Microscopy and NanoSIMS

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Outline

1. **Introduction: Images and imaging**
2. **Microscopy: optical/light microscopes**
3. **Microscopy: electron microscopes**
   - Principles
   - Scanning Electron Microscopy (SEM)
   - Transmission Electron Microscopy (TEM)
4. **Element specific (chemical) imaging**
5. **Secondary Ion Mass Spectrometry (SIMS)**
   - Static and dynamic SIMS
   - Ionization process in dynamic SIMS
   - History of dynamic SIMS instruments and size of the ion microprobe
5. **Nano Secondary Ion Mass Spectrometry (NanoSIMS)**
   - Principle
   - Ion sources
   - Ion transmission, lateral resolution, mass resolution, useful yield
6. **Preparation of (biological) samples**
   - Chemical preparation and cryo preparation
7. **NanoSIMS applications to nanoparticles from our research**

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Why do we like imaging ?

"A picture is worth a thousand words"

"Imaging is very popular"

Why do we like imaging ?

"A picture is worth a thousand words"

"An image shows more than 10,000 data"

One of the main goals of *visualization* is making it possible *to absorb* large amounts of data quickly.

Visualization through *visual imagery* has been an effective way to communicate both abstract and concrete information since the dawn of man. (*Wikipedia*)

Data and images

Example: Measurement of **relative intensities** of light

Presentation as:

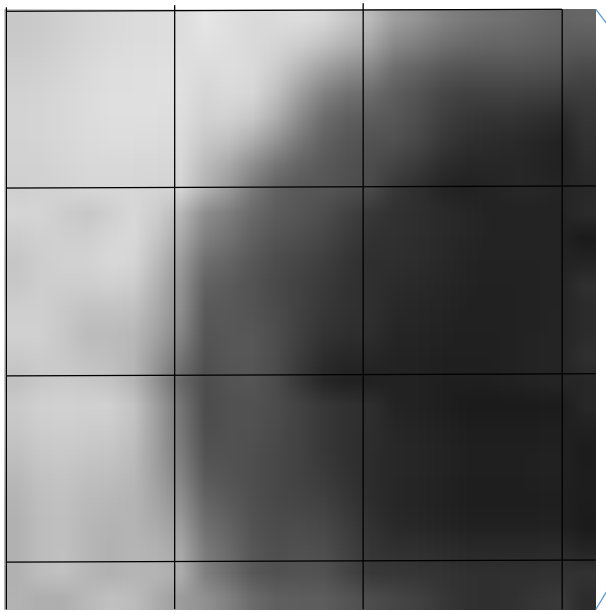
Data in a matrix

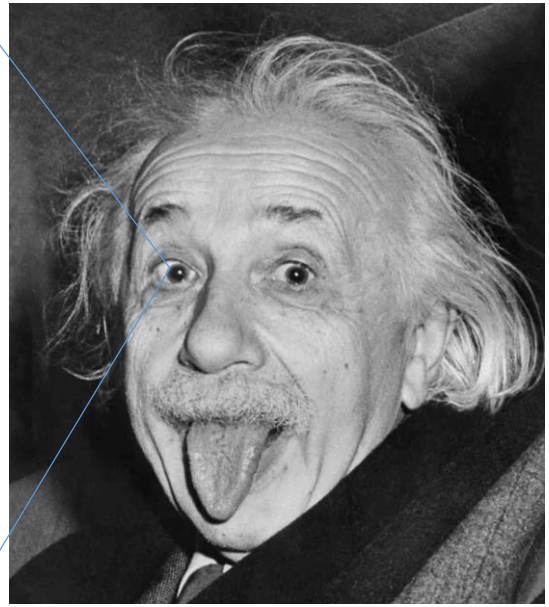
Shades of gray in an image

Whole picture

Defining the value of each pixel

	a	b	c	…
A	2.50	1.50	0.50	
B	2.35	0.80	0.30	
C	2.40	1.00	0.50	
…				





Abstract data

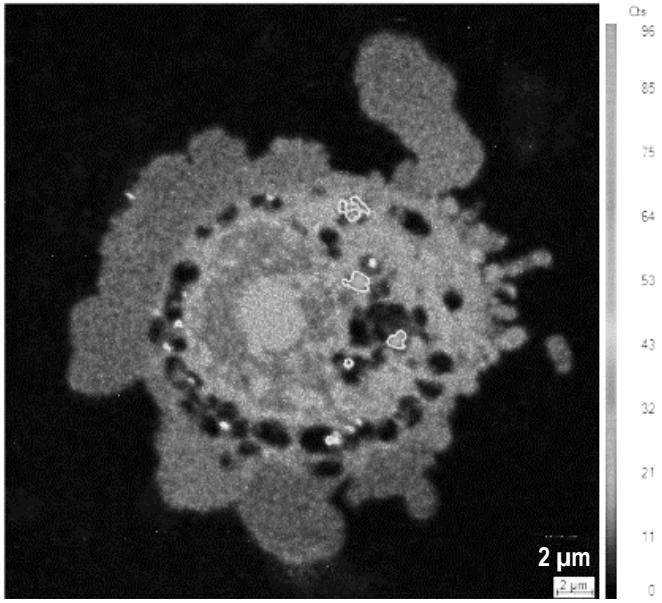
Absorbing quickly the whole information

Images and Analytical Sciences

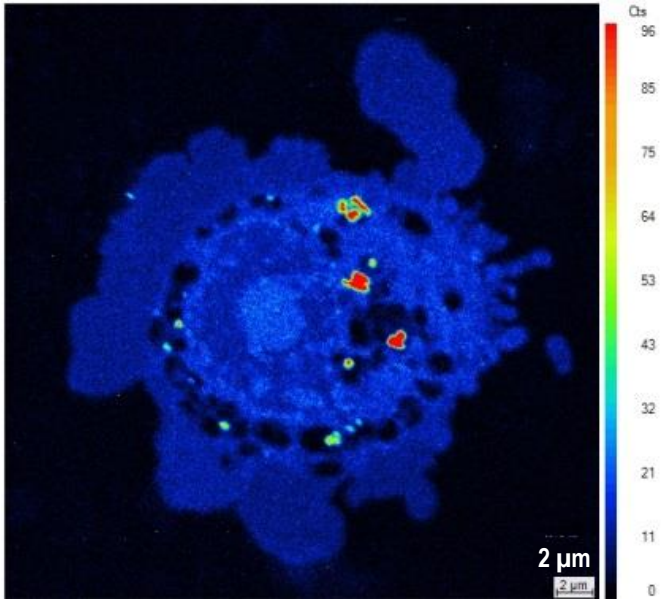
An image is more than a visual impression

$BaSO_4$ nanoparticle distribution in human lung cells analyzed with NanoSIMS

Grey scale



Colour scale



Different representations of an image possible – different visual impressions

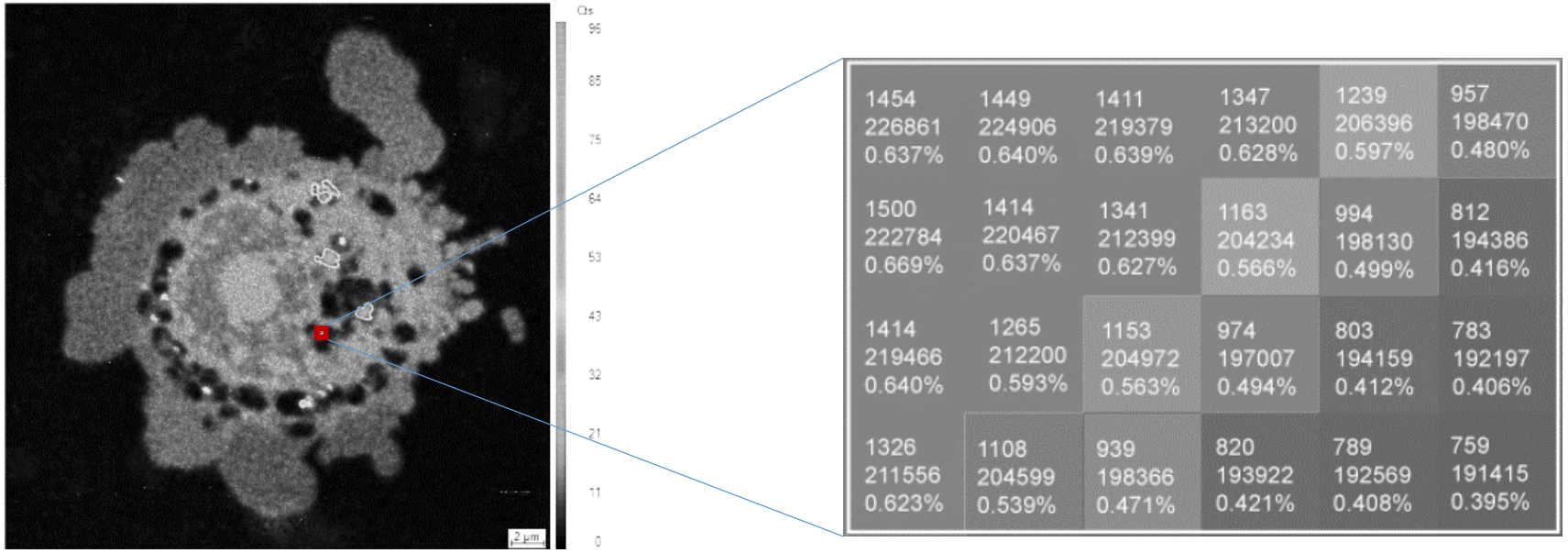
Behind each image is a matrix of analytical data

Images and Analytical Sciences

An image is more than a visual impression

… don't forget the data behind:

- matrix of data to be treated and interpreted
- raw data: measured intensities
- treated data: normalized intensities, intensity ratios, isotope ratios …



512 x 512 pixel: 262 144 data points

In general: Raw data obtained from detection of photons (visible light, UV, X-rays), electrons, atomic and molecular ions (mass spectrometry) …

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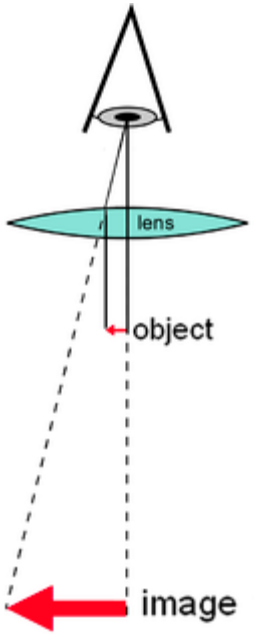
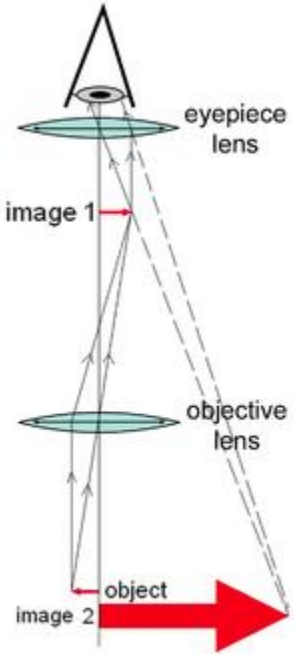
Observation of details in the micro world: Microscopy

Optical Microscope uses system of lenses and **visible light** to sharply magnify small detailed samples which is projected directly to the eye.



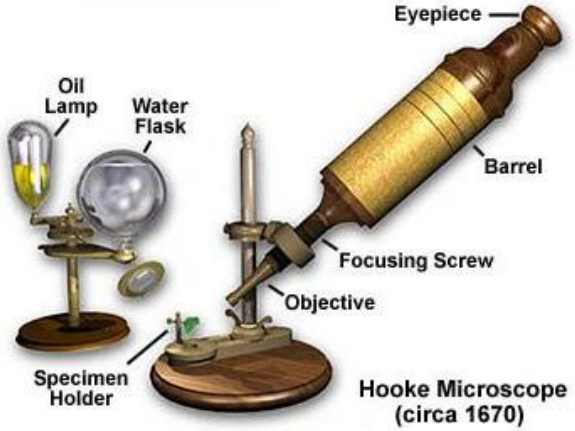
Simple microscope



Compound microscope

Observation of details in the micro world: development of optical microscopes

The First Compound Microscope (circa 1595)



Hooke Microscope (circa 1670)

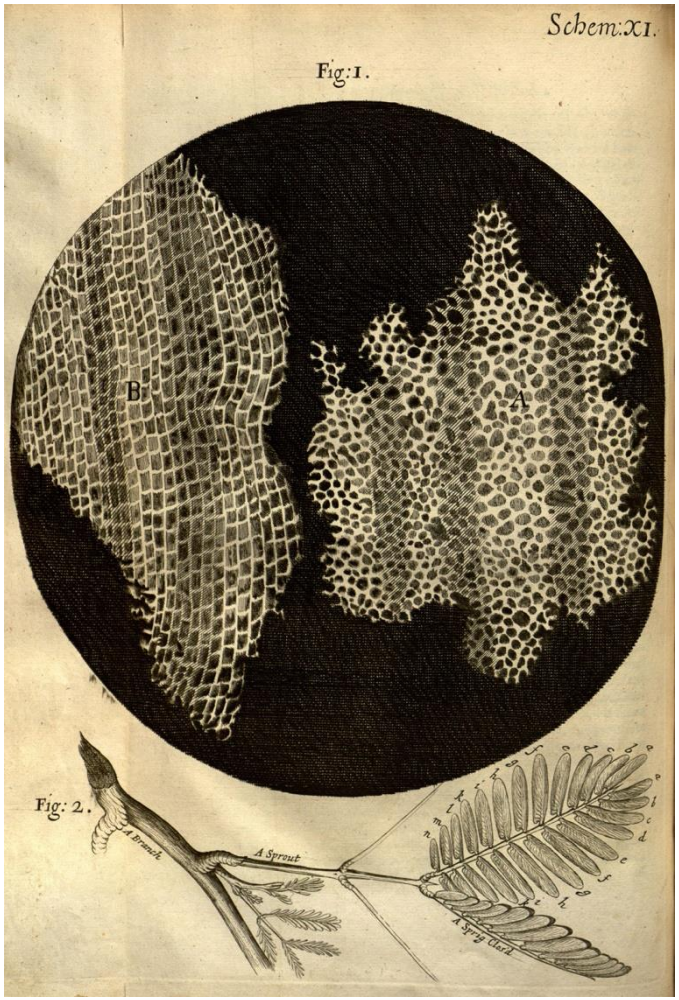
Leeuwenhoek Microscope (circa late 1600s)

Microscopes in the 16th and 17th century

Modern light microscope

Observation of details in the micro world: biology

Robert Hooke's microscope
17th century



Robert Hooke's drawings of the cellular structure of cork and a sprig of sensitive plant from *Micrographia* (1665).

Robert Hooke used a compound microscope to study cork from oak bark.

Hooke noted small geometric shapes which he names ***cells***, because they reminded him of small rooms where monks lived in a monastery

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Limit of resolution of optical microscope

The microscope uses visible light and visible light has a set range of wavelengths. The microscope can't produce the image of an object that is smaller than the length of the light wave, thus the wavelength of the visible light, which is 0.4-0.7 µm.

Any object that's less than half the wavelength of the microscope's illumination source is not visible under that microscope.

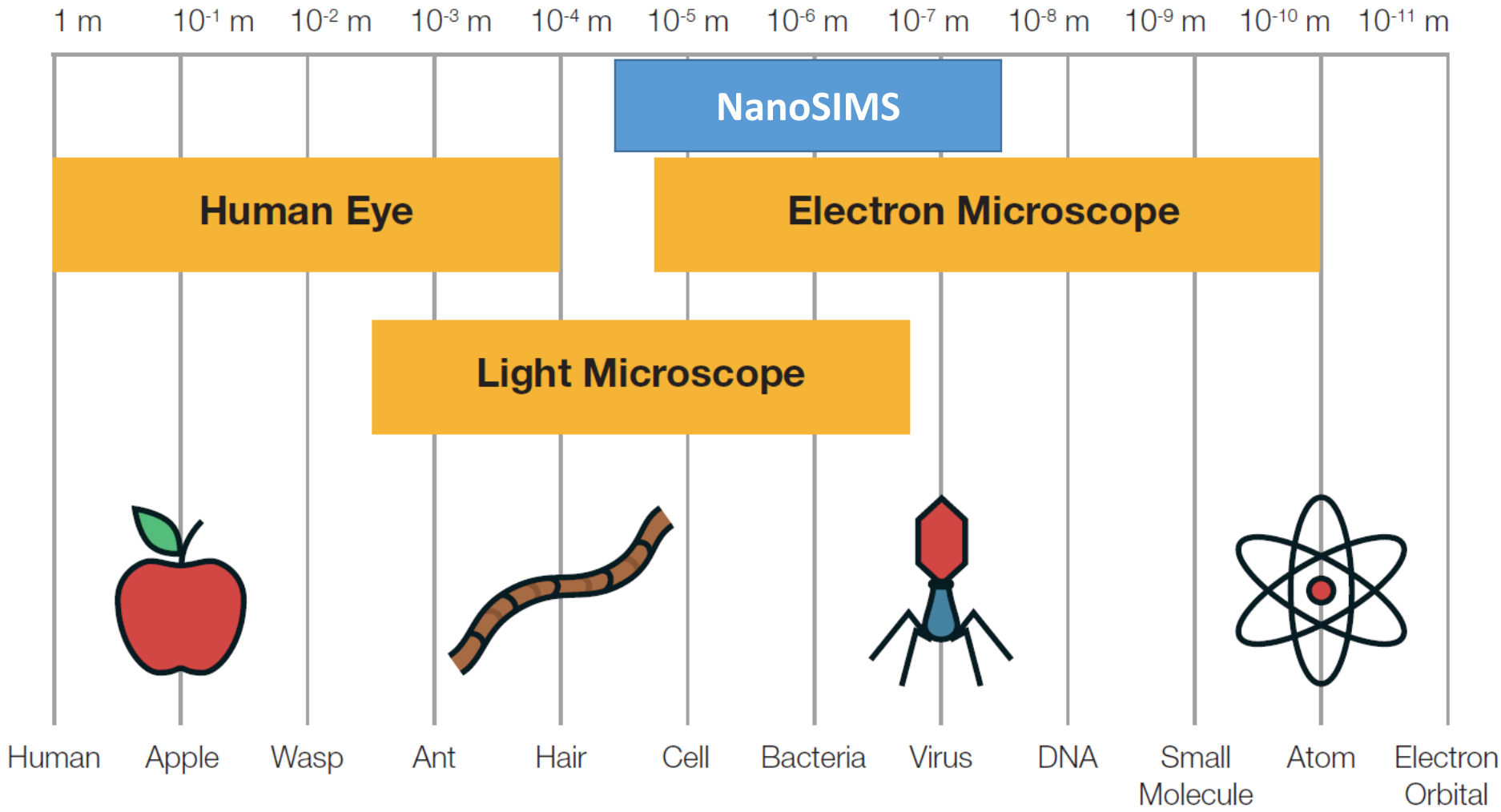
For example, when we can see green light (0.5 µm), the objects which can be observed cannot be smaller than 0.2 µm. Below this point, light microscope is not useful, as wavelength smaller than 400 nm is needed.

Increasing the resolution by electron microscopy

The waves that associate the electrons has smaller wavelength (up to 100,000 times shorter than that of visible light photons). Therefore we can use electrons using an electron microscope.

Electron microscopes can be used to visualize subcellular structures, viruses, molecules and even individual atoms.

