Project:

High Content Screening Multiparametric Assays for Cytotoxicity

Assessment of cellular responses to nanomaterials by means of HCS multiparametric assays: cell viability, cell permeability and mitochondrial membrane potential

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Contents

1 Introduction

The acute cytotoxicity of nanomaterials (or test substances) can be assessed following various exposure routes and exposure times using the High Content Screening (HCS) platform. Following exposure to the materials, the treated cells are stained with various dyes for subcellular compartments or cellular process, including for nuclei, cell membrane permeability, and mitochondrial function. Changes in the fluorescence intensity associated with these probes can give indications of the cytotoxicity of the materials, as a result. Consideration should be taken to ensure that experimental design, cellular models, methods of exposure, and analytical metrics are in line with OECD [1, 2] and European Food Safety Authority (EFSA) guidelines [3].

As such, this protocol describes the approach for testing the cytotoxic responses of human cell lines to nanoparticles (NPs) using the HCS platform by automated imaging and subsequent multiparametric image analysis. The introduction of a "nano" specific SOP enables and expands the use of existing HCS instrumentations for the titration, assessment and safety screening of nanomaterials, or their engineered forms.

As it is an image-based technique for single-cell quantitative analysis, it allows for the avoidance of artefacts and ambiguities in the results produced. Furthermore, it will also circumvent any interference issues originated by the nanomaterials / nanoparticles. The multiparametric nature of the analysis also enables the collection of large volumes of data, providing opportunities for data-mining or bioinformatic analytical approaches.

2 Principle of the Method

Procedures followed are in accordance with REACH on the hazard assessment of chemical entities [4-6] and aspects of the ISO 10993-5 guidelines for in vitro cytotoxicity assessment on the use of liquid samples [7].

The HCS assays detailed in this SOP relate to the measurement of nuclear area, cell permeability, and mitochondrial membrane potential using the Hoechst, Permeability, and Mitochondrial Membrane Potential dyes as part of the Cellomics ® HCS Multiparameter Cytotoxicity Kit. Cells are then imaged used an automated fluorescent microscope, imaging sufficient fields per well to obtain sufficient numbers of cells to be analysed to provide robust data. In the case of this SOP, cells are images using the Cytell Cell Imaging System, and analysis is conducted using the InCell Investigator 1.6 software package (all GE Healthcare, United States). Other imaging and analysis instruments and software packages can also be used. Other imaging and analysis instruments and software packages can also be used [8].

2.1 Changes in Nuclear Size/Morphology

Following a toxic insult, cells often undergo either necrosis or apoptosis, accompanied by changes in nuclear size and/or morphology, depending on the type of toxin [9]. Hoechst 33342 dye, one of the dyes used in this assay, labels DNA and emits a blue fluorescence. The assay uses the nuclear stain to identify individual nuclei and thus cells, and measures changes in nuclear size and morphology that are caused by toxic insult, such as valinomycin, a potent K+ ionophore.

2.2 Cell Membrane Permeability (CMP)

The cell membrane maintains cellular homeostasis by holding the varied constituents of the cell together, providing a specialized environment different from its extracellular surroundings, and providing a mechanism for the controlled exchange of its nutrients with its surroundings. Certain toxins can affect cell membrane integrity leading to the cell becoming permeable [10], eventually causing cell death. Healthy cells are nearly impermeable to the Permeability Dye used in this assay; however, after compromising the cell membrane's permeability, the dye stains the nucleus with a bright green fluorescence.

2.3 Changes in Mitochondrial Mass/Potential Intensity (MMPI)

Changes in mitochondria play a central role in apoptosis [11]. Mitochondria release apoptogenic factors through the outer membrane and dissipate the electrochemical gradient of the inner membrane, which is thought to occur via formation of the mitochondria permeability transition [12]. Mitochondria can proliferate during stimulation of apoptosis [13] leading to an increase in total mitochondrial mass. The Mitochondrial Membrane Potential Dye accumulates in healthy mitochondria, caused by its transmembrane potential, and is absent from depolarized mitochondria that result from a cytotoxic compound such as valinomycin.

