Scattering/Noise and Contrast Transfer and CTF Correction

Lecture 2
Dan Clare
DTP Course

Scattering
Radiation damage and noise
Types of Contrast/Transfer
The Weak Phase Object Approximation
Contrast Transfer Function
Determining Defocus
Tilted Images
Phase Plates
CTF Correction Methods

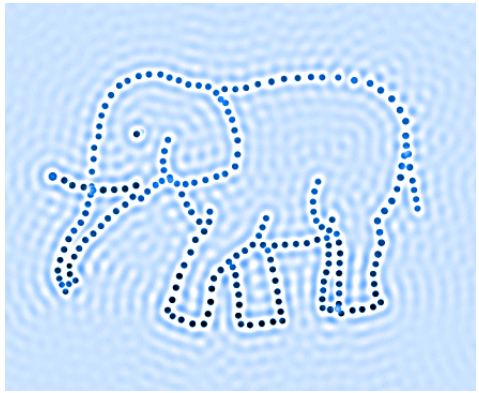


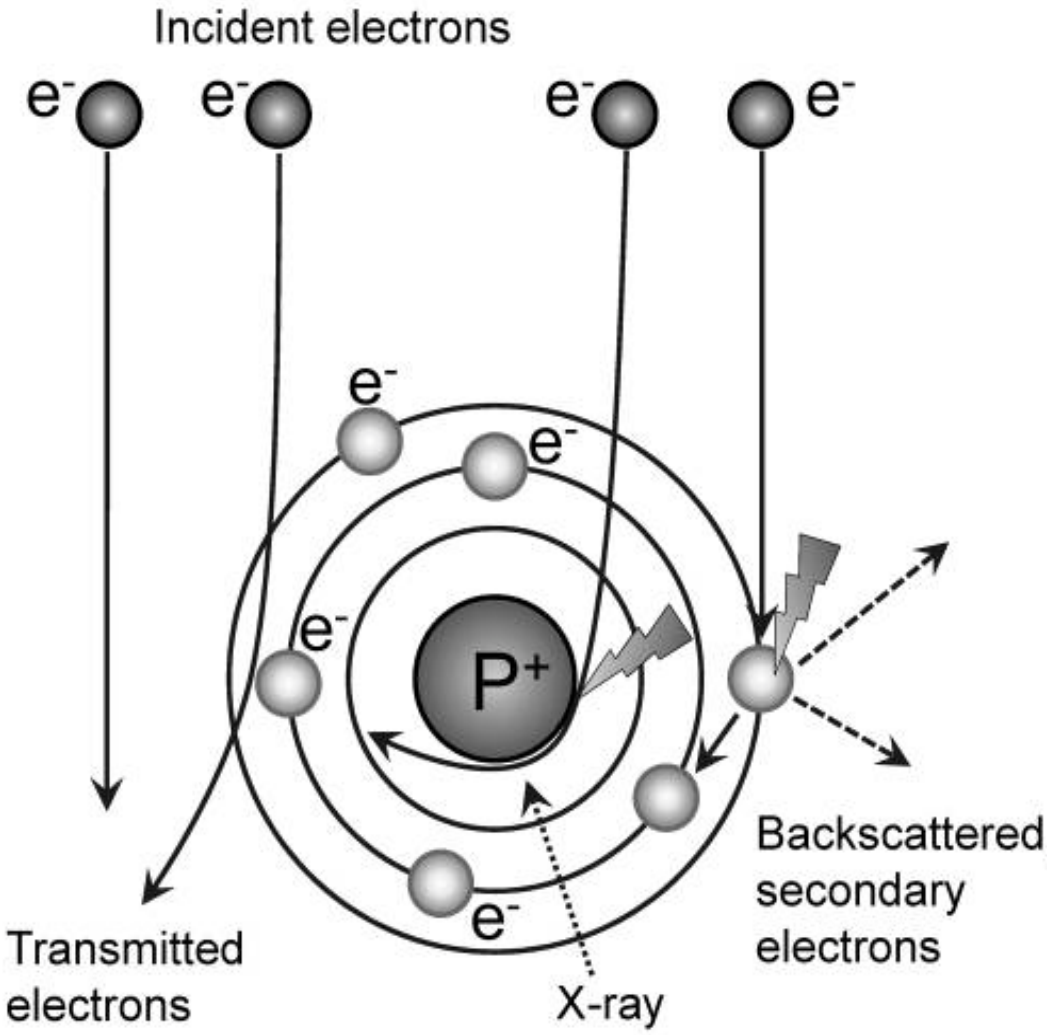
Image processing
for cryo microscopy

Thanks to:

EMBO

Practical Course
Birkbeck College London

Interactions of electrons with the specimen



Orlova and Saibil, 2011

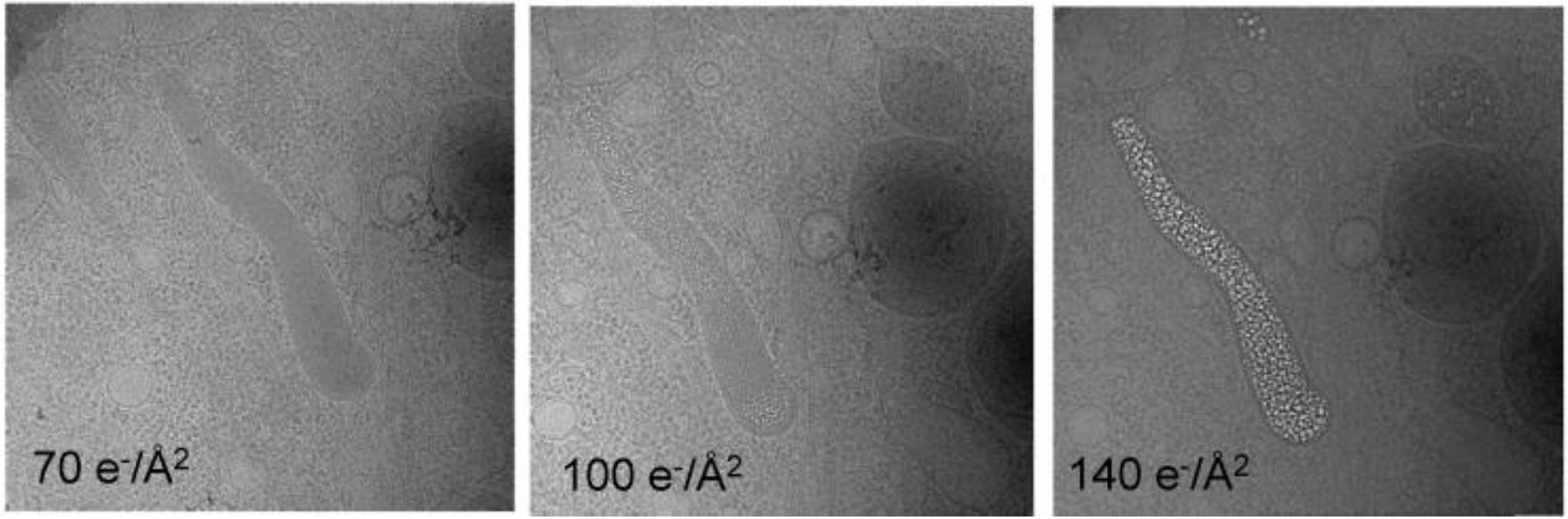
- For biological specimens, for every elastic scattering event that contributes positively to image formation, 3-4 inelastic scattering events also occur.
- Energy transfer from incoming electrons to the specimen can cause radiation damage.
- The angle that electrons are scattered at is proportional to the Z number of the atom – High Z = High angle
- Electron have the properties of both discrete particles and waves

Radiation damage

- Electrons are high energy particles that can transfer energy to the specimen and cause radiation damage.

- Electrons ionize the sample, breaking bonds and producing secondary electrons/free radicals.

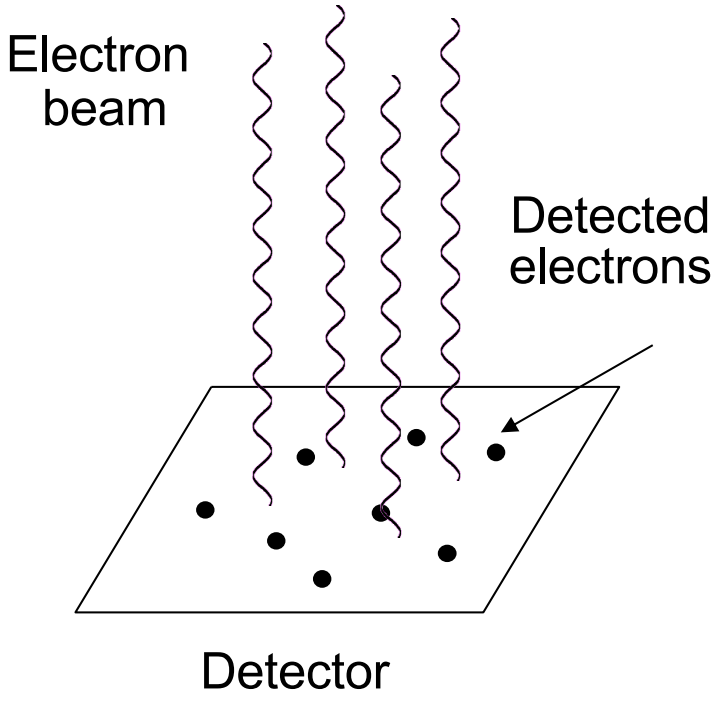
- These then migrate through the specimen and create further



Orlova and Saibil, 2011

What is noise ?

In structural biology, radiation damage limits the useful electron dose from ice-embedded specimens to **~10-30 electrons per Å^2**. Stochastic or **counting noise** arises from variations in the number of electrons which arrive at a particular point, and can be described by a Poisson distribution. The signal to noise ratio improves by a factor of $\sqrt{N}$ as the electron dose increases. However, the dose must be kept low to minimise **radiation damage** to the specimen. Electrons can transfer energy to the specimen, breaking bonds and causing mass loss in biological molecules. For analysis, we assume that the image is the **sum** of the structure information plus statistical noise.



electron dose = average (N) $\pm \sqrt{N}$

N = 100 electrons per unit area		
108	90	103
102	95	114
94	105	89

$\sqrt{N} / N = 10\%$

N = 1000 electrons per unit area		
1025	1007	980
967	894	1016
1010	1046	964

$\sqrt{N} / N = 3.3\%$

Signal, noise and detection

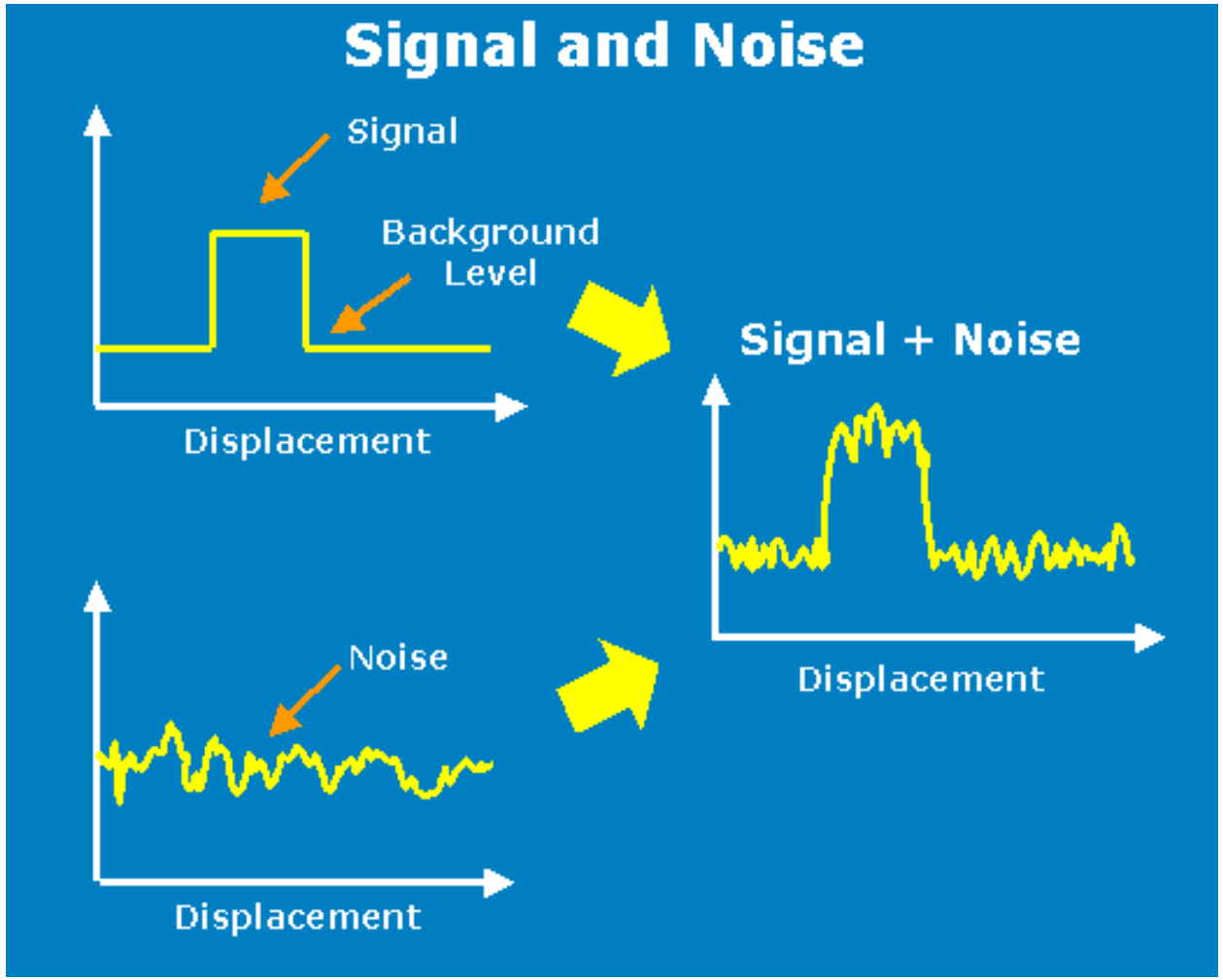
SNR: Signal-to-noise ratio

DQE: detective quantum efficiency

DQE gives a measure of how faithfully the signal is transmitted by the imaging system and is = **SNR_output²** **/ SNR _input²**

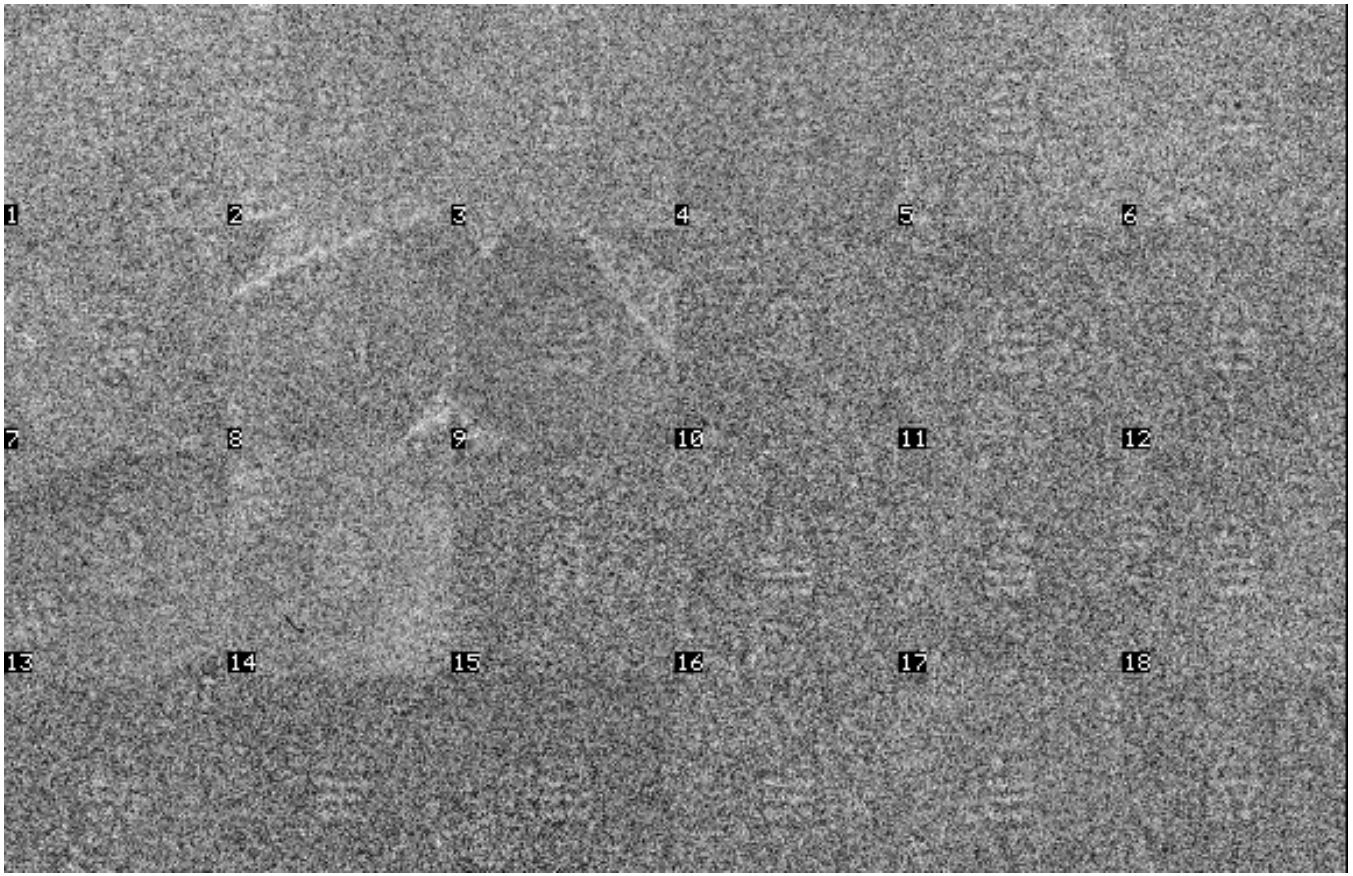
where output = digital image, input = electrons.

If DQE = 0.5, effectively half the electrons have been transmitted.



