A Bioreactor to Apply Multi-Modal Physical Stimuli to Cultured Cells

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Summary

Cells residing in the cardiac niche are constantly experiencing physical stimuli, including electrical pulses and cyclic mechanical stretch. These physical signals are known to influence a variety of cellular functions, including the secretion of growth factors and extracellular matrix proteins in cardiac fibroblasts, calcium handling and contractility in cardiomyocytes, or stretch-activated ion channels in muscle and non-muscle cells of the cardiovascular system. Recent progress in cardiac tissue engineering suggests that controlled physical stimulation can lead to functional improvements in multicellular cardiac tissue constructs. To

study these effects, aspects of the physical environment of the myocardium have to be mimicked *in vitro*. This protocol demonstrates how a specifically designed bioreactor system allows controlled exposure of cultured cells, during continuous live imaging, to cyclic stretch, rhythmic electrical stimulation, fluid perfusion, at user-defined temperatures.

Key Words: Bioreactor, excitable cells, cyclic stretch, electrical stimulation, perfusion, cell mechanics, electrophysiology, myocardium.

1. Introduction

The physical environment that cells experience in the cardiac niche influences many cellular functions. For instance, cyclic stretch has been shown to mediate growth factor secretion (1), and alter calcium-handling in cardiomyocytes (2) and production of extracellular matrix proteins in cardiac fibroblasts (3, 4). Similarly, isolated adult rat ventricular myocytes in primary culture subjected to continual electric field stimulation exhibit improved contractile properties and calcium-transient characteristics compared to unstimulated quiescent cells (5, 6). These studies highlight the important role of physical stimulation in sustenance of isolated cells and cultures.

Several "biomimetic" strategies have been applied to steer functional improvement of *ex vivo* cultivated 3D tissue constructs by mimicking aspects of the native myocardial environment. Stimuli applied include cyclic stretch (7), electrical pacing (8), "electro-tensile" stimulation (where electrical and mechanical stimuli are applied in controlled sequence) (9), constant fluid perfusion (10-12), oxygenation via carriers through channelled scaffold (12), and more recently, concerted medium perfusion in combination with electrical stimulation (13).

Various bioreactors have been designed to implement some of the above stimulation strategies on cultured cells or tissue constructs. Generally, though, bioreactors are either cyclic stretch or electrical stimulation chambers. Our custom reactor combines mechanical and electrical stimulation in one apparatus. It allows one to study the interplay of well-coordinated uniaxial cyclic stretch and electrical stimulation. Additionally, it is possible to adapt the perfusate's composition, temperature, and exchange rate. During experiments, the reactor is mounted on top of an inverted microscope for continuous imaging.

In the following, we describe the design and use of an improved version of the custom made bioreactor, first introduced in 2013 (14, 15) at the TU Dresden. We provide a step-by-step description of the protocols required to use this integrated system to study excitable and non-excitable cells.

2. Materials

2.1 List of Materials and Equipment

All parts with direct or indirect contact to the cells under investigation have to be sterilized before use (underlined in the list below). The assembly and loading of the reactor must be performed in a culture hood under sterile conditions. Subsequent experiments may be performed outside the hood in a reasonably clean room as usually maintained for tissue culture.

Experimental Setup

1. Equipment, devices and apparatuses for standard tissue culture work.
2. Polydimethylsiloxane polymer substrate (PDMS) mould [Fortech UG, TU Dresden].
3. Bioreactor, composed of the assembled chamber and driving unit [Fortech UG, TU Dresden]. Add-ons to the setup to seal the chamber [homebrew].
4. External electrical stimulation control box, cables and carbon electrodes. Photoelectric Sensor and cables [all Fortech UG, TU Dresden].
5. Motor control box and cables [Fortech UG, TU Dresden] to apply cyclic, uniaxial stretch.
6. 250 mL glass bottle with neck thread [Simax] and watertight lid with 4 sealable cap-ports [Schott]. All parts together serve as solution reservoir.
7. 3 x 2 mL serological pipette tips [Sarsted] for medium and inward gas handling.
8. Exit port with a serological pipette tip [Sarsted] and syphon [homebrew] for outward gas handling.
9. Carbogen (5% CO_2, 95% O_2) [BOC Medical] with 22 µm millipore filter [Merck Millipore] attached.
10. Carbogen sparger [BEOT].
11. Peristaltic pump [Gilson] for medium perfusion (rate up to 5 mL/s).
12. 2 x silicone tubes [Saint-Gobain], 6 mm outer diameter, 3 mm inner diameter, 75 and 100 cm long. The shorter tube is arranged between the medium reservoir and the peristaltic pump. The longer tube conducts carbogen gas into the reservoir.
13. 2 x silicone tubes [Saint-Gobain], 4 mm outer diameter, 3 mm inner diameter, 75 cm long each. The first tube is connected to the peristaltic pump and serves as forward supply for the chamber. The second is used as reverse supply.

