PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Tissue characterisation, nanomaterial treatment and toxicological assessment in 3D primary human liver microtissues

This is a SOP recommended for external use by PATROLS

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

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Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	05/06/2018	Initial Document	Ali Kermanizadeh
2.0	20/07/2018	Amended Protocol	Ali Kermanizadeh
3.0	17/11/2018	Amended Protocol	Ali Kermanizadeh
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1 Introduction:

It is essential to establish more advanced, physiologically relevant *in vitro* assessment tools for improved prediction of the adverse effects caused by chronic nanomaterial (NM) exposure in humans. The utilisation of human primary hepatic cells is the closest representative *in vitro* model for the human liver. However, these cells are phenotypically unstable in 2D cultures and have an extremely limited life span (typically no longer than 7 days - with continued reduced viability, functional and metabolic activity). Moreover, in most traditional 2D hepatic models, non-parenchymal cell (NPC) populations are not included or considered. In an attempt to address this issue, the use of scaffold free 3D liver microtissue (MT) model composed of primary human hepatocytes and primary human liver-derived NPC could be beneficial.

The following protocol outlines how commercially available 3D liver microtissues derived from primary human cells can be treated with NMs in order to assess impacts on viability and function.

1.1 Limits of the protocol:

- Microtissues are extremely expensive
- Cell culture is technically challenging
- Limited volumes of supernatant and cells
- There are complications in dosimetry in the model due to the shape of the well and unknowns about the rotation of the MT in the well over time.
- There is no physiologically relevant structure to the tissue - cells aggregate randomly

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	
Stage 2: Validated internal laboratory method	
Stage 3: Interlaboratory tested method	X
Stage 4: Method validated by Round Robin testing	
Standardisation plans	
Is the method considered for standardisation (OECD SPSF or similar)?	Maybe
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 Abbreviations:

CYP - Cytochrome P450

ELISA - Enzyme linked immunosorbent assay

KC - Kupffer cell

NMs - Nanomaterials

MT - Microtissue

NPC - Non-parenchymal cell

PBS - phosphate buffered saline

TBS - Tris buffered saline

3 Description of the Method:

3.1 Biological setting & test system used:

This SOP should be carried out under laboratory based conditions, with all work performed under sterile conditions and in a Class 2 Laminar Tissue Culture Hood.

3.2 Chemicals and reagents used:

Agarose - Sigma - A9539
Human liver maintenance medium AF - InSphero AG - CS-07-001a-01
40% formaldehyde solution - Sigma – 128775
10x phosphate buffered saline (PBS) - Sigma - P5493
Hematoxylin and eosin stains - Sigma - E4009 and H9627
10 x Tris buffered saline (TBS) - Sigma- T5912
Triton-X-100 - Sigma - T8787
ToxiLight™ bioassay kit - Lonza - LT07
Live/death cell staining kit - Abcam - ab115437
Albumin ELISA - e.g. Bethyl laboratories or R and D Systems
Caspase-Glo® 3/7 reagent - Promga - G8091
Midazolam - e.g. Sigma - BP722
Bond™ Epitope Retrieval Solution 2 - Leica
anti-CD68 antibody - i.e. Novocastra Laboratories Ltd
Appropiate secondary antibody for primary CD68 antibody
DAB substrate kit - i.e. Abcam

3.3 Apparatus and equipment used:

1.5 ml eppendorf tube - preferably with flat bottom - e.g. Eppindorf - 0030120086
Paraffin embedding cassettes e.g. Fischer Scientific - 10364511
Luminescence compatible 96 well plate
8 well microscopy chambers - Ibidi – 80826

Micropipettes
Centrifuge
Microcentrifuge
Plate shaker
Automatic cell counter or haemocytometer
-80°C freezer
Humidified incubator (37°C and 5% CO_2)
Inverted light microscope - e.g. ZEISS Axiovert 40 C light microscope
Inverted wide field fluorescence microscope - i.e. Leica AF6000
Tissue processor - e.g. Leica HistoCore PELORIS 3
Plate-reading luminometer

3.4 Reporting of protected elements:

This SOP does not have any associated patent restrictions, specific licenses, material transfer agreements

3.5 Health and safety precautions:

Standard health and safety precautions associated with working within a laboratory environment and performing mammalian cell culture, as described by the European Agency for Safety and Health at Work (https://osha.europa.eu/en/safety-and-health-legislation/european-guidelines), should be adopted when conducting this SOP. In addition, all health and safety precautions outlined in the MSDS data sheets associated with the specific chemicals required must also be followed.

