

Scanning X-ray Fluorescence Microscopy in Biology and the Life Sciences: Opportunities, Applications & Impact, Future

Stefan Vogt

**X-ray Science Division -
Advanced Photon Source**



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Outline

- Why study trace metals in biological systems ?
- Why use hard X-ray fluorescence microscopy ?
 - How does it relate to other techniques
- Scientific Applications:
 - Zn in heart muscle cells
 - TiO₂-DNA nanocomposites as intracellular tools

- Challenges & Future

Why Study trace elements / metals in biology and life sciences?

- Trace elements (**metals**) are **fundamental, intrinsic components** of biological Systems. estimated: 1/3 of all known proteins contain **metallofactors** as integral, catalytic components. These proteins often have regulatory or catalyzing functions, e.g.,
 - Cu binding chaperones (protein folding)
 - Zn in Zinc finger proteins: transcription factors in the cell nucleus
 - Fe in Haemoglobin; and necessary in Chlorophyll synthesis
- Metals can be **linked to disease**
 - Endogenous dysregulation, e.g., Alzheimer's, ALS
 - Exogenous uptake, e.g., Pb, As, Hg
- Metals can be made use of in **therapeutic drugs** and **diagnostic agents**
 - Cis-platin in chemotherapy
 - Sb to treat Leishmaniasis
 - Gd in Magnetic resonance imaging (MRI)

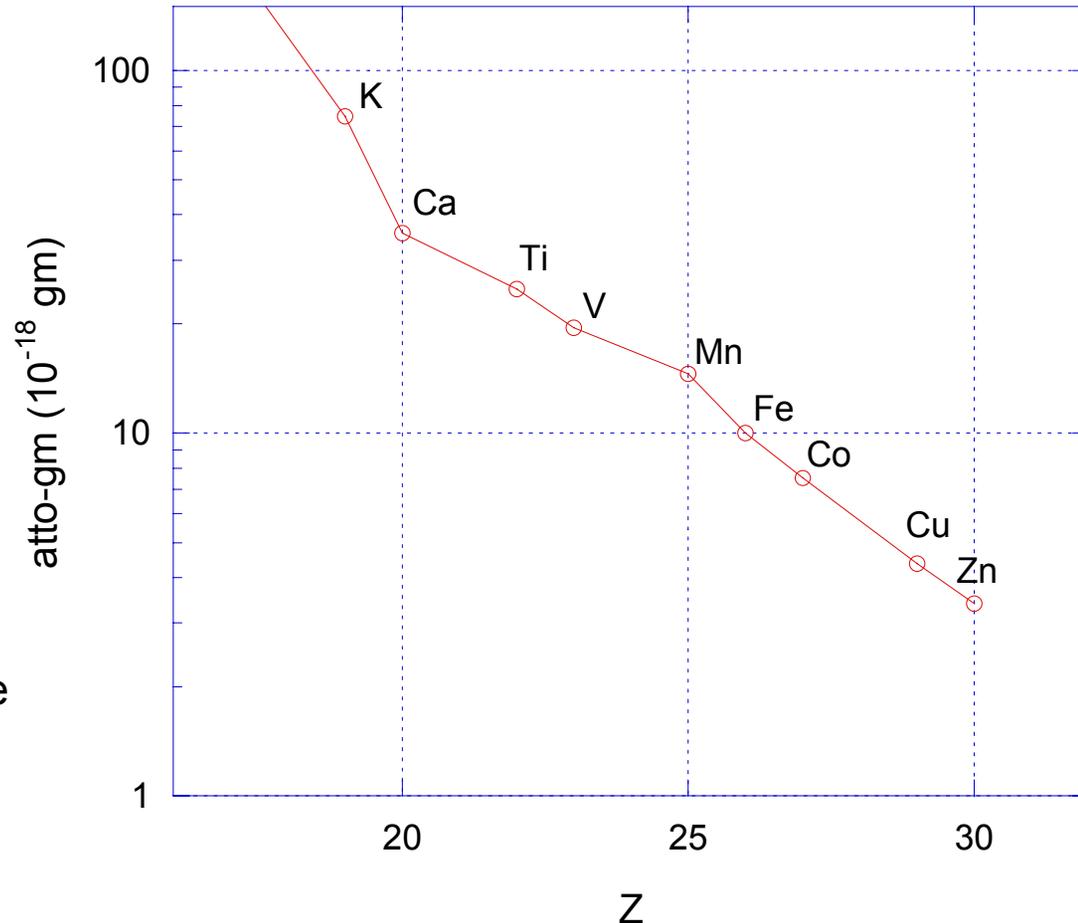
➔ ***study distribution and quantity of these elements within cells to understand how they act***



See e.g., Science 9 May 2003 (300 #5621) with Focus: "Metals Impacts on Health and Environment"

Why use x-ray-induced fluorescence to study trace metals?

- Simultaneously map 10+ elements
- No dyes necessary
- High signal/background ratio
 - sub-ppm (part-per-million) sensitivity, increasing with Z
- Little radiation damage
- Large penetration depth (> 100 μm)
 - study whole cells, w/o sectioning
 - study ‘thick’ tissue sections
 - possibility to study hydrated “natural” samples using cryo
- monochromatic incident beam: choose at which Z to stop excitation (e.g., excite As but not Pb)
- straightforward quantification
- Map chemical states by XANES
- Microspectroscopy / Spectromicroscopy



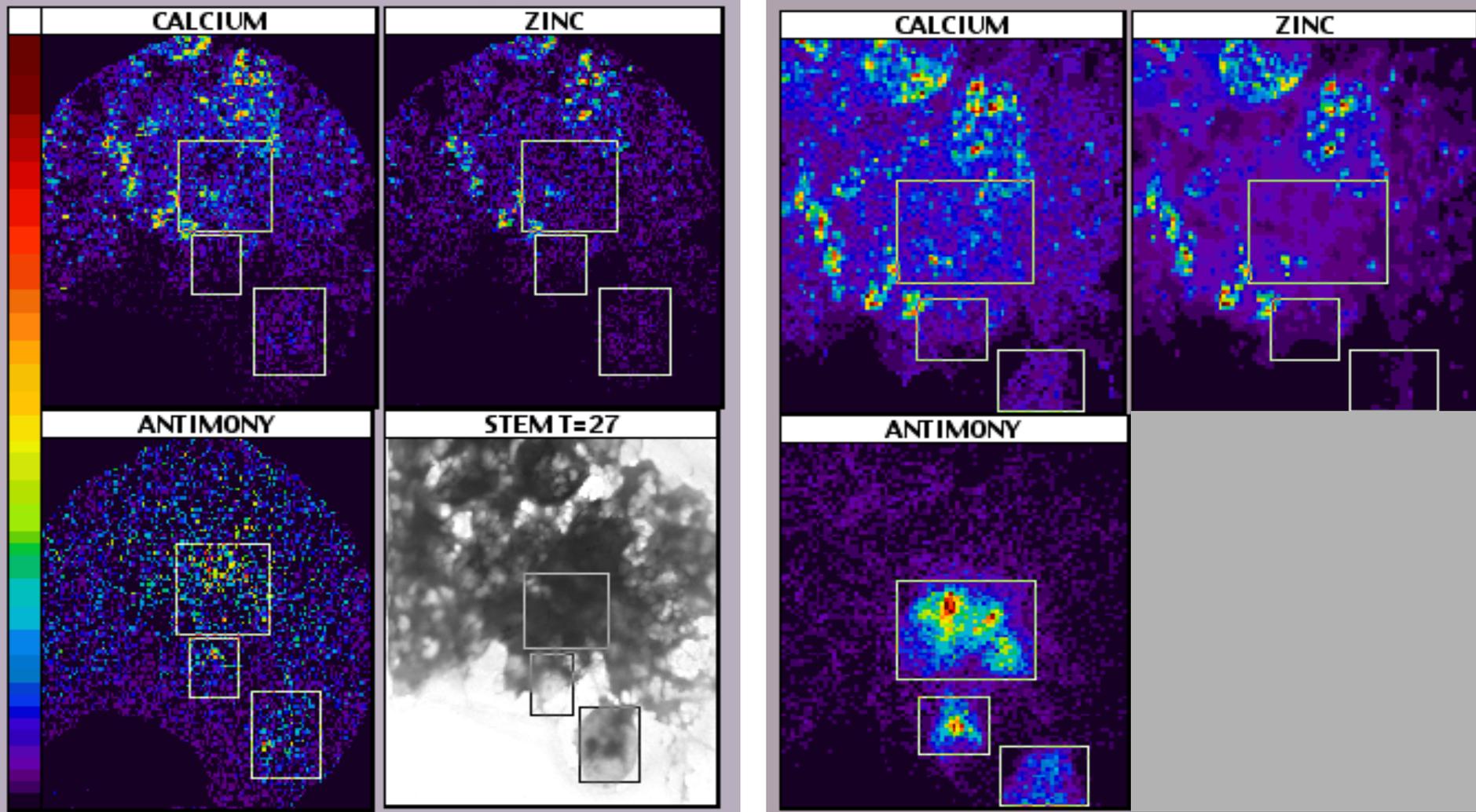
Detection Limit for Transition Elements: for 1 sec. acquisition time, $0.2 \times 0.2 \mu\text{m}^2$ spot, $E=10 \text{ keV}$