14. 40°C water bath with pump [Haake] for water jacketing and to warm the medium in the solution reservoir.
15. A fixation stage [homebrew] to stabilize the reservoir.
16. 3 silicone tubes [Saint-Gobain], 6 mm outer diameter, 3 mm inner diameter, 50 cm long each. Tubes are thermally insulated and used to circulate water through the water jacket.
17. Flow gauge indicator [Bürkle].
18. Tubing connectors [World Precision Instruments].

Data Acquisition

1. Inverted microscope [Olympus] equipped with long distance objectives 5x, 10x and 20x [Thalheim Spezial Optik] and an adapted microscope table [Fortech UG, TU Dresden].
2. Microscope Camera [Nikon].
3. Computer Workstation [HP] with Data Acquisition Software [Fortech UG, TU Dresden].

2.2 General Description of the Setup

The microscope stage can accommodate up to 4 bioreactors that can be used in parallel (14, 15). Each of the reactors consists of two parts: The bio-chamber and the driving unit (Figure 1). The chamber is perfused with culture medium and houses two stretchable PDMS membranes onto which cells of interest are cultured (see Note [1]). Two carbon electrodes are fitted in the chamber and allow for pacing with defined temporal off-set to the stretch protocol. Cell responses to a set of user-defined, ambient stimuli are monitored via long-working distance objectives and a camera connected to a computer for bright field time-lapse

imaging. All materials used for the construction of the chamber meet demands on biocompatibility and sterilisation. Stainless steel is used for rods, screws, the bottom framework and the lid. Silicone rubber is used for sealing in- and outlets and connections between microscope glass slides and the bioreactor housing. Polyether ether ketone (PEEK) polymer was selected for all other parts.

A 250 mL glass bottle containing 100 mL of suitable, bicarbonate buffered culture medium is held in a water bath set to 40 °C (Figure 1). A peristaltic pump, located between the water bath and the chamber of the bioreactor (see Note [2]), circulates the culture medium at a flow rate of 0.5 - 1.0 mL/s, to maintain the nutrient and gas supply in the vital range for the cells under investigation. The culture medium is bubbled in its reservoir with carbogen (95% O_2, 5% CO_2) to keep pH constant (see Note [3]). An exit tube with a syphon resting in ethanol (see Note [4]) ensures that there is no contamination from ambient atmosphere and indicates the gas exchange. Thermally insulated tubing conveys water from a temperature-controlled water bath to the fluid perfused lower section of the chamber (henceforth referred to as 'water jacket') in a closed loop configuration. Its purpose is to supply the top chamber with thermal energy to compensate for heat losses to the environment. A flow gauge (enclosed propeller) next to the bioreactor indicates water jacket's flow (see Note [5]).

2.3 Stretching

Torque from a stepper motor (Figure 2A) is converted into a uniaxial stretch pattern via an eccentric disk with cam (Figure 2A and 2B1) that acts on a PEEK cantilever (Figure 2B). This cantilever drives a shaft with one set of the PEEK brackets that hold the stretchable membranes. The second set of brackets, arranged in parallel, is fixed. The stretchable PDMS membrane is clamped between the two neighbouring brackets (Figure 2, 3A#3 and 3A#4).

