PATROLS
Advanced Tools for NanoSafety Testing

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 760813

ANNEX 3203

PATROLS Standard Operating Procedures (SOP)

SOP for assembly and use of DALI

This is a SOP recommended for external use by PATROLS

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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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Reviewed by:

WP3 members

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Version	Approval Date	Description of the change	Author(s) of change
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1.1	07/04/2020	Additional detail added form UNIPI and final comments implemented	Roberta Nossa
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2.1	25/08/2020	All comments integrated and SOP uploaded to server	

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1 Introduction:

DOMAIN: Advanced system for improving physiological relevance of the lung model by introduction of mechanical motion

Nanoparticles are widely used in industrial, household and medicinal applications. However, these dispersed particles can cause inflammation and stress in lung tissue, leading to the development of disease, such as asthma or chronic obstructive pulmonary disease. Moreover, their effect on human tissue is complex and not completely understood, since it is mediated by different factors, such as the humidity in the alveolar environment and the rhythmic contraction of the diaphragm. This rhythmic contraction generates a periodic change in the alveolar volume and the displacement of the alveolar wall, influencing nanoparticles (NPs) deposition, substances uptake and inflammatory response.

To understand and study the complex interaction between inhaled particles and lung tissue, both *in vitro* and *in vivo* models can be used. However, traditional *in vitro* models cannot reproduce the entire complexity of the alveolar environment, since they are not able to apply a mechanical cyclic strain to the cultured cells. On the other hand, *in vivo* models do not consider interspecies differences, which lead to a different response to drugs or nanoparticles due to a different physiology, resulting a non-predictive model of the human alveolar microenvironment. Moreover, animal tests are expensive and pose ethical problems.

To overcome these issues, an air-liquid interface bioreactor was developed, provided with a mobile elastic membrane to simulate physiological lung muscle stretching. This system, named DALI (Dynamic model for ALveolar Interface), consists of an aerosol generator and a bioreactor with a moving membrane placed between an air-liquid interface to study drug and nanoparticles deposition and passage. To mimic natural breathing, an external compressed air system was used to stretch the elastic membrane where alveolar epithelial cells are seeded. Finally, a Quartz Crystal Microbalance (QCM) was integrated to quantify the amount of aerosolized nanoparticles on the cell layer.

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1.1 Scope and limits of the protocol

This SOP was developed to provide a systematic description of the DALI system, therefore a guide for its usage.

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]

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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.

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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, 2.2.

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
DALI	Dynamic model for the ALveolar Interface
EtOH	Ethanol
PC	Polycarbonate
PDMS	Polydimethylsiloxane
QCM	Quartz Crystal Microbalance
UV	Ultraviolet

4 Principle of the Method:

These methods aim at describing the DALI system and all its components in detail: the bioreactor chambers, membrane holder, hydraulic circuit, aerosol nebulizer, QCM and control box.

5 Description of the Method:

5.1 Test system used:

This SOP should be carried out under laboratory-based conditions.

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5.2 Chemicals and reagents used:

Not applicable to this SOP.

5.3 Apparatus and equipment used:

DALI system equipped with:

- Two dual-flow bioreactors (Figure 1)

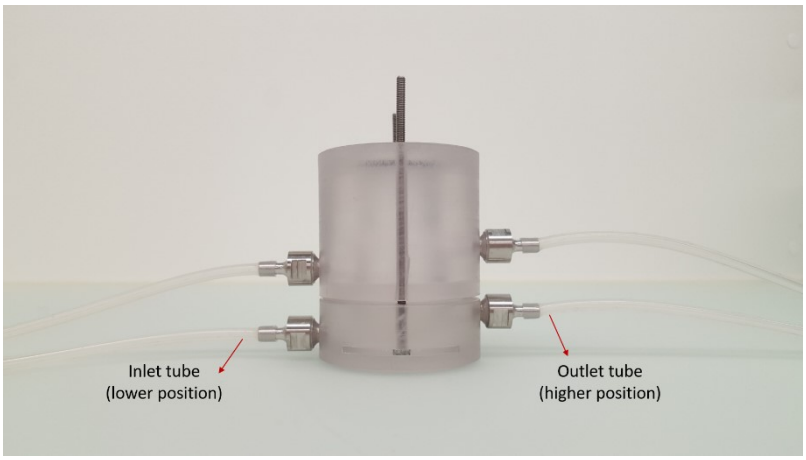

Figure 1 : Picture of the bioreactor

- Covers: "standard" cover and breathing cover (for air tightness) (Figure 2)

