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1 **Multimodal correlative microscopy for *in situ* detection and quantification of chemical elements in**
2 **biological specimens. Applications to nanotoxicology.**

3

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17

1 **Abstract**

2 Correlative microscopy is the application of two or more distinct microscopy techniques to the same region of a
3 sample, generating complementary morphological and structural information that exceeds what is possible with
4 any single technique to answer a biological question. We propose an approach based on a multimodal correlative
5 microscopy, *via* two imaging and analytical techniques: fluorescence microscopy (FM) and ion beam analysis
6 (IBA) to investigate *in vitro* nanoparticles (NPs) interactions. Indeed, the explosive growth in Nanotechnology
7 has led to their utilization in a wide range of applications from therapeutics to multimodal imaging labeling.
8 However, the risks for adverse health effects have not been clearly established. Detecting and tracking
9 nanomaterials in biological systems is thus challenging and essential to understand the possible NPs-induced
10 adverse effects. Indeed, assessing *in situ* NPs internalization at the single cell level is a difficult but critical task
11 due to their potential use in Nanomedicine. One of the main actual challenges is to control the number of NPs
12 internalized per cell. The data obtained by both FM (fluorescence microscopy) and IBA (ion beam analysis)
13 were strongly correlated in terms of detection, tracking, and colocalization of fluorescence and metal detection.
14 IBA not only provides the *in situ* quantification of exogenous elements in a single cell but also of all the other
15 endogenous elements and the subsequent variation in their cellular homeostasis. This unique property gives
16 access to dose-dependent response analyses and therefore new perspectives for a better insight on the effect of
17 metal oxide NPs on cellular homeostasis.

18

19 **Keywords**

20 Correlative Microscopy, Ion Beam Analysis, Nanoparticles Detection, Single Cell, *In Situ* Quantification.

21

22

1 **Introduction**

2 Correlative microscopy is usually considered as the application of two or more distinct microscopy techniques to
3 the same region of a defined biological sample, producing complementary morphological, structural and
4 functional information that exceeds what is possible with any single technique to answer a specific biological
5 question (Mironov and Beznoussenko 2009; Caplan et al 2011). The most commonly reliable correlative
6 combination, referred to as “Correlative Light and Electron Microscopy” or CLEM, combines light and
7 transmission electron microscopy. Light microscopy delivers functional information through fluorescent labeling
8 in both living and fixed samples while electron microscopy extends this information through detailed high-
9 resolution images of the same region of interest. Nevertheless, the study of the living is not only limited to
10 genes/proteins/functions and structural information. Indeed, as observed in biological specimens from bacteria to
11 human, metals and trace elements homeostasis play a key role in the cellular regulation physiology. The life of
12 an organism is critically dependent on the proper regulation of uptake, assimilation, intracellular distribution of
13 trace chemical elements, such as ions, metals or exogenous inorganic compounds. These elements, often at the
14 trace level, play a considerable role in physiology and pathology of biological systems. For example, iron is
15 required for oxygen delivery to tissue, the control of cell growth and differentiation and energy metabolism
16 (Andrews 2008; Waldvogel-Abramowski et al 2014). Zinc is involved in immunity, growth and development
17 (Nolte et al 2004), an enzyme cofactor and ensures the correct functioning of over 2000 transcription factors
18 (Takeda 2001; Jeong and Eide 2013). Calcium acts as a crucial second messenger and is involved in various
19 tissular and cellular processes such as muscle contraction (Kurebayashi and Ogawa 2001) and skin homeostasis
20 (Proksch et al 2008; Savignac et al 2014). Thus, the study of the cellular biochemistry in relation to the function
21 and structure in normal or physio-pathological conditions is a critical step toward a complete understanding of
22 the whole cellular metabolic processes. In that sense, it is mandatory to develop imaging and analytical methods
23 to investigate the *in situ* cellular content, the structural organization, and the function.

24 Only few methods give *in situ* and quantitative information about the whole composition of a sample. It is thus
25 essential to develop such a method, which considers biological samples in their whole integrity, mass, structure
26 and chemicals (trace elements and ions) and protein contents.

27 With the development of Nanosciences and Nanotechnologies, there is an increasing need in developing such
28 micro-imaging and micro-analytical method. Nanomaterials or nanoparticles (NPs) are defined as objects with at
29 least one of their dimensions ranging from 1 to 100 nm (Krug and Wick 2011). NPs have unique
30 physicochemical properties such as large surface area per gram, high surface/volume ratio, surface reactivity,

1 charge and shape, which make them extensively used in industry. As their size approaches that of proteins, DNA
2 and other biological species, they are used in bio-applications such as therapeutics (Agostinelli et al 2015),
3 antimicrobial agents (Rai et al 2009), transfection vectors (Jong and Borm 2008; Huang et al 2014) and for
4 fluorescent labeling (Le Trequesser et al 2013; Key and Leary 2014). Nevertheless, besides the numerous
5 physical and chemical advantages exhibited by NPs, the risks for adverse health effects due to prolonged or
6 repetitive exposures at various concentration levels in biological species and in the environment have not yet
7 been clearly established. It has been notified by different public authorities and governments worldwide that the
8 current methods for chemical safety assessments have to be modified and reevaluated to address the particular
9 characteristics of NPs and more especially, to assess the biological effects of these highly reactive materials
10 (Yun et al 2009). In particular, the behavior of NPs inside living cells and the associated specific metabolic
11 responses are, to date, not fully understood.

12 There is a real need for the development of analytical methods, able to *in situ* detect and quantify NPs, whatever
13 their size, nature and surface reactivity and whether they are native or functionalized.

