PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

PATROLS 4401

SOP for 3D high throughput screening of HepG2 cells

This is a SOP used by members of PATROLS only

Adapted from the NanoImpactNet SOP, Clift *et al* (Deliverable 5.4 under the European Commission's 7th Framework Programme, Grant Agreement 218539).

Authored by:

Vesa Hongisto and Roland Grafström, Misvik Biology Ltd

Reviewed by:

Shareen H. Doak (Swansea University)

Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	DD/MM/YYYY	Initial Document	

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1 Introduction:

DOMAIN: Human Toxicology

This SOP is based on the PATROLS 4104 “3D In Vitro HepG2 Spheroid Model” SOP and provided by S. V. Llewellyn from Swansea University. The goal of the “3D In Vitro HepG2 Spheroid Model” SOP was to provide advanced 3D hepatic cultures *in vitro* which can provide a more physiologically relevant assessment of the hazards associated with ENM exposures over both an acute and long-term, repeated dose regime. The goal of this SOP is to convert the “3D In Vitro HepG2 Spheroid Model” SOP in to a 384-well plate high throughput format that enables analysis of larger nanomaterial libraries in a robust manner while still making use of the more *in vivo* like spheroids cultures.

Use of the Nanogenotox dispersion SOP (The Nanogenotox standard operational procedure for preparing batch dispersions for *in vitro* and *in vivo* toxocological studies, version 1.2, 12 June 2018, K. A. Jensen) is needed to disperse the materials. Also, the probe sonicator is to be calibrated according to the NanoReg SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for *in vitro* and *in vivo* toxicological testing (version 1.1, 14 July 2018, K. A. Jensen).

1.1 Scope and limits of the protocol:

The SOP is intended for 384-well plate high throughput cytotoxicity assessment of HepG2 cells cultured in 3D spheroids and exposed to enginered nanomaterials.

As automated imaging of 3D spheres in 384-well high throughput format is not feasible, it was decided that the endpoints chosen should be population based endpoints that capture key toxicological events. All endpoints are luminescence

based and thus possible assay interference induced by nanomaterials might be hard to detect as no alternative measurement techniques are used. A higher baseline background of Caspase 3/7 activation, ROS and cytotoxicity was detected in 3D spheroid screens than in 2D screens carried out at the same time using the same assay setup, which might be an inherent property of the spheroid culture or an indication of stress induced by the protocol. In addition, the spheroid transfer protocol from growth plates to assay plates is error prone if the plates are not aligned perfectly and leaves some wells empty if not properly executed. The HepG2 spheroid model system has only been tested for exposures up to five days without medium change. Longer exposure times may be possible but require further testing.

Due to these limitations, this SOP should not be considered as a final HTS SOP for HepG2 spheroid screens, but rather a starting point for further assay development and a protocol that was used to create data within PATROLS.

1.2 Validation state of protocol:

Level of advancement towards standardization	**Level reached** (please mark only one with "X")
Stage 1: Internal laboratory method under development	x
Stage 2: Validated internal laboratory method	
Stage 3: Interlaboratory tested method	
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	N
Has the method been submitted for standardisation (to OECD, CEN, ISO,…) in its own right or as part of another standardisation project?	N
Is the method included in an existing standard (or ongoing standardisation work)	N

2 Terms and Definitions:

Nanomaterial

Material with any external dimension in the *nanoscale* or having internal structure or surface structure in the nanoscale.

Note 1 to entry: This generic term is inclusive of *nano-object* and *nanostructured material*.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.4]

Engineered nanomaterial

Nanomaterial designed for specific purpose or function

[SOURCE: ISO/TS 80004-1: 2016, definition 2.8]

Manufactured nanomaterial

Nanomaterial intentionally produced to have selected properties or composition.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.9]

Particle

Minute piece of matter with defined physical boundaries.

Note 1 to entry: A physical boundary can also be described as an interface.

Note 2 to entry: A particle can move as a unit.

Note 3 to entry: This general particle definition applies to *nano-objects*.

