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FUNDAMENTALS OF MICROSCOPY: THEORY

NRAMM Practical Course on

Electron Cryo-microscopy

Wed. Nov. 12, 2003

Robert M. Glaeser

THE ELECTRON MICROSCOPE HAS RECOGNIZABLE OPTICAL PARTS

- 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

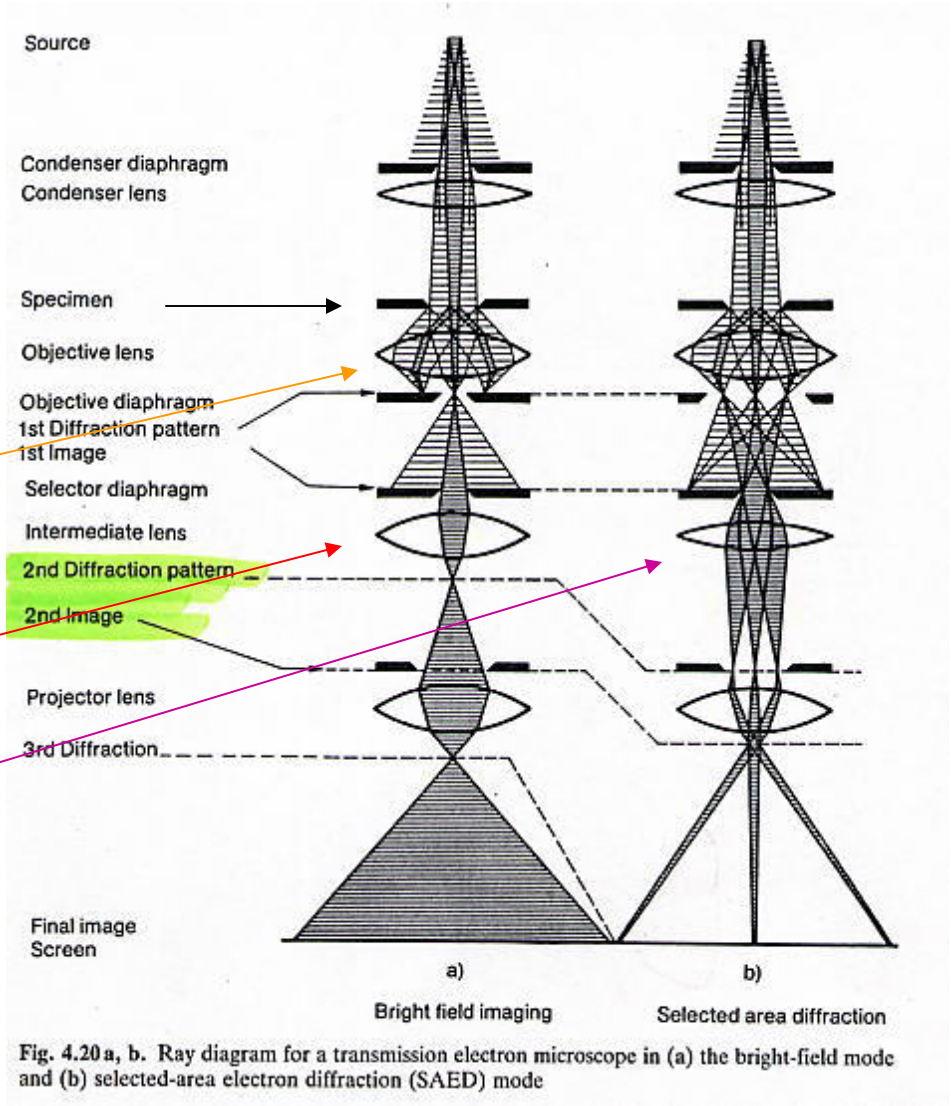
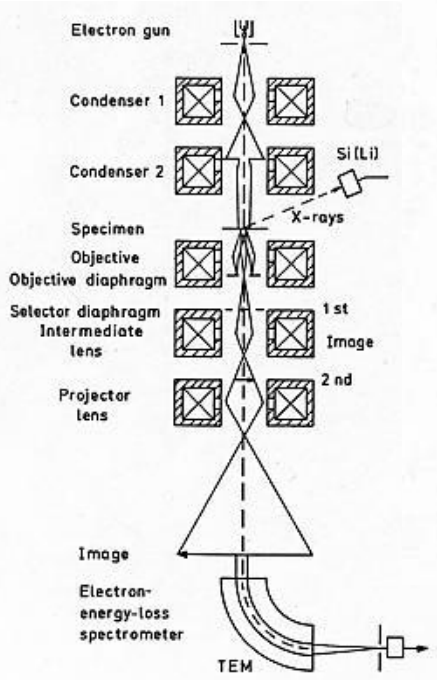


Fig. 4.20 a, b. Ray diagram for a transmission electron microscope in (a) the bright-field mode and (b) selected-area electron diffraction (SAED) mode

Reimer (1989) Transmission EM [Springer]

