Subcellular Imaging of Trace Metal Distribution and Chemistry by X-Ray Microfluorescence

***B. Lai*, S. Vogt, J. Maser, Z. Cai, D. Legnini**

Advanced Photon Source

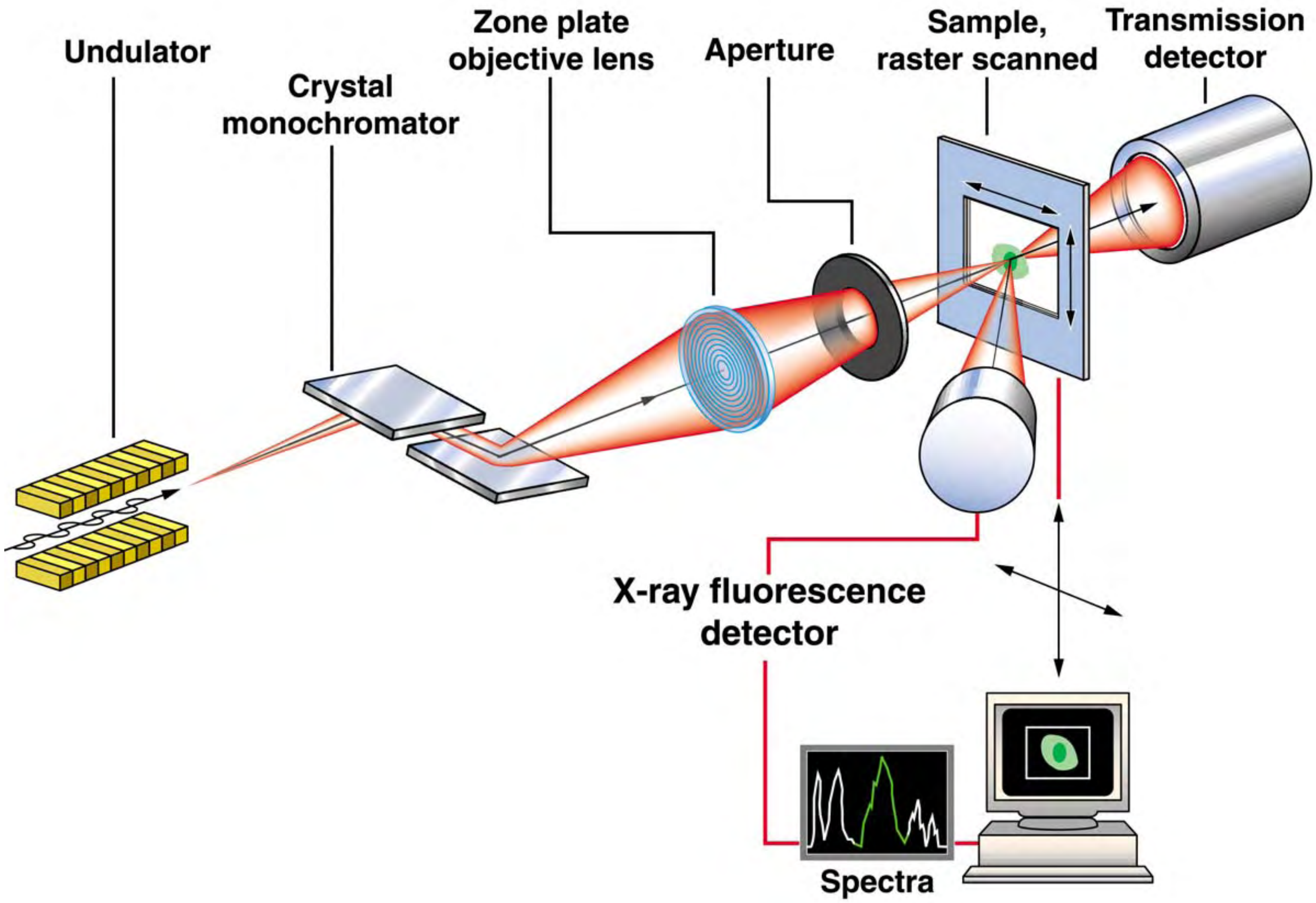
Outline:

1. **X-ray fluorescence microprobe**
   - **instrumentation**
   - **unique capabilities**

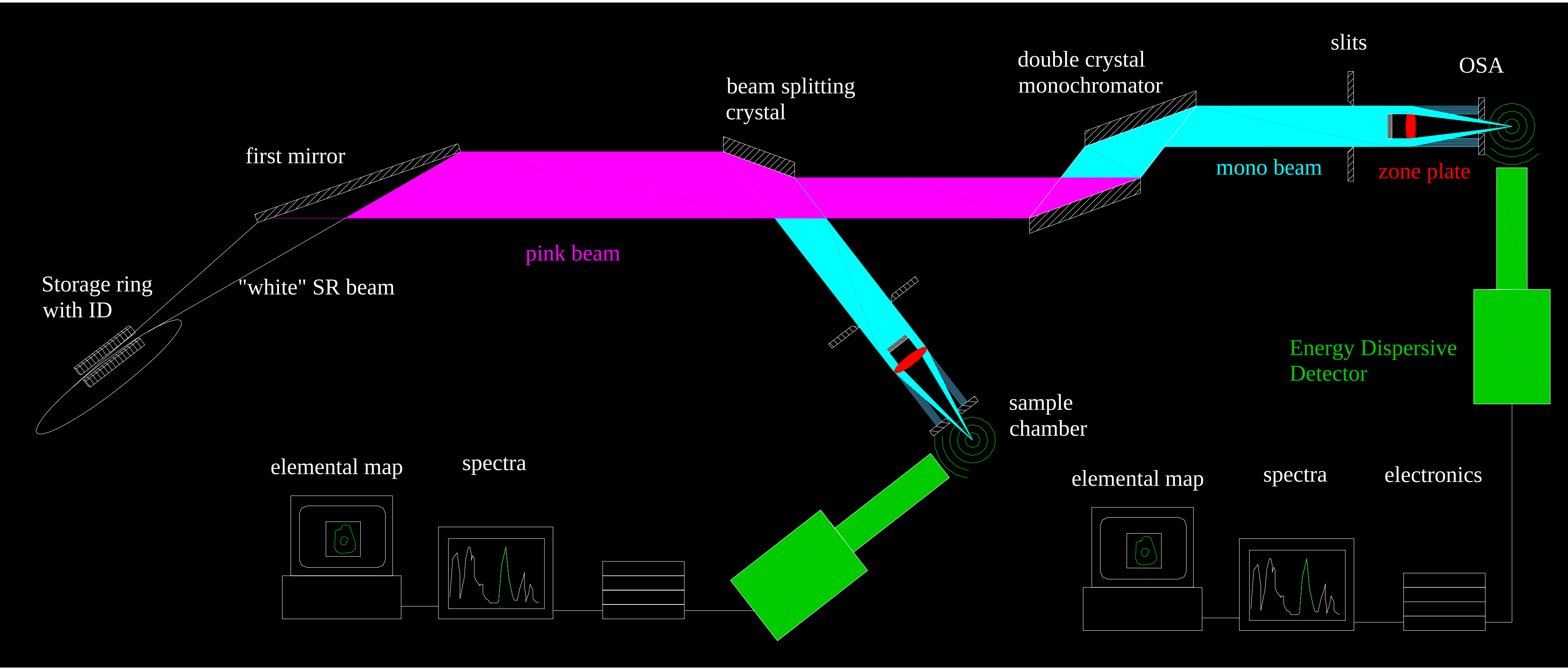
2. **Biological applications**
   - **exogenous elements**
   - **endogenous elements**

3. **Future prospects**
   - **resolution, detection limit, challenge**

Schematic of Scanning X-Ray Fluorescence Microprobe



Hard X-ray Microprobe Facility, APS sector 2

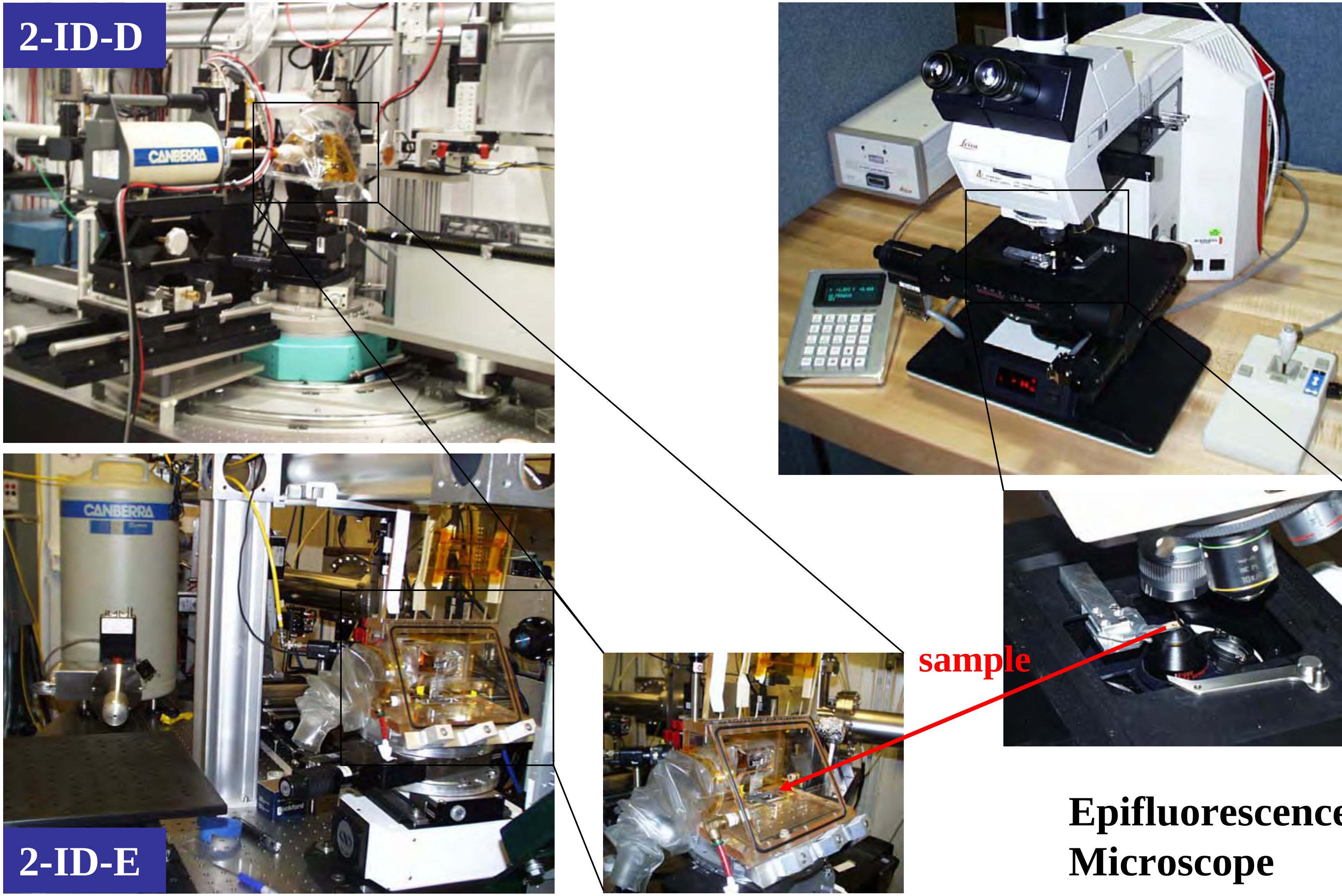


Main branch 2-ID-D: E = 5 – 30 keV, δ = 150 nm ↔ $2\cdot10^9$ phot/s, ~ 1,500 hrs