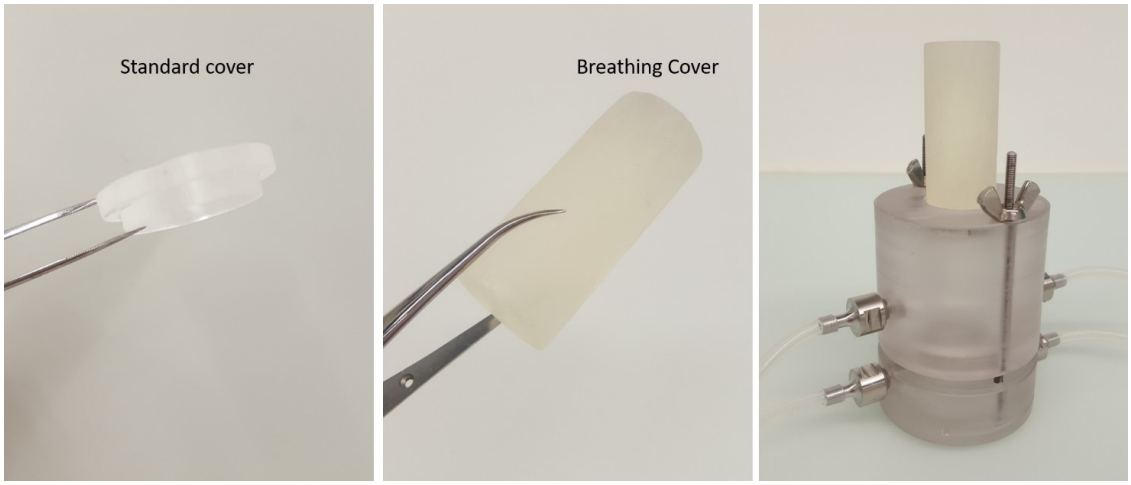

Figure 2 : Picture of the standard cover (to use without pressurization) and the breathing cover (to use during pressurization).

- Peristaltic pump
- Hydraulic circuit:
    - Pump tube (the one with clamps). Inner diameter 2 mm, outer diameter 4 mm (connectors 3/32). Material: silicone
    - Air flow tube (without clamps). Inner diameter 2 mm, outer diameter 4 mm (connectors 3/32). Material: silicone

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- Cell culture medium reservoir and its holder (Figure 3)

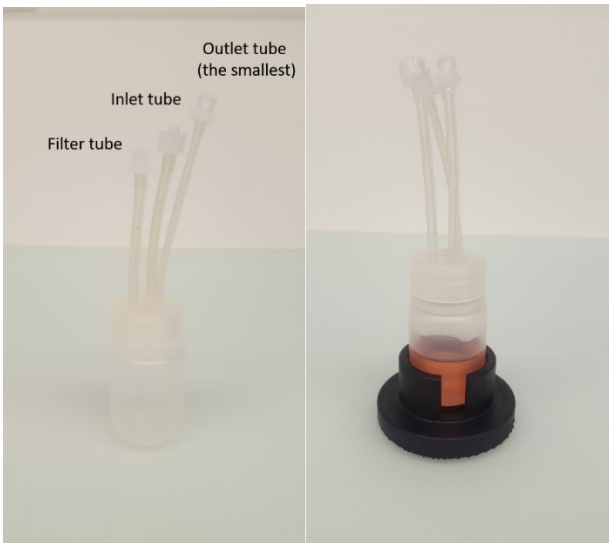

Figure 3 : Picture of the reservoir and its holder

- Control box containing an Arduino Micro board (Arduino, Interaction Design Institute, Ivrea, Italy) and two electropneumatic regulators (ITV0011-2BL, SMC, Italy) (Figure 4)