Thus, inward rotational motion transmitted by the cantilever translates into a lateral change in distance between the parallel edges of the two brackets (Figure 2B). The membrane, clamped between the brackets, experiences pseudo-uniaxial deformation, which is transferred to the cells attached to it. To improve homogeneity of strain, the short sides of the membrane have arc-shaped indentations with reinforced edges (Figure 2B2), which compensate for (some of) the Poisson's effects (see Note [6]). The stretch, stretch/relaxation velocities and frequency are highly adaptable by adjusting the settings of the stepper motor (0.25 – 4 Hz), while stretch waveform and amplitude can be altered by exchanging the eccentric disk with cam (0 – 20% ratio of deformation, Figure 2B1). A standard protocol used involves 10% stretch at 1 Hz for 24 h. Modifying the shape of the eccentric disk and cam adjusts the mechanic deformation behaviour of the setup: Smooth or steep slopes before and after the tip of the cam allows to accurately control stretch and relaxation kinetics. Transitions can be adjusted to match physiological cardiac deformations in this *ex vivo* system. On the other hand, increasing the diameter of the cam and creating a 'dip' instead of a 'bump' allows one to maintain cell stretch and apply a short relaxation, mimicking a different mechanical loading pattern. Stretch pattern options are thus infinite and easily adjustable.

2.4 Pacing

Homogeneous electrical stimulation is applied via carbon electrodes (Figure 3A#4) positioned in close proximity to the membrane holding brackets. The pattern of electrical stimulation is flexible by changing, in software, parameters for rate (for example to match mechanical protocols), pulse duration (1 – 10 ms), current amplitude (1 – 10 mA) and timing. Temporal interrelation of electrical and mechanical stimulation is controlled via photoelectric feedback from a sensor located on the axis of the motor (Figure 2A).

3. Methods

3.1 PDMS Membrane Preparation

Moulding

1. Make a solution of silicon: Dow Corning Sylgard 184; base:curing agent ratio = 10:1.

2. Poor this solution into a custom-made polycarbonate casting mould in a clean room environment (see Note [7]).

3. Leave the mould undisturbed for 72 h at room temperature to allow curing.

4. Store resulting PDMS substrates (2 cm x 1 cm x 50 mm) in a petri dish to prevent from dust contamination (see Note [8]).

The following steps are to be performed under a standard cell culture hood to work in very clean, preferably sterile, conditions.

Sterilisation

1. Incubate PDMS membrane in 70% ethanol for 30 min (see Note [9]).

2. Wash 2 times with sterile Phospahte-Buffered Saline (PBS).

3. Leave to dry for 10 min.

Coating

1. Coat PDMS substrates with a 1:100 mixture of gelatin, type I collagen, fibronectin or collagen-fibronectin. Use water as dilution medium (see Note [10]).
2. After 4 h, wash PDMS surfaces 3 times with PBS and seed cells of interest on coated substrates (see Note [11]).
3. After 12 h, visually inspect cell attachment to PDMS substrates. Wash samples 3 times with warm PBS (37 °C) to remove non-adherent cells.

3.2 Bioreactor Construction

1. Sterilise all parts of the bioreactor using a steam autoclave (121 °C, 200 kPa, 20 min) or incubating in 70% ethanol for 30 min (see Note [12]).
2. If using 70% ethanol for sterilisation, all parts need to be dried entirely in the hood.
3. Place the two 4 mm circular rubber rings (O-rings) and one 60 mm O-ring in the framework of the bioreactor to ensure a water tight seal can be created later for the water jacket (Figure 3A#1).
4. Next, insert the glass slide with the two circular openings for the water jacket, followed by the spacer with the rubber seal and the microscope glass slide (Figure 3A#2).
5. Take the central chamber's component (Figure 3A#6) hosting the two brackets. At this point, their clamps are not attached yet. Next, fit the carbon electrodes (Figure

3A#4) to the brackets with a medical suture, ensuring antiparallel current flow (see Note [13]). Make sure the wiring is fitted firmly into its opening from the inside of the chamber. The glued construction is depicted in Figure 3A#4 while Figure 3B#8 illustrates its assembled position (see Note [14]). Afterwards, the clamps (Figure 3A#3) are clipped onto their according brackets. Each combination of bracket and clamp will serve as a membrane holder later. However, PDMS membranes (Figure 3A#5) are not attached yet. Subsequently, prepare to seal the slide bearing by adding the 5 mm O-ring (Figure 3B#9), the PEEK shell (Figure 3B#10) and PEEK plate (Figure 3B#11). Fasten the screws (Figure 3B#12) in order to squeeze the O-ring into the surrounding construction until the opening is watertight. A good trade-off has to be found that ensure the friction levels for the driving shaft remain low. Fine tune the screws' position in step 8) (see Note [15]).

6. Check that both the bottom and top of the central chamber are equipped with a 60 mm O-ring each. Afterwards, the prepared central chamber's component is added to the preassembled bottom framework parts.

