SickKids
RESEARCH INSTITUTE

Optimizing cryo-EM image acquisition

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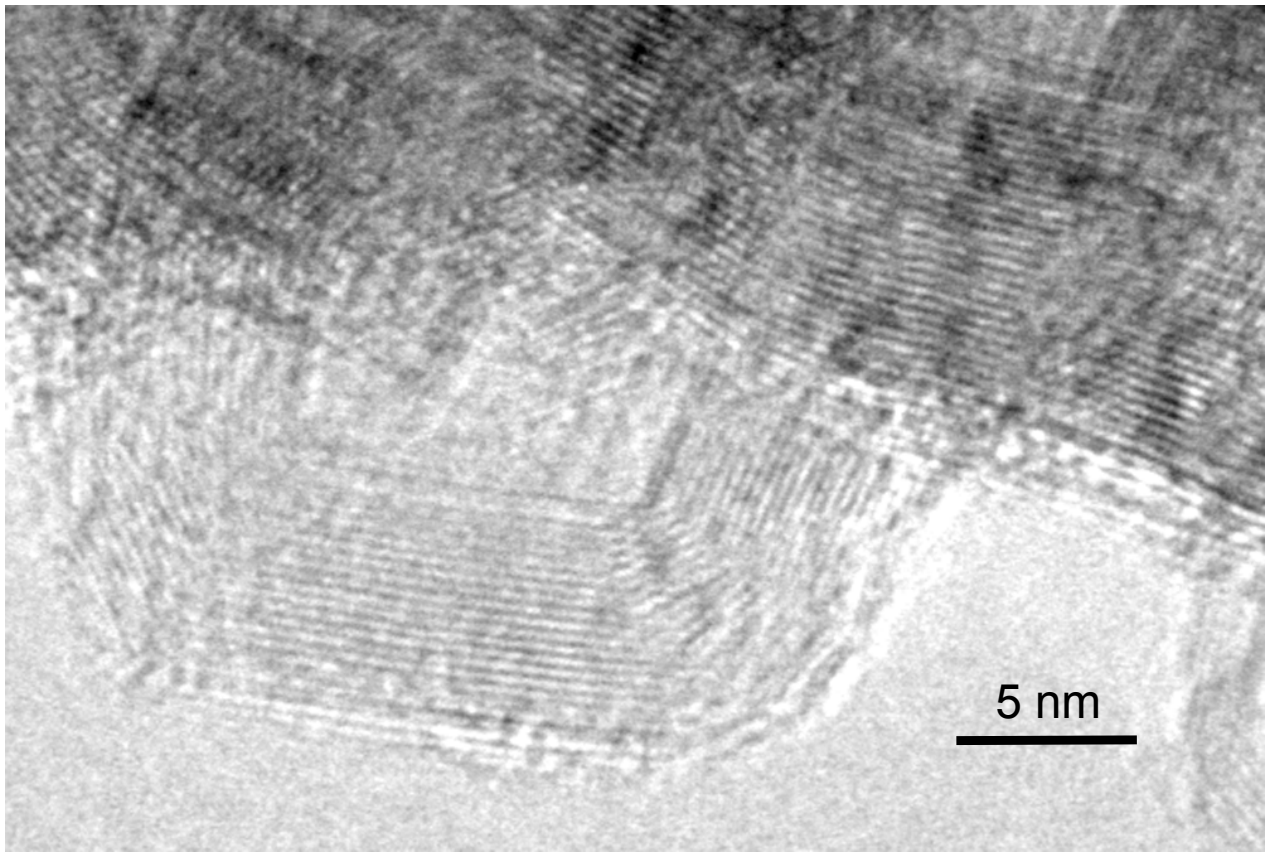
What's holding us back?

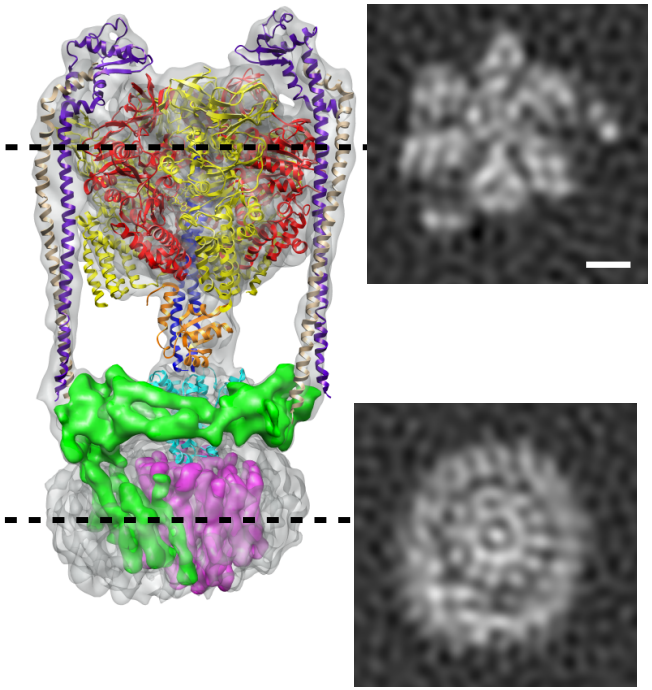
Fact 1: We know our microscope is capable of "atomic" resolution



Fact 2: Our best 3D maps are not at atomic resolution

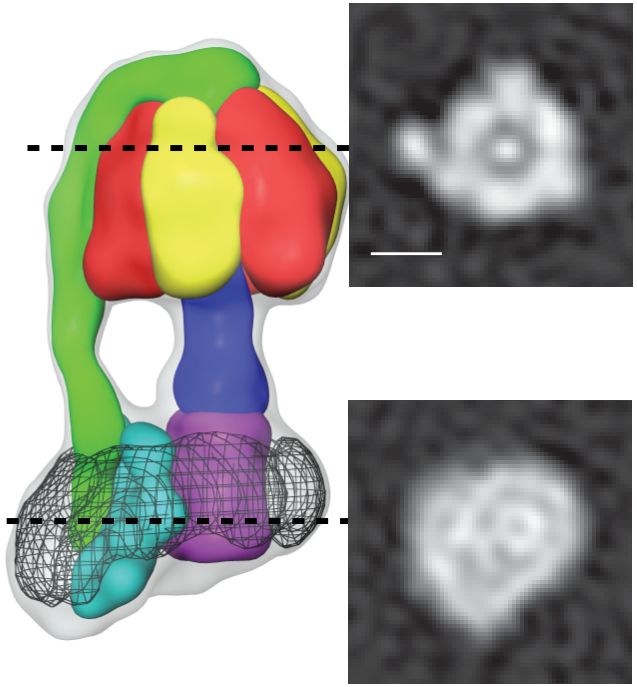
Why do different maps reach different resolutions?

9.7 Å



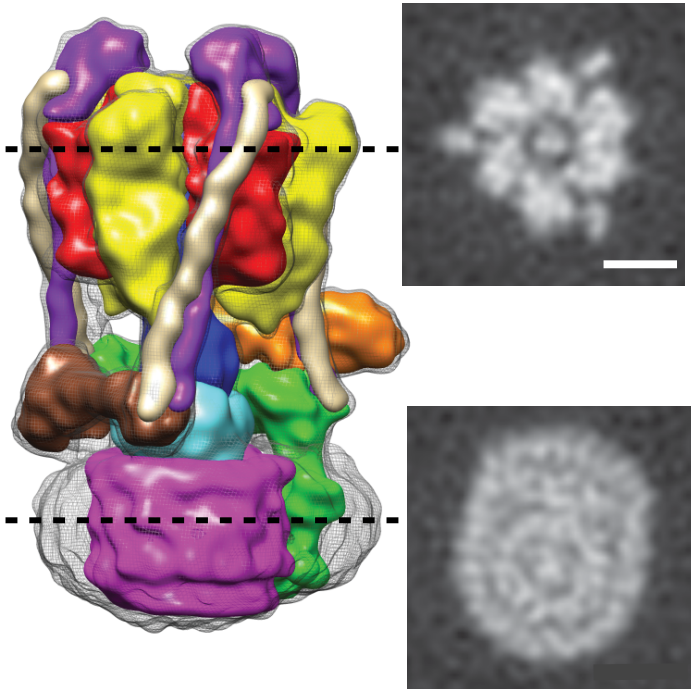
Lau and Rubinstein (2012). *Nature* **481**, 214-17.

18 Å



Baker, Watt, Runswick, Walker, and Rubinstein (2012). *PNAS* **109**, 11675-80.

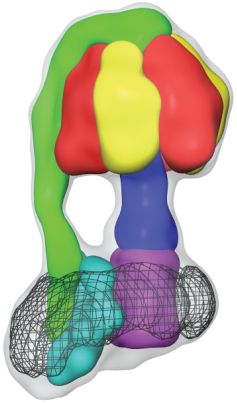
11 Å



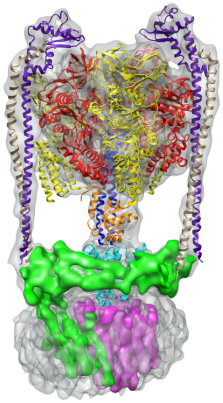
Benlekbir, Bueler, and Rubinstein (2012). *NSMB* **In Press.**

Particle image (pair) alignment accuracy

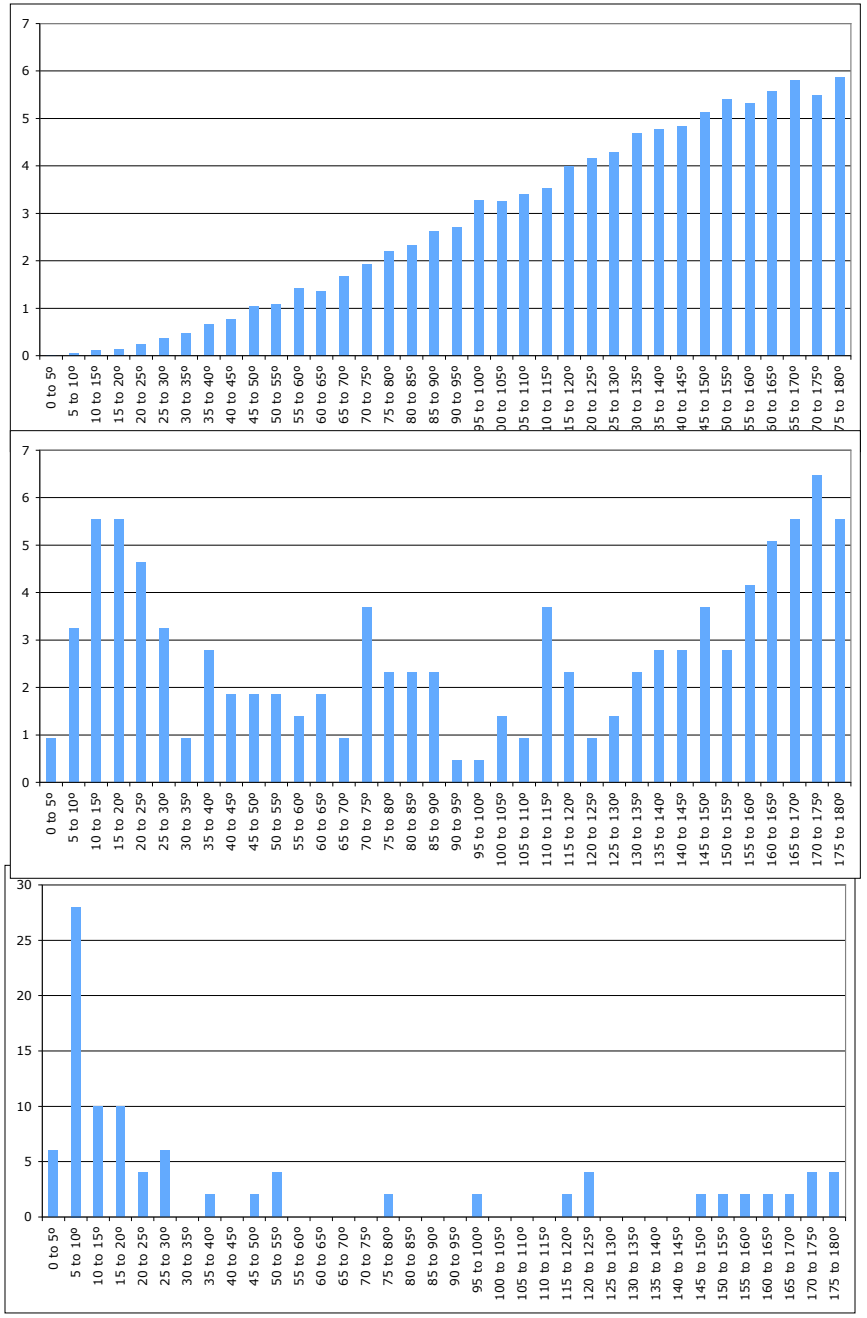
Random



18 Å



9.7 Å



Baker, Watt, Runswick, Walker, and Rubinstein (2012). *PNAS* **109**, 11675-80.

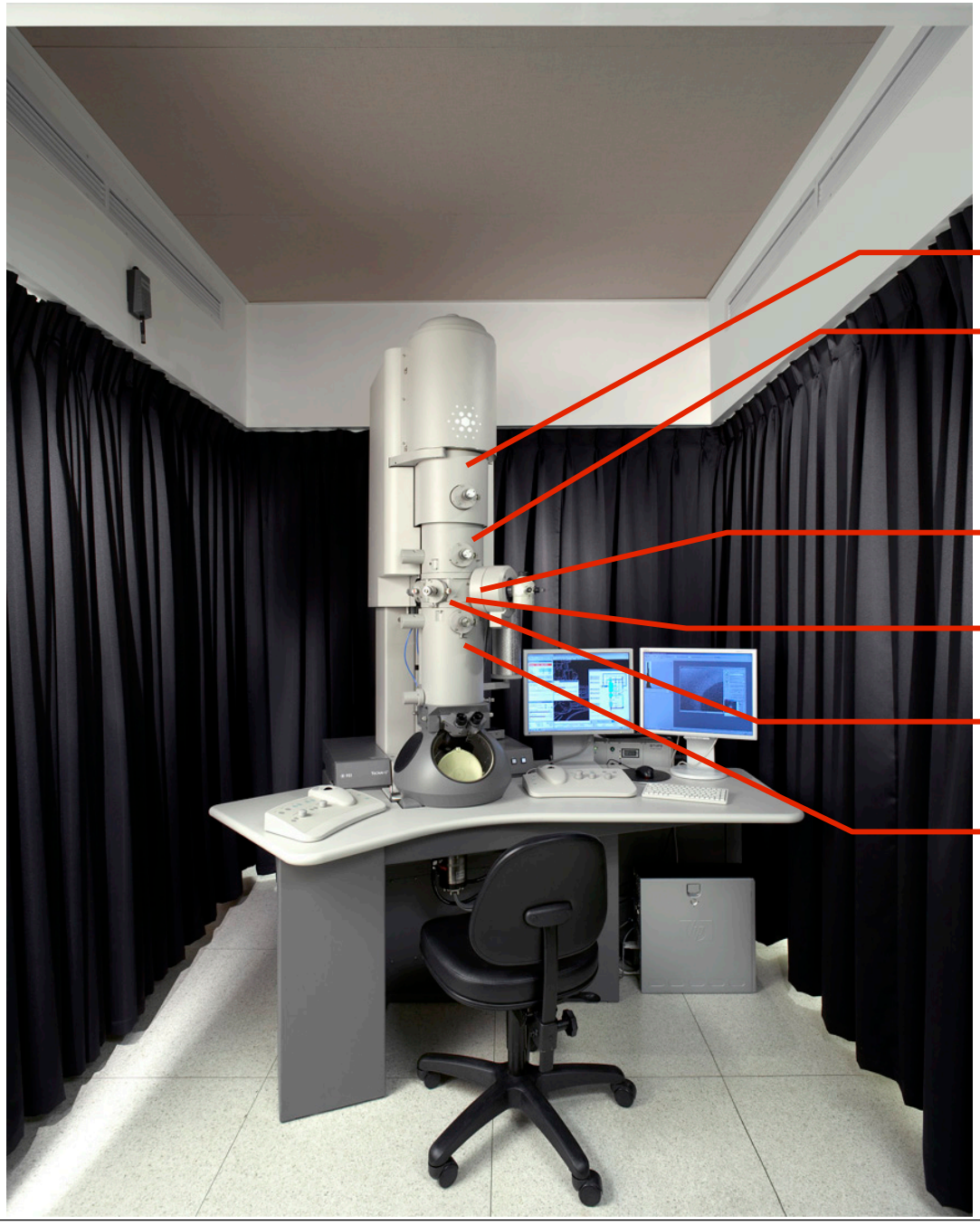
What is a good image?

- Contains high-resolution information that can be extracted by averaging

- Contains enough low-resolution information to allow alignment and coherent averaging

What is a good image?

