

Biophysical and physicochemical methods
for analyzing plants *in vivo* and *in situ* (III):
X-ray spectroscopy for
localising&quantifying metals
and for investigating metal ligands

X-ray spectroscopy on biological samples

General comments on sample preparation techniques

a) chemical fixation and resin embedding

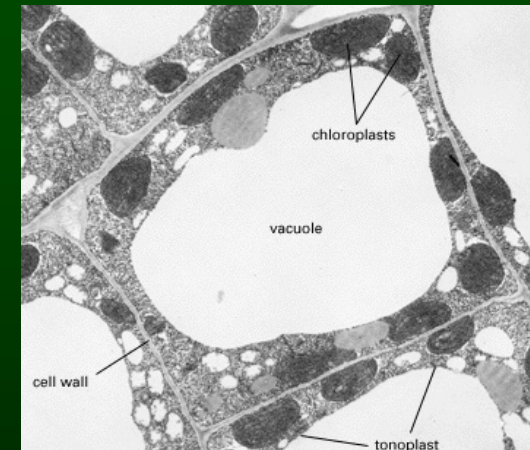
→ Advantages: over many years best established procedure in many laboratories

→ Disadvantages: Metals will inevitably be re-distributed → ARTEFACTS

b) freeze substitution or freeze drying

→ Advantages: less element re-distribution than in (a)

→ Disadvantages: still at least intracellular (vacuole → wall) re-distribution artefacts inevitable



c) frozen-hydrated tissues

→ Advantages: hardly any element-redistribution → METHOD OF CHOICE!

→ Disadvantages: Required rapid-freeze techniques and cryostage (→ expensive)

e) non-frozen fresh tissues

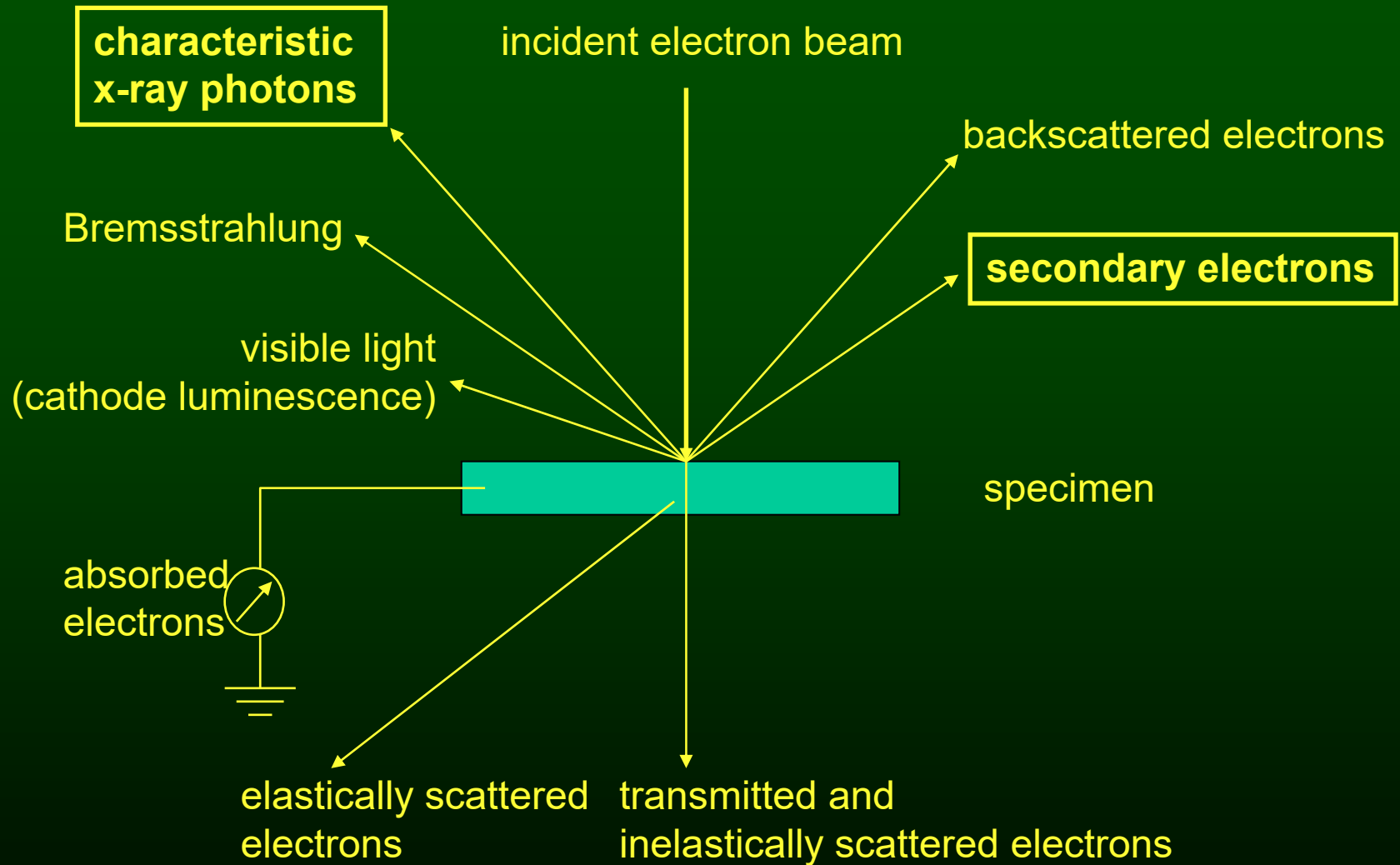
→ Advantages: NO preparation necessary, “*in vivo*” situation

→ Disadvantages: Strong beam damage → MORE artefacts than in (c)!

(1) X-ray emission spectroscopy
(a) Energy Dispersive X-ray Analyses (EDXA)
Use of an electron microscope as an X-ray spectrometer

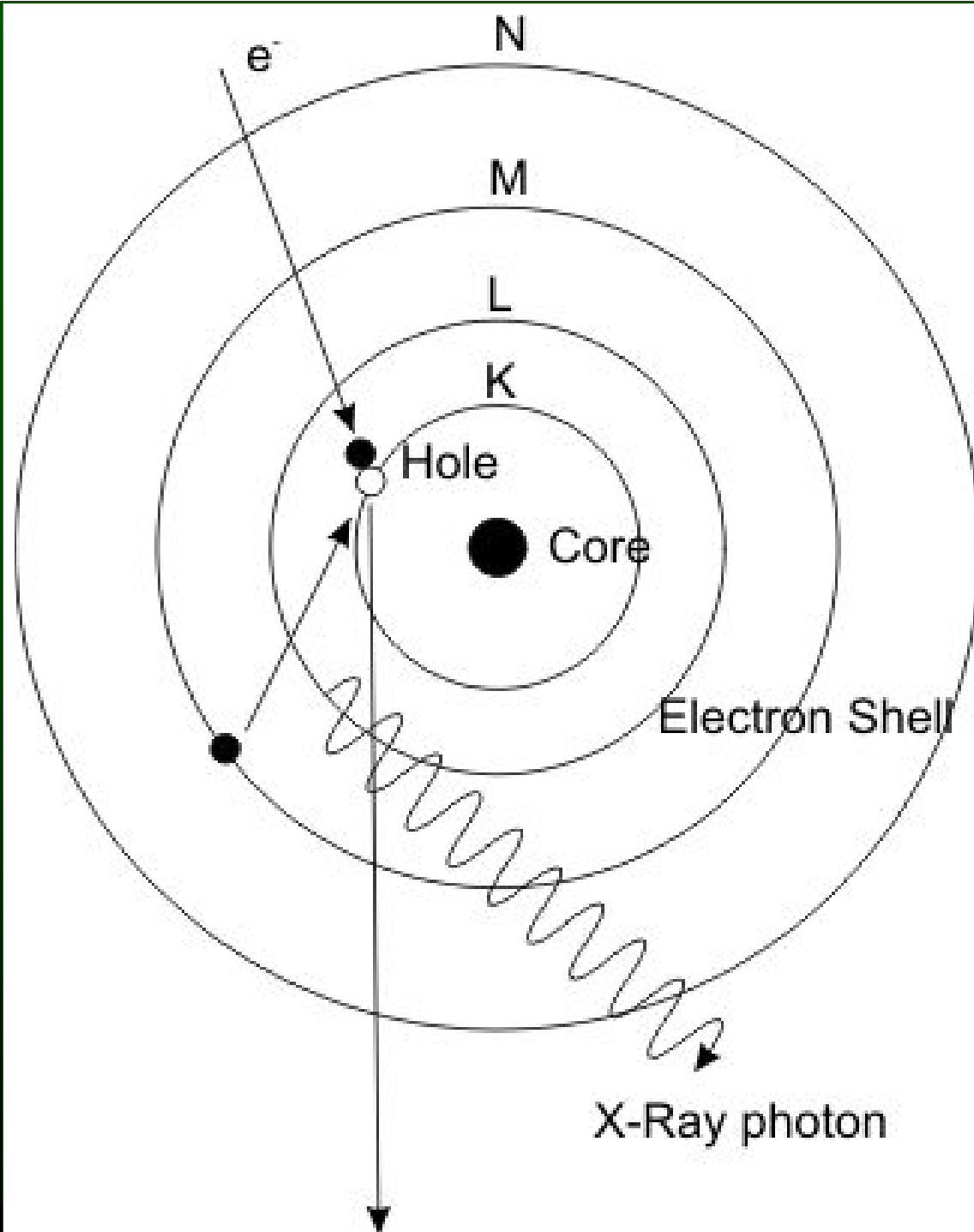
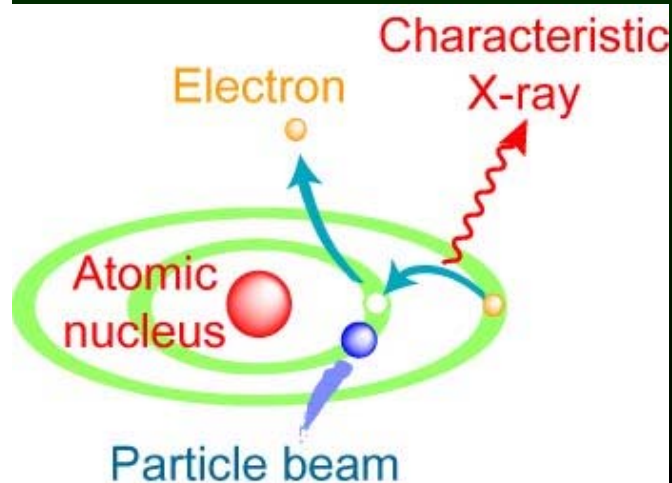


Signals generated in the scanning electron microscope (SEM)

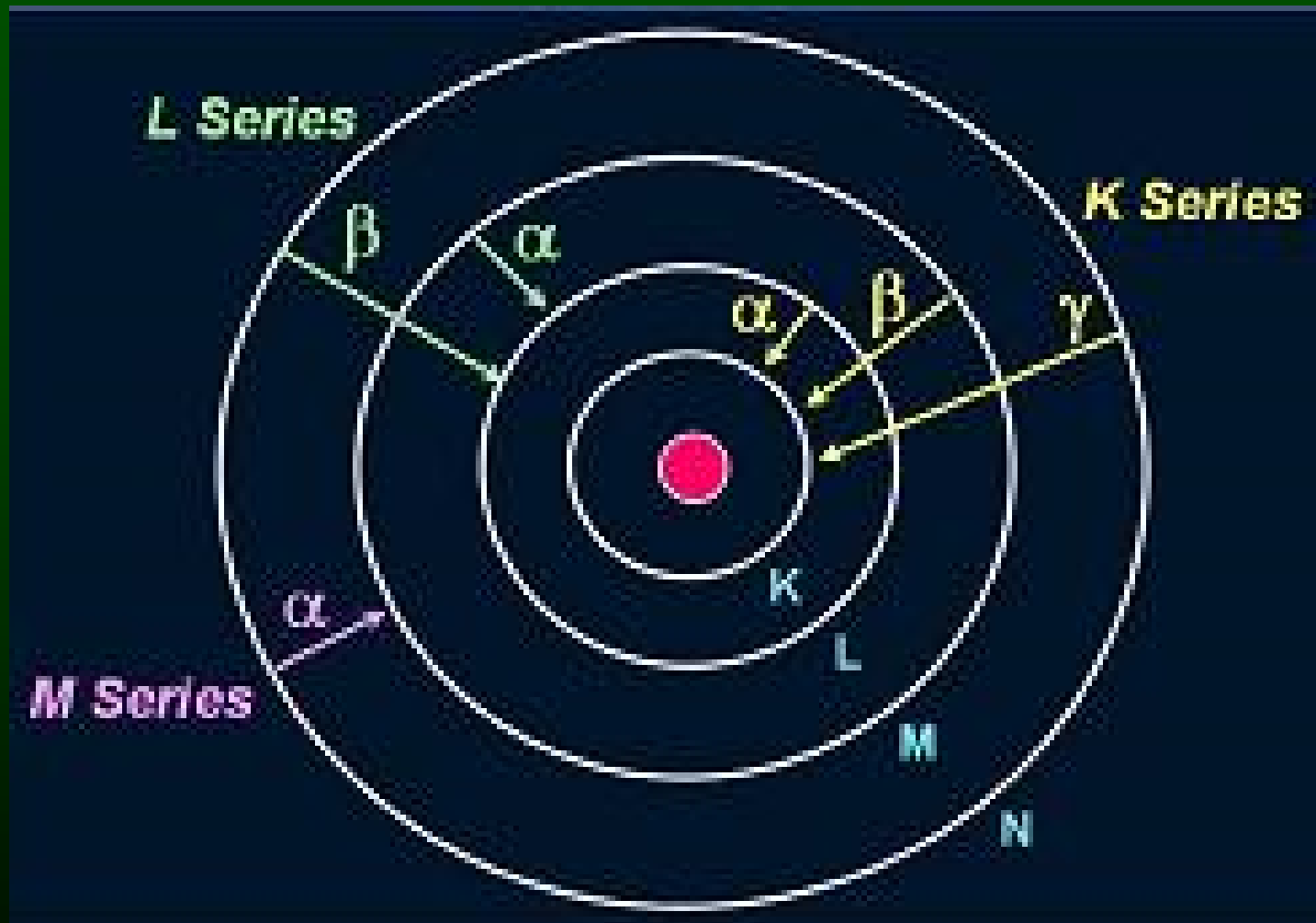


Principle of Energy Dispersive X-ray Analysis (EDXA)

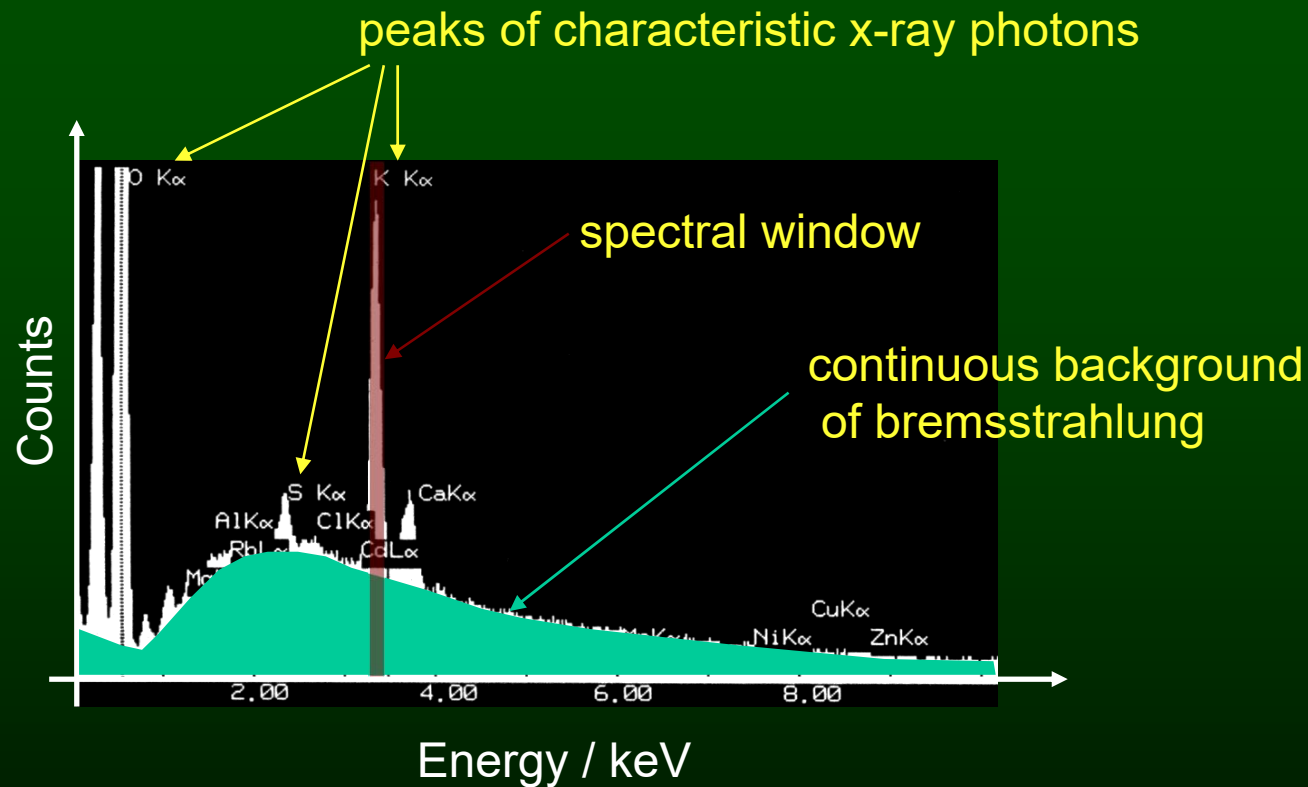
Principle of Particle Induced X-ray Emission (PIXE)



The origin of the different lines in an EDXA spectrum

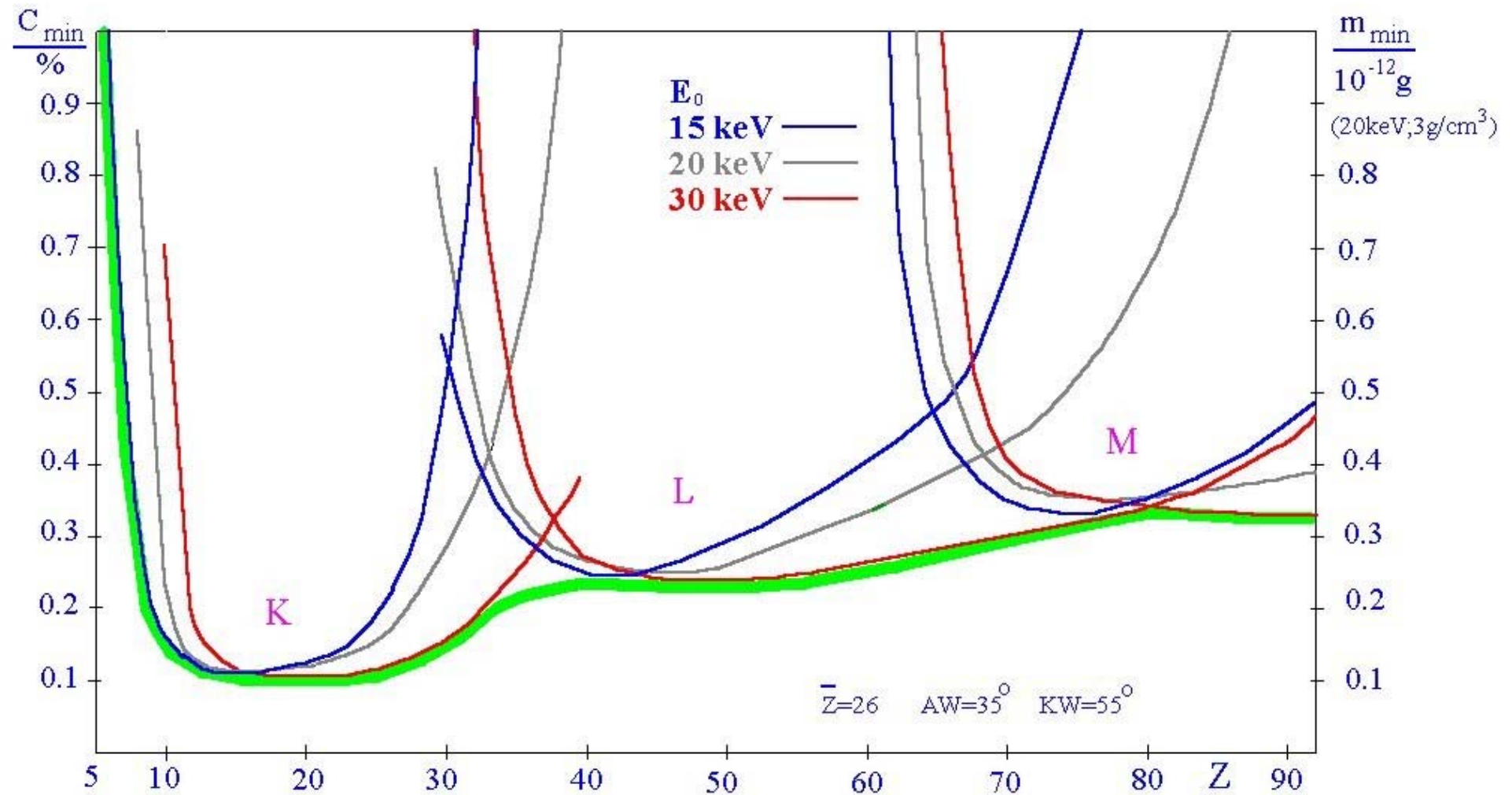


Analysis of EDXA spectra



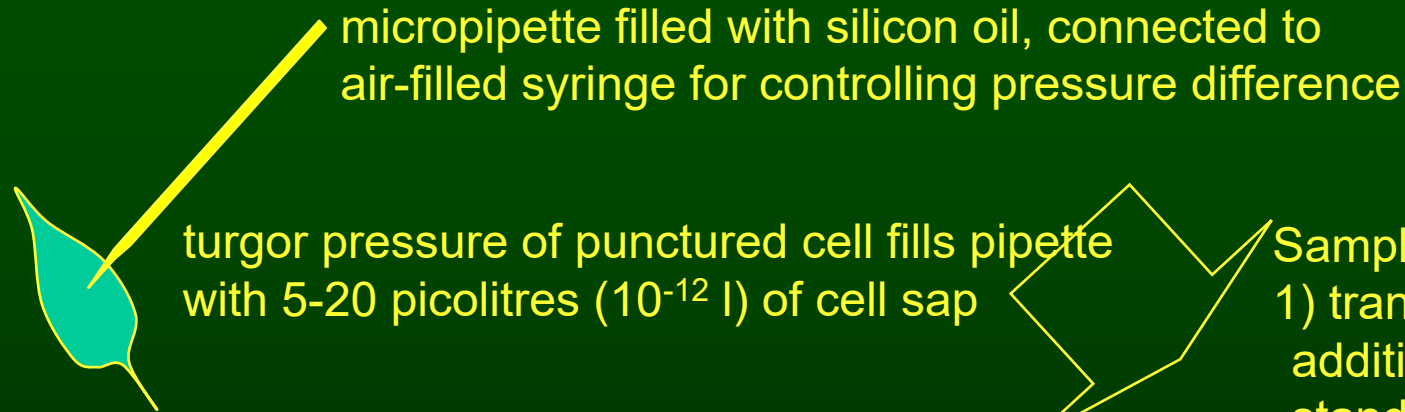
- Analysis:
- a) recording of complete spectrum, subtraction of background
--> quantification of peak areas by comparison to internal standard
 - b) recording of counts in spectral window
--> dot maps, line scans

Detection limits of EDXA



Methods of plant analysis using EDXA

Sampling of single-cells saps with micropipettes

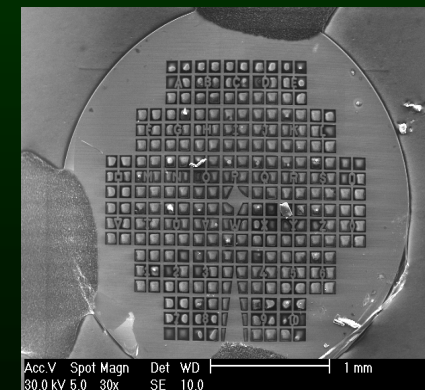
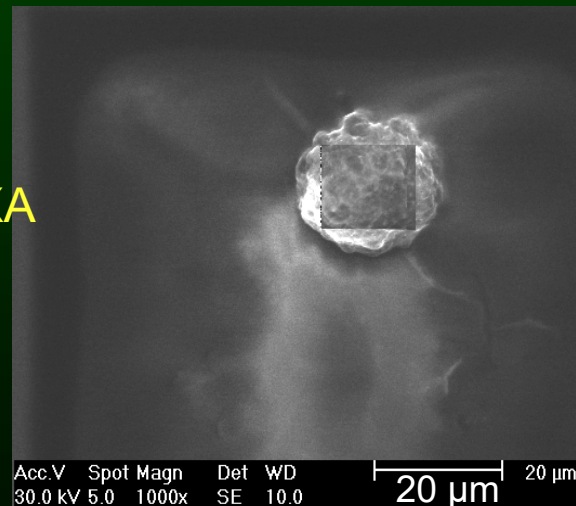


Sample preparation:

- 1) transfer to storage grid, addition of internal standard (e.g. RbF) and matrix (e.g. mannitol)
- 2) transfer to analysis grid, drying with isopentane

Analysis:

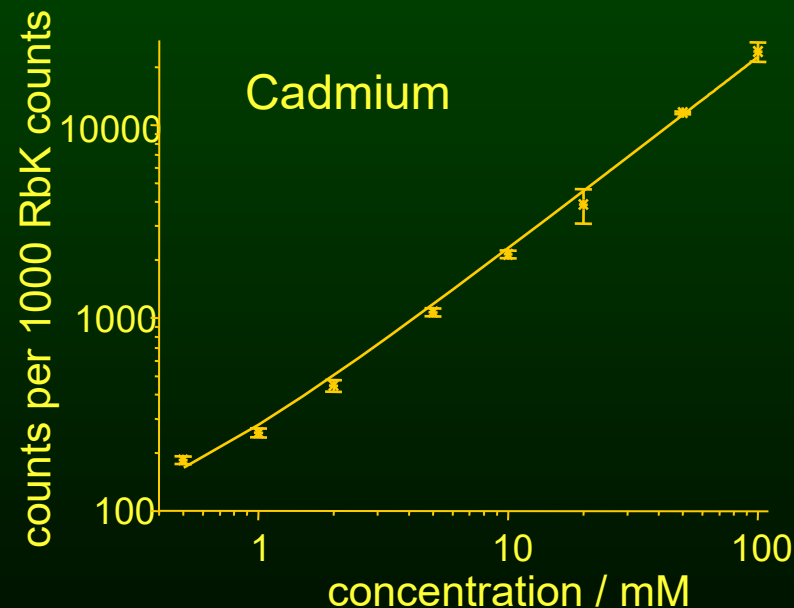
- 1) recording of EDXA spectra in SEM
- 2) data processing



Methods of plant analysis using EDXA

Quantification of elements in single-cells saps

- 1) net peak area is normalised by internal standard (an element not naturally present in the sample, e.g. Rb)
- 2) ratio obtained from 1) is quantified using calibration curve



Evaluation of the method

Advantages:

- potentially very accurate
- enables measurement of small concentrations

Disadvantages:

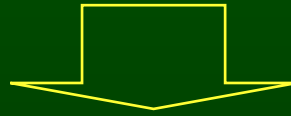
- only few types of cells are accessible to sampling with micropipettes
- risk of preparation artefacts
- no distinction between cytoplasm and vacuole, measurement of cell walls impossible
- very difficult to obtain information about heterogeneity of element distribution inside the analysed tissue

Methods of plant analysis using EDX or XRF

Freeze-fracturing

Excise sample from plant, mount in/on stub or vice.