14 Thus, we have developed an approach based on a correlative microscopy *via* the complementarity of two
15 imaging and analytical techniques: fluorescence microscopy (FM) and ion beam analysis (IBA) to detect and
16 quantify NPs at the cellular scale of biological models (from human *in vitro* cells to micro-organisms) and
17 investigate NPs exposure consequences and interactions with the living (Le Trequesser et al 2014a; Le
18 Trequesser et al 2014b). In this article, we will focus on the new possibilities offered by this method in terms of
19 local quantification of particle aggregates at the sub-cellular level.

20

21 **Results**

22 In order to demonstrate the capability of applying such correlative methods to follow NPs at the cellular level,
23 we exposed cells to a 2 $\mu\text{g}\cdot\text{cm}^{-2}$ -TiO₂ concentration and followed NPs in the living state as well as after the
24 fixation required for IBA analysis. To perform these different types of analyses on a same sample, we developed
25 (i) a sample holder dedicated to these experiments, (ii) sample preparations protocols adapted for multimodal
26 analysis and (iii) methods to precisely retrieve a specific region of interest on the different experimental setups
27 and make the correlation observations faster and easier.

28

29 1. Universal sample holder design dedicated to multimodal analysis

1 We designed a specific sample holder suitable for cell culture, cell handling/preparation on such a holder, and
2 multimodal analysis (**Fig. 1a (insert)**). It is designed for biological experiments: cell culture, *in vitro*
3 observations using routine optical microscopy as well as chemical elemental analysis and imaging. This sample
4 holder is made of a PEEK frame covered with a 2 μ m-thick polycarbonate foil. Cells are directly grown on this
5 polycarbonate foil. They are maintained in sterile culture conditions for several days and then used for the
6 different experimental conditions, such as NPs exposure (Le Trequesser et al 2014b). The thin polycarbonate foil
7 gives no X-ray signal during IBA analysis because it contains only C, N and O that produce X-rays that are
8 filtered out by the x-ray detectors. Although polycarbonate foil present local asperities and defaults, it does not
9 preclude its use for cellular observation or impact the quality of cell imaging, using either optical or ion
10 microscopy (**Fig. 1a** and **Fig. 1c**).

11

12 2. Live imaging, cellular compartments identification and NPs tracking

13 Fluorescent dye-modified TiO₂-NPs (TRITC-TiO₂-NPs) were designed, synthesized, and grafted with
14 fluorophores commonly used in biology such as Tetramethyl Rhodamine isothiocyanate (TRITC) (Wessendorf
15 and Brelje 1992). Such a surface chemical modification allows the detection and tracking of the NPs as well as
16 their *in situ* and *in vitro* localizations in living cells using FM (**Fig. 1b**, white arrows). The cell nucleus and
17 specific organelles such as the Golgi apparatus, lysosomes, early and late endosomes were stained using well
18 characterized fluorescent markers, the vital-dye Hoechst³³³⁴² (blue) and the transduced *CellLight*[®] technology
19 (green), respectively. This allowed to assess the intracellular localization of the TRITC-TiO₂ NPs (red), 24 hours
20 after exposure (**Fig. 1b**). NPs were exclusively found in the cytoplasm of exposed cells with no detection in the
21 cell nucleus. Moreover, NPs are randomly localized in the cytoplasm all around the nucleus with a close vicinity
22 to the Golgi apparatus (perinuclear region). NPs can be observed as “free” in the cytoplasm or associated with
23 endosomes (early and late) and lysosomes, in both living and fixed human cells (**Fig. 1b**, **Fig. 2** and **Fig. 3**).

24 TiO₂-NPs displayed the same localization whatever the cell type studied *i.e.* primary keratinocytes (PHFK) (**Fig.**
25 **1b**) and primary endothelial cells (HUVEC) (**Fig. 2** and **Fig. 3**). The combination of light and fluorescence
26 microscopy can discriminate between dense cellular structures and NPs aggregates that appear in cells as black
27 or red dots, respectively (**Fig. 1a** and **Fig. 1b**). Although light microscopy is very informative about the
28 localization of NPs inside exposed cells, it is still not possible to evaluate the exact number of NPs per cell. The
29 main difficulty concerning the quantification of NPs using fluorescence microscopy mainly results from the
30 uncertainty concerning the amount of fluorophores attached to a single NP and its bleaching stability during the

1 experiment. Because they are directly sensitive to the NPs with no need of an intermediate signal like
2 fluorescence of a grafted molecule, IBA methods are interestingly complementary to more conventional optical
3 microscopy. Furthermore, they can also be quantitative, giving access to information about the intracellular
4 biochemical content that is usually unknown.

5

6 3. Cryofixation of cells after live imaging

7 The main constraint in performing ion microscopy is the necessity to perform analysis under vacuum condition.
8 For this reason, we had to develop a protocol for cell fixation enabling to preserve its ultrastructure and its
9 biochemical integrity. Chemical fixation is known to modify the chemical composition of cells by replacement
10 of their cellular medium with a polymer aiming to preserve cellular ultrastructure. At the same time, the
11 evacuation of water releases free ions and species thus modifying its composition. This is the reason why it is
12 appropriate to give priority to physical fixation methods like cryogenic ones. These cryogenic procedures
13 provide rapid cessation of the cellular activity within the millisecond time scale (as compared to several seconds
14 for conventional fixation) (Carmona et al 2008). For sample preparation, we therefore established a two-step
15 protocol in which LM investigations of living cells were performed first, followed by a plunge freezing fixation
16 and dehydration at low temperature without addition of any fixative compounds (see Material and methods).

