THE UNIVERSITY of TEXAS

SCHOOL OF HEALTH INFORMATION SCIENCES AT HOUSTON

Image Formation in the Electron Microscope

For students of HI 6001-125

"Computational Structural Biology"

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http://biomachina.org/courses/structures/08.html

Microscope Optics

A microscope is an optical system which transform an "object" to an "image". The simplest microscopes are light microscopes. Very often one or several lenses are used to change the direction of (focus or spread) the light in order to enlarge/minimize the "object" and form an "image" at a certain place.

In order to see an "object" which is too small to be seen by our eyes, one needs to magnify the image. An example of magnifying an image by a lens is illustrated in Fig. 2.1:

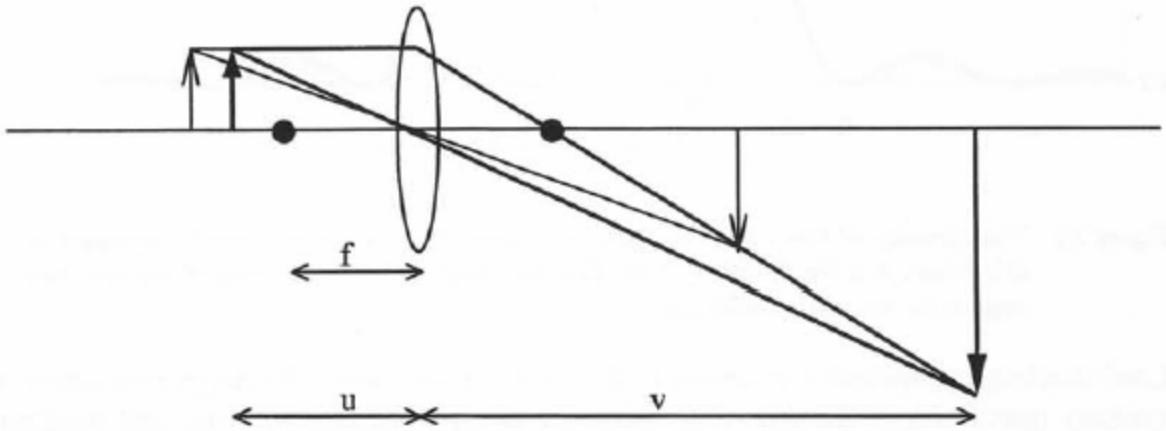


Figure 2.1 Ray diagram illustrating the formation of images by a lens. f = focal length, u = the distance between the object and the lens, v = the distance between the image and the lens.

The relation between the "object distance" u, the "image distance" v and the focal length f is:

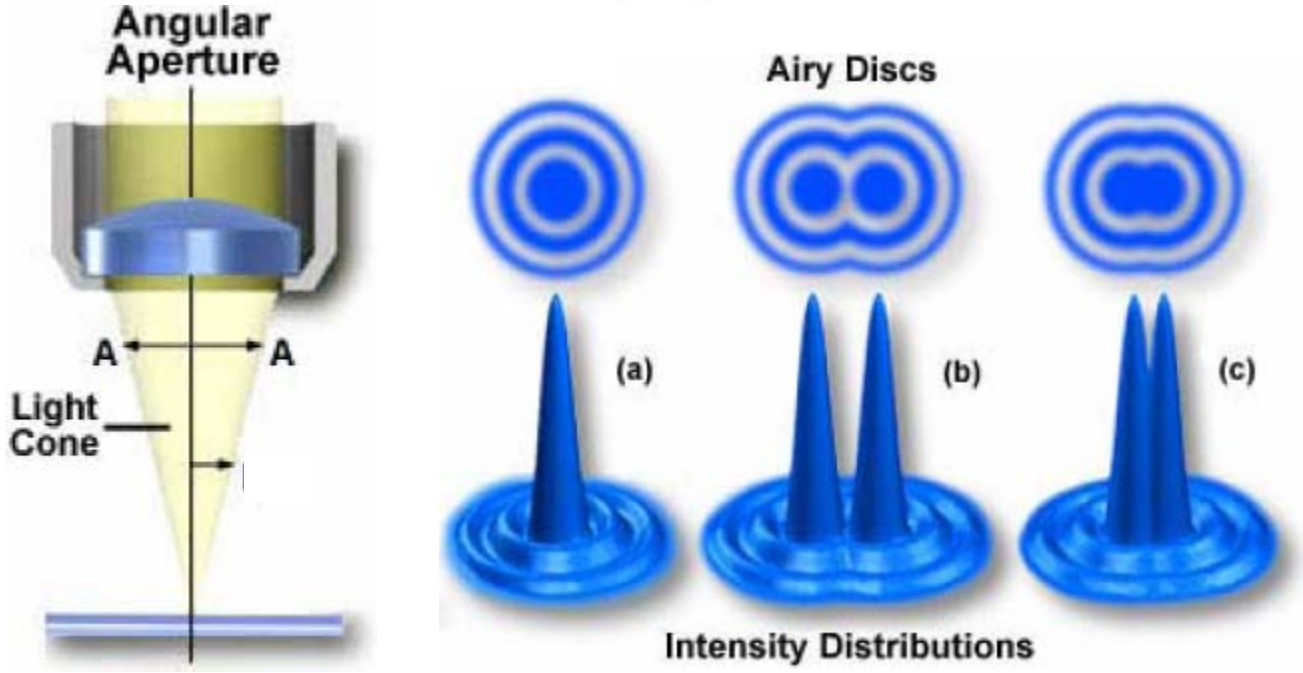
$$\frac{1}{f}=\frac{1}{u}+\frac{1}{v} \qquad (2\text{-}1)$$

The closer the object distance u is to the focal length f, the larger the image is magnified.

Optical Resolution

An image cannot be endlessly enlarged due to the limit of the resolution. "Resolution" is the closest distance between two points on the object which can be clearly seen through the microscope to be separate entities.

The resolution of a microscope is limited by several parameters. For light microscopes, the resolution is mainly limited by the wavelength of light. When light passes through an opening, such as a lens or an aperture, diffraction occurs so that a parallel beam of light is transformed into a series of cones, which are seen as series of circles (Airy's rings) in the image. Consequently a point object will form a disc instead of a sharp point in the image. The diameter of the disc is inversely proportional to the diameter of the lens or aperture. If two point objects are too close to each other, the discs formed will merge



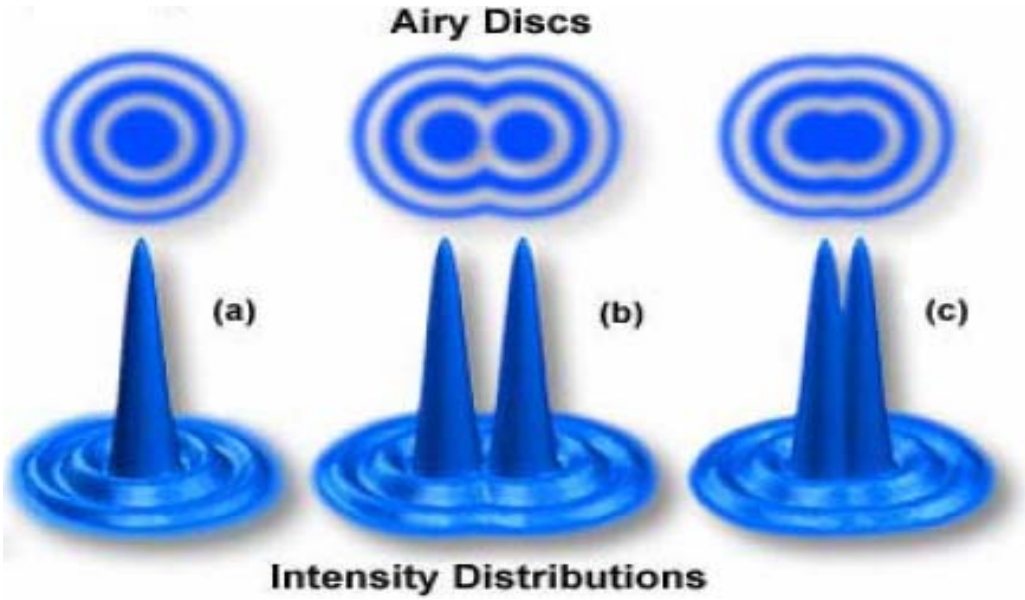
Airy Patterns

The central maximum of the Airy patterns is often referred to as an Airy disk, which is defined as the region enclosed by the first minimum of the Airy pattern and contains 84 percent of the luminous energy.

The Airy disk (typically termed a zero'th order maximum) is surrounded by concentric 1st, 2nd, 3rd, etc., order maxima of sequentially decreasing brightness.

If the separation between the two disks exceeds their radii, they are resolvable.

When the center-to-center distance between the zero'th order maxima is less than the width of these maxima, the two disks are not individually resolvable.

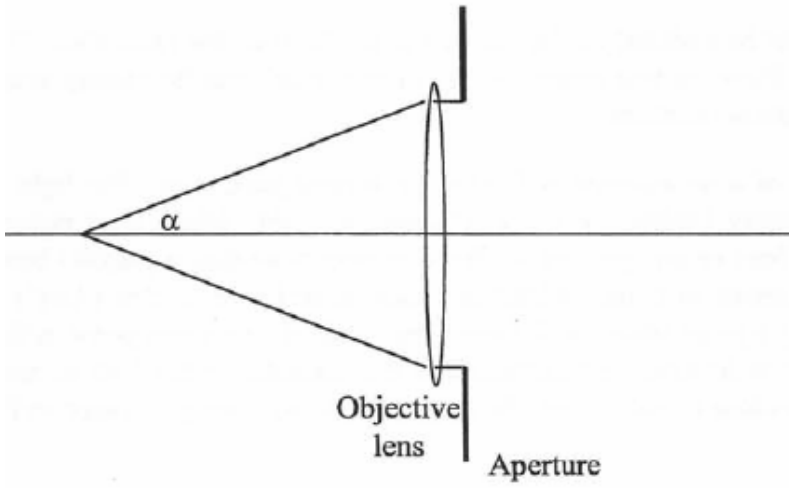


Rayleigh Criterion

Lord Rayleigh proposed a criterion to define the resolution - Rayleigh resolution: when the intensity maximum of the Airy disc from one point coincides with the first minimum of the Airy disc from the second point, then the two points can be just resolved. The Rayleigh resolution can be derived from diffraction theory to be:

$$r = \frac{d}{2} = \frac{0.61\lambda}{\mu \sin\alpha} \qquad (2\text{-}2)$$

where λ is the wavelength of the light and μ is the refractive index of the medium between the object and objective lens. α is the semi-angle above which the light is stopped by the aperture, see in Fig. 2.3.



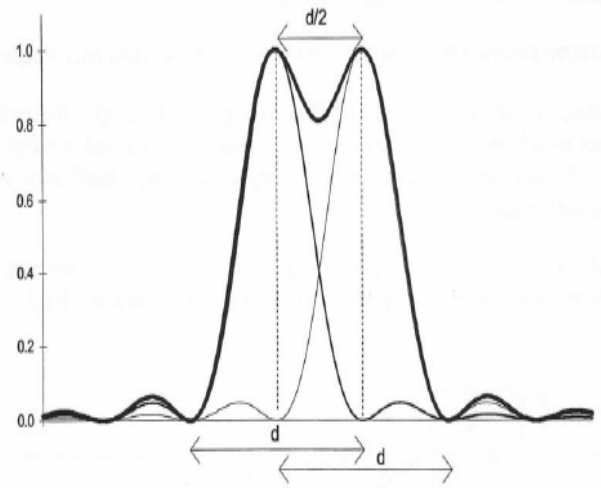
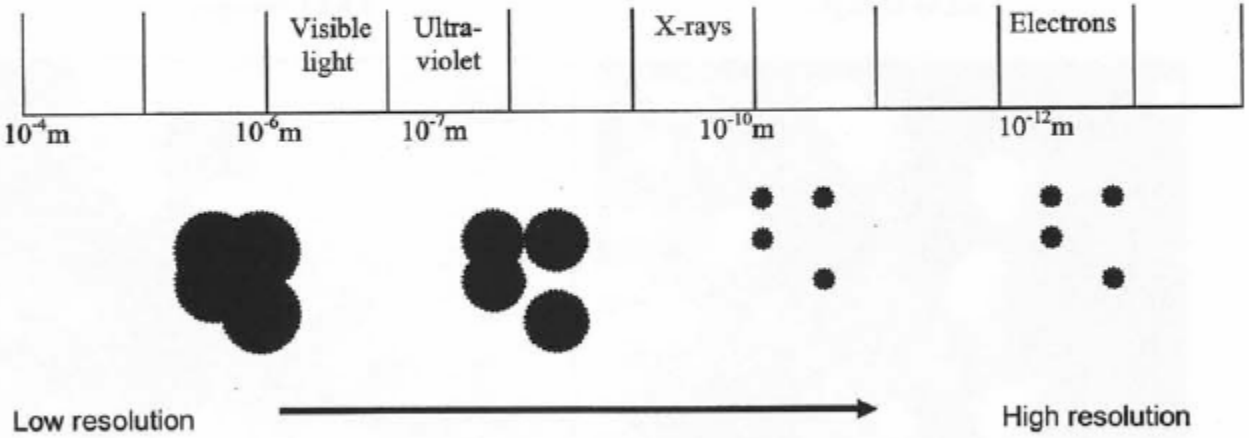


Figure 2.3 The definition of the semi-angle, α.

Electron Microscopes Provide Higher Resolution than Light Microscopes

The resolution can be increased (r decreased) by decreasing the wavelength λ or increasing μ or α. Since μ and $\sin\alpha$ have limited range, the most effective way of improving resolution is to decrease λ.

Light, or electron-magnetic waves, can be divided into different categories according to the wavelengths: visible light, ultraviolet light and X-rays.



The best resolution limit achievable for a light microscope, using green light and material with very high μ, is about 150 nm. X-rays, on the other hand, have very short wavelength. However, it is practically impossible to make a lens which can focus X-rays effectively, making an X-ray microscope is still a challenge for scientists and engineers.

Electrons are Both Particles and Waves

Electrons, like other particles, can be considered both as particles and waves. The wavelength of an electron depends on its velocity, v:

$$\lambda = \frac{h}{mv} \tag{2-3}$$

When an electron is accelerated through a potential difference U (volt), its wavelength can be calculated as follows:

$$\lambda = \frac{h}{(2emU + \frac{e^2U^2}{c^2})^{\frac{1}{2}}} = \left(\frac{1.5}{(U + 10^{-6}U^2)}\right)^{\frac{1}{2}} \text{nm} \tag{2-4}$$

where h is Planck's constant, c is the speed of light, e and m are the mass and charge of the electron. Typical values of the electron wavelengths used in a TEM are:

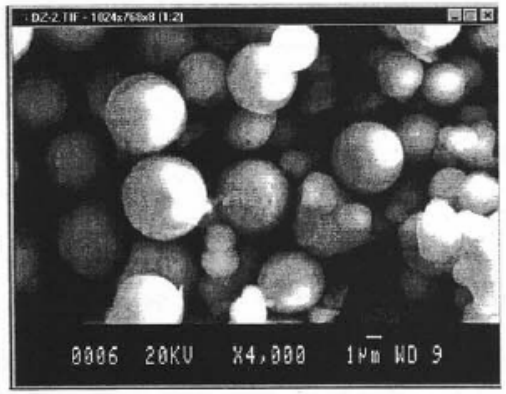
U (kV)	100	200	300	400	500	1000
λ (Å)	0.0370	0.0251	0.0197	0.0164	0.0142	0.0087

The wavelength depends on the accelerating voltage used. The higher the accelerating voltage, the shorter the wavelength.

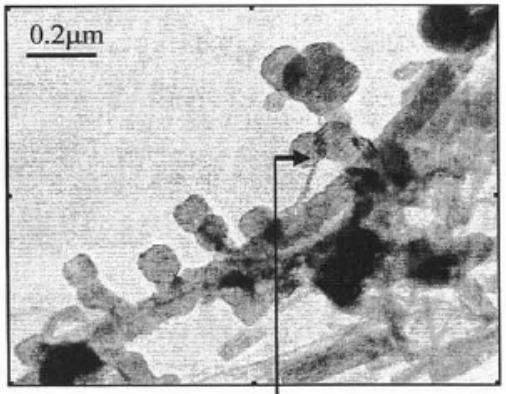
Electrons can be Focused by Lenses

Unlike X-rays, electrons are negatively charged, so they can be focused by electromagnetic or electrostatic lenses. It is possible to build electron microscopes, similar to light microscopes. The two main types of electron microscopes are scanning electron microscopes (SEM) and transmission electron microscopes (TEM).