[SOURCE: ISO 26824:2013, 1.1]

3 Abbreviations:

2D	Two dimensional
3D	Three dimensional
4NQO	4-Nitroquinoline 1-oxide
5-FU	Fluorouracil
CTG	CellTiter-Glo
DMEM	Dulbecco's Modified Eagle's – Medium
DMSO	DiMethyl SulfOxide
DNA	DeoxyriboNucleic Acid
EDTA	Ethylenediaminetetraacetic acid
ENM	Engineered NanoMaterial
FBS	Foetal Bovine Serum
HT	High Throughput
HTS	High Throughput Screening
LDH	Lactate DeHydrogenase
MMC	Mitomycin C
NM	NanoMaterial
PBS	Phosphate Buffered Saline
Pen/Strep	Penicillin/Streptomycin
polyHema	poly(2-hydroxyethyl methacrylate)
ROS	Reactive Oxygen Species
RT	Room Temperature

4 Principle of the Method:

This SOP is based on measurement of five cellular events using commercially available kits with luminescent readouts. CellTiter-Glo assay measures cellular ATP levels as surrogate readout for cell viability. Caspase 3/7-Glo assay measures caspase cleavage of the kit substrate. ROS-Glo measures the level of hydrogen peroxide in cell culture and Cytotox-Glo measures the relative number of dead cells in cell populations. More specifically, the cytotox assay measures the extracellular

activity of a dead-cell protease when the protease is released from membrane-compromised cells. In addition, the assay can be used to calculate cell viability by determining the signal associated with the total number of cells in each assay well and by subtracting the luminescent dead-cell signal from the total luminescent value.

Measurement of lactate dehydrogenase (LDH) levels (LDH-Glo assay) from cell culture medium is included in this SOP, but data from the assay did not meet quality criteria and was omitted from the analysis of results.

5 Description of the Method:

5.1 Biological setting & test system used:

- This SOP should be carried out under laboratory based conditions, with cell culture work performed under sterile conditions and in a Class 2 Laminar Tissue Culture Hood.
- Cell line utilised is a Human Caucasian Hepatocellular Carcinoma derived epithelial cell line, HepG2 (ECACC 85011430, Lot:17K028, passage number upon purchase: 100). Cryopreserved HepG2 cells were frozen down in 10% DMSO and 90% FBS. No mycoplasma contamination was detected using DNA-staining (Dapi). The cells were cultured for a maximum of 20 passages.
- For further information, please visit https://www.phe-culturecollections.org.uk/products/celllines/generalcell/detail.jsp?refId=85011430&collection=ecacc_gc

5.2 Chemicals and reagents used:

4-Nitroquinoline 1-oxide (Sigma-Aldrich, 442683)
Accutase Cell detachment solution (Thermo-Fisher Scientific, 00-4555-56)
Caspase-Glo 3/7 assay (Promega, G8092)
CellTiter-Glo Luminescent Cell viability assay (Promega, G7572)

CytoTox-Glo (Promega, G9291)
DMEM with 4.5g/L D-Glucose, L-Glutamine (GIBCO®, 41966-029)
Ethanol 99% (Altia, Finland)
FBS (GIBCO®, 10270-106)
Fluorouracil (Sigma-Aldrich, F6627)
LDH-Glo Cytotoxicity assay (Promega, J2380)
Mitomycin C (Sigma-Aldrich, Y0000378)
PBS pH 7.4 1X, MgCl2 and CaCl2 Free (GIBCO®, 14190-094)
Pen/Strep (GIBCO®, 15140-122)
polyHema (Polysciences Inc, 18894)
ROS-GLO H2O2 assay (Promega, G8821)
Trypsin-EDTA (GIBCO®, 5300-054)

5.3 Apparatus and equipment used:

10 cm Cell culture
384-well storage plates
384-well assay
Cell counter
Cell culture incubator, 37˚C, 5% CO2
Cell freezing aid
Electronic 16-channel pipette
GraphPad Prism 6.0 software
Heat sealer
Laminar tissue culture hood
Light microscope
Liquid dispensing station
Liquid handling station
Luminescent plate reader
Microsoft Excel software
Plate centrifuge
Probe sonicator
Tissue culture flask

Tube centrifuge
Ultrapure water
Water bath

5.4 Reporting of protected elements:

This SOP does not have any associated patent restrictions, specific licenses (excluding Microsoft office and GraphPad Prism software licences), material transfer agreements (excluded the use HepG2 cell line) or commercial purchase requirements required to perform the protocol described.

