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DOI
10.1109/MEMSYS.2018.8346549

Publication date
2018

Document Version
Final published version

Published in
2018 IEEE Micro Electro Mechanical Systems, MEMS 2018

A MULTIWELL PLATE ORGAN-ON-CHIP (OOC) DEVICE FOR IN-VITRO CELL CULTURE STIMULATION AND MONITORING

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ABSTRACT

This work presents the first multi-well plate that allows for simultaneous mechanical stimulation and electrical monitoring of multiple *in-vitro* cell cultures in parallel. Each well of the plate is equipped with an Organ-on-Chip (OOC) device consisting of a stretchable micro-electrode array (MEA). For the first time, a film assisted molding (FAM) process was employed to embed an OOC into a multi well plate format packaging. The functionality of the MEA in the device was assessed with electrochemical impedance spectroscopy. Moreover, the biocompatibility of the plate was demonstrated with cardiomyocytes derived from human induced pluripotent stem cells (iPSC) cultured in the wells.

INTRODUCTION

Pharmaceutical companies are currently relying on cell-based assays and animal models in their preclinical tests in order to predict drug responses in humans. However, these models do not always fully capture the human physiology and pathology, and are thus not sufficiently predictive [1]. Organ on Chips (OOC) are much more sophisticated *in-vitro* models that aim at improving the prediction capability of cell-based assays [1,2].

OOCs combine micro-fabricated chips and cell cultures. The dynamic environment provided by the chip mimics the physiological conditions in a functional unit of a human organ. The chip could facilitate growth, proliferation, differentiation and maturation of the cells, which may then better recapitulate *in-vivo* human responses [3].

However, the fabrication and assembling methods employed are very often time consuming and heavily dependent on manual assembly [2,3], which conflicts with the need of high throughput screening. Moreover, it is becoming more and more clear that the integration of sensors will be a crucial feature to monitor and acquire quantitative and qualitative data from the cell cultures [2,3]. Last but not least, OOCs are often not user-friendly and are not compatible with standard biological work-flows [4].

Cytostretch is a modular platform for OOC applications [5] previously applied as a Heart-on-Chip model by Pakazad et al. [6]. Unlike the OOCs previously developed, the Cytostretch chip is based on conventional cleanroom-compatible micro-fabrication processes, thus avoiding the labor-intensive fabrication steps often required in other OOC models. The Cytostretch key feature lies in the mechanical stimulation of the cell cultures [4] by means of a stretchable membrane. Moreover, due to the silicon based fabrication process, it can be easily equipped with a micro-electrode array (MEA) to record the electrical activity of the cells [5].

However, the initial concept still needed several manual handling steps. In fact, to culture, stimulate and monitor a cell monolayer, the Cytostretch needed to be manually loaded and unloaded in a system composed of a printed circuit board (PCB), multiple screws and a plastic well [6]. The assembly of the system drastically reduced the ease-of-use and might affect the reproducibility of the results. Last but no least, the previously used system was not compatible with a multi-well plate format and thus only useful for low throughput experiments.

Here we propose the integration of multiple Cytostretch chips on a novel multi-well plate (Fig. 1). The multi-well plate is in fact realized using film assisted molding (FAM) [8], a straightforward and monolithic packaging technique, which allows the formation of wells directly and automatically on top of the chip and the PCB. The resulting device allows for simultaneous mechanical stimulation and electrical monitoring of the cells. The multi-well plate was designed in order to be compatible with conventional readout equipment [9].

In order to mount four chips on a single plate, the Cytostretch chips were re-designed to a new format. The electrochemical performance of the MEA embedded in the new chip was characterized in order to assess its functionality. Moreover, in order to prove the biocompatibility of the chip, a preliminary biological assessment was performed by culturing human iPSC-

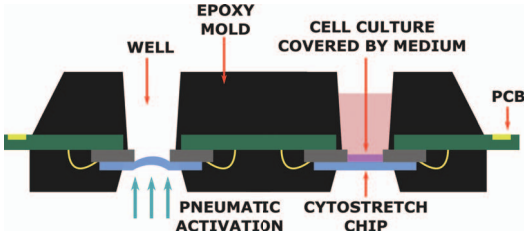

Figure 1: Cross section of the plate composed of Cytostretch chips glued and wirebonded on a Printed Circuit Board (PCB) and covered with molded wells. The stretchable membranes on the Cytostretch chips can be actuated by applying a pneumatic pressure to the backside of the plate.

