X-ray Microscopy

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Wilhelm Röntgen
Universität Würzburg
Dec. 1895

Michael Pupin
Columbia University/New York
Feb. 1896

"This is of the hand of a gentleman resident in New York, who, while on a hunting trip in England a few months ago, was so unfortunate as to discharge his gun into his right hand, no less than forty shot lodging in the palm and fingers. The hand has since healed completely; but the shot remain in it, the doctors being unable to remove them, because unable to determine their exact location. The result is that the hand is almost useless, and often painful." - Cleveland Moffett, McClure's Magazine, April 1896

The X-ray Microscope

It would be a big improvement on microscopes using light or electrons, for X-rays combine short wavelengths, giving fine resolution, and penetration. The main problems standing in the way have now been solved.

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THE X-RAY MICROSCOPE by Paul Kirkpatrick
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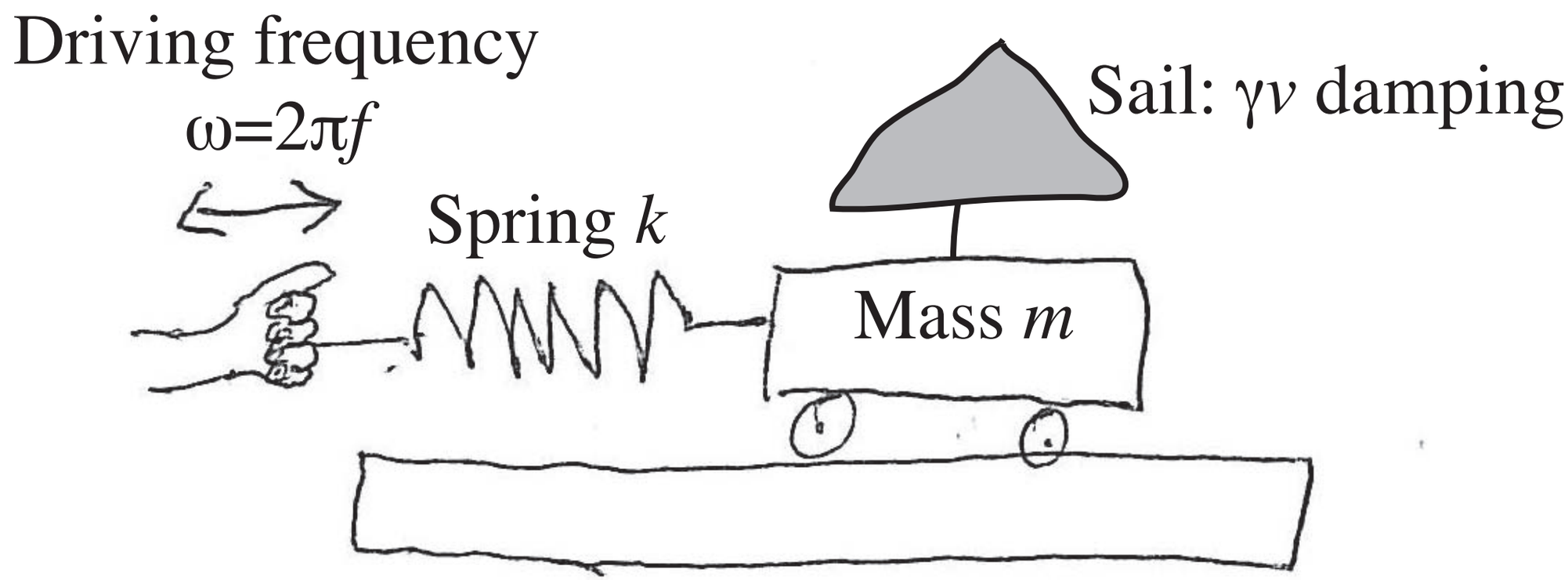
The refractive index

- Damped, driven harmonic oscillator



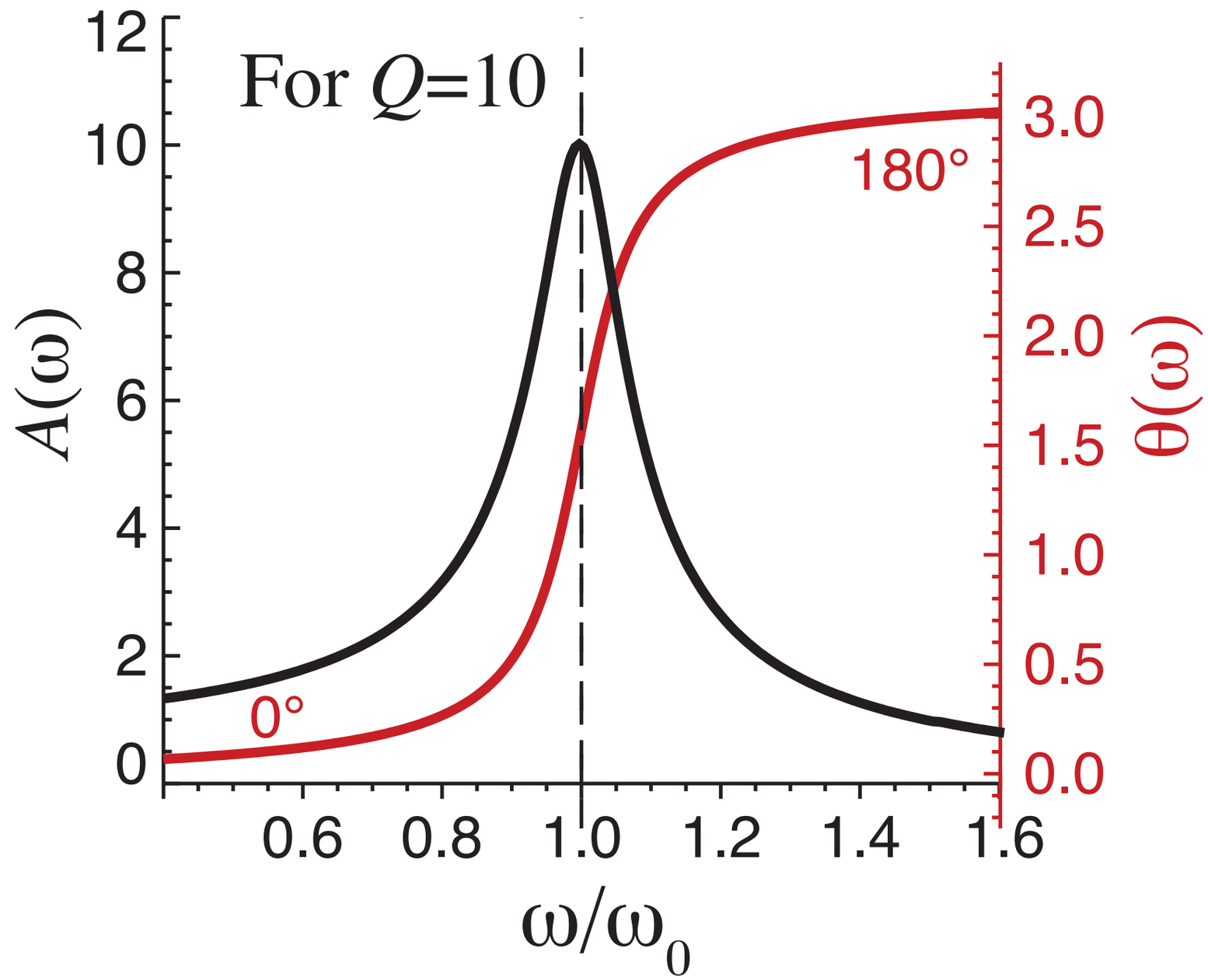
- Damped: scattering, absorption
- Driven: incident electromagnetic wave ω
- Harmonic oscillator: electronic quantum state with energy

$$\hbar\omega = \hbar\sqrt{k/m}$$

Damped, driven harmonic oscillator

- Single resonance: absorption peak, phase shift across resonance

$$Q = \omega_0/\gamma$$

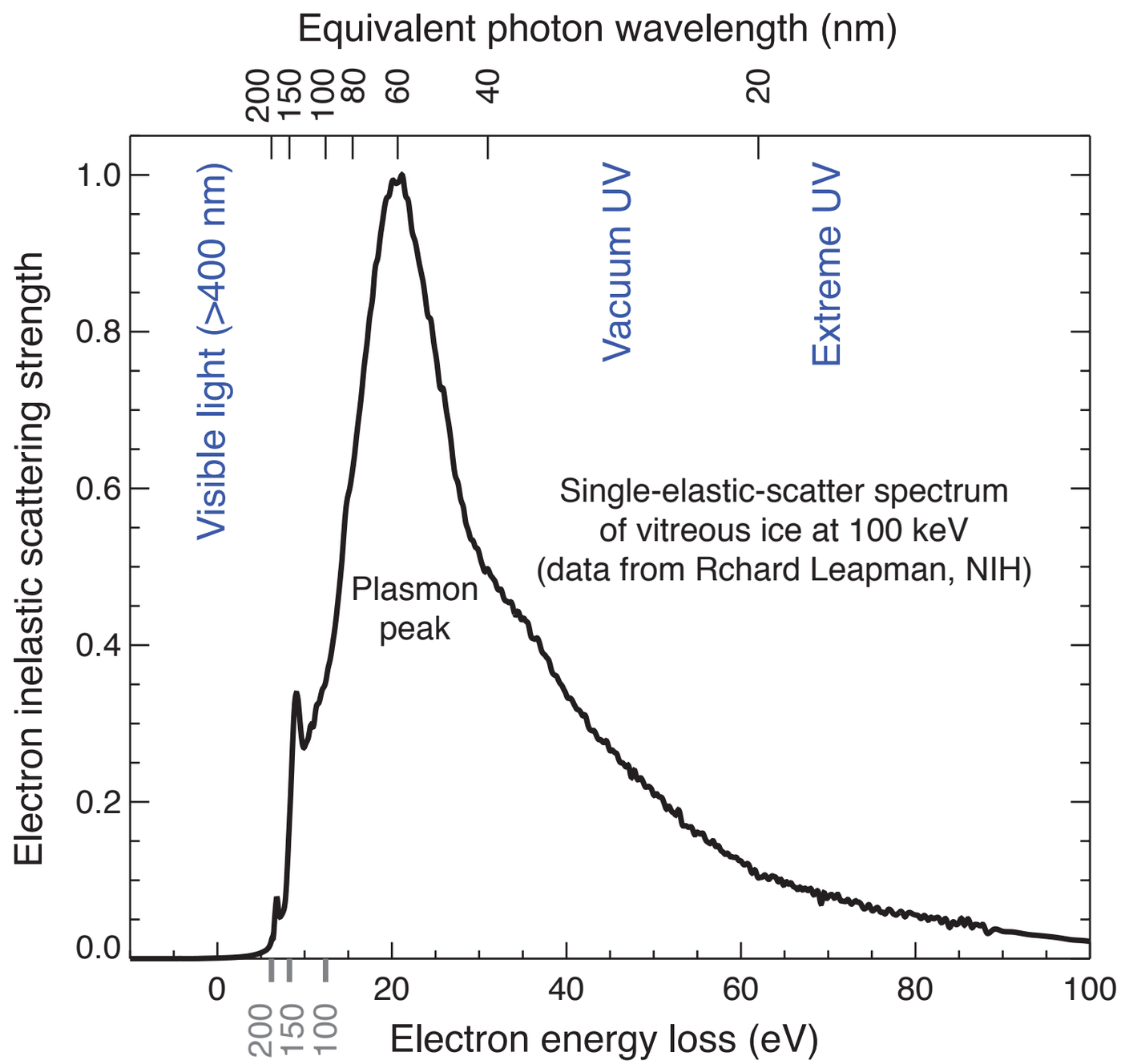


X-rays: the high frequency limit?

What's the dividing line between low and high frequency limits of refractive index? At what frequency are most of the oscillators?

Plasmon frequency

$\omega_p=(4\pi c^2 n_a r_e)^{1/2}$



Mysteries of the x-ray refractive index

Write refractive index as

$$n = 1 - \frac{n_a r_e}{2\pi}\lambda^2(f_1 + i f_2)$$

$$= 1 - \alpha\lambda^2(f_1 + i f_2)$$

where n_a=# atoms/volume, and r_e=2.818x10^{-15} m is the classical radius of the electron. Assumes exp[-$i(kx$-$\omega t)$] for forward propagation.

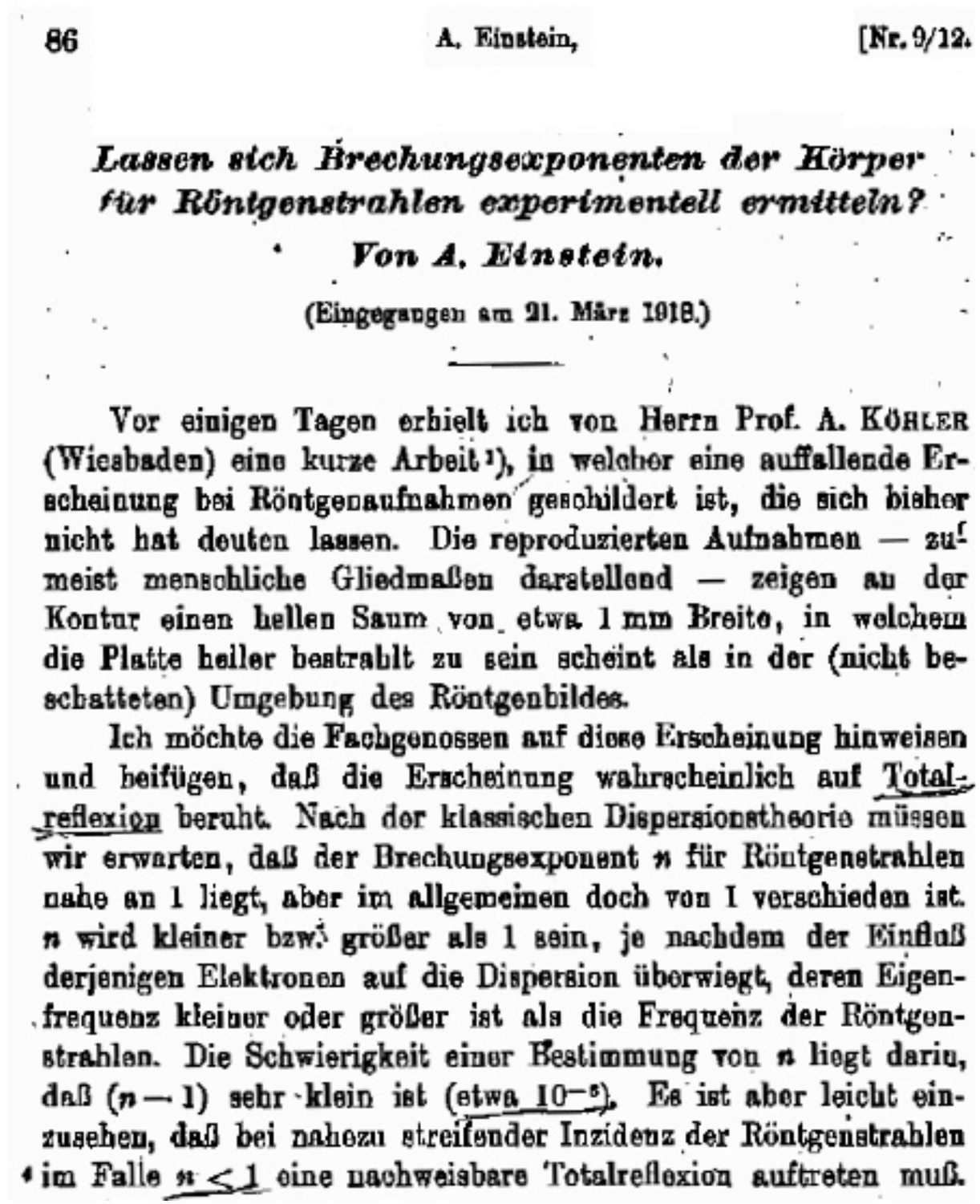
Also written as n=1-δ-iβ

Phase velocity is

$$v_p = \frac{\omega}{k} \simeq c(1 + \alpha\lambda^2 f_1)$$

Group velocity is

$$v_g = \frac{d\omega}{dk} \simeq c(1 - \alpha\lambda^2 f_1)$$



86 A. Einstein, [Nr. 9/12.

Lassen sich Brechungsexponenten der Körper für Röntgenstrahlen experimentell ermitteln?

Von A. Einstein.

(Eingegangen am 21. März 1918.)

Vor einigen Tagen erhielt ich von Herrn Prof. A. Köhler (Wiesbaden) eine kurze Arbeit[1]), in welcher eine auffallende Erscheinung bei Röntgenaufnahmen geschildert ist, die sich bisher nicht hat deuten lassen. Die reproduzierten Aufnahmen — zumeist menschliche Gliedmaßen darstellend — zeigen an der Kontur einen hellen Saum von etwa 1 mm Breite, in welchem die Platte heller bestrahlt zu sein scheint als in der (nicht beschatteten) Umgebung des Röntgenbildes.

Ich möchte die Fachgenossen auf diese Erscheinung hinweisen und beifügen, daß die Erscheinung wahrscheinlich auf Totalreflexion beruht. Nach der klassischen Dispersionstheorie müssen wir erwarten, daß der Brechungsexponent n für Röntgenstrahlen nahe an 1 liegt, aber im allgemeinen doch von 1 verschieden ist. n wird kleiner bzw. größer als 1 sein, je nachdem der Einfluß derjenigen Elektronen auf die Dispersion überwiegt, deren Eigenfrequenz kleiner oder größer ist als die Frequenz der Röntgenstrahlen. Die Schwierigkeit einer Bestimmung von n liegt darin, daß $(n-1)$ sehr klein ist (etwa 10^{-5}). Es ist aber leicht einzusehen, daß bei nahezu streifender Inzidenz der Röntgenstrahlen im Falle $n < 1$ eine nachweisbare Totalreflexion auftreten muß.

X-ray refractive index

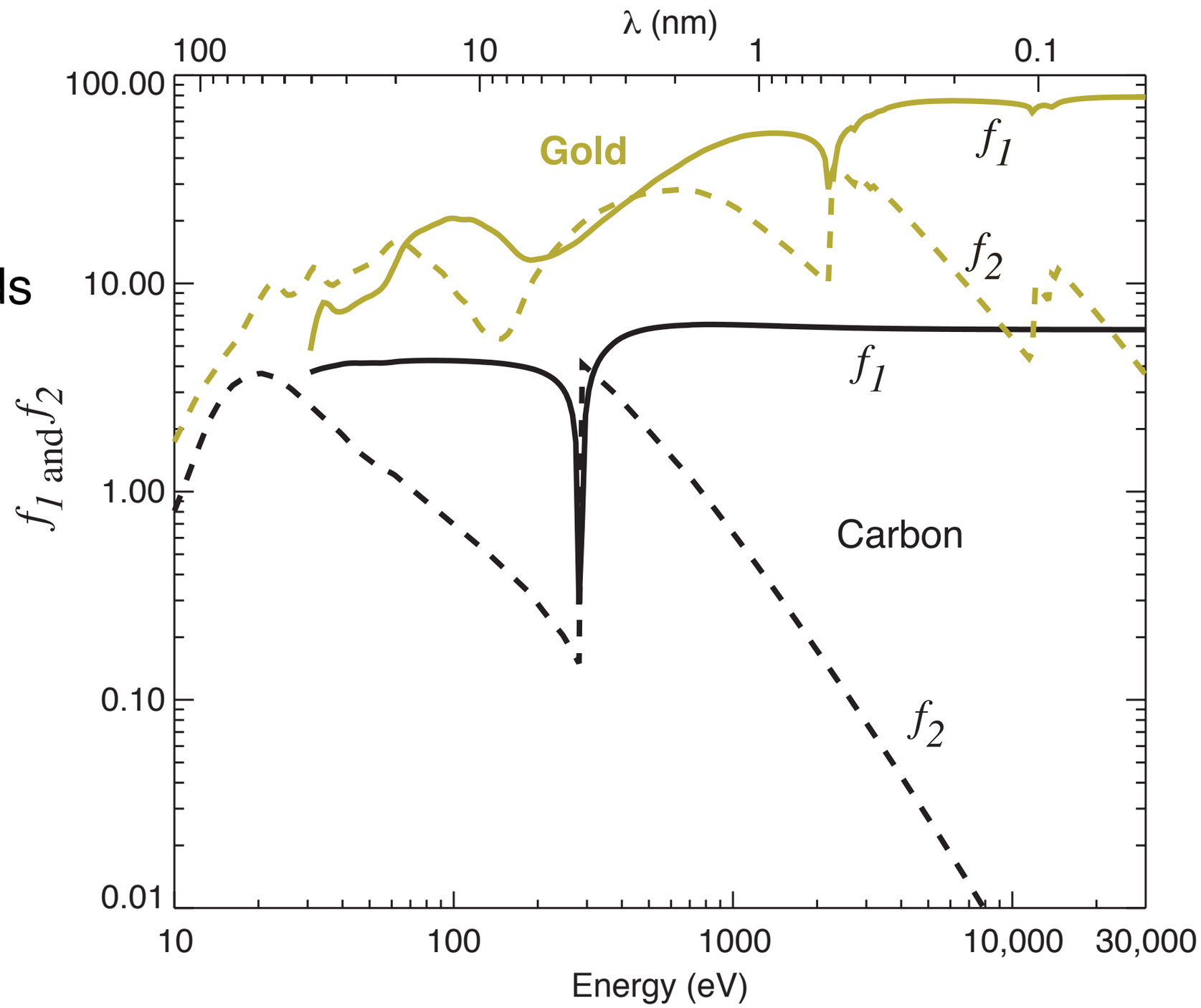
Refractive index of
$n=1-\alpha\lambda^2(f_1+if_2)$

Real part of oscillator strength f_1 tends towards atomic number Z

Imaginary part of oscillator strength f_2 declines as E^{-2}

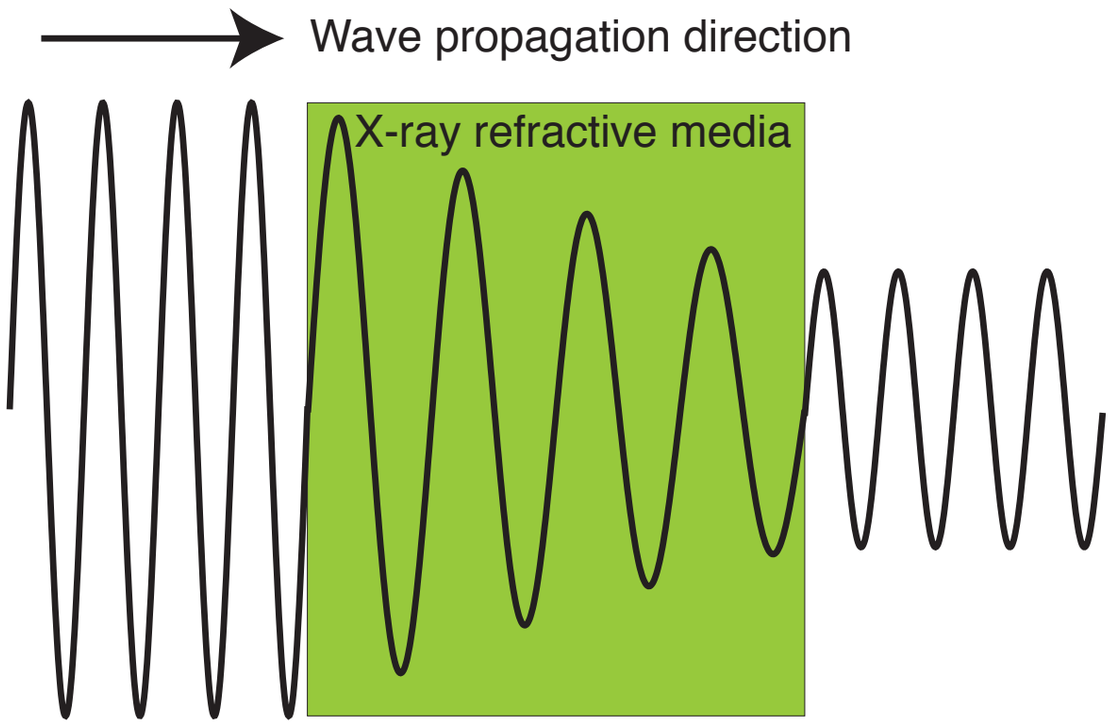
Phase exp[$-ink$] is advanced relative to vacuum by $2\pi\alpha\lambda f_1$

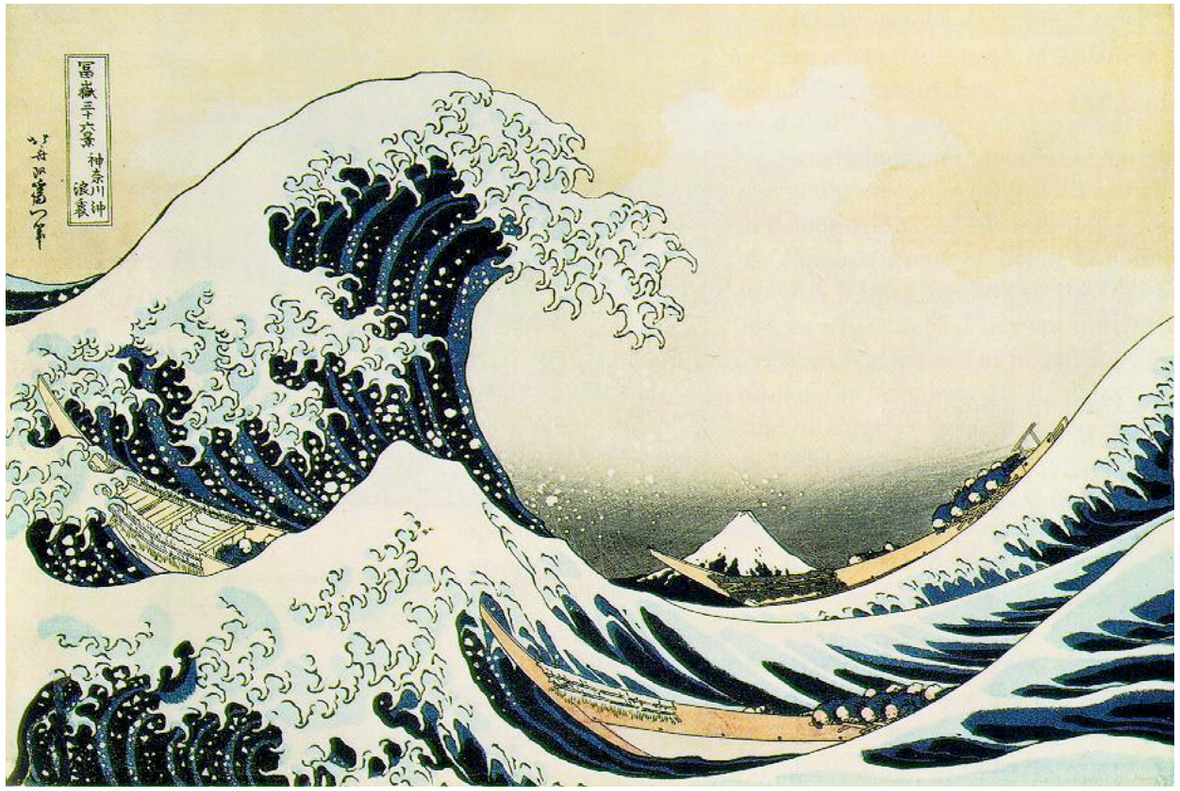
Intensity is decreased as exp[$-4\pi\alpha\lambda f_2$]



Data from http://henke.lbl.gov/optical_constants/

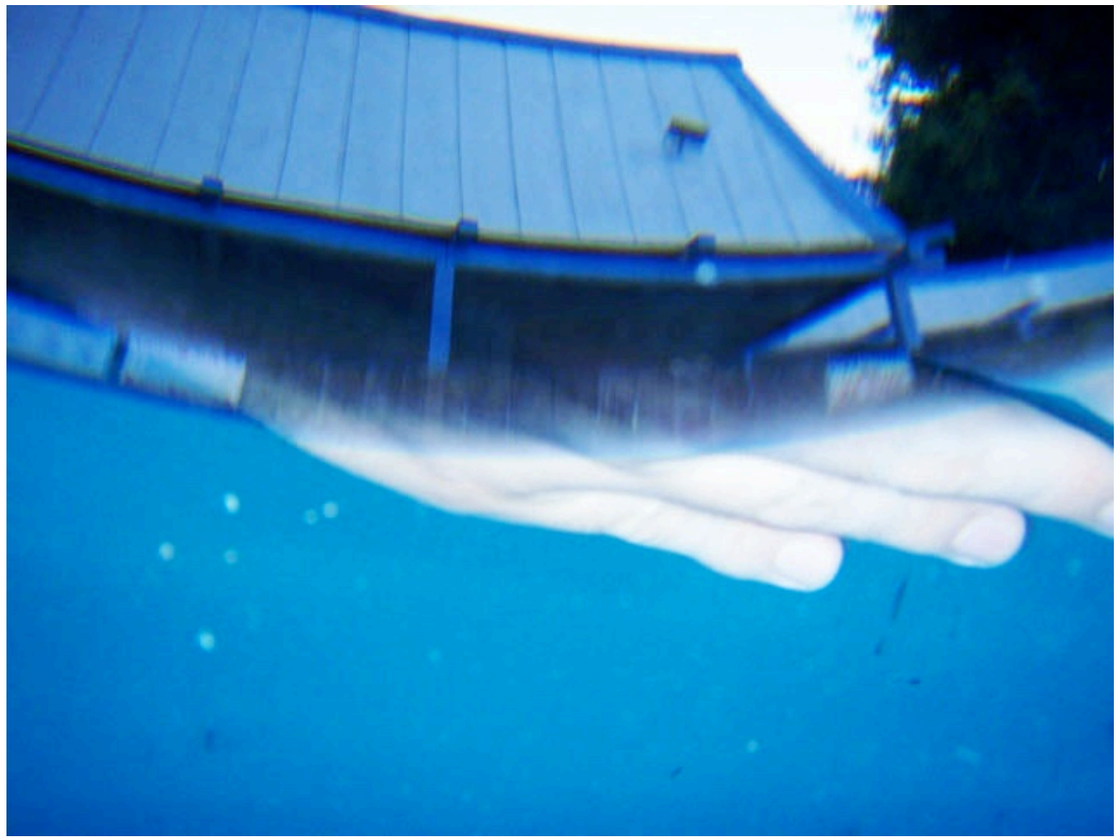
X rays in media

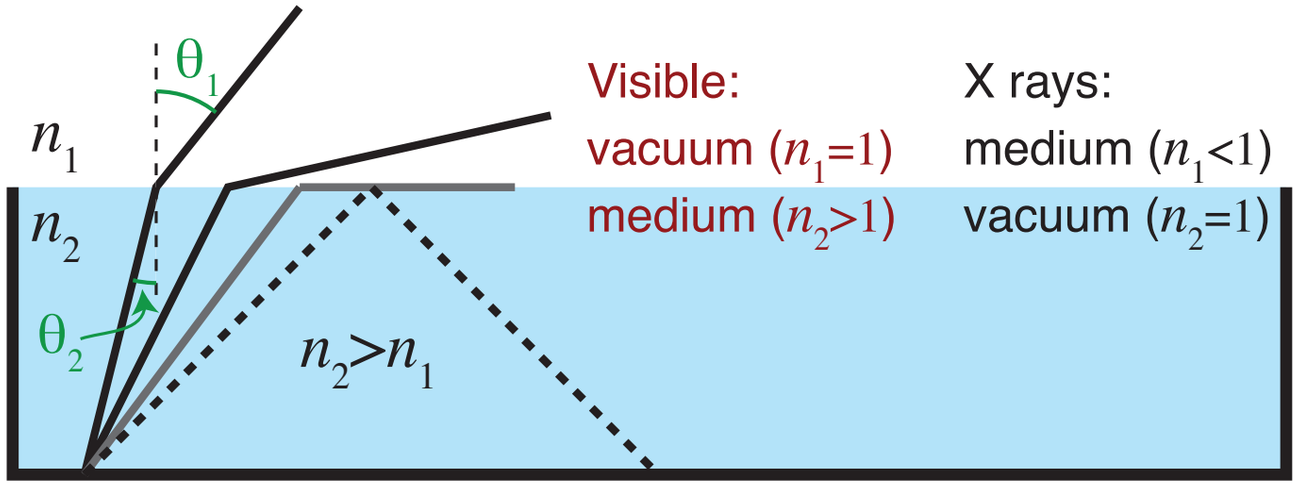


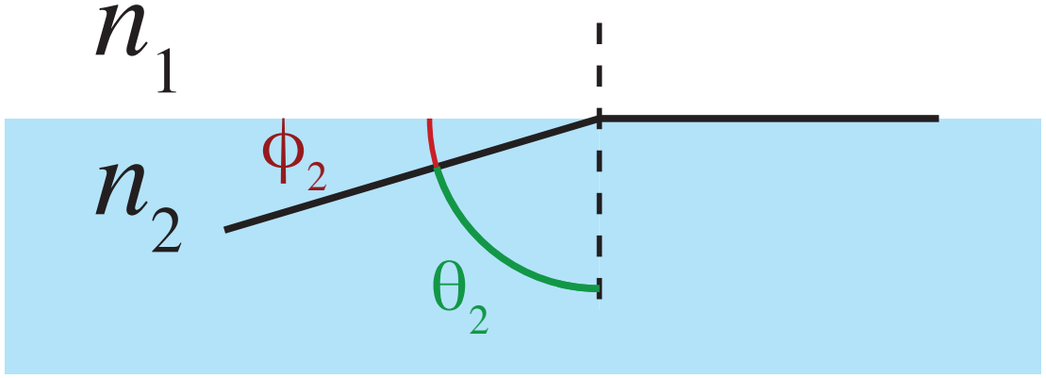


X-ray mirrors use total *internal* reflection!

- Total internal reflection happens when θ_1=90° in $n_1\sin\theta_1=n_2\sin\theta_2$, or $\theta_2=\mathrm{asin}(n_1/n_2)$.

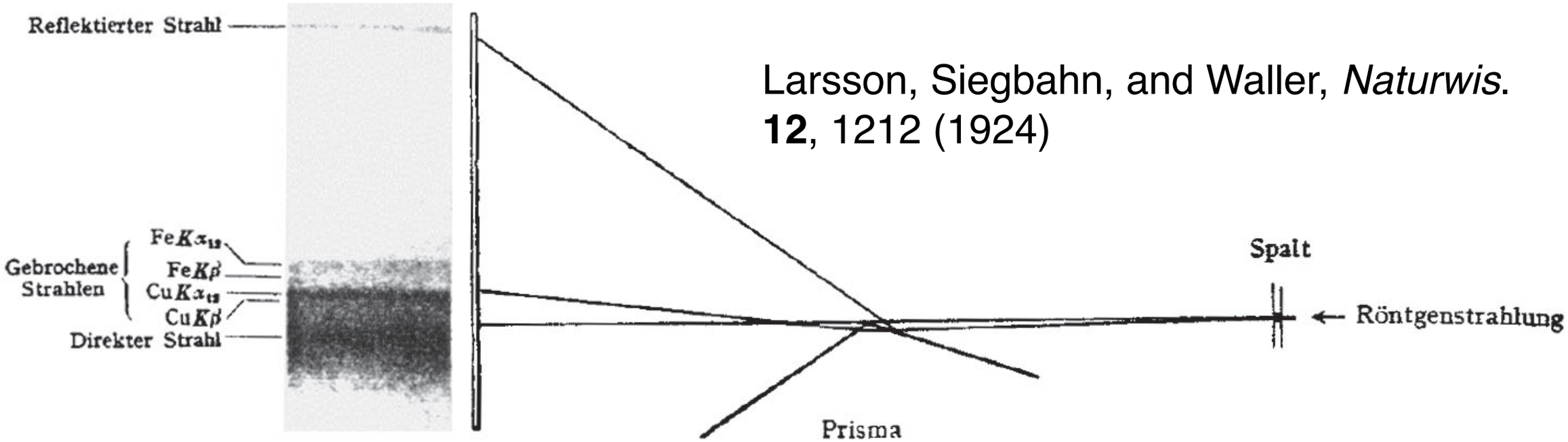




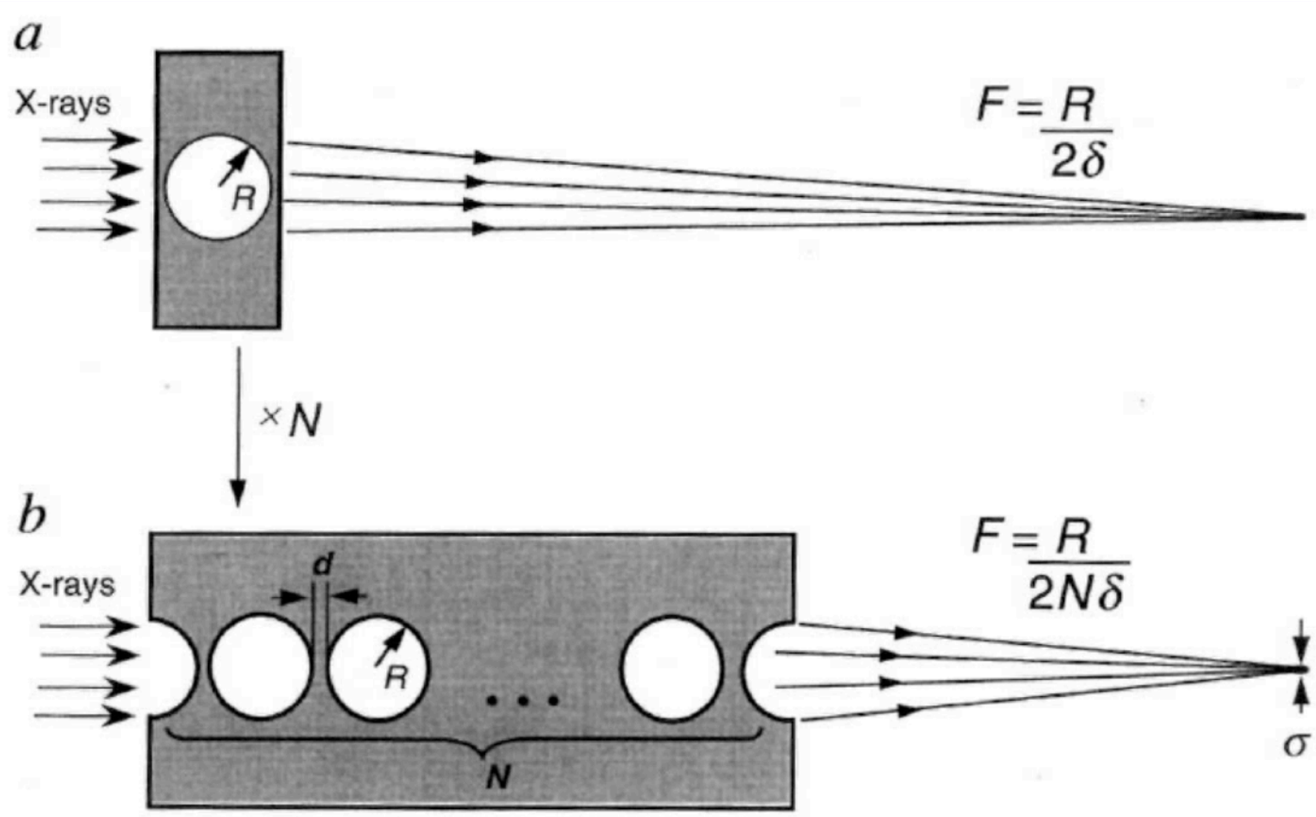


- Switch from angle θ_2 relative to normal incidence, to angle ϕ_2 relative to grazing incidence, or $\sin(\theta_2)=\cos(90°-\phi_2)=\sin(\phi_2)$
- We then have $n_1=n_2\sin(\phi_2)$ or with $n_2=1$ and $\sin(\phi_2)\approx1-(\phi_2)^2/2$ we have a grazing incidence critical angle of $\phi_2=\lambda(2\alpha f_1)^{1/2}$
- Note diffraction resolution limit of ϕ_2/λ is (almost) independent of wavelength!

X-ray refraction

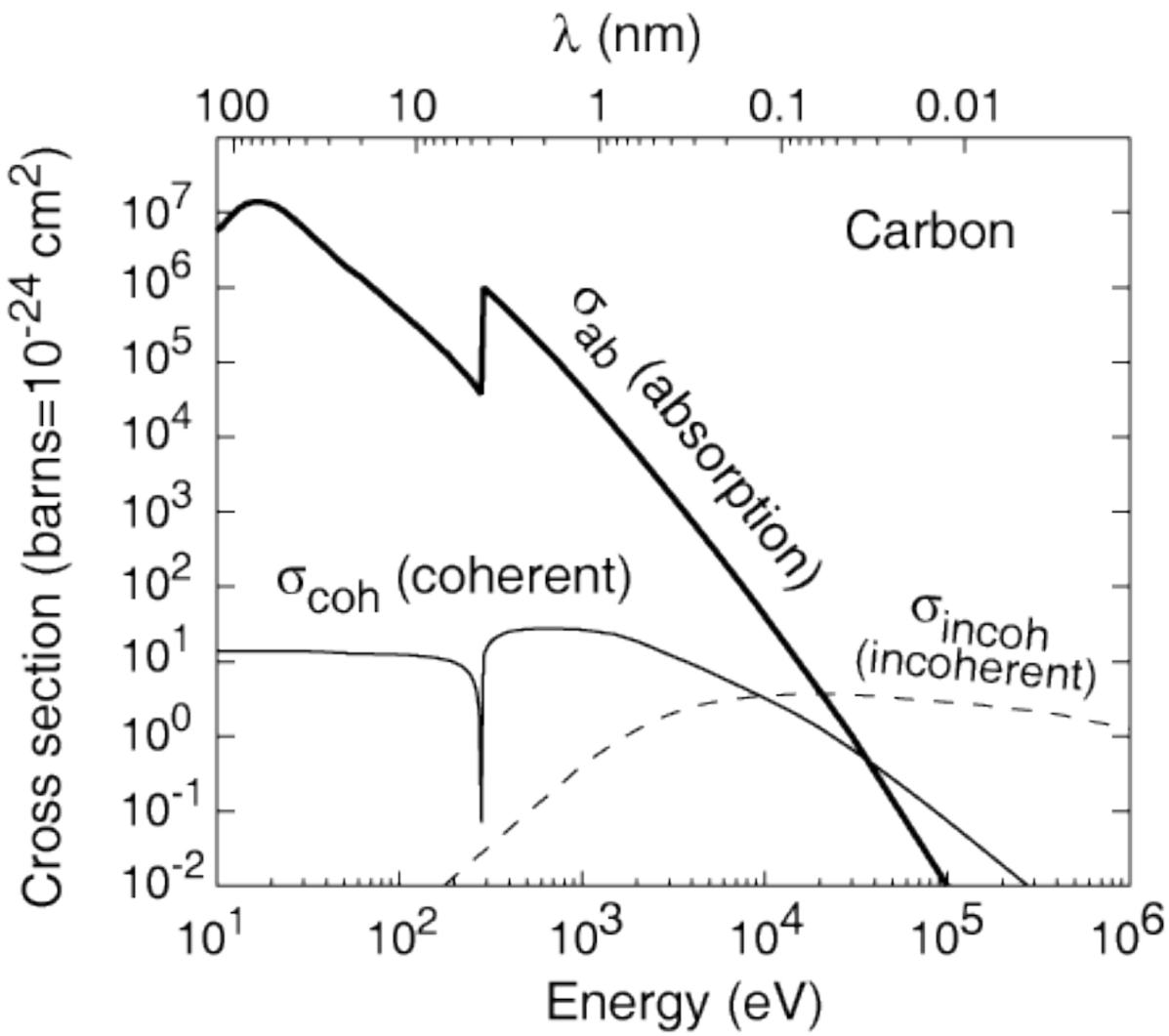


Larsson, Siegbahn, and Waller, *Naturwis.* **12**, 1212 (1924)



Snigirev, Kohn, Snigireva, and Lengeler, *Nature* **384**, 49 (1996). Earlier patent by T. Tomie.

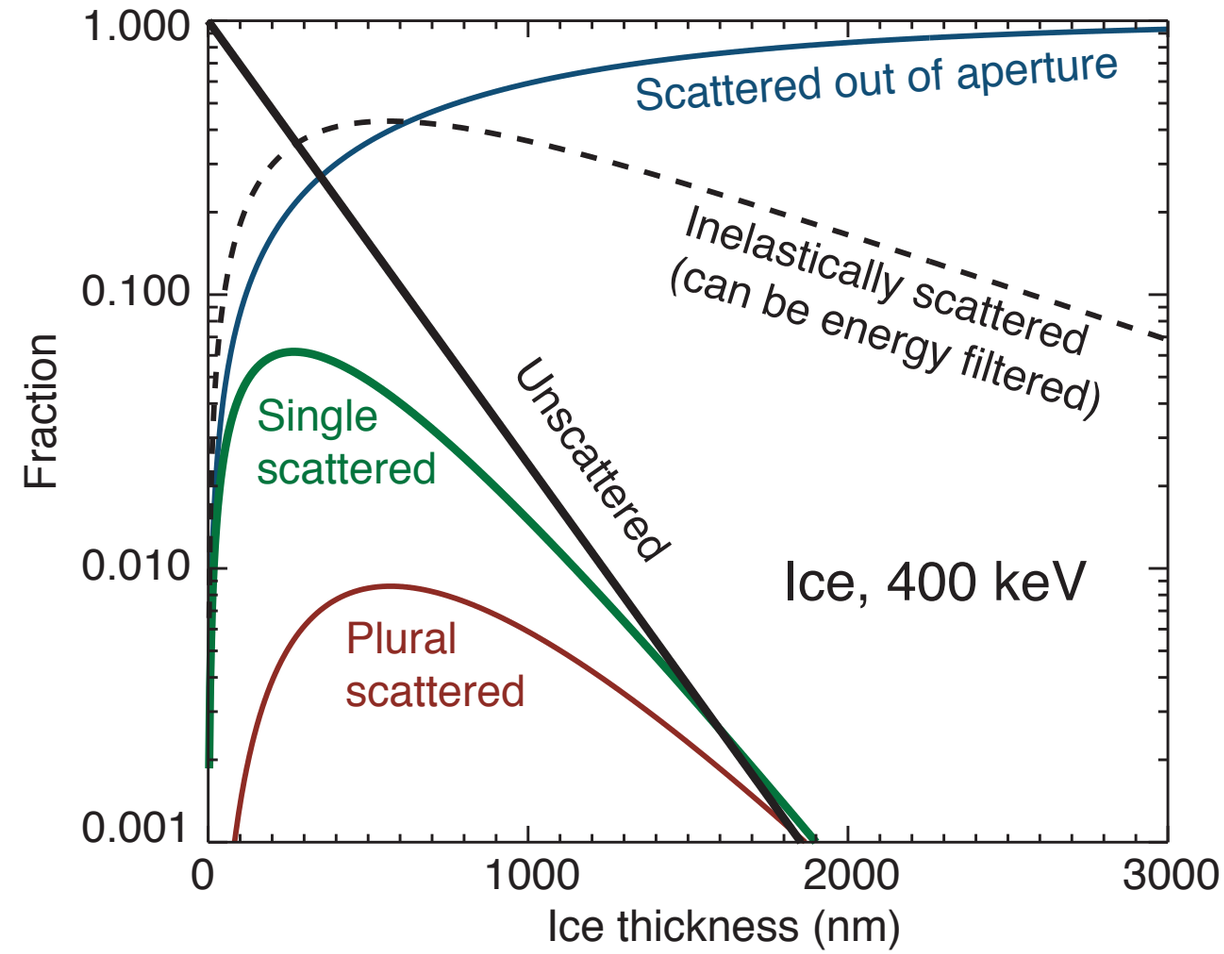
X rays



- Absorption dominates
- Inelastic scattering is weak
- No multiple scattering

There are no cloudy days for X rays!