3 Applicability and Limitations (Scope)

This SOP describes the *in vitro* methods for the evaluation of the cytotoxicity of nanoparticles using the HCS platform to measure nuclear area, cell permeability, and mitochondrial membrane potential. The protocol is based on the assessment of the A431 epidermoid carcinoma cell line, a cell model used to access skin exposure to the nanomaterial. Other cell lines can be used.

The assay, and this SOP, does not provide detailed mechanistic information of the toxic effect experienced by the cell.

Interference of the NP with the assay readouts should be considered, particularly if the NP under examination exhibits fluorescent properties (quantum dots for example), during experimental planning and interpretation of the results. Existing available literature should therefore be reviewed during design of experiments in order to evaluate the applicability of the assays. Alternatively, specific measurements should be carry out by incorporation of "no cells" controls which will allow for accounting of the additional contribution provided by the "nano" materials or particle. It is worth re-stating that the here proposed SOP, and technique adopted, can be used in a dual-measurement: single-cell quantitative fluorescence analysis and well-based fluorescence intensity averages. This is the main advantage of HCS techniques versus other cell-based approaches (e.g., MTT, WST-8, and others) for the cell viability assessment.

Test material dilutions are carried out in the presence of serum. This may cause aggregation of the test material, and so stability studies in such media should be carried out. Further details on experimental design and characterisation protocols can be found in scientific literature or by contacting EUNCL core expert teams. Methodology for the characterisation of test materials in complex media (such as cell culture medium) are available in EUNCL-PCC-21, EUNCL-PCC-22, EUNCL-PCC-23, and EUNCL-PCC-35.

Where appropriate serum-free medium should be considered for use, as variations and inconsistencies in serum quality can lead to unquantifiable uncertainties in experimental procedures and results [1].

Finally, the protocols for image analysis are specific to the InCell Investigator software. A comprehensive review of other HCS capable equipment can be found in the literature [8].

4 Equipment and Reagents

4.1 Equipment

- Fluorescence microscope – GE Healthcare Cytell Imaging system (or equivalent)
- Image analysis software – GE Healthcare InCell Investigator 1.6 (or equivalent)
- 96 well flat bottom polystyrene cell culture plate (Nunc, 167008)
- Incubator, 37˚C with 5% CO2 and 95% humidity

4.2 Reagents

- HCS Multiparameter Cytotoxicity Kit (Cellomics ®, 8400202)
    - Absorption/Emission maxima for dyes used:
        - Hoechst Dye = 350/461 nm
        - Permeability Dye = 491/509 nm
        - Mitochondrial Dye = 554/576 nm
- Positive control compound – valinomycin (Sigma, V3639-5ML)
- A431 (epidermoid carcinoma cell line) (ATCC CRL-1555)
- DMEM (Sigma, D5796)
- Fetal bovine serum (Sigma, F9665-500ML)
- Penicillin-streptomycin (Sigma, P4458-100ML)
- Dimethyl sulfoxide (Sigma, D5879)
- 37 % formaldehyde (Sigma, 252549-25ML)
- Dissociation reagent – TrypLE (or equivalent) (BioSciences, 0040090DG)
- PBS (Fischer, 092810305)

4.3 Reagent Preparation

4.3.1 Cell culture medium

The complete medium for A431 is prepared by adding 10% FBS and 1% Penicillin-Streptomycin to DMEM medium. The medium is stable for up to 4 weeks at 4˚C.

4.3.2 Positive control

Prior to nanomaterials exposure, it would be advisable to identify the assay response to the specific cell line adopted for the experimental investigation. Thus, a titration dose range finding has to be carried out on valinomycin where to identify the specific EC50 for the cell line in question. Typical dose range goes from 50 to 200 µM in at least 4 concentration doses. This will require a separate plate where to carry out the dose finding. Once this is identified

Prepare all positive controls fresh for each assay. Dilute valinomycin to desired concentration in cell culture medium. Stock solution used in this SOP is at 1 mg/mL concentration, equating to 899 µM. The concentration of valinomycin should be titrated to find the EC50 and EC90 concentration for each cell line, with EC50 being used as an internal plate control.

