nffa.eu

PILOT 2021 2026

DELIVERABLE REPORT

WP14 JA4 – A safe-by design platform for nanomaterials

D14.1
Definition of relevant safe-by-design protocols for access by users

Due date

M8

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

PROJECT DETAILS

PROJECT ACRONYM	PROJECT TITLE
NEP	Nanoscience Foundries and Fine Analysis - Europe| PILOT

GRANT AGREEMENT NO:	FUNDING SCHEME
101007417	RIA - Research and Innovation action

START DATE

01/03/2021

WORK PACKAGE DETAILS

WORK PACKAGE ID	WORK PACKAGE TITLE
WP14	JA4 - A safe-by design platform for nanomaterials

WORK PACKAGE LEADER

Hiram Castillo Michel (ESRF)

DELIVERABLE DETAILS

DELIVERABLE ID	DELIVERABLE TITLE
D14.1	Definition of relevant safe-by-design protocols for access by users

DELIVERABLE DESCRIPTION

This deliverable describes a set of workflows aiming for studying the interactions between ENMs and biological systems with an advanced 'safe by design' experimental platform.

DUE DATE	ACTUAL SUBMISSION DATE
M8 (31/10/2021)	

AUTHORS

Amenitsch H., R. Ciancio (CNR), E.T. Capria and H. Castillo Michel (ESRF), P. Colpo (JRC), S. Dal Zilio (CNR), T. F. Keller (DESY), C. Isaxon (Univ. Lund), D. Petrovykh, I Pinto, (INL) E. Stratakis (FORTH).

PERSON RESPONSIBLE FOR THE DELIVERABLE

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

Emmanuel Stratakis (FORTH)

NATURE

R – Report

DISSEMINATION LEVEL

☒ P - Public

☐ PP - Restricted to other programme participants & EC: (Specify)

☐ RE - Restricted to a group (Specify)

☐ CO - Confidential, only for members of the consortium

REPORT DETAILS

ACTUAL SUBMISSION DATE
08/11/2021

NUMBER OF PAGES
29

FOR MORE INFO PLEASE CONTACT

Hiram Castillo Michel (ESRF) hiram.castillo_michel@esrf.fr

VERSION	DATE	AUTHOR(S)	DESCRIPTION / REASON FOR MODIFICATION	STATUS
1.0	15/10/2021	Amenitsch H., R. Ciancio, E.T. Capria, H. Castillo Michel P.Colpo, S. Dal Zilio, T. F. Keller, C.Isaxon, D.Petrovykh, I Pinto, E. Stratakis.		Final
2.0	02/11/2021	Amenitsch H., R. Ciancio, E.T. Capria, H. Castillo Michel P.Colpo, S. Dal Zilio, T. F. Keller, C.Isaxon, D.Petrovykh, I Pinto, E. Stratakis	Contributions added	Final updated
2.1	08/11/2021	E. Stratakis	Minor revions	Submitted

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

CONTENTS

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

1 INTRODUCTION

Nanotechnology and nanomaterials are key players in the EU research and innovation processes having a tremendous impact on many domains of applications ranging from electronics, textiles, cosmetics, agriculture, and food to health applications. The next generation of nanomaterials i.e. smart nanomaterials is expected to represent a step forward by providing nanomaterials with new properties and functionalities and thus adding values to existing products and technologies with high benefits for human health and environment. Consequently, a large increase in the development and use of new kinds of engineered nanomaterials (ENMs) is expected in the near future. To favour safe innovation there is an urgent need for robust, standardized and advanced methods for nanomaterials safety assessments.
ENMs safety assessment remains a challenging task due to the complexity of nanomaterials since they may display a wide range of properties including size, shape, surface functionalisation, surface charge, that affect their way of interacting with biological systems such as biological fluids (protein-surface interactions) and living cells (cellular uptake, mechanisms of toxic response).
This report describes a set of workflows aiming for studying the interactions between ENMs and biological systems with an advanced 'safe by design' experimental platform.
The workflows consist in integrated sequences of steps for guiding users with different comprehensive analysis methods among a wide range of facilities available within the NEP consortium. This platform will support materials scientists to design new or improved materials with a safer approach from the early stage of ENMs synthesis and nanosafety researchers to elucidate the interactions and effects of ENMs with biological samples (human, animal or plant cells) in order to understand the life cycle (uptake, dispersion, accumulation) of ENMs in biological systems.

The workflows will provide the users a set of entry protocols/methods at different phases of the studies ranging from nanomaterials characterisation to fine cell interaction analysis (Figure 1):

- Selection and Characterisation of the ENMs before and after contact with cell culture media
- Selection of Cell models with respect to the mode of exposure (inhalation, dermal contact and oral ingestion)
- *In vitro* assays and end-points for the determination of cell viability versus ENMs exposure using standardised assays
- *In operando* analysis of the cell culture before, during and after exposure to the ENMs using advanced microscopy methods such as real-time confocal microscopy, and photo-acoustic microscopy. For this purpose, a device will be specially designed, consisting of an operando platform with a sealed exposure chamber, wherein a fluid (liquid or aerosol) containing the ENMs will be injected and will interact with the enclosed biological material; the cells will be maintained under conditions to ensure their welfare. The design of the system will ensure the compatibility between the exposure chamber, and the assays designed for different types of cell systems, in particular, those commonly used for nanotoxicology study. A patterned 2D or will be also tested to implement cell cultures with particular features using the expertise developed within the JA "Advancing Nano-Engineering for an Enhanced Transnational Access" to produce the substrates.
- Fine-analysis microscopy/spectroscopy on fixed cells. The sample will be fixed by the most appropriate techniques (dehydration, cryogenic, vacuum, anaerobic environment, etc.) and the regions of interest (ROIs) to be characterised at higher resolution will be defined and recorded thanks to an integrated positioning system compatible with the various facilities among them the ultimate high-resolution microscopy techniques (e.g. SEM, STEM, AFM, SAXS, X-ray fluorescence and tomography imaging, FIB-SEM). The space correlation system described above will be developed in synergy with the JA "Correlative Nano-spectroscopy and Nanodiffraction".

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

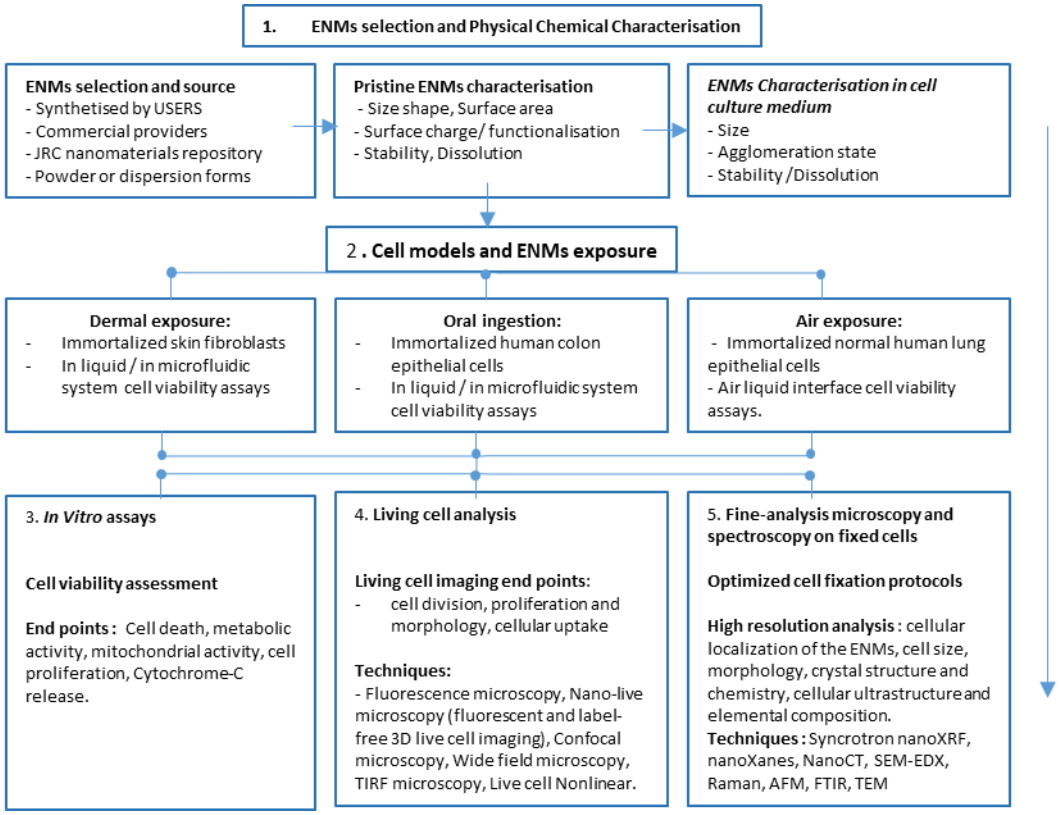

Figure 1: Workflow for ENMs safety assessment

The major modes of interactions between ENMs and cells that will be investigated are summarized in Figure 2. In addition to cell-specific parameters (e.g. cell type or cell cycle stage)[1,2], cellular uptake, targeting, intracellular trafficking and cytotoxicity of NPs can be modulated by their morphology and size[3,4,5] as well as by their physicochemical and surface properties[6,7,8].