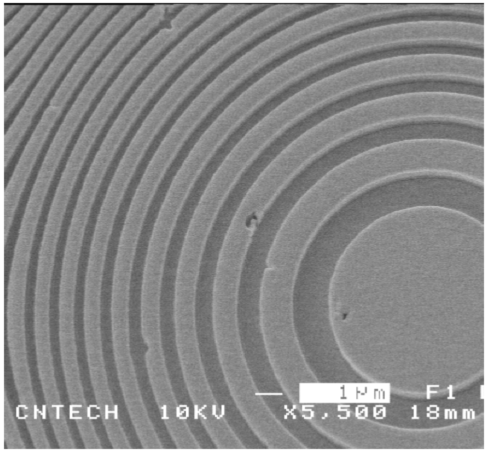
Side branch 2-ID-E: E = 7 – 17 keV, δ = 250 nm ↔ $5\cdot10^8$ phot/s, ~ 2,500 hrs

Integrated epi-fluorescence microscope

2-ID-D/E Hard X-ray Microprobe Facility



Performance of Fresnel Zone Plates



Parameters: Δr = 100 nm
f = 12.9 cm @ 8keV
N = 400 zones
160 μm diameter
1.6 μm thick Au

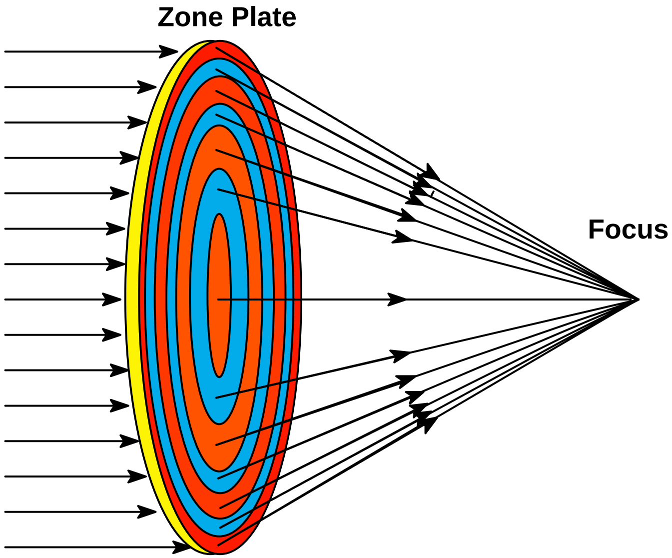
Spatial Resolution = 150 nm FWHM

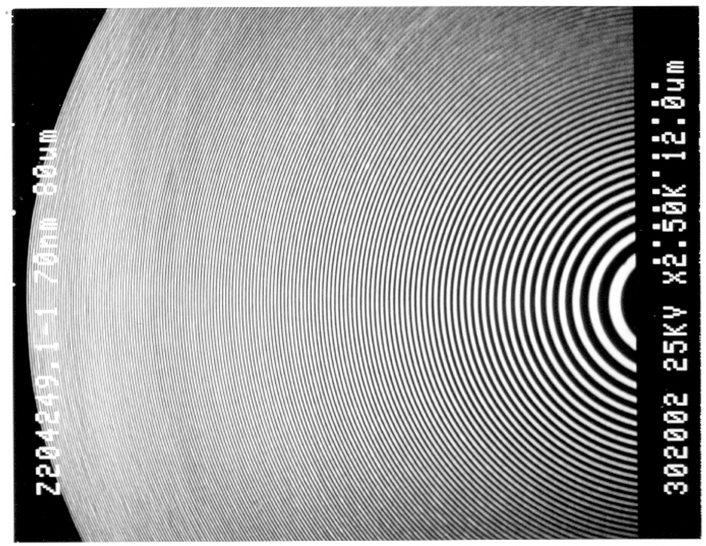
Efficiency = 20%

Flux density = 2 x 10^{11} photons/sec/μm^2/0.01%BW
= 2,000 photons/sec/$Å^2$

Flux density gain = 3 x 10^4

Challenge in Fabricating High Resolution Optics

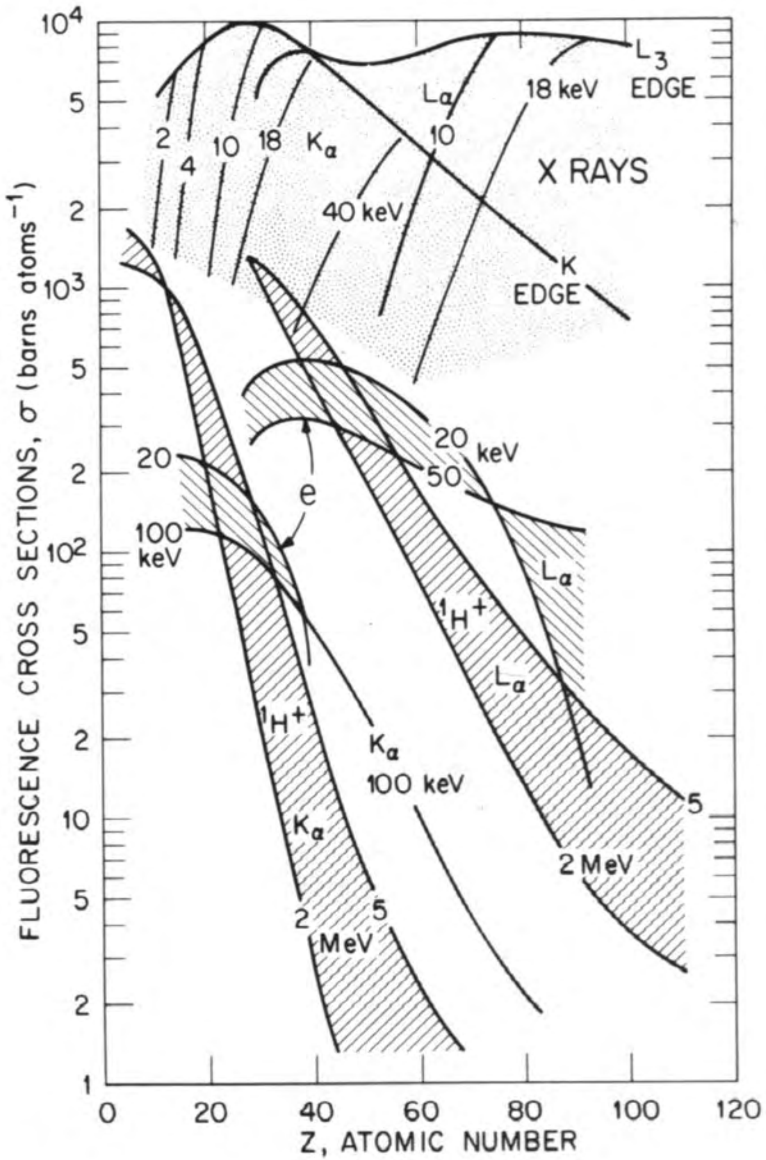




- Spatial resolution related to outermost zone width: $\delta = 1.22\ \Delta r_n$
- Good diffraction efficiency requires large thickness: t = 1.5 μm Au, @ 8 keV
- Nanostructuring challenge: Large aspect ratio $t/\Delta r_n \sim 2000$, $\Delta r_n = 0.8$ nm

⇒ **Need to invest in developing high resolution optics**

Why use x-rays to excite fluorescence for studying trace metals?



- Higher fluorescence cross sections
- Better signal/background ratio
  - ⇒ sub-ppm (part-per-million) sensitivity
  - ⇒ quantitative
- Less radiation damage
- Large penetration depth (> 100 μm)
  - ⇒ simple sample preparation
  - ⇒ can study whole cells, no thinning
  - ⇒ can study hydrated "natural" samples
- Selectively excite one particular element
- Map chemical states by XANES

