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Video Article

In Situ Detection and Single Cell Quantification of Metal Oxide Nanoparticles Using Nuclear Microprobe Analysis

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Abstract

Micro-analytical techniques based on chemical element imaging enable the localization and quantification of chemical composition at the cellular level. They offer new possibilities for the characterization of living systems and are particularly appropriate for detecting, localizing and quantifying the presence of metal oxide nanoparticles both in biological specimens and the environment. Indeed, these techniques all meet relevant requirements in terms of (i) sensitivity (from 1 up to 10 $\mu\text{g}\cdot\text{g}^{-1}$ of dry mass), (ii) micrometer range spatial resolution, and (iii) multi-element detection. Given these characteristics, microbeam chemical element imaging can powerfully complement routine imaging techniques such as optical and fluorescence microscopy. This protocol describes how to perform a nuclear microprobe analysis on cultured cells (U2OS) exposed to titanium dioxide nanoparticles. Cells must grow on and be exposed directly in a specially designed sample holder used on the optical microscope and in the nuclear microprobe analysis stages. Plunge-freeze cryogenic fixation of the samples preserves both the cellular organization and the chemical element distribution. Simultaneous nuclear microprobe analysis (scanning transmission ion microscopy, Rutherford backscattering spectrometry and particle induced X-ray emission) performed on the sample provides information about the cellular density, the local distribution of the chemical elements, as well as the cellular content of nanoparticles. There is a growing need for such analytical tools within biology, especially in the emerging context of Nanotoxicology and Nanomedicine for which our comprehension of the interactions between nanoparticles and biological samples must be deepened. In particular, as nuclear microprobe analysis does not require nanoparticles to be labelled, nanoparticle abundances are quantifiable down to the individual cell level in a cell population, independently of their surface state.

Video Link

The video component of this article can be found at <https://www.jove.com/video/55041/>

Introduction

Cellular homeostasis is determined by the uptake control, assimilation, and intracellular localization of different trace elements (ions, metals, exogenous inorganic compounds). These components are frequently in the form of traces, but nevertheless may have a considerable impact in the system physiology. Thus, the study of cell biochemistry in both normal and pathological/stressed situations is a key-step towards an overall understanding of cellular metabolic mechanisms. Therefore, the development of imaging and analytical techniques enabling the investigation of intracellular chemical abundances, structural organization and their related metabolic functions becomes necessary. Very few methods are able to provide an *in situ* quantitative piece of information concerning the overall chemical nature of a given sample. Apart from methods analyzing samples in the bulk form, *in situ* analyses consider biological samples in their integrality without losing mass and structural information, thereby preserving their constituent chemicals (trace elements and ions) and proteins. Furthermore, as the nanosciences continue to develop, improved imaging and analytical methods for environmental monitoring at the cellular scale will be necessary to observe and quantify nano-object behaviors and interactions.¹

Nanoparticles (NPs) have been defined as objects exhibiting at least one facial dimension in the range 1 and 100 nm.² Due to their particular physicochemical properties, NPs are extensively used in industry. NPs are employed in bio-applications and in nanomedicine.^{3,4} Despite the numerous physicochemical characteristics of NPs, they may generate some risks of adverse effects on human health and environment. These risks can be induced by both prolonged and repetitive exposures at various concentration levels and this has not yet been clearly

established.^{5,6,7,8} In particular, the fate of NPs inside cells and the associated cellular responses are, to date, not fully described. This is in part due to the scarcity of methods that allow the detection and quantification of internalized NPs in a single cell.⁹

The classical analytical tools used to estimate the cellular dose of nanoparticles are microscopies, mass spectrometry (MS), inductively coupled plasma MS (ICP-MS)^{10,11} and liquid chromatography MS (LC-MS), but they only provide useful information at the macroscopic scale. None of them can provide a precise evaluation of the subcellular NPs content nor the NPs distribution without the use of fractionation methods. A systematic assessment of the dose-response is thus impossible with these methods, as opposed to methods based on atomic spectroscopy such as nuclear microprobe analysis^{12,13}, synchrotron X-ray fluorescence microscopy¹⁴, and Secondary Ion Mass Spectrometry (SIMS).^{15,16} These methods are particularly interesting as they complement observations made using fluorescence microscopy, especially when NPs cannot be labeled with fluorescent molecules and are thus studied in their native state. To some extent, even when NPs are grafted with fluorophores, (i) quantification remains difficult because the tagging level per NP is unknown and (ii) the chemical modification of the NP surface may modify its cellular distribution.

In this article, we focus on a method based on a combination of nuclear microprobe techniques aiming at imaging the morphology and elemental composition of biological specimens in major, minor, and trace concentrations.

Nuclear microprobe analysis proves to be particularly suitable for the measurement of trace chemical elements in biological tissues. Both the beam lateral resolution (0.3 to 1 μm) and sensitivity in chemical element detection (from 1 to 10 $\mu\text{g}\cdot\text{g}^{-1}$ dry mass) are well suited for studies at the cellular level. Nuclear microprobe techniques are based on particles detection (photons, electrons, ions) emitted after the ion beam (typically running at MeV energies) interacts with atoms present in the sample. Interactions occurring in cells are mainly: 1) excitation/ionization of atoms followed by an emission of photons after atoms return to their fundamental state; and 2) diffusion of incoming particles leading to change in their energy and direction. The measurement of emitted particle energy allows the identification of atoms involved in the interaction. To perform mapping of elements, the ion microbeam is repeatedly scanned over the sample surface, often over an area of about 100 by 100 μm^2 containing several cells. Emitted particles are detected and their energy is recorded for each beam position. Sorting of particles according to the beam position, thus identifying the structure responsible for the emission of such particles is the aim of data treatment. Here, we precisely describe an approach based on fluorescence microscopy and nuclear microprobe analysis to detect as well as to quantify exogenous NPs at the cellular and sub-cellular scales, in order to investigate the consequences of NP interactions with living systems. We shall particularly focus on the opportunities offered by this method in terms of *in situ* quantification of titanium dioxide nanoparticles (TiO_2 NPs) aggregates at the subcellular level.

