4. Data Acquisition

Christos Savva

Outline

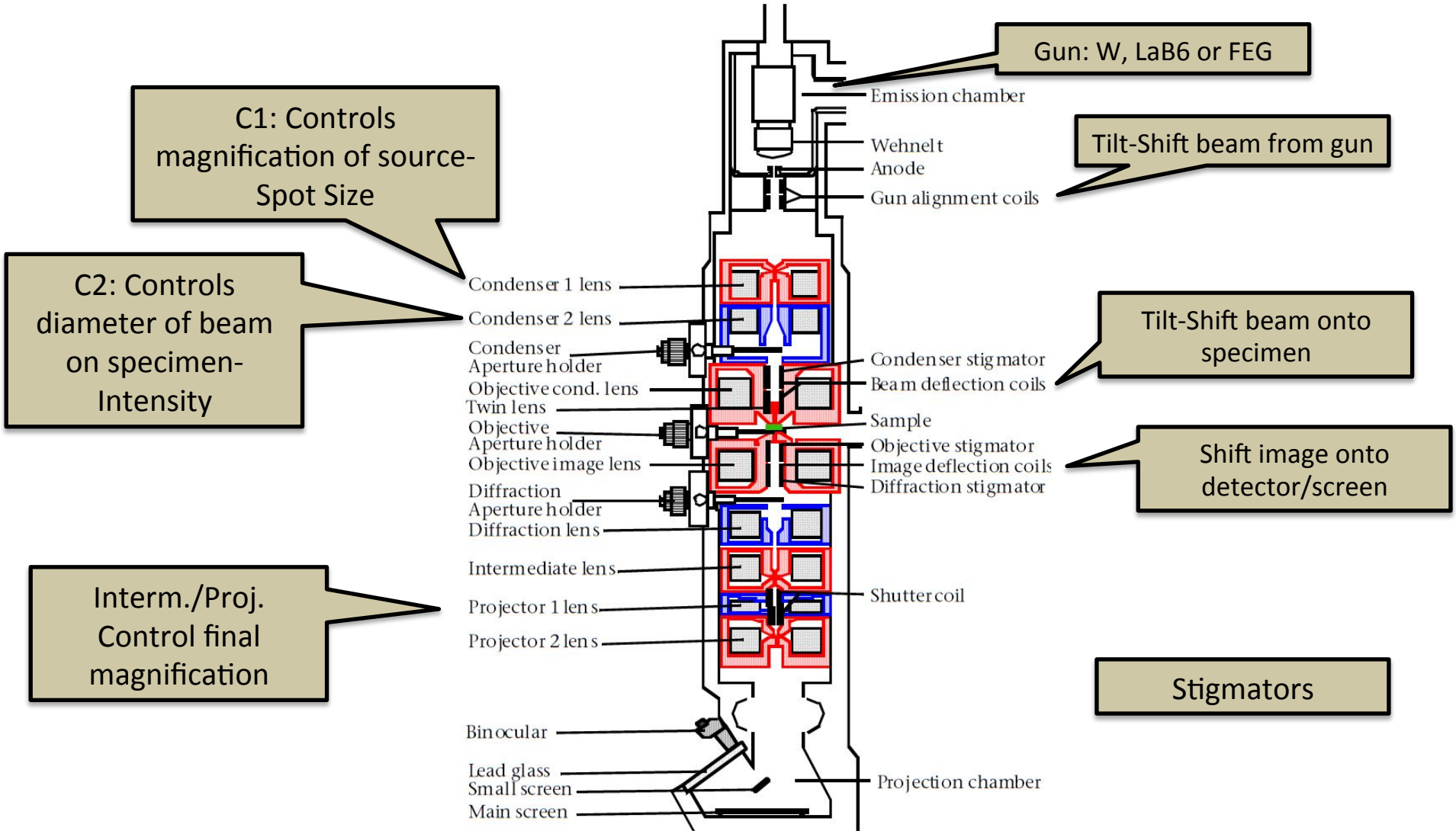
- Aligning the Microscope
- Choice of Microscope
- Choice of Detector
- Initial screening
- Data Collection/Software

Data Acquisition

Procedure

1. Load Samples
2. Align the microscope
3. Find a suitable grid
4. Setup data collection

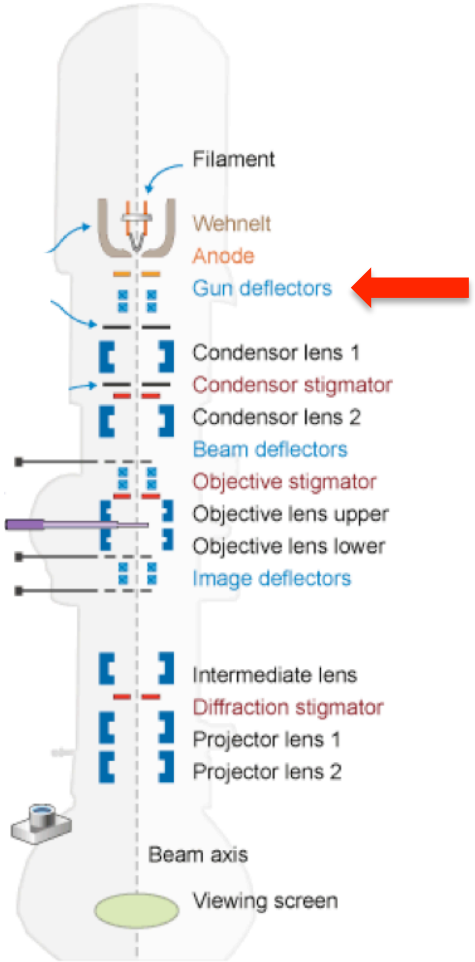
Microscope parts

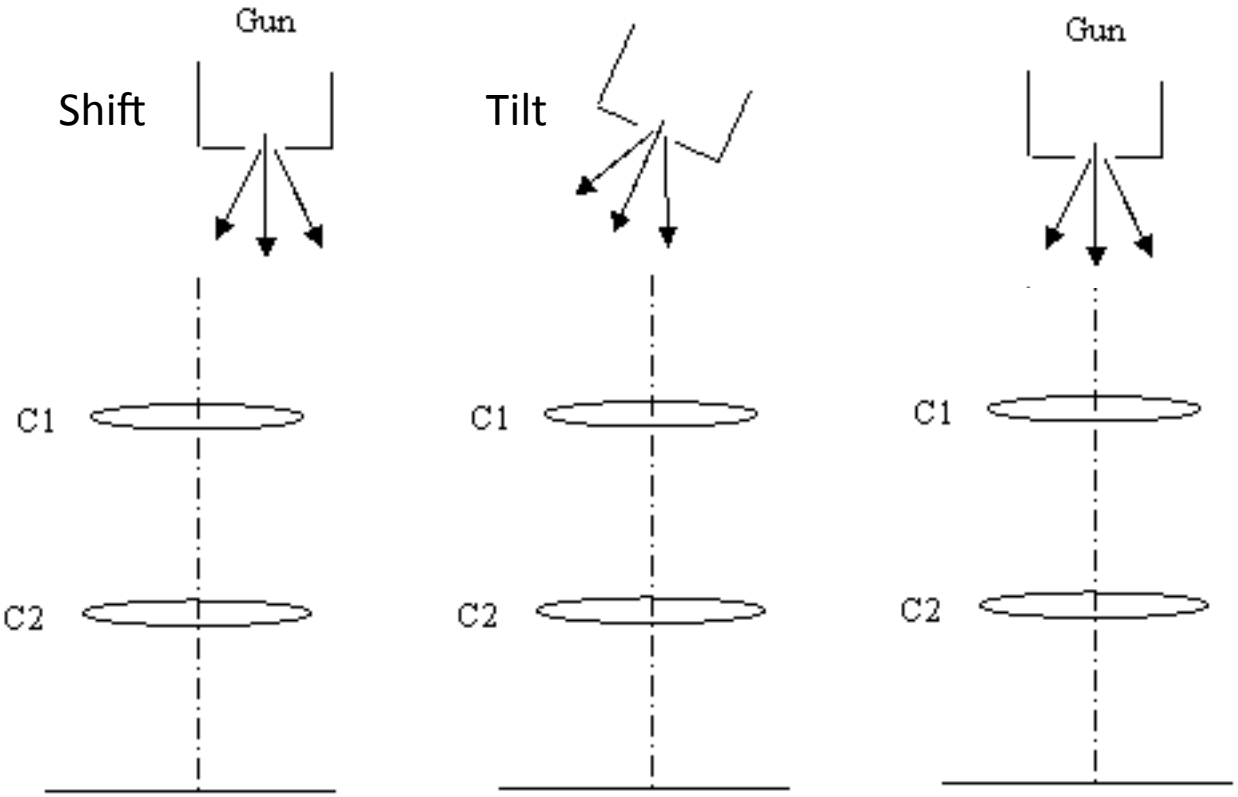


Microscope Alignments

- We fine-tune the alignments to ensure the best performance
- Gun alignment (fairly stable on FEG)
- C2 aperture centering
- Beam tilt
- Objective aperture centering
- Lens astigmatism (condenser and objective)
- Gain reference for detector (if required) and GIF tuning

Gun Alignment

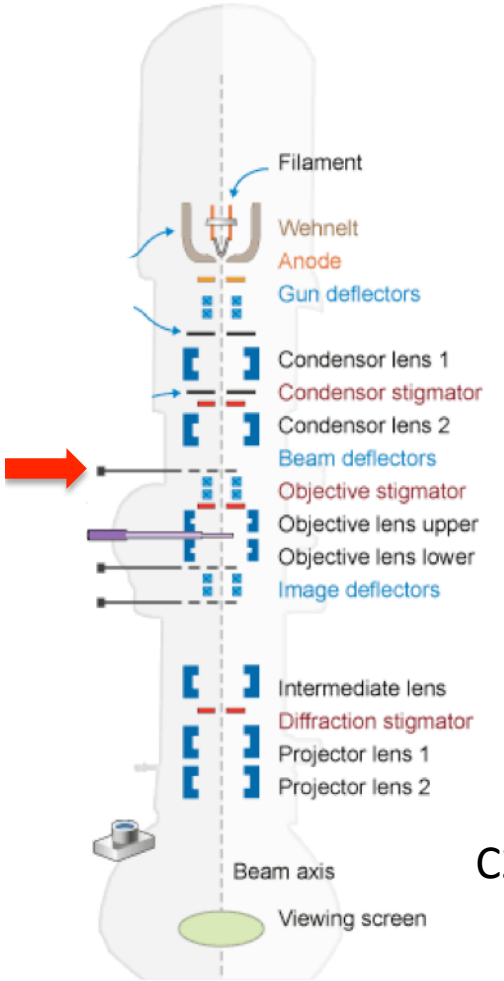




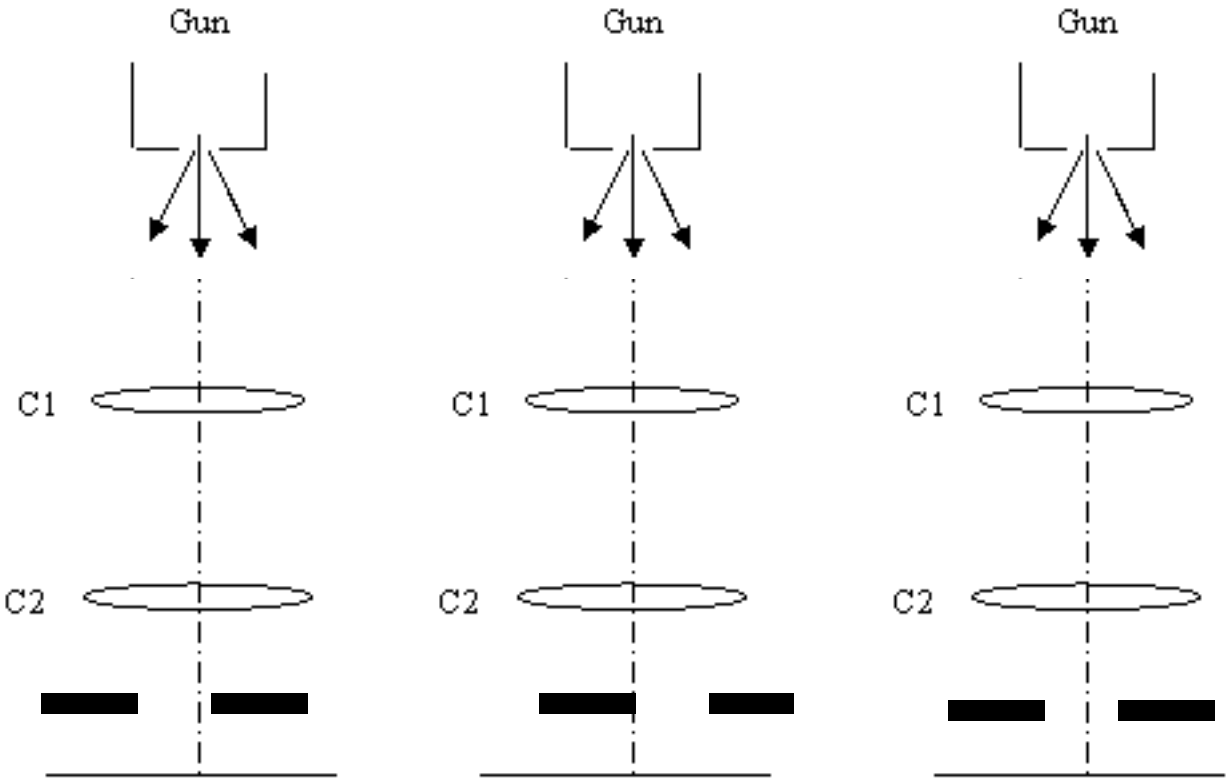
Check for maximum brightness and beam centering when C1 is weak (source larger)

www.rodenburg.org

C2 aperture centering

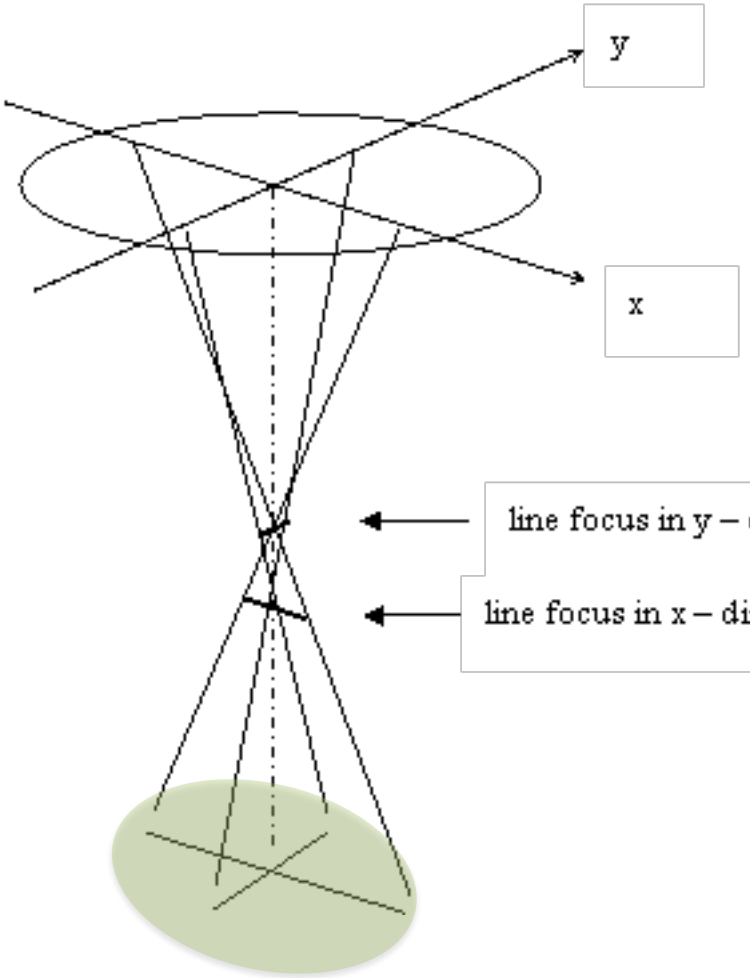


C2 ap.

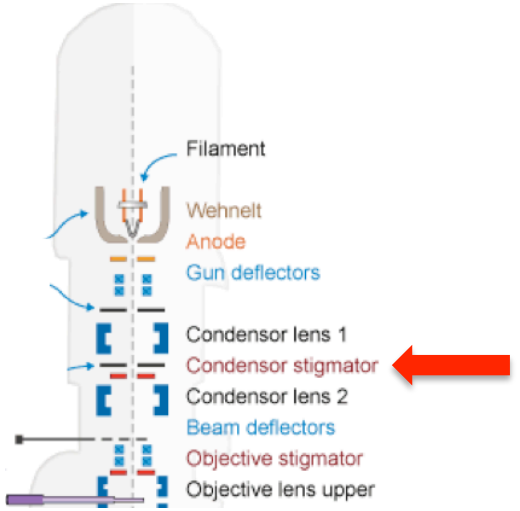


Check for co-centric beam expansion with screen

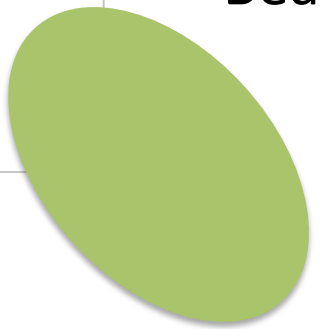
Condenser astigmatism correction



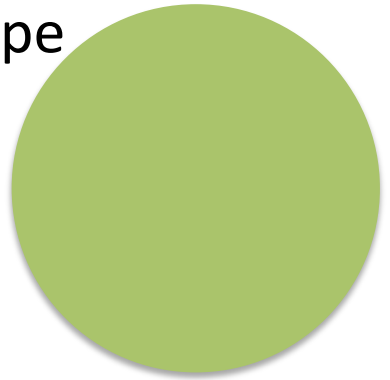
www.rodenburg.org



Beam shape



Astigmatic



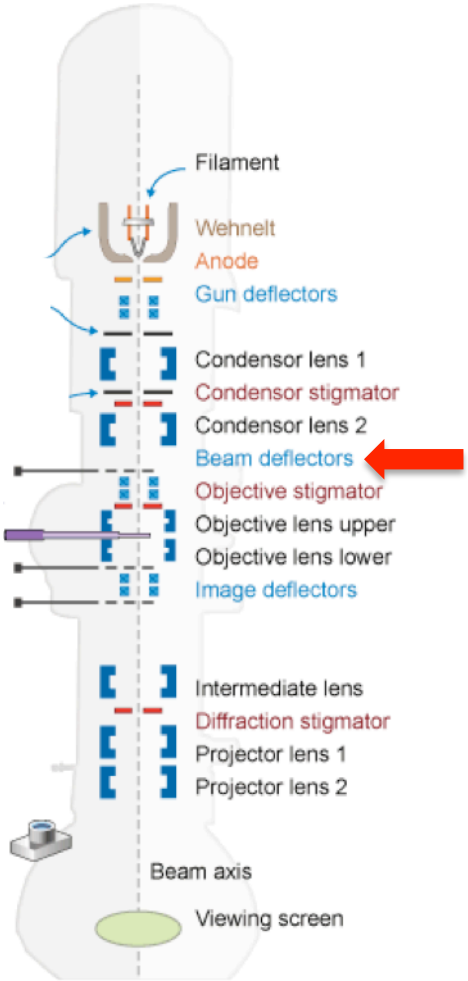
Non Astigmatic

Check for a circular beam

Beam Tilt correction

- Occurs when beam is not parallel to objective lens optical axis
- Two ways to correct (using beam deflection coils):
  1. Rotation center (wobble obj. lens current)
  2. Coma free alignment (tilt beam +/-)