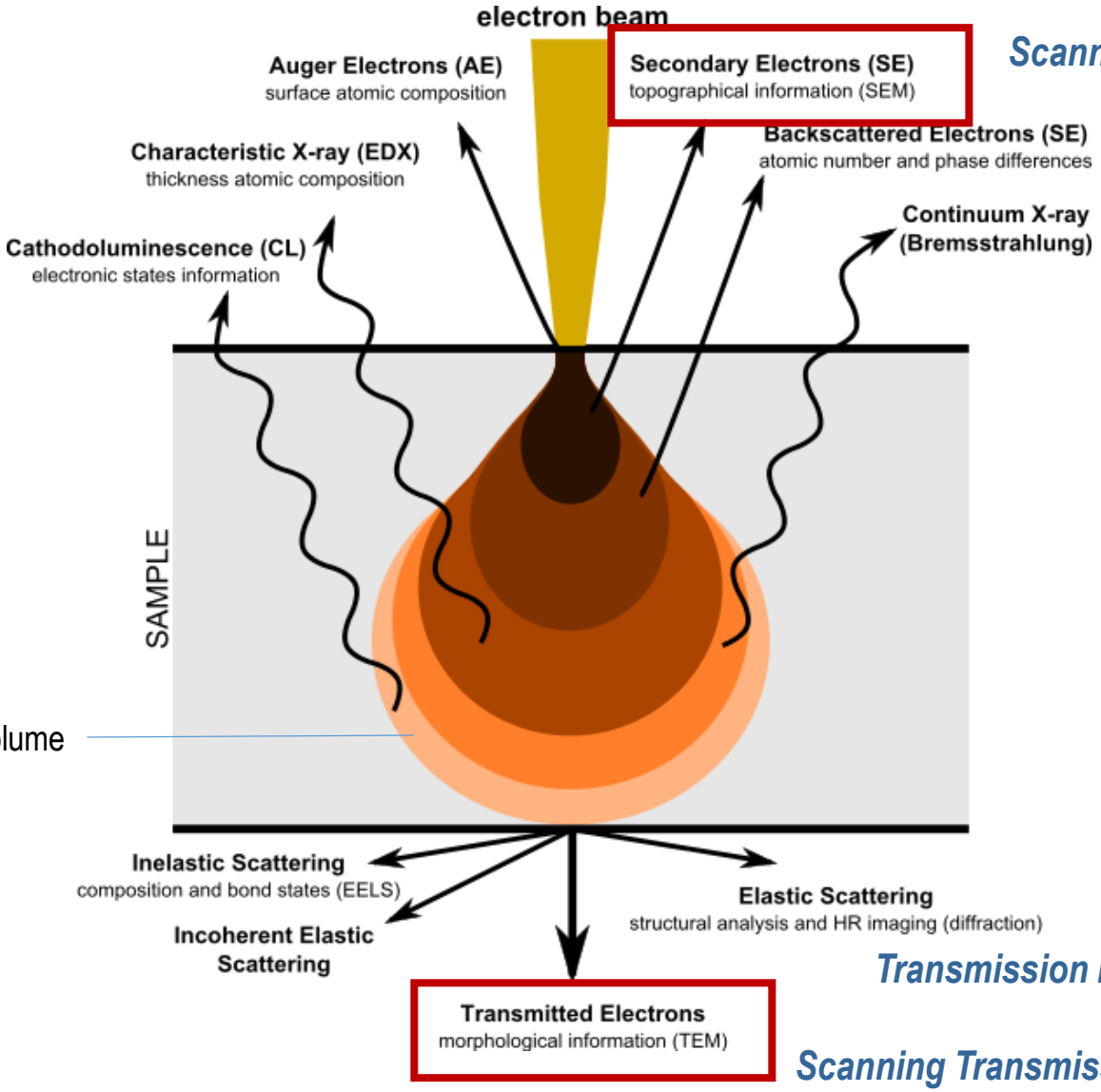
Comparing the scale of different microscopes



Nanoparticles

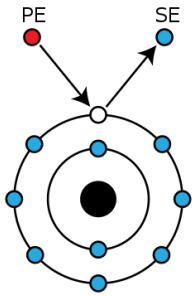
Electron microscopy

interaction of highly energetic electrons with matter



Electron interaction volume

Scanning Electron Microscopy (SEM)



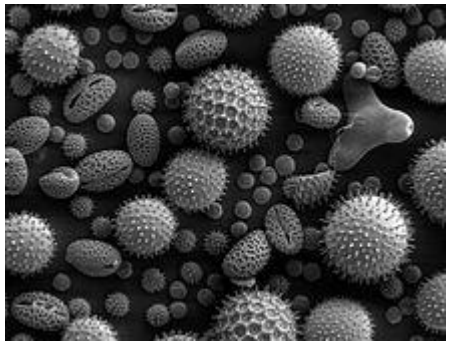
Transmission Electron Microscopy (TEM)

Scanning Transmission Electron Microscopy (STEM)

Electron microscopes: SEM and TEM

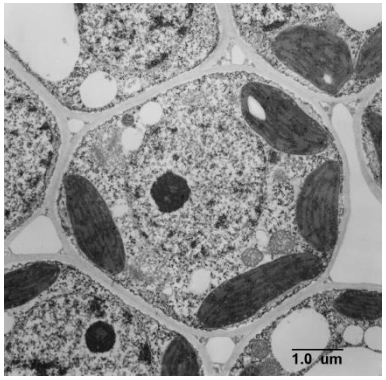
There are two basic types of electron microscopes:

- **Scanning Electron Microscopes (SEM)** allow imaging the surface of objects to study their shape



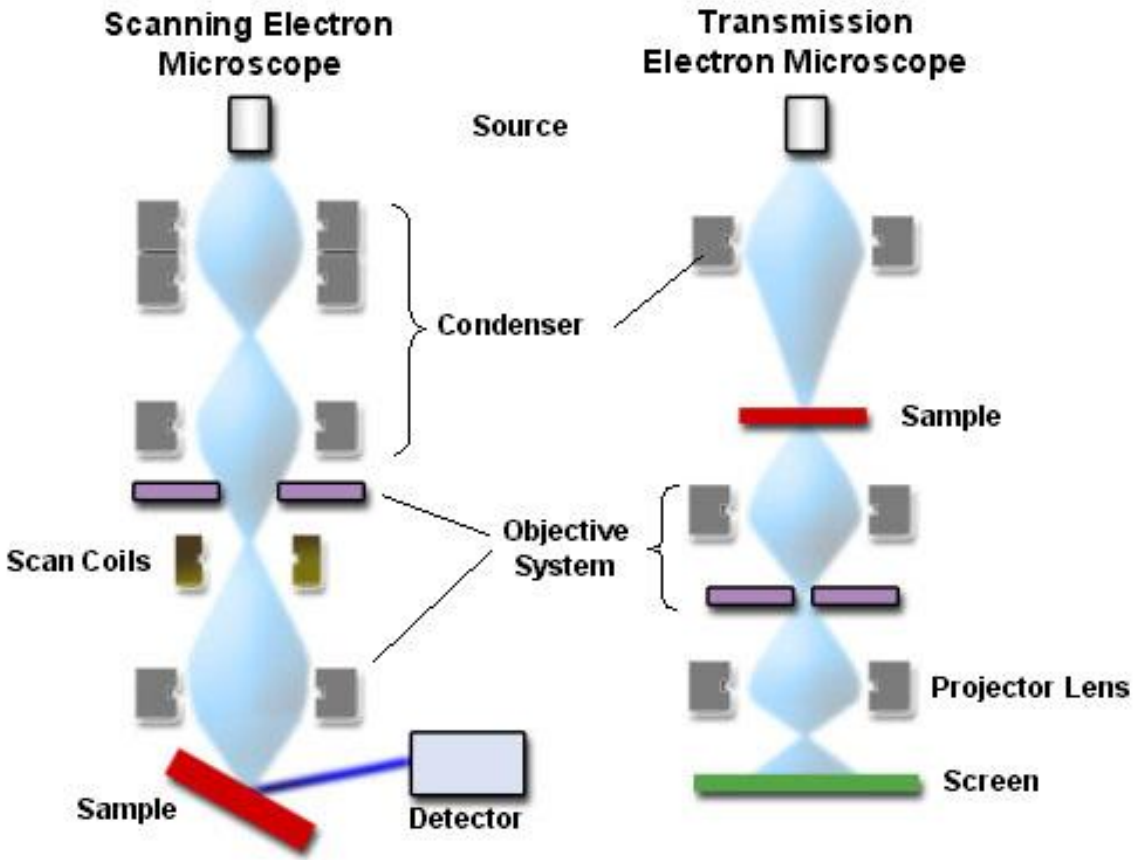
SEM image of pollen grains

- **Transmission Electron Microscopes (TEM)** allow to study the structures within objects



TEM image of a plant cell

Electron microscopes: instrumental principles



Spatial resolution:

1 nm typical HR instruments **0.1 nm**

0.4 nm *highest resolution achieved* *0.05 nm*

ZEISS Gemini SEM 300

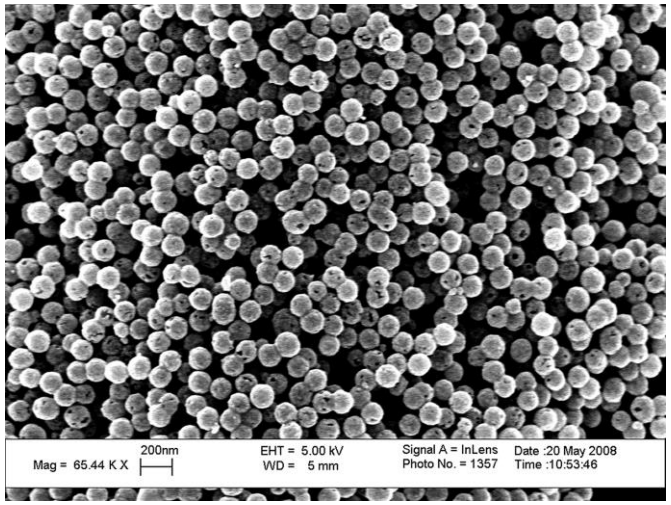
ThermoFisher Talos F200S G2

Scanning Electron Microscope

Transmission Electron Microscopy

	Scanning Electron Microscopes (SEM)	Transmission Electron Microscopes (TEM)
Electron stream	Fine, focused beam Scans over sample surface	Broad beam passes through thin sample
Image taken	Topographical/surface	Internal structure
Resolution	Lower resolution	Higher resolution
Magnification	Up to 2,000,000 times	Up to 50,000,000 times
Image dimension	3-D image of sample surface	2-D projection of sample
Sample thickness	Thin and thick samples, any thickness	Ultrathin samples only supported on TEM grids
Sample position	In the chamber of the bottom of the column	Halfway down column
Penetrates sample	No	Yes
Sample restriction	Less restrictive	More restrictive
Sample preparation	Less preparation required	More preparation required
Cost	Less expensive	More expensive
Speed	Faster	Slower
Operation	Easy to use	More complicated; requires training

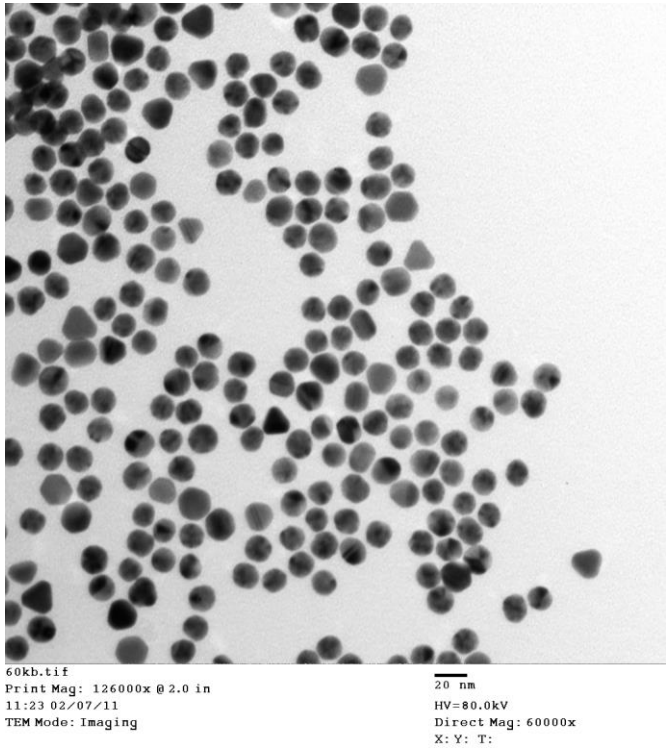
Examples of applications to nanoparticles



SEM image of Gold Nanoshells 120 nm

To create this scanning electron microscope image, gold nanoshells were dispersed in a drop of water which then dried on a glass microscope slide.

https://www.nisenet.org/catalog/scientific-image-gold-nanoshells-sem



TEM image of Gold Nanoparticles 15 -20 nm

https://www.nisenet.org/catalog/scientific-image-gold-nanoparticles-0

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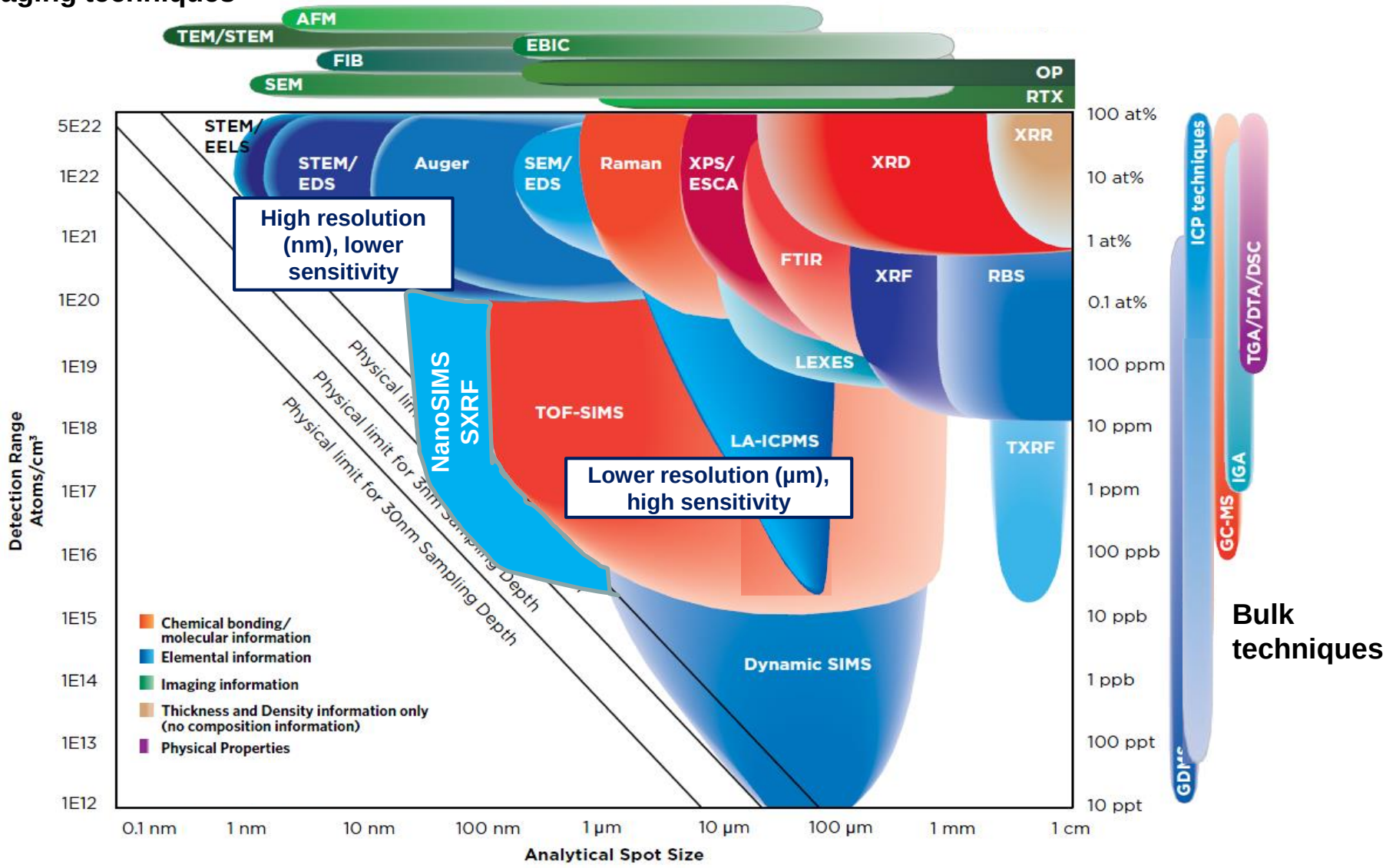
Element specific imaging

How to obtain element specific signals in each pixel of an image?

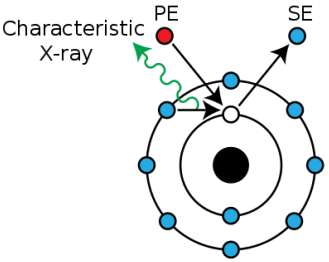
Combining microscopy/imaging with element/molecule specific techniques

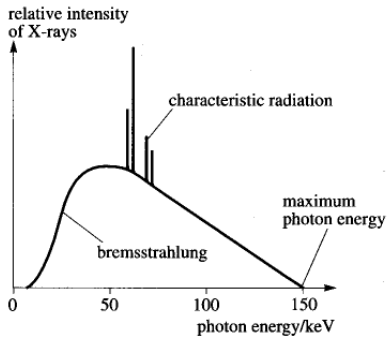
Imaging techniques

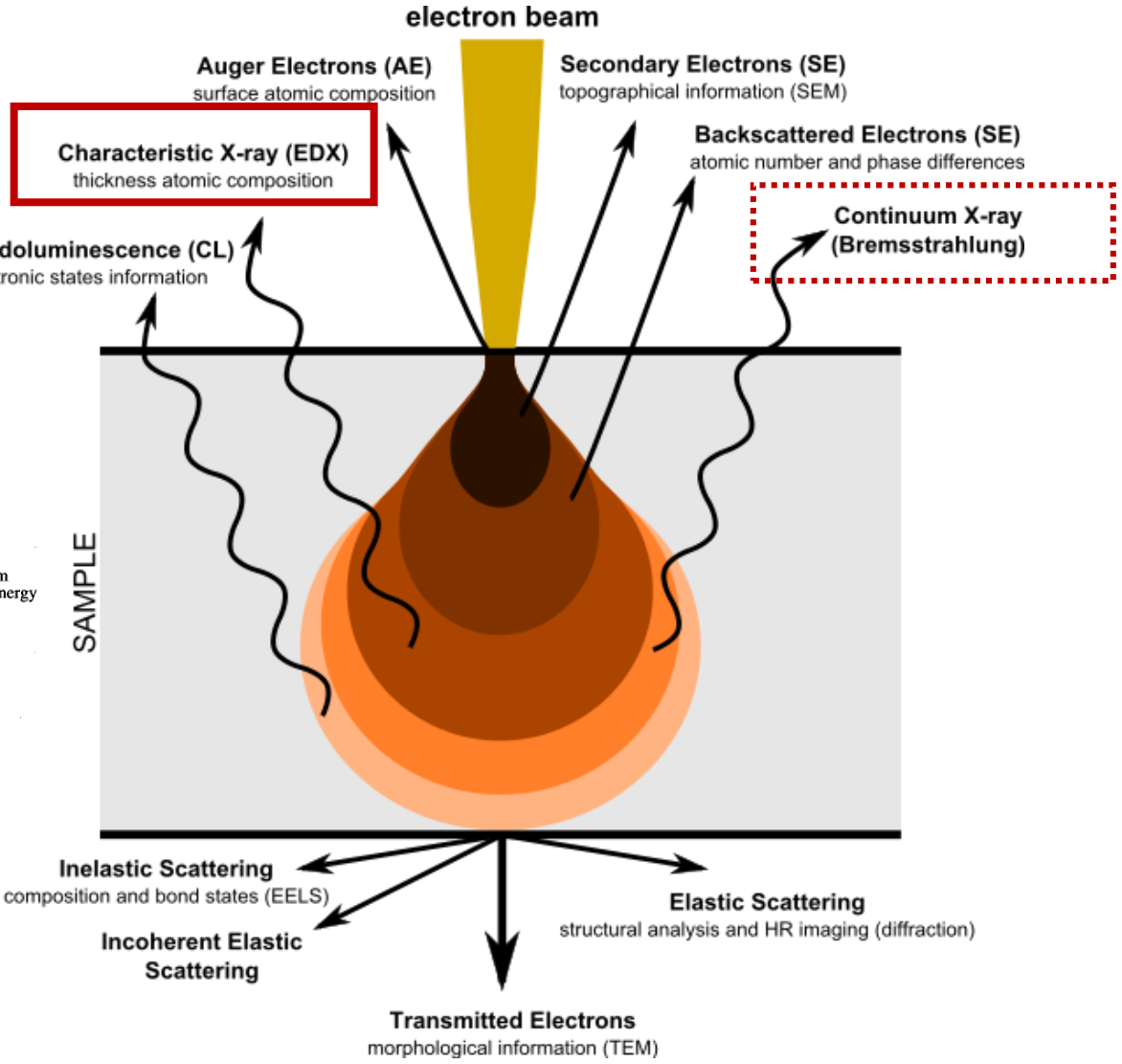


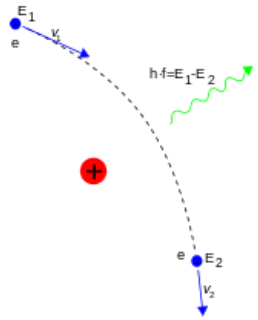
Bulk techniques

Getting chemical information:
SEM and TEM with Energy-dispersive X-ray spectroscopy (EDS, EDX, EDXS or X-EDS)









produced by a high-energy electron deflected in the electric field of an atomic nucleus

