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DELIVERABLE REPORT

WP14 JA4 - A safe-by design platform for nanomaterials

D14.2
Delivery of the operando microscopy station

Due date
M24

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

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WORK PACKAGE ID	WORK PACKAGE TITLE
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WORK PACKAGE LEADER

Dr. Hiram Castillo-Michel (ESRF)

DELIVERABLE DETAILS

DELIVERABLE ID	DELIVERABLE TITLE
D 14.2	Delivery of the operando microscopy station

DELIVERABLE DESCRIPTION

This deliverable involves the development of a sustainable biological substrate holder installed in-operando at a microscope for the live imaging of the interactions of cells on patterned substrates with and without nanoparticles exposure.

DUE DATE	ACTUAL SUBMISSION DATE
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NATURE

☐ R – Report

☒ P – Prototype

☐ DEC - Websites, Patent filing, Press & media actions, Videos, etc

☐ O – Other

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

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D14.2 DELIVERY OF THE OPERANDO MICROSCOPY STATION

Subtask 14.2.1: Development of a sustainable biological substrate holder

The ultimate goal of this subtask is to provide a platform to understand the phenomena occurring at the interfaces between contacted nanomaterials and biological materials, enabling users to implement a safe-by-design approach for new materials and to understand the life cycle of nanomaterials in biological systems. For this reason, a sustainable biological substrate holder incorporated patterned 2D and 2.5D substrates was developed and installed in a microscope for real-time imaging with and without nanoparticle (NP) exposure.

In particular, we successfully developed 2D (flat) and 2.5D (microgrooves) polydimethylsiloxane (PDMS) substrates that could be inserted in the biological substrate holder (µ-slide I Luer 3D microfluidic chamber, ibidi). The substrates were used in direct contact with an immortalized mouse fibroblast (NIH 3T3) cell line to investigate the interactions of the cells with the substrates and the effect of ZnO NPs under static and dynamic (under flow) conditions. Prior to using PDMS substrates, polyethylene terephthalate (PET) substrates (TCP coverslips) were also used as control samples. The main focus of the experiments was to evaluate the optimum ZnO concentrations that would not be cytotoxic in this system and the study of the cellular-nanomaterial-PDMS surface interactions under live imaging. There was a series of optimization steps that were performed to overcome some obstacles of the *in-vitro* experiments. All the above experiments were performed at FORTH.

Fabrication of the PDMS substrates (2D/flat and 2.5D microgrooves) via soft lithography

The PDMS mixture was prepared by mixing the PDMS prepolymer (base) and a curing agent (PDMS Sylgard®184) in a 10:1 ratio by weight. The PDMS degassed, to remove the bubbles generated during the mixing, and then this was poured into various master samples e.g. a well (from a 24 well plate), PET substrates with laser fabricated microgrooves and directly on ibidi µ-slide I Luer 3D microfluidic chambers. Different volumes of the PDMS solution were used to achieve the optimum sample thickness to fit in the microfluidic chambers. All of them were cured at 80 °C for 3 h (Vacucenter VC20 Vacuum Oven, Salvis). The samples are left to cool down and reach room temperature and then were peeled off from well plate, microfluidic wells and microgrooves and cut to fit properly on 5.4 mm x 4.0 mm x 0.8 mm height wells of microfluidic device. Figure 1 presents the PDMS samples fabricated via soft lithography.

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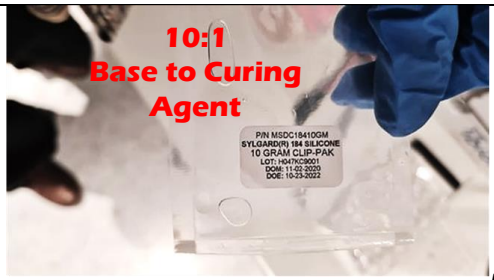


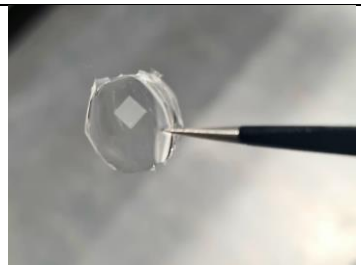
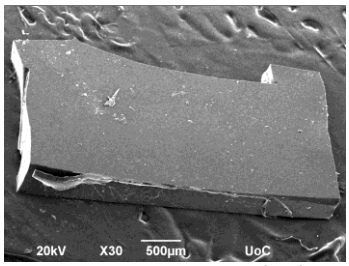
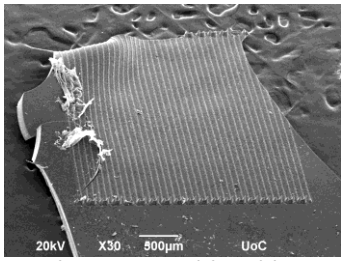


Photo of the PDMS flat sample (left) and replica sample (microgroooves) (right)

PDMS is the best material because:

- It's biocompatible
- It's transparent
- Hydrophobic; non-specific absorption of small molecules
- Easy hydrophilic modification for cell seeding;
- Easy to fabricate, deformable and cheap





SEM images of the PDMS flat (left) and replica (microgroooves) (right) (tilted view)

Figure 1: PDMS material before and after the soft lithography process performed. Photos and SEM images (tilted view) show the PDMS flat and microgrooves.

Preparation of the samples prior to the cell studies

The samples were left in UV light for 30 min to sterilize them. Laminin, which is an extracellular matrix multi-domain trimeric glycoprotein, was used to coat the samples (2 hours at 37°C, 5% CO_2 incubator).