Comparison with other techniques:

	spatial resolution	object thickness	resolution limitation	Advantages/Disadvantages
Light-microscope	200 nm	30 μm	wavelength	<ul style="list-style-type: none"> + changes in living cells can be monitored - need dyes, competition w. proteins +/- typically see ions, and not total content - quantification difficult
Hard X-ray-microprobe	150 nm	10 – 100 μm	currently optics	<ul style="list-style-type: none"> + no dyes (pot. close to native state) + low background, high sensitivity + simultaneously detect >10 elements + μ-XANES for chemical state mapping - long integration times
Analytical Electron-microprobe	20 nm	0.1 μm	object thickness	<ul style="list-style-type: none"> + high spatial resolution + simultaneously detect >10 elements - whole cells very difficult, sectioning necessary

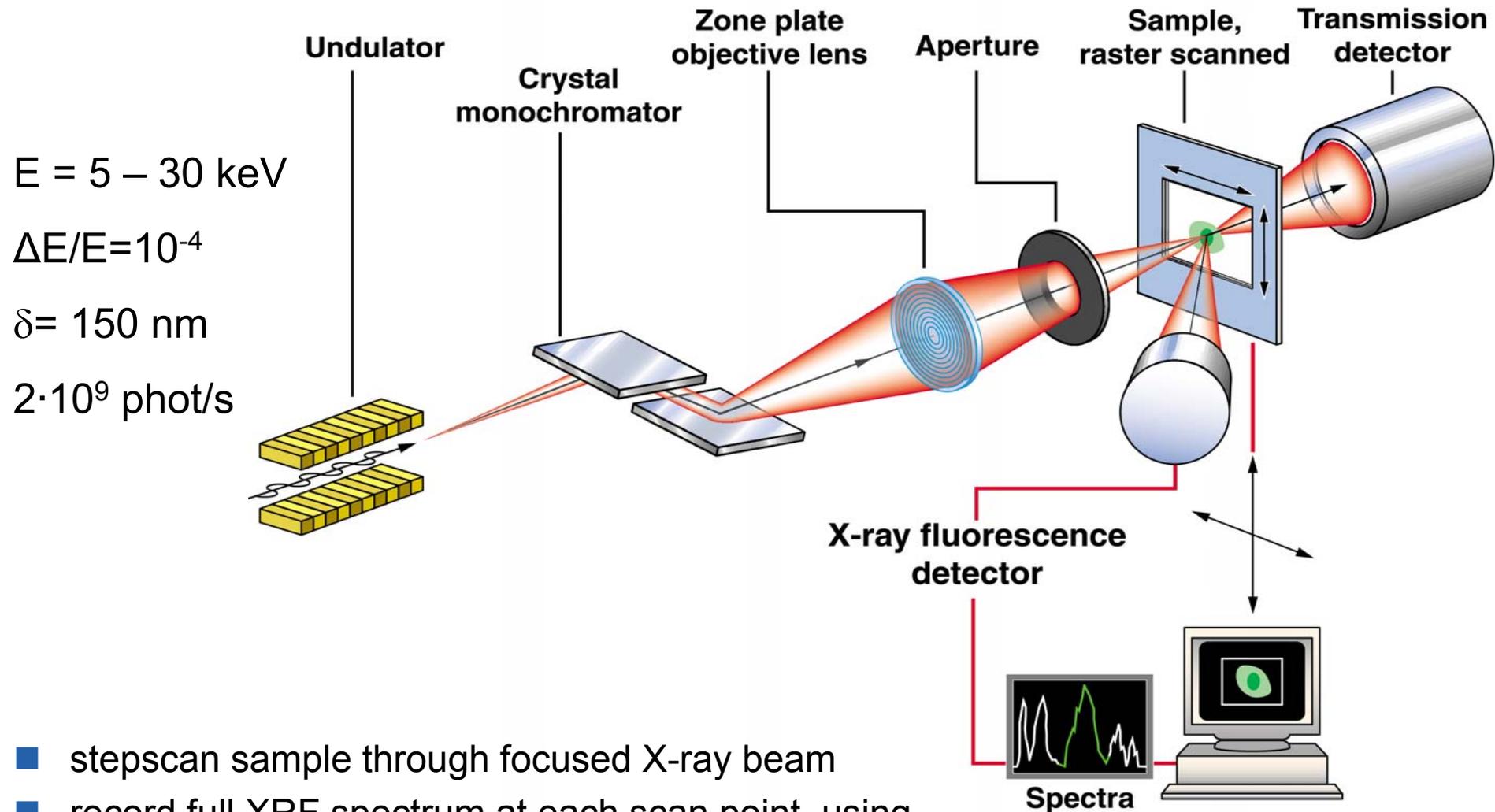
analytical electron microscope *hard X-ray microscope*

Collaboration with Ann LeFurgey and Peter Ingram, VA & Duke University



Elemental images of the same air-dried cells from several Sb-treated *Leishmania* amastigotes. Sb is much clearer visible in the x-ray microscope due to its greater sensitivity. Scan width: 10 μ m.

Schematic of a Hard X-Ray Microprobe

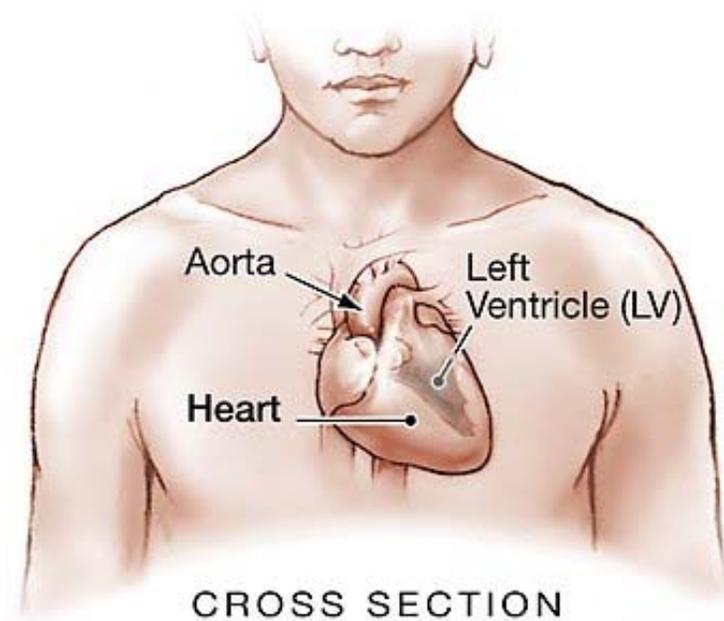


- stepscan sample through focused X-ray beam
- record full XRF spectrum at each scan point, using an energy dispersive detector (typically LN₂ cooled Ge, or SDD)
- compare specimen counts/spectra to calibration curve, to quantify to area density 8

Scientific applications

Cardiac Hypertrophy

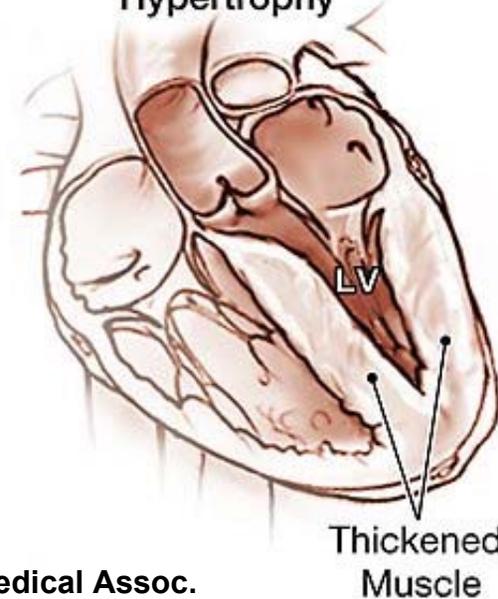
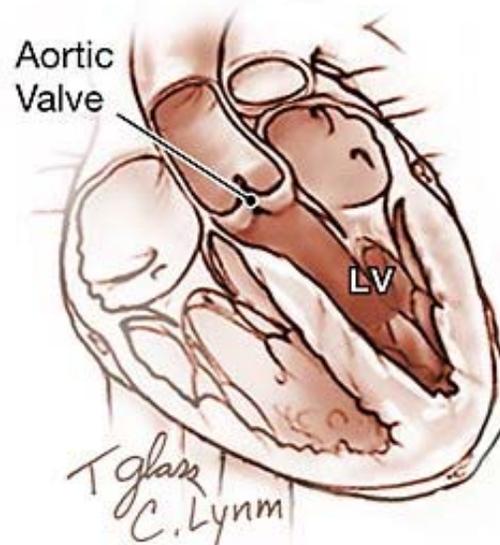
- Can be response to mechanical load (e.g., athletes) – can be good
- But: more often maladaptive process (e.g., due to high blood pressure), leading to
 - a decrease in size of chamber of heart
 - less pumped blood
 - heart cannot fully relax between beats
 - May lead to
 - *Sudden death*
 - *Progression to heart failure*



CROSS SECTION

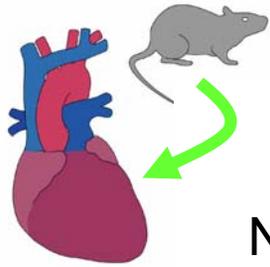
Normal Heart

Left Ventricular Hypertrophy



From American Medical Assoc.