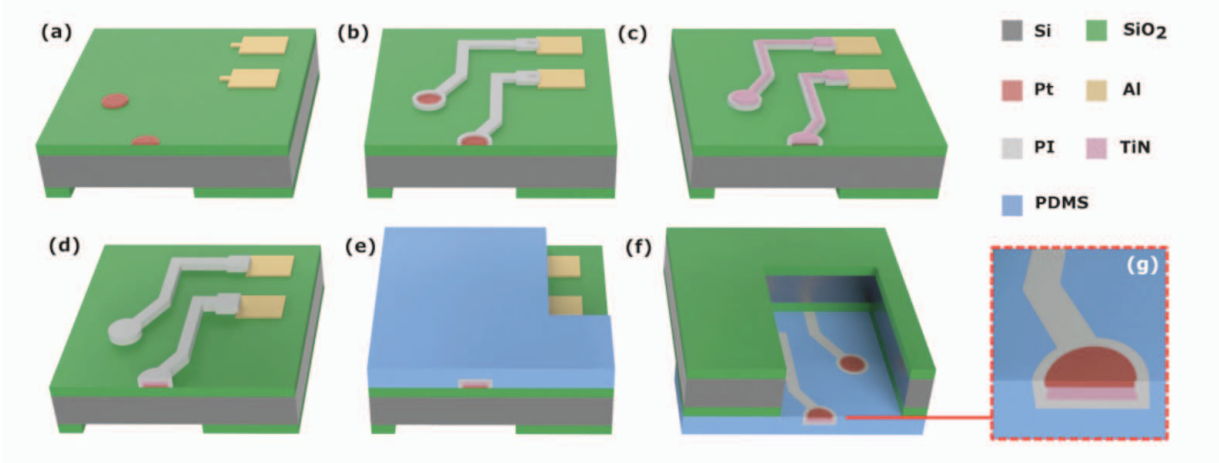

Figure 2: Process flow for the fabrication of the Cytostretch chips on Silicon (Si) wafer: (a) contact pads and Platinum (Pt) electrodes are fabricated on top of a 2 µm Silicon Oxide (SiO_2) layer. A 5 µm-thick SiO2 layer is deposited and patterned on the back of the wafer. (b) A 1 µm layer of Polyimide (PI) is deposited and patterned. (c) The 200 nm thick Titanium Nitride (TiN) metal lines are fabricated. (d) The second layer of PI is deposited and patterned to isolate the metal lines. (e) A Polydimethylsiloxane (PDMS) layer is deposited and patterned to access the contact pads. (f,g) The PDMS membrane is released by etching the Si under the PDMS layer making the electrodes accessible.

derived cardiomyocytes (Pluricyte® Cardiomyocytes) in the wells and monitoring them for seven days.

MULTI-WELL PLATE FABRICATION

Chip Manufacturing

The Cytostretch chips are fabricated on 4 inch silicon wafers. The process starts with the deposition of 2 and 6 µm of silicon oxide (SiO_2) by plasma-enhanced chemical vapor deposition (PECVD) on the front and back of the wafer, respectively. The SiO_2 layer on the back is patterned by dry-etching to define the membrane area. The process continues by sputtering a 1.5 µm-thick aluminum (Al) layer on the frontside of the wafer. The Al is then patterned by dry-etching to define the contact pads. Then, a 100 nm-thick platinum layer is evaporated on the wafer and patterned by lift off to form the electrodes of the MEA (Fig. 2a). Next, the metal lines extending from the contact pads to the Pt electrodes are fabricated. For this, an 800 nm-thick photosensitive polyimide (Fujifilm LT9305) layer is deposited by spin coating and patterned (Fig. 2b). Subsequently, a layer of 200 nm of TiN is sputtered on the frontside of the wafer, and patterned by dry etching (Fig. 2c). A second layer of 800 nm-thick polyimide is deposited and patterned to provide electrical insulation to the metal lines (Fig. 2d). Subsequently, a 10-µm-thick PDMS layer is deposited by spin coating on the frontside of the wafer at 6000 rpm for 60 s, and cured for 30 min at 90 °C. The contact pads are then open by patterning the PDMS layer by means of reactive ion etching using an Al layer as hard mask (Fig. 2e). Finally, the membrane is released by removing the Si and the SiO_2 layers underneath the membrane using deep reactive ion etching (DRIE) and buffered hydrofluoric acid (BHF), respectively (Fig. 2f,g).