- Contains high-resolution information that can be extracted by averaging
  (reduce drift, coherent illumination, parallel and untilted illumination, use little defocus, use low electron exposure)
- Contains enough low-resolution information to allow alignment and coherent averaging
  (use more defocus, use higher electron exposure)



Coherence

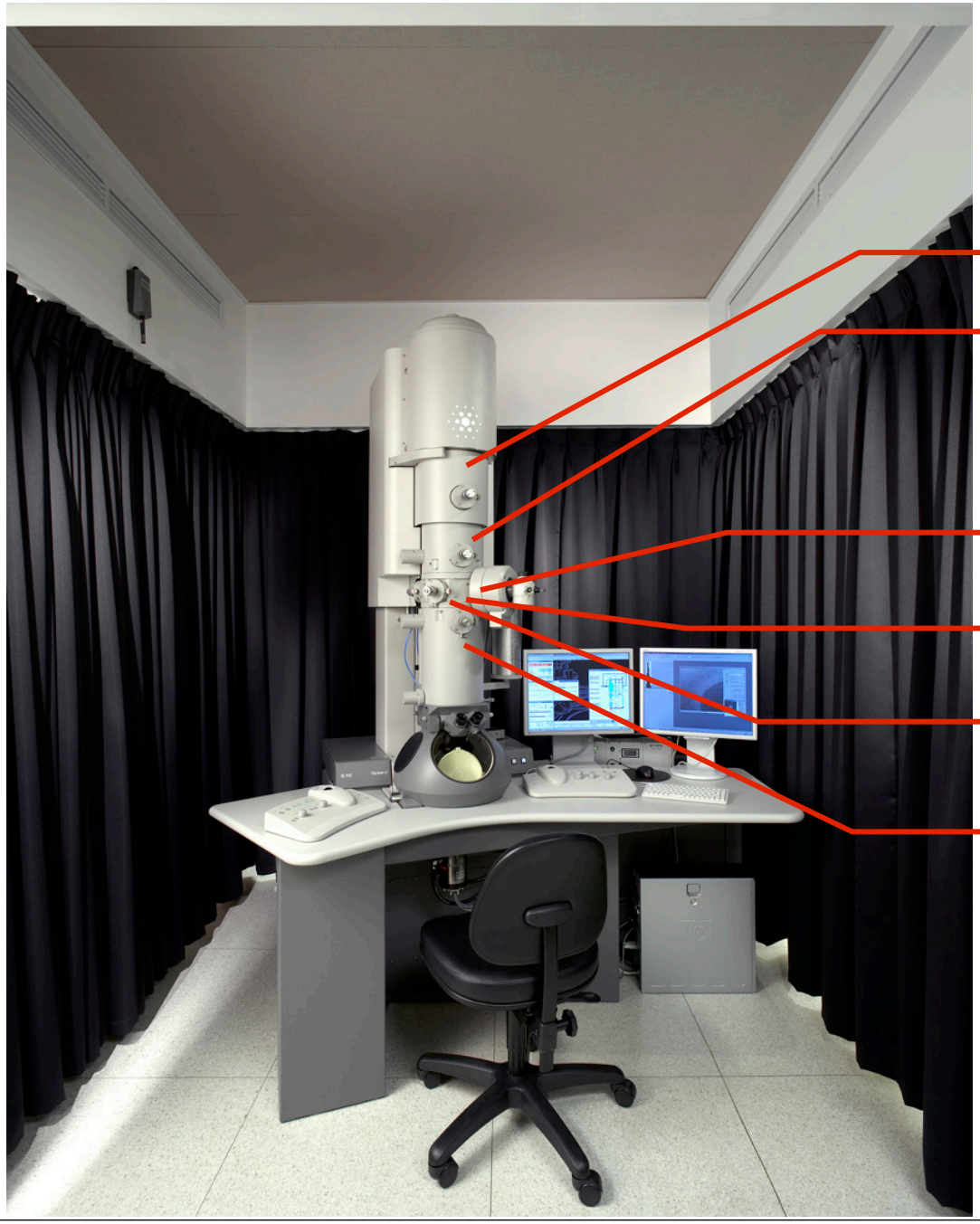
Beam tilt/Coma

Specimen drift

Radiation damage

Choosing defocus

Lens hysteresis


Gun
Condenser lenses
Specimen stage
Specimen
Objective lens
Projector lenses

Coherence and coma

Important terminology:

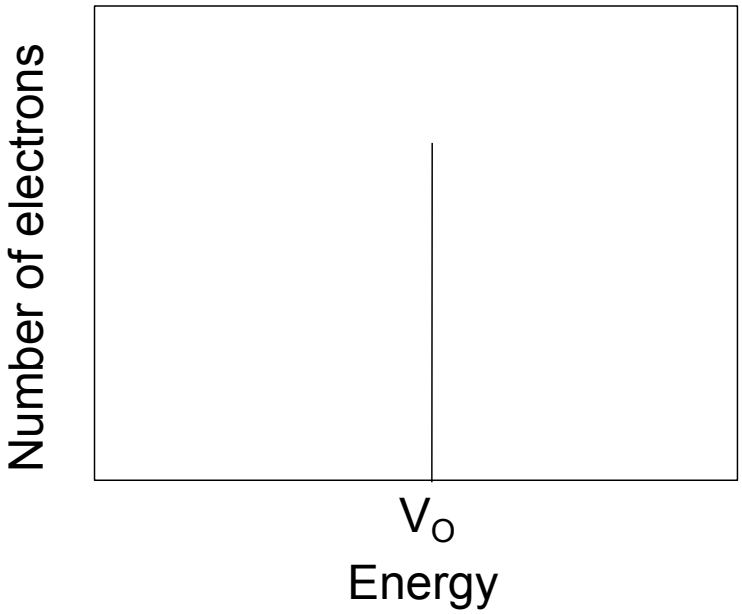
- Temporal and Spatial coherence
- On-axis coma and off-axis coma

Choices:

- Condenser lens 1 setting
- Condenser lens 2 setting
- Condenser lens 3 setting (if available)
- Condenser aperture
- Area of specimen irradiated

Temporal coherence

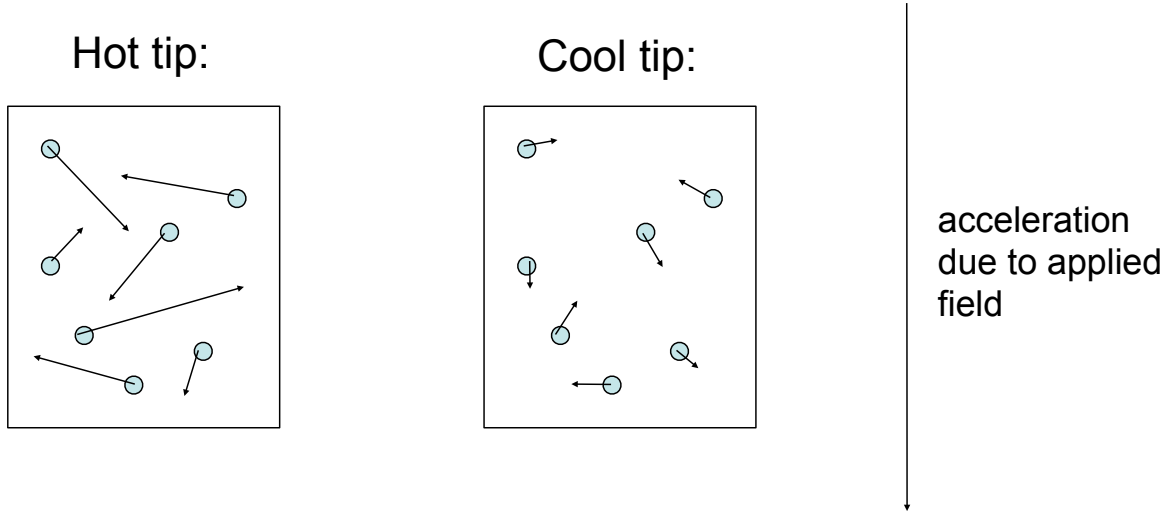
Ideal electron source:



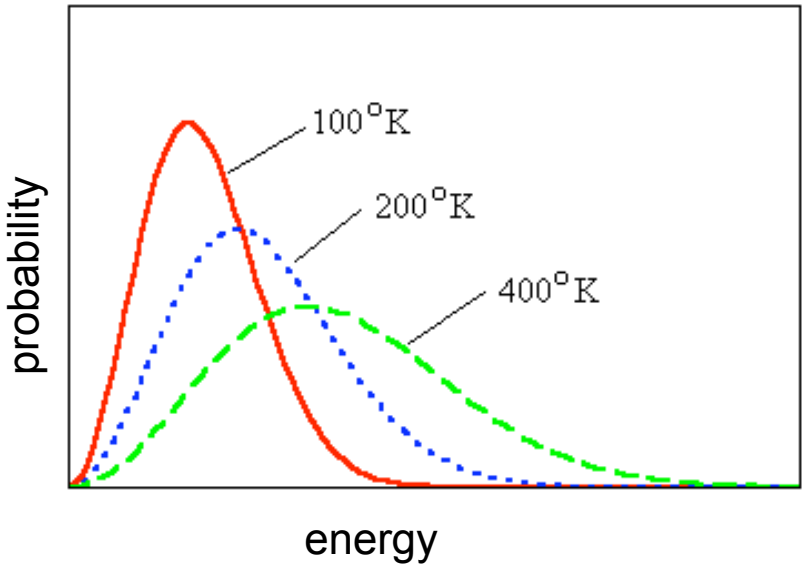
Ideally, electrons leaving the source will all be of the same energy and therefore the same wavelength.

In practice, there is always a spread of energies leaving the electron source.

Temperature dependance of electrons in beam



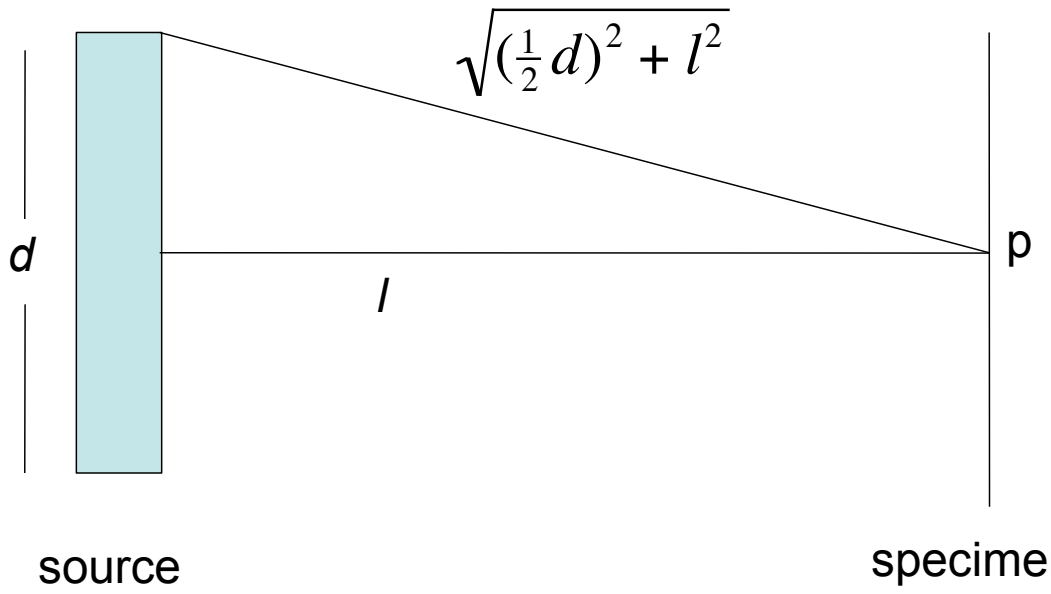
Energy of electron in direction of beam = Accelerating energy + Energy in Tip



An electron beam will have more temporal coherence when it is emitted from a cold tip

Spatial coherence

Ideally, the electron gun would be a point source, but in practice it has a finite size



At point "p", electrons from the centre of the source will have traveled distance *l*. Electrons from the edge of the source will have traveled $\sqrt{(\frac{1}{2}d)^2 + l^2}$

Electrons from edge will be out of phase with electrons from centre by $l - \sqrt{(\frac{1}{2}d)^2 + l^2}$

Conclusion: the smaller the tip, the better the spatial coherence of the source

Things that affect spatial coherence:

(1) The size of the tip in the gun

(2) The gun lens (FEG):

increased brightness=decreased spatial coherence

(3) The condenser 1 lens setting (i.e. spotsize):

increased brightness=decreased spatial coherence

(4) condenser 2 aperture size:

bigger aperture=decreased spatial coherence

General rule:

For a given gun, the higher you set the brightness, the less coherent your illumination will be.

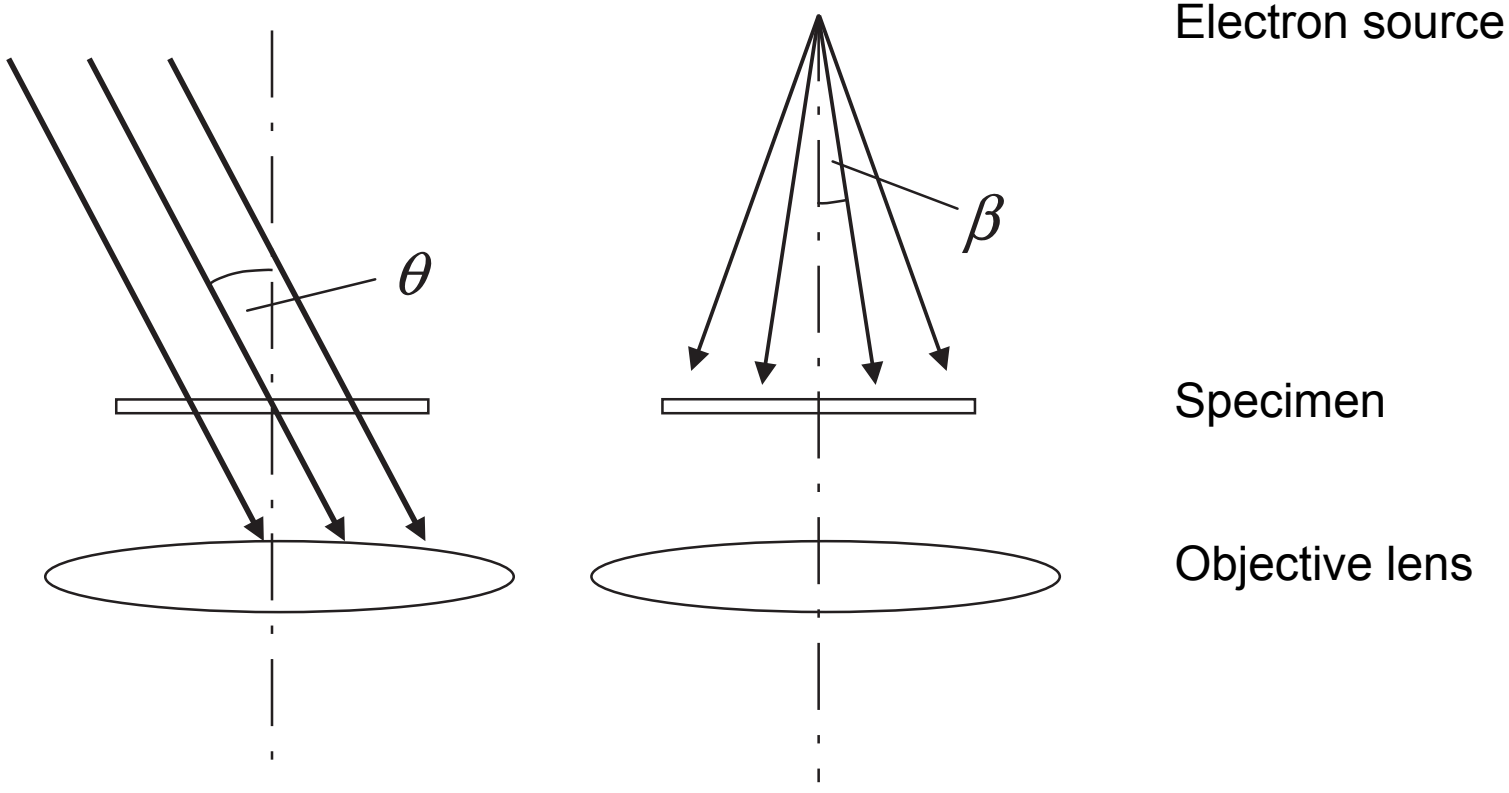
Natural assumption:

One should set the gun lens, C1 lens, C2 aperture, and C2 lens to provide just enough brightness to irradiate the area you want to irradiate. However...

Coma

Glaeser *et al.* (2011). Precise beam-tilt alignment and collimation are required to minimize the phase error associated with coma in high-resolution cryo-EM. *J. Struct. Biol.* 174, 1-10.