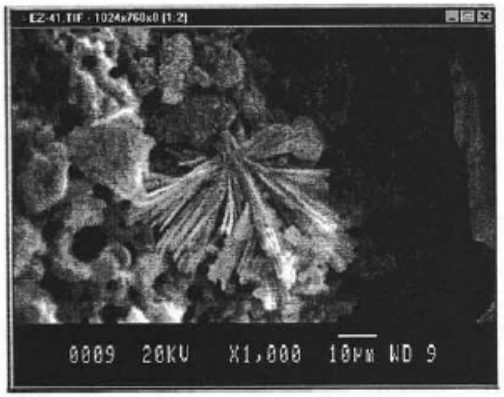
SEM images | **TEM images**



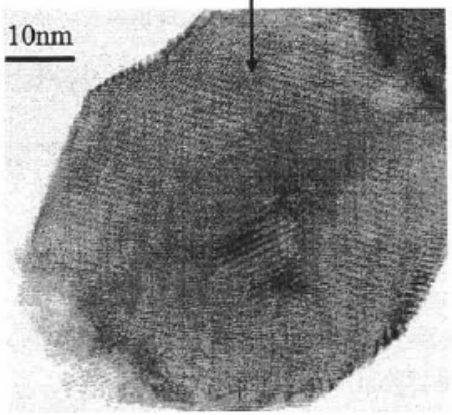
SiO_2 particles



Conventional TEM image of BaO



Titanium silicate



HREM image of BaO

Recognizable Similarities to Light Microscope

- ELECTRON "GUN" [equivalent to a light source]
- CONDENSOR LENS SYSTEM
- SPECIMEN STAGE
- OBJECTIVE LENS
- "PROJECTOR LENSES"
  - FURTHER MAGNIFY THE IMAGE,
  - OR RELAY AN IMAGE OF THE DIFFRACTION PATTERN THAT IS PRODUCED IN THE FOCAL PLANE OF THE OBJECTIVE LENS

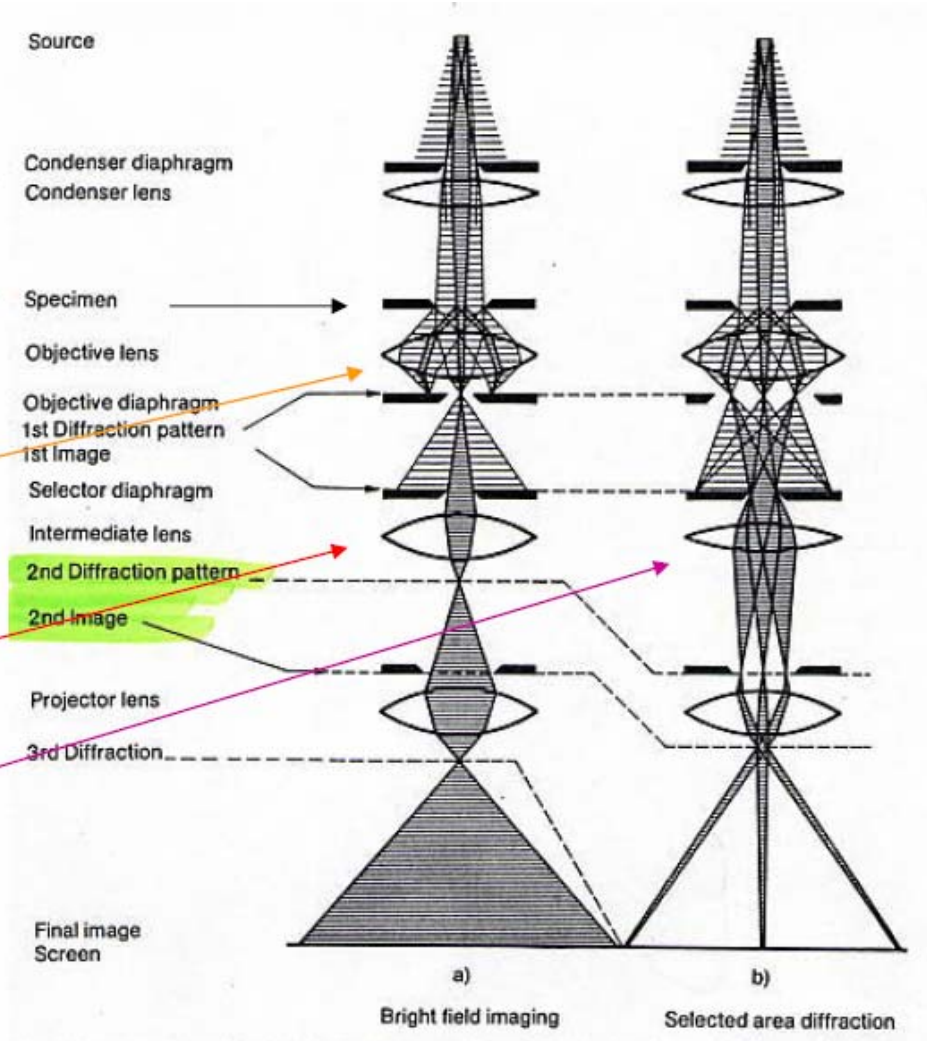


Fig. 4.20 a, b. Ray diagram for a transmission electron microscope in (a) the bright-field mode and (b) selected-area electron diffraction (SAED) mode

Reimer (1989) Transmission EM [Springer]

TEM: Illumination System

- **Electron gun**: the electron gun is the source of electrons. An electron gun plays an important role on the ultimate performance of the microscope. An optimal electron source should give high brightness, fine point source and low energy spread (high coherence).

  Three types of electron sources are available in TEM: the tungsten (W) hair pin filaments, LaB_6 single crystals and field emission guns (FEG), either cold or thermally activated. The vacuum needed increases with the brightness.

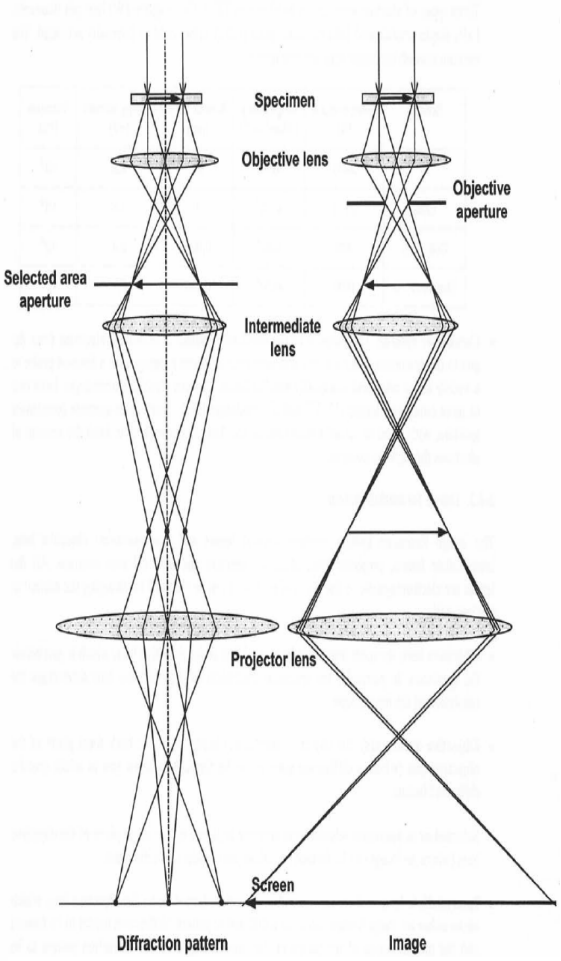
Source	Temperature (K)	Brightness ($A/cm^2\ sr^{-1}$)	Source size (μm)	Energy spread (eV)	Vacuum (Pa)
W	2800	$4x10^4$	50	3.0	10^{-2}
LaB_6	2100	$4x10^6$	1	1.5	10^{-5}
Cold FEG	293	$4x10^8$	0.005	0.4	10^{-8}
Thermal FEG	1800	$4x10^8$	0.020	0.7	10^{-9}

- **Condenser system:** The purpose of the condenser system is to deliver electrons from the gun to the specimen under various illumination conditions ranging from a focused probe to a widely unfocussed and essentially parallel beam. Modern electron microscopes have two or more condenser lenses (1^{st}, 2^{nd} and 3^{rd} condenser lens). There is an aperture (condenser aperture, with fixed or variable sizes) below the first condenser lens to limit the amount of electrons through the column.

TEM: Image Formation System

The image formation system contains several lenses and two apertures: objective lens, intermediate lenses, projector lens, objective aperture and selected area aperture. All the lenses are electromagnetic so the focus of each lens can be changed by changing the current in the lens coil.

- **Objective lens**: the most important lens in a TEM is the objective lens, which is just below the specimen. In particular the spherical aberration of the objective lens determines the resolution of the microscope.
- **Objective aperture(s)**: the objective aperture(s) is placed in the back focal plane of the objective lens (where a diffraction pattern can be formed) to allow you to select specific diffracted beams.
- **Selected area aperture**: selected area aperture is placed in the image plane of the objective lens (where an image can be formed) to allow you to select specific areas.
- **Intermediate lenses:** there are several intermediate lenses under the objective lens, which allow either an image (image mode) or a diffraction pattern (diffraction mode) to be formed and the magnification of the image or the camera length of the diffraction pattern to be varied.
- **Projector lens:** the projector lens is a special intermediate lens which projects the final image or diffraction pattern into the image recording system. The focus of the projector lens is fixed.



TEM: Recording Systems

Different systems are available for recording images and diffraction patterns. There are: photographic films, real time video CCD cameras, slow scan CCD cameras and image plates.

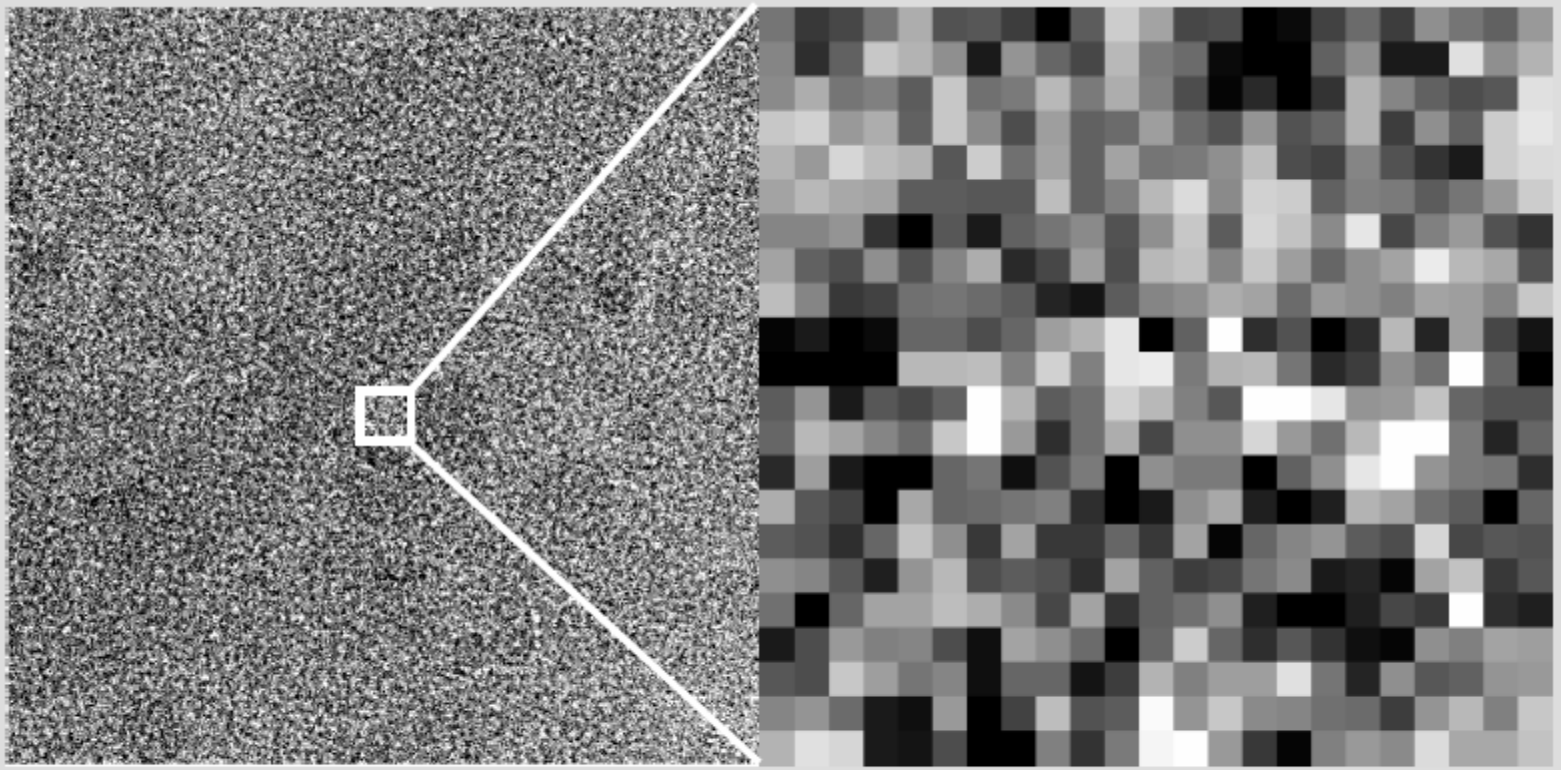
- **Photographic films**: photographic films are the most used medium for recording TEM images and ED patterns. They are relatively cheap, easy to use and store, give large field of view with high resolution.

- **Real time video CCD cameras:** real time video CCD cameras are mainly used for tuning the microscope and searching for crystal areas. Since the size is limited and the noise level is high, it is only used for data recording in some special cases, for example for recording reactions or changes of the specimen in situ in the microscope.

- **Slow scan CCD cameras**: slow scan CCD cameras have a large linear intensity response range and provide digitised images and diffraction patterns with high quality. They have become more and more common in modern microscopes for providing fast and high quality digitised images. The price is relatively high.

- **Image plates**: image plates are recorded in a similar way as photographic films, giving large field of view with high resolution. Image plates provide a very large linear intensity response range and are ideal for recording electron diffraction patterns in order to get accurate intensities.

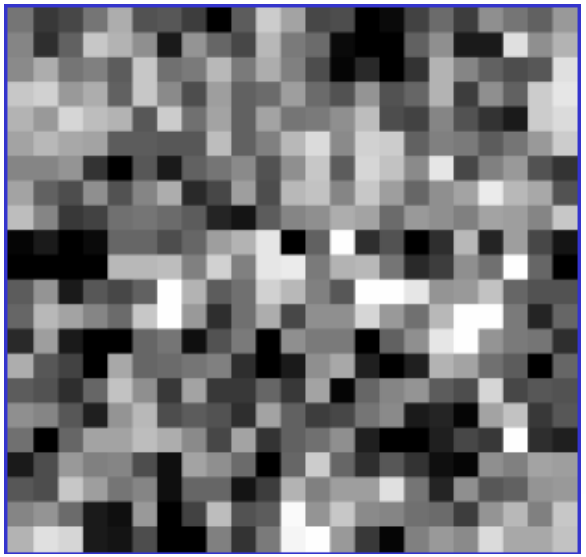
Digitization of Recorded Image



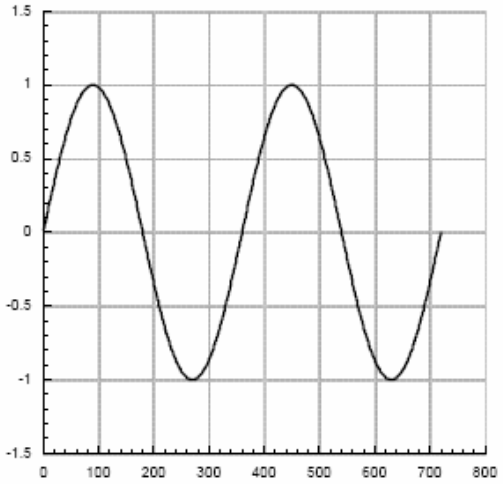
image

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

Nyquist Limit



Pixel size/magnification = sampling distance at the level of the specimen
For instance: pixel is 10µm, magnification was 50,000-fold ==> sampled @ 2Å/pixel



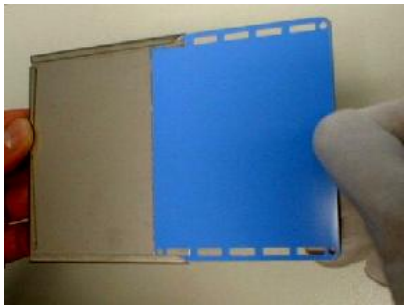
A plain sine-curve is defined by any pair of values taken from within one period
==> if the sampling in the image is @ 2Å per pixel, and 2 pixel values are needed to define frequencies, then the highest frequency that can be reliably described is 2*2=4Å

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

Overview of Recording Media

CCD: fixed pixel size (rather large, 15-25µm)
•need to increase magnification to get better resolution
•lowers the # of particles/unit cells to be imaged per image (poor SNR if 2D-crystal)
•need large, expensive CCD array (1K:$60K, 2K:$150K, 4K:$300K)
•low to intermediate acceleration voltage
•immediate feedback, FFT, use for adjusting scope
•MTF is 12-25% at Nyquist frequency, $(2 \text{ pixels})^{-1}$

Film: fixed grain size (~5µm, to get high speed)
•but, optical scanners are inexpensive now and can go as low as 5µm/step
•can choose pixel size to match problem
•can use smaller magnifications
•more particles/unit cells per film (good SNR if 2D-crystal)
•more particles with "identical" base parameter [defocus, magnification…])
•slow chemical processing times (dark room), but least expensive solution
•MTF depends on scanner, inexpensive scanners comparable to CCD.

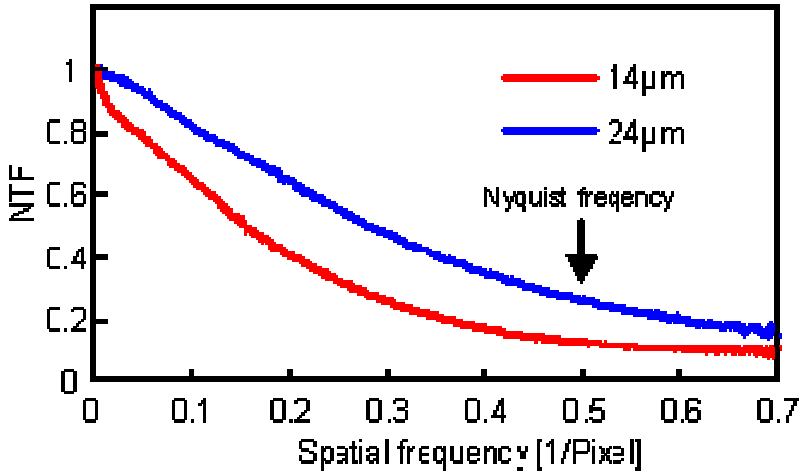


Imaging Plates: variable pixel size (15-50µm)
•full frame coverage
•can choose pixel size to match problem
•can use smaller magnifications
•best signal linearity (6 orders of magnitude – needed for diffraction patterns, not critical for direct imaging)
•intermediate processing times (reader)
•MTF is 38% at 25µm

Detector Resolution

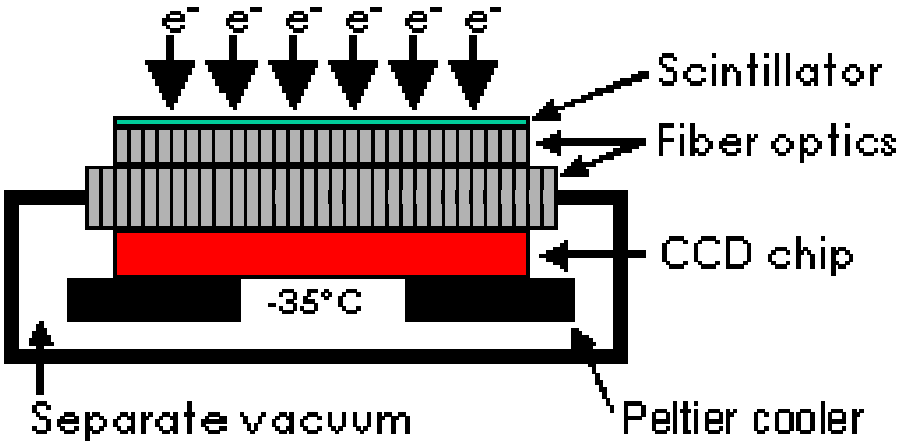
The resolution of any camera system can be expressed by the point spread function (PSF) or the modulation transfer function (MTF), the Fourier transform of the PSF. The MTF defines the ratio between input and output signal as a function of the spatial frequency. Higher spatial frequencies are attenuated due to cross-talk between pixels, resulting in loss of contrast.

The MTF of a CCD camera, for example, is determined by the pixel size, the type and quality of electron-optical coupling and the type and size of the scintillator:



MTF of TVIPS 2k CCD cameras with 14 µm and 24 µm pixel size.

Slow-Scan CCD Camera



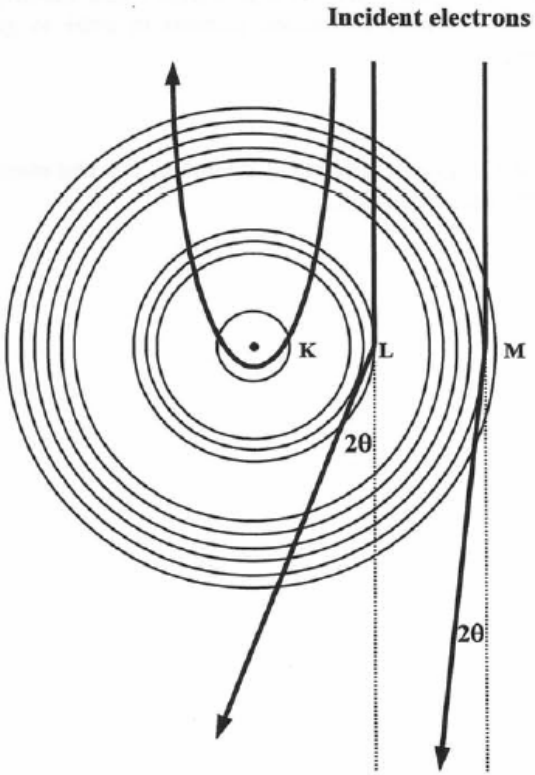
TVIPS Fiber-optically coupled CCD camera

The scintillator converts the electron image into a photon image. Fiber optics transfer this image to the CCD (charge coupled device) sensor where the photons generate electrical charge (CCD electrons). The charge is accumulated in the parallel register. During the readout, this charge is shifted line by line to the serial register from where it is transferred pixel by pixel to the output node and exits to the analog-to-digital converter. The main features of slow scan CCD cameras are high sensitivity, low noise, a high dynamic range and excellent linearity.

