen handling

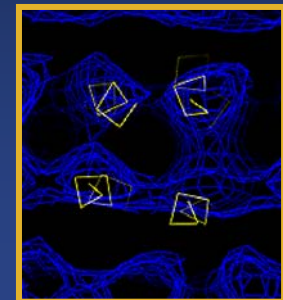
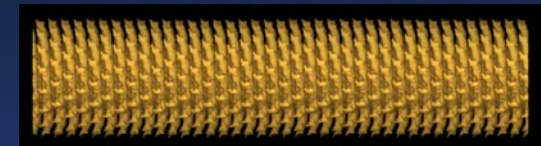
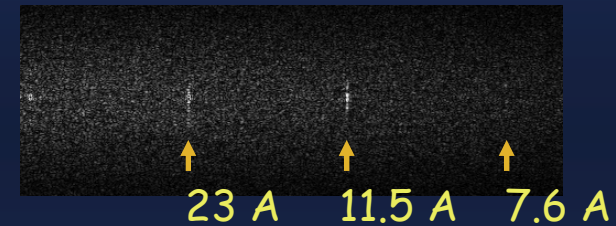
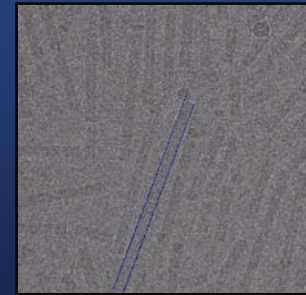
Data Collection

Image Evaluation

Boxing out

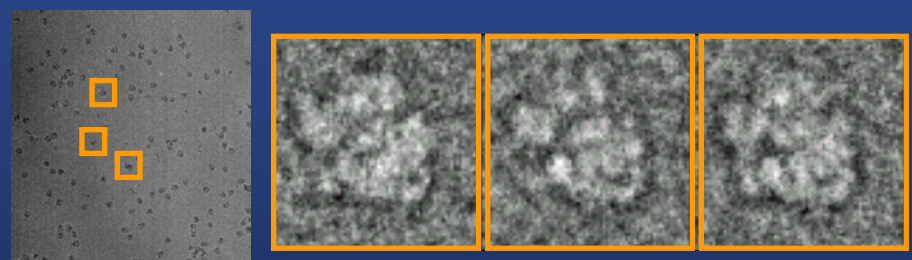
Processing (2d, icosahedral, helical, SP)

Fitting



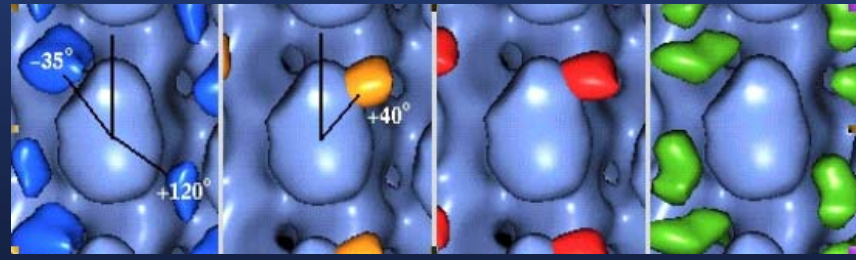
# Why do we need automation?

Low SNR  
Need averaging



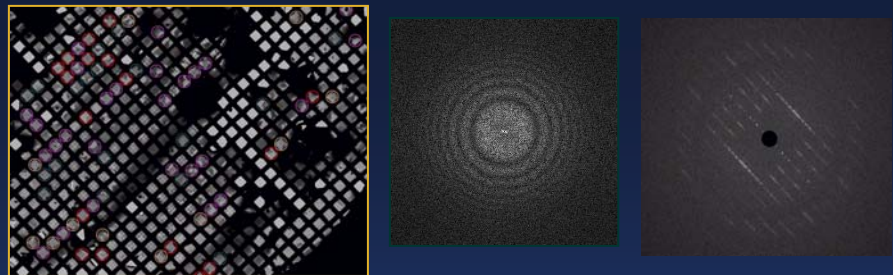
lots of data

Multiple conformational states



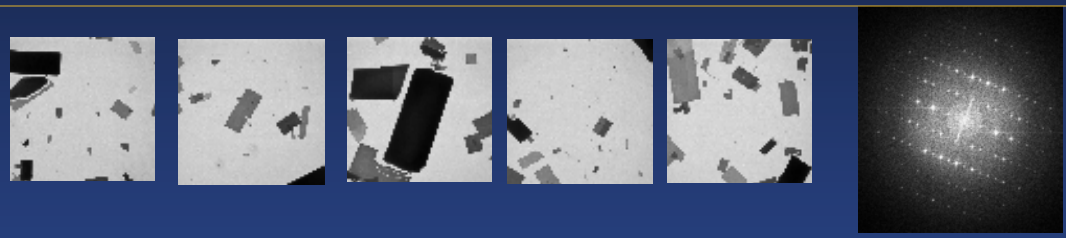
lots of experiments

Systematic evaluation and improvement of techniques



lots of luck

Screening trials



lots of patience

Challenging Technique

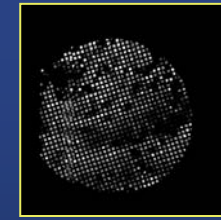
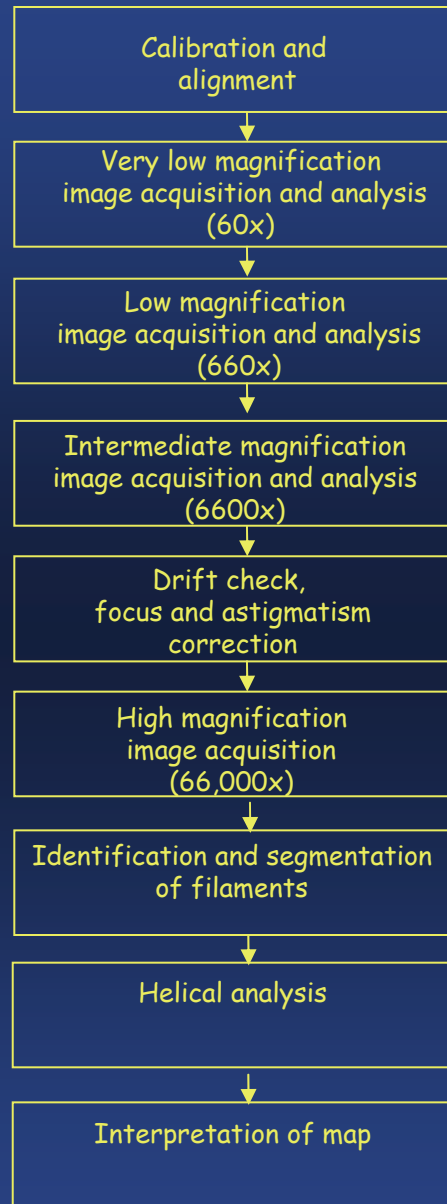


lots of skill

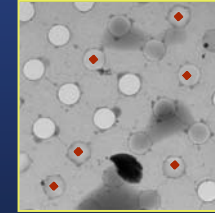


# Automated TMV data collection and reconstruction

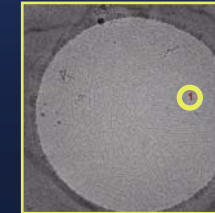
- Grid to map in <24 hours
- No user intervention except for refilling cryogenics



- Survey grid
- Identify potential grid squares
- Dose = 0



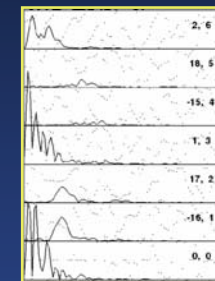
- Identify location of holes
- Estimate ice thickness
- Dose = 0.001 e/A<sup>2</sup>



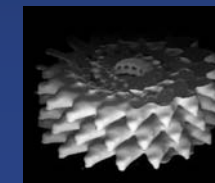
- Estimate probability of specimen
- Identify location of best specimen
- Dose = 0.1 e/A<sup>2</sup>



- Identify and segment filaments
- Dose = 10 e/A<sup>2</sup>

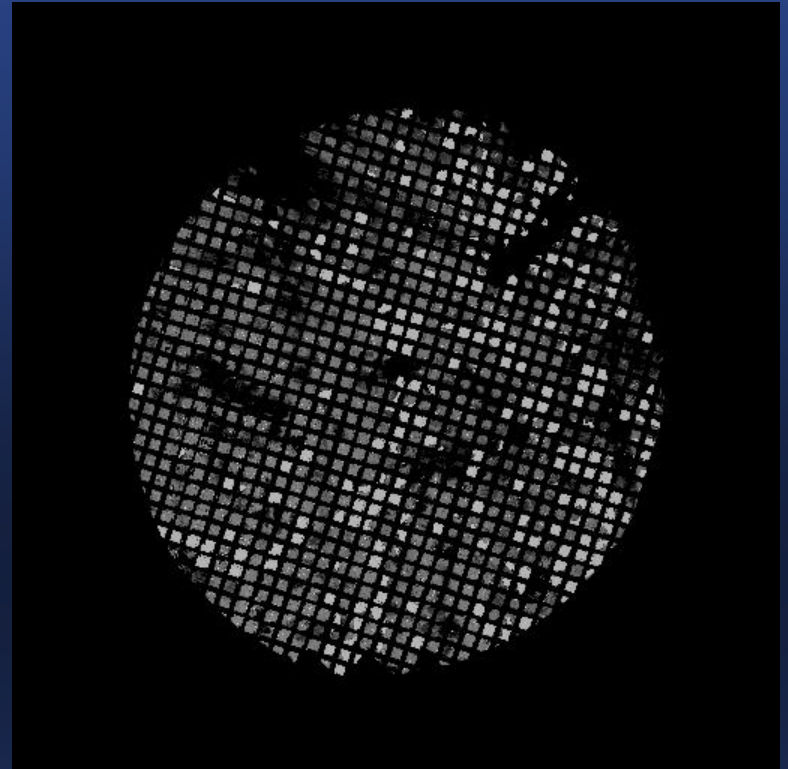


- Find layer lines
- CTF correction
- Tilt and shift correction
- Fitting and averaging

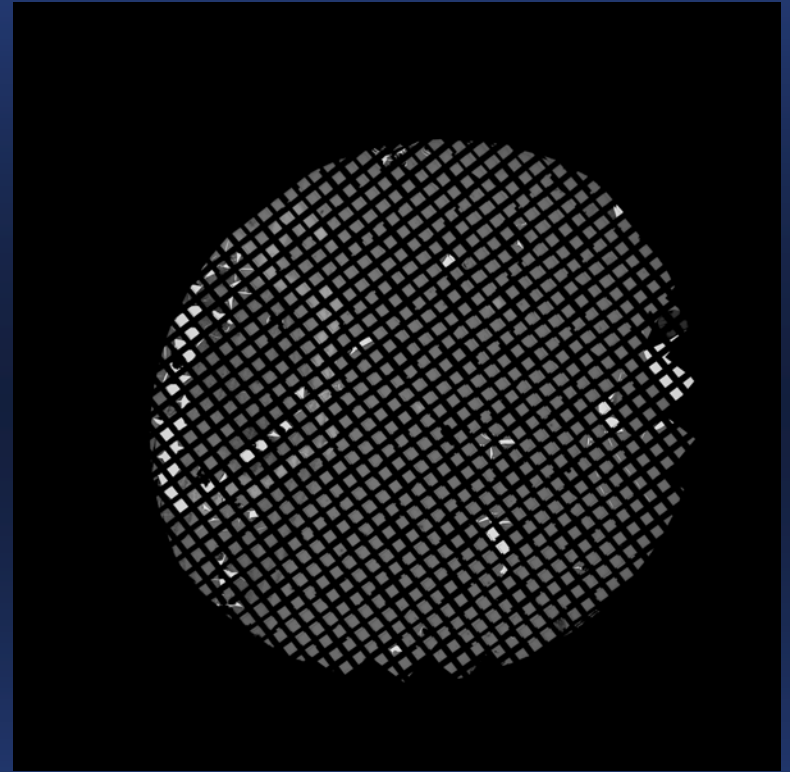
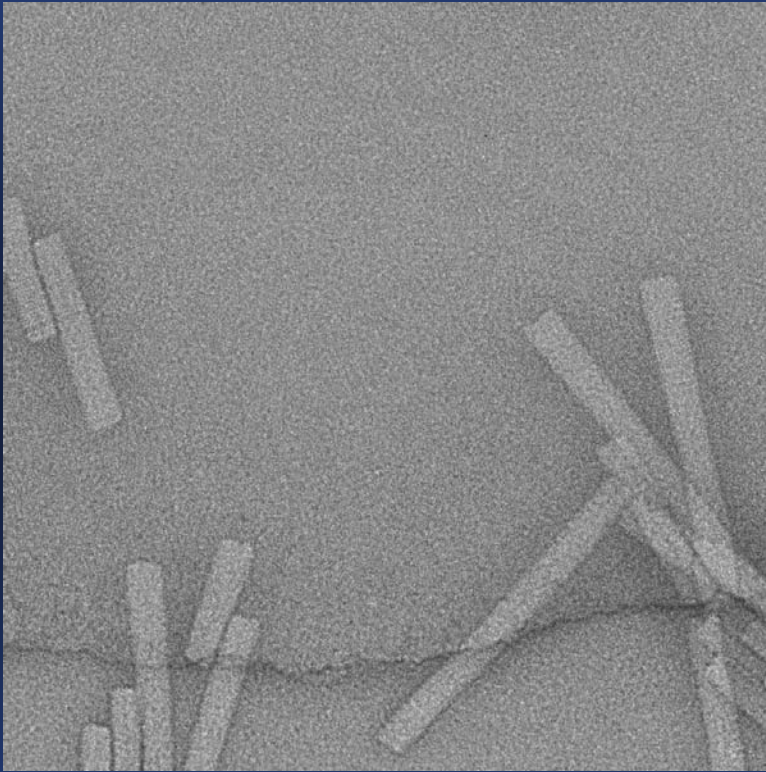




3mm



2mm



TMV in negative stain.  
180 Å diameter.



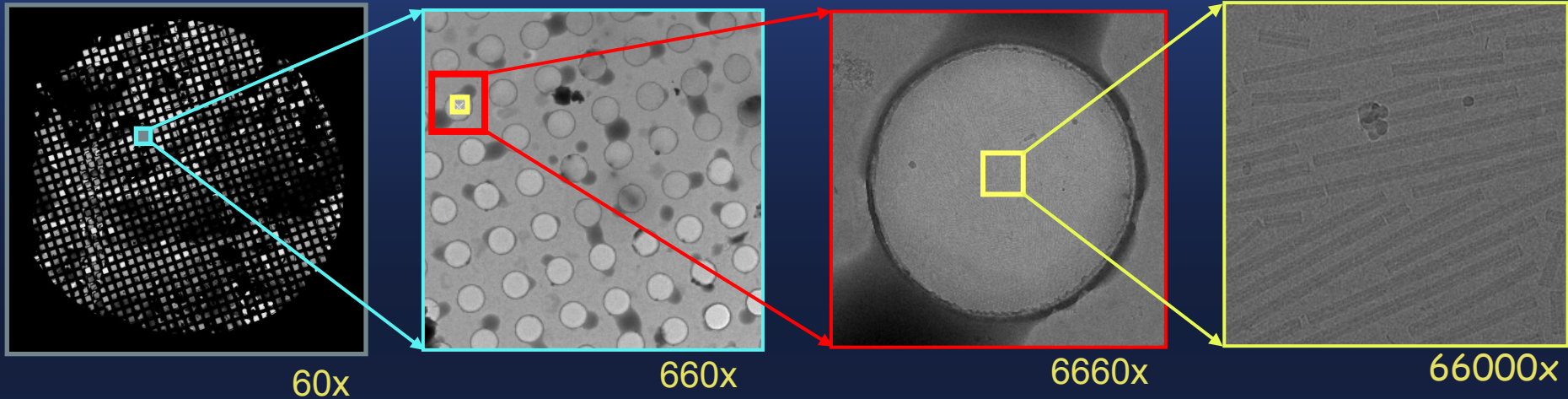
Son (1m) and Father (2m)



San Diego-Santa Monica (222Km)

# Automated cryoEM: A multiscale data collection problem

Total dose allowed:  $\sim 10 \text{ e}^-/\text{\AA}^2$



Grid  
2mm x 2mm

Grid square  
 $\sim 500$  squares/grid  
 $20 \mu\text{m} \times 20 \mu\text{m}$

Hole  
 $\sim 50$  holes / square  
 $2 \mu\text{m} \times 2 \mu\text{m}$

High mag image  
2K CCD:  $\sim 10$  / hole  
 $0.5 \mu\text{m} \times 0.5 \mu\text{m}$

25,000 holes / grid  
250,000 high magnification target areas / grid

Automation requires calibration:

