FUNDAMENTALS OF ELECTRON MICROSCOPY

THEORY

NRAM PRACTICAL COURSE

NOV. 2-10, 2005

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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

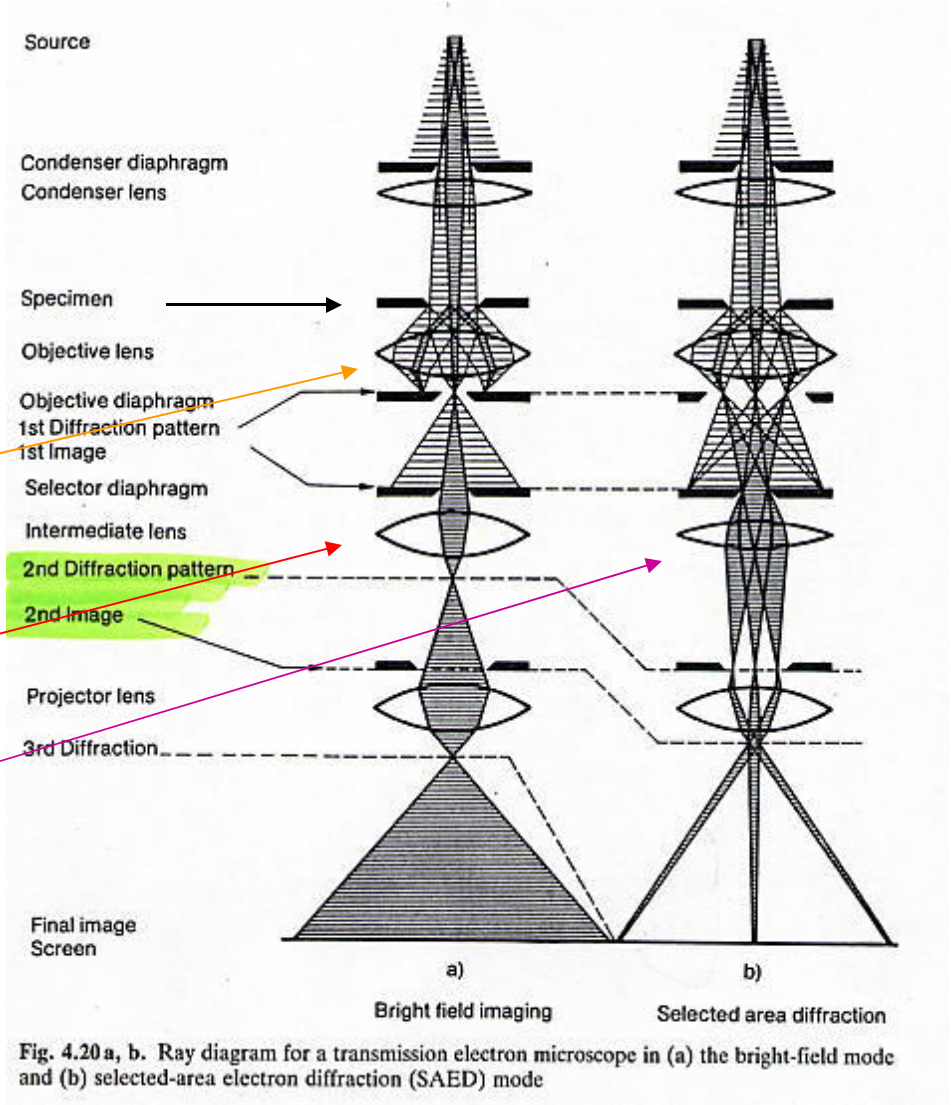


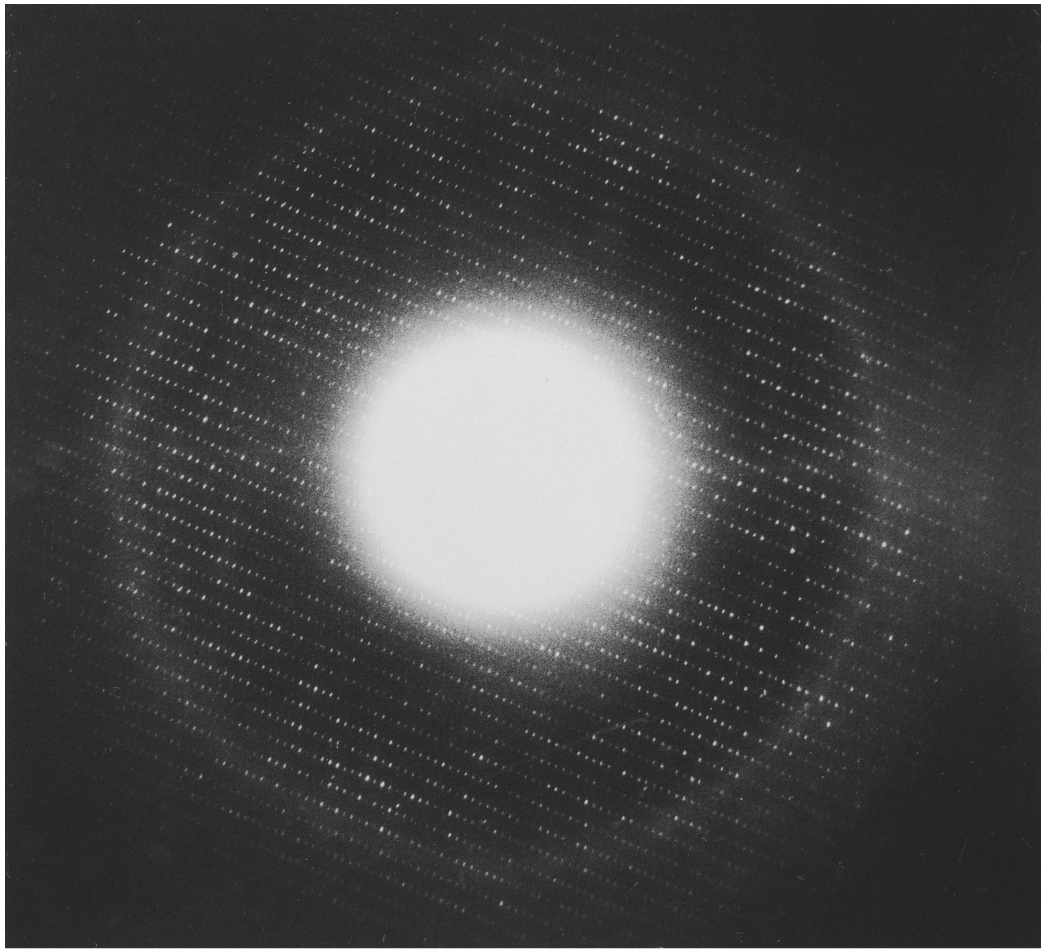
Fig. 4.20 a, b. Ray diagram for a transmission electron microscope in (a) the bright-field mode and (b) selected-area electron diffraction (SAED) mode