Electrons



- Inelastic scattering dominates (energy filters)
- Multiple scattering often present
- High contrast from small things

Poisson and Gaussian statistics

- Poisson distribution: if the average value over many, many tests is $\bar{n}$, what's the probability P of seeing n on one particular test?

$$P(n, \bar{n}) = \frac{\bar{n}^n}{n!} \exp(-\bar{n}).$$

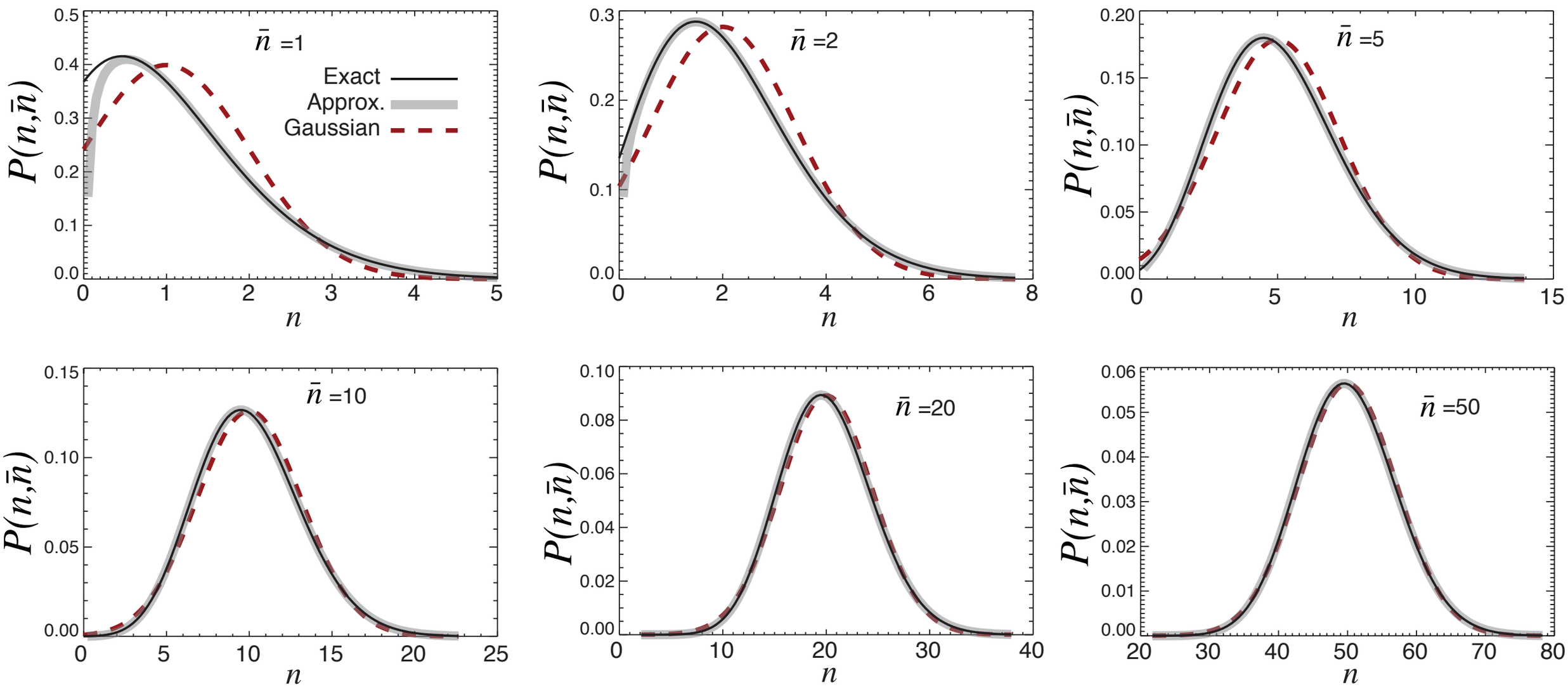
- Gaussian distribution: same meaning for $\bar{n}$, n, and P, but valid only for large $\bar{n}$:

$$P(n, \bar{n}) = \frac{1}{\sqrt{2\pi\bar{n}}} \exp\left[-\frac{(n-\bar{n})^2}{2\bar{n}}\right]$$

"Bell curve"

Comparing Poisson (exact) and Gaussian

- The Gaussian approximation works well for even very small values of $\bar{n}$



Signal to noise and exposure requirements

- Because we know that the x-ray refractive index is n=1-δ-$i\beta$, we can predict amplitude attenuation exp[-$k\beta t$] and phase advance exp[+$ik\delta t$] of a feature of thickness t.
- We can thus calculate unit-photon image intensities with, and without, a feature present over background as I_f and I_b, respectively.
- The average signal is then $\bar{n}|I_f$-$I_b|$ when each measurement is made with $\bar{n}$ incident photons.
- In the Gaussian approximation, the square root variance of each measure is $\sqrt{\bar{n}I}$, and photon fluctuations are uncorrelated from measurement to measurement.
- Thus the signal to noise ratio is

$$\mathrm{SNR} = \frac{\mathrm{Signal}}{\mathrm{Noise}} = \frac{\bar{n}|I_f - I_b|}{\sqrt{(\sqrt{\bar{n}I_f})^2 + (\sqrt{\bar{n}I_b})^2}} = \sqrt{\bar{n}}\frac{|I_f - I_b|}{\sqrt{I_f + I_b}} = \sqrt{\bar{n}}\,\Theta$$

X. Huang *et al.*, *Optics Express* **17**, 13541 (2009)

Signal to noise and exposure requirements

- Again, the signal to noise ratio is given by

$$\mathrm{SNR} = \frac{\mathrm{Signal}}{\mathrm{Noise}} = \frac{\bar{n}|I_f - I_b|}{\sqrt{(\sqrt{\bar{n}I_f})^2 + (\sqrt{\bar{n}I_b})^2}} = \sqrt{\bar{n}}\frac{|I_f - I_b|}{\sqrt{I_f + I_b}} = \sqrt{\bar{n}}\,\Theta$$

  where Θ=contrast parameter, I_f=intensity of feature, I_b=intensity of background.
- Note that this differers from fringe visibility $V=|I_{\max}-I_{\min}|/|I_{\max}+I_{\min}|$
- Required number of photons per pixel is then $\bar{n}=(\mathrm{SNR})^2/\Theta^2$
- Slope of SNR versus incident photons $\bar{n}$ on a log-log plot:

$$\log_{10}\mathrm{SNR} = \log_{10}(\sqrt{\bar{n}}\Theta) = \frac{1}{2}\log_{10}\bar{n} + \log_{10}\Theta$$

X. Huang *et al.*, *Optics Express* **17**, 13541 (2009)

Photons and SNR

- Again, required exposure is $\bar{n}=(\mathrm{SNR})^2/\Theta^2$
- What SNR is acceptable? Well, it depends... The usual criterion comes from the early days of television: A. Rose, “Television pickup tubes and the problem of vision,” pp. 131-166 in L. Marton, *Advances in Electronics* vol. 1 (Academic Press, 1948).
- The Rose criterion is SNR=5:1, so for 100% contrast objects we need 25 photons. Modern day electron microscopists often accept a SNR of 1.5:1, but we’ll use the Rose criterion.

SNR from unknown objects

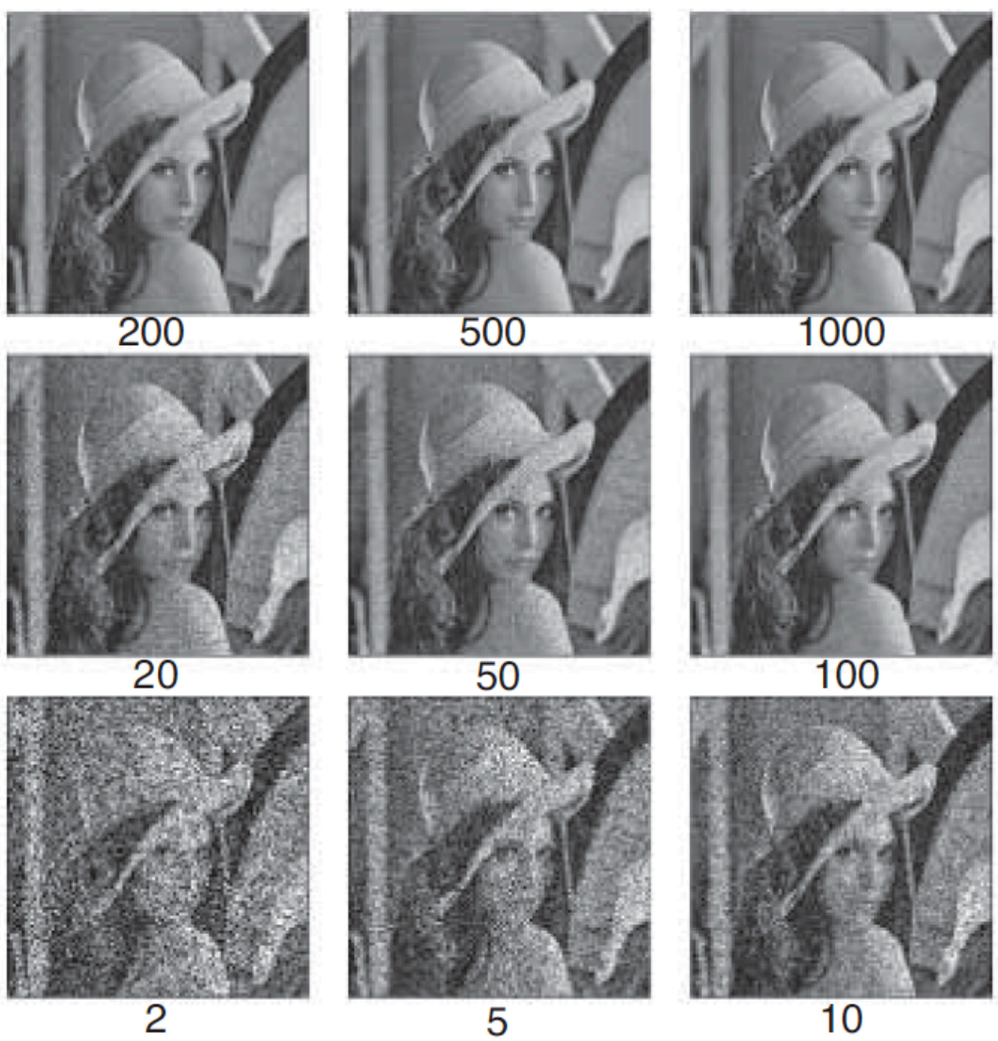
- What if we don't know I_f and I_b?
- Let's say we have two noisy images I_1 and I_2, but we know they are of the same object.
- Calculate correlation r: $$r = \frac{\langle (I_1 - \langle I_1 \rangle)(I_2 - \langle I_2 \rangle)^* \rangle}{\sqrt{\langle (I_1 - \langle I_1 \rangle)^2 \rangle \langle (I_2 - \langle I_2 \rangle)^2 \rangle}}$$
- We find $\mathrm{SNR} = \sqrt{r/(1-r)}$
- Note: square root of expression of Frank and Al-Ali, *Nature* **256**, 376 (1975)

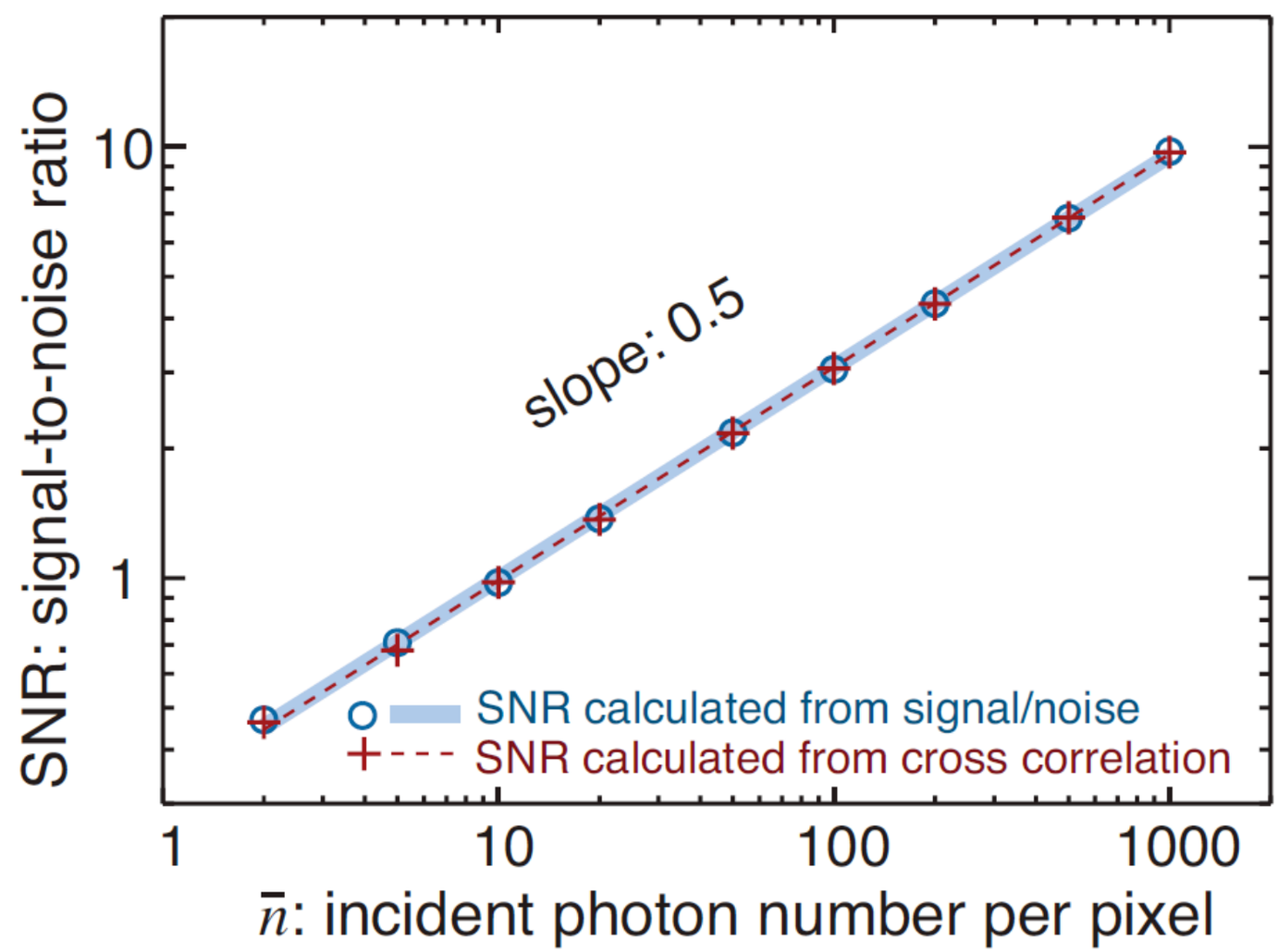
X. Huang *et al.*, *Optics Express* **17**, 13541 (2009)

Testing SNR scaling

- Again, we expect $\log_{10} \mathrm{SNR} = \frac{1}{2} \log_{10} \bar{n} + \log_{10} \Theta$
- Test correlation method $\mathrm{SNR} = \sqrt{r/(1-r)}$

Images I_1 with $\bar{n}$





X. Huang *et al.*, *Optics Express* **17**, 13541 (2009)

Calculating dose

- SI units for ionizing radiation: 1 Gray=1 J/kg=100 rad
- Lambert-Beer law with inverse absorption length μ(=1.3 mm for protein at 8.98 keV):

$$I = I_0 e^{-\mu x} \qquad \text{with} \qquad \mu = 2\frac{\rho N_A}{A} r_e \lambda f_2$$

- Energy per thickness with inverse absorption length μ:

$$\frac{dE}{dx} = h\nu \frac{dI}{dx} = h\nu\, \mu\, I_0 e^{-\mu \cdot 0} = I_0\, h\nu\, \mu$$

- Energy per mass (note: I_0 is effectively $\overline{n}$):

$$\frac{dE}{dm} = \frac{dE}{dx} \frac{1}{\text{Area} \cdot \rho} = h\nu\, \mu\, I_0 \frac{1}{\text{Area} \cdot \rho} = h\nu \frac{I_0\, \mu}{\text{Area} \cdot \rho}$$

Dose numbers

- G factor: number of bonds broken per 100 eV. G~5 for many organic molecules (room temp.)
- Quick estimate to break 1 bond per atom:

$$\frac{(20\ \text{eV/atom}) \cdot (N_A\ \text{atoms/mol}) \cdot (1.6 \times 10^{-19}\ \text{J/eV})}{(12\ \text{g/mol}) \cdot (10^{-3}\ \text{kg/g})} = 1.6 \times 10^{8}\ \text{Gray}$$

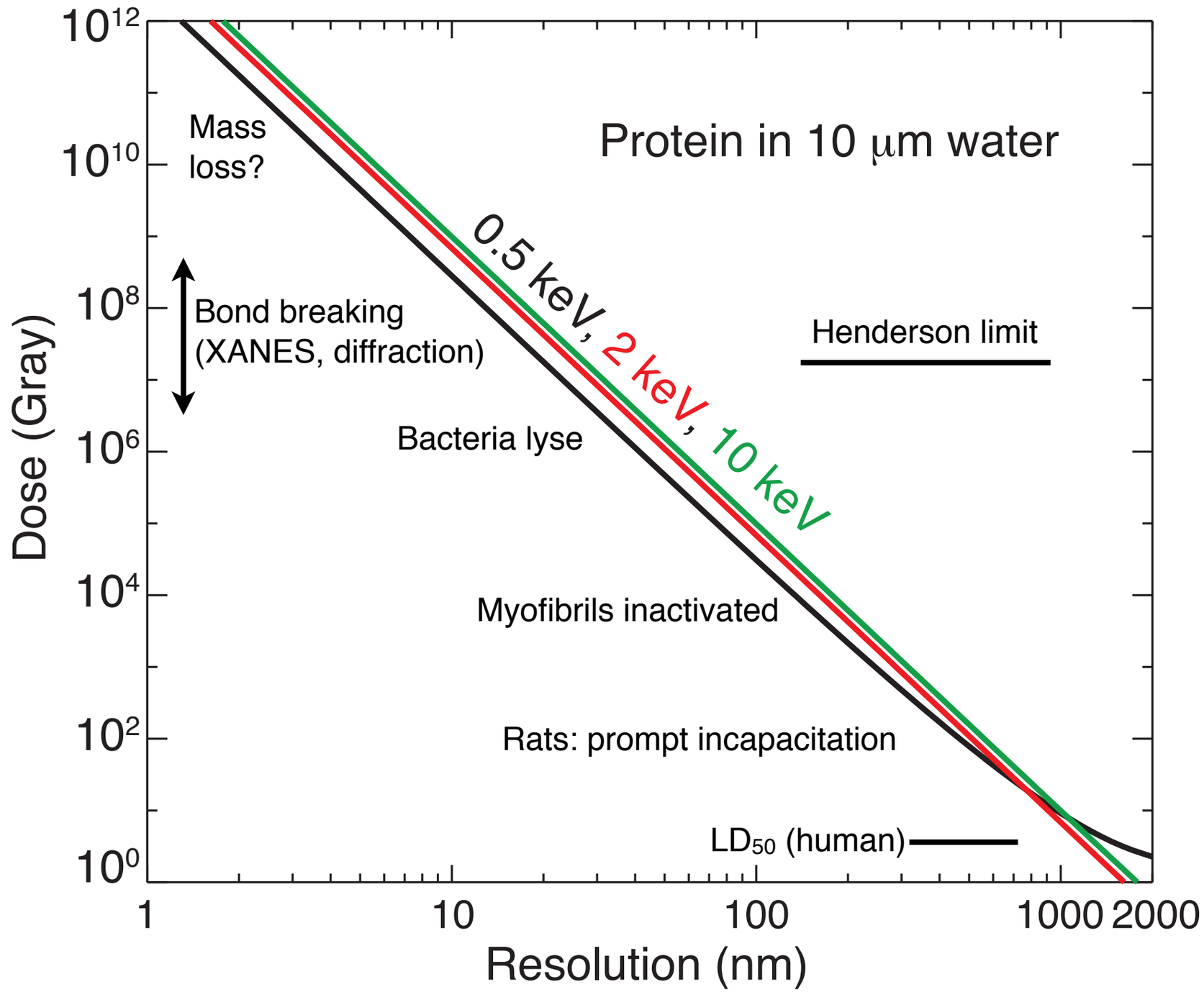
- Representative dose in crystallography:

$$\frac{10^{14}\ \text{photons}}{(50\ \mu\text{m})^2} \frac{(8979\ \text{eV/photon}) \cdot (1.6 \times 10^{-19}\ \text{J/ev})}{(1300\ \mu\text{m}) \cdot (1.35\ \text{g/cm}^3) \cdot (10^{-4}\ \text{cm}/\mu\text{m})^3 \cdot (10^{-3}\ \text{kg/g})} = 3.3 \times 10^{7}\ \text{Gray}$$

- "Henderson limit" [Henderson, *Q. Rev. Biophys.* **28**, 171 (1995)]: 2×10^7 Gray is when diffraction spots fade.
- X-ray microscopy: doses of 10^6-10^8 Gray are common, depending on resolution

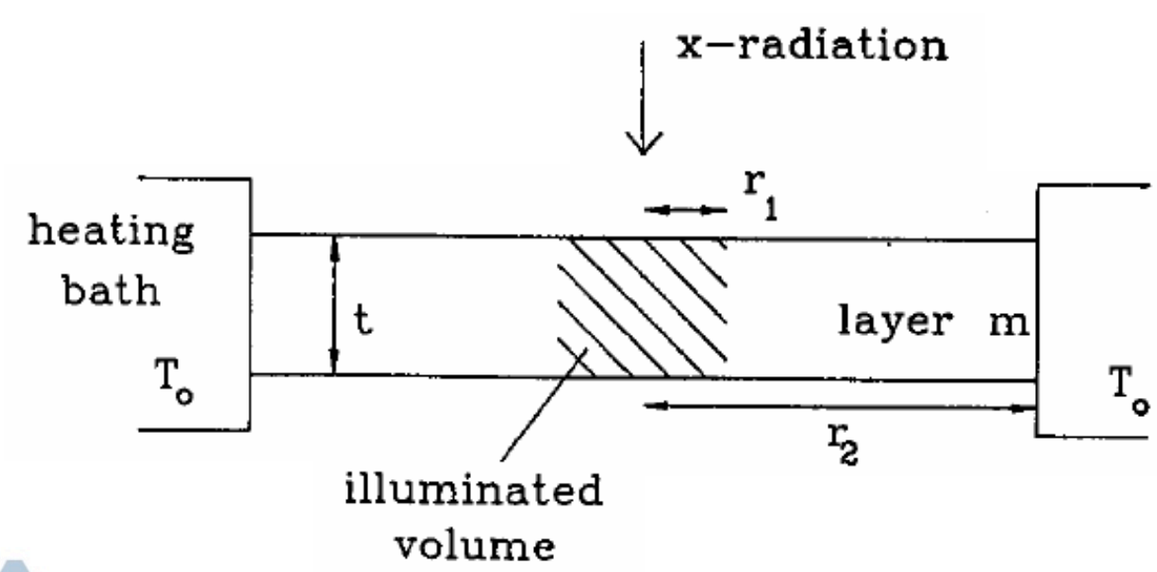
Dose versus resolution for x-ray imaging

- Calculation of radiation dose using best of phase, absorption contrast and 100% efficient imaging
- Things that can be done wet at room temperature:
  - bacteria at 50 nm resolution
  - small animals at micrometer resolution (followed by sacrifice)



Effects of 10^5 photons in $(10\ \text{nm})^3$

- With no cooling, the temperature rises due to absorption:
  - H_20@500 eV ⇒ 2300K
  - H_20@3 keV ⇒ 2200K
  - Si@10 keV ⇒ 7300K
- In scanning microscopes, localized heating with a thermal reservoir. Photon flux for ΔT=1K in 10 nm wide spot with r_2=100 μm:
  - H_20@500 eV: 4×10^{10} photons/sec
  - Si@10 keV: 2×10^{12} photons/sec

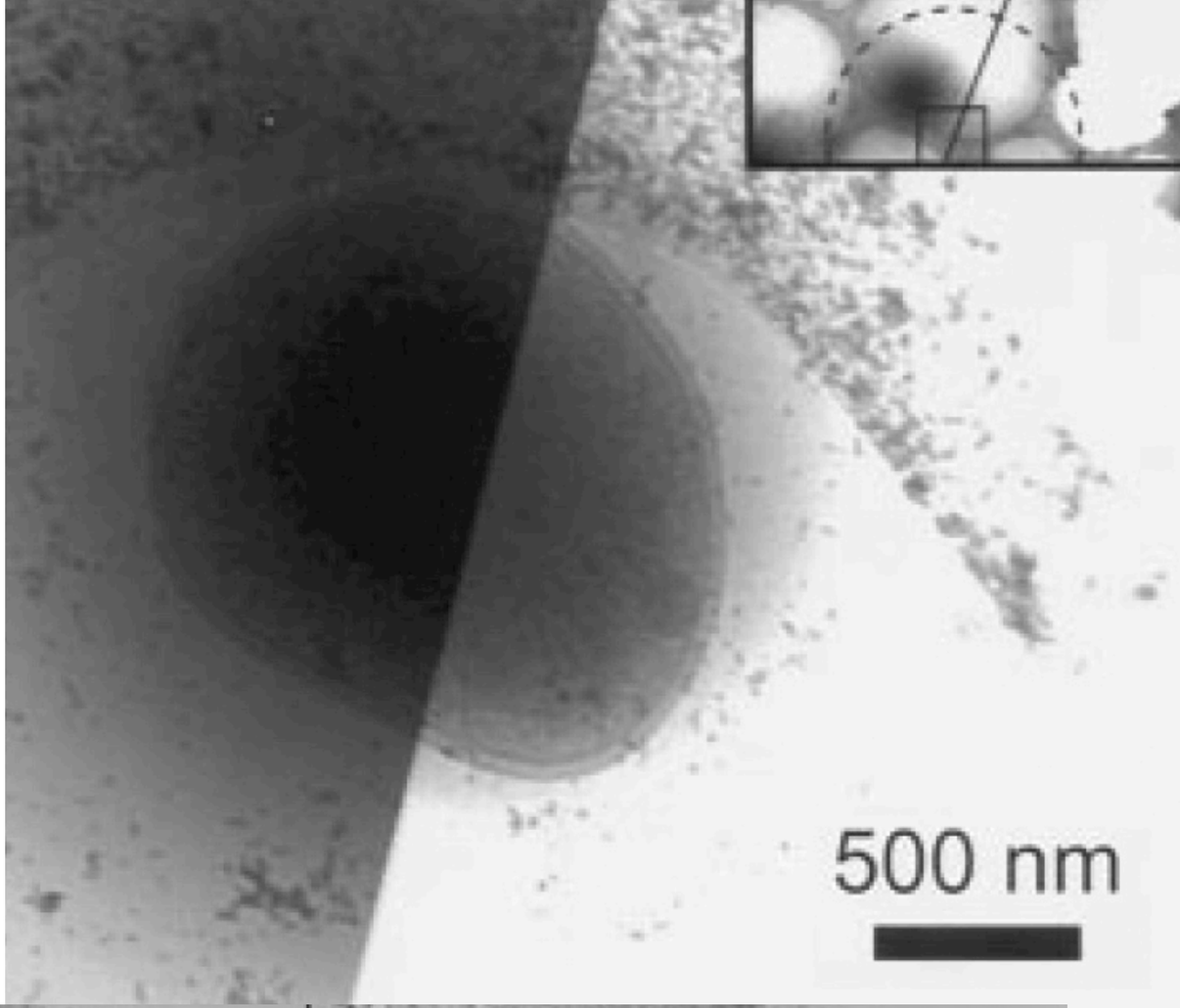


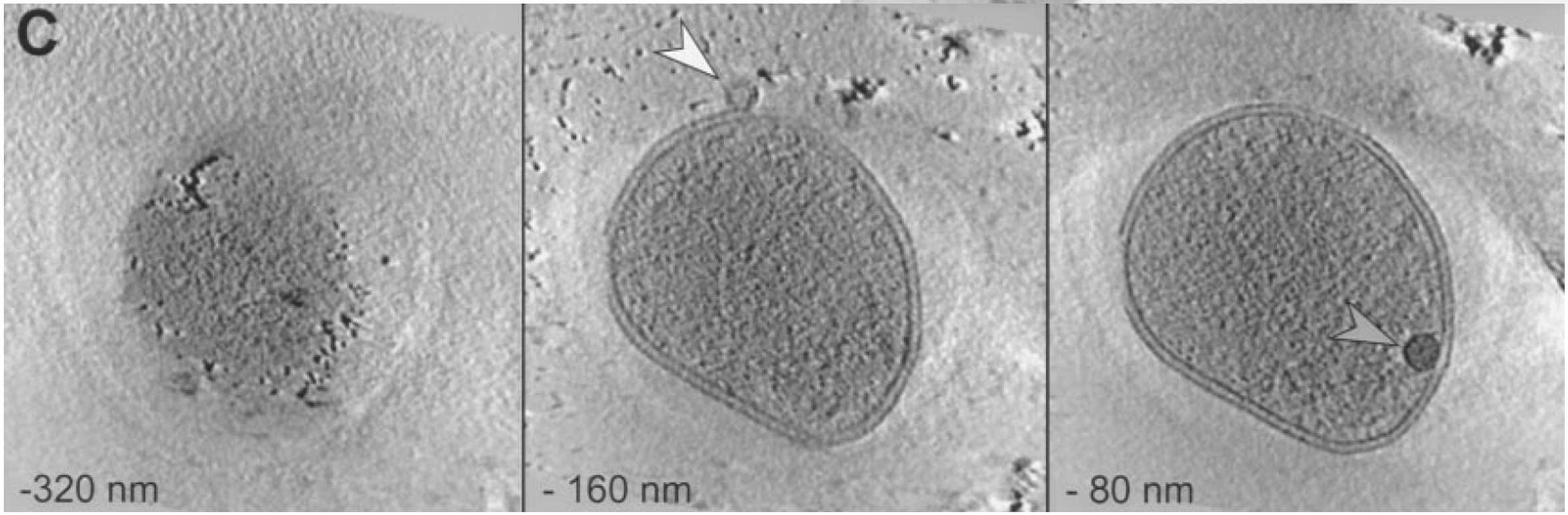
$$\Delta T = \frac{N}{t}\,\frac{h\nu\cdot\mu}{4\pi k}\left(1+2\ln\frac{r_2}{r_1}\right)$$

Greinke and Gölz, XRM 1991

Slow is good #1

- 3D imaging of complex objects: slices from tomography are *much* more revealing than single projections.
- Tomography requires multiple views of unchanged specimen.

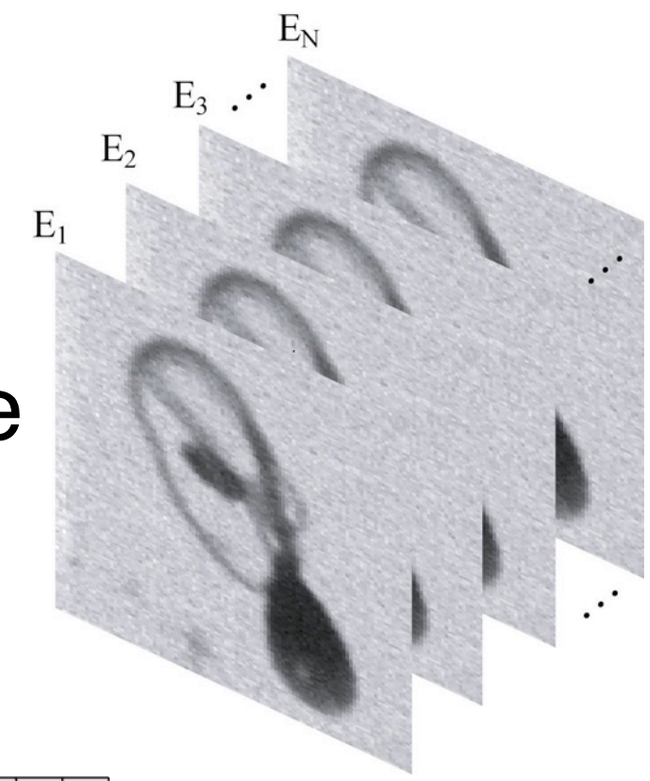


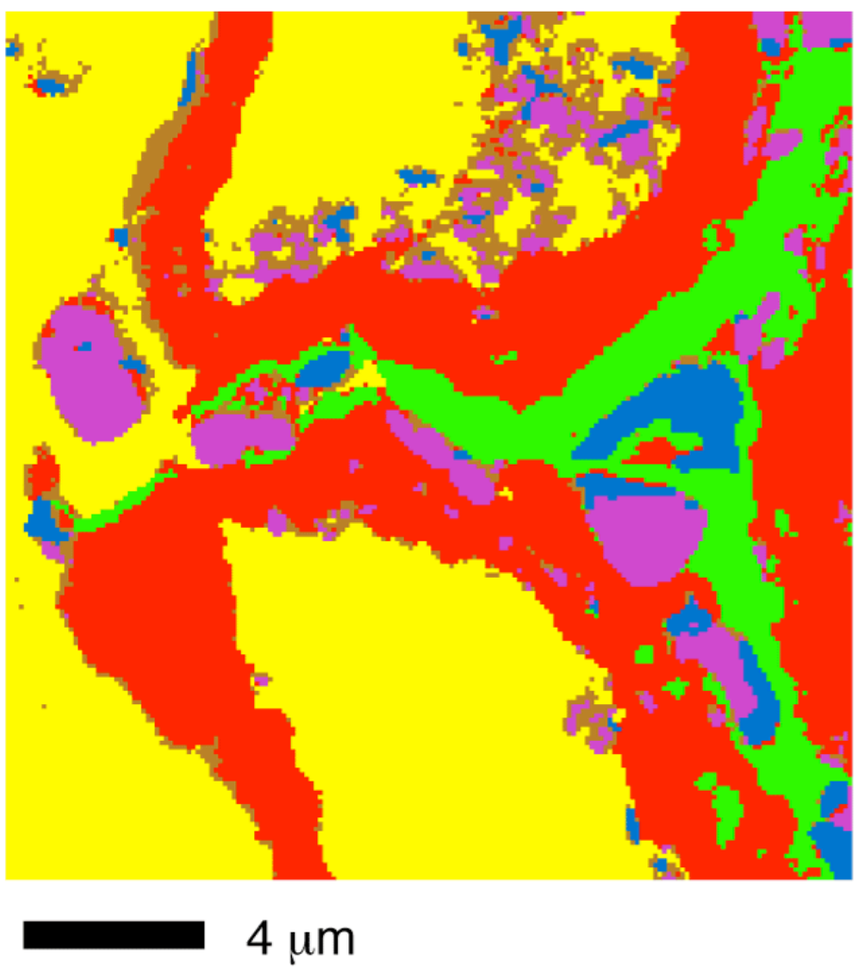


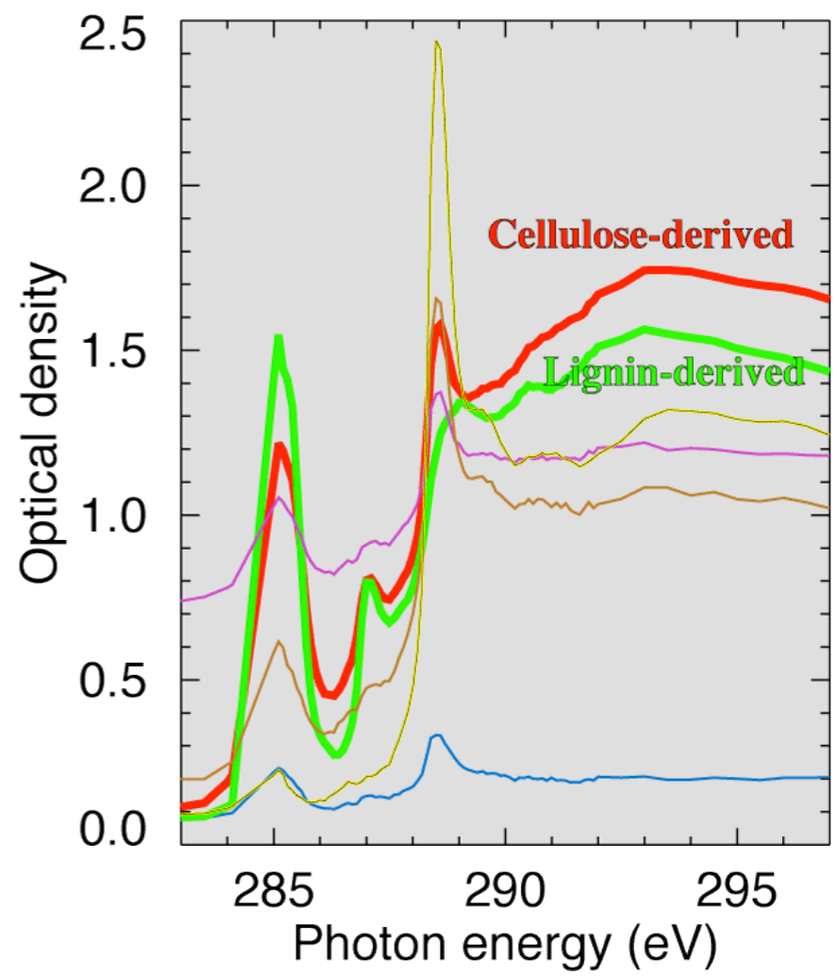
Grimm *et al.*, *Biophys. J.* **74**, 1031 (1998).

Slow is good #2

- Spectromicroscopy: learn about chemical speciation.
- Requires unchanged sample at multiple photon energies (or pink beam and spectrometer)

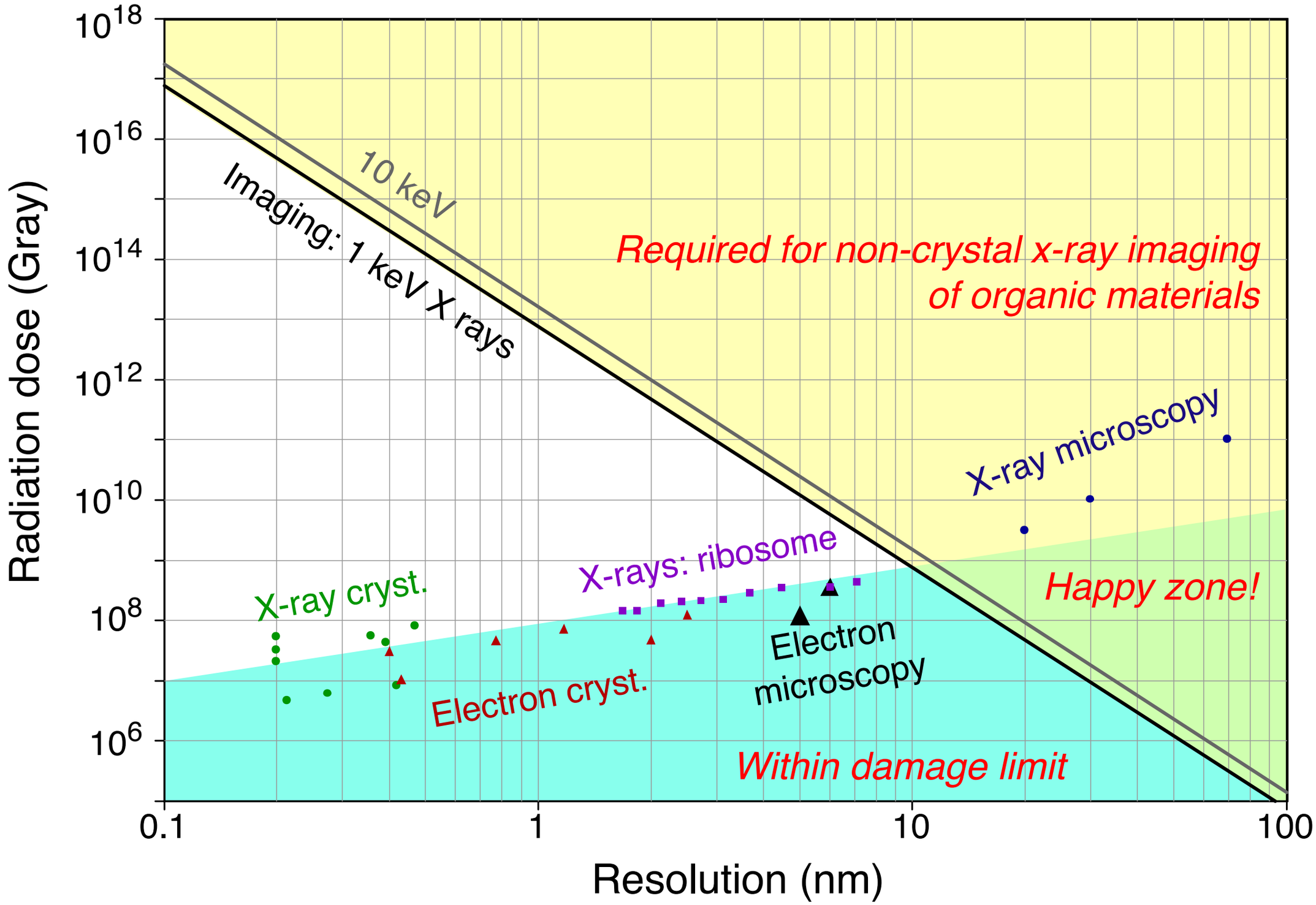






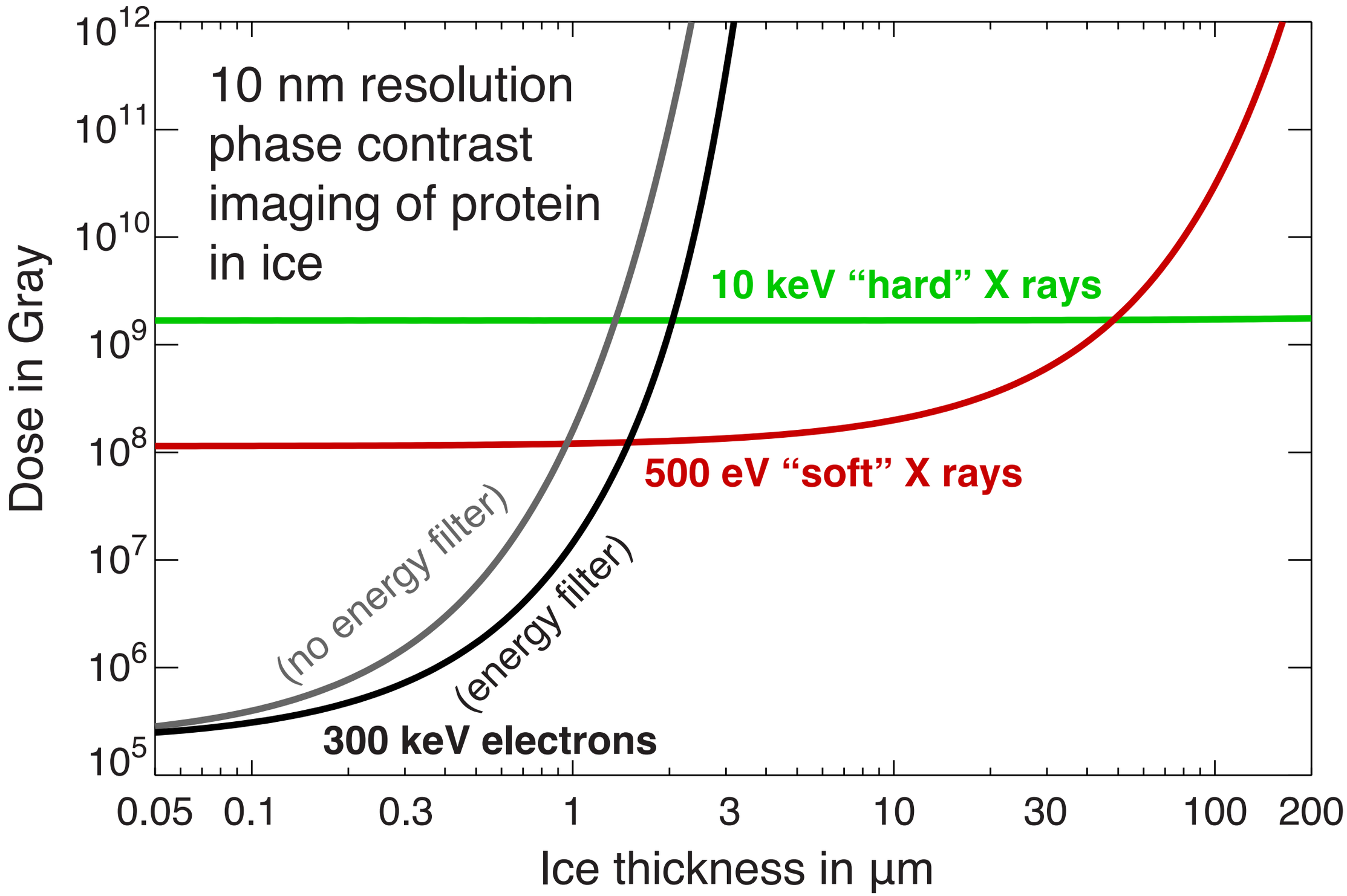
Lignin and cellulose in 400 million year old chert: Boyce *et al.*, *Proc. Nat. Acad. Sci.* **101**, 17555 (2004), with subsequent pattern recognition analysis by Lerotic *et al.*, *Ultramicroscopy* **100**, 35 (2004).

What's the limit for cells?



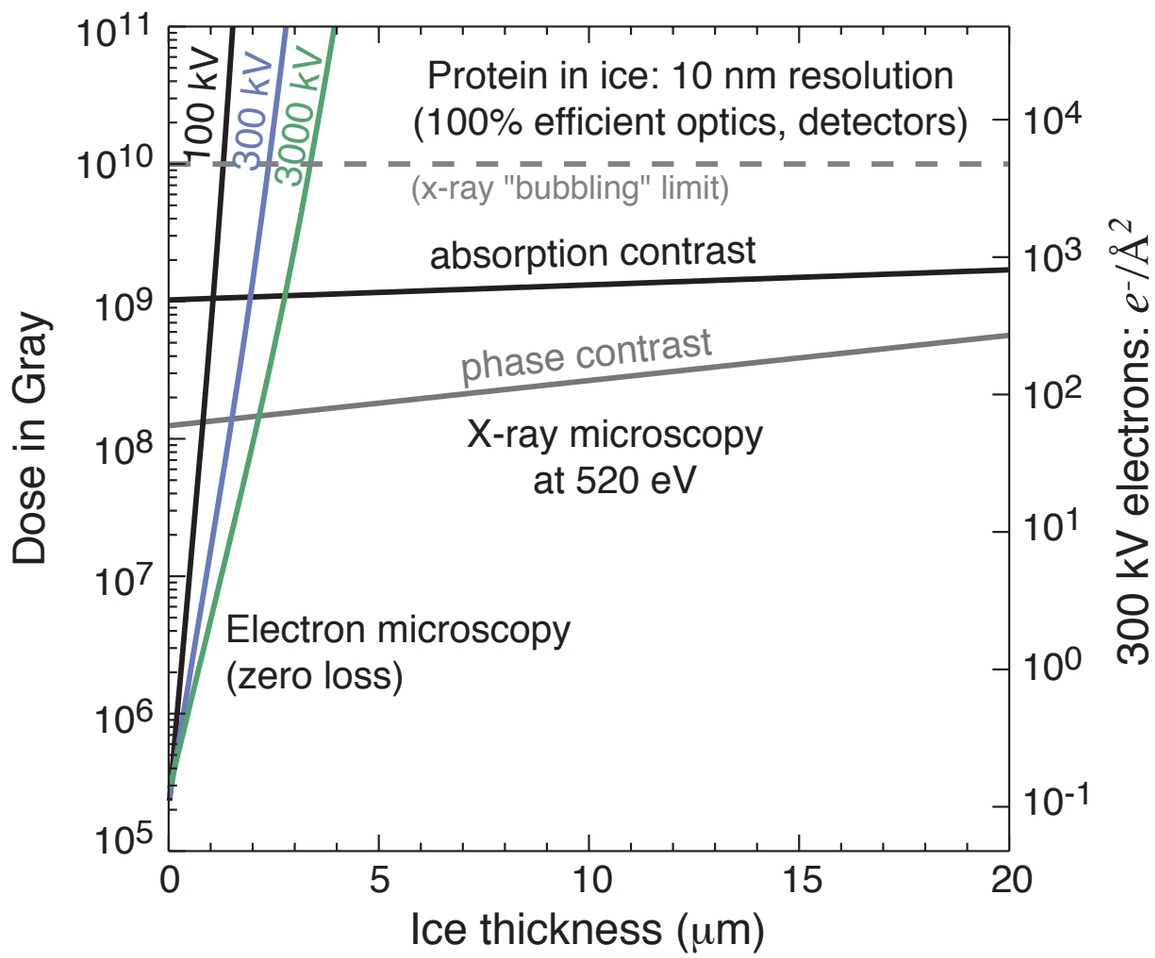
Howells et al., *JESRP* 170, 4 (2009)
See also Shen et al., *J. Sync. Rad.* **11**, 432 (2004)

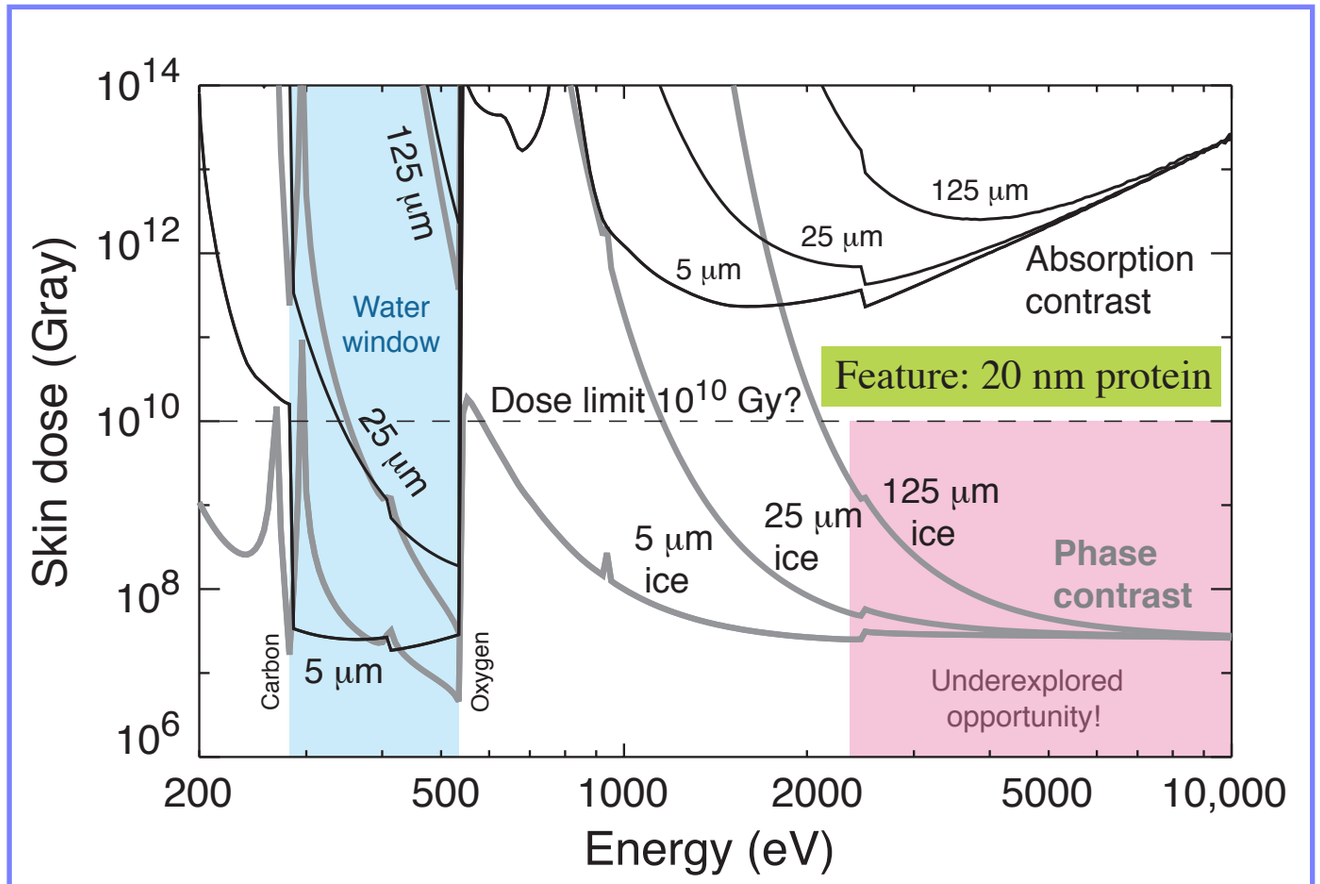
X rays and electrons: another look



X rays are better than electrons for thick specimens

- No more than 1 high-resolution image of wet, soft samples unless frozen
- At energies >3 keV, opportunities for thick specimens.