5.5 Health and safety precautions:

Prior to any use of this SOP a full risk assessment should be completed, considering all potential risks associated with chemicals equipment and use, in compliance with national regulation. Training of personnel should be completed before any person is working with the SOP.

5.6 Applicability:

This SOP is applicable and has been demonstrated for the following materials:

	Target (mg)	Measured (mg)	0.5 vol% EtOH (µl)	0.05% w/v BSA-water (µl)	Final max assay [C] (µg/ml)
DQ-12 - Silica	15.36	15.5	30.3	6024.4	256
JRCNM01005a - TiO_2 (NM-105)	15.36	15.5	30.3	6024.4	256
JRCNM01101a - ZnO (NM-111)	15.36	16.4	32.0	6374.2	256
JRCNM02102a - CeO_2	15.36	15.5	30.3	6024.4	256
JRCNM04000a - MWCNT (NM-400)	15.36	16.2	31.6	6296.5	256
JRCNM04002a - MWCNT (NM-402)	15.36	15.6	30.5	6063.3	256
JRCNM40011a - MWCNT (Mitsui-7)	15.36	16	31.3	6218.8	256
JRCNM50001a - $BaSO_4$ (NM-220)	15.36	16.3	31.8	6335.4	256
JRCNM62101a - ZNO (new NM-110)	15.36	15.8	30.9	6141.0	256
NM-105 - TiO_2	15.36	15.5	30.3	6024.4	256
NM-110 - ZnO	15.36	16.4	32.0	6374.2	256
NM-111 - ZnO	15.36	16.4	32.0	6374.2	256
NM-113 - ZnO	15.36	16.3	31.8	6335.4	256
NM-200- SiO_2	15.36	17.7	34.6	6879.5	256
NM-212 - CeO_2	15.36	16.6	32.4	6452.0	256
NM-220 - $BaSO_4$	15.36	16.3	31.8	6335.4	256
NM-300k - Ag (10% w/w)	640 mg/25ml	649.4	-	24717.8	256
NM-300k-DIS (liquid)	640 mg/25ml	644.3	-	24523.7	256
NM-302 - Ag-rod (8.3% w/w)	771.1 mg/25ml	773	-	29422.3	256
NM-302-DIS (liquid)	771.1 mg/25ml	776	-	29536.5	256
TiO_2 E171-Venator	15.36	17.7	34.6	6879.5	256
Sigma Silver #576832	15.36	16.1	31.4	6257.6	256
Carbon Black	15.36	15.7	30.7	6102.1	256
5-FU					13
4NQO					9.5
MMC					33.4

Table 1: Materials used in the SOP. *The measured weights and dispersion volumes are indicated together with the final maximum assay concentrations. NM-300k, NM-300k-DIS, NM-302 and NM-302-DIS were diluted in BSA-water to approximately 25 ml's and sonicated in 6 ml aliquots. Sigma Silver and the chemical controls were accuired from Sigma-Aldrich, Carbon Black from NRCWE and the rest through PATROLS web order site. The nanomaterials were stored in the dark, at room temperature.*

5.7 Reagent preparation:

5.7.1 Dispersion of nanomaterials:

Use the Nanogenotox dispersion SOP (The Nanogenotox standard operational procedure for preparing batch dispersions for *in vitro* and *in vivo* toxocological studies, version 1.2, 12 June 2018, K. A. Jensen) to disperse the test materials. Calibrate the sonicator according to NanoReg instructions provided in: SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for *in vitro* and *in vivo* toxicological testing (version 1.1 14 July 2018 K. A. Jensen).

Weigh ≥15.36 mg of each nanomaterial (see Table 1 for amounts used). Disperse by sonication for 9 min and 32 seconds to deliver 7056 J energy in 0.05% w/v BSA-water, to make 2.56 mg/ml stock solutions. Aliquot into 1.5-2 ml aliquots, snap freeze and store in -80°C.

