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A micro-incubator for cell and tissue imaging

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A low-cost micro-incubator for the imaging of dynamic processes in living cells and tissues has been developed. This micro-incubator provides a tunable environment which can be altered to study the response of cell monolayers for several days as well as relatively thick tissue samples and tissue engineered epithelial tissues in experiments lasting several hours. Samples within the incubator are contained in a sterile cavity closed by a gas permeable membrane. The incubator can be positioned in any direction and used on an inverted as well as on an upright microscope. The temperature is regulated with a Peltier system controlled with a sensor positioned close to the sample to be able to compensate for any changes in temperature. Rapid changes in the environment can be applied to the sample because of the fast response of the Peltier system and the sample's adaptations to induced changes in the environment can be monitored. To evaluate the performance of the micro-incubator we report on studies using cultured cells in monolayers, on monolayers of cells stretched to breaking point on a distensible membrane, on cells in open 3D fibrous scaffolds and on fluorescently labelled polymersome penetration into 3D tissue engineered oral mucosa.

keywords: cell, tissue, mucosa, culture, microscopy, polymersomes, electrospun, PDMS, incubator

INTRODUCTION

Conventional histology, immunohistochemistry or imaging of fixed samples give good information on the local morphology and structure of cells but are either not usable or exceedingly wasteful when trying to look at the development of tissues with time. To cope with this drawback live sample imaging has progressively become part of various characterization methods. From microrheology measurements over tens of minutes [1] to drug or gene delivery studies over several tens of hours [2], long term observations are required to characterize dynamic biological phenomena on a broad range of time scales.

To keep cells alive either “*ex vivo*” if part of a natural tissue or “*in vitro*” if cultured and grown in two dimensions (2D) or three dimensions (3D) within a synthetic extracellular matrix, physiological conditions need to be maintained with respect to temperature, oxygen availability and the metabolic nutrients required for an artificial environment .

Since the early 1950s, a variety of systems have been designed in parallel with improvements in microscopy and cell culture techniques [3]. However most of the available designs have been developed to meet a limited number of criteria [4; 5; 6]

We present a modular micro-incubator (m μ i) with an extended versatility thanks to the combination of a large set of features available only separately until now. The reusable m μ i is optimized in terms of mass and heat transfer, ease of assembly and cleaning, modularity and robustness. It offers the following features:

- Culture of cells in monolayers or on synthetic or natural tissue matrices over the course of several days.
- Imaging on any microscope, upright or inverted, thanks to the use of a gas permeable membrane to separate the culture medium from the ambient environment.

- Precise control of the temperature at the level of the sample to prevent any light heating or any cooling if using an oil immersion objective.
- Free choice of the substrate for the culture of cell monolayers. Possibility of cell stretching by growing cells on a distensible membrane.
- Independent perfusion of two different media onto each side of thick tissue samples if required.
- Use of the m μ i in an "open-dish" version if macromanipulation is required.
- Easy assembling of the m μ i in non sterile condition before autoclaving.

These features offer a broad spectrum of applications in experimental biophysics, and in the various fields of medical sciences: for instance cell stretching, cell motility along fibres, intracellular microrheology in variable environments and incorporation of fluorescently labelled nanocarriers in 3D tissue. A selection of different experiments chosen to demonstrate the capabilities of the m μ i are described in the supplementary information.

MATERIALS AND METHODS

Incubator's features

The m μ i is built from a series of different elements, some of which are optional (figure 1). They can be easily implemented depending on the needs of the user and as such contribute to the modularity of this incubator. Thanks to the silicon conical ring B at the centre of the m μ i several gaskets have been eliminated. The full height of the incubator is only 20 mm taking into account the thermal regulator K. The Autocad file with the detailed drawings of the incubator can be sent to the reader if required.

The global price for this m μ i comprising the temperature control system and the cost for the milling is approximately 500 Euros. The m μ i is much cheaper than many available commercial systems.

