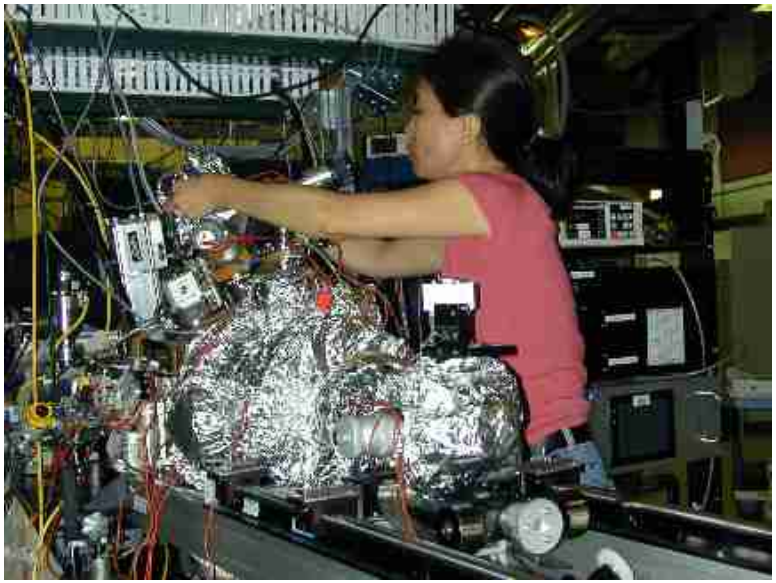
Diffraction microscopy of biological specimens: imaging of a freeze-dried yeast cell

David Shapiro, Tobias Beetz, Chris Jacobsen, Janos Kirz, Enju Lima, Huijie Miao, Aaron Neiman, David Sayre: Stony Brook

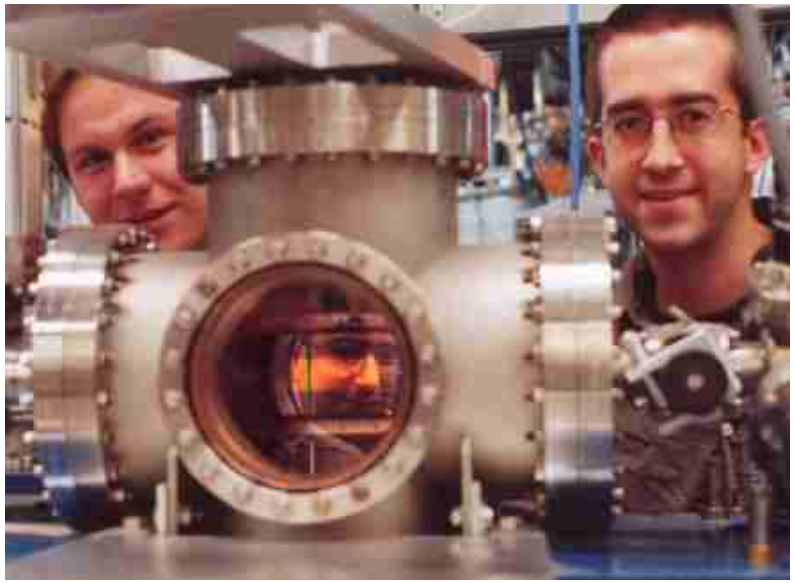
Pierre Thibault, Veit Elser: Cornell

Malcolm Howells: Advanced Light Source

(To be published in Proc. Nat. Acad. Sci.)



Enju Lima



Tobias Beetz

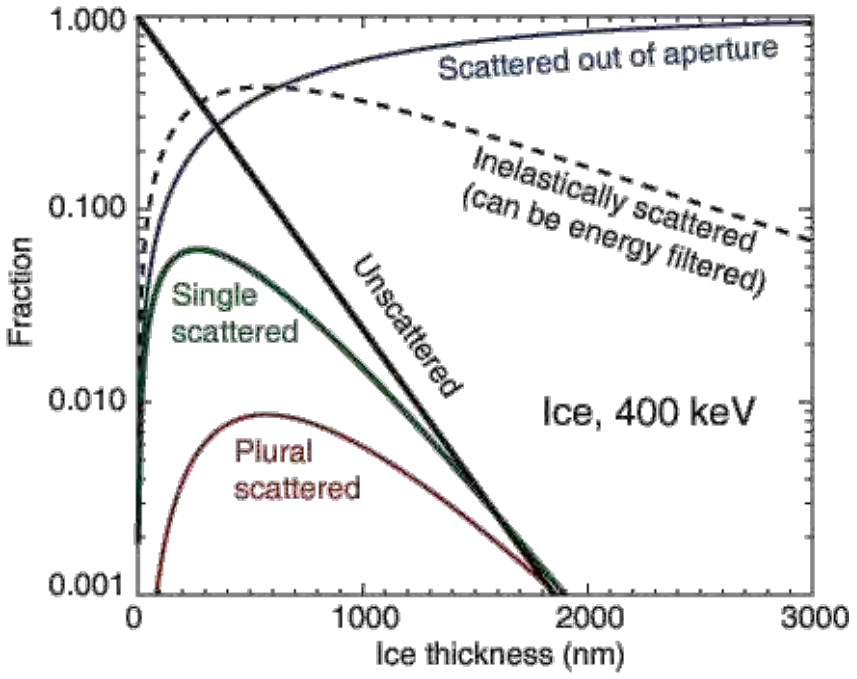
David Shapiro

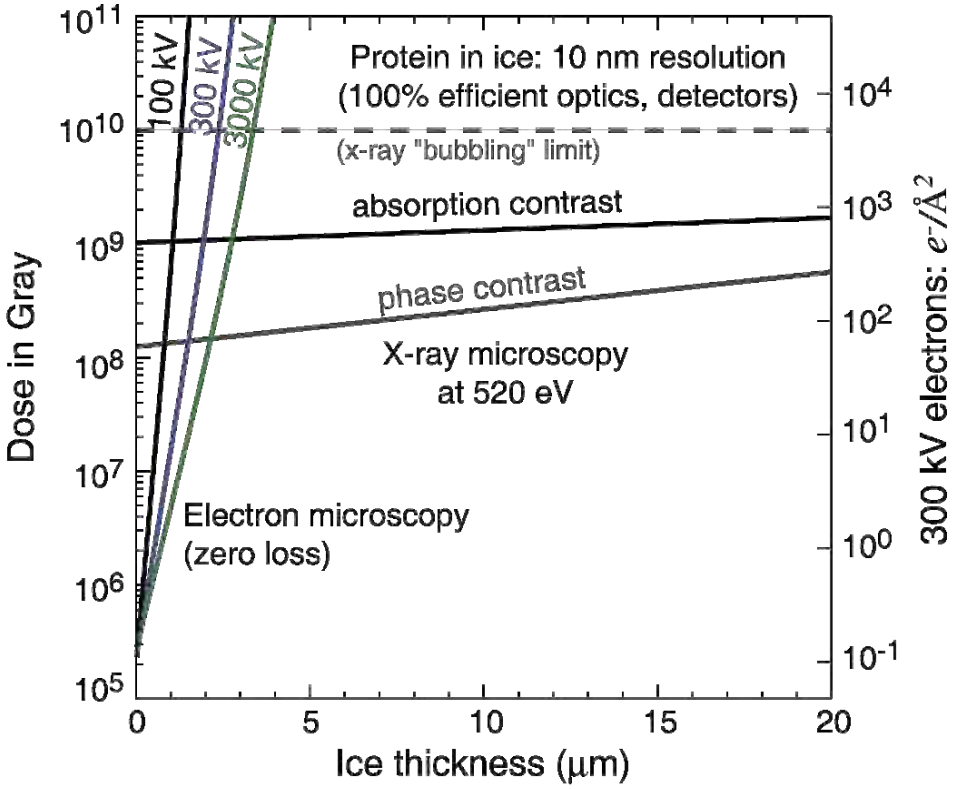
Synchrotron imaging in context

- Light microscopy:
  - **Live cells** at ~200 nm resolution. Approaching 50 nm with spatial light modulation! Gustafsson et al., UC San Francisco; Cremer et al., Heidelberg
  - Fluorescent labels for specific proteins (permeating, injected, or genetically "programmed in" such as Green Fluorescent Protein and its variants)
- Electron microscopy:
  - 0.1-0.2 nm resolution on robust, crystalline samples.
  - 0.5-1 nm resolution on many copies of identical molecules (single particle methods)
  - 6 nm resolution in tomography
  - Immunogold labeling

X rays and thick specimens

X rays better for thick specimens. Sayre et al., Science **196**, 1339 (1977); Schmahl & Rudolph in **X-ray Microscopy: Instrumentation and Biological Applications** (Springer, 1987)

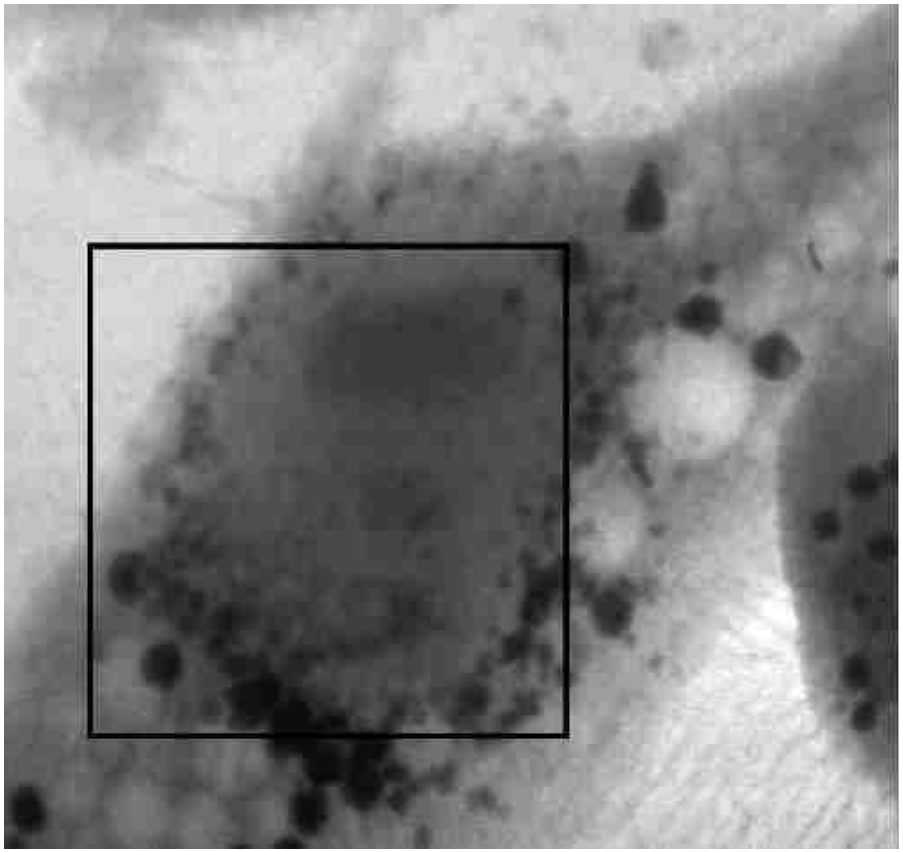




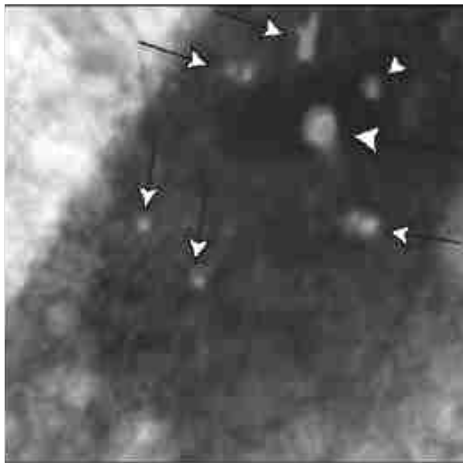
These plots: Jacobsen, Medenwaldt, and Williams, in **X-ray Microscopy & Spectromicroscopy** (Springer, 1998)

Radiation damage resistance of wet specimens at liquid nitrogen temperature

Left: frozen hydrated image after exposing several regions to ~10^{10} Gray



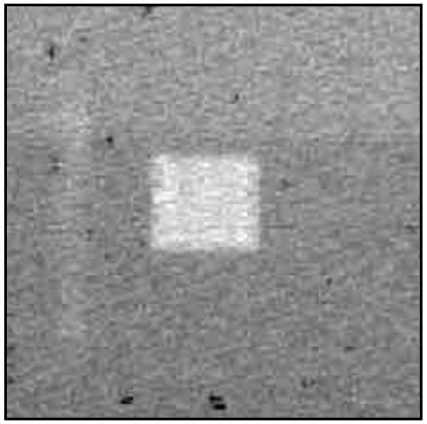
Right: after warmup in microscope (eventually freeze-dried): holes indicate irradiated regions!



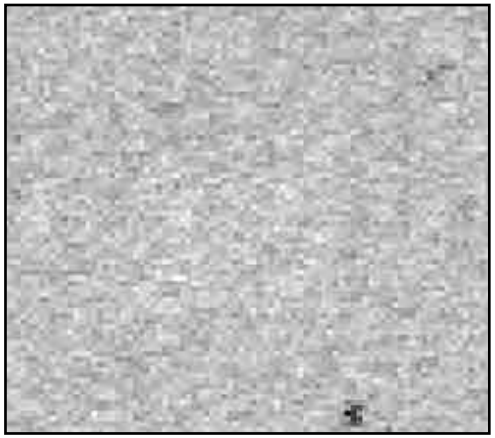
Maser et al., J. Micros. **197**, 68 (2000)

PMMA at room, LN2 temperature

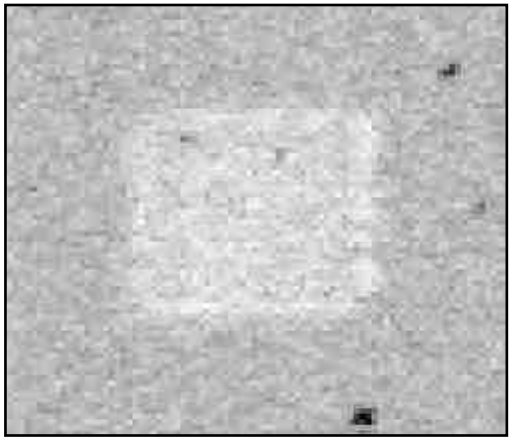
- PMMA: poly methyl methacrylate (plexiglass!) which is especially radiation sensitive – it's used as a resist for electron beam lithography
- Repeated sequence: dose (small square), spectrum (defocused beam). Low dose, larger area image at end.