Packaging

The packaging procedure starts by dicing the 4 inch wafer with an automatic dicing saw to obtain 100 Cytostretch chips. Four chips are mounted on a PCB with a fully-automatic pick-and-place system. The PCB is designed to fit into a MultiChannel System *in-vitro* recording system (MEA2100-System). The Al contact pads on the chip are subsequently wire-bonded to the PCB.

Finally, the PCB and the chips are encapsulated with an epoxy compound by means of Film-Assisted Molding (FAM), using a Boschman Unistar Innovate 2-FF system. FAM is a proprietary technology of Boschman Technologies [8] that guarantees a molding process without physical contact between the mold-tool and the

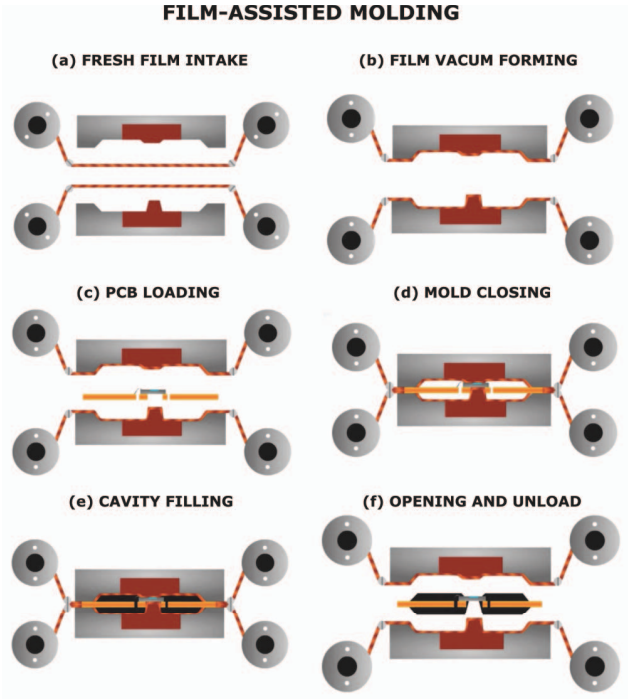

Figure 3: Process flow for the Film-Assisted Molding of the plate: (a) two Teflon films are rolled inside the mold, (b) the films are sucked into the inner surface of the mold, (c) the PCB is loaded inside the mold, (d) the mold is closed, (e) the liquefied epoxy material is injected in the mold and cured and (f) the mold is opened and the plate can be unloaded.