Magnification/Pixel Size

Dose rate

Goniometer

Image/beam tilts and shifts

Focus

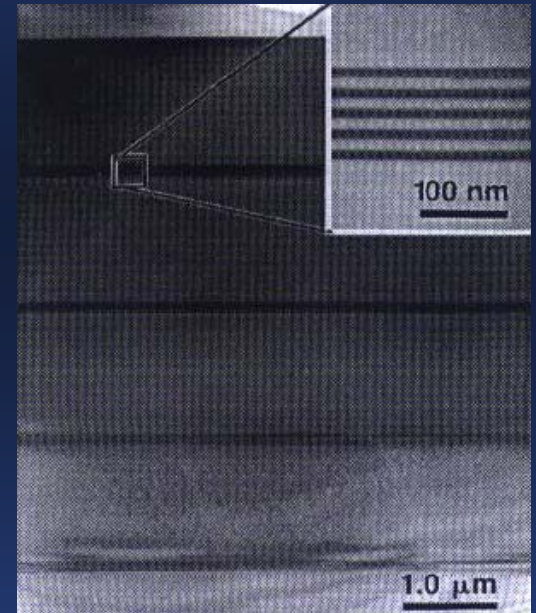
## Calibrations: Magnification/Pixel Size

Pixel size at specimen= (pixel size of camera/ magnification)

Standards for calibration:

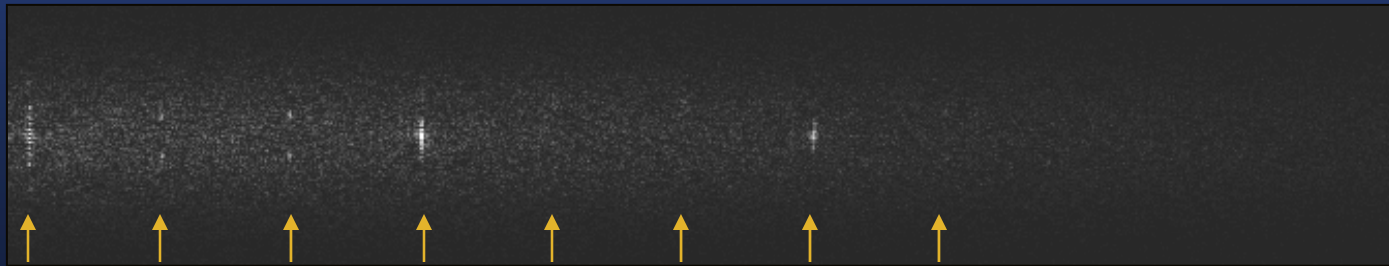
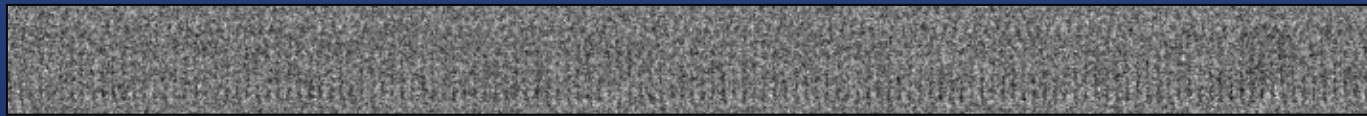
Diffraction gratings

Crystals: TMV, Catalase



Mag\*I\*Cal  
Standard

## Example of calculating pixel size from known diffraction spot:



LL 0 1 2 3 4 5 6 7  
69Å 34.5Å 23Å 17.3Å 13.8Å 11.5Å 9.86Å

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

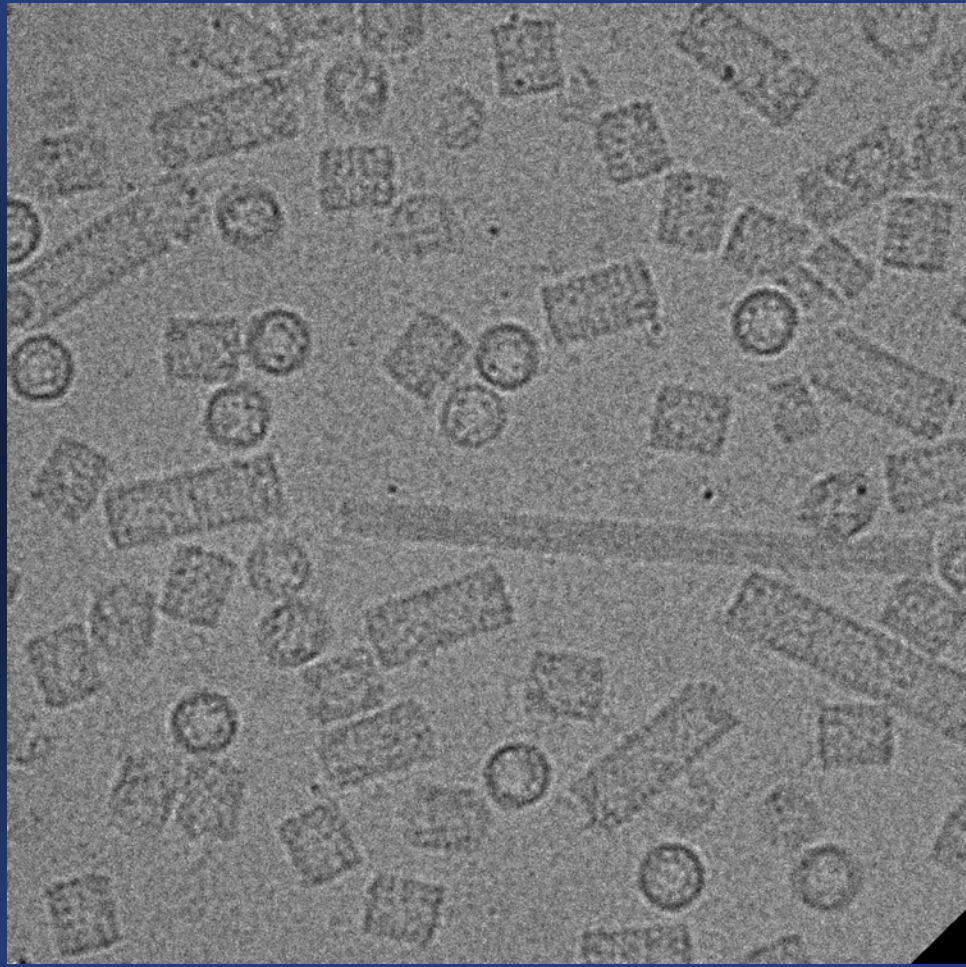
-> Pixel size at specimen =  $23\text{Å} (295/3072) = 2.21\text{Å}$

Microscope Nominal Magnification Setting: 62,000X

Camera pixel size is  $24\mu\text{m}$ .

-> Measured magnification is 109,000X ( $24\mu\text{m}/2.21\text{Å}$ )  
Additional post magnification of  $\sim 1.7\text{X}$  due to camera extension flange.





## Calibrations: Dose/ Beam Intensity

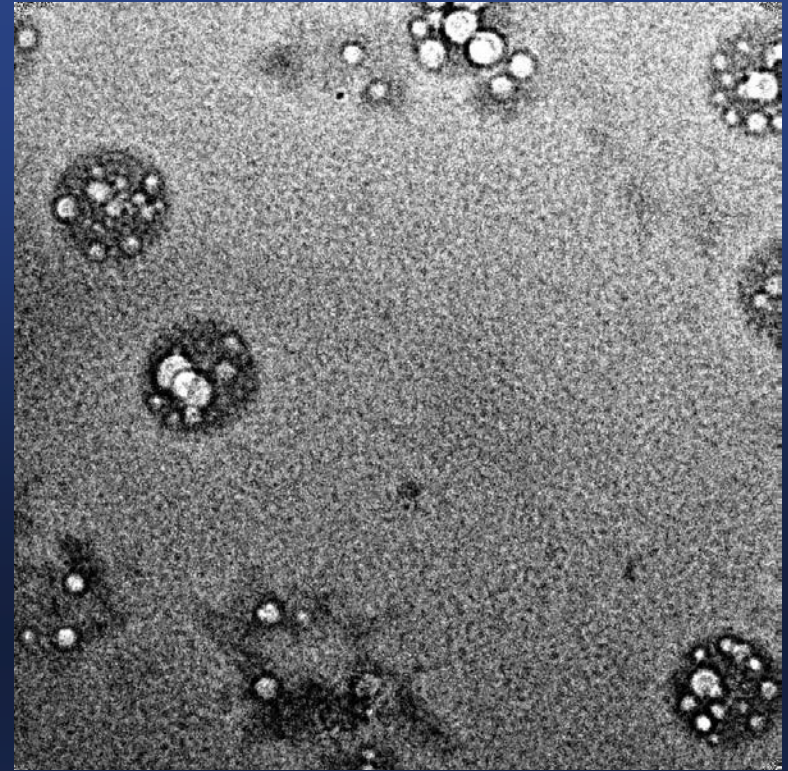
measured at the  
specimen

$$\text{Dose} = e^-/\text{\AA}^2$$

$$1 \text{ amp} = 6.25 \times 10^{18} e^-/s$$

Analytical Holder w/  
Faraday Cup

Screen Current



Dose  
measurements  
are critical!!



*Gatan Analytical Holder w/ integral Faraday Cup  
Picoammeter*

Measure dose rate from screen current:

$$\text{Dose (e}^{-}/\text{\AA}^2) = (K * M^2 * B) / A$$

$$K = 6.25 \times 10^{18} \text{ e}^{-}/\text{s}$$

B = beam current (amps)

A = Area on screen (m<sup>2</sup>)

B = C \* (measured screen current)

C = correction factor (measured as 1.04 on our T1)

## Gain Normalization and Flat Fielding of CCD camera

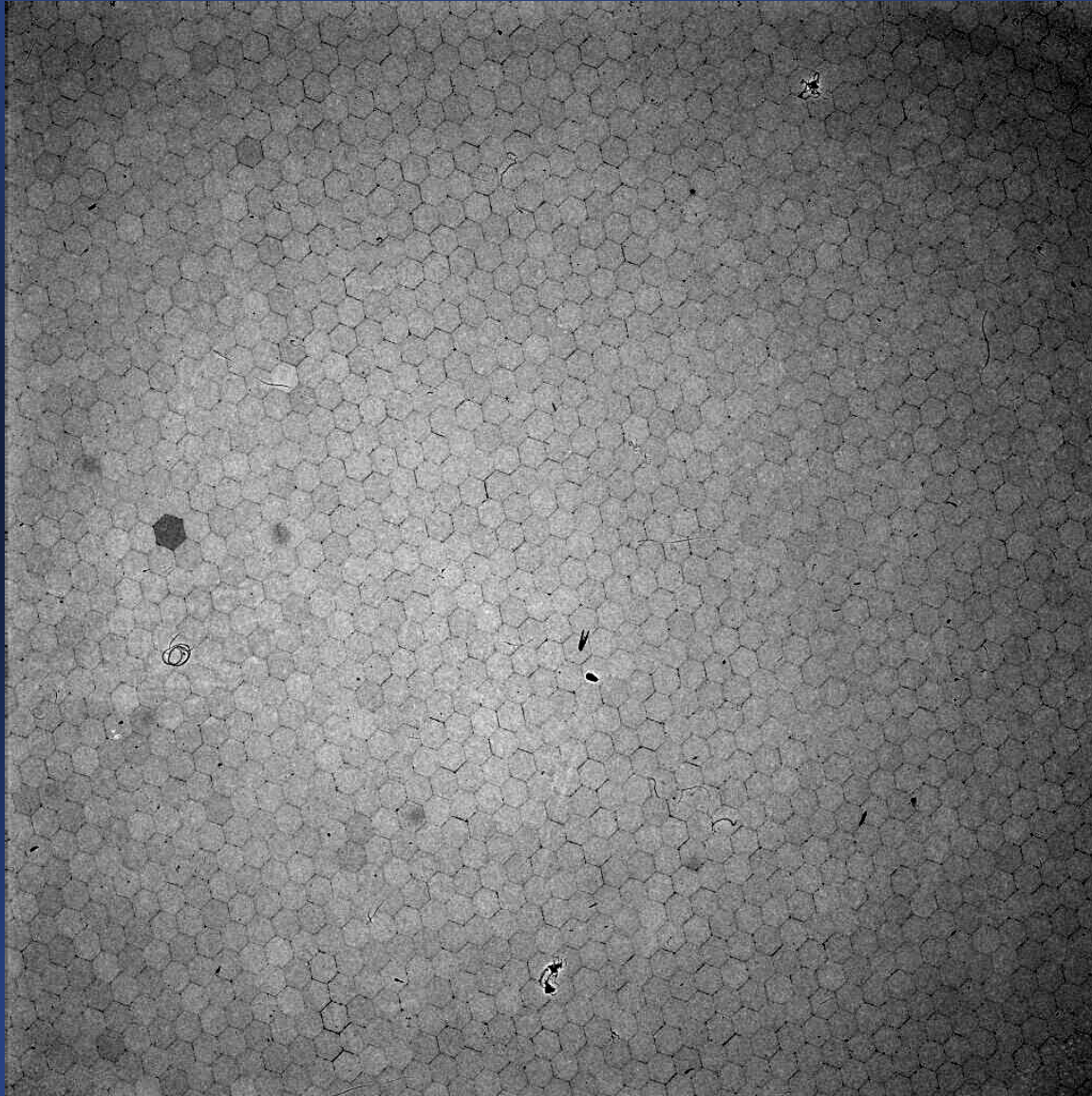
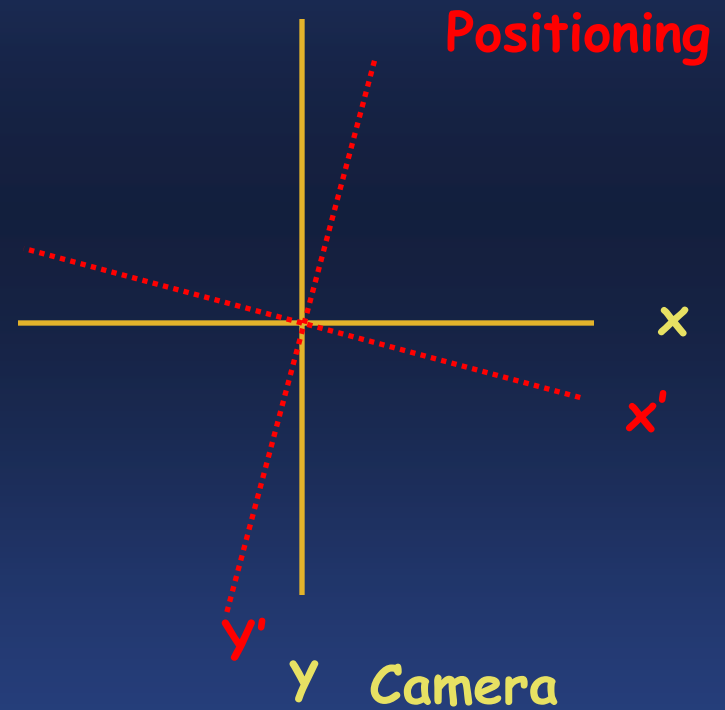
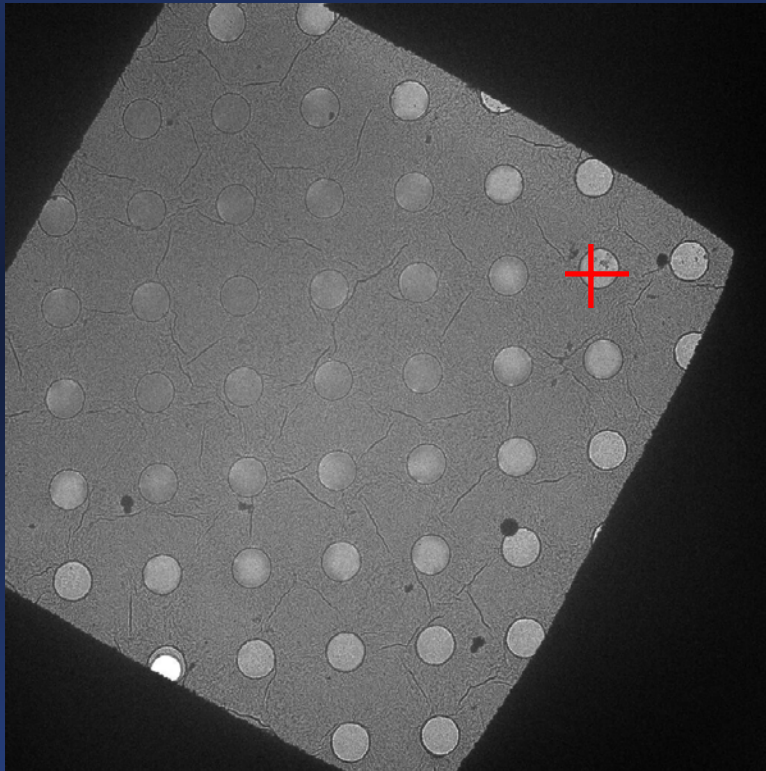
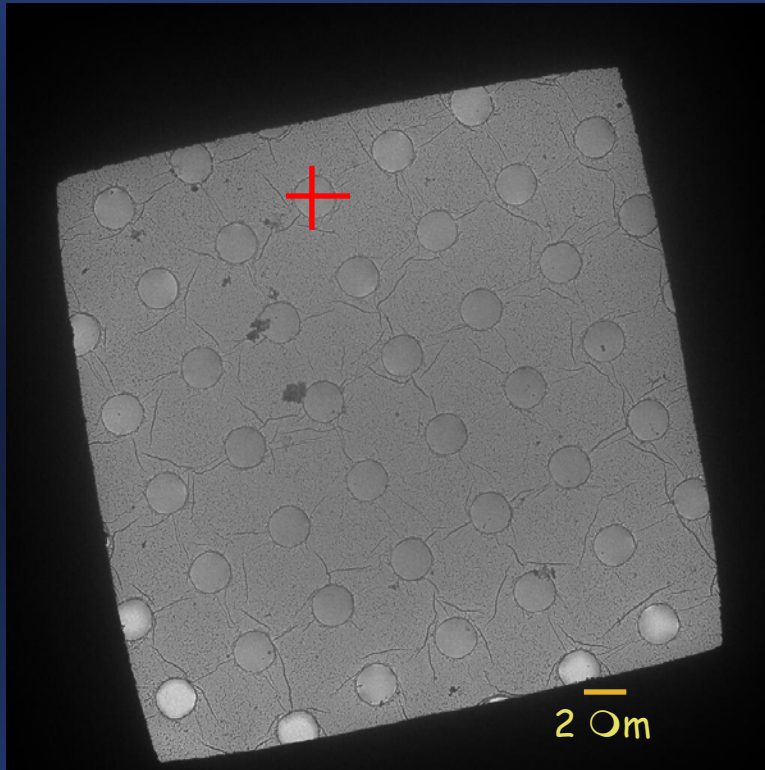


Image positioning calibration:

Calibrate positioning based on camera coordinate system.

