PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Guidance Document for co-culture of an lung epithelial cell-line (Calu-3), and macrophages derived from a monocytic cell line (dTHP-1)

This is a a) SOP used by members of PATROLS only or (b) 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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WP3 members and RR participants

Document History:

Version	Approval Date	Description of the change	Author(s) of change
1.0	06.04.2020	Initial Document	Hedwig Braakhuis
2.0	14.04.2020	Additional detail added from AMI	Anne Bannuscher
3.0	15.05.2020	Additional details added from SU	Kirsty Meldrum
3.1	27.07.2020	Comments from WP3 implimented	Kirsty Meldrum
4.0	30.07.2020	Edit of protocol to contain addition detail and evidence.	Kirsty Meldrum
4.1	30.09.2020	Harmonisation of SOPs	Kirsty Meldrum
4.2	21.10.2020	Additional information added for clarification	Kirsty Meldrum
5.1	14.12.2020	Harmonisation between MDM and dTHP-1 SOP completed	Kirsty Meldrum
5.1.1	12.01.2021	Additional harmonisation between MDM and dTHP-1 SOP completed	Kirsty Meldrum

Table of Contents

1 Introduction:

This SOP is specifically developed for the Round Robin (RR) testing of a co-culture lung model consisting of Calu-3 human bronchial epithelial cells and macrophages (dTHP-1). The co-culture model will be exposed to chemicals (acrolein, LPS and TNF-α) and to particles (DQ12 quartz) using air-liquid interface (ALI) exposure. ALI exposure is considered more relevant compared to submerged exposure. In this SOP, the VITROCELL® Cloud2 system (12-well version) equipped with Aeroneb Pro nebulizer, and quartz crystal microbalance (QCM) for measuring online deposition is used for the ALI exposures.

1.1 Scope and limits of the protocol

This SOP was established with the intention to be used by all participants of the RR study within the project PATROLS. This SOP provides instructions on how to culture the cells, aerosolize LPS, acrolein, TNF-α and DQ_{12} using the VITROCELL® Cloud2 system (12-well version).

Limitations:

To be able to provide a stable suspension of DQ_{12} (to achieve this, refer to SOP_PATROLS_Cloud_Aerosolization_DQ12 and NanoReg protocol: 'Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media' but **without** BSA dispersion).

There are also restrictions placed on the cell line which state the cells (Calu-3) are only permitted for use for research purposes only and proposed commercial uses must be negotiated with the Memorial Sloan-Kettering Cancer Center.

"The cells are distributed for research purposes only. The Memorial Sloan-Kettering Cancer Center releases the cells subject to the following: 1.) The cells or their products must not be distributed to third parties. Commercial interests are the exclusive property of Memorial Sloan-Kettering Cancer Center. 2.) Any proposed commercial use of these cells must first be negotiated with the Office of Technology Development, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Contact email: otd@mskcc.org »

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	
Stage 4: Method validated by Round Robin testing	x
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)	Y
If yes, specify	[standard reference number, eg. EN 17199-4]

2 Terms and Definitions:

Agglomerate

Collection of weakly or medium strongly bound *particles* where the resulting external surface area is similar to the sum of the surface areas of the individual components.

Note 1 to entry: The forces holding an agglomerate together are weak forces, for example van der Waals forces or simple physical entanglement.

Note 2 to entry: Agglomerates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.2]

Aggregate

Particle comprising strongly bonded or fused particles where the resulting external surface area is significantly smaller than the sum of surface areas of the individual components.

Note 1 to entry: The forces holding an aggregate together are strong forces, for example covalent or ionic bonds, or those resulting from sintering or complex physical entanglement, or otherwise combined former primary particles.

Note 2 to entry: Aggregates are also termed secondary particles and the original source particles are termed primary particles.

[SOURCE: ISO 26824:2013, 1.3, modified — Note 1 adapted.]

Nanoscale

Length range approximately from 1 nm to 100 nm

Note 1 to entry: Properties that are not extrapolations from larger sizes are predominantly exhibited in this length range.

[SOURCE : ISO/TS 80004-1: 2016, definition 2.1]

Nanotechnology

Application of scientific knowledge to manipulate and control matter predominantly in the *nanoscale* to make use of size- and structure-dependent properties and phenomena distinct from those associated with individual atoms or molecules, or extrapolation from larger sizes of the same material.

Note 1 to entry: Manipulation and control includes material synthesis.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.3]

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]

Nano-object

Discrete piece of material with one, two or three external dimensions in the *nanoscale*.

Note 1 to entry: The second and third external dimensions are orthogonal to the first dimension and to each other.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.5]

Nanostructure

Composition of inter-related constituent parts in which one or more of those parts is a *nanoscale* region.

Note 1 to entry: A region is defined by a boundary representing a discontinuity in properties.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.6]

Nanostructured material

Material having internal *nanostructure* or surface nanostructure.

Note 1 to entry: This definition does not exclude the possibility for a *nano-object* to have internal structure or surface structure. If external dimension(s) are in the *nanoscale*, the term nano-object is recommended.

[SOURCE: ISO/TS 80004-1: 2016, definition 2.7]

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]

Incidental nanomaterial

Nanomaterial generated as an unintentional by-product of a process.

Note 1 to entry: The process includes manufacturing, bio-technological or other processes.

Note 2 to entry: See “ultrafine particle” in ISO/TR 27628:2007, 2.21

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]

Substance

Single chemical element or compound, or a complex structure of compounds.

[SOURCE: ISO 10993-9:2009, definition 3.6]

3 Abbreviations:

ALI – air-liquid interface

BSA – bovine serum albumin

CCM - Cell Culture Medium for Calu-3

DQ12 - Dörntruper quartz 12

EDTA - ethylenediaminetetraacetic acid

ENM – engineered nanomaterials
FBS - fetal bovine serum
HBSS – Hank's balances salt solution
LDH – lactate dehydrogenase
LPS - lipopolysaccharide
M-CSF – macrophage colony stimulating factor
MEM – minimum essential medium
NEAA - Non-Essential Amino Acids
PBS – phosphate buffered saline
PMA- Phorbol-12-myristate-13-acetate
QCM – Quartz crystal microbalance
RPMI - Roswell Park Memorial Institute-1640 Medium
RR – Round Robin
TEER – trans epithelial electrical resistance
TEM – transmission electron microscopy
THP-1CCM - Cell Culture Medium for (d)THP-1
TNF-α – tumor necrosis factor alpha

4 Principle of the Method

This SOP aims to provide a comprehensive overview of all the steps in RR2: ALI exposure of Calu-3 and dTHP-1 macrophages using VITROCELL® Cloud system.

This protocol will be defined within five key stages:

1. Cell culturing
2. Preparing co-culture
3. Chemical and particle dispersion preparation
4. Preparing the device, aerosolization and cleaning
5. Read-out measuring

5 Description of the Method:

5.1 Biological setting & test system used:

This SOP should be carried out under controlled laboratory based conditions, with all work following safe handling of ENMs, particles and chemicals. In addition, cell culturing should be performed under sterile conditions and in a Class 2 Laminar Tissue Culture Hood, at Biosafety Level 1 (minimum).

- VITROCELL® Cloud12 system (VITROCELL®, Germany) equipped with
  - Quartz crystal microbalance (QCM)
  - Aeroneb® Lab nebulizer (4-6 µm pore size, Aerogen, Ireland)
  - This protocol referes to the Cloud2 system, which is designed for 12-well inserts.
- For further information:

https://www.vitrocell.com/inhalation-toxicology/exposure-systems/vitrocell-cloud-system/vitrocell-cloud-12

5.2 Chemicals and reagents used:

Chemicals, Nanomaterials and reagents are divided in three classes:

1) Recommendation of the supplier, alternative supplier is feasible, information about supplier and batch should be carefully documented in the raw data

2) Ordering from the defined supplier is mandatory, batch is flexible; information about the batch should be carefully documented in the raw data

3) Ordering from the defined supplier and the defines batch is mandatory. If the batch is not available, material can be exchanged between partners, if possible.