7. Add the second glass slide to complete the chamber, followed by a PEEK spacer and two metal lids in ascending size to allow the unit to be screwed closed (Figure 3A#7).

8. Circulate sterile PBS to flash any remaining ethanol. Adjust and assess the sealing of the driving shaft (see Note [15]). Once sorted, add and fasten the PEEK cantilever (Figure 3B#13).

9. Circulate with pre-warmed (37 °C) culture medium to check for leakages (see Note [16]).

10. Stop the circulation, open the top of the chamber and remove the culture medium filling the chamber.

11. Take out the central part of the chamber, flip it upside down and mount the membranes (pre-seeded with cells of interest) on the holders (see Note [17]).

12. Repeat steps 6) and 7) and while circulating culture medium mount the driving unit. The bioreactor is now complete (Figure 3C).

13. Fix the bioreactor to the stage of the inverted microscope.

14. Connect the chamber's water jacketed to the water bath (see Note [18]).

15. Connect the motor, the electrodes and the photoelectric sensor to their according control boxes for driving and coordinated pacing.

At this point, experiments can proceed for periods of up to 4 days (or more).

3.3. Improvements from the Original Design

One major amendment of the setup addresses the way in which the measurement chamber is encapsulated. Previously, gas exchange between the culture medium in the chamber and the surrounding incubator environment was promoted. Ventilation was possible through the feed

openings for the driving shaft and the pacing electrode connection wires. For long term experiments, these openings are prone to contamination, once the chamber is taken from the sterile incubator environment onto the microscope stage, as well as culture medium leakage. Thus, wires are glued into a shell (Figure 3A) and gas exchange with the environment is suppressed, once the chamber is fully assembled (Figure 3B#8). The slide bearing is equipped with a 5 mm O-ring from the outside of the chamber to seal the opening (Figure 3B#9). As the drive shaft needs to move easily, a shell (3B#10), plate (3B#11) and screws (3B#12) are used to secure the ring's position from the outside. Via the screws, holding pressure is adjusted such that the chamber is sealed but the shaft still able to move with low friction levels.

Another improvement addresses changes in the culture medium solution supply line. Previously, the solution was kept in a fridge, warmed up and used once before put to waste. We changed solution handling to a closed loop strategy in order to reduce costs and to be able to run long-term experiments, for example to test effects of (at times expensive) drugs.

To maintain the pH of the DMEM based culture medium (bicarbonate buffered) we bubble the perfusion solution in its reservoir with carbogen. To visually control gas exchange rate and avoid untoward pressure gradients in the system, gas is allowed to exit through a syphon filled with ethanol (Figure 1).

Furthermore, a holder for the reservoir bottle to be kept in place in the water bath has been added. This ensures upright alignment of the tubing at a reproducible height throughout and between experiments (not shown).

In addition to coordinated stretch and electrical stimulation, it is possible to grow cells on glass coverslips which can be placed in the central chamber's component (Figure 3A#6) section of the bioreactor, above the water jacket. This allows live imaging while pacing and controlling perfusion, solution composition, temperature and/or flow for short or long-term experiments.

Combining this set-up with a motorised computer-controlled microscope stage can allow acquisition of time lapse image series from different regions of interest and different incubators. This, together with an increase in the number of bioreactors used in parallel, would potentially allow use as a high-throughput system.

In the future, we intend to improve the shape of the chamber, to apply a controlled shear to cells cultured on PDMS membranes. Applying both shear and stretch while pacing and perfusing would open new avenues for research relevant for cardiovascular applications, and provide a better understanding of the role of a wider range of physical stimuli on cells.

4. Notes

[1] PDMS is used due to its ease of moulding, low cytotoxicity, oxygen permeability and transparency. Each bioreactor chamber can house 2 PDMS substrates at a time (Figure 1, 2A and 3A#5) thus all experiments can be performed in duplicate or one membrane can host control cells and the other mutant/treated cells for comparison within the same experiment.