ADDITIONAL REPRESENTATIONS OF THE ELECTRON MICROSCOPE AS AN OPTICAL INSTRUMENT



Reimer's book again

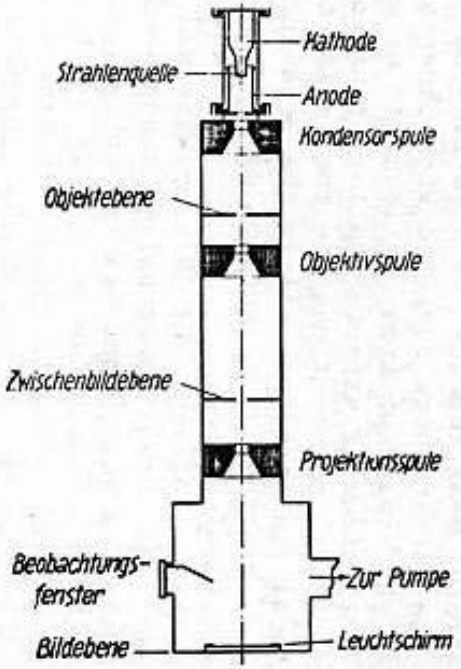
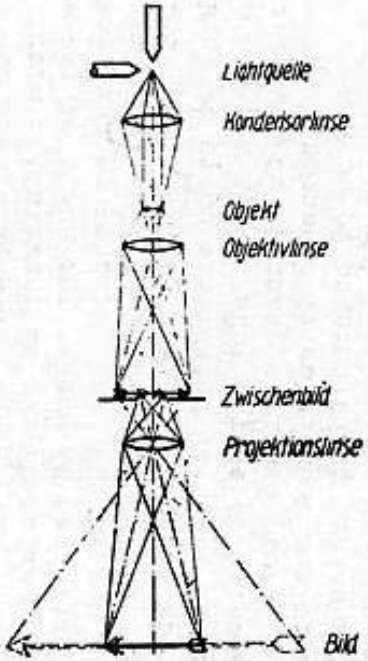


Fig. 1.3. Schematic ray path for a transmission electron microscope (TEM) equipped for additional x-ray and electron energy-loss spectroscopy



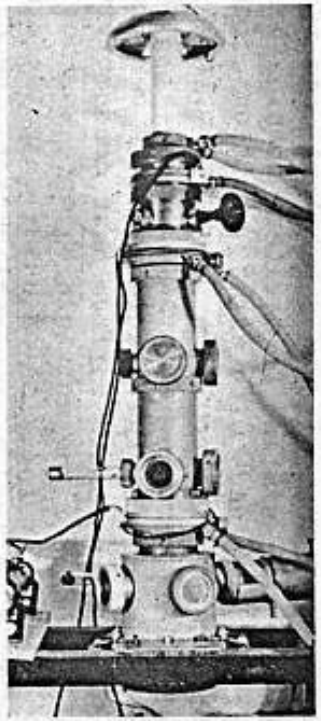
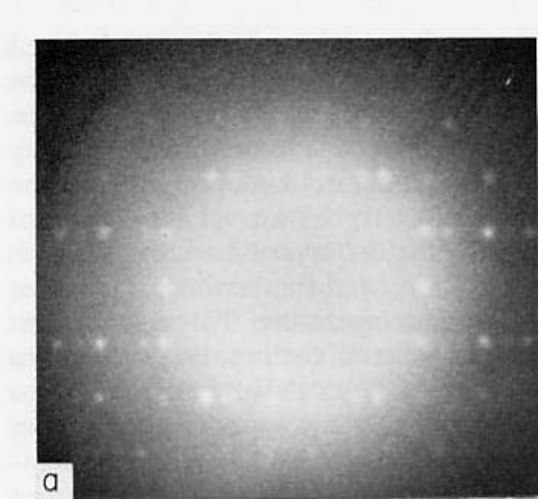


FIG. 6.--Ruska's 1934 microscope.