4.3.3 1X wash buffer

Add 20 ml 10X Wash Buffer to 180 ml ultrapure water for a final volume of 200 ml. Store buffer at 4°C for up to 7 days.

4.3.4 Live cell staining solutions

Add 117 µL of anhydrous DMSO to the Mitochondrial Membrane Potential Dye to make a 1 mM stock. Store the stock solution protected from light at -20°C. Add 2.1 µL Permeability dye to 6 ml of complete media that has been warmed to 37°C in a water bath just before use, and then add 21 µl of Mitochondrial Membrane Potential Dye.

4.3.5 Fixation solution

Add 2.5 ml of 37% formaldehyde to 22.5 ml of 1X Wash Buffer and heat to 37°C in a water bath just before use. Pre-warming the Fixation Solution is critical to maintaining cell integrity. Prepare solution just before each assay.

4.3.6 Nuclear staining solution

Add 5.5 µl Hoechst dye to 11 ml 1X Wash Buffer. Prepare solution just before each assay.

5 Procedure

5.1 General remarks

This procedure is based on guidelines set out in ISO 10993:5 for tests for *in vitro* cytotoxicity [7]. Time points and concentrations tested should be used that appropriate for the required outcome

5.2 Flow chart

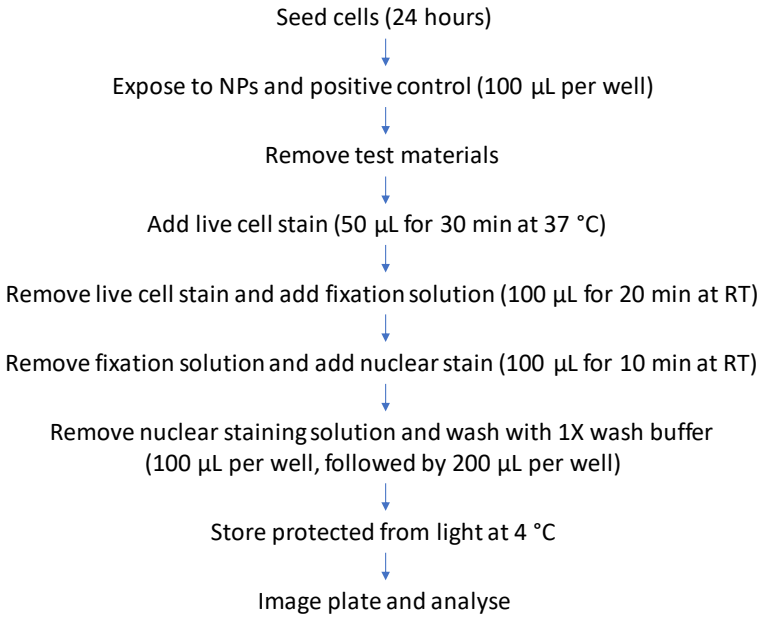

Figure 1: Brief outline of experimental workflow for HCS assay

5.3 Cell handling

The A431 cell line used in this study were obtained from the ATCC. Information relating to thawing, propagation, and creation of stocks is similar to that as described in EUNCL-GTA-001 and EUNCL-GTA-002. These stock ampoules serve as starting point for all experiments. After thawing of stock ampoules, cells are subcultured for 3 passages or more until enough cells are achieved (see Subcultivation). On the day of experiment, perform cell seeding as described in 5.4, continue with addition of samples the following day as described in 5.5.2.

5.3.1 Subcultivation

A431 cells are kept in a sub confluent state by routinely passaging twice or three times a week to seeding densities between 6 x 10^4 – 1 x 10^5 cells /cm^2.

Given volumes are for 75 cm^2 flask – proportionally reduce or increase amount of dissociation medium for culture vessel of other size.