Reimer (1989) Transmission EM [Springer]

ELECTRONS REALLY ARE WAVES

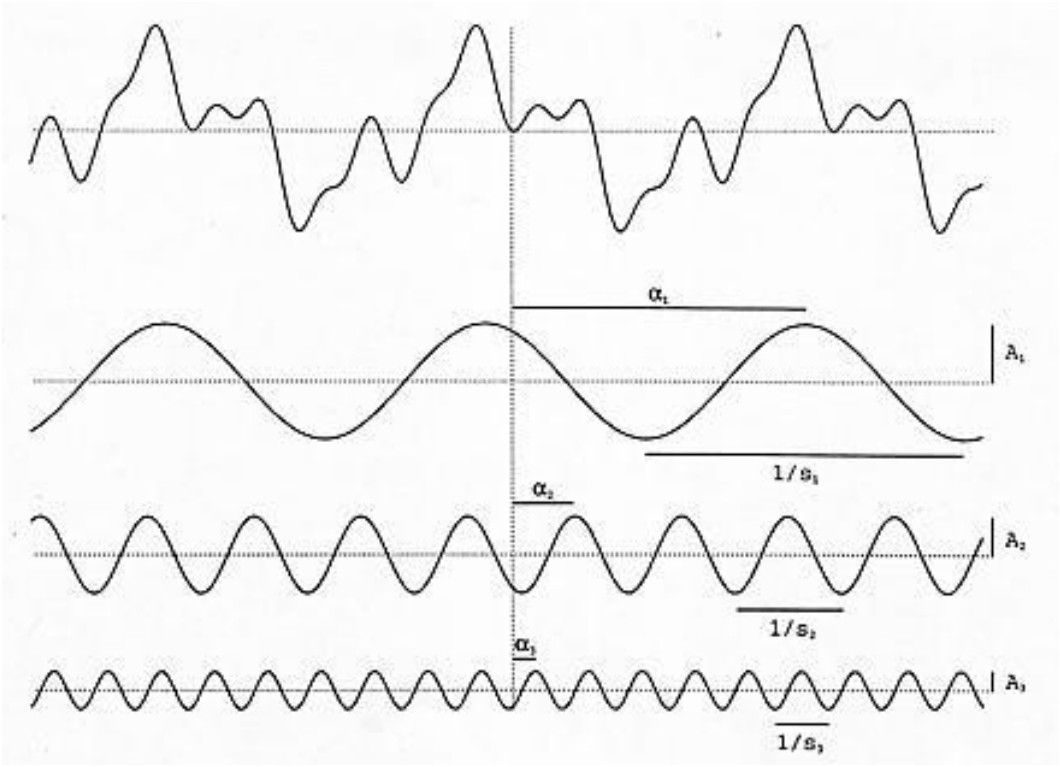
— AND DIFFRACTION IS IMPORTANT IN EM

- **Electrons produce diffraction patterns**
  - **just like those produced by x-rays**
- **Lens aberrations and defocus produce phase contrast**
  - **even though the intensity transmitted through the specimen is *almost* constant**
- **Heads up - electrons are also a flux of ionizing radiation …**