Marton (

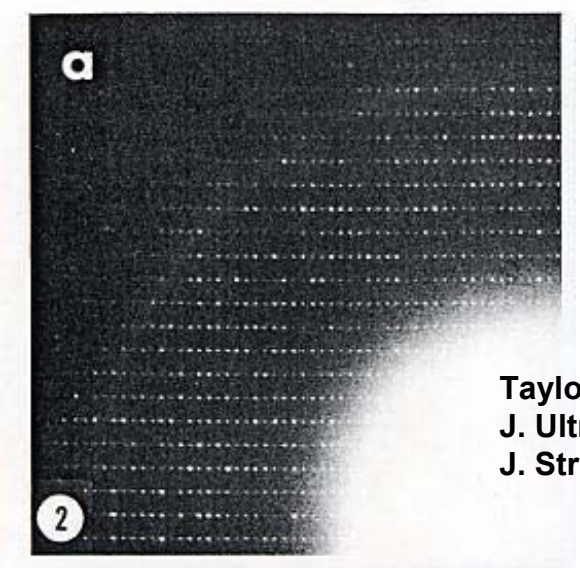
ELASTICALLY SCATTERED ELECTRONS ARE COHERENT WAVES

- 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

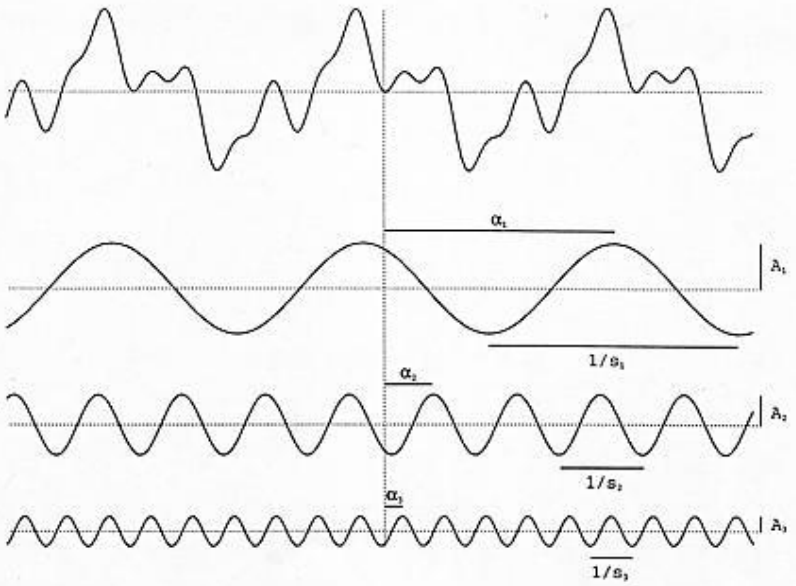


Unstained, frozen-hydrated catalase

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

RESOLUTION, SCATTERING ANGLE AND SPATIAL FREQUENCY

Chiu et al. (1993)
Biophys J. 64:
1610-1625



A "COMPLICATED" STRUCTURE

LOW-RESOLUTION FEATURES

HIGH-RESOLUTION FEATURES

FIGURE 1 An illustration of a one-dimensional object potential function v(x) at the top of the figure which can be decomposed into three cosine waves with different frequencies (s), amplitudes (A), and phases (α) with respect to a common origin. The mathematical expression is $v(x) = \sum A_i(s) \cos(2\pi S_i x + \alpha_i)$. (provided by A. Avila-Sakar and V. Mootha)

- **RESOLUTION, "d", AND SPATIAL FREQUENCY, "s = 1/d" ARE "THE SAME THING"**
- **SPATIAL FREQUENCY (RESOLUTION) AND SCATTERING ANGLE, θ, ARE CONNECTED BY BRAGG'S LAW: $1/d = 2/\lambda \sin \theta/2$**
- ***HIGH SCATTERING ANGLE MEANS HIGH RESOLUTION***

INELASTIC SCATTERING IN THE THIN-SAMPLE LIMIT

- **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!***

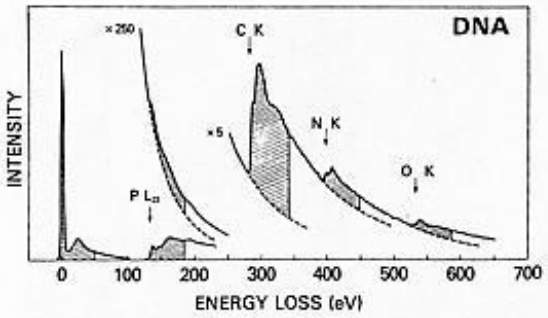


Fig. 2. Electron energy loss spectrum from DNA (extracted from herring sperm) showing zero-loss, plasmon, and P L_{23}, C K, N K and O K core edges. Inverse power law background extrapolation and integrated core edge intensities (shaded areas) are indicated. Background subtraction is illustrated for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

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

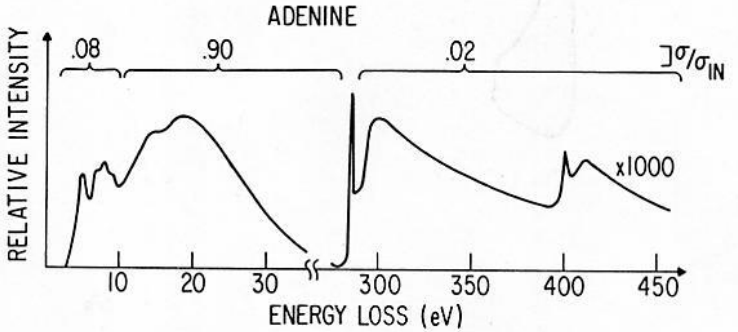


Fig. 1.4 The characteristic electron energy loss spectrum for an approximately 500-Å-thick film of the nucleic acid base, adenine ($C_5N_5H_5$), supported on a 30-Å-thick carbon substrate. The horizontal scale is the amount of energy lost by incident 25 keV electrons in traversing the film. The electron 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 IN THE THICK-SAMPLE LIMIT

- **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***

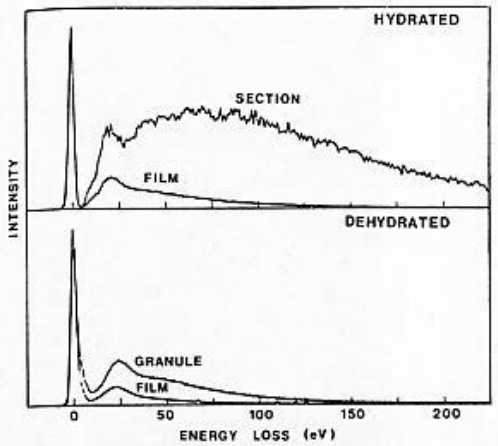
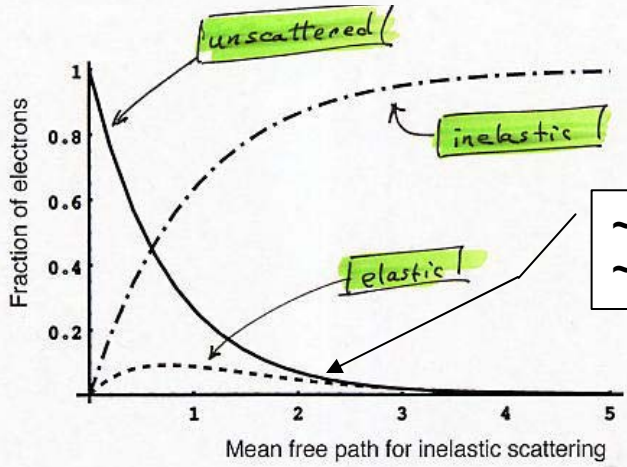


