IMpaCT
IMAGING LIFE FROM MOLECULES TO CELLS

Tomography Acquisition
-or-
The First "R" (of 3)

Sharon Grayer Wolf

Tomography workflow

1. Sample preparation (Eyal, Katya)
2. Correlative Light-Electron Microscopy -CLEM (Tali)
3. Data acquisition (Sharon)
4. Image processing (Nadav)

Data Acquisition

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1. Microscope Components important for Tomography workflow (EVERYTHING)
   a) Electron guns
   b) Higher Voltage for thick specimens
   c) Sample exchange system
   d) Excellent vacuum systems
   e) Stable optics
   f) Energy filter
   g) Phase plates
   h) Cameras/detectors

2. Tomography Acquisition
   a) Tilt schemes
   b) Dose considerations
   c) Defocus, magnification choices that affect resolution
   d) Data collection software

Electron Sources

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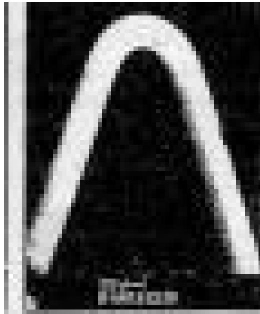
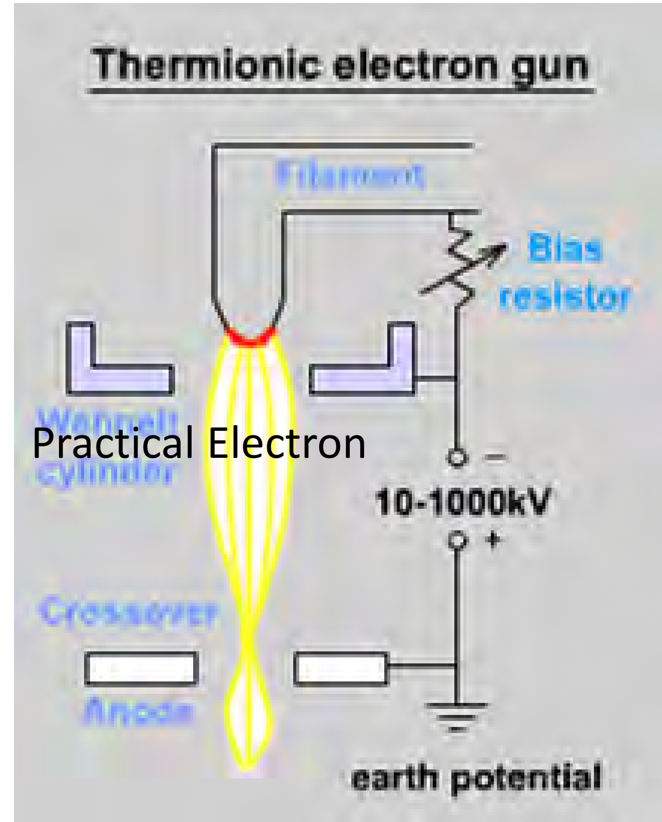
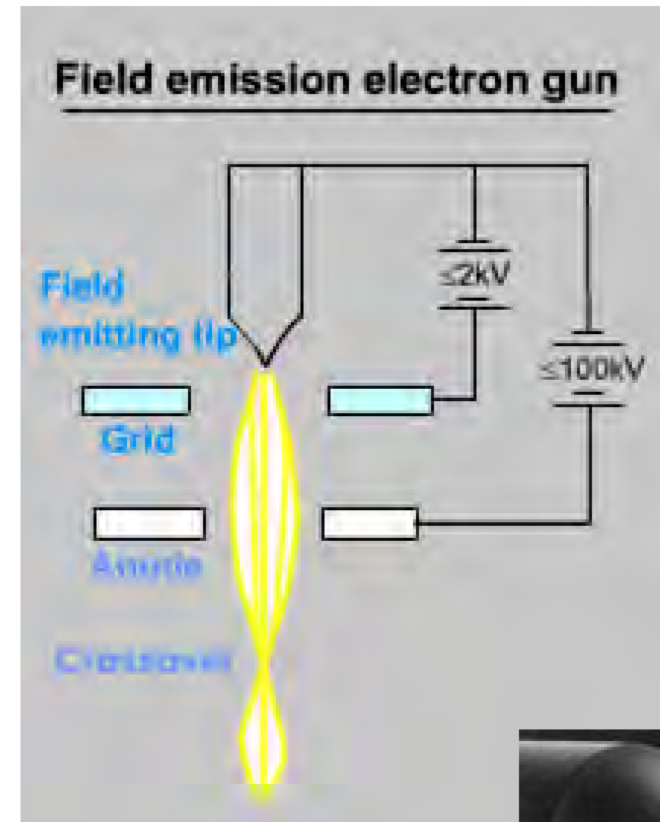
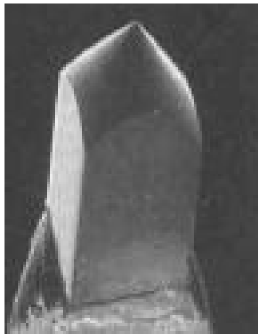


Tungsten

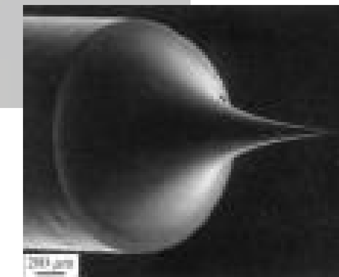


Practical Electron





LaB6



FEG

Practical Electron Microscopy and Database: www.globalsino.com/EM/

Electron Sources

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- Thermionic guns: Electron emission through heating.
- Field Emission Guns (FEG): Electron emission through applying an extraction voltage.

Properties	Tungsten	LaB6	FEG
Work function / eV	4.5	2.4	4.5
Temperature / K	2700	2000	1800
Energy spread / eV	3-4	1.5-3	0.4-1.5
Source size / nm	30000	5000	3-20
Maximum current / nA	1000	500	300
Brightness / A/m^2sr	10^9	$5x10^{10}$	10^{13}
Lifetime / h	100	500	> 1000

Electron Sources

Frensel fringes

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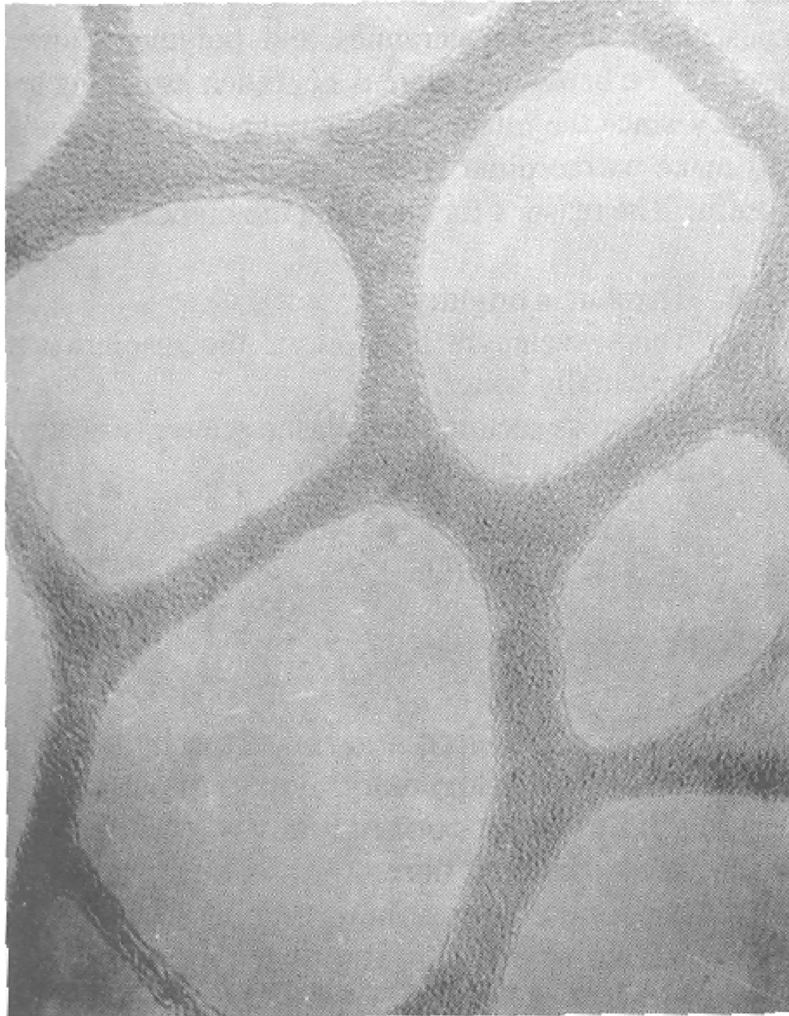
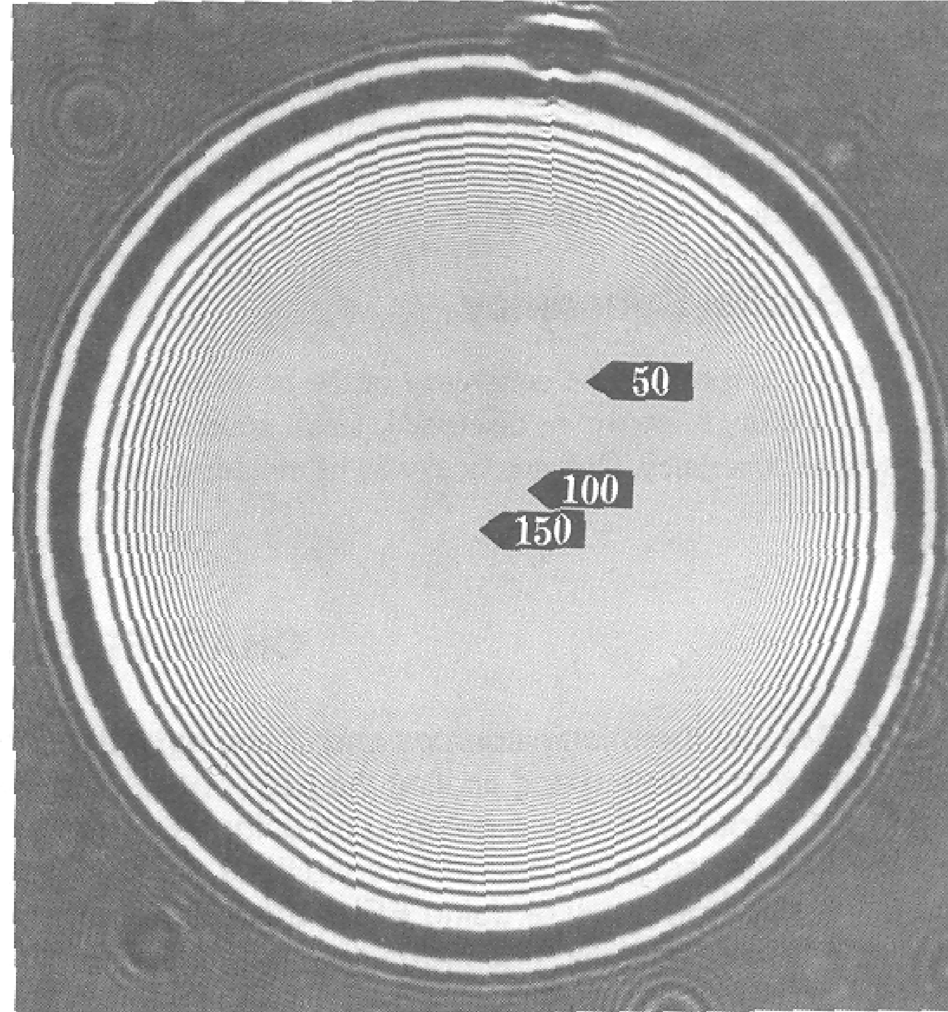
A Thermionic Source
(poor coherency)



A FEG
(high coherency)



Increasing voltage increases mean free path

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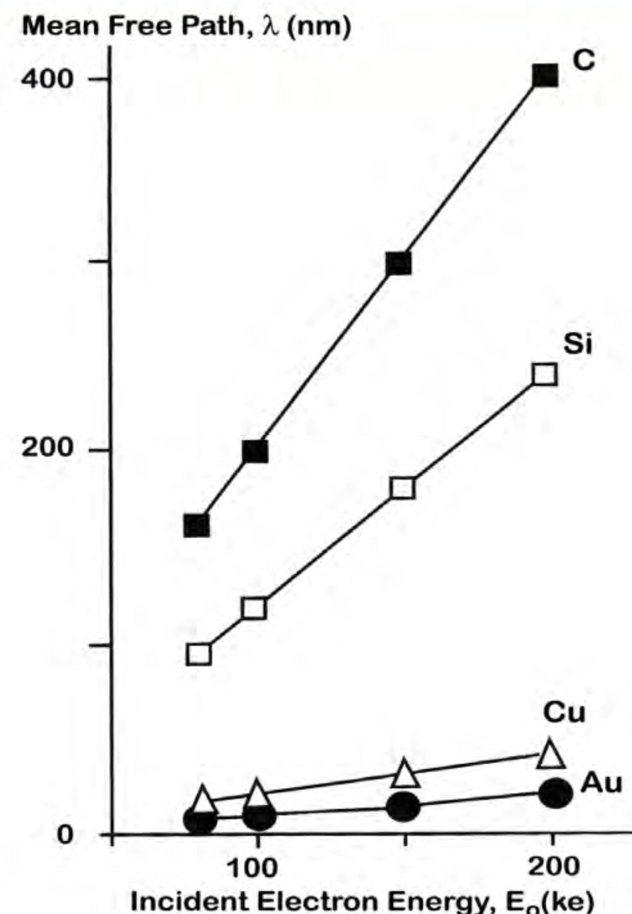
Elastic scattering

Screened relativistic Rutherford cross section

Can be plotted as an equivalent mean free path vs. incident energy

This gives you a good sense on allowable sample thickness!



https://nanohub.org/resources/4003/watch?resid=27358

Phase contrast in TEM images

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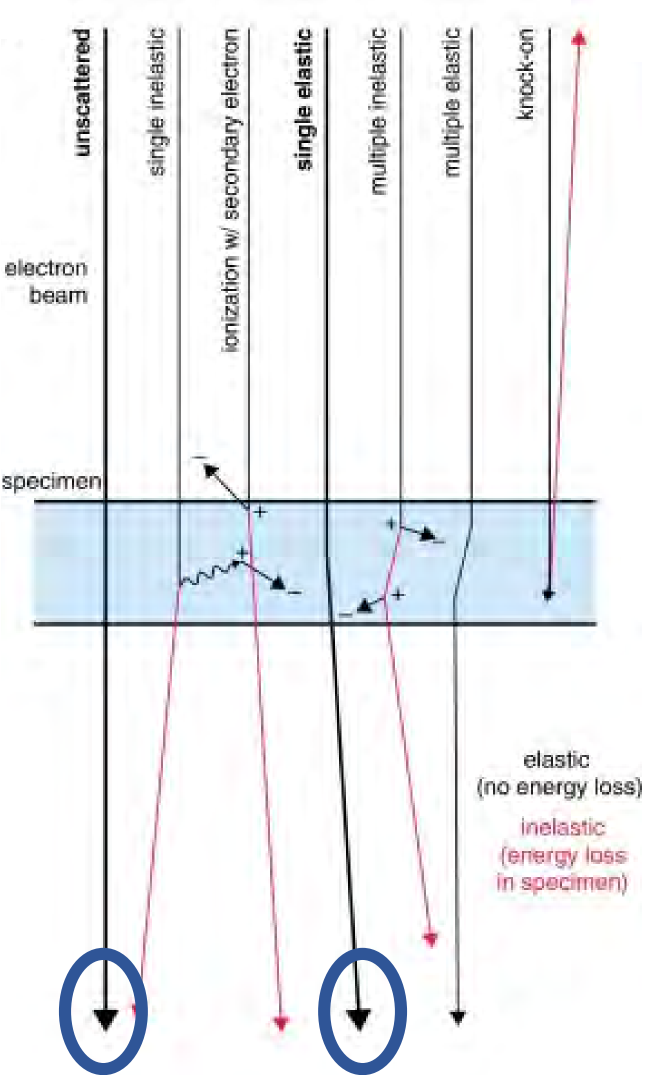
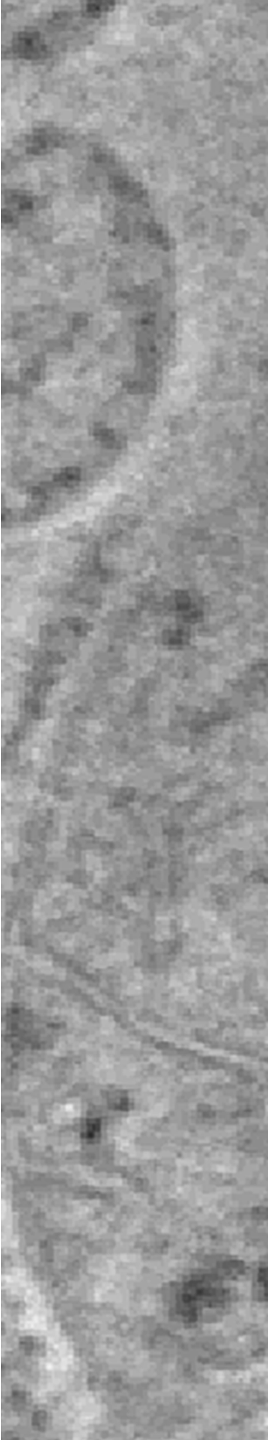


CryoTEM relies on *Phase Contrast which requires:*

COHERENT, ELASTIC SCATTERING

Russo, C.J. and L.A. Passmore, Curr Op Struct Biol, 2016. **37**: p. 81-89.



CryoTEM: Sample thickness is limited by the INELASTIC MEAN FREE PATH

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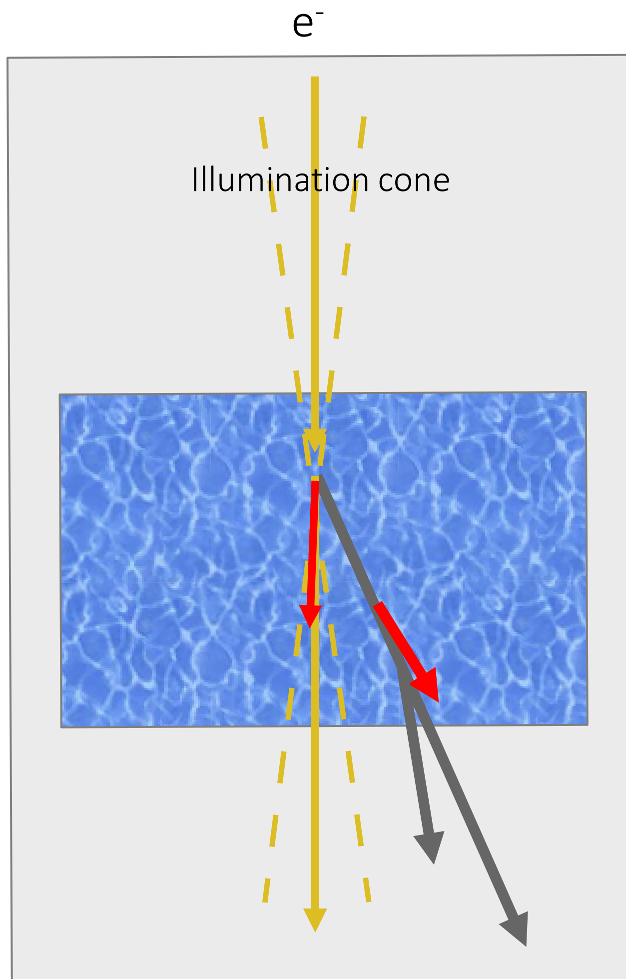

Elastic scattering

Inelastic scattering

Cross-section: elastic ~ $Z^{3/2}$
inelastic ~ $Z^{1/2}$

Ratio inelastic/elastic ~ 20/Z

Inelastic mean free path in vitrified water for 200kV

~ 250 nm

The “Natural” limit for cryoTEM Tomography

For thick samples,few “useful” (elastic) electrons remain:

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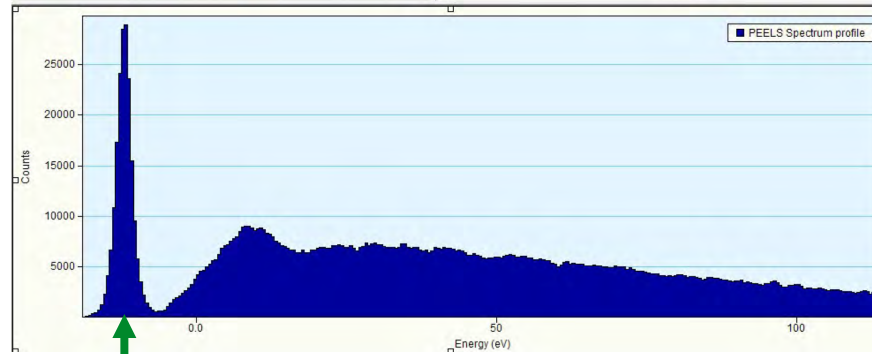
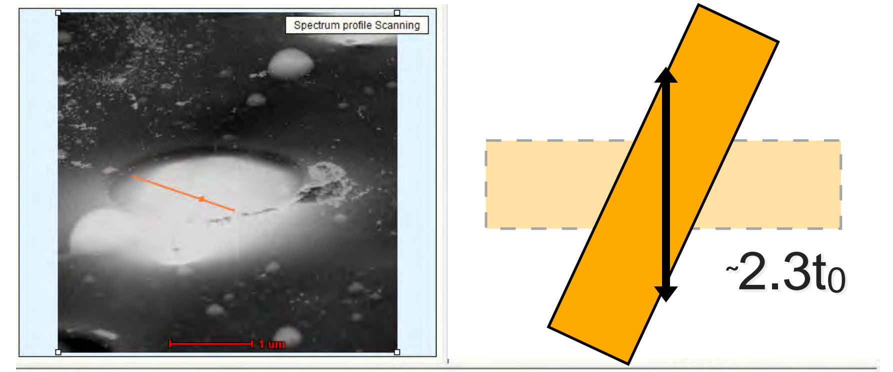
0 deg

