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Imaging and Data Assessment

V. Unger, 11/17/03

The Chapters

1. The good and evil of electrons, radiation damage and imaging modes

2. Images and Pixelation

3. The “you know who” of electron microscopy and why it’s not all bad

The "good and evil" of electrons

they interact ~10^5-fold stronger with matter than X-rays, and have a better ratio of "inelastic/elastic" scattering events (~ 3 inelastic/elastic for electrons versus ~10 inelastic/elastic for X-rays)

Why is this good?

- Higher success rate for elastic scattering events = less material needed to retrieve the structural information (remember: it's the elastic scattering that is useful).
- In theory, 12,000 - 20,000 molecules are sufficient to achieve near atomic resolution.
- In practice however ~10^6 - 10^7 (total) are required because of various limitations imposed by the instrument and the actual data (still compares favorably with the >10^{10} molecules that are required for making even a single small 3D-crystal suitable for X-ray analysis)

So, what is the "evil"?

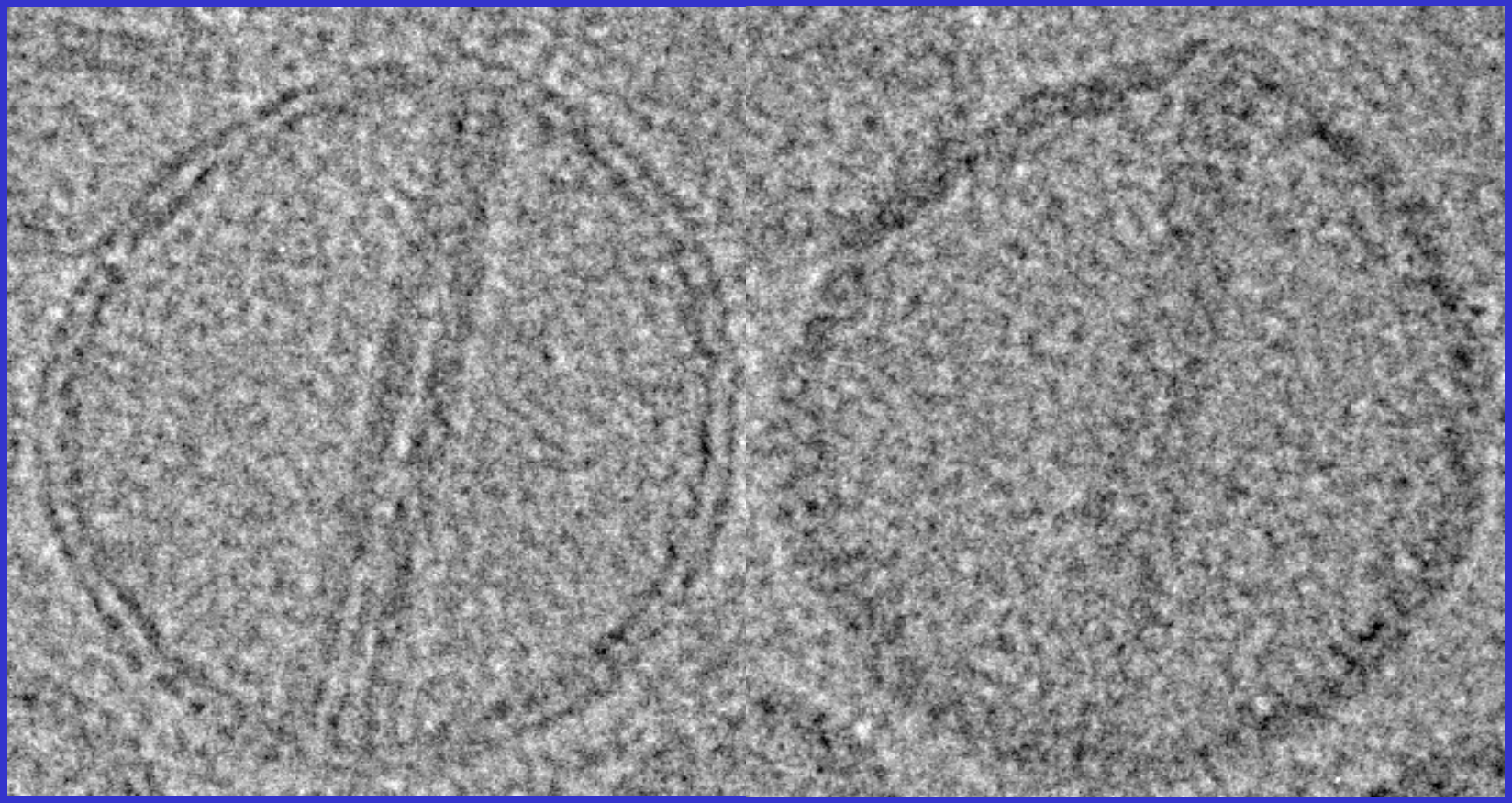
- The stronger interaction of electrons with matter ==> need thin specimen because the "mean free path" (= penetration) is short (typically a few 100Å). Besides absorption, electrons passing through a thick specimen will undergo secondary/tertiary/multiple scattering events which renders the image uninterpretable.

- Since the number of molecules we observe is small compared to X-ray experiments, **radiation damage** becomes a real problem, especially with vitrified samples that are needed for high resolution work on biological specimen. This can partially be overcome by "lose-dose" microscopy (= using only ~10 e/Å^2 for data collection) ==> however, the latter gives very poor signal-to-noise ratios especially for high spatial frequencies.

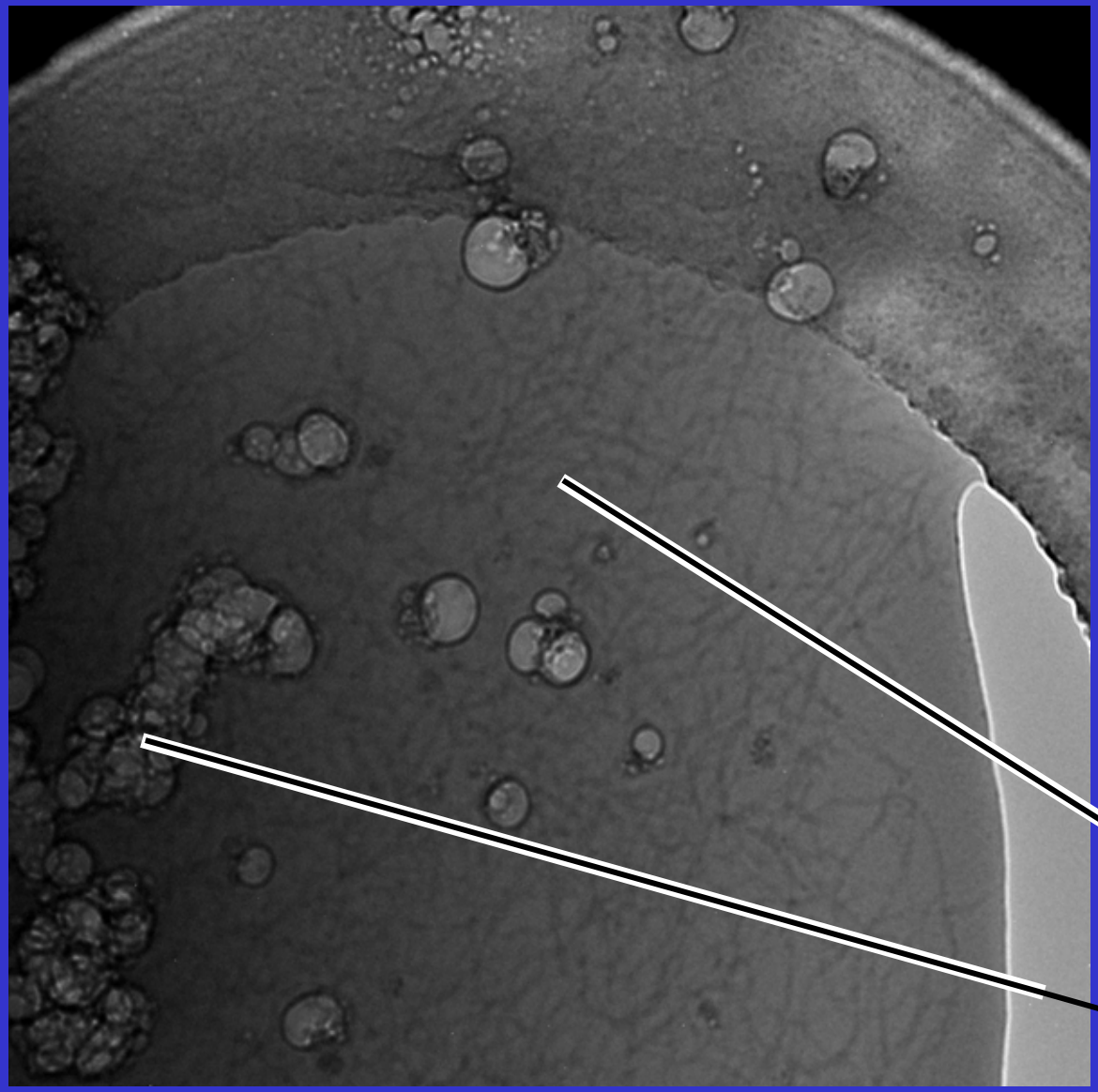
The Destructive Power of Electrons

After 0.2 sec

1 sec exposure



“Bubbling” … a sign of severe radiation damage

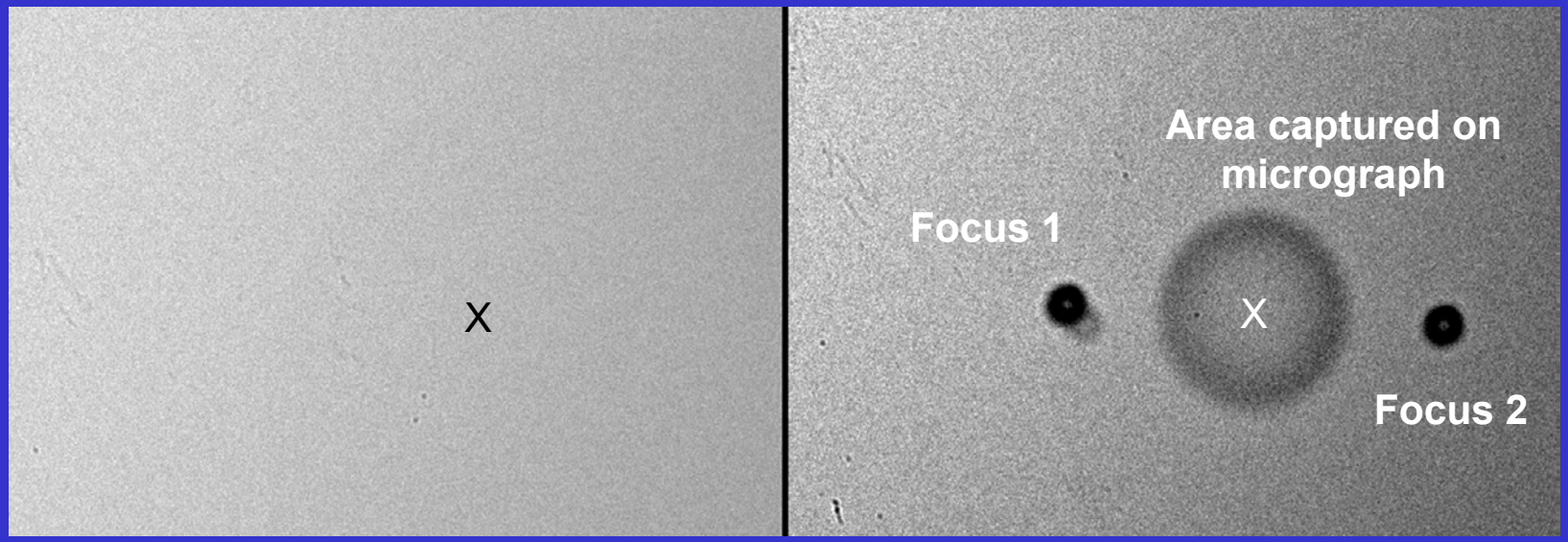


A sample of unstained amyloid materials after a few seconds of illumination with an electron beam

While some fibers can still be detected, “bubbling” within the field of view indicates total destruction of the sample