Electrons See the Electrostatic Potential

Electrons are negatively charged particles and they interact with atoms via Coulomb forces. The interactions are between the incident electrons and

- Valence electrons - low angle scattering
- Inner shell electrons - medium angle scattering
- The nucleus - high angle scattering



Shell	Sub-shell		No.
	KLM	spdf	e^-
K	K	1s	2
L	L1	2s	2
	L2	2p	2
	L3	2p, 2p	4
M	M1	3s	2
	M2	3p	2
	M3	3p, 3p	4
	M4	3d, 3d	4
	M5	3d, 3d, 3d	6

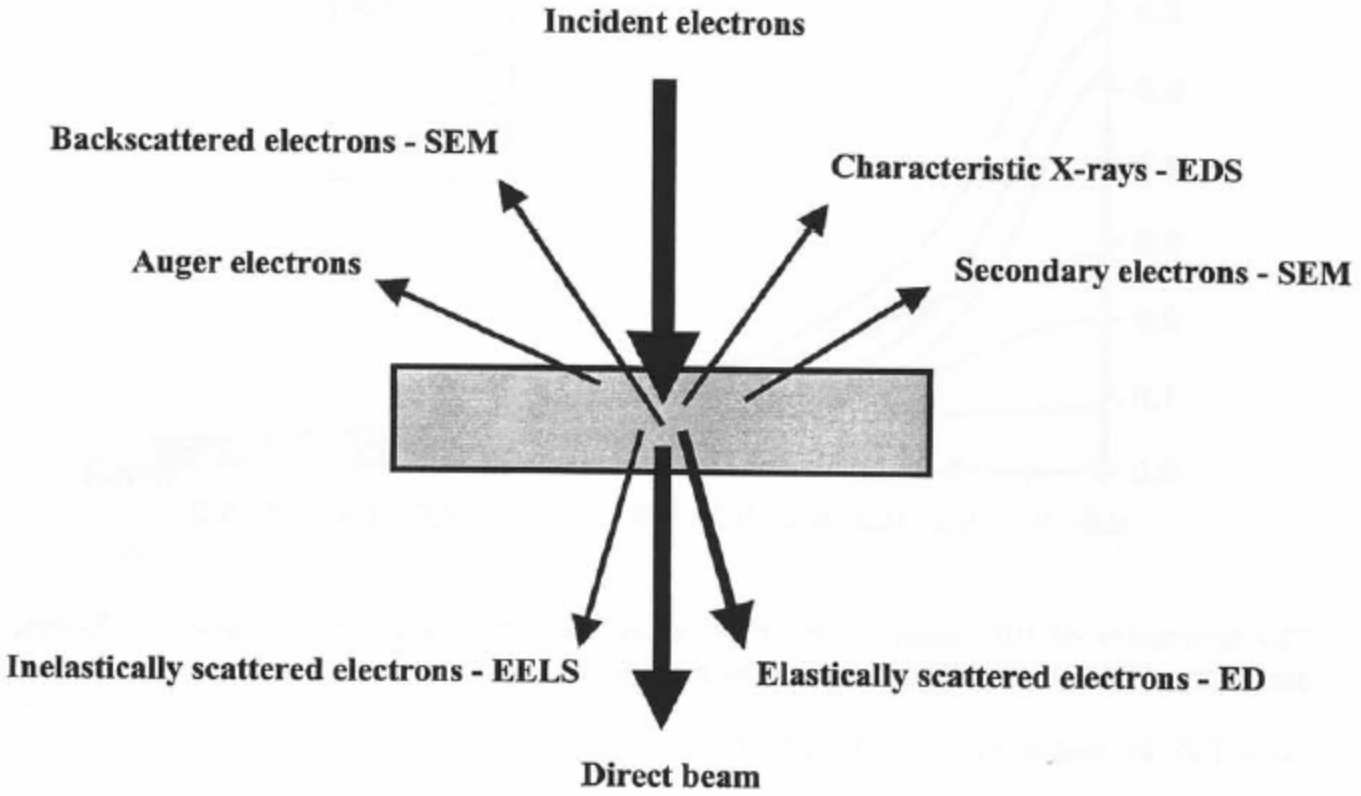
Electrons interact with both the electrons and nuclei, while X-rays interact only with electrons and neutrons only with nuclei. Thus electrons "see" the electrostatic potential, X-rays the electron density and neutrons the nuclei in a specimen.

Electrons are scattered much more strongly than X-rays or neutrons:

Because of the strong interaction between electrons and matter, the sample has to be very thin in the TEM to allow electrons to pass through. The maximum thickness is less than 500 Å for materials containing heavy elements and more for those containing light elements such as organic molecules.

Scattering and Energy Loss

When electrons pass through a thin specimen, most of them will not be scattered (direct beam). A large portion will be scattered elastically, i.e. without energy loss. Others will remove the inner shell electrons of an atom. The energy needed to kick out an electron from an atom is taken from the incident electron which thus suffers an energy loss of typically 1-10% of its energy. Various secondary signals will be generated when electrons fall back to fill the empty orbitals.

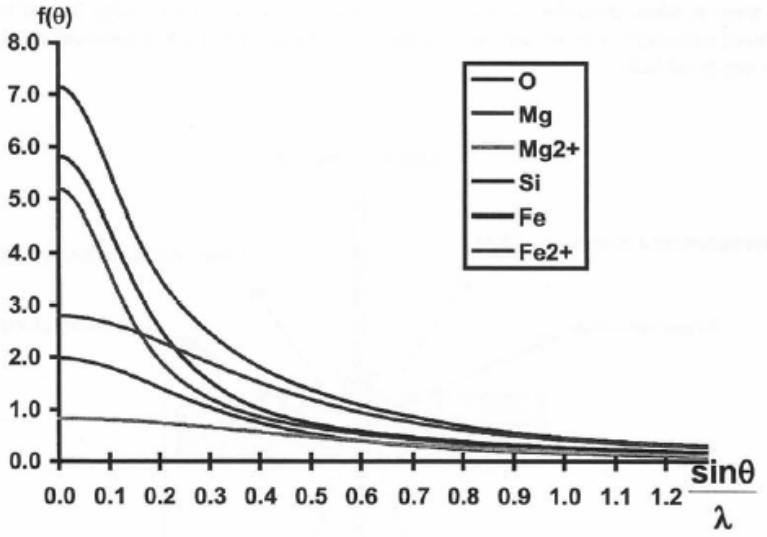


Atomic Scattering Factors for Electrons

The atomic scattering factors for electrons f(θ) represent the probabilities that an electron is scattered into a specific direction (2θ with respect to the incident beam) when it passes near an isolated atom. They can be derived from the atomic scattering factors for X-rays fx(θ) by the Mott-Bethe formula:

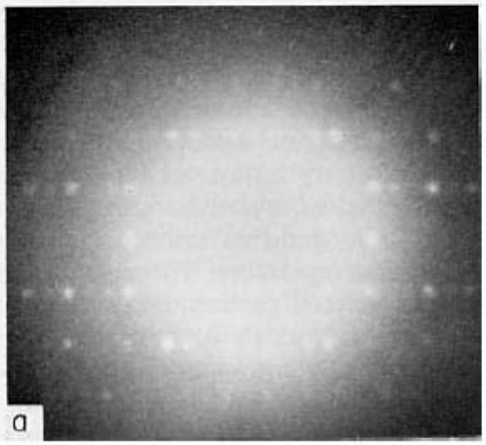
$$f(\theta) = 0.023934\left(\frac{\lambda}{\sin\theta}\right)^2 (Z - fx(\theta)) \qquad (3\text{-}1)$$

where fx(θ) is calculated from the Schrödinger wave equation. f(θ) for neutral and some of the ionized atoms are listed in the International tables for Crystallography, Volume C.



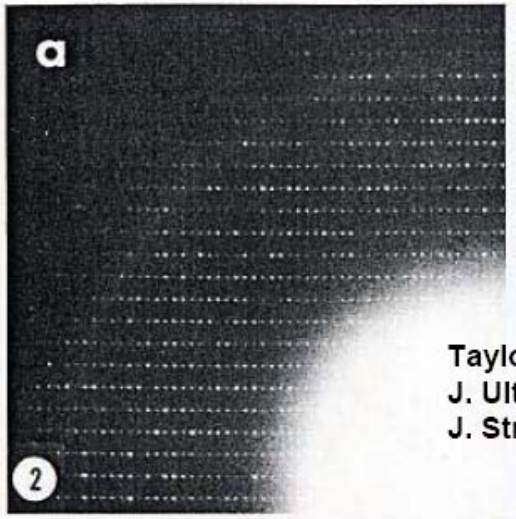
Elastically Scattered Electrons are Coherent Waves

- ELASTICALLY SCATTERED ELECTRONS PRODUCE DIFFRACTION PATTERNS FROM PROTEIN CRYSTALS
- ONLY ELASTICALLY SCATTERED ELECTRONS CONTRIBUTE TO THE THEORETICAL IMAGE INTENSITY
- INELASTICALLY SCATTERED ELECTRONS PRODUCE AN UNWANTED BACKGROUND
- THEY ARE ONLY A MINOR NUISANCE IN IMAGES OF THIN SPECIMENS, HOWEVER



Negatively stained catalase

Glaeser & Hobbs (1975) J. Microsc. 103:209-214



Unstained, frozen-hydrated catalase

Taylor & Glaeser (1976) J. Ultrastruct Res. (now J. Struct. Biol) 55:448-456

Inelastic Scattering – Thin Sample

- **MOST ELECTRONS PASS THROUGH A THIN SPECIMEN *WITHOUT* BEING SCATTERED**
- **INELASTIC SCATTERING IS 3X AS MUCH AS ELASTIC SCATTERING, BUT THAT DOESN'T MATTER IN THE END**
  - ***EXCEPT FOR SPECIMEN DAMAGE!***

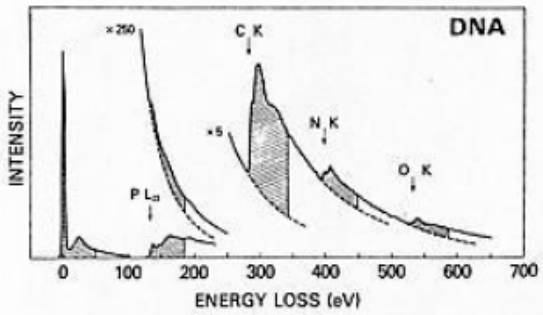


Fig. 2. Electron energy loss spectrum from DNA (extracted from herring sperm) showing zero-loss, plasmon, and P L_{23}, C K, N K and O K core edges. Inverse power law background extrapolation and integrated core edge intensities (shaded areas) are indicated. Background subtraction is illustrated for phosphorus L_{23} edge. Beam energy was 100 keV and collection angle was 30 mrad.

Leapman et al. (1988) Ultramicroscopy 24:251-268

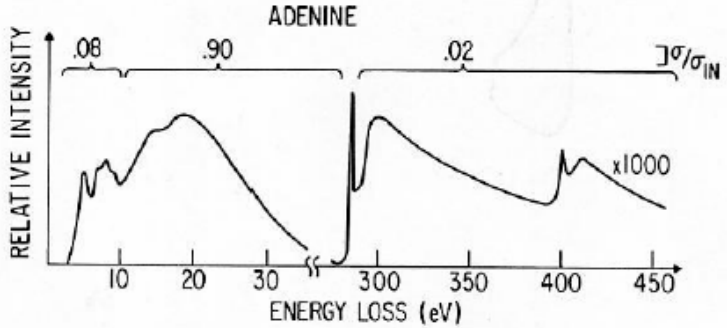


Fig. 1.4 The characteristic electron energy loss spectrum for an approximately 500-Å-thick film of the nucleic acid base, adenine ($C_5N_5H_5$), supported on a 30-Å-thick carbon substrate. The horizontal scale is the amount of energy lost by incident 25 keV electrons in traversing the film. The electron intensity was obtained by detecting only those electrons scattered in the forward direction. The numbers over the brackets indicate the fraction of the total inelastic scattering cross section which occurs in the respective energy loss regions (from Isaacson, 1975b). The peaks near 285 and 395 eV correspond to the *K*-shell excitation of the carbon and nitrogen atoms, respectively, while the region less than 50 eV corresponds mainly to valence shell excitations and ionizations.

Isaacson (1977) In "Principles and Techniques of Electron Microscopy" (Hayat, Ed.) , Vol. 7 Van Nostrand Reinhold Co.

Inelastic Scattering – Thick Sample

- **WHEN THE SPECIMEN BECOMES "TOO THICK", ESSENTIALLY *ALL* OF THE ELECTRONS WILL HAVE BEEN INELASTICALLY SCATTERED**
- **THE IMAGE FORMED BY THIS SPREAD OF INELASTICALLY SCATTERED ELECTRONS IS VERY POOR, INDEED**
- **THUS, REMOVAL WITH AN ENERGY FILTER IS GOOD, BUT**
- **AFTER A THICKNESS OF ~2 MEAN FREE PATHLENGTHS (for inelastic scattering) THE REMAINING IMAGE IS STILL TERRIBLE – *TOO FEW ELECTRONS REMAIN***

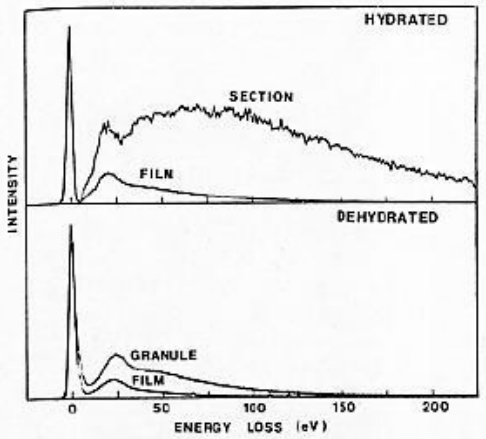
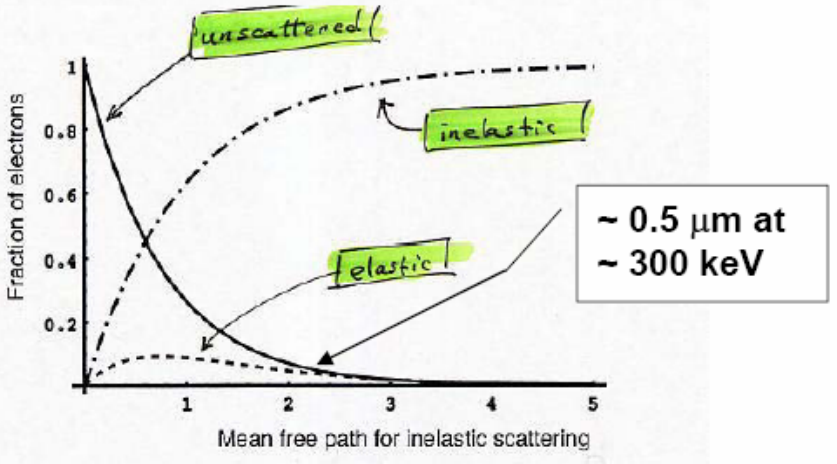
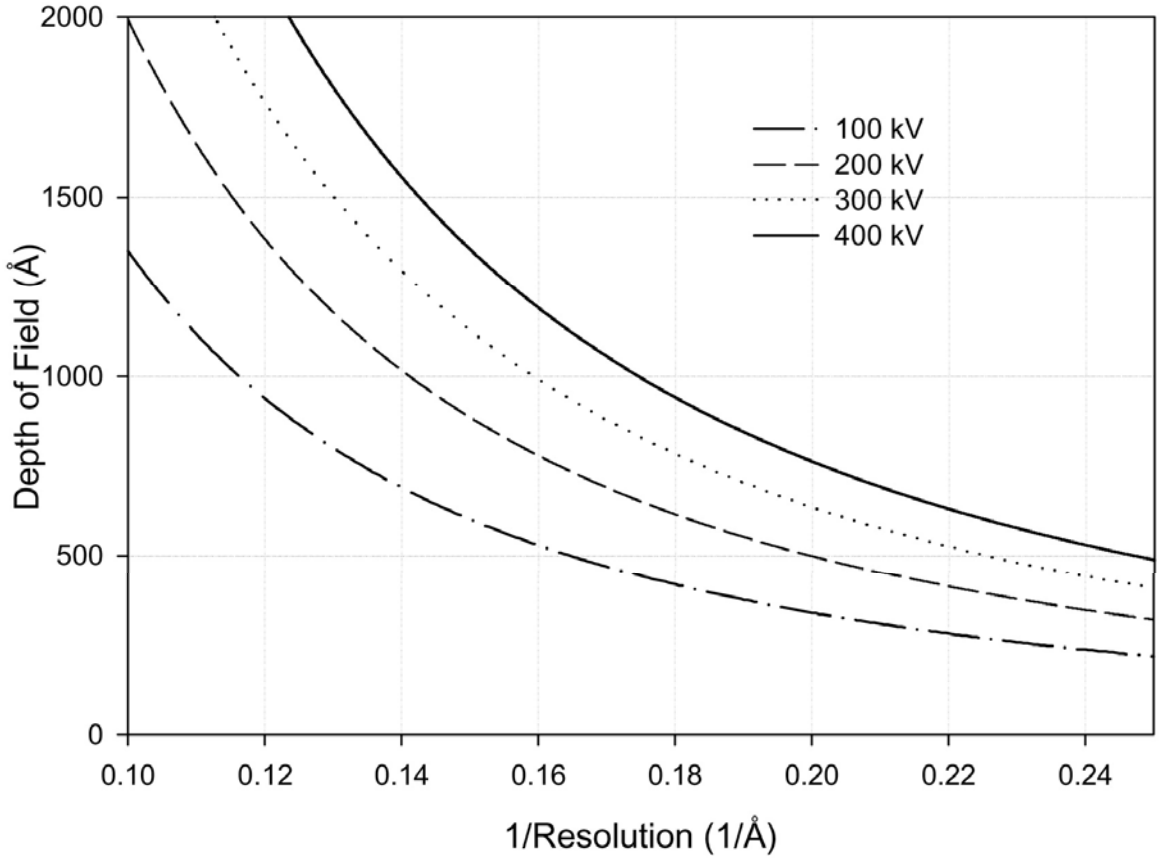


Fig. 11. Low-loss spectra from hydrated and dehydrated section in figs. 9 and 10. Spectrum from hydrated section, kept at −150 °C, recorded in TEM mode with an area including the entire cell. Spectrum from dehydrated section recorded at room temperature with STEM raster contained within single secretory granule marked by arrow in fig. 10. Spectra were also obtained from support film under the two sets of conditions.