Rotation Center



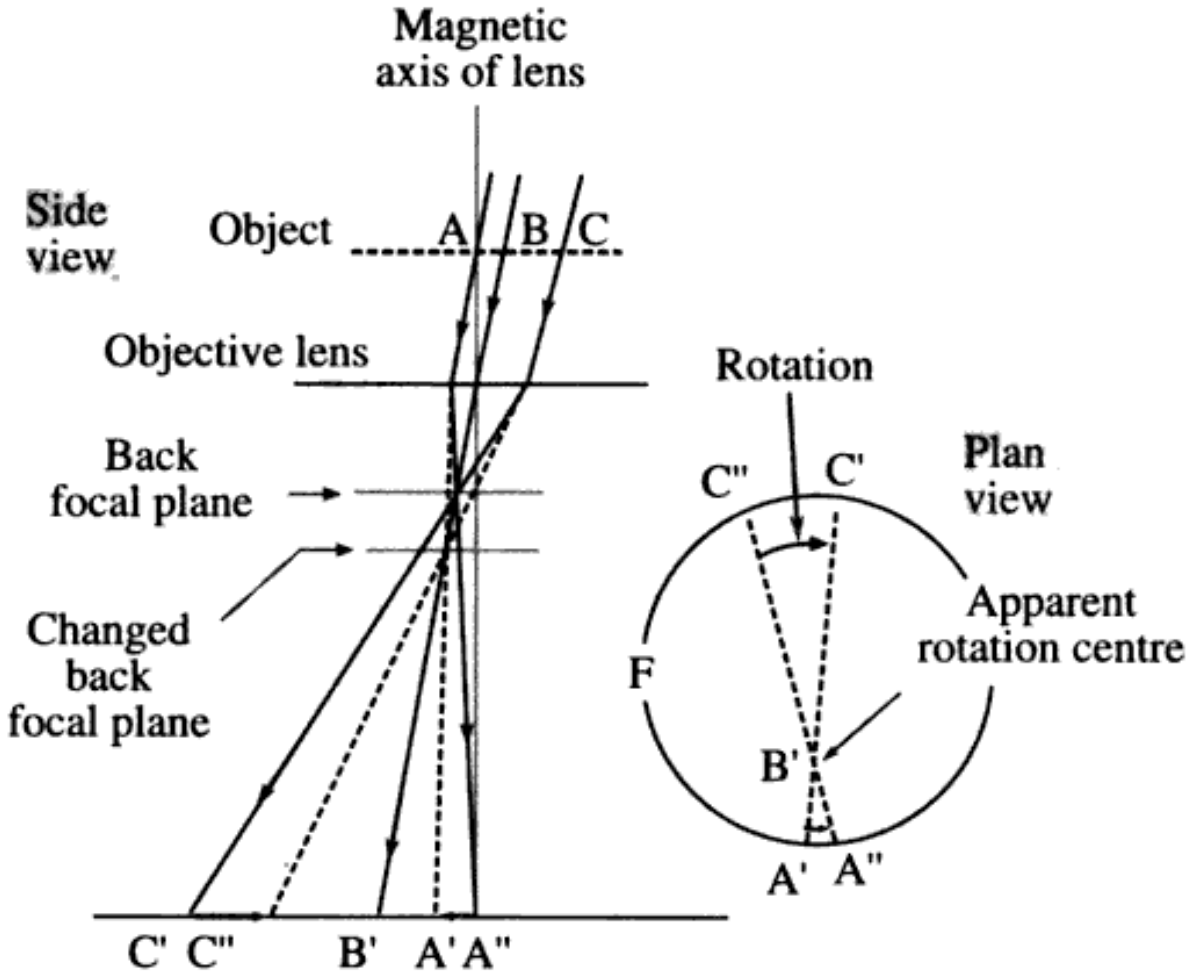
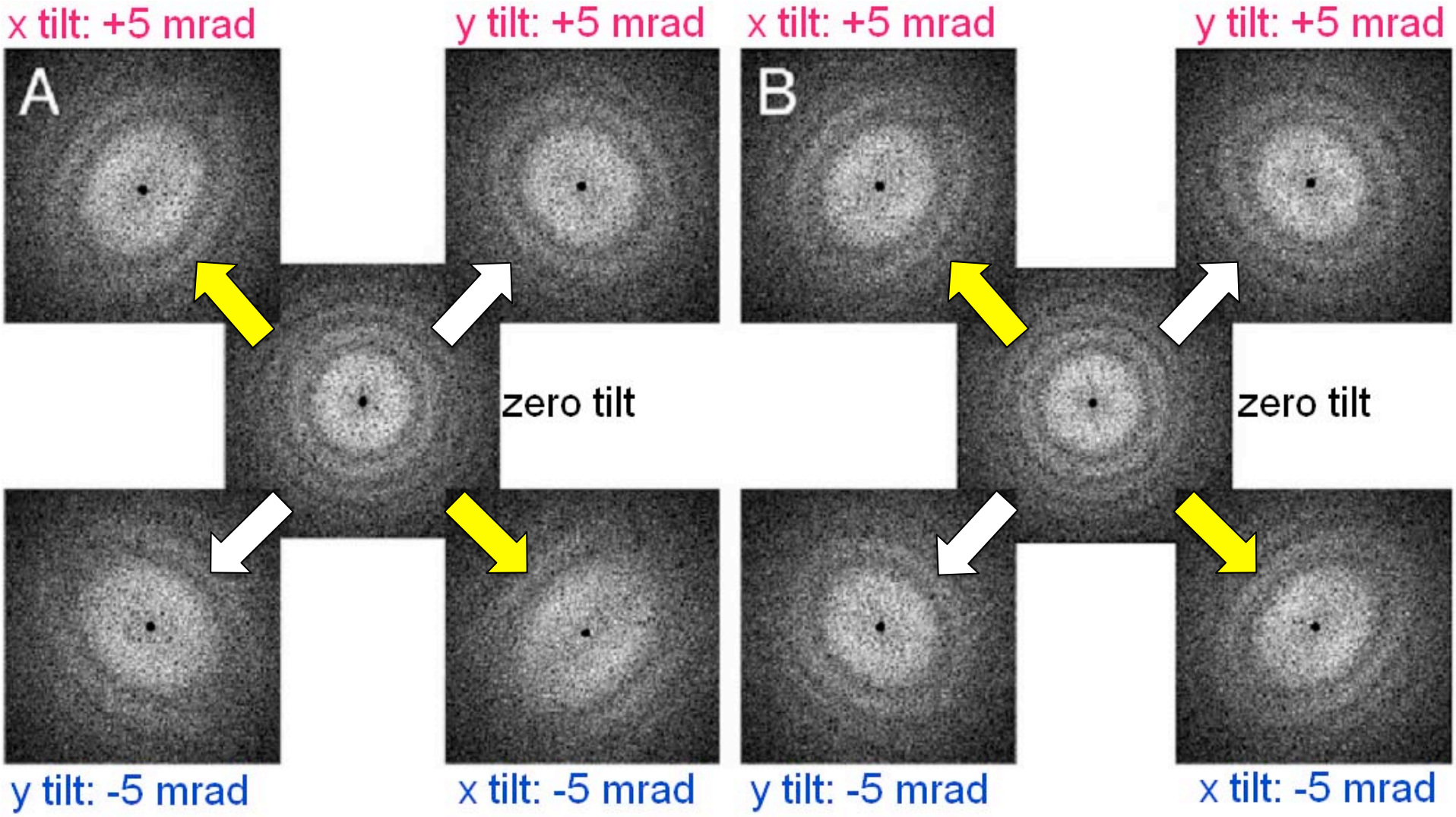


image rotation center should coincide with screen center

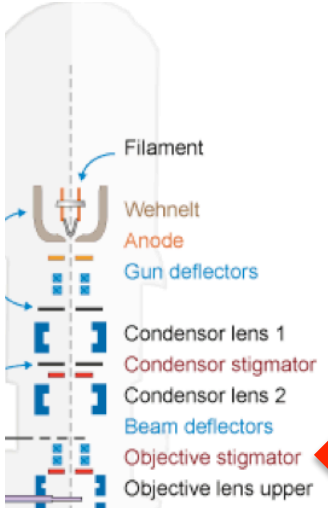
Williams and Carter, 2013

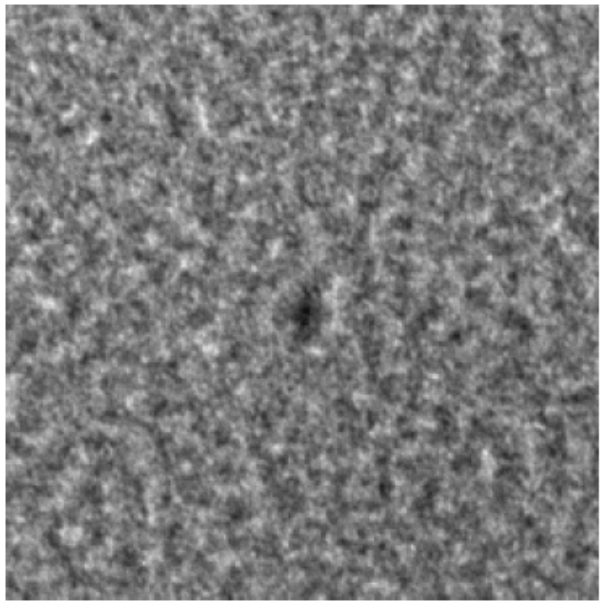
Coma-free alignment

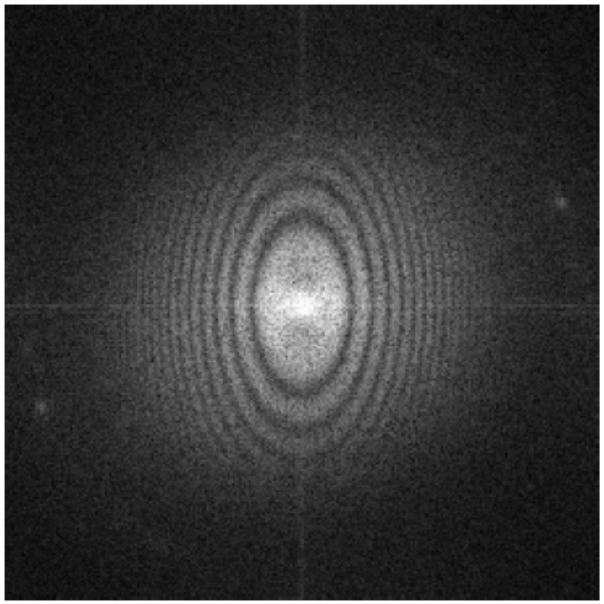


Objective lens astigmatism

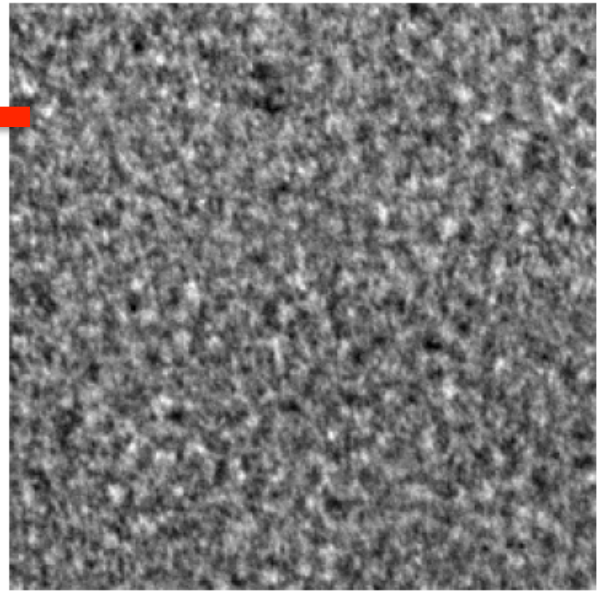
Astigmatic

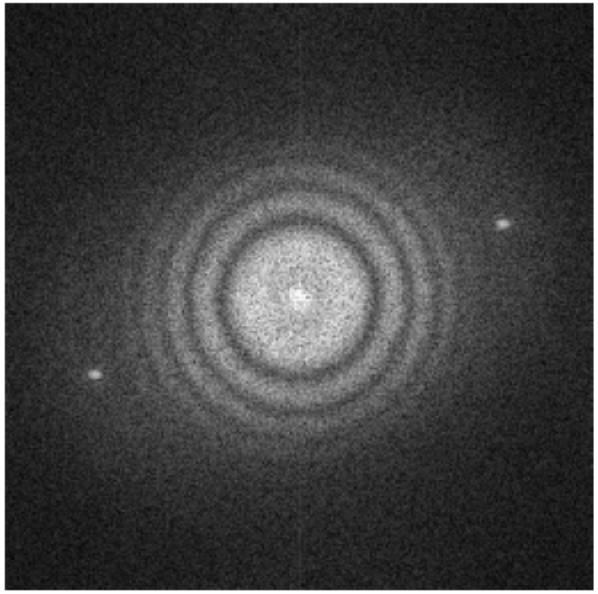






Non Astigmatic



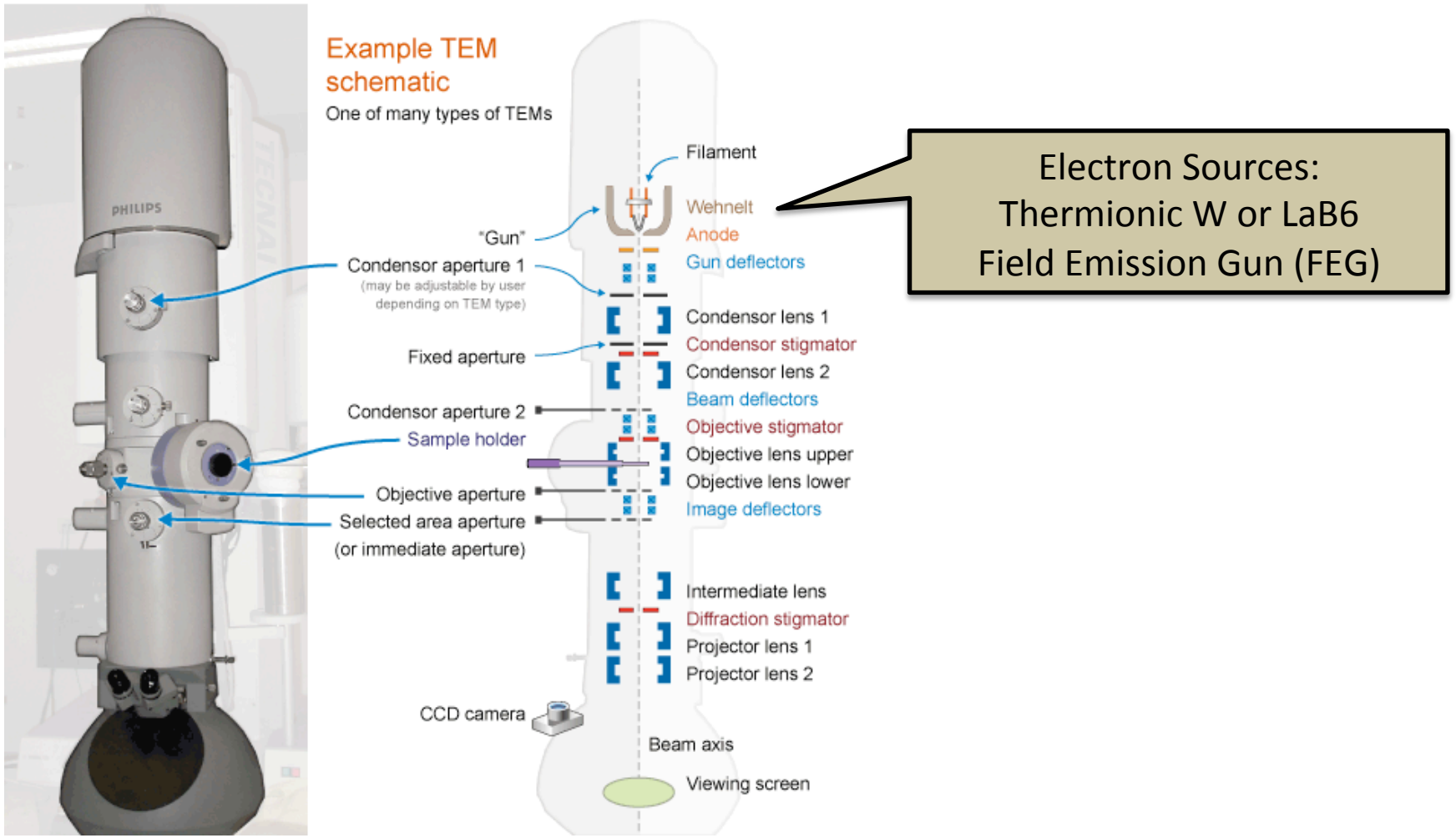


Obtain circular Thon rings

FEI co.

Microscope Alignments

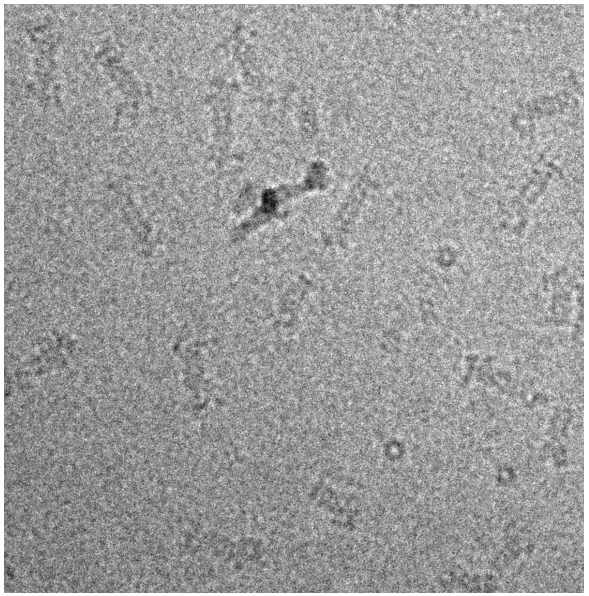
Screening-Choice of Microscope

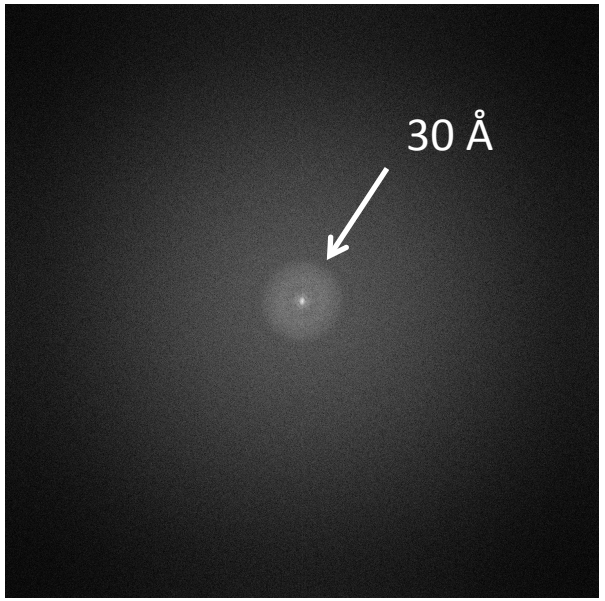


http://www.ammrf.org.au

Screening-Large Complexes

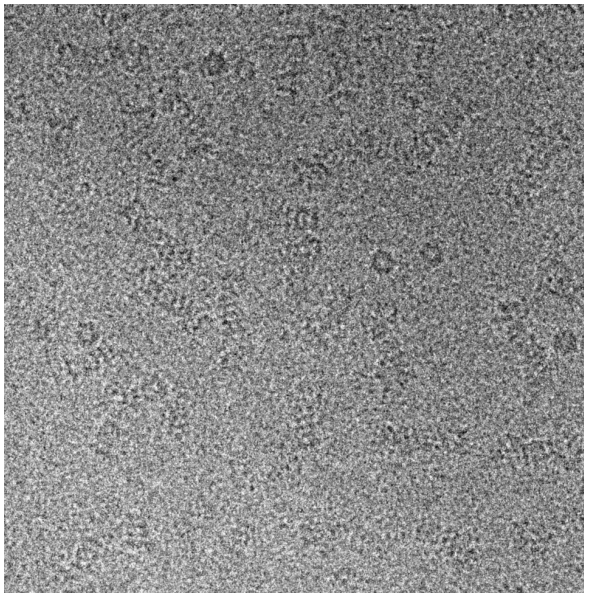
26S Proteasomes
(Δf=-2.5 µm)
FEI Spirit 120 kV
Thermionic gun
Ultrascan CCD

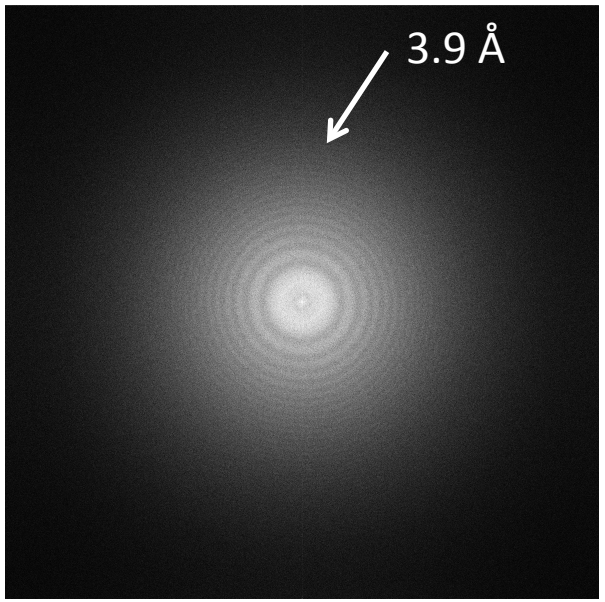




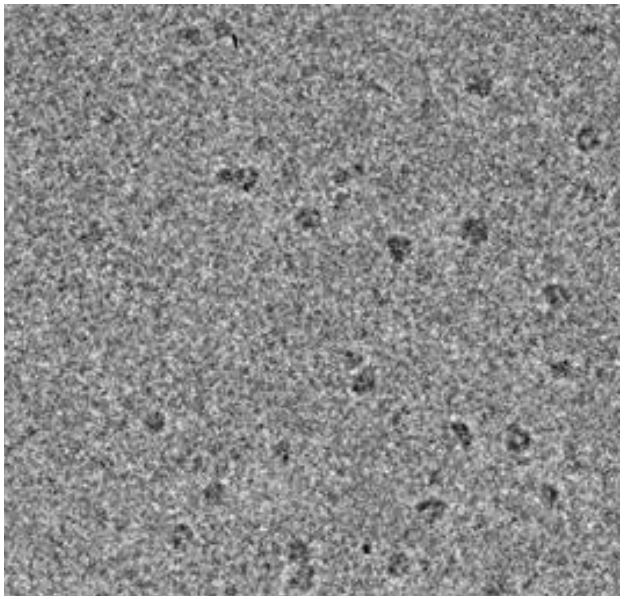
26S Proteasomes
(Δf=-2.5 µm)
FEI Titan Krios 300 kV
Field emission gun
Falcon II

(Alice Clark, 2014)

