Radiation damage limits to X-ray imaging, and their (partial) mitigation

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Signal to noise and required number of photons

- Simple photon statistics with known contrast:

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

where Θ=contrast parameter, I_f=intensity of feature, I_b=intensity of background.

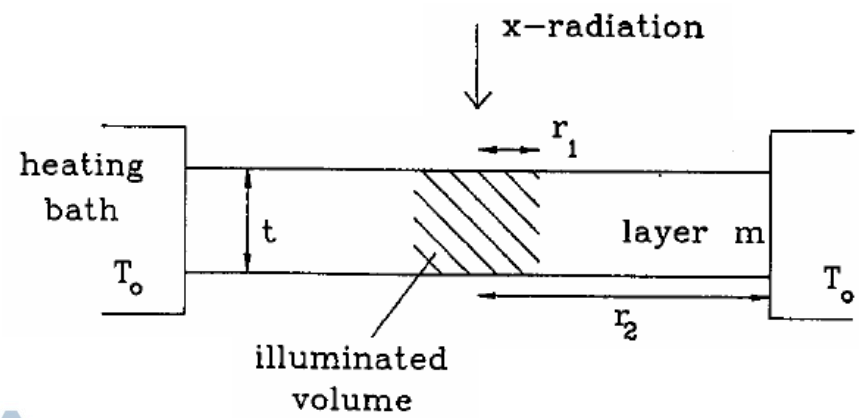
- Thus required number of incident photons $\bar{n}$ is

$$\bar{n} = \frac{\mathrm{SNR}^2}{\Theta^2}$$

- Example: 10 nm protein in ice at 520 eV via absorption contrast
  - Protein has linear absorption coefficient (LAC) of 1/9.900 µm, so 10 nm has I_f = exp[-0.010/9.900]=0.99899
  - Ice has LAC of 0.717 µm, so 10 nm has I_b = exp[-0.010/0.717]=0.98615
  - Contrast parameter is Θ=(.99899-.98615)/(.99899+.98615)$^{1/2}$=.00911
  - So with SNR=5 one requires $\bar{n}$=(5)^2/(.00911)^2=3x10^5 incident photons
- See e.g., Sayre *et al.*, *Ultramicroscopy* **2**, 337 (1977); Sayre *et al.*, *Science* **196**, 1339 (1977)

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 (3 µWatts)
  - Si@10 keV: 2×10^{12} photons/sec (3 mWatts)



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

Greinke and Gölz, XRM 1991

Radiation 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:

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

- Energy per mass:

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

Dose numbers

- G factor: number of bonds broken per 100 eV. G~5 for many organic molecules (room temp.)
- Break 1 bond per atom (Henderson limit):

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

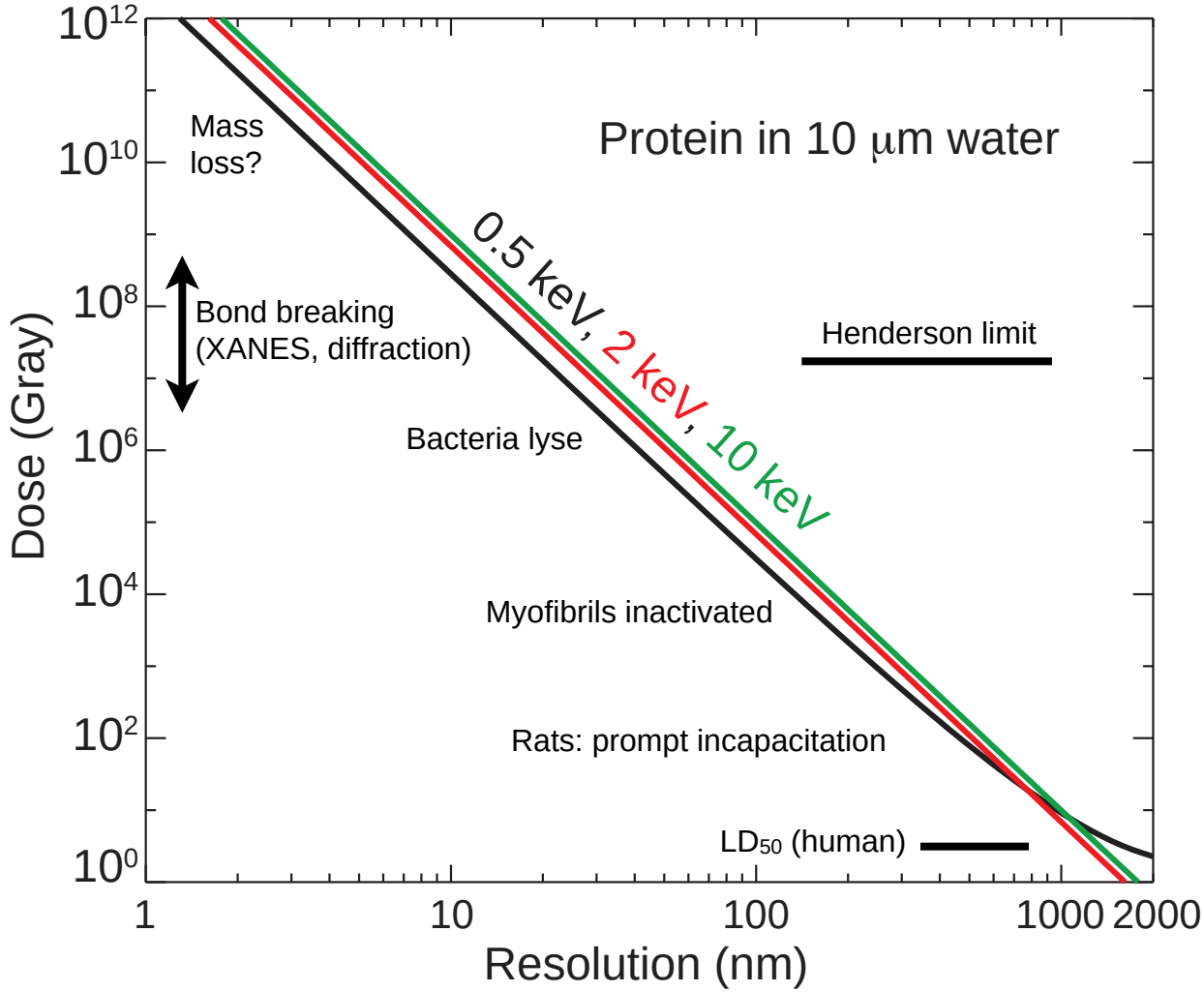
- Representative dose in crystallography:

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

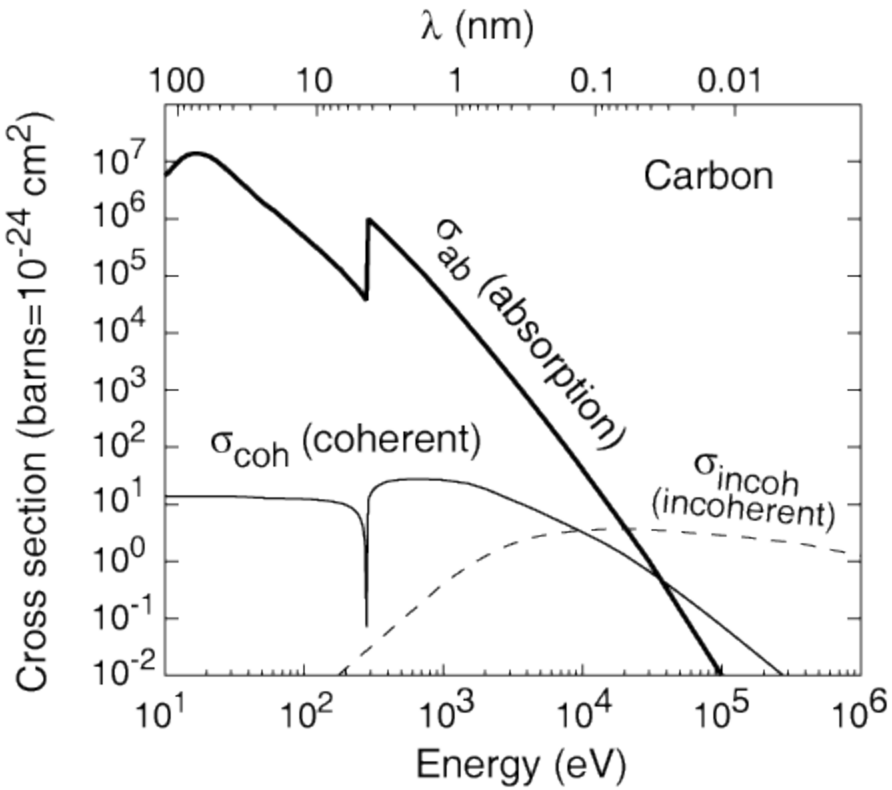
- X-ray microscopy: doses of 10^6-10^8 Gray are common, depending on resolution

Dose versus resolution for wet soft materials

- Calculation of radiation dose using best of phase, absorption contrast and 100% efficient imaging.
- In a 3D world, high resolution structures are also thin, with lower contrast.
- Things that can be done wet at room temperature:
  - bacteria at 50 nm resolution
  - small animals at micrometer resolution (followed by sacrifice)
  - At LD_{50}, half die!



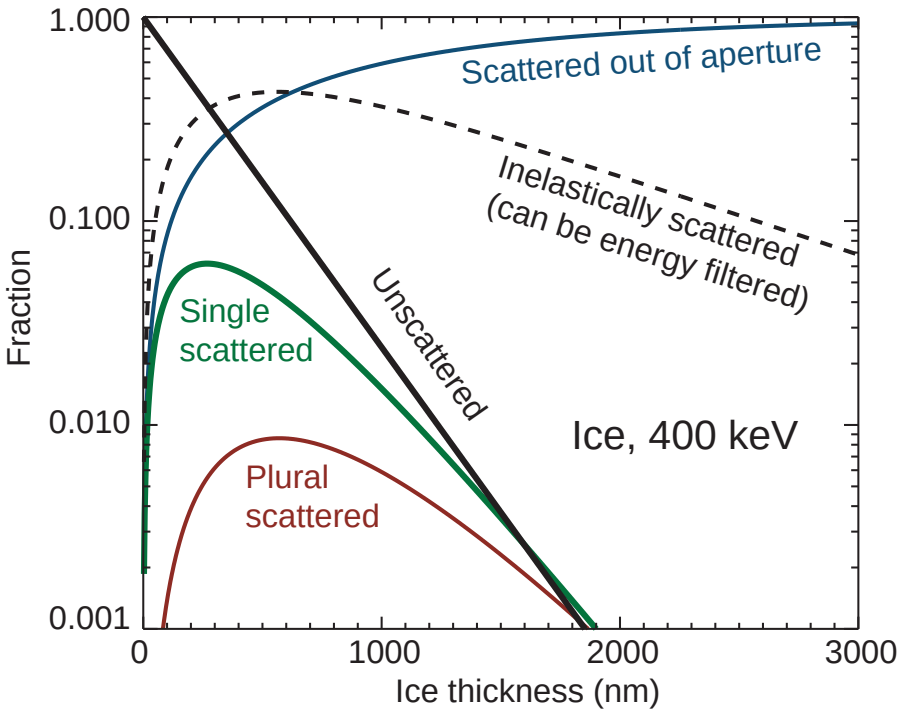
X rays



- 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

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: $10^3 \bullet 10^6$ eV into $(2\ \text{Å})^3$, or 2×10^{13} Gray

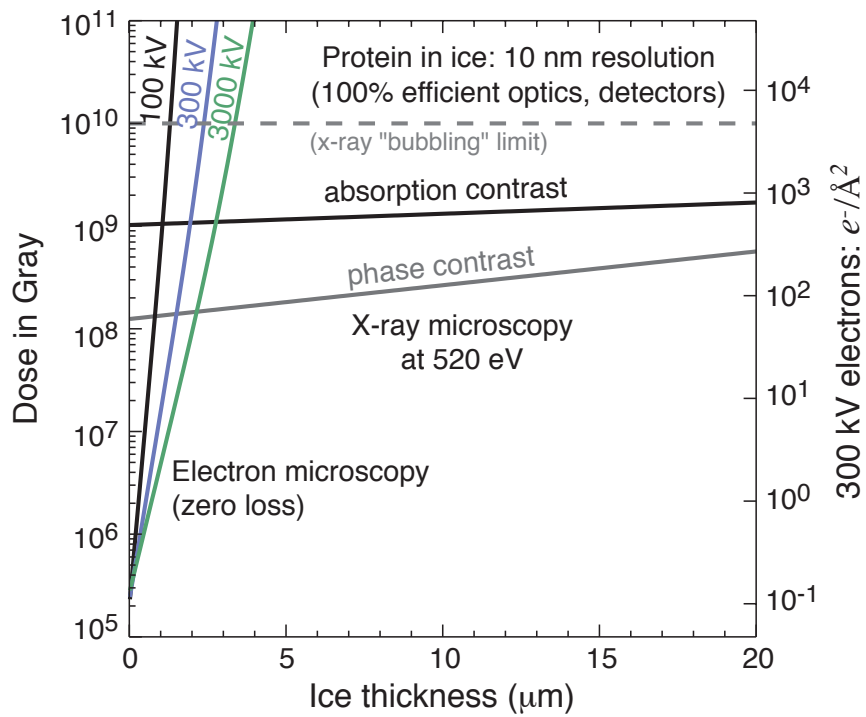
100 keV electrons

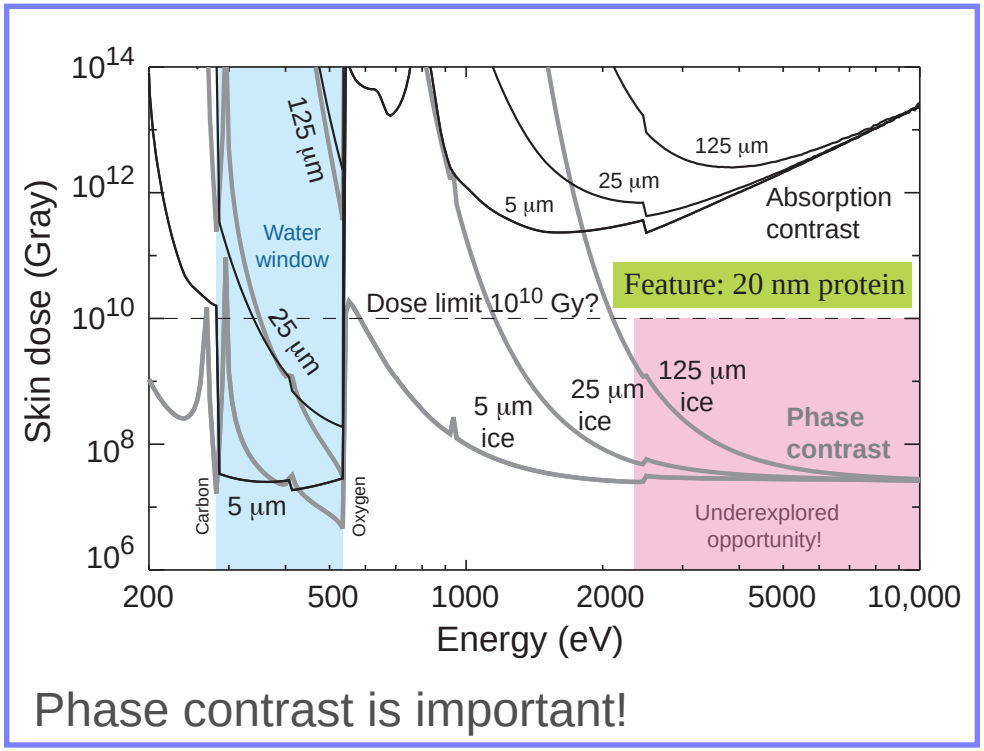
- About 2.5 inelastic scatters per elastic scatter
- About 45 eV deposited per inelastic scatter
- Therefore about 10^2 eV deposited per elastic scatter
- A thousand scattered electrons: $10^3 \bullet 10^2$ eV into $(2\ \text{Å})^3$, or 2×10^9 Gray

- 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 rays are better than electrons for thick specimens

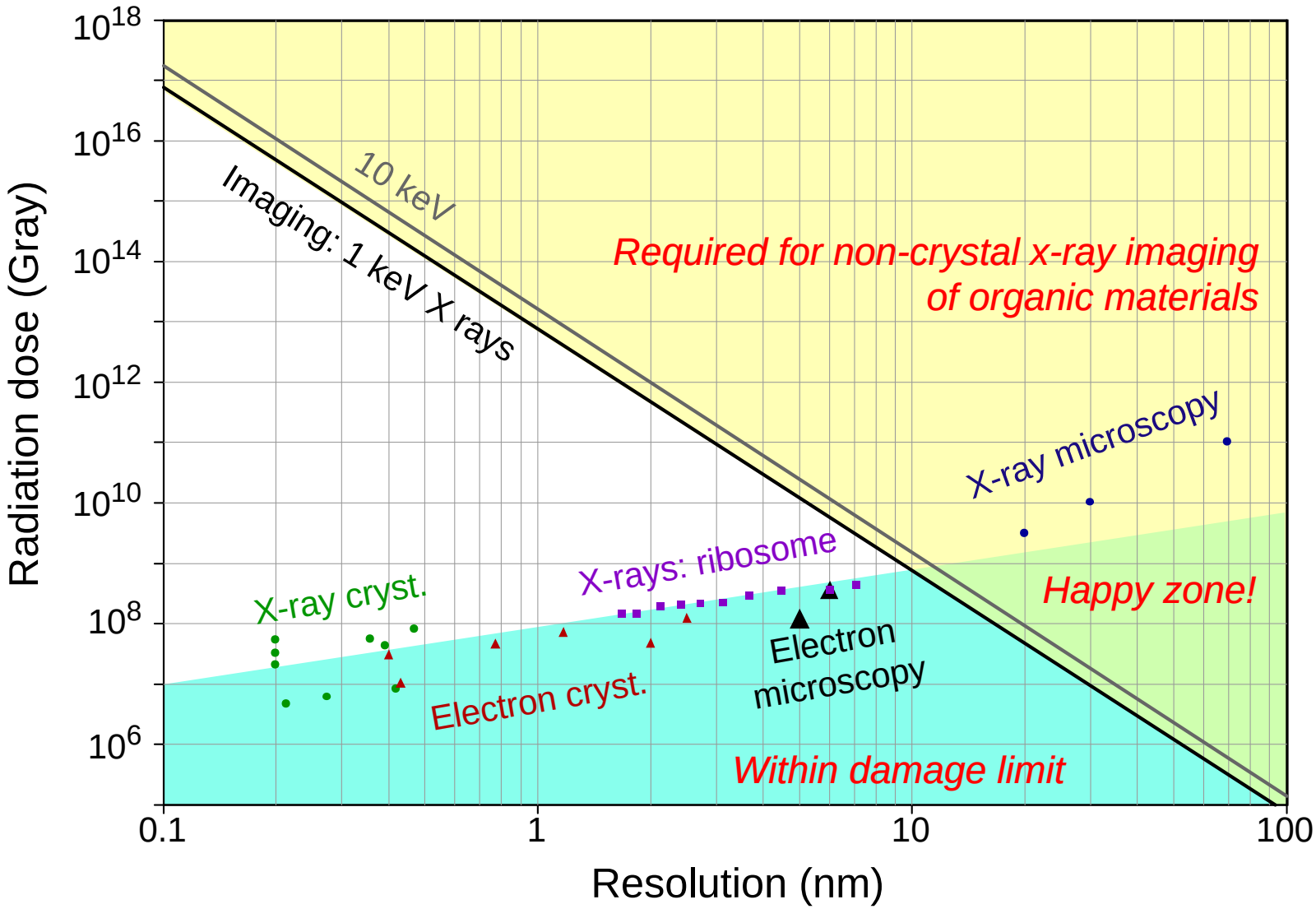
- No more than 1 high-resolution image of wet, soft samples unless frozen
- At energies >3 keV, opportunities for thick specimens.





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

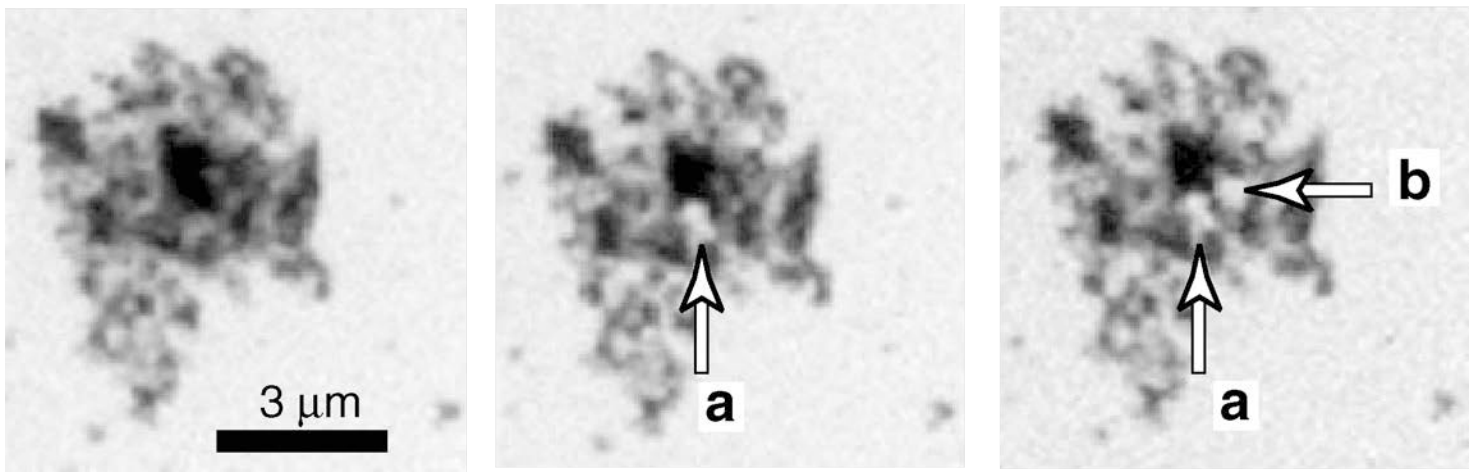
What's the limit for cells?



Howells *et al*., *JESRP* 170, 4 (2009)

See also Shen *et al*., *J. Sync. Rad*. **11**, 432 (2004)

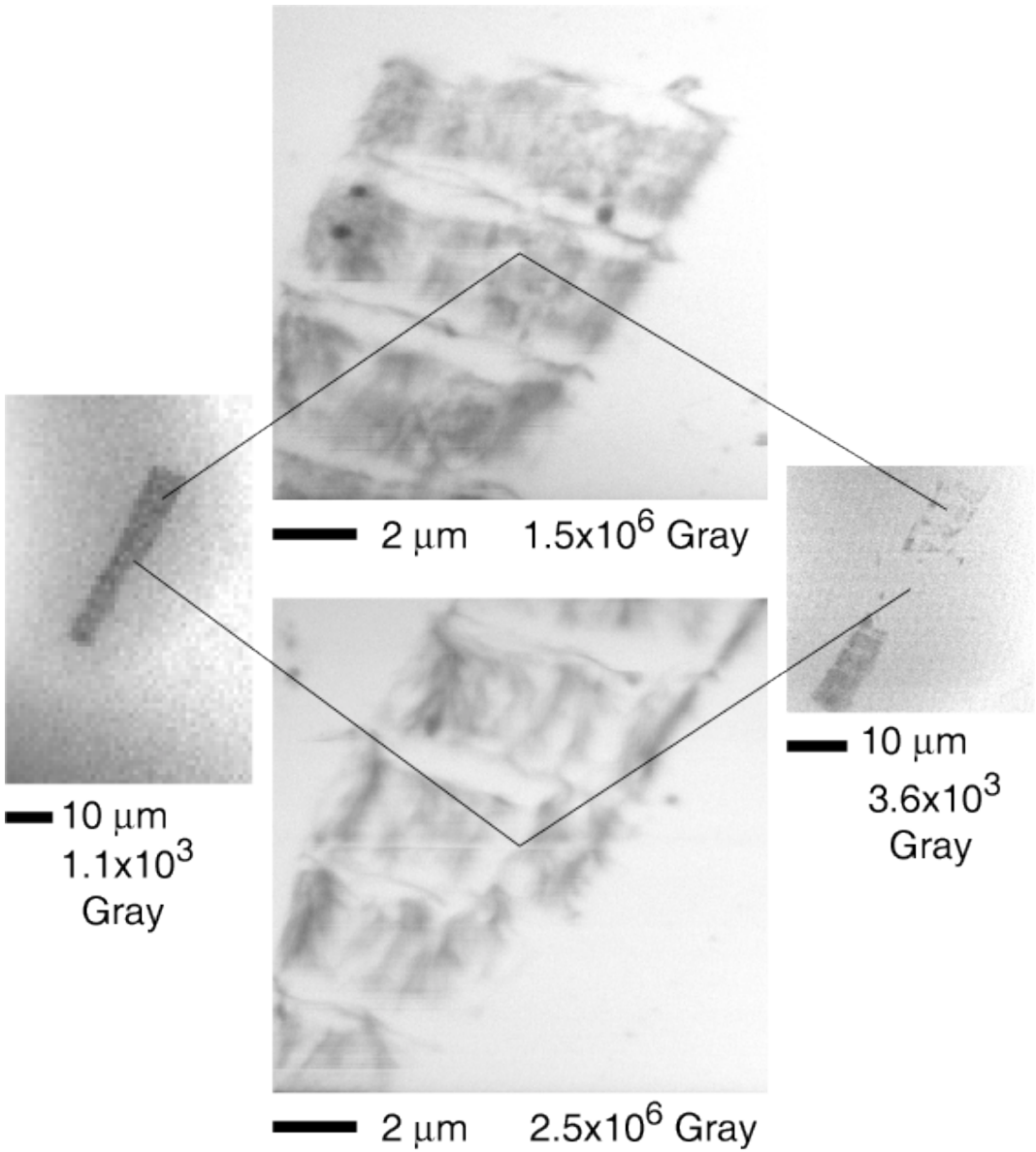
Spectromicroscopy can be damaging!



