A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***Ion Beam Microscopy***
Subtitle	
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Objective

Ion Beam Microscopy (IBM) techniques, such as micro-resolved proton-induced X-ray emission (μPIXE) and Rutherford backscattering (μRBS), are powerful tools for spatially resolved element analysis. IBM techniques were applied to quantify nanoparticles (NPs) uptake as well as cellular elements at the single-cell level.

Principle of the method

A proton beam (2.25 MeV) is used for scanning the sample in the xy-plane at a resolution of about 1 μm. With the combination of μPIXE and μRBS it becomes possible to determine the concentration of cellular elements (for example, P, S, Ca, K, Zn, and Fe), with a sensitivity in the ppm range. The simultaneous application of μRBS and μPIXE methods delivers unique information on the genuine concentration and distribution of NMs down to the single-cell level. IBM allows visualization and quantification of a wide range of NMs in tissues and cells. Moreover, μRBS can reveal the distribution of NMs in the z-direction with an accuracy of about 100 nm. The method distinguishes between NMs that are internalized and those that are attached on the outside of the plasma membrane, owing to the loss of energy of back-scattered protons from NMs located inside cells.

Equipment & chemicals

1. Ion beam accelerator (LIPSION, 3.5 MV Singletron™, company: High Voltage Engineering Europa B.V.)
2. Metal frame: 2 x 1.5 cm (for culture cells); 3.5 x 2 cm (for lung slices)
3. custom-made plastic holder containing 2 rings for
4. 4μm thick Polypropylene foil (GoodFellow, UK)
5. Carbon tape, double sided (Baltic Präparation e.K., Germany)
6. DePeX (SERVA Electrophoresis GmbH, Germany)
7. Superfibronectin from human plasma (S5171, Sigma, St. Luis, MO)
8. Dulbecco`s Phosphate Buffered Saline (PBS w/o Ca/Mg) (Biowest, France)
9. Methanol
10. Xylol

Settings for IBM measurements

- Ion beam: protons
- Ion beam energy: 2.25 MeV
- PIXE detector: Canberra, Meriden, CT, USA
  consists of Germanium crystal (100 mm^2 active area)
  covered with 60µm polyethylene layer
- RBS detector: Canberra PIPS-detector
- Vacuum chamber: 1 x 10^{-6} Torr
- Scan size: culture cells: 12.5µm x 12.5µm; 24µm x 24µm;50µm x 50µm
  Lung tissue: up to 400µm x 400µm
- Acquisition software: MpSys

Calibration of PIXE detector

ASTIMEX standards were applied for the calibration of PIXE detector. The standards are surface exposed and epoxy embedded mineral compounds covering the energy region up to 18 keV. The smallest possible sample-detector distance of 30.3 mm was adjusted for the calibration procedure to avoid screening of the active surface of RBS detector by the PIXE detector.

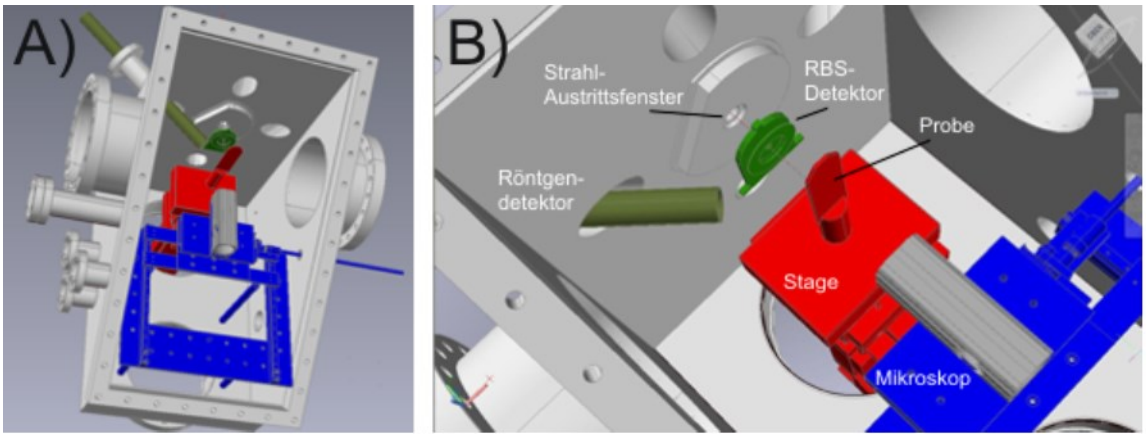

Fig 1 Ion Beam detection setup at Lipsion.

A) Overview of sample chamber structure.

B) Detailed arrangement of detectors and important devices in sample chamber (designed by **Dipl.-Ing. Joachim Starke** by means of Software AutoCAD).

A minimal distance is desirable, because the x-ray yield is reciprocal proportional to the squared distance. Figure 1 shows the experimental setup. The RBS detector detects backscattered protons from the tissue at 174° and the PIXE detector is positioned at 39° for measuring the photon emission.

Proton beam current

The calculation of sample thickness and composition (C, N, O & H) from µRBS spectra, which will be used as input parameter for the subsequent calculation of element concentrations from PIXE, requires at least a charge per sample of 1,0 – 2,0 µC.

An individual adjustment of the beam current and a consideration of the material dependent dead time of detector were required for analyses of culture cells exposed to different NPs. An proton beam current between 400 – 1000 pA should be applied.

The optimum of tissue slice thickness was established for reducing the acquisition time as well. A sample thickness of 7 µm was a good compromise between signal intensity and measuring time by applying ca. 600 pA

Sample preparation

1. Culture cells
   - Preparation of plastic holders: fix polypropylene foil between the two plastic rings without producing folds
   - Sterilization of polypropylene foil: 30 min UV-light & ethanol
   - Coating of polypropylene foil with superfibronectin (5µg/ml in PBS): incubation 2h at 37°C
   - Wash with PBS, cultivation of cells in culture medium (cell density: 75000 cells/ml, 2ml per plastic holder)
   - After NP exposure: wash 3-times with PBS
   - Add ice-cold Methanol (-20°C) at 4°C for 15 min
   - Wash twice with ice-cold Methanol (-20°C), dry sample at room temperature
   - Application of polypropylene foil to metal frames by means of double adhesive and conducting carbon tape, store samples at 4°C

2. Tissue slices
   - Paraffin-embedded tissues: removal of paraffin by means of xylol (3-times, 10min)
   - DePeX embedding
   - Peel off tissue and mount on metal frames with double adhesive carbon tape

Data analysis

Extracted µRBS spectra were analyzed by means of SIMNRA (Matej Mayer, MPI, Germany) to determine accumulated charge, area density (atoms/cm^2) and element matrix composition. These parameters were used as input for µPIXE analysis by means of GeoPIXE software (CSIRO Earth Science and Resource Engineering, Australia) to quantify element concentration.