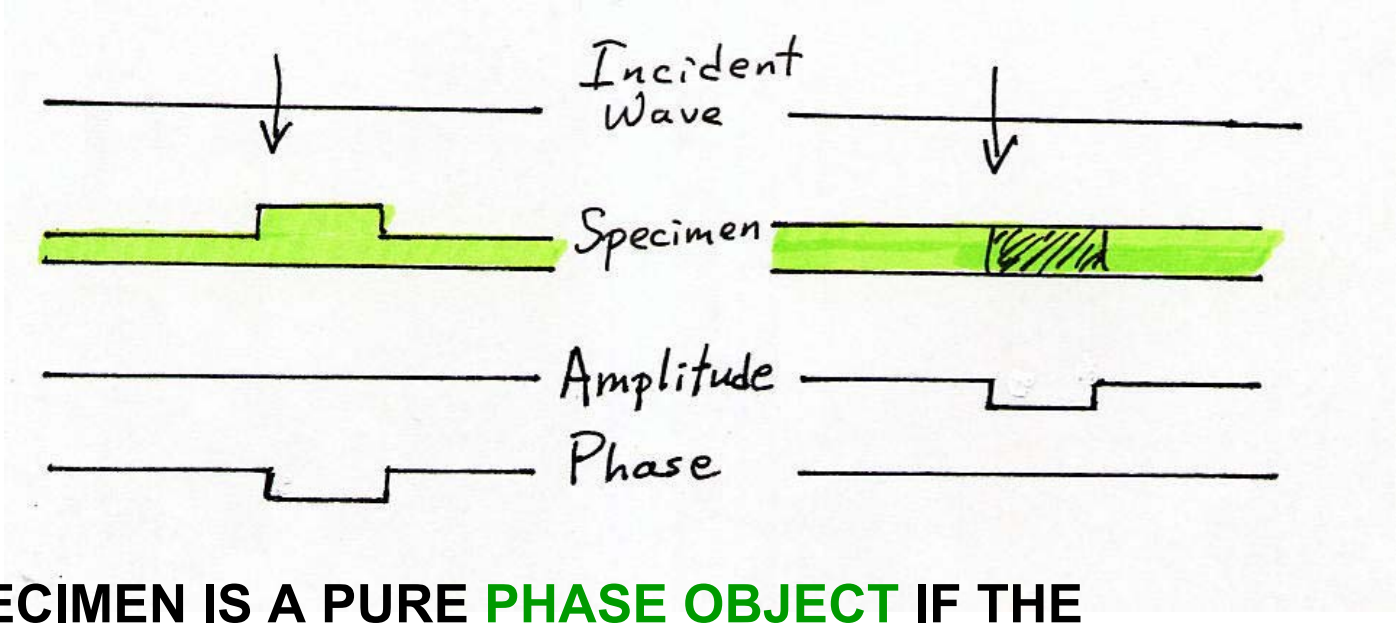
Fig. 11. Low-loss spectra from hydrated and dehydrated section in figs. 9 and 10. Spectrum from hydrated section, kept at −150 °C, recorded in TEM mode with an area including the entire cell. Spectrum from dehydrated section recorded at room temperature with STEM raster contained within single secretory 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



~ 0.5 μm at ~ 300 keV

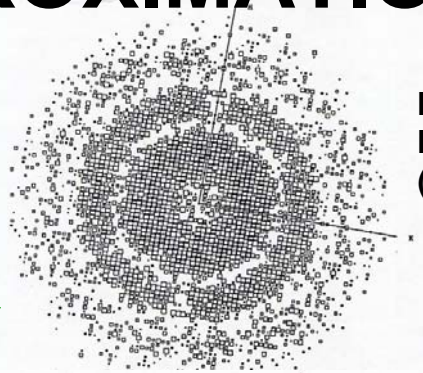
IMAGE CONTRAST REFLECTS CHANGES IN BOTH THE PHASE AND THE AMPLITUDE OF THE ELECTRON WAVES



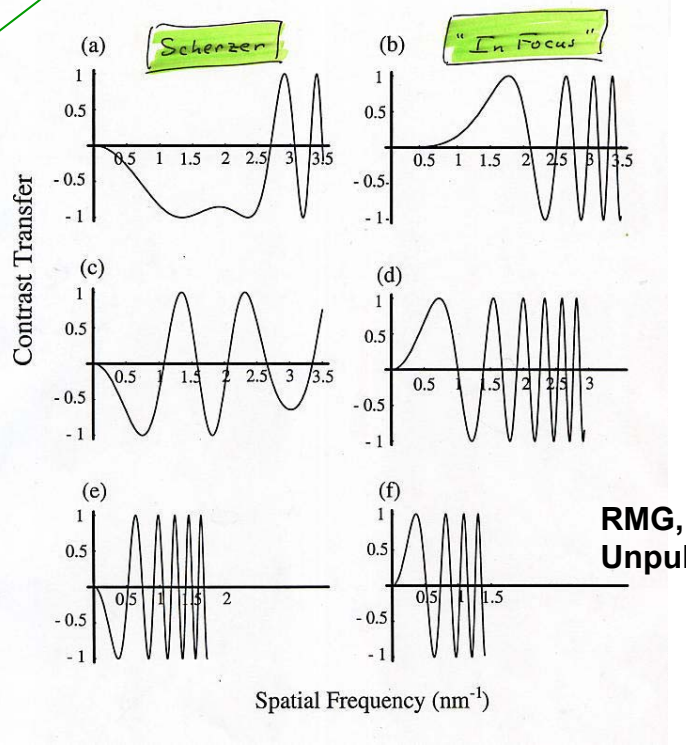
- A SPECIMEN IS A PURE PHASE OBJECT IF THE TRANSMITTED AMPLITUDE IS CONSTANT BUT PHASE IS NOT
- A SPECIMEN IS A PURE AMPLITUDE OBJECT IF THE TRANSMITTED PHASE IS CONSTANT BUT AMPLITUDE IS NOT
- REAL OBJECTS ARE ALWAYS MIXED, BUT AMPLITUDE CONTRAST IS VERY WEAK IN CRYO-EM SPECIMENS