Electron Diffraction Pattern of Catalase

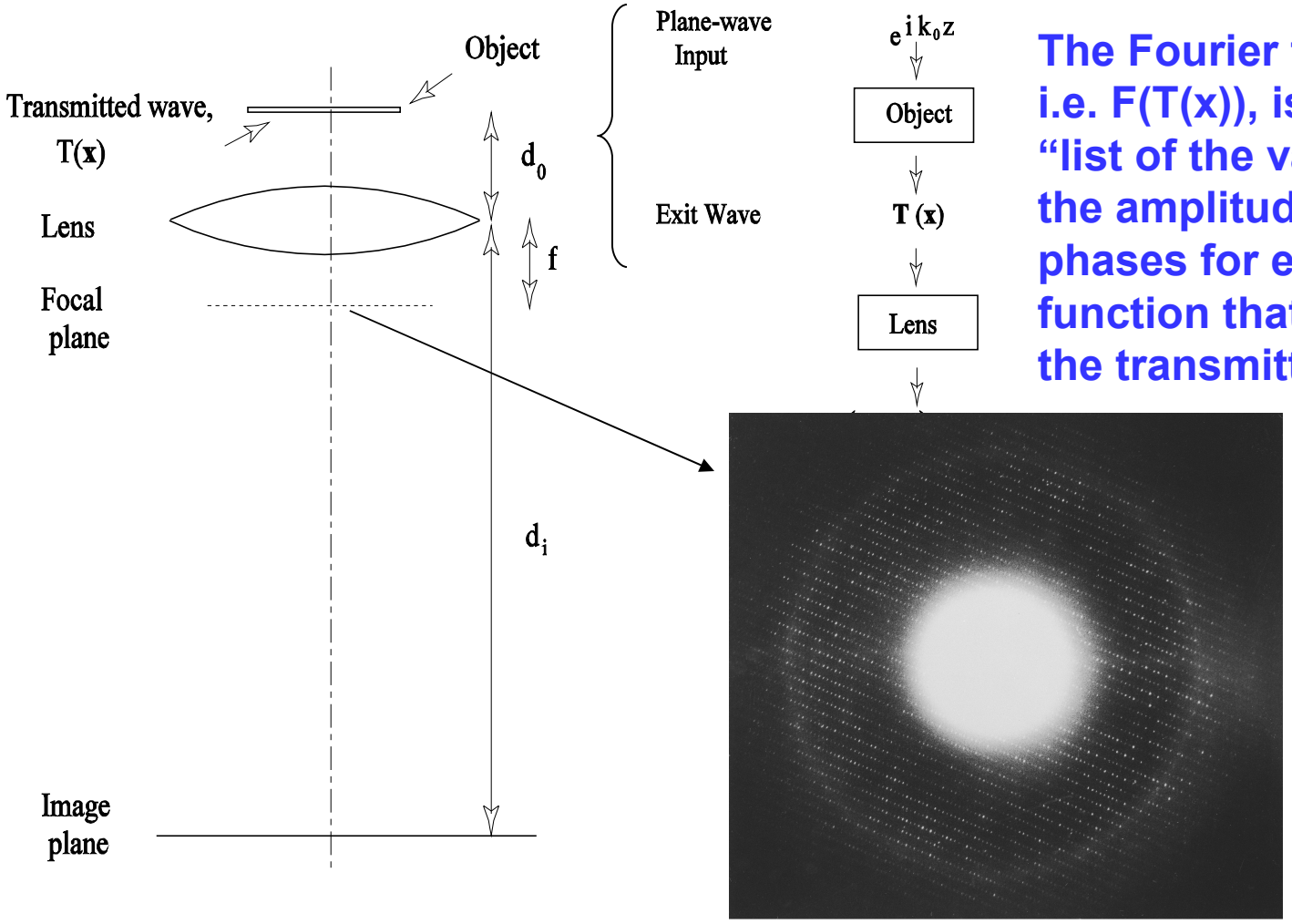
EACH SCATTERED BEAM IN THE DIFFRACTION PATTERN CONTRIBUTES A SINE-FUNCTION IN THE IMAGE



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

- Each sine-function has its own amplitude and phase
  - Larger scattering angles correspond to higher resolution
- The sine-functions add up to give a complicated function
  - e.g. the image of a molecule
- Crystals help to explain these concepts
  - but everything remains the same when there is no crystal

THE SCATTERED ELECTRON WAVE FUNCTION IS THE FOURIER TRANSFORM OF THE TRANSMITTED ELECTRON WAVE



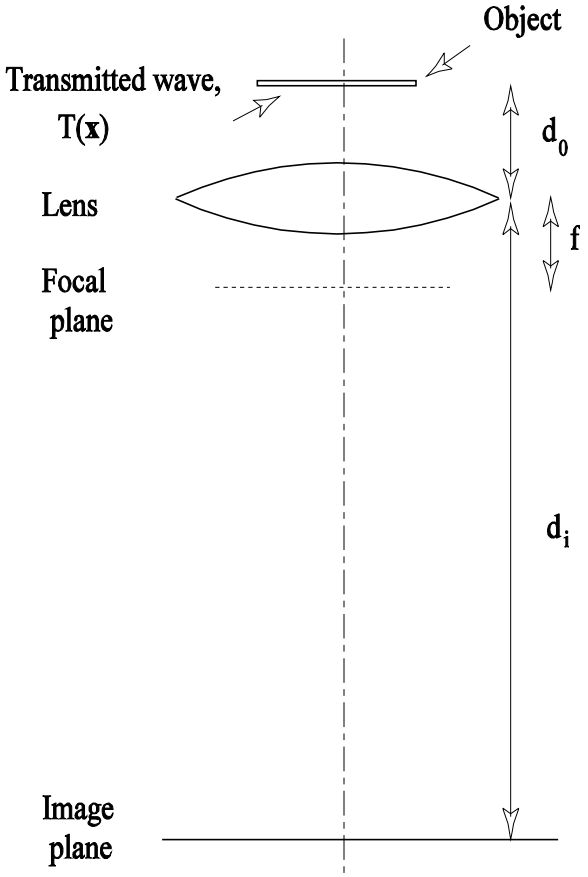
The Fourier transform, i.e. F(T(x)), is simply a "list of the values of the amplitudes and the phases for every sine function that makes up the transmitted wave"

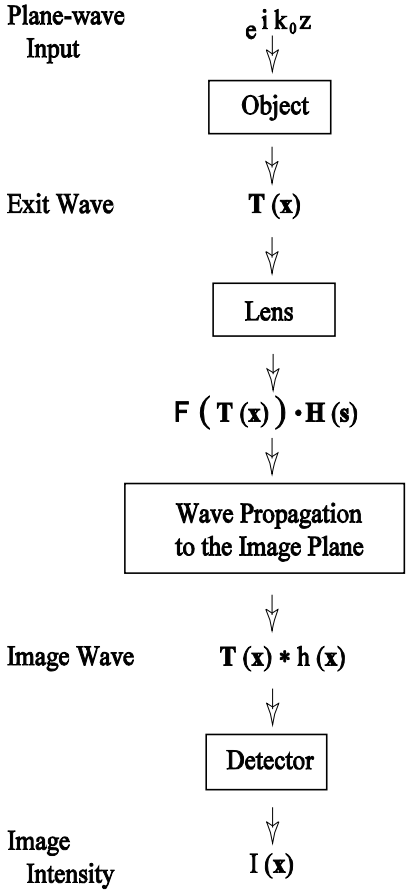
ABBE'S THEORY OF IMAGE FORMATION

APPLIES TO ELECTRON WAVES JUST AS IT DOES TO LIGHT

- **The scattered wave is the Fourier transform of the wave function transmitted through the object**
- **The lens of a microscope inevitably applies some aberration function, H(s), to the scattered wave**
- **The wave function in the image is the *INVERSE* operation (inverse Fourier transform)**
  - **But now the inverse step is applied to the *aberrated* wave function, so the result is not the same as the original, transmitted wave**
- **The image intensity is the square of the image wave function**

