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X-ray fluorescence microscopy with a nanoprobe – Opportunities and requirements from a life sciences perspective

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X-ray Science Division

Advanced Photon Source

Acknowledgements

- Lydia Finney, Barry Lai, Martin de Jonge, Joerg Maser, Konstantin Ignatyev, Qun Shen; ANL
- Christian Holzner, Benjamin Hornberger, Chris Jacobsen, Stony Brook Univ.
- Julia Diaz, Georgia Institute of Technology
- Hugh Harris, Peter Lay, Univ. Sydney
- Peter Ingram, Ann LeFurgey, Duke Univ / VA
- Brad Palmer Univ. Vermont
- Tanja Paunesku, Gayle Woloschak, Northwestern Univ
- Tom O'Halloran, Northwestern Univ

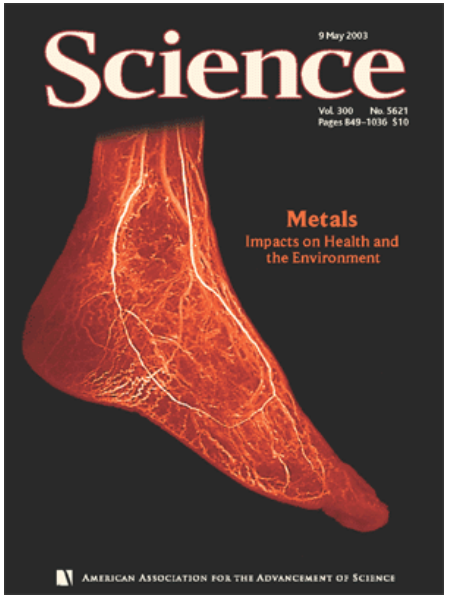
$$$: U.S. Department of Energy, Office of Science,
Office of Basic Energy Sciences

Outline

- Introduction
- X-ray fluorescence microscopy
- Instrument considerations
  - (optics) Hanfei Yan later today
  - (detectors) BNL excellently positioned – P. Siddons *et al*
  - Cryo (Chris Jacobsen later today)
  - Radiation damage & sensitivity
  - Visualizing ultrastructure (Chris Jacobsen later today)
  - Tomography
- Science examples
  - Cr carcinogenesis
  - Nanocomposites
  - Avoiding oxidative stress – radiation resistance
- Conclusion

Why Study trace metals in environmental and life sciences?

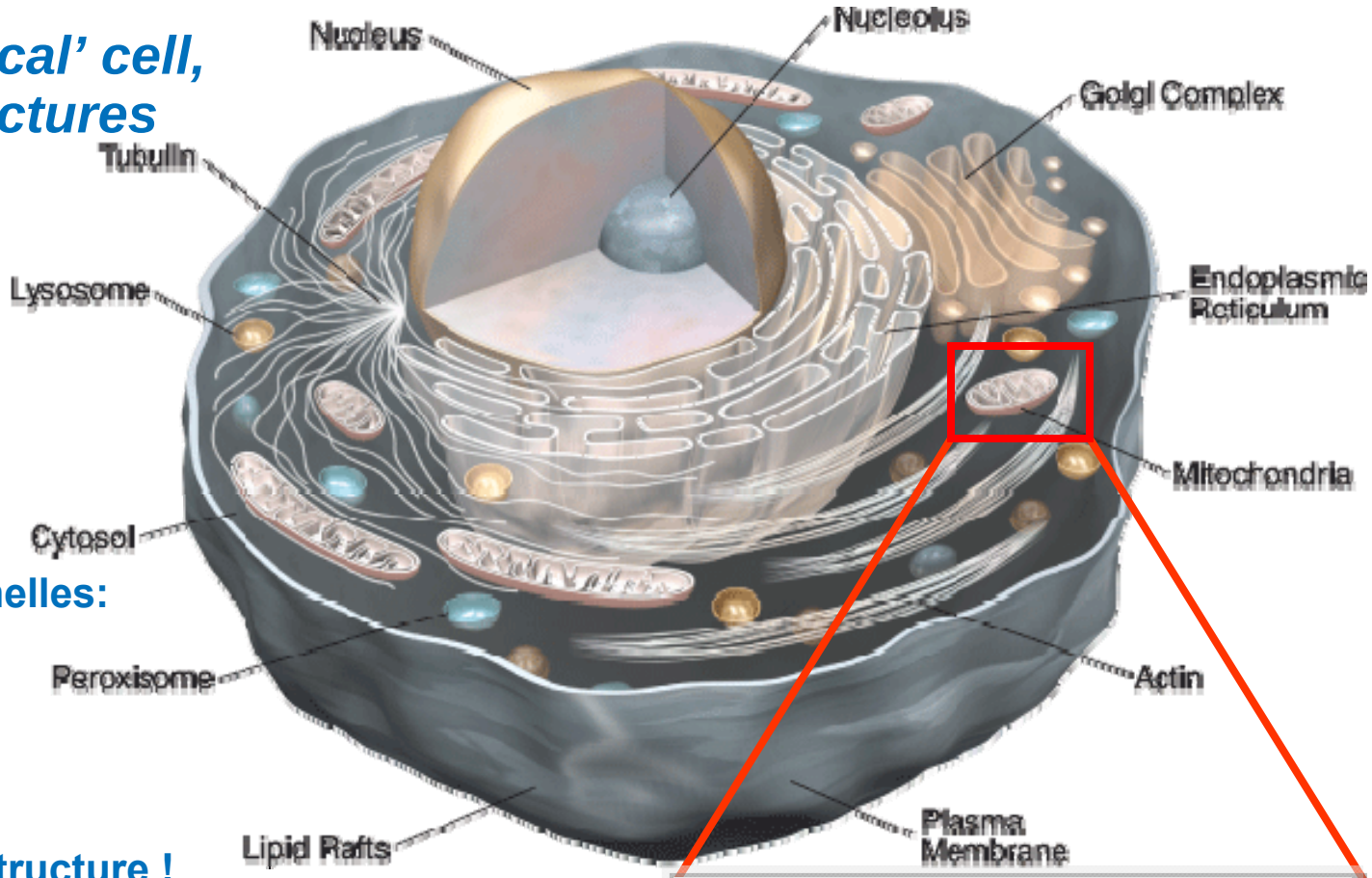
- Trace elements (metals) are fundamental, intrinsic components of biological Systems. estimated: 1/3 of all known proteins contain metalcofactors as integral, catalytic components. These proteins often have regulatory or catalyzing functions, e.g.,
  - Zn in Zinc finger proteins: transcription factors 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, Wilson disease (Cu accumulation)
  - Exogenous uptake, e.g., Pb, As, Hg
  - Bio-remediation
- Metals can be made use of in therapeutic drugs and diagnostic agents
  - Cis-platin in chemotherapy
  - Gd in Magnetic resonance imaging (MRI)
  - Novel bio-inorganic nanoparticles
    - *Nanomedicine: multifunctional nanovectors ideally combining targetting, therapy (e.g., Pt, TiO_2) and diagnosis (e.g., Gd)*



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

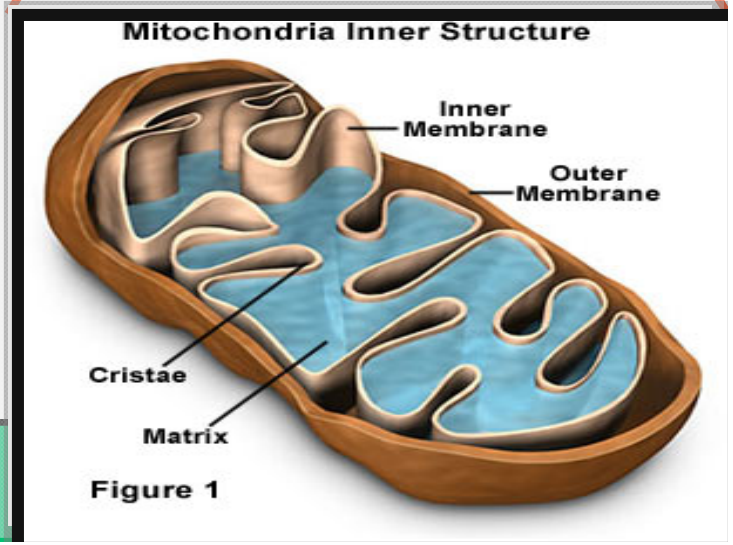
➔ ***study distribution and quantity of these elements within cells to understand how they act***