The EDX/XRF spectrum of the vice must not interfere with that of the sample!



Shock-freeze the sample, e.g. in **melting nitrogen slush (NOT regular liquid nitrogen!)**, transfer to cooled (-170°C) preparation chamber



Fracture / cut sample

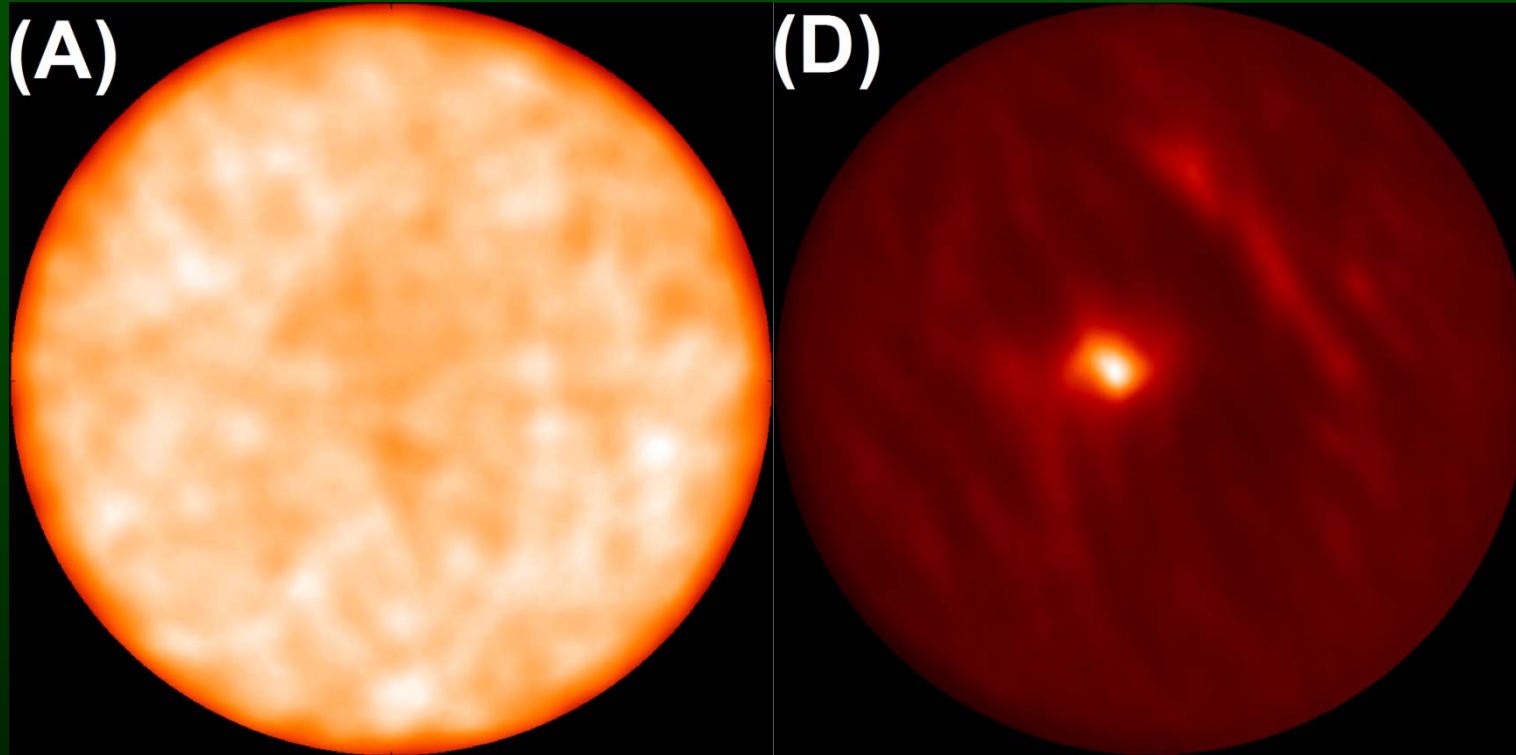


For EDX: Produce conductive sample surface by evaporating carbon wire



Transfer to cooled (-150°C) sample stage or cryostream, analyse

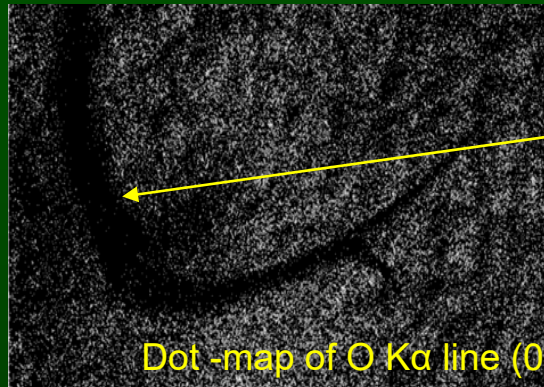
Tomographic X-ray emission spectroscopy (μ -XRF): Artefacts of slow freezing



→ shock-freezing in supercooled isopentane led to homogeneous standards (left), freezing in LN₂ was too slow due to gas layer and led to de-mixing during the freezing process (right)

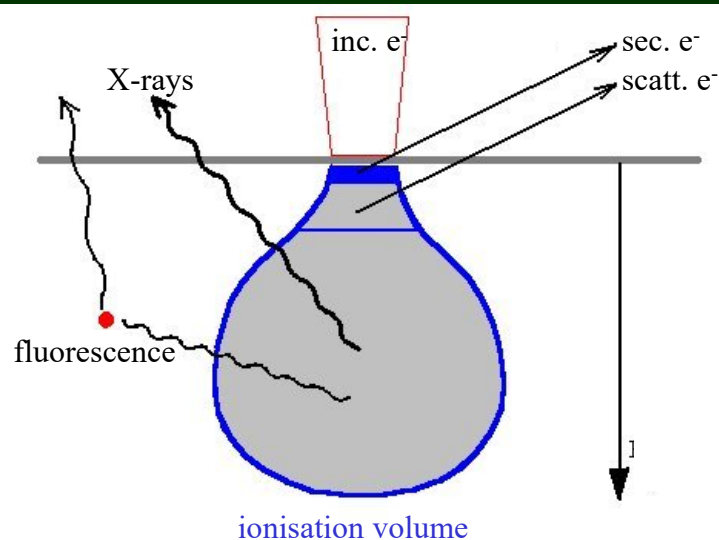
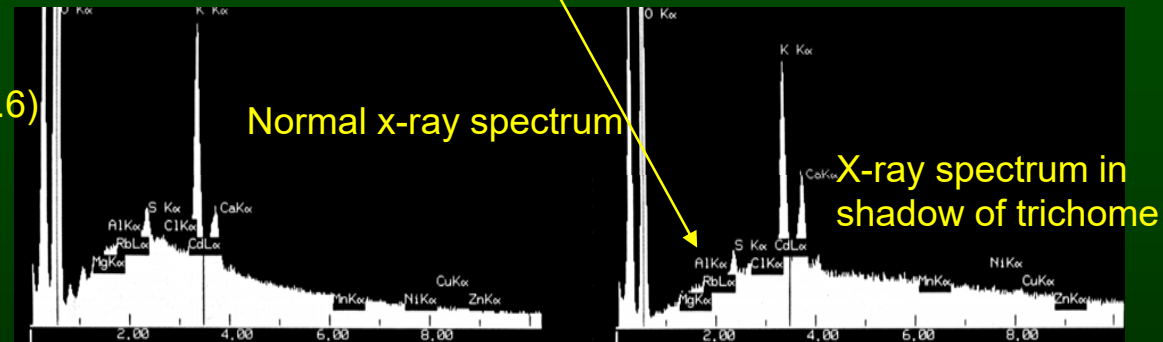
Methods of plant analysis using EDXA

Analysis of bulk-frozen samples



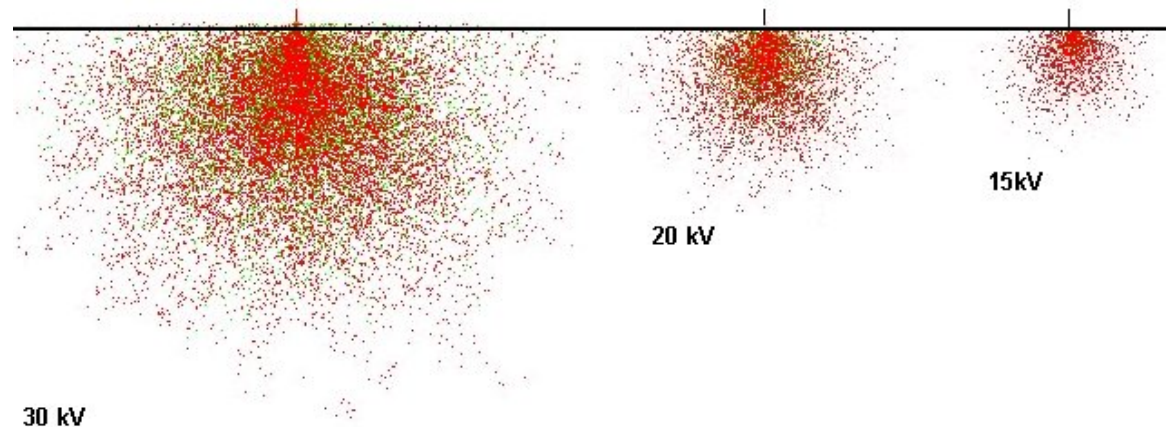
Effect of shading

shading inside a sample leads to absorption of low-energy x-rays



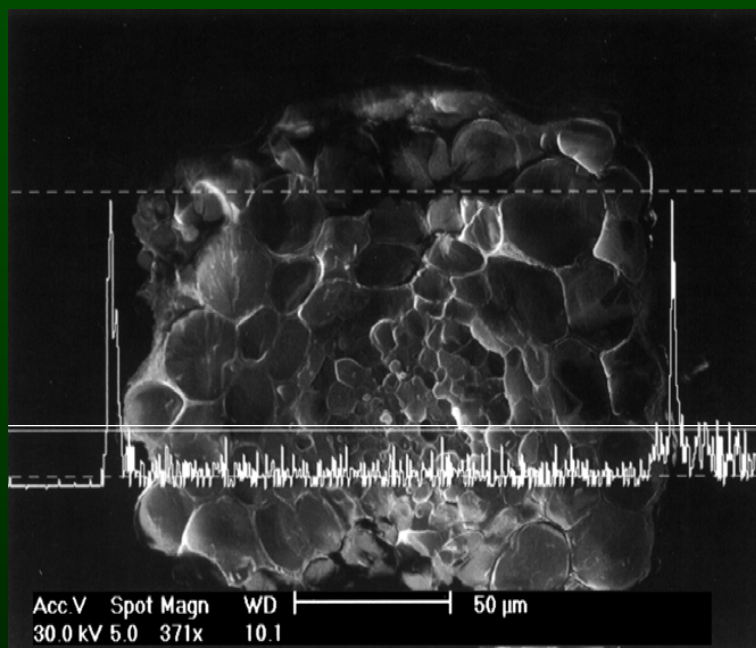
Effect of acceleration voltage

high acceleration voltage leads to deeper penetration into the sample!



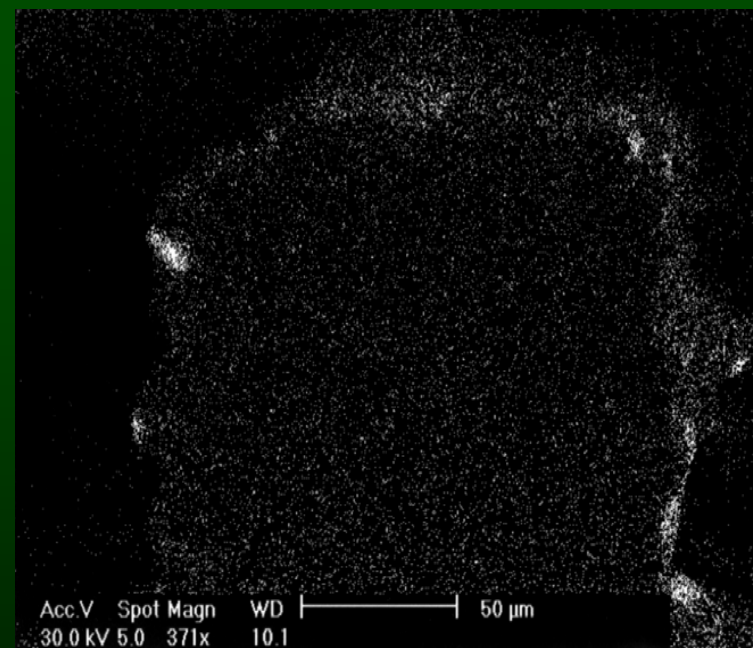
Methods of plant analysis using EDXA

Qualitative and semi-quantitative analysis of bulk-frozen samples



Line scans

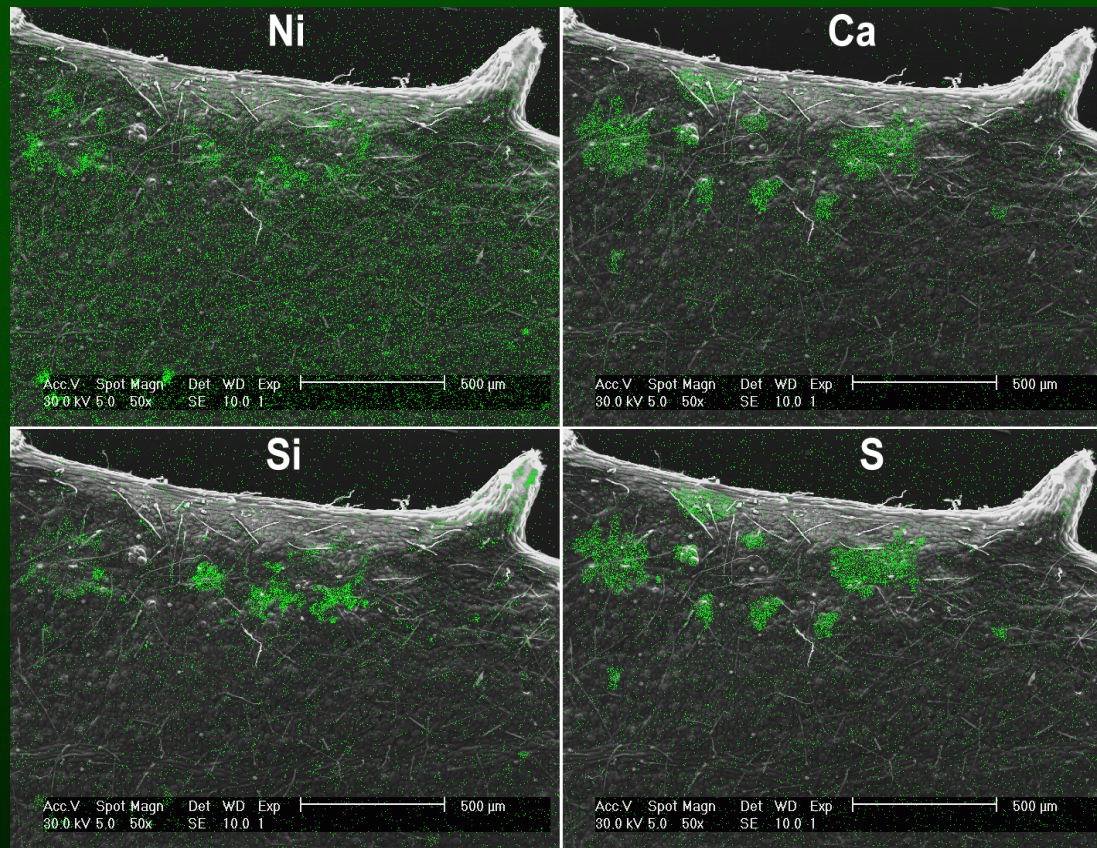
Scan of the Zn K alpha line (0.6x half width) along the straight line. Amplitude represents the counts/s inside the selected spectral window.



Dot maps

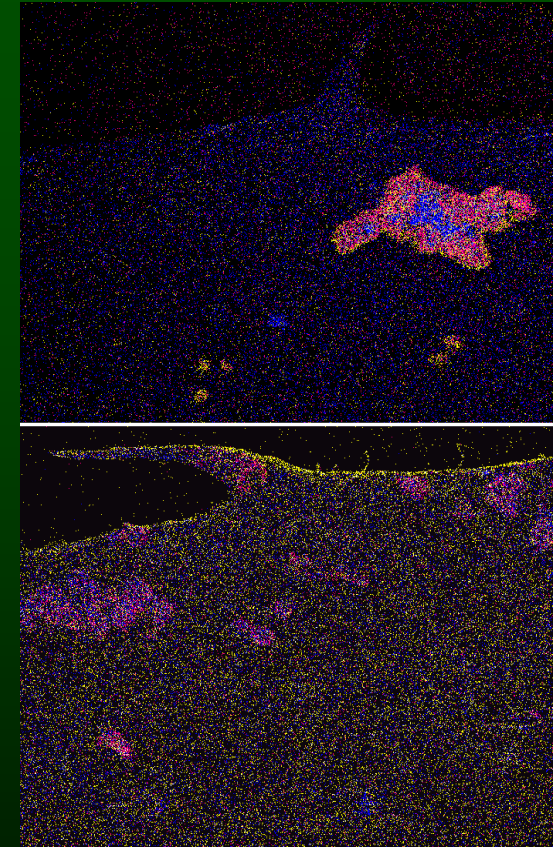
Scan of the Zn K alpha line (0.6x half width) over the whole image. Each dot represents one x-ray count inside the selected spectral window.

EDXA imaging application example: Ni silicate accumulation in cell walls of *Berkheya coddii*



Dot maps (K α lines) of the upper side of a *Berkheya coddii* leaf. Quantitative relation between Si and Ni in metal accumulation spots: $3.5 (\pm 1) \text{ Si / Ni}$ ($P = 0.0055$)

Küpper H (2001, doctoral thesis). UFO Atelier für Gestaltung und Verlag, Allensbach (ISBN 3-935511-07-8)4



Dot maps (K α lines) showing the development of metal accumulation spots.

Up: senescent leaf,
down: young-mature leaf;
blue: Ca, *red*: Mn, *yellow*: Ni

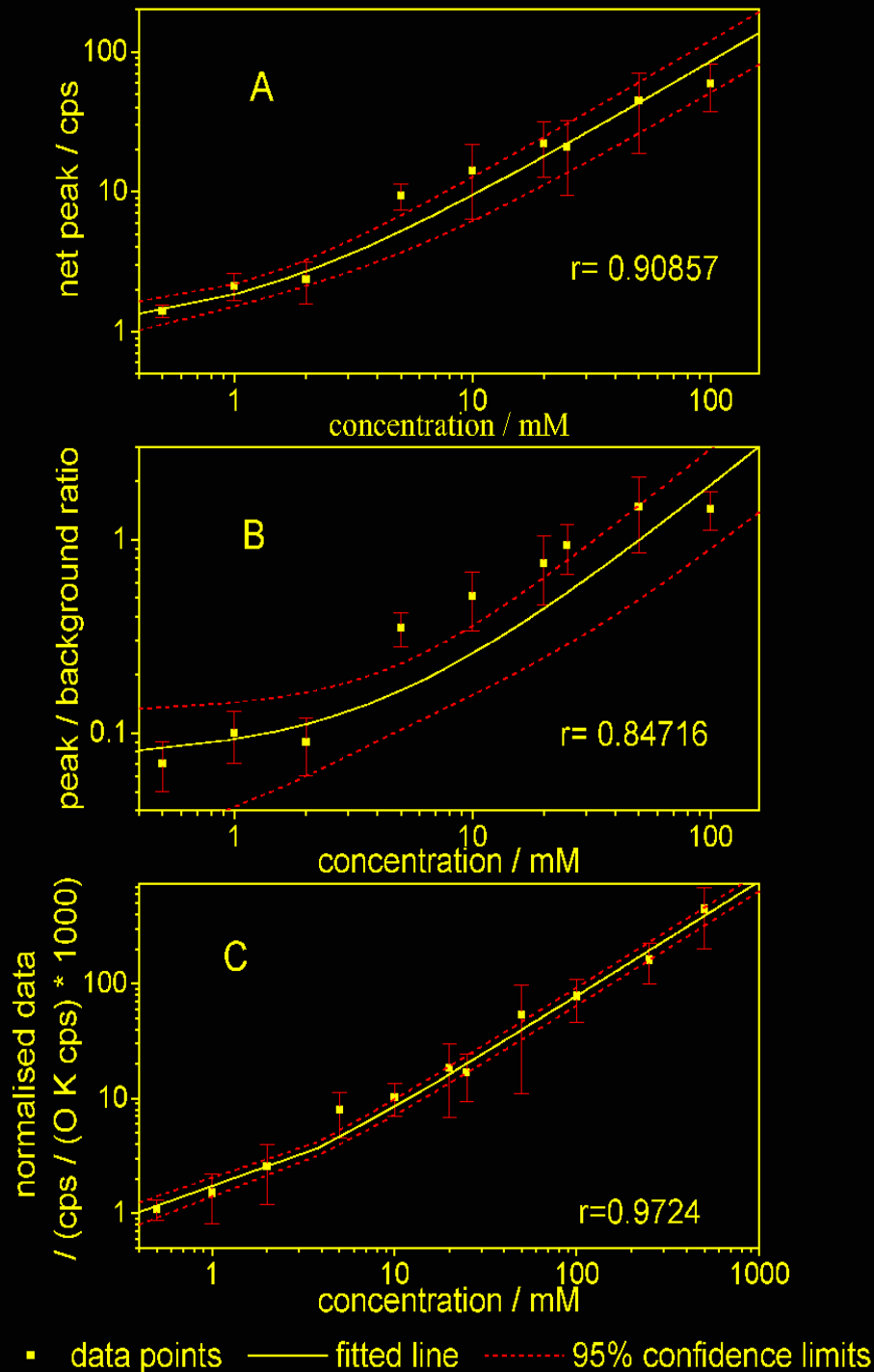
EDXA

Quantitative analysis of bulk-frozen samples

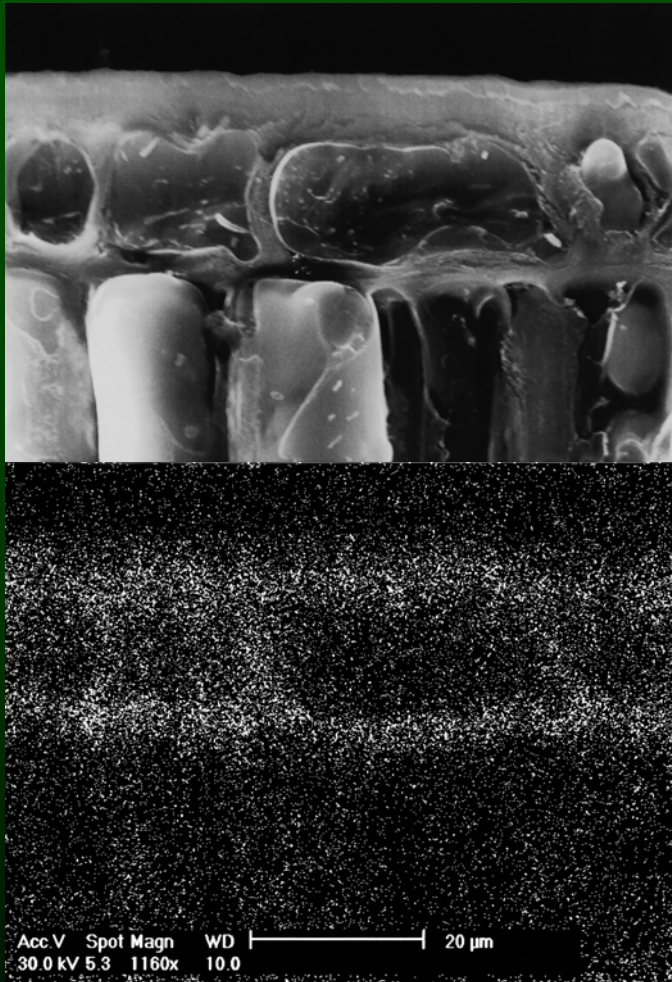
Counts in spectra (A)

can be normalised to either the background (B) or an internal standard. The oxygen K α line has proven to be a reliable internal standard in bulk-frozen samples, in particular in aqueous compartments like vacuoles (C).