1. Remove and discard culture medium.
2. Wash the cell layer twice by gently rinsing it with 10-15 mL preheated (37˚C) PBS.
3. Add 2.0-3.0 mL dissociation reagent (e.g TrypLE®), incubate at 37˚C for 5 minutes, and gently knock culture flasks to detach most of the cells.
4. Resuspend cells in complete cell culture medium to stop trypsination.
5. Transfer and dilute in new culture vessels.
6. Incubate the culture at 37˚C in a humidified atmosphere with 5% CO2 in a suitable incubator.

5.4 Cell Seeding

1. Harvest cells from prepared flasks, the cells should be cultivated for minimum 3 passages before use for experiment.
2. Count cell number using a coulter counter or haemocytometer.
3. Dilute cells to a density of 1 x 10^5 cells/mL in cell culture media.
4. Plate 200 µL cells/well per plate per time point (4, 24 hr, etc).
5. Incubate plates for 24 hr at 5% CO2, 37˚C and 95% humidity.

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B		Test Material Conc 6			Test Material Conc 6 (NSC)			Negative Control CMP/MMPI stain			Cell BGC	
C		Test Material Conc 5			Test Material Conc 5 (NSC)			Negative Control CMP stain			Cell BGC	
D		Test Material Conc 4			Test Material Conc 4 (NSC)			Negative Control MMPI stain			Cell BGC	
E		Test Material Conc 3			Test Material Conc 3 (NSC)			Positive control CMP/MMPI stain(EC90)			Val EC50	
F		Test Material Conc 2			Test Material Conc 2 (NSC)			Positive control CMP stain only (EC90)			Val EC50	
G		Test Material Conc 1			Test Material Conc 1 (NSC)			Positive control MMPI stain (EC90)			Val EC50	
H												

Figure 2: Representative plate layout for Live/Dead assay. NCS: No cell control, BGC: background control (no stains), Val EC50: valinomycin EC50 internal control. Outer wells are filled with PBS to minimise possibility of edge effects.

5.5 Assay procedure

5.5.1 Procedural Notes

1. Do not allow plate wells to become dry at any time during the protocol.
2. Perform all steps at room temperature unless otherwise indicated.
3. The protocol is optimized for A431 cells seeded in 96-well plates. Using conditions other than those indicated may necessitate optimization.
4. The DMSO concentration exposed to the cells at less than < 0.1%.
5. For best results, use low velocity fluid dispensing (≤ 4 ml/minute) with an automatic pipetting device.
6. For best results, aspirate with a weak vacuum. Toxic compounds may weaken cell adherence to the substrate, thus aspiration with moderate and strong vacuums will cause significant cell loss. To minimize cell loss, add and remove solutions at the edge of the well while tilting the plate without ever touching the bottom of the plate.
7. Please refer to the guide and instructions provided with the software and the HCS instrument for optimal implementation of the assay.

5.5.2 Procedure for 4, 24 hr, etc. exposure

1. Dilute test compounds to appropriate concentration in complete culture medium. Add 100 µL of the test compound or the positive control compound (120 µM valinomycin) to desired wells.
2. Incubate plates at 37°C in 5% CO_2 for 24 hours.
3. Remove test compound and add 100 µL of appropriate Live Cell Staining Solution to each well, expect background control.
4. Incubate plates at 37°C in 5% CO_2 for 30 minutes.
5. Gently aspirate media and staining solution from each well and add 100 µL of warmed (37°C) Fixation Solution.
6. Incubate plates for 20 minutes at room temperature.

7. Gently aspirate Fixation Solution and add 100 µL of 1X Wash Buffer to each well.
8. Remove buffer and add 100 µL of Nuclear Staining Solution to each well, incubate for 10 minutes at room temperature, protected from light.
9. Gently aspirate the Nuclear Staining Solution and wash wells once with 100 µl 1X Wash Buffer. Gently aspirate the wash and fill wells with 200 µl 1X Wash Buffer.
10. Seal plate and evaluate on the Cytell, imaging 12 fields per well at random locations.
11. Analyse images using InCell Investigator
12. Store sealed plates in the dark at 4°C.
13. Experiment should be completed in triplicate

5.6 Qualitative image acquisition protocol using Cytell Imaging system

Note: The following procedures are specific to the InCell Investigator software (GE Healthcare). Other image analysis programs are also available, and as such methods of analysis may differ, according to the instrumentation supplier.