ZnO NPs: Characteristics and Preparation

One of the characteristics of ZnO NPs is the formation of agglomerations. Environmental conditions such as pH, ionic strength and natural organic matter content seem to affect dissolution and agglomeration rate [1]. Also, different synthesis methods and capping agents could influence the agglomeration, particle size and morphology of fabricated ZnO NPs [2]. The type of ZnO NPs, in the form of powder, that is going to be used in this study is NM-110, provided by JRC to FORTH. Figure 2 shows some general characteristics of the specific ZnO group. Their size could range from 30 - 150 nm but there are mainly present as clusters. It seems also to form different shapes like a rod, star-like and isometric and their cluster size distribution has a broad range of 100 - 1000 nm (Figure 2c). DLS measurements are also depicted below (Figure 2d). The mean cluster size is approximately 275 nm. Table 1 shows the size and dispersion stability of NM-110 in DI water.

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[1] Domingos R., Rafiei Z., Monteiro C.E., Khan M., Wilkinson K.J., Agglomeration and dissolution of zinc oxide nanoparticles: role of pH, ionic strength and fulvic acid, Environmental Chemistry 2013 10(4) 306-312 https://doi.org/10.1071/EN12202

[2] Hozyen HF, Ibrahim ES, Khairy EA, El-Dek SI. Enhanced antibacterial activity of capped zinc oxide nanoparticles: A step towards the control of clinical bovine mastitis. Vet World. 2019 12(8) 1225-1232. doi: 10.14202/vetworld.2019.1225-1232

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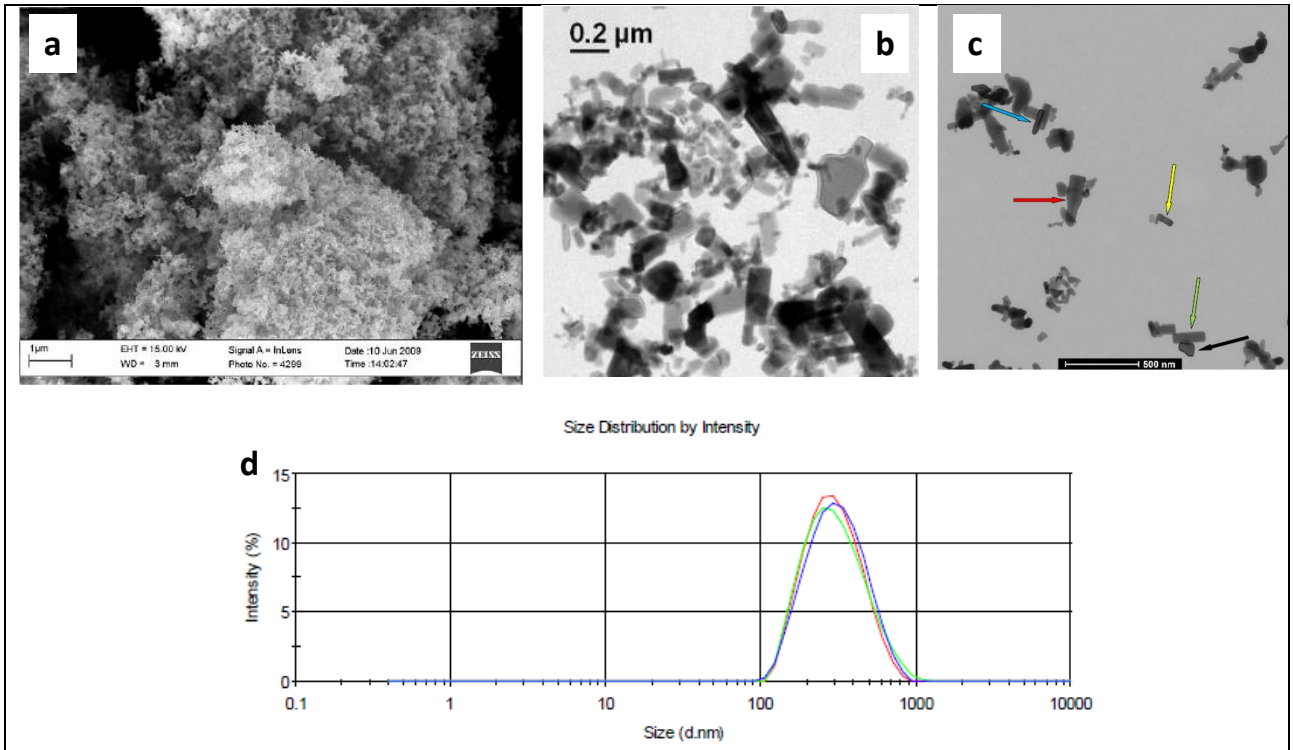

Figure 2: (a) SEM image of NM-110, with high agglomeration of particles; (b) TEM image of NM-110, showing again high agglomeration; (c) Different size and shapes of the primary particles of NM-110. Bottle-like (red arrow), rod-shaped (yellow arrow), larger (green arrow) and smaller (blue arrow) rectangular NP and angular NP (black arrow) were observed among other shapes; (d) Dynamic Light Scattering measurements for NM-110 ZnO NPs. Mean size distribution.

Table 1: Measurements about characteristic of size and dispersion stability of NM-110.

NM-110 Methods	**DI water**
Dynamic Light-Scattering - DLS	275 nm
Centrifugal Particle Sedimentation - CPS	193±3 nm
Dispersion stability	4038 min

The NM-110 ZnO NPs do not have a coating. Its initial condition was powder (Figure 3). The dilution of ZnO NPs was performed in PBS at 1% ZnO in PBS (10 mg/mL). From these initial concentrations, lower concentration dispersions were prepared (Table 2).

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Figure 3: NM-110 ZnO NPs in their initial condition.

The further dispersion of ZnO NPs solution on cell culture medium was followed in order to perform the *in-vitro* cytotoxicity studies. Dulbecco's Modified Eagle Medium (DMEM) high glucose without fetal bovine serum and 1% PS mixed with ZnO solution in different dilutions. The main objective of this study was to find out the concentrations of the ZnO solution that were not toxic to the cells. Table 2 demonstrated the different concentrations of ZnO NP powder diluted in 1 ml of PBS (1x). Serial dilutions of the ZnO NPs concentrations were further performed at 1:10 and 1:2 in DMEM medium without (w/o) FBS and PS.

