IN-FOCUS PHASE CONTRAST:

EXPECTED IMPROVEMENTS (THE GOOD)
REQUIRED OPTICS (THE BAD)
RESEARCH CHALLENGES (THE UGLY)

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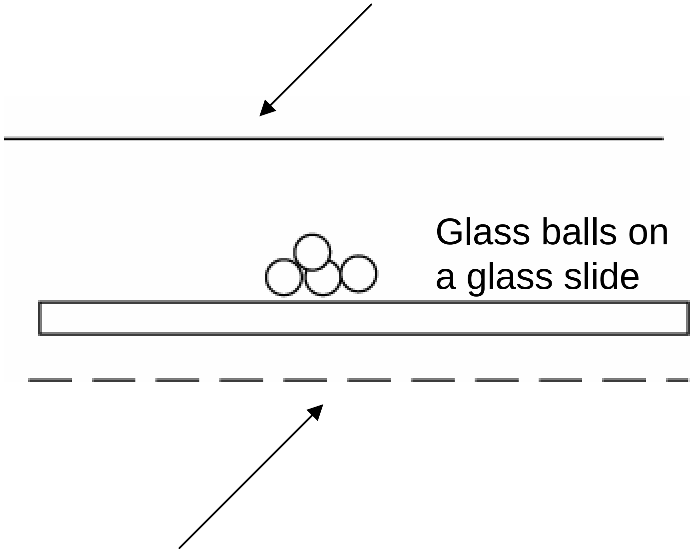
BIOLOGICAL MACROMOLECULES ARE WEAK PHASE OBJECTS

- **Electrons are not appreciably absorbed in thin biological specimens**
- **The intensity transmitted through the specimen thus shows "no" contrast**
- **There is, however, substantial elastic scattering**
  - **This is due to the fact that the phase of the exit wave is spatially modulated (no longer a plane wave)**

Light-microscope Analogy

Uniform incident Intensity and phase



Uniform transmitted Intensity, but variable optical path length (i.e. variable phase)

BUT DO WE REALLY NEED PHASE-CONTRAST OPTICS?

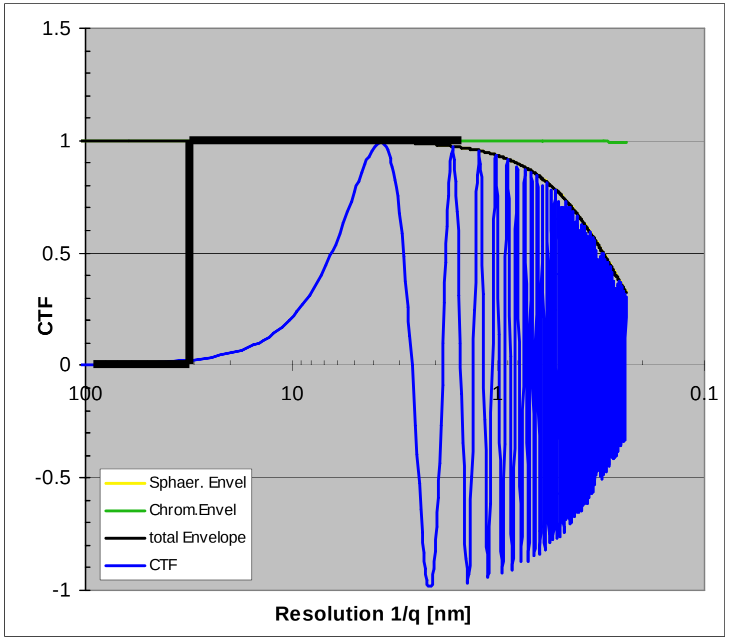
For a perfectly magnified image of the exit wave, "… the phase object is absolutely invisible 'in the ideal case.' Of course the practical microscopist has never been content with this; as a matter of fact, he has never found it out! Without realizing it, he has always turned the fine adjustment – that is, put the object a little out of focus – in order to see the tricky transparent details."

F. Zernike (1955) How I discovered phase contrast. Science 121:345-349 (Nobel acceptance speech)

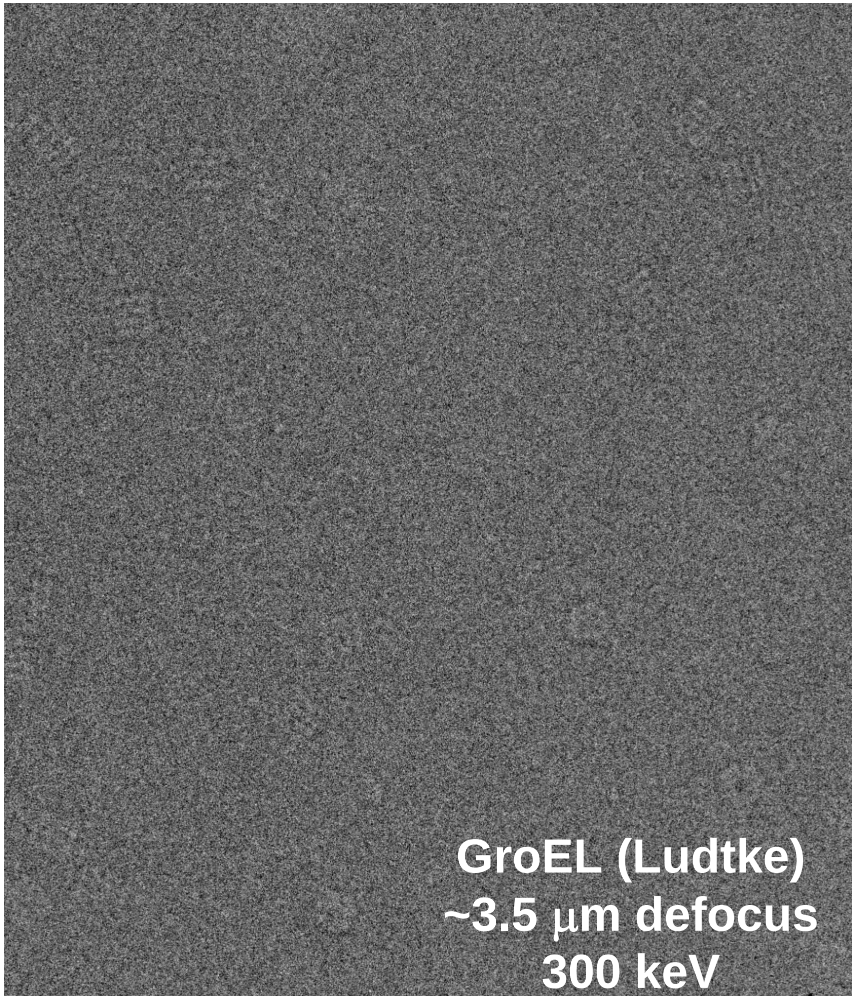
In the same paper Zernike also wrote "How quick we are to learn – that is, to imitate what others have done or thought before – and how slow to understand – that is, to see the deeper connections. Slowest of all, however, are we … in applying old ideas to a new field."