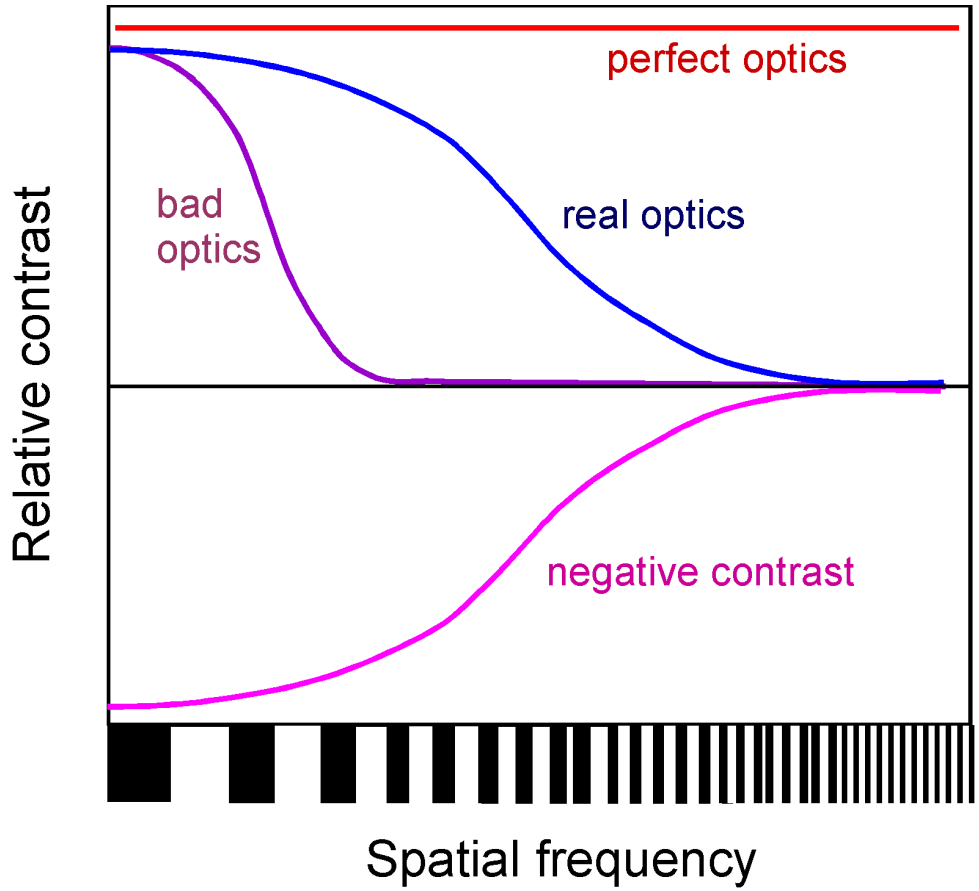
Noise reduction by averaging

Raw images



Averages of 2 5 10 25 200 images

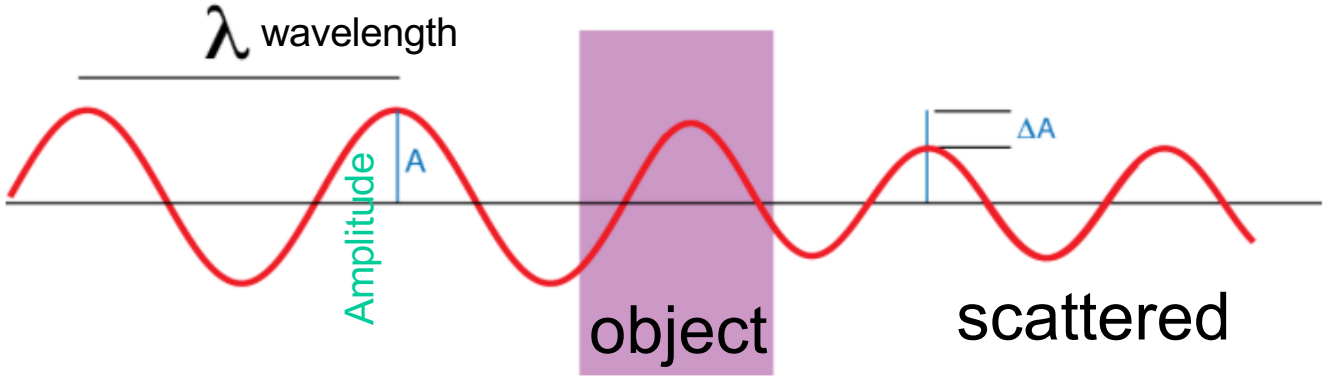
Contrast Transfer



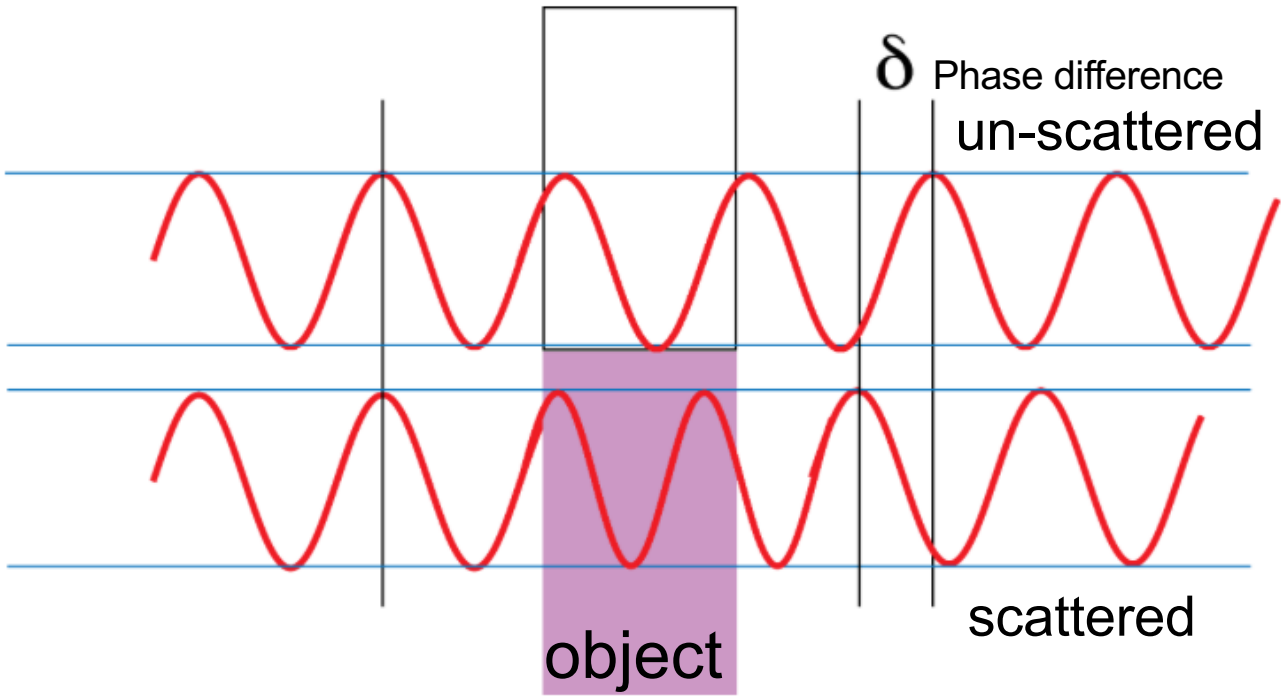
perfect optics | real optics | bad optics | negative contrast

Types of Contrast in TEM

Amplitude Object



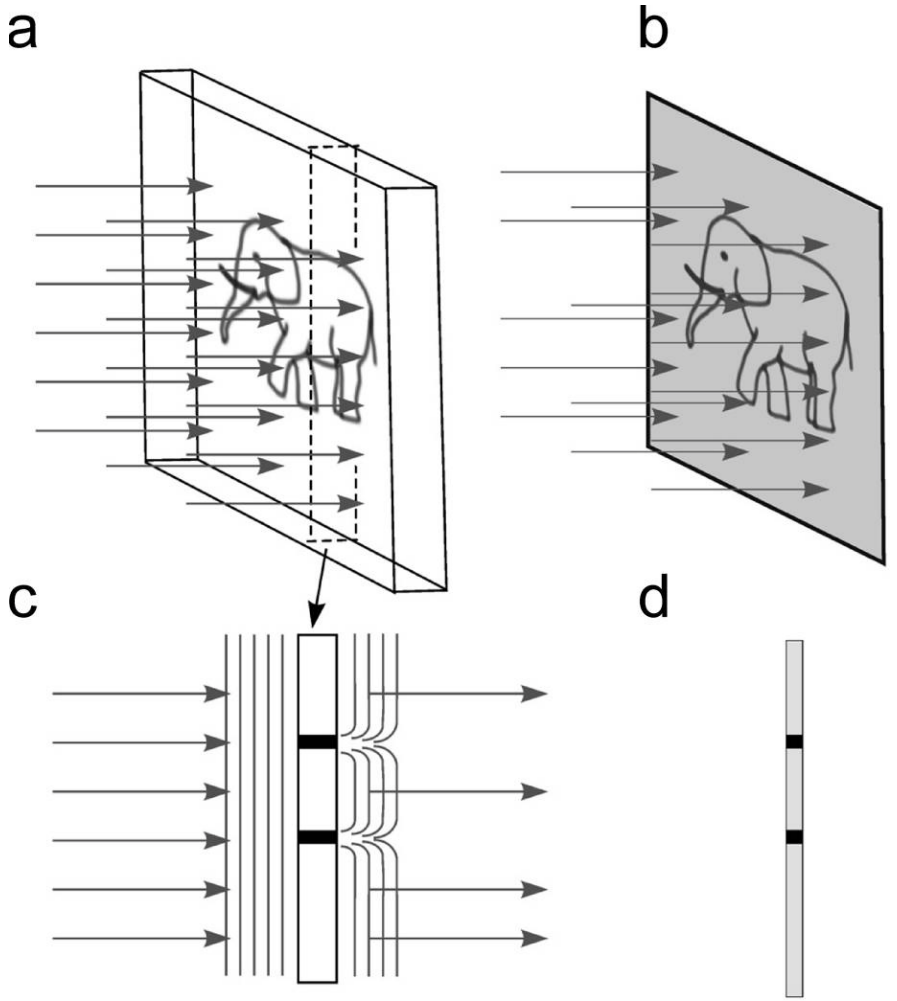
Phase Object



Types of Contrast in TEM

Amplitude contrast

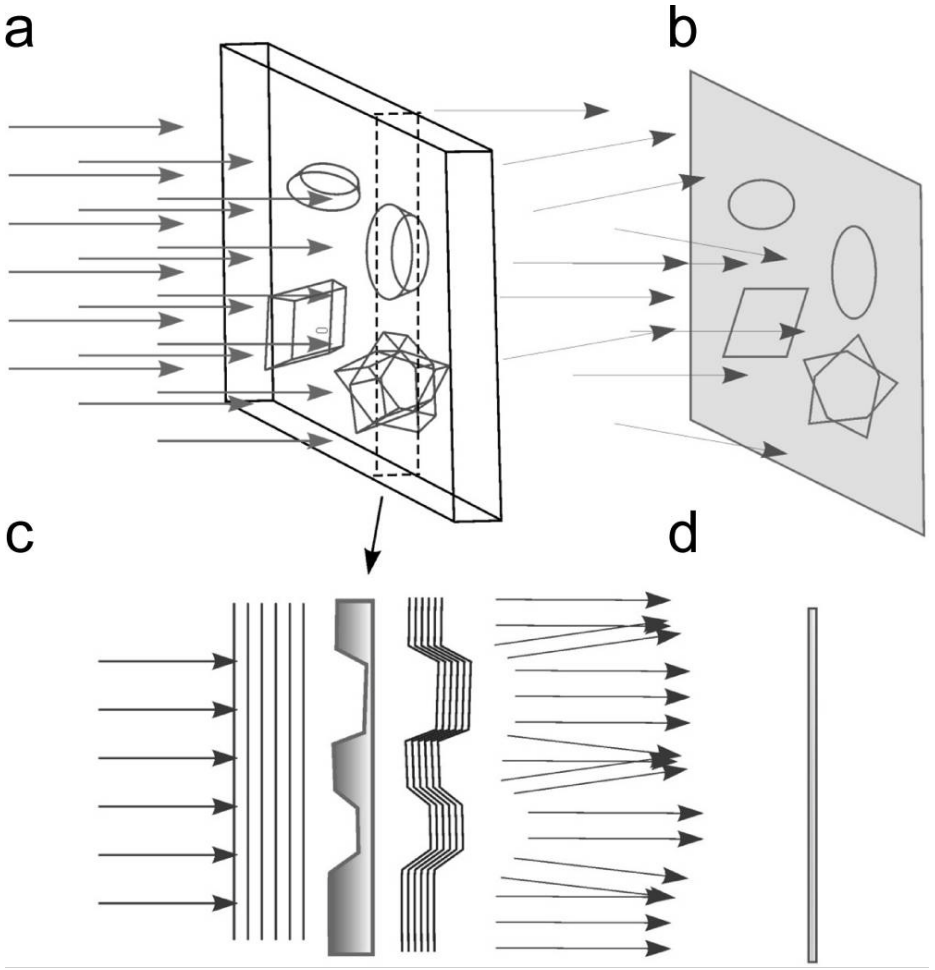
Negative stain EM



- Electron absorbed by sample

Phase contrast

Cryo-EM



- Change in path length of scattered electrons

Orlova and Saibil, 2011

The Weak Phase Object Approximation

Review of :

EM image = projected electron scattering density of object modified by the CTF of the objective lens

If the object is thin and weakly scattering (ie made of light atoms), a simplified form of the CTF function can be derived.

The phase shift $\Phi(\mathbf{r})$ from a **weak phase object** is small, and the wave expression $\psi \exp [i\Phi(\mathbf{r})]$ can be approximated by the series

$$\psi [1 + i\Phi(\mathbf{r}) - \tfrac{1}{2} \Phi(\mathbf{r})^2 + 1/3 \Phi(\mathbf{r})^3 - \ldots]$$

Because the phase shift is small, the 3rd order and higher terms can be ignored.

This approximation, combined with the phase shift introduced by **spherical aberration of the objective lens**, leads to the expression for the *phase contrast transfer function*, given on the next slide.

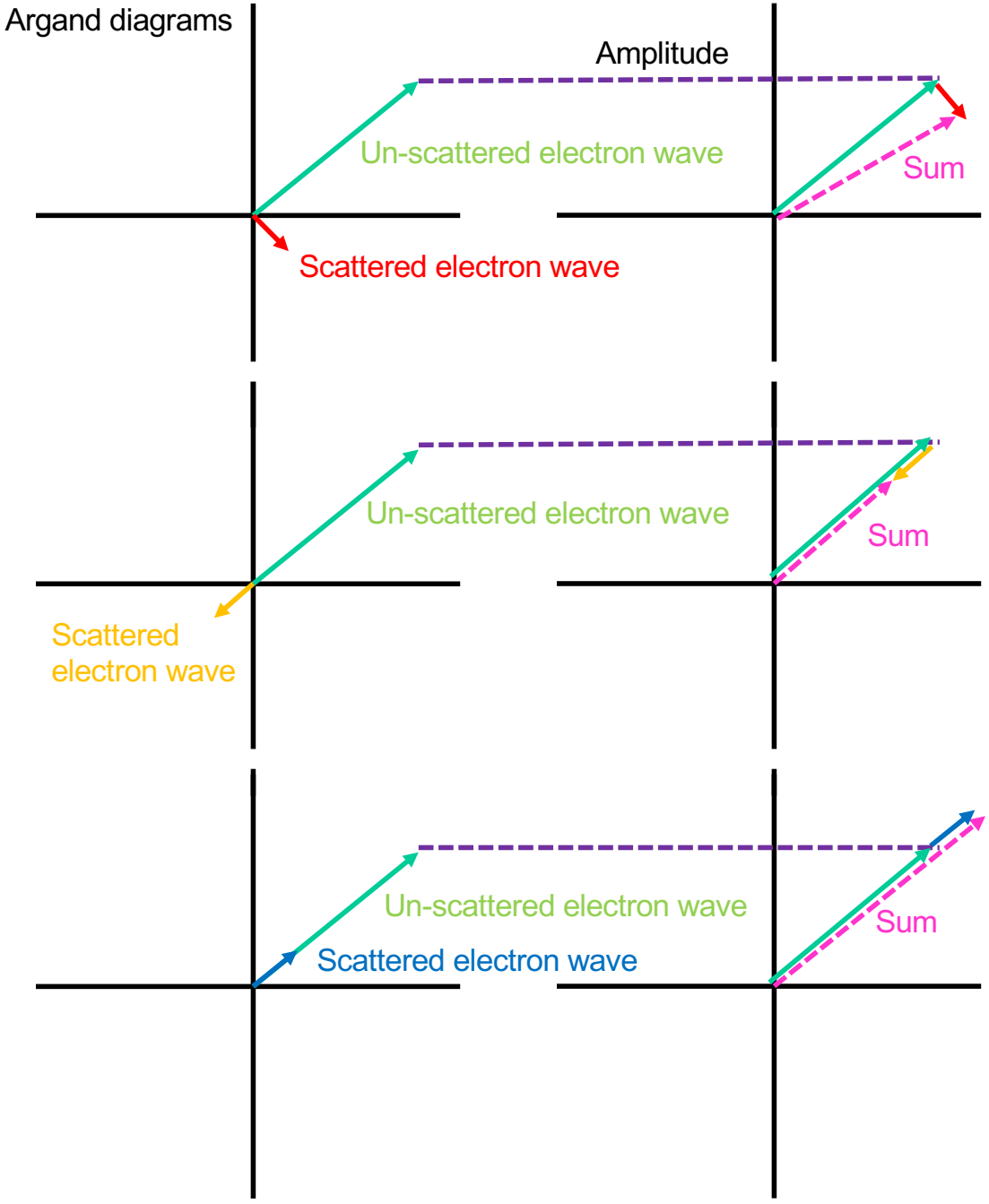
Phase CTF formula from the Weak Phase Object Approximation

$$\text{Phase CTF} = -2 \sin [\pi(\Delta z \lambda q^2 - C_s \lambda^3 q^4/2)]$$

C_s – spherical aberration coefficient
ΔZ – defocus
q – spatial frequency
λ – electron wavelength

- The only variable that we have to determine during CTF determination is the defocus (varied during the experiment)

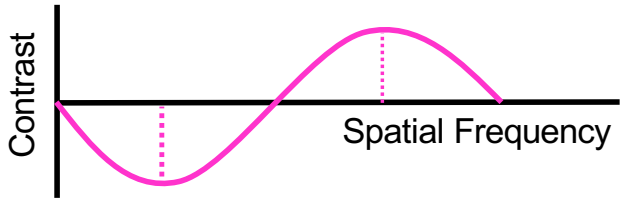
Phase Contrast in TEM



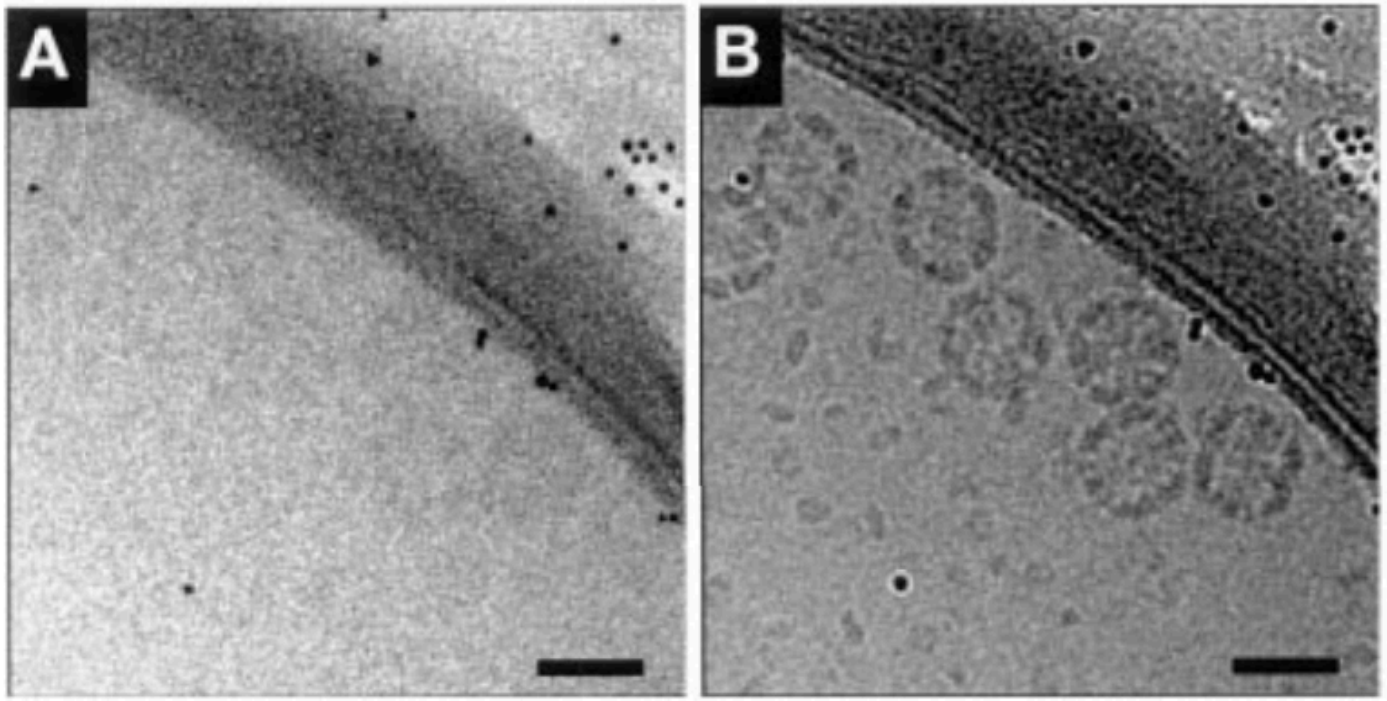
No additional phase shift from the path length difference between the scattered and un-scattered electron wave (low scattering angles) = no contrast

Additional 90° phase shift from the path length difference between the scattered and un-scattered electron wave = negative contrast

Additional 270° phase shift from the path length difference between the scattered and un-scattered electron wave = positive contrast



Why Defocus an Image?