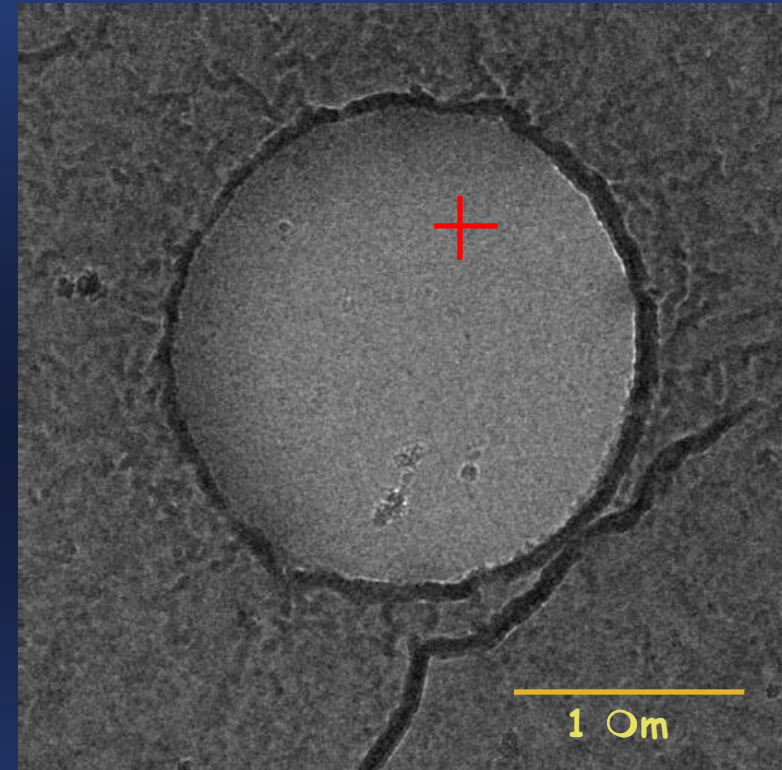


## Calibrations: Image Positioning



Use goniometer for  
moves  $> \sim 5 \mu\text{m}$

Accuracy  $\sim 100 \text{ nm}$ .

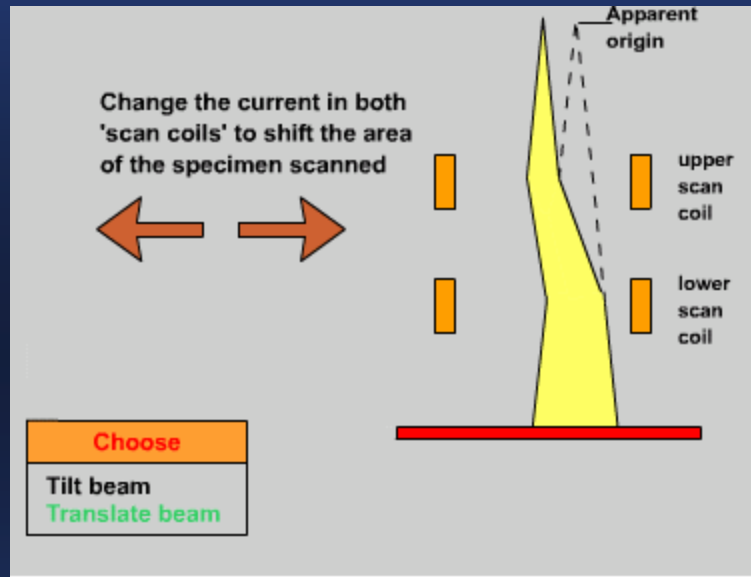


Use image shifts for  
moves  $< \sim 5 \mu\text{m}$

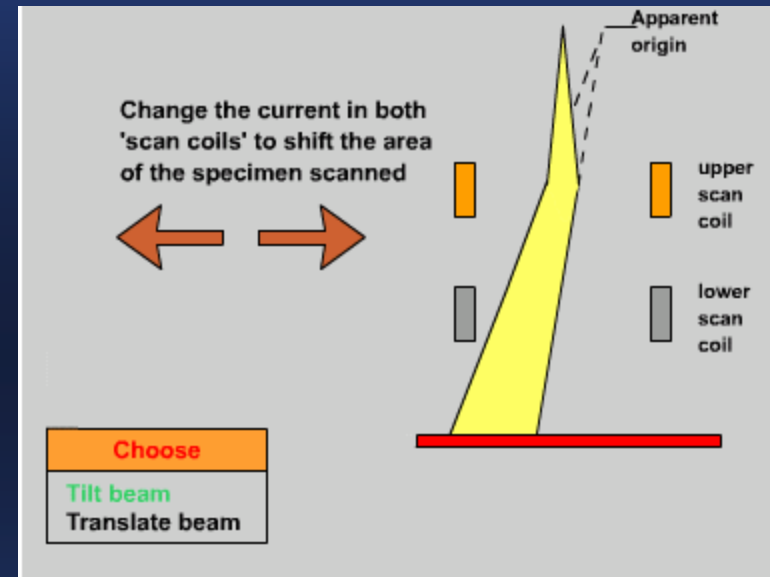
Accuracy  $\sim 1 \text{ nm}$

# Calibrations: Positioning and tilt using scan coils

## Beam translation



## Beam tilt

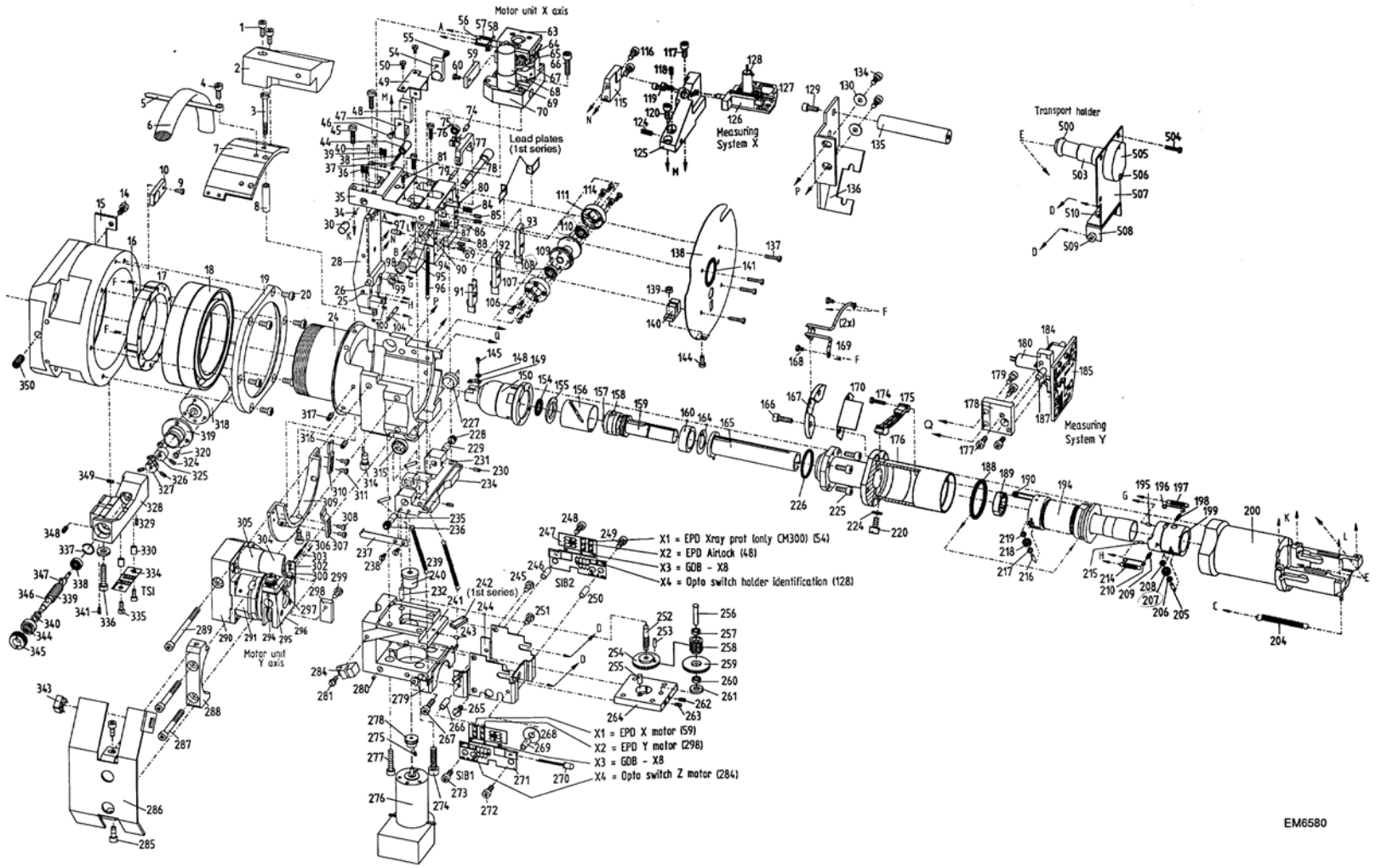


Simulations at <http://www.matter.org/tem>

Two sets of deflection coils enable translation of the beam across the specimen without apparently changing the angle of incidence or tilt the beam without changing its position on the specimen.



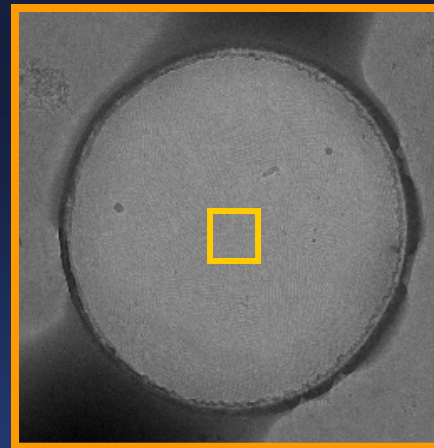
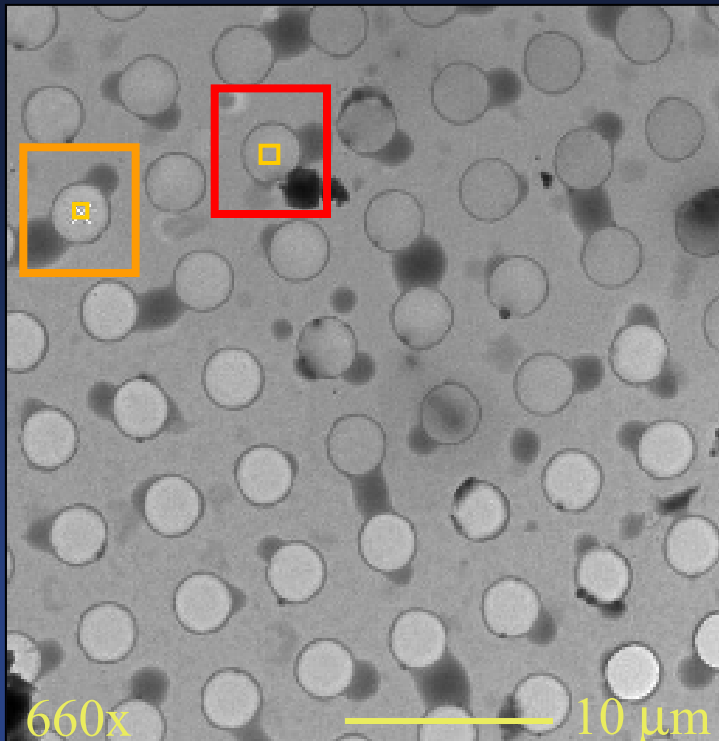
# FEI Compustage



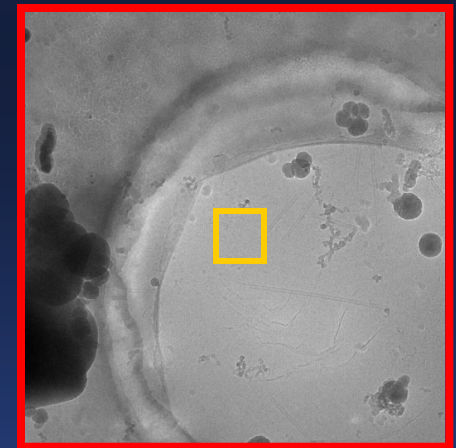
# Automation: Goniometer Positioning Accuracy

Problem: Accurately move stage to predicted location on the image.

CM200 compustage error is  $\sim 10\%$  of distance moved (1  $\mu\text{m}$  in a 10  $\mu\text{m}$  move). We need to improve this 10x.



Accurate relocation



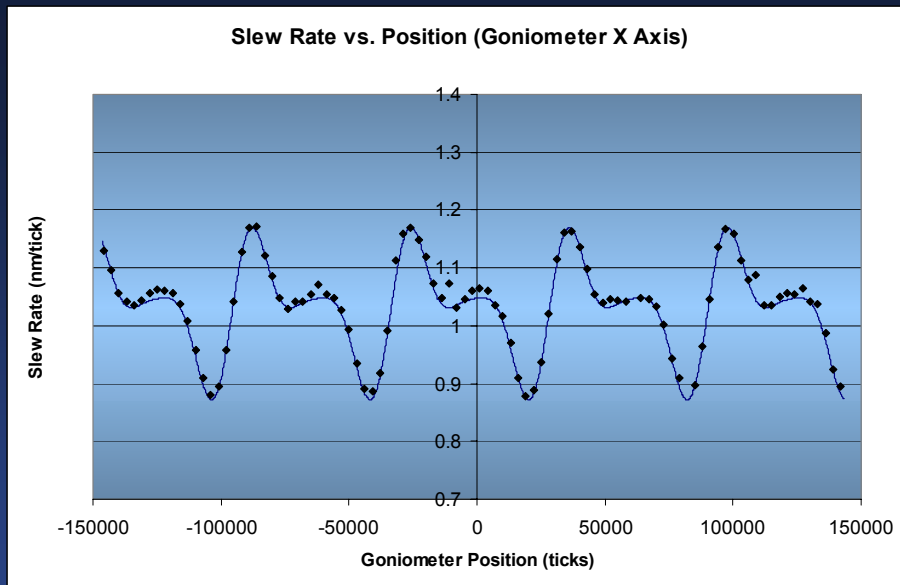
1  $\mu\text{m}$  error

□ - FOV at 38,000x on 1Kx1K camera.