- Study: polyacrylimide-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)

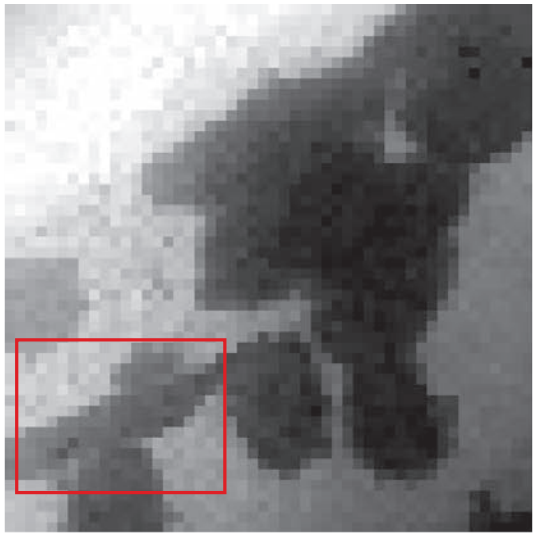
Muscle damage

- Images: dragonfly flight muscle, with Clara Franzini-Armstrong
- At 10^4 Gray, myofibrils stop contracting in the presence of ATP. Bennett *et al*., *J. Micros*. **172**, 109 (1993)



Initially living cells at room temperature

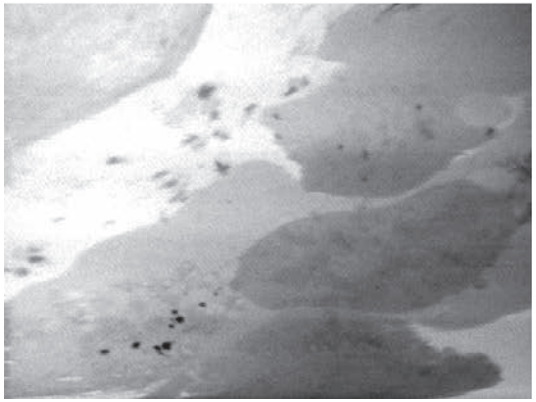
- Chinese hamster ovarian (CHO) fibroblasts in culture medium with periodic reflow.
- "Red for dead" fluorescent dyes used to confirm viability over hours with no x-ray exposure.
- Imaged in a soft x-ray scanning transmission microscope (NSLS X1A)



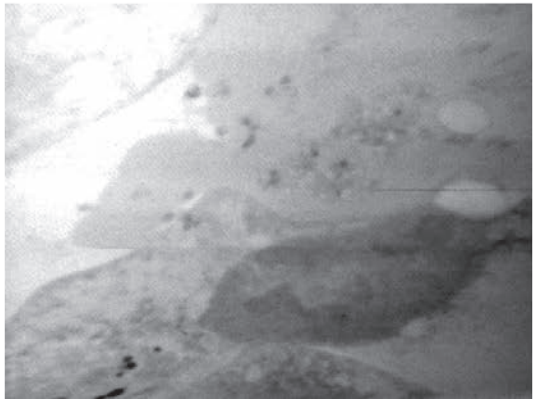
10 μm
6.0×10^{2} Gray, ET=2 minutes

Experiment by V. Oehler, J. Fu, S. Williams, and C. Jacobsen, Stony Brook using specimen holder developed by Jerry Pine and John Gilbert, CalTech. In Kirz, Jacobsen, and Howells, *Quarterly Reviews of Biophysics* **28**, 33 (1995).

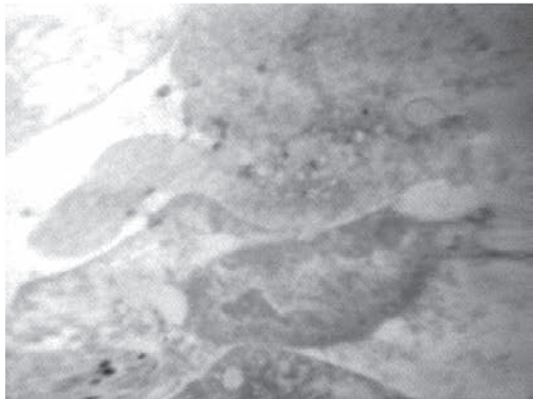
1 Gray=1 Joule/kg absorbed



5 μm
1.2×10^{5} Gray, ET=9.5 min



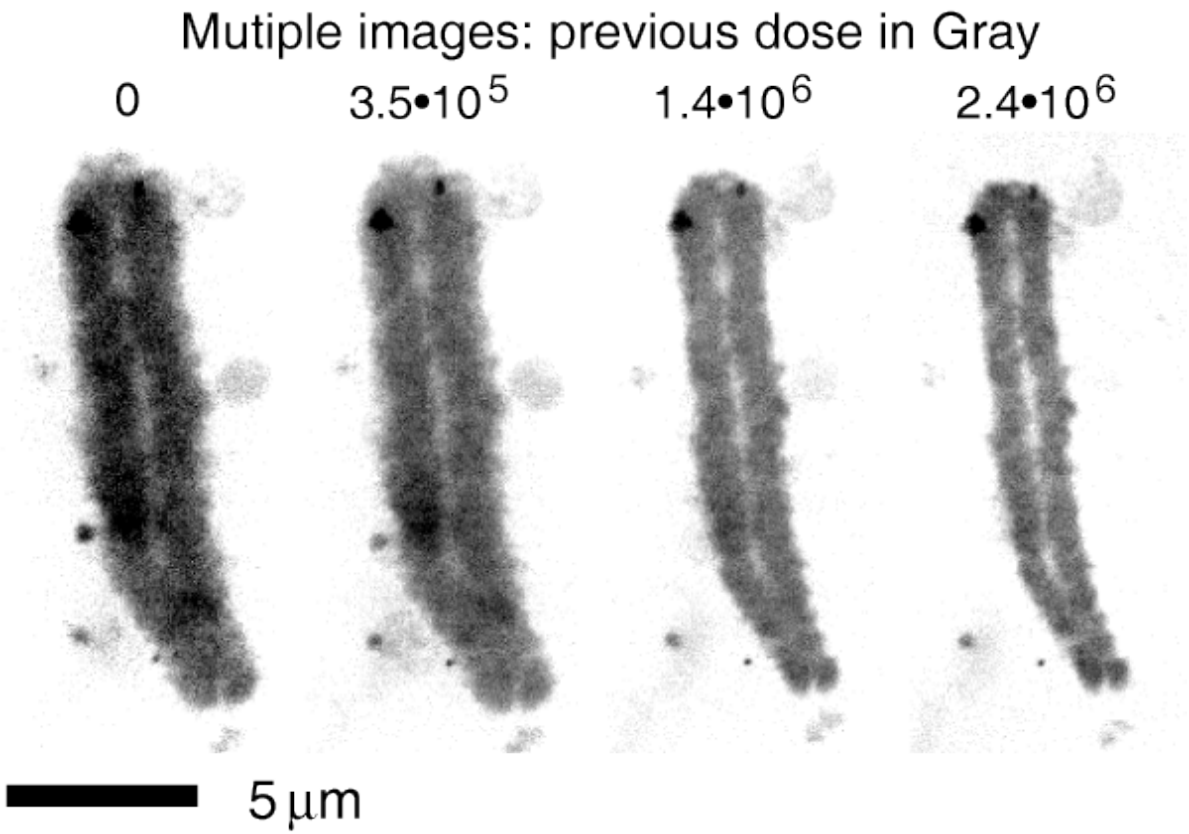
5 μm
2.4×10^{5} Gray, ET=17 min



5 μm
3.7×10^{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

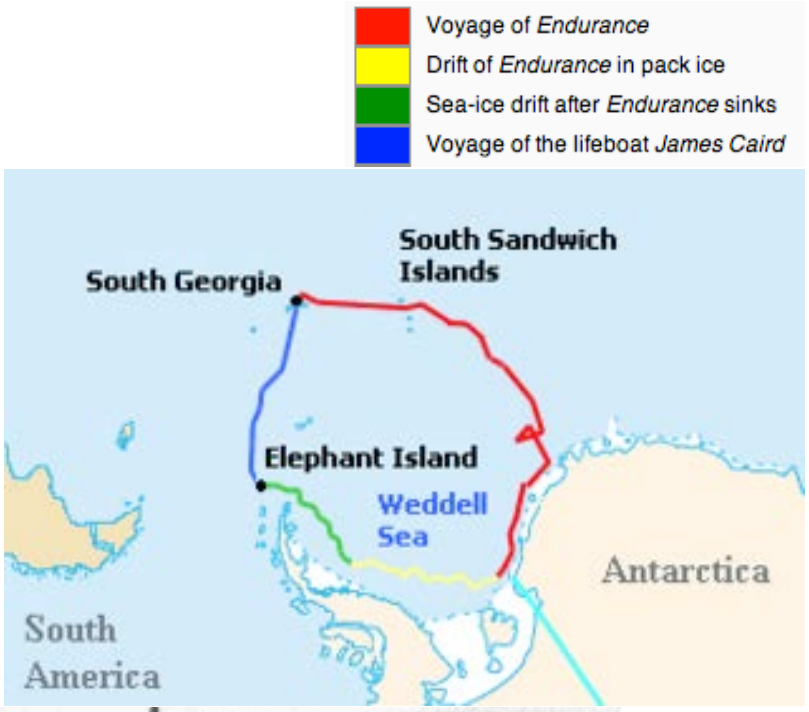


Limerick #1

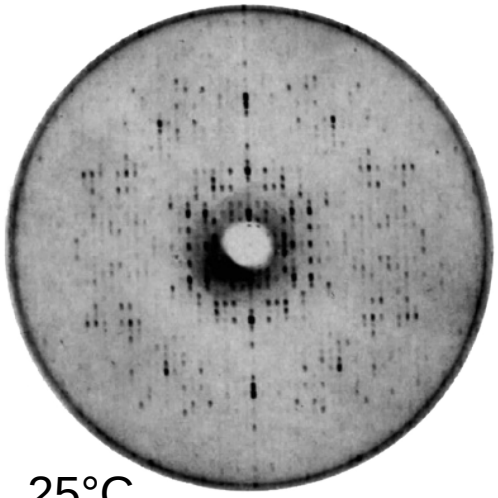
My data collection was shorted
Cells and crystals became all distorted
Due to very large dose
They all gave up the ghost
So my experiments they were all thwarted!

Ernest Shackleton, *Endurance*, 1914-1916

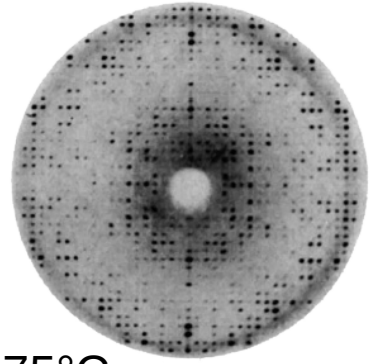
MEN WANTED
for hazardous journey, small wages, bitter cold, long months of complete darkness, constant danger, safe return doubtful, honor and recognition in case of success.
Ernest Shackleton 4 Burlington st.



Cryo crystallography



25°C



-75°C

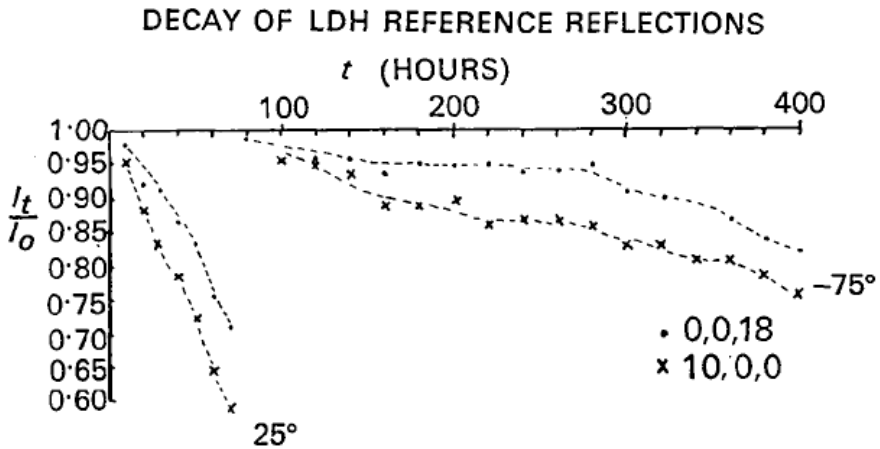


Fig. 5. The ratio I_t/I_0 for two reference reflections plotted as a function of exposure time for a typical native and frozen crystal. I_t represents the intensity at time t. Results for 0,0,18 and 10,0,0 are shown with dots and crosses respectively.

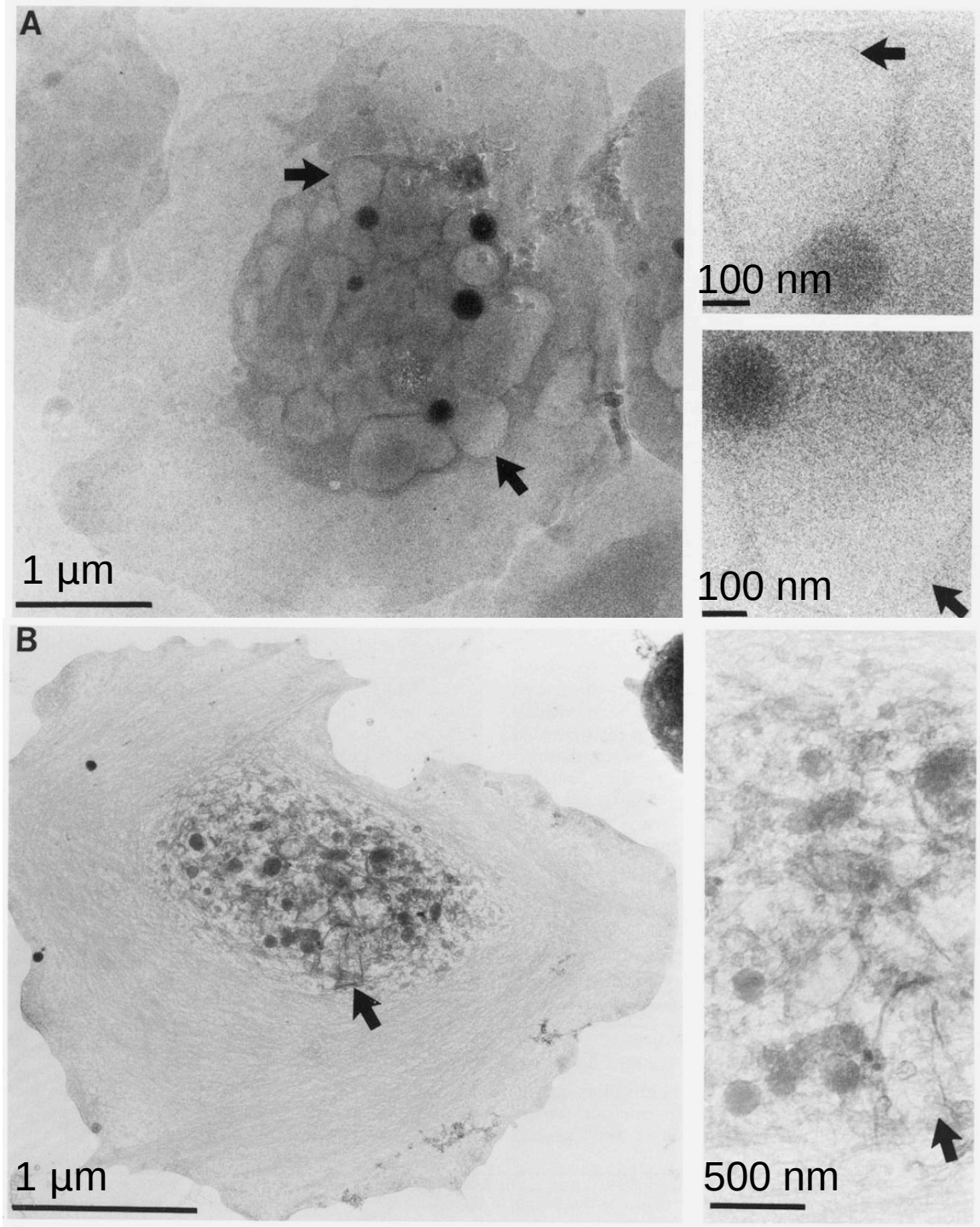
Acta Cryst. (1970). B**26**, 998

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)

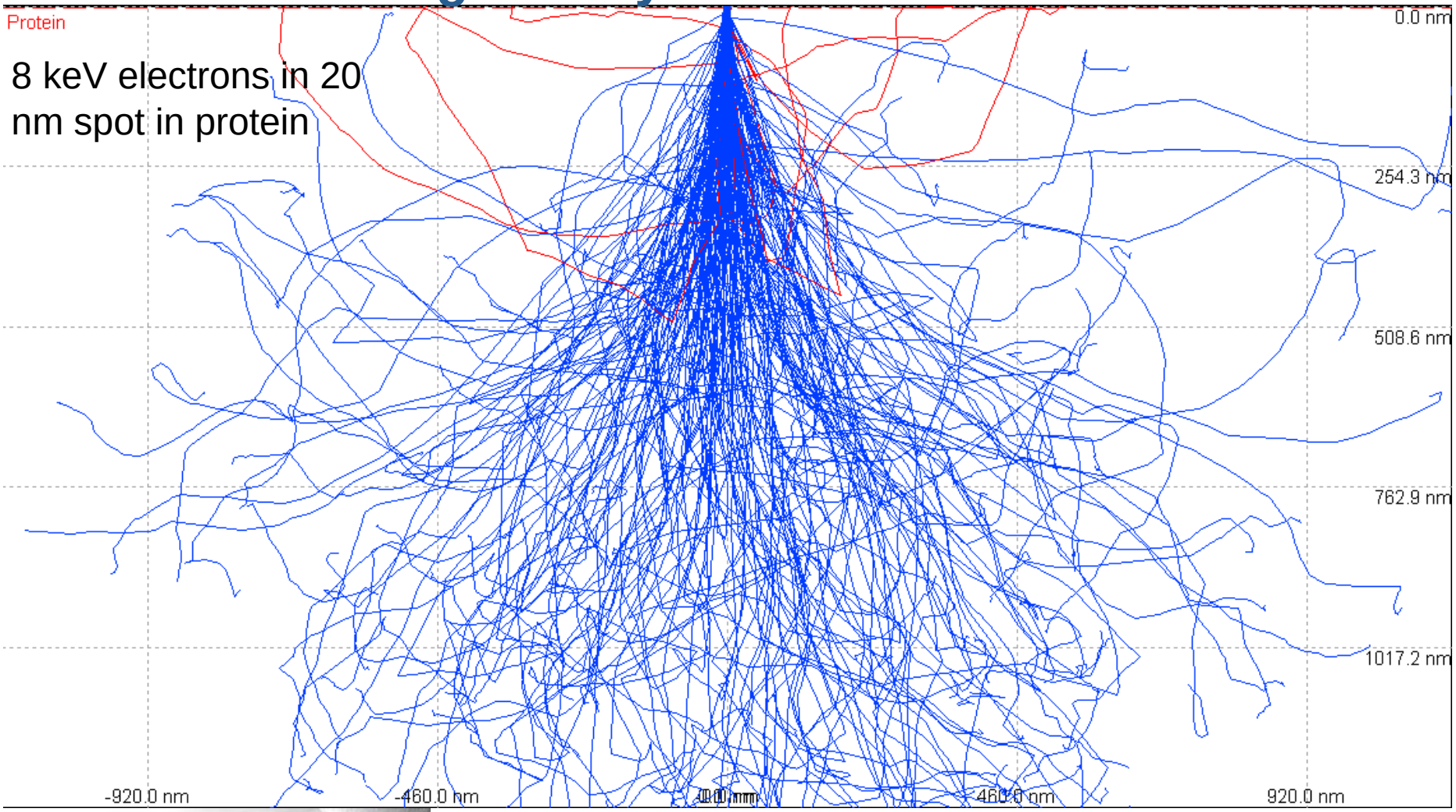


Frozen hydrated

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)

Radiation damage: X rays to electrons to broken bonds

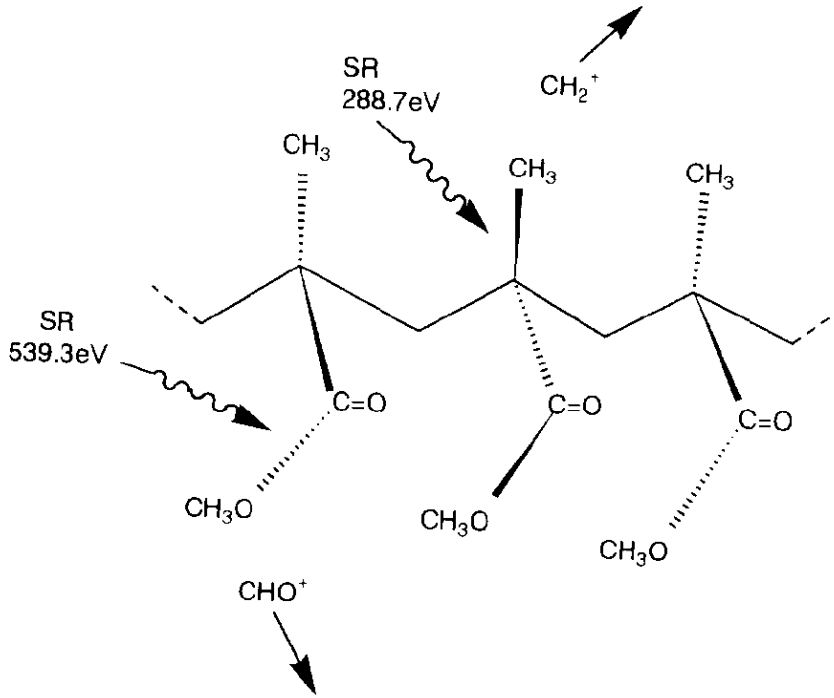


8 keV electrons in 20 nm spot in protein

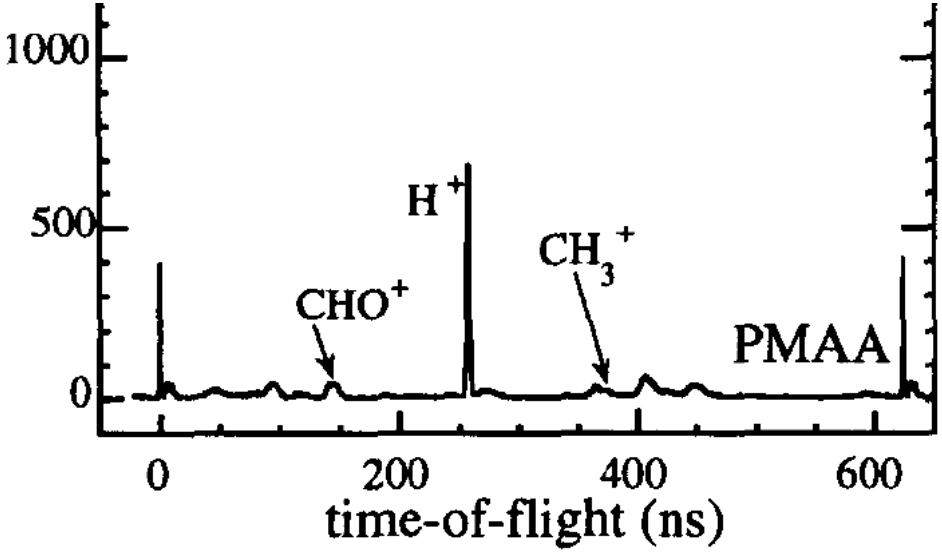
Casino
monte CArlo SImulation of electroN trajectory in sOlids

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

Radiation damage studies: poly (methyl methacrylate) or PMMA



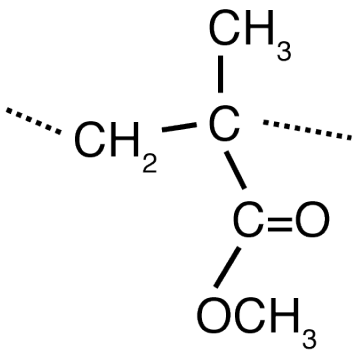
Tinone *et al.*, *Appl. Surf. Sci.* **79**, 89 (1994)



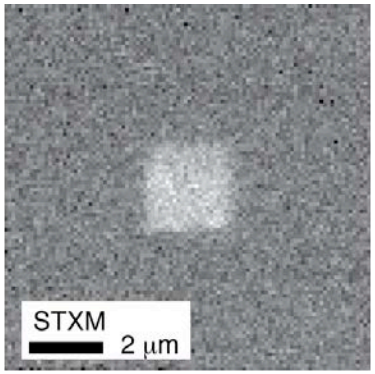
Tinone *et al.*, *J. Electron Spect. Rel. Phen.* **80**, 117 (1996).

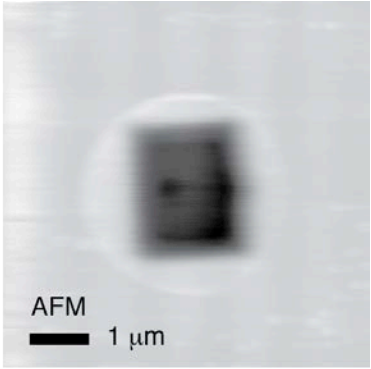
X-ray irradiation: poly(methyl methacrylate) (PMMA)

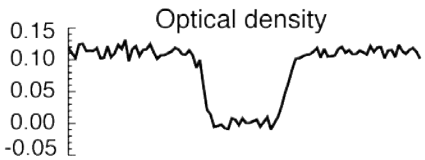
- Polymer that is especially radiation sensitive – it's used as a resist for electron beam lithography
- X. Zhang *et al.*, *J. Vac. Sci. Tech. B* **13**, 1477 (1995)
- Fine step size, high flux image for dose
- Slightly defocused beam for low dose image off XANES peaks
- At end, AFM for thickness

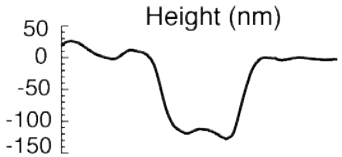


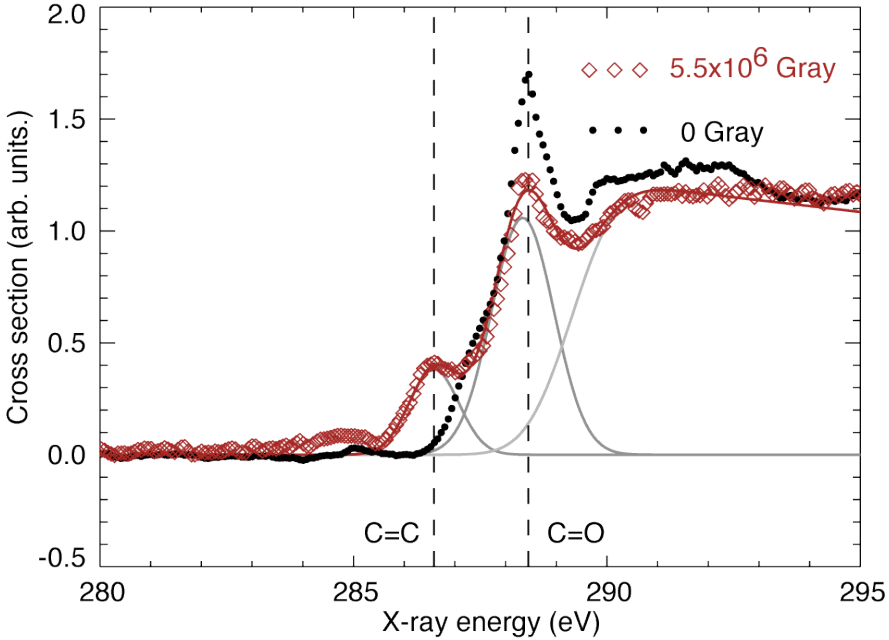
- Defocused beam for spectrum
- Gaussian fit to measure peak strengths at XANES resonances







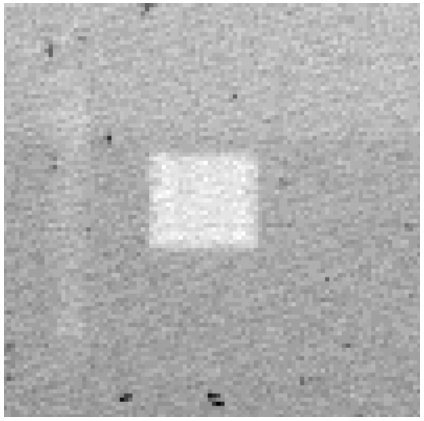




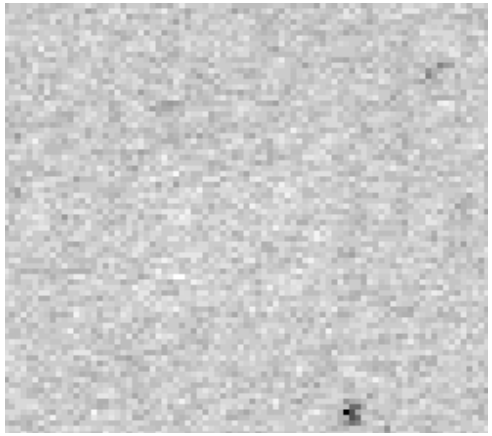
PMMA at 300 K and 110 K

PMMA in a cryo STXM [NSLS X1A; Maser *et al.*, *J. Micros.* **197**, 68 (2000)]

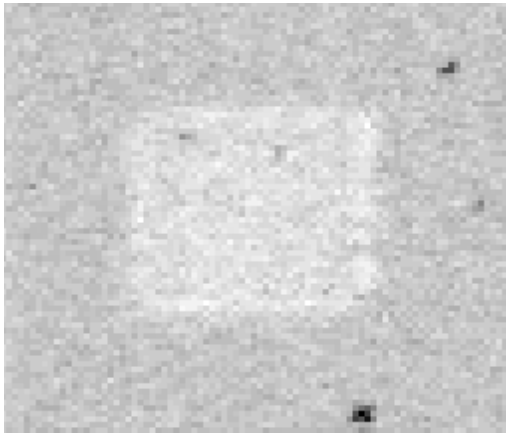
Repeated sequence: dose (small square), spectrum (defocused beam). Low dose, larger area image at end.