THE IMAGE WAVE IS THE INVERSE FOURIER TRANSFORM OF THE SCATTERED (AND ABERRATED) ELECTRON WAVE

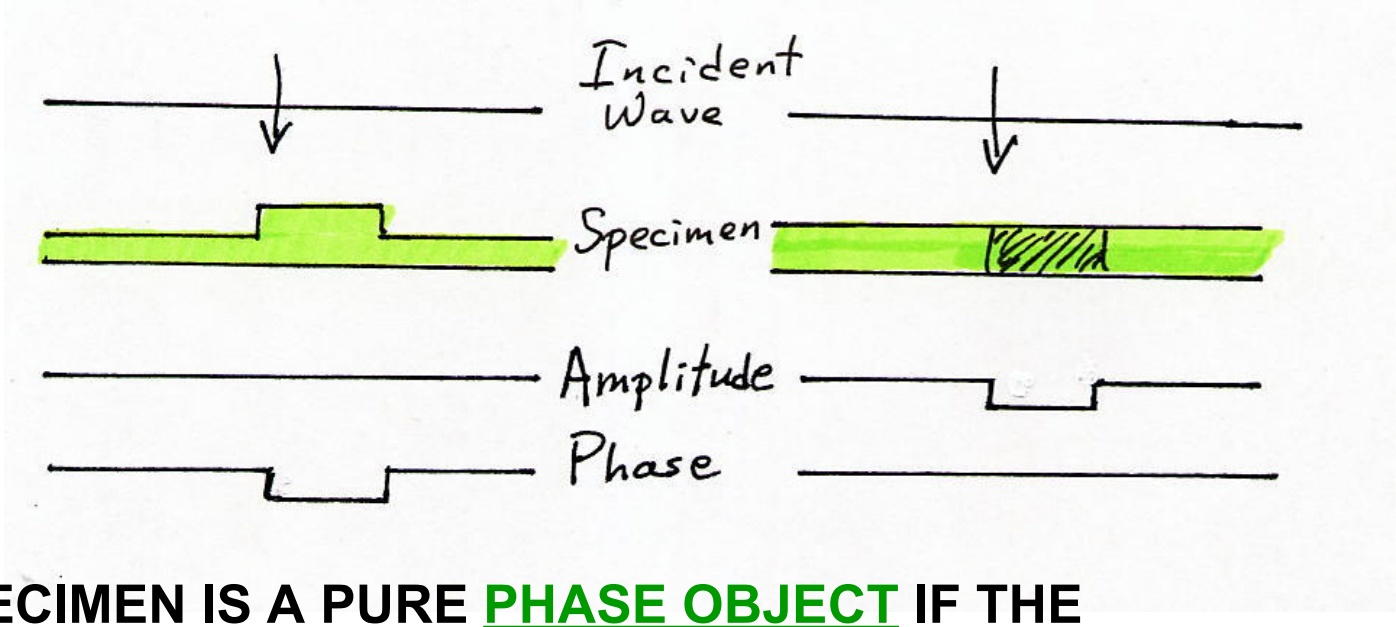




H(s) represents the wave aberration (and the effect of a limited lens-aperture)

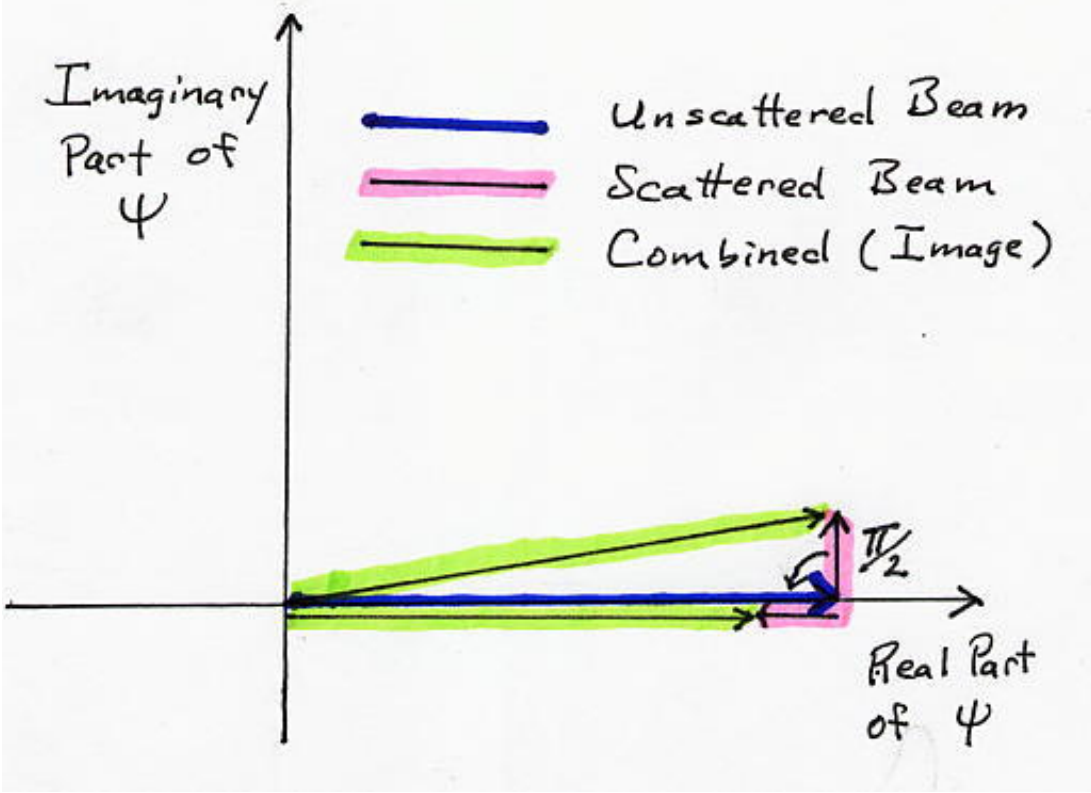
h(x) is the *point spread function* of the image wave function –
It is the inverse Fourier transform of H(s)

