PATROLS
Advanced Tools for NanoSafety Testing

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PATROLS Standard Operating Procedures (SOP)

Guidance Document for Round Robin study #2: Exposure of *in vitro* co-culture lung model at the air-liquid interface using VITROCELL® Cloud System

SOP_PATROLS_RR2_lung model

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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WP3 members and RR participants

Document History:

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2.0	14.04.2020	Additional detail added from AMI	Anne Bannuscher
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5.0	22.06.2020	Comments from RR participants integrated Comments from AMI integrated	Hedwig Braakhuis
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7.0	05.11.2020	Comments from AMI, BASF, and SU integrated	Anne, Barbara D, Barbara B, Kirsty and Rob

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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 primary macrophages. The co-culture model will be exposed to a positive control (lipopolysaccharide (LPS)) and to particles (TiO_2 and DQ12 quartz) using air-liquid interface (ALI) exposure. ALI exposure reflecting the air-blood barrier is considered more relevant to mimic *in vi*vo compared to submerged exposure (Loret, Peyret et al. 2016). In this SOP, the VITROCELL® Cloud system equipped with an Aeroneb Pro nebulizer, and a 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 participants of the RR study within the project PATROLS. This SOP provides instructions on how to culture the cells, and aerosolize LPS, TiO_2 and DQ_{12} using the VITROCELL® Cloud system.

Limitations:

The Calu-3 cell line is a bronchial epithelial cell line and therefore less suitable to model processes occurring in the alveoli. While the cell line is widely used in research because of several favourable characteristics, it should be mentioned that the use of a cell line does not grasp the complexity of the lung, several of the characteristics of primary cells, or interindividual variability. TiO_2 and DQ_{12} have to be dispersed in liquid. To be able to provide a stable dispersion, the SOP_PATROLS_Cloud_Aerosolization_DQ12 and NanoReg protocol: 'Protocol for producing reproducible dispersions of manufactured nanomaterials in environmental exposure media' are used, **without** BSA dispersion. For more details on particle dispersion, see section 9.3 of this SOP.

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]

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.

3 Abbreviations

ALI – air-liquid interface
BSA – bovine serum albumin
DQ12 - Dörntruper quartz 12
EDTA - ethylenediaminetetraacetic acid
ENM – engineered nanomaterials
FBS - foetal bovine serum
HBSS – Hank's balanced salt solution
LDH – lactate dehydrogenase
LPS - lipopolysaccharide
M-CSF – macrophage colony stimulating factor
MEM – minimum essential medium
NEAA - non-essential amino acids
PBMs – peripheral blood monocytes
PBS – phosphate buffered saline
QCM – quartz crystal microbalance
RR – round robin
TEER – trans epithelial electrical resistance
TEM – transmission electron microscopy
WST-1 - water-soluble tetrazolium salt-1

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 macrophages using VITROCELL® Cloud system.
This protocol will be divided into 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

5 Description of the Method

5.1 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.

- VITROCELL® Cloud12 system (VITROCELL®, Germany) equipped with
    - Quartz crystal microbalance (QCM)
    - Aeroneb® Lab nebulizer (4-6 µm pore size, Aerogen, Ireland)

    - This protocol refers to the Cloud 12 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, Nanomaterials 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.

- CD14+ magnetic beads (130-097-052, Miltenyi, Germany) - 2
- DMSO (e.g. D2438, Sigma Aldrich) - 1
- DQ_{12} (IOM, Edinburgh) - 3
- EDTA (E6758-100g, Sigma-Aldrich, Switzerland) - 1
- ELISA kit (e.g. eBioscience) - 1
- 70% Ethanol - 1
- FBS (758093, Greiner bio-one) - 1
- HBSS (1x) (14175, GIBCO®) - 2
- Isotonic sterile 0.9% NaCl solution - 1
- LDH cytotoxicity detection kit (No. 11 644 793 001, Roche Diagnostics GmbH, Mannheim, Germany) - 2
- L-Glutamine (25030081, GIBCO®, Switzerland) - 1
- LPS (L4391, Sigma) - 2
- Lymphoprep™ (1114547, Alere Technologies AS, Norway) - 2
- M-CSF (130-096-485, Miltenyi, Germany) - 2
- MEM (1x) + GlutaMAX (41090, GIBCO®) - 2
- NEAA solution (100x) (11140, GIBCO®) - 2
- PBS pH 7.4 1X, $MgCl_2$ and $CaCl_2$ Free (14190-094, GIBCO®, Switzerland) - 1
- Penicillin/Streptomycin (100X) (15140, GIBCO®) - 1
- RPMI 1640 (42401-018, GIBCO®, Switzerland) - 2
- Triton X-100 (93426, Sigma) - 1
- TiO_2 (NM-105, Fraunhofer) - 3
- Trypan Blue Solution (CAS# 72-57-1) (T8154-100mL, Sigma Aldrich®, UK) - 1
- 0.05% Trypsin-EDTA (1x) (25300, GIBCO®) - 1
- Ultrapure water - 1
- WST-1 cell proliferation kit (No. 11644807001, Roche Diagnostics GmbH, Mannheim, Germany) - 2