Figure 2 from manuscript:

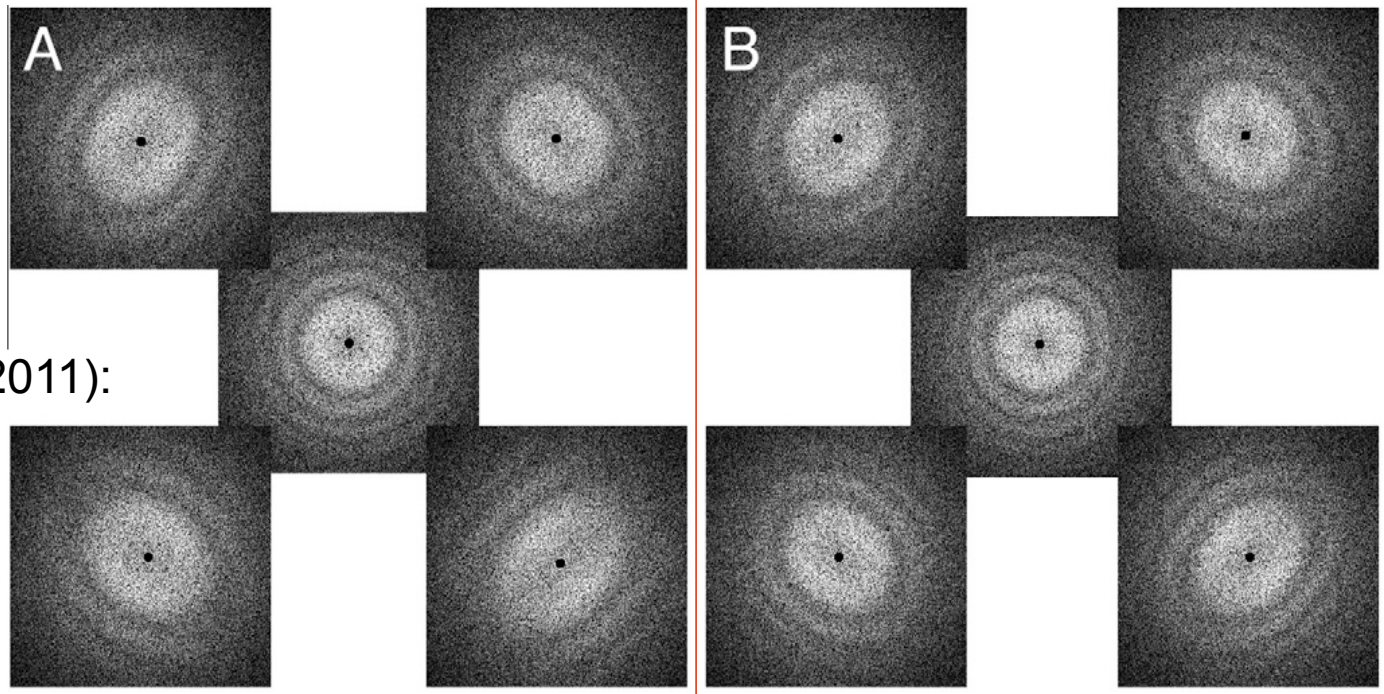


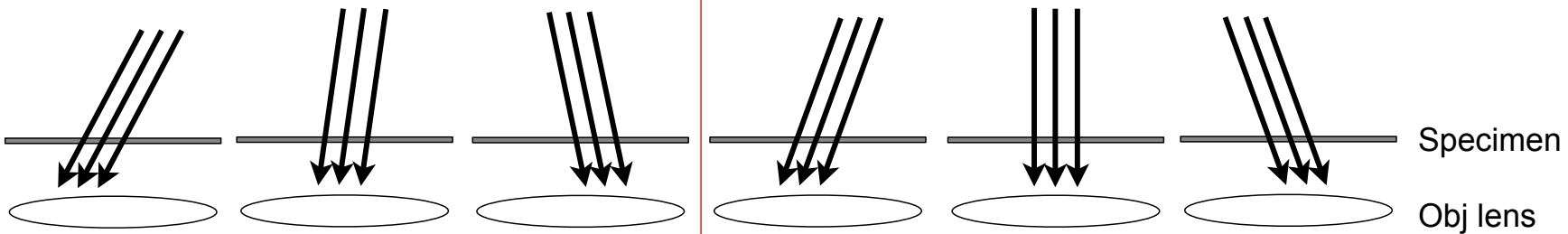
On-axis coma (beam tilt)

Off-axis coma (divergent/convergent illumination)

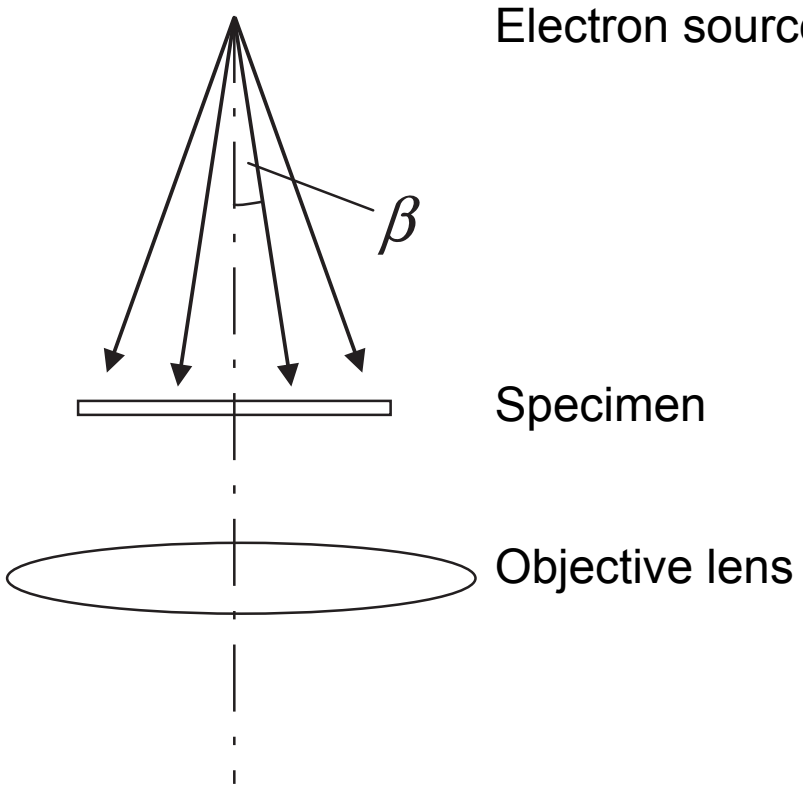
Correction of on-axis coma by “coma free alignment”

Figure 3 from Glaeser *et al.* (2011):





Minimize off-axis coma



Off-axis coma
(divergent/convergent illumination)

Obtaining parallel illumination with a C3 lens:

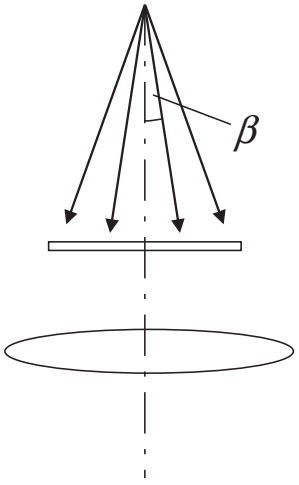
Wobble objective lens (focus) while adjusting C3 lens. The size of the illuminated remains constant when illumination is parallel. (haven't actually done myself)

Obtaining parallel illumination without a C3 lens:

Choose an appropriate C2 aperture and area to illuminate with the C2 lens

FEI Titan/Zeiss Libra microscopes have a 3rd condenser lens.
JEOL?

Maximum off-axis coma with a 2 condenser lens microscope



Divergence/convergence angle " β"

Equation 5 from Glaeser *et al*. (2011)

$$\beta_{max} = \frac{\left(\frac{D_{C2}}{M_{C2}} - D\right)}{2d}$$

β_{max} is the maximum tilt angle (at edge of irradiated area)

D_{C2} is the diameter of the C2 aperture

M_{C2} is the demagnification of the C2 aperture onto front focal plane of objective lens by microscope

D is the diameter of the region irradiated on specimen

d is the distance from the specimen to the virtual image of the C2 aperture

Microscope	M_{C2} (micro-probe)	M_{C2} (nano-probe)
FEI T12 (twin)	10.7	40.2
FEI F20 (twin)	5.2	40
FEI Polara/F30 (twin)	5.2	35

50 µm C2 aperture in microprobe mode is sufficient for ~5.5 Å resolution on F20 and ~4.5 Å resolution on F30 with almost any illumination area

Suggestions:

Coherence:

- Use the brightest electron source you can afford: Tungsten<LaB_6<FEG<X-FEG(?)
- Use a combination of Condenser 1 lens and Condenser 2 aperture that gives you only the brightness you need

Coma:

- Perform the coma-free alignment on your microscope
- Ensure you have a sufficiently parallel beam by choosing an appropriate C2 aperture and beam diameter at the specimen (depends on resolution desired)
- Use a microscope with continuously adjustable 3rd condenser lens

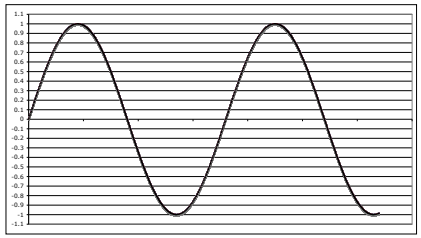
Drift

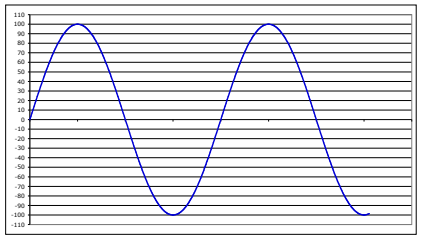
Image movement (drift) creates blurred images (loss of high-resolution detail)

How much drift is acceptable?

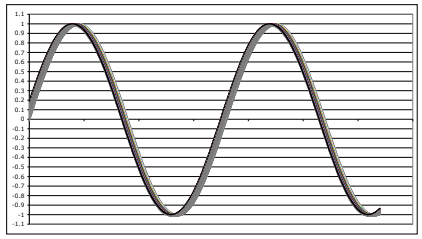
Consider averaging 100 waves of wavelength λ with different drifts from wave 1 to wave 100

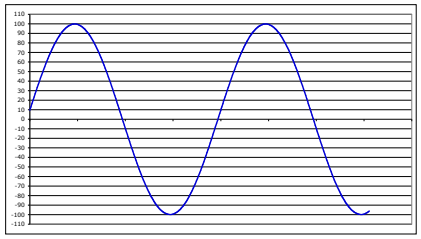
0 drift



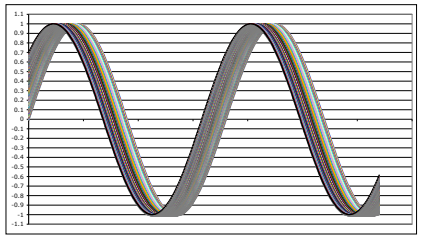


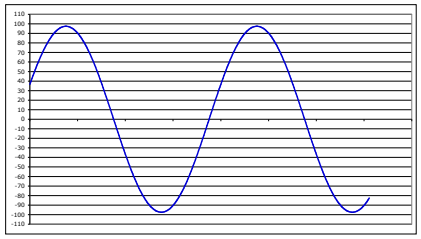
λ/32 drift



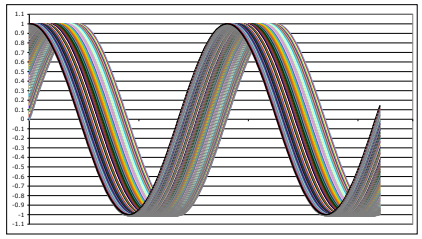


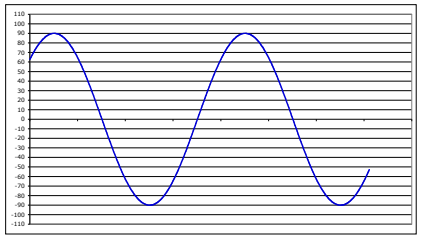
λ/8 drift



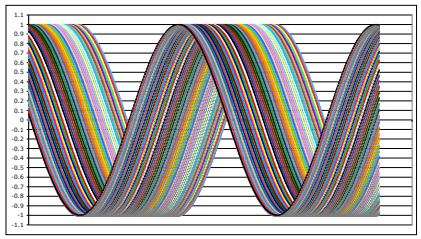


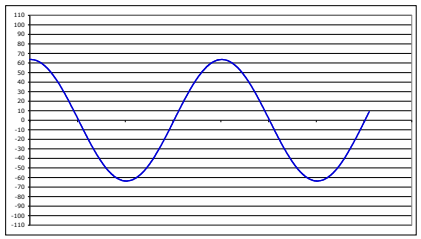
λ/4 drift



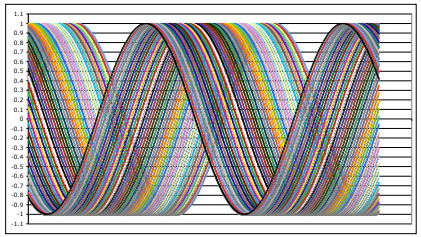


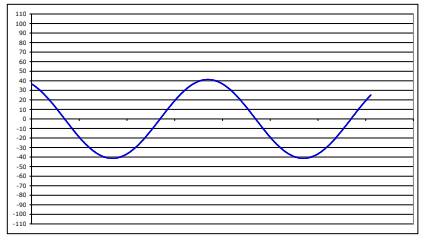
λ/2 drift



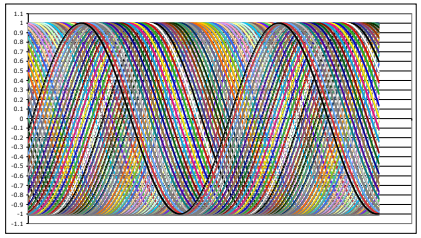


2λ/3 drift





λ drift

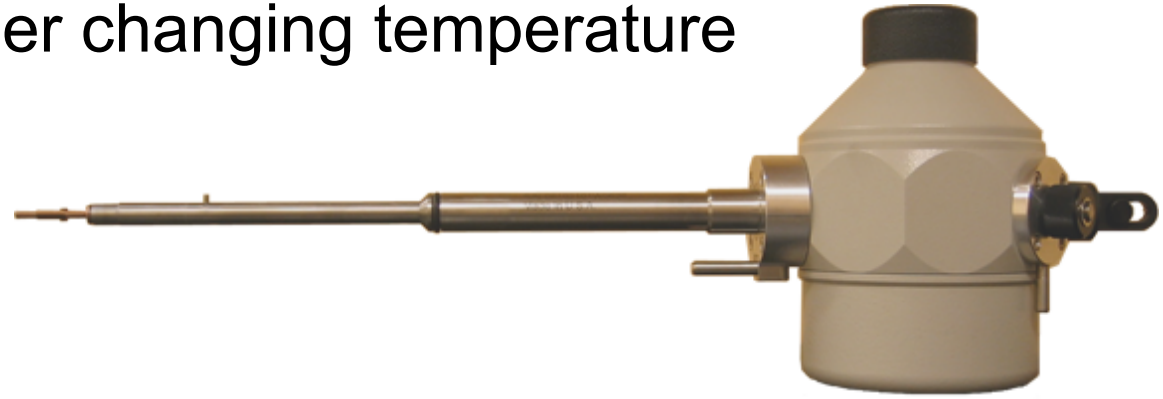


Things to realize:

1) Adding any two waves of wavelength λ produces a 3rd wave of wavelength λ
2) Adding a series of ‘drifting’ waves changes the amplitude of the sum wave
3) Adding a series of ‘drifting’ waves changes the position of the maximum of the sum wave

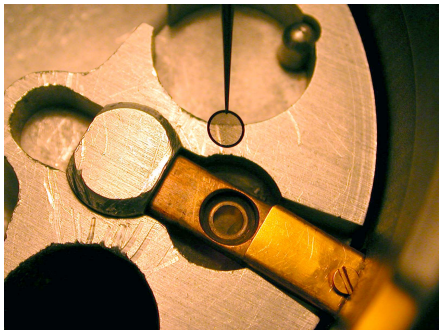
Sources of specimen drift:

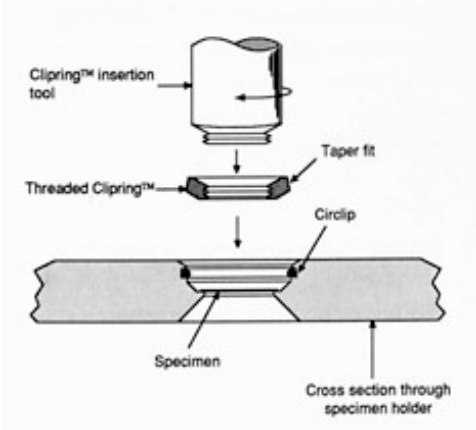
1) Cryoholder changing temperature

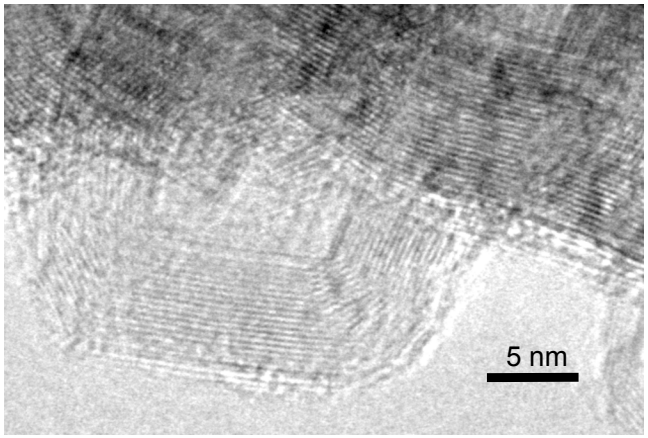


2) Boiling in cryoholder Dewar

3) Unsecured grid

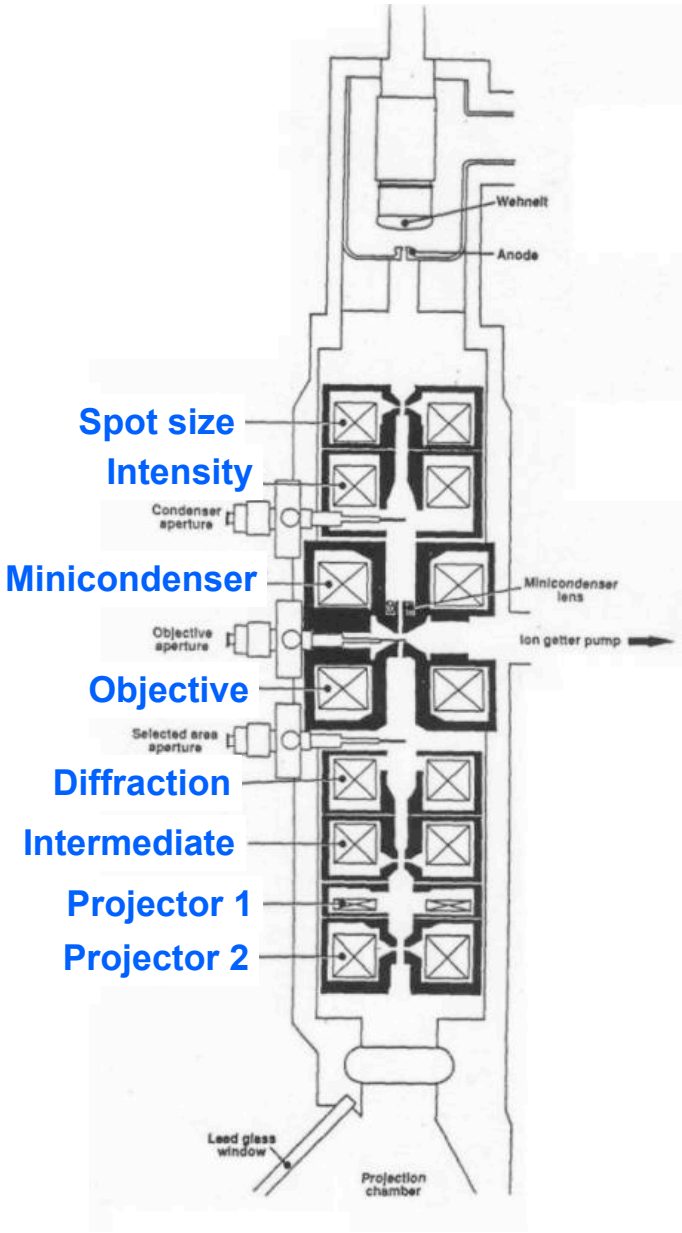






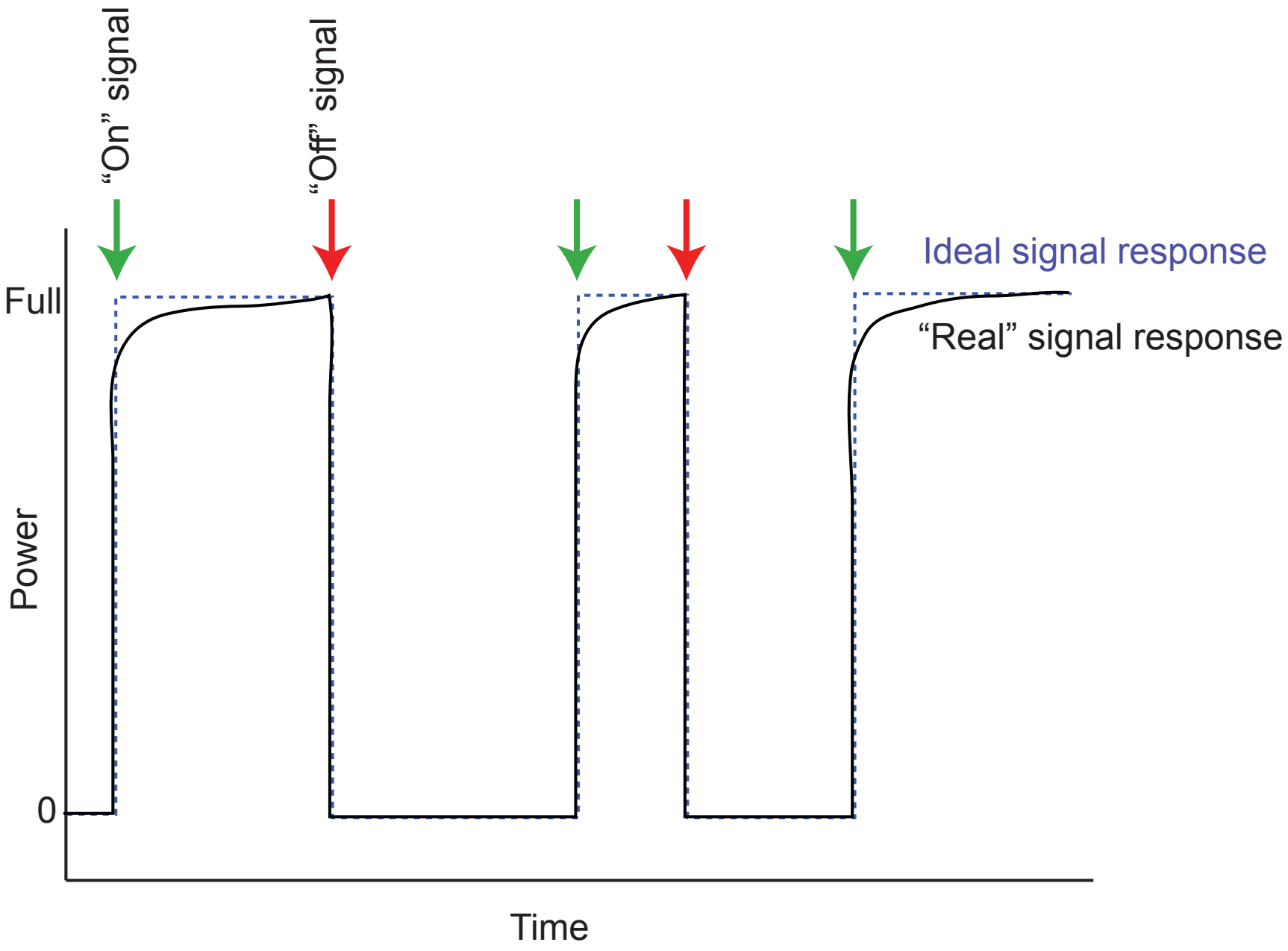
4) Unstable microscope stage or holder or stage/holder combination

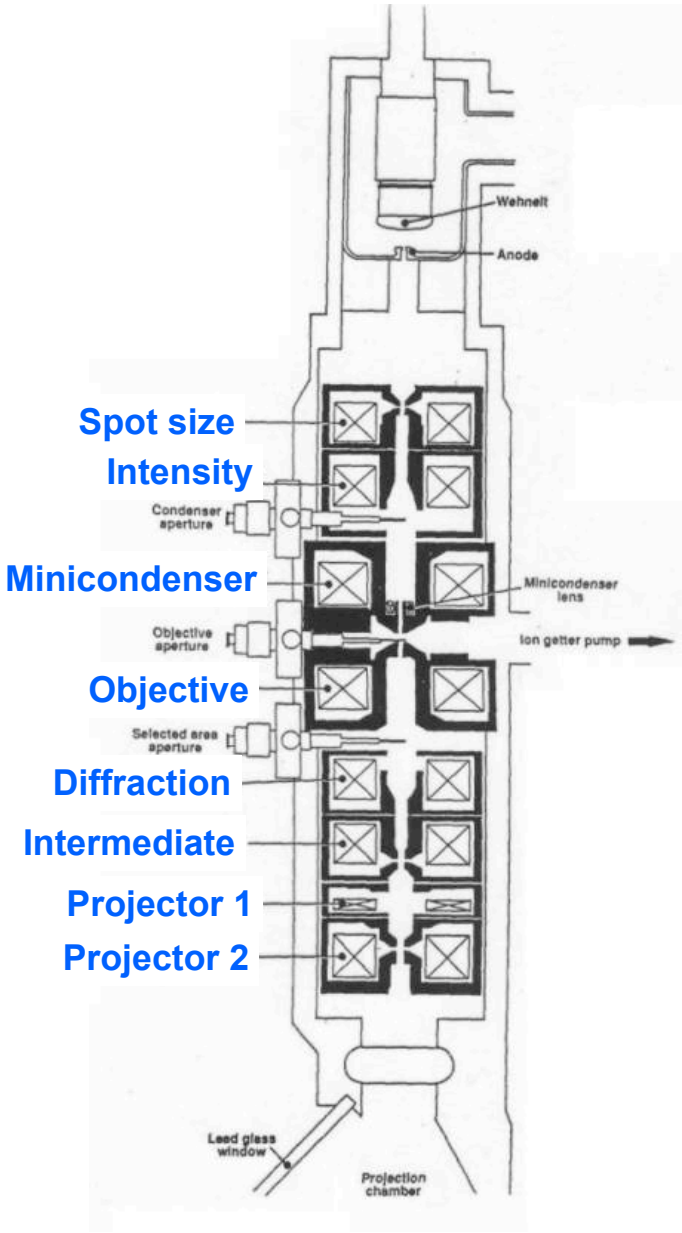
Changing magnification - a source of image drift?



Lens	Exposure (SA 50000x)
Spot size	14.31%
Intensity	50.89%
Minicondenser	83.92%
Objective	88.41%
Diffraction	65.84%
Intermediate	65.14%
Projector 1	90.73%
Projector 2	77.10%

*Values for Toronto FEI F20 microscope


"On" signal
"Off" signal
Ideal signal response
"Real" signal response
Full
Power
0
Time



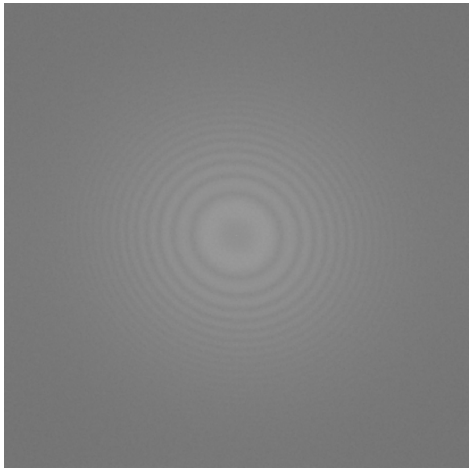
Lens	Exposure (SA 50000x)	Search (LM 2100x)
Spot size	14.31%	14.31%
Intensity	50.89%	50.42%
Minicondenser	83.92%	83.92%
Objective	88.41%	6.00%
Diffraction	65.84%	34.82%
Intermediate	65.14%	87.91%
Projector 1	90.73%	0.01%
Projector 2	77.10%	89.54%

Suggestions for ensuring a stable specimen and image:

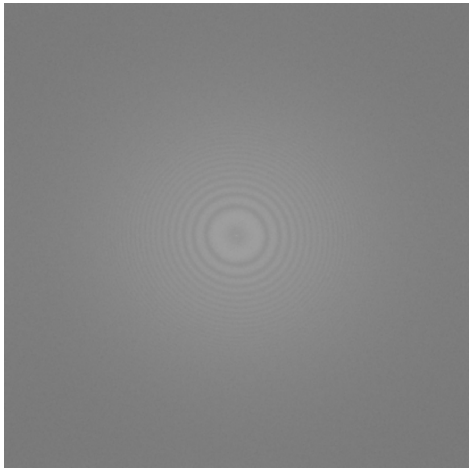
- Ensure cryoholder clamps grid properly
- Ensure cryoholder is stable in the microscope
- Use a defocused diffraction pattern for searching rather than a low-magnification microscope mode
- Use a DDD in movie mode to correct drift computationally?

Defocus

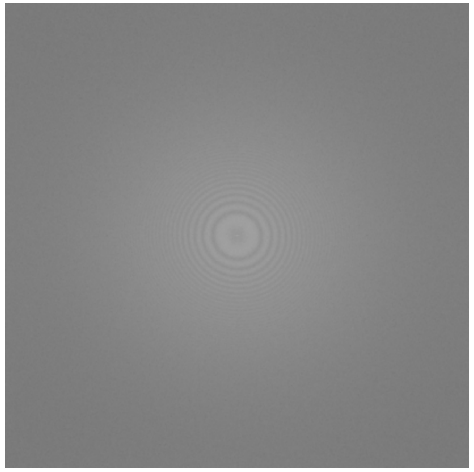
Effects of defocus



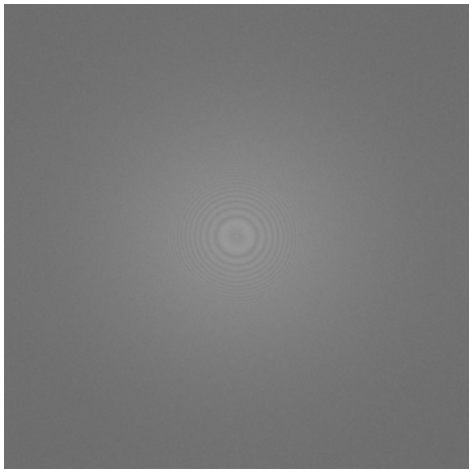
~1 μm



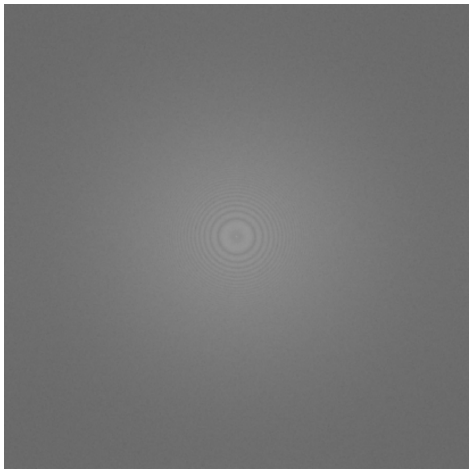
~2 μm



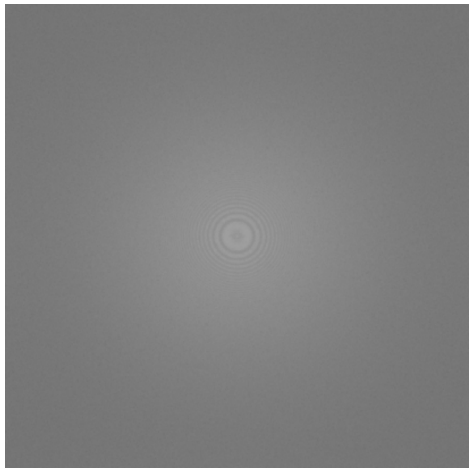
~3 μm



~4 μm



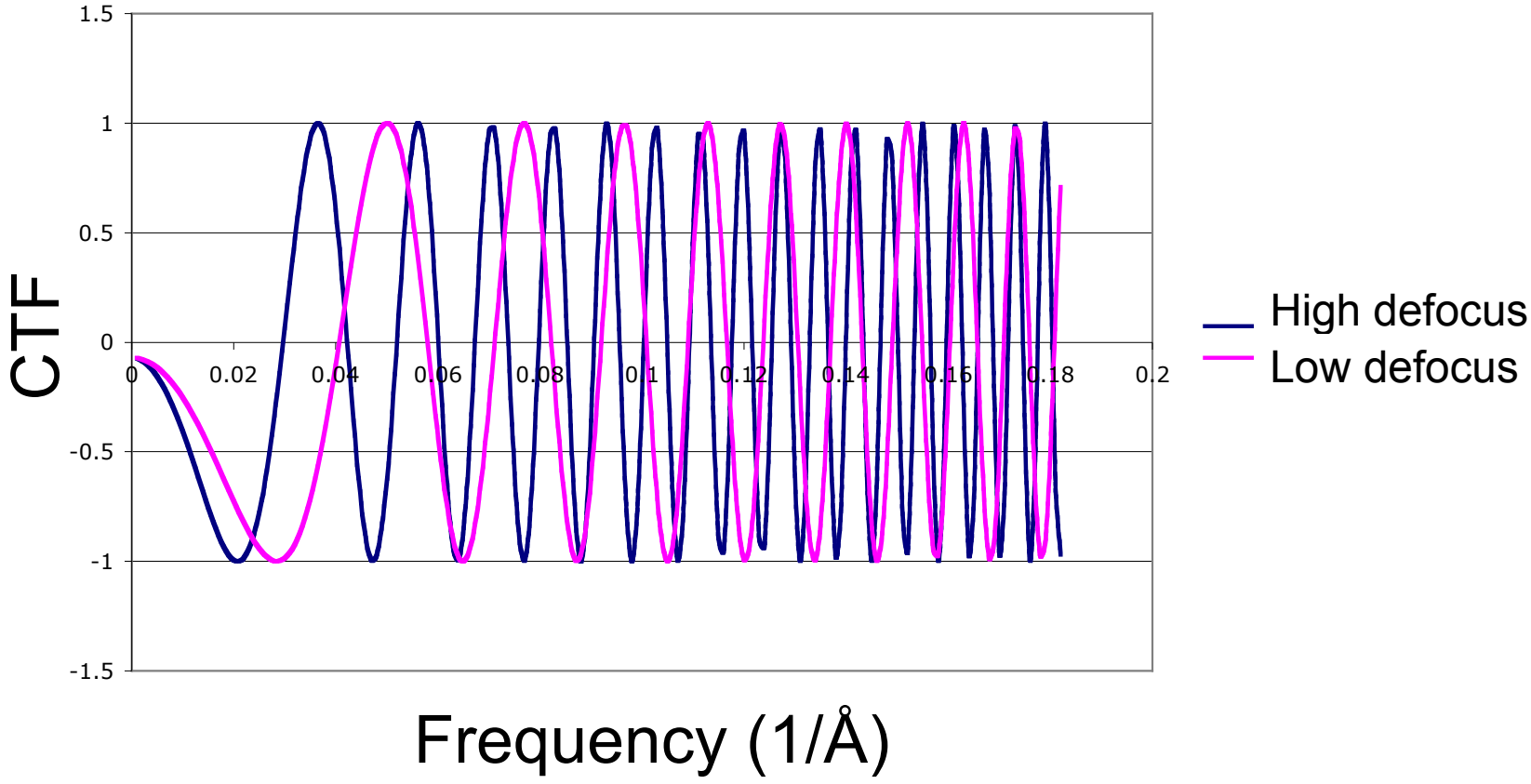
~5 μm



~6 μm

Image: exposure 12 $e^-/Å^2$, 1.4 Å/pixel, 24 e^-/pixel, 200 kV, record on F20 with DE-12
FFT: 2048x2048 pixels, compressed 5x5