We try to understand properties of cardiac hypertrophy on the cellular level

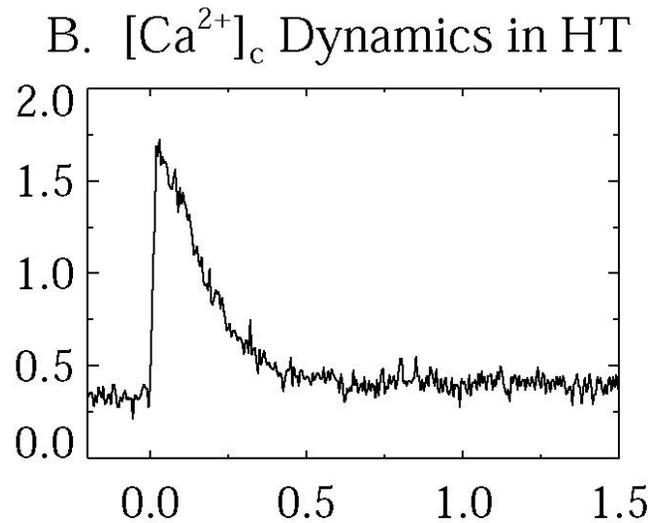
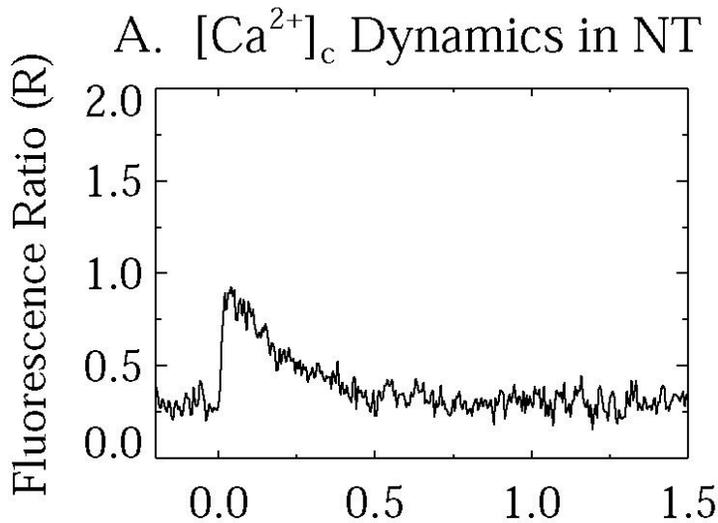
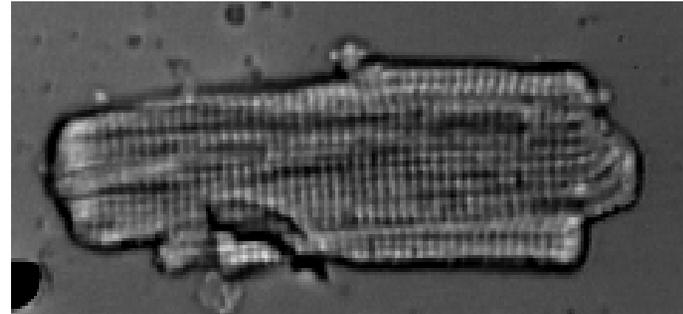
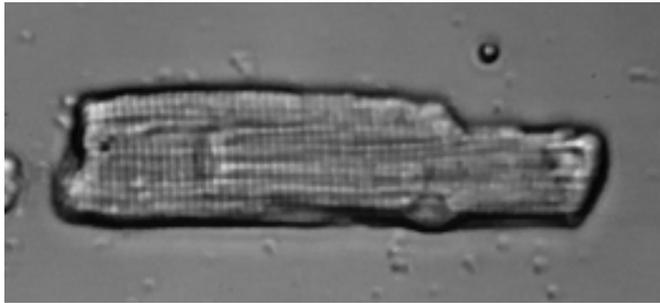


Normal

120 μm x 25 μm x 10 μm

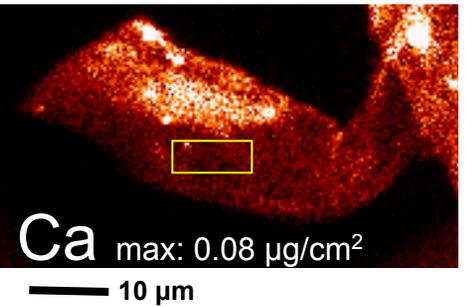
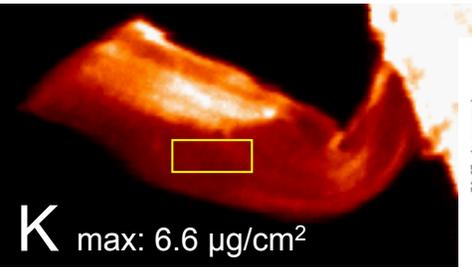
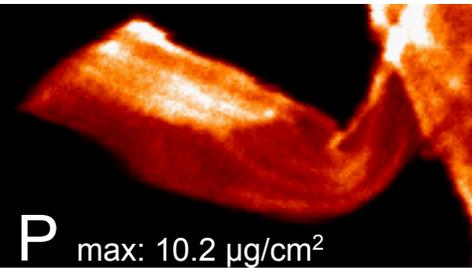
Cardiac hypertrophy due to bigger cardiac myocytes (heart muscle cells)

Hypertrophic Compensation for High Blood Pressure:
120 μm x 30 μm x 12 μm

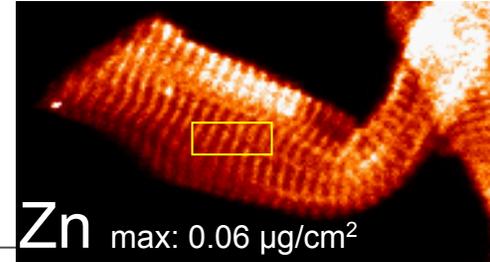
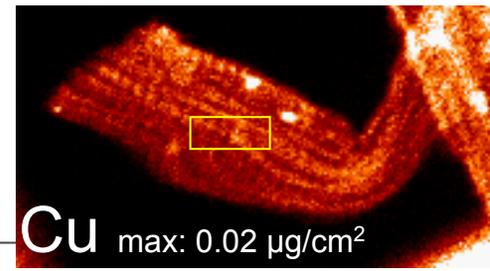
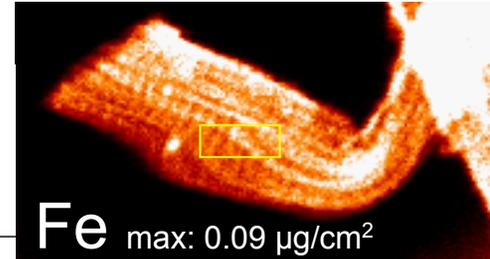
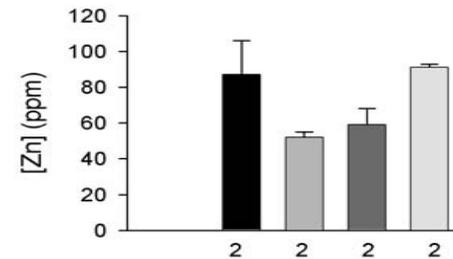
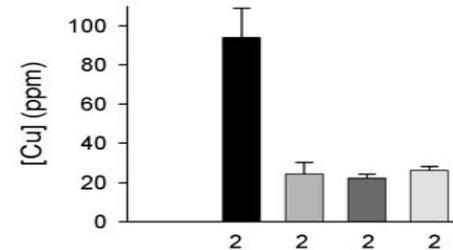
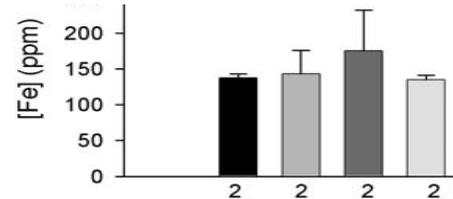
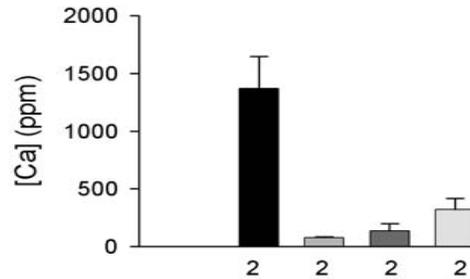
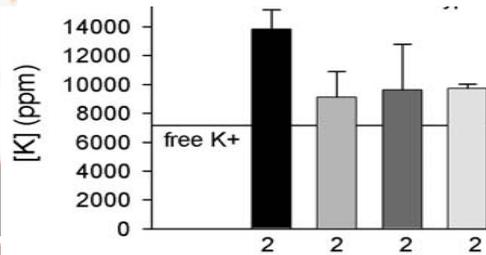


$[\text{Ca}^{2+}]_{\text{max}}$ is enhanced in compensated, hypertrophied myocytes.