IMAGE CONTRAST COULD BE INCREASED BY A LARGE FACTOR; CORRUPTION OF HIGH-RESOLUTION FEATURES COULD BE ELIMINATED

Contrast transfer oscillates when objective-lens defocus is the main source of phase contrast



Whereas in-focus phase contrast should produce a flat contrast-transfer function



IN-FOCUS PHASE CONTRAST REALLY WORKS AS EXPECTED !

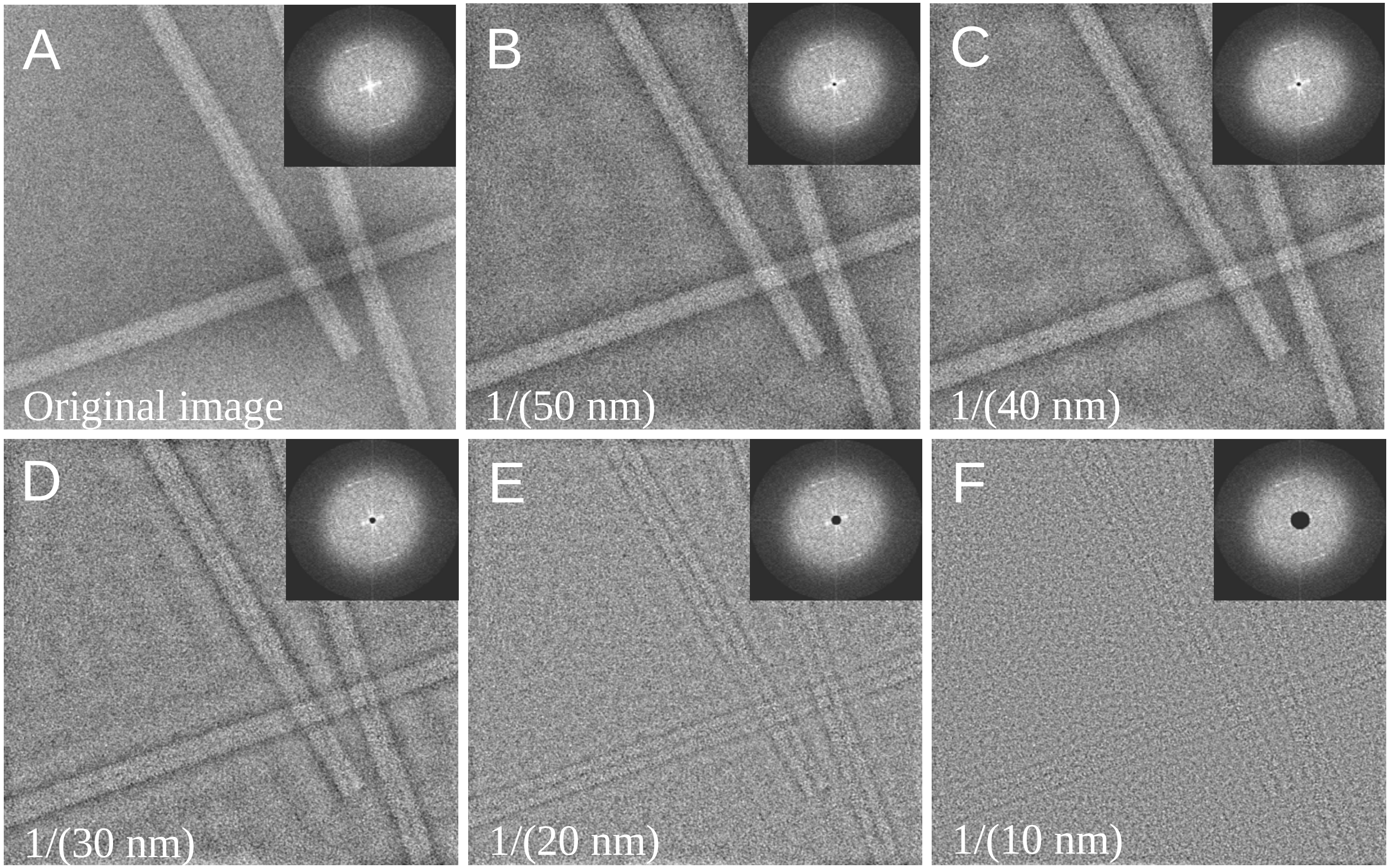
Comparison on the left is courtesy

K. Nagayama & R. Danev,

Okazaki Center for Integrative Bioscience

- **GroEL, unpublished**
- **Objective aperture covered with a thin carbon film with an 0.25 μm-radius hole**
  - **300 keV**
  - **f = 5 mm**

BUT IT REQUIRES THAT THE "CUT-ON" FREQUENCY BE AT LEAST 1/(30nm)



D. Typke, unpublished

THE GOOD
(EXPECTED IMPROVEMENTS)

- **It should be easy to box particles as small as 200 kDa**
  - **Since images will be close to focus, correcting a rapidly oscillating CTF at high resolution is no longer a limitation**
- **Information “delocalization” is no longer a problem**
  - **Caveat: it still is a problem at very high resolution, due to spherical aberration**
- **It may be possible to subclassify particles in a heterogeneous population**
  - **With greater accuracy and**
  - **With greater sensitivity (smaller differences)**

WHAT LAB, GIVEN THE CHOICE, WOULD ACTUALLY BUY THIS IF THEY COULD HAVE THAT?