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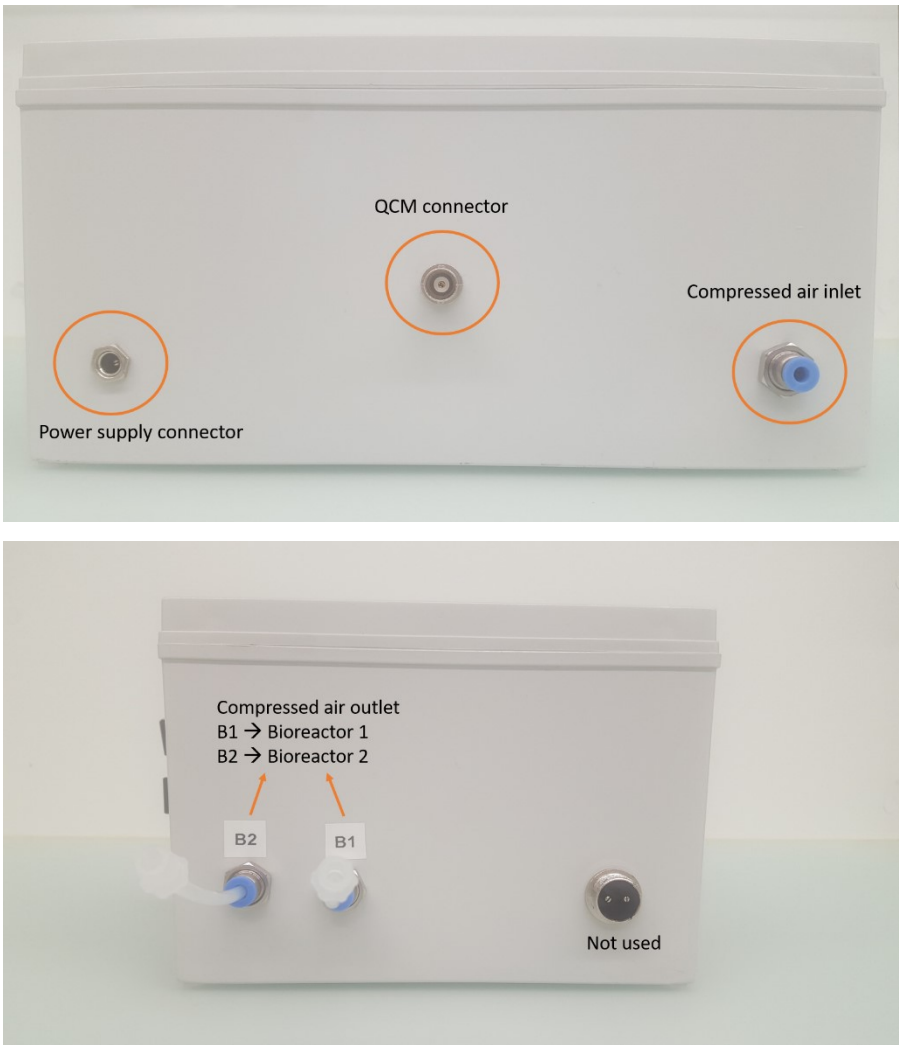

Figure 4 : Pictures of the control box (Front view, back view and lateral view)

- Membrane with its holder

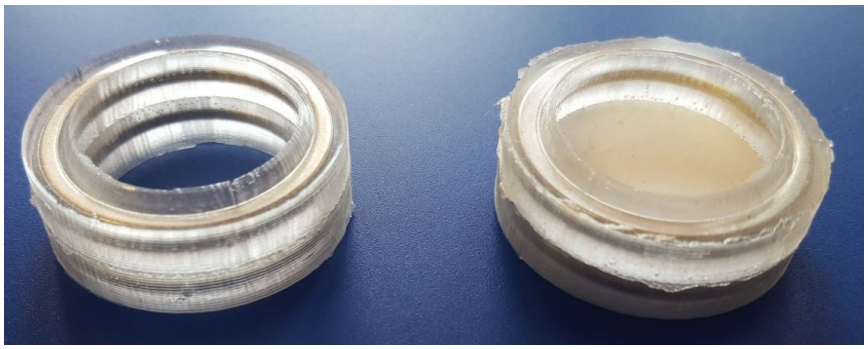

Figure 5 : Picture of the membrane holder

- Quartz Crystal Microbalance (QCM)
- A nebulizer (Aeroneb Pro, Aerogen, Galway, UK) and its controller (Aerogen® Pro-X, Aerogen, Galway, UK)

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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.

5.5 Health and safety precautions:

Standard health and safety precautions associated with working within a laboratory environment, 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.