Reminder: a 'typical' cell, and its (sub) structures

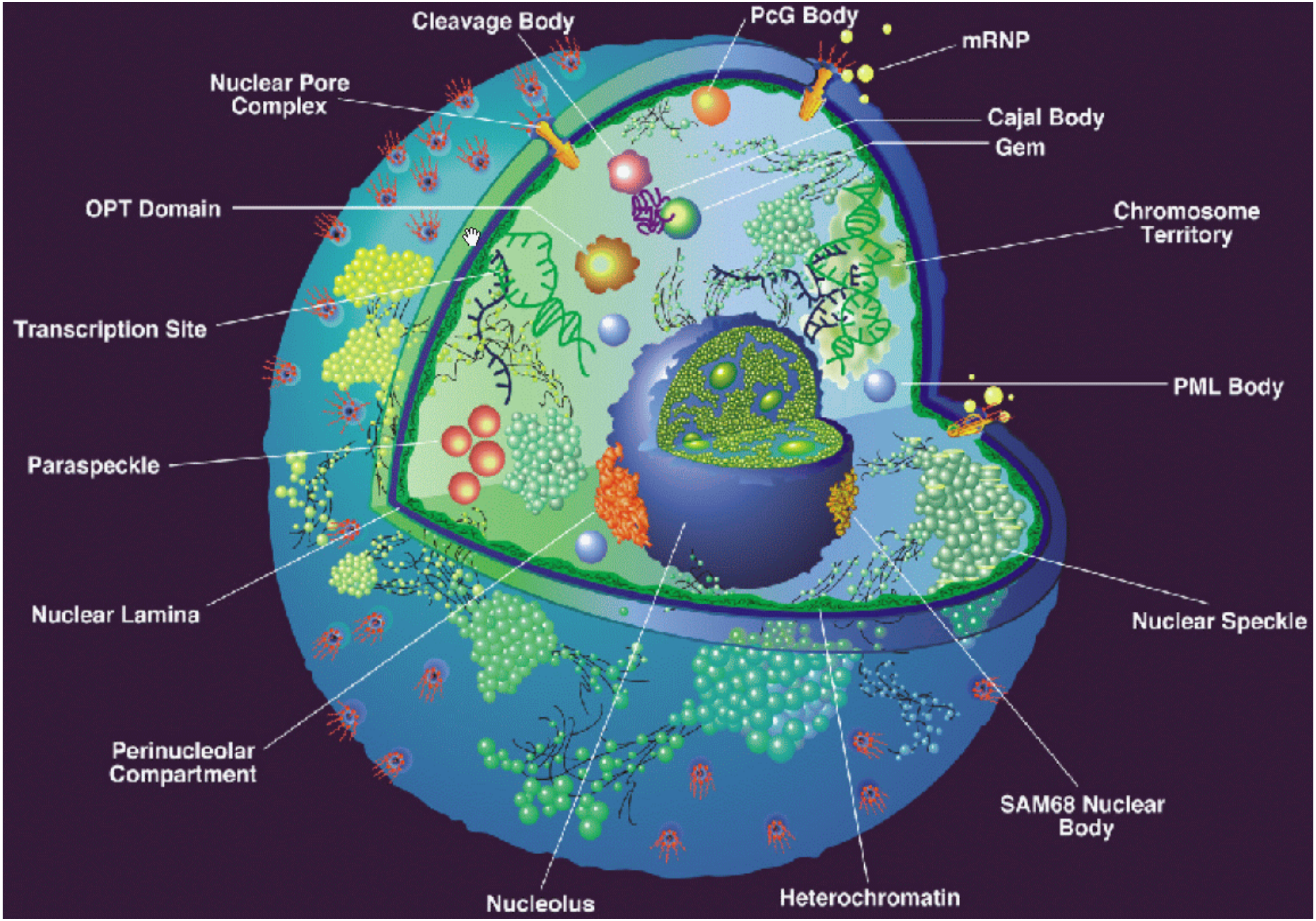


Typical sizes of cell structures and organelles:

- **nucleus: 2-5 µm**
- **nucleolus: 1 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)**
- **microtubuli: 20 nm diam. (cytoskeleton)**
- **double membrane thickness: 8 nm**



The cell nucleus also has structure:



Periodic table highlighting X-ray fluorescence

K-line Fluorescence typically used

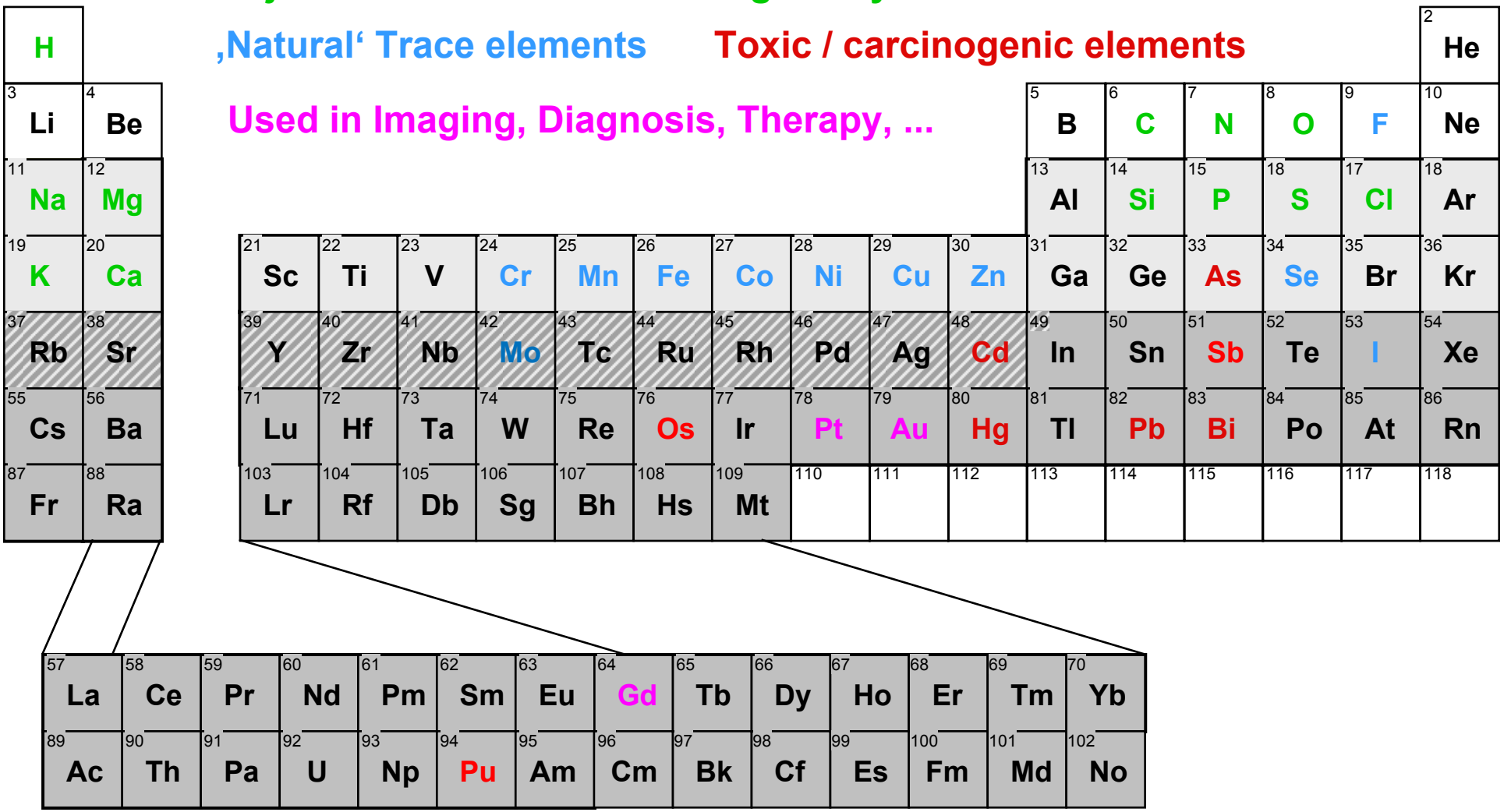
L-line Fluorescence typically used

Major/minor elements in Biological Systems

‚Natural' Trace elements

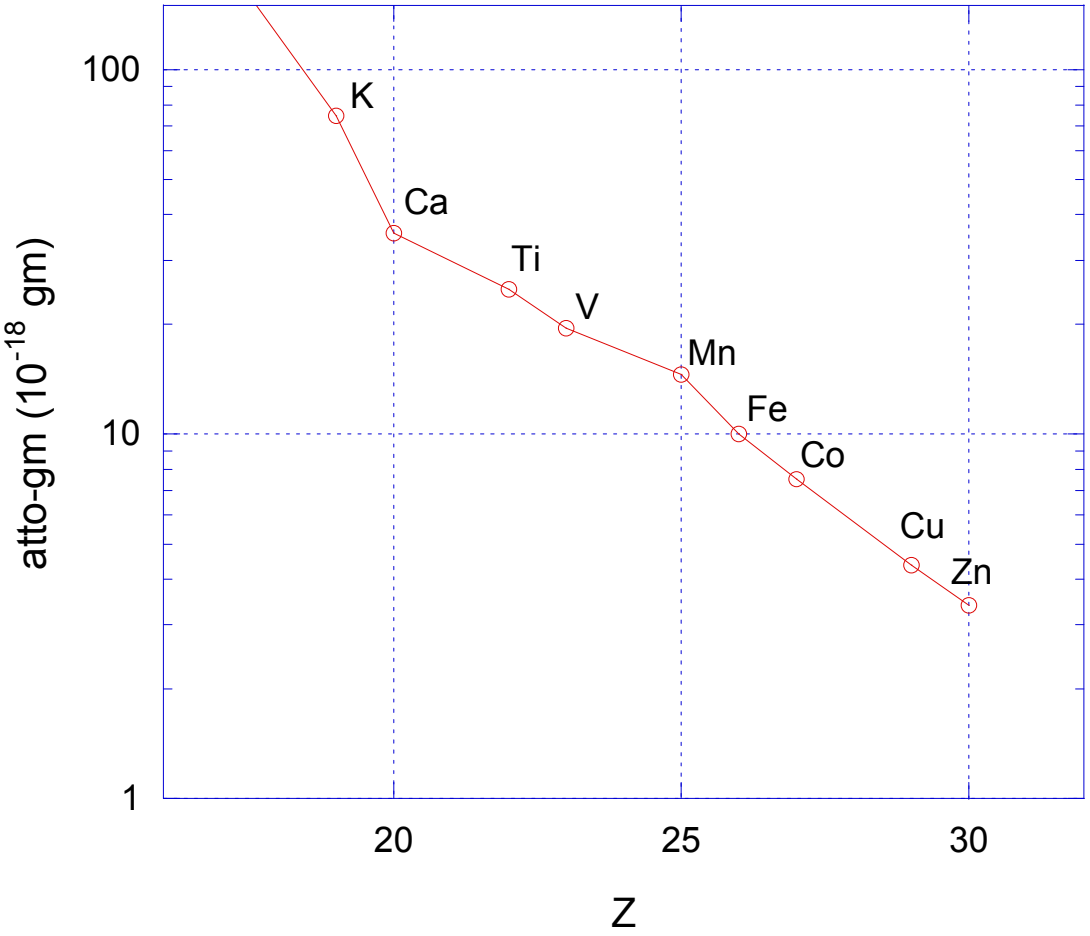
Toxic / carcinogenic elements

Used in Imaging, Diagnosis, Therapy, ...



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 x 0.2 μm² spot, E=10 keV

The right tool for the job ?

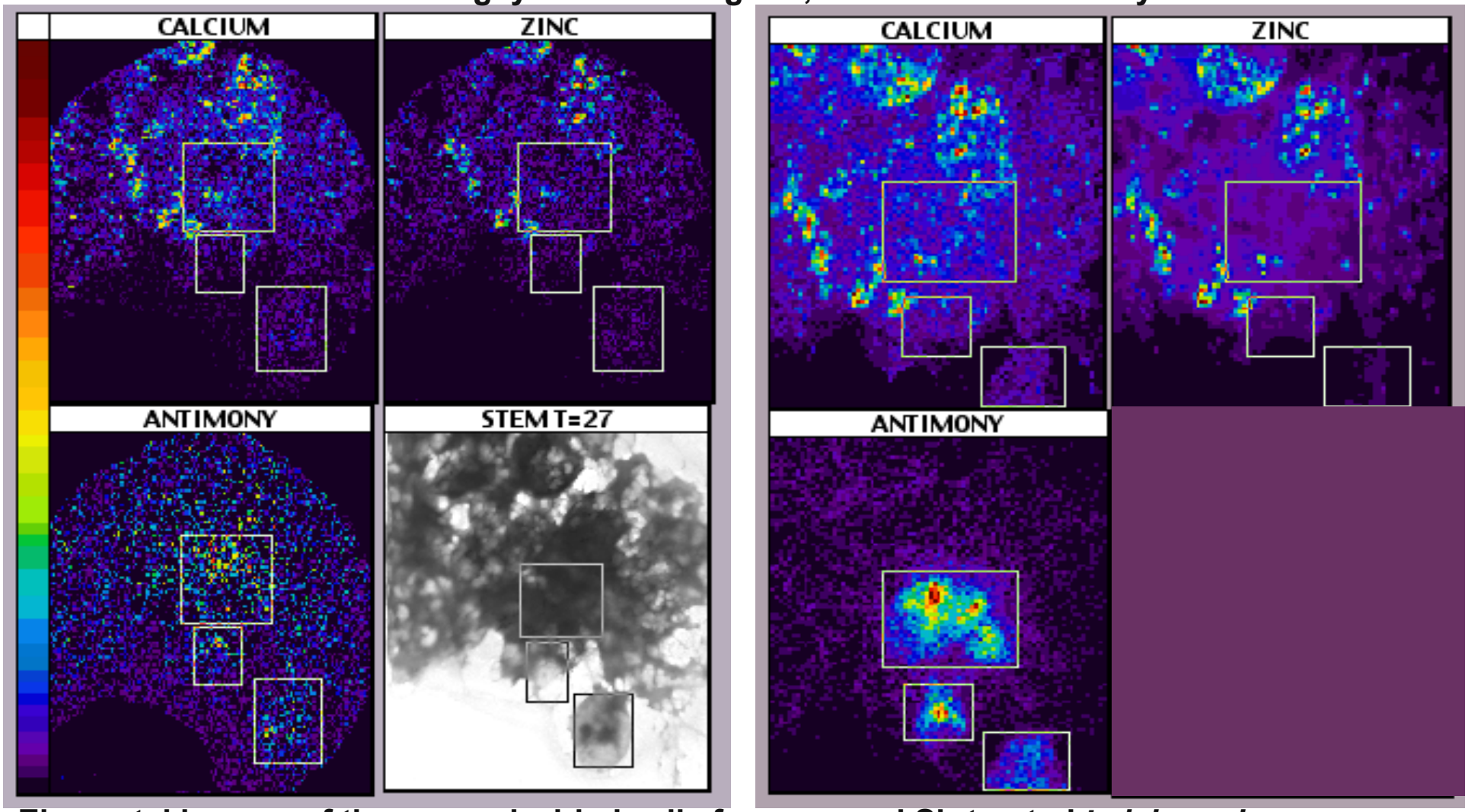
from www.cartoonstock.com/

Comparison of some techniques for trace element mapping:

	Spatial Resol.	object thick.	Res. Limit.	Advantages/Disadvantages
Light-microscope	200 nm	30 µm	Wave-length	+ changes in living cells can be monitored, but competition w. proteins +/- only see ions (in solution), and not total content - need dyes - quantification difficult
Hard X-ray-microprobe	**200 nm-20nm**	**10 µm**	**Curren-tly Optics**	**+ no dyes, visualize total elemental content** **+ very high sensitivity, low background, selective excitation, little rad. Damage** **+ high penetration depth (but limited DOF for high res)** **+ simultaneously detect >10 elements, select excitation** **+ µ-XANES for chemical state mapping, -slow**
Analytical Electron-microprobe	20 nm	0.1 µm	object thickn.	+ high spatial resolution + simultanously detect >10 elements - thick samples very difficult, sectioning necessary - slow - radiation damage
EELS/ EFTEM	2 nm	0.005 - 0.05 µm	Rad. Damage	+ very high spatial resolution - require ultrathin sections - only some elements readily accessible (e.g., P, Fe) -co-registration can be difficult (EFTEM), slow (EELS)

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: 10μm.

Instrumentation considerations

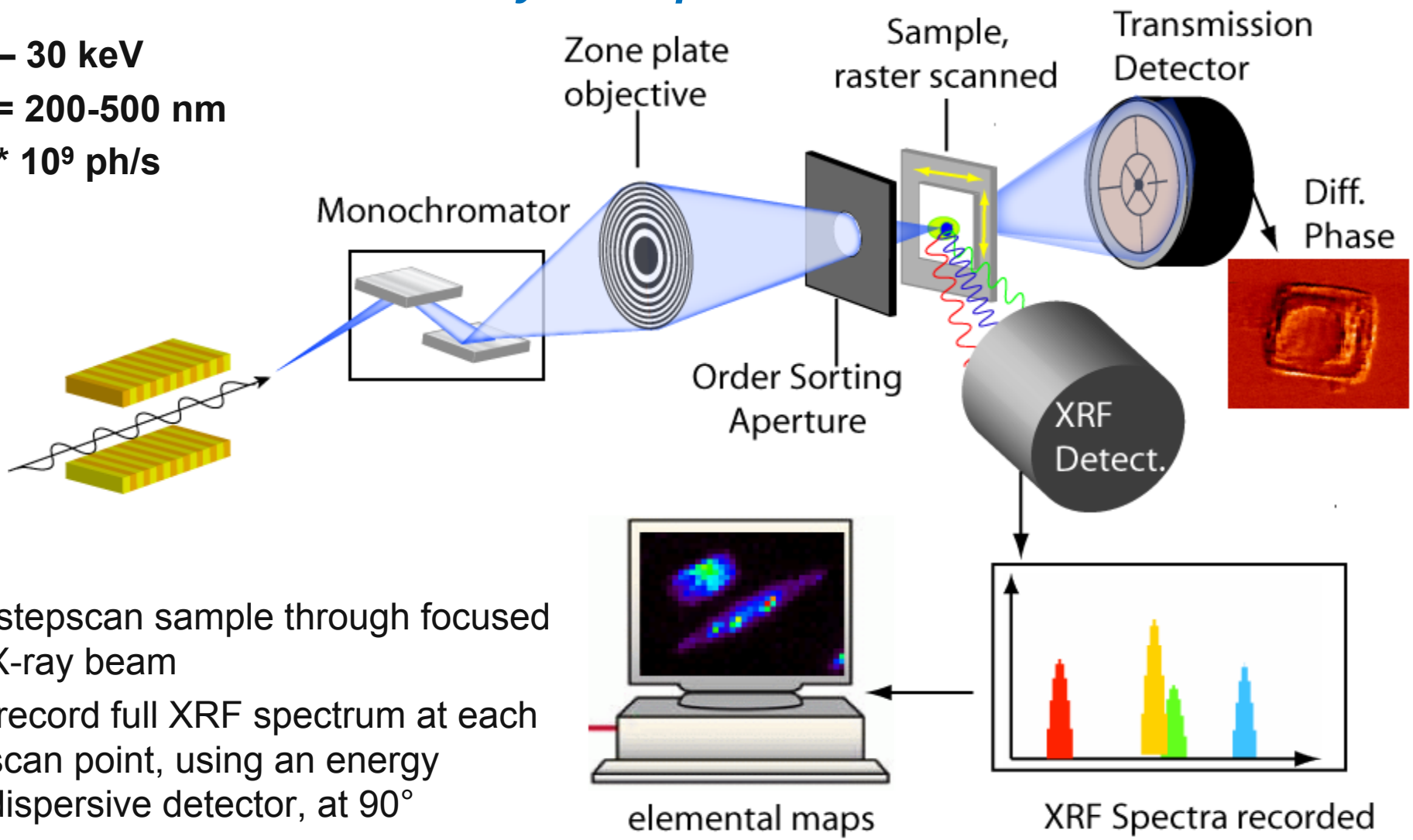
Schematic of a Hard X-Ray Microprobe

schematic NOT to scale !!

5 – 30 keV

δ = 200-500 nm

5 * 10^9 ph/s



- stepscan sample through focused X-ray beam
- record full XRF spectrum at each scan point, using an energy dispersive detector, at 90°

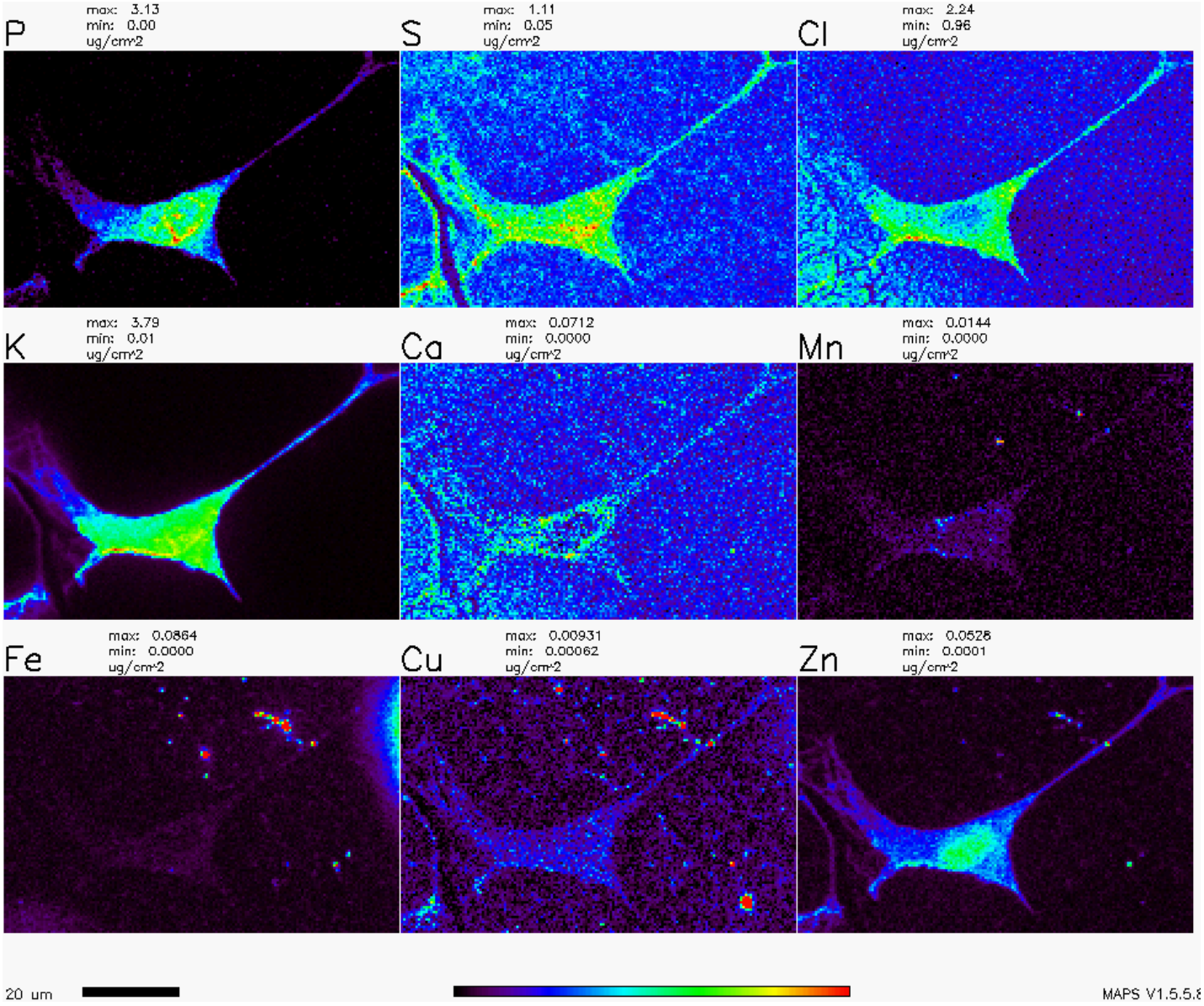
- Straightforward quantification: compare specimen counts/spectra to calibration curve, to quantify to area density