Protocol

1. Sample Holder Preparation

1. Sample holder design and preparation

1. Manufacture a sample holder by drilling a 5 mm x 5 mm square in a 1-mm thick PEEK frame.
2. Clean by rinsing with ethanol 70% (v/v) and keep in sterile plates until ready to use.

Note. A sample holder appropriate for cell culture and cell handling is required. It needs to be designed for cell culturing, *in vitro* observations with optical microscopy, and nuclear microprobe analysis and imaging. This holder is made of a PEEK frame¹³.

2. Sample holder preparation

1. Prepare the Formvar solution by dissolving 1 mg of Formvar powder in 100 mL of chloroform.
2. Stretch the polycarbonate foil on a metallic frame so that it presents no fold.
3. Use a sterile tip to spread the Formvar solution around the sample holder hole.
4. Put down delicately the sample holder with glue onto the stretched polycarbonate foil (a 5 mm x 5 mm square).
5. Repeat the previous sequence to prepare one sample holder for each replicate.

Note. The polycarbonate (PC) film is biocompatible, thick enough and resistant to the different protocols and analytical constraints.

Caution- Chloroform is toxic. Avoid inhalation, ingestion or contact with skin. Always use a properly functioning chemical fume hood and appropriate filters.

3. Sample holder sterilization

1. Place the mounted sample holder in a conical flask with 50 mL of ethanol 70% (v/v) under agitation at 180 rpm and +37 °C overnight, using an incubator/agitator.
2. Rinse the holder several times in sterile distilled water.
3. Air dry them in sterile conditions and expose them to ultraviolet light (germicidal lamp from Biosafety bench, class II) for 30 min on each side.
4. Keep the samples in sterile 12-well plates (one sample holder per well) until ready to use.

Note: Multiple sample holders can be stored at room temperature in sterile and dry 12-well plates sealed with parafilm for several days.

2. Growth of Cells in the Appropriate Sample Holder.

Caution: Protocol must be carried out in a biosafety laminar flow bench (Class II) to exclude contaminating micro-organisms. Handle antibiotics (e.g. penicillin, streptomycin) with gloves. Respect best practices when handling biological materials (Cell lines, genetically modified derived human cells).

Critical: The cell lines used should be checked to ensure that they are not infected with *Mycoplasma*.

1. Transfect U2OS cell line with plasmid bearing Matrix-roGFP^{17,18,19} using a transfection method in accordance to protocol established by manufacturer.
2. To confirm transfection with the plasmid-carrying the biosensor and to prevent plasmid loss, select and maintain the transfected cells on the appropriate culture medium.
3. Visualize transfected cells by fluorescence microscopy to ensure the transfection has occurred and to confirm the expression of the fluorescence and the display of a reticular and tubular mitochondrial network¹⁹.
Pause point: Cells can be frozen in liquid nitrogen for several years.
4. Use the transfected cell population by culturing the cells in an appropriate medium in order to obtain sub-confluent cell populations. Grow cells at 37 °C, 5% (v/v) CO₂ in a saturated water atmosphere.
5. Seed cells in a concentration, such as they are at 80% confluence the day of fixation. Typically, a concentration of 500 cells per μL could be used. Harvest cells using 400 μL of Trypsin-EDTA 0.25% (v/v) and keep for 3 min at 37 °C. Stop trypsin action with 1 mL of culture medium. Pellet the cells by centrifugation for 5 min at 230 x g and +4 °C, then remove supernatant, and add the desired volume of fresh culture medium.
Note: The protocol is applicable for all cellular types and the number of cells seeded is dependent on the cell size and on cell doubling time.
Caution: Avoid subjecting trypsin, antibiotics, and serum to repeated freeze-thaw cycles. Keep them sterile. Divide the stock solution into aliquots and freeze them at -20 °C. Store the aliquots until the expiration date. Carefully rinse the cell pellet in order to completely remove the trypsin. Traces of residual trypsin will delay and decrease the plating efficiency.
6. Count the cells and perform a dilution with fresh complete culture medium kept at 37 °C in order to obtain a cell suspension with 500 cells per μL .
Note: The concentration of the cell suspension has to be adapted as a function of the cell type, in order to plate a 40 μL drop.
7. Plate a 40 μL drop onto the center of the polycarbonate foil. Carefully place the sample for 2 h at +37 °C, 5% (v/v) CO₂ in a saturated water atmosphere.
Critical: Once the sample is placed in the incubator, limit as much as possible any mechanical movement to favor fast cell attachment. According to the cell type, it could be necessary to incubate cells longer than 2 h for optimal plating efficiency. Check that the incubator atmosphere is well saturated to prevent drops from evaporating.
8. Check that cells are well-attached on the polycarbonate foil using an optical microscope. Gently add 2 mL of fresh complete medium and keep for 24 h.

