ANNEX 3205
Technical Description

Biological testing of the DALI System

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1 Scope of the Annex:

This Annex was developed to describe the biological tests performed to validate the DALI membrane and the entire system from a biological point of view.

2 Abbreviations:

DALI	Dynamic model of the ALveolar Interface
PBS	Phosphate-Buffered Saline
PFA	Paraformaldehyde
UV	Ultraviolet

3 Principle of the Method:

This document details the biological experiments performed with the DALI system. Firstly, the biocompatibility and cell adhesion of the membrane material were investigated, then the biocompatibility of the bioreactor materials. Finally, dynamic cell culture tests were performed to evaluate the effect of the cyclic mechanical strain applied to the cells.

4 Description of the Method:

4.1 Biological setting & test system used:

The described procedures should be carried out under laboratory-based conditions, with all work performed under sterile conditions and at a minimum of Biological Safety Level 1 conditions.

Cell line utilized is a Human Caucasian lung Carcinoma derived epithelial cell line, A549 (ATCC CCL-185).

For further information: https://www.lgcstandards-atcc.org/products/all/CCL-185.aspx?geo_country=gb

4.2 Chemicals and reagents used:

- For A549 sub-culturing refer to SOP_PATROLS_A549
- Roswell Park memorial Institute-1640 Medium (RPMI-1640)

- 70% ethanol
- PBS w/o Ca2+ and Mg2+ (Cat No. D8537, Sigma)
- Alamar Blue: In Vitro Toxicology Assay Kit, Resazurin based (Cat No. TOX8, Sigma)
- 4% (w/v) Paraformaldehyde (PFA) (Cat No. 158127, Sigma)
- Phalloidin-iFluor 488 Reagent (Cat No. ab176753, Abcam)
- DAPI (4' 6-diamidino-2-phenylindole) (Cat No. D9542, Sigma)
- Trypan blue solution (Cat No. T6146, Sigma)

4.3 Apparatus and equipment used:

- For A549 sub-culturing refer to SOP_PATROLS_A549
- DALI system equipped with:
    - Two dual-flow bioreactors
    - Peristaltic pump (Ismatec IPC 4, IDEX Health & Science, Germany)
    - Hydraulic circuit (cell culture medium reservoir + silicone tubes)
    - Control box containing an Arduino Micro board (Arduino, Interaction Design Institute, Ivrea, Italy) and two electropneumatic regulators (ITV0011-2BL, SMC, Italy)
    - Membrane with its holder

  For DALI assembly, refer to Annex 3_SOP for assembly and use of DALI.
- Fluorecent based microscope.
- Spectrophotometer.

4.4 Reagent preparation:

For reagent preparation used for A549 sub-culturing refer to SOP_PATROLS_A549.

4.5 Procedure:

4.5.1 Preliminary evaluation of cell adhesion on membrane at UNIPI

4.5.1.1 Methods

Before performing experiments with the DALI system, biocompatibility and cell adhesion of the electrospun membrane (made of 1:1 Bionate®:gelatin, refer to Annex 4_TD_Preparation and mechanical characterization of Bionate membranes) were first investigated.

For A549 sub-culturing refer to SOP_PATROLS_A549. Cells were cultured on the fabricated samples for 1 week, and the Alamar Blue cell viability assay was performed to monitor cell growth, thus verifying the suitability of the membranes as cell culture support for the alveolar barrier. From a sheet of Bionate®:gelatin material, samples were prepared cutting a piece of 10x10 mm. They were placed within a 24-well plate and sterilised dipping the samples in 70% ethanol for 24 hours. Then, they were washed three times with PBS and maintained in PSB until cell seeding. Three different densities of A549 cells were seeded carefully on the membranes (150 000, 200 000 and 250 000 cells/cm2), in triplicate, adding 2 mL of cell medium. The negative control was A549 cells seeded directly on a 24-well plate at the same density, under the same environmental conditions.

