A common European approach to the regulatory testing of nanomaterials

Standard Operating Procedure

Title	***Standard Operation Procedure and background documentation for label-free nanotoxicity assessment by impedance-based flow cytometry***
Subtitle	
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Standard Operation Procedure (SOP) and background documentation for label-free nanotoxicity assessment by impedance-based flow cytometry

1. Background

AmphaZ30 (Amphasys AG, Lucerne, Switzerland) is an impedance flow cytometer for high-throughput single-cell characterization without optical components. Single cells pass through a micrometer-sized channel in a chip equipped with microelectrodes (Figure 1). The electrical impedance changes when a cell passes through the applied alternating current (AC) field, permitting cell detection and impedance measurement. The measured impedance is used to assess cellular size, membrane capacitance, and cytoplasm resistance [1, 2].

Figure 1: Impedance flow cytometer Ampha Z30 (left) and microfluidic chip (right).

2. Protocol

2.1. Cell culture, exposure to nanomaterials, and sample preparation

Materials

- U937 human monoblastoid cells (ATCC)
- Nanomaterial stock dispersion (2.54 mg/mL)
- Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies-Gibco) supplemented with 10% Fetal Bovine Serum (FBS) (GE Healthcare - HyClone™) and 1% Penicillin-Streptomycin (P/S) (10.000U/mL penicillin and 10.000 µg/mL streptomycin), (GE Healthcare - HyClone™)
- Trypan blue solution 0.4% (Invitrogen™, Molecular Probes™)
- Countess™ cell counter and cell counting chamber slides (Invitrogen™)
- Phosphate-buffered saline (1x PBS, Life Technologies-Gibco)
- Pipette and pipette tips (sterile)
- Nunc™ cell culture treated EasYFlasks™ 175 cm^2 (T_{175}) (Thermo Scientific)
- Nunc™ cell culture treated Multidishes 6-well dish (Thermo Scientific)
- Falcon tubes sterile, 15 and 50 mL, (Sarstedt AG & Co)
- Tumor necrosis factor-α (TNF-α) human recombinant (Sigma Aldrich)
- Cycloheximide (Cx) solution (Sigma Aldrich)
- Tube rotator SB3 (Bibby Scientific Limited)
- Sterile hood
- Polystyrene box with ice

Cell culture

U937 cells were cultured in T_{175} flasks in DMEM with supplements (see Materials section above) in a humidified atmosphere at 37°C and 5% CO_2. For each experiment cells were collected, centrifuged (160 g, 10 min), and then re-suspended in DMEM. Cells were then counted and their viability assessed using Trypan Blue Dye Exclusion and an automated cell counter (Countess™). U937 cells (viability above 90%) were seeded in two identical 6-well culture dishes at 50.000 cells/cm^2 (Figure 2).

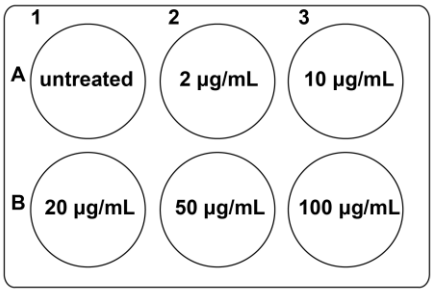

Figure 2: Plate layout for cell seeding in 6-well culture dishes.

Nanomaterial dilution in medium and cell exposure

At 24 hrs after seeding, the U937 cells were exposed to five nanomaterial (NM) concentrations: 2, 10, 20, 50, 100 µg/mL corresponding to 1, 5, 10, 25, and 50 µg/cm^2. Unexposed cells served as negative controls, cells kept at 70ºC for 30 min served as positive control for necrosis, while cells treated to TNF-α (100 UI/mL) and Cx (1 mg/mL) for 4 hrs were used to verify the placing of apoptotic cells in histograms and dotplots. An NM-stock dispersion of 2.56 mg/mL was prepared as specified in the generic NANOGENOTOX protocol using a 130-watt probe sonicator (VCX130, Vibra-Cell, 130W, Sonics & Materials) with a 12.8 mm replaceable tip for 16 min at 22% of the maximal amplitude. Next, a 100-µg/mL dispersion in medium was prepared by adding the calculated amount of stock dispersion to the medium (1.76 mL batch dispersion in 43.24 mL medium) and mixed using a tube rotator at 40 rpm for 2 min (outside the sterile bench), then a dilution row was prepared (Figure 3).