Effects of defocus:



Condition	Advantages	Disadvantages
High defocus	• More contrast at low resolution for particle image selection • More contrast at low resolution for image alignment	• More severe coherence envelope at high resolution • Faster oscillations at high resolution makes CTF correction more difficult
Low defocus	• Slower oscillations at high resolution make CTF correction more robust • Less severe coherence envelope provides higher contrast at high resolution	• Little contrast at low resolution for particle image selection • Less contrast at low resolution for image alignment

Suggestions:

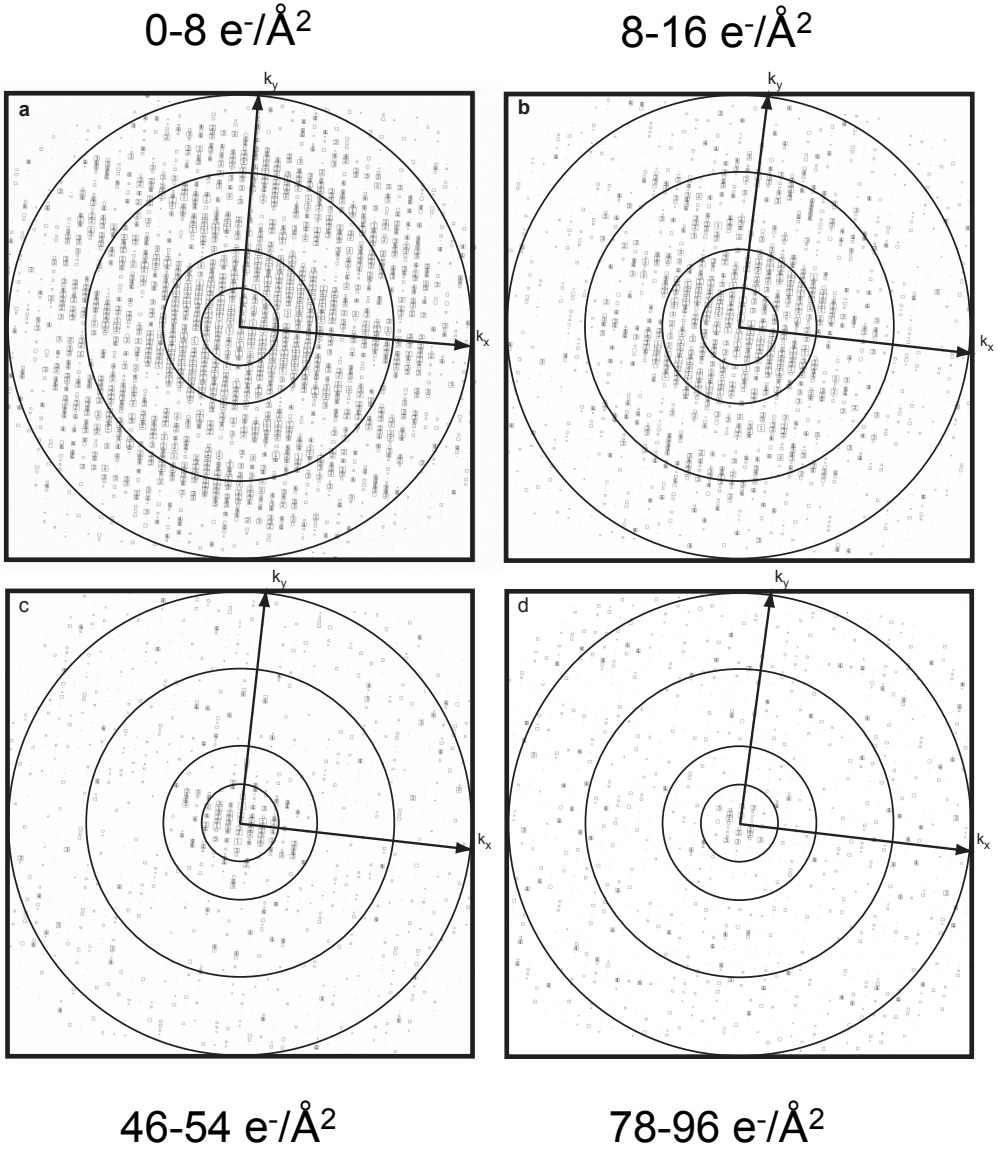
Defocus

- Use as much defocus as you need to select and align particles (but not more)

Radiation damage

(note: no discussion of “what is radiation damage”)

Effects of radiation damage



We all know that radiation damage destroys high-resolution information

Baker, Smith, Bueler and Rubinstein (2010). *J Struct Biol* **169**, 431-7.

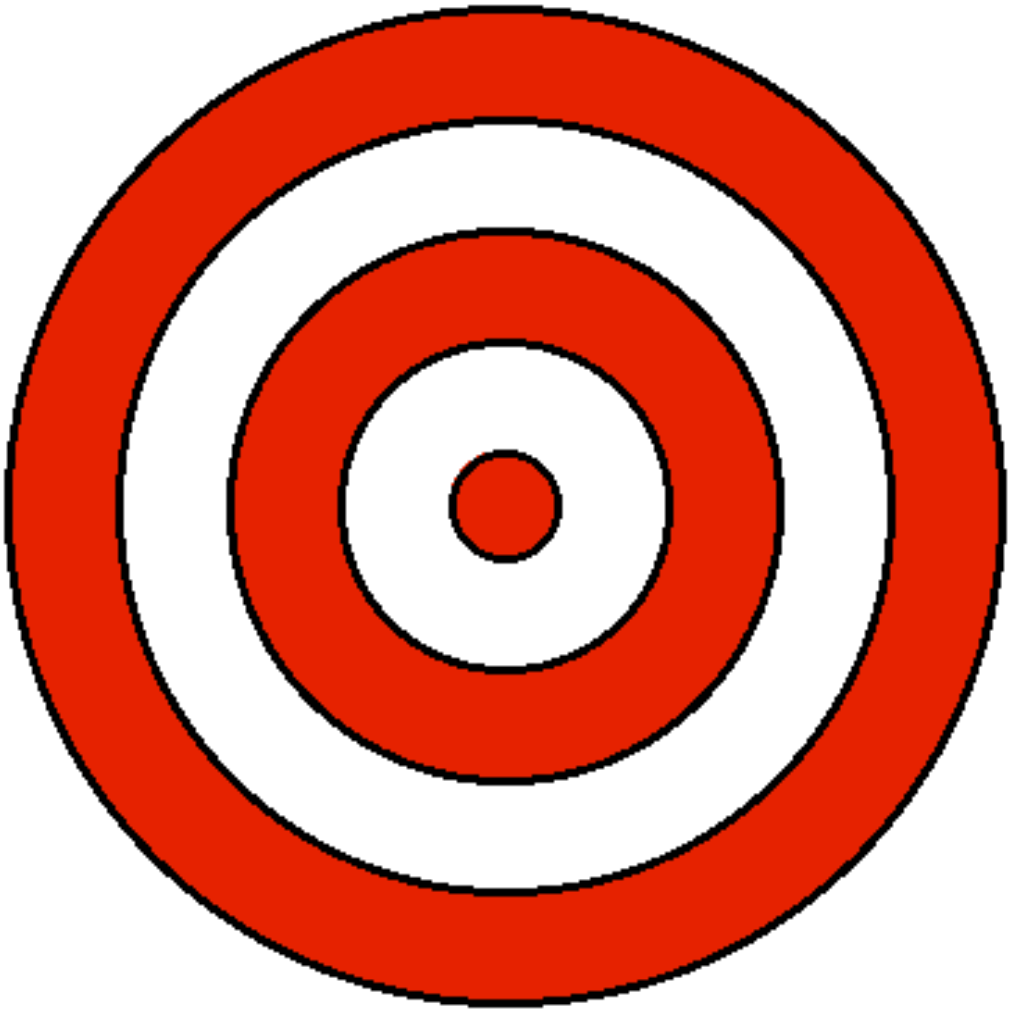
Important terminology:

- Cross section of interaction
- Elastic and inelastic interactions
- Exposure and dose
- Linear energy transfer

Choices:

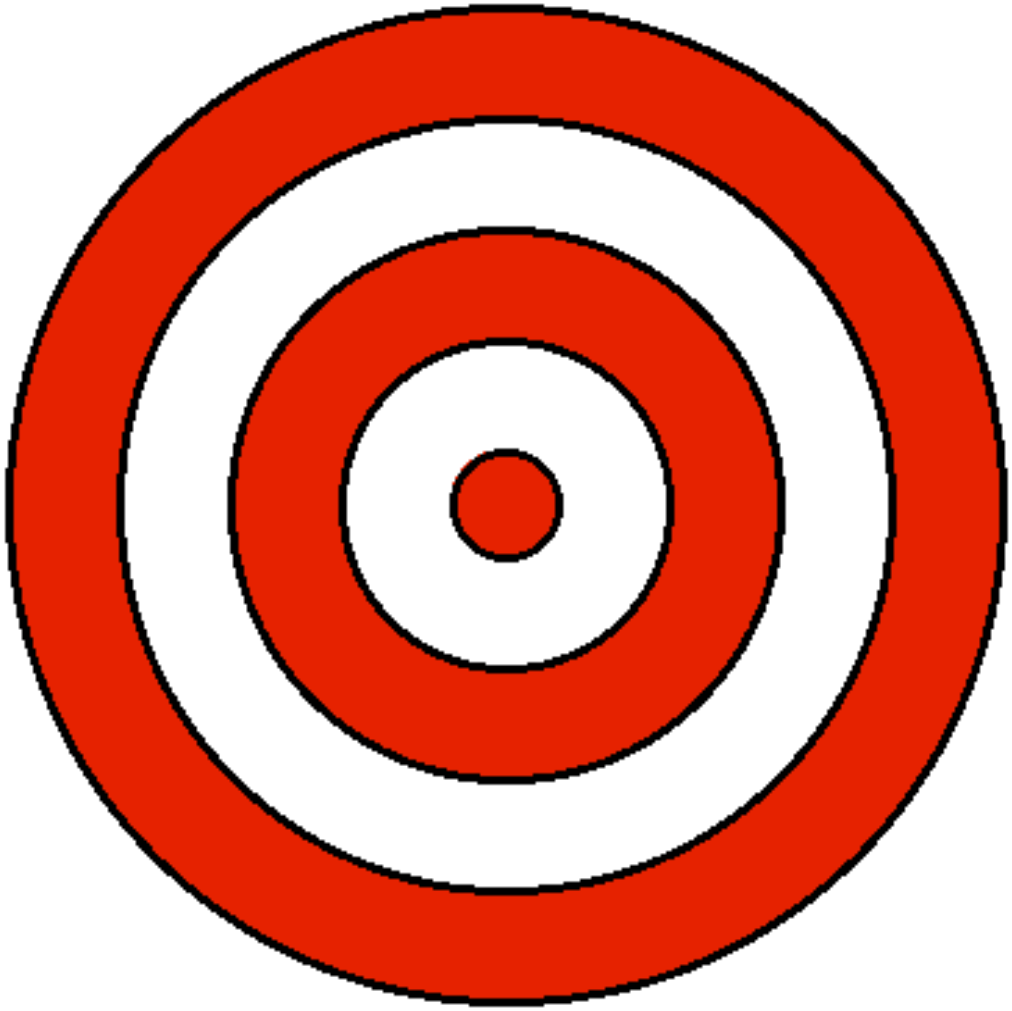
- Microscope voltage
- Electron exposure

Cross-section of interaction: likelihood of electron interacting with the specimen

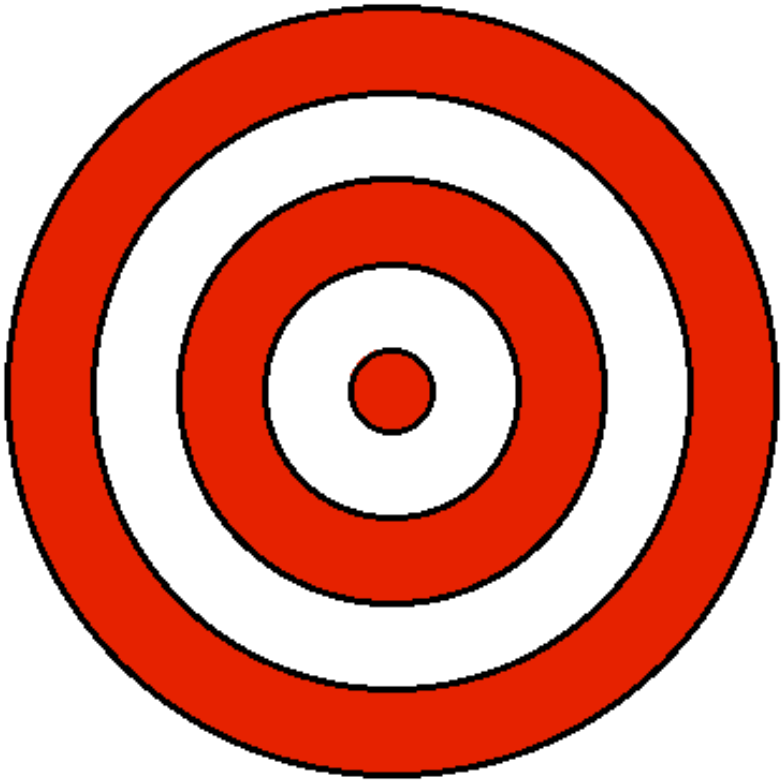


Unit: barn (10^{-28} m^2)