t_0

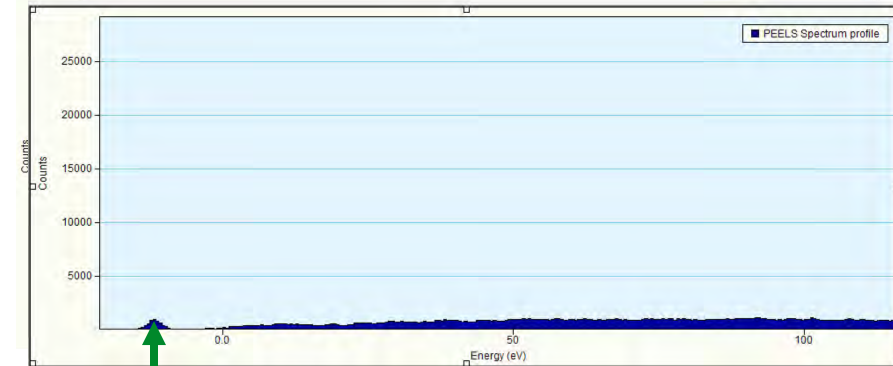


elastic scattering

65 deg tilt



$\sim 2.3t_0$



EELS SPECTRA

Energy Filter

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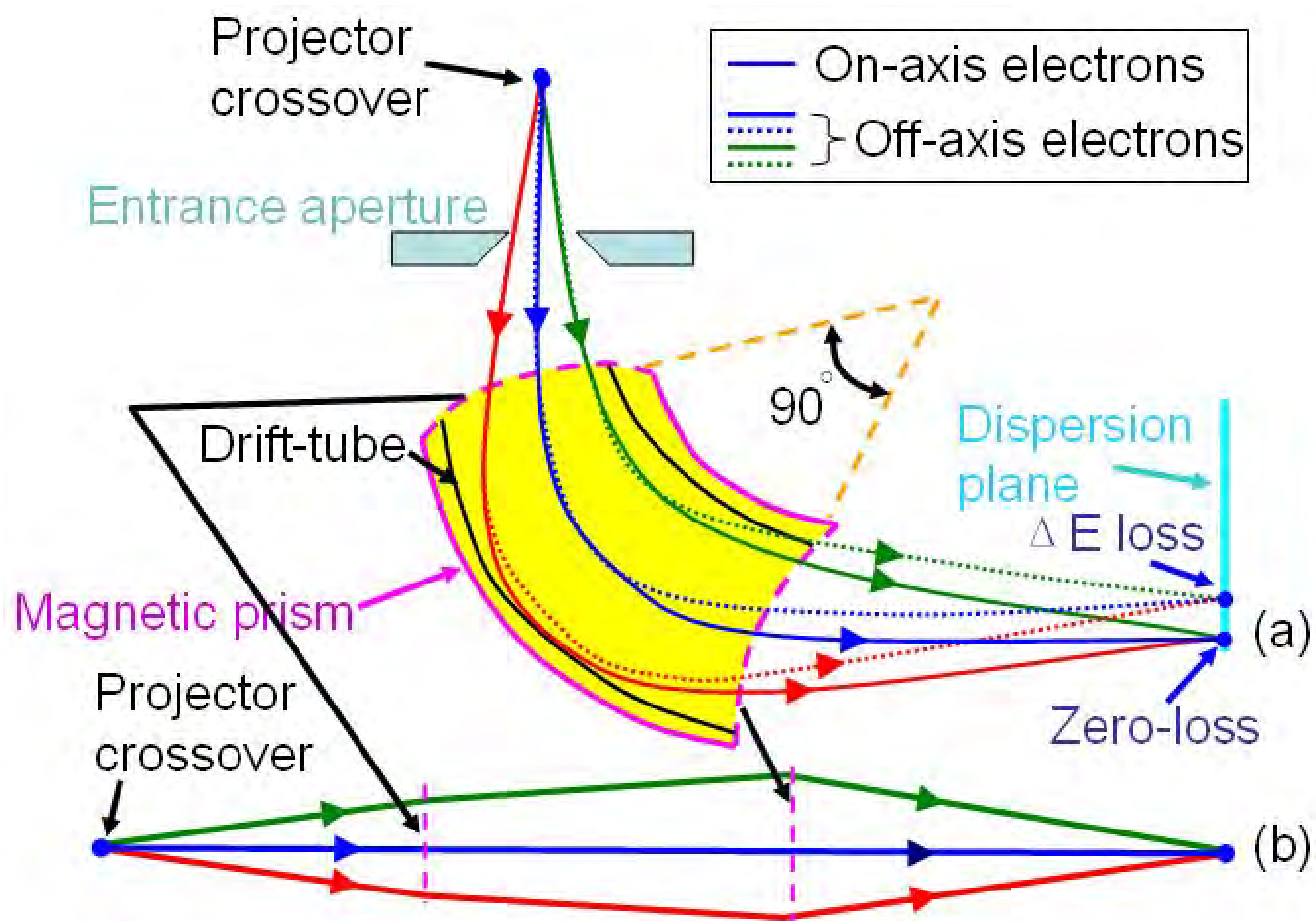
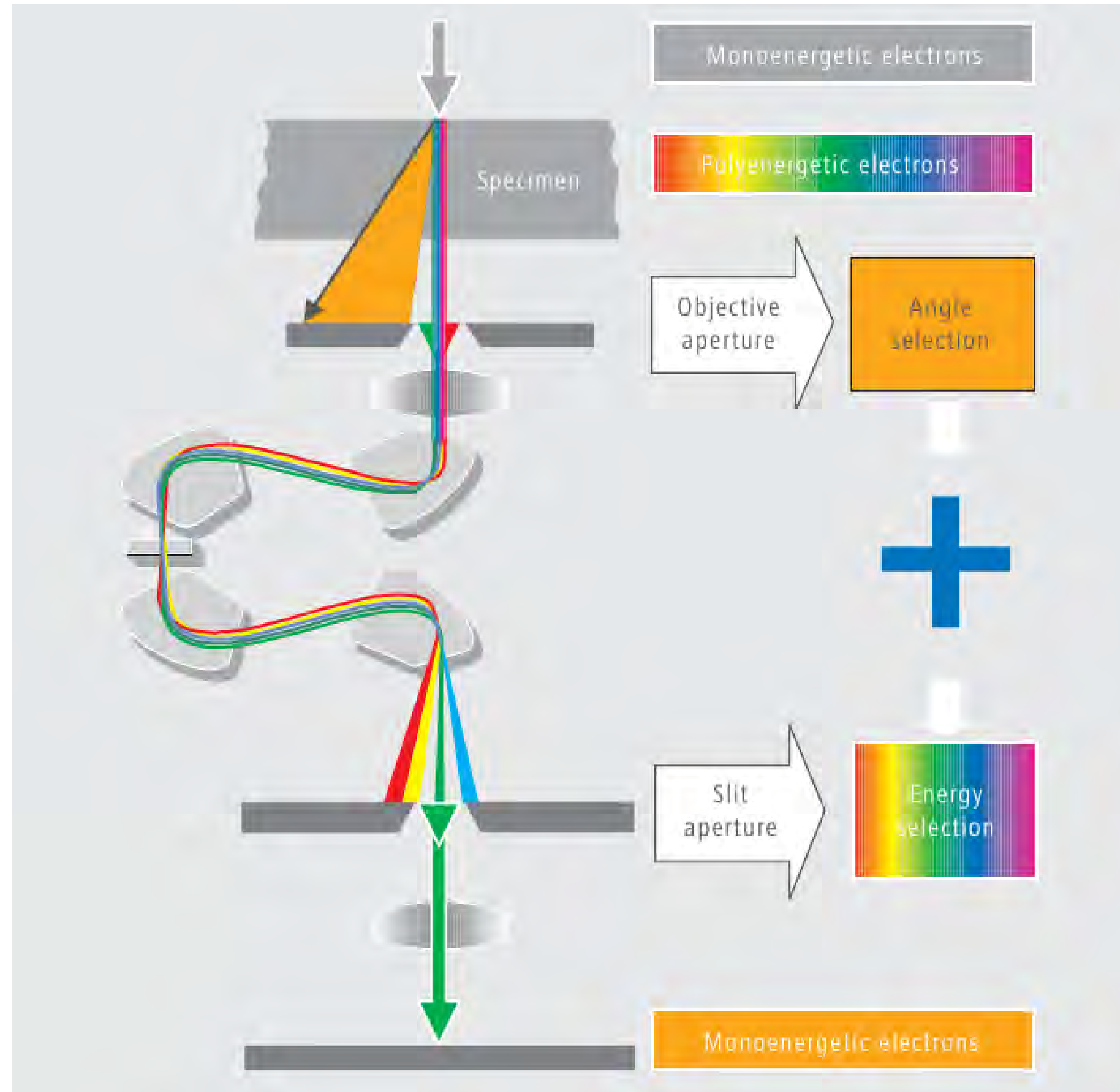


https://www.globalsino.com/EM/page4780.html

Energy Filter

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



ZEISS

Why Phase Plates? Enhanced Contrast

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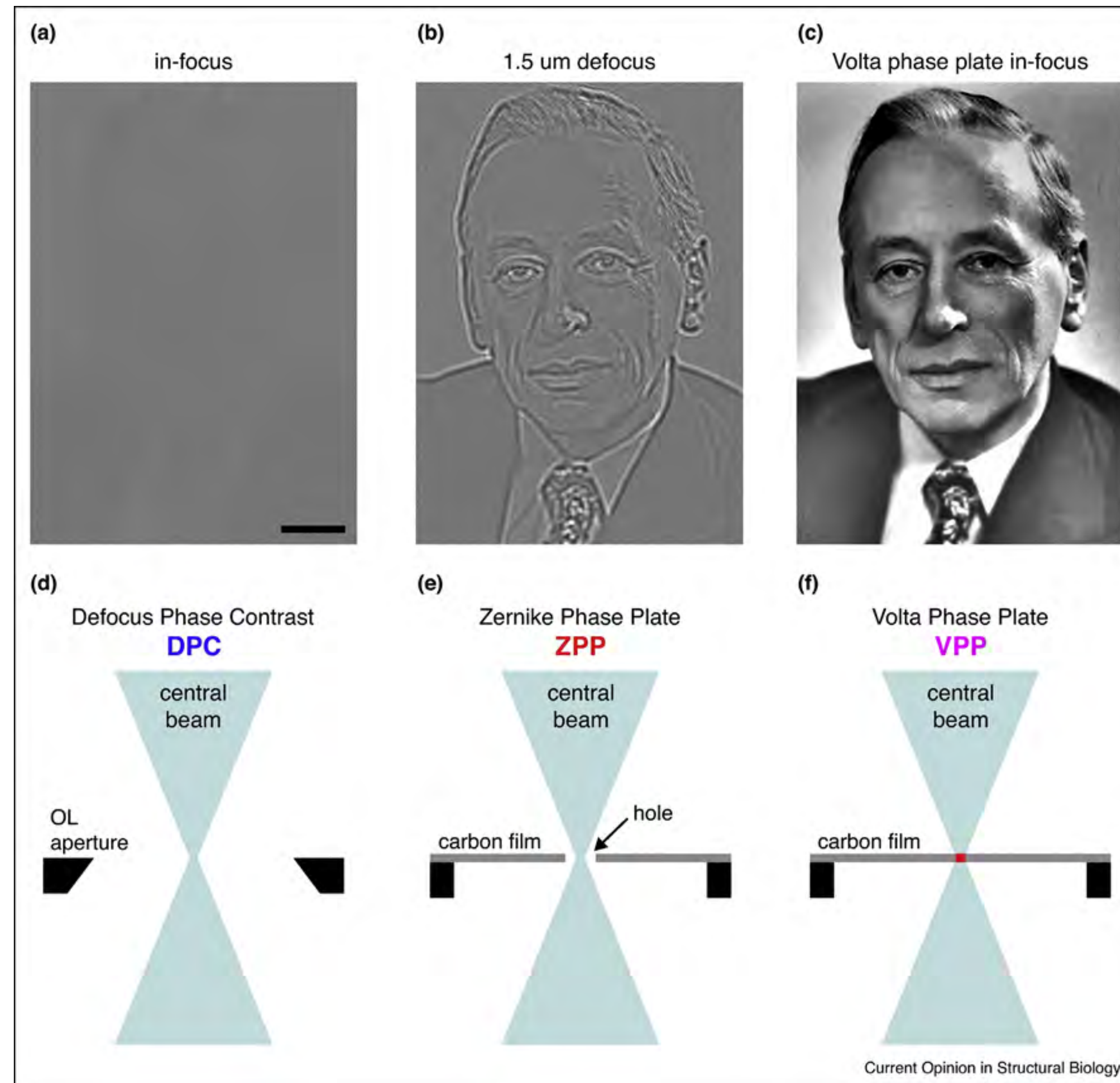

Danev R and Baumeister W, Curr Opin Struct Biol **46,** 87-94 (2017)

Why Phase Plates? Enhanced Contrast

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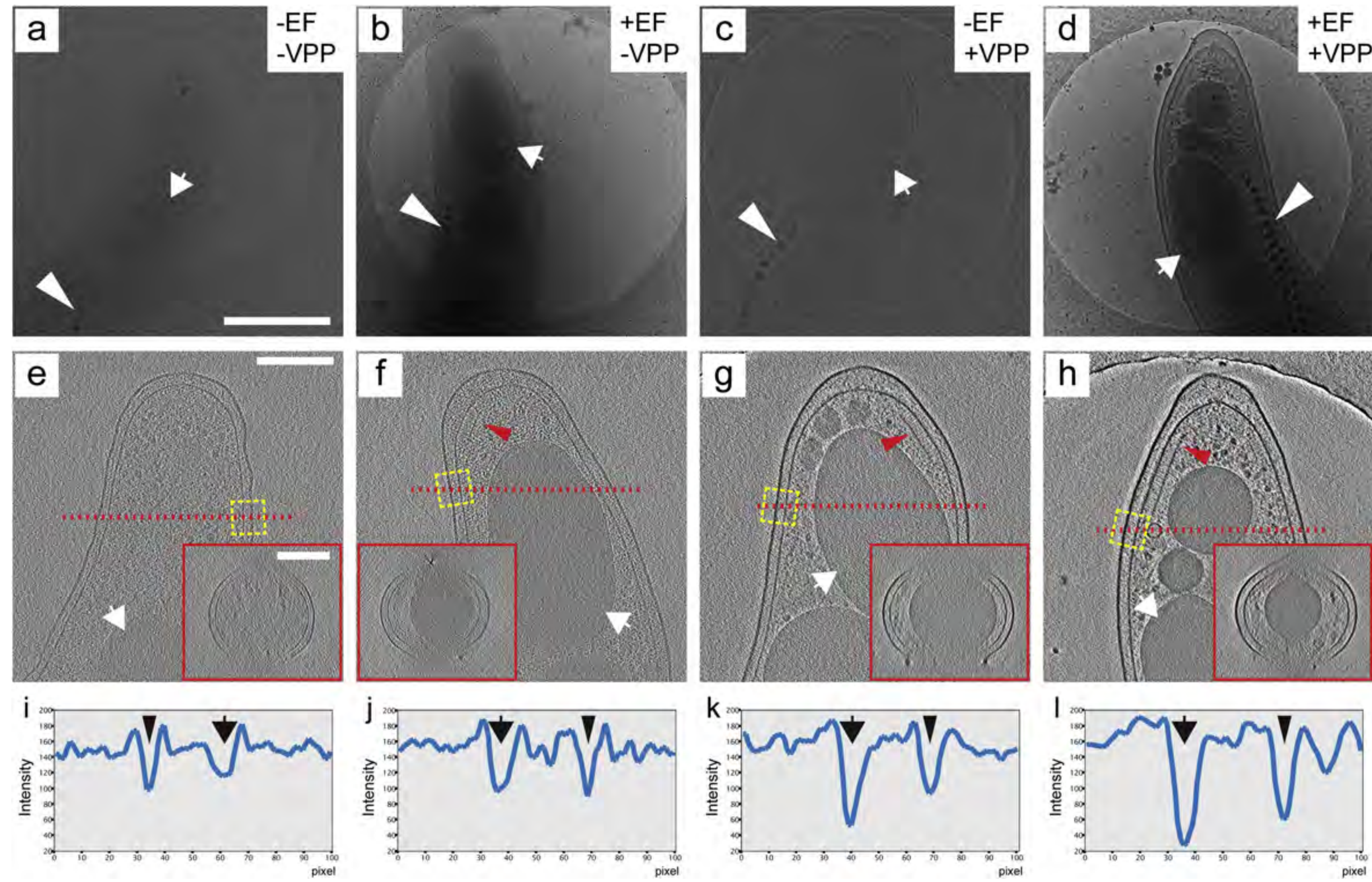
IMAGING LIFE FROM MOLECULES TO CELLS



Fukuda Y et al, J. Struct Biol **190(2),** 143-154 (2015)

Cryo-Specimen holders - side entry

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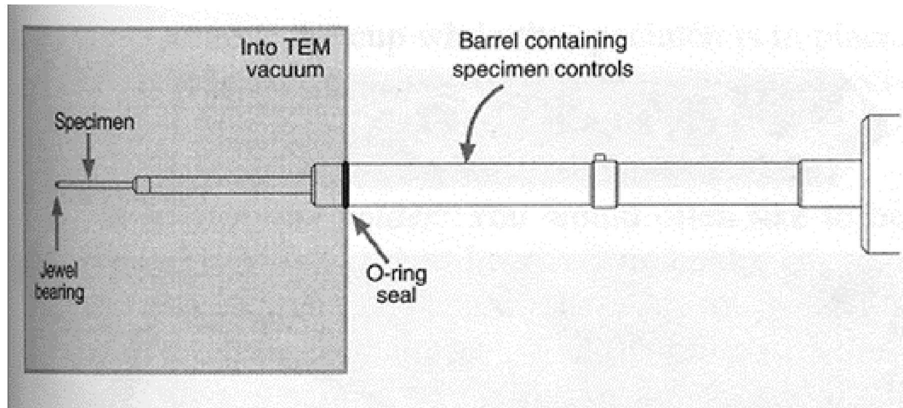

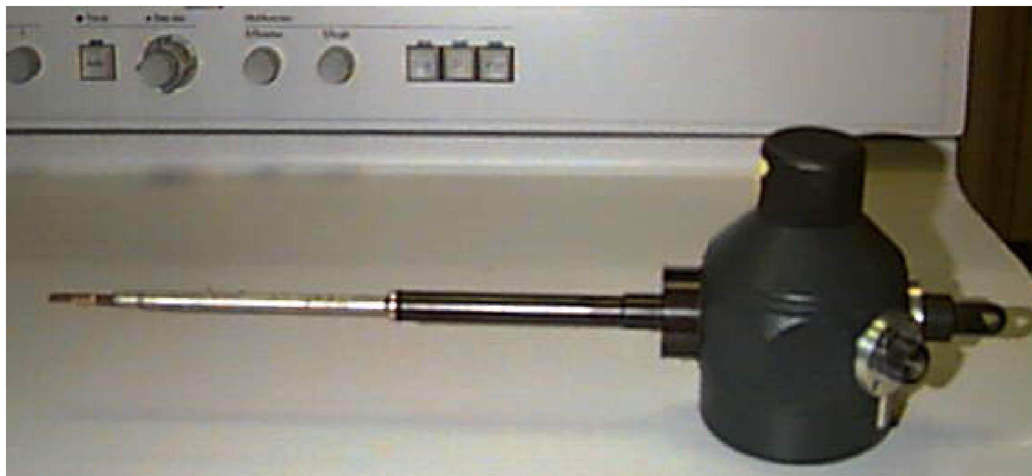

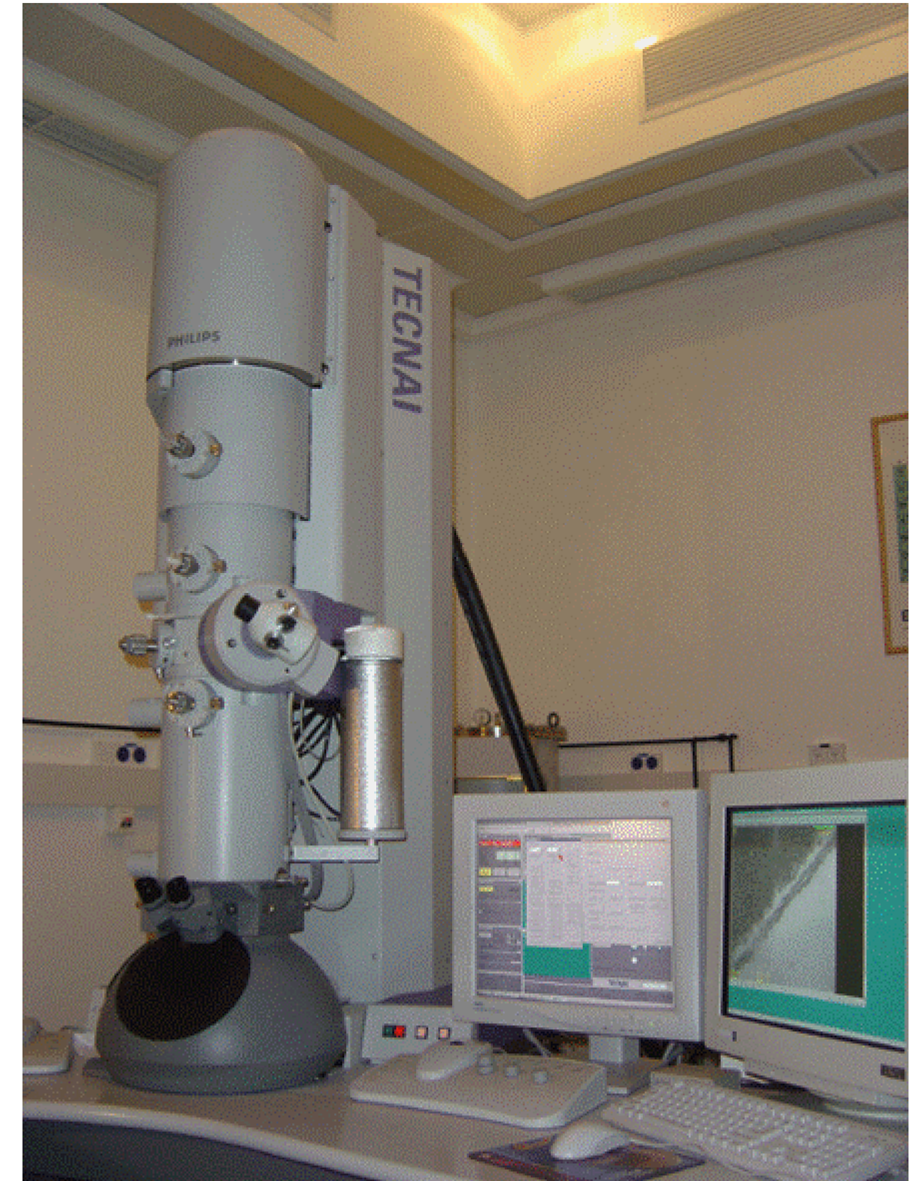

Cryo-Specimen holders – cartridge systems

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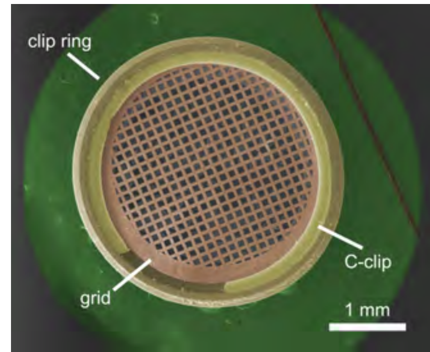