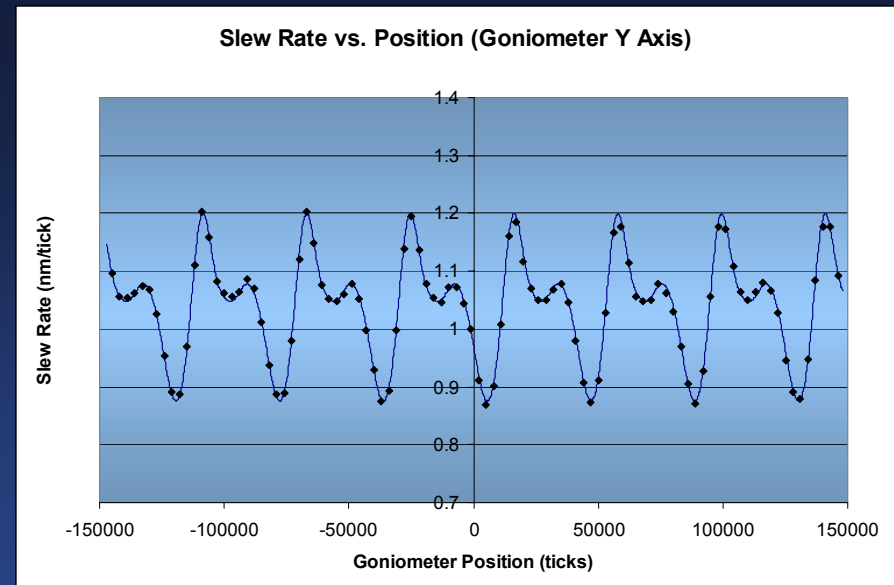


# Characterization of Goniometer Slew Rate

- 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:



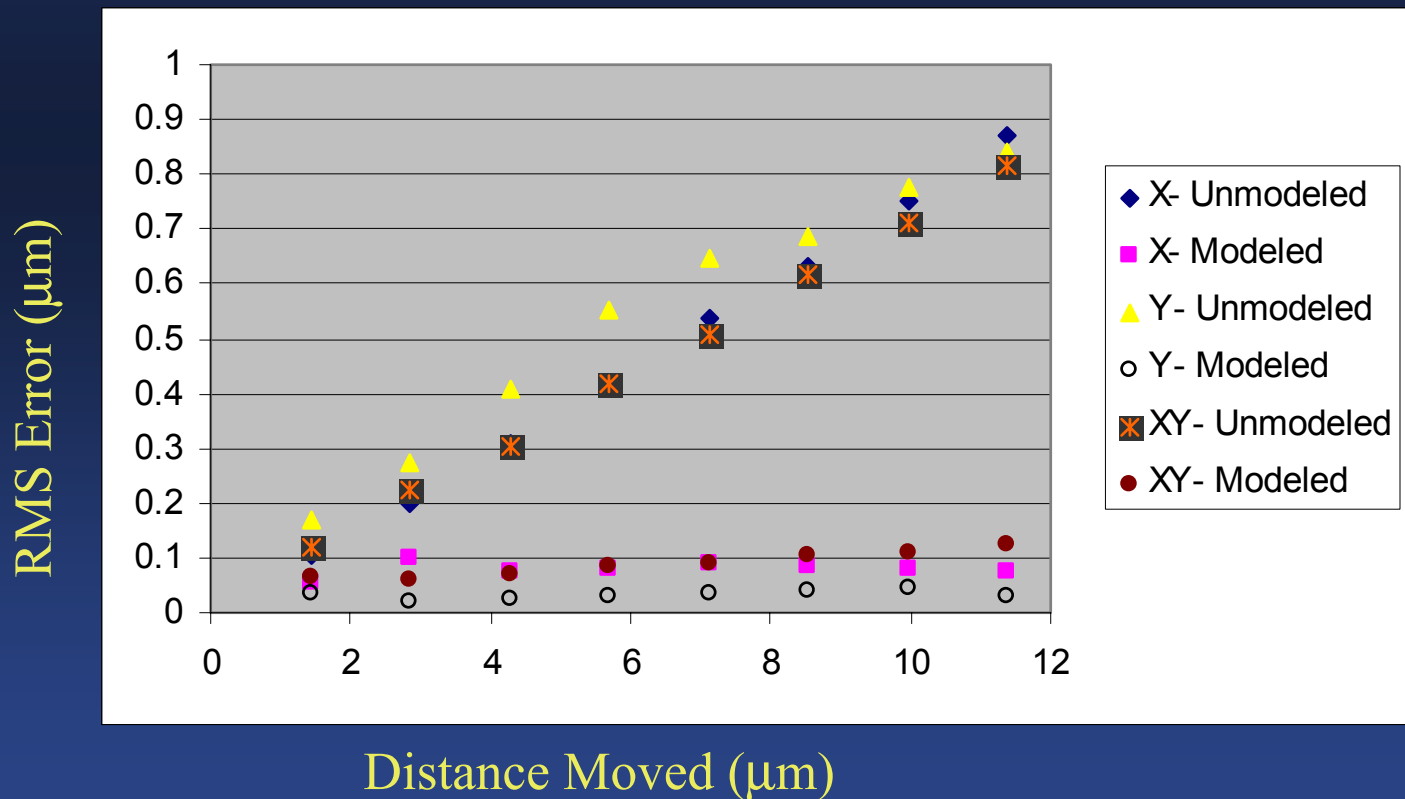
X axis (period =  $61.9 \mu\text{m}$ )



Y axis (period =  $41.6 \mu\text{m}$ )

# 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)



## Sample Preparation: Freeze using Vitrobot



Joel Quispe's baseline freezing conditions:

Temperature: 4 °C

Humidity: 100%

2 s blot

1 blot

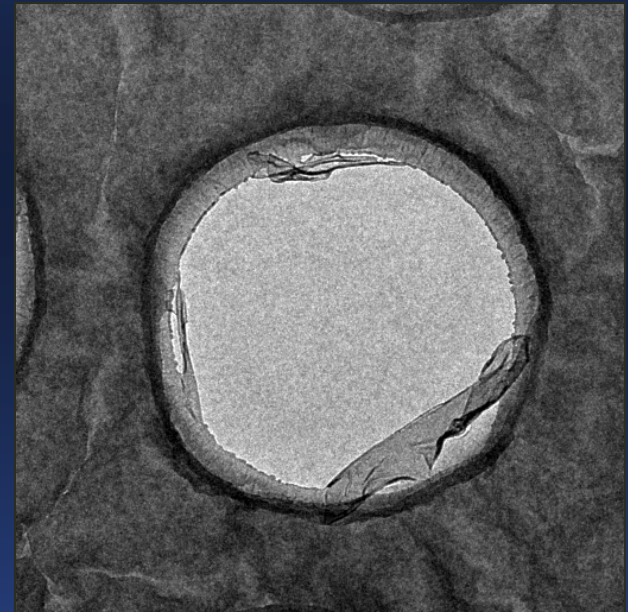
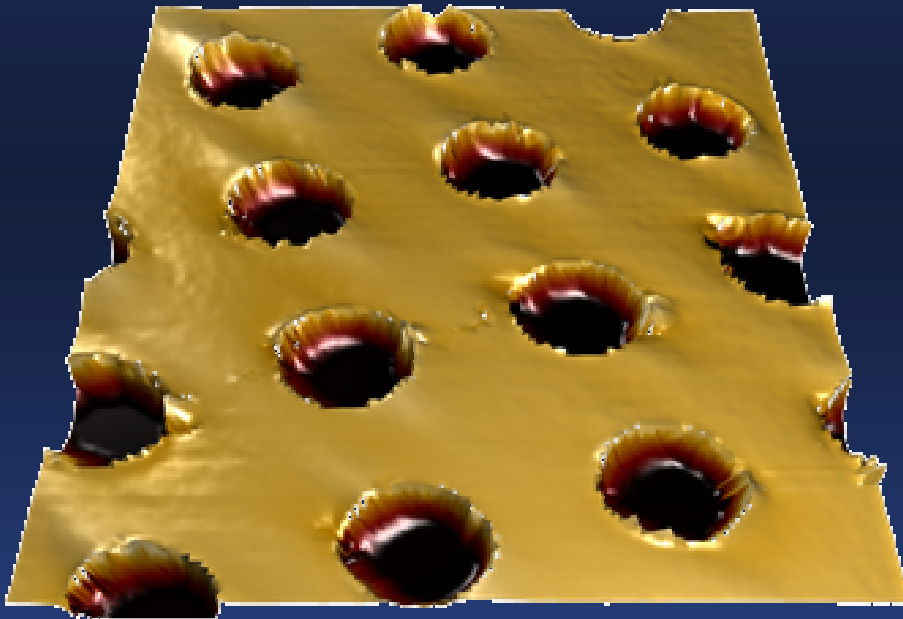
0 mm offset

0 s drain time

4 ml of sample on Qfoil grid

# Quantifoil Grids

- Perforated support foils with pre-defined hole size, shape and arrangement (Ermantrou et al., Ultramicroscopy 74)

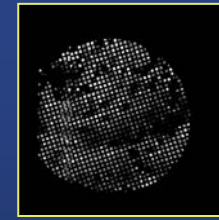
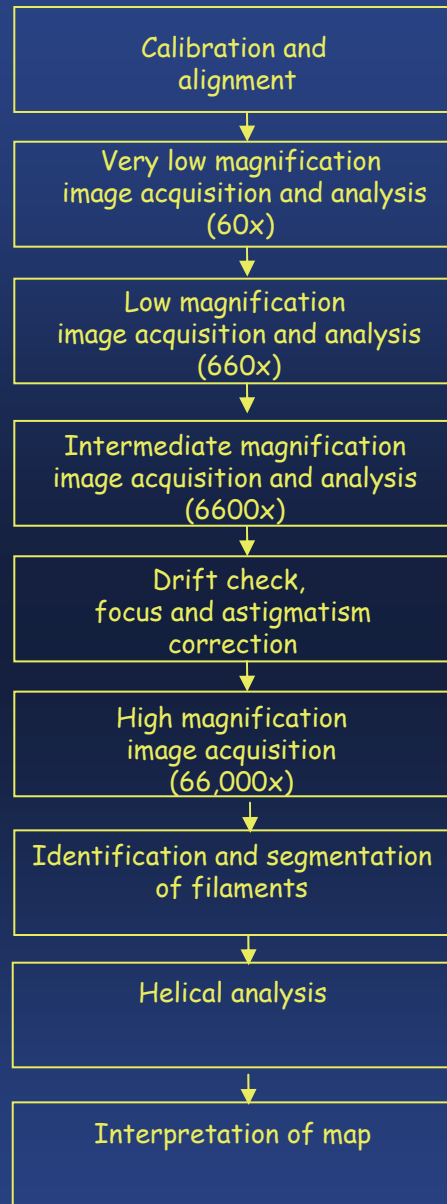


Hole diameter:  $\sim 2 \mu\text{m}$

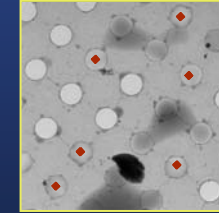
Hole spacing:  $\sim 4 \mu\text{m}$

# Automated TMV data collection and reconstruction

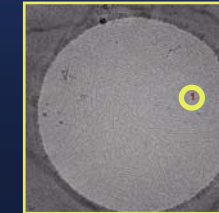
- Grid to map in <24 hours
- No user intervention except for refilling cryogenics



- Survey grid
- Identify potential grid squares
- Dose = 0



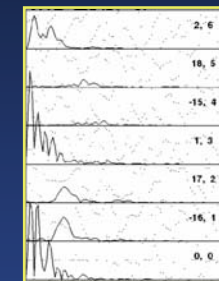
- Identify location of holes
- Estimate ice thickness
- Dose = 0.001 e/A<sup>2</sup>



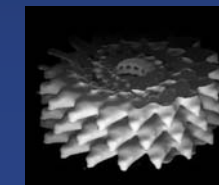
- Estimate probability of specimen
- Identify location of best specimen
- Dose = 0.1 e/A<sup>2</sup>



- Identify and segment filaments
- Dose = 10 e/A<sup>2</sup>

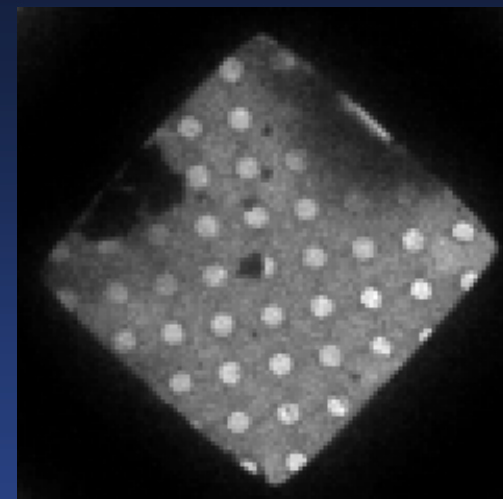
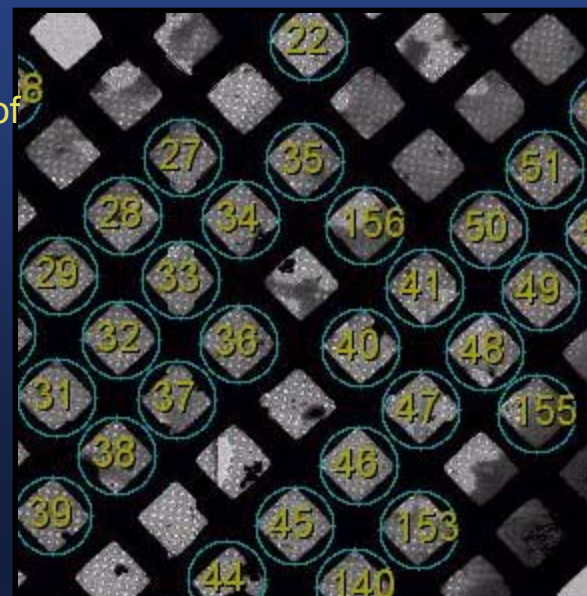
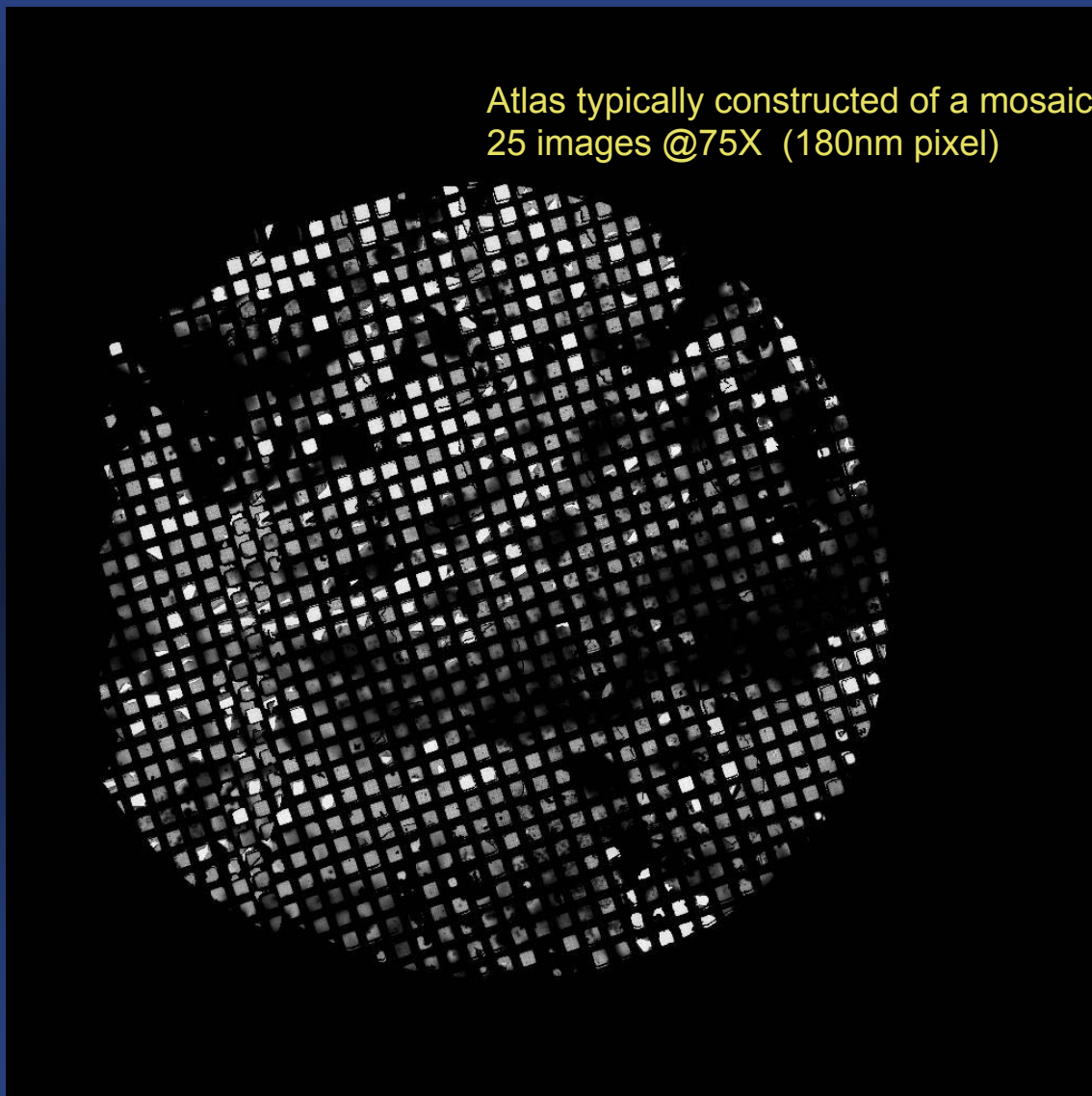