CRYO-EM IS BASED UPON THE WEAK PHASE-OBJECT APPROXIMATION

- $T(x,y) = \exp[i\,\phi(x,y)]$
  $\sim 1 + i\,\phi(x,y)$
  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)



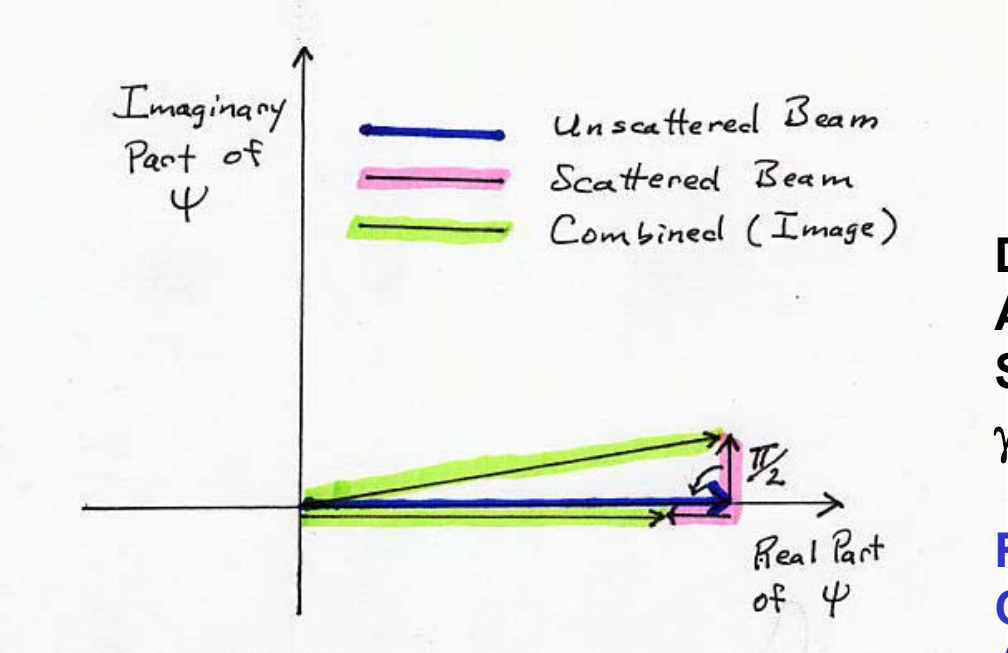
Downing & Jap
PhoE porin image
(unpublished)