Elemental content of cardiac myocytes

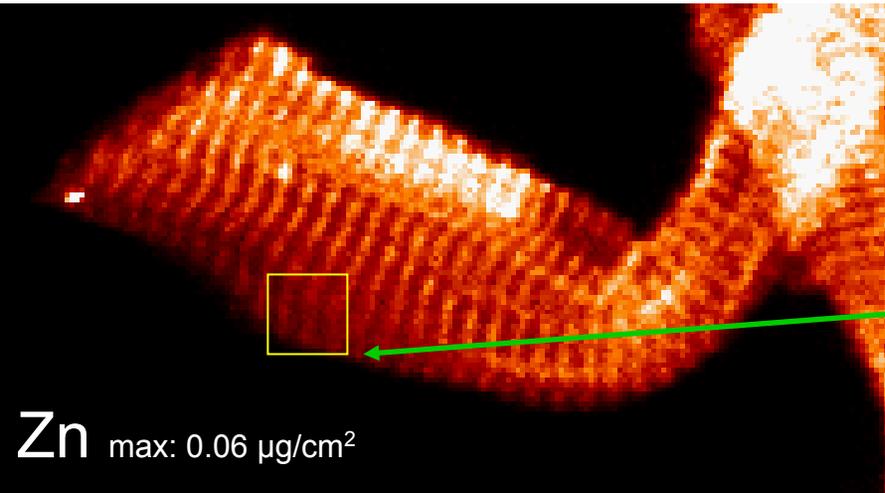


WKY: Normal
 WKHT: Hypertrophied
 WKHA: Hypertrophied
 SHR: Hypertrophied

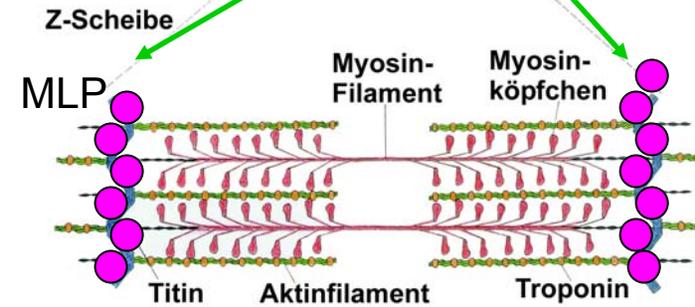
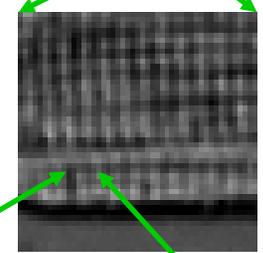
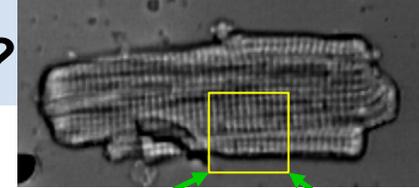


Surprisingly total [Ca] is significantly reduced in hypertrophied myocytes.

What are these striations in Zn ?



Zn max: 0.06 $\mu\text{g}/\text{cm}^2$

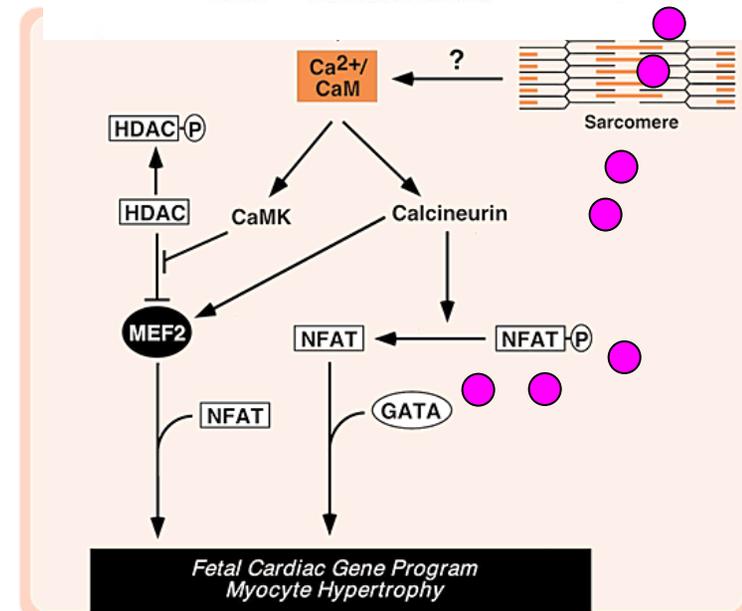


Zn striations occur at $\sim 1.6 \mu\text{m}$ intervals, which corresponds to one complete sarcomere. Zn seems to co-localise to I-band – is Muscle LIM Protein (MLP) responsible ?

MLP is also implicated as stress sensor. MLP released by injury is thought to activate GATA-4 and gene expression.

Is Zn enhancing response ('loading' Zn-finger proteins) ?

But, question remains: where exactly does Zn go ?

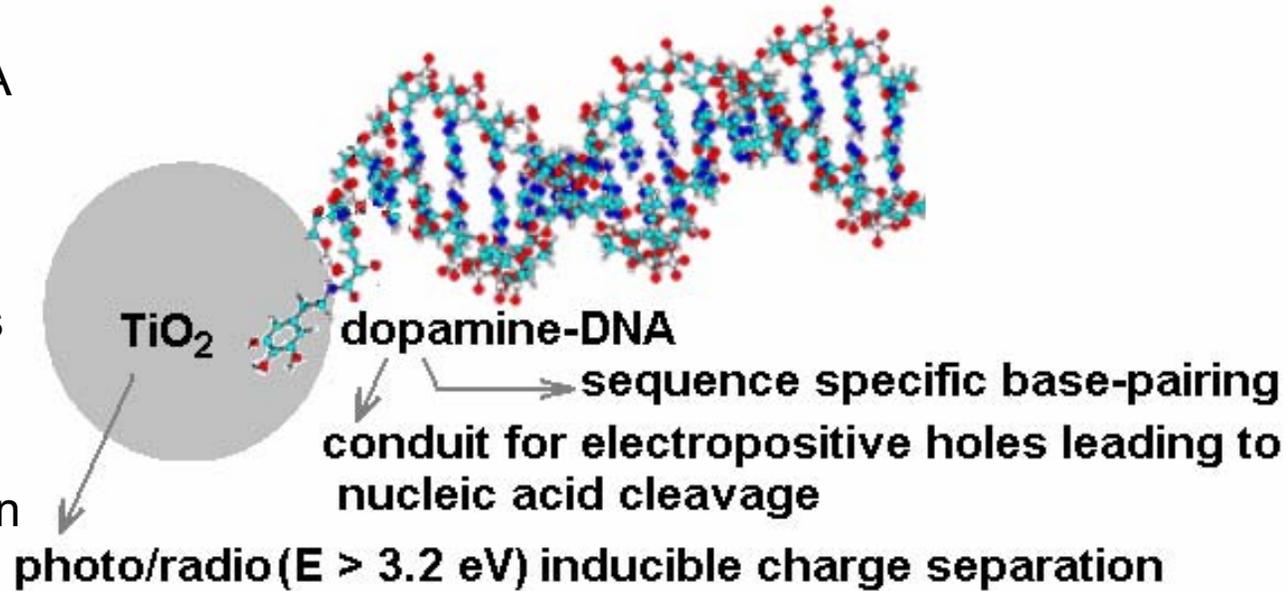


*TiO₂-DNA nanocomposites as
intracellular probes and tools*

T. Paunesku, S. Vogt, J. Maser, B. Lai, K. Thurn, C. Osipo, H.
Liu, P. Ingram, A. LeFurgey, and G. **Woloschak**

Ti nanocomposites as intracellular probes and tools

- attach TiO₂ nanoparticle (4.5 nm diameter) to DNA
- combine DNA biochemistry with semiconductor properties of TiO₂
- → carrier-particle that can bind to a specific chromosomal region w/ ability to cleave it upon illumination

