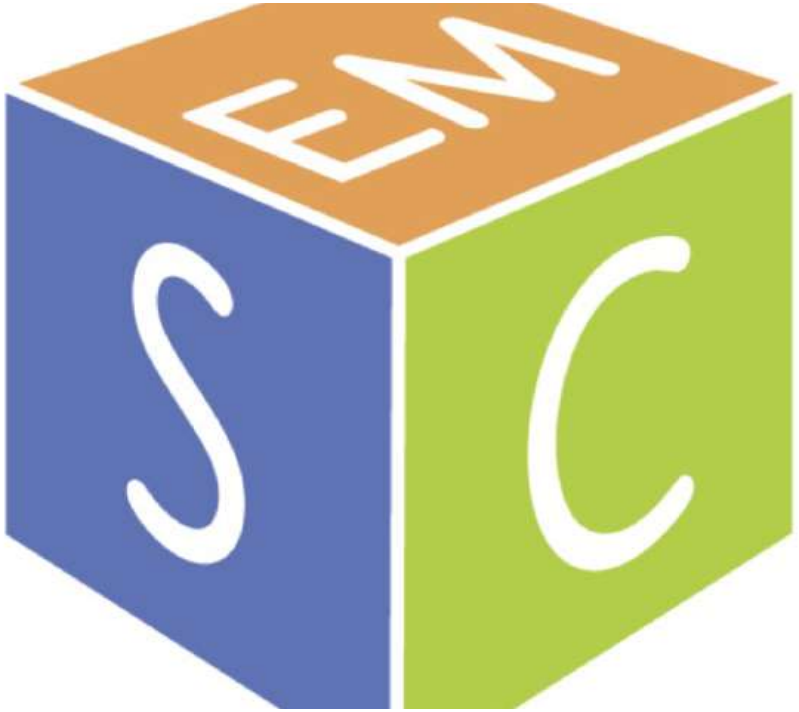


New cryo-EM hardware and supporting a facility

Winter Course 01 - 23 - 2023

Michael Alink

Krios 7:
4th Generation Krios-TEM

- Designed for high throughput
- Fringe Free Imaging (FFI)
- Aberration free Image shift (AFIS)
- Falcon IV camera
- Electron event representation (EER)

Microscope top - bottom

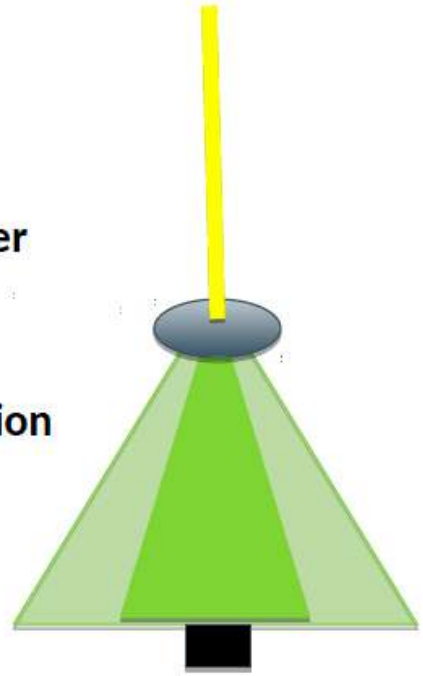
Source: gun

Illumination: condenser

Imaging: objective

magnification: projection

detection: camera's



Some more facts

- A Titan is NOT a Tecnai! (ThermoFisher is not ZEISS or JEOL or Hitachi): the optics are different!

- COLUMN alignments are not the same as

DIRECT alignments

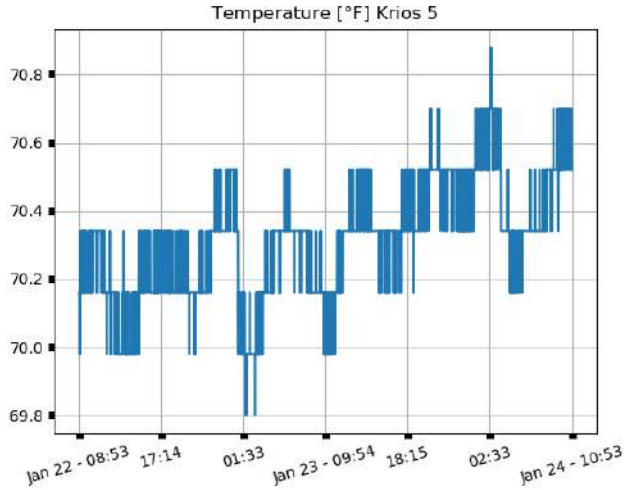
What NOT to do:

-You do NOT need to realign your microscope daily just out of routine!

-If you do not EXACTLY know what an alignment step does:

DON’T DO ALIGNMENTS!

- The user is often more unstable than the microscope or the room

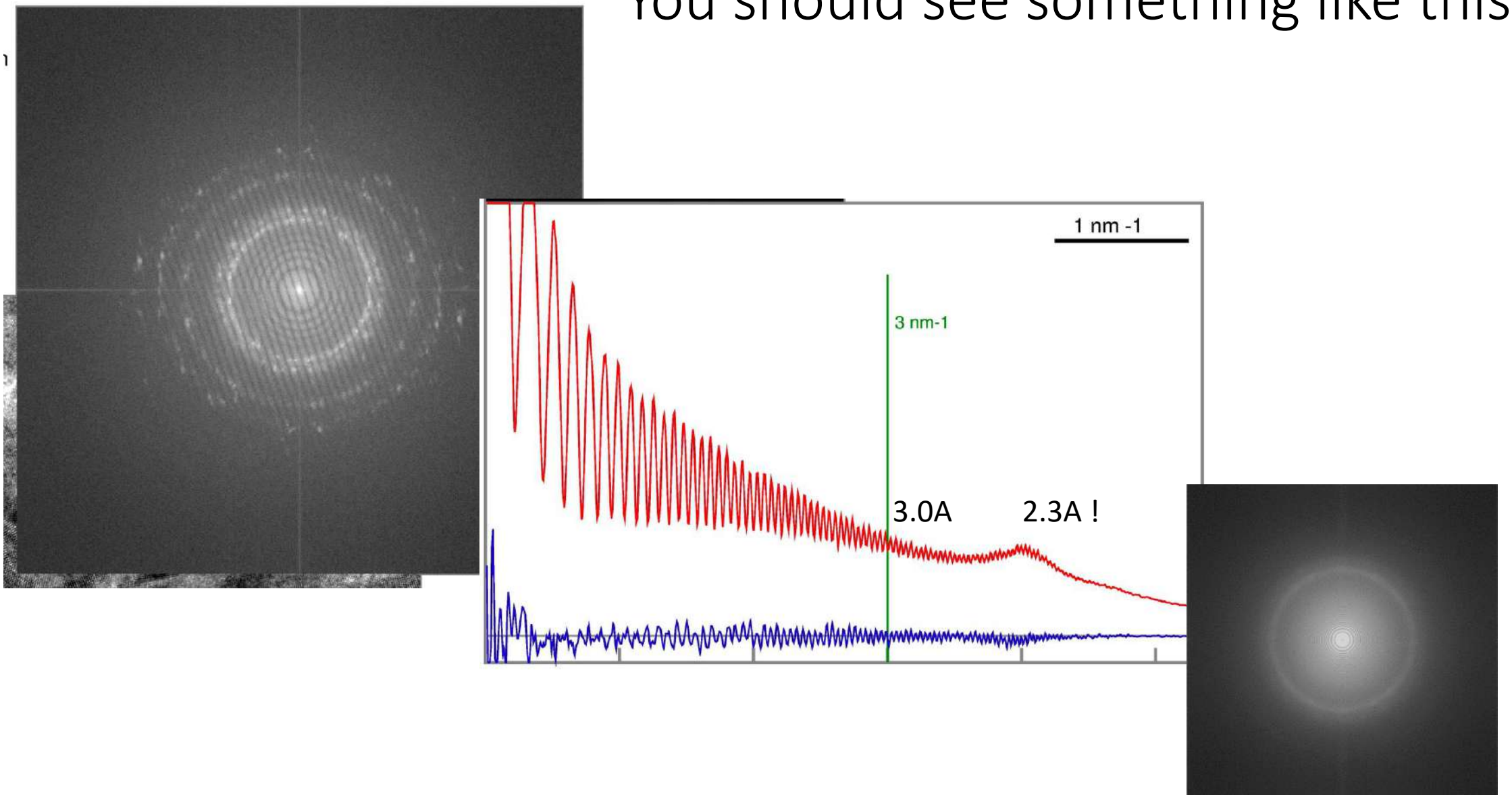


How to setup you’re scope

You should be:

- At Eucentric height
- At Eucentric focus
- Parallel
- Objective stigmated
- Coma free
- Dose rate

You should see something like this:



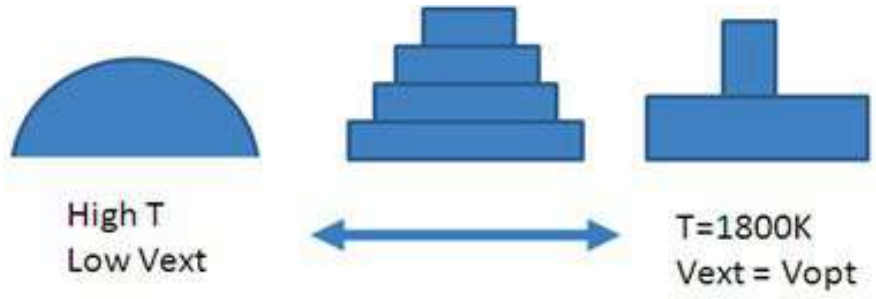
Gun: stability, brightness, dE

If the temperature is low, and the extraction voltage is high

- The tip end form will evolve in a single facet. This end form is stable resulting in a stable current

If the temperature is too high or if the extraction voltage is too low

- The tip end form will evolve into a blunt round shape (low brightness)



Gun: stability, brightness, dE

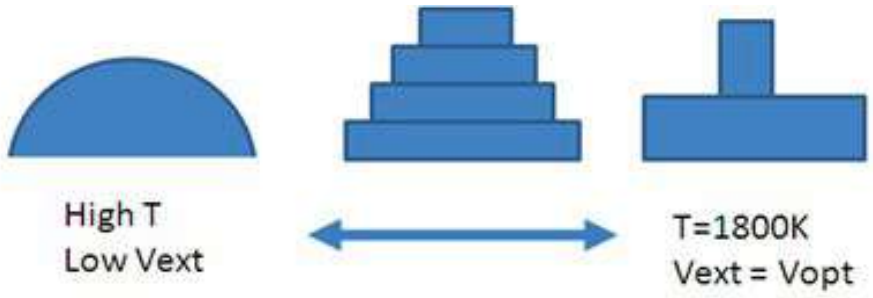
emission characteristics with high brightness

- But too high extraction voltage increases energy spread of electrons

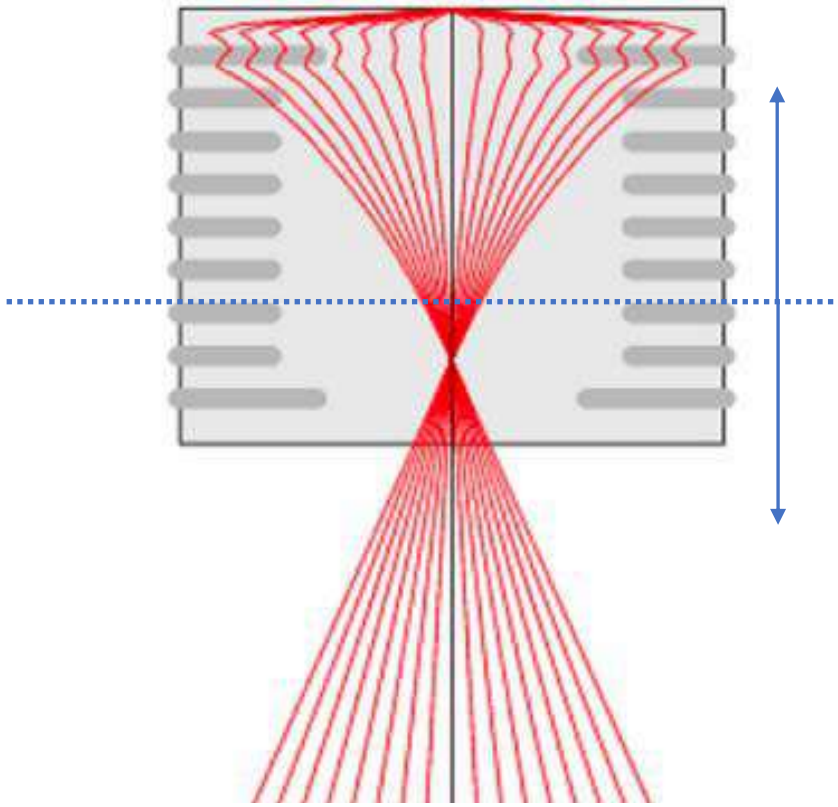
  Too low temperature makes the emission sensitive for pressure variations

- Compromise: T=<1800K. Screen current checked with spot = gun = 1. Screen current about 50nA

  acceptable dE & good Brightness & reasonable stability



Gun settings



Do you know what happens when you:

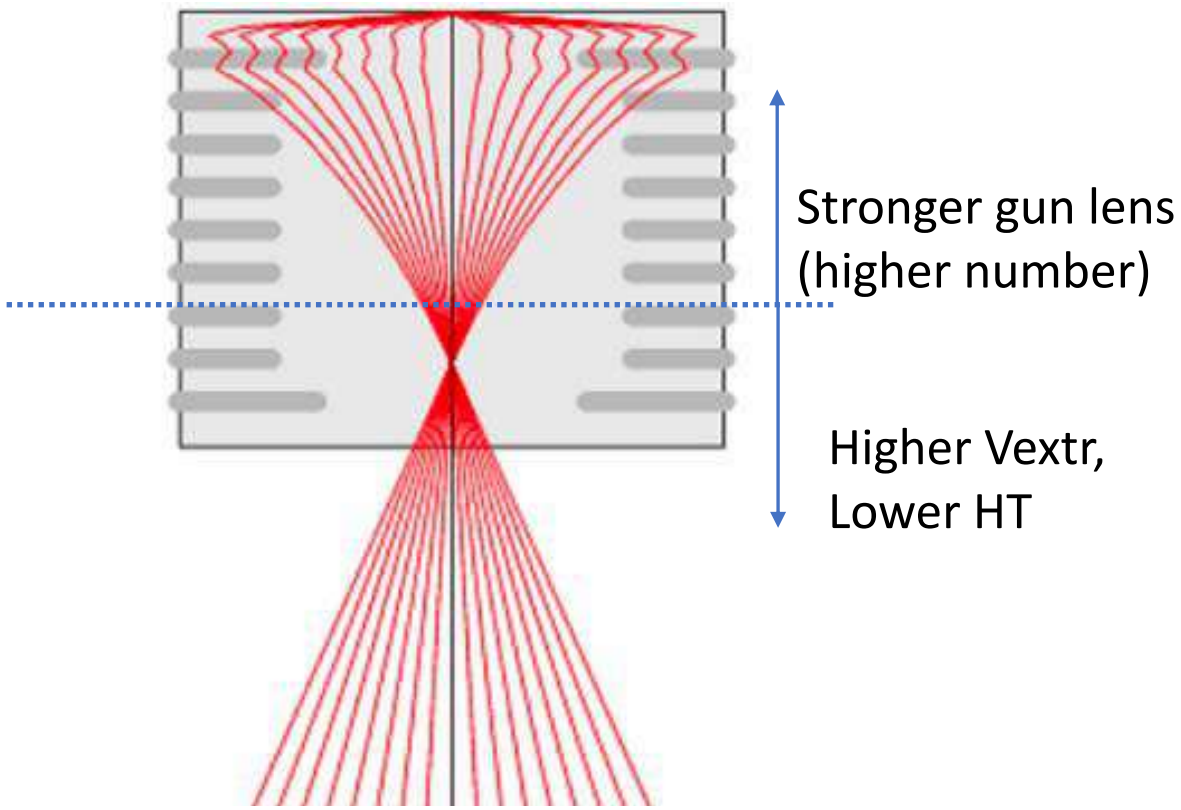
- Change the HT?
- Change the extractor?
- Change the gun lens?

- Do not work with the cross-over (XO) outside of the gun lens: increase in Vextr and decrease in HT need to be compensated by a stronger GL

- If you do not do STEM: DON’T TOUCH THE GUN SETTINGS

depending on the HT you work at, GL not to the max.

Gun settings



Do you know what happens when you:

- Change the HT?
- Change the extractor?
- Change the gun lens?

If your answer was NO:

DON’T TOUCH THE GUN SETTINGS

Gun alignment

Not that unstable that you need to do this daily!