[1] Mahmoudi M, Saeedi-Eslami SN, Shokrgozar MA, Azadmanesh K, Hassanlou M, Kalhor HR, Burtea C, Rothen-Rutishauser B, Laurent S, Sheibani S, Vali H: Cell "vision": complementary factor of protein corona in nanotoxicology. Nanoscale. 2012, 4: 5461-5468.
[2] Kim JA, Åberg C, Salvati A, Dawson KA. Role of cell cycle on the cellular uptake and dilution of nanoparticles in a cell population. Nat Nanotechnol. 2011, Nov 6;7(1):62-8.
[3] Wang J, Jensen UB, Jensen GV, Shipovskov S, Balakrishnan VS, Otzen D, Pedersen JS, Besenbacher F, Sutherland DS. Soft interactions at nanoparticles alter protein function and conformation in a size dependent manner. Nano Lett. 2011, 11: 4985-4991.
[4] Tenzer S, Docter D, Rosfa S, Wlodarski A, Kuharev J, Rekik A, Knauer SK, Bantz C, Nawroth T, Bier C. Nanoparticle size is a critical physicochemical determinant of the human blood plasma corona: a comprehensive quantitative proteomic analysis. ACS Nano. 2011, 5: 7155-7167.
[5] Shang W, Nuffer JH, Muniz-Papandrea VA, Colon W, Siegel RW, Dordick JS. Cytochrome c on silica nanoparticles: influence of nanoparticle size on protein structure, stability, and activity. Small. 2009, 5: 470-476.
[6] Verma A, Stellacci F. Effect of surface properties on nanoparticle-cell interactions. Small. 2010, 6: 12-21.
[7] Nel AE, Mädler L, Velegol D, Xia T, Hoek EM, Somasundaran P et al. Understanding biophysicochemical interactions at the nano–bio interface. Nat Mater. 2009, 8(7): 543–557.
[8] Tenzer S, Docter D, Kuharev J, Musyanovych A, Fetz V, Hecht R, Schlenk F, Fischer D, Kiouptsi K, Reinhardt C. Rapid formation of plasma protein corona critically affects nanoparticle pathophysiology. Nat Nanotechnol. 2013, 8: 772-781.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

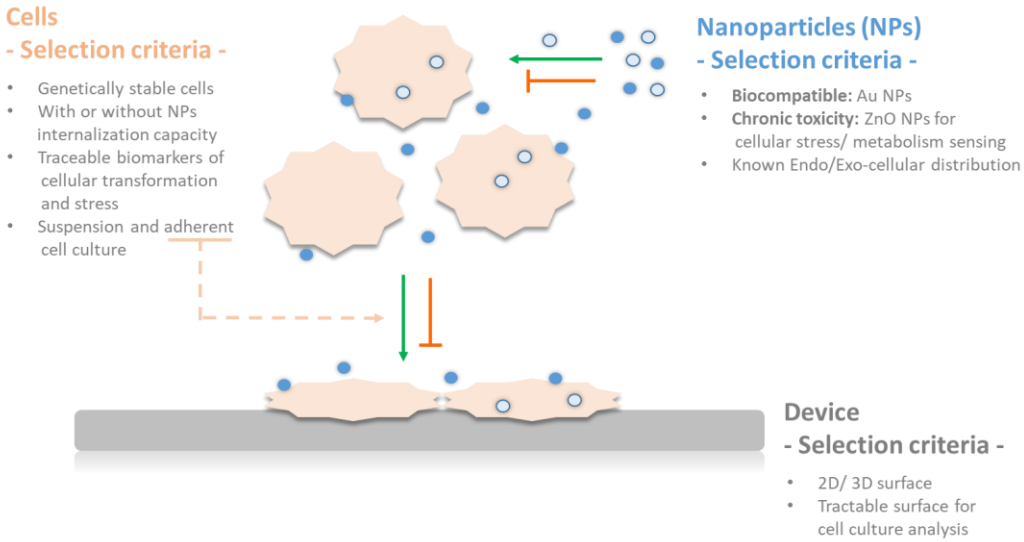

Figure 2: Modes of interactions between ENMs and cells

Different types of ENMs with enhanced or suppressed cellular uptake have been reported in the literature [9,10]. For example, shape and size-dependent uptake in different relevant cell lines has been observed for Au[11,12,13], mesoporous silica[14,15], and iron oxide[16] ENMs influencing uptake efficiency, internalization pathway selection, intracellular localization and cytotoxicity. Hence, knowledge of the underlying mechanisms involved in ENM uptake is crucial for assessing their fate and toxicity. Considering the variety of ENMs and the complexity of the biological probes and settings, NFFA envisions a model workflow to improve the quantitative understanding of interaction between ENMs and cells based on well characterized and ENMs, targeted cells and analytical techniques available via NEP participants.

[9] Li LW, Mu QX, Zhang B, Yan B. Analytical strategies for detecting nanoparticle-protein interactions. Analyst. 2010, 135: 1519-1530.
[10] Saptarshi S, Duschl A, Lopata A. Interaction of nanoparticles with proteins: relation to bio-reactivity of the nanoparticle. J Nanobiotechnol. 2013, 11: 26-10.
[11] Malugin A, Ghandehari H. Cellular uptake and toxicity of gold nanoparticles in prostate cancer cells: a comparative study of rods and spheres. J Appl Toxicol. 2010, 30: 212-217.
[12] Chithrani BD, Ghazani AA, Chan WCW. Determining the size and shape dependence of gold nanoparticle uptake into mammalian cells. Nano Lett. 2006, 6: 662-668.
[13] Wang SH, Lee CW, Chiou A, Wei PK. Size-dependent endocytosis of gold nanoparticles studied by three-dimensional mapping of plasmonic scattering images. J Nanobiotechnol. 2010, 8: 33-10.1186/1477-3155-8-33.
[14] Lu F, Wu SH, Hung Y, Mou CY. Size effect on cell uptake in well-suspended, uniform mesoporous silica nanoparticles. Small. 2009, 5: 1408-1413.
[15] Shapero K, Fenaroli F, Lynch I, Cottell DC, Salvati A, Dawson KA. Time and space resolved uptake study of silica nanoparticles by human cells. Mol BioSyst. 2011, 7:371–378.
[16] Huang J, Bu L, Xie J, Chen K, Cheng Z, Li X, Chen X. Effects of nanoparticle size on cellular uptake and liver MRI with polyvinylpyrrolidone-coated iron oxide nanoparticles. ACS Nano. 2010, 4: 7151-7160.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

2 ENMS SELECTION AND PHYSICAL CHEMICAL CHARACTERIZATION

In this workflow, the ENMs may come from different sources i.e. synthetized by users or a third research institution, purchased from commercial providers or collected from JRC nanomaterials repository. ENMs may be in form of powders or dispersed in liquid. The ENMs safety must be assessed according to the mode of exposure i.e. in liquid for dermal exposure and oral ingestion and in aerosol for air inhalation exposure. Optimized dispersing procedures from powders to liquids and in aerosols must be developed and used according to the needs. References to standard protocols of dispersion can be found in **Annex 1**.
In order to perform a reliable ENMs safety assessment, careful and extensive characterization of ENMs behaviour both before and during toxicity assessment is needed [17, 18, 19,20]

For pristine ENMs, the characteristics to be determined are:

- Size distribution and shape determination using Electron microscopy methods and small angle X-ray/neutron scattering
- Hydrodynamic diameter determination by using centrifugal or light scattering based methods
- Volume specific Surface area
- Surface charge
- Stability in liquids
- Dissolution rate

Additional characterisation for determining the behaviour of ENMs in cell culture medium is crucial. Cell culture media are complex media containing serum proteins and biomolecules that covers the ENMs upon interactions to form a protein corona modifying the biological identity of the nanomaterials affecting their stability (including dissolution), state of agglomeration and mode of cellular interactions.
Some relevant ISO standards and reports on ENMs characterisation are listed in **Annex 1**.
Techniques needed for the ENMs Physical chemical characterization are available in the NFFA-Pilot catalogue in particular in Structural & Morphology Characterization techniques families (https://www.nffa.eu/offer/area/?id=6246)

3 CELL MODELS AND ENMS EXPOSURE

One of the major goals of this Joint Action is to provide a platform capable of advancing our knowledge about the interactions and effects of ENMs with biological systems. Specifically, human cell lines representative of three major routes of exposure to nanomaterials: skin, lungs, and gastrointestinal tract will be studied.

---

[17] Bouwmeester H, Lynch I, Marvin HJ, Dawson KA, Berges M, Braguer D, Byrne HJ, Casey A, Chambers G, Clift MJ, Elia G, Fernandes TF, Fjellsbø LB, Hatto P, Juillerat L, Klein C, Kreyling WG, Nickel C, Riediker M, Stone V. Minimal analytical characterization of engineered nanomaterials needed for hazard assessment in biological matrices. Nanotoxicology. 2011 5(1):1-11.
[18] DeLoid, G., Cohen, J., Pyrgiotakis, G. *et al.* Preparation, characterization, and *in vitro* dosimetry of dispersed, engineered nanomaterials. *Nat Protoc* 12**,** 355–371 (2017).
[19] Halamoda-Kenzaoui B, Ceridono M, Colpo P, Valsesia A, Urban P, Ojea-Jimenez I, et al. Dispersion behaviour of silica nanoparticles in biological media and its influence on cellular uptake. PLoS ONE. 2015;10:e0141593.
[20] Rischitor G, Parracino M, La Spina R, Urbán P, Ojea-Jiménez I, Bellido E, et al. Quantification of the cellular dose and characterization of nanoparticle transport during in vitro testing. Part Fibre Toxicol. 2016;13:47.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

Furthermore, three assay configurations in relation to the mode of exposure can be envisaged as described in Figure 3.

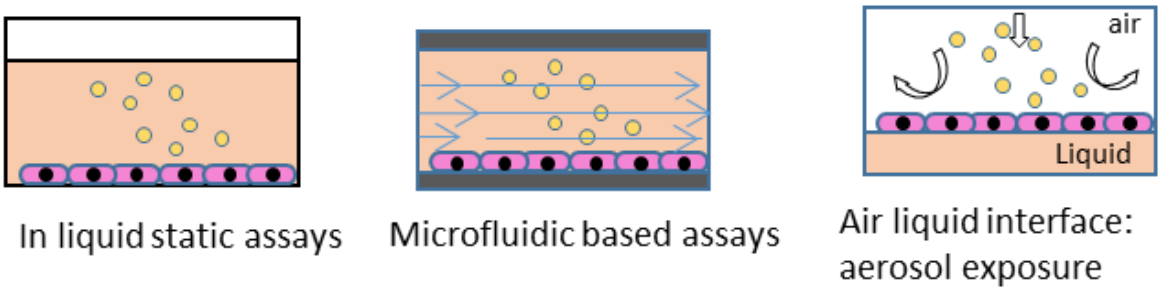

Figure 3: Cell assays configuration.