3. Nanoparticles Preparation and Exposure

NOTE: Fluorescent dye-modified TiO₂ NPs were designed, synthesized, and chemically modified with tetramethyl rhodamine isothiocyanate (TRITC).^{20,21} This surface modification allows nanoparticle detection, tracking and localization *in situ* and *in cellulo* in both living and fixed cells or in multicellular organisms.^{12,13,18}

Caution: Nanomaterials and Nanoparticles must be handled with care. Avoid inhalation, ingestion or contact with skin. To prevent dissemination in air, nanoparticles are maintained in solution (ultrapure water).

1. Prepare a suspension of 1 mg.mL⁻¹ of TiO₂ NPs in ultrapure water. Disperse the TiO₂ NPs using intense 1-min sonication pulses at RT (750 W, 20 kHz, amplitude: 30%) using an ultra-sound generator and a dedicated conical microprobe.
2. Dilute TiO₂ NPs at the desired concentration in the culture medium in order to obtain an exposure suspension of 4 $\mu\text{g} \cdot \text{cm}^{-2}$ (final concentration). Replace medium with the appropriate volume of medium containing NPs on cells and mix gently for homogeneous distribution of the TiO₂ NPs. Prepare control cells similarly without the addition of TiO₂ NPs.
3. Incubate the cell populations for 24 h at 37 °C, 5% (v/v) CO₂ and a saturated water atmosphere.

4. Paraformaldehyde fixation and fluorescence microscopy.

1. Prepare a fresh solution of paraformaldehyde solution (PFA, 4% w/v) buffered in Phosphate Buffer Saline (PBS, pH 7.4).
 1. Dissolve 4 g of PFA powder in 100 mL of PBS. Heat the solution to 65 °C while stirring using a magnet and a magnetic stirrer in a fume hood. Increase the pH by adding 1 M NaOH one drop at a time until the solution clears. Cool the solution to room temperature and adjust the pH to 7.4. Use the freshly prepared solution immediately or keep at +4 °C and protect from light.
Note: Pre-made PFA solutions could be used however an extemporaneous preparation of PFA guarantees better fixation quality and long-term conservation of the fixed samples.
Caution: PFA is toxic; avoid inhalation, ingestion or contact with skin. Always use a properly functioning chemical fume hood and appropriate filters.
2. Once, the incubation time has elapsed, remove the cell culture medium containing the TiO₂ NPs. Rinse the cell populations once with fresh culture medium and once with PBS (2 mL). Remove PBS, rinse quickly with an aliquot of fresh and cold PFA (4% w/v, +4 °C).
 1. Add PFA (2 mL, 4% w/v, +4 °C). Incubate 15 min at room temperature.
 2. Remove the PFA, rinse the cells with PBS (2 mL, 3 times, 5 min) under agitation.
Pause point: These chemically fixed samples can be used for the "plunge-freeze" procedure after fluorescence imaging (go to step 5) or used only for fluorescence imaging (go to step 4.3).
3. Stain the nucleus with Hoechst³³³⁴² incubating the cells for 10 min in PBS. Hoescht³³³⁴² is used at a final concentration of 500 nM. After incubation remove the solution and rinse with PBS.
Caution: Hoechst³³³⁴² is cell permeant and may be toxic. Avoid direct contact, and use gloves while preparing and using Hoechst³³³⁴².
Critical: Ensure that the Hoechst³³³⁴² staining solution is freshly prepared and maintained in sterile conditions.
4. Process fixed samples for *in situ* and single cell imaging using fluorescence microscopy (**Figure 1**).

5. "Plunge-Freezing" Fixation and Dehydration

1. Prepare an aluminum transfer plate by cooling it in liquid nitrogen. Store the plate into a box filled with liquid nitrogen and maintain the plate surface above the liquid in the cold nitrogen vapor.
Note: The transfer plate can be made of any metallic plate (here, we used aluminum) that could be cooled down to liquid nitrogen temperature, and that can enter the freeze-dryer sample chamber.
Critical: The box should be kept closed as much as possible in order to prevent water vapor deposition onto the cold plate surface. The sample stored on the plate during preparation should not be covered by liquid nitrogen.
2. Rinse cells once in culture medium and then twice more, briefly, in sterile and ultrapure water to get rid of any trace of remaining extracellular salts.
Critical: Ensure that the media are freshly prepared and maintained in sterile conditions. Heat all the media at 37 °C before being used. The rinse must be very brief (few seconds) and the excess of liquid on the samples removed as fast as possible.
3. Plunge-freeze the cells at -150 °C in liquid nitrogen-chilled 2-methylbutane during 30 s and place them onto the aluminum transfer plate. Store samples here during the preparation of all other samples. When samples are all cryofixed, transfer all-at-once by placing the transfer plate in a freeze-dryer.
Critical: The overall cryofixation process should not last more than 20 min in order to prevent structural modifications to appear in samples temporarily stored in the transfer box.
4. Freeze-dry the samples using the following sequences: 1) perform a primary desiccation for 12 to 24 h (-99 °C, 10^{-3} mbar) at low pressure and low temperature, then 2) perform a secondary desiccation phase for at least 24 h, increasing the plate temperature to +40 °C while keeping the pressure low (+40 °C, 10^{-3} mbar).
Caution: Liquid nitrogen is extremely cold and can cause severe frostbite or eye damage upon contact. Use adapted bench top containers for transport and wear safety equipment (cryogenic gloves, eye and face protection).
Caution: 2-Methylbutane is extremely flammable. A harmful contamination of the air can be reached rather quickly on evaporation of this substance at +20 °C. Avoid inhalation, ingestion or contact with skin. Use breathing and eye protection, and protective gloves. Always use a properly functioning chemical fume hood. May be stored at +4 °C.
Critical: Use an appropriate thermometer (-150 °C) to monitor the 2-Methylbutane temperatures during the "Plunge-freeze" session. The 2-methylbutane must be kept cool in a liquid phase. Transferring cryofixed samples to the ambient atmosphere may cause cellular damage due to the temperature increase or water vapor condensing on the sample surface. Accordingly, the transfer should be made as fast as reasonably possible (30 s)
Pause point: After cryofixation, samples can be stored at room temperature for several days in sterile and dry conditions (protected from dust and moisture). Carefully handle the samples with fine forceps and place them in a sterile 12-well plate sealed with parafilm. Desiccant can be used to dry the atmosphere.

