

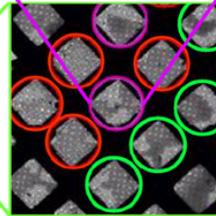
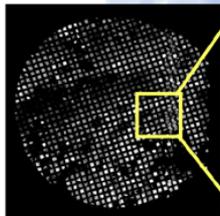
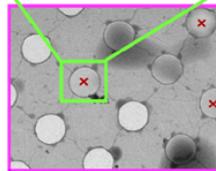
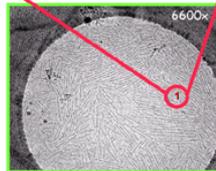
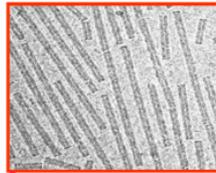
AMi Automated Molecular Imaging Group

LEGION

The Scripps Research Institute



ami.scripps.edu



Automation I: Data Collection

Clint Potter

NRAMM
CryoCourse
11/2005



Automated Molecular Imaging Group:



Jim Pulokas



Denis Fellmann



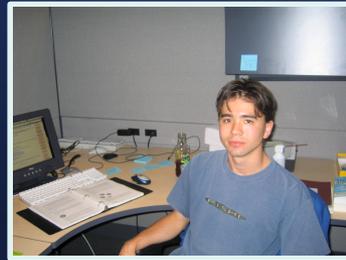
Joel Quispe



Anchi Cheng



Scott Stagg



Craig Yoshioka



Gabriel Lander



Satya Mallick



Christian Suloway



Phil Mercurio



Jill Krawczyk



Bridget Carragher



Clint Potter



Step 1 Sample Requirements



0.6 or 1.6ml Eppendorf Tubes

- 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 μ l of sample + 1-5 mls of dilution buffer.



10:20 AM Delivery

Step 2



GO

10:30 AM

Step 3 Examine Map



1:10 PM



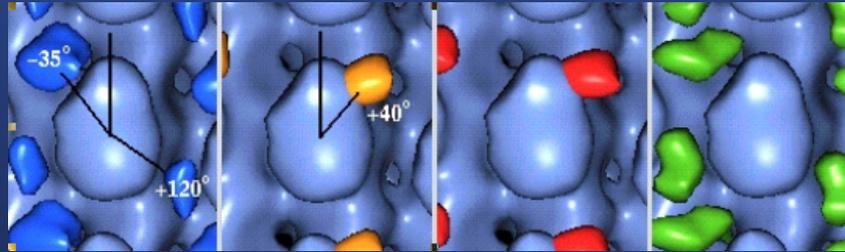
Step 4 Structure Determination



4:50 PM

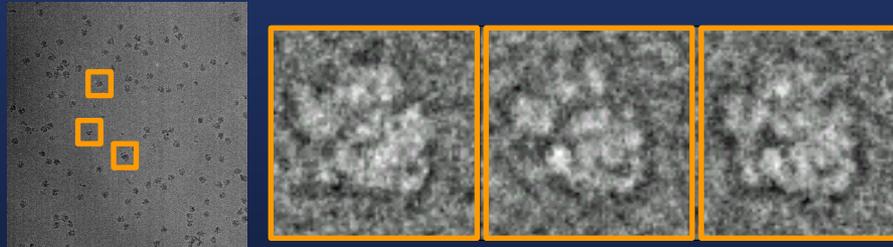
Why do we need automation in molecular microscopy?

Multiple conformational states



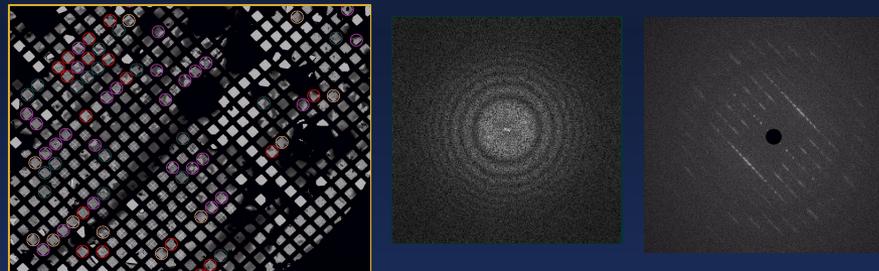
lots of experiments

Low SNR
Need averaging



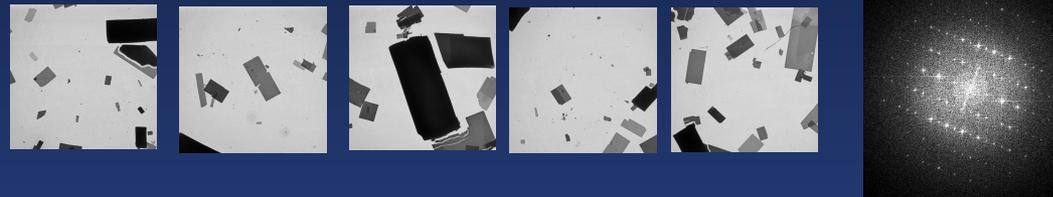
lots of data

Systematic evaluation and improvement of techniques



lots of luck

Screening trials



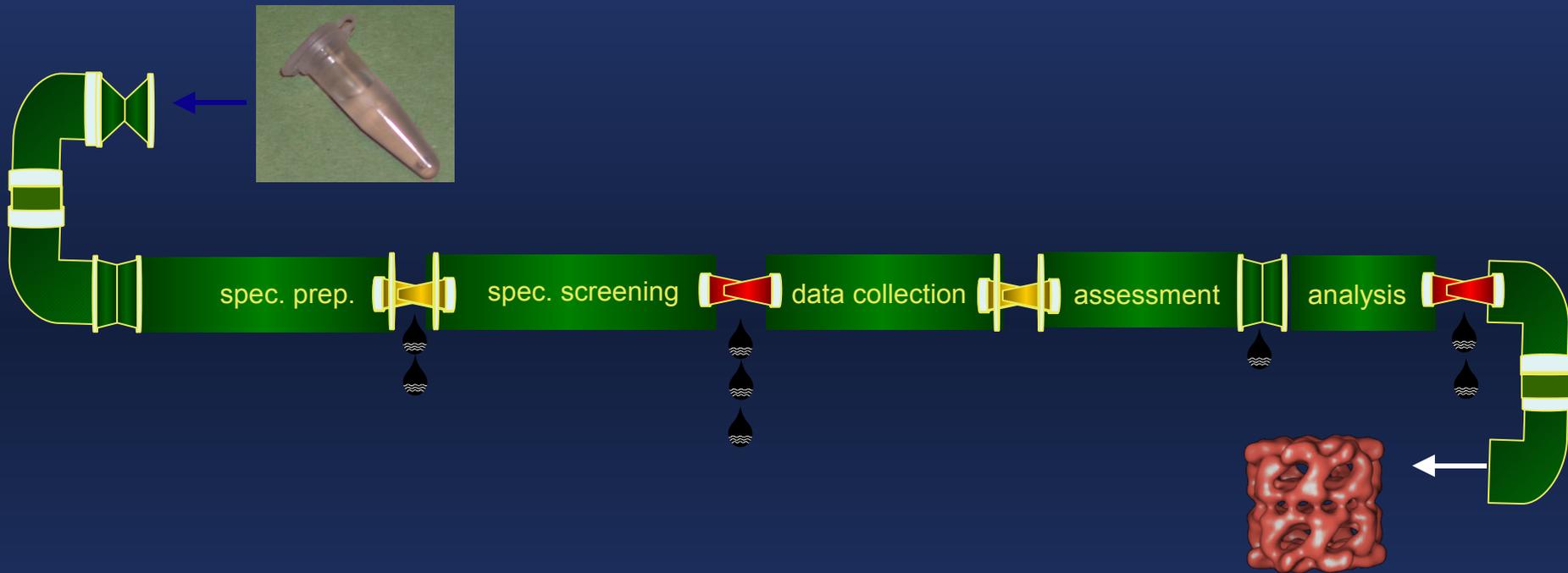
lots of patience

Challenging technique

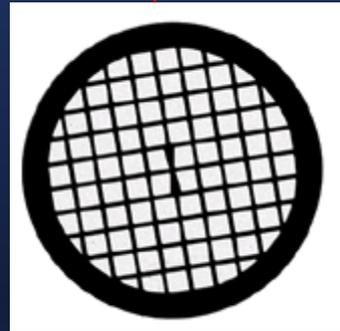


lots of skill

Automated Pipeline for Molecular Microscopy

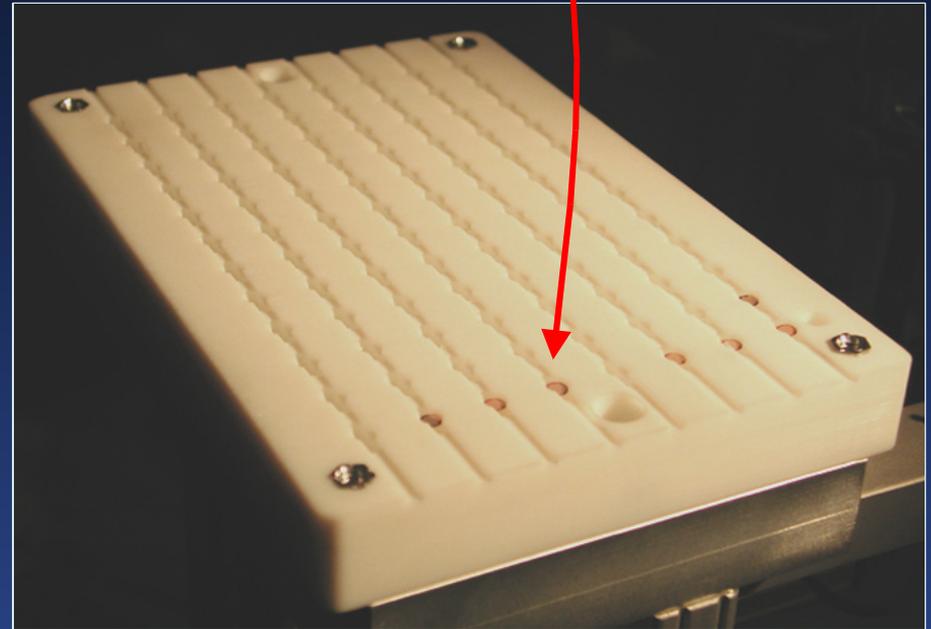


Specimen Screening :

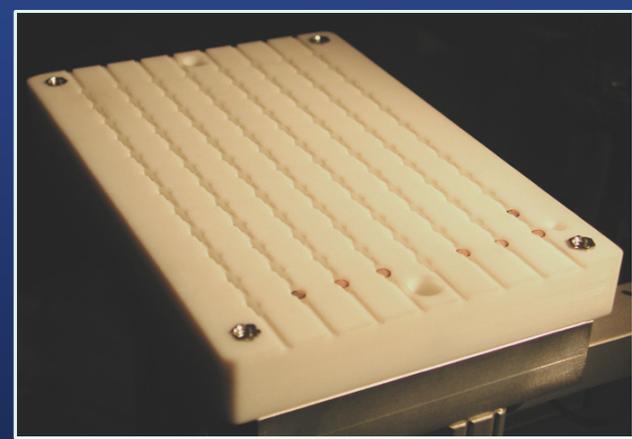


Crystallization Screening Trial

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



Robotic screening



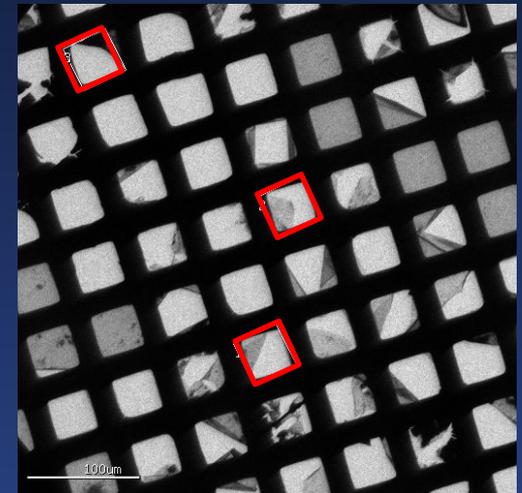
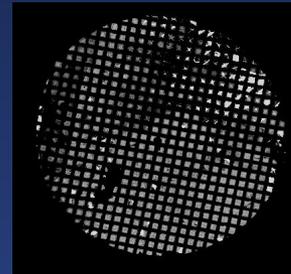
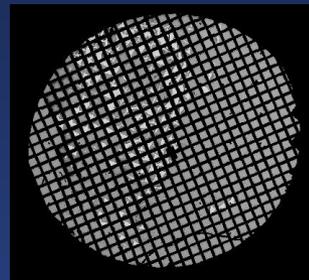
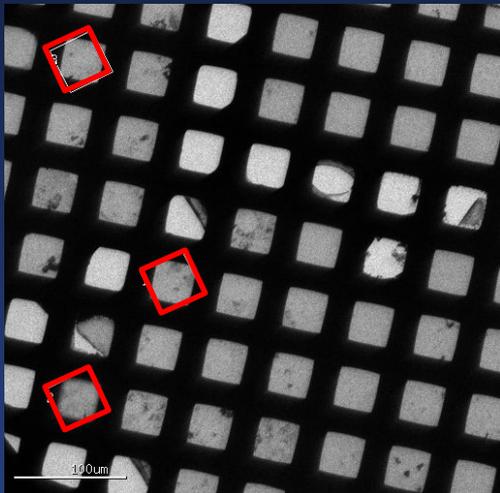
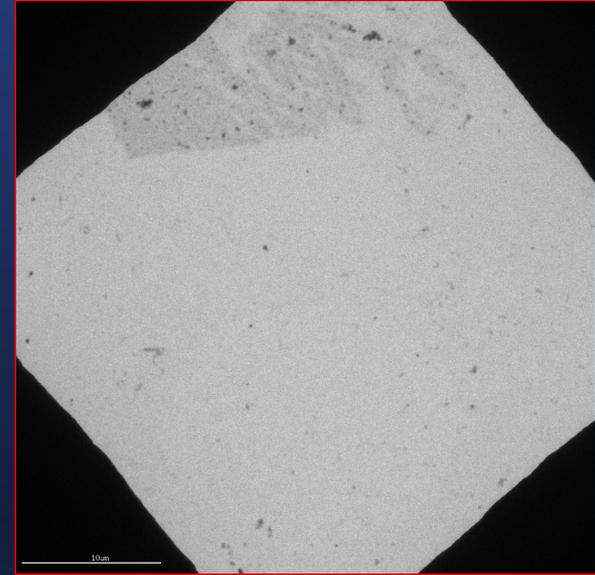
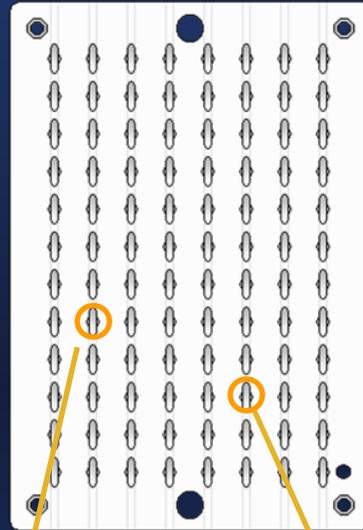
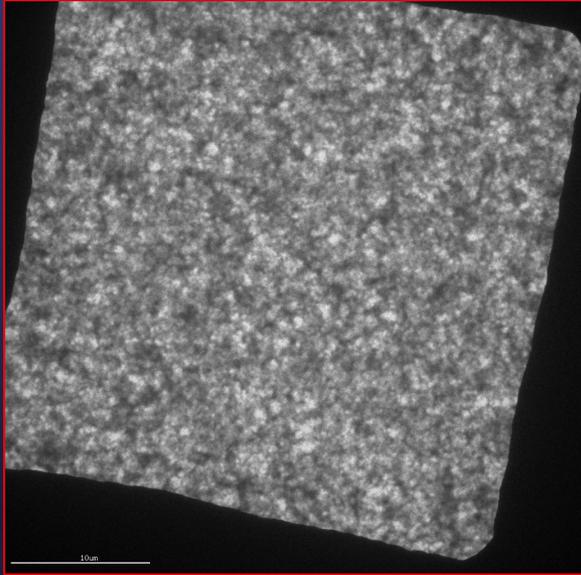
Stain not ice!

