# uBeat® Platform Setup

# User Guide

BiomimX

The purpose of this document is to provide guidelines for first time users. This user manual covers the setup and operation of uBeat® Platform.

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## 1. Who is BiomimX®

**BiomimX®** is a pioneer in the development of predictive models of human organs and pathologies that will revolutionize the way to test new drugs. By integrating 3D cell culture and mechanical stimulation, BiomimX® proposes a new generation of beating Organs-on-chip recapitulating with unprecedented precision the mechanically active human microenvironment.

## 2. Platform description

**uBeat® Platform** supports the culture of three biologically independent 3D microtissue models within a single device. uBeat® Platform is able to provide a controlled mechanical stimulation to cell constructs by means of the patented uBeat® technology (see section 3 for further details). Specifically, uBeat® Platform features hanging pillars that confine the cell-laden hydrogel in the central part of the culture chamber (*central channel* in Figure 1). The central channel is flanked by two *lateral channels* for culture medium support. The geometrical characteristics of the pillars identify two different product types namely uBeat® Stretch and uBeat® Compress.

Each culture chamber of uBeat® Platform is equipped with:

- 2 ports for cell-laden gel injection (*Inlet and Outlet* in Figure 1),
- 4 reservoirs for culture medium replacement/biochemical conditioning (*Medium reservoirs A, B, C, D* in Figure 1), connected with the lateral culture medium channels within the culture chamber.

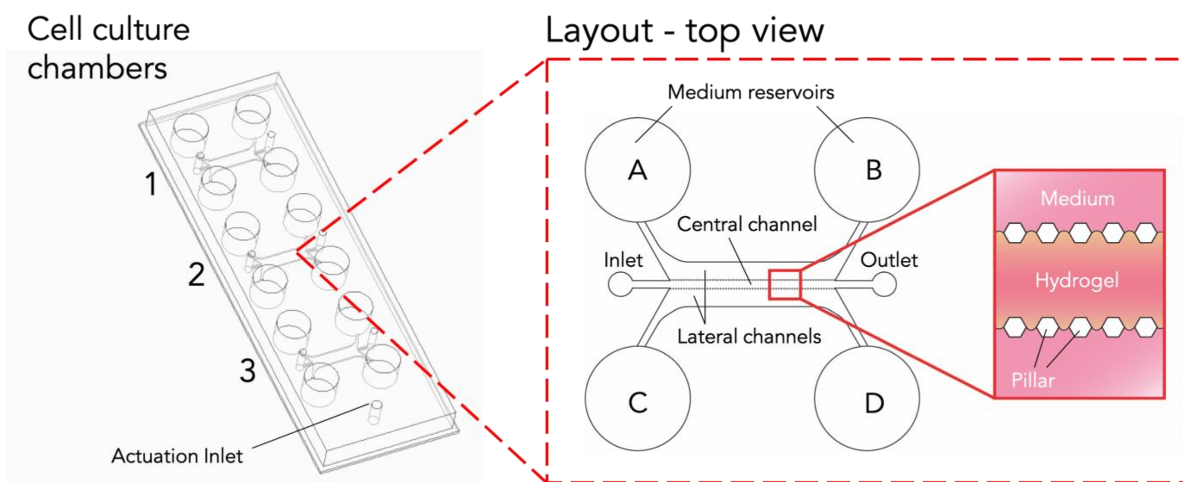


Figure 1 – a) uBeat® Platform houses three biologically independent microtissues **(1,2,3).** The actuation inlet is located at the extremity of the platform. b) Top view sketch of a cell culture chambers with representative hexagonal - shaped hanging pillars.

## 3. uBeat® Technology

The core technology of BiomimX®, **uBeat®**, provides 3D cell constructs with a controlled and tunable mechanical stimulation. uBeat® brings the *in vivo* mechanical microenvironment into your *in vitro* models, allowing to recapitulate human organs' function[1,2] and pathological states[2,3] with an unprecedented level of precision and accuracy.

Depending on the uBeat® Platform product type, the mechanical stimulation can be:

- **Confined compression** (uBeat® Compress);
- **Uniaxial stretching** (uBeat® Stretch).

The stimulation pattern can be personalized by means of a control system connected to the platform and to a compressed air source: **uBox** (see section 8 and 17 for further details).

uBeat® Compress and uBeat® Stretch are available with different stimulation intensities:

- **uBeat® Compress10**: 10% confined compression;
- **uBeat® Compress30**: 30% confined compression;
- **uBeat® Stretch10**: 10% uniaxial strain.

## 4. Content of Starting package

The Starting package includes:

- uBox package
- 3 uBeat® Platform kits (9 mini-kits)

### Content of uBox package

- uBox
- uBox instructions
- uBox connecting pieces
- uBox tools kit: uBox power supply, 4x three-way stopcocks, uBox pneumatic tube with control valve

### Content of uBeat Platform kit

uBeat Platform Kit includes 3 mini-kits, as shown in Figure 2.

### Content of mini-kit

- 4x sterile uBeat® Platforms. (Each uBeat® Platform is provided with a serial number, which you should keep track of)
- 1x sterile uCase
- Tools (4x 0.2µm sterile filters, 4x sterile caps)
- uBeat Platform mini-kit instructions

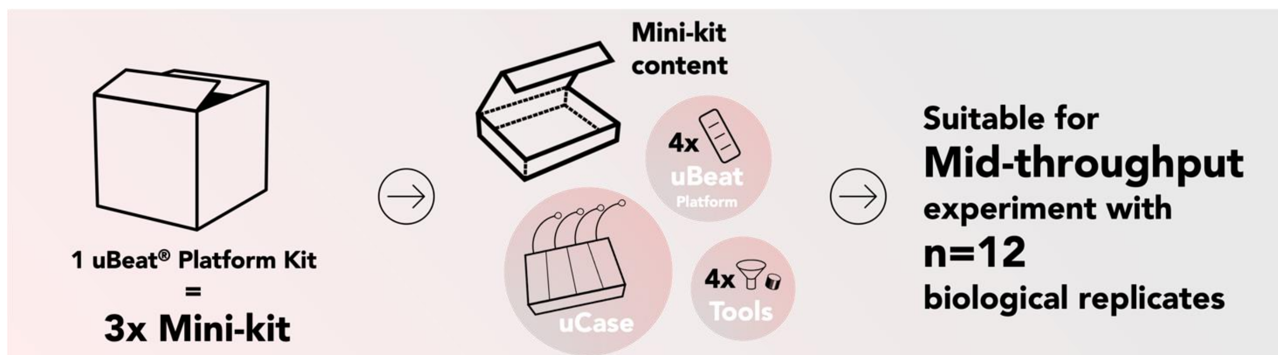


Figure 2 – Content of a uBeat® Platform Kit

### uBeat Platform Trial Kit

If your order includes the uBeat Platform Trial Kit, please refer to Appendix C for its description and protocols.

## 5. Content of additional kit

Each uBeat® Platform additional kit includes 3 mini-kits (see Figure 2).

### Content of mini-kit

- 4x sterile uBeat® Platforms. (Each uBeat® Platform is provided with a serial number, which you should keep track of)
- 1x sterile uCase
- Tools (4x 0.2µm sterile filters, 4x sterile caps)
- uBeat Platform mini-kit instructions

## 6. Site requirements

In this section you find the technical site requirements for the usage of BiomimX Setup presented in this User Guide.

| | |
|---|---|
| Voltage required for power supply * | 110-120V, 60Hz or 220-230V, 50Hz |
| Compressed air type | Just compressed air. Not Nitrogen, not Oxygen, not $CO_2$ |
| Compressed air pressure level | Min: 1 bar (14.5 psi) Max: 1.8 bar (26.1 psi) |
| Compressed air tubing diameter | ¼” or 3/8” or ½” are all compatible |
| Distance uBoX – incubator ** | Max: 4 m |
| Distance uBoX – Compressed air source | No limitation |

(*) please note that the power supply is provided by BiomimX

(**) please note that uBoX should be located on a stable plane outside the incubator. The ideal positioning would be on the opening side of the incubator door.

System parts' dimension:

- uBoX: L 16cm, W 15cm, H 10.5cm
- uBoX tubing (connects uBoX to uCase), it is available in 3 dimensions on demand:
    - Small: 1m
    - Medium: 2m
    - Large: 4m
- uCase: multiwell plate standard size

The user must provide an installation laboratory that meets the following requirements:

- Laminar flow hood;
- Pipettes (e.g., 10µl, 20µl, 200µl and 1000µl) with corresponding sterile tips;
- Sterile syringes (suggested volume 1ml);
- $CO_2$ incubator;
- Compress airline or portable compressor;
- Tube to connect uBoX and compress airline;
- Sterile tweezers;

## 7. Unpacking instructions

a) Inspect the exterior of the packaging for possible damages. If any sign of damage is present, please contact the responsible shipping company.
b) Remove all the components (i.e., uBox box and mini-kit boxes) from the shipping box and lay them out on a stable and horizontal surface. Keep the original shipping boxes in case you need to return the system for servicing or repair.
c) Inspect all the components for possible damages or defects. If any sign of damage is present, please contact us and the responsible shipping company.
d) To preserve sterility, please store uBeat® Platforms in their original envelop at room temperature.

## 8. uBox installation

uBox is the system able to provide pressurized air to uBeat Platforms and allows the operator to choose among preset mechanical stimulation patterns (i.e., frequency, duty cycle and on-off timing, reported in section 17).

uBox needs to be connected both to a compressed air source and a power source; the following list shows all the components needed to assemble the whole system:

a) Power supply (provided with uBox).
b) Compressed air source in close proximity to cell culture incubator, better if including a pressure regulator.
c) uBox connecting pieces: 3/8"-1/4" adapter, 1/2"-1/4" adapter, ¼" tubing, three cable ties, male luer connector (provided with uBox).

## HOW TO ASSEMBLE THE SYSTEM

**Notice**: Before starting the experiment, we recommend installing the uBoX system to ensure everything is functioning properly.