Room temperature:
mass loss
immediately visible



LN2 temperature:
no mass loss
immediately visible

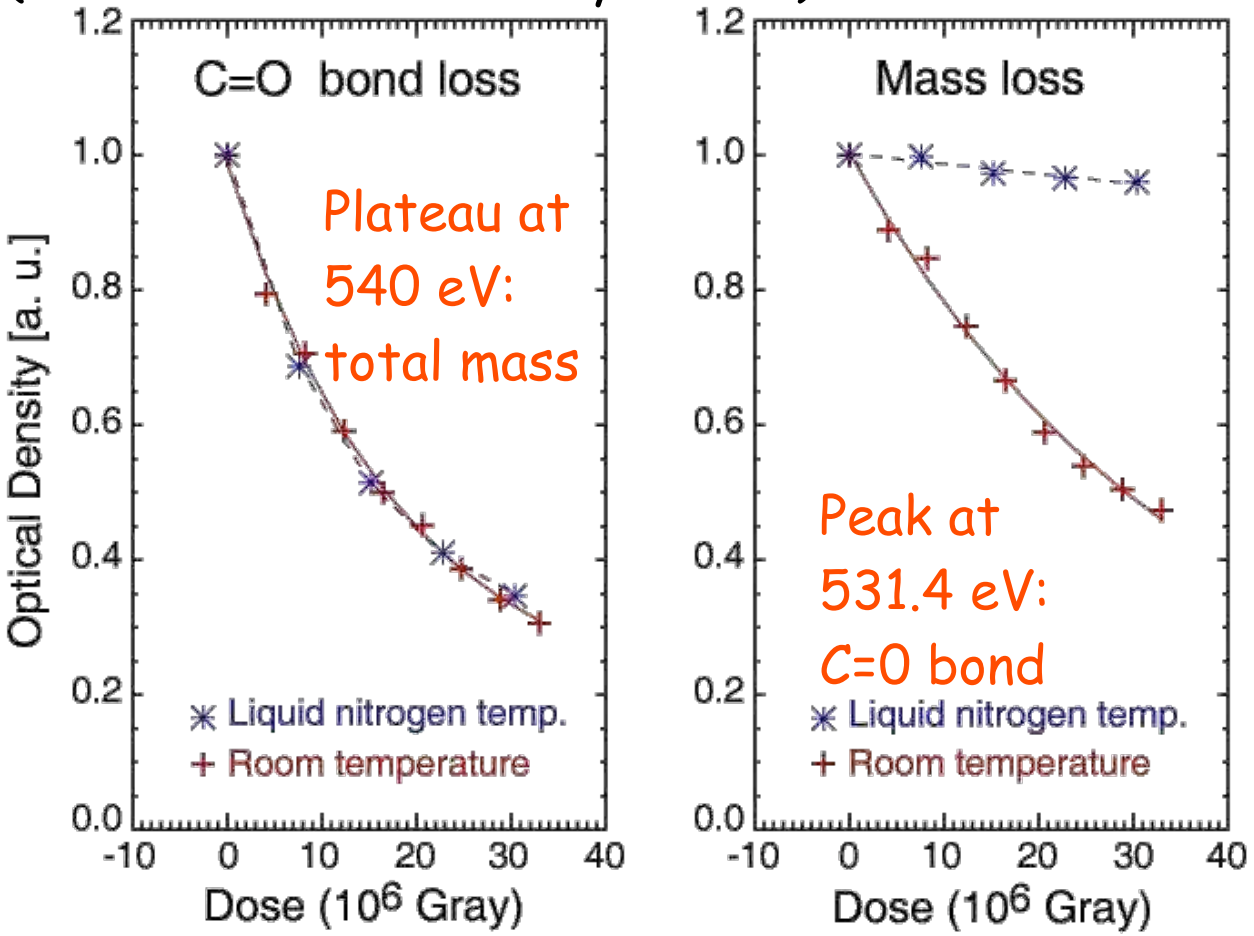


After warm-up:
mass loss becomes
visible

Beetz and Jacobsen, J. Synchrotron Radiation **10**, 280 (2003)

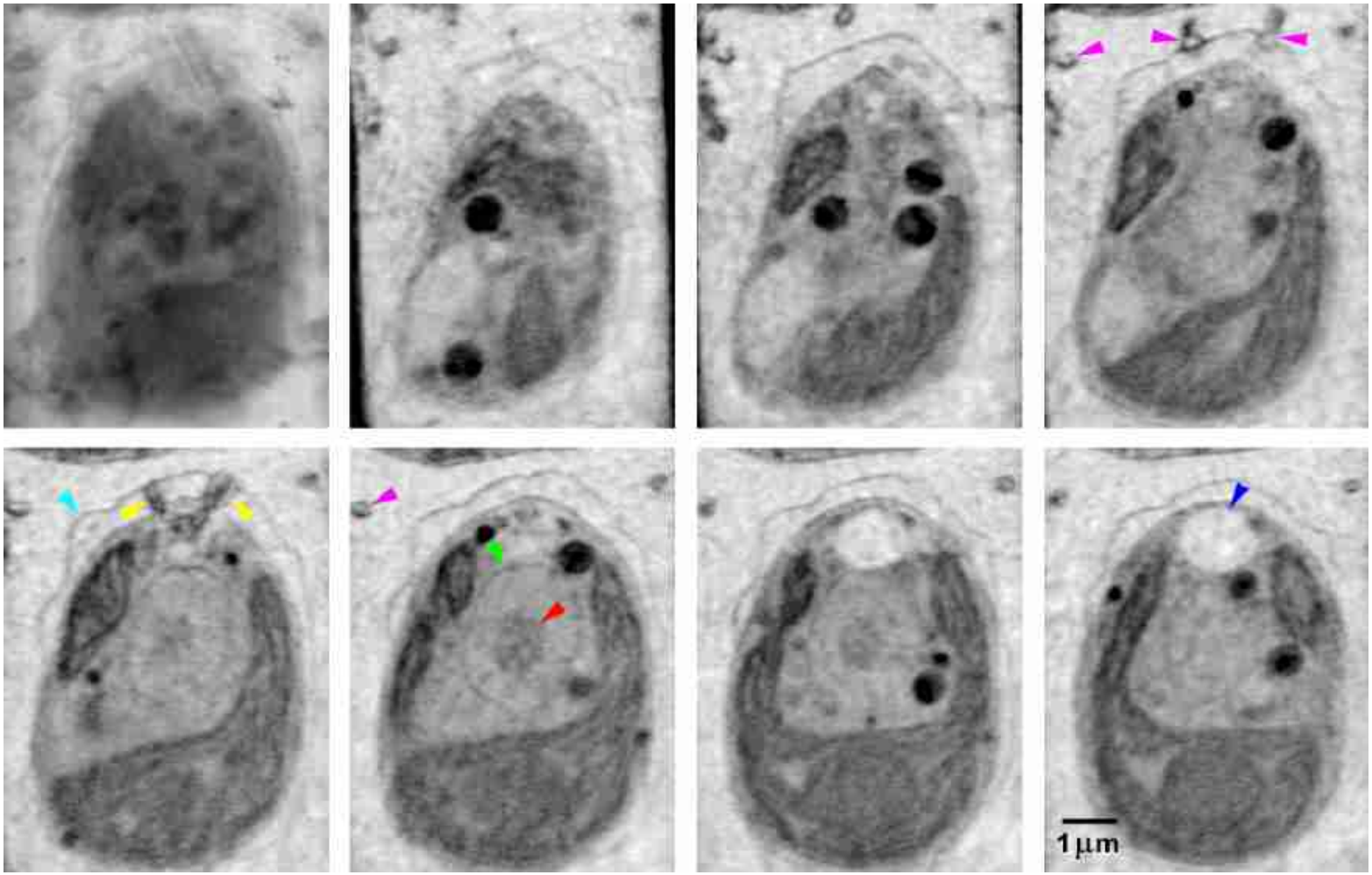
Results from fitting spectra

LN_2 temp: protection against mass loss, but not against breaking bonds (at least C=O bond in dry PMMA)



Beetz and Jacobsen, J. Synchrotron Radiation **10**, 280 (2003)

Soft x-ray tomography of algae shock-frozen in liquid ethane: Slices through a tomographic reconstruction



Vacuole

Nuclear membrane

Nucleolus

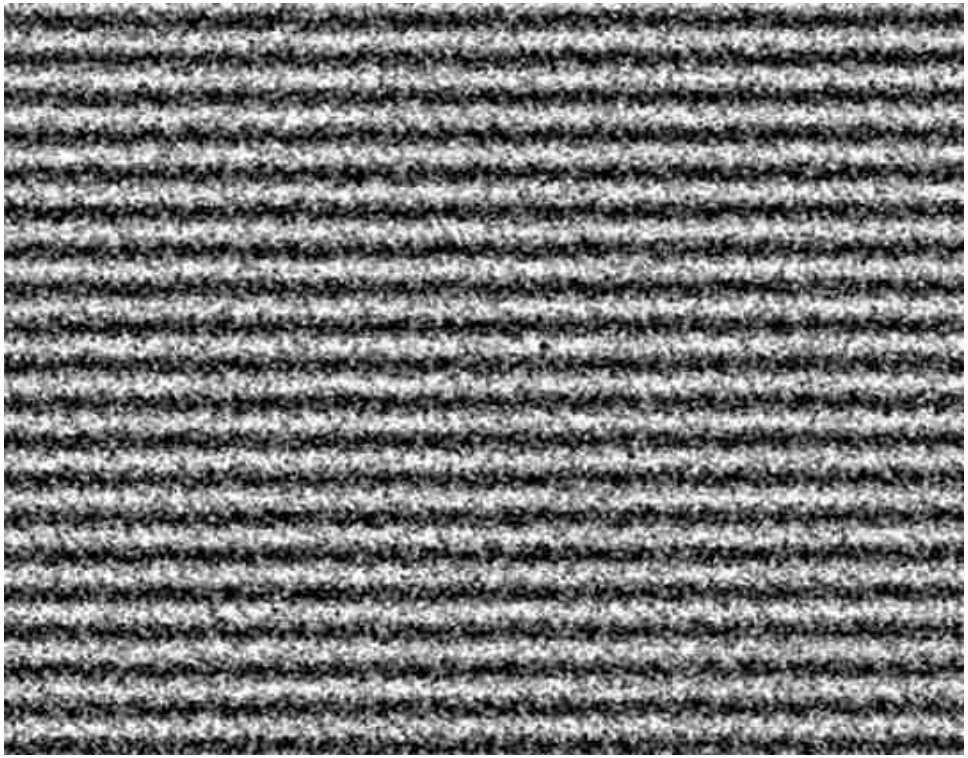
Flagellar roots and neuromotor

Cell wall

Flagella

Weiss, Schneider et al., Ultramicroscopy **84**, 185 (2000).
See also Larabell and Le Gros, Molecular Biology of the Cell **115**, 957 (2004)

Resolution frontier of zone plates



But efficiency only ~3%. Other results: 9.2% at 20 nm: Peuker, Appl. Phys. Lett. **78**, 2208 (2001)

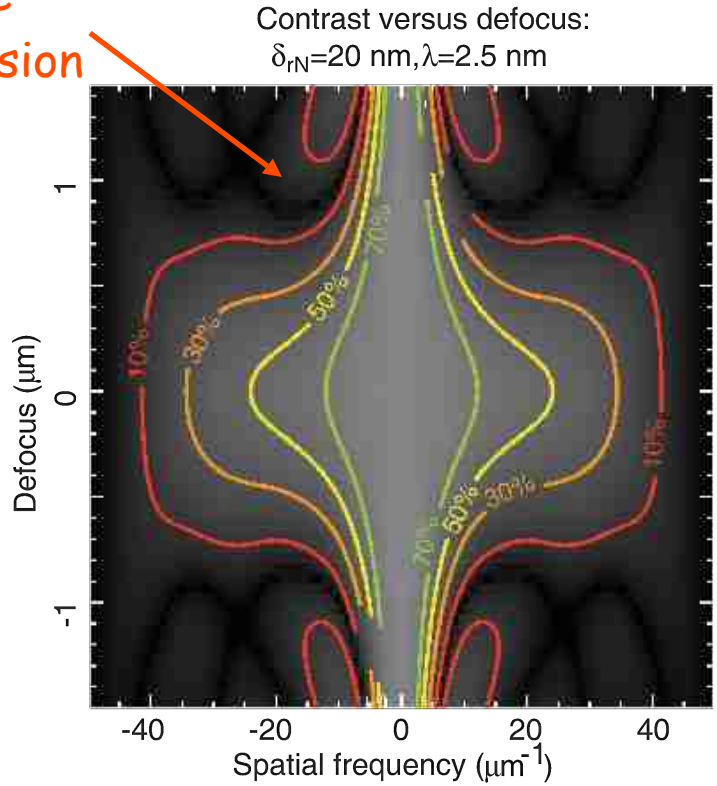
15.1 nm half-pitch multilayer slice imaged with a 15 nm outermost zone width zone plate. Chao et al., CXRO/LBL. Previous work: see Chao et al., Opt. Lett. **28**, 2019 (2003)

Depth of field limit to conventional tomography

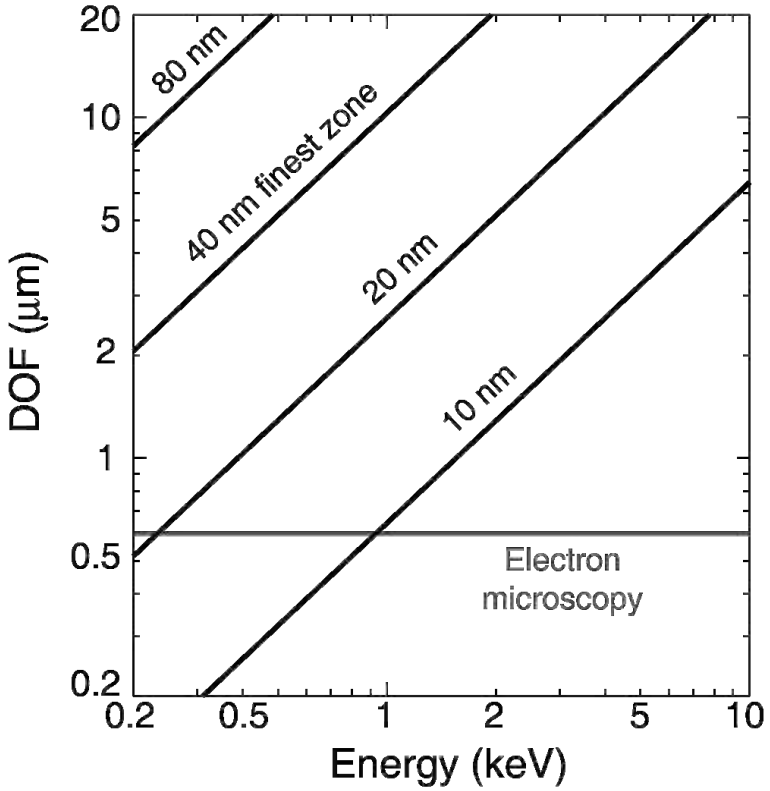
Transverse: $\delta_t \Rightarrow \frac{\lambda}{4\theta} = \frac{dr_N}{2}$