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

Leginon Image Viewer - Mozilla Firefox

File Edit View Go Bookmarks Tools Help

http://cronus3/dbem/3wviewer.php

<summary>

all

GridID01141_Insertion5_026gr_1sq_3hl.mrc
GridID01141_Insertion5_026gr_1sq_2hl.mrc
GridID01141_Insertion5_024gr_1sq_012hl.mrc
GridID01141_Insertion5_024gr_1sq_011hl.mrc
GridID01141_Insertion5_024gr_1sq_010hl.mrc
GridID01141_Insertion5_024gr_1sq_9hl.mrc
GridID01141_Insertion5_024gr_1sq_8hl.mrc
GridID01141_Insertion5_024gr_1sq_7hl.mrc
GridID01141_Insertion5_024gr_1sq_6hl.mrc
GridID01141_Insertion5_024gr_1sq_5hl.mrc
GridID01141_Insertion5_024gr_1sq_4hl.mrc
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GridID01141_Insertion5_024gr_1sq_2hl.mrc
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GridID01141_Insertion5_023gr_1sq_011hl.mrc
GridID01141_Insertion5_023gr_1sq_010hl.mrc
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GridID01141_Insertion5_023gr_1sq_4hl.mrc
GridID01141_Insertion5_023gr_1sq_3hl.mrc
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GridID01141_Insertion5_022gr_1sq_013hl.mrc
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GridID01141_Insertion5_022gr_1sq_011hl.mrc
GridID01141_Insertion5_022gr_1sq_010hl.mrc
GridID01141_Insertion5_022gr_1sq_9hl.mrc
GridID01141_Insertion5_022gr_1sq_8hl.mrc
GridID01141_Insertion5_022gr_1sq_7hl.mrc
GridID01141_Insertion5_022gr_1sq_6hl.mrc
GridID01141_Insertion5_022gr_1sq_5hl.mrc
GridID01141_Insertion5_022gr_1sq_4hl.mrc
GridID01141_Insertion5_022gr_1sq_3hl.mrc
GridID01141_Insertion5_021gr_1sq_015hl.mrc
GridID01141_Insertion5_021gr_1sq_014hl.mrc
GridID01141_Insertion5_021gr_1sq_013hl.mrc

05apr21a - Lipid Tube Screening #2

View 1
gr no filter bin:auto jpg80
grid# 96 info mag: 56 defocus: 0.0000 μm
pixelsize: 145.5960 nm
05apr21a_GridID01141_Insertion005_00023g

Main View
hl filter:default bin:1 png
grid# 96 info mag: 5000 defocus: -50.0000 μm pixelsize: 1.6307 nm
05apr21a_GridID01141_Insertion005_00023gr_00001sq_00007hl.mrc

View 3
sq no filter bin:auto jpg80
grid# 96 info mag: 550 defocus: -500.0000 μm pixelsize: 14.8244 nm
05apr21a_GridID01141_Insertion005_00023g

200 μm

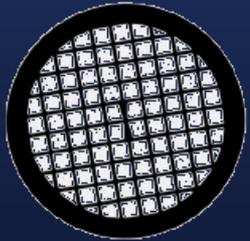
2 μm

20 μm

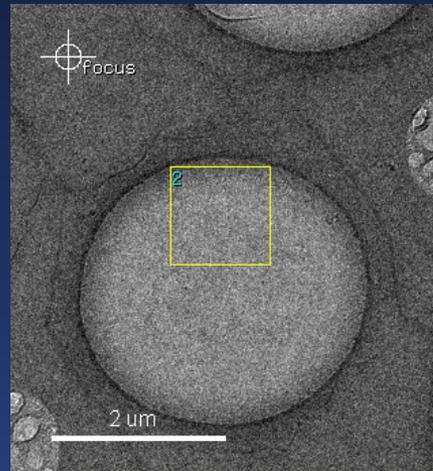
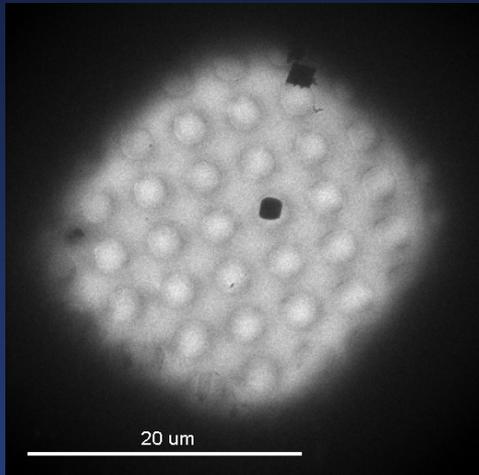
Collaboration: Josh Chappie, Ron Milligan, Sandy Schmid, TSRI

Transferring data from cronus3...

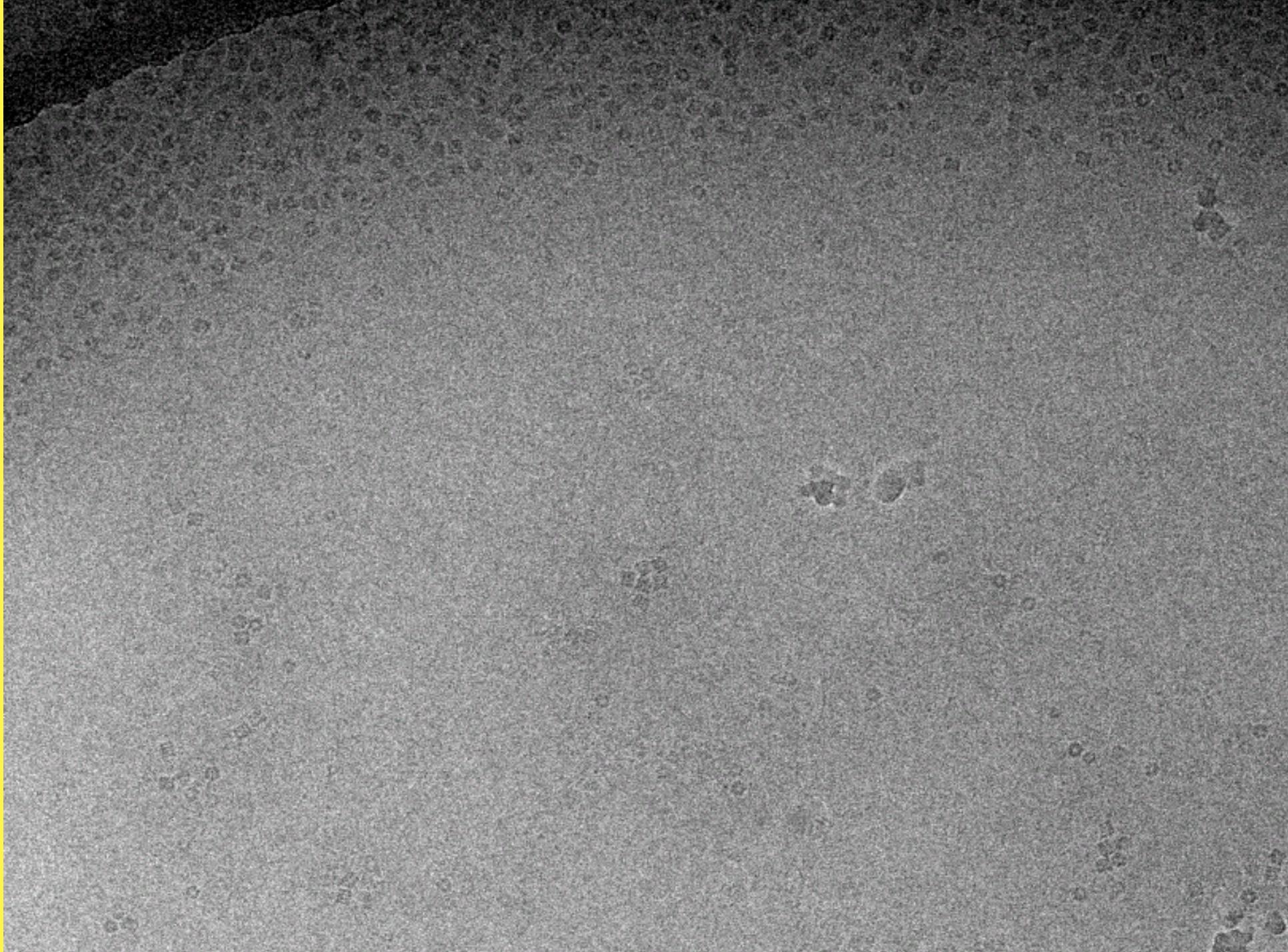
Specimen Preparation



Plasma cleaner

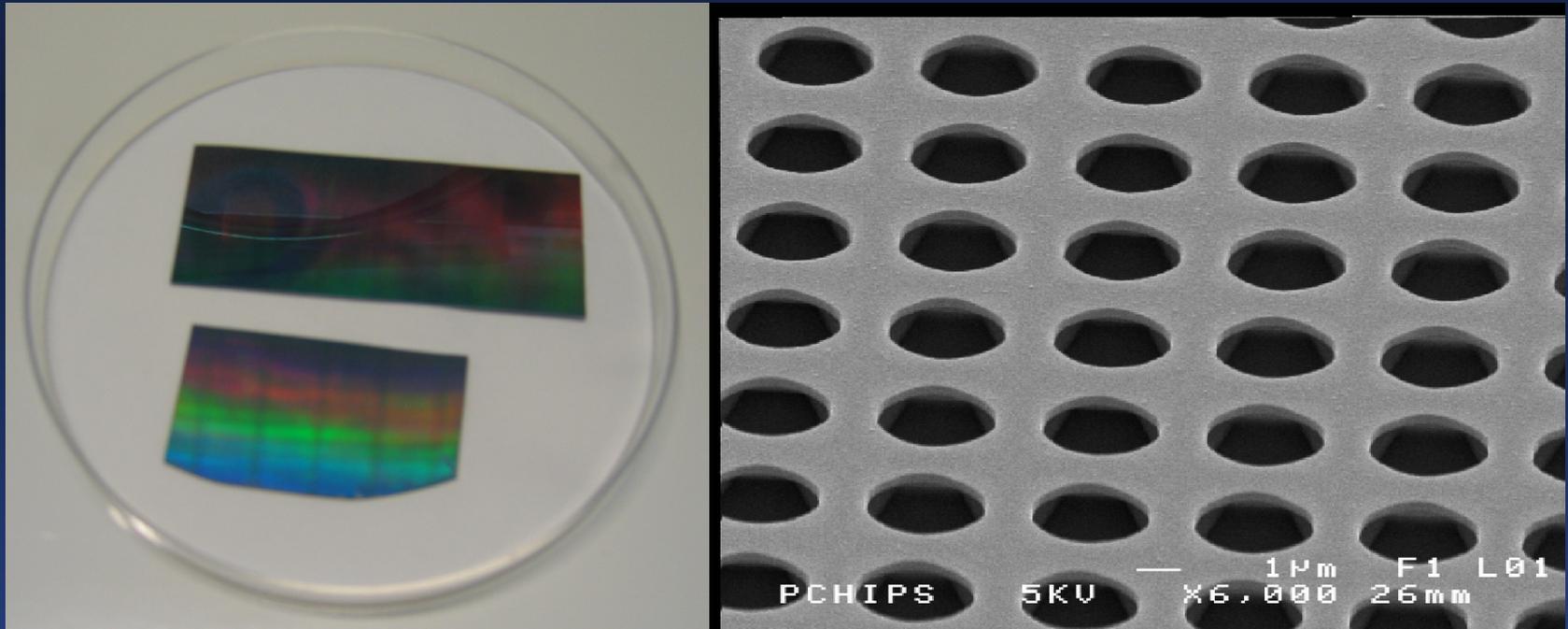


Vitrobot

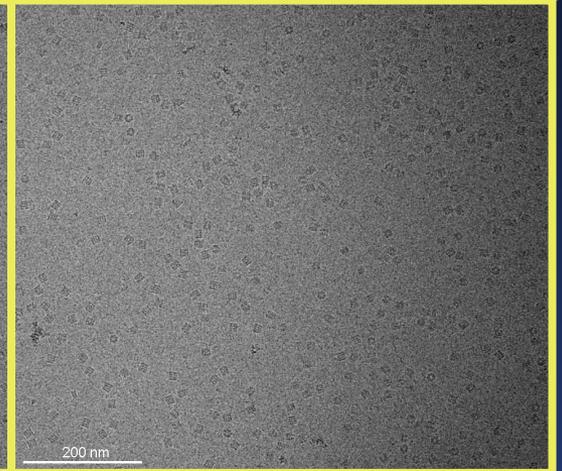
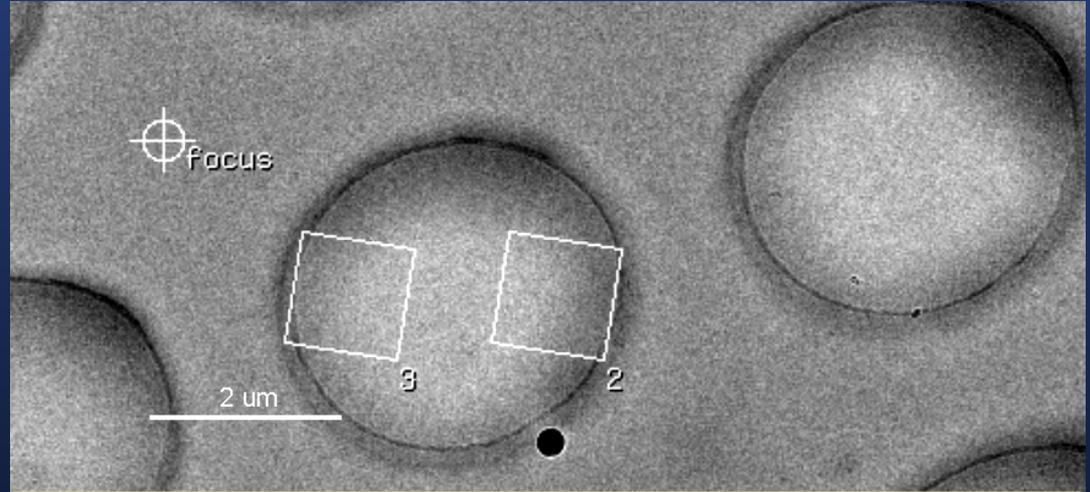
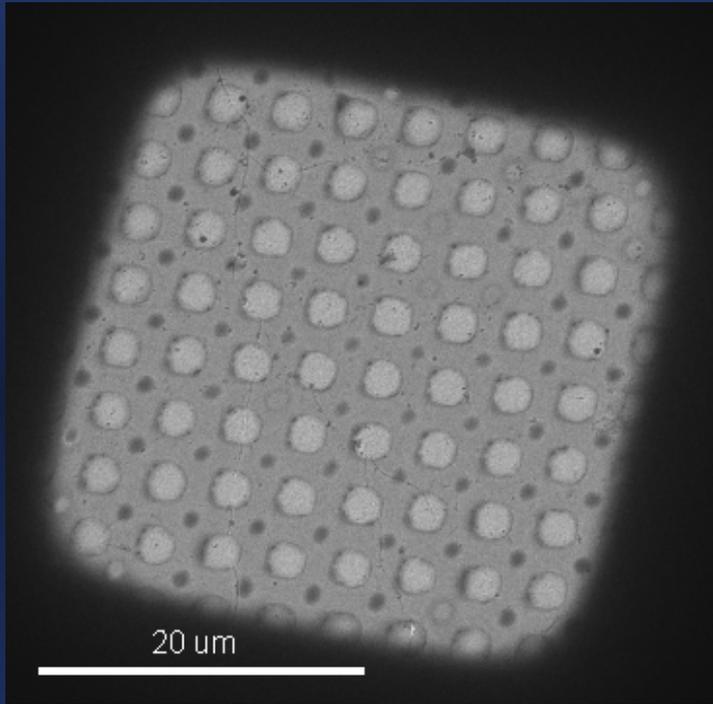