Every lab that is currently doing Cryo-Bio EM will be in the queue to purchase a microscope that is capable to deliver "that"

Indeed, like light microscopes, ALL biological research microscopes will be sold with Zernike phase contrast as standard equipment

MAJOR IMPROVEMENTS ARE EXPECTED IN BOXING AND CLASSIFYING PARTICLES

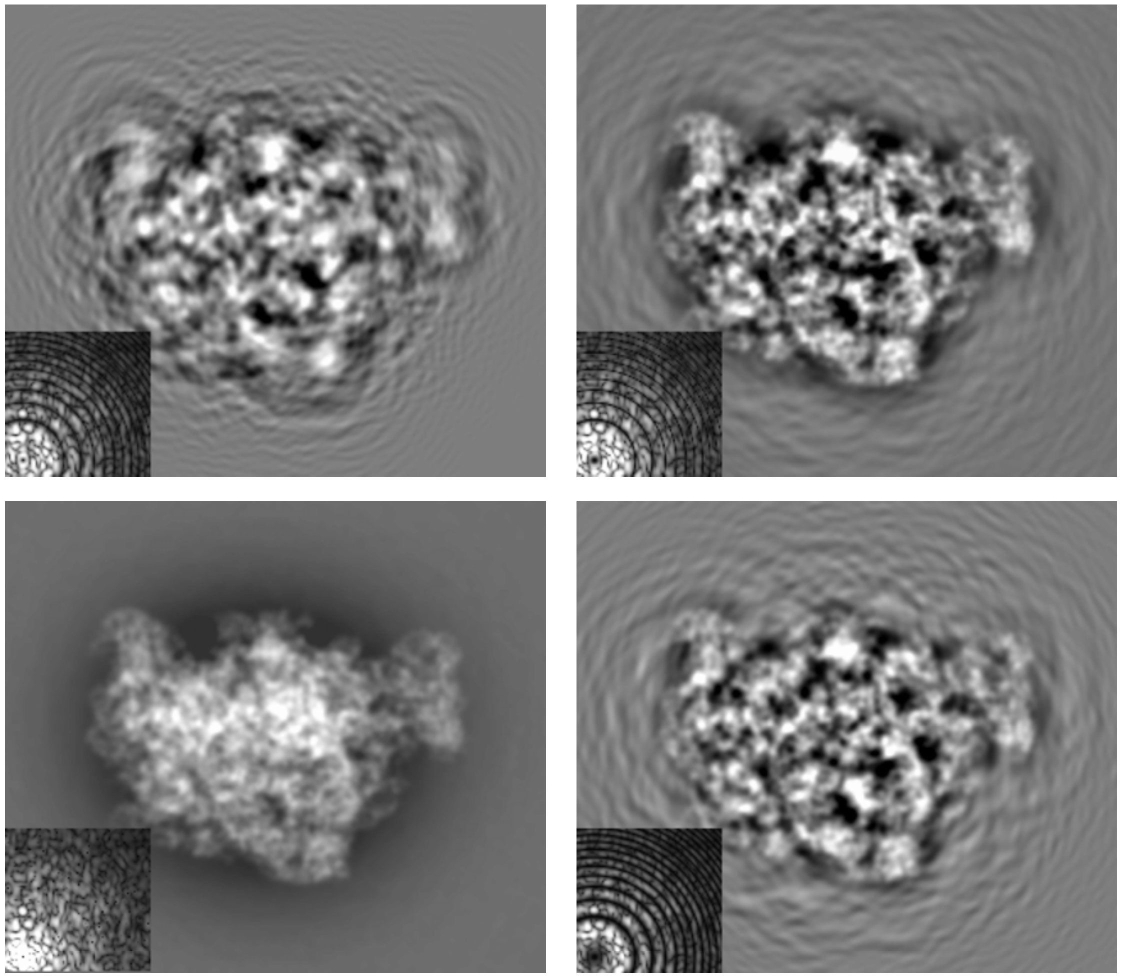
- Particles as small as ¼ the size of GroEL
  i.e. Mr ~200 k
  should be easy to identify and “box”
  without the limitations of CTF oscillations

- Subtle conformational subclasses will be MUCH easier to identify

K. Nagayama & R. Danev, Okazaki Center for Integrative Bioscience

DELOCALIZATION IS STILL NOT FULLY RESTORED, EVEN BY WIENER-FILTER CTF-CORRECTION

Simulation using a large macromolecular complex with coordinates taken from the PDB



Initial image
3 μm defocus

Image restoration achieved for S/N = 3

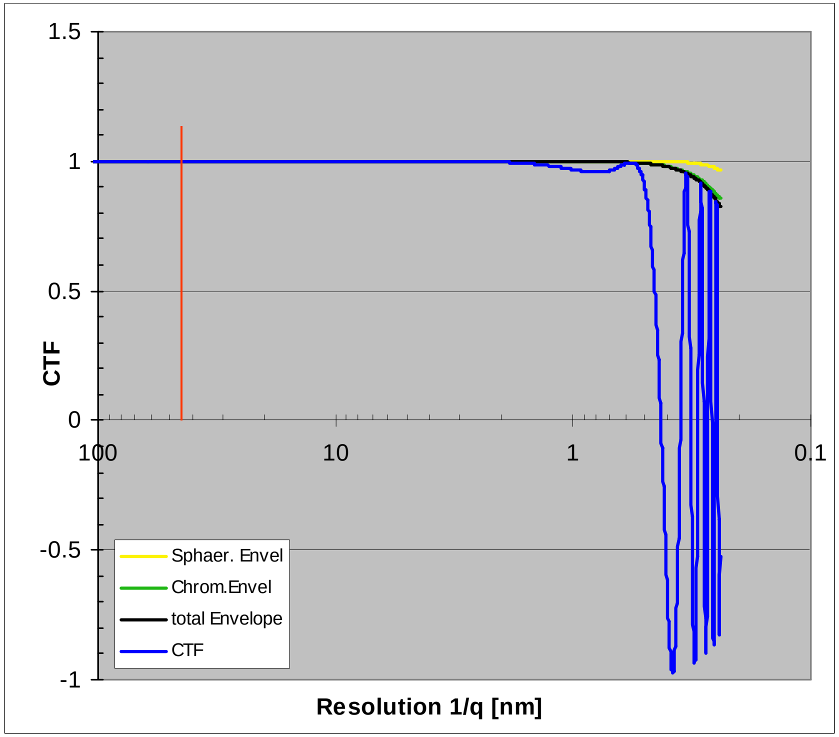
"Perfectly" restored image S/N = 30
Effectively the same as an in-focus phase contrast image