- Find layer lines
- CTF correction
- Tilt and shift correction
- Fitting and averaging



# Constructing a grid atlas (~60x) and targeting “good” squares

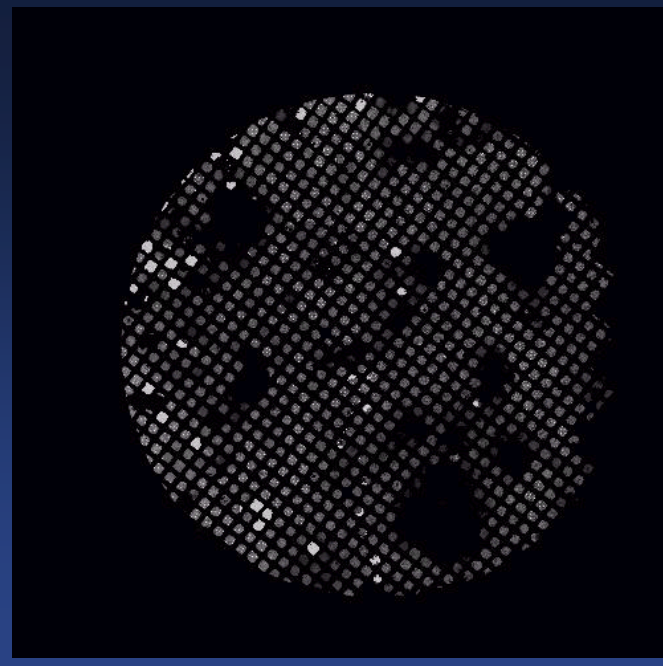
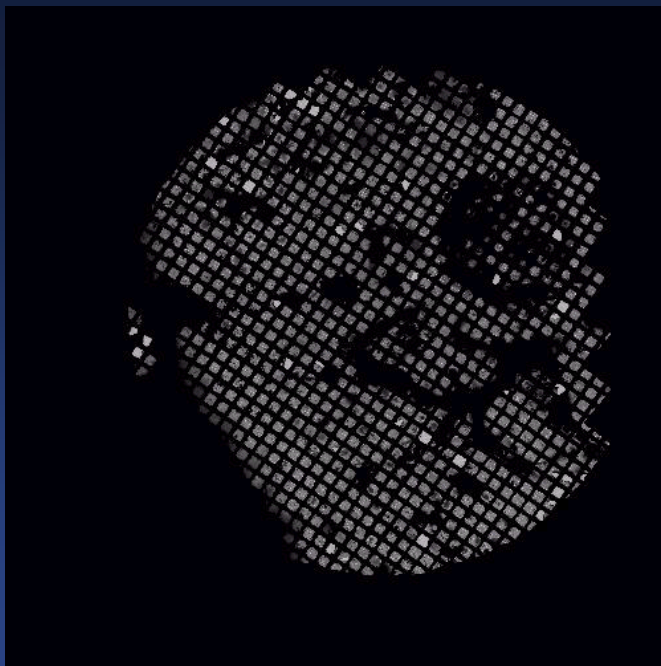
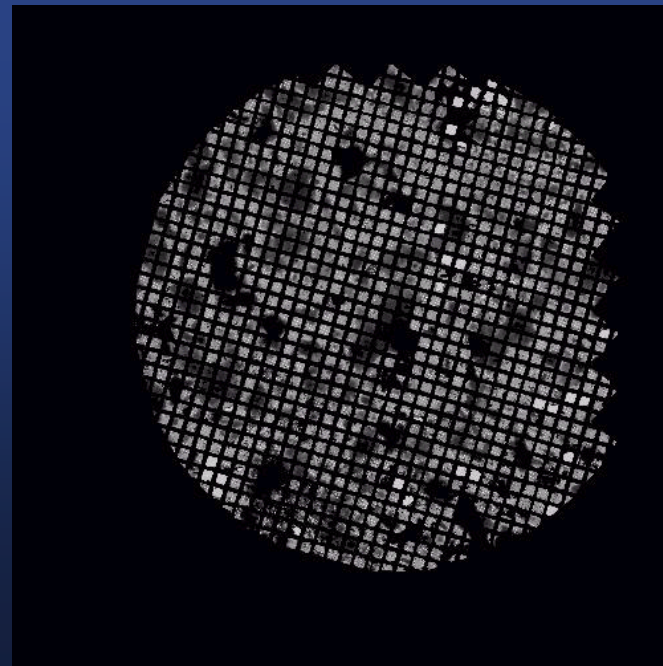
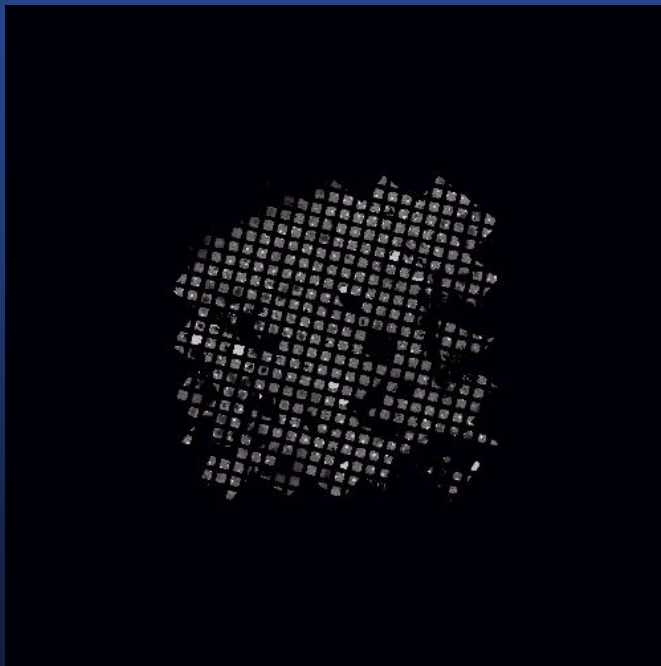
Atlas typically constructed of a mosaic of  
25 images @75X (180nm pixel)

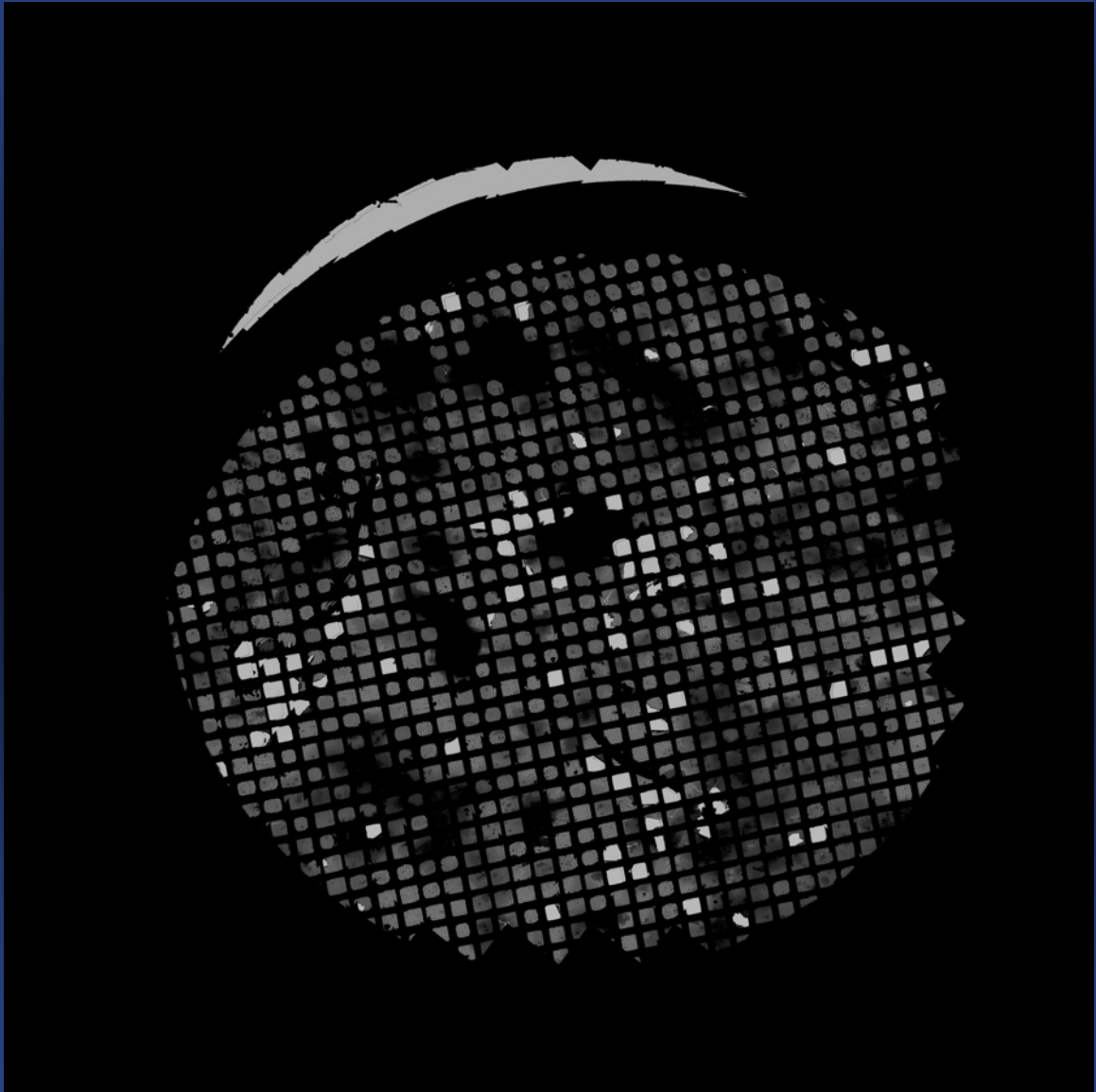


Total dose accumulated:  $\sim 0 \text{ e}^-/\text{\AA}^2$

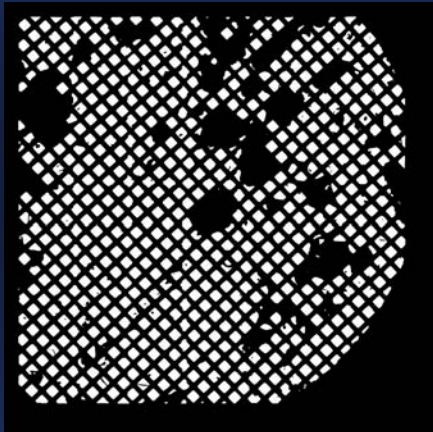
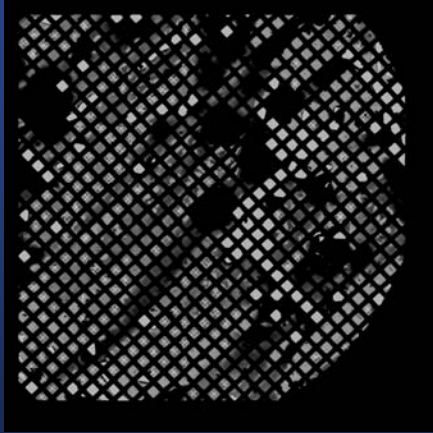
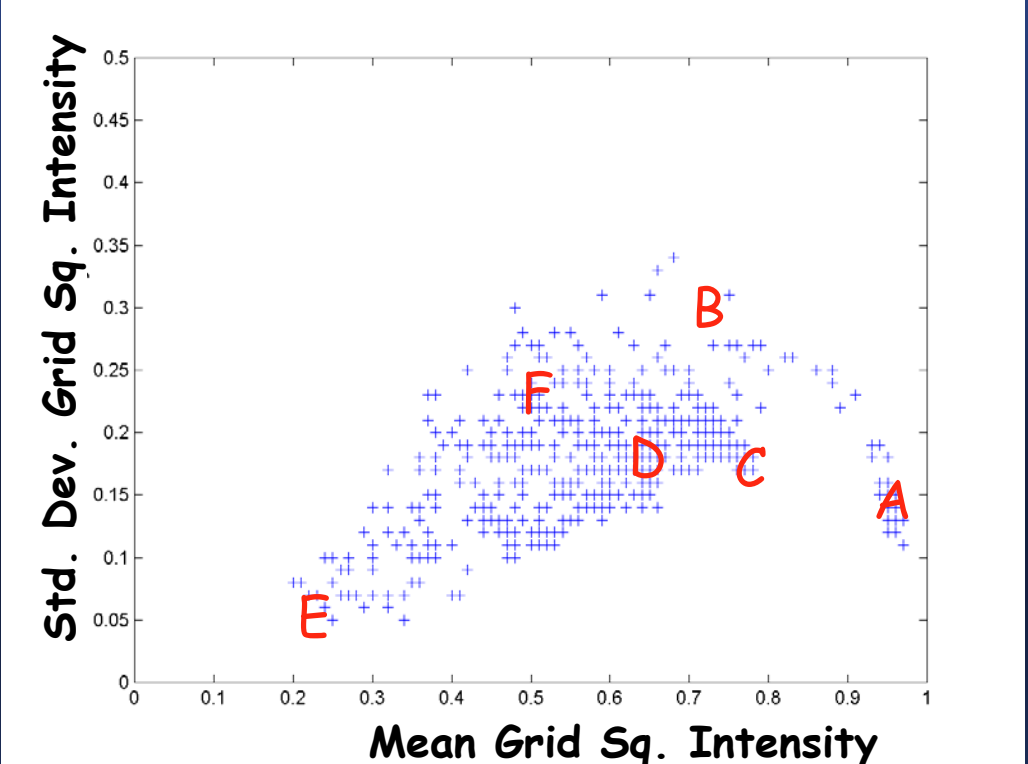
Full resolution







# Grid Atlas Analysis



A

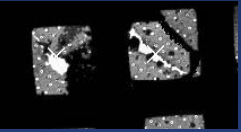
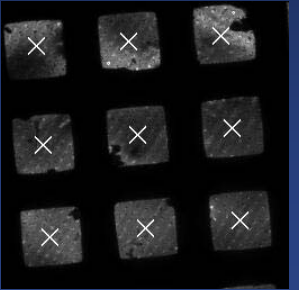
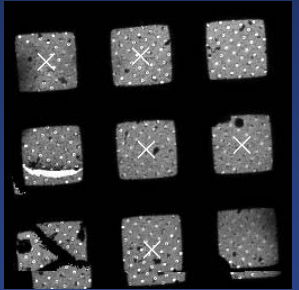
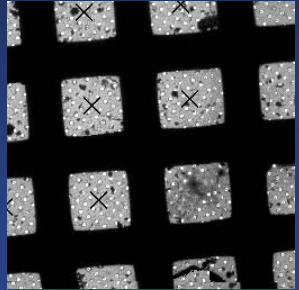
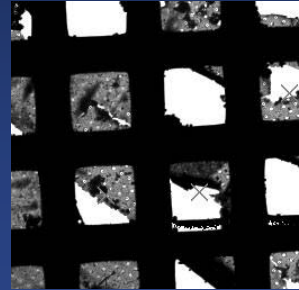
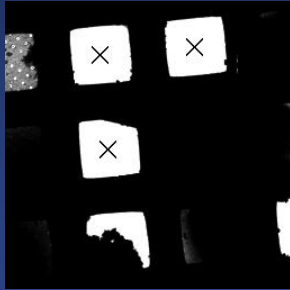
B

