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Microtiter plate-sized standalone chip holder for control of physiological conditions inside closed microfluidic cell culture systems, made from gas-impermeable materials.
Microtiter plate-sized standalone chip holder for microenvironmental physiological control in gas-impermeable microfluidic devices

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We present a microtiter plate-sized standalone chip holder for precise control of physiological conditions inside closed microfluidic cell culture systems, made from gas-impermeable materials. Specifically, we demonstrate the suitability of the holder to support cell growth in a glass chip, to allow time-lapse imaging of live cells and the creation of a hypoxic environment, all relevant for applications in regenerative medicine research.

Microfluidic systems are successfully applied to create tailored chemical and physical microenvironments with high fidelity at the micrometre scale, enabling in-depth analysis of cell behaviour, in a milieu that mimics their natural biological environment. As such, microfluidic systems have become highly relevant tools for fundamental (cell) biological studies, diagnostics, and pharmacological screens. Furthermore, as we recently discussed, microfluidic systems may become highly relevant tools for fundamental (cell) biological studies, diagnostics, and pharmacological screens. As such, microfluidic systems have become highly relevant tools for fundamental (cell) biological studies, diagnostics, and pharmacological screens. We present a microtiter plate-sized standalone chip holder for precise control of physiological conditions inside closed microfluidic cell culture systems, made from gas-impermeable materials. Specifically, we demonstrate the suitability of the holder to support cell growth in a glass chip, to allow time-lapse imaging of live cells and the creation of a hypoxic environment, all relevant for applications in regenerative medicine research.

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Chip holder

The chip holder consists of an aluminium chamber, into which the chip can be inserted, allowing for fluidic interfacing using a manifold connected to an array of capillaries. The whole platform was designed, fabricated and assembled in-house. Aluminium was chosen for its high thermal conductivity and machinability. The whole aluminium chamber is used as a heat sink and is kept at a stable temperature using two square 20 mm Peltier elements (MULTICOMP, Farnell, The Netherlands) placed on either side of the chamber, and each containing an attached outer heat sink (Fig. 1A). The chamber and heat sinks are thermally insulated using polyoxymethylene (POM) spacers. Since the whole device is precision milled, it is fully symmetrical and involves a relatively small temperature difference (37°C - RT = 12 K), with negligible temperature gradient over a distance of 1 mm, as measured using conventional probes (<±0.05°C). To ensure stable temperature and fast response, the Peltier elements are powered by a computer programmable controller, capable of delivering pulse-width modulated and polarizable output (TCM, Electro Dynamics Ltd, Southampton, United Kingdom). The feedback temperature sensor (NTC) is placed on the side of the chip (Fig. 1B-C) not to block the light, and an offset of 0.8 K was determined using an external temperature probe near the micro chamber. This resulted in a stable temperature at a desired value (37°C) with a tested SD of ± 0.003°C over a period of 20 h. This feature is particularly important for the stability of the cell culture, and imperative for the stability and predictability of the fluidics.

Gas exchange is ensured through the microfluidic connections with the chip inside the closed chip holder, using perfluoroalkoxy (PFA) capillaries (150 µm ID and 360 µm OD PFA HP Plus, DuPont, USA), each having an internal volume of 6 µL (Fig. 1B-C). By controlling the flow rate through the tubing, the residence time of the medium inside the tubing can be set to ensure gas equilibrium is reached before medium enters the chip (for detailed calculation to determine minimum residence time, please see supplementary information). Fused silica capillaries (200 µm ID and 360 µm OD, PolyMicro, France) are used for outlet connections and collection of (waste) medium, since medium is passed only once. Even though fluoropolymers are not known for their inherent gas-permeability, they provide a good compromise between chemical resistance, thermal stability and gas exchange. The gas mixture is provided by 5% CO2 supplemented compressed air (Linde Group, The Netherlands) and reduced oxygen mixture is obtained by partial mixing with N2. To provide sufficient humidity, to avoid possible evaporation through the PFA tubing (Fig. 1D), the gas mixture is flown through a water bubbler before entering the chip holder.