[2] After leaving the pump, the tubing is dipped in the water bath to compensate for heat loss occurring at the level of the pump. The tubing section in between the water bath and bioreactor is kept as short as possible to avoid heat loss. The tubing used in this section has a small open diameter in order to increase the flow rate in this section and thus reduce the time for heat exchange. Temperature maintenance is further improved by application of insulating material around that tube. The pump is installed upstream of the chamber to generate a slightly positive pressure in the chamber when feeding culture medium. Having the pump downstream of the chamber would create a slightly negative pressure which would increase the contamination risk if the chamber is not fully airtight. Having a slightly positive pressure increases the risk of perfusate leaks, but this should not prevent the experiment from continuing (assuming the leak is not massive); this is in contrast to the alternative scenario (solution contaminated), which would end experiments in all simultaneously perfused chambers. Finally, hydrostatic pressure should be considered when the system is installed. We found that the tendency for the chamber to leak was increased when the level of the bioreactor was about 20 cm lower relative to the perfusate level in the reservoir; this should be avoided.

[3] A Millipore filter is applied before the carbogen gas bubbles the solution to ensure sterile conditions.

[4] To avoid hyper-pressure in the system, the tubes exit point should be close to the liquid surface (volume of ethanol between 1 – 5 mL). A positive pressure in the reservoir avoids infiltration and contamination from outside pathogens.

[5] The propeller indicator needs adjustment and fixation such, that the in- and outlet are aligned horizontally at the top of the device. That way, small air bubbles are transported further into the reservoir directly. This prevents gas accumulation in the indicator and, as a consequence, ensures functionality.

[6] Poisson's effect is leading to compression perpendicular to the stretch direction. This effect is compensated for by the membranes due their sophisticated design. Validation was done with ARAMIS surface studies as presented in (14, 15).

[7] Pay close attention to not get any dust on the PDMS membranes. Dust particles are extremely hard to remove once attached.

[8] To reduce the risk of contamination, it is recommended to seal the dish with parafilm.

[9] Activation of the membranes surface via plasma treatment can be done but is not considered a mandatory procedure. In general, activation is used to treat materials that are normally hydrophobic. After treatment with plasma, they become more hydrophilic and more susceptible to chemical modifications or coating with proteins. However, adhesion of cells after coating on both the ozone plasma treated membranes and raw membranes yield comparable results in our hands when cultivating cardiac fibroblasts.

[10] We found that PDMS membranes had to be coated as our cells of interest (fibroblasts and cardiomyocytes) did not adhere otherwise.

[11] As a positive control, seed the same numbers of cells on a standard 24 well plate. Both the culture plate and the PDMS substrates have an effective growth area of 2 cm^2.

[12] Sterilisation is a crucial step. The bioreactor is made of numerous parts and if the entire assembly is not perfectly clean, contamination is likely to occur. Contamination is not only prone to waste of resources and time, but may yield misleading results.

[13] The carbon electrodes have to be knotted to the PEEK brackets, and electrically connected in an antiparallel fashion, to allow for a rather homogeneous electrical field distribution (14). The electrodes need to be clamped to the brackets facing the inside of the chamber.

[14] Fitting the wiring appropriately is crucial for sealing and thus obtaining a watertight chamber. Fluid tightness is important to avoid leakages and contamination.

[15] In order to avoid leakage and spill of PBS in the lab, the adjustment of the screws shall be done by using negative pressure in a hood in sterile conditions. Fill up the chamber of the fully assembled reactor with PBS via a syringe. Once all air is removed, block the outlet of the main chamber. Connect a 2 mL syringe to the inlet and apply negative pressure to the chamber by pulling on it. The appearance of air bubbles inside the chamber indicates that the chamber is leaking. The according position of the leak can be derived during visual inspection. If this happens at the opening for the driving shaft, the screws have to be fastened further. Perform multiple iterations. It is advised to apply and hold negative pressure via the syringe for a few seconds and rotate the driving shaft. Fine-tune the adjustment such that a

good trade-off between friction level and water tightness is found. This adjustment is very crucial for the success of the experiment.

[16] Leakages are usually the result of incorrectly mounted parts or missing rubber O-rings. The mounting step is crucial as it is a precondition for the entire experiment. Cell culture medium is used to assess the setup, as the solution is coloured and leakages are spotted more easily than using PBS.

[17] When attaching the PDMS membranes, the eccentric disc shall be fastened to avoid accidental movements and damage to the cells. Instead of PDMS membranes, other deformable constructs, tissue sections, or trabecular muscle samples may be clamped and studied.