Longitudinal: $\delta_l \Rightarrow \frac{\lambda}{\theta^2} = 8dr_N \frac{dr_N}{\lambda}$

Phase inversion



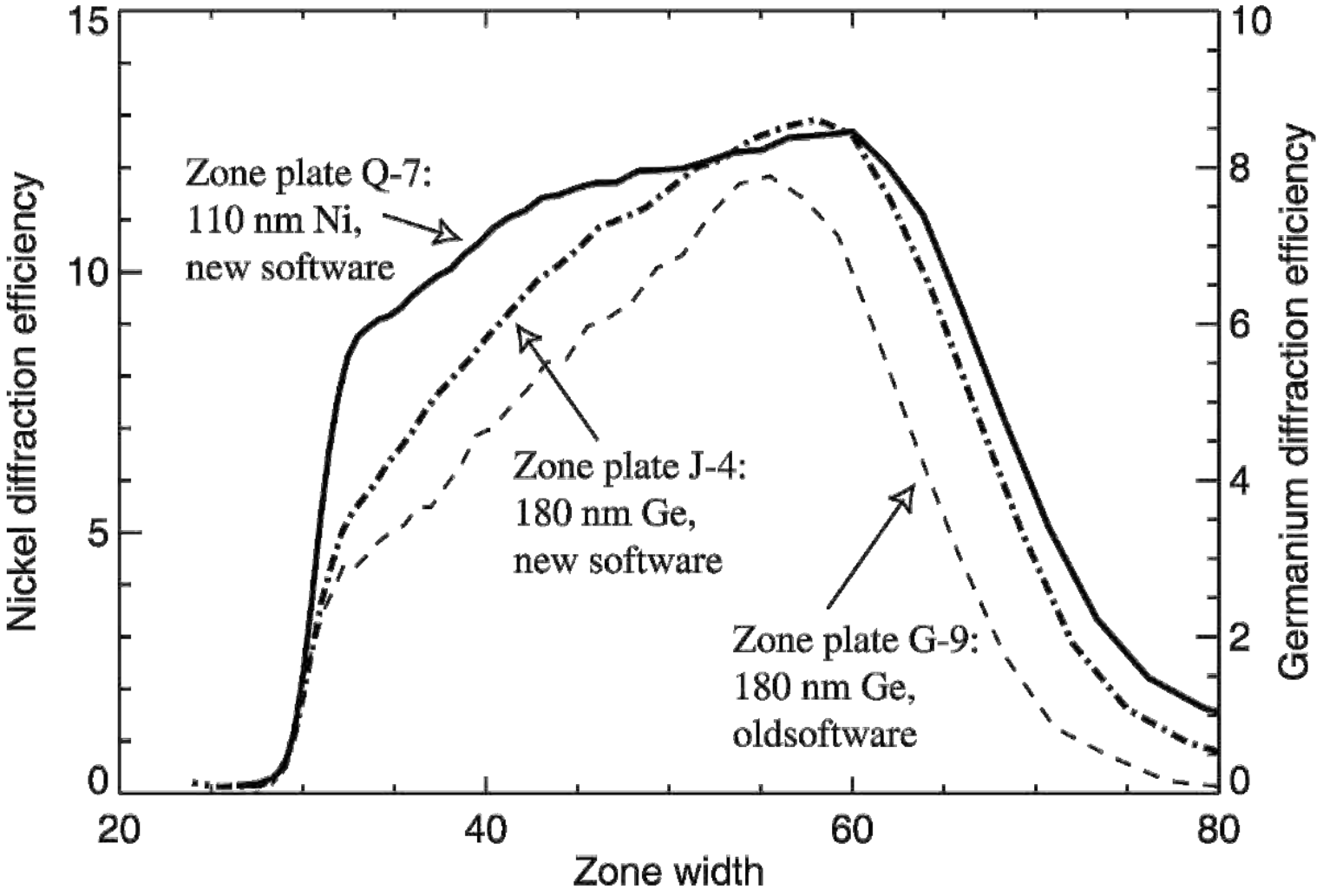
MTF of a 20 nm zone plate @ 0.5 keV



Decreased monochromaticity increases DOF but also reduces transfer function. Schneider et al., Surf. Rev. Lett. **9**, 177 (2002)

Through-focus deconvolution on a complex object requires exact knowledge of partially coherent transfer function

Limitations of zone plates

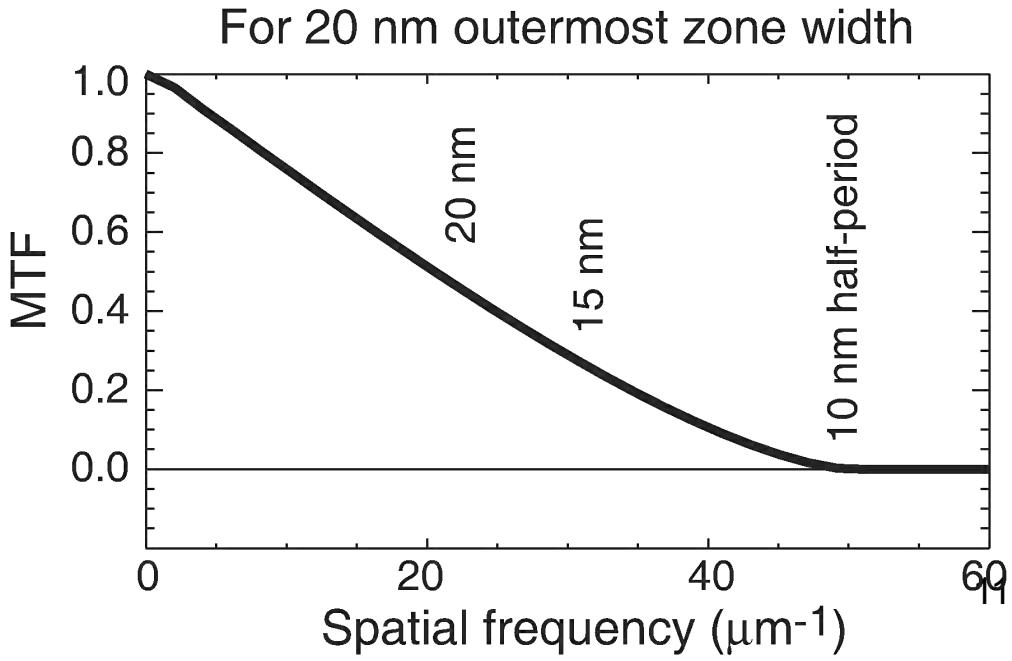


Spector et al., J. Vac. Sci. 1997

Radiation damage sets the ultimate resolution limit

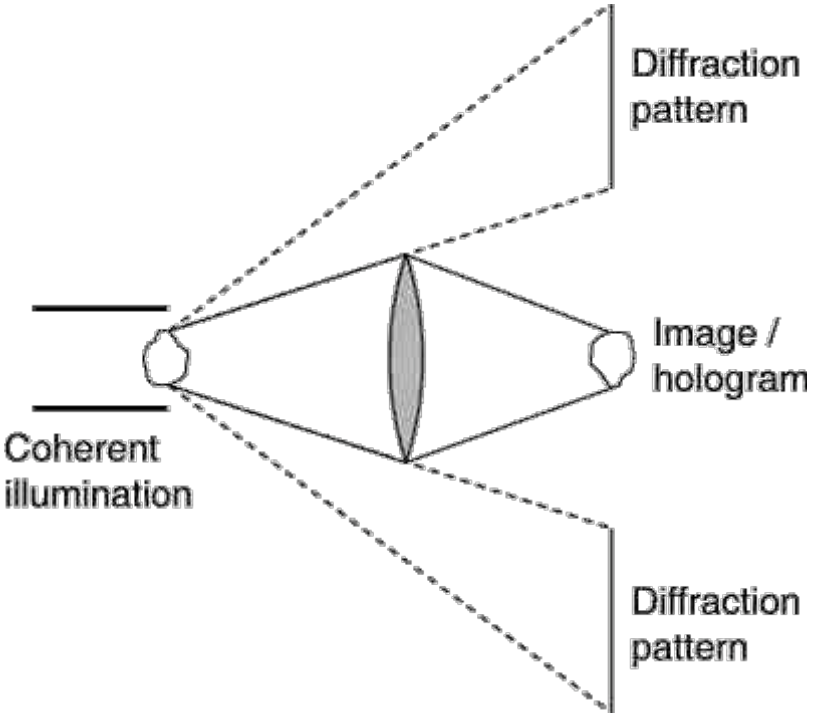
- For many specimens, radiation damage sets the ultimate limit on achievable resolution
- With 20 nm zone plates, have 8x loss for diffraction efficiency, 2x loss for window transmission, 5x loss for modulation transfer function (MTF) at 15 nm feature size.
  - On-Bragg (tilted) zone plates will help: Maser, Opt. Comm. **89**, 355 (1992); Hambach and Schneider, J. Vac. Sci. Tech. B **17**, 3212 (1999).
- Can we avoid this 80x signal loss, and go beyond the limits of available optics?

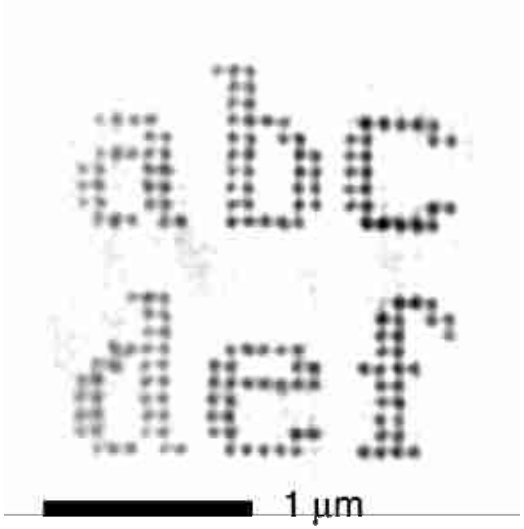
(MTF=modulation transfer function)



Can we beat the losses of lenses?

- Proposed by Sayre (in Schlenker, ed., Imaging and Coherence Properties in Physics, Springer-Verlag, 1980)
- Previous experiments by Sayre, Kirz, Yun, Chapman, Miao





First x-ray reconstruction: Miao et al., Nature **400**, 342 (1999)

Phase matters

Image→ Fourier transform→ zero magnitude or phase→ inverse Fourier transform

Malcolm Howells at La Clusaz

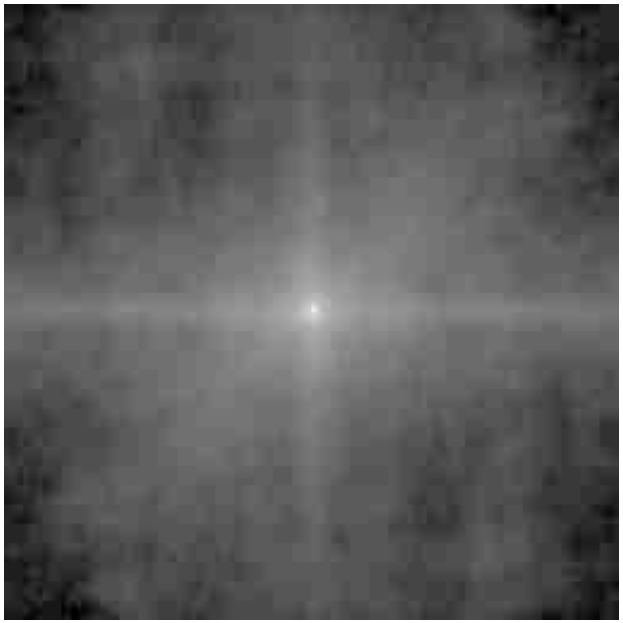


Image using only Fourier magnitudes

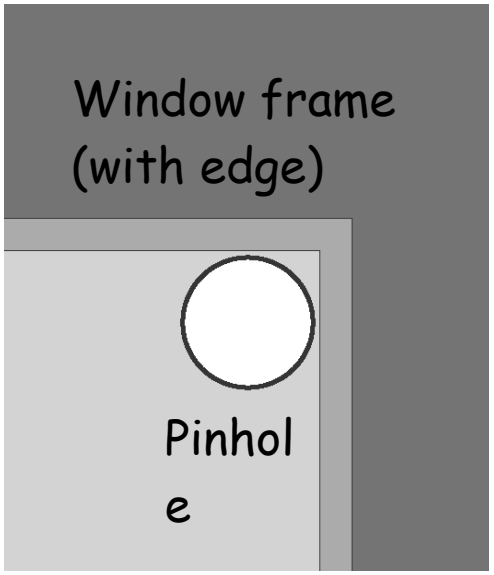
Image using only Fourier phases

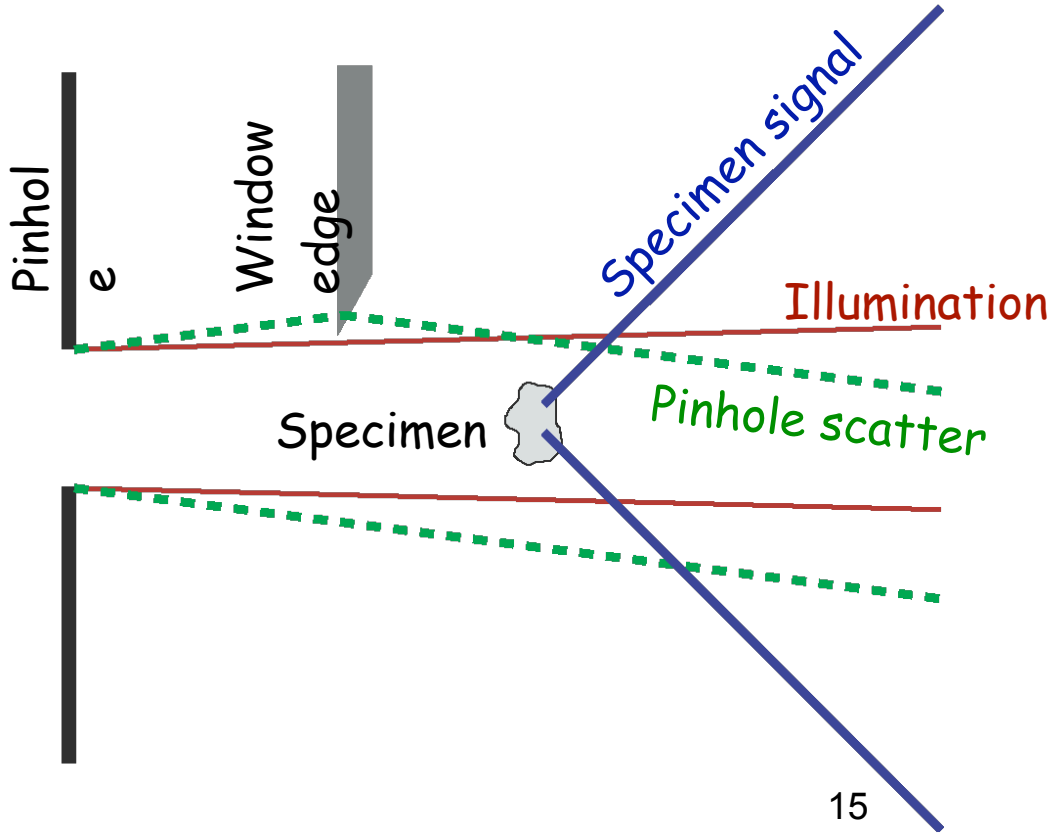
Wrapping our tentacles around the problem