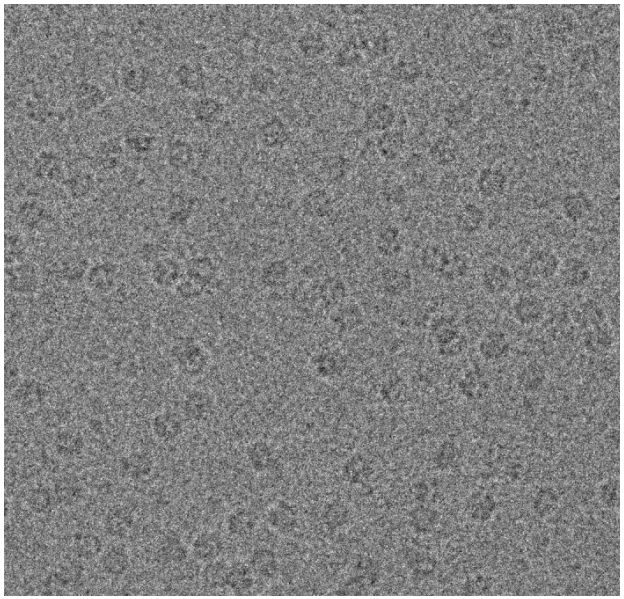




Screening-Small Complexes



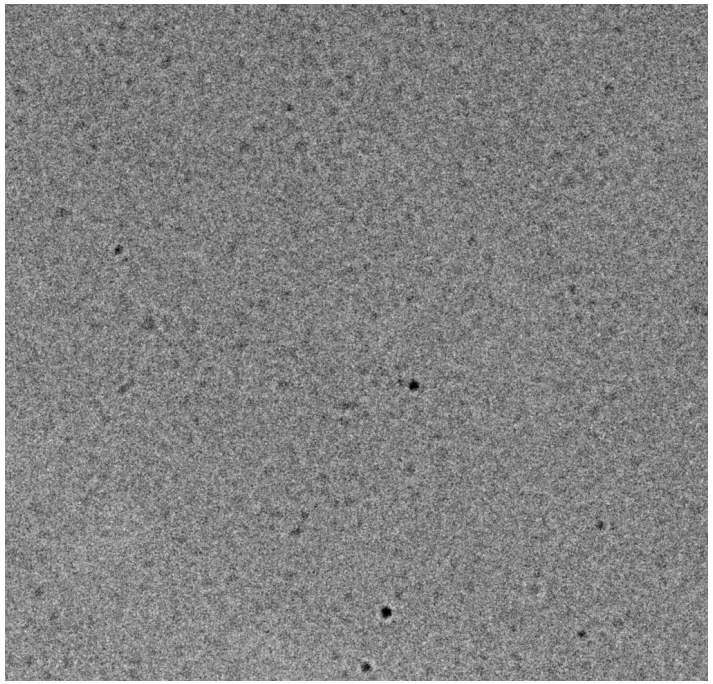
T12 120 kV, Tungsten, Ultrascan CCD



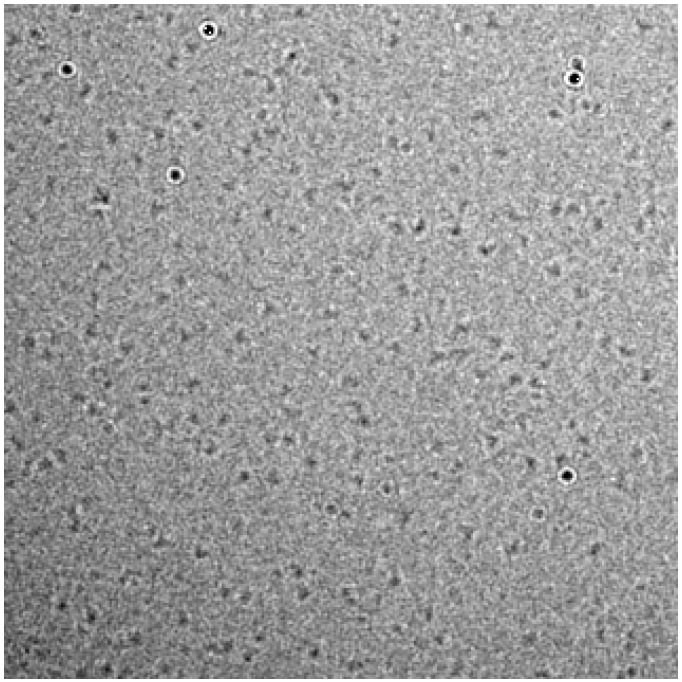
Krios 300 kV, FEG, K2

Lysenin, 315 kDa on graphene oxide (Christos Savva, 2015)

Screening-Small Complexes



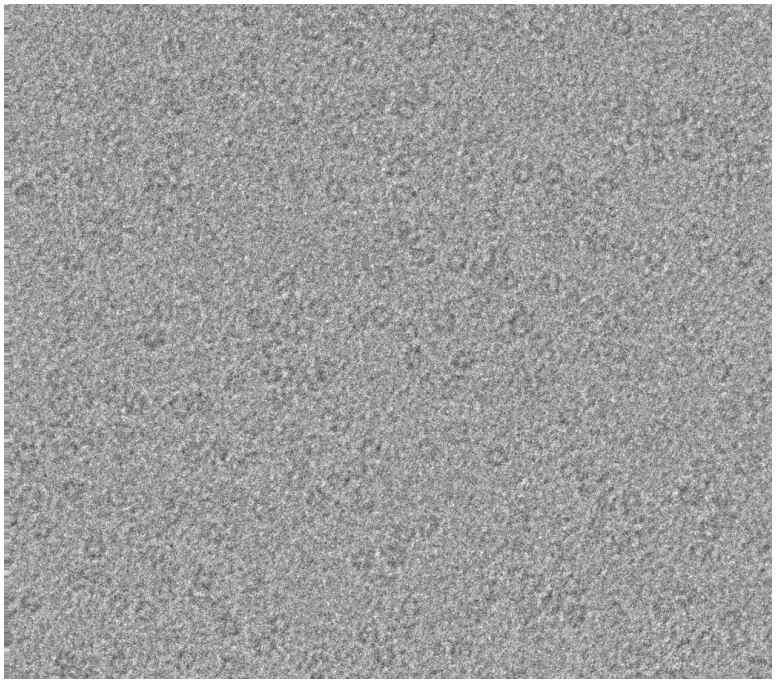
Spirit 120 kV, Tungsten, Ultrascan CCD



200 kV F20, FEG with Falcon II

85 kDa protein in ice
(Chris Hill, 2017)

Screening-Small Complexes



200 kV F20, FEG with Ultrascan CCD

(NetB, 235 kDa on carbon, Christos Savva, 2012)

Choice of Microscope: Screening

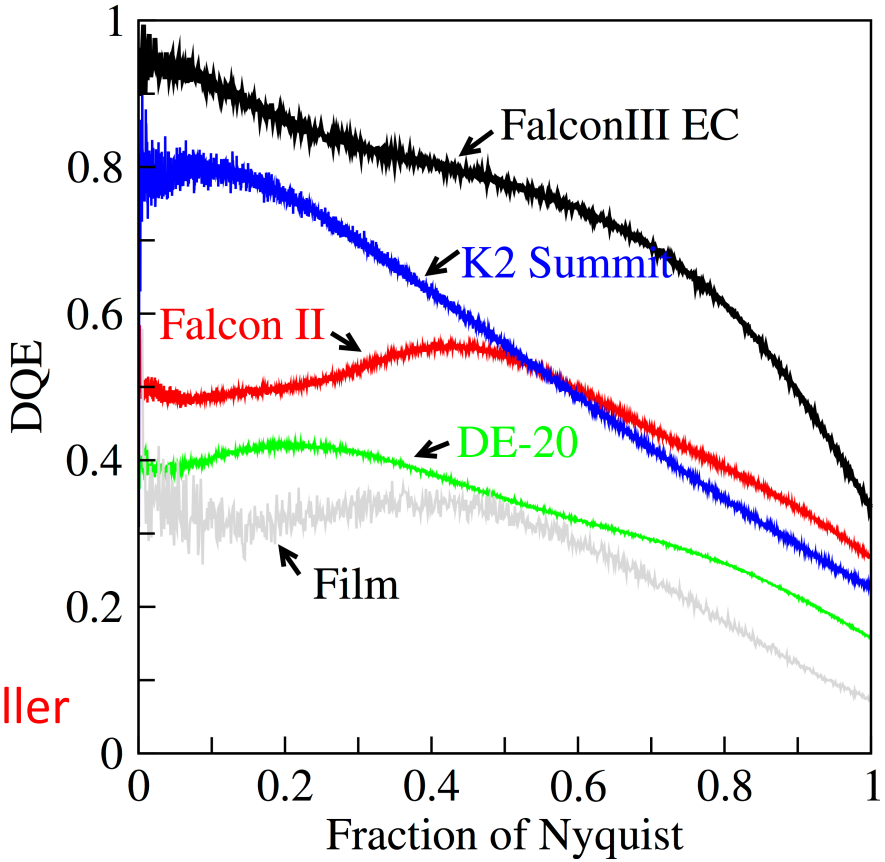
- For screening a FEG source is optimal
- Larger complexes can be screened with a thermionic source
- Smaller <300 kDa complexes can be seen if sample behaves

In the near future

- Affordable 100 kV FEG microscope or ability to retrofit a FEG on to a thermionic TEM
- Detector with 40% DQE (signal to noise) at half Nyquist
- 1400 frames/second

Choice of Detector

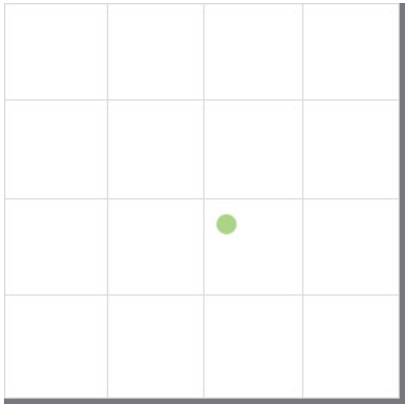
- Detective Quantum Efficiency
- DQE =SNR^2o/SNR^2i
- A measure of the signal to noise ratio degradation
- Perfect detector has DQE of 1



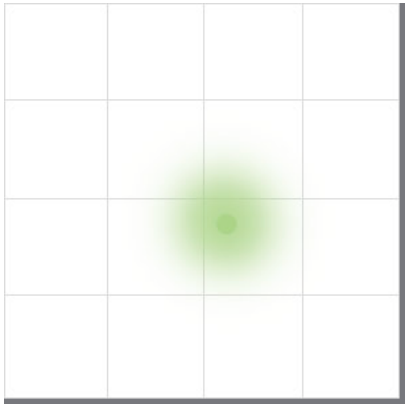
Counting detector advantageous for smaller complexes > boost low frequencies

Greg McMullan

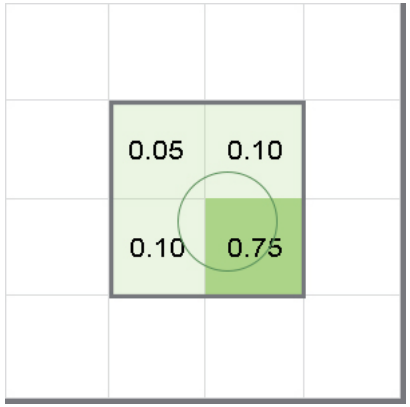
Integration vs Counting



Electron enters detector.



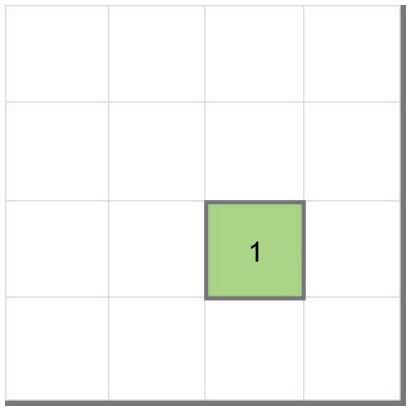
Electron signal is scattered.



Charge collects in each pixel.

Integration

- Short exposures
- High Dose-rates applications
- Lower DQE

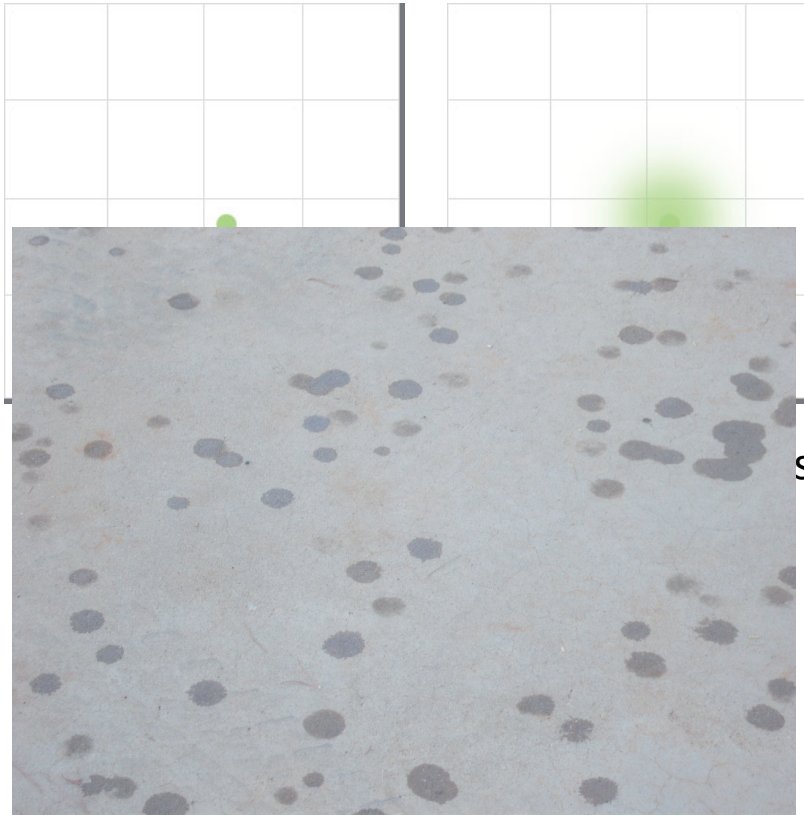


Events reduced to highest charge pixels.

Counting

- Very low dose rate (0.5-5 e-/pixel/sec)
- Fast frame rates
- Long exposures
- Higher DQE

Integration vs Counting



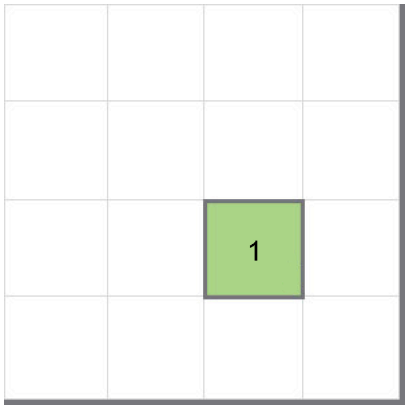
Integration
- Short exposures
- High Dose-rates

(0.5-5 e-/pixel/sec)
- Fast frame rates
- Long exposures
- Higher DQE

Events reduced to highest charge pixels.

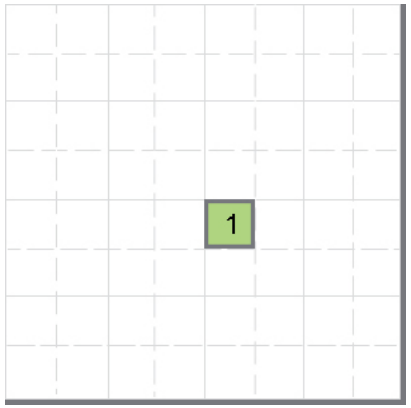
Super-resolution

Counting



Events reduced to highest charge pixels.

Counting with Super-resolution

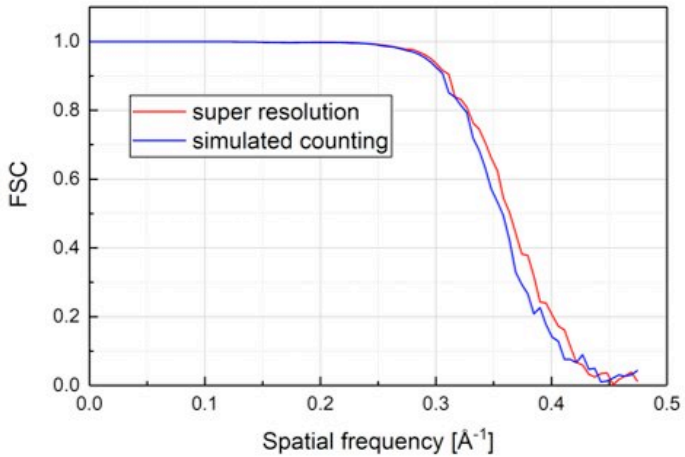


Events Events localised to sub-pixel accuracy.

- Super-resolution with Fourier cropping “binning” reduces effect of aliasing at Nyquist thus reducing noise
- e.g. at a nominal 105kX Nyquist is 2.3Å
- Will slow data collection => less particles

Is Super-resolution on K2 worth it?

	Super resolution	Simulated counting (2x binned movies)
Sample	20S proteasome	
Symmetry	D7	
Physical pixel size (Å)	1.054	
Frames	24	
Exposure time (s)	12	
Doserate (e^- pix^{-1} s^{-1})	3.6	
Total dose (e^- $Å^{-2}$)	39	
Extracted particles	142,918	142,133
Final particles (after Class3D)	83,127	69,994
Resolution (Å)	2.43	2.53
B-factor ($Å^2$)	-85.4	-89.9
Resolution vs physical Nyquist (%)	86.7	83.3



Rado Danev @RadoDanev · Jun 10

Replying to @RubinsteinJohn @Cannabatopia and 2 others

The results are in and the effect is even smaller than what I expected. SuperRes 86.7% of phys. Nyq.; Simulated counting 83.3% 🤔

pic.twitter.com/crCD7hXiMr

Unpublished Data

Gatan K2 Summit

- 400 frames/sec (40 frames/sec output)
- Counting and Super-resolution modes
- Optimal dose rates <5 e-/physical pix/second
- Typical exposures 8-16 seconds

e.g. at 105 kX 5e-/pix/sec= 3.8 e-/A^2/Sec

12 sec exp: 45.6/A^2 total dose

0.3 sec/frame = 40 frames, 1.14 e-/A^2/frame

FEI Falcon II

- 17 frames/sec
- Linear mode only
- Optimal dose rate 2-3 e-/pixel/frame
- Typical exposures 1-2 sec

e.g. at 59 kX 3e-/pix/frame= 1.7e-/A^2/frame

or 28.9 e-/A^2/sec

FEI Falcon III

- 40 frames/sec
- Linear and counting modes (Super-resolution soon-> Greg)
- Optimal dose rate:
  - Linear: 2-3 e-/pixel/frame
  - Counting: 0.5-0.7 e-/pix/sec (Greg: 0.5-0.5 e-/pix/sec)
- Typical exposures:
  - Linear: 1-2 sec
  - Counting: 60 sec

Counting: e.g. at 75 kX 0.6e-/pix/sec= 0.52e-/A^2/sec

=> 60 sec exposure = 31.5e-/A^2 total dose

Linear: e.g. at 59 kX 3e-/pix/frame= 1.7e-/A^2/frame

or 68 e-/A2/sec

Data Collection-Which Microscope?