17

18 4. IBA microscopy and quantification of nanoparticles at cellular scale

19 After cryofixation and lyophilisation, the samples were analyzed by IBA to obtain precise quantitative data on
20 their elemental chemical composition. Contrast in Scanning Transmission Ion Microscopy (μ -STIM) images is
21 due to local differences in density and allow the detection of cell structures such as nucleolus and cytoplasm
22 (**Fig. 1c**) (Barberet et al 2011). Comparing Figures 1a and 1c, the nucleolus can be easily detected suggesting
23 that this nuclear region is dense. In the cytoplasm, “vacuoles” can be easily defined from both *in vitro* analysis
24 and cryofixed sample. Unfortunately, NPs suggested as dark dots on phase contrast imaging (**Fig. 1a**) could not
25 always be seen using μ -STIM rendering. This is because STIM contrast is not specific of NPs but to average
26 local thickness. As cells are seen through the polycarbonate foil, changes in support thickness or density are
27 liable to confusion with cellular structures. This limit can eventually be compensated by a better spatial
28 resolution (100-200 nm) of the beam compared to optical microscopy (200-400 nm).

29 Particle-Induced X-rays Emission (μ -PIXE) analysis provides both the chemical composition of the sample
30 (PIXE spectrum, **Fig. 4**), and the elemental maps of chemical elements (**Fig. 1d-f**). During the interaction with

1 the proton beam, the chemical elements present in the cells, follow a schematic excitation-desexcitation process
2 that eventually ends up with the emission of a photon with an energy characteristic of the atomic number of the
3 excited element. The sum of all the emitted photon events builds a characteristic peak spectrum (**Fig. 4**), usually
4 considered as a chemical fingerprint of the sample. The usual experimental setups and detectors used for PIXE
5 experiments allow quantifying at the same time all the elements heavier than Na with a 1 to 10 $\mu\text{g}\cdot\text{g}^{-1}$ of dry
6 mass detection limit. Accuracy in measuring elements is usually driven by charge collection and detector
7 efficiencies and is about 20%.

8 We used ion microscopy to observe the distribution of cellular elements like phosphorus and potassium and to
9 quantify the intracellular amount of TiO_2 NPs with titanium mapping. Chemical element maps are computed
10 after sorting photons according to the beam position at the time of recording and using an energy window
11 centered around a specific element (**Fig. 4**) Maps usually represent the number of detected events at the beam
12 position and can be quantitative; noises and background can be filtered out. Inversely, chemical element maps
13 can be sorted in order to retrieve the local PIXE spectrum required for quantification.

14 Here, phosphorus is found homogeneously distributed in the cell with a higher concentration in the nuclear area
15 (**Fig. 1d and Fig. 2d**). Potassium (**Fig. 1e**) and sulfur (**Fig. 2e**) are homogeneously distributed in the cytoplasm.
16 Titanium is located in the cytoplasmic perinuclear region as aggregates (**Fig. 1f and Fig. 2f**), as previously
17 observed using LM and FM (**Fig. 1a and Fig. 1b**). NPs displayed the same perinuclear localization whatever
18 their surface state *i.e.* functionalized (**Fig. 2a**) or native (**Fig. 2f**). The average content of titanium in the cell
19 presented here is very low ($5.6 \text{ ng}\cdot\text{cm}^{-2}$) compared to the $2 \mu\text{g cm}^{-2}$ exposure dose of the cell population (**Fig. 4**).
20 In the aggregates found around the nucleus, the number of particles varies from 200 NPs to 9,000 NPs, giving
21 locally exposure dose as much as twice the average exposure dose (**Fig. 5**). In the meantime, no trace of titanium
22 was detected in control cells confirming that the titanium distribution observed by μ -PIXE has to be attributed to
23 the TiO_2 NPs (**Fig. 1f**, white arrows) and in good agreement with our previous observations by conventional FM
24 (**Fig. 1b**). In addition, μ -PIXE reveals additional NPs sites of accumulation not detected by FM. The cellular
25 internalization of NPs has already been shown by high-resolution 3D imaging using confocal microscopy with
26 fluorescent modified NPs and TEM analysis on ultra-thin sections (Simon et al 2011; Stefančíková et al 2014).

27

28 **Discussion**

29 IBA can provide useful information that other imaging techniques are not able to do, especially at the sub-
30 cellular level. Indeed, beyond its imaging ability, IBA also provides a quantification of chemical elements

1 constituting the sample under study, whatever its nature. In this case, it can analyze biological samples and focus
2 on a chosen region of interest of a single cell. Its combination with other techniques provides both a
3 morphological cellular imaging and precise quantitative data on the elemental chemical composition of the
4 sample. These techniques present the following advantages (i) a sample preparation which does not require
5 chemical fixation, staining nor sectioning; (ii) large observable areas with the option to focus on a given region
6 of interest (ROI) of the sample; (iii) the quantification of the chemical elements content with a sensitivity of a
7 few μg per gram ($\mu\text{g}\cdot\text{g}^{-1}$); (iv) the analysis of the overall cell volume with a sensitivity to cell density changes
8 which allows the identification of not only the different cell compartments (nucleus, nucleolus, cytoplasm), but
9 also any dense structure with a size comparable to that of the beam diameter (about 300 nm spatial resolution).
10 As shown in the example here, this ability to observe and quantify NPs within individual cells allows addressing
11 the particular questions of bio-accumulation and bio-persistence of endogenous/exogenous chemical elements
12 such as metal oxide NPs.
13 This is a crucial challenge for further applications in biomedicine. The accurate determination of dose when
14 studying NPs internalization in cells is essential for quantitative NPs toxicology and pharmacology. Up to now,
15 most of the known methods cannot accurately reflect the concentration of internalized NPs in a single cell. There
16 are numerous analytical tools, including microscopy, mass spectrometry (MS), inductively coupled plasma MS
17 (ICPMS), liquid chromatography MS (LC-MS) and radioactive isotope which are used to estimate the cellular
18 dose of NPs but provide information at a macroscopic level. None of them can provide as IBA techniques a
19 precise identification of cellular dose as well as distribution giving access to a further systematic consideration of
20 dose-response assessment. Moreover, many inorganic NPs suffer from a lack of fluorescence in the optical
21 regime and cannot be followed by light microscopy in their native state. To overcome this point, NPs are often
22 functionalized with fluorescent dyes but (i) quantification remains difficult because the level of tagging of a
23 single NP is usually unknown, and (ii) the chemical modification of the surface of a NP may alter its cellular
24 distribution as compared to that of a bare one.