Room temperature: mass loss immediately visible



LN2 temperature: no mass loss immediately visible

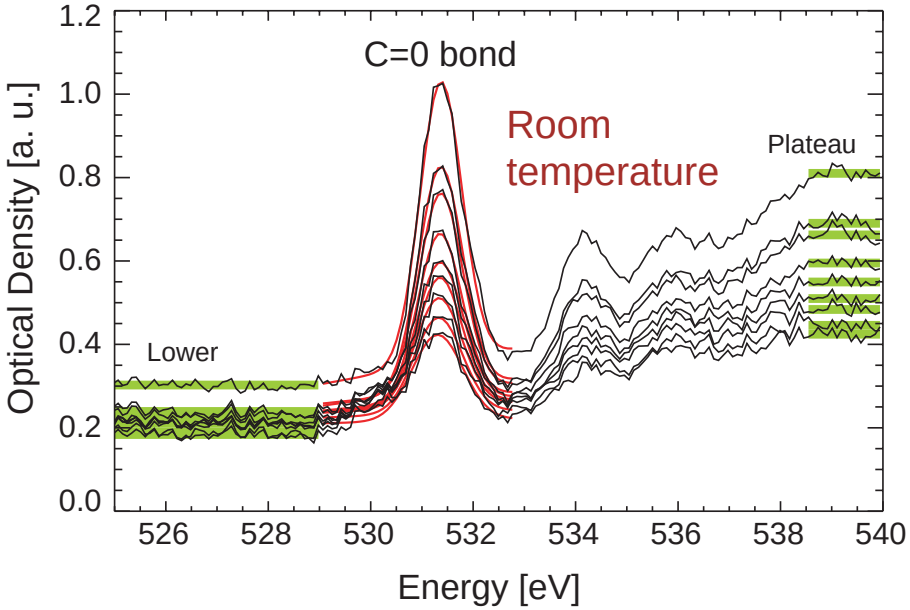


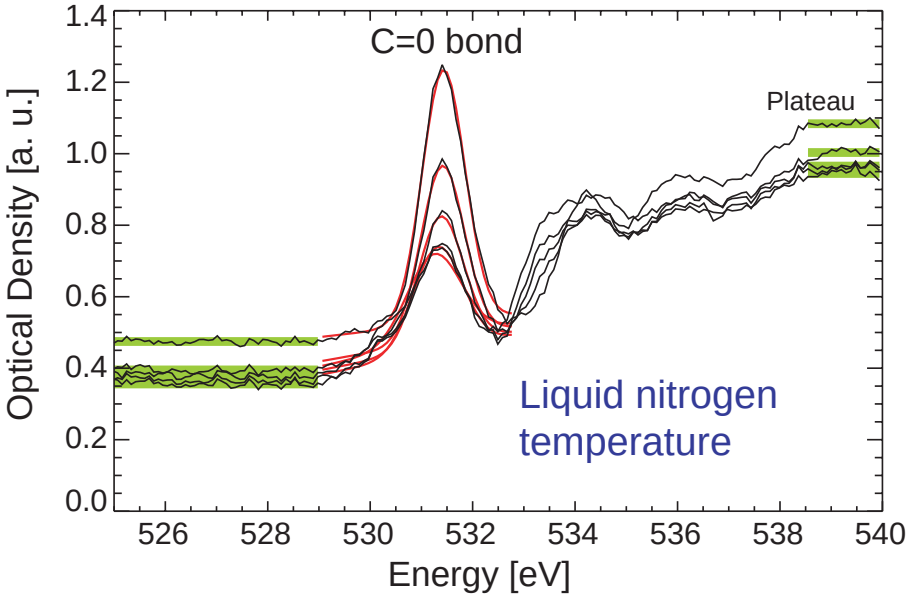
After warm-up: mass loss becomes visible

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

PMMA at 300 K and 110 K: x-ray absorption near-edge spectra

- Peak at 531.4 eV: C=0 bond
- Plateau at 540 eV: total mass (plus some emphasis on oxygen σ* bonds)

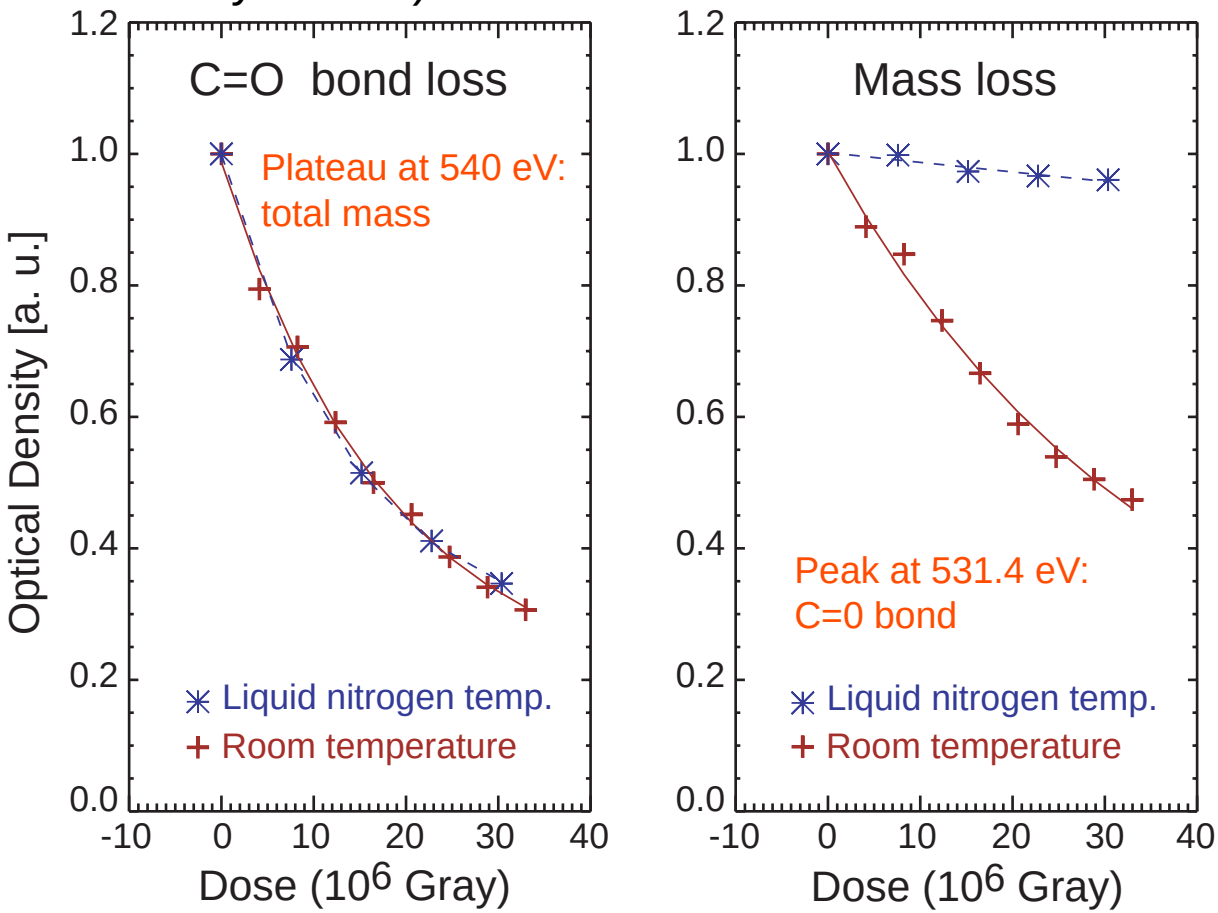




Beetz and Jacobsen, *J. Synchrotron Radiation* **10**, 280 (2003)

PMMA at 300 K and 110 K: 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)

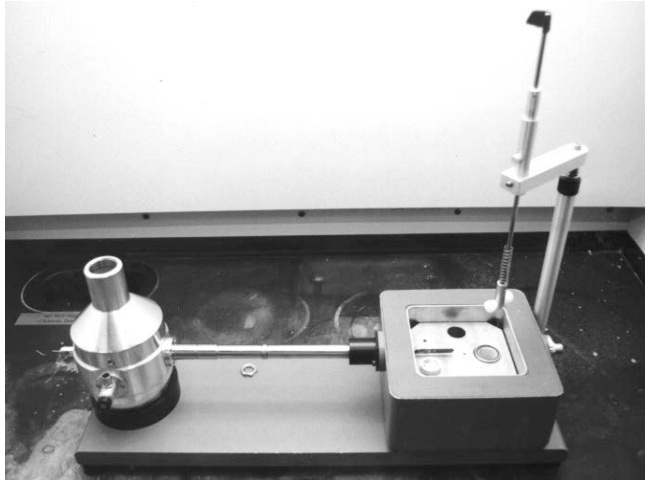
Limerick #2

I’m despondent, and such a sad bloke
As my wildest dreams go up in smoke
We have all racked our brains
Hoping for damage-free XANES
But the cold bonds they still wind up broke

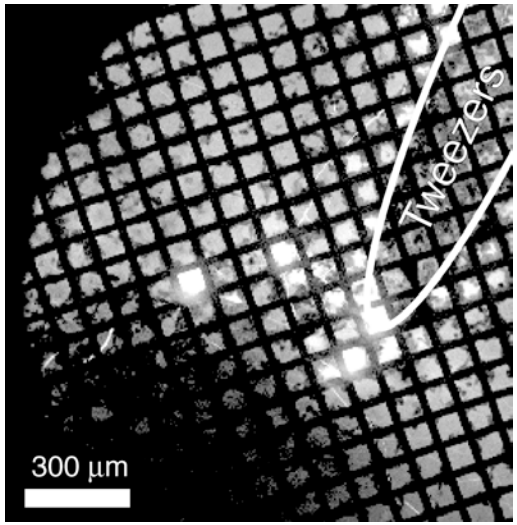
Frozen hydrated specimens

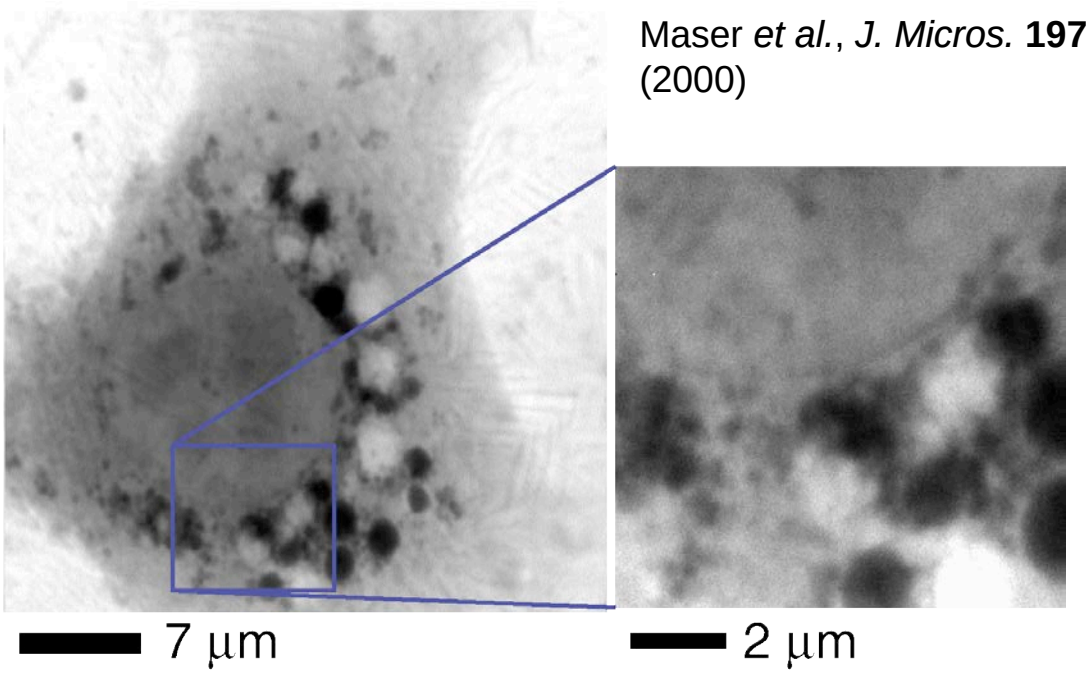
Grids with live cells are

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

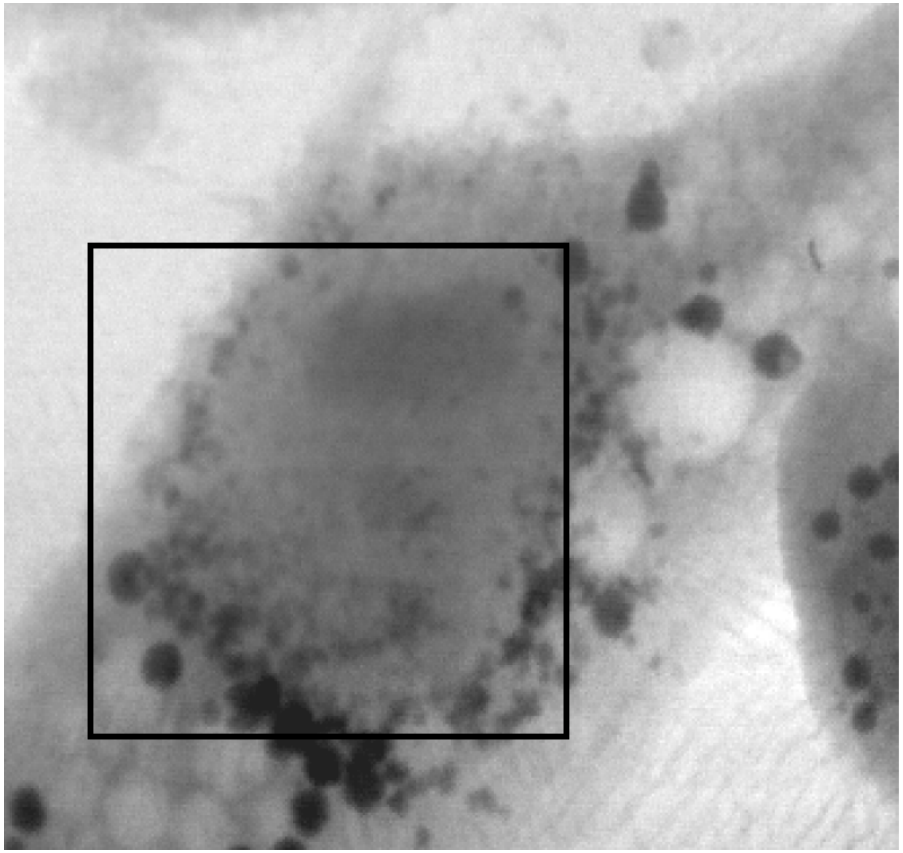


Maser *et al.*, *J. Micros.* **197**, 68 (2000)

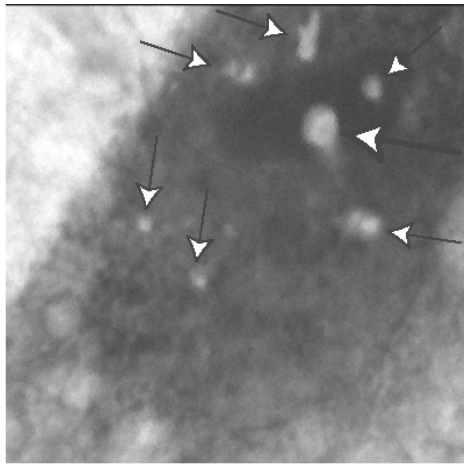




Radiation damage resistance of wet specimens at liquid nitrogen temperature



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



7 μm

Frozen hydrated image after exposing several regions to ~10^{10} Gray

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

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

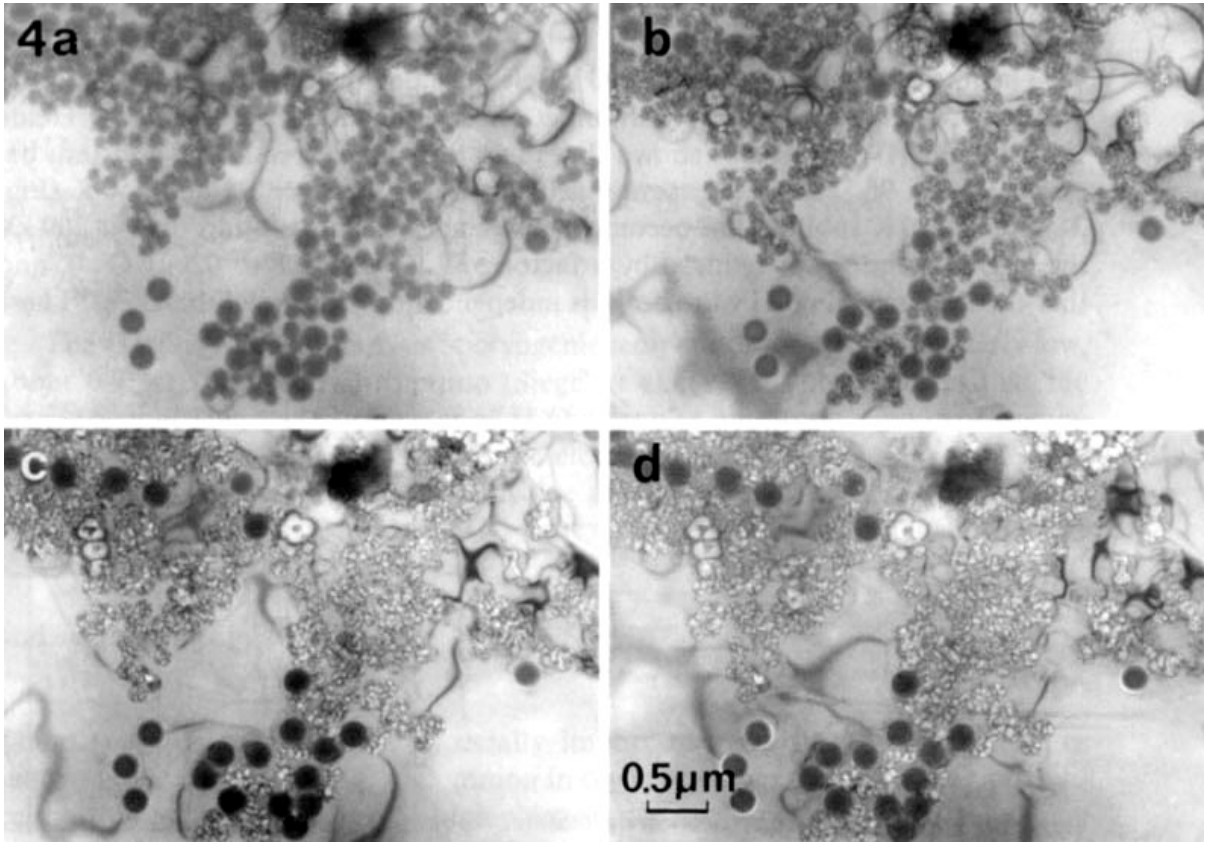
Electrons: frozen hydrated

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)

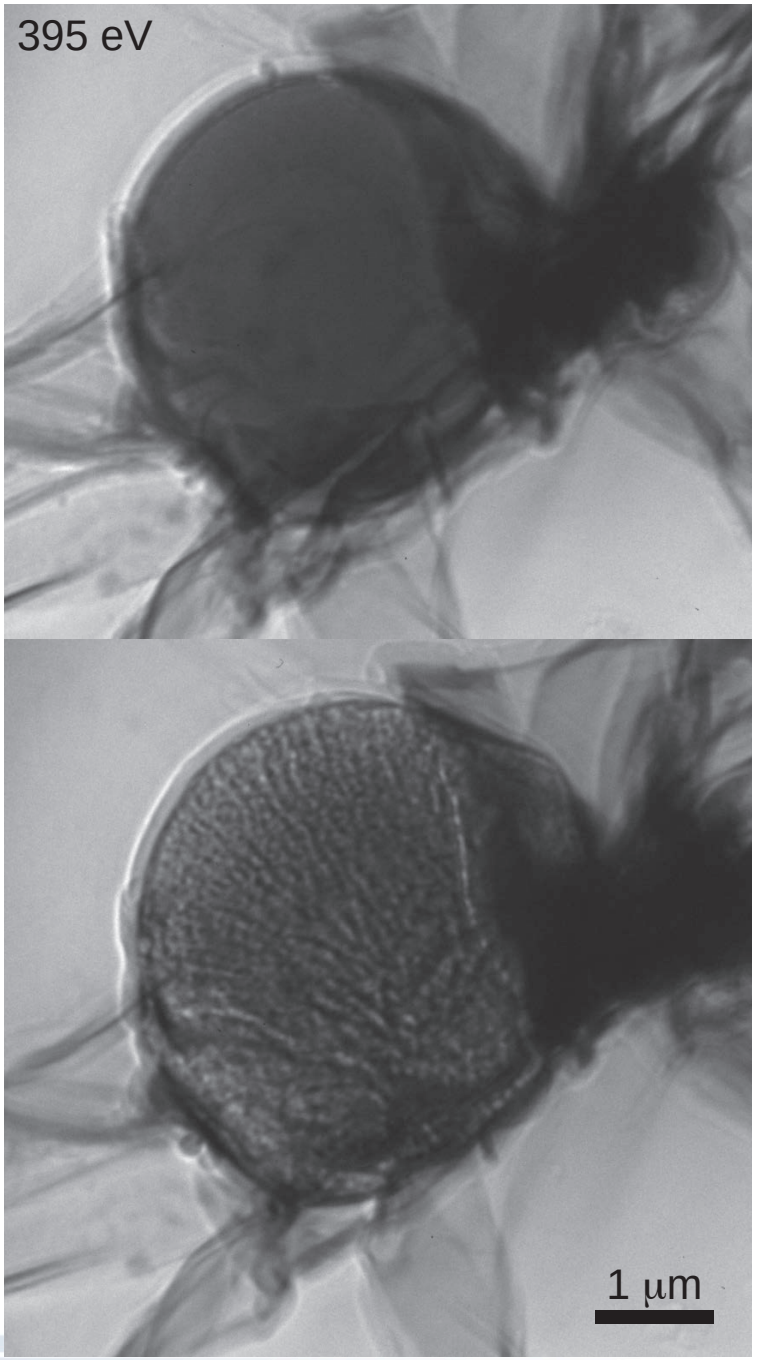


"Bubbling" dose in cryo electron microscopy: ~1000 e-/nm^2 or about 3x10^7 Gray. Bubbles: hydrogen gas [Leapman, *Ultramic.* **59**, 71 (1995); Sun *et al.*, *J. Mic.* **177**, 18 (1995)]