Courtesy Dr. M. Pelletier

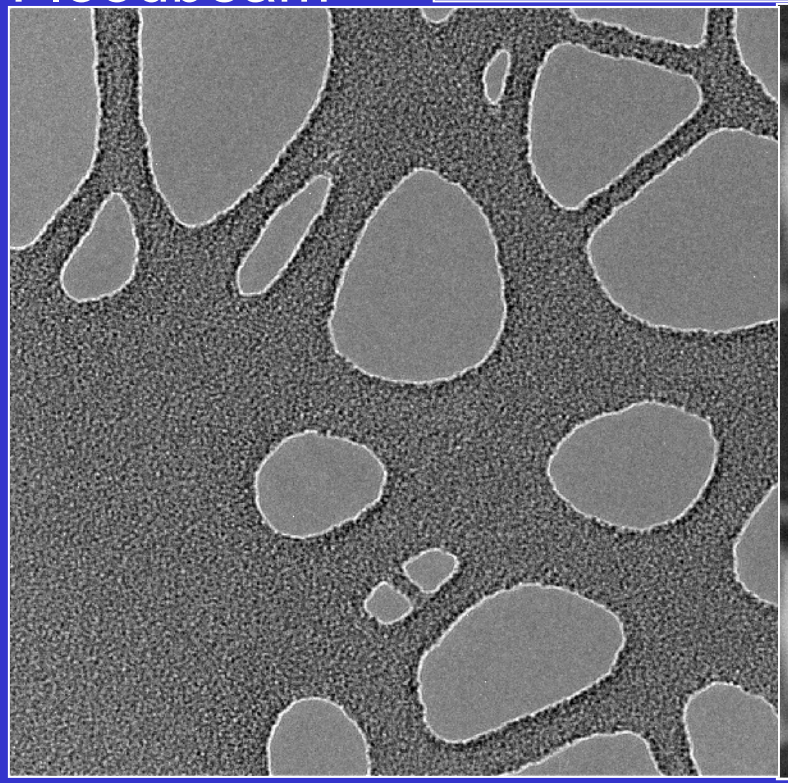
Principle Of Low-Dose Microscopy



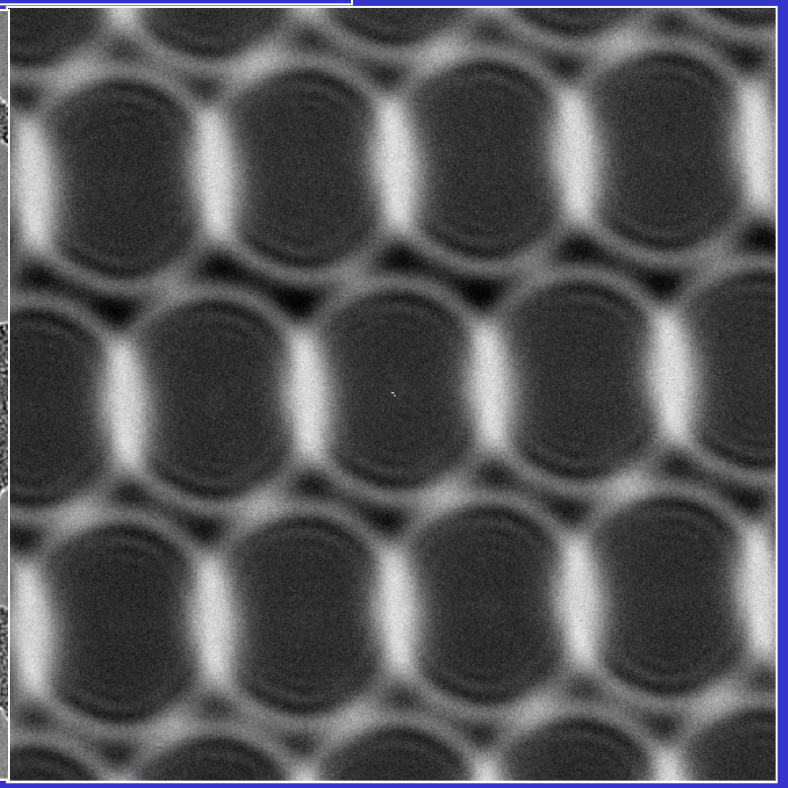
Appearance of trehalose dried down on a carbon film (left). The sugar allows to demonstrate how “low-dose” microscopy is done (right). Let X be the area of interest (for instance a crystal or virus/single particle). Prior to taking a picture some parameters such as “defocus” and “astigmatism” need to be adjusted. To avoid destruction of the specimen, any adjustments are made on small areas (Focus 1 and 2) located adjacent to the area that will be photographed. In the example, the trehalose burned as it was exposed at high magnification (220kx, Focus 1 and 2). Similarly, by exposing the area to be captured for about 30 seconds at 52,000 fold magnification.

Two Modes of Imaging

Floodbeam



Spotscan



Generally applicable

Advantages over floodbeam: least charging; less sensitive to specimen movement; allows dynamic focussing.

Disadvantages compared to floodbeam: only good for crystals so far. Can produce strange artifacts in calculated diffraction pattern

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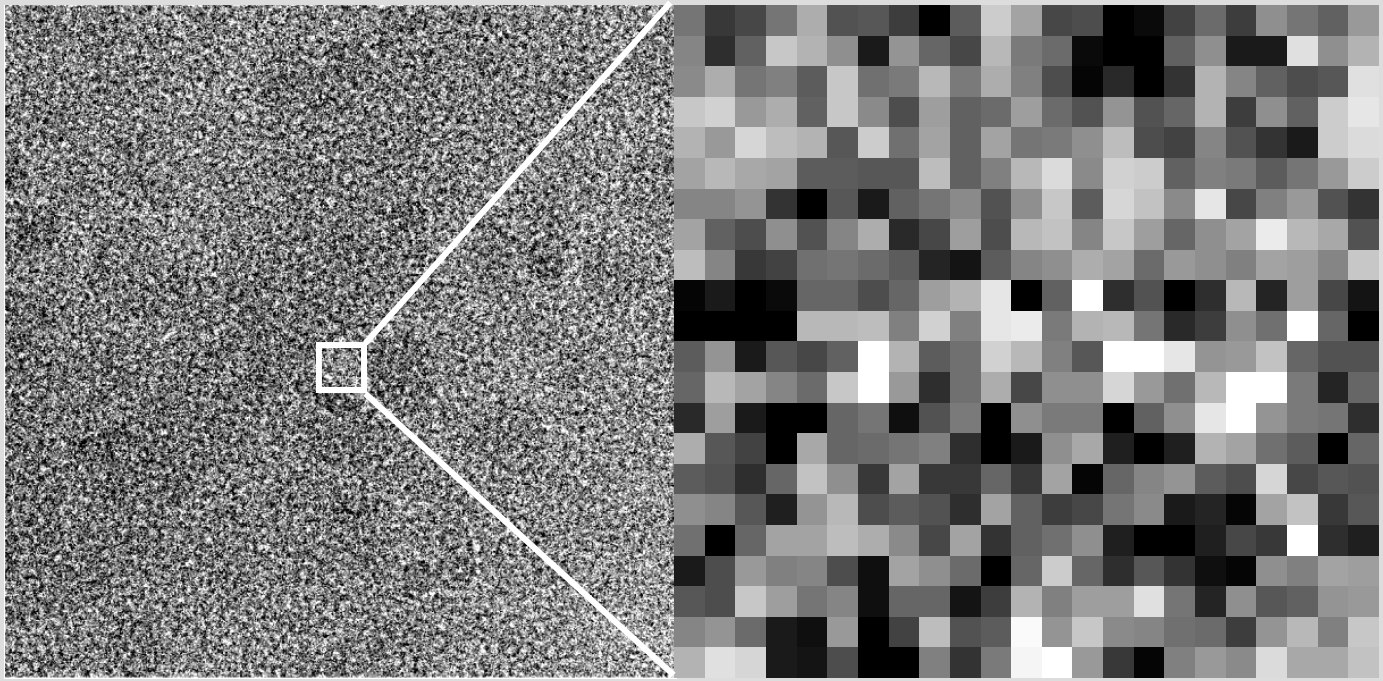
Electron Micrographs Need to be Digitized Prior to any Image Processing



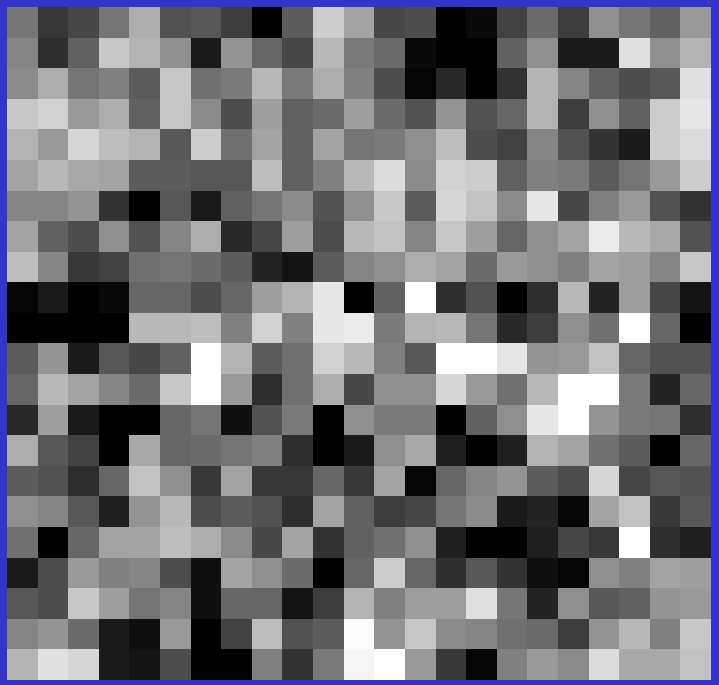
image

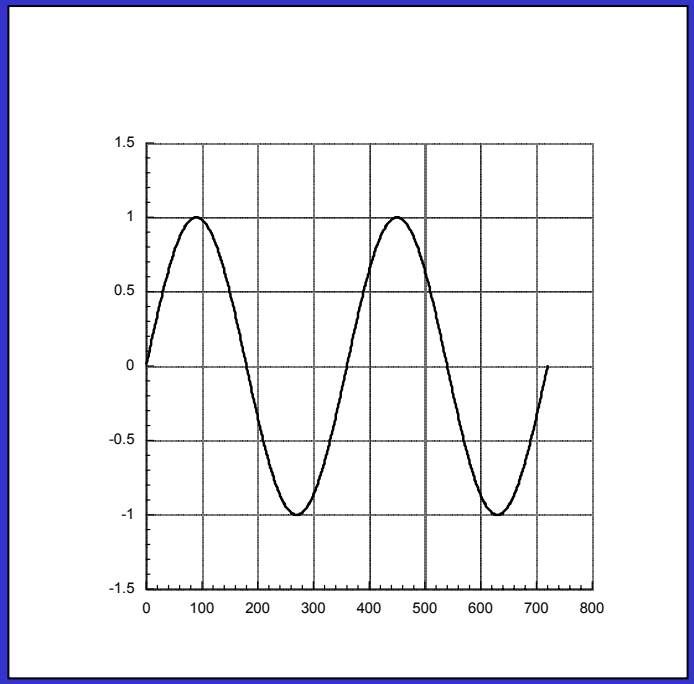
magnified sample of image showing how the image is formed by a grid of 10x10µm pixel

The Nyquist limit

Pixelsize/magnification = sampling distance at the level of the specimen

For instance: pixel is 10µm, magnification was 50,000-fold
==> sampled @ 2Å/pixel