Try to avoid spot 1

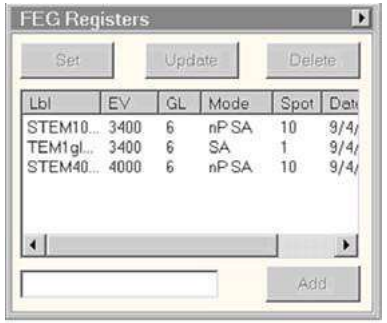
Align only when needed: whole gun alignment

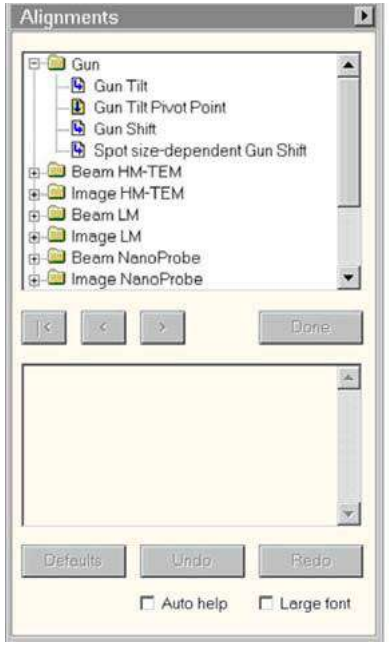
The Gun XO is the most important step,

therefore only do a complete gun alignment

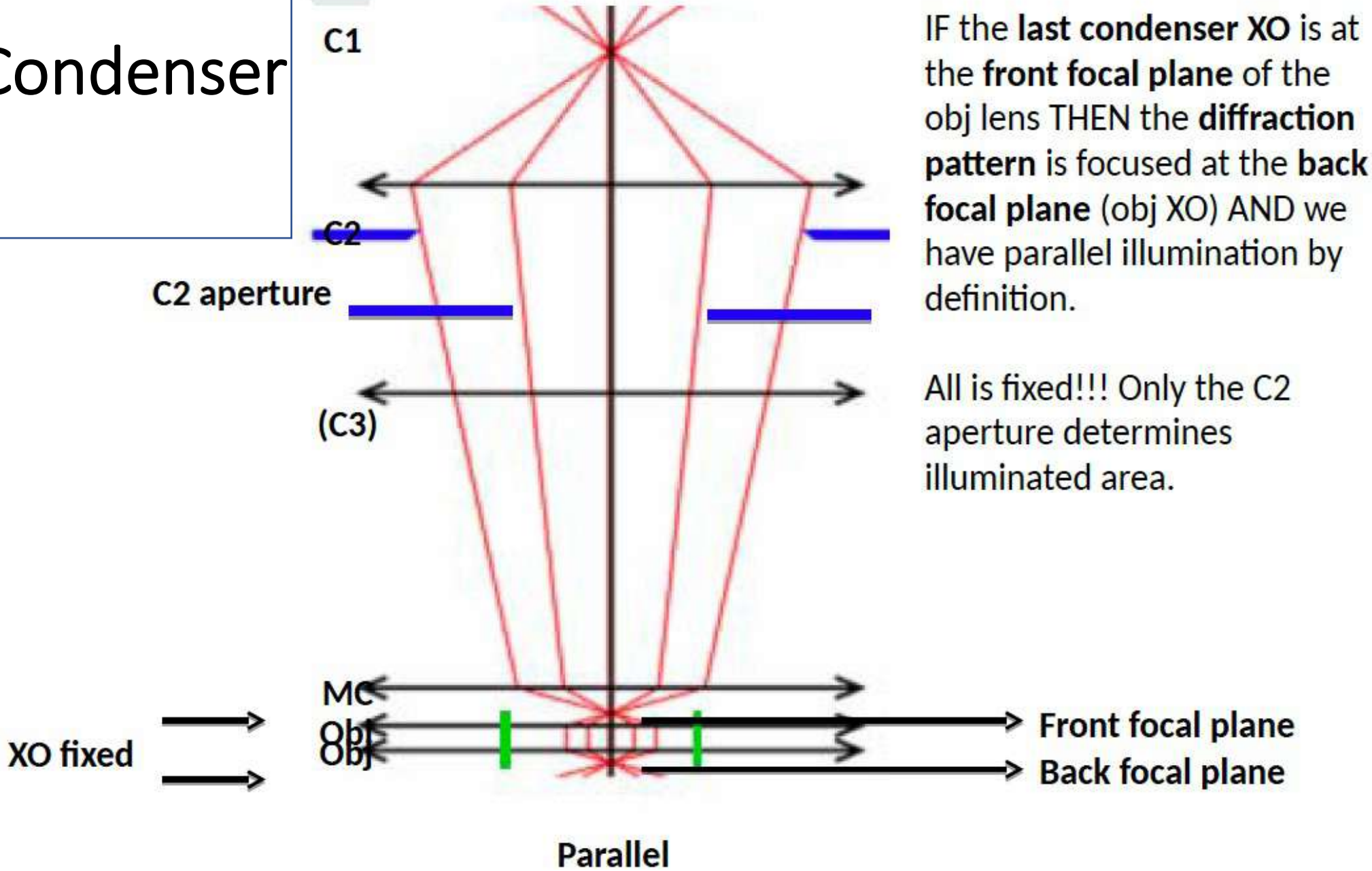
Avoid direct gun alignments

- Save a gun alignment in the FEG registers.
- That is what they are for! (delete the old one)





Condenser



IF the **last condenser XO** is at the **front focal plane** of the obj lens THEN the **diffraction pattern** is focused at the **back focal plane** (obj XO) AND we have parallel illumination by definition.

All is fixed!!! Only the C2 aperture determines illuminated area.

Condenser system

IF the last condenser XO is at the front focal plane of the obj lens THEN the diffraction pattern is focused at the back focal plane (obj XO) AND we have parallel illumination by definition.

- How to find parallel illumination?

Where is the back focal plane?

How to put your eye site at the back focal plane?

How do you know you are focused at the back focal plane (BPF)?

What is also in the back focal plane?

How to focus the back focal plane?

How to find the front focal plane?

When am I parallel?

Condenser system

- How to find parallel illumination

Where is the back focal plane? Where the diffraction pattern lies.

How to put your eye site at the back focal plane (BPF)? Switch to diffraction

How do you know you are focused at the BPF? Find something fixed in the BFP

What is also in the back focal plane? The objective aperture

How to focus the back focal plane? Sharpen the obj aperture with focus

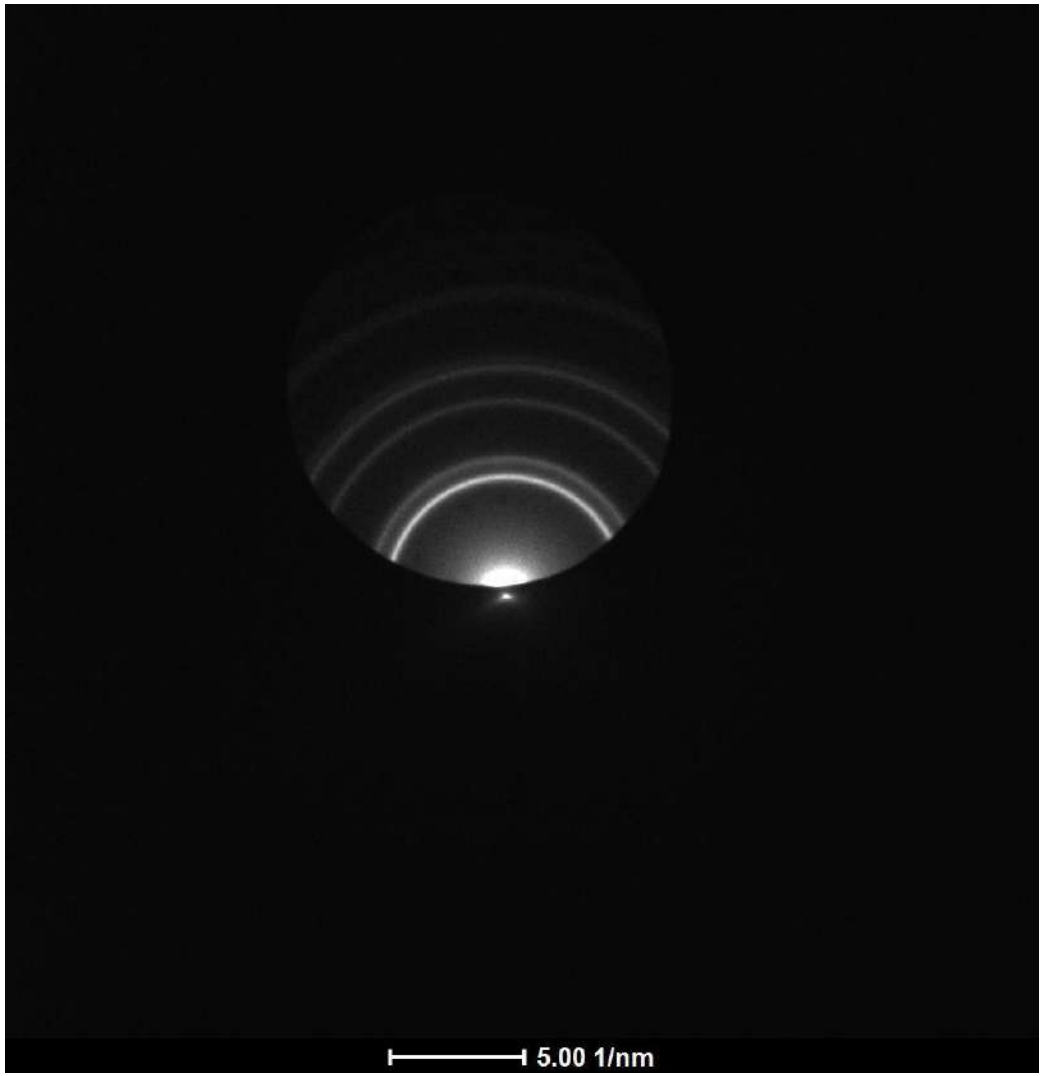
How to find the front focal plane? Change intensity till diffraction pattern is focused

When am I parallel? When simultaneous the diffraction pattern and the obj aperture are focused

Parallel illumination

- With FOCUS sharp objective aperture with INTENSITY
- sharp diff pattern

-DO NOT TURN THE TWO BUTTONS AT THE SAME TIME
-Sharp diffraction pattern with blurry obj aperture is NOT parallel!



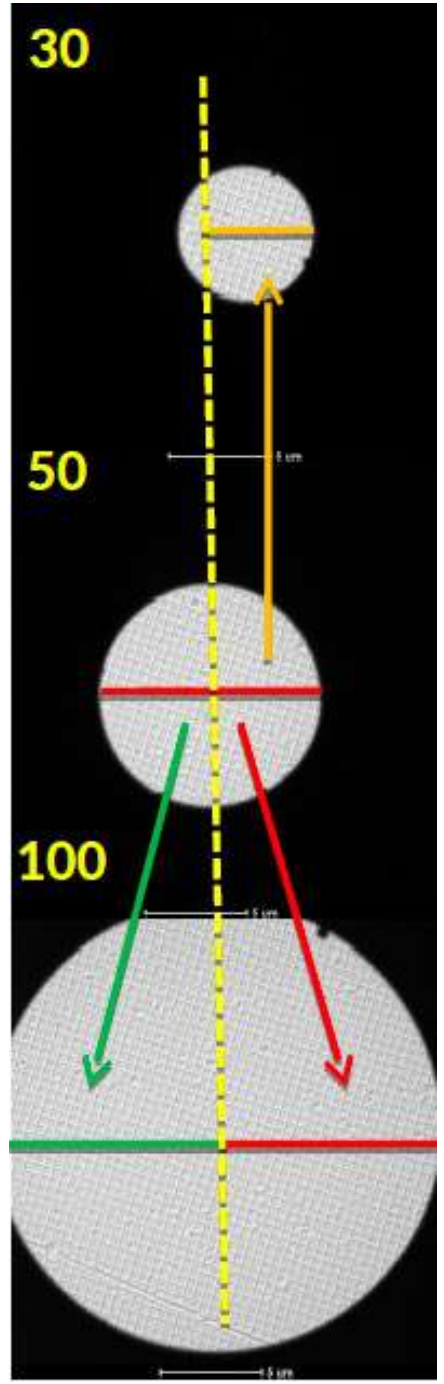
Rules of parallel illumination

Parallel settings should not change when:

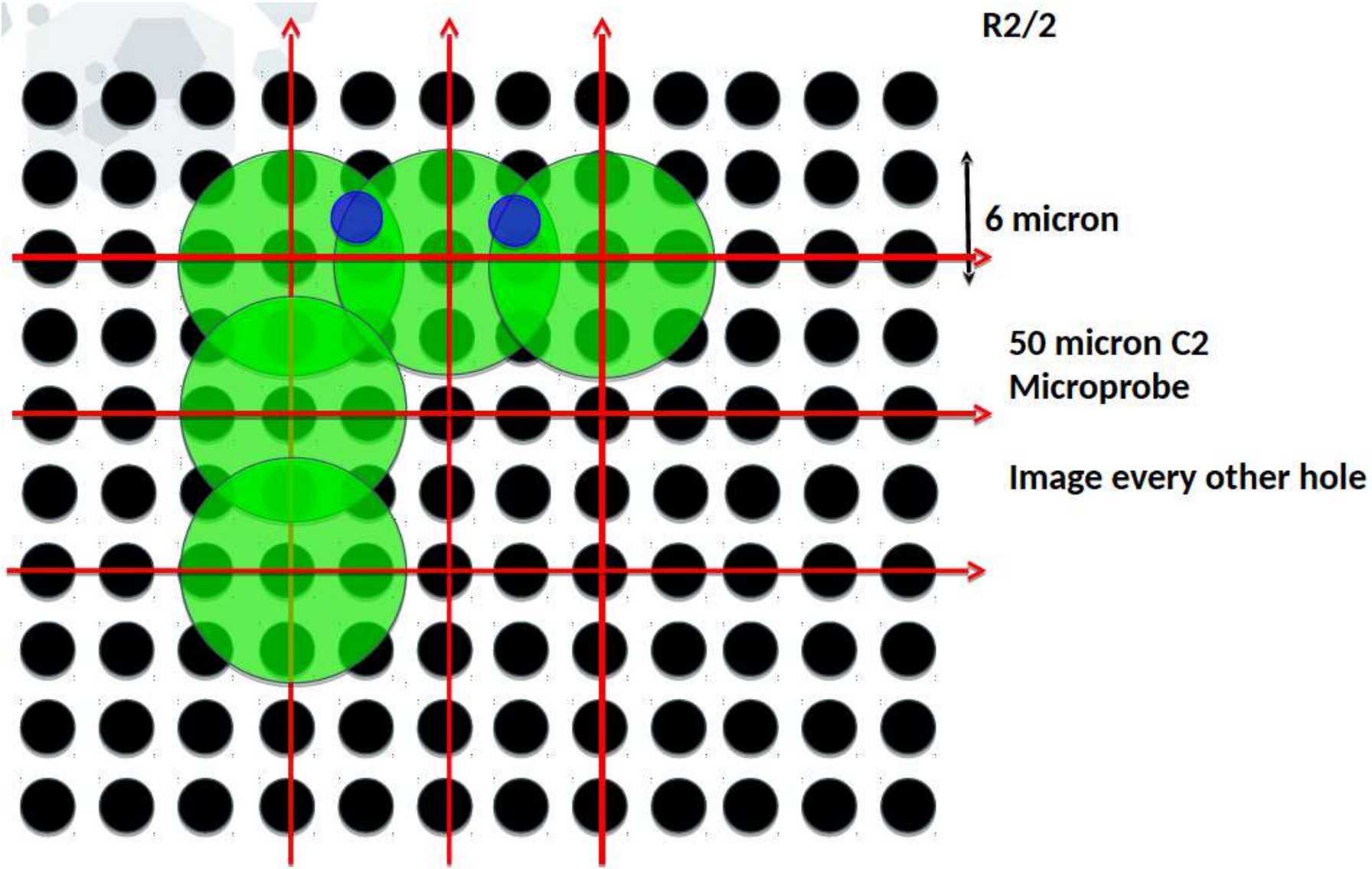
- changing spot size only
- changing magnification only
- changing C2 aperture

While being parallel beam size can only change when:

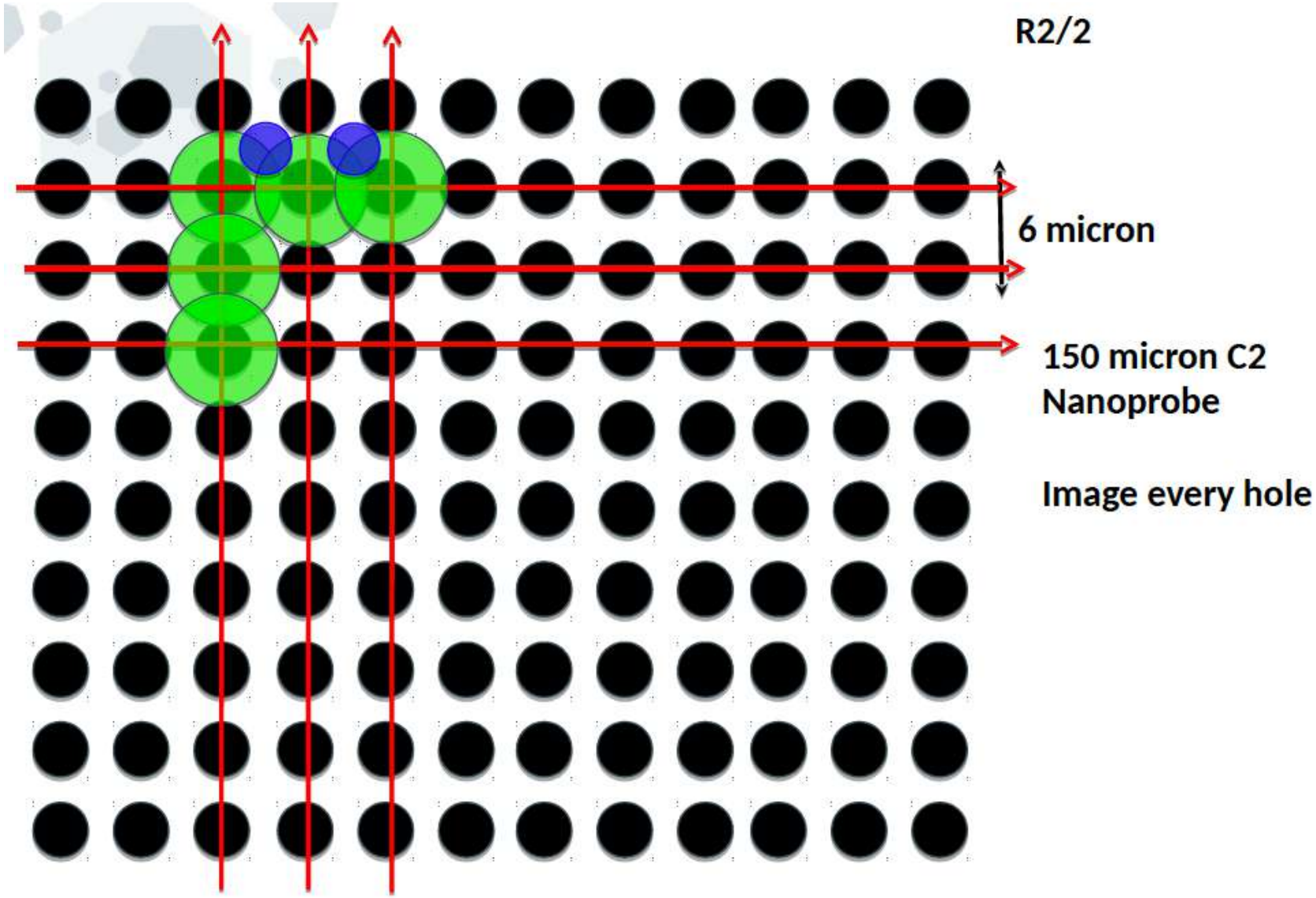
- changing C2 aperture
- and should be consistent (50->100 micron should be 2x larger beam)
- Beam sizes should be constant for each C2 aperture when:
- changing spot size
- changing magnification