5.7.2 Cell Culture Medium:

In preparation for the SOP to be performed, it is advised that the cell culture medium is prepared and pre-warmed at 37˚C for 30 minutes prior to use. Cell culture medium can be prepared as follows:

- Into a 500ml of DMEM, add 50ml FBS and 5ml Pen/Strep. The order of addition does not need to be specific, but the medium needs to be mixed by inverting the medium bottle after each addition.

5.7.3 PolyHema coating of 3D assay plates:

- For 12 plates measure 200 ml of 99% Ethanol + 11.04 ml of sterile filtered milliQ water + 9.256 ml of polyHema to a glass bottle in this order. Mix well with a magnetic stirrer. Dispense to plates.
- Use 35 µl per 384-well for coating.

- Use a dedicated multi-drop casette for dispensing polyHema mixture to plates.
- After dispension dry the plates by placing a KIMwipe paper towel in between the lid and the plate to speed up the drying.
- Let dry for 1 week. The coated plates can be store at RT for extended periods of time.

5.7.4 Preparation of nanomaterial master plates:

- Use Eppendorf deepwell plates as master plates (see Figure 1 for plate layout).
- Add 160 µl of sterile water to wells : A1+J1+P1 + A7+J7+P7 + A13+J13+P13 + A19+J19+P19 (= control rows).
- Add 128 µl of sterile water to columns 2-6 + 8-12 + 14-18 + 20-24.
- Add 160 µl of the ENM / chemical stock to highest concentration wells (column 1 + 7 + 13 + 19).
- Mix column 1 samples, take 32 µl from column 1 to column 2, mix well, take 32 µl to column 3, mix, 32 µl to next - up to column 6.
- This gives 1/5 dilution of the samples. Final concentrations : 256, 51.2, 10.24, 2.048, 0.4096 and 0.08192 µg/ml.
- Repeat dilutions steps with columns 7-12, 13-18 and 19-24.
- The final volume is 128 µl in each well (take out 32 µl from the last columns of the dilution series (columns 6+12+18+24)).
- 11 assay plates at 10 µl per well can be prepared from one master plate; 4 master plates are needed.
- 1408 µl of 10x stock is needed (2.56 mg/ml) for the whole screen which is < ¼ of the dispersion.
- Heat seal the master plates with a plate sealer (ThermoFisher Scientific) and freeze.

5.7.5 Preparation of nanomaterial assay plates:

- Thaw master plates to room temperature, spin down at 100g for 30 seconds and remove the seal.
- Use Eppendorf Eppmotion to disperse 10 µl per well from the master plates to both polyHema coated (for 3D cultures) and non-coated (2D cultures) 384-well plates (Greiner µ-clear plates) in the following way:
    - Mix quarter 1 of master plate using pipette and mix program (volume 50 µl, mix 40 µl, repeat 7 times).
    - Pipette 10 µl per assay plate from quarter 1 (volume 20 µl, mix 15 µl, 1x). Mix in between each assay plate to keep the materials well suspended. Be very carefull with "blow out": Do not blow out inside liquid to avoid bubbles, and take the plate further away for the final stage of blow out. Repeat for quarters 2,3 and 4.
- Heat seal the assay plates and store frozen.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24
A	water						water						water						water					
B	1) DQ-12						14) NM-212 (CeO2)						1) DQ-12						14) NM-212 (CeO2)					
C	2) JRCNM01005a (TiO2, =NM-105)						15) NM-220 (BaSO4)						2) JRCNM01005a (TiO2, =NM-105)						15) NM-220 (BaSO4)					
D	3) JRCNM01101a (ZnO, =NM-111)						16) NM-300k (Ag)						3) JRCNM01101a (ZnO, =NM-111)						16) NM-300k (Ag)					
E	4) JRCNM04000a (MWCNT, =NM-400)						17) NM-300k-DIS						4) JRCNM04000a (MWCNT, =NM-400)						17) NM-300k-DIS					
F	5) JRCNM04002a (MWCNT, =NM-402)						18) NM-302 (Ag-rod)						5) JRCNM04002a (MWCNT, =NM-402)						18) NM-302 (Ag-rod)					
G	6) JRCNM40011a (Mitsui-7, NRCWE-006)						19) NM-302-DIS						6) JRCNM40011a (Mitsui-7, NRCWE-006)						19) NM-302-DIS					
H	7) JRCNM50001a (BaSO4, =NM-220)						20) TiO2 E171-Venator						7) JRCNM50001a (BaSO4, =NM-220)						20) TiO2 E171-Venator					
I	8) JRCNM62101a (ZnO)						21) JRCNM02102a (CeO2)						8) JRCNM62101a (ZnO)						21) JRCNM02102a (CeO2)					
J	water						water						water						water					
K	9) NM-105 (TiO2)						22) Sigma Silver #576832						9) NM-105 (TiO2)						22) Sigma Silver #576832					
L	10) NM-110 (ZnO)						23) carbon black (Calibrate dispersion #21)						10) NM-110 (ZnO)						23) carbon black (Calibrate dispersion #21)					
M	11) NM-111 (ZnO)						C2) 5-FU 100mM final						11) NM-111 (ZnO)						C2) 5-FU 100mM final					
N	12) NM-113 (ZnO)						C3) 4NQO 50uM final						12) NM-113 (ZnO)						C3) 4NQO 50uM final					
O	13) NM-200 (SiO2)						C4) MMC 100uM final						13) NM-200 (SiO2)						C4) MMC 100uM final					
P	water						water						water						water					