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 (e.g. Clean Air 4082 SHR4); or Laminar Class II Tissue Culture Hood (Scanlaf Mars) - 1
- Cell Freezing Aid (Mr Frosty, 5100-0001, Thermo Fisher Scientific, UK) - 1
- 12-well cell culture inserts (0.4 μm pore size, polyester)(CLS 3460, Corning) - 2
- Cell scraper (nonspecific, for example 353085, VWR, Switzerland) - 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) or Merck Millicell ERS-2 Voltohmmeter MERS00002 - 1
- Liquid Nitrogen or Ultra low freezer (-130°C) - 1
- Sterile filtration cup for vacuum filtration, 0.2 μm pore size (nonspecific, for example 10040-446, VWR, Switzerland) - 1
- Sterile Lab Bottle compatible with Filtration cup (min. 100 mL) - 1
- Haemocytometer (Bürker-Turk) - 1
- 37˚C and 5% CO_2 ISO Class 5 Hepa Filter Incubator (Binder or NUAIRE™ DHD Autoflow) - 1
- Light Microscope (e.g. Olympus, CKX41 or Axiovert 40C, Zeiss, Germany) - 1
- Microbalance - 1
- MultiStand Macs (Macs Cell Separator, 130-042-303, Miltenyi, Germany) - 2
- LS Columns (130-042-401, Miltenyi, Germany) - 2
- 9.4 cm Square, Petri Dish (688161) or Labmaterials PDIN-150-036 glass petri dish 150 mm base - 1
- P20, P200 and P1000 micropipettes (e.g. Biohit) - 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.) - 1
- VITROCELL® Cloud12 - 2
- Aeroneb nebulizers (4-6 μm pore size, IV1232 Vitrocell) - 2
- Water Bath (37˚C) - 1

- Water bath sonicator (no need to be specific, for example Elmasonic P30H cleaning unit, 100 W, 37 kHz, 30% (Elma Schmidbauer GmbH, Germany)) - 1

5.4 Reporting of protected elements

To the best of our knowledge, this SOP does not have any associated patent restrictions, specific licenses, material transfer agreements or commercial purchase requirements required to perform the protocol described. However, the following applies to the Calu-3 cells:

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

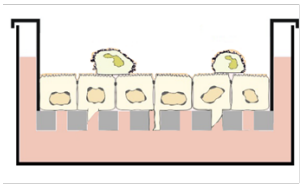
5.5 Health and safety precautions

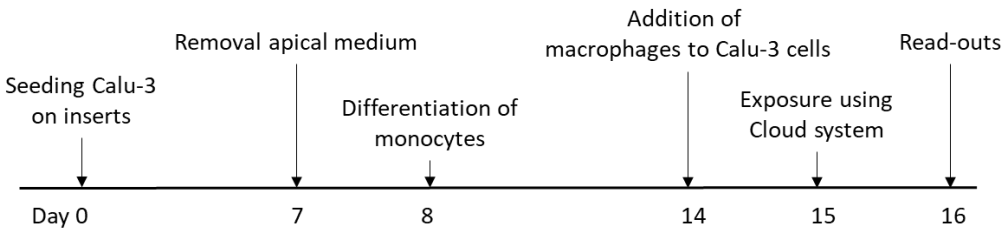
Working with non-tested human blood samples involves specific care to prevent transmission of HIV and Hepatitis B and C. The use of protective measures such as gloves, gowns, masks, and eye protection as well as vaccination against the Hepatitis B virus is mandatory. These protections reduce the risk of exposing the skin or mucous membranes to potentially infectious fluids. Avoid using sharp, or fragile utensils to minimize potential injury.

In addition, standard health and safety precautions associated with working within a laboratory environment, performing mammalian cell culture and handling ENMs, 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 be followed.

DQ_{12} is a non-nanosized crystalline silicon dioxide particle which can induce inflammation and fibrosis in humans upon inhalation. Therefore, suitable protective equipment such as a lab coat, goggles, gloves and a respirator must be worn.