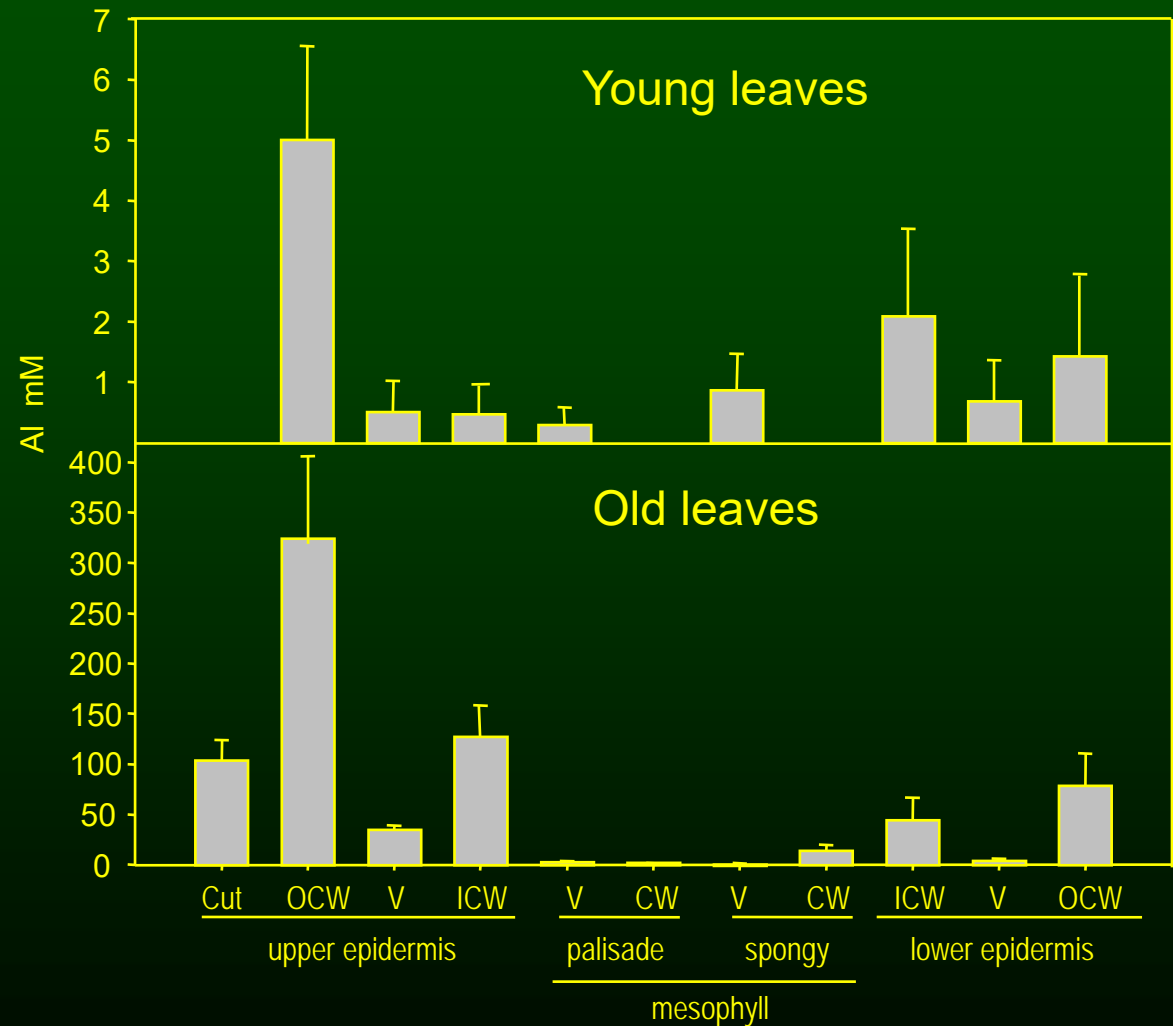
Küpper H, Lombi E, Zhao FJ, McGrath SP
(2000) *Planta* 212, 75-84



EDXA quantification application example: Al accumulation in epidermal cell walls of tea (*Camellia sinensis*)



Electronoptical picture of an old *C. sinensis* leaf (upper epidermis) and dot map of the Al K α line



Methods of plant analysis using EDXA

Analysis of bulk-frozen samples

Evaluation of the freeze-fracturing method

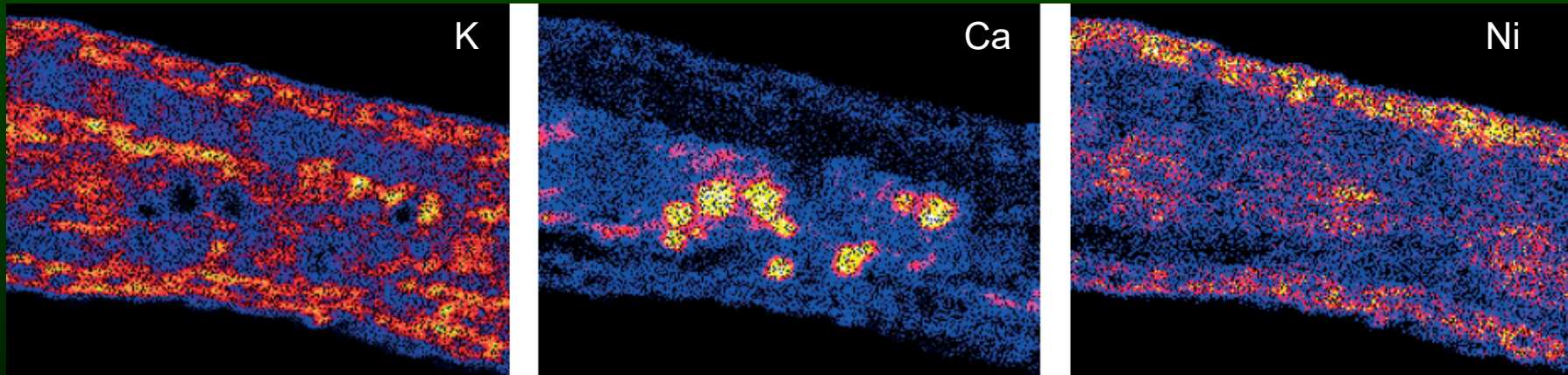
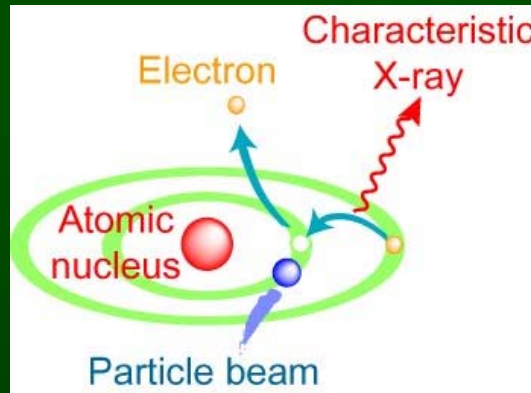
Advantages:

- All types of cells and tissues can be analysed
- *In situ*-analysis with very little risk of preparation artefacts
- Easy analysis of the heterogeneity of element distribution, by use of dot-maps even in an imaging way

Disadvantages:

- Limited sensitivity (min. 1mM) and accuracy (shading)
- Elements in dead tissues with low water content cannot be reliably quantified

(1) X-ray emission spectroscopy (b) Proton induced X-ray emission (PIXE) imaging



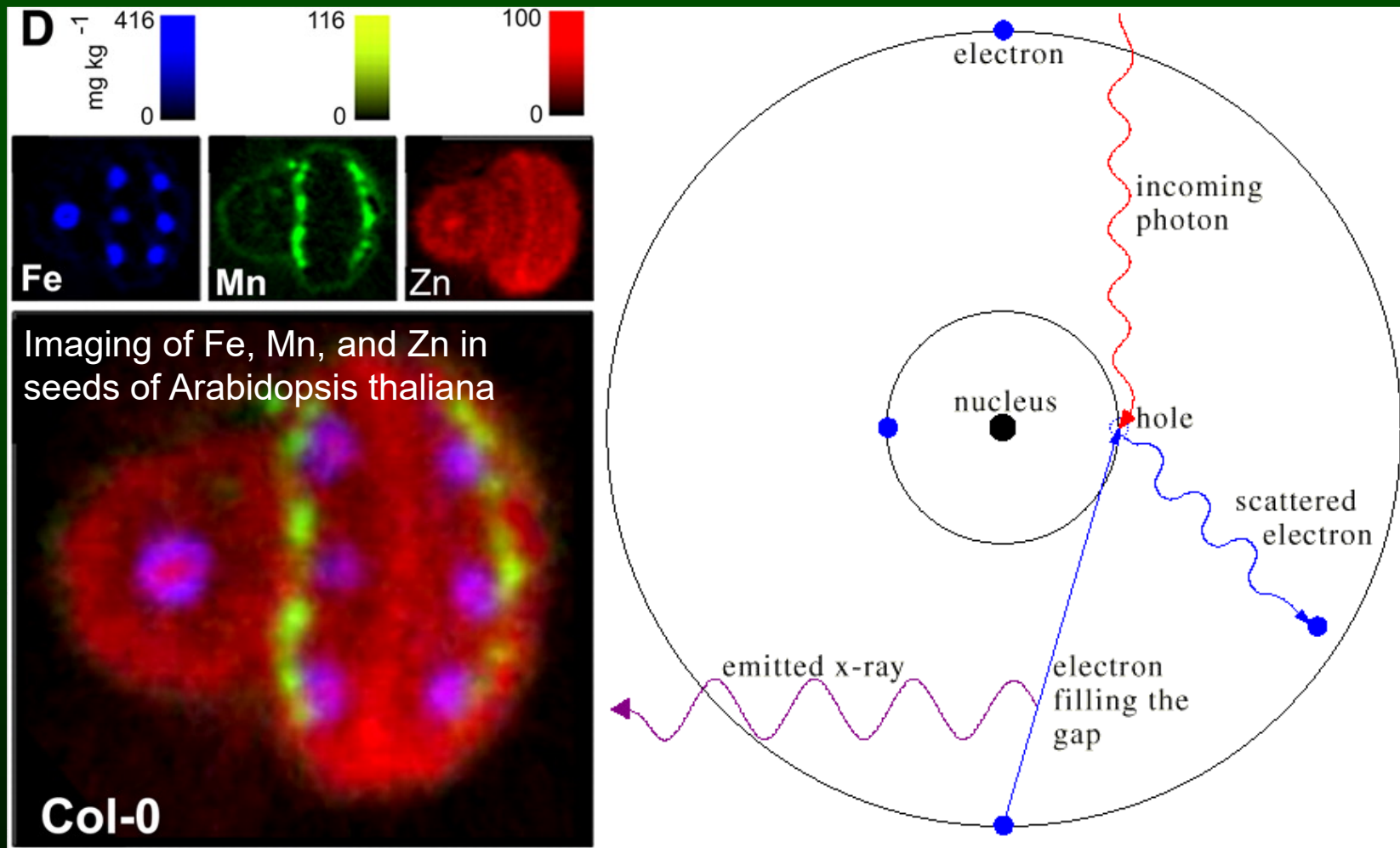
From: Siegle R, Kachenko AG, Bhatia NP, Wang YD, Ionescu M, Singh B, Baker AJM, Cohen DD, 2008, X-ray spectrometry 37, 133-6

Imaging of potassium, calcium and nickel in a leaf of *Hybanthus floribundus*

→ more sensitive than EDX, but no observation of frozen-hydrated samples (samples have to be freeze-dried) → increased risk of artefacts

Tomographic X-ray emission spectroscopy: principle of X-ray fluorescence imaging (XRF)

From:
Kim SA,
Punshon T,
Lanzirotti A,
Li L,
Alonso JM,
Ecker JR,
Kaplan J,
Guerinot ML,
2006,
Science 314,
1295-8

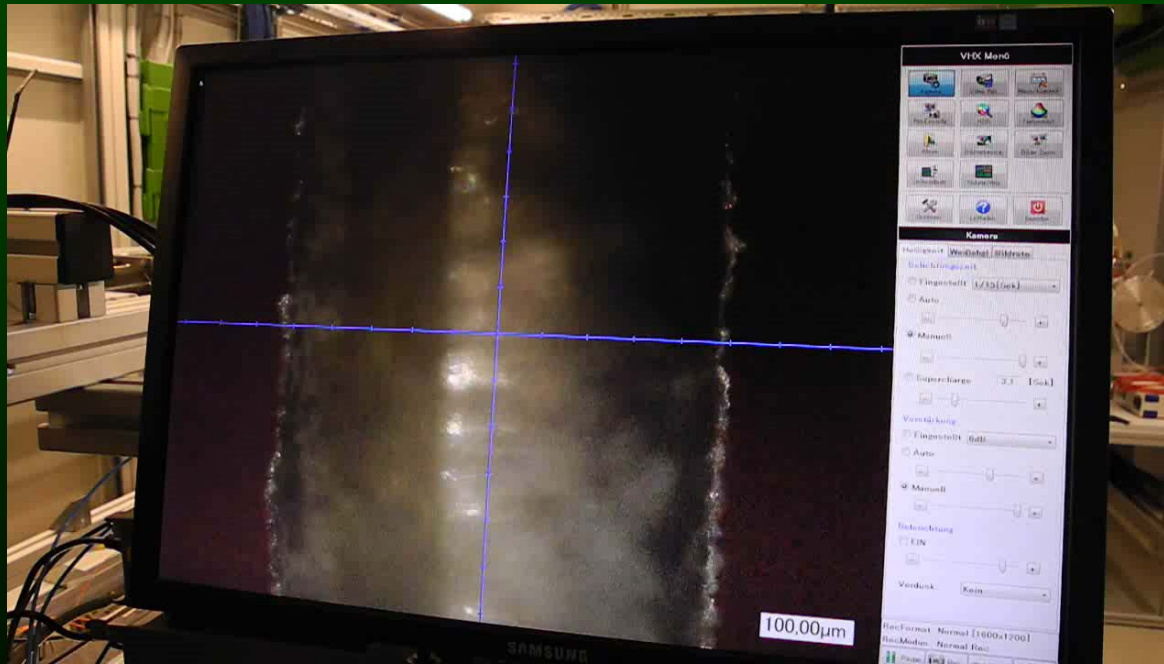


- MUCH more sensitive than EDX and PIXE, but in contrast to EDX it requires a synchrotron for excitation at resolutions $<15\mu\text{m}$ (limit of current commercial lab sources)
- limitation of beamtime

Tomographic X-ray emission spectroscopy:

Why is tomography better than 2D mapping for biological samples?

- MAIN advantage for our purposes: no thin sectioning necessary for large samples (e.g. roots, leaves)
 - drastic reduction of sample preparation artefacts
 - analysed optical slice of sample is protected by surrounding sample and mounting capillary, therefore less damage during storage, transport and analysis

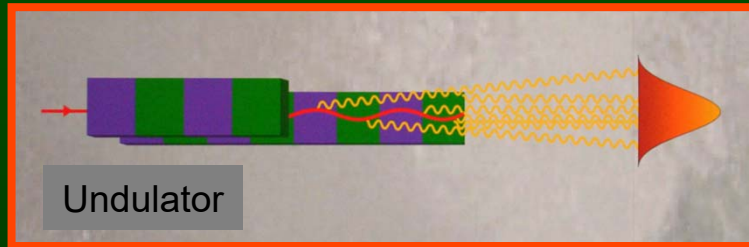


From work of Mishra S, Alfeld M, Sobotka R, Andresen E, Falkenberg G, Küpper H on As-stress in *Ceratophyllum demersum* measured at the the PETRA III P06 beamline of DESY (Hamburg)

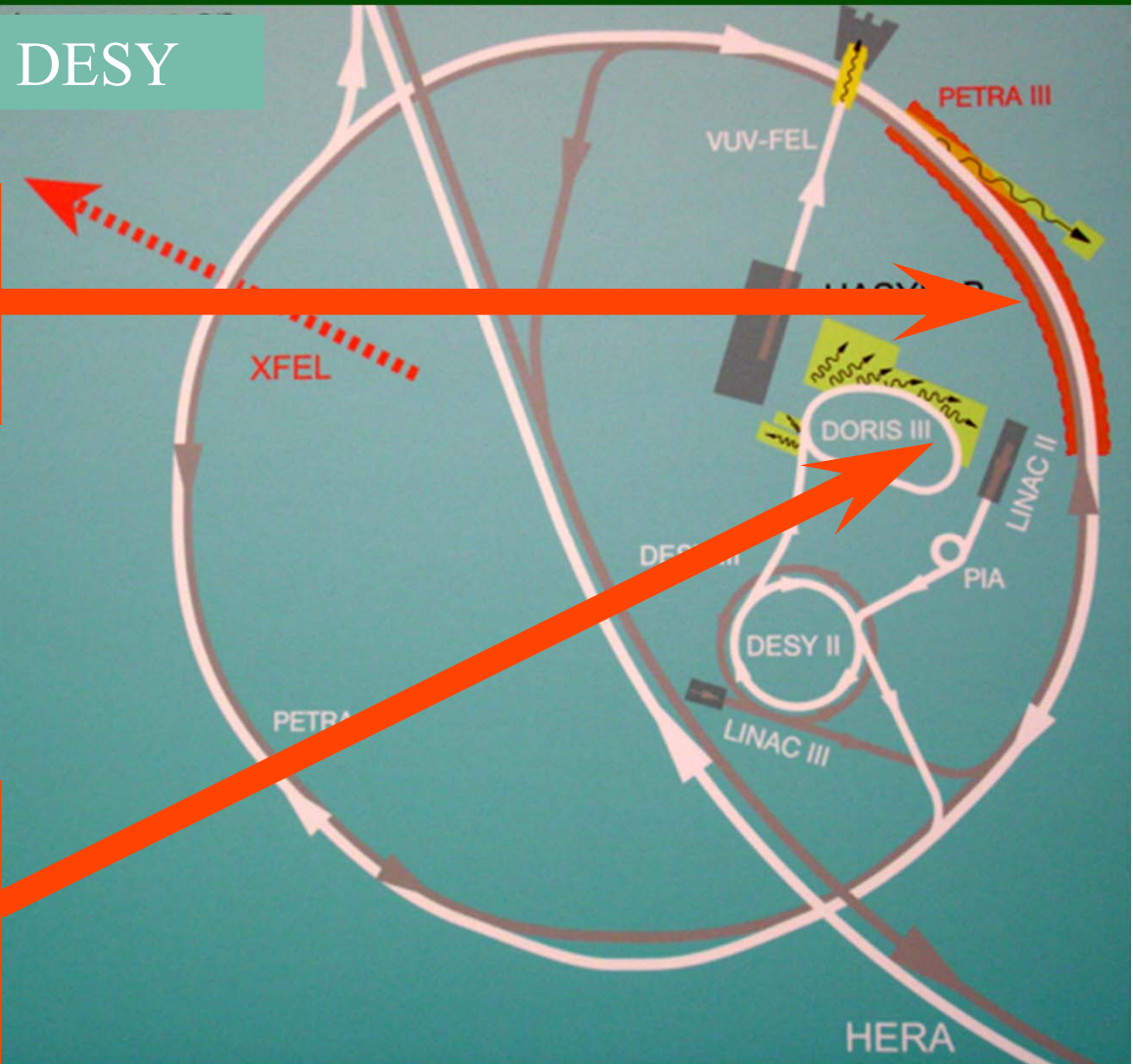
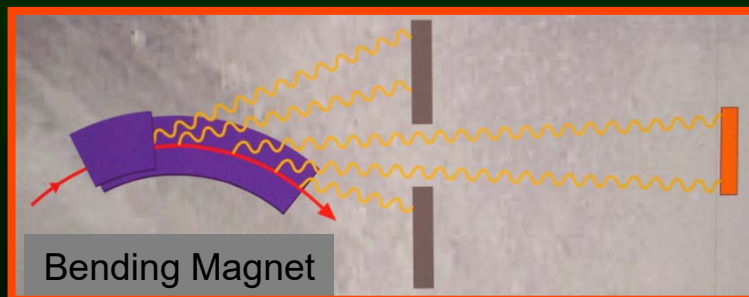
- For small samples (e.g. microalgae): 3D analysis possible

Where we measure (I): Synchrotrons

P06 beamline at PETRA III, microprobe, focussing with KB mirror system, $0.6\ \mu\text{m}$ beam size, Maia detector



Beamline L at DORIS, focussing with single-bounce capillary, $10\ \mu\text{m}$ beam size, 2 Vortex SDD detectors

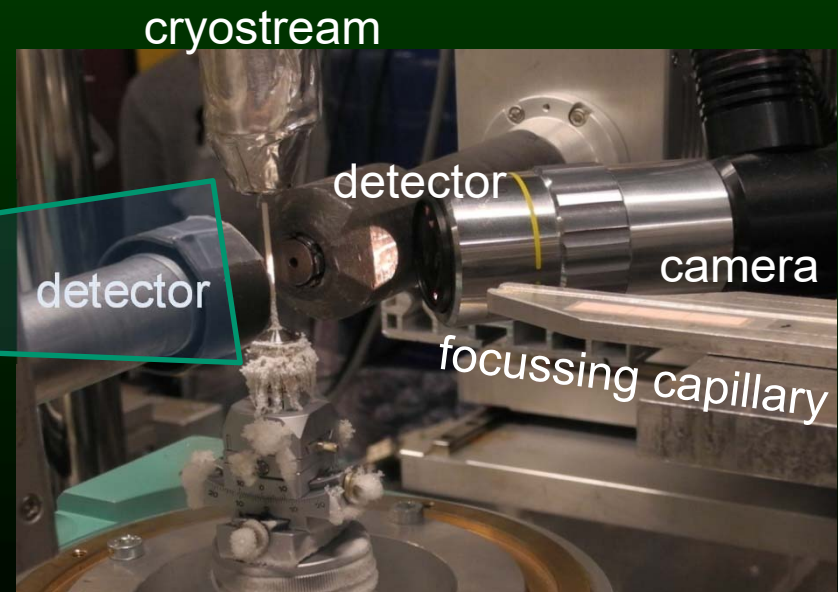
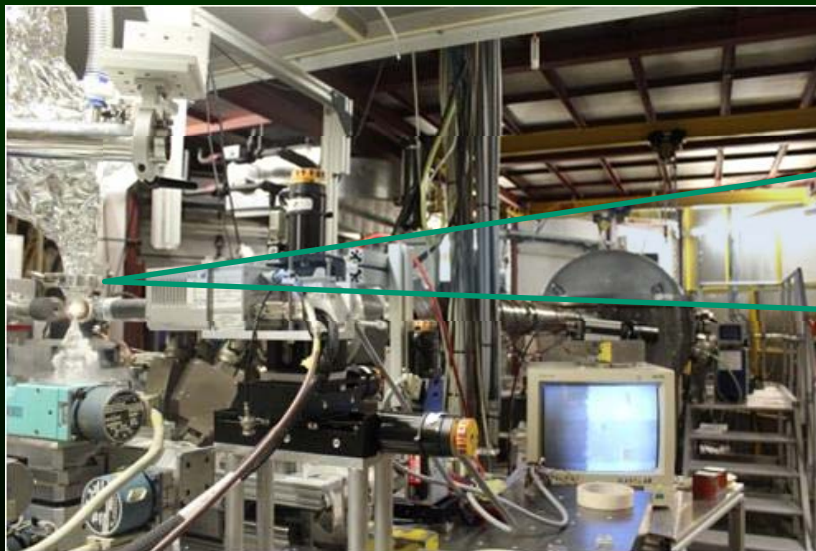