C

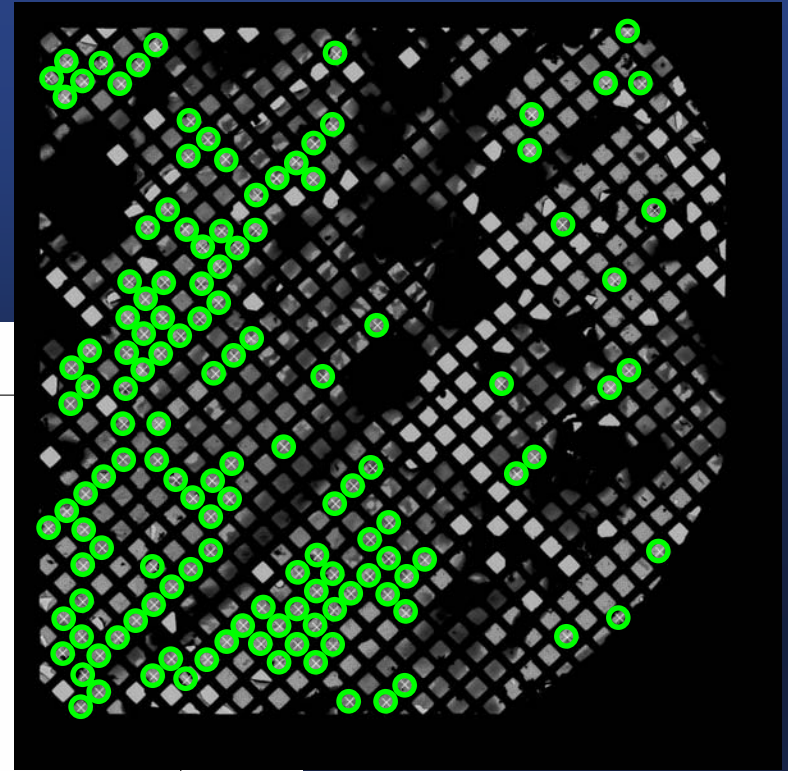
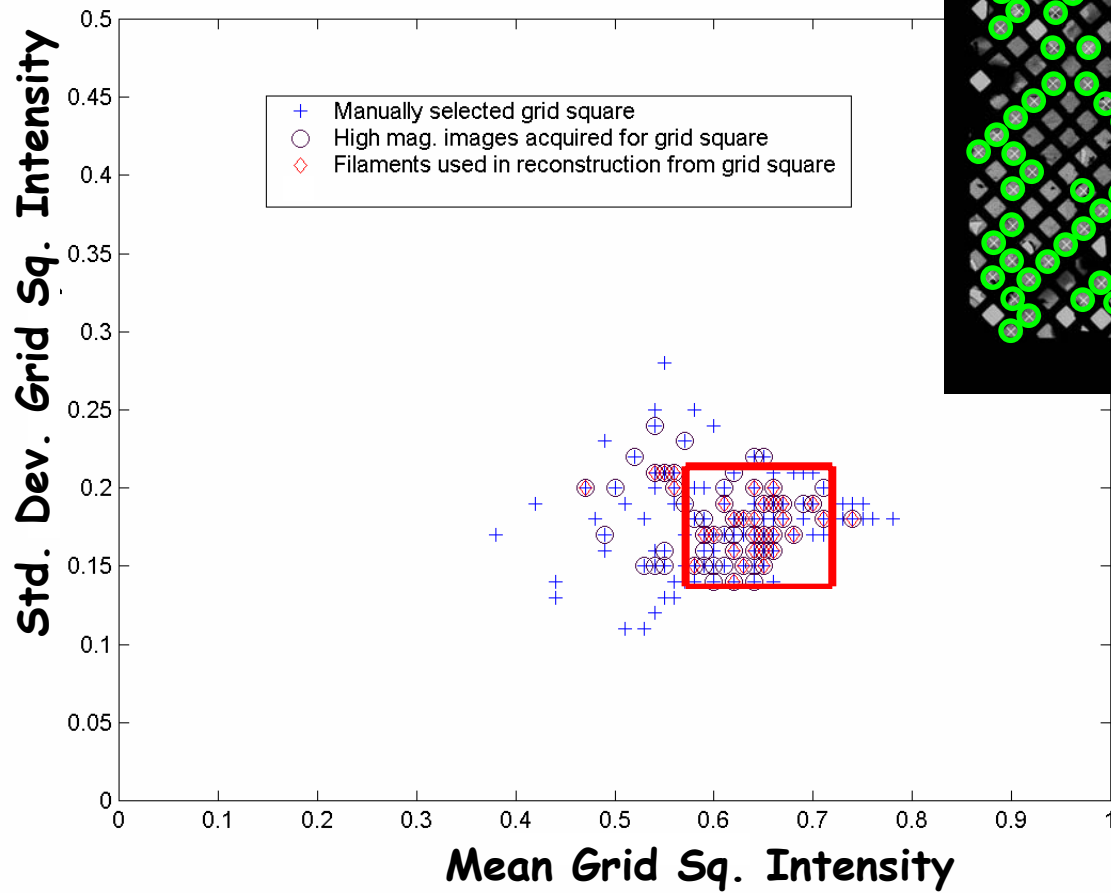
D

E

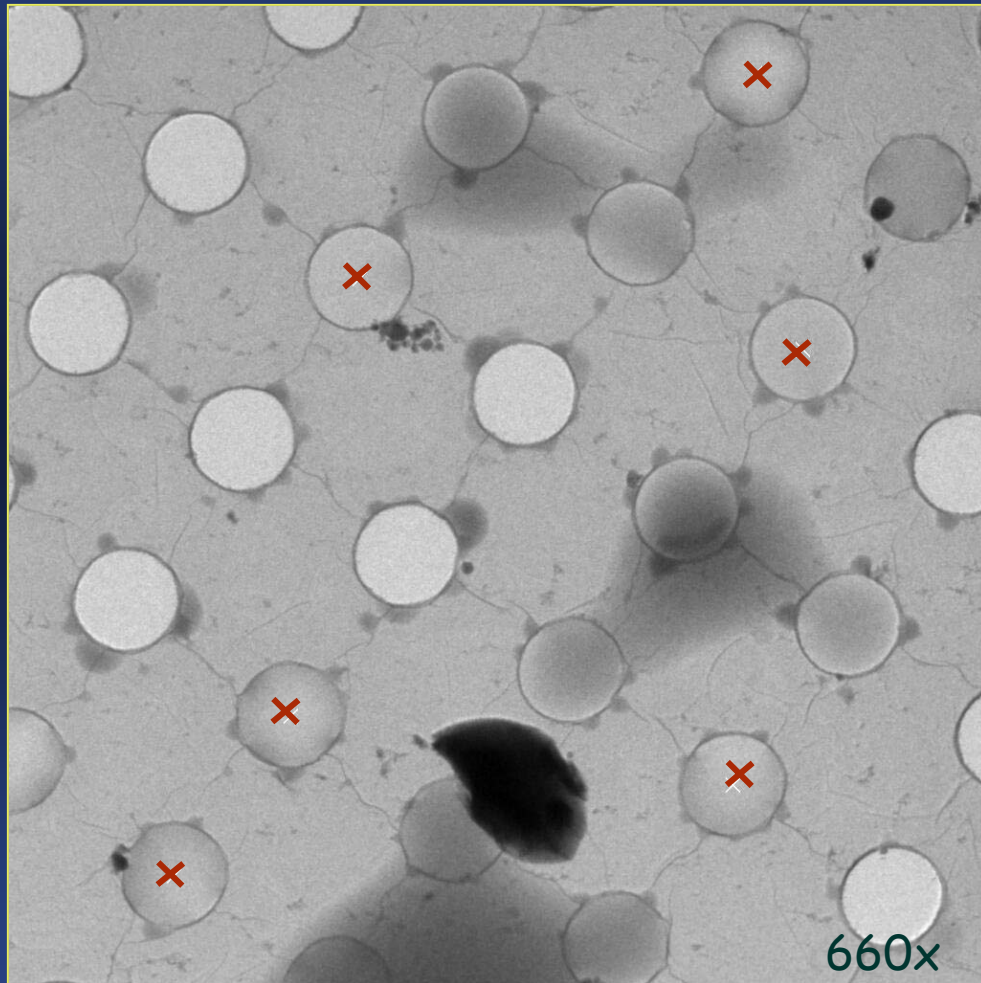
F



# Classification of "good" grid squares



# Finding "good" holes at low magnification (LM)



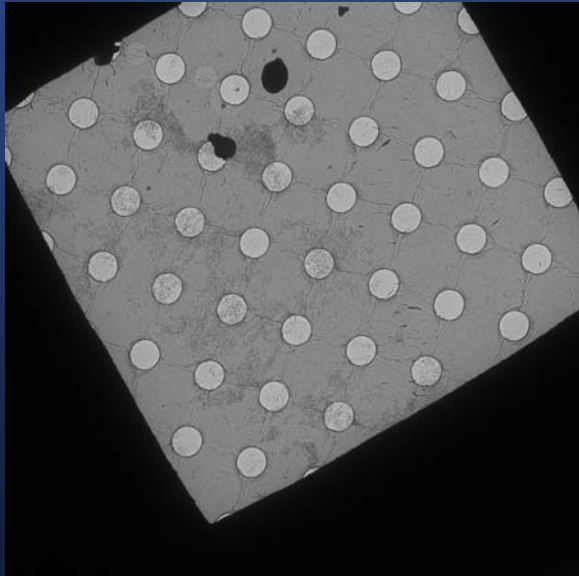
1. Find holes
  - Template matching
  - Find local lattice
2. Estimate ice thickness

Results:

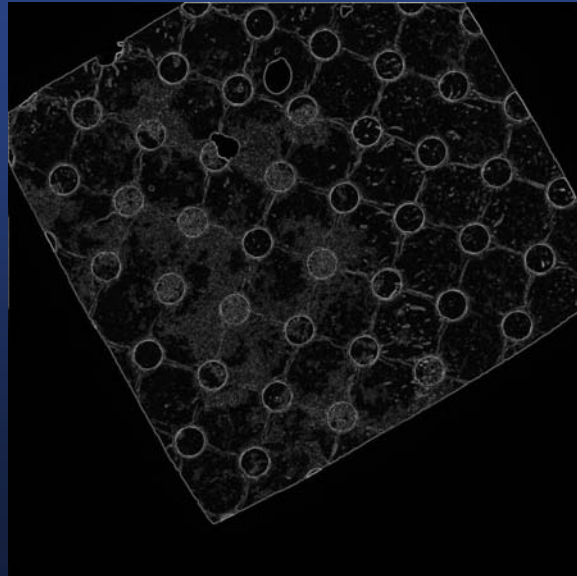
Appropriate holes identified  
95% of the time.

Consistent and reproducible.

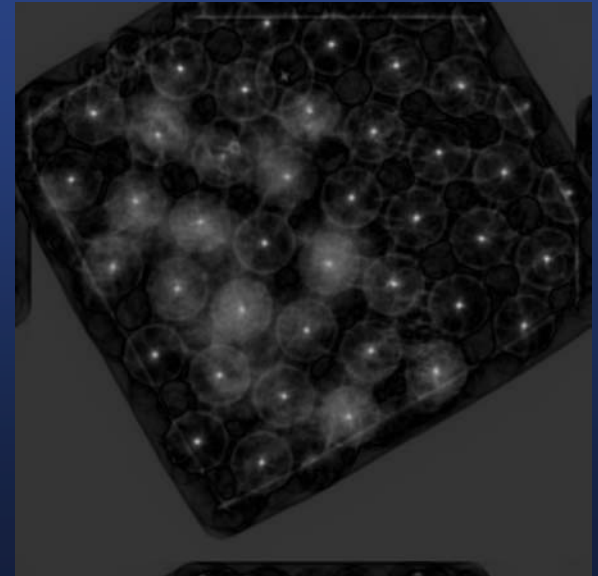
Original



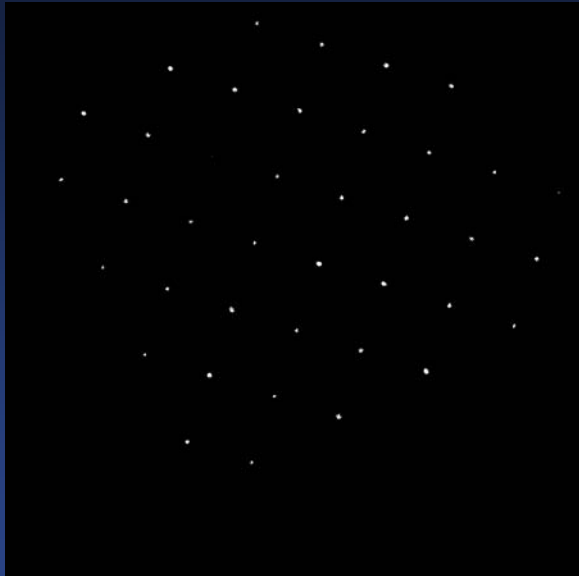
1. Edge enhanced



2. Correlation w/ ring



3. Threshold corr. map



4. Lattice test

## 5. Threshold on estimated ice thickness

$$t \approx K \ln(I_0/I)$$

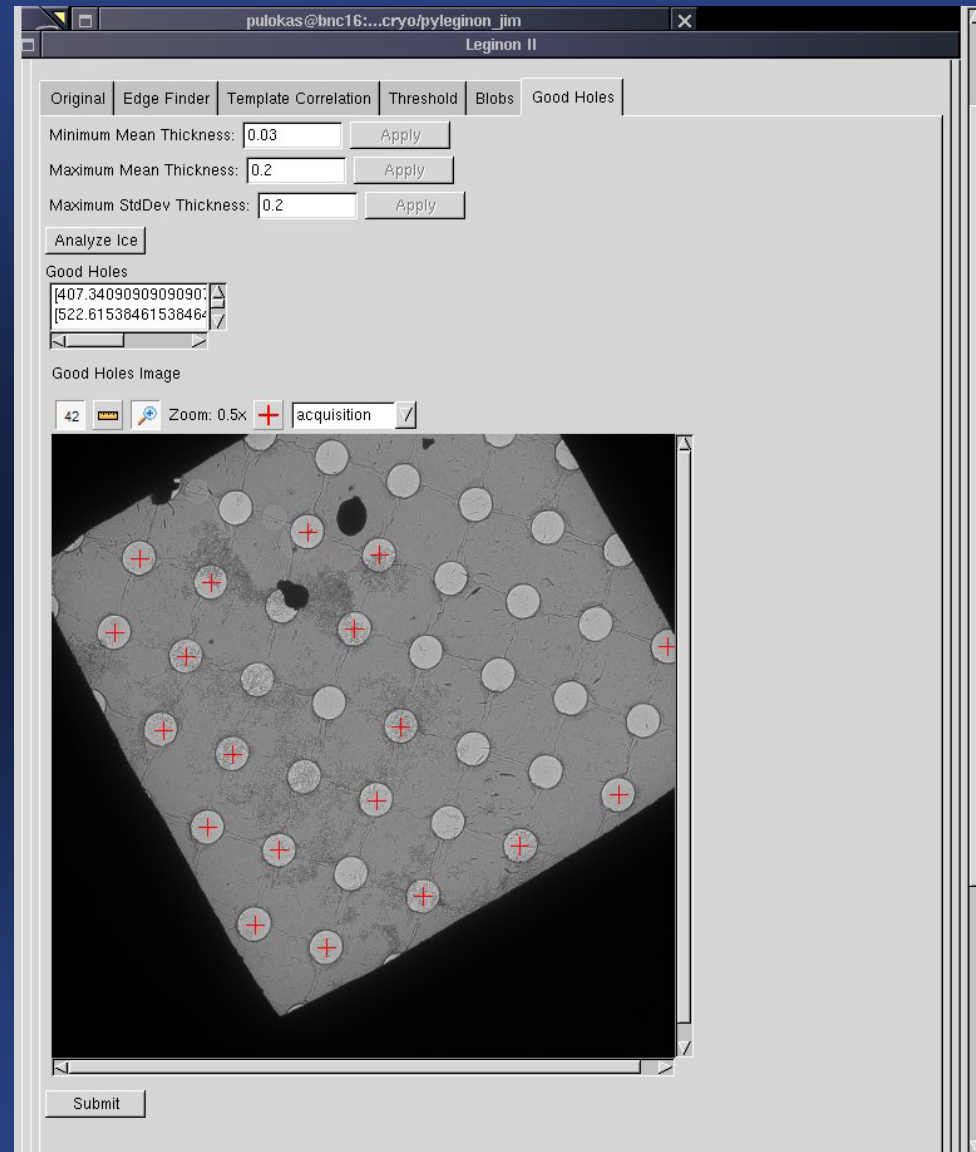
Where  $0.2 < K < 1.0 \mu\text{m}$

$I_0$  = Intensity of unattenuated hole

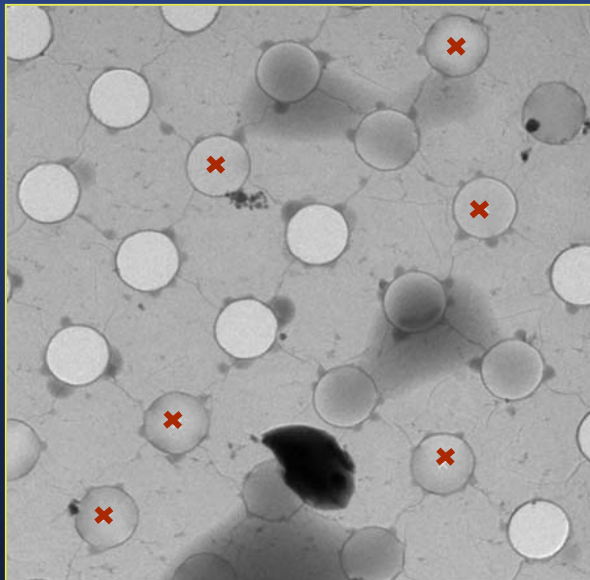
$I$  = Intensity of attenuated hole.