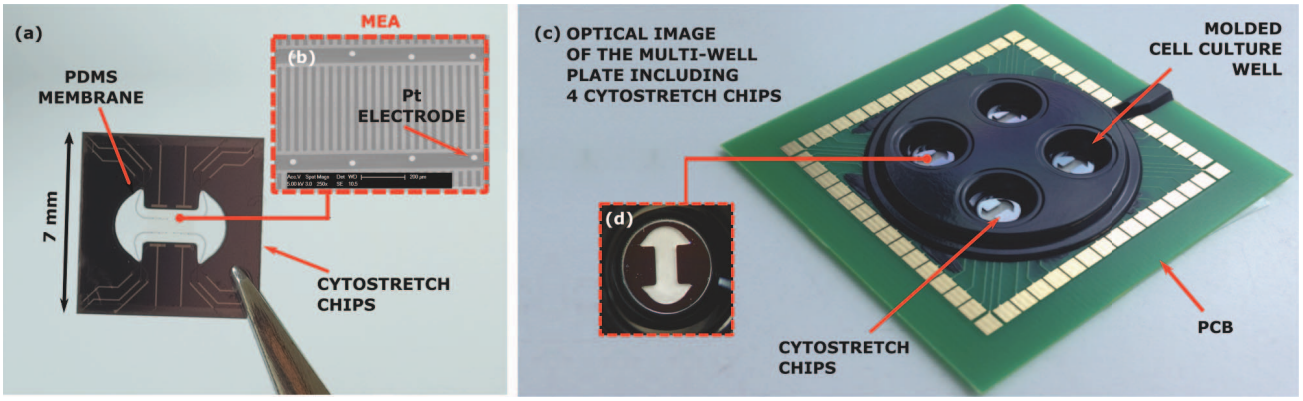

Figure 4: (a) Optical image of one Cytostretch chips including the MEA embedded in a stretchable PDMS membrane. The chip includes 12 circular electrodes (diameter: 30 µm, pitch 100 µm). (b) SEM image of Pt MEA. (c) Optical image of the multi-well plate consisting of four Cytostretch chips mounted on a PCB and encased by the molded wells (d) Optical image of the bottom of a well consisting of one of the chip.

epoxy. This is achieved by two Teflon foils (Fig. 3a) that are sucked to the inner surfaces of the mold (Fig. 3b). The PCB is then inserted in the tool (Fig. 3c,d) and the epoxy material liquefied by heat and pressure is then forced into closed mold cavities and held there until the epoxy is solidified (Fig. 3e). A Sumitomo G700 serie epoxy material was used with a process temperature of 175ºC and an end-cure pressure of 45 bar. The in-mold cure time used for this application was 80 sec.

The mold was customized in order to create the open-wells on top of the four chips, where the cells will be seeded and to protect the wirebonds from the humid environment of a cell culture incubator. After opening the mold, the encapsulated products are unloaded (Fig. 3g). Next, the vacuum is removed, and the foils are transported and renewed so that a new cycle can start without the need for a manual cleaning step.

CHARACTERIZATION

Electrochemical Characterization

The electrochemical performance of the MEA embedded in the Cytostretch was characterized by performing electrochemical impedance spectroscopy (EIS) on the Pt electrodes embedded in the membrane before mounting the chip on the plate. A measurement set-up similar to the one presented in [9] was used. The PDMS membrane on the Cytostretch chip was covered by Phosphate-Buffered Saline (PBS). A Silver/Silver chloride (Ag/AgCl) electrode and a Pt strip were used as reference and counter electrode, respectively.

EIS tests were performed with an Autohom Metrolab galvanostat/potentiostat (PGSTAT302N) with FRA32M Module. The amplitude of potential variations between working and reference electrode was equal to 50 mV and the stimulation frequency was swept between 0.01 and 10 kHz. The output current was monitored during the measurements to detect potential non-linearity caused by high-amplitude stimulations.

Cell culture

A preliminary biological assessment of the novel plate was performed by culturing human iPSC-derived Cardiomyocytes (Pluricyte® Cardiomyocytes, Ncardia, Belgium) in the wells (100.000 cells/cm^2) and monitoring them for seven days. Cell culture was performed according to manufacturer's protocol under standardized cell culture conditions (37°C, 5%CO_2, ~100% relative humidity).

Cell and monolayer appearance was monitored with light microscopy and beating of the cardiomyocytes was further assessed using a calcium sensitive dye (FLIPR Calcium 6 Assay Kit, Molecular Devices).

RESULTS

Figure 4 shows an optical image of the silicon chip (Fig. 4a) and a SEM image of the stretchable microelectrodes (Fig. 4b). The final plate after FAM is shown in Figure 4c. The FAM molding did result in a clean chip surface without epoxy residues on the chip-surface (Fig. 4d). Both sides of the chip where clamped with high precision without inducing too much stress on the chip that remained intact during the FAM. Standard transfer molding process conditions for the epoxy molding compound were used to mold the chips to assure a perfect adhesion between the epoxy mold and the chip.