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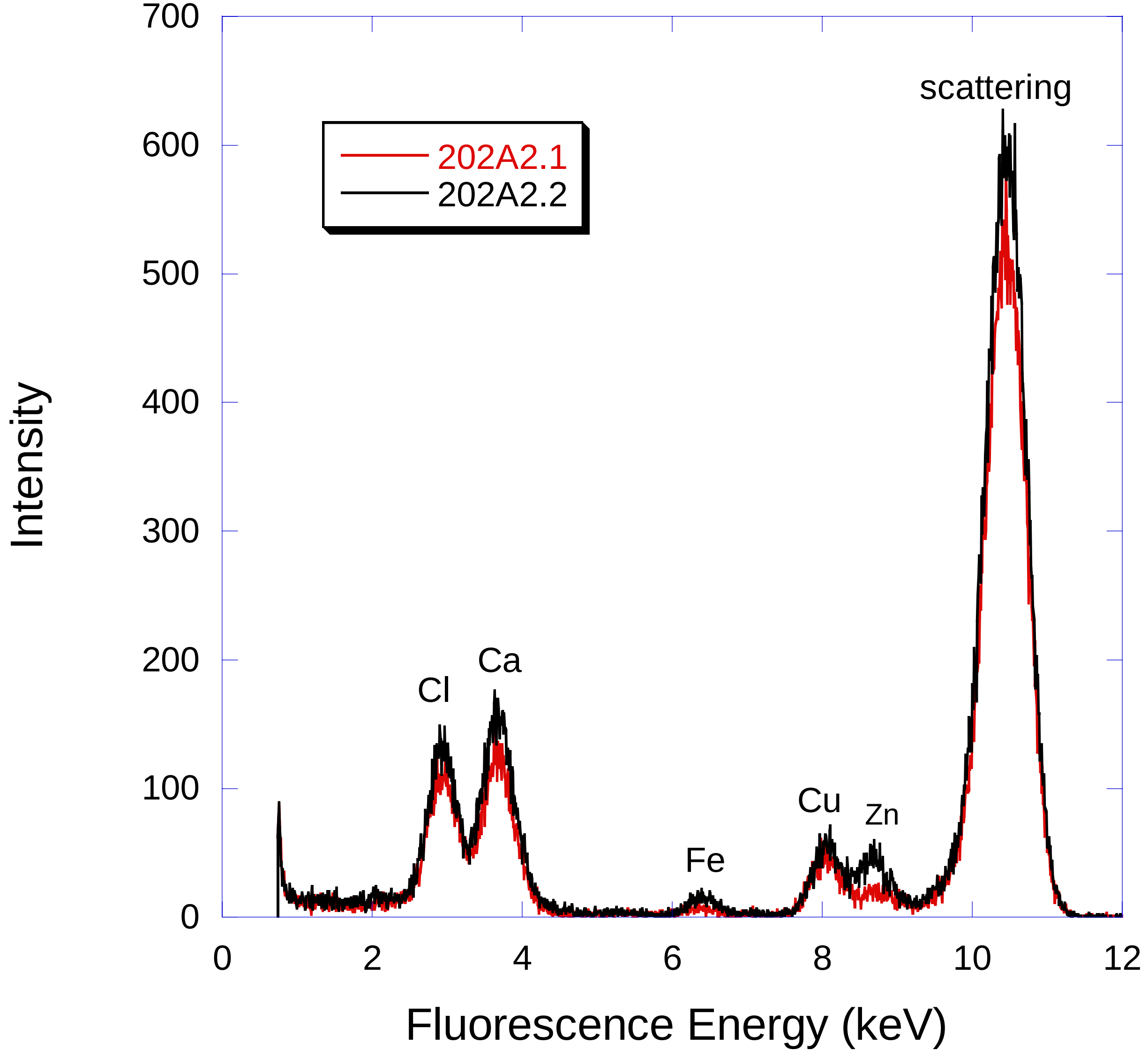
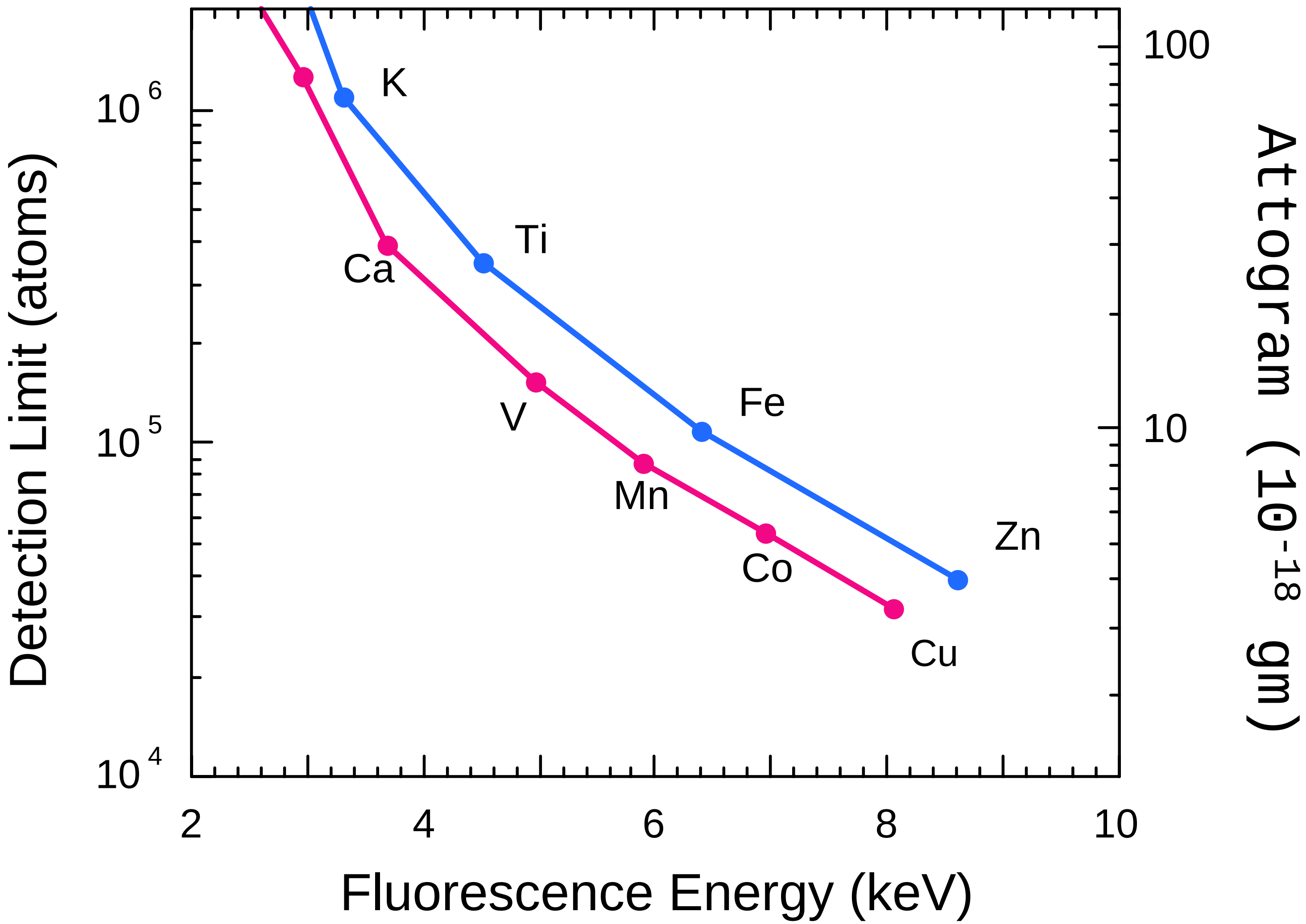
* C.J. Sparks, Jr., in Synchrotron Radiation Research (Plenum Press) 1980.

Fluorescence spectra from tissue section (5-μm thick)



Detection Limit for Transition Elements

for 1 sec. acquisition time, 0.2 x 0.2 μm^2 spot, E=10 keV



X-ray fluorescence microscopy to study trace metals:

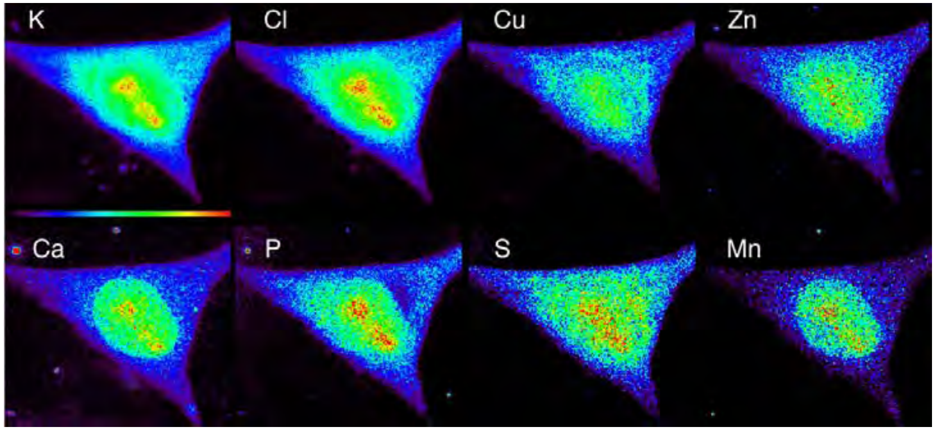
Pro:

- ≈ 10 part-per-billion (ppb) are detectable for Z < 40,
  ≈ 1 part-per-million (ppm) for heavy elements
- No fluorescence dyes, markers, nor labeling
- Minimal sample preparation, allow hydrated/*in situ* studies.
- Direct parallel acquisition of > 10 elemental images
- 200 nm spatial resolution obtained routinely
- Chemical states, e.g. Cr (VI) vs Cr (III), Pt (IV) vs Pt (II), can be revealed

Con:

- Long integration time (1-2 hours for a set of images)
- Non-specific to binding partners (which protein?)

Trace elements/metals in biology & life sciences:



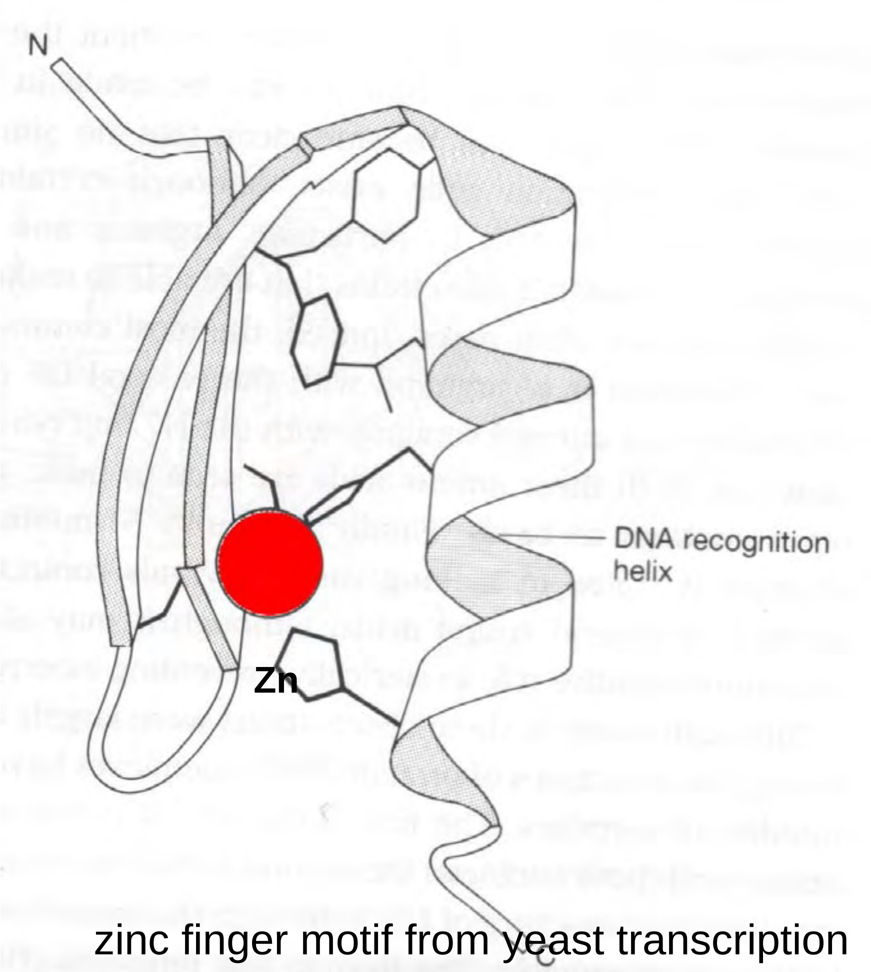
- essential cofactors in proteins
- linked to diseases
- in therapeutic drugs
- as intracellular labels

Why study trace metals in cells ?