Leapman et al. (1988) Ultramicroscopy 24:251-268



The Allowable Thickness is Also Resolution and Energy Dependent

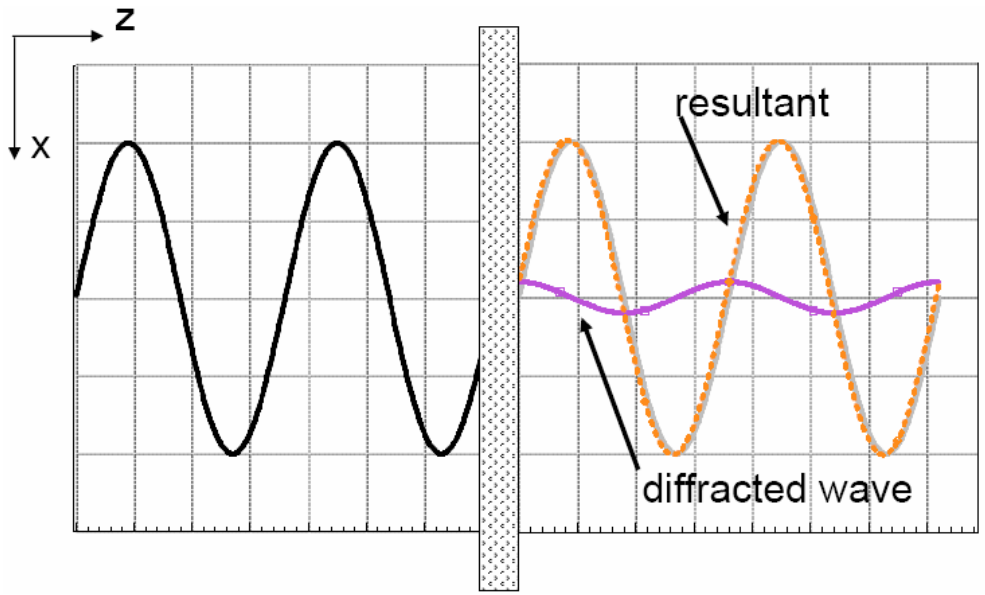


Necessary Assumptions:

- Image is a true 2-D projection of the 3-D object with the same focus throughout
- Only elastically scattered electrons form the image

The Phase Object

In cryo EM thin specimens do not absorb electrons, instead most of the electrons pass through the sample. The resultant wavefront emerges with almost the same amplitude, but has suffered a small phase shift proportional to the projection of the Coulomb potential. This can be reconstructed vectorially by interfering the undiffracted beam with a diffracted beam of low intensity that is shifted by ~90° ($\pi/2$) with respect to the undiffracted beam.



This is BAD news for imaging because in order to record a signal we need differences in amplitude…

Coulomb Wavefront Distortion

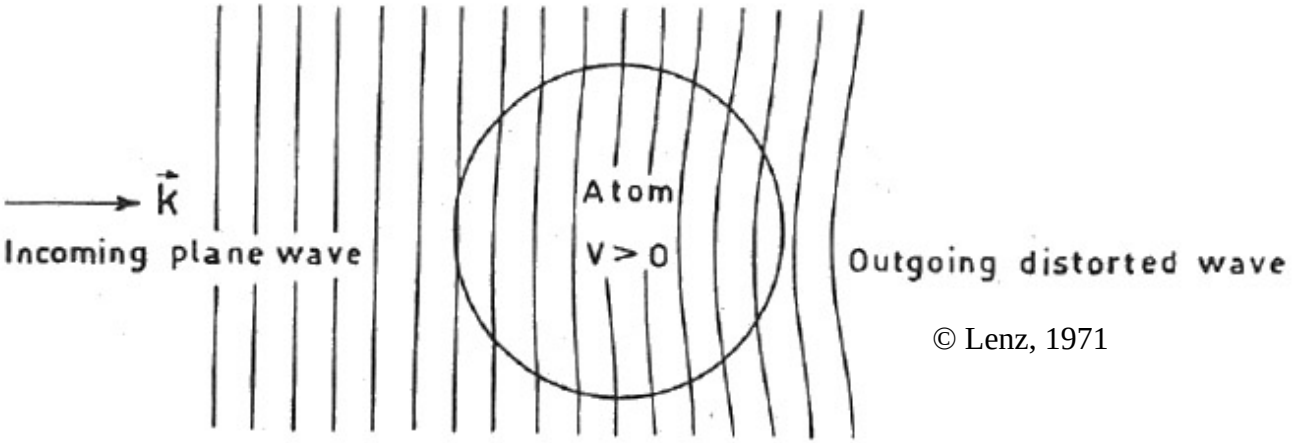


Fig. 1. – Interaction of atom and electron wave.

Object Coulomb potential function $V(x_o, y_o, z_o)$

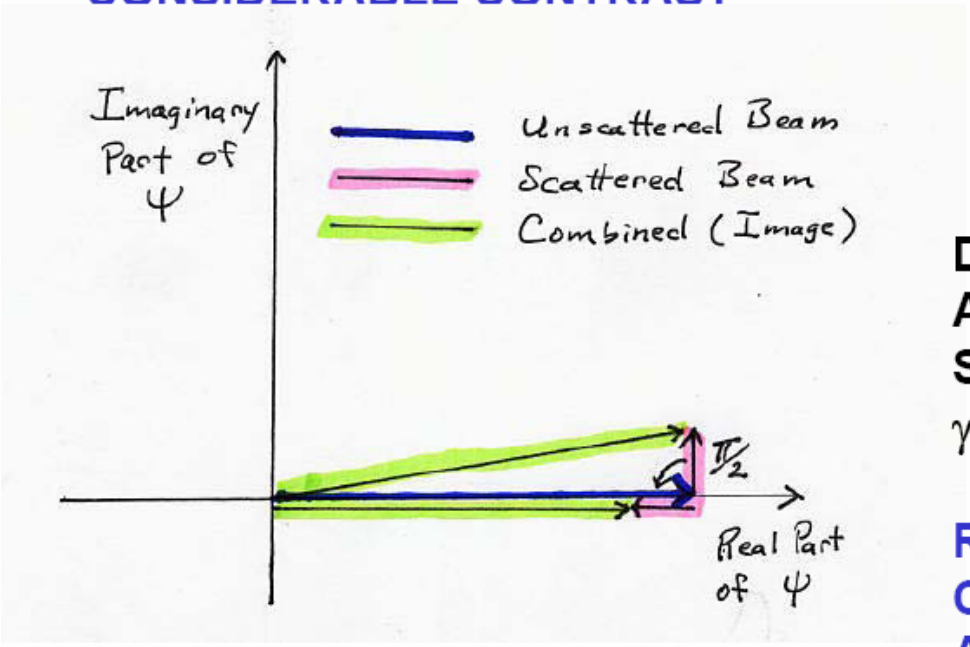
Object transmitted wave function $\Psi_o(x_o, y_o)$

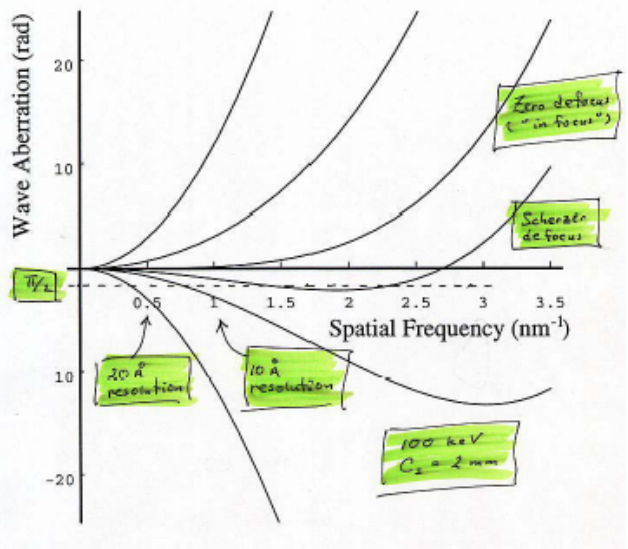
$$\Psi_o(x_o, y_o) \simeq 1 + i\sigma v(x_o, y_o)$$

$$v(x_o, y_o) = \int V(x_o, y_o, z_o)\, dz_o$$

Phase Objects Require an Additional Phase Shift to be Seen

- **THE SCATTERED BEAM GIVES NO CONTRAST FOR A PHASE OBJECT BECAUSE IT IS π/2 OUT OF PHASE WITH THE UNSCATTERED BEAM**
- **APPLYING AN ADDITIONAL π/2 PHASE SHIFT CAN THUS PRODUCE CONSIDERABLE CONTRAST**



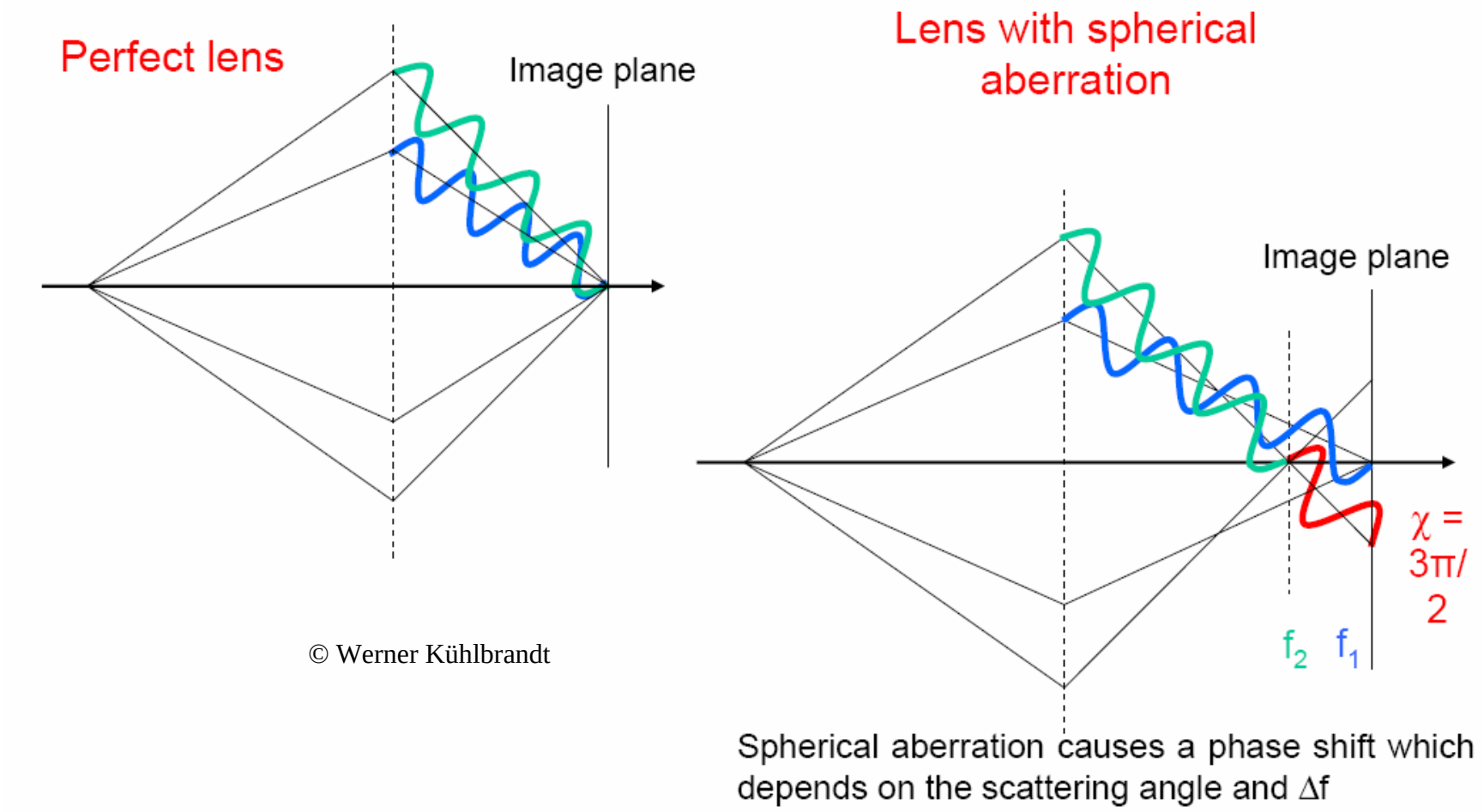


DEFOCUS AND SPHERICAL ABERRATION IMPOSE A PHASE SHIFT

$$\gamma(s) = 2\pi[\, C_s/4\, \lambda^3 s^4 - \Delta Z/2\, \lambda\, s^2 \,]$$

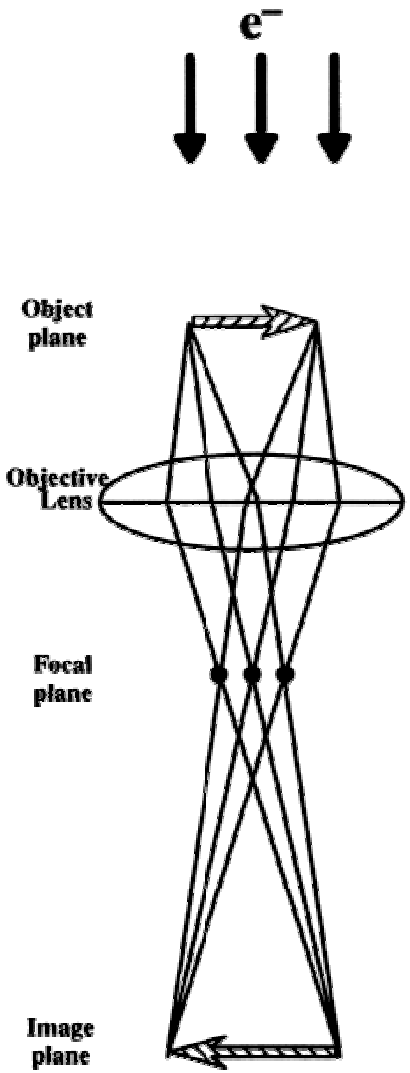
RESOLUTION-ZONES OF HIGH CONTRAST CAN BE "TUNED" BY ADJUSTING THE DEFOCUS

Spherical Aberration C_S



- Typical defect of electron lens, contributes to phase shift
- Modern lens designs seek to compensate this
- Normally C_S not modifiable, fine-tuning of CTF is done by adjusting defocus ΔZ

Phase Object Approximation



Object Coulomb potential function $V(x_o, y_o, z_o)$

Object transmitted wave function $\Psi_o(x_o, y_o)$

$$\Psi_o(x_o, y_o) \simeq 1 + i\sigma v(x_o, y_o)$$

$$v(x_o, y_o) = \int V(x_o, y_o, z_o)\, dz_o$$

Phase shift $\gamma(S)$ *introduced by objective lens*

$$\gamma(S) = 2\pi\left(\tfrac{1}{4} C_s \lambda^3 S^4 - \tfrac{1}{2} \Delta Z \lambda S^2\right)$$

Diffraction wave function $\Psi_d(S_x, S_y)$

$$\Psi_d(S_x, S_y) = F(S_x, S_y)\ exp\ (i\gamma(S))$$

$$F(S_x, S_y) = \mathcal{F}\ [\Psi_o(x_o, y_o)]$$

Diffraction intensity $I_d(S_x, S_y) = \Psi_d(S_x, S_y)\ \Psi_d^*(S_x, S_y)$

Image wave function $\Psi_i(x_i, y_i)$

$$\Psi_i(x_i, y_i) = \mathcal{F}^{-1}\ [\Psi_d(S_x, S_y)]$$

Image intensity $I_i(x_i, y_i)$

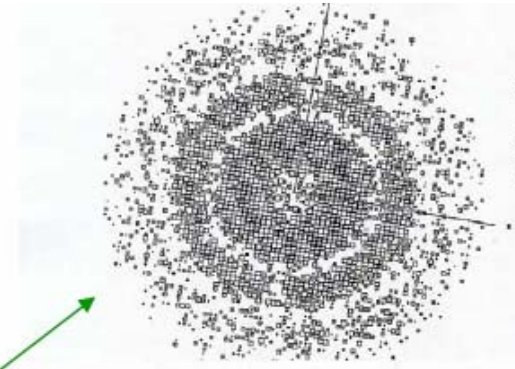
$$I_i(x_i, y_i) = \delta(0,0) - 2\sigma v(x_i, y_i) * \mathcal{F}^{-1}\ [sin\ \gamma(S)]$$

Computed diffraction wave function $T(S_x, S_y)$

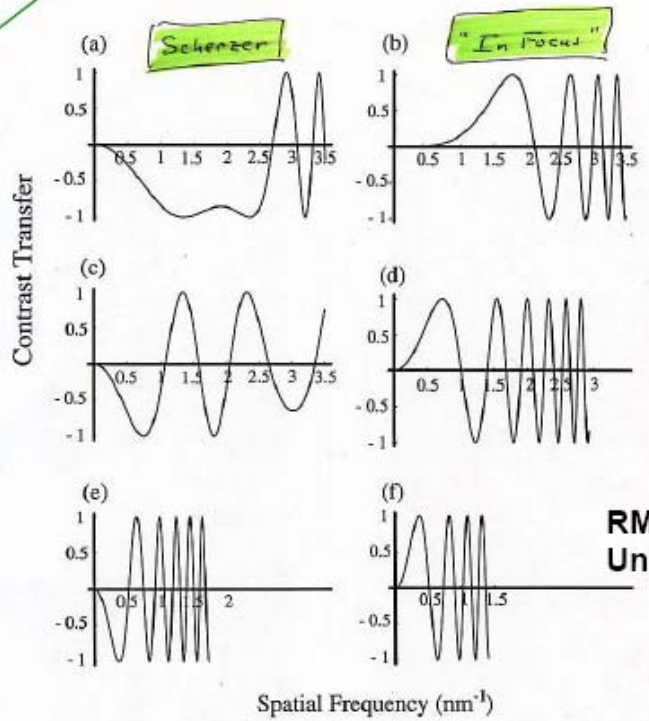
$$T(S_x, S_y) = \mathcal{F}\ [I_i(x_i, y_i)]$$
$$= \delta(0,0) - 2\ F(S_x, S_y)\ sin\ \gamma(S)$$

The Phase Contrast Function

- $T(x,y) = \exp[i\,\phi(x,y)]$
  $\sim 1 + i\,\phi(x,y)$
  WHERE $\phi(x,y)$ IS PROPORTIONAL TO THE COULOMB-POTENTIAL "DENSITY" OF THE OBJECT
- WHEN THIS LINEAR APPROXIMATION IS VALID, THE FOURIER TRANSFORM OF THE IMAGE INTENSITY IS PROPORTIONAL TO
  Sin $\gamma(s)$ {*FT* [object]}
- SIN $\gamma(s)$ OSCILLATES BETWEEN +/- 1.0
- SIN $\gamma(s)$ IS KNOWN AS THE PHASE CONTRAST TRANSFER FUNCTION (CTF)