3.6 Nanomaterials used/handling procedures:

1. NMs to be prepared following the NANOGENOTOX dispersion protocol (Appendix 1) (stock solution 1 mg/ml).
2. Following the sonication step, transfer all samples to ice before being diluted in human liver maintenance medium just prior to the experiments.

3. Add the NM to the wells at a total volume of 50 µl.
4. Include negative (cell culture medium) and positive (1% Triton-X) controls (or other appropriate controls for different toxiclogical end-points).

Note: For each treatment, five wells are used and all experiments are repeated on three separate occasions.

3.7 Procedure:

3.7.1 Liver MT maintenance:

1. Maintain the human liver in human liver maintenance medium AF at 37°C, 5% CO_2 95%.humidity.
2. Exchange old medium (70 µl per well) with new preheated medium (37°C) on the day of arrival.
3. The medium is exchanged every 2 days thereafter (50 µl per well) until the experiments are commenced.

3.7.2 Morphological characterisation of 3D human liver MT:

3.7.2.1 Hematoxylin and eosin staining:

1. The MT are harvested at the required time-points by placing the pipette tip close to the bottom of the well using a 1 ml pipette tip and aspirating 50 µl of medium supernatant. Alternatively, a multi-channel pipette can be used for parallel harvesting of multiple MTs (aspirate 1.2-1.5x sample volume at medium aspiration speed)
2. Transfer the MT into a 1.5 ml Eppendorf tube (as described in step 1)
3. Allow the MT to settle to the bottom of the tube by gravity (5 min).
4. Optional: centrifuge for 10 seconds at minimum speed of 200 RCF. Use a centrifuge with fixed angle horizontal rotor, if available. Otherwise spin twice, with turning the tube by 180° in between spins.
5. Carefully aspirate the supernatant and discard it.

6. Fix the MT by the addition of 500 µl 4% formaldehyde (diluted in distilled water) solution into the reaction tube for at least 1 hr at room temperature or overnight at 4°C.
7. Wash 2 times with 1 ml PBS (centrifugation step between washed - 200 RCF).
8. Prepare 2% agarose solution in PBS, boil and allow to cool to approximately 50-60°C with constant stirring.
9. Remove PBS from MT.
10. Carefully pipette 600 µl agarose solution into the tube containing the MT.
11. Immediately spin the sample for 2 seconds using a centrifuge with a horizontal rotor. If no horizontal centrifuge is available, centrifuge twice for 2 seconds, with turning the tube by 180° in between spins.
12. For easier removal of the agrose plug from the tube, insert a short string immediately after the centrifugation and before the agarose is completely solidified.
13. Let the agarose plug solidify on ice for 5 min.
14. Add 500 µl PBS and place at 4°C for intermediate storage (stable for a minum of a week).
15. Remove the agarose plug with a disposable plastic pipette by generating gentle suction (or by pulling the thread).
16. Transfer the agarose plug with the MT pellet into a conventional paraffin embedding cassette.
17. Dehydrate the tissue sample in a tissue processor using a standard dehydration program.
18. Embed the agarose plug into a paraffin embedding mould with the MT pellet facing the bottom of the mould. Make sure the agarose plug stays upright until paraffin starts to solidify from the bottom.
19. Section and stain paraffin sections with H&E

3.7.2.2 Optional: immunohistochemistry staining for KCs is performed with a CD68 antibody:

1. For CD68 staining (to identify location of KCs), the samples are treated with Bond™ Epitope Retrieval Solution 2 for 30 mins at 100°C.
2. The samples are treated with anti-CD68 antibody (i.e. Novocastra Laboratories Ltd, USA - NCL-L-CD68) (1:150) for 30 min at 25°C.
3. Next, a horseradish peroxidase conjugated secondary antibody is applied (i.e. human anti mouse IgG2a: HRP - AbD Serotec, UK) - 1 hr incubation at room temp.
4. The samples are rinsed three times using 5x TBS and developed using a DAB substrate kit according to manufacturer's instructions.
5. The MT can be visualised utilising a light microscope

Note: It is recommended to harvest >20 MTs per sample. This will ensure a sufficient number of MTs for IHC staining and assessment of biomarker expression

3.7.3 Toxicological anlysis:

3.7.3.1 Adenylate kinsase (AK) assay:

The cell membrane integrity can be evaluated utilising a Lonza ToxiLight™ bioassay kit.