(1) X-ray emission spectroscopy

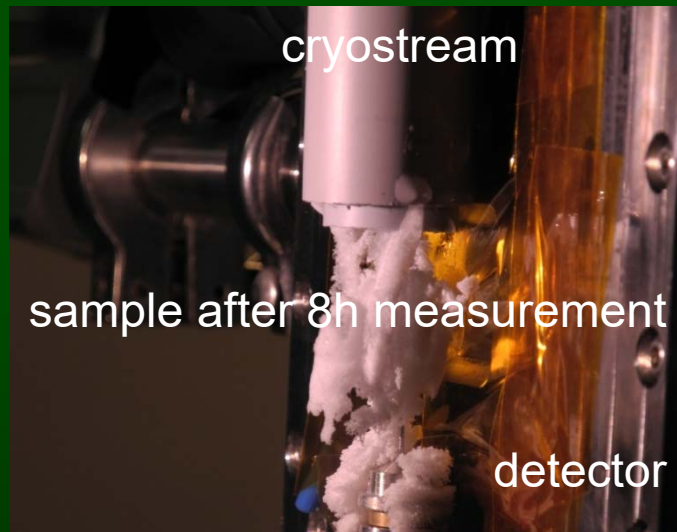
(c) μ -XRF: Sample preparation and measurement



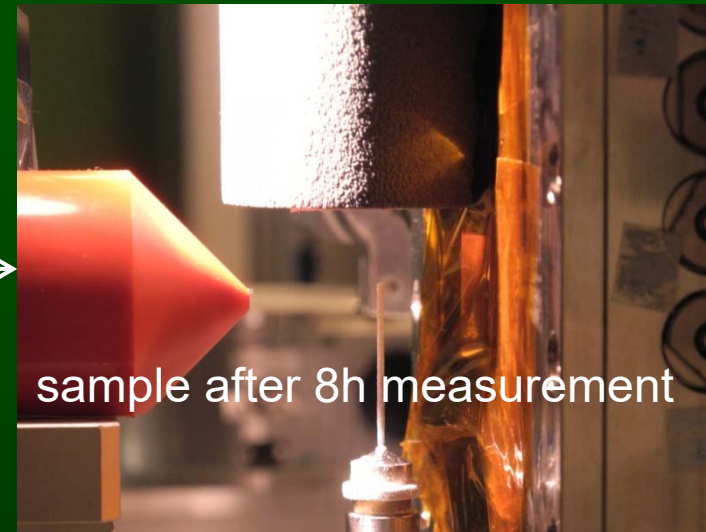
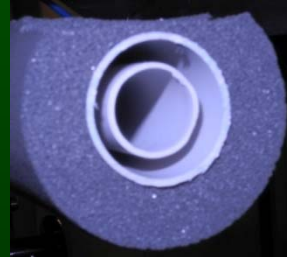
From work of Mishra S, Wellenreuther G, Küpper H
on As-stress in *Ceratophyllum demersum*
measured at the DESY (Hamburg)



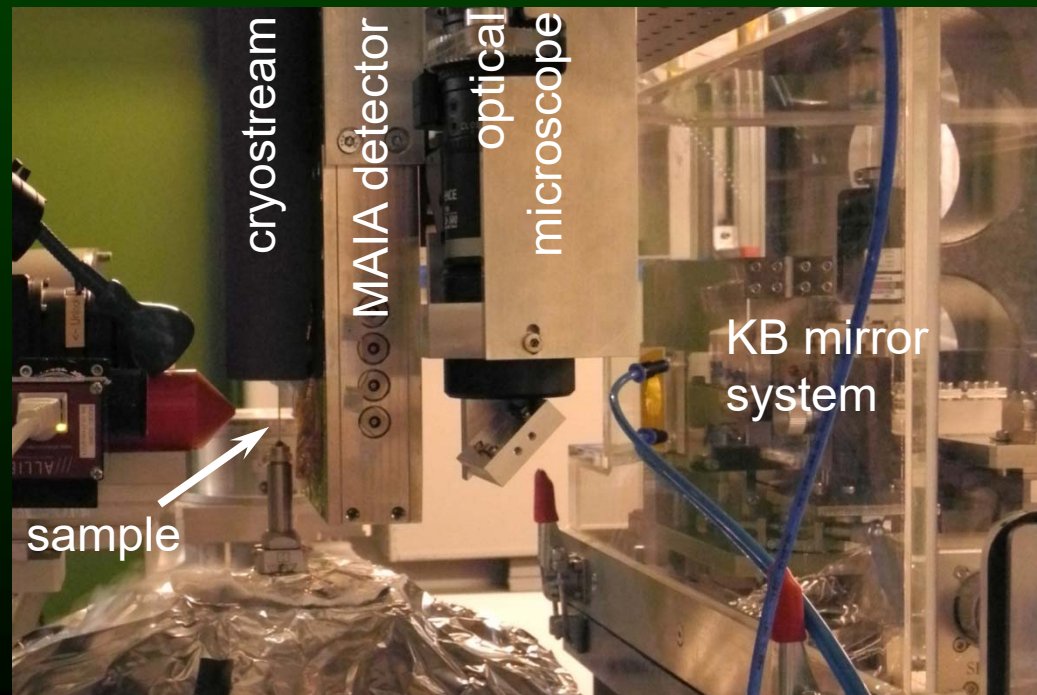
Tomographic X-ray emission spectroscopy (μ -XRF): Cryostream extension for a bulky MAIA detector



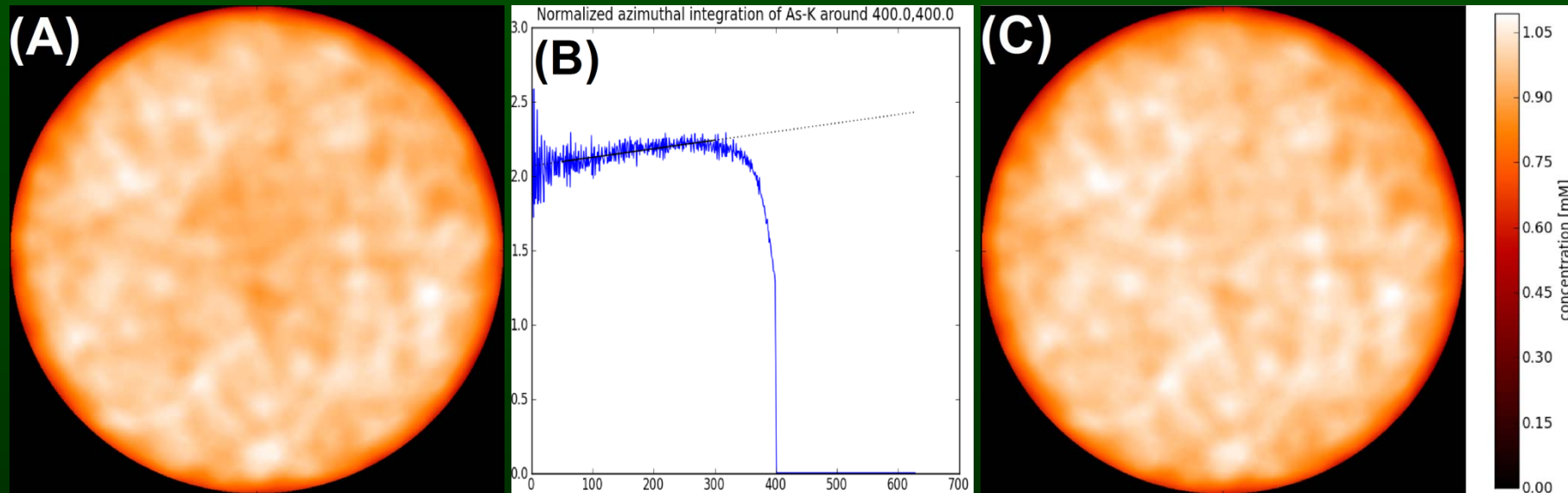
improved flow
and insulation
of cryostream
extension



From work of Mishra S, Alfeld M, Sobotka R,
Andresen E, Falkenberg G, Küpper H on As-
stress in *Ceratophyllum demersum*
measured at the the PETRA III P06
beamline of DESY (Hamburg)

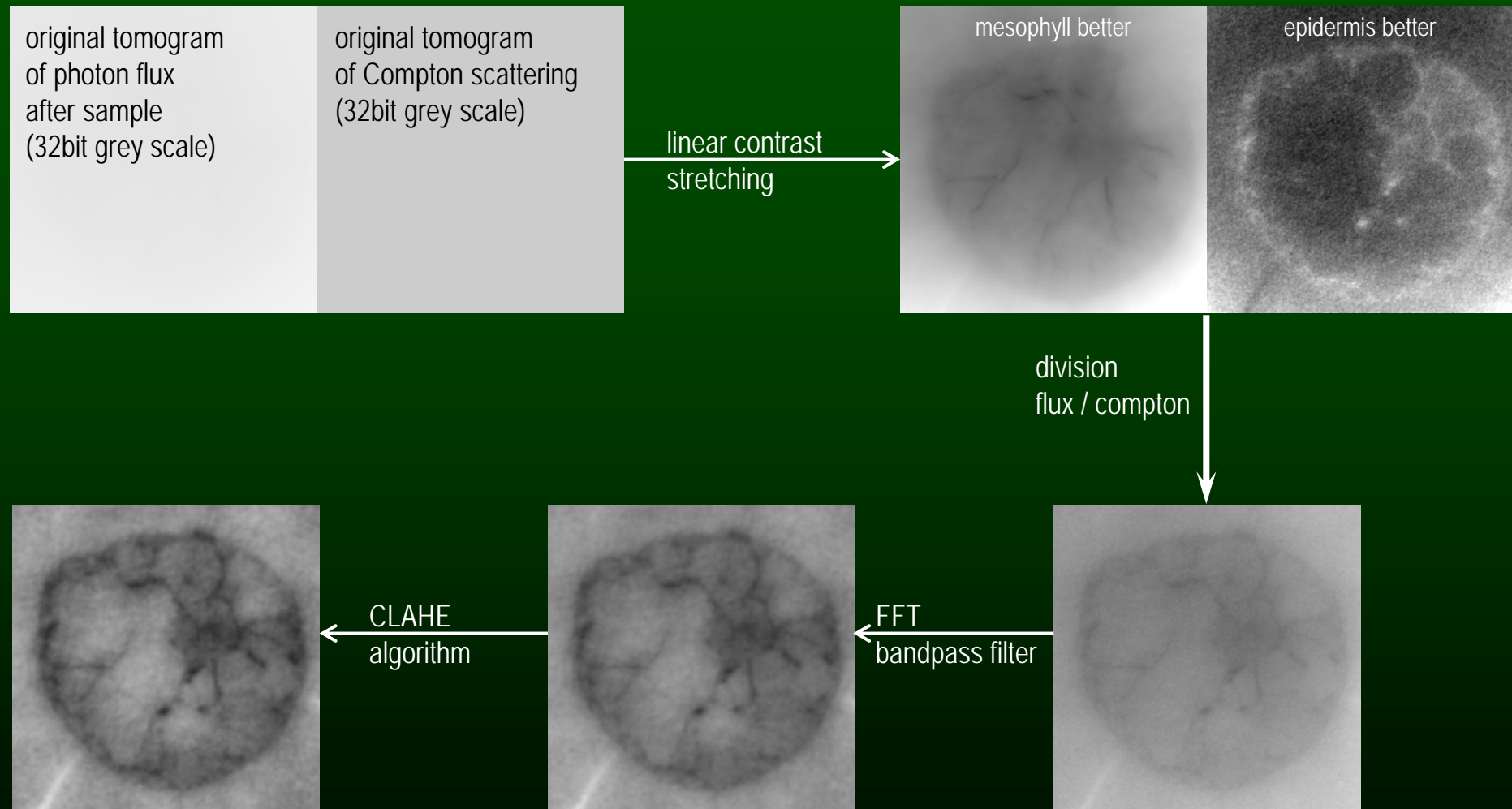


Tomographic X-ray emission spectroscopy (μ -XRF): Quantification with correction of self-absorption

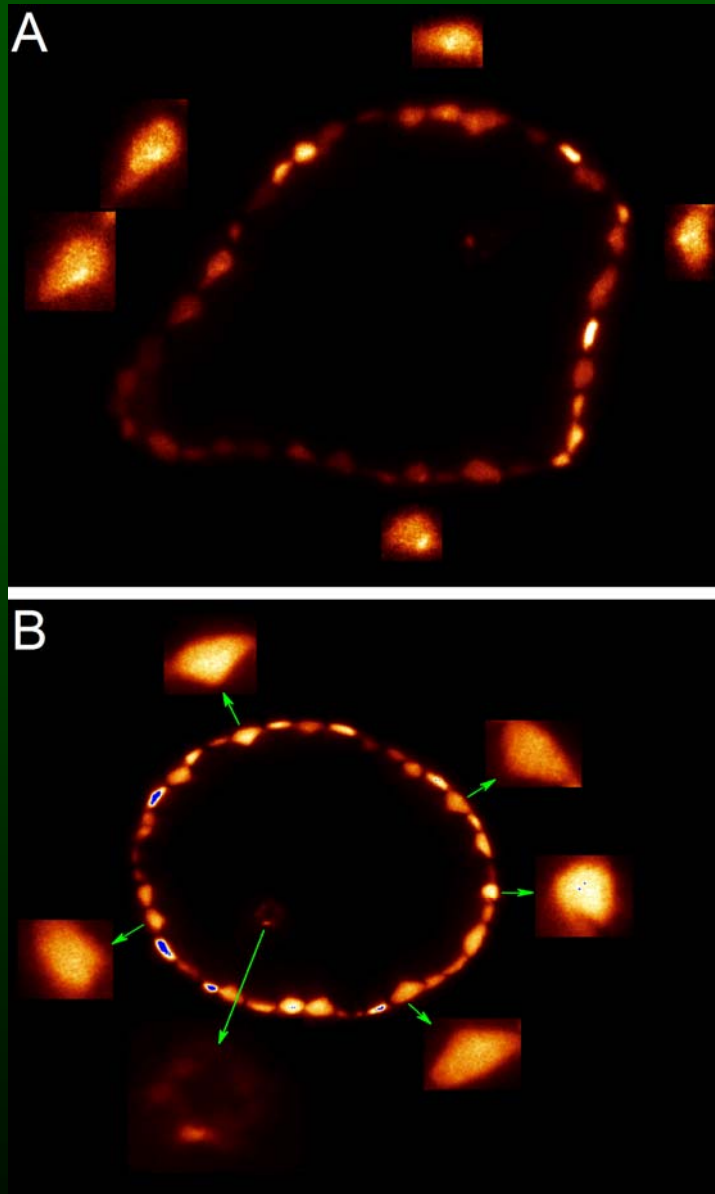


- intensity artefacts due to absorption inside the sample were corrected by taking the fluorescence intensity distribution in homogeneous standards resembling the shape of the sample as a reference
- for minimising disturbance by background and neighbouring emission lines, full spectral deconvolution/fitting was used (in PyMCA for SDD data, Geopixe for Maia data).
- for minimising reconstruction artefacts, the MLEM (maximum likelihood expectation maximization) algorithm was applied

Tomographic X-ray emission spectroscopy (μ -XRF): Using Flux and Compton tomograms for showing tissue structures in frozen-hydrated plant samples



Sub-cellular distribution of As in *C. demersum* leaves



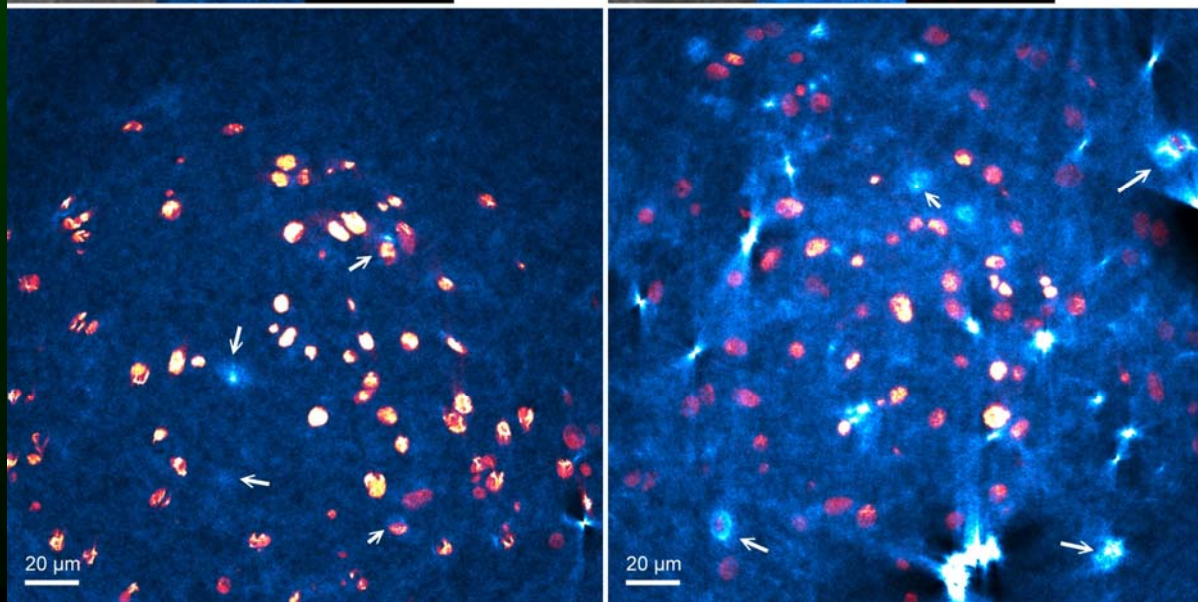
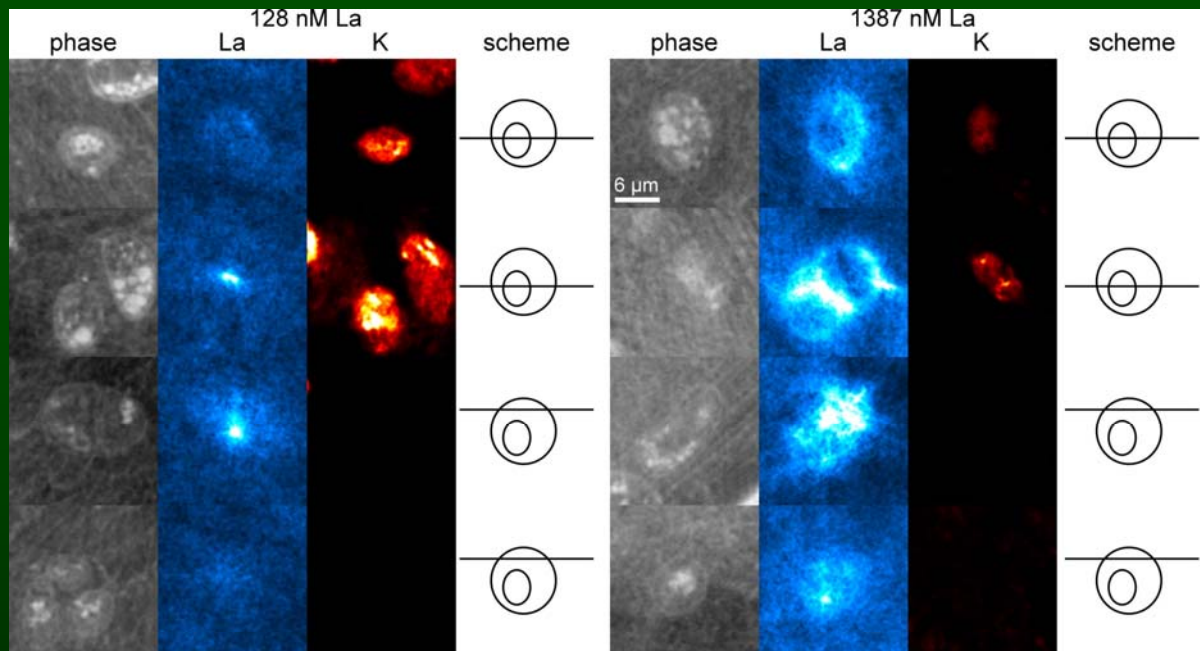
2 phase response to As toxicity

- A) Initially, at sublethal concentrations, As is accumulated mainly in the nucleus
→ genotoxicity (besides inhibition of Chl biosynthesis)
- B) At lethal concentration, As fills the whole cell
→ various types of damage

Sub-cellular distribution of La (measured at La K α with 0.2 μ m resolution at beamline ESRF ID16A)

Response to La toxicity

- A) At sublethal La concentrations, La is usually accumulated inside the cells, often in small spots (biominerals?)
- B) At lethal concentration, essential elements become released through leaky membranes, La binds in the whole cytoplasm



Ashraf N, Vitova M, Cloetens P, Mijovilovich A, Bokhari SNH, Küpper H* (2021) Effect of nanomolar concentrations of lanthanum on *Desmodesmus quadricauda* cultivated under environmentally relevant conditions. *Aquatic Toxicology* (accepted for publication)

Where we measure μ XRF (II): At home

Customised Bruker Tornado M4 machine in our lab

- Purpose: Imaging measurement of element distribution ($Z \geq 11$ Na) with tissue-level (15-20 μ m) resolution *in vivo*

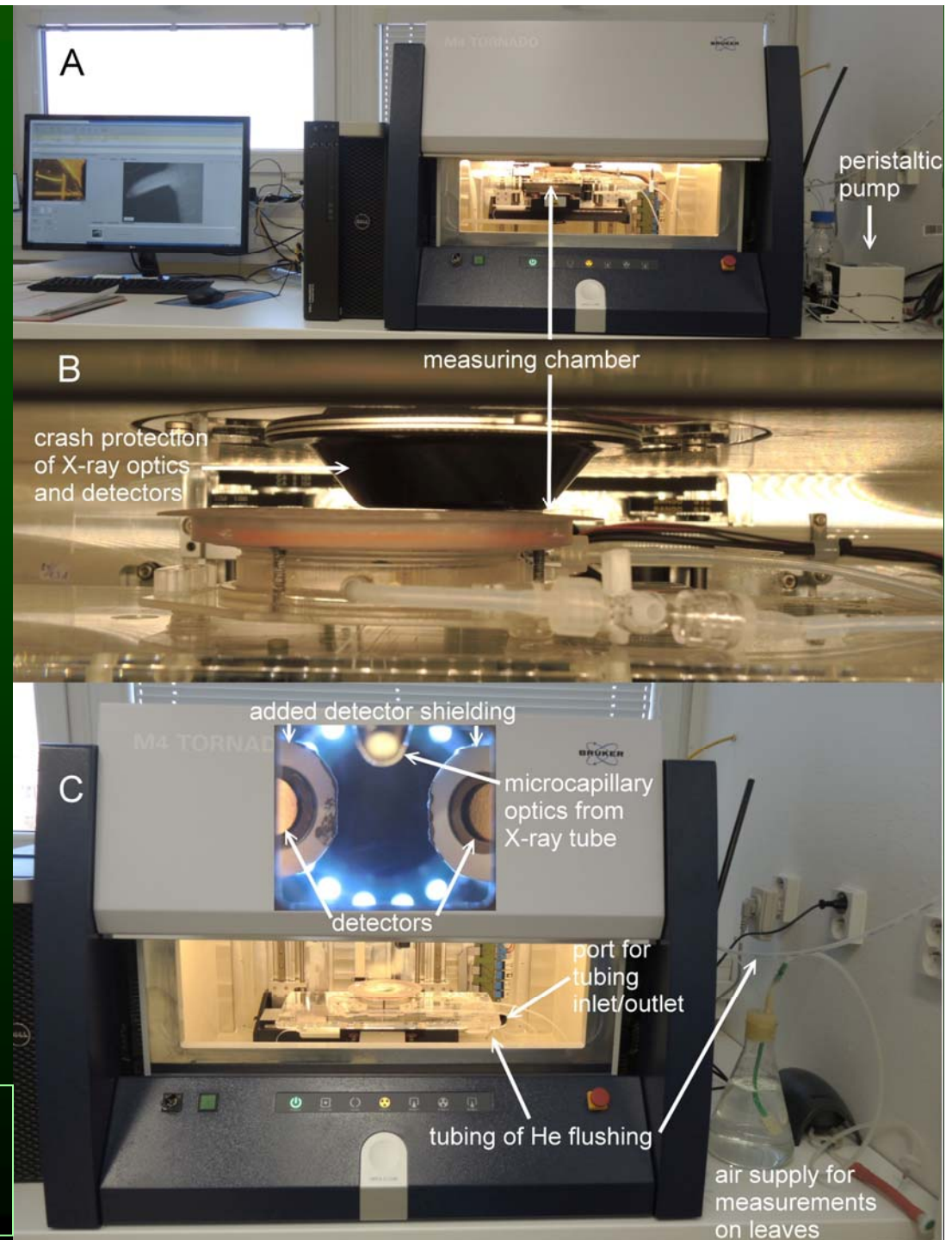
- Special features:

- biology-optimised configuration with 2 SDD detectors of together 120 mm² active area and fast readout electronics

- custom-made special shielding to reduce background counts in the range of the trace elements that are important for our work

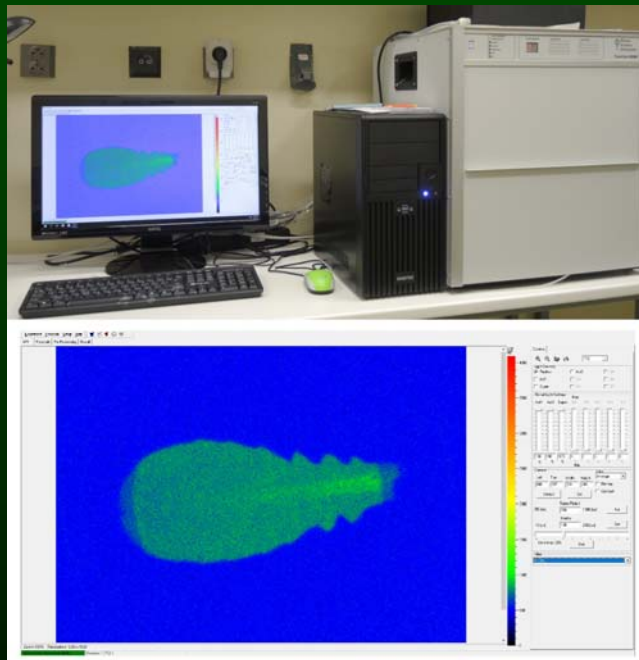
- custom-made measuring chamber for keeping samples alive