Dose to yeast at the nitrogen edge

~10^7 Gray

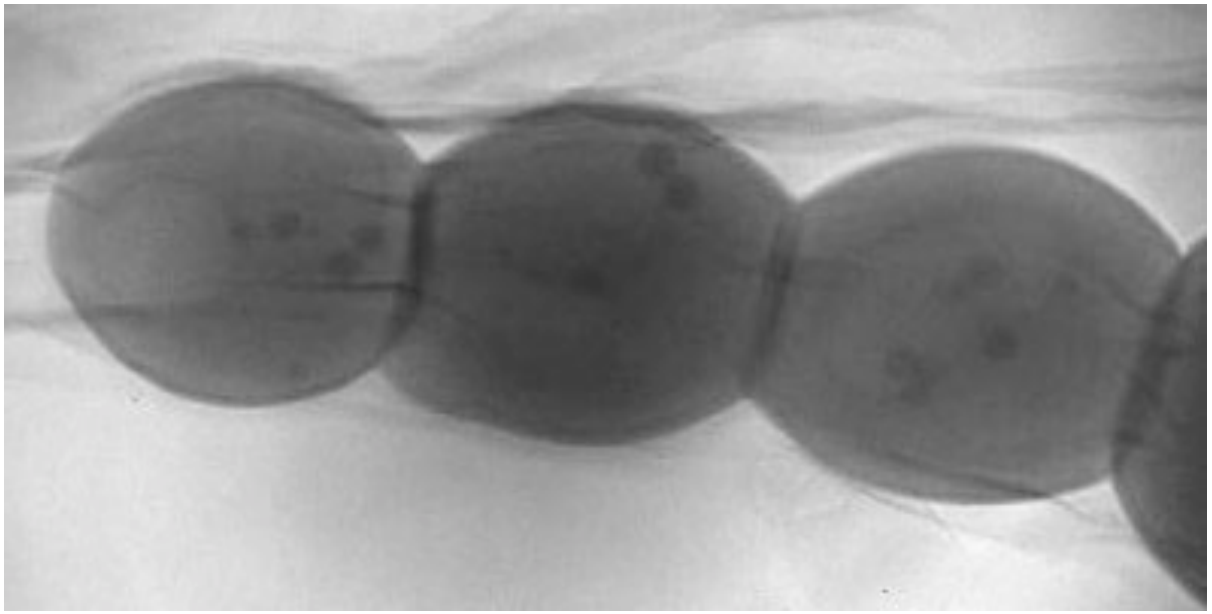
~10^9 Gray



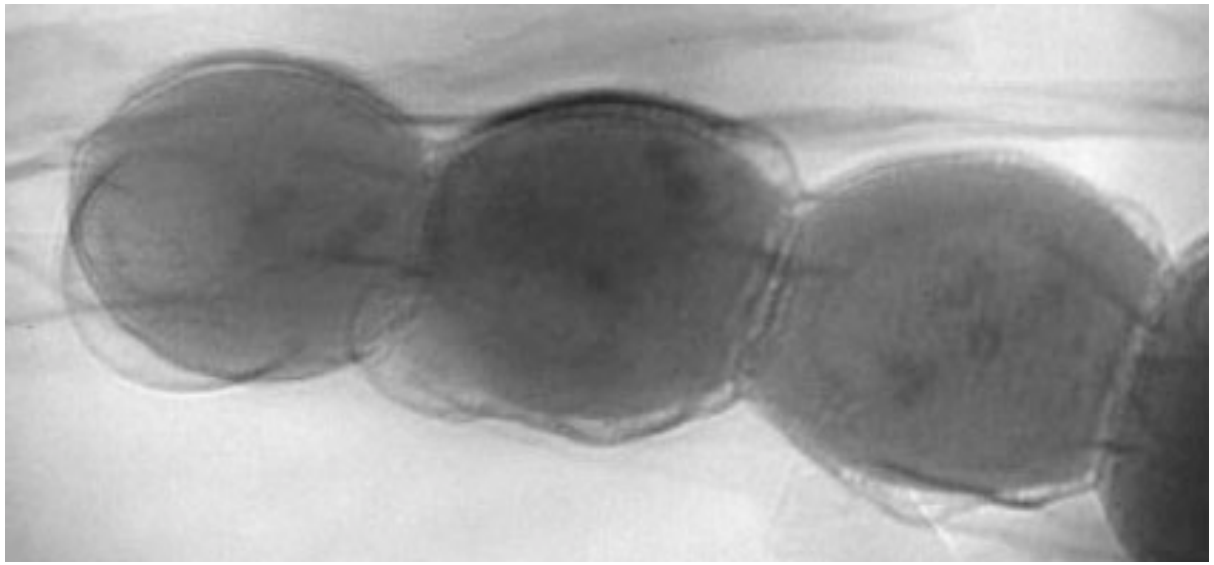
BESSY II TXM
Nelson, Holzner, Steinbrener, Jacobsen (Stony Brook)
Schneider, Heim, Guttmann (BESSY II)

Dose to yeast at the oxygen edge

~10^7 Gray

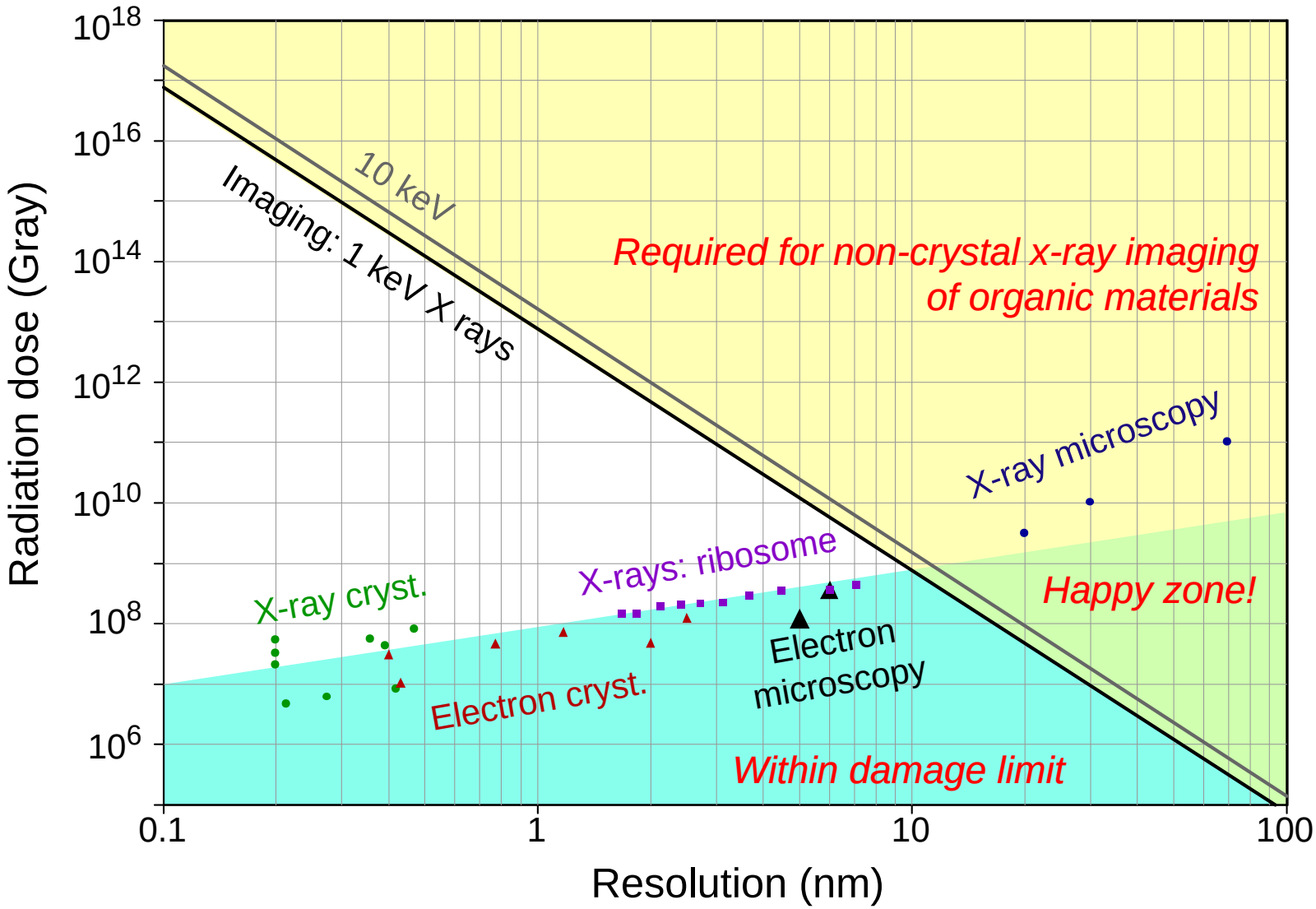


~10^9 Gray



BESSY II TXM
Nelson, Holzner, Steinbrener, Jacobsen (Stony Brook)
Schneider, Heim, Guttmann (BESSY II)

What's the limit for cells?



Howells *et al.*, *JESRP* 170, 4 (2009)

See also Shen *et al.*, *J. Sync. Rad.* **11**, 432 (2004)

J. Synchrotron Rad. (2014). **21**, 66–75

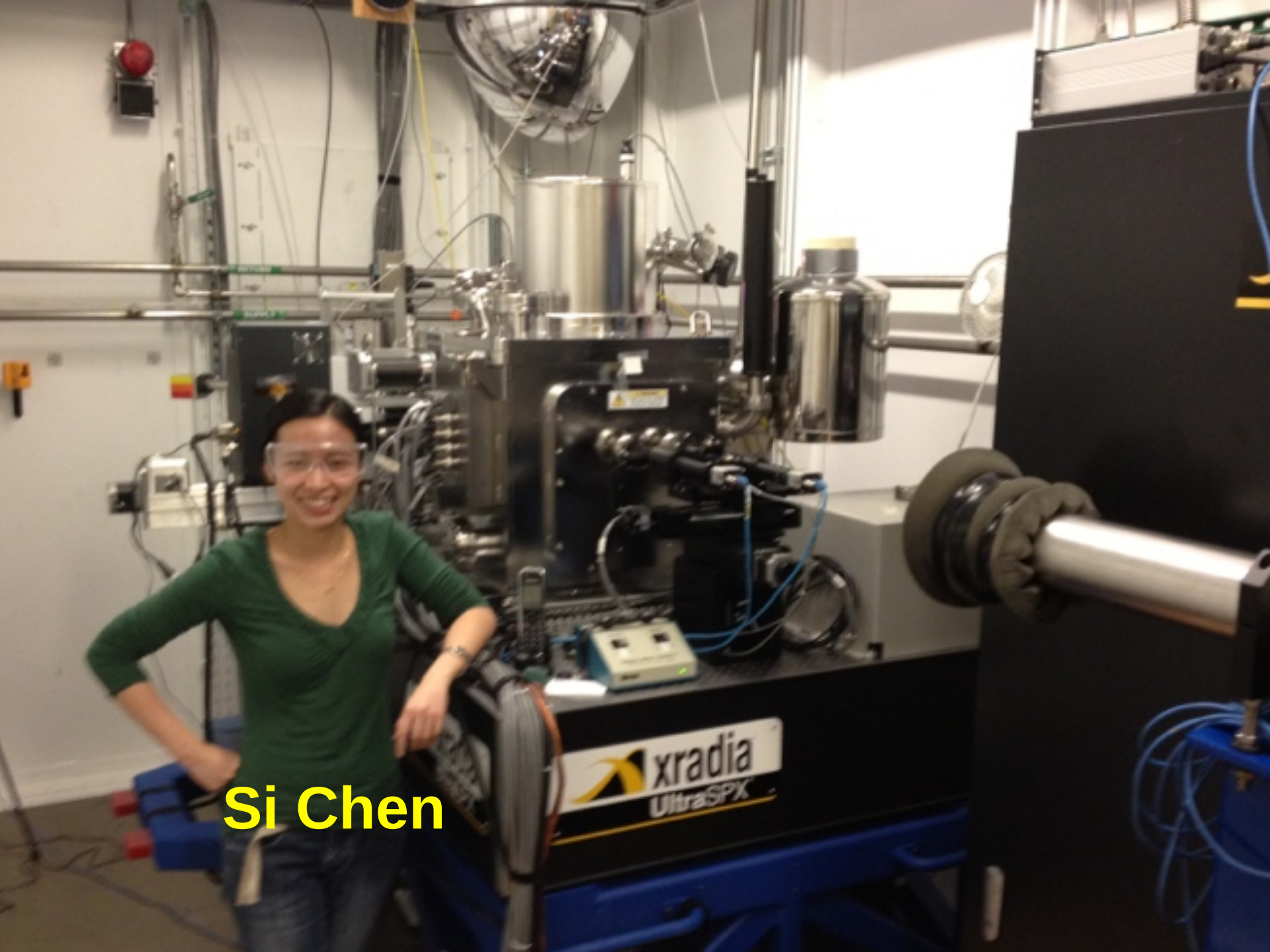
The Bionanoprobe: hard X-ray fluorescence nanoprobe with cryogenic capabilities

xradia is now ZEISS

S. Chen,[a*] J. Deng,[b] Y. Yuan,[c] C. Flachenecker,[d] R. Mak,[e] B. Hornberger,[d] Q. Jin,[e] D. Shu,[a] B. Lai,[a] J. Maser,[a] C. Roehrig,[a] T. Paunesku,[c] S. C. Gleber,[a] D. J. Vine,[a] L. Finney,[a] J. VonOsinski,[f] M. Bolbat,[f] I. Spink,[d] Z. Chen,[d] J. Steele,[d] D. Trapp,[d] J. Irwin,[d] M. Feser,[d] E. Snyder,[d] K. Brister,[f] C. Jacobsen,[a,b,e,g] G. Woloschak[c] and S. Vogt[a]

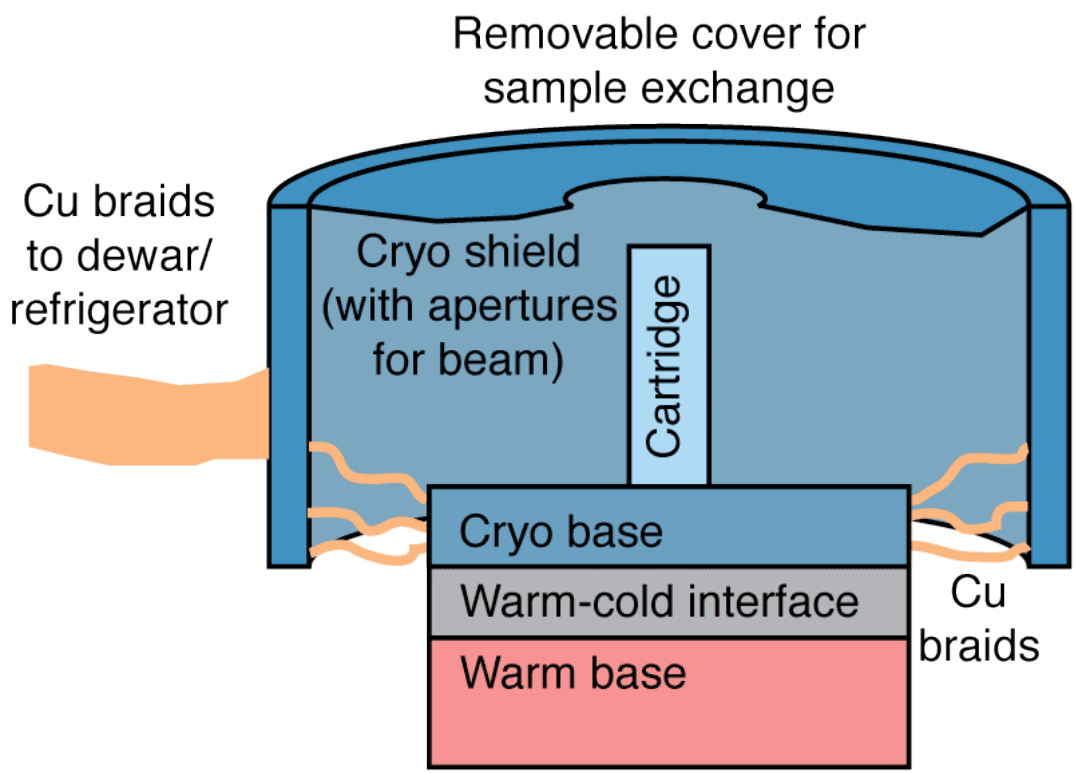
[a]X-ray Science Division, Advanced Photon Source, Argonne National Laboratory, Argonne, IL 60439, USA, [b]Applied Physics, Northwestern University, Evanston, IL 60208, USA, [c]Department of Radiation Oncology, Northwestern University, Chicago, IL 60611, USA, [d]Xradia Inc., Pleasanton, CA 94588, USA, [e]Department of Physics and Astronomy, Northwestern University, Evanston, IL 60208, USA, [f]Northwestern Synchrotron Research Center, Argonne, IL 60439, USA, and [g]Chemistry of Life Processes Institute, Northwestern University, Evanston, IL 60208, USA.
*E-mail: sichen@aps.anl.gov

Funded by NIH Grant to Gayle Woloschak *et al.*
Argonne technical lead: Stefan Vogt


xradia
UltraSPX
Si Chen

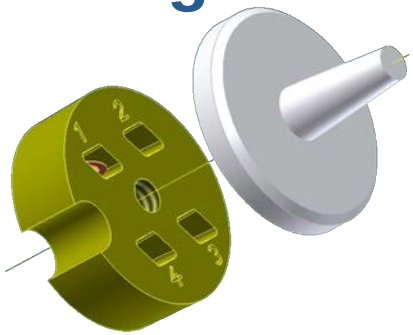
Xradia/Zeiss cryo sample stage scheme

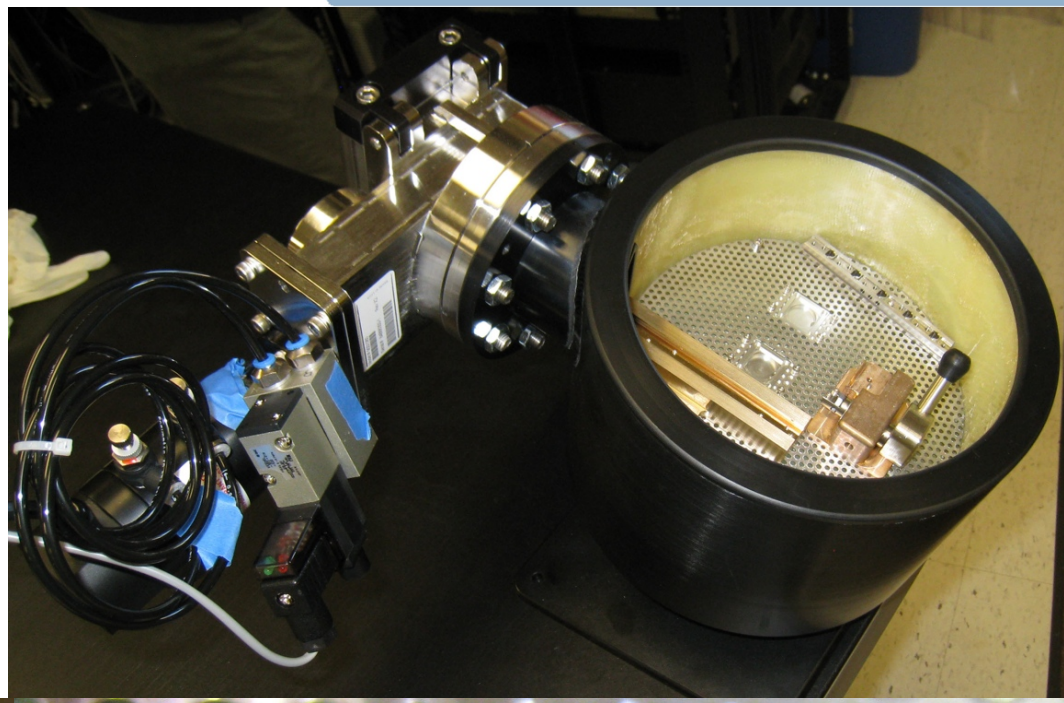
- “Strong” coupling of external dewar or closed-cycle refrigerator to cryo shield.
- “Weak” coupling of cryo shield to cold base and specimen-containing cartridge

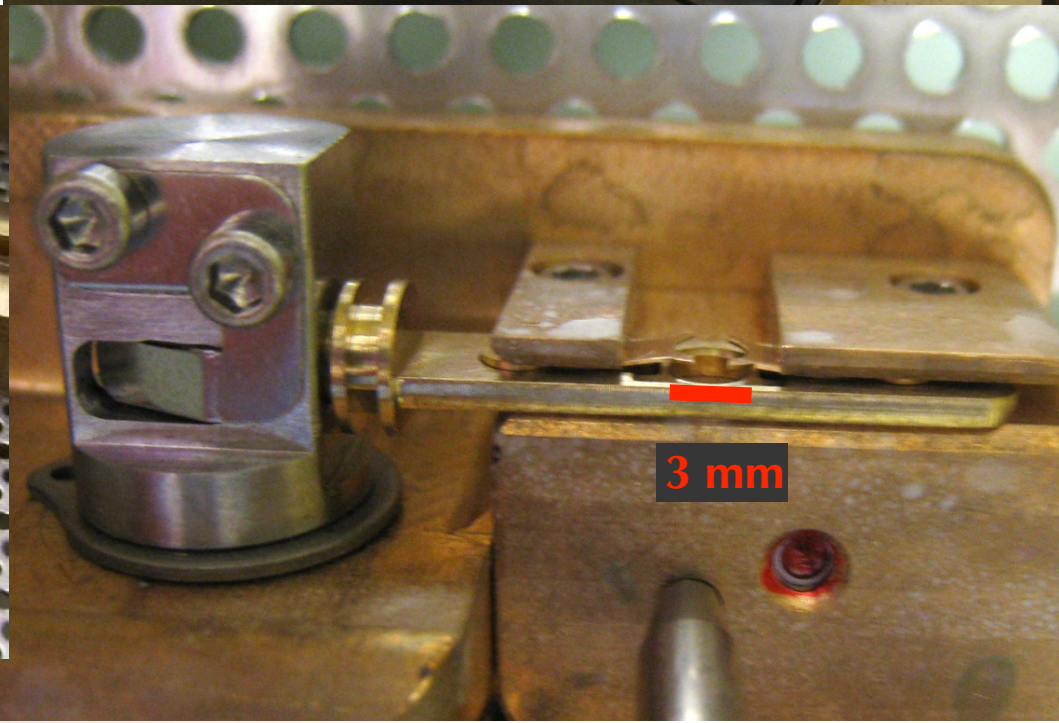


Approach developed by C. Jacobsen, D. Trapp, M. Howells, W. Yun, M. Feser at Xradia in 2007