6. Nuclear Microprobe Analysis

NOTE: Nuclear Microprobe Analysis was carried out at the microbeam line of AIFIRA (*Applications Interdisciplinaires des Faisceaux d'Ions en Région Aquitaine*) using the complementary ion beam analytical techniques Particle Induced X-ray Emission (μ -PIXE) and Scanning Transmission Ion Microscopy (μ -STIM). The facility is based on a 3.5 MV particle accelerator delivering light ion beams in the MeV energy range.^{22,23}

Pause point: AIFIRA is an ion beam facility hosted by the University of Bordeaux that offers an access to national and international teams after scientific evaluation of the proposed experiment.

1. Mount the PEEK sample holders on an apertured plate using double-sided tape.
2. Place the plate supporting sample in a vertical plane perpendicular to beam direction.
3. Close the analysis chamber and vacuum the analysis chamber.
4. Scanning Transmission Ion Microscopy (μ -STIM)
 NOTE: STIM is used to record areal density maps of cells after conversion of energy loss to cellular mass, taking advantage of the fact that the energy loss is proportional to the sample areal density (expressed in $\mu\text{g}\cdot\text{cm}^{-2}$).
 1. Use a 2 MeV Helium (He^+) microbeam as a probe with a size in the focal plane of around 300 nm in diameter and at low fluency (2000 ions $\cdot\text{s}^{-1}$). Measure the energy of the transmitted ions with a planar silicon detector (PIPS detector, 25 mm², 11 keV energy resolution @ 5.4 MeV), placed behind the sample in the beam axis.
5. Particle induced X-ray Emission (μ -PIXE) and Rutherford Backscattering Spectrometry (μ -RBS).
 NOTE: μ -Pixe and μ -RBS analyses provide the spatial distribution and quantification of chemical elements with a sub-micrometer resolution.
 1. Use a 1.5 MeV proton (H^+) microbeam (50-150 pA), focused down to a diameter of 1 μm and scan over the same cells of interest spotted by STIM from 4 to 8 hours and around 100 x 100 μm^2 .
 2. Collect induced X-ray photons emitted from atoms present in the sample (heavier than Na) by a high-resolution Si(Li) solid-state detector (145 eV energy resolution, @Mn-K α) positioned at 45° from incoming beam axis. Use the detected photon energy to identify the nature of emitters (e.g. Z of element) and X-ray intensity to determine the element concentration.
 3. Simultaneously collect back-scattered protons at -135° with a silicon detector (partially depleted detector, 25 mm², 11 keV FWHM @ 5.4 MeV) to measure the total number of incoming particles and to normalize X-ray intensities.
Critical: A collection of certified calibration standards for atomic concentration quantification is used in order to calibrate the X-ray detector response. Reference materials are a collection of thin atomic films deposited on 6.3 μm thick Mylar foils. Emitted X-Ray energies range from 0.6 (Li, K line) to 20.2 keV (Rh, K line).

7. Data analysis

1. Reconstruct chemical elemental maps using the IBA-J plugin for *ImageJ*.²⁴
NOTE: Dedicated software in the form of a plugin for ImageJ has been developed to process raw nuclear microprobe analysis data. Raw data correspond to a list of events arising from particle beam interaction with cells that are recorded along with the beam position as a chronological sequence.
 1. Use the IBA-J plugin to sort events according to their type: transmitted ion energy (STIM), backscattered ion energy (RBS) or X-ray photon energy (PIXE). Then, process separately each type of data.
 2. Calculate STIM area density map
 3. Calculate average X-ray photon energy spectra and define for each chemical element of interest an energy window in order to retrieve the element spatial distribution.
 4. Define regions of interest (ROI) (e.g. individual cells, dense structure, aggregates, etc.) and calculate the corresponding x-ray spectra.
2. Analyze the corresponding RBS spectra in order to calculate the total number of incident particles²⁵.
3. Fit X-ray spectra using the Gupix software²⁶ in order to determine the element concentration for each ROI.
NOTE: Typical limit of detection (LOD) for the method is in the range 1 to 10 $\mu\text{g.g}^{-1}$ in dry mass depending on the atomic number of elements (LOD is decreasing with Z).