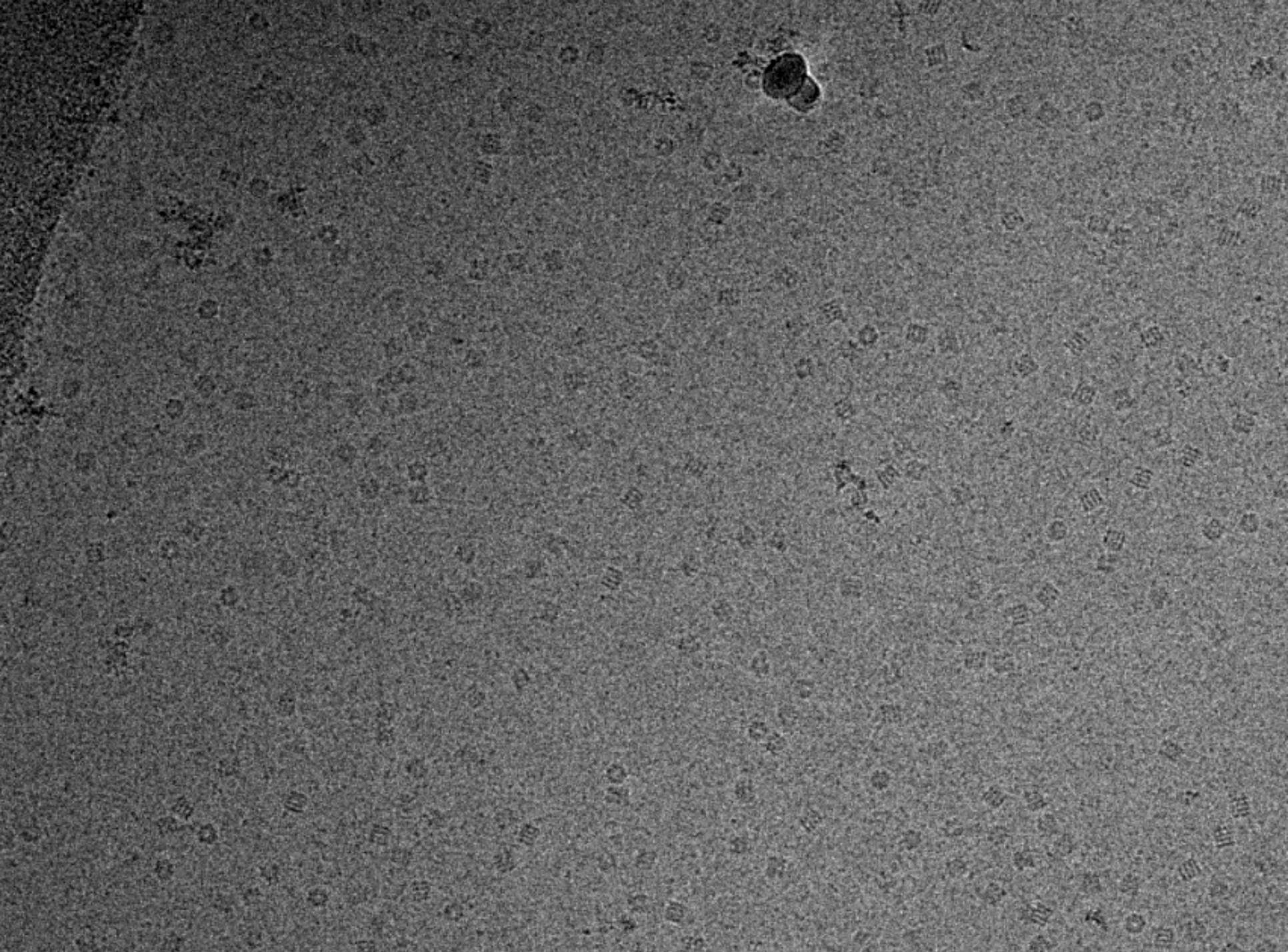
New technique for making an array of holey carbon film

- 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 be made.
- Make a carbon replica of the template using Victawet as a releasing agent.

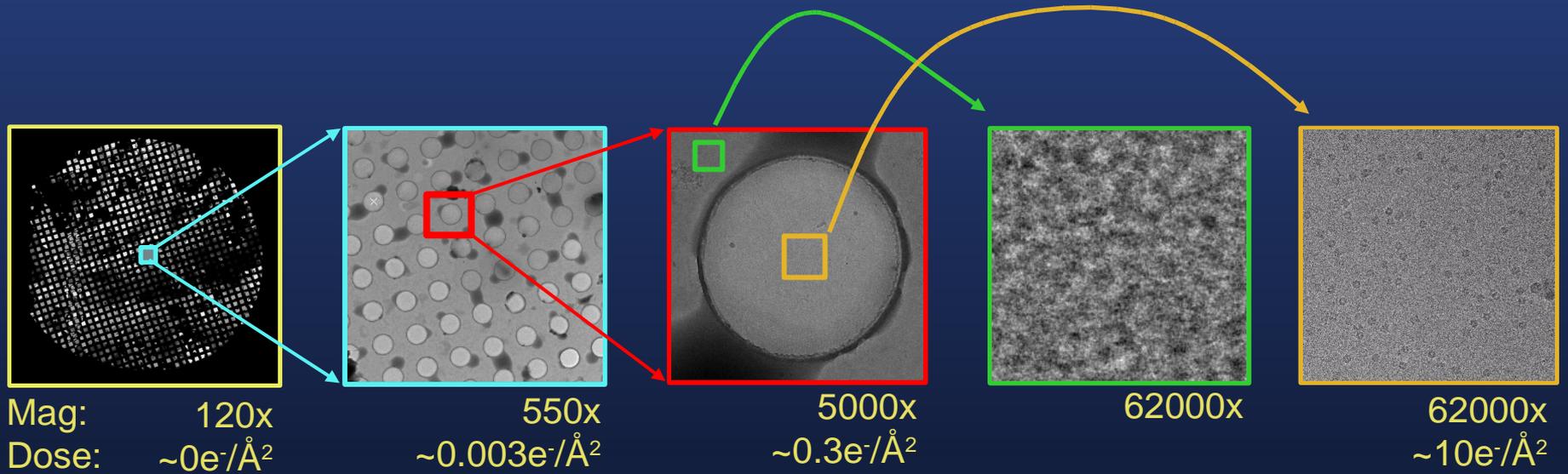


Vitreous ice across JAHC's





Automated image acquisition: A multiscale targeting problem



Grid
2mm x 2mm

Grid square
 ~ 500 squares/grid
20 μm x 20 μm

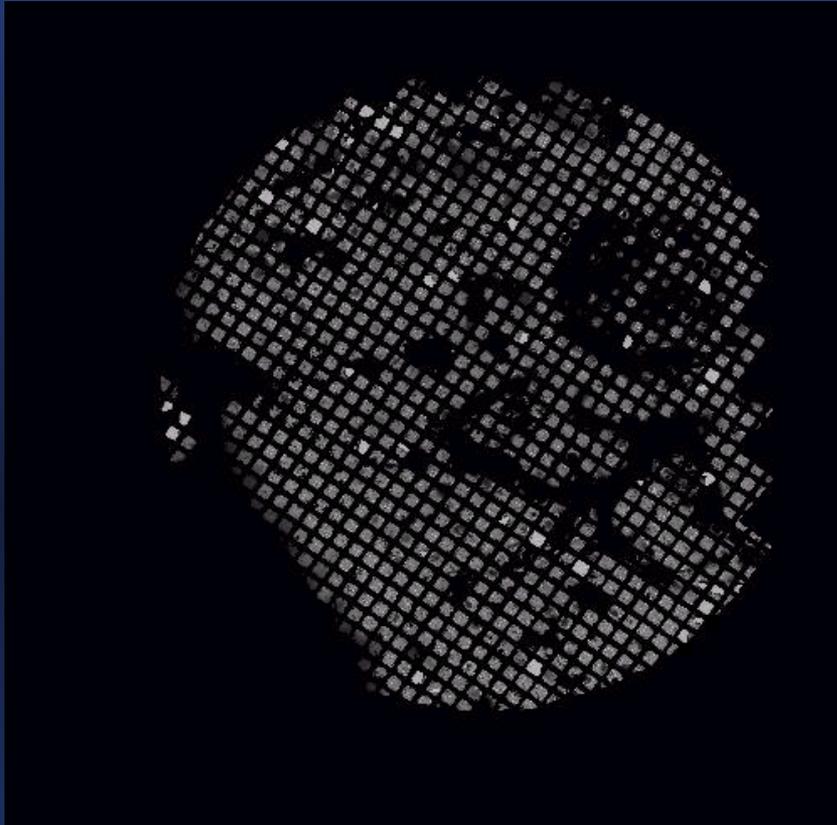
Hole
 ~ 50 holes / square
2 μm x 2 μm

High mag image
4K CCD: ~ 6 / hole
0.5 μm x 0.5 μm

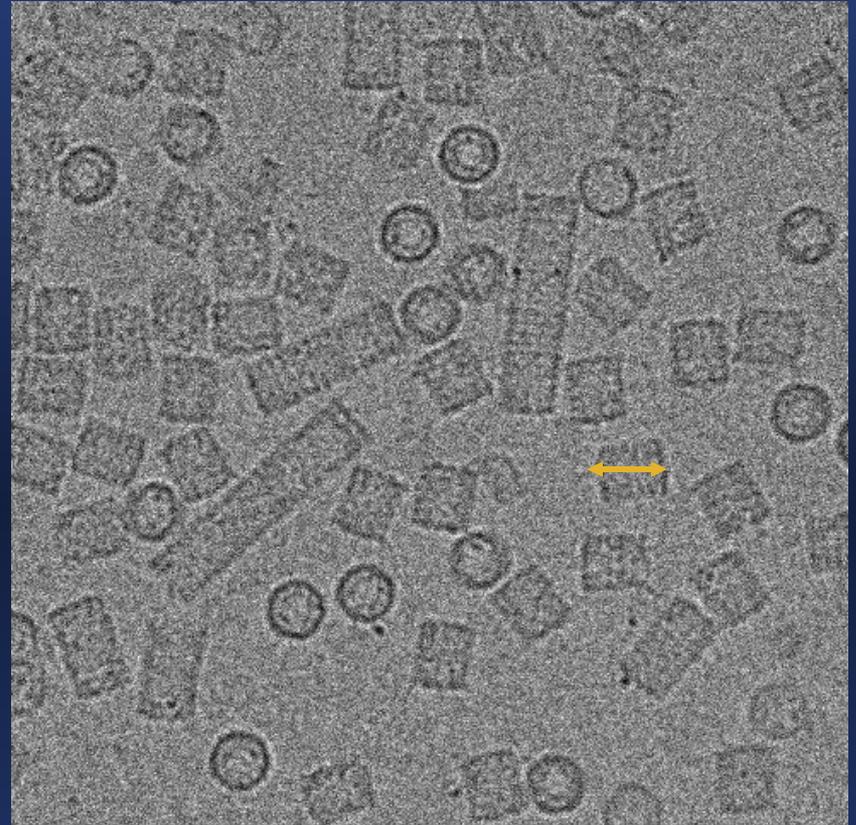
$\sim 25,000$ holes / grid

$\sim 150,000$ high magnification target areas / grid

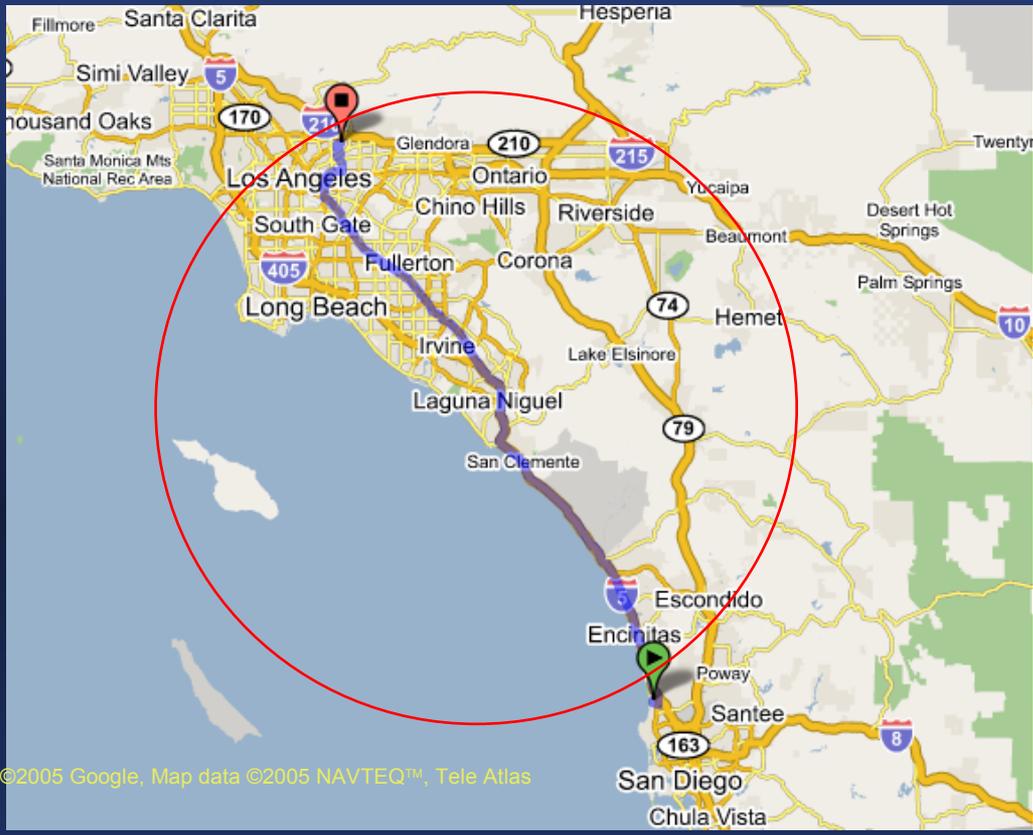
Total dose allowed: $\sim 10 e^-/\text{\AA}^2$