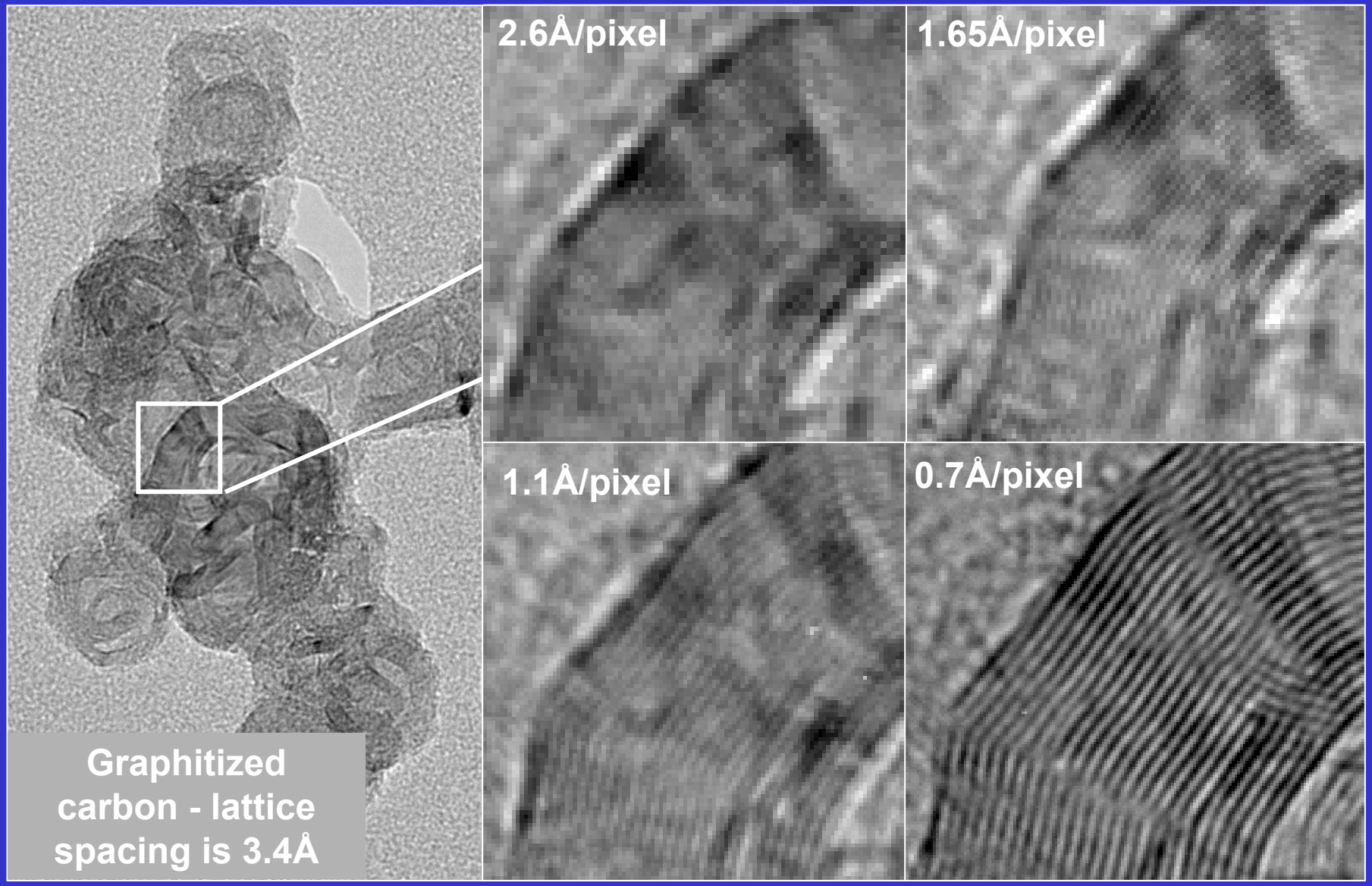


A plain sine-curve is defined by any pair of values taken from within one period

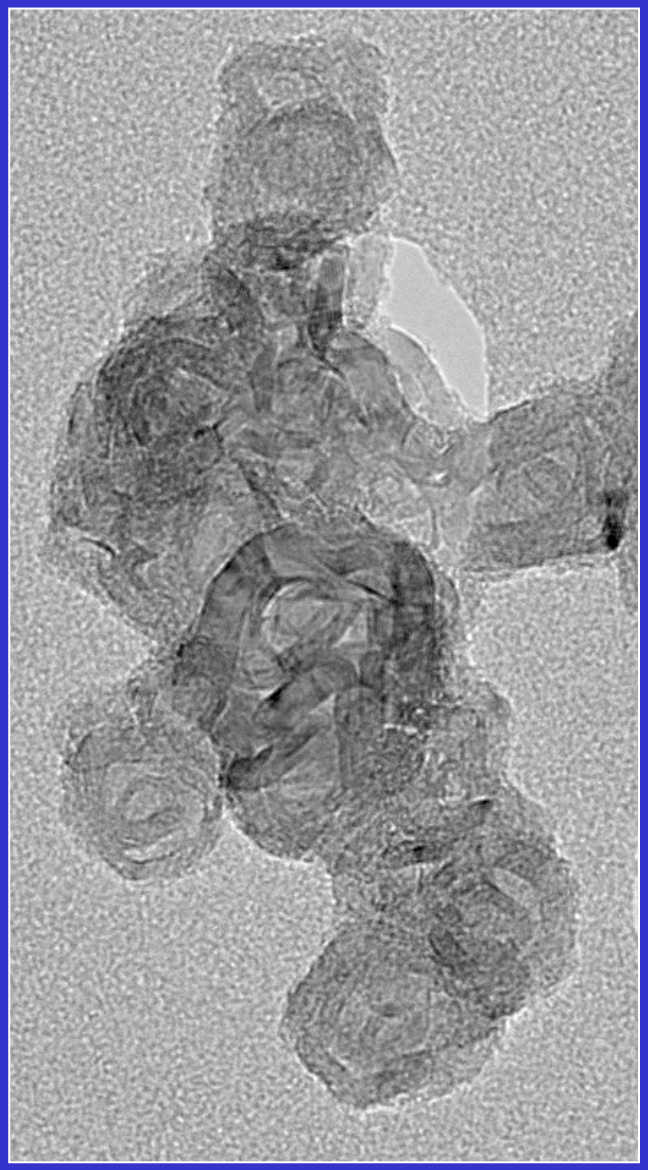
==> if the sampling in the image is @ 2Å per pixel, and 2 pixel values are needed to define frequencies, then the highest frequency that can be reliably described is 2*2=4Å

This relation is referred to as the Nyquist limit and says that an image needs to be sampled at at least twice the frequency of the highest resolution to be obtained

RESOLUTION-how does it impact on imaging?



RESOLUTION-how does it impact on imaging?



CCD: fixed pixelsize (rather large, ~15µm)

--> need to increase magnification to get better resolution

--> lowers the # of particles/unit cells to be imaged per image (☹ SSN if 2D-crystal)

--> need large CCD array to get work done (☺ manufacturer, ☹ budget)

need low to intermediate acceleration voltage.

Film: fixed grain size (~5µm, to get high speed)

--> ultimately becomes limiting.

But, optical scanners can go as low as 5µm/step --> can choose pixelsize to match problem.

Can use smaller magnifications --> more particles/unit cells per film (☺ SSN if 2D-crystal, ☺ more particles with "identical" base parameter [defocus, magnification…])

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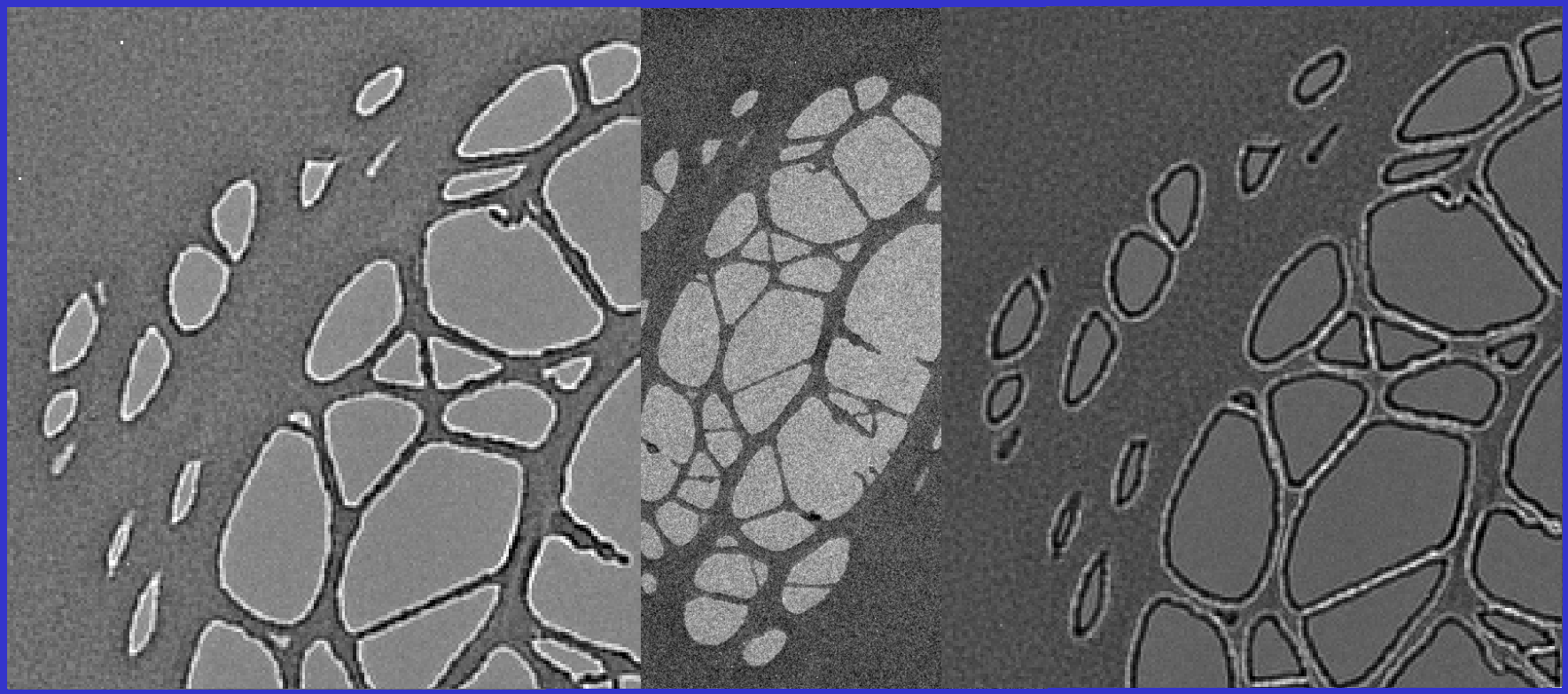
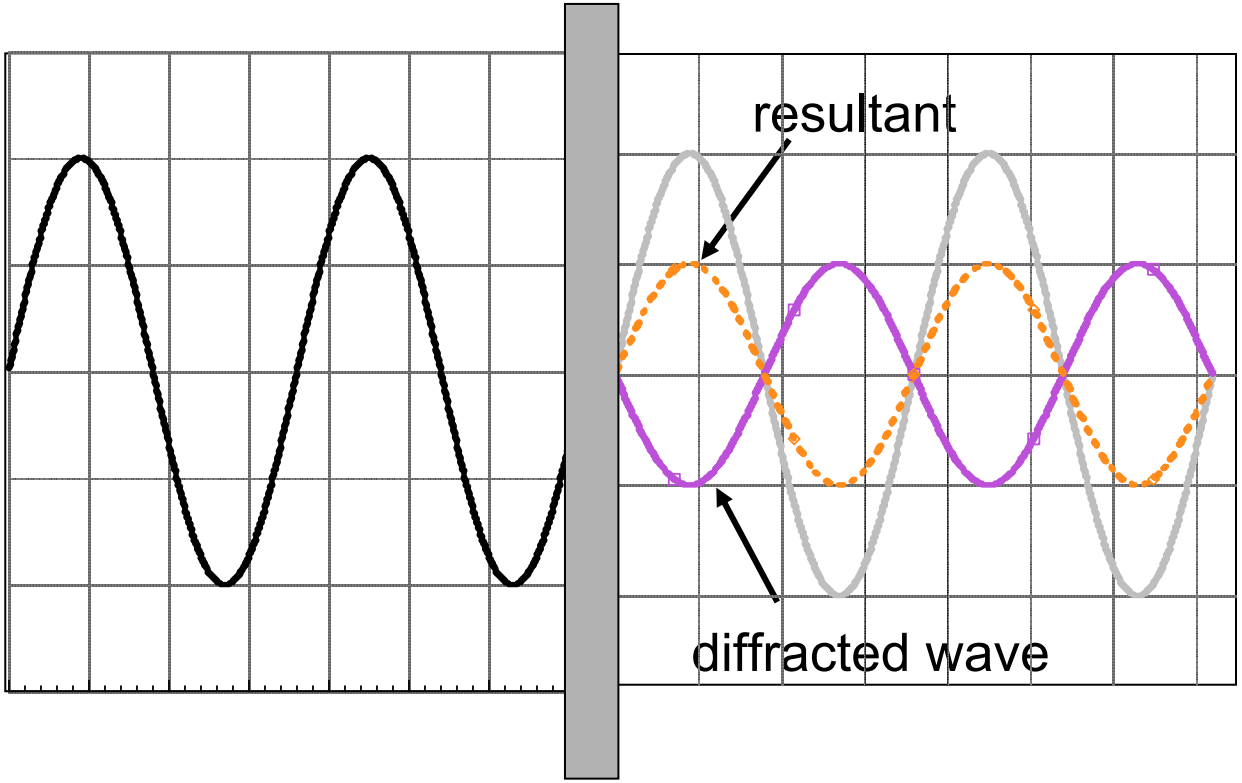
What it true?



What sunglasses have to do with CTF…..

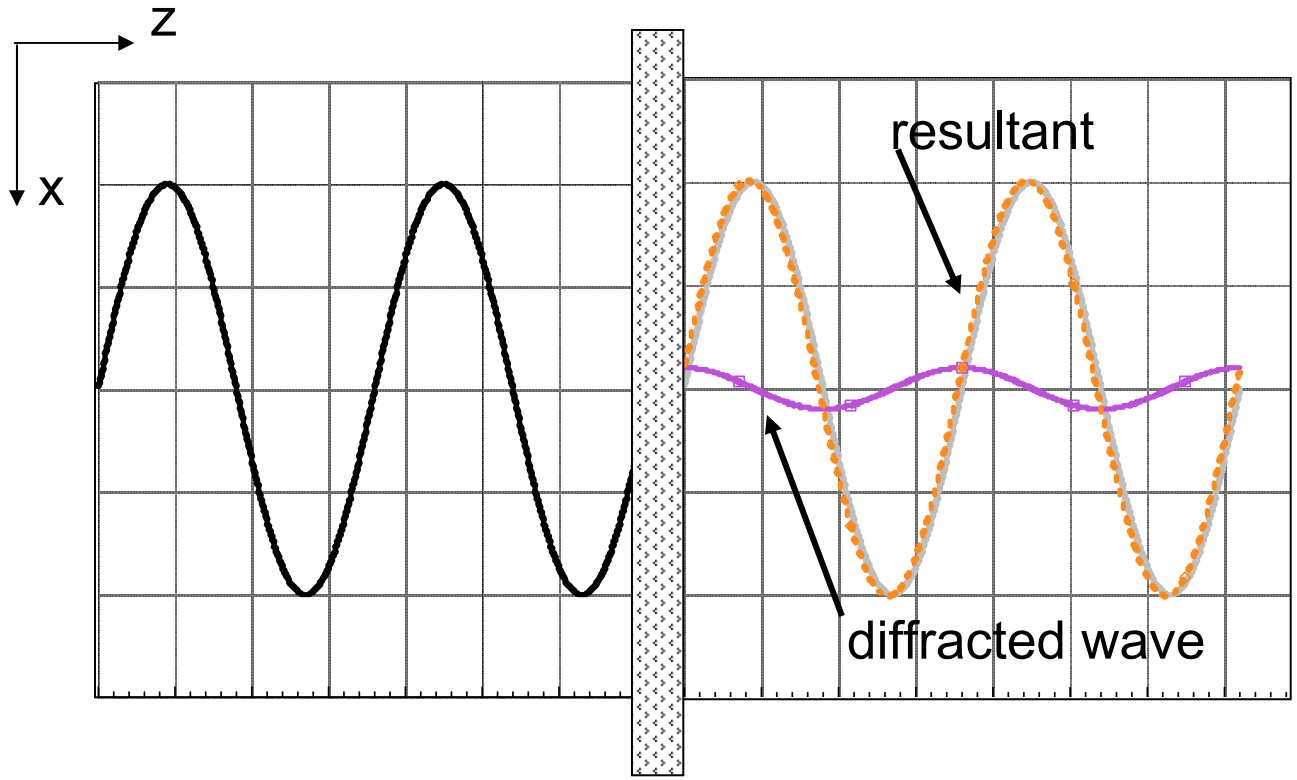


An amplitude object causes contrast by absorbing some of the incident light. In terms of waves, the resultant is the sum of the undiffracted beam and a diffracted beam that is shifted by 180˚ and has lost intensity due to absorption.