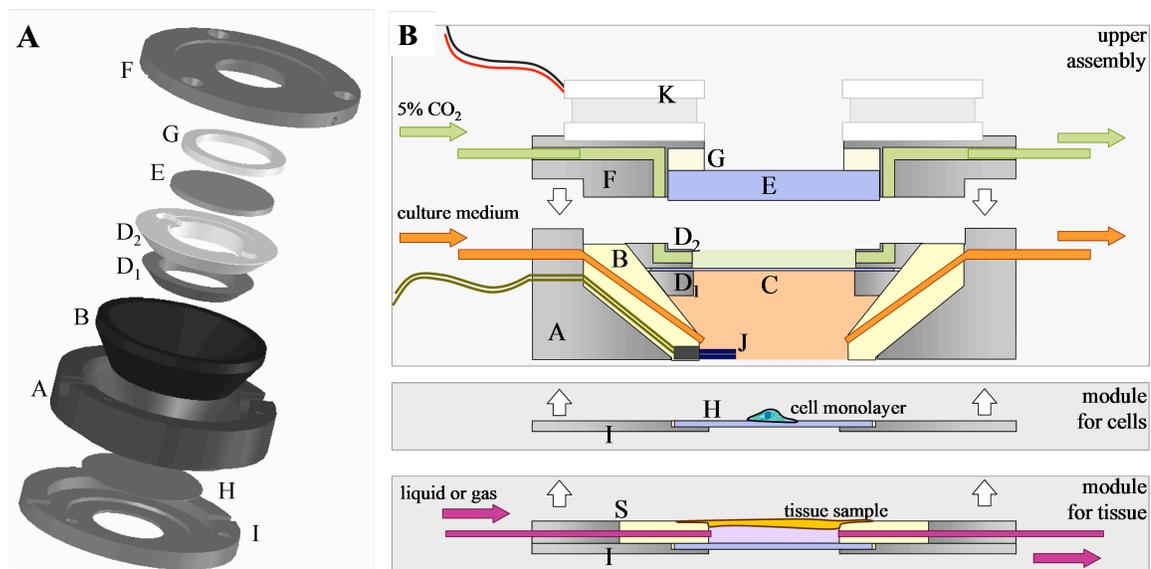


Figure 1: A) Exploded view of the the microincubator. B) Schematic of a cut of the modular microincubator, the upper part, the middle part and the two versions for the lower part are separated to make the visualisation easier. Legend: A stainless stell container, B silicone conical ring, C permeable membrane, D stainless steel rings, E circular glass cover, F incubator lid, G elastomer gasket, H circular substrate, S optional elastomer spacer, I incubator base, J platinum resistance thermometer, K thermal regulator.

Description of the incubator's elements

A. An open **stainless steel container** (height 7 mm, outer diameter 36 mm), is machined to accommodate the silicone ring B. The container confers rigidity and conducts the heat all around the incubator. Compared to brass or aluminium, stainless steel is a good compromise in terms of oxidation resistance and thermal conductivity.

B. The **silicone conical ring** (flexiPERM ConA, Greiner bio one) is the core of the incubator. It defines a cell growth cavity filled with culture medium and an upper adjacent gas-filled cavity used for the aeration of the medium. The silicon ring acts as a gasket as well between the majority of the elements. Two Teflon capillaries, 0.8 mm in diameter are inserted in the silicone ring B. These capillaries allow one to inject or circulate the culture medium as required. To mount these capillaries a syringe needle is used to pierce two small holes in B along one diameter through which the capillaries are inserted. The elasticity of B ensures the sealing around the capillaries. The portion of each of these capillaries which is outside B is protected by thick resistant silicone tubes mounted around the capillary and clamped between the stainless steel ring A and the incubator lid F.

C. A **permeable membrane** (biofolie 25, Greiner bio one) separates the 2 mm thick liquid cavity from the gaseous cavity. The height of the liquid cavity is similar to the height of culture media normally above cells in routine non-perfused cell culture. On the lower side of the membrane the pH of the sterile medium is maintained at a physiological level due to the circulation on the upper side of the membrane of a 5% CO₂ atmosphere. The incubator can also be placed upside down and mounted on an upright microscope with the liquid cavity above the gaseous cavity.

D. The membrane is clamped between two **small stainless steel rings** (a lower ring D₁ and an upper ring D₂) pressed against the internal wall of the silicon ring B. The external conical shape of the rings defines the vertical position of the membrane/rings sandwich.

E. A 1 mm thick **circular glass cover** (cut from a standard microscope slide) is centered on top of the upper ring. The gaseous cavity is thus contained between the membrane and the glass. The gas circulation is carried out through two holes drilled in the lid F which sits on top of two apertures machined in the upper ring D₂.

F. The **incubator lid** covers the circular glass E, the small upper ring D, the upper surface of the silicon ring B and the container A. The gaseous cavity is partly sealed at the interface between the lid and the silicon ring and partly sealed at the interface between the lid and an elastomer gasket G (polydimethylsiloxane, sylgard 184, Dow Corning). The lid is fixed against the container A with three countersunk M2 screws.