1. Transfer 20 µl of cell supernatant from liver MT to a luminescence compatible plate.
2. Transfer 80 µl of AK detection buffer to all wells.
3. Incubate plates for 5 min at room temperature
4. Measure luminescence using a standard plate-reading luminometer.

3.7.3.2 Live/dead staining:

In order to get pictorial representations of NM induced MT cell death a live/dead cell staining kit (i.e Abcam) can be utilised. The kit uses a cell permeable green

fluorescent dye (Ex/Em - 488/518 nm) to stain live cells, while dead cells are stained via propidium iodide (Ex/Em - 488/615 nm).

1. Transfer MT to 8 well microscopy chambers (using a 1 ml pipette).
2. Add 200 µl of the staining solution to all samples.
3. Incubate for 15 min at 37°C.
4. Tissues can be observed under an inverted wide field fluorescence microscope. In all instances, images are captured on a 2D plane, and then as a z-stack before undergoing a 3D de-convolution and represented as a final 3D reconstituted image.

3.7.3.3 Albumin production:

After exposure, the supernatants (from both the control and treated cells as described above) are collected and stored at -80ºC.

1. Centrifuge the supernatant at 1000 g
2. Transfer supernatant to new tube to remove NMs and cell debris.
3. Dilute the supernatant two fold in 1xPBS
4. Albumin levels determined by ELISA according to the manufacturer's instructions

3.7.3.4 Caspase activity assay:

1. Before starting the assay, Caspase-Glo® 3/7 reagents (Promega) are prepared according to manufacturer's instructions.
2. Mix the reagents thoroughly and allow to equilibrate to room temperature.
3. Remove the MT plates from the incubator and allow to equilibrate to room temperature.
4. Add 50 µl of Caspase-Glo® 3/7 reagent to all appropriate wells.
5. Gently mix contents of wells using a plate shaker at 500 rpm for 5 min.
6. Incubate the plates at room temperature for 90 min in the dark.
7. Transfer entire well content into to a luminescence compatible plate
8. Measure the luminescence in a standard plate-reading luminometer.

3.7.3.5 CYP3A4 measurements:

1. 3D InSight™ human liver MT are incubated in 50 µl medium containing 8 µM of midazolam for 24 hr as a probe substrate for CYP3A4 activity. The amount of midazolam-1'-hydroxylation is assessed at required time-points. For all time points three single MT can be analysed as replicates for each time point.
2. Harvest the supernatant and store at -80°C.
3. The midazolam-1-hydroxylation in cell culture supernatants can be quantified (i.e. outsourced to Pharmacelsus® - GmbH, Germany)).

NOTE: Cytochrome P450 enzymes metabolize approximately 60% of all prescribed drugs, with CYP3A4 responsible for about half of this metabolism (Zanger *et al.* 2014).

4 Data Analysis and Reporting of Data:

The data generaed data using the above SOP have been distribted to WP7 of PATROLS project (Dissemination, exploitation and knowledge transfer) and published below.

5 Publications:

Kermanizadeh A, Brown DM, Moritz W, Stone V. 2019. The importance of inter-individual Kupffer cell variability in the governance of hepatic toxicity in a 3D primary human liver microtissue model. Nature Scientific Reports 13: 7295.

Kermanizadeh A, Berthing T, Wheeldon M, Whyte G, Vogel U, Moritz W, Stone V. 2019. Assessment of nanomaterial-induced hepatotoxicity using a 3D human primary multi-cellular microtissue exposed repeatedly over 21 days - the suitability of the in vitro system as an in vivo surrogate. Particle and Fibre Toxicology 19: 42.

Kermanizadeh A, Løhr M, Roursgaard M, Messner S, Gunness P, Kelm JM, Møller P, Stone V, Loft S. 2014. Hepatic toxicology following single and multiple exposure of engineered nanomaterials utilising a novel primary human 3D liver microtissue model Particle and Fibre Toxicology 11: 56.

6 References:

Zanger UM, Klein K, Thomas M, Riegler JK, Tremmel R, Kandel BA, Klein M, Magdy T. 2014. Genetics, epigenetics, and regulation of drug-metabolizing cytochrome p450 enzymes. Clinical Pharmacology and Therapeutics 95: 258-261.

7. Appendix:

Nanogenotox dispersion protocol