25 **Conclusions**

26 This work highlighted the suitability of using IBA with other techniques in a correlative study concerning the
27 future assessments of NPs interactions with living cells. The correlative approach gives information on the
28 impacts of these NPs in terms of detection, identification, localization and quantification at the single cell level
29 of both native and chemically modified NPs. Cells were grown on a single specific sample holder allowing the

1 successive *in vitro* analysis of human cells (PHFK and HUVEC) exposed to TiO₂ NPs with FM and IBA. The
2 set-up allowed a dual analysis with *in vitro* and cryofixed images of the same set of cells. The procedure used to
3 prepare the samples was chosen as to avoid the resin embedding thus allowed to study by IBA the ion
4 homeostasis alteration due to the exposure to NPs. Both techniques, FM and IBA, gave corroborative results
5 showing that the fluorescent functionalization of NPs to see them is not a mandatory process and that any sample
6 can be observed whatever its state surface. This then opens the field to toxicological study on native NPs (not
7 functionalized). IBA main interest here is that it provides critical data concerning the local quantification of all
8 the chemical elements including exogenous ones (metals and metal oxides) when present in regions of interest.
9 As such, this specific technique may become a much useful tool as compared to more classical approaches for
10 two reasons: (i) the latter are based on chemical analysis and mass spectrometry which provide information at
11 the macroscopic level; (ii) IBA can provide new reply to the problematic of dose-dependent response analyses
12 and a better understanding of metal oxide NPs toxicity by a follow-up of the concentration of endogenous
13 elements.

14

15 **Materials and Methods**

16 **Synthesis of fluorescent dye-modified TiO₂-NPs.** TiO₂ P25 AEROXIDE[®] nanoparticles kindly provided by
17 Degussa/Evonik consist in a mixture of anatase (80 %) and rutile (20 %) phases (specific surface area of 55 m².g⁻¹,
18 25 nm ±10 nm). Their surface modification was performed according to published procedure (Simon et al.,
19 2011). Briefly, 600 mg of TiO₂-NPs were mixed with 3 mL of ammonium hydroxide 25 % (v/v), 100 µL 3-
20 aminopropyltriethoxysilane and absolute ethanol and stirred at room temperature for 48 h. The suspension was
21 then heated at 100 °C for 2 h under reflux. The white powder was then washed 5 times with absolute ethanol.
22 The powder was then added to a 30 mL of Na₂CO₃ (0.01 M) aqueous solution containing 2.5 mg of
23 tetramethylrhodamine-isothiocyanate (TRITC) and stirred for 48 h. NPs were finally washed several times with
24 a Na₂CO₃ (0.01 M) solution and then with milliQ water.

25 **Cell exposure.** Primary human foreskin keratinocytes (PHFK) or human umbilical vein endothelial cells
26 (HUVEC), derived from normal human tissue, were obtained from Invitrogen (Cergy Pontoise, France). They
27 were respectively grown in Defined Keratinocyte-SFM or M200 complemented with LSGS (Gibco, Life
28 Technologies) with 100 mg.mL⁻¹ penicillin/streptomycin at 37°C in a 5% (v/v) CO₂ humidified atmosphere.
29 Passages are realized at 80% confluency. The suspensions of fluorescent dye-modified TiO₂-NPs were prepared
30 ultrapure water, at a final concentration of 1 mg.mL⁻¹. Suspensions were then sterilized *via* autoclaving, and

1 hereby known as “stock suspensions”. TiO₂-NPs were dispersed by high intensity sonication at room
2 temperature (RT) using a probe for 1 min before cell exposure. Stock suspensions were diluted at the appropriate
3 concentration in defined culture medium in order to obtain exposure suspension at 2 µg.cm⁻² (final
4 concentration).

5 ***Labeling of cellular organelles and nucleus of PHFK and HUVEC.*** Before NPs exposure, cells were exposed
6 to the *CellLight*[®] BacMan reagent of interest (40 µL) as recommended by the suppliers (Life Technologies).
7 Here, we used *CellLight*[®] BacMan reagent to label specific organelles such as the Golgi apparatus, lysosomes,
8 early and late endosomes. *CellLight*[®] technology is a modified baculovirus, non-replicating in mammalian cells,
9 and expressing a fusion construct of proteins targeted to specific intracellular structures and a green fluorescent
10 protein (GFP). After the desired time of incubation with 2 µg.cm⁻² fluorescent dye-modified TiO₂-NPs, cells
11 were rinsed 3 times with fresh culture medium.

12 For confocal microscopy acquisitions, cells were fixed with 2 % (w/v) paraformaldehyde (Sigma, St Quentin-
13 Fallavier, France) in PBS (pH 7.4, without Ca²⁺ and Mg²⁺, Invitrogen, Cergy Pontoise, France) for 30 minutes at
14 4°C. Then, a cell permeabilization was performed using a solution containing 0.2 % (v/v) Triton X-100 (Sigma,
15 St Quentin Fallavier, France) in PBS for 15 min. Cells were then rinsed 3 times with PBS and nuclei stained
16 with 10 µM Hoechst³³³⁴² in PBS. Hoechst³³³⁴² is a vital DNA stain that binds preferentially to A-T base-pairs.
17 Slides were mounted using Prolong Gold Antifade Reagent (Invitrogen, Cergy Pontoise, France) and visualized
18 on Leica DMRE TCS SP2 AOBS confocal microscope.