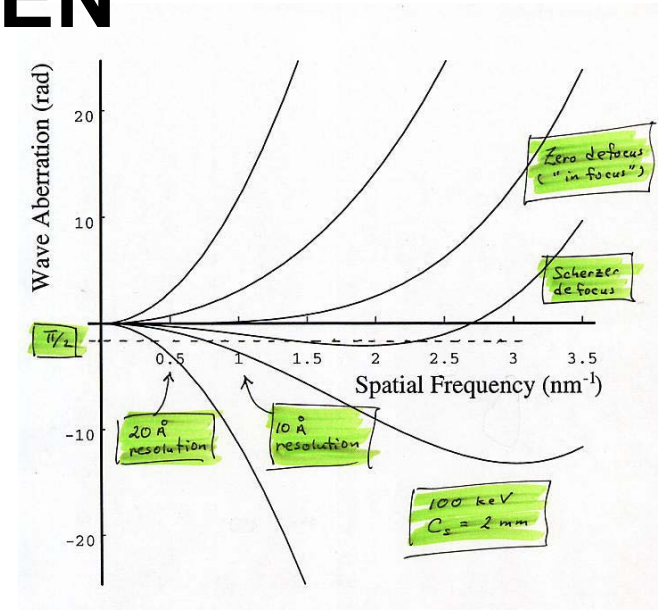


RMG,
Unpublished

PHASE-CONTRAST OBJECTS REQUIRE A 90-DEGREE PHASE SHIFT TO BE SEEN

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





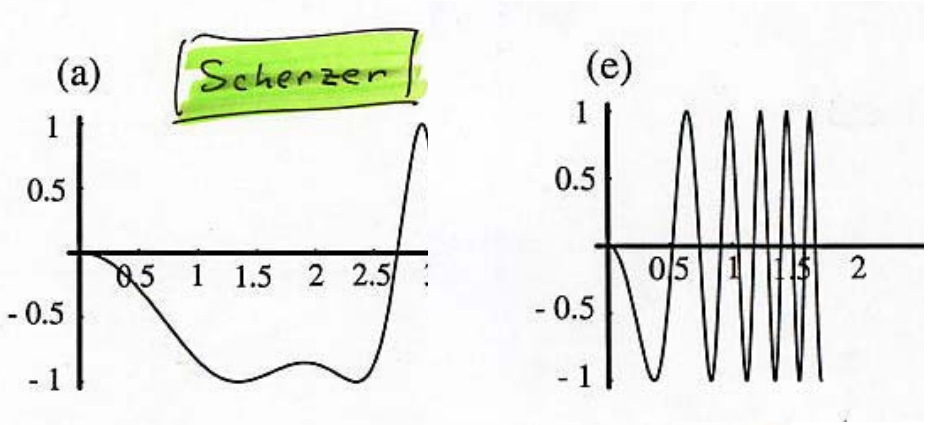
DEFOCUS AND SPHERICAL ABERRATION IMPOSE A PHASE SHIFT

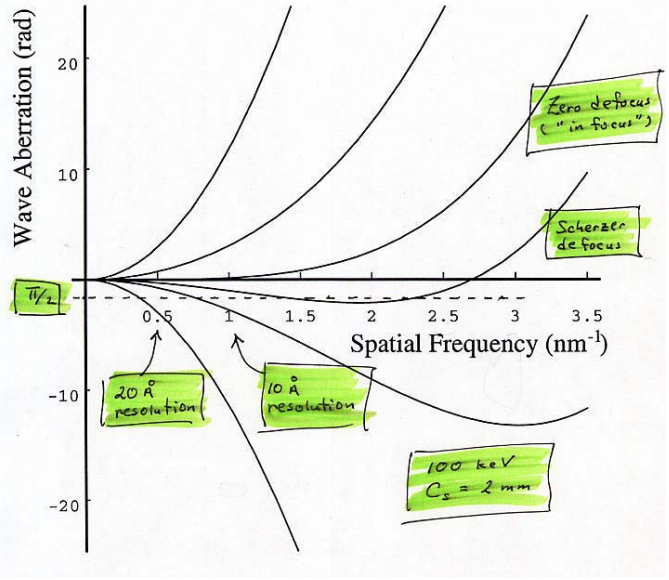
$$\gamma(s) = 2\pi[\, C_s/4\, \lambda^3\, s^4 - \Delta Z/2\, \lambda\, s^2\,]$$

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

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

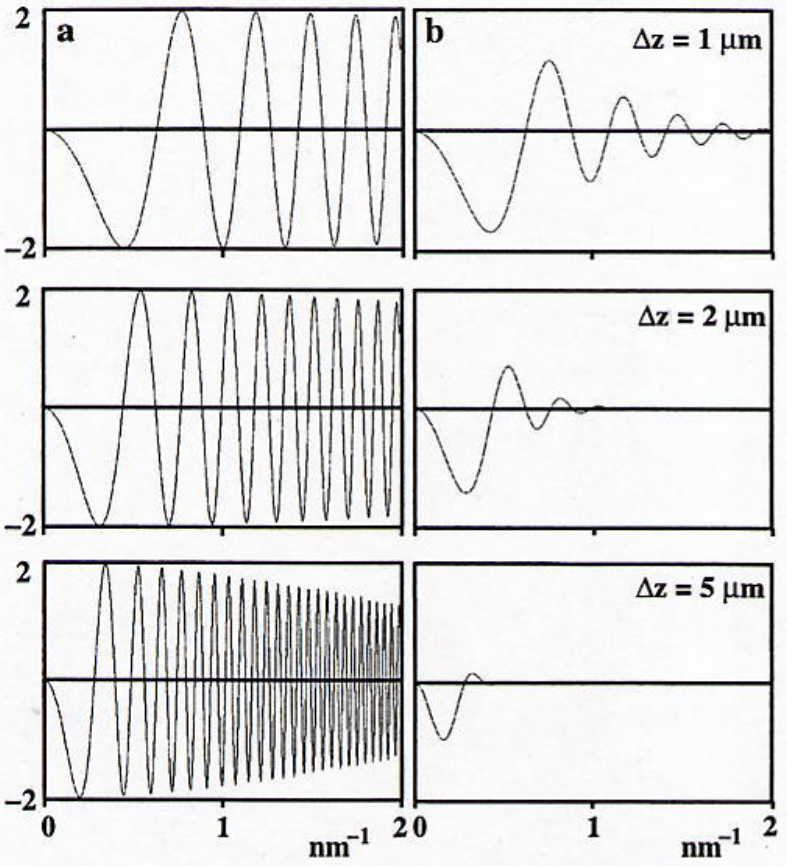
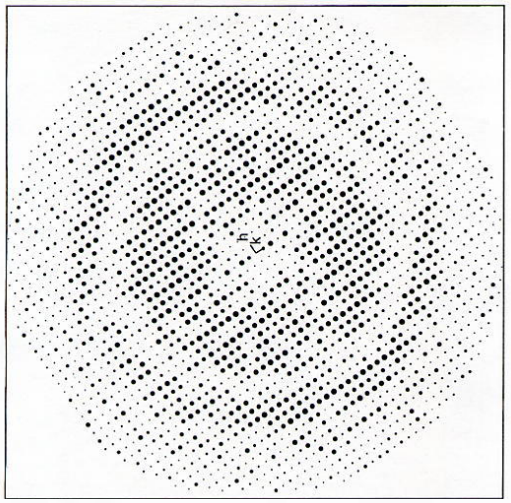
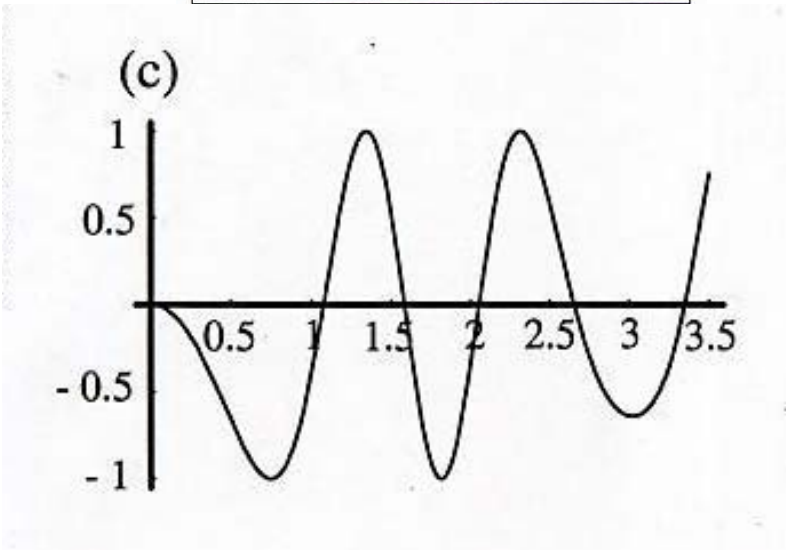