6 Schematic overview





For a typical experiment, 18 inserts are needed:

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

7 Cell culturing

7.1 Reagent preparation

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

Minimum essential medium (MEM) + GlutaMAX supplemented with

- 1% Pen-Strep (corresponds to 100 U/ml Penicillin and 100 µg/ml Streptomycin);
- 1% NEAA solution;
- 10% FBS (heat inactivated, please follow the ATCC protocol for heat inactivation; (https://www.atcc.org/~/media/PDFs/Culture%20Guides/AnimCellCulture_Guide.ashx, page 19))

Culture medium and supplements do not require pre-heating before supplementation but should be stored at between 4-6°C after preparation. Can be kept for 3 months at this temperature.
Antibiotics are added to the medium to reduce bacterial contamination.
It should be noted that the CCM for Calu-3 cells used in this SOP, is different from the one advised by ATCC.
From a 500 mL medium bottle, first remove 60 ml. Then add:

- 5 mL Penicillin-Streptomycin;
- 5 mL NEAA;
- 50 mL FBS;

The order of adding the ingredients is not important, except that FBS should be added the last. The medium needs to be mixed after each addition.

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 (CCM) for primary macrophages

RPMI supplemented with

- 1% Pen-Strep (corresponds to 100 Units/mL Penicillin and 100 µg/mL Streptomycin)
- 1% L-glutamine (corresponds to 2 mM L-Glutamine)
- 10% FBS (heat inactivated, please follow the ATCC protocol for heat inactivation (https://www.atcc.org/~/media/PDFs/Culture%20Guides/AnimCellCulture_Guide.ashx, page 19))

Culture medium and supplements do not require pre-heating before supplementation but should be stored at 4-6°C after preparation and can be kept for 3 months at this temperature.
Antibiotics are added to the medium to reduce bacterial contamination.

From a 500 mL medium bottle, first remove 60 ml. Then add:

- 5 mL Penicillin-Streptomycin
- 5 mL L-glutamine
- 50 mL FBS

The order of adding the ingredients is not important, except that FBS should be added the last. The medium needs to be mixed after each addition.

The term CCM is used for the complete cell culture medium with all additions. To avoid confusion between the two types of CCM (for Calu-3 vs. primary macrophages), in this SOP the CCM for primary macrophages is denoted MDM-CCM.

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

7.2 Cell culturing procedure Calu-3

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 1).

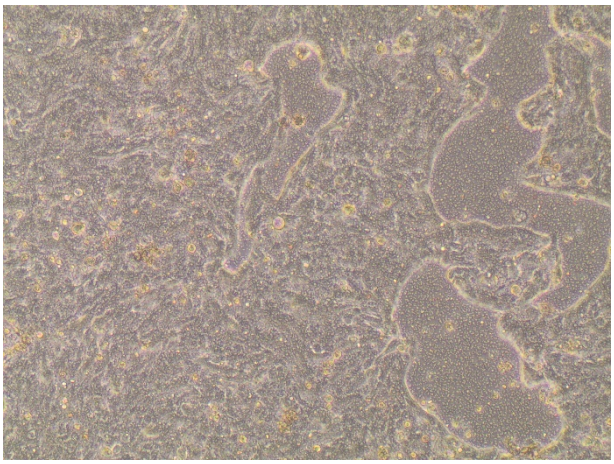


Figure 1. Calu-3 cells at 60-80% confluency

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 10-15 min (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 2) using a cell counter.

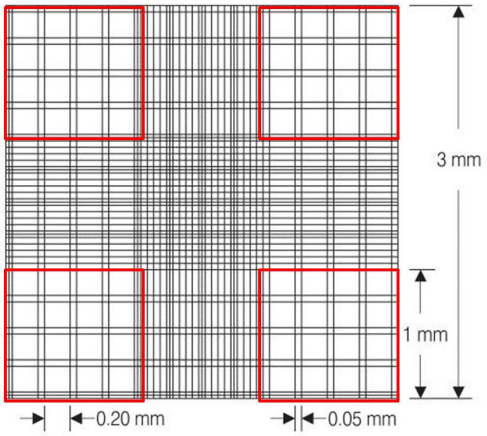


Figure 2. Counting cells using a haemocytometer

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.
   Note: since monocytes have to be thawed the next day (in order to obtain MDM), measure the TEER. The goal is to see whether the formation of barrier function is going as planned, and thus whether thawing the monocytes is in order. It is sufficient to check only a few wells (randomly).
   Note: the size and reproducibility of the TEER should be established in earlier experiments using Calu-3 cells only.
3. Remove the CCM from both compartments (apical and basolateral).
4. 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.
5. At this point cells are apically exposed to air, which is referred as culturing at the air-liquid interface (ALI).
6. Culture cells at the 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).
   Note: the value and reproducibility of the TEER should be established in earlier experiments using Calu-3 cells only. The aim should be a TEER >500Ωxcm^2 and a CV <20%. See the PATROLS SOP: Guidance Document for cell culture of lung epithelial cell-line (Calu-3).
7. Change the basolateral CCM every 2-3 days.
8. The Calu-3 cells at the ALI can be used for a maximum of 6 weeks. However, it is preferable to add the macrophages to the Calu-3 cells, 2 weeks after starting the Calu-3 cell culture (so,

according to the schematic view above). In case the TEER value is below the acceptance threshold (500 Ωxcm^2), or in case of an insufficient number or quality of macrophages implicating new thawing and differentiation of monocytes, the macrophages can be added to the Calu-3 cell culture at a later time point. Do not delay adding MDMs to the Calu-3 cells any longer than necessary.