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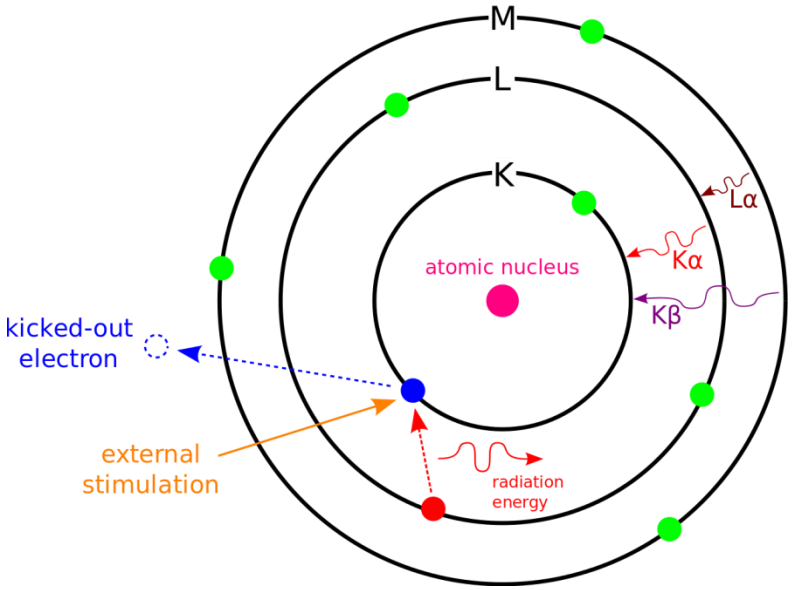
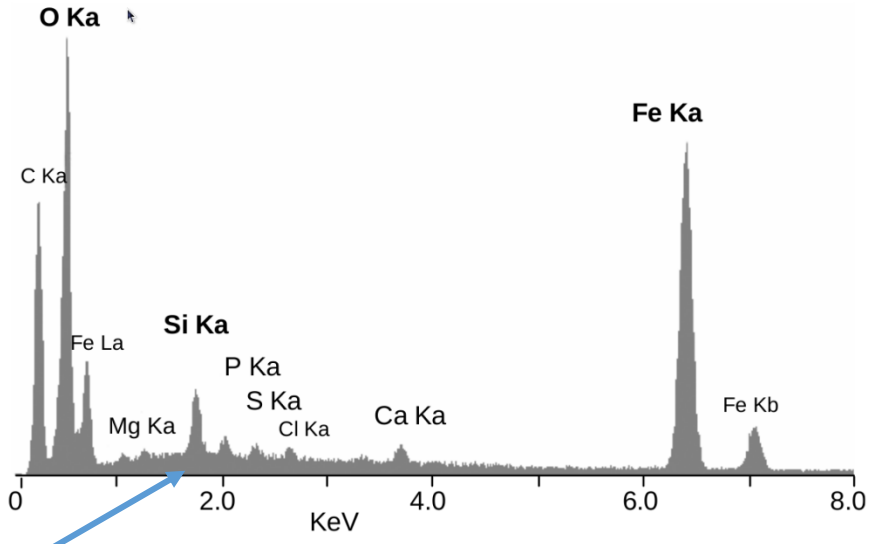


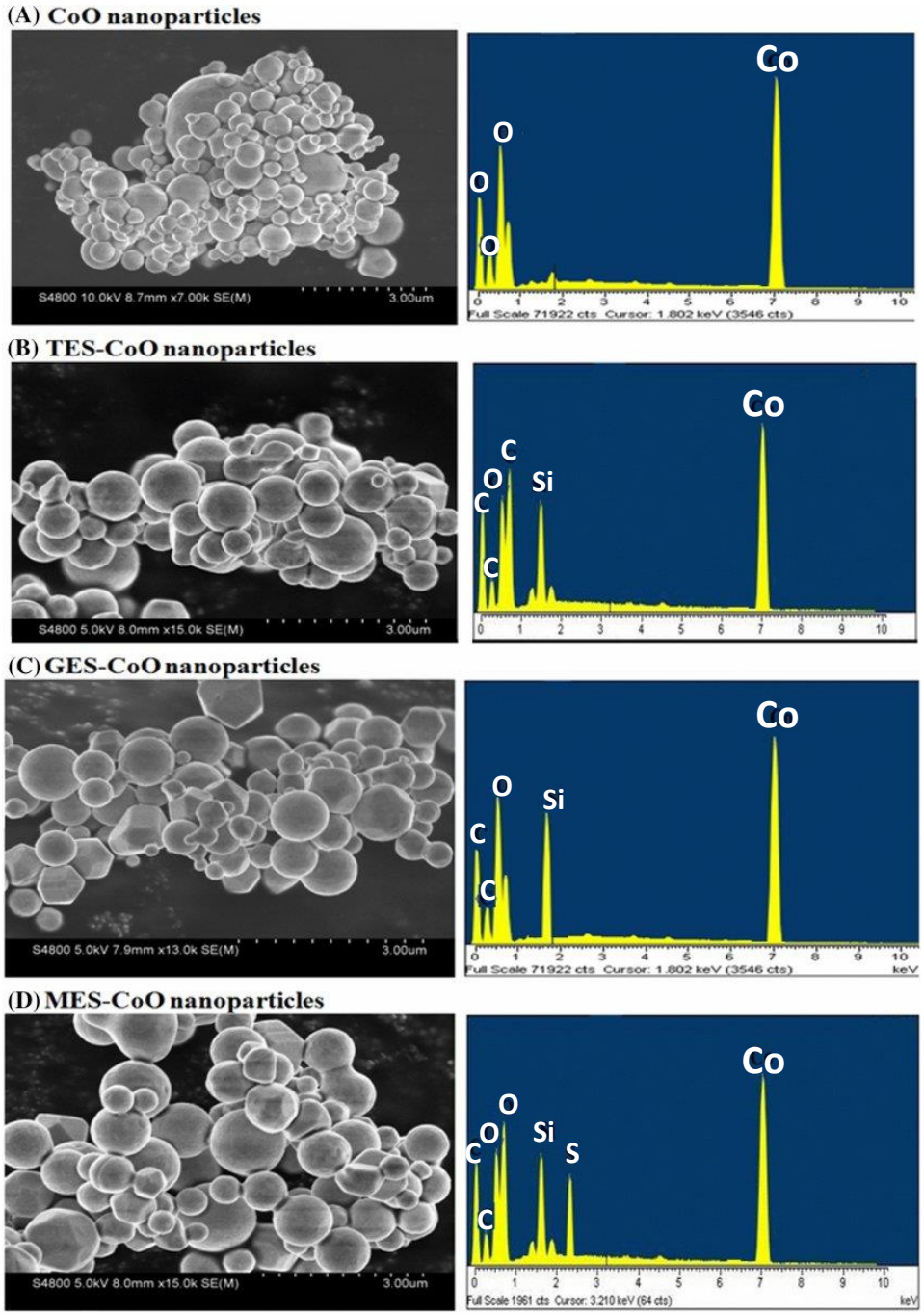


Background due to Bremsstrahlung

Characteristics

- Spatial resolution : down to 5 nm
- Sensitivity g/kg
- Detection of metals and non-metals

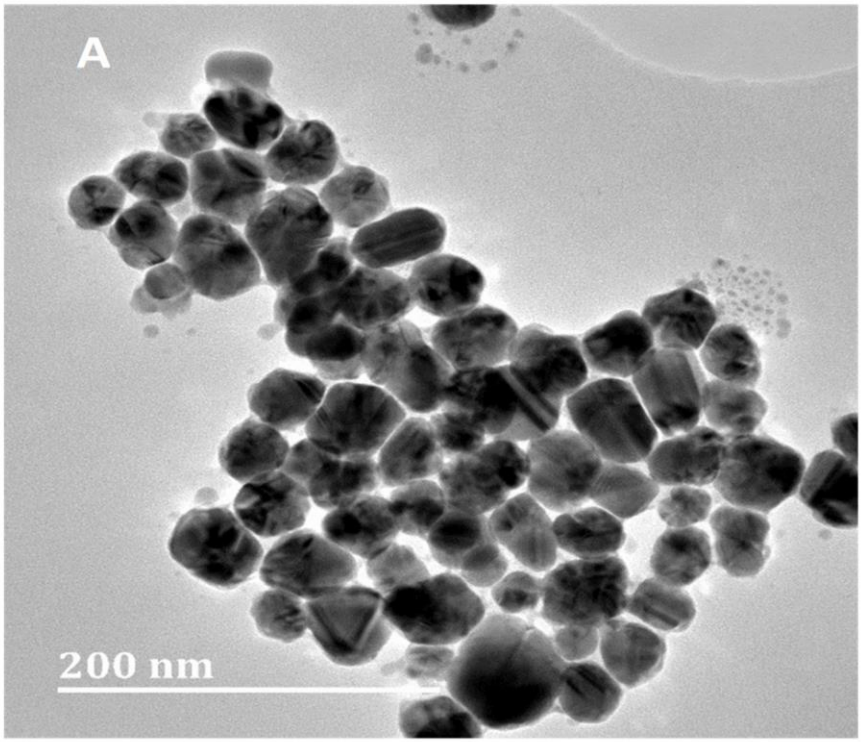
EDS:	Energy Dispersive Spectroscopy
EDXS:	Energy Dispersive X-ray Spectroscopy
X-EDS:	X-ray Energy Dispersive Spectroscopy
EDX:	Energy Dispersive X-ray analysis

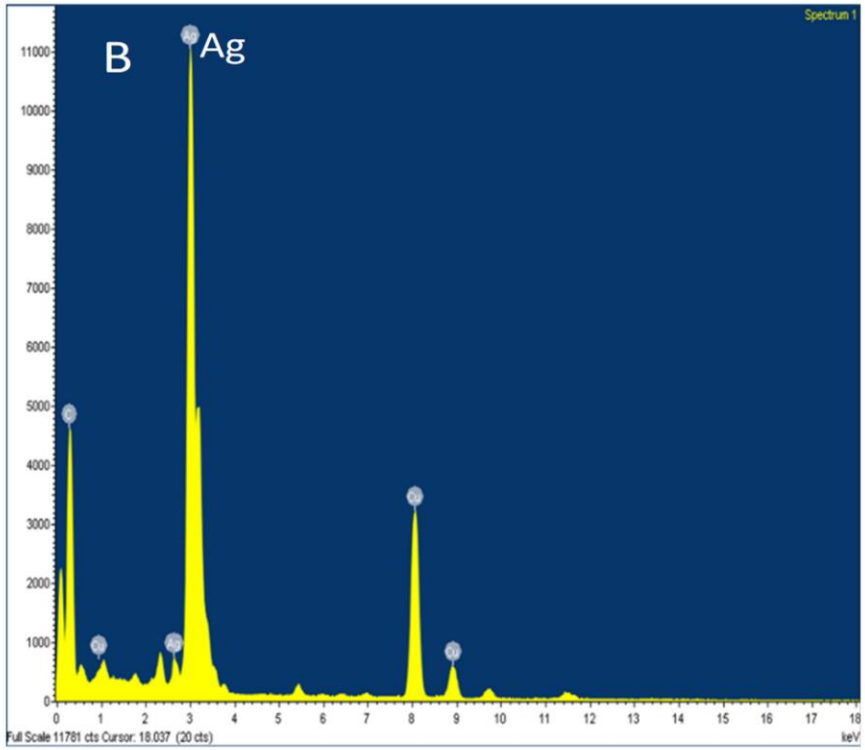


SEM/EDX analysis of
(a) CoO nanoparticles
and silane modified
(a) TES-CoO,
(b) GES-CoO,
(c) and MES-CoO
nanoparticles

Xavier, Joseph Raj. (2021). Enhanced Protective and Mechanical Properties of Polypyrrole Coatings Modified by Silane/CoO Nanocomposite on AZ91 Mg Alloy in Chloride Media. Journal of Bio- and Tribo-Corrosion. 7. 10.1007/s40735-021-00479-7.

TEM/EDX analysis of silver nanoparticles





Analysis of chemical synthesis of silver nanoparticles. Images showing the TEM picture of silver nanoparticles (A) and EDX pattern (B)

Murei, Arinao, Karen Pillay, Patrick Govender, Ntevheleni Thovhogi, Wilson M. Gitari, and Amidou Samie. 2021. "Synthesis, Characterization and In Vitro Antibacterial Evaluation of Pyrenacantha grandiflora Conjugated Silver Nanoparticles" Nanomaterials 11, no. 6: 1568. https://doi.org/10.3390/nano11061568

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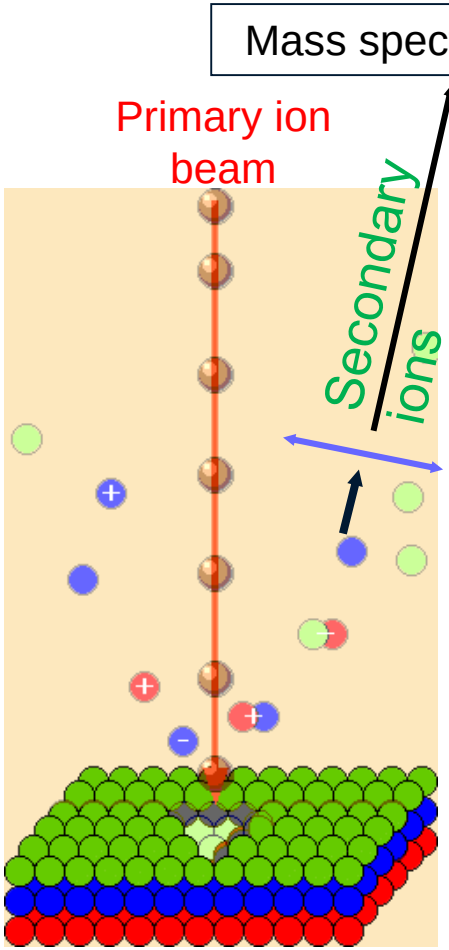
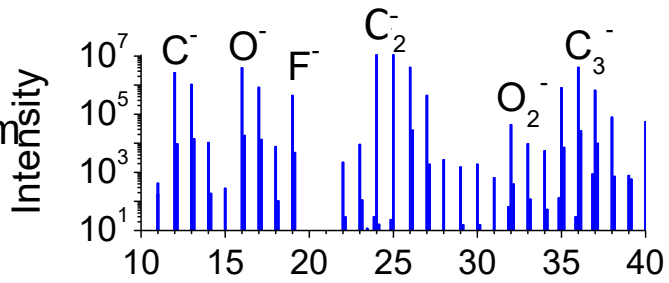
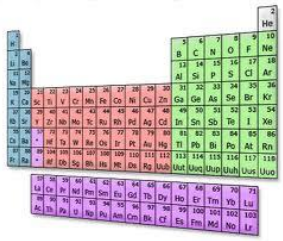
SIMS : Secondary Ion Mass Spectrometry

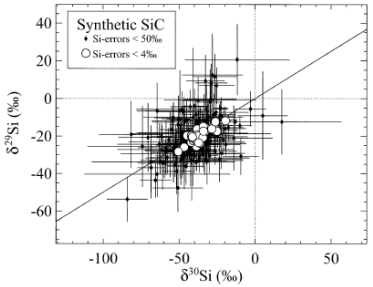


Mass spectrum

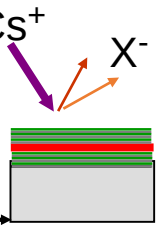


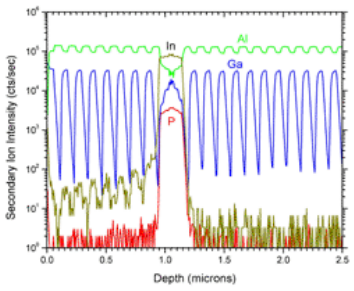


Isotopic measurement



Depth profiling ~nm





Imaging ~50 nm

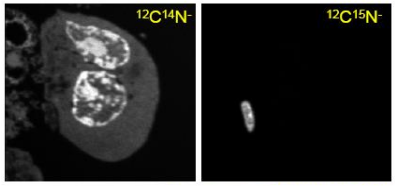
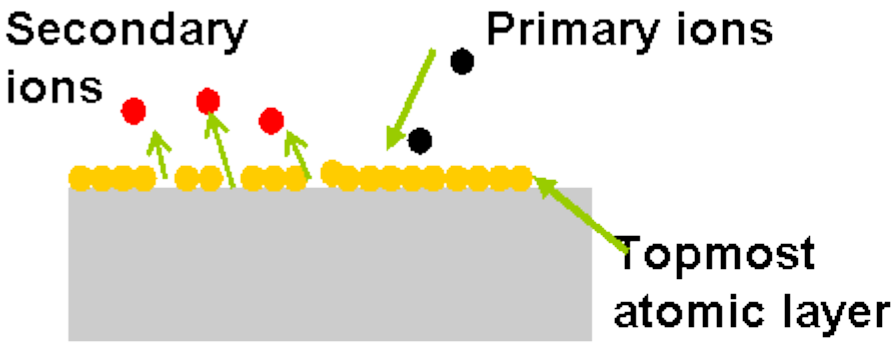
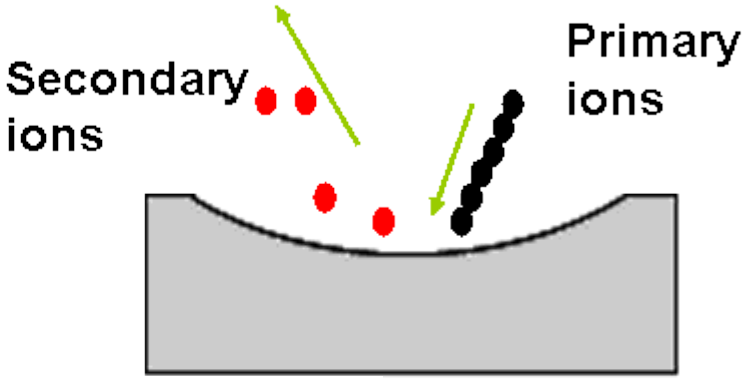


Image 3D

Static and dynamic SIMS



Static SIMS : Secondary ions are ejected only from the topmost atomic layer



Dynamic SIMS : Top few monolayers are removed because of sputtering caused by the high dose of primary ions

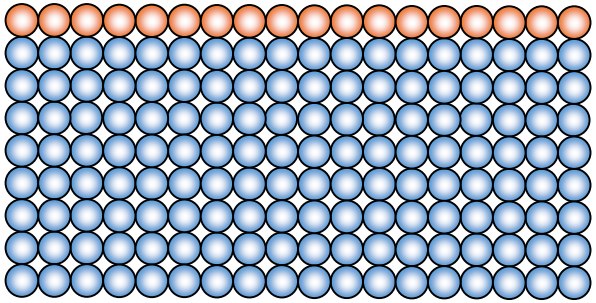
- Low energy, pulsed primary ion beam
- Time of flight mass spectrometer (TOF-SIMS)

- High energy, high density primary ion beam
- Magnetic sector mass spectrometer

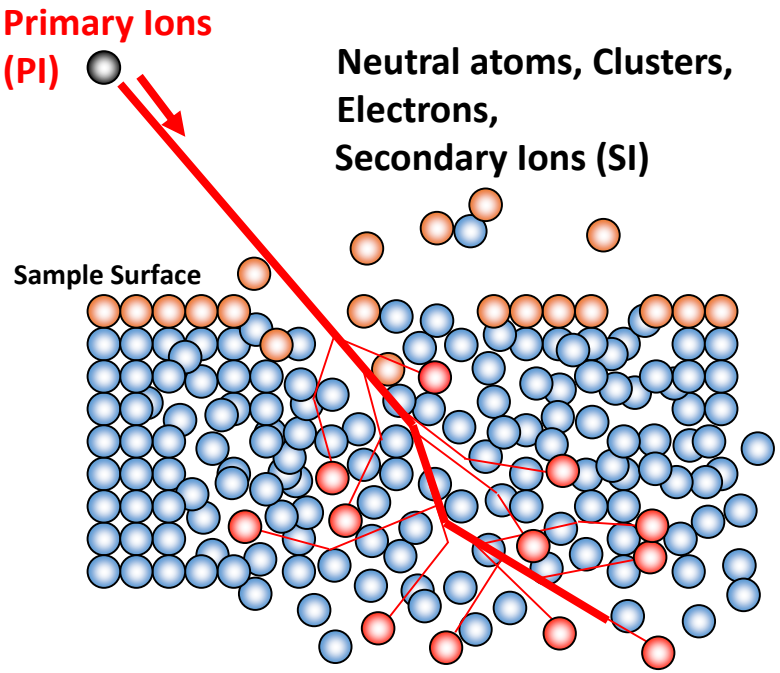
Ionization process in dynamic SIMS

- Samples analyzed under **ultra high vacuum** (UHV)

Sample Surface

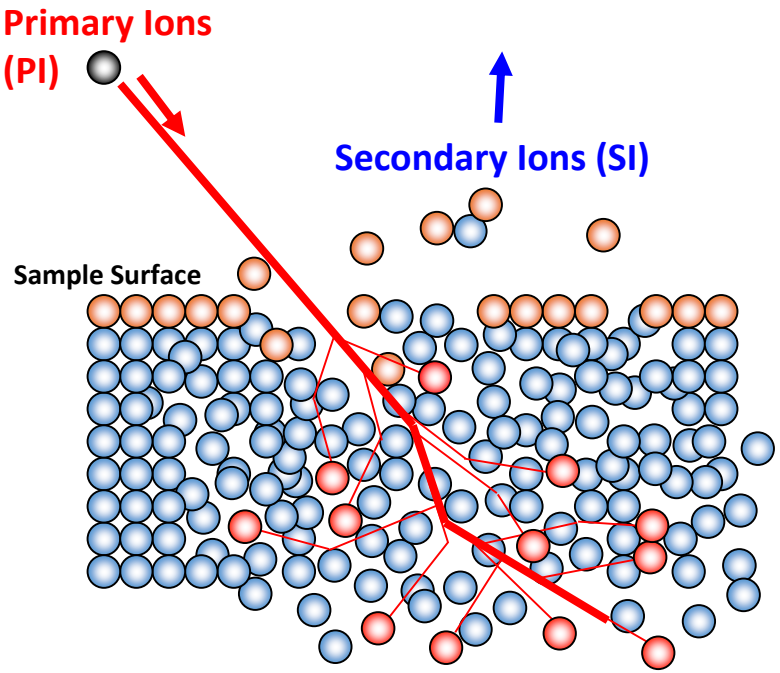


Ionization process in dynamic SIMS



- Samples analyzed under **ultra high vacuum** (UHV)
- **Bombardment** by focused **Primary Ions** (**PI**):
  - **Collision cascade** (10-20nm depth) with simultaneous Implantation and Sputtering.
- **All molecules are broken**, single neutral atoms, clusters and electrons are ejected.