Representative Results

Cell culture and fluorescence imaging of fluorescently labeled TiO_2 NPs

We designed a sample holder adapted for cell culture, cell handling as well as multimodal analysis. Specifically, it was important that the holder permitted routine optical microscopy as well as nuclear microprobe analysis and imaging. This sample holder is based on a 2- μm thick polycarbonate foil deposited on a PEEK frame. Cells are directly grown on polycarbonate, in sterile culture conditions for several days and then used for different experimental settings, such as NPs exposure.

The chemical surface modification with fluorophores (TRITC) allowed the detection of the TiO_2 NPs as well as their *in situ* and *in vitro* localization in living or in paraformaldehyde fixed cells using fluorescence microscopy. The cell nucleus and mitochondria were stained using the vital-dye Hoechst³³³⁴² (blue for the nucleus) and the transfected Matrix-roGFP (green for mitochondria), respectively. This multiple staining allowed the intracellular localization of the TRITC- TiO_2 NPs (by its red emitting fluorescence) 20 h after exposure. NPs were only found in the cytoplasm of exposed cells with no detection in the nucleus. NPs were randomly localized in the perinuclear region of the cytoplasm and totally excluded from the mitochondria (no overlapping between TRITC and GFP signals).

Although fluorescence microscopy is very useful to localize NPs inside exposed cells, it is not able to assess the exact number of NPs per cell. The main difficulty of fluorescence microscopy concerning the quantification of NPs is linked to the uncertainty about (i) the number of fluorophores attached to a given NP and the bleaching stability of the chosen fluorophore during the observation and (ii) the aggregation state of the NPs inside the cell.

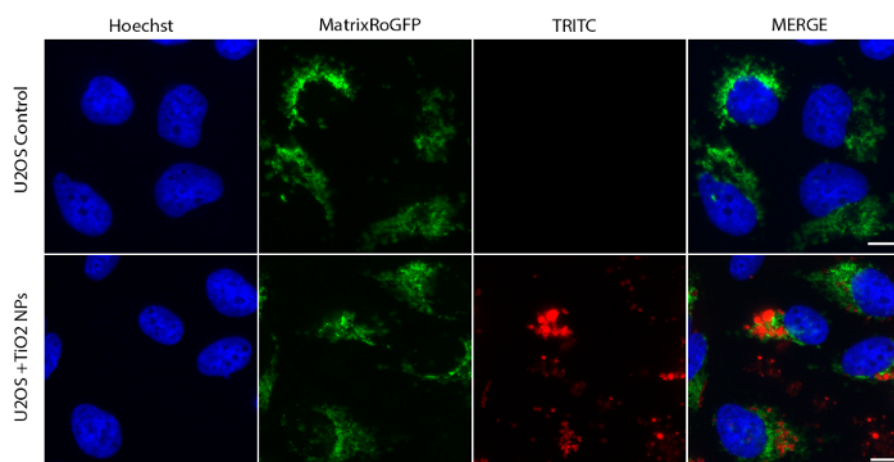


Figure 1: *in vitro* and *in situ* fluorescence imaging of U2OS transgenic cells expressing Matrix-RoGFP and exposed to TRITC- TiO_2 NPs. U2OS cells (top) marked with Hoechst³³³⁴² (blue), Matrix-roGFP (green) are exposed to 4 $\mu\text{g.cm}^{-2}$ TRITC-labeled TiO_2 nanoparticles (red) for 20 h. Observations indicate that TiO_2 NPs aggregate in cells in a perinuclear region. Scale bar: 10 μm . [Please click here to view a larger version of this figure.](#)

To overcome these shortcomings, nuclear microprobe analysis techniques provide a complementary approach to the conventional optical microscopy due to their sensitivity to the NPs which prevents the use of any intermediate signal like fluorescence from a grafted dye. Furthermore, they are also fully quantitative, providing data about (i) the sub-cellular biochemical content that is otherwise unknown, and (ii) the intracellular quantity of NPs at the single cell level.

Cryofixation of cells after live imaging.

The highest constraint in performing nuclear microprobe analysis is to work under vacuum conditions. We have developed a cell fixation protocol enabling the preservation of the biological ultrastructure and the biochemical integrity of the biological specimen. Chemical fixation is known to modify the trace element composition of cells because it requires the replacement of their cellular medium by a polymer used to preserve the cellular ultrastructure. Moreover, the removal of water also releases free ions and other species, which modifies the overall sample composition. Hence, it becomes mandatory to give priority to a physical fixation method, notably cryogenic methods. These cryogenic procedures induce a swift cessation of the cellular activity and this, in the millisecond time scale.

Nuclear microprobe analysis microscopy and quantification of NPs at the cellular scale.

Scanning transmission ion microscopy (μ -STIM) and Particle-induced X-ray emission (μ -PIXE) analysis were performed on samples after cryofixation and dehydration to obtain precise quantitative data on their elemental chemical composition.

μ -STIM images revealed the local differences in density and allows the detection of cell structures such as the nucleus and cytoplasm. Although the beam lateral resolution enables the observation of dense structures as narrow as 300 nm wide, like thin aggregates visible here in the cytoplasm, the STIM methods cannot discriminate between NP aggregates and other dense cellular structures. This is because, like for transmission electron microscopy (TEM), the physical process leading to variation in transmitted energy is the interaction of the incoming ion with the atomic electron cloud. Unlike TEM analysis however, because the entire cell volume is analyzed, local thickness variations prevent discrimination between high-Z structures and a local increase of cellular density.