Note: it may be advised to perform a trial differentiation of frozen monocytes to MDM, prior to setting up the Calu-3/MDM co-culture. When successful, it is expected that subsequent differentiations from the same batch of frozen monocytes will be similarly successful. To evaluate the quality of the MDM, check their number, viability, and morphology.

7.3 Cell culturing procedure primary cells:

7.3.1 Isolation of PBMs from human buffy coat

The procedure was originally published (Lehmann, Brandenberger et al. 2010), however several modifications in the protocol were made. Publication online (Barosova, Drasler et al. 2020)

NOTE: Due to the experimental planning, primary monocyte isolation should be done in advance. Monocytes shall be frozen (for up to 12 months) and thawed for differentiation 6 days before adding them to the Calu-3 layer. Fresh monocytes may be used as well.

Preparation of reagents

1. Prepare 100 mL of MACS buffer per buffy coat - 0.5% BSA (in PBS) with 2mM EDTA (adjust to pH 7.2, keep in fridge, sterile filter (0.22 µm). Keep at 4 °C throughout the procedure.
2. Prepare cell culture medium (MDM-CCM, i.e. RPMI 1640 medium with 10% FBS, 1%L-Glutamin and 1% Penicillin-Streptomycin, ca 100mL per buffy coat), see section 7.1.2.

Isolation of the PBMs (peripheral blood monocytes):

1. Use scissors to cut open the hose end of the bag containing the buffy coat.
2. Distribute the buffy coat by pouring the bag's contents through the bag's duct directly into two conical centrifuge 50 mL tubes (~25 mL each).Gently pour or pipette PBS into the tubes to reach 50 mL volumes. Mix the contents by turning the tube gently upside down 3x.
3. Divide the buffy coat-PBS mixture into four new 50 mL conical centrifuge tubes by pipetting 25 mL of the mixture in each fresh tube.
4. Slowly lay 13 mL of Lymphoprep underneath the blood-PBS mixture and repeat this for all four tubes.

- Use a 10 mL pipette. The filled pipette has to be removed from the pipette boy and immediately plugged with the thumb to keep the Lymphoprep from rinsing out. The full pipette tip is placed at the bottom of the falcon and the Lymphoprep is allowed to flow below the blood-PBS mixture. Do not empty the pipette completely, about 1 mL will stay in it (avoid bubbling, cover end with thumb when removing it from the falcon tube).
- Lymphoprep addition can also be done the other way around, place 13 mL Lymphoprep and slowly add 25 mL buffy coat on top.

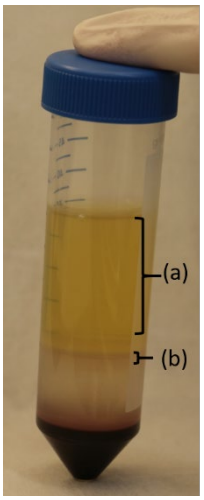


Figure 3: Cell layers separated by Lymphoprep during centrifugation at step 5.

5. Centrifuge for 20 min at 1000 g, 25 °C, and press "SLOW" (i.e. slow breaking) button. Use holders with protective lids for centrifugation.
6. Use a serological pipette, remove the upper layer (containing plasma and most of the platelets, Figure 3 (a)). Using a fresh serological pipette, remove the PBM layer (a whitish turbid small fraction of about 2-3 mm in thickness, shown at Figure 3 (b)) with a 5 mL pipette (avoid to transfer erythrocytes), pool PBM from two tubes in one 50 mL tube > two tubes in total.
7. Fill up the two tubes with PBS to 50 ml.
8. Centrifuge for 8 min at 500 g, 18-20 °C (use regular centrifuge speed from this step on).
9. Remove the supernatant, re-suspend the cells with a few mL of PBS and pool them all in one tube, and finally fill up again with PBS to 50 ml.
10. Add 5 µL of the cell suspension to 45 µL trypan blue (final dilution of 1:10) and count the cells.
11. Centrifuge for 8 min at 500 g, 18-20 °C
12. Count the cells (PBM) with the cell counter or haemocytometer (see section 7.2.2), and calculate the volume required.

Total cell number cT: (cells/mL x 50 mL) =_____

Needed MACS volume (15): $((cT/10^7) \times 80)$ =_____ µl

Needed bead volume: $((cT/10^7) \times 10)$ =_____ µl

Needed MACS volume (21): $((cT/10^7) \times 50)$ =_____ µl

CD14 positive selection

<u>Magnetic labelling:</u>

13. Gently open the lid of each tube, then remove and discard the supernatant using a serological pipette without disturbing the pellet.
14. Re-suspend cell pellet in MACS buffer (80 µL MACS buffer per 10^7 total cells).
15. Add MACS beads (10 µL per 10^7 total cells).