Ionization process in dynamic SIMS

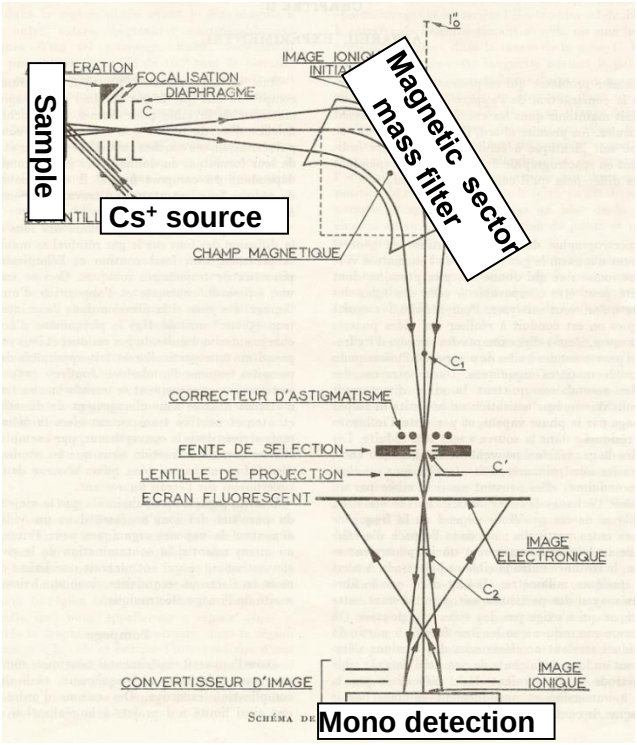


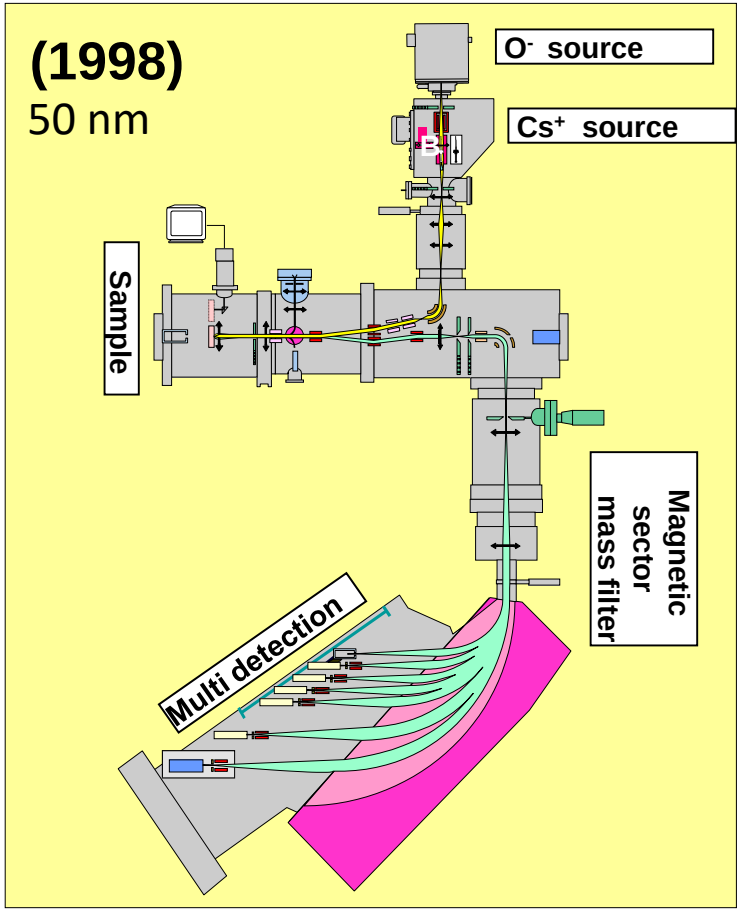
- Samples analyzed under **ultra high vacuum** (UHV)
- **Bombardment** by focused **Primary Ions** (**PI**):
  - **Collision cascade** (10-20nm depth) with simultaneous Implantation and Sputtering.
- **All molecules are broken**, single neutral atoms, clusters and electrons are ejected.
- A **small fraction is ionized** (+ or - charge): **Secondary Ions** (**SI**) available for **Mass Spectrometry**.

The Secondary Ions, characteristic of the local composition, are collected, then separated in a magnetic sector analyzer according to their **mass/charge** ratio: SIMS reveals **elemental** (H included) and **isotopic** surface composition

Consequences for analysis: samples must be stable in ultra high vacuum and flat!

History of dynamic SIMS instruments (CAMECA)

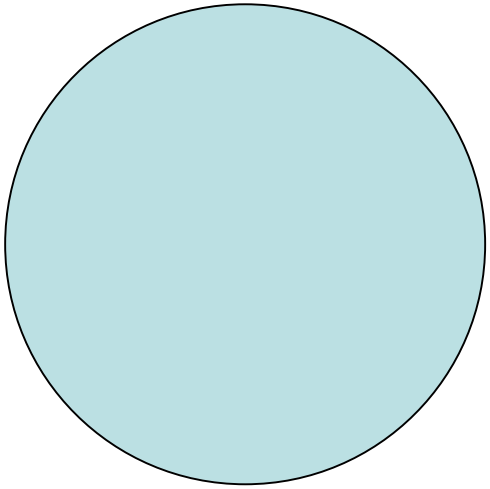




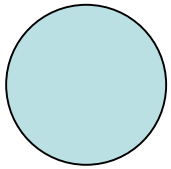
Cameca SMI 300, 1968

Cameca NanoSIMS50, 2000

The beam size of the ion microprobe



IMS 3f Ion Microprobe **1970 ‘**
2 – 3 µm



IMS 4f **1980 ‘**
500 nm

IMS 6f / Tof-SIMS ***250 nm*** **1990 ‘**

NanoSIMS50/50L ***50 nm*** **2000 ‘**

Orion HIM-SIMS ***20 nm*** **2010 ‘**

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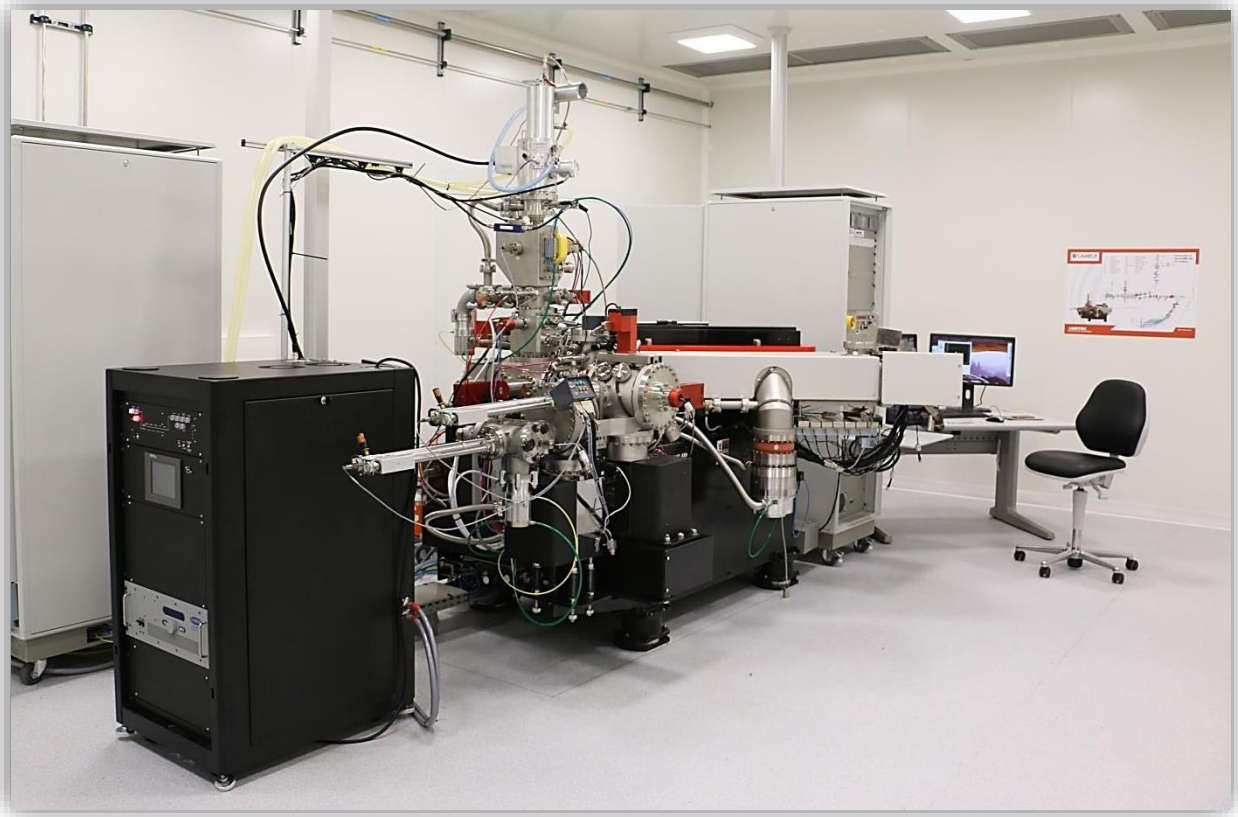
Nano Secondary Ion Mass Spectrometry (NanoSIMS)

The NanoSIMS 50L instrument
part of the Mass Spectrometry Center in Pau, France **(MARSS)**

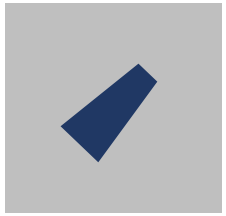


- **High lateral resolution: 50nm in Cs^+, 40nm in O^-**
- **High Sensitivity *together with* High Mass Resolution *and* small spot size**
- **Parallel Detection: 7 masses**

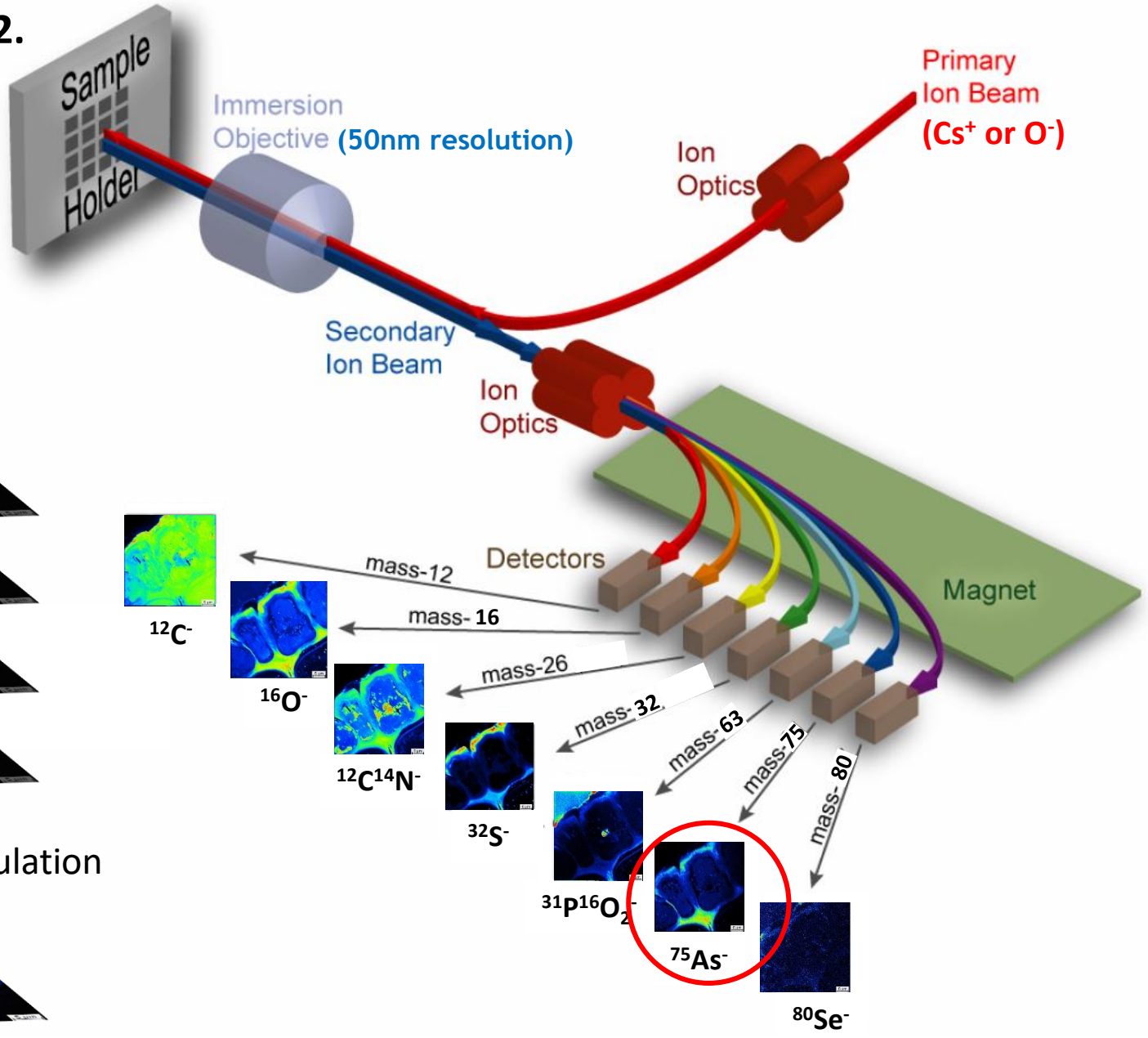
The NanoSIMS: a scanning Ion Microprobe with a multicollection mass spectrometer

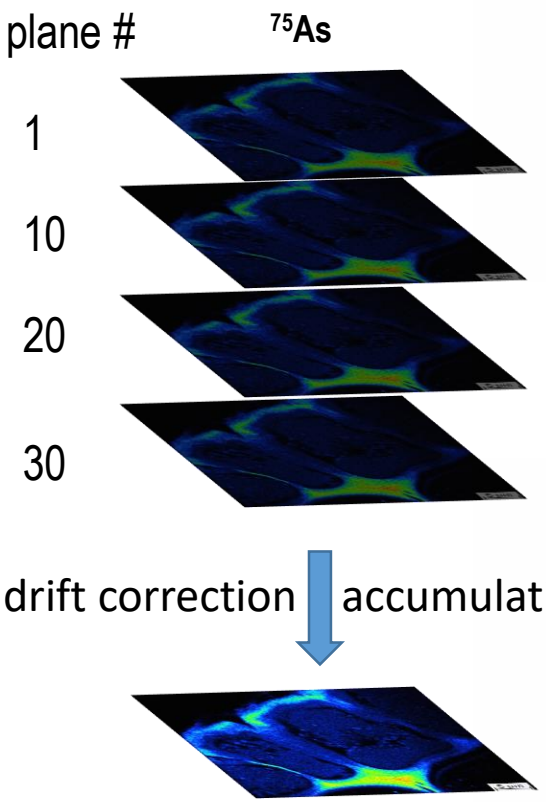
1.

Observation
with CCD camera



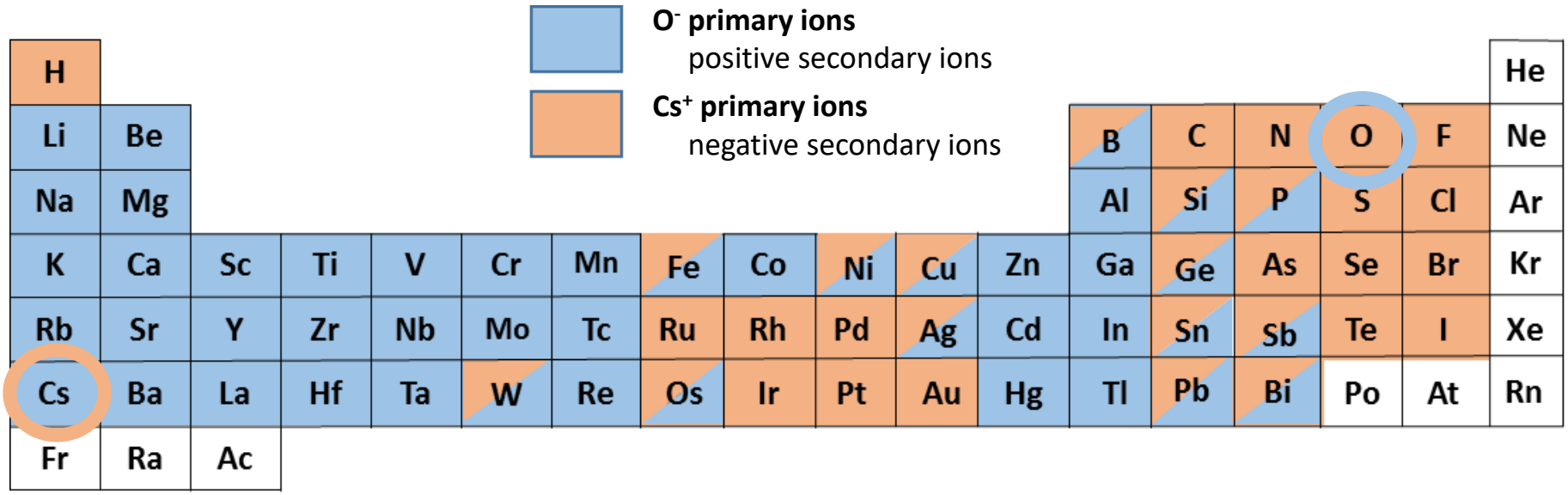
2.





Schematics from: M. Steinhauser et al., Nature, Vol 481, 26 January 2012

Primary Ion Beam - Secondary Ion Yields



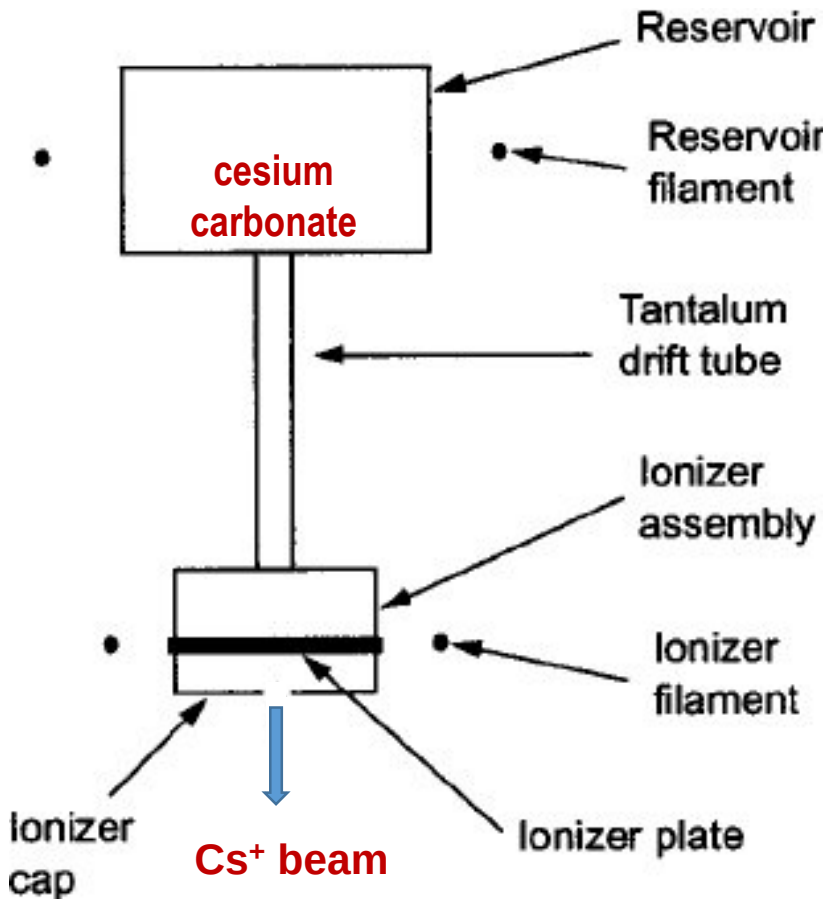
Cs^+ primary ion source

Classic NanoSIMS application (e.g. cell imaging):
C, N (via CN^-), **O, S, P, Se** and their stable isotopes for tracer studies.