19 For correlative microscopy experiments, cell culture medium was then supplemented with Hoechst³³³⁴² at a final
20 concentration of 10 µM. The cells require no permeabilization for labeling, but do require good physiologic
21 conditions since the dye internalization is an active process. Cells were then incubated at 37 °C for 1 h and
22 rinsed 3 times with fresh medium to remove any excess of Hoechst³³³⁴². The cells were then observed before and
23 after freeze-drying using an optical microscope Zeiss AxioObserver Z1 (Carl Zeiss MicroImaging, GmbH). EC
24 Plan-Neofluar 20x/0.50 Ph2 M27 objective was used for phase contrast and fluorescence imaging. Fluorescence
25 imaging was performed with different excitation/emission filters according to the considered fluorochrome
26 (GFP, Rhodamine, Hoechst³³³⁴²).

27 ***Cell preparation for high-resolution ion beam microanalysis (IBA).*** Cells were cultured directly onto ion beam
28 microprobe sample holders as adapted from previous studies. This sample holder is composed of a 2 µm-thick
29 polycarbonate films tightly stuck on a PEEK support (PolyEther Ether Ketone). Polycarbonate film was chosen
30 for its biocompatibility and mechanical properties. In addition, this polymer is transparent and shows high

1 resistance to ion beam without stopping it. PEEK, a colorless thermoplastic organic polymer, resistant to thermal
2 degradation (suitable for numerous cycles of sterilizations by autoclaving) as well as to attack by both organic
3 and aqueous environments, is very robust and widely used in bio-medical applications (medical implants). The
4 polycarbonate film was chosen for its biocompatibility and for its ability to resist to different protocols and
5 analytical constraints. In addition, polycarbonate is a transparent polymer, which also shows a high resistance to
6 ion beam without stopping it. The choice of the support in PEEK was essential to allow multi-treatments and
7 multi-analyses on the same sample without degradation. Briefly, cells were directly grown at high confluence on
8 2 μm -thick polycarbonate foil for 24 hours in appropriate culture medium, and then exposed to TiO_2 -NPs for 24
9 h. Control cells were prepared similarly with no addition of TiO_2 -NPs. Cells were rinsed twice in culture
10 medium, rinsed in phosphate saline buffer (PBS) and very briefly rinsed in ultrapure water to remove excess of
11 extracellular salts from PBS. Finally, cells were plunge-frozen at $-120\text{ }^\circ\text{C}$ into liquid nitrogen chilled
12 isopentane, and freeze-dried at $-35\text{ }^\circ\text{C}$ for 96 h.

13 ***Single cell recognition during multimodal analyses.*** Correlative microscopy is limited by the ability to perform
14 single cell recognition and identification in the separate microscopes (Liv et al 2013). Considering the size of a
15 cell, relocating a sample requires a precision of a few tens of micrometers or below. The use of the same
16 microscope stage for the different analysis would in principle solve this problem, but this is not possible because
17 each microscope has different requirements in terms of dimensions, positioning, vacuum, alignment, *etc.* For
18 this reason, using a coordinate system relative to the sample holder is more appropriate. In our case, living cells
19 were observed and their position recorded using images of the complete sample holder ($5\times 5\text{ mm}^2$) by LM/FM
20 microscopy. This was possible using a motorized microscope stage (AxioObserver Z1, Carl Zeiss
21 MicroImaging, GmbH) that enables fast tiled acquisition and provides stitching procedures for post-acquisition
22 large-scale image reconstruction. In such a way, cells of interest could be identified individually either relative to
23 any other cell or to the frame. The operation was repeated after freeze-drying in order to maximize our ability to
24 retrieve cells because lyophilization modifies cell opacity. The interest of imaging the entire cell frame is that it
25 enables to retrieve cells on any type of microscope. On the nuclear microprobe set-up, a procedure of alignment
26 of the ion beam with the online visible light microscope is performed with a motorized sample stage with sub-
27 micrometer accuracy in sample positioning. This enables to spot any cell either by moving the sample or by
28 moving the beam.

29 ***Ion beam microanalysis (IBA).*** Chemical element imaging was carried out at the microprobe beam line of
30 AIFIRA using complementary ion beam analytical techniques (μ -PIXE, μ -RBS and μ -STIM). The facility is

1 based on a 3.5 MV (HVEE, The Netherlands) Singletron particle accelerator delivering light ion beams in the
2 MeV energy range. For Scanning Transmission Ion Microscopy (μ -STIM), a 2 MeV He^+ beam was used as a
3 probe with a size in the focal plane around 300 nm in diameter and at low fluence ($2000 \text{ ions}\cdot\text{s}^{-1}$). The energy of
4 the transmitted ions is measured with a planar silicon detector (Canberra PIPS detector, 25 mm^2 , 12 keV energy
5 resolution @5.5 MeV), placed at 0° on the incoming beam axis. For μ -PIXE and μ -RBS, a 1.5 MeV H^+
6 microbeam, focused down to a diameter of about 1 μm , was scanned over areas of interest using a fast
7 electrostatic scanning system. Measurements on narrow areas containing a limited number of cells (usually 1 to
8 10) were performed. This spatial resolution enabled to discriminate the cytosol and nuclear areas of the cells.. In
9 the case of μ -PIXE, typical beam currents in the range of 50–150 pA were used. Induced X-rays emitted from
10 light ions present in the sample (from Na to Ti) were collected by a high-resolution Si (Li) solid-state detector
11 (Oxford Instruments, 145-eV energy resolution, @Mn-K α) positioned at 135° . Simultaneously, back-scattered
12 protons were detected at -135° by a silicon detector (Canberra, partially depleted detector, 25 mm^2 , 12 keV
13 resolution @ 4.5 MeV). To provide elemental maps, 256×256 pixels matrices were calculated from μ -PIXE
14 measurements. A simple energy windowing was used to select the X-ray lines and construct yield maps from
15 events stored in the list files. For the extraction of quantitative results, X-rays (μ -PIXE) and backscattered
16 particles energy spectra (RBS) were obtained from the regions of interest. This operation was carried out using
17 the Supavisio software. X-ray spectra were fitted using the Gupix software and the RBS spectra were treated
18 using the SIMNRA software.
19
20