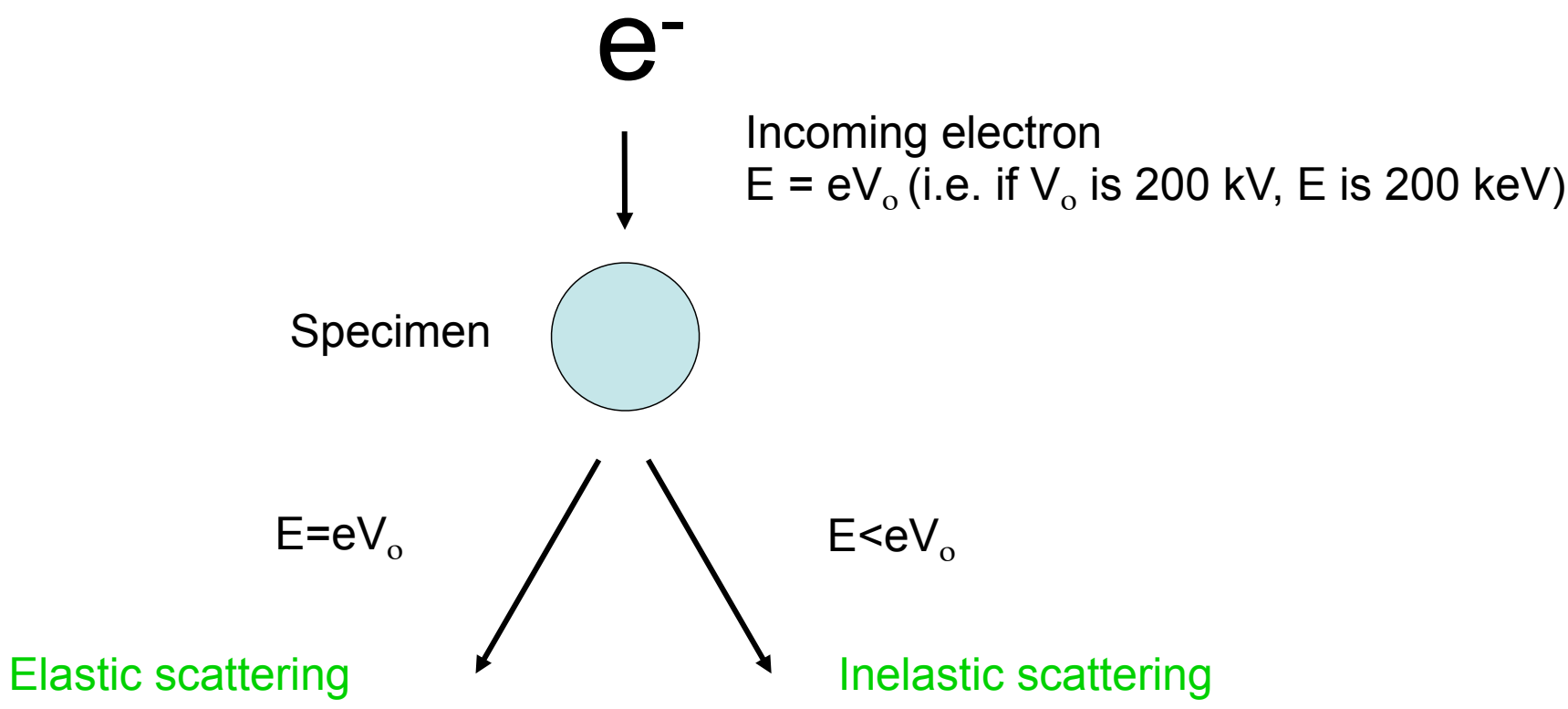
electrons have different cross sections of interaction at different accelerating voltages



100 kV



300 kV



'Knock on damage' (knocking an atom out of its chemical bonds) is a type of elastic event that damages the specimen but cross section is very low for biological specimens at relevant voltages

Exposure: number of electrons incident on specimen ($e^-/Å^2$)

- Ideally measured with a Faraday cup
- Can get a reasonable estimate from a calibrated phosphor screen

Dose: Amount of energy absorbed by specimen (eV/g; J/kg; Gy)

1 eV = 1.602 x 10^{-19} J

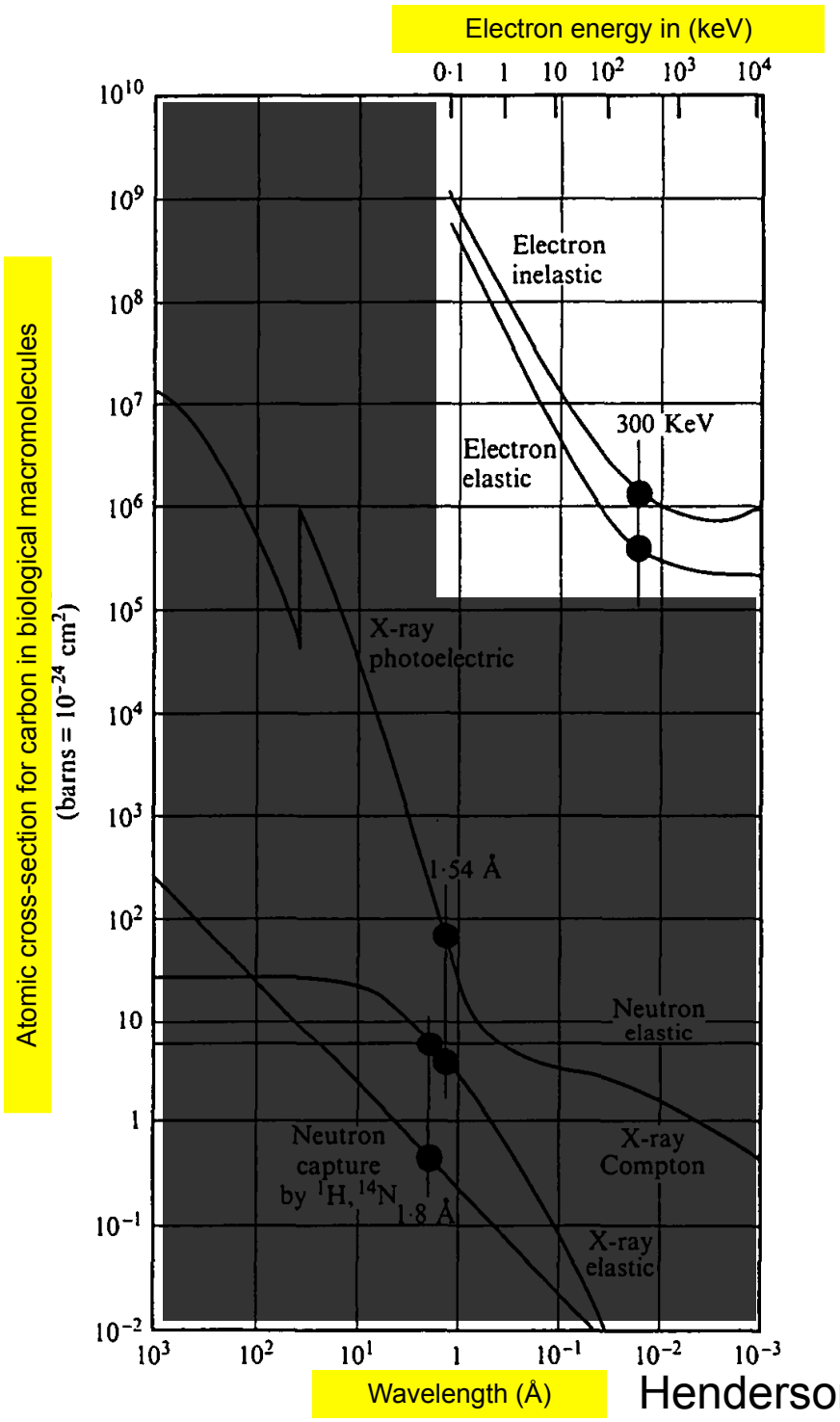
- Will be different for different electron energies
- Will depend on the specimen

Convert between exposure and dose with the Linear Energy Transfer (LET)

- Will depend on the specimen
- Approximations for protein LETs (Glaeser *et al*., 2007):
  - 4.1 MeV cm^2/g at 100 keV
  - 2.8 MeV cm^2/g at 200 keV
  - 2.3 MeV cm^2/g at 300 keV
  - 1.8 MeV cm^2/g at 1 MeV

Exposure LET Dose

$$\frac{N}{Å^2} \times \frac{Å^2}{cm^2} \times \frac{MeV\ cm^2}{g} \times \frac{J}{MeV} \times \frac{g}{kg} = N\ \frac{J}{kg}$$



- Damaging inelastic interactions decrease at higher voltage
- Useful elastic interactions decrease at higher voltage
- Ratio of inelastic:elastic stays approximately constant (~3:1)
- Amount of energy per inelastic interaction stays approximately constant (~20 eV)

Henderson (1995)

Handy “equivalent exposure calculator”

“Equivalent exposures” result in the same number of scattering events and the same amount of radiation damage at different voltages

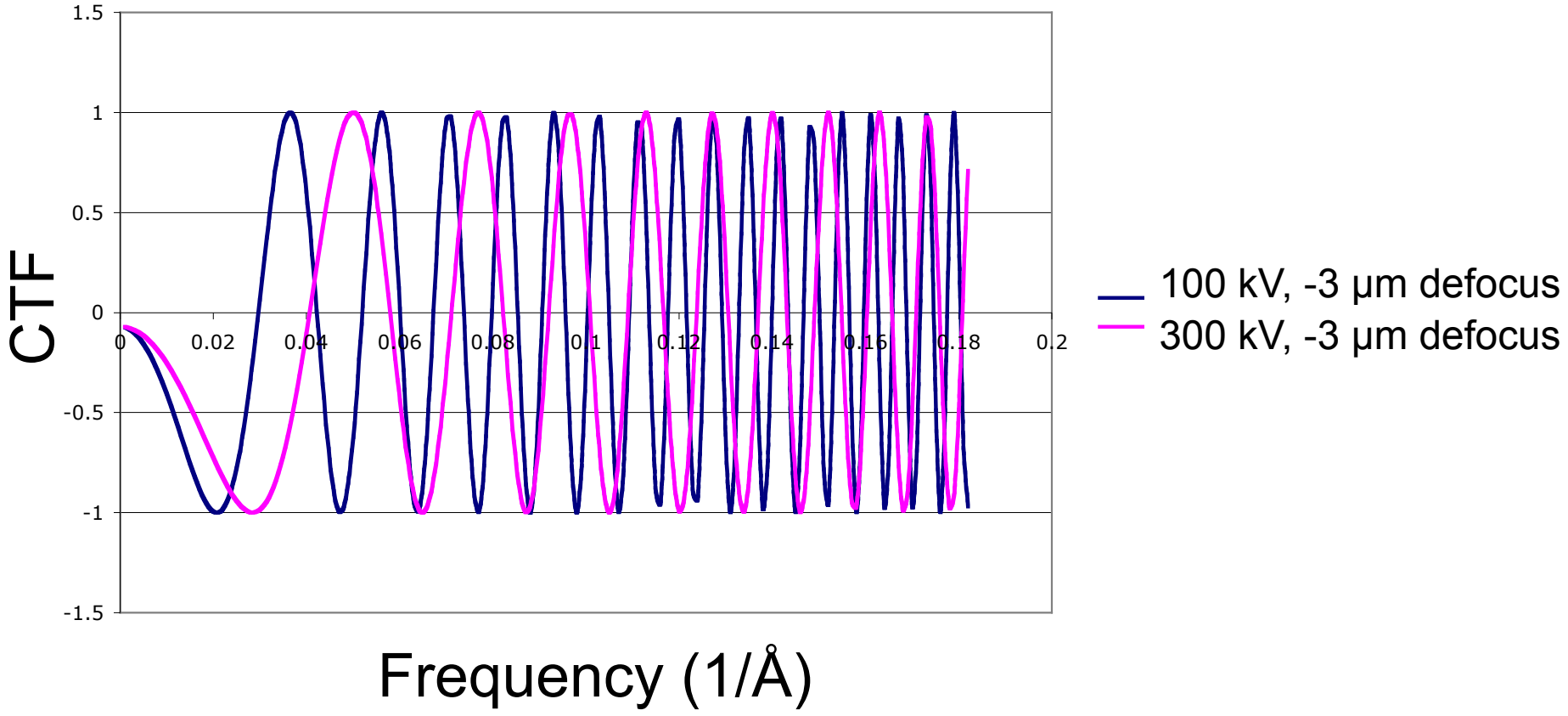
Voltage at which exposure wanted

Voltage at which exposure known

	100 kV	200 kV	300 kV	400 kV	1000 kV
100 kV	1.0	1.5	1.8	2.0	2.3
200 kV	0.68	1.0	1.2	1.3	1.5
300 kV	0.56	0.82	1.0	1.1	1.3
400 kV	0.51	0.75	0.91	1.0	1.2
1000 kV	0.44	0.64	0.78	0.86	1.0

Based on linear energy transfers from Glaeser (2007)

CTF also changes with accelerating voltage



Handy “equivalent defocus calculator”

“Equivalent defocuses” result in the same amount of low-resolution contrast at different voltages

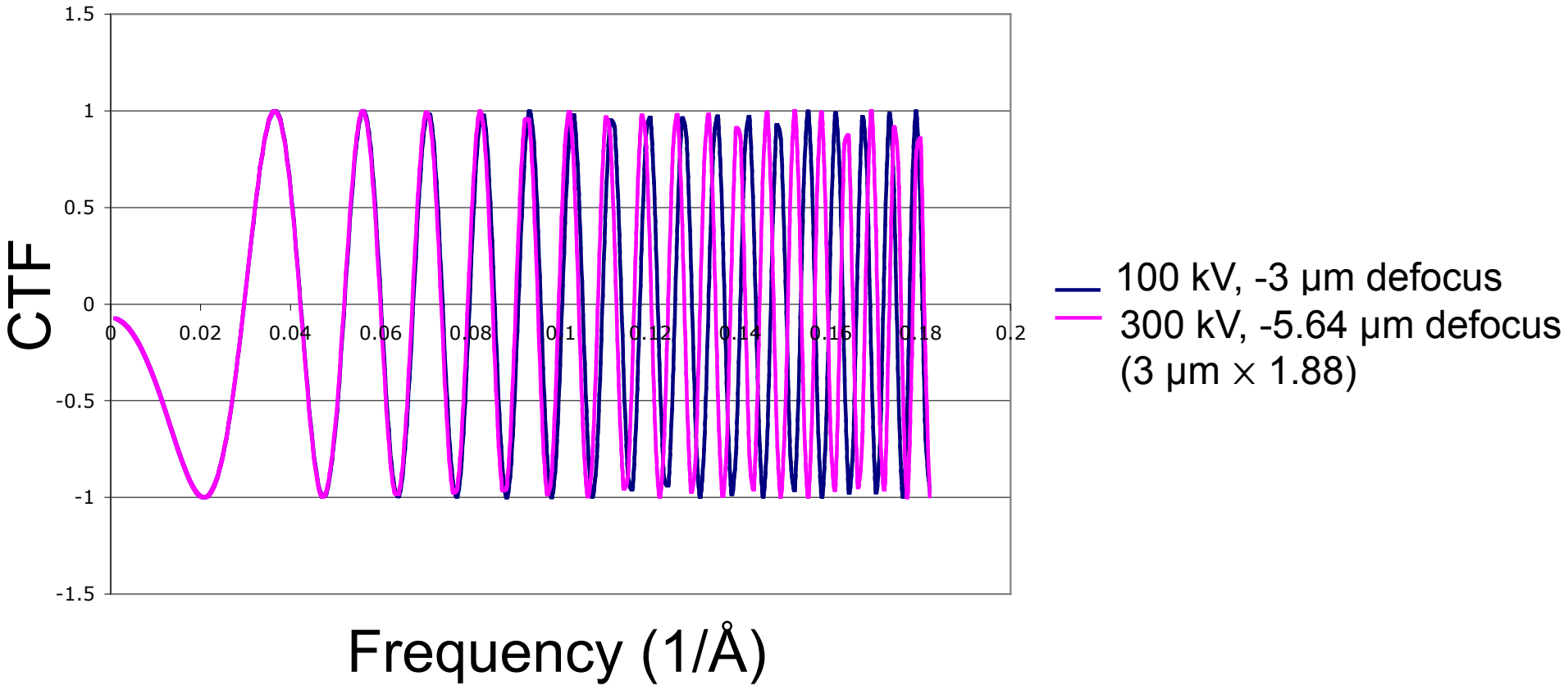
Voltage at which defocus wanted

Voltage at which defocus known

	100 kV	200 kV	300 kV	400 kV	1000 kV
100 kV	1.0	1.47	1.88	2.26	4.25
200 kV	0.678	1.0	1.27	1.53	2.89
300 kV	0.532	0.785	1.0	1.20	2.26
400 kV	0.443	0.633	0.832	1.0	1.89
1000 kV	0.235	0.347	0.442	0.530	1.0

New defocus must keep product of λ and Δz constant

Matching CTFs at different voltages



The CTFs still differ at high resolution because of the different combination of λ and C_s.