At days 1, 5 and 7 of culture, cells were incubated at 37 ºC for 3 hours with the Alamar Blue reagent (Sigma, Italy) diluted 10X in cell culture medium. In this period cells converted the compound resazurin into resorufin (see Sect 4.2). The fluorescence signal of the latter was measured with a spectrophotometer (PerkinElmer, USA) at 595 nm. Results were obtained by plotting the fluorescent signal versus compound concentration.

At the end of the culturing period, cells were fixed with 4% PFA for 20 minutes at room temperature and rinsed two times with PBS 1X. Cells were then labelled with 1X Phalloidin-iFluor 488 Reagent or 40 minutes, and rinsed for three times in PBS 1X. Finally, cells were labelled with followed by DAPI (4' 6-diamidino-2-phenylindole; 1µg/mL in PBS) for 10 minutes, and then rinsed for two times in PBS 1X. The seeded samples were assessed with visualization with a confocal microscope (A1 Confocal Microscope System, Nikon Italy).

4.5.1.2 Results and discussion

To assess the metabolic activity of the A549 cells, the biological samples were incubated with resazurin for 3 hours at the culture days 1, 5 and 7, to measure their metabolic activity. As presented in Figure 1 the converted units of resazurin increased over time, reflecting the gradual cell growth, as expected.

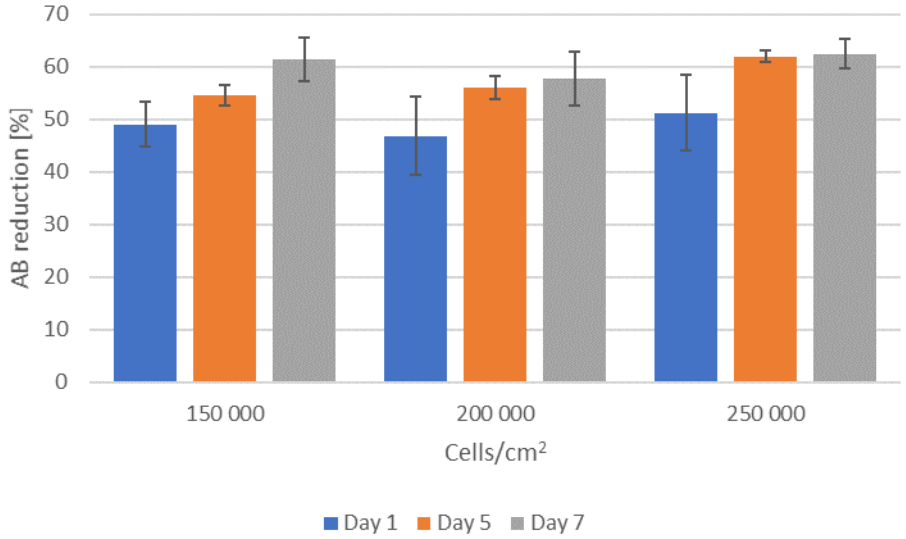

Figure 1: Percentage of Alamar Blue reduction with respect to TCP controls during the culturing period (at 3 different time-points of the cell culture: 1, 5 and 7 days).

Samples seeded with 150 000, 200 000 and 250 000 cells/cm^2 show comparable cell metabolic activity during the entire culture period. After culturing the cells for 7 days, the samples were fixed and stained with DAPI (nuclei) and Phalloidin (cell cytoskeleton). Figure 2 shows the cells forming a compact monolayer on the substrate, meaning that the fabricated membranes allowed cell growth. Cells were also shown to penetrate the fibers (not shown).