O^- primary ion source

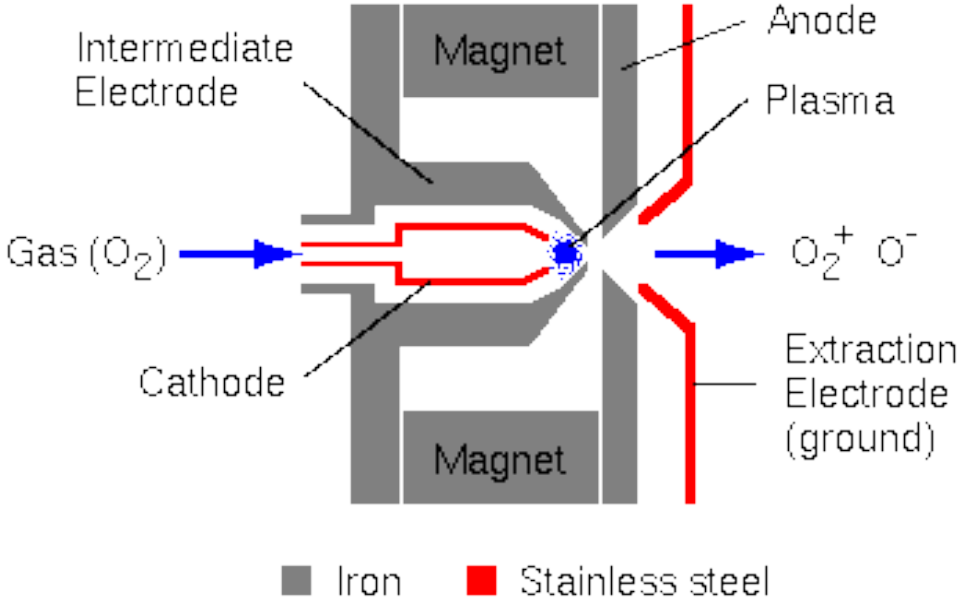
Imaging of major and trace metals is possible:
Ca, Mg, Al, Mn, Cr, Cu, Fe, Ni …

Cesium (Cs^+) primary ion source



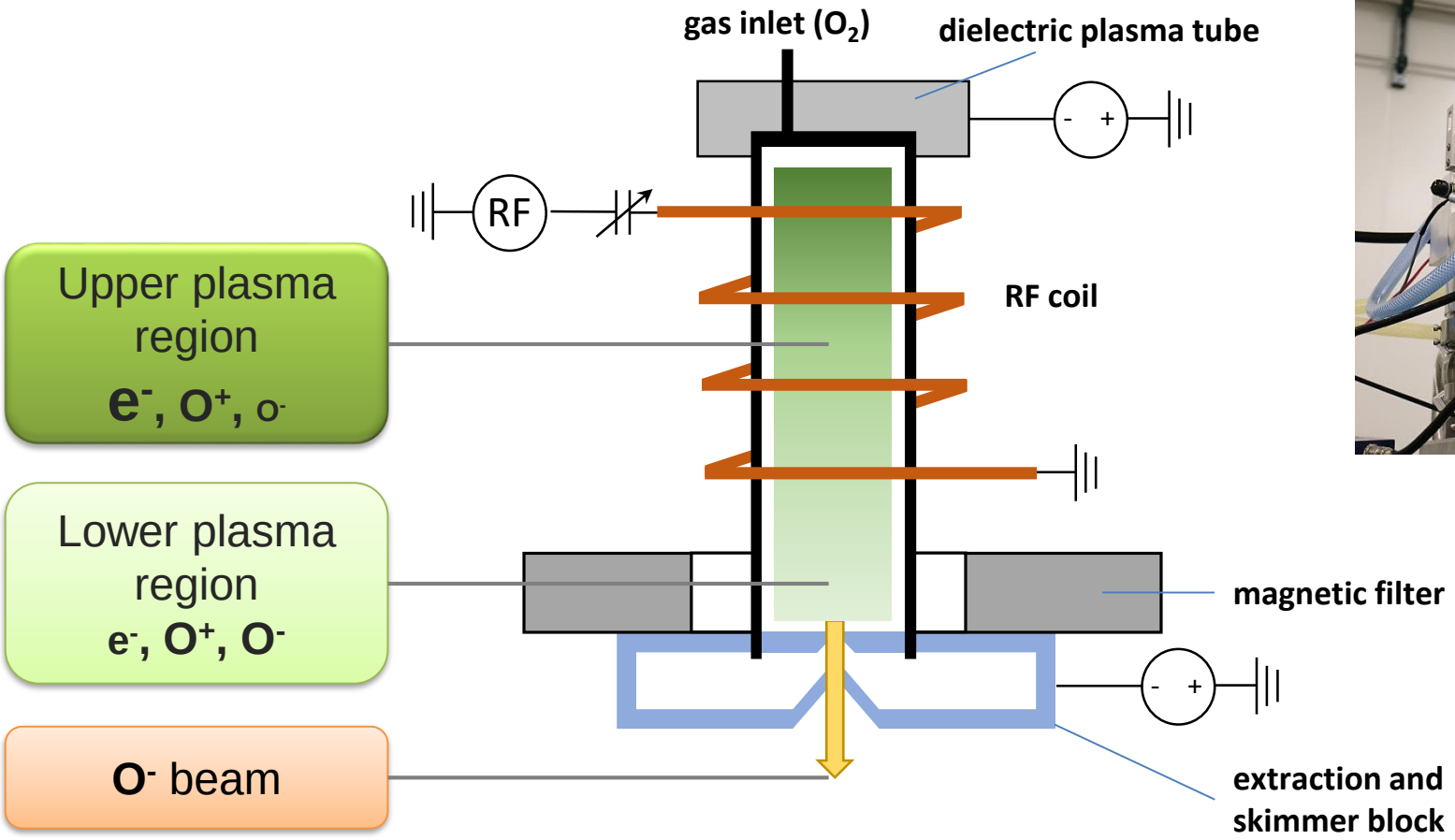
Cesium carbonate reservoir for 1 year operation

Conventional Duoplasmatron O^- primary ion source



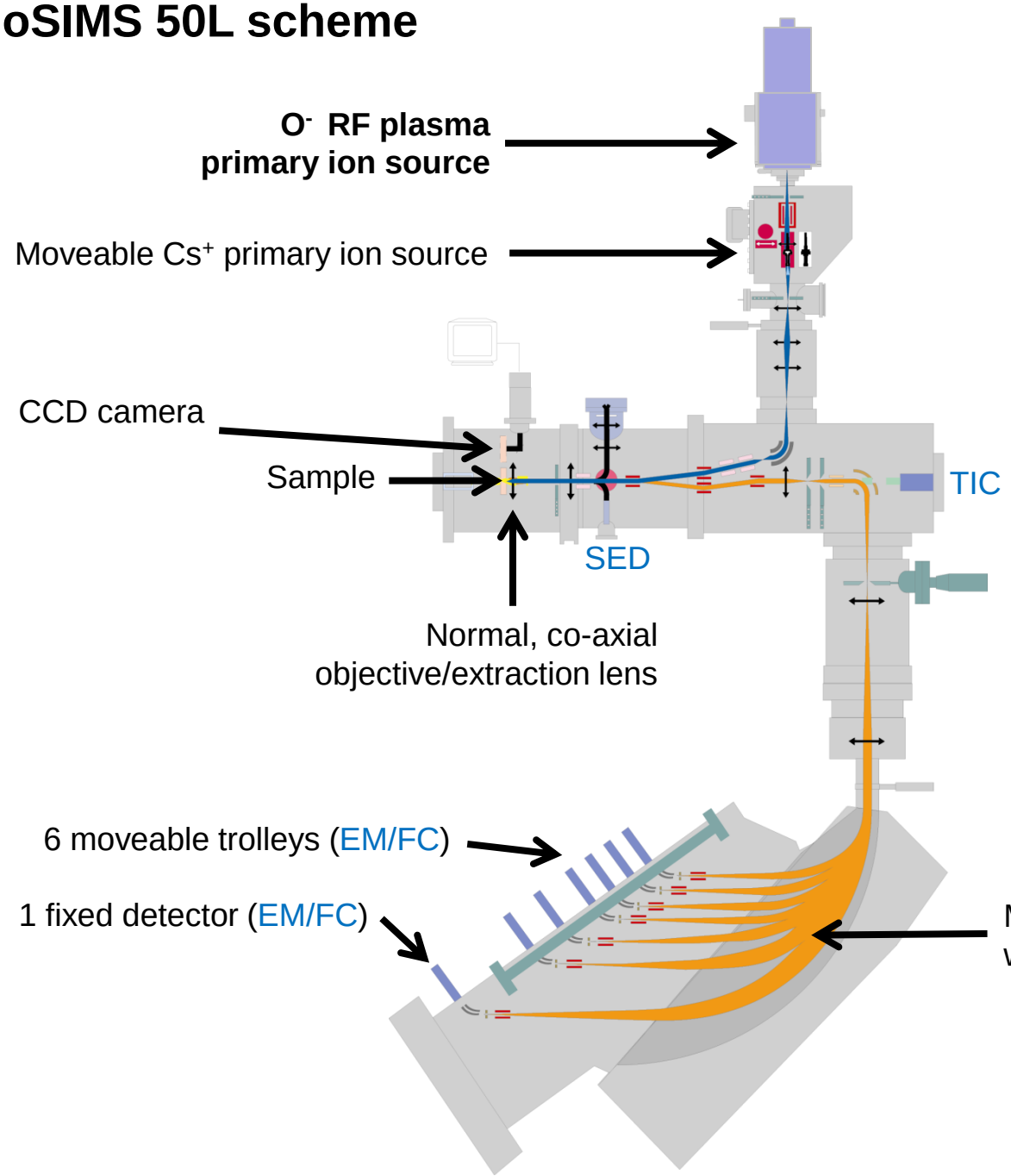
- Low beam density
- Low lateral resolution 200 – 300 nm
- Low long term stability – frequent maintenance necessary

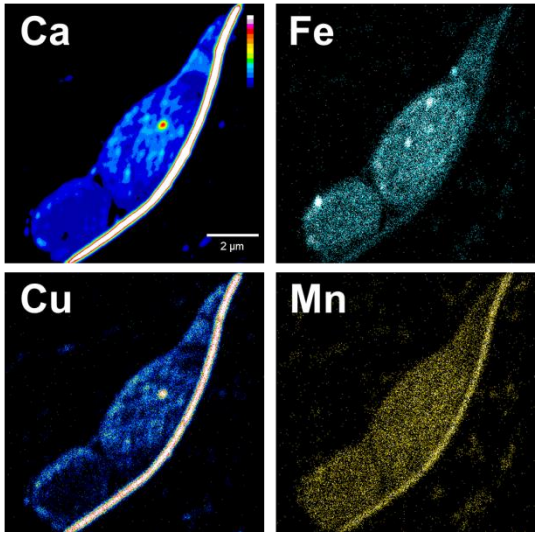
New O^- RF plasma primary ion source on NanoSIMS



- **Higher beam density** = better sensitivity for (trace) metals (Ca, Fe, Cu, Mn….)
- **Higher lateral resolution** : **40 nm**
  = sharper images enabling the observation of smaller details
- **Long term stability** – less maintenance

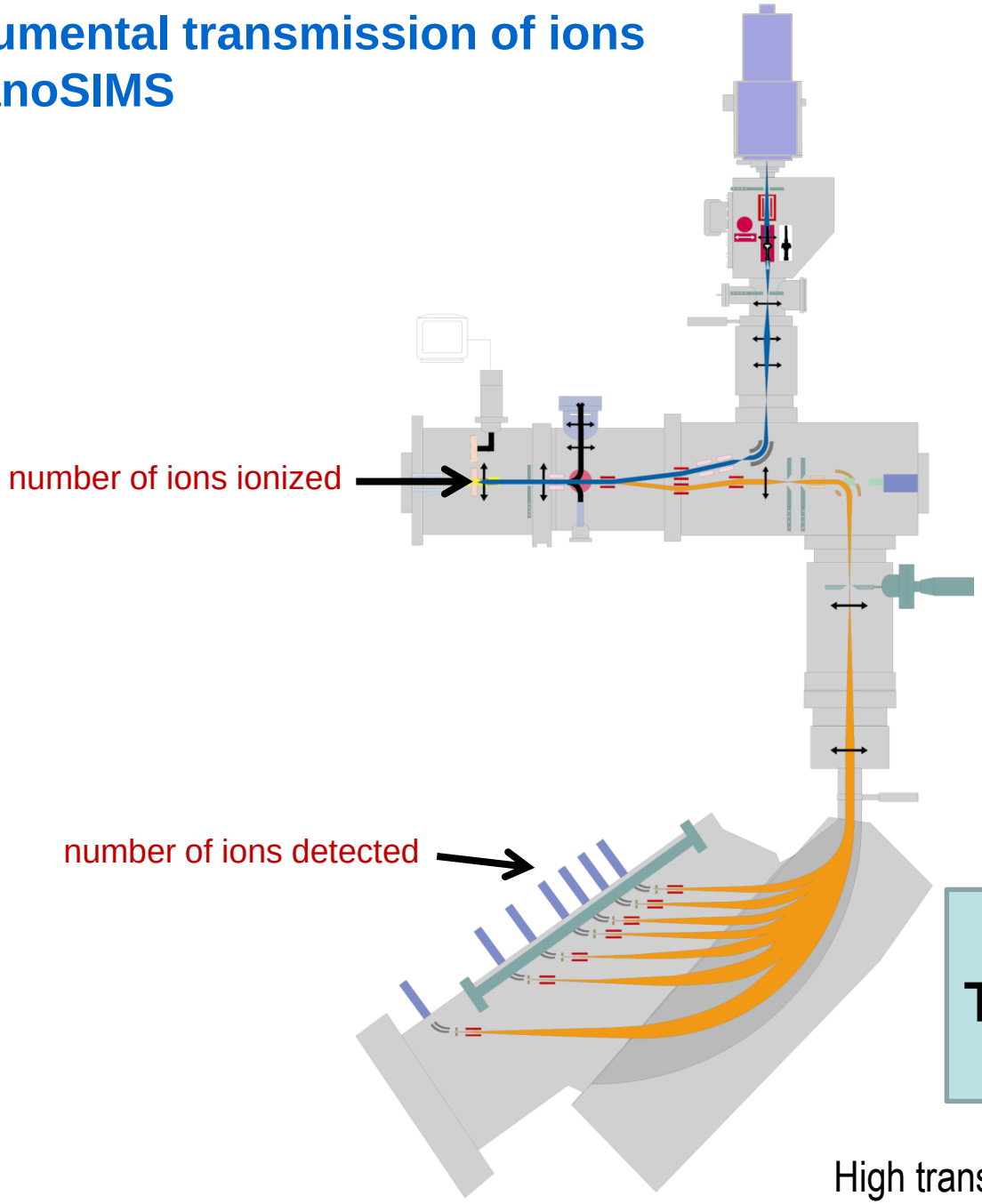
NanoSIMS 50L scheme





Essential trace metals in a chloroplast (*A. thaliana*)

Instrumental transmission of ions in NanoSIMS



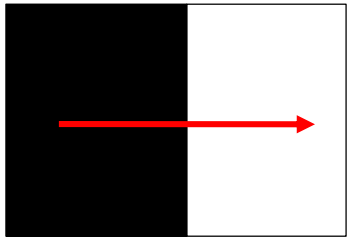
$$T = \frac{\text{nb ions } \textbf{detected}}{\text{nb ions } \textbf{ionized}}$$

High transmission in NanoSIMS > 80%

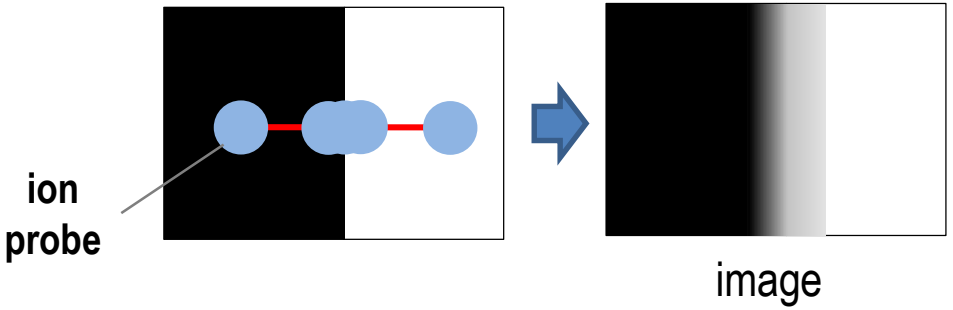
Resolution in NanoSIMS

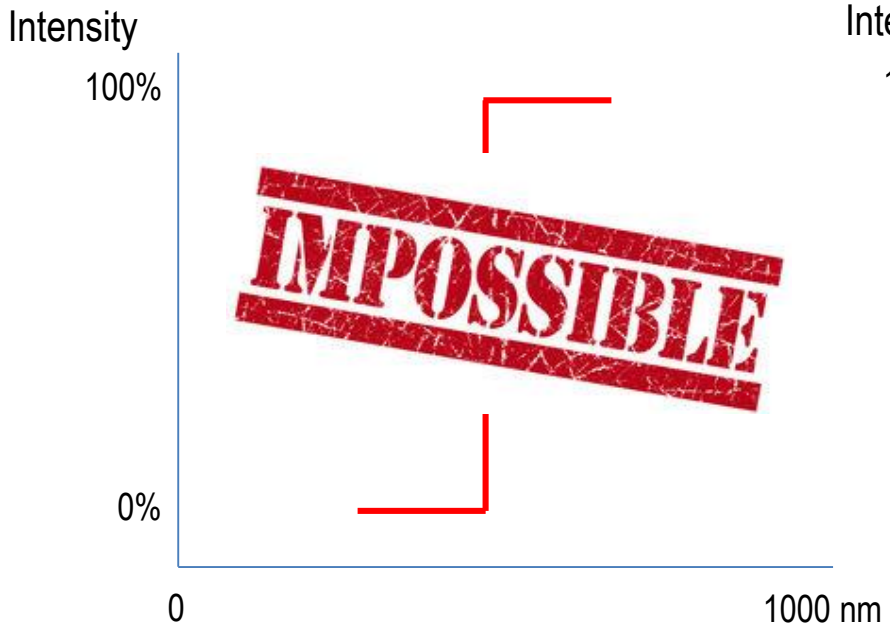
- **Lateral resolution** (image resolution) depending of the ion beam size
- **Mass resolution** depending on the mass spectrometer

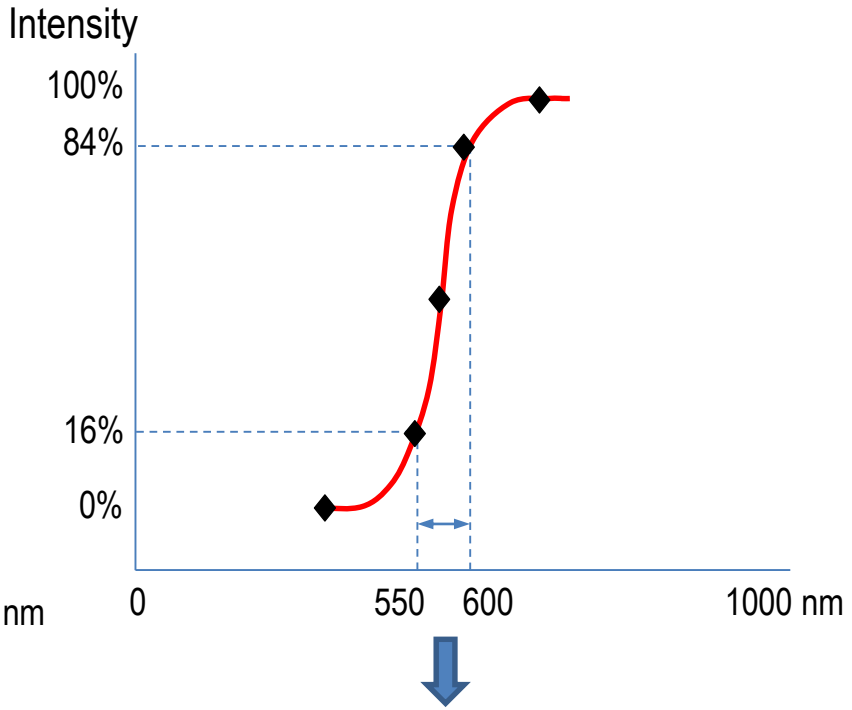
Determination of the ion beam size (probe size) in SIMS: line scan at edge, 16-84% criterion - information on minimal lateral resolution