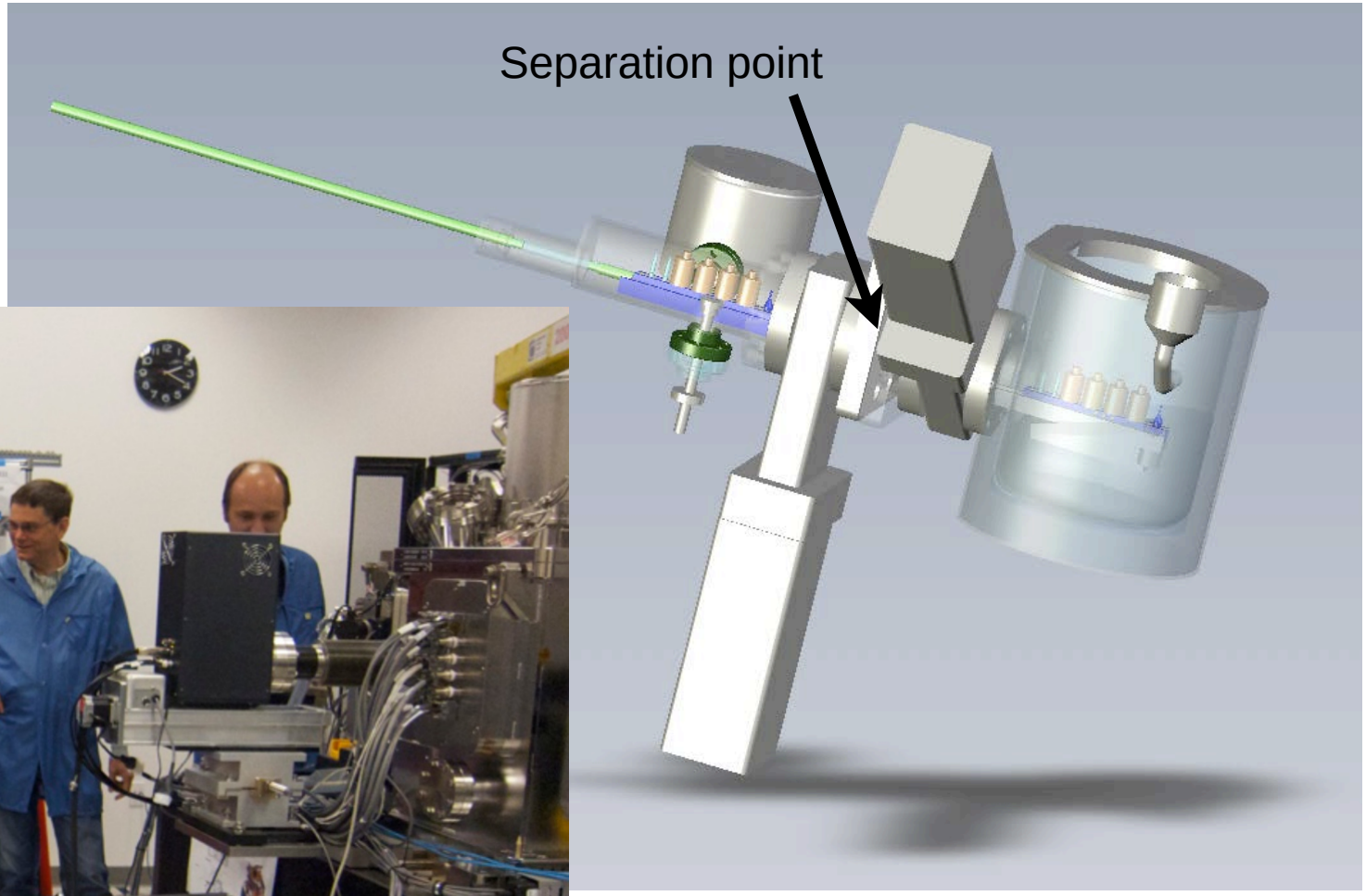
Sample loading

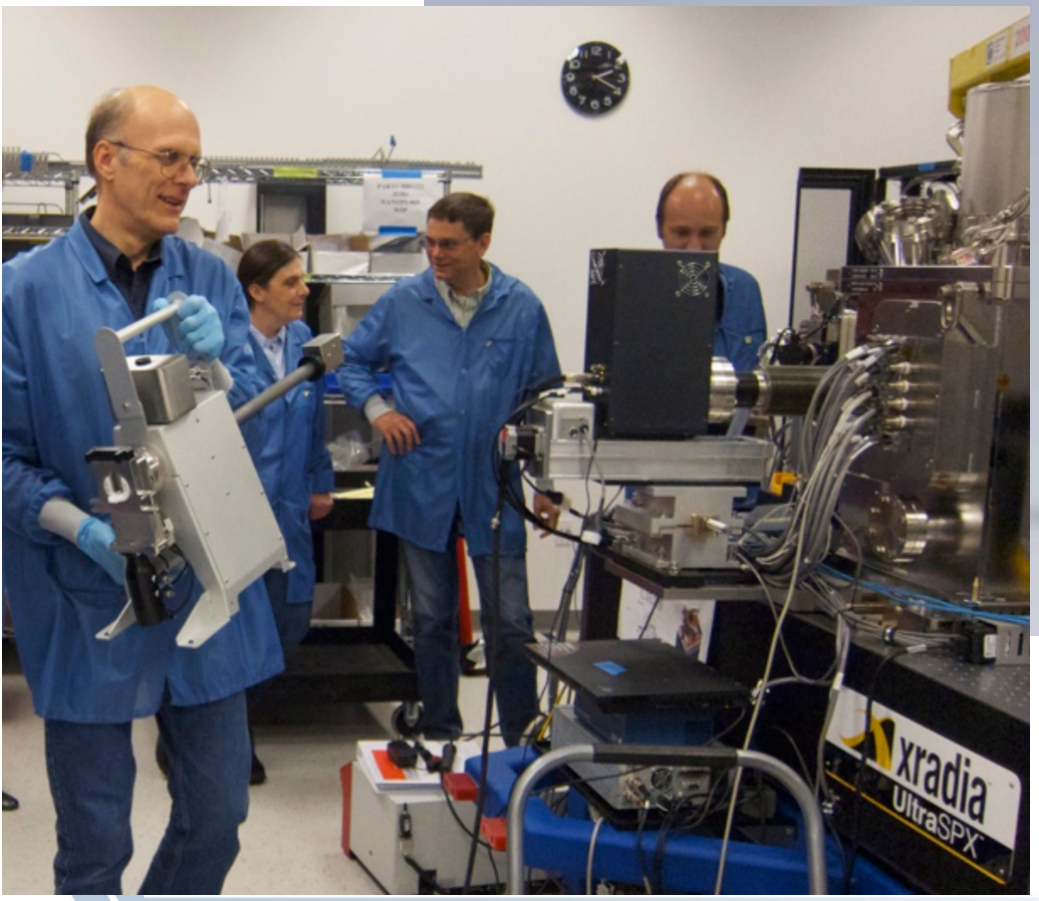


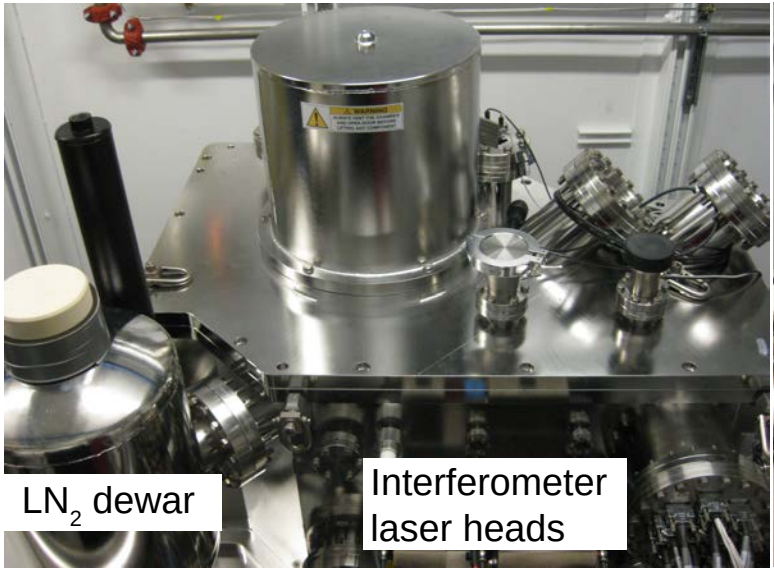


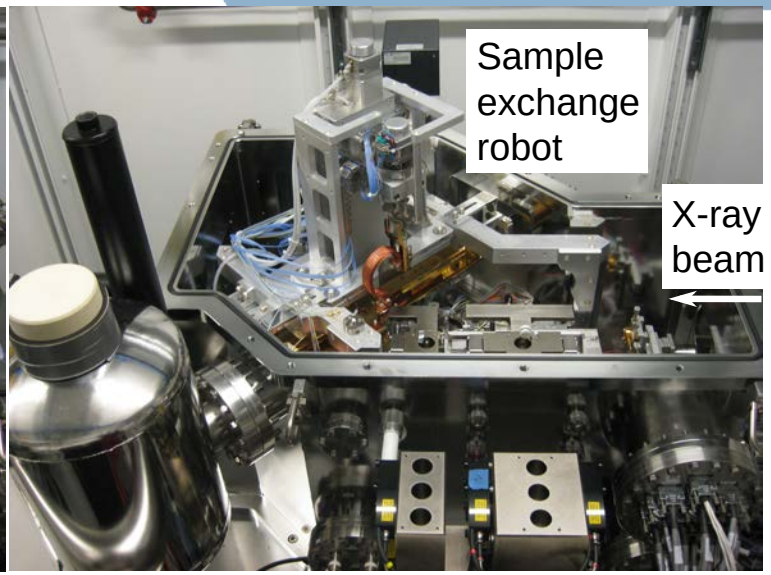


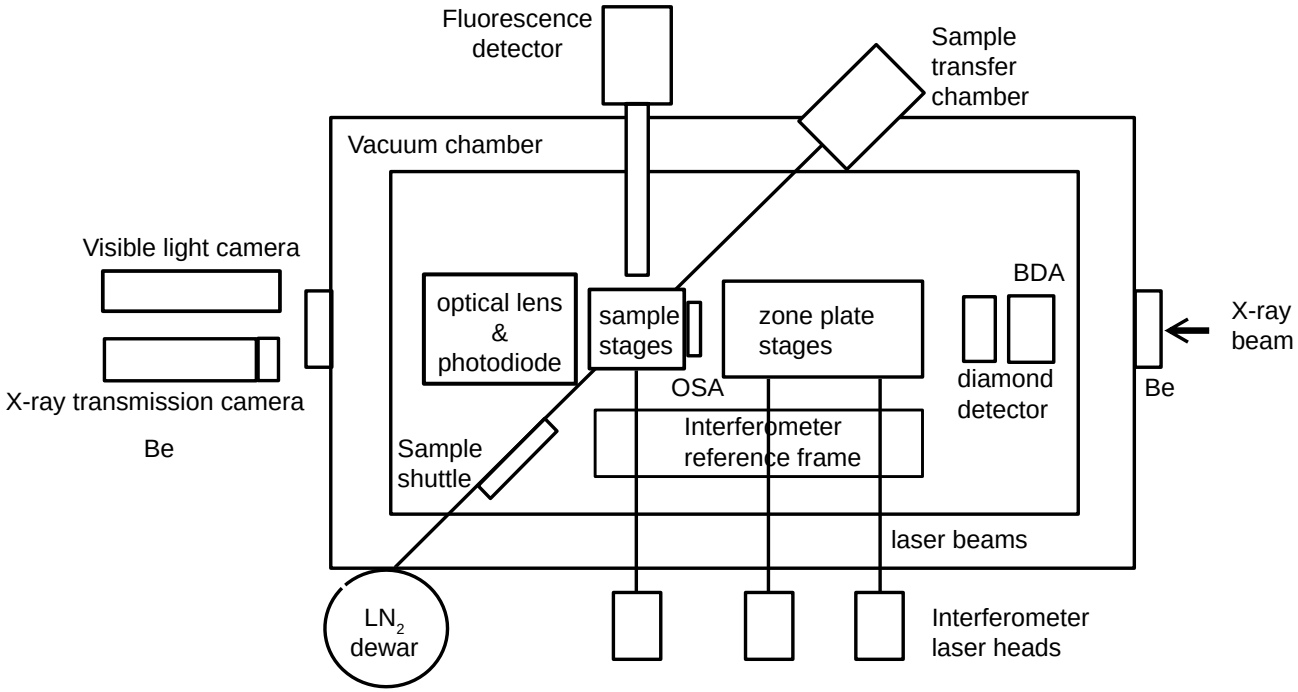
Transfer chamber

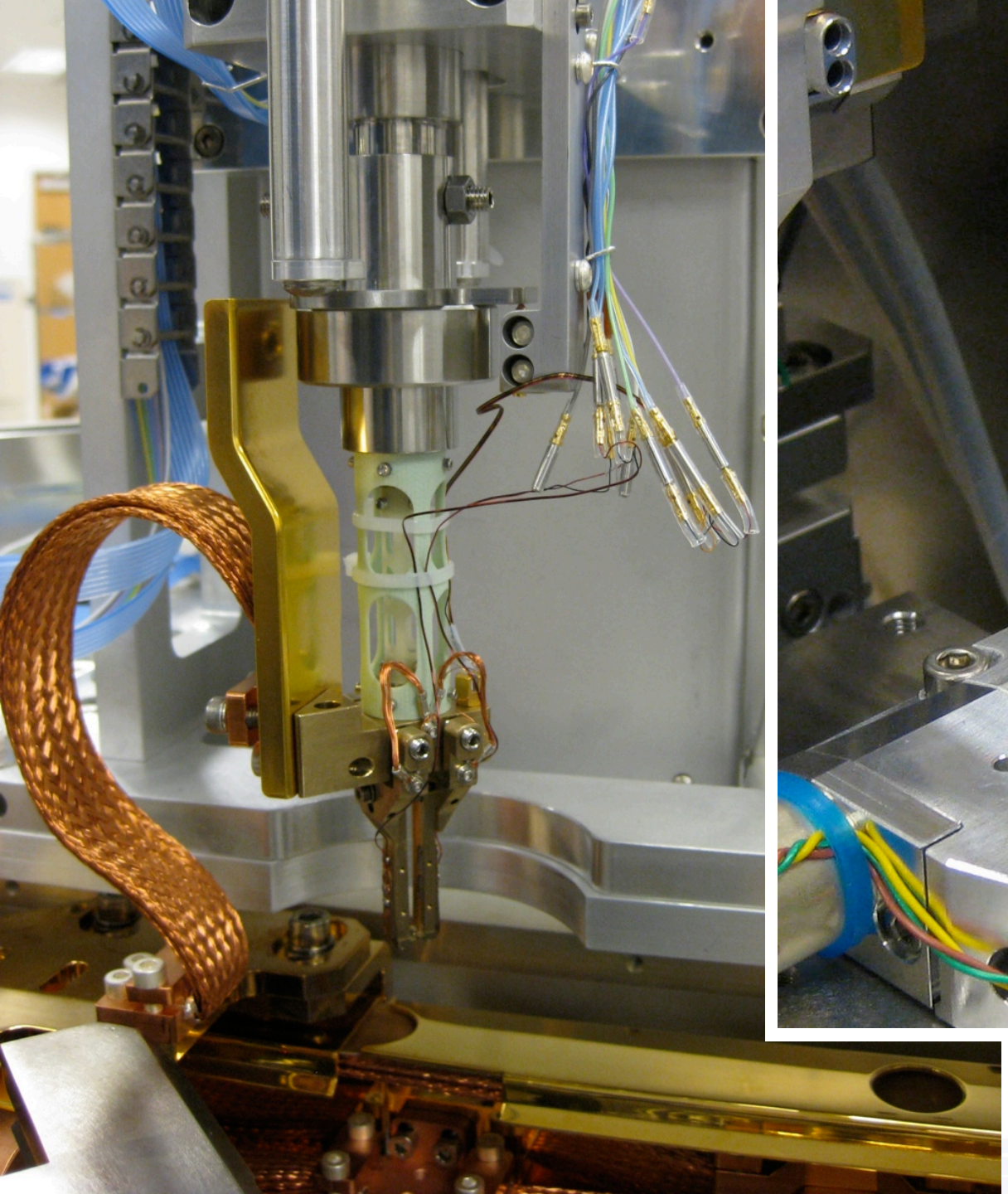


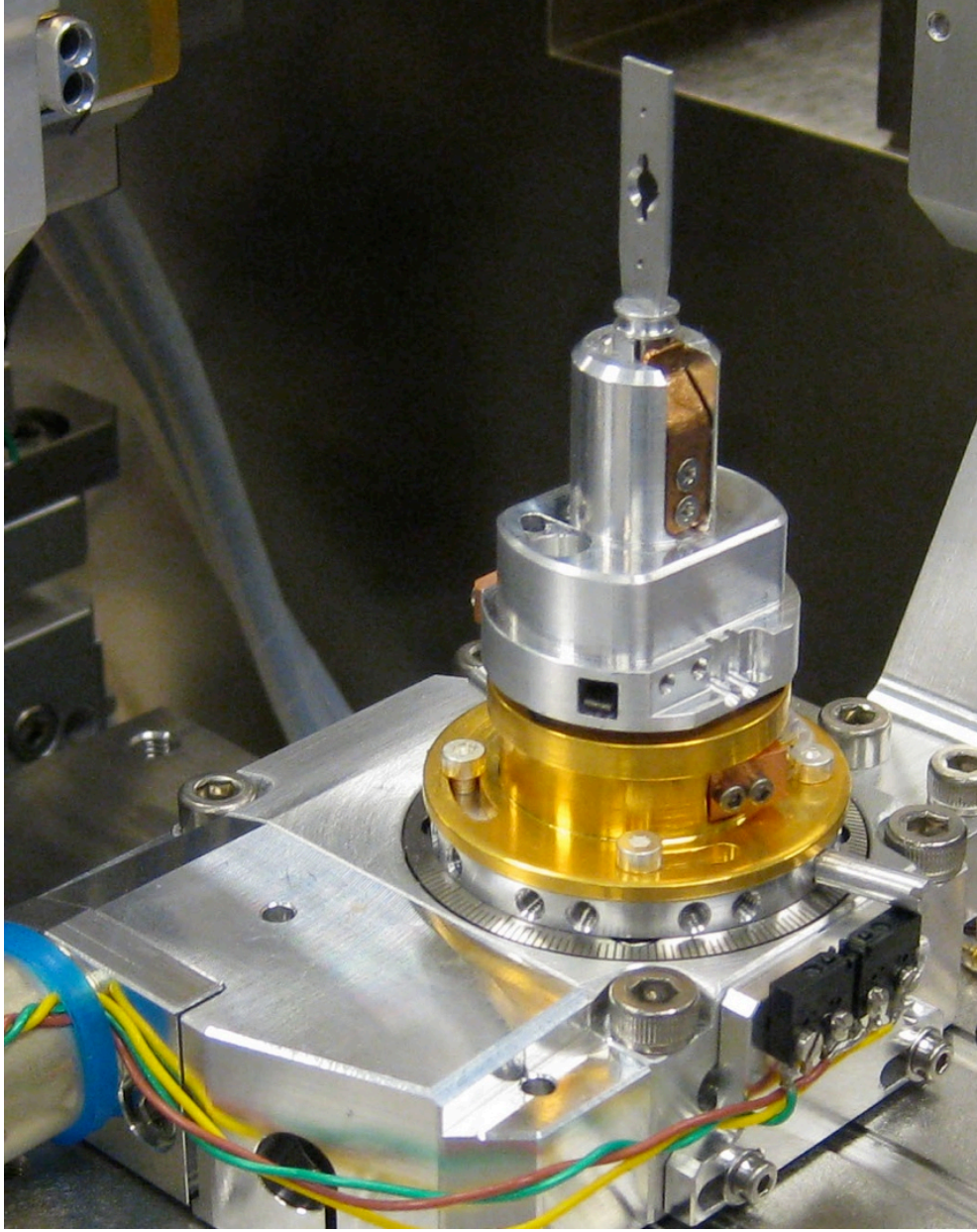



LN$_2$ dewar
Interferometer laser heads

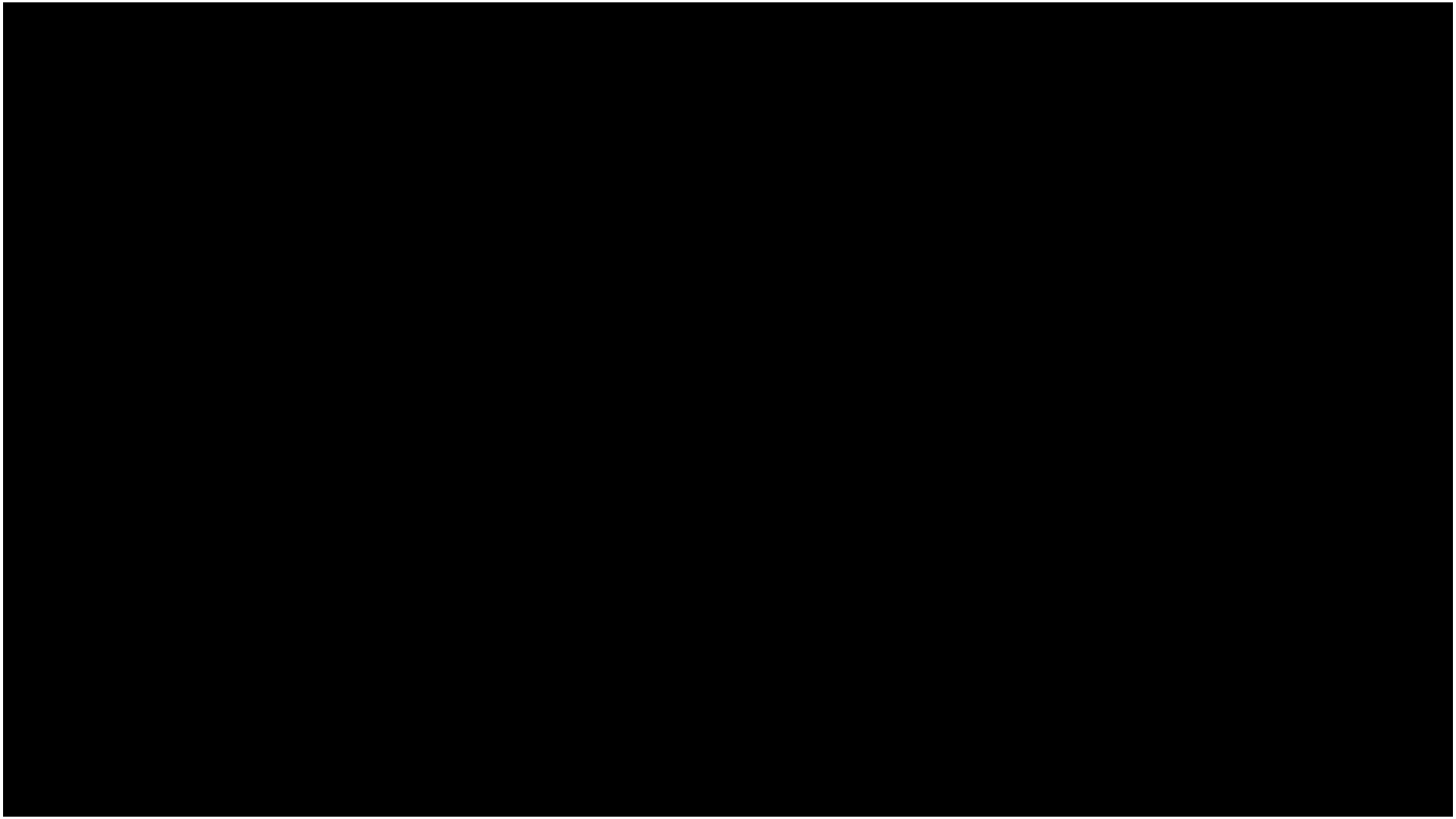

Sample exchange robot
X-ray beam


Fluorescence detector
Sample transfer chamber
Vacuum chamber
Visible light camera
X-ray transmission camera
Be
optical lens & photodiode
sample stages
zone plate stages
BDA
diamond detector
OSA
Be
X-ray beam
Sample shuttle
Interferometer reference frame
laser beams
LN$_2$ dewar
Interferometer laser heads





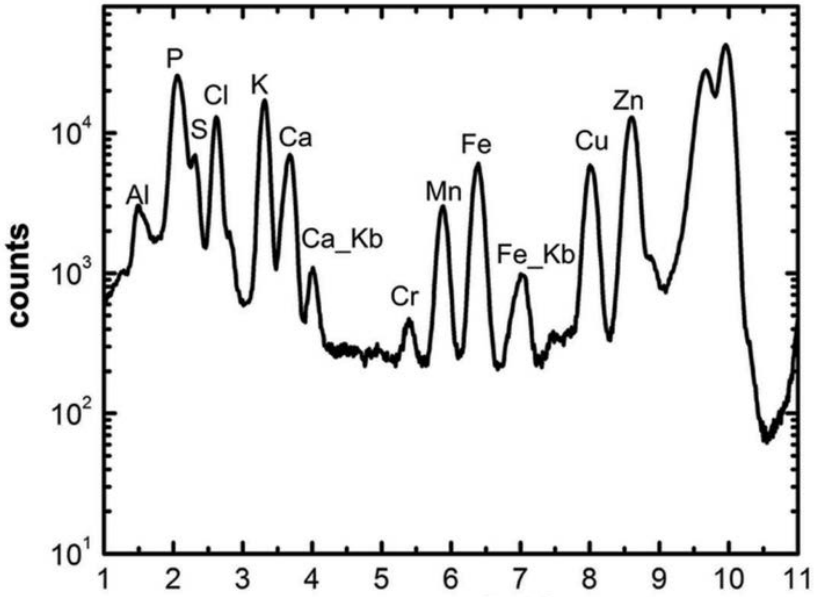
Cryo robot in action



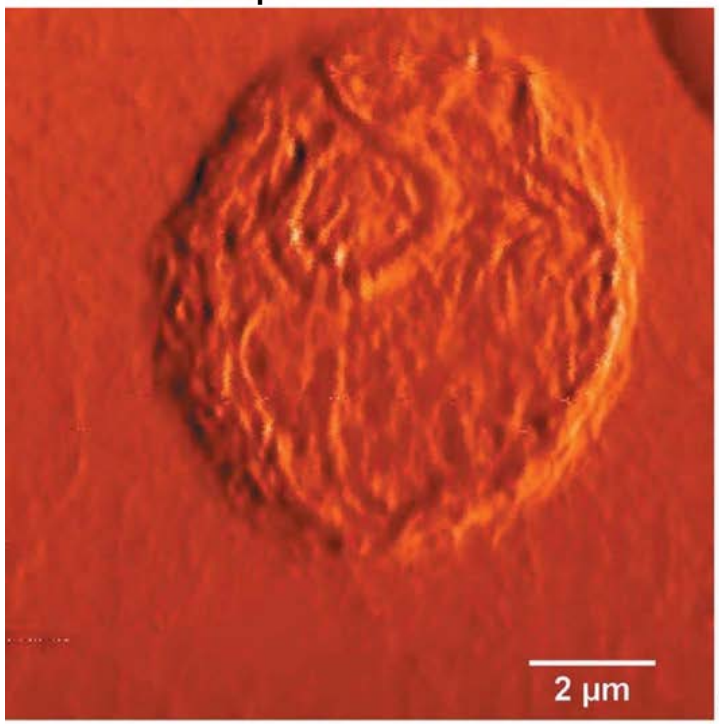
10x actual speed

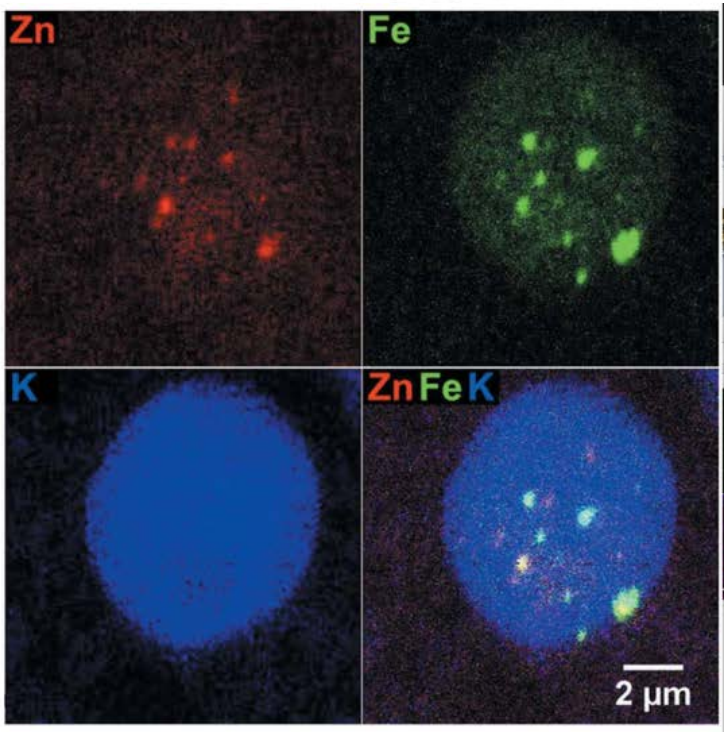
Silverman Hall
Northwestern University
H
Li
Na
Mg
K
Ca
Mn
Fe
Co
Cu
Zn
Ga
Mo
Tc
Ag
Pt
C
N
O
F
P
S
As
Gd