Mijovilovich A, Morina F, Bokhari SNH, Wolff T, Küpper H* (2020) Analysis of trace metal distribution in plants with lab-based microscopic X-ray fluorescence imaging, Plant Methods, DOI: 10.1186/s13007-020-00621-5

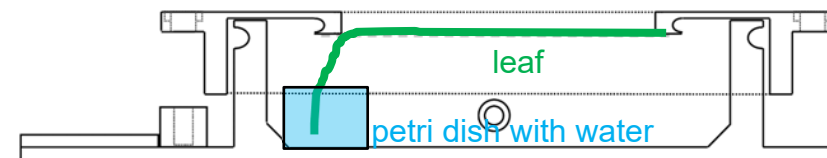
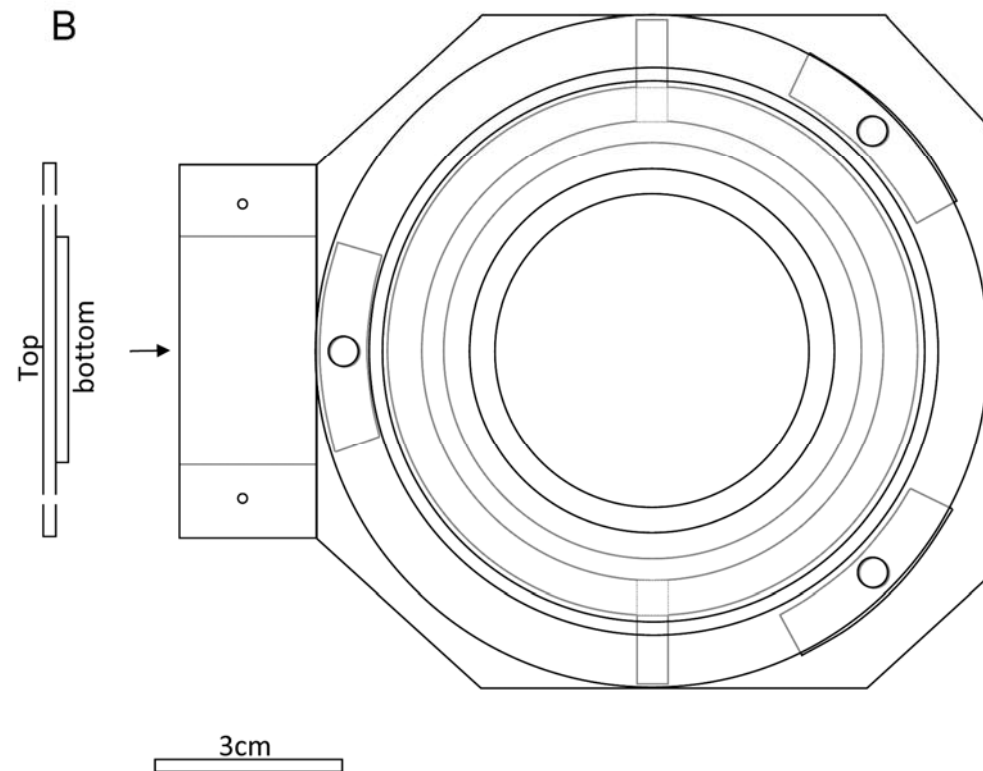
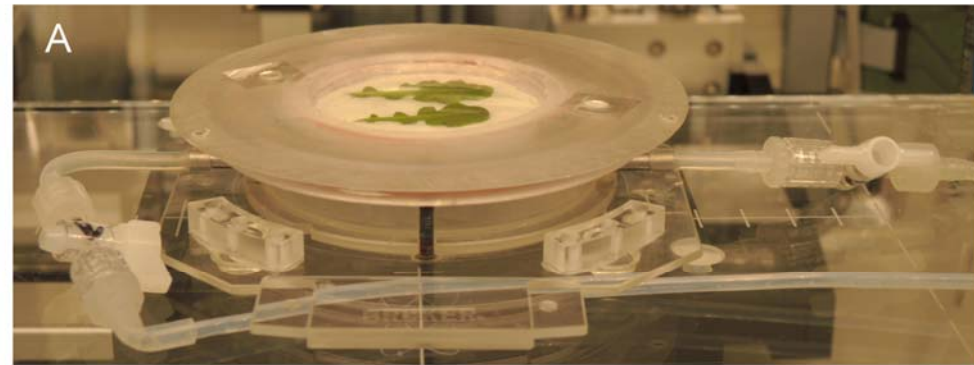


Where we measure μ XRF (I): Customised Bruker Tornado M4 in our lab

Sample preparation and
photosynthesis measurement



OJIP: Küpper H, Benedikty Z, Morina F, Andresen E, Mishra A, Trtílek M (2019) Plant Physiology 179, 369-381.
 μ XRF: Mijovilovich A, Morina F, Bokhari SNH, Wolff T, Küpper H* (2020) Plant Methods, DOI: 10.1186/s13007-020-00621-5

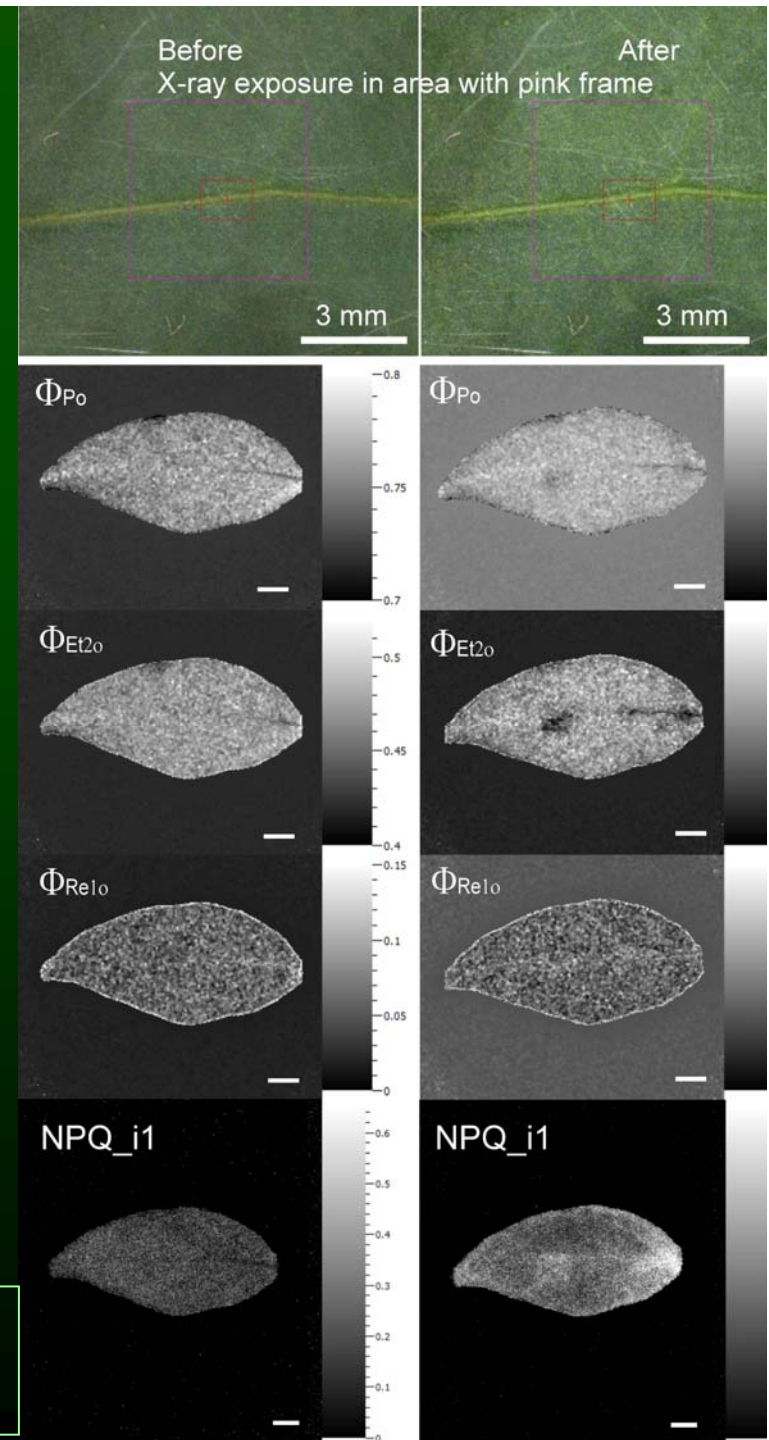


Verification of sample vitality during measurement in the in-house X-ray fluorescence microscope for analysis of metal localization in living tissues

Measurement of photosynthetic activity by direct fast imaging of OJIP chlorophyll fluorescence kinetics

→ Samples stay vital even after 20h total measurement time, but small effects on electron transport and non-photochemical quenching can be seen

Mijovilovich A, Morina F, Bokhari SNH, Wolff T, Küpper H* (2020) Analysis of trace metal distribution in plants with lab-based microscopic X-ray fluorescence imaging, Plant Methods, DOI: 10.1186/s13007-020-00621-5

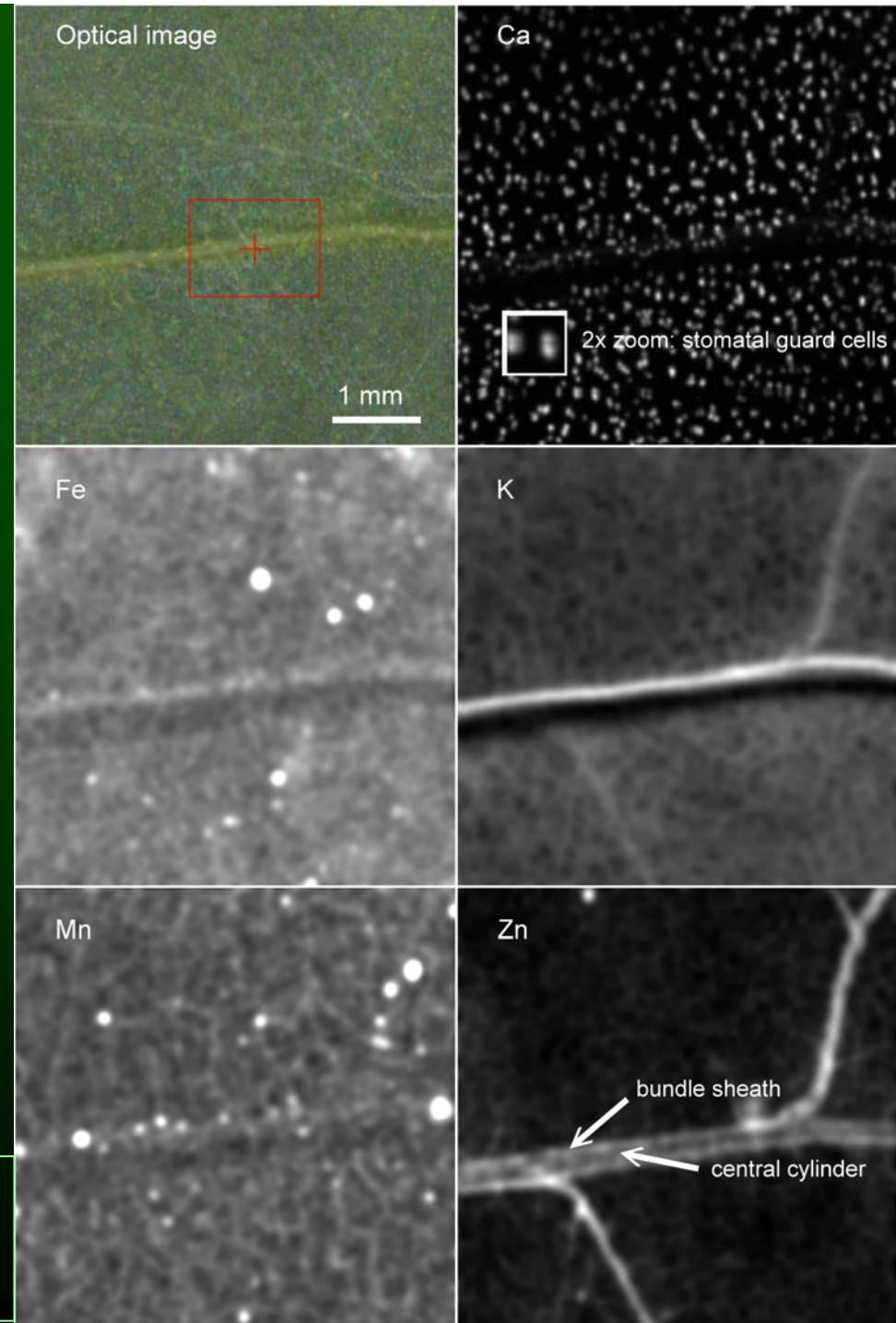


Examples of the use of the in-house X-ray fluorescence microscope for analysis of metal localization in living tissues:
***Capsicum annuum* (pepper) leaves**

→ The sensitivity of the machine is sufficient for visualising trace metals in non-accumulator crop plants

→ The spatial resolution (15 μm beam size) is sufficient for imaging metal distribution between tissues and larger cells

Mijovilovich A, Morina F, Bokhari SNH, Wolff T, Küpper H* (2020) Analysis of trace metal distribution in plants with lab-based microscopic X-ray fluorescence imaging, Plant Methods, DOI: 10.1186/s13007-020-00621-5

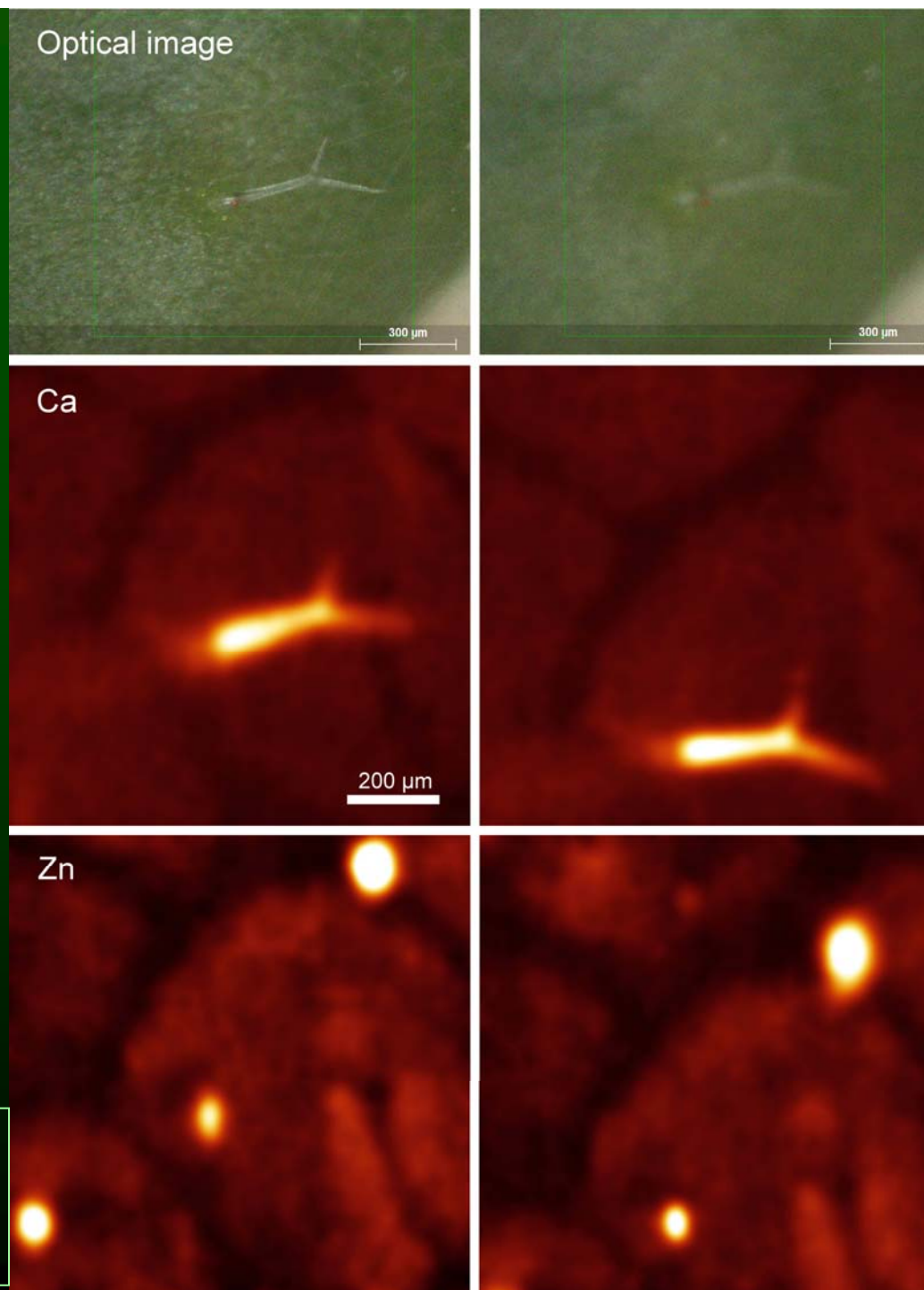


Examples of the use of the in-house X-ray fluorescence microscope for analysis of metal localization in living tissues: *Arabidopsis halleri* leaves

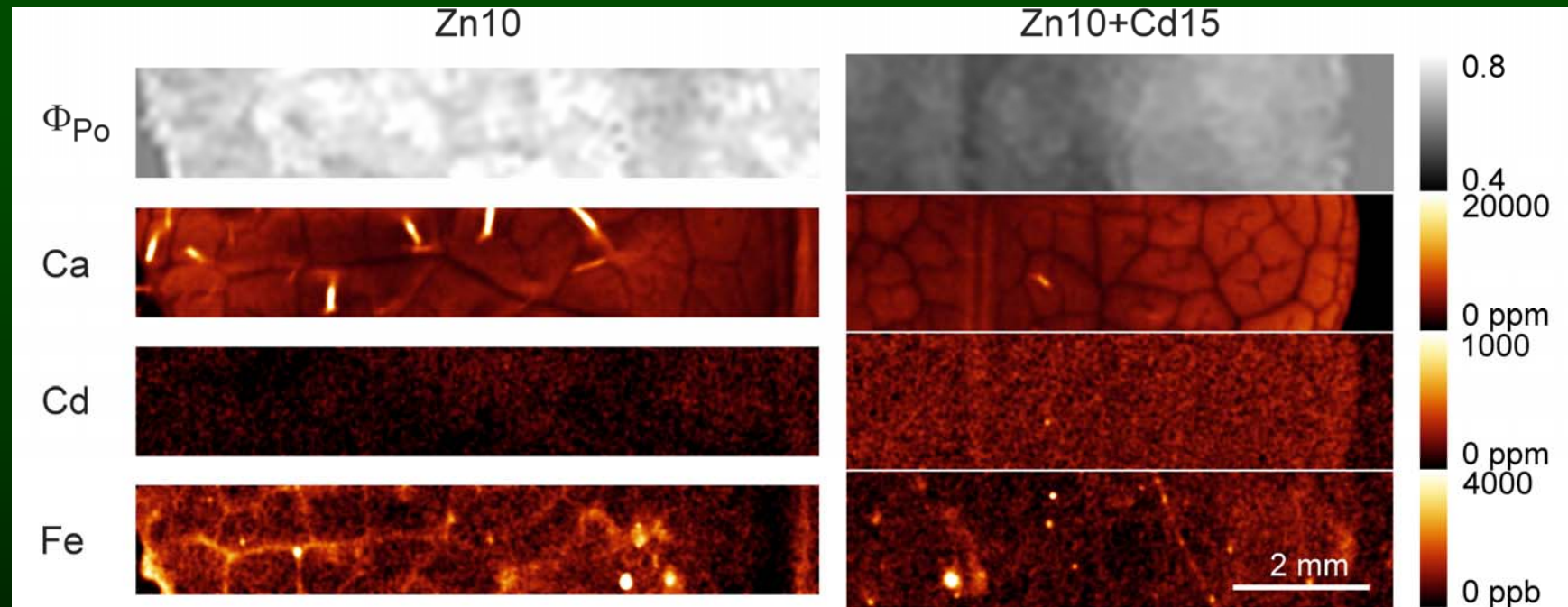
→ de-focussing affects the resolution of the optical image more than the μ XRF maps

→ Due to the geometric arrangement of the optical camera vs. the X-ray optics (polycapillary), de-focussing leads to a shift of the μ XRF maps relative to the optical image.

Mijovilovich A, Morina F, Bokhari SNH, Wolff T, Küpper H* (2020) Analysis of trace metal distribution in plants with lab-based microscopic X-ray fluorescence imaging, Plant Methods, DOI: 10.1186/s13007-020-00621-5



Examples of the use of the in-house X-ray fluorescence microscope for analysis of metal localization in living tissues: Cd accumulation vs. PSII efficiency in *Arabidopsis halleri* leaves

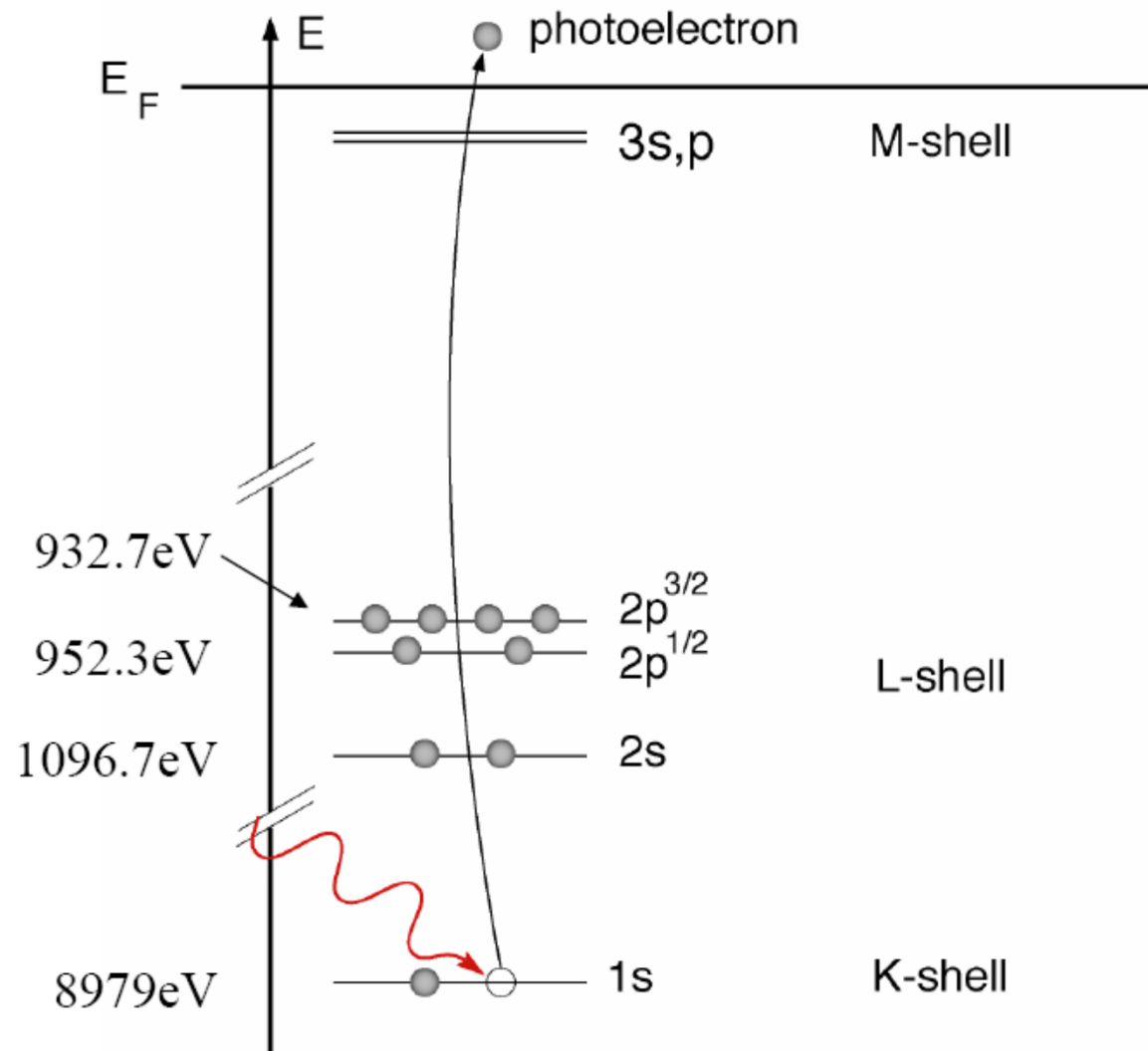


→ In vivo measurement allows for direct correlation between physiology and metal accumulation!