Cryogenic sample preservation & imaging

Need cryo to preserve specimen structure & chemistry (unaltered) at high spatial resolution

-must allow loading of prefrozen samples

HMVEC cell, plunge frozen in liquid ethane, freeze dried

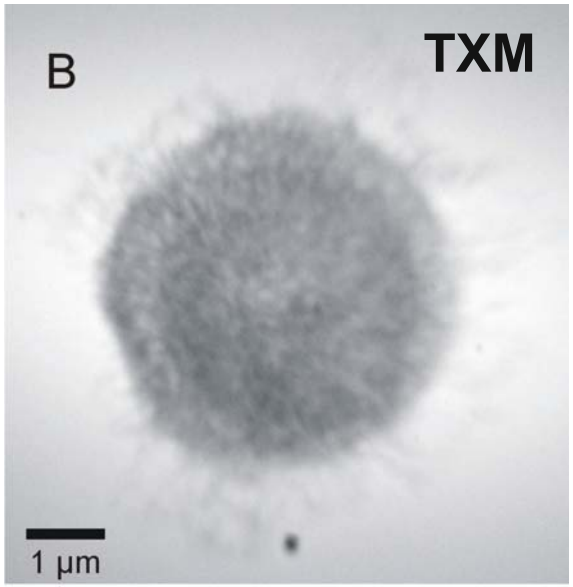


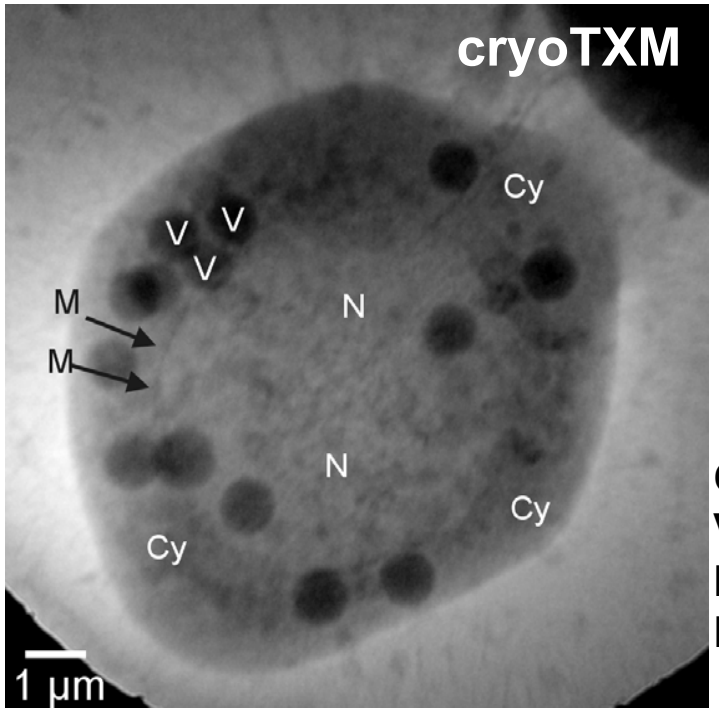
Sample Preservation !

- 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.





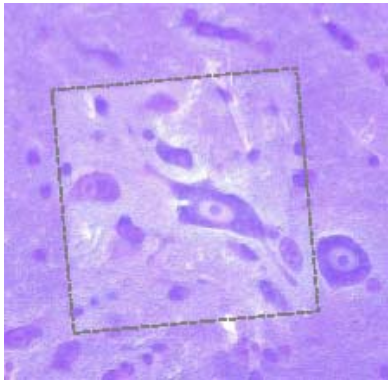
- *Drosophila melanogaster* cell, in vitrified ice, imaged @ 0.5 keV with the Goettingen TXM @ BESSY I. S. Vogt, *et al*

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

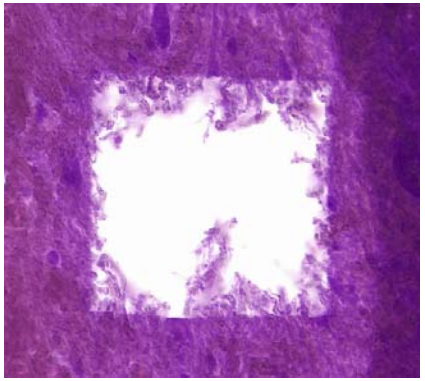
Sensitivity, spatial resolution and radiation damage:

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

Fixed (p-formaldehyde), paraffin, scanned, rehydrated



Freeze dried (unfixed), scanned, rehydrated



minimum detectable Zn [#atoms], in 1s or limited by rad damage:

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

- **Today** (100 mA, 3.0 nm,UA, L=2.4 m)
- XRF detector collects 6% of 4πSR

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

- APS upgrade, 40x more coherent flux
- plus XRF detector collects 30% of 4πSR

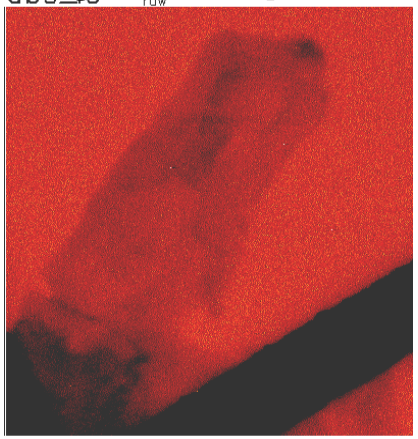
	Spot size		
sample thickness [um]	200 [nm]	20 [nm] (0.03s)	5 [nm] (0.002s)
0.1 [um]	180	6	4
10 [um]	1800	50	25

Depth of field:

10keV	200 [nm]	20 [nm]	5 [nm]
DOF +/-[um]	433	4.3	0.3

What about ‚soft' (low-Z) structures ?