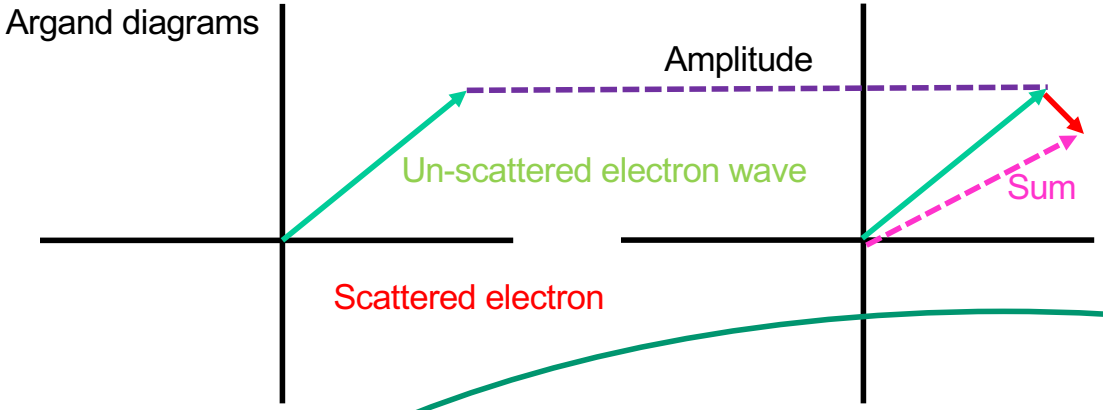


2 μm

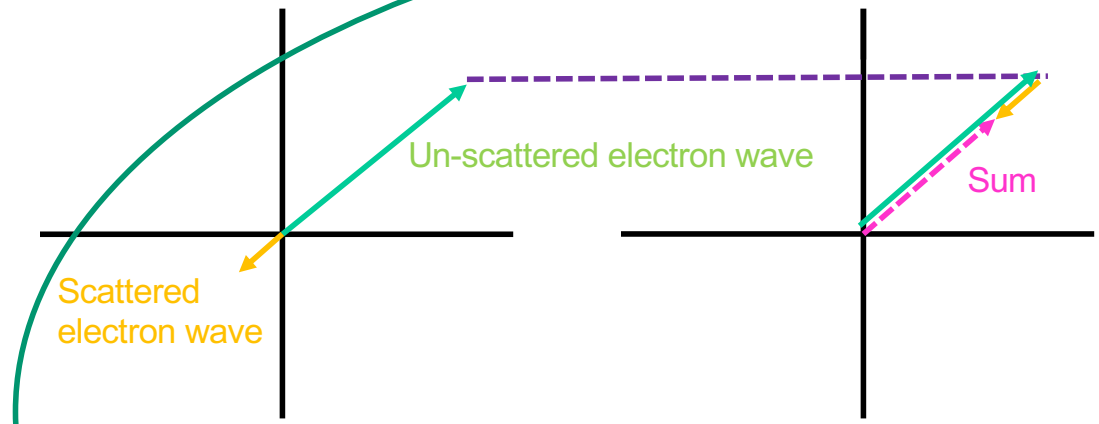
7 μm

Tricorn protease, Walz, J et al (1997) Mol Cell 1, 59-65

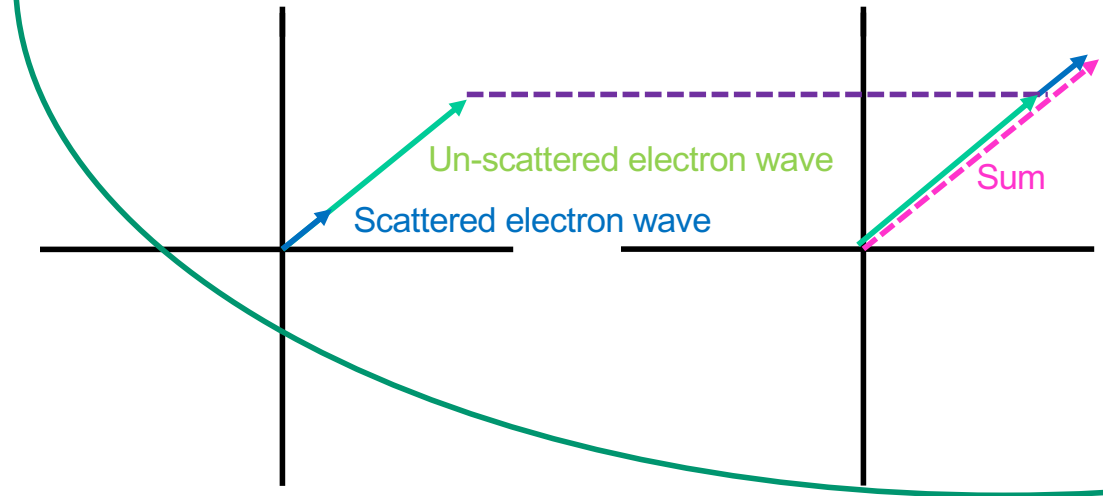
Phase Contrast in TEM



No additional phase shift from the path length difference between the scattered and un-scattered electron wave (low scattering angles) = no contrast

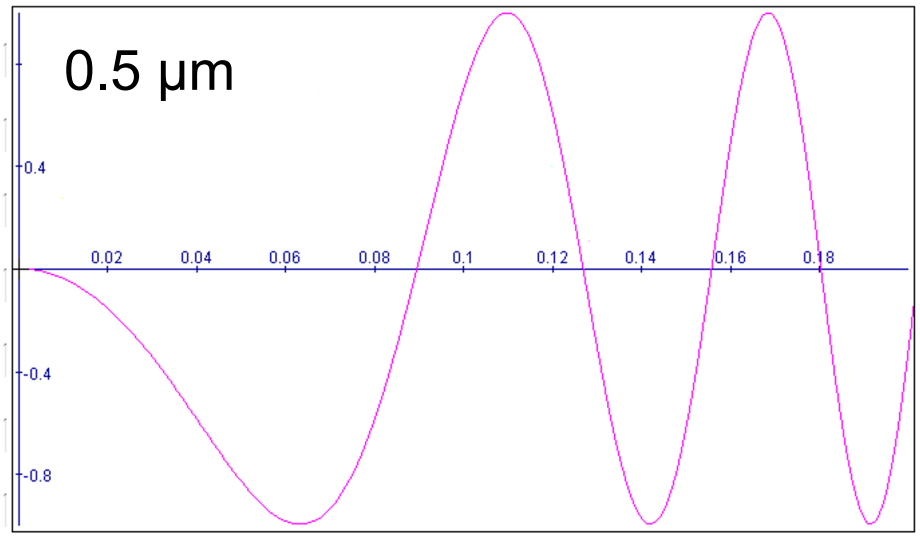


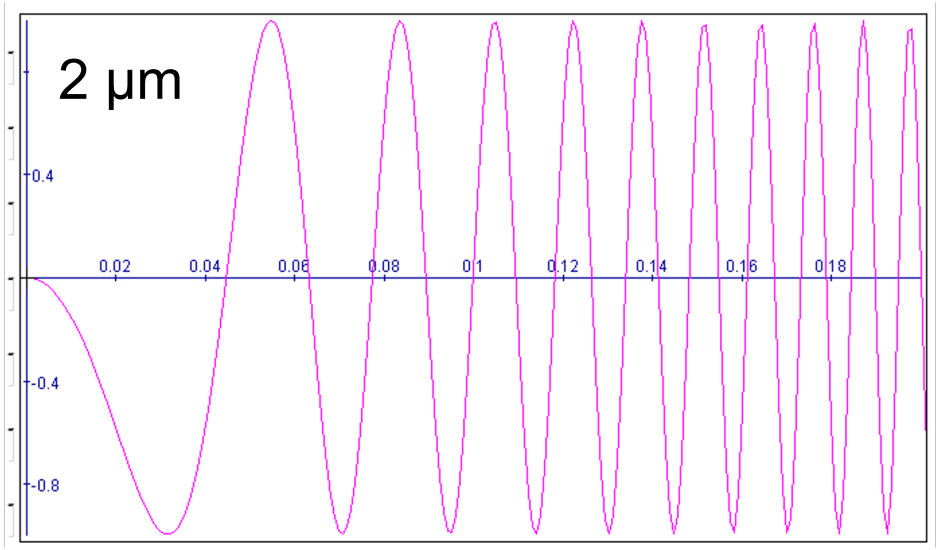
Additional 90° phase shift from the path length difference between the scattered and un-scattered electron wave = negative contrast
Shifted towards lower spatial frequencies

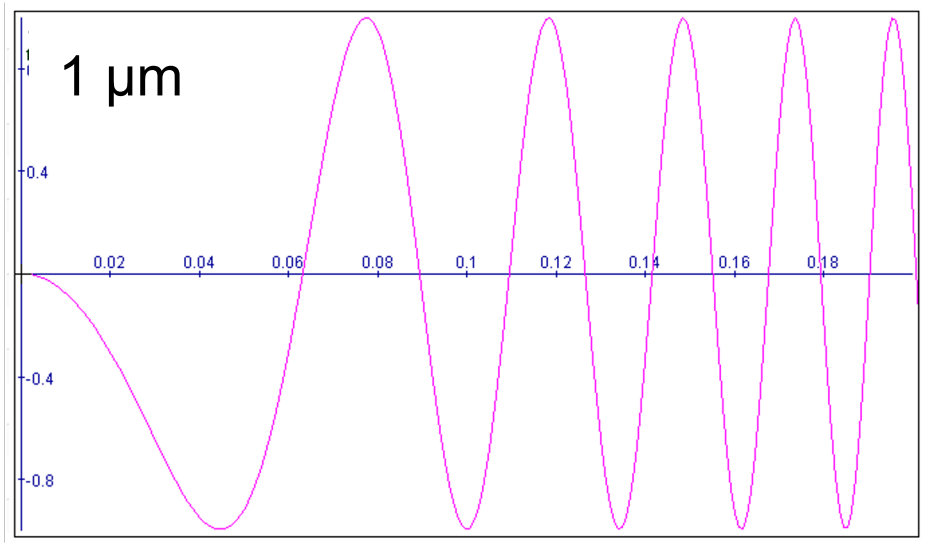


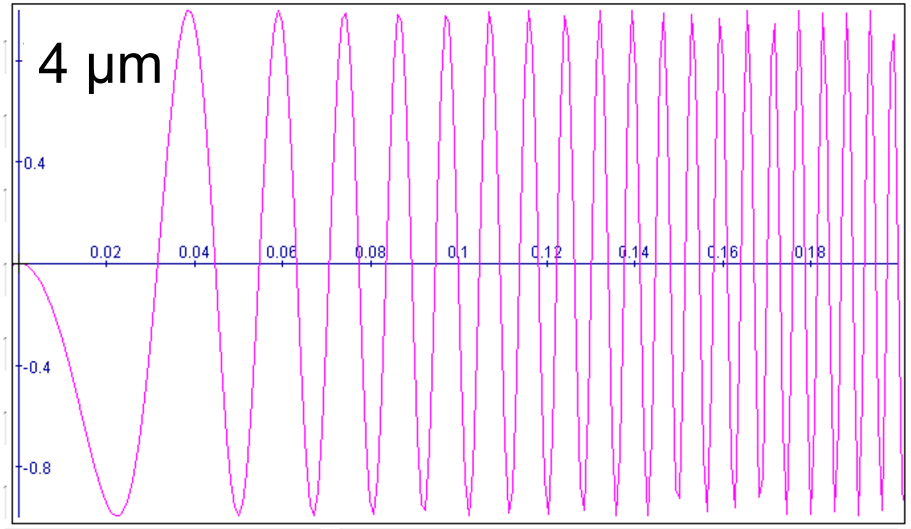
Additional 270° phase shift from the path length difference between the scattered and un-scattered electron wave = positive contrast
Shifted towards lower spatial frequencies

Ideal CTF curves

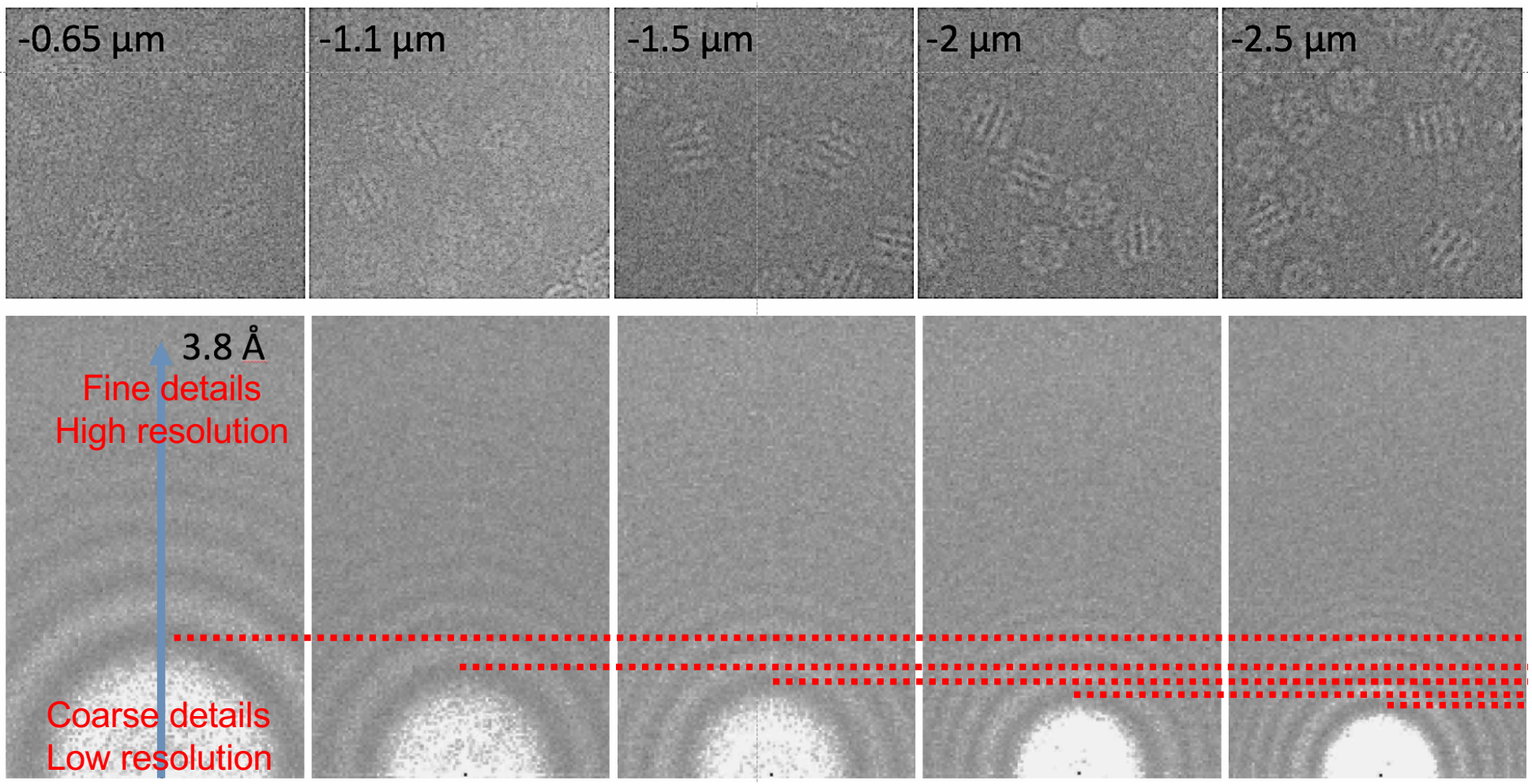








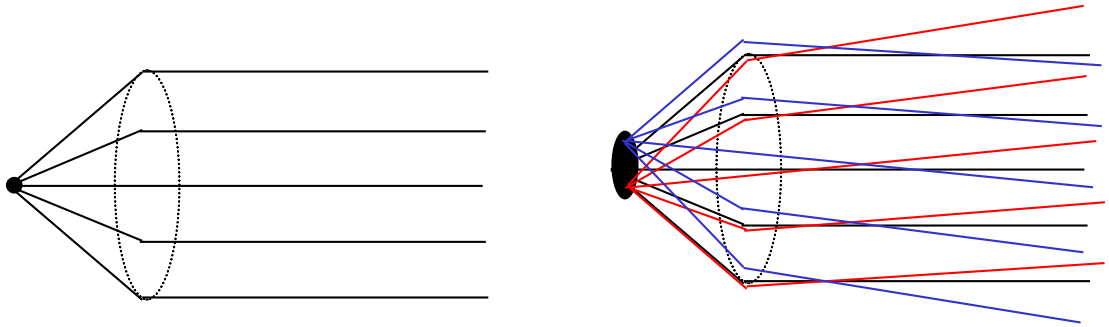
Defocus Generates Contrast



- As more defocus is used the relative phase shift between the scattered and un-scattered electron waves at low spatial frequencies are increased making it easier to see your object.
- However this increases the number of zero and contrast reversal!