**Notice**: Never start a uBoX program without connecting it to the compressed air source. Proceed according to the following instructions for the proper uBoX installation.

First, uBox must be placed next to the incubator, better on the side where it opens, and nearby a power source. **When connecting uBox, be sure that the compressed air is off.** Tubing must be long enough to connect uBoX to the compressed air source (e.g., 1 – 6 m) and should be arranged so to avoid the risk of lab operators being hampered.

a) Connect a tube (e.g., 1 – 6 m long, 3/8" diameter) to the compressed air source and ensure a tight connection. If necessary, use the uBox connecting pieces we provided to adapt the diameter of your tubing. This tube must end with the male luer connector that we provide within uBox connecting pieces (Figure 3, a).
b) Connect the tube ending with the male luer connector (tubing 'a' in Figure 3) to the 'IN' port of uBox.

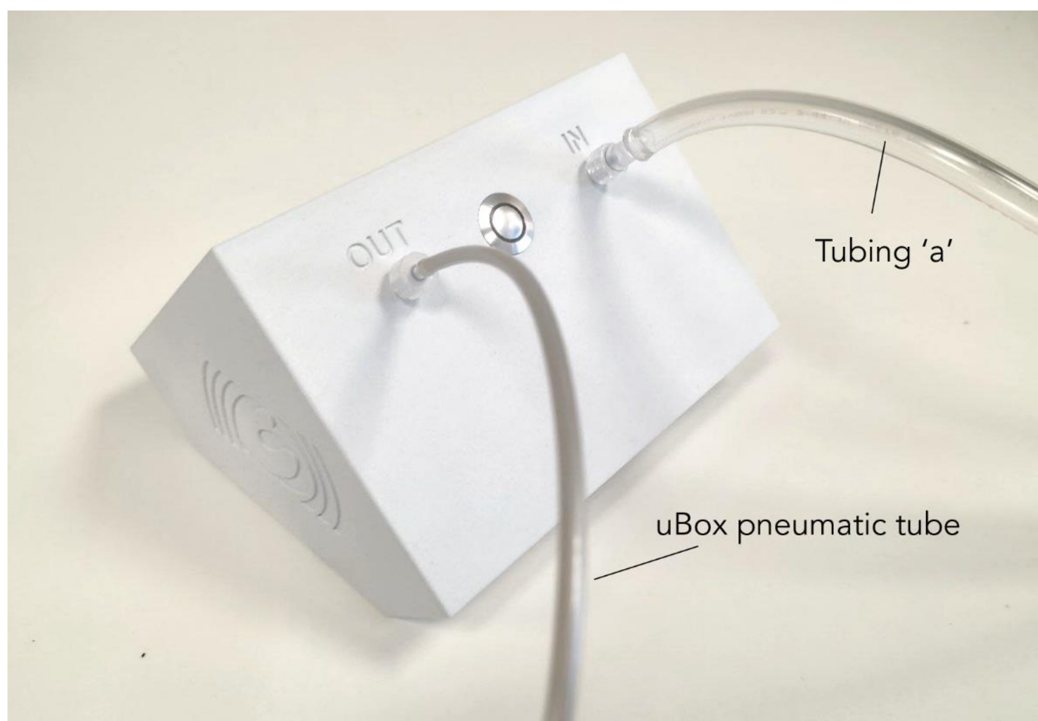


Figure 3 – uBox tubing connections. 'a' is the tube ending with a male luer that connects the compressed air source to the inlet port of uBox. 'uBox pneumatic tube' is the tube which connects uBox to the uCase and it is provided within the kit.

Now that uBox system is installed, you can proceed.
From now on procedures and protocols will be described considering a mini-kit as a reference.

# 9. Standard procedure for cell seeding – Mini-Kit

a) Unpack the uCase envelop in a laminar flow cabinet in sterile conditions and place it in front of you.

b) Unpack each uBeat® Platform from its bag and transfer it into the uCase by means of sterile tweezers. **Remember to keep track of the serial number of each device**: we suggest you write it on the lid of uCase with a marker. The Actuation Inlet of each platform shall face towards the tubing (as shown in Figure 4), but do not connect them yet.

c) Pair each needle of the uCase with the caps provided within the mini-kit to keep a sterile environment.

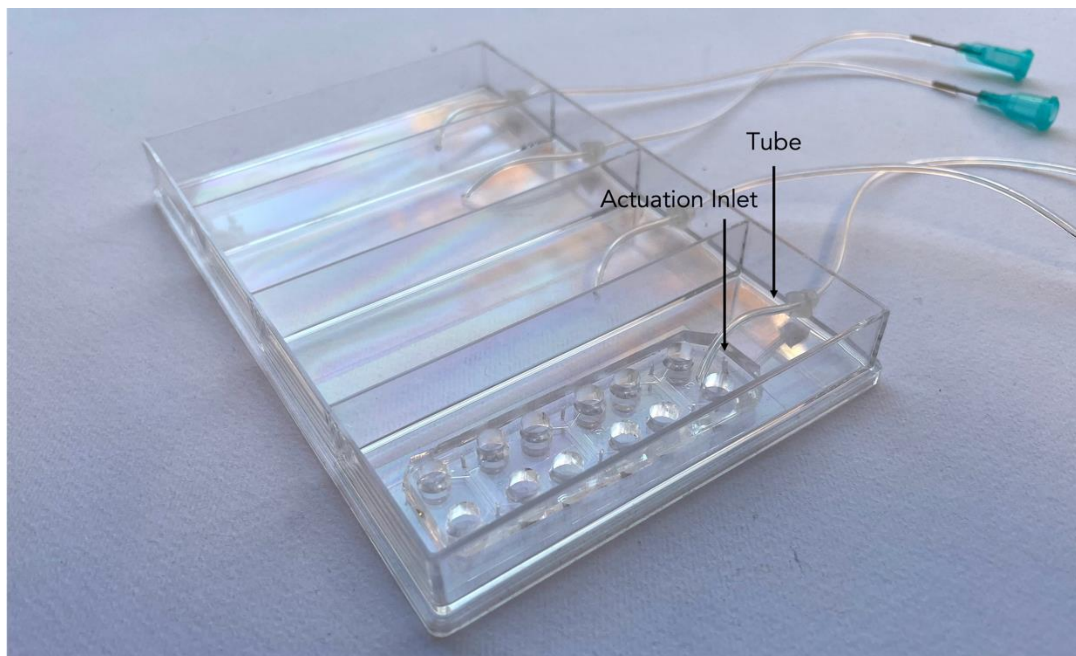


Figure 4 – Proper positioning of uBeat® Platform within a uCase compartment.

d) As summarized in Figure 5, prepare your favourite cell-laden hydrogel to be inoculated in the device: a total solution of 10µl is enough to seed one uBeat® Platform. Adjust cell density (cell density that has been tested in our models ranges between 10 and 100 M cells/ml) and hydrogel concentration according to your specific application (an example for cartilage formation in fibrin gel is provided in section 16).

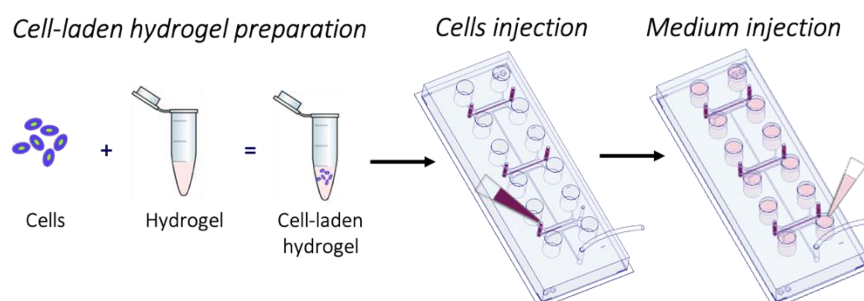


Figure 5 – Schematic process to seed cells into uBeat® Platform

Depending on the cross-linking time of the selected hydrogel, larger hydrogel's aliquots may be prepared. For hydrogels with cross-linking times >10 minutes, a cumulative aliquot (i.e. 30µl, enough to seed 3 devices) can be prepared. For hydrogels with cross-linking times <10 minutes, we recommend preparing single 10µl aliquots at a time to avoid undesired polymerization while injecting into the device.

e) Seed all the three cell culture chambers by slowly injecting the cell-laden hydrogel solution through the inlets by using a pipette tip (see Figure 6).

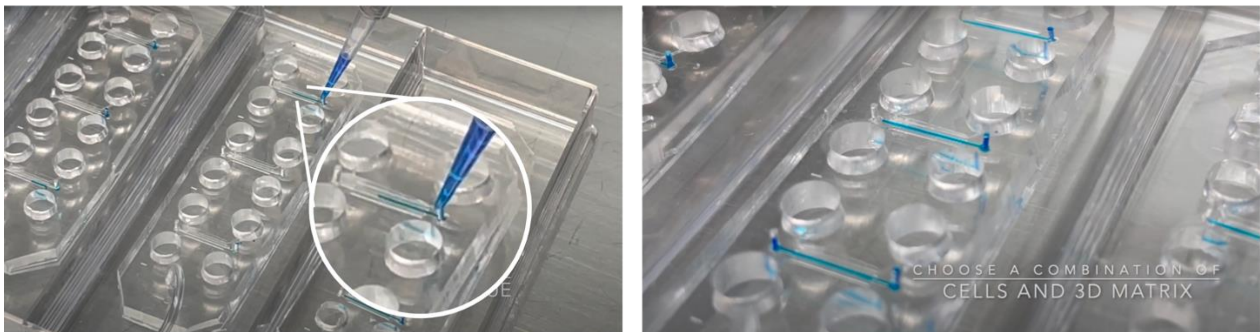


Figure 6 - Hydrogel injection procedure. A blue hydrogel advancing in the central channel of a cell culture chamber (left) and blue hydrogels filling all three cell culture chambers of a uBeat Platform (right).

f) Check under an optical microscope the outcome of the seeding. As shown in Figure 7, the seeding is successful when the cell-laden hydrogel is confined between the two rows of pillars, without leakages towards the lateral channels. Leakage may occur when an excess of pressure is applied while injecting the hydrogel solution inside the platform. Indeed, we suggest maintaining the same pressure on the pipette while injecting the cell-laden hydrogel in each channel.