G. The **elastomer gasket** (polydimethylsiloxane, sylgard 184, Dow Corning) is mounted between the lid and the circular glass E.

H. Cells are cultured onto a **circular substrate**, 19 mm in diameter, composed of any material thinner than 500 µm. Unlike culture chips, there are no constraints on the choice of the mechanical, physical and chemical properties of the substrate. From standard rigid round glass coverslips, functionalized as desired, to a thin stretchable elastomer membrane, one can use chemically or topographically micropatterned substrates [7] or even superimpose two different materials such as a coverslips and electrospun fibres (see supplementary document). For study in reflection mode of a **3D tissue**, the substrate is simply replaced by the sample of interest with an additional elastomer spacer S and a cover slip.

S. The **optional elastomer spacer** creates an additional cavity useful for the study of 3D tissue. One side of the tissue sample is in contact with this additional cavity while the other side is in

contact with the cavity formed by the silicone ring B. In this configuration an additional stainless steel ring the same height as the elastomer spacer is mounted around it. As desired, medium, dye or gas can be injected or circulated within the additional cavity through two Teflon capillaries connected to the protective thick silicone tubes.

I. The **incubator base** machined in stainless steel closes one side of the system. The substrate or the tissue sample is clamped between the base I and the silicone ring B. The base is fixed against the container A with three countersunk M2 screws.

J. A **platinum resistance thermometer** (Pt 1000, 1266941 Farnell) is fixed on the edge of the silicone ring within a small drop of elastomer. The sensitive part of the sensor, protected by a thin layer of elastomer, comes directly in contact with the substrate or the tissue sample. The temperature measured is thus the temperature sensed by the specimen.

K. The **thermal regulator** is a central hole Peltier stage (RH1.4-14-06L Melcor) the upper side of which is connected to an aluminium heat sink. The thermal regulator is mounted on top of the lid (with a thin layer of thermal grease in between) and powered through a PID (proportional integral derivative) controller (MTTC-1410 Melcor) with a feedback loop based on the signal given by the temperature sensor J.

Preparation of the incubator

Prior to first use, the upper elements of the m μ i, A to G and J are put together in an easy-to-handle assembly. Afterwards this assembly is unmounted only every ten experiments to change the membrane C. On this occasion, all the above mentioned parts are thoroughly cleaned, first with pure water, then with ethanol and finally brushed with soap. Parts are again cleaned with water, ethanol, and finally rinsed with water. Parts are then reassembled and autoclaved.

If required the m μ i can also be operated in an "open-dish" version on an inverted microscope. For this purpose one removes the membrane C, the rings D, the elastomer gasket G and the glass E from the upper assembly. One can then access the sample through the aperture of the lid F.

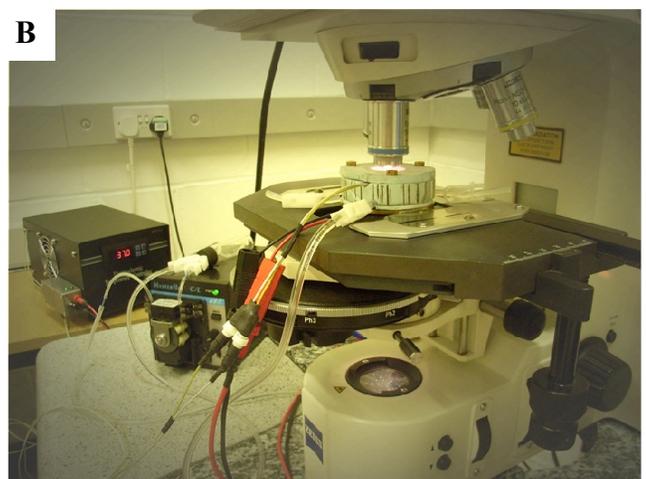
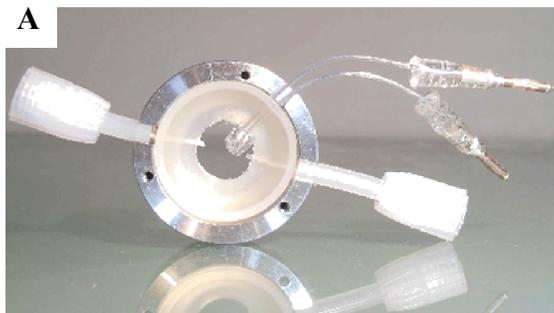


Figure 2: A) View of the stainless steel container A and the silicone conical ring B with the Pt temperature sensor J fixed to its side. B) View of the experimental setup with the micro-incubator enclosed in solid insulating foam. The thermo controller is at the back and the peristaltic pump for the CO₂ circulation is in the middle of the picture.