16. Mix well and incubate for 15 min (at 2-8 °C).
17. Fill the tube up to 50 mL with MACS buffer.
18. Centrifuge for 8 min at 500 g, 18-20 °C.
    a. Prepare magnetic separation (wash 1 min before end of spinning, see point 23).
19. Remove supernatant completely.
20. Re-suspend the cell pellet in MACS buffer (500 µL MACS buffer per 10^8 cells).

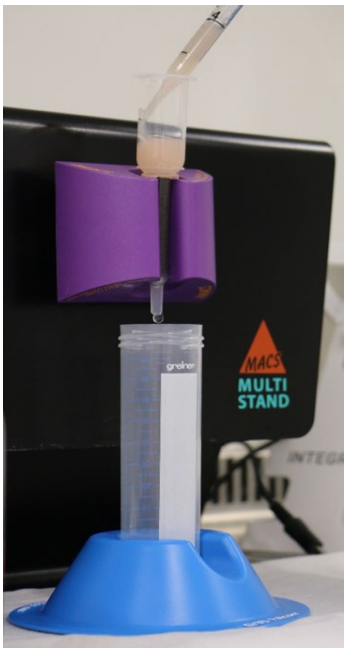


Figure 4: Magnetic separation (step 21).

<u>Magnetic separation:</u>

21. Place the column in the magnetic field; place a 50 mL tube under the column (Figure 4).
22. Prepare column by rinsing with 3 mL of buffer.
    - Also add 1 mL to a 15 mL falcon tube.
23. Apply cell suspension to column (max $2x10^9$ cells).
24. Collect unlabelled cells that pass through (in the 50 mL Falcon tube placed below).
25. Wash 3x with each 3 mL buffer (9-12 mL in total) → apply new buffer when the column reservoir is empty, avoid drying out the column.
26. Remove the column from the separator and place it on the 15 mL tube (the one with 1 mL of buffer).
27. Add 5 mL to the column and flush out the magnetically labelled cells by firmly pushing the plunger into the column.

Cell differentiation:

28. Add 5 µL of the cell suspension to 45 µL trypan blue (final dilution of 1:10).
29. Centrifuge for 8 min at 500 g, 18-20 °C.
30. Count the cells with the cell counter (remember to multiply the result from the cell counter by the dilution factor and calculate the MDM-CCM volume required for a density of 10^6 cells/mL).

Total cell number cT: (cells/mL x 6) =_____

Needed volume of MDM-CCM: ((cT/10^6) x 1) =_____ ml

31. Remove the supernatant.
32. Continue with steps 33 – 37 to freeze PMCs.

7.3.2 PBM freezing

33. Resuspend the cell pellet in a cryoprotective medium (here, FBS and dimethyl sulfoxide [DMSO; cytotoxic]) at a ratio of 9:1 (v/v) by pipetting a volume of prewarmed FBS. This

corresponds to a final cell concentration of 6 x 10^6 cells/mL, considering a further addition of 10% DMSO (v/v).

34. Mark the desired number of cryovials in the laminar flow hood (i.e., record the date, isolation code, and number of cells).
35. Pipette 0.9 mL of cell suspension in pure FBS (here, 6 x 10^6 cells in 0.9 mL of FBS) to each cryovial. Subsequently, slowly pipette 0.1 mL of DMSO and mix the suspension well by turning the cryovials up and down 3x.
36. Transfer the cryovials to a cell-freezing container and immediately set it to -80 °C for 24 h.
37. After 24 h, remove the cryovials from the -80 °C freezer and container and place them into the liquid nitrogen tank suitable for cell storage.

7.3.3 PBM seeding

38. Warm all required reagents to 37 °C in a water bath (~20-30 min).
39. Prepare a 6 well plate (number of plates depends on cell number of thawed cells) with 2 mL of MDM-CCM/well under aseptic conditions and place in the incubator (5% CO_2, 37°C) for a minimum of 15 min to allow the pH to settle in preparation for new cells. A specific pH level is not required, instead the equilibration period is to ensure there is not a significant change in pH after the initial seeding that might lead to cell shock.
40. Remove one vial of cells from liquid nitrogen storage and gently swirl in a 37 °C water bath (to ensure uniform thawing of the cell suspension) ensuring the O-ring and lid is not submerged (to reduce the potential for contamination) until the contents are almost thawed (between 1 and 2 min).
41. 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.
42. Pipette slowly (drop by drop) the contents of the vial into a centrifuge tube with 9 mL of prewarmed MDM-CCM and centrifuge at 200 g for 5 min.
43. Discard the supernatant into waste and re-suspend (via pipetting up and down) the cell pellet in 2 mL of prewarmed MDM-CCM.
44. Count the cells (see section 7.2.2).
45. Transfer the re-suspended cells into the pre-prepared MDM-CCM in 2x 6-wells: calculate the amount of MDM-CCM needed to reach a cell density of 3*10^6 cells/well and 10^6 cells/mL and add then growth factors to the MDM-CCM: final concentration of 10 ng/mL M-CSF. It should be mentioned that 3*10^6 cells/well is not a strict number as the efficiency of differentiation to MDMs varies between experiments. Incubate the culture at 37 °C and 5% CO_2.
46. Let the cells differentiate for 6 days in the incubator (37°C, 5% CO_2) without changing MDM-CCM. Do not let them differentiate for more than 8 days.