grid: ~2 mm



particle : ~20 nm



Southern California: ~200 km

**Waldo (~2m)
(1m)**

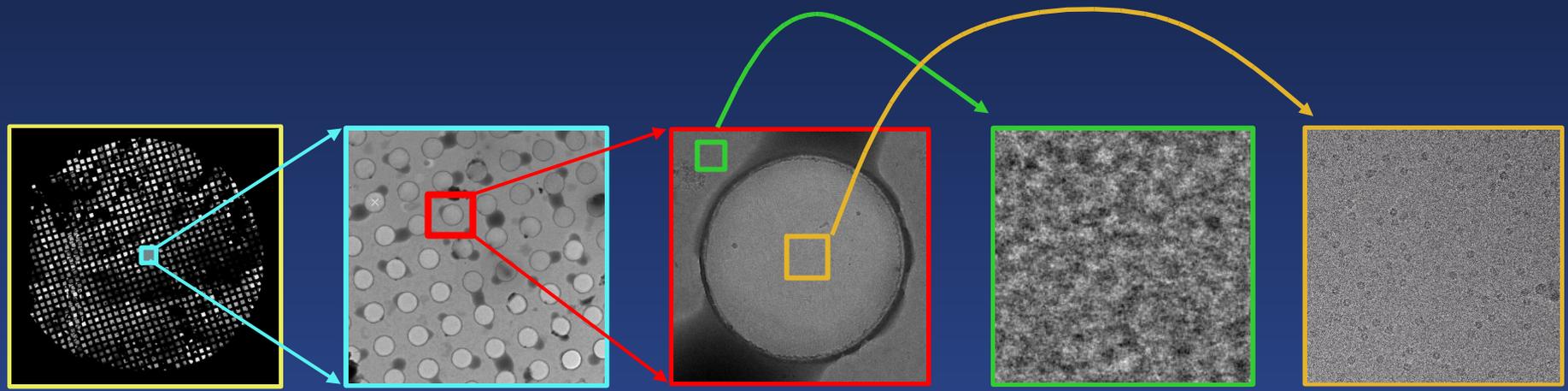
Accurate targeting across a magnification range of ~600 requires accurate and stable calibrations, precisely defined relationships between preset magnification settings, and well defined targeting parameters.

Calibrations:

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

Presets:

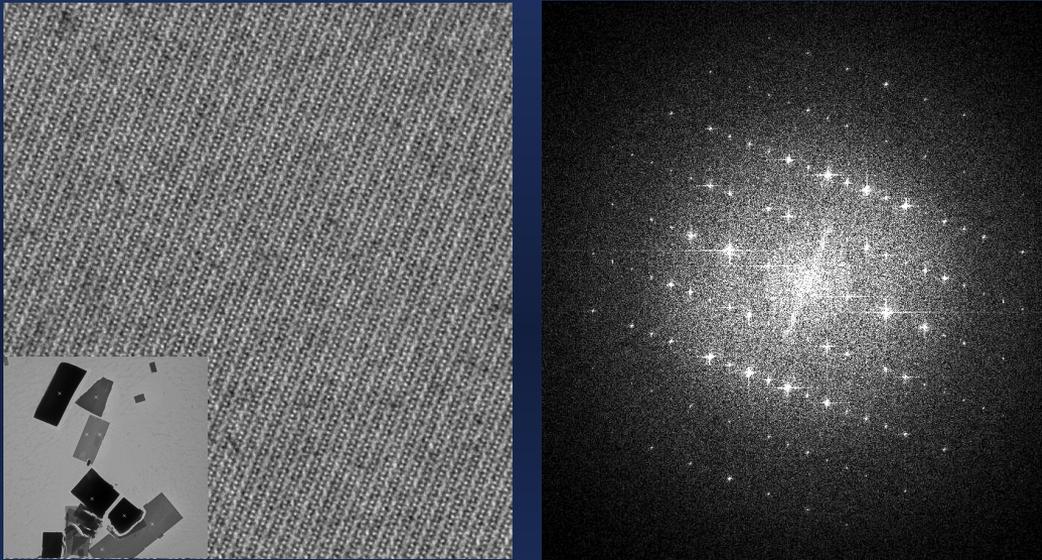
- Atlas
- Square
- Hole
- Focus
- Image



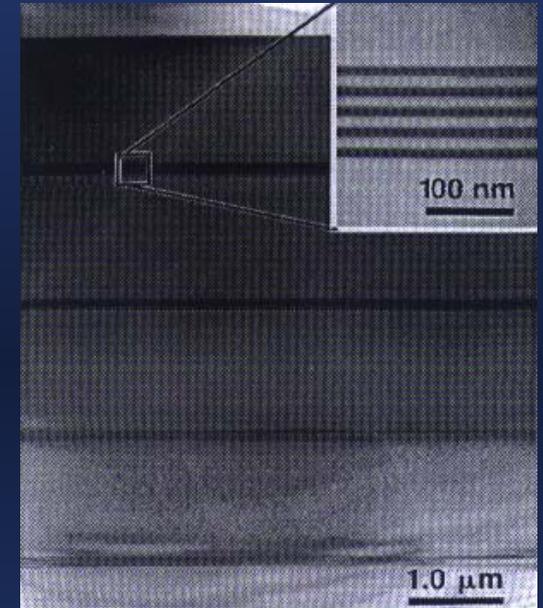
Calibrations: Magnification/Pixel Size @ Specimen

Calibration standards:

Crystals (catalase / TMV)



Diffraction gratings

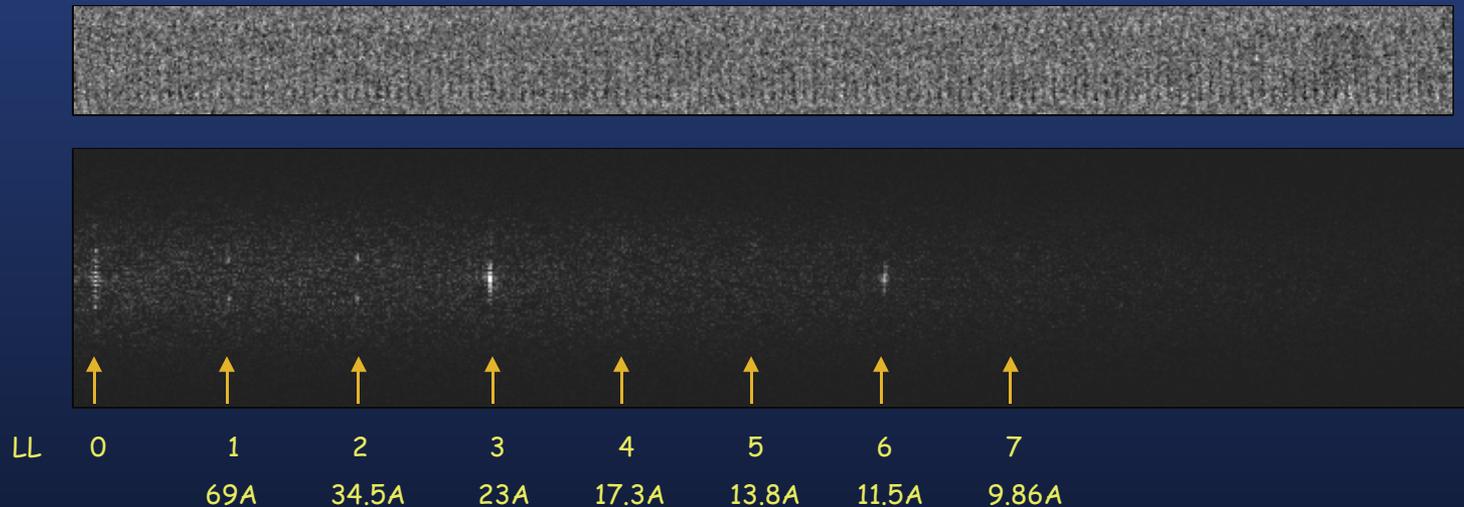


Wrigley, N. G. The lattice spacing of crystalline catalase as an internal standard of length in electron microscopy. *J Ultrastruct Res* 24, 454-64 (1968).

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

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

Microscope Nominal Magnification Setting: 62,000X

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

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

Calibrations: Dose -> Beam Intensity

measured at the specimen

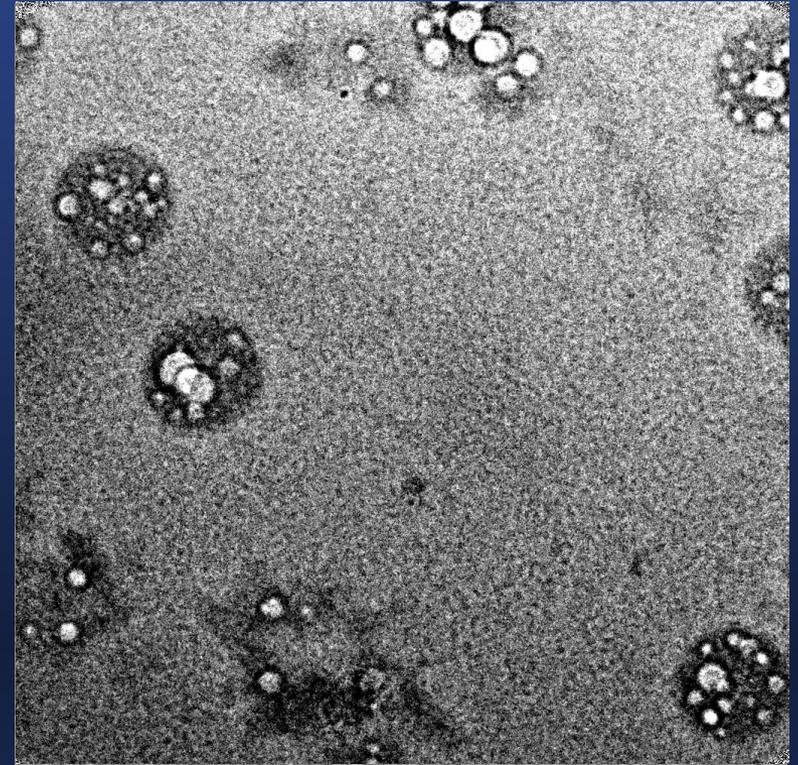
dose in $e^-/\text{\AA}^2$

dose rate in $e^-/\text{\AA}^2/\text{sec}$

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

Analytical Holder w/ Faraday Cup

Screen Current



Measure dose rate from screen current:

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

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

B = beam current (amps)

A = Area on screen (m^2)

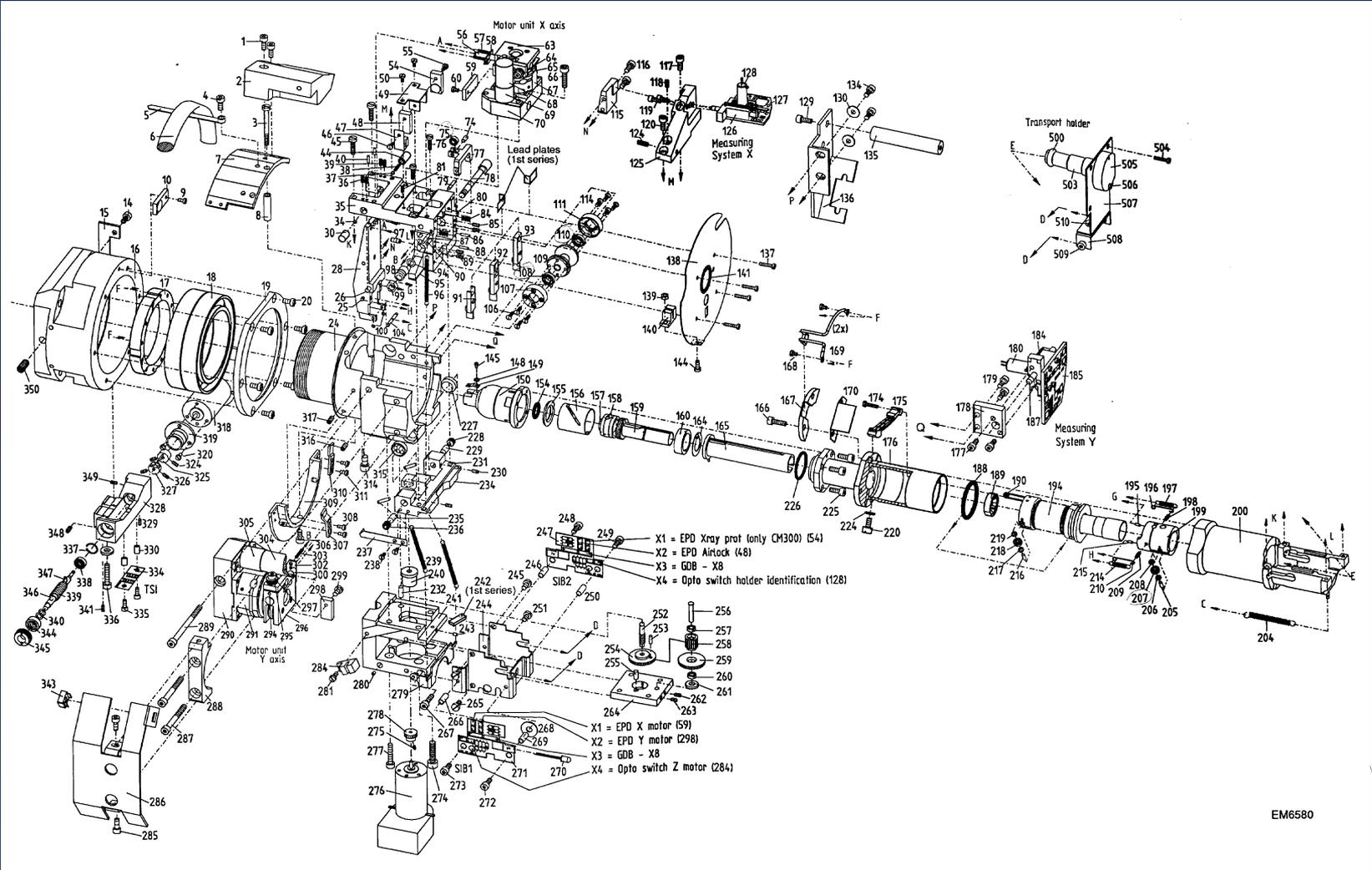
B = C * (measured screen current)

C = correction factor (1.04 on our T1)



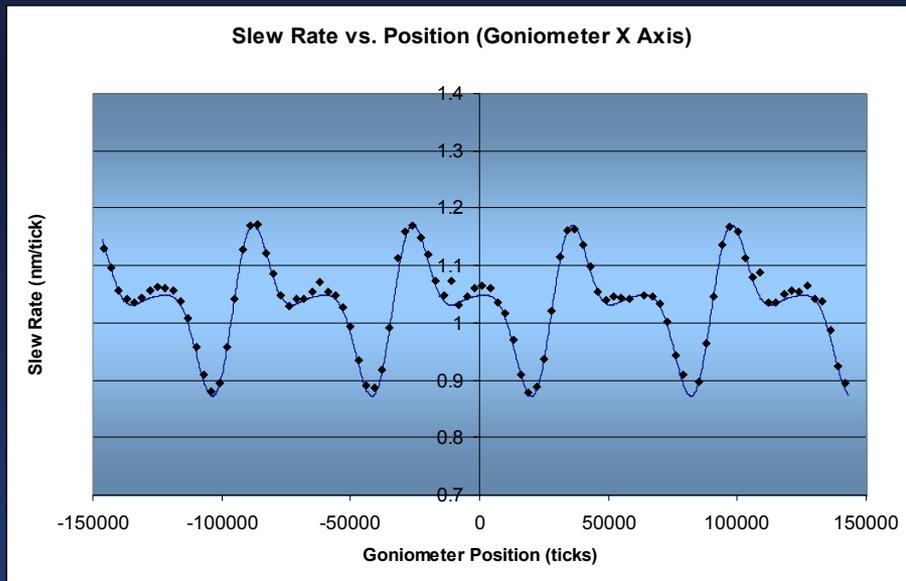
Dose measurements are critical!!