1. **Estimated 1/3 of all known proteins contain metal cofactors. These proteins often have regulatory or enzymatic functions:**

- Ca in calcium-binding proteins: second messenger pathways, e.g. Troponin C in muscle
- Fe in Hemoglobin; and necessary in Chlorophyll synthesis
- Cu binding chaperones (protein folding)
- Zn in Zinc finger proteins: transcription factors in the cell nucleus

- **At the same time: most essential trace metals toxic at higher concentrations (e.g., Cu, Se)**



zinc finger motif from yeast transcription factor SWI5
[from Encylopedia of Molecular Biology]

Why study metals in diseases ?

2. **Several diseases either linked to or suspected to be linked to metals directly or indirectly, e.g.**

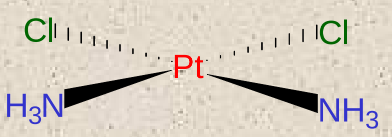
   - Alzheimers Disease [Al, Fe, Cu]
   - Lewy Body Diseases (including Parkinson disease) [Al, Fe, Cu, Hg, Cd]
   - Amyotrophic lateral sclerosis (ALS or Lou Gehrig's disease) [Cu, Zn]

Science 9 May 2003 with Focus: "Metals: Impacts on Health and Environment"

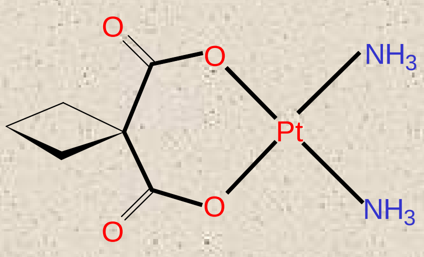
Why study metals in medicine ?

3. Pt-based chemotheraputic drugs in the clinic

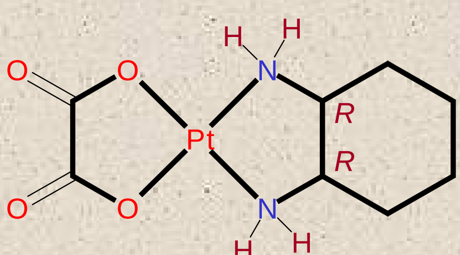
Cisplatin: \$280 million



Carboplatin: \$993 million



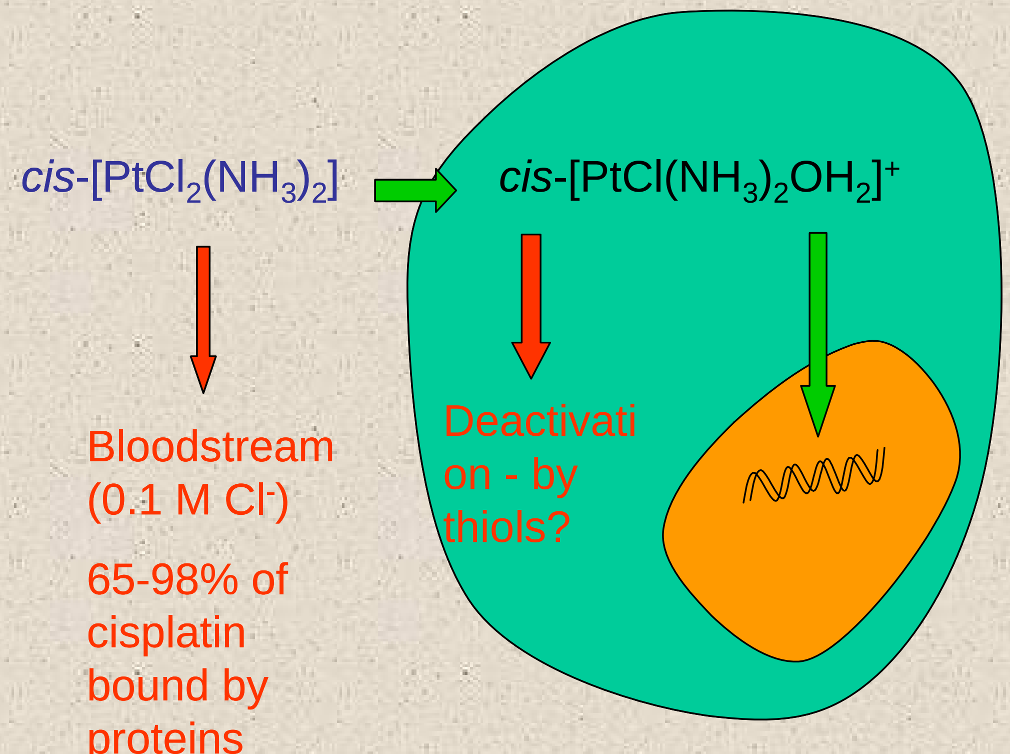
Oxaliplatin: \$111 million



1999 First quarter results annualised

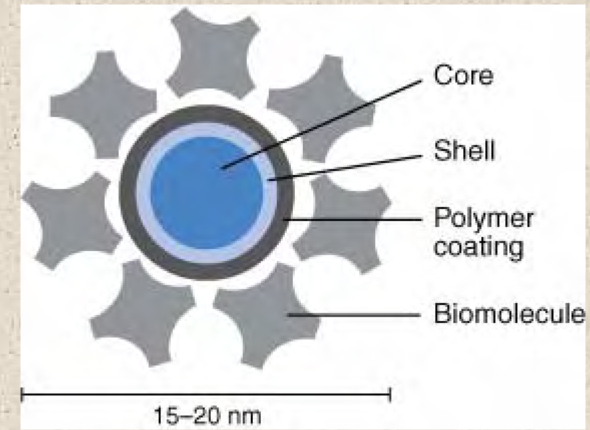
Cis-Pt coordinates to DNA, inhibits replication and transcription of DNA, and leads to apoptosis

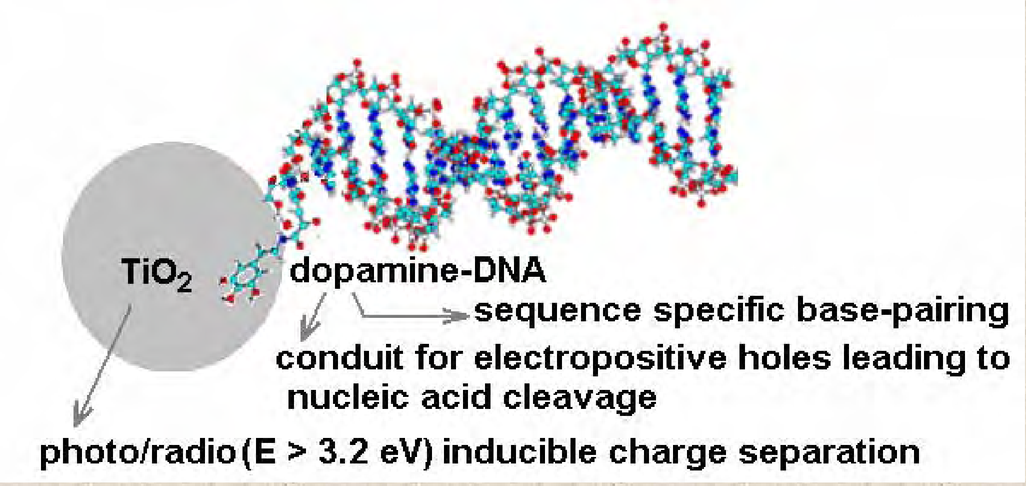
But: very few cisplatin molecules reach DNA targets

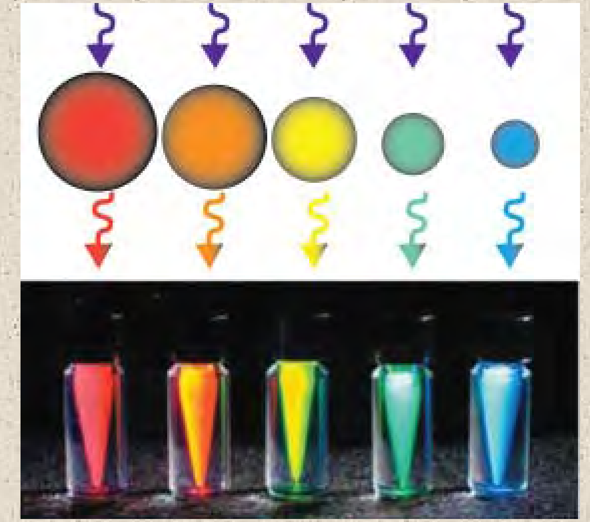


Why study trace metals in life sciences ?

4. Qdot/Nanocomposite as intracellular label/tool







- attach TiO_2 nanoparticle (4.5 nm diameter) to DNA
- combine DNA biochemistry with semiconductor properties of TiO_2
- ➔ carrier-particle that can bind to a specific chromosomal region w/ ability to cleave it upon illumination

Biological Applications of μ-XRF

High resolution and high elemental sensitivity of the x-ray microprobe provide a new tool for biological studies:

Exogenous:

- Microbial analyses/Environmental studies
- Metal toxicity/Carcinogenesis
- Chemotherapeutic drugs
- Nanobiotechnology

Endogenous:

- Metalloproteins, metallothionein
- Infectious parasites/Anti-parasitic drugs
- Cell differentiation
- Neurodegenerative diseases

Intracellular Distribution of Copper

- Copper is an essential trace element for all life forms
- Catalyze the production of highly reactive oxygen species

  ⇒ oxidative damage to lipids, proteins, DNA, etc
- Defects in regulatory processes may led to:
  - ✓ Menkes syndrome
  - ✓ Wilson’s disease
  - ✓ Amyotropic lateral sclerosis (ALS)
  - ✓ Alzheimer’s disease
- Cellular uptake, trafficking, storage need to be understand
- A novel Cu(I) fluorescent sensor (CTAP-1) was recently developed.

  ⇒ Does it reflect the true cellular distribution?