Chlamydonomas reinhardtii at 110 K

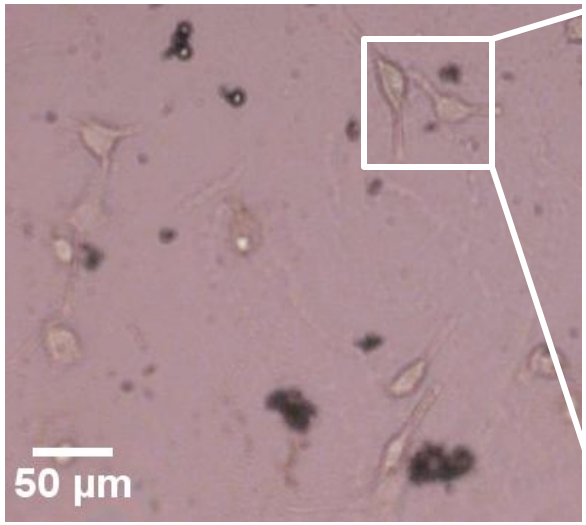


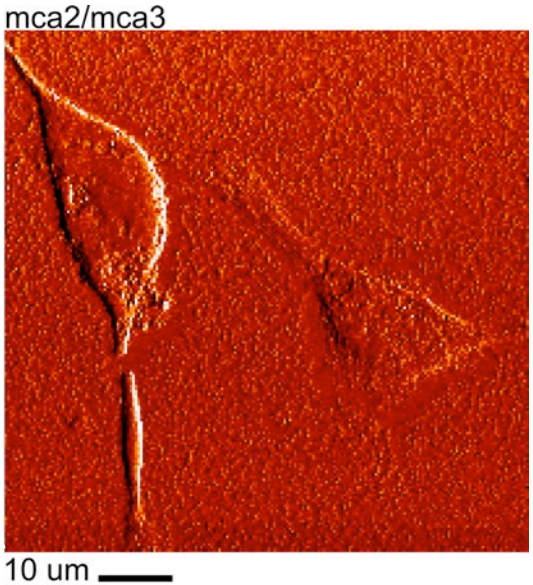
Differential phase contrast



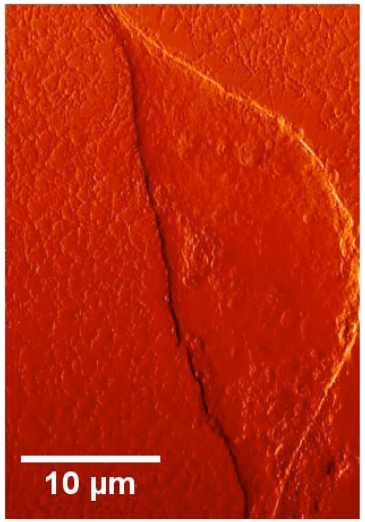


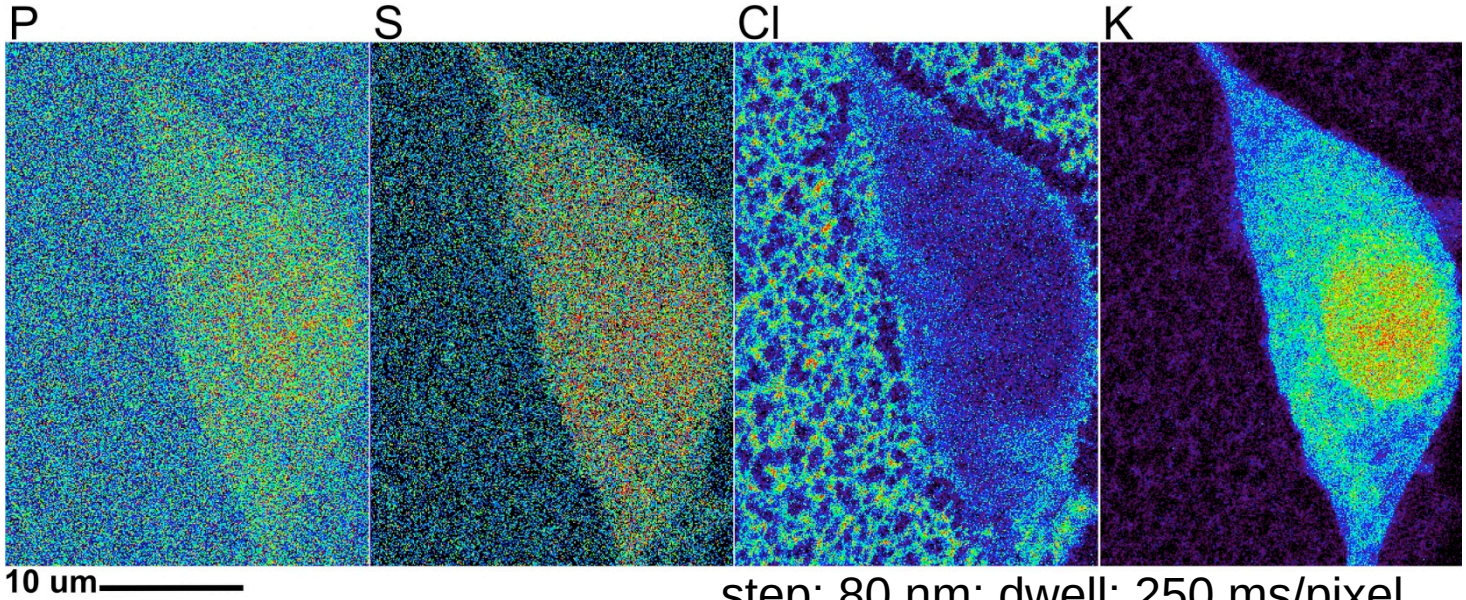
Mouse fibroblast: frozen hydrated





fly-scan mode
area: 70 µm x 70 µm
step: 0.35 µm
dwell time: 50 ms/pixel

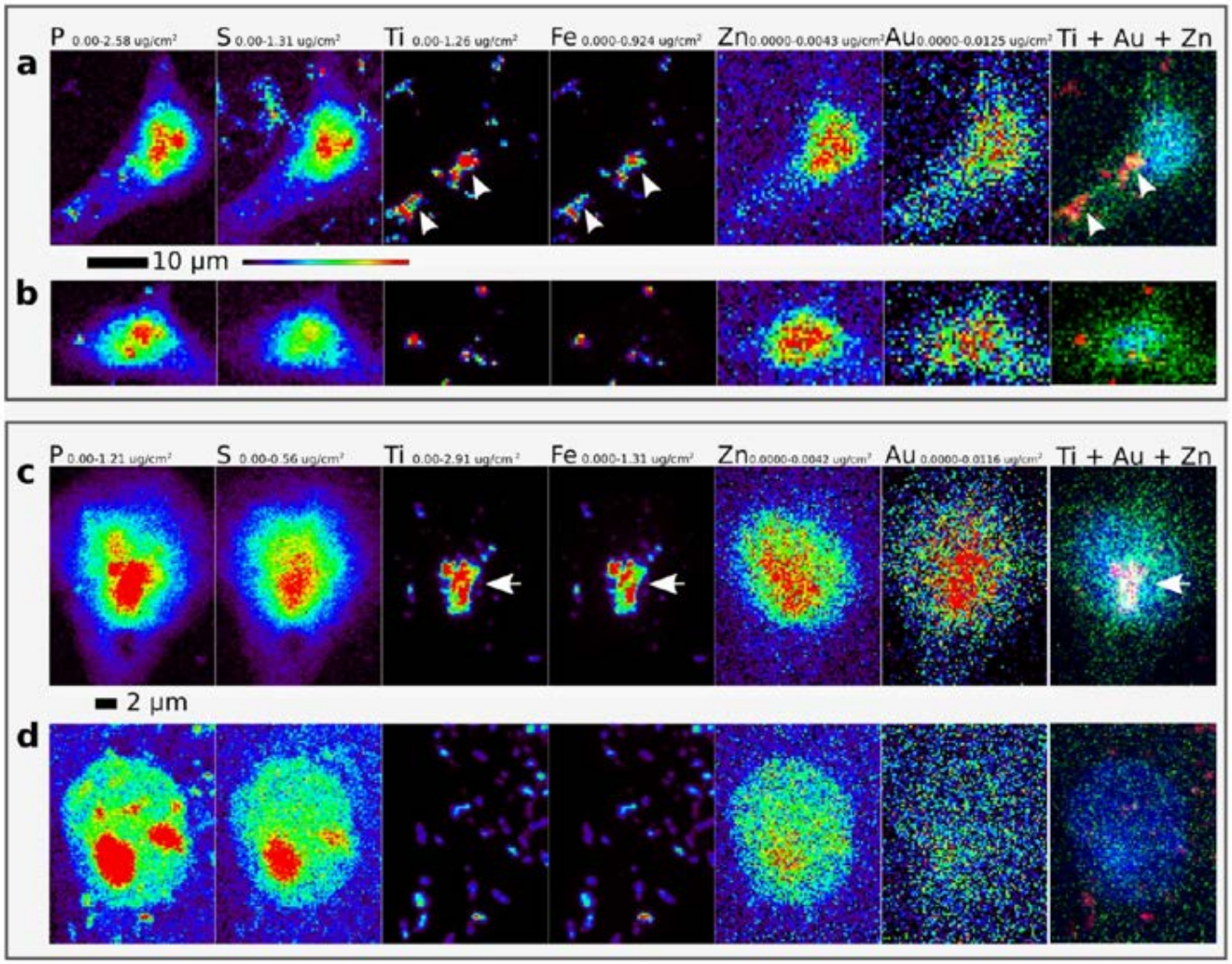




step: 80 nm; dwell: 250 ms/pixel

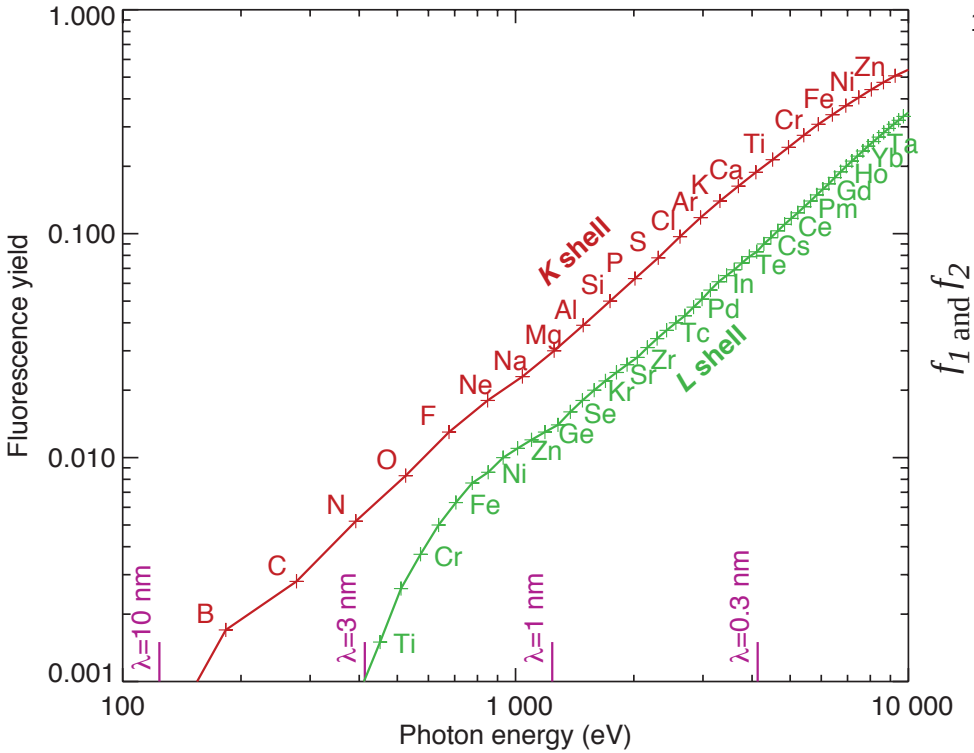
Targeted drug delivery using $Fe_3O_4@TiO_2$

- Yuan *et al.*, *ACS Nano* **7**, 10502 (2013); Woloschak lab +Argonne/NU
- Nanoparticles targeted to epidermal growth factor receptor (EGFR) for photostimulated cancer therapy
- First bionanoprobe tomography shows nanoparticles reaching nucleus

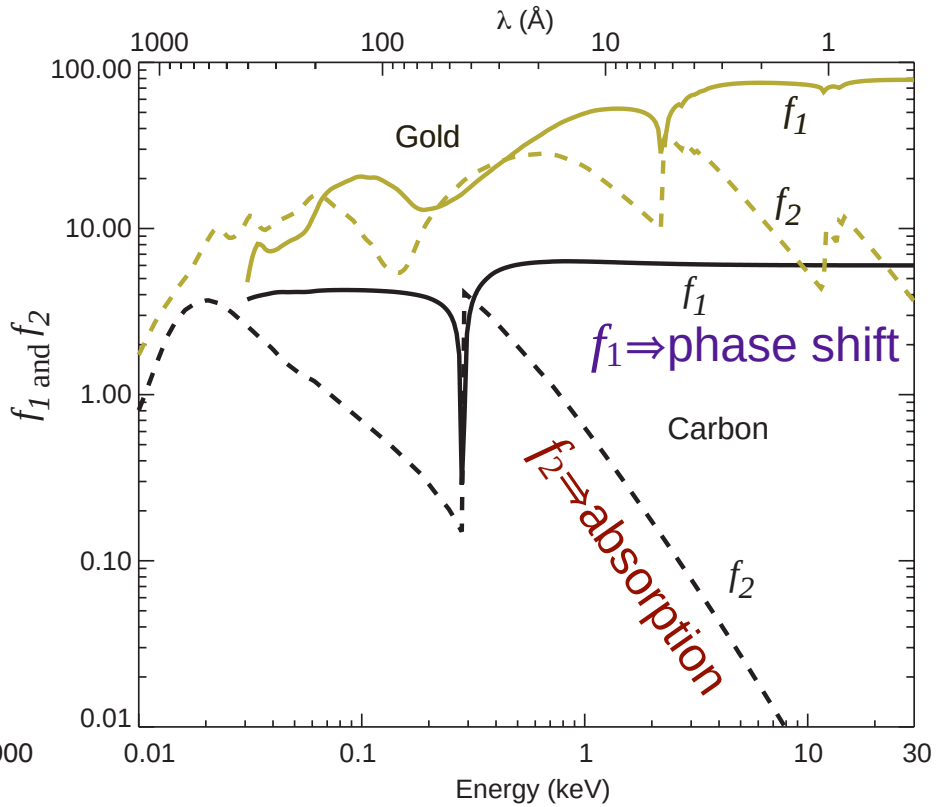


How to see the soft stuff?

Fluorescence is weak for light materials



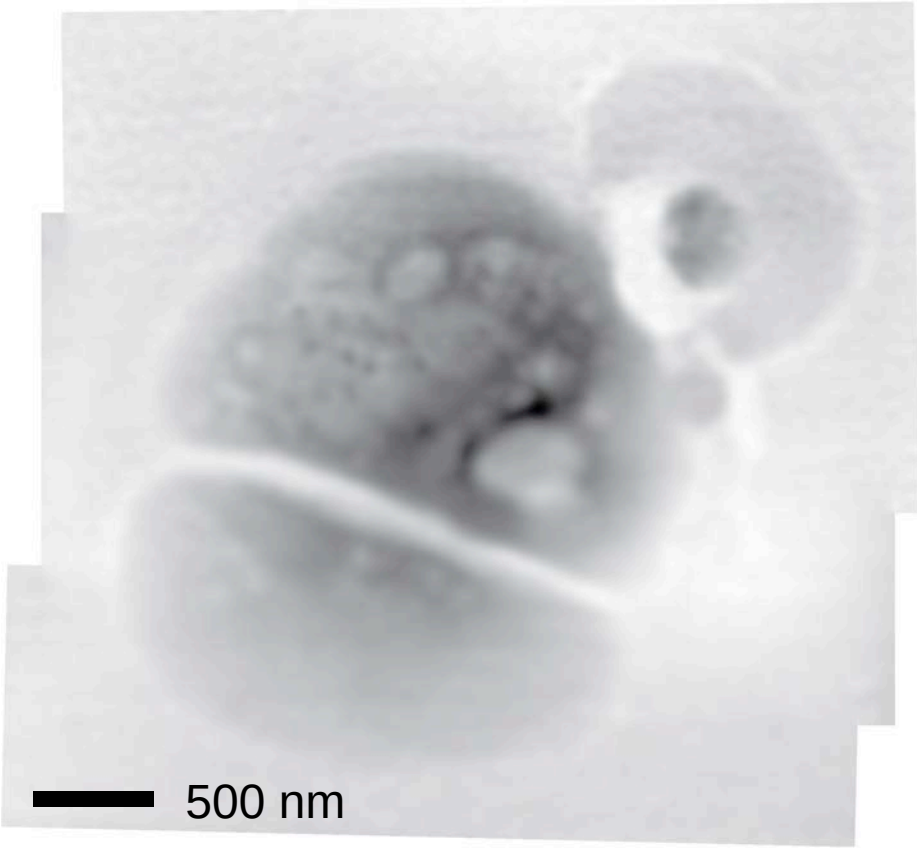
Phase contrast is much stronger than absorption

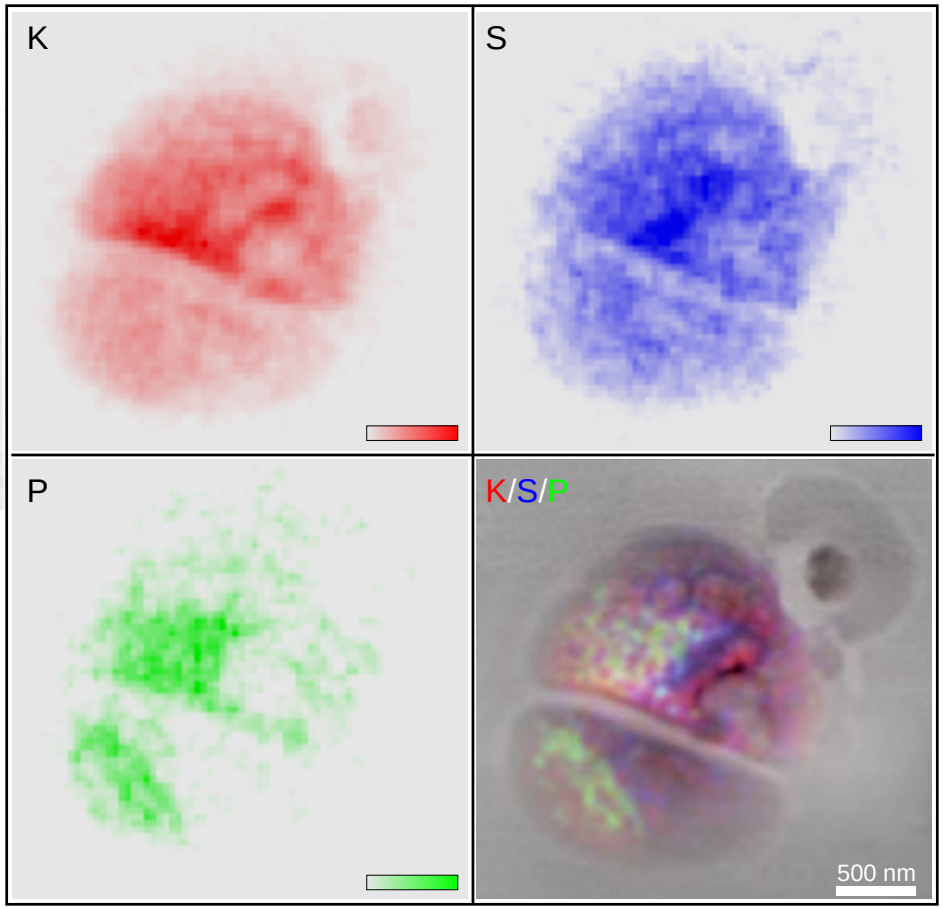


Refractive index $$n = 1 - \frac{n_a r_e}{2\pi}\lambda^2 (f_1 + i f_2)$$

Junjing Deng

Ptychography and fluorescence: structure, and chemistry



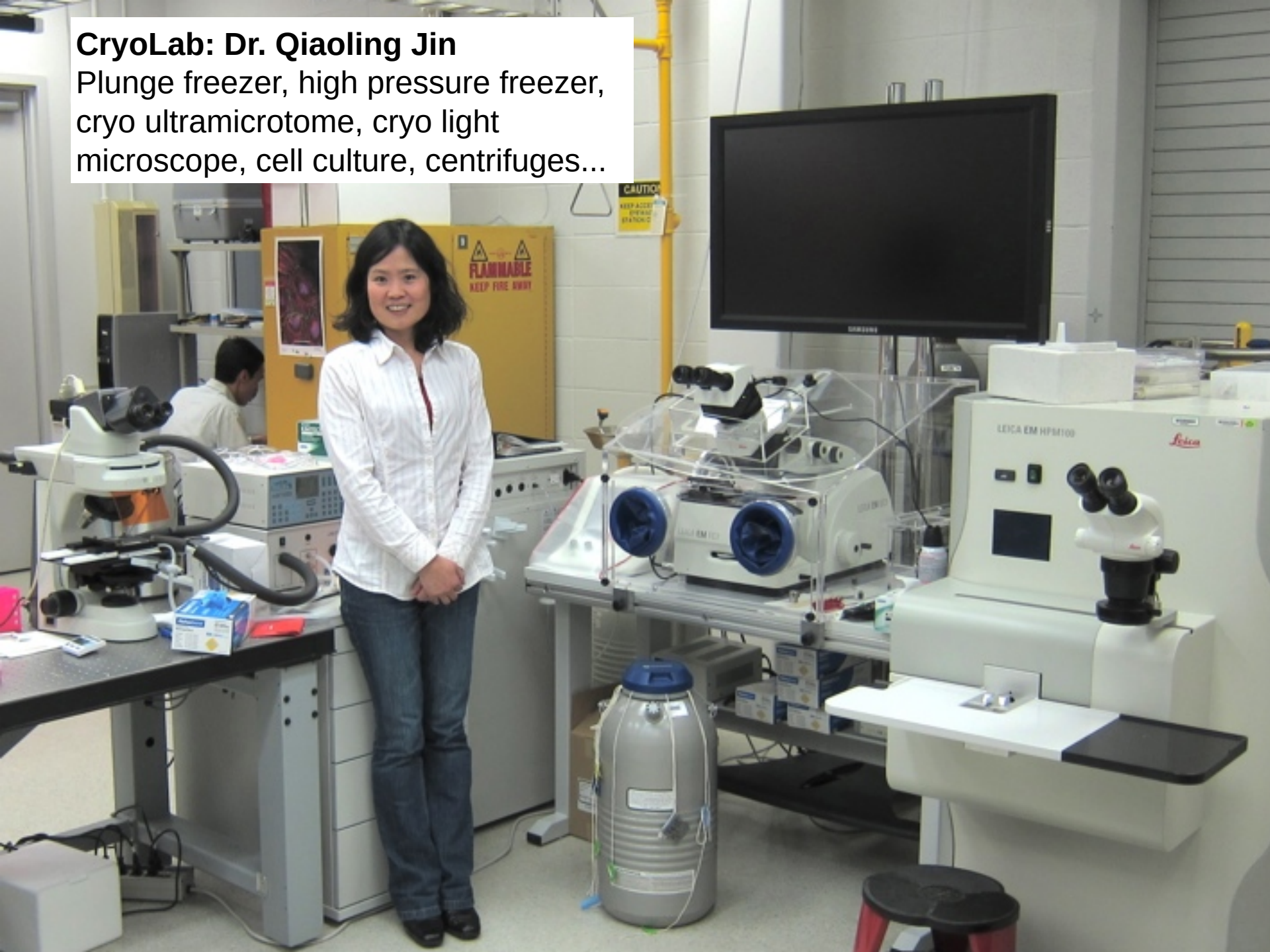


Marine algae: *Ostreococcus* sp., class *Prasinophycae*, common name chlorophyta. Imaged frozen hydrated, at 5 keV photon energy, with 12 nm reconstruction pixel size

First combined cryo high spatial resolution ptychographic image simultaneous with elemental maps from x-ray fluorescence. Junjing Deng, David Vine, Youssef Nashed, Tom Peterka, Si ChenQiaoling Jin, Chris Jacobsen. Argonne Lab/Northwestern University. BioNanoprobe, LS-CAT beamline.

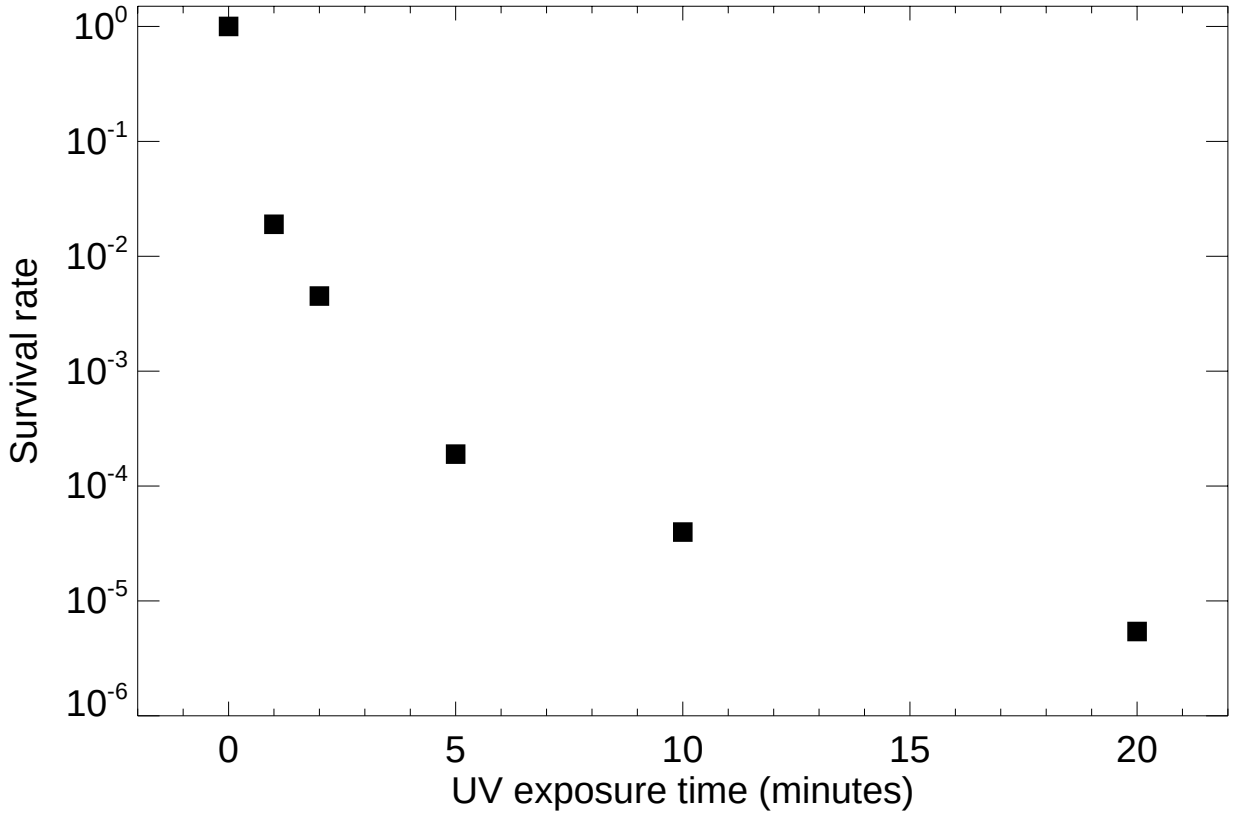
CryoLab: Dr. Qiaoling Jin

Plunge freezer, high pressure freezer, cryo ultramicrotome, cryo light microscope, cell culture, centrifuges...