The 96 well plate is placed into the stage of the HCS system and an automated imaging protocol created. The software allows for the selection of wells to be imaged, and the number of fields to be imaged per well. 10x magnification is used to obtain the optimal resolution while ensuring the number of cells imaged is maximized.

The focus settings and exposure times are selected automatically by the system. These can then be fine-tuned manually using the integrated controls. The user is required to check the focus and exposure times for each fluorescent channel separately to ensure optimal settings are used. It is good practice to verify that the exposure settings are optimizing for both the untreated and positive control wells to allow for appropriate image analysis following completion of the imaging. Acceptance criteria for image suitability are available from the various instrument manufacturers, with a comprehensive discussion of methods for detection out-of-focus and over saturated images being available within the scientific literature [14]. Singly stained positive and negative controls are included to ensure appropriate cellular/organelle staining has been achieved.

It should also be noted that experimental data generated, including images, must remain in compliance with FDA 21 CFR Part 11 regulations.

5.7 Quantitative image analysis using InCell Investigator software

Note: The following procedures are specific to the InCell Investigator software (GE Healthcare). Other image analysis programs are also available, and as such methods of analysis may differ, according to the instrumentation supplier.

Analysis of the images requires the classification of the of each wavelength used (each dye) as a specific designation. This enables the segmentation of the image based on the colour channels.

- Hoechst (blue channel, Wave 1: nucleus) represents the nuclei and is used as the basis of the image segmentation, marking the location of each cell and enabling the quantification of cell counts. The change in nuclear area can also give information relating to the viability of the cell (see section 2.1)

- Cell permeability dye (green channel, Wave 2: cell) is designated as a “cell mask”. Detection, and colocalisation of this dye within the nuclei allows the user to distinguish non-viable cells (see section 2.2)
- Mitochondrial membrane potential intensity (red channel, Wave 3: organelle) is classified as an “organelle”. The fluorescent intensity, and subsequent changes, reflects the polarization state of the mitochondria (see section 2.3)

Nuclei were segmented using top-hat segmentation, enabling the accurate selection of closely positioned nuclei. Minimum nuclear size was determined through selection of the smallest nuclei using the arrow tool, which estimates the size based on local background intensity, and returns the measured object’s area in square microns.

Cell segmentation (cell permeability dye) used the region growing method by which the boundaries of the cell were well defined. For this parameter, data was generated based on the intensity of dye which overlaid with the nuclear segmentation, and provided the positive readout for cytotoxicity.

The segmentation of organelles defining mitochondrial staining was by the multiscale top-hat method, whereby the detection of inclusions was confined to the cytoplasm.

Data generated by the defined multi-target analysis included cell count, nuclear area, nuclear localised cell staining (nuclear/cell membrane permeability) and organelle intensity (mitochondrial membrane potential). Results were exported as tab-delimited text allowing analysis of the various parameters.

During analysis, the InCell software generates real time heatmaps, matching the experimental design graphically illustrating the live/dead outputs, thus showing preliminary results. It is also possible to generate further graphical plots to show statistical significance and trends by importing and processing the data file with other data visualisation tools (e.g., GraphPad Prism, R, MATLAB, Origin, and Microsoft Excel).

It should also be noted that quantitative image analysis must remain in compliance with FDA 21 CFR Part 11 regulations.

The Z’-factor of the assay can also be calculated, where Z’ =1 is an ideal assay, Z’=0 is suitable for binary yes/no responses, and Z’ < 0 in unacceptable. The Z-factor is calculated as shown below:

$$Z'factor = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}$$

Where μ_p and σ_p are the mean and standard deviation values of the positive control (or treated samples) and μ_n and σ_n are those of the negative control [14]. A Z’ Factor > 0.5 is an determined as being an “excellent assay for screening” [15].