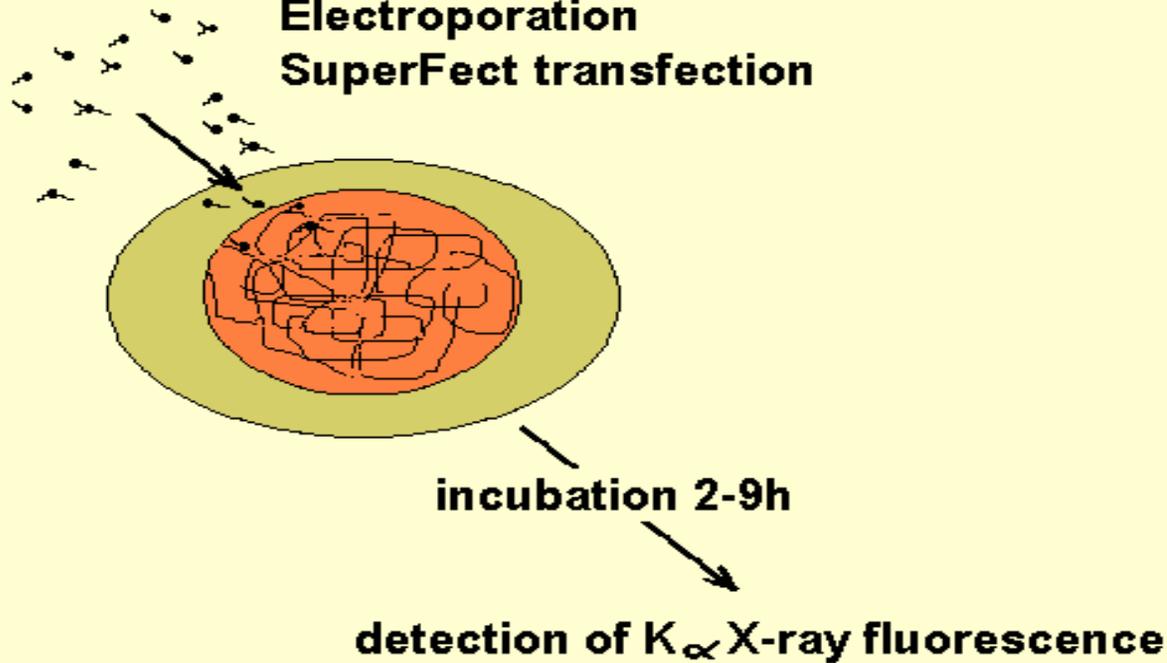


T. Paunesku *et al*, Nature Materials 2, 343-346 (01. May 2003)

T. Paunesku *et al*, submitted

APS Experiments

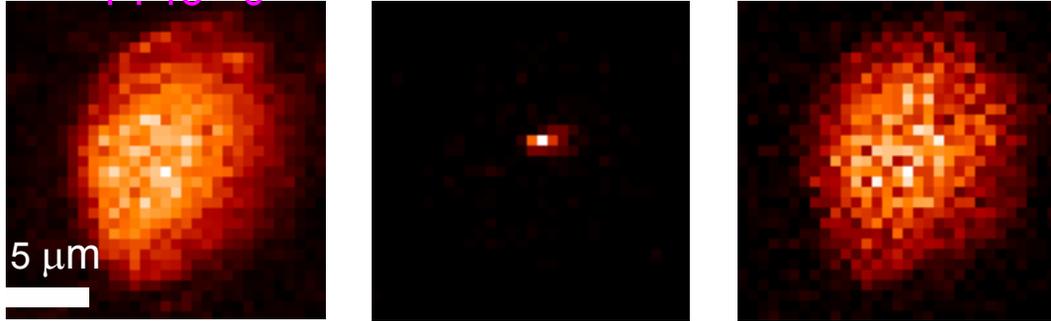
**Electroporation
SuperFect transfection**



- transfect mammalian cells with TiO_2 -DNA nanoparticles, incubate
- target appropriate cells using VLM, record cell positions
- map elemental distributions (Si, P, ..., Ti, ... Zn) in hard X-ray microprobe

TiO₂-DNA nanocomposites as intracellular probes

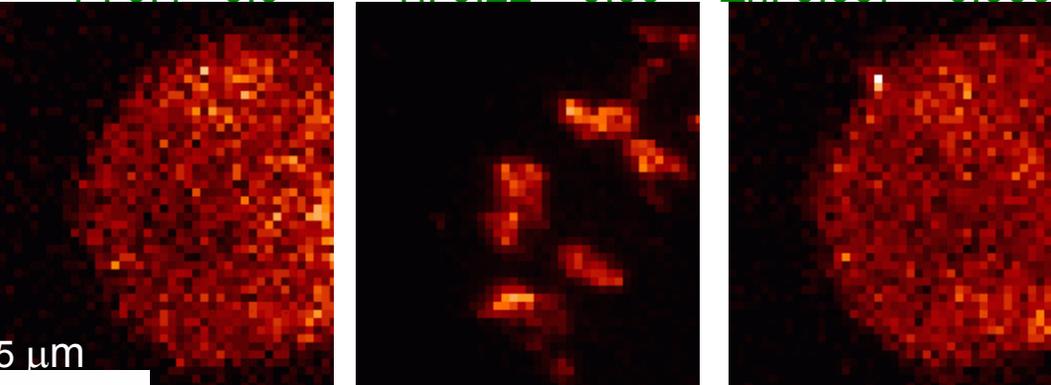
A: P: 13 - 0 Ti: 0.25 - 0.00 Zn: 0.039 - 0.001



Units: μg/cm²

- **A:** scan of a MCF7 cell transfected with nanocomposites targeted to nucleolus

B: P: 0.4 - 0.0 Ti: 0.22 - 0.00 Zn: 0.007 - 0.000



- **B:** scan of a PC12 cell transfected with nanocomposites targeted to mitochondria

But, question remains:
how do nanocomposites localise
WITHIN mitochondria or nucleolus ?

Conclusion:

- Can target specific organelles with TiO₂-DNA nanocomposites
- Currently 10-20% transfection efficiency

Future: Nanocomposites as tools for Gene therapy ?

- Correct defective genes responsible for disease development, e.g.,
 - *destroying mutated and dominant genes (e.g., oncogenes)*
 - *replacing mutated and recessive genes (e.g., tumor suppressor genes)*

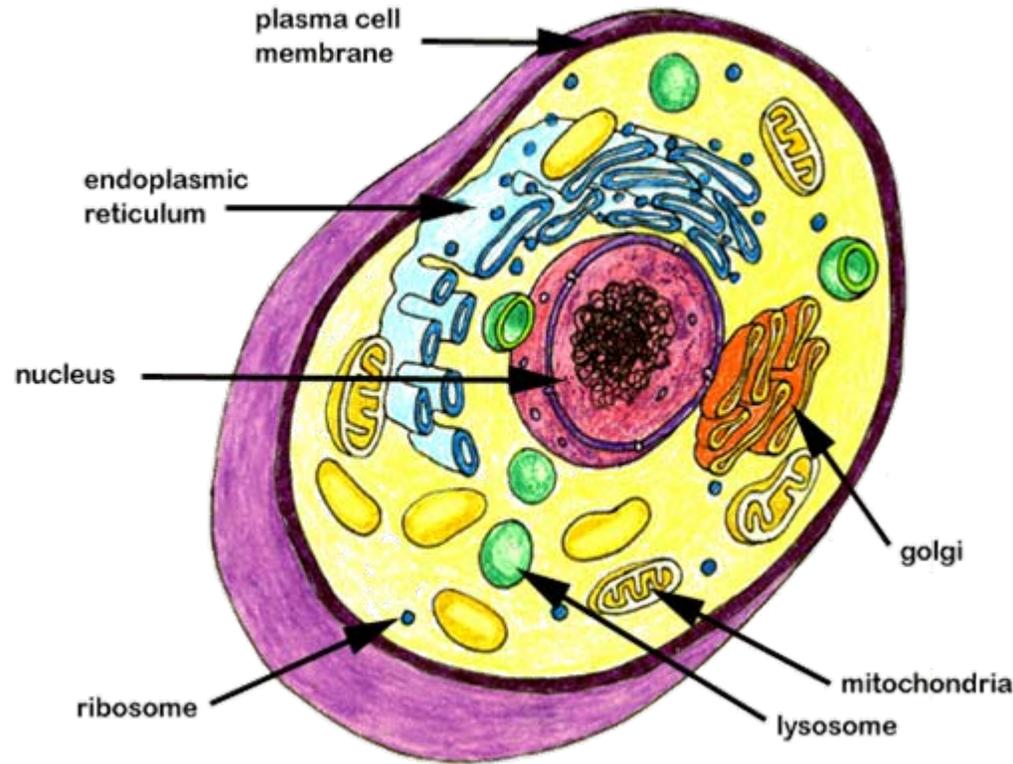
Future and Challenges

Challenge: high spatial resolution

Typical sizes of cell structures and organelles:

- nucleus: 2-5 μm
- mitochondrion: 0.5x2 μm (cellular respiration)
- ribosome: 25 nm (protein synthesis from mRNA)
- chromatin fiber: 20 nm diam. (DNA double helix on histones)
- microtubuli: 20 nm diam. (cytoskeleton)
- membrane thickness: 8 nm