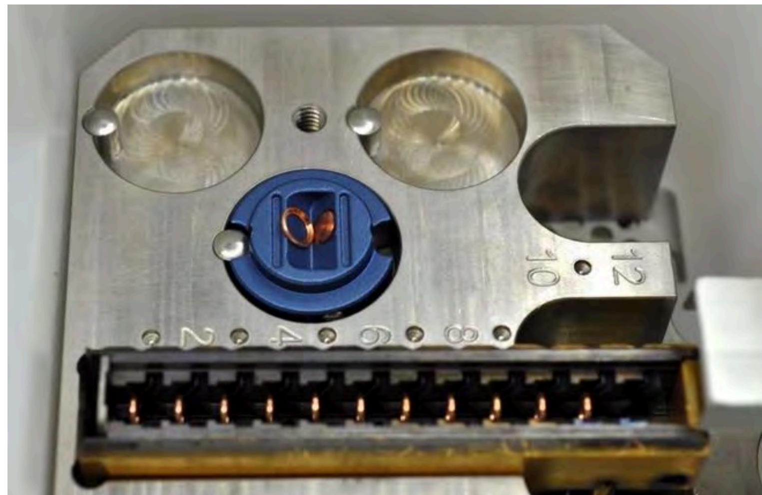

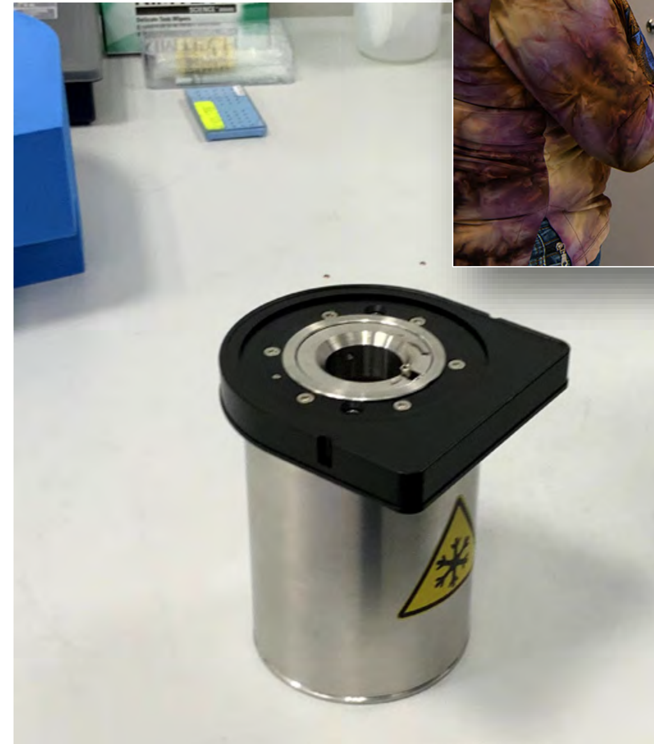

Charge-Coupled Device (CCD) cameras

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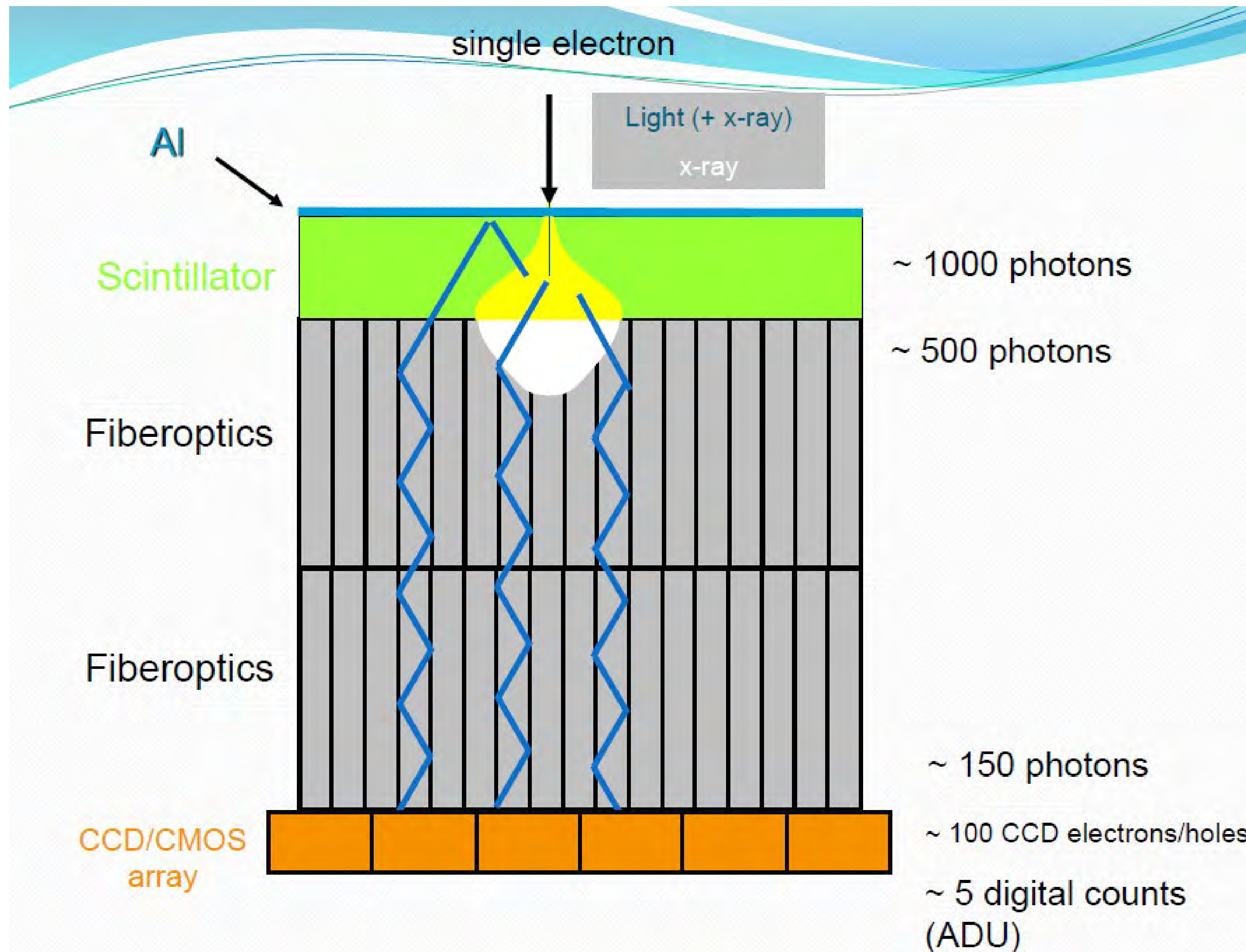
Electrons are converted into photons of light before being detected.



Courtesy of Hans Tietz, TVIPS

CMOS (complementary metal-oxide semiconductor) cameras

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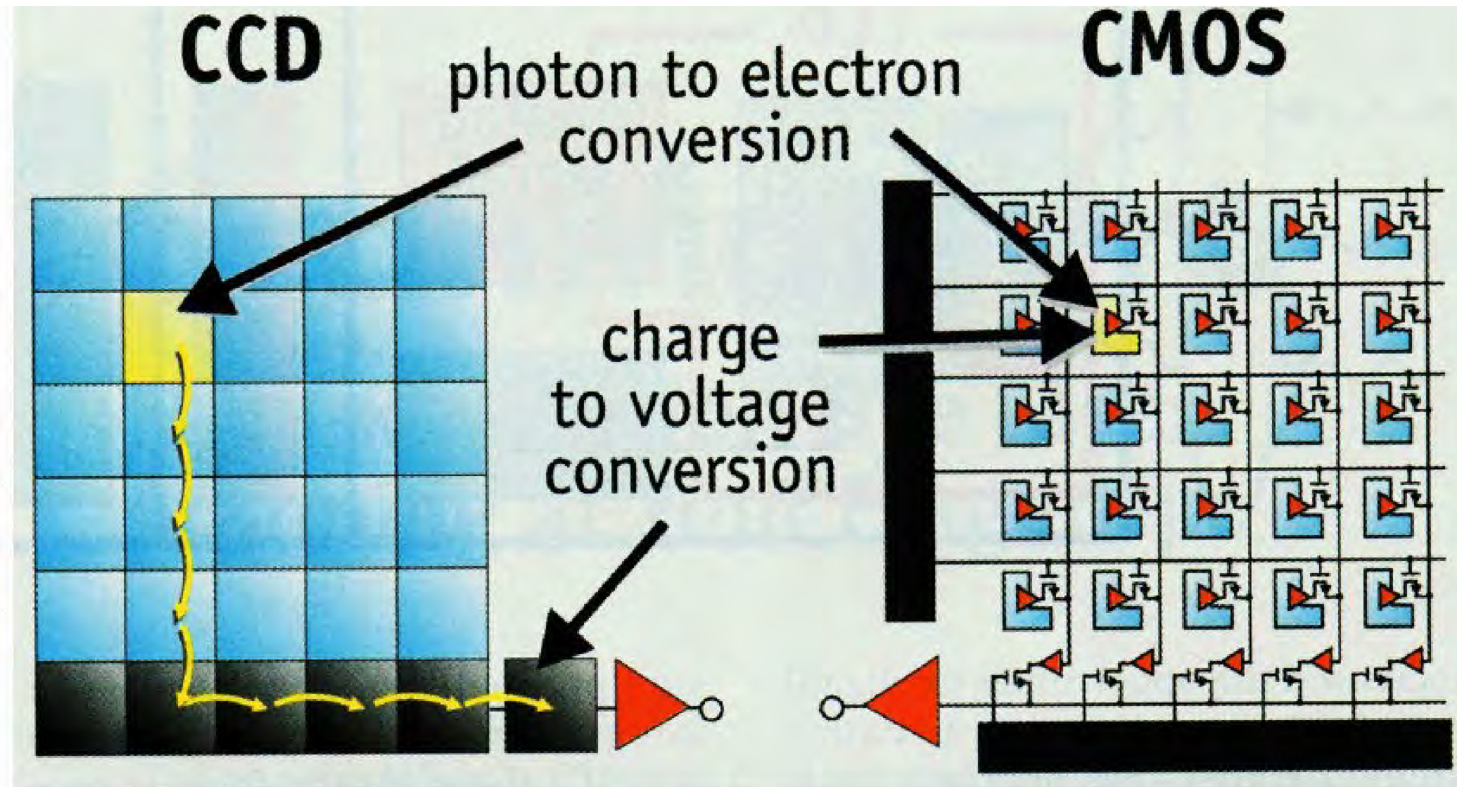

CCDs move photogenerated charge from pixel to pixel and convert it to voltage at an output node. CMOS imagers convert charge to voltage inside each pixel.

http://meroli.web.cern.ch/meroli/lecture_cmos_vs_ccd_pixel_sensor.html

Why Direct Electron Detection?

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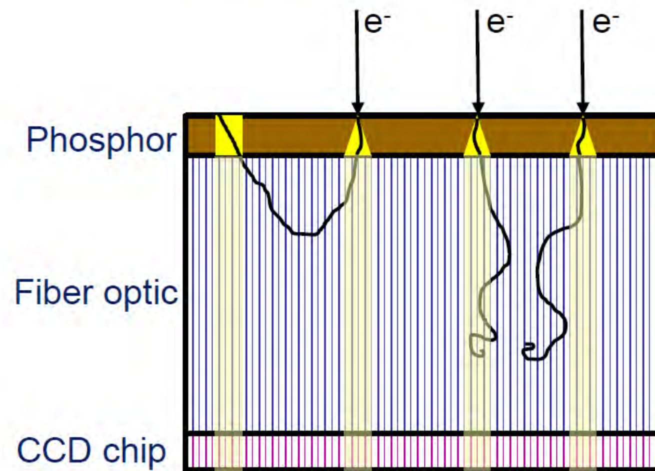
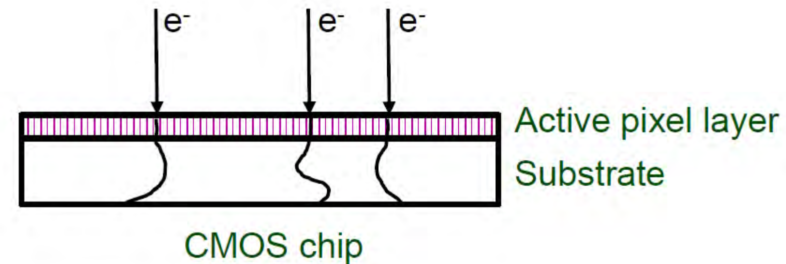
CCD Camera

- Signal from one electron spreads in the scintillator and is recorded in several pixels
- At higher voltages, 20-30% of electrons scatter back into phosphor in the wrong place
  - Spurious signals ruin resolution and efficiency

Direct Detector

- Signal from one electron is generated mostly within one pixel
- Backscatter is much less if the substrate is thin enough





David Mastronarde, 2019 Vienna Course on Tomography

Why Direct Electron Detection?

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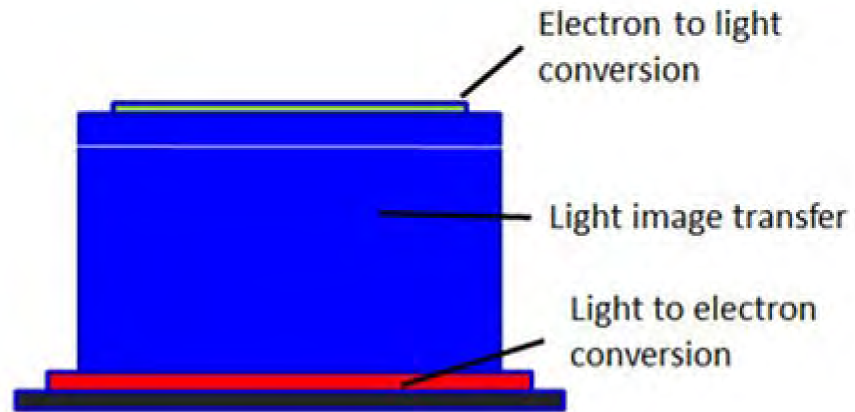

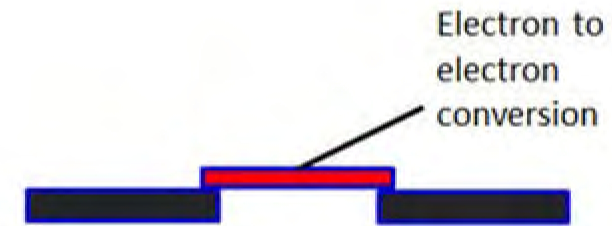

Indirect (left) and Direct (right) detection and Monte Carlo simulations of electron tracks within the device

From Gatan Inc. website

Electron Counting

- Single electron events can be distinguished if frames are read out fast enough and number of electrons in each is low enough
- Electron counting helps by about a factor of 2 because it:
  - Reduces blurring from signals spreading to more than one pixel
  - Eliminates effects of variable sized packets of signal and of readout noise, which impair the signal-to-noise ratio

Single electron events in one frame from an active pixel sensor

McMullan et al., 2009, Ultramicroscopy 109: 1411–1416

Why Direct Electron Detection?

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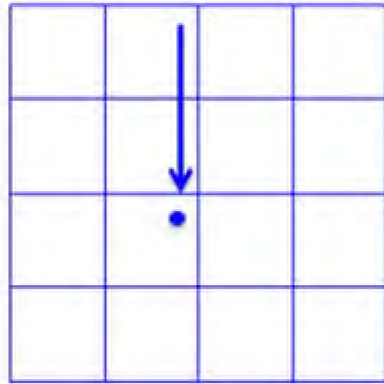
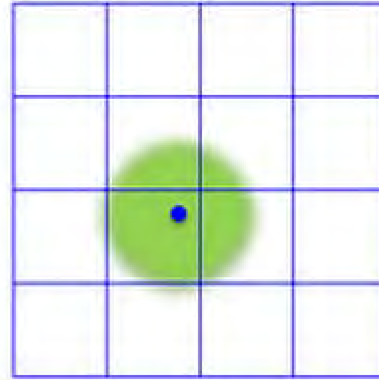
Single electron counting brings noise down to practically zero

Standard Integrated Mode

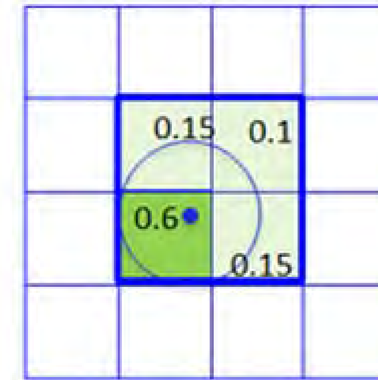
1. Electron enters detector



2. Signal Is Scattered

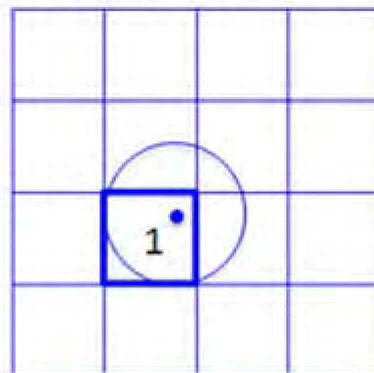


3. Charge collects in each pixel



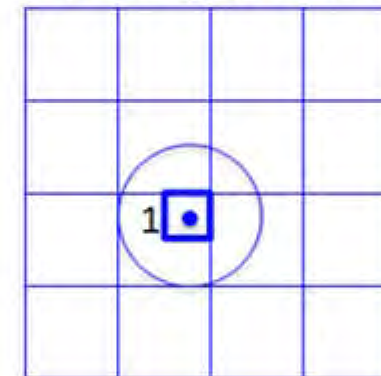
Single Electron Counting Mode

4a. Events are reduced to the highest charge pixels



From Gatan Inc. website

4b. Events are localized with sub-pixel accuracy



Direct Electron Detector (DED) cameras

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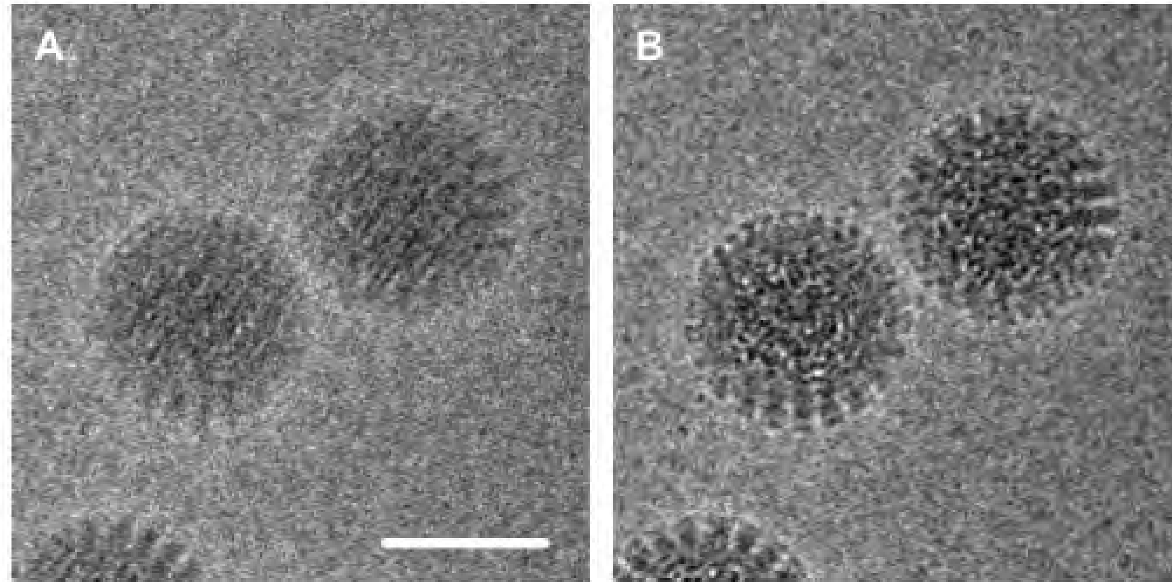

Figure 1. Electron micrograph of double-layered rotavirus particles frozen in a thin layer of amorphous ice. The image was recorded by the author and co-workers using the DE-12 direct electron detector (Direct Electron, San Diego, United States) in movie mode at 40 frames per second. In panel A, 60 frames have been averaged without alignment, resulting in an image that is blurred due to beam-inducted movement. In panel B the frames have been aligned to compensate for this movement, which results in an image with significantly reduced blurring and improved contrast. The alignment method used here involved tracking the movement of the particles (***Brilot et al., 2012***); the alignment method used by Bai et al. used additional statistics to predict the movement of the particles caused by the electron beam. Scale bar = 50 nm.

Image formation in STEM – incoherent detection, particle nature

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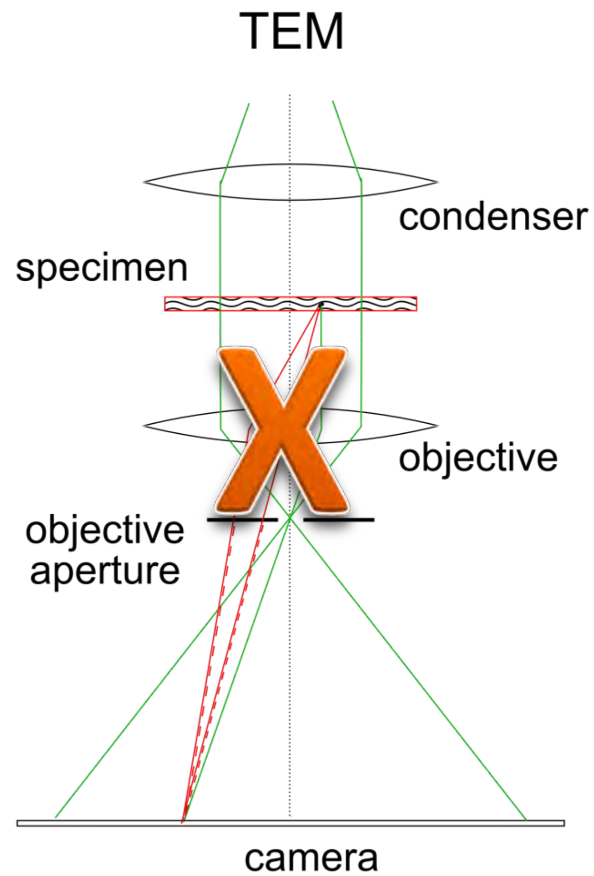

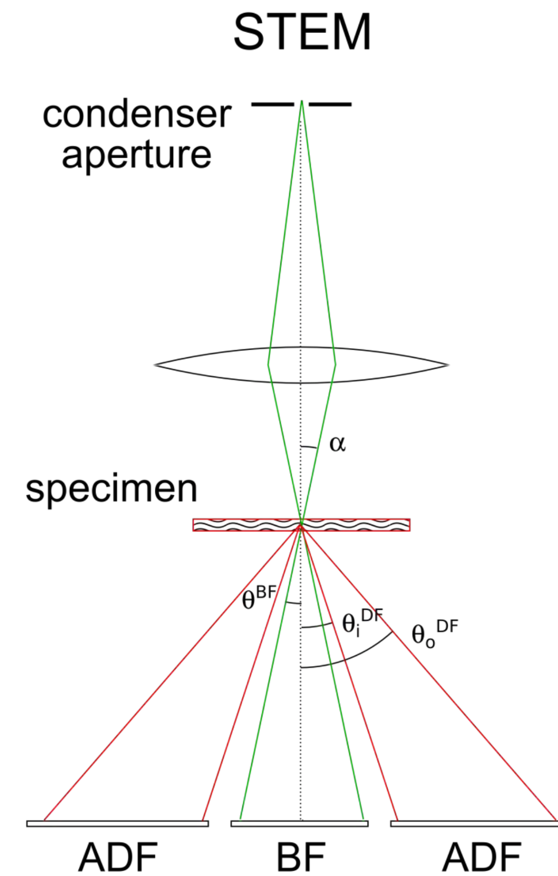

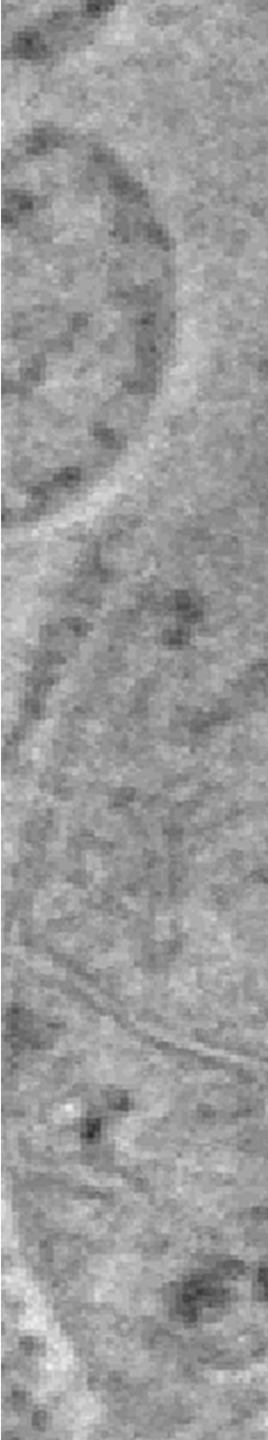

CryoTEM: Sample thickness is limited by the INELASTIC MEAN FREE PATH

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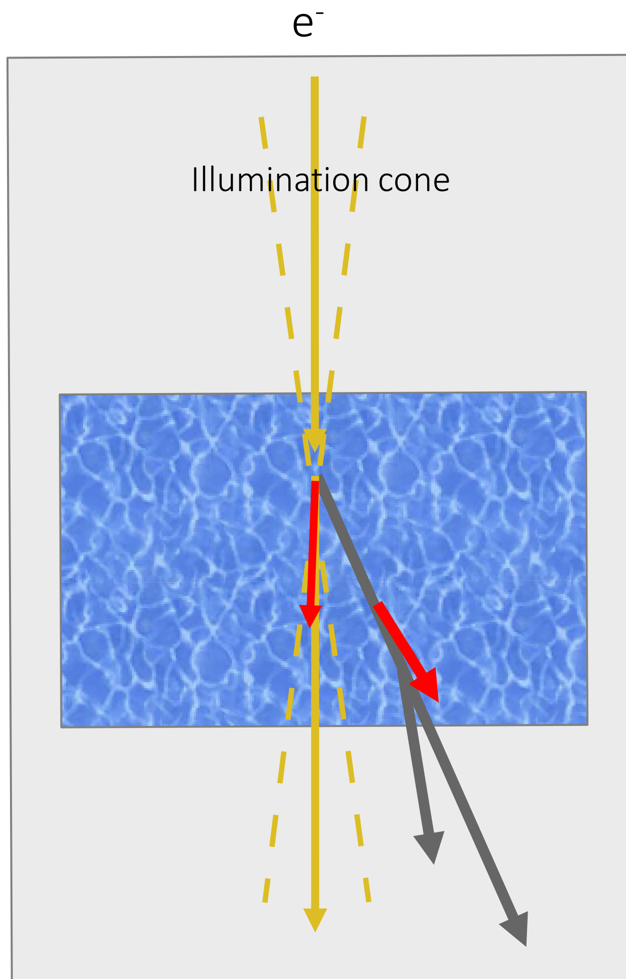