Causes of CTF decay

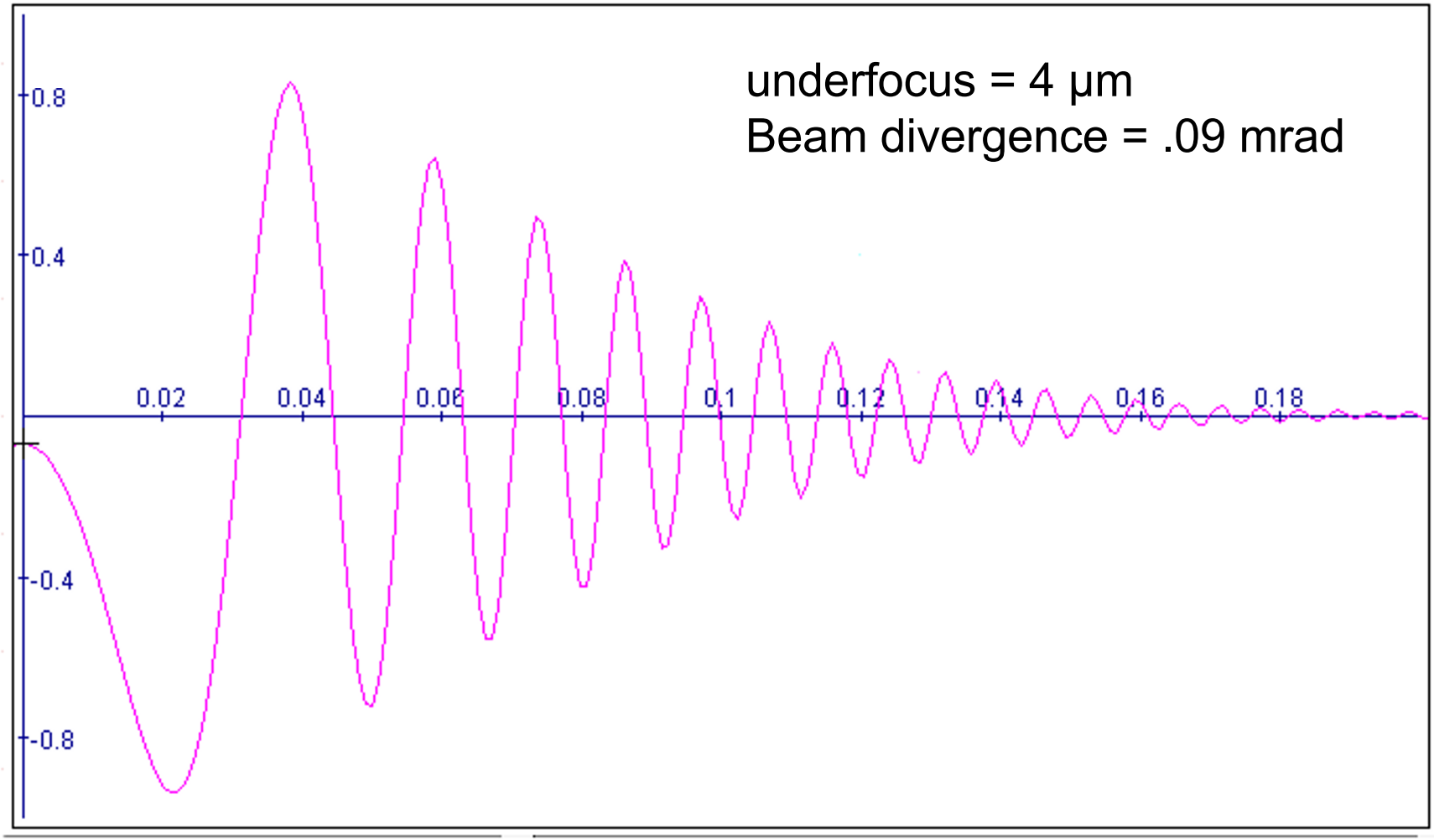
- Loss of spatial coherence - source size



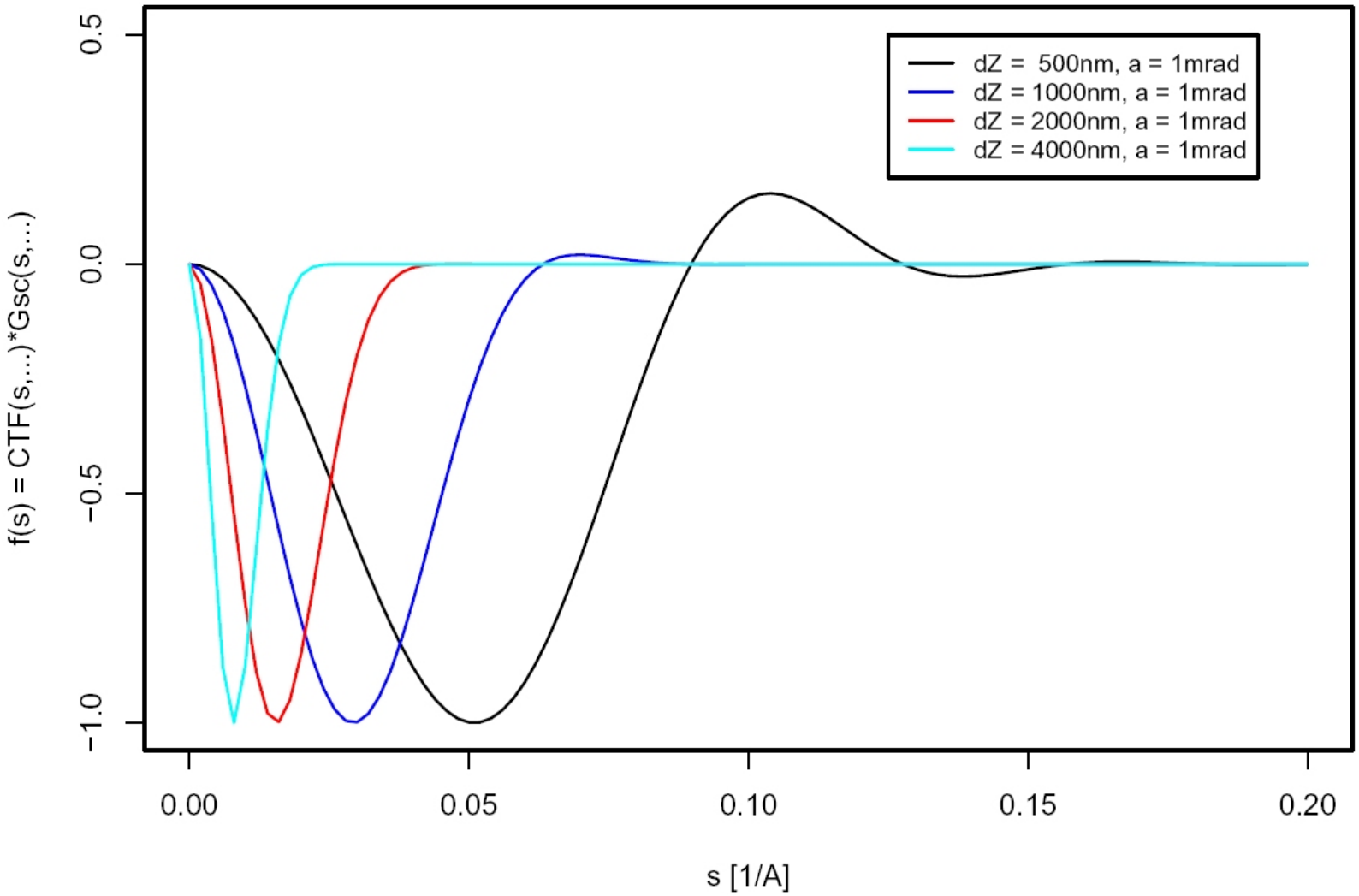
FEG Tungsten

- Image drift (*Motion correction*)
- Thick ice
- Specimen charging (*Grid type and support*)
- Chromatic aberration - variation in voltage
- Variation of lens current

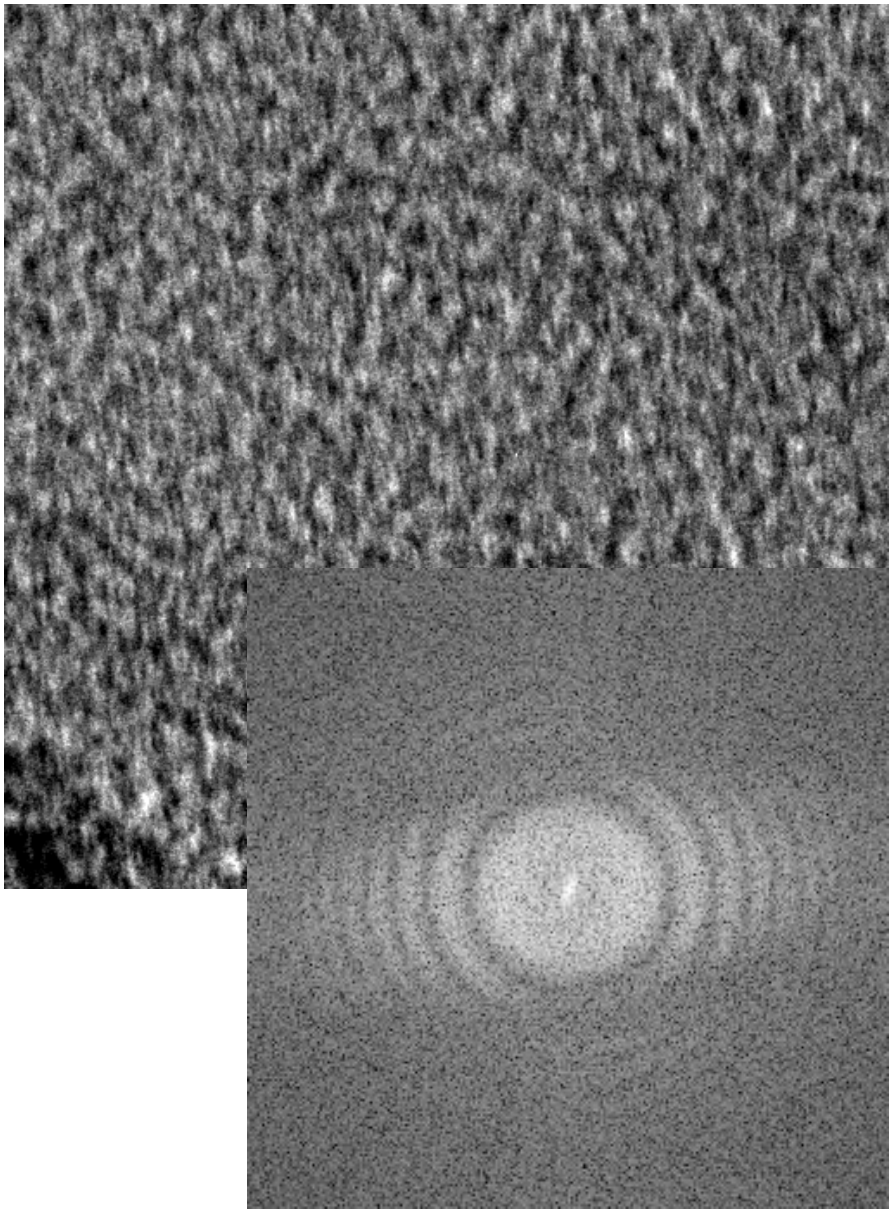
Decay caused by loss of spatial coherence

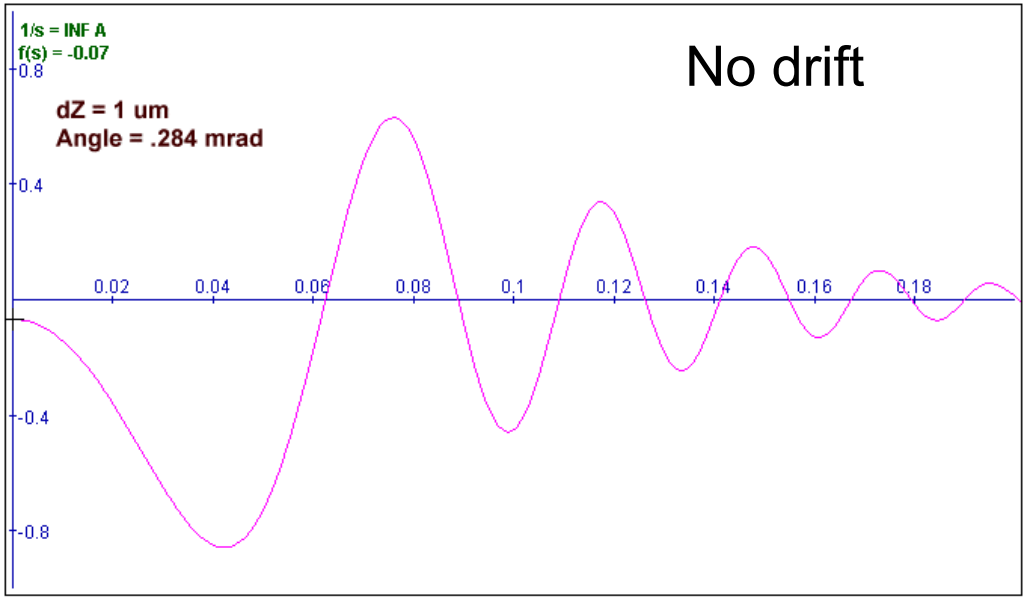


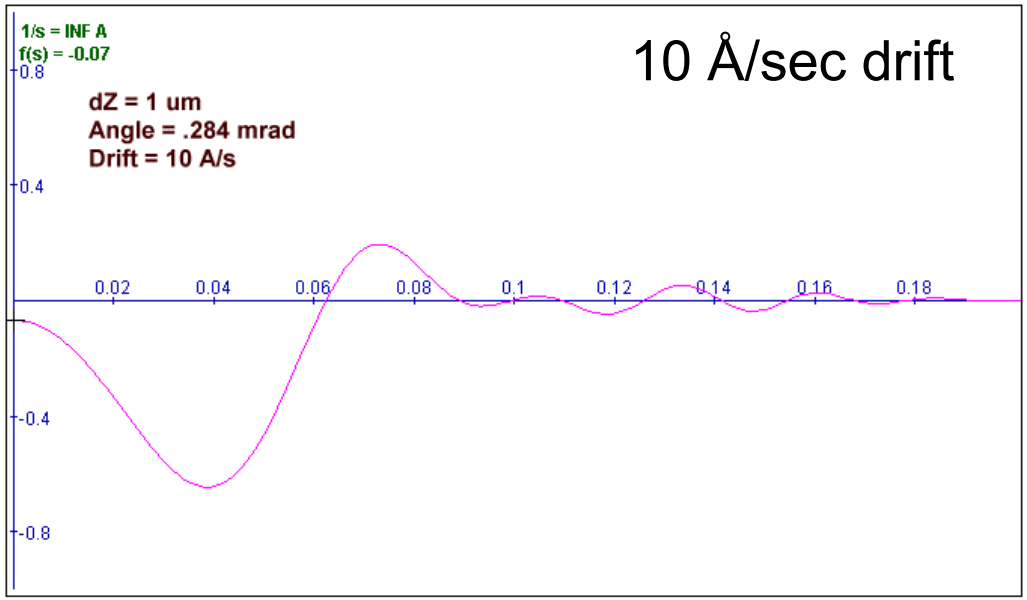
underdefocus = 0.5 - 4 µm
Beam divergence = 1 mrad



Effects of drift on CTF







Problem of the past with motion correction?