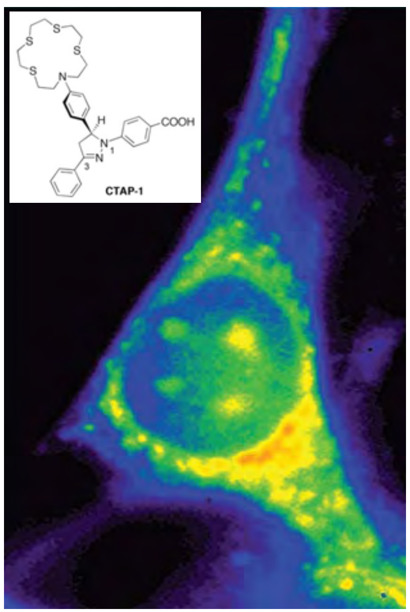
XRF:

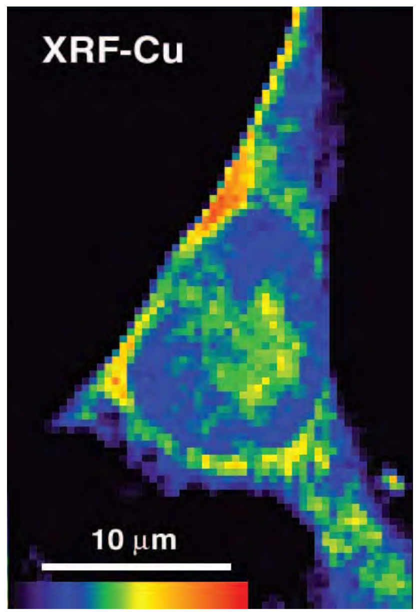
1) Validate Cu sensor CTAP-1

2) Quantify cellular Cu

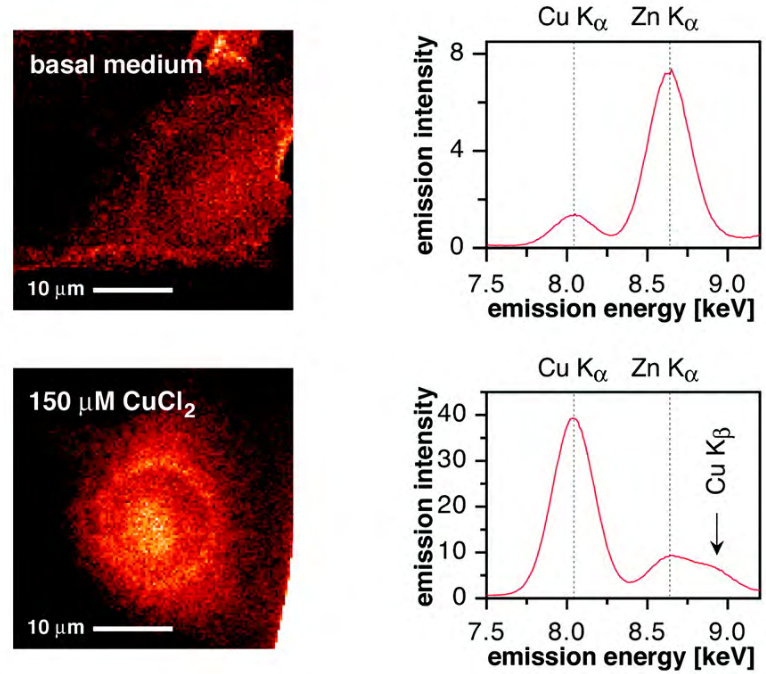
Optical fluorescence from CTAP-1:
<u>Chelatable Cu</u>

Cu XRF:
<u>Total Cu</u>



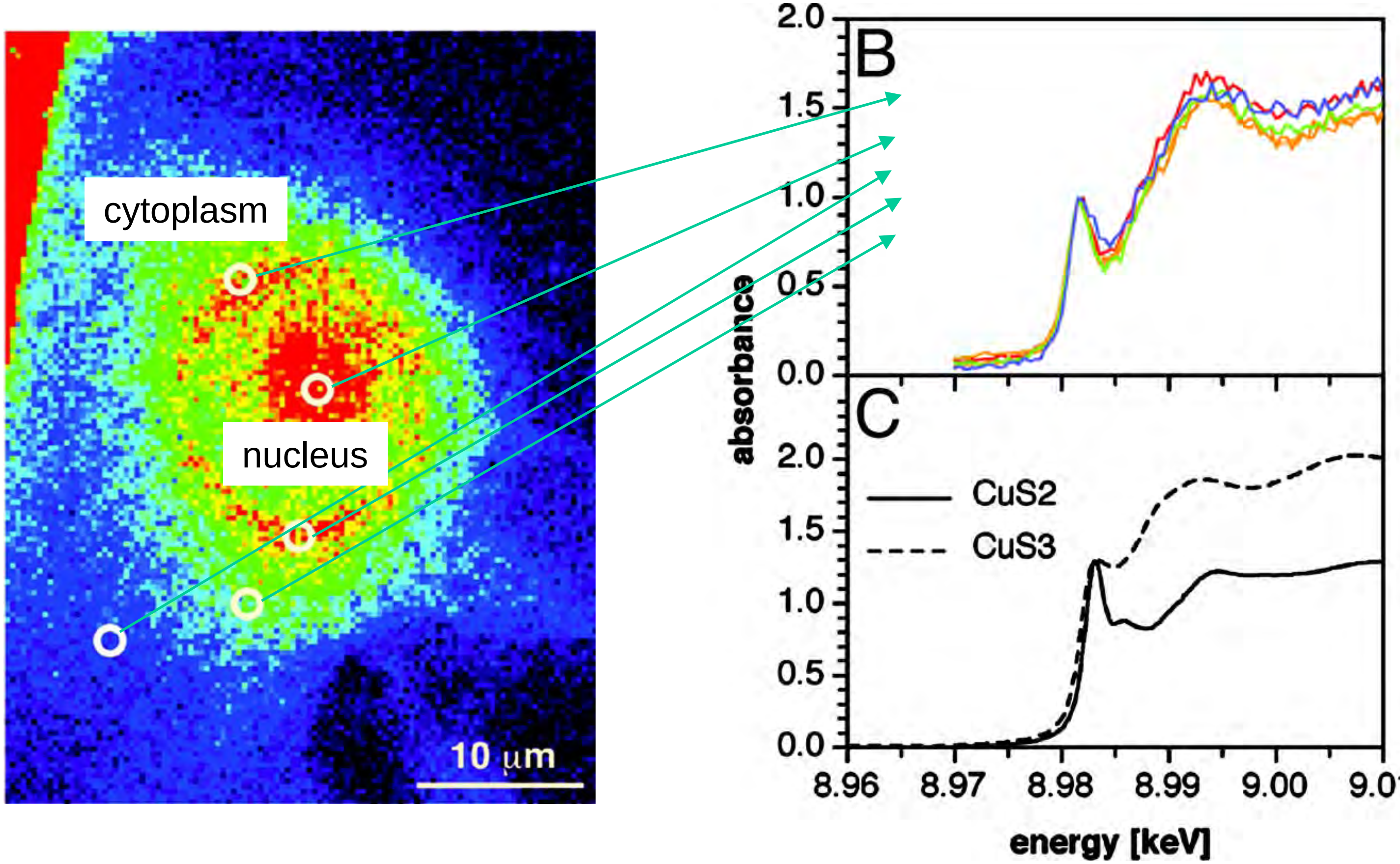


Mouse fibroblast cell + 150 µM $CuCl_2$



L. Yang, et al., Proc. Natl. Acad. Sci. 102, 11179 (2005)

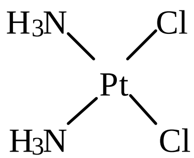
μ-XANES indicated Cu(I), confirming the reducing cellular environment



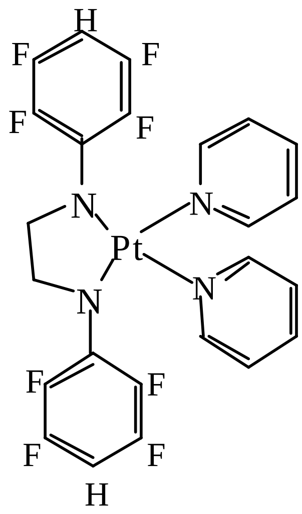
L. Yang, et al., Proc. Natl. Acad. Sci. 102, 11179 (2005)

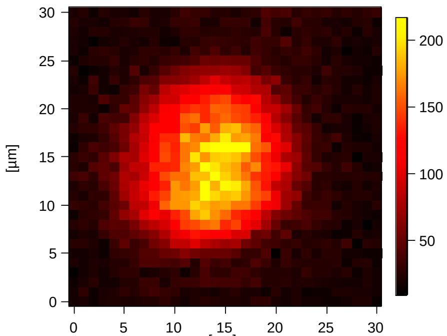
Cisplatin derivative to overcome tumor resistance