[18] To ensure cells are kept within a suitable temperature range during the entire experiment, the water bath and pump shall be switched on well before cells are put in the chamber (minimum 20 min earlier). The bypass can be used before the chamber is installed on the microscope's table to circulate warm water through the tubing. This allows the system to warm up and reduce the time needed to reach the targeted temperature range later, once the fluid perfused section of the chamber is connected.

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Figure Legends

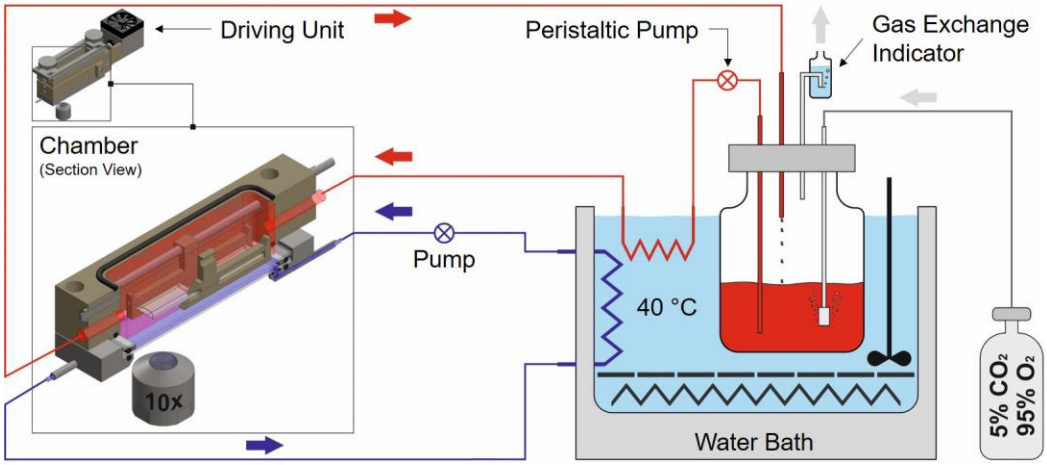

Figure 1

Figure 1: General schematic of the setup. The bioreactor, composed of the driving unit and the chamber (left hand side), is connected to a water bath for temperature control and to a bottle of culture medium for cell perfusion. This bicarbonate buffered solution is bubbled

with carbogen to keep the pH constant. The bioreactor's fan, stepper motor, pacing electrodes and photoelectric sensor are connected to their external control boxes (not shown).