Specific parameters and protocols based on the up-to-date literature on nanosafety and aligned with the state of the art, in particular with the outcomes of the previous international projects and the proposed protocols, e.g., standardized by the International Organization for Standardization (ISO) or validated by the Nanotechnology Characterization Laboratory (NCL) of the US the National Cancer Institute (NCI) are outlined in **Annex 2**.

Furthermore, the standardized protocols will be adapted to the needs of NEP users and to take advantage of the techniques available via NEP participants.

Cell lines (Table 1):

Genetically stable and human telomerase reverse transcriptase (hTERT) immortalized cell lines with robust and homogenous growth capacity will be used to provide consistent and reproducible results on safety and analysis of interactions between ENMs and cells. Immortalized skin fibroblasts (e.g., BJ-5ta), immortalized normal human lung epithelial cells (e.g., NuLi-1), and immortalized human colon epithelial cells (e.g., HCEC-1CT) will be used following established cell-culture protocols and controls. Additional cell lines commonly used in published studies, e.g., A549 or Caco-2, may be used in comparative experiments.

CELL LINES	PROVIDERS
hTERT-immortalized foreskin fibroblast cell line, ***BJ-5ta***	https://www.atcc.org/products/crl-4001 https://cientificasenna.com/wp-content/uploads/2018/02/hTERT.pdf
Human airway epithelial (HAE) cell line NuLi-1	https://cientificasenna.com/wp-content/uploads/2018/02/hTERT.pdf https://www.atcc.org/products/crl-4011
Immortalized human colon epithelial cells (e.g., HCEC-1CT)	https://www.evercyte.com/medizin/cell-lines/immortalized-human-somatic-cells/hcec-1ct-170.html https://www.pharmaceutical-networking.com/wp-content/uploads/2017/06/In-vitro-propagation-of-HCEC-1CT-cells-Protocol.pdf
A549 Human Caucasian lung carcinoma ECACC 86012804	ECACC 86012804 https://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=86012804&collection=ecacc_gc
CaCo2 Human Caucasian colon adenocarcinoma DSMZ	https://www.dsmz.de/collection/catalogue/details/culture/ACC-169

Table 1: Cell lines proposed to be used in the workflow.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

4 *IN VITRO* ASSAYS FOR ENMS SAFETY ASSESSMENT

In vitro assays are cornerstone for assessing potential toxic effects of advanced materials and in particular nanomaterials[21]. These assays provide information on the ENMs concentration threshold guarantying cell viability (IC_{50} inhibition coefficient by 50%). Different assays are available for addressing different end-points: Cell death, metabolic activity, mitochondrial activity, cell proliferation, Cytochrome-C release.

4.1 Submerged exposure based *in vitro* assays

In these assays, cells are cultured in multi well plates and exposed to ENMs for different period of times (constant doses, repetitive doses). Main assays can be categorised versus their detection principles e.g. Dye exclusion assays, Colorimetric assays, Fluorometric assays: Alamar Blue assay, Luminometric assays: ATP assays. However, due to the special properties of these materials (including their high surface area), ENMs may show unexpected interferences with assay reagents in classical *in vitro* toxicity assays. It is widely acknowledged that each *in vitro* test system has to be evaluated for each ENM type to accurately assess their toxicity.[22] [23]

Parameters in toxicity assessment and assays

The NFFA portfolio includes different *in vitro* assays to assess mechanisms of toxicity, including:

- General cytotoxicity (e.g., cell viability, membrane integrity and permeability): using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) –based assays, LIVE/DEAD Cell Imaging assay and Celltox DNA assay;
- Apoptosis: using Caspase 3,3/7 activation assays;
- Oxidative stress: using Glutathione, Lipid peroxidation, Reactive oxygen species-based assays;
- Autophagy: using autophagic dysfunction and MAP LC3I to LC3II conversion-based assays;
- Genotoxicity and DNA damage: using micronucleus assays.
- Alamar Blue cell health assay that uses the reducing power of living cells to quantitatively measure viability.
- ATP assay: Luminometric assays provide fast and simple determination of cell proliferation and cytotoxicity in mammalian cells
- Cell proliferation: Colony Forming Efficiency (CFE) is label free assay that measures the ability of a single cell to form a colony.

Some relevant information about *in vitro* assays is presented in **Annex 2.**

Cell substrates:

[21] Guggenheim, E. J., Milani, S., Röttgermann, P. J. F., Dusinska, M., Saout, C., Salvati, A., Rädler, J. O., & Lynch, I. (2018). Refining in vitro models for nanomaterial exposure to cells and tissues. *NanoImpact*, *10*, 121-142.

[22] Kroll, A., Pillukat, M.H., Hahn, D. *et al.* Interference of engineered nanoparticles with in vitro toxicity assays. *Arch Toxicol* 86**,** 1123–1136 (2012)

[23] Rösslein M., Elliott J.T, Salit M., Petersen E.J., Hirsch C., Krug H.F., and Wick P., Use of Cause-and-Effect Analysis to Design a High-Quality Nanocytotoxicology Assay, *Chem. Res. Toxicol.* 2015, 28, 1, 21–30

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

Main assays formats are multi-well plastic plates, or PDMS substrates, glass slides, with or without functionalization. The surfaces used for the *in vitro* assays must be transparent since main microscopy analyses are performed in transmission mode. The standard format of *in vitro* assays are multi-well plastic plates (96-48-24-12-6 wells) and plastic petri dishes.

4.2 Air liquid interface-based assays

Air liquid interface assays are used for assessing the effects of air exposure of ENMs in mimicking respiratory exposures. Different methods to introduce aerosolized ENMs to cells cultured at the air-liquid-interface (ALI) have been developed. [24] The Nano Aerosol Chamber for In Vitro Toxicity (NACIVT) set-up available in NFFA portfolio for toxicity testing of airborne particles is presented in Figure 4. Information regarding protocols used for cell cultures can be found in the literature[25].

The particles are deposited onto cell cultures (for example small airway epithelial cells) directly from the aerosol phase, mimicking real life respiratory exposure. The set-up has the following features:

- Electrostatic deposition of nanoparticles (NPs) – with deposition fractions similar to human lungs
- Controlled temperature and relative humidity ensure biological conditions
- Table-top size and transportable – can be used for in vitro studies e.g. at workplaces
- Collected particles from any environment can be re-aerosolized and used in the NACIVT

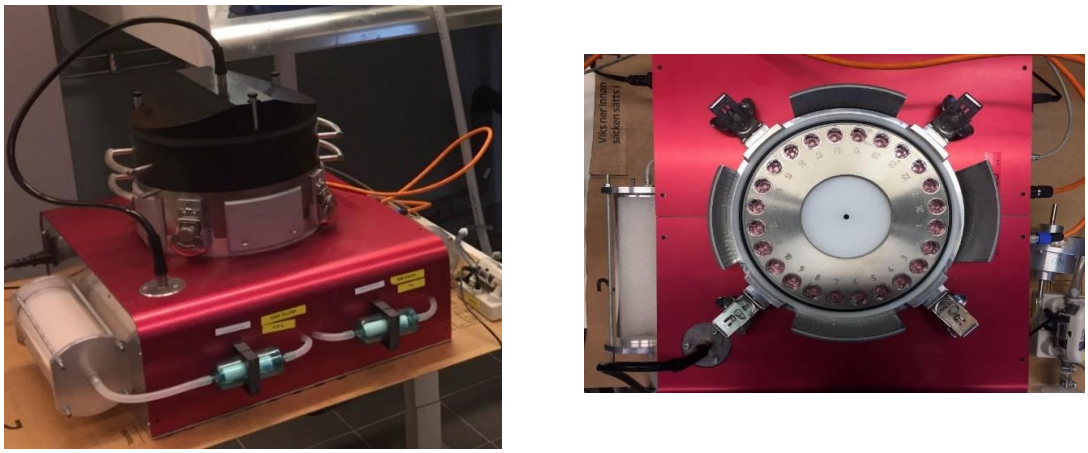

Figure 4: Nano Aerosol Chamber for In-Vitro Toxicity (NACIVT)

Cell substrates:

The membranes used for cell culture Transwell polyethylene terephthalate (PET) membranes with a pore size of 0.4 µm. Selection guide of permeable support can be found in most of cell plastic ware manufacturers.

Cell analysis

[24] Jeannet N., Fierz M., Kalberer M., Burtscher H. and Geiser M. (2015) Nano Aerosol Chamber for In-Vitro Toxicity (NACIVT) studies, Nanotoxicology, 9:1, 34-42, DOI: 10.3109/17435390.2014.886739

[25] Lovén K.,Dobric J.,Bölükbas D.A,Kåredal M.,Tas S.,Rissler J., Wagner D.E. and Isaxon C. Toxicological effects of zinc oxide nanoparticle exposure: an in vitro comparison between dry aerosol air-liquid interface and submerged exposure systems. Nanotoxicology , 2021, VOL. 15, NO. 4, 494-510, DOI: 10.1080/17435390.2021.1884301

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

Cell analysis can be done by fluorescent microscopy. The corresponding protocols are described in **Annex 3**. Cell viability is assed with the WST-1 assay, cytotoxicity with LDH, and cytokine release by multiplex Luminex as described in the Lovén et al.