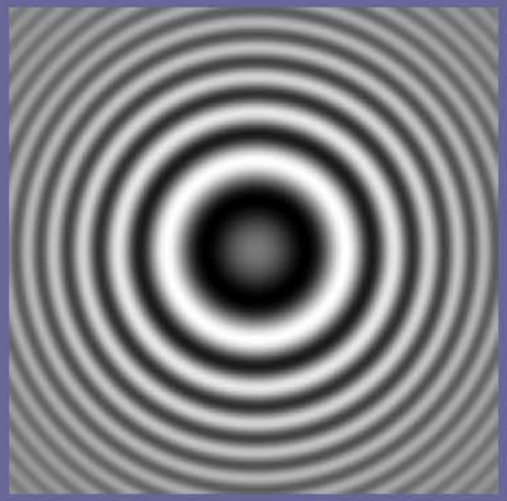
Downing & Jap
PhoE porin image
(unpublished)



RMG,
Unpublished

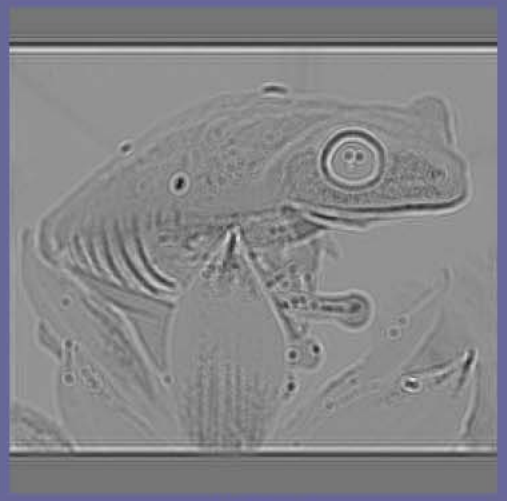
original object

x

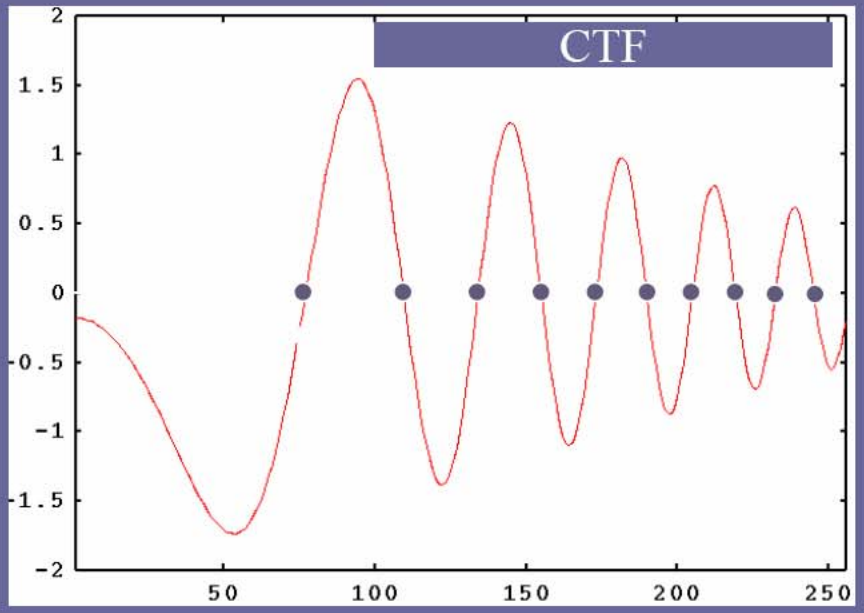


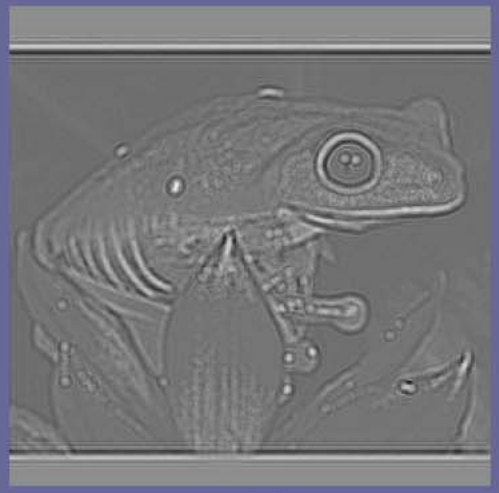
CTF for $\Delta z = 2.500$ µm

=



cryo-EM image

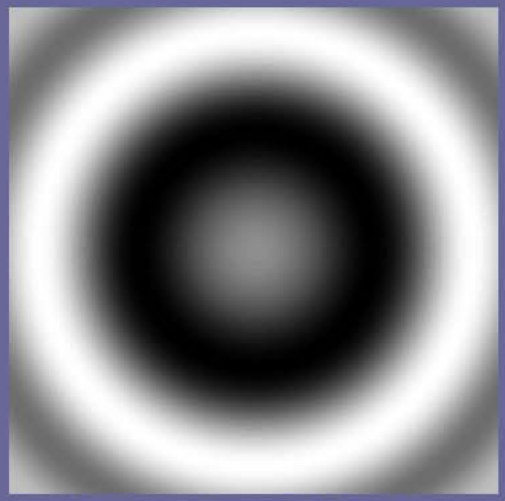




cryo-EM image,
contrast-inverted

original object

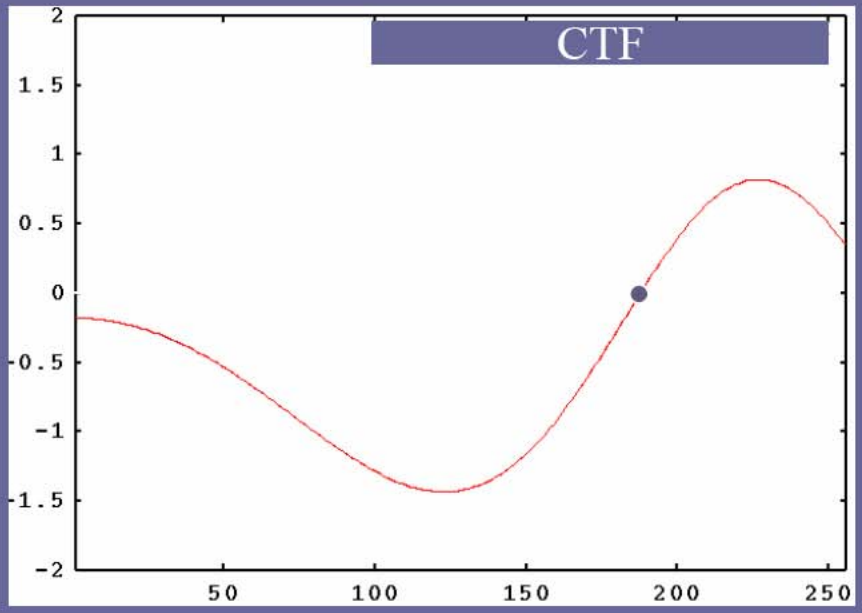
x



CTF for $\Delta z = 0.400$ µm

=

cryo-EM image

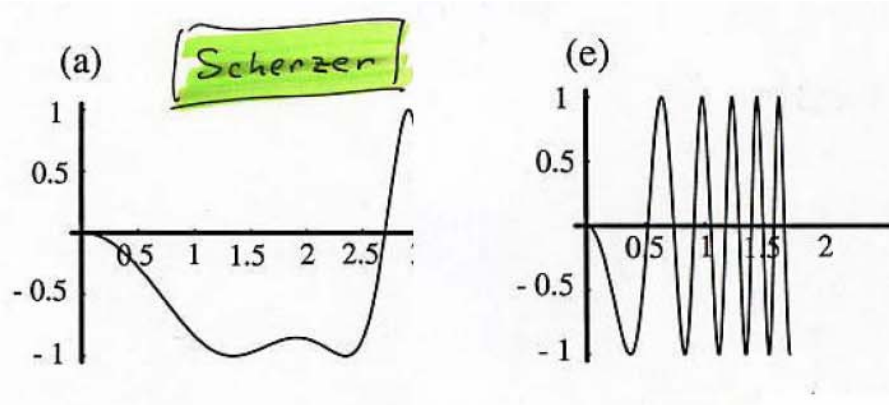


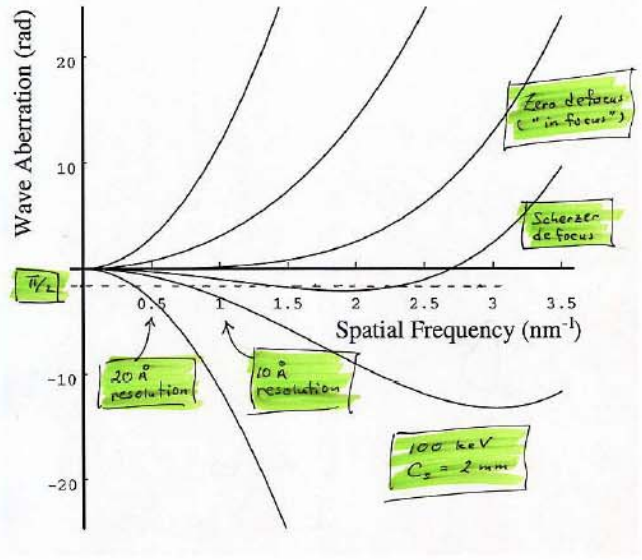
cryo-EM image,
contrast-inverted

High Defocus Gives "Good Contrast" – But at a Cost

- **ONE IS TEMPTED TO USE HIGH DEFOCUS VALUES BECAUSE LOW RESOLUTION IS ALL THAT ONE CAN SEE BY EYE**
- **WHILE HIGH DEFOCUS MAKES IT POSSIBLE TO SEE THE OBJECT, IT ALSO CAUSES RAPID OSCILLATIONS [CONTRAST REVERSALS]**
- **THE RAPID CONTRAST REVERSALS ARE DUE TO THE STEEP INCREASE IN**

$$\gamma(s) \sim \pi \Delta Z \lambda s^2$$





Rapid Oscillation of the CTF Causes a Loss of Signal

- **THE FUNDAMENTAL PROBLEM IS IMPERFECT SPATIAL COHERENCE, EXPRESSED AS**
  - **FINITE SOURCE SIZE,**
  - **OR NON-PARALLEL ILLUMINATION**
- **THE FIELD EMISSION GUN (FEG) GIVES SUFFICIENT INTENSITY EVEN WITH HIGHLY PARALLEL ILLUMINATION**
- **TEMPORAL COHERENCE (ENERGY SPREAD) IS ALSO A LIMITATION AT HIGHER RESOLUTION**

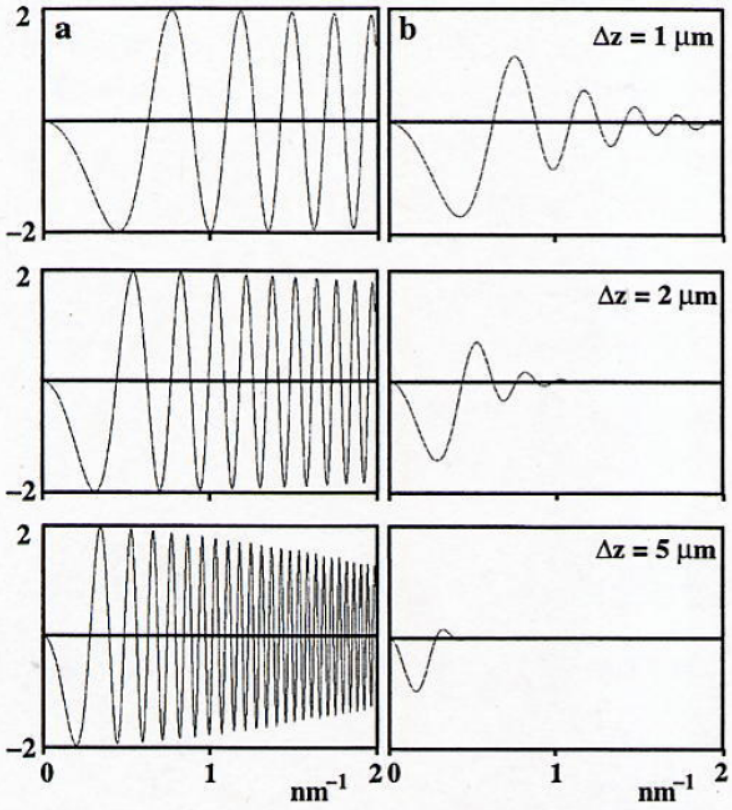
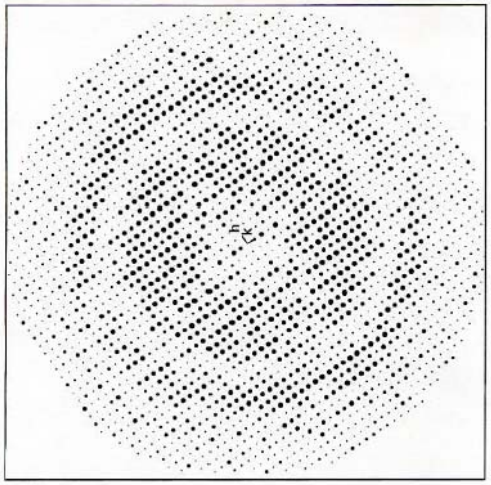
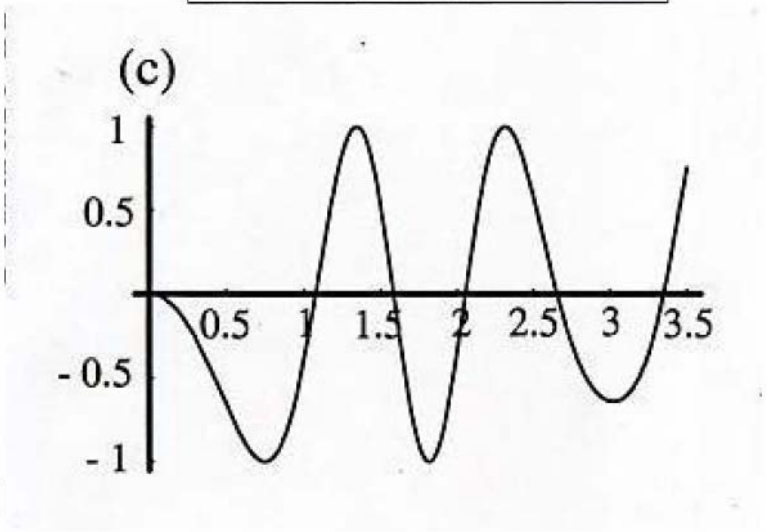


FIG. 4.10. Comparison of transfer functions. FEG versus LaB_6 (a) FEG, U = 200 kV (b). LaB_6, U = 200 kV. Defocus values 1.0, 2.0, and 5.0 μm, respectively. An energy spread of 0.8 eV (2.0 eV) and an emission angle of 0.02 mrad (0.3 mrad) was assumed for the FEG (LaB_6) cathode.

Effect of Imperfect Coherence and Contrast Reversal Can Be Partially Corrected

- ONE MUST FIRST SEE (OR PREDICT?) THE LOCATION OF THE "ZEROS" IN THE CTF
  - THEY ARE APPARENT IN THE FOURIER TRANSFORM OF THE TUBULIN CRYSTAL ON THE RIGHT
  - THEY ARE SIMILARLY APPARENT IN AREAS WITH AMORPHOUS CARBON, etc.
- SIMPLY CHANGE THE SIGN OF THE FOURIER TRANSFORM IN "EVEN" ZONES OF THE CTF; THE SAME CAN BE DONE FOR NON-CRYSTALLINE OBJECTS
- BE AWARE THAT ASTIGMATISM INVALIDATES APPLICATION OF CIRCULAR SYMMETRY
- COMPENSATION FOR THE AMPLITUDE OF THE CTF AND THE ENVELOPE FUNCTION IS ALSO POSSIBLE DURING COMPUTATION





Computation of Averaged Power Spectrum

For each micrograph …

1) Divide field into overlapping subfields of ~512 x 512

2) Compute FFT for each subfield

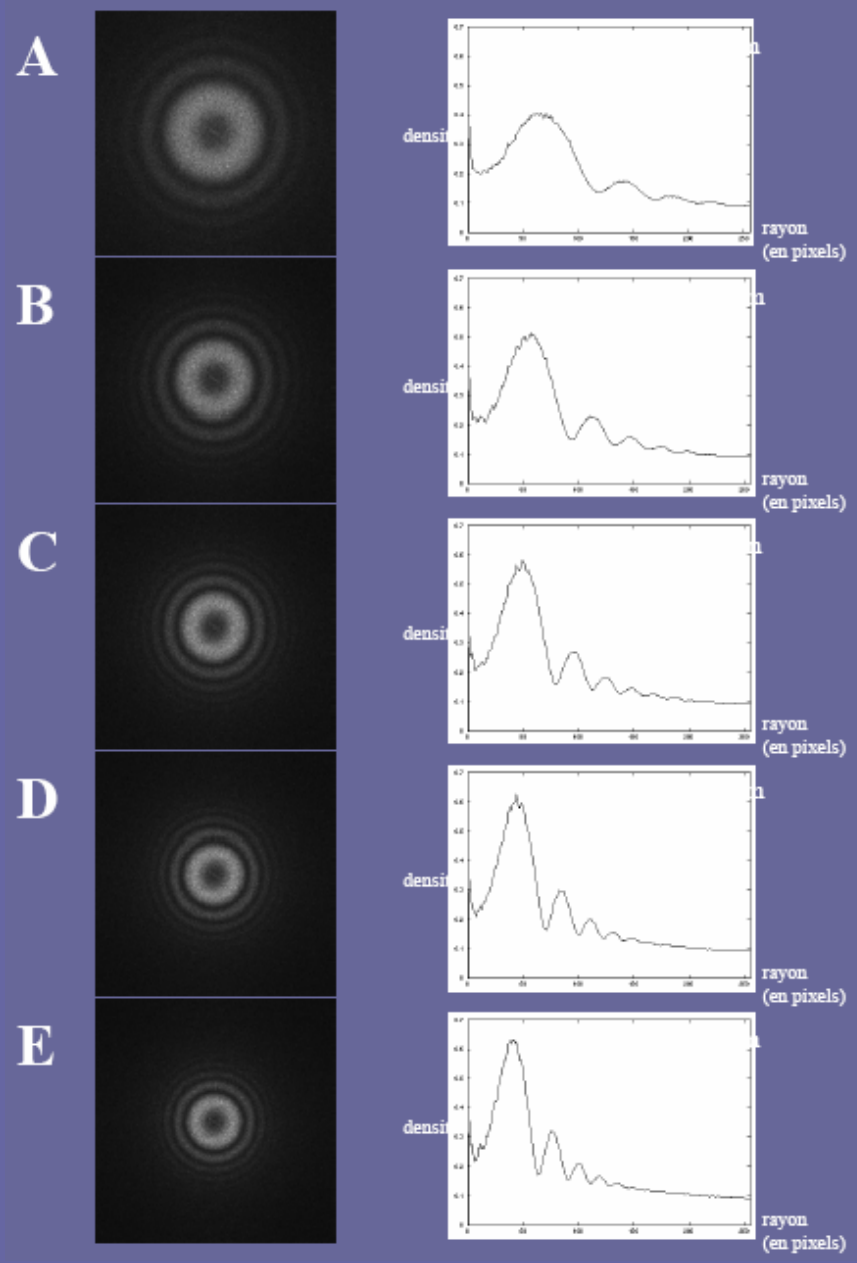
3) Compute $|F(\mathbf{k})|^2$ for each subfield

4) Form average over $|F(\mathbf{k})|^2$ of all subfields => averaged, smoothed power spectrum