Image restoration achieved for S/N = 0.3
Cannot expect better than this for images where the contrast comes from defocus

Downing & Glaeser, In Preparation

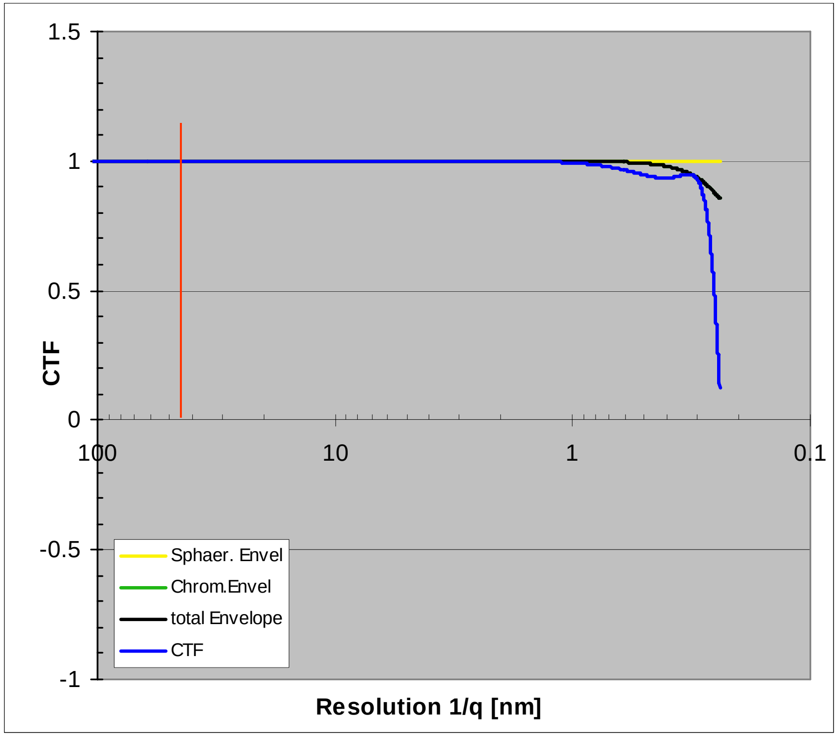
A PROPOSED f = 20 mm DESIGN MIGHT EXCEED THE REQUIREMENTS OF THE CRYO-BIO COMMUNITY

Standard 100 keV Cryo-Bio



Δz = 60 nm
Cs = 10 mm
Band pass 40 nm – 0.5 nm

Advanced 100 keV Cryo-Bio



Δz = 20 nm
Cs = 1 mm (Cs corrector)
Band pass 40 nm – 0.3 nm

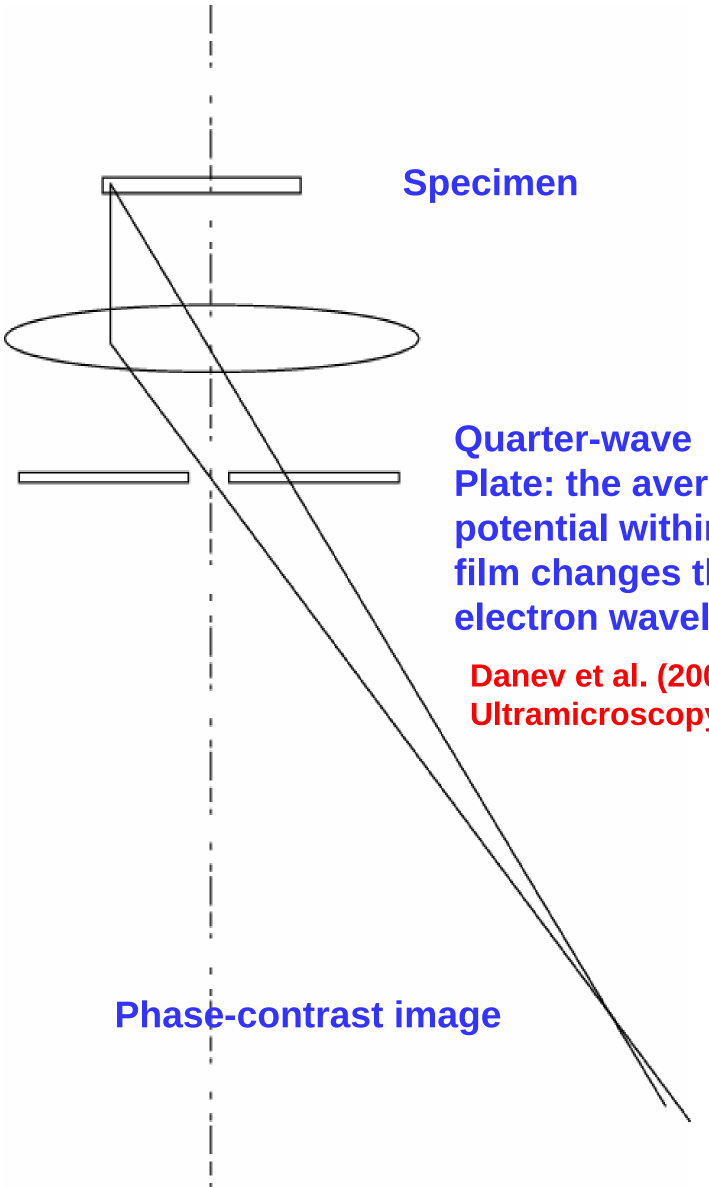
100 keV; $\Delta E \cdot Cc$ = 5 eV-mm (gun monochromator); $\alpha = 2\times10^{-5}$ rad