(Porquerolles)

Removing scatter from pinholes

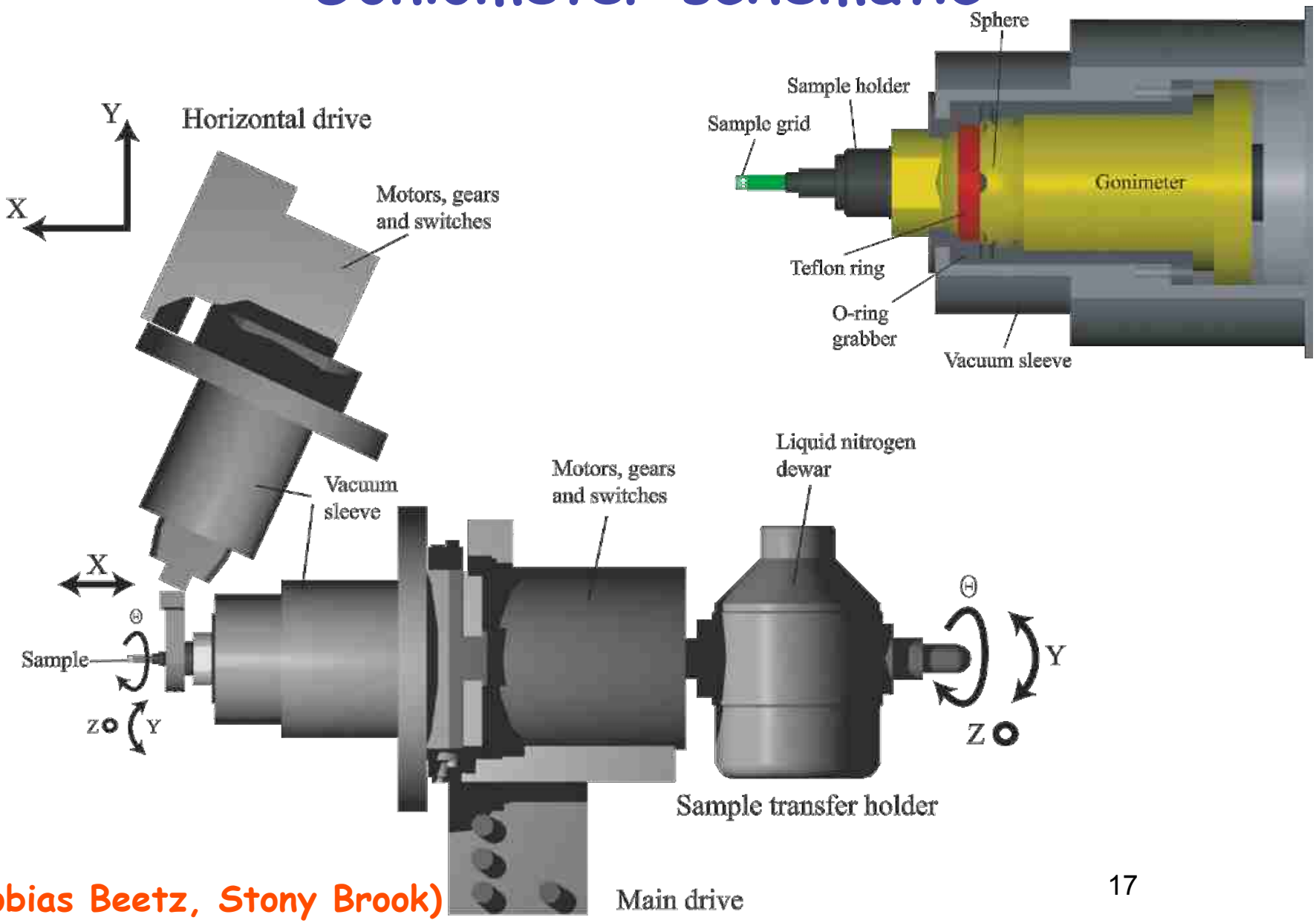
- Pinholes have scatter; can overwhelm weak diffraction.
- Use a "soft," refractive corner to limit to one quadrant (idea due to H. Chapman, then at Stony Brook)





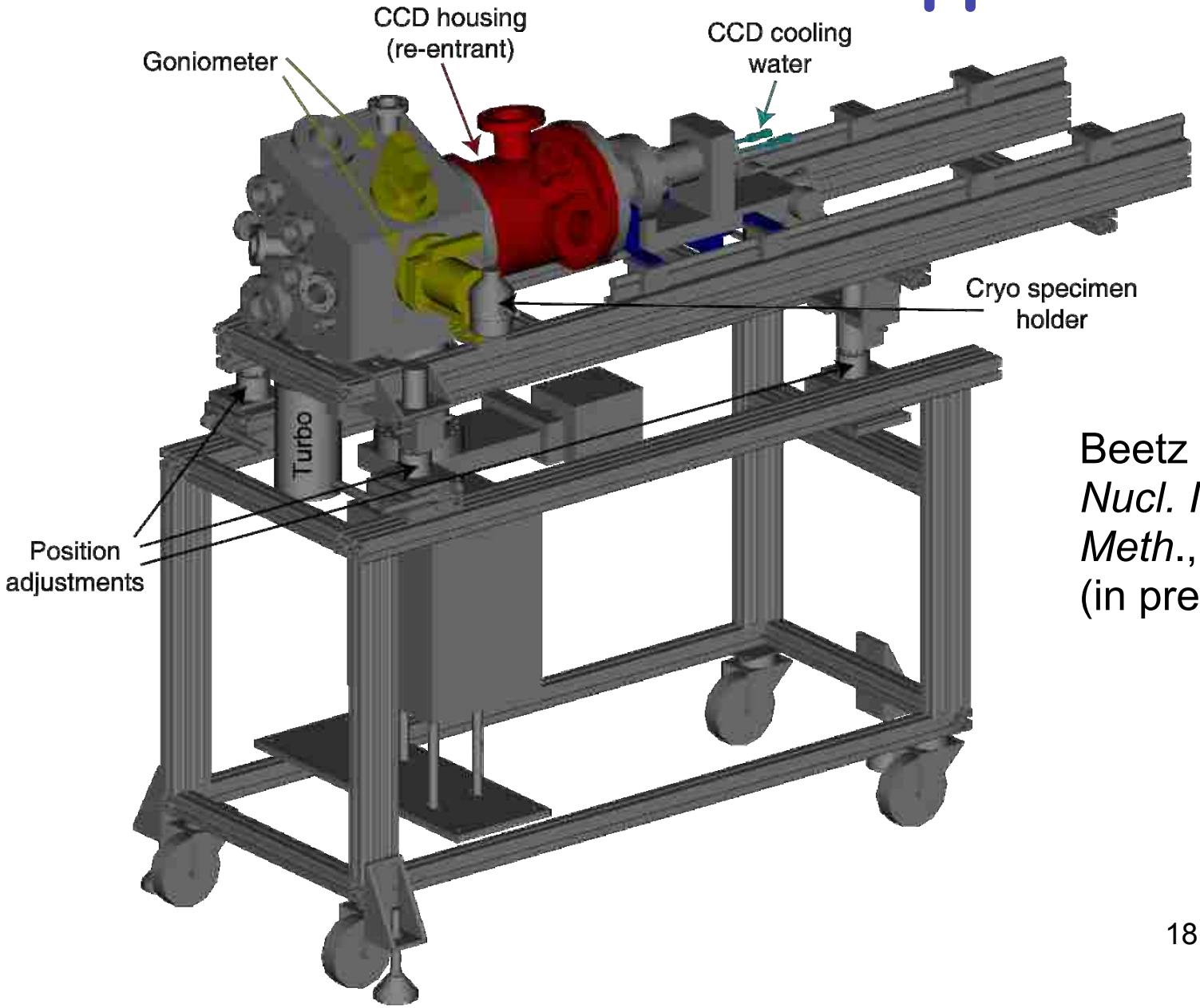
Gatan 630 cryo holder

Goniometer schematic



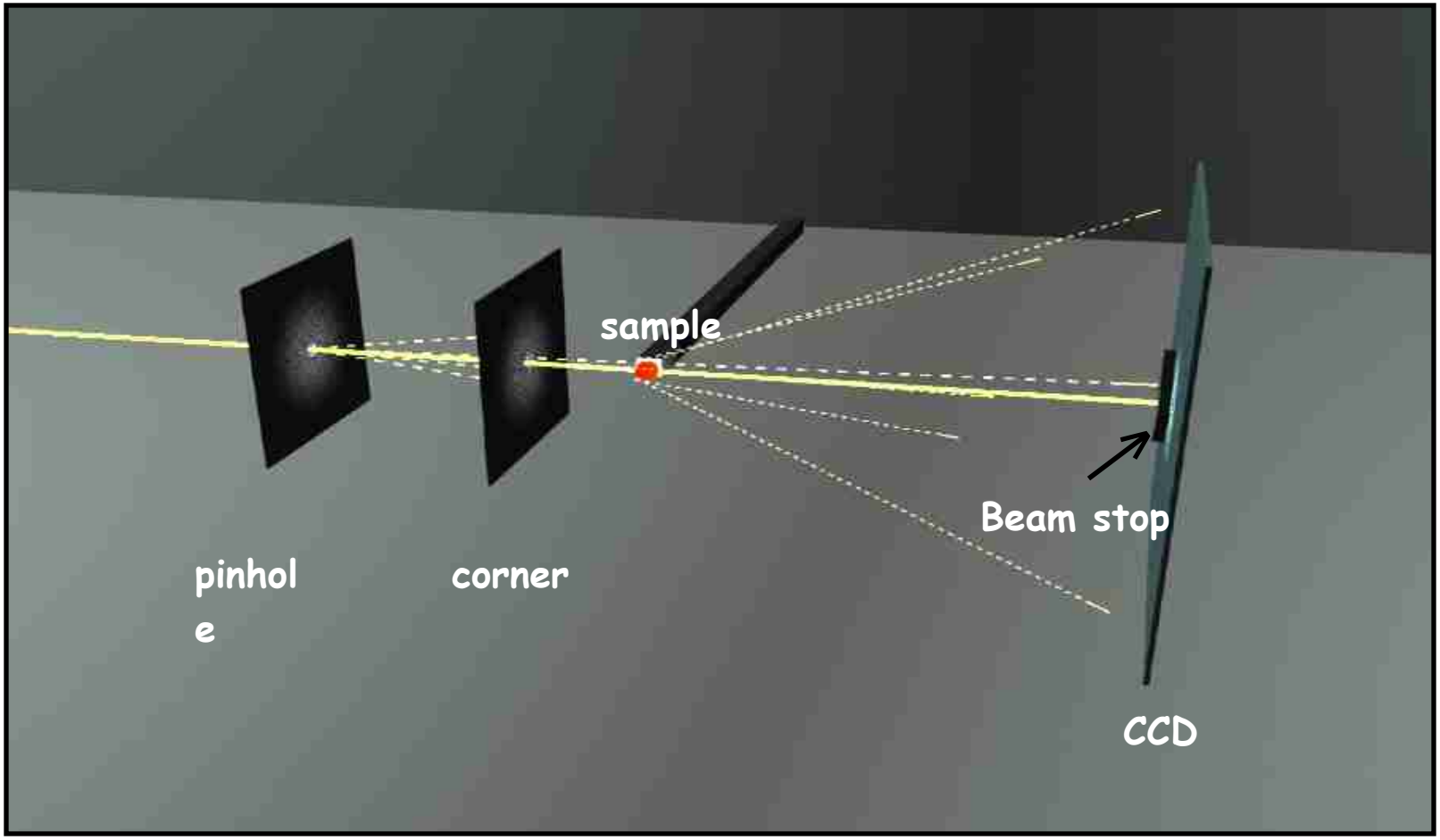
(Tobias Beetz, Stony Brook)

ALS beamline 9.0.1 apparatus



Beetz *et al.*, *Nucl. Inst. Meth.*, 2005 (in press)

Experimental setup

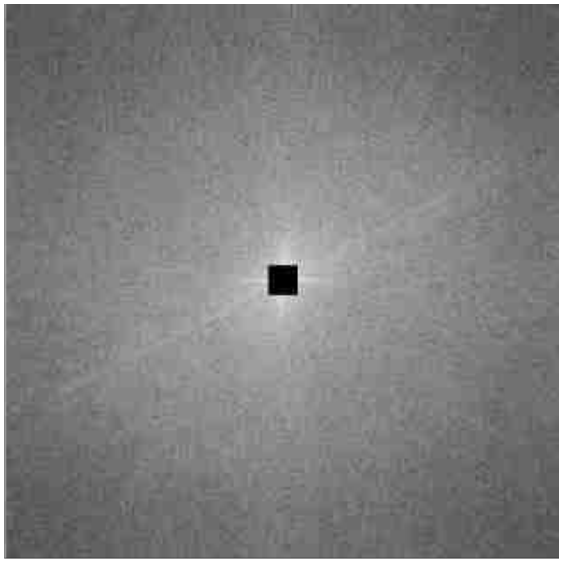


Inside vacuum chamber

(Enju Lima, Stony Brook)

Effect of missing low spatial frequencies

Meghan Sumner



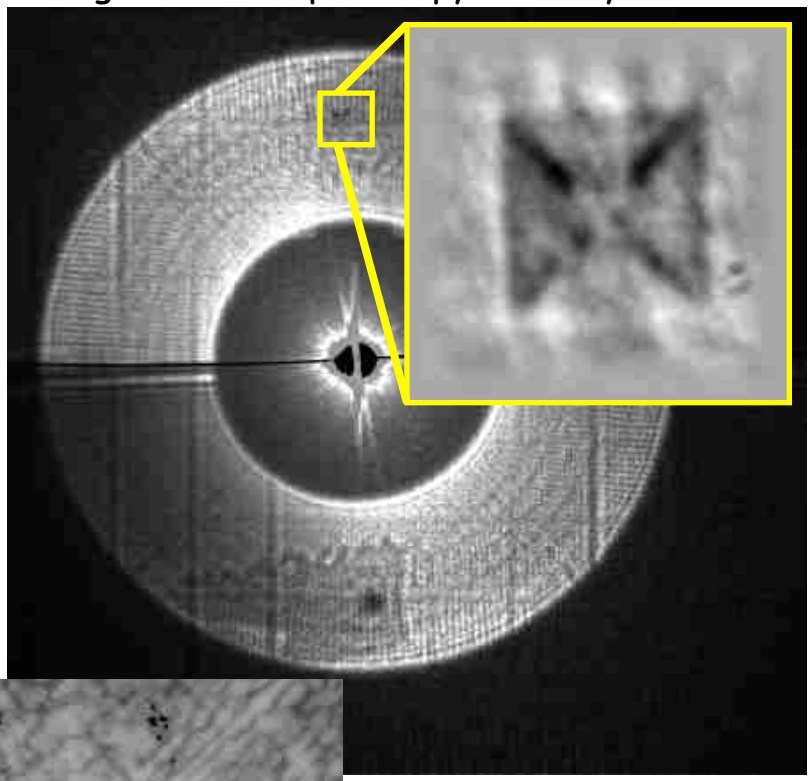
(Tobias Beetz, Stony Brook)