1 **Author Contributions**

2 The manuscript was written through contributions of all authors. All authors have given approval to the final
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4

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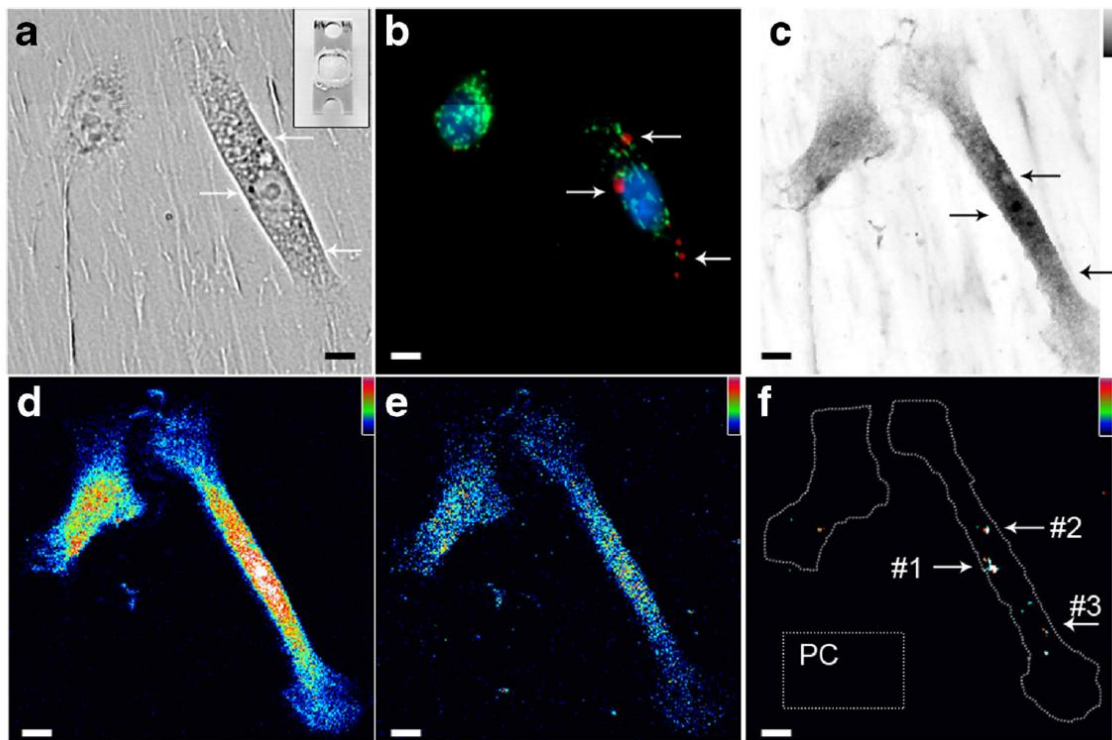
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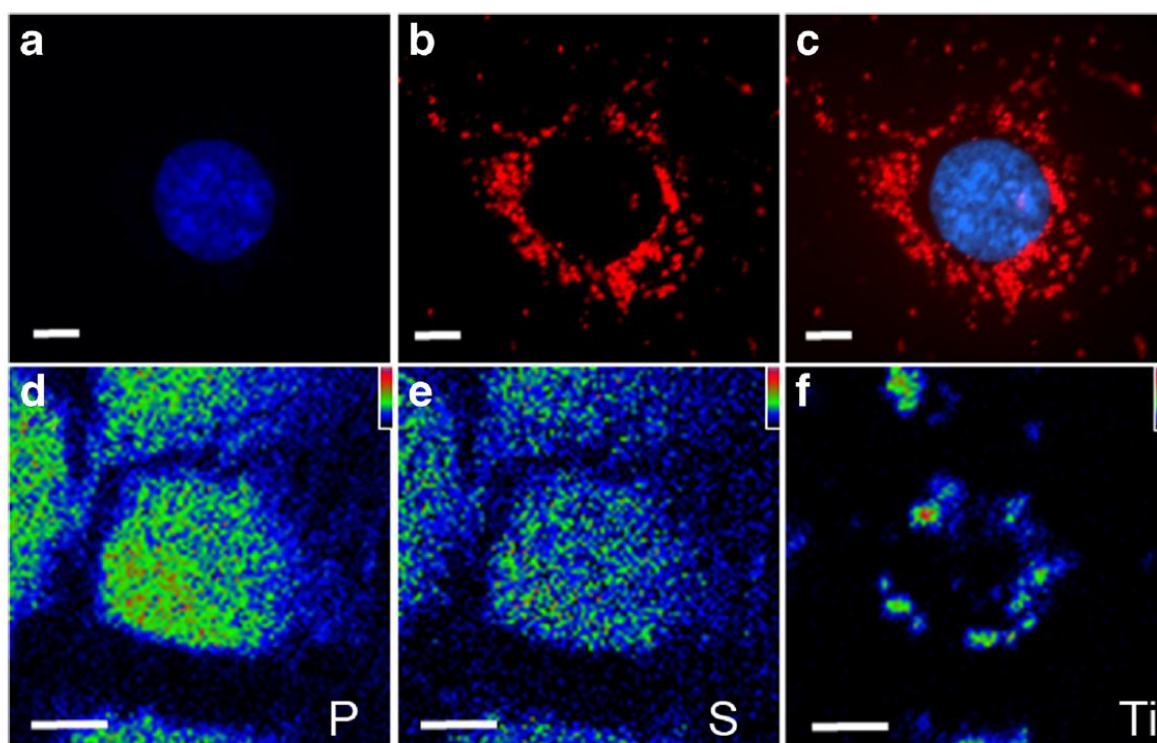
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Figures Captions