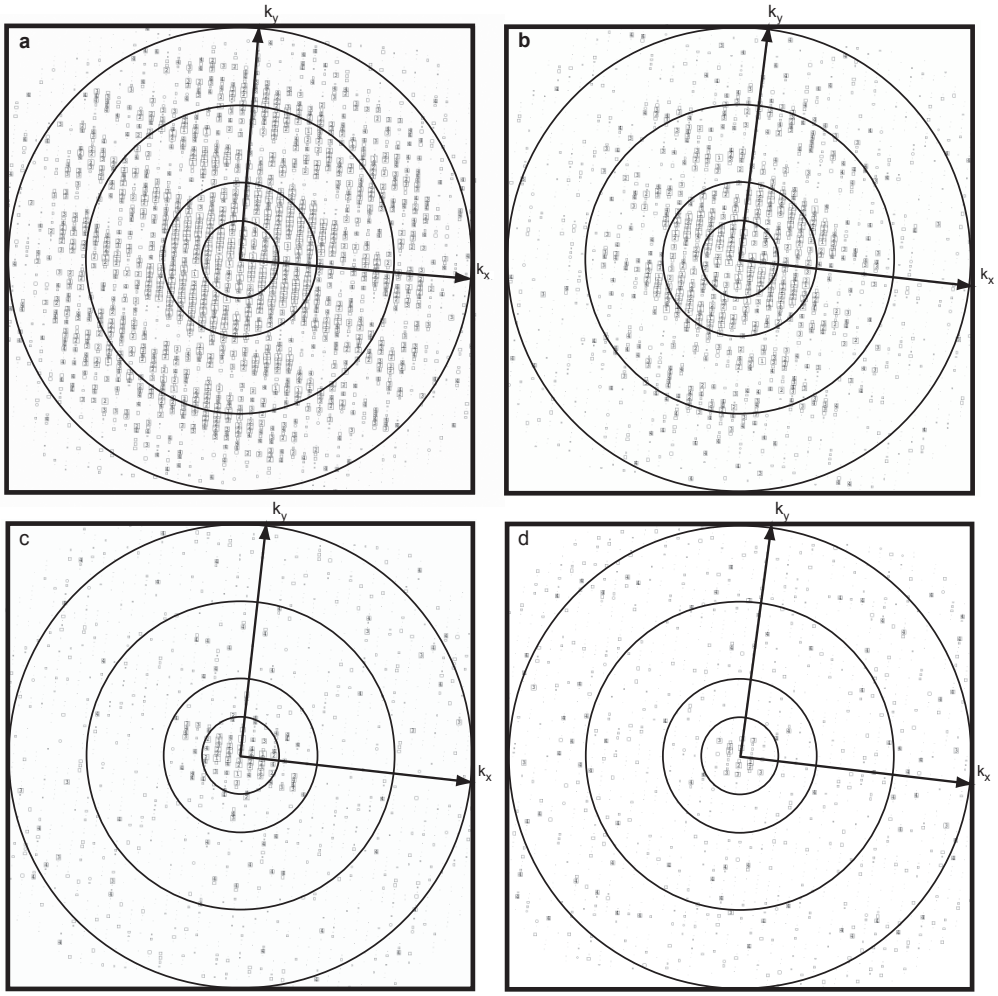
Other voltage considerations

- Ewald sphere curvature is better at higher voltages (high resolution)
- Beam tilt is less severe at higher voltages (high resolution)
- Fewer multiple scattering events at higher voltages (thick specimens)
- Some detectors work better (better DQE) at higher voltages (DDDs)
- Some detectors work better (better DQE) at lower voltages (Film, CCDs)

Optimizing signal-to-noise ratios in images

0-8 $e^-/Å^2$

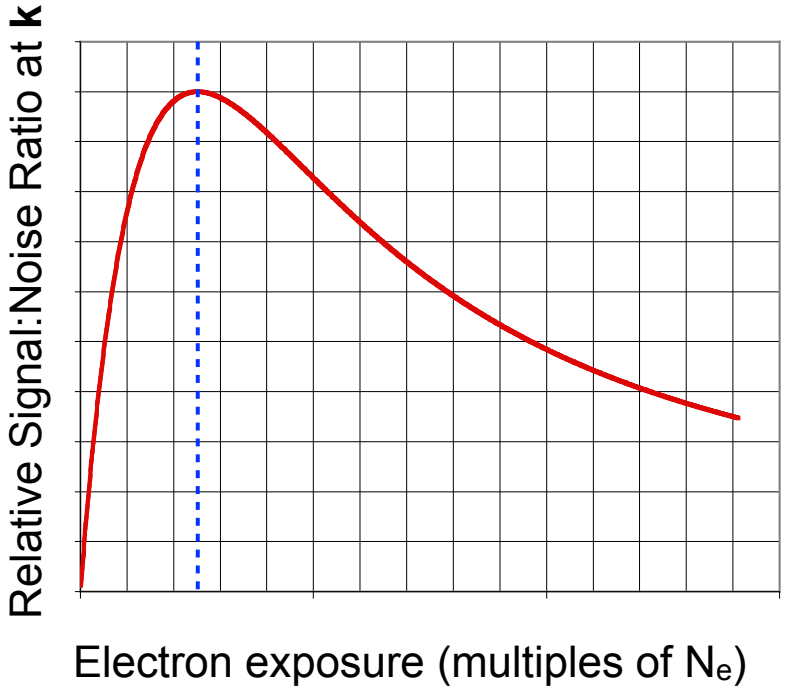
8-16 $e^-/Å^2$



46-54 $e^-/Å^2$

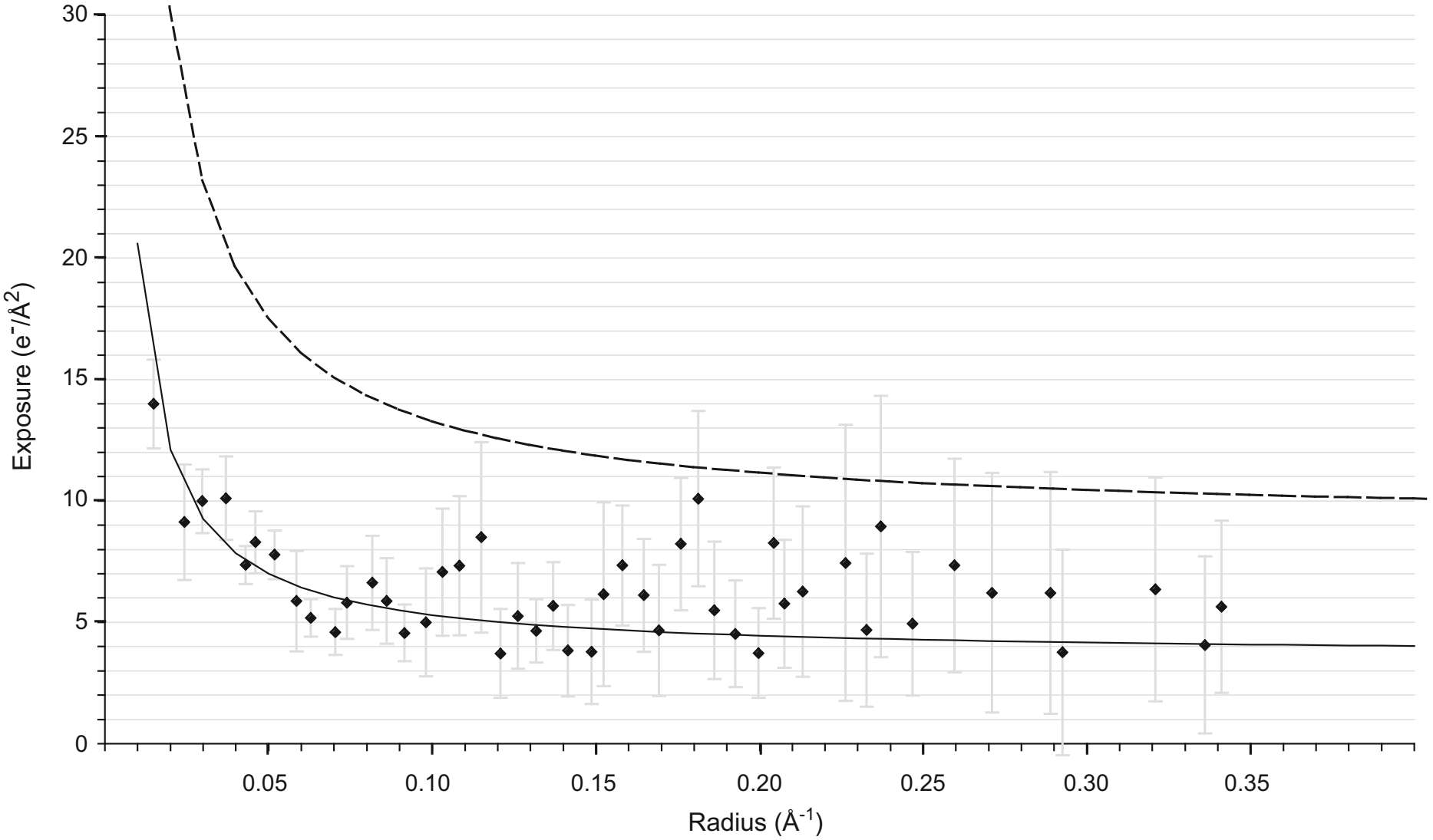
78-96 $e^-/Å^2$

Baker, Smith, Bueler and Rubinstein (2010).
J Struct Biol **169**, 431-7.



Hayward and Glaeser (1979).
Ultramicroscopy **4**, 201-10.

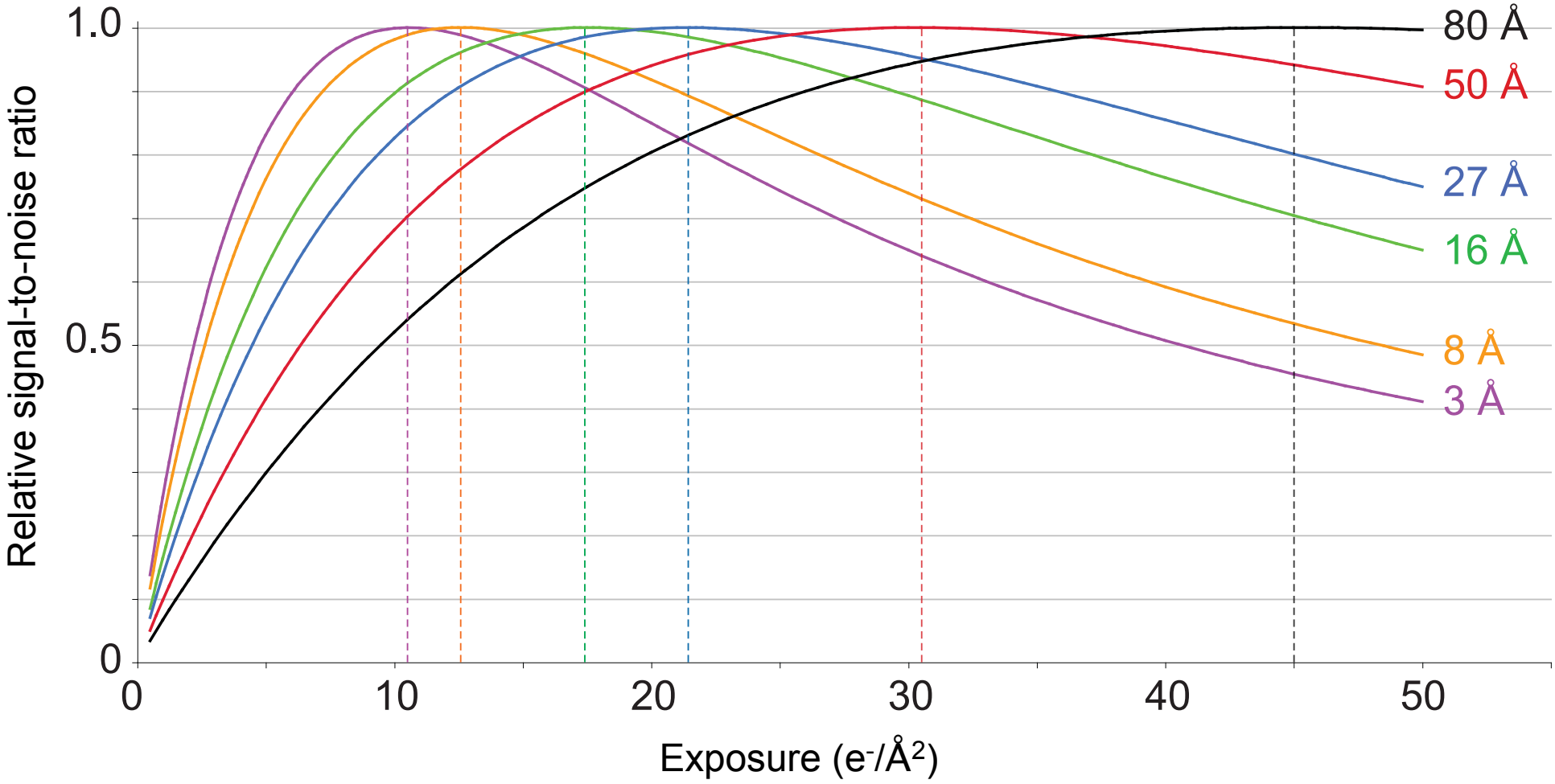
Optimal weighting for radiation damage



Baker, Smith, Bueler, and Rubinstein (2010), *J. Struct. Biol.*, **169**, 431-7.

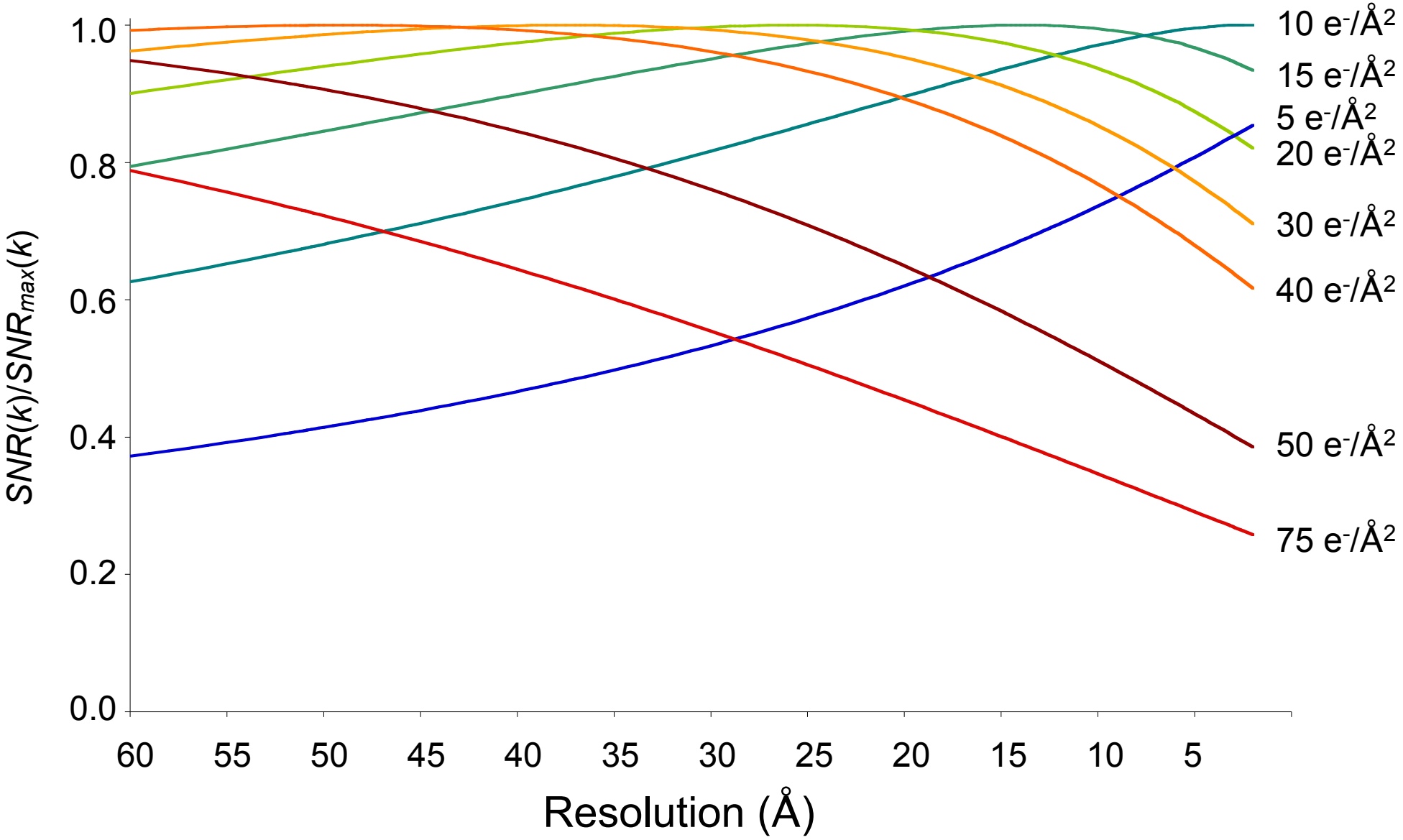
Baker and Rubinstein (2010), *Method Enzymol* **481**, 373-90.