5.6 Nanomaterials used / handling procedures:

Not applicable to this SOP.

5.7 Reagent preparation:

Not applicable to this SOP.

5.8 Procedure:

5.8.1 The DALI system: an introduction

Figure 6 schematizes the DALI system. The upper chamber (or apical chamber, Figure 6b) contains filtered atmospheric air, simulating the air side of the air/liquid interface (alveolus); the bottom chamber (basolateral chamber, Figure 6a) contains the cell culture media, mimicking the blood side of the ALI (capillary). The membrane is the support on which cells are seeded and separates the two compartments of the bioreactor, like the alveolar barrier that separates the two compartments of the alveolus. A peristaltic pump (Figure 6d) placed within the hydraulic circuit allows the cell culture media flow from a reservoir (Figure 6c) to the bottom chamber, reproducing the blood flow through the capillaries that surround the alveoli.

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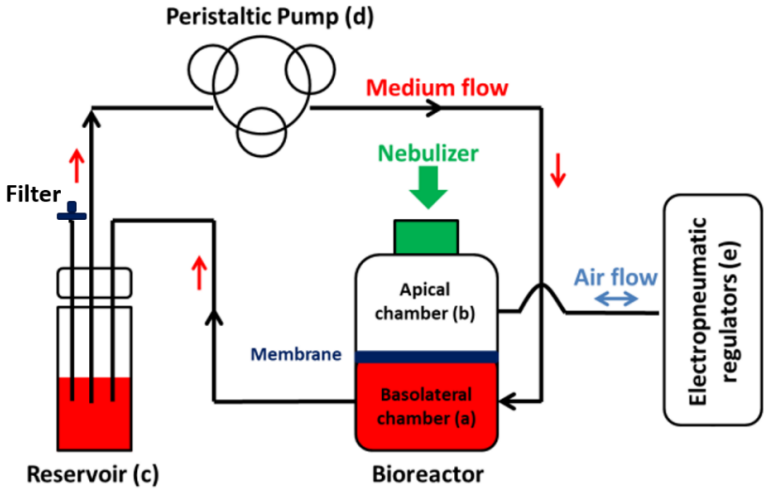

Figure 6: On the top: schematic view of the entire DALI system, which include a two-compartment bioreactor – (a) and (b) are respectively the basolateral and the apical chamber, a reservoir (c) from which the media is suck by a peristaltic pump (d), and a electropneumatic regulator (e) that introduce air in the upper chamber, allowing membrane stretching.

Figure 7 shows the scheme of the key elements of the alveolar interface adapted to the DALI system. These key elements are the cell culture media flow (Figure 7A), lung breathing motion (Figure 7B) and aerosol deposition (Figure 7C).

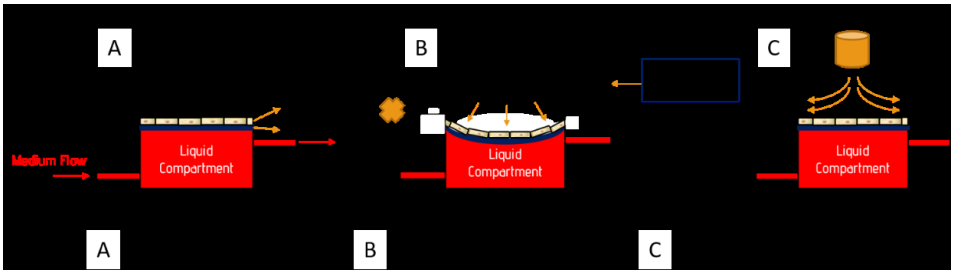

Figure 7: Key elements of the DALI system: A) ALI with media flow, B) stretching of the membrane and C) aerosol deposition on the cell layer.

To reproduce lung breathing motion, an external source of controlled compressed air increases the pressure on the upper side of the membrane (Figures 6e and 7B), allowing its displacement. Lung expand and recoil frequency is 0.2 Hz (5 seconds), meaning that the inhalation and exhalation phases last 2.5s each [1]. Therefore, the device is able to apply this cyclic motion. Moreover, the DALI system apply to the membrane different linear strains, allowing to mimic different conditions, both pathological than physiological.