5 LIVE CELL ANALYSIS

Advanced techniques of live cell imaging enable to determine the effects of the ENMs interactions with cells and understand the potential mechanism of toxicity. Live cell-imaging techniques available in the NFFA catalogue are describe below, classified in labelled and label free techniques. These techniques are based on advanced imaging microscopies (using two- or three- photon excitation), which are appropriate for the all-optical minimally invasive, high-resolution (~400nm), depth (>500µm) monitoring of living cells and tissues for long periods of time. Apart from multi-photon fluorescence microscopy, live cell imaging can be further enriched with polarization-resolved Second Harmonic Generation (P-SHG), polarization-resolved Third Harmonic Generation (P-THG) imaging. Consequently, in addition to exogenous fluorescence markers (e.g. dyes) live cell-imaging can probe endogenous contrast agents. Furthermore, it provides theoretical tools for the utilization of quantitative imaging biomarkers by using P-SHG or P-THG imaging techniques.

5.1 Biological sample preparation

The general concept of the workflow selected for initial implementation is to use the combination of model cells and ENMs that will provide biological and physicochemical signatures across the multiple complementary techniques described in this document. Specifically, gold and ZnO NPs have been selected as models of NPs that are expected to induce, respectively, minimal and chronic cytotoxicity. Furthermore, the expected mechanism of cytotoxicity induced by ZnO NPs is via the production of reactive oxygen species (ROS). The ROS mechanism can be tracked in live cells via both optical microscopy and *in vitro* assays. This mechanism is also expected to involve surface modification of the ZnO NPs and their compartmentalization inside the cells, i.e., phenomena that will be observable using the complementary fine-analysis techniques.

5.2 Live cell imaging techniques

Techniques proposed in this workflow (Table 2) are:
Fluorescence microscopy, Nanolive microscopy (fluorescent and label-free 3D live cell imaging), Confocal microscopy, Wide field microscopy, TIRF microscopy, Live cell Nonlinear microscopy (2p and 3p Fluorescence, Polarized 2nd Harmonic Generation, Polarized 3rd Harmonic Generation)
The analyses can be made in static (in a multiwall plate) or in flow using a microfluidic device.

TECHNIQUES	LABEL	LABEL FREE METHOD / AUTOFLUORESCENCE BASED METHODS
Fluorescent microscopy	x	x
Nanolive microscopy	x	x
Confocal microscopy	x	
Wide field microscopy	x	
TIRF	X	x
Live cell Nonlinear microscopy (2p and 3p Fluorescence, P-SHG, P-THG)	x	x

Table 2: Live cell-imaging techniques

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

Main end-points for live cell imaging are: Cell division/proliferation, nuclear size, cytoskeleton organization and dynamics, Fluorescent ENMs aggregates, Cell uptake quantification and intracellular localisation using fluorescent labelled ENMs.

Cell substrates:

Main analysis substrates include glass, multi-well plastic plates, or PDMS, with or without functionalization and glass-bottom petri dishes. More advanced substrates platforms can be used for single cell analysis and better simulate cell microenvironment, such as:

- Functionalised surfaces for cell micropatterning,
- micro- and nano-patterns of ECM proteins
- 2.5 D micro- and nano-structures.

5.2.1 Custom-built, laser raster-scanning, multiphoton microscope (FORTH)

The custom-build multiphoton microscope (Figure 5a) operated at FORTH has the capability of recording 2-photon and 3-photon excited fluorescence, as well as polarization-resolved second harmonic generation (P-SHG) and polarization-resolved third harmonic generation (P-THG), by using 4 detectors and appropriate optics [1-6]. The multiphoton imaging workstation is also equipped with an incubator at the sample plane (Figure 4b) which provides temperature and CO_2 control, allowing long-lasting live cell imaging, in either static cultures or under microfluidic flow (i.e. dynamic cultures).[26], [27] [28] [29] [30] [31]

[26] S. Psilodimitrakopoulos, A. Orekhov, L. Mouchliadis, D. Jannis, G.M. Maragkakis, G. Kourmoulakis, N. Gauquelin, G. Kioseoglou, J. Verbeeck, E. Stratakis, Optical versus electron diffraction imaging of Twist-angle in 2D transition metal dichalcogenide bilayer superlattices, *npj 2D Materials and Applications* (2021)5:77.

[27] Three-dimensional characterization of collagen remodeling in cell-seeded collagen scaffolds via polarization second harmonic generation," Dionysios Xydias, Georgios Ziakas, Sotiris Psilodimitrakopoulos, Andreas Lemonis, Eleni Bagli, Theodore Fotsis, Achille Gravanis, Dimitrios S. Tzeranis, and Emmanuel Stratakis, *Biomed. Opt. Express* 12, 1136-1153, 2021.

[28] Leonidas Mouchliadis, Sotiris Psilodimitrakopoulos, George Miltos Maragkakis, Ioanna Demeridou, George Kourmoulakis, Andreas Lemonis, George Kioseoglou & Emmanuel Stratakis, Probing valley population imbalance in transition metal dichalcogenides via temperature-dependent second harmonic generation imaging, *npj 2D Mater. Appl* 5, 6, 2021.

[29] Alexandra Kourgiantaki, Dimitrios Tzeranis, Kanelina Karali, Sotirios Psilodimitrakopoulos, Konstantina Georgelou, Ioannis Yannas, Emmanuel Stratakis, Kyriaki Sidiropoulou, Ioannis Charalampopoulos, Efstathia Bampoula, and Achille Gravanis, "Neural Stem Cell Delivery via Porous Collagen Scaffolds Promotes Neuronal Differentiation and Locomotion Recovery in Spinal Cord Injury, *Nature Regenerative Medicine*, 5, 12 2020.

[30] S. Psilodimitrakopoulos, L. Mouchliadis, I. Paradisanos, A. Lemonis, G. Kioseoglou and E. Stratakis, Ultrahigh-resolution non-linear optical imaging of the armchair orientation in 2D transition metal dichalcogenides,*Light: Science & Applications*, 7, 18005, 2018.

[31] Dionysios Xydias, Georgios Ziakas, Sotiris Psilodimitrakopoulos, Andreas Lemonis, Eleni Bagli, Theodore Fotsis, Achille Gravanis, Dimitrios S. Tzeranis, and Emmanuel Stratakis, Three-dimensional characterization of collagen remodeling in cell-seeded collagen scaffolds via polarization second harmonic generation, *Biomed. Opt. Express* 2, 1136-1153, 2021.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

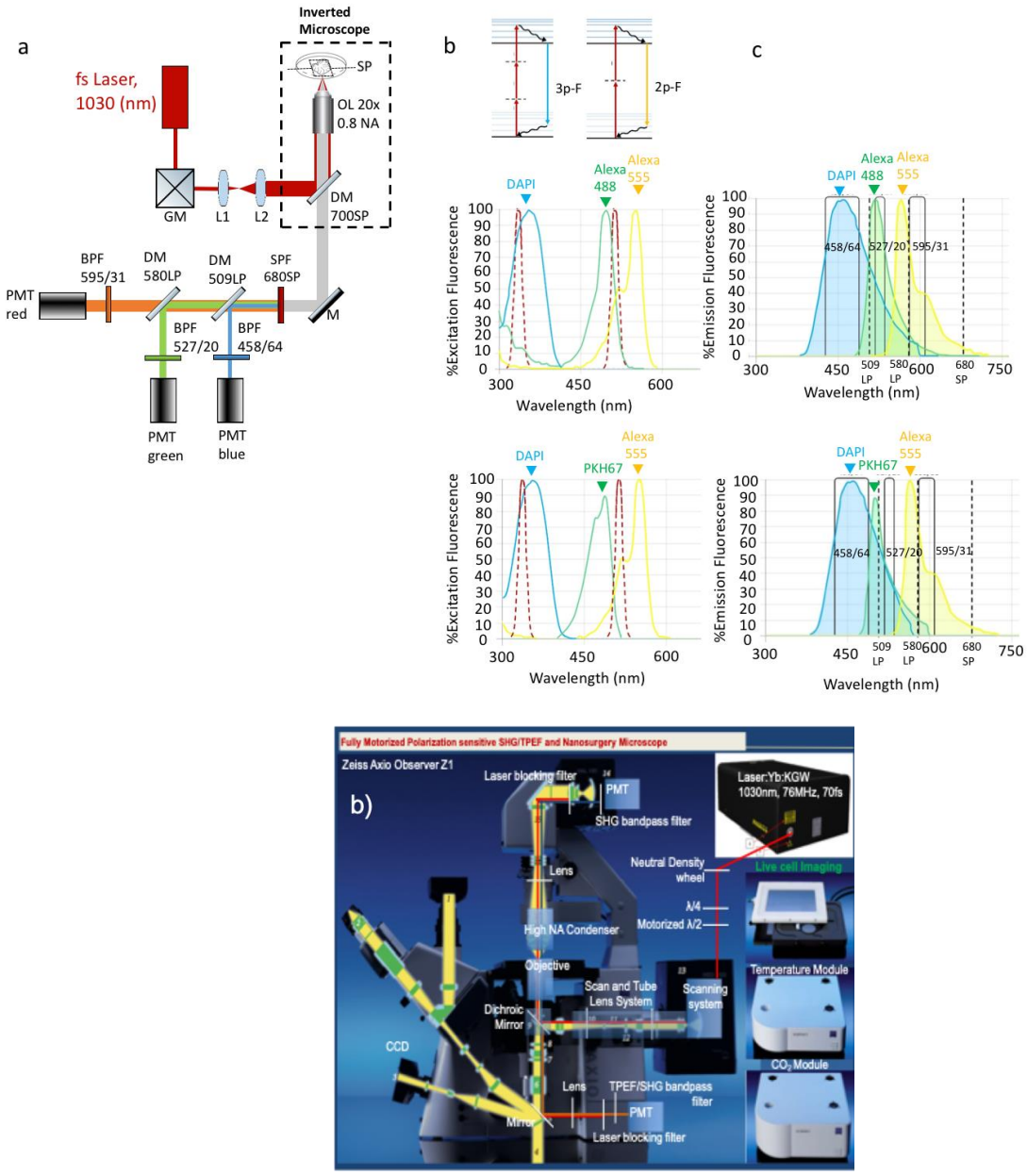

Figure 5: a) Schematic representation of the experimental setup for simultaneous 3p-F and 2p-F imaging microscopy. b) Multiphoton microscope supporting live cell-imaging, using an incubator (temperature and CO_2 control) at the sample plane.