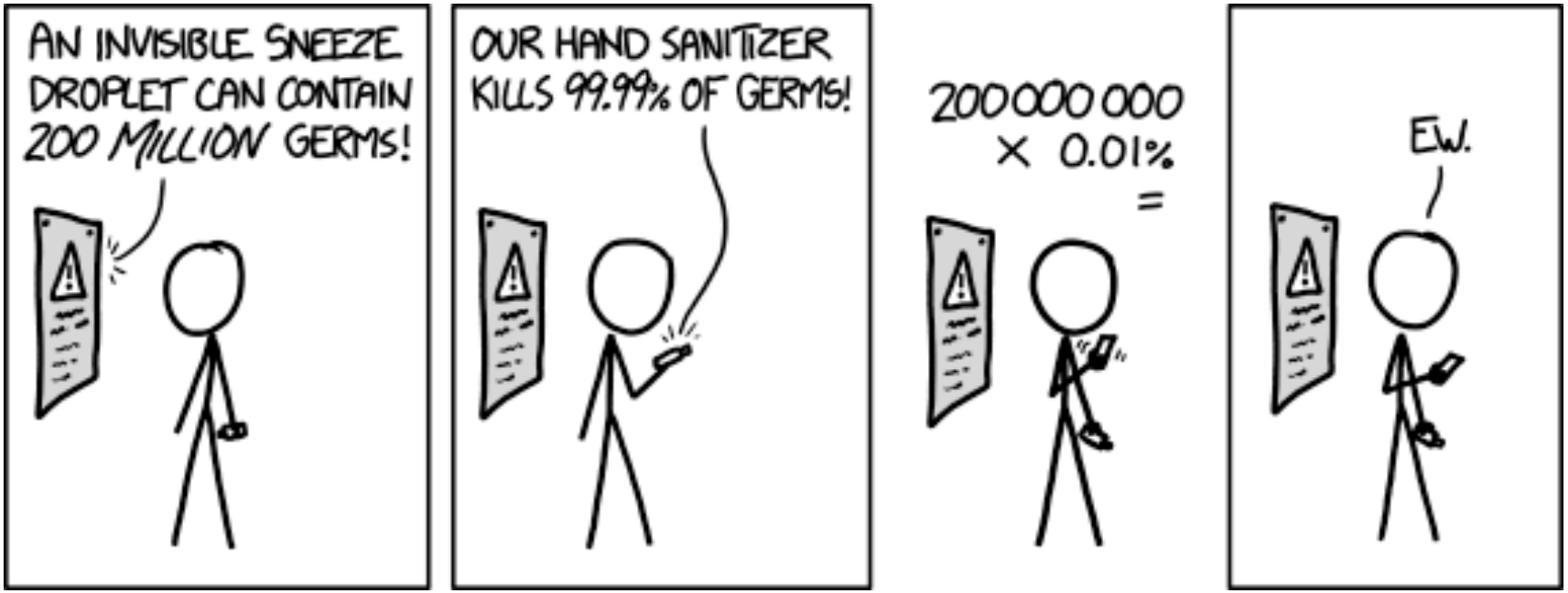


Making Biosafety level 2 samples safe to work with

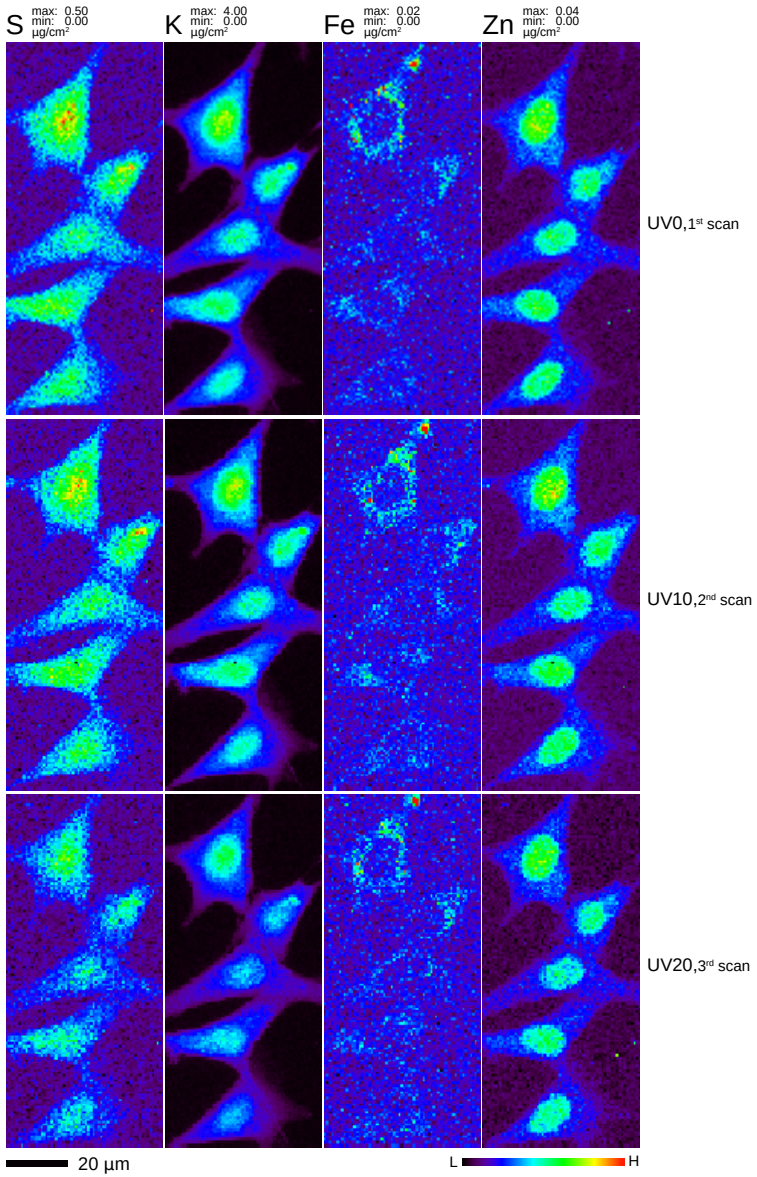
- Ultraviolet germicidal irradiation of frozen hydrated samples under LN_2
- Qiaoling Jin *et al.*, in preparation
- Survival rate of T7 bacteriophage under LN_2



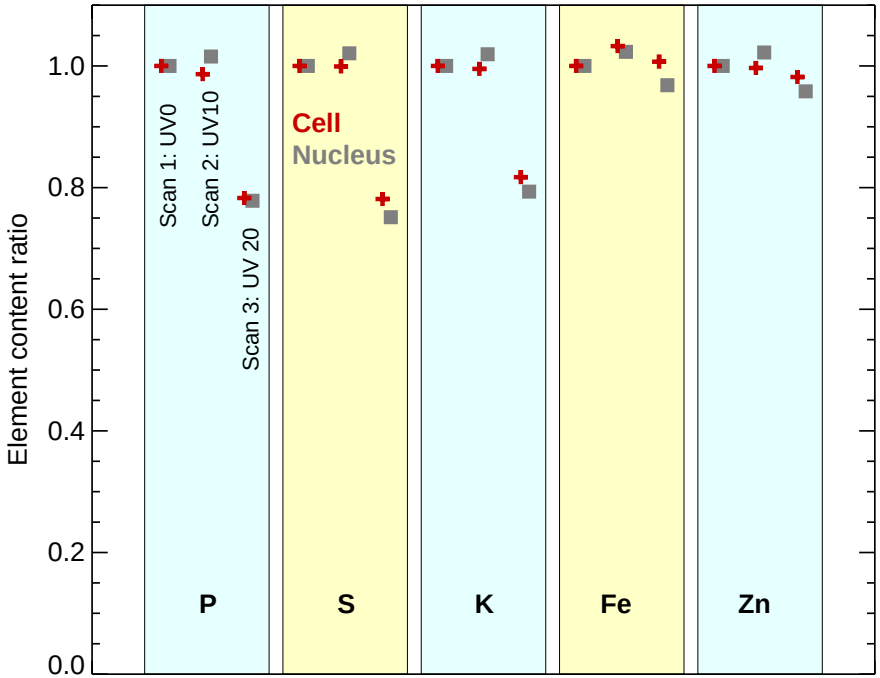
http://www.xkcd.com/1161/



UV sterilzation does not affect metal content



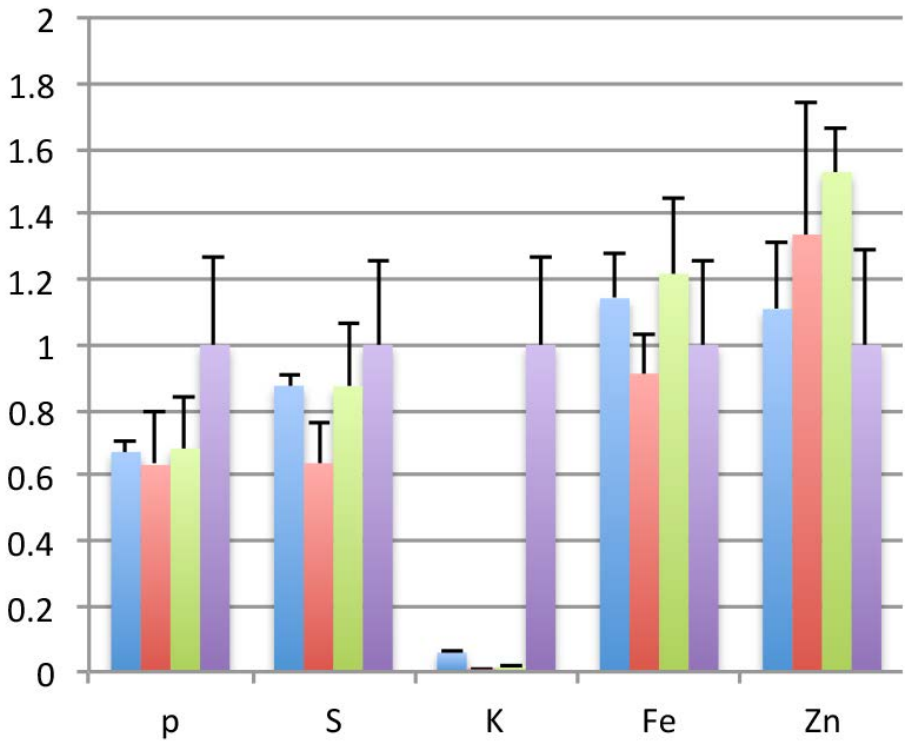
Separate experiment:

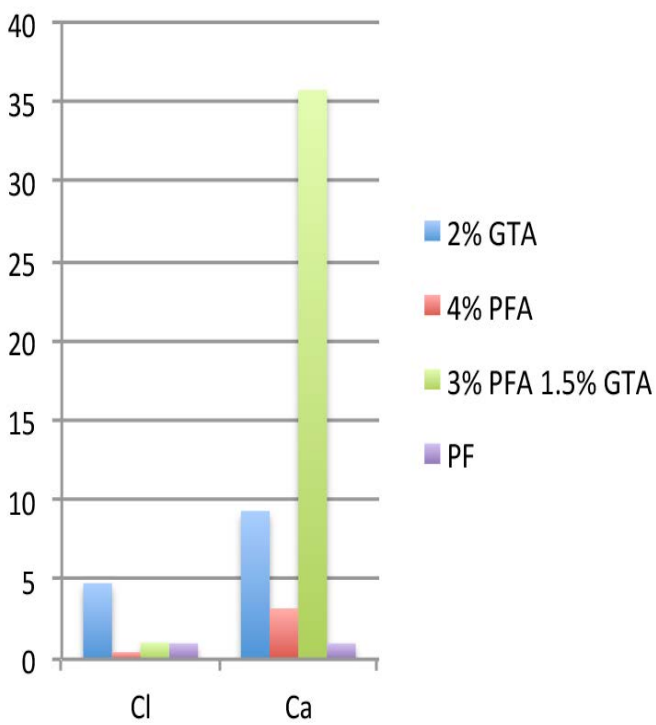


800 nm pixel size, cryo jet/2-ID-D nanoprobe (Q. Jin, B. Lai *et al.*)

Cryo versus chemical fixation: elemental content

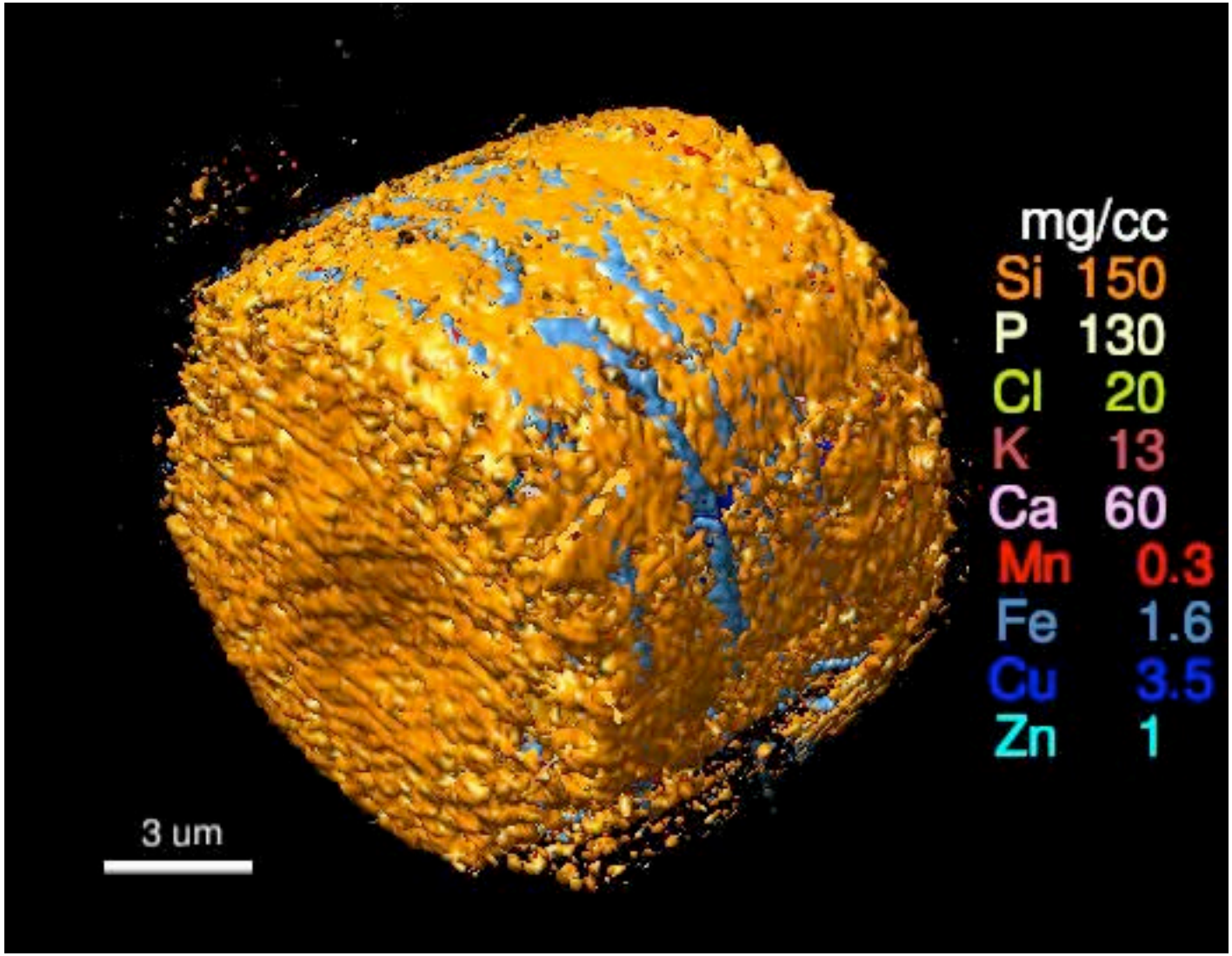
- Dr. Qiaoling Jin; experiments in progress





GTA: glutaraldehyde
PFA: paraformaldehyde
PF: plunge frozen

Fluorescence tomography



Cyclotella meneghiniana, de Jonge *et al.*, *Proc. Nat. Acad. Sci.* **107**, 15676 (2010).
36 hours data collection for 24 tilt angles; room temperature, dehydrated

Low-dose fluorescence tomography

- Dose fractionation [Hegerl and Hoppe, *Z. Natur.* **31**, 1717 (1976)] provides a way to do fluorescence tomography at higher speed and with lower dose: divide the signal needed for a 2D view among all the 3D projections!
- Differential phase contrast allows you to align low-dose fluorescence projections with ~30x better precision.

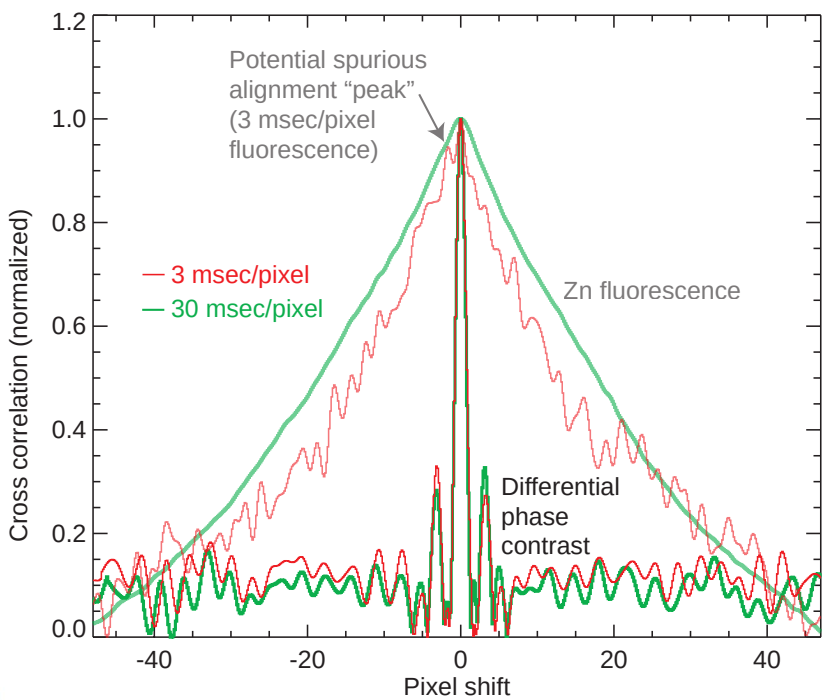
J. Synchrotron Rad. (2014). **21**, 229–234

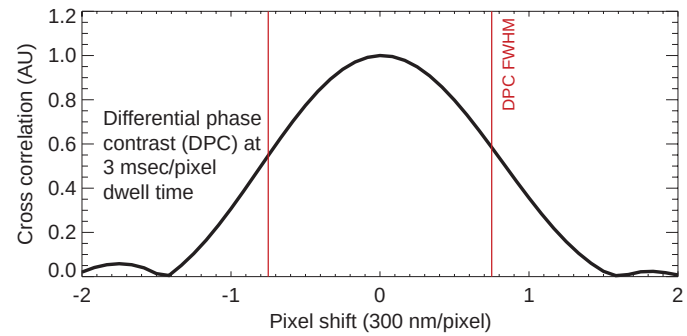
Alignment of low-dose X-ray fluorescence tomography images using differential phase contrast

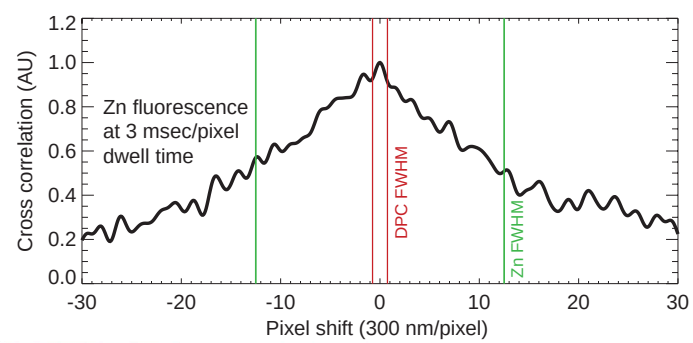
Young Pyo Hong,[a]* Sophie-Charlotte Gleber,[b] Thomas V. O'Halloran,[c] Emily L. Que,[c] Reiner Bleher,[d] Stefan Vogt,[b] Teresa K. Woodruff[e] and Chris Jacobsen[b,a]

[a]Department of Physics and Astronomy, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA, [b]Advanced Photon Source, Argonne National Laboratory, 9700 South Cass Avenue, Argonne, IL 60439, USA, [c]Department of Chemistry, Northwestern University, 2145 Sheridan Road, Evanston, IL 60208, USA, [d]Department of Materials Science and Engineering, 2220 Campus Drive, Evanston, IL 60208, USA, and [e]Department of Obstetrics and Gynecology, Northwestern University, 303 East Superior Street, Chicago, IL 60611, USA.

*E-mail: younghong2015@u.northwestern.edu



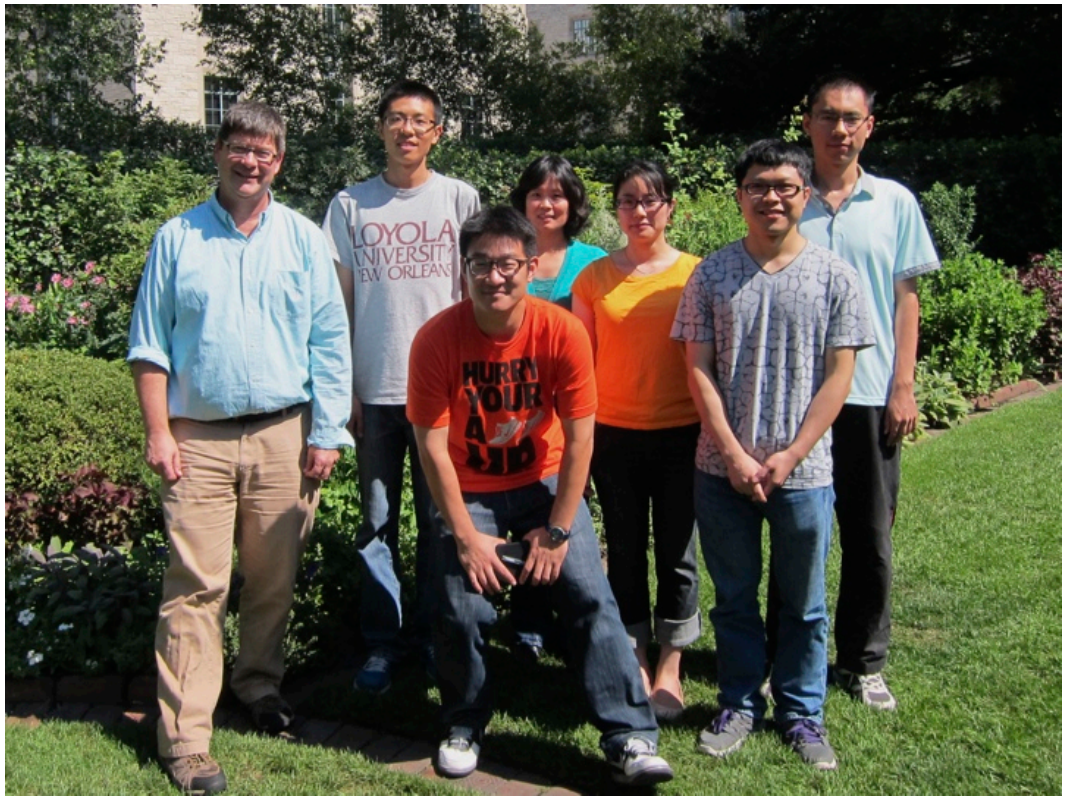




Limerick #3

Using cryo, or so I am told
Allows exposures to be much more bold
Radicals I do tease
To be stuck in a freeze
So my samples they like to be cold!

Thanks to all!



- NU Physics XRM group: Yue Sun, Young Pyo Hong, Qiaoling Jin, Rachel Mak, Junjing Deng, Kenan Li, Daikang Yan (not shown)
- Former Stony Brook group!
- APS X-ray Microscopy and Imaging Groups
- Xradia→Carl Zeiss X-ray Microscopy
- Woloschak, O'Halloran, and Woodruff labs, Northwestern University
- Support: APS, Argonne LDRD, NIH NIGMS

www.alcor.org

"Cryonics is a speculative life support technology that seeks to preserve human life in a state that will be viable and treatable by future medicine [which] will include mature nanotechnology"

"Following vitrification, neuropatients are placed in individual aluminum containers"

"Cryonics cannot work for anyone who is truly brain dead"

The Frozen Dead (1966)

Futurama's producer and lead writer

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)

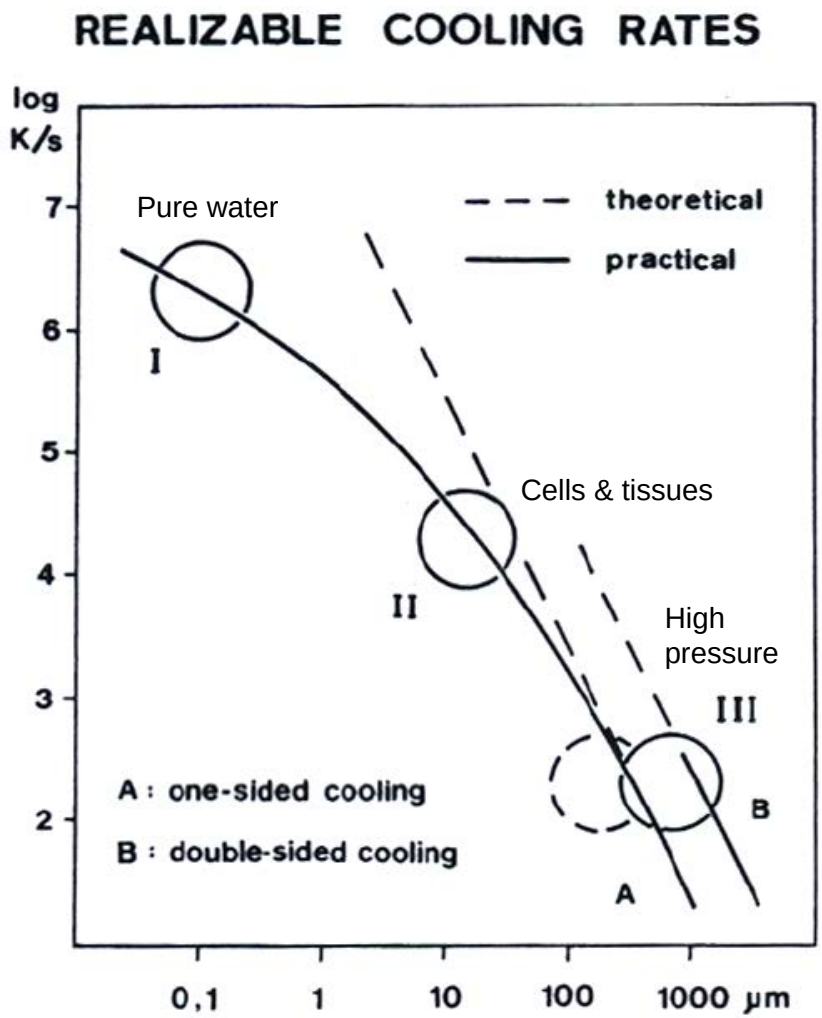
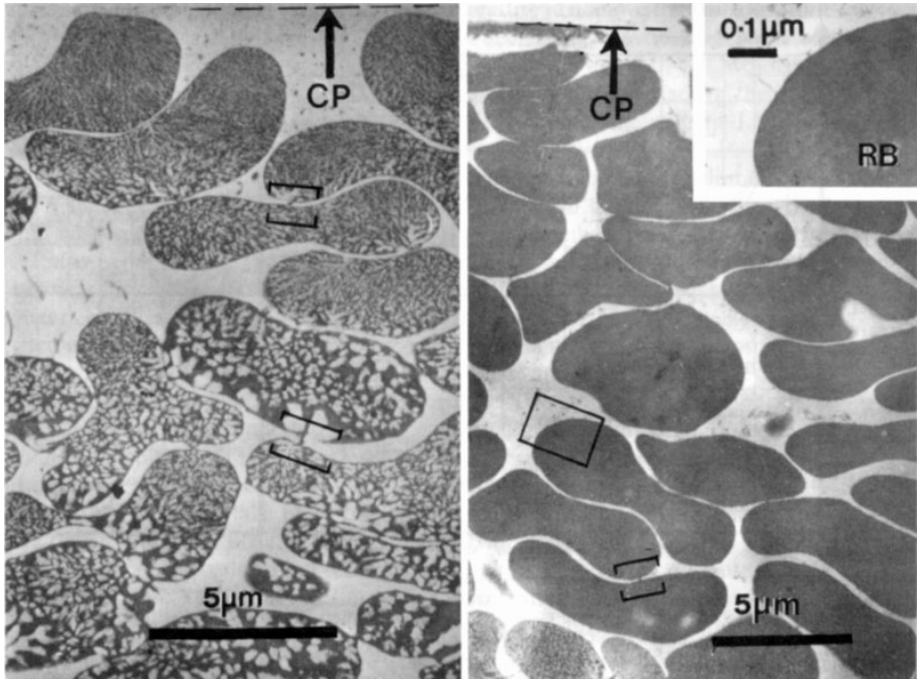
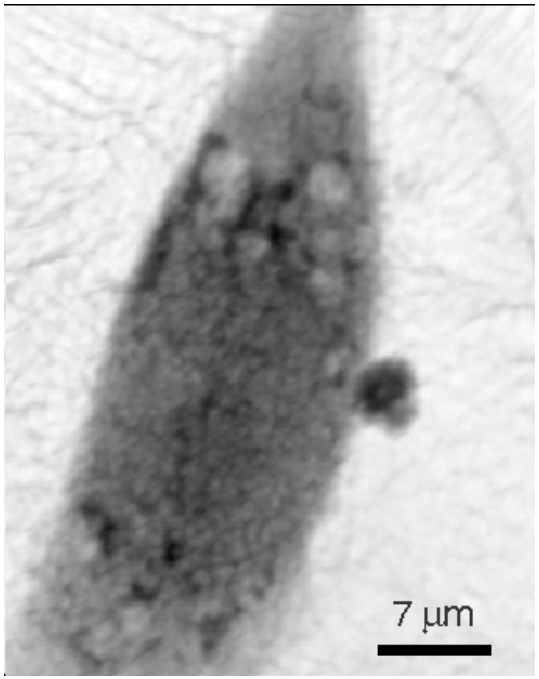