RESULTS

The m μ i for the observation of cell monolayers

Our system can be used as a complete cell incubator (into which cells grow *de novo* after initial seeding) as well as a microscope incubator (into which cells are transferred after culture in a

routine way). As such our system offers more possibilities than a standard microscope incubator.

Cells cultured *de novo* in our microincubator are maintained in the same culture conditions from start to end, especially during the observations on the microscope. With a standard microscope incubator cells are submitted to a sharp change of environment during their transfer from the laboratory incubator into the microscope incubator. This change of environment can induce a bias on the cell behavior. Such a bias is avoided with our microincubator.

For a culture *de novo* in the m μ i, the user places the substrate which has been chosen at the centre of the base I and adds the upper assembly on top of it. The base I and the upper assembly are bound together with screws. If the substrate can bear high temperature the m μ i is sterilized by autoclaving once assembled. If the substrate is sensitive to temperature the m μ i (part I and upper assembly separated) is presterilized and assembled with the substrate in a sterile environment. Once the m μ i is sterile, cells suspended within the culture medium are injected in the silicone ring B cavity with the capillaries. The air contained in the cavity needs to be removed if the m μ i is to be used on an upright microscope. Before placing the chamber in the desired orientation, the system is left on its base for 20 min so that the cells adhere to the substrate at the physiological temperature (37°C). Cell can be cultured in the m μ i for several days until they reach confluency, as illustrated in section 1 of the supplemental document.

Although culture conditions are better controlled with a culture *de novo* in the m μ i, it may be useful to prepare several cell cultures in a standard incubator and then to place the best sample within our incubator for observations on a microscope. In this configuration, cells are moved into the m μ i on their initial substrate without any passage. The substrate needs only to be thinner than 500 μ m and circular (19mm in diameter). The m μ i is presterilized. Under sterile conditions, the substrate with the cells is rapidly placed at the center of the incubator base I. The upper assembly of the m μ i is screwed on top of the base and the chamber is filled with liquid through the capillaries.

Whatever the type of culture chosen, complex substrates can be used. Patterned substrate [7] or composite substrate (see supplemental document section 1) can easily be implemented into the m μ i. Deformable or stretchable substrates are also perfect candidates for the m μ i. As described in section 2 of the supplemental document, cells attached onto a stretchable substrate in the m μ i can be stressed in a well controlled manner within a well defined environment.

The m μ i for the study of tissue samples

Study of cell monolayers is particularly useful to understand cell behavior, especially at the single cell level. However tissue samples with a 3D arrangement of cells are far more realistic from a biological point of view and their study is particularly instructive if medical applications are envisioned. The m μ i has been designed to accommodate tissue samples.

For the study of a tissue sample, the upper assembly, the base I and the elastomer and stainless steel spacers S are sterilized separately. Under sterile conditions, the upper assembly is placed upside down and the cavity located above the membrane, in this configuration, is filled with the required media. The sample is then gently deposited onto the surface of the media, taking care not to trap any bubble below the sample. Once the sample completely covers the silicone ring B, the elastomer spacer S is placed on top of it. The stainless steel spacer is added around the elastomer spacer and the system is closed placing a coverslip between the elastomer spacer and the base I. The system is bound with screws through the stainless steel spacer.

The m μ i can be used to study the delivery, the transport and the impact of bioactive molecules through a complex tissue sample. The study of the diffusion within a tissue of delivery vectors such as polymersomes is, for instance, the type of experiment that can be carried out using the m μ i (see supplemental document section 4). The m μ i is an experimental tool which offers the possibility to quantify the diffusivities and absorption constants within a tissue sample.

Adjustment of the chemical environment

The μ pi has been designed to be able to switch easily from one medium composition to another using the injection capillaries. The μ pi has also been designed with a volume of medium in the silicon ring large enough to be able to maintain the same chemical composition for a long time. Without any circulation, a cell monolayer is able to grow normally for 3 days and a tissue sample for roughly 12 hours. The specimens can thus be cultured in the absence of shear stresses, which are generally present in a culture chip [8]. Moreover the risks of contamination are notably limited when the sterile medium is not circulated.

Nevertheless for many applications the medium composition will need to be changed. With the injection capillaries the medium contained in the incubator can easily be renewed to be able to work along extended time course or chemically changed if needed. One can typically study the response of the cells to the addition of a bioactive species (see supplemental document section 4) and more generally study the sensitivity of the cell to their chemical environment in terms of chemical, physical or mechanical response.