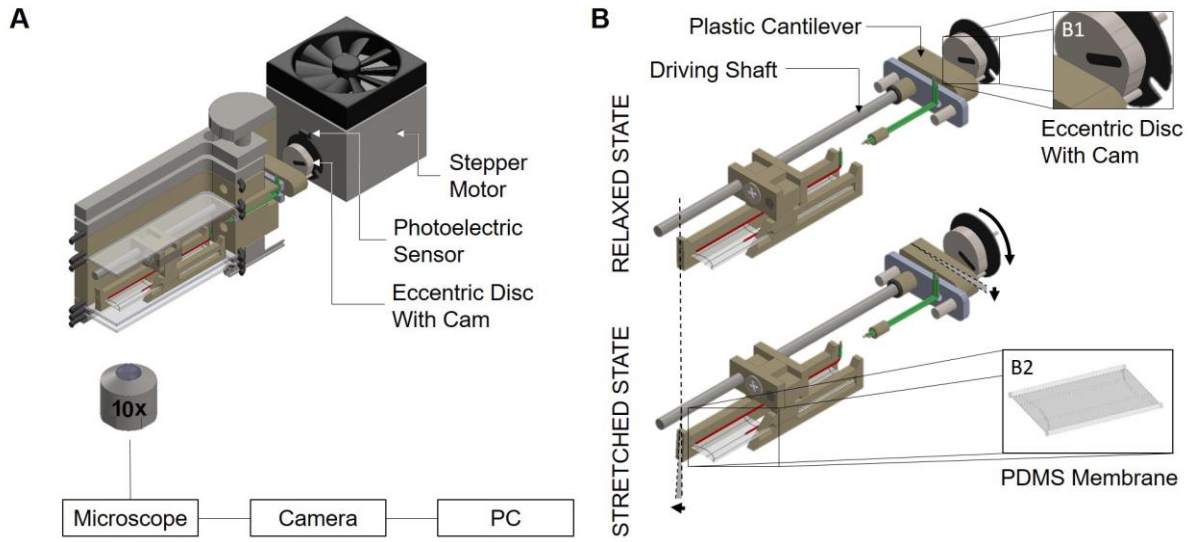

Figure 2

Figure 2: Key components of the bioreactor. (A) Section view of the chamber connected to the driving unit located above a microscope's objective. The subsequent signal acquisition chain is sketched underneath. (B) View of the inside components of the chamber allowing cyclic, uniform uniaxial stretch. The eccentric disk with cam (B1) rotates and acts on the PEEK cantilever which transmits the movement to a shaft that drives the left membrane holder apart from the right, fixed one. This introduces stretch to the membrane. (B2) Focus on a carefully designed PDMS membrane which is designed to compensate for Poisson's effect through arc-like reinforcements on both sides.

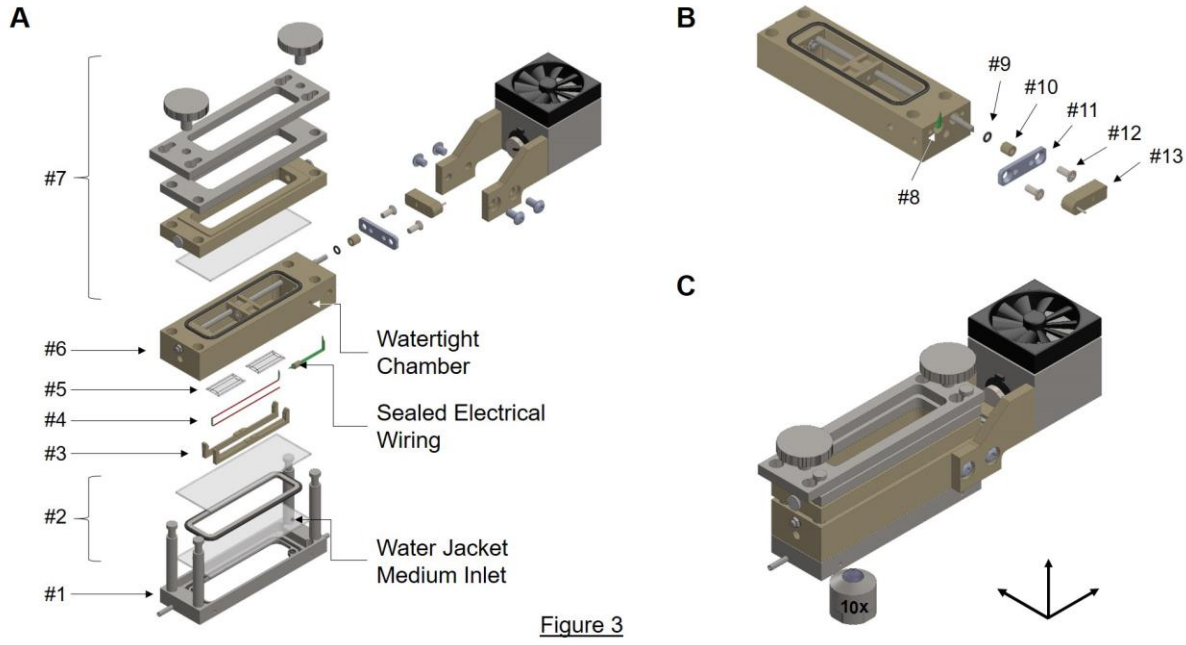

Figure 3: Bioreactor assembly. (A) Exploded view of the chamber for step by step assembly. #1: Stainless steel constituting the frame of the chamber and allowing water circulation for the water jacket. #2: The water jacket. The machined microscope glass slide with two holes serving as in- and outlet for the water jacket at the bottom (the left hole is hidden). The inside of the water jacket is framed to the sides by the spacer surrounded by a rubber seal. It is closed by an off-the shelf microscope glass slide. #3: Membrane holder clamps. #4: Carbon electrodes and wiring. #5: PDMS Membranes. #6: Main part of the chamber hosting the shaft which transmits the motor's energy to the membrane holders. #7: Top glass that seals the chamber and screw top allowing controlled tightening of the whole setup. (B) Depiction of the seal of the driving shaft. #9: 5 mm O-Ring. #10: PEEK shell. #11: PEEK plate. #12: M3, steel screws. #13 PEEK cantilever. (C) The complete bioreactor ready to be used in isometric representation. Arrows serve as scale reference where each arrow indicates a length of 3 cm.