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**

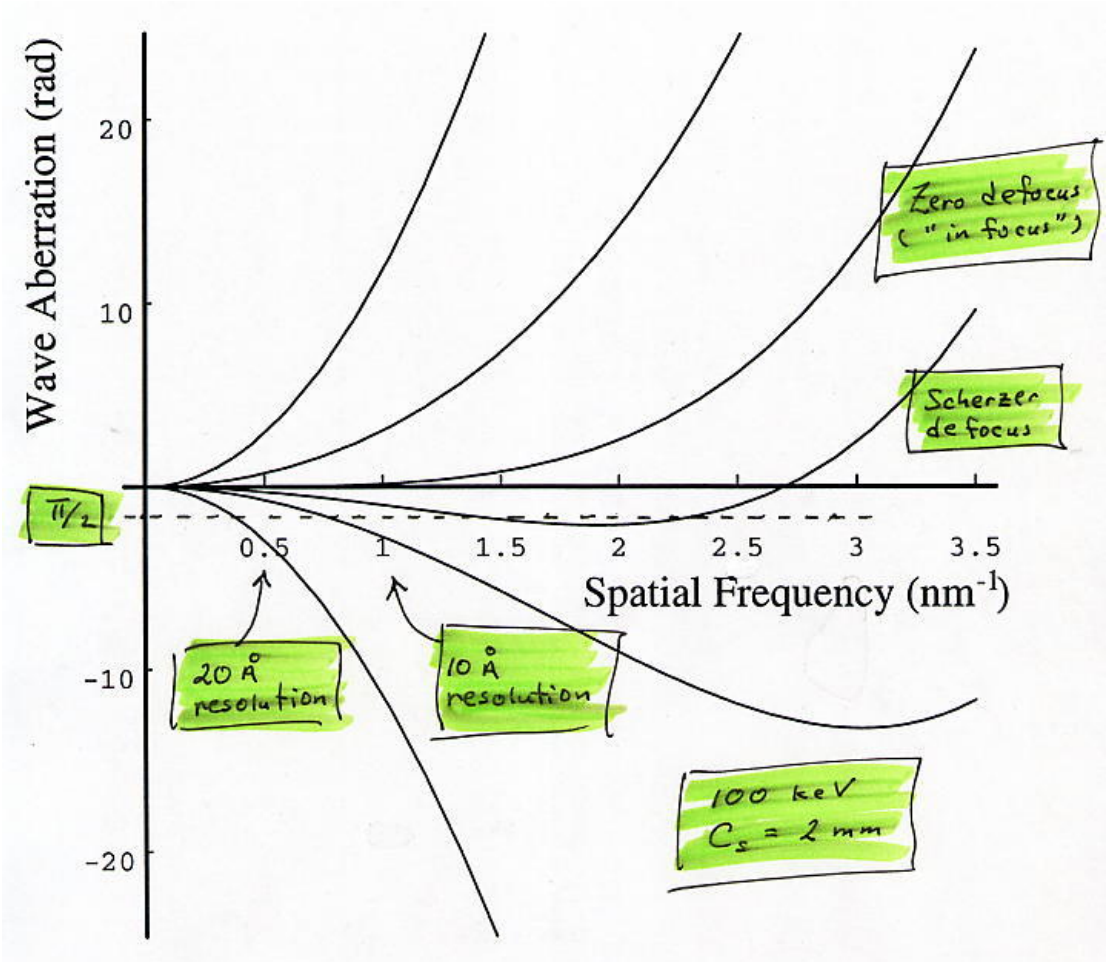
PHASE-CONTRAST OBJECTS REQUIRE A π/2 PHASE SHIFT TO BE SEEN



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

DEFOCUS AND SPHERICAL ABBERATION CHANGE THE PHASE OF THE SCATTERED ELECTRON WAVE

- **Defocus and spherical aberration combine to change the phase**
  - **just as happens in the phase-contrast light microscope**
- **The "wave aberration" is not a uniform 90-degree phase-shift as it is in the Zernicke phase-contrast microscope, however**



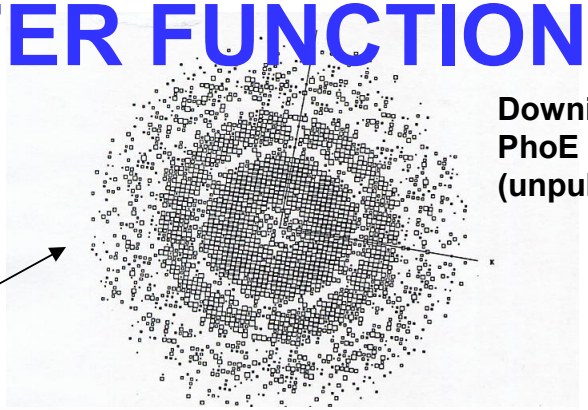
H(s) = exp i{γ(s)}, and γ(s) = 2π[C_sλ^3/4 s^4 – ΔZλ/2 s^2]