Table 2: Different concentrations of ZnO NP powder were diluted in 1mL PBS.

Concentrations of ZnO in PBS	**OR**	µg/mL
1%		10000
0.5%		5000
0.01%		100
0.0075%		75
0.0050%		50
0.0025%		25
0.001%		10
0.0001%		1

In-vitro cytotoxicity study: MTT assay – Viability Assay

The MTT assay is used to measure cellular metabolic activity as an indicator of cell viability, proliferation and cytotoxicity. It is a colorimetric assay and it is based on the reduction of yellow tetrazolium salt (3- (4,5-dimethyl-2-thiazolyl) -2,5-diphenyl-2H-tetrazolium bromide or MTT) to purple by the action of mitochondrial succinate dehydrogenase in the mitochondria of living cells. The darker the solution, the greater the number of viable, metabolically active cells.

Cytotoxicity of the ZnO NP solution

In these experiments, NIH 3T3 cells were seeded in a concentration of 50000 cells/mL in high glucose DMEM medium with 10% FBS and 1% PS in order to have an 80% cell confluency

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(overnight). Various ZnO concentrations (w/o FBS_PS) were added to the cells and left for 24 hours at 37°C, 5% CO_2 in a humidified environment. MTT assay (5 mg/mL in PBS) was performed after 24 hours in order to obtain which of the ZnO dilutions were toxic and which were not. MTT solution was added on DMEM-HG w/o FBS_PS (50:50) and left for 3 hours in the incubator. DMSO was added to dissolve the insoluble purple formazan product into a colored solution (purple). The absorbance of wells was measured at 545 nm wavelength at an ELISA plate reader. The data as Optical Density (OD) measurements were in .txt files and exported into an excel file for further processing. Figure 4 shows the three experiments performed to optimize the MTT protocol in order to obtain the optimum non-toxic ZnO NPs concentrations on the mouse fibroblasts after 24 hours.

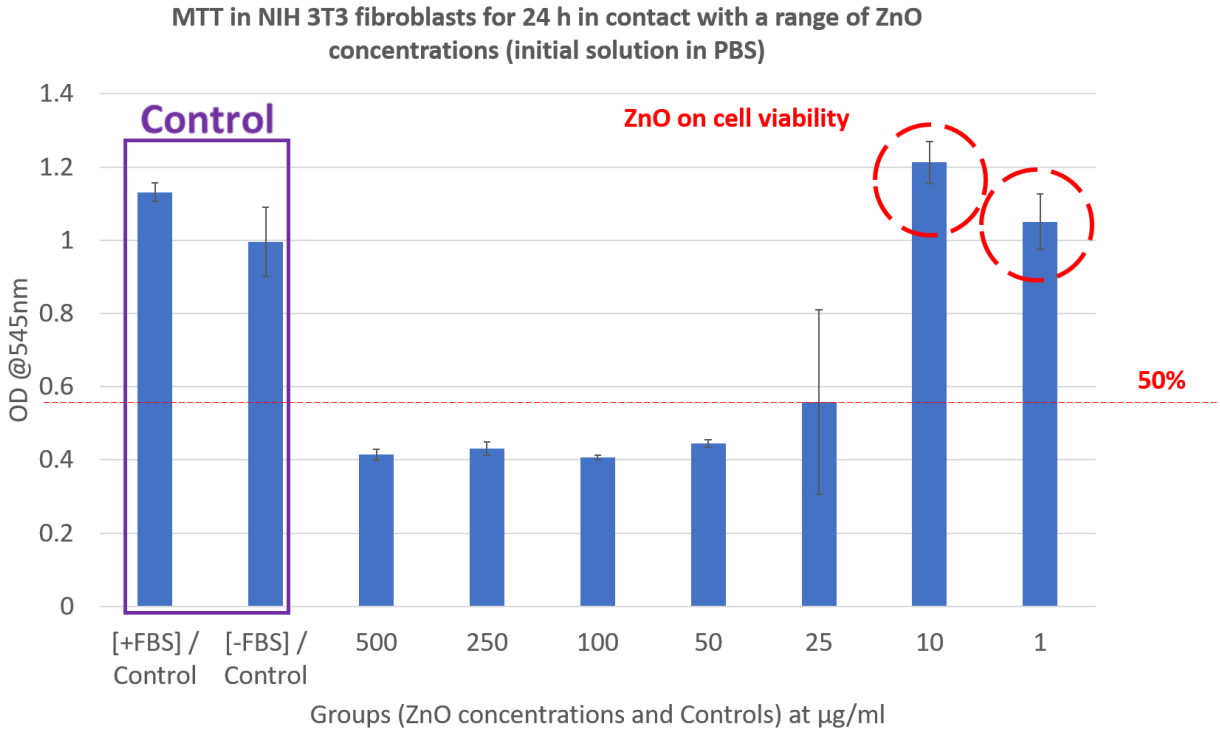

Figure 4: MTT assay on various ZnO NPs concentrations with NIH 3T3 fibroblasts for 24 hours; An optimization process took place on serial dilutions of the ZnO NP concentrations in order to obtain the non-toxic concentrations for the cells under static conditions.

According to Figure 4, it was observed that all the different ZnO concentrations after 24 hours had an effect on cells' viability and specifically, and most of the ZnO concentrations (25-500µg/ml) decreased the cell viability up to 50%. The 1-10µg/ml ZnO concentrations show cell viability close to the TCP control. This range of ZnO concentrations will be taken into account for further investigation of cell studies under flow conditions with NLOM microscope.

Cell Viability of ZnO NP concentrations on PDMS substrates via Live Dead Assay

This method provides a quick and easy two-color assay to discriminate viable and non-viable cells in a population based on plasma membrane integrity and esterase activity. Live cells are stained with Calcein (green fluorescent protein) while Ethidium homodimer-1 (EthD-1) dye is used to monitor dead cells. It enters cells with damaged membranes producing a bright red fluorescence.