- 300 kV FEG with a direct electron detector

Polara 1: Falcon III in linear mode only

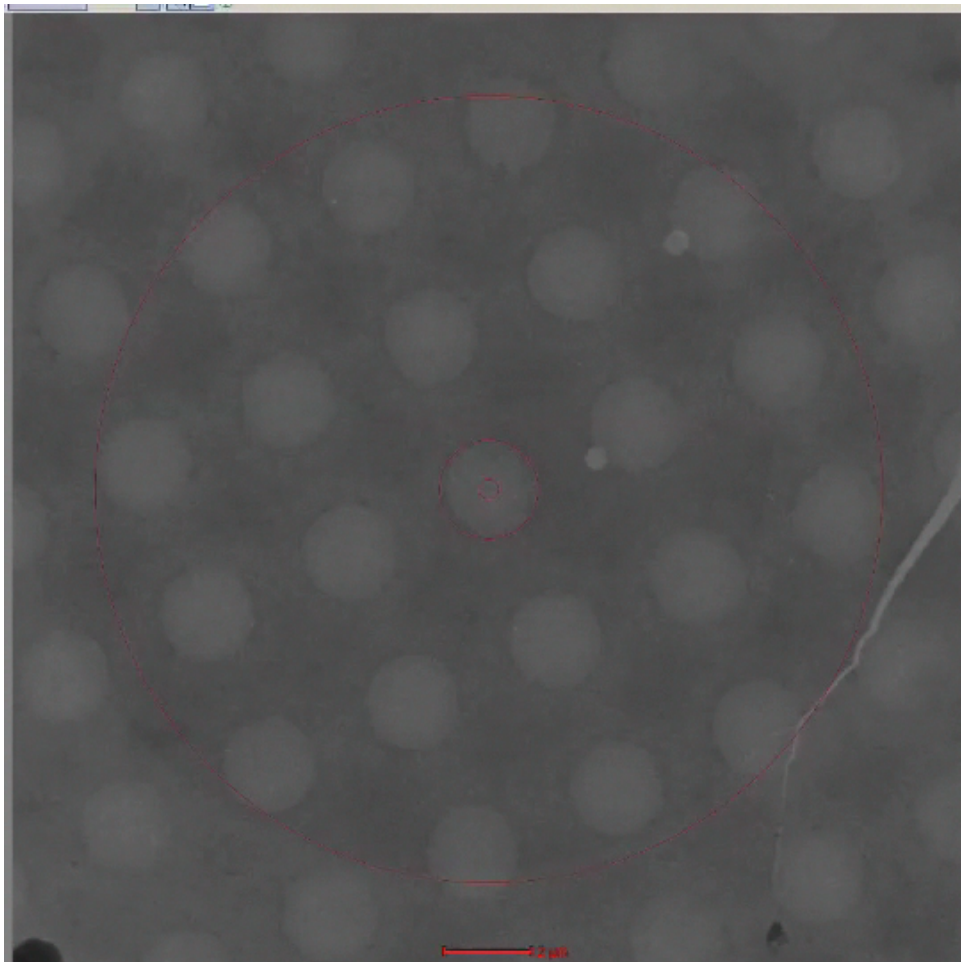
Polara 2 : Coming soon with K3

Krios 1: Falcon III EC and Quantum/K2

Krios 2: Falcon III EC and Quantum/K2, Volta phase plate

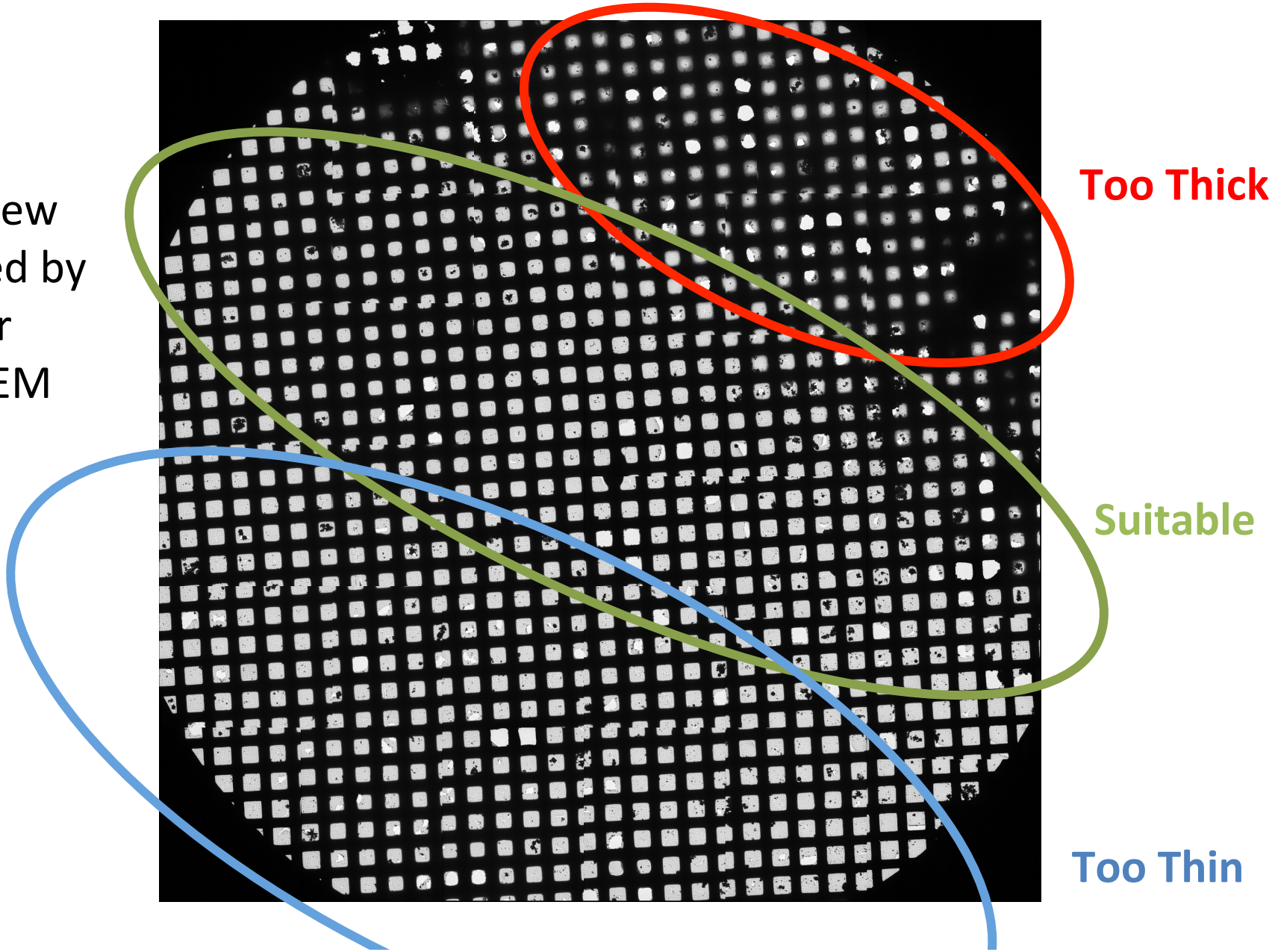
Initial screening

- Find the good areas at low mag
- Drop the mag (quick way) or do an Atlas (slow way)
- Use Low Dose in TUI to screen suitable squares
- Save good squares for data collection

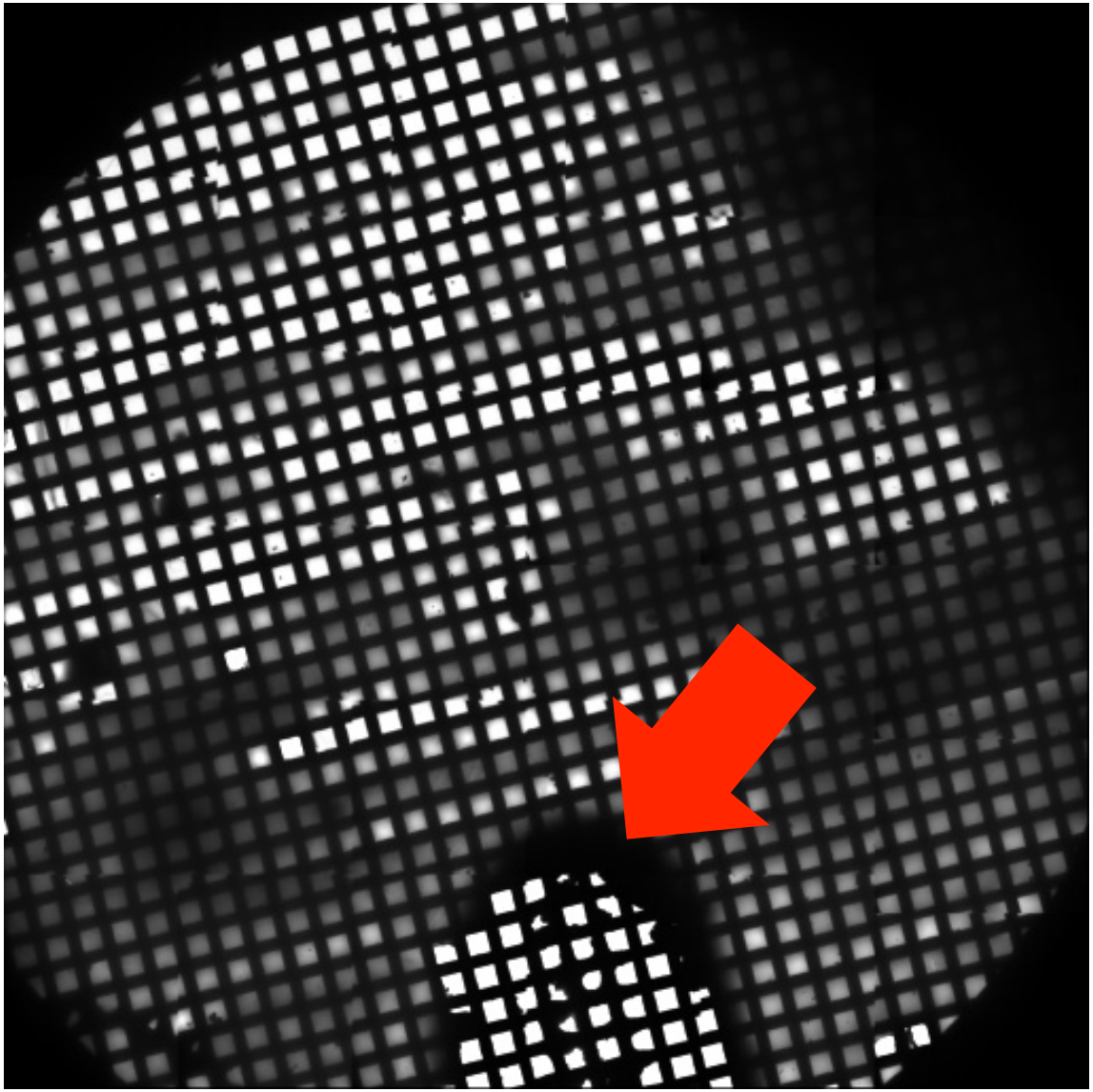


Grid overview

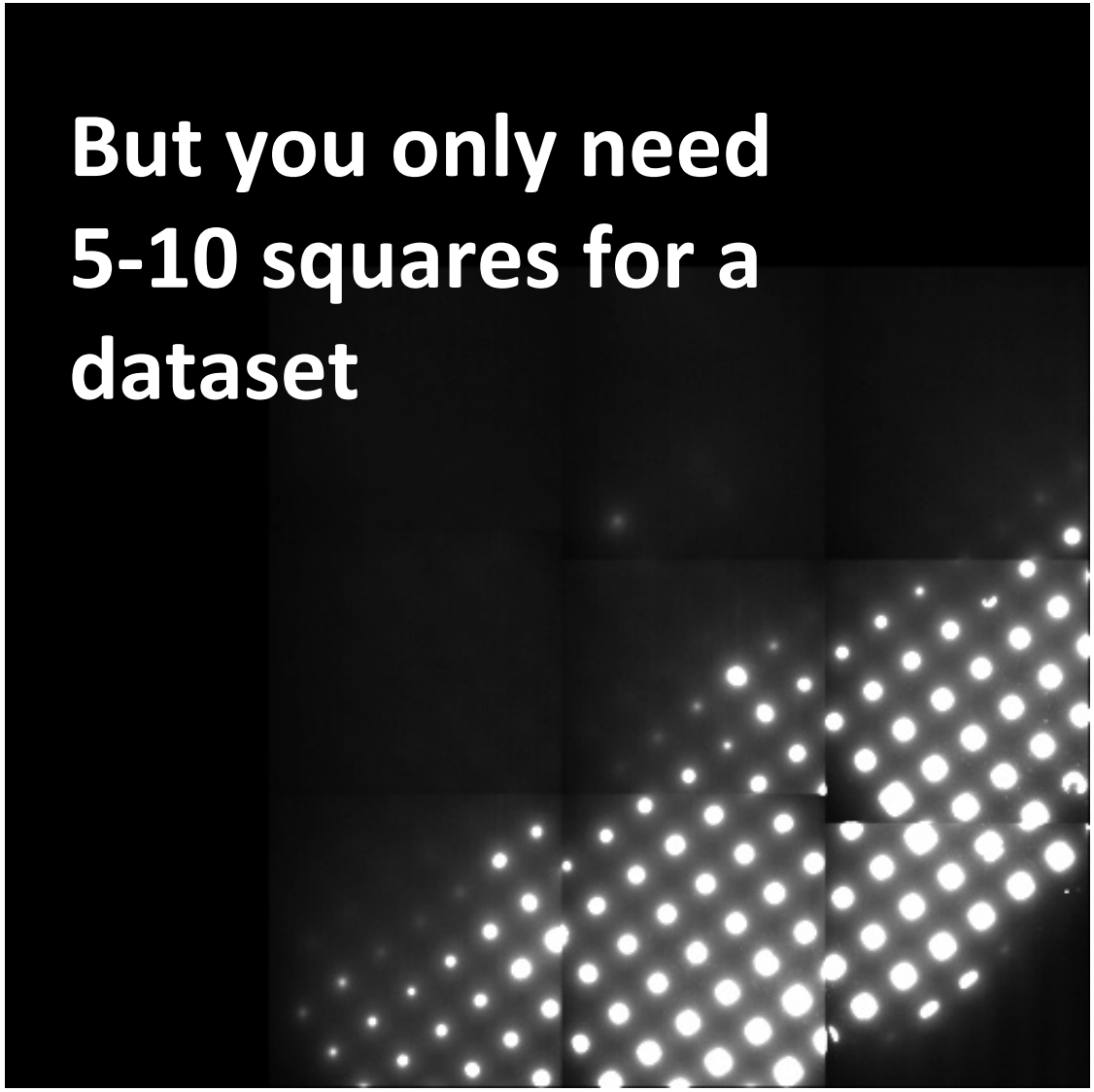
Grid overview created by EPU or SerialEM