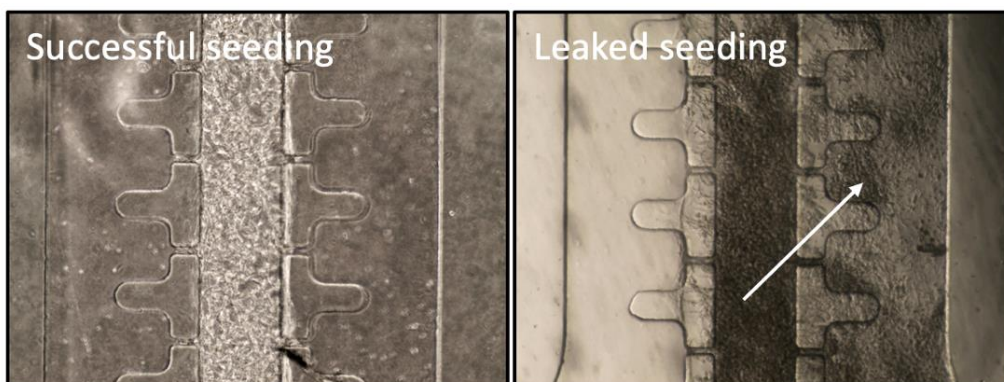


Figure 7 – Examples of successful and leaked seeding.

During injection, you can visually check that the hydrogel solution is confined in the central channel, between the two rows of hanging pillars by slightly tilting the uCase under the light (see Figure 8).

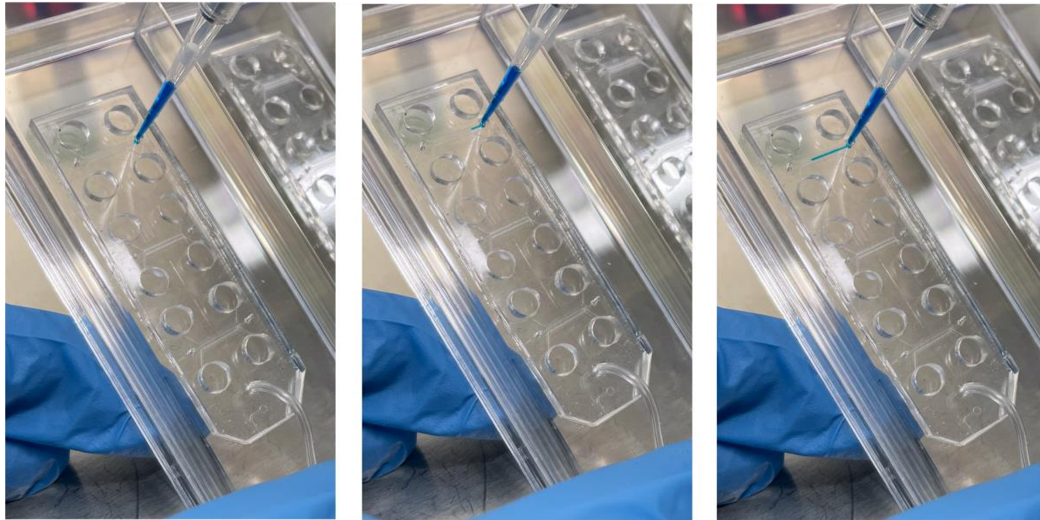

Figure 8 – uCase handling during injection. Slightly tilting the uCase can help visualize the injection.

g) Allow the gel to cross-link as from the specific procedure for the selected hydrogel.

Put some PBS drops around the device inside the uCase during this process, to compensate for evaporation, especially if the cross-linking occurs at 37°C inside the incubator.

h) Once the hydrogel is cross-linked, add the appropriate cell culture medium in the medium reservoirs by using a 200µl pipette tip. By directing the tip in the correspondent channels, force the medium from reservoir A (Figure 1) until you see medium exiting in reservoir B (or vice versa). Repeat the operation from reservoir C to D. Once the entire lateral channels are filled, add more medium to fill each reservoir. Please consider using 300µl of medium culture/chamber.

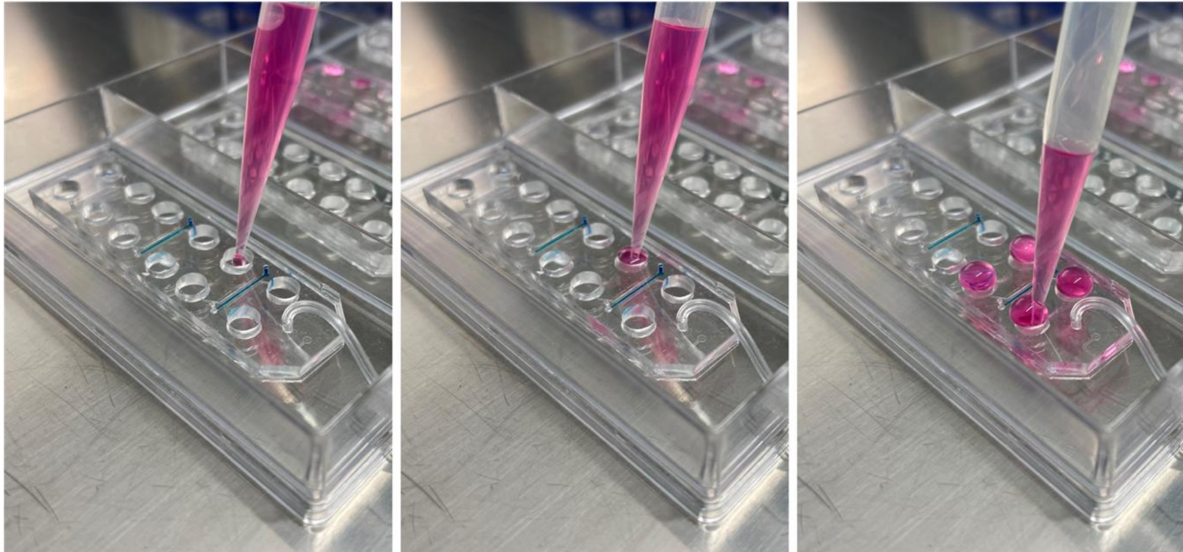

Figure 9 – Medium reservoirs' filling with 1000µl pipette.

In case the medium injection in lateral channel results difficult, as an alternative, you can cut under sterile conditions a 1000µL pipette tip to fit the reservoir diameter and force the medium inside the lateral channels by applying a positive pressure as depicted in Figure 10. As described before, force the medium into reservoir A to go to B (or vice versa) and into reservoir C to go to D.

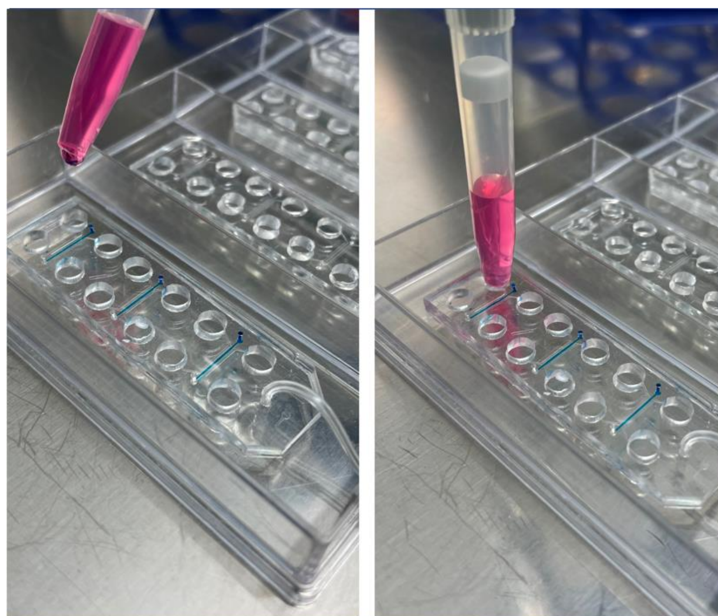

Figure 10 – Medium injection in the lateral channels using a truncated 1000 µl pipet tip.

i) Incubate uCase containing uBeat® Platforms in a humidified incubator at 37 °C and 5% $CO_2$.

j) At this point, you can either pre-culture the microtissues in uBeat® Platforms in static condition or start immediately the desired mechanical stimulation (instructions provided in the next sections 10 and 11), depending on your experimental setup.

k) Change the medium with a frequency depending on the specific application. In section 16, we provide a possible protocol for the generation of a cartilage construct in fibrin gel.

## 10. STANDARD PROCEDURE FOR ACTUATION CHAMBERS FILLING

a) For all the uBeat Platforms that need to be mechanically stimulated, un-pair the corresponding cap from the uCase needles in a sterile environment.

b) Fill a sterile syringe with sterile PBS (e.g., 1 ml) and pair it with one of the needles exiting from the uCase (Figure 11, a).

c) Inject the PBS through the tube until it reaches the end (Figure 11, b).

d) By helping with sterile tweezers, fit the tube extremity inside the actuation inlet of the corresponding uBeat® Platform (Figure 11, c).

e) Once the tube is connected to the uBeat® Platform, unpair the syringe and repeat the previous steps (a-d) for all the remaining uBeat® Platforms that need to be mechanically stimulated.