X-ray absorption

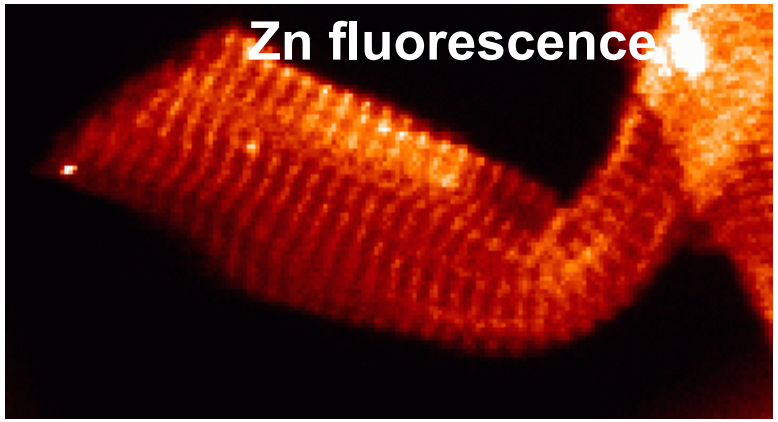


Hard X-ray microscopy: great sensitivity for medium/high Z elements, but mapping of biological mass and structure (mostly C,N,H) difficult:

- very low photoelectric absorption
- very low fluorescence yield

at the same time:

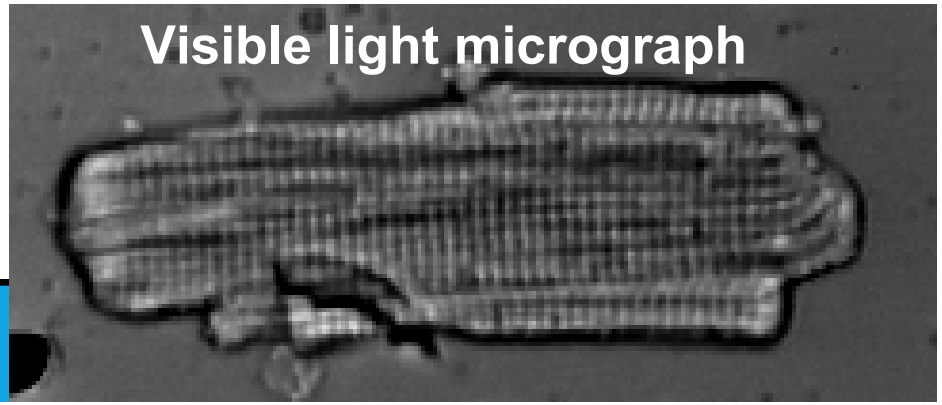
• exact correlation of elemental maps with biological structure critical !!



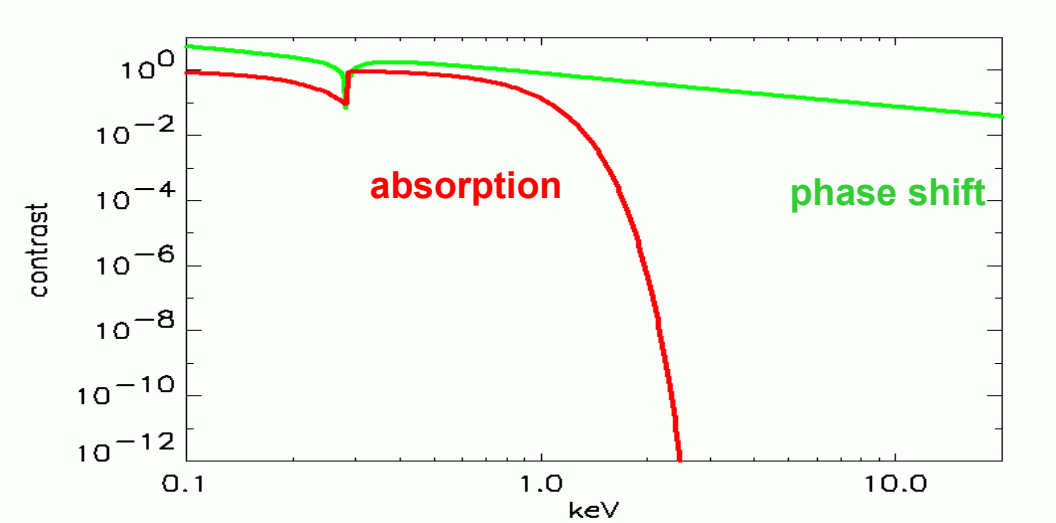
How to correlate element distribution with low-Z structure ?

Are these the same striations ???

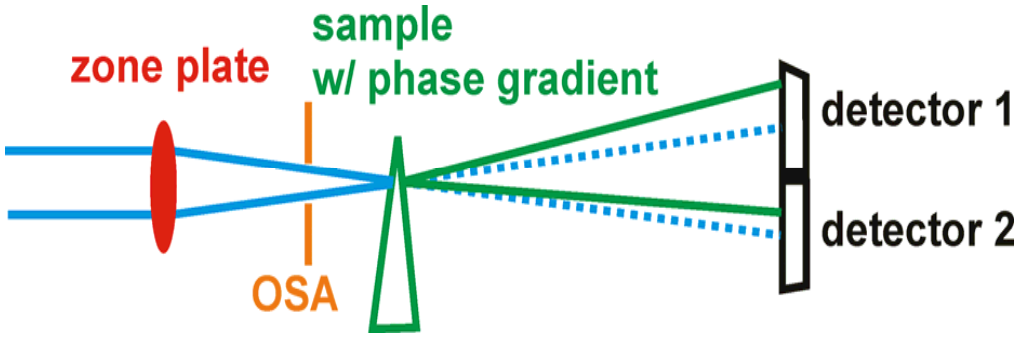
How to determine metal concentrations (normalize metal content by mass)?



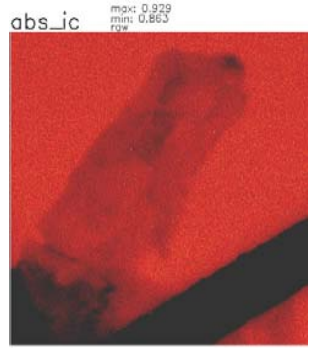
Differential Phase Contrast to co-localize structure with elemental content & acquire fast overview scans in scanning microprobes



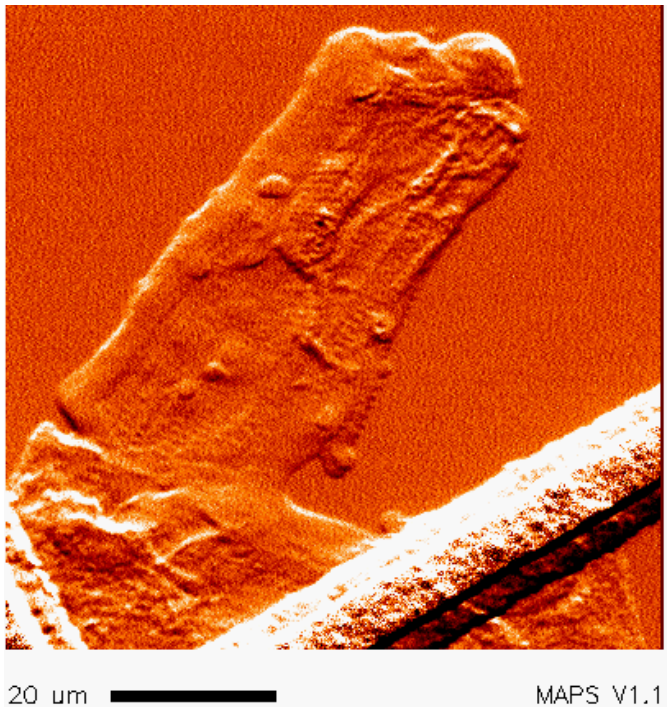
phase and absorption contrast for 1 µm thick carbon structure



to visualize cell structure in hard X-ray microscopy, use phase contrast instead of absorption, e.g., for scanning probe: differential phase contrast



DPC image of cardiac myocyte: shows striations caused by the regular arrangement of myofilaments.