0.05% Trypsin-EDTA (1x) (5300-054, GIBCO®, Paisley, UK) -1

70% Ethanol - 1

Accutase (00-4555-56, Thermo Fisher Scientific) - 1

DMSO (e.g. D2438, Sigma Aldrich) - 1

DQ12 (IOM, Edinburgh) – 3

EDTA (E6758-100g, Sigma-Aldrich, Switzerland) - 1

ELISA kit (e.g. eBioscience) - 1

Human TNFalfa ELISA Ready-SET-Go!	Thermo Fisher Scientific	Invitrogen	88-7346-88	10 x 96 tests
Human IL-8 ELISA Ready-SET-Go!® (2nd Generation)	Thermo Fisher Scientific	eBioscience	88-8086-88	10x96tests
Human IL-12 p70 Elisa Ready-Set-Go Kit Invitrogen	Thermo Fisher Scientific	eBioscience	88-7126-88	10x96=1 kit
Human IL-10 Elisa Ready-Set-Go Kit Invitrogen	Thermo Fisher Scientific	eBioscience	88-7106-88	10x96=1 kit
Human IL-1beta ELISA Ready-SET-Go!	Thermo Fisher Scientific	Invitrogen	88-7261-88	10 x 96 tests
Human IL-6 ELISA Ready-SET-Go!	Thermo Fisher Scientific	Invitrogen	88-7066-88	10 x 96 tests

FBS (758093, Greiner bio-one or 10270-106, GIBCO®, Switzerland) - 1

HBSS (1x) (14175, GIBCO®) - 1

Isotonic sterile 0.9% NaCl solution - 1

L-Glutamine (25030081, GIBCO®) - 1

LDH cytotoxicity detection kit (No. 11 644 793 001, Roche Diagnostics GmbH, Mannheim, Germany) - 2

LPS (L4391, Sigma) – 2

MEM (1x) + GlutaMAX (41090, GIBCO®) –2

NEAA solution (100x) (11140, GIBCO®) - 2

PBS pH 7.4 1X, MgCl2 and CaCl2 Free (14190-094, GIBCO®) - 1

Penicillin/Streptomycin (100X) (15140-122, Penicillin/Streptomycin 100X or 10,000U/mL, GIBCO®, Paisley, UK) - 1

PMA (phorbol 12-myristate 13-acetate; CAS Number 16561-29-8) (Sigma P8139-1MG or P8139-5MG) - 1

RPMI 1640 (21870076, GIBCO®, UK) –2

WST-1 cell proliferation kit (No. 11644807001, Roche Diagnostics GmbH, Mannheim, Germany) - 3

Triton X-100 (93426, Sigma) - 1

TiO2 (NM-105, Fraunhofer) - 3

Trypan Blue Solution (CAS# 72-57-1) (T8154-100mL, Sigma Aldrich®, UK) - 1

Ultrapure water - 1

5.3 Apparatus and equipment used:

Apparatus and equipment are divided in two classes:

1) Recommendation of the supplier, alternative supplier is feasible, information about supplier and batch should be carefully documented in the raw data

2) Ordering from the defined supplier is mandatory, batch is flexible; information about the batch should be carefully documented in the raw data

All tissue culture equipment is from Greiner Bi-One, UK unless stated otherwise.

Biohazard downflow cabinet - 1

Cell Freezing Aid - 1

12-well cell culture inserts (0.4 μm pore size, polyester)(CLS 3460, Corning) - 2

Cell scraper - 1

Centrifuge (Eppendorf) - 1

50 mL and 15 mL Centrifuge Tubes - 1

Sterile 2.0 mL Cryo vials - 1

0.2 mL and 1.0 mL Eppendorf Tubes - 1

EVOM2™ Epithelial Voltohmmeter supplemented with STX2 Chopstick Electrode Set (World Precision Instruments Inc., FL, USA) - 2

Liquid Nitrogen or Ultra low freezer (-130°C) - 1

Sterile filtration cup for vacuum filtration, 0.2 μm pore size - 1

Sterile Lab Bottle compatible with Filtration cup (min. 100 mL) - 1

Haemocytometer - 1

37˚C and 5% CO2 ISO Class 5 Hepa Filter Incubator - 1

Light Microscope - 1

Microbalance - 1

P20, P200 and P1000 micropipettes - 1

Filtered, Sterile 20 µl, 200 µl and 1000 µl Pipette tips - 1

Pipette Controller - 1

2 mL, 5 mL, 10 mL and 25 mL Pipettes - 1

12-wells plates (734-1598, Corning) - 1

Probe Sonicator - 1

Spectrophotometer - 1

T-25 (690175), T-75 (658175) and T-175 (660175) Tissue Culture Flask - 1

20 mL Scint-Burk glass pp-lock+Alu-foil vials (WHEA986581; Wheaton Industries Inc.) - 2

VITROCELL® Cloud12 - 2

Aeroneb nebulizers (4-6 µm pore size, IV1232 Vitrocell) - 2

Water Bath (37˚C) - 1

Water bath sonifier - 1

5.4 Reporting of protected elements:

There are restrictions associated with this SOP as previously indicated (Section 1.1). They are licenced for use within research institutions and not within industry.

From section 1.1

“There are also restrictions placed on the cell line which state the cells (Calu-3) are only permitted for use for research purposes only and proposed commercial uses must be negotiated with the Memorial Sloan-Kettering Cancer Center.

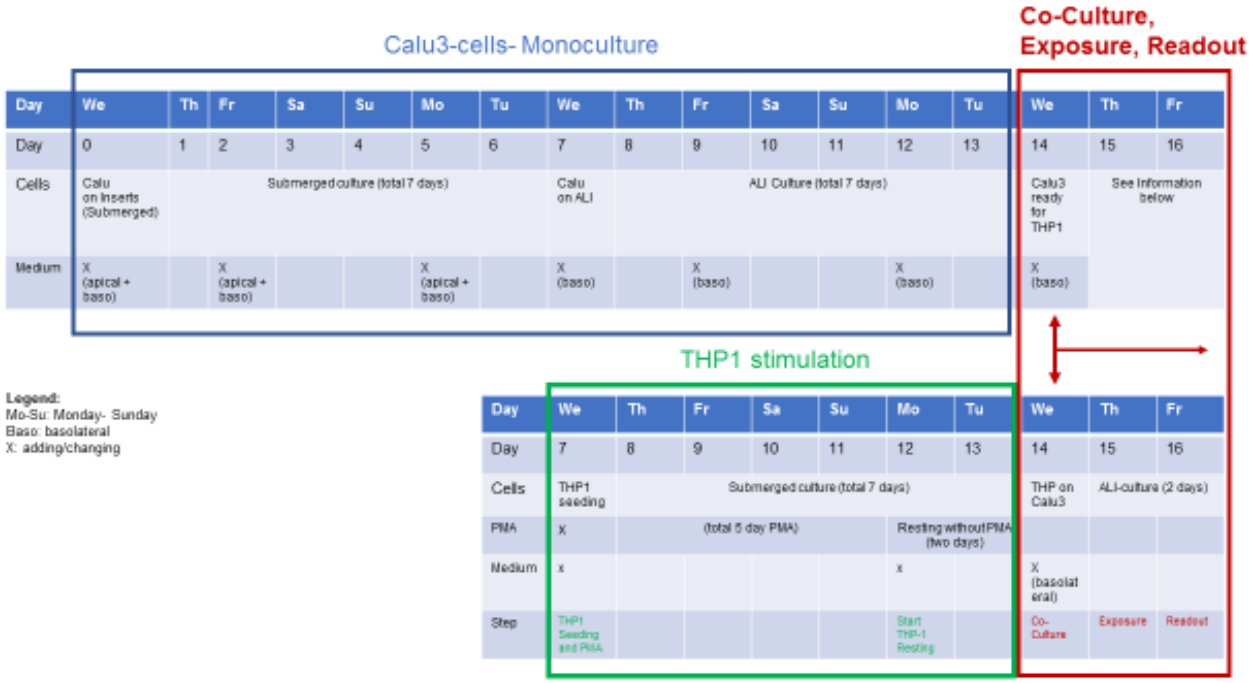
“The cells are distributed for research purposes only. The Memorial Sloan-Kettering Cancer Center releases the cells subject to the following: 1.) The cells or their products must not be distributed to third parties. Commercial interests are the exclusive property of Memorial Sloan-Kettering Cancer Center. 2.) Any proposed commercial use of these cells must first be negotiated with the Office of Technology Development, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10065. Contact email: otd@mskcc.org »

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.