→ need spatial resolution of <20 nm

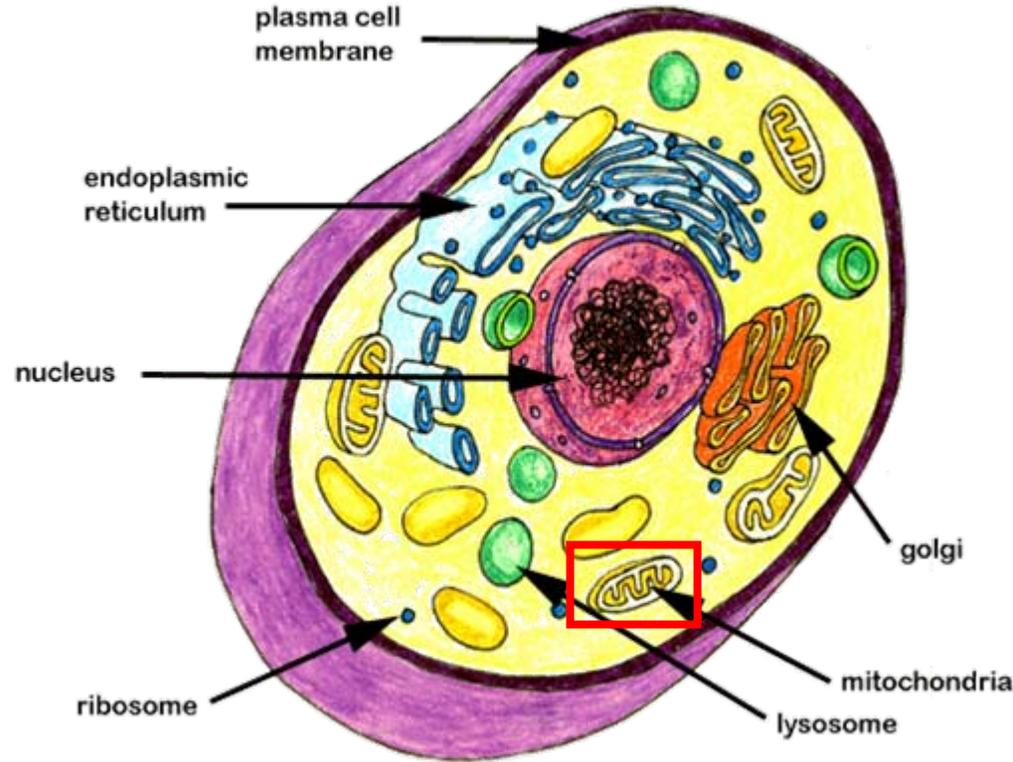


Challenge: high spatial resolution

Typical sizes of cell structures and organelles:

- nucleus: 2-5 μm
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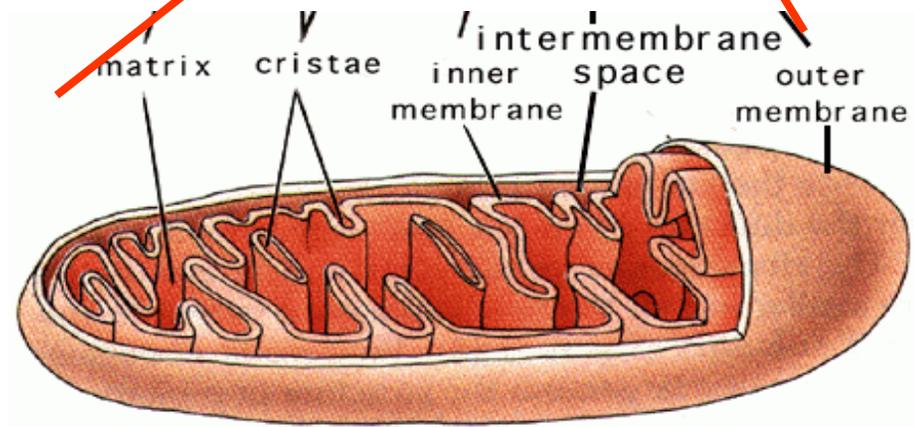
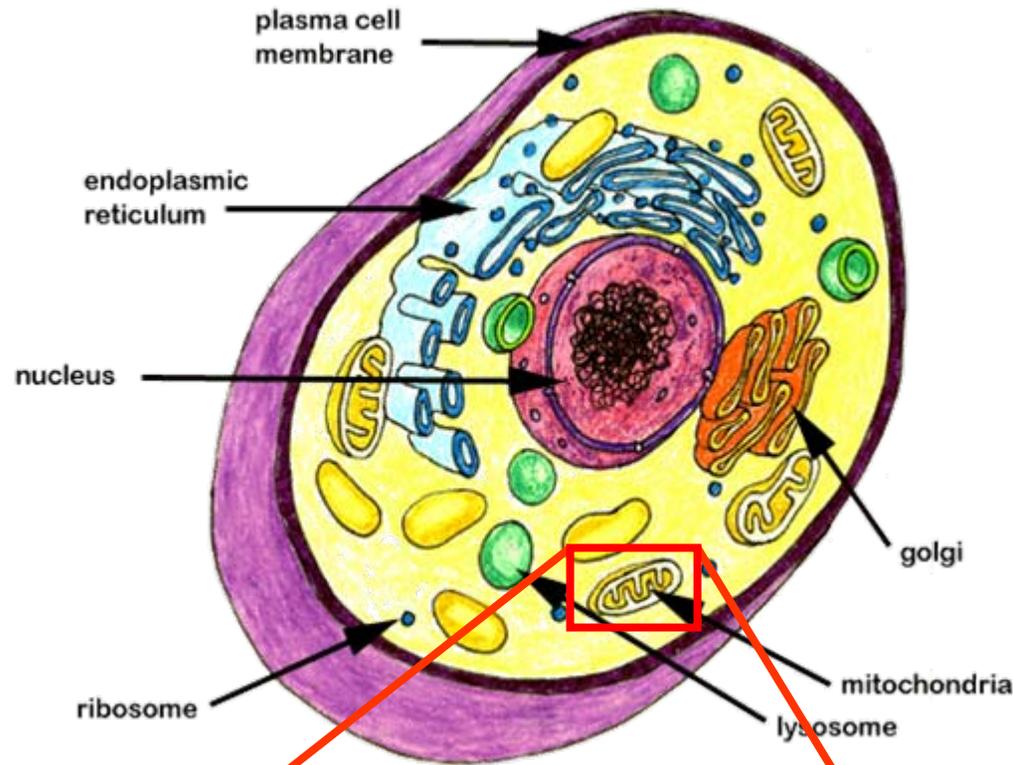


Challenge: high spatial resolution

Typical sizes of cell structures and organelles:

- nucleus: 2-5 μm
- **mitochondrion: 0.5x2 μm (cellular respiration), w/ substructure !**
- ribosome: 25 nm (protein synthesis from mRNA)
- chromatin fiber: 20 nm diam. (DNA double helix on histones)
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- membrane thickness: 8 nm