Z’-factors for the individual dye components are listed below, as provided by the manufacturer and extensively reported for high content screening methodologies. It should be noted that these data are based on the HepG2 cell line, but can act as guidelines for the suitability of the assays:

Target	Z'-factor (mean ± SD)
Nuclear area	0.58 ± 0.04
Cell membrane permeability	0.87 ± 0.07
Mitochondrial membrane potential	0.31 ± 0.20

It should also be noted that experimental data generated, including images, must remain in compliance with FDA 21 CFR Part 11 regulations.

5.8 Calculations

All samples, positive, negative, and NP controls are run in triplicate, with each well will be subtracted from its respective cell-free blank to account for any fluorescence that may be due to the test material. The average of these three values should be used in the equations below, as specified by the assay developer.

$$\%\ MMPI = \frac{Mean\ sample\ fluoresence - cell\ free\ sample\ blank}{Mean\ Fluorescence\ Intensity\ of\ Untreated\ Control} \times 100$$

$$\%\ CMP = \frac{Mean\ sample\ fluoresence - cell\ free\ sample\ blank}{Mean\ Fluorescence\ Intensity\ of\ Untreated\ Control} \times 100$$

$$\%\ Nuclear\ Area = \frac{Mean\ sample\ nucelar\ area}{Mean\ nuclear\ area\ of\ untreated\ Control} \times 100$$

$$\%\ Cell\ Count = \frac{Mean\ sample\ cell\ count}{Mean\ cell\ count\ of\ untreated\ control} \times 100$$

- Mean, SD and %CV should be calculated for each set of samples tested.
- Results should be plotted and presented as mean ± standard error of the mean for repeated experiments.
- The sample EC50 values can be calculated by monitoring the decreases in cell count or nuclear area, or by monitoring changes in cell membrane permeability or mitochondrial membrane potential, as the test material concentration increases. It is recommended to generate a nonlinear fit (based on at least three independent measurement for each datapoint) calculating the EC50 as the concentration of the test material that induces half the maximum response for the given parameter. Below is a dose-response curve for changes in nuclear radius over increasing valinomycin concentrations (provided as examples)

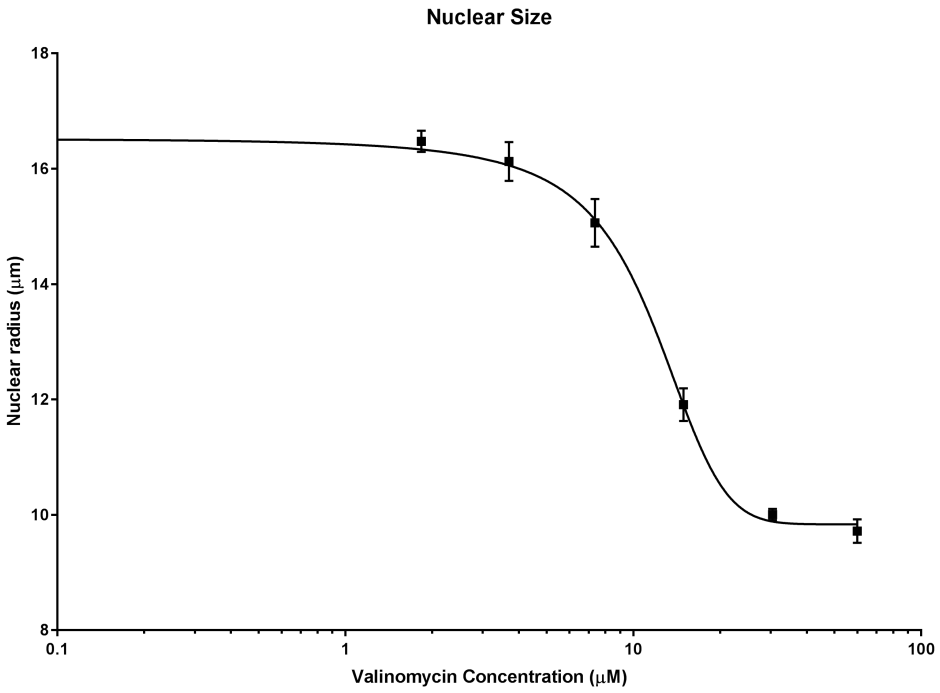

Figure 3: Representative dose-response curve generated for cells treated with varying concentration of valinomycin. The EC50 can be calculated as the concentration that induces a decrease in nuclear radius by 50 %.