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.

Trypan Blue solution is a known teratogen, so it is advised that any pregnant individuals take extra caution when handling the chemical and should follow additional health and safety guidelines stated by the Occupational Safety and Health



Administration (OSHA) in SOP 3.21 Reproductive Toxins, Mutagens, Teratogens and Embryotoxins – Procedures for Safe Handling and Storage. However, alternative cell viability assays (*e.g.* Eryothrosyn B) could be used instead if preferred.

6 Overview

Figure 1. Overview of seeding and exposure times.

For a typical experiment (n=1 and 1 concentration), 20 inserts are needed:

- Calu-3 monolayer clean air controls: 3
- Calu-3 monolayer incubator control: 1
- Calu-3 monolayer exposed cells: 4
- Calu-3 monolayer LDH max: 1
- Calu-3 monolayer reserve cells: 1
- Calu-3 + macrophages clean air controls: 3
- Calu-3 + macrophages incubator control: 1
- Calu-3 + macrophages exposed cells: 4

- Calu-3 + macrophages LDH max: 1
- Calu-3 + macrophages reserve cells: 1

7 *Cell Culturing (monocultures)*

7.1 Reagent preparation:

7.1.1 Cell culture medium (CCM) for Calu-3 cells

Minimum essential medium (MEM) + GlutaMAX (410900-36, Gibco) supplemented with:

- 1% Penicillin-Streptomycin (corresponds to 100 U/ml Penicillin and 100 µg/ml Streptomycin)
- 1% Non-Essential Amino Acids (NEAA) solution
- 10% FBS

Complete medium should be stored at between 4-6°C after preparation, and can be kept for 3 months at this temperature.

It should be noted that the CCM for Calu-3 cells used in this SOP, is different from the one advised by ATCC.

E.g. To a 500ml medium bottle remove 60ml of medium and add:

- 5ml Penicillin-Streptomycin
- 5ml NEAA
- 50ml FBS

Add the supliments in the order provided above. The medium needs to be mixed via invertion after each addition (make sure the lid is on before doing this!).

The term CCM is used for the complete cell culture medium with all additions.

In the lab journal make a note of the brand, catalogue number and batch number of FBS!!

7.1.2 Cell Culture Medium ((d)THP-1CCM) for (d)THP-1

RPMI (RPMI 1640 (21870076, Gibco) supplemented as above (Section 7.1.1), with the exception of NEAA and add 5ml of L-Glutamine instead.

This medium is used for both the growth and the differentiation of the THP-1s while in a monoculture.

7.2 Cell culturing procedure for Calu-3 epithelial cells

Publication online (Braakhuis, He et al. 2020)

7.2.1 Thawing Cells

1. Warm all required reagents to 37°C in a water bath (~20-30 min).
2. Remove one vial of cells from (-130°C) storage and gently swirl in a 37°C water bath (to ensure uniform thawing of the cell suspension) ensuring the O-ring and lid are not submerged (to reduce the potential for contamination) until the contents are almost thawed (between 1 and 2 min).
3. Remove the vial from the water bath and decontaminate with 70% ethanol, ensuring again that the decontaminant does not interact with the lid and the O-ring.

From here on, all steps need to be completed under aseptic conditions.

4. Transfer the cells into 14 mL prewarmed CCM in a T75 flask and incubate the culture at 37°C and 5% CO_2. NOTE cells from ATCC have to be seeded into T25 flasks in 5 mL to allow better cell contact and growing.
5. Change the CCM every 2-3 days. CCM is poured off and 15 mL of fresh CCM is pipetted into the flask and the flask placed back into the incubator.

7.2.2 Sub-Culturing

Calu-3 cells (ATCC HTB-55) are provided in cryovials frozen previously in liquid nitrogen with the number of passages identified. Keep a record of the passage number in the database of the -130°C freezer.

Calu-3 cells are maintained in T75 or T175 cell culture flasks, in which the cells are passaged at 60%-80% confluency every 7 days with CCM renewal every 2-3 days. CCM is poured off and fresh CCM (T75=15 mL and T175=25 mL) is pipetted into the flask and the flask is placed back into the incubator.

Cells should be passaged at least 2 times after thawing, before using in experiments or before freezing, and they should be passaged no more than 25 times in total (recovery of cells using trypsin takes longer after this passage has been reached, and therefore it is suggested to discard cells at this point).

Protocol

1. Confirm if flask is 60%-80% confluent by checking under a light microscope (See Figure 2).

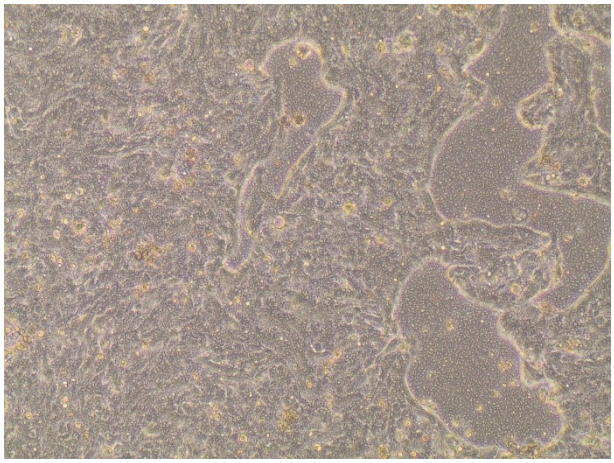


Figure 2. Calu-3 cells at 60-80% confluency

The following steps are completed under aseptic conditions

2. Remove the CCM.
3. Wash the cells with 5 mL of 1xHBSS two times, discard the HBSS after each wash. HBSS removes serum which inhibits trypsin.
4. Add 3 mL Trypsin-EDTA and place flask back into the incubator at 37°C and 5% CO_2 for 10-15min (checking after 10 min) ensuring the cells have become detached from the flask surface.
5. Add 6 mL (double the Trypsin-EDTA volume originally added) of CCM to the flask and gently rock the flask to ensure proper mixing. This is to ensure the trypsin has been neutralised by the FBS in the CCM and its actions on the cells are halted.

   NOTE If trypsin is allowed to remain in contact with the cells for too long they will not re-attach when put into a new cell culture flask.
6. Pour the complete contents of the flask into a 50 mL centrifuge tube.
7. Centrifuge the cells for 5 min at 130 x g, ensure that the centrifuge is correctly balanced.
8. Return the vial containing the cells back to aseptic conditions and remove the supernatant gently, without disturbing the pellet. The supernatant can be poured off and the remainder pipetted off ensuring the pellet is not disturbed.

9. Resuspend the cell pellet in 1 mL of CCM by pipetting up and down until all cells are suspended (no pellet or cell agglomerates can be observed). Additional CCM can be added to dilute the cell suspension.
10. Count the cells (dead and alive) in 1 mL of CCM using a haemocytometer. To achieve this;

a. Take the haemocytometer out of the box and place the cover slip using breath vapour.
b. Determine cell viability using 0.4% trypan blue solution. Add one volume of trypan blue to one volume of cell suspension (for example 10 µL of trypan blue mixed with 10 µL of re-suspended cells).
c. Remove 10 µL of the sample and disperse it underneath the cover slip.
d. Count the cells in the 4 large squares (in red) (see Figure 3) using a cell counter.