PHASE CONTRAST IS USUALLY DESCRIBED IN TERMS OF A CONTRAST TRANSFER FUNCTION

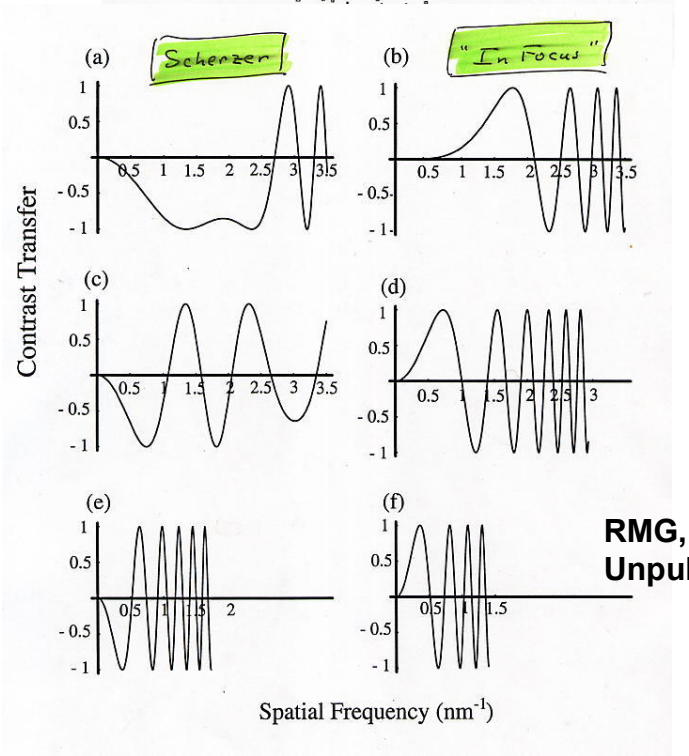
- **THE FOURIER TRANSFORM OF THE IMAGE INTENSITY IS PROPORTIONAL TO**

  Sin γ(s) {*FT* [object]}

- **SIN γ(s) is itself the *FT* of a point spread function for the image <u>intensity</u>, which is derived from h(x) mentioned in slide #7**
- **SIN γ(s) IS KNOWN AS THE PHASE CONTRAST TRANSFER FUNCTION (CTF)**



Downing & Jap PhoE porin image (unpublished)

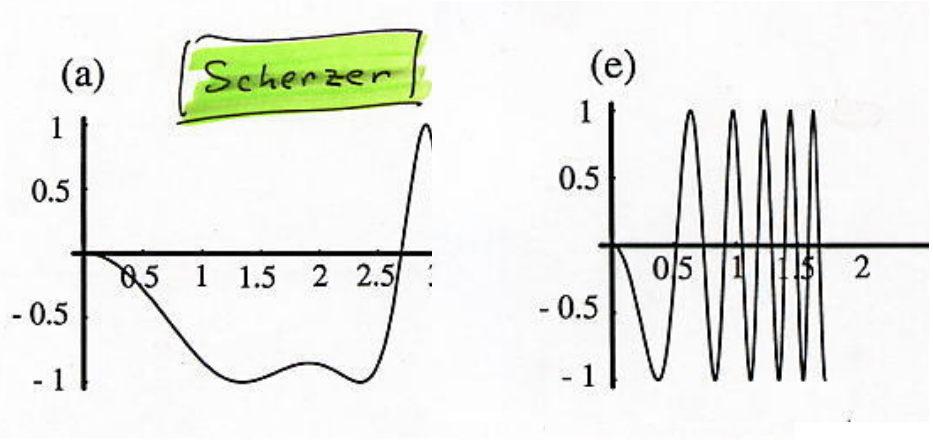


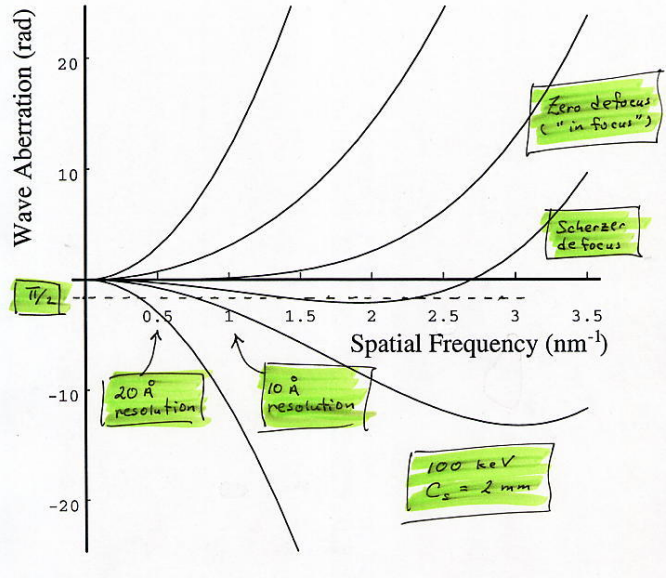
RMG, Unpublished

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**
- **THE RAPID CONTRAST REVERSALS ARE DUE TO THE STEEP INCREASE IN**

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

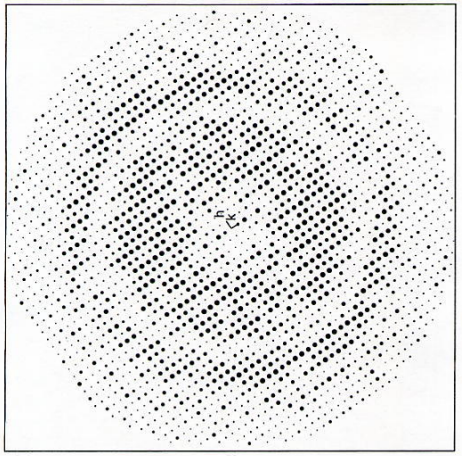




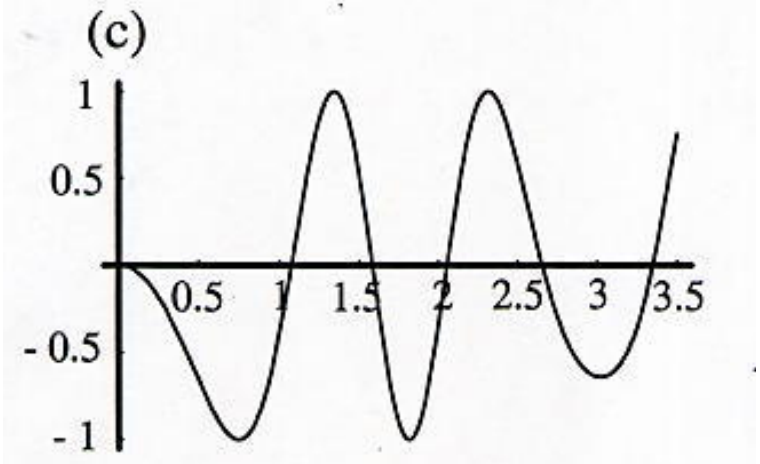
IMAGES LOOK "ROUGHLY" LIKE A PROJECTION OF THE OBJECT