Phase contrast is important!

These plots: based on Jacobsen, Medenwaldt, and Williams, in **X-ray Microscopy & Spectromicroscopy** (Springer, 1998). See also Sayre *et al.*, *Science* **196**, 1339 (1977).

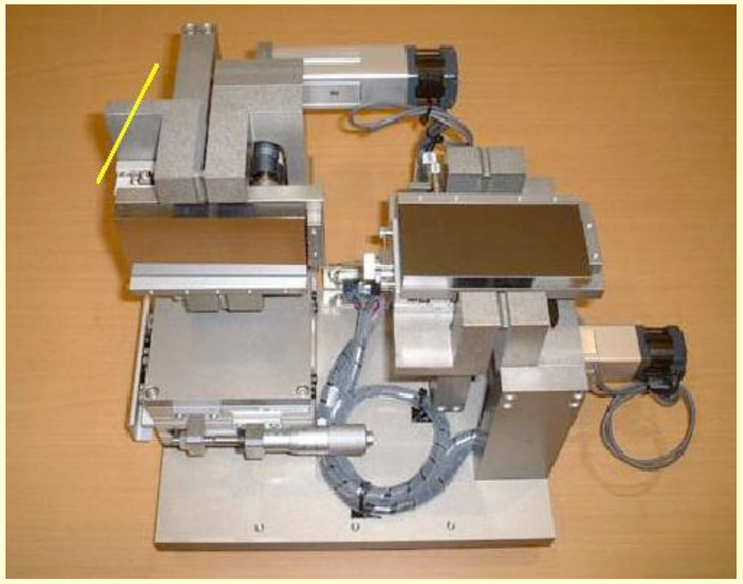
Reflective x-ray optics

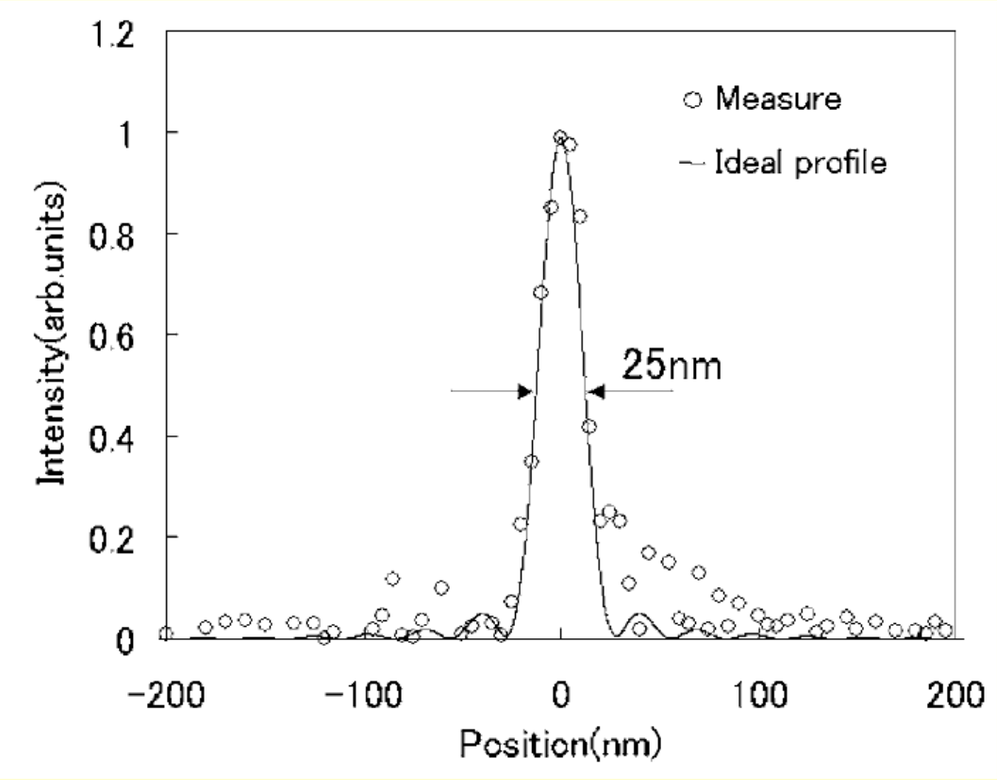
- Grazing incidence critical angle for mirror reflection:

$$\cos\theta_C = 1 - \alpha\lambda^2 f_1 \quad \text{or} \quad \theta_C \approx \lambda\sqrt{2\alpha f_1}$$

- Use two orthogonal cylinders for 2D focusing: Kirkpatrick and Baez, *J. Opt. Soc. Am.* **38**, 766 (1948)

Mimura, Yamauchi *et al.*, *Appl. Phys. Lett.* **90**, 051903 (2007): 25 nm using elastic emission machining.

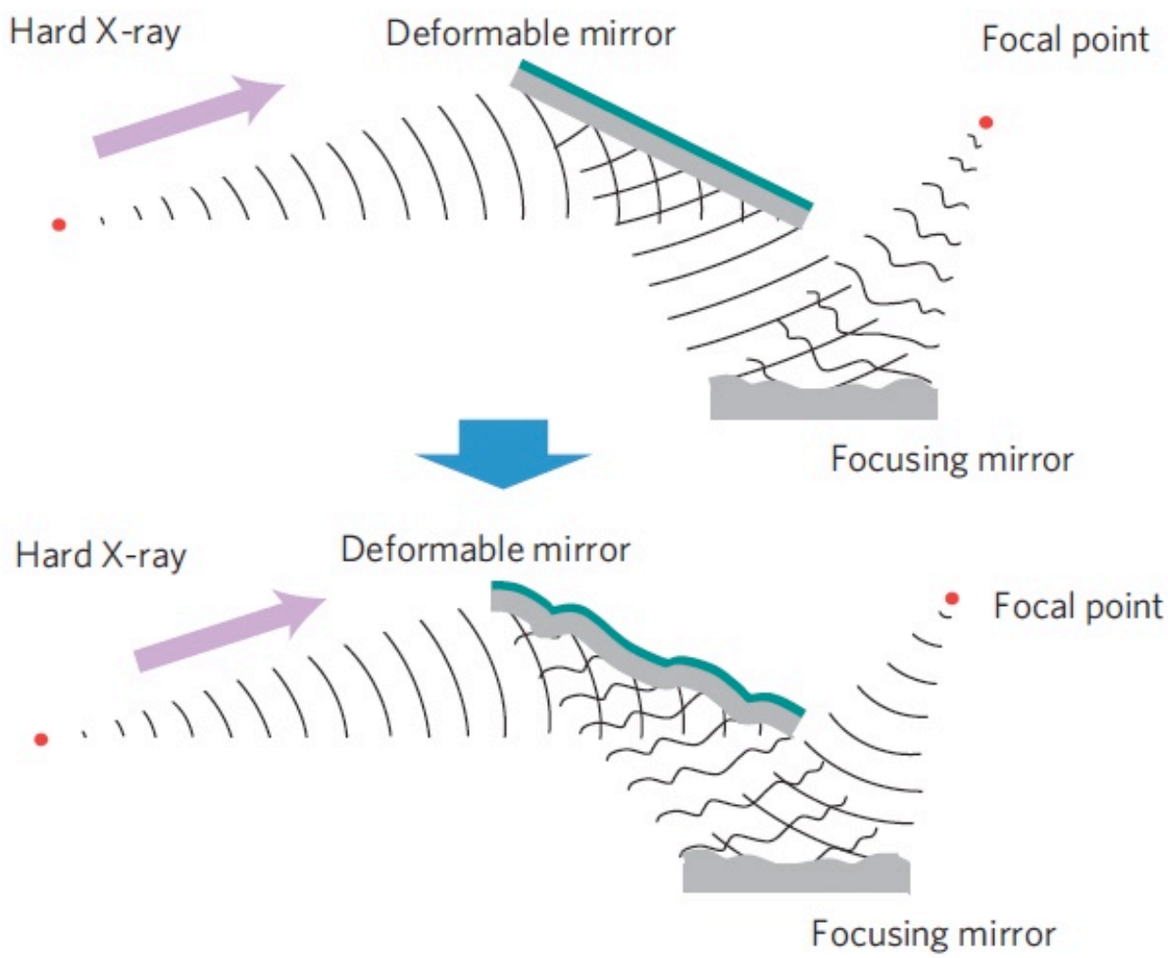


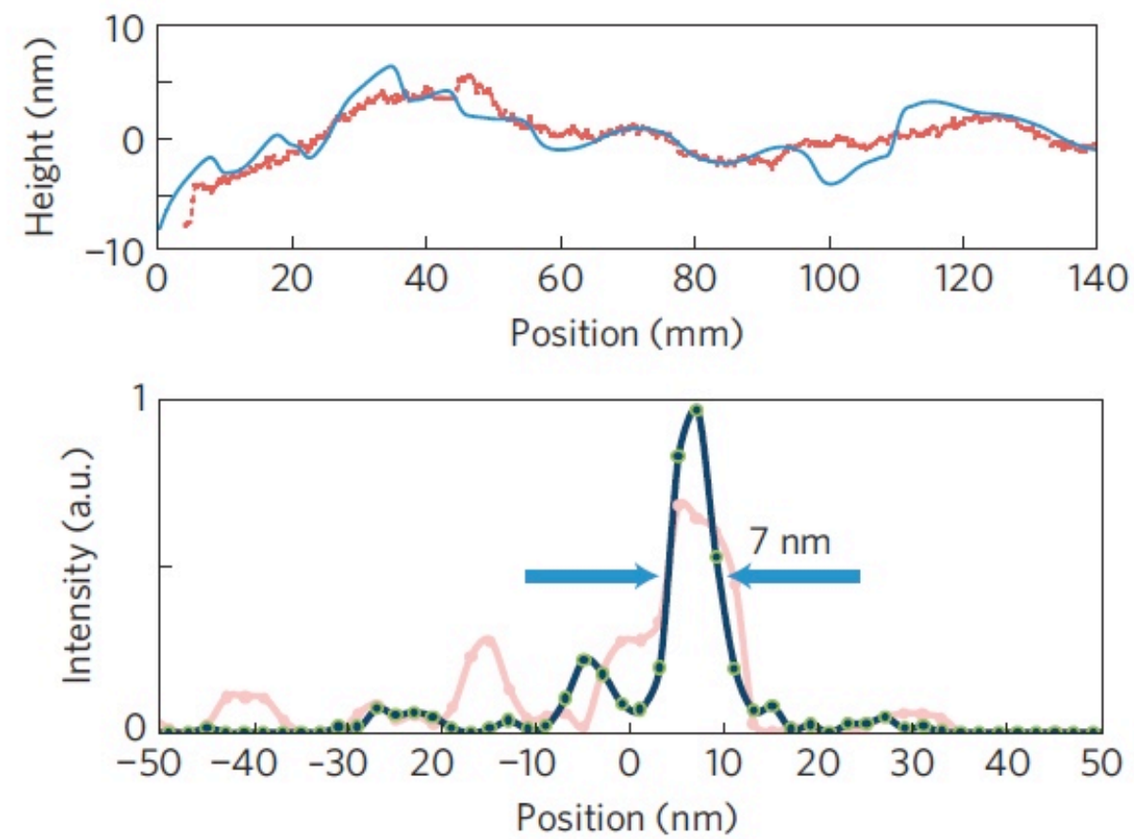


Synthetic multilayer mirrors: work well at approx. λ≥10 nm

Reflective optics

- Ultimate limit for single reflection: $[A/8\rho N_A r_e f_1]^{1/2}$ or about 3 nm (Pt, 10 keV)

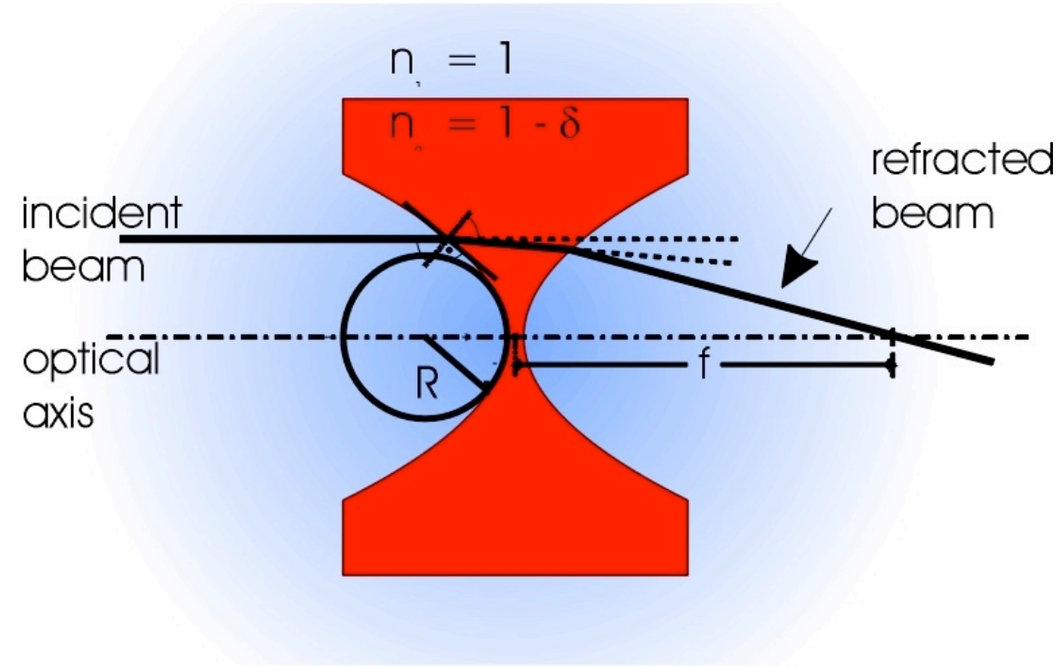




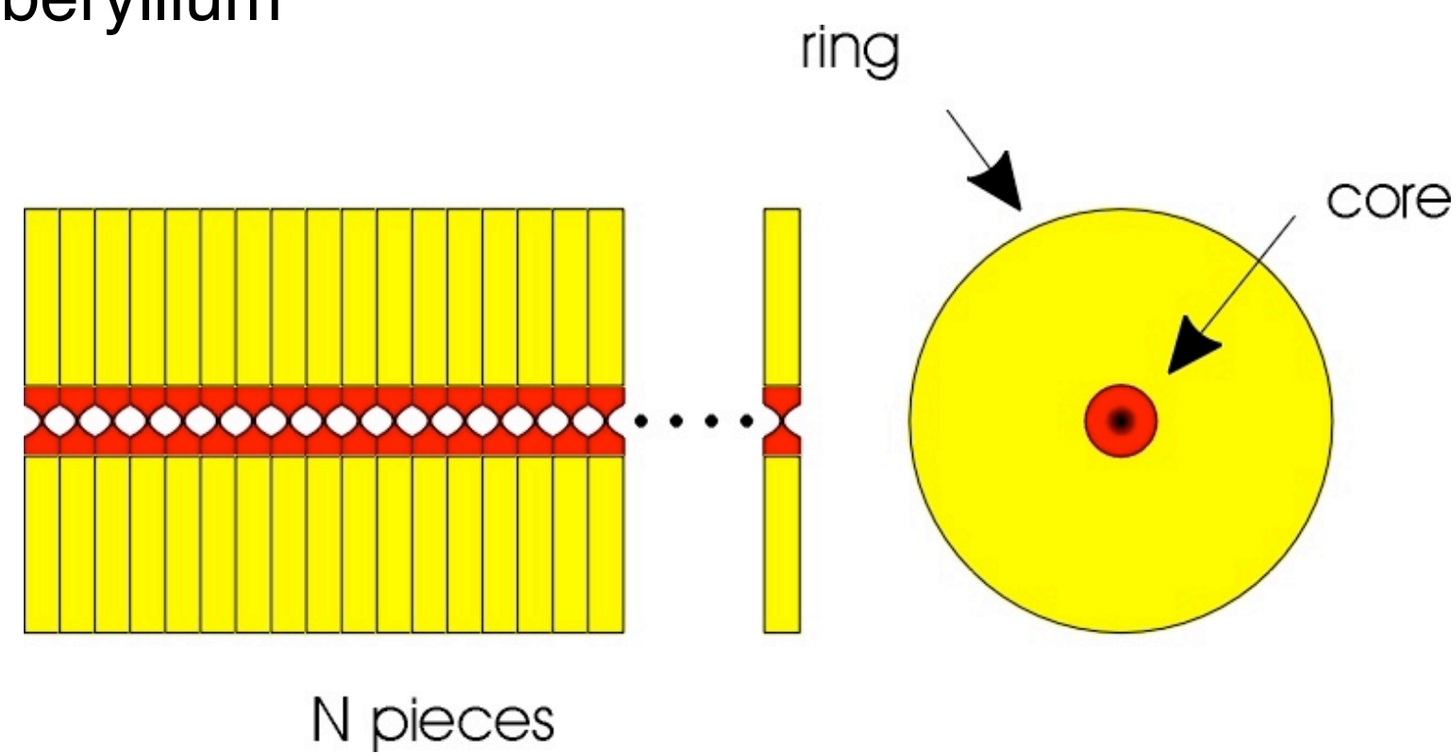
Mimura *et al.*, *Nature Physics* **6**, 122 (2010)

Compound refractive lenses

- Röntgen tried to make lenses, but found no focusing.
- Focal length of one lens is long – so combine many lenses! Tomie; Snigirev *et al.*, *Nature* **384**, 49 (1996); Lengeler *et al.*, *J. Synch. Rad.* **9**, 119 (2002).
- Resolution approaching 100 nm at 5-10 keV with parabolic beryllium lenses



Compound refractive lenses at Universität Aachen



Atomic resolution imaging: electrons or photons?

10 keV photons

- About 100 absorption events per elastic scatter
- About 10 keV deposited per absorption
- Therefore about 10^6 eV deposited per elastic scatter
- A thousand scattered photons: $10^3 \cdot 10^6$ eV into $(2\ \text{Å})^3$, or 2×10^{13} Gray

100 keV electrons

- About 2.5 inelastic scatters per elastic scatter
- About 45 eV deposited per inelastic scatter
- Therefore about 10^2 eV deposited per elastic scatter
- A thousand scattered electrons: $10^3 \cdot 10^2$ eV into $(2\ \text{Å})^3$, or 2×10^9 Gray

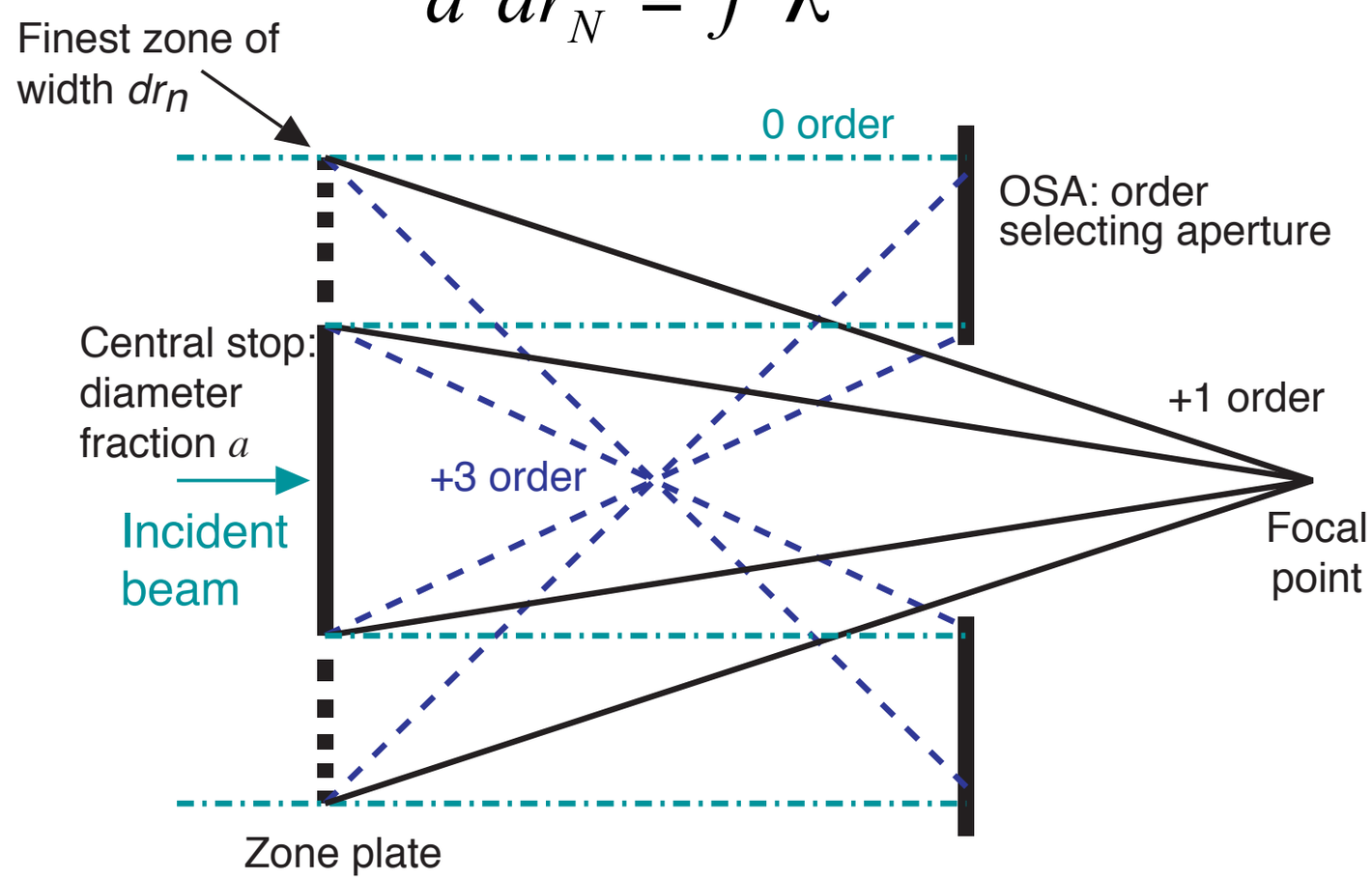
- Electrons are better than photons for atomic resolution imaging: J. Breedlove and G. Trammel, *Science* **170**, 1310 (1970); R. Henderson, *Q. Rev. Biophys*. **28**, 171 (1995).
- X-ray crystallography's answer: spread the dose out over many identical unit cells
- X-ray Free Electron Lasers: get image in <100 fsec, before damage

X-ray focusing: Fresnel zone plates

- Diffractive optics: radially varied grating spacing
- Largest diffraction angle is given by outermost (finest) zone width dr_N as $\theta=\lambda/(2dr_N)$
- Rayleigh resolution is 0.61 $\lambda/(\theta)=1.22dr_N$
- Zones must be positioned to ~1/3 width over diameter (10 nm in 100 μm, or 1:10^4)

Diameter *d*, outermost zone width dr_N, focal length *f*, wavelength λ:

$$d\ dr_N = f\ \lambda$$



Central stop and order sorting aperture (OSA) to isolate first order focus

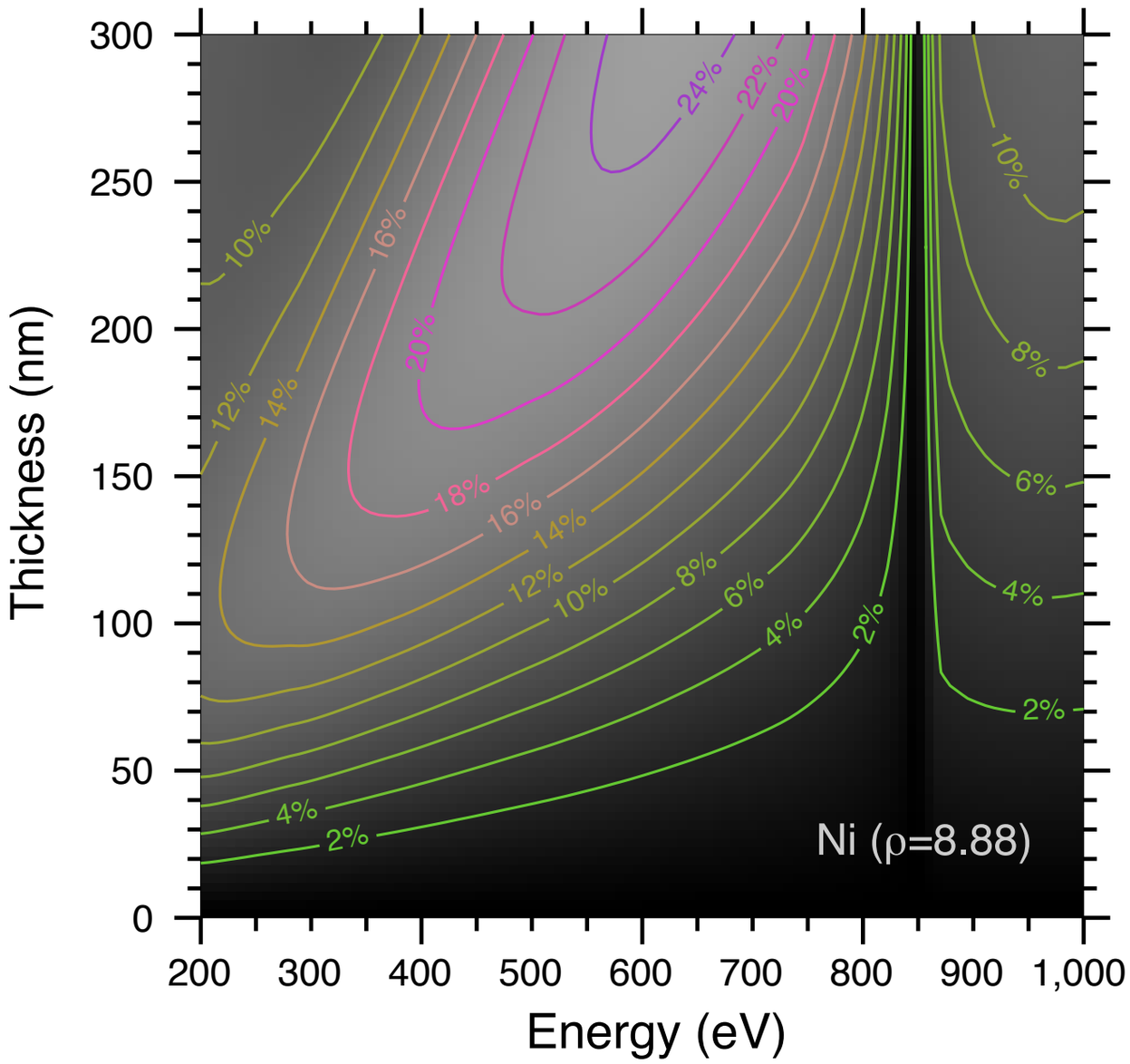
Fresnel zone plate images

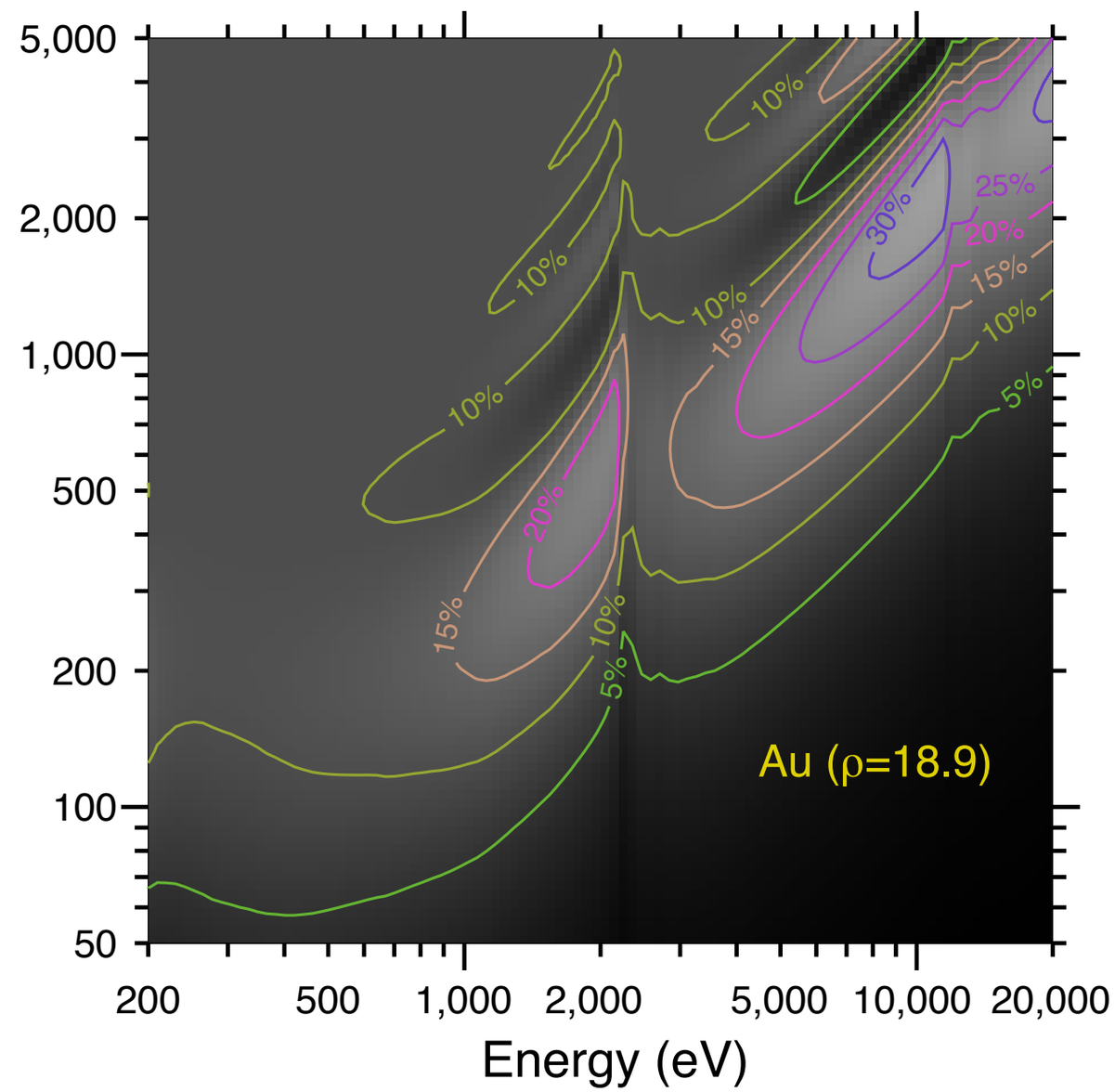
R. W. Wood (1898): zone plate figure drawn with a pen and a compass! Photographically reduced

PLATE 2. ZONE-PLATE, FROM A DRAWING.

Zone plate efficiency and thickness

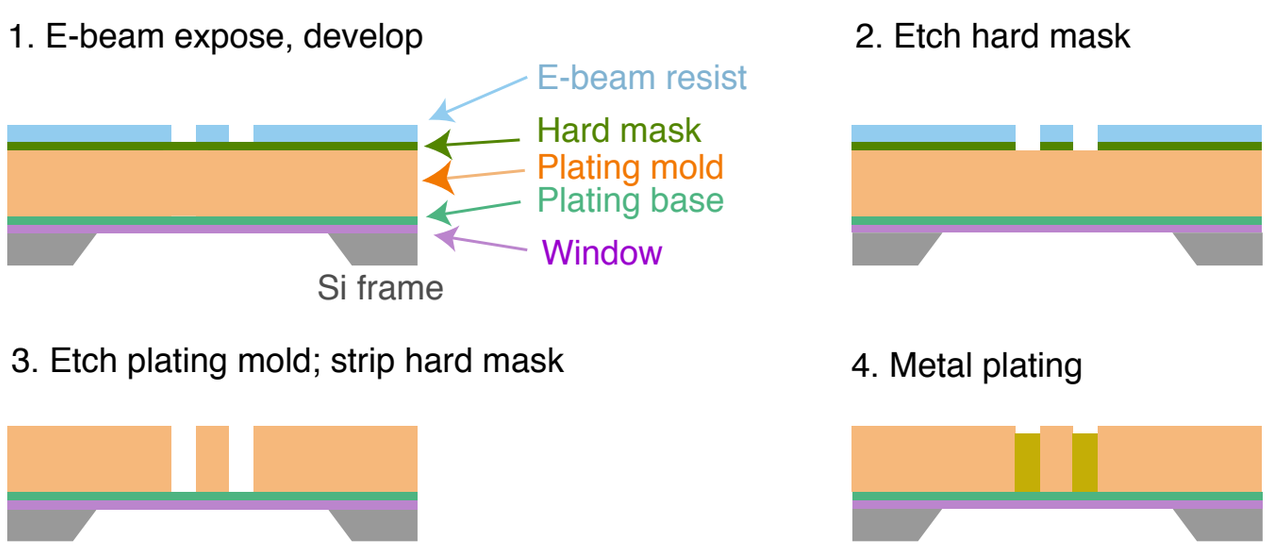
For binary zones, 1:1 mark:space ratio.
See Kirz, *J. Opt. Soc. Am.* **64**, 301 (1974)





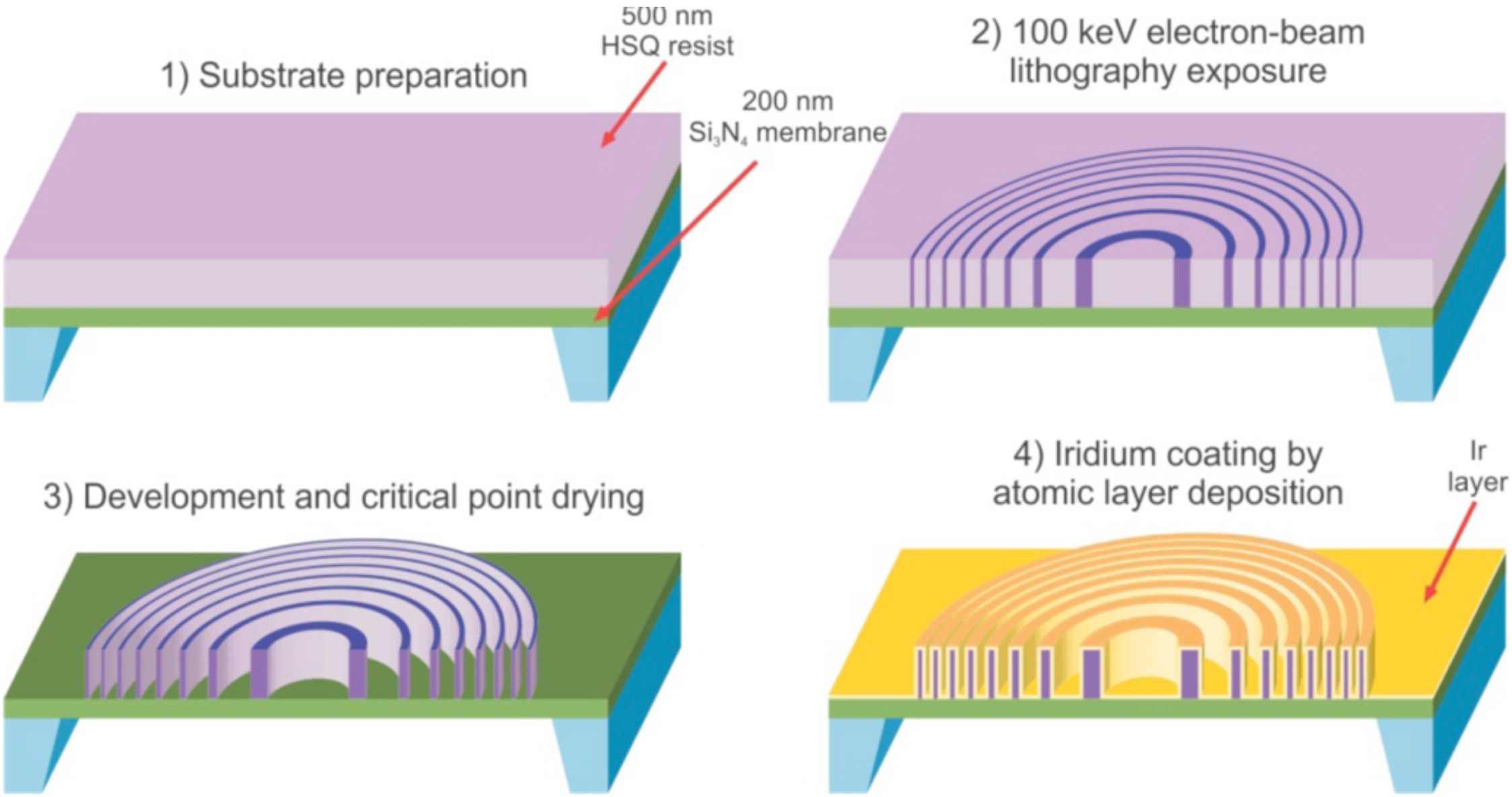
Zone plates by electron beam lithography

- Electron beam lithography: produces the finest possible structures (other than what nature can be persuaded to make by itself)
  - Example: JEOL JBX-9300FS: 1 nA into 4 nm spot, 1.2 nm over 500 μm, 100 keV
- Electrons scatter within resist, so highest resolution is only within ~100 nm thickness.
- Use directional etching methods like reactive ion etching for thick structures

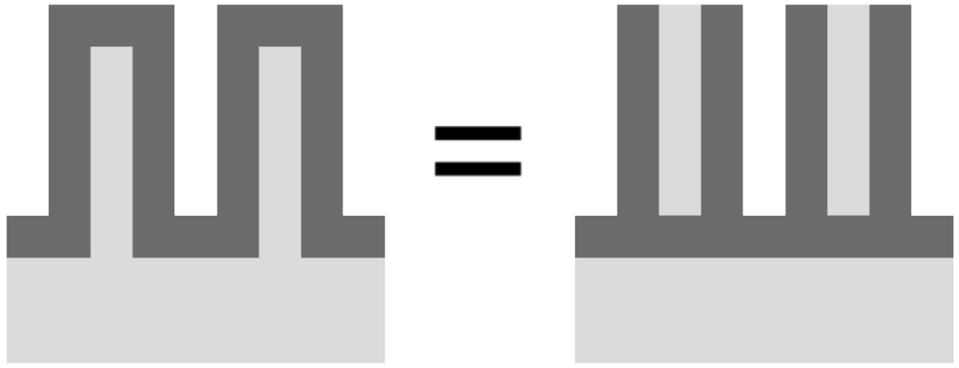


A. Stein and JBX-9300FS

Improving single zone plates: zone doubling



Jefimovs *et al.*, *Phys. Rev. Lett.* **99**, 264801 (2007).



Zone plate microscopes

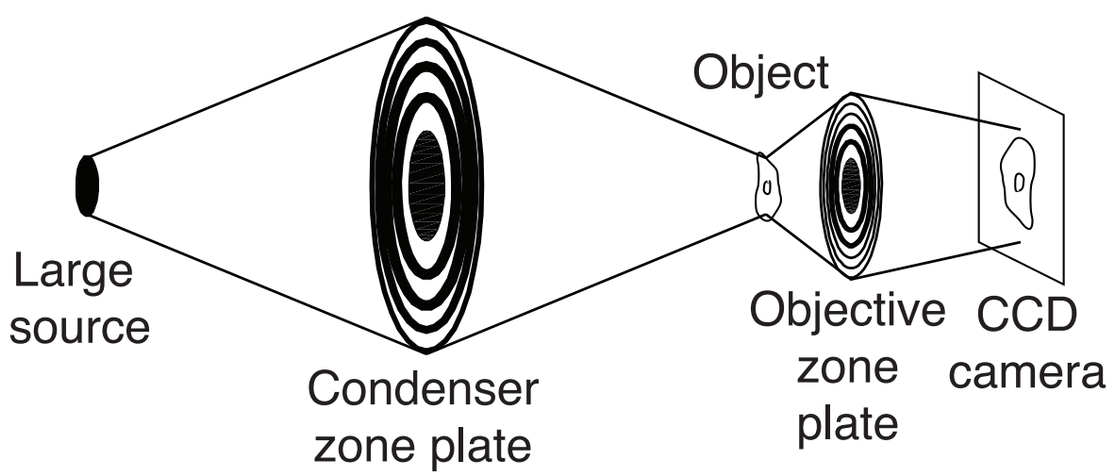
TXM

- Incoherent illumination; works well with a bending magnet, with fast imaging
- More pixels (e.g., 2048^2)
- Moderate spectral resolution in most cases - but new instrument at BESSY, Berlin!

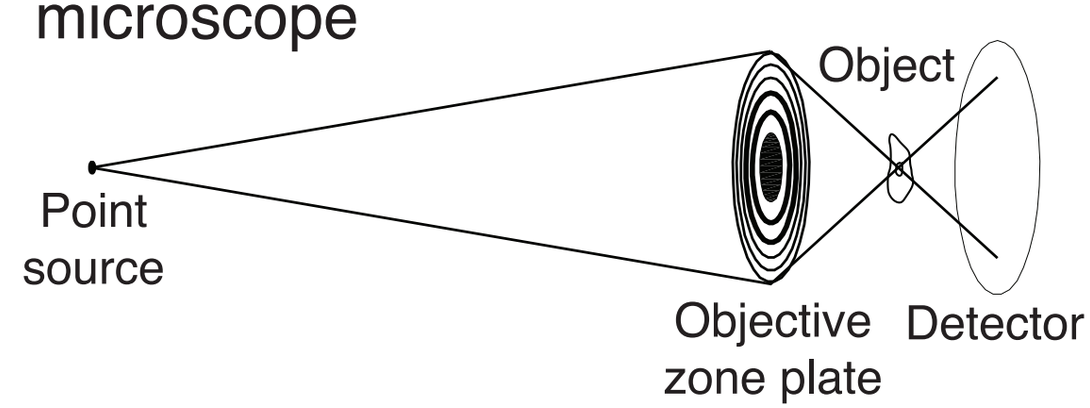
STXM

- Coherent illumination; works best with an undulator
- Less dose to sample (~10% efficient ZP)
- Better suited to conventional grating monochromator [high E/(ΔE)]
- Microprobes: fluorescence etc.