FIG. 4.10. Comparison of transfer functions. FEG versus LaB_6 (a) FEG, $U = 200$ kV (b). LaB_6, $U = 200$ kV. Defocus values 1.0, 2.0, and 5.0 μm, respectively. An energy spread of 0.8 eV (2.0 eV) and an emission angle of 0.02 mrad (0.3 mrad) was assumed for the FEG (LaB_6) cathode.

CONTRAST REVERSAL CAN BE CORRECTED COMPUTATIONALLY

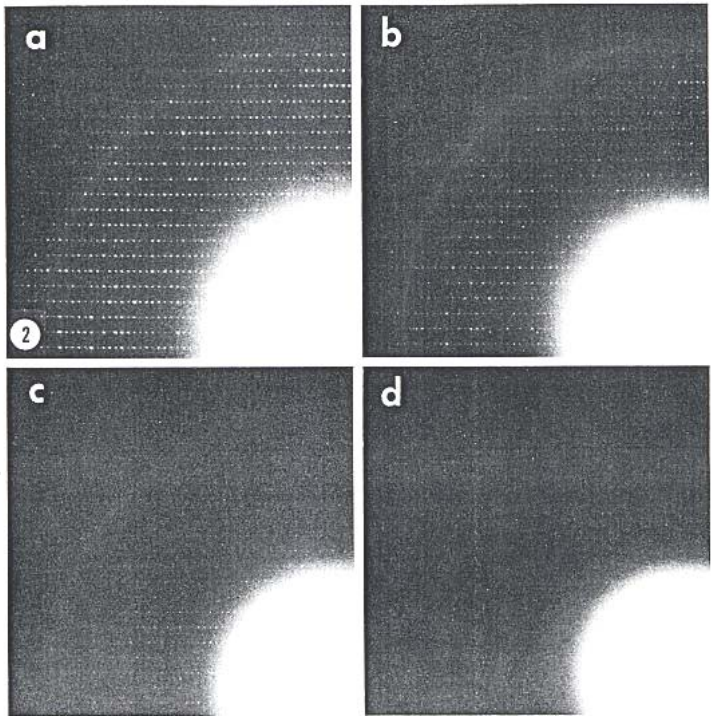
- ONE MUST FIRST SEE (OR PREDICT?) THE LOCATION OF THE "ZEROS" IN THE CTF
  - THEY ARE APPARENT IN THE FOURIER TRANSFORM OF THE TUBULIN CRYSTAL ON THE RIGHT
  - THEY ARE SIMILARLY APPARENT IN AREAS WITH AMORPHOUS CARBON, etc.
- SIMPLY CHANGE THE SIGN OF THE FOURIER TRANSFORM IN "EVEN" ZONES OF THE CTF; 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