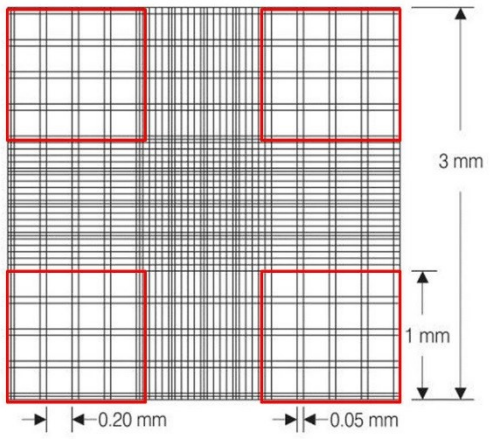


Figure 3. Counting cells using a haemocytometer

11. Using the following calculation, count the number of living cells (not labelled with trypan blue) that are in your sample.

Total cells/mL= counted cells (4 squares) × dilution factor ÷16 × 25 × 10'000

The term "dilution" refers to how many times the stock solution was diluted in trypan blue (in this case 2x) and "counted cells" here refers to the number of cells in the 4 big squares. Using the following calculation, count the percentage of viable cells:

Viable cells % = Living cells number (white)/Total cells number (white+blue)

Alternative methods and automated systems can be used alternatively as soon as they show robust measurement (e.g. CASY, Luna FL, EVE-MC). The method should be documented in the raw data.

The seeding density for cell passage not only depends on the size of flask and number of flasks required, but also on the cell properties. For example, due to the low growth rate, it is better to passage Calu-3 cells once a week with 60%-80% confluency. So the seeding number should be around 6.0×10^6 per T175 flask and 2.0×10^6 per T75 flask. Using the number of living cells calculated above, determine the dilution required with the below calculation to get the correct cell number in the new culture flask.

$C_1V_1 = C_2V_2$

Where:

C_1 = the current concentration

V_1 = the current volume with the current concentration

C_2 = the desired concentration

V_2 = the desired volume

12. Suspend the cells into the CCM volume required and add the cell suspension into each flask (as previously stated in section 5.8.2).
13. Gently rock the flask and then place it back into the incubator (37°C and 5% CO_2).
14. Replace with fresh CCM every 2-3 days and subculture when they reach 60%-80% confluency.

Tips: Passage number of Calu-3 cells should keep below P25 for experiments especially under ALI testing as cell morphology starts to change after P25.

7.2.3 Seeding Cells onto Culture Inserts

Protocol

The following is performed under aseptic conditions

1. Prepare cell suspension with known concentration following steps 1 – 11 from sub-culturing protocol (see section 7.2.2).
2. Dilute cells to a concentration of $2.24x10^5$ cells/ml in prewarmed CCM.
3. Take a cell culture plate with 12-wells inserts and place under aseptic conditions.
4. Fill the basolateral side with 1.5 mL pre-warmed CCM.
5. By pipetting up and down carefully mix the cell suspension. Pipette 500 µl of cell suspension (i.e., 112,000 cells/insert which is equivalent to 100,000 cells/cm^2) on the top of the membrane in the cell culture insert.
6. Cover the cell culture plate and place back into the incubator at 37°C and 5% CO_2.
7. Change the CCM every 2-3 days.

8. Let the cells become sub-confluent for 7 days under submerged conditions and continue to culture the cells at the air-liquid interface (section 7.2.4).

7.2.4 Culturing Cells at Air-Liquid Interface

The following steps are performed under aseptic conditions

1. Cells are growing on the cell culture inserts as prepared in “Seeding Cells on Cell Culture Inserts section 7.2.3”.
2. Let the cells become sub-confluent for 7 days under submerged conditions.
3. Check the TEER values of the inserts here before switching to an ALI. The goal is to see whether the formation of barrier function is going as planned.
4. Remove the CCM from both compartments (apical and basolateral).
5. Apply 1.5 mL of pre-warmed CCM to the basolateral side of the well. The CCM should touch the membrane from the bottom, but not leak onto the top of the insert.
6. At this point cells are apically exposed to air, which is referred as culturing at the air-liquid interface (ALI).
7. Culture cells ALI for 7 days prior to exposures in the incubator at 37°C and 5% CO_2. This time is needed to achieve a TEER of >500 Ωxcm^2. (See section 11.1 on how to measure TEER). In case the TEER is below 500 Ωxcm^2, the cells should not be used.
8. Change the basolateral CCM every 2-3 days.
9. The cells can be used at the ALI for 6 weeks. Preferably, add the macrophages to the Calu-3 cells, 2 weeks after starting the Calu-3 cell culture (according to the schematic view above). In case the TEER value is below the acceptance threshold (500 Ωxcm^2), or of an insufficient number or quality of macrophages implicating new thawing and differentiation of the monocytes, the macrophages can be added to the Calu-3 cell culture at a later time point. This delay should not be longer than necessary.

7.3 Cell culturing procedure for THP-1 cells

Please note – these cells are suspension cells and therefore the process for culturing them is different from the Calu-3 cells.

7.3.1 Thawing cells

Follow all steps outlined in Section 7.2.1 up to and including step 3.
Then:

1. Pipette slowly (drop by drop) the contents of the vial into a centrifuge tube with 9ml of prewarmed (d)THP-1CCM and centrifuge at 1200 rpm for 5 min.
2. Pellet the cells at 1200 rpm for 5 mins
3. Discard the supernatant into waste (this is laboratory specific so follow laboratory specific protocols) and re-suspend (via pipetting up and down) the cell pellet in 1ml of prewarmed (d)THP-1CCM
4. Dilute the cell suspension by adding 4 ml THP-1CCM

5. Transfer the re-suspended cells into a T25 flask and incubate the culture at 37°C and 5% CO_2

Allow two days to grow in a T25 flask before transferring to a larger T75 flask to expand cell cultures.

6. Change the cell culture medium every 2-3 days. Cells are spun into a pellet by pouring contents of flask into a 15ml centrifuge tube and centrifuge at 1200rpm for 5mins. The pellet is then resuspended in the fresh (d)THP-1CCM, pipetted up and down to ensure mixing and then pipetted into the flask (final volume 15 ml for a T75 and 25 ml for a T175) and the flask placed back into the incubator.

7.3.2 Sub-Culture

Cells should be passaged at least 2 times after thawing, before use in experiments or before freezing, and they should be passaged no more than 25 times in total (minimal published references show data beyond this point, and cell doubling time may decrease beyond this point). The cells should be subcultured when the cell concentration reaches a maximum of $8x10^5$ cells/mL.Do not allow the cell concentration to exceed 1 x 10^6 cells/mL. (From ATCC protocol).

Protocol:

1. Visually check the (d)THP-1CCM colour and cells under the light microscope (Figure 4)

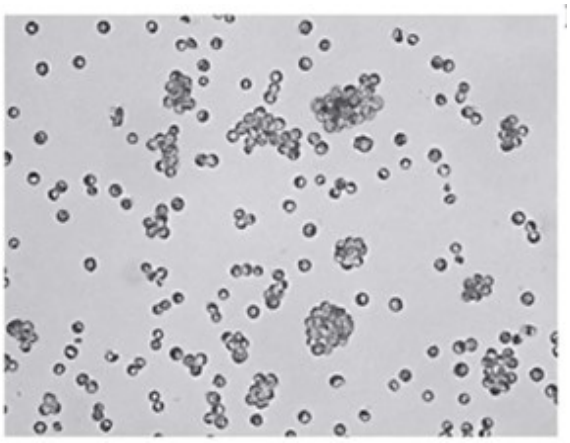


Figure 4 – THP-1 cells in culture

From here on, all steps need to be completed under aseptic conditions.