6 Quality Control, Quality Assurance, Acceptance Criteria

- Assay wells exposed to a series of dilutions of valinomycin should be used as internal quality controls. The EC50 value can be used in monitoring the assay performance.
    - If the value lies outside the linear response of the EC50 curve then the assay has to be repeated.
- The nuclear area, and cell viability of the internal valinomycin positive control should be less than 50%
    - If not, the assay has to be repeated since either the cell did not respond as from cell technical specification or the assay did not work (see point above).
- Assays in which the internal controls are outside of these ranges should be discarded.
    - If the internal control estimation is out of the specification then the validity for the testing of the nanomaterials are in question. As above, the entire, assay, including positive controls, should be repeated.
- A 30 % reduction in cell viability 20 % change in mitochondrial fluorescence intensity is deemed to be a marker of cytotoxicity [7, 16]
    - The cell supplier technical specification should indicate or else please check literature for different cell viability cut offs.
- The nuclear area, mitochondrial membrane potential intensity of the internal valinomycin positive control should be less than 50%, with the cell membrane permeability being above 150 %.

- If the values are out of the indicated percentages, the assay has to be repeated since either the cell did not respond as from cell technical specification or the assay did not work (see point above).
- Good practice in multiparametric analysis or titration study is to ensure consistency between the data generated and the recorded images where the data is processed.
  - If exceeding cell fragmentation is present in the well, please revise your cell seeding density or check for exceeding nanomaterial aggregation (not quantified during physico-chemical characterisation).
  - Nanoparticle aggregates could be counted as cells or organelles depending on the size of aggregates and sensitivity of the analytical thresholds.
  - If fluorescent data generated does not align with the images recorded that there may be possible fluorescent interference or saturation points within the selected fields of view where to process the data. This could be either an outlier or systematically present across all wells. The common procedure here is to repeat the assay by revising the cell seeding and or nanomaterial test concentrations.

7 Health and Safety Warnings, Cautions and Waste Treatment

Please refer to available H.S.E information for any nano formulations evaluated in the assays. Note that some of the listed reagents are hazardous and must be handled with precaution. Please refer to safety data sheets for each reagent, wear protective equipment, and dispose waste according to local regulations.

8 Abbreviations

NP: Nanoparticle

RT: Room temperature

CMP: Cell membrane permeability

MMPI: Mitochondrial membrane potential intensity

PBS: Phosphate buffered saline

SD: standard deviation

% CV: Percentage coefficient of variation

FBS: fetal bovine serum

EC: Effective concentration

9 Related Documents

Table 1:

Document ID	Document Title	URL
EUNCL-GTA-001	LLC-PK1 Kidney Cytotoxicity Assay	http://www.euncl.eu/about-us/assay-cascade/PDFs/Toxicology/EUNCL_GTA_001.pdf?m=1468937871&
EUNCL-GTA-002	HepG2 hepatocarcinoma cytotoxicity	http://www.euncl.eu/about-us/assay-cascade/PDFs/Toxicology/EUNCL_GTA_002.pdf?m=1468937871&
EUNCL-PCC-21	Measuring NP aggregation propensities with DLS	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL-PCC-021.pdf?m=1468937870&
EUNCL-PCC-22	Size, Size Distribution	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL-PCC-022.pdf?m=1468937868&
EUNCL-PCC-23	Particle Tracking Analysis	http://www.euncl.eu/about-us/assay-cascade/PDFs/PCC/EUNCL_PCC_023.pdf?m=1526712237&
EUNCL-PCC-35	Drug release in complex media	

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