Since the device investigates the effects of aerosolised particles on the cells, it is provided with a nebulizer directly connected to the upper chamber (Figure 7C). In

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order to facilitate aerosol deposition procedures, a commercial nebulizer was used and directly connected to the apical chamber.

A QCM was integrated to the DALI system, to quantify the amount of aerosolized nanoparticles, allowing to evaluate their interactions with the cells.

Finally, a control unit was designed to allow the operator to regulate the stretching level of the membrane and quantify particle deposition.

Table 1 summarizes the key elements of the DALI system, reporting the designed solution for each of them.

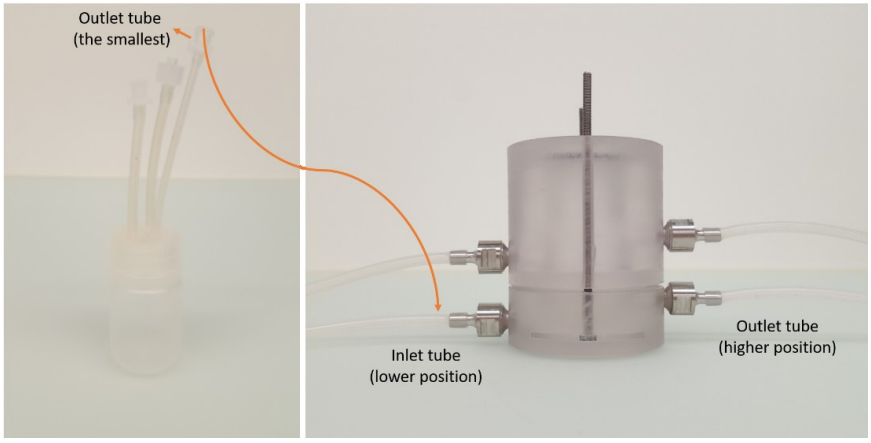
Table 1: Key elements of the DALI system with their designed solutions.

Key elements of the DALI system	Design solution
Air/liquid interface	Modular bioreactor (2 chambers)
Cell culture media flow	Hydraulic circuit equipped with peristaltic pump, reservoir and tubing.
Lung breathing motion	External source of controlled compressed air
Aerosol deposition system	Commercialised aerosol device
Quantification of aerosolized particles	A QCM

5.8.2 DALI system assembly

The procedure for the assembly of the DALI system for dynamic cell culture experiments is the following:

1. Place the reservoir in its holder
2. Connect the outlet tube of the reservoir (the smallest one) to the inlet tube of the bioreactor (tube in the lower position)



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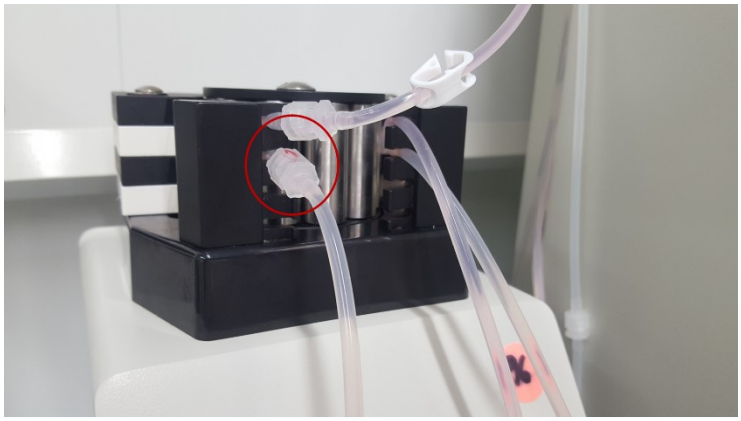
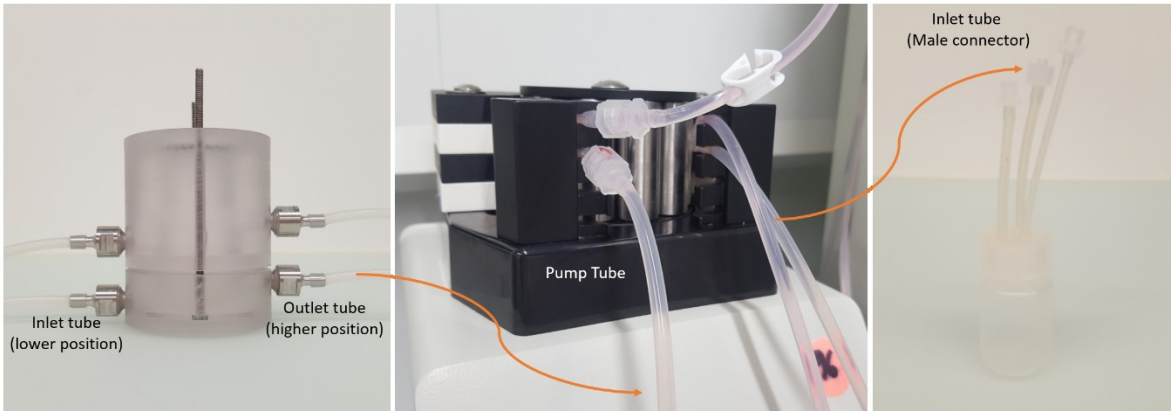
3. Put the *pump tube* in the peristaltic pump → mark the connector that is used to fix the tube to the pump (do this to know how to place again the *pump tube* after moving everything in the incubator)