2. Transfer the cell suspension from the flask to a 50ml centrifuge tube
3. Centrifuge at 1200 rpm for 5 min (24°C; the setting of “accelerate” should be 8 (on a scale of 0-10); the setting of “brake” should also be 8 (on a scale from 0-10)
4. After centrifugation, pour off the supernatant, use a pipette to remove any excess supernatant

5. Add 1ml (d)THP-1CCM to the pellet and re-suspend using a pipette and count as previously outlined in section 7.2.2
6. For continuing cell culture, prepare a cell suspension concentration of $2x10^5$ cells/mL. The total volume per T75 flask is 15ml
7. Add the required volume of complete cell culture medium into a new flask and then add the cell suspension. Mix well by pipetting up and down.
8. Label the flask with cell type, operator's name, date, and passage number

7.3.3 Differentiation of THP-1 to dTHP-1

In the flow cabinet, prepare the PMA stock solution by adding 10ml ethanol to 1mg of PMA to give a stock solution of 100µg/ml of PMA. Further dilute the stock solution by adding 100µl into 10ml of medium to give a working stock of 1µg/ml. A final concentration of 30ng/ml is used to differentiate the THP-1s (300µl of working stock into 10ml of cell solution). Store both stocks at -20°C before use.

7.3.3.1 Addition of PMA

From here on, all steps need to be completed under aseptic conditions

1. Spin down THP-1 cells and count the pellet (as outlined in section 7.2.2)
2. Thaw the PMA working stock solution at room temperature. When thawed, invert the tube several times
   Caluclate the number of differentiated cells required for experimental work and add 10% (to allow for cell death and loss)
   Cells are seeded within a T25 flask @ $5x10^5$cells/ml in a maximum of 10ml per flask, with 300µl of working stock solution PMA added per 10ml of THP-1CCM
   Any remaining PMA needs to be replaced back into the -20°C freezer
3. The flasks are then replaced into the incubator for 5 days, checking their morphology after 24 hours (Figure 5)

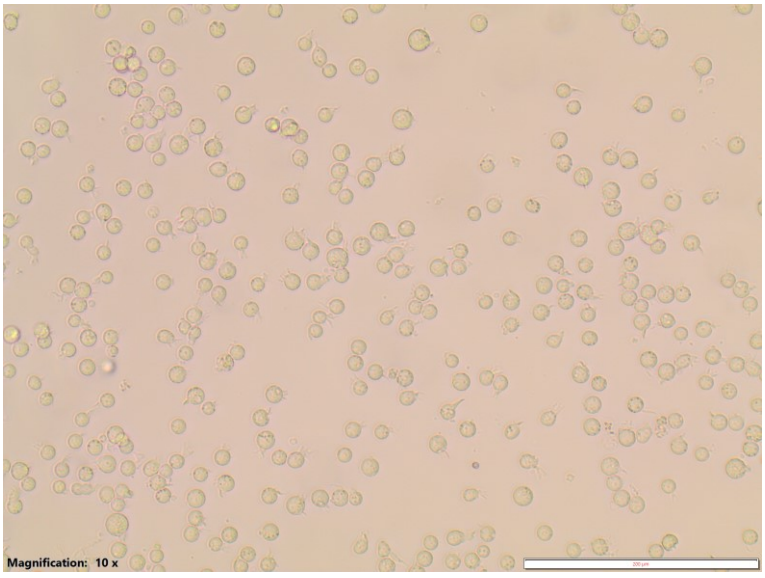


Figure 5. PMA-treated THP-1 cells after 48 hours at $1x10^5$cells/ml.

4. After 5 days of incubation with PMA, the (d)THP-1CCM medium is replaced with fresh (d)THP-1CCM and the cells incubated for 48 hours
5. After 48 hours of incubation with the fresh (d)THP-1CCM the cells are then removed using accutase
    a. The (d)THP-1CCM is removed to waste and the flask washed 3 times with PBS (the dTHP-1 are adherent after the addition of PMA)
    b. 5ml/T25 flask of accutase is added and the flask replaced into the incubator for 5-10mins
    c. 10ml of (d)THP-1CCM was added to the flask and the contents of the flask poured into a 15ml tube and centrifuged to obtain a pellet
    d. The dTHP-1 is counted once again (as outlined in section 7.2.2) and resuspended in (d)THP-1CCM ready to be seeded onto the transwell inserts

Overall the THP-1 cells require 5 days incubation in PMA, 48 hours recovery in fresh THP-1CCM, and 24 hours recovery at an ALI.

Please note – there are numerous protocols avalible that implement PMA to differentaiate THP-1 cells to dTHP-1. For this specific study it was decided (TC on the 27th May 2020 and 10th July 2020) that the differentiation protocol implemented within RIVM would be the one to use for this protocol (as it has previously been used with Calu-3 cells specifically (van Helden, van Leeuwen et al. 2008, Daigneault, Preston et al. 2010, Velde, Wilbers et al. 2015)).

8 Preparing the co-culture

Co-culture assembling of dTHP-1 with Calu-3 cells – all cells are in CCM medium throughout the co-culturing process.

Before the addition of macrophages measure the TEER of each well to ensure the TEER value is high enough to continue.

Epithelial cell-macrophage co-culture

The following steps are completed under aseptic conditions

The following is for 12 well transwell inserts (1.12cm^2 growth area)

1. Take the pelleted dTHP-1 and resuspend at $1.4x10^5$ cells/ml
2. Add 100µl of the cell suspension onto the apical side of the 12 well insert ($1.4x10^4$ cells/well)
3. Make sure the inserts are placed in the centre of each well (there are grooves the inserts sit on the plate) and then place back into the incubator (37°C and 5% CO_2).
4. Allow 2 hours for the dTHP-1 cells to adhere to the Calu-3 cells (Table 1)
5. Transfer the co-culture model to ALI by discarding both basal and apical CCM and adding 1.2ml of CCM to the basal side only

Table 1. dTHP-1 cells from the waste apical medium 2 hours after seeding onto A549 epithelial cells were counted and the cell viability (%) and cell loss (%) were calculated. Medium was removed (in order to switch the culture to ALI) and cells in apical medium were counted. These are the cells that have not adhered to the epithelial cells on the inset. Cell viability was calculated using trypan blue, and cell loss was calculated from the original seeding number onto the insert and what remained within the waste apipcal medium. N=6

	Average	*SD*
Viability (%)	34.39771	9.501981
Cell Loss (%)	0.513333	0.067132

9 Chemical and particle dispersion preparation

9.1 LPS preparation

Dilute LPS to a concentration of 175 µg/mL in ultrapure water. This corresponds to a deposited dose of about 0.25 µg/cm^2 after a single nebulization of 200 µL. LPS can be prepared as stock solution and frozen in aliquots to avoid any additional thaw-freeze-cycle.

9.2 Particle dispersion preparation

This part of the SOP is based on *´The NANOGENOTOX standard operational procedure for preparing batch dispersions for in vitro and in vivo toxicological studies´* that is optimized within the NanoReg project (12 June 2018). The DQ_{12} and TiO_2 dispersions are prepared **without** BSA.

Weighing of DQ_{12} and TiO_2 should be performed in a ventilated weighing box, glove box or fume hood designed for sensitive weighing with an accuracy of at least 0.1 mg or better. Ensure appropriate safety clothing.

1. Using a microbalance, weight the respective amount of DQ_{12}/TiO_2 in a glass vial corresponding to a minimum of 4 ml to a maximum of 10 ml (e.g. 10.24 mg corresponding to the final volume of 4 ml leading to a final concentration of 2.56 mg/ml).
2. In this protocol EtOH pre-wetting is used for all materials to harmonize the treatment for all powder materials. EtOH pre-wetting is introduced to enable dispersion of hydrophobic materials in water-based systems. 0.5 vol% EtOH (96% or higher) is used for pre-wetting. This corresponds to 4 mL x 0.5/100 = 0.020 mL (20 µl) EtOH.
3. Tilt the scintillation vial with pre-weighed powder so the material is gathered in a small area at the vial bottom and carefully open the vial.
4. Tilt the scintillation vial ca. 45° and add 20 µl EtOH drop-by-drop onto the particles in the vial by pipette. Let the EtOH move back and forth over the

particles for ca. 1 min until EtOH has interacted with the powder or mixed with the liquid dispersion.