Figure 1. Assay plate layout for the 3D / 2D screens

5.8 Procedure:

5.8.1 Culture of cryopreserved HepG2 cells and preparation of frozen cell stocks:

- Pre-warm both cell culture medium and Trypsin-EDTA in a 37°C water bath.
- Remove one vial of HepG2 cells from -80 freezer and thaw in a 37°C water bath for 2-3 minutes, whilst gently swirling the vial to allow for uniform thawing

of the cell suspension. Take care not to submerge the vial above the O-ring in order to reduce the potential for contamination.

- Once thawed, remove the vial from the water bath and spray generously with 70% Ethanol to decontaminate the outer surface if the vial before placing under a sterile Laminar Tissue Culture hood.
- Carefully pipette the contents of the cryovial of HepG2 cells into a falcon tube containing 9ml of warm cell culture medium.
- Using a 10 ml strippette, transfer 10 ml of the cell suspension into a T-25 flask and incubate the culture for 3 Days (from seeding) at 5% CO_2 and 37°C until ~80% confluency is reached before undergoing sub-culture into a larger T-75 flask.
- To subculture into a T-75 flask, discard cell medium, rinse the cells with room temperature 1xPBS and add 1.5 ml Trypsin-EDTA, ensuring to cover the entire surface of the cells and incubate cells for 6-8 minutes at 37°C and 5% CO_2.
- Following this, gently tap the flask to dislodge the cells from the bottom of the flask and then add 1.5 ml of 1X PBS at room temperature to dilute Trypsin.
- Transfer the cell suspension into a 15ml centrifuge tube.
- Centrifuge the diluted cell suspension at 113 g (900 rpm) for 3 minutes.
- Discard the supernatant by aspiration and re-suspend cell pellet in 1 ml of 1X DMEM cell culture medium. Suspend the culture by pipetting 50x with a 1ml pipette followed up by 50x with a 200 µl pipette to ensure complete dissociation.
- Transfer cell suspension into a T-75 flaks and incubate at 37°C and 5% CO_2 for 3-4 Days.
- To prepare frozen cell stocks repeat sub-culture steps stated above, except following centrifugation and dissociation to a single cell suspension bring the total volume up to 10 ml with DMEM complete medium, suspend and take a 20 µl aliquot for cell counting.
- Pipette 20 µl of cell suspension in to a counting chamber and count the cell number using Nexcelom bioscience Cellometer mini automated cell counter.
- Prepare a HepG2 cell suspension with 50% HepG2 medium, 50% freeze

medium (7.5% DMSO final) at concentration of $2.0x10^6$ cells/ml and freeze the cells at a rate of -1°C/minute using Corning CoolCell freezing containers.