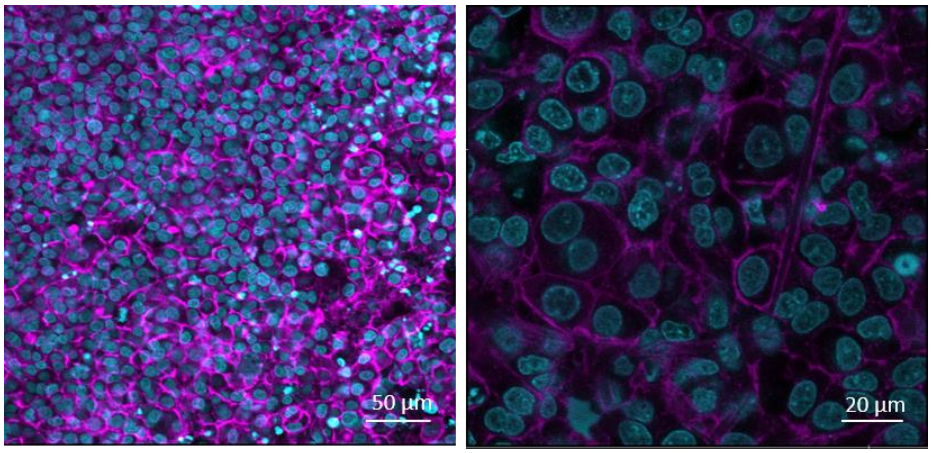

Figure 2: Confocal images of A549 cells cultured on a sample for 7 days and stained with DAPI (cyan, nuclear stain) and phalloidin (magenta, actin fibers). Cell density: 150 000 cells/cm2. 20X magnification on the left, 40X magnification on the right.

The experiments show that in resting conditions, the electrospun membrane selected for the DALI prototype supports A549 adhesion.

4.5.2 Bioreactor biocompatibility evaluation at UNIPI

4.5.2.1 Materials and Methods

After verifying the cell adhesion of the electrospun membrane, the impact of the bioreactor and of the membrane holder upon cell cultures was investigated with the Alamar Blue Assay. Samples were prepared cutting a 24-mm diameter disc from a sheet of the electrospun material, trying to avoid cutting samples from the edges where the membrane is thinner. Before cell seeding, membranes were fixed in their holder and sterilized, dipping the samples in 70% ethanol for 15 minutes and washing them 2 times with PBS. A549 cells were cultivated for 5 days in three different conditions:

- *Holder condition*: 3 membranes fixed in the holder (two magnets covered by PDMS, see Annex 2 for images and details) and placed within a 6-well plate.
- *Bioreactor condition*: 3 membranes fixed in the holder and placed within the bioreactor. No media flow or stretching was applied.
- *Control condition*: cells seeded directly on the bottom of 3 wells of a 6-well plate.

The cell seeding density was 150 000 cells/cm^2.(see Sect. 4.5.1.2). All the samples were cultivated under submerged conditions. Concerning *Holder* and *Bioreactor conditions*, cells were seeded on the top side of the membrane (facing the apical side when placed within the bioreactor). In *Control condition,* the cells were covered with 3 mL of culture media; in *Holder condition*, 3mL were added under the membrane and 2 mL over the cells. Finally, in *Bioreactor condition*, the bottom chamber was filled with 9 mL of media and 2 mL were added on the top of the membrane. Cell culture media was changed after 2 days. At day 5 of culture, the Alamar Blue assay was proceeded as mentioned in section 4.5.1.1. At the end of the culturing period, cells were fixed and stained with DAPI and Phalloidin as described in section 4.5.1.2. The samples were assessed with confocal microscope images.

4.5.2.2 Results

After 5 days of culture, A549 cells cultivated in the three different mentioned conditions (*Control condition*, *Holder condition* and *Bioreactor condition*) were fixed and stained with DAPI and Phalloidin. Figure 3 shows that cells grew well in all the three conditions, attesting that the holder and bioreactor materials are biocompatible and promote cell growth.