Line scan at edge

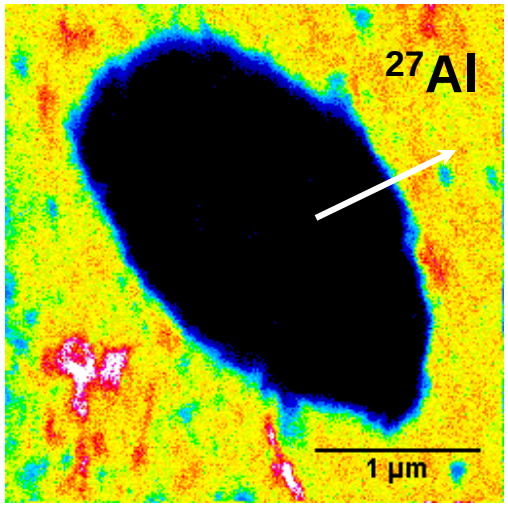


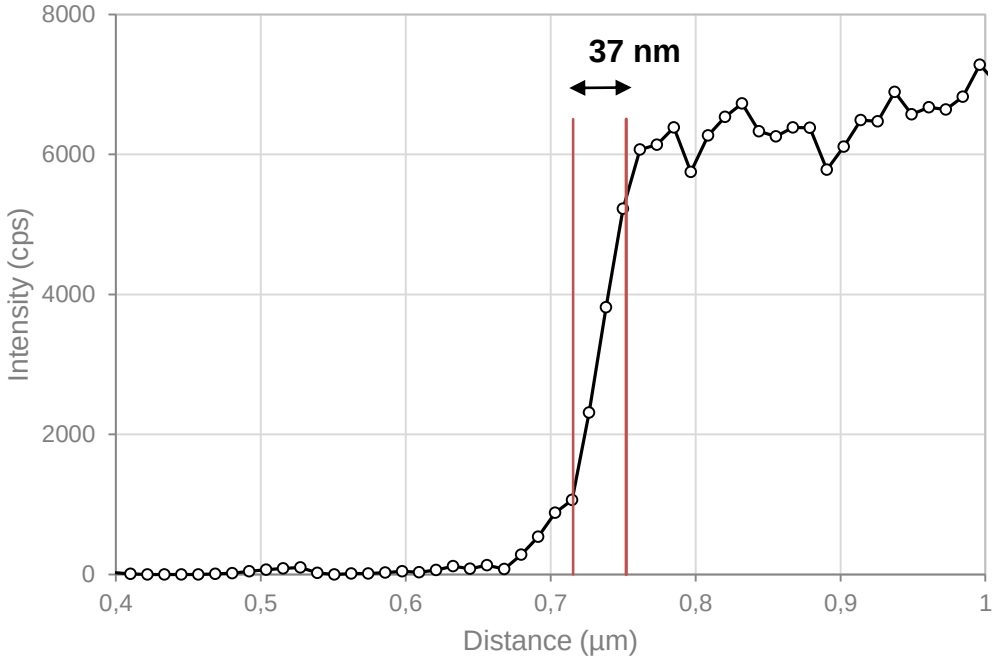




Probe size – minimal lateral resolution: 50 nm

Determination of the size of the O^- primary ion beam in NanoSIMS (probe size)

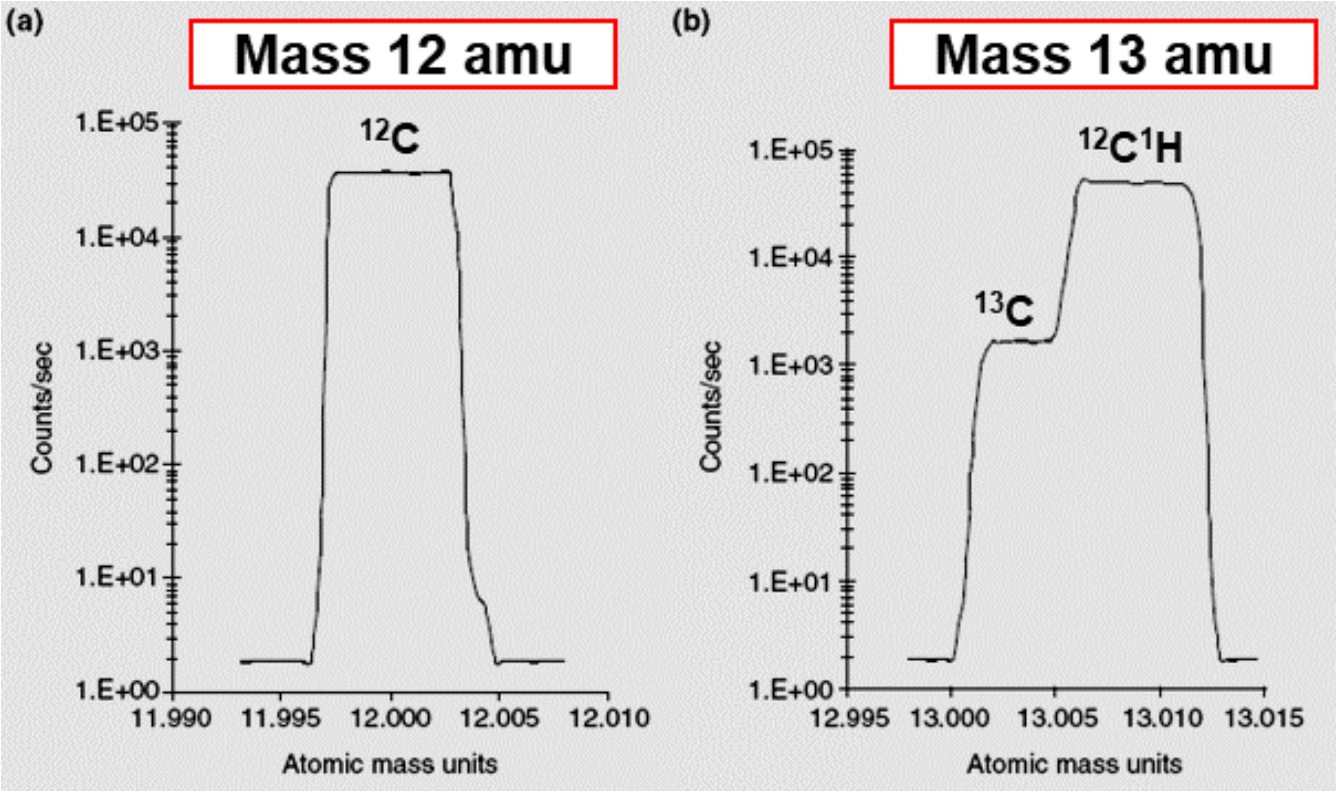




Al/Si oxide grain sample
Image size: 3 x 3 µm
Probe intensity: 0.15 pA

Line scan (left image) showing **intensity variation** from **16 to 84 %:** determination of **probe size** (minimal possible lateral resolution)

Mass Resolution in NanoSIMS



- In SIMS mass interferences are usually present at each unit mass.
- High Mass Resolution is necessary to resolve such mass interference
- **Mass resolution (dM/M) 3000 to 9000** possible
  (Note the log. scale and flat top peak shape)

The uniqueness of the NanoSIMS is to keep nearly full Transmission (= High Sensitivity) at High Mass Resolution *together* with High Lateral Resolution (< 50nm).

Curves extracted from: High-resolution quantitative imaging of mammalian and bacterial cells using stable isotope mass spectrometry. C. Lechene et al, Journal of Biology 2006, Volume 5, Article 20.

Other points to consider:

Signal intensity (useful yield) depending also on

- **Chemical element**
- **Matrix**

Characteristics of NanoSIMS

- **Allmost all Elements** (from H, D, T,… up to Pu), but with different sensitivity
- **High Sensitivity**: down to ppb in spot analysis, ppm in imaging,
- **High resolution imaging**: down to **40** nm lateral resolution, access to **3D** analysis with depth resolution of 10-15nm.
- **Isotopic analysis:** e.g. metabolic pathways and activity in biology

- **Quantification difficult**: matrix effects
- **Sample preparation** for biological samples is challenging

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Preparation of (biological) samples for NanoSIMS

NanoSIMS analyses require :

- **Flat samples** to avoid artifact during ionization
- **Dehydrated samples** stable in ultra-high vacuum (10^{-11} mbar)
- **Conductive sample surfaces** to avoid charging effects from the ion beam

How these requirements can be compatible with biological cells or tissue ?

Sample preparation methods for transmission electron microscopy can be adapted for NanoSIMS

Biological sample preparation (similar to TEM)

Analysis at room temperature **under vacuum**: sample must be **dehydrated and fixated**

Chemical fixation

Glutaraldehyde
Formaldehyde
Osmium tetroxide

Cryofixation

high pressure freezer
tissues
(up to 6 mm diameter,
200 µm thick)

Dehydration

Solvent baths (acetone or ethanol/water)
with increasing solvent concentrations

Dehydration

Cryo-substitution
lyophilization

Resin embedding

Solvent baths with increasing
resin concentrations

Resin embedding

Solvent baths with increasing
resin concentrations

Ultramicrotomy

300 nm sections for NanoSIMS
70 nm sections for TEM/X-EDS

Equipment at Bordeaux Imaging Center

Biological sample preparation (similar to TEM)

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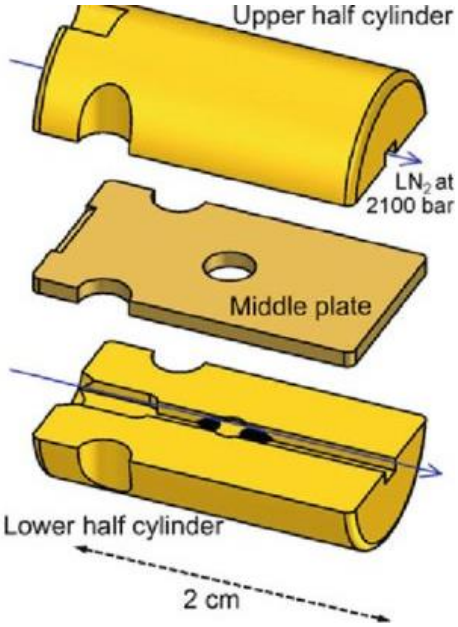
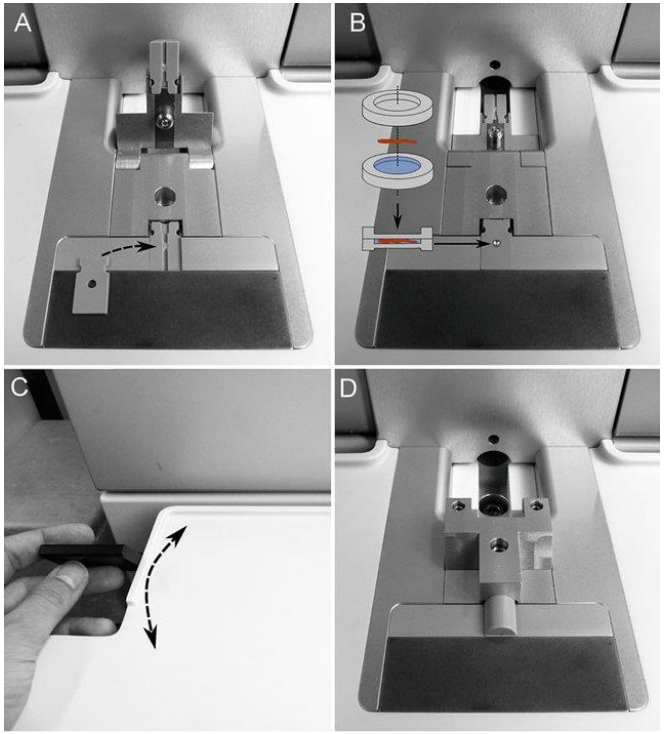
Equipment at Bordeaux Imaging Center

Cryofixation by high pressure freezing

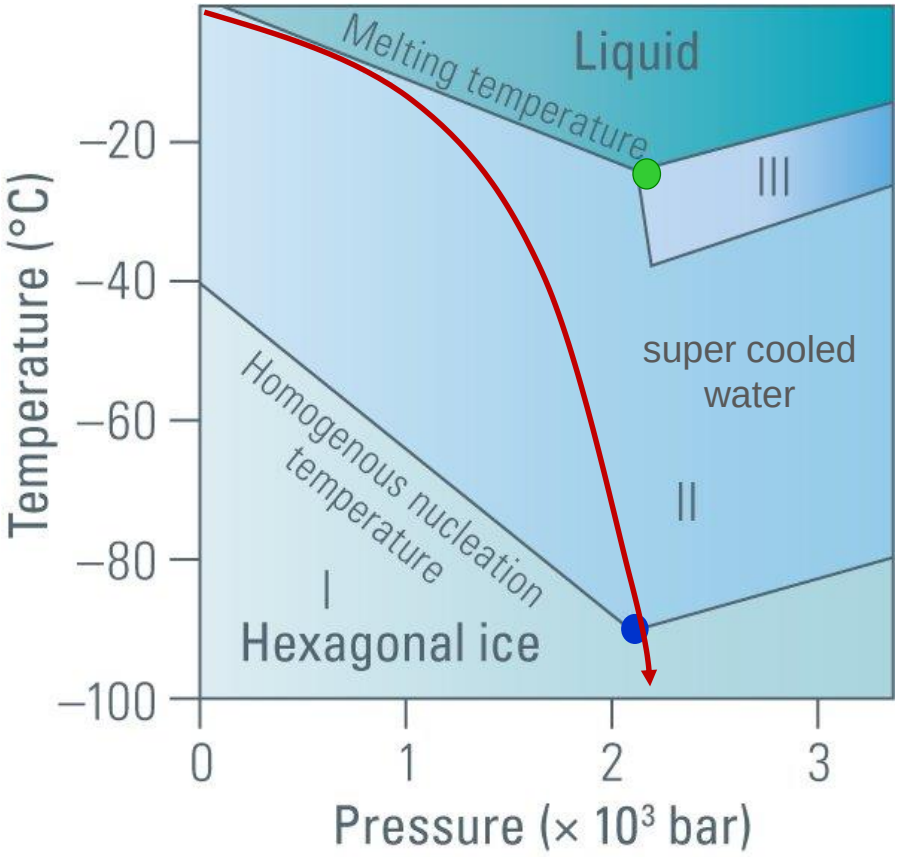




High pressure freezer Leica HPM 100

Cryofixation by high pressure freezing

States of water depending on pressure and temperature



At a pressure of 2045 bar the melting point of water is lowered to -22 °C ● and the temperature for homogenous nucleation is reduced to -92 °C.●

Kanno H et al. Science 189: 880–881 (1975)

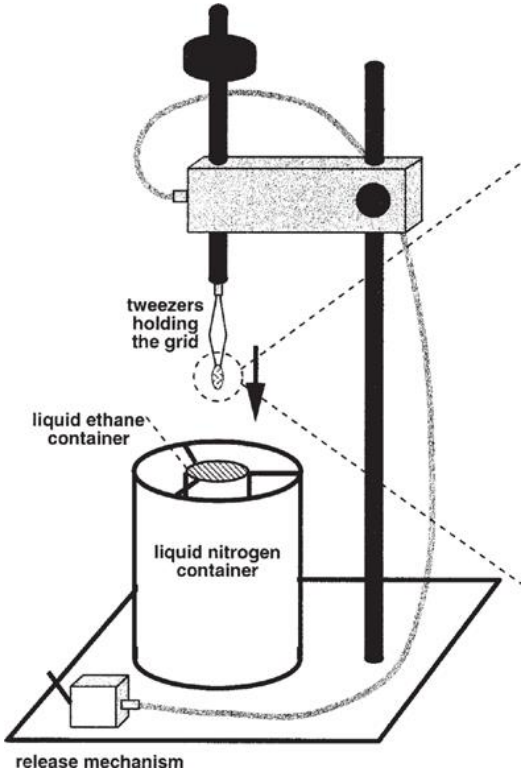
High pressure freezing allows synchronized pressurization (2100 bar) and cooling of the sample within **20 ms** in a highly reproducible manner:

(1) lowering of the freezing point,
(2) reduction in the rate of ice crystal formation, and
(3) slowing of the growth of ice crystals

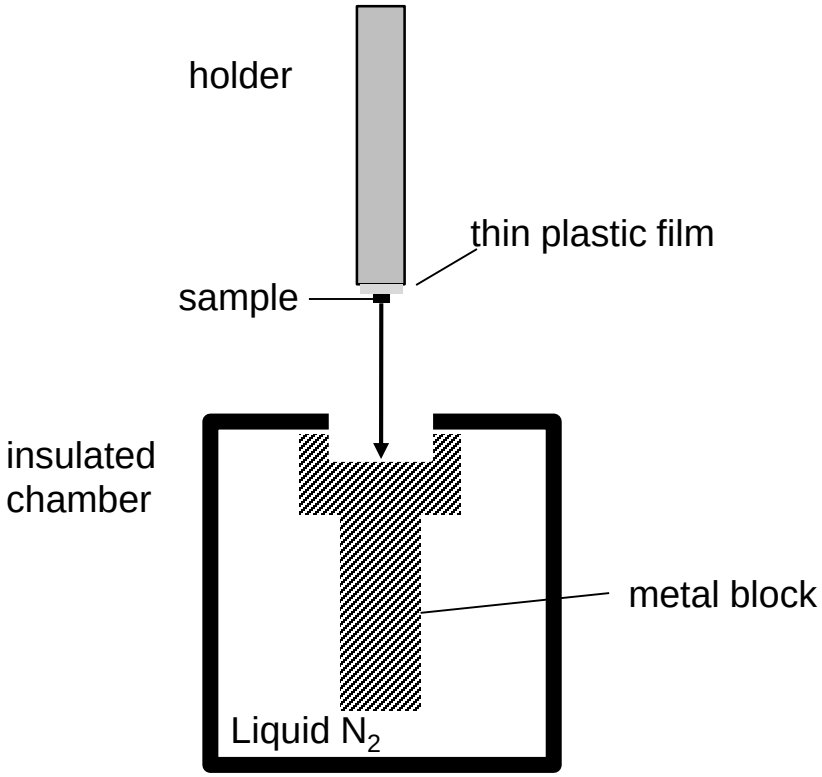
At 2100 bar water is 1500 times more viscous than at atmospheric pressure. This reduced considerably formation of ice crystals. Amorphous ice is formed.

⇨ Water is transformed in the vitreous state (amorphous ice) and thus the **cellular ultrastructure is fixed** and preserved.

Cryofixation by plunge freezing

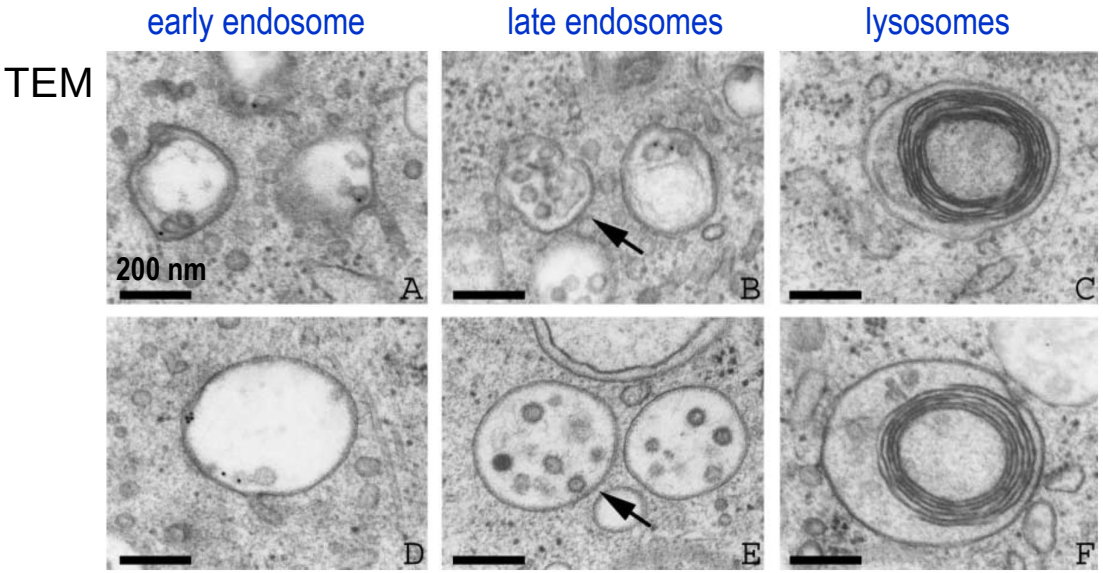


Cryofixation by slamming



Why cryofixation?

- Reduced Fixation Artifacts
  - Membrane blisters
  - Mesosomes
  - Nuclear equivalent
- Reduced Shrinkage



Chemical fixation

Cryo fixation

J. Murk et al., J. Microsc. 212 (2003), 81–90

- Reduced extraction of cellular components
  - Lipids
  - Proteins
  - Proteoglycans
  - Metals

Biological sample preparation (similar to TEM)

Analysis at room temperature **under vacuum**: sample must be **dehydrated and fixated**

less redistribution of highly diffusable trace metals !