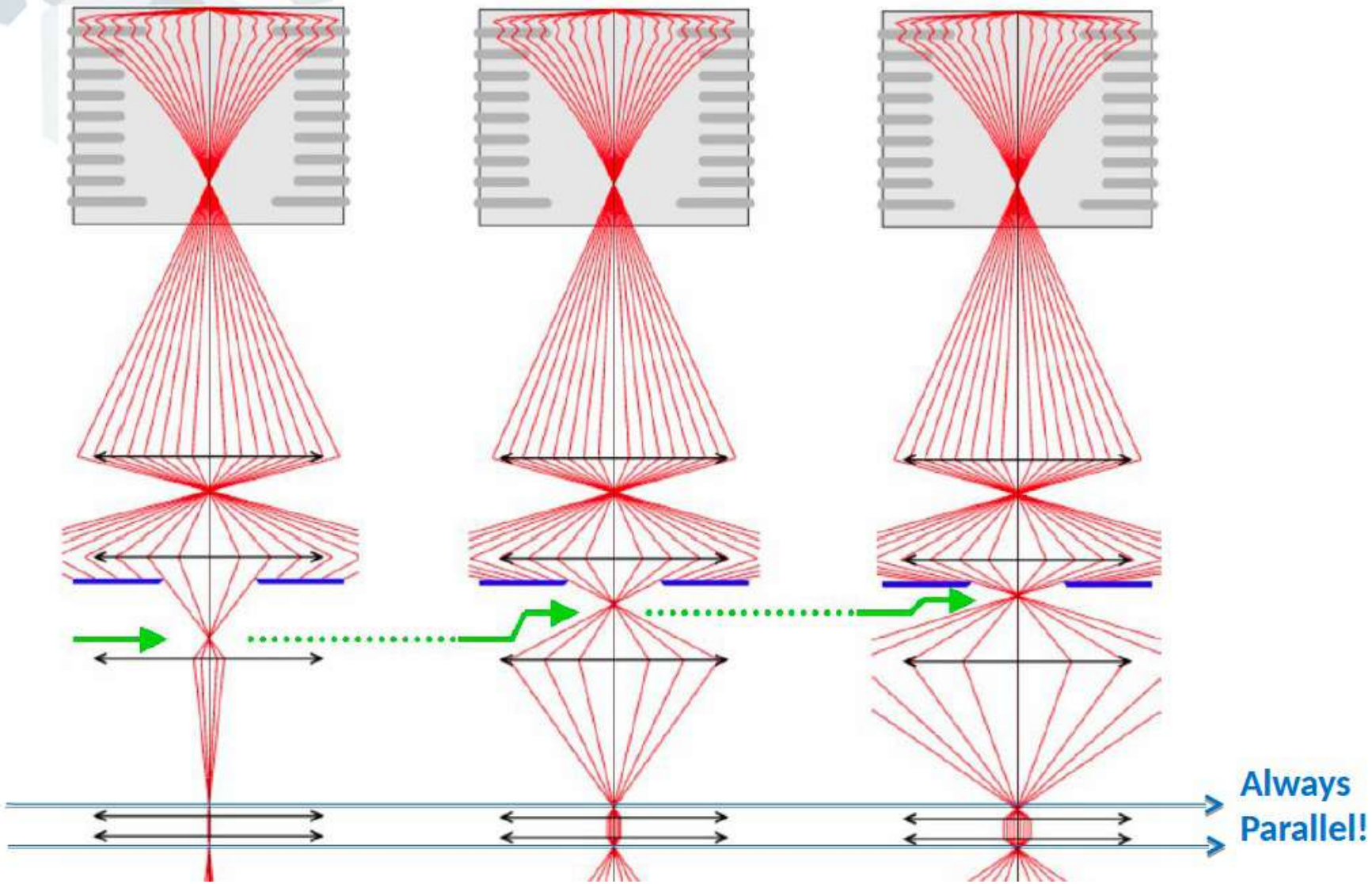
Micro-probe (Glacios)



Nano-probe (Glacios)



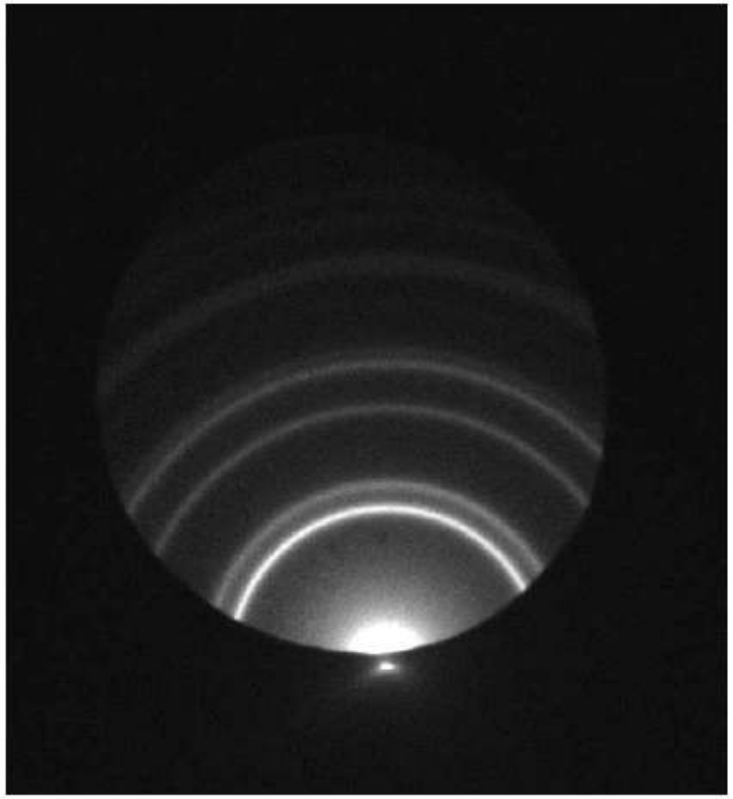
3 Condenser zoom system (Titan)



3 Condenser zoom system (Titan)

-Whenever in the parallel range on a titan,
    -pressing eucentric focus
    -inserting objective aperture
    -pressing diffraction

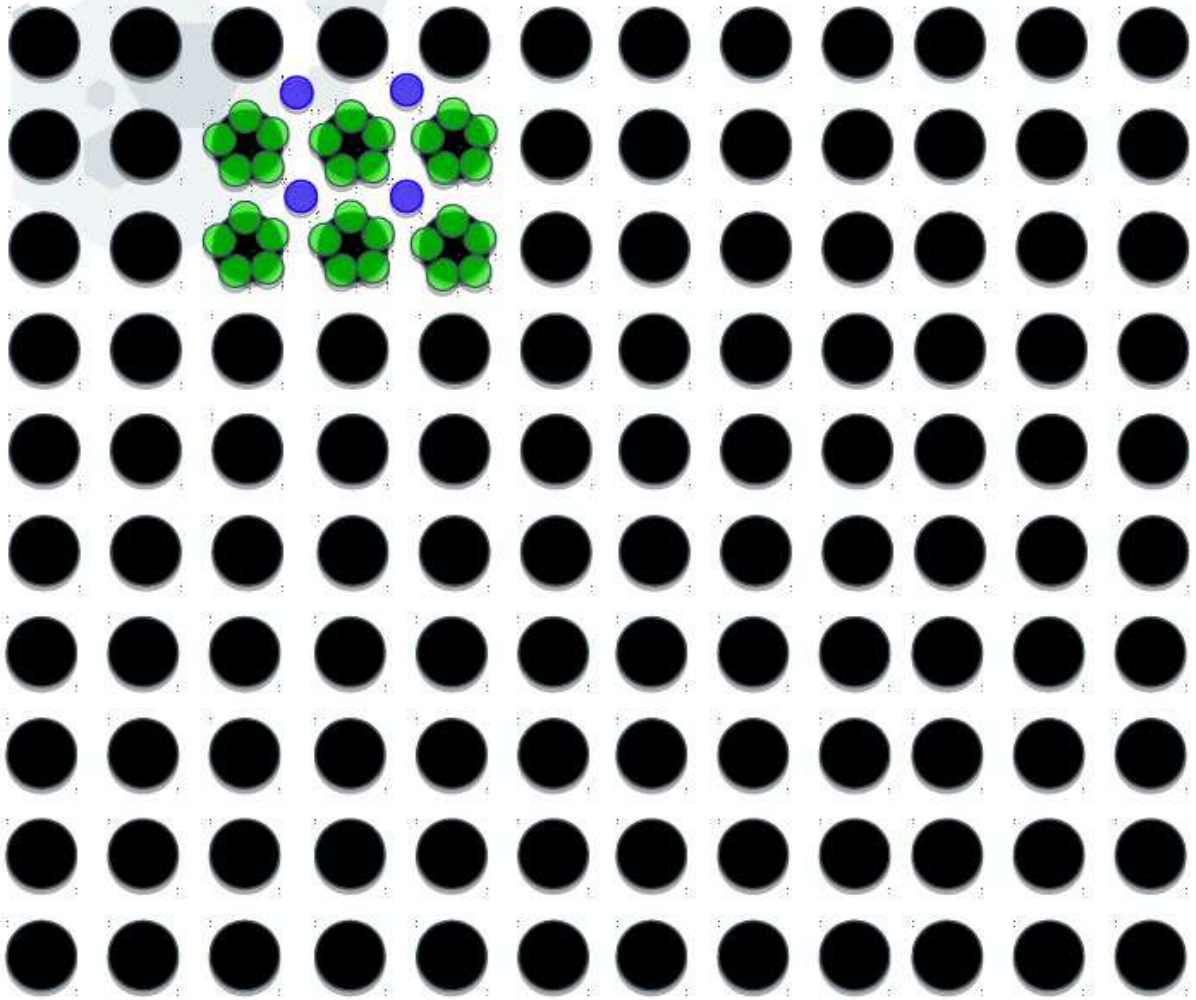
MUST result in the right image! (or very close to)



The image **MUST** remain focused in diffraction AND on the objective aperture when changing intensity and/or spotsize (apart from becoming more intense).

IF NOT: IT DID NOT LEAVE THE FACTORY LIKE THAT!

Nanoprobe (Krios)

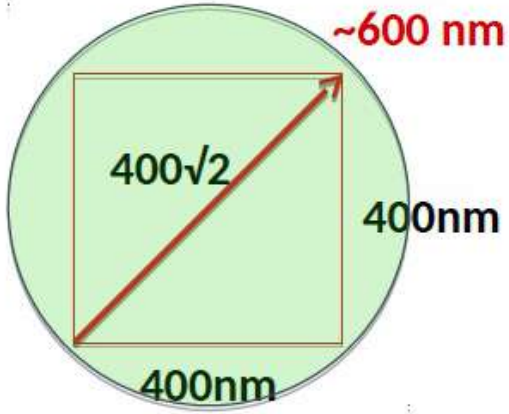


R2/2

6 micron

Any beam size:

4K camera: 1Å pixel

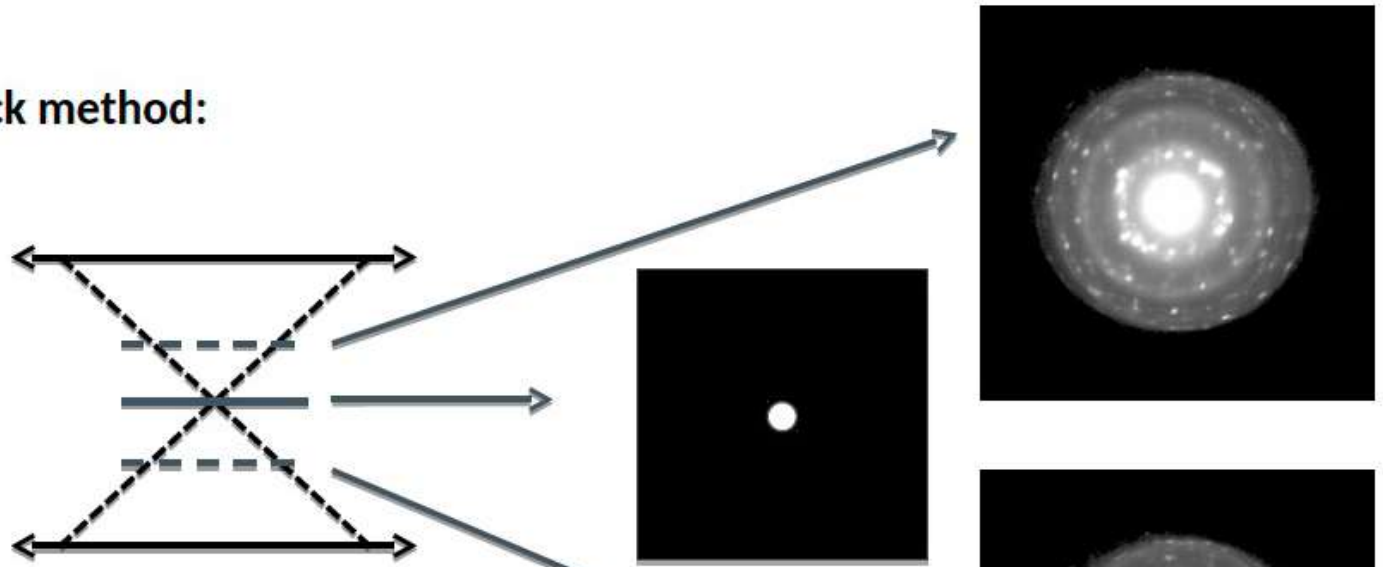


No compromise on dose rate, no wasted area, perfectly parallel, less strain on grid quality (one good grid square is enough)

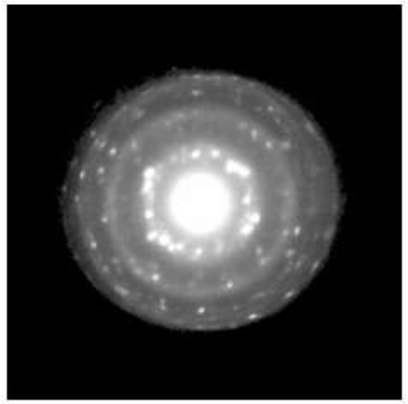
Tips and tricks: eucentric

-Press Eucentric focus
-Always set the sample at Eucentric height (ALPHA-wobbler or quick method)

Quick method:



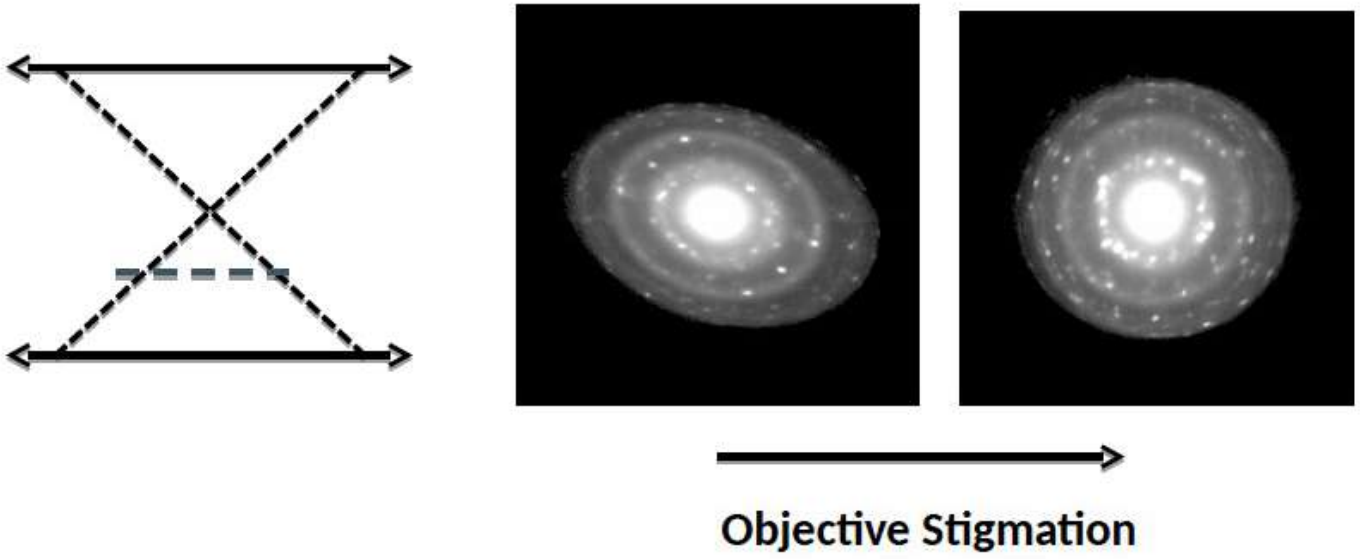
-Moving Z reveals a caustic ring
-Turning Focus has the same effect only now changing XO up and down



Tips and tricks: stigmation

-When at Eucentric height and Eucentric focus lower Z to reveal the caustic
-Adjust the caustic ring to be round with objective stigmator
-Stigmator values should not be larger than 0.1. at intermediate mag 30-60kx it should not be much off from zero!

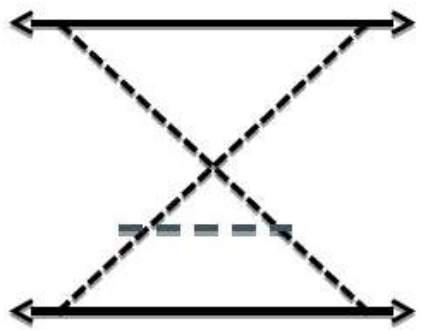
Quick method:

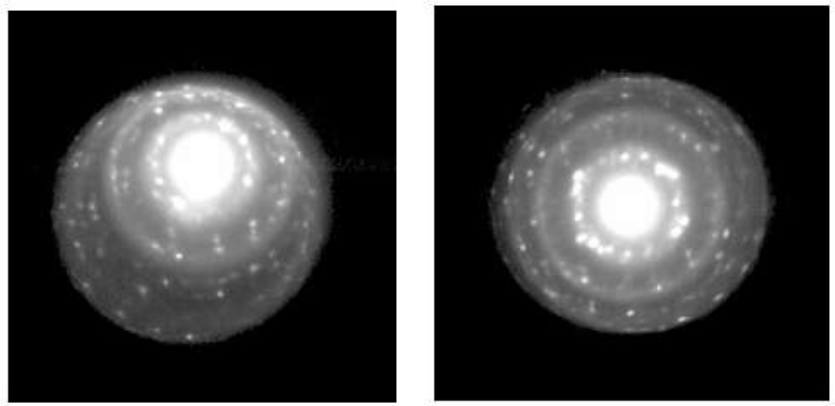


Tips and tricks

-When at Eucentric height and Eucentric focus lower Z to reveal the caustic
-Activate Rotation center and stop the wobbler using the course focus button
-Center the central spot in the middle of the caustic using the MF buttons (rot center)
-Coma free alignment and Rotation center both use the same button! You can not optimize both!