4. Connect one end of the *pump tube* to the outlet tube of the bioreactor (higher position) and the other end to the reservoir



5. Fill a tube with 35 mL of medium
6. Put the membrane holder inside the lower chamber

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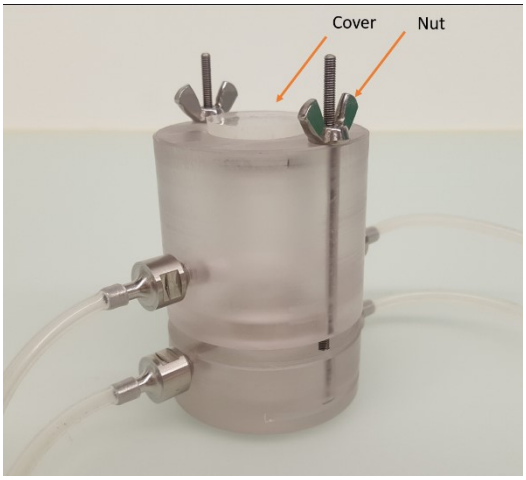
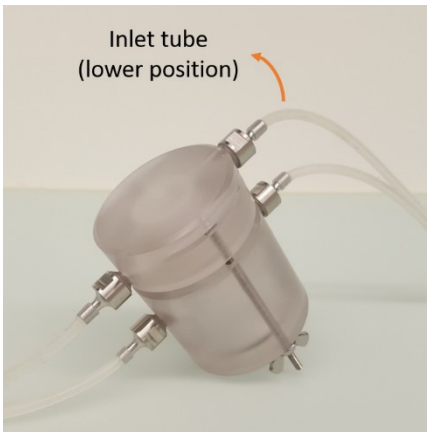
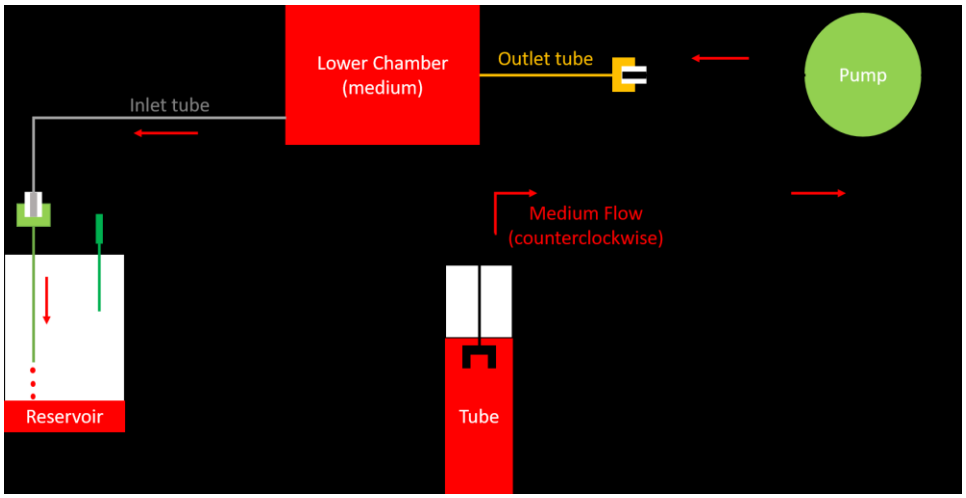
7. Close the bioreactor with the upper chamber, the cover and the nuts.