Cisplatin

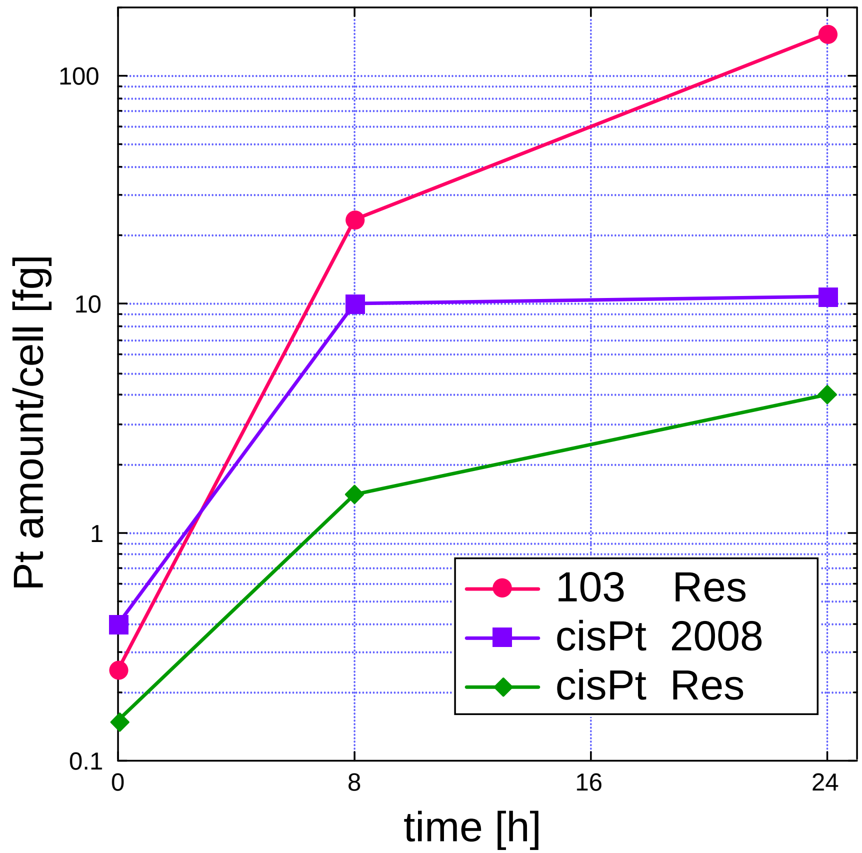


Derivative Pt 103



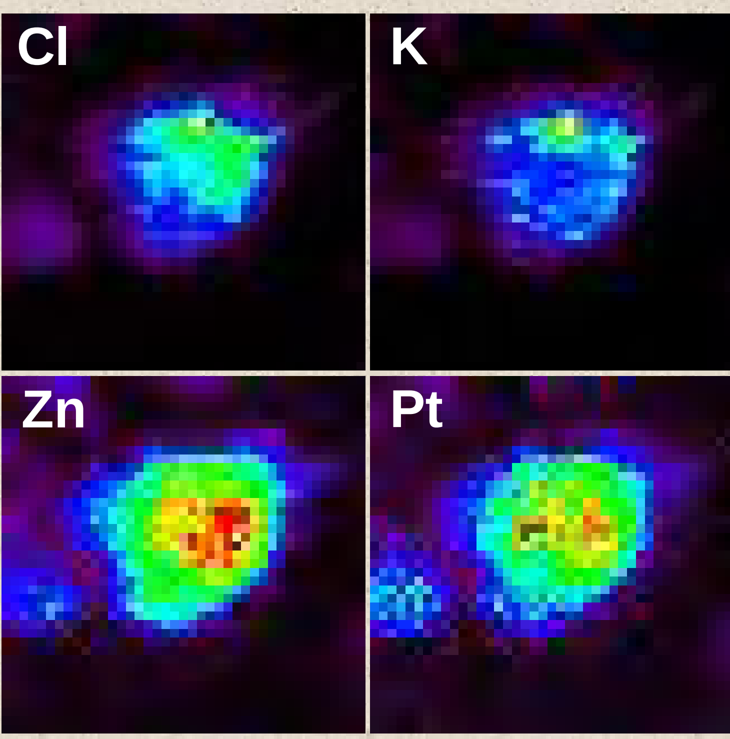


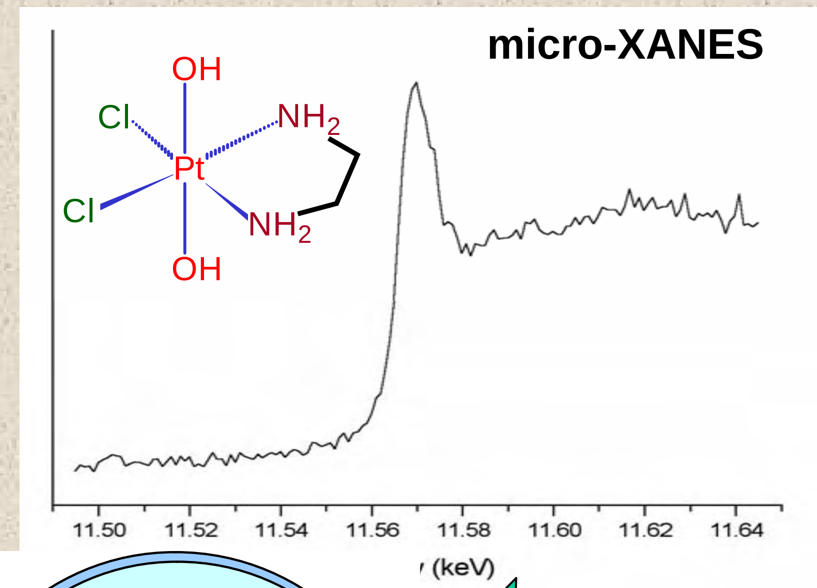
Study of cisplatin uptake and effectiveness of derivative Pt 103

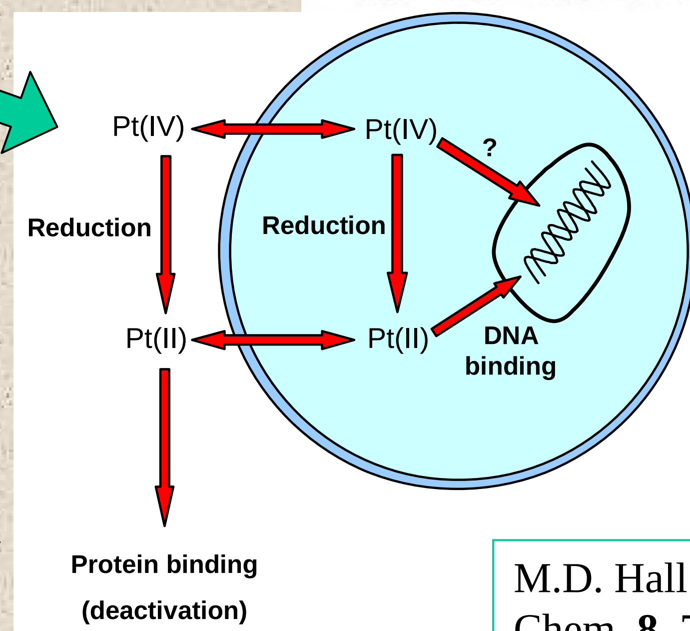


P. Ilinski *et al.*, Cancer Research **63**, 1776-1779 (2003)

Pt(IV) complexes: Metabolic pathway





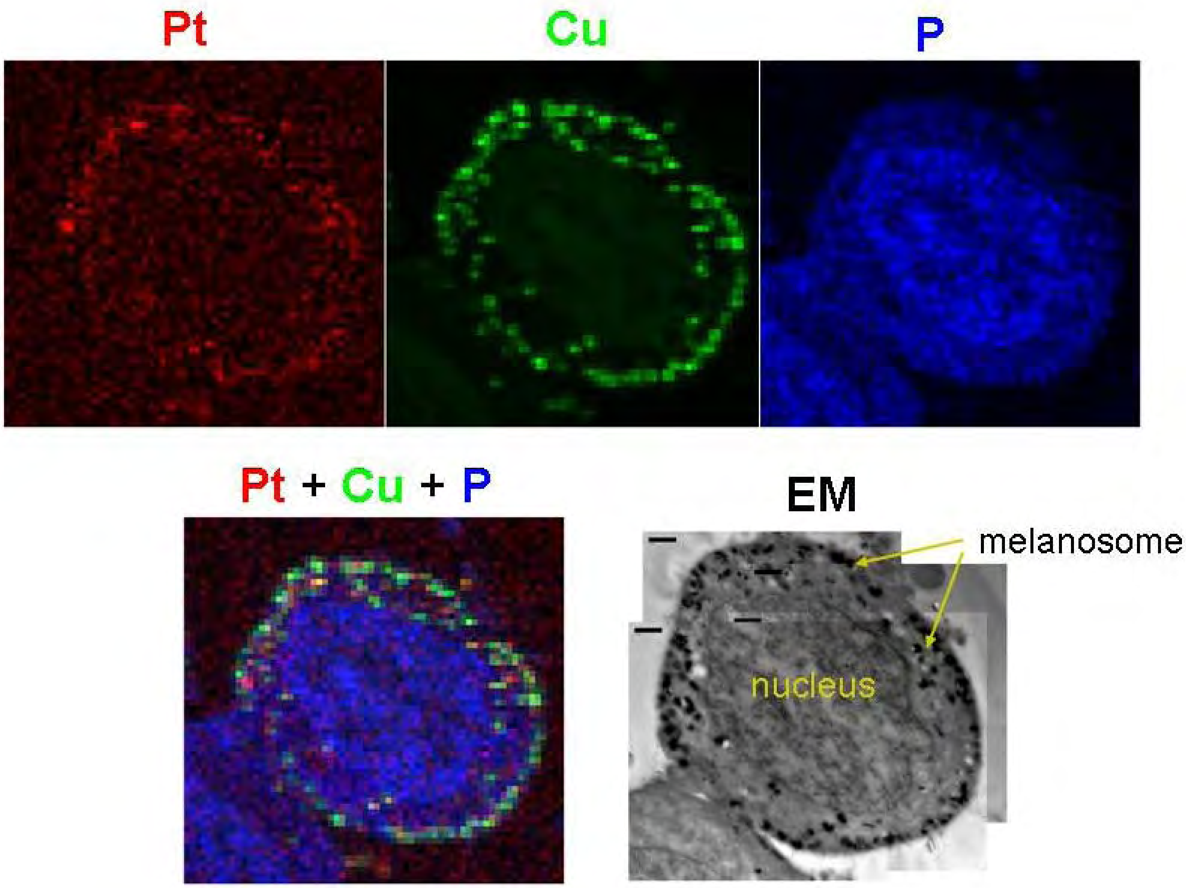


Pt(IV) complexes are more inert:

- **less likelihood of deactivation**
- **potentially fewer side effects**
- **possibility of selective activation**

M.D. Hall *et.al.*, J. Biol. Inorg. Chem. **8**, 726-732 (2003)