In this set of experiments, the main objective was to investigate the NIH 3T3 cell viability on 2D and 2.5D PDMS substrates after exposure to ZnO NPs after 24 hours. These data are in sequence with

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the MTT measurements. The fibroblasts are seeded in a concentration of 50000 cells/mL in high glucose DMEM medium with 10% FBS and 1% PS onto PDMS flat and microgrooved substrates to reach an 80% confluency (overnight). Selected ZnO NPs concentrations (taken from the MTT results) were added and left for 24 hours. Live/Dead reagent solution was prepared (2 μL of EthD-1 and 0.5 μL Calcein added on 1 mL PBS) and a specific volume was added to each well for 30 min at room temperature. The images were taken by a fluorescence inverted microscope and specifically, a fluorescence light source detecting green (GFP filter) and red fluorescent (Rhodamine filter) were used. Image processing was done using FIJI/ImageJ NIH software.

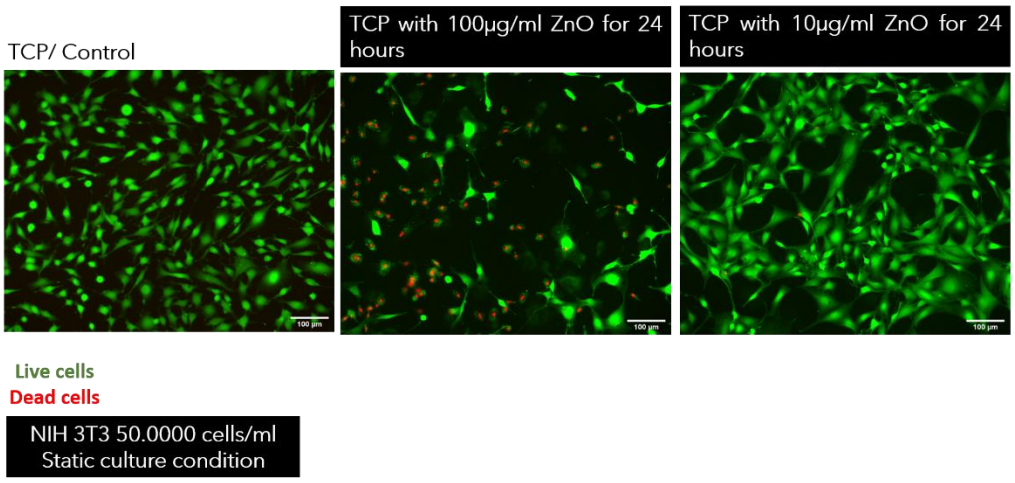

Figure 5(a): Live/Dead Assay of various ZnO NP concentrations on mouse fibroblasts after 24 hours.

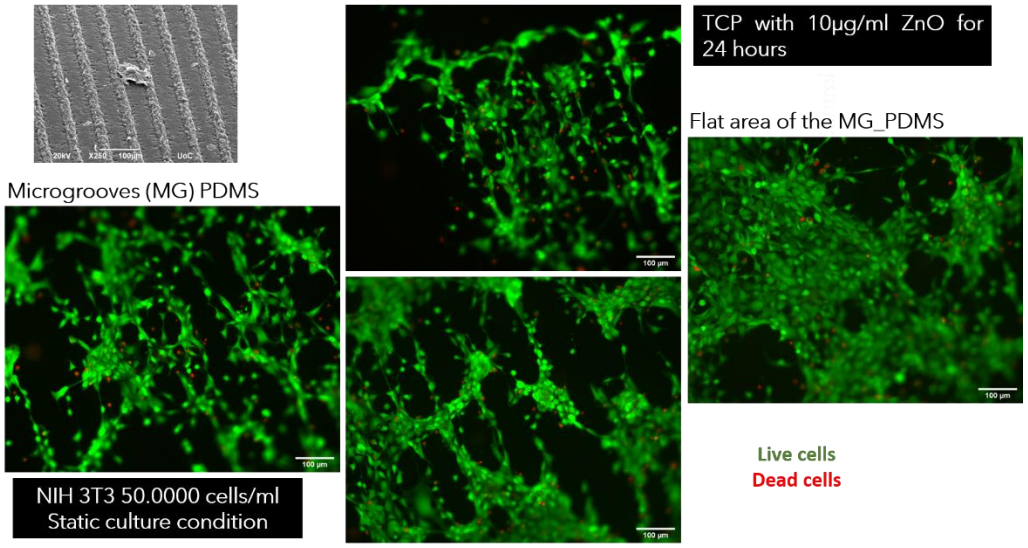

Figure 5(b): Live/Dead Assay of 10 μg/ml ZnO NP concentrations on mouse fibroblasts adhered on PDMS substrates (2D and 2.5D) after 24 hours.