Fig. 1. The *curves* show the dependence of the cooling rate ($K\,s^{-1}$) on the distance (µm) of the specimen region from the cooled surface. *I* Range of vitrified pure water. *II* Range of vitrified animal cells and tissues. *III* Range of specimens vitrified under high pressure

Freezing: same final temperature, different cooling rates

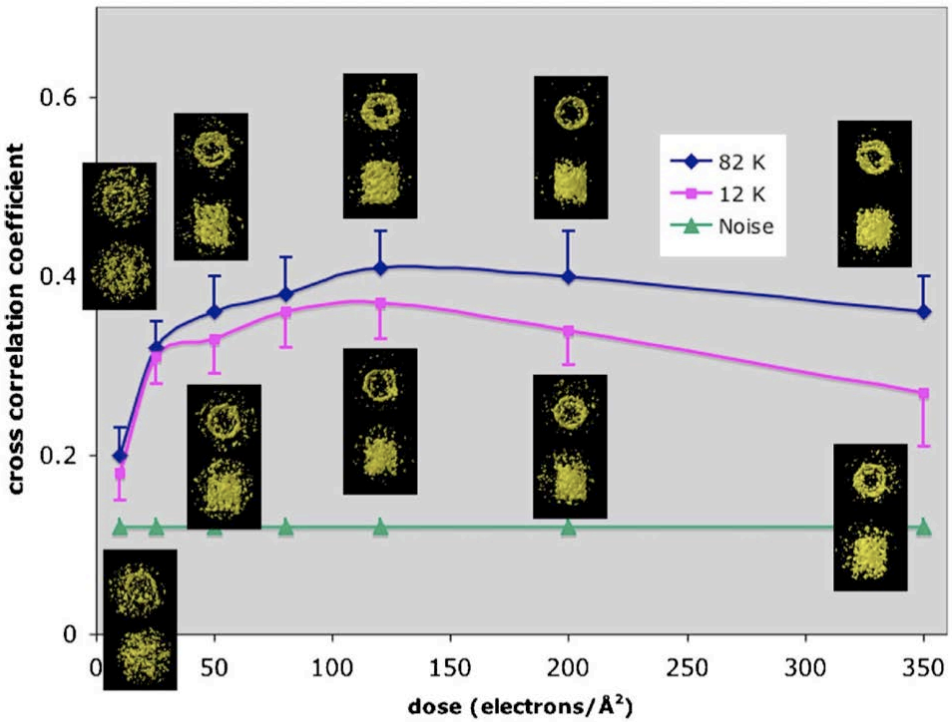


Plunge into liquid ethane but at different speeds (erythrocytes, thin sectioned). From Steinbrecht and Zierold, **Cryotechniques in Biological Electron Microscopy** (Springer, 1987)

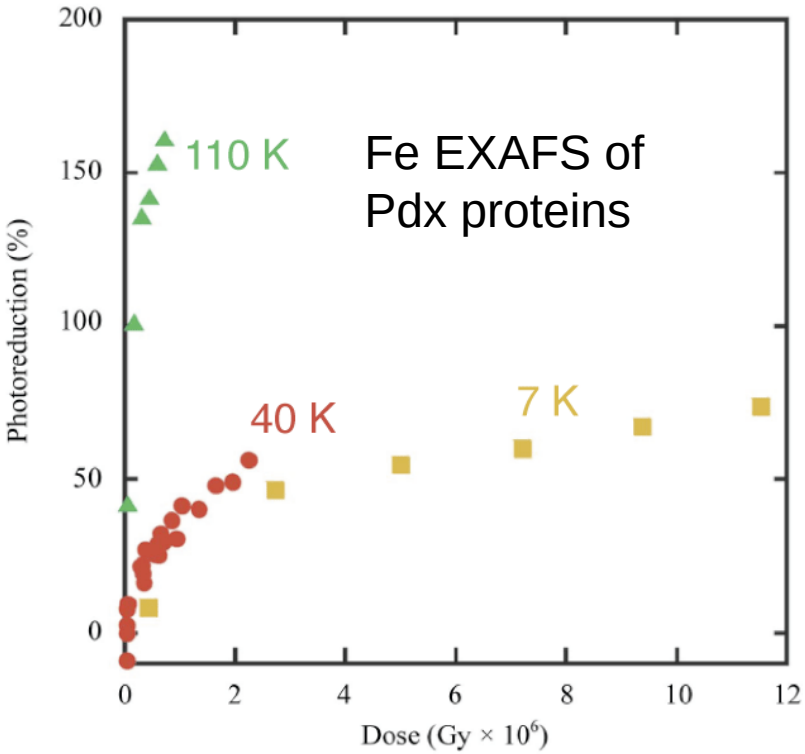


Fibroblast plunged into LN_2 (Wang, Maser, Osanna *et al.*)

How cold?



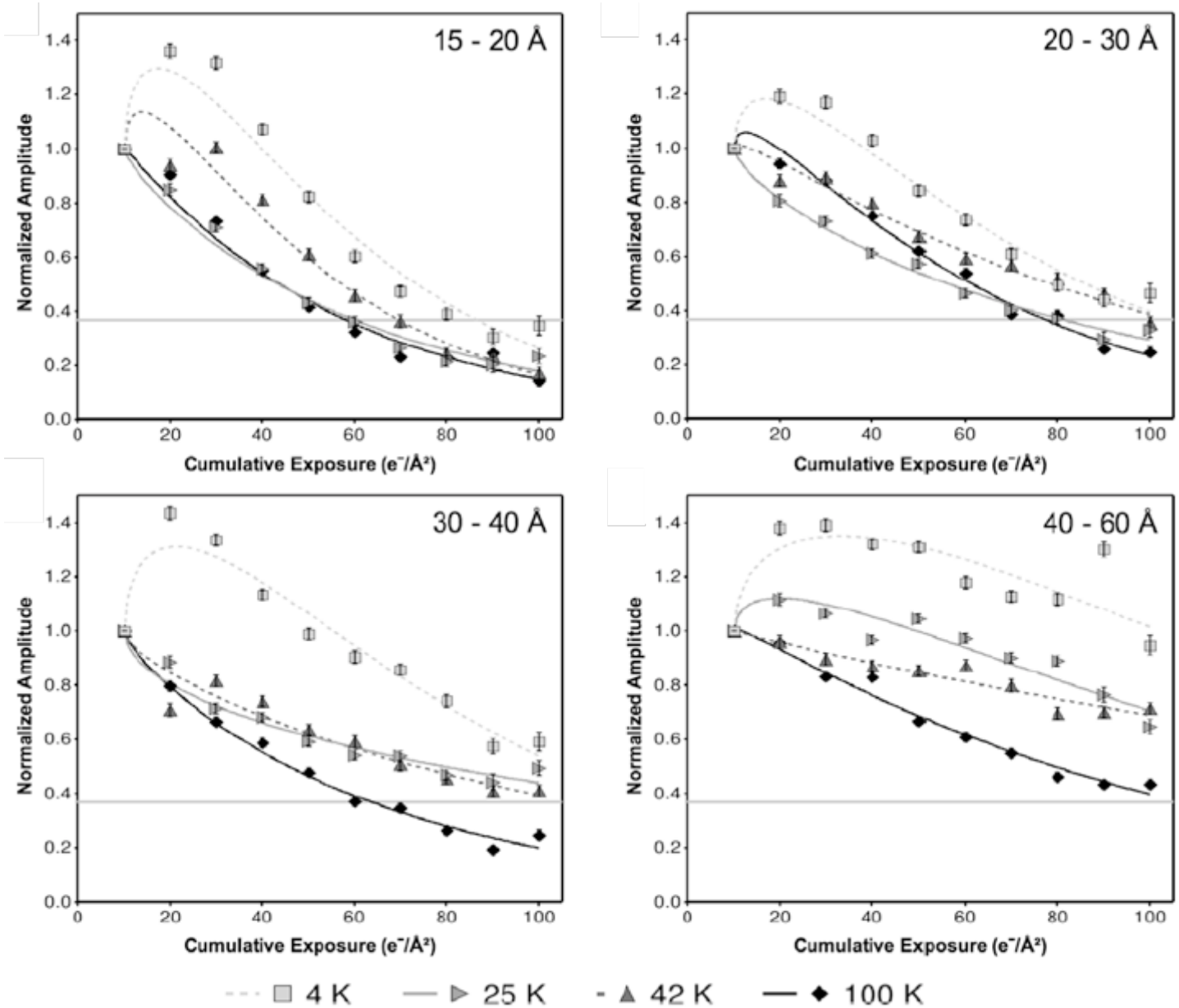
Iancu, Wright, Heymann, and Jensen, *J. Struct. Bio.* **153**, 231 (2006)



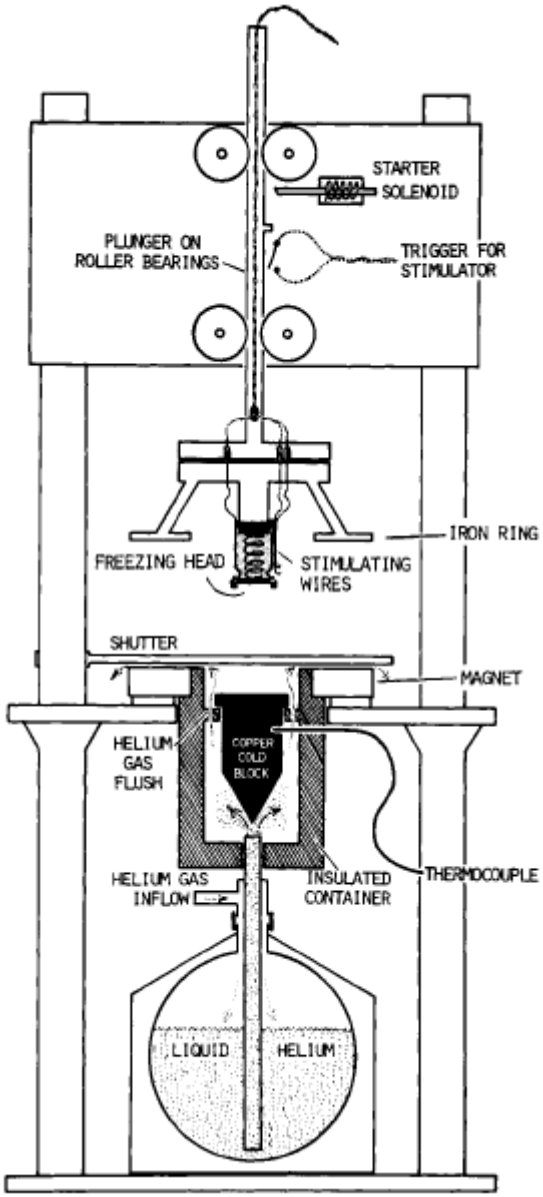
Corbett, Latimer, Poulos, Sevrioukova, Hodgson, and Hedman, *Acta Cryst. D* **63**, 951 (2007)

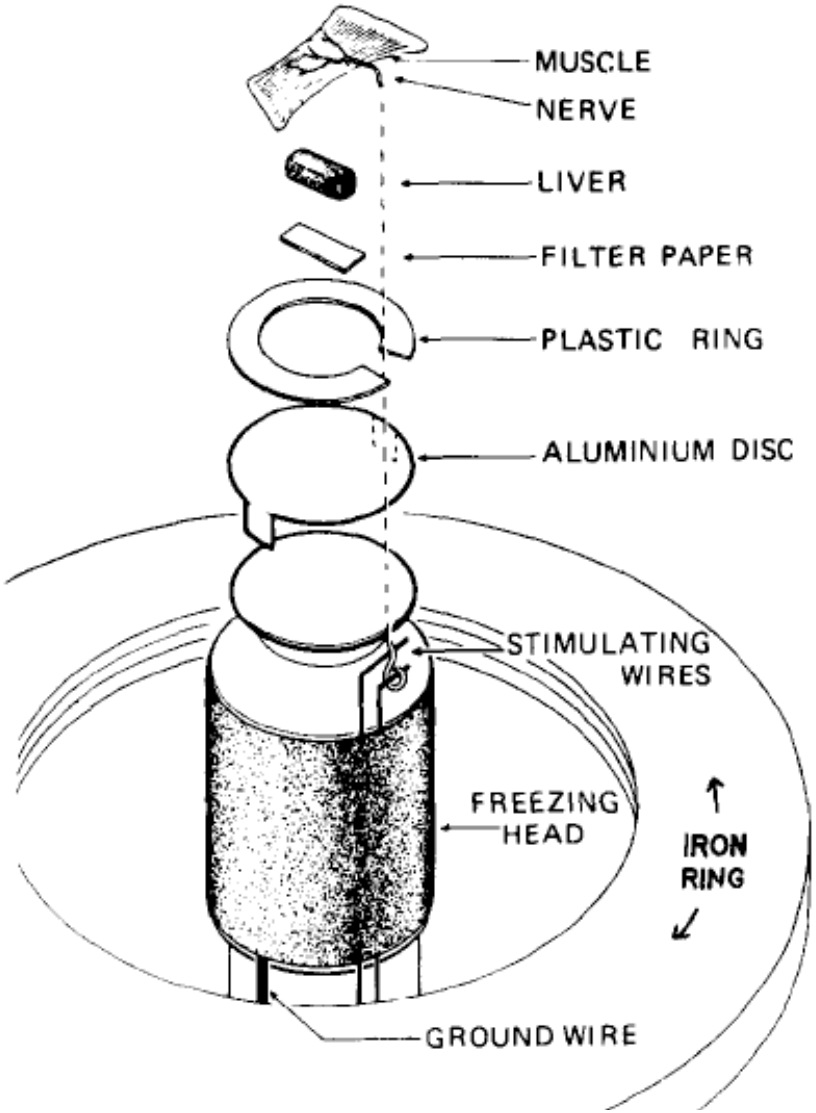
How cold? Bammes 2010

- Bammes, Jakana, Schmid, Chiu: *J. Struct. Bio.* **169**, 331 (2010)
- Frozen hydrated catalase crystals
- 100 $e^-/Å^2$ at 300 keV: ~2×10^8 Gray



Research into timed freezing methods

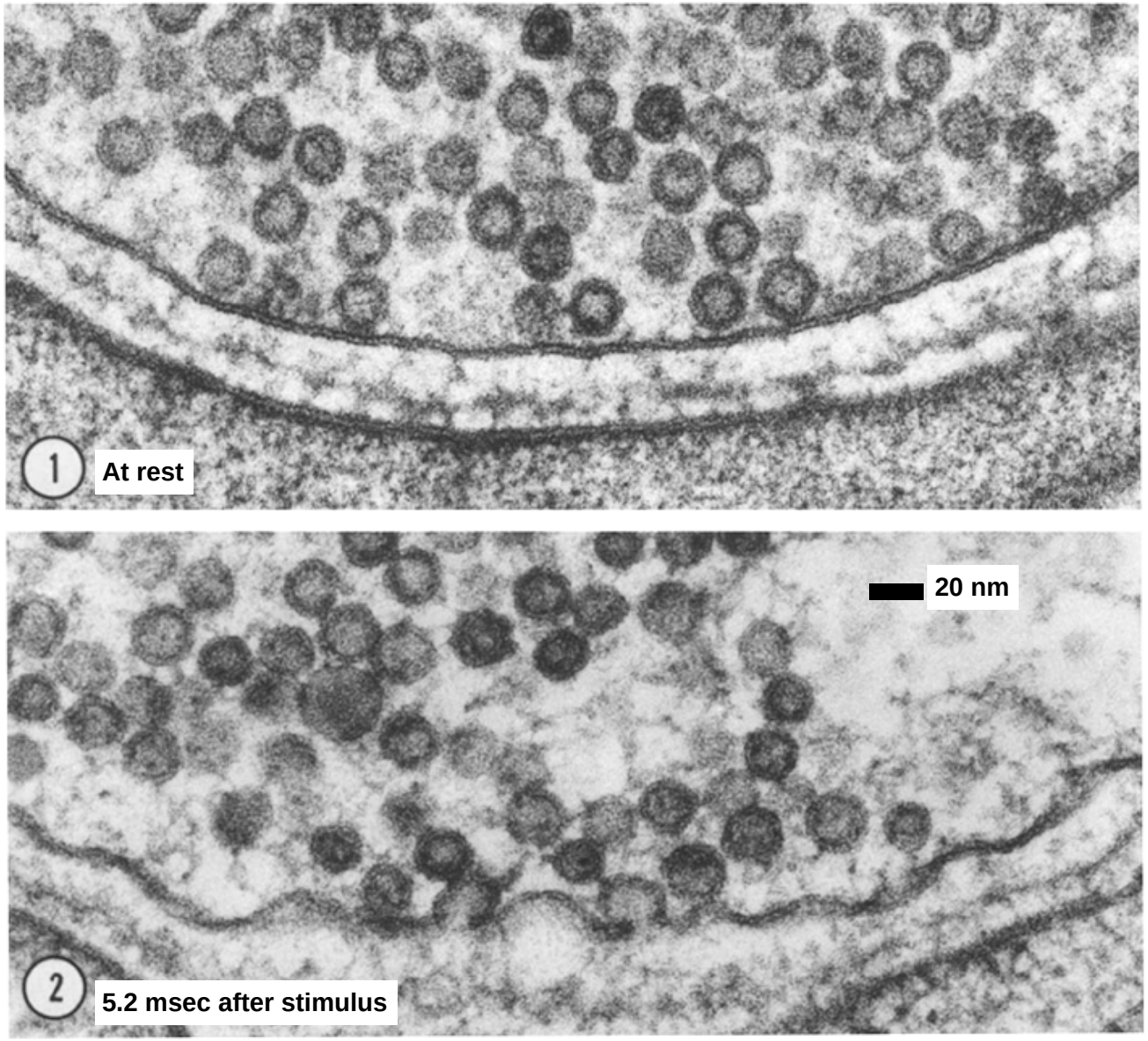




J.E. Heuser, T.S. Reese, M.J. Dennis, Y. Jan, L. Jan, and L. Evans, "Synaptic vesicle exocytosis captured by quick freezing and correlated with quantal transmitter release," *J. Cell Biology* **81**, 275 (1979)

Electrochemical stimulation: dynamics of exocytosis

- J.E. Heuser and T.S. Reese, “Structural changes after transmitter release at the frog neuromuscular junction,” *J. Cell Bio.* **88**, 564 (1981).
- Rapid freezing after stimulus, followed by fixation, embedding, sectioning, electron microscopy.



FIGURES 1 and 2 High magnification views of freeze-substituted nerve terminals at rest (Fig. 1) and stimulated in 4-AP at 5.2 ms before freezing (Fig. 2). Many pockets occur in the stimulated nerve terminal, which is sectioned through an active zone. The smaller of these pockets have the same diameter and curvature as synaptic vesicles. The larger, shallower pockets look like collapsing vesicles. × 180,000.

Heuser and Reese results

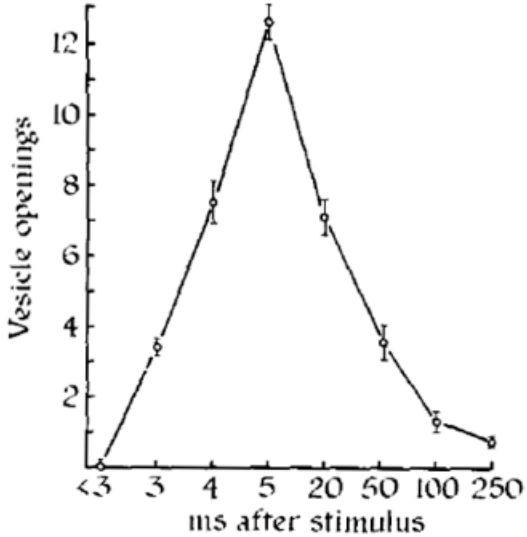


FIGURE 10 Vesicle openings per micrometer of active zone at different times after a single nerve stimulus in 4-AP. Note the nonlinear time scale. Because an active zone is considered to surround each ridge in the presynaptic membrane, the number of vesicle openings on both sides of the ridge were included. The bars are standard errors relative to the number of active zones and "ms after stimulus" is the interval between stimulation and contact with the freezing block.

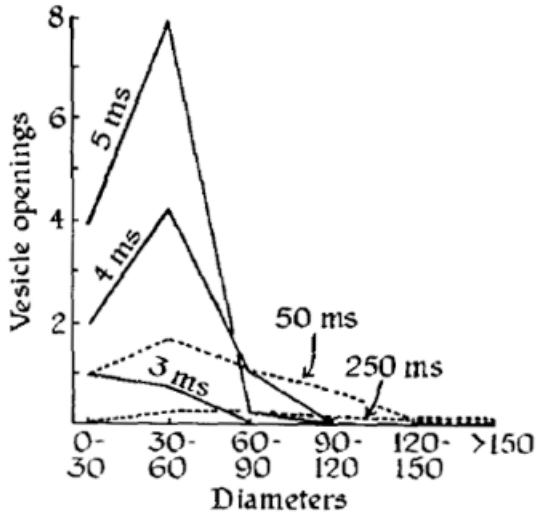


FIGURE 11 Number of vesicle openings per micrometer of active zone classified by diameter at different times after a single nerve stimulus in 4-AP. The diameters were measured on the set of micrographs used for Fig. 10, but the sample was smaller because it was more difficult to find vesicle openings suitable for measurement than for counting. As discussed in the text, the trend is for later times to show larger openings.

- "Vesicle openings begin to appear at the active zones of the intramuscular nerves within 3-4 ms after a single stimulus. The concentration of these openings peaked at 5-6 ms, and then declined to zero 50-100 ms later."
- "At the later times, vesicle openings tended to be larger. Left behind at the active zones, after the vesicle openings disappeared, were clusters of large intramembrane particles."
- J.E. Heuser and T.S. Reese, "Structural changes after transmitter release at the frog neuromuscular junction," *J. Cell Bio.* **88**, 564 (1981).

Cryopuncher: diffusible ions in tissues

- K. Zierold, “The cryopuncher: a pneumatic cryofixation device for x-ray microanalysis of tissue specimens,” *J. Microscopy* **171**, 267 (1993).
- J. A. Quaedackers *et al.*, *Nucl. Inst. Meth. B* **158**, 399 (1999): figure at right.

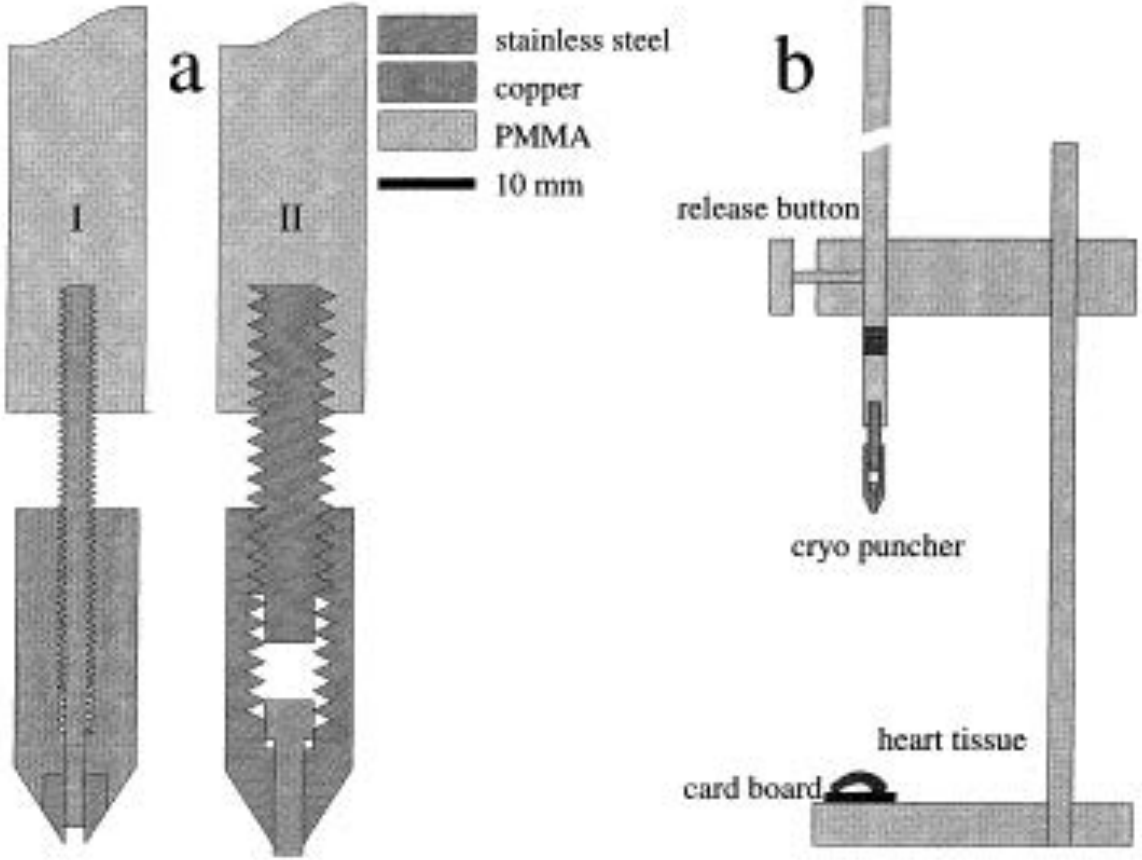


Fig. 1. (a) Cross-sections of both the cryopuncher described by Zierold (I) and the adapted cryopuncher (II) developed at the Eindhoven University of Technology used in later experiments. (b) Experimental set-up to drop the cryopuncher in heart tissue.