5. Screw on the lid and gently mix the EtOH and powder by simultaneous gently tapping the vial on the table-top while rotating the tilted 45° vial from side to side between your fingers for approximately one minute.
6. Add 980 µl 2 ultrapure water by pipette while slowly rotating and swirling the 45° tilted scintillation glass.
7. Add the remaining 3 ml ultrapure water by pipette along the sidewalls of the scintillation vial to wash down test material that may be stuck to the sidewalls and collect the it in the fluid at the vial bottom.
8. Place the vial on ice for at least 5 minutes while the sonicator and ice-water is prepared.

The samples are continuously cooled in an ice-water bath to minimize heat development during sonication. For the ice-water bath, add pre-cooled MilliQ to the insulated box (e.g., styrofoam) with ice in order to ensure a more direct cooling of the sample.

9. Fill a 250 ml glass beaker with ice and place it upside-down in an insulation box (e.g., styrofoam).
10. Add ca. 85-90 vol% ice into the insulation box.
11. Add ca. 10-15 vol% cold (e.g. refrigerated) water into the insulation box.
12. Carefully place the glass scintillation vial with powder on top of the upside-down glass-beaker and pack the ice-water around the vial to keep the dispersion cooled. One may fix the vial using a clip or burette holder to ensure that the vial is not moving during sonication.
13. Insert the sonication probe as close as possible one third into the dispersion. Never sonicate with the probe immerses it less than the upper quarter and never lower than half-way into the dispersion.
14. Start sonication and run according to *NANoREG SOP for probe-sonicator calibration of delivered acoustic power and de-agglomeration efficiency for in vitro and in vivo toxicological testing* (e.g. for 16 min with amplitude setting

10%), while controlling that the sonication probe does not touch the walls of the scintillation vial.

15. Remove the scintillation vial and add the lid.
16. Clean the sonication probe by sonication for 5 minutes (similar sonication settings) with the probe fully immersed in a 50:50 water-EtOH (>96%) mixture followed by rinsing in EtOH using a dispenser and a collection bottle underneath. The probe is allowed to air-dry in the fume-hood. Other in-house cleaning methods may also apply.
17. Use the dispersion within 1 hours. The stock dispersions are meta-stable due to the relatively high particle concentrations. Suspensions normally remain stable for 30 to 60 minutes depending on the NM. To ensure homogeneity and representative dosing, one should always gently shake or Vortex the stock dispersion at low to intermediate speed for 10 sec before use. It has been shown that this, normally re-establishes the original dispersion characteristics.
18. Re-homogenization of the dispersions may also be done by use of ultrasound bath treatment. However, the result may vary with type of equipment and position in the bath and the dispersion qualities should in this case be followed carefully.
19. Prepare a 500 µg/ml dilution from the stock solution.

10 Preparing the device, aerosolization and cleaning

10.1 Prior nebulization

1. Sterilize VITROCELL® base module and cover top with 70% ethanol.
2. Figure 6 shows the VITROCELL® Cloud system with its all parts.
3. Assemble the QCM, insert it to the VITROCELL® Cloud system and connect it with the oscillator.
4. Turn on the VITROCELL® Cloud heating unit and wait until the temperature reaches 37°C.
5. Warm the LPS to room temperature and prepare a fresh TiO_2/DQ_{12} dispersion. Shortly sonicate to redisperse the sedimented/agglomerated particles if necessary. See RR1 or steps 17. and 18. in the previous section.

6. Keep nebulizer for 15 min in a beaker containing 70% ethanol. After 15 min remove the nebulizer, and let it dry.
7. When the nebulizer is dry, rinse the nebulizer in ultrapure water, and subsequently nebulize 3x300 µl of ultrapure water to remove all the residuum from the nebulizer.
8. Do the performance check of each nebulizer used for the exposures. It is recommended to use one single nebulizer for each material and a separate one water for water controls in order to prevent cross-contamination.

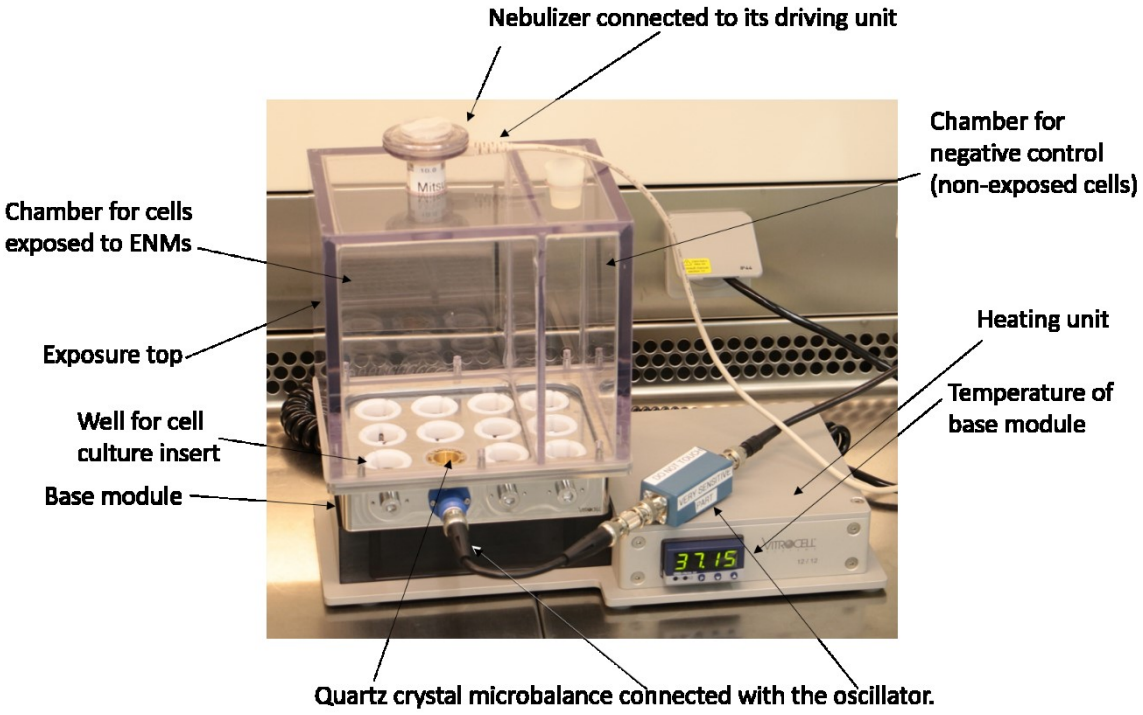


Figure 6: The VITROCELL® Cloud system.

10.2 Nebulization

The following procedure (step 1-5) has to be performed when nebulizing new material for the first time, and should be performed regularly before each experiment to test the proper performance of the nebulizer. Please perform it for each nebulizer used in RR2.

1. Rinse the reservoir of nebulizer with ultrapure water.

2. Nebulize 200 µl of ultrapure water containing 2 µl of isotonic NaCl solution, and measure the time needed for nebulization of all the content (using a stopwatch).
3. If possible, do the same for LPS and the TiO_2/DQ_{12} dispersions, and measure time for complete nebulization (using a stopwatch). If it is not possible, use the performance check using water with NaCl to control the proper performance of the nebulizers.
4. Regularly check the nebulizer performance following steps 1 – 3, also between subsequent nebulizations. Values for 200 µl of ultrapure water and of particle dispersions are acceptable between 15-60 s (0.2 – 0.8 ml/min); after the determination, the values for water and DQ_{12}/TiO_2 nebulization should be within 10% of the determined value. If not, the nebulizer may be blocked or aged and needs to be cleaned / replaced.