5.2.2 Live cell imaging techniques in a microfluidic system (FORTH)

In the framework of WP 14, a microfluidic system will be designed at FORTH to perform live cell imaging under dynamic cell culture conditions. Microfluidic devices provide a more realistic environment for biological research, as they are related to scales found in biological systems (micro- and nano-). Cells are in the micrometer range, typically around 10 - 15 μm. Unlike conventional static cultures, microfluidic cell cultures permit precise control of the microenvironment (e.g. changes in the flow rate) that influence biochemical and mechanical factors in a cell and, thus, cell functionality [32]. In particular, cell cultures under microfluidic flow reflect more appropriately the *in vivo* environment of cells in tissues such as the physiological fluid flow inside the body, consistent

[32] C. Zhang, D. Van Noort, Cells in microfluidics, Top. Curr. Chem. 304 (2011) 295–321.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

nutrient delivery, effective waste removal, and mechanical stimulation due to fluid shear forces.[33] Laminar flow regimes, small length scales, and diffusion dominated mass transport characterize the microfluidic devices resulting in a more *in vivo*-like environment.[34] The microfluidic chamber including the substrate with the cells will be installed in a microscope for real-time imaging under flow conditions. The cross-section image of the microfluidic chamber, containing the substrate with cells, where the flow occurs is also illustrated (Figure 6b).

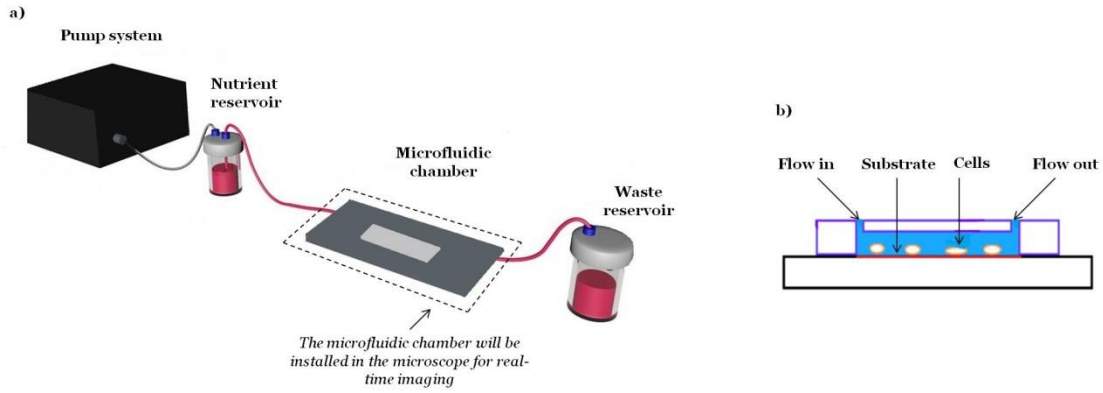

Figure 6: (a) Schematic illustration of the microfluidic system. It will be composed of a pump system connected with a nutrient reservoir, a microfluidic chamber containing the cells and the substrate, and a waste reservoir. (b) Cross-section image of the microfluidic chamber, containing the substrate and cells, where the flow occurs.

Live-cell imaging experiments will be performed in the microfluidic chamber, including the substrate with cells, to examine the cell viability (cytotoxicity, cell adhesion, proliferation), as well as, the uptake of NPs under flow conditions. For this reason, live-cell imaging fluorescent dyes or fluorescent/transfected cells or fluorescent ENMs will be used. Different flow rates will be used to examine the cell viability, as well as, the ENMs uptake.
As illustrative examples, Figures 7 and 8 show images from the in-situ monitoring of mesenchymal stem cells (MSCs) and NIH 3T3 cells, respectively, cultured on polymeric microgrooves (MG) in the microfluidic chambers under static conditions.

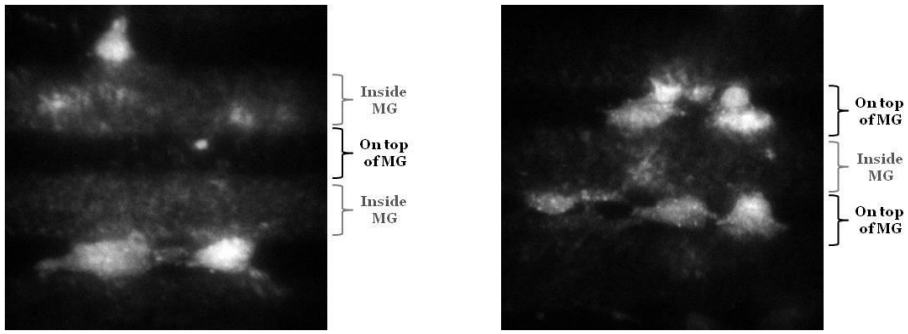

Figure 7: Live-cell imaging of mesenchymal stem cells (MSCs) cultured on polymeric microgrooves (MG) in the microfluidic chamber. Fluorescent dye acridine orange was used.

[33] M. Mehling, Microfluidic cell culture, Curr. Opin. Biotechnol. 25 (2014) 95–102.
[34] M. Karimi, S. Bahrami, H. Mirshekari, S.M.M. Basri, A.B. Nik, A.R. Aref, M. Akbari, M.R. Hamblin, Microfluidic systems for stem cell-based neural tissue engineering, Lab Chip. 16 (2016) 2551–2571.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

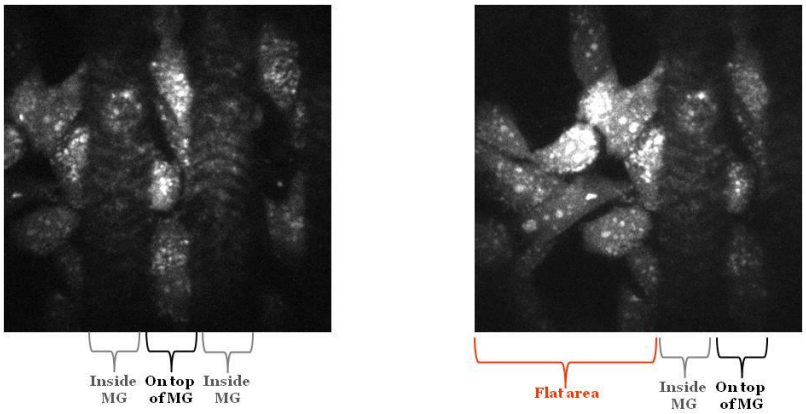

Figure 8: Live-cell imaging of NIH 3T3 cells cultured on polymeric microgrooves (MG) in the microfluidic chamber. Fluorescent dye acridine orange was used.

5.3 Advanced live cell-imaging by STEM/SEM

The study of cell physiological processes at nanoscale is very challenging. Electron microscopy is a potential candidate for this purpose although the needed electron dose to obtain contrast is well above the lethal dose. Operando STEM will be attempted (DESY and CNR-IOM) in static conditions relying on microreactors based on sealed SiN membranes, in order to explore the live functions of the biological systems in liquid conditions by means of low voltage STEM / SEM. To this end, an in-depth study will be conducted within WP14 in order to identify suitable protocols for sample preparation to preserve the living cell functions after fixation on the SiN membranes. The best conditions for the direct observation of biological systems will also be investigated, with the aim of identifying possible information and the limits of this methodological approach.
Starting from earlier studies with commercial holders [35] and based on the above results, novel approaches will be pursued to design multifunctional devices with SiN membranes suitably modified to perform in operando study of the biological systems in STEM/SEM, integrating with GISAXS/GIWAXS or other synchrotron based techniques.

5.4 X-ray fluorescence live cell imaging

X-ray fluorescence imaging using X-rays generated by a synchrotron source can provide the spatial distribution of elements in thicker samples. The lateral resolution can reach the micro-to-nanometer regime. Typically, samples are investigated in transmission. In situ analysis in biological environments is principally possible in appropriate sample cells.[36,37]

Description of main requirements in term of analysis and samples preparation

The optimal sample thickness is determined by the X-ray absorption / attenuation length and the X-ray scattering cross-section to create sufficient contrast. Due to the complexity of the experiments it is strongly recommended to discuss details of the experiment with the local contact person.

Type of Substrates used

[35] Niels de Jonge and Diana B. Peckys *ACS Nano* 2016 *10* (10), 9061-9063
[36] Sanchez-Cano, C. et al., X-Ray-Based Techniques to Study the Nano–Bio Interface. *ACS Nano* 2021, *15* (3), 3754–3807.
[37] Zhang, R.; Li, L.; Sultanbawa, Y.; Xu, Z. P. X-Ray Fluorescence Imaging of Metals and Metalloids in Biological Systems. *Am. J. Nucl. Med. Mol. Imaging* 2018, *8* (3), 169–188

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

In transmission measurements with the X-ray beam passing through the substrate, parasitic scattering shall be avoided to reduce the background.

6 FINE-ANALYSIS ON FIXED CELLS.

This section describes different techniques available in the NFFA catalogue for fine analysis of fixed cell that are proposed in the workflow for ENMs safety assessment.
Cells fixation protocols suitable to be used within the workflow are listed in **Annex 4**.

6.1 SEM/EDX of stained/fixed cells with optional SEM/EDX slicing tomography (DESY Facility).

Scanning electron microscopy *(SEM)* is a technique of choice for determining cell morphology and probing the inside of whole cells, giving information on organelles and internal structure and possible ENMs uptake [38]. In a SEM, a beam is scanned over the sample surface in a raster pattern while a signal from secondary electrons (SE) or Back-scattered electrons (BSE) is recorded by specific electron detectors. The electron beam, which typically has an energy ranging from a few hundred eV up to 40 keV, is focused to a spot of about 0.4 nm to 5 nm in diameter. Latest generation SEMs indeed can achieve a resolution of 0.4 nm at 30 kV and 0.9 nm at 1 kV. Beyond the ability to image a comparatively large area of the specimen, SEM can be equipped with a variety of analytical techniques for measuring the composition, crystallographic phase distribution and local texture of the specimen.