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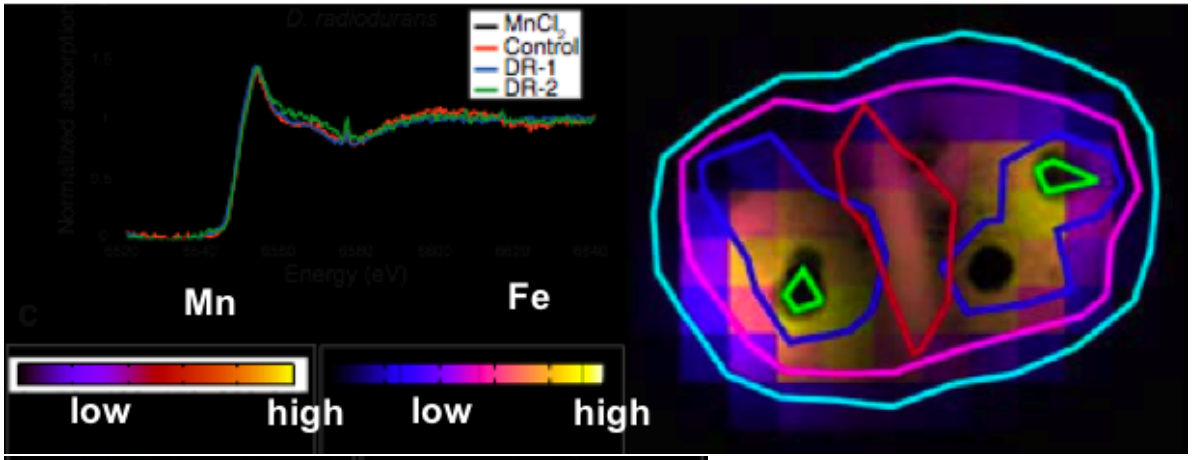
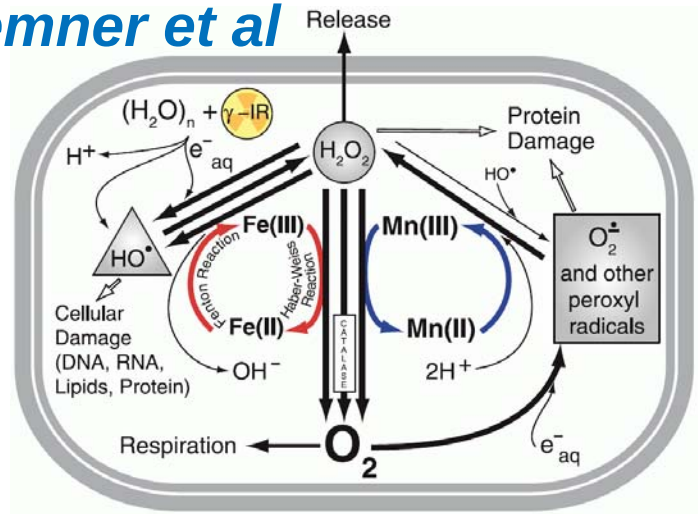
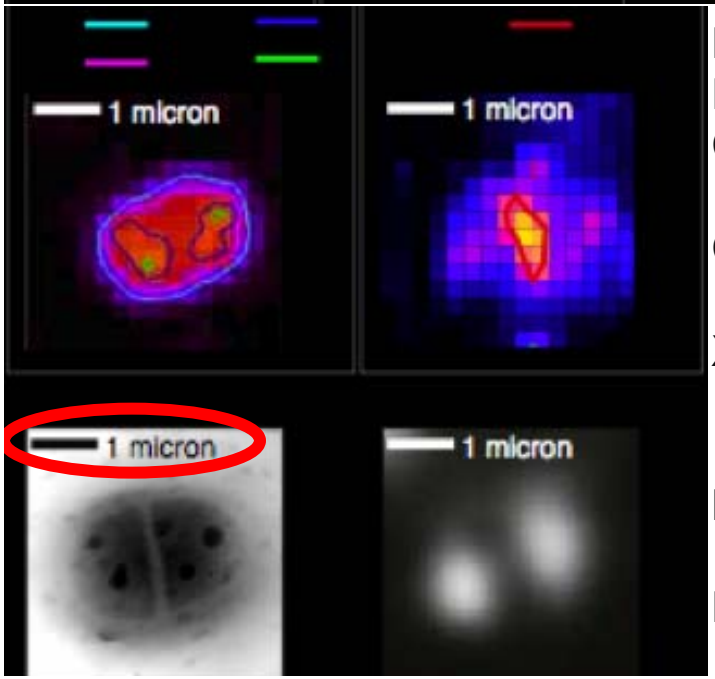
Other approaches to visualise ultrastructure also possible, e.g., soft x-ray microscopy, or coherent diffraction (e.g., C. Jacobsen talk)

Bacterial resistance to radiation

Kemner et al







Radiation-induced cell death often attributed to DNA damage

Fe-facilitated Fenton reaction accentuates damage

Cells with high Mn/Fe ratios (D. radiodurans) resistant to radiation

Cells with low Mn/Fe ratios (S. oneidnesis) less resistant to radiation

XRF microscopy shows

- Mn ubiquitous throughout cell
- Fe (Fenton reaction) between cells

Mn XAFS shows Mn2+ throughout cell

- facilitates superoxide scavanging

Radiation damage caused by protein oxidation during irradiation

Higher spatial resolution: could probe the space between bacteria, their membranes, and single nanosized biomineralization products – something that is currently not possible …

Visualizing single nanoparticles in cells

- Numerous developments to create functional nanocomposites that combine properties for
  - imaging (in its application to humans, e.g. Gd as a contrast agent for MRI)
  - therapy (e.g., TiO_2 with photo-induced cleavage of DNA)
  - targeting (e.g., sequence specific DNA, visualize via optical fluorescence)

(e.g., nanocomposites that target specific genes in cancer cells, can destroy an oncogene, and be visualised ny NMR)

- But: before being able to test on subject, need to confirm in vitro:
  - Do the nanocomposites enter the cells ?
  - Do they 'find' the right target ?
  - Do they ONLY interact with the right target (e.g., toxicity) ?
  - Do the different components remain joined ?

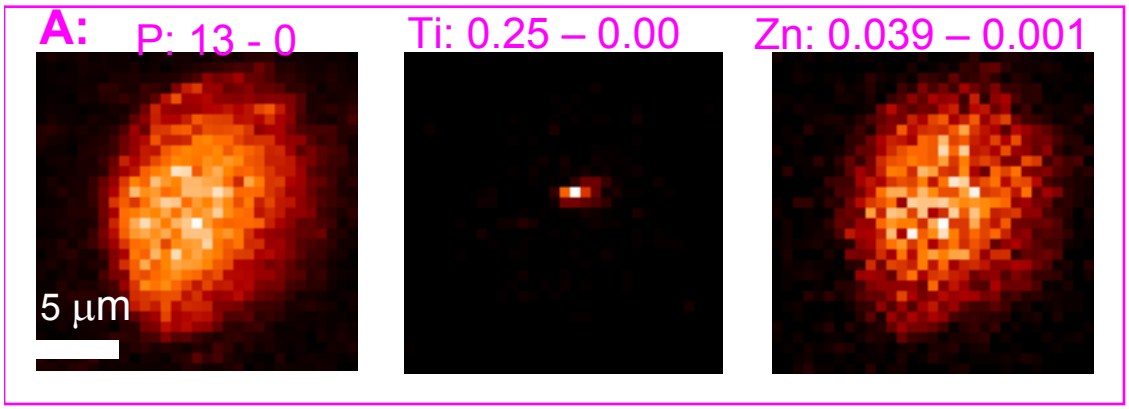
⇒ Need to be able to find and localise a single nanocomposite with 'just a few' active metals.