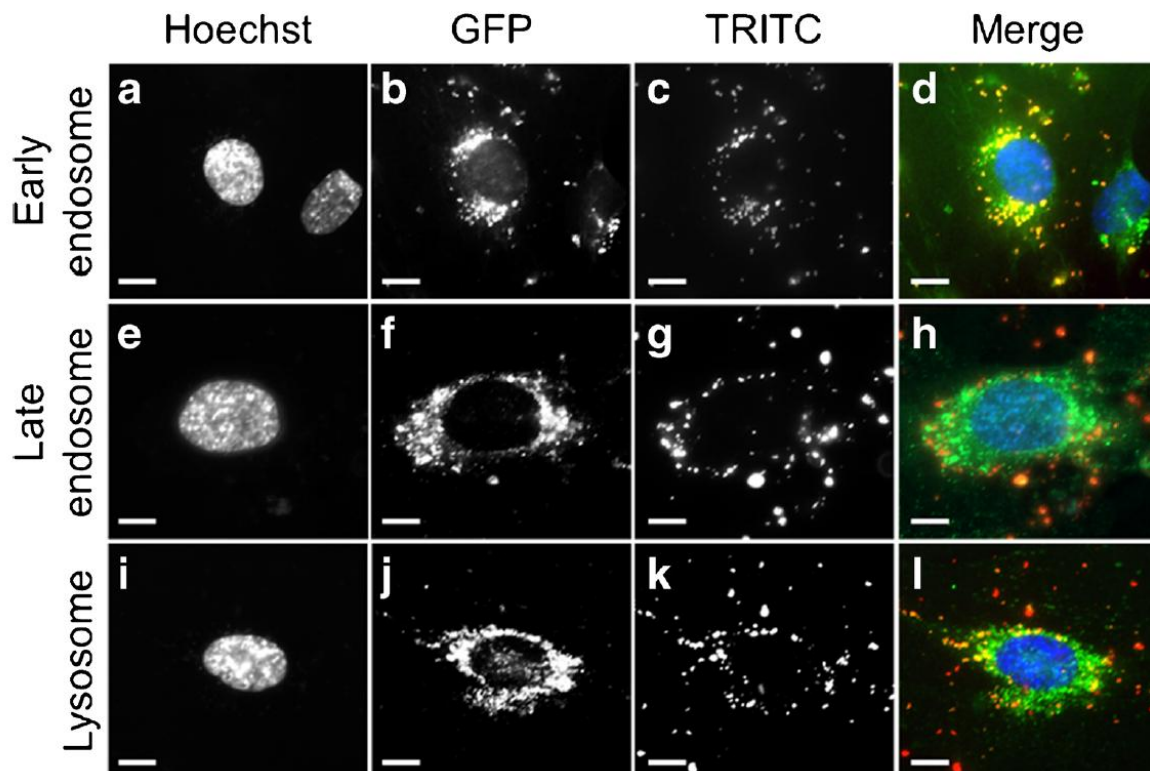
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Fig. 1 Correlative microscopy: from *live in vitro* microscopy to IBA. **(a)** Bright field image of primary keratinocytes plated on 2 μm -thick polycarbonate foil, (insert: picture of the sample holder) **(b)** *in vitro* fluorescence imaging of TRITC-TiO₂ NPs loaded cells. Hoechst³³³⁴² (blue) counterstains DNA. TRITC-TiO₂ NPs are shown in red and the Golgi apparatus (*CellLight@Golgi-GFP*) is displayed in green. **(c)** μ -STIM performed on the same freeze-dried cells once cryofixed and lyophilized. Density scale ranges from white (minimum) to black (maximum). **(d, e, f)** μ -PIXE maps of phosphorus, potassium and titanium, respectively. The arrows locate the NPs under the different techniques. Element yields shown in pseudo-color ranging from black (minimum) to pale gray (maximum). Scale bars: 10 μm .