Even if the medium is not circulated, it needs to be aerated at least to control the pH level. For this purpose a 5% CO₂ atmosphere is circulated on the upper side of the membrane. An empty medium bottle (known to be not permeable to CO₂) is used as a container and filled with the controlled atmosphere provided in any standard laboratory incubator. It is worth keeping in mind that the gas circulation system does not need to be sterile thanks to the presence of the permeable membrane. The atmosphere is circulated through the μ pi with a peristaltic pump (PMM-500-030Q, Fisher) at a low flow rate of 0.1 mL min⁻¹. Low CO₂ permeability tygon tubes are used to connect the gas container to the μ pi through two small Peek tubes mounted into the lid F.

If the μ pi is used with a tissue sample, the chemical compositions on each side of the tissue sample can even be changed independently. The spacer S permits indeed the addition or the circulation of a gas or a liquid on the lower side of the tissue which may be different from the liquid contained in the silicone ring B. (see supplemental document section 4)

Control and change of the thermal environment

In order to control the temperature precisely, the thermometer is pressed against the substrate or the tissue sample when the microincubator is closed. The measured temperature is then the same as the temperature sensed by the cells.

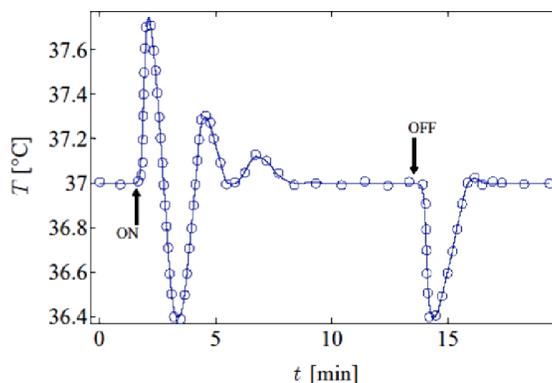


Figure 3: Thermal response of μ pi when the halogen lamp of the microscope is suddenly switched on full power and then switched off. Each transient regime lasts less than 5 min.

In the steady-state regime, the temperature can be adjusted with fluctuations limited to less than 0.05°C.

Sudden heating or cooling subsequent to a change in the experiment configuration (injection of fluid, lighting, contact with an oil immersion objective, etc.) are efficiently corrected (see figure 3). As the thermal regulation is maintained with the use of the Peltier stage, working temperatures higher or lower than ambient temperatures can be chosen.

A controlled change of temperature can as well be applied in order to study the thermal sensitivity of a tissue or cells. Transient behaviors can be characterized during cooling from a physiological temperature or heating toward a physiological temperature (see supplementary

document section 3).

DISCUSSION

The aim of this work was to develop an inexpensive reusable microincubator for non-invasive microscopy studies of living biological specimen. Unlike most commercial systems the microincubator was designed to accommodate various kind of specimens, from 2D cell monolayers to 3D tissues samples. The m μ i provides a precisely controlled environment, with a specific thermal regulation: on the one hand the temperature is measured in the vicinity of the specimen itself, on the other hand the temperature is adjusted using a Peltier stage. Compared to other available systems this particular thermal regulation offers a very fine control of the temperature of the specimen itself, both in static and transient thermal regimes. The presence of a permeable membrane as a separator between the culture medium and the gaseous atmosphere is another specificity of our system. Thanks to this membrane our microincubator can be used in any orientation and the sterility of the liquid region in contact with the cell is more easily maintained.

Mammalian cells and more generally any living samples are dynamic systems characterized by a broad range of time scales. The m μ i allows us to access this dynamic behavior with studies of mechanisms which occur over intervals as short as tens of ms, typically associated with the local intra-cellular activity, or as long as several days associated with the formation of multicellular structures.

A particular feature of living systems is their capability to adapt to their environment [1]. Thanks to sensitive perception and regulation mechanisms, living systems are able to react when their environment changes. The m μ i allows us to mimic changes in the environment by mechanical stresses with isotropic stretching of adherent cells, by sudden temperature changes, or by chemical stresses with changes of the culture media composition. Essentially the environment can be modified as desired while the specimen is visualized on a microscope. Thus it is possible to study in real time the underlying mechanisms which generate transient regimes of adaptation of cells or tissues to a new environment.

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COMPETING INTERESTS STATEMENT

The authors declare no competing interests.

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