Although amplitude contrast does contribute to the image, most of the contrast is generated by phase contrast

Phase Contrast

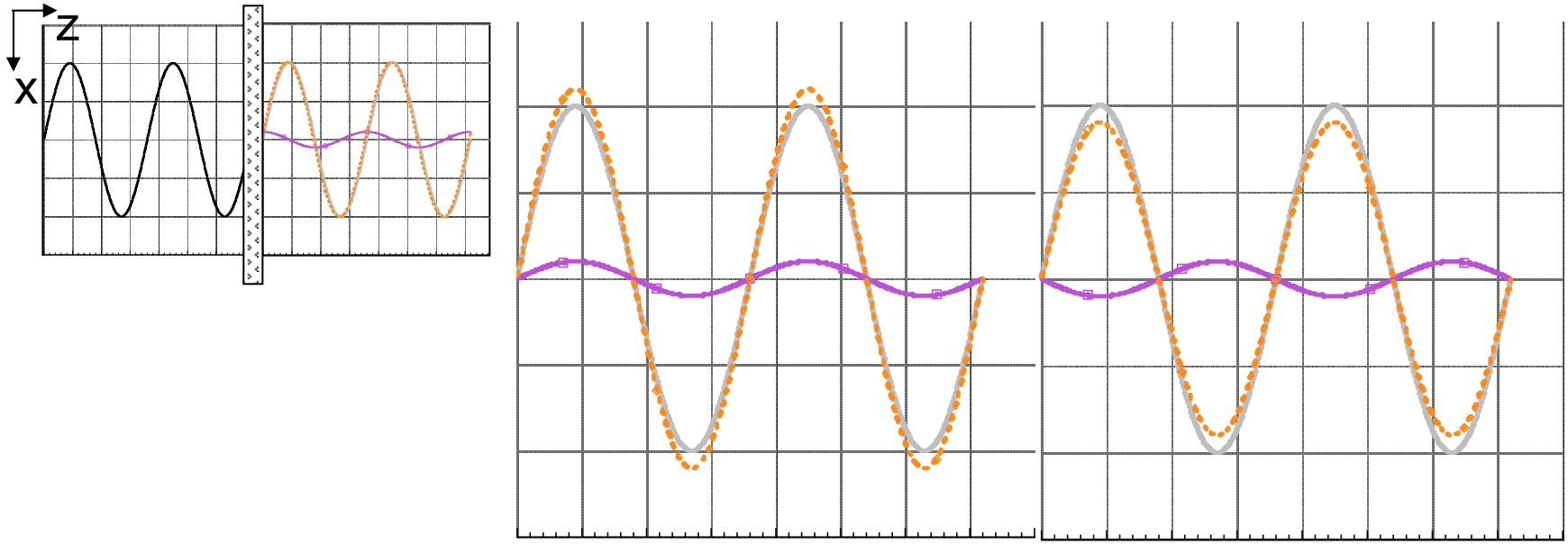
A phase object behaves drastically different. In this case, no light is absorbed and the resultant wavefront emerges with almost the same amplitude, but has suffered a small phase shift. This can be reconstructed vectorially by interfering the undiffracted beam with a diffracted beam of low intensity that is shifted by ~90˚ with respect to the undiffracted beam.



This is BAD news for imaging because in order to record a signal we need differences in amplitude! In light microscopy we use a phase plate that brings the undiffracted and diffracted beams into register and also reduces the intensity of the undiffracted reference beam. So, how do we solve this problem in the EM?

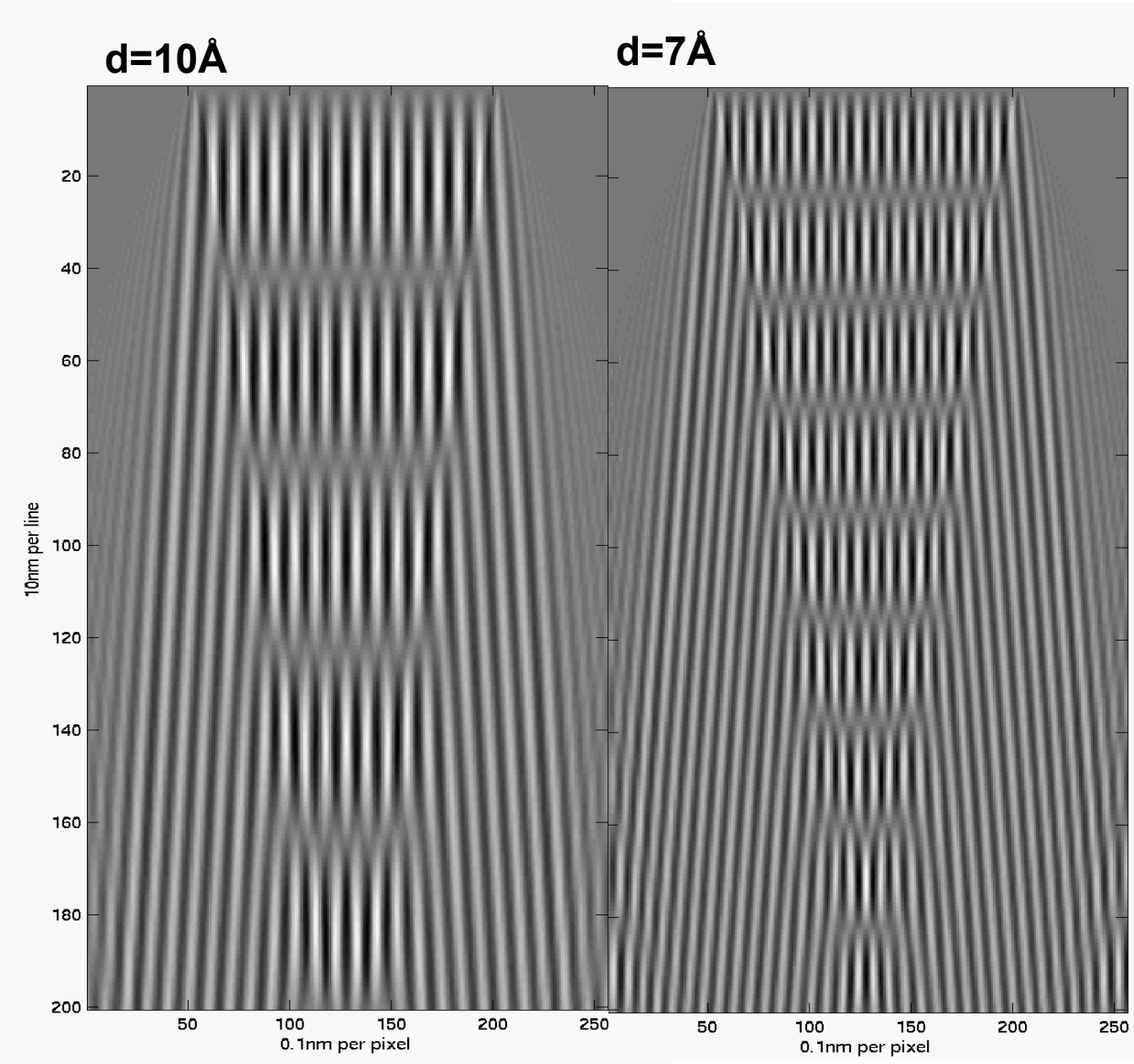
We can exploit two things to turn the original phase signal into an amplitude difference.

First: the objective lens forces the undiffracted and diffracted beams to interfere. Why does that generate contrast? It does because the diffracted beams propagate at an angle to the reference beam, and hence their pathlengths differ.



The difference in pathlength cause the register between the undiffracted reference beam and the diffracted beams to change. Consequently, interference between the beams will alternate between constructive and destructive.

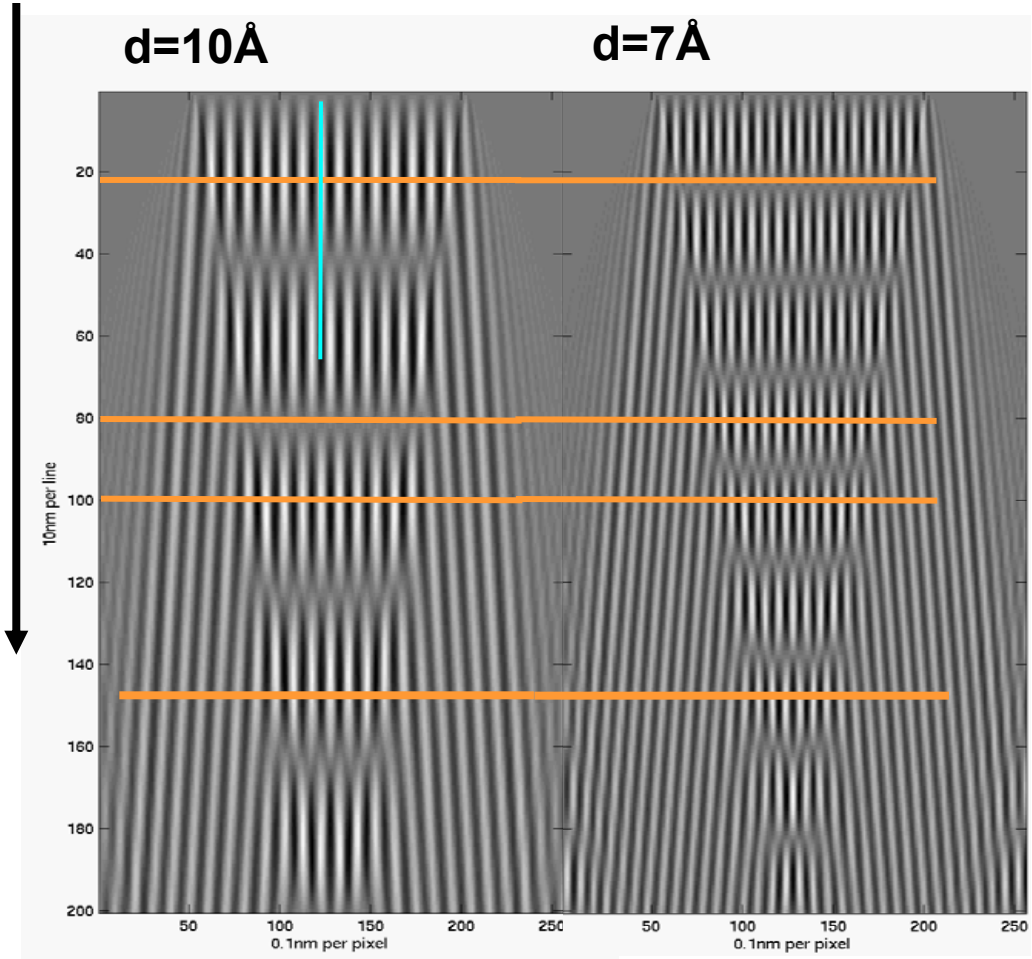
Simulated Interference between the Undiffracted Reference Beam and Diffracted Beams at Two Different Spatial Frequencies



200kV electrons

Courtesy
Dr. F. Sigworth,
Yale Univ

Distance from specimen increases



Courtesy
Dr. F. Sigworth, Yale Univ

These interference patterns reveal three important points:

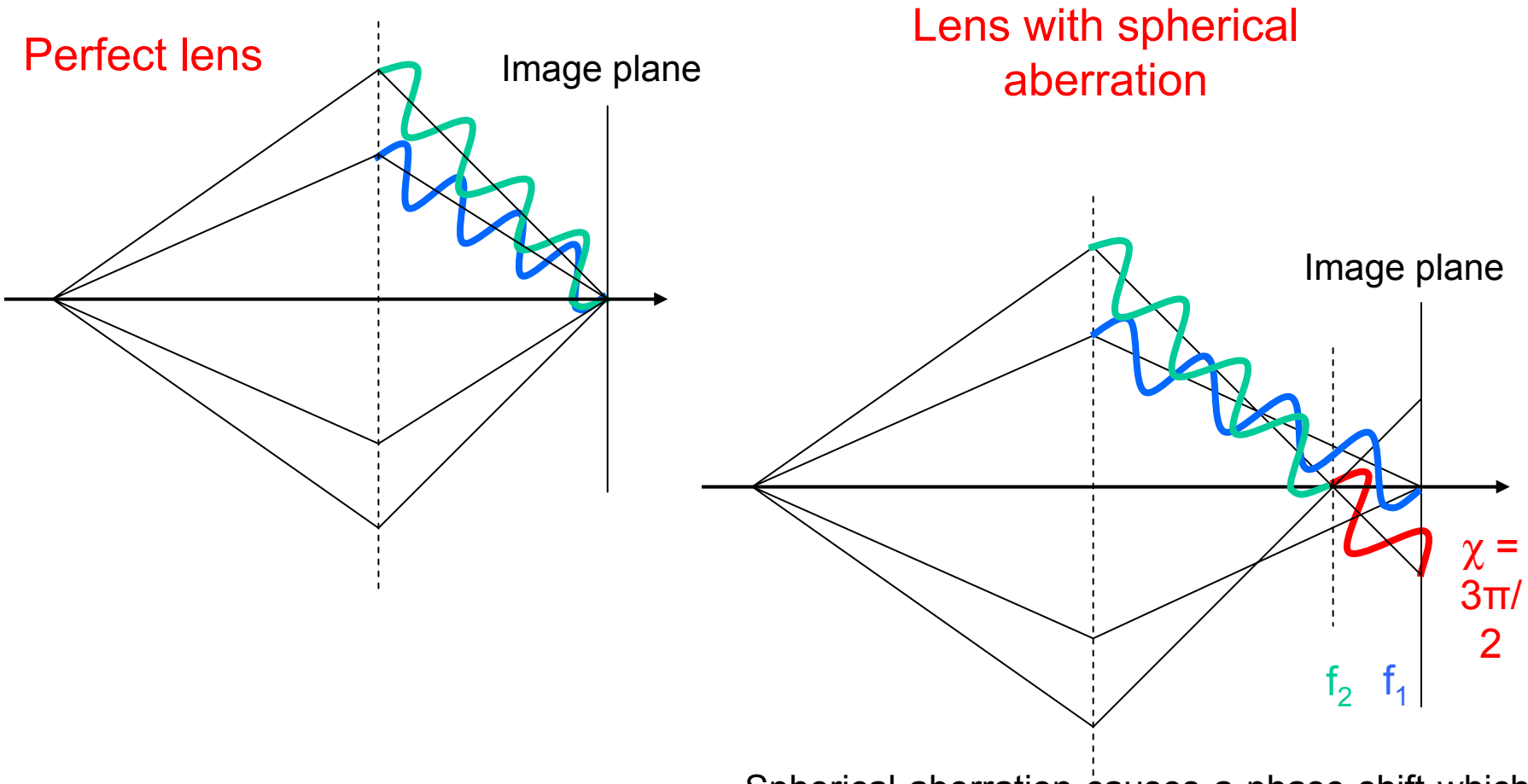
- The **magnitude of contrast** for each spatial frequency depends on where behind the specimen plane we take a look at it. The latter we choose by defocussing!

- The contrast of a spatial frequency is not the same at all distances away from the specimen, it keeps alternating between negative and positive contrast.

Since our samples scatter at all possible frequencies at once, what is the consequence for the images we record?

- The interference patterns show that our image will be a “compromise”! Some frequencies will be strong, others will be weak or absent, and yet others will have reversed contrast!

A second factor contributing to phase contrast is the lousy quality of electromagnetic lenses!



Spherical aberration causes a phase shift which depends on the scattering angle and Δf

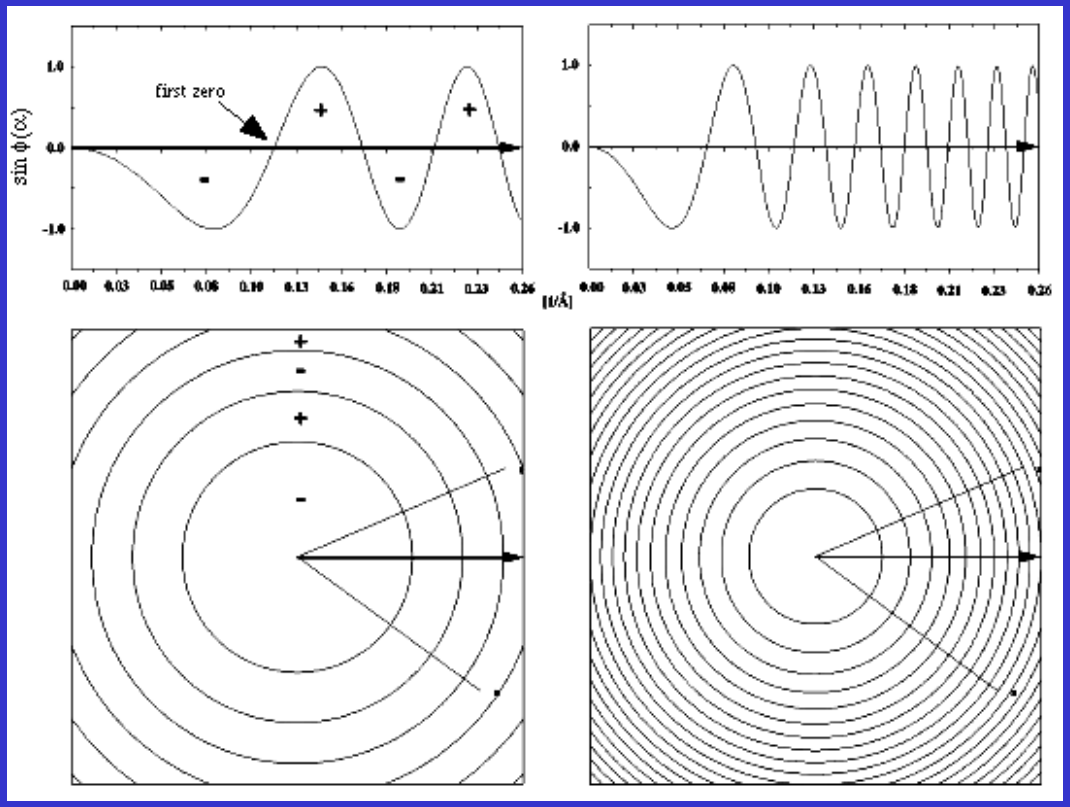
Courtesy Dr. W. Kühlbrandt, MPI for Biophysics, Frankfurt/Germany

SUMMARY CTF

The phase shift $\Phi(\alpha)$ introduced to the scattered waves depends on the wavelength (λ), spherical aberration of the objective lens C_s, the diffraction angle (α) and the underfocus (Δf).

$$\Phi(\alpha) = \frac{2\pi}{\lambda}(0.25 \bullet C_s\alpha^4 - 0.5 \bullet \Delta f\alpha^2)$$

This phase shift (also known as CTF= contrast transfer function) modulates the FT of the image in an oscillating manner described by [sin $\Phi(\alpha)$].



The simulated curves are for 3000 and 6000Å of underfocus respectively, an accelerating voltage of 200keV (λ=0.025Å) and a C_s=2mm

These lower two panels demonstrate how the CTF would look like in the FT of the image. Circles represent [sin $\Phi(\alpha)$] =0 Frequencies where [sin $\Phi(\alpha)$]<0 contribute with reversed contrast to the image. Therefore, the phases of reflections in these regions need to be adjusted by 180˚

CTF-”Correction”

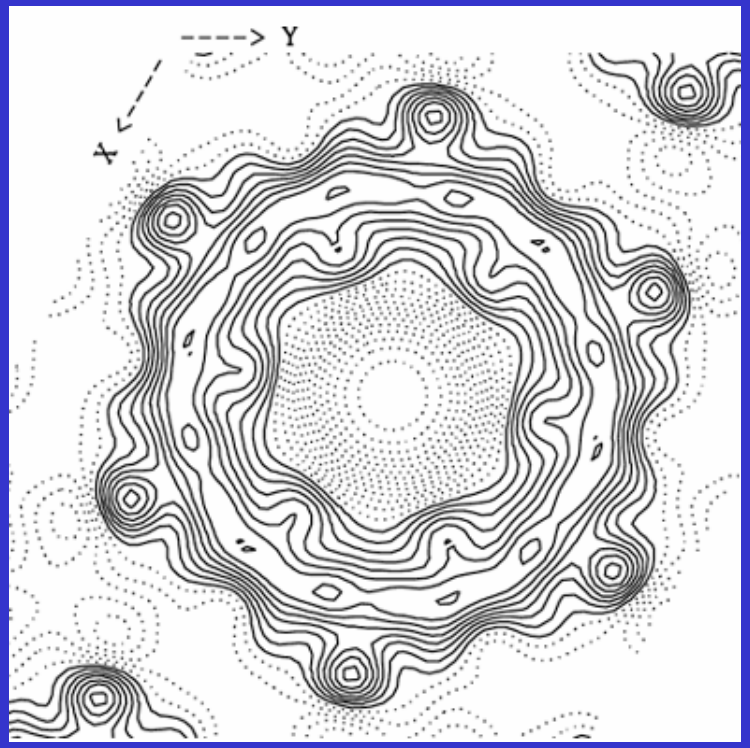
The effect of the CTF on an object/image can easily be simulated. **But:** the reverse is not true. Once an image has been recorded, it cannot be fully corrected for the effect of the CTF because some information has been “wiped out” by the objective lens.

Still, since we know how the CTF modulates the transform, we can partially correct the effect.

Need to consider two components: Amplitude and Phase

Even without “longwinded” argument, we know that the correction of the phases will turn out to be more important because it’s the phases that carry the information about the structure.

Amplitudes



Real space map obtained by Fourier summation

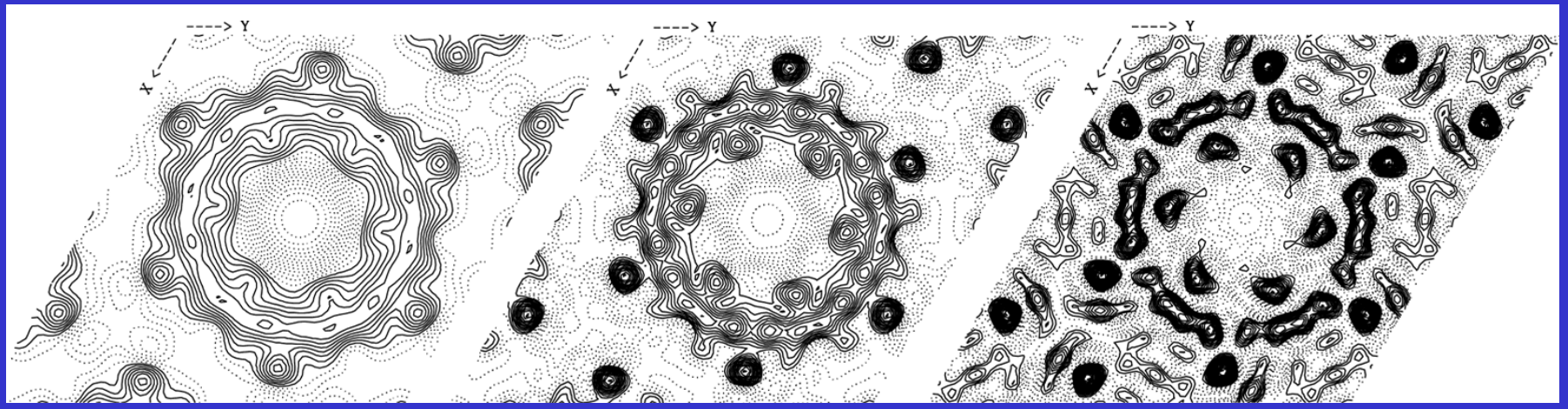
(H,K,L)	amp	phase	FOM
1 0 0	2566	180	99.5
1 1 0	12424	180	99.9
1 2 0	777	180	99.5
1 3 0	1123	0	99.7
1 4 0	208	0	73.9
1 5 0	605	0	99.0
1 6 0	670	180	99.2
1 7 0	250	180	99.6
1 8 0	350	0	94.3
1 9 0	77	180	59.8
1 10 0	140	0	13.3
2 0 0	9265	180	99.9
2 1 0	1971	0	99.8

And so on…..

What happened??

Couple of things…first, crystals are less well ordered at higher resolutions --> signal to noise down; and second, CTF-dependent fall-off of image amplitudes (envelope function) past 10Å downweights high resolution terms

Effect of Image Sharpening (B-factor)



B=0 B= -350 Å^{-2} unity amplitudes

$$sf = e^{-B/4d^2}$$

d= resolution [Å]

→ Example: 6Å, B=-350
sf=11
Compare: unity amplitude
sf=110 B=-680

CTF-Correction: AMPLITUDE

Crystals/Helices

If possible, collect electron diffraction data

If image-derived amplitudes are used

- Correct for CTF-amplitude modulation by: (amplitude/CTF), limit to maximum scaling of 5x to avoid “infinite” amplitudes close to CTF-zero’s.

- Correct for CTF-dependent fall-off (=envelope function) by applying inverse B-factor to data (exact amount not critical unless very high resolution data are available).

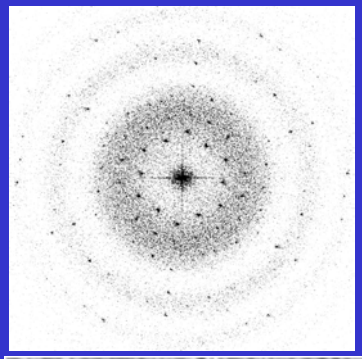
Applying B-factor works because phase is more important, and crystals/helices provide good statistics on reliability of phase data in averaged data set.