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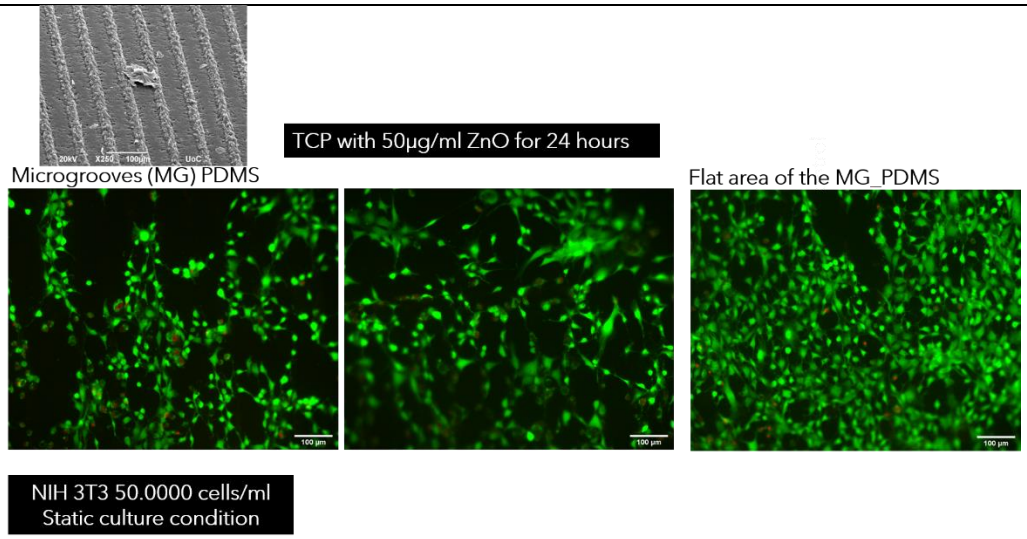
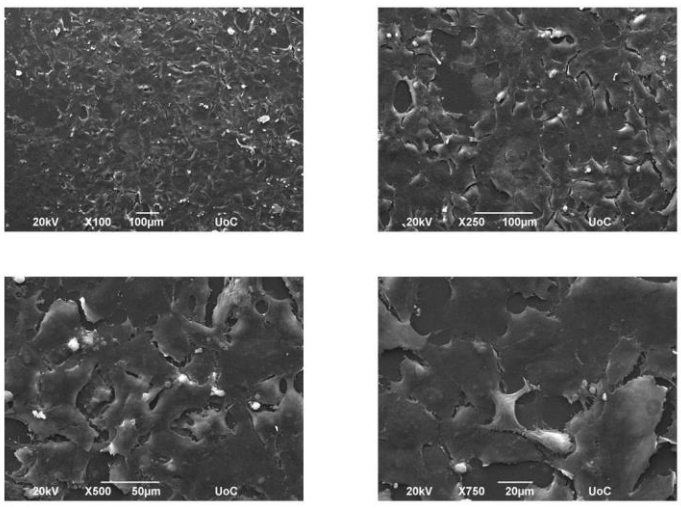


Figure 5(c): Live/Dead Assay of 50µg/ml ZnO NP concentration on mouse fibroblasts adhered on PDMS substrates (2D and 2.5D) after 24 hours.

According to Figure 5, viable fibroblasts are observed in all the groups at the 24 hours in contact with ZnO concentrations of 10, 50 and 100µg/ml (selected concentrations from the MTT results). All the ZnO concentrations above 50µg/ml were toxic even for 24 hours. In PDMS flat substrates, the fibroblasts were adhered and proliferated randomly. For the PDMS microgrooved substrates, the cells adhered and grew in the direction of the microgrooves.

SEM observation of NIH 3T3 cells cultured on PDMS substrates in the presence of ZnO NPs

50000 NIH 3T3 cells/mL were cultured on the PDMS flat and microgrooved substrates. After 24 hours of culture, 10µg/ml ZnO concentration in DMEM-HG medium w/o FBS w/o PS (1/10) was added to the samples. After 2 days of culture, the samples were dried in a critical point drier. NIH 3T3 cells seemed to attach strongly and proliferated well on the samples after the addition of ZnO NPs in this concentration.



(a) Flat PDMS

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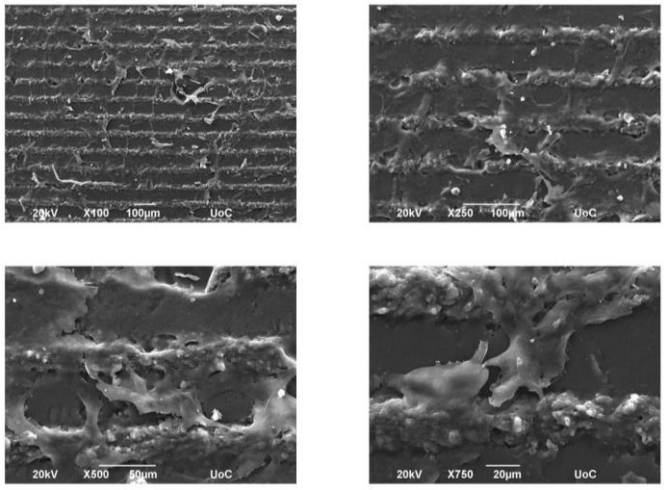

(b) PDMS microgrooved substrate

Figure 6: SEM observation of NIH 3T3 cells cultured on PDMS flat (a) and microgrooved substrates(b) in the presence of ZnO NPs under static conditions.

Observation of ZnO NPs with NLOM microscope

At the same time with the *in-vitro* cell studies, the detection of NPs dispersed in PBS was investigated with the NLOM microscope under static conditions. This step was performed in order to make sure that the tested ZnO NPs concentrations can also be observed by the NLOM microscope. At first, images and z-stacks were taken from one drop of the initial dilutions of 10 mg/mL and 5 mg/mL in glass and after that on PDMS. According to Figure 7, the ZnO concentrations were easily detected and many agglomerations were observed.