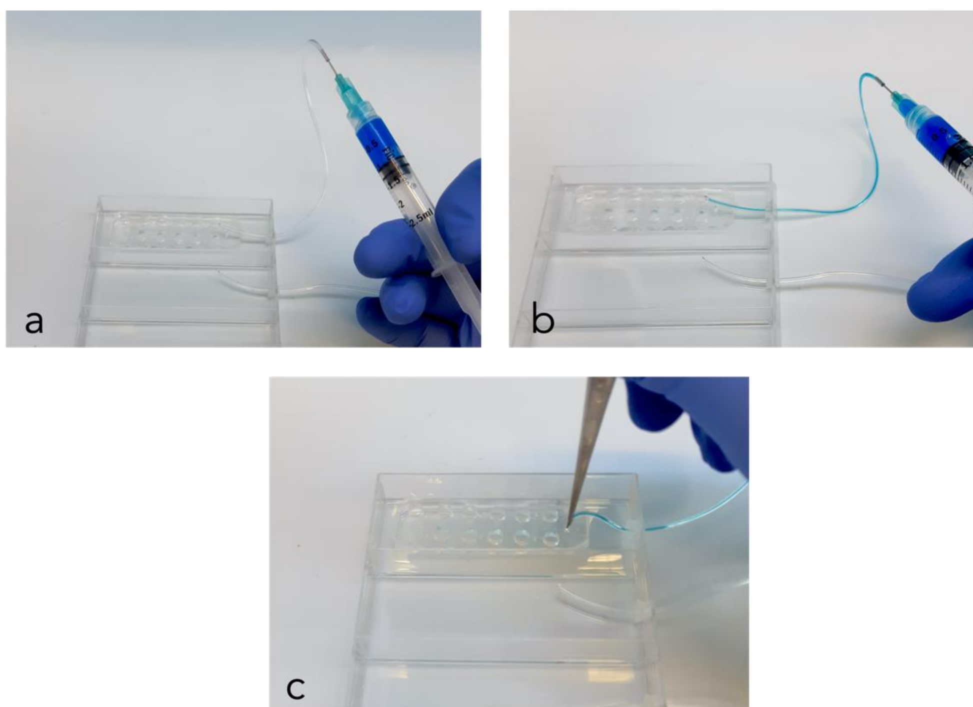


Figure 11 – Standard procedure for actuation filling. a) Use a syringe to aspirate PBS (represented in blue in the picture) and then pair it with one of the needles. b) Inject PBS until it reaches the tube extremity. c) Insert the tube in the actuation inlet.

f) When all the tubes have been plugged, pair each needle with a sterile filter (provided within the mini-kit) and a three-way stopcock (provided within uBox tools kit) as shown in Figure 12.

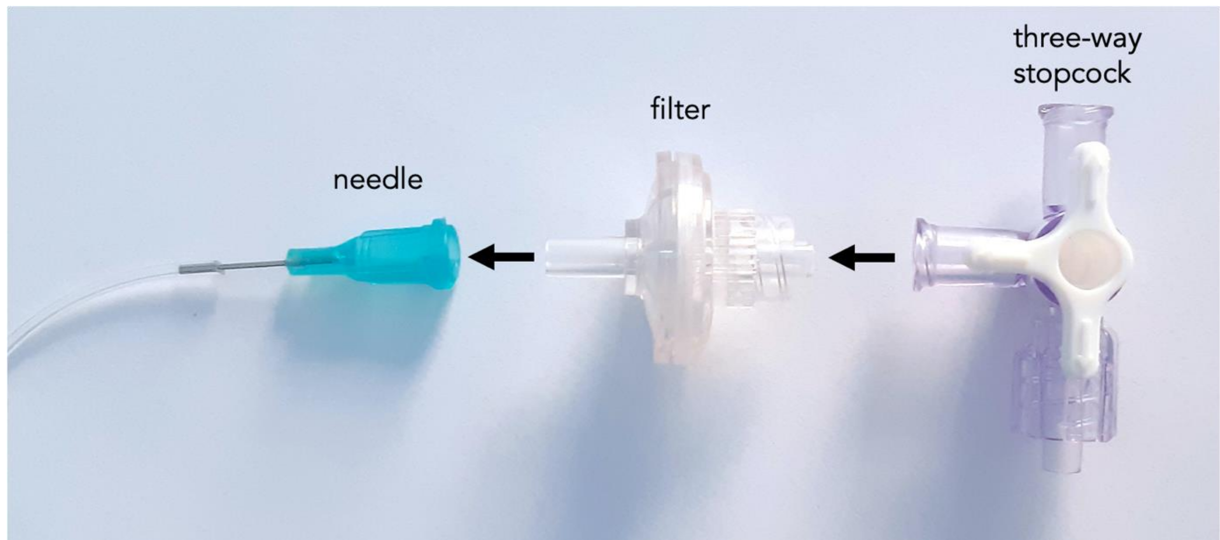


Figure 12 – Assembly of needle, filter and three-way stopcock.

g) Repeat this procedure for each needle, then connect the four stopcocks to create a ramp (pay attention to close the ramp at one extremity as displayed in Figure 13).
h) Incubate the uCase in a humidified incubator at 37 °C and 5% $CO_2$.

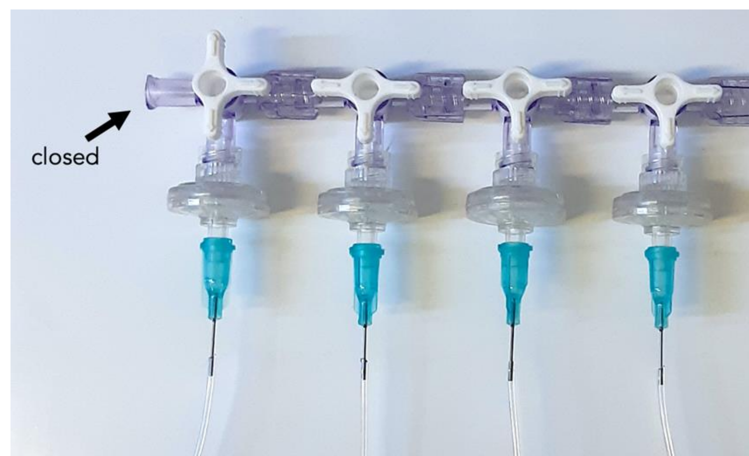


Figure 13 – Assembly of the ramp. Pay attention to set the valves as displayed in figure, so that air can pass through each needle.

After having prepared the uCase (see section 10) and installed uBox (see section 8 ), all you need to do is to connect them to each other.

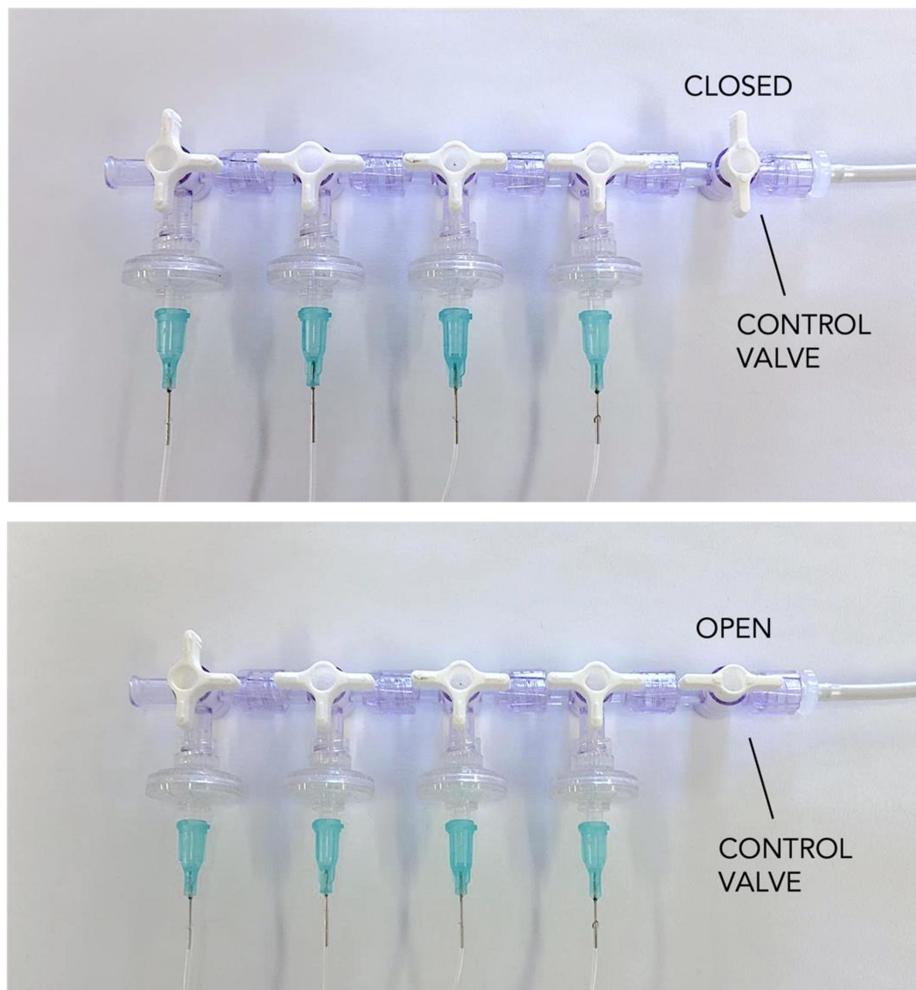


Figure 14 – Assembly of the ramp with the uBox pneumatic tube, to connect uCase with uBox: closed configuration (top) and open configuration (bottom).

a) As displayed in Figure 14, connect the ramp of the uCase to the uBox pneumatic tube, which ends with a CONTROL VALVE to allow/block the access to the air flow. **Be careful, the CONTROL VALVE (Figure 15, in red) must remain closed (Figure 14, top) during this setup.** It must be opened only during the filling and the stimulation stages.

b) Connect the uBox pneumatic tube (see Figure 15) to 'OUT' port of uBox and close the incubator. This tube is meant to pass through the incubator door, so pay attention not to accidentally occlude it.