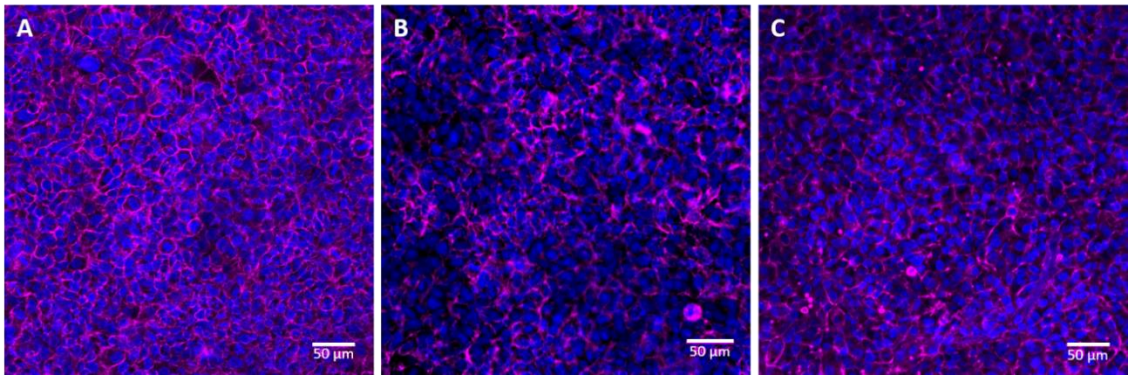

Figure 3: Confocal images of A549 cells cultured for 5 days and stained with DAPI (blue, nuclear stain) and phalloidin (magenta, actin fibers). A) Control condition, B) Holder condition and C) bioreactor condition. Magnification 20X.

Furthermore, to assess the metabolic activity of the A549 cells, the Alamar Blue assay was performed (Figure 4). Viability resulted slightly higher in the bioreactor than the holder, even if the sample in the bioreactor was also fixed in a holder. This difference could be due to the heterogeneity of the electrospun membranes, which could have a different porosity. In fact, during electrospinning, fibers deposit randomly on the collector, which results in slight variations between samples .

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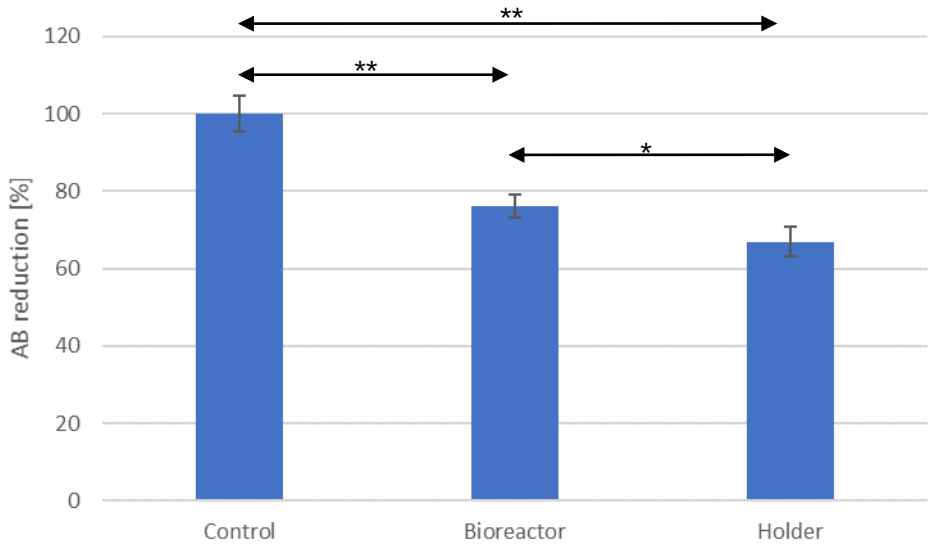

*Figure 4: Percentage of Alamar Blue reduction respect the control after 5 days of culture. * p < 0.01, ** p < 0.0001.*

4.5.3 Dynamic cell culture experiments with DALI system performed at AMI

Bioreactors are often used in advanced *in vitro* models because they can provide cells with an appropriate pattern of physical and chemical stimuli that better reproduce the physiological environment [1]–[7]. In the DALI system, the presence of media flow and membrane stretching are the physiological elements that are addressed. Cell culture media flow enhances deliver essential nutrients and oxygen to the cell culture compared to static culture. Additionally, the flux of cell culture medium mechanically stimulates the cultured cells by providing shear stress. On the other side, different studies underline the strong influence of mechanical forces on lung cells and tissues, especially on growth and repair metabolic pathways, surfactant release, injury, and inflammation. For example, mechanical stretch of cultured type II cells was shown to stimulate changes in surfactant secretion [8]–[10], cell injury or death [8], [11]–[13], permeability [14]–[17], and cell migration [18]. Hence the importance of design a system able to apply physiological and pathological levels of stretching.