5.8.2 Sub-culture of HepG2 cells:

- Discard existing media, wash cells to remove all traces of existing media by rinsing the flask twice with room temperature 1X PBS solution.
- Once washed, add 1.5 ml of Accutase cell detachment solution, ensuring to cover the entire surface of the cells and incubate cells for 6-8 minutes at 37°C and 5% CO_2.
- Following this, gently tap the flask to dislodge the cells from the bottom of the flask and then add 1.5 ml of 1X PBS at room temperature to dilute Accutase.
- Transfer the cell suspension into a 15ml centrifuge tube.
- Centrifuge the diluted cell suspension at 113 g (900 rpm) for 3 minutes.
- Discard the supernatant by aspiration and re-suspend the cell pellet in 1 ml of 1X DMEM cell culture medium. Suspend the culture by pipetting 50x with a 1ml pipette followed up by 50x with a 200 µl pipette to ensure complete dissociation.
- Transfer 1:3-1:6 of the cell suspension into a 10 cm dish and incubate at 37°C and 5% CO_2 for 3-4 Days, then sub-culture again as they reach ~80% confluency.

5.8.3 HepG2 Spheroid Preparation:

- Repeat Sub-Culture steps stated above, except following centrifugation and dissociation to a single cell suspension bring the total volume up to 10 ml with DMEM complete medium, suspend and take a 20 µl aliquot for cell counting.
- Pipette 20 µl of cell suspension in to a counting chamber and count the cell number using a Nexcelom bioscience Cellometer mini automated cell counter (or equivalent cell counter).
- Prepare a HepG2 cell suspension with DMEM cell culture medium set at a

concentration of $5x10^4$ cells/ml to get 1000 HepG2 cells per 20 µl culture (or $2x10^5$ cell/ml for 4000 cells per 20 µl).

- Mix the cell suspension thoroughly to ensure all cells are fully suspended within the media.
- Pipette 20 µl of cell suspension per well to Thermo Fisher Scientific microarray polypropylene 384-well plates (AB-1055) using a multidrop liquid handling station.
- Place the plates into an incubator at 37°C and 5% CO_2 for 3 days prior to spheroid transfer to assay plates.

5.8.4 Seeding for the parallel HepG2 2D screens:

- Parallel 2D HepG2 screens were carried out together with the 3D screens.
- Day 3 post seeding of the 3D spheroids HepG2 cells grown as monolayers in 10 cm dishes were detached and counted and 1000 cells per 384-well added to non-polyhema coated assay plates.

5.8.5 HepG2 spheroid transfer:

Day 3 post seeding, the spheroids are transferred into the wells of 384-well assay plates all of which have been previously coated with polyhema prior to addition of nanomaterials to the plates. The polyhema coating prevents the HepG2 spheroids from adhering to the base of wells and forming a cellular monolayer enabling thus anchorage independent growth and retaining their 3D spheroid structure.

- On Day 3 post seeding, remove the 384-well plate containing the HepG2 spheroids out of the incubator and carefully flip the plate and place on top of an assay plate containing the nanomaterial library (in 10 µl). Place the spheroid plate *exactly* on top of the assay plate so that position of each well matches to ensure transfer of spheroids form each well. Attach the plates together by wrapping Parafilm around the plate edges. Transfer the cell to assay plate by centrifugating the plate “sandwich” down at 100g for 30 seconds. Add in 70 µl of fresh culture medium with multidrop liquid handling

station to allow five day exposures without a need for medium change.

- Note: AB-1056 384-well polypropylene plate has smaller well diameter than AB-1055 and would be the preferred choice to grow the cells in due to easier transfer. These plates were unfortunately only available from Thermo Fisher scientific with over six-month delivery time.

5.8.6 HepG2 spheroid endpoint measurements:

Following either chemical or nanomaterial exposure treatments, both cell culture medium or the spheroids can be analysed. Two assay plates were prepared for each timepoint and culture (24hour, 120 hour, 3D and 2D cultures).