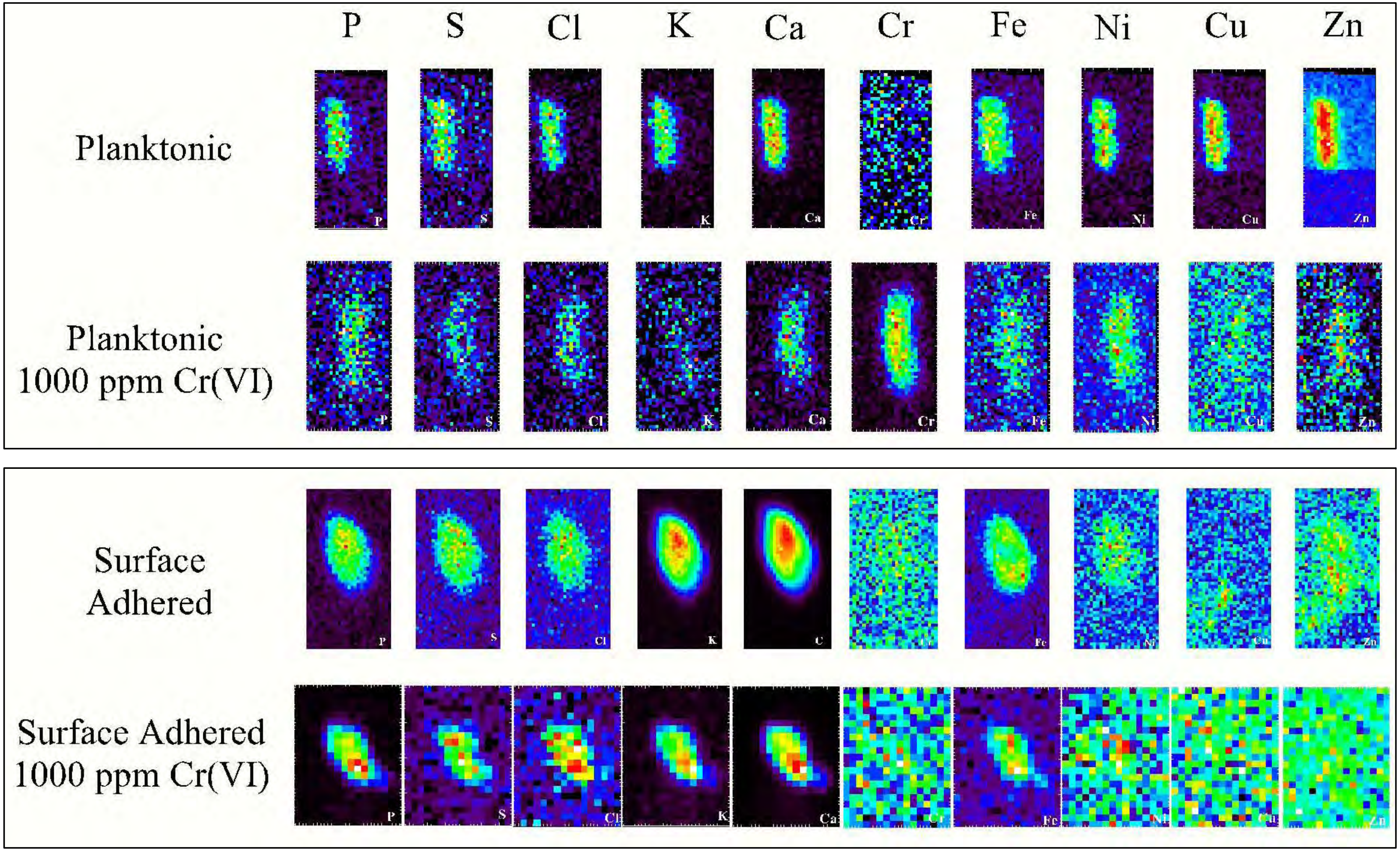
Multidrug Resistance Mechanism of Malignant Melanomas



K.G. Chen et al., *Proc. Natl. Acad. Sci.* **103**, 9903-7 (2006)

- Melanomas possess intrinsic resistance to radiation- and chemo-therapy
- Melanoma cells MNT-1 were treated with cisplatin CDDP, a cytotoxic drug
- μ-XRF reveals that cisplatin was sequestered in melanosomes, a pigmented subcellular organelle
- This suggests novel approaches to modulate chemosensitivity of melanoma cells

Single hydrated bacteria treated with Cr(VI)



K. Kemner et. al., Science 306, 686 (2004)

Quantitative elemental analysis

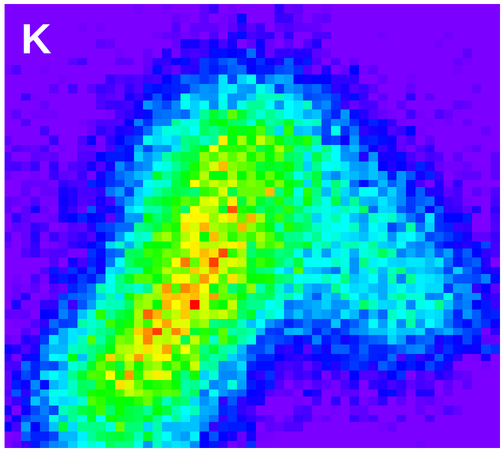
	[P]	[S]	[Cl]	[K]	[Ca]	[Cr]	[Mn]	[Fe]	[Co]	[Ni]	[Cu]	[Zn]
Planktonic (5)	16,048 (2,446)	6,625 (1,117)	8,421 (2,628)	3,604 (1,173)	3,815 (392)	9 (2)	22 (4)	156 (23)	190 (37)	120 (33)	201 (46)	1,175 (176)
Planktonic, Cr(VI) added at 1000 ppm (6)	6,156 (1,034)	3,719 (1,516)	3,908 (1,814)	2,201 (1668)	673 (230)	949 (323)	22 (4)	58 (29)	13 (12)	26 (18)	105 (76)	94 (30)
Planktonic, Cr(VI) added at 25 ppm (12)	8671 (4097)	3201 (1092)	994 (421)	19 (9)	42 (36)	34 (15)	3 (1)	195 (68)	7 (2)	2 (1)	ND*	11 (5)
Surface-adhered (8)	661,032 (139,416)	ND	ND	ND	570,855 (92,831)	32 (10)	40 (7)	360 (216)	14 (7)	26 (10)	0 (14)	25 (13)
Surface-adhered, Cr(VI) added at 1000 ppm (10)	419,034 (362,728)	ND	ND	ND	427,987 (147,983)	24 (15)	23 (8)	326 (177)	12 (7)	18 (9)	2 (5)	15 (7)
LB growth mediim solution (3)	17.8168 (0.1619)	3.028 (0.006)	NA	228.91 (3.37)	7.5915 (0.0986)	0.0194 (0.0002)	0.0181 (0.0002)	0.4034 (0.0049)	0.0138 (0.0001)	0.0036 (0.0001)	0.0253 (0.0004)	5.4162 (0.0064)

*[Cu] could not be determined accurately due to elevated [Cu] in the grid for this particular sample.

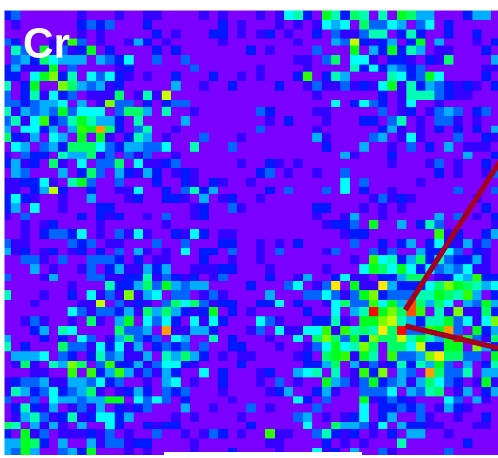
=> Assume $CaPO_4$ moiety, extracellular deposit ~ 0.4 μm thick

<u>Hydrated</u> *Pseudomonas fluorescens* treated with Cr(VI)

K

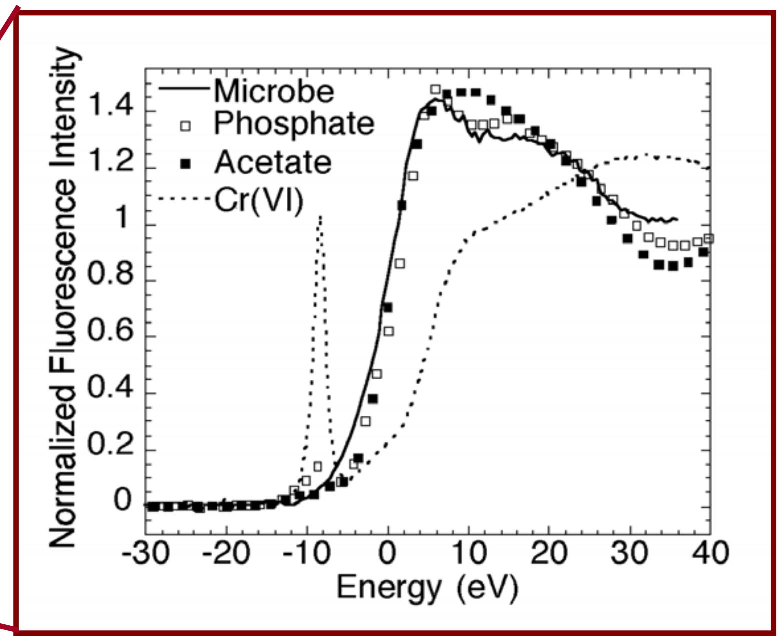


Cr



5 µm

Probe local chemistry *in-vivo* with micro-XANES: Cr(VI) → Cr(III)