COMPUTATIONAL RESTORATION IS NECESSARY FOR QUANTITATIVE WORK, HOWEVER

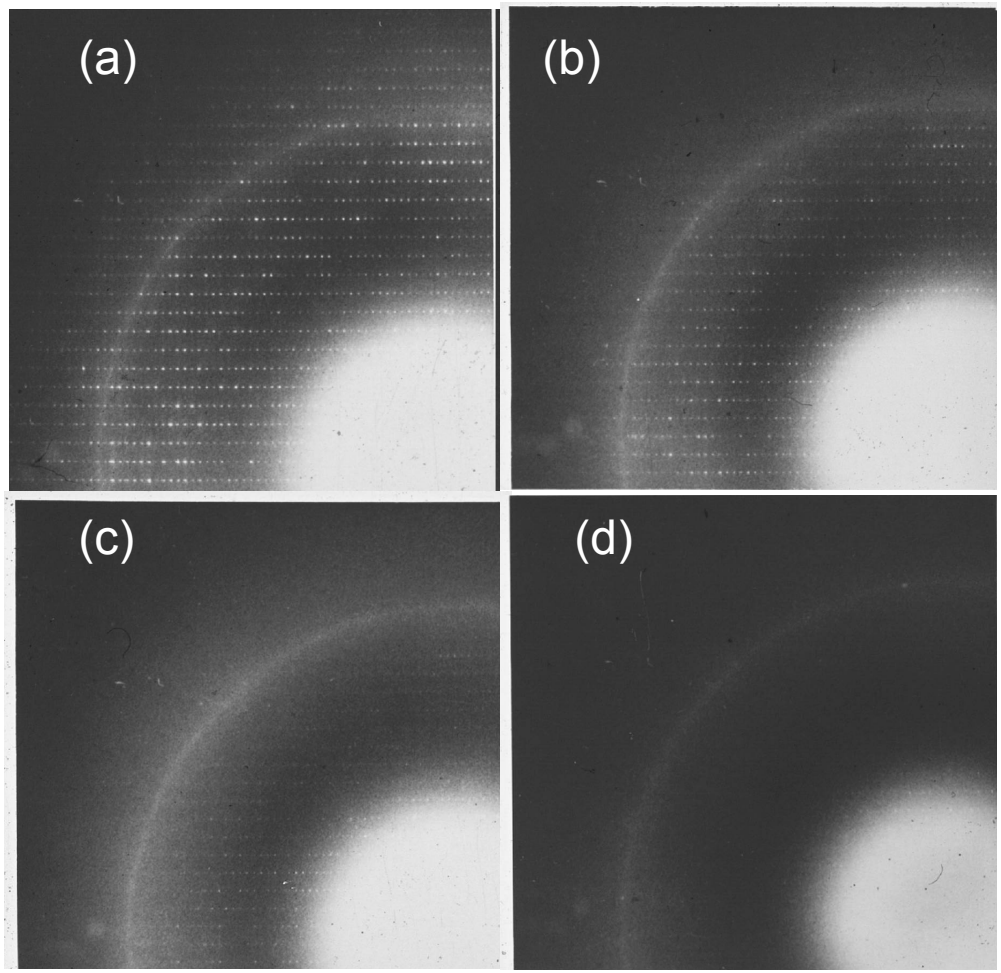
- ONE MUST FIRST LOCATE THE "ZEROS" IN THE CTF
  - THEY ARE APPARENT IN THE FOURIER TRANSFORM OF THE TUBULIN CRYSTAL ON THE RIGHT
  - THEY ARE SIMILARLY APPARENT IN AREAS WITH AMORPHOUS CARBON, etc.
- SIMPLY CHANGE THE SIGN OF THE FOURIER TRANSFORM IN "EVEN" ZONES OF THE CTF
- BE AWARE THAT ASTIGMATISM INVALIDATES APPLICATION OF CIRCULAR SYMMETRY
- COMPENSATION FOR THE AMPLITUDE OF THE CTF AND THE ENVELOPE FUNCTION IS ALSO POSSIBLE DURING COMPUTATION



Courtesy of Ken Downing



RADIATION DAMAGE: ELECTRONS ARE A FLUX OF IONIZING RADIATION



- **Biological macromolecules are destroyed by radiation damage**
  - **Remember – there is a one-to-one connection between spots in the scattered wave and sine-functions in the image**
- **Images must thus be recorded with "safe" electron exposures**
  - **< 10e/A^2 at 100 keV**
  - **< 20e/A^2 at 300 keV**
- **Bubbling sets in at doses about 3X higher than that**

SAFE ELECTRON EXPOSURES RESULT IN INSUFFICIENT STATISTICAL DEFINITION OF HIGH-RESOLUTION FEATURES

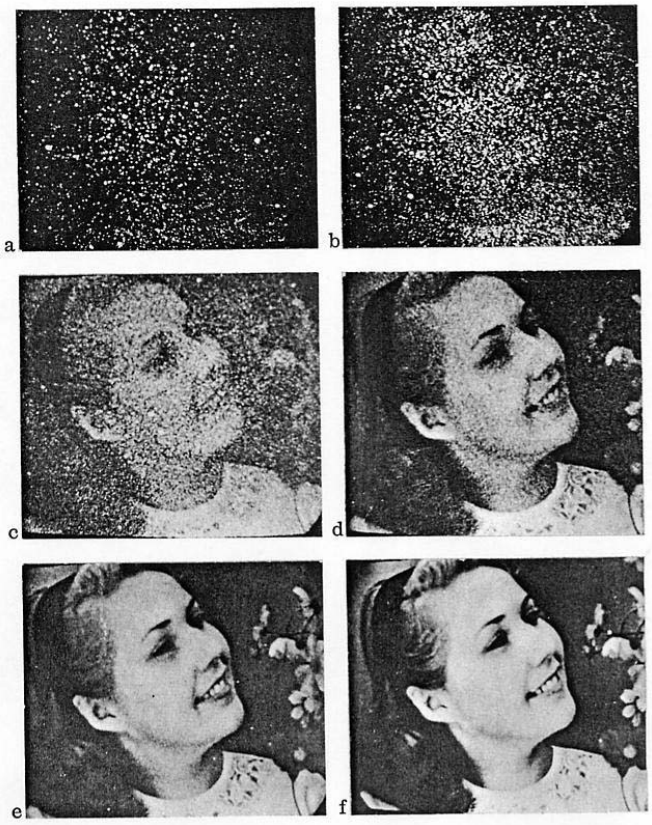
- **ALBERT ROSE DETERMINED A QUANTITATIVE RELATIONSHIP BETWEEN FEATURE SIZE AND VISUAL DETECTABILITY:**