FEI Compustage

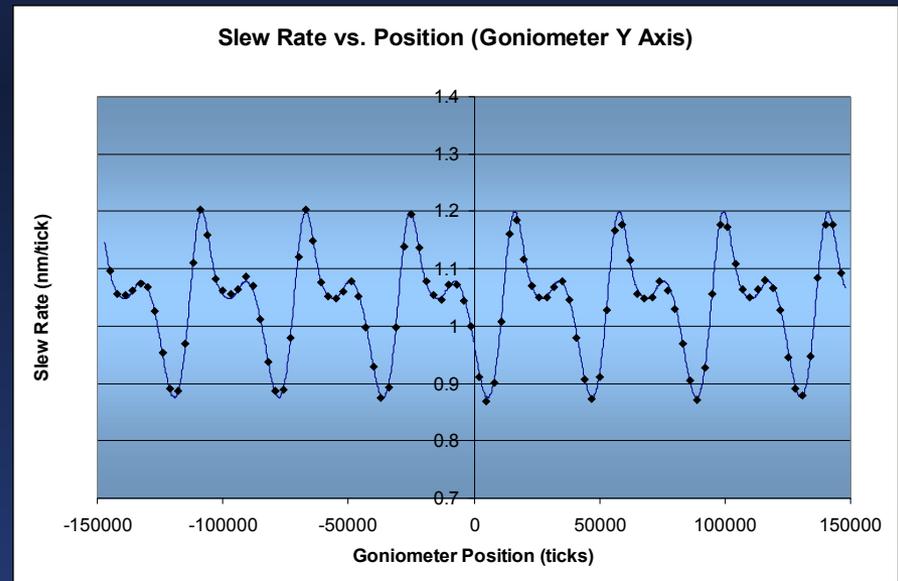


Characterization of Goniometer Slew Rate

- 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:



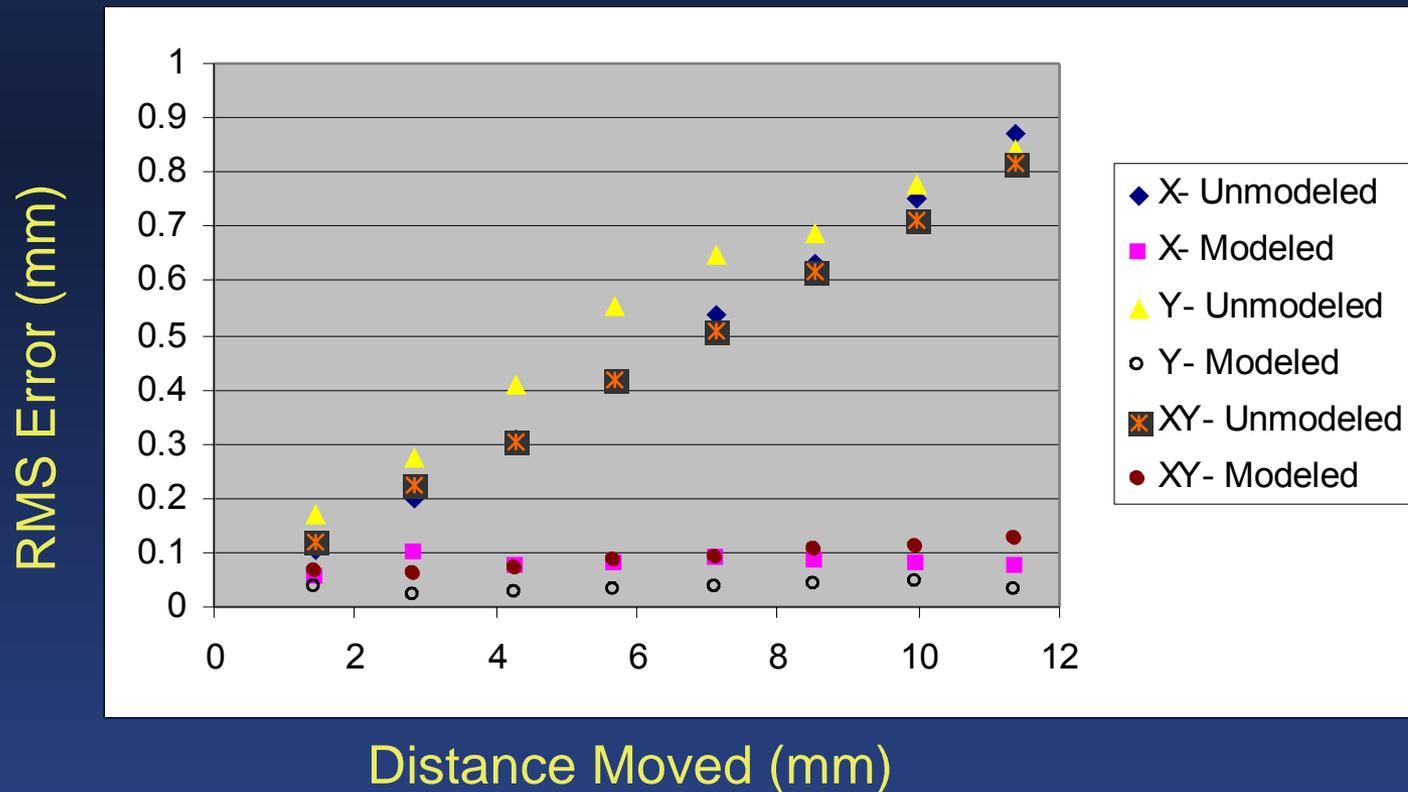
X axis (period = 61.9 mm)



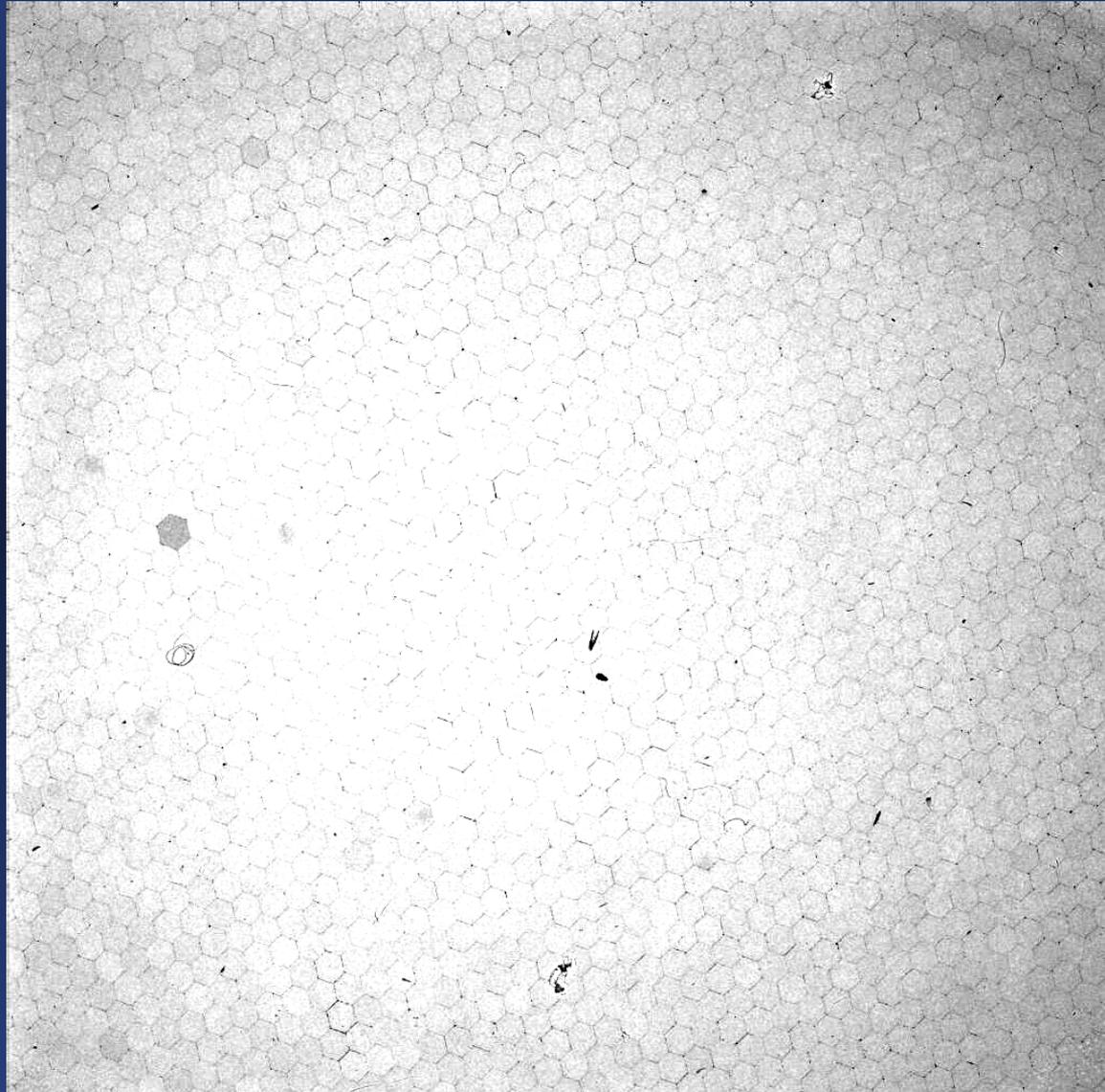
Y axis (period = 41.6 mm)

Goniometer Modeling: Results

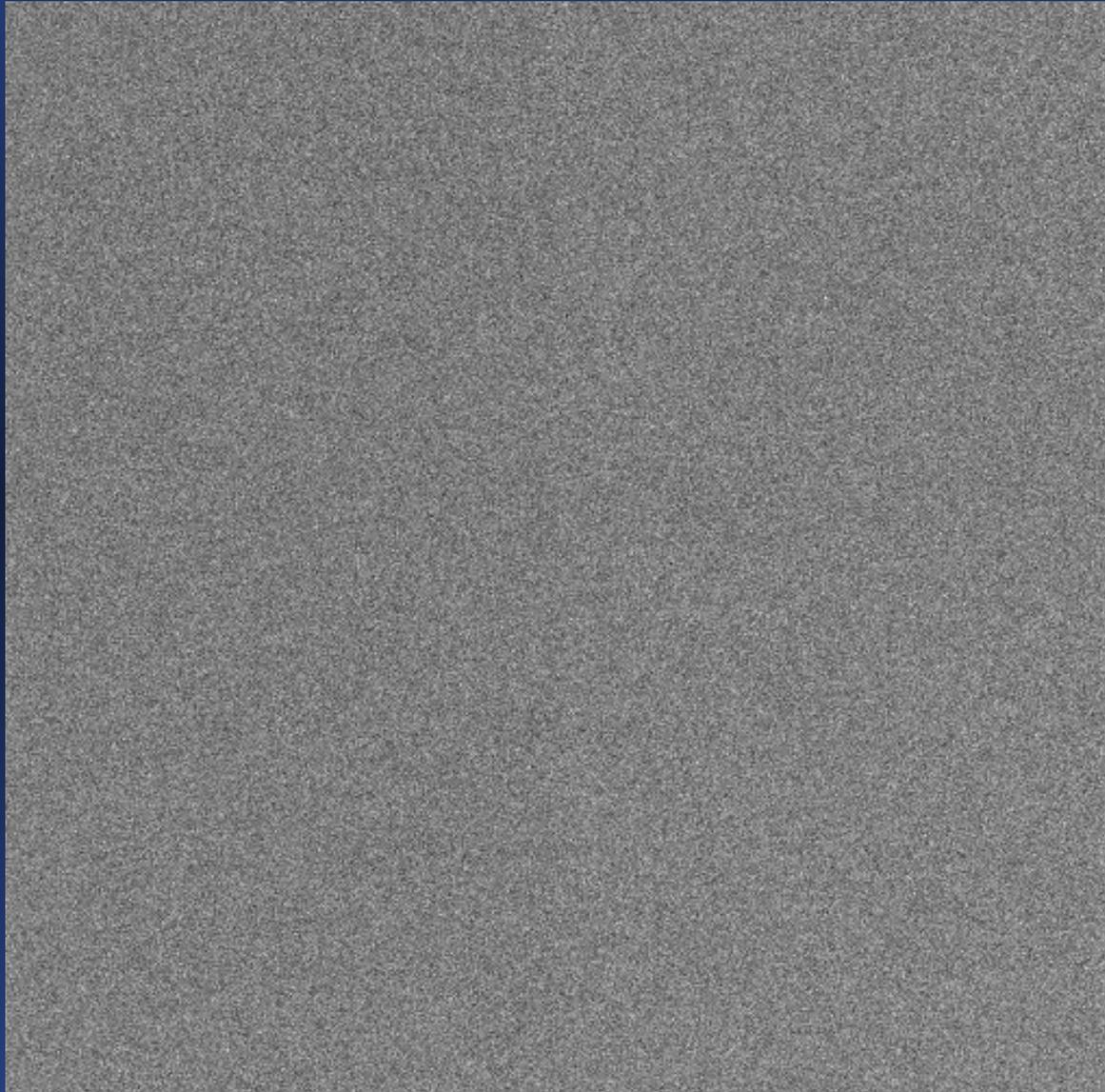
- 2 CompuStages characterized with similar results.
- Slew rate for X and Y modeled with Fourier Series
- Validation: RMS error as a function of distance moved over range of goniometer:
- Pulokas et al., J. of Struct. Biology, 128, p.250-256 (2000)



