

Standard Operating Procedure

Title	High Content Analysis-based nanotoxicity assessment (HCA)
Subtitle	Standard Operation Procedure (SOP) and background documentation for High Content Analysis-based nanotoxicity assessment
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Owner and co-owner(s)	Kevin Hogeveen, ANSES
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1 Background

The Arrayscan VTi High Content Imaging platform (Thermo Scientific) is an automated fluorescence microscope and camera system dedicated to automated cellular imaging and analysis. The cell analysis software coupled to the instrument enables automated, quantitative cellular and subcellular imaging and analysis in multi-well plate formats. Image acquisition, image analysis and quantification of fluorescence in various cellular compartments are performed simultaneously. This approach, adapted for cultured cells in a monolayer, requires immunofluorescence labeling or incubation with fluorescent probes prior to imaging with the High Content Imaging platform.

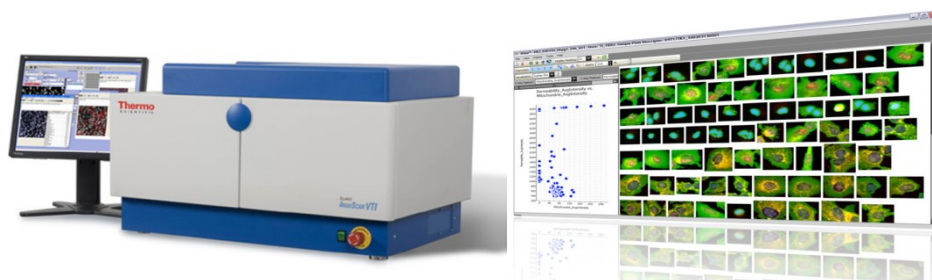


Figure 1. Array scan VTi High Content Imaging Platform

2 Protocol

2.1 Nanoparticle dispersion

The NANOGENTOX protocol was used for nanoparticle dispersion. Briefly, particle powder was pre-wetted in 0.5% absolute ethanol in a scintillation vial and dispersed at a final concentration of 2.56 mg/mL in 0.05% BSA in ultra-pure water by sonication in ice for 16 min at 400W using a Branson sonicator equipped with a 13 mm probe diameter.

Following MNM dispersion, MNMs were diluted at concentrations of 1, 5, 10, 25, 50 and 100 µg/mL in cell culture media.

2.2 Cell culture and treatment

A549 and Caco-2 cells were cultured in complete DMEM at 37 C with an atmosphere of 5% CO₂. Media was changed every 2-3 days and cells were passaged two times per week. For High Content Analysis experiments, cells were plated at a density of 10 000 cells/well in 96-well plates 24 hours prior to treatment. Cells were treated for 24 hours at concentrations of MNMs of 0, 1, 5, 10, 25, 50 and 100 µg/mL. Each concentration was tested in triplicate wells.

2.3 Immunofluorescence

Materials

- PBS (Phosphate Buffered Saline) pH7.4
- Formaldehyde solution 4%
- PBS Triton X-100 0.2% (permeabilisation buffer)
- PBS Tween-20 0.05% (washing buffer)
- PBS Tween-20 0.05% 1% BSA (blocking buffer, antibody dilution buffer)

Protocol

- Wash cells 3 times with PBS
- Fix cells with 4% formaldehyde for 15 minutes at RT
- Remove PFA and wash 2 times with PBS
- Permeabilize cells with PBS + 0,2% Triton X-100 for 10 minutes at RT
- Wash cells 3times with PBS-Tween 20 0.05%
- Block with PBS Tween 20 0.05% + 3% BSA for 60min at RT
- Remove blocking solution and add primary antibody diluted (1:1000) in PBS Tween 20 0.05% + 3% BSA at 4°C for 16h.
- Wash cells 3 times with PBS-Tween 20 0.05%
- Add secondary antibody diluted (1:1000) in PBS Tween 20 0.05%+ 3% BSA for 60 minutes at RT
- Wash cells 3 times with PBS Tween 20 0.05%
- Stain with DAPI (diluted in PBS 1µg/mL) for 5 min at RT
- Wash cells 2 times with PBS

NOTE: Fluorochromes associated with the secondary antibodies need to be chosen carefully in order to avoid interference in certain wavelengths. For TiO₂ MNMs, DAPI and FITC channels should be avoided.

2.4 High Content Analysis

Cellular imaging was performed with an Arrayscan VTi High Content Imaging platform. Ten fields per well were imaged with a 10X objective. Images were analyzed with the Target Activation BioApplication of the HCS Studio software. Briefly, individual cells were identified based on DAPI staining and regions of interest for fluorescence quantification were selected in each channel based on the localization of the marker to be analyzed. When applicable, positive cells were defined as cells with fluorescence 2 standard deviations above the mean fluorescence of negative control cells.