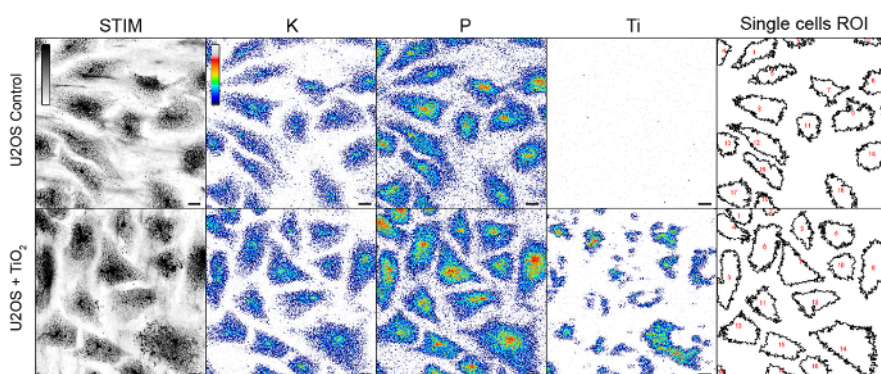


Figure 2: Images of density and elemental distribution obtained by μ -STIM and μ -PIXE on cryo-fixed U2OS cells. U2OS control cells (up) are compared to exposed cryo-fixed U2OS cells (exposed to $4 \mu\text{g}\cdot\text{cm}^{-2}$ TiO_2 NPs, down) and observed using nuclear microprobe analysis/microscopy. STIM microscopy (left, grayscale maps) revealed dense intra- or extra-cellular structures (nucleus, salt aggregates, nanoparticles). The spatial resolution (300 nm) is comparable to fluorescence microscopy and shows structures such as NP aggregates in the perinuclear region. Identification of structures based only on their density may nevertheless be ambiguous. The μ -PIXE elemental maps of K, P and Ti (thermal color scale) are complementary to μ -STIM maps. They can be used to ensure the presence of NPs in cells. Each cell can be individually analyzed in terms of element concentration (see **Figure 3**). Scale bars: 10 μm . Color scales range from minimum (blue) to maximum intensity (gray). [Please click here to view a larger version of this figure.](#)

As illustrated in **Figure 2**, the μ -STIM rendering allows the recognition of individual cells within both a population and also intracellular sub-compartments such as the nucleolus and nucleus. Unfortunately, and as mentioned previously, NPs could not always be detected using μ -STIM reconstruction.

Particle-induced X-ray emission (μ -PIXE) analysis provides not only the chemical composition of the sample but also their elemental mapping (**Figure 2**). During the interaction with the exciting beam, the chemical elements undergo atomic excitation-de-excitation processes that eventually lead to the emission of a photon which exhibits the characteristic energy of the atomic number of the excited element. A characteristic peak spectrum is built from the sum of all the emitted photon events and is considered a chemical signature of the sample.

Standard experimental setups and detectors used for μ -PIXE experiments allow simultaneous quantification of all elements heavier than Na with a 1 to $10 \mu\text{g}\cdot\text{g}^{-1}$ dry mass detection limit. Accuracy in measuring elemental concentrations is usually limited to around 20% due to charge collection and detector efficiencies.

In this study, the distribution of elements like potassium and phosphorus and to quantify the intracellular amount of TiO_2 NPs with titanium mapping is observed by nuclear microprobe analysis. Chemical element maps are computed after the photons are sorted according to the beam position at the timing of recording and the selection of an energy window centered around a specific element. Maps are representative of the number of detected events at the beam position and are quantitative. Both noise and background are numerically simulated and filtered out. Furthermore, chemical mapping can be used to extract the local PIXE spectrum required for quantification.

As illustrated in **Figure 2**, phosphorus is homogeneously distributed in the cell with, as expected, a much higher concentration in the nuclear area. Potassium is homogeneously distributed in the cell volume. Titanium is located in the cytoplasmic perinuclear region in the form of aggregates, as previously observed using fluorescence microscopy (**Figure 1**). NPs displayed the same perinuclear localization whatever their surface state: functionalized (**Figure 1**) or native (**Figure 2**). Meanwhile, no trace of titanium was detected in control cells confirming that the titanium distribution observed by μ -PIXE must be attributed to the TiO_2 NPs, in agreement with our previous analyses by fluorescence microscopy.

In addition, as illustrated in **Figure 2**, it is possible to extract the intracellular distribution of NPs and quantitative NPs concentrations from specific regions of interest. Based on the STIM maps and in correlation with the phosphorus/potassium distributions, single cell analysis is possible.

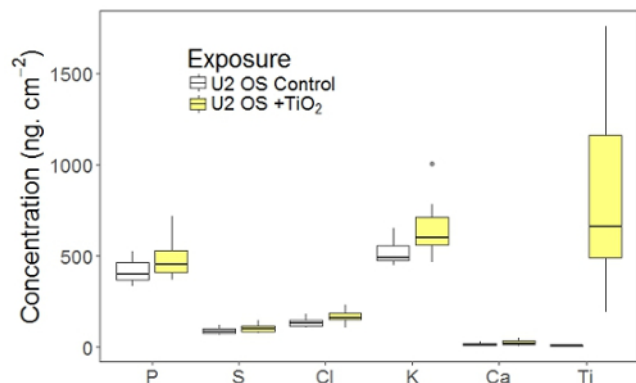


Figure 3: Single cell quantitative analysis using μ -PIXE. Individual X-ray spectra calculated for cells shown in **Figure 2** can be fitted in order to determine the element concentration at the cellular level. This feature is particularly interesting for NP analysis where cellular concentrations usually show strong variations inside the same population. For example, for the same mean exposure, the Ti concentrations range from 0.2 up to 1.8 $\mu\text{g}\cdot\text{cm}^{-2}$. Controls correspond to untreated cell populations. Exposure dose: 4 $\mu\text{g}\cdot\text{cm}^{-2}$. Boxplots represent the distribution of individual cellular concentrations with median value (horizontal line) and bars extending toward lowest and highest measured values. Control cells: N=14; Exposed cells: N=16.