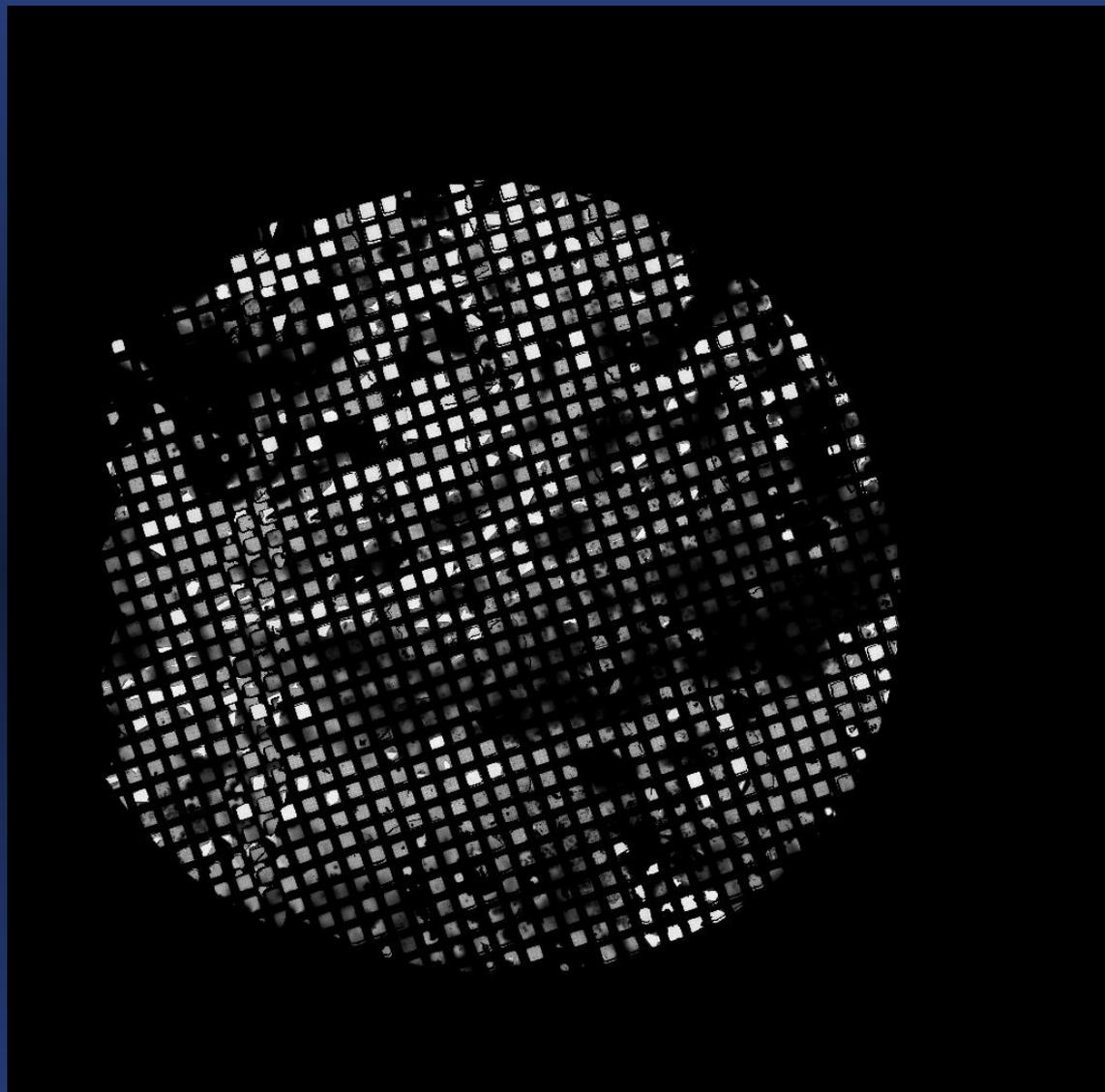
Calibrations: Gain Normalization and Flat Fielding of CCD camera



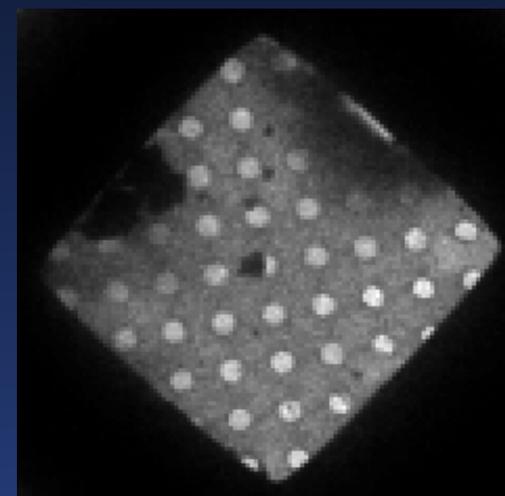
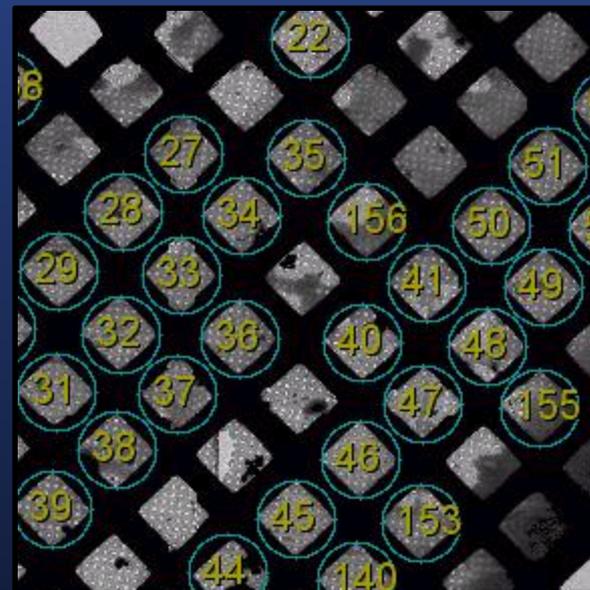
Calibrations: Gain Normalization and Flat Fielding of CCD camera



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



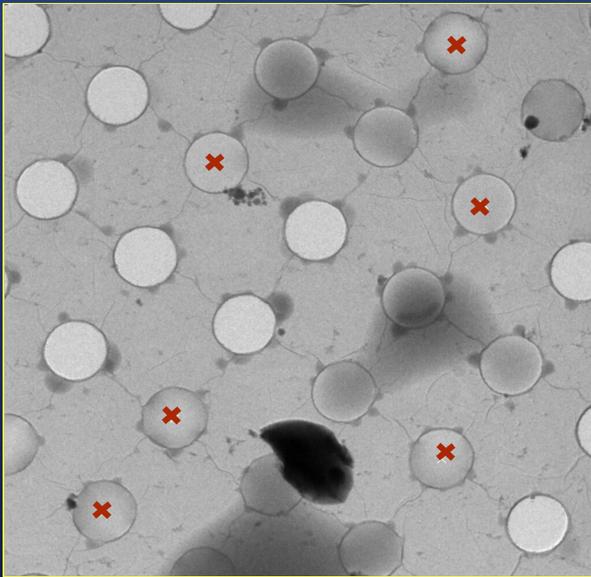
Atlas typically constructed of a mosaic of 25 images (180nm / pixel)



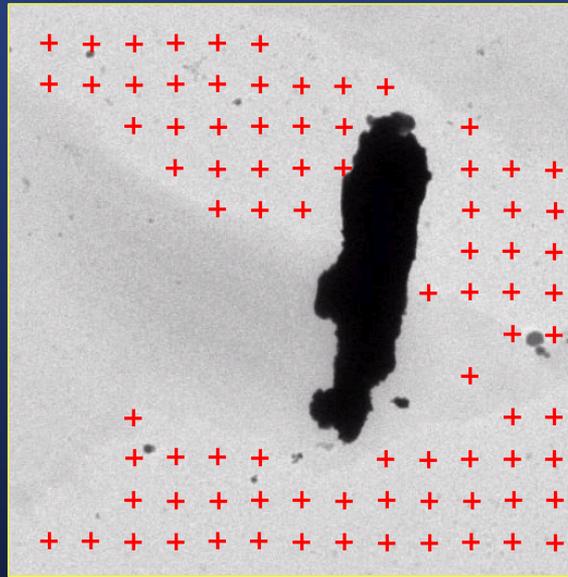
Full resolution

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

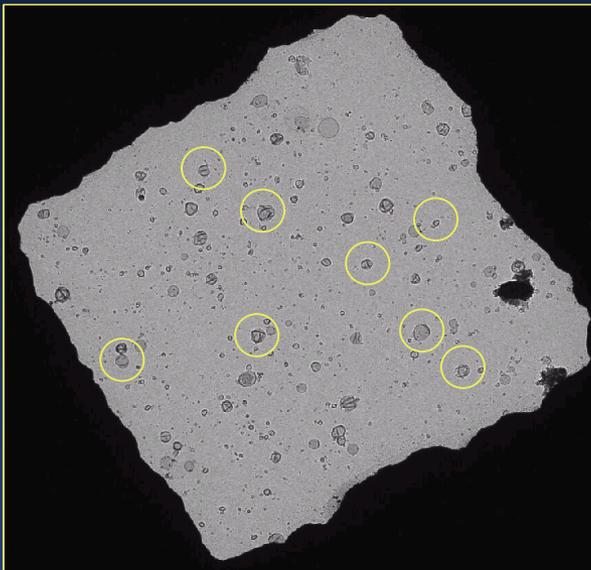
2. Finding targets at low magnification (~600x)



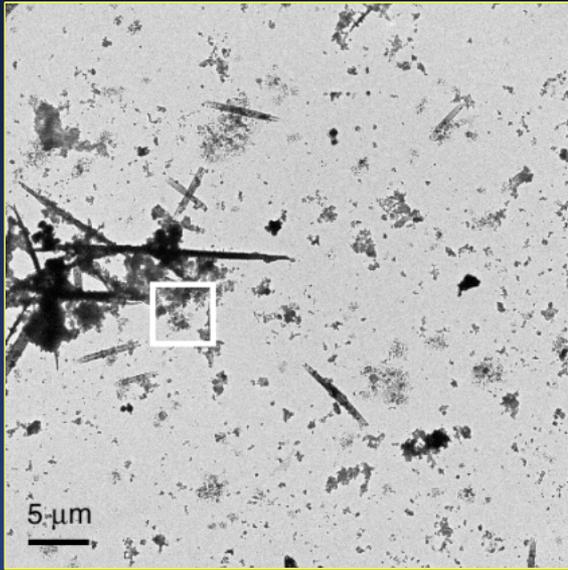
TMV



RNA PolIII



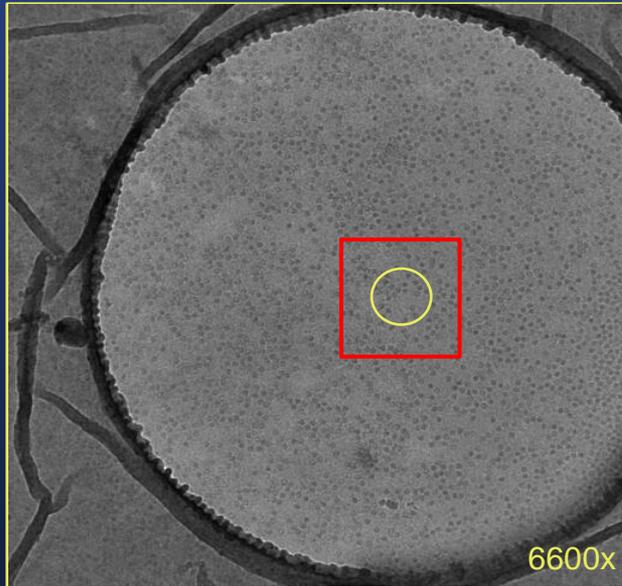
Rhodopsin



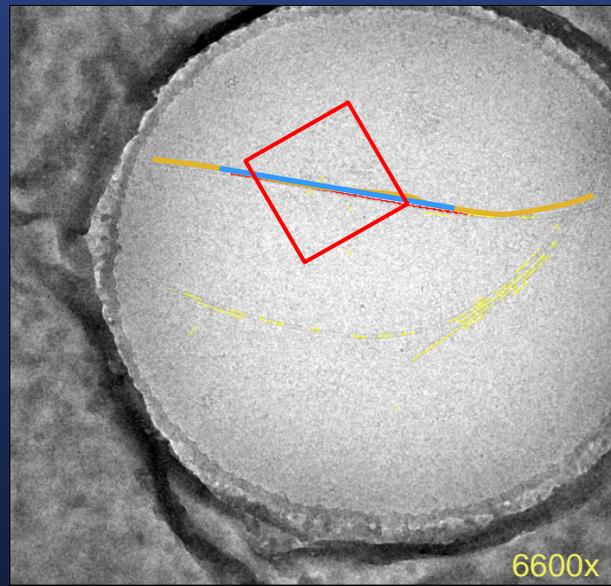
Connexin

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

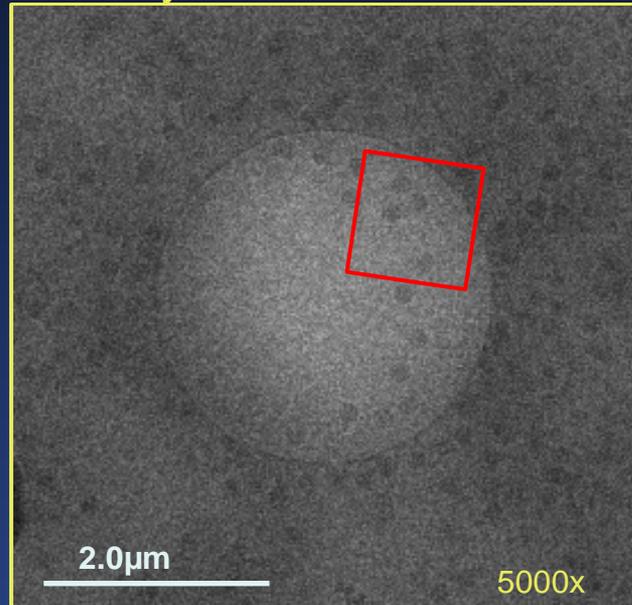
3. Finding targets at intermediate magnification (~6000x)