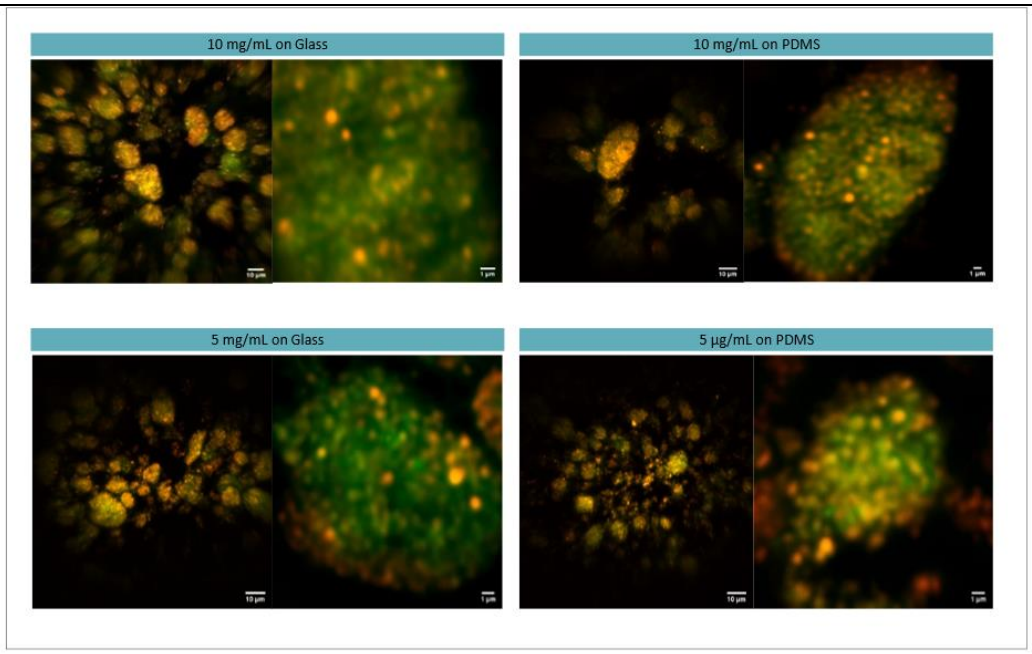

Figure 7. NLOM images of 10 mg/mL and 5 mg/mL of ZnO NPs diluted on PBS on the top of glass and 10 mg/mL and 5 µg/mL of ZnO NPs diluted on PBS on the top of PDMS.

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Under the optimization process, smaller concentrations were prepared in order to observe if agglomerations were reduced or become smaller (Table 2). One drop of each of them was placed above a PDMS scaffold and images were taken from the NLOM microscope (Figure 8). The ultrasonication of the ZnO NP concentrations in PBS at an ultrasonicator bath for 30 min with no temperature and the lower ZnO concentrations improved the agglomeration issue.

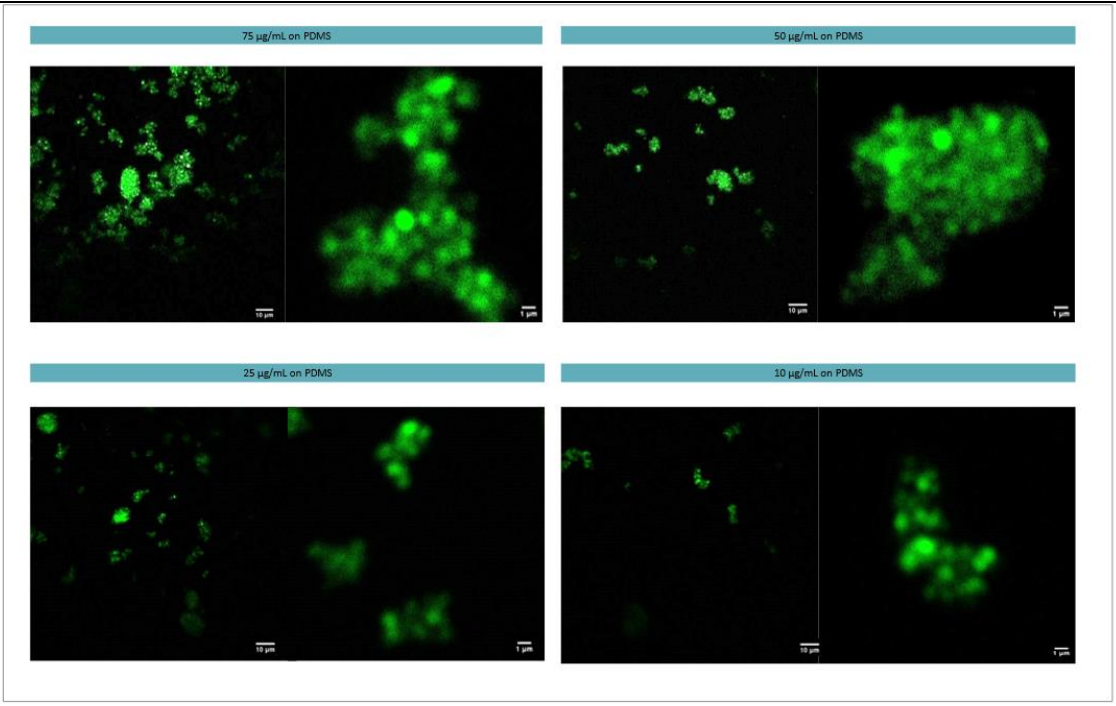

Figure 8. NLOM images of ZnO NPs of 2V and 0.3V in different concentrations.

Observation of fixed NIH 3T3 cells cultured on PET substrates with/without ZnO NPs with the NLOM microscope

Prior to using the PDMS substrates, we cultured NIH 3T3 cells on PET substrates and observed them with the NLOM microscope to optimize some of the parameters of the experiment. 50000 cells/mL were cultured on the substrates. NIH 3T3 cells attached strongly and proliferated well on them. After 24 hours of culture, 0.0001% (1μg/ml) ZnO NPs in DMEM-HG medium w/o FBS w/o PS were added to some of the substrates. After 48 hours of culture, the samples were fixed with PFA and stained with the fluorescent dyes i) Alexa Fluor® 568 Phalloidin (cytoskeleton) and ii) DAPI (nuclei). Then, we proceeded with the observation. We successfully observed the nuclei, the cytoskeleton of the cells, as well as, the ZnO NPs using the NLOM microscope (Figure 9).