THE BAD
(REQUIRED OPTICS)

- **Carbon-film phase plates**
  - **Some signal is lost due to electron scattering from the atomic structure of the film**
- **Electrostatic phase plates**
  - **Einzel “lens”; Drift tube**
  - **Optical microfabrication cannot make devices that are small enough to use with standard objective-lens focal lengths of ~3 mm**
- **Any type of phase-contrast aperture**
  - **Yet another fiddly element to keep properly aligned**
  - **Forces one to use illumination that is as parallel as it should be**

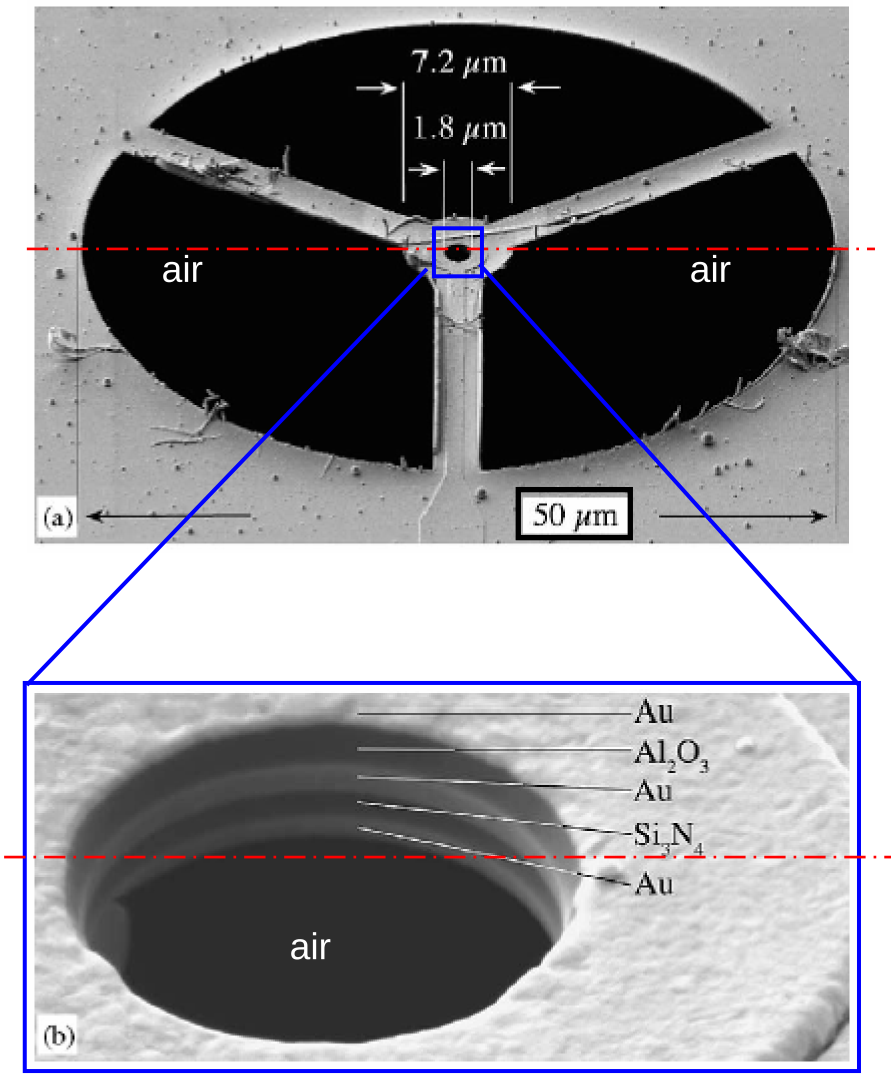
HOW THE DANEV / NAGAYAMA RESULTS WERE ACCOMPLISHED

- **The quarter-wave plate is a thin film (~30 nm) of evaporated carbon**
- **An 0.5 μm diameter hole is drilled with a focused ion beam**
- **Down-side is that >1/4 of the scattered electrons are lost by being scattered a second time**
  - **i.e. by the carbon film**



Danev et al. (2001) Ultramicroscopy 90:85-89

Electrostatic (Boersch) Phase Plate



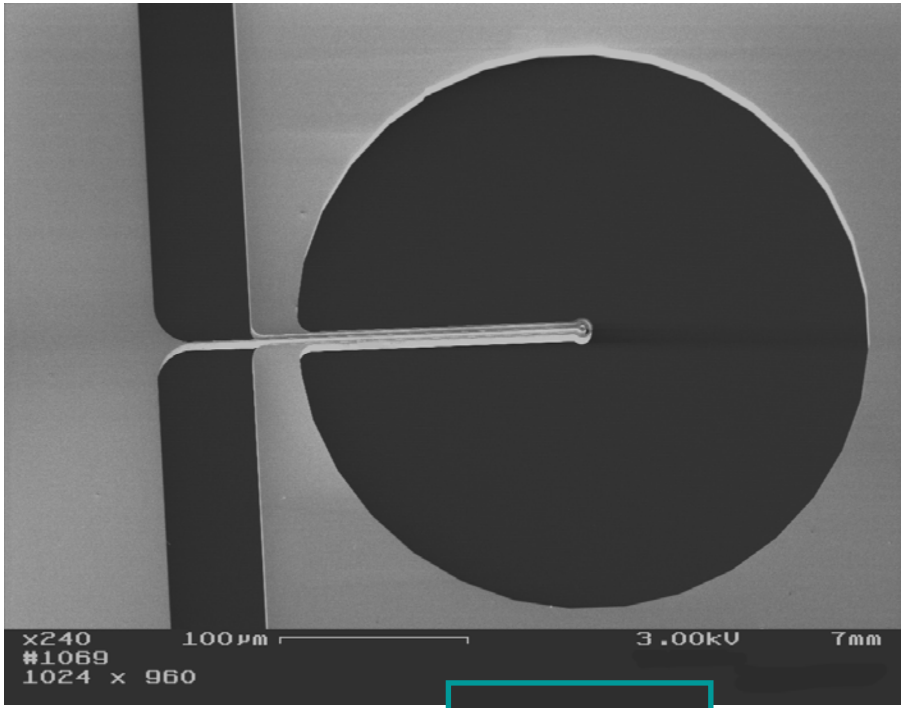
E.Majorovits et al./Ultramicroscopy 2006