During the training period at AMI, the effects of the cell culture media flow and mechanical stretch were evaluated in the bioreactor, cultivating A549 cells in three different conditions:

- *Control condition*: cells seeded on a 6-well Transwell insert. This is the 'gold standard'.
- *Flowing condition*: cells seeded on a membrane placed within the bioreactor. Cell culture media flow is present, but no membrane stretching.
- *Breathing condition*: cells seeded on a membrane placed within the bioreactor. Both cell culture media flow and membrane stretching are present.

As before, samples were prepared cutting a 24-mm diameter disc from a sheet of the electrospun material. Before cell seeding, membranes were fixed in their holder and sterilized, dipping the samples in 70% ethanol for 15 minutes, washing them 2 times with PBS, and exposing to the UV rays for 15 minutes each side (as instructed by the supplier). For both the insert and the membranes, a cell density of $0.24X\ 10^6$ cell/cm^2 was used, as per protocols established at AMI. Considering *Flowing* and *Breathing condition*s, cells were seeded facing the apical side of the bioreactor. Two experiments were performed: the first consisted in comparing the *Control* with the *Flowing condition*, in order to evaluate if the cell culture media flow maintain the integrity of the cell monolayer; the second consisted in comparing all the 3 culturing conditions, to evaluate also the effect of the mechanical cyclic strain.

As mentioned, in the first experiment *Control* and *Flowing Conditions* were compared. A549 were seeded on a Transwell insert and on a membrane placed within a bioreactor. Then, the bioreactor was connected to the hydraulic circuit as shown in Figure 5 the reservoir was filled with 35 mL of medium and flow was applied at a rate of 400 µL/min (maximum flow rate used in the work of Giusti *et al.* [19]), using a peristaltic pump.

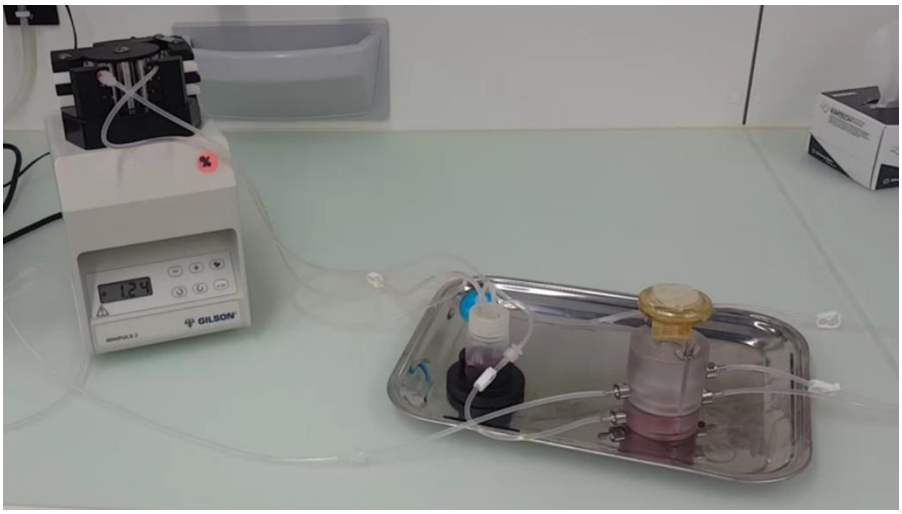

Figure 5: Experimental setup of the Flowing condition, showing the peristaltic pump, the reservoir and the bioreactor.