→ need spatial resolution of <20 nm

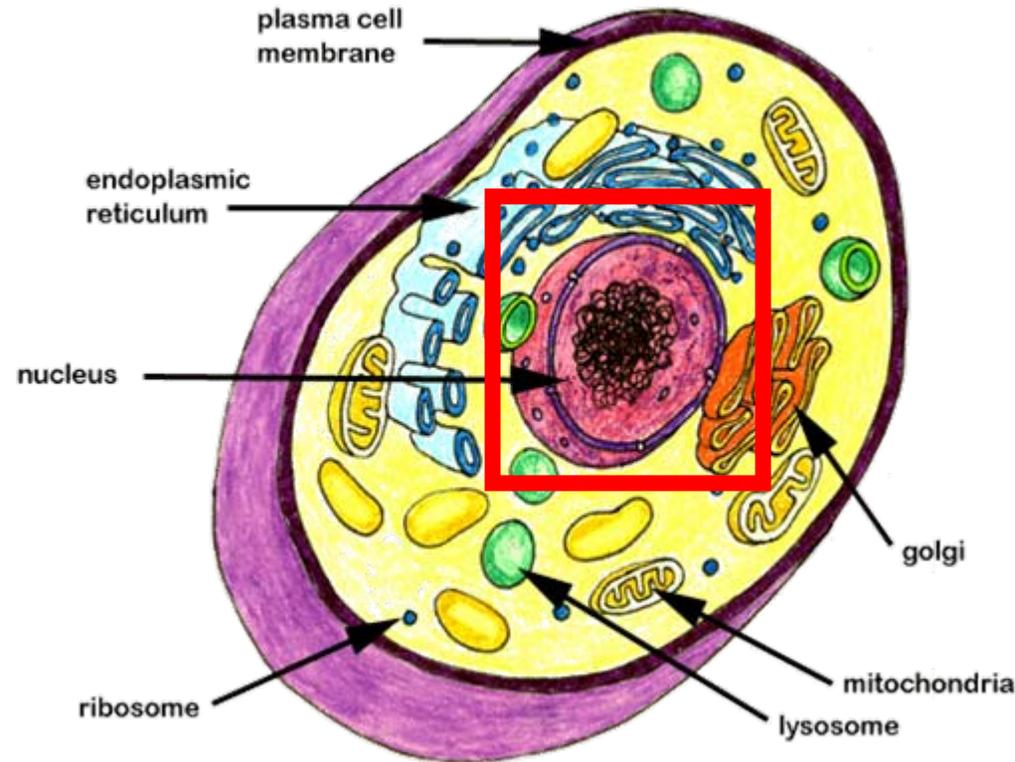


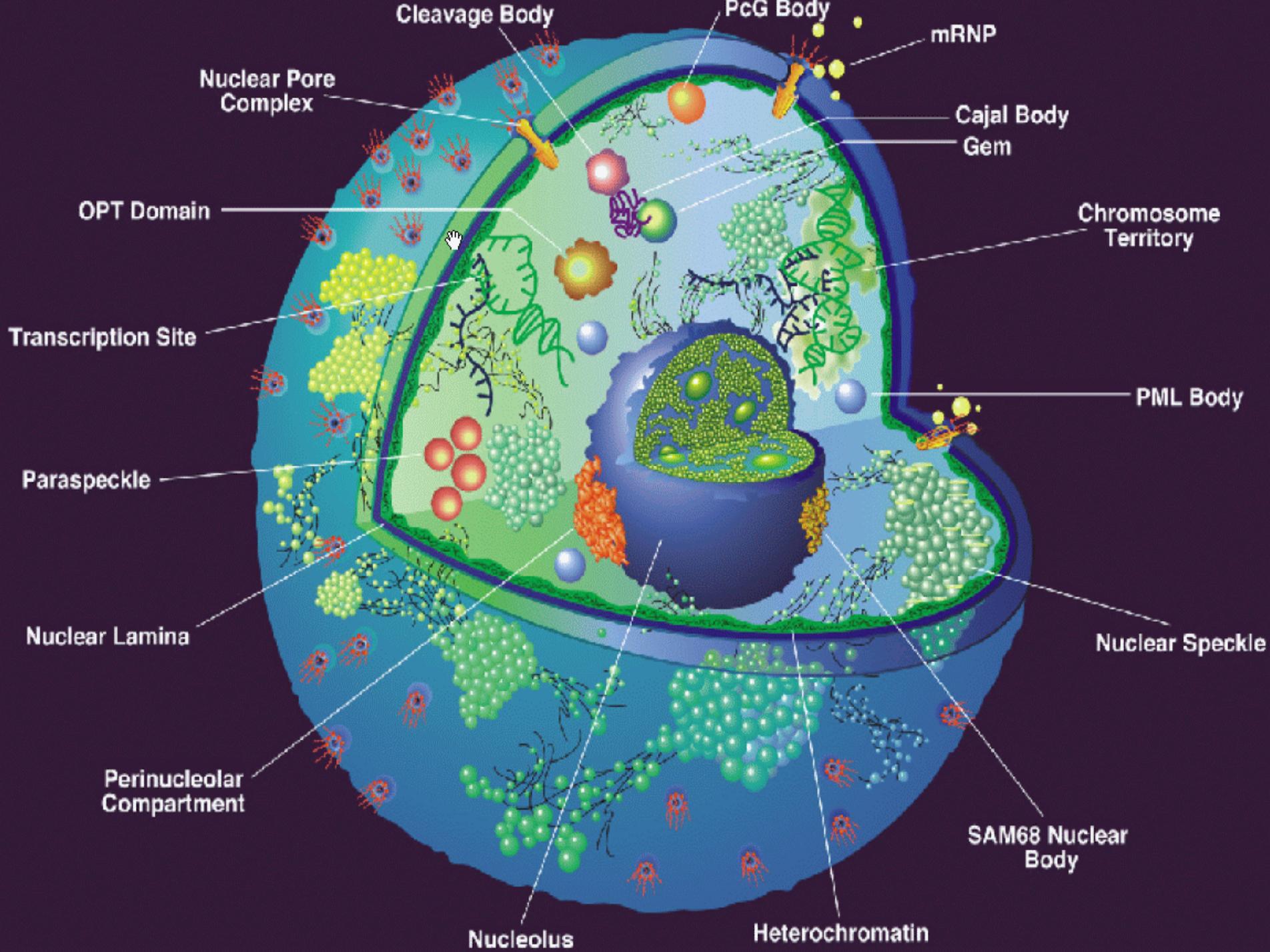
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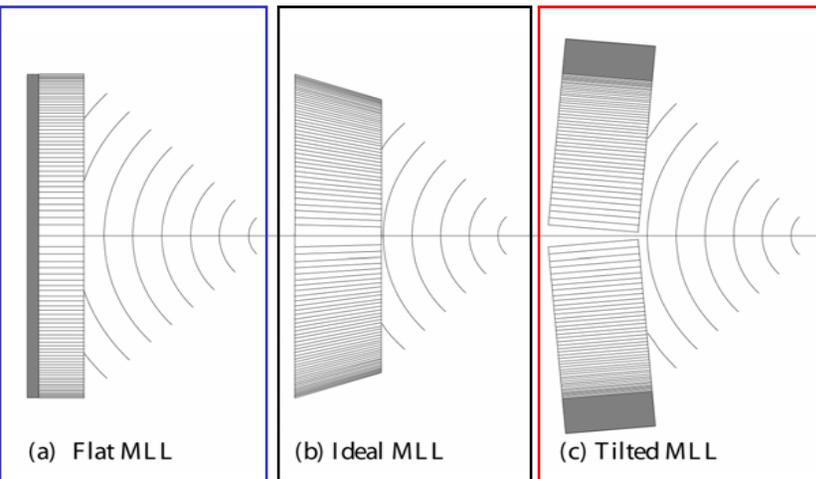




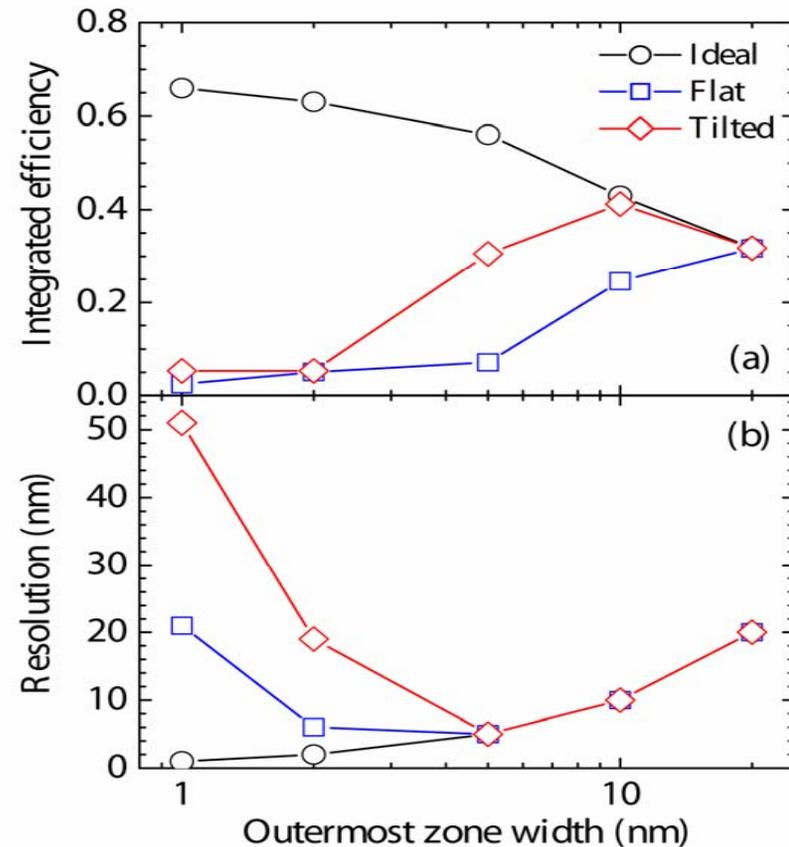
Spatial resolution, sensitivity & radiation damage

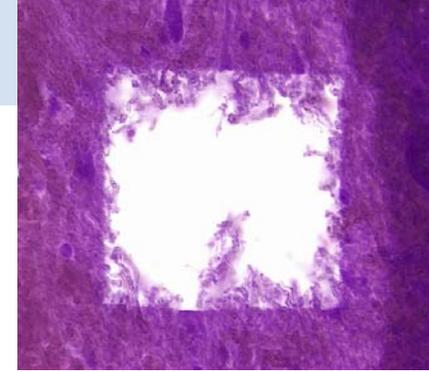
Spatial resolution in a microprobe

- To achieve diffraction limited spot size, **must** illuminate X-ray optics coherently
- => any increase in brilliance directly increases focused flux accordingly
- Currently, high-res X-ray optics ‘routinely’ achieve $\leq 200\text{nm}$; demonstrated on Japanese 1km beamline: $40\text{nm} \times 30\text{nm}$
- ANL demonstrated with new Multilayer Laue Lens $< 30\text{ nm}$ in 1D (Maser *et al*)



- Ideal structure:
 - Resolution approaching 1 nm feasible,
 - Diffraction efficiency (2D) $> 50\%$
- Tilted MLL: $\delta = 5\text{ nm}$ feasible





Sensitivity, spatial resolution and radiation damage:

- Exciting optics developments: <10 nm spatial resolution seems achievable, but what about radiation damage ?
- From soft X-ray microscopy, Limit is $\sim 10^{10}$ Gy, corresponding to:
 - focused flux density of 10^{13} ph/s/ μm^2 at 10keV (we currently have 10^{11} ph/s/ μm^2)

minimum detectable Zn [#atoms], limited by rad damage:

- Today (100 mA, 3.0 nm, UA, L=2.4 m)
- XRF detector collects 6% of $4\pi\text{SR}$

- Upgraded (200 mA, 1.0 nm, UA, L=8.0 m), = 40x more coherent flux
- plus assume XRF detector collects 30% of $4\pi\text{SR}$

	Spot size		
sample thickness [μm]	200 [nm]	20 [nm]	5 [nm] (0.1s)
0.1 [μm]	3500	35	15
10 [μm]	26000	260	60

	Spot size		
sample thickness [μm]	200 [nm]	20 [nm] (0.03s)	5 [nm] (0.002s)
0.1 [μm]	180	6	4
10 [μm]	1800	50	25

10 keV incident beam energy, biological sample in water (frozen hydrated)

APS upgrade vs detectors & optics ?