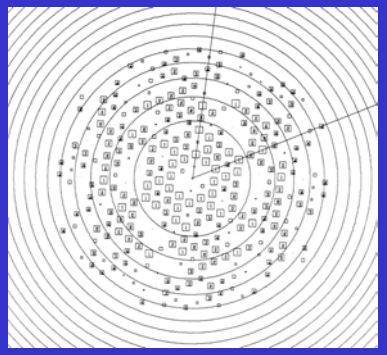
Single Particle

Cannot collect electron diffraction data, but since resolutions often are on the low side, image-derived amplitudes are just fine. Do not even need to try correcting CTF-modulation of amplitudes because at low resolutions subtle changes to amplitudes do not make much difference to the appearance of the structure. So, in terms of amplitude correction, easiest solution is to record images at different underfocus values, which will “equalize” the amplitude-modulation caused by the CTF.

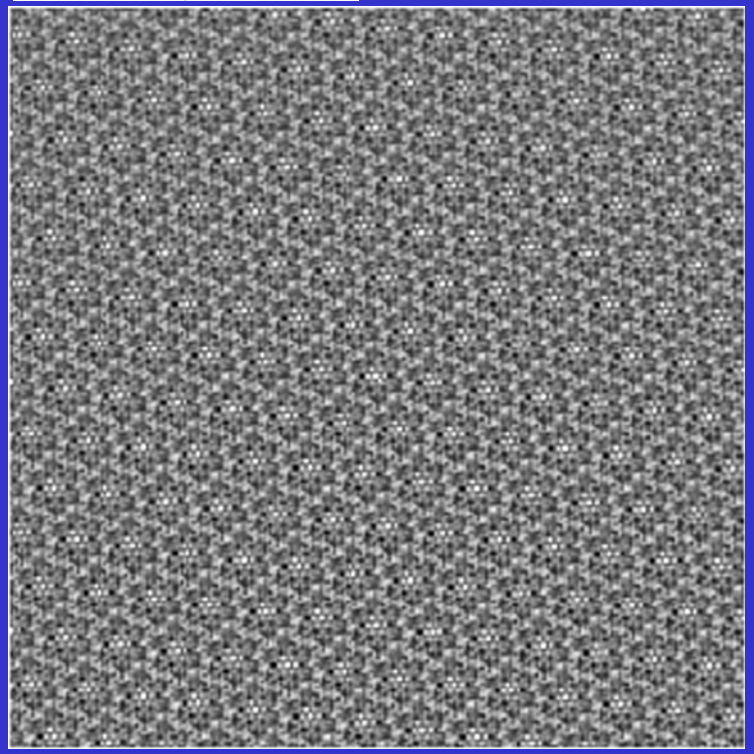
Situation different when going to higher resolutions. In this case, one would need to consider fall-off, and CTF-induced modulation of amplitudes for each individual particle, and the final structure. That does not seem trivial.

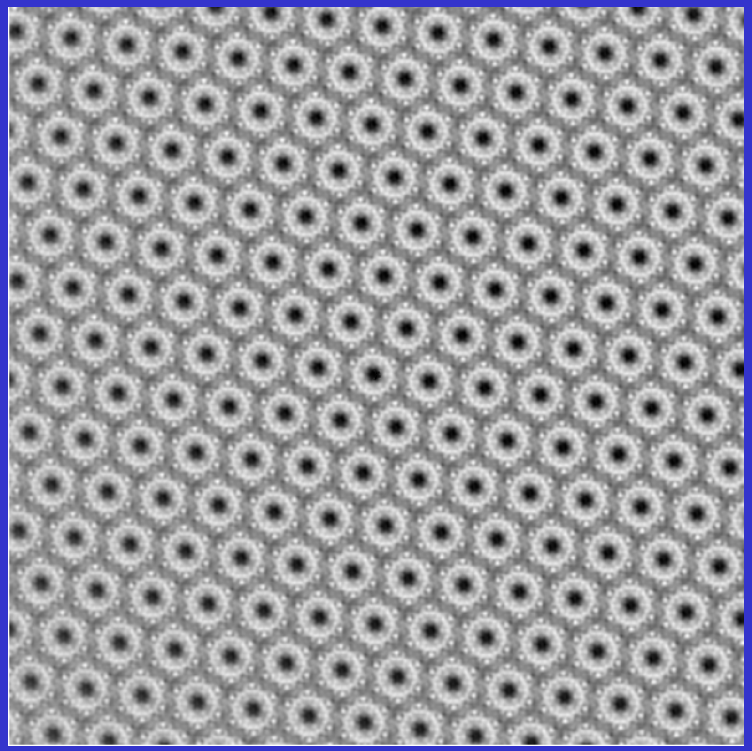
Phases





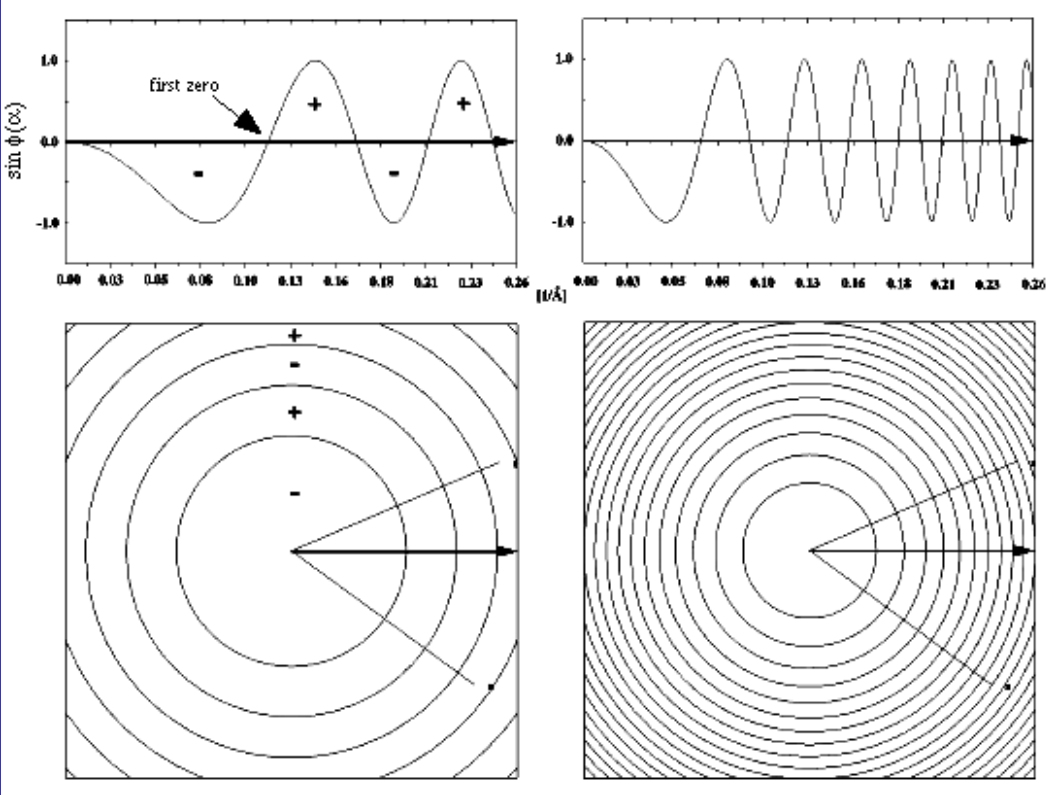
After Correction of Phases For CTF





Correcting the Phases for the Effect of the CTF

$$\Phi(\alpha) = \frac{2\pi}{\lambda}(0.25 \bullet C_s \alpha^4 - 0.5 \bullet \Delta f \alpha^2)$$



Frequencies where [sin $\Phi(\alpha)$]<0 contribute with reversed contrast to the image. Therefore, the phases of reflections in these regions need to be adjusted by 180˚

CTF-Correction: PHASE

Crystals/Helices

Easily achieved and reliable (especially for crystals) because the relevant information is contained in discrete spots/layer lines

Phase of spots represent average of entire crystal, that is: local changes need not be considered unless the sample is highly tilted (CTF--> TTF [tilt transfer function]). Latter irrelevant for helices because no tilt required for structure determination.

Note: minimize number of contrast reversals by recording images at low defocus

Single Particle

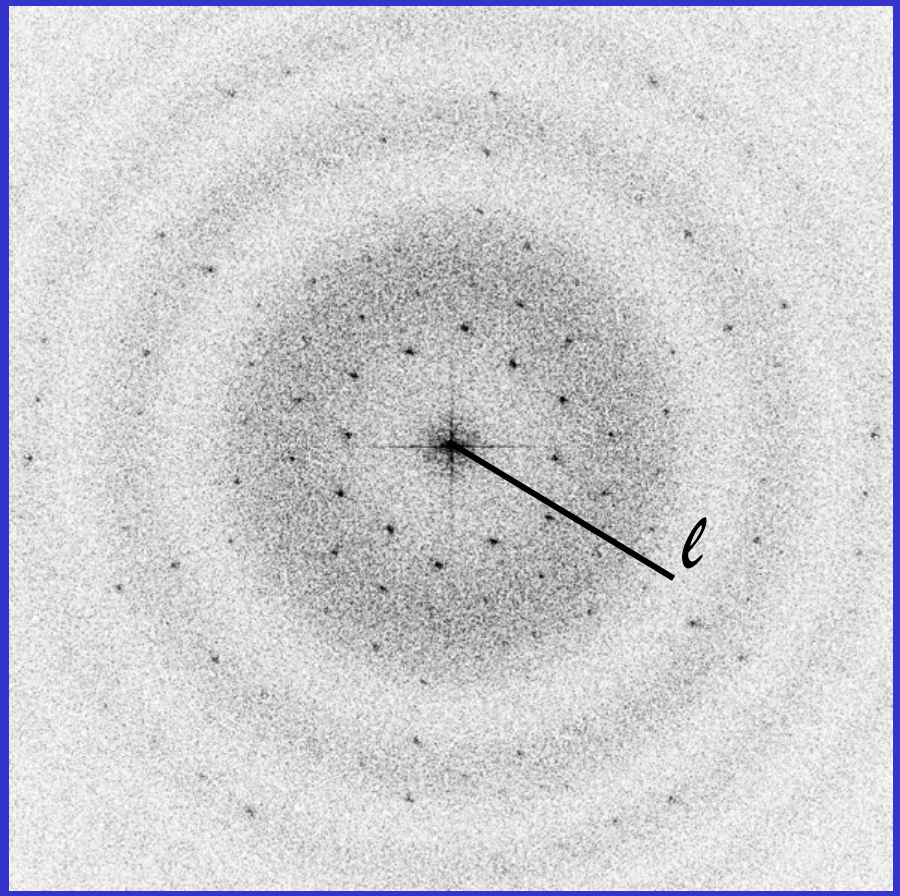
Phase correction for single particles is a "fishing expedition". The most commonly applied "solution" is to do "phase flipping", that is: the overall defocus is determined and the entire image is corrected in a uniform way.

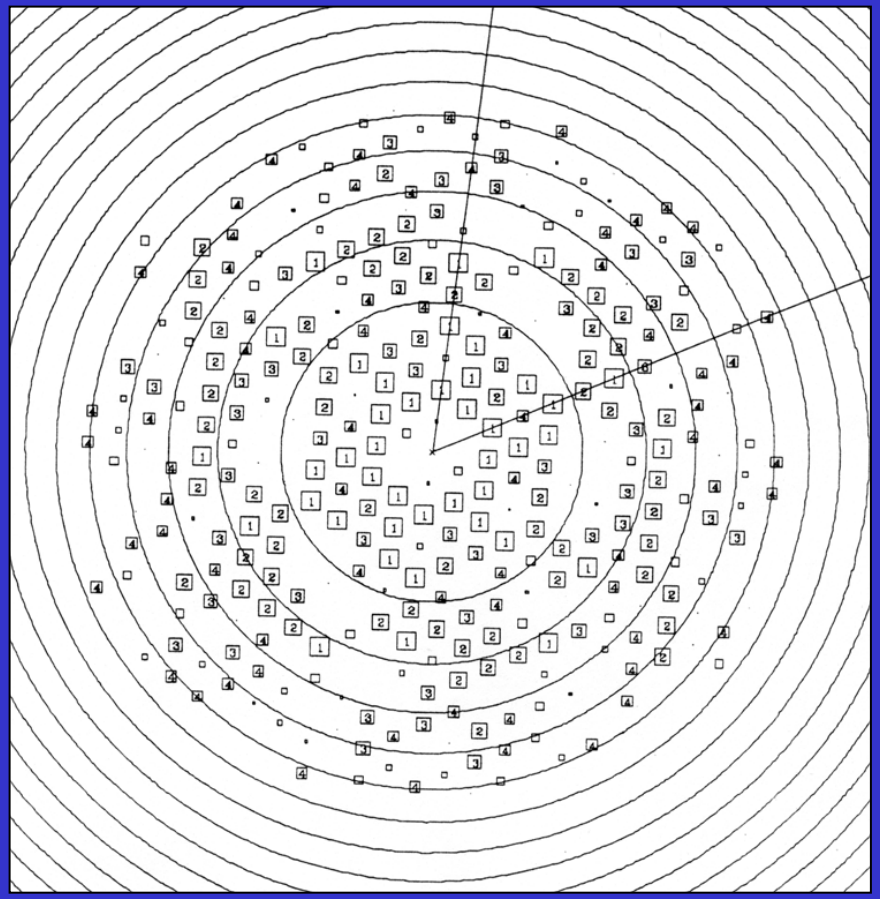
While absolutely necessary, this approach has limitations:

- Not all programs can handle astigmatism
- Local defocus can be different across image (and so can astigmatism)

--> in principle would want to correct each particle image for defocus and astigmatism, especially when resolution goes close or past 10Å. Would have to be based on phases. Problem with that is that signal to noise ratio (= reliability of phase) is bad for image of a single particle

CTF-"correction"





Calculated FT of image

$$1/d[\mathring{A}^{-1}] = \frac{\ell \bullet XMAG}{p_y \bullet step[\mu m] \bullet 10{,}000}$$

Xmag: magnification
p_y: transform size (y-axis)
step: digitizing stepsize

For the correct estimate of the underfocus, the simulated CTF will match that actually observed in the FT of the image

Sounds like CTF is absolutely awful -
But, sometimes the CTF can actually be useful too

The CTF is useful to determine specimen tilt

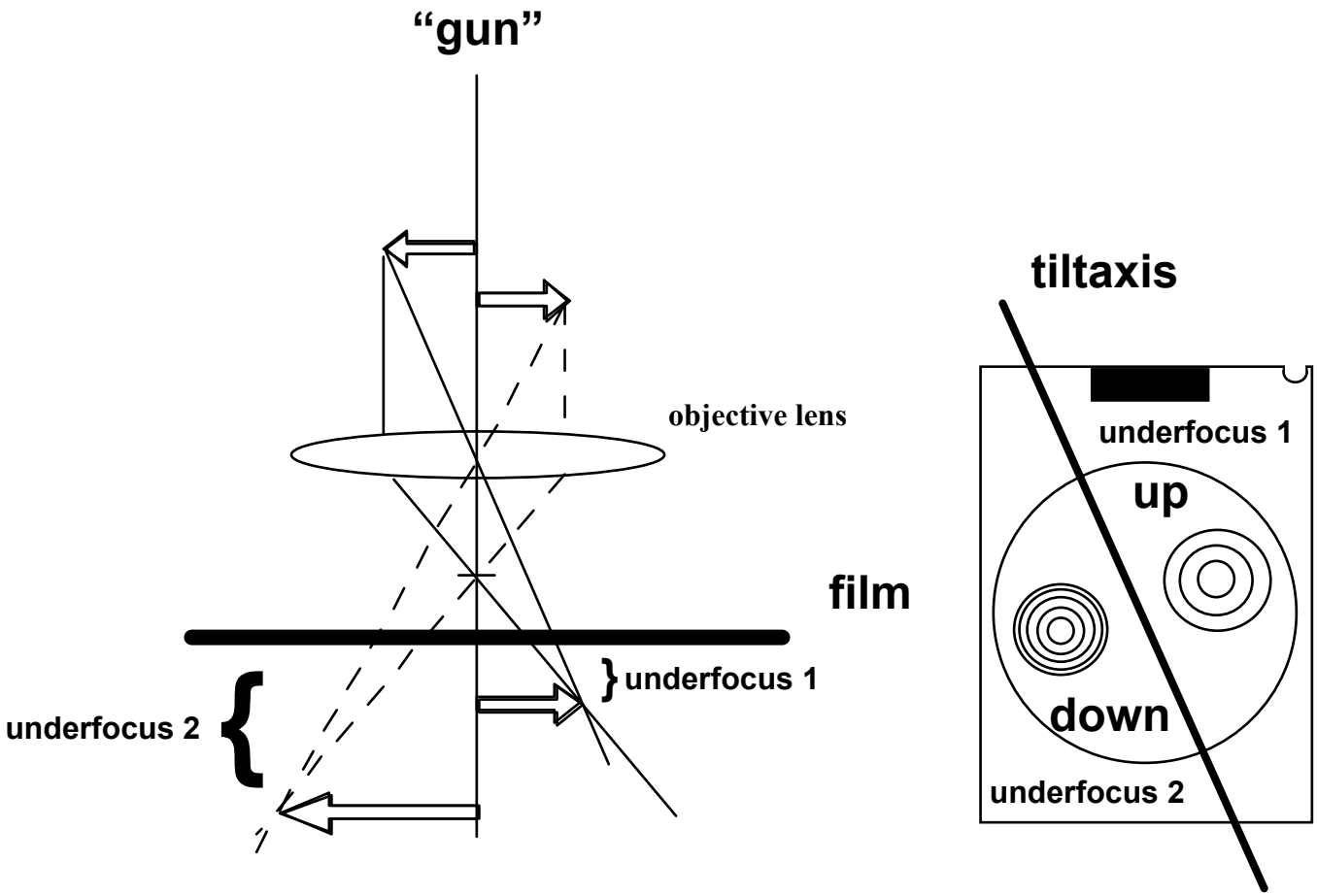
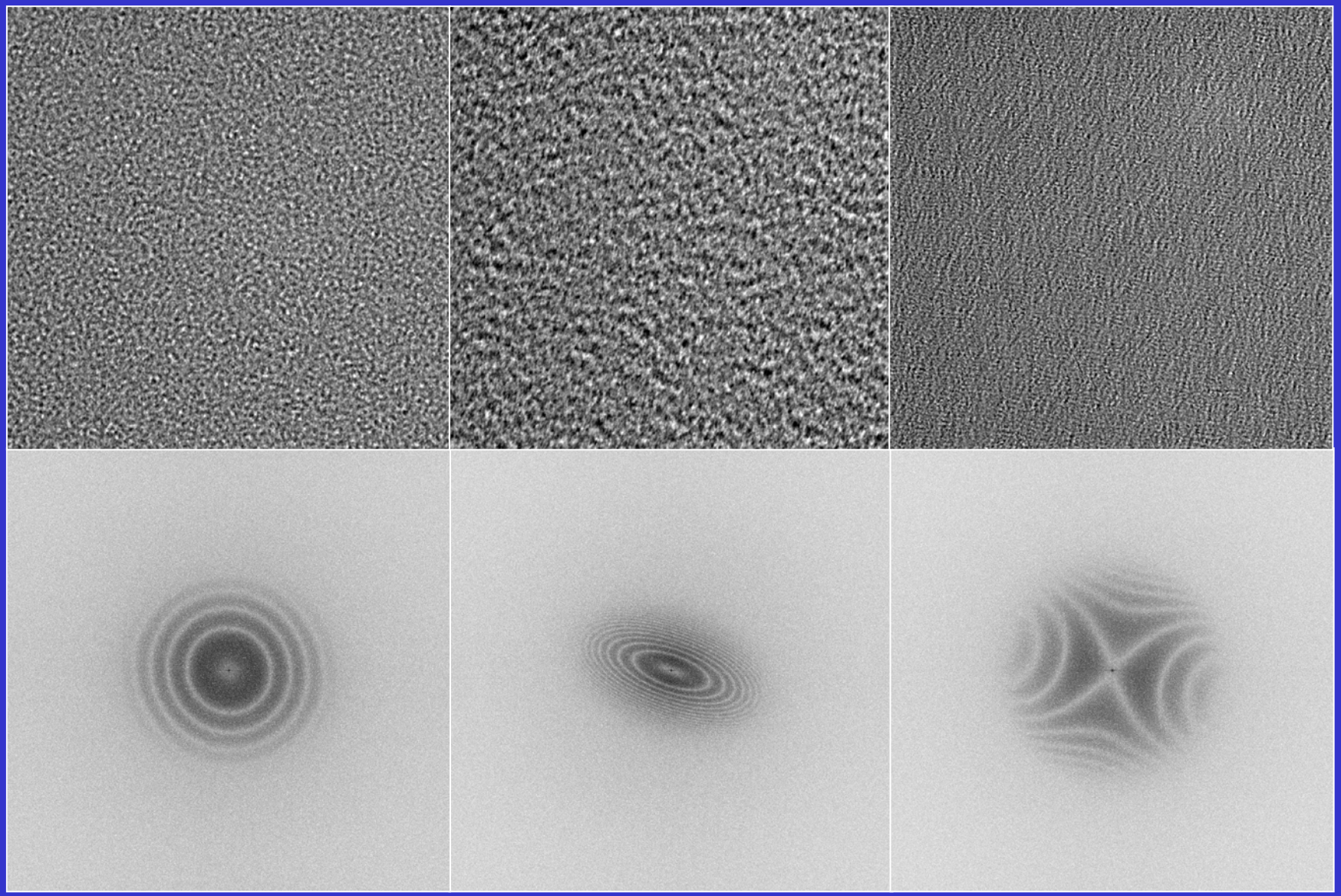
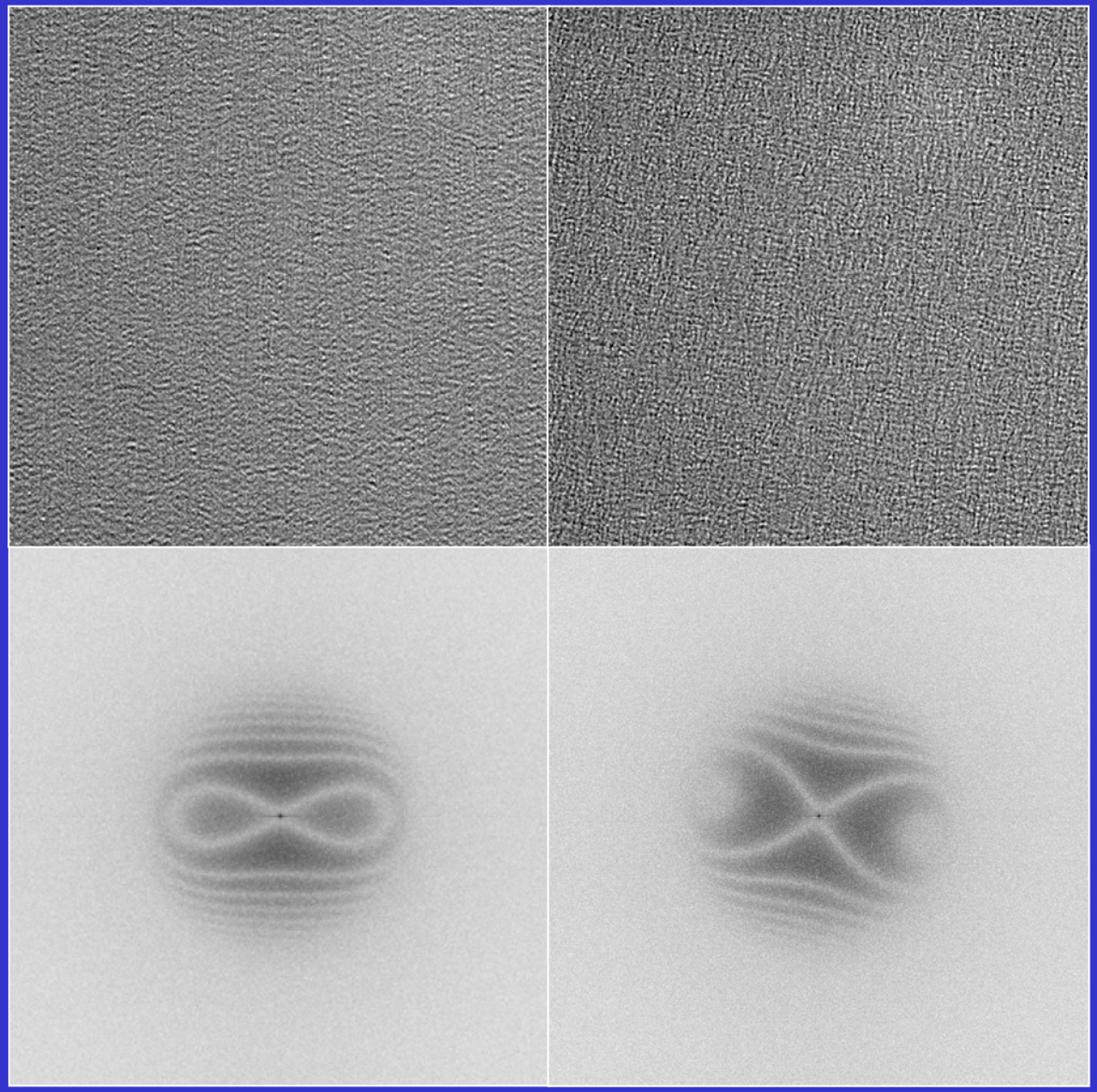


Fig.13: Correlation between specimen height in column and observed underfoucs on film
The strongly simplified ray diagram (left) illustrates that parts of the specimen which are higher up in the column will be imaged at a lower underfoucs if the objective lens current is held constant to image the two positions of the specimen. In the optical diffraction this relation can be directly observed by comparing the Thon ring pattern on either side of the tilt axis. The part of the micrograph that displays fewer and more separated Thon rings is at lower underfocus. Accordingly, this part of the specimen was higher up in the column.