c) Open the compressed air valve and (if present) set the integrated pressure regulator at 1 bar (see Figure 15, *integrated pressure regulator*). **Remember to keep the CONTROL VALVE closed during this phase.**

d) Turn on uBox, start the FILLING PROGRAM (see section 17), and open the CONTROL VALVE. **We suggest to run the FILLING PROGRAM twice to ensure the actuation compartment to be completely filled.**

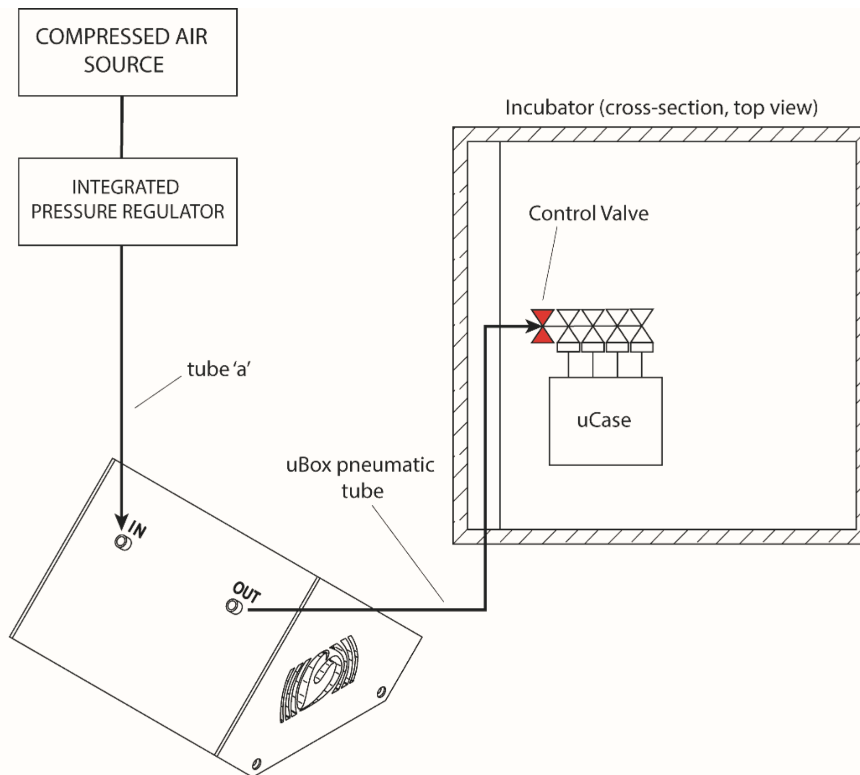


Figure 15 – Schematic view of uBox system, which displays all the connections.

e) Once the filling is completed, close the CONTROL VALVE.

f) Disconnect uCase from the uBox pneumatic tube and check under the microscope if bubble formation occurred during the filling process.

g) Check the lateral channels of each chamber of the chips and if some bubbles are stuck inside, proceed as it follows:

- remove the medium from the reservoirs;
- Force the fresh medium into the channels and fill the reservoirs as explained in section 9, h).

h) Place the uCase inside the incubator, connect it with the uBox pneumatic tube and set the desired stimulation program (**check section 17 to see which programs are available**).

i) Open the CONTROL VALVE.

## 12. Standard procedure for medium change

Frequency of medium change depends on the specific application. In **section 16**, we provide a possible protocol for the culture of cartilage construct in fibrin gel.

a) Keep the stimulation program going on uBox (don't press 'STOP'), close the CONTROL VALVE.

b) Disconnect the uCase from the uBox pneumatic tube and bring it inside the biological hood.

c) Remove the culture medium from all reservoirs.

Do not use vacuum to avoid the introduction of undesired air bubbles in the lateral medium channels.

d) Fill each culture chamber with 300µl of fresh culture medium (0.9ml of medium is enough to fill one uBeat® Platform).
e) Incubate the uCase containing the uBeat® Platforms in a humidified incubator at 37 °C and 5% $CO_2$ and connect it with the uBox pneumatic tube.
f) Open the CONTROL VALVE to proceed with the stimulation program.

## 13. How to stop a single device for general analysis

According to your experiment, it could happen that you want to select one or more devices to be stopped for an end-point analysis (e.g., PCR, Immunofluorescence), while keeping the remaining devices present in the uCase in culture. If this is your case, please follow the protocol listed below:

a) Close the CONTROL VALVE.
b) Disconnect the uCase from uBox pneumatic tube.
c) Bring the uCase inside the biological hood. By helping with sterile tweezers, remove the tube from the actuation inlet of the device/s you have chosen and transfer it/them into sterile Petri for your analysis.
d) Close the specific stopcock/s of the device/s you have disconnected. **Remember: the airflow must be able to pass through the stopcocks of the remaining devices that you want to keep in culture, so pay attention on the way you arrange the stopcocks' valves (see a representative configuration in Figure 16).**

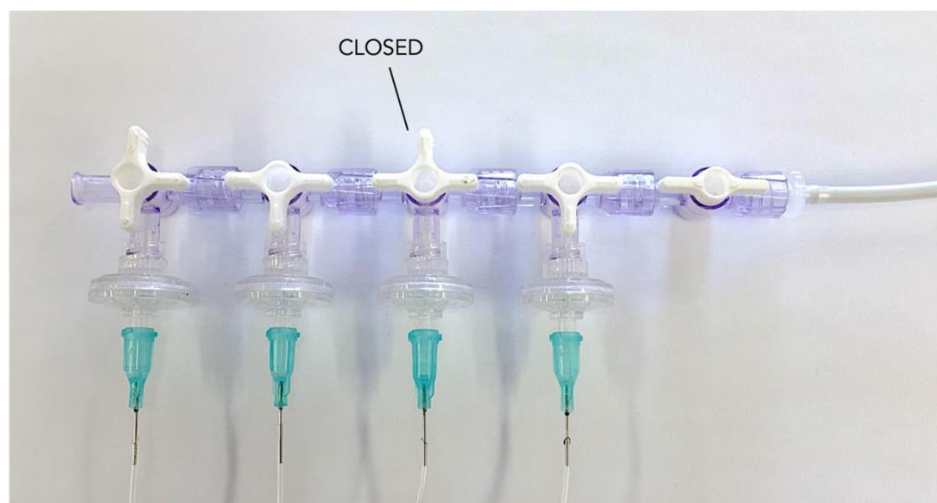


Figure 16 – A representative configuration where one of the stopcocks is closed (so the corresponding device can be disconnected) without blocking the airflow for the other devices.

e) Incubate the uCase containing the remaining uBeat® Platforms in a humidified incubator at 37 °C and 5% $CO_2$ and connect it with uBox pneumatic tube.
f) Open the CONTROL VALVE.

## 14. Standard procedure for collecting the material for qRT-PCR analysis

a) If you want to stop only some of the four devices present in the uCase and keep the others in culture, follow the indications listed in section 13. Otherwise, close the CONTROL VALVE, disconnect the uCase from the uBox pneumatic tube and bring it inside the biological hood.
b) Completely remove the culture medium from all reservoirs.

> Do not use vacuum to avoid the introduction of undesired air bubbles in the lateral medium channels.

c) Fill the reservoirs A and C (Figure 1) with PBS and wait 5 minutes to let it flow within the culture chamber.
d) Remove PBS from all reservoirs and repeat the operation at point c, this time filling reservoirs B and D (Figure 1).

> Do not use vacuum to avoid the introduction of undesired air bubbles in the lateral medium channels.

e) Remove the PBS from all reservoirs.

> If available, use the vacuum to completely aspirate the PBS from all the reservoirs and lateral medium channels.

f) Fill 3 Eppendorf tubes (volume 1.5ml) with 400 µl of TRI Reagent each. As an alternative, you can use a commercial RNA extraction kit, following the instructions for the buffer volume to prepare for this step. We have already used the following commercial extraction kits:

- **Total RNA purification micro kit, cat. 35300, Norgen.**
- **ReliaPrep RNA Cell Miniprep System, cat. Z6011, Promega.**
- **miRNeasy Mini Kit, Cat. No. / ID: 217084, QIAGEN.**

g) Disassemble the uBeat® Platform (as shown in the Figure 17) by carefully detaching the glass coverslide (1). Then, peel off the top layer (2-4). The gel will remain on the top layer entrapped within the two hanging posts rows.

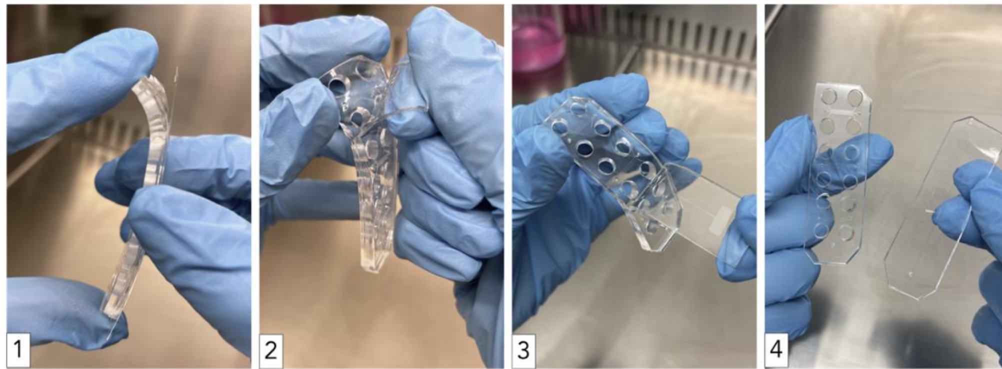


Figure 17 – Processing uBeat® Platform device to expose the micro-constructs in order to collect them for subsequent RNA extraction.

h) For each chamber, aspirate 5 µl of TRI Reagent (or the buffer of choice matching your specific selected RNA extraction method) from one Eppendorf tube and put it on the construct. Disrupt the sample mechanically with the pipette tip.

i) Aspirate the solution and drop it back to the corresponding original Eppendorf tube (the disrupted construct will turn white when in contact with TRI Reagent/buffer).