Quick method:

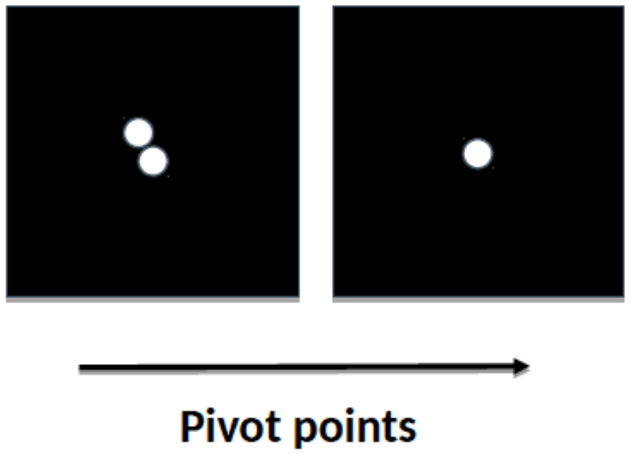




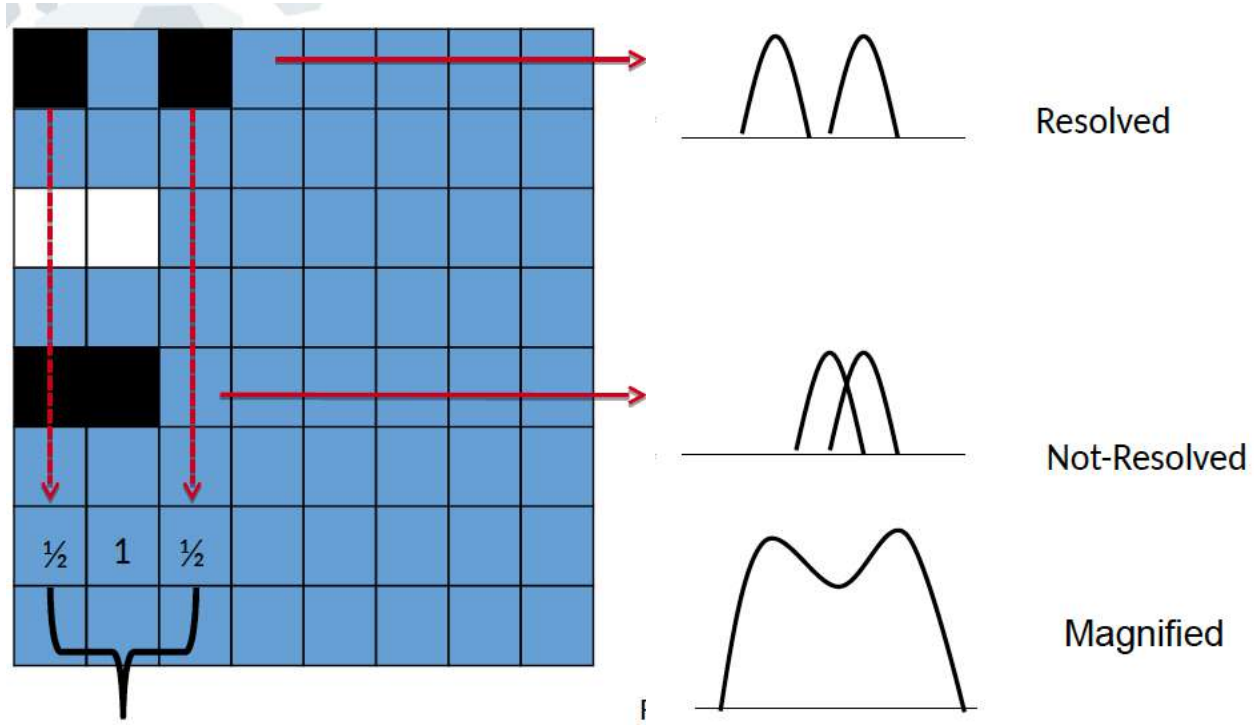
Rotation center/Coma

Tips and tricks

-Bring the sample back to Eucentric height with Z-height (caustic back to a spot)
-Check pivot points at Eucentric focus
-Be aware that the PP are focus dependent!
-As for SPA you change the focus the PP will change! (Unavoidable), therefore never correct PP at a specific focus other than Eucentric focus
-Since PP are focus dependent, the beam will move while imaging at different defocus settings! This is not beam instability, it's a fact of life.
-Your parallel beam size should therefore be chosen a bit larger than the size of the diagonal of the camera surface!



Resolution



2 pixels = smallest distance to be resolved = Nyquist

Full Nyquist = 1 (normalized) = highest resolution at given magnification

Example: 75000 mag, pix size = 1A :

Ny = 2A , ½ Ny = 4A , 1/4 Ny = 8 A

How to setup your scope -recap

This should not take more than 15 minutes:

- Determine the desired resolution,

sensitivity of the camera: pixelsize

- Pixel size determines the magnification

(example 1A on Titan is 60kx), set mag!

- Insert cross-grating
- Press Eucentric Focus (~80% titan, 90% Tecnai)
- Set Eucentric Height using Alpha- wobbler for course

and then using a focused spot

- Lower Z Height to show caustic ring
- Center spot in the middle of caustic ring: rotation center
- Make caustic ring round: objective astigmatism
- Bring Z-height back to a spot
- Check Pivot Points

How to setup your scope

Check parallel illumination condition

- Switch to diffraction
- Insert objective aperture
- Focus objective aperture with focus
- Focus diffraction pattern with intensity
- ON A TITAN, simply set the beam size slightly larger than the camera diameter
- then press diffraction and insert objective aperture: BOTH should be focused by definition!

You should now be:

At Eucentric height

At Eucentric focus

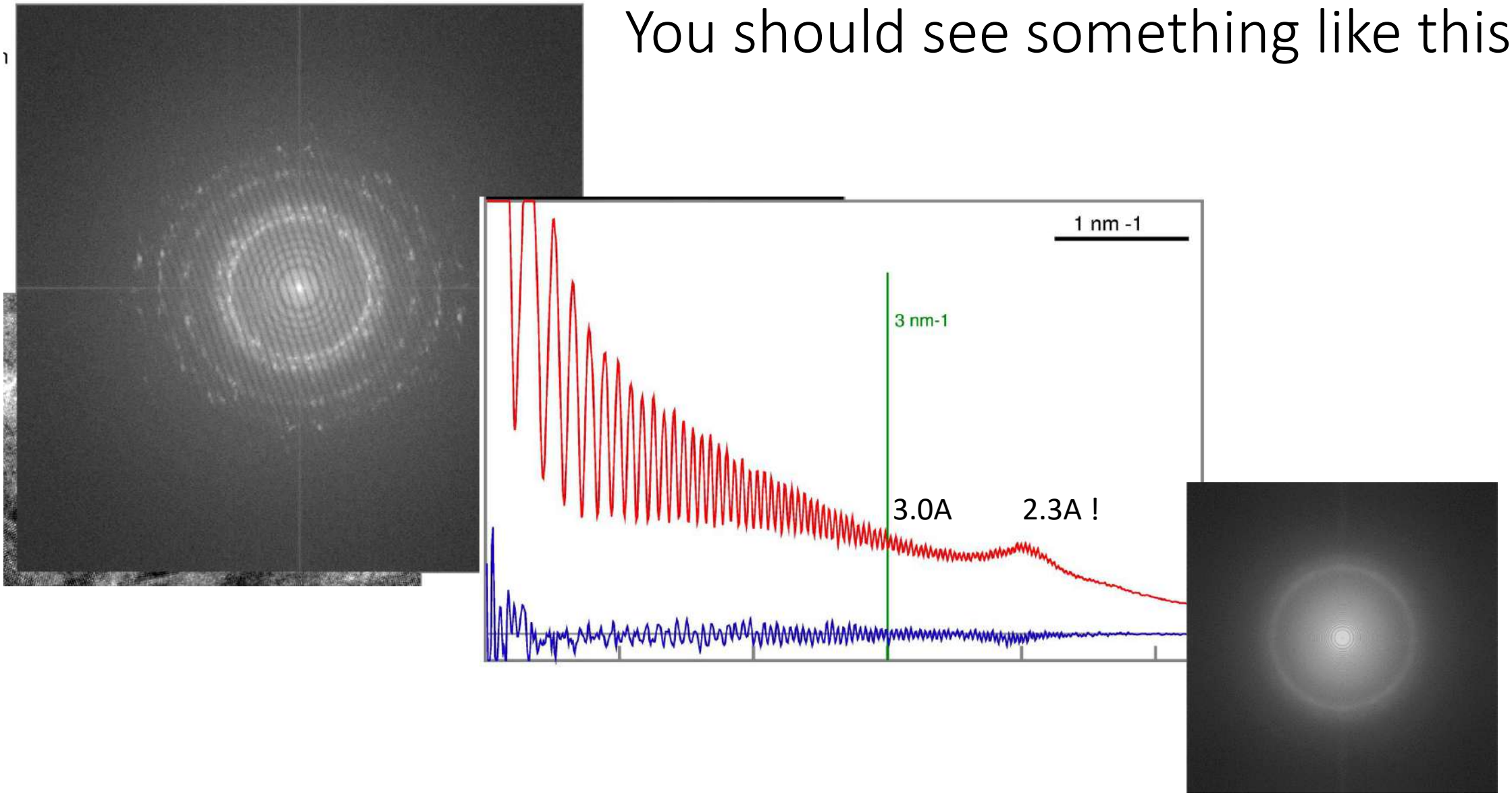
Parallel

Illuminating the detector fully

Roughly stigmated

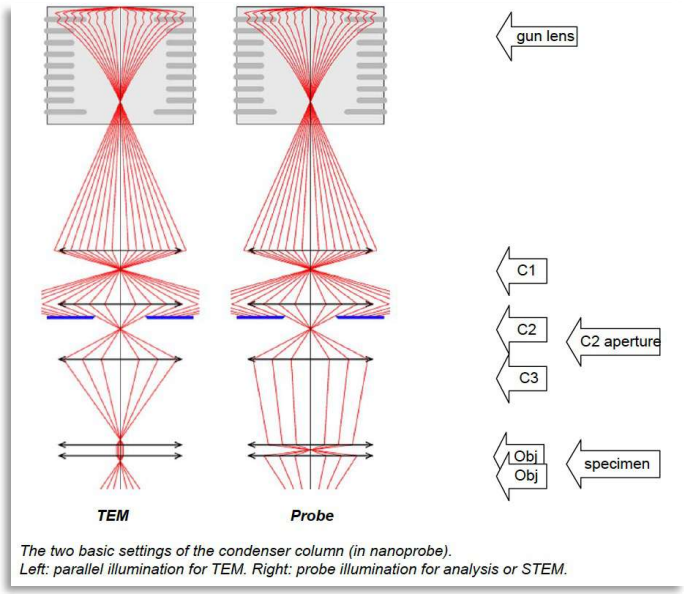
Roughly coma free

And then:
You should see something like this:

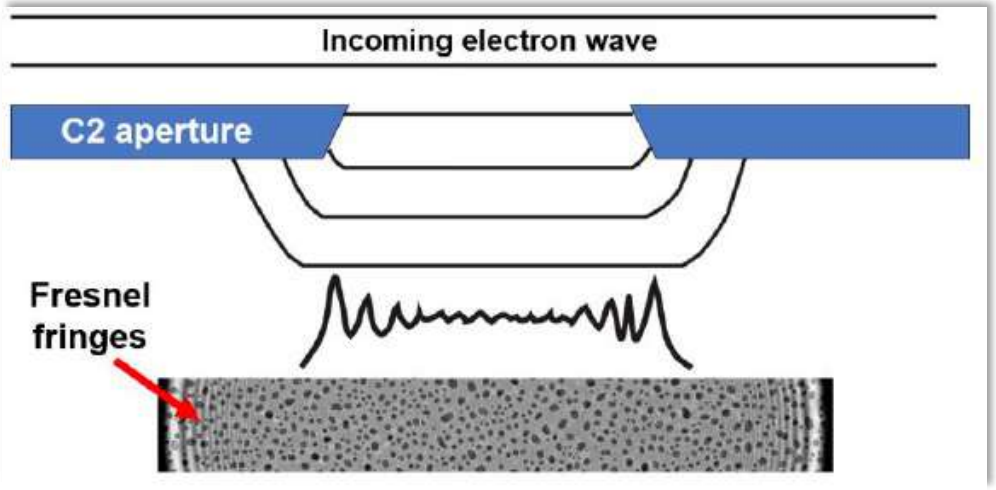


Fringe Free Imaging (FFI)

Electron diffraction

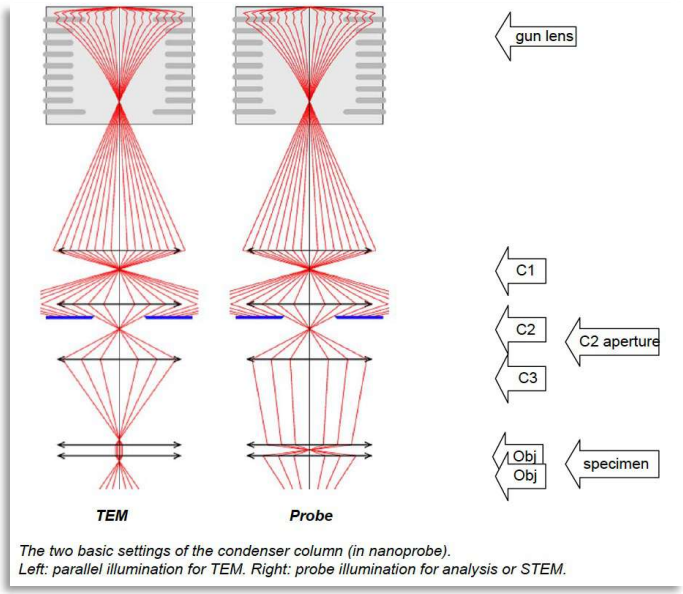


The two basic settings of the condenser column (in nanoprobe).
Left: parallel illumination for TEM. Right: probe illumination for analysis or STEM.



Fringe Free Imaging (FFI)

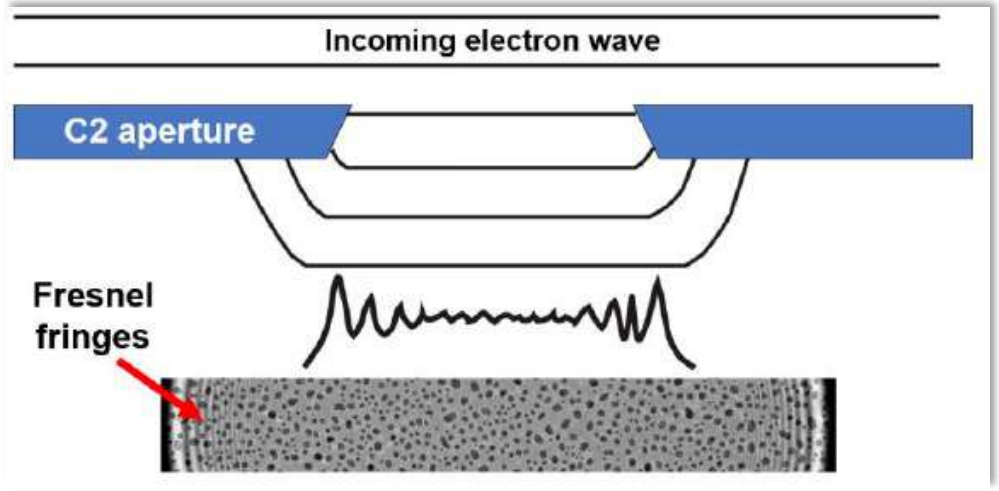
Electron diffraction



The two basic settings of the condenser column (in nanoprobe).
Left: parallel illumination for TEM. Right: probe illumination for analysis or STEM.