Chemical composition analysis can be performed by Energy Dispersive X-ray Spectroscopy (EDS), which relies on the generation of an X-ray spectrum from the entire scan area of the SEM. An EDS detector mounted in the SEM chamber collects and separates the characteristic X-rays of different elements into an energy spectrum and EDS system software is used to analyse the energy spectrum in order to determine the abundance of specific elements. EDS can be used to find the chemical composition of materials down to a spot size of a few microns and to create element composition maps over a much broader raster area. (e.g., https://www.nffa.eu/offer/area/technique/?id=6267)

Main requirements in term of analysis and biological samples preparation.

Biological samples can be analysed using SEM/EDX on fixed cells. For this purpose, the biological sample must be decontaminated and fixed following standard protocols. In addition, standard staining procedures are recommended for contrast enhancement in weakly scattering samples.

Any substrate material can be used. Conductive substrates are preferred to avoid charging or any needs of a sputter coated carbon or a thin metal film.

[38] Ishak M.I., Jenkins J., Kulkarni S., Keller T.F., Briscoe W.H., Nobbs, Su B.,Insights into complex nanopillar-bacteria interactions: Roles of nanotopography and bacterial surface proteins,Journal of Colloid and Interface Science,Volume 604,2021,Pages 91-103.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

6.2 Scanning Electron Microscopy (CNR –IOM facility)

Ex-situ characterization of the biological systems interacting with the ENMs will be performed by Scanning Electron Microscopy (SEM) at CNR-IOM. In particular, cells will be analyzed after chemical fixation on dedicated supports compatible with SEM to inspect the intrinsic properties of the nanomaterial and its support-dependent properties.

To this end, a ZEISS Supra 40 equipped with an Everhart Thronley secondary electron detector as well as with an InLens and Backscattered detectors will be used and operated at low energy in order to find the best compromise between appreciable contrast from the biological cells and low electron dose on the examined objects. This will enable to track also the ENMs inclusion into the biological systems and determine their functional behavior at the different steps of the workflow.

Ex-situ, analysis of the cells at specific steps of the workflow will be also performed in SEM configuration at the Dual-Beam (ZEISS XB1540) of the CNR-IOM for a comparative study of the cells with the embedded NPs under supercritical drying fixation or cryogenic conditions, with the possibility of in-situ cross sectioning by Ga based FIB preparation.

Biological Sample preparation and cell fixation

The sample preparation will be performed in collaboration with specialized NFFA partner.

- New protocols for TEM sample preparation will be explored and optimized, in order to ensure the cell fixation on TEM support grids. This will open the possibility to explore the prepared sample in STEM (Scanning Transmission Electron Microscopy) at low voltage (30KeV) in the SEM by means of the annular STEM detector which is the advanced complement of the SEM at CNR-IOM.
- **Critical point dryer** (Tousimis Smdri-PVT-3D) allows to achieve the precondition of water removal from the sample by replacing it with an increasing alcohol concentration. Liquid CO_2 is then employed for the drying of the sample without being exposed to a phase boundary (liquid/gaseous), avoiding cell membranes disruption.
- **Cryo-stage integrated in Dual-Beam microscope** permit the direct inspection of the cells through the fast freezing of the sample. Quorum PP3010 Cryo-SEM/Cryo-FIB/SEM Preparation System is design for a highly automated, gas-cooled Cryo-SEM preparation and Cryo Transfer System suitable for the observation of biological, wet or beam sensitive specimens. Cryo SEM electron microscopy permit to overcome the conventional preparation methods, such as chemical fixation, for the direct observation of specimens in their natural hydrated state.

6.3 Atomic force microscopy on fixed cells

AFM is a surface sensitive technique permitting to obtain a microscopic image of the topography of a material surface e.g. a fixed cell, adsorbed proteins or nano-bio interfaces in generale.[39]
Typical lateral image sizes are within a range of only a few Nanometers to several tenth of Micrometers, whereas height changes of less than a nanometer may be resolved. A fine tip attached to a cantilever is scanned across the material surface and enables to measure height changes via a laser that is reflected from the rear side of the cantilever onto a segmented photodiode. The position of a laser spot on the photodiode permits to track height changes as e.g. due to a nano-particle on the surface or an atomic terrace of a single crystal surface. A feedback loop controls the tip-surface distance and therefore ensures stable imaging conditions.

[39] Keller T.F., Schönfelder J., Reichert J., Tuccitto N., Licciardello A., Messina G.M.L , Marletta G., and Jandt K.D, How the Surface Nanostructure of Polyethylene Affects Protein Assembly and Orientation. *ACS Nano* 2011, *5* (4), 3120–3131.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

Different operation modes like contact or non-contact mode can be used to optimize the imaging conditions with highest lateral resolution on one hand and least sample interaction on the other hand. Additional surface properties may be obtained for each point of the scan like friction force by lateral force imaging and magnetization properties by magnetic force imaging. Elasticity maps of heterogeneous sample surfaces can be obtained by non-contact phase imaging utilizing the phase shift arising from the local penetration behaviour of the tip into the surface. (e.g., https://www.nffa.eu/offer/area/technique/?id=6264)

Main Sample preparation requirements

Biological samples must be decontaminated and fixed following standard protocols. AFM requires flat substrates. Typical model substrates are freshly cleaved Mica, Highly Ordered Pyrolytic Graphite (HOPG) or Silicon wafers. More relevant substrates (for adsorption / adhesion studies) may be used, provided they are sufficiently flat.

6.4 X-ray microscopy (ESRF facilities)

Synchrotron based X-ray based microscopy is a powerful tool for the characterization of nanomaterials in cells. The use of a highly coherent bright synchrotron source allows X-ray microscopy to reach nano-scale resolution (down to ~20nm depends on beamline characteristics) in several detection modes (absorption, emission, and phase contrast) with great sensitivity.
The morphology of the sample can be studied in 2D and 3D mode by measuring the absorption of the sample using a focused X-ray beam (Scanning X-ray microscope SXM) or by projection imaging. The SXM mode is advantageous (though is slower) as it allows register the emission of X-rays (X-ray fluorescence XRF) at each pixel of the image (the resolution limited by the size of the probe) and it allows to have simultaneous detection of several elements depending on the selected excitation energy. Moreover, the electronic structure of the sample (e.g a hot spot where ENMs are detected) can be investigated by means X-ray absorption spectroscopy near edge structure (XANES) which is sensitive to the oxidation state and coordination geometry of the element of interest.

Three beamlines are proposed to be chosen in function of the objectives of the experiment and the type of nanomaterial for this workflow. Beamline ID21 capable of performing cryogenic and room temperature 2D imaging of cells with resolution of ~300nm (~100nm after upgrade in 2022) with XRF detection mode and XANES capabilities in the energy range from 2-10keV. Beamline ID16B for 2D imaging of cells with resolution of ~60nm with XRF detection mode and XANES capabilities in the enrgy range from 7-33keV. In phase contrast mode 3D imaging is available with resolution of ~50nm. Beamline ID16A capable or performing cryogenic and room temperature 2D/3D imaging with XRF detection mode and phase contrast at fixed energies 17keV and 33keV.

Biological Sample preparation and cell fixation

The preferred substrate for X-ray imaging analysis is SiN windows. The cells can be prepared using vitrification cryo-fixation protocols (e.g. using the vitrobot plunger from thermo scientific) followed by freeze drying if the analysis is to be done at room temperature.[40] These above-mentioned preparation protocols are applied for whole cell imaging, however analysis can be done on ultra-thin sections of cells by means of high-pressure freezing followed by freeze substitution (acetone with or

[40] Bissardon, C., Reymond, S., Salomé, M., André, L., Bayat, S., Cloetens, P., Bohic, S. Cell Culture on Silicon Nitride Membranes and Cryopreparation for Synchrotron X-ray Fluorescence Nano-analysis. J. Vis. Exp, (154), e60461, doi:10.3791/60461 (2019).

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

without OsO_4) and resin embedding,[41] mounted on SiN windows. Chemical fixation protocols followed by resin embedding also allow to produce ultra-thin sections for X-ray imaging analysis and correlative studies with TEM.[42] Ultra-thin sections can be obtained on cryofixed cells (e.g. using high pressure freezing) and analysed at beamlines with cryogenic stages (ID21/ID16A).

6.5 Multi-screening of ENMs formulations prior cell interaction with SAXS (ELETTRA, ESRF, SOLEIL)

Interaction of ENMs with cell culture media including serum is an important investigation to understand the underlying mechanisms of the toxicity and internalization. SAXS is the technique of the choice for a fast screening of the morphology of ENMs in cell culture media with and without serum or any other application vehicle under various conditions (pH, storage time, temperature, etc.) before the interaction with cells.[43] [44].

Here, the high throughput auto-sampling capabilities at the SAXS beamline[45] (ELETTRA), BioSAXS beamline ESRF, Swing beamline will be used to reveal shape, size distribution, the aggregation state and interaction with the media at various concentrations in the range of 0.1 untill 10 mg/ml. The ENMs solutions prepared with specific protocols (degradation, toxicity tests) will be loaded into 96 well plates (volume about 100µl), mounted and automatically measured three times.

The acquired SAXS data will be analysed applying the standard methods for such type of investigations, in which the form factor will reveal shape and size distribution and the structure factor the state of aggregation and interactions with the media. This will provide information of the ENMs morphology interacting with the model cell lines.