10.3 Cell exposures to LPS/TiO_2/DQ_{12} aerosol

1. Rinse the reservoir of nebulizer with ultrapure water and dry it with lint-free cloths. Place the nebulizer on top of the exposure chamber.
2. Prepare a solution of ultrapure water or LPS, or TiO_2/DQ_{12} dispersion containing 1% of isotonic NaCl solution (v/v), e.g. 2.5 µl in 250 µl in a separate Eppendorf tube and pipette 200 µl to the nebulizer (make sure the liquid covers the nebulizer's mesh).
3. Before exposing the cells to particles, one signal for water needs to be measured for each experiment using the nebulizer for water only. This is to have a reference for water-exposed cells and as blank to show a clean and working system (values below 250 ng/cm^2 are accepted).
4. Add in the TEM grid holder (with a clean set of grids for each particle nebulisation) for future analysis of deposition. Fill the remainder of wells with 3.1 ml CCM. Place the inserts into the wells. Cover the base module with the exposure top. A scheme of the following procedure is provided in Figure 7.

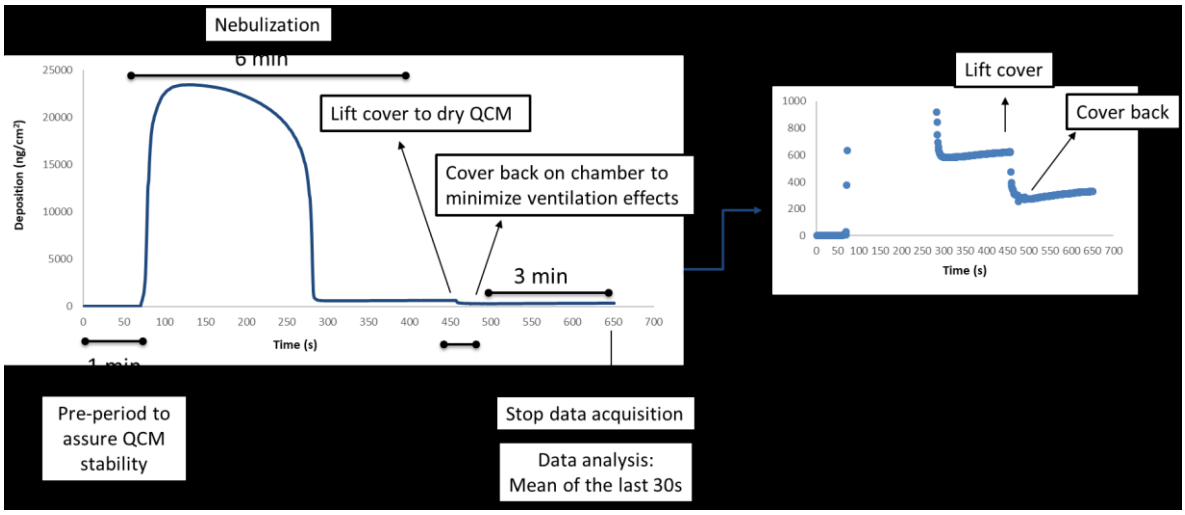


Figure 7: Typical time course of QCM signal and the time periods applied.

5. Start the data acquisition of the microbalance and wait until the signal is stable (typically ca. 30 s, but can be longer), set the signal to zero and wait another 30 s to verify stability of the zero point signal (the signal should be in the range of ± 25 ng/cm2). If this is not the case, set the QCM signal to zero again and wait for another 30 s to verify stability of the signal.

   Note: It is recommended to zero the signal once again after starting the data acquisition.

6. Measure the signal for 1 minute before starting the nebulization (next step). The fluctuation should not be more than 25 ng/cm^2 in that one minute. If it is, the QCM needs to be changed/cleaned.
7. Start the nebulization process by plugging the nebulizer into the driving unit and at the same time start the timer (t = 0 min). Carefully measure and collect the time needed for nebulization.

   Note: The acceptable time range for nebulizing 200 µl of water/suspension is between 15-60 s (0.2 – 0.8 ml/min).

   Note: The output rate should not exceed 10% deviation from the original output rate of the individual nebulizer, and this should be measured for all the subsequent nebulizations. Prolonged nebulization time can be a sign of a blocked nebulizer; therefore it needs to be cleaned thoroughly (ultra-bath sonication, see section Cleaning) or replaced.
8. Wait for 6 min to ensure a complete settlement of the test substance to the inserts and QCM. A drop of the signal should be visible.

Note: If the curve is not dropped within this time frame, extend this period to 10 min and follow this procedure for all nebulizations of the same material

9. Lift the exposure top for 1 min allowing the QCM to dry.
10. After 1 min place the exposure top back onto the device.

    Note: The reason behind covering the device is to minimize the effects of ventilation and temperature fluctuation on the measured QCM values.
11. Wait for 3 min and stop the data acquisition.
12. For DQ_{12} and TiO_2, perform nebulization of 500 µg/ml (2- or 3-times) in order to obtain the desired deposited doses (see Table 2). It is more important to reach the desired deposited dose than to follow the time of nebulizations. It is highly important to have comparable deposited doses and thus biological responses.

    Note: For subsequent nebulizations of the same particle solution concentration: in order to achieve a higher deposition, subsequent nebulizations are possible, but to a maximum of 4. After the first nebulization, check the output rate of the nebulizer. If this is within 10% of the initial output rate, nebulize 1x 200 µl ultrapure water with salt (not into the cloud) and continue with the second nebulization. If not, place the nebulizer in a beaker with ultrapure water or 70% ethanol and sonicate it in an ultrasonic bath for 5-10 min and proceed. Do not stop the data acquisition and do not clean the QCM in between (possible to a maximum deposition of ~1-1.5 µg/cm^2). The measured deposited dose should be approximately twice the first measured dose. If not, the nebulizer may be blocked and must be cleaned carefully, see section 10.4 (Cleaning) . In case of subsequent nebulizations, the inner walls of the exposure chamber need to be wiped with 70% ethanol.

    Note: If the time is exceeding, put the nebulizer in a beaker with ultrapure water or 70% ethanol and sonicate it in an ultrasonic bath for 5-10 min. Then, measure the output rate again. If nebulizing time is not improving, clean more thoroughly or replace the nebulizer.

*Table 2. Example of layout of inserts in the Cloud system. * There are only 3 positions for controls: The Calu-3 monolayer and the Calu-3 + macrophages co-culture are exposed to ultrapure water in two rounds: first the monolayer and next the co-culture.*

Exposure to LPS/ TiO_2/DQ_{12}			Exposure to ultrapure water*
Calu-3	Calu-3	Calu-3	Calu-3 / Calu-3 + macrophages
Calu-3	Calu-3 + macrophages	Calu-3 + macrophages	Calu-3 / Calu-3 + macrophages
Calu-3 + macrophages	QCM	Calu-3 + macrophages	Calu-3 / Calu-3 + macrophages

13. When exposure is finished, place cell culture inserts back to the cell culture plate and transfer the plate back to the incubator.
14. Lift the exposure top and clean the QCM if exposure of one substance is finished. The QCM needs to be changed for each substance i.e. LPS, TiO_2, DQ_{12}. These QCMs can be cleaned (see section 1.4 (Cleaning)) and re-used.
    Note: The quartz crystal of the QCM needs to be replaced when scratches are observed on the surface and/or if a stable signal cannot be achieved after the crystal and base module have been heated up appropriately.
15. Data analysis should be performed on the mean of the last 30 values.
16. If the experiment is finished, follow to 10.4 (Cleaning). If further exposures have to be done, rinse the nebulizer reservoir, wipe the chamber and module base with ethanol, let them dry and then follow steps 1 – 14 until the experiment is finished.
17. Incubate the inserts at 37 °C for 24 hours.