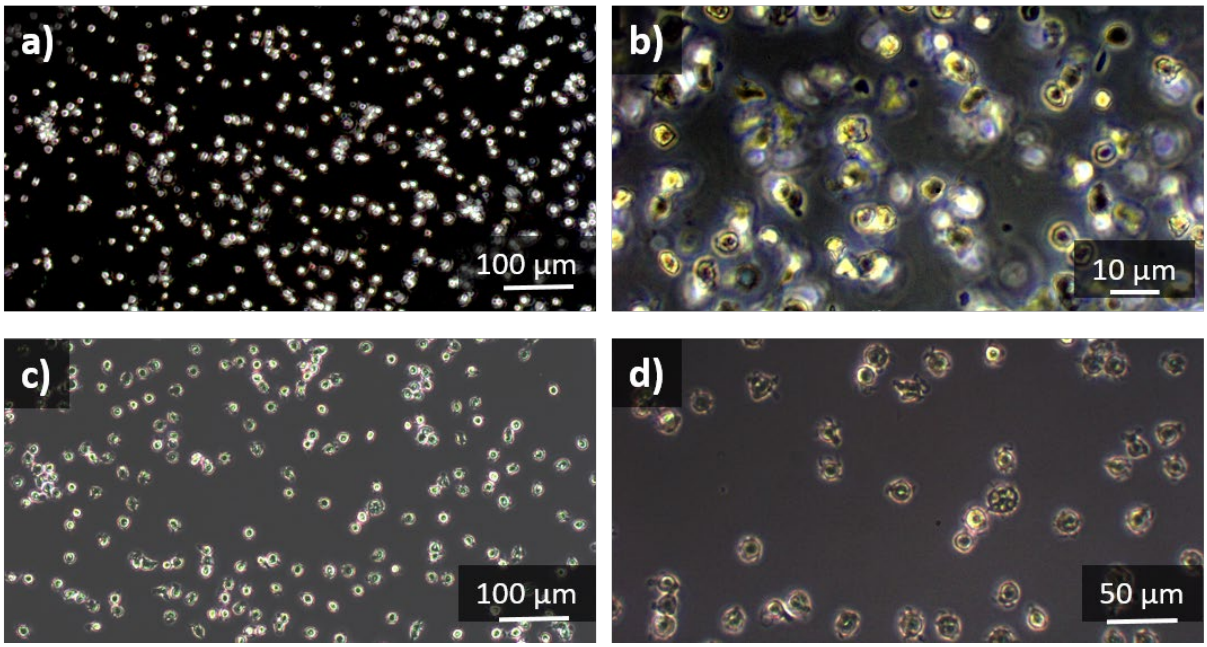


Figure 5: Images of (a, b) PBMs directly after seeding, (c, d) differentiated MDMs 6 days after seeding, using 10 ng/mL M-CSF.

8 Preparing co-culture

8.1 Co-culture assembling of MDMs with Calu-3

8.1.1 Epithelial cells -macrophages

1. 6 days after PBMs culturing in RPMI + M-CSF, visually check MDMs under a phase contrast microscope (preferably take pictures), to confirm that MDMs have attached to the plate, keep their round shape and that no contamination has occurred.

The following steps are completed under aseptic conditions

2. Add 1 mL of MDM-CCM, and using a cell scraper, gently scrape MDMs attached to the bottom of each well. Gently wash the well and harvest all the cells into a Falcon tube. Mix cells from all the wells into one Falcon tube.
3. Add the supernatants (containing the floating MDM), centrifuge for 5 min at 200 g and discard the MDM-CCM into a waste vessel.
4. Carefully resuspend the MDM in 1 ml MDM-CCM.
5. Count the cells (dead and alive) using a haemocytometer as described in 7.2.2. Do not proceed when the viability is below 70%.

   Using the live cell number calculated above, determine the dilution required to get a cell concentration of 196 000 MDMs/ml.

   Note: the desired concentration is 25,000 cells/cm^2 corresponding to 28,000 cells for a 12-wells insert (1.12 cm^2) in 0.2 mL MDM-CCM. However, not all MDMs will attach. Therefore an additional 40% is added (total of 39,200 cells).