K. Kemner, ANL-ER

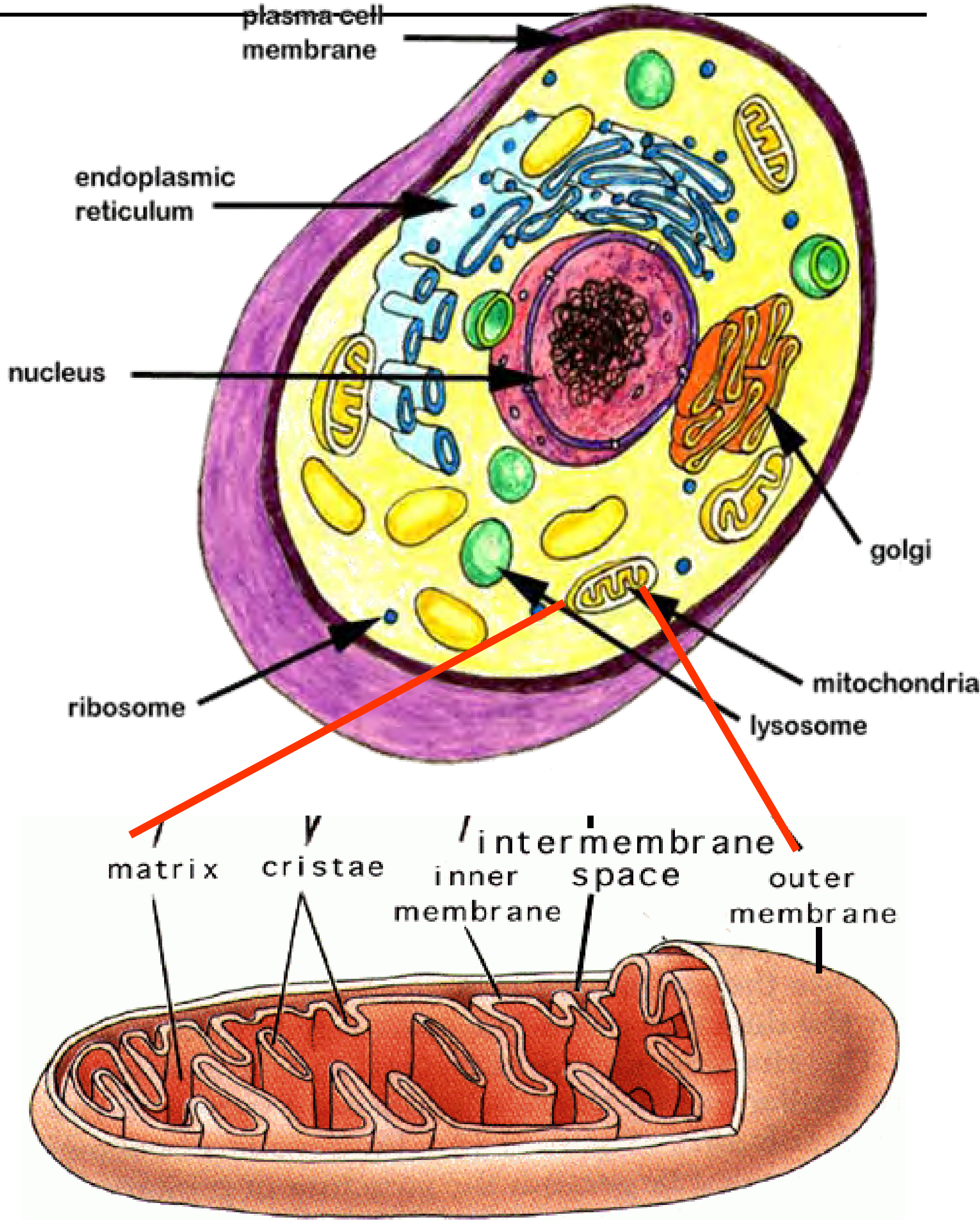
Future Prospects

Increased spatial Resolution

Typical sizes of cell 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)**
- **microtubuli: 20 nm diam. (cytoskeleton)**
- **membrane thickness: 8 nm**

➔ **1 nm can resolve individual macromolecules**



Detection Sensitivity

- **Current detection limit for Zn: $3x10^{-18}$gm or 28,000 atoms (1 sec)**
- **ERL:**

Coherent flux = $5x10^{14}$ ph/s/0.1%

Focused flux = $1x10^{13}$ ph/s/0.01%

Focused flux density = $1x10^{19}$ ph/s/μm^2/0.01%

(10^8 times higher than 2-ID-D)

Can detect one Zn atom in 100-nm thick sample within 1 msec,

but dose ~ 10^{13} Gy!

Increased Throughput

High variability in most organisms, therefore need good statistics

- **Current image acquisition time per cell ~ hours**
- **Signal increase ~ 10,000x: ERL ~ 1,000x**

  detector ~ 10x

  ➔ 100x faster (same SNR)

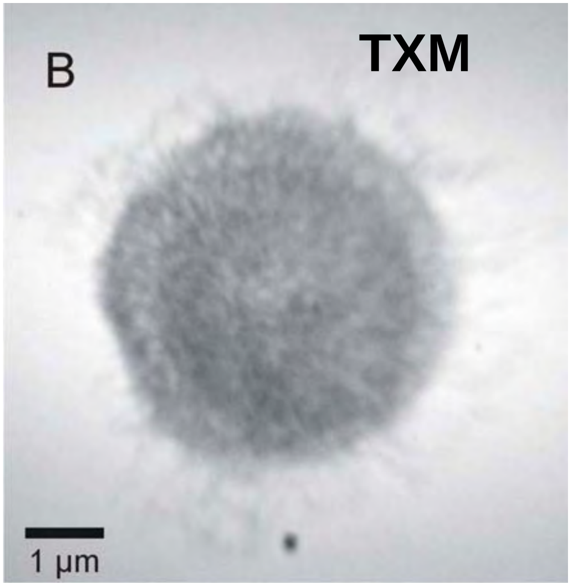
current resolution ➔ 1,000 cells/day, using fly scan

Sample Preservation !

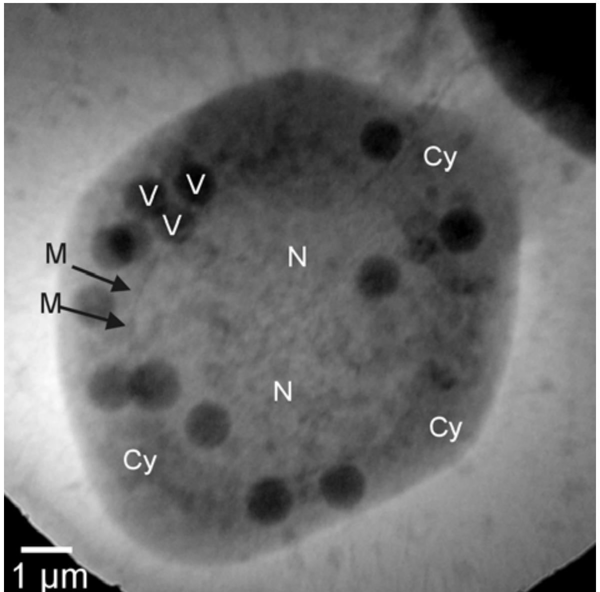
- **study cells / tissues as close to their native, hydrated state as possible:**
  - avoid artifacts introduced by chemical fixation / drying
- **reduce radiation damage, in particular to oxidation state**

➔ **elemental mapping of rapid frozen samples at cryogenic temperatures (LN2)**

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



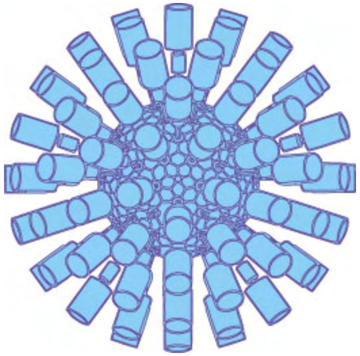
cryoTXM



- *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

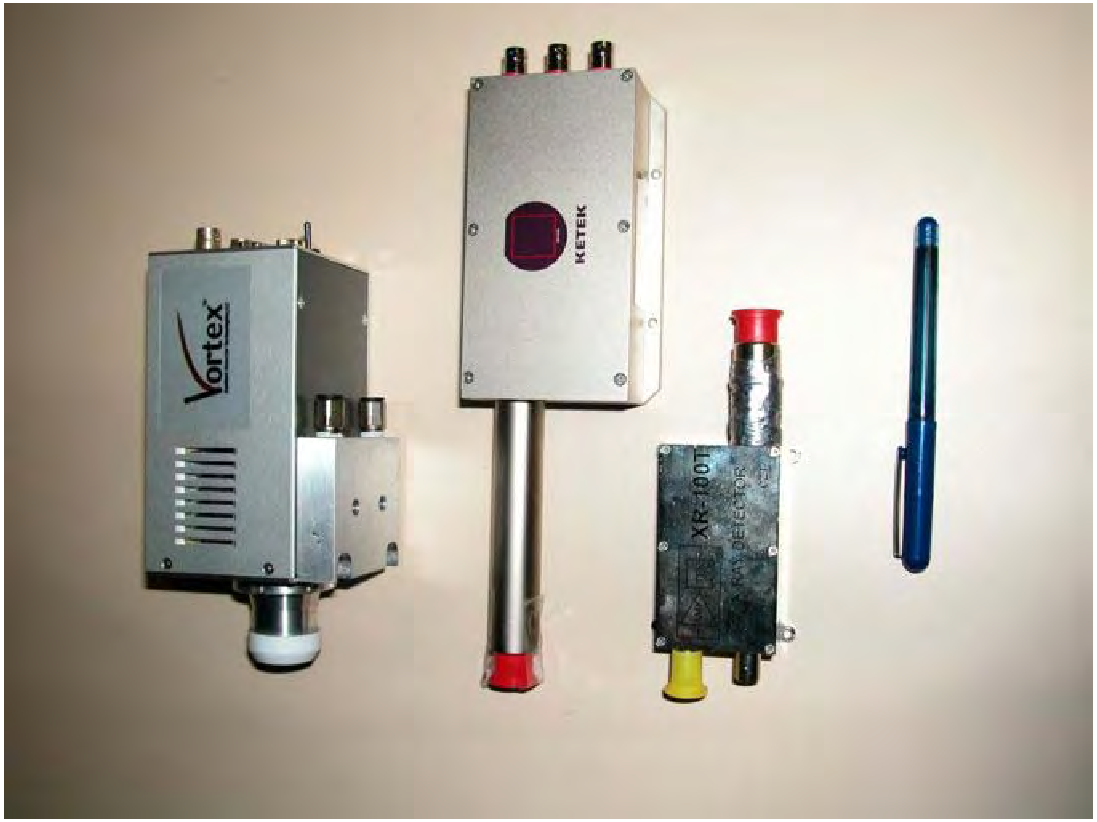
Single element detector captures only <u>5%</u> of 4π!!



GammaSphere consists of 110 solid state detectors

Possible solution: Silicon Drift Detectors

1. compact (stacking in a smaller volume)
2. higher count rate (> 10^5 cps)
3. low maintenance (no LN2)



Instrumentation requirements

- Increase spatial resolution
  - Better zone plates
  - Stability of beam & microprobe
  - Environment control
- Specimen preparation
  - Cryogenic sample handling
    - *Ultrastructure preservation*
    - *Reduce drying/radiation artifacts*
  - Ability to incubate and handle live cells
- Detector
  - Large angular acceptance

Conclusions

1. **X-ray fluorescence microscopy offers:**
    - Quantification to < ppm level
    - Spatial resolution ~ 150 nm
    - Large penetration depth => simple sample preparation
    - Reveal chemical state, Cr(VI) vs Cr(III), Pt(IV) vs Pt(II)

2. **Track in-situ cellular distribution of metal complexes:**
    - trace metal nutrients
    - nanoparticles
    - environmental contaminants, carcinogens, therapeutic agents
    - metalloproteins

3. ERL:

- Resolution for individual macromolecules
- Sensitivity to detect single metal atom
- 1000 cells/day at reduced resolution

4. Challenges:

- High resolution focusing optics
- Stable beam & microprobe
- Detector acceptance ~ 2π
- Sample preservation & damage control

Acknowledgement

Microprobe

Z. Cai, J. Maser, S. Vogt, D. Legnini, W. Yun, P. Ilinski, A. Stampfl

Zone Plate Optics

W. Yun (Xradia)

Biological Applications

K. Kemner, S. Kelly (bacteria)

M. Hall, T. Hambley, D. Philips, T. Talarico (cisplatin and Pt(IV))

K.G. Chen, R. Leapman, M.M. Gottesman (melanomas)

C. Fahrni, R. McRae (Cu pool)