To measure CellTiter-Glo (CTG), Caspase 3/7-Glo and LDH-Glo from assay plate number 1:

- Add 2 µl of 10% Triton x-100 per 100 µl to wells P19-24 for 10-15 minutes to be used as maximum LDH release controls for calculation of % cytotoxicity.
- Spin at 50g for 30 seconds to ensure mixing of Triton x-100 with the medium.
- After 10-15 minutes carefully remove the supernatant using an Eppdendorf Eppmotion liquid handling station, leaving spheroids in 15 µl of medium (suction height setting calibrated to leave 15 µl of liquid to the wells).
- Transfer the medium to new 384-well plates (Corning 3570) for LDH release study.
- Add 8 µl per well of Caspase-Glo to columns 13-24 and 8 µl per well of CTG to columns 1-12 with an electronic 16-channel pipette.
- Measure luminescence when Caspase 3/7-glo has been applied for 30 minutes using a Labrox plate-reader (300 ms measurement time using an IR-blocker).
- While waiting for the Caspase / CTG measurement use “aspirate 10 µl + Dispense x5” routine on the Eppmotion station to mix supernatants meant for LDH analysis on quarter of a plate at time. After mixing aspirate 10 µl of supernatant and transfer to a new 384-well plate containing 60 µl of LDH

storage buffer (=1:7 dilution). Mix five times and transfer 10 µl to a new plate containing 60 µl of LDH storage buffer (1:49 dilution of supernatant). Mix the final 1:49 dilution for five times. Repeat for plate quarters 2,3 and 4.

- Transfer 8 µl of 1:49 diluted supernatant to a new plate (columns 1-12 2D samples, columns 13-24 3D samples)
- Add 8 µl per well of LDH mix (mix 4.2 ml of LDH detection enzyme mix + 21 µl reductase substrate for 2x half-plates of 2D and 3D cultures).
- Dilute HepG2 medium 1:7 + 1:7 to make corresponding medium only controls.
- Add 8 µl of 1:49 diluted medium to 8x 384-plate wells and add 8 µl of LDH mix.
- Incubate for 30 minutes and read luminescence as above.
- Heat-seal the undiluted, 1:7 and 1:49 diluted supernatant-plates. Store at -20°C for possible further analysis.

To measure ROS-Glo and Cytotox-Glo from assay plate number 2:

- Spin the plates (2D+3D cultures) at 50 g for 30 seconds.
- Aspirate supernatant off using an Eppendorf Eppmotion at 11.1 cm setting, leaving the spheroids in 15 µl of medium.
- Discard the supernatant.
- Add 2.5 µl per well of H_2O_2-substrate to columns 1-12 of both plates.
- Spin down at 50g for 30 seconds.
- Incubate at +37°C, 5% CO_2 for 5 hours.
- Add ROS-Glo detection solutions (mix 4 ml ROS-Glo + 40 µl d-cysteine + 40 µl signal enhancer for 2x half-plates) 8 µl per well to columns 1-12.
- Add 8 µl per well of AAF-Glo (Cytotoxicity) to columns 13-24 (4 ml in total).
- Measure luminescence (at 20 minutes for ROS-Glo and 15 minutes for AAF-Glo).
- Add 8 µl per well of cytotoxicity lysis buffer (4 ml of assay buffer + 26 µl of Digitonin (concentration)) to measure total dead cell protease signal from lysed and dead cells and incubate for 15 minutes.
- Measure luminescence as detailed above.
- Add 15 µl of HepG2 medium to 8x 384-wells.

- Add 8 µl of AAF-Glo reagent and incubate for 15 minutes.
- Measure background signal from the medium using the luminescence measurement approach detailed above.

5.8.7 Testing for nanomaterial interference:

A decrease in Cytotox-Glo signal was detected when control wells were compared to wells with all tested ZnO particles as well as all silver particles (including inhibitory effects with the silver 300k dispersant). Normalisation of the data to cell viability values from the same assay greatly improved the issue. Also, high luminescence signal from the cytotox assay, which was in the same plate as the ROS-Glo samples, interfered with the significantly lower ROS-Glo signal (signal carryover form neighbouring wells despite using black wells to overcome the issue). All ROS samples from neighbouring column (column 12) were omitted from the analysis (the lowest concentration samples). Although not direct interference, but rather a result of the material property, TiO_2 increased cell viability especially at the 24 hour timepoint in 3D cultures. 0-hour CTG and Caspase-Glo interference tests (where cells are added to the assay plate as usually and then CTG/Caspase-activity is directly measure) was carried out with 2D cultures and although a CTG signal decrease was observed with silver nanomaterials indicative of assay interference, the effect was not evident in later timepoints and CTG data correlated well with flourenscent cell number measurements obtained from Dapi staining. Thus, it was concluded that the early interference was not present at later timepoints. No interference was observed in the Caspase-3/7-Glo assay.