TXM: transmission x-ray microscope

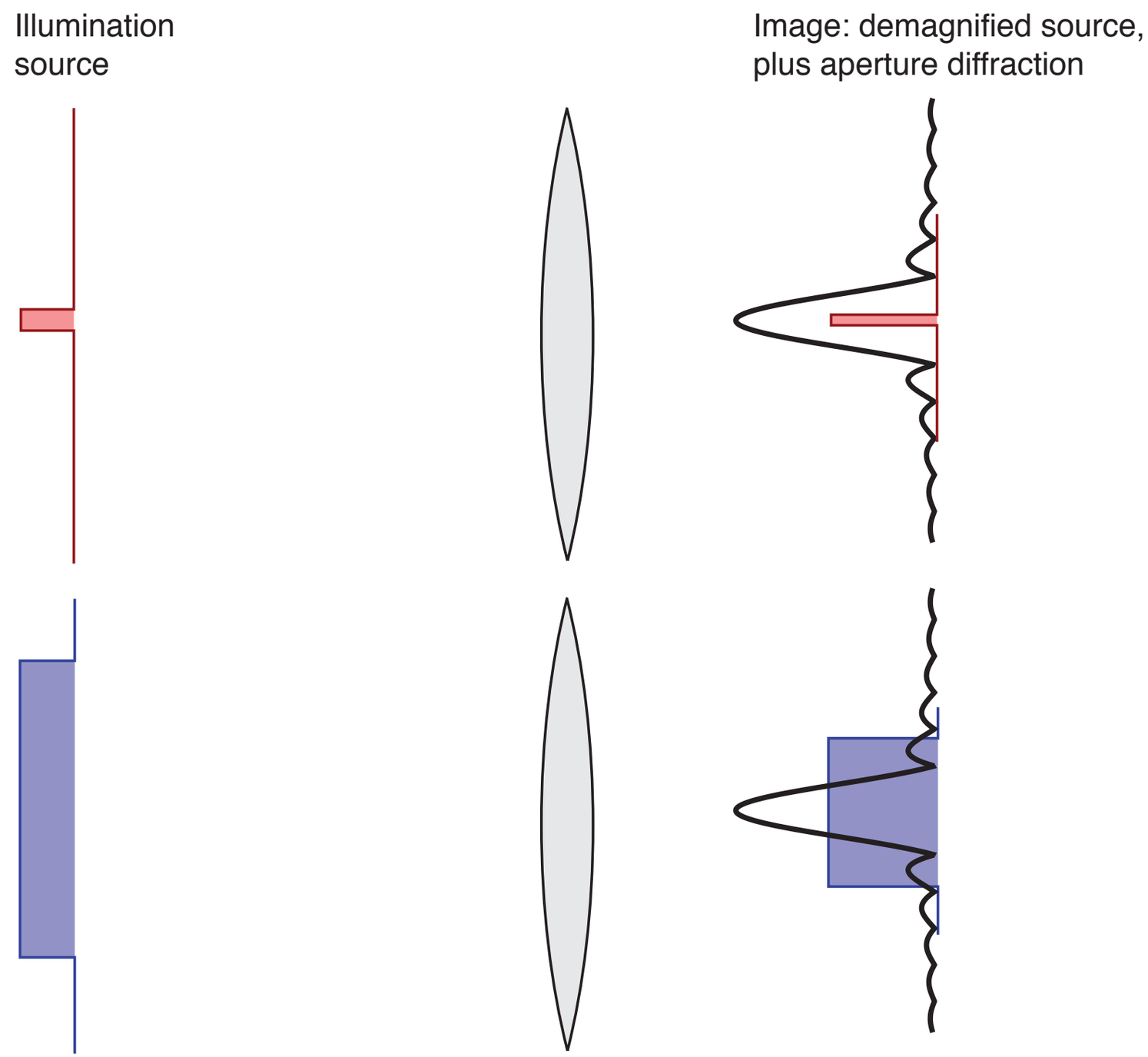


STXM: scanning transmission x-ray microscope



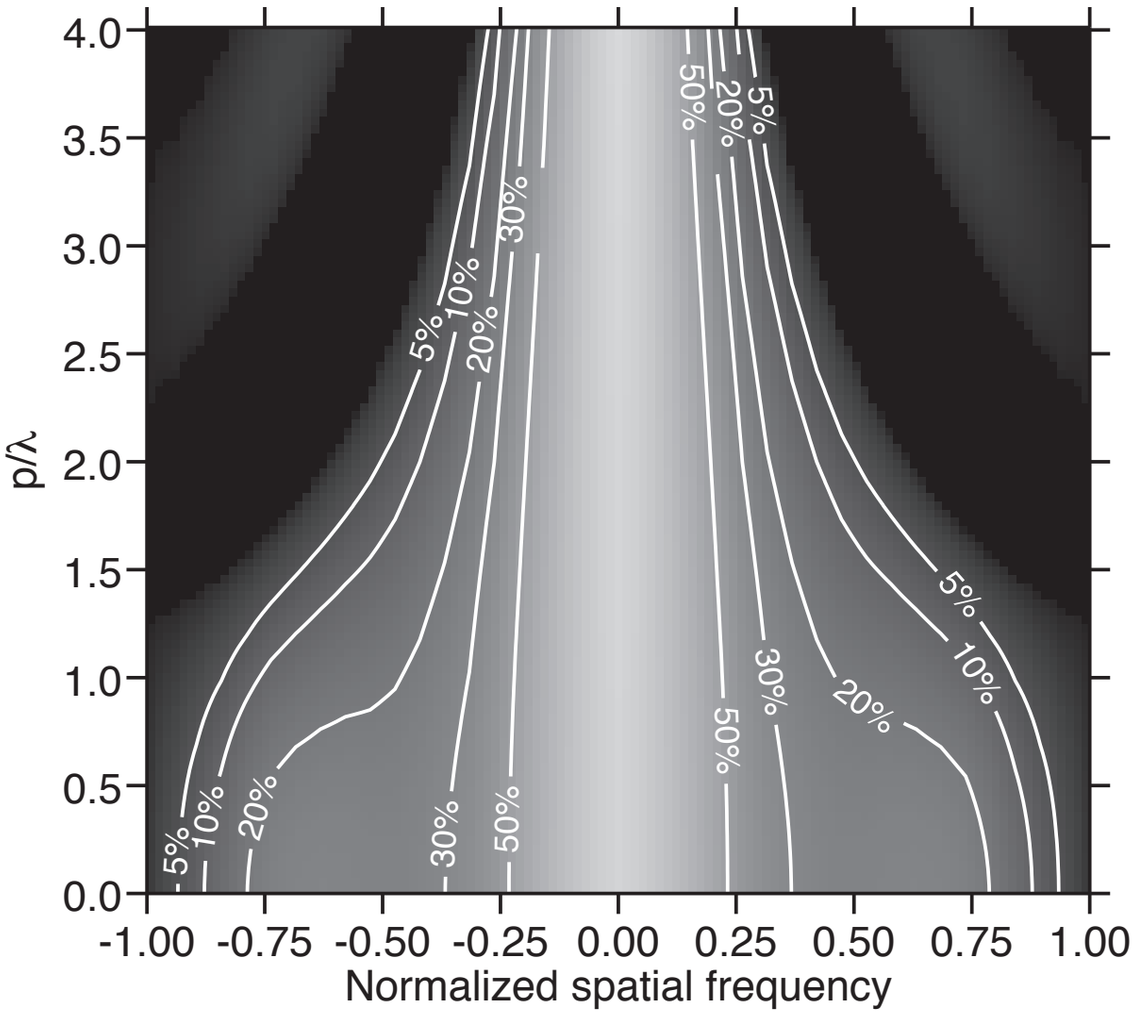
Scanning microscopes require coherent illumination

- Liouville's theorem: you can't reduce phase space without doing work (hard with photons!)
- Phase space of a diffraction limited lens with numerical aperture θ: $(2\theta)\cdot(2\cdot 0.61\lambda/\theta)=2.44\lambda$
- Thus need to limit source phase space to ~λ both in x and y
- Coherent flux is brightness$\cdot\lambda^2$
- See Kondratenko and Skrinsky, *Opt. Spectr.* **42**, 189 (1977)

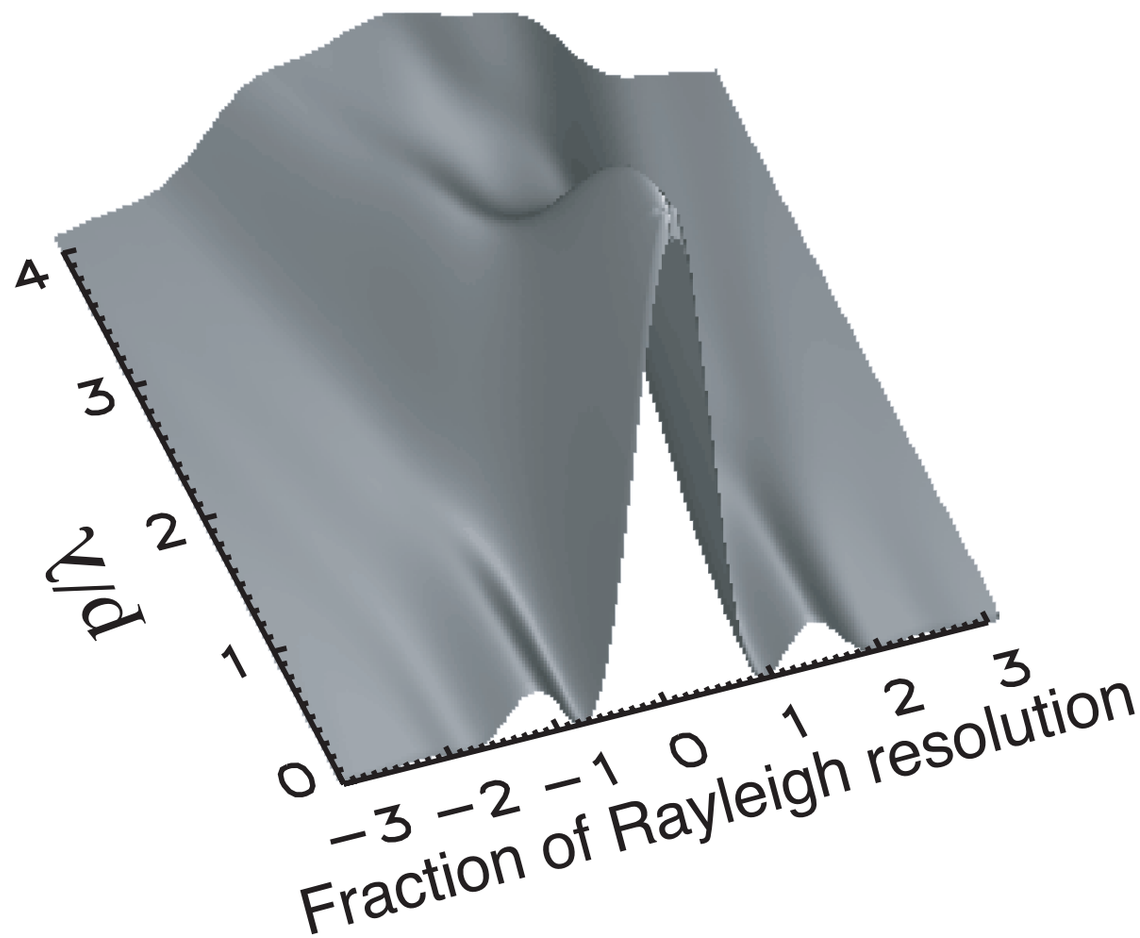


Phase space parameter p=(optic dia.)·(subtended angle)

How close must $p=h\theta$ be to λ?



Effect on modulation transfer function MTF (50% central stop)

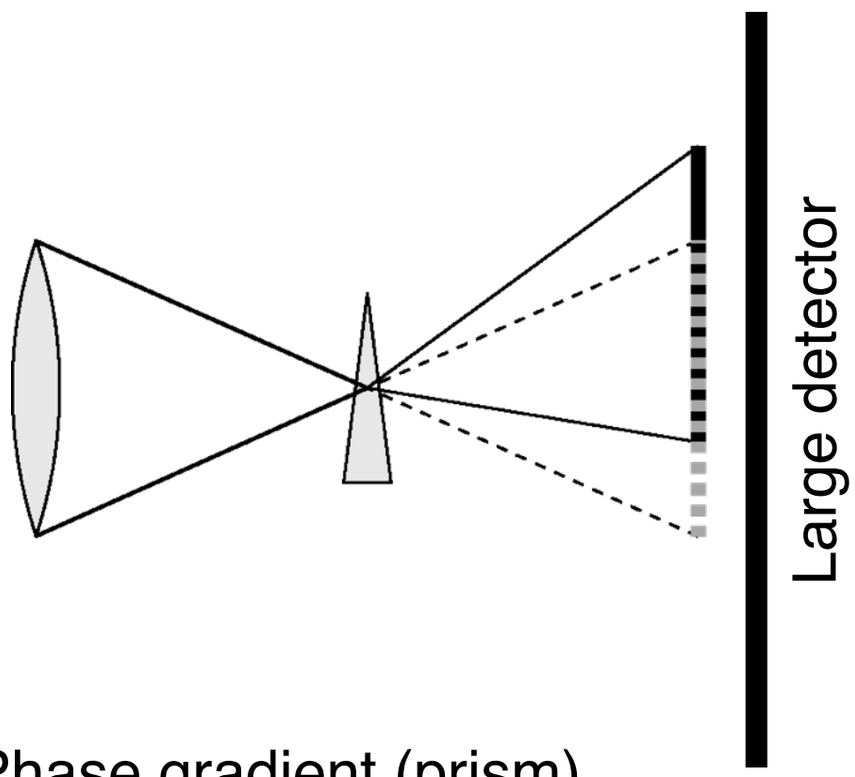


Effect on point spread function PSF (50% central stop)

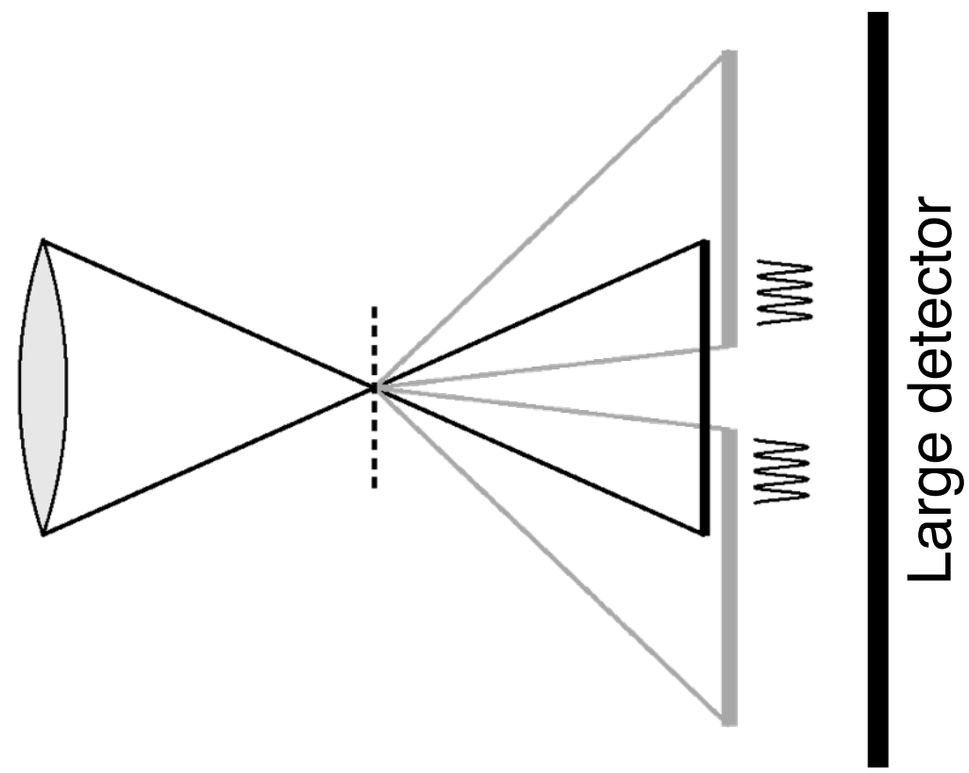
Jacobsen *et al.*, *Ultramicroscopy* **47**, 55 (1992); Winn *et al.*, *J. Synch. Rad.* **7**, 395 (2000).

STXM: contrast depends on detector

- Large area detector: sensitive only to absorption
- Point detector on-axis: coherent imaging
- Detector with restricted or segmented spatial response: some degree of phase contrast



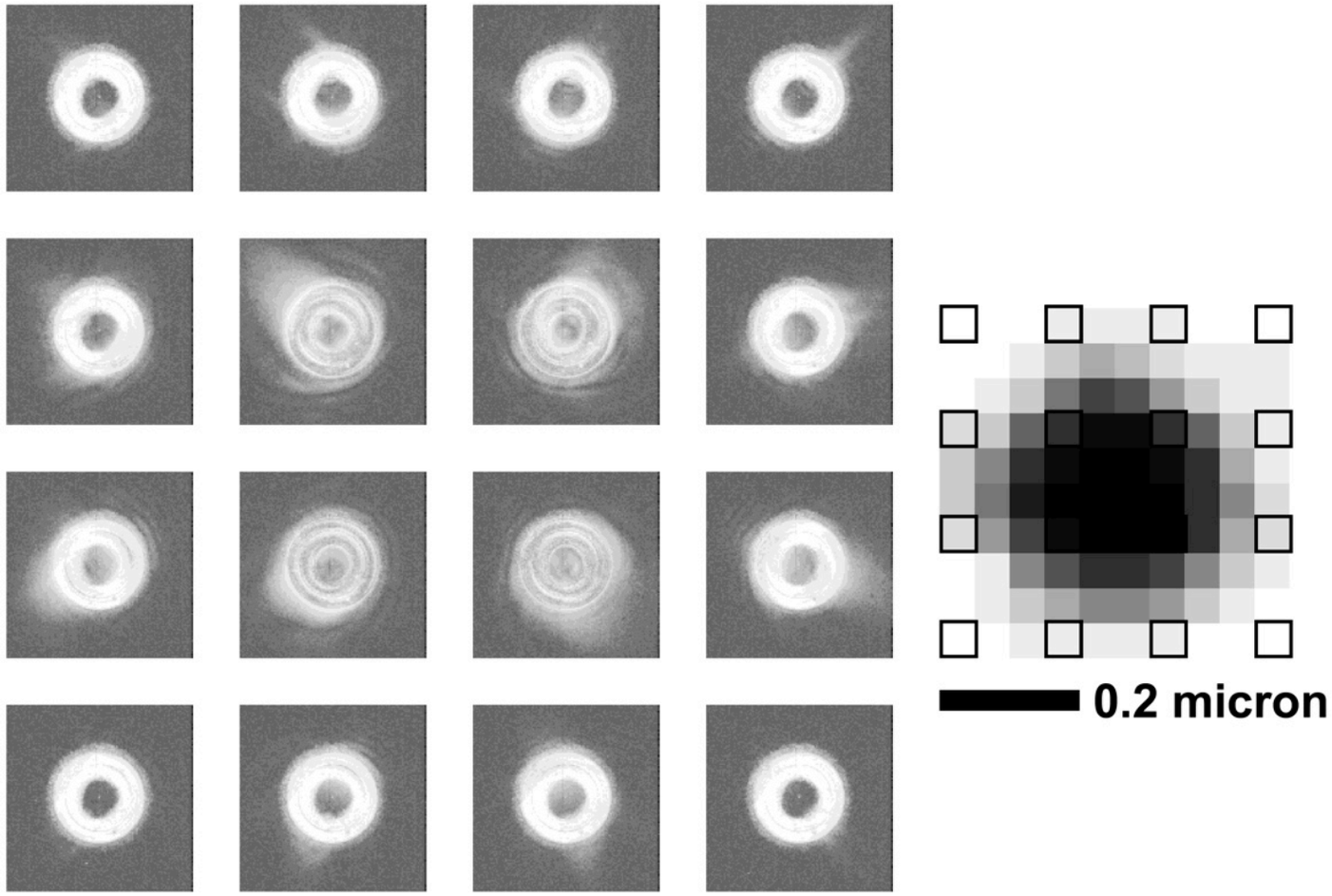
Phase gradient (prism)



Spatial frequency in object
See e.g., Spence and Cowley, *Optik* **50**, 129 (1978); Nellist *et al.*, *Nature* **374**, 630 (1995).

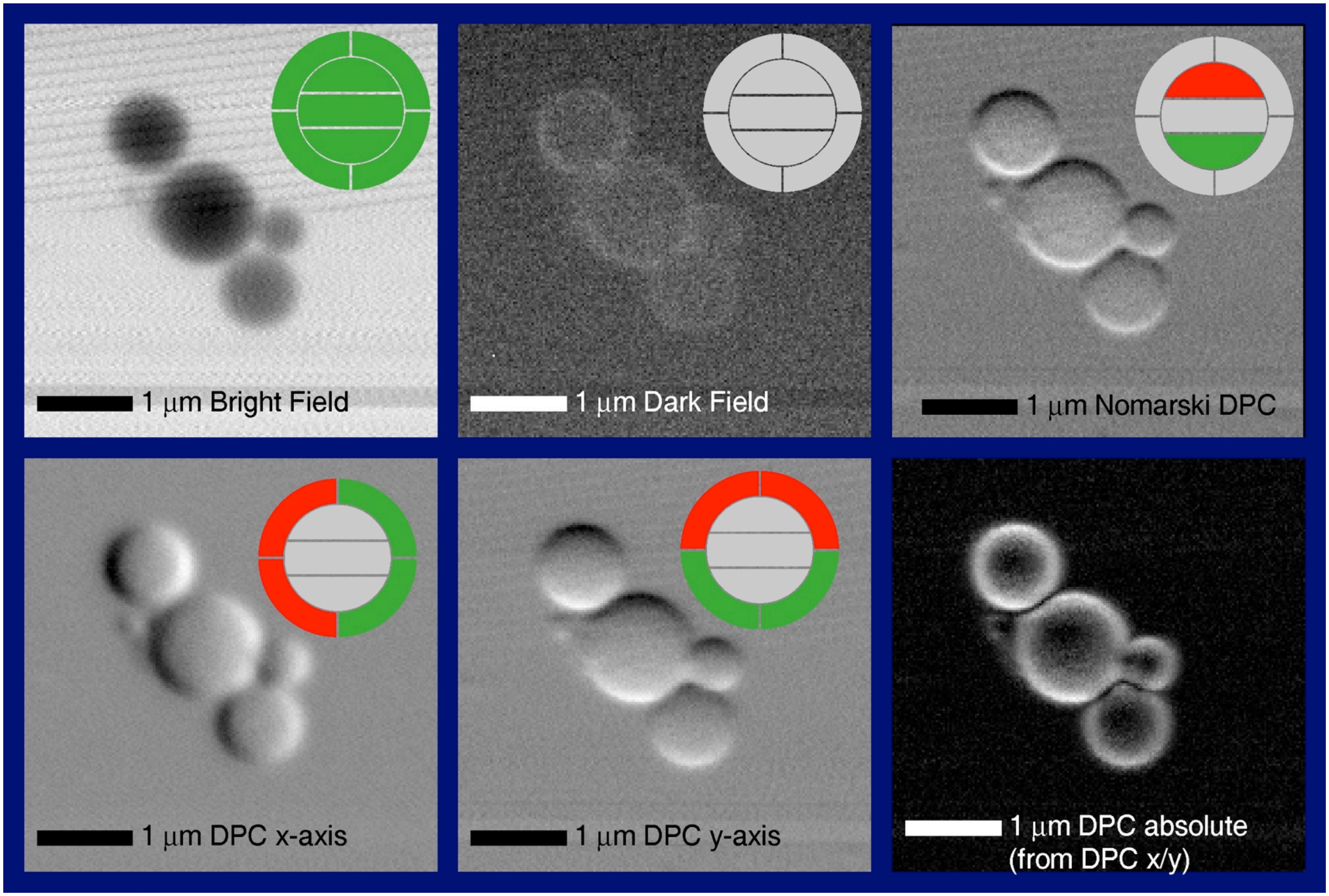
STXM: CCD as the ultimate detector

Record microdiffraction pattern per pixel; Wigner phase reconstruction. Chapman, *Ultramicroscopy* **66**, 153 (1996). Shown below: polystyrene sphere raw data (which was reconstructed to give amplitude and phase)



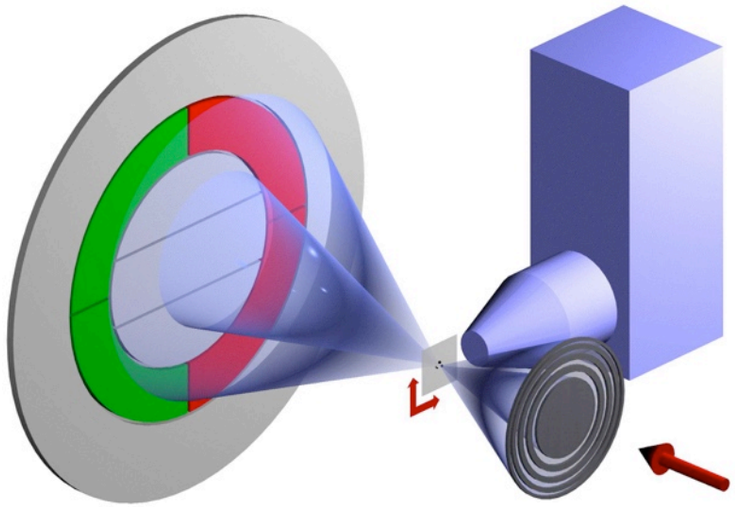
Simultaneous Availability Of Contrast Modes

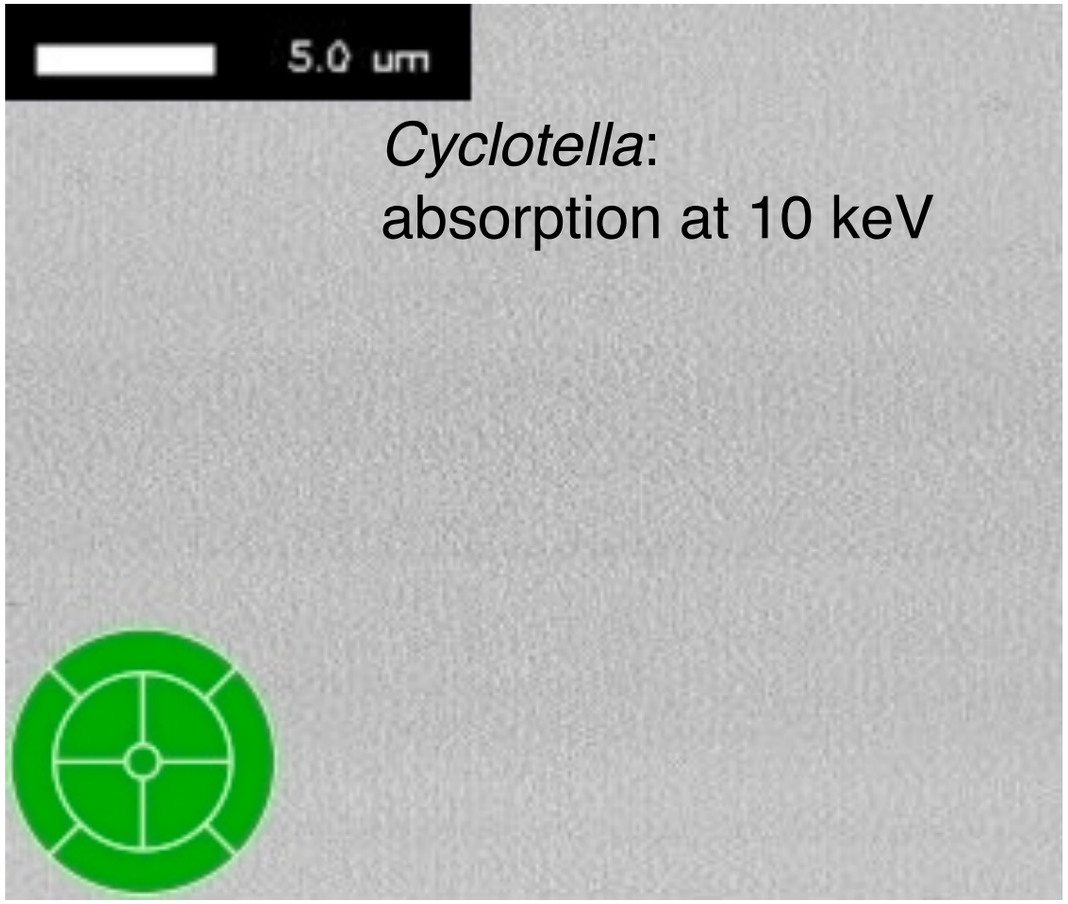
- Silica spheres 1 μm diameter or less
- Differential phase contrast filters out intensity fluctuations of the source!

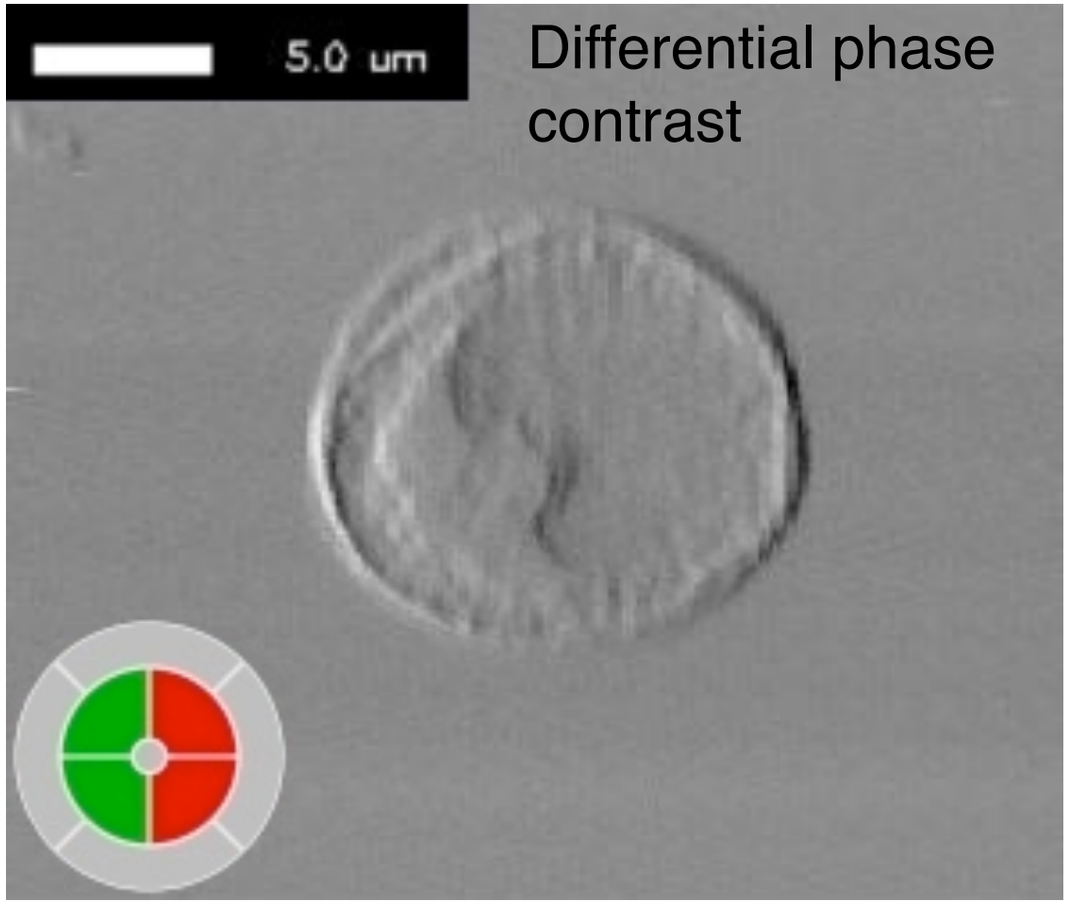


What's missing? Phase contrast for low-Z

- Segmented x-ray detector (Stony Brook, BNL, Max Planck silicon lab).
- Acquire simultaneously with fluorescence
- Fourier filtering, Fourier integration for absolute phase contrast.
- Sensitivity: ~$\pi/180$.





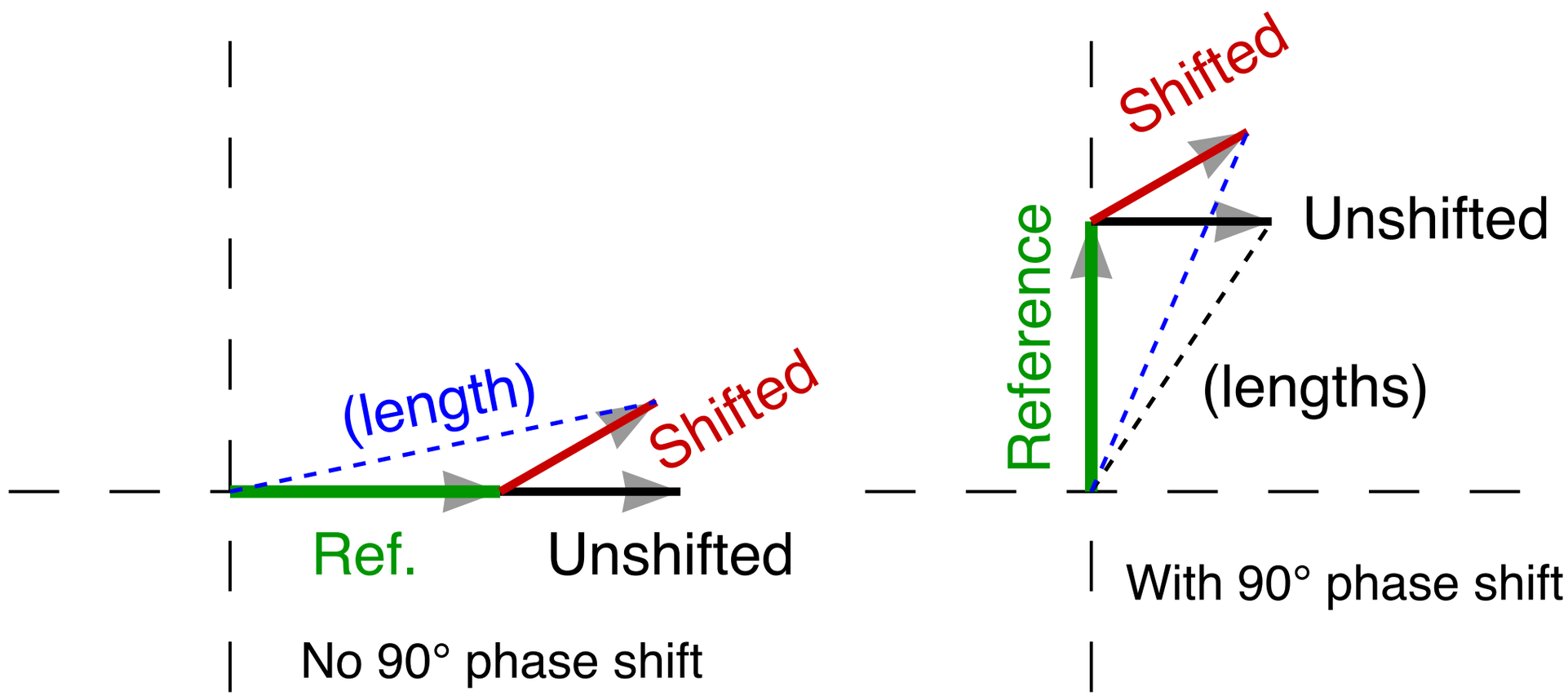


Hornberger *et al.*, *Ultramic.* **107**, 644 (2007); Feser *et al.*, *Nucl. Inst. Meth. A* **565**, 841 (2006); de Jonge *et al.*, *Phys. Rev. Lett.* **100**, 163902 (2008)

Turning phase variations into intensity variations

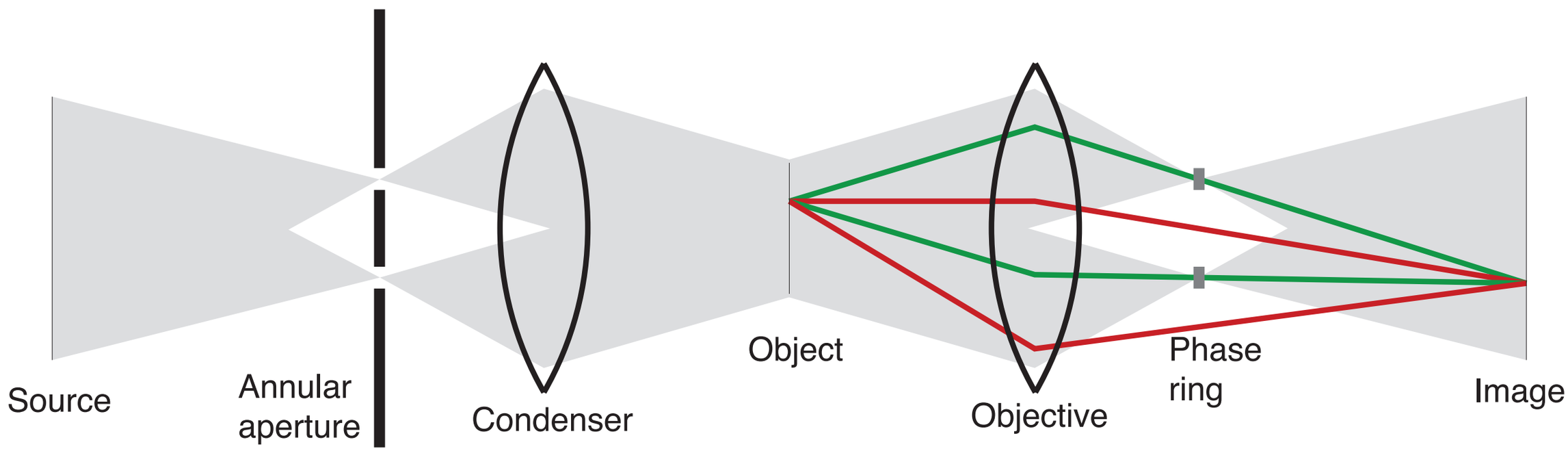
- Familiar principle: make two waves interfere (Reference, and Shifted-by-phase-feature).
- If the “reference” wave is phase shifted by 90°, small phase variations produce larger variations in intensity.

Change phase into intensity by mixing with a reference wave



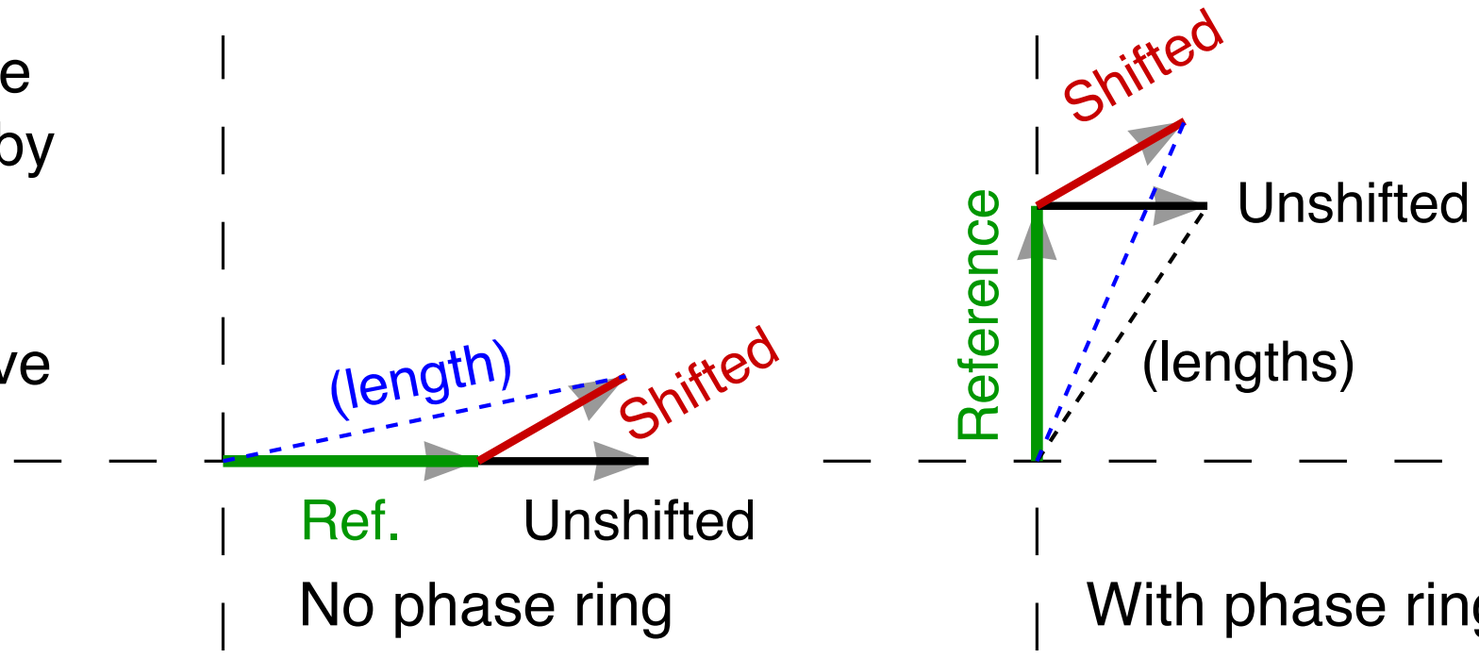
Beamsplitting: diffraction from the object

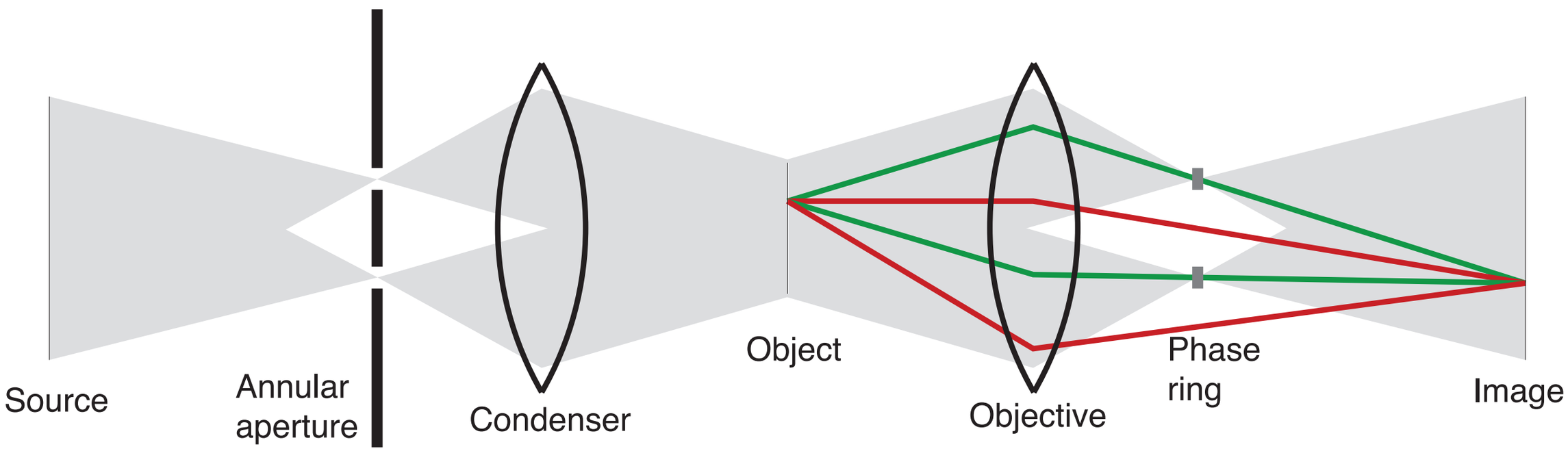
- Image annular aperture (the reference beam) from condenser back-focal plane to objective back-focal plane.
- Structure in the specimen diffracts light to different angles than the illumination, so that the specimen beam is spatially separated from the reference beam in the back focal plane.



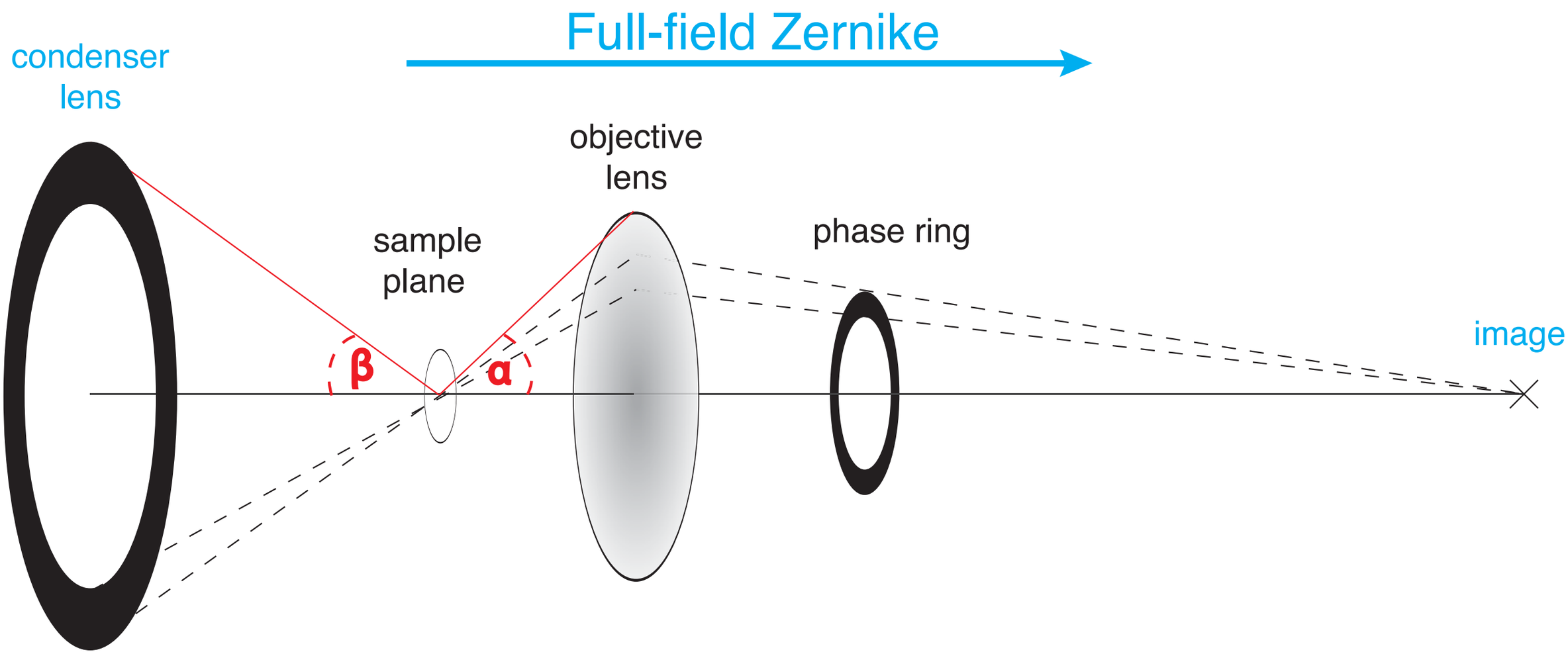
Zernike phase contrast: putting it all together

Change phase into intensity by mixing with a 90°-shifted reference wave



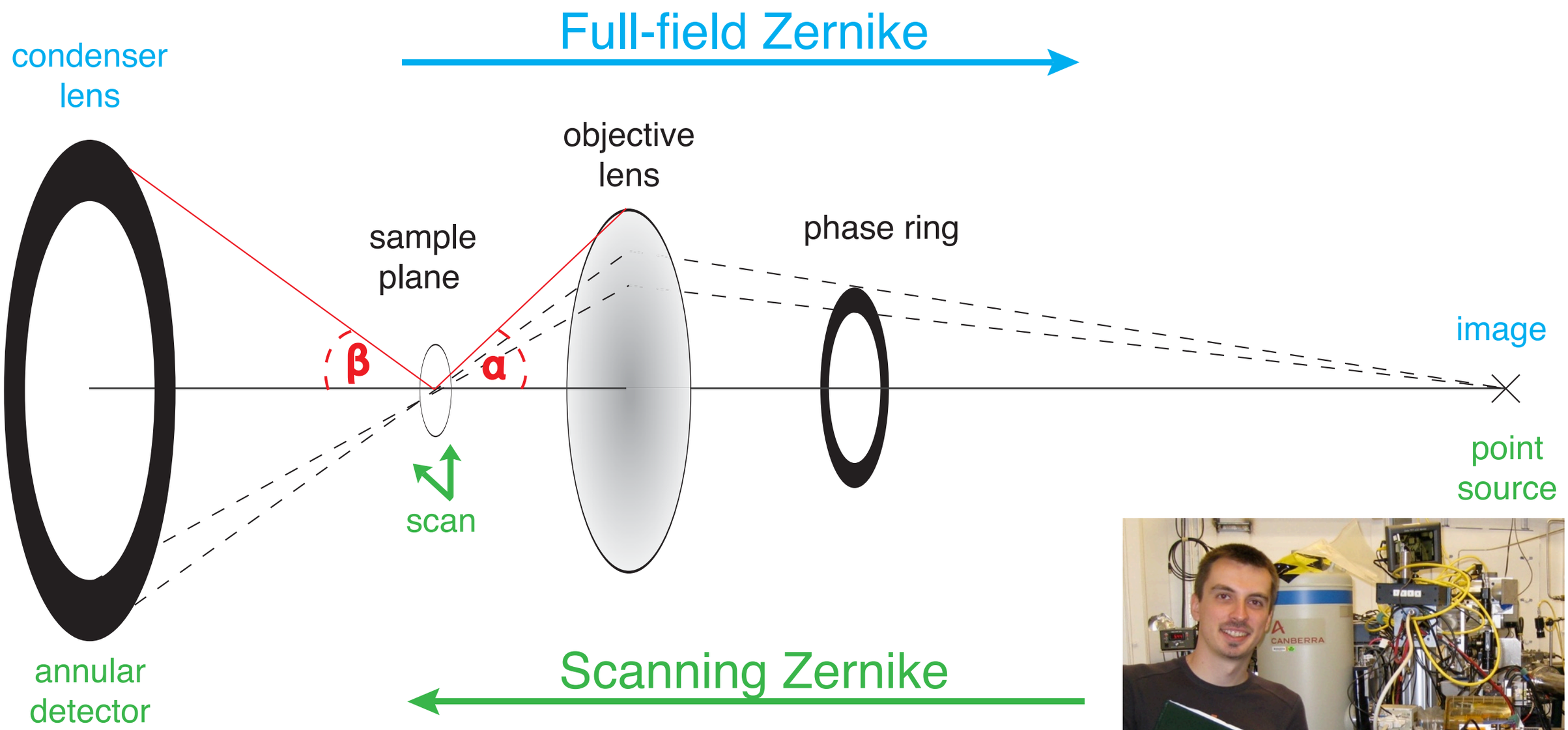


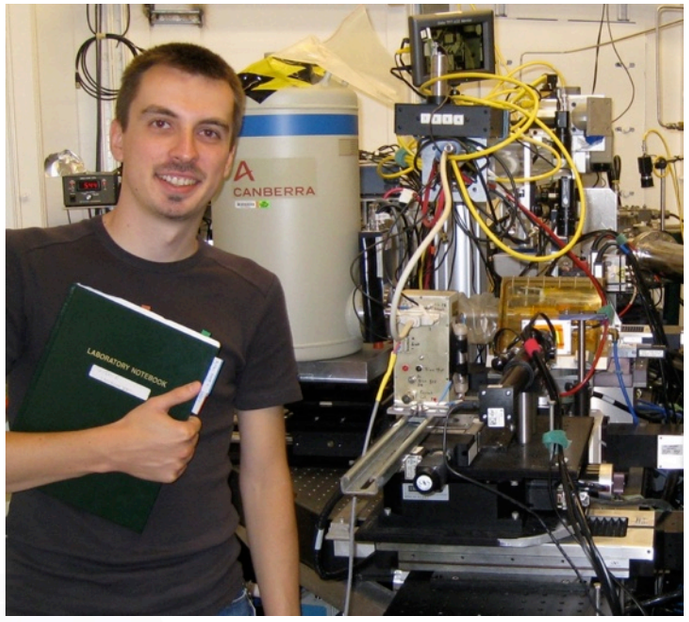
Zernike in a full field microscope



Zernike in a scanning x-ray microscope!