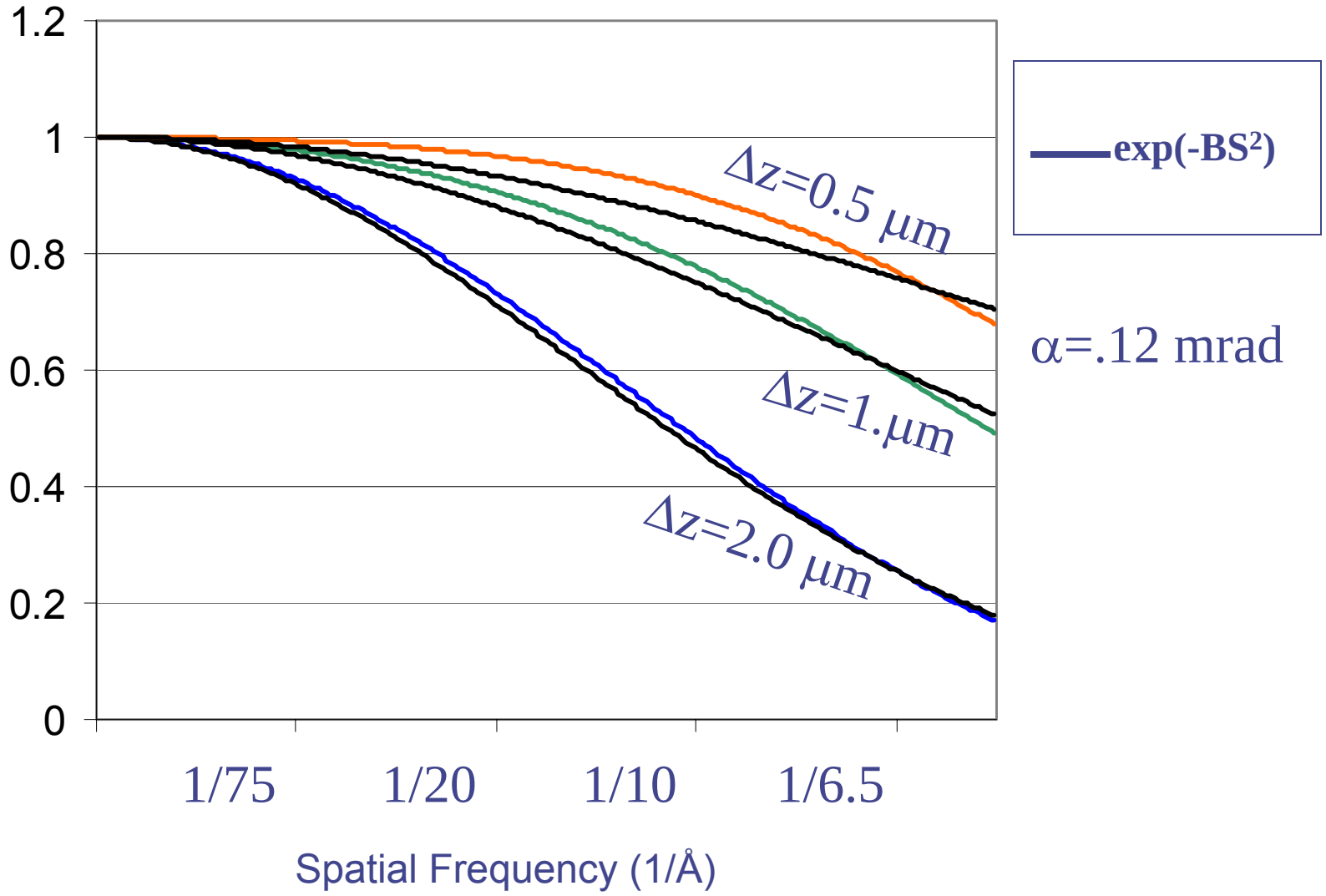
5) Take square root of result => “power spectrum” with reduced dynamic range

6) Form azimuthal average => 1D profile, characteristic for the micrograph, ready to be compared with CTF

Gallery of Power Spectra at Different Defocus



Fitting the Spatial Coherence Envelope Function with exp(-BS^2)



CTF Simulation and Fitting

$$CTF(s) = -A\left[(1-Q^2)^{1/2}\sin(\gamma) + Q\cos(\gamma)\right]$$

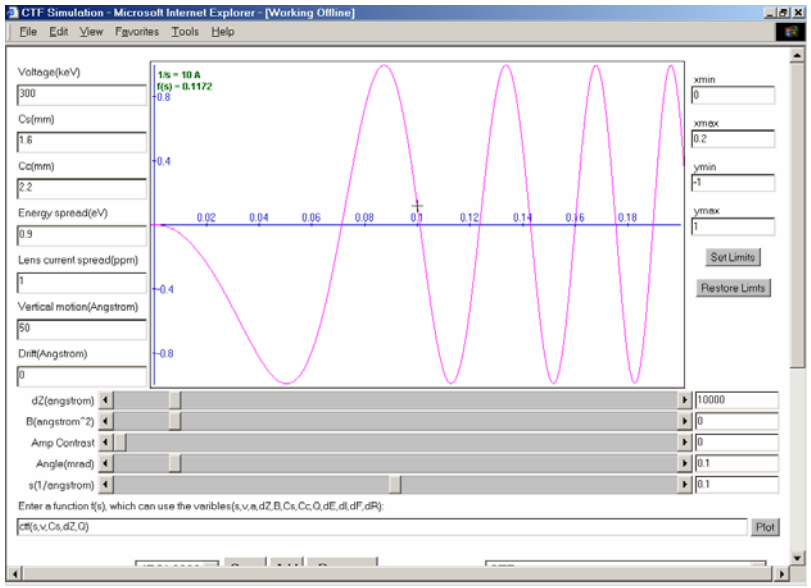
scaling factor, varies among micrographs

wave-length

defocus, varies among micrographs

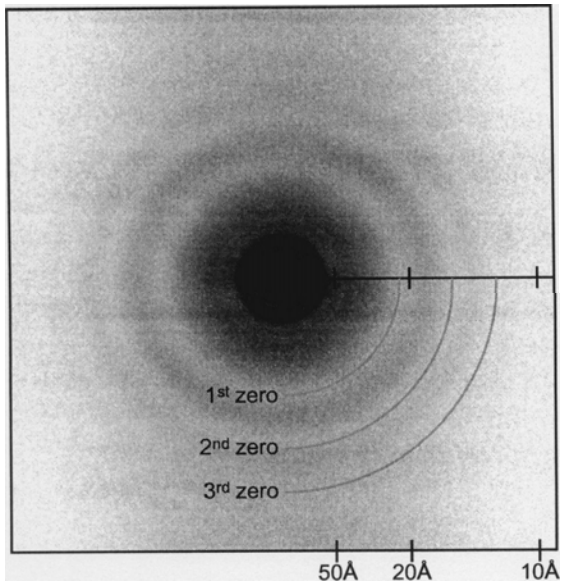
amplitude contrast, ≈constant

$$\gamma(s) = -2\pi\left(C_s\lambda^3 s^4/4 - \Delta Z\,\lambda s^2/2\right)$$



http://ncmi.bcm.tmc.edu/~wjiang/ctf

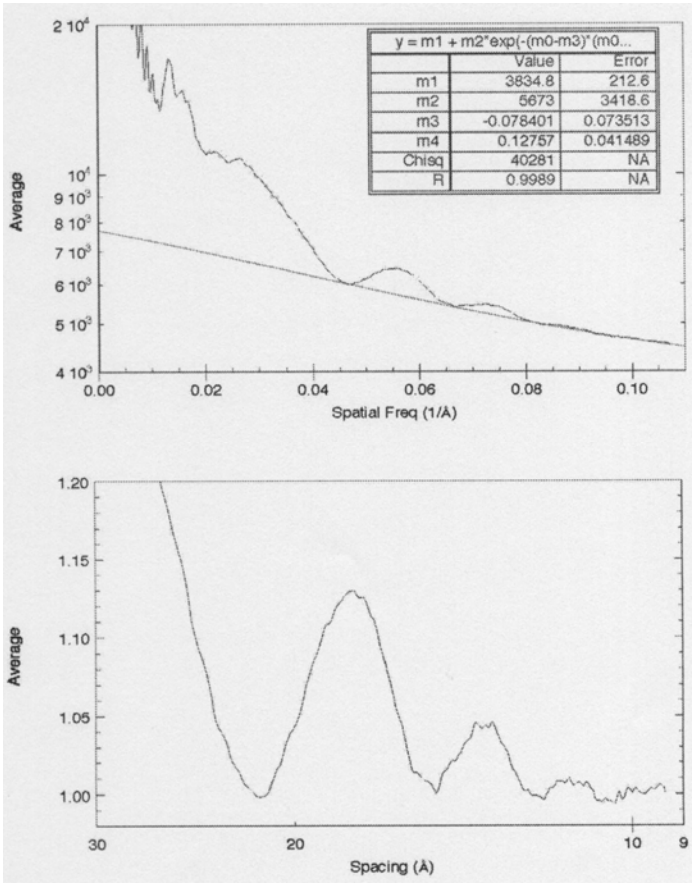
Example



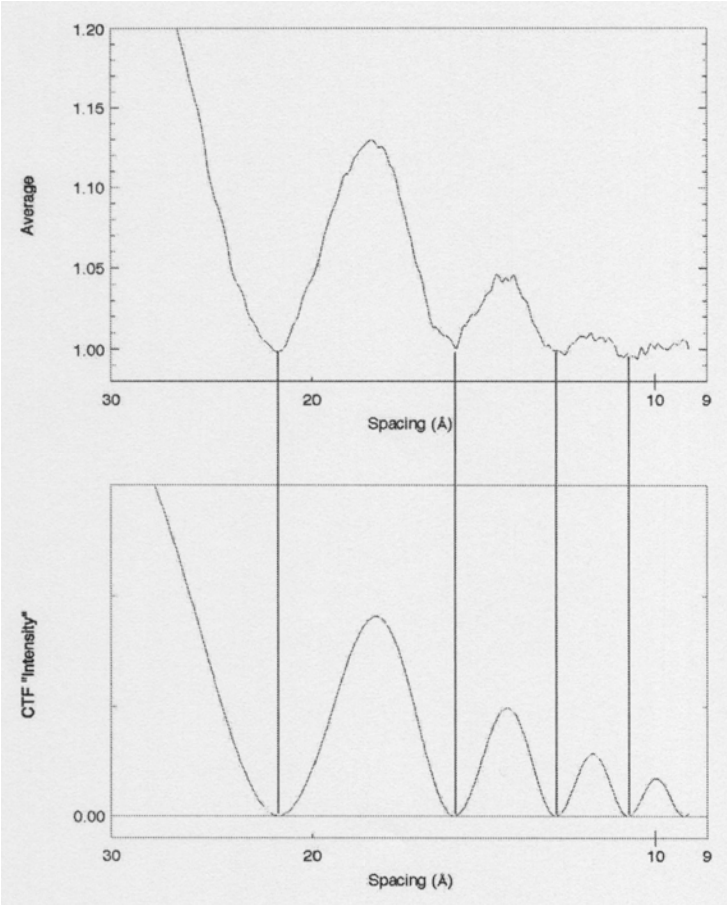
Power Spectrum Image
Sum of Fourier Transforms (amplitudes) from all particle images from one micrograph. Light-grey rings indicate weak signal, caused by minima in the contrast transfer function (CTF).

PS Profile
Amplitude of PS Image as a function of spatial frequency. Decay is fit with a Gaussian.

PS Profile - corrected
After removal (division) of Gaussian decay.
Minima represent positions of "phase reversal" - see next page.



Example

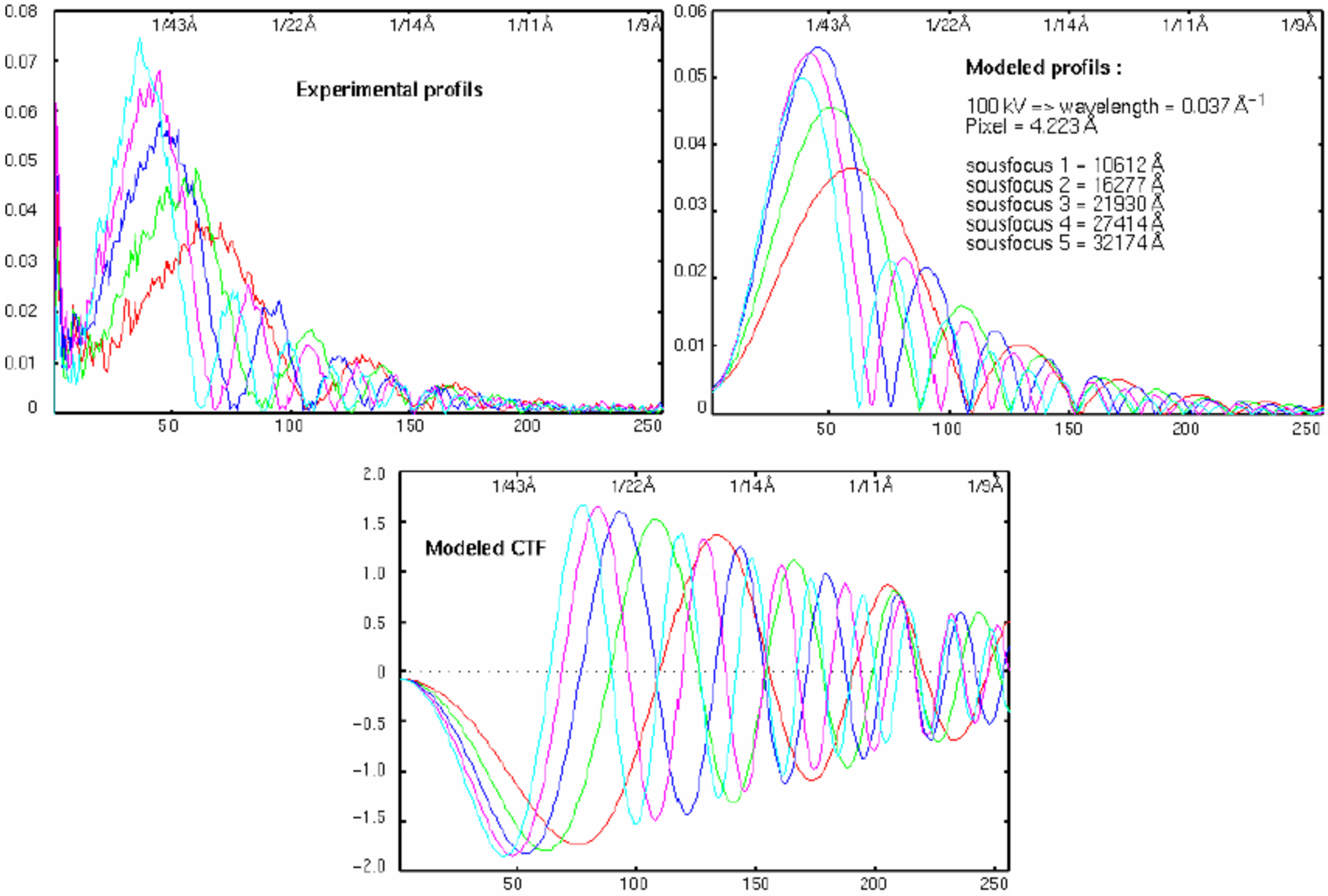


CTF measured from images (see previous page): Thon rings clearly identifiable.

CTF estimated
The microscope settings (accelerating voltage, defocus) and observed image decay were used to calculate this theoretical squared CTF “Intensity” curve.

Use this information to flip phases and to amplify high frequencies (careful…). This is a partial correction because information at zeros of CTF is lost.

Multiple Defocus Groups



Combining Multiple Defocus Groups

- **Coverage of large defocus range required**
- **Data collection must be geared toward covering range without major gap**
- **Characterizing all particles from the same micrograph by the same defocus is OK up to a resolution of ~1/8 $Å^{-1}$.**

Sequence of steps:

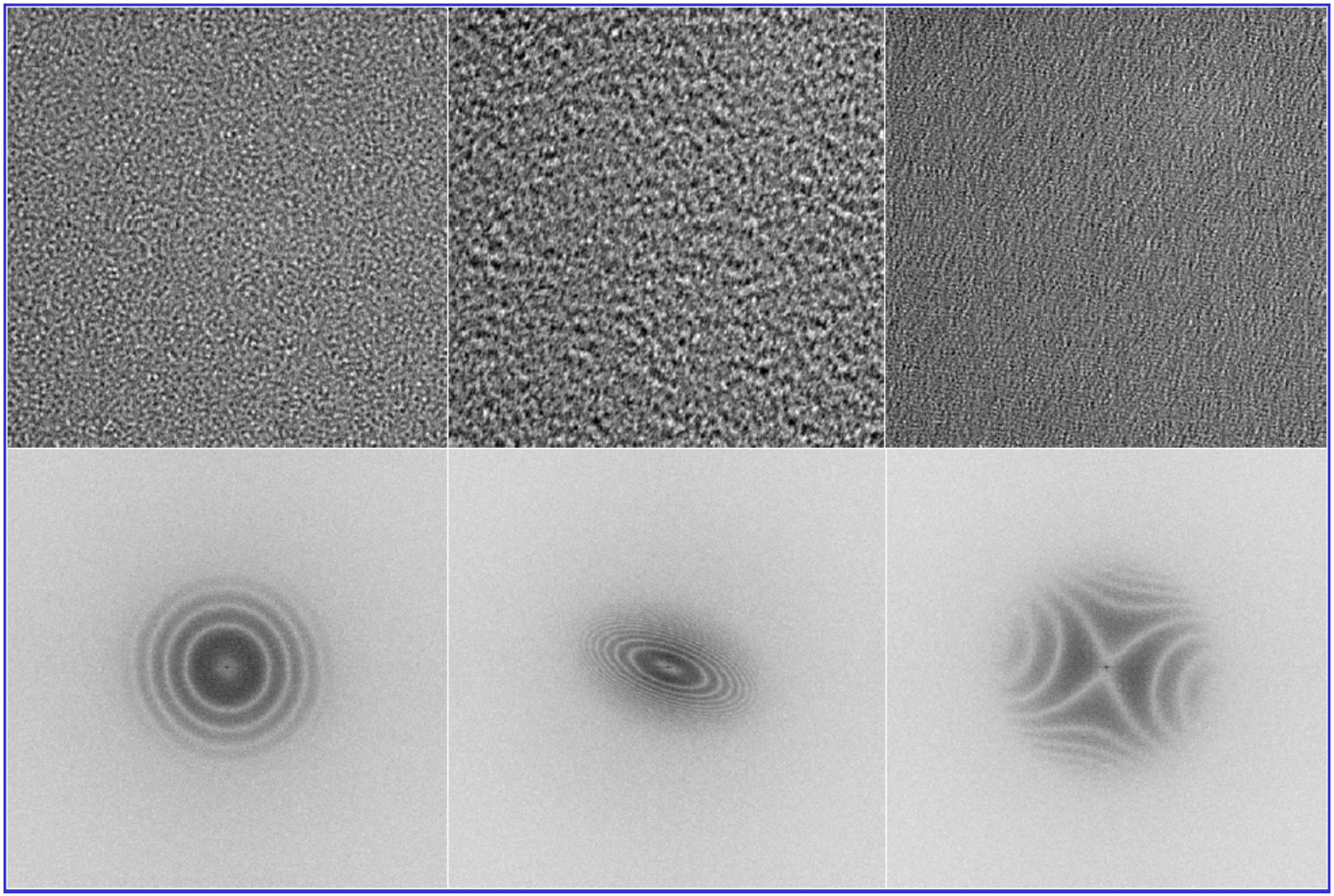
1) Determine defocus for each micrograph