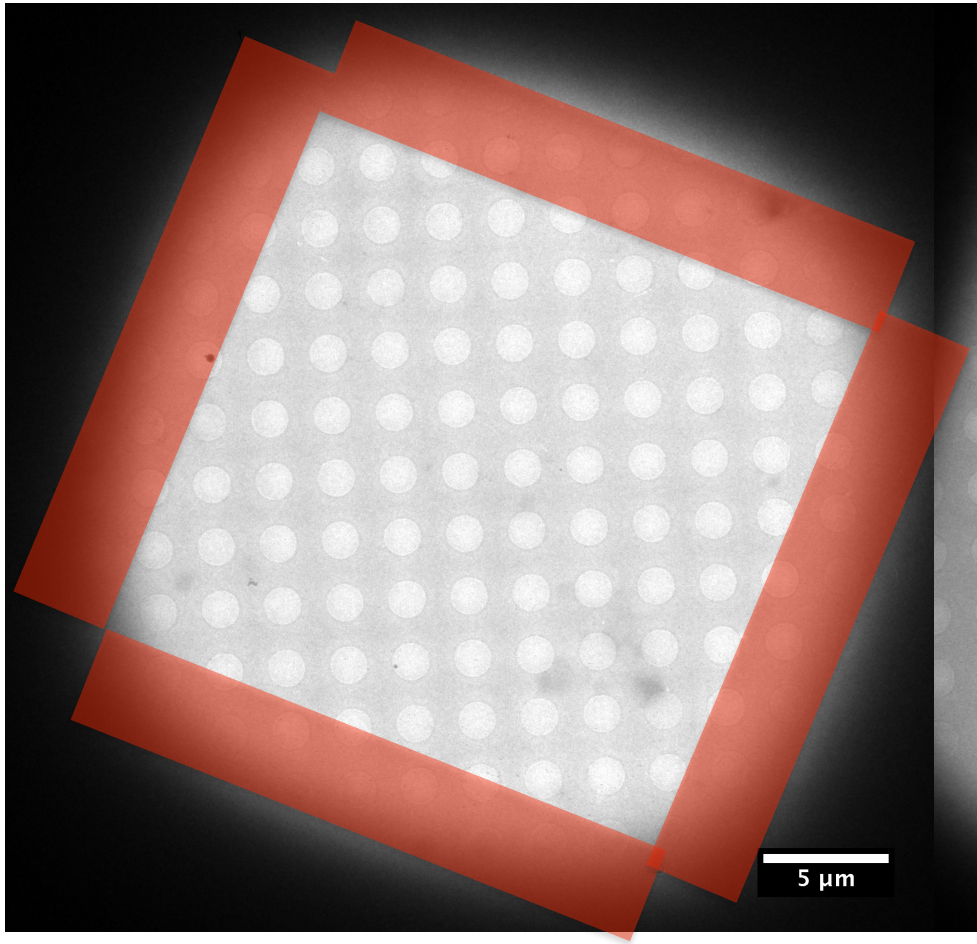
Grid overview



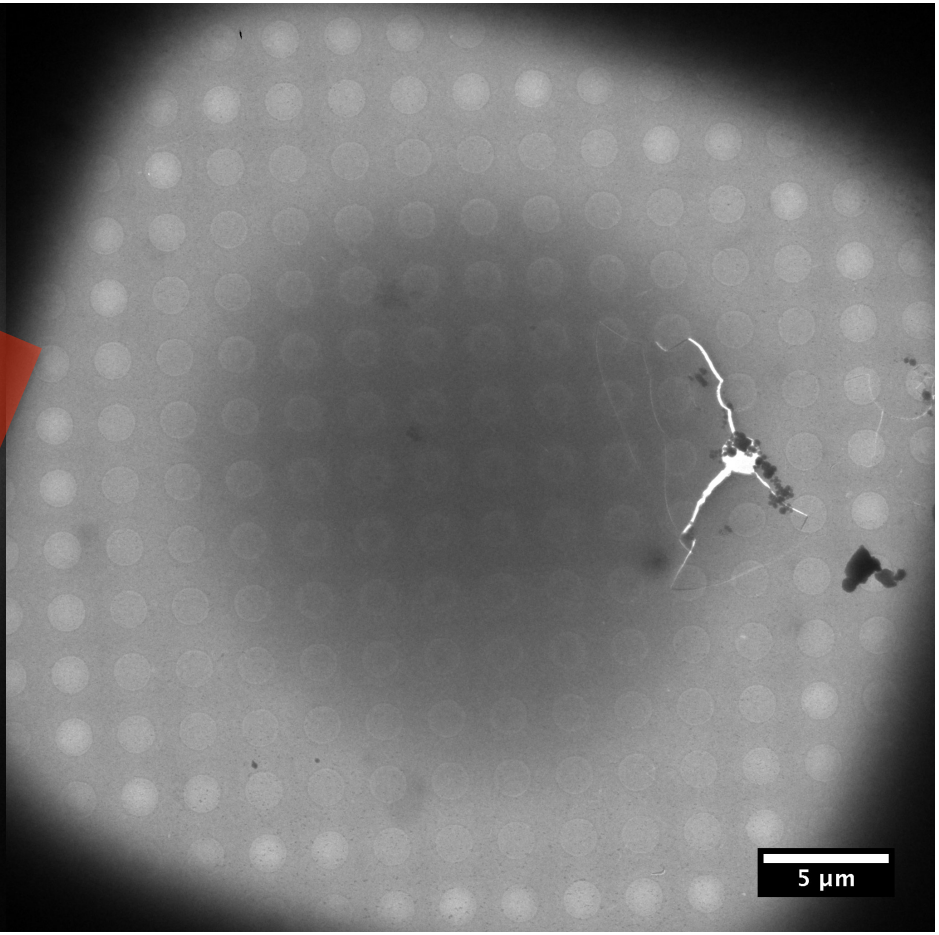
Grid overview



Finding the right Ice thickness

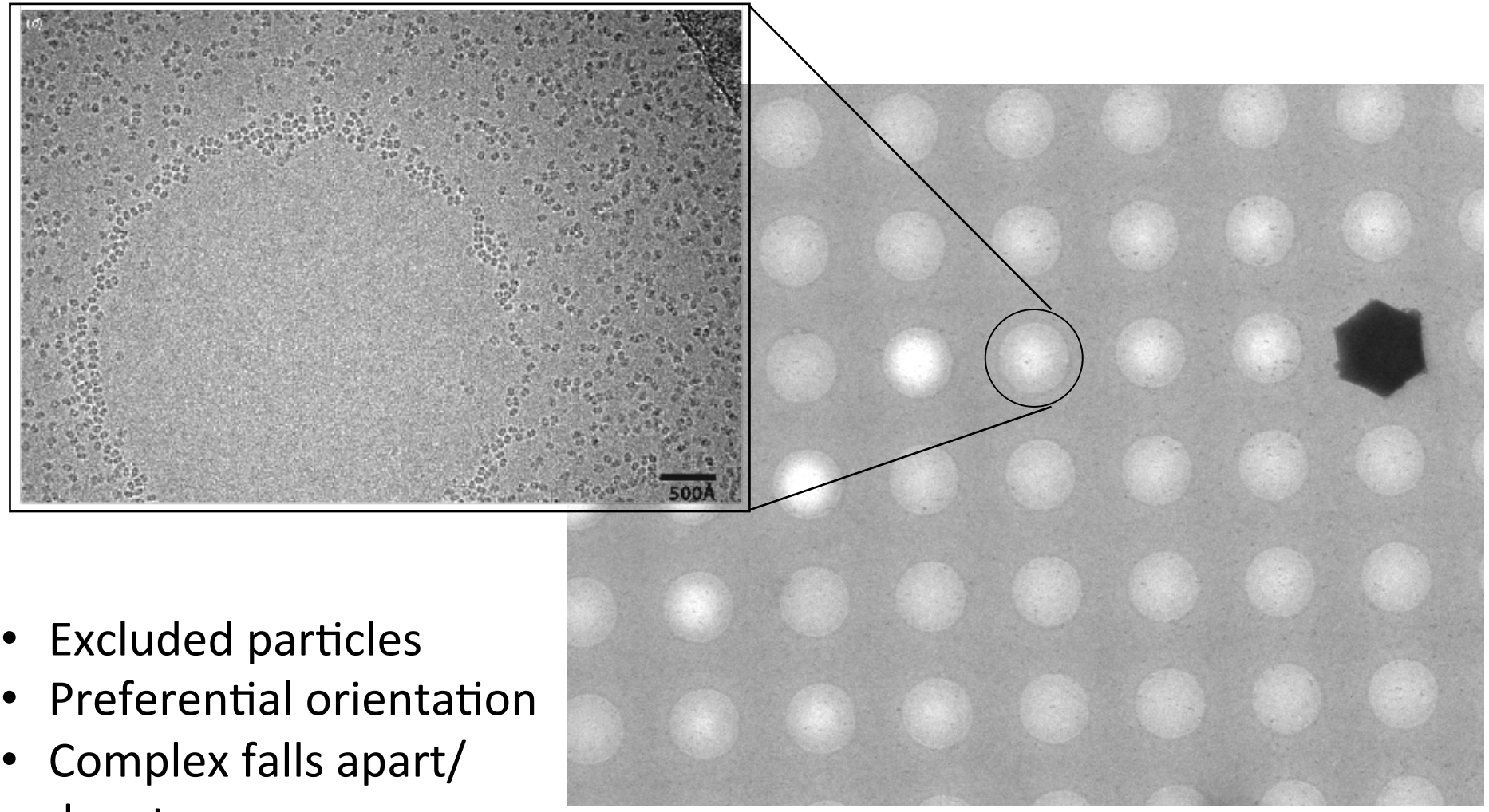


Suitable Ice



Thick Ice

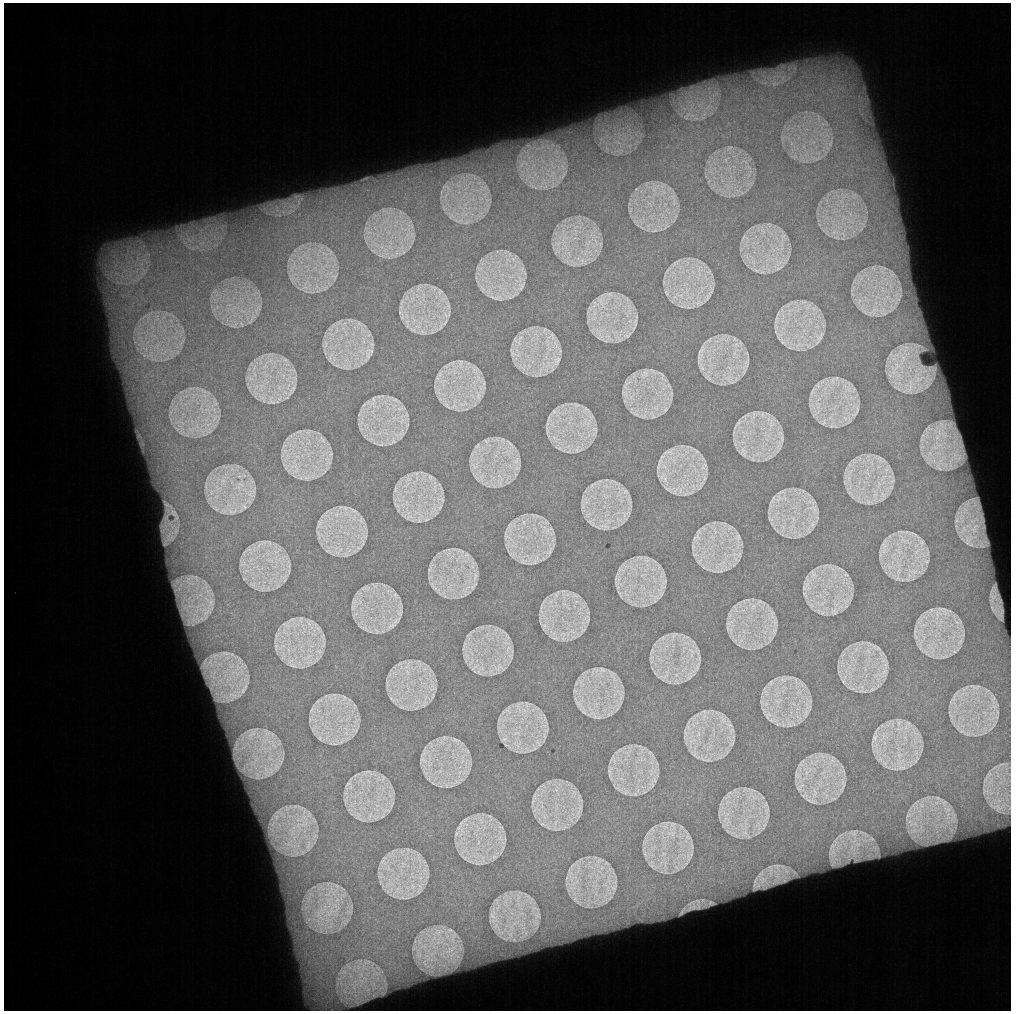
Finding the right Ice thickness

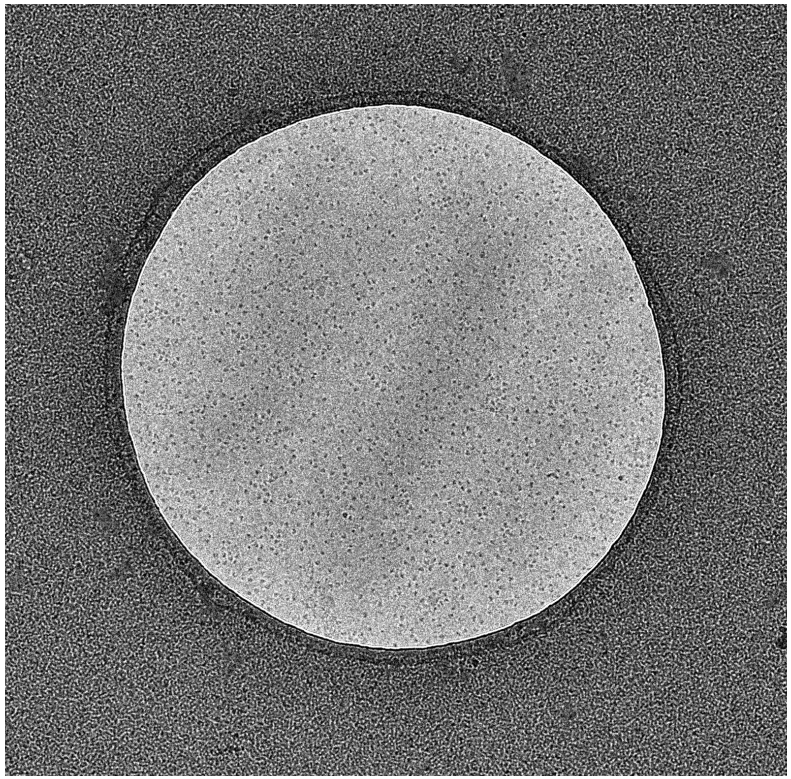


- Excluded particles
- Preferential orientation
- Complex falls apart/ denatures

Thin Ice

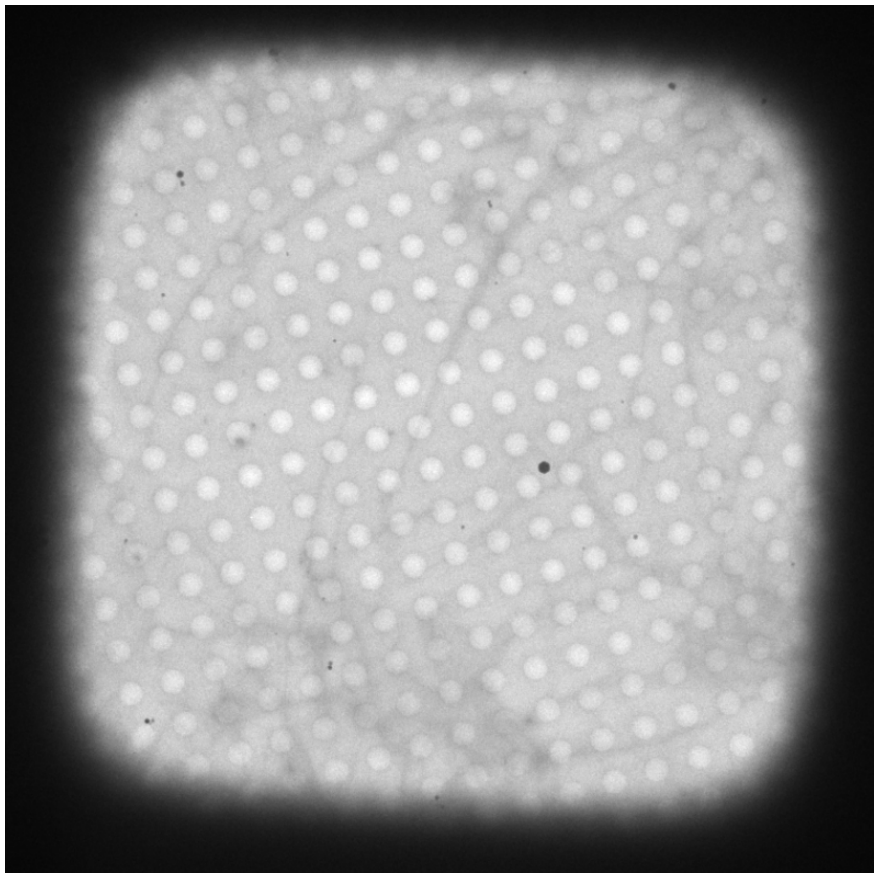
Ice appearance with overlaid carbon

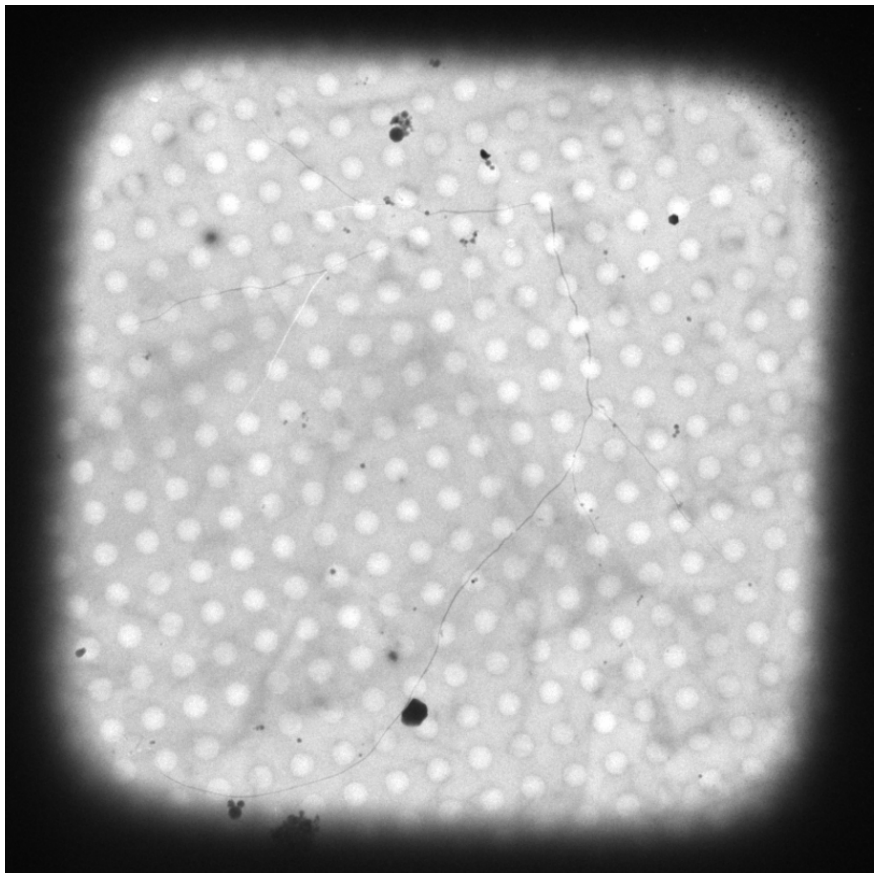




Rebecca Voorhees, 2016)

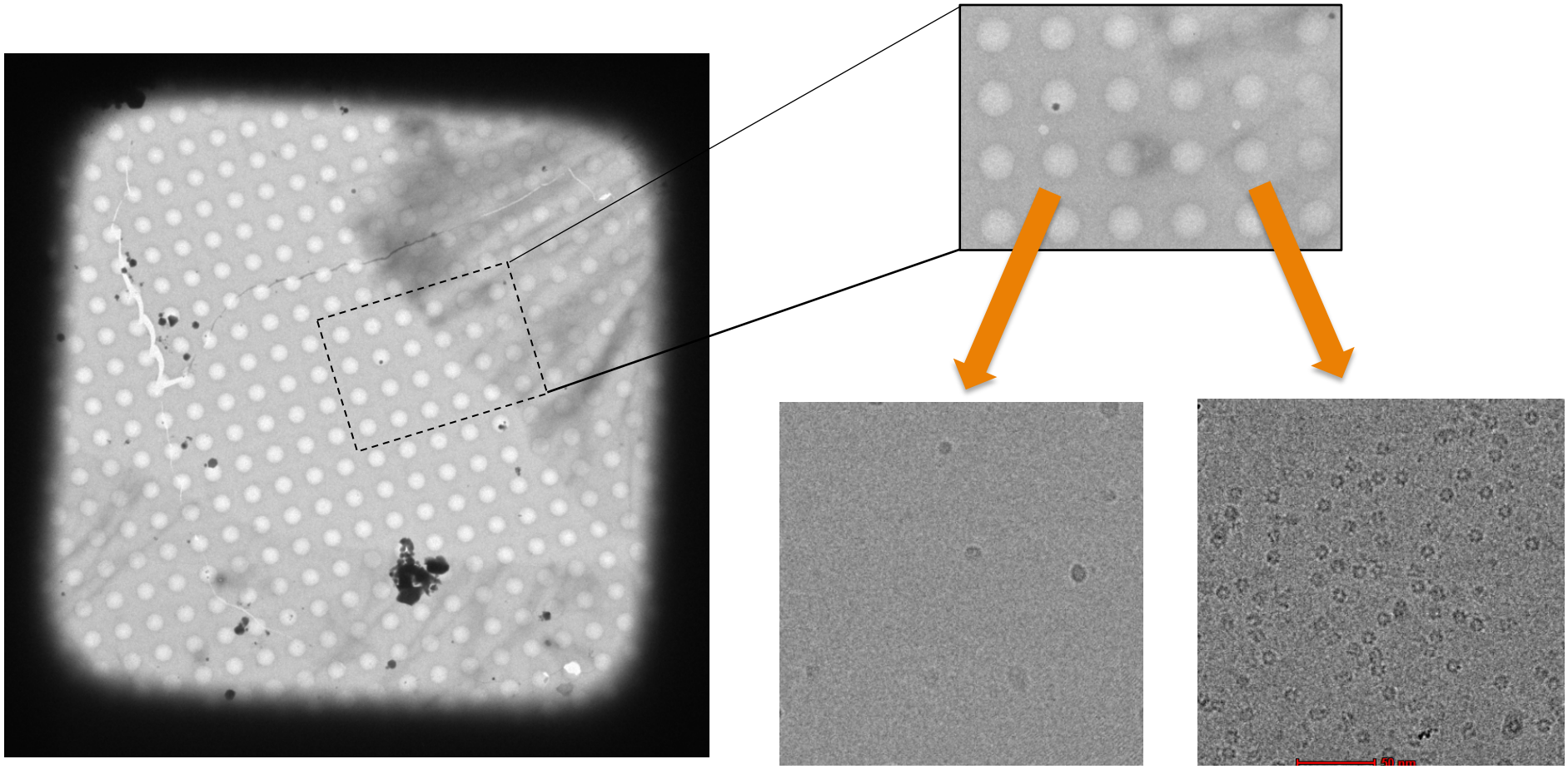
Ice appearance with graphene oxide





Christos Savva, 2017

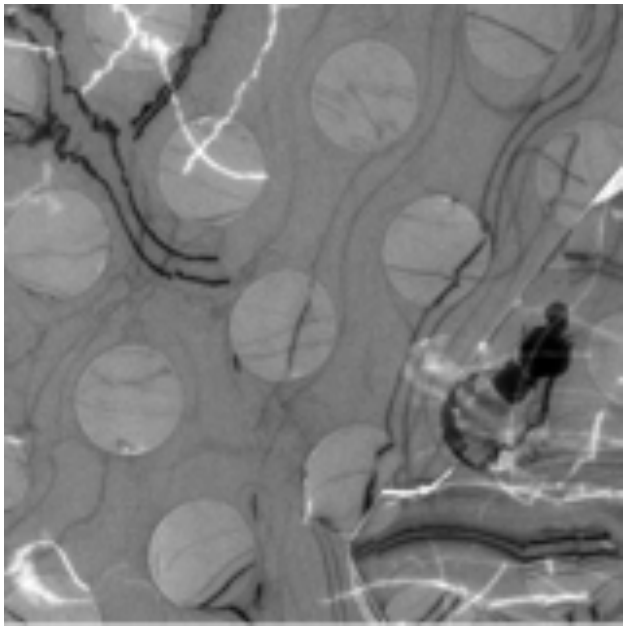
Incomplete graphene oxide coverage



Christos Savva, 2017

Crystalline Ice

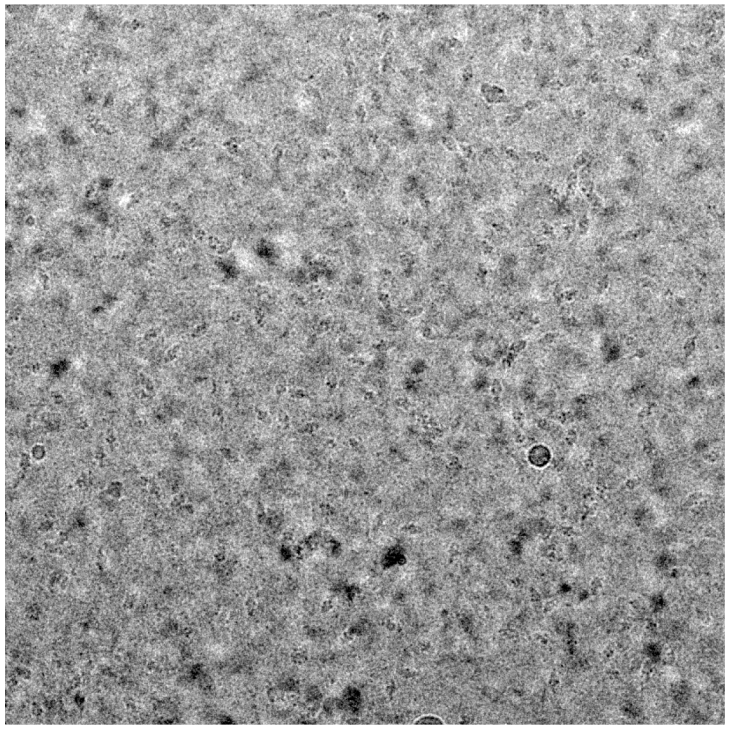
Causes:
- Cryogen temperature above ~-150°C
- Warm-up during after freezing and transfer to the holder
- Vacuum crash



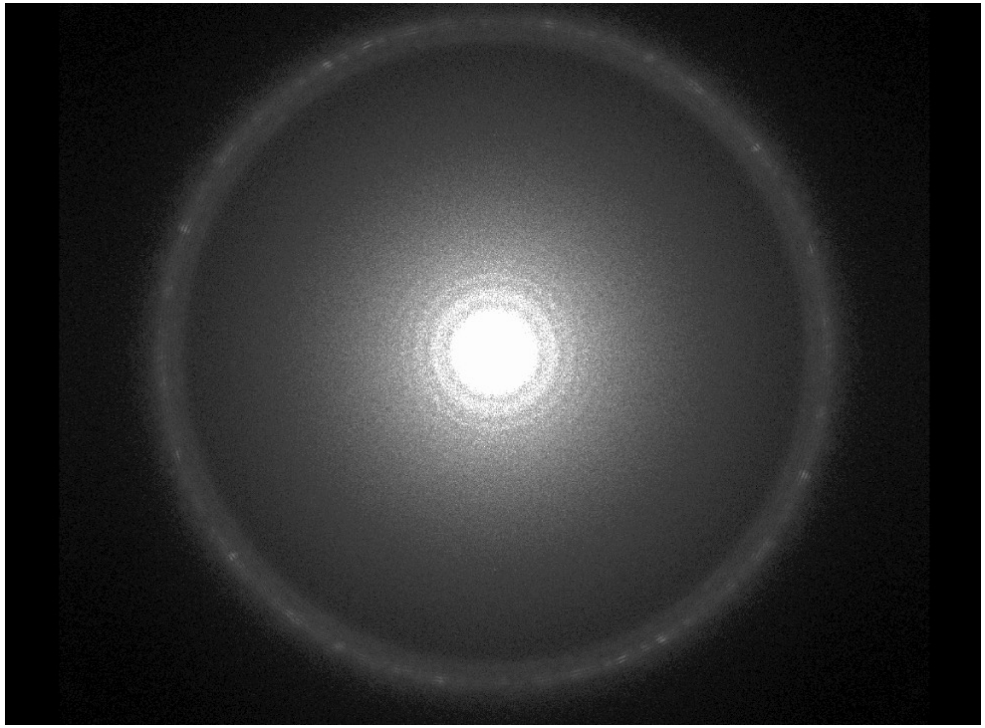
Chris Russo

- Obvious at 2,500X with CCD camera
- Tilt stage +/-10° and the stripes change
- Avoid the edges of grid squares

Crystalline Ice



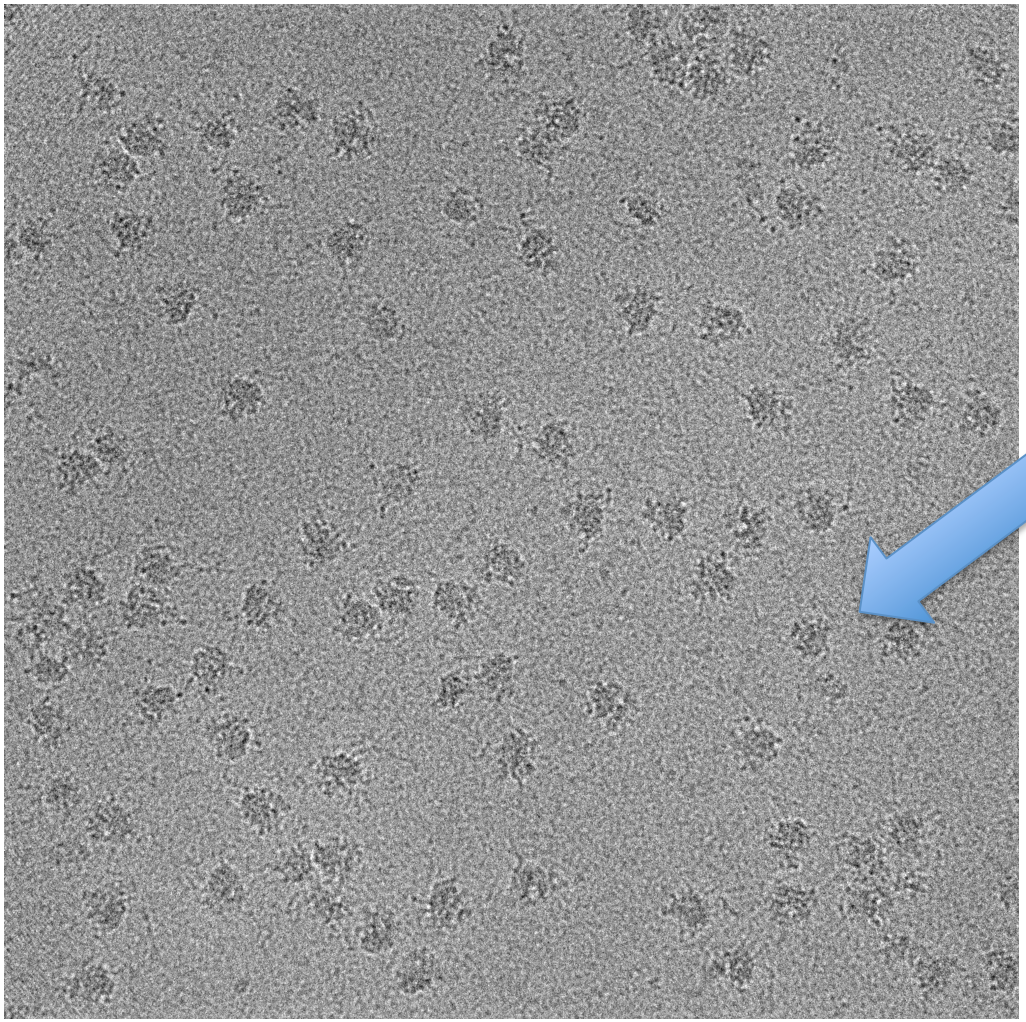
50,000 X



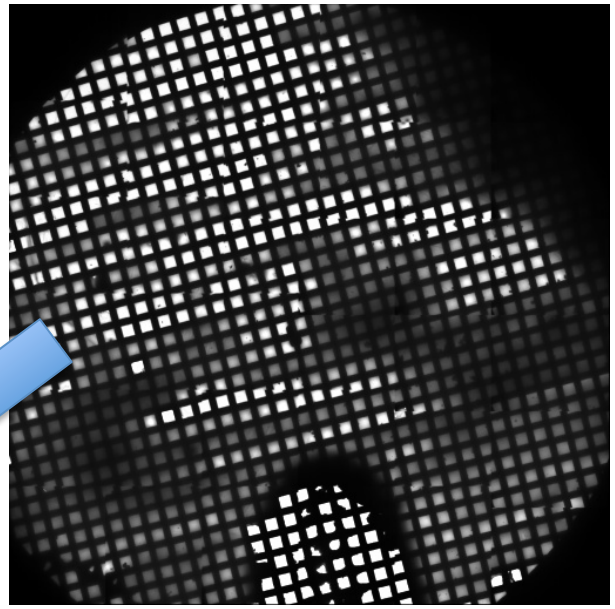
FFT of Micrograph

Chris Russo

End result



Collect some data!