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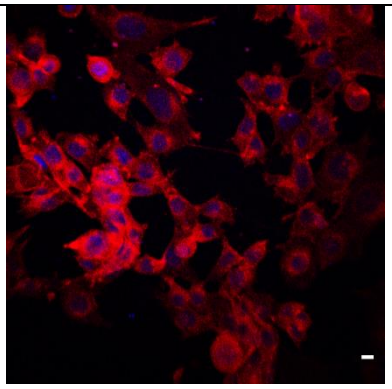
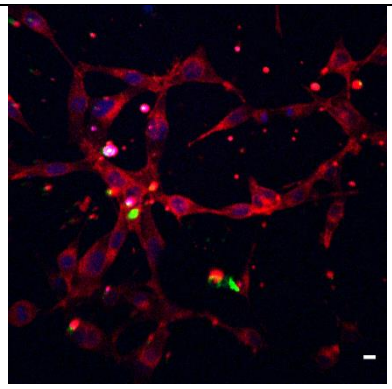
Fixed samples		
	DAPI (nuclei) + Alexa Fluor® 568 Phalloidin (cytoskeleton)	DAPI (nuclei) + Alexa Fluor® 568 Phalloidin (cytoskeleton) + 0.0001% ZnO NPs

Figure 9: Observation of fixed NIH 3T3 cells cultured on PET substrates with/without ZnO NPs, under static conditions, with the NLOM microscope. Scale bar: 10 μm.

Observation of live NIH 3T3 cells cultured on PET substrates with ZnO NPs with the NLOM microscope

Similarly, 50000 NIH 3T3 cells/mL were cultured on the PET substrates. After 24 hours of culture, 0.0001% (1μg/ml) ZnO NPs in DMEM-HG medium w/o FBS w/o PS were added to the substrates. After 48 hours of culture, the samples were stained with the fluorescent dyes i) Biotracker 555 Orange Cytoplasmic Membrane dye and ii) Hoechst (nuclei). As shown in Figure 10, we could observe the nuclei, the membrane of the live cells, as well as, the ZnO NPs at the cells using the NLOM microscope in agreement with the fixed samples.

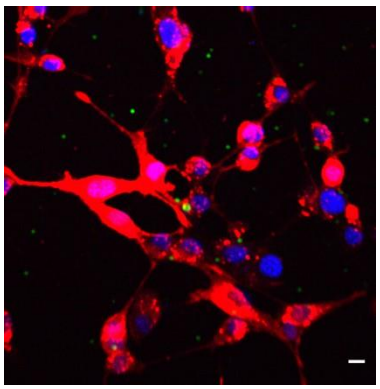
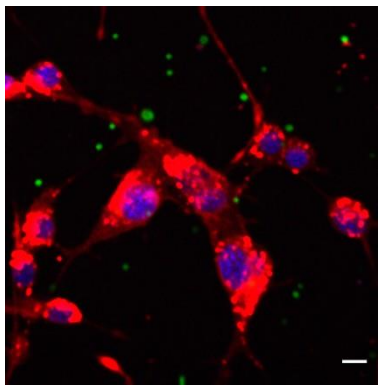
Live samples		
	Hoechst (nuclei) + Biotracker 555 Orange Cytoplasmic Membrane dye + 0.0001% ZnO NPs	

Figure 10: Observation of live NIH 3T3 cells cultured on PET substrates with ZnO NPs, under static conditions, with the NLOM microscope. Scale bar: 10 μm.

Observation of live NIH 3T3 cells cultured on PDMS flat and microgrooved substrates with ZnO NPs inside ibidi μ-slide I Luer 3D microfluidic chamber with the NLOM microscope

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After optimizing some parameters of the experiment using the PET substrates, we cultured 400000 NIH 3T3 cells/mL on the PDMS flat and microgrooved substrates inside the ibidi µ-slide I Luer 3D microfluidic chamber, component of the final demonstrator (Figure 11). NIH 3T3 cells attached strongly and proliferated well on the PDMS substrates. Fluorescent dyes Biotracker 555 Orange Cytoplasmic Membrane dye and Hoechst were used. As depicted in Figures 12 and 13, we could observe the nuclei, the membrane of the live cells, as well as, the ZnO NPs at the cells cultured on the substrates inside the µ-slide I Luer 3D microfluidic chamber with the NLOM microscope.

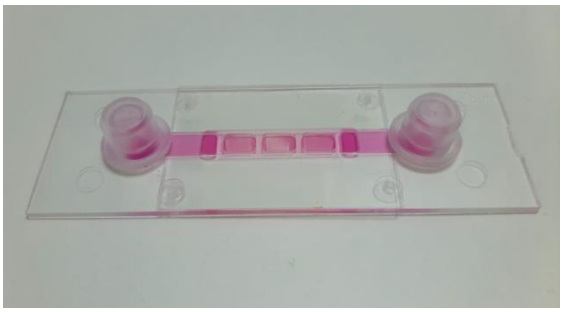


Figure 11: Illustration of ibidi µ-slide I Luer 3D microfluidic chamber (component of the final demonstrator). The well dimensions are 5.4mm x 4.0mm and the well height is 0.8mm.

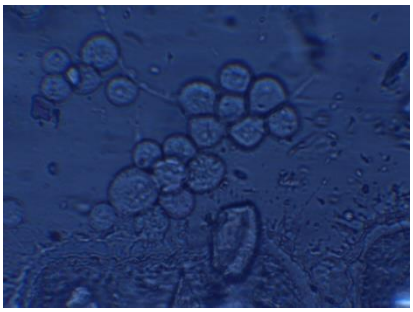
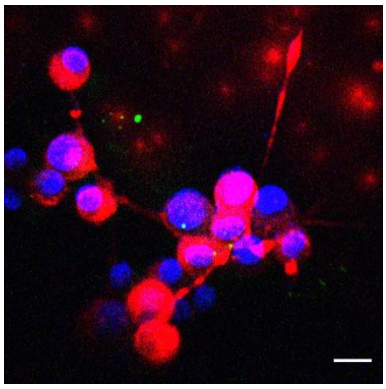
Live samples		
	CCD camera	Hoechst (nuclei) + Biotracker 555 Orange Cytoplasmic Membrane dye + 0.0001% ZnO NPs
	PDMS flat substrate	

Figure 12: Observation of live NIH 3T3 cells cultured on PDMS flat substrate inside ibidi µ-slide I Luer 3D microfluidic chamber, under static conditions, with the NLOM microscope. Scale bar: 10 µm.