Elastic scattering

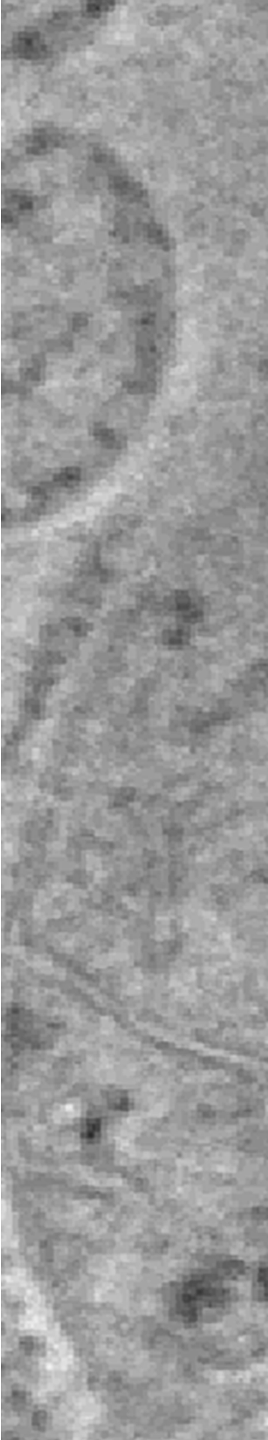
Inelastic scattering

Cross-section: elastic ~ $Z^{3/2}$
inelastic ~ $Z^{1/2}$

Ratio inelastic/elastic ~ 20/Z

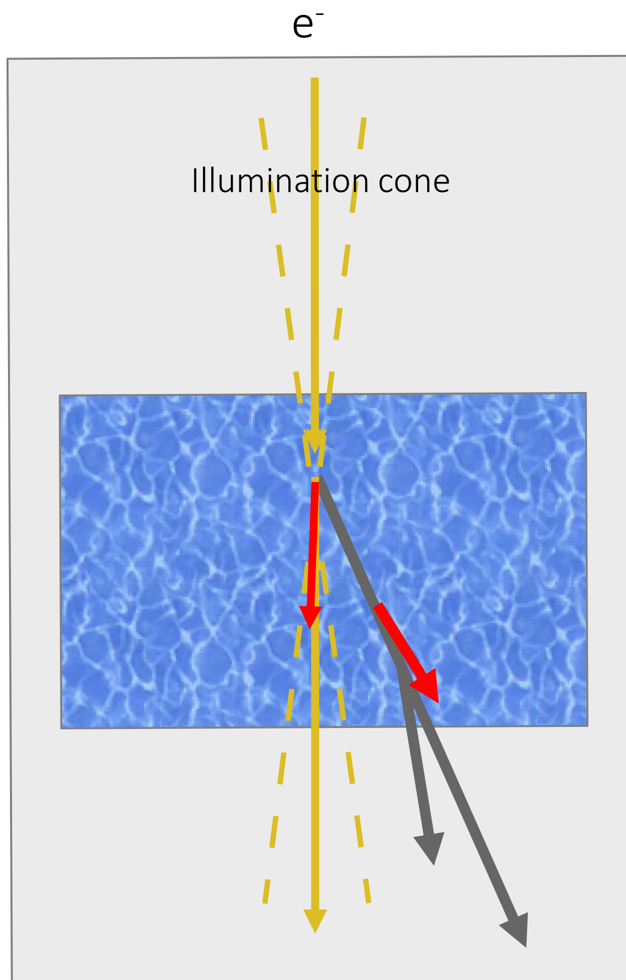
Inelastic mean free path in vitrified water for 200 kV
~ 250 nm

The “Natural” limit for cryoTEM Tomography



CryoSTEM: Sample thickness is limited by the ELASTIC MEAN FREE PATH

IMAGING LIFE FROM MOLECULES TO CELLS



Elastic scattering

Inelastic scattering

Cross-section: elastic ~ $Z^{3/2}$

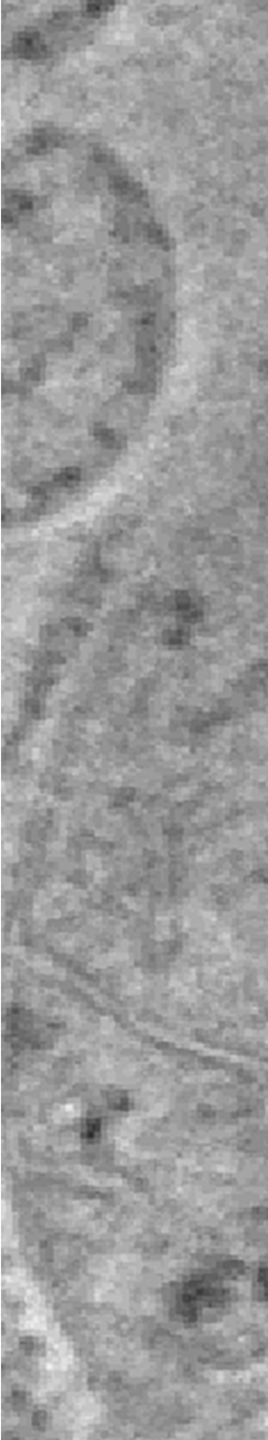
inelastic ~ $Z^{1/2}$

Ratio inelastic/elastic ~ 20/Z

Elastic mean free path in vitrified water for 200 kV

~ 750 nm

The “Natural” limit for cryoSTEM Tomography



CryoSTEM: Efficient use of electrons

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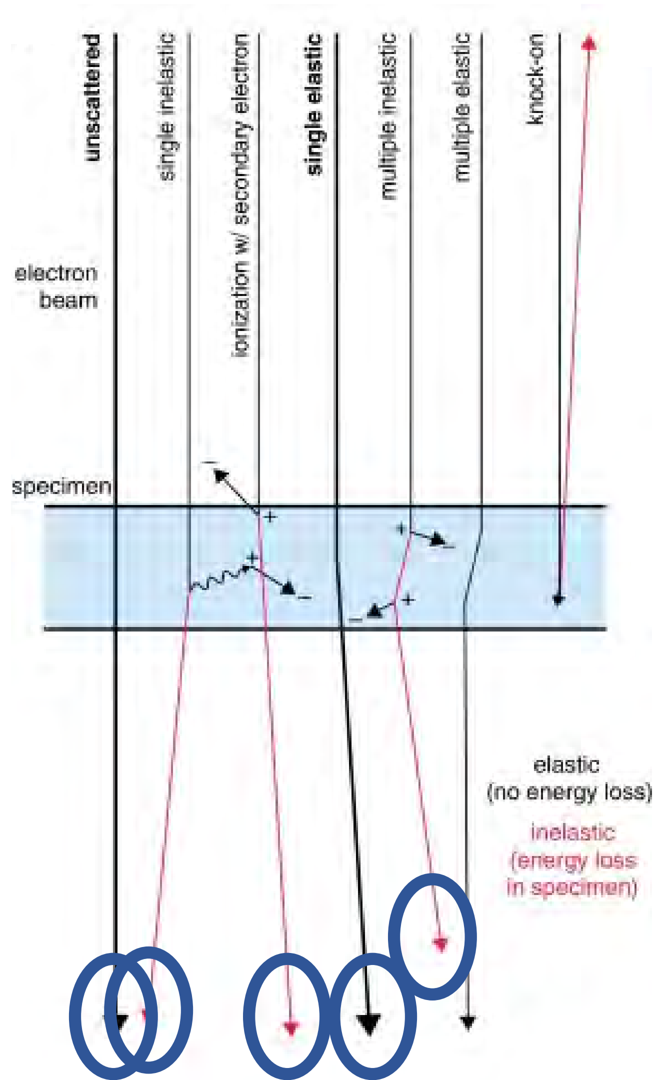
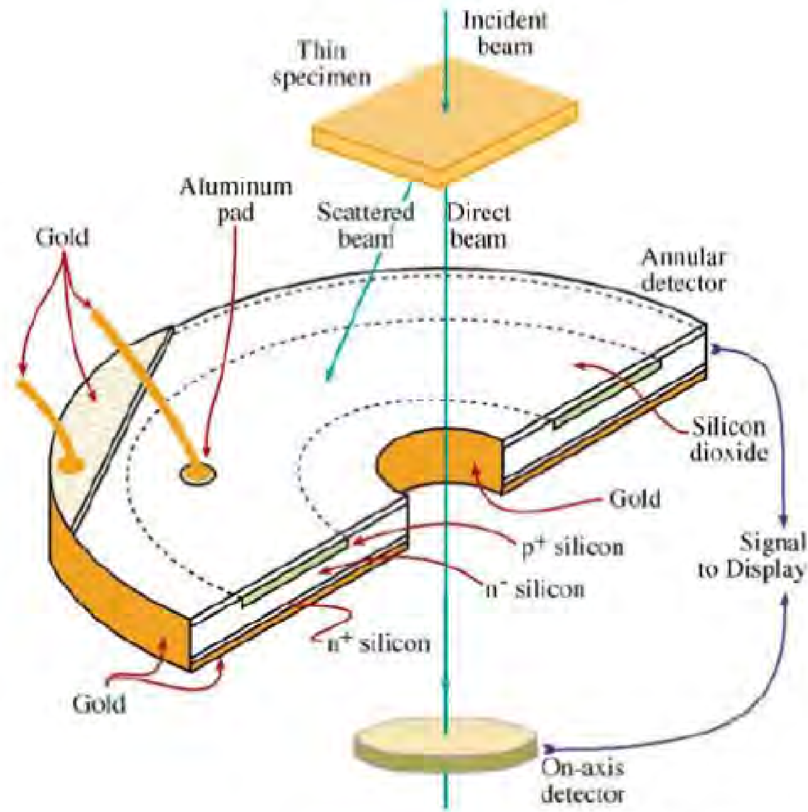


CryoSTEM relies on *Amplitude Contrast which requires:*

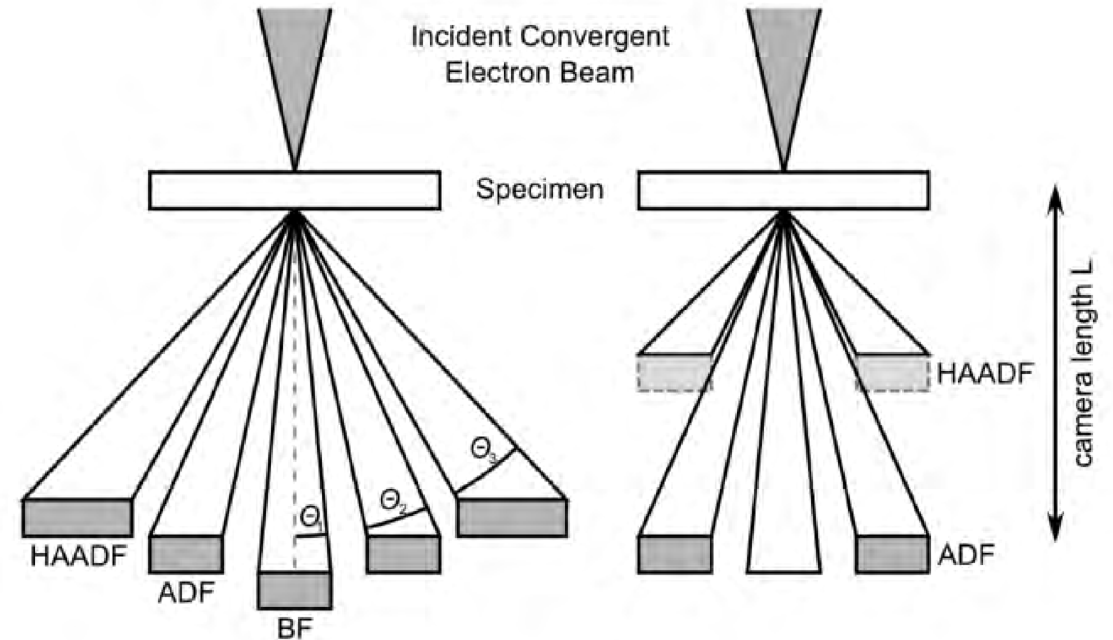
INELASTIC or ELASTIC SCATTERING (but only one elastic scattering event)

Russo, C.J. and L.A. Passmore, Curr Op Struct Biol, 2016. **37**: p. 81-89.

STEM Detectors



http://www.globalsino.com/EM/page4996.html



STEM Phase-contrast Detectors

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4 quadrant (Krios)

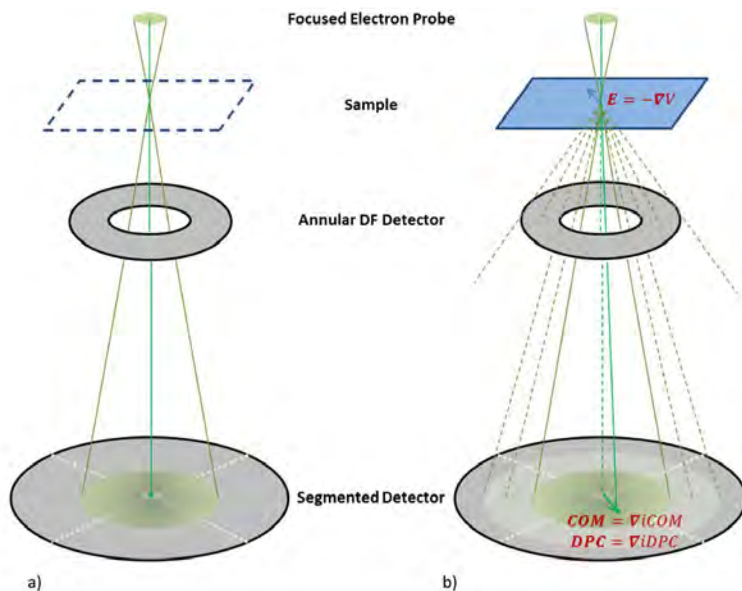
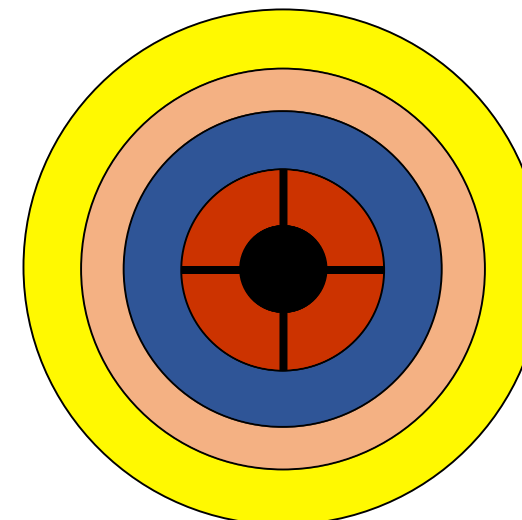


Figure 1. Schematic representation of the experimental setup. (**a**) Focused probe without sample. (**b**) Focused probe with a sample.

"**i**ntegrated **D**ifferential **P**hase Contrast (iDPC)
Yücelen et al, Sci Rep 2017

4-quadrant and annular rings (F20)



Under development
Michael Elbaum, Lothar Houben, El Mul company

Simultaneous BF and DF data collection.
Dynamic Focus
Agrobacteria

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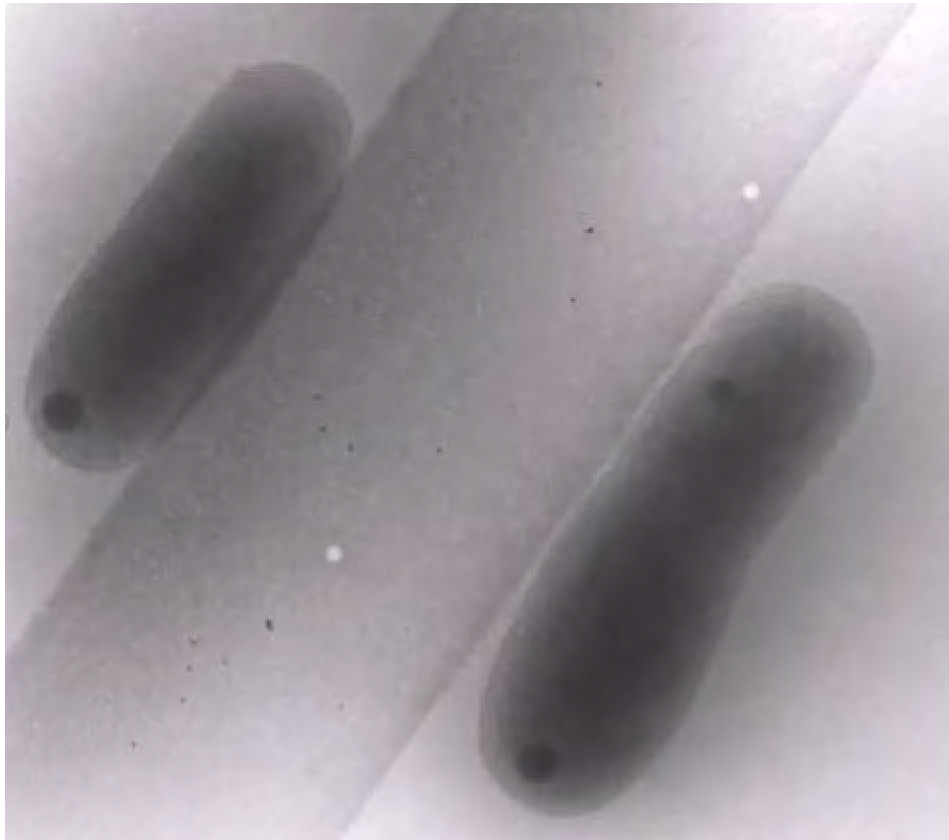
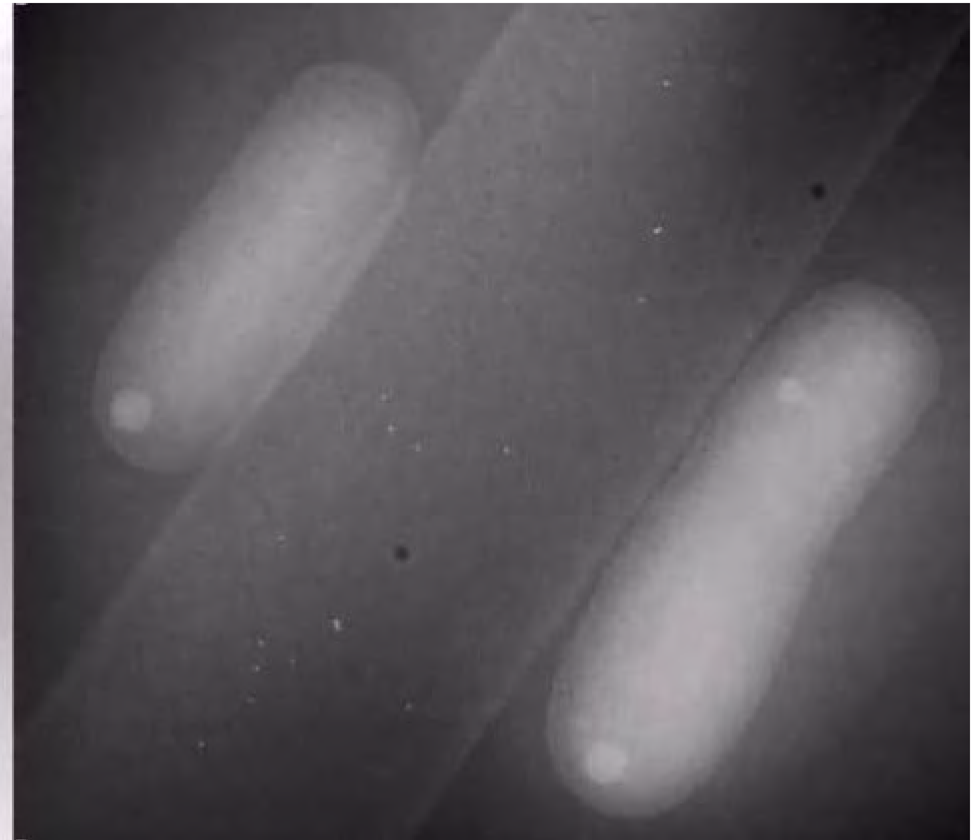
bright field



dark field



Wolf, Houben & Elbaum, Nature Methods 2014

Human WI-38 fibroblasts, ECM, 750 nm thick

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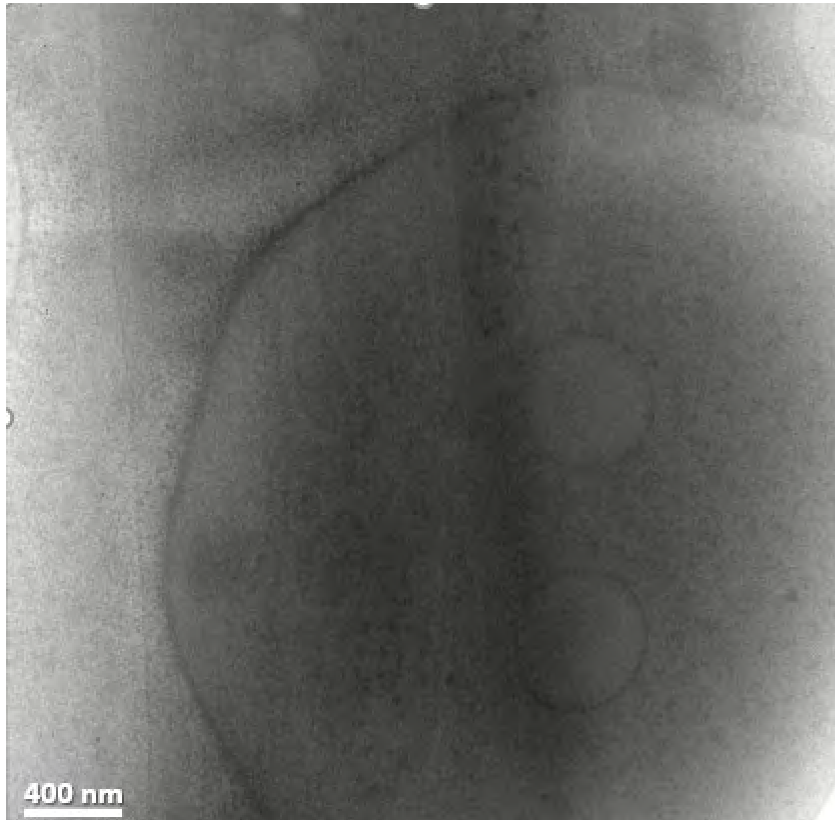
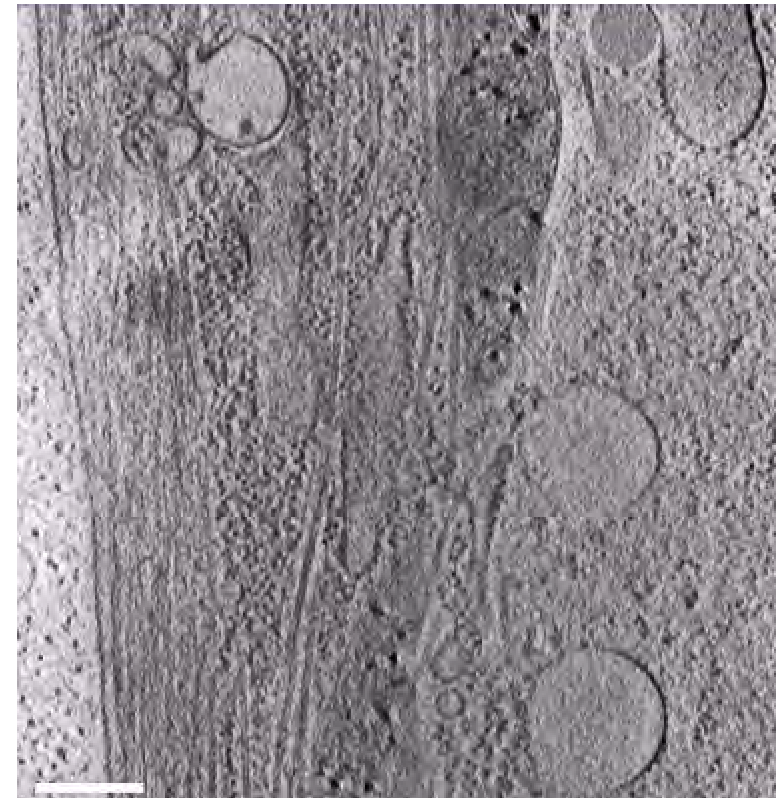
Deborah Fass, Michael Elbaum