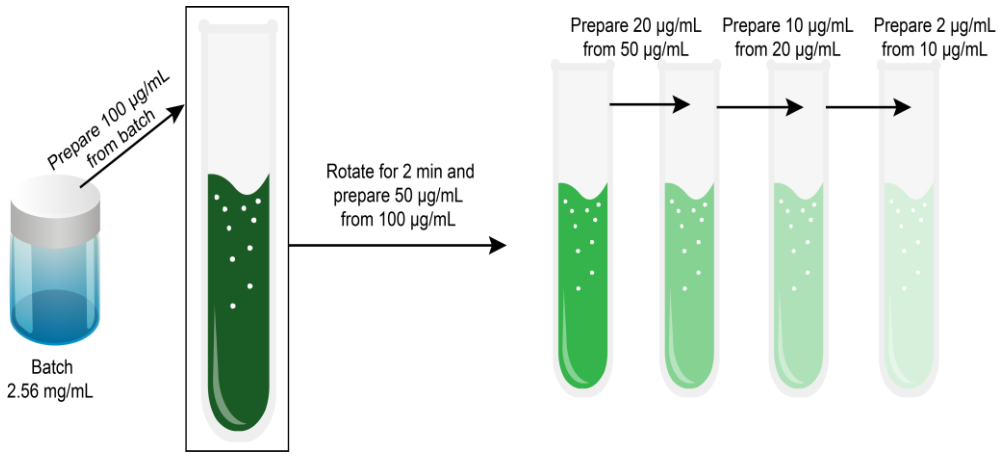

Figure 3: Preparation of NM-dilutions in cell culture medium for cell exposure.

Prior to exposure, the U937 cells were centrifuged in the culture dish at 250 g for 5 min, then the supernatant was carefully removed and the NM dispersions in the 5 different concentrations were added using a total volume of 4.75 mL.

Sample preparation for AmphaZ30 reading

After the 24 hrs exposure to NMs, the cells were spun down in the culture dish (250 g, 5 min), the supernatant was carefully removed and 1 mL PBS was added to each sample. Subsequently, cells were transferred to 1.5 mL Eppendorf tubes and centrifuged (250 g, 5 min), then the supernatant was removed and cells were re-suspended in 100 µL PBS. All samples were kept on ice during measurements.

2.2. Impedance measurements using Ampha Z30

Materials

- 0.2 µm-filtered 1x Sucrose buffer (97 g/L in distilled water = 0.28 M) solution (prepared every two weeks and stored at 4°C)
- AmphaChip 50 µm (Amphasys AG)
- AmphaClean (Cleaning Solution, Amphasys AG)
- Cleaning Chip (Amphasys AG)
- Eppendorf tubes
- 0.2 µm-filtered distilled water

Ampha Z30 settings and measurements

First, the system was rinsed 3-4 times using the rinse cycle implemented in the software (AmphaSoft-1.2.8, Amphasys AG). The settings for the measurements are summarized in Table 1.

Table 1: Ampha Z30 settings

Chip	50 µm
Frequencies [MHz]	0.5, 2, 6, 12
Amplifier	3
Modulation	3
Demodulation	2
Trigger level	0.02
Measured no. cells	20.000
Size of tubing set	Medium
Pump speed [rpm]	80-100

After applying the settings, 400 µl (ratio 1:4) of 1x sucrose buffer was added to each Eppendorf tube (stored on ice) and mixed by pipetting, resulting in a measurement buffer of 80% sucrose buffer and 20% PBS. Untreated viable cells were used as negative control and necrotic cells (previously kept in a water bath for 30 min at 70°C) were used as positive controls. All measurements were done in duplicate and repeated at least three times.