Accordingly, we have not only quantified the average content of titanium in a cell population but also shown the titanium distribution per cell in one population in a specific experimental condition. The median content of titanium measured here is quite low (500 $\text{ng}\cdot\text{cm}^{-2}$) as compared to the 4 $\mu\text{g}\cdot\text{cm}^{-2}$ exposure dose of the cell population (**Figure 3**) and variation between cells is large (Ti concentrations range from 0.2 up to 1.8 $\mu\text{g}\cdot\text{cm}^{-2}$ according to the analyzed cells). We also noticed an increase of intracellular ions such as potassium and calcium in exposed samples suggesting a cellular alteration homeostasis induced by the presence of TiO_2 NPs, as previously described by several authors.^{21,27}

Discussion

We describe a method providing useful information beyond what is possible with other imaging techniques, especially at the subcellular level. In addition to its imaging ability, nuclear microprobe analysis also offers possibilities of quantification of chemical elements entering in the composition of a biological sample. In the present work, we studied human cell populations and focused down to the analysis of a chosen region of interest based on a single cell exposed to TiO_2 NPs. Its combination with other techniques provides both morphological cellular imaging and precise quantitative data on the elemental chemical composition.

To successfully apply nuclear microprobe analysis, it is mandatory to respect the following points to avoid potential pitfalls. First, it is imperative to use a cryogenic protocol for the cell fixation in order to retain its ultrastructure and its biochemical integrity. Second, it is also mandatory to perform analysis of thin samples with thickness below 20 μm . If needed, sectioning of cryofixed samples could be performed. Samples should absolutely be kept frozen. Third, it is also important to keep in mind that nuclear microprobe analysis reveals a two-dimensional representation of biological samples. Thus, the chemical element distribution obtained is a two-dimensional projection that could possibly introduce misinterpretations. This limitation will be bypassed in the future by the use of tomographic acquisition.

Nuclear microprobe analysis also has limitations. It has to be stressed that its main constraint is the need to perform analysis under *vacuum*. Therefore, it is imperative to use cell fixation protocols that preserves cells ultrastructure and biochemical integrity. It is therefore necessary to test the resistance of the biological samples to the cryogenics procedure (without the addition of chemical resin). This could limit the use of nuclear microprobe analysis.

Another limitation is the accessibility to the facilities for carrying out nuclear microprobe analysis. The assigned beam times are therefore sometimes limited, and this is an additional constraint for this kind of time-consuming experiment. Several hours are often needed for one acquisition.

This technique is different from other analytical methods, including microscopy, mass spectrometry (MS), inductively coupled plasma MS (ICP-MS), liquid chromatography MS (LC-MS), and radioactive isotope. The latter are indeed used to estimate the cellular dose of chemical elements but they are only able to provide information at a macroscopic scale *i.e.* on a macroscopic amount of a sample. None of them can give access, as nuclear microprobe analysis does, to a precise detection and mapping of a subcellular dose of specific chemical elements (ions, metal or metal oxide NPs). These data help accessing to a further systematic study of dose-response evaluation.

The precise estimation of the dose when studying the internalization of NPs in cells is crucial from both quantitative NP toxicology and pharmacology points of view. As suggested by the large discrepancy in the observed NP contents, which shows a 10-fold difference between the minimum and maximum observed concentrations (**Figure 3**), the mean cellular concentration might not be a relevant parameter to describe the phenomenon of particle exposure. This is particularly true when a threshold effect is supposed to take place because inhomogeneous dose exposure could lead to contradictory observations. Since the fractionated nature of nanoparticles appears clearly at the cellular level, this study therefore poses again the question of the relevance of methods based on analyzing global variables in addressing questions around the behavior of cells exposed to inhomogeneous doses of contaminants.

As illustrated in the case studied here, observation and quantification of NPs within individual cells allow us to better understand the bioaccumulation of endogenous/exogenous elements such as metal oxide NPs. This is a critical task for further applications of NPs in biomedicine, where a poor understanding of the underlying NP distribution in cells could lead to misinterpreted results.

This protocol highlights the suitability of using nuclear microprobe analysis with other techniques for future assessments of NP interactions with biological specimens. The quantitative approach provides information about the impact of such NPs in terms of detection, identification, localization, and quantification at the level of one single cell.

Disclosures

The authors have nothing to disclose.