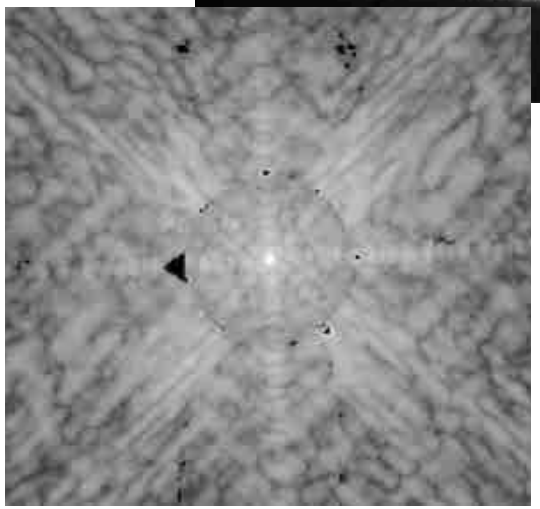
Zone plate imaging

Zone plate can be inserted behind specimen by motor control.
However:

- 20 nm zone plate with central stop, JBX-6000 stitching errors (S. Spector)
- At 16 cm CCD distance; optical magnification gives only 100 nm pixels

Image of H. Chapman's pyramid by T. Beetz





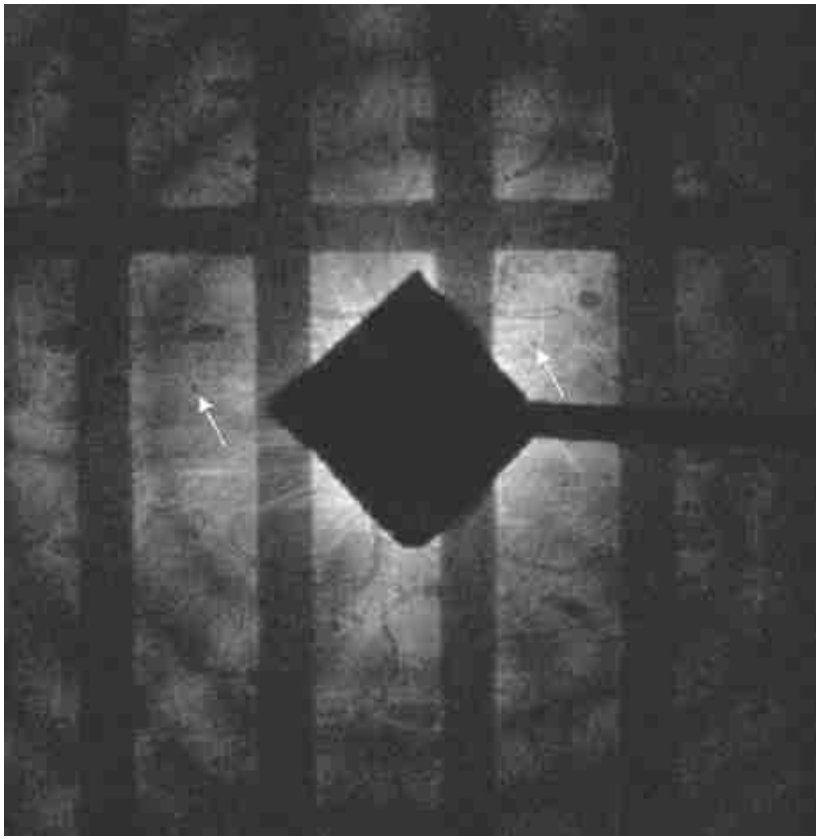
Patch in low spatial frequencies with zone plate image? Counterproductive thus far!

When hopes are dashed on the rocks, you have to find a way out

(Porquerolles)

Finding specimens

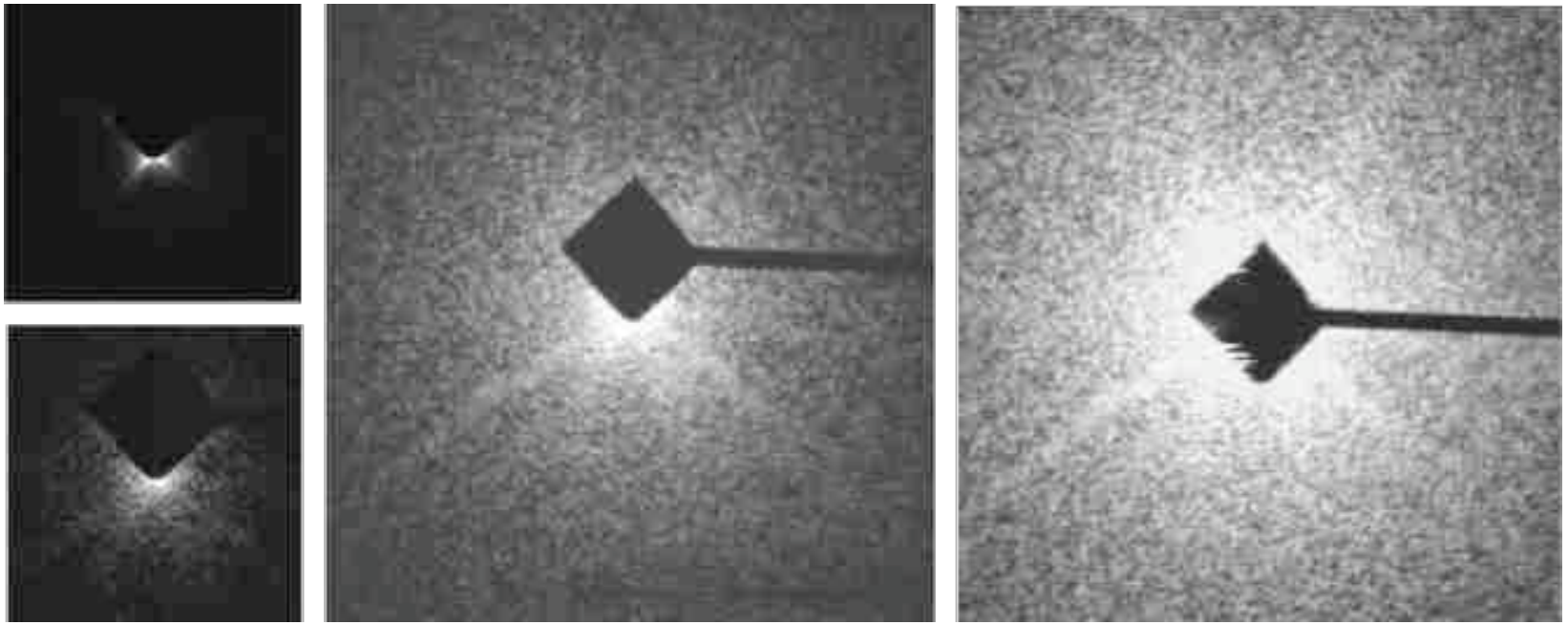
- "Point projection" microscope from moving coherence pinhole upstream.
- Not a good way to evaluate preparations; poor feedback loop!
- Future solution: build a crude scanning microscope into apparatus



Yeast cells on a grid

Dealing with the CCD limits

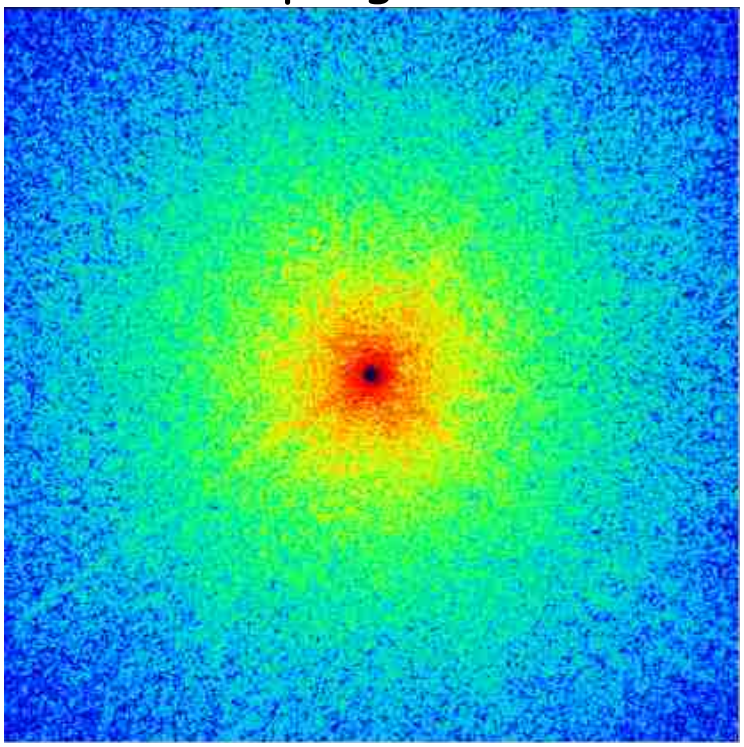
- ~Direct detection: 100 e^-/photon; ~10^5 e^- full well capacity; ~10^3 dynamic range.
- Diffraction data drops off $\propto f^{-4}$
- Move beamstop and merge exposures

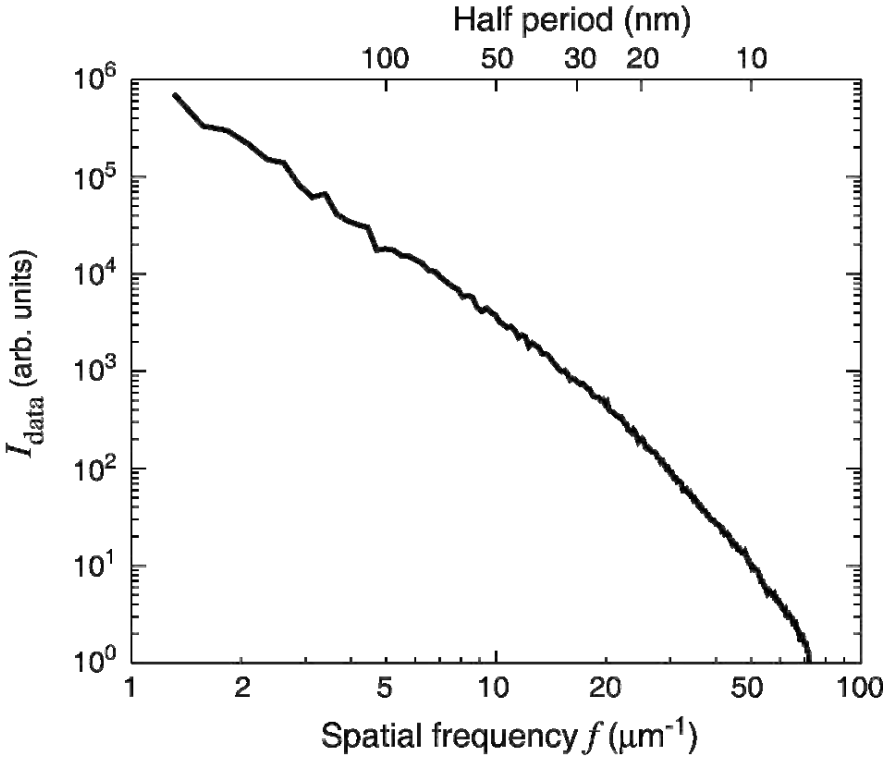


Future: use a "Tower of Hanoi" beamstop?

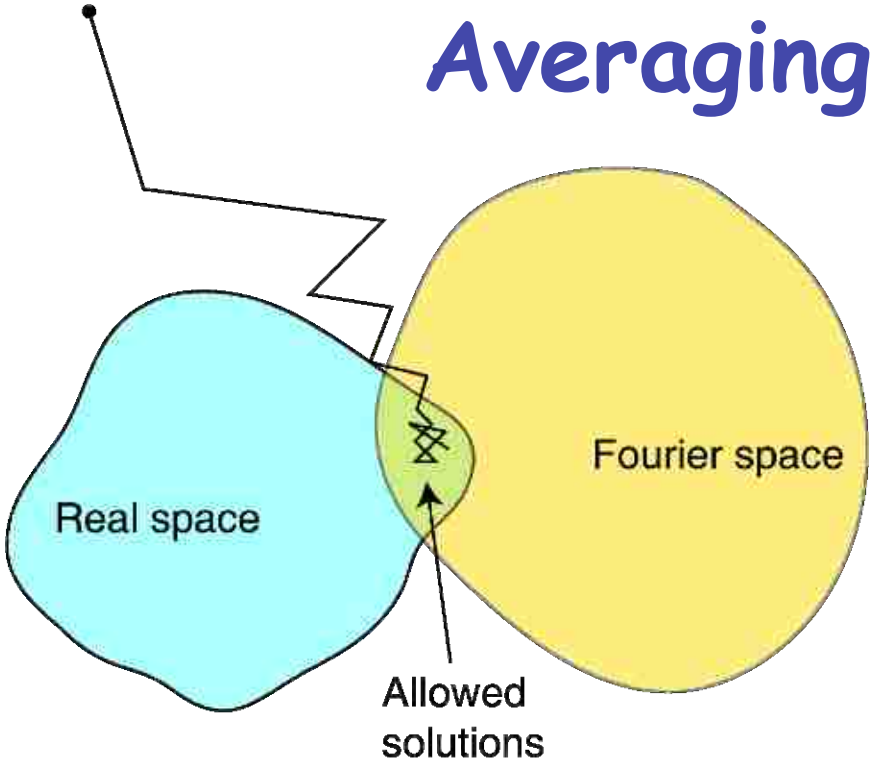
Diffraction from a yeast cell

- ALS beamline 9.0.1 operated at 750 eV
- Total dose to freeze-dried, room temperature cell around 10^8 Gray
- Oversampling ratio is about 9 in each dimension