Morina F, Küpper H* (2020) Direct inhibition of photosynthesis by Cd dominates over inhibition caused by micronutrient deficiency in the Cd/Zn hyperaccumulator *Arabidopsis halleri*. Plant Physiology and Biochemistry 155_252-261 (DOI: <https://doi.org/10.1016/j.plaphy.2020.07.018>)

X-ray absorption (I)

Cu



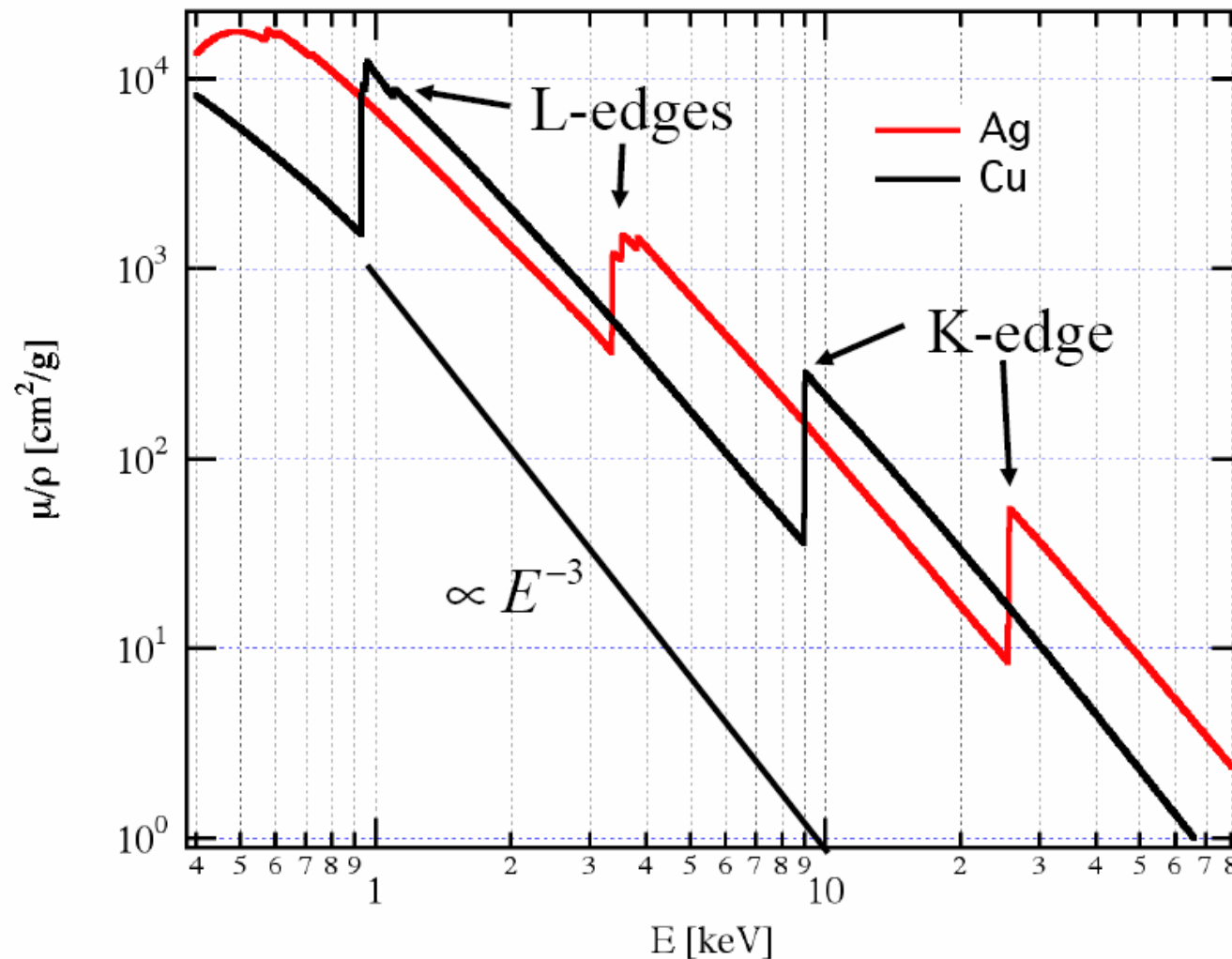
- Electrons in atoms are arranged in shells with different atom-specific binding energies: K, L, M
- Atomic electron can absorb x-rays if:

$$E_{\text{photon}} > E_{\text{ionization}}$$

(Pauli-principle)

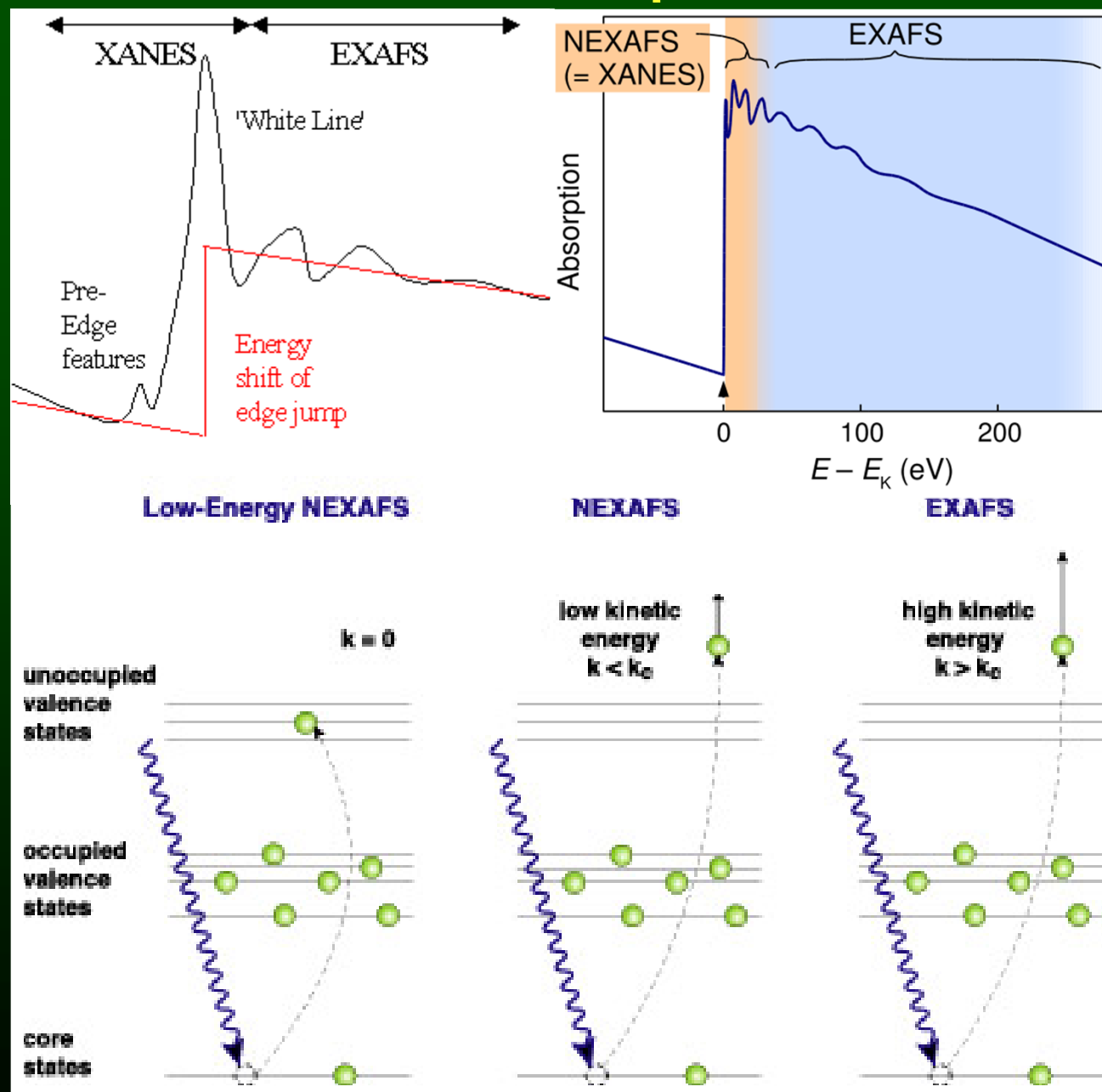
X-ray absorption (II)

$\mu(E)$: linear absorption coefficient



- mainly atomic effect
- strong dependence on energy:
 $\propto E^{-2.78}$
- strong dependence on atomic number:
 $\propto Z^{2.7}$
- inner shell electr. contribute most strongly

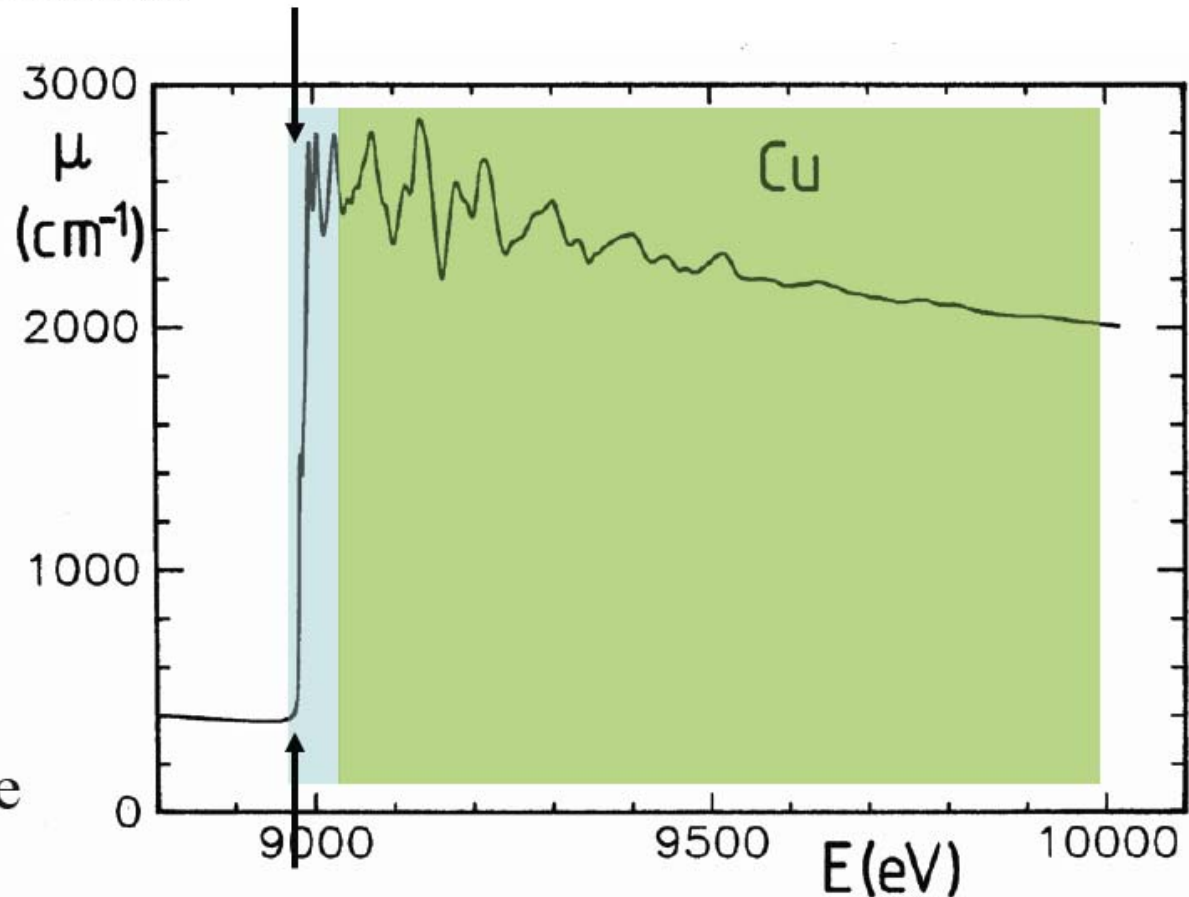
XAS techniques



What can we learn from XAS?

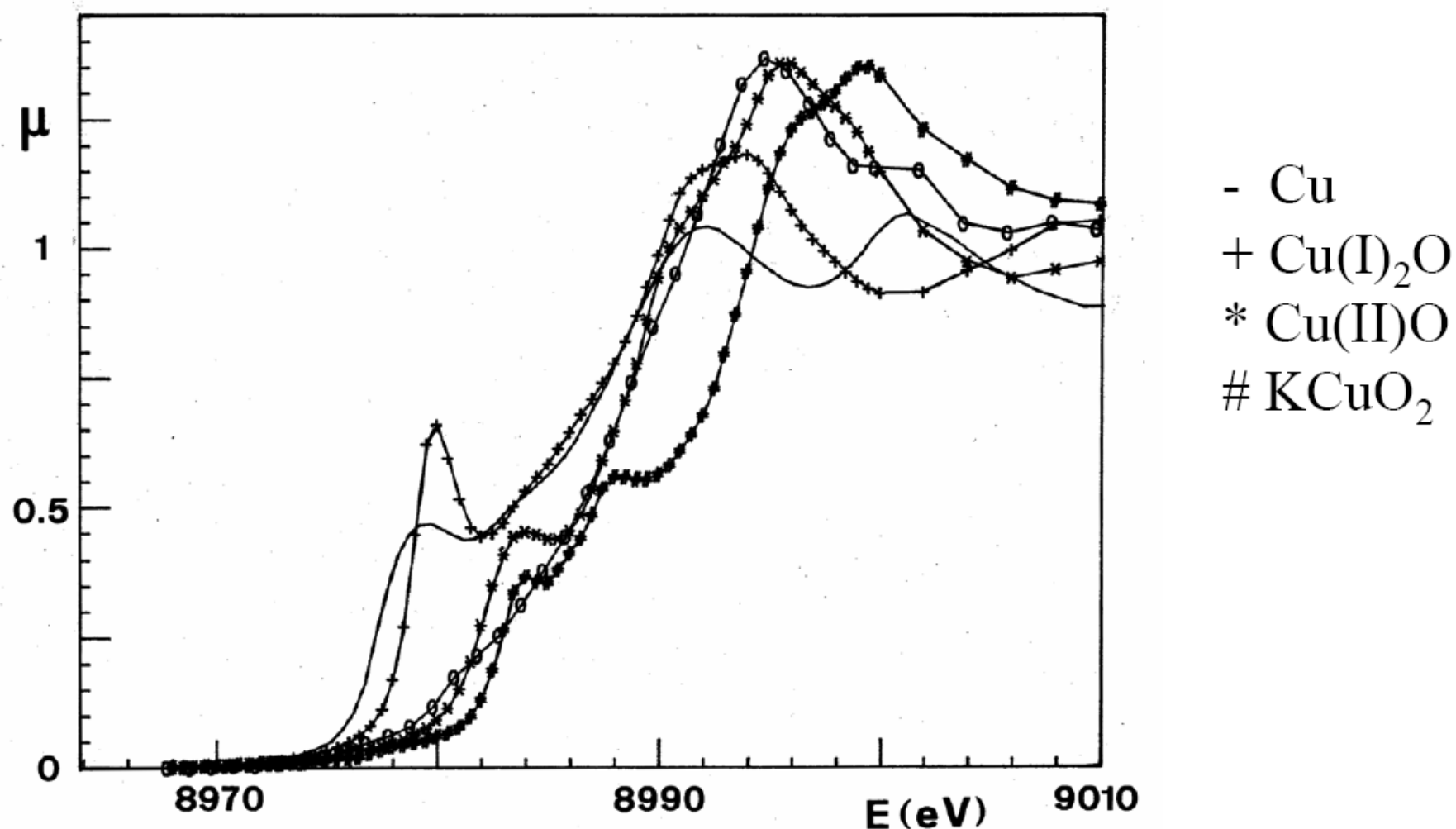
Three different characteristics:

- edge position:
oxidation state
- near edge spectrum
(XANES):
local projected
density of states
- extended fine structure
(EXAFS):
local neighborhood
of atomic species



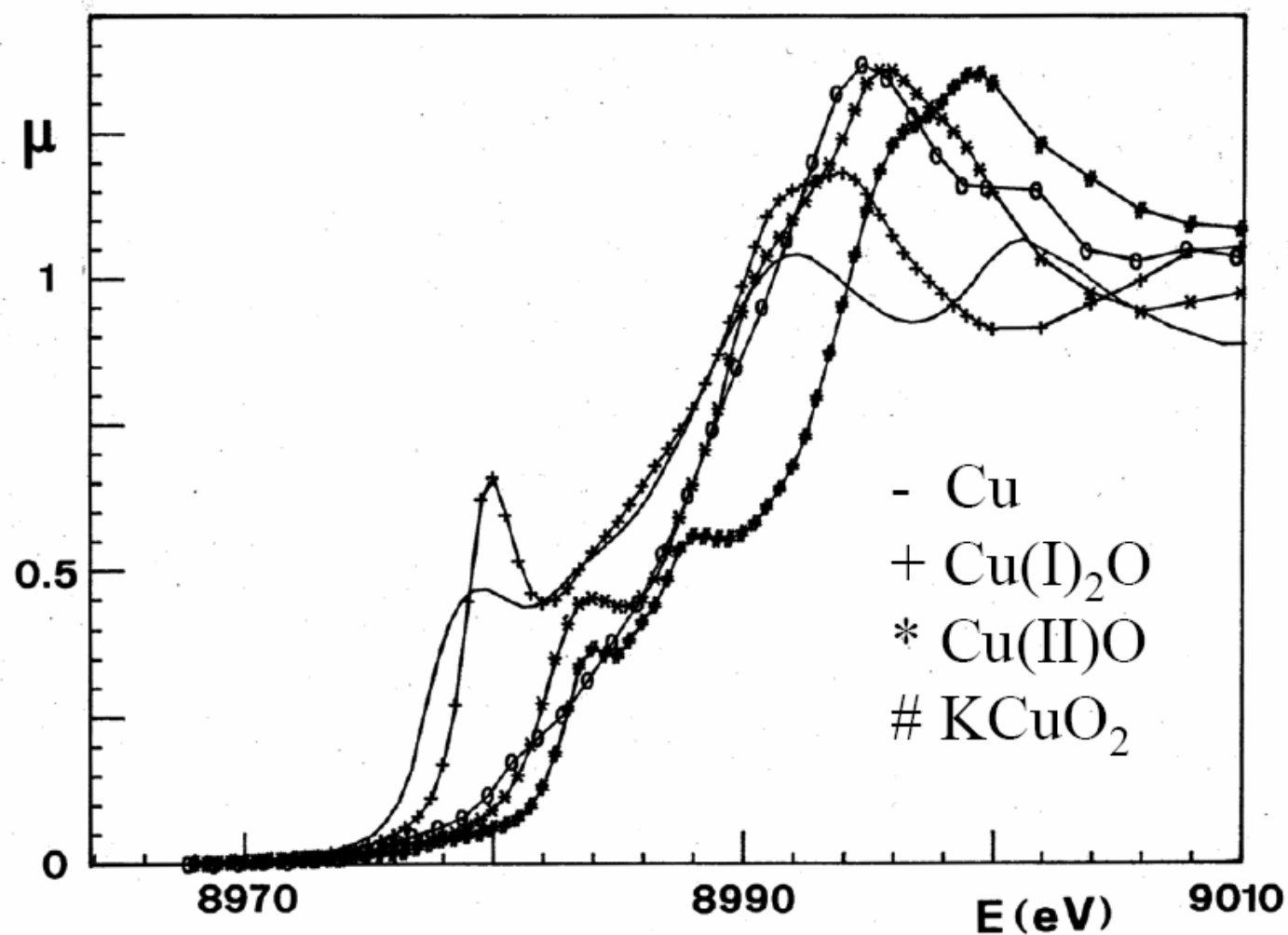
Example of what can we learn from XANES (I)

Edge is shifted to higher energy with increasing formal valence:



Example of what can we learn from XANES (II)

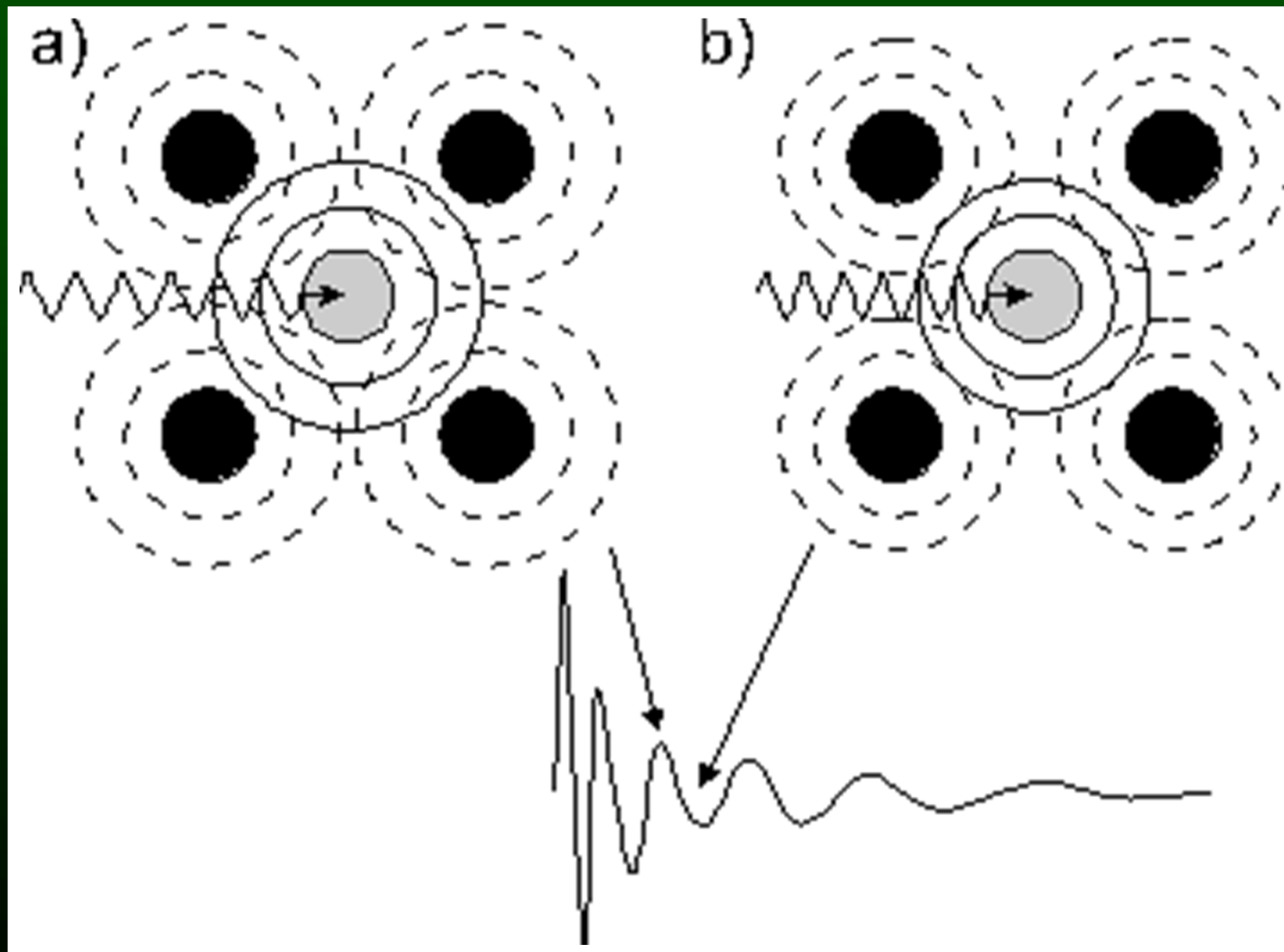
Finger print for chemical state of element of interest:



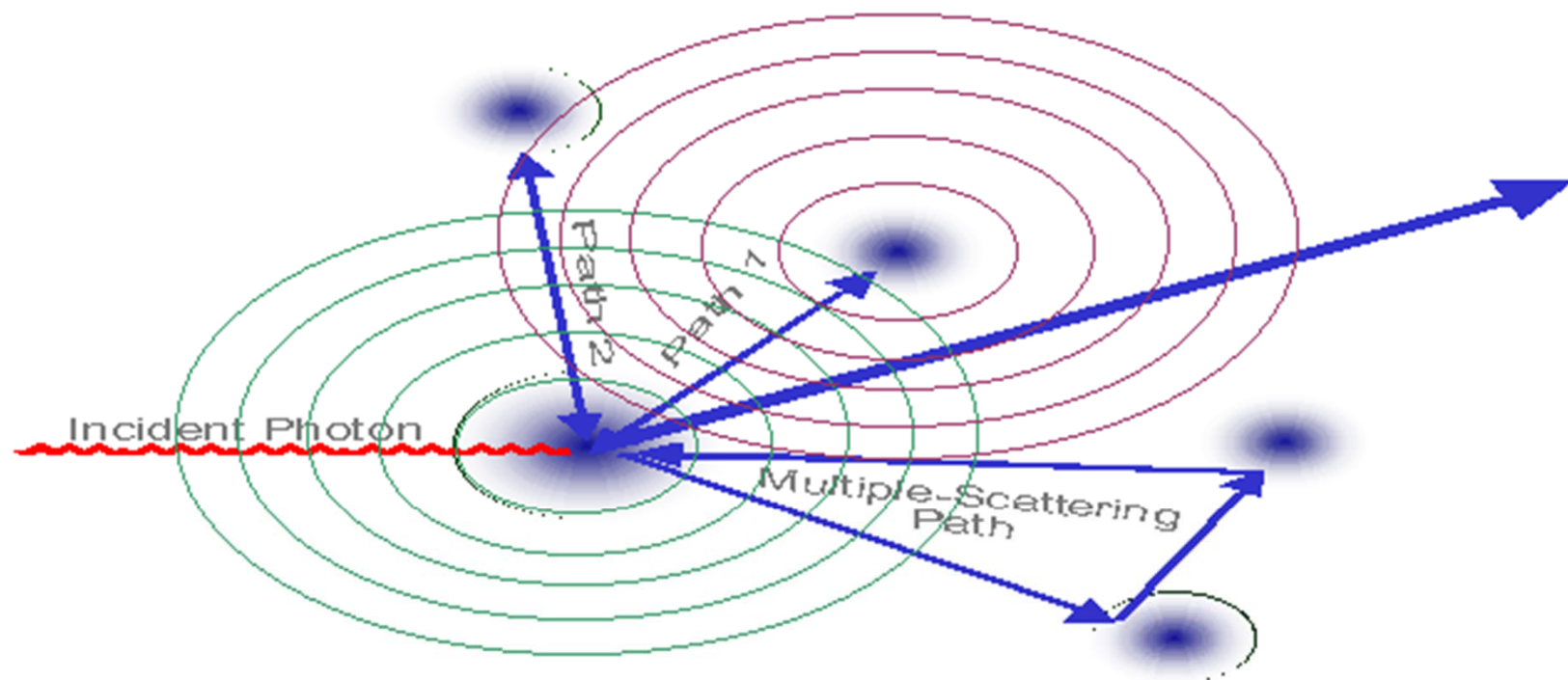
determine
concentration
of chemical
compounds
in mixtures

example:
inhomogeneous
specimens

Principle of Extended X-ray Absorption Fine Structure (EXAFS)