- Electron: wave - particle duality
- Electrons get diffracted through C2 aperture
- Upper pole piece of the objective lens (OBL) produces an image of the C2 aperture.
- This image does not always coincide with the sample plane
- Simultaneous imaging of the focused sample and the out-of-focus C2 aperture by lower pole piece OBL

When the C2 aperture is imaged out-of-focus, wave interference at the edge of the condenser beam appears as Fresnel fringes (see figure bottom left)

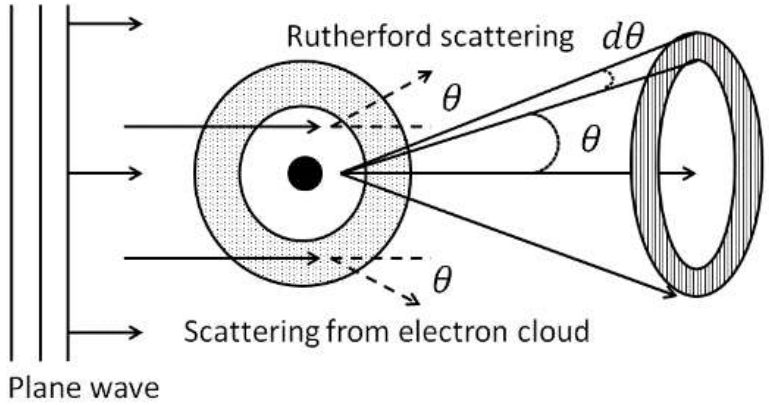


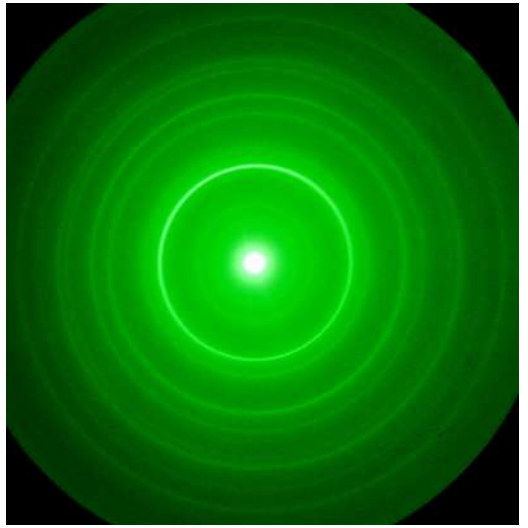
Electron diffraction

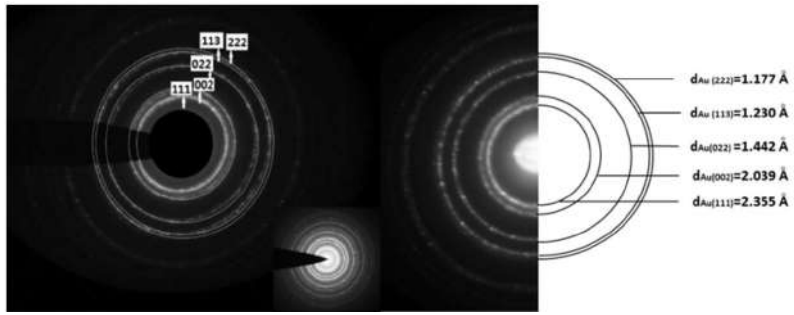
- Electron: wave - particle duality
- Rutherford scattering
- Crystal planes and interplanar spacing are shown by Miller indices. Bragg law:

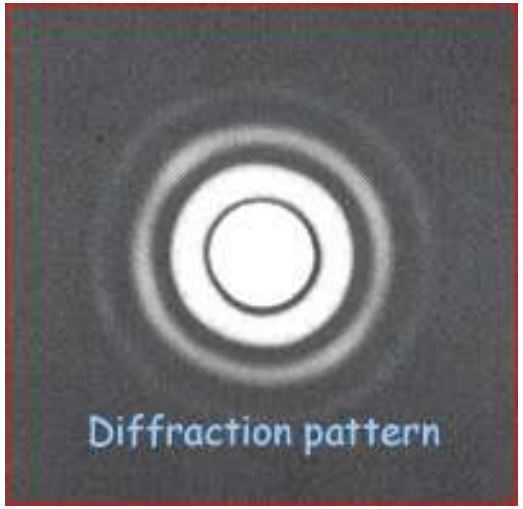
  $2 \times d(hkl) \times \sin(\theta) = n \times \lambda$

- Bottom: The ring diffraction pattern from a polycrystalline pure gold film with an f.c.c crystal structure.





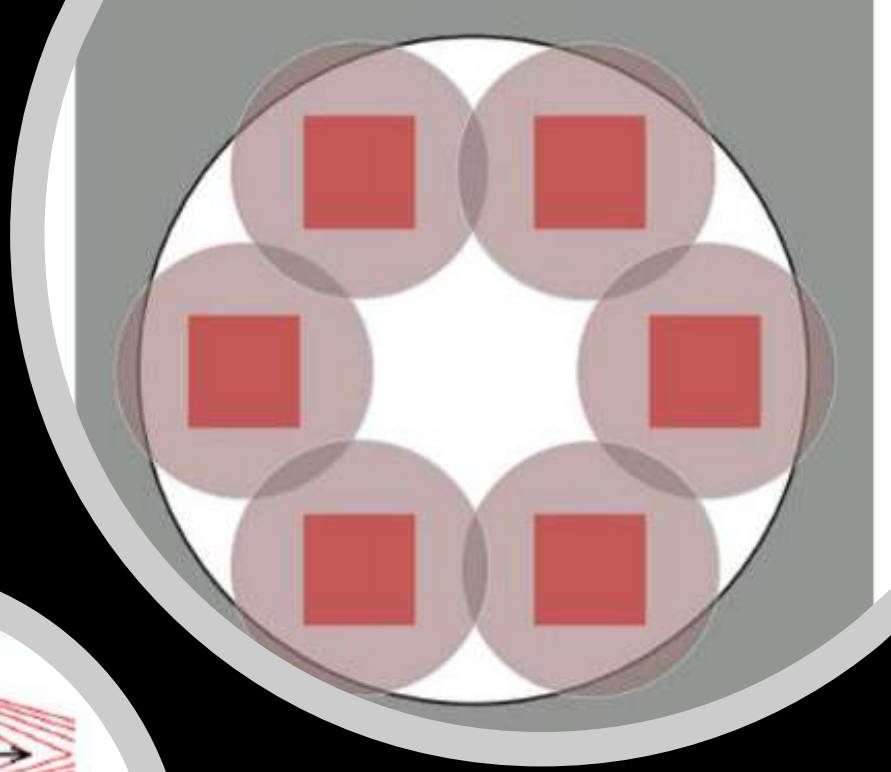


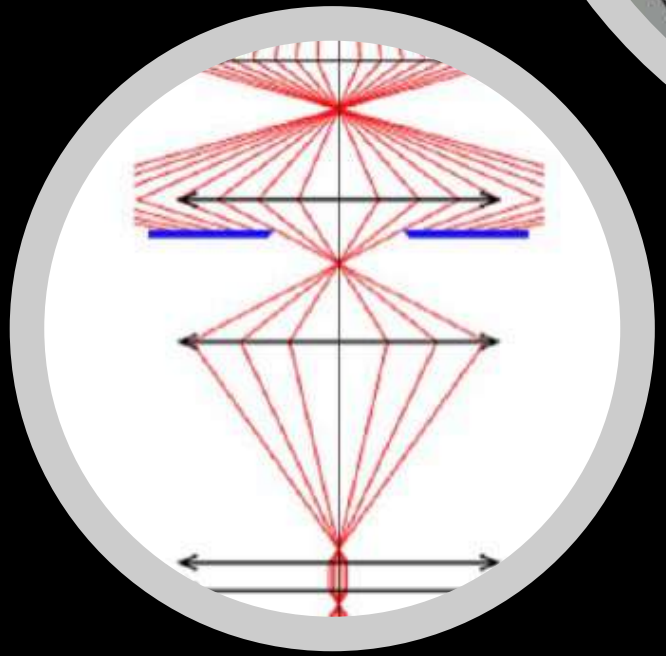


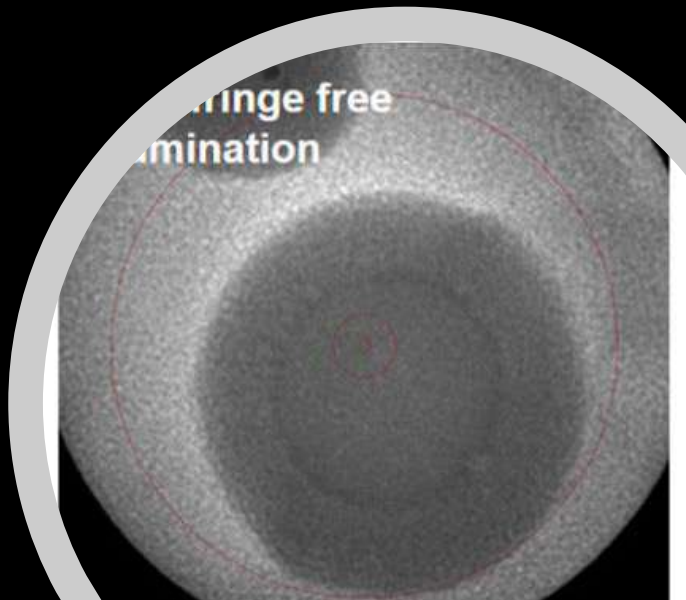
Wave interference => Fresnel fringes

- Fresnel fringes reduce the useful beam area and limit the number of images which can be recorded from a single hole (Fig. C).
- To minimize Fresnel fringes: adjust stage position to bring the specimen to the image plane of the C2-aperture
- The lower OBL pole piece strength is adjusted so that the sample is focused on the camera imaging plane.
- Now both the C2 aperture and the sample will be in focus and no, or very few, Fresnel fringes will be visible in the image recorded by the camera (see figure to the right).

Claim is it allows reduction of the beam size and more images can be acquired from a single hole.

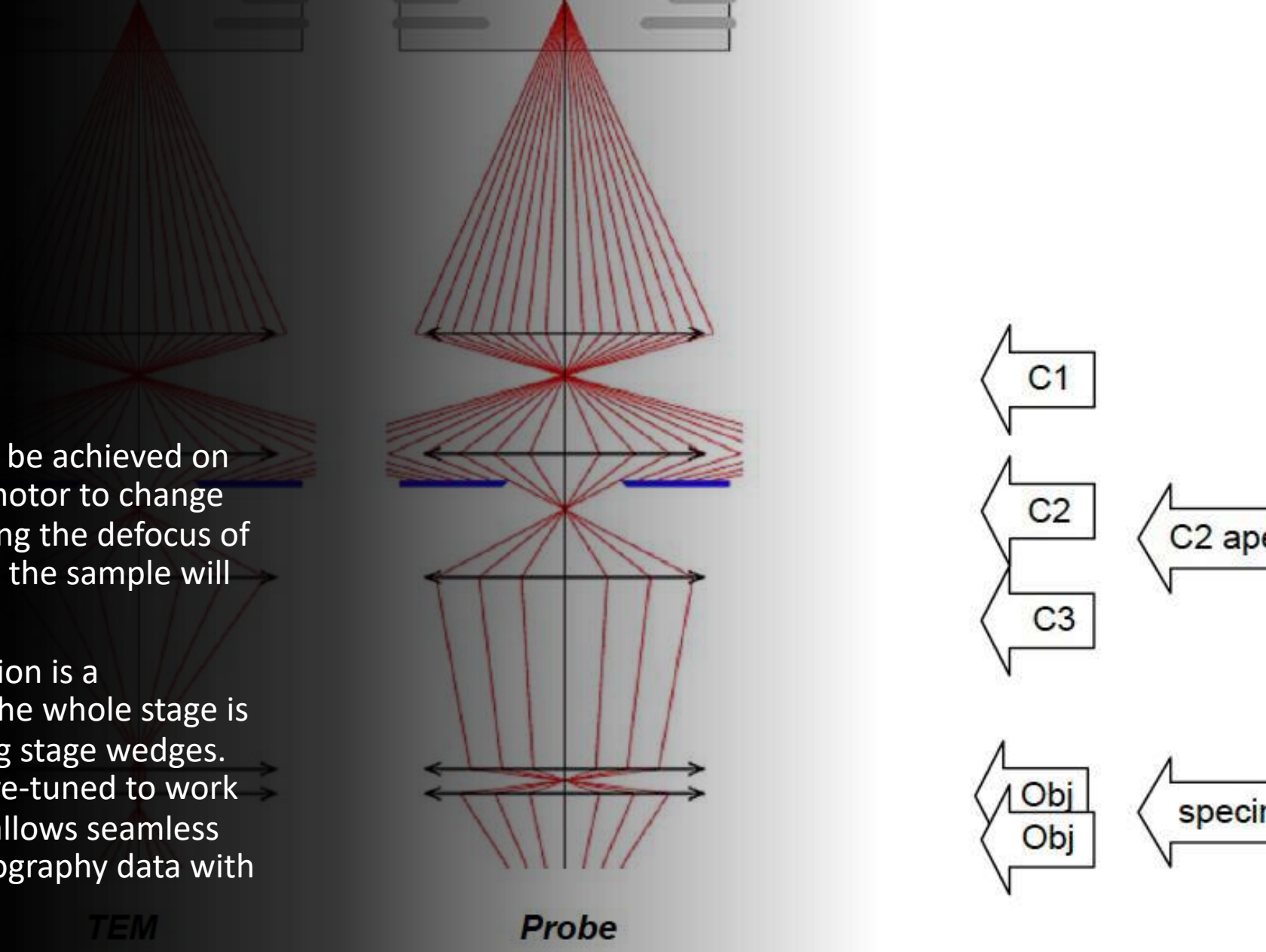






Compatibility

- In theory fringe free imaging (FFI) can be achieved on any microscope by driving the stage motor to change sample height to C2 focus and adjusting the defocus of the sample. However, this means that the sample will no longer be at eucentric height.
- Thermo Fisher Scientific FFI modification is a mechanical stage adjustment where the whole stage is lowered to the required Z-height using stage wedges. The tilt axis and eucentric height are re-tuned to work optimally at that stage location. This allows seamless acquisition of single particle and tomography data with FFI.



basic settings of the condenser column (in nanoprobe).
parallel illumination for TEM. Right: probe illumination for analysis or STEM.

TEM system compatibility

- FFI is provided as a standard feature on every Krios G4.
- Earlier Krios models (G1, -2, -3(i)) need to undergo modifications in order to operate in FFI mode.
- On Generation 1 Krios, FFI is only compatible with the plan 3 Autoloader systems.
- The FFI modifications are not provided on Glacios and Arctica TEM systems.
- FFI can only be used in nanoprobe (nP) mode.

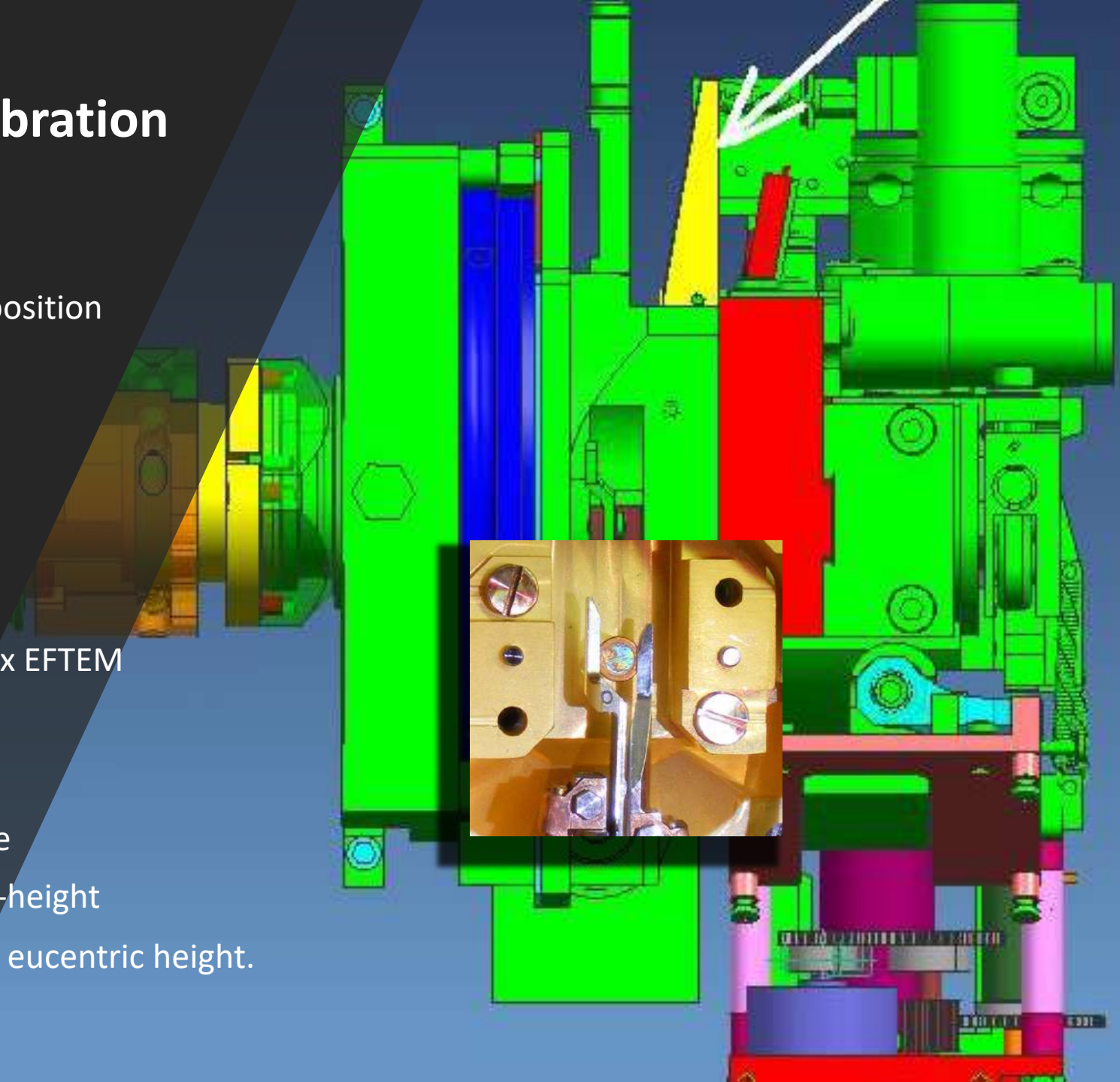
FFI installation and calibration

field upgrade

- physical adjustment of the stage position
  (~60 µm displacement)
- Re-alignment of the Autoloader.

Procedure:

- nP parallel illumination,
- 96kx magnification in TEM or 165kx EFTEM
- beam diameter set to ~600nm
- Objective lens excitation increased
  until Fresnel fringes are not visible
- Image focused by lowering stage Z-height
- Stage wedges adjusted to redefine eucentric height.