CSTET BF tilt series



CSTET BF reconstruction, 30 nm thick slices



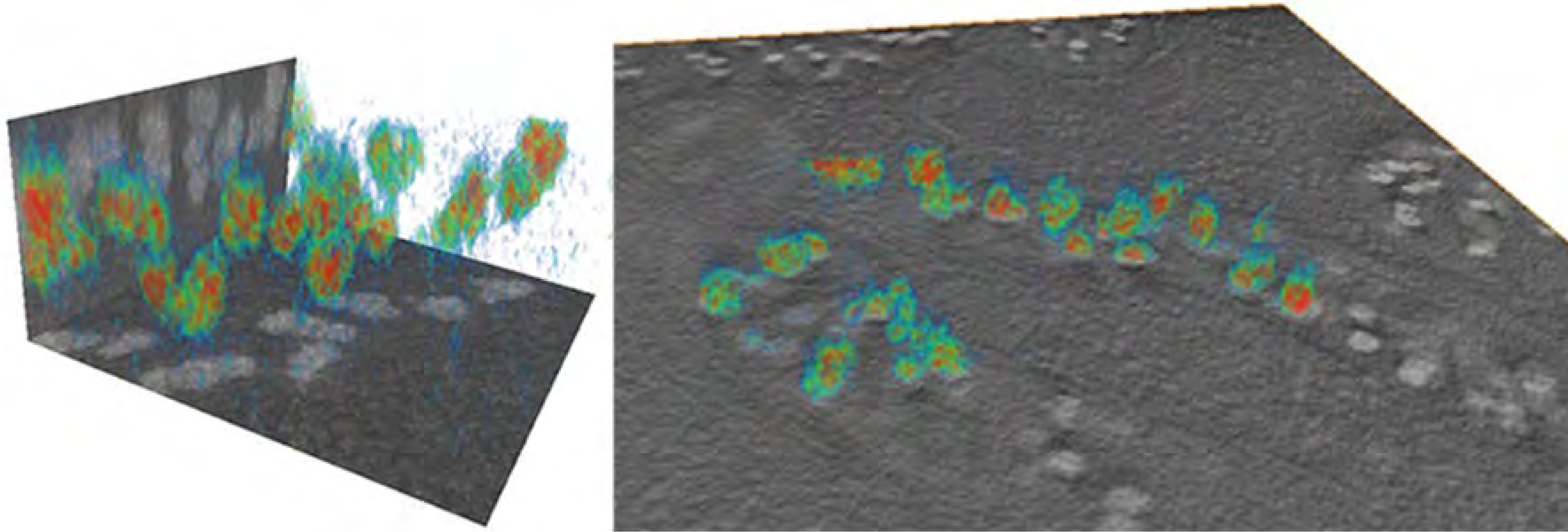
-65º → +70º, every 2º, every 1.5º above 50º

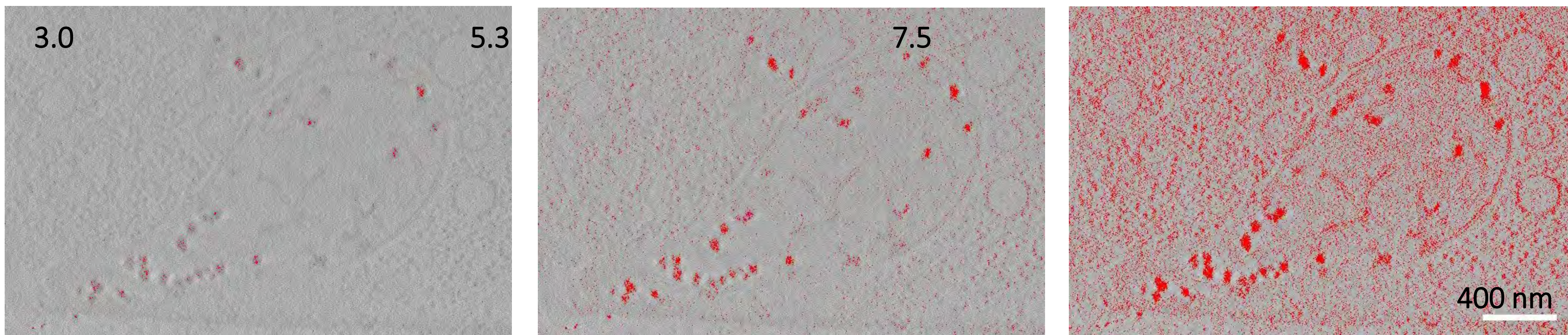
Wolf et al, eLIFE. 6:e29929.

Granule Heterogeneity

Typical density 1.0 to 1.3 gm/cm^3 (ACP = 2.0)

IMAGING LIFE FROM MOLECULES TO CELLS
IMpaCT



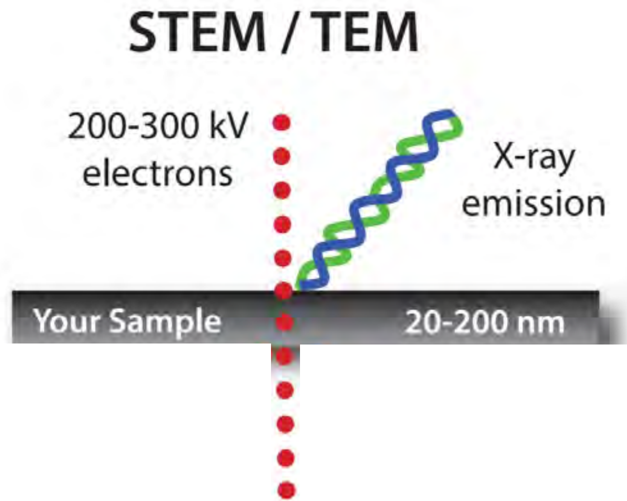


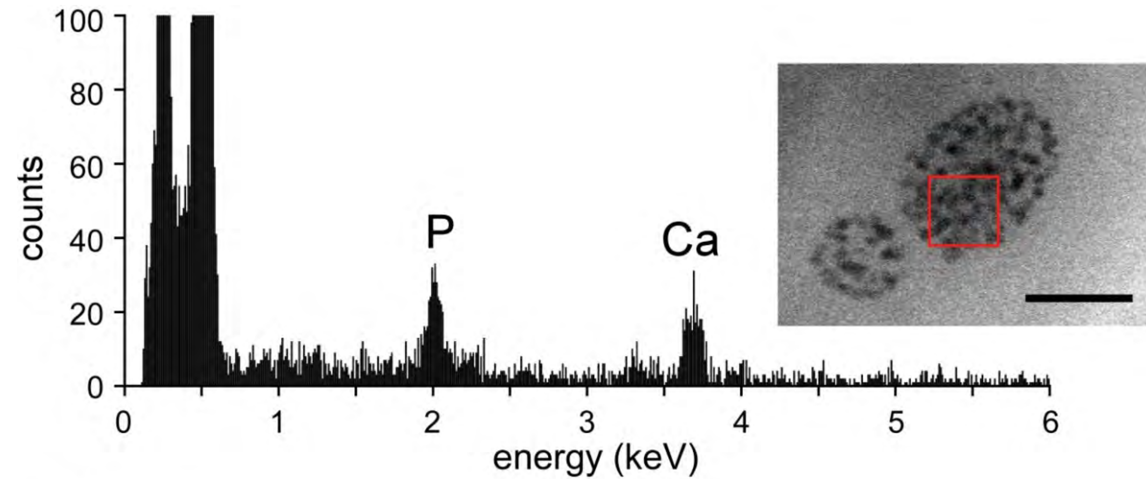
Dense parts of granules | Densest regions of ribosomes | All ribosomal volumes

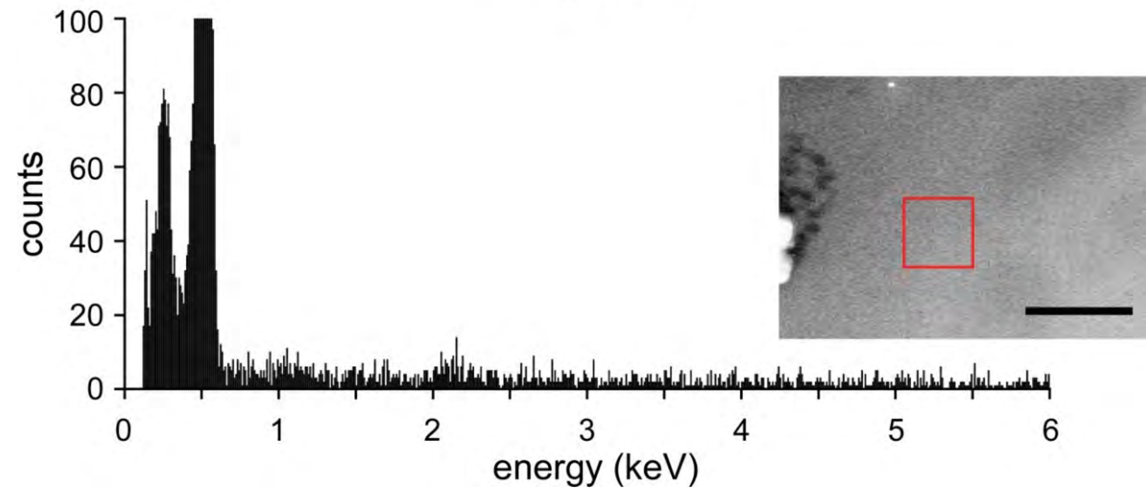
Wolf et al, eLIFE. 6:e29929.

EDX shows Ca, P

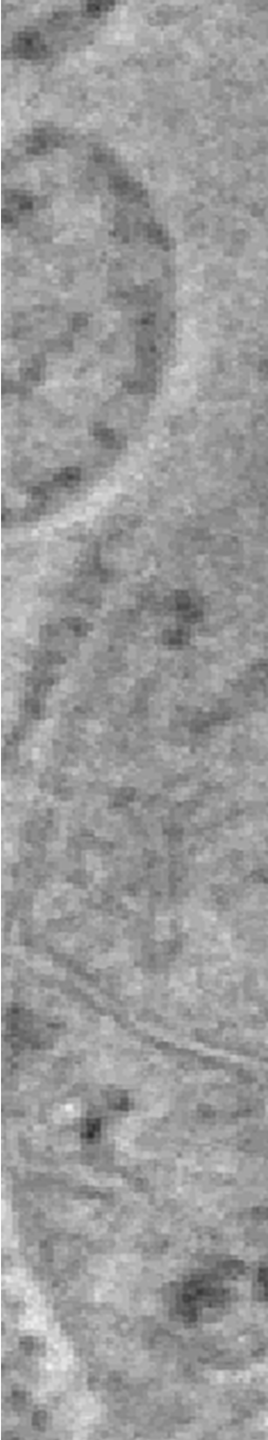
IMPACT
IMAGING LIFE FROM MOLECULES TO CELLS







Wolf et al, eLIFE. 6:e29929.



Microscopes – which one to use?

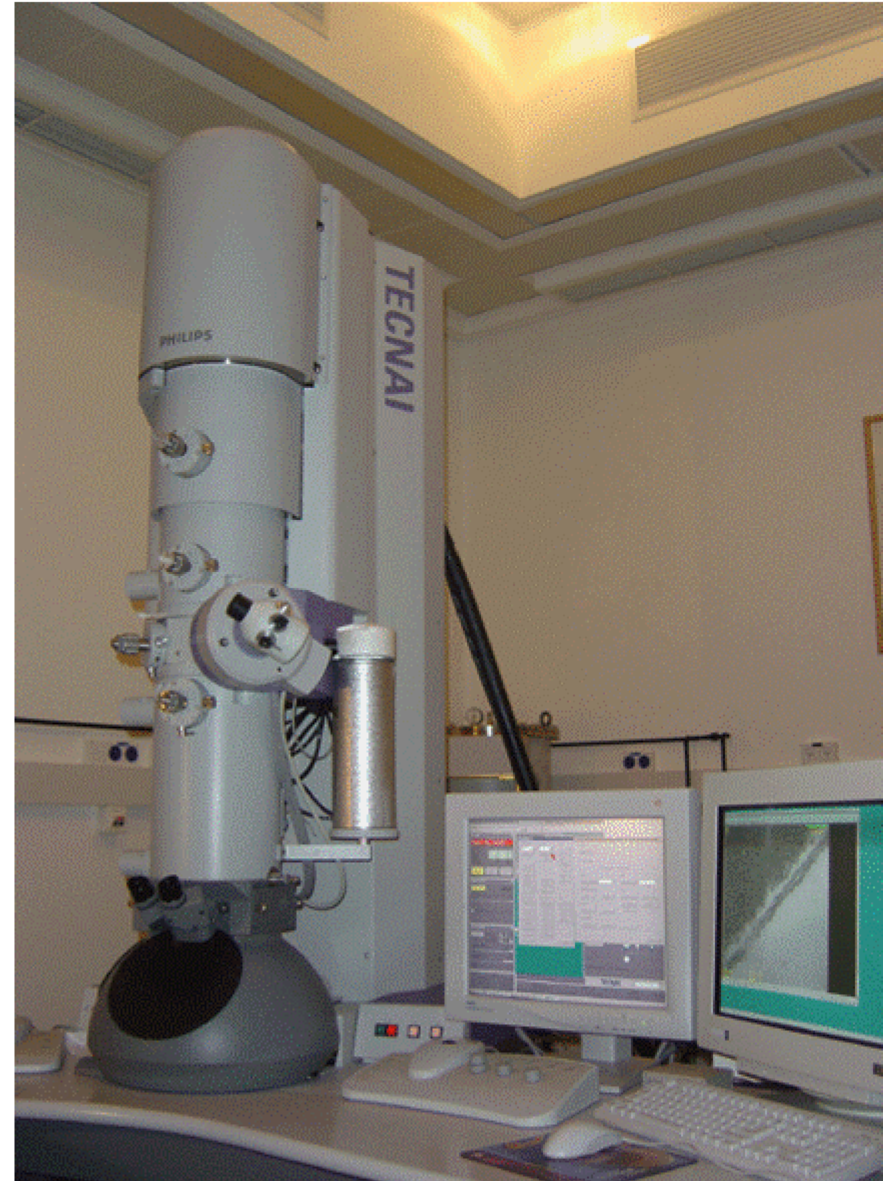
IMpaCT
IMAGING LIFE FROM MOLECULES TO CELLS

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS

Tecnai T12

Good:
- LaB6 source
- Decent vacuum system
- Very sensitive CMOS camera (TVIPS)
- Cryo-blades

Bad:
- LaB6 source
- Only 100 kV

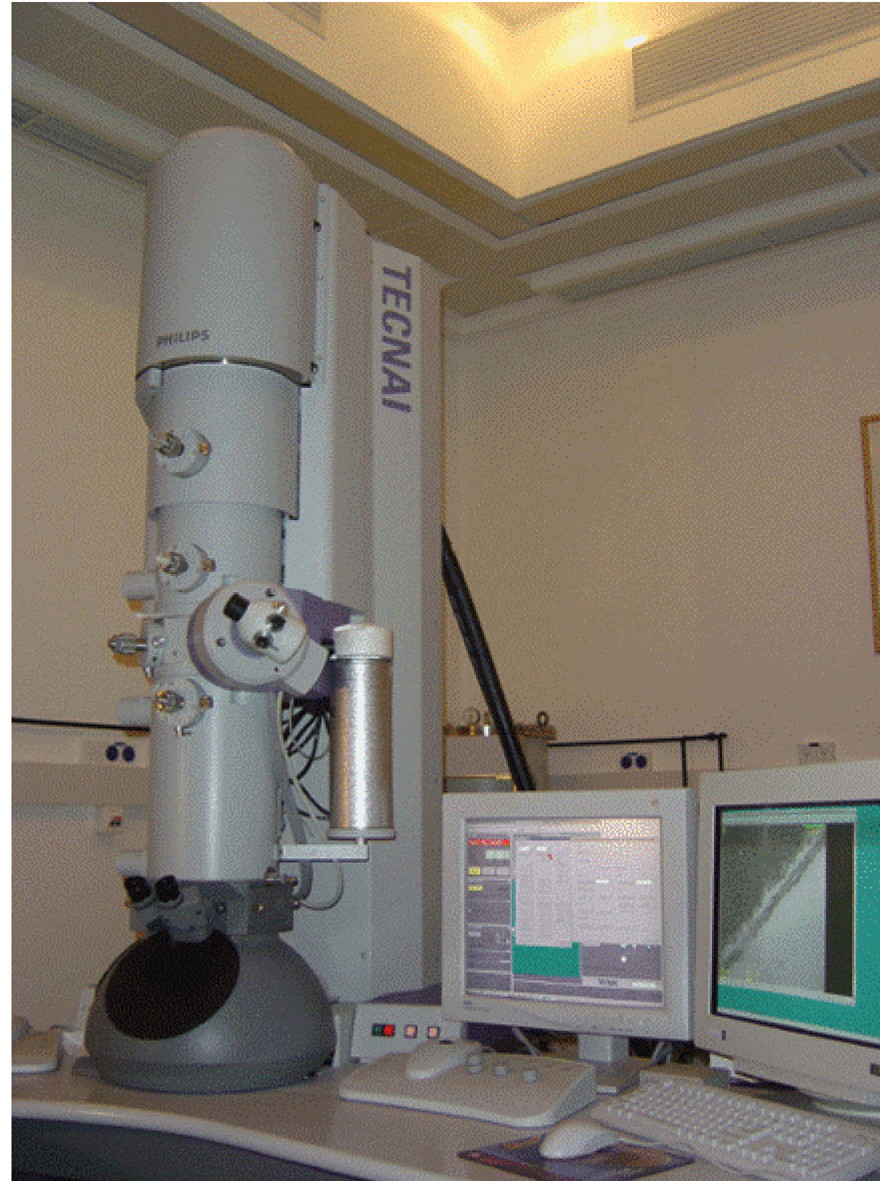


Tecnai T12

IMpaCT
IMAGING LIFE
FROM MOLECULES
TO CELLS

In practice:

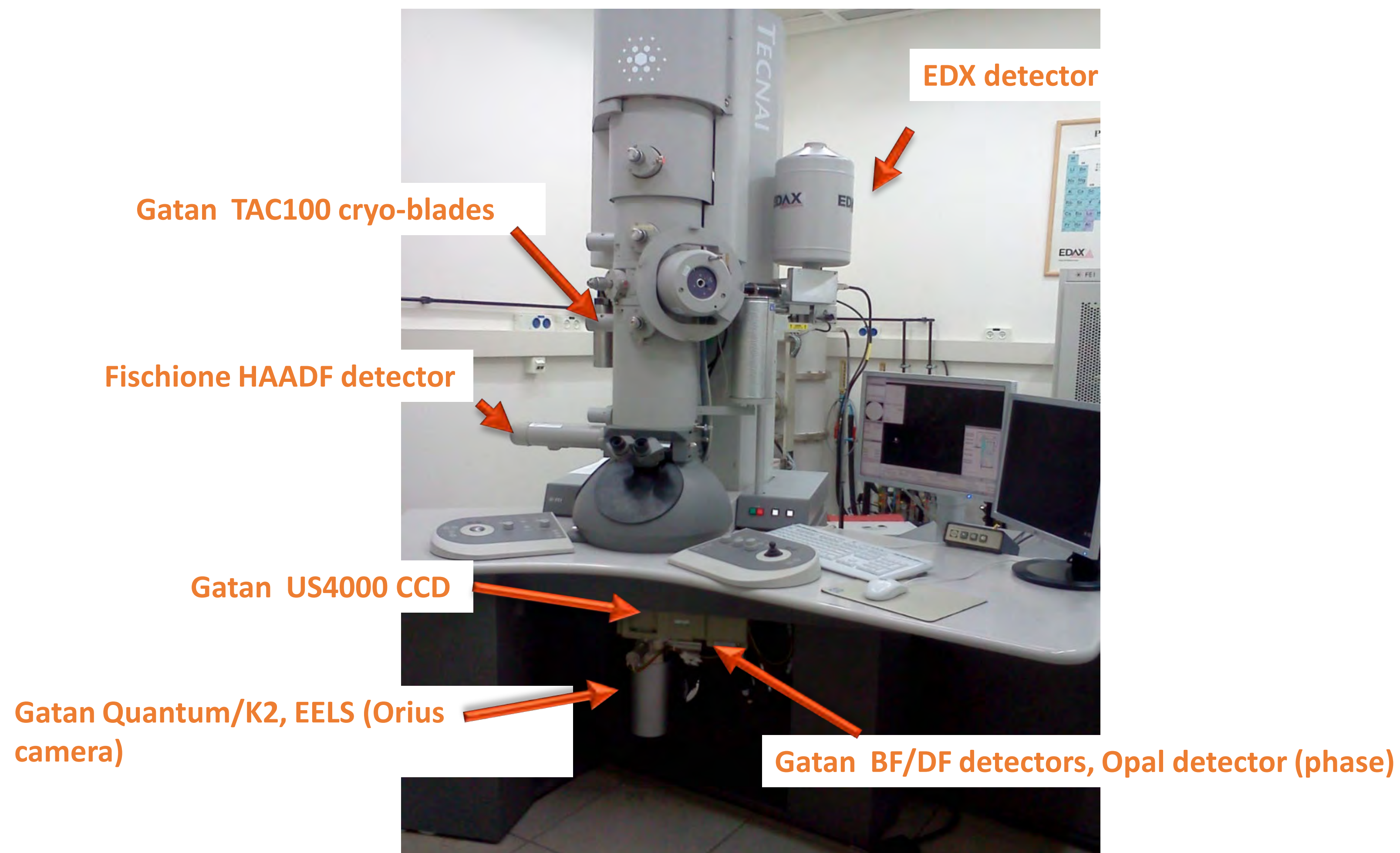
Used for screening and high quality TEM imaging of cryo-samples, negative stain samples, general biological TEM



Tecnai F20

Good:
- FEG source
- Decent vacuum system
- Direct Electron Detector for TEM
- Easy Switching from TEM to STEM
- Multiple STEM detectors
- Stable optics
- 200 kV
- Energy filter for tomography

Bad:
- Side entry holder
- Cryoblades and vacuum system not adequate enough for long-term cryo-sessions
- No convenient automated liquid N2 filling system
- Complicated interfaces with multiple computers
- Communication issues