Samples can be used immediately or stored at -80°C.

If you decide to use a commercial RNA extraction kit, you can avoid opening the device as detailed in Figure 17 and directly inject the buffer in the medium channels. After washing with PBS (steps b-e), fill the medium reservoirs with the lysis buffer and incubate for 3 minutes at room temperature. At this point you should be able to observe the disruption of the micro-tissues under the microscope. Wait few more minutes if you are not sure about the outcome. Retrieve the buffer with the cells as it's explained in the caption of Figure 18. **Please consider that if you use this method there is the chance that some biological material gets lost, so we suggest to collect the material from 2 chambers as one single sample**.

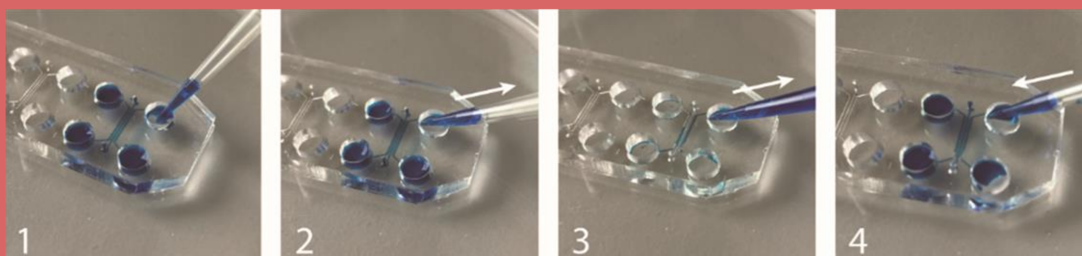


Figure 18 - Procedure for cell collection with lysis buffer (in blue) without openin the device. 1) Collect and transfer the buffer from one reservoir to the Eppendorf tube; 2) Position the pipette tip as shown in the figure and start aspirating from the channel inlet; 3) Keep aspirating until the chamber is empty; 4) Inject the buffer back into the chamber without moving the pipette tip from that position. Repeat three times the alternation aspirate-inject, then transfer the buffer with cells in the Eppendorf tube. To maximize the collection of cells, we suggest to repeat this procedure again from the beginning by filling all the reservoirs with buffer from the same Eppendorf tube and following steps 1 to 4.

## 15. Standard procedure for performing immunofluorescence analysis

If you want to stop only some of the four devices present in the uCase and keep the others in culture, follow the indications listed in section 13. Otherwise, close the CONTROL VALVE, disconnect the uCase from the uBox pneumatic tube and bring it inside the biological hood.

### *Fixation*

a) Remove the culture medium from the reservoirs.

> During all the staining procedure, do not use vacuum to avoid the introduction of undesired air bubbles in the lateral medium channels.

b) Fill the reservoirs A and C (Figure 1) with PBS and wait 5 minutes to let it flow within the culture chamber.

c) Remove the PBS from all reservoirs and repeat the operation at point b), this time filling reservoirs B and D (Figure 1).

d) Remove the PBS from all reservoirs.

e) Fill the device with Formalin/PFA at 4%. Put Formalin/PFA in reservoirs A and C (≈80µl/reservoir) and wait 5 minutes to let it flow within the culture chamber.

f) Remove the Formalin/PFA and repeat the operation at step e), this time filling reservoirs B and D.

g) Remove the Formalin/PFA from all reservoirs.

h) Fill all the 4 reservoirs with Formalin/PFA (≈80µl/reservoir).

i) Put the device at 4°C overnight, or according to your specific protocol.

j) Repeat the PBS washing described in b) and c).

k) Fixed uBeat® Platforms can be stored in the fridge at 4°C up to 4 months, but they have to be in a PBS bath (fill a Petri dish with PBS until uBeat® Platform is completely covered to avoid evaporation and close it with Parafilm).

To staining a dense matrix, like in the case of uKnee model, we suggest to open the device to expose the micro-tissues and drop all the solutions listed in the following paragraphs directly on them. More details are explained in the caption of Figure 19.

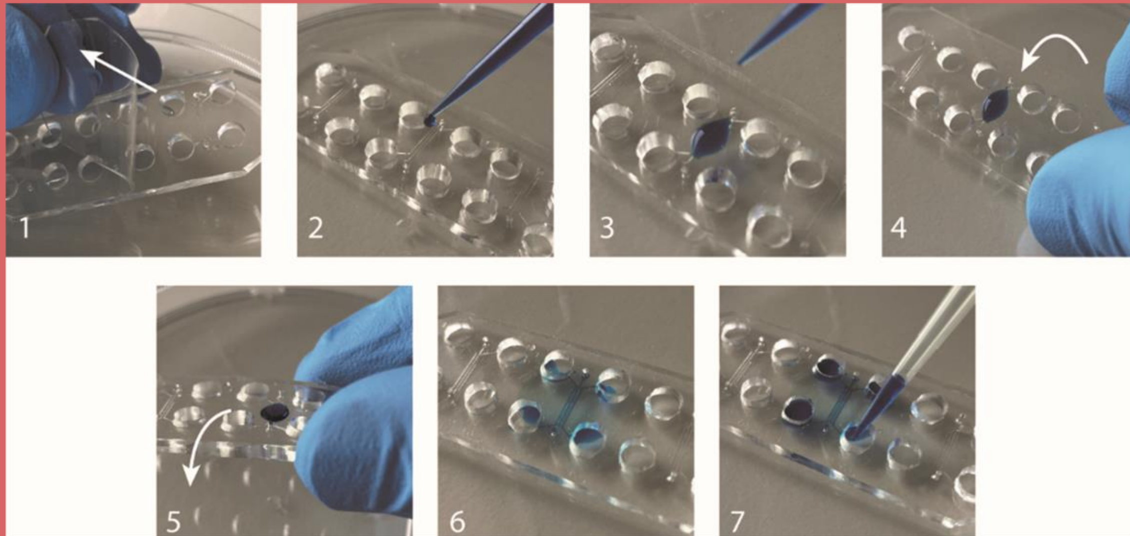


Figure 19 – Procedure for staining microtissues by opening the device. 1) Remove the glass coverslip and the PDMS membrane to expose the micro-tissues; 2-3) drop 20 µl of the solution (e.g., permeabilization sol., blocking sol., antibodies etc.) right on top of the microtissue; 4-5-6) flip the device and place it on a clean coverslide or on a clean Petri Dish surface; 7) Refill the rest of the solution in the reservoirs for the incubation.

### Permeabilization

Avoid this step if you are performing an extracellular staining.

a) Remove the PBS from the reservoirs.
b) Select the permeabilization solution according to the staining to be performed (e.g., Tween 0.2% v/v or Tryton-X 0.1% v/v). Fill reservoirs A and C (≈80µl/reservoir) with the selected solution and wait 5 minutes to let it flow within the culture chamber.
c) Remove the permeabilization solution and repeat the operation at point b), this time filling reservoirs B and D.
d) Remove the permeabilization solution from all reservoirs.
e) Fill all the 4 reservoirs with permeabilization solution and incubate at room temperature for 10 minutes.
f) Remove the permeabilization solution from all reservoirs.

### Blocking of unspecific sites

a) Fill the device with the blocking solution (BS) selected according to the staining to be performed (e.g., PBS, 3% v/v of Goat serum, 0.2% v/v Tween). Put the BS in reservoirs A and C (≈80µl/reservoir) and wait 1 minute.
b) Remove the BS and repeat the operation at point a), this time filling the reservoirs B and D.

c) Fill all the 4 reservoirs with BS and incubate at room temperature for 1 hour.
d) Remove the BS solution from all reservoirs.

### Primary antibody

a) Fill the device with primary antibody solution prepared according to the specific staining to be performed (e.g., diluted in BS). Put the primary antibody solution in reservoirs A and C (≈25µl/reservoir) and wait 5 minutes.
b) Remove the solution from all reservoirs.
c) Fill all the 4 reservoirs with primary antibody solution (≈50µl/reservoir) and incubate at 4°C overnight.

### Secondary antibody

**Protect from light from now on!**

a) Remove the primary antibody solution from all reservoirs.
b) Wash with BS for at least four times: put the BS in reservoirs A and C (≈80µl/reservoir) and wait 15 minutes.
c) Remove the BS and repeat the operation at point b), this time filling reservoirs B and D.
d) Repeat operations b) and c).
e) Remove the BS.
f) Fill the device with secondary antibody solution prepared according to the specific staining to be performed (e.g., diluted in BS). Put the secondary antibody solution in reservoirs A and C (≈25µl/reservoir) and wait 5 minutes.
g) Remove the solution and fill again all the 4 reservoirs with secondary antibody solution (≈50µl/reservoir) and incubate at 4°C overnight.

### Nuclear staining and final washing

a) Remove the secondary antibody solution from all reservoirs.
b) Wash with PBS for at least four times: put the PBS in reservoirs A and C (≈80µl/reservoir) and wait 15 minutes.
c) Remove the PBS and repeat the operation at point b), this time filling reservoirs B and D.
d) Repeat operations b) and c).
e) Remove the PBS.
f) Fill the device with a nuclear staining solution (e.g., DAPI). Put nuclear staining solution in reservoirs A and C (≈25µl/reservoir) and wait 5 minutes.
g) Remove the solution and fill again all the 4 reservoirs with nuclear staining solution (≈50µl/reservoir) and incubate according to manufacture instruction of the specific nuclear staining.
h) Wash with PBS: put the PBS in reservoirs A and C (≈80µl/reservoir) and wait 15 minutes.
i) Remove the PBS and repeat the operation at point h), this time filling reservoirs B and D.
j) Repeat operations h) and i).
k) Remove the PBS.

l) Store stained uBeat® Platforms in a PBS bath at 4°C protected from light, if not immediately imaged. **It is recommended to stain the chip no more than 1 week before imaging for an optimal signal quality**.