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References

1. Krug, H. F., Wick, P. Nanotoxicology: An Interdisciplinary Challenge. *Angew. Chem. Int. Ed.* **50** (6), 1260-1278 (2011).
2. Van Hove, M. A. From surface science to nanotechnology. *Catalysis Today.* **113** (3-4), 133-140 (2006).
3. Le Trequesser, Q., Seznec, H., Delville, M.H. Functionalized nanomaterials: their use as contrast agents in bioimaging: mono- and multimodal approaches. *Nanotox. Rev.* **2** (2), 125-169 (2013).
4. Oberdorster, G. Safety assessment for nanotechnology and nanomedicine: concepts of nanotoxicology. *J. Int. Med.*, 89-105 (2009).
5. Savolainen, K. *et al.* Nanotechnologies, engineered nanomaterials and occupational health and safety - A review. *Saf. Sci.* **48** (8), 957-963 (2010).
6. Savolainen, K., Alenius, H., Norppa, H., Pylkkanen, L., Tuomi, T., Kasper, G. Risk assessment of engineered nanomaterials and nanotechnologies - A review. *Toxicol.* **269** (2-3), 92-104 (2010).
7. Arora, S., Rajwade, J. M., Paknikar, K. M. Nanotoxicology and in vitro studies: The need of the hour. *Toxicol. . Appl. Pharm.* **258** (2), 151-165 (2012).
8. Donaldson, K. Resolving the nanoparticles paradox. *Future Med.* **1** (2), 229-234 (2006).
9. Schaumann, G. E. *et al.* Understanding the fate and biological effects of Ag- and TiO₂-nanoparticles in the environment: The quest for advanced analytics and interdisciplinary concepts. *Sci. Total Environ.* **535**, 3-19 (2014).
10. Olesik, J. W. *Inductively Coupled Plasma Mass Spectrometers. Treatise on Geochemistry.* Elsevier Ltd.: (2014).
11. Krystek, P. A review on approaches to bio-distribution studies about gold and silver engineered nanoparticles by inductively coupled plasma mass spectrometry. *Microchem. J.* **105** (November 2011), 39-43 (2012).
12. Le Trequesser, Q. *et al.* Multimodal correlative microscopy for in situ detection and quantification of chemical elements in biological specimens. Applications to nanotoxicology. *J. Chem. Biol.* **8** (4), 159-167 (2015).
13. Le Trequesser, Q. *et al.* Single cell in situ detection and quantification of metal oxide nanoparticles using multimodal correlative microscopy. *Anal. Chem.* **86** (15), 7311-7319 (2014).
14. Ackermann C.M., Lee S., Chang, C.J. Analytical Methods for Imaging Metals in Biology: From Transition Metal Metabolism to Transition Metal Signaling. *Anal. Chem.* **89**(1), 22-41 (2017).
15. Legin, A. A. *et al.* NanoSIMS combined with fluorescence microscopy as a tool for subcellular imaging of isotopically labeled platinum-based anticancer drugs. *Chem. Sci.* **5**, 3135 (2014).
16. Lanni, E.J., Rubakhin, S.S., Sweedler, J.V. Mass spectrometry imaging and profiling of single cells. *J. Proteomics.* **75**(16), 5036-5051 (2012).
17. Waypa, G. B. *et al.* Hypoxia triggers subcellular compartmental redox signaling in vascular smooth muscle cells. *Circ. Res.* **106** (3), 526-535 (2010).
18. Dooley, C. T. *et al.* Imaging Dynamic Redox Changes in Mammalian Cells with Green Fluorescent Protein Indicators. *J. Biol. Chem.* **279** (21), 22284-22293 (2004).
19. Hanson, G. T. *et al.* Investigating Mitochondrial Redox Potential with Redox-sensitive Green Fluorescent Protein Indicators. *J. Biol. Chem.* **279** (13), 13044-13053 (2004).
20. Chen, X., Mao, S. S. Titanium dioxide nanomaterials: Synthesis, properties, modifications and applications. *Chem. Rev.* **107** (7), 2891-2959 (2007).
21. Simon, M., Barberet, P., Delville, M.H., Moretto P., Seznec, H. Titanium dioxide nanoparticles induced intracellular calcium homeostasis modification in primary human keratinocytes. Towards an in vitro explanation of titanium dioxide nanoparticles toxicity. *Nanotox.* **5** (June), 125-139 (2011).
22. Sorieul, S. *et al.* An ion beam facility for multidisciplinary research. *Nucl. Instr. Meth. Phys. Res., B.* **332**, 68-73 (2014).
23. Barberet, P. *et al.* First results obtained using the CENBG nanobeam line: Performances and applications. *Nucl Instr Meth Phys Res B.* **269** (20), 2163-2167 (2011).
24. Devès, G. *et al.* An ImageJ plugin for ion beam imaging and data processing at AIFIRA facility. *Nucl. Instr. Meth. Phys. Res. B.* **348**, 62-67 (2015).
25. Mayer, M. *SIMNRA User's Guide.*, Report IPP 9/113, Max-Planck-Institut für Plasmaphysik, Garching, Germany, (1997).

26. Campbell, J.L., Boyd, N.I., Grassi, N., Bonnicksen, P., Maxwell, J.A.. The Guelph PIXE software package IV. *Nucl. Instr. Meth. Phys. Res. B.* **268** (20), 3356-3363 (2010).
27. Yu, K., Chang, S., Park, S. J., Lim, J., Lee, J. Titanium Dioxide Nanoparticles Induce Endoplasmic Reticulum Stress-Mediated Autophagic Cell Death via Mitochondria- Associated Endoplasmic Reticulum Membrane Disruption in Normal Lung Cells. *PLoS ONE.*, 1-17 (2015).