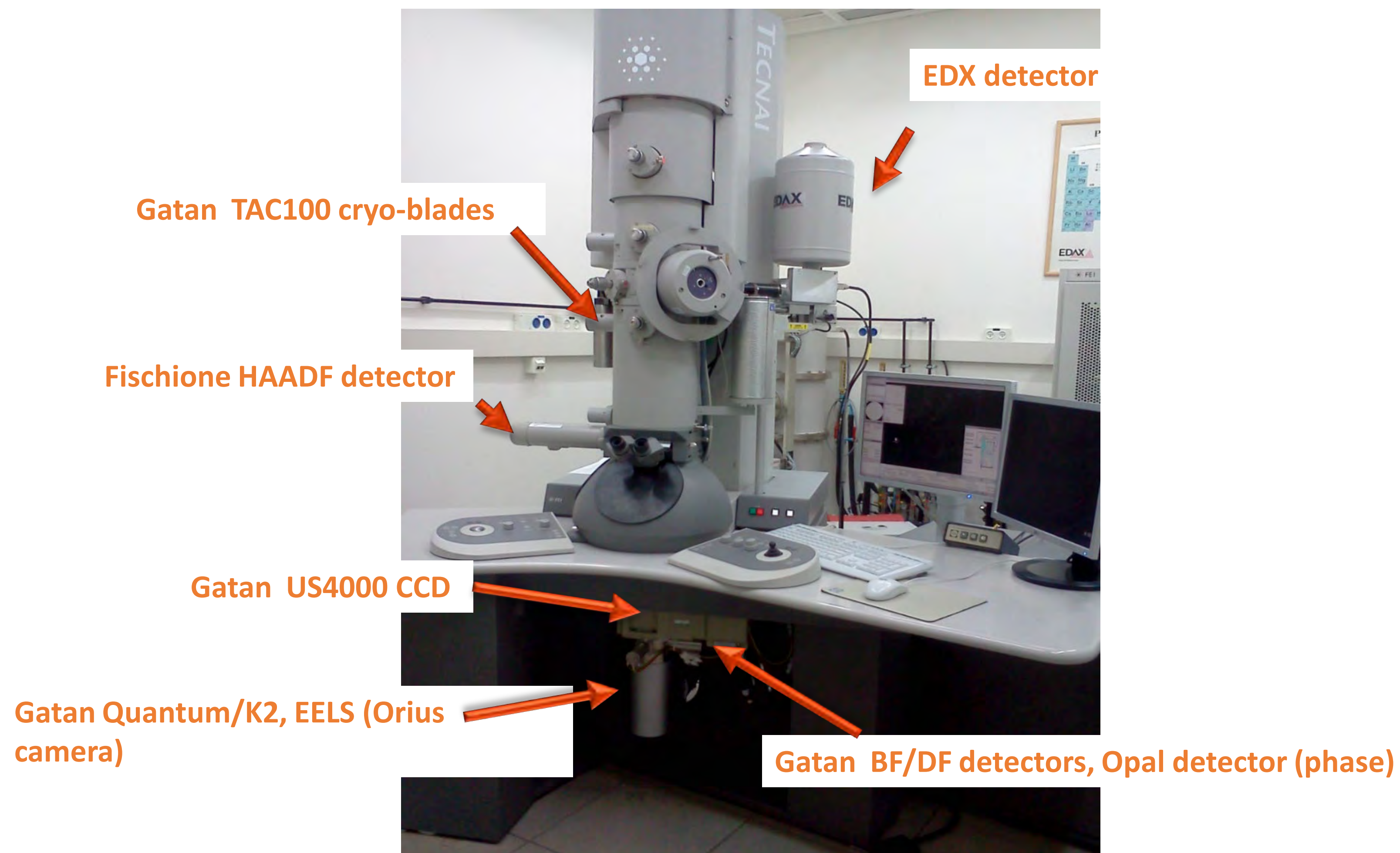
Tecnai F20

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS

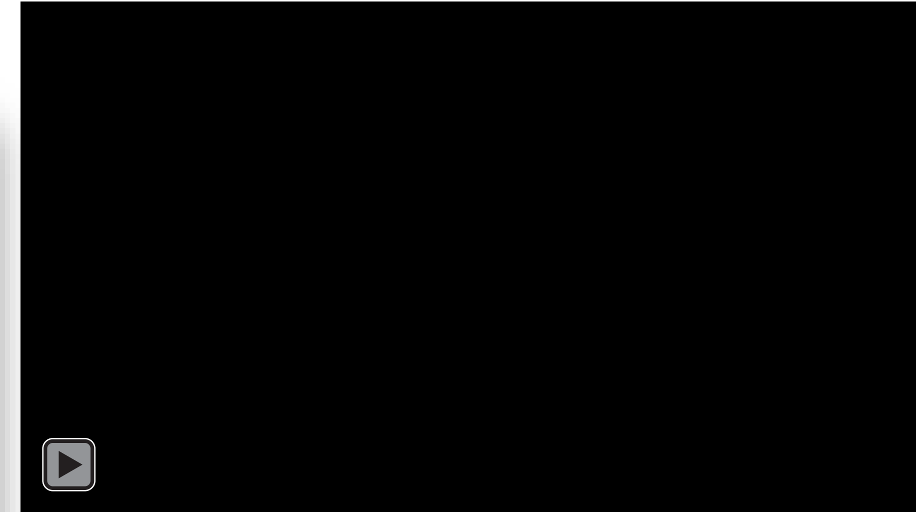
In practice:

Not usually used for data collection for cryo-samples.

Used for data collection for plastic sections, TEM and STEM.



IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



Talos Arctica

- FEG
- Stable Optics
- 2-condenser lens system
- 200 kV
- Gatan CMOS camera (Oneview)
- STEM imaging

Adjacent operator room

Titan Krios

- FEG
- Super Stable Optics – power lenses
- 3-condenser lens system
- 300 kV
- Two DED’s (Falcon III and GIF/K3)
- Energy Filter (with K3)
- STEM imaging

Data Collection - Considerations

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS

What total dose? Resolution vs signal-to-noise

What tilt range? Completeness of information vs dose and speed

What angular increment? Resolution vs dose and speed

What order to collect the images? Speed, reliability, optimal dose, sample distortion…

What magnification? Resolution and DQE vs field of view

What defocus? High-frequency information vs low frequency information

John Briggs Lecture, MRC Cryo-EM course 2017
https://www2.mrc-lmb.cam.ac.uk/research/scientific-training/electron-microscopy/

What Magnification?

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The Shannon Theorem

If a function $x(t)$ contains no frequencies higher than B hertz, it is completely determined by giving its ordinates at a series of points spaced $1/(2B)$ seconds apart.

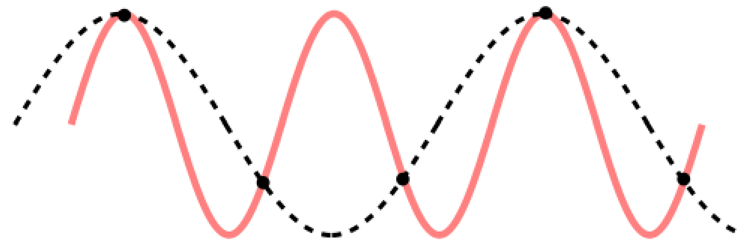
The Nyquist Limit

$$B \leq \frac{f(s)}{2}$$

f(s) is the sampling rate

Example of undersampling:

Two different sine waves can accurately describe the sampled data. So data is not adequately sampled for this frequency.



How much do we have to sample to get a desired resolution?

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS

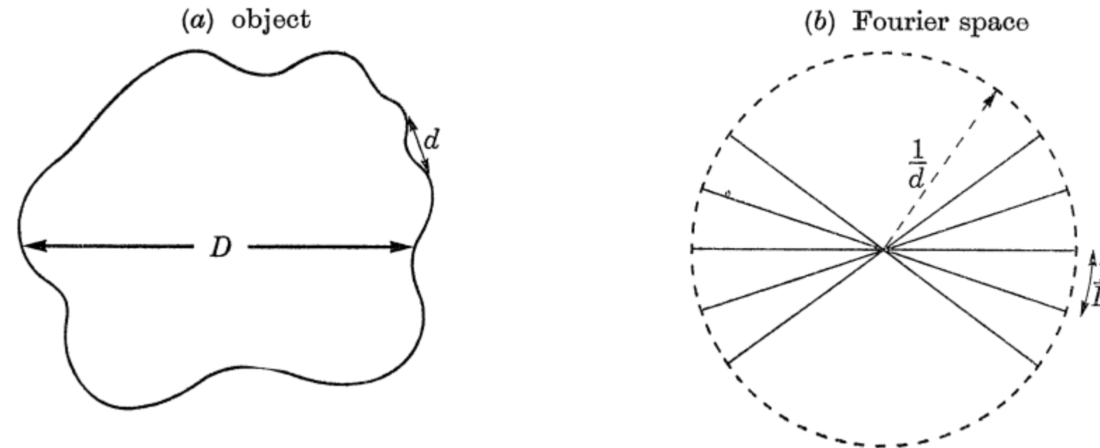
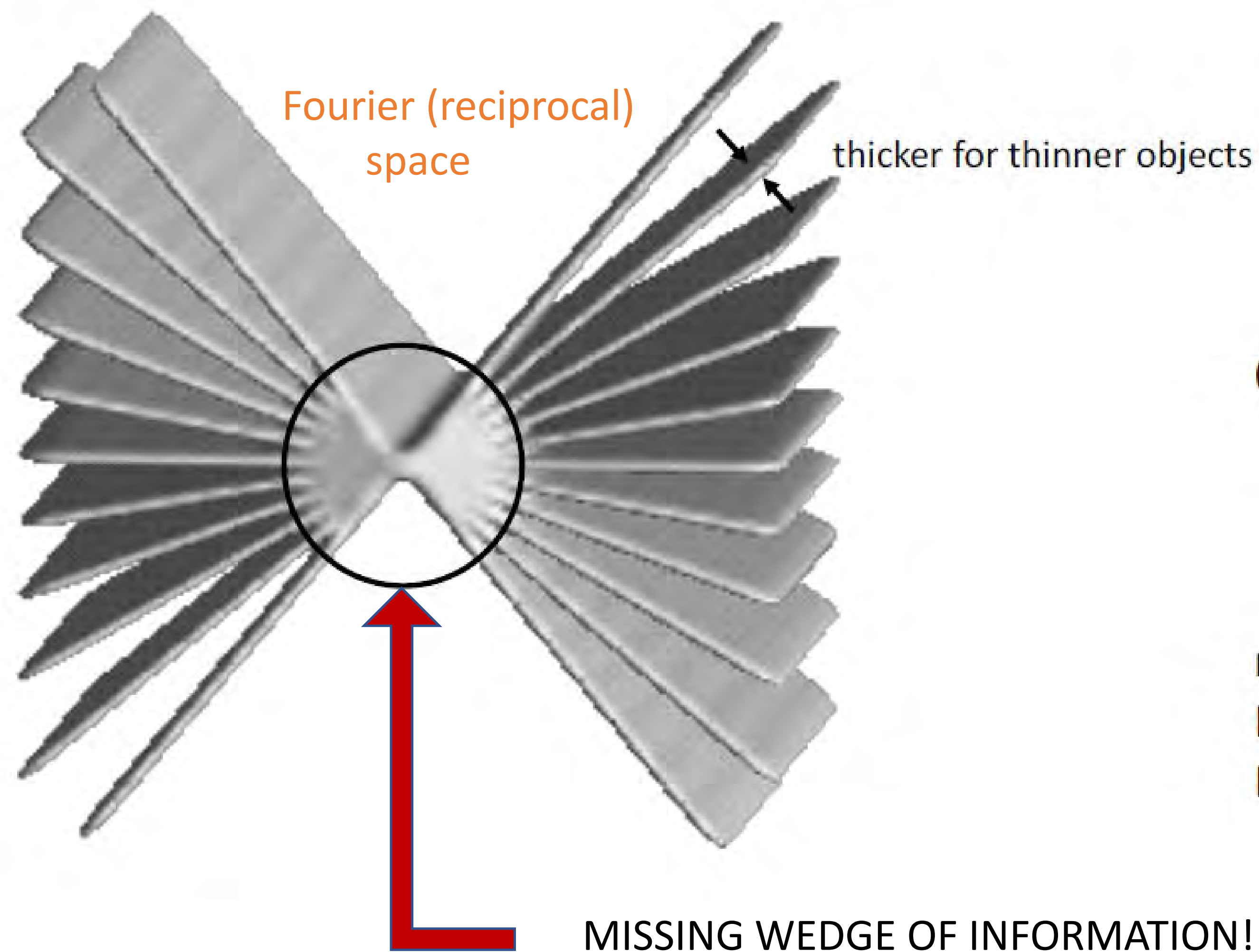


FIGURE 1. (*a*) An asymmetric particle of maximum diameter D containing features on a scale d. (*b*) Two-dimensional illustration of the 'filling-up' of Fourier space by central sections. Data must be collected to a radius of $1/d$ if the reconstruction is to show detail to a resolution d. The angular separation is decided as follows. The Fourier transform of a particle of diameter D does not, on the average, change appreciably over a distance $1/D$ in a Fourier space. There will thus be a sufficient number of sections if their intersections with a circle of radius $1/d$ are spaced at intervals at least as small as $1/D$. The minimum number of views, n, is therefore equal to $n = \pi(1/D)/(1/d) = \pi D/d$. For a particle of diameter 20 nm, to be solved to a resolution of 2 nm, $n \approx 30$. The value of n will in practice need to be greater since the calculation assumes that all the data can be collected by a set of tilts about a single axis covering a range of $\pm 90°$. This is impossible in practice. Limited ranges of tilts about more than one axis will have to be employed, and the strategy of data collection can be worked out by the methods given by Crowther, DeRosier & Klug (1970).

Optical Diffraction and Filtering and Three-Dimensional Reconstructions from Electron Micrographs
Author(s): A. Klug
Source: *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*, Vol. 261, No. 837, A Discussion on New Developments in Electron Microscopy with Special Emphasis on their Application in Biology, (May 27, 1971), pp. 173-179

How much do we have to sample to get a desired resolution?

IMAGING LIFE FROM MOLECULES TO CELLS



MISSING WEDGE OF INFORMATION!

Crowther Criterion

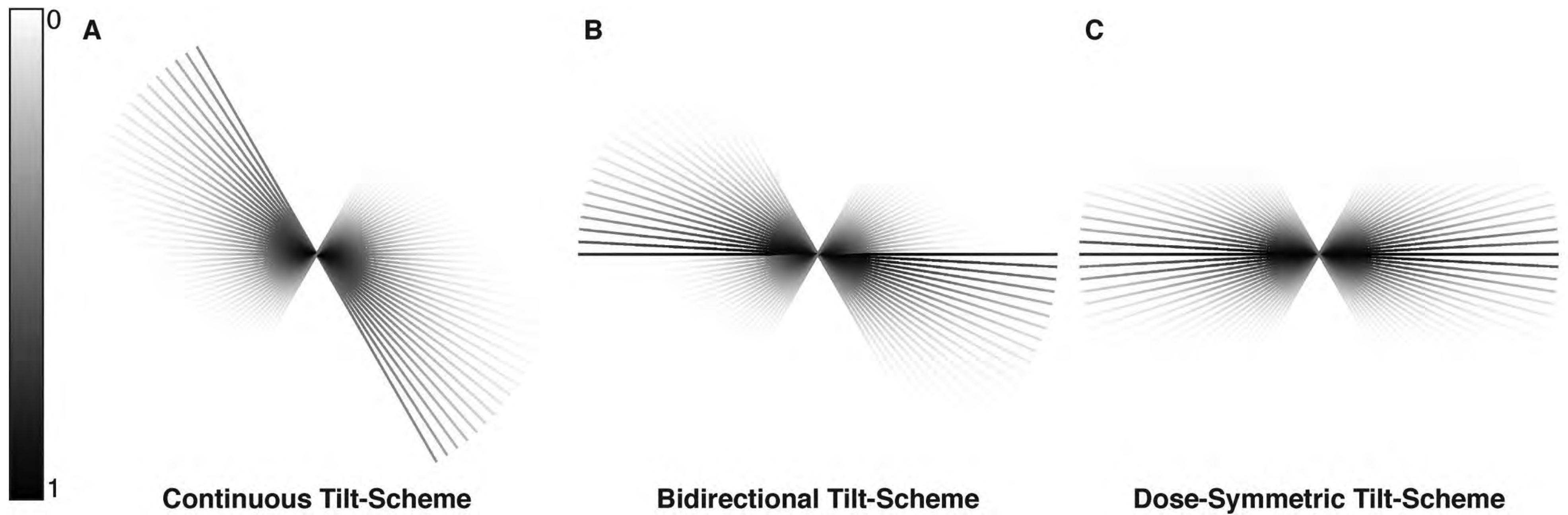
$$r = \pi D/N$$

r: Resolution limit
N: Number of projections
D: Object thickness

John Briggs Lecture, MRC Cryo-EM course 2017
https://www2.mrc-lmb.cam.ac.uk/research/scientific-training/electron-microscopy/

Tilt Schemes

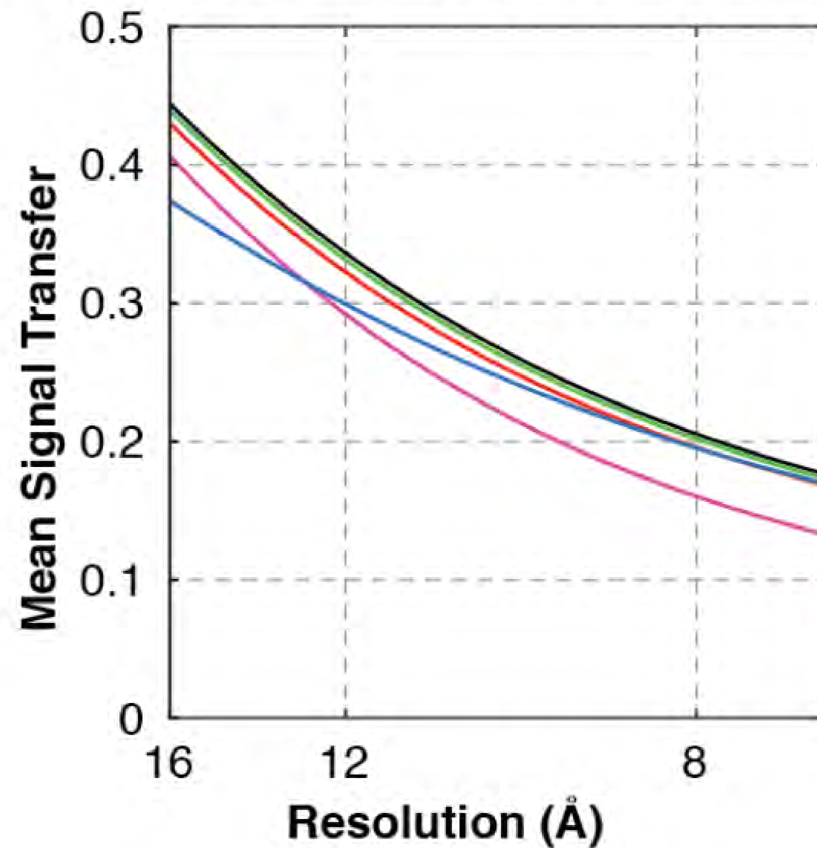
IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



Hagen WJH, Wan W and Briggs JAG, J. Struct Biol. 197(2), 191-198 (2017)

Tilt Schemes – signal transfer

IMpaCT
IMAGING LIFE FROM MOLECULES TO CELLS



Continuous

Bidirectional from 0

Bidirectional from -21

Bidirectional from -21 deleting second branch

Dose-symmetric

Hagen WJH, Wan W and Briggs JAG, J. Struct Biol. 197(2), 191-198 (2017)

Data Collection - Considerations

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS

	Strategy to increase contrast	Strategy to increase resolution	Strategy to deal with thick samples
Voltage	Low (200 keV) as thickness allows	High (300 keV)	High (300 keV)
Total electron dose	High (>100e-/A2)	Low (<50e-/A2)	High (>100e-/A2)
Defocus	High (−5um to −15um)	Low (−2um to −4um)	High (−5um to −15um)
Magnification	Low (more e- per pixel)	High (pixel size half Nyquist)	Low (more e- per pixel)
Tilt range	Low (maximise e- dose per projection image)	Higher (decrease missing wedge)	Low (extreme tilts will be even thicker)
Tilt increment	Large (more e- per image)	Small (sample fine details but not important for subtomogram averaging)	Large (facilitates higher SNR, enabling alignment of tilt series)
Tilt scheme	Not relevant	Dose symmetric	Dose symmetric
Phase plates	Always use if possible	X Always use if possible	Always use if possible
Energy filter	Always use if possible	Always use if possible	Always use if possible

Imaging mode: STEM TEM STEM

Adapted from: Ferreira J.L., Matthews-Palmer T.R.S., Beeby M. (2018) Electron Cryo-Tomography. In: Hanssen E. (eds) Cellular Imaging. Biological and Medical Physics, Biomedical Engineering. Springer, Cham.

TFS Tomography Interface - ATLAS

TFS Tomography Interface Presets – Search/Template

STEM Tomography

Preparation (STEM) | Atlas | Auto Functions | Tomography Calibrations | Tomography | Visualizer

Presets Search / Templat

Atlas
Overview / Positioning
Search / Template
Eucentric Height
Tracking
Exposure
Focus
Drift

Resolution 1024 x 1024
Dwell Time (µs) 4.00
Est. Frame Time (s) 5.97
Scan Settings

Get NanoProbe [BF]
Set (µm) 26.799
Optics Settings

Preview Stop
Acquisition

Calibrate Image Shifts
Image Filters Settings

Messages
2 Errors 0 Notifications
Image shift Calibration missing for specified imaging context. Magnification: 4525 (x)
Image shift Calibration missing for specified imaging context. Magnification: 4525 (x)

Status
4525 (x)
Acquiring image..
Ready!
Setting Optics..
Acquiring image..
Ready!
Setting Optics..
Acquiring image..
Ready!