ELECTROSTATIC PHASE PLATES AVOID RESCATTERING OF A FRACTION OF THE ELECTRONS

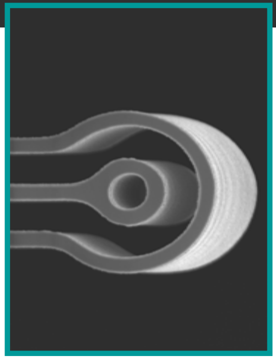
- **The einzel lens has a biased electrode that is shielded top and bottom by grounded electrodes**
- **Unscattered electrons go through the tiny hole that is on-axis, and experience a phase shift due to the applied voltage**
- **Scattered electrons pass by and experience no phase shift**
- **First proposed by Boersch in 1947**

JIAN JIN (LBNL) PROPOSED A SHIELDED "DRIFT TUBE" AS AN ALTERNATIVE DESIGN

- **The focused beam of unscattered electrons goes through the axis of the biased, inner cylinder**
- **A grounded "guard-ring" electrode shields the open area of the objective aperture**
  - **The scattered electrons thus do not experience a phase shift**
- **Electrostatic modeling shows that fringing fields are weak for an aspect ratio >10:1**

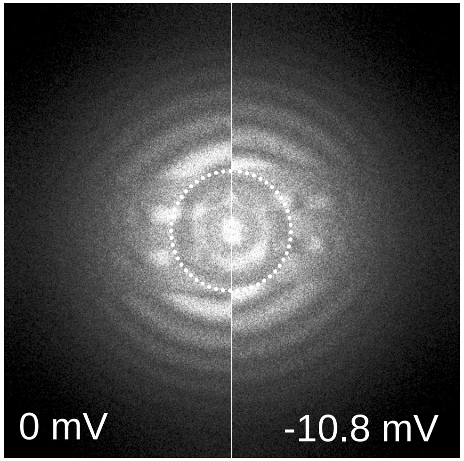


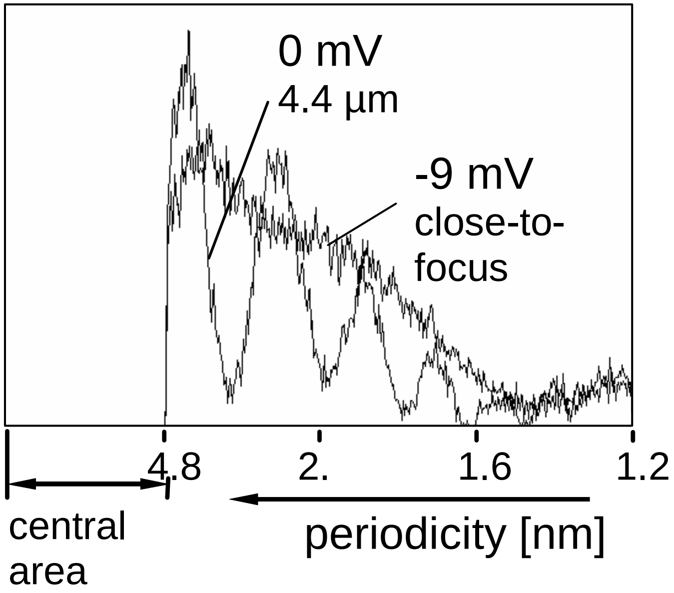
Cambie et al. (2007) Ultramicroscopy 107:329-339



EXPERIMENTAL DEMONSTRATION THAT THE DRIFT-TUBE DESIGN WORKS AS EXPECTED

- **Thon rings are shifted by "π/2" when the drift tube is biased by ~11 mV**
  - **The electrode structure shows up with Friedel symmetry in the power spectrum, of course**
- **The background-subtracted power spectrum is "flat" at zero defocus**
  - **The envelope is similar to that for a defocused image taken with no bias on the drift tube**
  - **The poor envelope is due to many limitations of our old 100C**
    - **Purposely long focal length; tungsten filament; known charging of the device**





STANDARD FOCAL LENGTHS ARE TOO SHORT FOR MICROMETER-SIZED ELECTRODES

EXAMPLE

- **f = 3 mm**
- **λ = 2 pm (300 keV)**
- **R = 2 μm**
- **$S_{CUT\text{-}ON}$ = 1/(3 nm)**