Further reading:

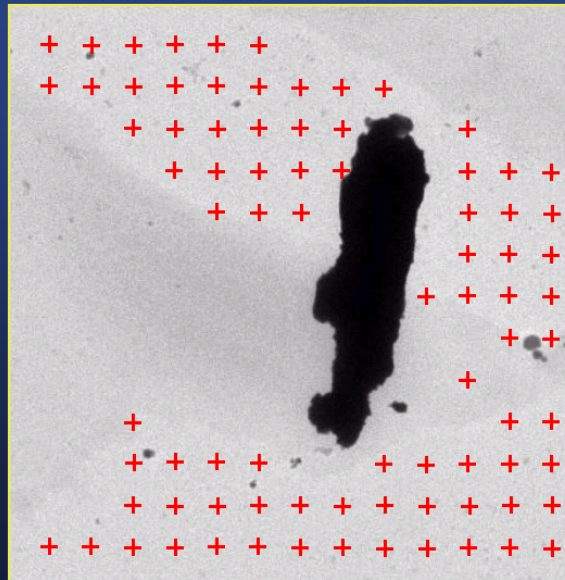
Feja and Aebi, *J. Microscopy*,  
193,15-19 (1999)



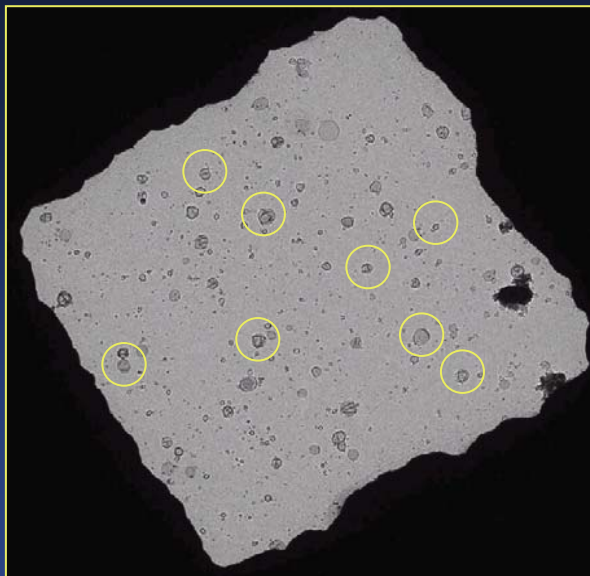
# Finding targets at low magnification (~600x)



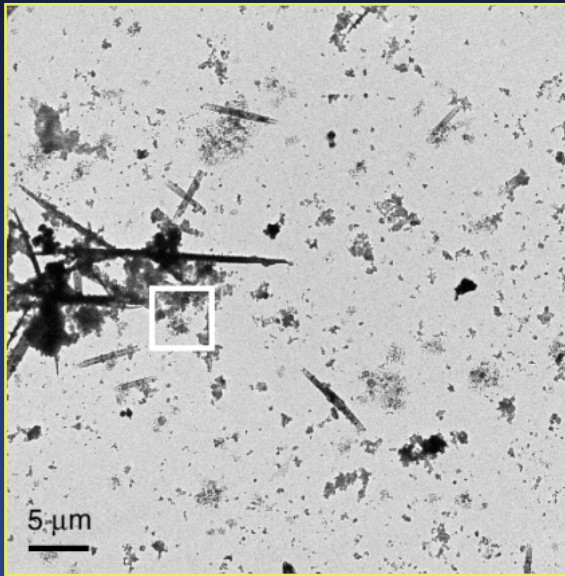
TMV



RNA PolIII



Rhodopsin



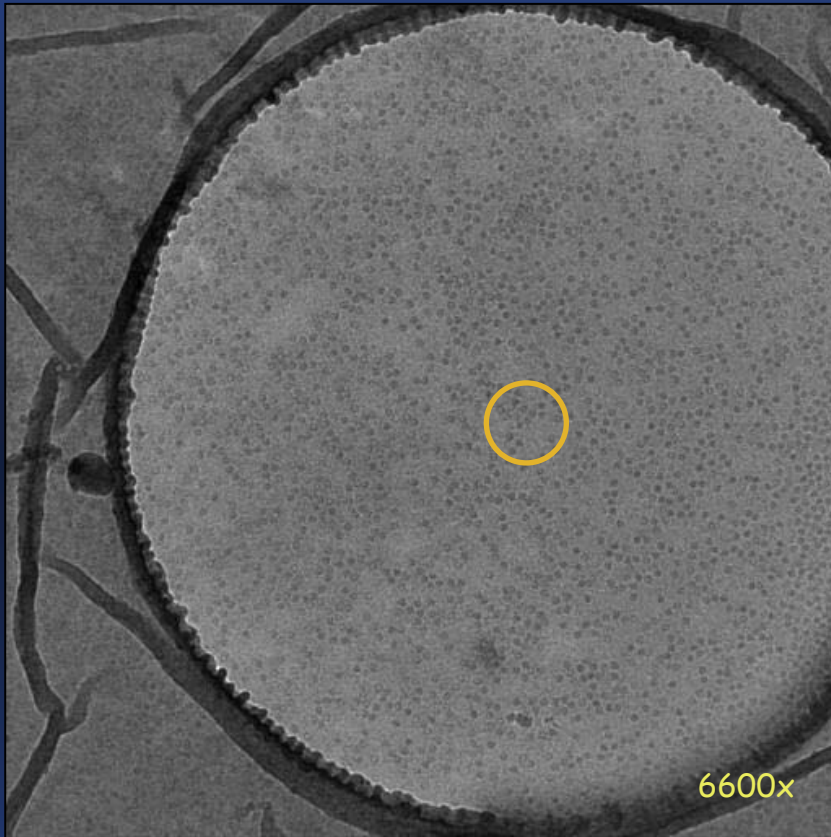
Connexin

Dose accumulated:  $\sim 0.003 \text{ e}^-/\text{\AA}^2$

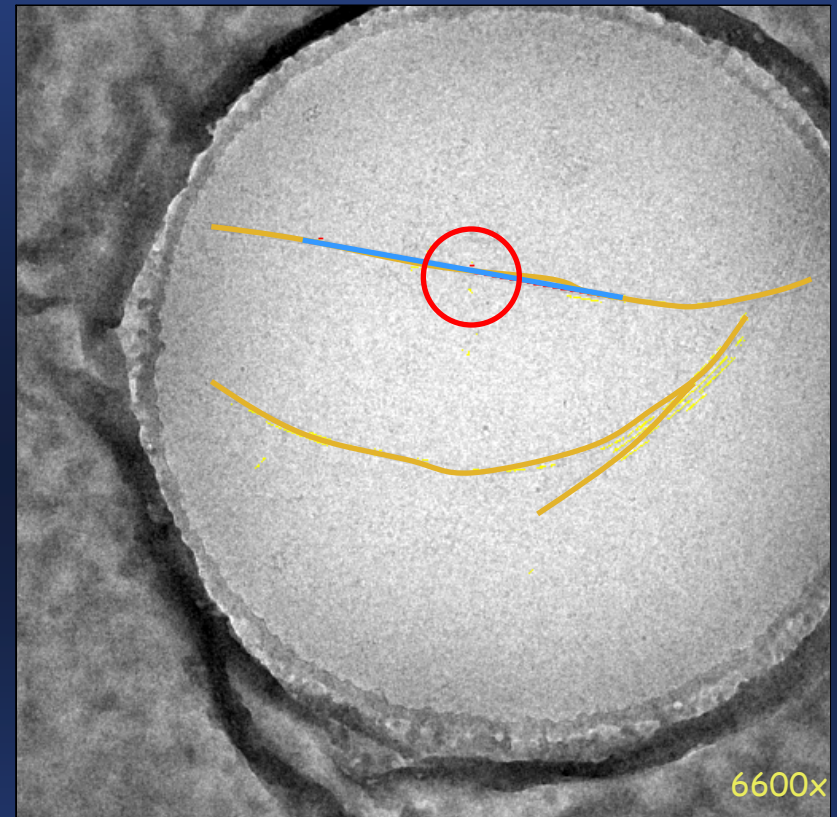


# Targeting within hole at intermediate magnification (IM)

Strategy depends on sample



Single Particles

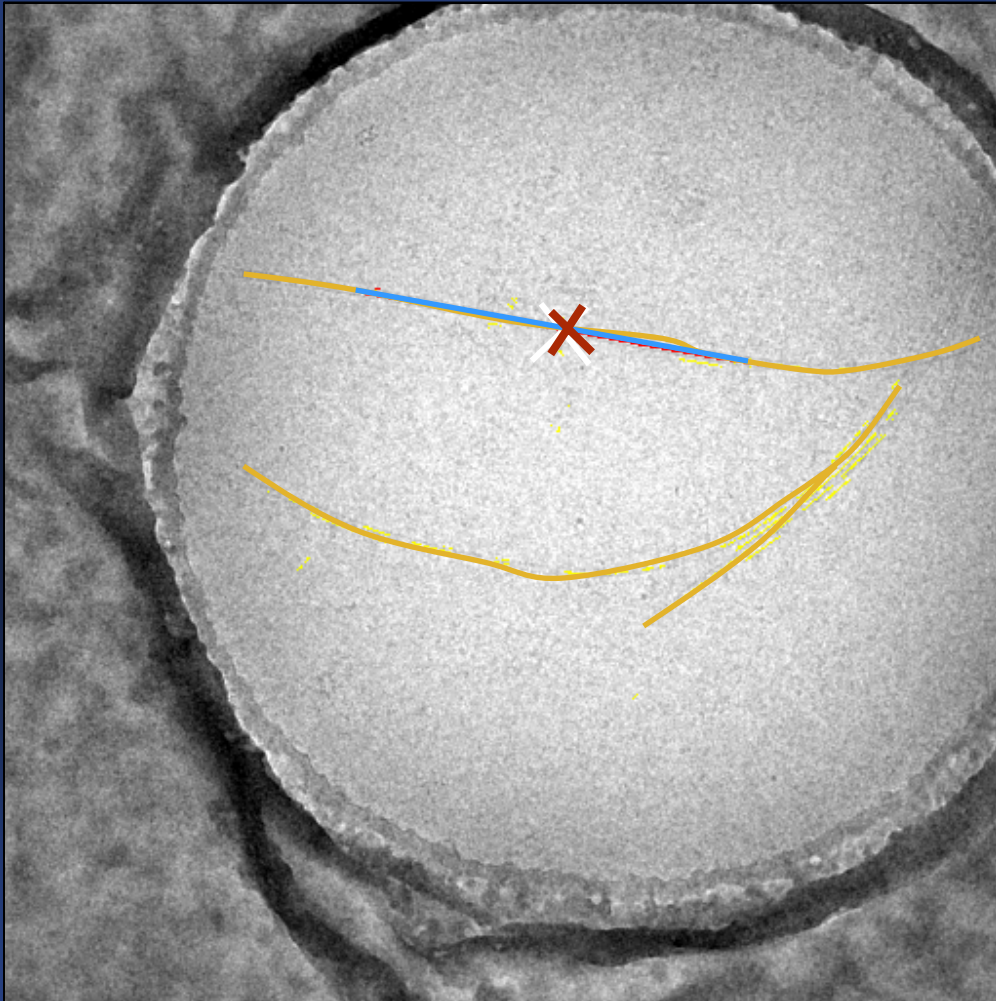


Microtubules

Intermediate magnification image analysis:

- Filaments present?
- Filaments suitable (length, straightness)?
- Best filament to target at high magnification?

# Filament identification and selection



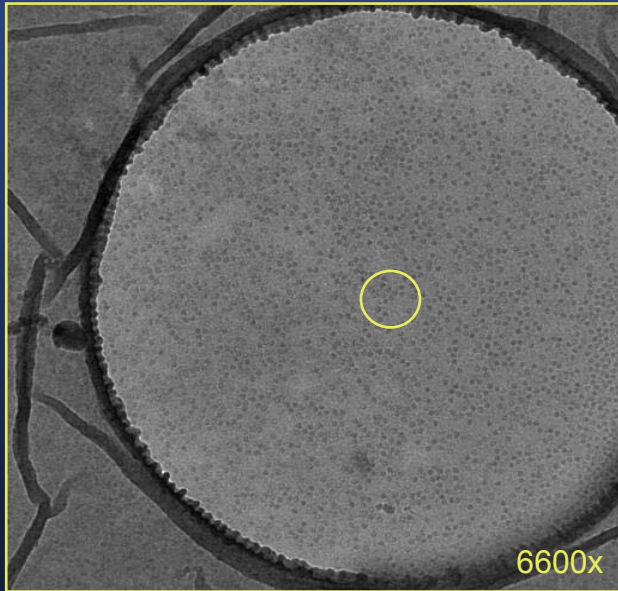
Methods:

Rotationally oriented template matching.

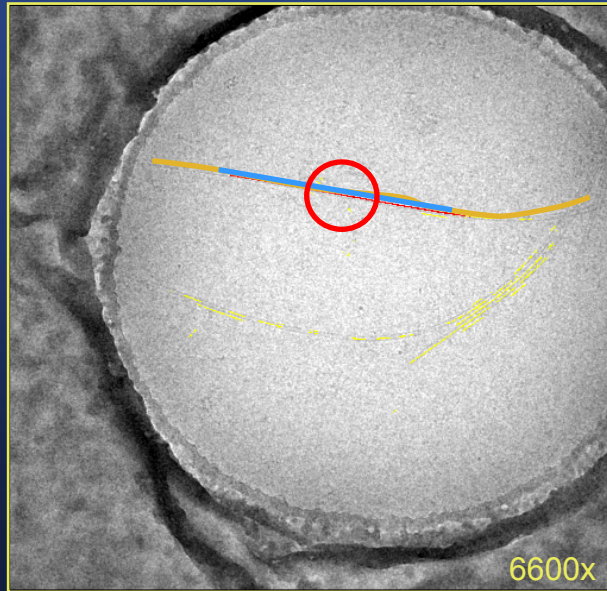
Hough transform to determine best straight segment.

Target center of straight segment.

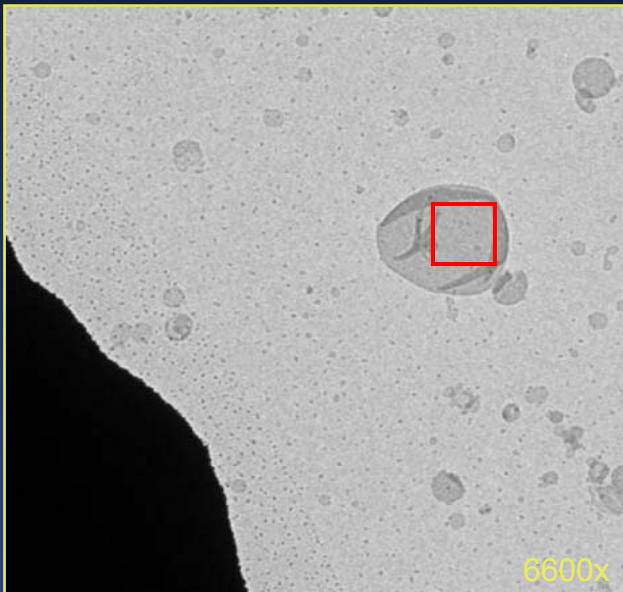
# Finding targets at intermediate magnification (~5000x)



