OPTICAL CLEARANCE OF SPHEROIDS ON CHIP

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ABSTRACT

Spheroidal clusters of cells are mimicking physiological tissues, while retaining the advantages of in vitro cell culture in terms of simplicity and reproducibility.[1] Here, we integrated an optical tissue clearing process, named CLARITY[2] on a microfluidic large-scale integration (mLSI) chip platform in order to facilitate rapid immuno-staining of proteins in spheroids. With this method we overcome the drawbacks of densely packed lipid bilayers that act as diffusion barriers for molecular probes and the limits of optical penetration of light into spheroids.

KEYWORDS: Spheroids, CLARITY, Microfluidic large-scale Integration, Immuno-staining

INTRODUCTION

The potential of spheroids for *in vitro* screening has been widely recognized, and recent developments in the field of microfluidics have shown that spheroids can be rapidly and reproducibly formed on chips.[3]

For analysis of biomolecules in deeper tissue structures, however we are confronted with an optical and diffusion problem. Densely packed lipid bilayers are diffusion barriers for molecular probes, so antibody-based protein staining methods lead to inhomogeneous labeling in the deeper layers and can require days to clear non-specific binding. Additionally, photons scatter strongly at the lipid-aqueous interface of the cell membrane, which limits the optical penetration of light into cell spheroids or tissue to depths of 50-100 µm. To solve this problem we integrated a recently developed tissue clearance method, named CLARITY[2], on a mLSI chip. Spheroids used for clearing are formed by human derived adipose stem cells (hASC) and feature a size of about 250 µm in diameter.

THEORY: mLSI chip

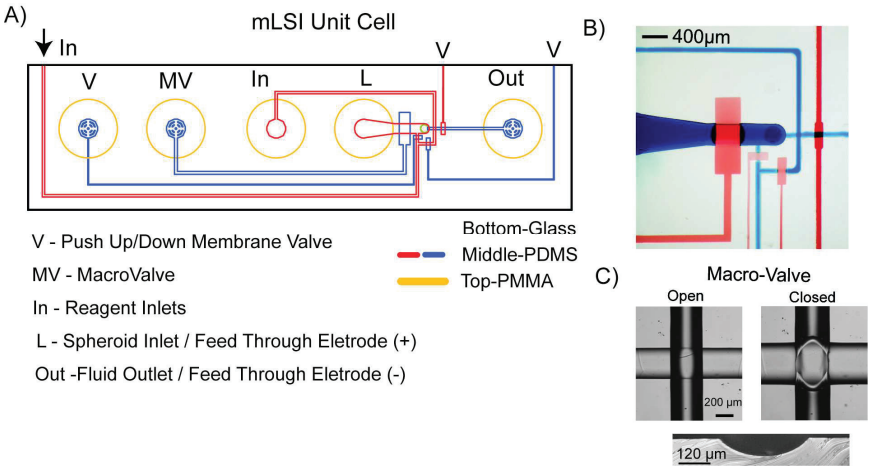

Figure 1: Design of the microfluidic large-scale integration chip platform. A) Flow logics of a unit cell, which can be arrayed on a mLSI chip. B) Real chip image. C) Example of a 'MacroValve', with a size of 360x360 µm for spheroid loading.

Figure 1 shows the microfluidic chip used to perform the clearing of the spheroids. The chip was prototyped in PDMS, where the molds were manufactured from PMMA by precision milling. This was required in order to manufacture semi-half round channels for the spheroid inlet channels with a height and width > 200 µm. A 'Macrovalve' with a size of 360x360 µm in each unit cell allows the loading of the spheroid into the microfluidic chip. With increasing spheroid size the height of the loading channel has to be increased. The height of positive resists in standard photolithography is limited to ~100 µm.

EXPERIMENTAL

The clearing procedure on the microfluidic chip consists of four main process steps. Figure 2 shows those steps for the spheroids integrated on chip. 1) Spheroids are loaded, trapped and perfused. 2) Spheroids are fixed with a hydrogel solution. Polymerization and cross-linking of the cellular content to the embedding hydrogel is achieved by increasing the temperature. 3) Lipid molecules are excluded from the crosslinking step and are extracted. 4) Thereafter, the refraction index difference on the cell membranes is reduced, and probes can access deeper layers.