Reciprocity. Suggested by Wilson & Sheppard (1984); US Patent 4,953,188, Siegel, Schmahl, and Rudolph (1990); but not realized.



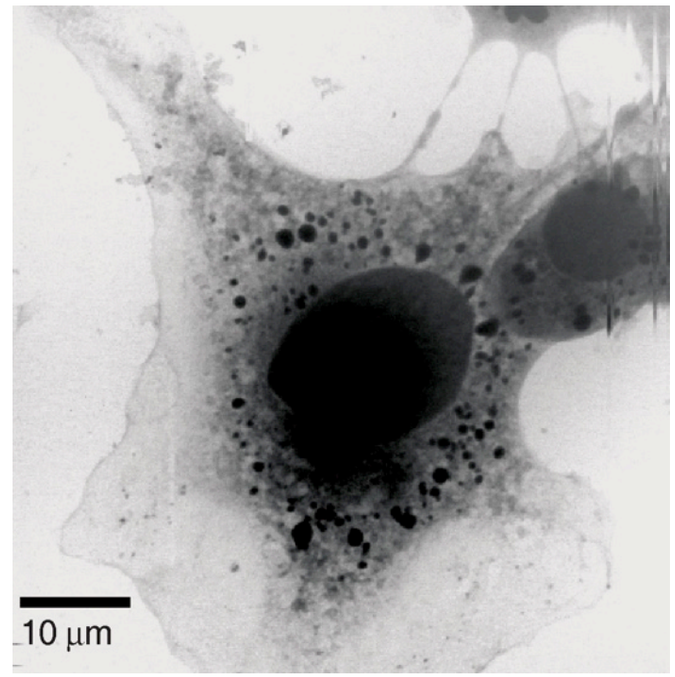


C. Holzner, M. Feser, B. Hornberger, S. Vogt, C. Jacobsen, *Nature Physics* **6**, 883 (2010)

2D imaging with Stony Brook STXMs

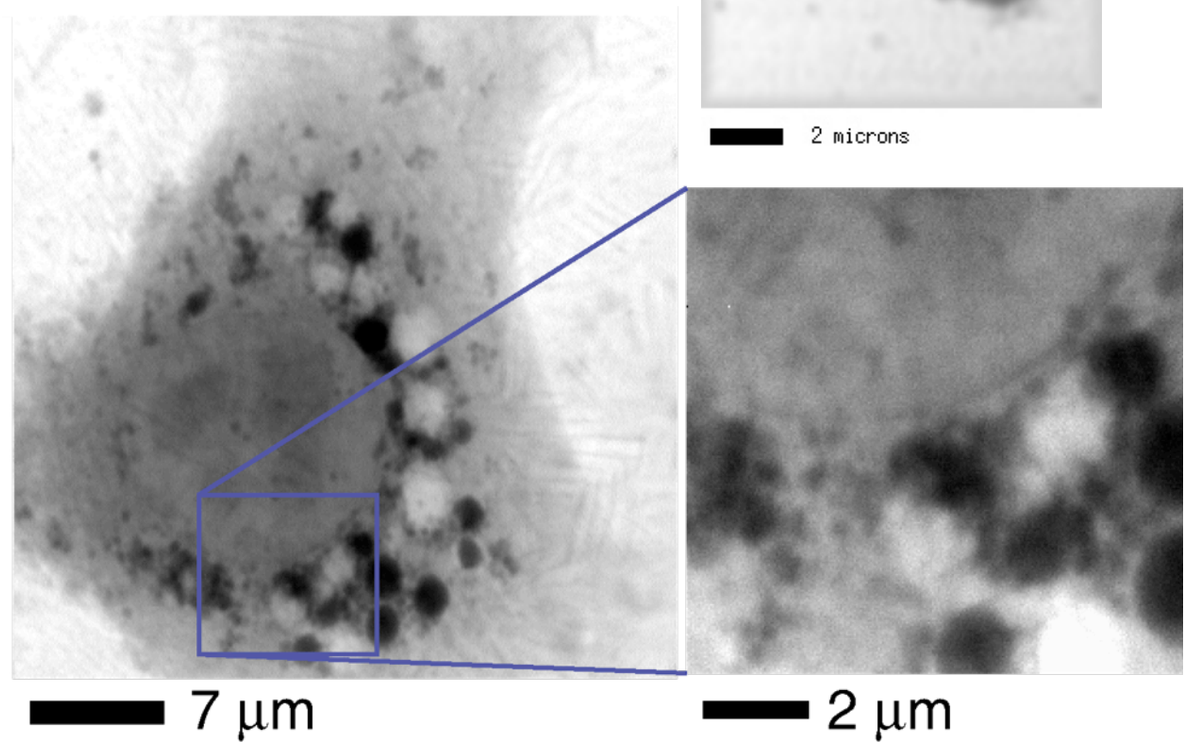
2D imaging is moderately useful but...

- Cannot track fluroescently-labeled proteins in living cells
- Resolution is inferior to cryoEM, though do not need to section
- Best utility may lie beyond simple 2D imaging



NIL 8 fibroblast (wet, fixed): Oehler *et al.*

Human sperm (unfixed): Wirick, Fleckenstein, Sheynkin *et al.*



Fibroblast (frozen hydrated): Maser *et al.*, *J. Micros.* **197**, 68 (2000)

Anilao, Philippines, 17-Oct-2010

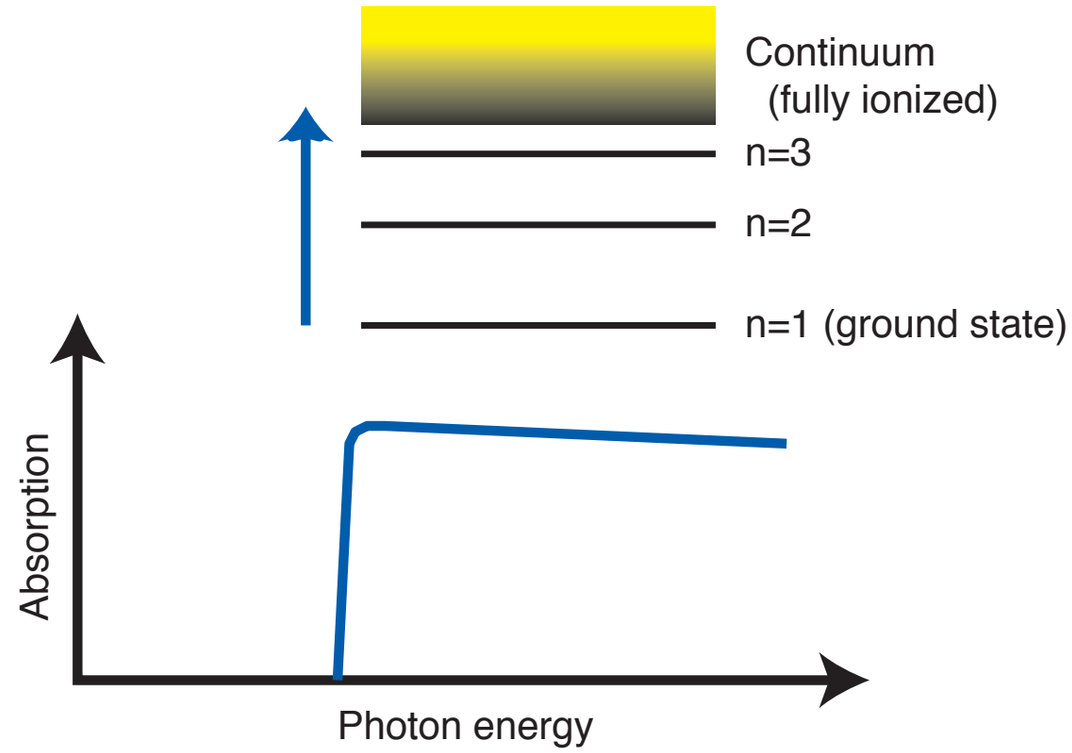
Absorption edges

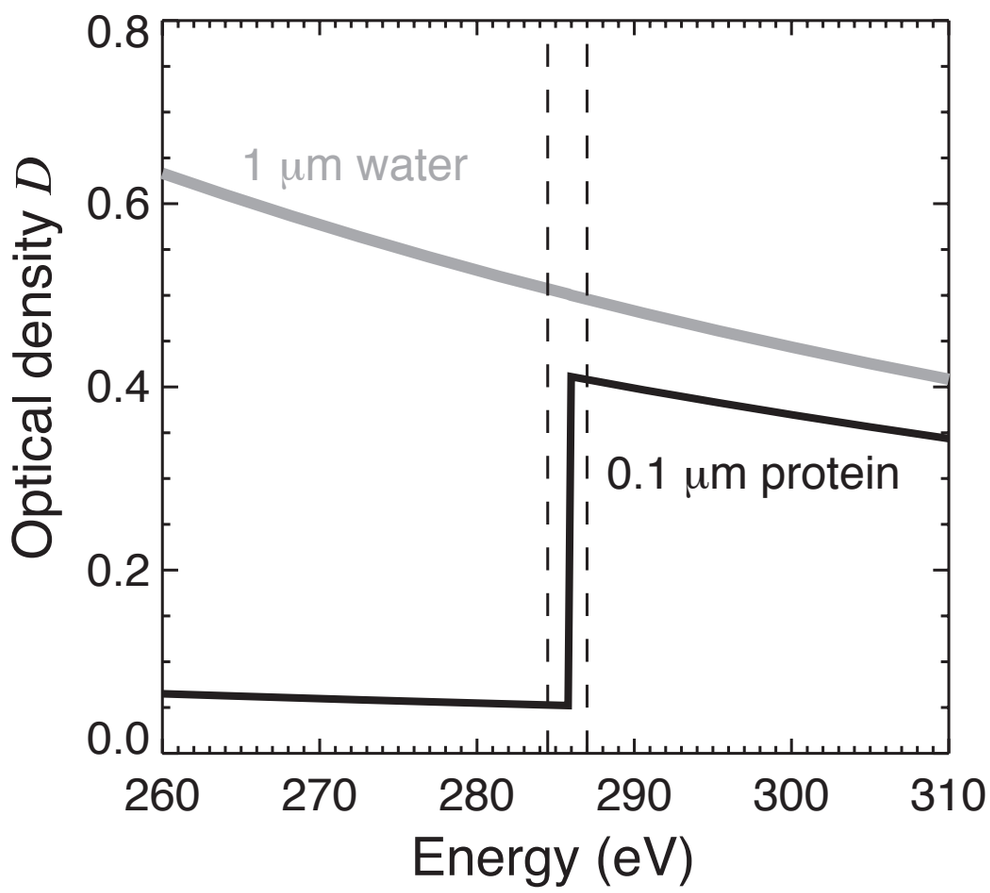
Lambert-Beer law: linear absorption coefficient μ

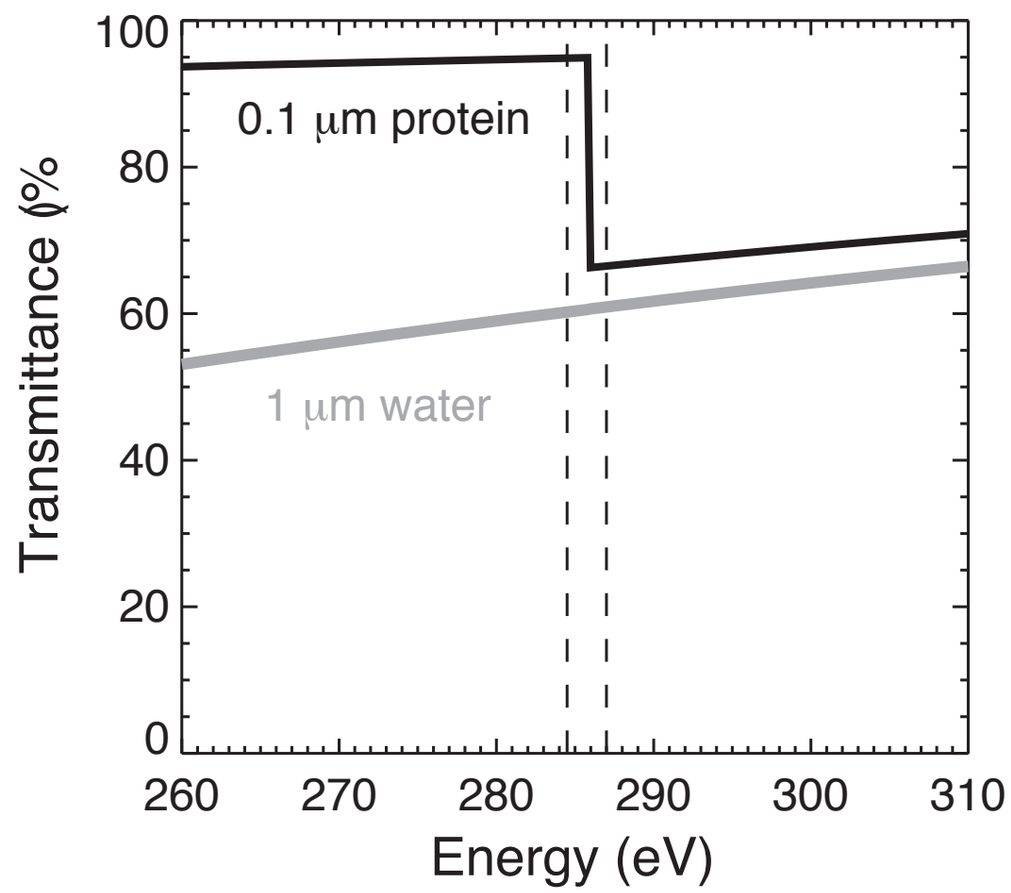
$$I = I_0 e^{-\mu(E)\cdot t} = I_0 e^{-D(E)}$$

This coefficient makes a jump at specific elemental absorption edges!

This example: 0.1 μm protein in water

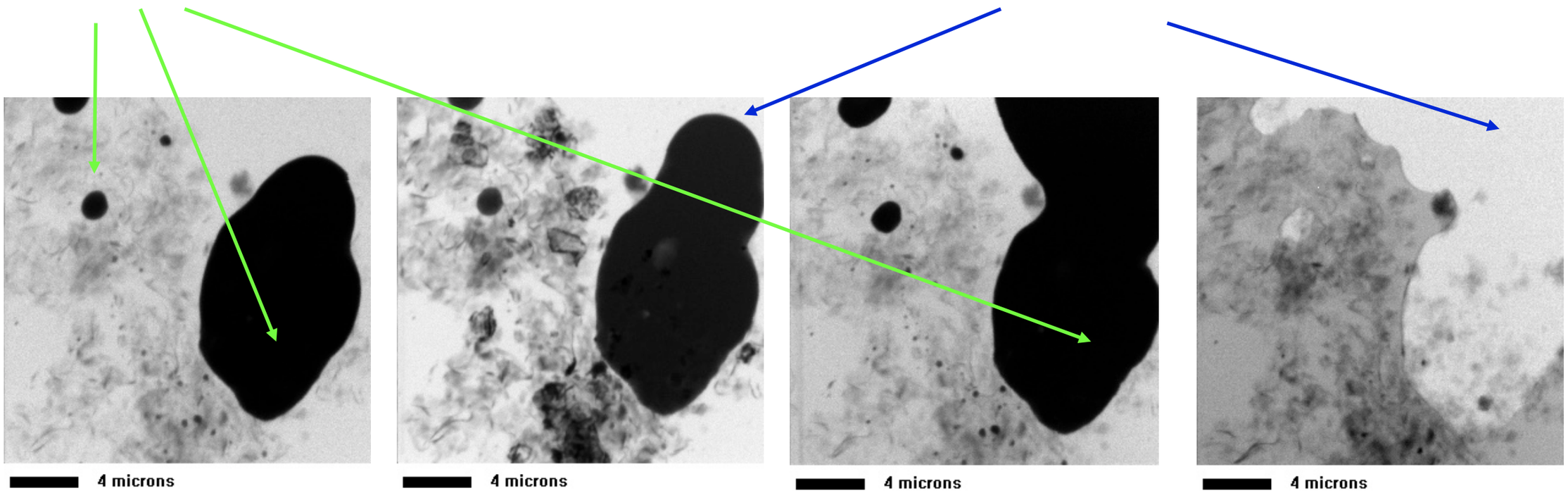






X-ray microscopy of colloids

- U. Neuhäusler (Stony Brook/Göttingen), S. Abend (Kiel), G. Lagaly (Kiel), C. Jacobsen (Stony Brook), Colloid and Polymer Science **277**, 719 (1999)
- Emulsion: water, oil droplets, clay, and layered double hydroxides (LDH)
- "Caged" part of oil droplet remains fixed; "uncaged" part can disperse



346 eV: calcium weakly absorbing. Clays and LDHs absorb equally

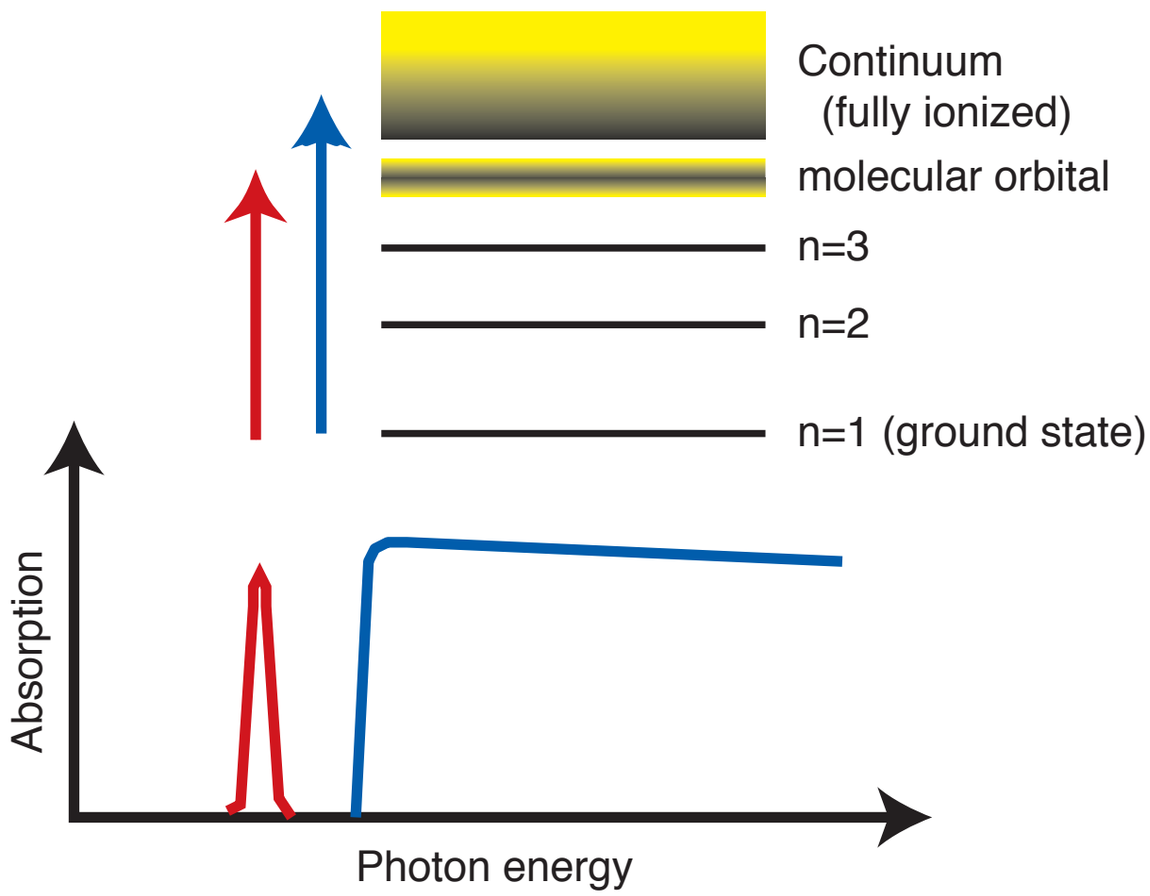
352.3 eV: calcium strongly absorbing. Calcium-rich LDHs are highlighted.

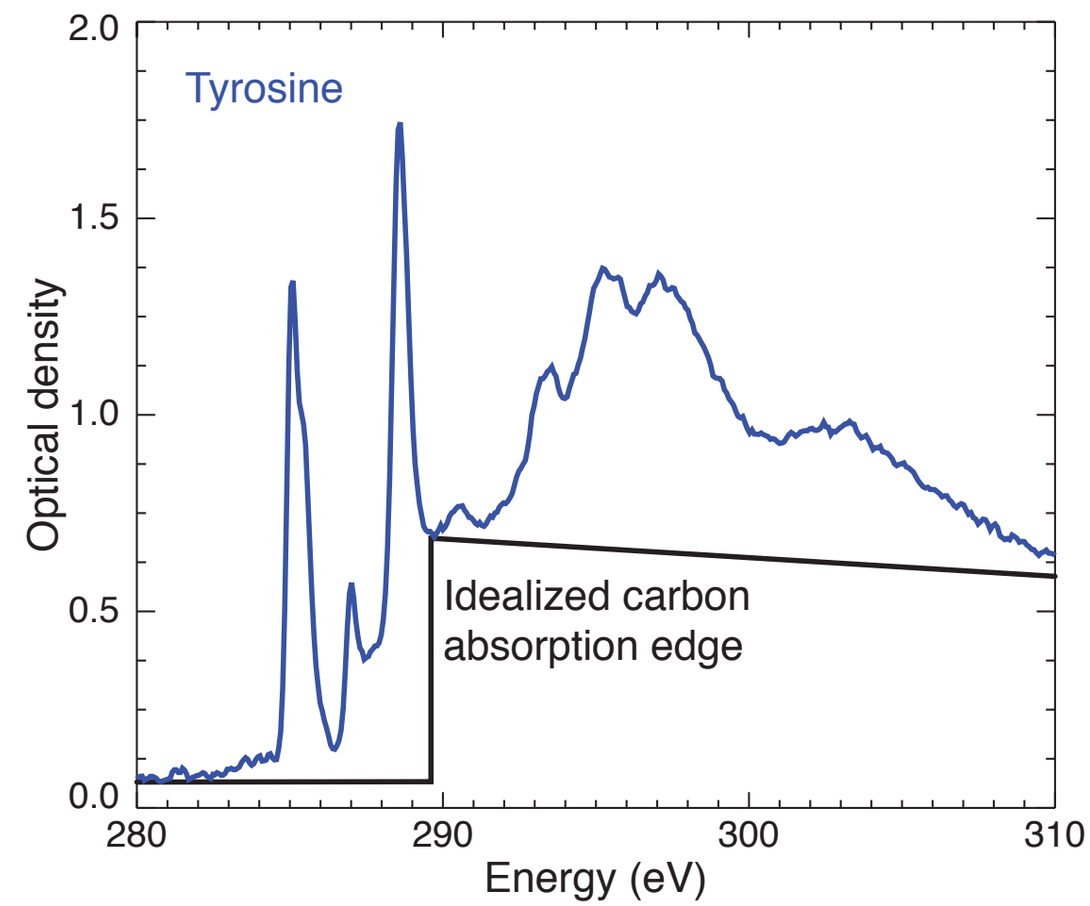
290 eV: carbon strongly absorbing

284 eV: carbon (oil drop) weakly absorbing

Near-edge absorption fine structure (NEXAFS) or X-ray absorption near-edge structure (XANES)

- Fine-tuning of the x-ray energy near an atom's edge gives sensitivity to the chemical bonding state of atoms of that type
- First exploitation for chemical state transmission imaging: Ade, Zhang *et al.*, *Science* **258**, 972 (1992) – Stony Brook/X1A



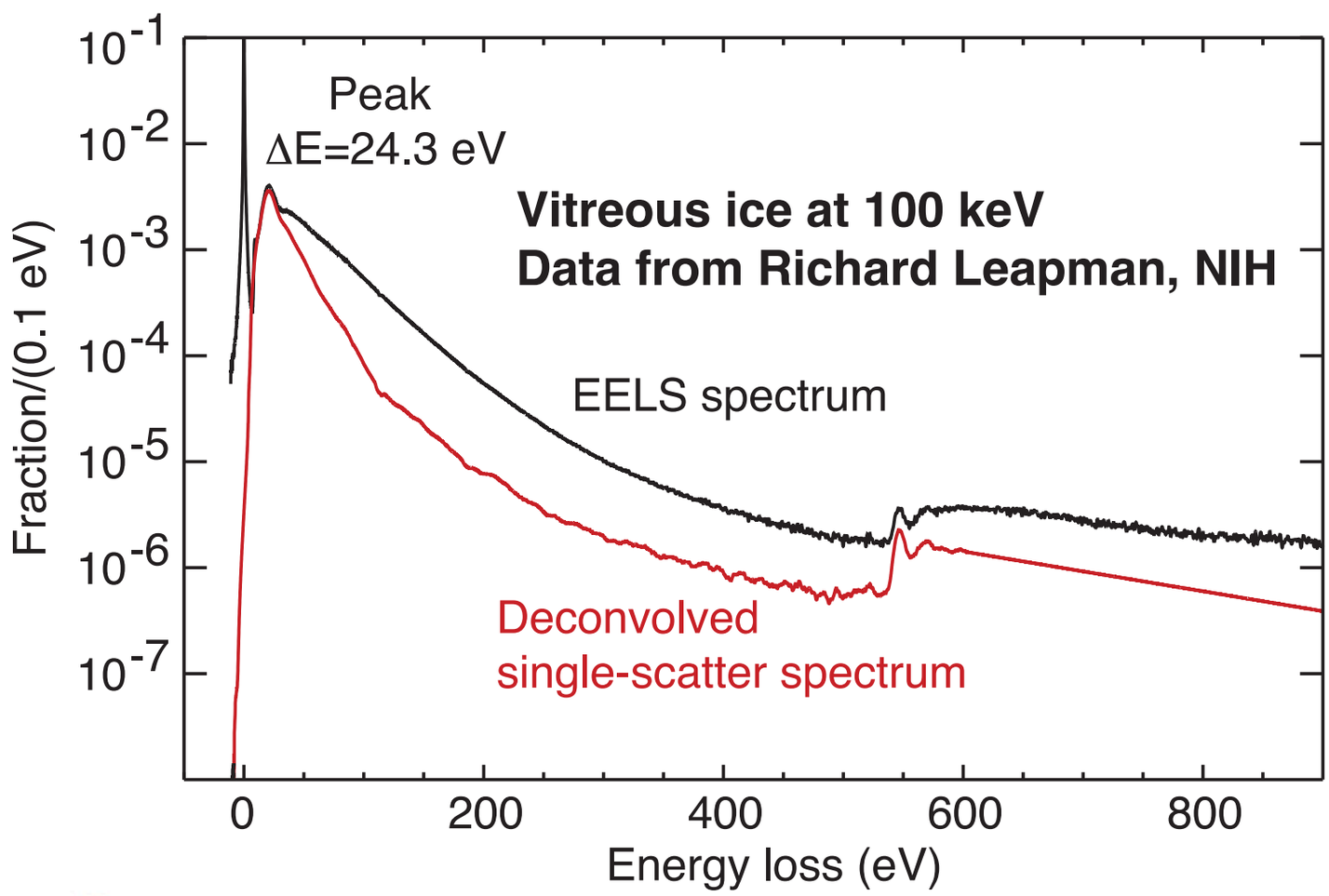


Compared with UV "tickling" of molecular orbitals, core-level electrons come from a single, well-defined state!

Near-edge spectroscopy: ELNES and XANES

ELNES (electron **E**nergy **L**oss)
- Plural inelastic scattering
- Many elements at once - but plasmon modes are always excited (damage)
- ΔE was ~0.6 eV, but now <0.1 eV in some cases

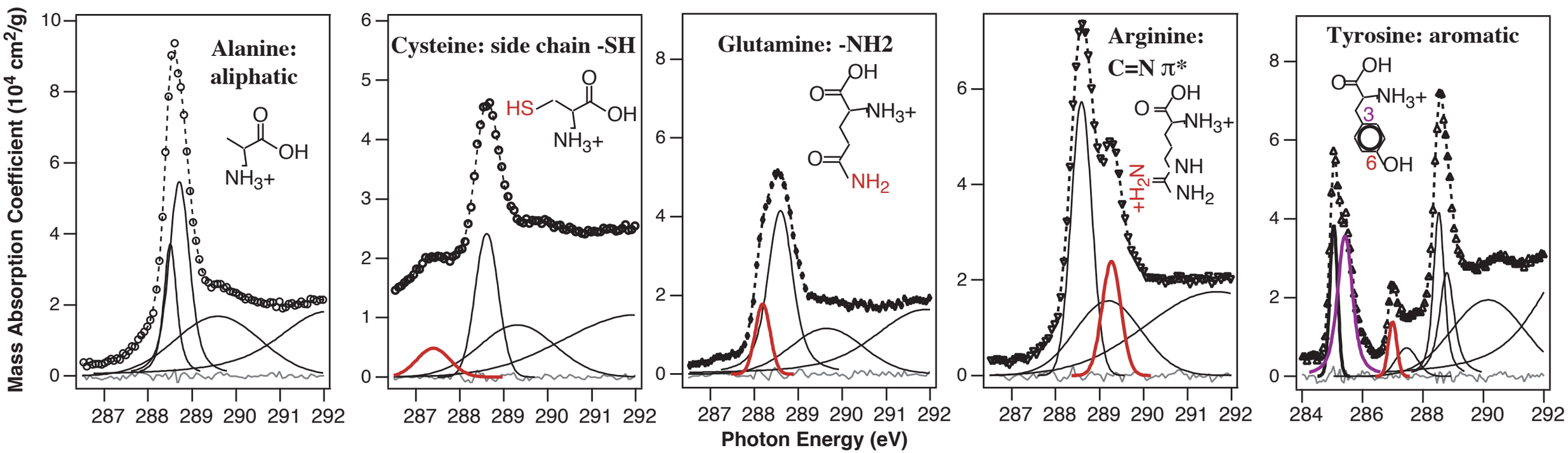
XANES (**X**-ray **A**bsorption)
- No plural scattering
- One element at a time - slow but less damage
- ΔE of 0.05-0.1 eV is common



Electrons ~1000x more damaging:
- Isaacson and Utlaut, *Optik* **50**, 213 (1978)
- Rightor *et al.*, *J. Phys. Chem. B* **101**, 1950 (1997)

C-XANES of amino acids

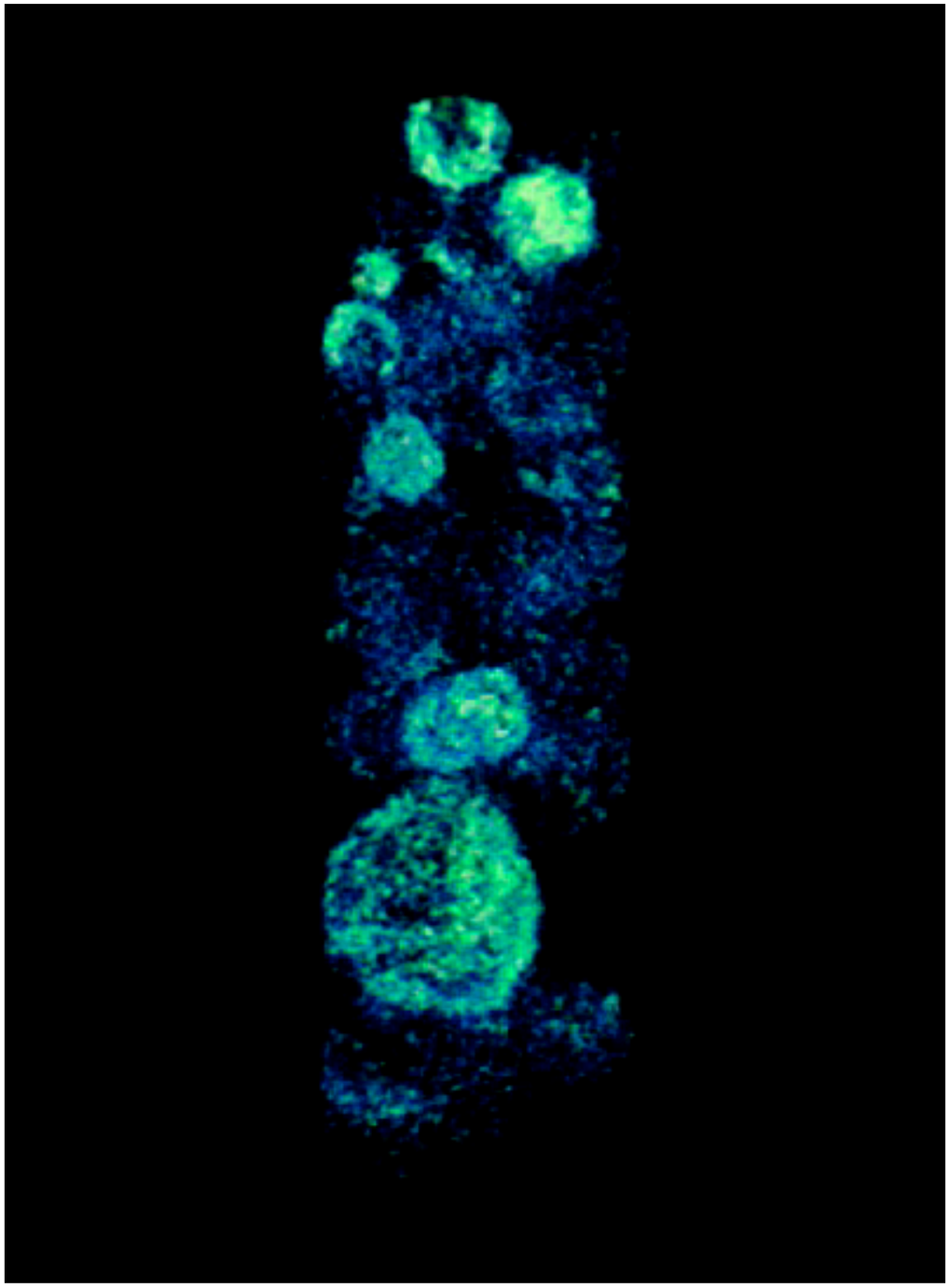
- K. Kaznacheyev *et al.*, *J. Phys. Chem.* **A 106**, 3153 (2002)
- Experiment: K. Kaznacheyev *et al.*, then at Stony Brook
- Theory: O. Plashkevych, H. Ågren *et al.*, KTH Stockholm; A. Hitchcock, McMaster



Polymers: see e.g., Dhez, Ade, and Urquhart, *JESRP* **128**, 85 (2003)

XANES tomography: chemical states in 3D

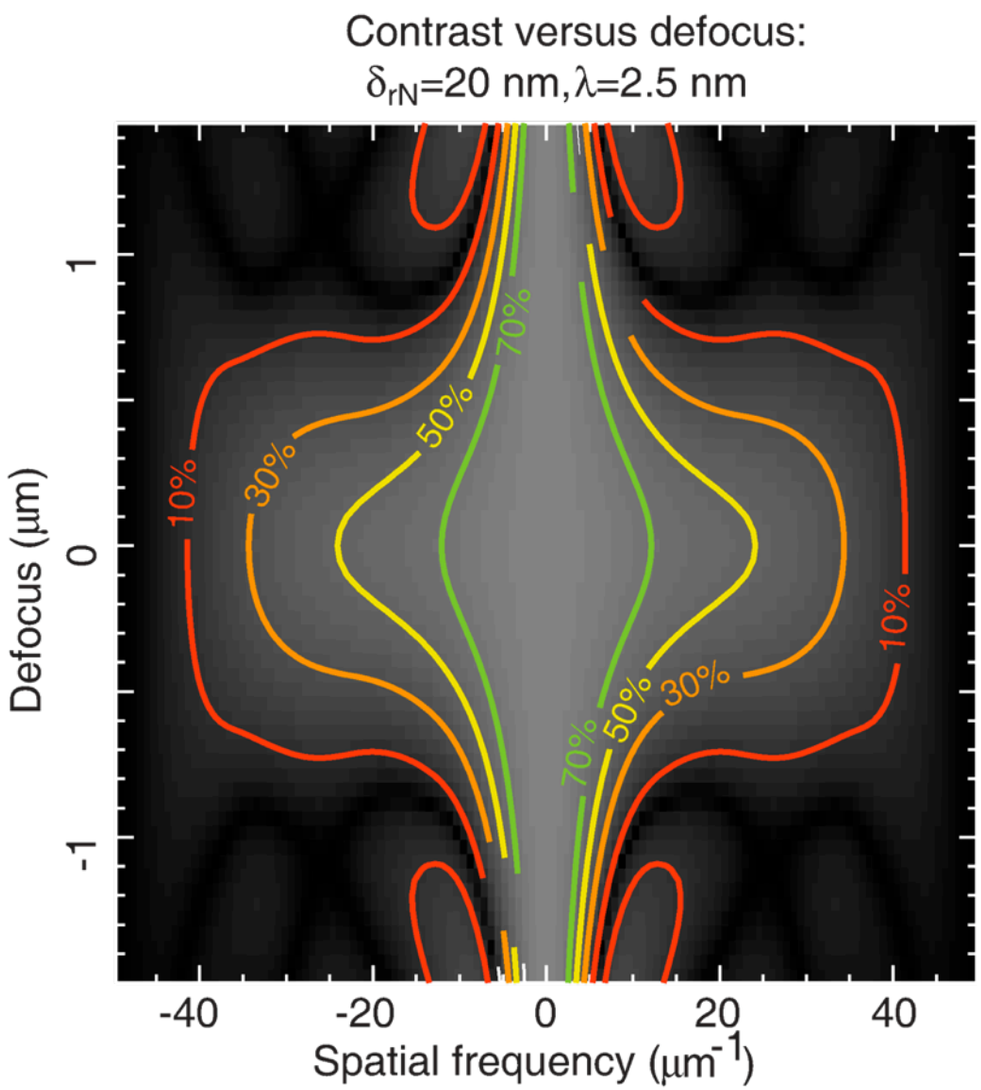
- Johansson, Hitchcock *et al.*, *J. Synch. Rad.* **14**, 395 (2007).
- This example: acrylates in polymer microspheres.
- Experiments are *slow* at present-day synchrotrons.
- Radiation damage? Dose fractionation?

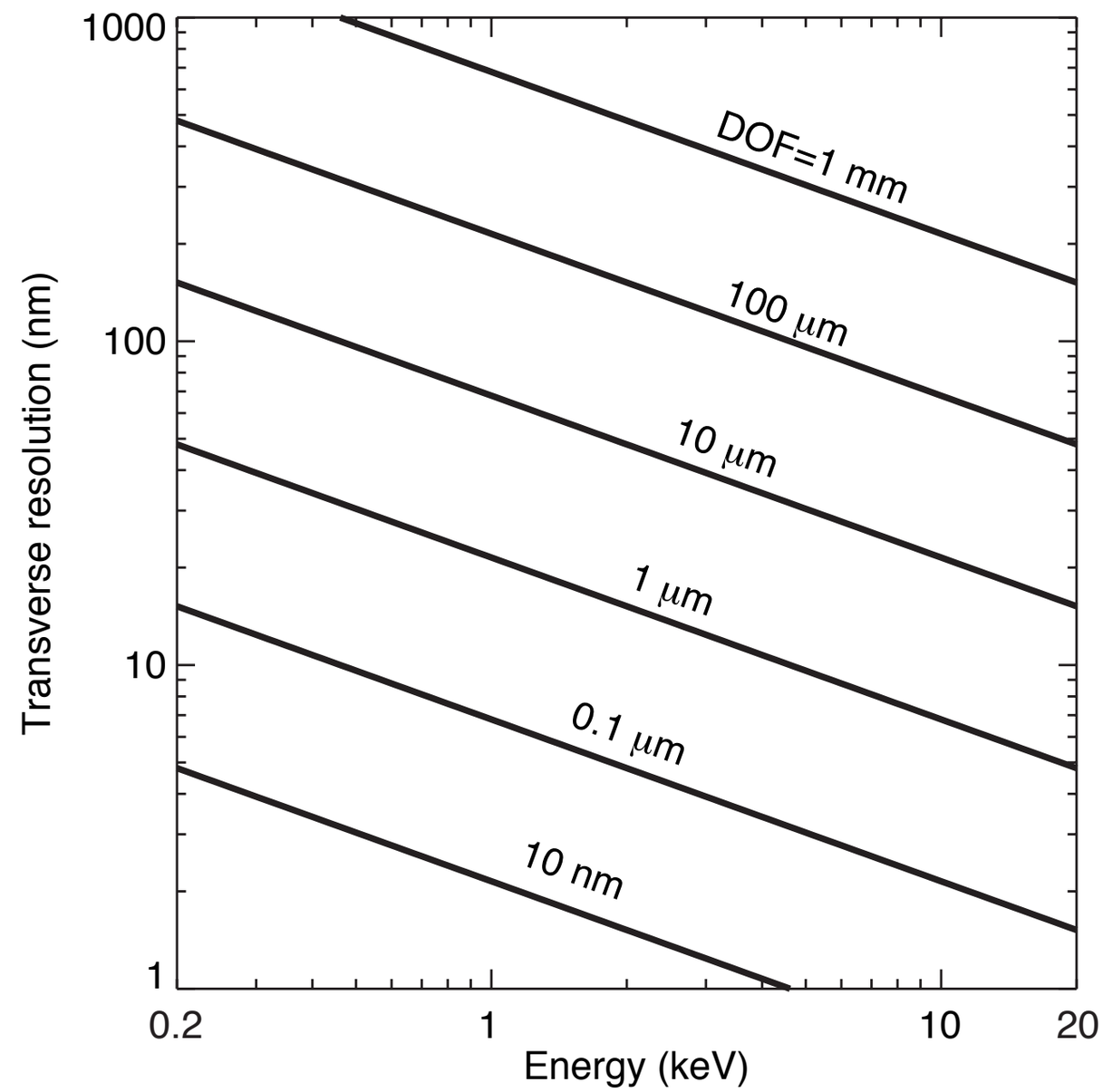


Depth of focus

Transverse: $$\Delta_t \Rightarrow \frac{\lambda}{4\theta} = \frac{\Delta_{rN}}{2}$$

Longitudinal: $$\Delta_\ell \Rightarrow \frac{\lambda}{\theta^2} = 4\Delta_{rN}\frac{\Delta_{rN}}{\lambda}$$





Silverman Hall
Northwestern University
H
Li
Na
Mg
K
Ca
Mn
Fe
Co
Cu
Zn
Ga
Mo
Tc
Ag
Pt
C
N
O
F
P
S
As
Gd

Transition metals in cells

- ~1/3 of proteins are metalloproteins.
- Cells prefer to maintain a narrow rang of transition metal concentrations[e].
- Iron plays a metabolic role (also Friedrich's ataxia)
- Copper affects angiogenesis[f] (also Wilson's disease)
- Chromium and Vanadium-based drugs considered for diabetes treatment[g]

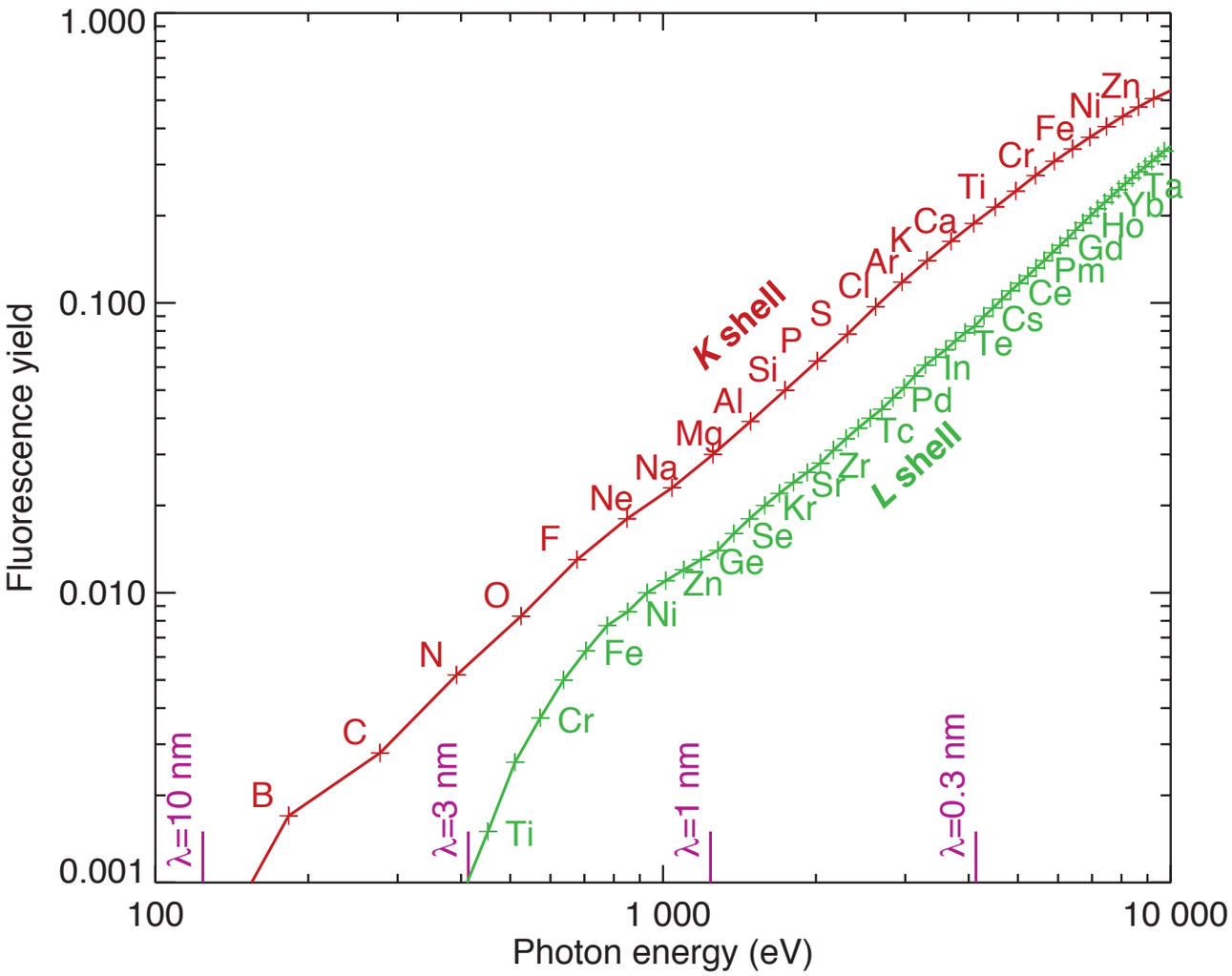
Bulk concentrations: but cell-to-cell, and intracellular variations?