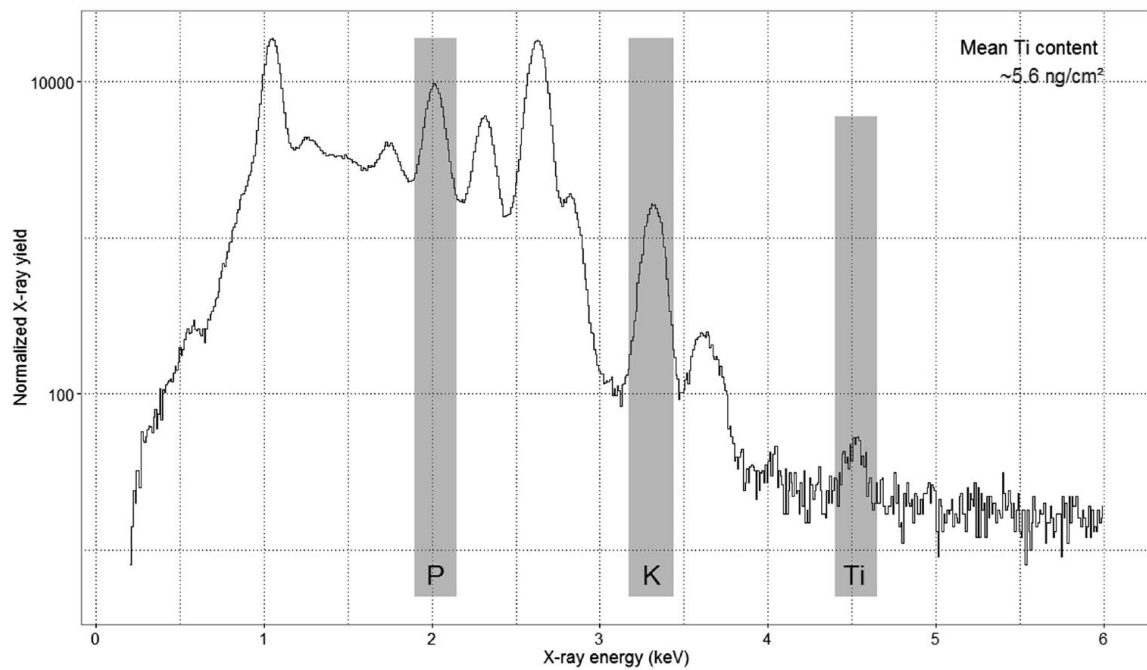
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 2 **Fig. 2** HUVEC cells exposed to $2 \mu\text{g}\cdot\text{cm}^{-2}$ of TiO_2 NPs (**a, b, c**) Confocal fluorescence imaging of loaded cells.
 3 Hoechst³³³⁴² (blue) counterstains DNA and TRITC- TiO_2 NPs are shown in red. (**d, e, f**) μ -PIXE maps of
 4 phosphorus, sulfur and titanium elements, respectively. The two microscopic techniques revealed an exclusive
 5 perinuclear localization of the TiO_2 -NPs. Element yields shown in pseudo-color ranging from black (minimum)
 6 to pale gray (maximum). Scale bars: 10 μm .

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2 **Fig. 3** TRITC-TiO₂ NPs subcellular localization. HUVEC cells were transduced to label (a-d) early endosomes,
3 (e-h) late endosomes and (i-l) lysosomes (green staining). Hoechst³³³⁴² (blue) counterstains DNA and TRITC-
4 TiO₂ NPs are shown in red. TRITC-TiO₂ NPs display no specific localization in the different organelles tested.
5 NPs associated with the considered tagged organelles are depicted in yellow, “free” NPs are detected as red dots.
6 Scale bars: 10µm.

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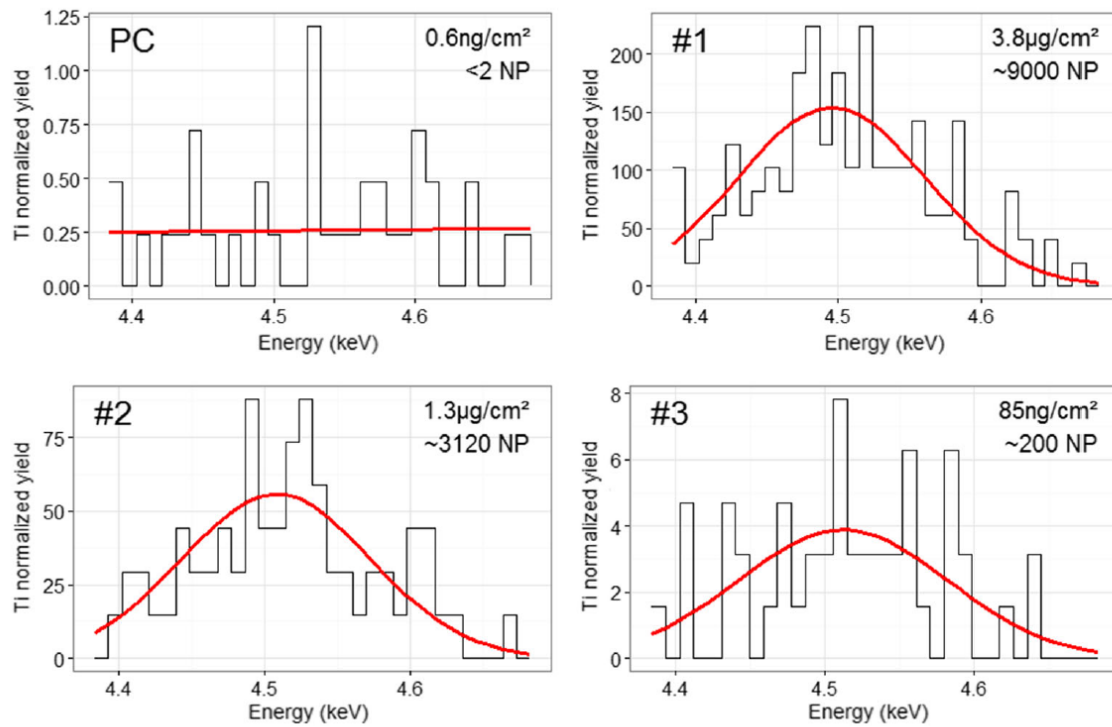
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2 **Fig. 4** Single cell μ-PIXE spectrum corresponding to cell showed in **fig. 1** (right). Energy windows (grays)
 3 around peaks of phosphorus, potassium and titanium were used to compute the distribution of elements shown
 4 **on fig. 1d, 1e and 1f**. Quantification of elements indicates that the average Ti content in this cell is very low
 5 compared to average exposure of cells ($2 \mu\text{g}\cdot\text{cm}^{-2}$).

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1

2 **Fig. 5** Ti peaks extracted from regions of interest on Ti map (**Fig. 1f**) corresponding to polycarbonate support
 3 (PC) taken outside the cell of interest and cellular aggregates #1, #2 and #3 as shown in **Fig. 1f**. Peak yield
 4 fitting (red) enables to convert peak intensity into Ti concentration and calculate the equivalent number of NPs
 5 present in the dedicated region of interest.

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