The whole platform has the footprint of a microtiter plate (127.5 mm x 85.5 mm) and a height of 32 mm. These dimensions make it compatible with a variety of read-out equipment meant for microtiter plates. For live imaging, the chip holder is placed in a microtiter plate microscope (BD Pathway 435, USA) capable of fully automated fluorescence and bright-field microscopy (Fig. 1D) or in a fully automated DIC/confocal microscope (NIKON Ti-Eclipse with A1 confocal, Japan).

Glass chips

Two different chip designs were tested to demonstrate the effectiveness of the platform: (i) a device with two 650 µm x 4 mm rectangular chambers connected to two supply channels (Fig. 2A), via an array of 1 µm x 3 µm x 10 µm channels; and (ii) a device with a 650 µm square chamber surrounded by four supply channels (Fig. 2C), also connected to the chamber by a similar array of microchannels (Fig. 2D). Thereby, diffusion is dominant over convection between the channel and the chamber due to the high relative resistance of the connecting channels. This configuration allows for shear stress-free culture of cells, which is essential for delicate human cells.
The microdevices were fabricated in glass, as described in detail in the online supplementary information. In short, the microfluidic features of the chip were fabricated in 0.21 mm glass, using an elaborate two-step wet etching process to successfully create structures with two different heights. Thereafter, the glass substrates were thermally bonded with 1.1 mm glass substrates with powder blasted via-holes. Finally, devices were diced in 2 cm square dies (Fig. 2B).

Fig. 2 (A) Schematic diagram of the rectangular chamber, showing the flow directions in the supply channels and cell loading channels, with as inset an enlargement and side-view of the small channel array. (B) Photograph of the 2 cm square die with two rectangular chambers. (C) Schematic diagram of the square chamber. (D) Bright-field microscopy image of a glass chip, depicting the chamber with the surrounding supply channels, with as inset an enlargement of the smaller channels that connect the supply channel with the chamber.

Cell culture

To demonstrate the possibility to perform live or time-lapse imaging, murine myoblastic C2C12 cells were cultured over 2 days and imaged using an automated DIC microscope (Nikon Ti Eclipse). C2C12 cells were cultured in Dulbecco’s-Minimum Essential Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 100 mg mL⁻¹ penicillin and 100 mg mL⁻¹ streptomycin (Pen/Strep, Invitrogen). After expansion culture, they were dissociated using trypsin (Invitrogen), resuspended to a concentration of approximately 5 million cells ·mL⁻¹, and injected into the chip using a syringe through the cell loading channels into the chambers. Cells were left to proliferate for 2 days, while flowing cell culture medium in the supply channels (single pass), at a flow rate of 8 µL·h⁻¹ using a precession syringe pump (Nexus Performance, Chemyx, USA). Fig. 3A presents snapshots taken from a 2 day time-lapse series on the growth of C2C12 cells in the square chamber at different time points: at time point 0, just after loading of the cells, and at time points 22 and 48 h, where the attachment and spreading of cells can be observed, as well as the formation of a cell monolayer.

To demonstrate culture of more delicate human cells, osteoblastic MG-63 cells were cultured for 2 days, subsequently fluorescently stained with Phalloidin (cytoskeleton, F-actin) and DAPI (nuclei), and imaged by fluorescence confocal microscope (Nikon Ti Eclipse with A1). MG-63 cells were cultured in αMEM supplemented with 10% FBS and Pen/Strep. After culture, cells were rinsed with phosphate buffered saline (PBS, Invitrogen), and fixed with 4% paraformaldehyde (PFA, Sigma-Aldrich) for 10 min, before being stained in situ by flushing the staining solutions through the chamber via the cell loading channels. As shown in Fig. 3B, MG63 cells were successfully stained inside the chip, which demonstrates the suitability of the platform for in situ cell staining and fluorescence imaging.