This dynamic regimen was maintained for 1 day. In particular, cells were cultured for 2 hours in submerged conditions (*Control condition*: 3 mL basal and 2 mL apical, *Flowing condition*: 9 mL in the bottom chamber, 2 mL apical), then the medium in the apical sides of the Transwell and bioreactor was removed to establish the air-liquid condition. After 24 hours, we evaluated cells viability with Trypan blue, a dye used as stain to selectively colour dead cells blue. Live cells with intact cell membranes are not coloured, since Trypan blue is not absorbed; however, it crosses the membrane in a dead cell. Hence, dead cells appear as a distinctive blue colour under a microscope, and it is possible to know the viability of the sample counting the number of coloured cells. A high cell viability was found both for *Control* and *Flowing* experiments, as shown in Table 1. This means that cell culture media flow did not cause a detachment of the cell layer from the membrane, not even during the first period of cell culture, when cells are not confluent.

Table *1: Results of the viability assay performed with the Trypan blue, comparing Control and Flowing conditions.*

	Live cells	**Dead cells**	**Tot. cells**	**Viability**
Control cond.	$6.8 \cdot 10^5$	$7.1 \cdot 10^4$	$7.5 \cdot 10^5$	91%
Flowing cond.	$8.2 \cdot 10^5$	$8.6 \cdot 10^4$	$9.0 \cdot 10^5$	91%

Having verified that the cell culture media flow did not have a negative effect on the cell culture, another experiment comparing the three culturing conditions (*Control*, *Flowing* and *Breathing condition*) was performed. A549 were seeded on a 6-well Transwell insert and on 2 membranes (top side) fixed in their holders and placed

within a 6-well plate. Considering *Control condition*, cell culture media was changed every 2 days. Cells were cultured for 1 day in submerged conditions (3 mL basal, 2 mL RPMI apical), then the holders were transferred to the bioreactors filled with fresh media, and liquid was removed from the apical side to establish the air-liquid interface. At this point, also for the control was established the air-liquid interface. After 24 hours of air-liquid static condition, the membranes in the bioreactor were split in two condition: stretched and not stretched. The setup was the same as the previous experiment: the bioreactors were connected to a fluidic circuit consisting of a reservoir filled with cell medium and a peristaltic pump for media flow; Considering *Breathing condition,* the bioreactor was connected also to the pressurized air circuit as shown in Figure 6.

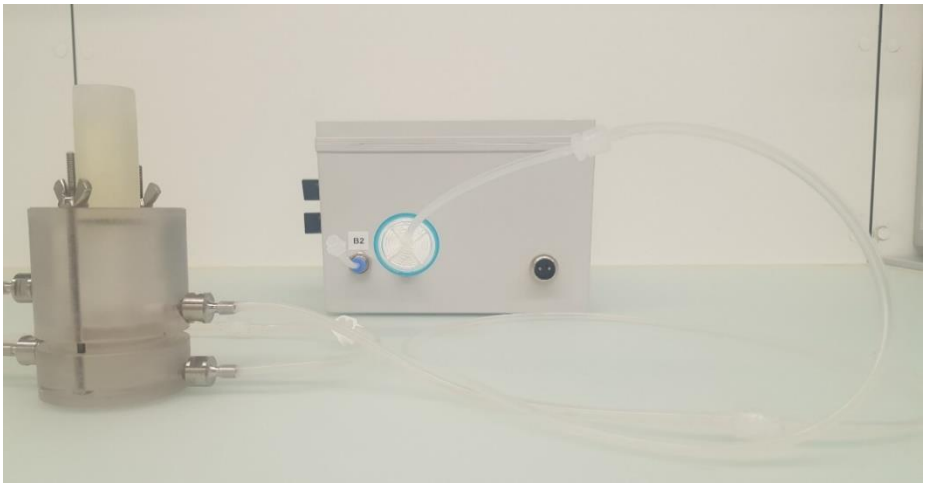

Figure 6: Bioreactor connected to the pressurized air circuit for the membrane stretching.