2) Define defocus groups, by creating supersets of particles from micrograps in a narrow range of defoci

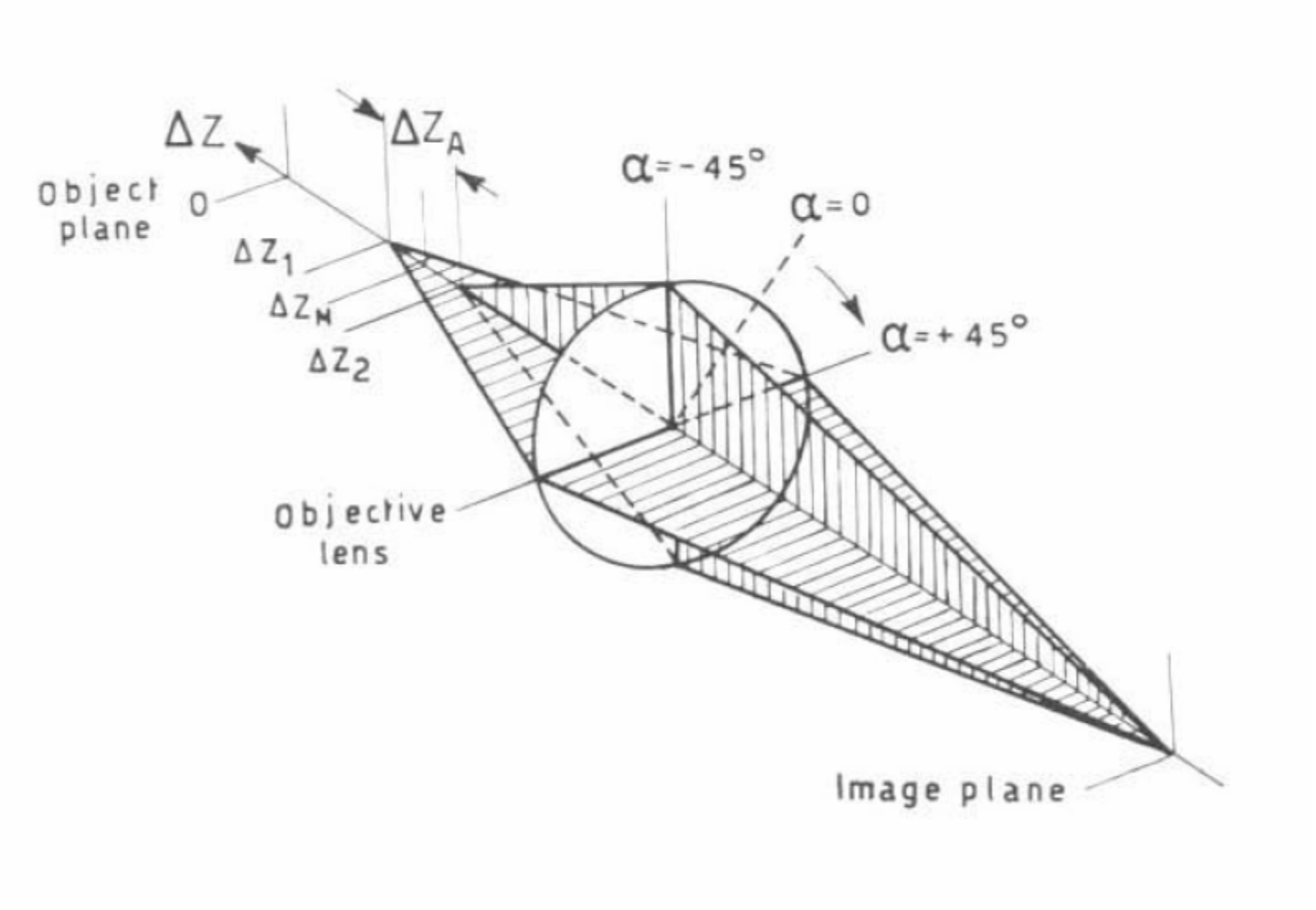
3) Process particles separately, by defocus group, till the very end (3D reconstruction by defocus groups)

4) Compute merged, CTF-corrected reconstruction. E.g., Wiener filtering.

Astigmatism



Astigmatism



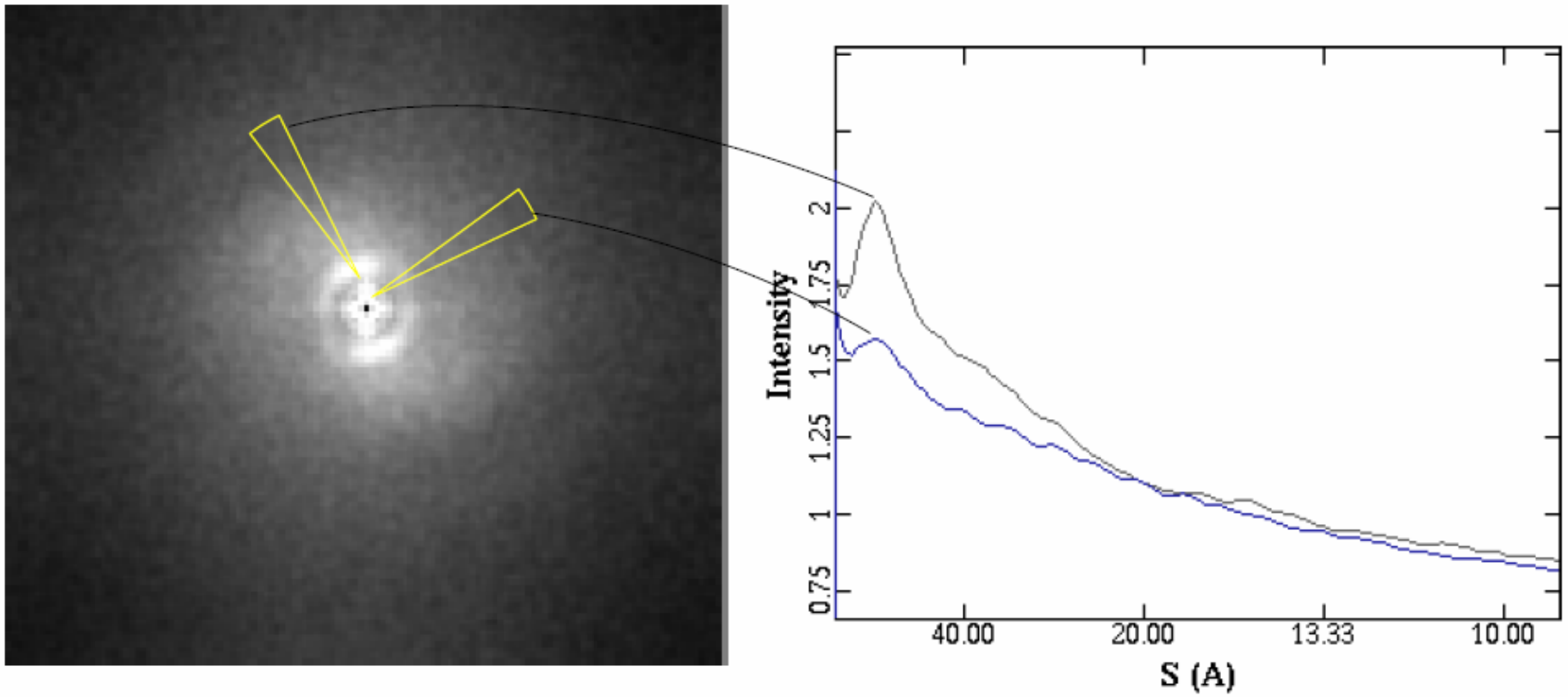
Astigmatism

$$\Delta Z_{eff}(\alpha) = \Delta Z_m + (\Delta Z_a \sin 2\alpha)/2$$

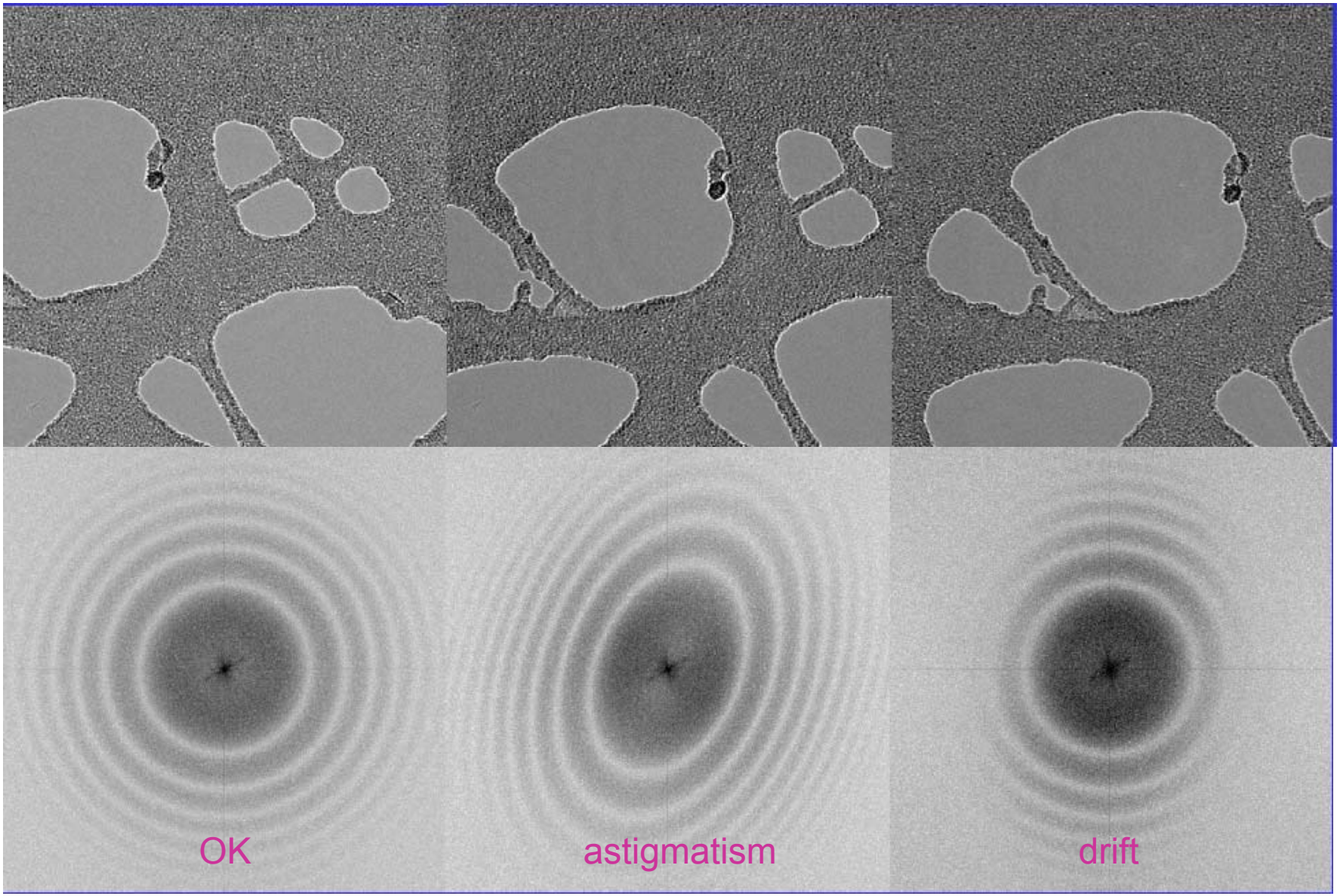
$$\Delta Z_m = (\Delta Z_1 + \Delta Z_2)/2$$

$$\Delta Z_a = \Delta Z_1 - \Delta Z_2$$

Astigmatism



Can You Tell the Difference?



CCD vs. Film, Revisited

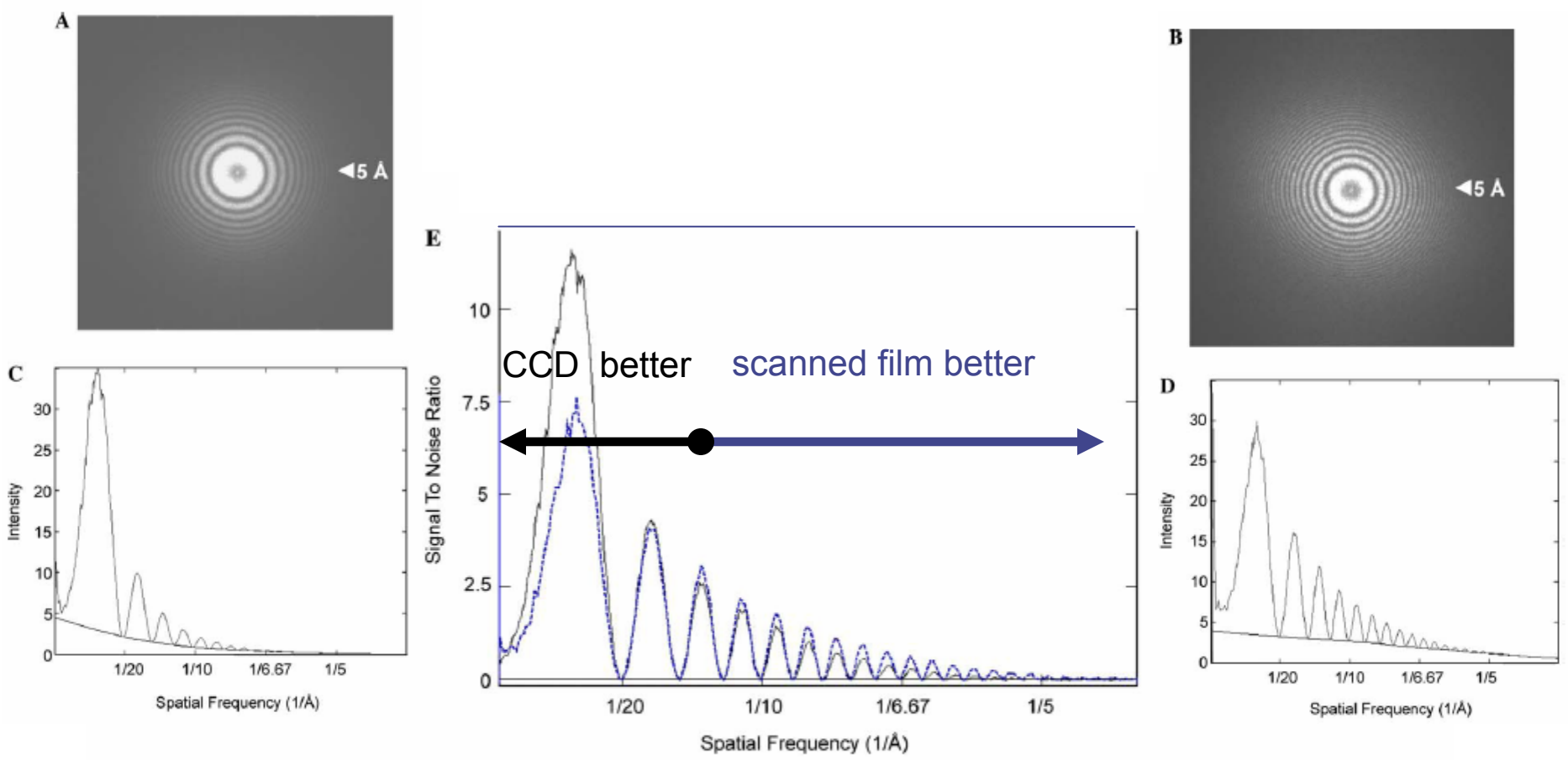


Fig. 2. Characterizing the CCD Camera. Images of amorphous carbon film recorded on CCD and photographic film followed by digitization in a Zeiss scanner. (A) 2-D power spectrum calculated from amorphous carbon film imaged on CCD. (B) 2-D power spectrum calculated from amorphous carbon film imaged with photographic film and scanned using a Zeiss scanner at 7 μm. (C–D) 1-D power spectrum and noise profile estimate calculated by rotationally averaging (A) and (B), respectively, and fitting a line through the zeroes of the contrast transfer function. Signal-to-noise ratio (SNR) comparison between images of amorphous carbon film collected on CCD and film is shown in (E). In a solid line is the SNR calculated from data collected on CCD. Shown in a dotted line is the SNR calculated from digitized data collected on film.