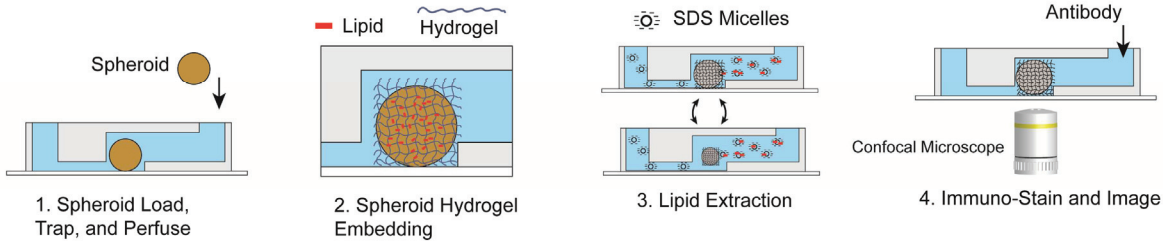

Figure 2: Process overview for optical clearing of spheroids on a microfluidic large-scale integration chip platform with (i) spheroid trapping, (ii) fixation and hydrogel embedding, (iii) lipid extraction and (iv) protein staining.

After introduction of the spheroids formed by human adipocyte stem cells by standard procedures[1], they are trapped in a large round chamber that penetrates through both layers of the microfluidic chip, where the outlet channel has only a height of ~40µm. Afterwards, the spheroids are perfused with paraformaldyhde (4%) together with the hydrogel monomer solution (10% Acrylamid/Bis) for 2h. After a wash step with PBS the thermal polymerization initiator VA-044 in PBS buffer was introduced to crosslink all proteins and DNA. Spatial hydrogel polymerization within the spheroid took place upon raising the chip temperature over 37°C for 3h. The lipid content from the spheroid was removed by alternating the pH value of the media surrounding the spheroids, which leads to a change in the osmotic pressure and thus induces a swelling and shrinking on the spheroids. The spatial order of the biomolecules is not affected by the 'pumping' in size.

RESULTS AND DISCUSSION

The aforementioned swelling is characteristically shown in Figure 3 during one cycle by alternating the pH value of the surrounding media. The osmotic pressure leads to a swelling of the spheroid tissue at pH 8.5 and shrinking at pH 5.5. The cycling of the pH decreased the required time to clear the tissue from 14 days to 24 hours, which corresponds to 40 pH cycles.

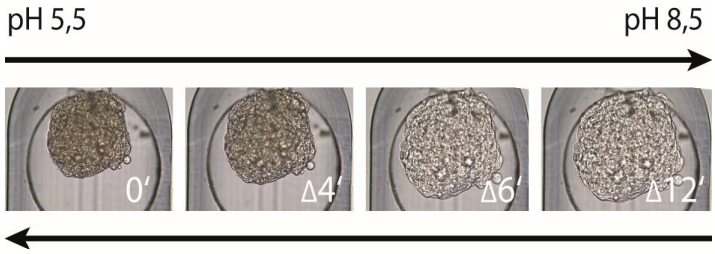

Figure 3: Alternating the surrounding pH value in order to swell and shrink the spheroids in one cycle.

After clearing the spheroids on chip, we stained them with GAPDH for 1 h and compared how deep the antibody penetrates through a cleared and a non-cleared sphere. Figure 4 shows brightfield and fluorescence images of both types of spheres and shows that the penetration depth of the antibody is much higher on a cleared sphere.

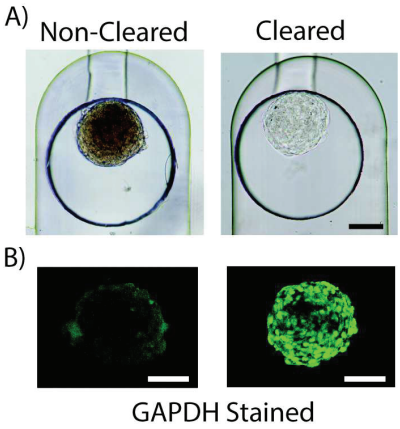

Figure 4: Brightfield and confocal fluorescence image of cleared and non-cleared spheroids. After clearing, the spheroids were immuno-stained with a GAPDH antibody for 1 h in PBS. Images were taken 150 µm deep in the spheroids. All scale bars correspond to 100 µm.

CONCLUSION

We successfully integrated the process steps for rapid optical clearing of spheroids on a microfluidic chip platform. We demonstrated that it is possible to crosslink the cellular content of cells to a hydrogel and extract the lipid membranes. In future perspective we aim to use the chip for quantitative protein analysis in 3D tissue modes my immunofluorescence microscopy

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