Don’t judge a grid by its Atlas!

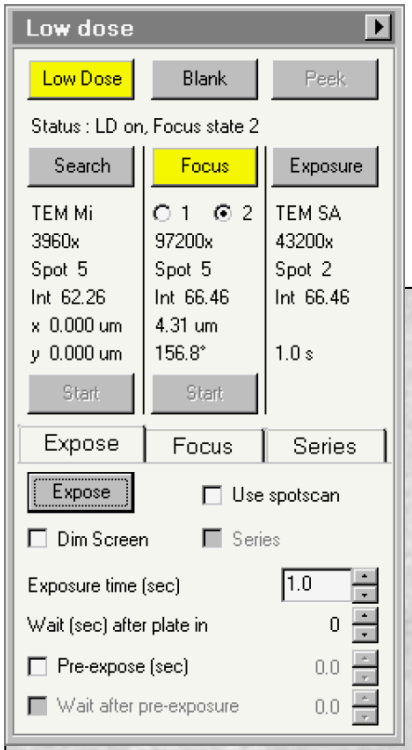
(Rebecca Voorhees, 2016)

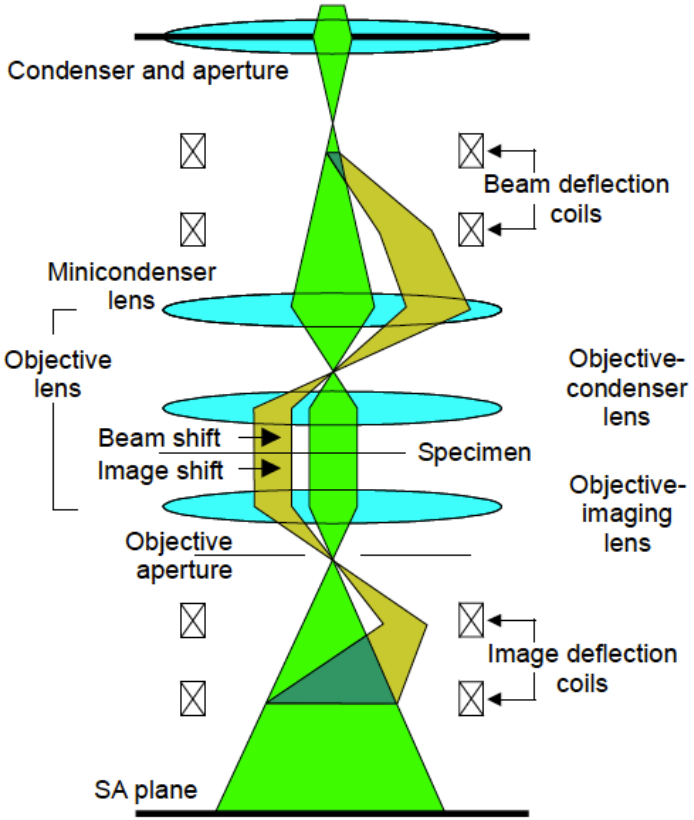
Data Collection Software

- Manual: FEI low dose in TUI
- Semi-automated: UCSF Image 4
- Automated:

- EPU: FEI
- Serial EM: Boulder (David Mastronarde)
- Leginon: (NRAMM)

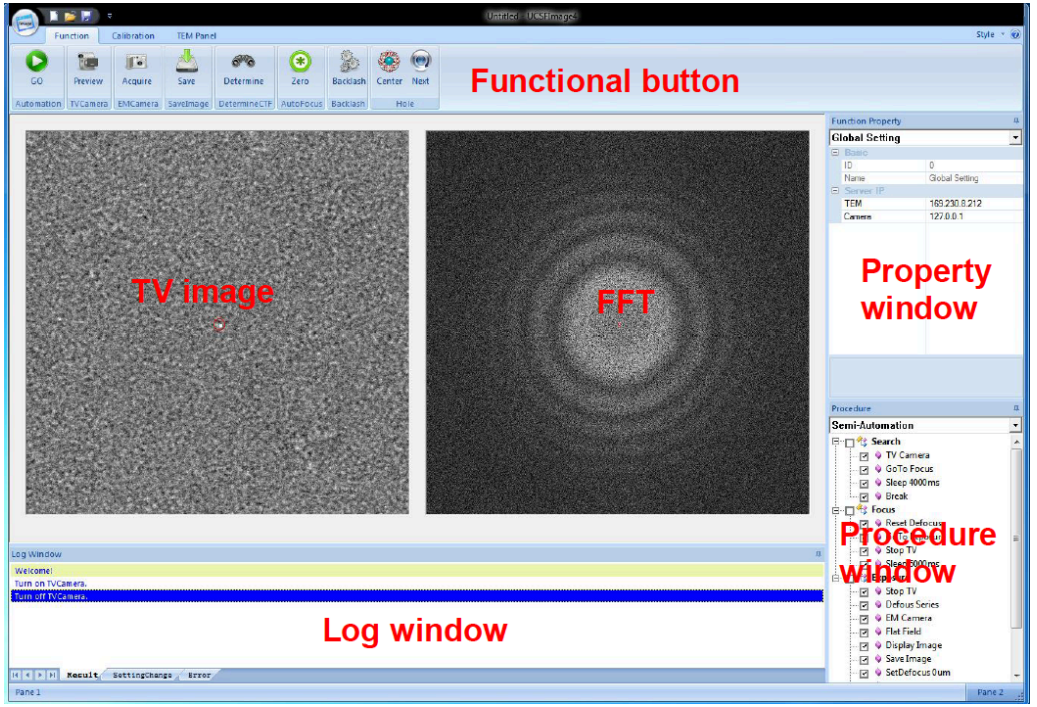
Low dose Data acquisition





Semi-Automated: UCSF Image 4

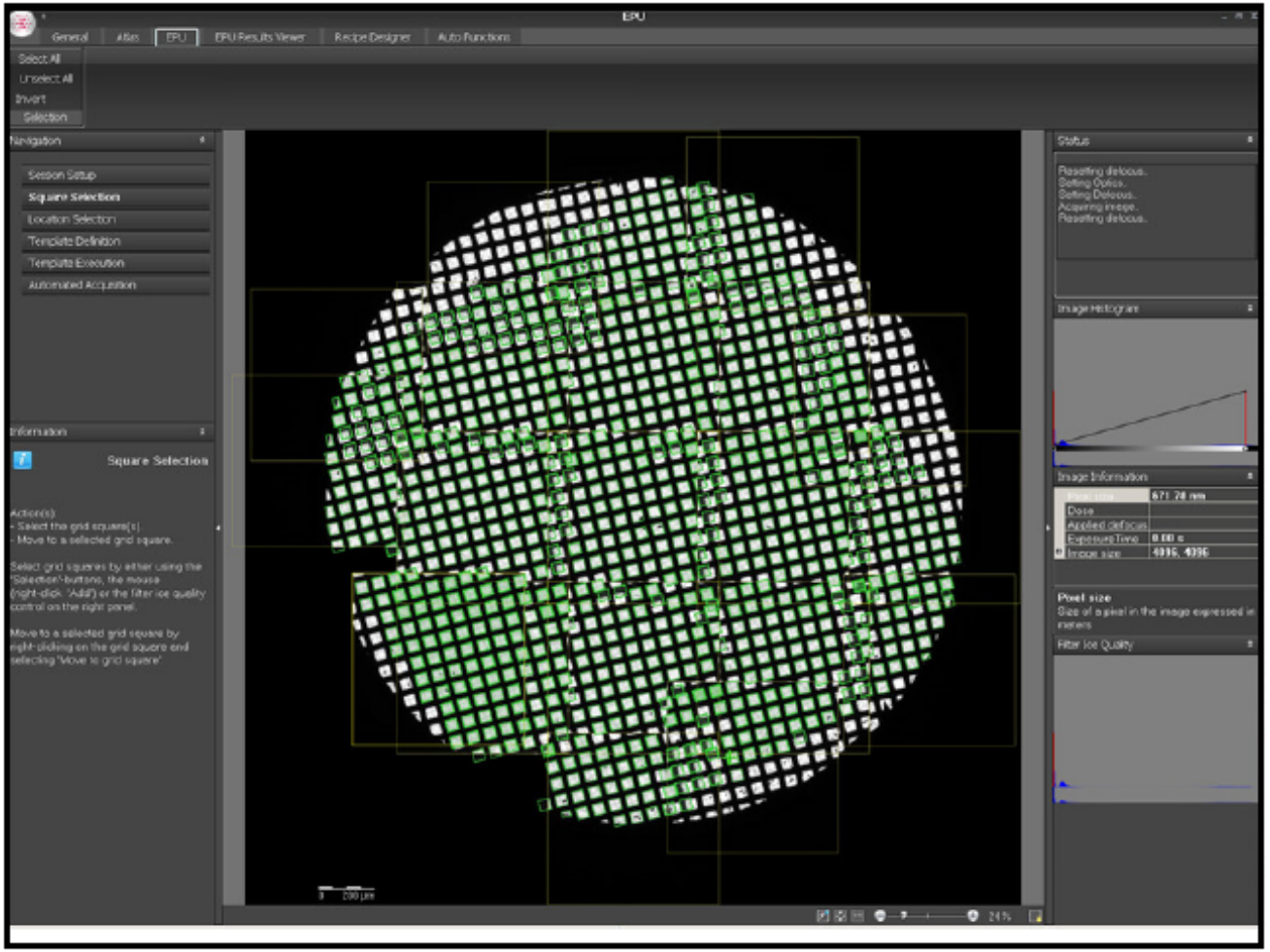
- Interface for controlling the FEI Low Dose routine
- Allows movement from one hole to the next and image recording
- Works with K2
- Cannot set-up multiple grid-squares



Automated Data Acquisition
FEI-EPU

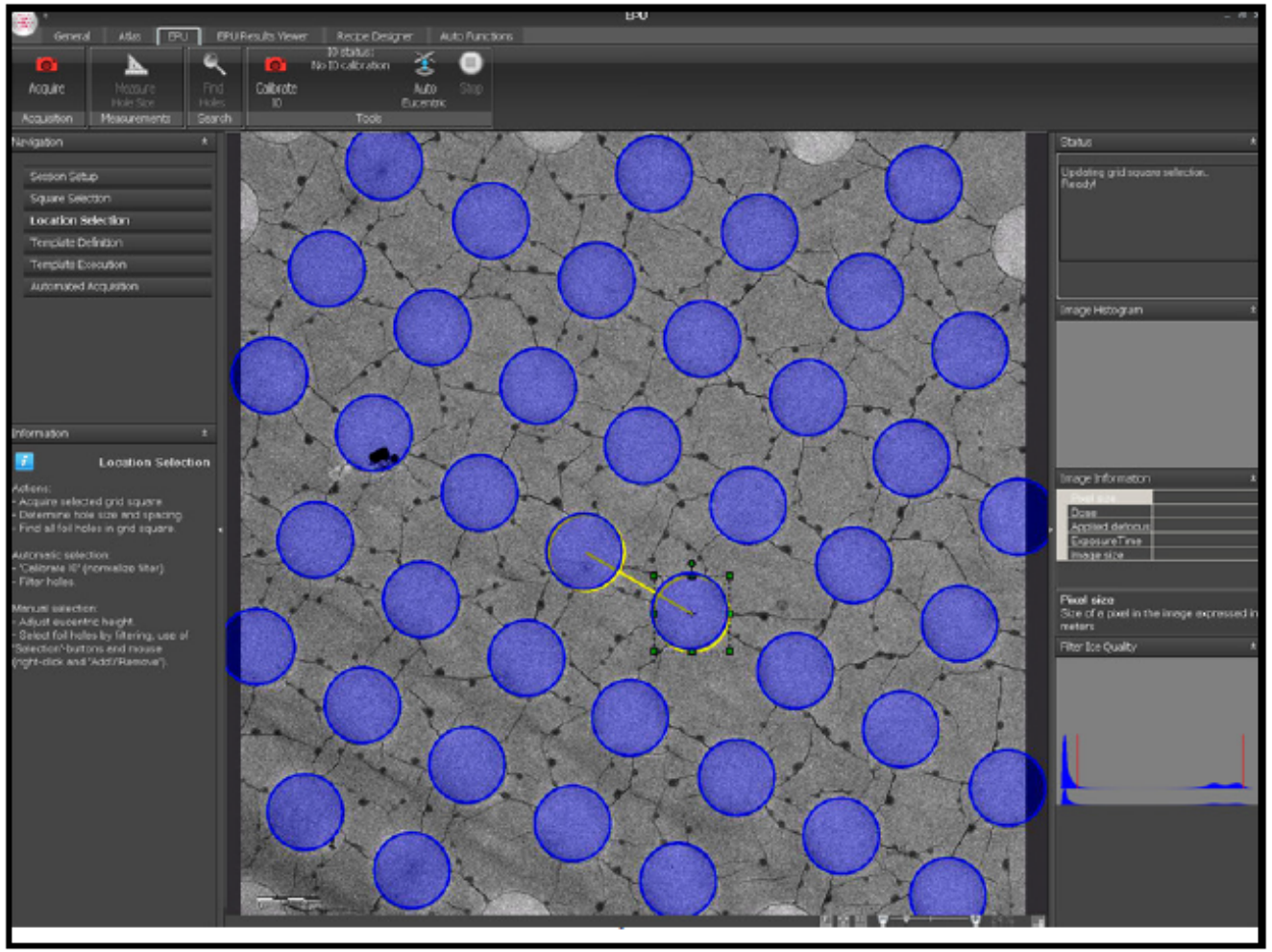
- EPU- Latin *E Pluribus Unum* — “out of many, one“

- Atlas: Overview of the grid
- User selects suitable squares



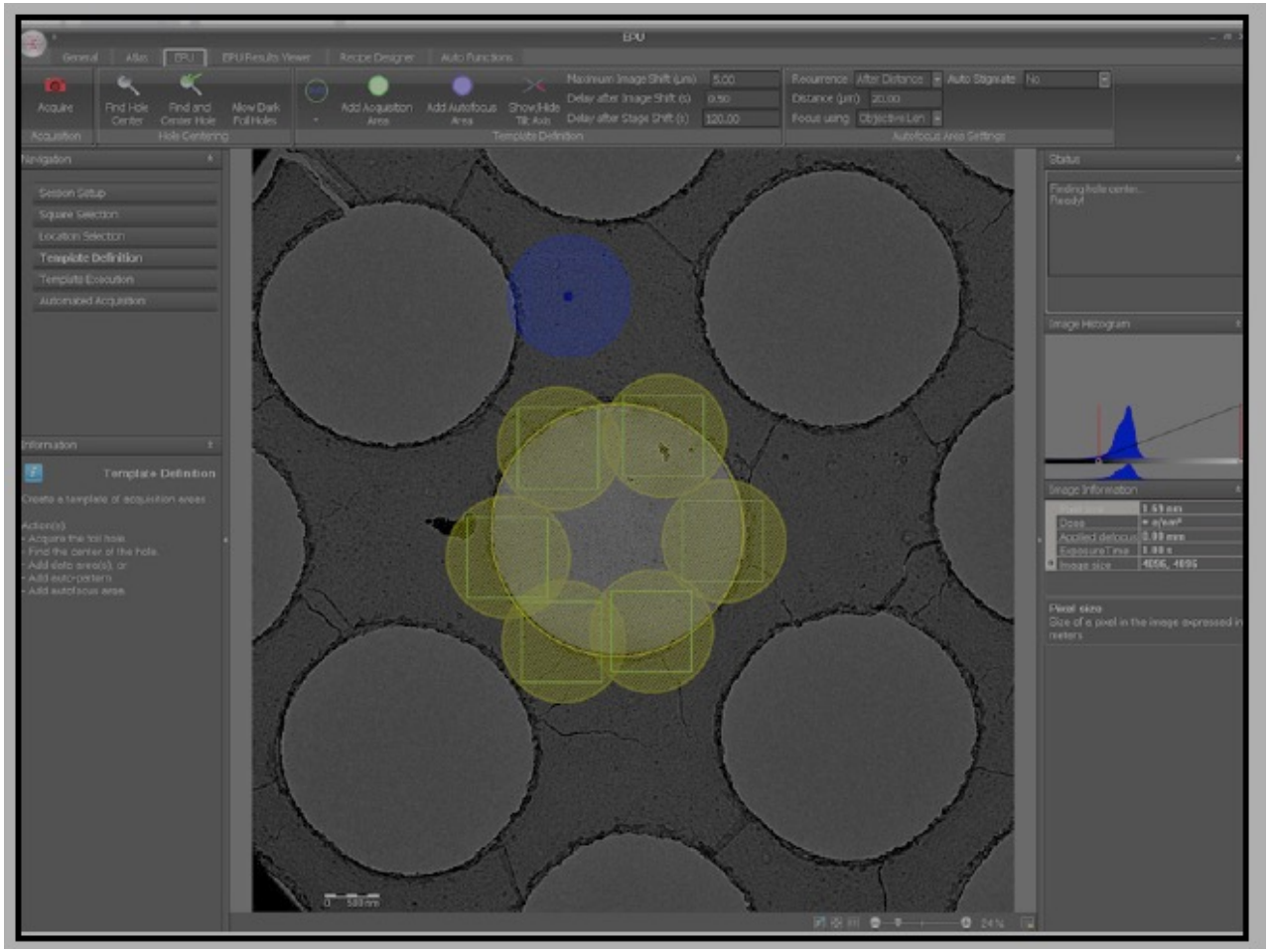
Automated Data Acquisition
FEI-EPU

- Gridsquare-Overview of each selected gridsquare
- User selects suitable holes



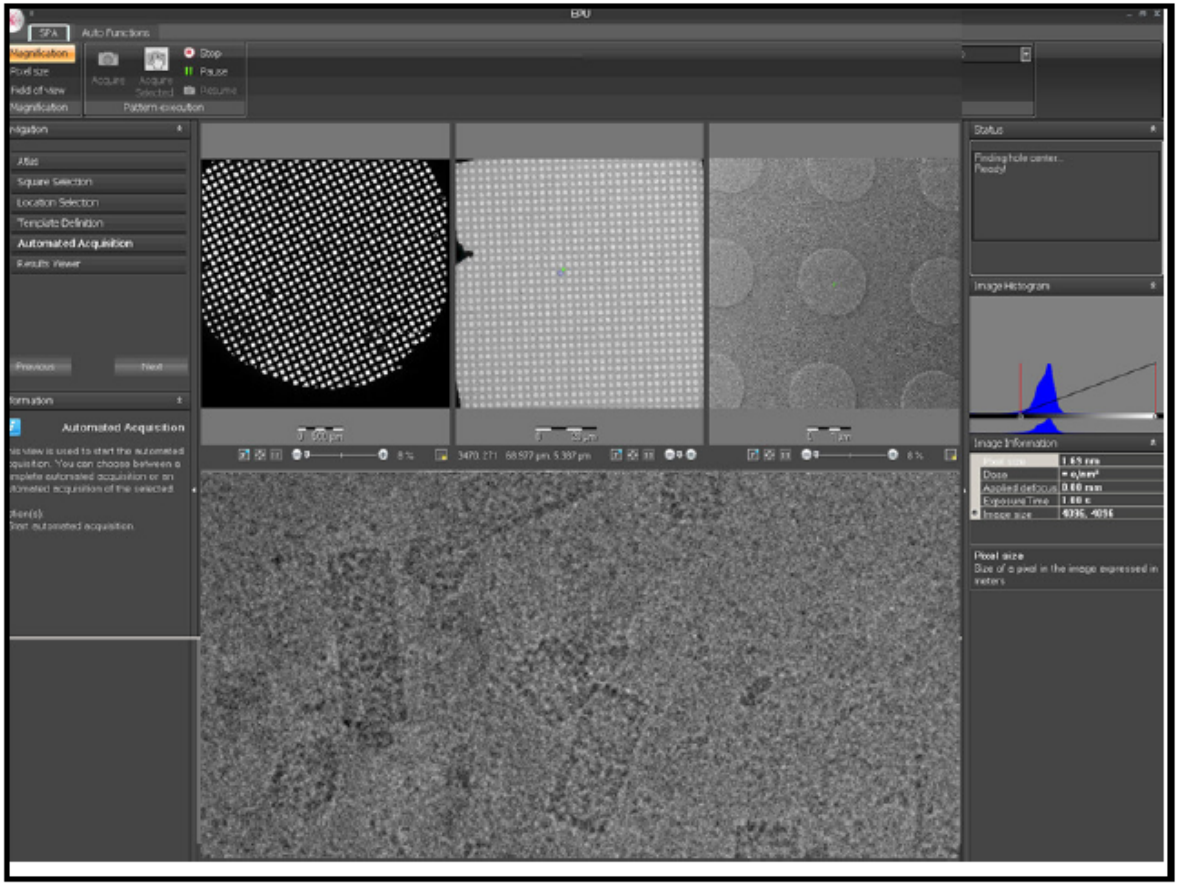
Automated Data Acquisition
FEI-EPU

- Template definition
- User setup of Exposure, Focus and Drift tracking
- Easy setup of multiple exposures/hole