The stretching magnitude was set to a 5% linear strain, corresponding to the normal breathing condition [20], and maintained up to 3 days in air-liquid dynamic conditions. During the culturing period, the medium within the bioreactors (both for *Flowing* and *Breathing conditions*) was not changed because the 35 mL within the reservoir combined with the fluidic flow were enough to guarantee fresh medium to the cells. After 3 days, the stimuli were stopped, and the membranes removed from the bioreactors. Samples were fixed and stained with DAPI and Phalloidin. Morphological analysis with confocal images were used to evaluate the effect of stretching, related to the *Flowing* and *Control conditions*. As shown in Figure 7, the images demonstrate that for *Control* and *Flowing conditions* (A and B respectively in Figure 7) cells were able to proliferate on the membrane retaining their typical morphology and forming a homogeneous cell layer. However, Figure 7 C shows that the sample cyclically

stretched was not able to sustain a homogeneous cell layer on the substrate, even though the some cells still remained adherent on the membrane.

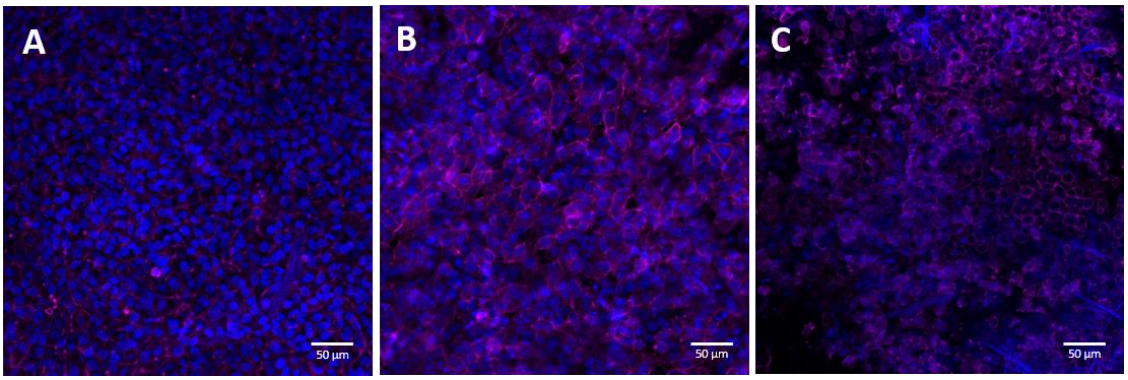

Figure 7: Confocal images of A549 cells cultured for 5 days and stained with DAPI (blue, nuclear stain) and phalloidin (magenta, actin fibers). A) Control condition, B) Flowing condition and C) Breathing condition. Magnification 20X.

This could be due to an excessive strain applied to the cells during the cyclic stretching, meaning that the cell culture protocol still needs to be optimised. Surface treatment of the membrane could be performed, in order to enhance the adhesion of the cells to the substrate, especially when they are cyclically stimulated and more easily subject to detachment. Alternatively, other membrane compositions or materials with similar mechanical properties could be employed in the DALI system.

4.6 Conclusion

Biological studies demonstrated the cytocompatibility of the materials used for fabricating the bioreactor and the membrane holder (PC and PDMS). These experiments proved that the device was able to maintain a sterile environment during cell culture. Moreover, the materials do no affect cell vitality (Figure 2); in addition, confocal images showed that cells formed a homogeneous monolayer on the substrate (*Holder* and *Bioreactor conditions* in Figure 3). Morphological analysis with confocal images demonstrated also that, during the *Flowing condition*, cells were able to proliferate on the membrane retaining their typical morphology and began to form a compact cell layer. This suggests the suitability of the system for the culture of epithelial cell at air-liquid conditions under flow. Finally, biological experiments suggest the feasibility of the system for stimulating epithelial cells. Even though an optimisation of the cell culture protocol still needs to be performed, the DALI bioreactor can provide cyclic stretch during the entire culture period, demonstrating its reliability from an engineering point of view.

5 References

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