In Figure 5 the Bode plot of the impedance spectra of one of the Pt electrodes is shown. At 1 kHz the average impedance calculated over five Pt microelectrodes is 870 kΩ. The impedance trend and the recorded value is

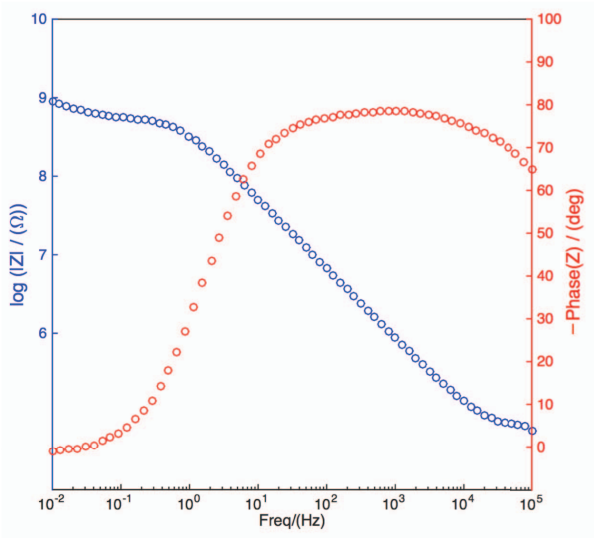

Figure 5: (a) Average bode plot of impedance spectra of five Pt electrodes (705 µm^2 geometric surface area). The average electrode impedance (1 kHz) is 870 kΩ.

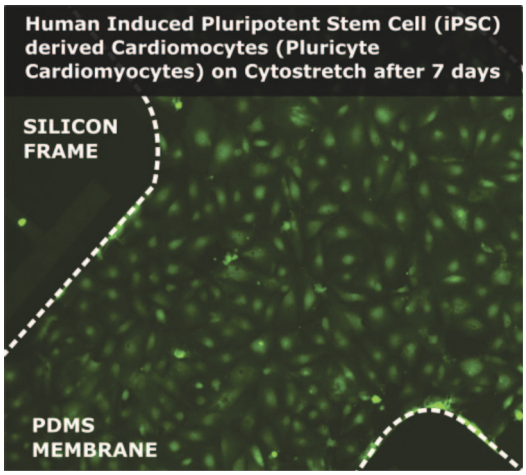

Figure 6: Microscopic image of hiPSC-derived cardiomyocytes cultured on the stretchable membrane for 7 days. Cells were stained with Calcium 6 dye (Molecular Devices). The Cardiomyocytes continue to appear normal and viable under fluorescent imaging even after seven days.

comparable to the one previously presented by Pakazad et al. [6].

The biological results achieved after applying the mentioned protocol shows cardiomyocytes which appeared overall normal and viable under fluorescent imaging (Fig. 6) up to seven days of culturing. However, no spontaneous beating of the cells was observed throughout the study. The reason for this has to be further investigated. During the cell culture, no leakage was observed, indicating adequate adhesion at the interface molded epoxy material, which forms the the wells, and the Cytostretch chips.

CONCLUSIONS

A novel multi-well plate that can be used to simultaneously mechanically stimulate, and electrically monitor *in-vitro* cell cultures was designed, fabricated and characterized. The plate includes an optimized version of the previously presented Cytostretch, mounted on a PCB. The wells and the insulation of the wirebonding were directly molded on top of the PCBs using a fully automatic film assisted molding technology. This is the first time that such a packaging technique is employed for an OOC device, and this work demonstrates that FAM can be a valuable option for directly molding a cell culture environment on top of OOC devices, creating a muti-well plate platform. The dimensions of the PCB and the mold can be easily re-defined in order to fit more Cytostretch in one plate, which will eventually be fabricated with a high-throughput format.

The new version of the Cytostretch chip was electrochemically characterized and the presented results showed an impedance in line with previously presented results.

This novel plate provides monolithic and robust biocompatible packaging for the Cytostretch. This was tested by culturing human iPSC-derived cardiomyocytes (Pluricyte® Cardiomyocytes) in the molded wells. Unlike most of the previously presented OOCs, the cells are seeded in open-wells by simply using a pipette. The plate has proven to provide easy cell culturing, sampling and inspection. The presented results show that the plate is biocompatible even though further investigations are needed to determine the cause of the absence of spontaneous beating of the cardiomyocytes.

The combination of high quality microfabrication and standard assembly and conventional packaging techniques are an important step forwards toward high-throughput Organ-on-Chip applications.

ACKNOWLEDGEMENTS

The authors would like to thank the Institute for human Organ and Disease Model Technologies (hDMT). Moreover, the authors gratefully acknowledge the technical support and advice of the staff at the Else Kooi Laboratory (EKL). This work was performed in the framework of the ECSEL JU InForMed Project (grant no. 2014-2-662155).

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