Image Histogram
Enable auto update

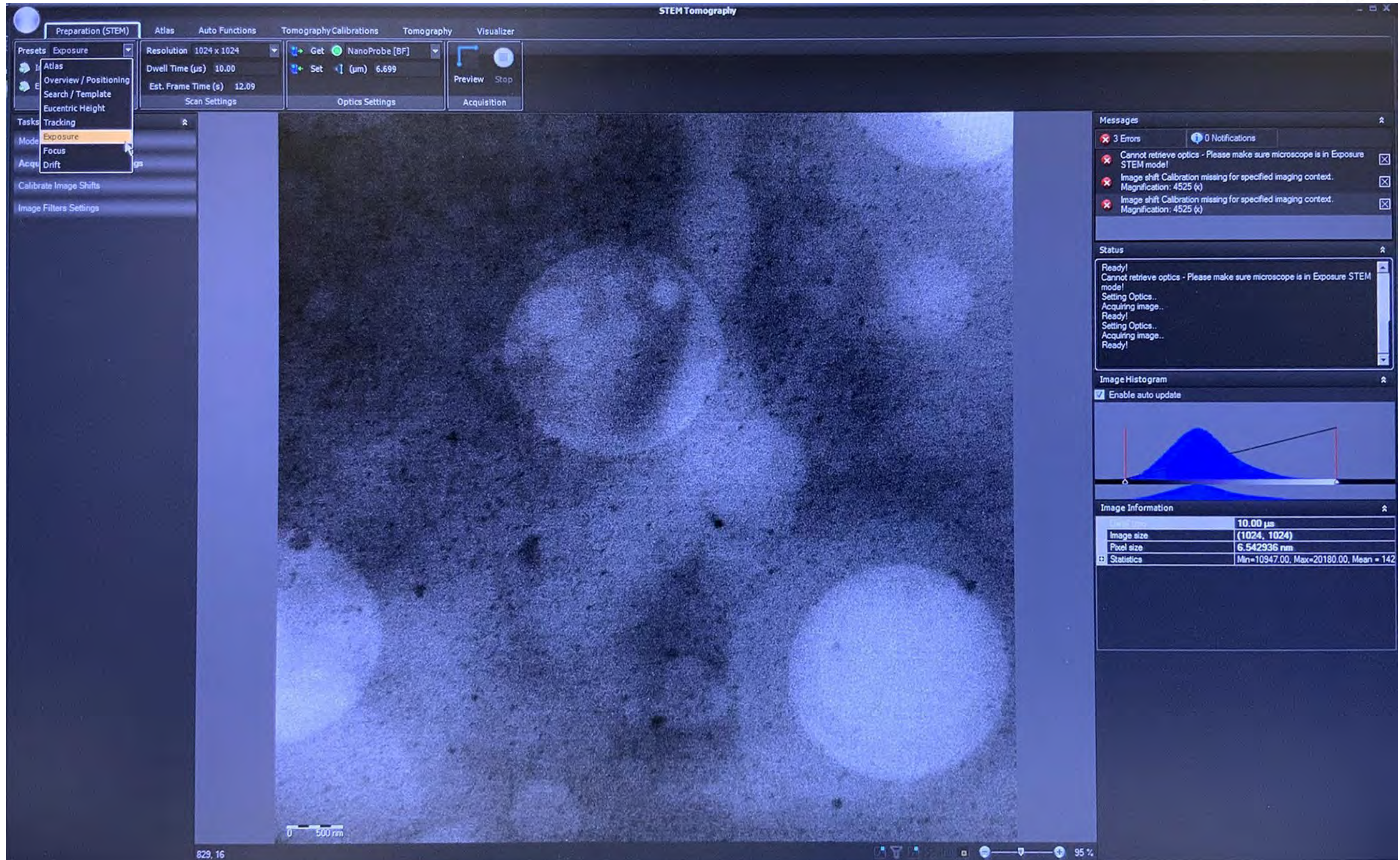
Image Information

Dwell time	4.00 µs
Image size	(1024, 1024)
Pixel size	26.171744 nm
Statistics	Min=6470.00, Max=22051.00, Mean = 1553

95 %

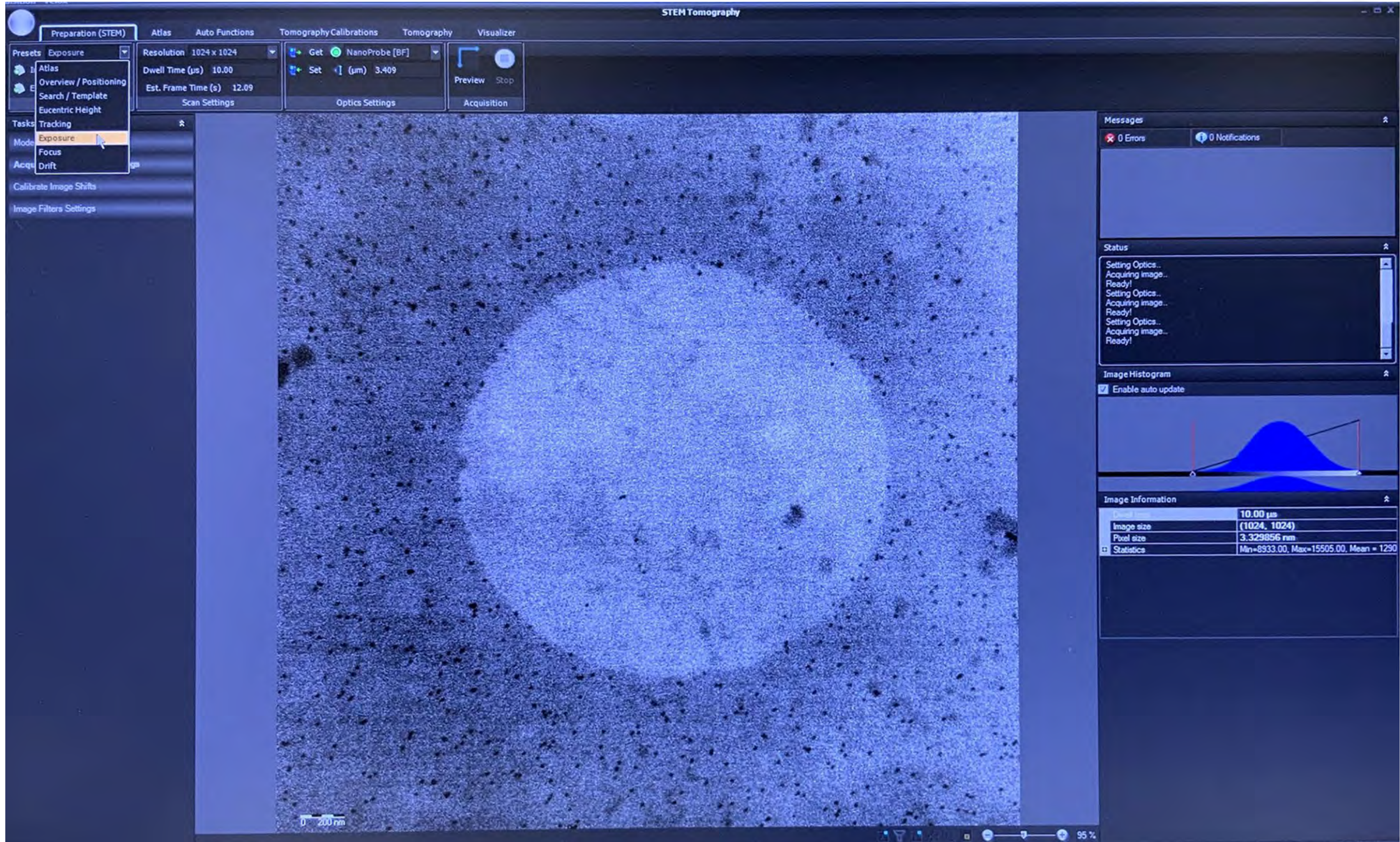
TFS Tomography Interface   Presets – Exposure

IMaCT IMAGING LIFE FROM MOLECULES TO CELLS



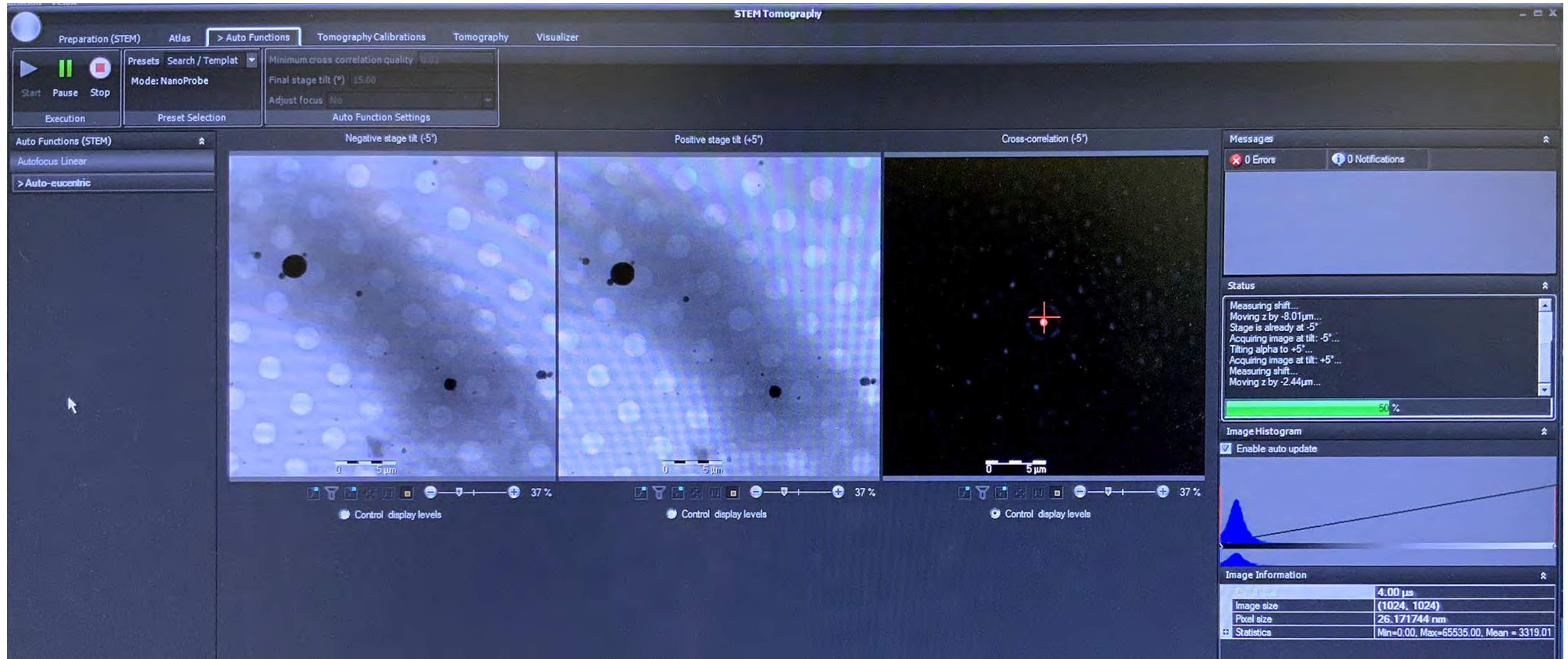
TFS Tomography Interface   Presets – Exposure

IMPACT
IMAGING LIFE FROM MOLECULES TO CELLS



TFS Tomography Interface   Autofxns – Eucentric height

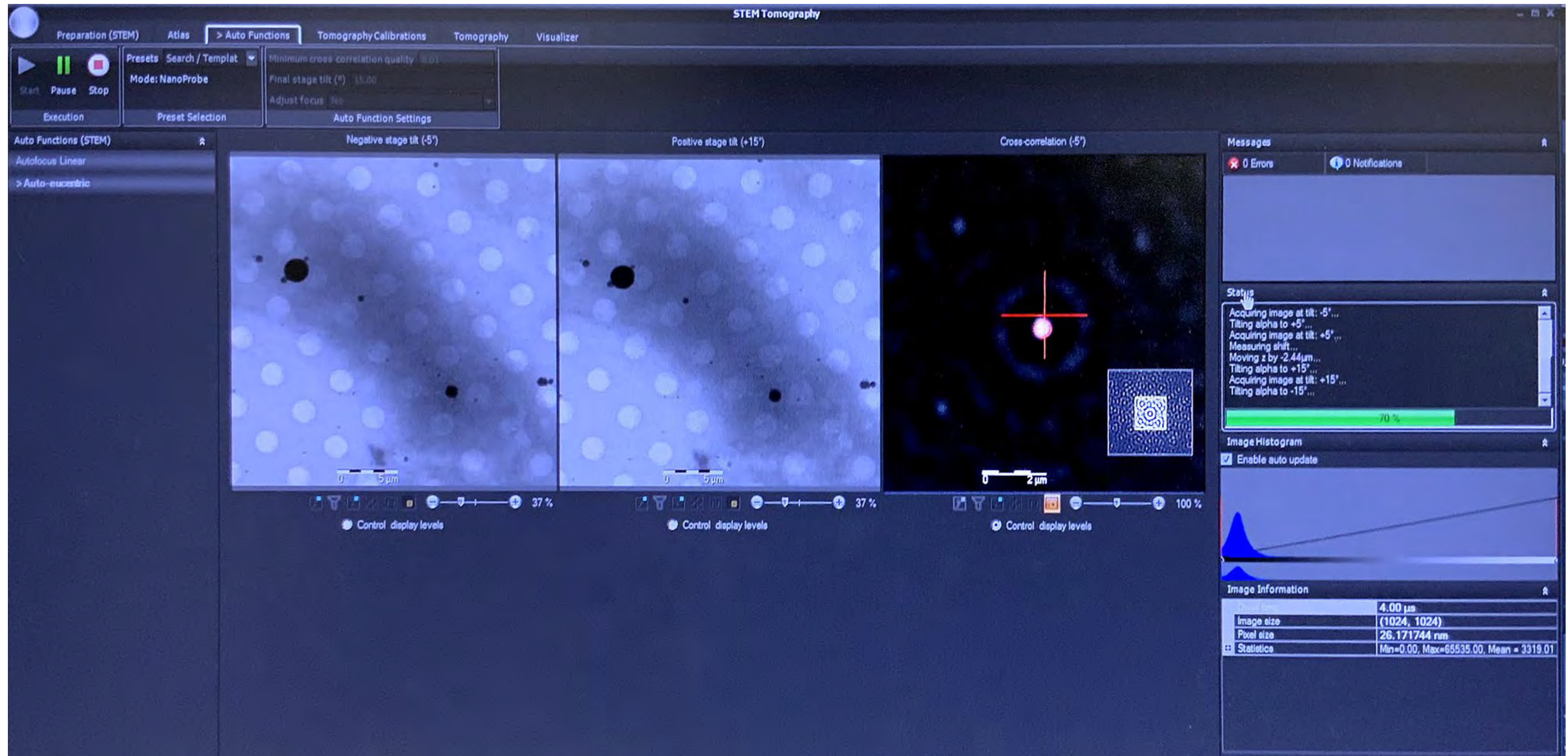
IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



TFS Tomography Interface

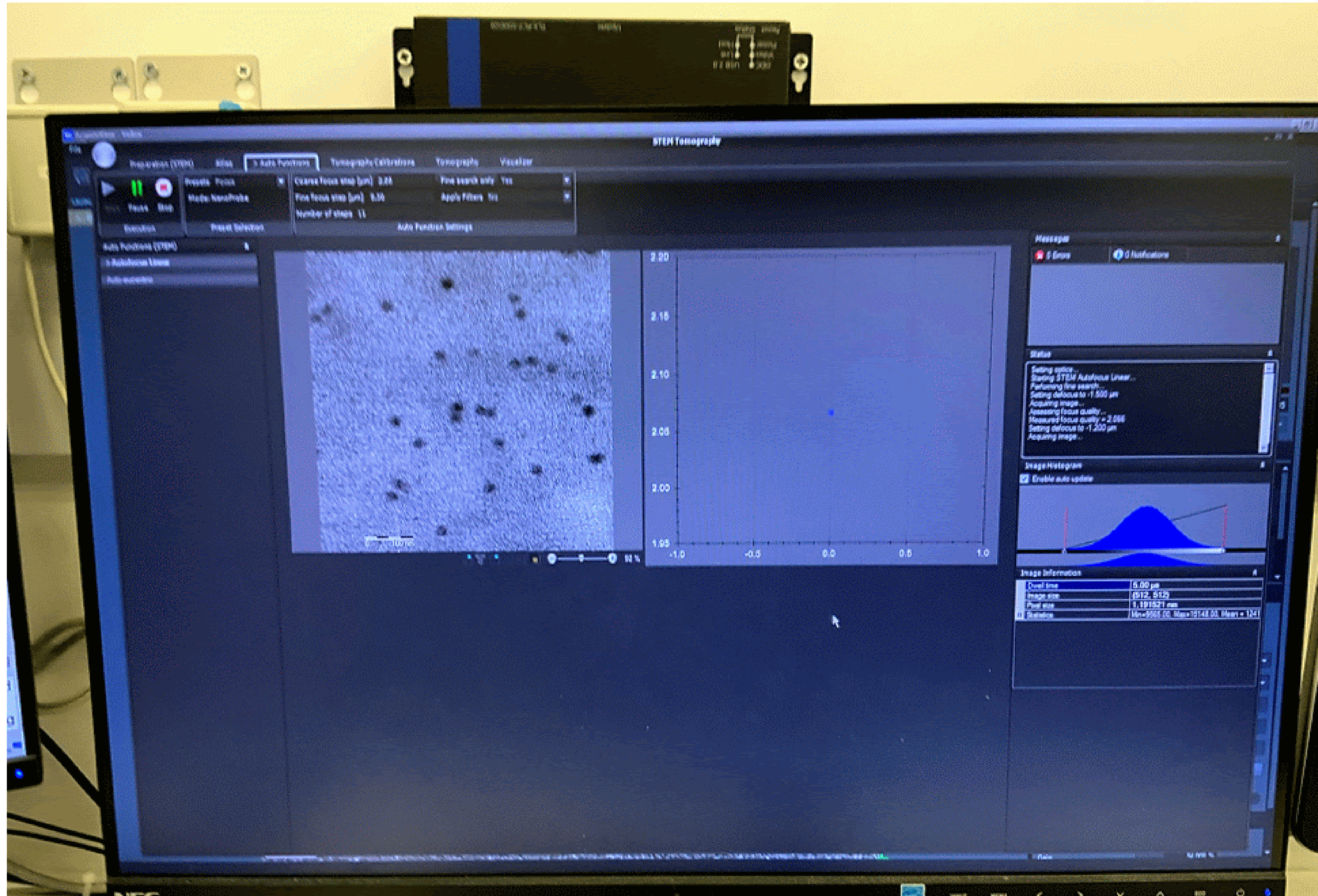
Autofxns – Eucentric height

IMpaCT
IMAGING LIFE FROM MOLECULES TO CELLS



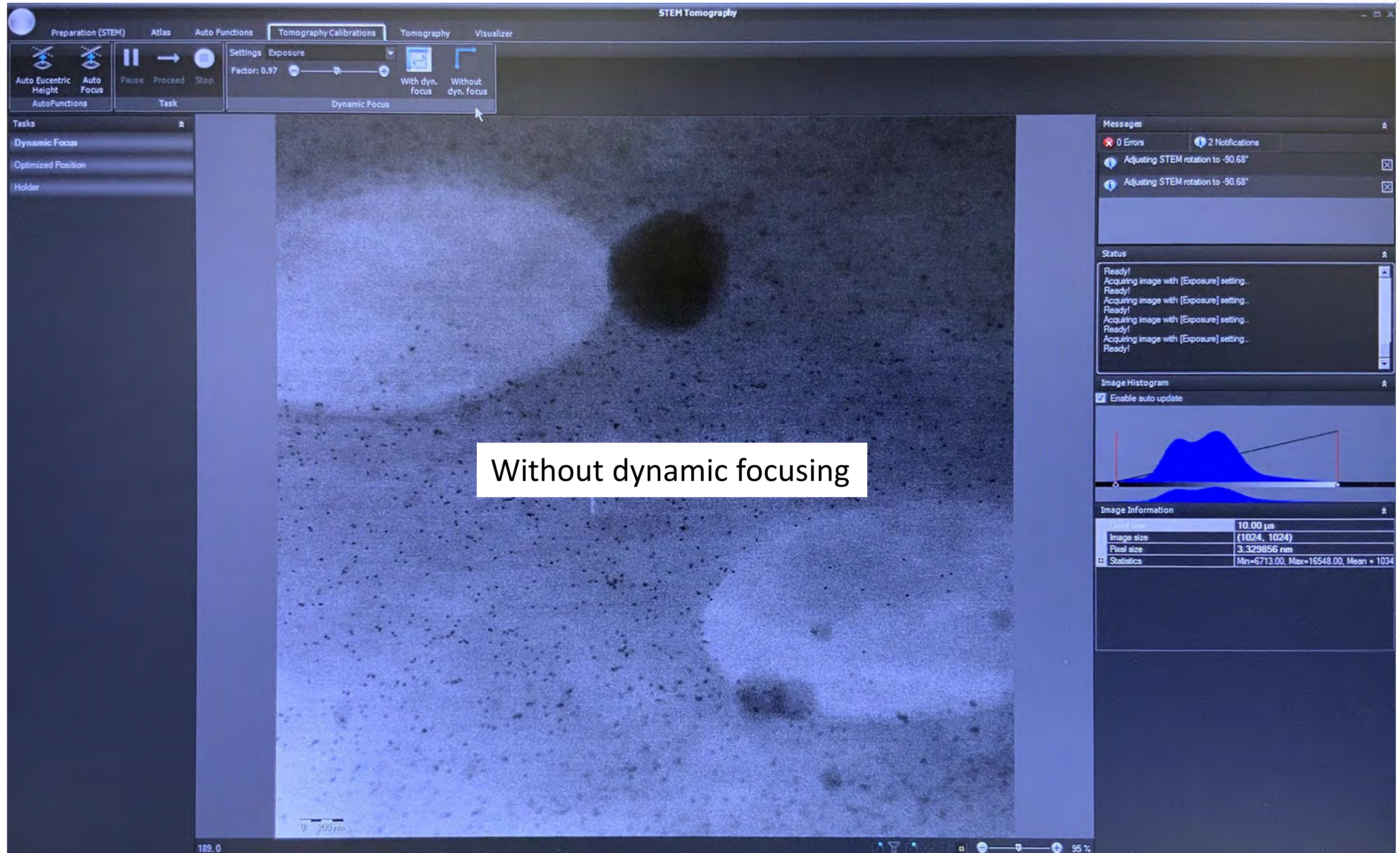
TFS Tomography Interface Autofxns – focusing

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



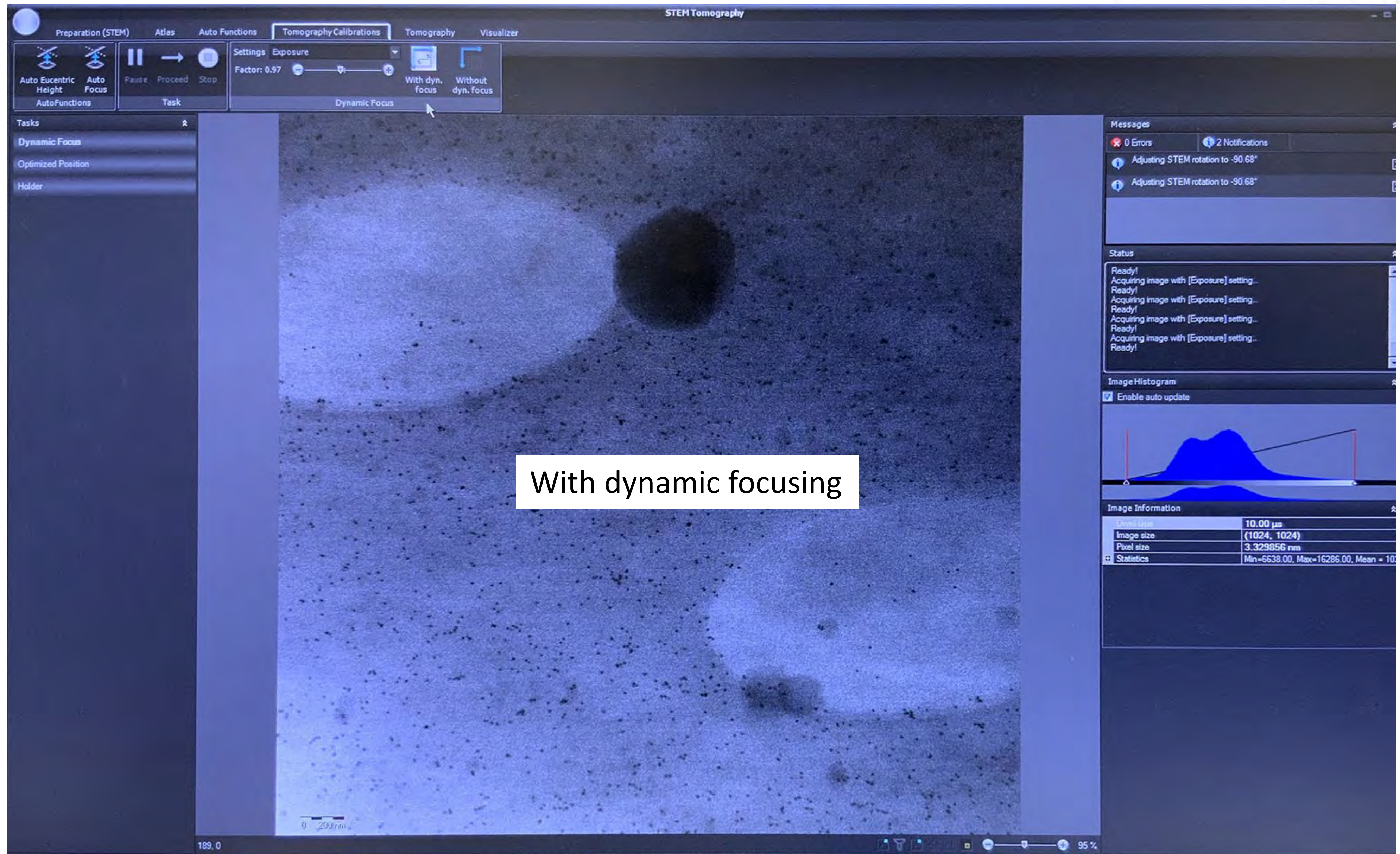
TFS Tomography Interface STEM dynamic focusing