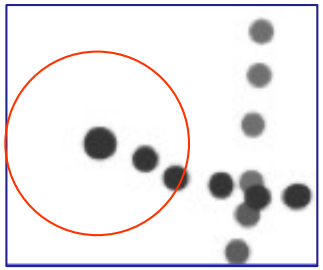
The CTF is the FT of the Point Spread Function

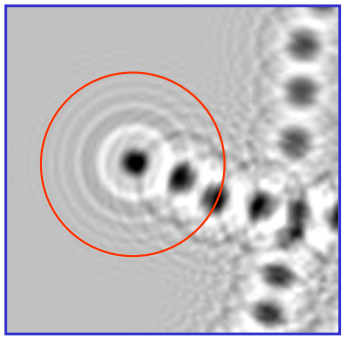
In focus

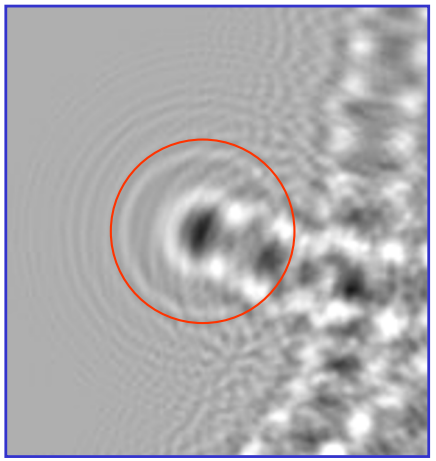
Defocus 1

Defocus 2

Point spread functions





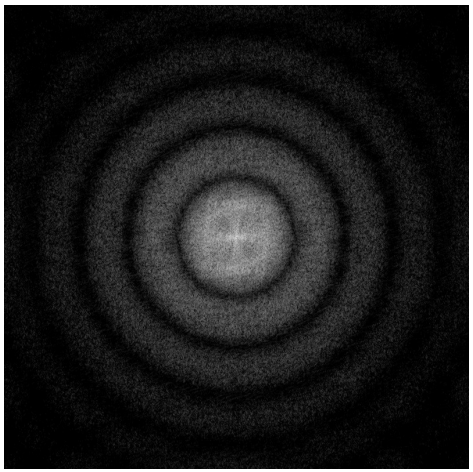


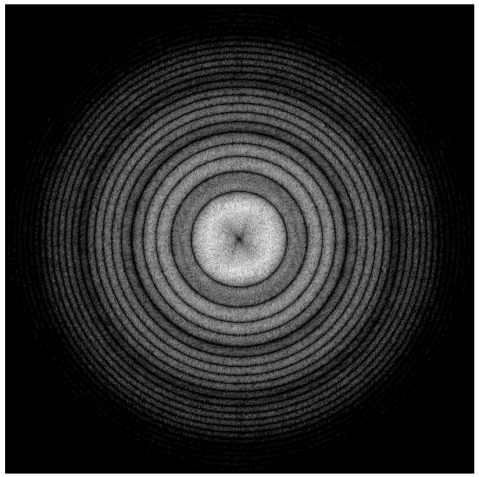
PSF

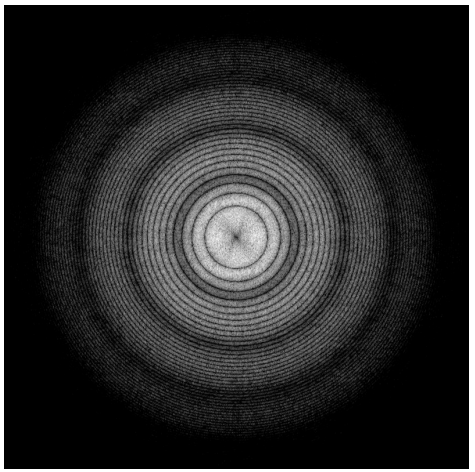
FT

CTF

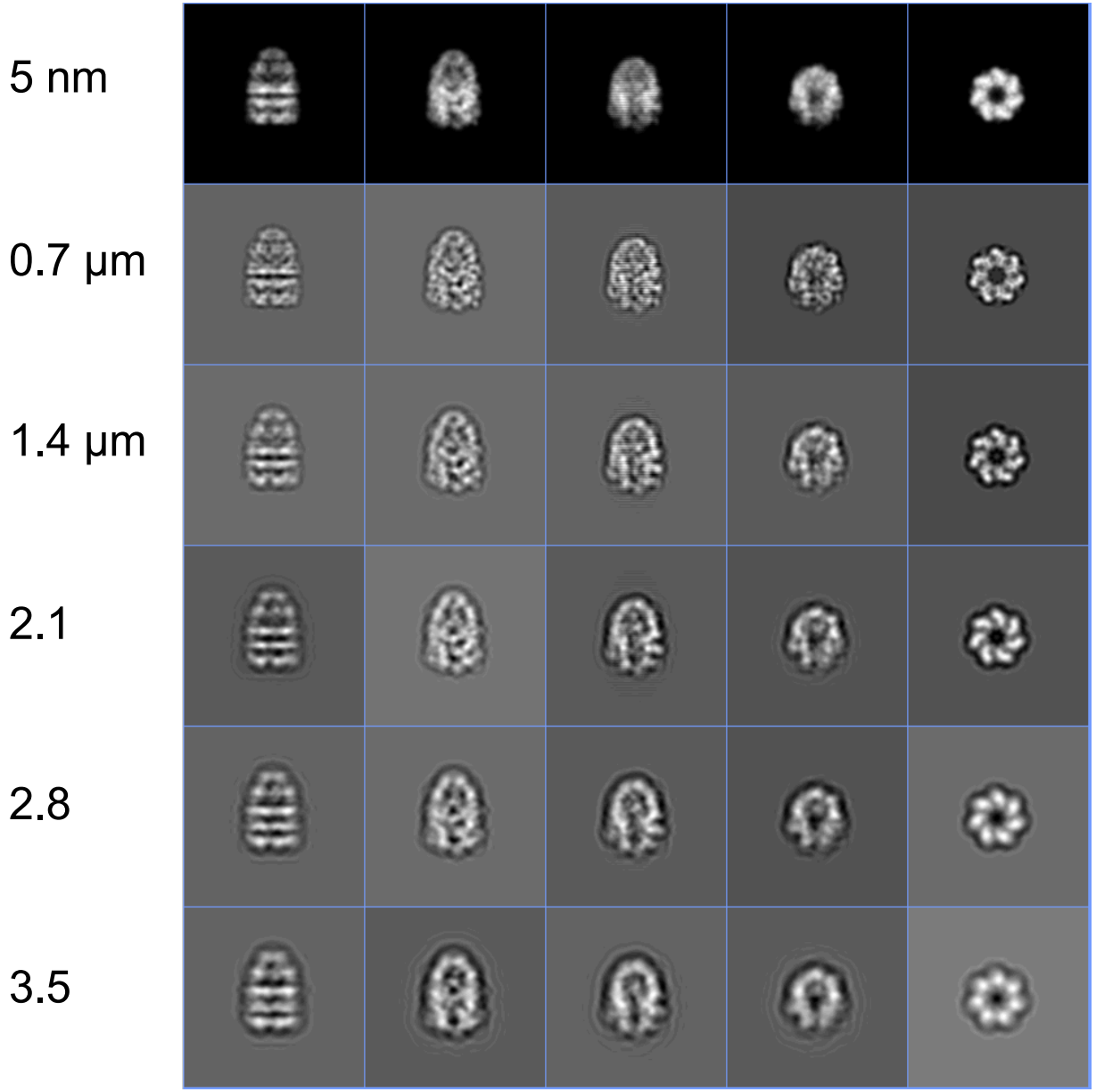
Diffraction patterns





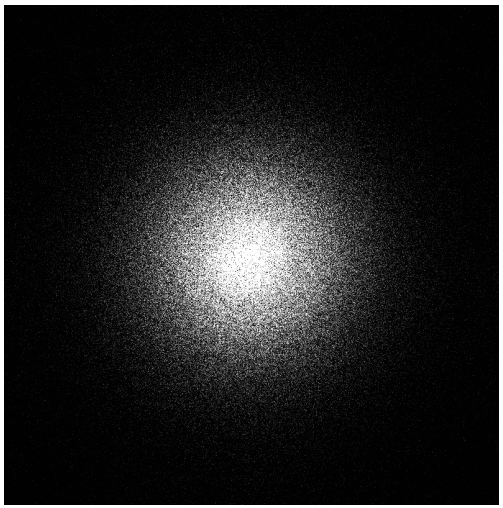


Effects of CTF on 2D projections



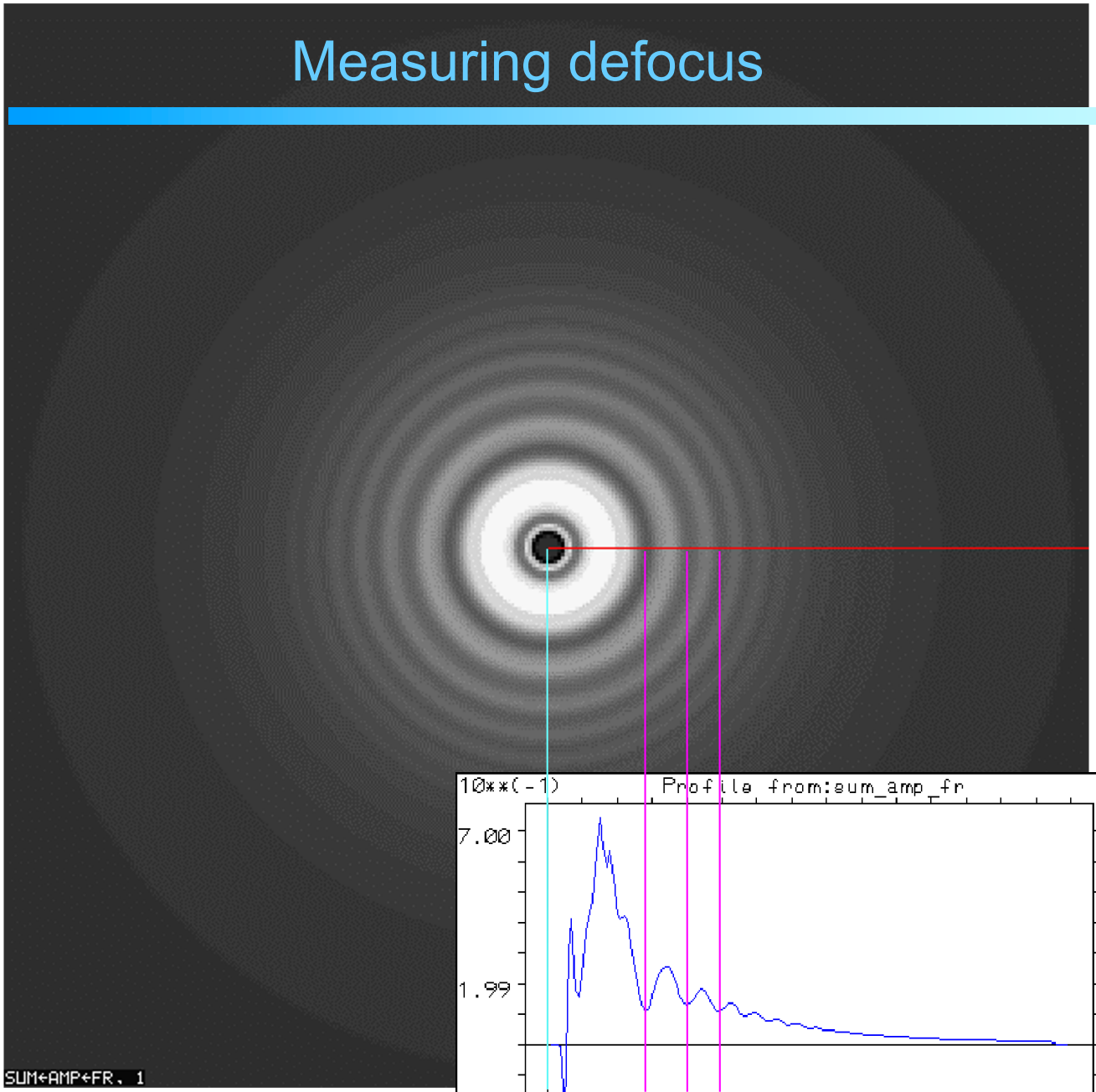
Why don’t I see Thon rings???

- Ice too thick
- No carbon in image
- Too little specimen – vitreous ice alone does not give Thon rings!* (and too thin ice excludes sample)
- Too close to focus on a non-FEG source



*Not in all circumstances McMullan et al., 2015

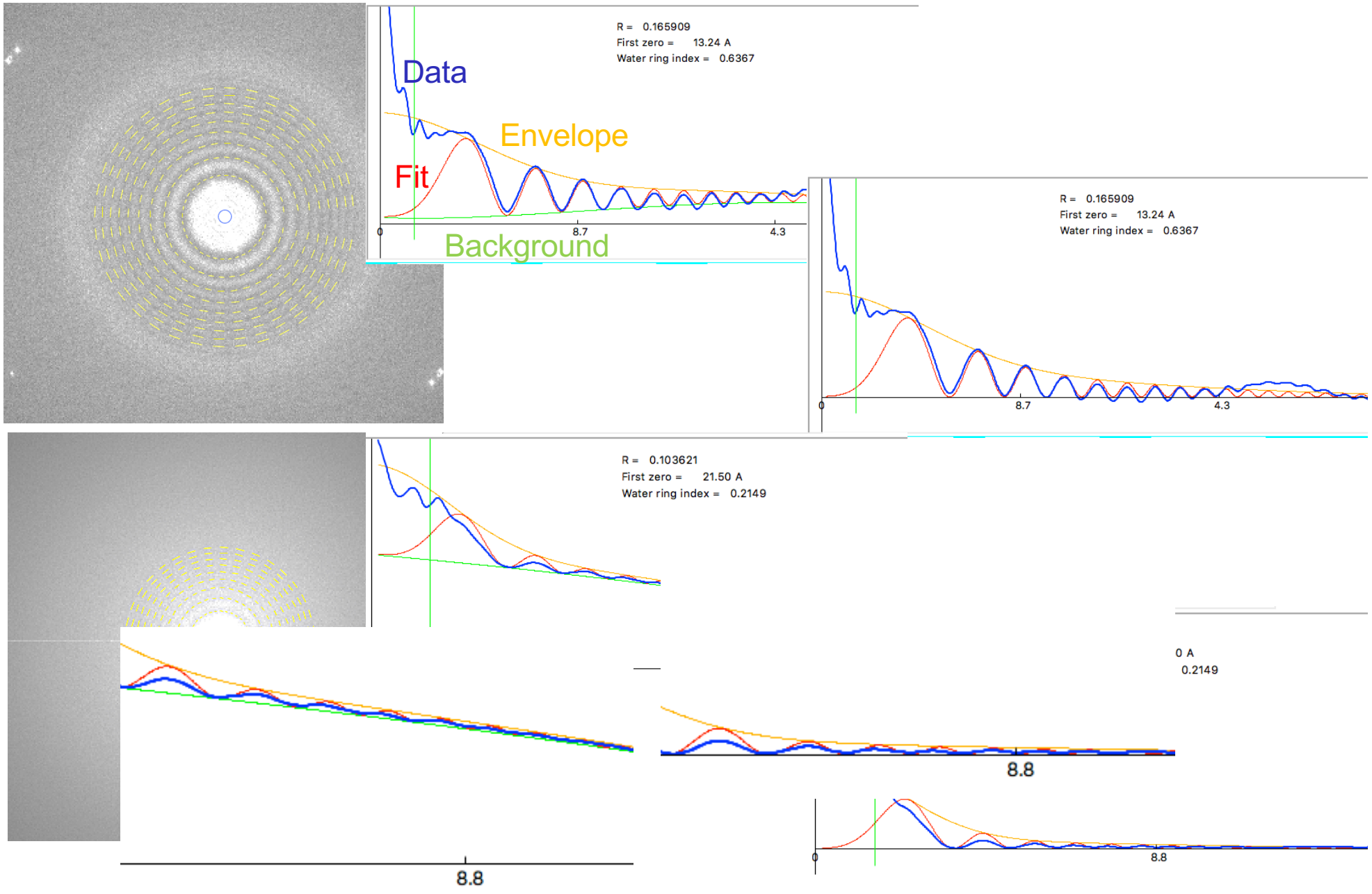
Measuring defocus



Rotationally averaged total sum of image power spectra; band-pass filtered

Profile of the averaged spectrum

CTF ripples are superimposed on a large background of incoherent scattering, noise and other features



Procedures for measuring defocus and CTF correction

EMAN2 - evalimage graphical interface
http://blake.bcm.edu/emanwiki/EMAN2/Programs/e2evalimage

Bsoft – Nice graphical interface and can be used for CTF correction

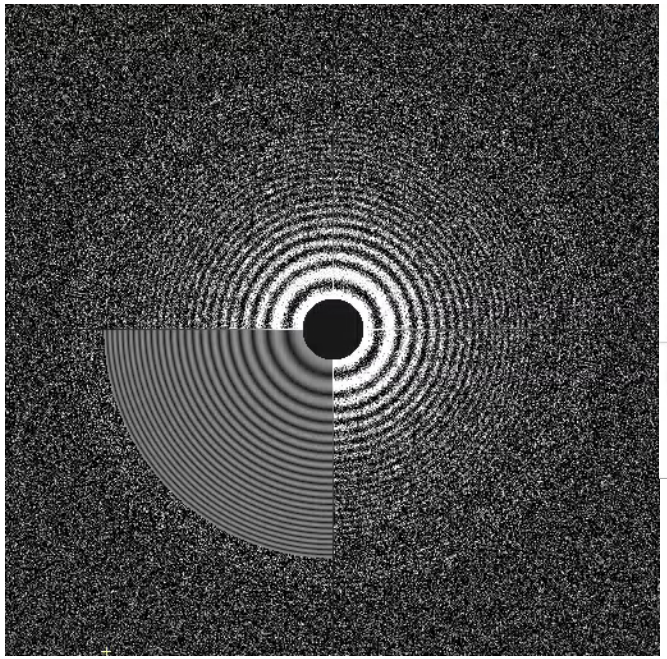
CTFFIND4 – graphical/automated
Chops up areas into boxes
Uses estimate of starting defocus
Searches over a specified range of defocus
Estimates astigmatism
Gives split display output for verification of result
http://grigoriefflab.janelia.org/ctffind4

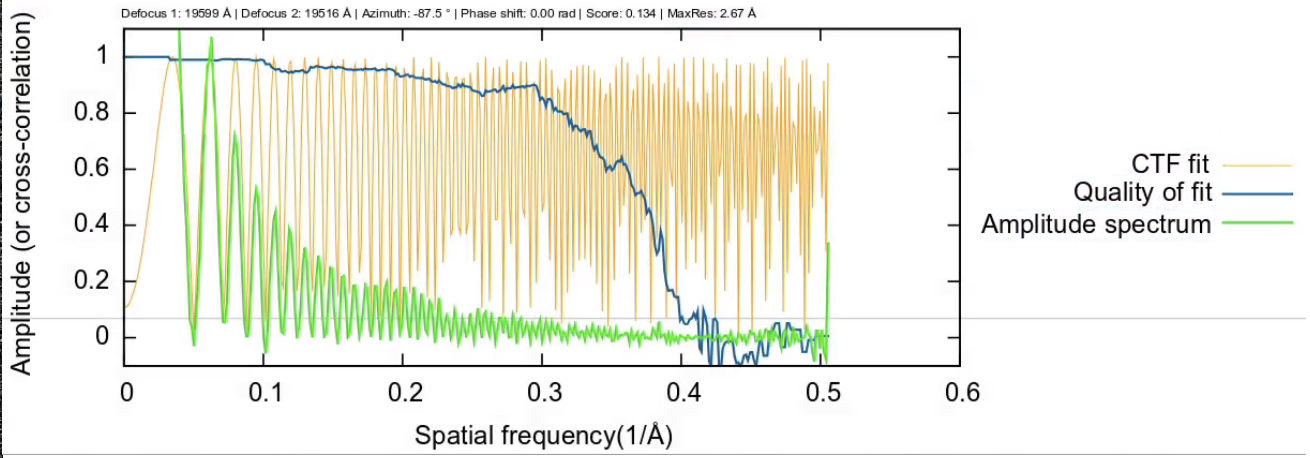
GCTF – GPU accelerated CTF determination with local refinement. Zhang (2016) JSB 193, 1-12.

IMOD – Defocus determination on single tilts or groups of tilted images and strip based correction on individual tilt images**.**

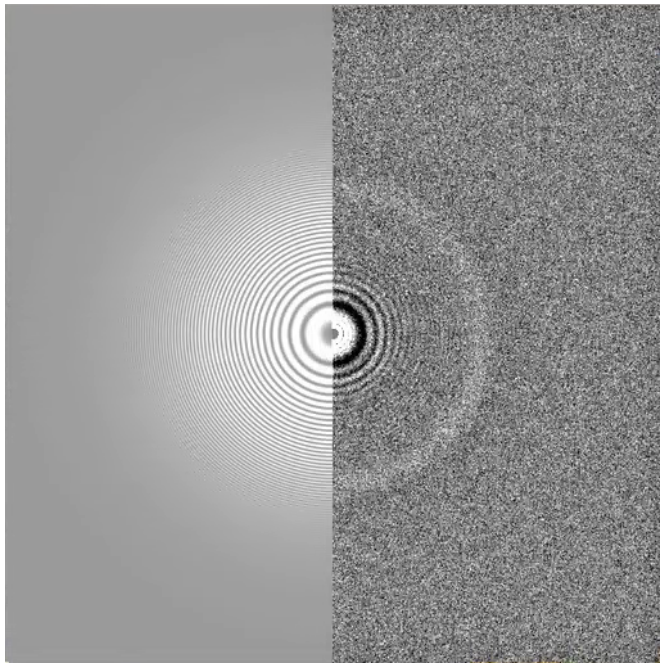
Nova CTF – 3D CTF correction for subtomogram averaging. Separate correction of each subtomogram. Turonova et al (2017) JSB.