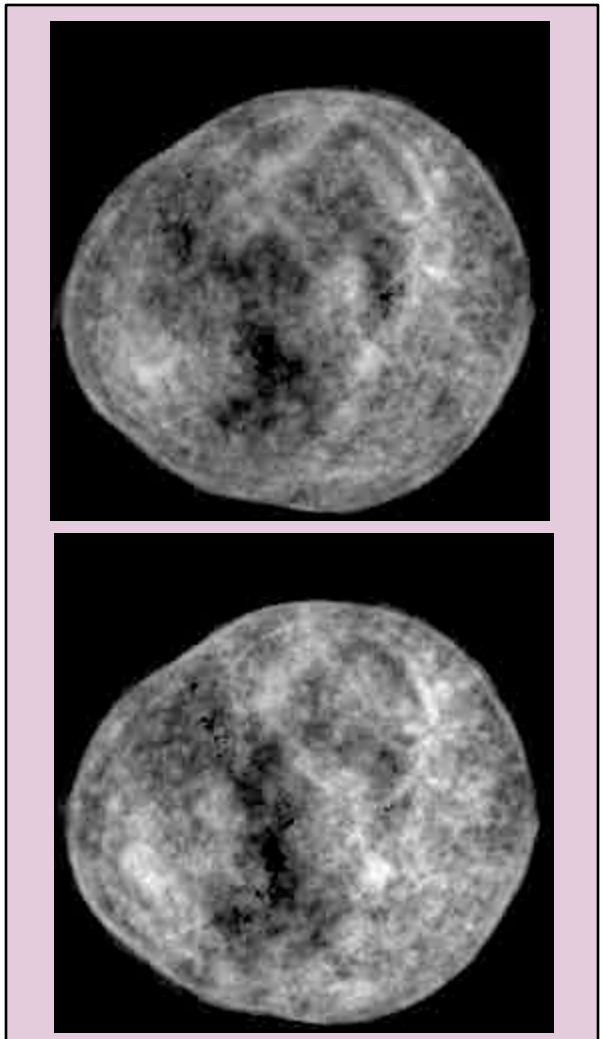




Averaging iterates



- Ideally, there is a single point of best agreement between various constraints.
- Noise and incomplete constraints only provide a neighborhood of acceptable solutions. Choosing one iterate is unwarranted.
- Averaging many iterates:
  - Reproducible information is reinforced
  - Non-reproducible information is suppressed
- Elser and Thibault, Cornell

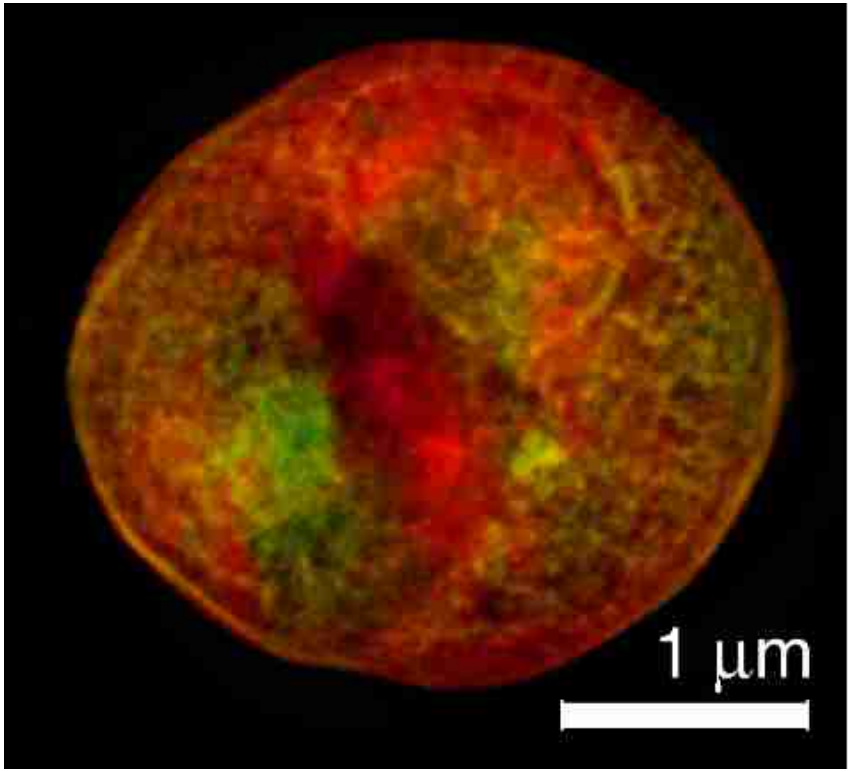


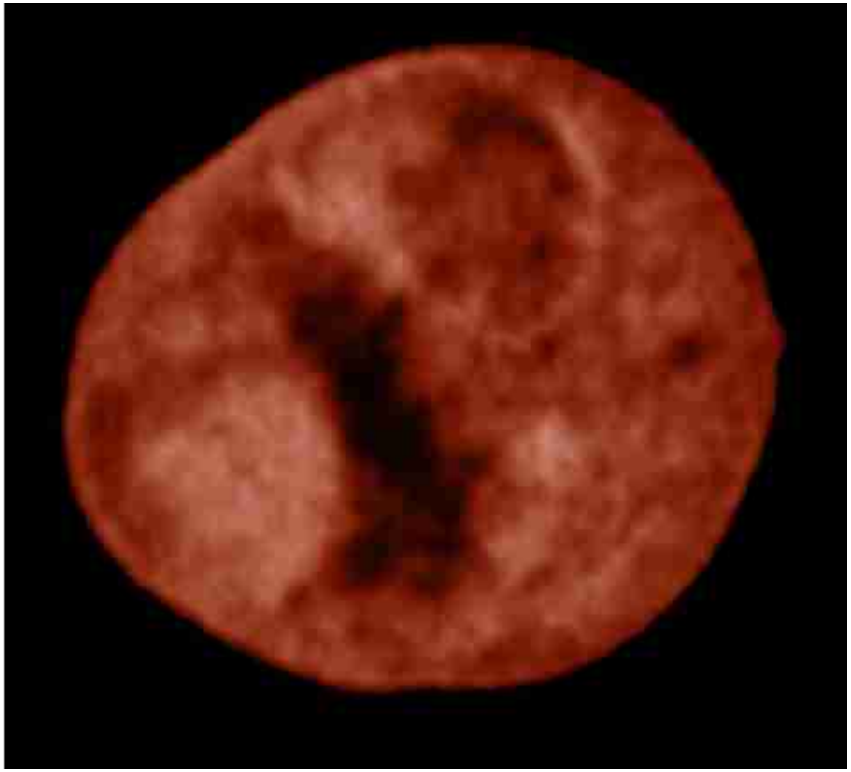
Two individual iterates

Reconstruction

- Done first by Thibault and Elser; then by Shapiro, Lima
- Difference map algorithm
- Hand-drawn tight support based on autocorrelation and reconstruction attempts
- Shrink-wrap was not used because it started to "eat into" the cell but this could probably be improved.

Comparison with a microscope





Diffraction reconstruction (data taken at 750 eV; absorption as brightness, phase as hue).

Stony Brook/NSLS STXM image with 45 nm Rayleigh resolution zone plate at 520 eV (absorption as brightness)

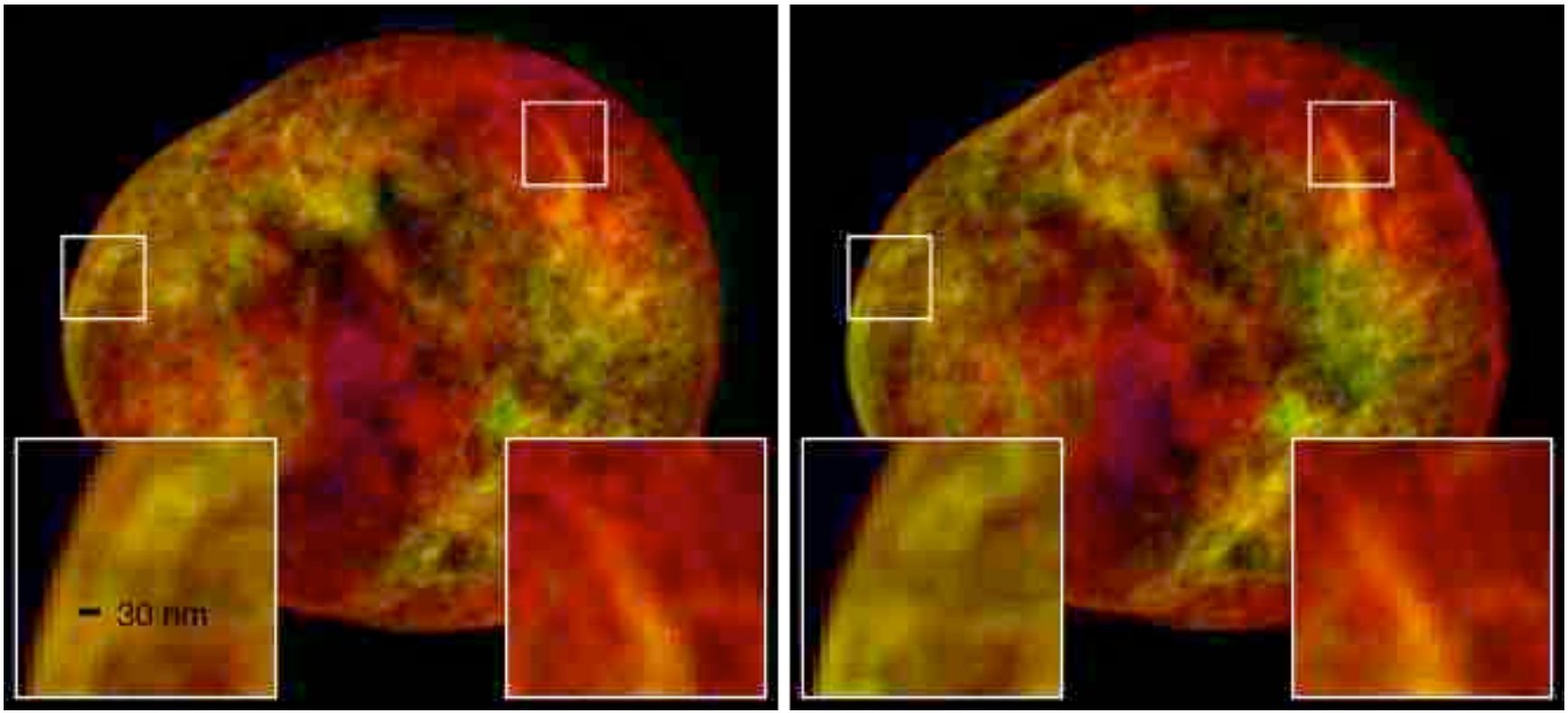
Using color to represent phase

(Porquerolles)

Are we only seeing things we want to see?

(HMS Rhone, British Virgin Islands)