Viability assay

Cell viability was assessed using a standard live/dead assay on Chinese hamster ovary (Cho) cell line, cultured in DMEM with 10% FBS and Pen/Strep. All channels and the chamber were first flushed with PBS, and subsequently a mixture of 2 µM calcein AM and 4 µM ethidium homodimer-I (EthD-I, Invitrogen), to stain live and dead cells respectively, was introduced into the chamber via the cell loading channels, and left for 20 min, before washing again with PBS. Samples were imaged using an epi-fluorescence microscope (TS100, Nikon, Japan), and overview images created by stitching multiple images together. After 5 days of culture, in the rectangular culture chamber, the large majority of cells were found viable, with few dead cells homogenously spread through the chamber (Fig. 3C-D), similar to conventional cell culture, proving that cells could be kept viable inside the platform for multiple days. Due to the seeding method, the entrance and exit of the chamber show slightly more red fluorescence due to cell debris flowing back from the dead-volume of the loading channels.

Fig. 3 (A) Bright-field microscopy images taken from 2 day time-lapse series of C2C12 cells at different time points, with t=0 taken just after cell loading. (B) Confocal fluorescence microscopy image of MG63 cells in the square chamber after 2 days of proliferation, with stained nucleus (DNA, DAPI, blue) and cytoskeleton (F-actin, Phalloidin, green). (C) Bright-field microscopy stitched image of the rectangular chamber with Cho cells after 5 days of culture. (D) Epi-fluorescence image of the same Cho cells with live/dead staining, live cells being green and dead cells red.

Hypoxia assay

Oxygen is a critical nutrient for cell survival, which is likely the first one to become limited due to its high uptake and low solubility. Therefore, it is frequently used as a model nutrient. When cells are subjected to low oxygen conditions (hypoxia), numerous genes are up- or down-regulated, like angiogenesis related genes to promote cell survival. To study this, a Cho cell line (Cho HRE-GFP cells)
transfected with a green fluorescence protein (GFP) hypoxia reporter, was employed to demonstrate the ability of our microfluidic platform to recapitulate normal and hypoxia conditions. The cells fluoresce in case of limited availability of oxygen. More specifically, hypoxia-inducible factor-1 (HIF-1) translocates into the nucleus and binds to the hypoxia responsive element (HRE) promoter, thereby activating GFP transcription (for details on transfection and clonal procedure, please see the earlier work by Liu and co-workers).13

Cho cells were cultured in the chip, under normal (20%) and reduced (4%) oxygen conditions inside the chip holder, using regular culture medium supplied at a flow rate of 1 µL·h⁻¹ through the supply channels. Under these conditions, the residence time of 6 h, was sufficient for the medium to reach equilibrium through the PFA tubing. The cells were introduced in the chamber by the cell loading channels and left to attach under normoxic conditions for 8 h. Cell culture either continued at 20% O₂, or the culture condition was switched to hypoxic, whereby the gas mixture flowing in the chip holder contained approximately 4% O₂. After only 22 h of culture under hypoxia, cells showed increased expression of green fluorescence protein (Fig. 4D-F), demonstrating that by varying the oxygen level inside the chip holder, cells in the culture chamber of the chip are affected. In contrast, after 48 h of culture in normoxia, limited or no green fluorescent was observed (Fig. 4A-C), showing that under normal conditions, the cells inside the gas-impermeable glass chip have sufficient amount of oxygen not to turn to a hypoxic state. These results showed that the platform presented here can be utilized in a standalone fashion with chips made of gas-impermeable materials and that gas composition inside the chip can be varied for mimicking specific in vivo conditions, like hypoxia.

Conclusions

Here we have presented a standalone chip holder, particularly meant for microfluidic platforms made from gas-impermeable materials, to control the cell physical microenvironment without the necessity of using a separate incubator. The system is compatible with standard imaging laboratory equipment, making it suitable for live or time-lapse imaging. Various adherent cell types have been successfully cultured over a period of at least 5 days, and both stained and imaged in situ. The platform also proved to be suitable to control gas tension, e.g., to induce hypoxic conditions inside the microfluidic chip to reproduce in vivo conditions. In the future, this chip holder will particularly be applied to screen interactions between cells and biomaterials for regenerative medicine applications inside microfluidic platforms.

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Notes and references