- Most of the improvement in sensitivity comes from detector and optics improvement. What role does APS (ring & ID upgrade) play ?

SPEED !!! - only this will make certain experiments feasible

- Example scan of a cell nucleus (5x5 microns)
 - Upgraded (200 mA, 1.0 nm, UA, L=8.0 m)
 - plus assume XRF detector collects 30% of 4π SR
- Today (100 mA, 3.0 nm, UA, L=2.4 m)
- XRF detector collects 6% of 4π SR

dwel time [s]	resolution [nm]	scan time [h]
1	200	0.2
1	20	17.4
0.1	5	27.8

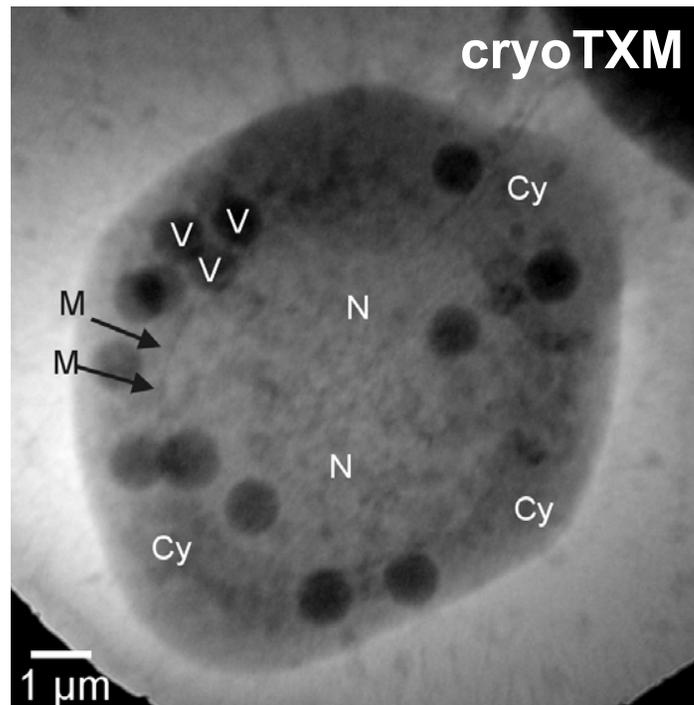
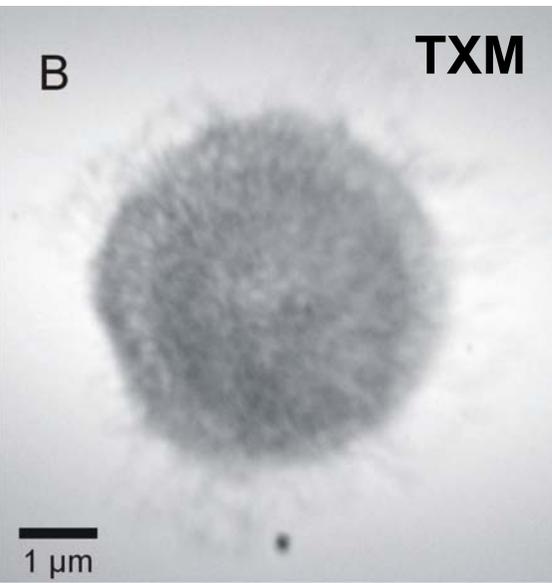
dwel time [s]	resolution [nm]	scan time [h]
1	200	0.2
0.03	20	0.5
0.002	5	0.6

Other Future requirements:

Sample Preservation !

- study cells / tissues as close to their native, hydrated state as possible:
 - avoid artifacts introduced by chemical fixation / drying
 - reduce radiation damage, in particular to oxidation state
- ➔ elemental mapping of rapid frozen samples at cryogenic temperatures (LN2)

D. *Melanogaster* cell, chemically fixed, extracted, at room temp.



- *Drosophila melanogaster* cell, in vitrified ice, imaged @ 0.5 keV with the Goettingen TXM @ BESSY I.

Cy: cytoplasm
V: vesicle
M: nuclear membrane
N: nucleus

Summary: Hard x-ray fluorescence microscopy for biological systems

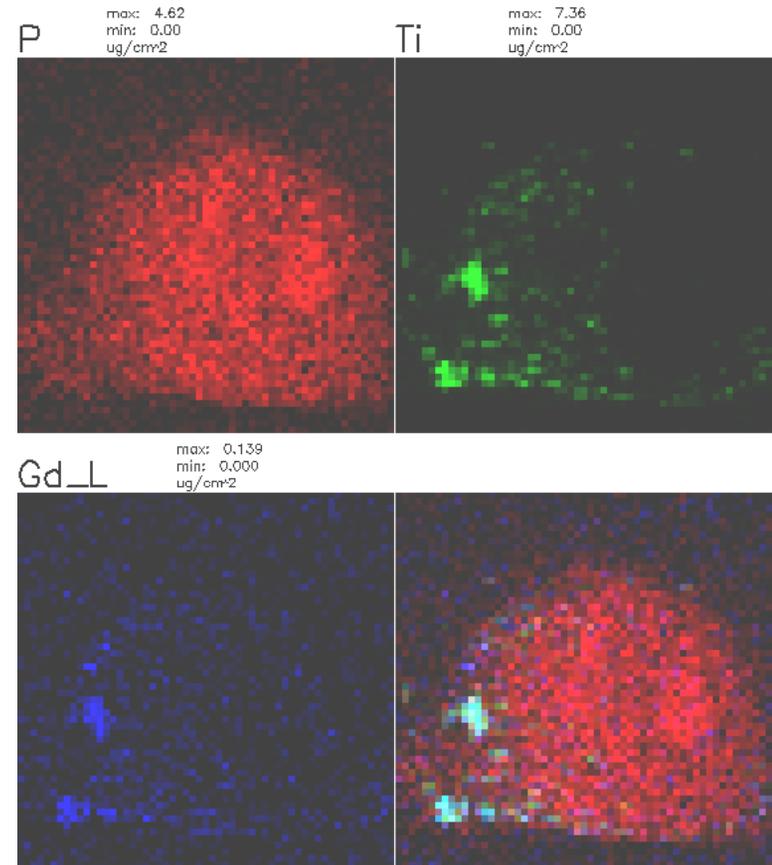
- Focus hard x-rays on sample, raster scan sample through focal spot, collect characteristic x-ray fluorescence at each position to determine elemental content
- Current spatial resolution: 150 nm
- Large penetration depth \Rightarrow Unsectioned cells, tissue section
- X-ray induced x-ray fluorescence
 - *High trace element sensitivity (10^{-18} g) for medium to high Z elements*
 - *Quantification to ppm level for most metals*
 - *Combine with micro-XANES to determine speciation*

APS upgrade:

- Is required, to allow acquisition of high resolution data in realistic timeframe
- But needs to be accompanied by improvements in
 - Optics (high spatial resolution, stability)
 - Specimen Preparation / Environment (cryo)
 - Detectors (large solid angle)
 - Data analysis (quantification, automation)
 - Correlative experiments with other techniques (IR, visible light, EM, ..)

5 nm spatial resolution then seems achievable, with sensitivity down to ~3 Zn atoms, for biological samples

Enable future experiments, that, detect and map single nanovectors in cells and tissues (e.g., combine Gd base MRI contrast agent, with TiO₂-DNA active component)



Proposed new beamline: The BioNanoprobe

- Dedicated to Life Sciences
- **Energy range: 5-35 keV, optimised for 5-20 keV (Ti – I K edge spectroscopy, Cs – U L edge spectroscopy)**
- **mapping mode: lateral spatial resolution: ≤ 20 nm (5nm) , estimated minimum detection level: ~ 5 Zn atoms in 1s in a thin sample (~ 3 Zn in <10 ms).**
- **μ -XANES mode: lateral spatial resolution: ≤ 50 nm (high spectral resolution, crystal monochromator)**
- cryogenic specimens, **in-vacuum**
 - reduce **radiation damage**
 - **avoid** chemical fixation, and correlated **artifacts**
- **fluorescence tomography**