Thonrings - A Useful Analytical Tool: ASTIGMATISM

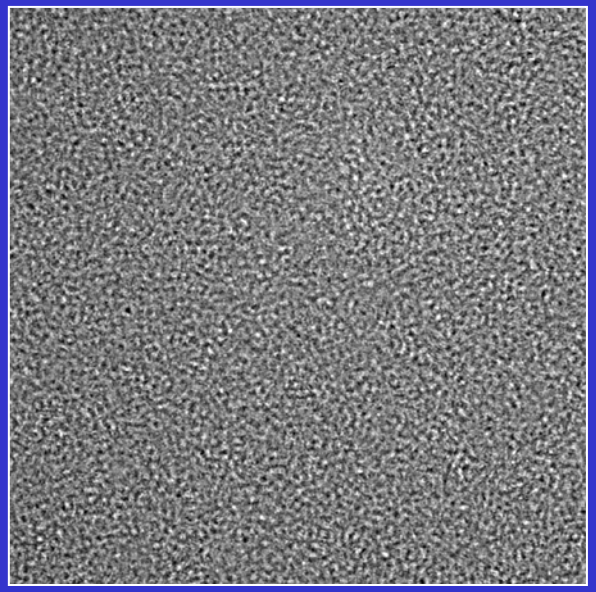


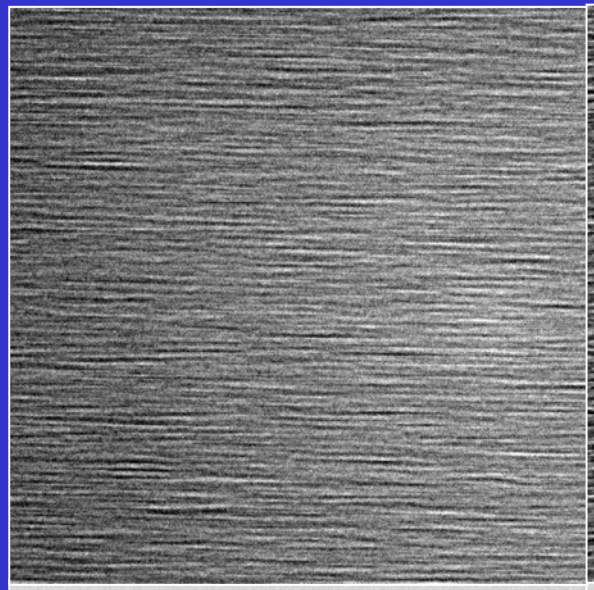
…more ASTIGMATISM

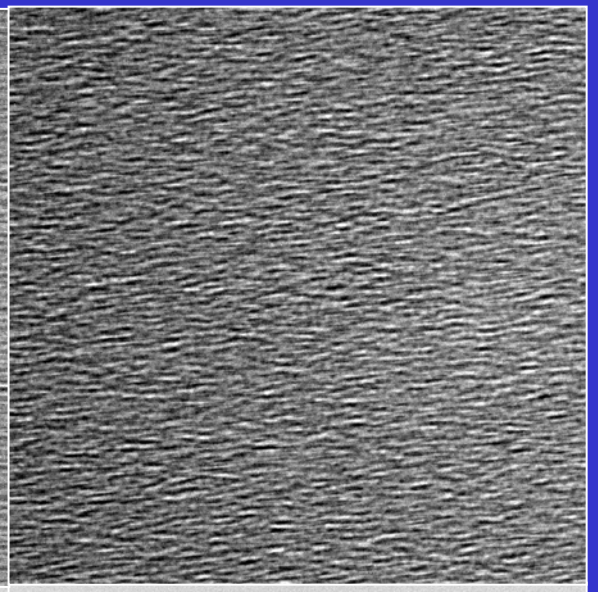


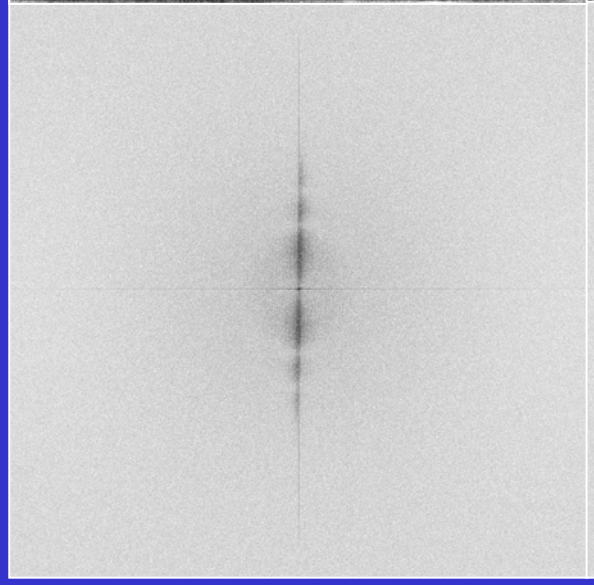
Correction of those is no fun…..it's far easier and faster to just correct for astigmatism during the focussing step

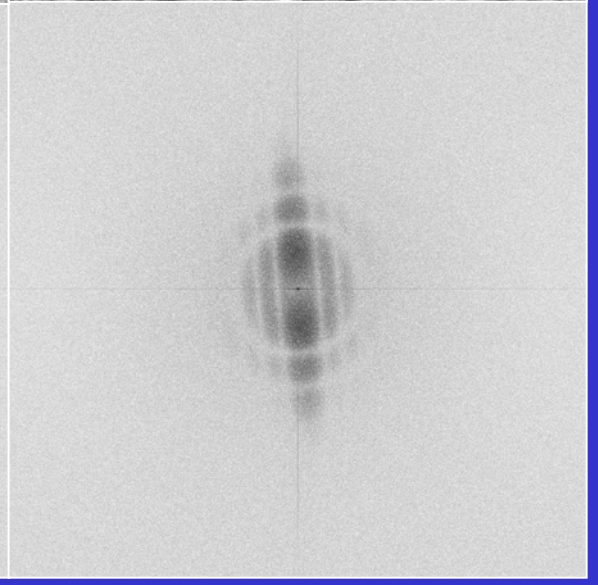
……..what's this?



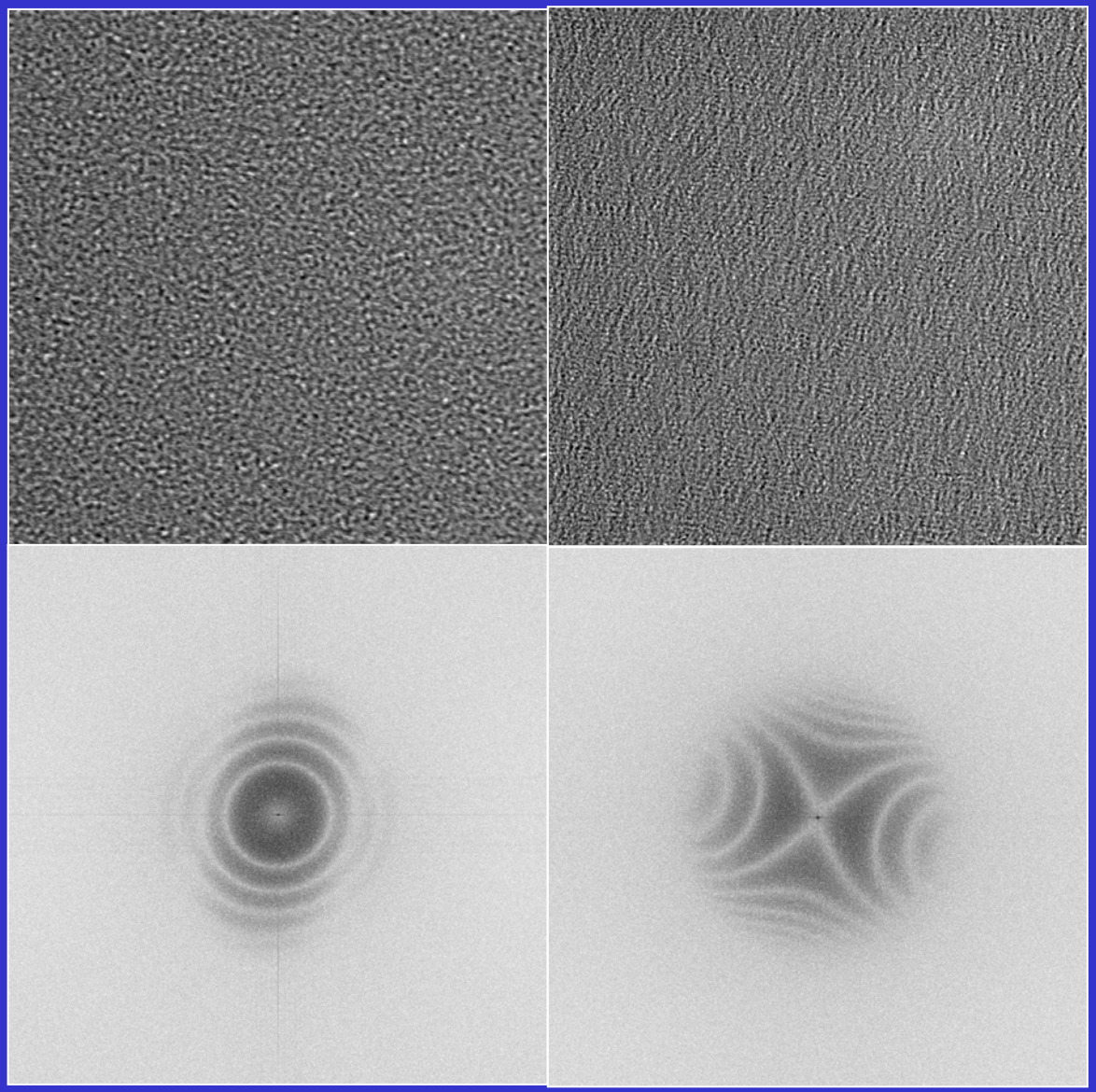








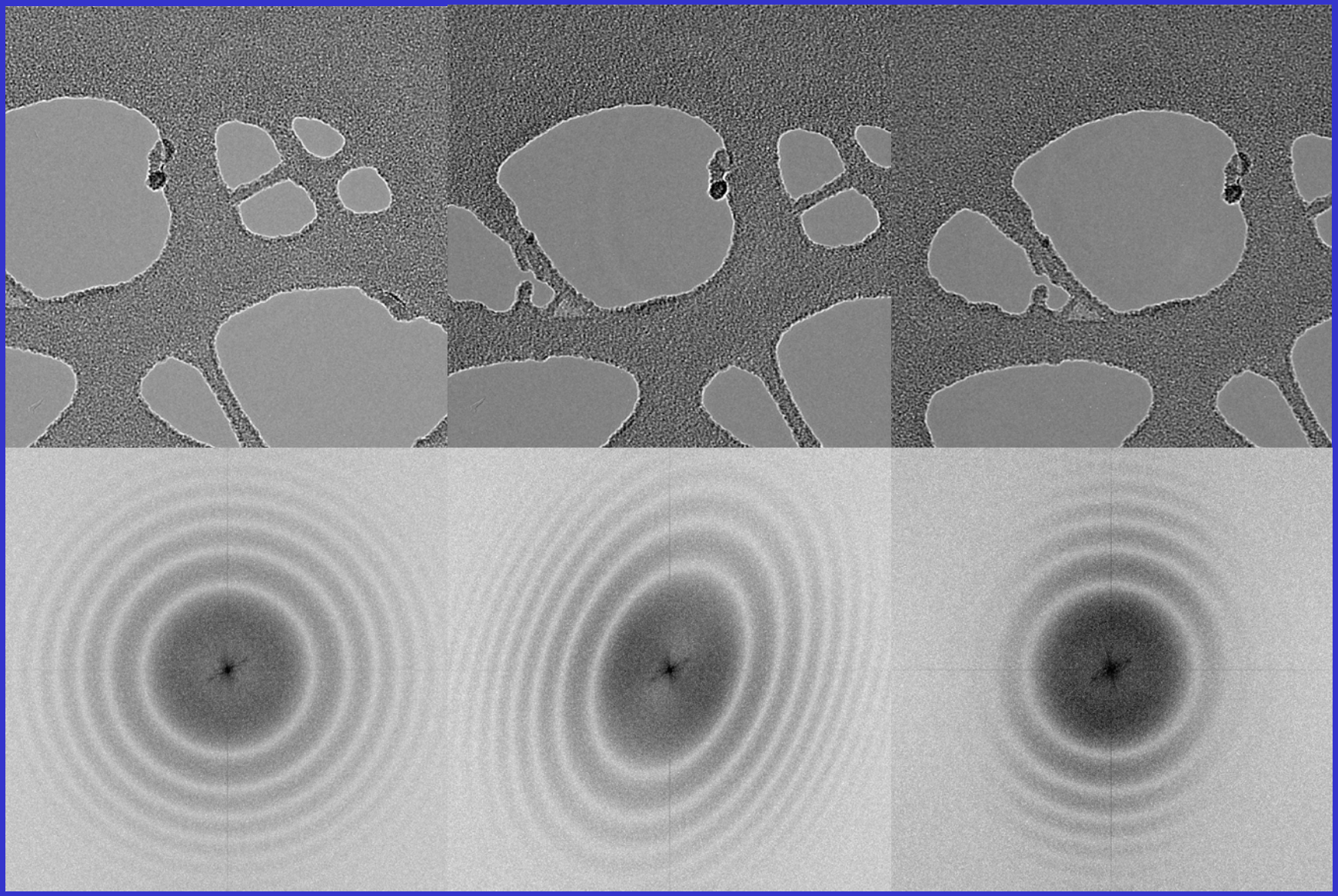
This……does not really look all that different to this



These few examples illustrate that in real-space, it is not always easy to distinguish between different imaging defects. However, the CTF- allows us to determine the cause of defects very easily.

The CTF, however, tells us immediately whether an image is a “lost cause”. In contrast to astigmatism, drift cannot be “fixed” computationally after the picture has been taken. The only “fix” for drift is patience and/or automation coupled with better specimen stages.

….and at low magnifications, we depend even more on the CTF to tell us about images



THE END