5.9 Quality control & acceptance criteria:

5-FU, MMC and 4NQO were used as positive controls in all the assays. Statistically significant (students t-test of the 4 replicates versus non-treated controls) response with at least two of the controls with a minimum of two concentrations was used as acceptance criteria for the assays.

Response values above two standard deviations from the median of controls were considered as positive.

Transfer of nanomaterials from source wells to assay wells was not 100% successful due to unavailability of optimal source plates and thus data from empty wells were omitted from analysis.

In the LDH-Glo assay the maximum LDH release control values induced by addition of Triton x-100 to control wells were in many cases smaller than median controls values, so due to lack of positive response from the maximum response controls the data was left out of the analysis. Also, response from the positive controls (5-FU, MMC, 4NQO) did not meet acceptance criteria.

6 Data Analysis and Reporting of Data:

- Export data from the Labrox plate reader to excel files.
- Tranform the data from plate format to list format using a transformation template.
- Annotate the data.
- Express the data plate-wise as % of median controls (water only).
- Make dose-response curves of data (median of biological replicates) for visual inspection of the results.
- Calculate a modified Tox5 score by calculating AUC, maximum effect and the 1st significant effect from the data:
    - Calculate median response of the four biological replicate screens from the % of median control data.
    - Remove values from wells in which transfer of spheroid from source plate to assay plate has not succeeded using: IF(AND(AT4<(MEDIAN(AT4:AW4)/2), AT4<20, MEDIAN(AT4:AW4)>25),"",AT4). This removes values that are clearly

smaller than the median of the four biological replicates.

- Normalize cytotoxicity data to cell viability data (from the cytotox-Glo assay) to get a specific cytotoxicity signal, which is not influenced by cell number.
- Calculate “median of controls + 2x standard deviation” cut-off values for each timepoint.
- Calculate p-values for each concentration by comparing values from four replicates to corresponding controls (water only) using student’s T-test.
- Eliminate p-values from responses smaller than “median of controls + 2x standard deviation” (biologically insignificant responses).
- Determine the first significant effect concentration for each nanomaterial.
- Determine the maximum effect for each nanomaterial.
- Determine Area Under Curve (AUC) for each nanomaterial using data larger than “median of controls + 2x standard deviation” using GraphPad Prism 6.0 software.

- Take the three calculated parametres to ToxPi software (Reif et al., 2013), (Marvel et al., 2018), (Reif et al., 2010) as individual slices so that each parameter will be normalized separately. Use square root normalization for AUC and maximum effect data and -log10(x)+6 normalization for 1st significant effect data to shift data closer to normal distribution.
- Collect the normalized parameter values from ToxPi and take them back to ToxPi by combining parameters from a single timepoint and endpoint into a single slice. Collect the final ToxPi scores with 95% confidence intervals and ranks.
- Use ToxPi to do hierarchical clustering of the data to find common toxicity mechanisms.

7 Publications:

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8 References:

Marvel, S.W., To, K., Grimm, F.A., Wright, F.A., Rusyn, I., Reif, D.M., 2018. ToxPi Graphical User Interface 2.0: Dynamic exploration, visualization, and sharing of integrated data models. BMC Bioinformatics 19, 80. https://doi.org/10.1186/s12859-018-2089-2

Reif, D.M., Martin, M.T., Tan, S.W., Houck, K.A., Judson, R.S., Richard, A.M., Knudsen, T.B., Dix, D.J., Kavlock, R.J., 2010. Endocrine profiling and prioritization of environmental chemicals using ToxCast data. Environ. Health Perspect. 118, 1714–1720. https://doi.org/10.1289/ehp.1002180

Reif, D.M., Sypa, M., Lock, E.F., Wright, F.A., Wilson, A., Cathey, T., Judson, R.R., Rusyn, I., 2013. ToxPi GUI: an interactive visualization tool for transparent integration of data from diverse sources of evidence. Bioinforma. Oxf. Engl. 29, 402–403. https://doi.org/10.1093/bioinformatics/bts686