Chemical fixation

Glutaraldehyde
Formaldehyde
Osmium tetroxide

Cryo fixation

high pressure freezer
tissues
(up to 6 mm diameter,
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Dehydration

Solvent baths (acetone or ethanol/water)
with increasing solvent concentrations

Dehydration

Cryo-substitution
lyophilization

LIMITATION of NanoSIMS:
Direct analysis of frozen hydrated samples not possible

Resin embedding

Solvent baths with increasing
resin concentrations

Resin embedding

Solvent baths with increasing
resin concentrations

Ultramicrotomy

300 nm sections for NanoSIMS
70 nm sections for TEM/X-EDS

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Preparation of (biological) samples for NanoSIMS

NanoSIMS analyses require :

- **Flat samples** to avoid artefacts during ionization

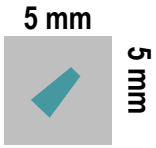
  Sections prepared with an ultramicrotome or polishing

- **Dehydrated samples** stable in ultra-high vacuum (10^{-11} mbar)

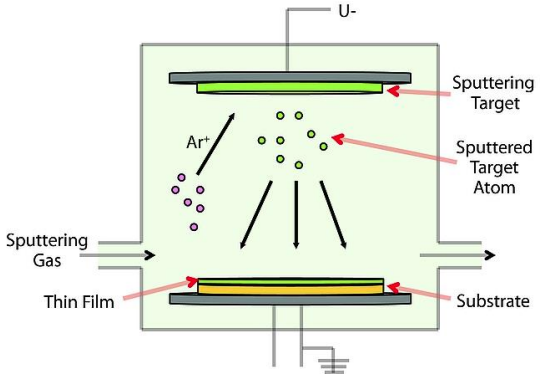
  Dehydrated and embedded in epoxy resin

- **Conductive sample surfaces** to avoid charging effects from the ion beam

  Ultrathin sections (< 500 nm) placed on conductive silicon wafer pieces



Thicker samples are metal (Au, Pt) coated with sputter coater (nm), similar to SEM



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How nanoparticles accumulate in human lung cells

In collaboration with : **Sarah Thomas, Felix Glahn, Gerd Hause, Martin Herzberg, Dirk Dobritzsch**
Martin-Luther Universität Halle-Wittenberg, Germany

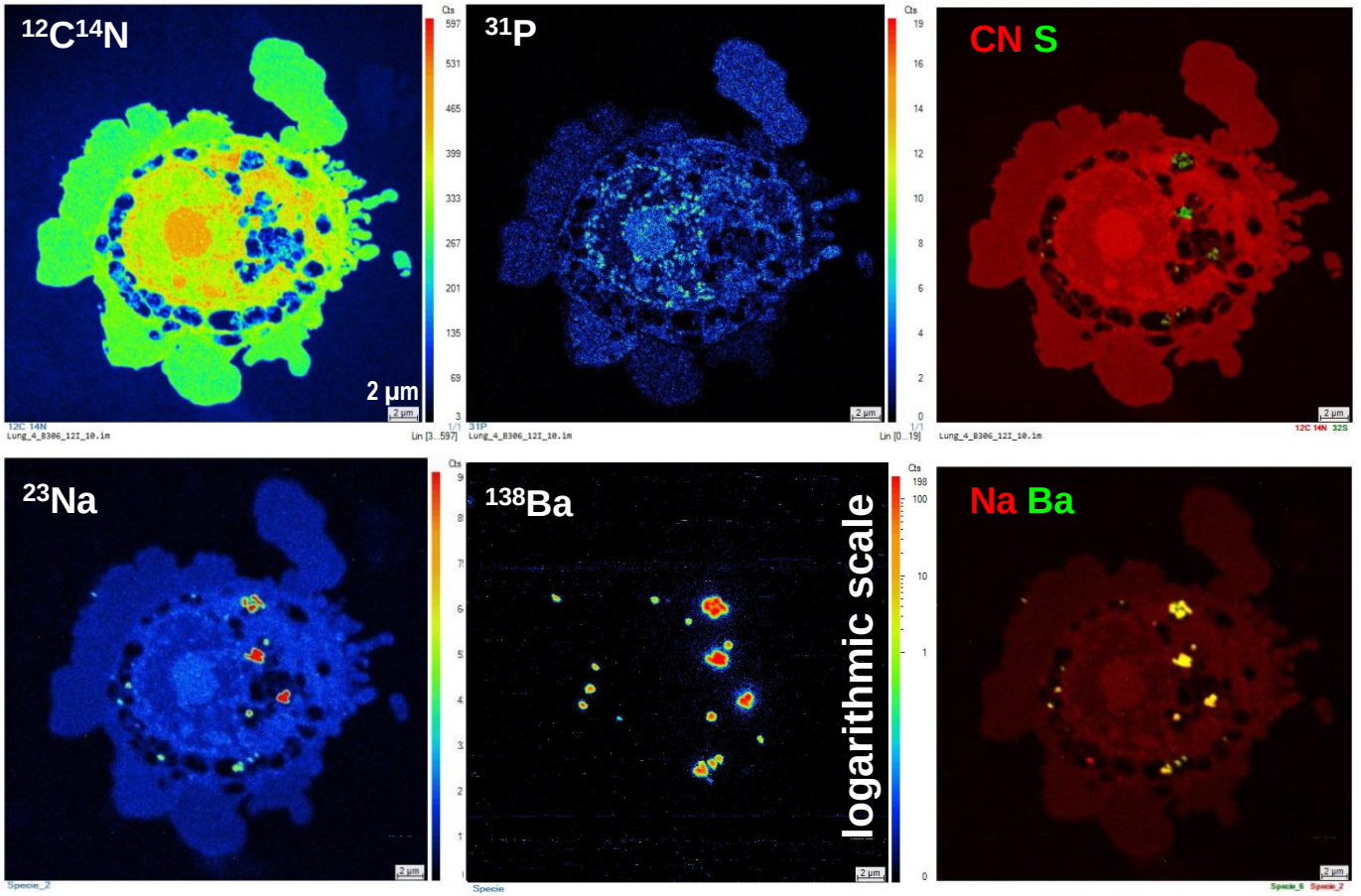
Exposure of cultured normal human bronchial epithelial cells (NHBEC) to $BaSO_4$ nanoparticles

- Human bronchial cells were exposed to 0.1 and 0.01 mg/ml $BaSO_4$ nanoparticles for 72 hours.

0.1 mg/ml $BaSO_4$

Cs^+ source

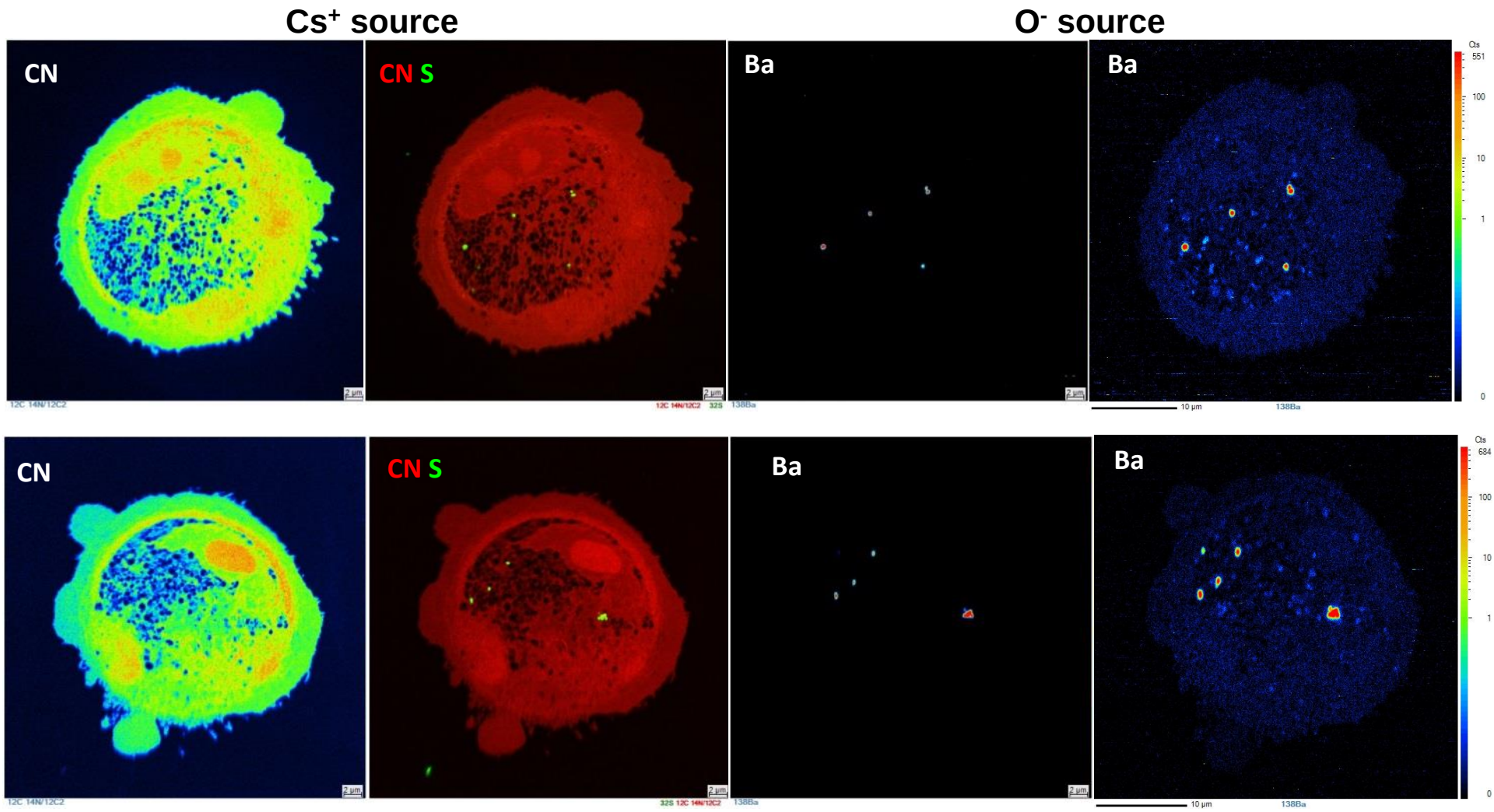
O^- source



Images 35 x 35 µm
Acquisition 45 min
512x512 pixel

➢ **Nanoparticles accumulate in the cytosol, but do not enter the cell nucleus**

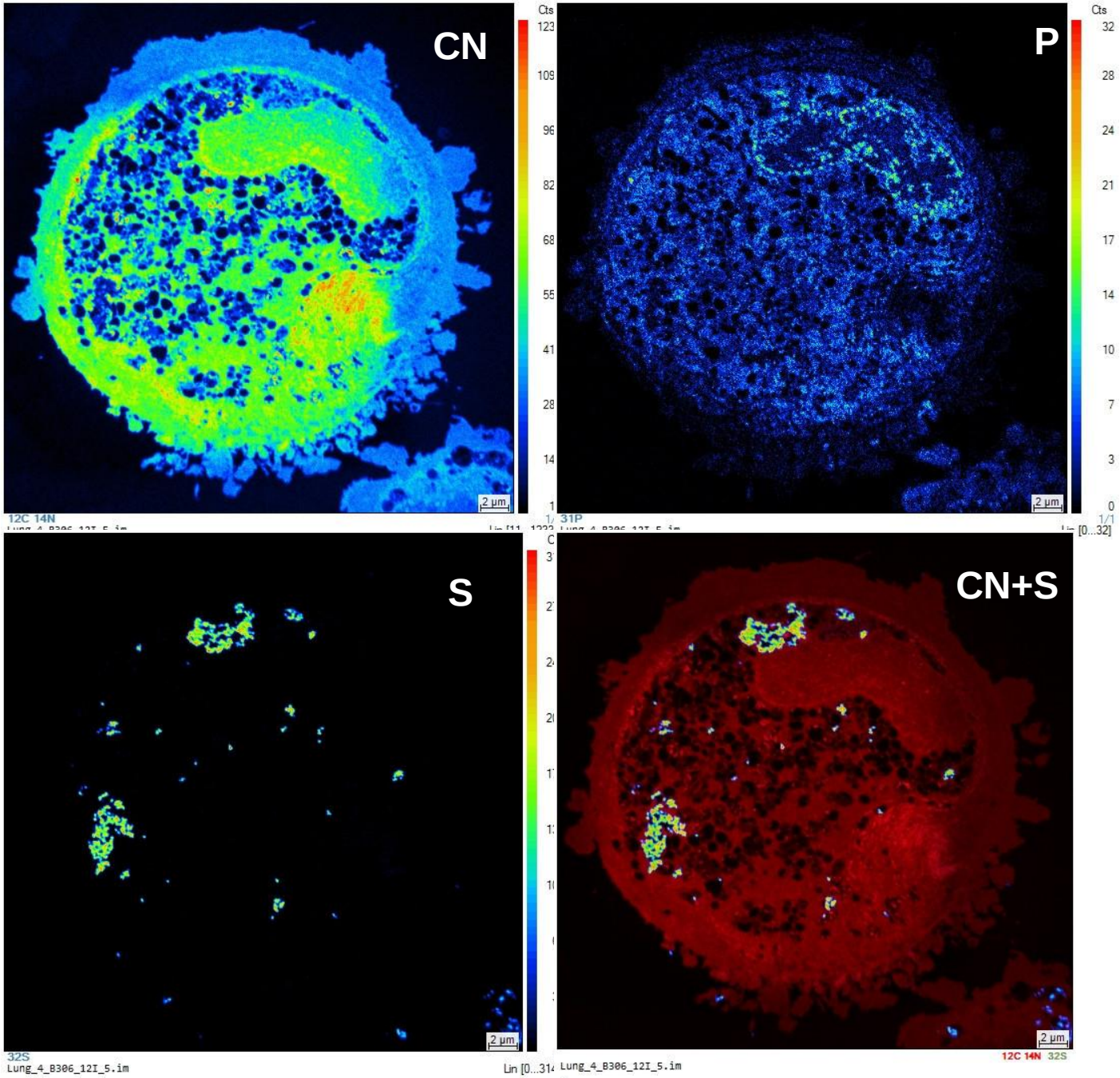
Human bronchial cells exposed to 0.01 mg/ml $BaSO_4$ nanoparticles for 72 hours



- A part of the $BaSO_4$ Nanoparticles are dissolved and distributed in the entire cell
- A rough quantification of the Ba signal reveals: about 30 – 50 % of the Nanoparticles are dissolved, only 3 – 7 % of the dissolved Ba is located around the particles, the majority is distributed in the entire cell.

Cells exposed to $BaSO_4$ nanoparticles, **0.1mg/mL** during 72 h

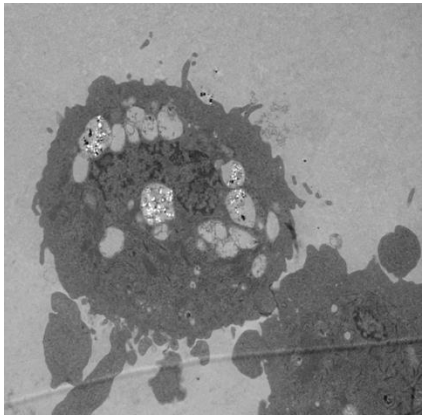
Images 35 x 35 µm
Acquisition 45 min
512x512 pixel

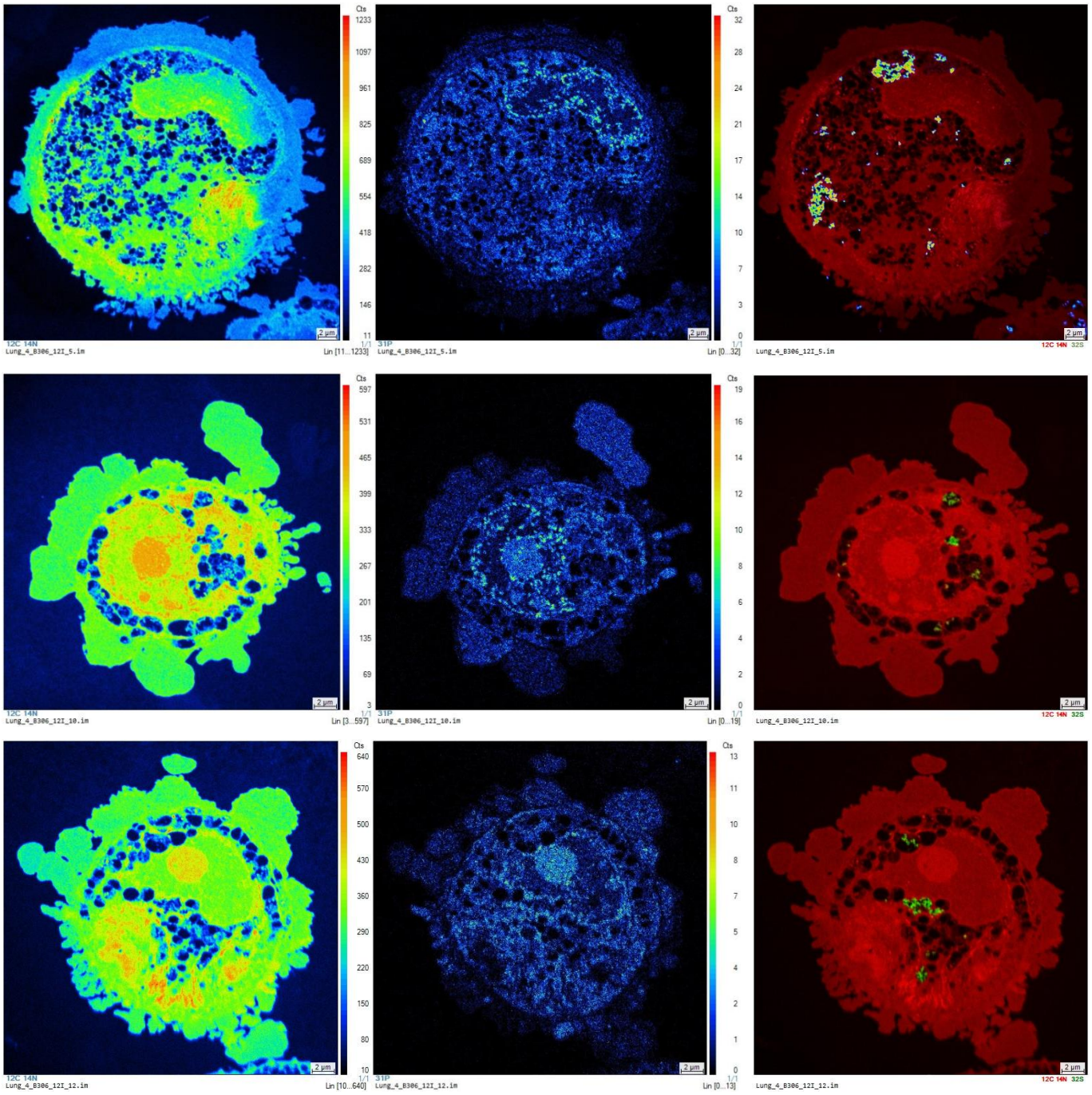


Exposure to 0.1 mg/mL $BaSO_4$ NP:

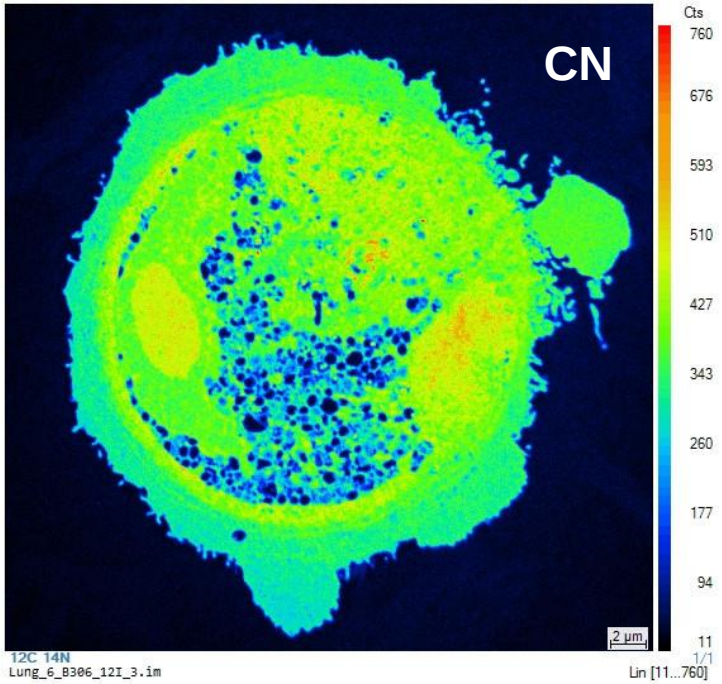
NPs do not enter cell nucleus

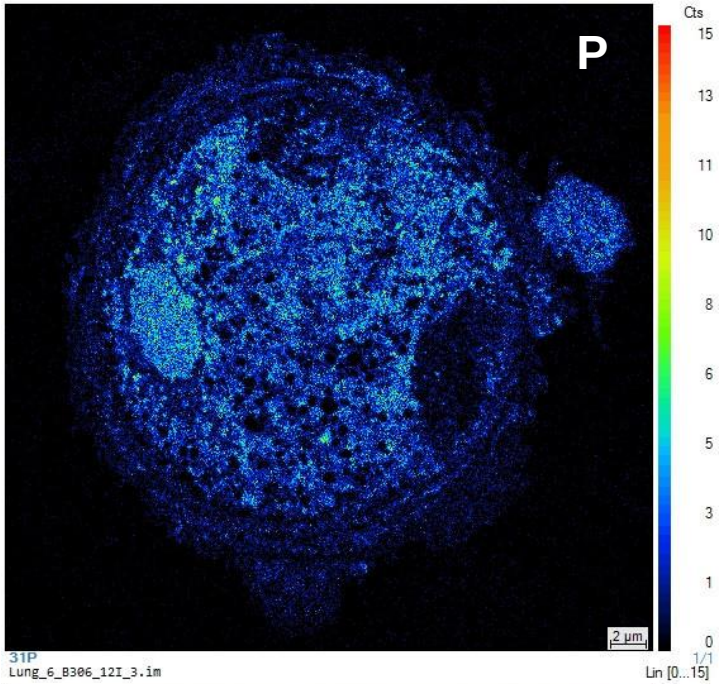
Confirmation by TEM



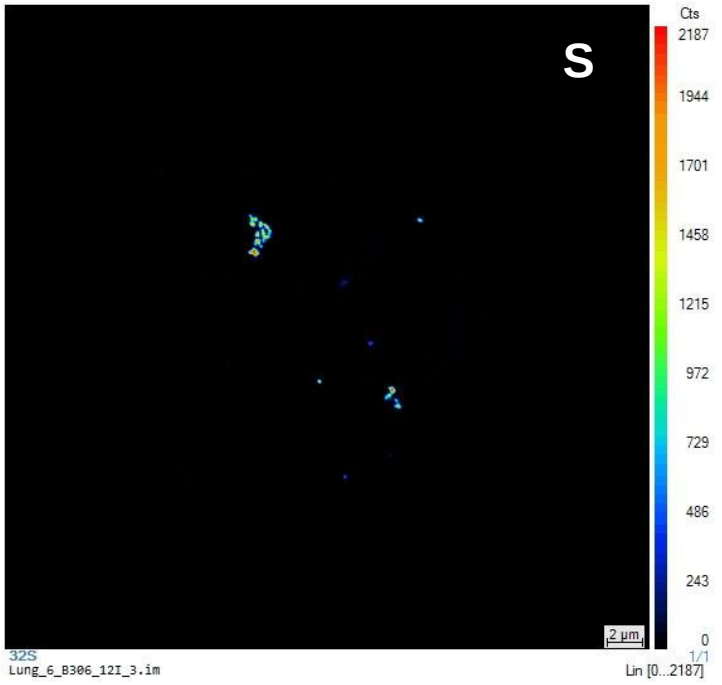


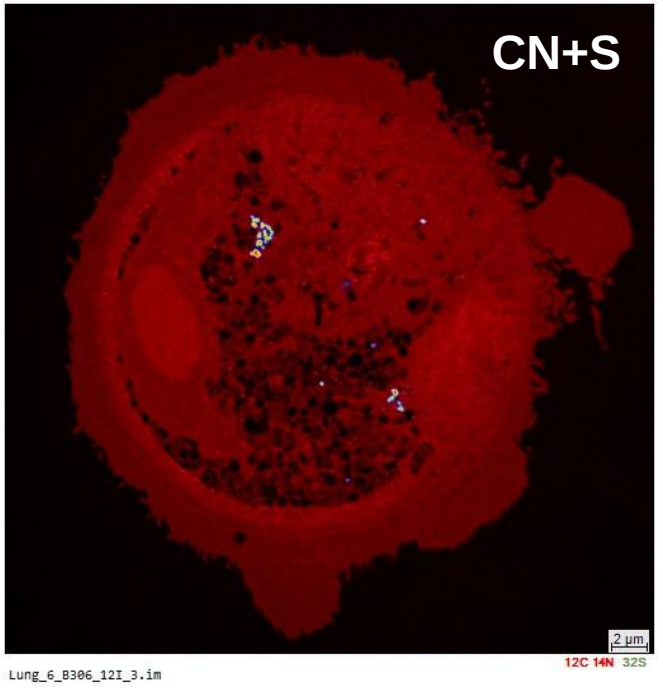
Cells exposed to $BaSO_4$ nanoparticles, **0.01mg/mL** during 72 h