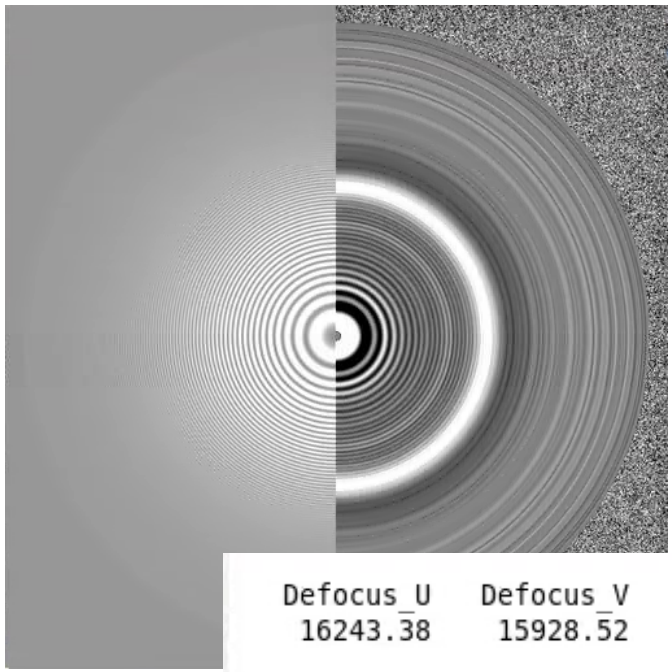
CTFFIND4 and GCTF output






```
Estimated defocus values        : 19599.48 , 19516.48 Angstroms
Estimated azimuth of astigmatism: -87.53 degrees
Score                           : 0.13367
Pixel size for fitting          : 1.400 Angstroms
Thon rings with good fit up to  : 2.7 Angstroms
CTF aliasing apparent from      : 3.9 Angstroms
```

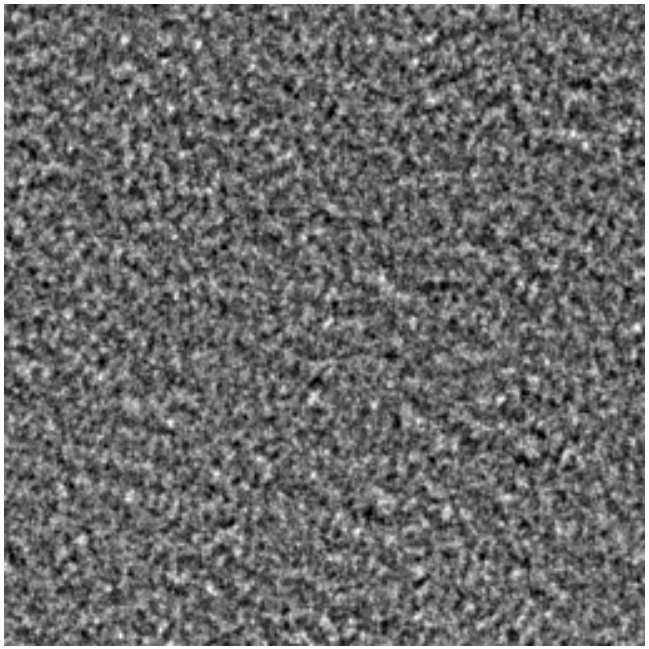


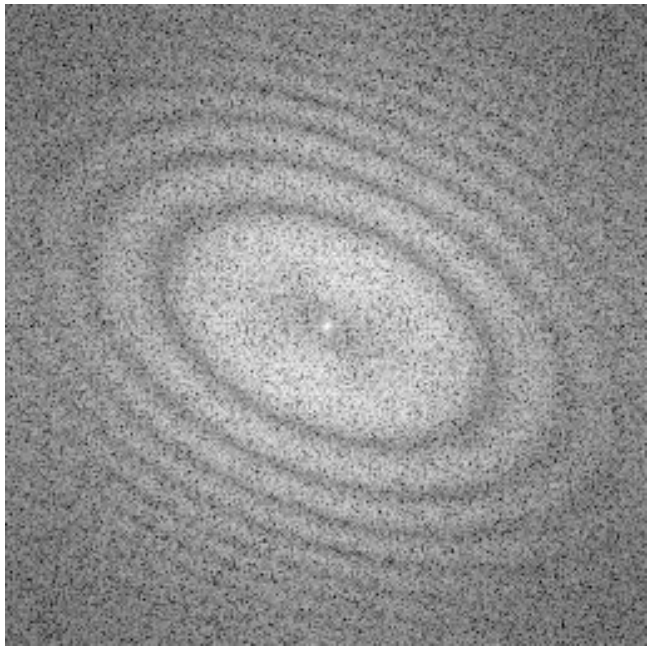


```
Defocus_U   Defocus_V       Angle        CCC
 16243.38    15928.52       86.72    0.109494  Final Values
```

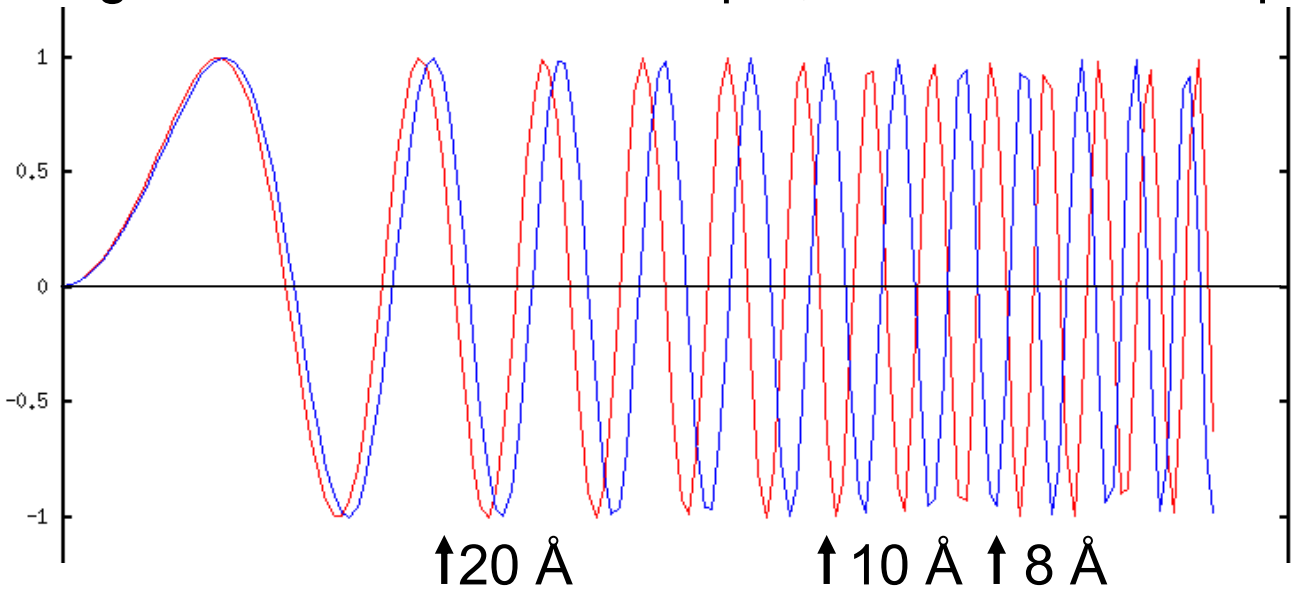
- Both CTFFIND 4 and GCTF have multiple outputs to allow you to asses the quality of the CTF estimation!

Astigmatism

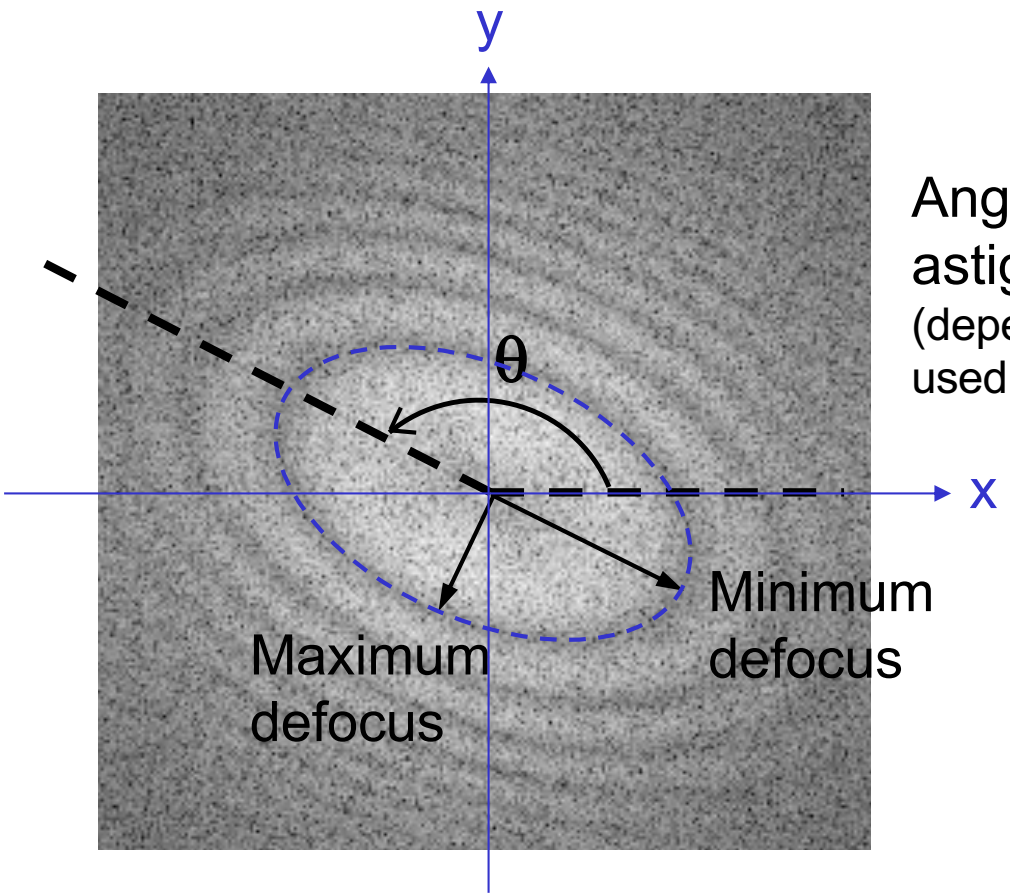




Astigmatic: defocus 1 = 4.41 µm, defocus 2 = 4.14 µm



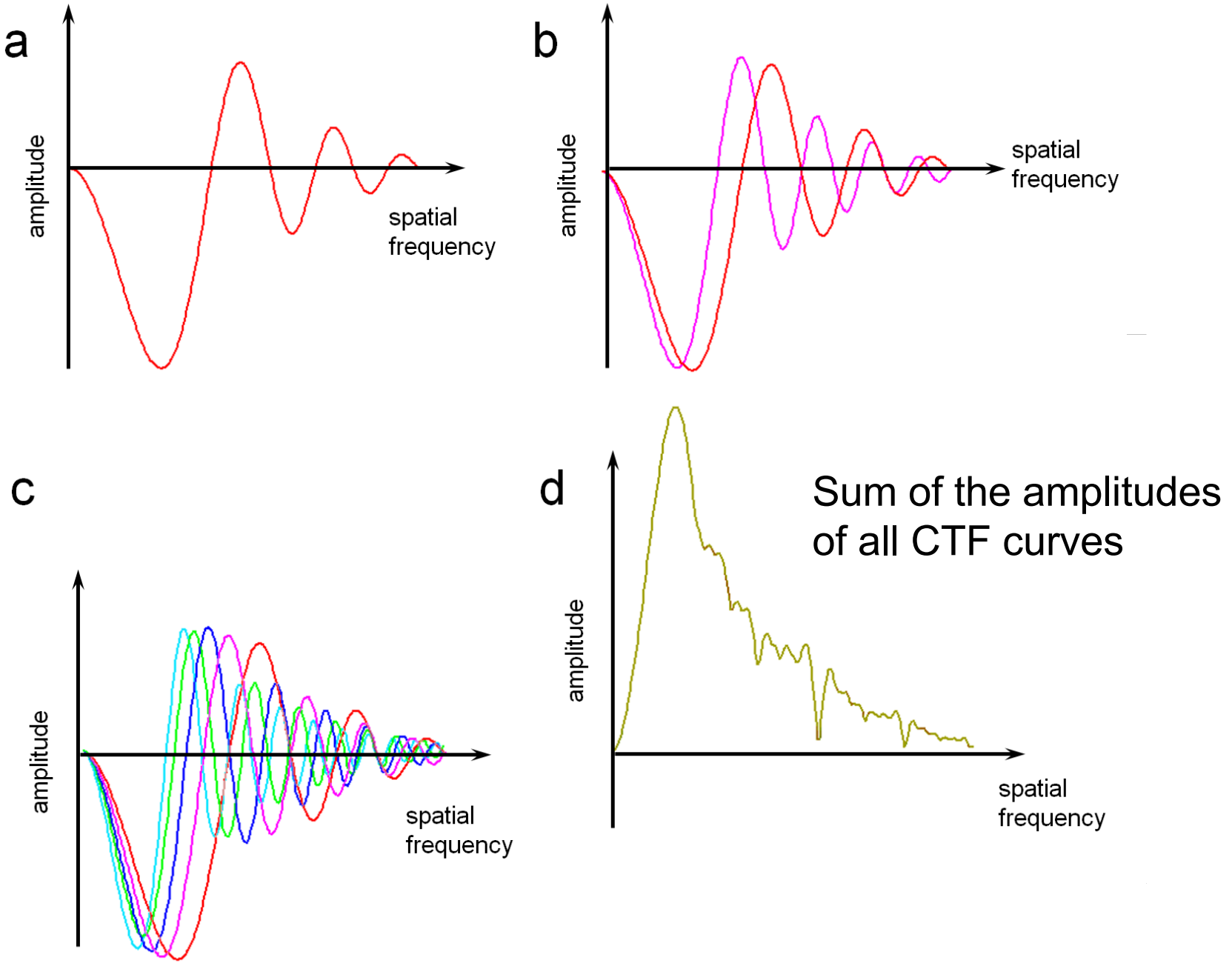
How to measure an astigmatic CTF



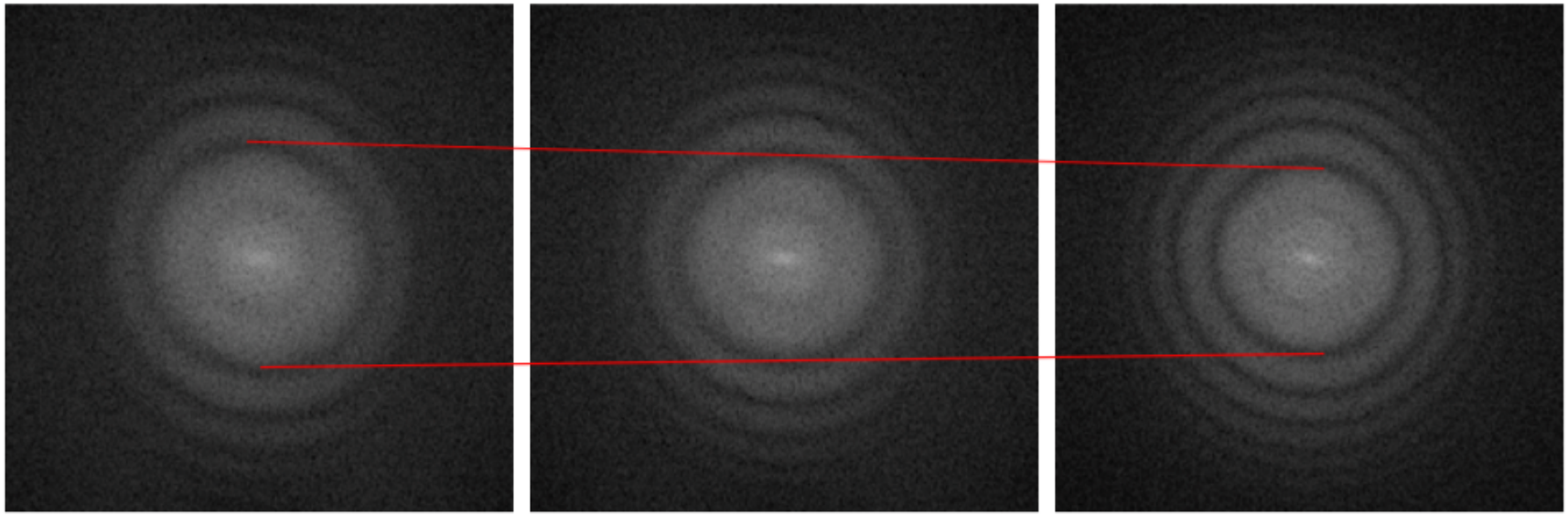
Angle of astigmatism, θ
(depends on convention used by your program)

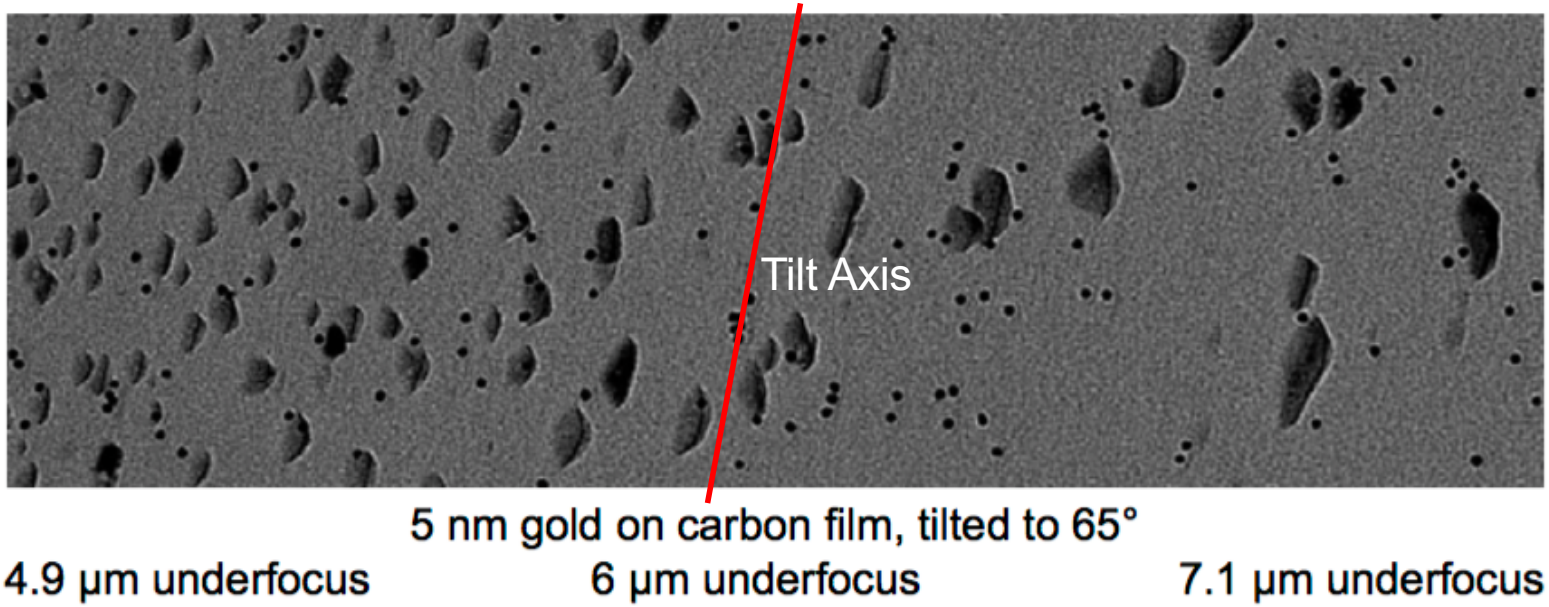
The ellipse must be fitted or measured in sectors to get the degree and angle of astigmatism so that the zeroes can be correctly determined for all directions.