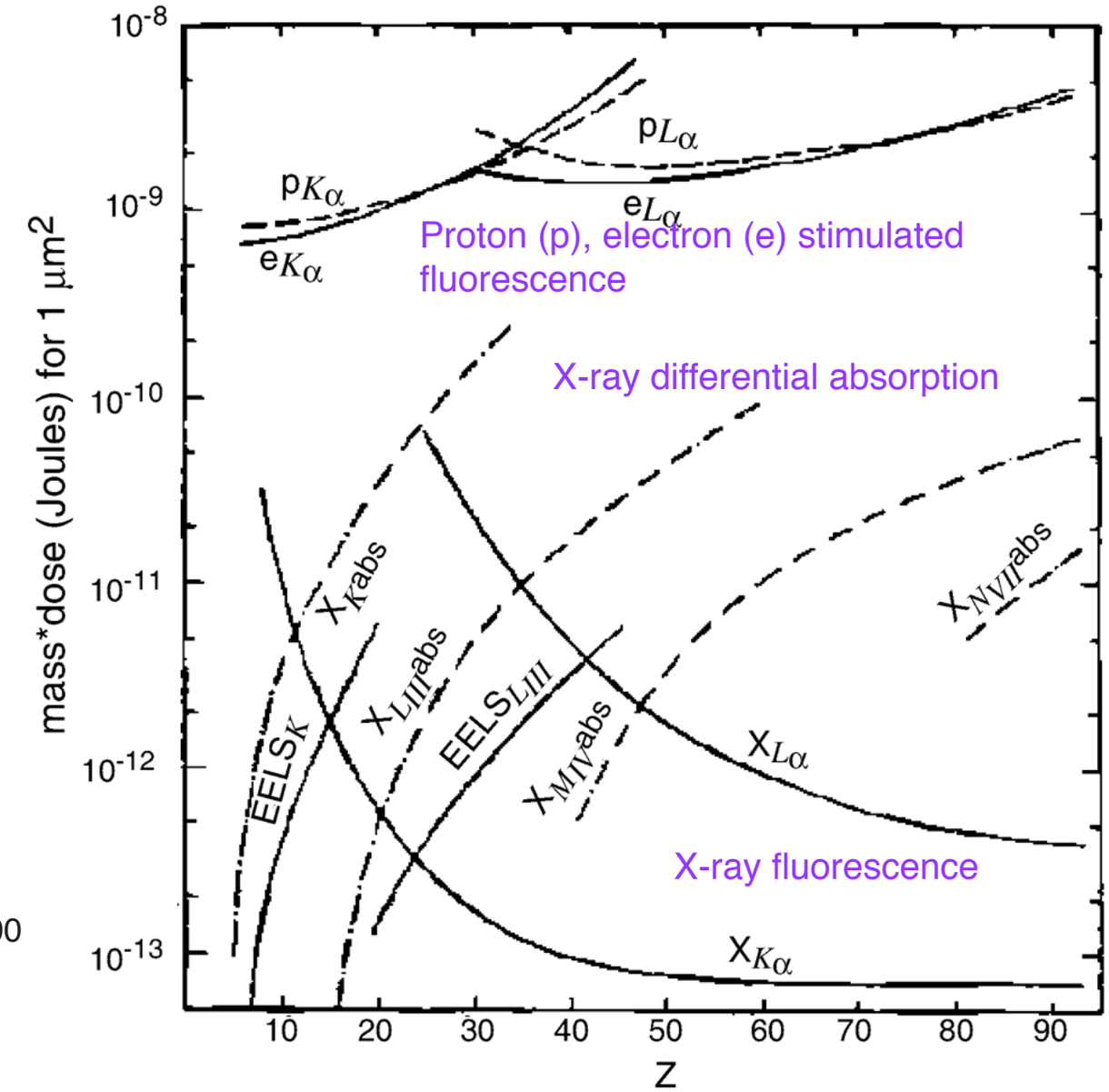
Table from Alison Kim

	mM		
	26 Fe	29 Cu	30 Zn
E. coli[a]	0.3	0.04	0.2
S. cerevisiae[b,c]	0.2	0.01	0.4
mouse fibroblasts[c,d]	0.5	0.04	0.6
red blood cells[c]	**12.5**	0.01	0.2

[a] Outten *et al*. *Science* **292**, 2488-2492 (2001)
[b] MacDiarmid *et al*. *EMBO J.* **19**, 2845-2855 (2000)
[c] Unpublished data courtesy of R. Marvin (O'Halloran Group)
[d] Suhy *et al*. *J. Biol. Chem.* **274**, 9183-9192 (1999)
[e] Colvin *et al., Eur. J. Pharmacol.* **479**, 171-185 (2003)
[f] Finney *et al.*, *PNAS* **104,** 2247 (2007)
[g] Levina and Lay, Dalton Transactions 40, 11675 (2011)

Detecting trace elements: x-ray fluorescence

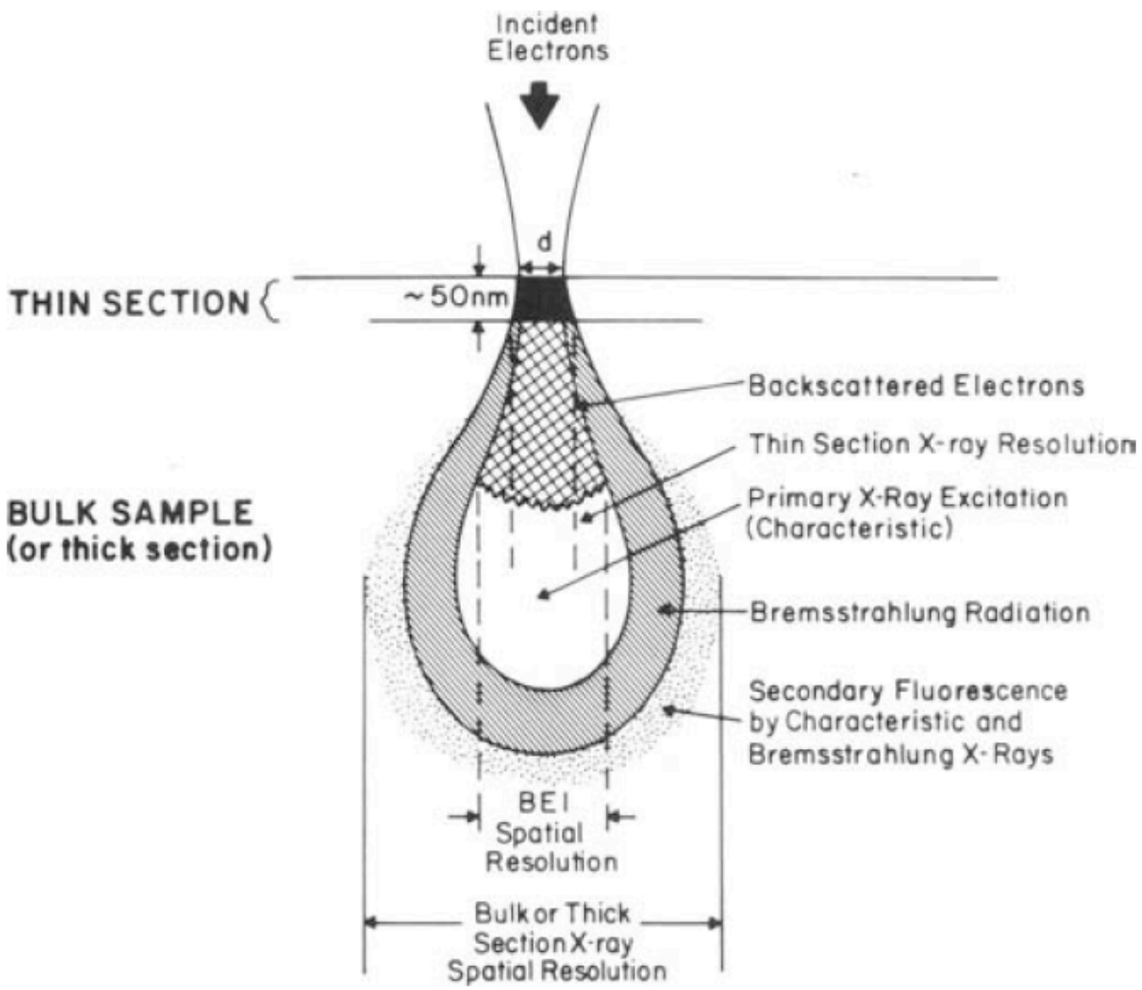


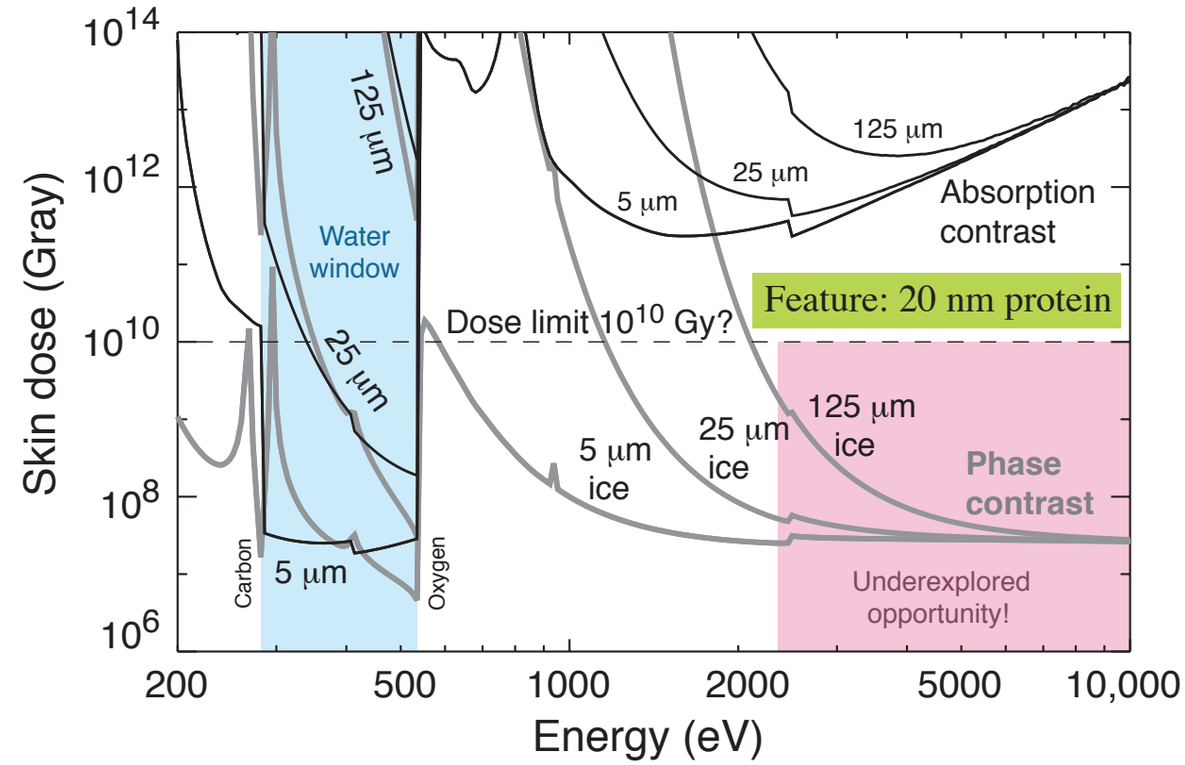


X-ray fluorescent photons (fluorescence yield) can "escape" from deep within cells; Auger electrons (1-fluorescence yield) cannot.

J. Kirz, in *Scanning Electron Microscopy* **2**, 1980, p. 239, for organic "matrix".
EELS=electron energy loss spectroscopy.
Hard-to-find reprint: http://tinyurl.com/2fx94m6

"Hard", multi-keV x-ray microscopy





Electron beams broaden in thick specimens due to sidescattering; x-ray beams do not. LeFurgey and Ingram, *Environmental Health Perspectives* **84**, 57 (1990).

Depth of focus (DOF) goes like (transverse resolution)$^2/\lambda$:

25 nm, 0.5 keV: 4 μm

25 nm, 10 keV: 80 μm

Resolution versus zone plate outermost zone width Δ_{rN}:

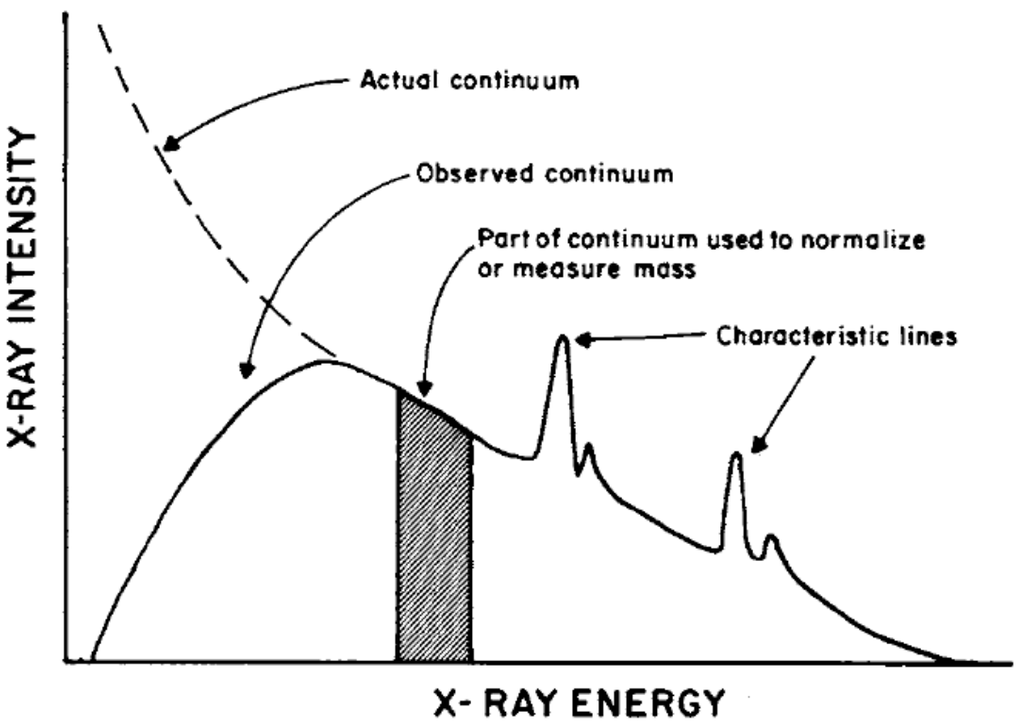
Transverse: $$\Delta_t \Rightarrow \frac{\lambda}{4\theta} = \frac{\Delta_{rN}}{2}$$

Longitudinal: $$\Delta_\ell \Rightarrow \frac{\lambda}{\theta^2} = 4\Delta_{rN}\frac{\Delta_{rN}}{\lambda}$$

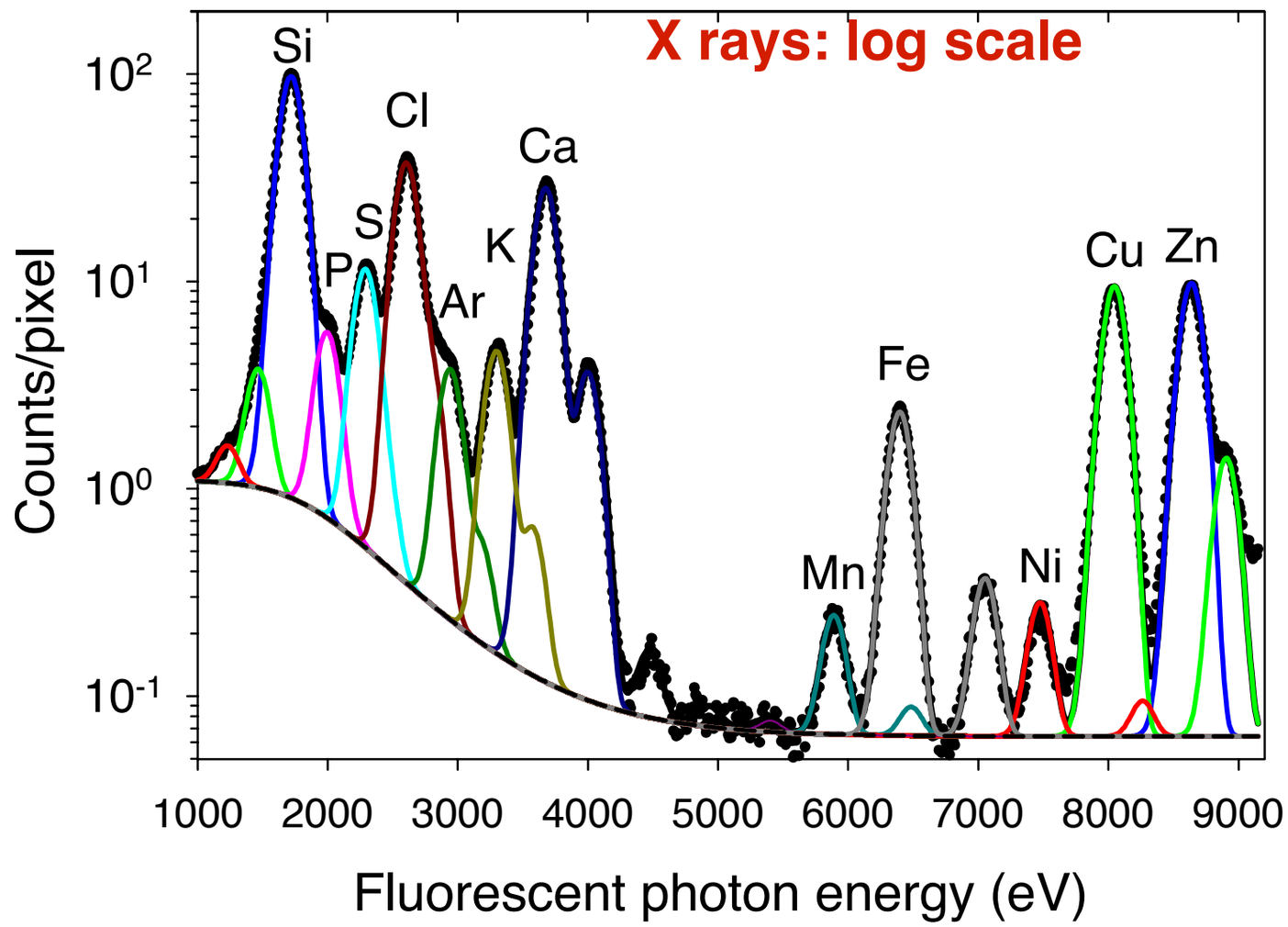
Exciting x-ray fluorescence

X rays and protons produce a dramatically lower continuum background, increasing sensitivity (but proton microprobes induce much more damage)

Electrons: linear scale

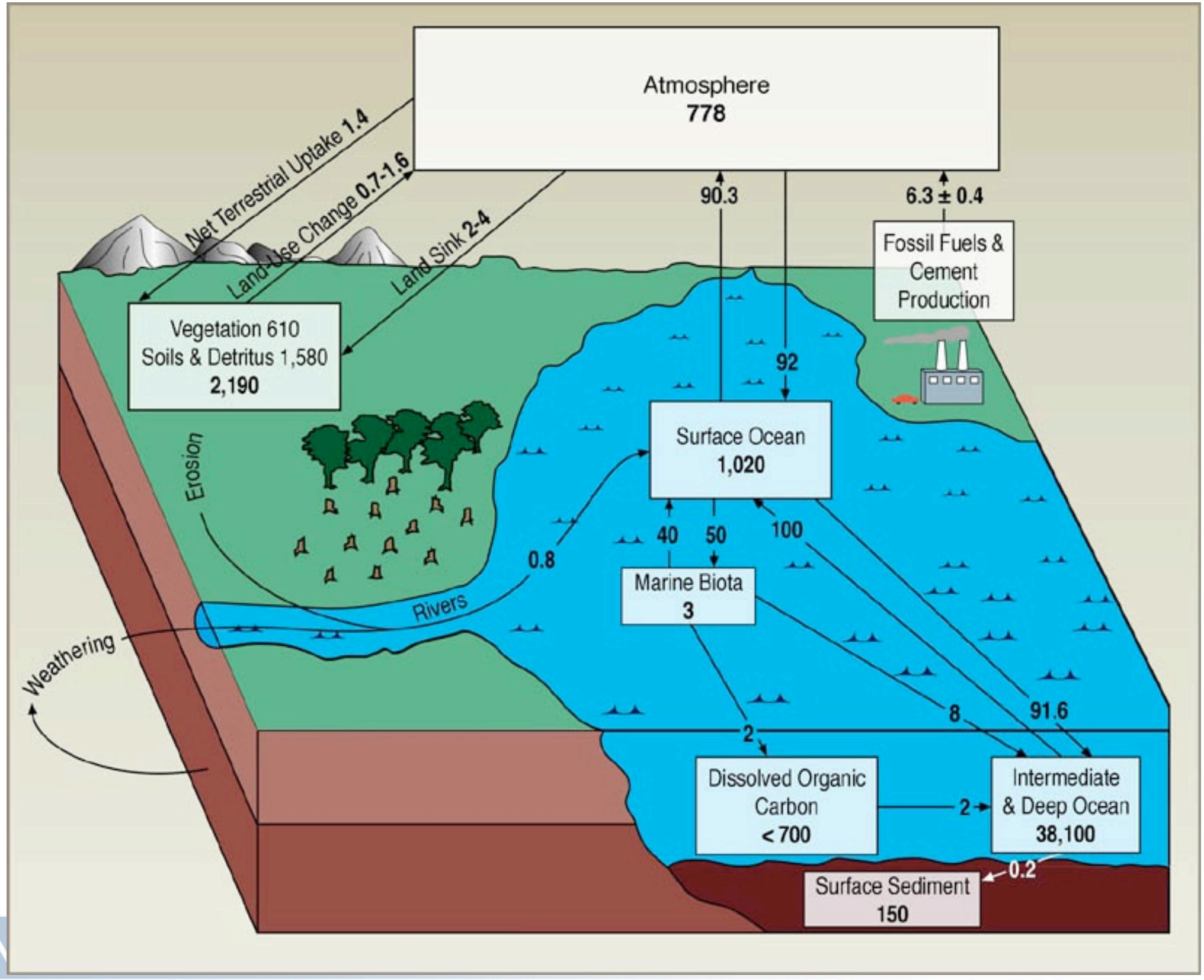


LeFurgey and Ingram, *Environmental Health Perspectives* **84**, 57 (1990)



Twining *et al.*, *Anal. Chem.* **75**, 3806 (2003). Analysis approach: Vogt, Maser, and Jacobsen, *J. Phys. IV* **104**, 617 (2003).

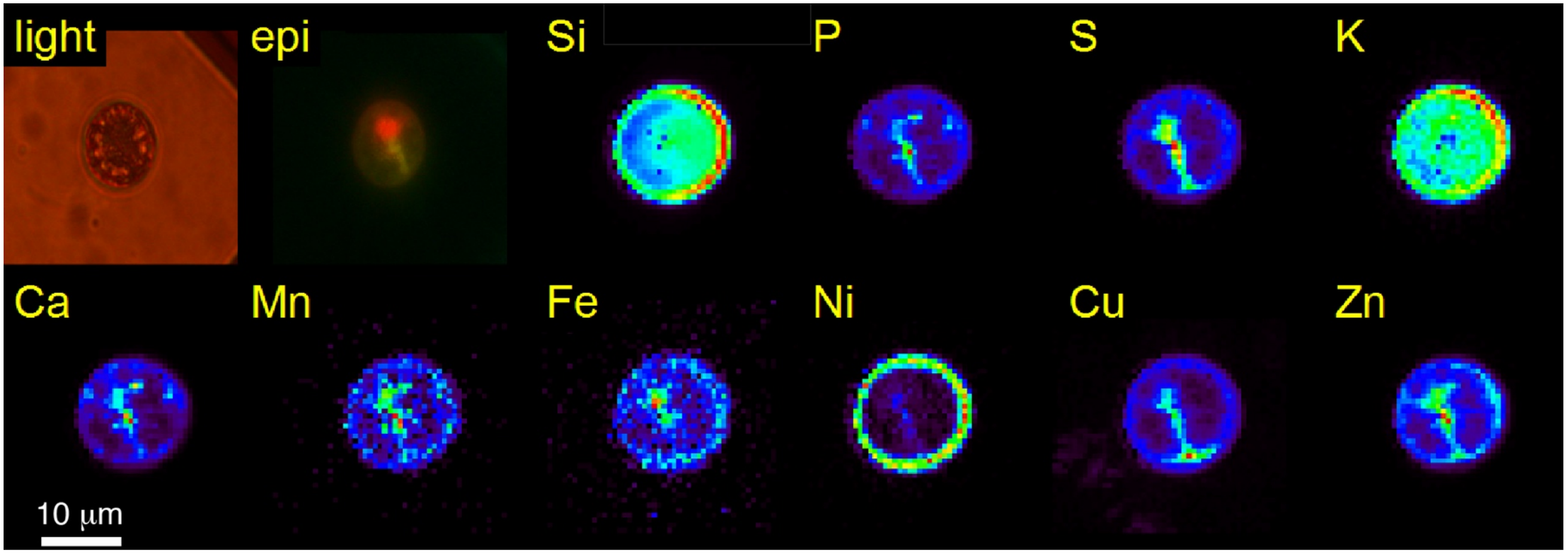
Global carbon cycle



petagrams, petagrams/year (climatescience.gov)

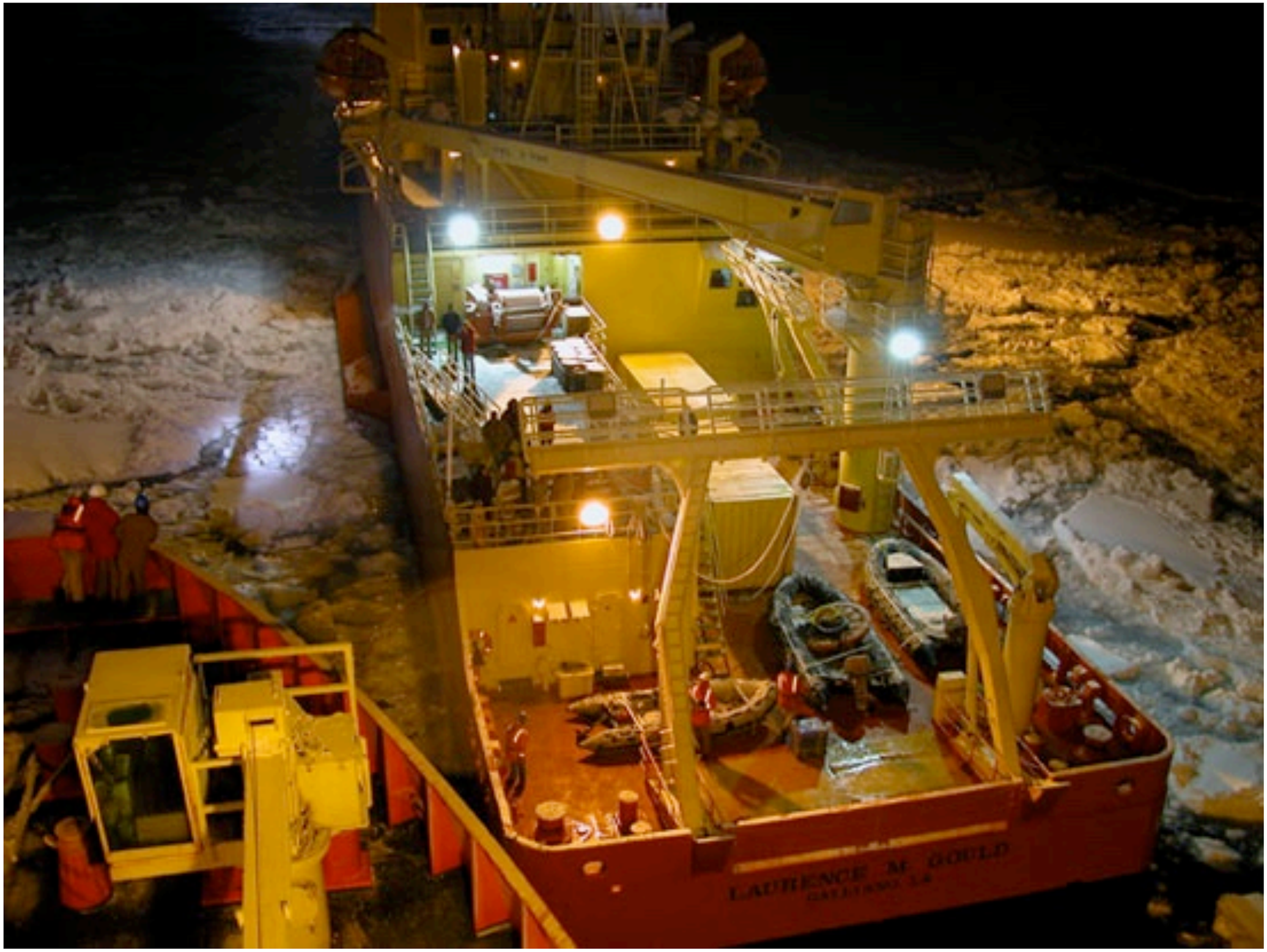
Iron and carbon in the ocean

- Seed Southern Pacific with bioavailable iron to increase CO_2 uptake?
- Requires understanding of iron and carbon uptake in phytoplankton; combine fluorescence with phase contrast.

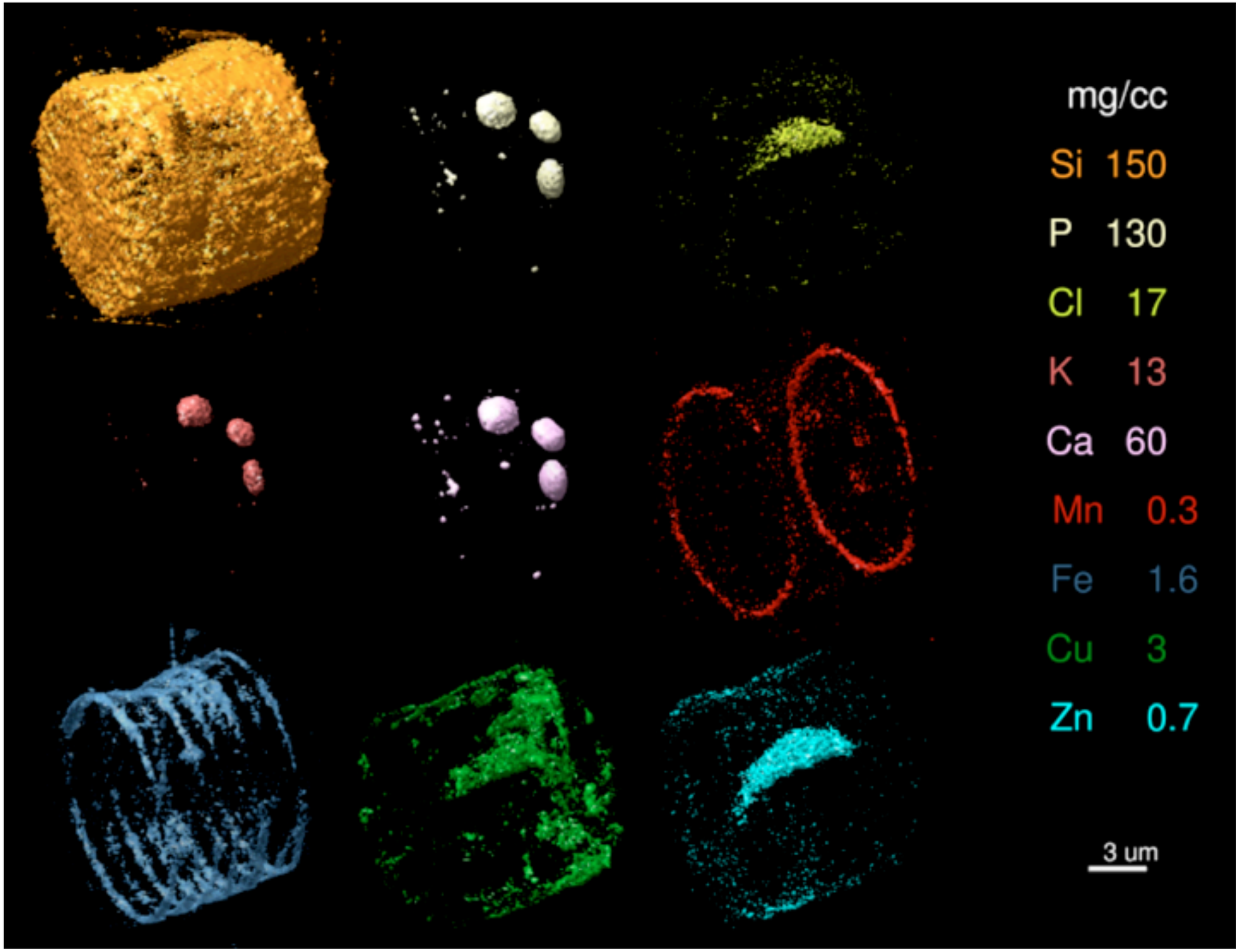


B. Twining, S. Baines, N. Fisher, J. Maser, S. Vogt, C. Jacobsen, A. Tovar-Sanchez, and S.Sañudo-Wilhelmy, *Anal.. Chem.* **75**, 3806 (2003)

Cruising the Southern Pacific

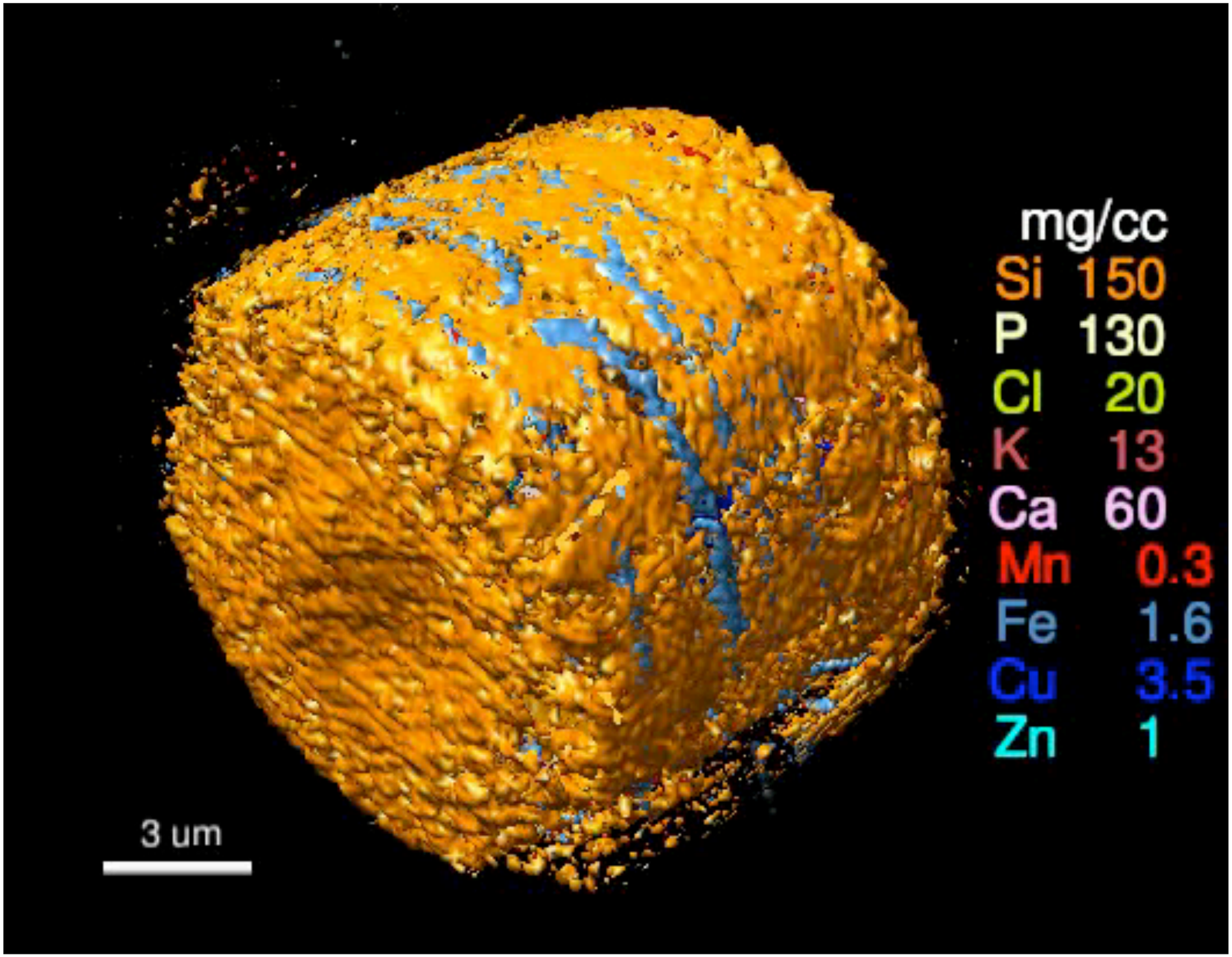


Quantitative 3D fluorescence of a diatom



M. de Jonge, C. Holzner, S. Baines, B. Twining, K. Ignatyev, J. Diaz, D. Howard, A. Miceli, I. McNulty, C. Jacobsen, S. Vogt, *Proc. Nat. Acad. Sci.* **107**, 15676 (2010)

Fluorescence tomography

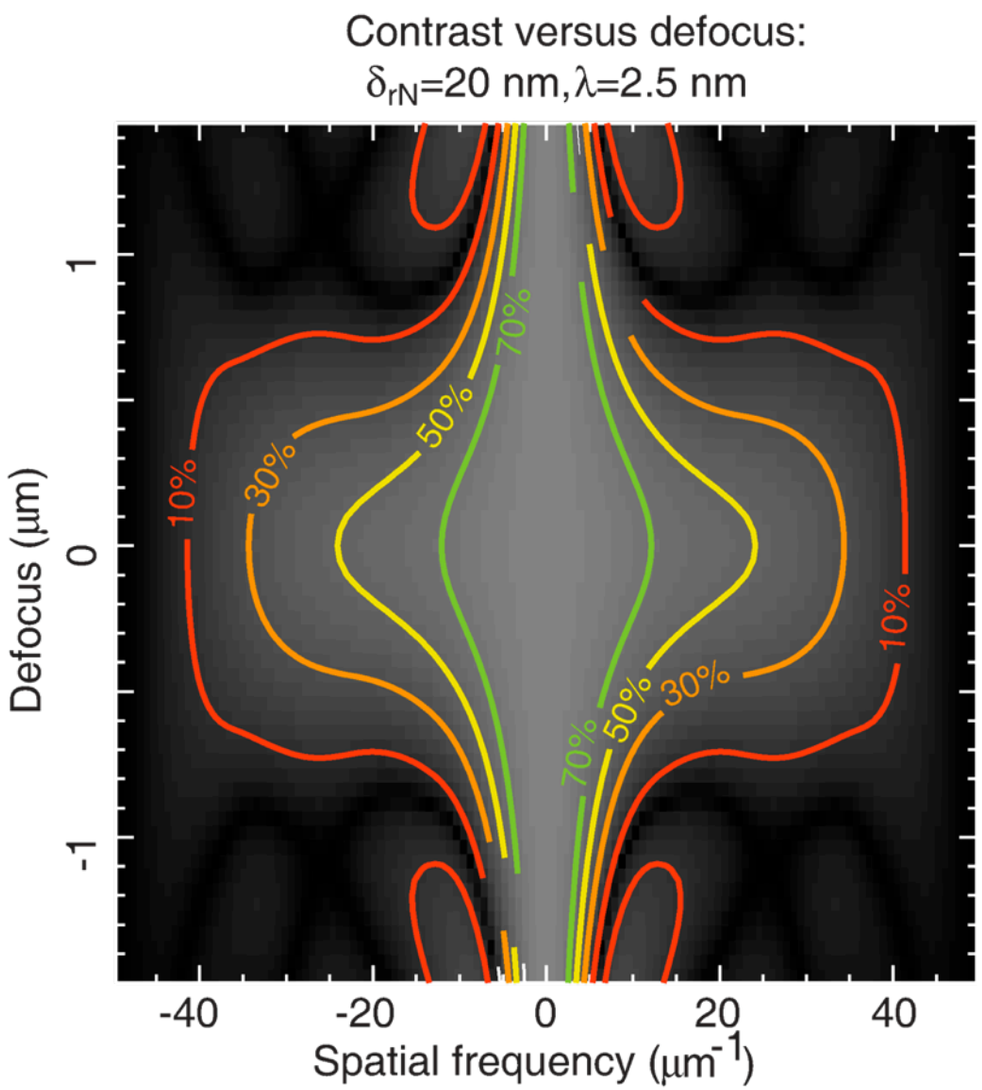


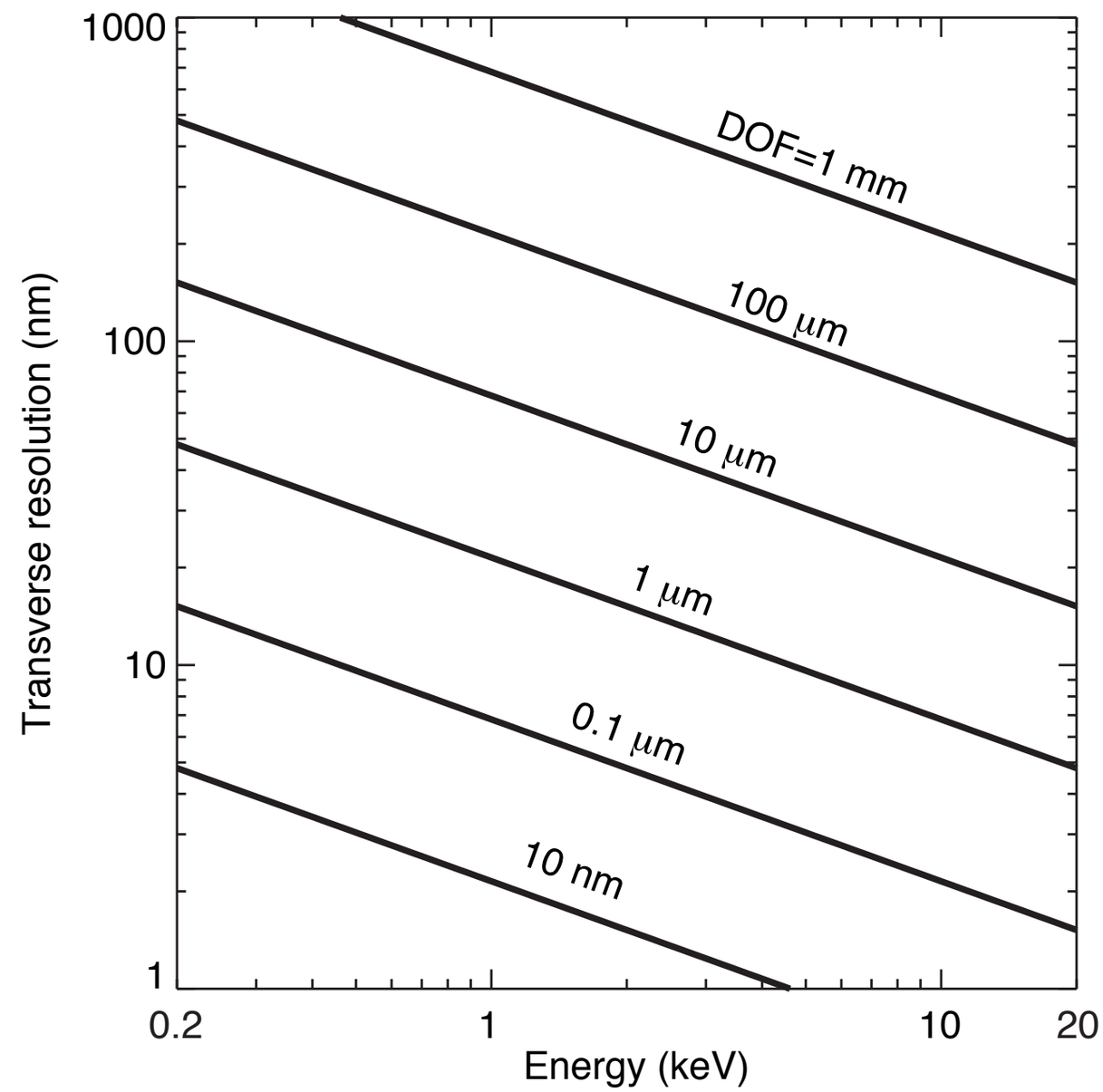
de Jonge *et al.*, *Proc. Nat. Acad. Sci.* **107**, 15676 (2010). Next: phase contrast for alignment, dose fractionation for fluorescence.

Depth of focus

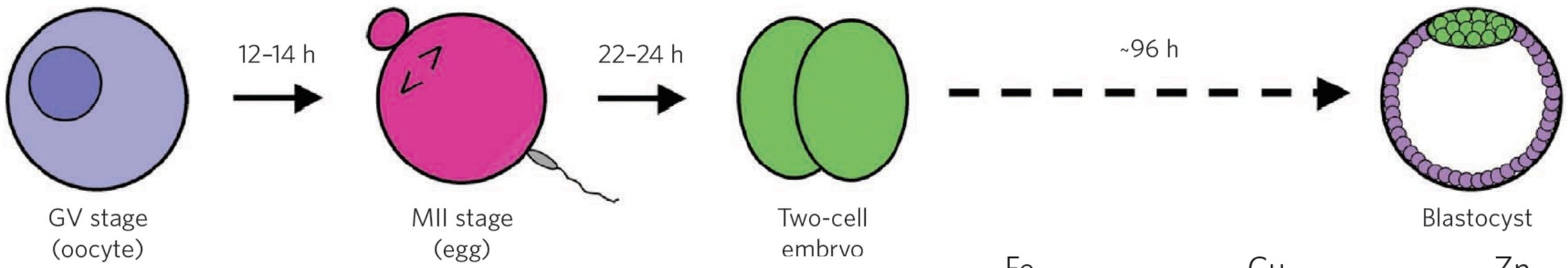
Transverse: $$\Delta_t \Rightarrow \frac{\lambda}{4\theta} = \frac{\Delta_{rN}}{2}$$

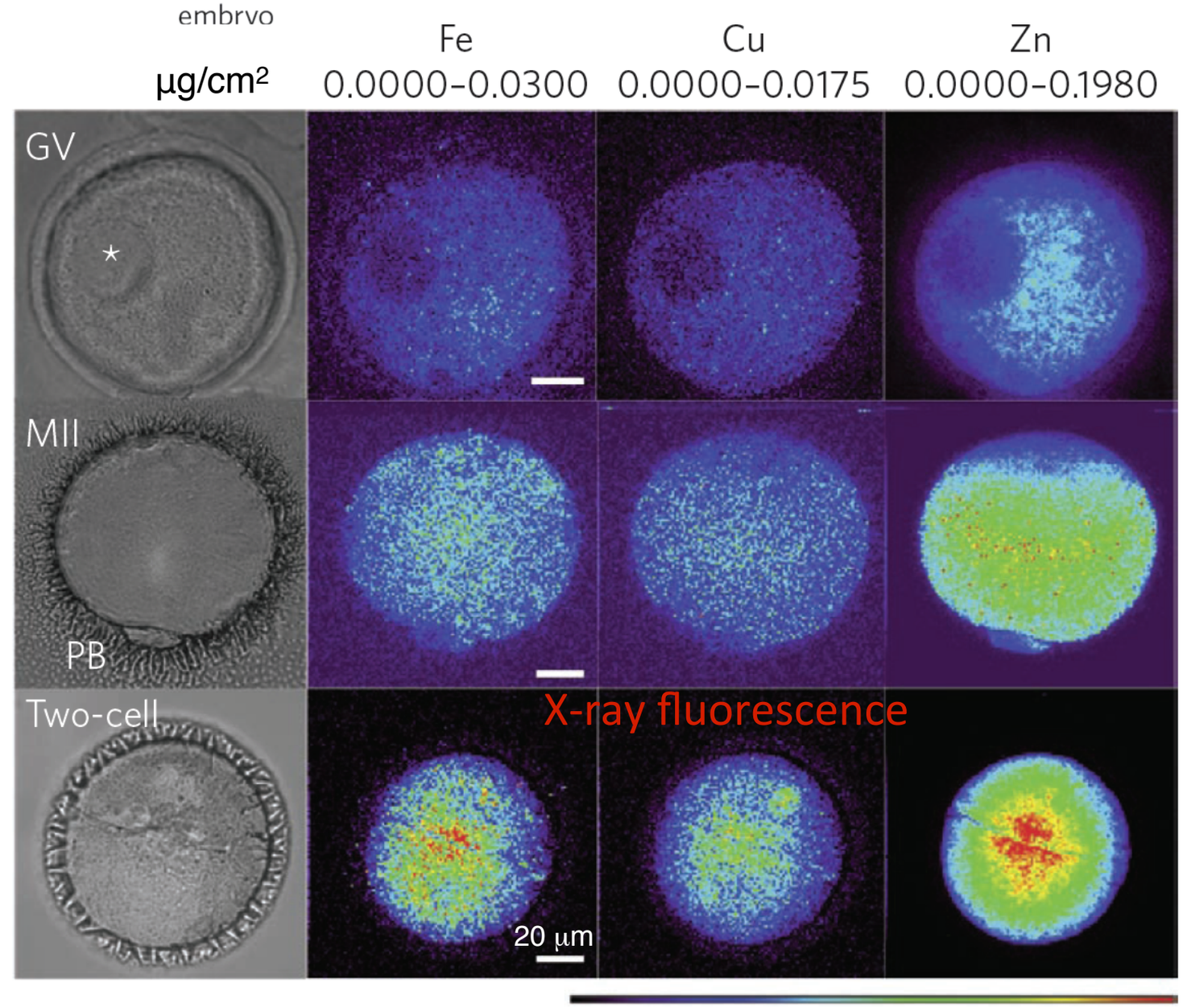
Longitudinal: $$\Delta_\ell \Rightarrow \frac{\lambda}{\theta^2} = 4\Delta_{rN}\frac{\Delta_{rN}}{\lambda}$$





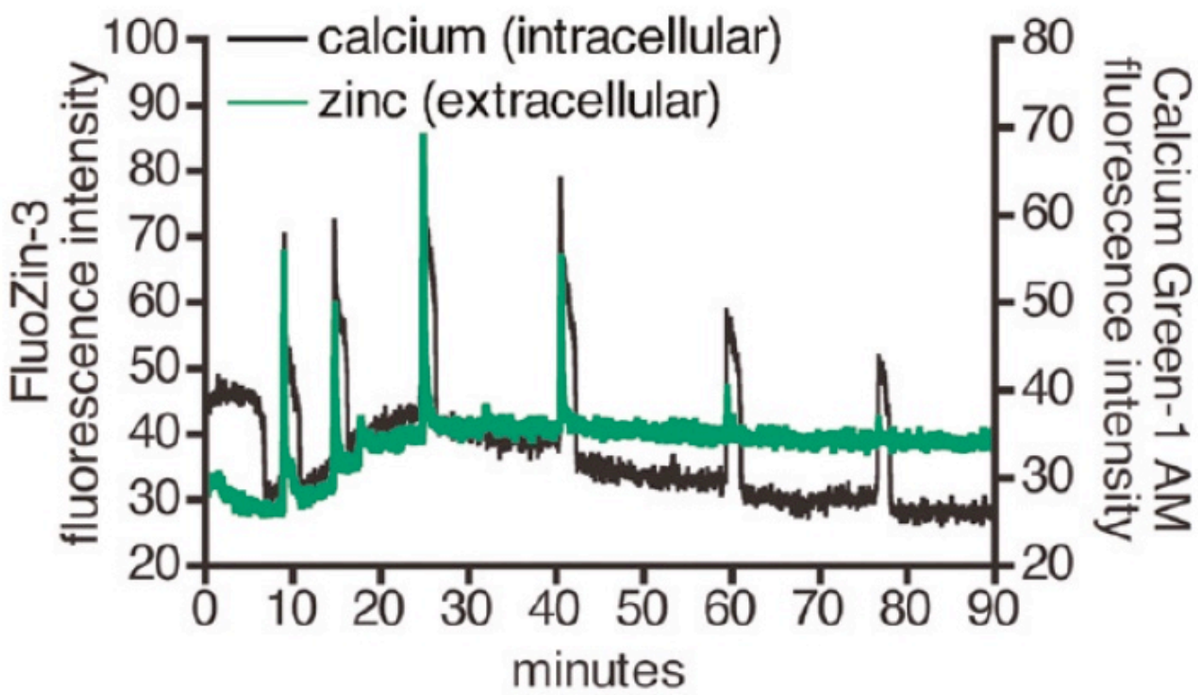
Zinc and development

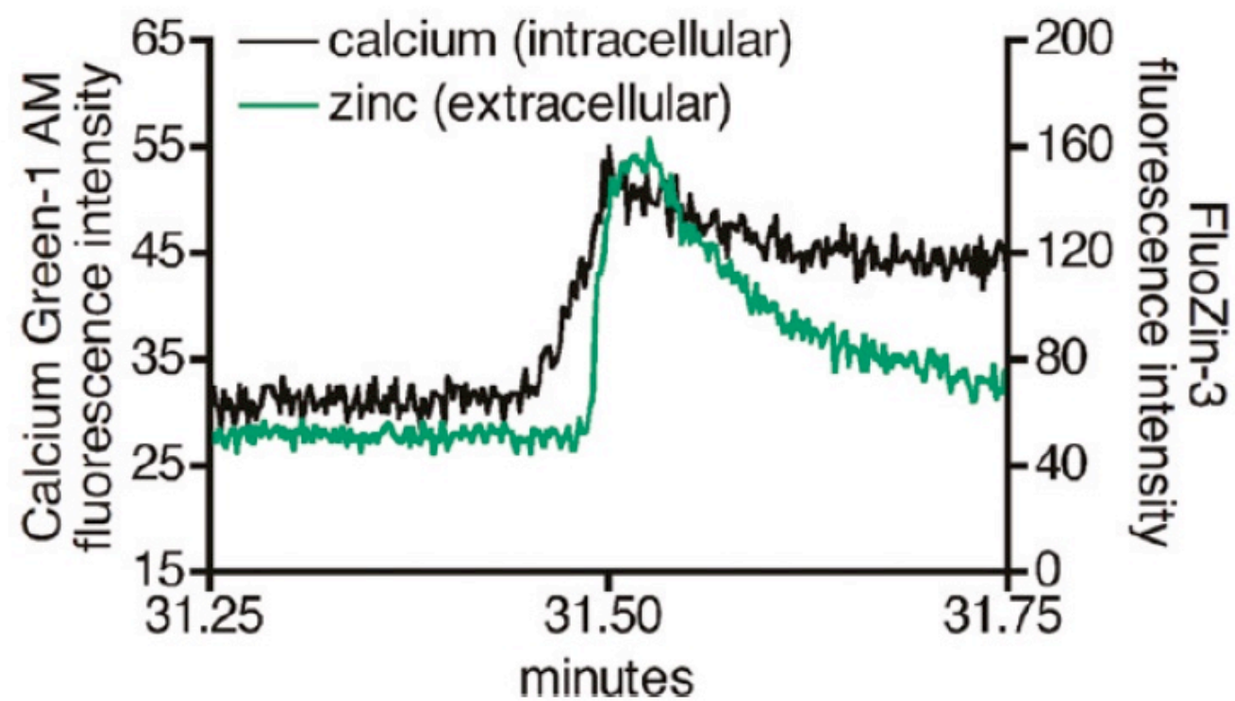




- Zinc is collected (10^{10} atoms!) during metaphase II arrest, before fertilization.
- Chelation (tying zinc up) halts division.
- Oocyte supplies zinc bolus as maternal legacy to the embryo?
- Kim *et al.*, *Nature Chem. Bio.* **6**, 674 (2010).