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



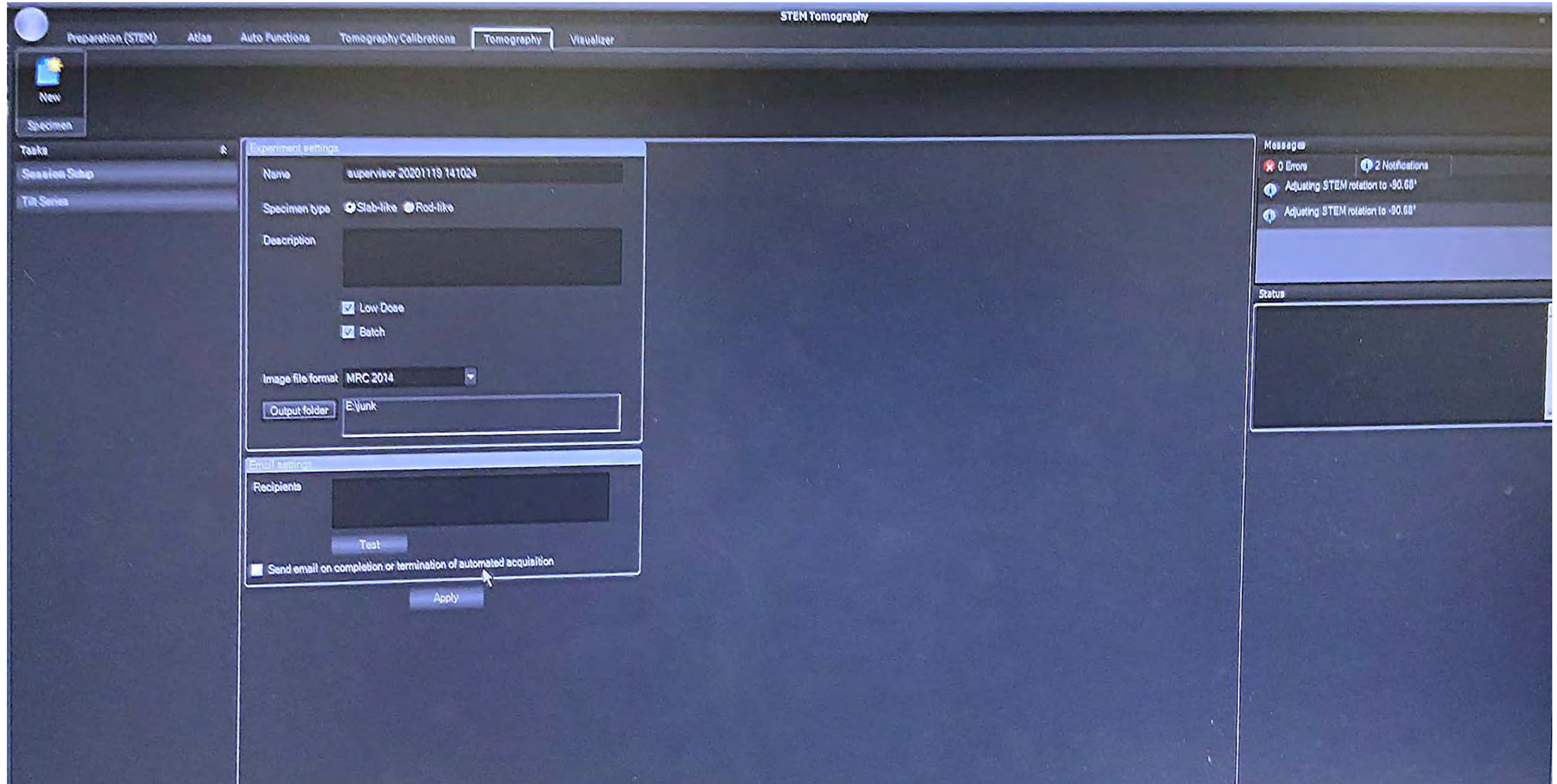
TFS Tomography Interface STEM dynamic focusing

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



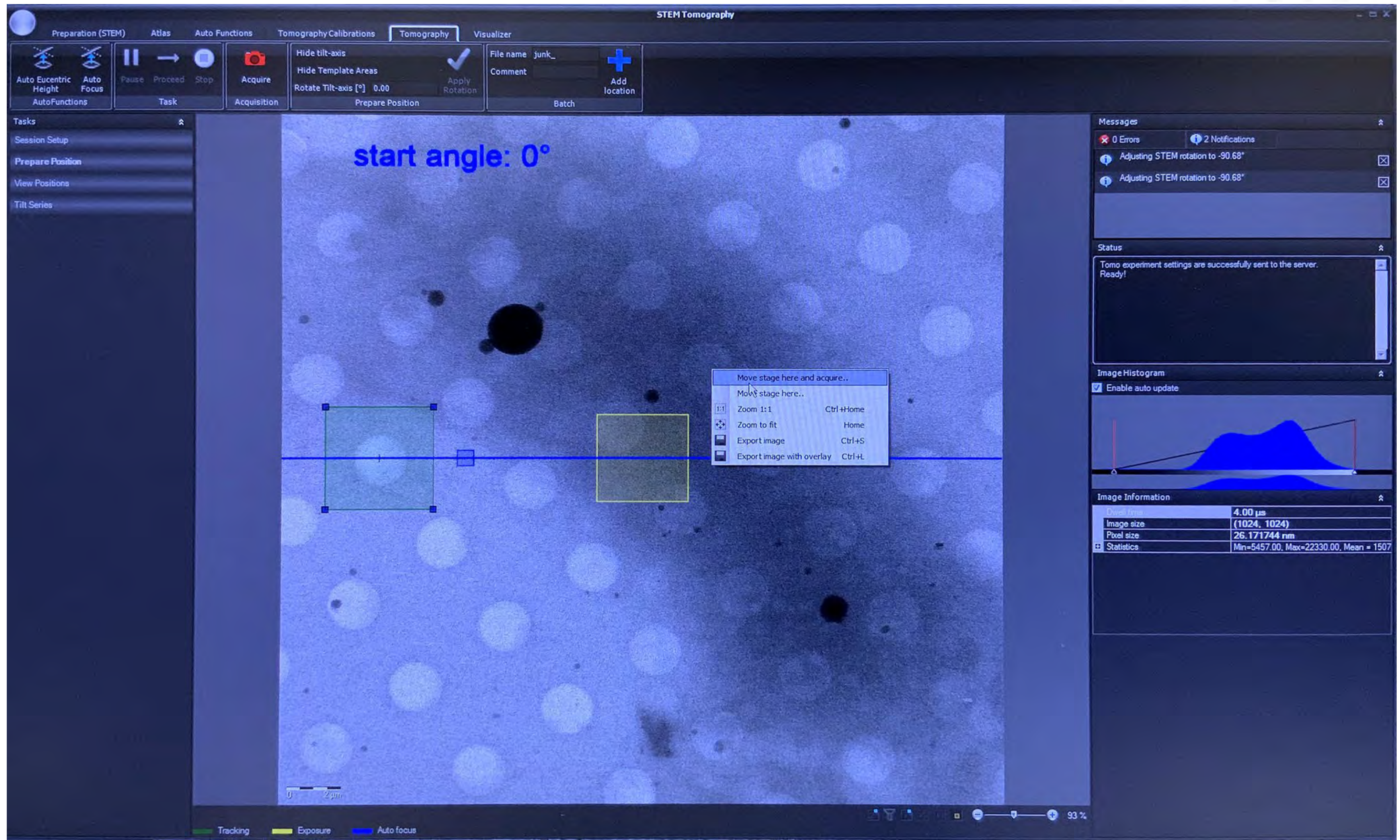
TFS Tomography Interface - Set up Tomography

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



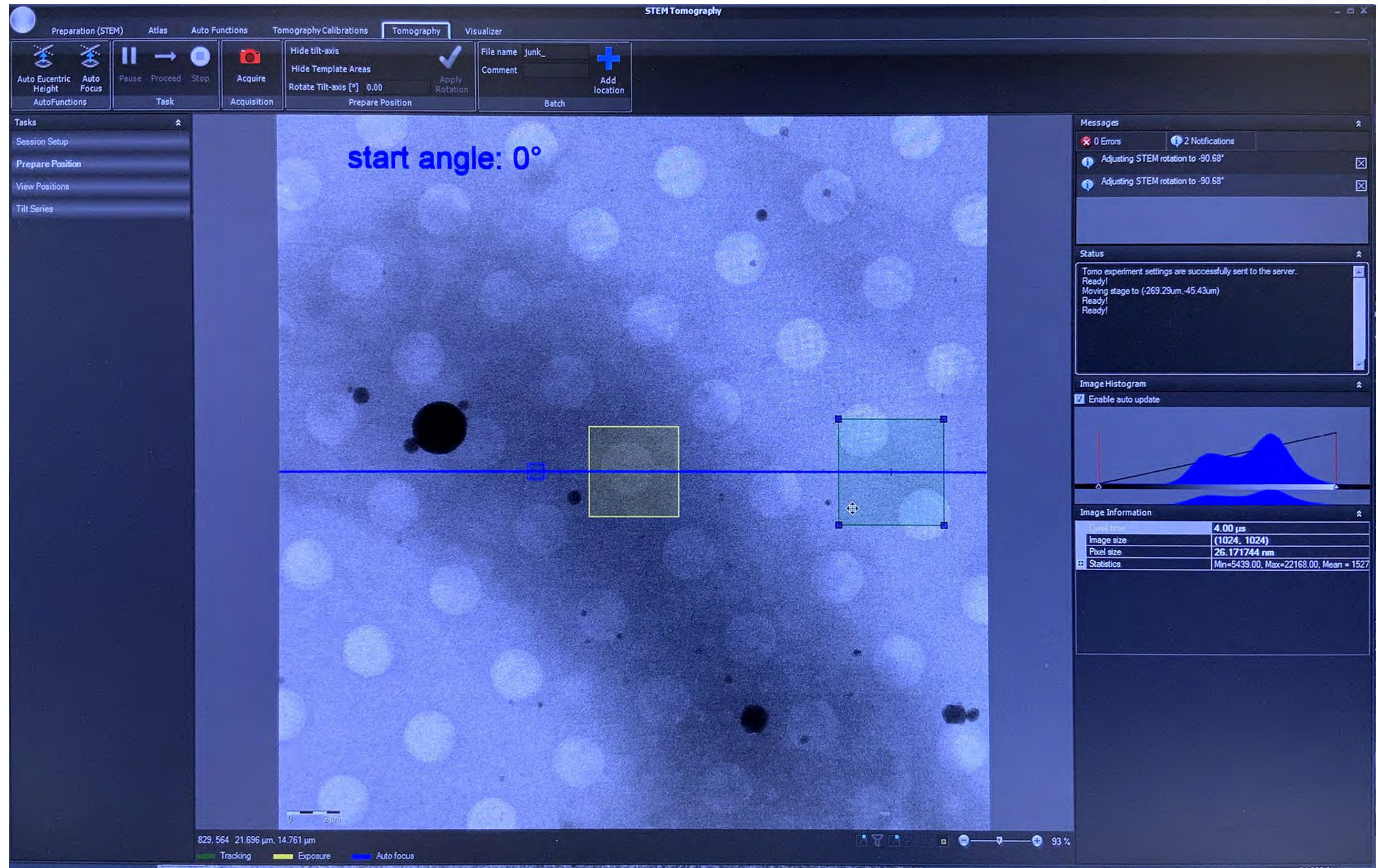
TFS Tomography Interface - Prepare Position

IMPACT
IMAGING LIFE FROM MOLECULES TO CELLS



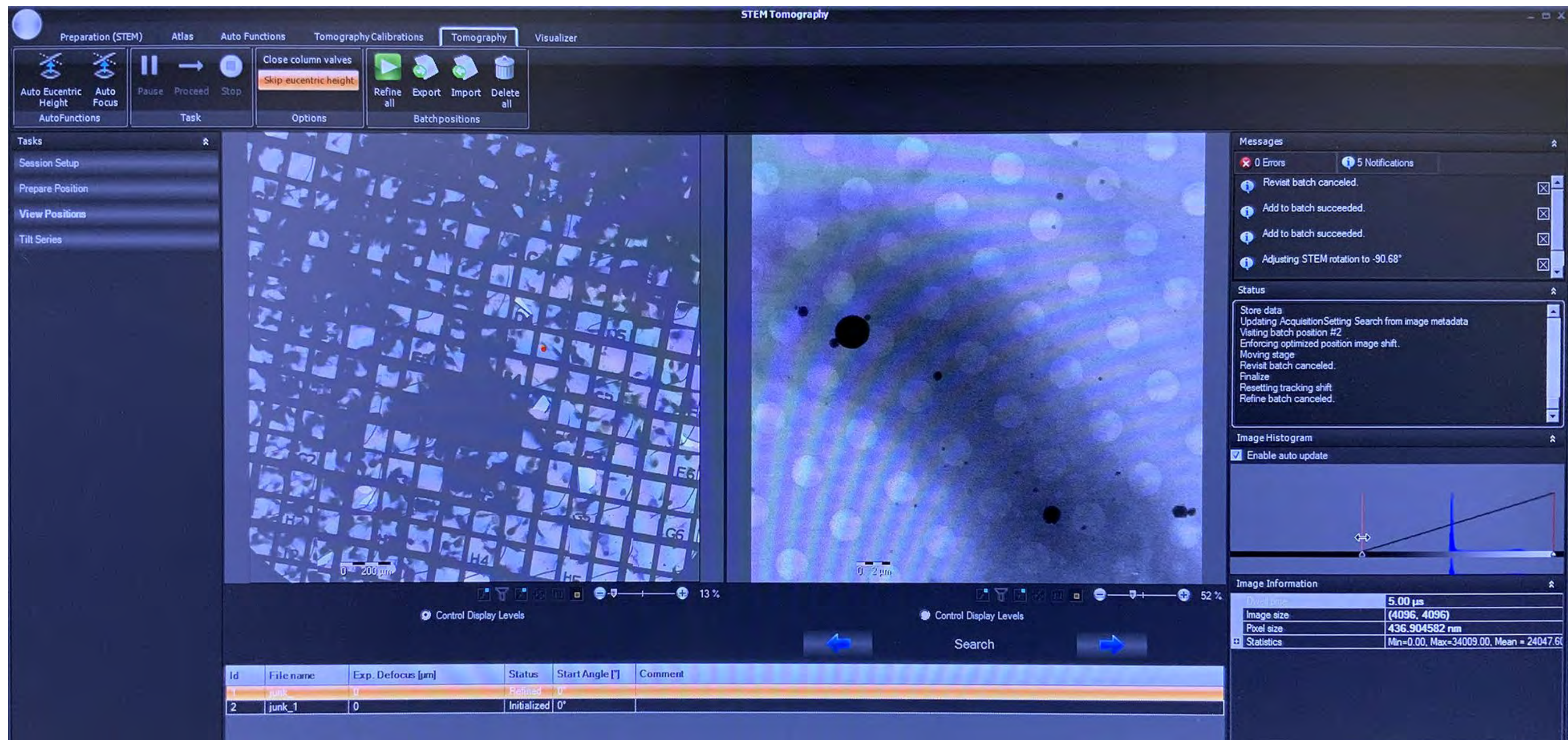
TFS Tomography Interface - Prepare Position

IMPACT IMAGING LIFE FROM MOLECULES TO CELLS



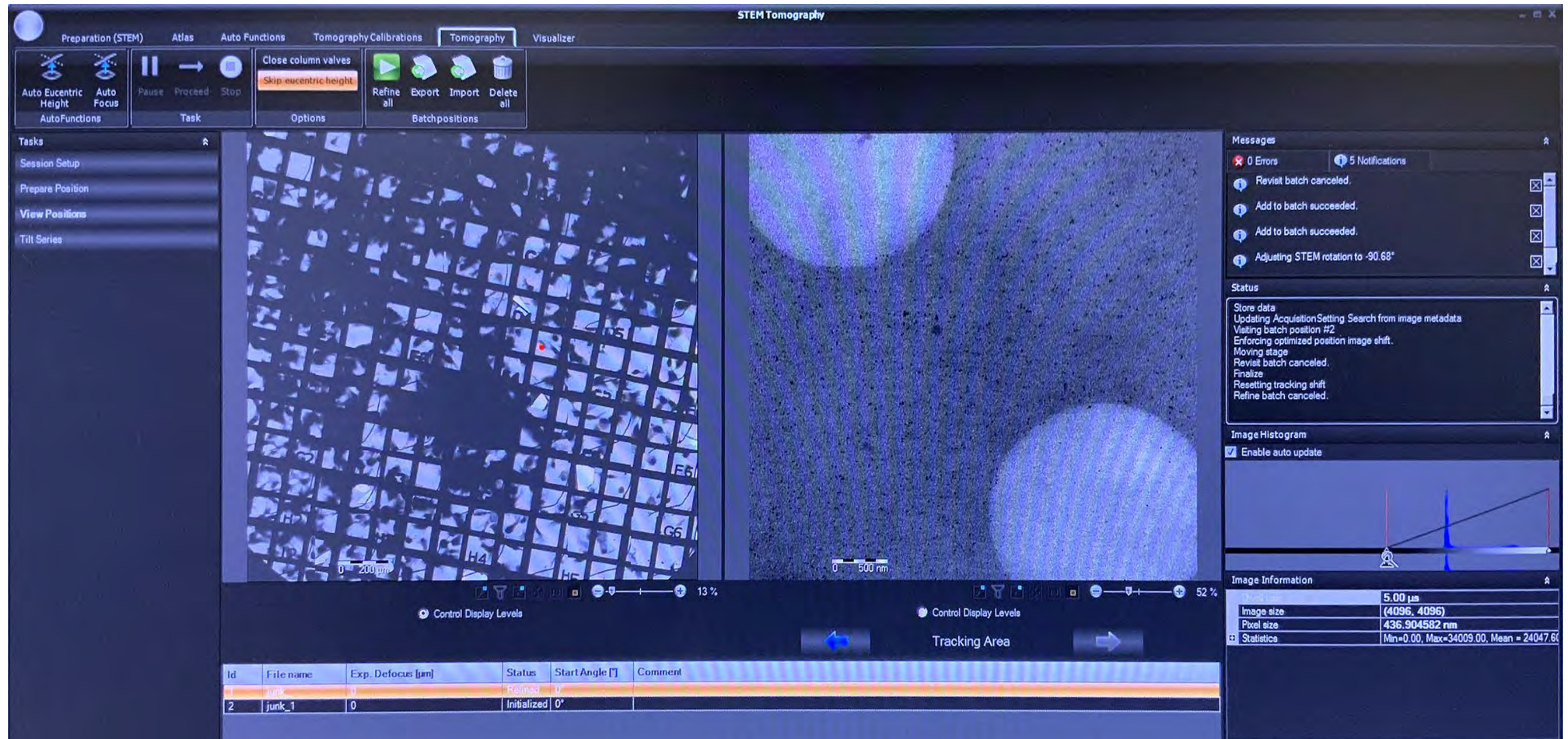
TFS Tomography Interface - Refine Positions

IMPACT
IMAGING LIFE FROM MOLECULES TO CELLS



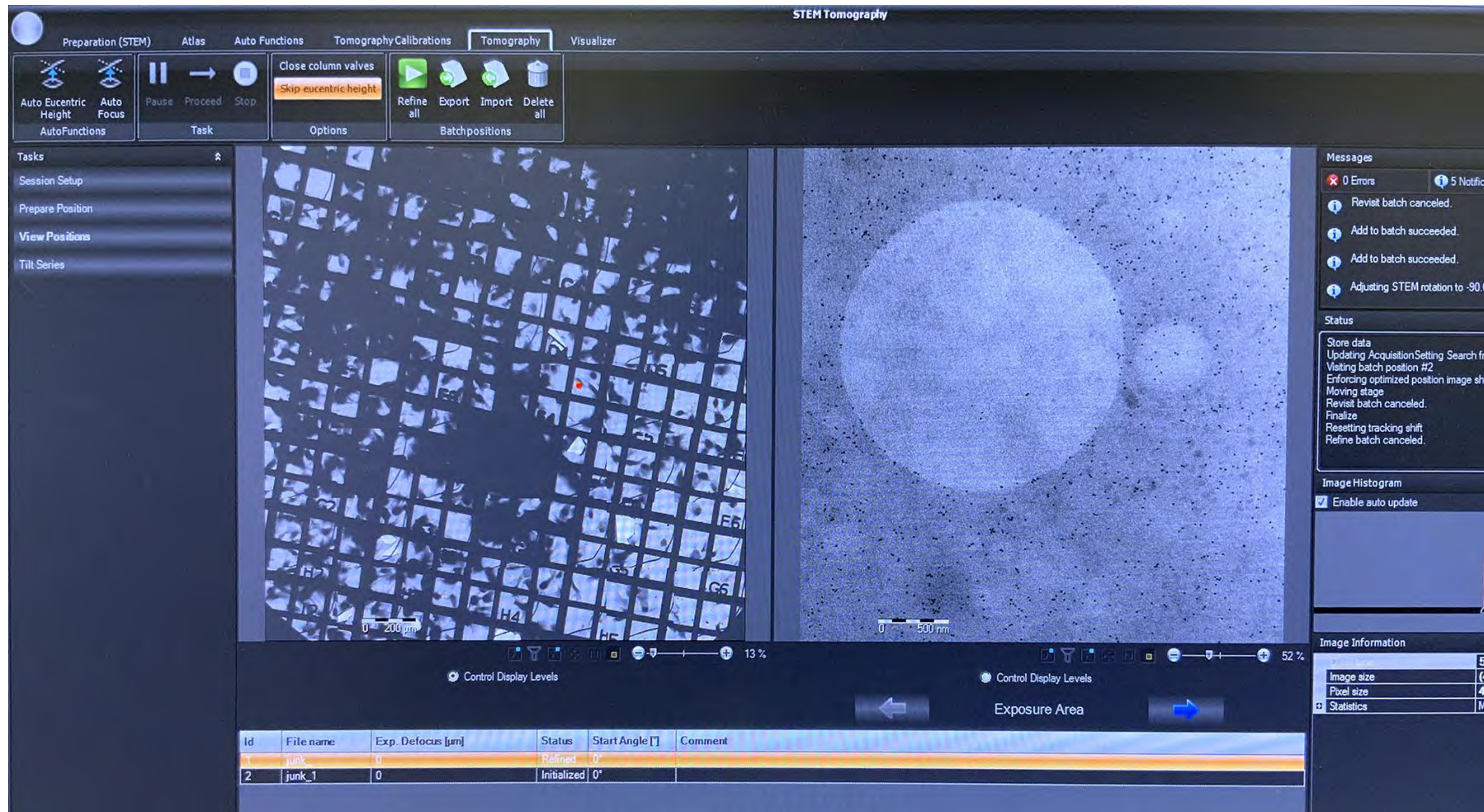
TFS Tomography Interface - Refine Positions

IMpaCT
IMAGING LIFE FROM MOLECULES TO CELLS



TFS Tomography Interface - Refine Positions

IMpaCT IMAGING LIFE FROM MOLECULES TO CELLS



TFS Tomography Interface - Tilt Series Parameters

IMpaCT
IMAGING LIFE FROM MOLECULES TO CELLS

Tilt Series Acquisition

Start Angle [°]	-30.00
Max. Negative Angle [°]	-65.00
Max. Positive Angle [°]	65.00
Stage Relaxation Time [s]	3.00

☐ Partial tilt series

Tilt Scheme

◉ Linear ○ Saxton

Low Tilt Step [°]	2.00
High Tilt Switch [°]	80.00
High Tilt Step [°]	1.00

Focus

☑ Use Dynamic Focus

Defocus (at 0°) [µm] 0.000

Detectors

☑ Use Additional Detectors

☑ BF ☐ DF2 ☐ DF4

☑ HAADF

Acquisition Output

Output File Name: Dummy

Apply Reset

TFS Tomography Interface - Tilt Series Corrections

IMpaCT
IMAGING LIFE FROM MOLECULES TO CELLS

Auto focus

Check focus before acquisition

Auto focus periodicities:

High tilt 3

Low tilt 3

Switch angle [°] 10.00

Tracking

Track after acquisition

Track before acquisition

'Tracking before' periodicities:

High tilt 100

Low tilt 1

Switch angle [°] 20.00

Holder predictions

XY and Focus

Auto zero loss

Adjust

Periodicity 100

Apply Reset

TFS Tomography Interface - Acquisition

IMAGING LIFE FROM MOLECULES TO CELLS

References – emphasis on cellular tomography

- Chapter 10, Biomedical Electron Microscopy, “Microscopy”, Maunsbach and Afzelius https://doi.org/10.1016/B978-012480610-8/50012-3 (1999)

  Old book with great images demonstrating various optical phenomena, using biological sections as examples.

- Transmission Electron Microscopy (textbook), DB Williams & CB Carter (2009)

  Excellent general chapters for electron optics.

- Cellular Imaging: Electron Tomography and Related Techniques, Ed. E Hanssen, Springer (2018).

  Recent book covering the field.

- Methods in Cell Biology, Volume 152: Three-Dimensional Electron Microscopy, Ed. T Muller-Reichert & G Pigino (2019).

  Another recent book including practical guidelines in many chapters.

- Electron Tomography Methods for Three-Dimensional Visualization of Structures in the Cell, 2nd Edition, Ed. J Frank, Springer (2006).

  The basic tomography principles