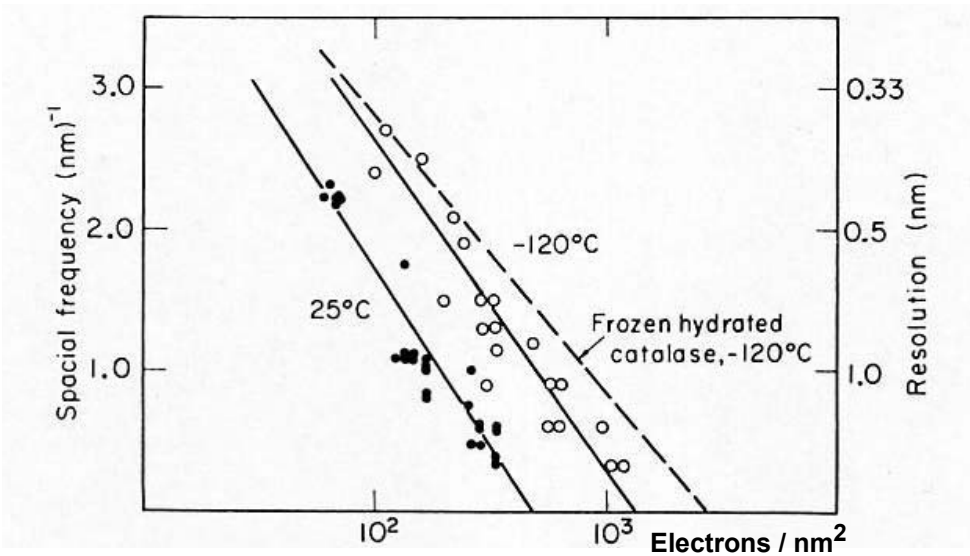


IMAGES MUST BE RECORDED WITH VERY LOW ELECTRON EXPOSURES

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



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

SHOT NOISE LIMITS THE RESOLUTION AT WHICH YOU CAN SEE THINGS

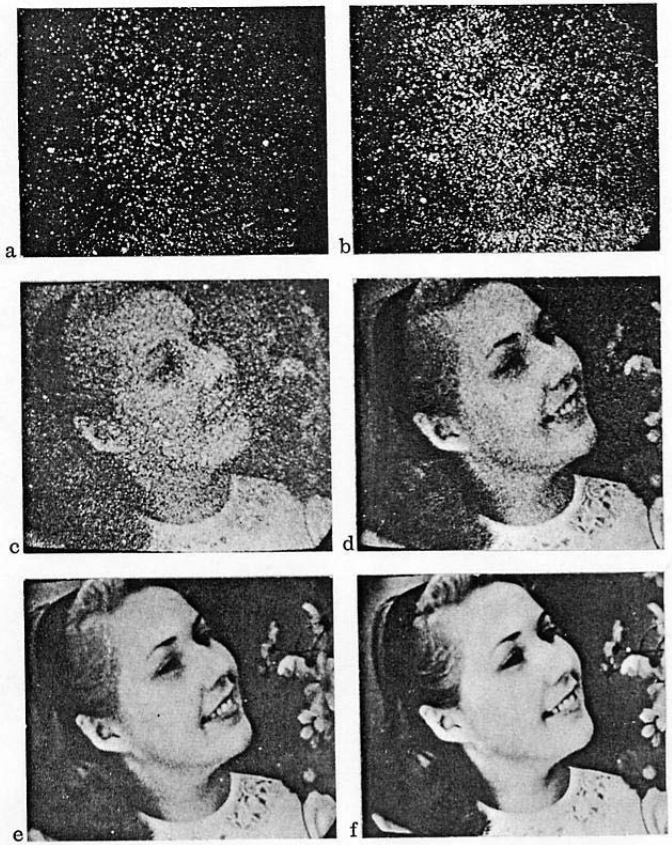
- ALBERT ROSE DETERMINED A QUANTITATIVE RELATIONSHIP BETWEEN FEATURE SIZE AND VISUAL DETECTABILITY:

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

  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 ORDERED ASSEMBLIES

- **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**

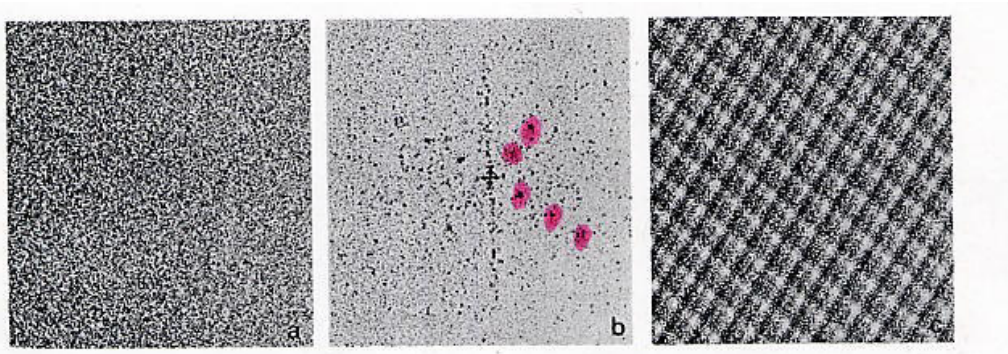


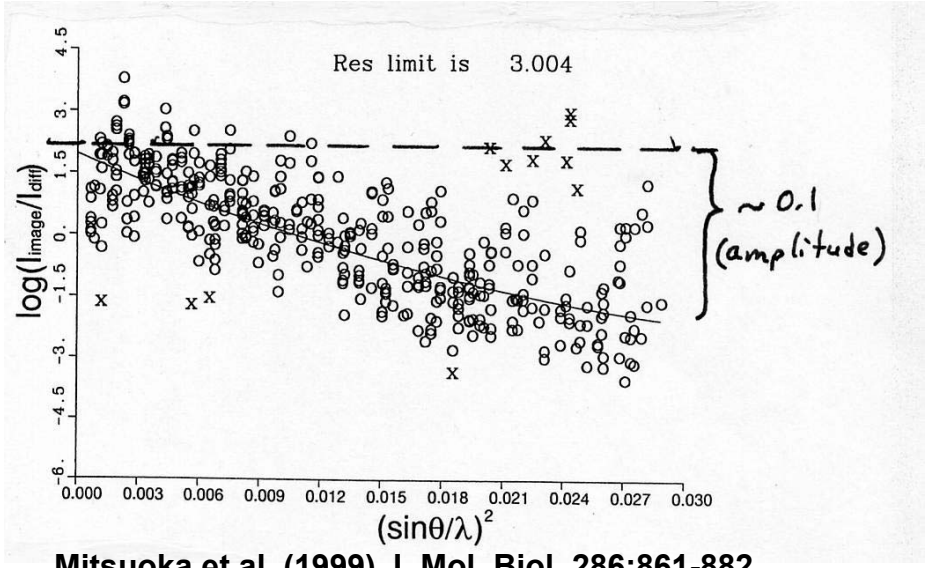
Fig. 5. (a) The Z-modulation display of a statistically noisy image of a carbon replica of an optical diffraction cross grating, recorded with an image intensifier; (b) power spectrum of the statistically noisy image; (c) the spatially averaged image.

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 HAVE EXPECTED IT TO BE

- 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

THE POWER OF SINGLE-PARTICLE, REAL-SPACE AVERAGING WILL ONLY KEEP GETTING BETTER!