Optimizing signal-to-noise ratios in images



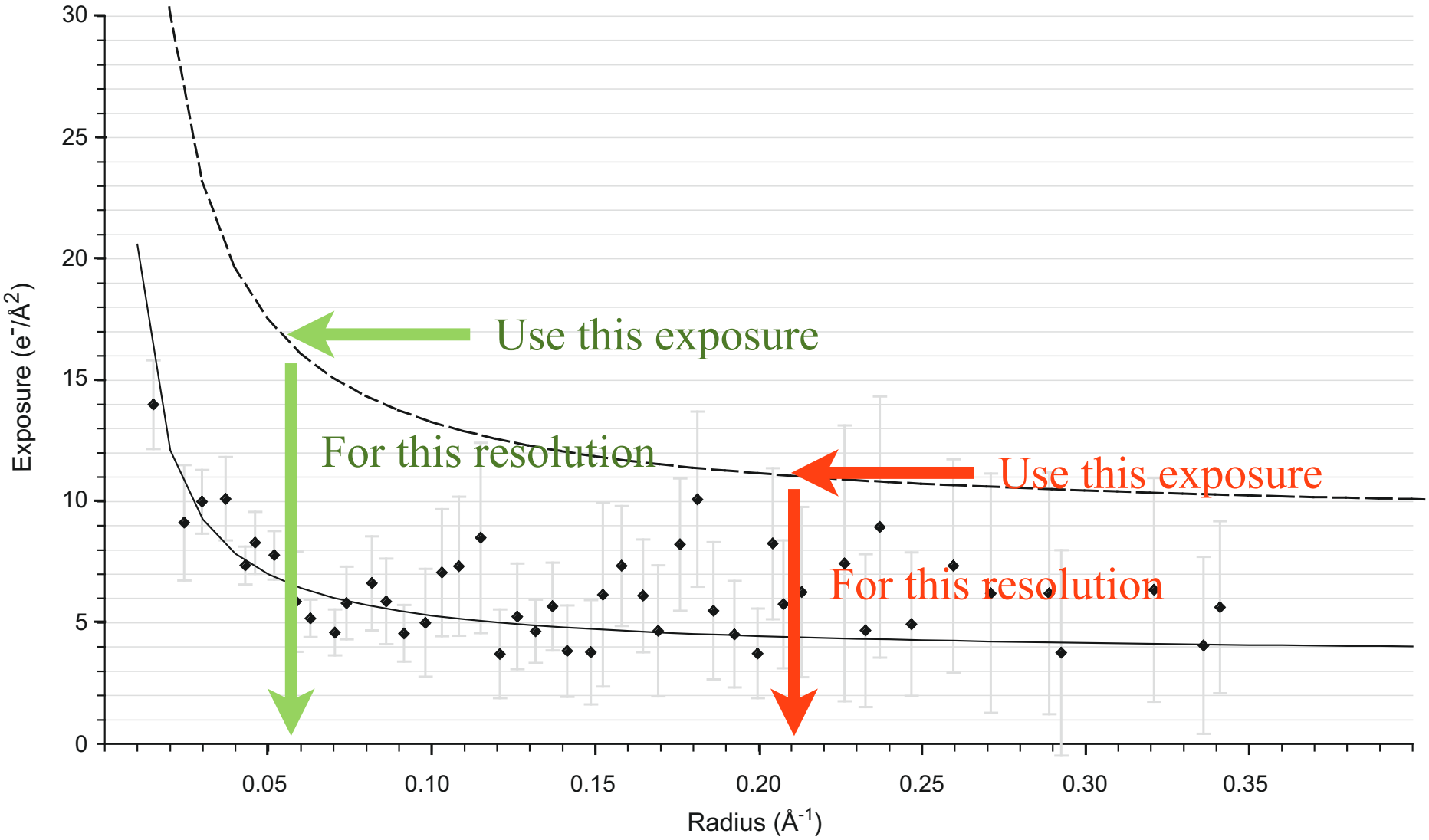
Baker, Smith, Bueler, and Rubinstein (2010), *J. Struct. Biol.*, **169**, 431-7.
Baker and Rubinstein (2010), *Method Enzymol* **481**, 373-90.

Optimizing signal-to-noise ratios in images



Baker and Rubinstein (2010), *Method Enzymol* **481**, 373-90.

Optimal weighting for radiation damage



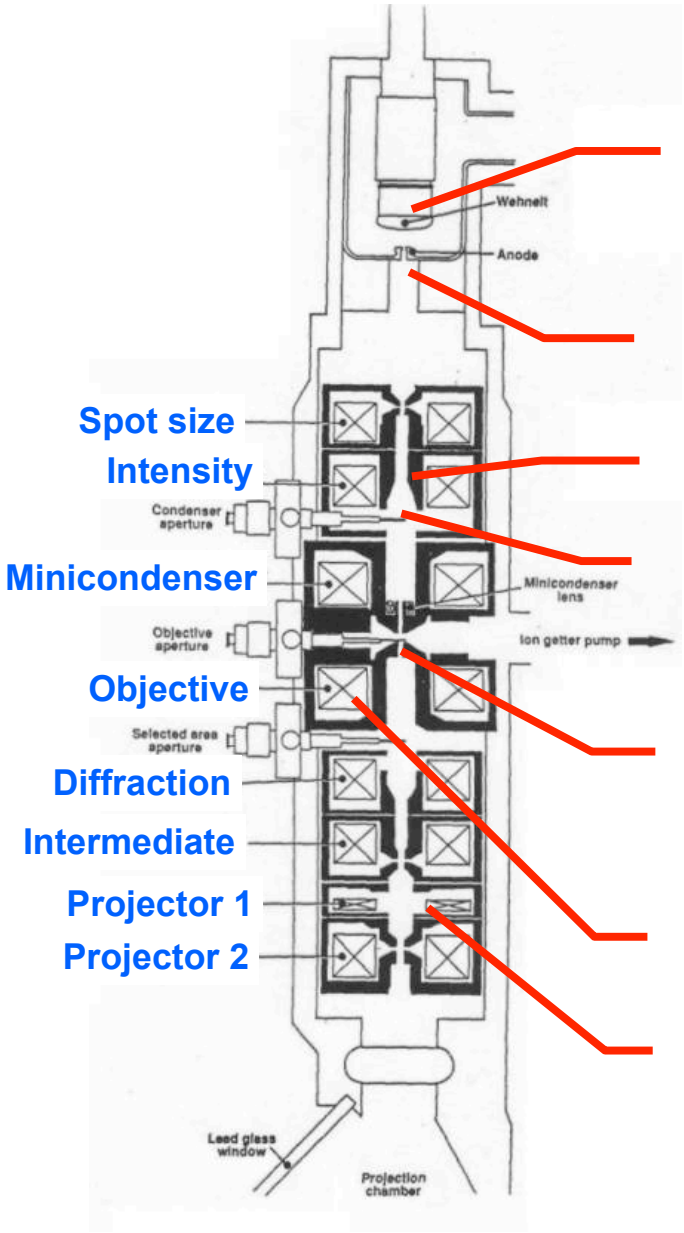
Baker, Smith, Bueler, and Rubinstein (2010), *J. Struct. Biol.*, **169**, 431-7.

Baker and Rubinstein (2010), *Method Enzymol* **481**, 373-90.

Suggestions:

- Use enough exposure to determine particle orientations (but not more)
- Use a DDD in movie mode to optimally weight the exposure at different resolutions

Optimizing image acquisition: be deliberate



Use a gun that gives you good spatial and temporal coherence

Choose your electron exposure depending on your objectives (consider voltage)

Align microscope to prevent on-axis coma

Use a C2 aperture and C2 lens setting to avoid off-axis coma

Ensure specimen does not drift

Choose your defocus (consider voltage)

Avoid turning projector lenses on and off

Acknowledgements:

Current members:
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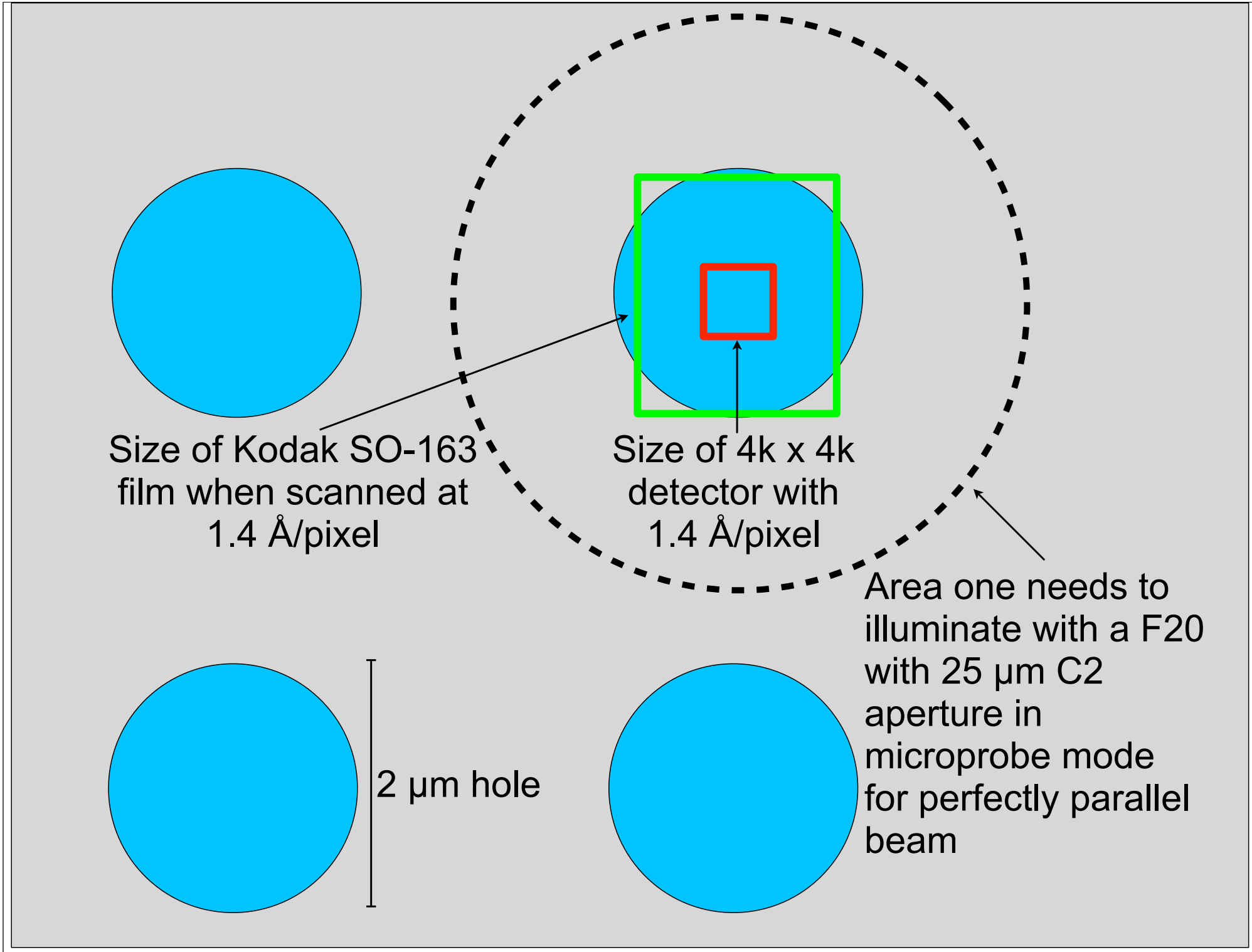
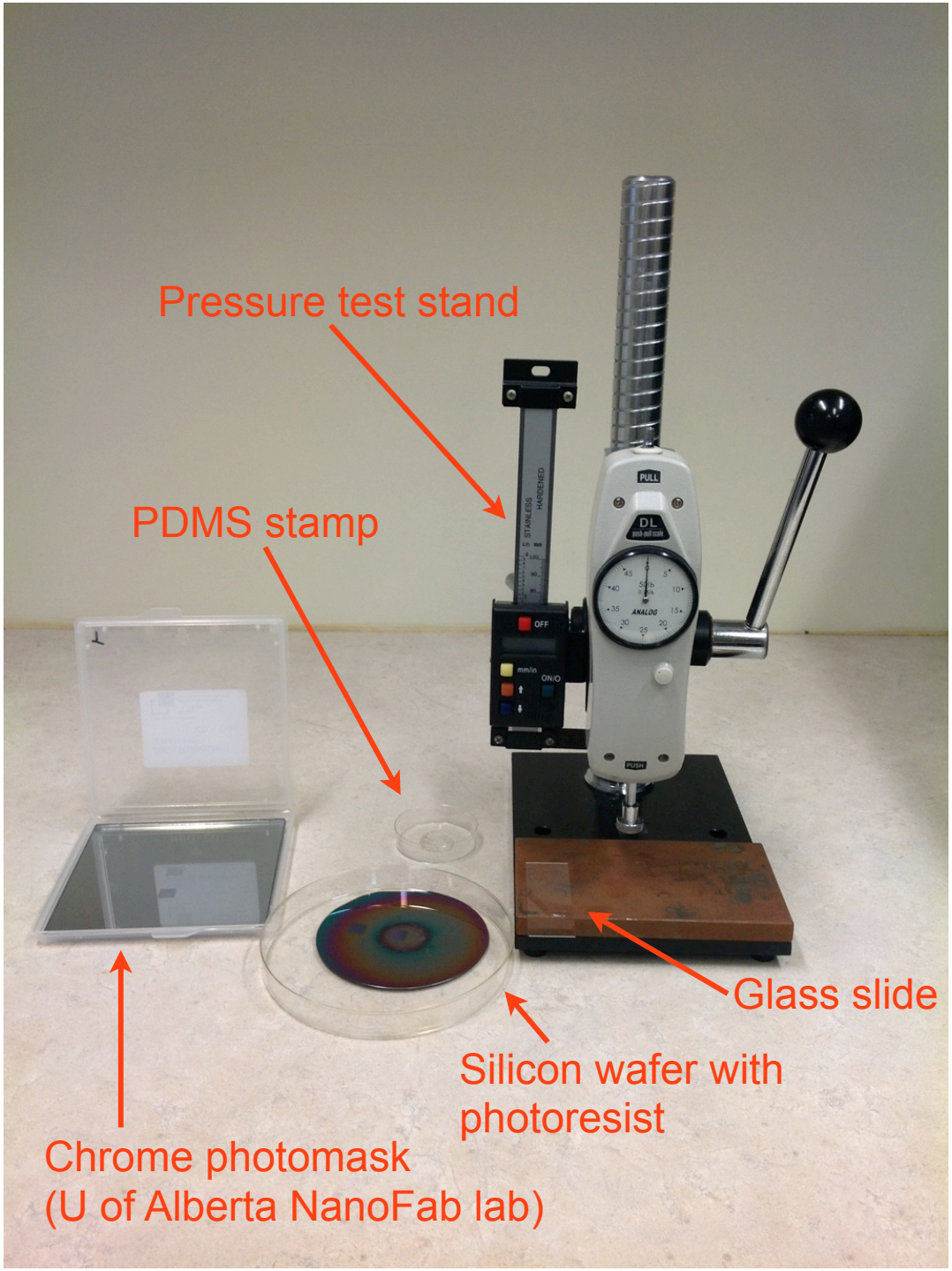
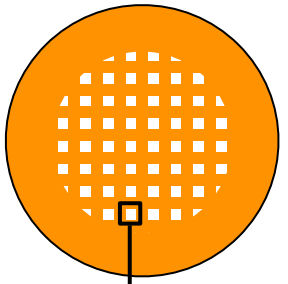
Shawn Keating
Michael Latham
Jianhua Zhao

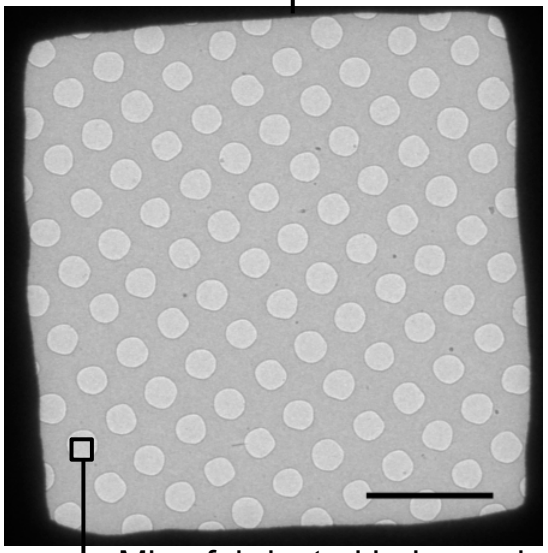
Past members:
Lindsay Baker
Wilson Lau
Nawaz Pirani
Jana Tuhman


Size of Kodak SO-163 film when scanned at 1.4 Å/pixel
Size of 4k x 4k detector with 1.4 Å/pixel
Area one needs to illuminate with a F20 with 25 µm C2 aperture in microprobe mode for perfectly parallel beam
2 µm hole

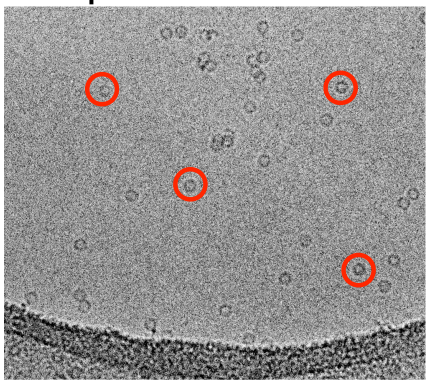




400 mesh
Cu/Rh EM grid



Microfabricated holey carbon film
Chester, Klemic, Stern, Sigworth and Klemic (*Ultramicroscopy* **107,** 685-91, 2005).



Apoferritin in ice