$$S_{CUT-ON} = \frac{R}{\lambda f}$$

$R = electrode\_radius$

$\lambda = wavelength$

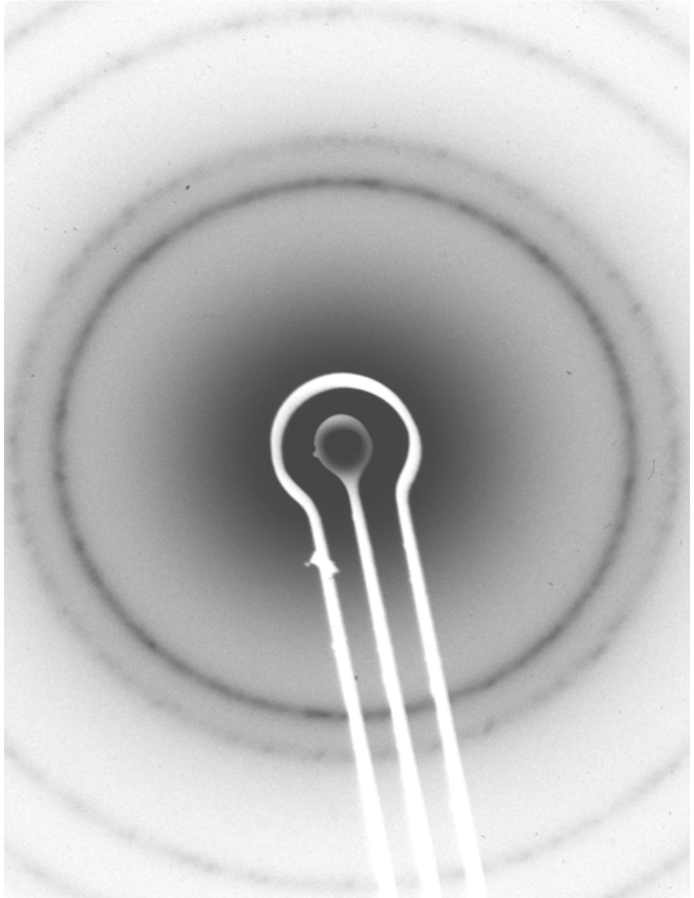
$f = focal\_length$

The cut-on frequency in this example is still about a factor of 10 too high

since we require that $S_{CUT\text{-}ON} < 1/(30\ nm)$

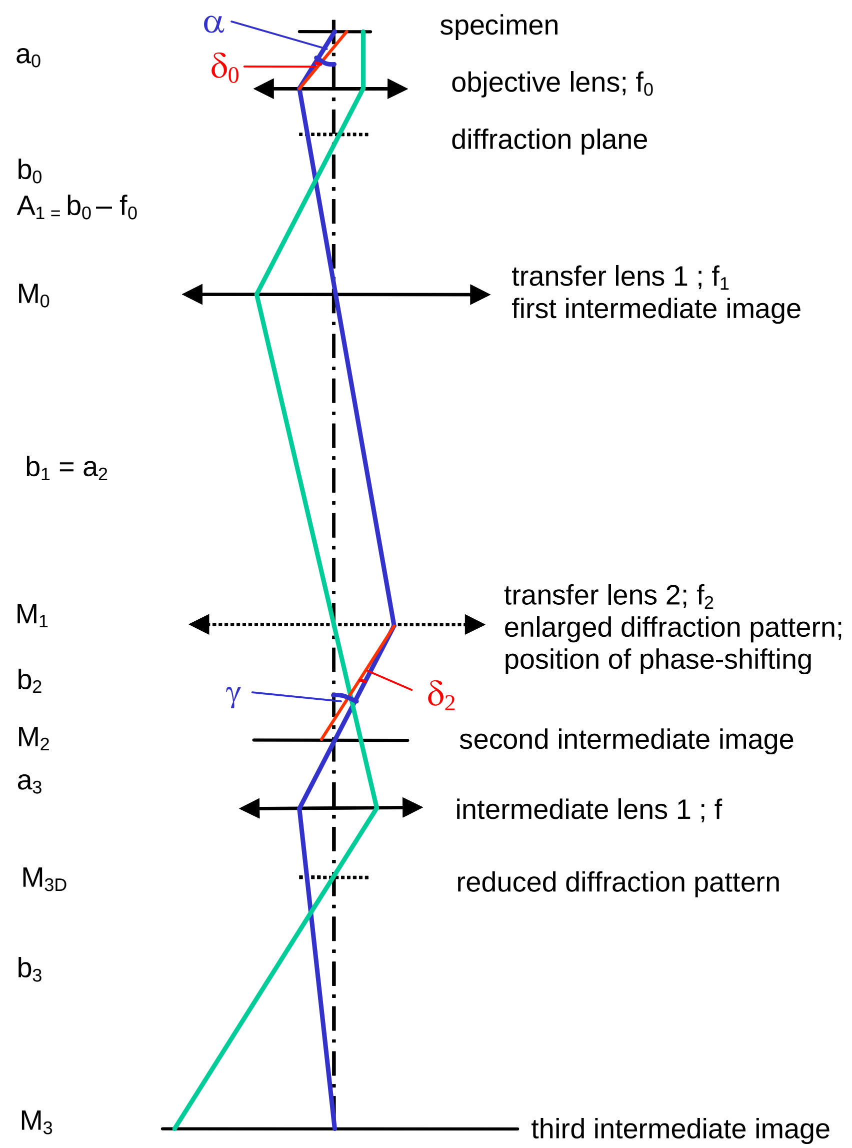
EXAGGERATED EXAMPLE TO ILLUSTRATE THE CHALLENGE OF MATCHING THE SIZE OF THE DEVICE TO THE SCALE OF THE DIFFRACTION PATTERN

- **In this example the cut-on frequency is ~1/(0.6 nm)**
  - **First diffraction ring from gold is at 1/(0.235 nm)**
- **While it is possible to reduce the device size by 5-fold, it will also be necessary to increase the focal length about 10-fold**



MAGNIFYING THE DIFFRACTION PATTERN WITH A RELAY LENS IS THE SIMPLEST WAY TO INCREASE THE FOCAL LENGTH

- **The transfer doublet does increase both Cs and Cc, however**
  - **Add a Cs corrector if your work can justify it**
  - **Use a gun monochromator to compensate for the increased Cc**
- **It also requires an additional step of lens-alignment**
  - **Should be incorporated in automated microscope operation**