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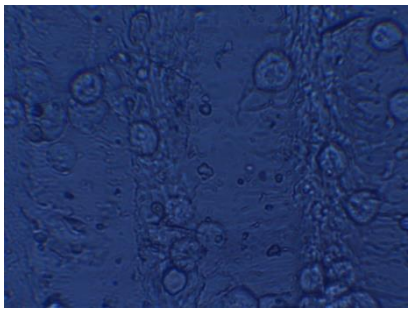
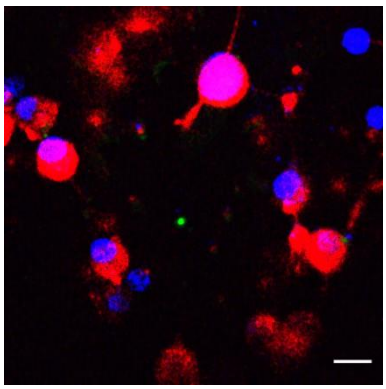
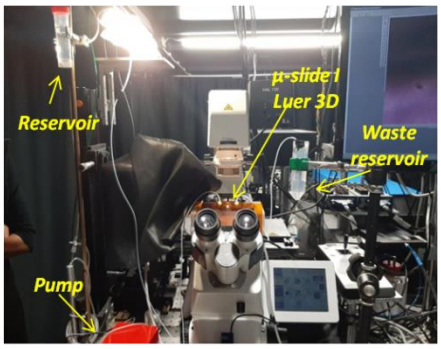
<table>
<tr><td rowspan="3">Live samples</td><td>  </td><td>  </td></tr>
<tr><td>CCD camera</td><td>Hoechst (nuclei) + Biotracker 555 Orange Cytoplasmic Membrane dye + 0.0001% ZnO NPs</td></tr>
<tr><td colspan="2">PDMS microgrooved substrate</td></tr>
</table>

Figure 13: Observation of live NIH 3T3 cells cultured on PDMS microgrooved substrate inside ibidi µ-slide I Luer 3D microfluidic chamber, under static conditions, with the NLOM microscope. Scale bar: 10 µm.

Observation of ZnO NPs in the µ-slide I Luer 3D microfluidic chamber containing the PDMS microgrooved substrates under flow conditions with the NLOM microscope

We set up the microfluidic system for live imaging observation. The microfluidic system is composed of the pump system connected with a reservoir, the ibidi µ-slide I Luer 3D microfluidic chamber and the waste reservoir (Figure 14). We inserted inside the ibidi µ-slide I Luer 3D microfluidic chamber the PDMS microgrooved substrates. A continuous flow (rpm 1) of ZnO NPs (0.001% - 10µg/ml in PBS) was used. As depicted in Figure 15, we could observe the ZnO NPs inside the µ-slide I Luer 3D containing the substrates under flow conditions with the NLOM microscope (final demonstrator). The analytical experimental setup of NLOM microscope was described in D14.1 (submitted).



Microfluidic system

Pump

µ-slide I Luer 3D microfluidic chamber containing PDMS substrates inside microscope's incubator

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Figure 14: Illustration of the microfluidic system. This system is the final demonstrator.

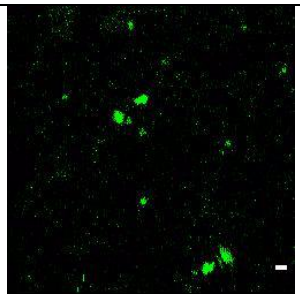

Figure 15: Observation of ZnO NPs in the µ-slide I Luer 3D microfluidic chamber containing the PDMS microgrooved substrates under flow conditions with the NLOM microscope. Scale bar: 1 µm.

Observation of live NIH 3T3 cells cultured on PDMS flat and microgrooved substrates inside ibidi µ-slide I Luer 3D microfluidic chamber with the NLOM microscope

We cultured 600000 NIH 3T3 cells/mL (for more confluence) on the PDMS flat and microgrooved substrates inside the ibidi µ-slide I Luer 3D microfluidic chamber (Figure 16). NIH 3T3 cells attached strongly and proliferated well on the PDMS substrates. The next day, a continuous flow (rpm 1) of cell culture medium was used. As illustrated in Figure 16, we could observe the cells adhered and grew on the system under flow conditions using the NLOM microscope (CCD camera). The experiments under flow with NPs are ongoing, however, they need further optimization.

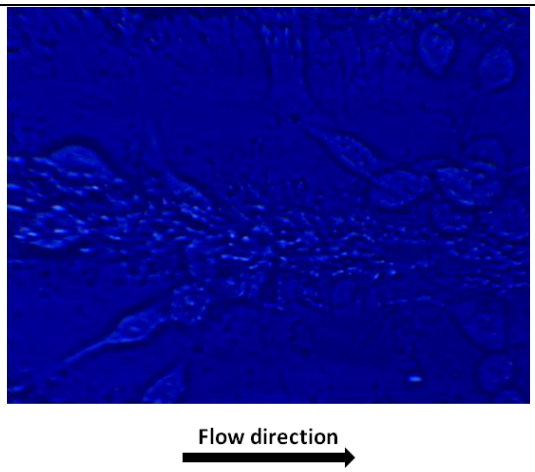

Figure 16: Observation of live NIH 3T3 cells cultured on PDMS microgrooved substrate inside ibidi µ-slide I Luer 3D microfluidic chamber, under flow conditions, with the NLOM microscope (CCD camera).

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Conclusions

We successfully developed PDMS flat and microgrooved substrates that could be inserted in the biological substrate holder (µ-slide I Luer 3D microfluidic chamber, ibidi). Moreover, we examined the optimum ZnO concentrations that would not be toxic to NIH 3T3 cells cultured on PDMS flat and micropatterned substrates in conventional wells plates and in the biological substrate holder for a short-time period. Finally, we successfully set up a microfluidic system containing the biological substrate holder for live imaging observation of the cellular-nanomaterials-PDMS surface interactions.

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