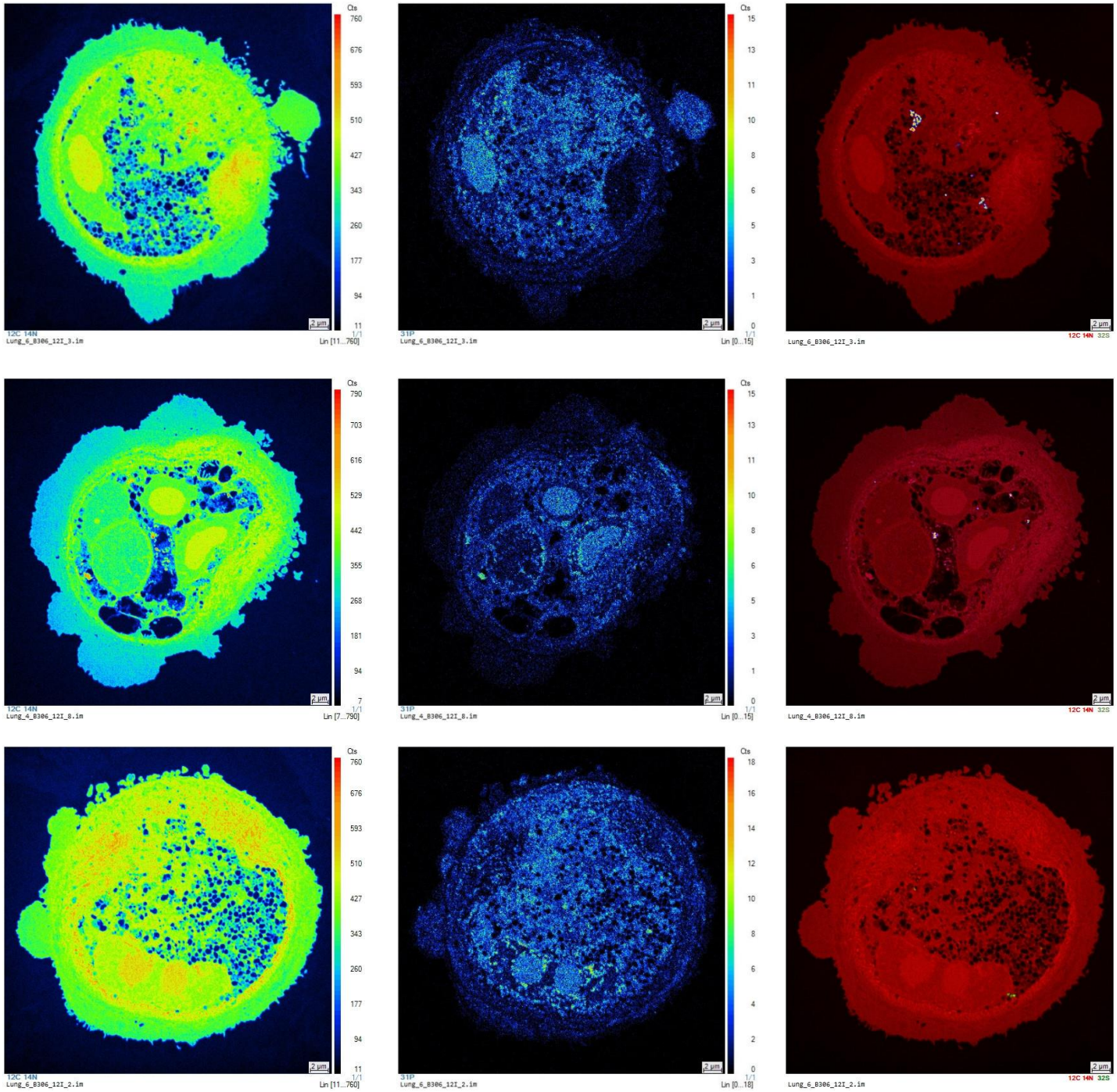
Images 35 x 35 µm
Acquisition 45 min
512x512 pixel





Exposure to 0.01 mg/mL $BaSO_4$ NP:

NPs do not enter cell nucleus



Detection of HgSe particles in whale liver tissue

In collaboration with : **Lhiam Paton, James Hall, Andrew Brownlow, Eva M. Krupp, Jörg Feldmann**
Aberdeen University, UK, and Graz University, Austria

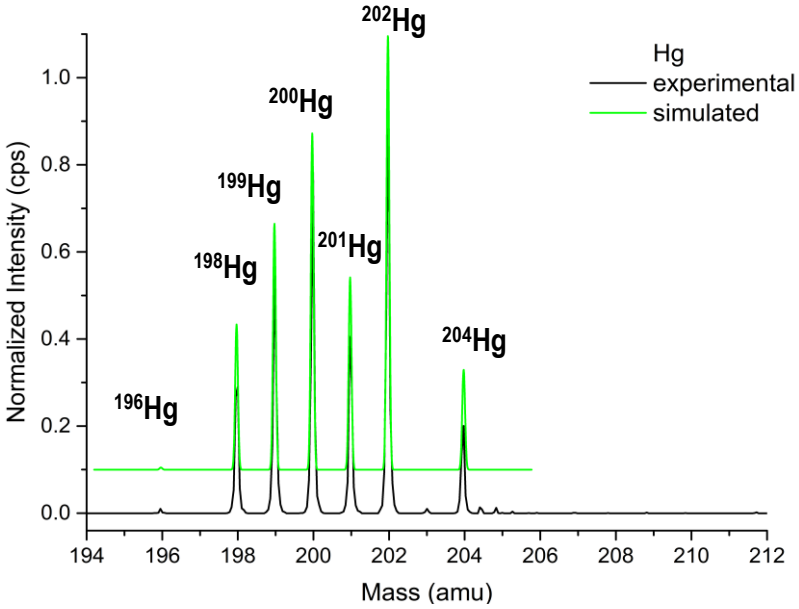
Liver tissue was obtained from a Sperm Whale (*Physeter macrocephalus*) stranded at the Scottish coast.

Ardersier Beach
57°33'47.5"N 4°02'24.5"W

- Many cetacean species such as the sperm whale are known to bioaccumulate excessive concentrations of mercury.
- Selenium can behave in an antagonistic way be reducing the toxic effects of mercury within the mammal by forming insoluble HgSe crystals within cells thus these mammals are able to survive for much longer periods of time.
- Objective was the first NanoSIMS detection of HgSe particles in whale liver. Important method development was necessary for Hg detection by NanoSIMS (**first Hg data at all**).

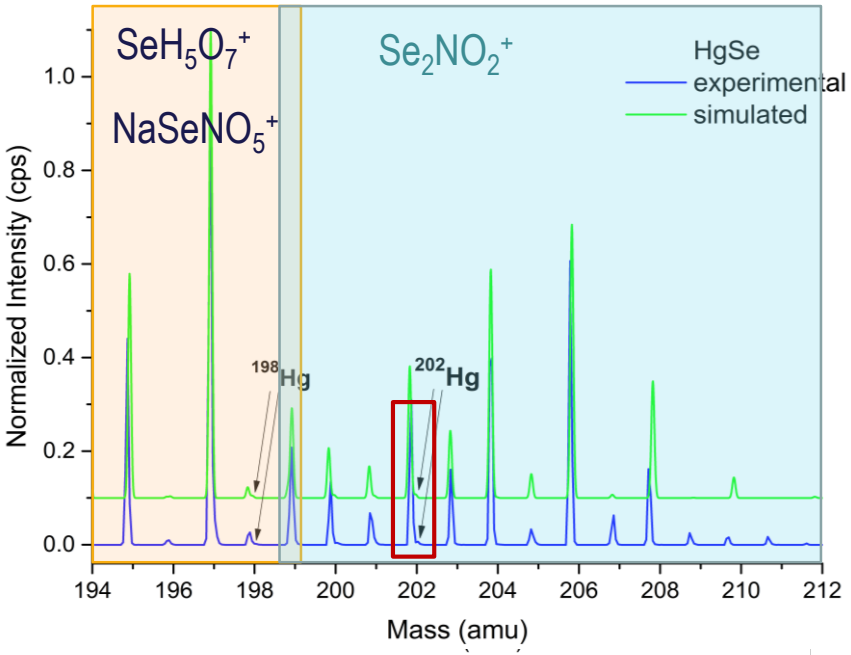
Method development for mercury detection by NanoSIMS

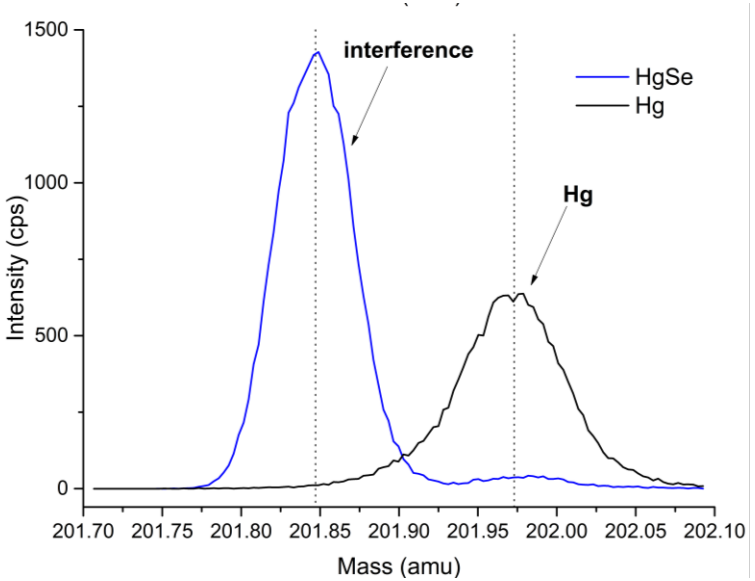
Detection of Hg isotopes in a mercury standard



NanoSIMS spectra obtained by B field scan mass resolution 2500

Analysis of nanocrystalline HgSe



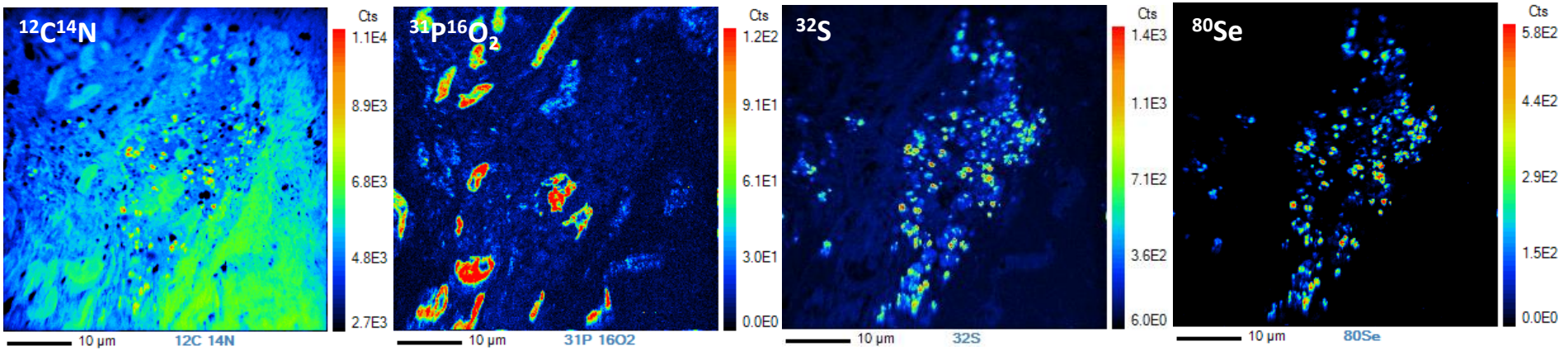


High resolution NanoSIMS spectra obtained by E field scan at the the detector mass resolution 3200, interferences are separated

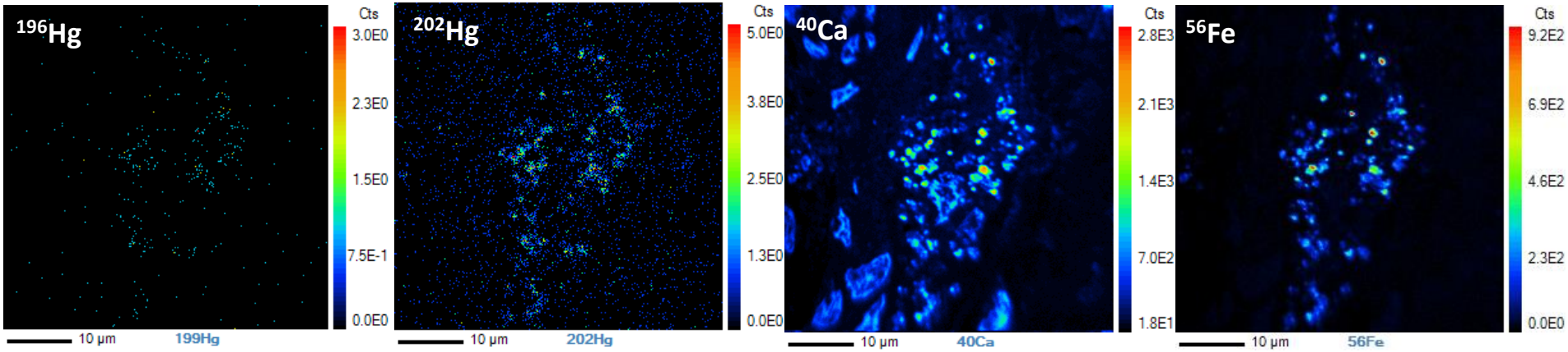
Subirana, MA., Paton, L., Hall, J., Brownlow, A., Krupp, E., Feldmann, J., Schaumlöffel, D. (2021), *Anal. Chem.*, **93,** 12733-12739

Elemental imaging of whale liver tissue

Cs^+ source



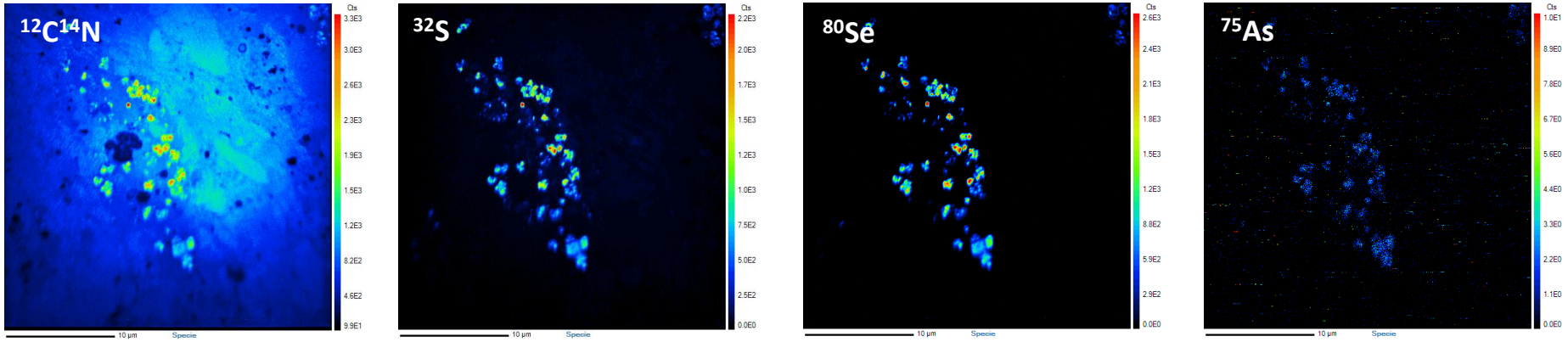
O^- source



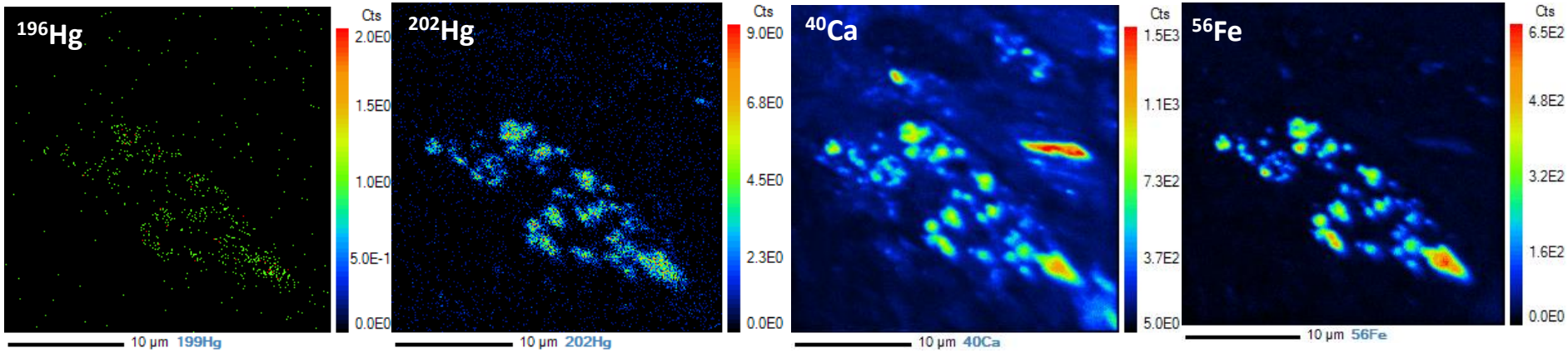
- Hg detection required 18h of signal accumulation
- Accurate Hg detection was confirmed by 2 mercury isotopes
- Hg and Se are colocalized suggesting HgSe particles
- Particles seem to accumulate other metals, too, such as Ca and Fe
- Particles are colocalized with nitrogen and sulfur: implication of proteins?

Elemental imaging of whale liver tissue

Cs^+ source



O^- source



- Second example confirming the observation
- Hg signal is more clear, however, low count rates after 18h of signal accumulation
- In addition, the accumulation of arsenic was observed at the HgSe particles

Conclusions

- Microscopy and element specific imaging techniques can give fascinating insights in samples at the nano- and micrometer level. Beyond the visual impression, the full data matrix should be explored.

- The development of the sample preparation strategy is most important to obtain reliable data, especially for biological samples.

- Electron microscopy provides the highest resolution down to the subnanometer level. In combination with EDS element specific detection is possible, but the sensitivity is limited compared to NanoSIMS:
    - TEM and SEM: higher spatial resolution
    - NanoSIMS: higher sensitivity for element detection

- NanoSIMS enables new applications for nanoparticle localization and trace element detection in cells and tissues:
    - dissolution of barium sulfate nanoparticles
    - first mercury detection by NanoSIMS and HgSe localization

Questions and Discussion ?

Co-funded by the Horizon 2020 Framework Programme of the European Union under the grant N° *952306*