10.4 Cleaning

1. Nebulize at least 3x 200 µl of ultrapure water containing 1% (v/v) isotonic NaCl.
2. Put the nebulizer in a beaker with ultrapure water in a water bath sonicator (e.g. Elmasonic P30H cleaning unit, 100 W, 37 kHz, 30% (Elma Schmidbauer GmbH, Germany)) for 10 min.

3. Rinse the nebulizer with ultrapure water.
4. Put the nebulizer in a beaker with 70% ethanol and sonicate in water bath sonicator (e.g. for 10 min).
5. Clean VITROCELL® base module and cover top with 70% ethanol.
6. QCM Crystal: Used, dirty crystals can be collected, cleaned and re-used, if no scratches on the surfaces are visible. For cleaning, put them in ethanol for 0.5 min to 5 min (depends on the degree of impurity/dirt) and wipe them carefully afterwards. Check their performance afterwards and trash them if the signal oscillates too strongly, loss of signal is present or scratches on the surface are visible.
7. VITROCELL® base module and exposure top can be washed in dishwasher (recommended). Do NOT clean with acetone.
8. VITROCELL® base module can be autoclaved (recommended on regular basis, i.e., every week, but not necessary after each experiment) following intervals and temperatures listed, i.e., minimum warm-up of 30 min, autoclaving at 121°C for a minimum of 20 min, minimum cool-down time in the autoclave of 30 min.

10.5 LPS/ TiO_2/DQ12 concentrations applied and deposition measured

Dispersion	Concentration (µg/mL)	Deposition (ng/cm^2)±SD	Nebulization	Target depositions in RR2 (ng/cm^2)
LPS	175	250	1x	250
DQ_{12}	500	516.0 ± 32	2-3x	1000
TiO_2	500	394.8 ± 121	2-3x	800

Note: If the concentration 500 µg/ml of TiO_2NM-105 is causing blocking of the nebulizer, the nebulizer needs to be thoroughly cleaned by sonicating in water, water plus soap, 70% ethanol (each 60 min) and the concentration 250 µg/ml shall be measured.

11 Read-outs

Measurements are performed at 24 hours after the start of the exposure. This incubation time allows cells to respond to the exposure. The exception here is TEER measurement which must be completed both BEFORE and after the exposures.

11.1 Collecting samples for cytokine measurements

1. At 24 h after the start of the exposure, take the plate with inserts from the incubator.
2. Add 500 µL of pre-warmed CCM to the apical side of the inserts.
3. Incubate the inserts in the incubator for 30 minutes at 37 °C.
4. Use this time frame to measure TEER values of all inserts.

11.2 Measuring TEER

1. Take an Epithelial Voltohmmeter supplemented with Chopstick Electrode Set and charge the battery system overnight.
2. Disconnect the Voltohmmeter from the charger and connect the chopstick electrode.
3. Clean the electrode with 70% ethanol.
4. Place the electrode in the CCM by putting the longer electrode in the external CCM until it touches the bottom of the dish and putting the shorter electrode in the CCM without touching the membrane (Figure 8). Using a single position for the electrode is sufficient.
5. Start with an empty insert without cells. Wait until the measurement stabilizes (about 3-4 seconds) and write down the resistance in Ohms. This measurement is the resistance of the insert membrane without any cells (i.e., blank resistance).
6. Measure the TEER of each insert. Usually, a single measurement per insert is sufficient. In case the measurement does not stabilize within 3-4 seconds, a second measurement can be performed. This is done by placing the electrode at another position of the insert. In case multiple measurements per inserts are performed, the average TEER will be used for later analysis.
7. Repeat the measurement for each insert and subtract the blank resistance to obtain the true resistance.
8. Multiply the true resistance with the surface area of the insert (1.12 cm^2) to obtain the corrected values in Ohm x cm^2.

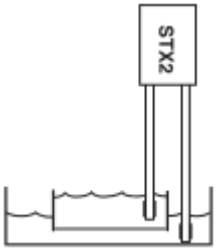


Figure 8. Correct positioning of the electrode in the insert to measure TEER.

11.3 Sample collection for measuring LDH release and cytokines

1. Take the inserts from the incubator and collect both apical and basolateral CCM separately.
2. Store 120 µl at 4˚C for measuring basal LDH release and the remainder below -20 ˚C for cytokine analysis.

11.4 Measuring LDH release

1. Use collected samples. NOTE: Samples can be stored at 4 °C, do not freeze samples before LDH measurement. Measuring one technical replicate is sufficient.
2. Include an LDH max sample:
    a. Use a control insert and lyse the cells using 500 µl lysis buffer (2% Triton X-100) on the apical side of the inserts for 5 minutes.

    Note: longer periods of lysis (up to 20 min) have also found necessary.

    b. Use the lysate to measure the total LDH release – again do not freeze this.
3. Take LDH reagent components from the freezer or fridge.
4. Take 100 µL of each collected sample and also from the lysate (for total LDH release) and load in a 96-well plate.
5. Prepare reaction reagent according to the manufacturer's instructions, this is the reaction mix.
6. Add 100 µL of the reaction mix to the samples in the 96-well plate and incubate in the dark for 15 minutes at room temperature.

7. After 15 minutes, check the absorbance at a wavelength of 490 nm. If the LDH max samples give a signal of around 1, stop the reaction by adding stop solution: 50 µL HCl per well. Alternative ways of measurement are endpoint measurements at various timepoints (e.g. after 0, 5, and 10 min) and kinetic measurements (e.g. at 0-5 min and 5-10 min).
8. Measure absorbance at a wavelength of 490 nm.
9. Correct all measurements for the background of the culture medium and normalize the values to the LDH max value.

11.5 Measuring WST-1 conversion

For 1 out of 4 inserts per treatment, fixation can be performed WITHOUT WST-1 (due to possible interferences). AMI offers to analyse 1 treated insert per exposure (8 wells in total per lab). The protocol for fixation is given below.

1. Remove all culture medium.
2. Prepare WST-1 reagent by diluting 10X in CCM.
3. Add 500 µL of the reagent mix to the apical side of each insert and incubate for 20 -60 min (depending on the development of the absorbance).
4. After incubation, add 100 µL of each insert to a 96-wells plate, as well as some WST-1 reagent alone as a control.
5. Measure absorbance at a wavelength of 440 nm and a reference wavelength of 620 nm.

Paraformaldehyde (PFA) fixation:

- Wash cells 3x with PBS
- Fix with 4 % PFA for 15 min at room temperature
- Wash 3x with PBS, seal with parafilm and keep at 4 ˚C until staining/sending to AMI.

11.6 ELISA panel

IL-1β, IL-6, IL-8, IL-10, and TNF-α will be measured using an ELISA (e.g. eBioscience) or a Multiplex system (e.g. Luminex (BioRad)). FACS-based assays are optional, as long as at least 3 RR partners use an ELISA or Multiplex system.

Measuring one technical replicate is sufficient.

11.7 Collection samples for deposition measurement

This is optional for TiO_2.

1. Make sure all samples have been collected.
2. Lyse the cells by adding lysis buffer (2% Triton X-100) for 5 minutes.
3. Collect the lysate and store at a temperature below -20 C for later analysis (e.g. ICP-MS).

11.8 Quality control & acceptance criteria:

Visual confirmation of Calu-3 and dTHP-1s shape using light microscope. If possible, it is recommended to take phase contrast images of Calu-3 cells and dTHP-1s.

12 Data Analysis and Reporting of Data:

Use the data template for RR2 PATROLS to report all raw data.

13 Publications:

https://www.jove.com/video/61090/multicellular-human-alveolar-model-composed-epithelial-cells-primary

https://www.jove.com/video/61210/an-air-liquid-interface-bronchial-epithelial-model-for-realistic

14 References

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15 Appedix

Form 1.

Calu-3 cells:

Lot number of cells used:……

Passage of cells used:……..

THP-1 cells:

Lot number of cells used:……

Passage of cells used:……..

FBS:

Supplier of FBS used:……

Lot number of FBS used:……..