Fringe Free illumination

differences when compared to

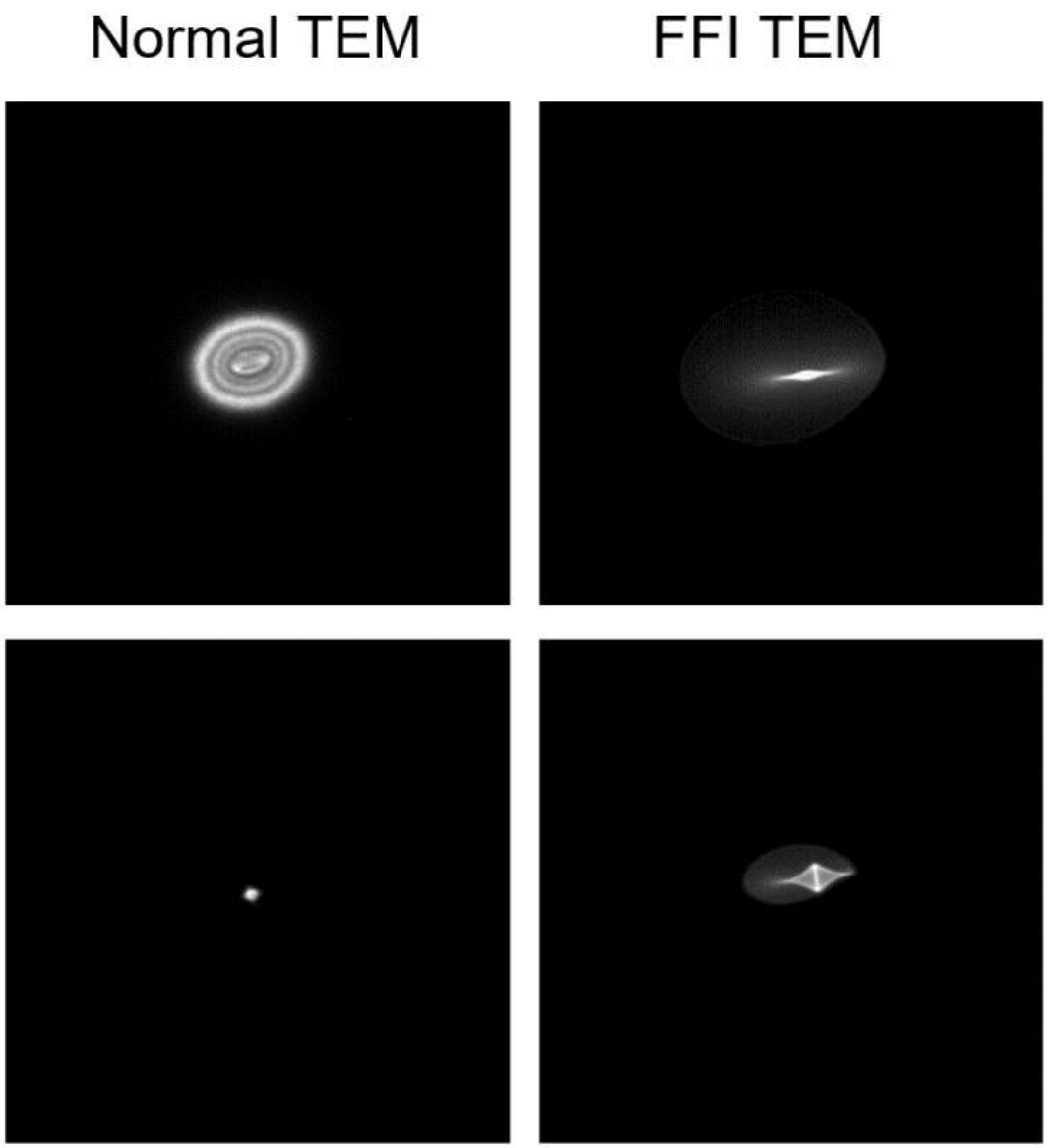
normal TEM illumination

When the stage height is re-adjusted to meet the FFI requirements, the electron beam has a different appearance when condensed to a spot.

It is not as small and round anymore.

However, the appearance of the beam does not influence the performance of the system in TEM mode.

Condenser astigmatism is now best optimized by adjusting the stigmators to create a round beam in parallel mode, instead of optimizing the spot shape by condensing the beam.



Fringe Free illumination

differences cont'ed

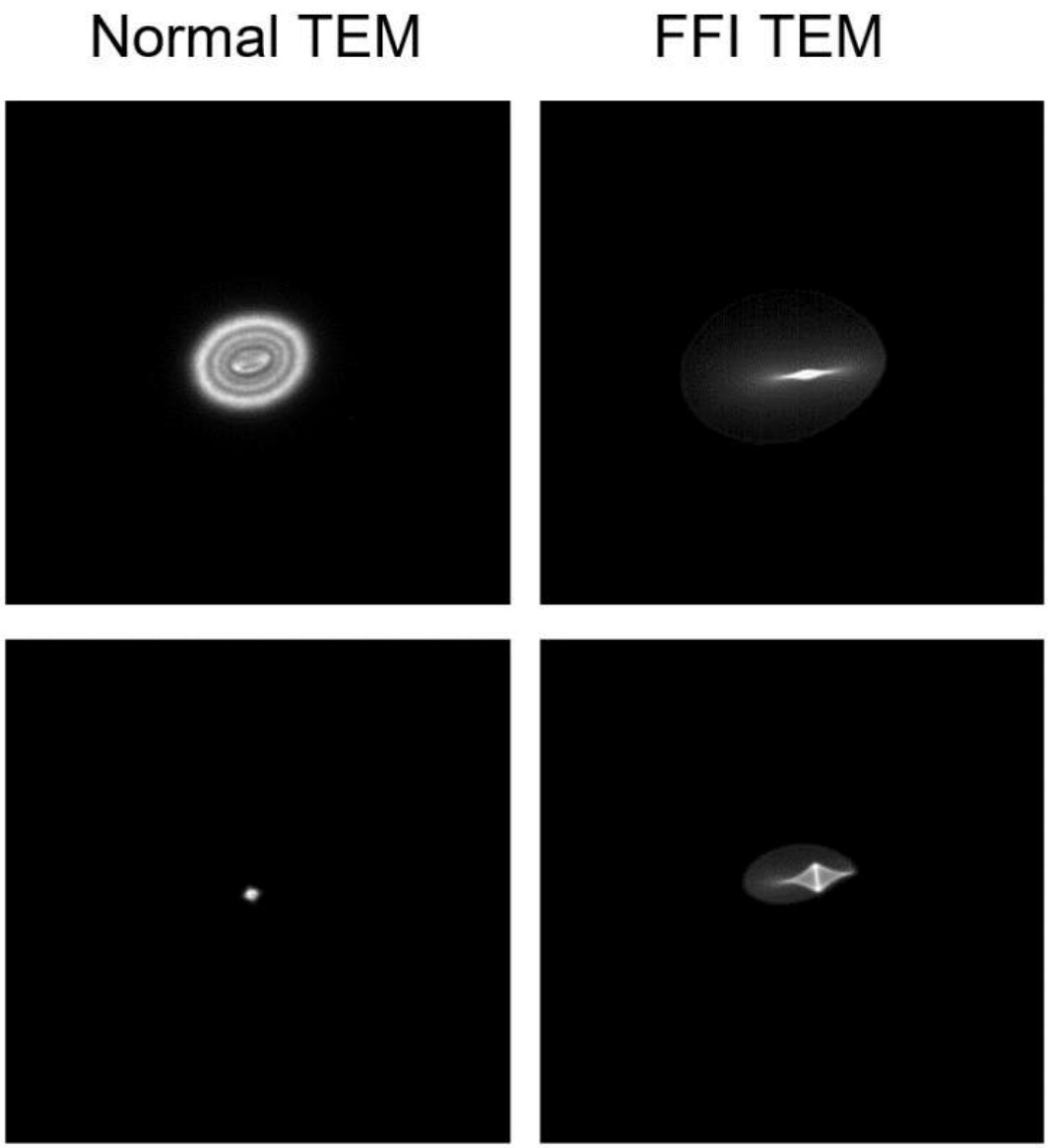
Magnification range and defocus

FFI alignments are done at medium-high SA magnifications (96.000x) corresponding to a beam diameter of around ~600 nm.

When the beam diameter is varied by adjusting the condenser lenses, this also affect the focus of the C2 aperture and hence the quality of FFI with just several more fringes appearing in the image.

Beam diameter changes are needed when working at different magnifications

This will not limit the use of FFI for magnifications that are typically utilized for SPA experiments with a narrow beam diameter. A similar effect will occur when applying an additional defocus with the objective lens. Such a defocus will change the focus of the C2 aperture and introduce some fringes around the edge of the beam



Fringe Free illumination

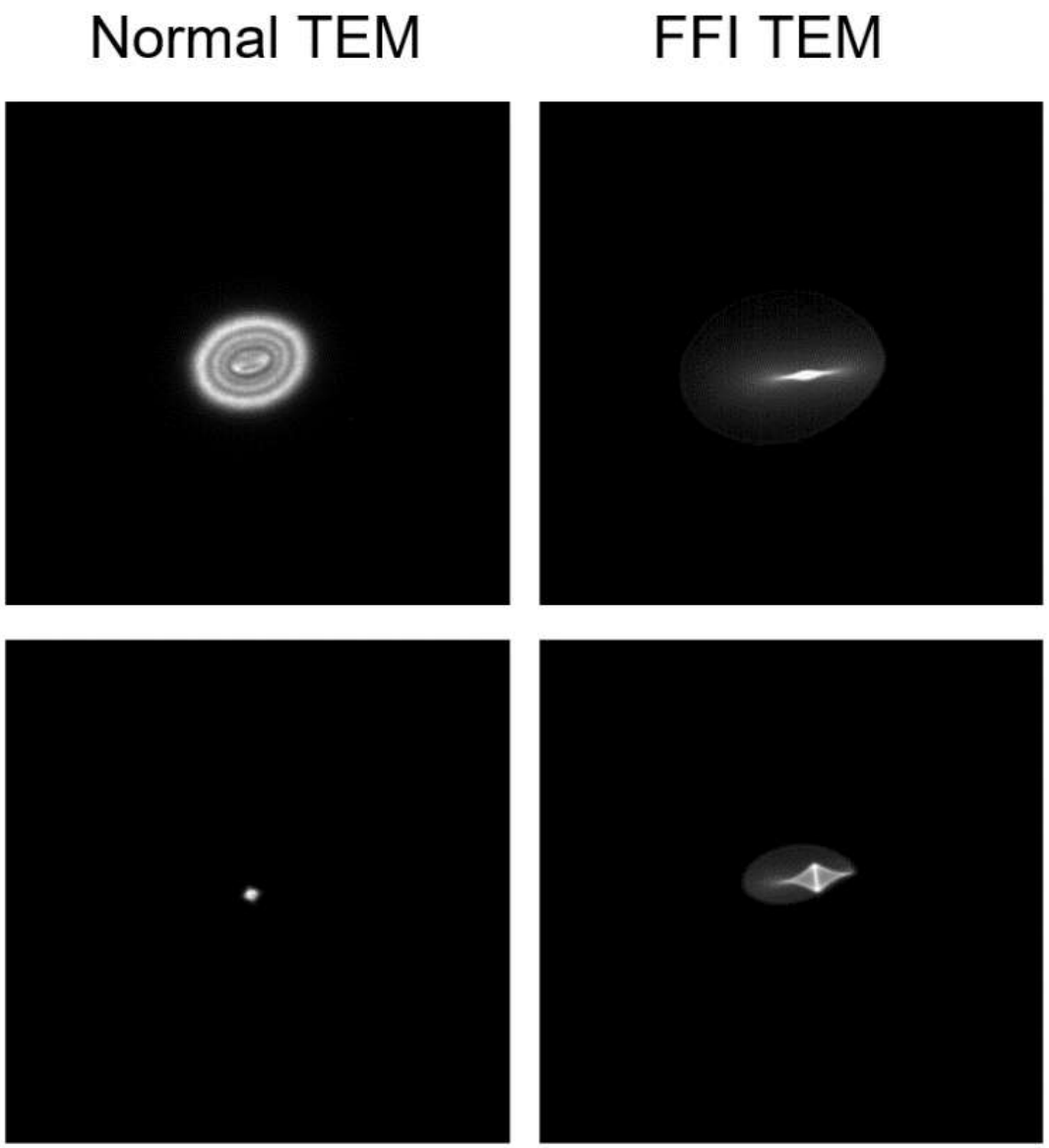
differences cont’ed

Direct alignments:

On systems with FFI configuration, the recommended way to adjust beam tilt pivot points for TEM experiments is to work with a parallel beam and change the magnification to allow displaying the full beam on the flu cam viewer. Pivot points are then adjusted by minimizing the beam movement during the alignment.

Note:

On systems with an anti-contaminator aperture (e.g., Krios G3i), the beam can be cut-off at low magnifications below 5kx.