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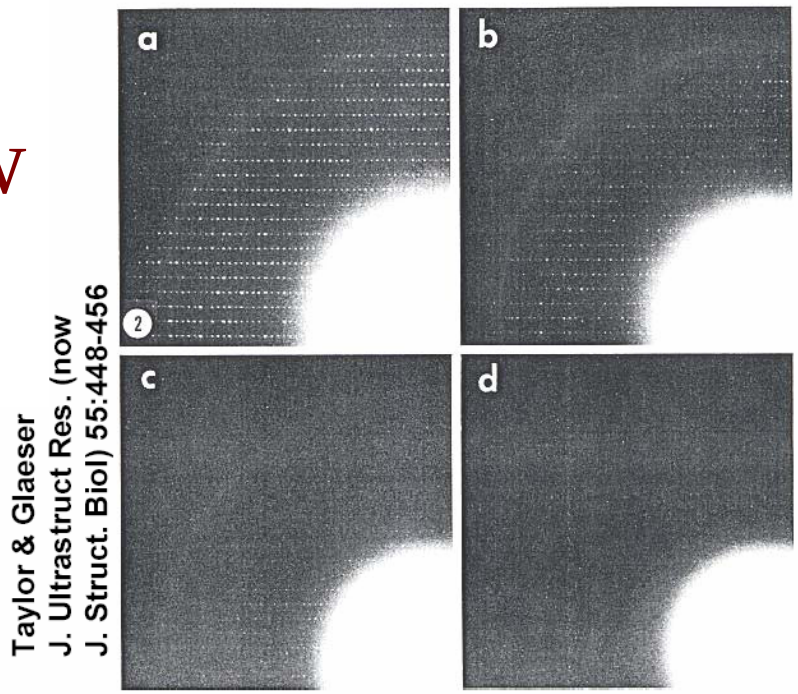
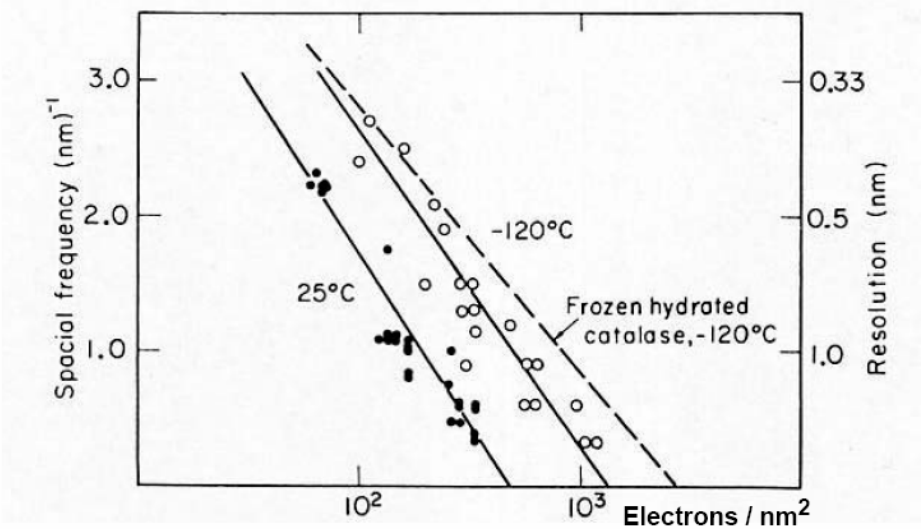
Images Must be Recorded with Very Low Electron Exposures

- PROTEIN STRUCTURES
- DISINTIGRATE AS RADIATION DAMAGE PROGRESSES
- LOW-RESOLUTION FEATURES LAST LONGER THAN HIGH-RESOLUTION FEATURES
- THE CRITICAL DOSE FOR RADIATION DAMAGE IS ~"THE SAME" FOR ALL PROTEINS AND ALL EMBEDDING MEDIA AT LOW TEMPERATURE
- BUBBLING SETS IN AT ~ 30 e/A^2 (AT 100 keV)
- THE SMALL NUMBER OF ELECTRON "COUNTS" RESULTS IN LARGE STATISTICAL FLUCTUATIONS FROM ONE PIXEL TO THE NEXT

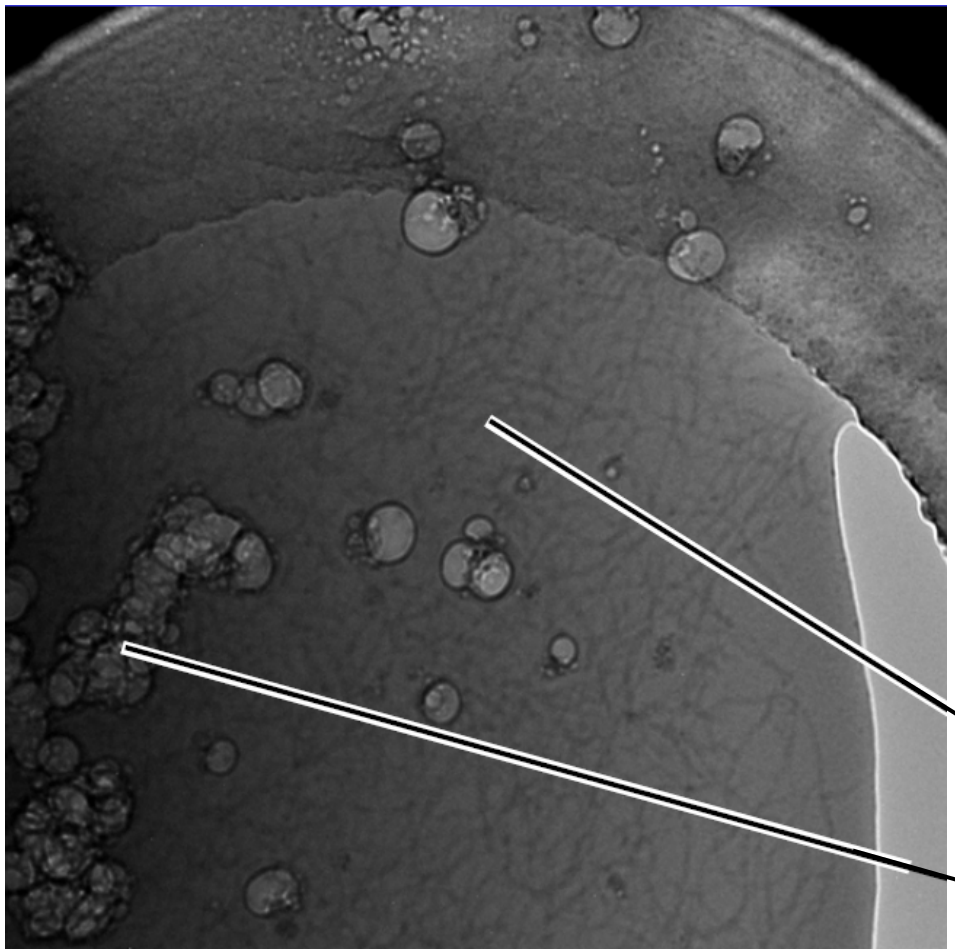


Taylor & Glaeser
J. Ultrastruct Res. (now
J. Struct. Biol) 55:448-456



Glaeser & Taylor (1977) J. Microsc. 112:127-138

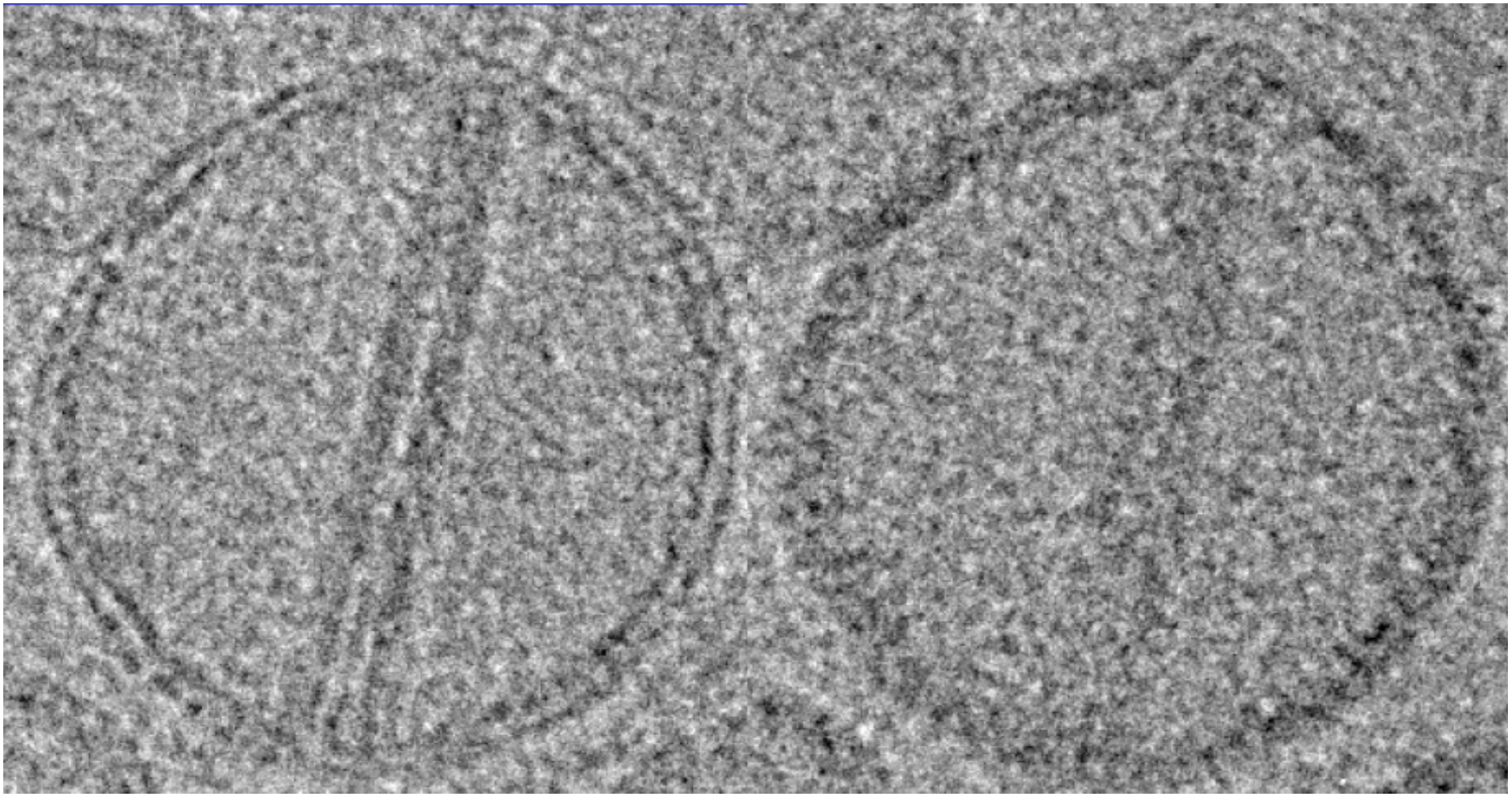
Bubbling: A Sign of Radiation Damage



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

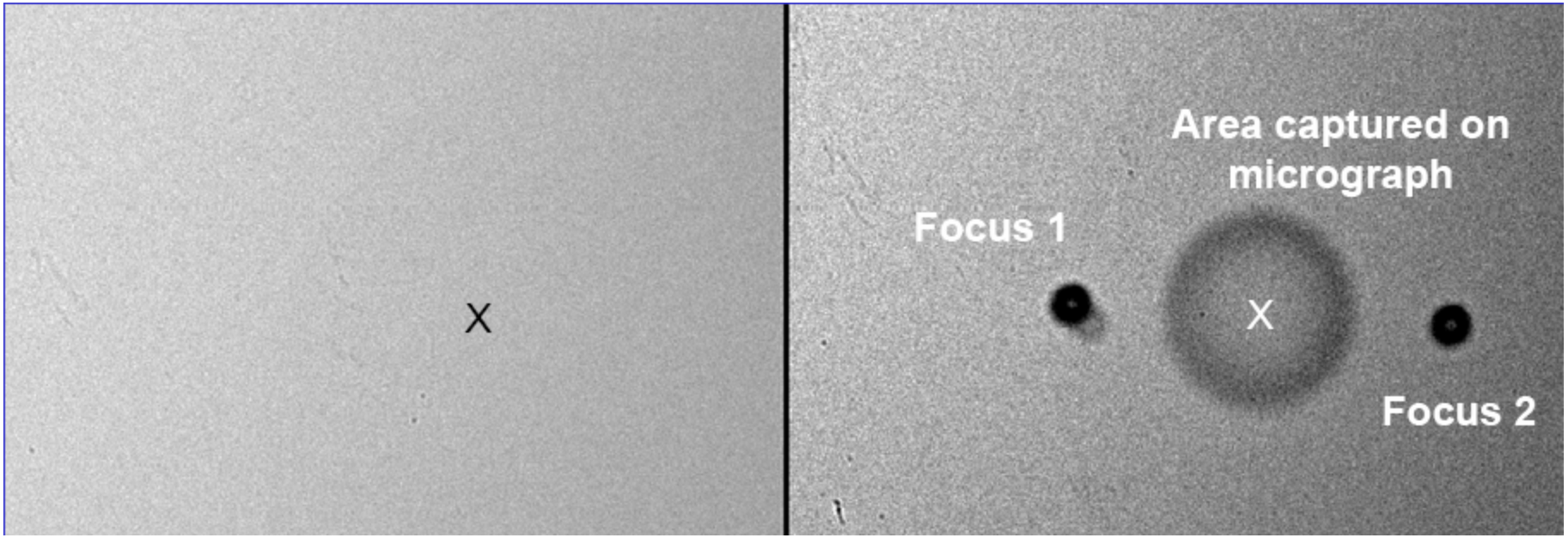
Destructive Power of Electrons



after 0.2 sec

1 sec exposure

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.

Resolution Limits Due to Shot Noise

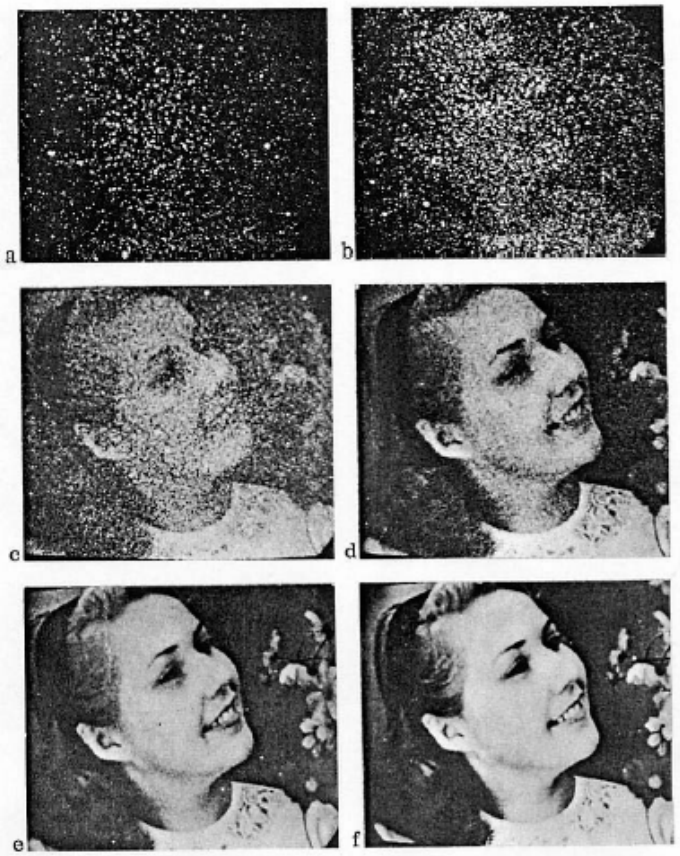
- **ALBERT ROSE DETERMINED A QUANTITATIVE RELATIONSHIP BETWEEN FEATURE SIZE AND VISUAL DETECTABILITY:**

  $$dC > 5 / (N)^{1/2}$$

  WHERE "N" IS THE NUMBER OF QUANTA PER UNIT AREA

- **FEATURES SMALLER THAN 25A MAY NOT BE DETECTABLE FOR EXPOSURES AS LOW AS 25 e/A^2**

- **THE ONLY WAY TO OVERCOME THIS LIMITATION IS TO AVERAGE INDEPENDENT IMAGES OF IDENTICAL OBJECTS**



Picture	Number of photons	High-light brightness, foot-lamberts
a	3×10^3	10^{-6}
b	1.2×10^4	4×10^{-6}
c	9.3×10^4	3×10^{-5}
d	7.6×10^5	2.5×10^{-4}
e	3.6×10^6	1.2×10^{-3}
f	2.8×10^7	9.5×10^{-3}

Rose (1973) Vision: human and electronic. Plenum

Averaging Images of Identical Objects is Easy for Crystals

- **AVERAGING CAN BE DONE IN REAL SPACE**

- **BUT IT IS EVEN EASIER TO DO IT IN FOURIER SPACE**
  - INFORMATION ABOUT FEATURES IN THE IMAGE THAT ARE PERIODIC MUST APPEAR IN THE DIFFRACTION SPOTS
  - NON-PERIODIC "NOISE" IS DISTRIBUTED UNIFORMLY AT ALL SPACIAL FREQUENCIES
  - YOU ELIMINATE MOST OF THE NOISE IF YOU USE JUST THE DIFFRACTION SPOTS TO DO AN INVERSE FOURIER TRANSFORM

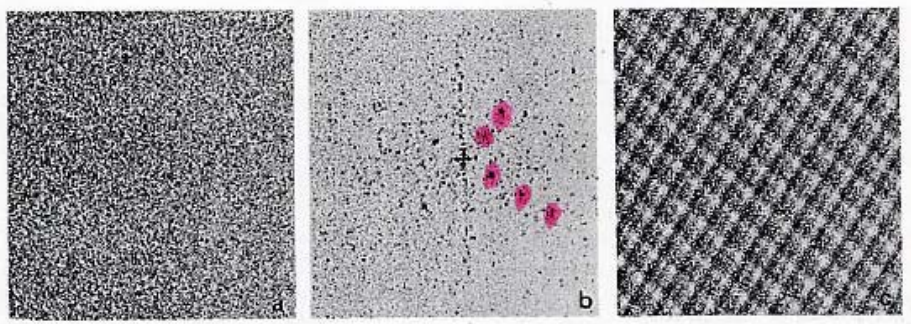


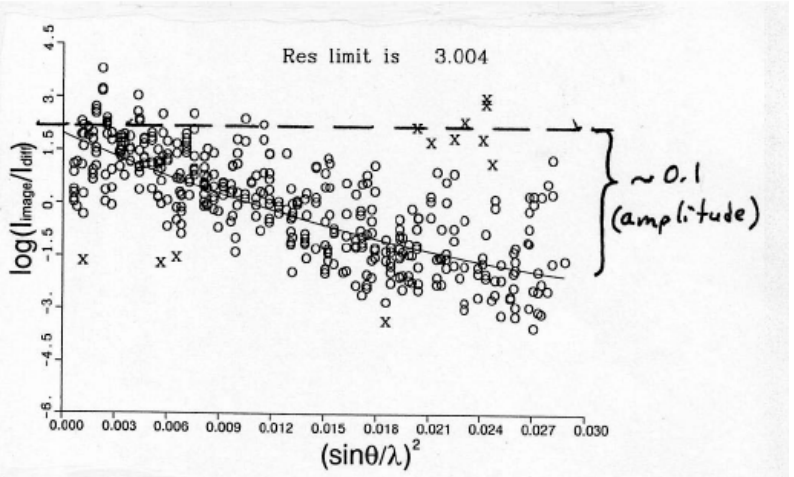
Fig. 5. (a) The Z-modulation display of a statistically noisy image of a carbon replica of an optical diffraction cross grating, recorded with an image intensifier; (b) power spectrum of the statistically noisy image; (c) the spatially averaged image.

Kuo & Glaeser (1975) Ultramicroscopy 1:53-66

- **AVERAGING A 100X100 ARRAY (i.e. 10^4 PARTICLES) PROVIDES THE NEEDED STATISTICAL DEFINITION REQUIRED FOR ONE VIEW (PROJECTION) AT ATOMIC RESOLUTION**

Real Space Averaging is More Powerful Than You Might Expect

- ALIGN IDENTICAL PARTICLES IN IDENTICAL VIEWS BY CROSS CORRELATION, AND DO SO AT ATOMIC RESOLUTION, EVEN THOUGH THE IMAGE IS NOISY
- CROSS CORRELATION WORKS BETTER, THE BIGGER THE PARTICLE IS, BECAUSE THERE IS "MORE MASS TO BE CORRELATED"
- PERFECT IMAGES WOULD PRODUCE ATOMIC RESOLUTION FROM ~12,000 PARTICLES AS SMALL AS Mr = 40,000
- INCREASE BOTH FIGURES BY 100X IF C = 0.1 WHAT IT SHOULD BE
- CONTRAST *IS* 0.1 "WHAT IT SHOULD BE" IN CURRENTLY RECORDED DATA
  - YONEKURA/NAMBA RESULT REQUIRED SELECTION OF PARTICLE-IMAGES THAT WERE MUCH BETTER THAN THE AVERAGE
- BEAM-INDUCED MOVEMENT (CHARGING) IS THOUGHT TO BE THE CURRENT LIMITATION



Mitsuoka et al. (1999) J. Mol. Biol. 286:861-882

Resources

Handouts:

•Mauro Gemmi, “Image Formation”, Electron Crystallography School 2002, Tampere Finland

Journal Articles:

•Zhu et al., J. Struct Biol. 118, 197-219, 1997 (excellent review of CTF)
•Saad et al, J. Struct Biol. 133, 32-42, 2001(Chiu lab CTF model)
•Wade, Ultramicroscopy 46, 145-156, 1992 (pp. 145-149 recommended as review)

Book Chapters:

•Lenz, pp. 541-569 in “Electron Microscopy in Material Science”, U. Valdre, editor, Academic Press 1971 (detailed theory of image formation, historic interest)