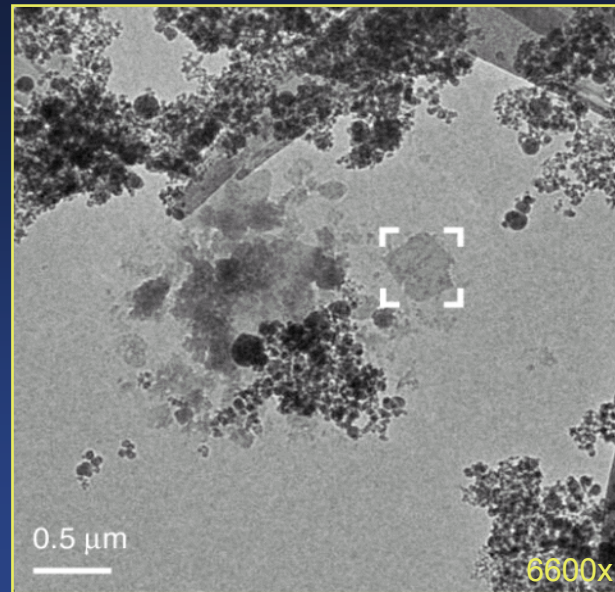
Hemocyanin



Microtubules



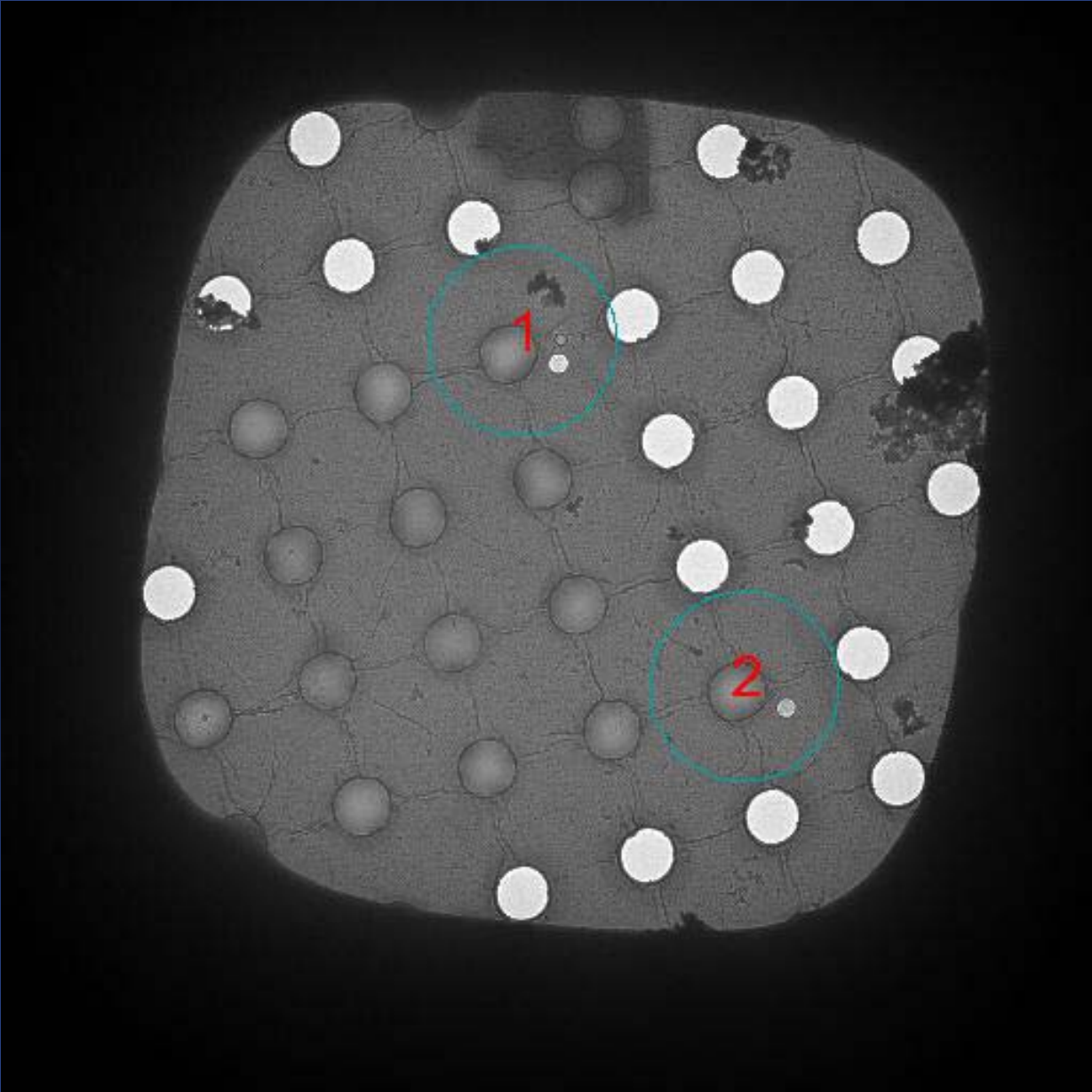
Rhodopsin



Connexin

Dose accumulated:  $\sim 0.3 \text{ e}/\text{\AA}^2$

# Low dose drift check and focus



## Drift measurement using cross correlation

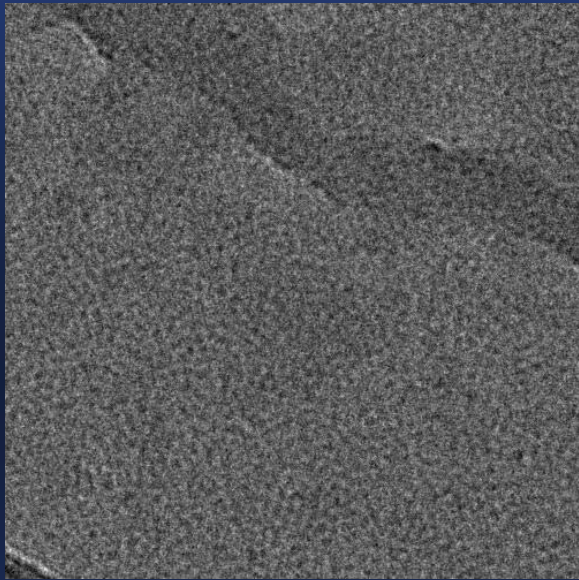


Image1

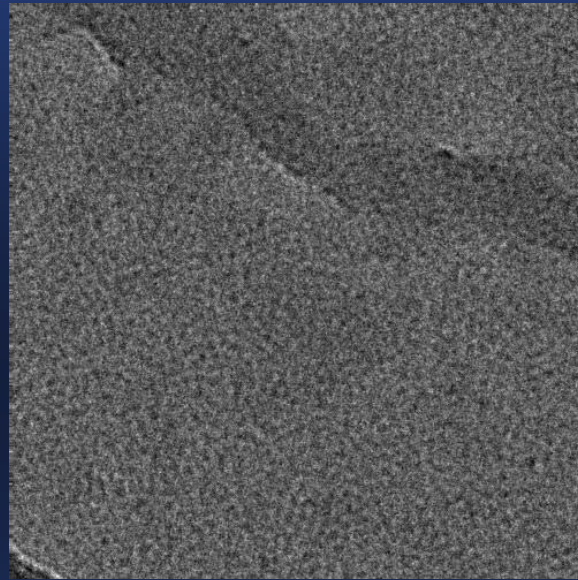


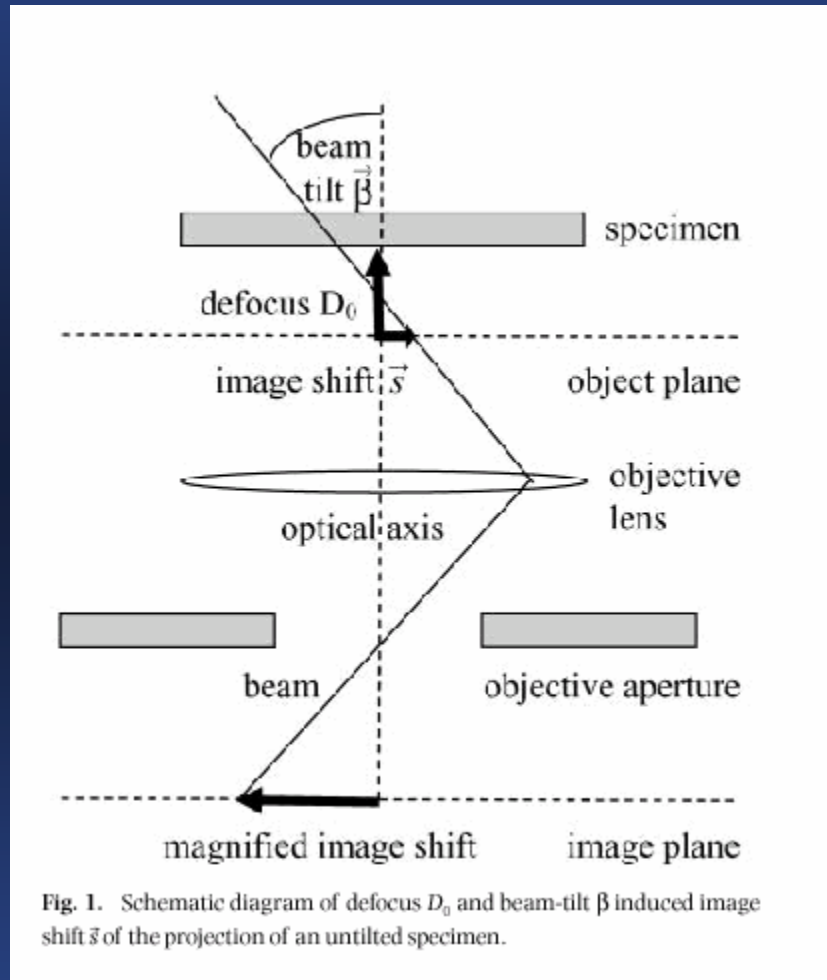
Image2



CC

Typical drift tolerance:  $< 2/s \text{ \AA}$  w/ 0.5s exposure

## Autofocus Technique:



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

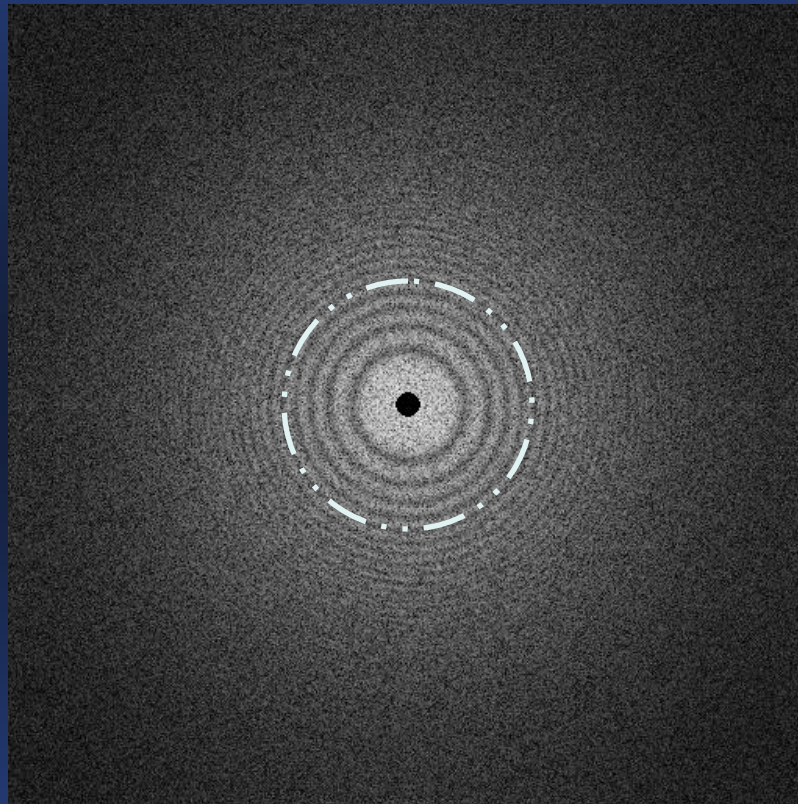
$$S = D_0 * \tan ( B )$$

Reference:

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

# Auto focus to within 100nm

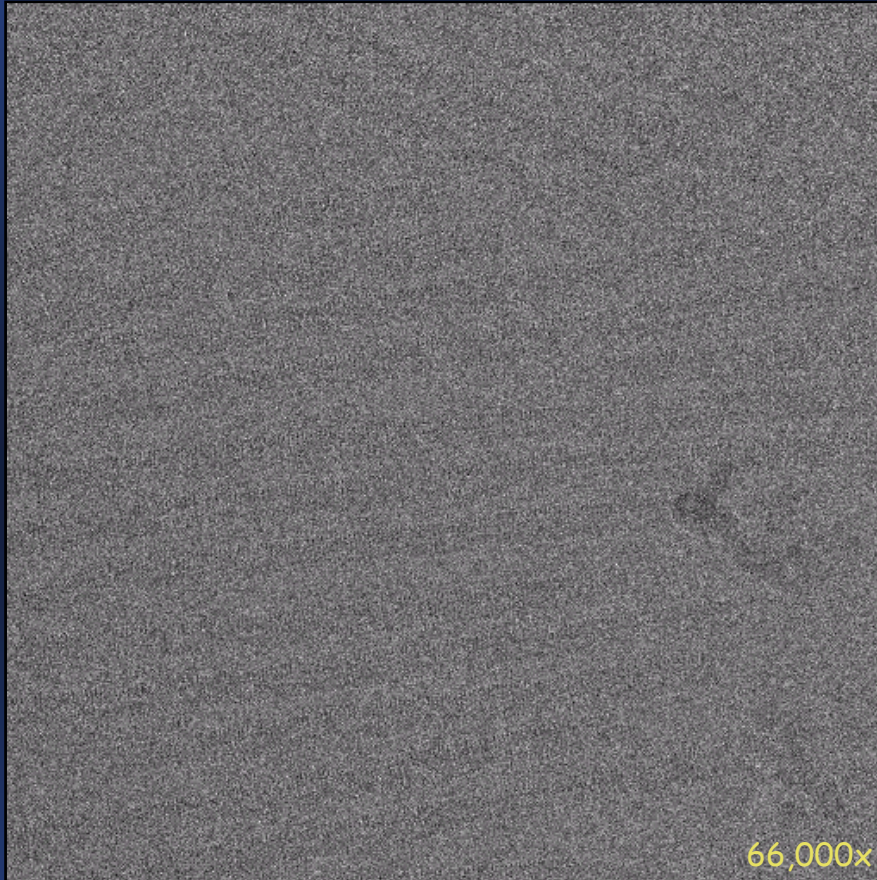
Defocus =  $2.95 \pm 0.08 \mu\text{m}$



02may14a

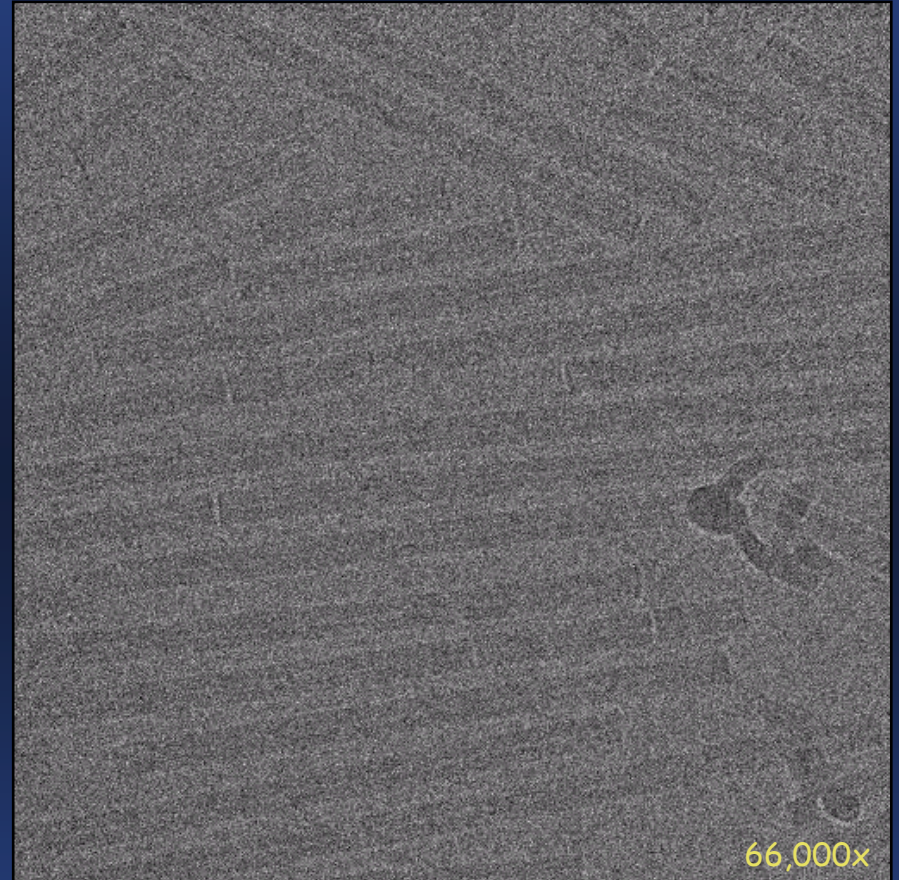
High magnification images are acquired as defocus pairs (or sequences).

Exposure #1



Nominal defocus = -300nm  
Accumulated dose = 12 e/A<sup>2</sup>

Exposure #2



Nominal defocus = -2000nm  
Accumulated dose = 23 e/A<sup>2</sup>



# Leginon Typical "Big" Experiment

03oct15a

107 grid squares

150 holes

300 high mag defocus pairs

5000 images

20GB of data

22 hour data collection

144 experiments over last 12 months.

# Automated Data Collection Software for Molecular Microscopy

Leginon (AMI Group@TSRI):

Potter, et al. Ultramicroscopy, 77 (1999) 153-161  
Carragher, et al., J. Struct. Biology 132 (2000) 33-45  
Zhu, et al. J. Struct. Biology 135 (2001) 302-312  
Available from <http://nramm.scripps.edu>

Autoem (Subramaniam Group at NIH):

Zhang, et al, J. Struct. Bio, 135 (2001) 251-261

James (JEOL Automated Microscope Expert System):  
National Center for Macromolecular Imaging

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