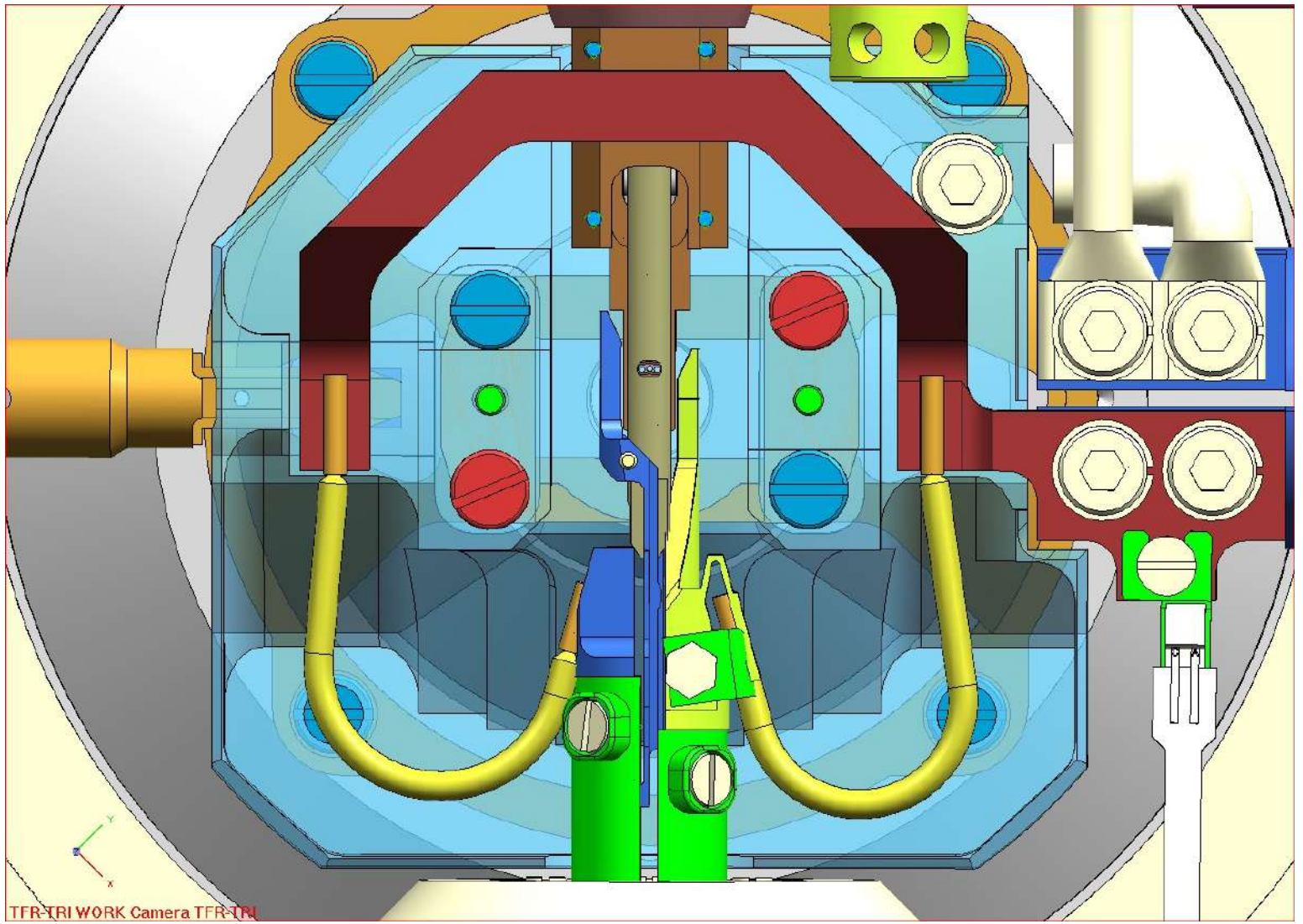
Backup slides

Cryo box design

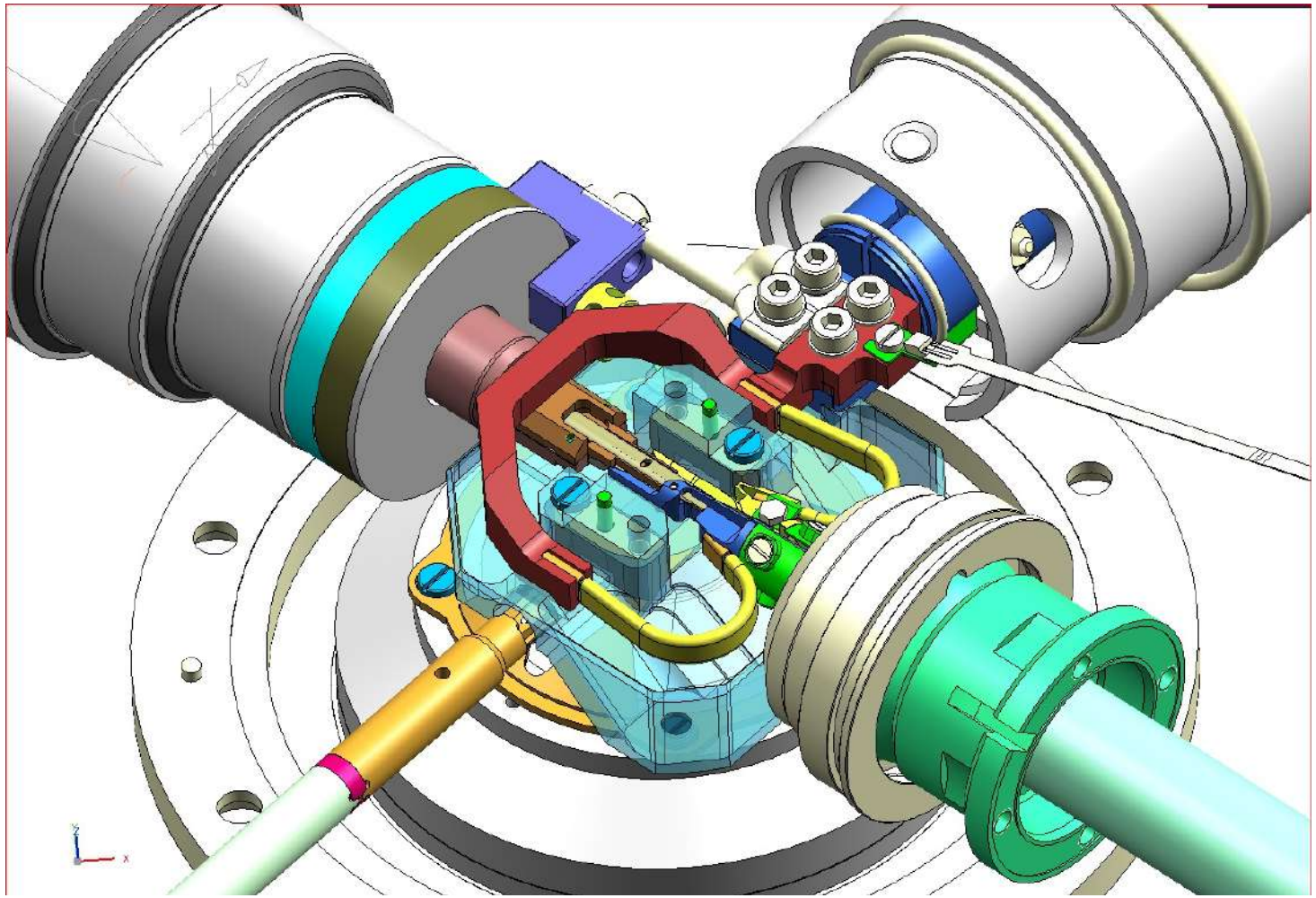
New holder

temp sensor

obj. aperture



Cryo box design

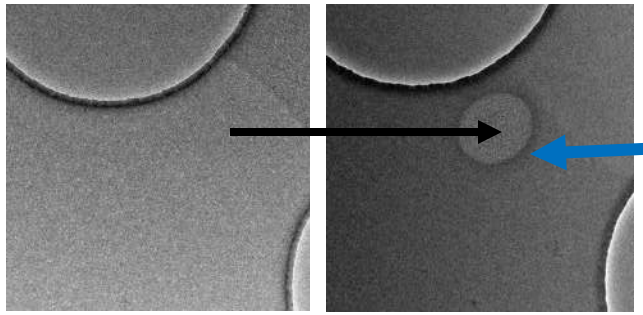


Cryo Box and single axis holder

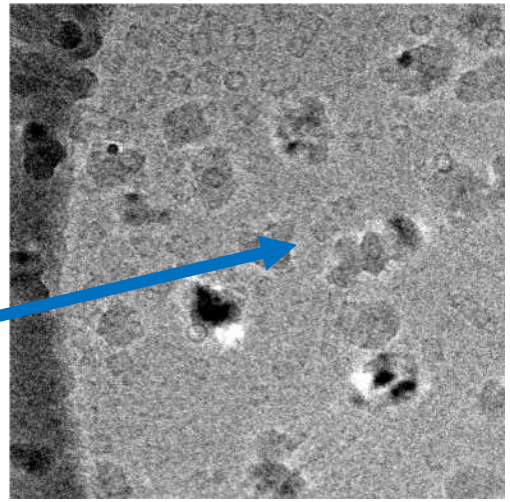
- **Low sample temperature**
- No negative influence on optics performance
- Improved interfacing/tolerances
- Improved mechanical robustness
- Improved tomography spec
- Serviceable
- Lower price

Sample Quality – Types of contamination

Column



Column / Autoloader



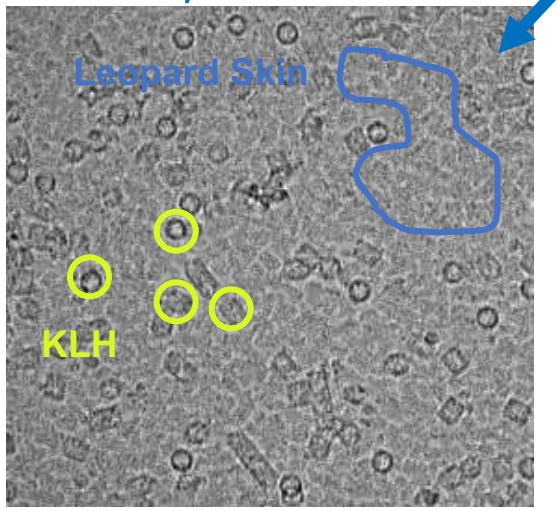
Lifetime

Ice Growth

Leopard Skin

Black Spots

Column / Autoloader

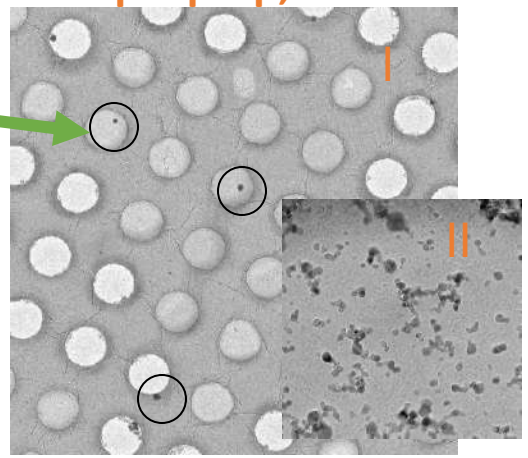


All: surface contamination

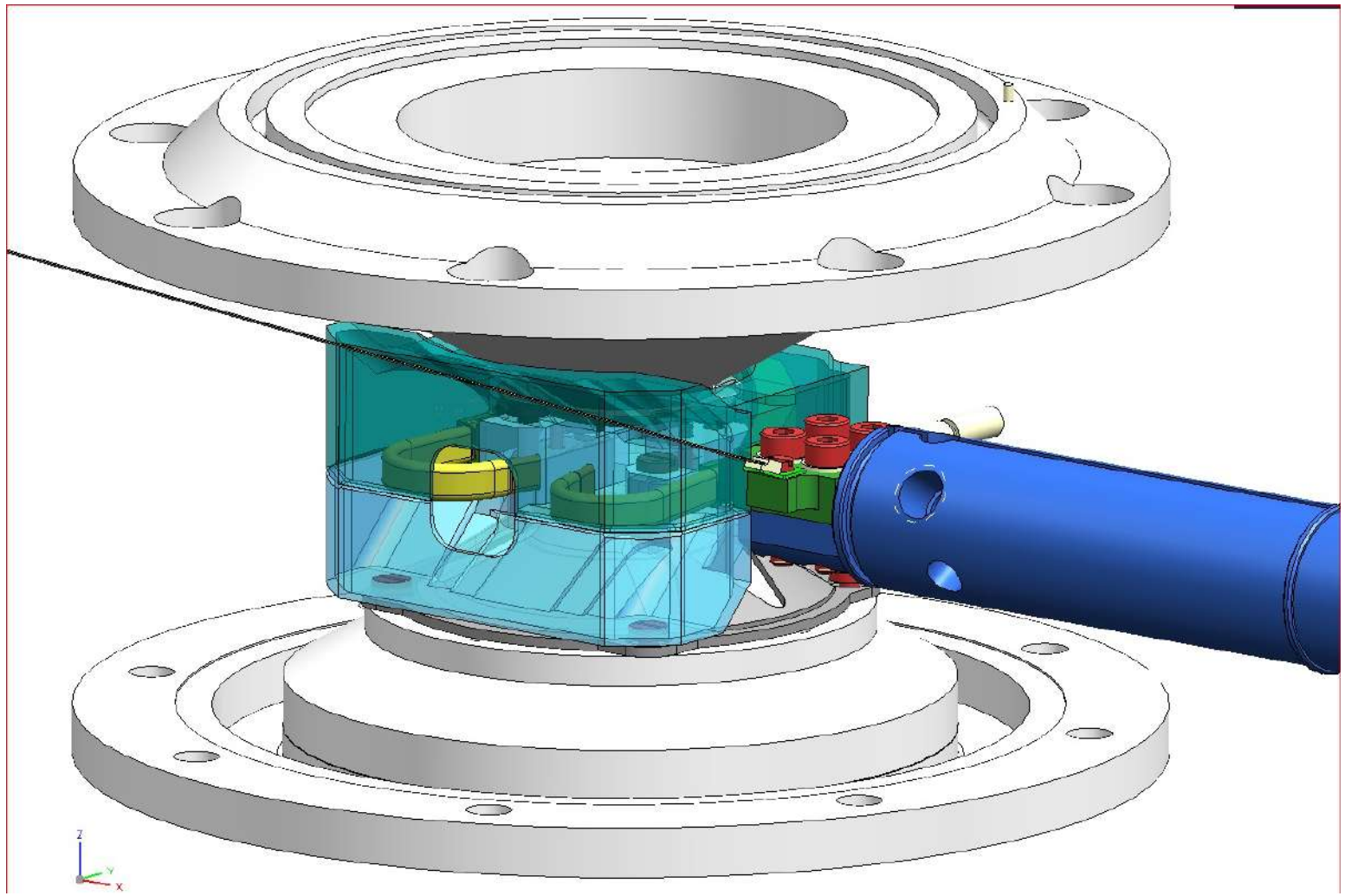
Initial

Transfer Ice I&II

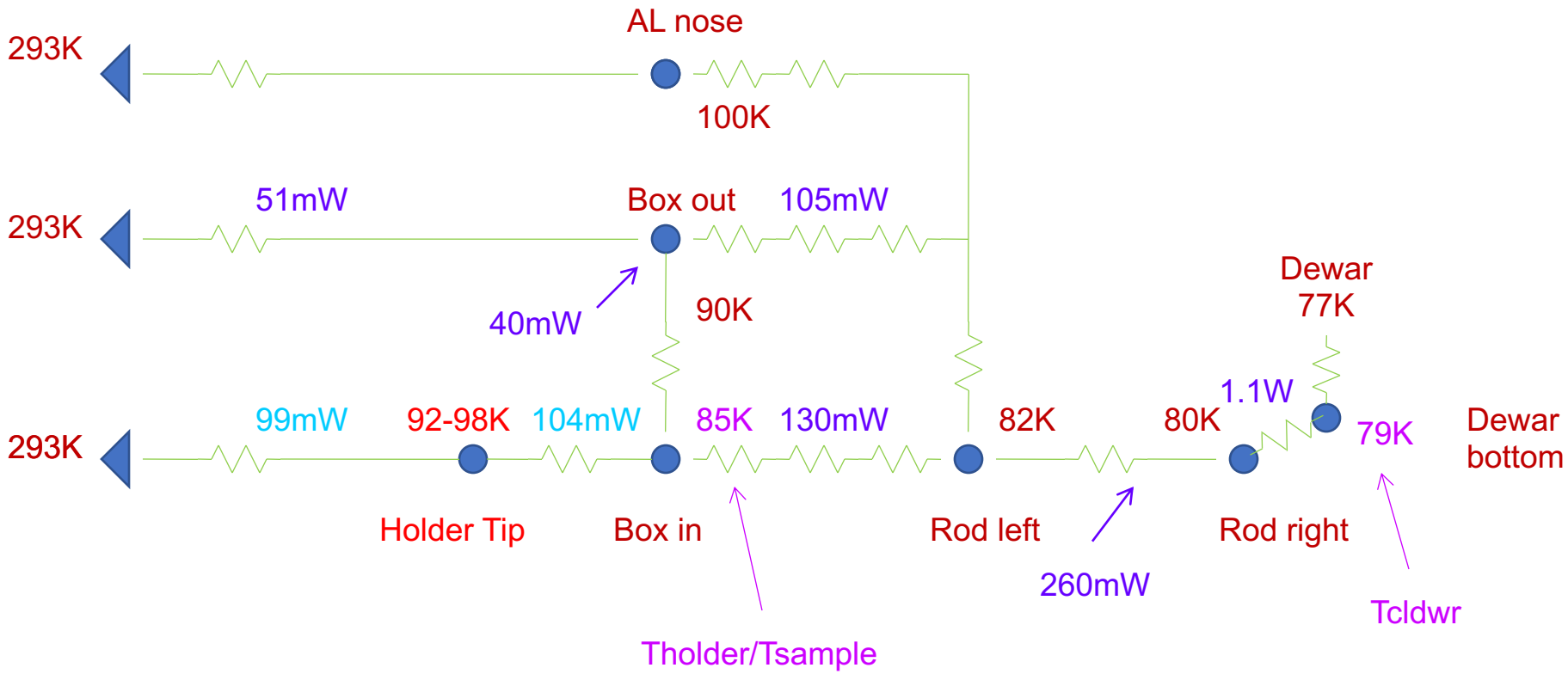
Sample prep, transfer



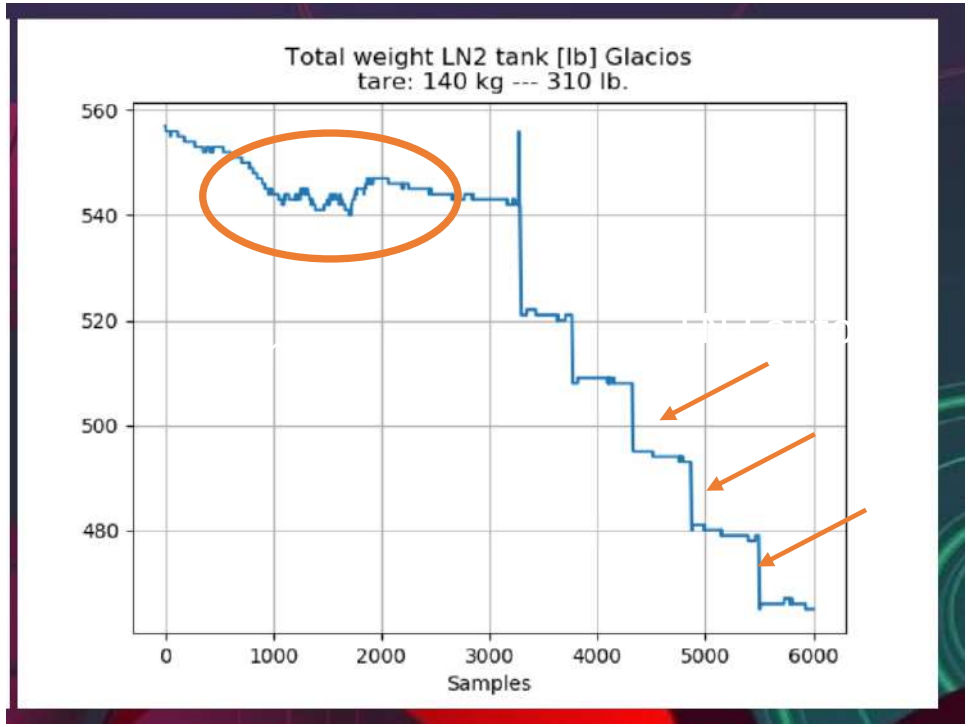
Cryo box design



Thermal model current configuration

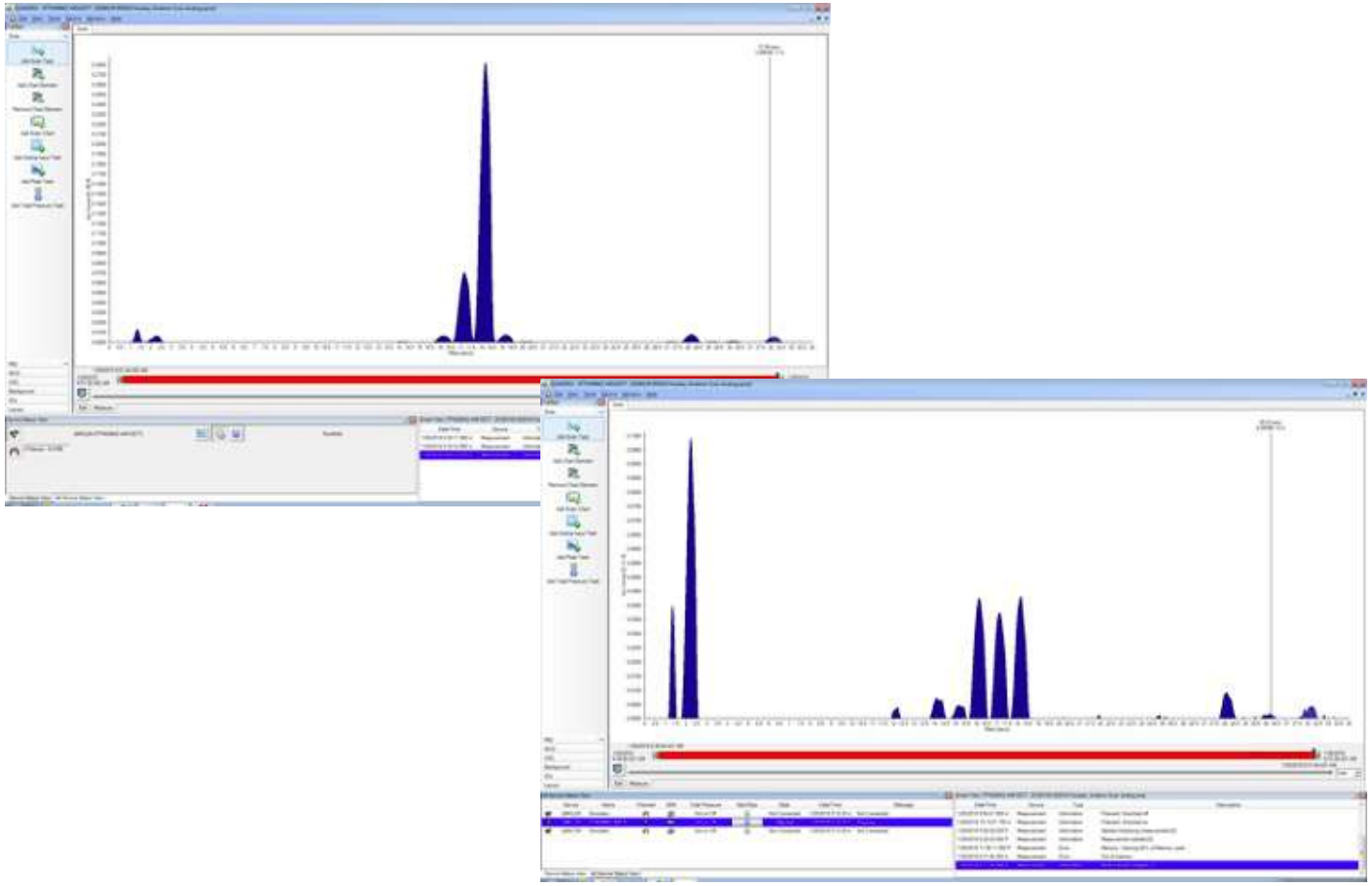


Backup slides



Tank failure:

- Frozen valve
- Excessive ice built up
- Too little pressure



RGA

- Residual
- Gas
- Analyzer

Electron Event Representation (EER)

- Conventional representations of cryoEM movies store pixel intensities for each exposure fraction

- In contrast, in EER each electron detection event is recorded as a tuple of position and time (x,y,time), indicating where and when the electron was detected on the sensor

- storing EER data removes the need to decide on an exposure fractionation strategy during imaging

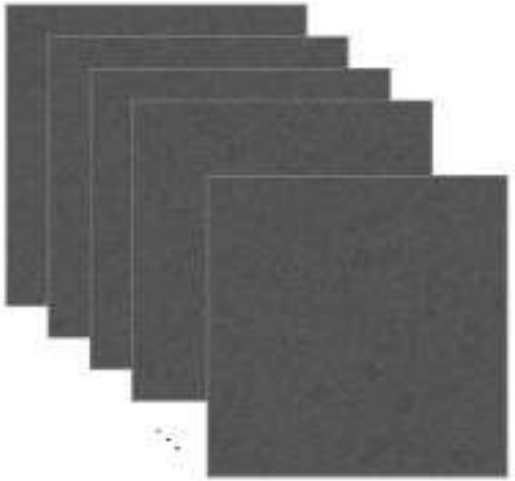
- Enables optimal correction of specimen motion.