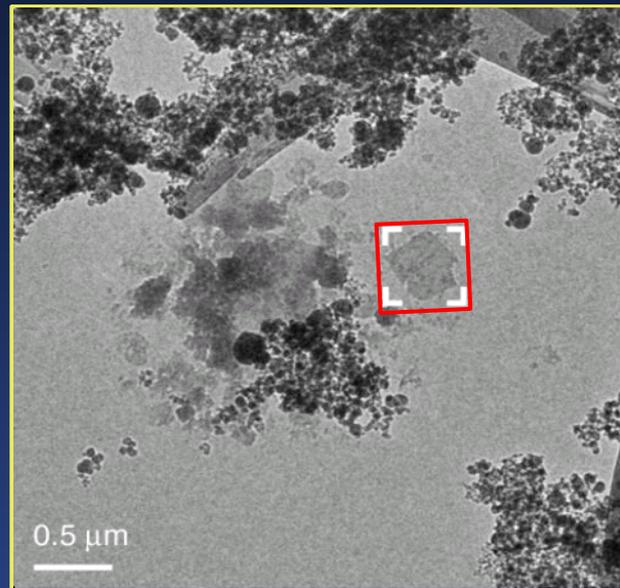
Hemocyanin



Microtubules



Large virus

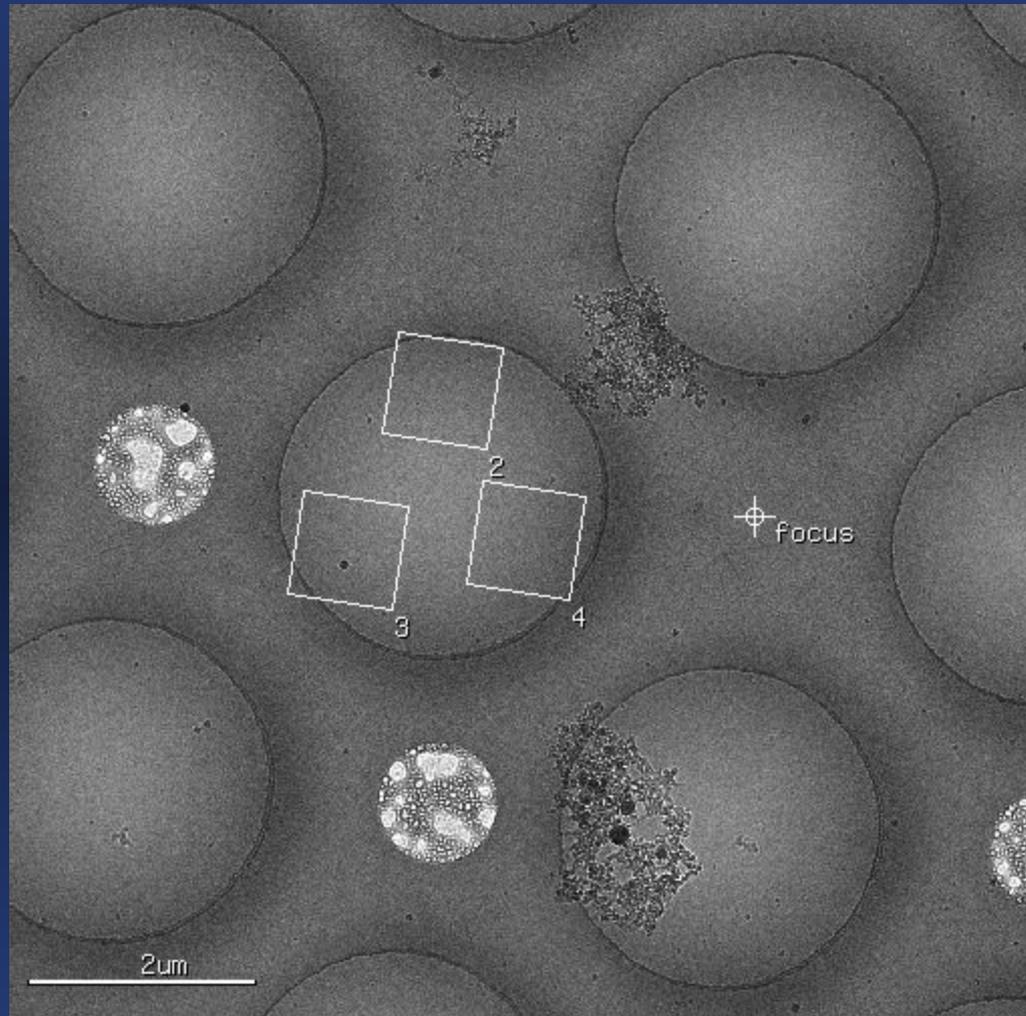


Connexin

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

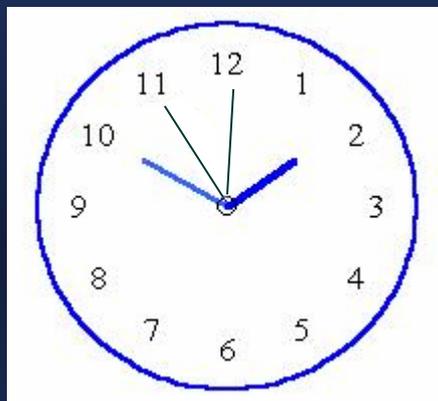
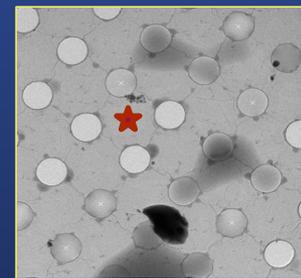


Image1(time1)

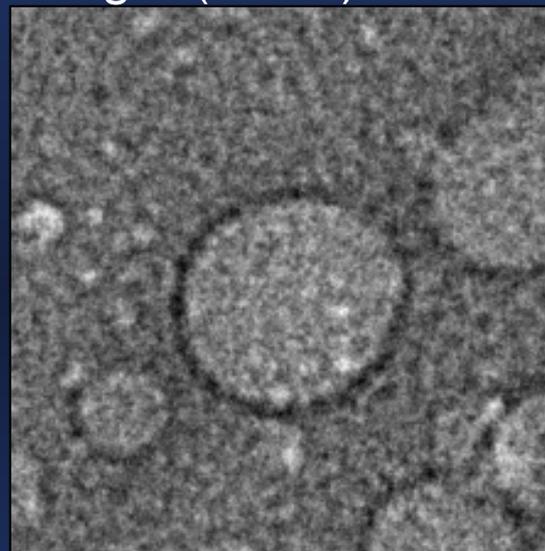


Image2(time2)

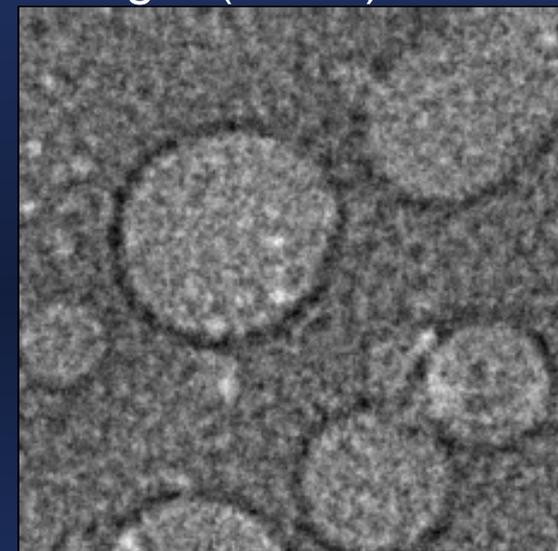


Image displacement

Image displacement(time) \propto (drift)

Focus and astigmatism measure

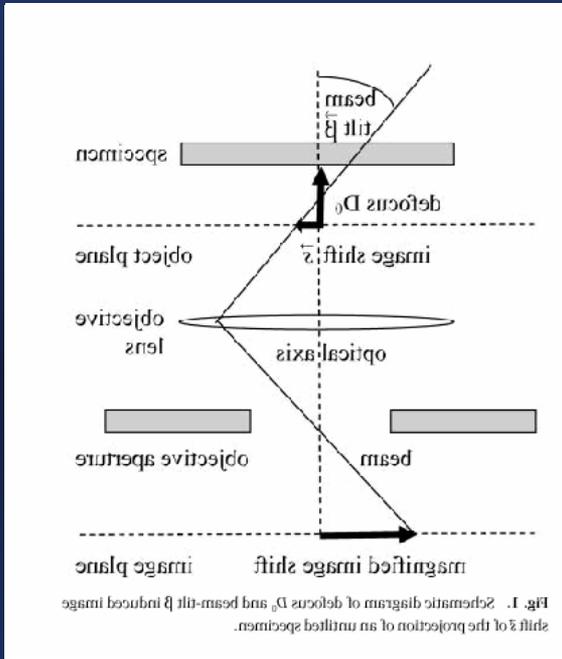
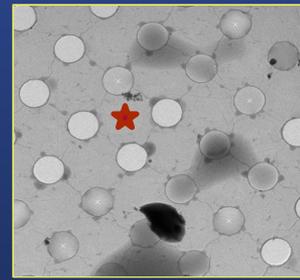


Fig. 1. Schematic diagram of defocus D_0 and beam-tilt β induced image shift of the projection of an untitled specimen.

Image1 (tilt1)

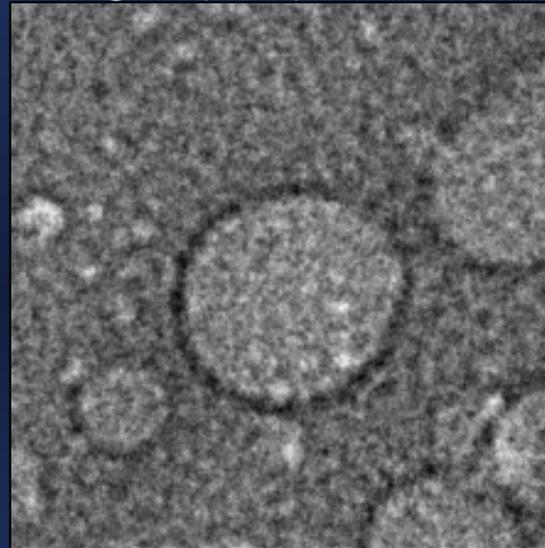


Image2 (tilt2)

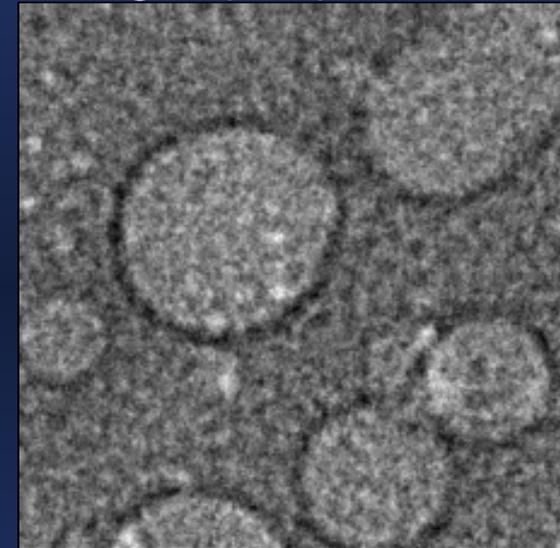


Image displacement

Ziese et al, J. Microsc. 211, 179 (2003)

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

Image displacement (beam tilt) \propto (defocus)

Drift measurement using cross correlation

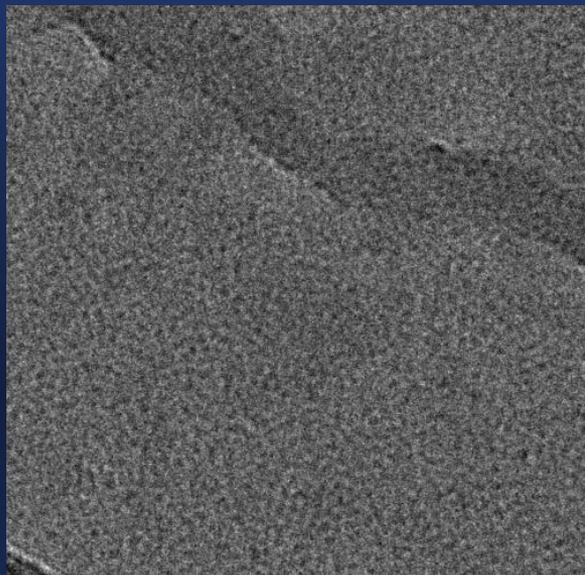


Image1

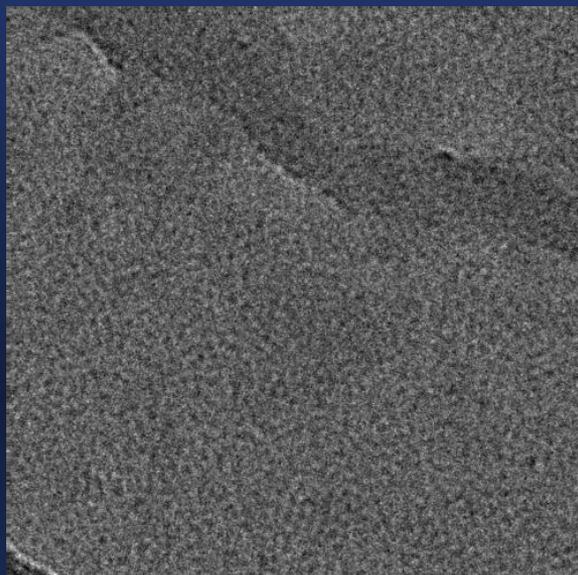


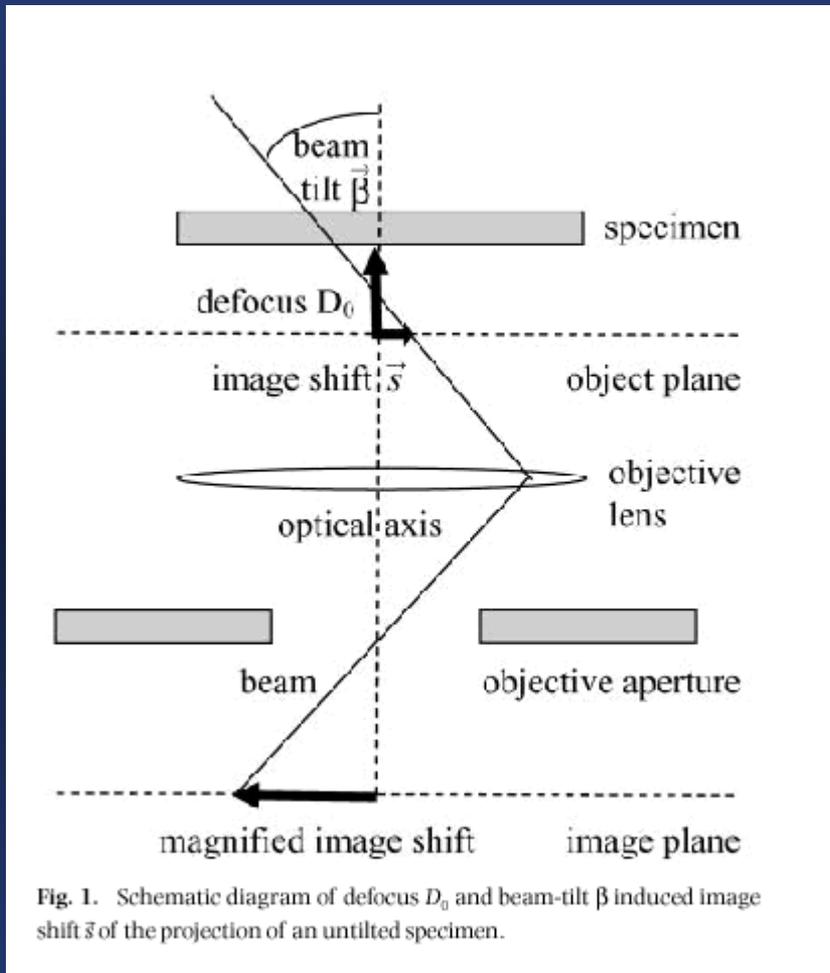
Image2



CC

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

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

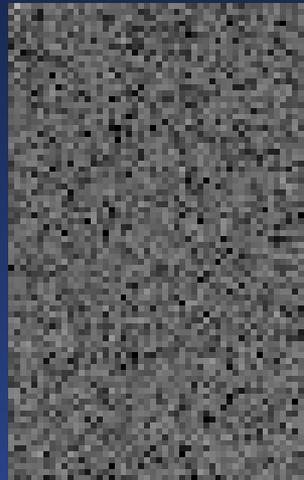
Reference:

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

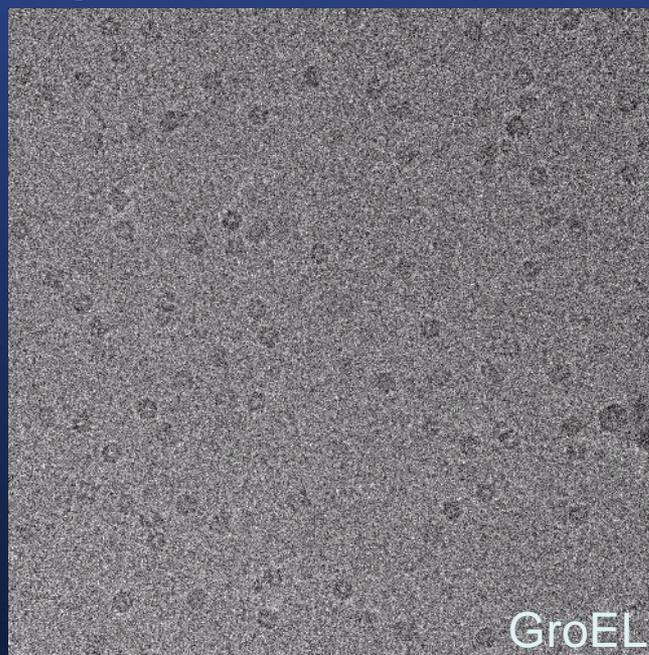
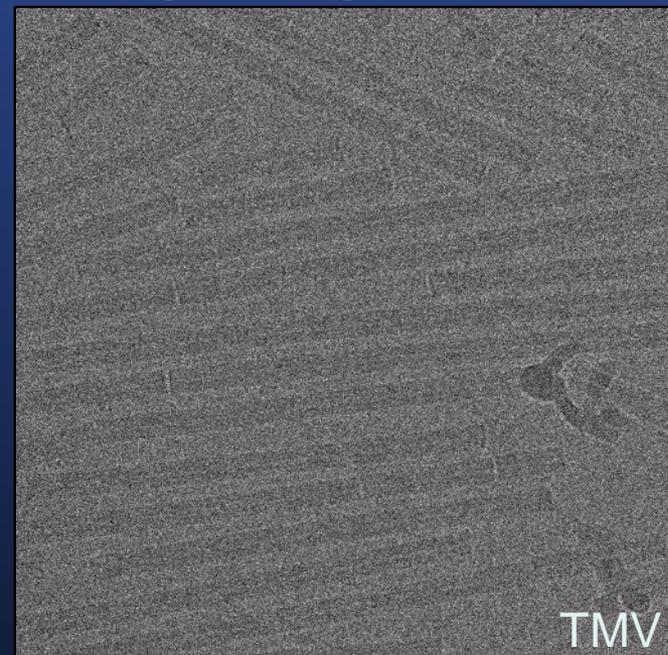
Autofocus accurate to within $\sim 150\text{nm}$

Defocus = $2.1 \pm 0.13 \mu\text{m}$

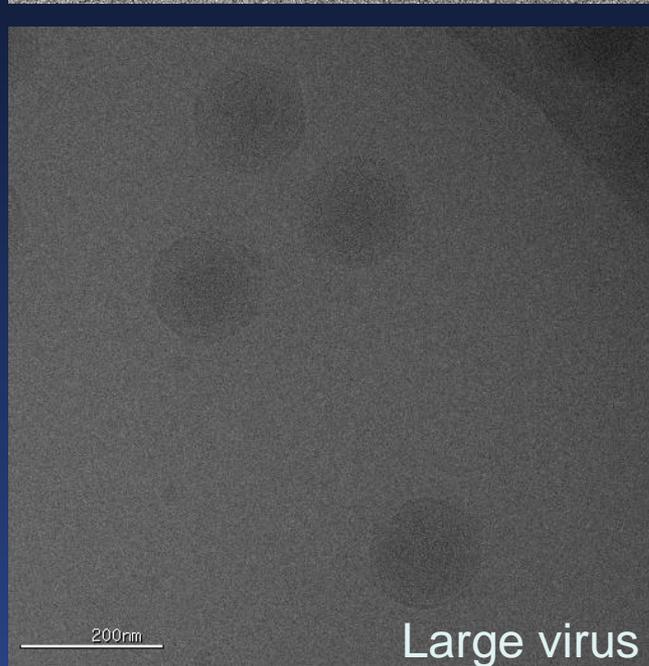
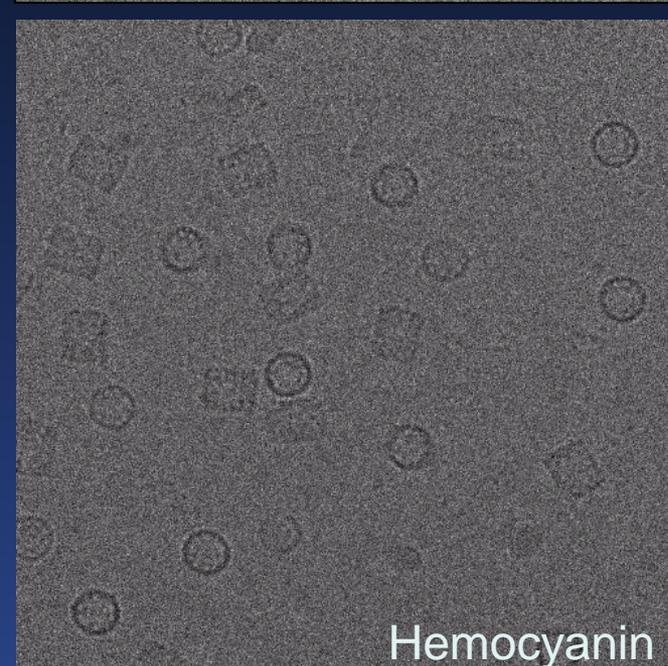
05may19a



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



Acquired as defocus pairs or sequences



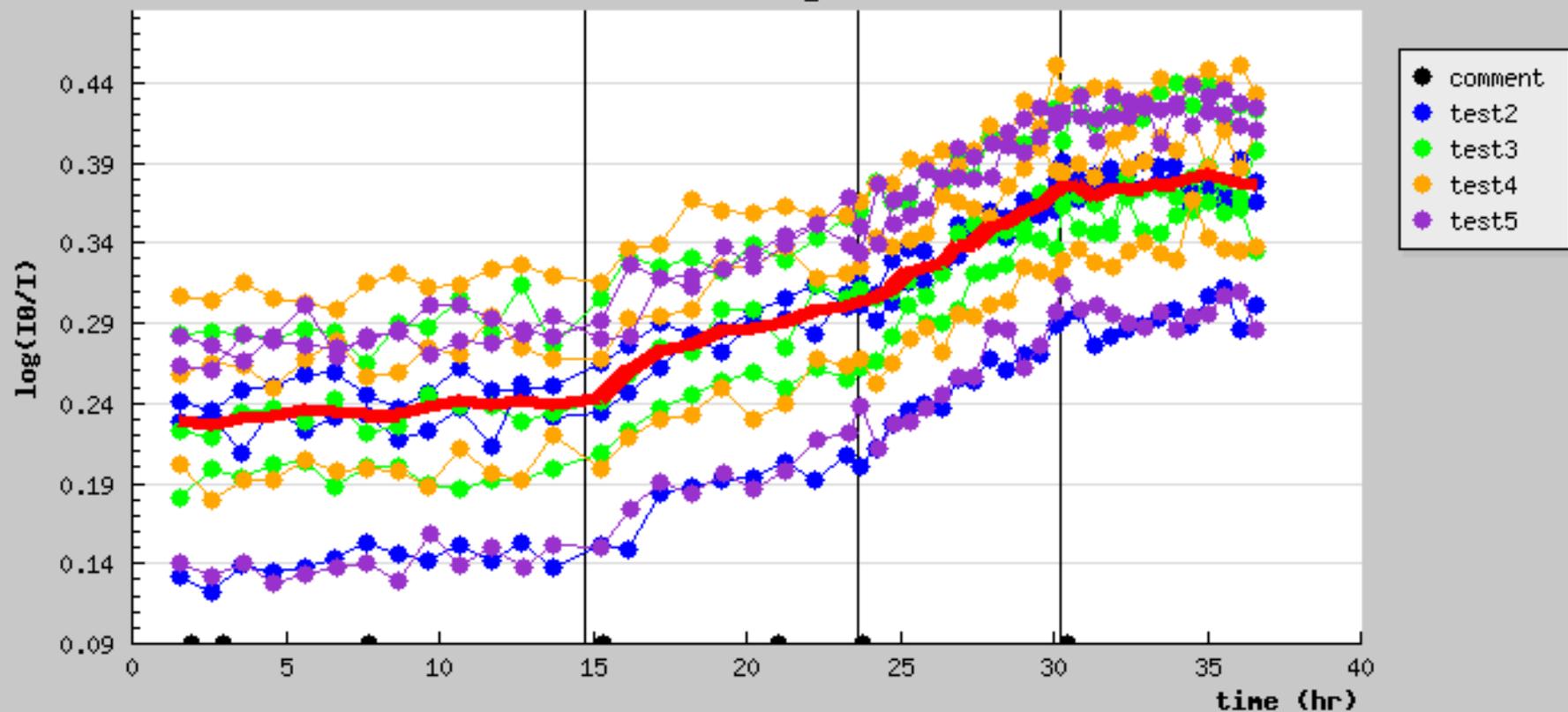
Total dose: $\sim 10 \text{ e}/\text{A}^2$

Leginon Observer Interface (LOI)

The image displays the Leginon Observer Interface (LOI) with six panels, each showing a different sample and its corresponding parameters. Each panel includes a title bar with 'View X', a toolbar with icons for information, zoom, pan, and window management, and a header area with sample name, magnification, defocus, and pixel size. The main area shows the sample image with a scale bar and various annotations.

- View 1:** Sample: enr. Parameters: mag: 50000, defocus: -1.0000 μm , pixelsize: 0.1630 nm. Image shows a textured surface with a 200nm scale bar.
- View 2:** Sample: hole. Parameters: mag: 5000, defocus: -150.0000 μm , pixelsize: 1.6358 nm. Image shows a textured surface with a 2 μm scale bar, a 'focus' label, and numbered boxes (2-8).
- View 3:** Sample: sq. Parameters: mag: 550, defocus: -2000.0000 μm , pixelsize: 14.8709 nm. Image shows a square grid pattern with a 20 μm scale bar and numbered boxes (1-4).
- View 4:** Sample: foc. Parameters: mag: 50000, defocus: -2.0000 μm , pixelsize: 0.1630 nm. Image shows a textured surface with a small dark spot.
- View 5:** Sample: foc. Parameters: mag: 50000, defocus: -2.0000 μm , pixelsize: 0.1630 nm. Image shows a textured surface with a 20nm scale bar.
- View 6:** Sample: grid. Parameters: mag: 120, defocus: 0.0000 μm , pixelsize: 68.1583 nm. Image shows a grid pattern with a 100 μm scale bar and numbered boxes (1, 2).

Contamination sessions: Starting from 05aug28b
ice grid



Film

-

+

+

-

Aperture

+

+

-

+

Leginon Database: Images and Acquisition Parameters

- Multi-scale: Keeps track of relationships between scales.

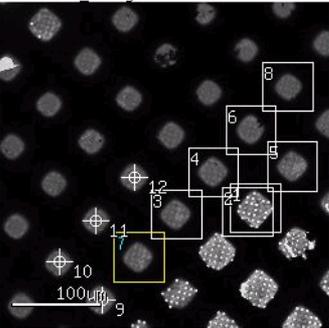
04mar16a - PBCV-1, 25mg/ml, blot 2.5sec., new batch from J. Gurgon in Nebraska

View 1

grid adjust

mag: 120 defocus: 0.0000 μm pixelsize: 93.0800 nm

04mar16a_0036grid.mrc

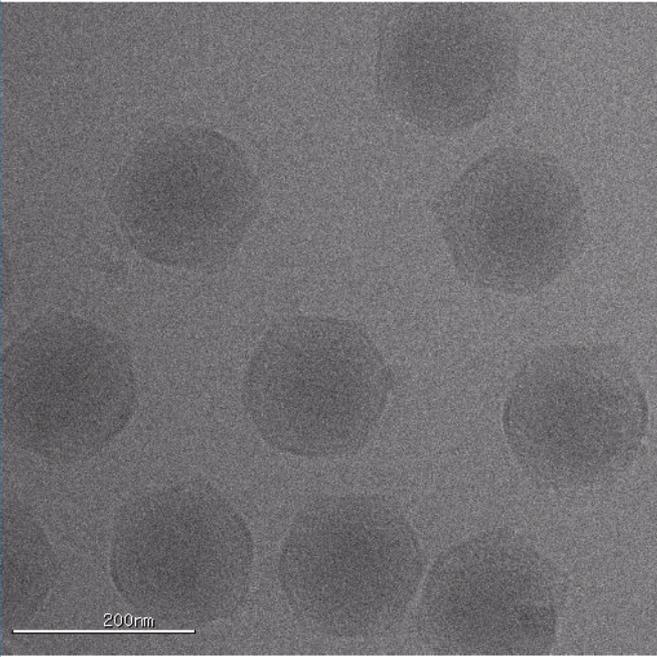


Main View

efar adjust

mag: 62000 defocus: -2.0000 μm pixelsize: 0.1791 nm dose: 10.6336 $\text{e}^{-}/\text{\AA}^2$

04mar16a_0036grid_0007sq_0003hole_0002efar.mrc



View 3

hole adjust

mag: 5000 defocus: -150.0000 μm pixelsize: 2.2328 nm

04mar16a_0036grid_0007sq_0003hole.mrc



Image Report: 04apr07a_00046grid_00019sq_00005hole_00003efar.mrc - Microsoft Internet Explorer

Created with HyperSnap-DX 4
To avoid this stamp, buy a license at <http://www.hyperionics.com>

Address: <http://cronus3.scripps.edu/dbem/imgreport.php?id=332878&preset=efar>

General	
Filename:	04apr07a_00046grid_00019sq_00005hole_00003efar.mrc
Size:	513 kB
Acquired:	2004-08-04 17:44:45
Path:	/ami/data04/leginon/04apr07a/
Session:	04apr07a - PBCV-1, 25 mg/ml, 200kV, qfoil R2/4, blot 3.5 secs., good grid
Instrument:	Tecnai 1 - Tecnai F20 and Gatan 4k

Image Information	Mrc Header Information
imageid: 33287	nx: 512
preset: efar	ny: 512
dimx: 512	mode: MRC_MODE_UNSIGNED_SHORT
dimy: 512	alpha: 90
binning: 8	beta: 90
high tension: 200000 V	gamma: 90
mag: 62000	amin: 26886
defocus: -1.0000 μm	amax: 45680
pixelsize: 0.1791 nm	amean: 0.162334442139
	xorigin: 2.34128947419E-41
	yorigin: 35943.1914062

Parent Image Information	Image Relations
parentid: 33282	grid: 04apr07a_00046grid.mrc
parentimage: 04apr07a_00046grid_00019sq_00005hole.mrc	sq: 04apr07a_00046grid_00019sq.mrc
parentpreset: hole	hole: 04apr07a_00046grid_00019sq_00005hole.mrc
parenttype: acquisition	enr: 04apr07a_00046grid_00019sq_00005hole_00003enr.mrc
parentnumber: 3	fcc: 04apr07a_00046grid_00019sq_00005hole_00001fcc.mrc
targetx: 388	last: < back
targety: 165	
targetdim: 41.078715669086	
targetdiam: 58.094076824089	

Thumbnail



Data Tree view >