There are two options to measure the TEER: (1) Before addition of MDM, (2) In wells that do not receive MDM.
Ad (1) Carefully pipette (slowly on the wall) 0.5 mL CCM on the apical side of each insert without disturbing the already present epithelial layer. Measure TEER. Carefully remove the CCM. Carefully pipette (slowly on the wall) 0.2 mL of cell suspension (containing 39,200 cells) on the apical part of each insert without disturbing the already present epithelial layer. In the wells that will contain only Calu-3 cells, carefully pipette 0.2 ml MDM-CCM (without cells).
Ad (2) Carefully pipette (slowly on the wall) 0.5 mL of cell suspension (containing 39,200 cells) on the apical part of each insert without disturbing the already present epithelial layer. In the wells that will contain only Calu-3 cells, carefully pipette 0.5 ml MDM-CCM (without cells). Measure TEER only in the wells without MDM and take these values as representative for wells containing the primary macrophages.

6. Gently rock the inserts, make sure they are placed in the centre of each well and then place back into the incubator (37 °C and 5% CO_2).
7. Let MDMs attach for 4 h and then transfer the co-culture model to ALI by discarding both basolateral and apical CCM. From here on, use only CCM for Calu-3 cell culture, not MDM-CCM! Add 1.5 mL of CCM to the basolateral side only.
8. Co-culture model can be used for exposure after a specific time at ALI, depending on epithelial cell type (consult with corresponding SOP), for Calu-3 after 24 h.

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 DQ12/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 min 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 h. The stock dispersions are meta-stable due to the relatively high particle concentrations. Suspensions normally remain stable for 30 to 60 min 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 s 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 re-disperse 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.

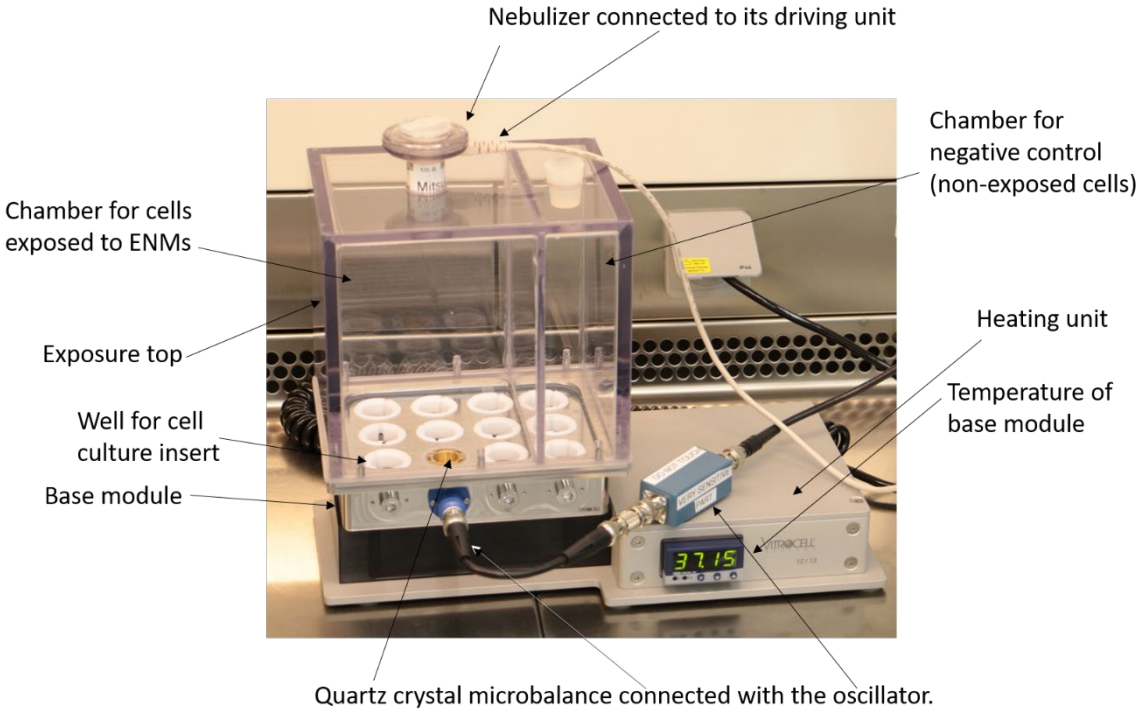


Figure 6: The VITROCELL® Cloud system.

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 nebulisations. 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 $DQ12/TiO_2$ 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/DQ12 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 TiO2/DQ12 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 without cells 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. Fill the 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.