6.6 Revealing the intracellular state of ENMs with SAXS (ELETTRA, ESRF, SOLEIL)

The internalization of ENMs into cells lead frequently to the formation of larger ENM clusters within the intracellular compartments such as lysosomes. One method to investigate such structures in cells being in their liquid state without fixation is SAXS [46] , in which the cells lines will be detached

[41] Decelle J., Stryhanyuk H., Gallet H., Veronesi G., Schmidt M., Balzano S., Marro S., Uwizeye C., Jouneau P-H, Lupette J., Jouhet J., Maréchal E., Schwab Y., Schieber N.L, Tucoulou R., Richnow H., Finazzi G., Musat M, Algal Remodeling in a Ubiquitous Planktonic Photosymbiosis,
Current Biology,2019, Volume 29, Issue 6. Pages 968-978

[42] Tardillo Suárez V., Gallet B., Chevallet M., Jouneau PH, Tucoulou R. , Veronesi G., Deniaud A., Correlative transmission electron microscopy and high-resolution hard X-ray fluorescence microscopy of cell sections to measure trace element concentrations at the organelle level,
Journal of Structural Biology,2021, Volume 213, Issue 3.

[43] F. Giulimondi , L. Digiacomo , D. Pozzi , S. Palchetti , E. Vulpis , A. L. Capriotti , R. Z. Chiozzi , A. Laganà , H. Amenitsch , L. Masuelli , M. Mahmoudi , I. Screpanti , A. Zingoni and G. Caracciolo , *Nat. Commun.*, 2019, 10 , 1 —11

[44] Le Goas M, Roussel T., Kalbazova M., Carrière D., Barruet E., Geertsen V. , Fadda G.C., Testard F., Carrot G.,Renault J.P. Combining surface chemistry modification and in situ small-angle scattering characterization to understand and optimize the biological behavior of nanomedicines *J. Mater. Chem. B*, 2020,8, 6438

[45] Haider R et al. Journal of Applied Crystallography, 2021, 54, pp. 132-141

[46] Mulens-Arias V., Balfourier A. , Nicolás-Boluda A. , Carn F. and Gazeau F. Endocytosis-driven gold nanoparticle fractal rearrangement in cells and its influence on photothermal conversion, Nanoscale, 2020, 12, 21832–21849

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

from their support and cleaned following standard protocols in the literature. The SAXS measurements will be performed on concentrated cells prepared through centrifugation of the X-ray capillaries filled with 100 µl cell solutions. The aggregation as well as the structure of the ENMs will be determined by applying appropriate SAXS models for the interpretation of the obtained SAXS data.

7 CONCLUSIONS

This report presents a set of entry protocols/methods to be used for studies focused on the understanding of the mechanism of interactions between ENMs and biological systems. The different phases of analysis ranging from nanomaterials characterisation to fine cell interaction analyses are described and related to the set of techniques available in the NFFA catalogues.

The current activities in this work package are moving towards the demonstration of one ENMs characterization workflow. This workflow is illustrated in Figure 9.

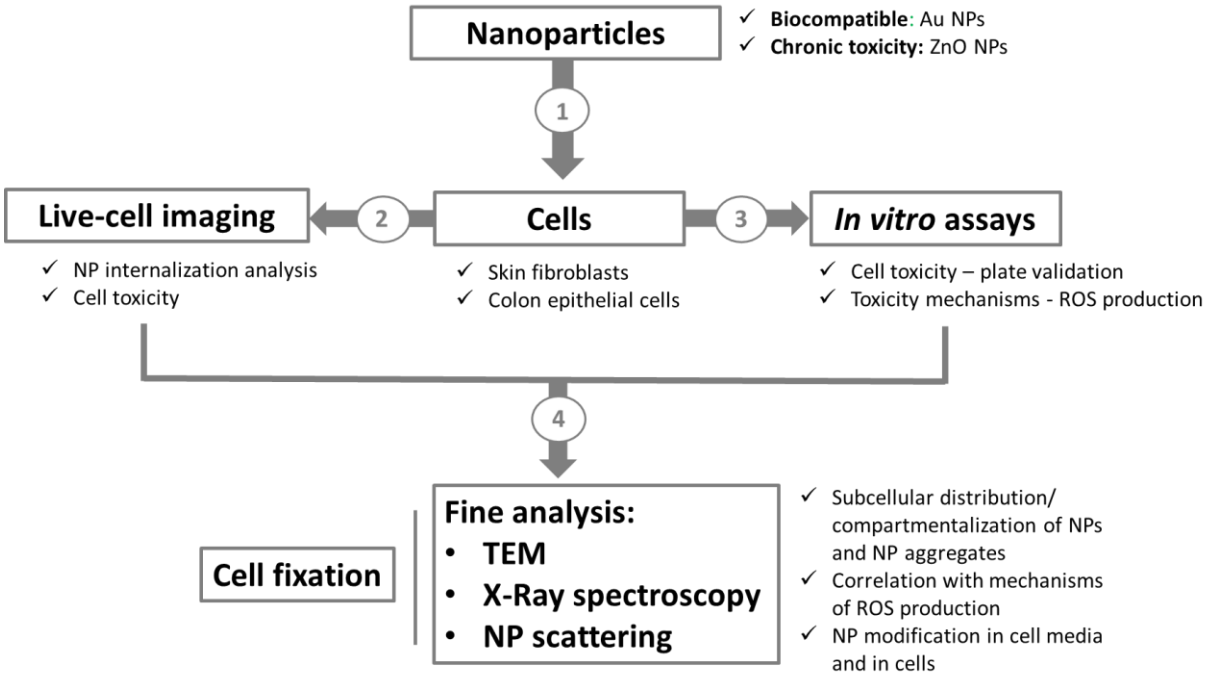

Figure 9: Workflow of characterization for Au and ZnO nanoparticles (NPs) within the NFFA catalogues.

The general concept of the workflow selected for initial implementation is to use the combination of model cells and ENMs that will provide biological and physicochemical signatures across the multiple complementary techniques described in this document. Specifically, gold and ZnO NPs have been selected as models of NPs that are expected to induce, respectively, minimal and chronic cytotoxicity. Furthermore, the expected mechanism of cytotoxicity induced by ZnO NPs is via the production of reactive oxygen species (ROS). The ROS mechanism can be tracked in live cells via both optical microscopy and *in vitro* assays. This mechanism is also expected to involve surface modification of the ZnO NPs and their compartmentalization inside the cells, i.e., phenomena that will be observable using the complementary fine-analysis techniques.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

A combination of two high resolution microscopies is proposed for the fine analysis. X-ray micro-spectroscopy at 500nm resolution at ESRF (France) beamline ID21 will allow to localize the ZnO NPs in the fibroblast cells and determine the amounts of Zn ions that can be potentially released from these NPs. Thanks to the chemical specificity of X-ray Absorption Spectroscopy at the nano-scale we could distinguish between ZnO species and ionic allowing to obtain spatially resolved information about the transformation of these NPs in cellular compartments. Electron based microscopy at IOM-CNR will complement this information with higher resolution imaging (~2nm) and will allow to observe structural damage to the cells and localize the ZnO NPs thanks to X-ray fluorescence detection. The samples will be prepared at JRC-Ispra (Italy) by chemical fixation/dehydration followed by epoxy resin embedding and sectioning with an ultra-microtome.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

ANNEX 1: ENMS CHARACTERISATION

A. ISO Standards on particle sizing

Sampling and sample preparation

ISO 14887:2000: Sample preparation - Dispersing procedures for powders in liquids Methods of dispersion for assessment of dispersion

Kaur, I., Ellis, L. J., Romer, I., Tantra, R., Carriere, M., Allard, S., Mayne-L'Hermite, M., Minelli, C., Unger, W., Potthoff, A., Rades, S., Valsami-Jones, E. Dispersion of Nanomaterials in Aqueous Media: Towards Protocol Optimization. *J. Vis. Exp.* (130), e56074, doi:10.3791/56074 (2017).

Sizing

- ISO 9276-2:2014: Representation of results of particle size analysis - Part 2: Calculation of average particle sizes/diameters and moments from particle size distributions
ISO 9276 -6:2008: Representation of results of particle size analysis –Part 6: Descriptive and quantitative representation of particle shape and morphology

Electron microscopy

ISO 13322 - 1:2014, Particle size analysis – Image analysis methods – Part 1: Static image analysis methods

Particle tracking analysis

ISO/DIS 19430, Determination of particle size distribution – Particle tracking analysis

Inductively coupled plasma–mass spectrometry

ISO/TS 19590:2017, Nanotechnologies Size distribution and concentration of inorganic NPs in aqueous media via single particle inductively coupled plasma mass spectrometry

Centrifugation based methods

ISO 13318 - 1:2001, Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 1: General principles and guidelines

ISO 13318 -2:2007: Determination of particle size distribution by centrifugal liquid sedimentation methods –Part 2: Photocentrifuge method

ISO 13318 - 3:2004, Determination of particle size distribution by centrifugal liquid sedimentation methods – Part 3: Centrifugal X-ray method

Light scattering based methods:

ISO 13321:1996, Particle Size Analysis –Photon Correlation Spectroscopy
ISO 22412:2017, Particle size analysis – Dynamic light scattering (DLS)
ISO 13320:2009*, Particle size analysis - Laser diffraction methods.*
ISO 17867:2015, Particle size analysis –Small -angle X-ray scattering

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

BET, Brunauer-Emmett-Teller :

ISO 9277:2010: Determination of the specific surface area of solids by gas adsorption – BET method

B. JRC reports :

1. Singh C. et al., NM-Series of Representative Manufactured Nanomaterials. Zinc Oxide NM-110, NM-111, NM-112, NM-113 Characterisation and Test Item Preparation. EUR 25066 EN, Publications Office of the European Union, Luxembourg, 2011, ISBN 978-92-79-22215-3, ISSN 1831-9424, doi:10.2787/55008. JRC 64075.
2. Singh C. et al., NM-Series of Representative Manufactured Nanomaterials Cerium Dioxide, NM-211, NM-212, NM-213. Characterisation and test item preparation, EUR 26649 EN, Publications Office of the European Union, Luxembourg, 2014, ISSN 1831-9424 (online), Doi:10.2788/80203, JRC89825.
3. Rasmussen K. et al., NM-Series of Representative Manufactured Nanomaterials, Titanium Dioxide, NM-100, NM-101, NM-102, NM-103, NM-104, NM-105: Characterisation and Physico-Chemical Properties, EUR 26637 EN , Publications Office of the European Union, Luxembourg, 2014,ISSN 1831-9424 (online) , doi: 10.2788/79554 (online). JRC 86291
4. Drewes C.C et al, Physicochemical characterisation of gold, silica and silver NPs in water and in serum-containing cell culture media, EUR 29054 EN, Publications Office of the European Union, Luxembourg, 2018, ISBN 978-92-79-77705-9, doi 10.2760/818663, PUBSY No. JRC110379.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

ANNEX 2: CELL MODELS AND *IN VITRO* ASSAYS

Cell models and studies

ISO 10993-5 was prepared by Technical Committee ISO/TC 194, Biological evaluation of medical devices. Parts 5 and 12 are the standard guidelines used as references.