CTF curves from different images– what range do we need



Defocus and Thon ring variations in tilted samples



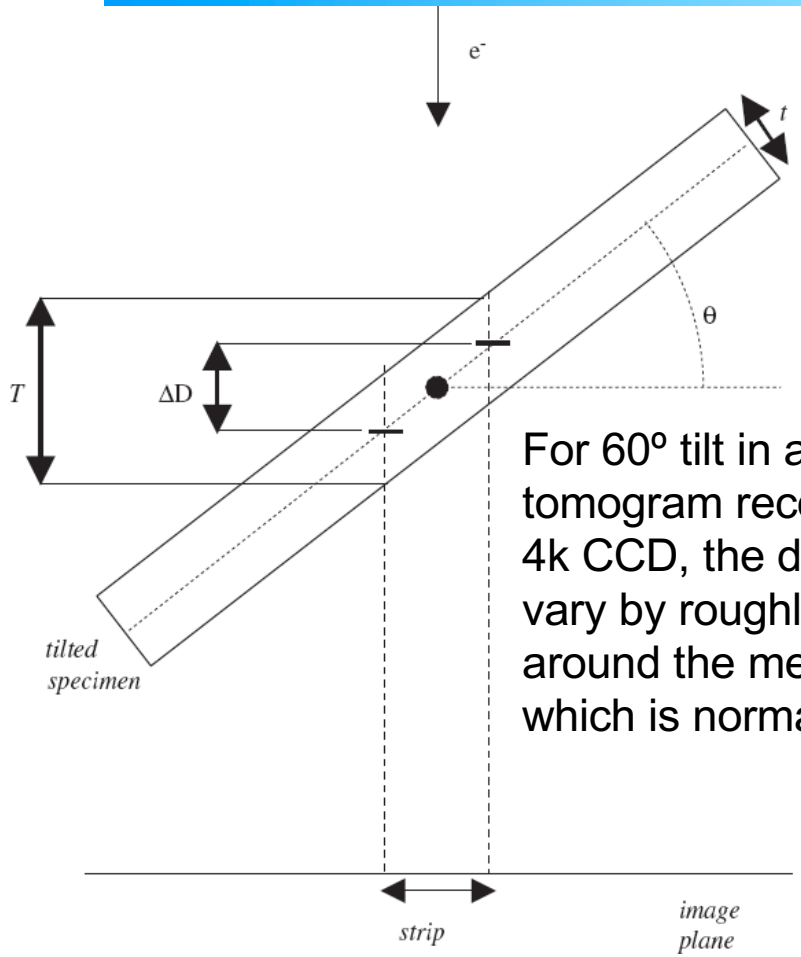


5 nm gold on carbon film, tilted to 65°

4.9 µm underfocus 6 µm underfocus 7.1 µm underfocus

http://bio3d.colorado.edu/RML_2017/2017_IMOD_PEET_Workshop/Lectures/CTFcorrInIMOD.pdf

Tilt geometry and defocus



For 60º tilt in a typical tomogram recorded on a 4k CCD, the defocus will vary by roughly ±1 μm around the mean value, which is normally 4-15 μm.

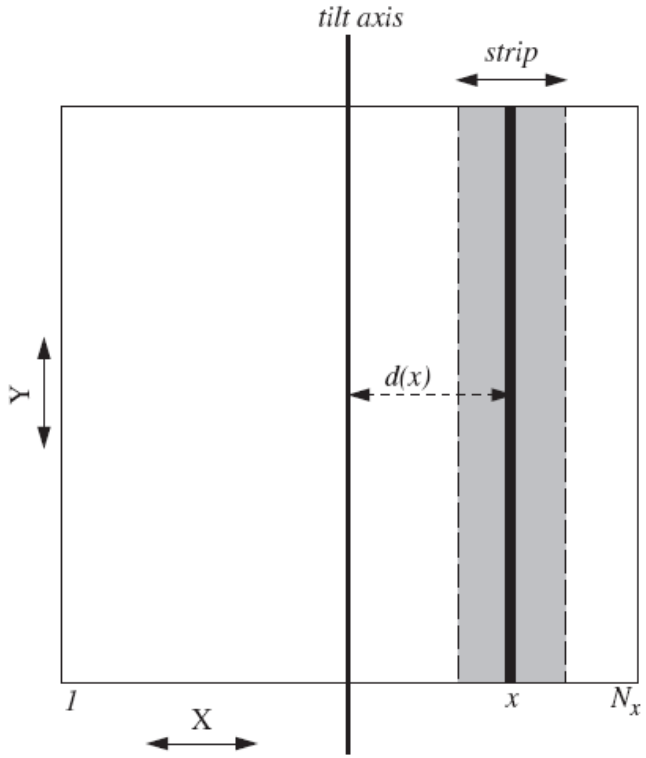
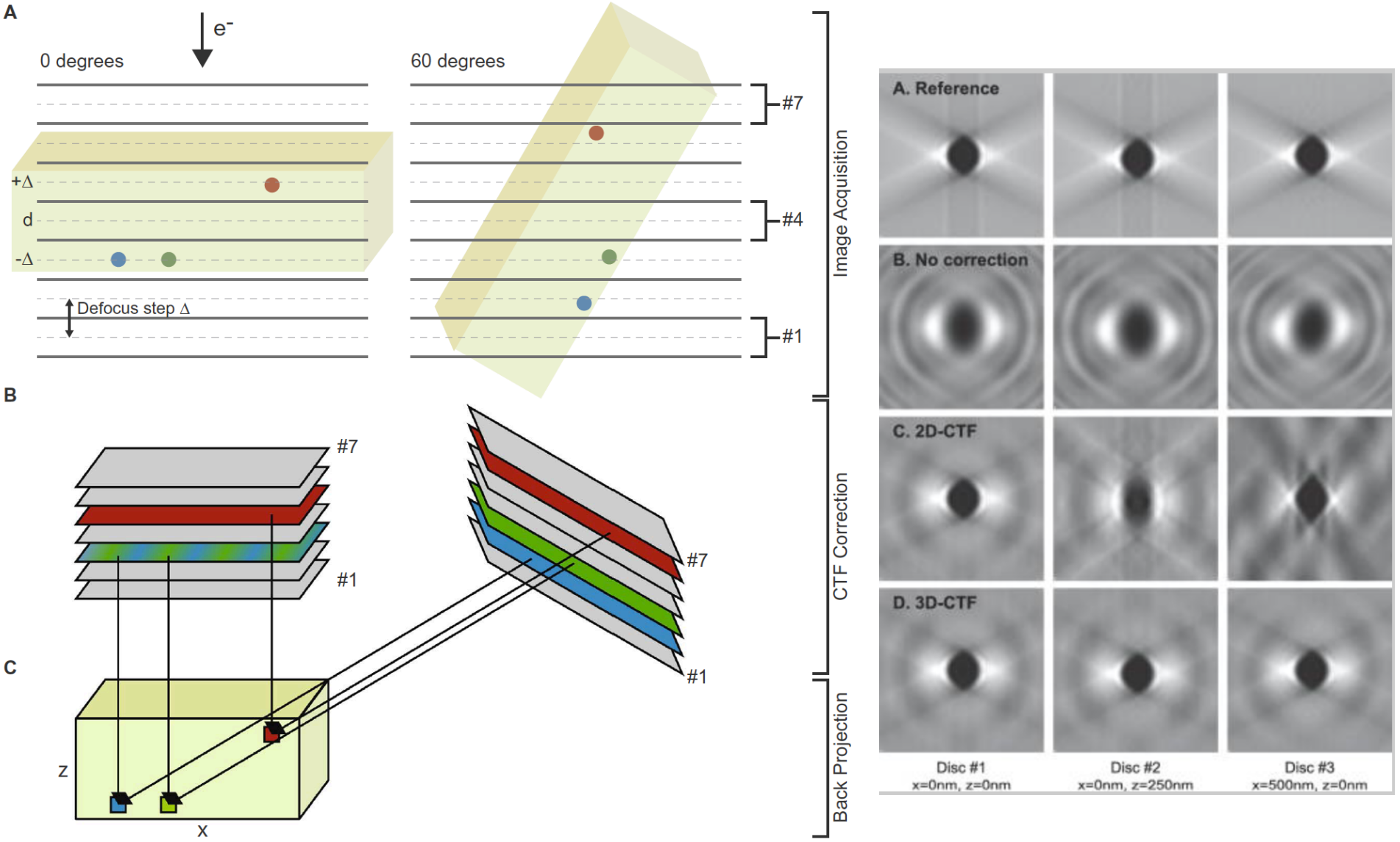


Fig. 1. Computation of a strip in an image of a tilted specimen. ΔD represents the defocus range considered as a single defocus value, T denotes the defocus range in the strip, t is the thickness of the specimen and θ is the tilt angle. The tilt axis runs perpendicular to the sheet and is marked by the black circle in the middle of the specimen slab.

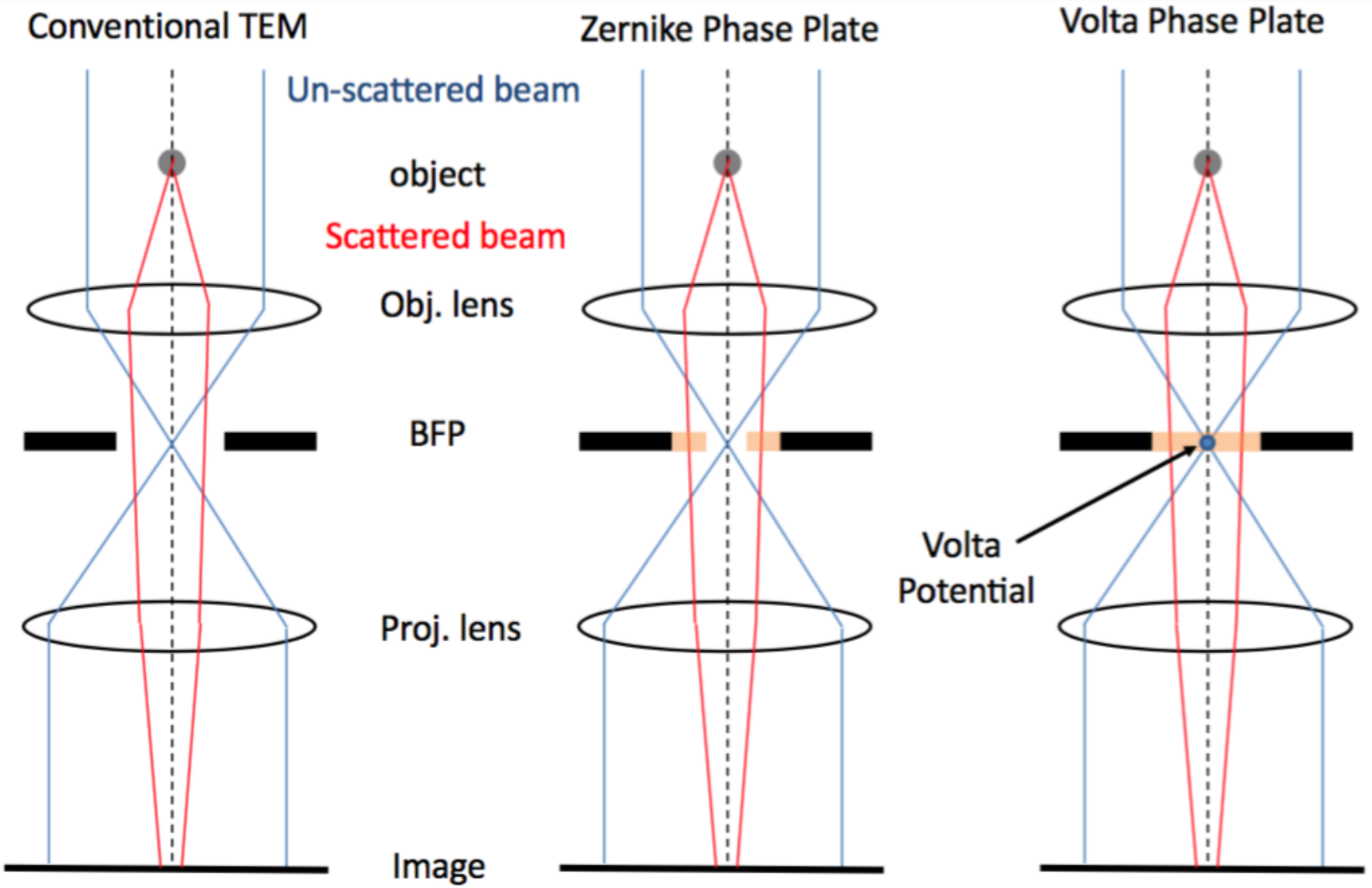
Fig. 4. Extraction of a strip with a single effective defocus value from a tilted specimen. The square represents an image acquired from the tilted specimen. The tilt axis runs along the Y-axis. x denotes the index of the x-line around which the strip is extracted. $d(x)$ represents the distance from the x-line to the tilt axis.

from Fernandez, Li & Crowther (2006) CTF determination and correction in electron cryotomography. Ultramicrosc. 106, 587-596. Strip CTF correction is implemented in IMOD