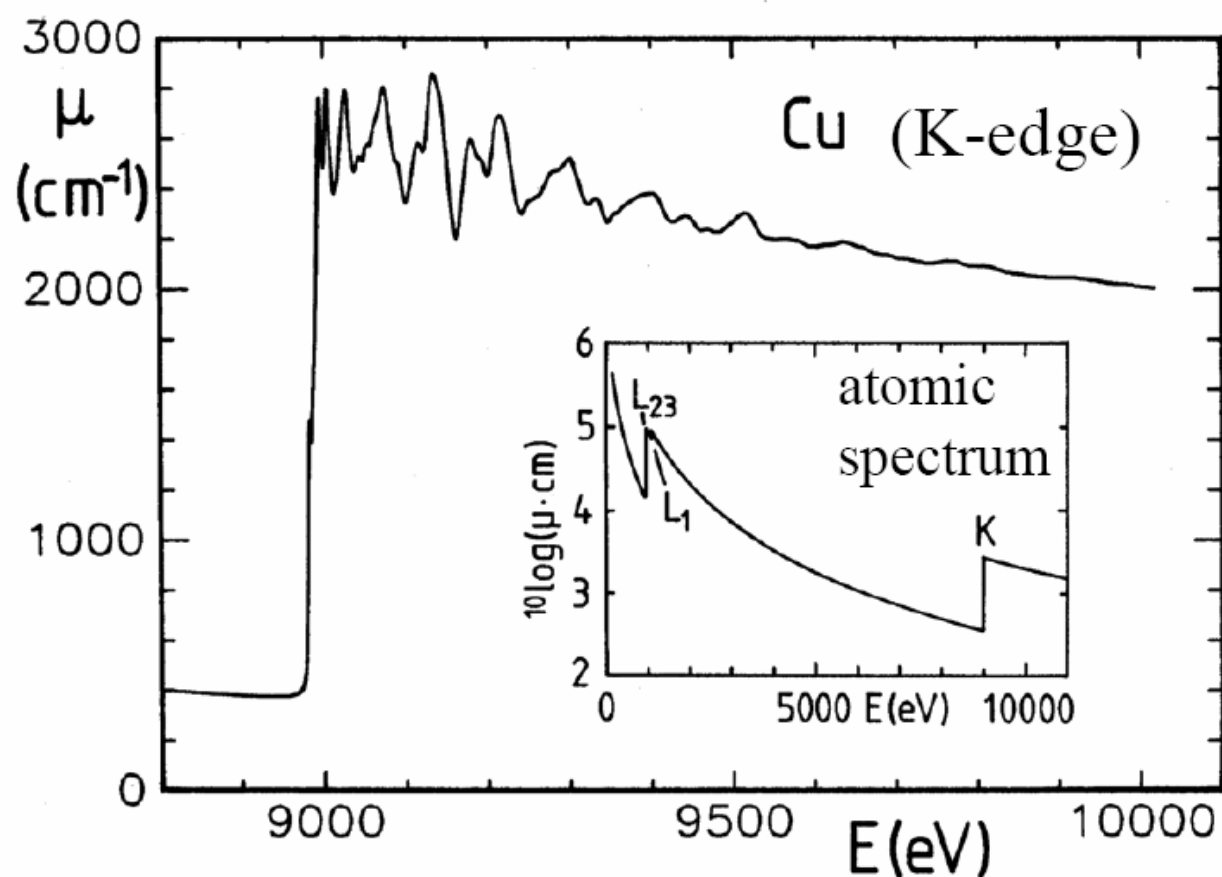
Principle of single vs. multiple scattering contributions in EXAFS



Effects of single vs. multiple scattering contributions in EXAFS

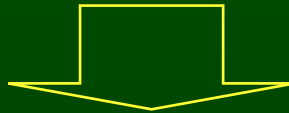
$\mu(E)$: linear absorption coefficient

metallic
Cu:



Preparation of plant material for XAS (EXAFS and XANES)

Excise sample from plant



Freeze the sample in melting nitrogen slush



grind sample in mortar cooled by dry ice

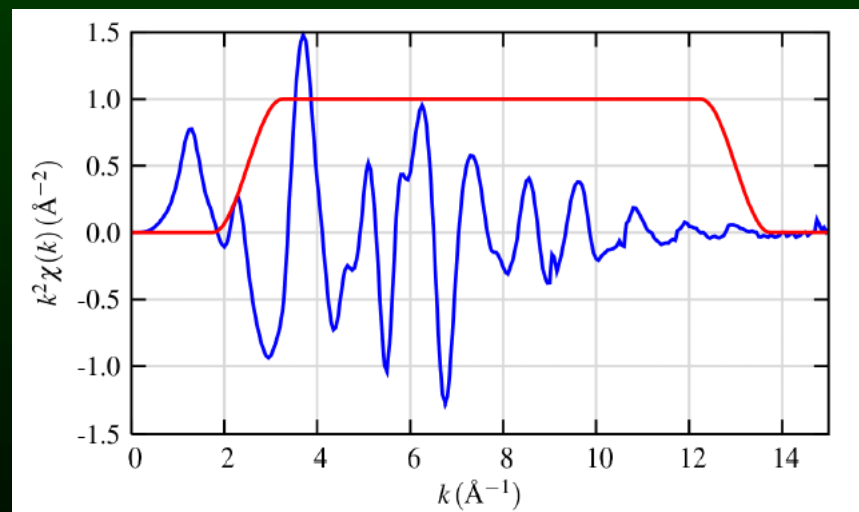
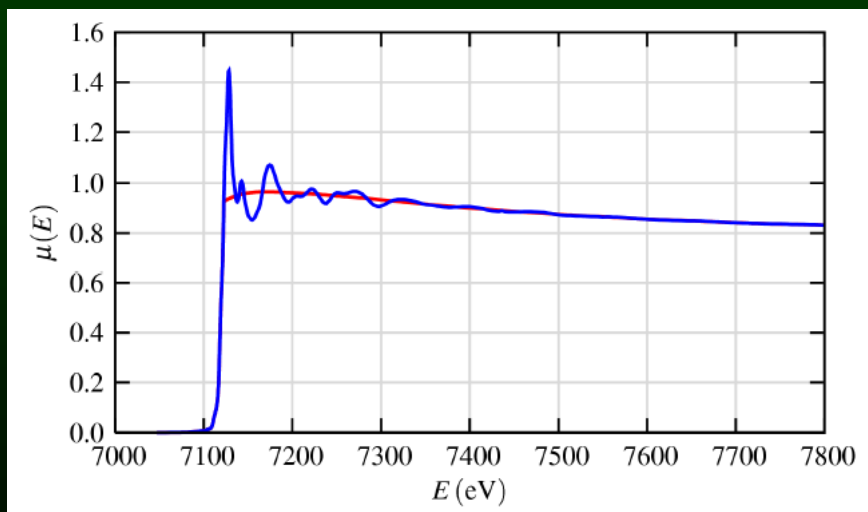
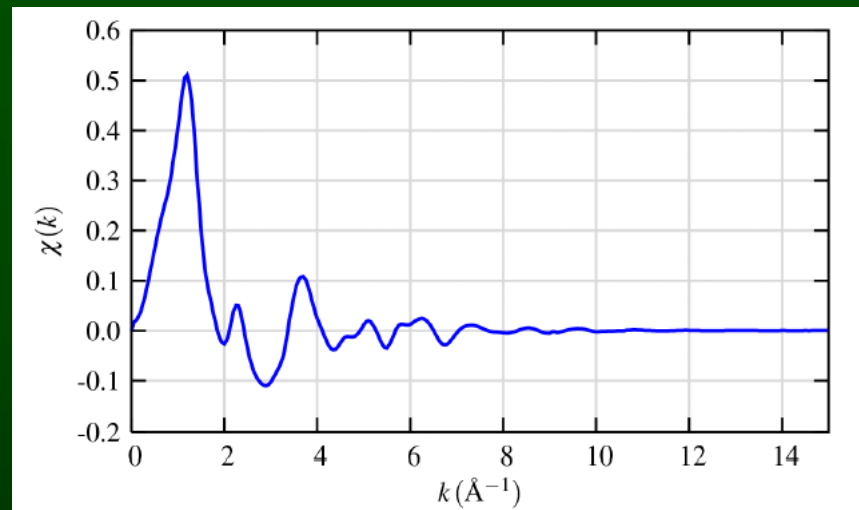
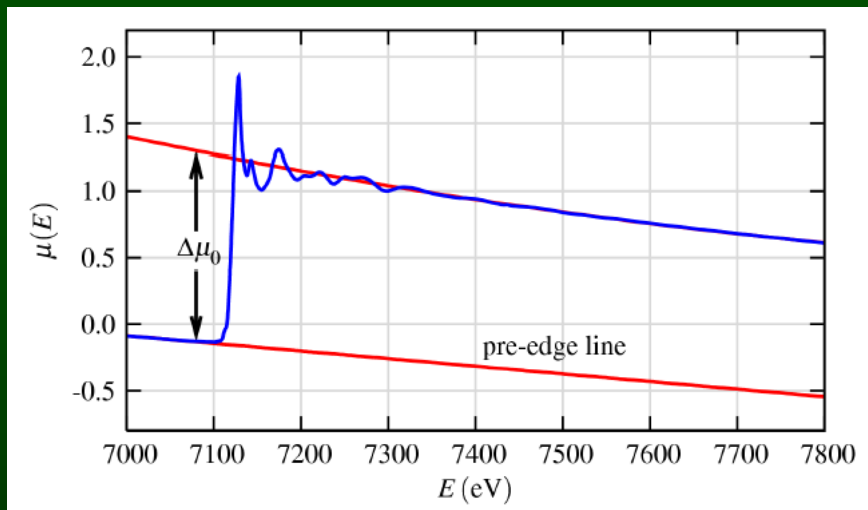


fill the still frozen-hydrated powder into an EXAFS cuvette, seal with Kapton tape
The EXAFS spectrum of the cuvette must not interfere with that of the sample!

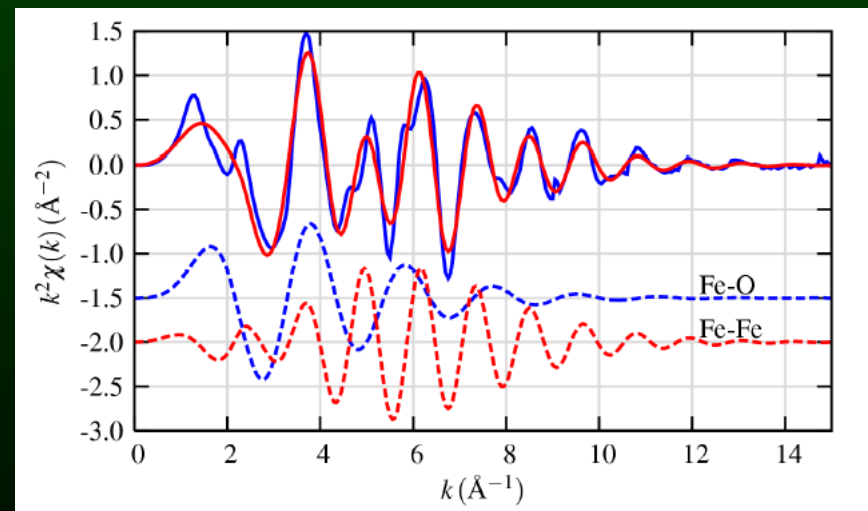
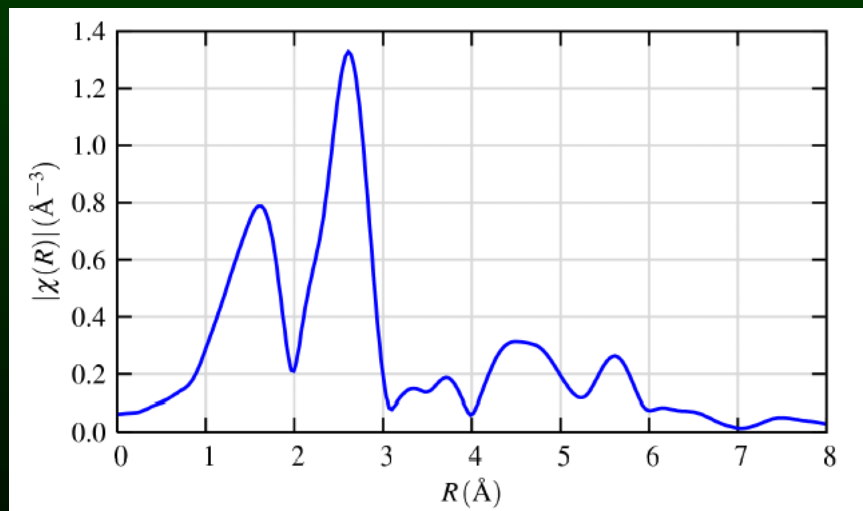
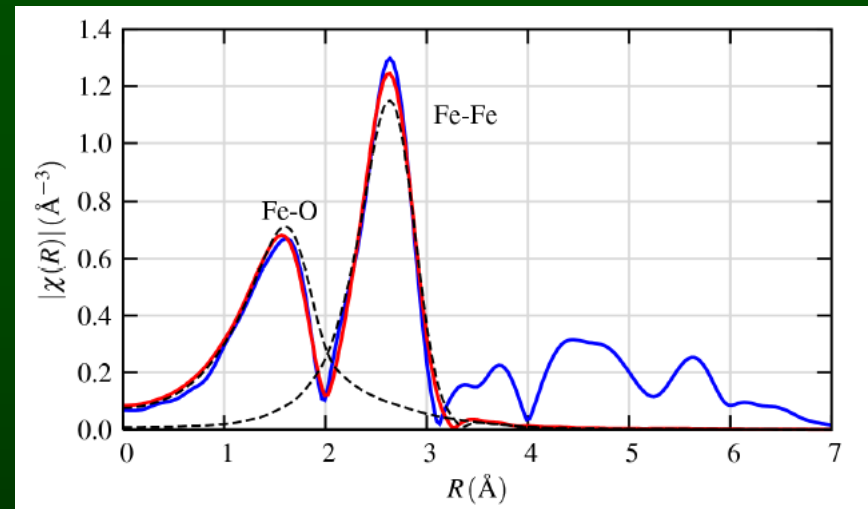
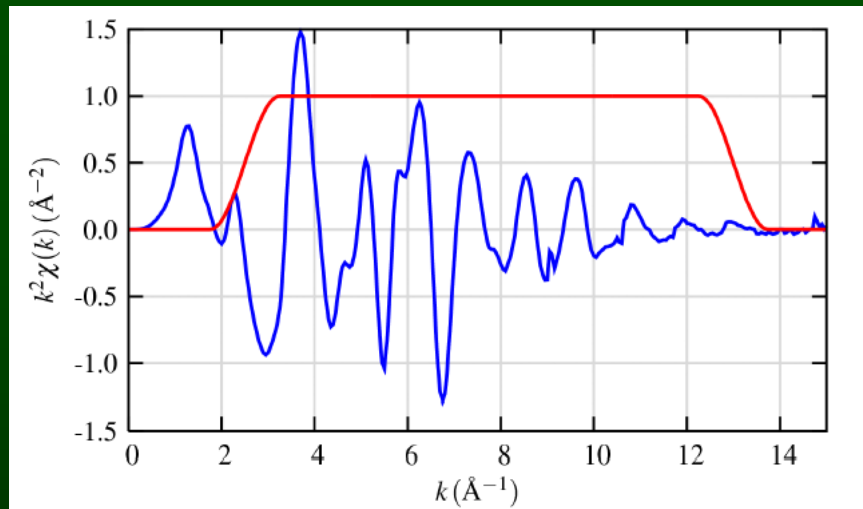


Transfer to cooled (20 K) sample holder of beamline, analyse

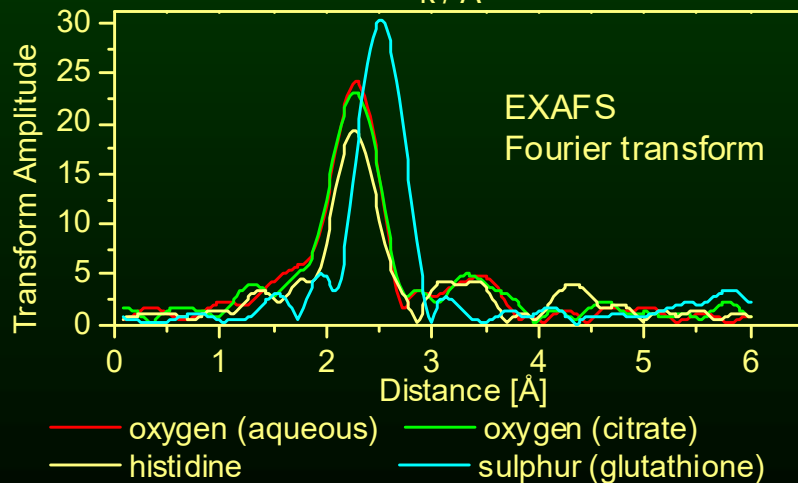
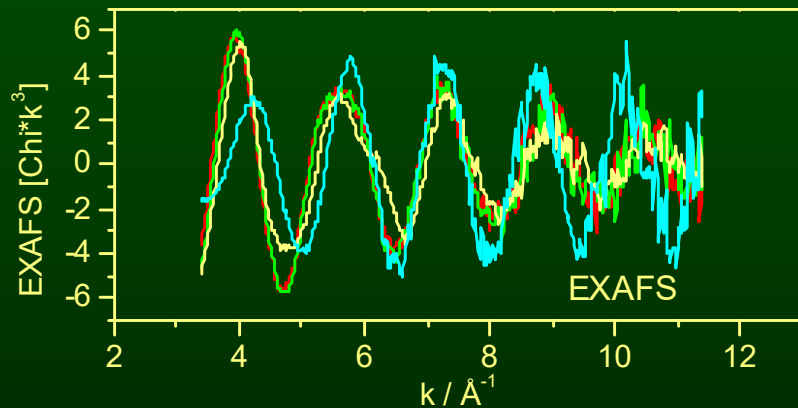
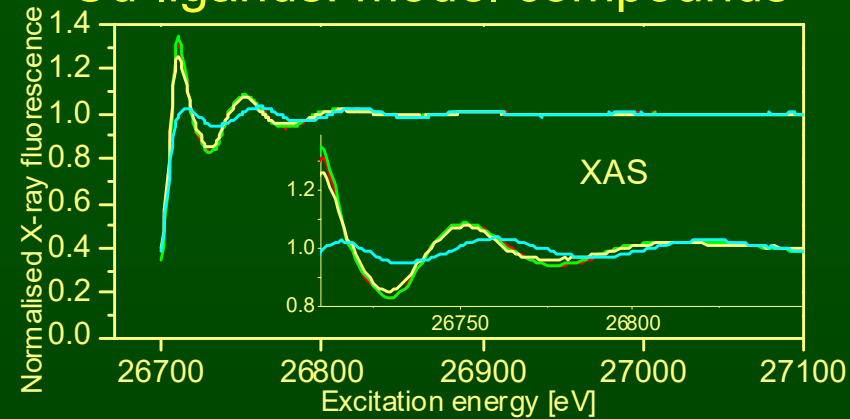
Analysis of EXAFS data (I)



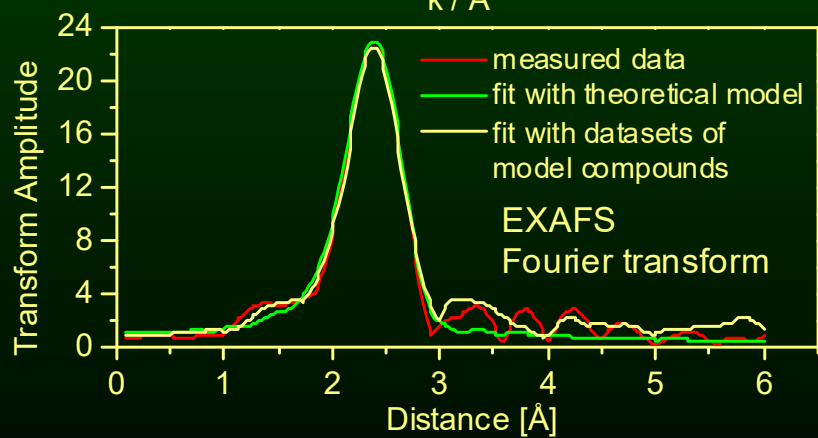
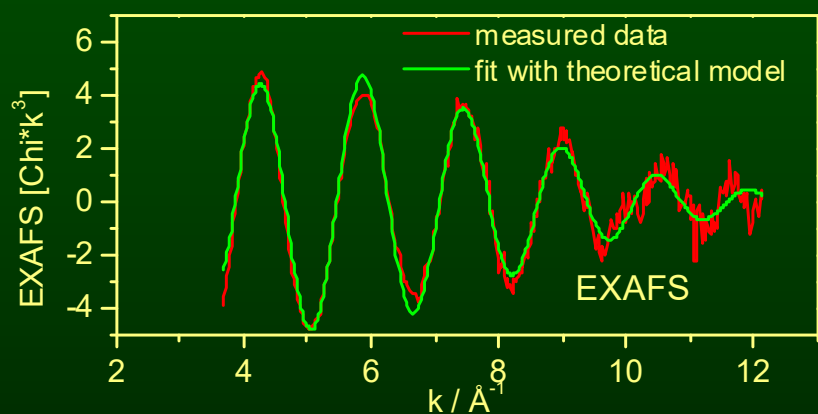
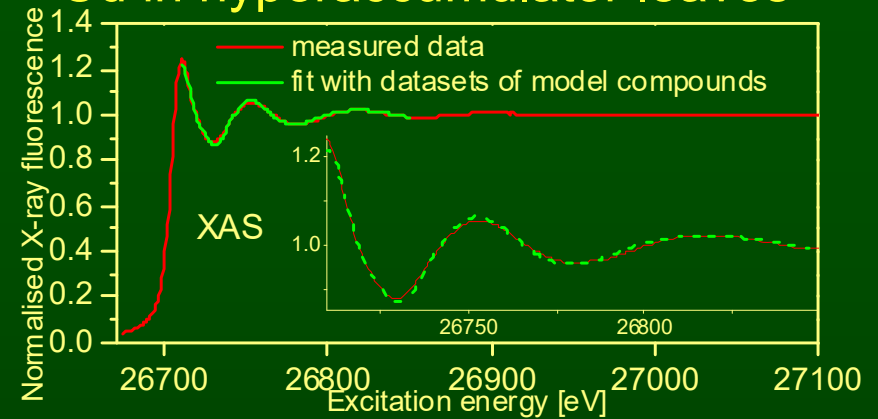
Analysis of EXAFS data (II)