  $$d\,C > 5 / (N)^{1/2}$$

  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

CRYSTALS MAKE IT "EASY" TO AVERAGE LARGE NUMBERS OF INDEPENDENT IMAGES

- **AVERAGING CAN BE DONE IN REAL SPACE**
- **BUT IT IS EVEN EASIER TO DO IT IN FOURIER SPACE**
  - **INFORMATION ABOUT FEATURE 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**

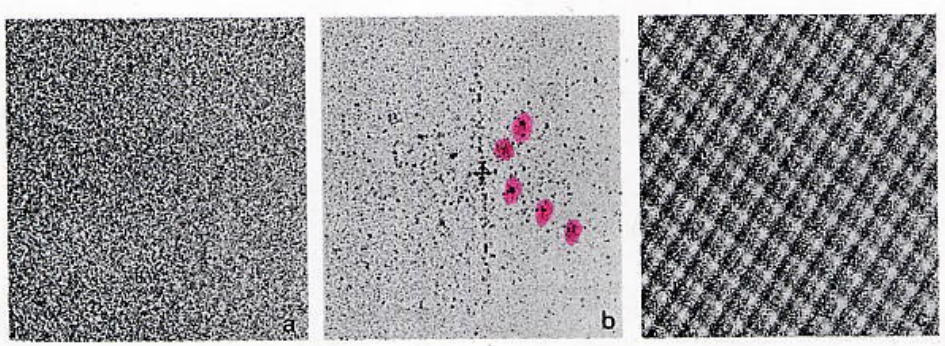


Fig. 5. (a) The Z-modulation display of a statistically noisy image of a carbon replica of an optical diffraction cross grating, recorded with an image intensifier; (b) power spectrum of the statistically noisy image; (c) the spatially averaged image.

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**

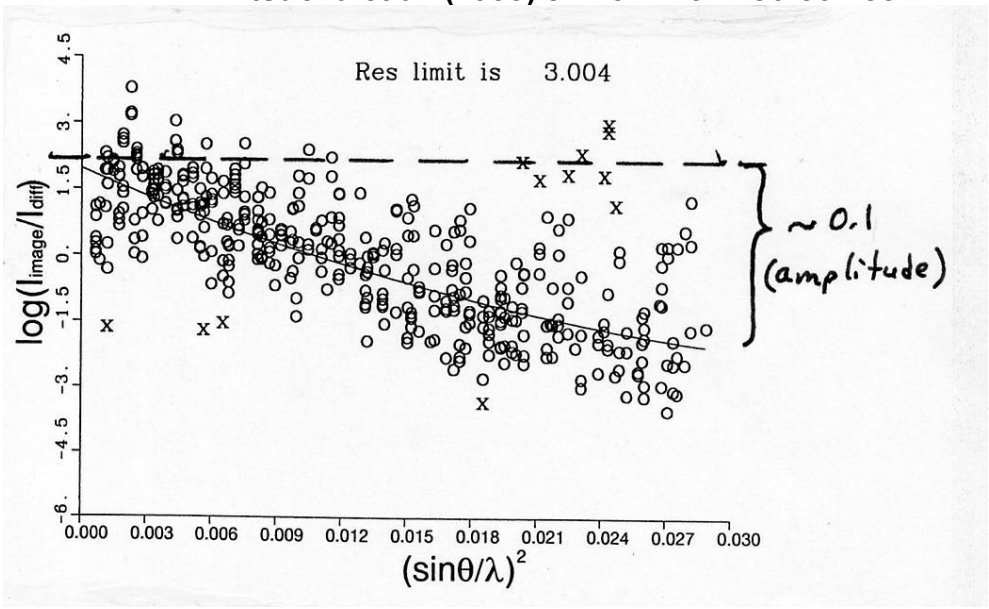
CRYSTALS ARE NOT NECESSARY

- ALIGN IDENTICAL PARTICLES IN IDENTICAL VIEWS BY CROSS CORRELATION
- 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 [HENDERSON (1995) QUART. REV. BIOPHY.]
- COMPUTATIONAL ALIGNMENT IS EQUIVALENT TO CRYSTALLIZATION *IN SILICO*

MOST IMAGES CAPTURE ONLY 10% (OR LESS) OF THE SIGNAL THAT IS IN THE SCATTERED WAVE FUNCTION

- **BEAM-INDUCED MOVEMENT IS THOUGHT TO BE THE CURRENT LIMITATION**

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



- **CONTRAST *CAN BE* OCCASIONALLY CLOSE TO "WHAT IT SHOULD BE" IN CURRENTLY RECORDED DATA, HOWEVER**

  YONEKURA/NAMBA RESULT REQUIRED SELECTION OF PARTICLE-IMAGES THAT WERE MUCH BETTER THAN THE AVERAGE

EVEN "ROUTINE" CRYO-EM OF BIOLOGICAL MACROMOLECULES IS CURRENTLY *BRILLIANT*

- **Chain-trace models by 2-D electron crystallography**
- **Accurate docking of atomic models of components into large, macromolecular complexes**
- **Whole-cell tomographic imaging at ~5 nm resolution**

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

- **Automated data-collection will make it trivial to collect data sets of 10^5 to 10^6 particles**
- **Computer speed is keeping up with the size of data sets and the demands of higher resolution (well, at least we are trying to make it so …)**
- ***SOMEONE* is bound to solve the problem of beam-induced movement … (and when that happens, watch out for what cryo-EM will be able to do!)**