Automated Data Acquisition
FEI-EPU

- Data collection begins
- EPU manuals for K2 and Falcon available on our Calpendo website



EPU Summary

- Ease of use and reliability
- Now also works with K2 detector in GMS 2.33
- Newest version allows phase plate selection and beam-tilt offset adjustment
- Speed: For 1 exposure/hole:
  - ~ 30 images/hour for K2 in counting
  - ~ 30 images/hour for Falcon III counting
  - ~ 45 images/hour for Falcon II/III linear

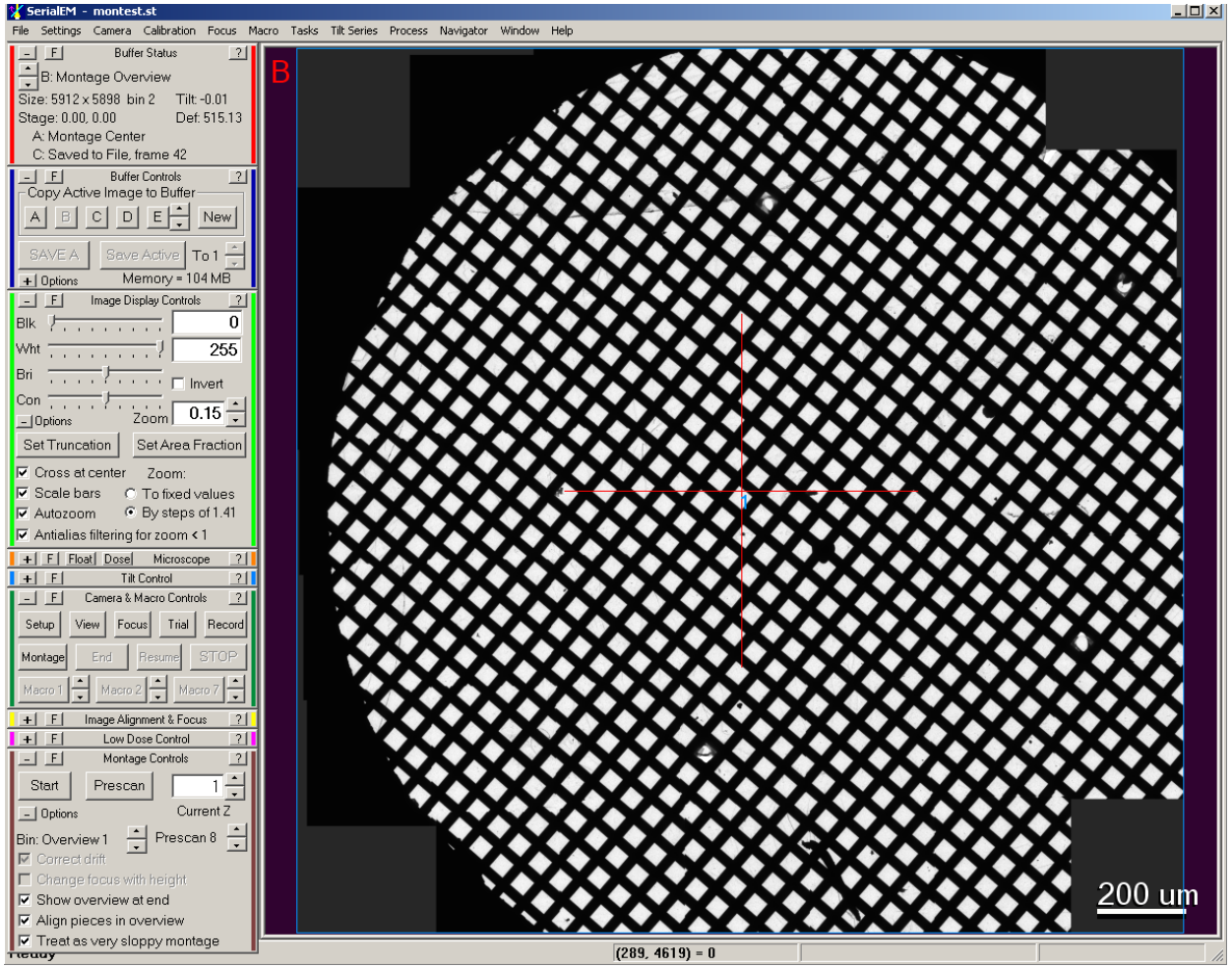
Automated Data Acquisition
Serial EM

- Software developed for tilt-series collection
- Scripts written for single particle collection- (Chen Xu, Wim Hagen…..)
- Library for sharing scripts:

  https://serialemscripts.nexperion.net/

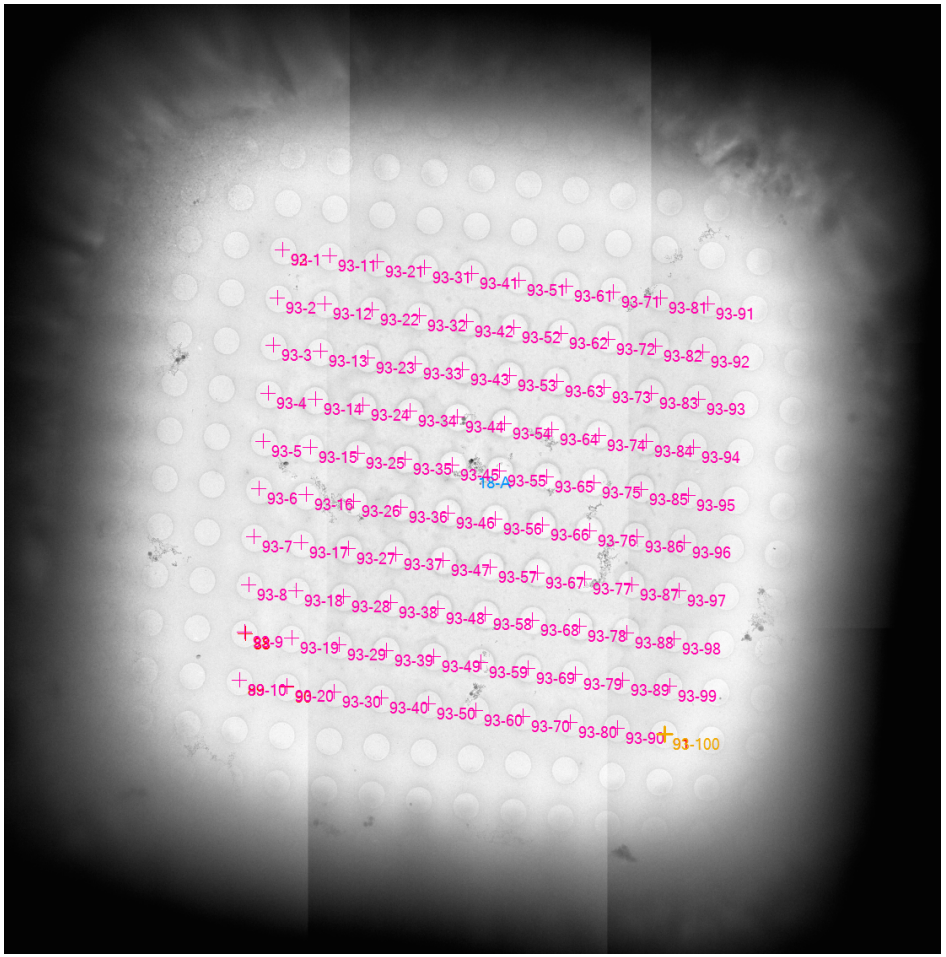
Automated Data Acquisition
Serial EM

- User acquires a grid map (Atlas)
- Good squares are selected
- Maps of grid-squares are acquired



Automated Data Acquisition
Serial EM

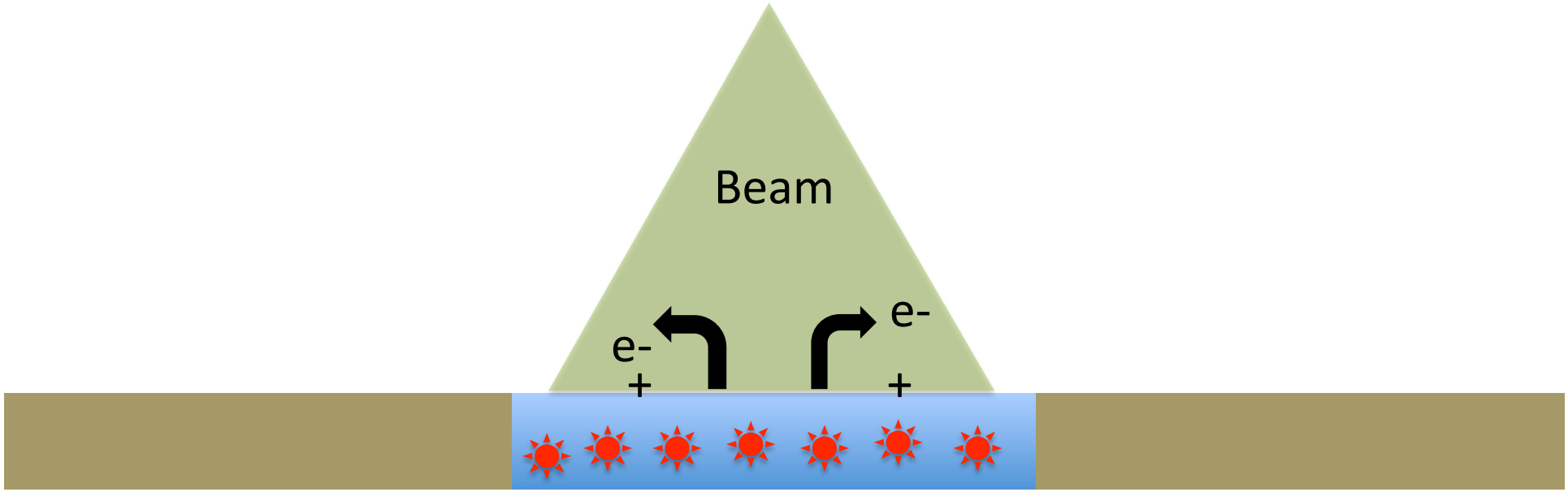
- A grid of points is added in each square e.g. 10x11
- Points may also be added manually
- Points have to be manually removed



Serial EM Summary

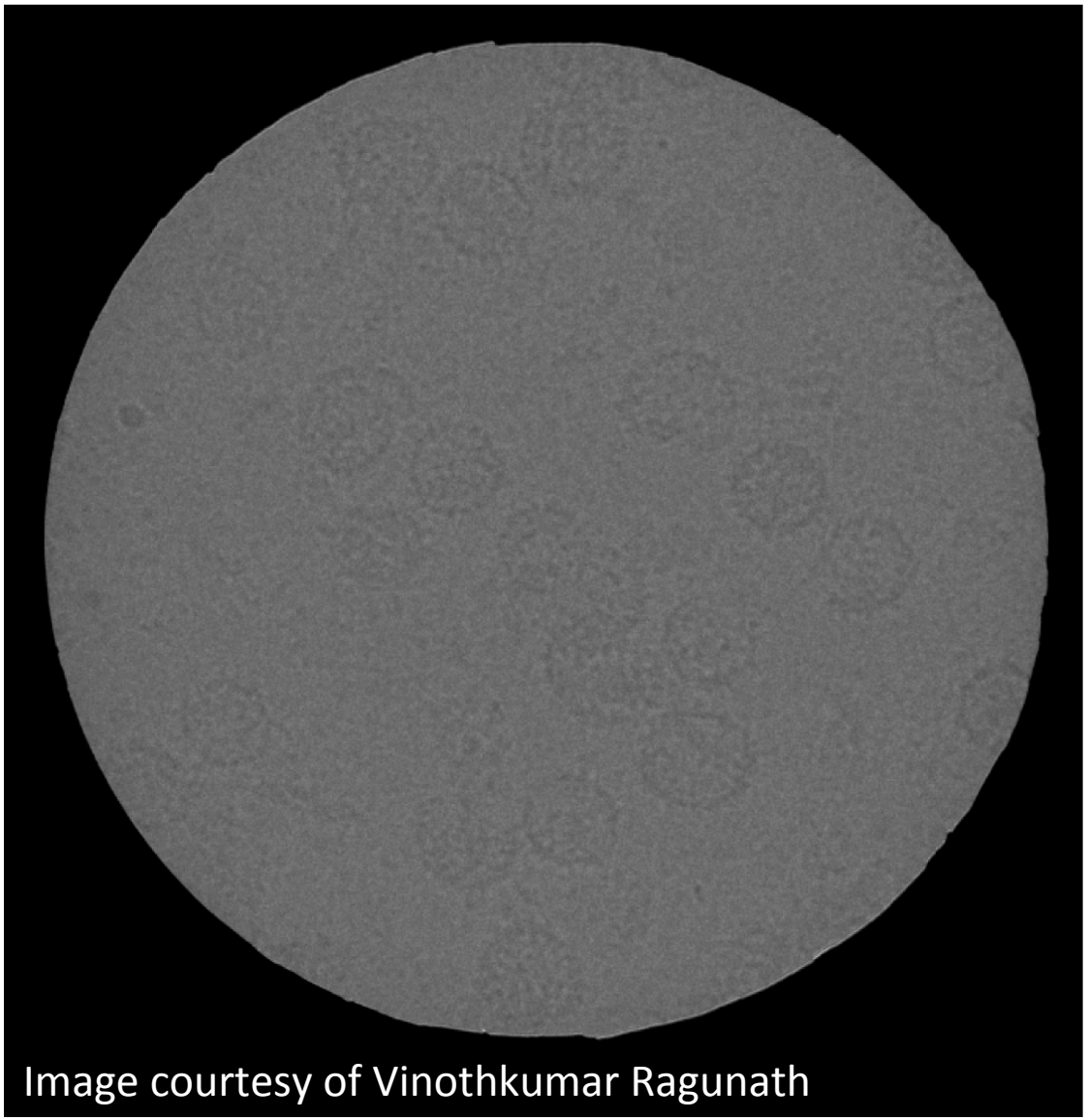
- Free and very flexible/customizable
- Works with Quantum/K2. Falcon movie frames not saved (testing in progress)
- File size: Uncorrected counting images can be saved as 4-bit Tiffs (LWZ) and post-processed later.
- Counting images are anti-aliased
- Speed: ~40 images/hour in counting; ~30 images/hour in Super-resolution.
- Works on FEI, Hitachi and JEOL microscopes
- New routines for auto-stigmation and auto-comma

Other considerations : Charging

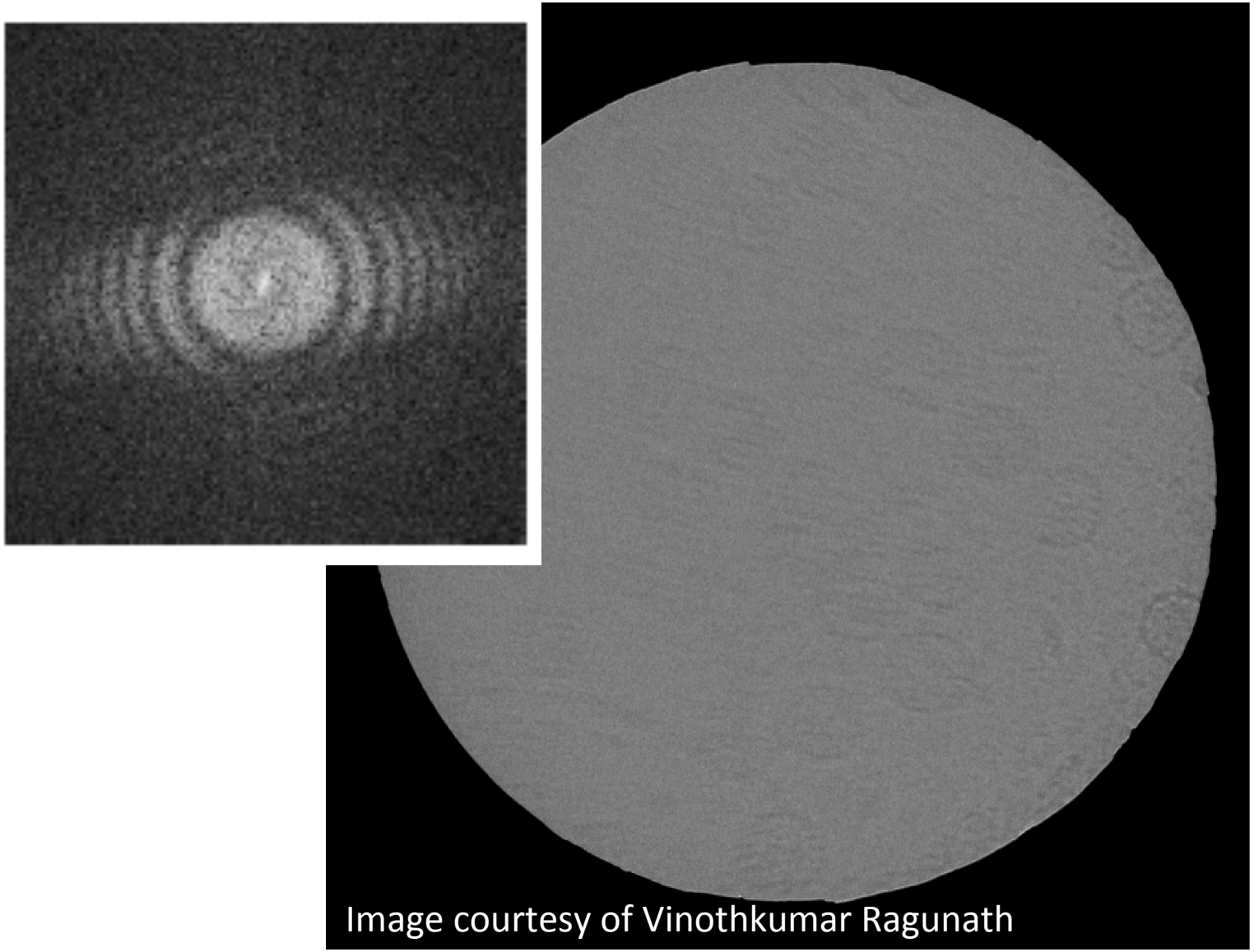


- Occurs due to insulating properties of ice
- Carbon or graphene overlaying can decrease charging