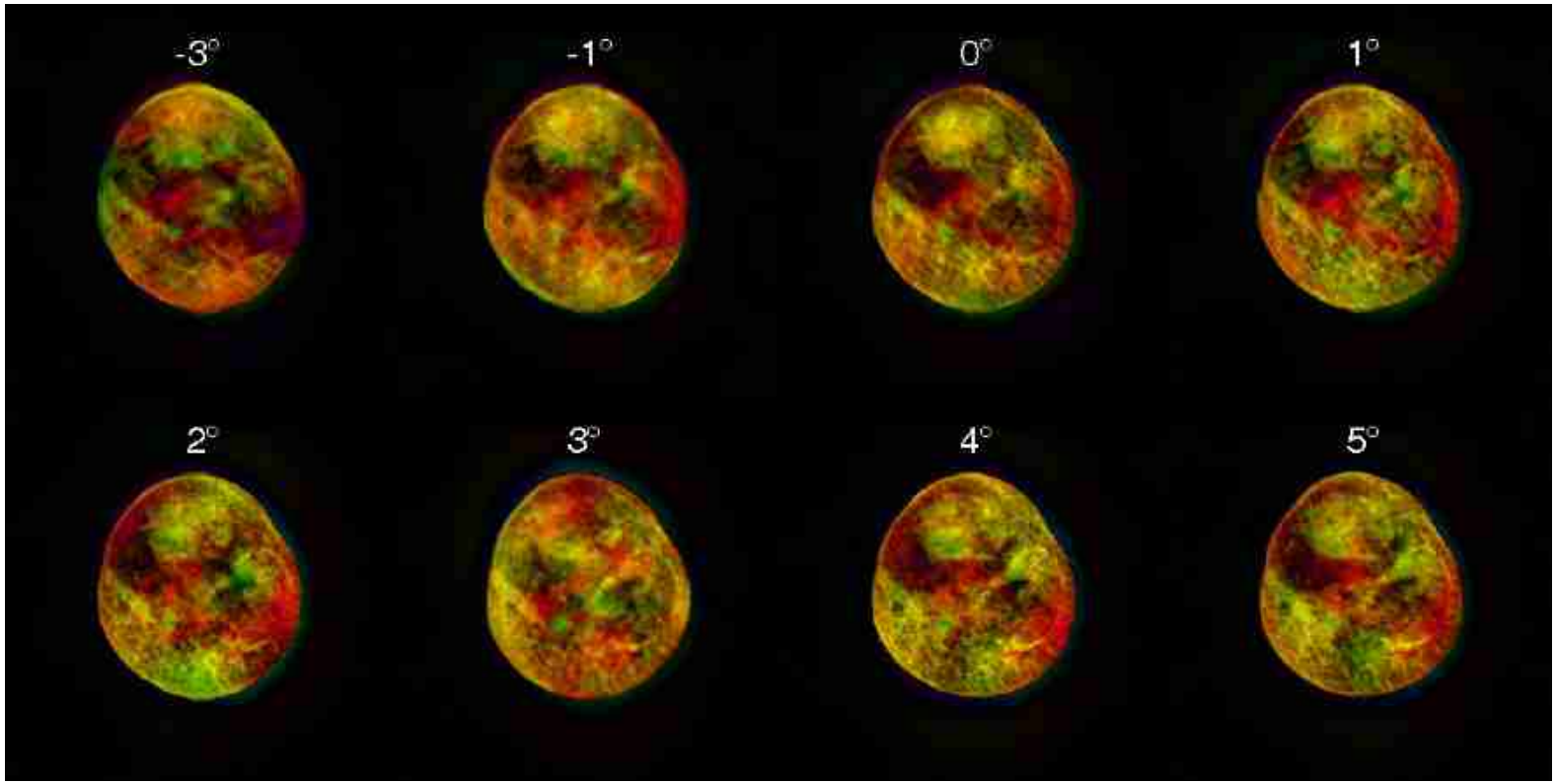
Data taken 1° apart



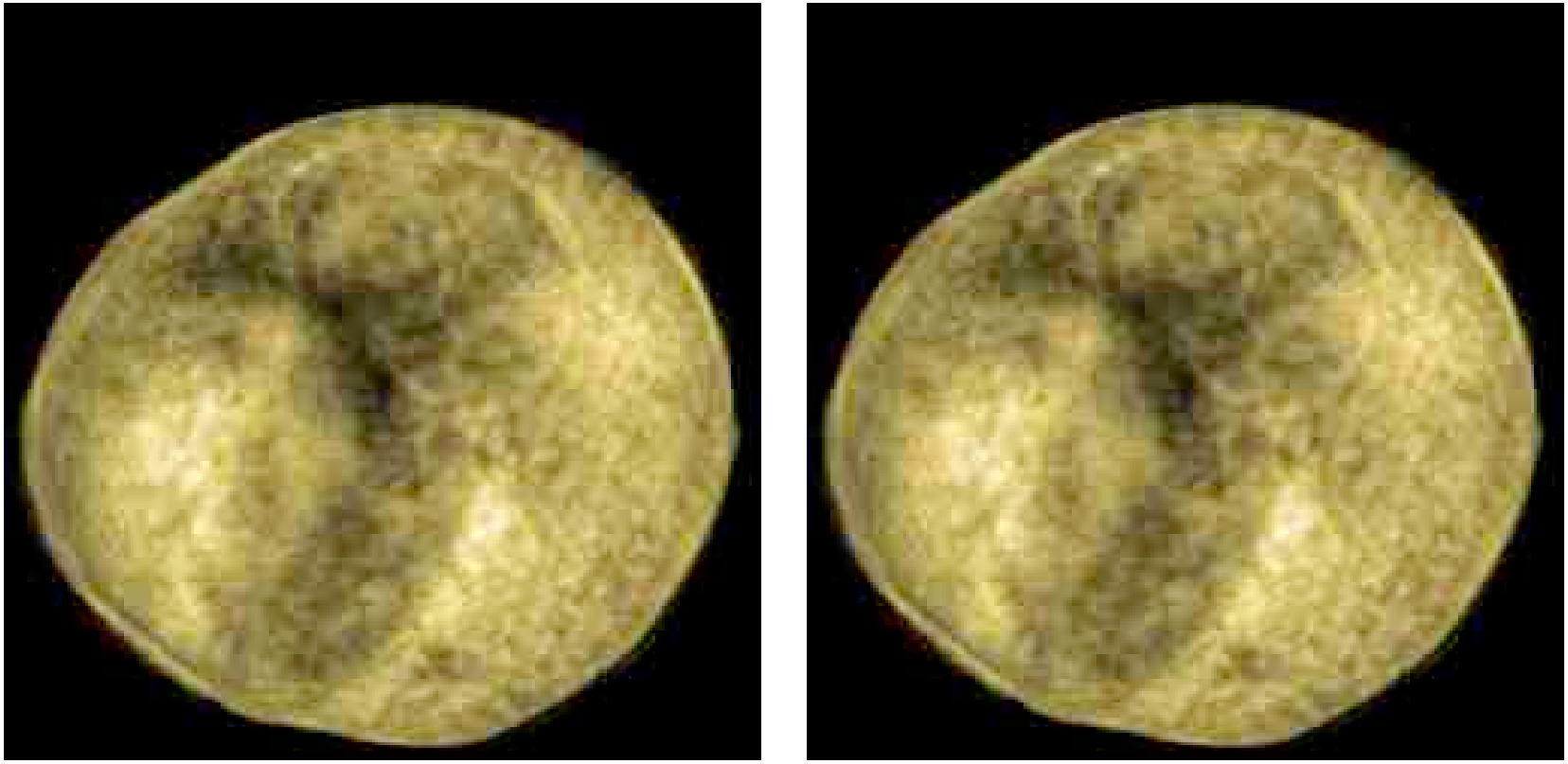
Separate reconstructions of separate data sets give reconstructions that are quite consistent

Tilt series

This sample: only an 8° tilt range. 2D reconstructions on each projection.



Different starting random phases



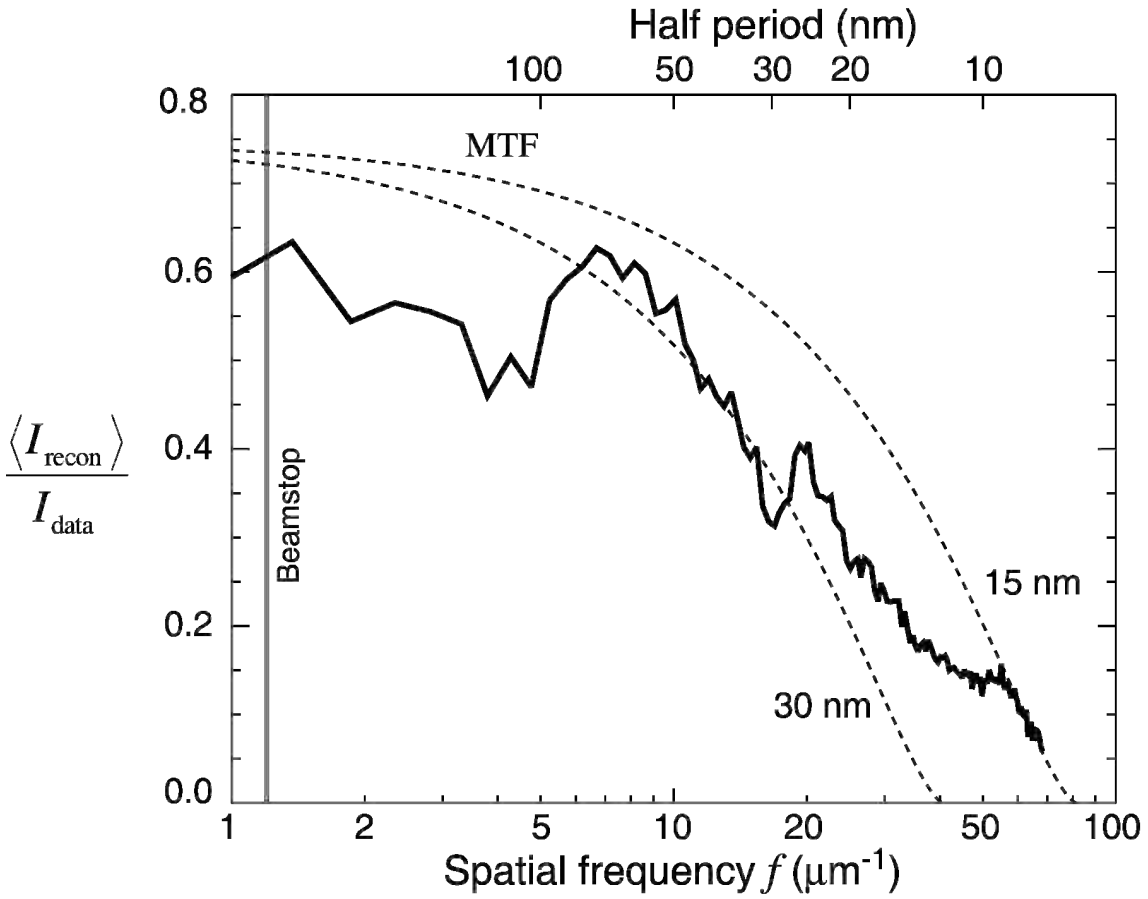
Two separate runs of algorithm with different random starting phases. In both cases, 125 iterates spaced 40 iterations apart were averaged (E. Lima).

Diving deeper into reconstructions

(Erik Jacobsen, HMS Rhone, British Virgin Islands)

What is the resolution?

- We do not claim maximum angle of speckles as the resolution of the reconstructed image
- Instead, one can look at how much iterate preserves Fourier intensities at various spatial frequencies – like an MTF
- Elser and Thibault, Cornell



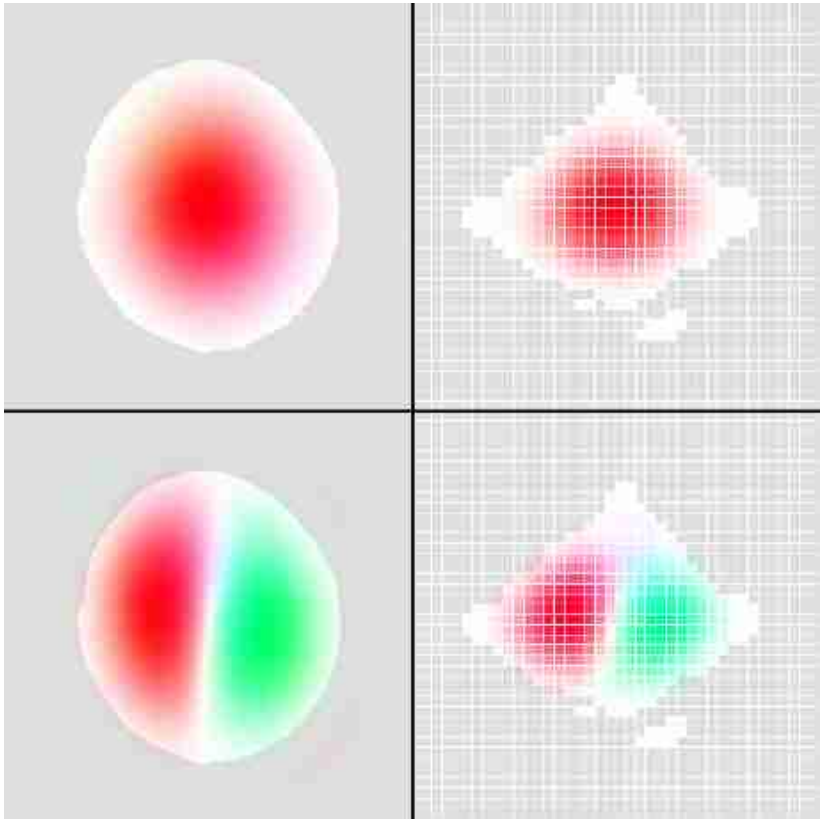
Problems we wish we didn't have…

(The IGeSA beach)

Have my jellyfish! I'm not sellyfish… Ogden Nash

We miss those low frequencies…

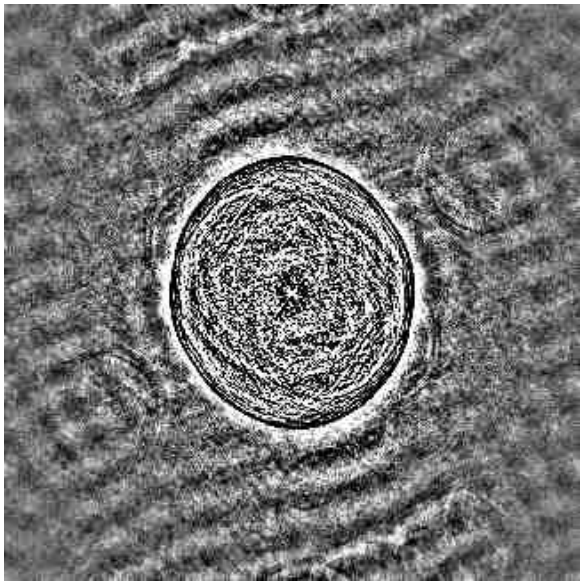
Unconstrained modes in the reconstruction. See poster by Thibault et al.



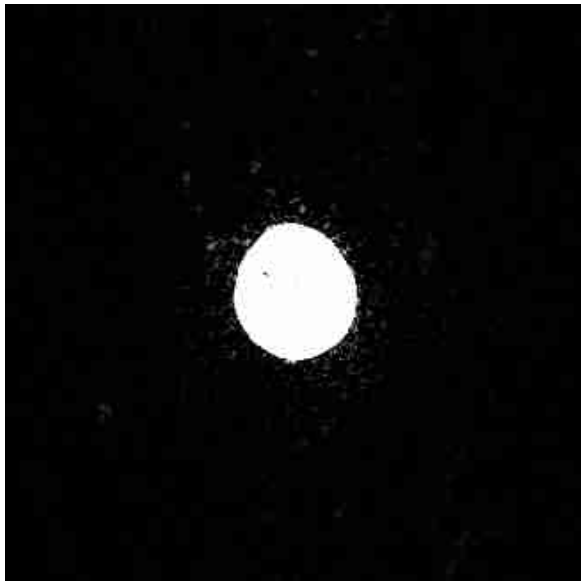
Real space Fourier space

Is it really empty outside the support?

(Elser and Thibault, Cornell)



Real part of the (Laplacian filtered) autocorrelation of the yeast cell data showing evidence for the presence of dirt around the cell.

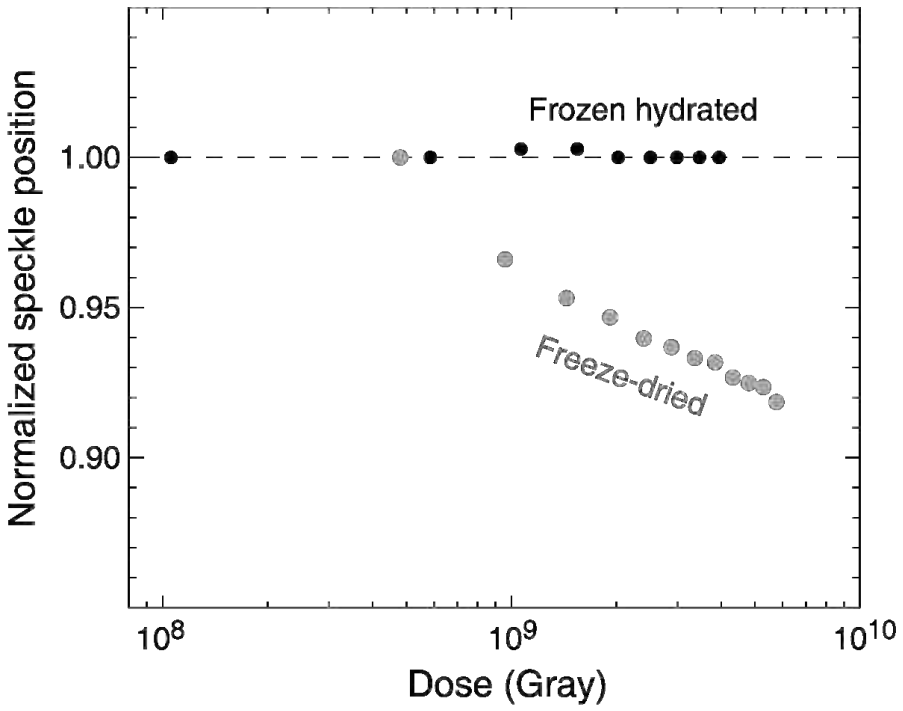


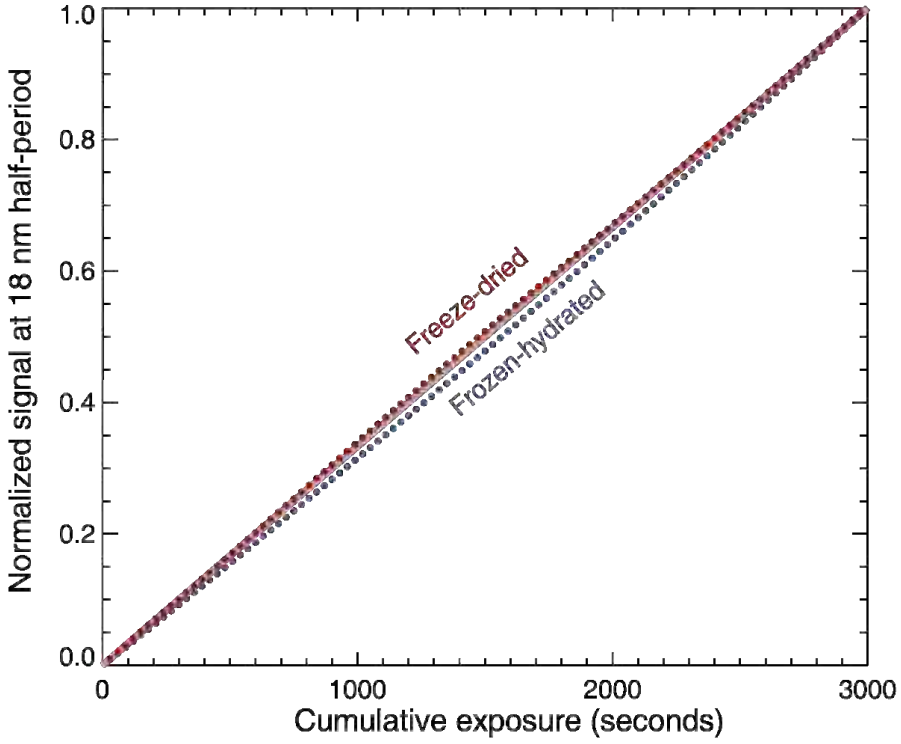
"Dirt" recovered around the yeast cell. A threshold-based support has been added to the fixed support of the cell.