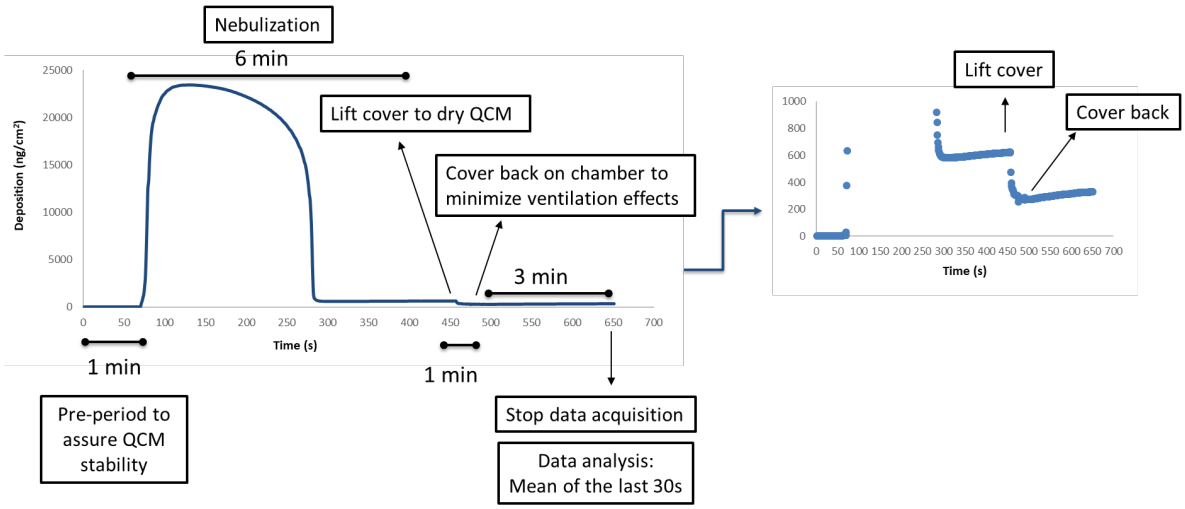


Figure 7: Typical time course of QCM signal and the time periods applied.

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 nebulisations. 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 nebulisations 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 DQ12 and TiO_2, perform nebulization of 500 µg/mL (2- or 3-times) in order to obtain the desired deposited doses (see Table 1). It is more important to reach the desired deposited dose than to follow the time of nebulisations. It is highly important to have comparable deposited doses and thus biological responses.
    Note: For subsequent nebulisations of the same particle solution concentration: in order to achieve a higher deposition, subsequent nebulisations 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 nebulisations, the inner walls of the exposure chamber need to be wiped with 70% ethanol in between.
    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 (see section 6.3) or replace the nebulizer.

Exposure to LPS/ TiO_2/DQ12			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

Table 1. 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.

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, DQ12. 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) (assessed by AMI in RR1)	Nebulization	Target depositions (ng/cm^2)
LPS	175	250	1x	250
DQ12	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.

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 7). 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.

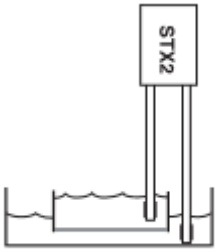


Figure 7. Correct positioning of the electrode in the insert to measure TEER.

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 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 min.
    Note: longer periods of lysis (up to 20 min) have also found necessary.
    b. Use the lysate to measure the total LDH release.
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 10-15 min at room temperature.
7. After 15 min, 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.
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 (Bio-Rad)). 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 20 min.
3. Collect the lysate and store at a temperature below -20 C for later analysis (e.g. ICP-MS).

12 Quality control & acceptance criteria

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

13 Data Analysis and Reporting of Data

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

14 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

15 References

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Lehmann, A., C. Brandenberger, F. Blank, P. Gehr and B. Rothen-Rutishauser (2010). A 3D model of the human epithelial airway barrier. Alternatives to animal testing. L. R. Yarmush ML, Artech House**:** 239-260

Loret, T., E. Peyret, M. Dubreuil, O. Aguerre-Chariol, C. Bressot, O. le Bihan, T. Amodeo, B. Trouiller, A. Braun, C. Egles and G. Lacroix (2016). "Air-liquid interface exposure to aerosols of poorly soluble nanomaterials induces different biological activation levels compared to exposure to suspensions." Particle and Fibre Toxicology **13**(1): 58.

Other useful sources:

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

https://www.hemocytometer.org/hemocytometer-protocol/

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ROTHEN-RUTISHAUSER, B. M., KIAMA, S. G. & GEHR, P. 2005. A Three-Dimensional Cellular Model of the Human Respiratory Tract to Study the Interaction with Particles. *Am J Resp Cell Mol,* 32, 281-289.

PATROLS SOP. Guidance Document for cell culture of lung epithelial cell-line (Calu-3).

PATROLS SOP. Guidance Document for DQ12 and TiO2 Aerosolization using VITROCELL® Cloud System.