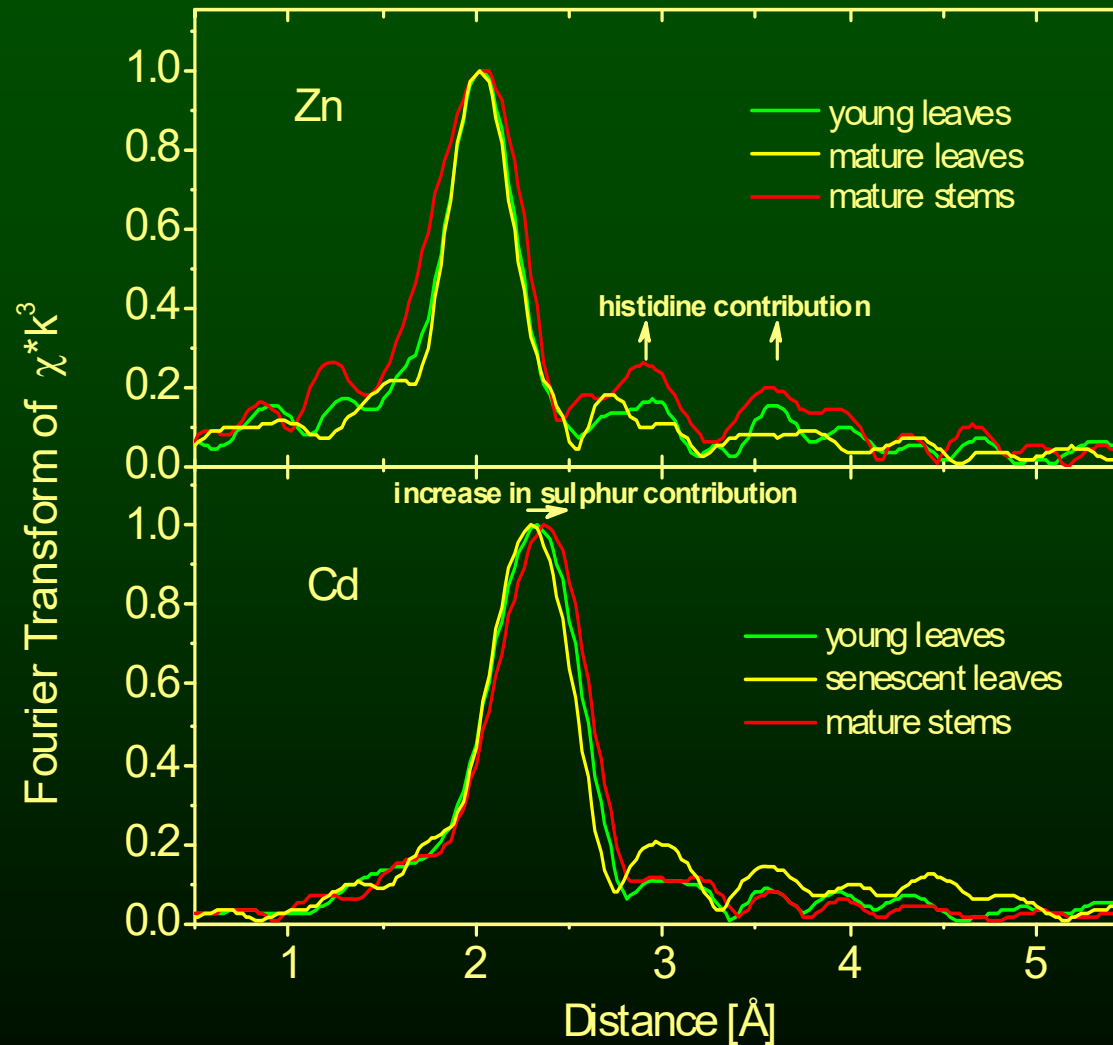
Cd-ligands: model compounds



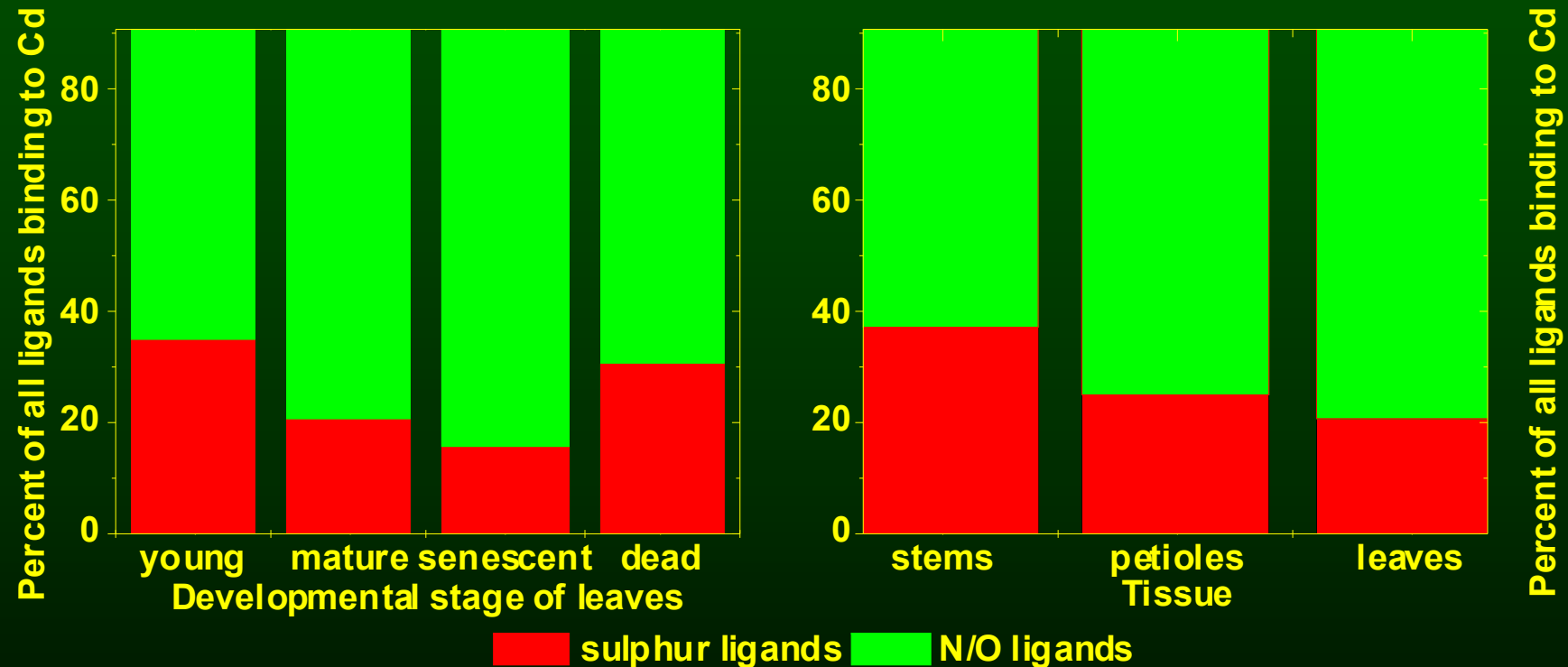
Cd in hyperaccumulator leaves



Application example: Speciation of cadmium and zinc hyperaccumulated by *Thlaspi caerulescens* (Ganges ecotype)

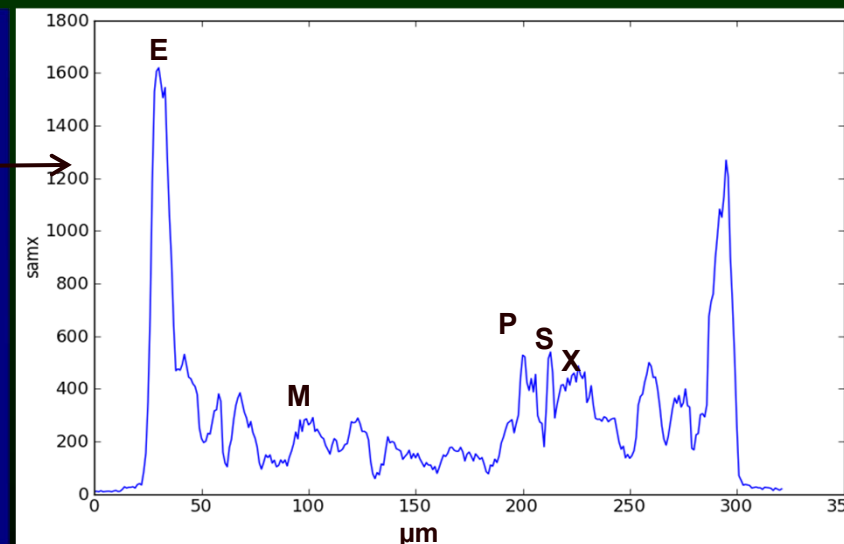
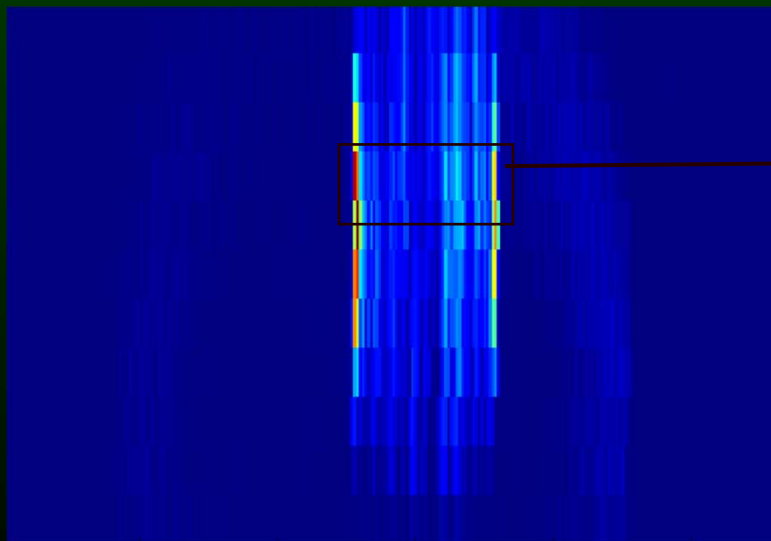
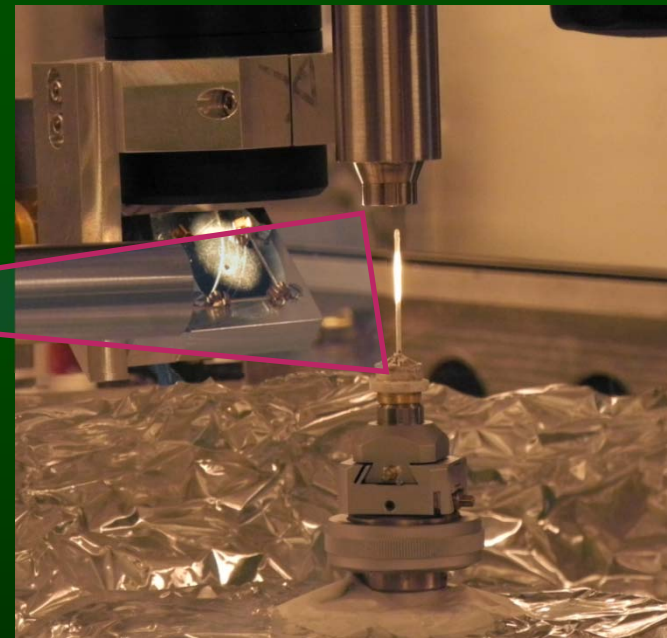
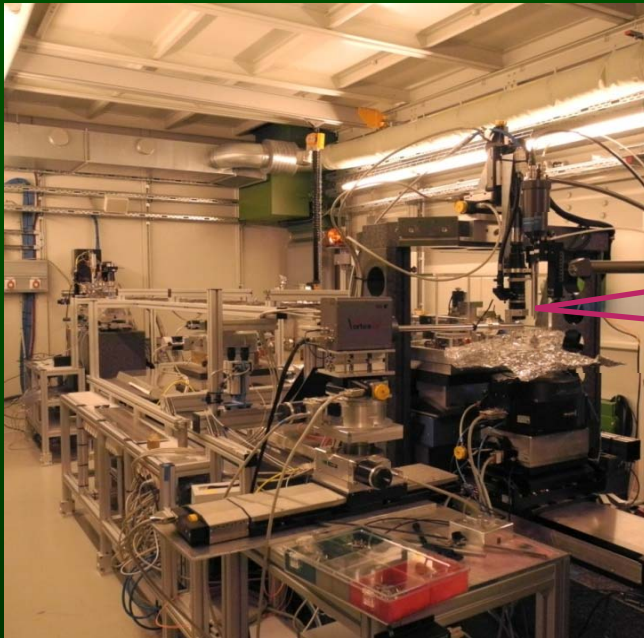


Application example: Speciation of cadmium and zinc hyperaccumulated by *Thlaspi caerulescens* (Ganges ecotype)

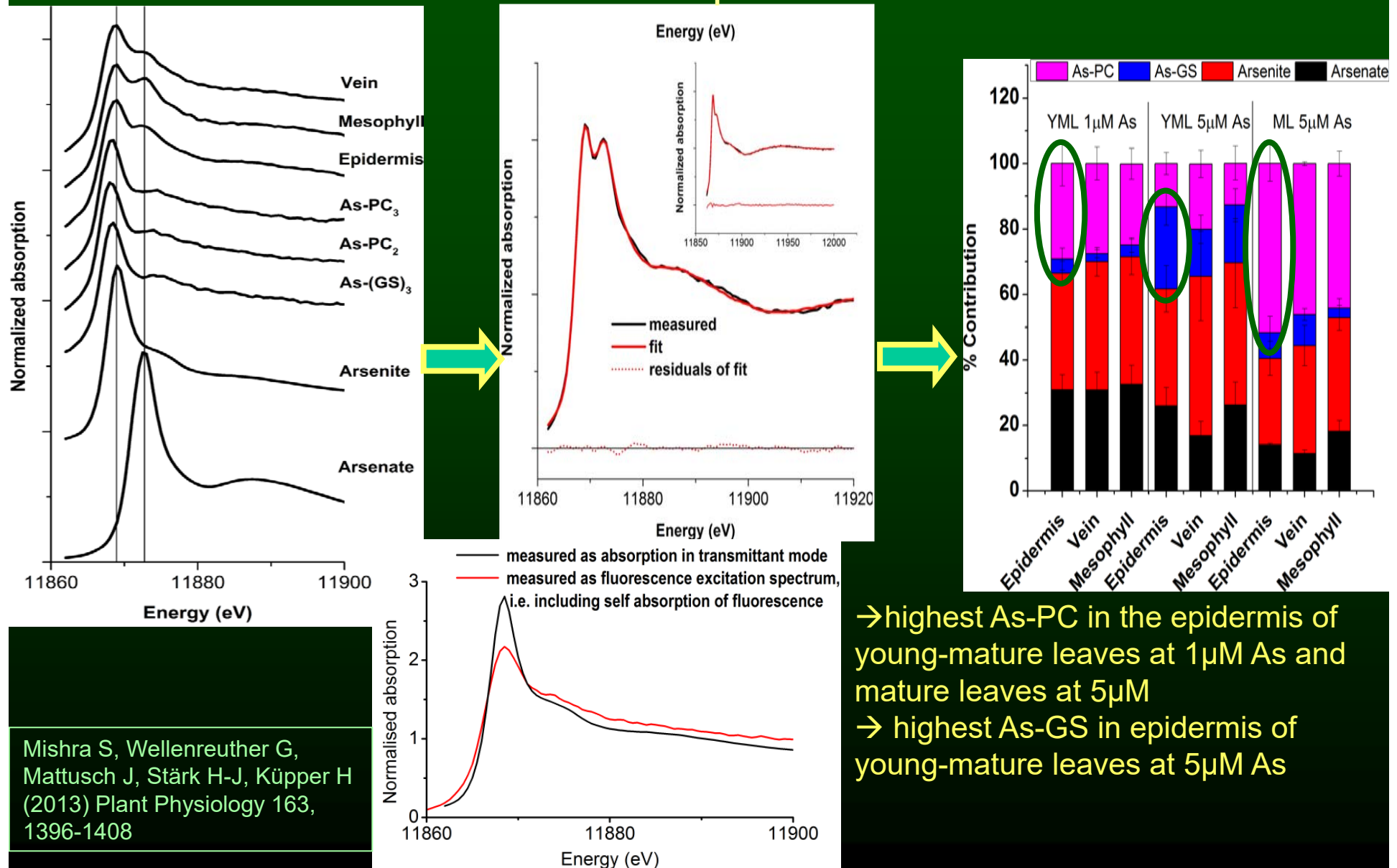


Microscopic X-ray absorption spectroscopy

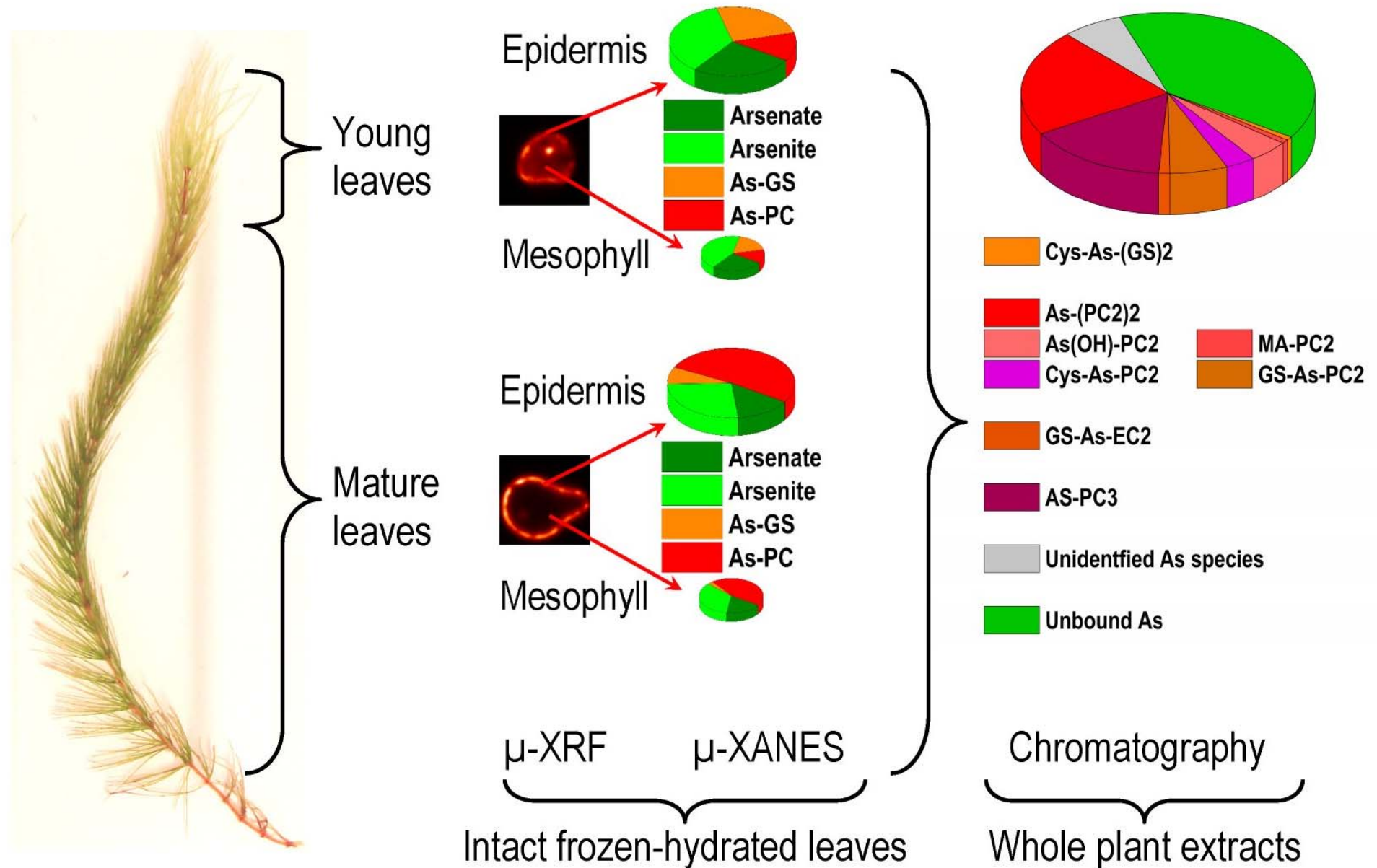
Confocal μ -XANES: Sample mounting and measurement



Tissue specific As speciation through confocal μ -XANES: linear combination fitting with correction for absorption and baseline drift



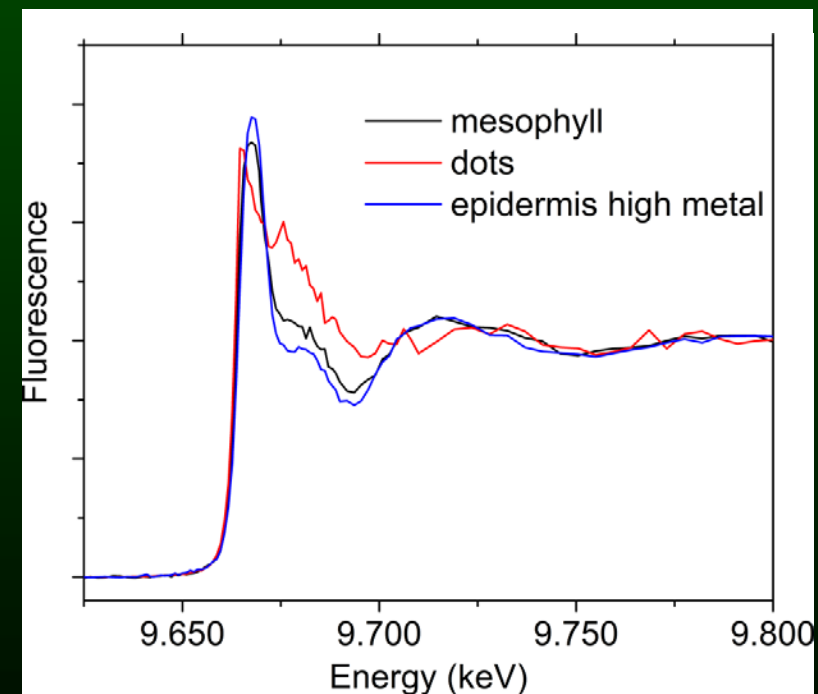
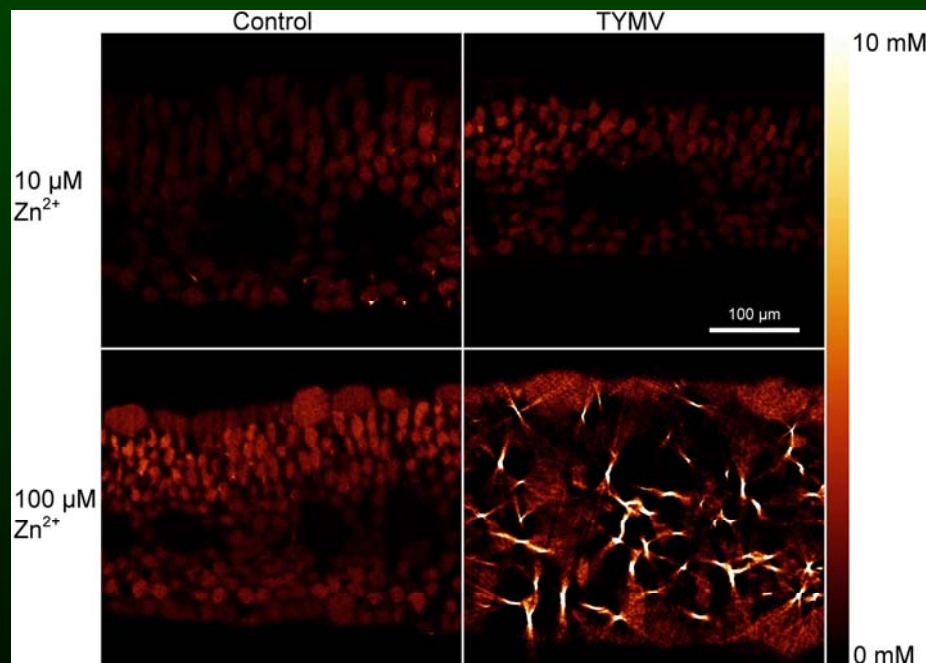
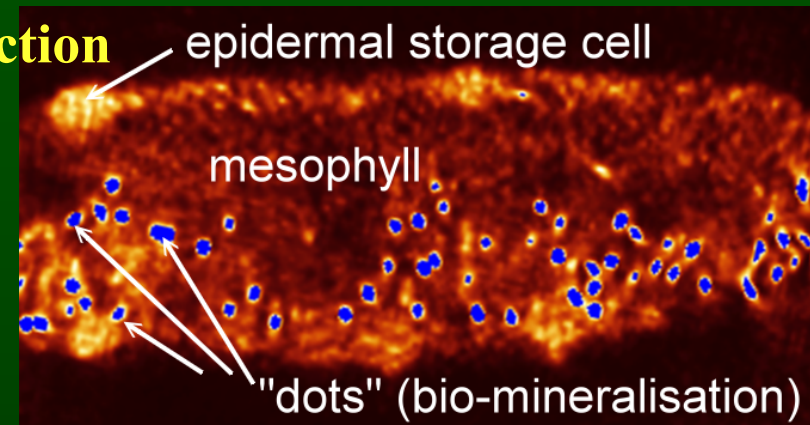
Arsenic: comparison of μ XRF & μ XANES with chromatography



Tissue specific Zn speciation through μ -XANES tomography

Biomining in response to virus infection

→ Infection with Turnip Yellow Mosaic Virus leads to enhanced biomineralization as revealed by μ XRF and μ -XANES tomography



X-ray spectroscopy on biological samples

General *technical* conclusions

a) Sample preparation

- For analysing metals in biological samples, organisms need to be treated with metal concentrations they encounter in real life if meaningful results should be obtained
- Samples should be prepared by shock-freezing and kept frozen-hydrated to minimise the risk of artefacts of element re-distribution and changes in speciation

b) Measurement

- Compared to mapping of thin sections, tomography of larger samples minimises sample preparation (sectioning) and therefore the risk of artefacts
- Detectors with large solid angle increase the ratio of signal / beam damage

c) Data analysis

- Standards having the same shape and average composition as the samples allow for good correction of absorption effects

**All slides of my lectures can be downloaded
from my workgroup homepage**

Biology Centre CAS → Institute of Plant Molecular Biology → Departments
→ Department of Plant Biophysics and Biochemistry,
or directly

http://webserver.umbr.cas.cz/~kupper/AG_Kuepper_Homepage.html