Nova CTF and point spread functions

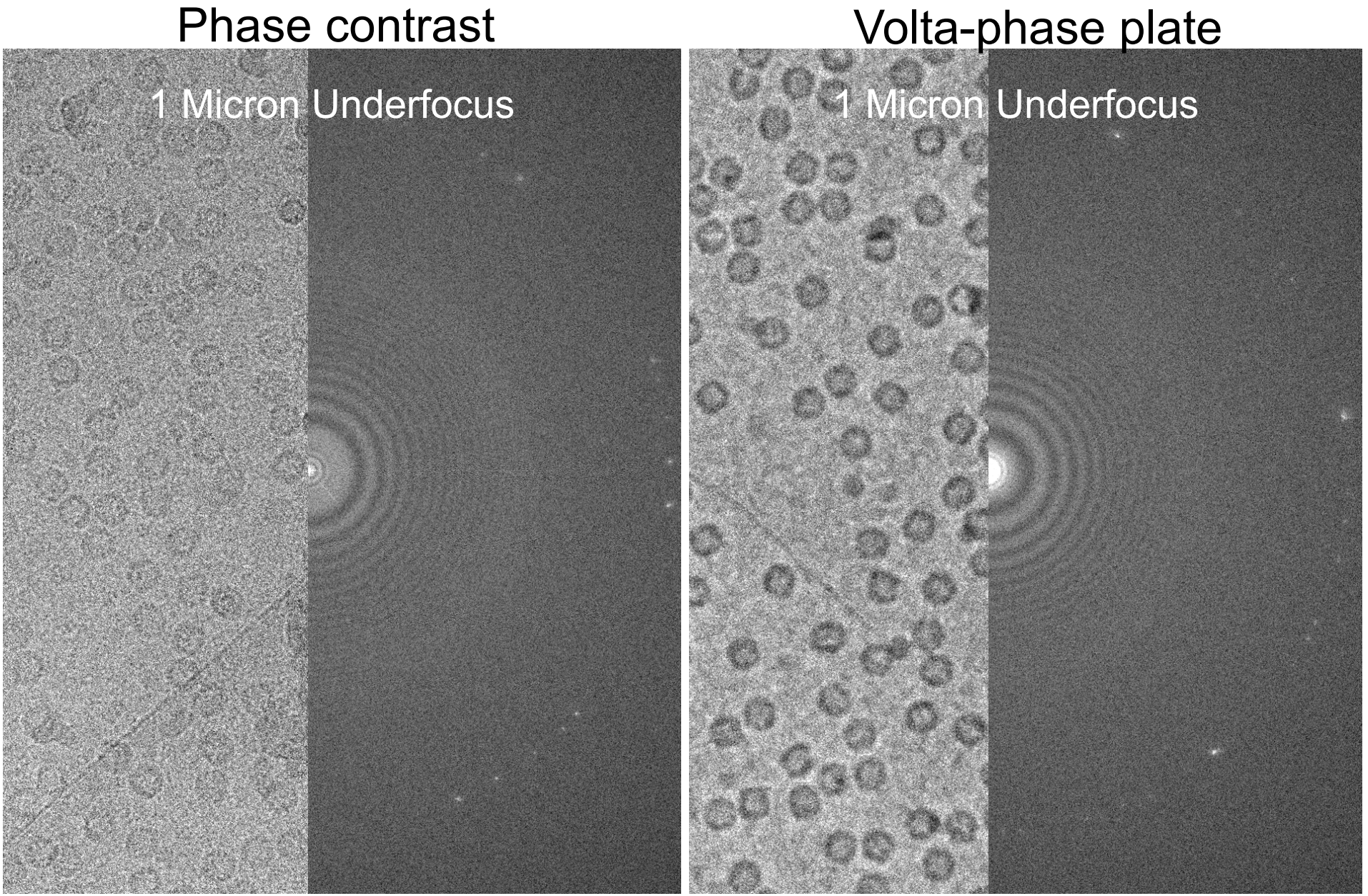


Phase plates



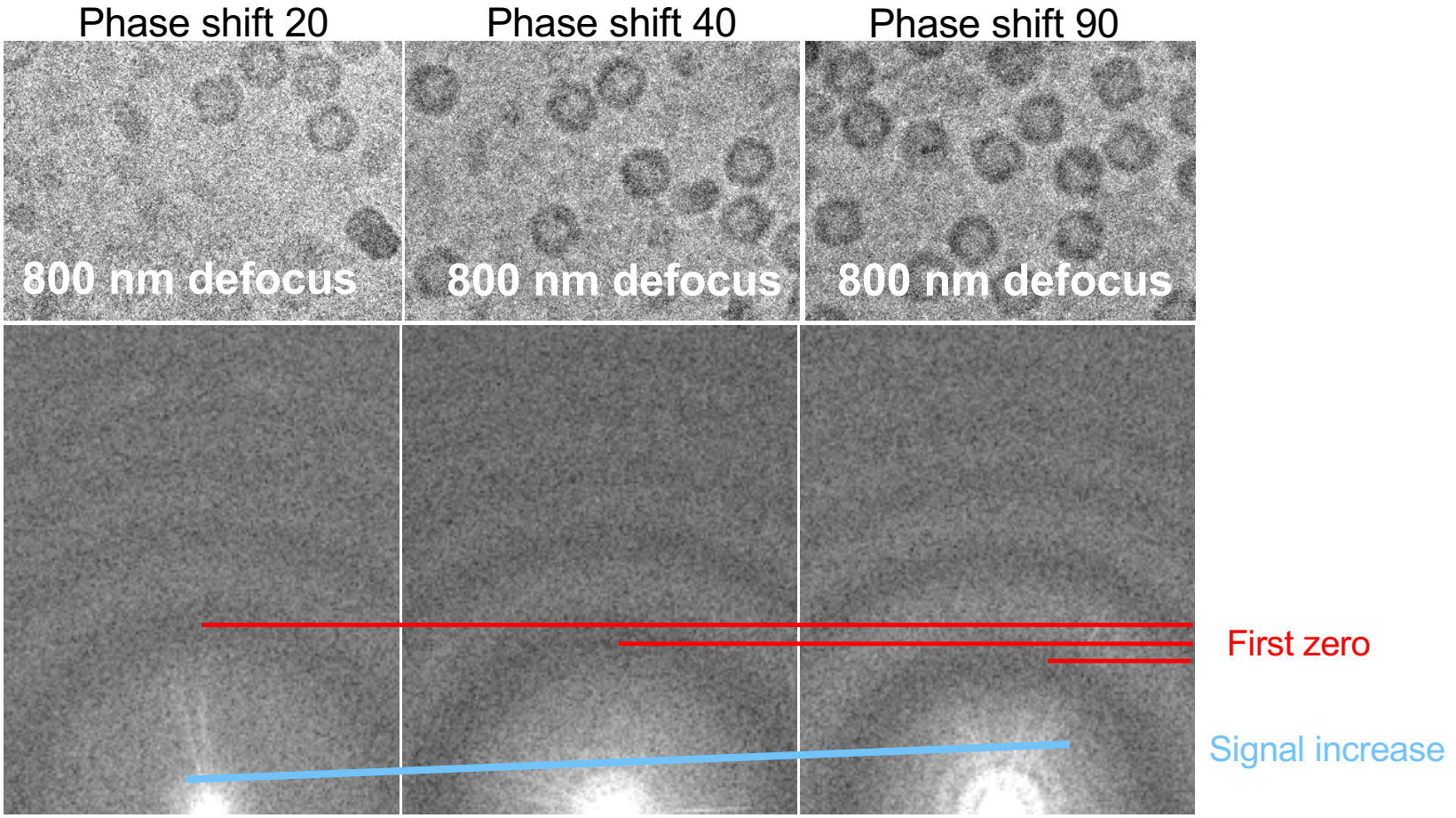
Thanks to Christos Savva for the slide

Volta Phase plates – effect on contrast and CTF



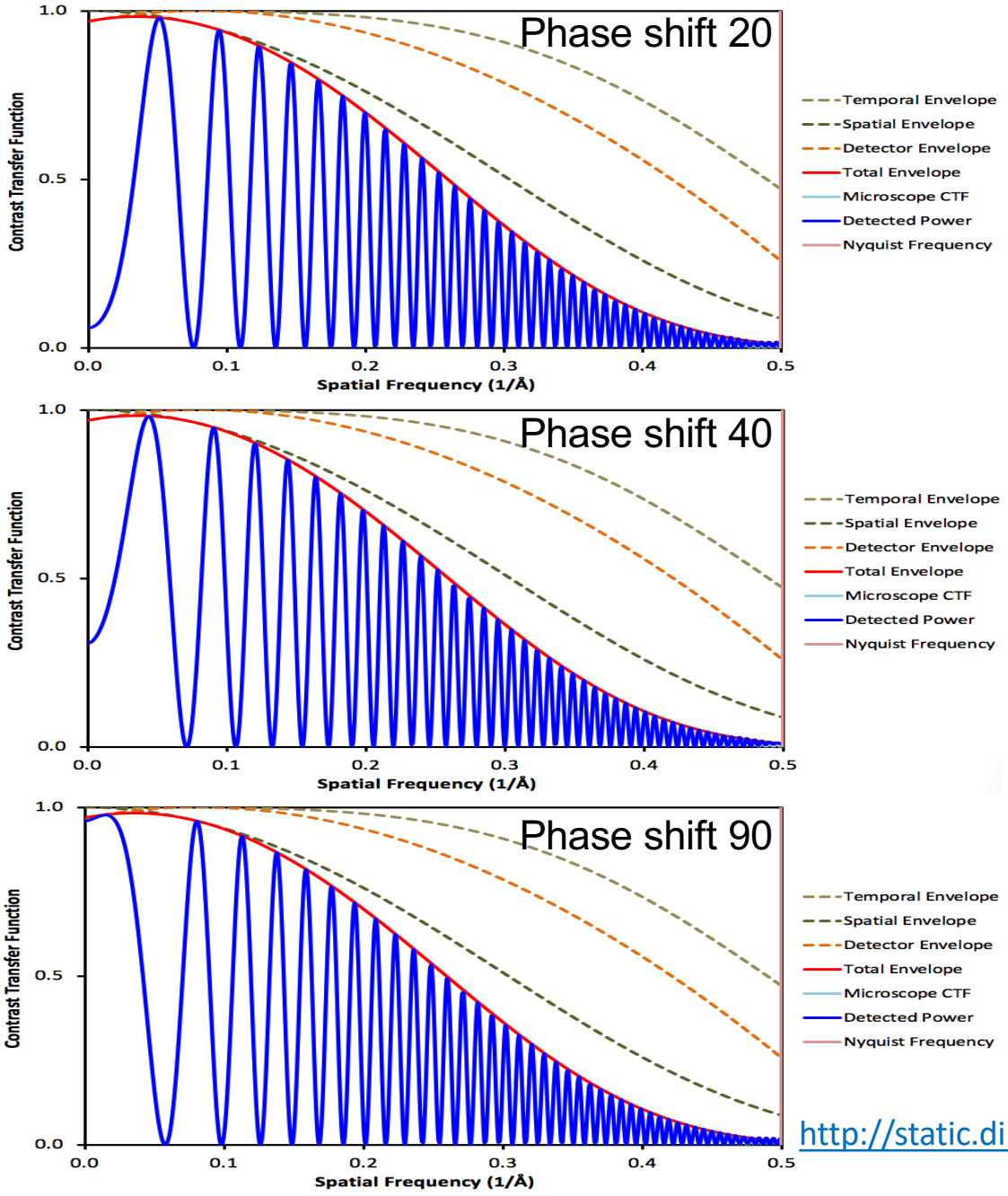
- Huge increase in low frequency contrast (low resolution features)

Volta Phase plates – effect on contrast and CTF continued

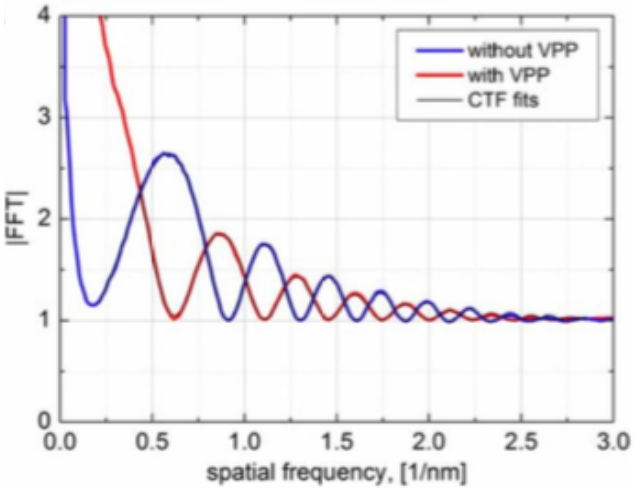


- Increasing phase shift improves contrast as the interference between the un-scattered and scattered electrons, particularly at low spatial frequencies, is increased.
- The CTF zero's shift towards the centre of the power spectra as the phase shift increases (increase in the relative speed of un-scattered electrons as the phase shift increase).
- Limited defocus values can be used as the variation in phase shift will change the zero positions for a set defocus.

Volta Phase plates – effect on contrast and CTF continued



The problem with VPP



~18% signal loss with VPP

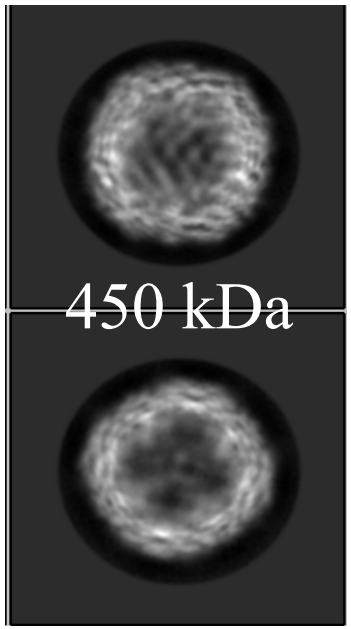
Danev et al., 2014 PNAS

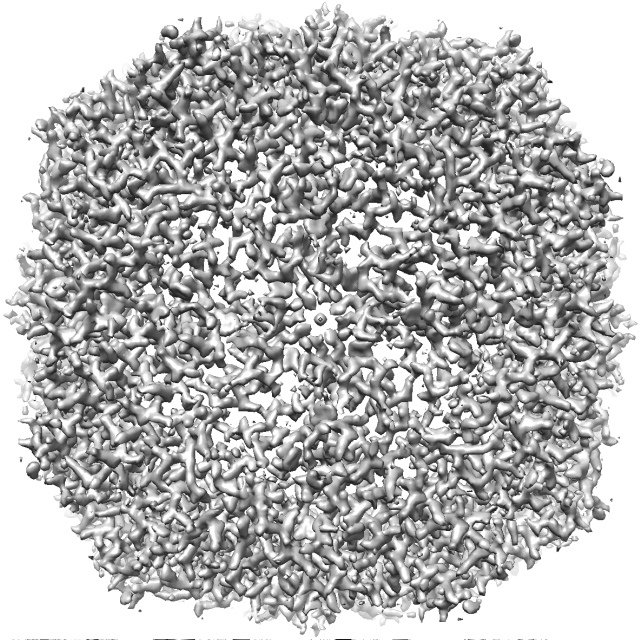
Sin to Cos!

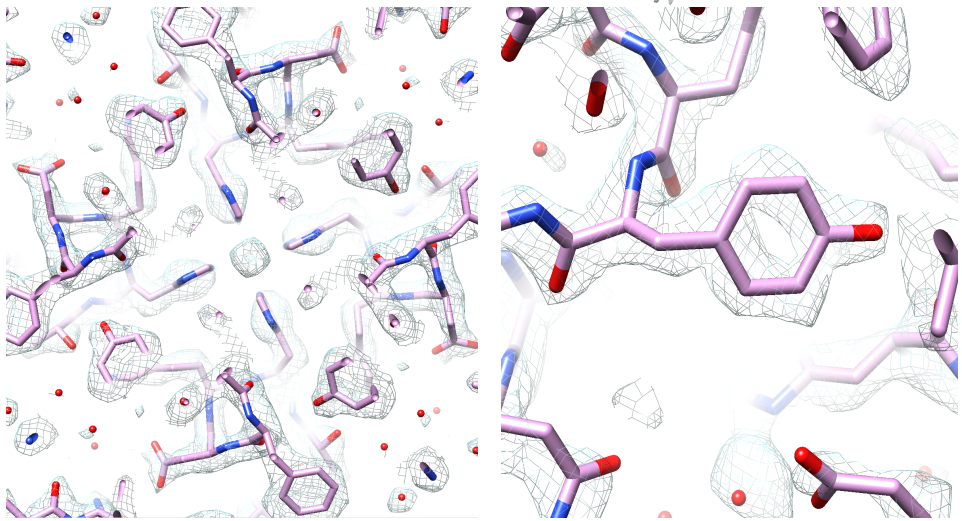
http://static.directelectron.com/HelpfulTools/CTFSimulation.xlsx

Volta Phase plates

2.2 Å Reconstruction of Apoferritin



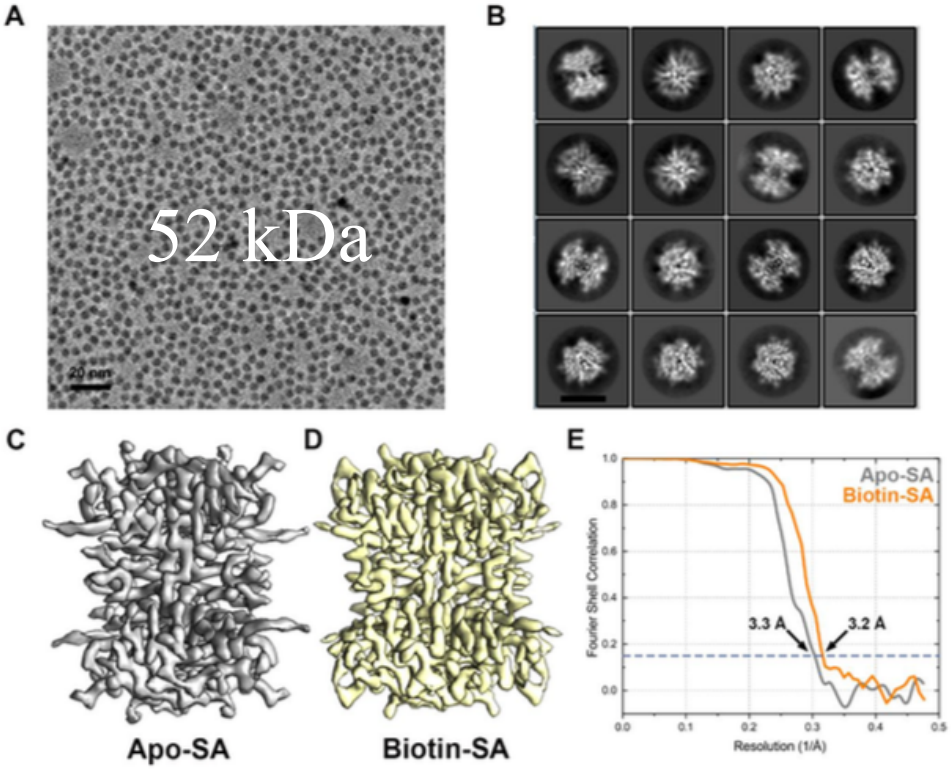


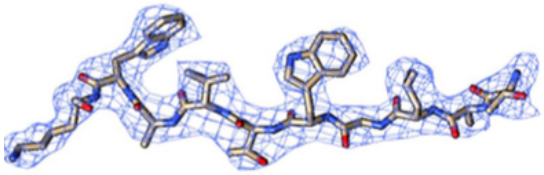


*Thanks to Christos Savva for the protein and Yuriy Chaban for the GO grid prep

3.2 Å Reconstruction of Streptavidin

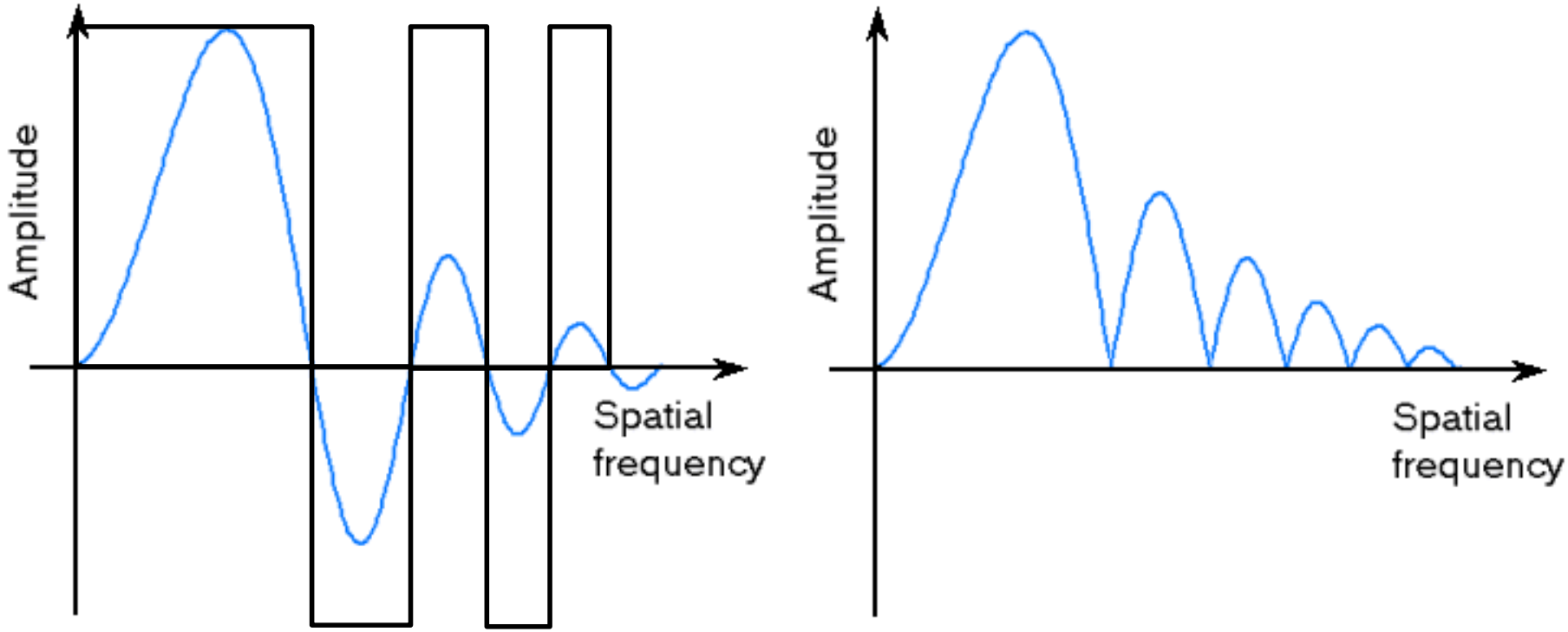
Fan et al., 2018





- Structure of proteins smaller than 70 kDa now possible with the Volta phase plate

Methods of CTF correction



1. Phase flipping - can be done on raw images

Multiplication in Fourier space by a simple box function = 1 and -1

Full CTF correction

2. Full restoration of amplitudes: Multiply each image FT by its own CTF, then add up all the equivalent views and divide the sum by the sum of all the CTF's squared, plus a constant related to the signal:noise ratio (Wiener factor) to avoid division by zero.

$$FT\_Merged\_class = \frac{\sum_{i=1,N} FTclass_i . CTF_i}{\sum_{i=1,N} (CTF_i^2) + w}$$

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