— Part 1: Evaluation and testing within a risk management process
— Part 2: Animal welfare requirements
— Part 3: Tests for genotoxicity, carcinogenicity and reproductive toxicity
— Part 4: Selection of tests for interactions with blood
— Part 5: Tests for in vitro cytotoxicity
— Part 6: Tests for local effects after implantation
— Part 7: Ethylene oxide sterilization residuals
— Part 9: Framework for identification and quantification of potential degradation products
— Part 10: Tests for irritation and skin sensitization
— Part 11: Tests for systemic toxicity
— Part 12: Sample preparation and reference materials
— Part 13: Identification and quantification of degradation products from polymeric medical devices
— Part 14: Identification and quantification of degradation products from ceramics
— Part 15: Identification and quantification of degradation products from metals and alloys
— Part 16: Toxicokinetic study design for degradation products and leachables
— Part 17: Establishment of allowable limits for leachable substances
— Part 18: Chemical characterization of materials
— Part 19: Physico-chemical, morphological and topographical characterization of materials [Technical Specification]
— Part 20: Principles and methods for immunotoxicology testing of medical devices [Technical Specification]

Methods/protocols (adapted from NCL and ISO databases):

- MTT/LDH assays: Stern ST, Adiseshaiah PP, Potter TP, NCL Method GTA-1: LLC-PK1 Kidney Cytotoxicity Assay.
https://ncl.cancer.gov/resources/assay-cascade-protocols
DOI: 10.17917/Q6EC-XC97
https://ncl.cancer.gov/sites/default/files/Pharmacology%20and%20Toxicology%20of%20Nanomedicines.pdf
- LIVE/DEAD Cell Imaging assay
https://www.thermofisher.com/pt/en/home/references/protocols/cell-and-tissue-analysis/protocols/live-dead-cell-imaging-kit-488-570.html
- Celltox DNA assay.
https://www.promega.com/-/media/files/resources/protocols/technical-manuals/101/celltox-green-cytotoxicity-assay-protocol.pdf
- Caspase 3 assay: Stern ST, Potter TP, NCL Method GTA-6, HepG2 Hepatocarcinoma Apoptosis Assay.
https://ncl.cancer.gov/resources/assay-cascade-protocols
DOI: 10.17917/3F7T-M688

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

- Caspase 3/7 activation assay: Stern ST, Neun BW, NCL Method GTA-14: Hep G2 Hepatocarcinoma Homogeneous apoptosis Assay.
https://ncl.cancer.gov/resources/assay-cascade-protocols
DOI: 10.17917/95VC-ZP26
- Glutathione assay: Stern ST, Potter TM, Neun BW, NCL Method GTA-3: Hep G2 Hepatocyte Glutathione Assay.
https://ncl.cancer.gov/resources/assay-cascade-protocols
DOI: 10.17917/EHR9-HB86
- Lipid peroxidation assay: Stern ST, Potter TP, Neun B, NCL Method GTA-4: Hep G2 Hepatocyte Lipid Peroxidation Assay.
https://ncl.cancer.gov/resources/assay-cascade-protocols
DOI: 10.17917/H65D-SJ70
https://www.thermofisher.com/order/catalog/product/C10445#/C10445
- Reactive oxygen species assay: Stern ST, Zolnik BS, NCL Method GTA-7: Hepatocyte Primary ROS Assay.
https://ncl.cancer.gov/resources/assay-cascade-protocols
DOI: 10.17917/0WF6-GX40
- Autophagic dysfunction assay: Stern ST, Neun BW, NCL Method GTA-12: Autophagic Dysfunction in LLC-PK1 Cells.
https://ncl.cancer.gov/resources/assay-cascade-protocols
DOI: 10.17917/YPTH-N396
- MAP LC3I to LC3II conversion assay: Stern ST, McLeland C, Rodriguez J, NCL Method GTA-11: Autophagic Dysfunction Assay: Qualitative Analysis of MAP LC3-I II Conversion By Western Blot.
https://ncl.cancer.gov/resources/assay-cascade-protocols
DOI: 10.17917/WY7Z-MD10
- Micronucleus assays: ISO OECD. Test no. 487: in vitro mammalian cell micronucleus test. OECD, Paris.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

ANNEX 3: PROTOCOLS FOR CELL STAINING

- Cell proliferation/division: BioTracker NTP-Transporter-based assay
https://www.sigmaaldrich.com/PT/en/technical-documents/technical-article/cell-culture-and-cell-culture-analysis/imaging-analysis-and-live-cell-imaging/cell-tracking-lipophilic-membrane-dyes
- Cell migration and adhesion: Wound Healing and individual cell-tracking assays
https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6587234/pdf/fcell-07-00107.pdf
- Cell morphology and Cytoskeleton organization: Cell plasma membrane, Actin and Microtubule imaging assays
https://www.cytoskeleton.com/live-cell-reagents/memglow-membright-fluorogenic-membrane-probes
https://www.cytoskeleton.com/tubulins/live-cell-imaging-tubulin
- Nanoparticle interaction and entry into cells: Nanolive label-free live cell imaging, fluorescent NPs-cell interaction/internalization analysis
www.nanolive.ch/applications/overview/cell-material-interactions/
https://doi.org/10.1039/C4CS00392F

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

ANNEX 4: PROTOCOLS FOR CELL FIXATIONS.

Cell fixation for Electron Microscopy (at LUND University)
Be careful when you work with PFA, it is toxic!

1. Pre-warm PBS to room temperature.

2. Carefully wash the cell layer in the insert with 100 µl PBS on both sides of the membrane three times.
3. Add 100 µl EM fixing solution (2 % PFA and 2 % glutaraldehyde in Sorensen phosphate buffer) on top of the cells. Incubate in room temperature for 1 h.

4. Pipette away the solution and wash with 100 µl Sorensen phosphate buffer for 1 min three times.
5. Add 200 µl Sorensen phosphate buffer and leave at 4°C.
6. Look at the samples one time every day; do not let the samples dry out. If needed, add more Sorensen phosphate buffer.

7. Store the liquids at 4°C.

Cell fixation for Fluorescent Microscopy (at LUND University)*Be careful when you work with Formalin, it is toxic!*

1. Pre-warm PBS to room temperature.

2. Carefully wash the cell layer in the insert with 100 µl PBS on both sides of the membrane three times.
3. Add 100 µl FM fixing solution (10 % Formalin in PBS) on top of the cells. Incubate in room temperature for 1 h.

4. Pipette away the solution and wash with 100 µl PBS for 1 min three times.
5. Add 200 µl PBS and leave at 4°C.
6. Look at the samples one time every day; do not let the samples dry out. If needed, add more PBS.

7. Store the liquids at 4°C.

Cell fixation for Scanning Electron Microscopy (SEM) (at FORTH)

- After the selected times points, the cells will be fixed following a specific fixation protocol. The protocol should be performed at 4°C:
- Remove the cell culture medium from samples and wash them twice (x2) with 0.1M sodium cacodylate buffer (SCB) for 5min each.
- Fix them with a solution of 2.5% glutaraldehyde (GDA)/ 2.5% parafolmadehyde (PFA) in 0.2M SCB for 30 min.
- Wash the samples twice (for 10 min each time) with SCB
- Dehydration phase: A graded series of 30%, 50%, 70%, 90%, and 100% EtOH for 10 min each
- The standard critical point dryer protocol is used for hard materials eg silicone, polymer, metals

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project

• Please perform the steps below under a chemical hood: (for sensitive materials eg biodegradable polymers): Immerse the samples in hexamethyldisilizane (HDMS)/ EtOH (50:50) solution for 30 min twice
• Final step is the 100% HDMS for 20 min twice
• Remove the HDMS and let the samples dry completely overnight under the chemical hood.
• Prior to SEM imaging, the samples should be sputter-coated with a 10-15nm film of Au (BAL-TEC SCD 050).

Cell fixation for Confocal Microscopy (at FORTH)

After the selected times points, the cells will be fixed following a specific fixation protocol.

The protocol should be performed at room temperature (RT):
• Remove the cell culture medium from samples and wash them twice (x2) with PBS (pH = 7.4) for 5min each.
• Fix them with 4% paraformaldehyde (PFA) for 15 min
• Wash them again with PBS twice for 5 min each
• Treat them with Triton-X100 0.1% solution in PBS for 5 min in order to permeabilize cell membranes.
• Wash them again with PBS twice for 5 min each
• Block them using 2% Bovine serum albumin (BSA) blocking buffer in PBS solution for 30 min
• Incubate the samples with the first antibody (specific concentration) overnight at 4oC.
• Next day, wash them with PBS and incubate with the secondary antibody (specific concentration) for 2 h.

Nuclear staining carried out by 4,6-diamidino- 2-phenylindole (DAPI 1:10.000 in PBS) is the final step after you transfer the samples on microscope slides for observation using a 'Leica SP8' laser scanning confocal microscope.

This initiative has received funding from the EU's H2020 framework program for research and innovation under grant agreement n. 101007417, NFFA-Europe Pilot Project