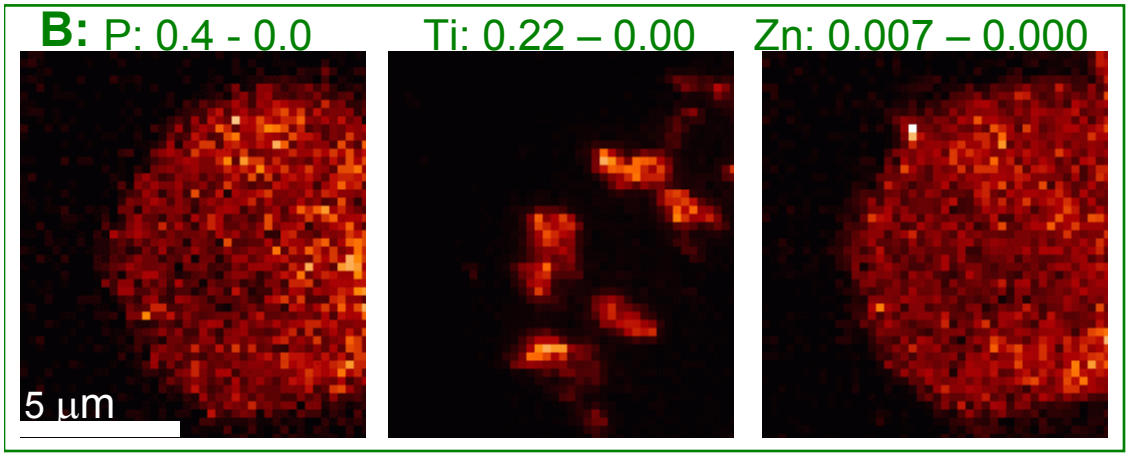
⇒ For sufficient sensitivity, need small (<20nm) beam

⇒ To determine localisation precisely, need <10nm beam (8nm membrane double layer)

⇒ Need to image several whole cells (10x10 μm^2), ideally tomographically

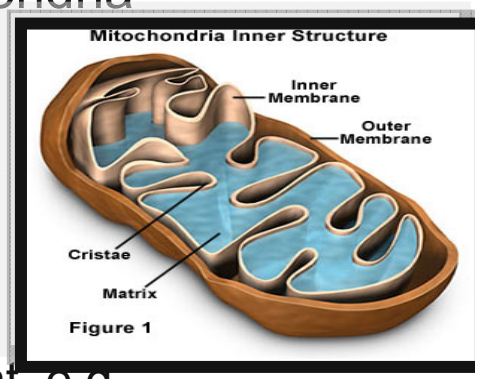
TiO_2-DNA nanocomposites as intracellular probes





Units: μg/cm^2

- A: scan of a MCF7 cell transfected with nanocomposites targeted to nucleolus
- B: scan of a PC12 cell transfected with nanocomposites targeted to mitochondria



Promising Future: Nanocomposites as tools for Gene therapy ?

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

But: need to be able to RESOLVE cellular targets of nanocomposites, to determine specific localisation, and ability to 'see' single nanocomposites

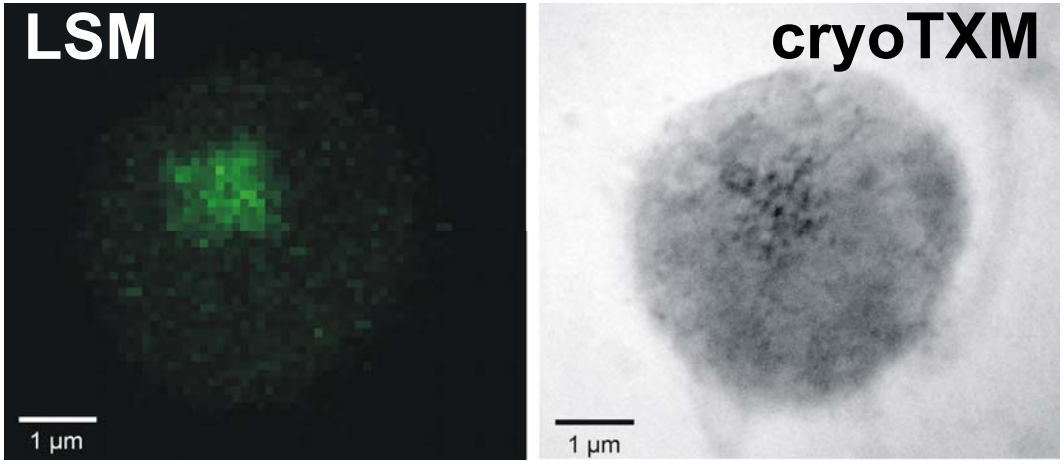
Paunesku *et al*, Nano Lett. 2007; Paunesku et al, Nat. Mat. 2003

Some requirements:

- Energy range: 2-30 (10-13) keV
- High spatial resolution (e.g. <=10 nm)
  - BUT complemented by lower resolution (e.g., 100nm, 1 um), higher flux objectivs
- Tomography
  - Need to use dose fractionation
  - Automated alignment / data acquisition (~>=1000 projections!)
- Detectors:
  - need large solid angle XRF detector to mitigate radiation damage (30% of 4πsr)
    - *Space around sample is tight; problematic to get XRF out (at 90º) for high NA lens, detectors US or DS suffer from increased scatter, reduced sensitivity*
    - *Multi element SDD, fast readout (including 'list mode' that allows combining 'fly scans' with full spectral information. (dev. P. Siddons BNL, C. Ryan, CSIRO)*
  - Need to visualize specimen structure (low Z), e.g., using differential phase contrast in transmission (collab. with C. Jacobsen et al)
- Specimen Environment & Preparation
  - Must have Cryogenic specimen environment
  - Must allow cryogenic sample transfer
  - In line visible light microscope (ideally w. optical fluorescence)

Some requirements (2):

- Specimen Preparation
  - Should have sample prep facilities (ideal: high pressure freezing)
- Enable correlative experiments with other techniques (IR, visible light, EM, soft X-rays...)
  - In particular, Fluorescence light microscopy (e.g., GFP)
  - Common mounting system (kinematic mounts), compatible also with other BLs



- data acquisition and data analysis
  - semi-automation (both acquisition and analysis)
  - GOOD user interface
- Staff
  - adequate staffing level
  - some background/interest in life sciences
  - need to advise users in experiment planning, sample prep, data acquisition, analysis AND interpretation. NOT sufficient to send user home with data!

Some examples of future applications requiring high spatial resolution:

- Environmental Sciences
  - Allow study of metal-influenced process on and near bacterial surfaces => improve our understaning on how they interact and influence their environment
- Biology and Life Sciences
  - Map ‘natural’ metals within organelles.
  - Potential to detect and localise individual or at least small clusters of metalloproteins in cells, providing a very exciting tool for cell/molecular biology
    - *in particular: could now probe interactions at cell interfaces and membranes*
- Biomedical
  - Probe elemental content of cytoplasma (host cell), vesicles (phagosome), as well as parasites, to significantly improve our understanding of infectious diseases
- Nanoscience / Nanomedicine
  - Enable future experiments, that detect and map single nanovectors in tissues, cells and organelles. In particular, multifunctional nanovectors that combine targeting (e.g., DNA), therapy (e.g., Pt, TiO2) and diagnosis / imaging (e.g., Gd); correlate exactly to target (<=10nm) – currently impossible
    - *Verify functionality, study mode of action, side effects / toxicity, ….*

<10 nm spatial resolution seems achievable, with sensitivity down to <5 Zn atoms, for thin biological samples. (Limiting factor: radiation damage)

Outlook & Future:

- Outlook is bright, significantly increasing spatial resolution for x-ray microprobes seems possible
- Great science that can make excellent use of improved resolution

- But, spatial resolution must be matched by other instrument improvements
  - Useability (instrument & subsequent analysis!)
  - Sensitivity (detectors)
  - Facilitate correlative work with other technqiues