Specimen Charging

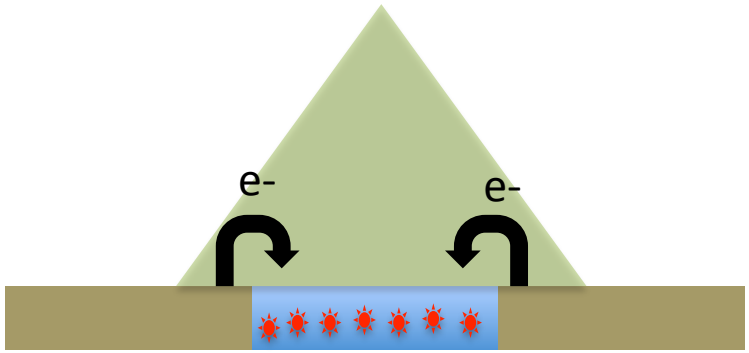


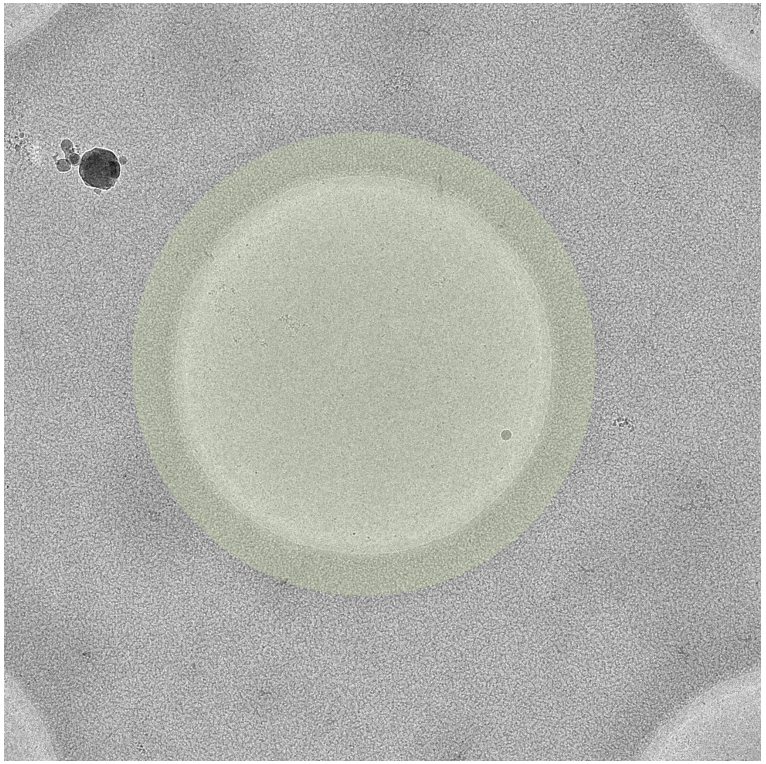
Specimen Charging



How to avoid Charging

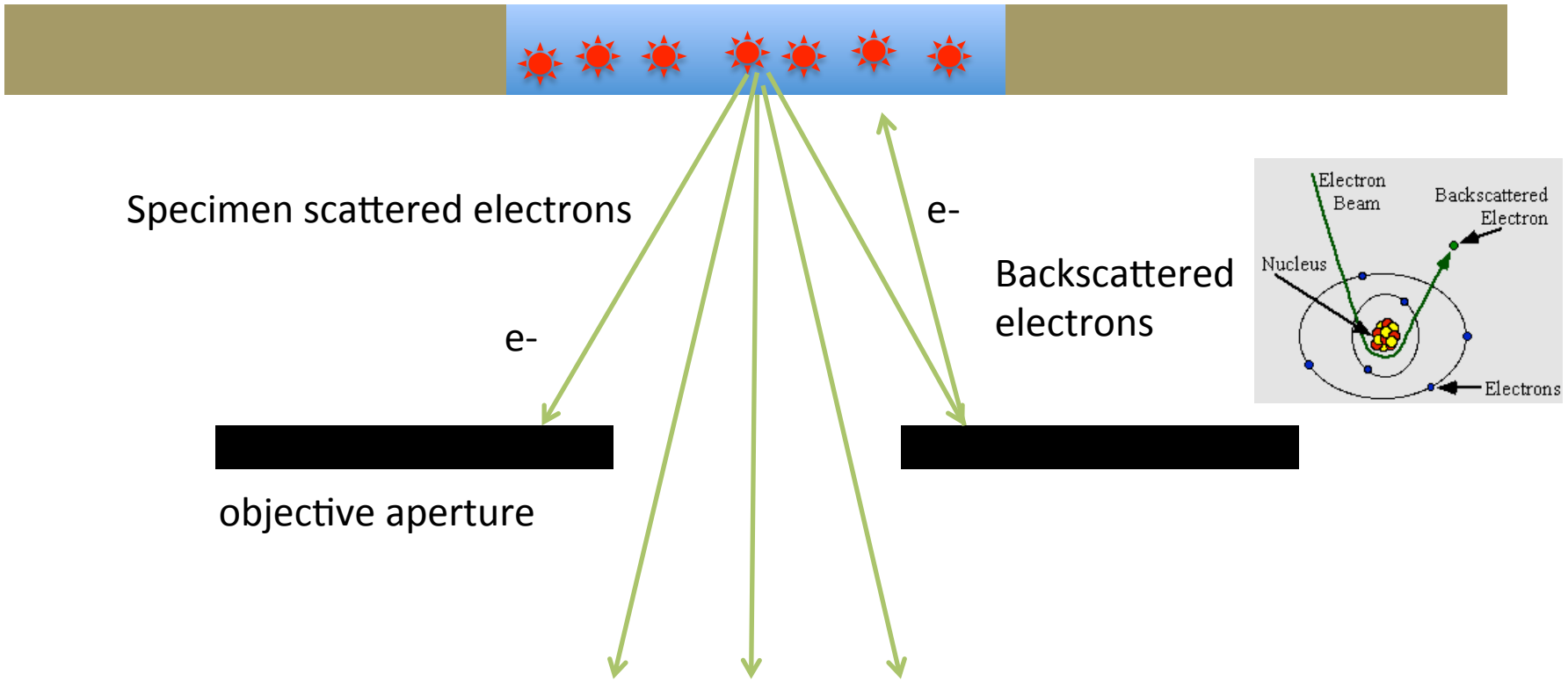
1. Illuminate an area larger than the hole or contact the carbon edge





How to avoid Charging

2. Use an objective aperture



Objective Aperture

- Can increase contrast and reduce charging
- Affects astigmatism> Check it after you insert

Consider resolution cut-off. e.g on Krios:

30 µm > 4 Å
70 µm > 1.8 Å
100 µm > 1.3 Å

Other considerations

What Magnification to use? E.g. for Falcon II/III on Krios

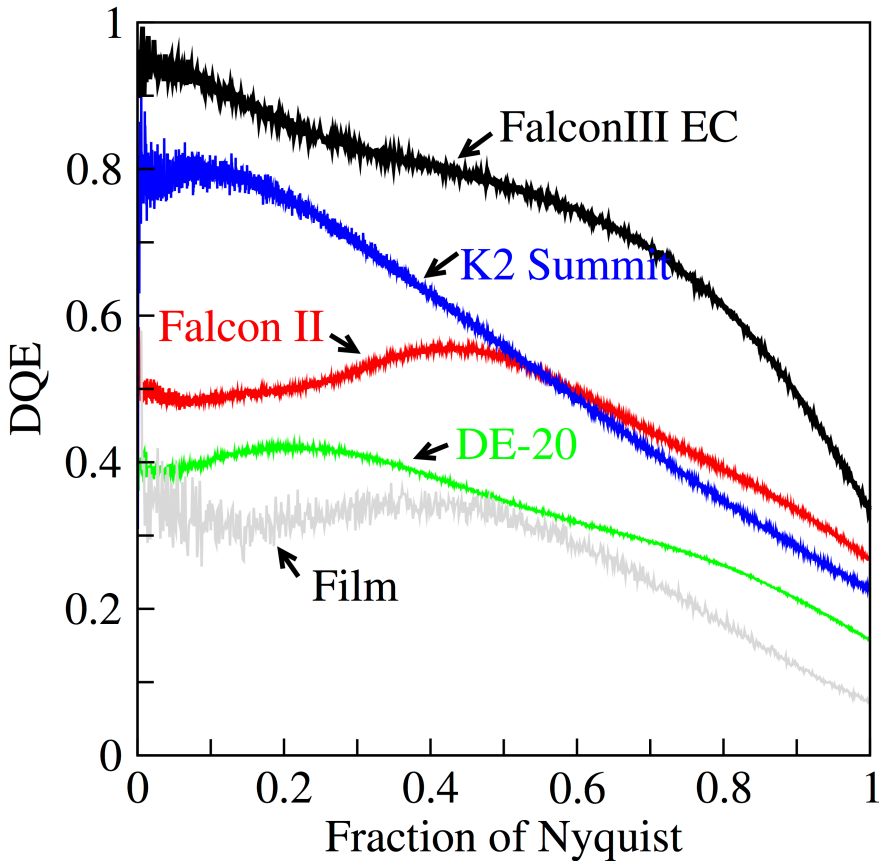
At 47Kx Nyquist is 3.54Å (50,000 ptcls)

At 59Kx Nyquist is 2.68Å (28,735 ptcls)

At 75Kx Nyquist is 2.14Å (18,337 ptcls)

Depends on:

- Sample stability/ heterogeneity
- Resolution/ Biological question



Remember:

Set-up eucentric height accurately and record an image for mag. Calibration (Ultra-Au or cross-grating)

Other considerations

- What defocus range to use?
  Generally ~1.5µm-4µm, 0.3 µm intervals

In counting mode particles visible at lower defocus

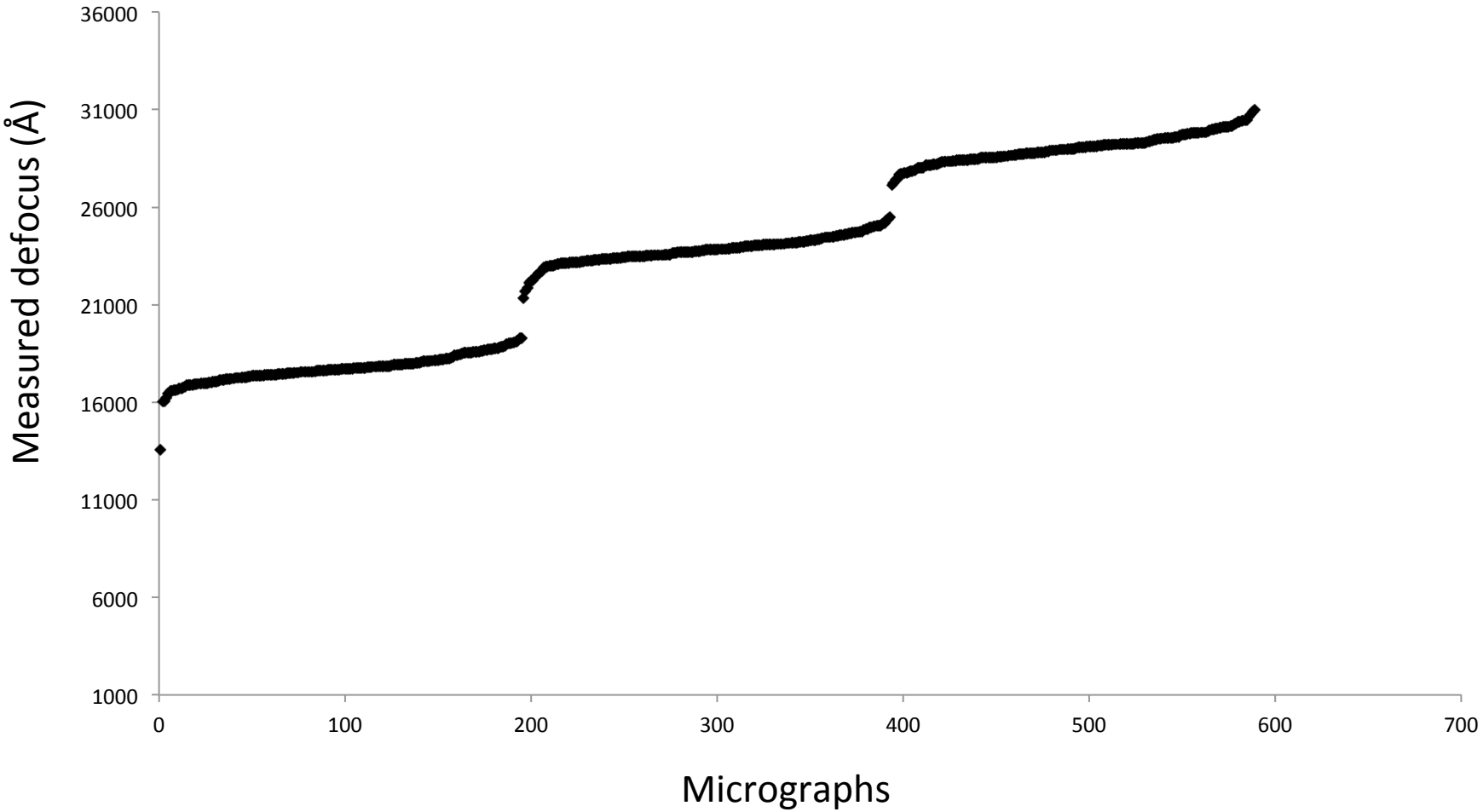
Always check if you can see your particles at the low defocus values!

For smaller particles use higher dose >60 e-A^2

Beware of Einstein from noise!

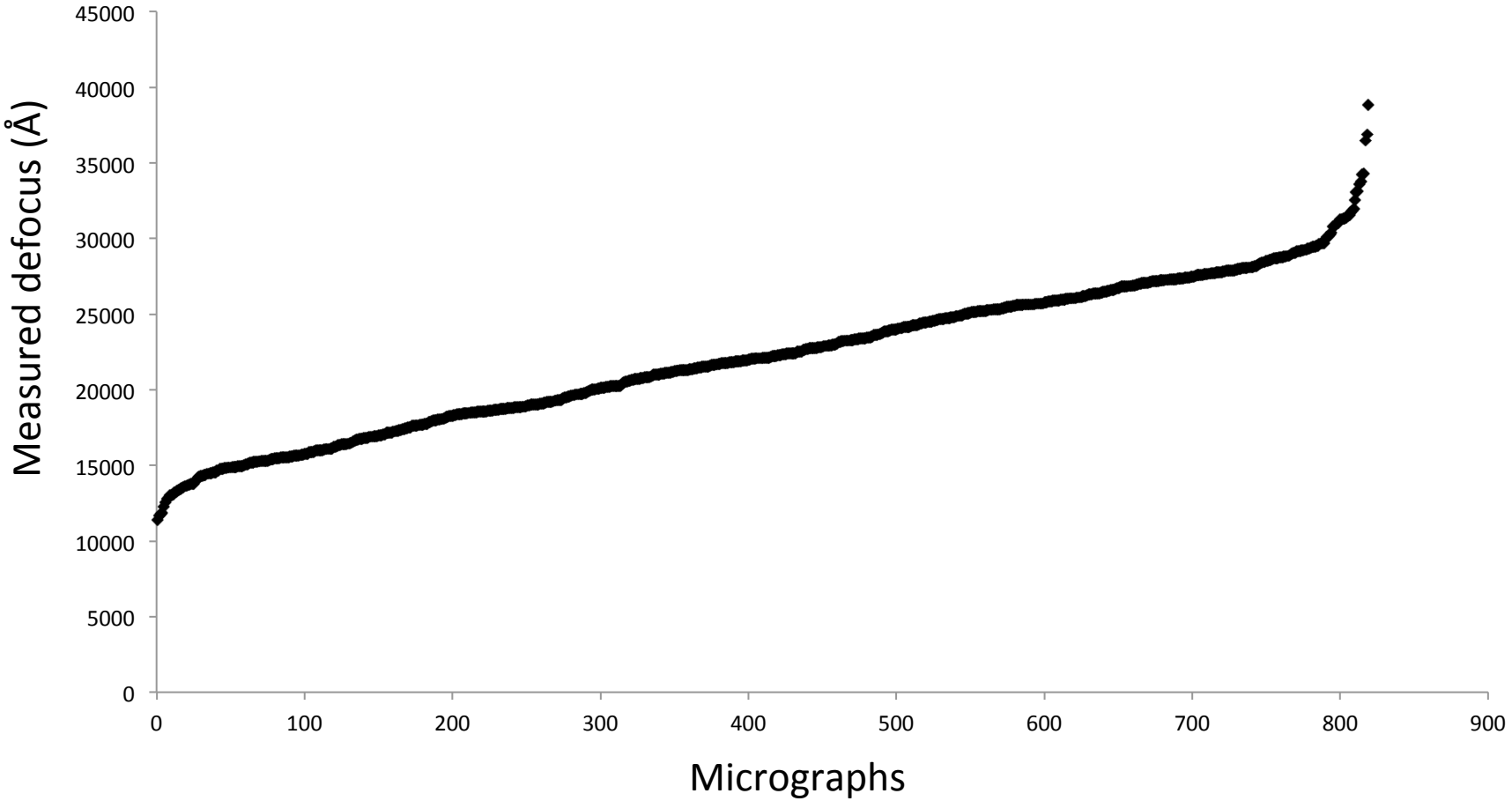
Defocus spread: EPU

Values selected: -1.8µm, -2.3µm, -2.8µm, Focusing at every hole



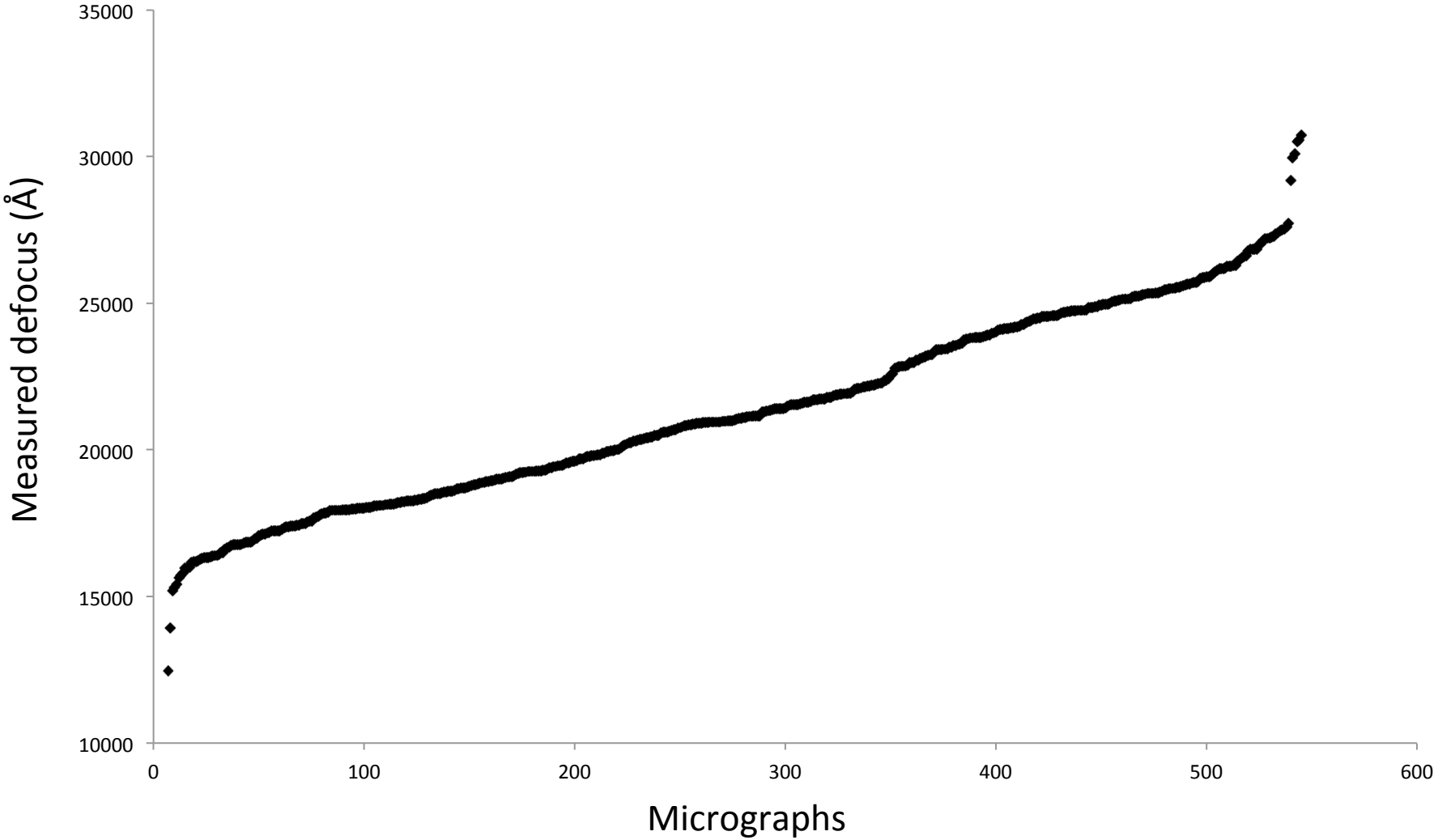
Defocus spread: EPU

Values selected: -2.3, -2,6, -2.9 ,-3.2 and- 3.4, Focusing after every 8µm distance

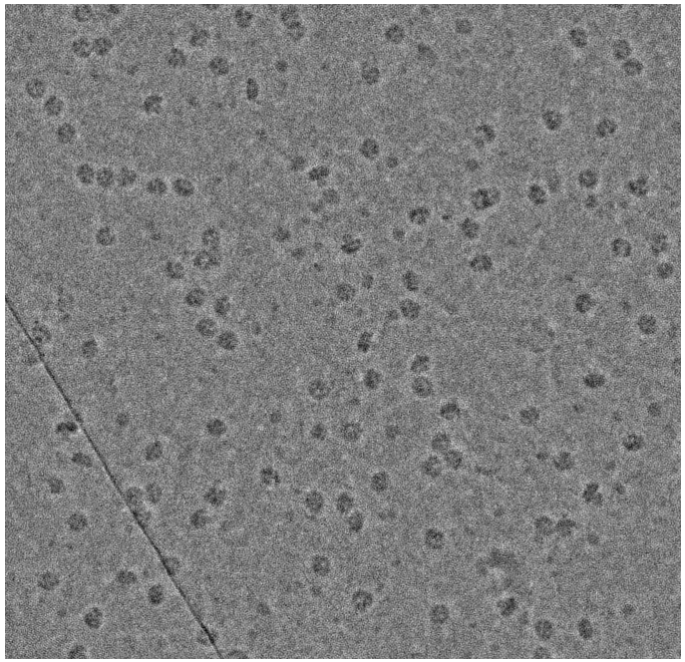


Defocus spread: Serial EM

Values selected: -1.7µm, -2.0µm, -2.3µm, -2.6µm, Focusing in every hole

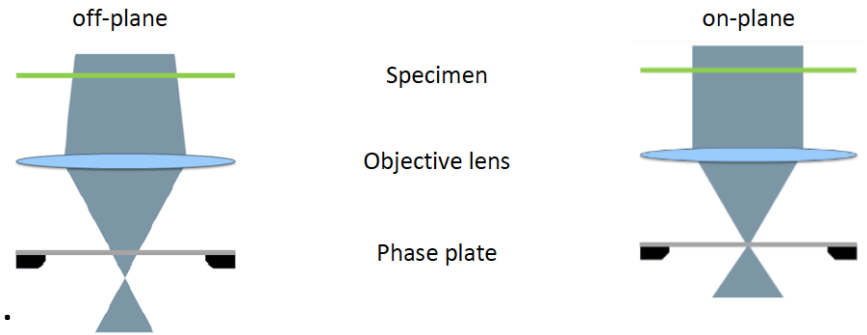


Volta Phase Plate



Krios 2, Falcon 3 in counting mode
Lysenin at 3.9Å from ~5000 particles
(Rafa Leiro/Christos Savva)

- Complexes smaller than 150 kDa could benefit
- Required alignments for single particle: Setup in-plane condition and reduce condenser astigmatism
- For tomography beam-shift pivot points also require adjusting



Danev, R., Baumeister, W.

Acknowledgements

- Thanks to Greg, Giuseppe Shaoxia, Vinoth, Sjors, Lori and Chris for input
- Other LMB members for the wealth of knowledge
- Toby and Jake

- More movies available on:
- EPU data collection
- Phase plate setup
- GIF tuning and gain reference acquisition (K2)