8. Turn the bioreactor → the inlet tube (the lower) must be on top (**important to avoid air bubbles formation in the lower chamber**)



9. Disconnect the *pump tube* from the reservoir and put its female-end inside the tube with media. Start the pump (i.e. counterclockwise) and run it until the filling of the entire circuit.



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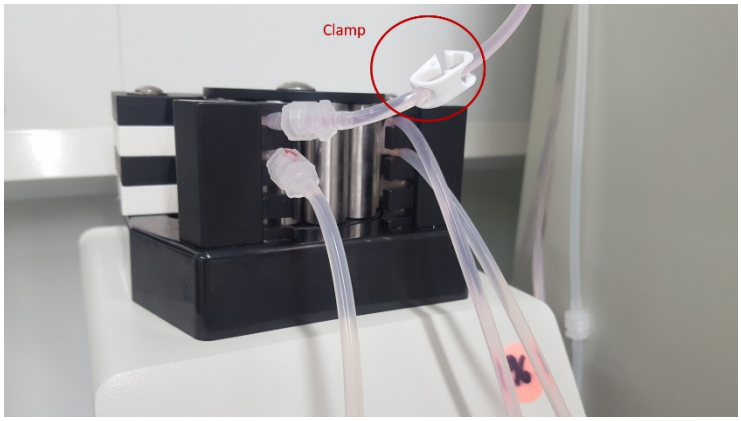
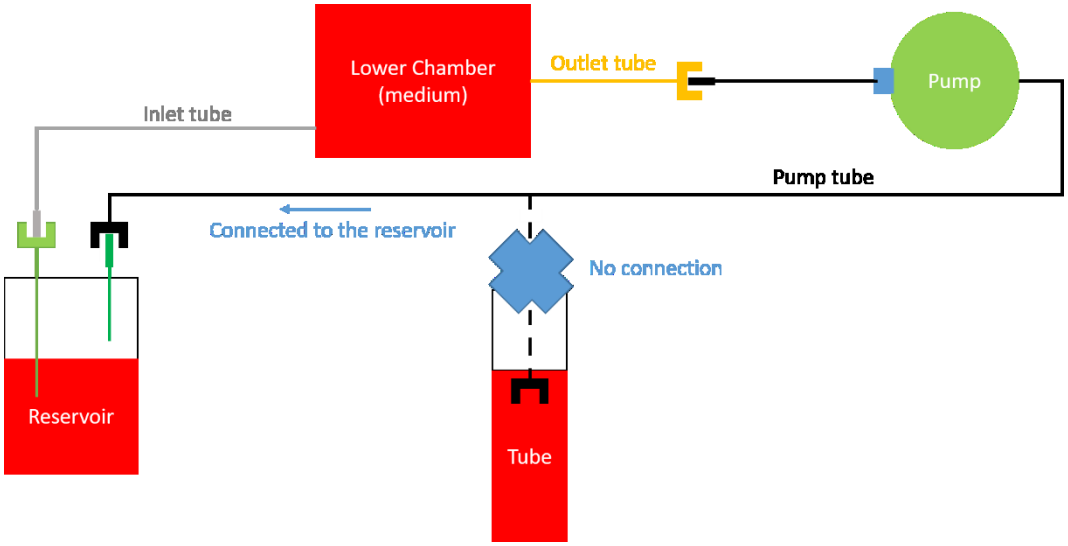
10. Stop the pump and **close all the clamps (important to avoid bubble formation inside the circuit)**



11. Reconnect the female end of the *pump tube* to the reservoir



12. If the pump will go inside the incubator: move the pump, the reservoir and the bioreactor into the incubator, then skip to passage "15", otherwise:
13. Remove the *pump tube* from the pump, put the bioreactor and the reservoir in the incubator and then let the tube pass through the incubator hole
14. Put again the tube in the pump (pay attention to the direction, use the mark on the connector as guide)
15. **Open the clamps**
16. Reverse the rotation of the pump and start it (with AMI's pump use 0.24 rpm) → Flow rate is 400 µL/min.