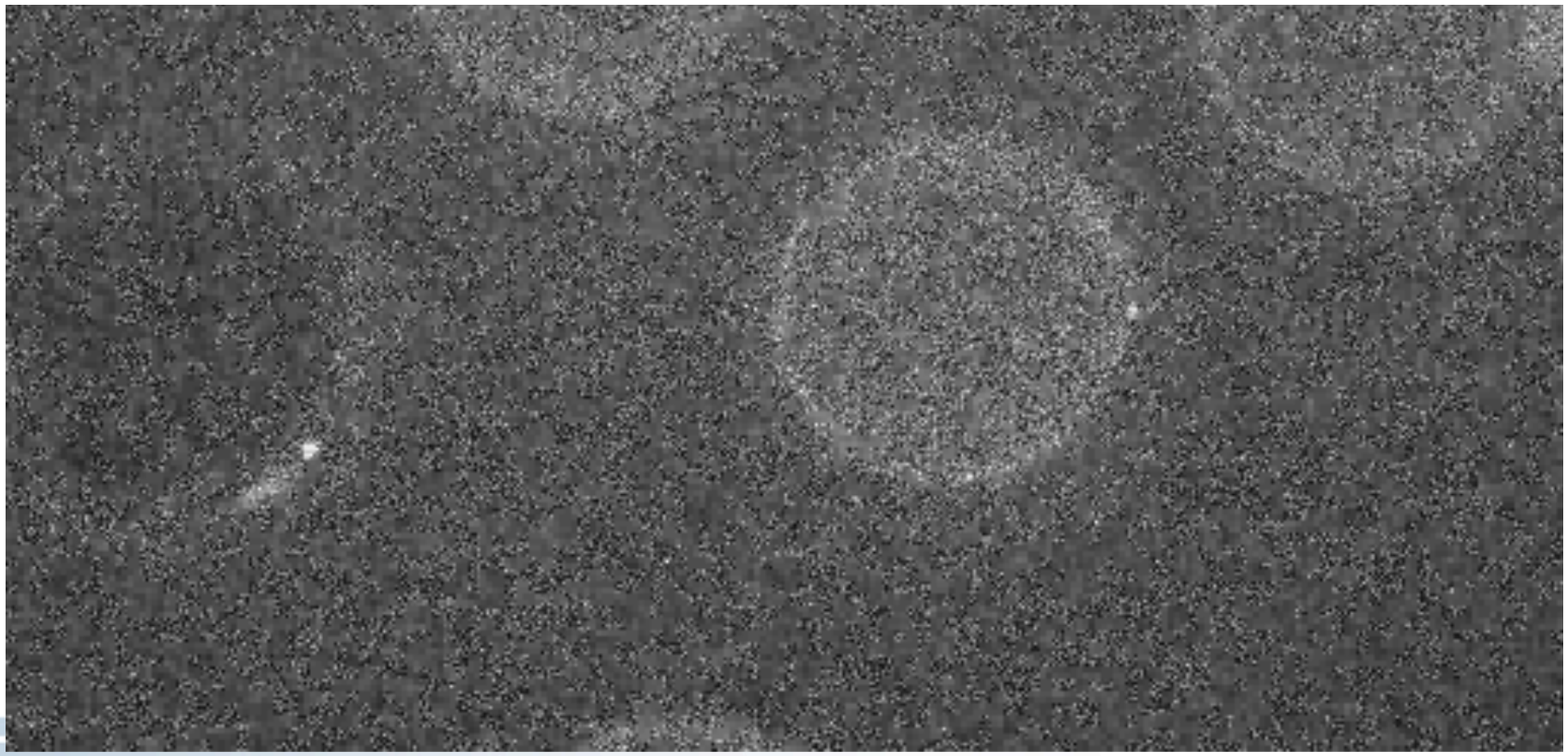
Zinc sparks





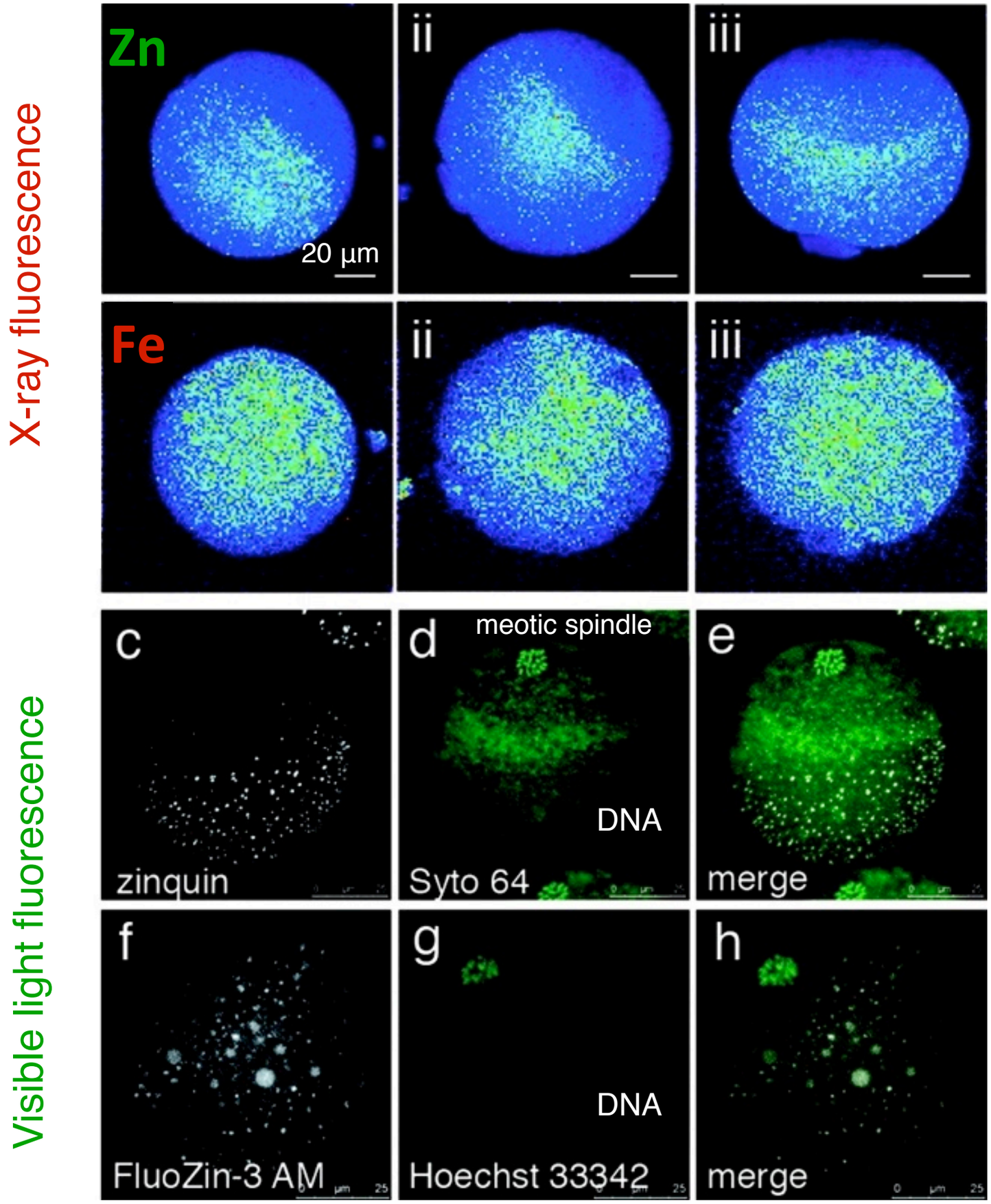
Real time: ~20 minutes.

- Kim *et al.*, *ACS Chem. Bio.* **6**, 716 (2011).

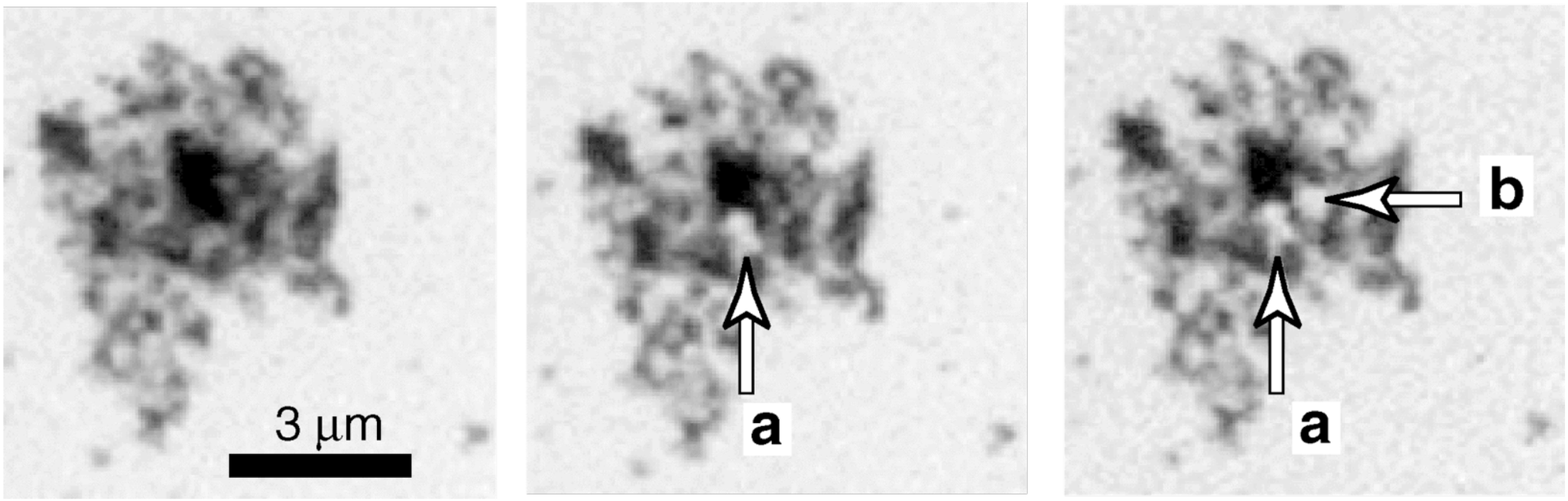


Zinc: non-uniform distribution during metaphase II

- X-ray fluorescence (top) confirms indications from fluorophores (zinquin and FluoZin).
- Indicates zinc-rich compartments in oocytes (lipoproteins?)
- Zinc regulates early meiotic inhibitor Emi2?
- Kim *et al.*, *ACS Chem. Bio.* **6**, 716 (2011).

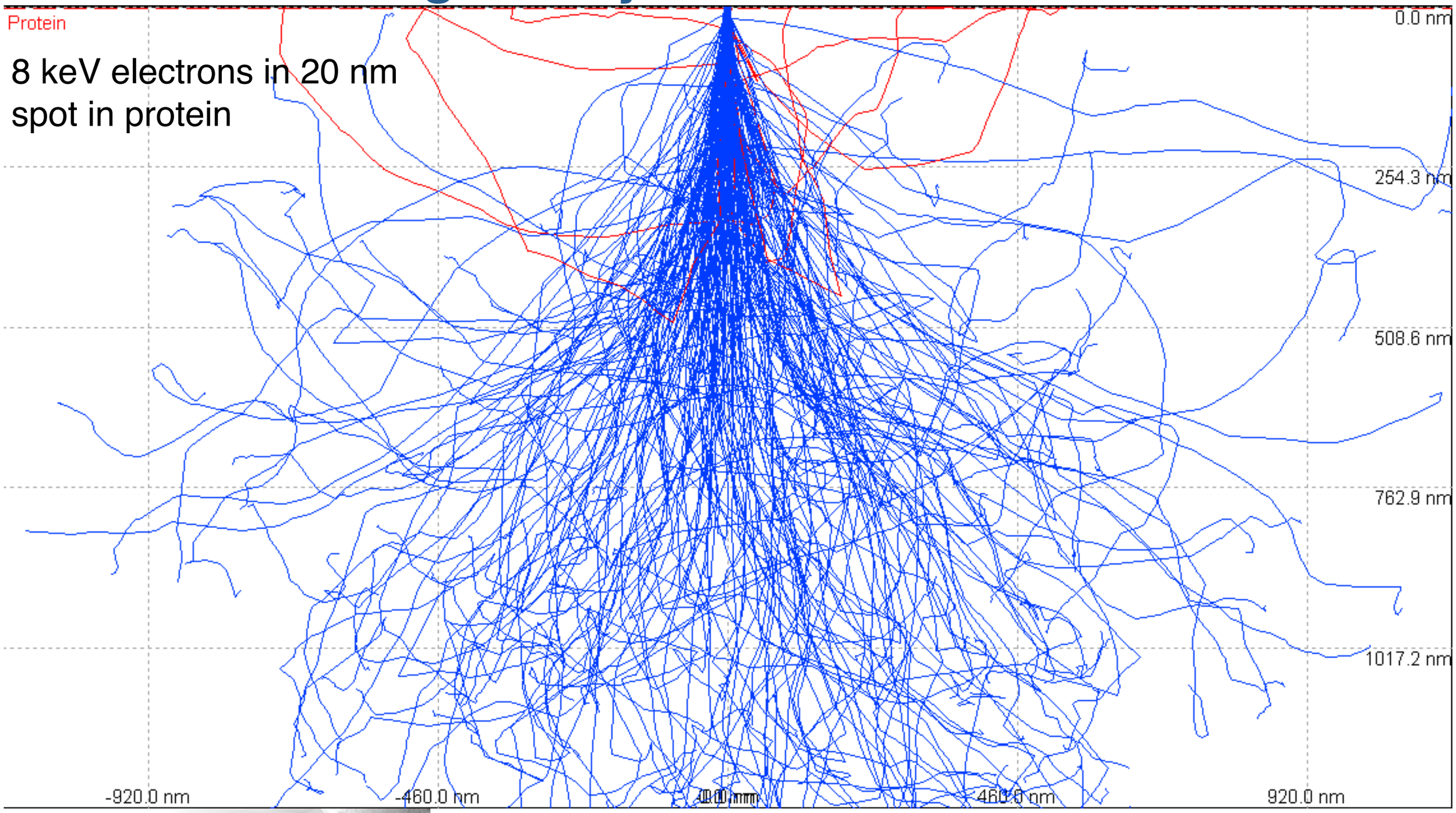


Spectromicroscopy can be damaging!



- Study: polyacrilimide-induced flocculation of clays (irrigation of loamy soils)
- Mass loss is visible after acquiring spectra with focused beam (wet sample at room temperature)
- U. Neuhaeusler, PhD Thesis (Stony Brook/Göttingen)

Radiation damage: X rays to electrons to broken bonds



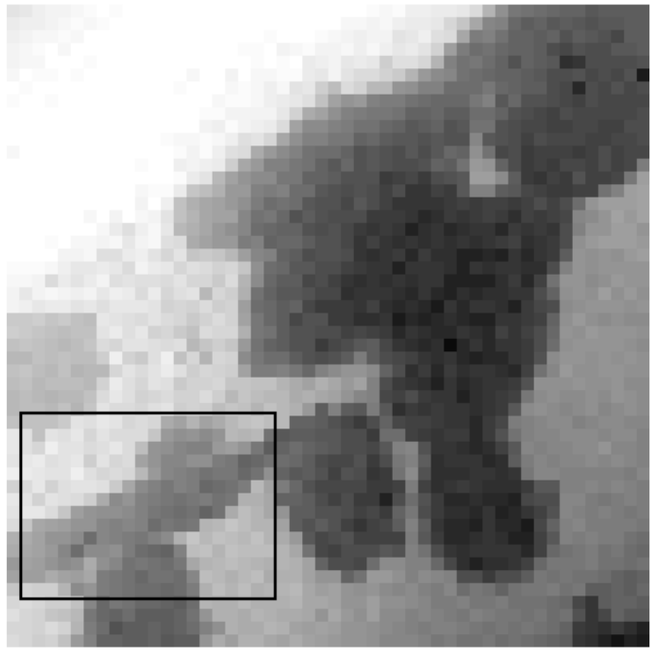
8 keV electrons in 20 nm spot in protein

Casino
monte CArlo SImulation of electroN trajectory in sOlids

http://www.gel.usherbrooke.ca/casino/

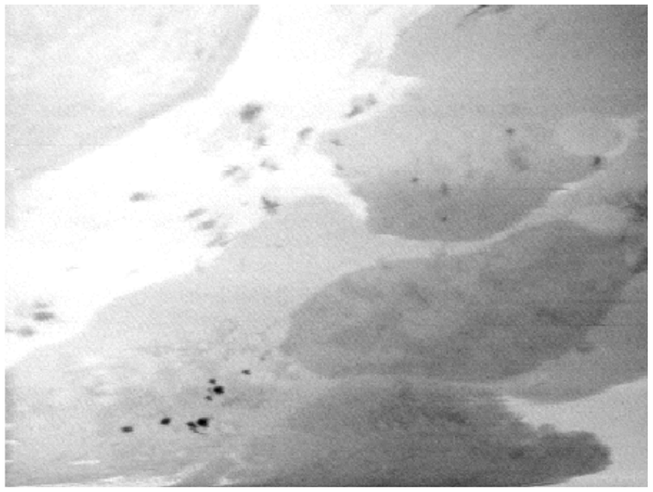
Radiation damage on (initially) living cells

- X-rays are ionizing radiation. The dose per high resolution image is about 100,000 times what is required to kill a person
- Makes it hard to view living cells!

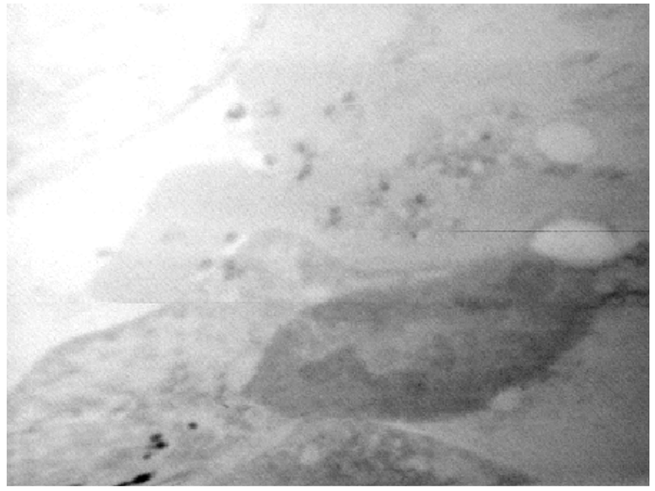


Experiment by V. Oehler, J. Fu, S. Williams, and C. Jacobsen, Stony Brook using specimen holder developed by Jerry Pine and John Gilbert, CalTech

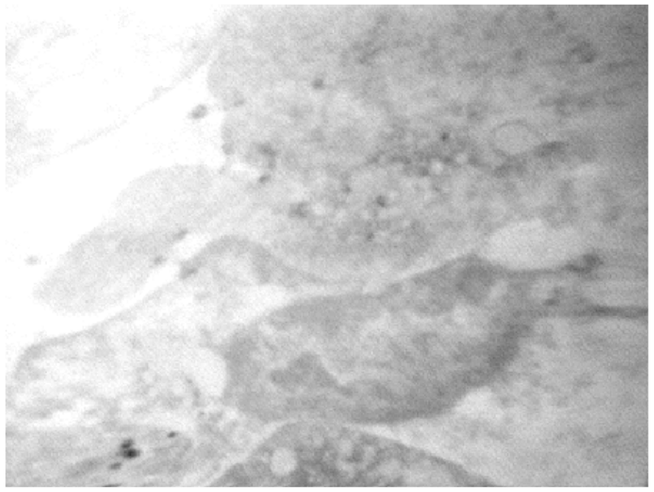
10 μm
$6.0 \cdot 10^2$ Gray, ET=2 min.



5 μm
$1.2 \cdot 10^5$ Gray, ET=9.5 min.



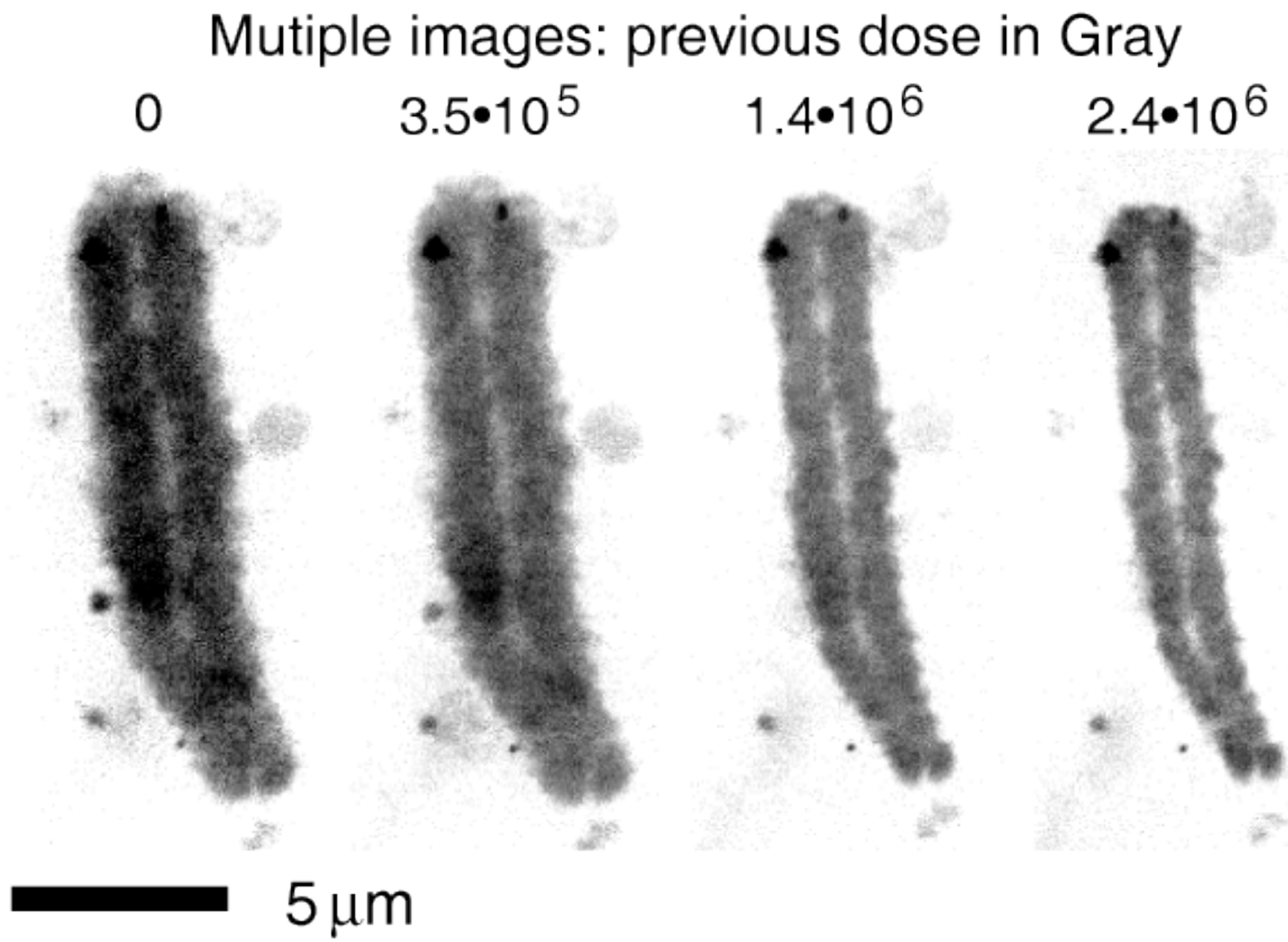
5 μm
$2.4 \cdot 10^5$ Gray, ET=17 min.



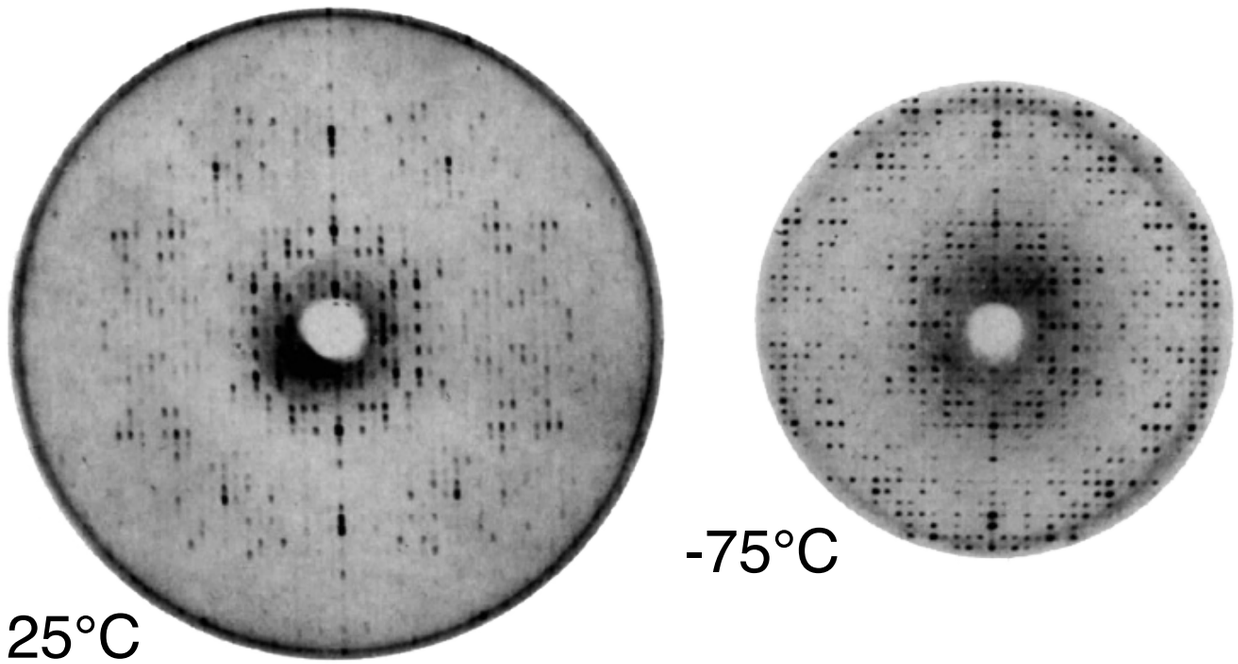
5 μm
$3.7 \cdot 10^5$ Gray, ET=24.5 min.

Wet, fixed samples: one image is OK

- Chromosomes are among the most sensitive specimens.
- *V. faba* chromosomes fixed in 2% glutaraldehyde. S. Williams *et al*., *J. Microscopy* **170**, 155 (1993)
- Repeated imaging of one chromosome shows mass loss, shrinkage



Cryo crystallography



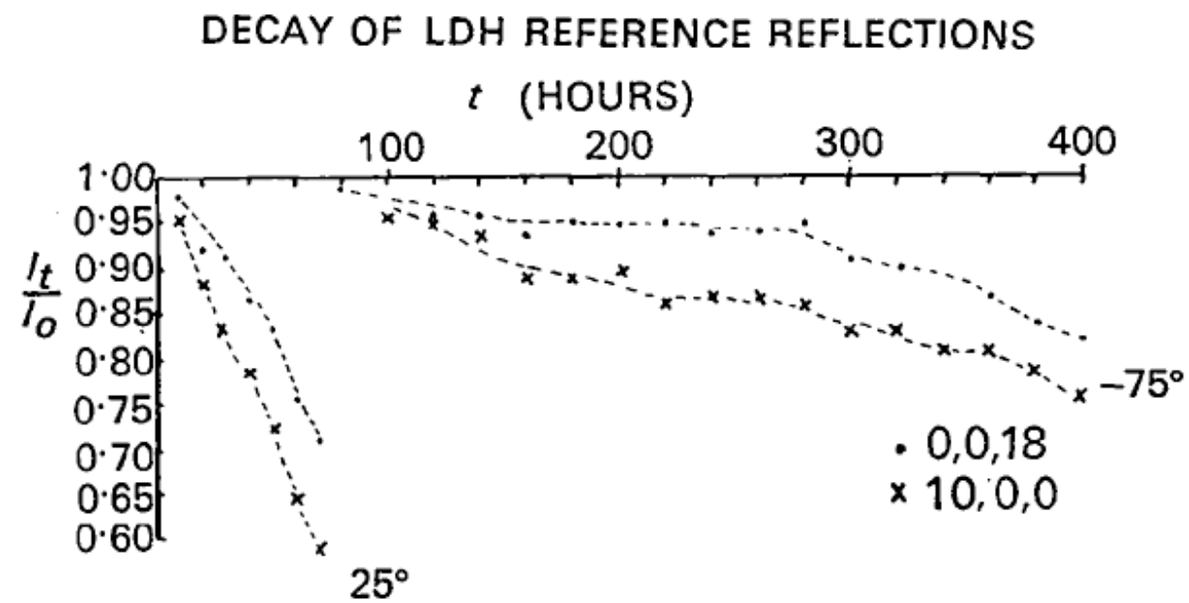


Fig. 5. The ratio I_t/I_0 for two reference reflections plotted as a function of exposure time for a typical native and frozen crystal. I_t represents the intensity at time t. Results for 0,0,18 and 10,0,0 are shown with dots and crosses respectively.

Acta Cryst. (1970). B**26**, 998

Crystallographic Studies on Lactate Dehydrogenase at -75°C

By David J. Haas* and Michael G. Rossmann

Crystals of lactate dehydrogenase (LDH) were frozen by equilibration in a sucrose–ammonium sulfate solution, and then dipping into liquid nitrogen. The rate of radiation damage for frozen crystals was tenfold less than for crystals at room temperature. The physical properties of frozen crystals are discussed. Analysis of 3·5 Å data collected at −75°C for native LDH and two heavy atom derivatives showed that these derivatives retained their isomorphism in the frozen state.

See also Low, Chen, Berger, Singman, and Pletcher, *PNAS* **56**, 1746 (1966)

Cooling without ice crystal formation

- Slow cooling with cryoprotectants: protein crystals, sperm and egg preservation
- Rapid cooling without ice crystals: no biochemical or structural changes
  - Limited by heat capacity and heat of vaporization of cryogen, and thermal conductivity of specimen.
  - 10^4 K/s means msec freezing time
  - This figure: H. Moor, “Theory and Practice of High Pressure Freezing,” in R.A. Steinbrecht & K. Zierold, **Cryotechniques in Biological Electron Microscopy** (Springer, 1987)

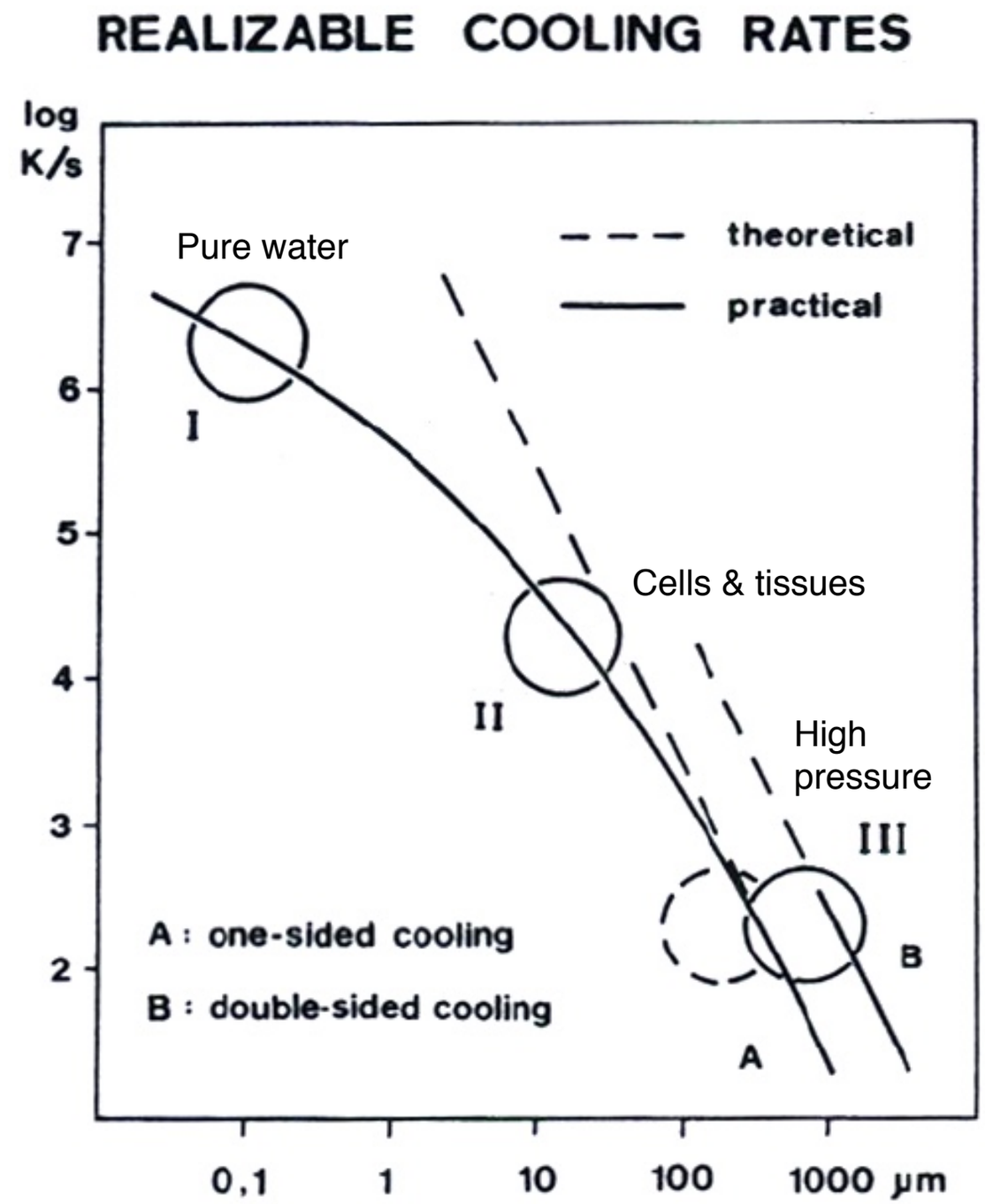


Fig. 1. The *curves* show the dependence of the cooling rate ($K\,s^{-1}$) on the distance (µm) of the specimen region from the cooled surface. *I* Range of vitrified pure water. *II* Range of vitrified animal cells and tissues. *III* Range of specimens vitrified under high pressure

High pressure cooling of loop-mounted crystals

Kim, Kapfer, and Gruner, *Acta Cryst. D* **61**, 881 (2005)

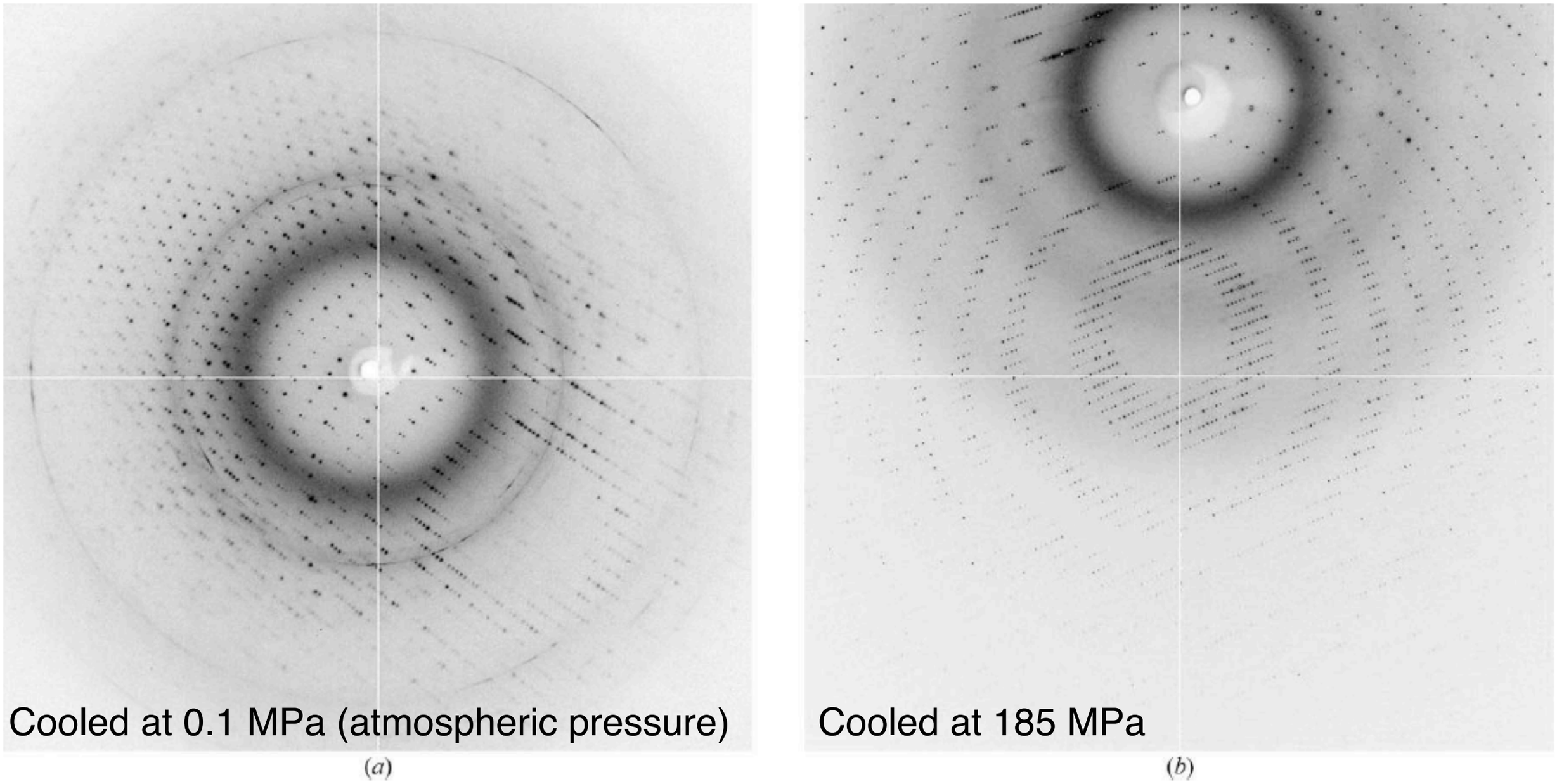
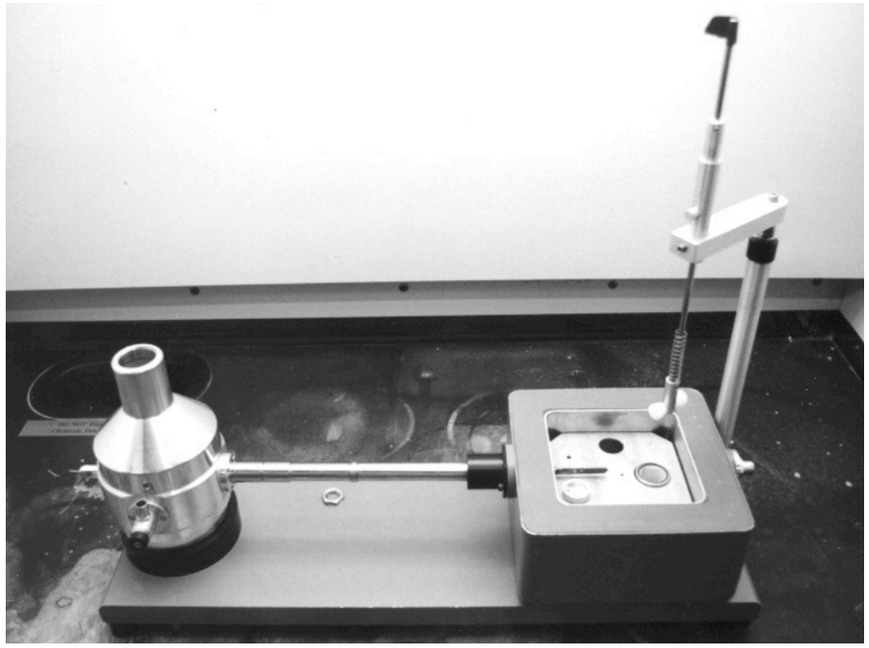


Figure 4
Thaumatin. (*a*) Diffraction image of a crystal flash-cooled at ambient pressure ($\lambda = 0.9795$ Å, beam diameter = 100 µm, $\Delta\varphi = 1.0°$, $d = 200$ mm, 20 s). Ice rings are seen. The diffraction resolution is 1.8 Å and the mosaicity is 1.29°. (*b*) Diffraction image of a crystal pressure-cooled at 185 MPa ($\lambda = 0.9186$ Å, beam diameter = 100 µm, $\Delta\varphi = 1.0°$, $d = 175$ mm, 15 s). The diffraction resolution reaches 1.15 Å and the mosaicity is 0.11°.