To achieve highest magnifications during imaging, disassemble the uBeat® Platform peeling the top layer off (as described in Figure 17). The gel will remain on the top layer entrapped within the pillars' rows. At this point, gently lay down the top layer on a microscope coverslide (with the construct facing down), on a drop of PBS.

## 16. EXAMPLE: GENERATION AND MATURATION OF 3D HUMAN OSTEOARTHRITIC (OA) CARTILAGE MICRO-CONSTRUCTS

### *CHEMICAL AND REAGENTS*

- Fibrinogen, human type I from human plasma (Sigma Aldrich, cat. no. F3879-250MG)
- Thrombin from human plasma (Sigma Aldrich, cat. no. T6884-100U)
- Aminocaproic acid (Sigma Aldrich, cat. no. A2504-25G)
- NaCl powder
- $CaCl_2$ powder
- Demineralized water

### *PREPARATION AND STORAGE OF FIBRIN HYDROGEL'S COMPONENTS*

a) **Fibrinogen** solution preparation (100mg/ml) in NaCl 0.9% w/v
   - Prepare NaCl 0.9% (w/v) in demineralized water.
   - Sterilize the solution using a 0.2-µm filter and store it at room temperature.
   - Dissolve 250 mg of fibrinogen powder in 2.5 ml sterile NaCl solution (0.9% w/v).
   - Prepare aliquots (~50µl) and store the aliquots in -20°C.

b) **Thrombin** solution preparation (100U/ml) in $CaCl_2$ 40mM
   - Prepare $CaCl_2$ at 40mM.
   - Sterilize the solution using a 0.2-µm filter and store it at room temperature.
   - Dissolve 100 UN of thrombin in 1 mL of sterile $CaCl_2$ solution.
   - Prepare aliquots (~20µl) and store the aliquots in -20°C.

c) **Aminocaproic acid** preparation (100mg/ml) in PBS
   - Dissolve 300mg of aminocaproic acid in 3ml of sterile PBS.
   - Sterilize the solution using a 0.2-µm filter.
   - Store in the refrigerator (@4°C).

### *2D EXPANSION OF HUMAN ARTICULAR CHONDROCYTES*

- Thaw the human Articular Chondrocyes (hACs), ideally after 7-7.5 doublings, and plate them with a seeding density of 5000-5500 cells/cm2.
- Use Expansion medium for these steps (e.g., Composition of expansion medium: DMEM High Glucose supplemented with 1 mM sodium pyruvate, 10mM HEPES Buffer solution, 1% PS100 U/ml penicillin, 100µg streptomycin, 0.292 mg/ml L-glutamine, 10% Fetal Bovine Serum, 1ng/ml of transforming growth factor-β1 and 5 ng/ml of fibroblasts growth factor-2)
- Culture the cells changing the medium every 3 days.
- Harvest the cells when they reach 80% of confluence.

### *SEEDING OF HUMAN ARTICULAR CHONDROCYTES-LADEN FIBRIN MICRO-CONSTRUCTS*

a) Unpack uCase and each uBeat® Platform envelop inside a laminar flow cabinet in sterile conditions. Close each needle with a sterile cap and carefully transfer the 4 uBeat® Platforms in the uCase by means of sterile tweezers as described in section 9.

b) Prepare a cell suspension of hACs.
c) Put stock solutions of Fibrinogen, Thrombin and Aminocaproic acid in ice.
d) Dilute the Fibrinogen solution to a final concentration of 20 mg/ml in PBS and keep it in ice *(e.g., dilute 50 µL of fibrinogen stock solution (100 mg/ml) in 200 µl of PBS).*
e) Dilute the Thrombin solution to a final concentration of 5 U/ml in culture medium (i.e., DMEM) and keep it in ice (*e.g., dilute 10 µl of Thrombin stock solution 100 U/ml in 95 µl of DMEM).*
f) Prepare 10 µl of cell suspension in 5U/ml Thrombin solution at a cell density of $100x10^6$ cells/ml (i.e., 2x the targeted cell density of $50x10^6$ cells/ml): put $1x10^6$ in a sterile 0.5ml Eppendorf tube, centrifuge it to form a cell pellet, carefully re-suspend the cell pellet in 8 µl of Thrombin solution at 5 U/ml (considering cell pellet of approximately 2 µl, for a total of 10 µl of cell suspension in Thrombin).
g) Mix 1:1 the cell suspension in 5U/ml Thrombin solution with the 20mg/ml Fibrinogen solution to obtain 10 µl of cell-laden fibrin and immediately inject the cell-laden fibrin solution in the three culture chambers of uBeat® Platform.
*i.e., mix 5 µl of 20mg/ml Fibrinogen solution with 5 µl of cell suspension in Thrombin*

Since fibrin cross-linking is very fast, it is recommended to prepare maximum 10 µl of cell laden hydrogel at the time. This is enough to seed the three chambers of uBeat® Platform.

In this step, the mixing should be very careful but quick (i.e., mix the solution by pipetting 3 times up and down).

Keep all the aliquots in ice for all a) to g) steps.

h) Check under an optical microscope the outcome of the seeding.
i) Make small drops of PBS all around the uBeat® Platform.
j) Incubate uCase containing uBeat® Platform for 8 minutes (37°C, 5% $CO_2$) to allow fibrin crosslinking.
k) Inject the chondrogenic differentiation medium supplemented with 2 mg/ml of ACA into the reservoirs and fill the wells.
*e.g., dilute 100 µl of ACA stock solution in 4.9 mL of media.*
*e.g., Composition of chondrogenic differentiation medium: DMEM containing 2% fetal bovine serum, 4.5 mg ml−1 d-glucose, 0.1 mM non-essential amino acids, 1 mM sodium pyruvate, 100 mM HEPES buffer, 100 U ml−1 penicillin, 100 µg ml−1 streptomycin and 0.29 mg ml−1 l-glutamine, and supplemented with 0.1 mM ascorbic acid 2-phosphate, 10 µg ml−1 insulin and 10 ng ml−1 TGF-b3.*

l) Incubate uCase containing the uBeat® Platform in a humidified incubator at 37 °C and 5% $CO_2$.

> Put some PBS drops around the device inside the uCase during this process, to compensate for evaporation, especially if the cross-linking occurs at 37°C inside the incubator.

m) Change medium every other day. ACA concentration should follow the following table:

| Day 0 (seeding) | Day 2 | Day 4 | Day 6+ |
|---|---|---|---|
| 2 mg/ml | 1.6 mg/ml | 1.2 mg/ml | 1 mg/ml |

n) A mature cartilage construct is obtained after 14 days of culture[2].

### INDUCTION OF OA TRAITS INSIDE THE MICRO CARTILAGE CONSTRUCTS

a) After 14 days of culture in static conditions, fill the uBeat® Platforms actuation chambers and connect uCase to the uBox system as detailed in section 11.
b) Upon filling, in order to induce OA traits in your micro cartilage constructs, start the mechanical stimulation following the 'uKNEE' program (detailed in section 17) for 7 days.
c) During these 7 days, change the chondrogenic differentiation medium (supplemented with ACA 1mg/ml) every other day as detailed in section 11.
d) After 7 days, you have generated a micro cartilage model featuring OA traits[2].

## 17. Appendix A – uBox programs

In this section, the parameters pre-set for two default stimulation programs 'uHEART', 'uKNEE', 'uGUT' and 'uSCAR' together with 'FILLING' program will be detailed.

**Notice**: Never start a uBoX program without connecting it to the compressed air source.

Each 'setting screen' of the three programs shows two sets of information:

- Stimulation: where frequency (Hz) and duty cycle (%) are indicated.
- Timing: where the timing pattern of the specific program is indicated.

### Filling

- Timing: 30 min

### uHeart

- Frequency: 1 Hz.
- Duty Cycle: 50%.
- Timing: On forever (no pattern).

### uKnee

- Frequency: 1 Hz.
- Duty Cycle: 50%.
- Timing: 2 hours (ON) – 4 hours (OFF) – 2 hours (ON) – 16 hours (OFF).

### uGut

- Frequency: 1 Hz.
- Duty Cycle: 50%.
- Timing: On forever (no pattern).

### uScar

- Frequency: 1 Hz.
- Duty Cycle: 50%.
- Timing: On forever (no pattern).

### Custom

The custom program allows you to set key parameters of the stimulation to get the desired pattern. You can set up to 3 Custom Patterns, for each pattern it is possible to customize:

- Waveform: Square wave vs Sin wave.
- Frequency: from 0 Hz to 3 Hz.
- Duty cycle: from 0 % to 100 %. Only available for Square waveform.
- Timing: ON/OFF block and REST block.

# 18. Appendix B– Parallelization Branch

The Parallelization Branch is not included in our standard offer (i.e., Starting Package/Additional Kits), but it can be purchased separately when there is the need of culturing more uCases in parallel.

### Included in the package:

- Parallelization Branch
- 16 sterile three-way stopcocks
- 1 closing cap

### Description

The Parallelization Branch allows to stimulate up to 4 uCases simultaneously.

It is a tubing-valves system that is connected to the main control valve (provided with the uBox pneumatic tube in the Starting Package and described in section 11 step a, User Guide) at one extremity and to 1-4 uCases at the other ones (see Figure 20, lines 1-4). The so obtained branched connections ensure an equal stimulation to all the connected devices.