NOTE:
It is very important to clean the system after each experiment twice using the rinse cycle, as implemented in the software. Next, use the cleaning chip for disinfection of the fluidic system with ethanol (70%). In addition, choose “instrument clg” in the software and clean the system with AmphaClean.

3. Analysis

Measurements were stored as *.fcs-files and analysed using the FlowJo software (FlowJo, LLC). Negative and positive control samples were plotted as histograms over the impedance angle at 6 MHz and were used to set gates for viable and necrotic cells (Figure 4). These gates were applied to all samples within one experiment. Finally, the median of the percentage of viable cells and its standard error were calculated in Microsoft® Excel® for Mac 2011, Version 14.6.7.

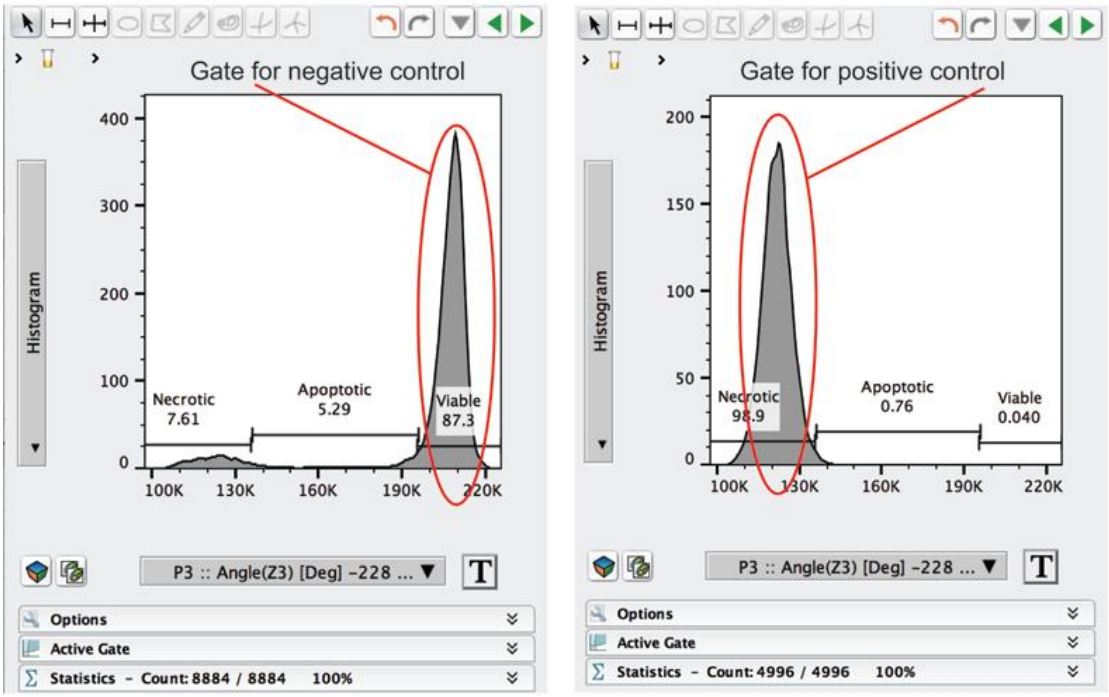

Figure 4: Histogram of positive and negative controls to set gates. These gates were applied to all samples within one experiment.

References

[1] Chen J., Xue C., Zhao Y., et al. Microfluidic Impedance Flow Cytometry Enabling High-Throughput Single-Cell Electrical Property Characterisation, Int. J. Mol. Sci. 2015, 16, Doi:10.3390/ijms16059804

[2]http://www.amphasys.com/sites/default/files/Amphasys_IFC_Impedance_Flow_Cytometry_for_Label_Free_Single_Cell_Analysis.pdf