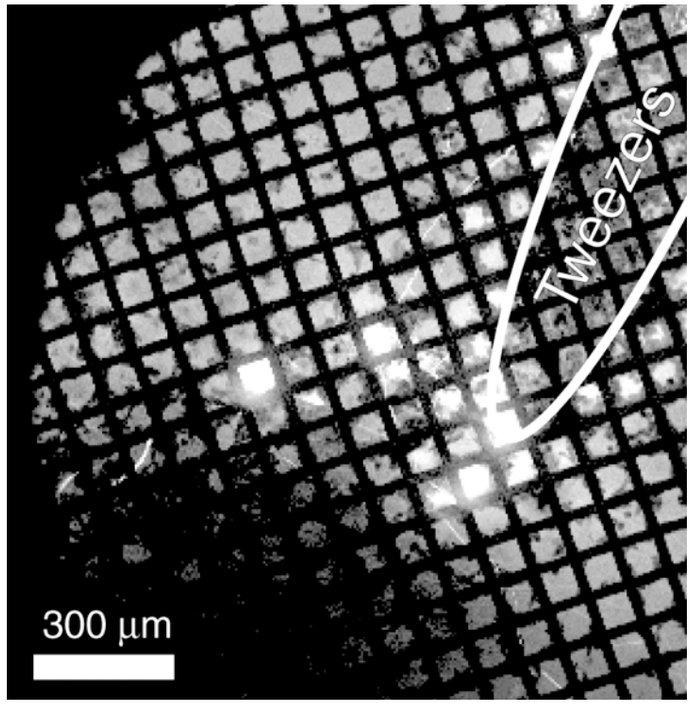
Frozen hydrated specimens

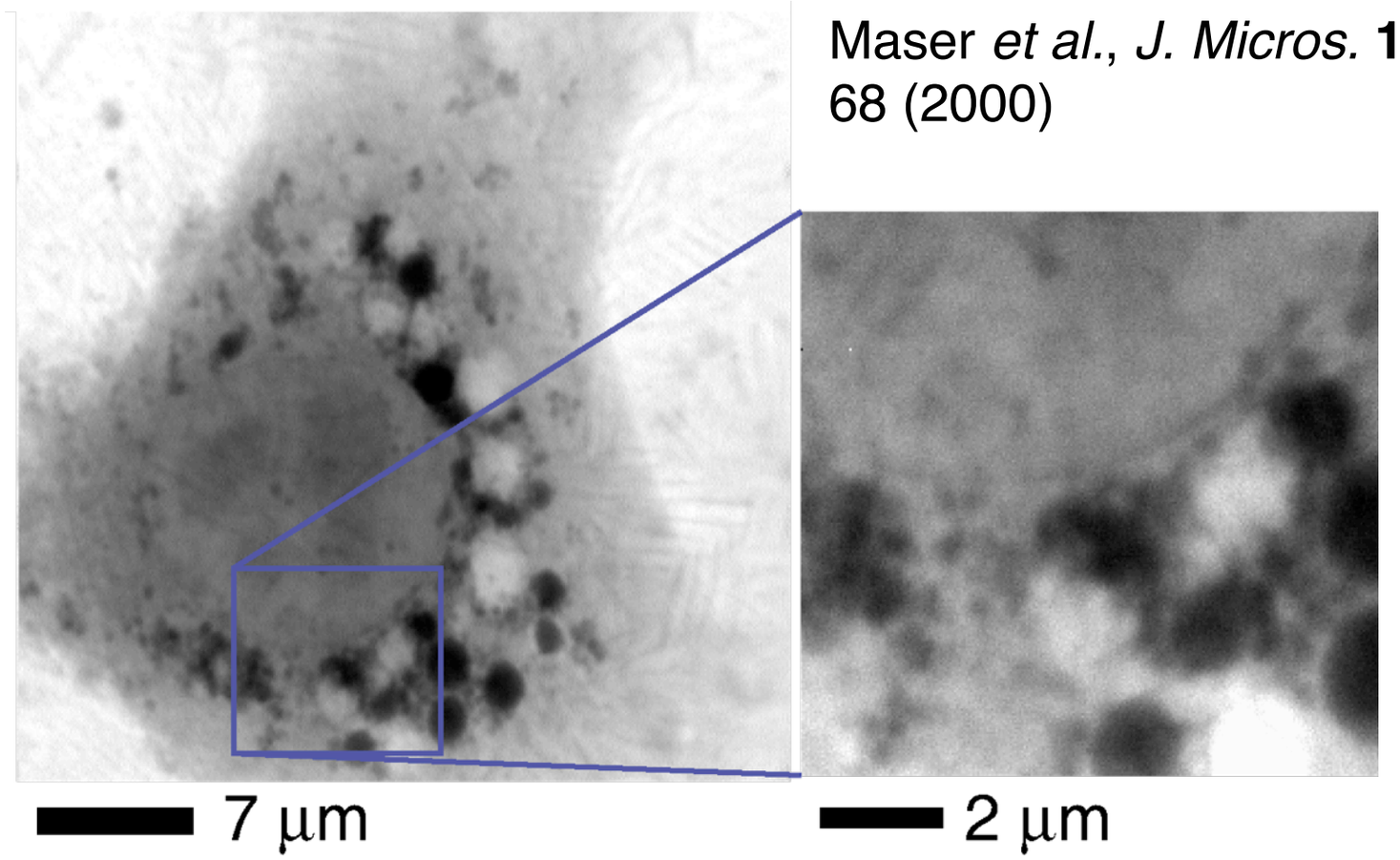
Grids with live cells are

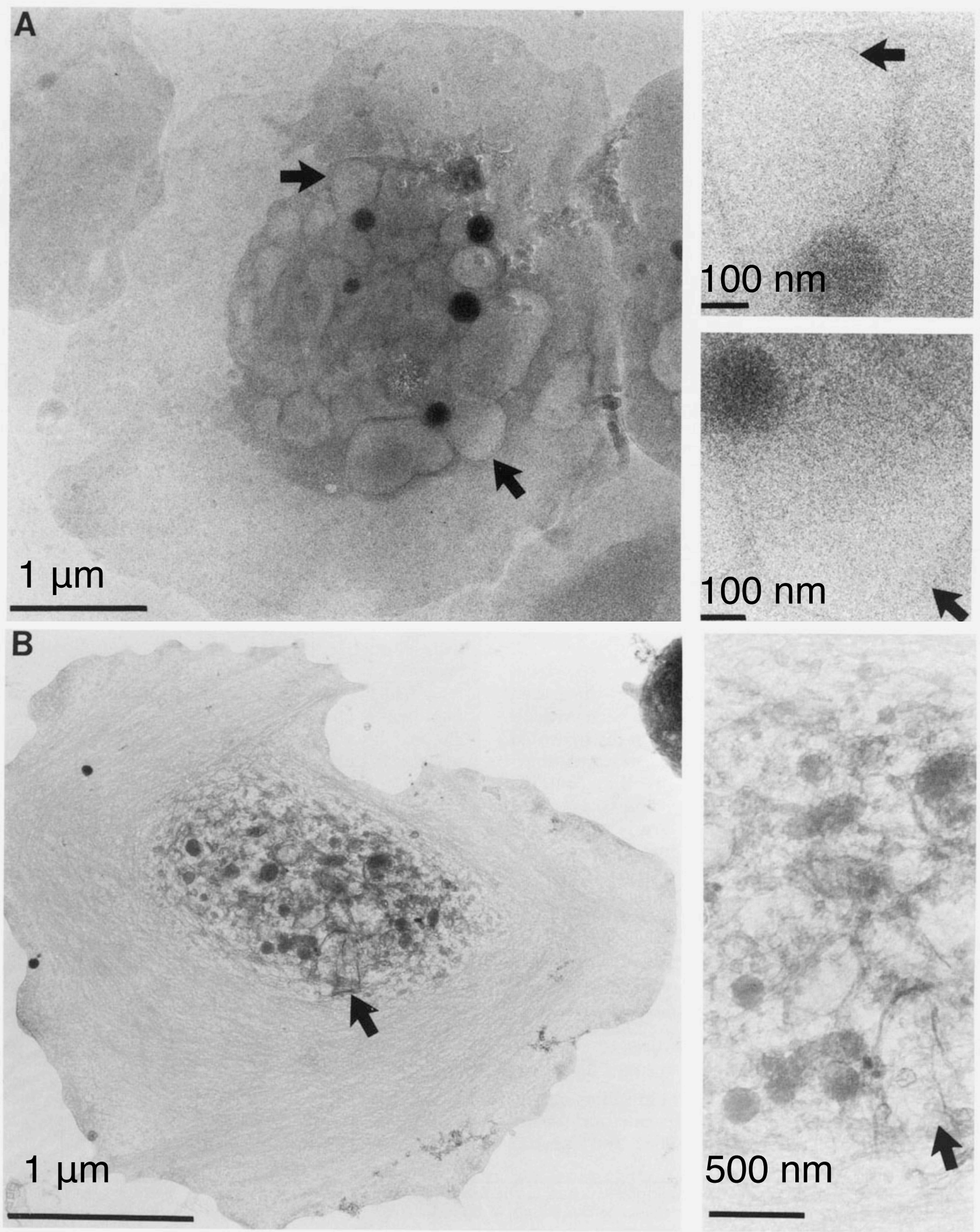
- Taken from culture medium and blotted
- Plunged into liquid ethane (cooled by liquid nitrogen)
- Loaded into cryo holder



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







Frozen hydrated

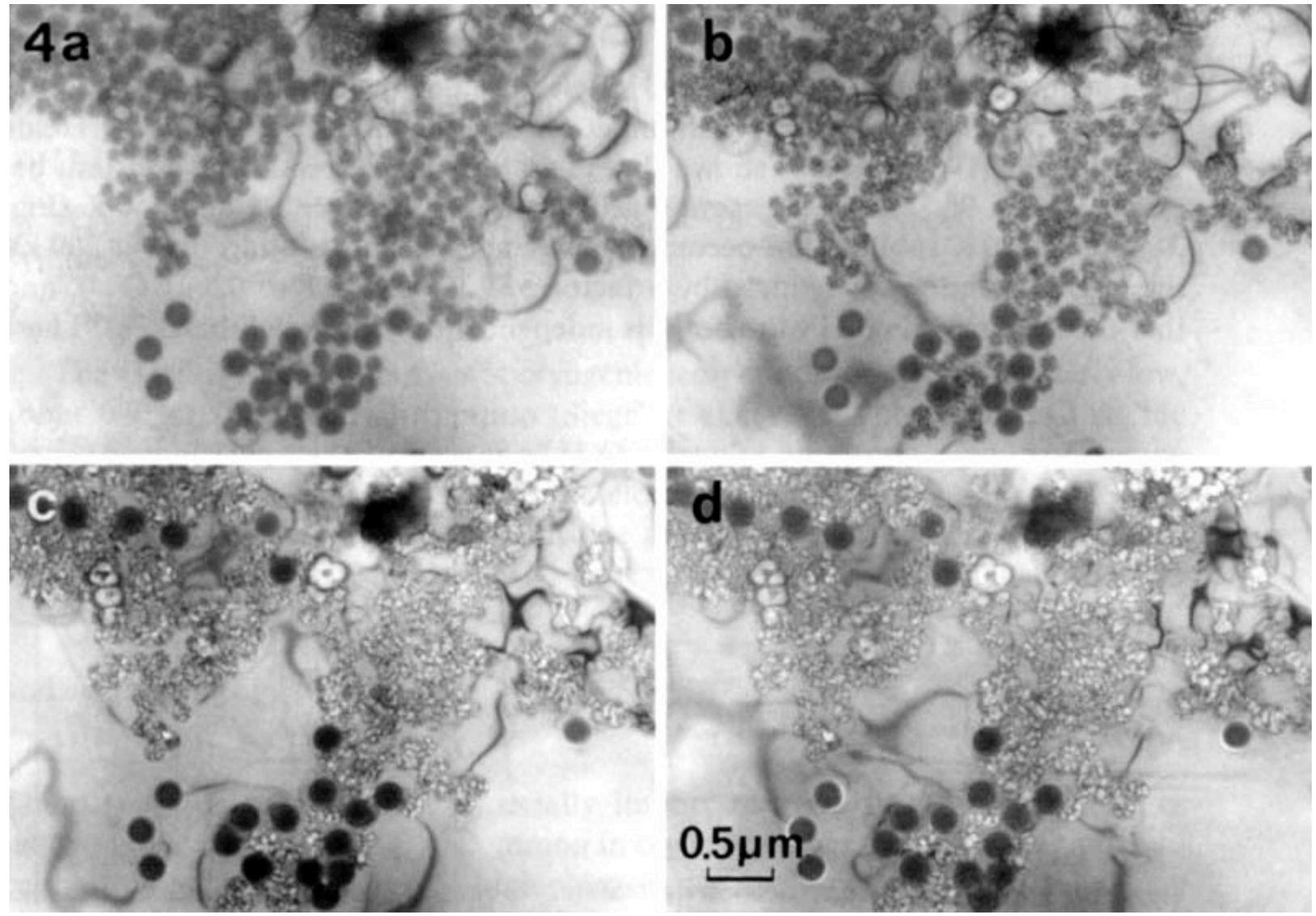
2% glutaraldehyde fix
1% OsO_4 postfix
critical-point dry

- Human blood platelets
- 1 MeV transmission electron microscope (JEOL-1000)
- O'Toole, Wray, Kremer, and McIntosh, *J. Struct. Bio.* **110**, 55 (1993)

Electrons: frozen hydrated

Polymer spheres in amorphous ice viewed with low dose rate at 100 keV
Smaller spheres: PMMA
Larger spheres: PS
Doses are in Gray

From Y. Talmon, in Steinrecht and Zierold, **Cryotechniques in Biological Electron Microscopy** (Springer, 1987)

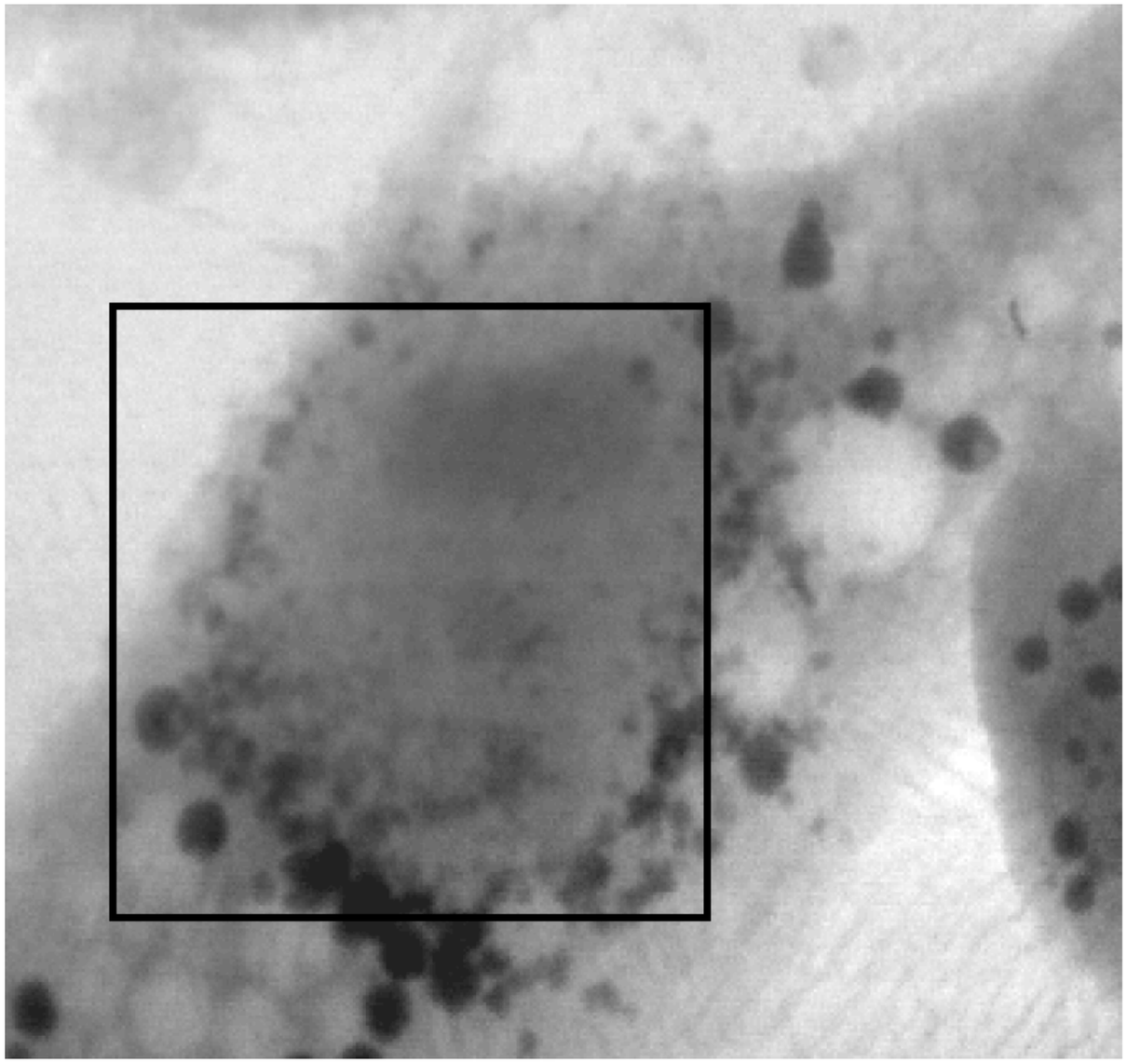


"Bubbling" dose in cryo electron microscopy: ~1000 e-/nm^2 or about 3x10^7 Gray. Bubbles: hydrogen gas [Leapman, *Ultramic.* **59**, 71 (1995); Sun *et al.*, *J. Mic.* **177**, 18 (1995)]

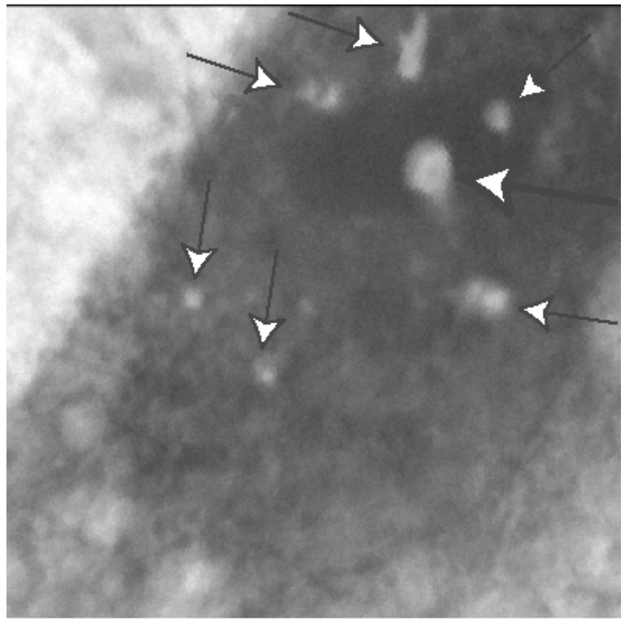
Radiation damage resistance in cryo

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

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



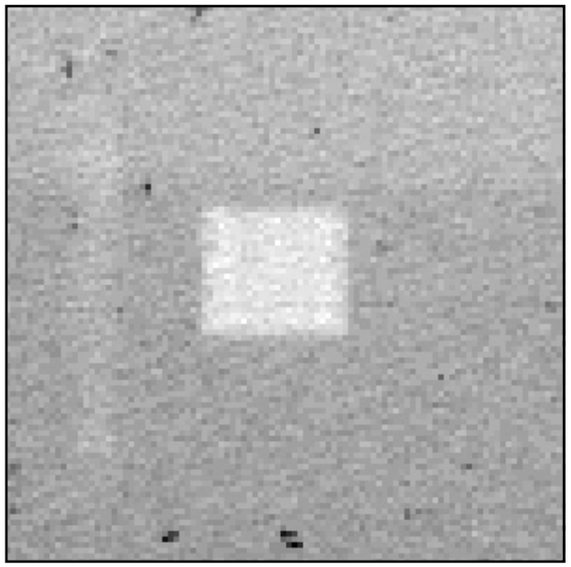
Maser *et al.*, *J. Micros.* **197**, 68 (2000)



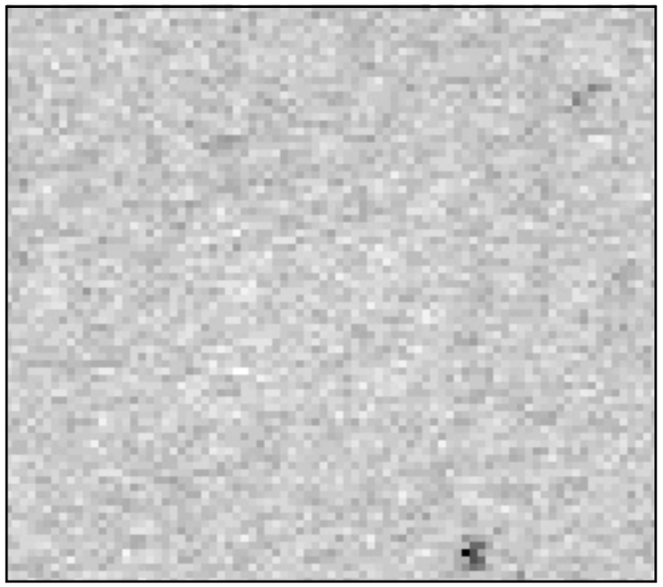
7 μm

PMMA at room, LN2 temperature

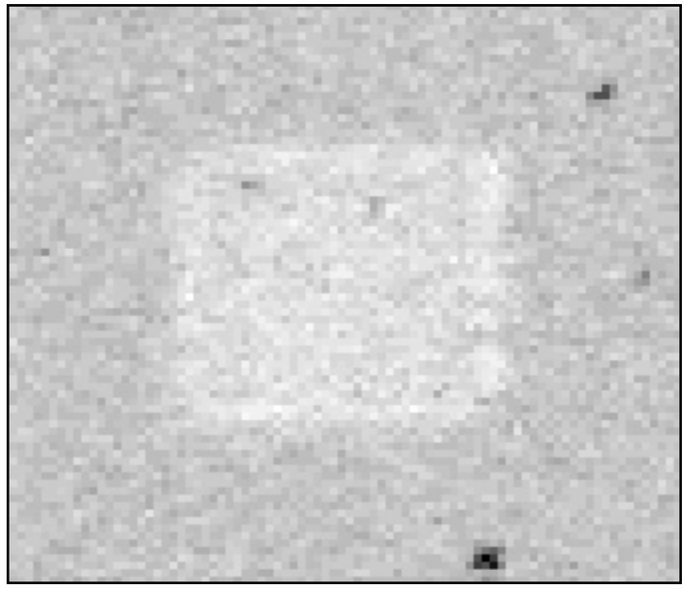
- Beetz and Jacobsen, J. Synchrotron Radiation **10**, 280 (2003)
- Repeated sequence: dose (small step size, long dwell time), spectrum (defocused beam)
- Images: dose region (small square) at end of sequence



Room temperature: mass loss immediately visible



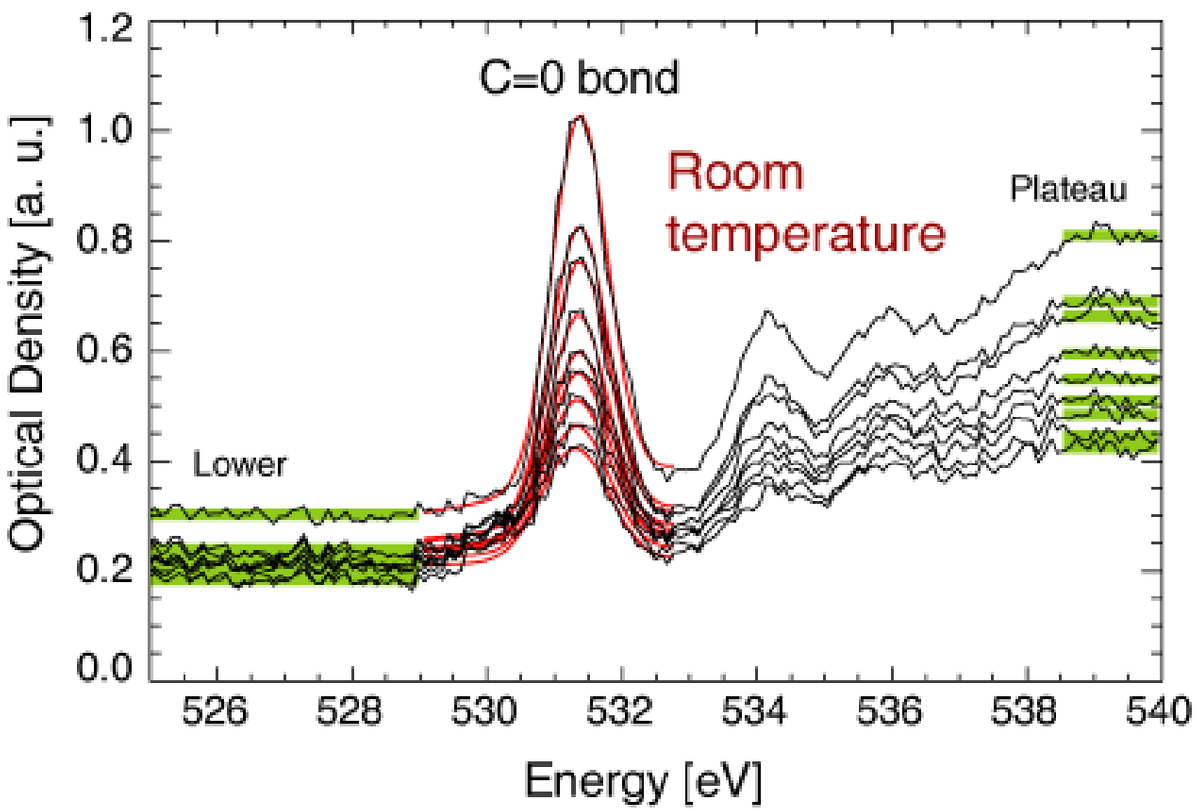
LN2 temperature: no mass loss immediately visible

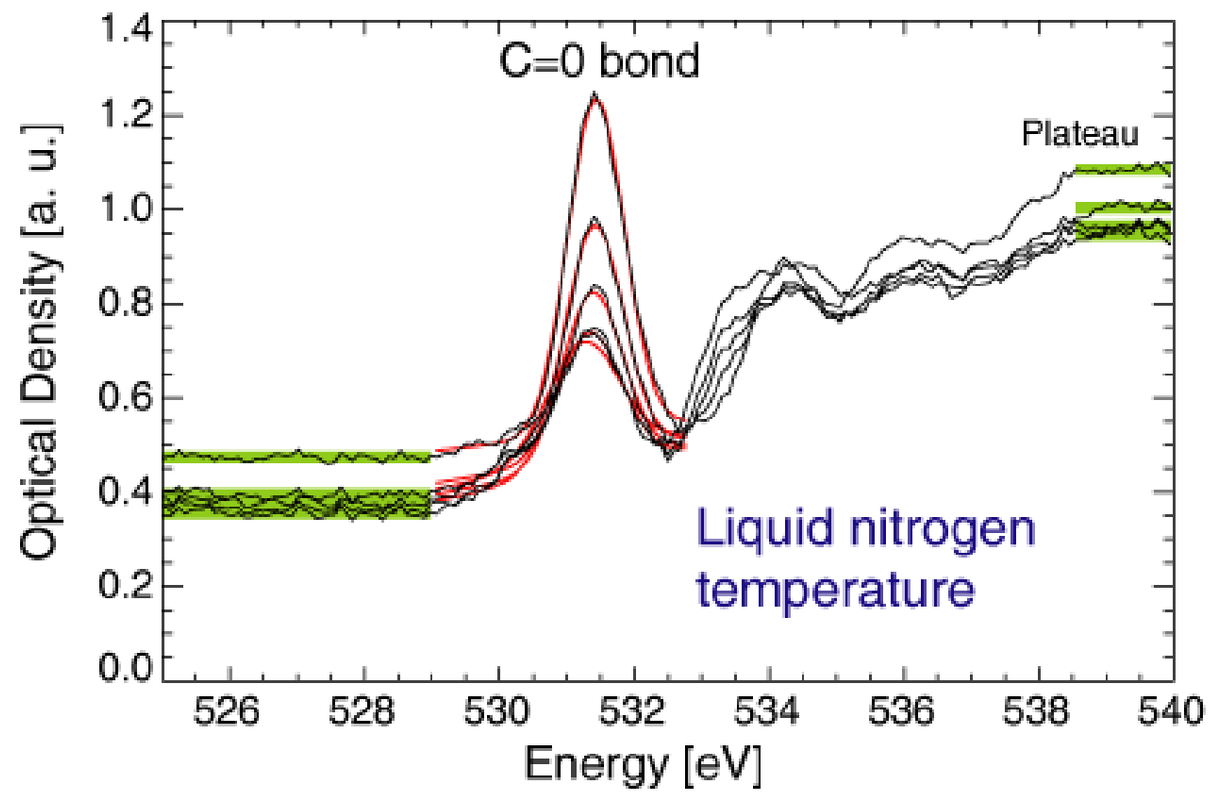


After warm-up: mass loss becomes visible

PMMA at LN2, room temperature: XANES spectra

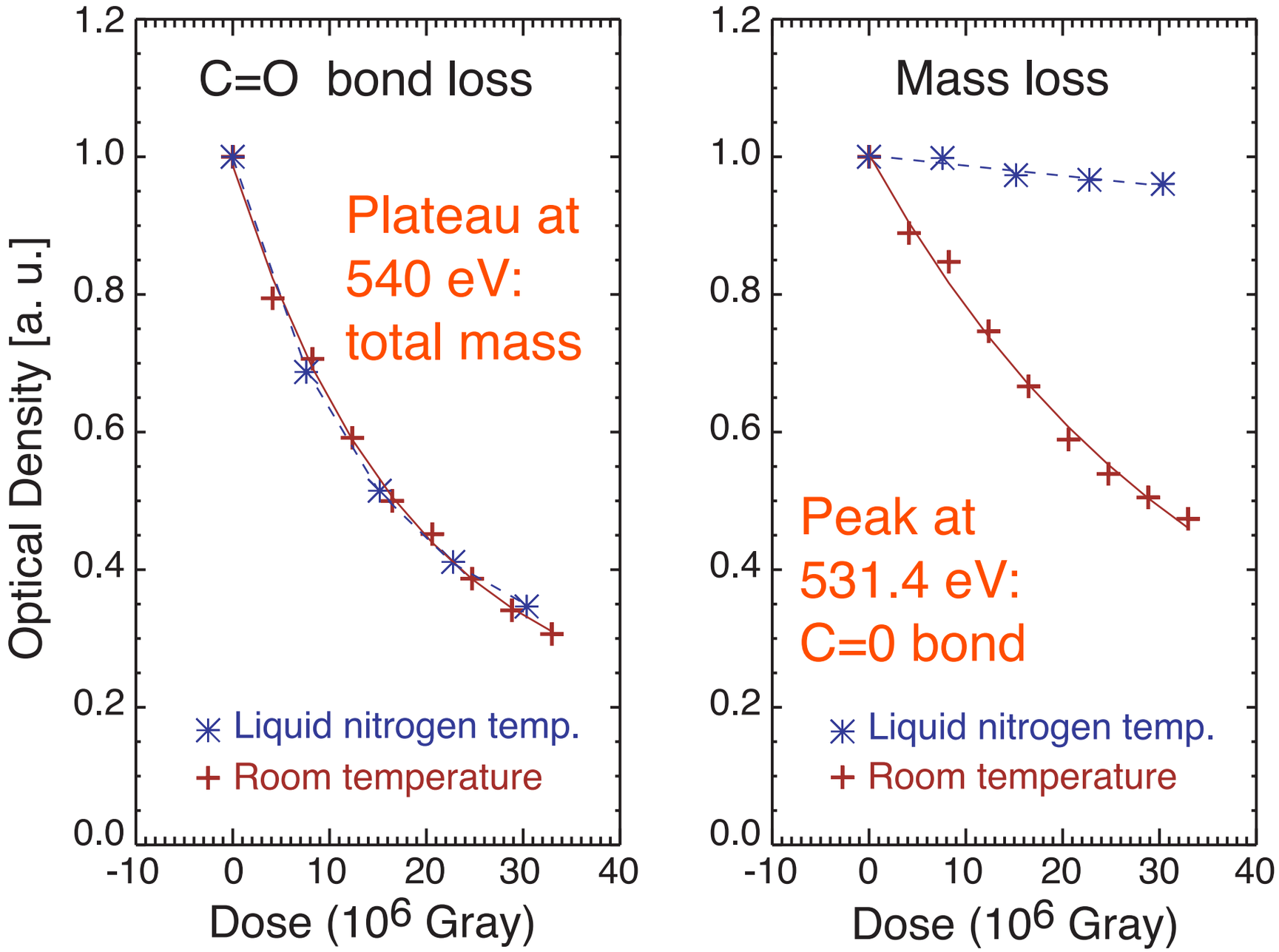
- Peak at 531.4 eV: C=0 bond
- Plateau at 540 eV: total mass (plus some emphasis on oxygen σ* bonds)
- Beetz and Jacobsen, *J. Synchrotron Radiation* **10**, 280 (2003)





Results from fitting spectra

LN_2 temp: protection against mass loss, but not against breaking bonds (at least C=0 bond in dry PMMA)



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

The Ramen noodle model of radiation damage

Macromolecular chains with no “encapsulating” matrix
(dry, room temperature wet)

The Ramen noodle model of radiation damage

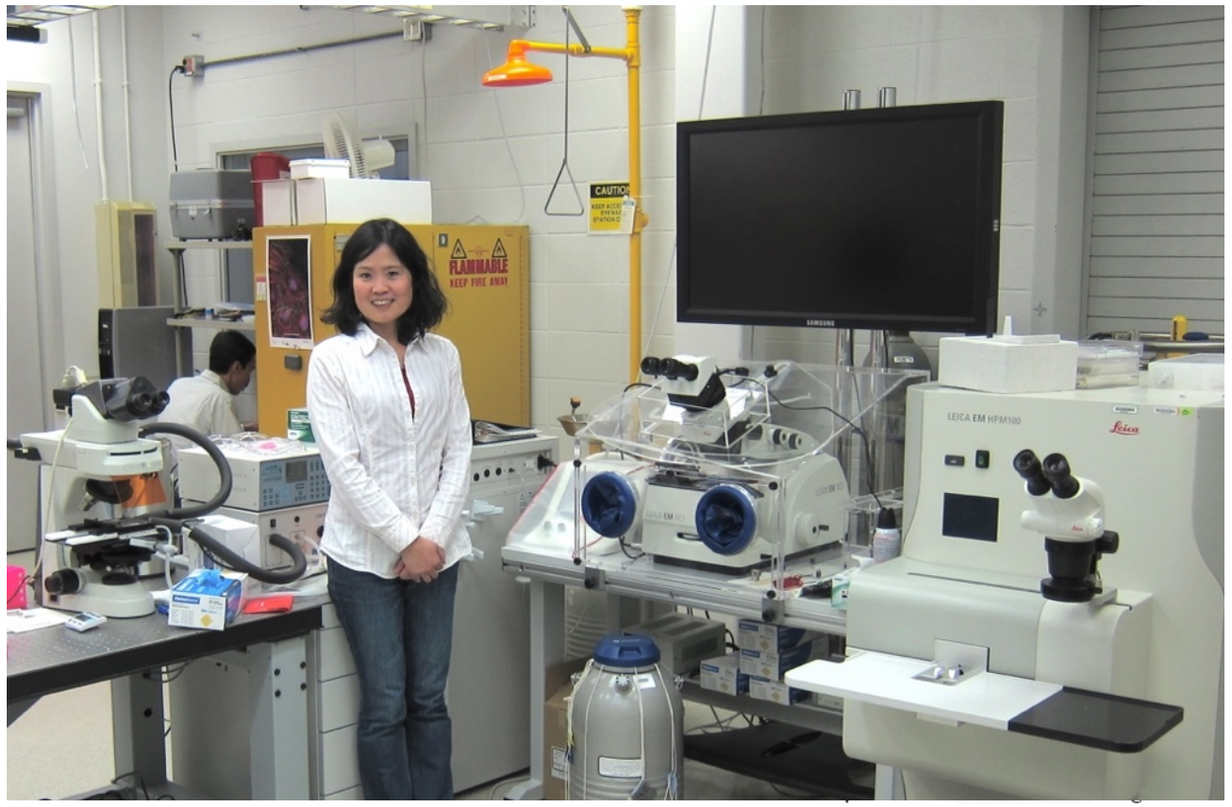
Macromolecular chains in an “encapsulating” matrix
(frozen hydrated)

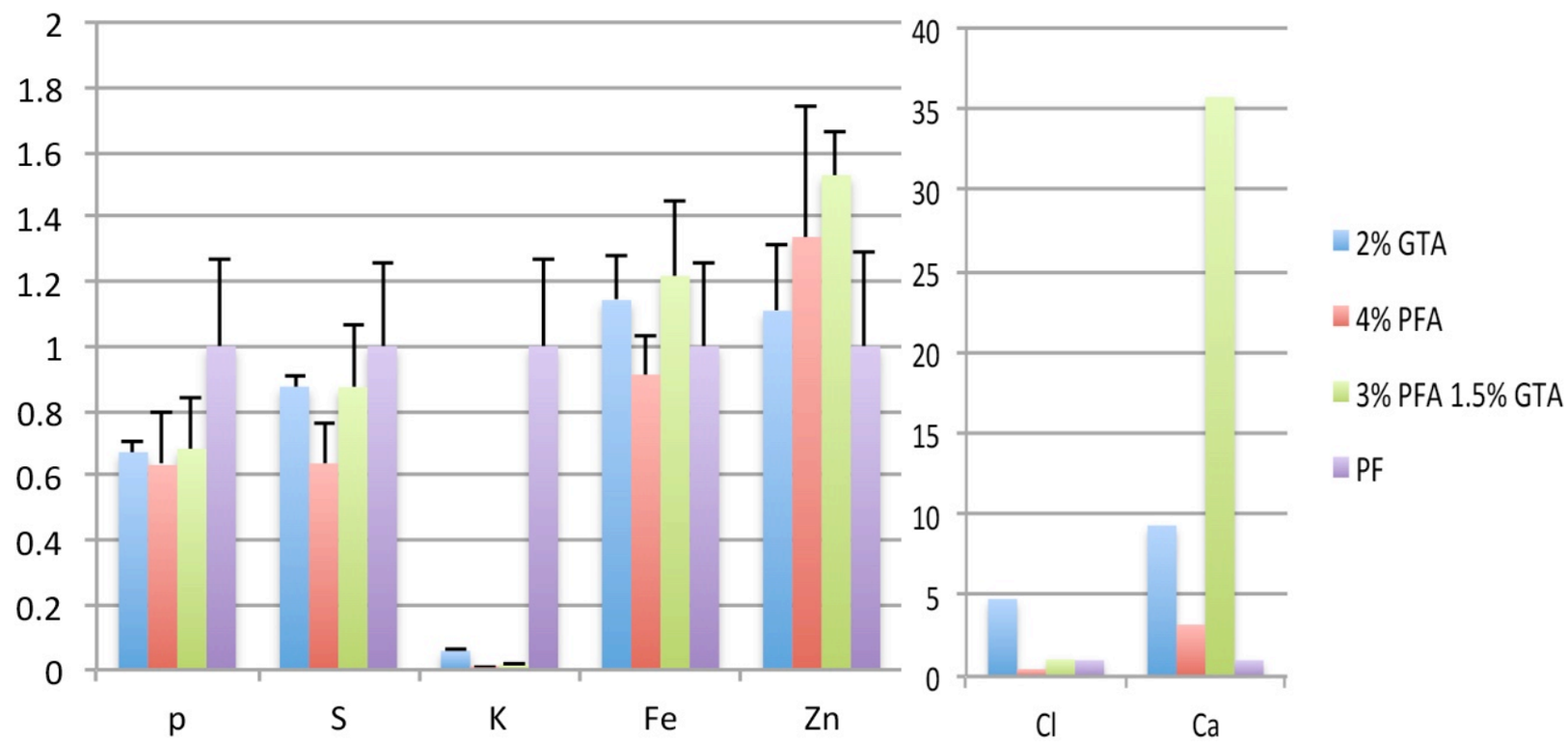
The Ramen noodle model of radiation damage

Actual noodles *were* harmed during the filming of this movie

CryoLab at Argonne

- Dr. Qiaoling Jin
- High pressure freezer (Leica HPM 100)
- Cryo ultramicrotome (Leica UC7)
- Cryo light microscope (Instec/Nikon)
- Robotic plunge freezer (FEI Vitrobot)
- NIH R01 GM104530 (Jacobsen, Woloschak)





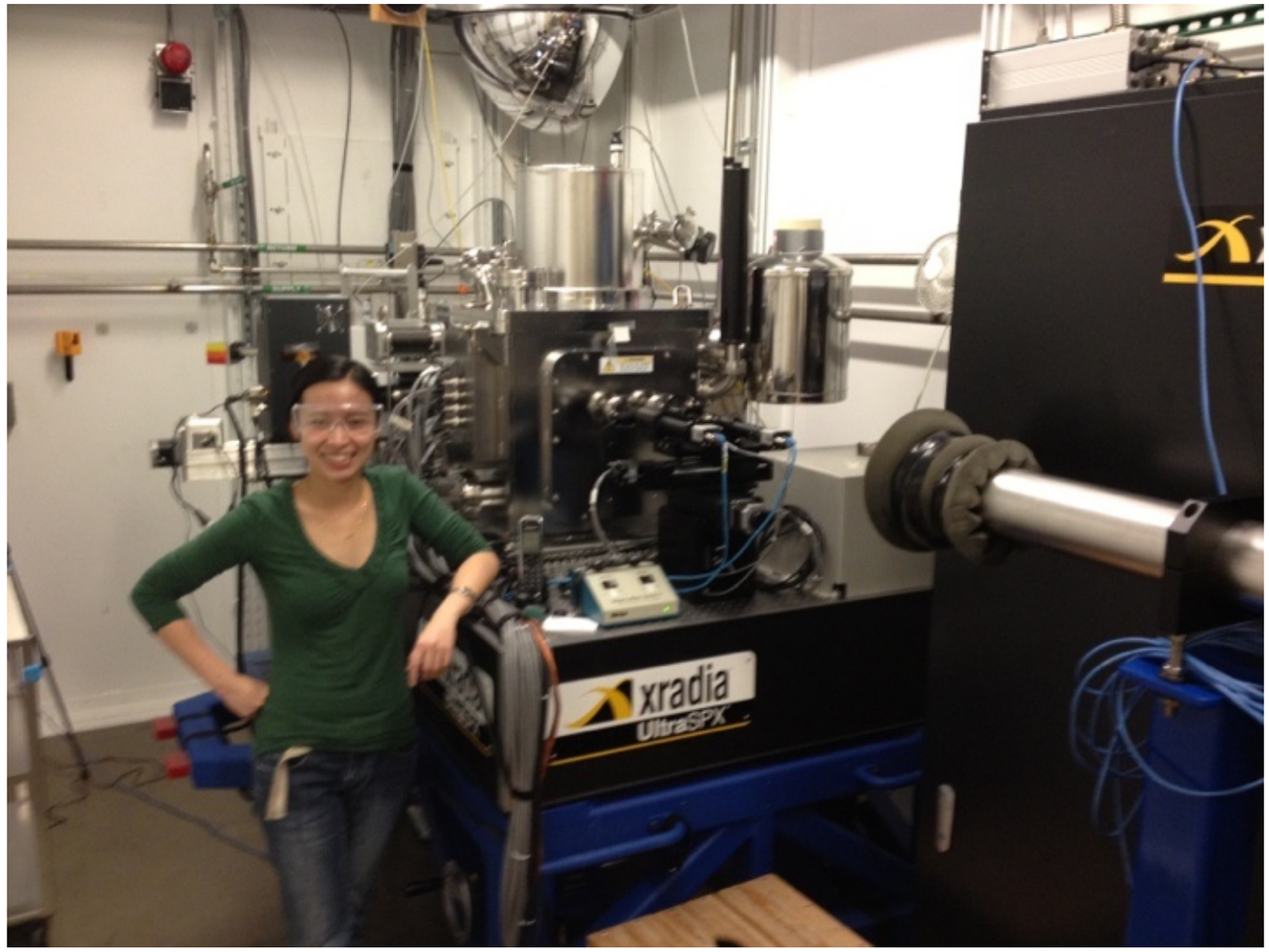
GTA: glutaraldehyde

PFA: paraformaldehyde

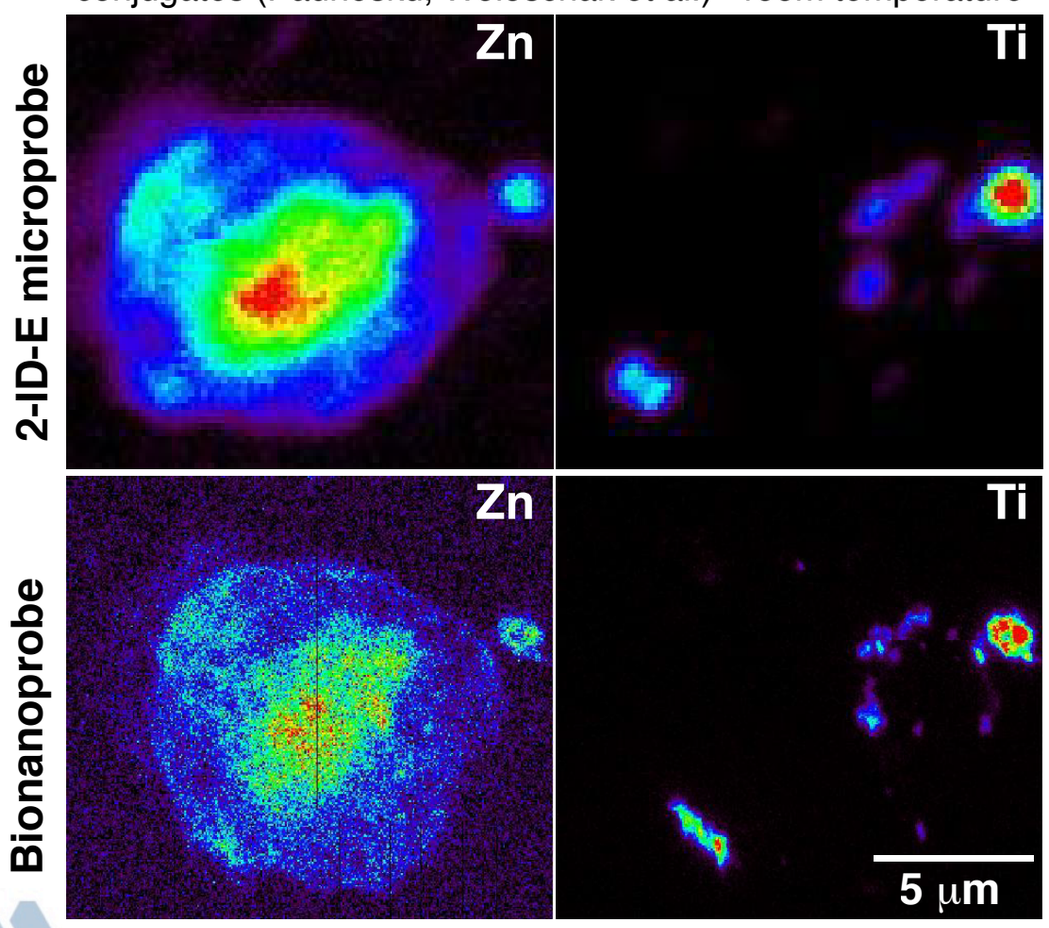
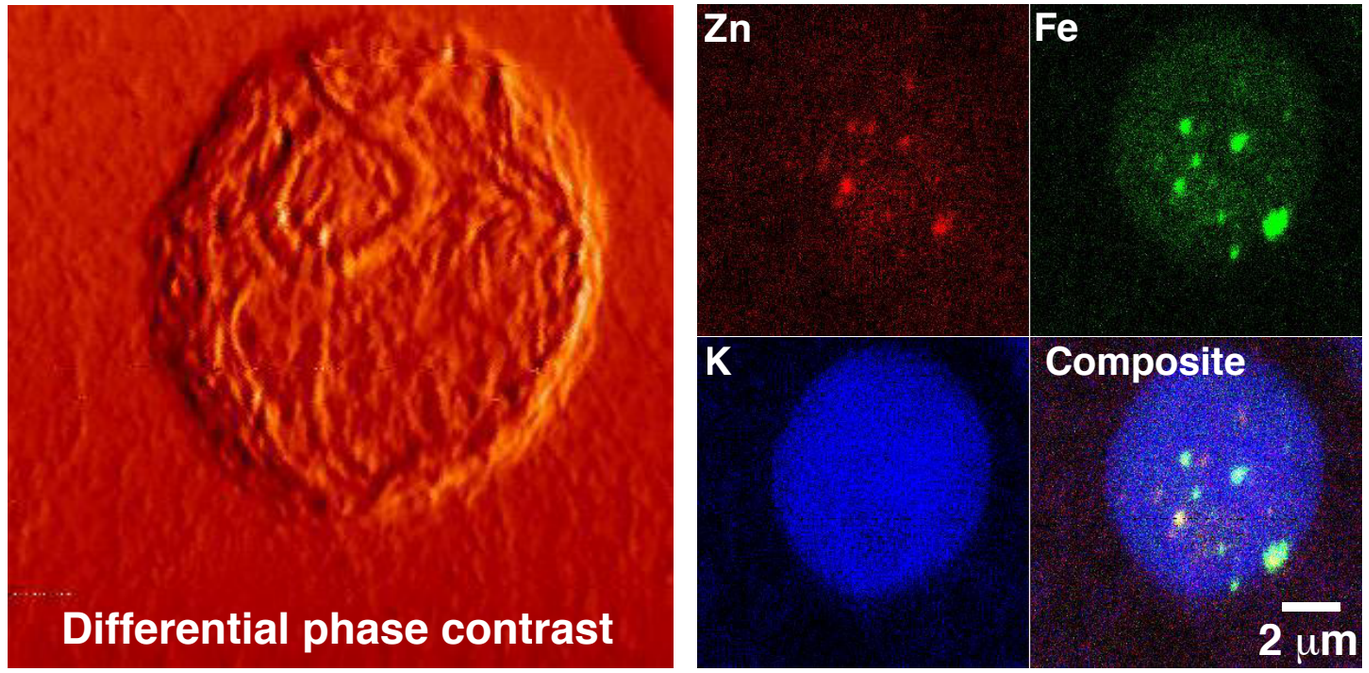
PF: plunge frozen

Bionanoprobe

- Dr. Si Chen (bionanoprobe), Dr. Qiaoling Jin (cryo prep R&D), APS Microscopy Group, instrument from Xradia (now Carl Zeiss XRM), LS-CAT Beamline
- NIH ARRA to Woloschak *et al.*
- Cryo ptychography w/fluorescence: NIH to Jacobsen *et al.*



Paraffin-embedded HeLa transfected with TiO_2-DNA conjugates (Paunesku, Woloschak et al.) - room temperature



Chlamydonomas reinhardtii at 110 K