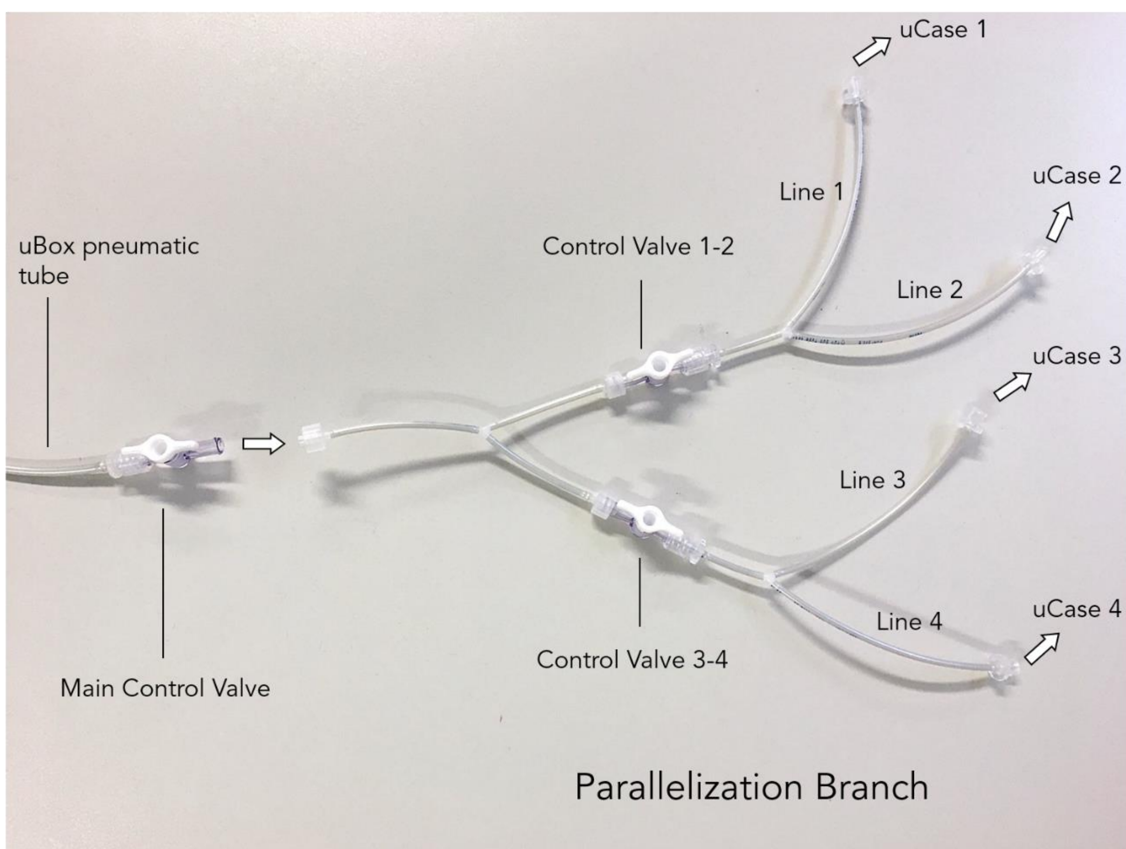


Figure 20 – Representative picture of the parallelization branch.

The presence of two additional control valves (i.e., Control Valve 1-2, Control Valve 3-4), that can be independently opened and closed, allows to separately command lines 1-2 and lines 3-4 (see Figure 20).

The different possible combinations make it easy to:

- Set-up experiments with four uCases running in parallel (Figure 21a: Control Valve 1-2 and 3-4 opened, all Lines are in pressure);
- Unplug during the culture period a portion of the uCases when mechanical stimulation is active (e.g., for medium change in the devices, optical inspection of microtissues, or other applications) or set-up experiments with just 2 uCases at a time (Figure 21b: Control Valve 1-2 opened and Control Valve 3-4 closed, Lines 1 and 2 are in pressure while Lines 3 and 4 are closed; Figure 21c: Control Valve 1-2 closed and Control Valve 3-4 opened, Lines 1 and 2 are closed while Lines 3 and 4 are in pressure);
- Unplug during the experiment all four uCases during the culture period when mechanical stimulation is active (e.g., for medium change in the devices, optical inspection of microtissues, or other applications) (Figure 21d: Control Valve 1-2 and 3-4 closed, all lines are closed).

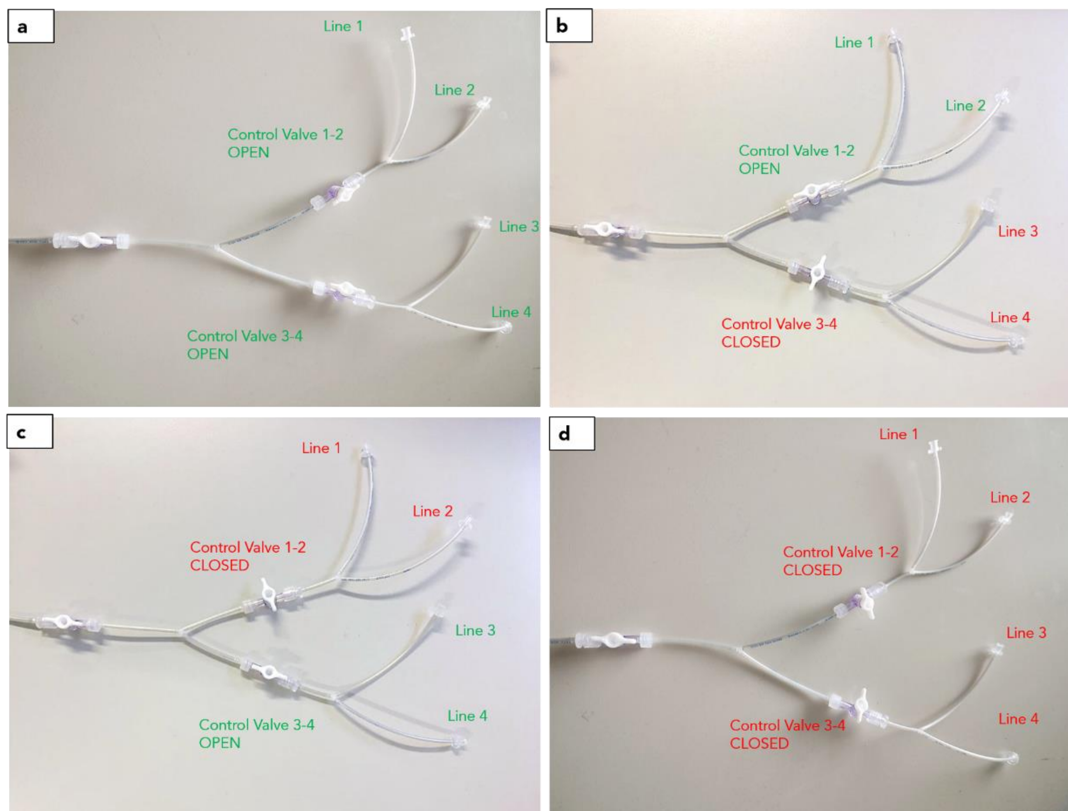


Figure 21 – Usage of Control Valves 1-2 and 3-4 to control the lines.

## Procedure

After having prepared all the uCases (see section 10) using the sets of four three-way stopcocks provided with the Parallelization Branch, and after having installed uBox (section 8), you can proceed connecting them.

1) Connect the main Control Valve (already connected to uBox pneumatic tube) to the single male luer-lock connector of the Parallelization Branch. Keep the main Control Valve closed during this stage (as indicated in section 11 step a).

2) Connect each three-way stopcocks' ramp of the 4 uCases to each Line.
   If you are connecting two uCases, we suggest connecting them to Line 1 and 2, closing the Control Valve 3-4 (Figure 21b).
   If you are connecting three uCases, connect them to Line 1, 2 and 3, keeping Control Valves 1-2 and 3-4 opened and closing Line 4 with the closing cap supplied with the parallelization branch (Figure 22) .
   If you are connecting four uCases, connect them to each line and leave the Control Valves 1-2 and 3-4 opened (Figure 21 a).

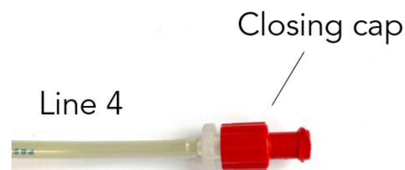


Figure 22 – The closing cap allows to culture three uCases using the parallelization branch by closing line 4.

3) Now the set-up is ready to be connected to uBox: follow indications described in section 11 (starting from step b).

The Parallelization Branch allows the user to easily organize the uCases' disposition inside the incubator in different ways. uCases can be either placed on top of each other or side by side and they can be arranged in pairs either on one single incubator shelf or on two shelves sufficiently closed to each other.

# 19. Appendix C – uBeat Platform Trial kit

This kit allows the user to become familiar with Organ-On-Chip technology as well as with seeding, culturing and readouts procedures.

## Content of uBeat Platform Trial Kit

Similarly, to the uBeat Platform Kit, also the uBeat Platform Trial Kit consists of 3 Mini-Kits. Each Mini-kit contains:

- 4x sterile uBeat® static Platforms (each uBeat® static Platform is provided with a serial number, which you should keep track of);
- uBeat® Platform mini-kit instructions.

## Usage of uBeat Platform Trial Kit

For unpacking the mini-kit boxes please follow section 7 instructions.

For seeding and culturing the devices please follow section 9 instructions. Please, consider that in order to use this Trial Kit the uCase is not needed, so it is not provided. The devices can be placed **individually** inside a Petri dish and we suggest to write on the Petri lid their serial number. Keep culturing your devices in the Petri dishes paying attention to put some PBS (e.g., 1ml) around the device, to prevent medium evaporation, and to refill it every two-three days.

**Important: the devices included in the uBeat Platform Trial Kit are static, meaning that their simplified design is not compatible with mechanical stimulation.**

# 20. References

[1] A. Marsano, C. Conficconi, M. Lemme, P. Occhetta, E. Gaudiello, E. Votta, G. Cerino, A. Redaelli, M. Rasponi, *Lab Chip* **2016**, *16*, 599.

[2] P. Occhetta, A. Mainardi, E. Votta, Q. Vallmajo-Martin, M. Ehrbar, I. Martin, A. Barbero, M. Rasponi, *Nat. Biomed. Eng.* **2019**, *3*, 545.

[3] P. Occhetta, G. Isu, M. Lemme, C. Conficconi, P. Oertle, C. Räz, R. Visone, G. Cerino, M. Plodinec, M. Rasponi, *Integr. Biol.* **2018**, *10*, 174.



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