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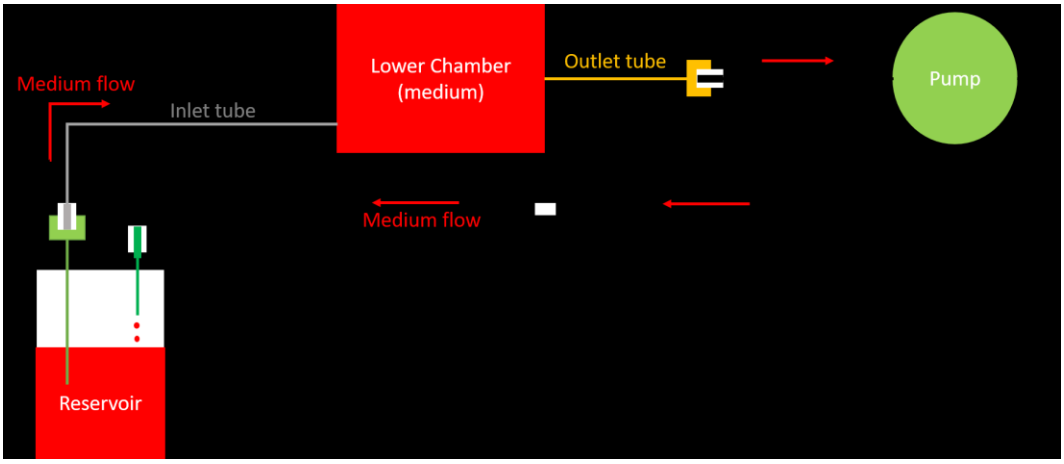

5.8.3 DALI system sterilization

- Sterilize the bioreactor (lower chamber, upper chamber, *pump tube*, *air tube*, cover for the upper chamber) → different options:
    a. Autoclave – not the magnets (Membrane holder)
    b. Ethylene oxide
    c. With 70% EtOH + UV light
- Sterilize the 3D printed covers for the air tightness: UV light (not ethanol cause it dissolves them)
- Sterilize the membrane holder:
    a. put each magnet in well of a 6-well plate
    b. dip in a 70% EtOH in deionized water solution (V/V) for 15 minutes;
    c. let it dry under a laminar ventilation hood;

5.9 Quality control & acceptance criteria:

The DALI system satisfies the following specifications:

- The biocompatibility, thanks to the use of biocompatible materials, such as PC and PDMS.
- The sterility, thanks to the use of sterizable materials. Moreover, the culturing chambers were designed in order to facilitate cleaning procedures (i.e. rounded edges). Finally, the tightness of the bioreactor prevents the contamination of the cells.

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- The compatibility with the laboratory procedures: the bioreactor is easy to handle and assemble under the hood, preventing its contamination during experiments.
- Cross-contamination is avoided thanks to the use of one specific bioreactor (culturing chamber) for each sample.
- The fluidic dynamic culture is performed by placing a peristaltic pump within the hydraulic circuit, which allows cell culture media flow.
- The physical stimulation that mimic the breathing movement is given by the pressurisation of the upper chamber by the electropneumatic regulator.
- The device is small and can be easily placed under the hood and inside the incubator.
- The control box was designed to easily control the experimental setup and monitor device operations without the help of a computer (stand-alone system), using a user-friendly interface.
- The system presents an air/liquid interface, allowing reproducing *in vivo* physiological conditions of the alveolar interface.
- It is provided with a QCM to quantify the amount of aerosolized nanoparticles.

6 Data Analysis and Reporting of Data:

Not applicable to this SOP.

7 Publications:

Not applicable to this SOP.

8 References

[1] M. Felder *et al.*, "Impaired wound healing of alveolar lung epithelial cells in a breathing lung-on-a-chip," *Front. Bioeng. Biotechnol.*, vol. 7, no. JAN, pp. 1–5, 2019, doi: 10.3389/fbioe.2019.00003.

[2] D. A. Buttry and M. D. Ward, "Measurement of Interfaclal Processes at Electrode Surfaces with the Electrochemical Quartz Crystal Microbalance," *Chem. Rev.*, 1992, doi: 10.1021/cr00014a006.

[3] K. G. Birukov *et al.*, "Magnitude-dependent regulation of pulmonary endothelial cell barrier function by cyclic stretch," *Am. J. Physiol. - Lung Cell. Mol. Physiol.*, 2003.