- In addition, EER files record super resolution information in images, allowing 3D reconstruction beyond the Nyquist frequency.

A

Single frame representation

B

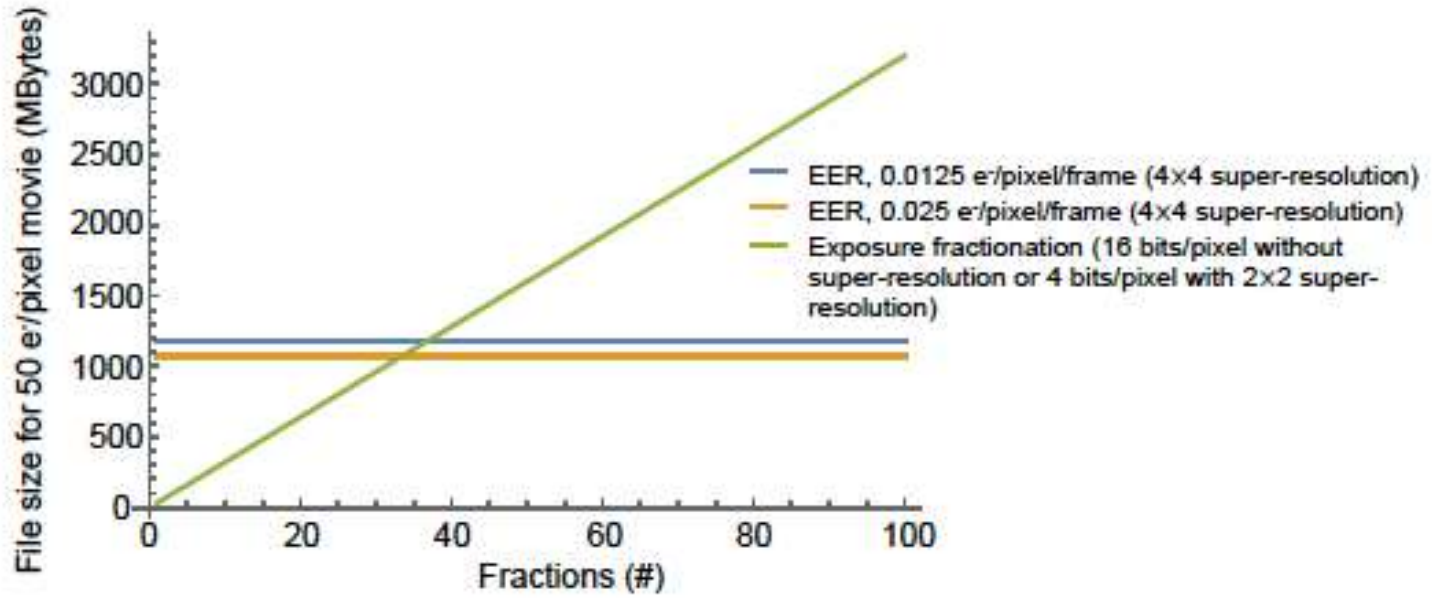


Exposure fractionation representation

C

x	y	t
3953.24	2845.63	1
919.78	1447.39	1
3864.43	348.13	1
3606.05	1539.54	1
1758.86	2971.55	1
1749.18	596.72	1
3342.11	3967.5	1
...	...	...
3983.58	531.96	N

D



Cold FEG slides

The cold field emission gun (CFEG) is a type of electron source in which the gun tip (emitter) is kept at ambient room temperature and only the electrostatic field is used to extract electrons.

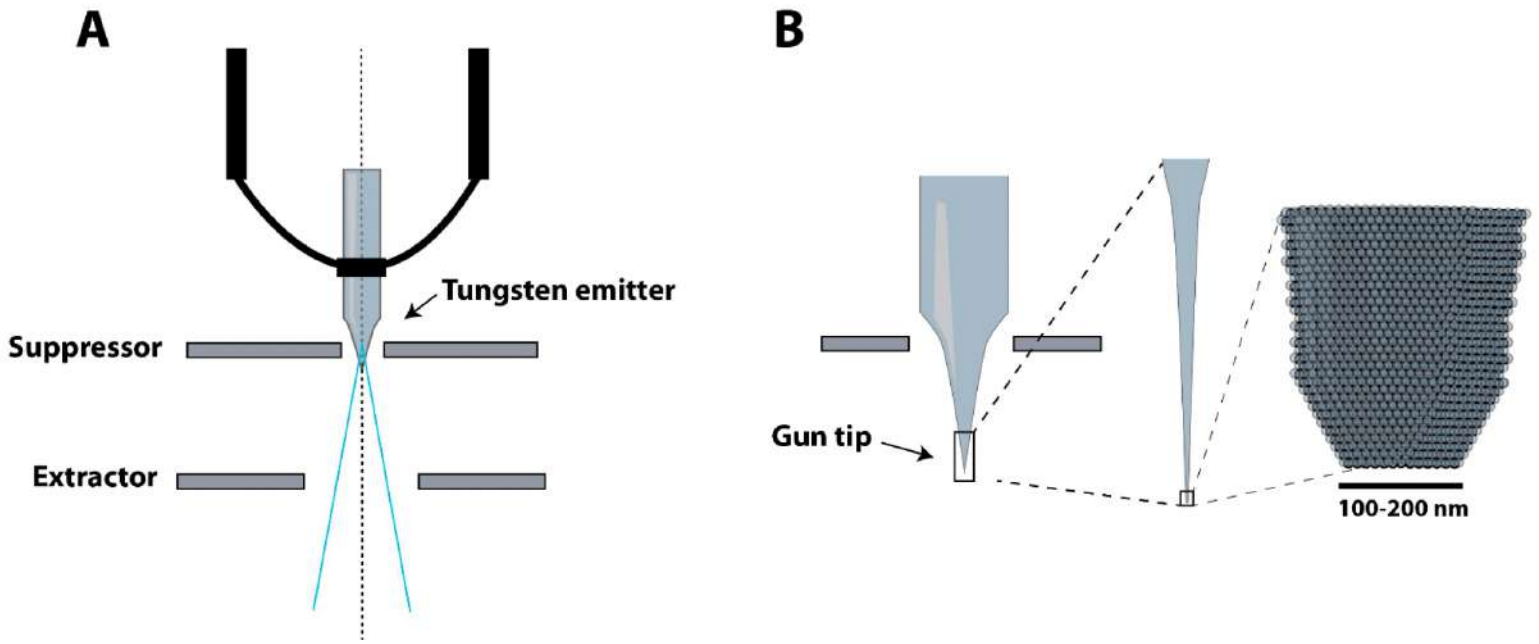


Figure 1 CFEG design. A) Schematic representation of the main CFEG components. B) Schematic representation of a CFEG emitter tip and thickness.

CFEG design

no filament current
no heating of the tip

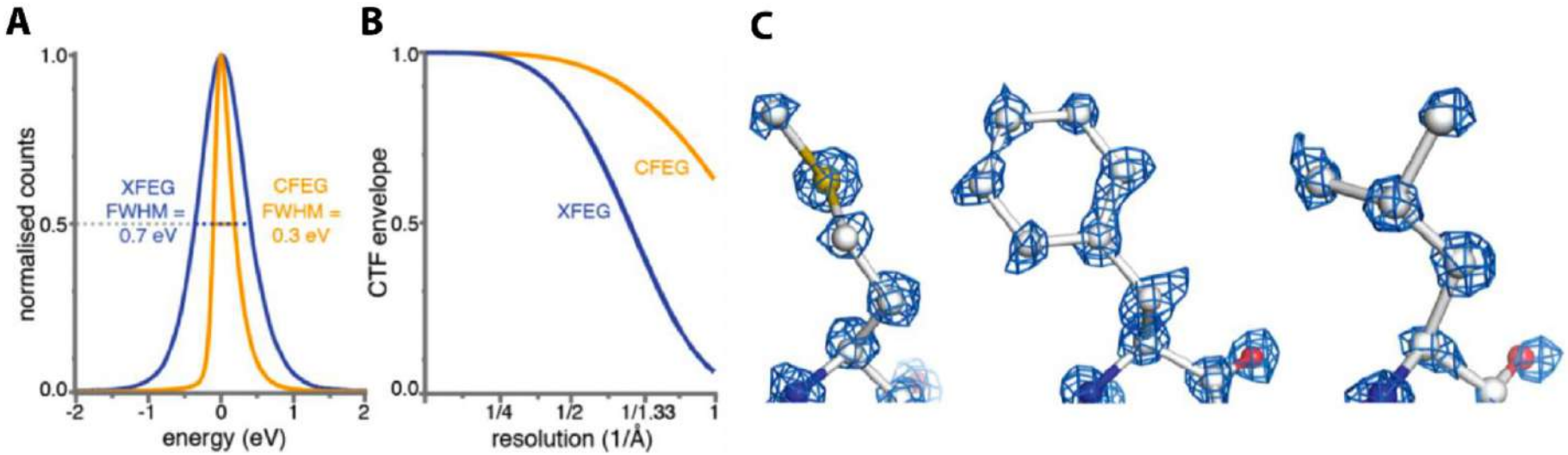


Figure 2. CFEG offers lower energy spread and higher resolution reconstructions. A) Energy spread of the XFEG (blue) and the CFEG (orange), with the full width of the curves at half their maximum value (FWHM). B) Theoretical CTF envelope functions for the XFEG (blue) and the CFEG (orange). C) EM densities for methionine, phenylalanine and leucine (left to right) residues from an apoferritin structure solved to 1.2 Å. At this resolution each atom is resolved as a separate sphere. Panels A-C were adapted from Nakane, T., Kotecha, A., Sente, A. et al. Single-particle cryo-EM at atomic resolution. Nature **587,** 152–156 (2020).

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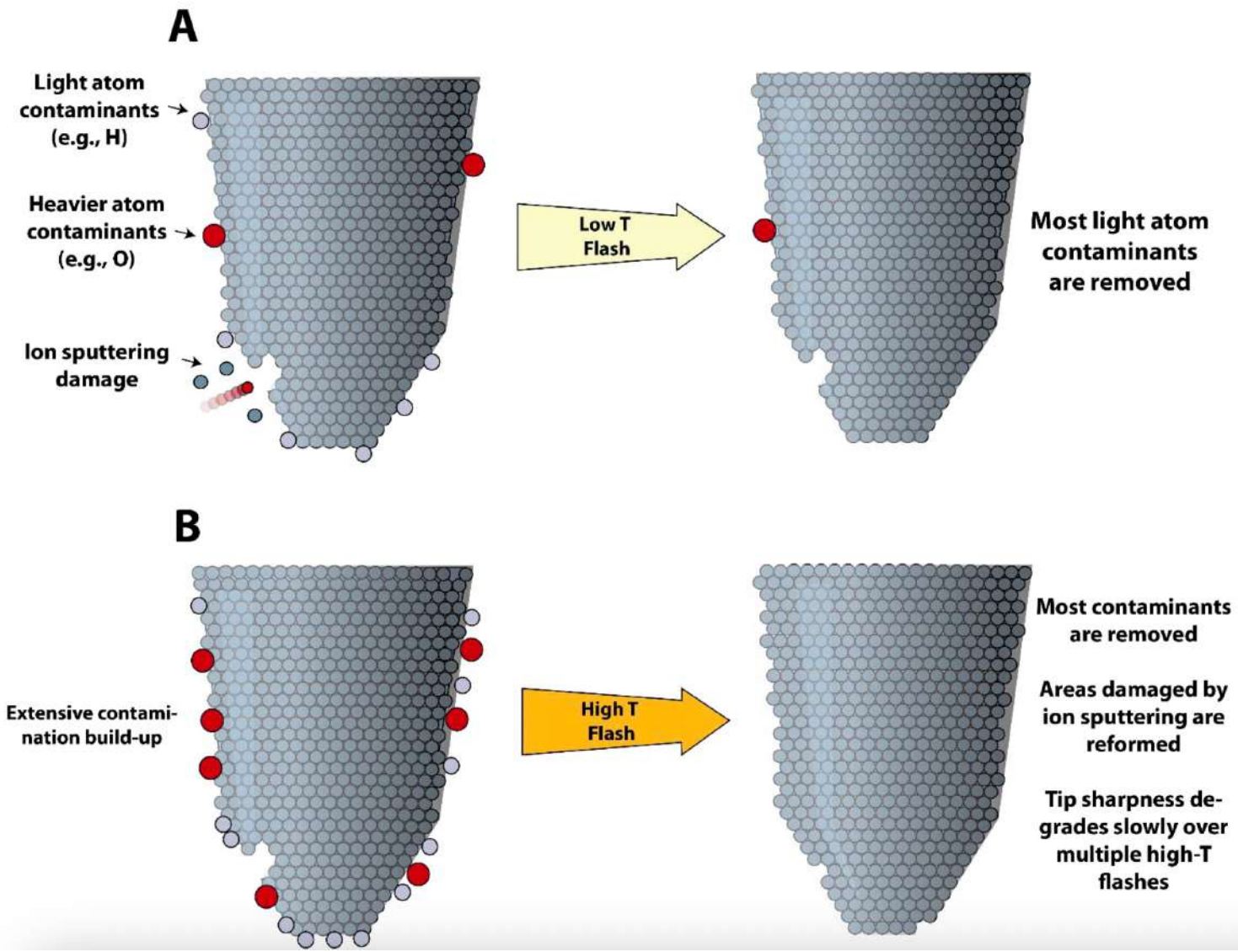


Low T flash:
<1000*C.

vs.

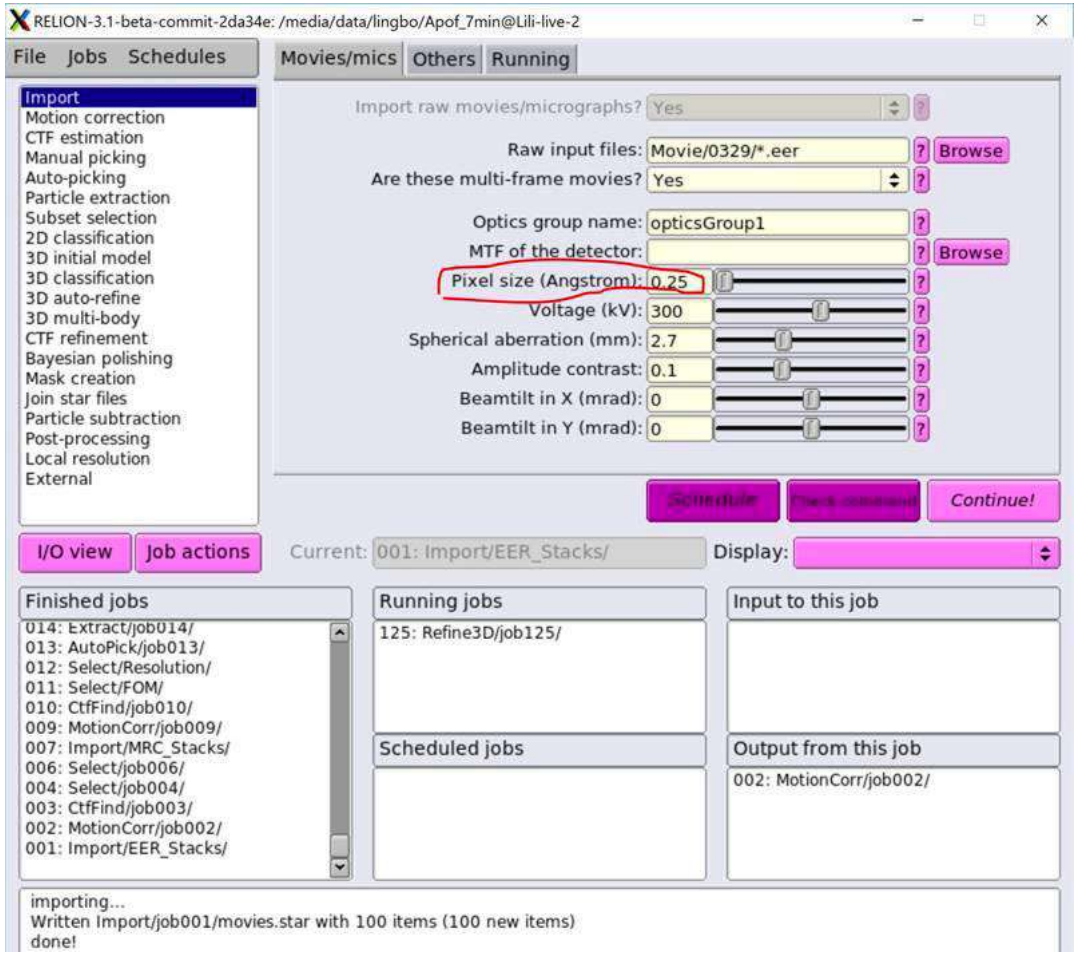
High T flash
> 1000*C.

EER data

- EER data is stored as super-resolution. So, when importing movies, the input pixel size is half of physical pixel size (reported by EPU).

A separate branch of Relion (Relion-EER) is needed to read .eer files.

Instructions for download and compilation are here: https://github.com/3dem/relion/tree/devel-eer



Next Generation Cryo-TEM hardware

- Still LN2!
- Still very cool