Problems looming in the distance…

(Wreck Alley, British Virgin Islands)

Frozen hydrated?



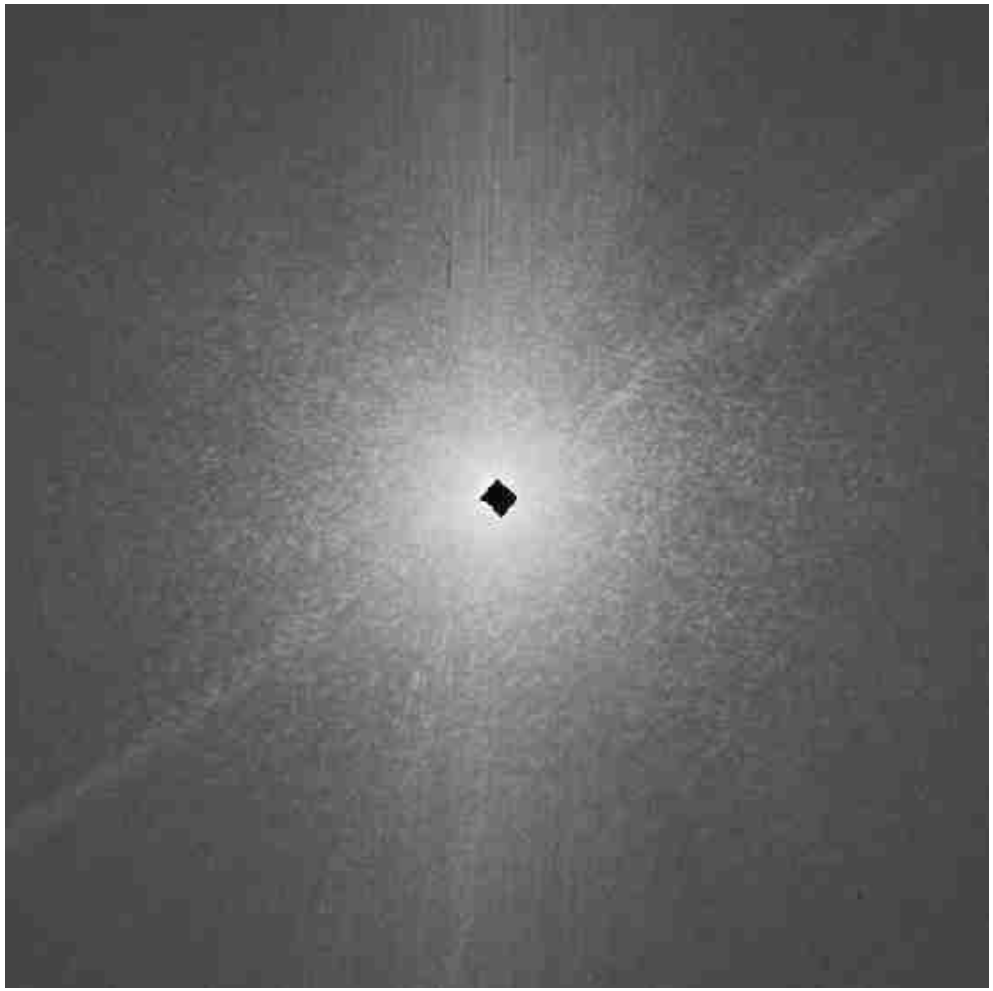


Frozen hydrated specimens don't shrink in the beam (freeze-dried specimens do)

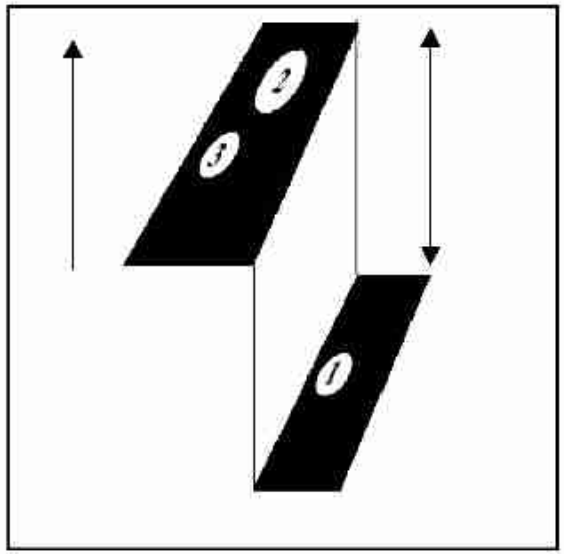
Scattering power is linear with dose thus far in both cases

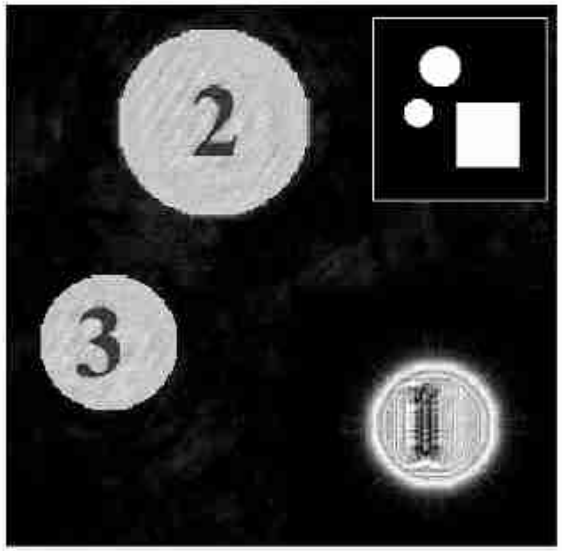
Frozen hydrated!

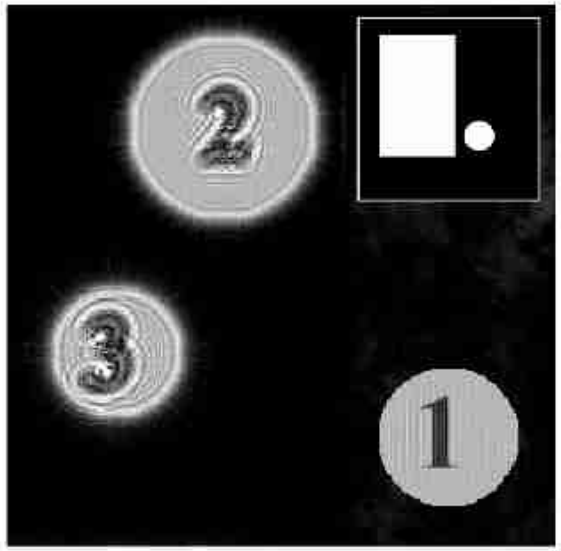
Specimen preparation has been challenging… E. Lima and X. Huang, May 2005, at 520 eV.



In 2D, what depth plane are we reconstructing?



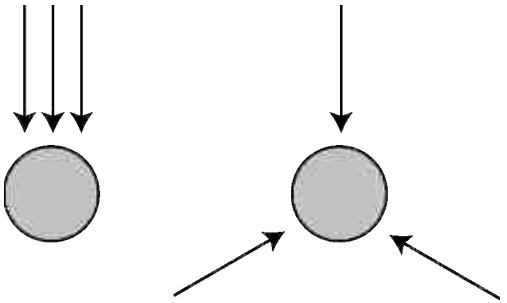




Spence et al., Phil. Trans. R. Soc. London A **360**, 875 (2002)

3D reconstructions

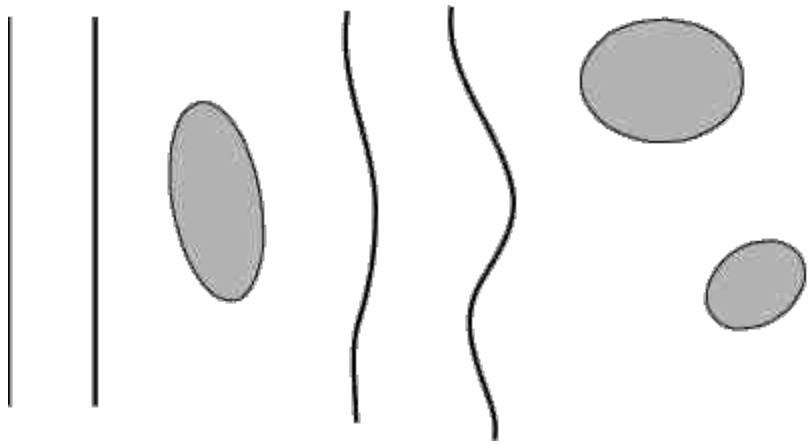
- Thanks to Chapman et al., we know it will work on our apparatus!
- Thanks to "dose fractionation", we know we don't need many more photons. Hegerl and Hoppe, Z. Naturforschung **31a**, 1717 (1976); McEwen et al., Ultramic. **60**, 357 (1995).



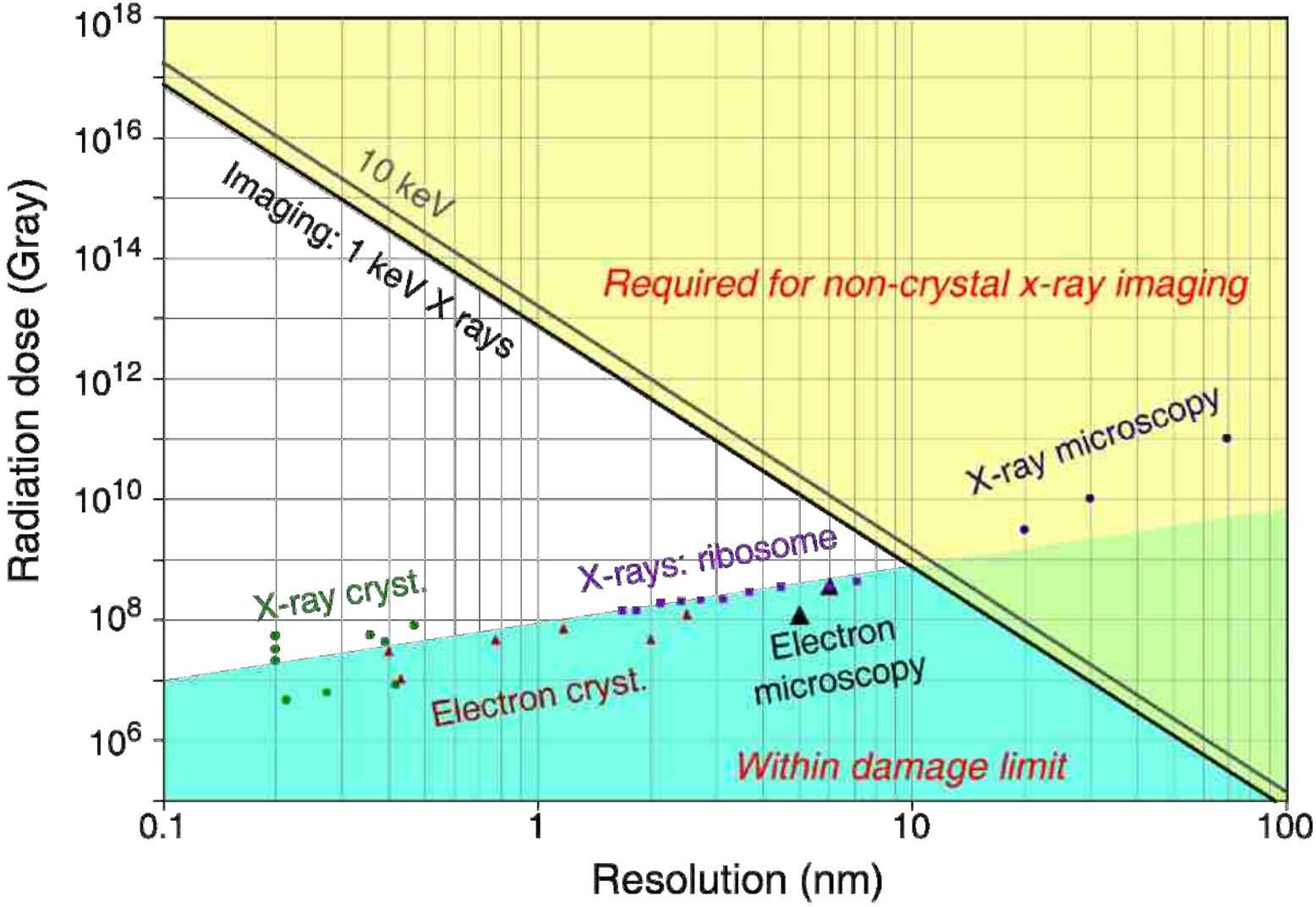
- We need to do more to automate our data taking, and work around CCD dynamic range limits (Tower of Hanoi?)
- Iterative phasing works better in 3D!
- A voxel is thinner than a pixel and thus less complex

Will we need to be Born again?

- Illumination of downstream planes can be corrupted by upstream objects
- Multislice calculations can be used to evaluate this problem; see poster by Thibault et al.
- Going to higher photon energies will help, though contrast then goes down



What's the limit for cells?



Howells et al., JESRP (submitted)

Thanks for your attention!

(HMS Rhone, British Virgin Islands)

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