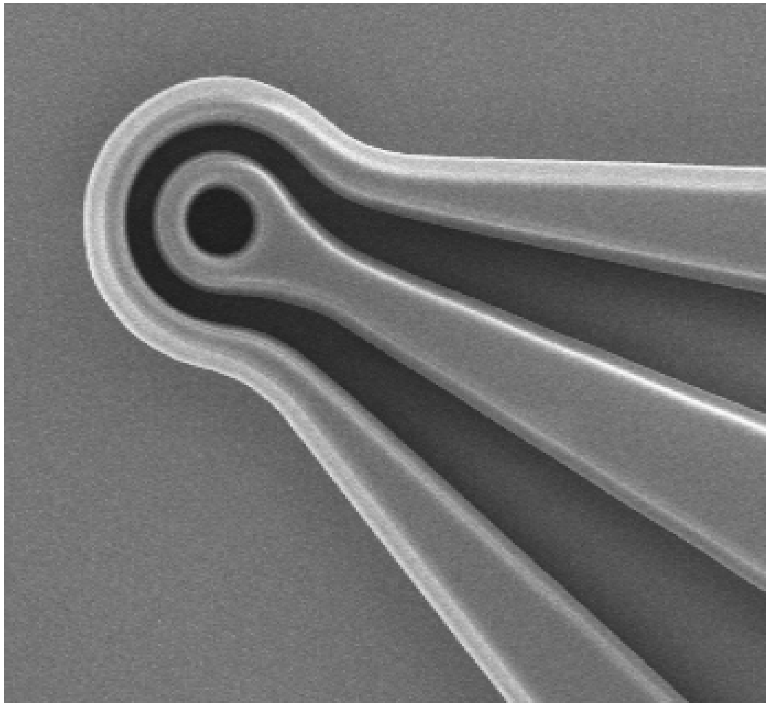
D. Typke, unpublished

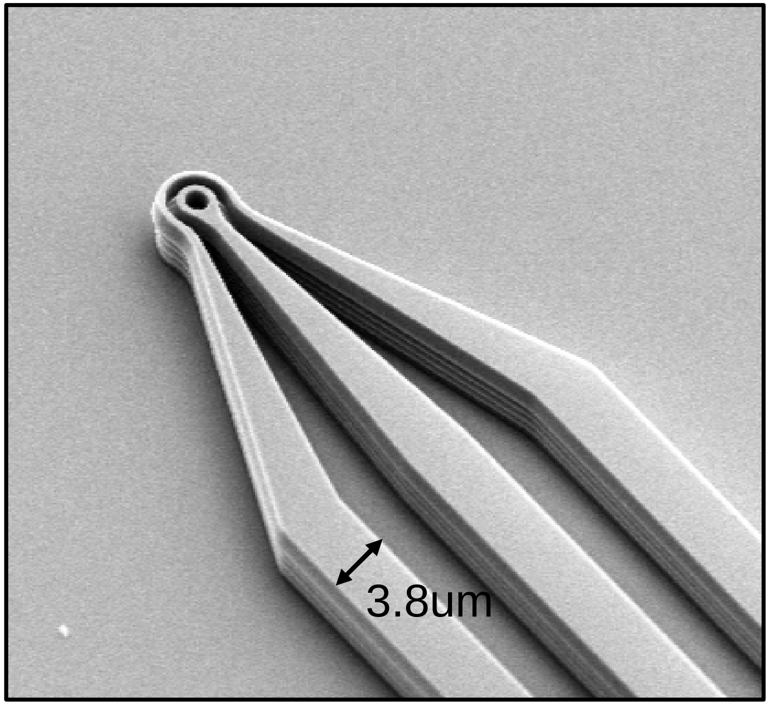
THE UGLY
(RESEARCH CHALLENGES)

- **Will UV-photolithography succeed to bring the device size down to <2 μm?**
- **Can technology be put in place that will rigorously prevent contamination and charging of the device when it is hit by the unscattered beam?**
  - **Heating >200 C?**
  - **Bake-out followed by cooling?**
  - **Continuous oxygen-plasma cleaning?**
- **Can use of the device be made effectively "transparent" to the user**
  - **Use of feed-back repositioning will help**
  - **Full automation of data collection will really be the key**

UV PHOTOLITHOGRAPHY

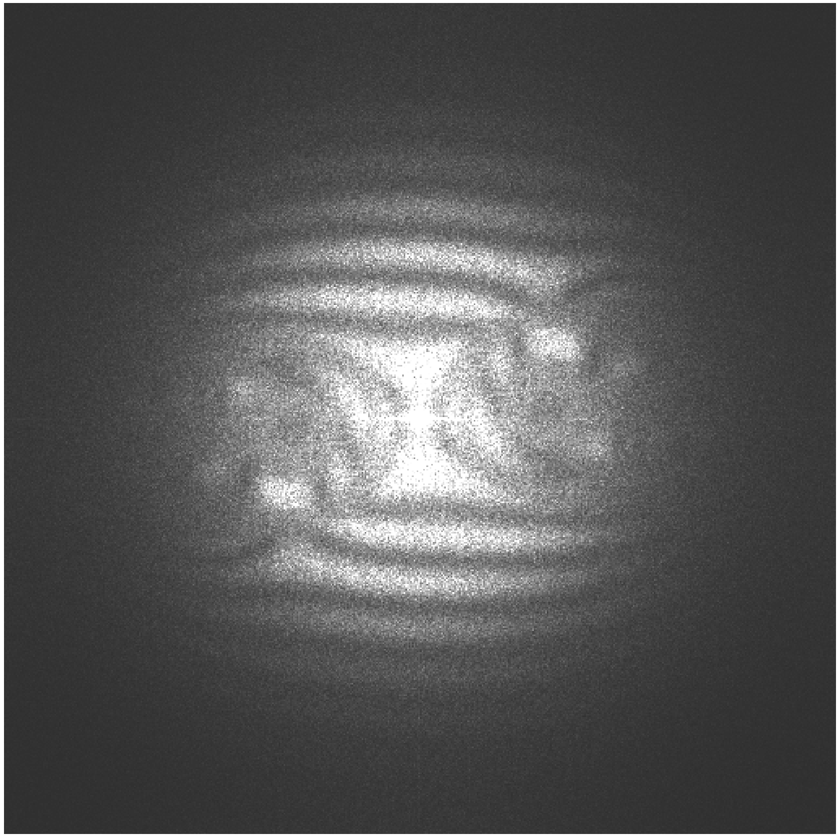
RESULTS OF A FABRICATION PROTOTYPE INDICATE THAT FEATURE SIZES ~0.4 μm AND R = 1.8 μm WILL BE PRACTICAL





CHARGING IS DREADFUL WHEN NO PRECAUTIONS ARE TAKEN TO AVOID IT

- **Severe astigmatism develops as the device approaches the focused, unscattered beam**
  - **This effect can be used as a zeroth order test for charging**
- **The final criterion must be whether the envelope function is the same for images taken with and without the device in place**



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