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Corso di Dottorato	Physics
Ciclo	32
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Titolo Progetto	Femtosecond laser based smart procedures for the fabrication of polymeric lab on a chip devices

In piena coerenza con le attività previste dal progetto nell'ambito del quale è stata finanziata la borsa di dottorato, si presenta la relazione annuale dell'attività svolta.

OGGETTO:

Data:2-7-2020

Firma dottorando:

Dipartimento Interateneo di Fisica "*Michelangelo Merlin*"

Dottorato di Ricerca in Fisica XXXII ciclo

PhD: Udith Krishnan

3rd year activity report

1. Introduction

Lab on a chips (LoCs), defined as devices in which multiple laboratory techniques are integrated in a chip of few square centimeters, have tremendous potential for application in various fields of chemistry and life sciences. In the last years, polymeric LoCs have generated a huge interest because of their competitiveness in terms of production costs, production times and easy way to go from an idea to a physical chip. Different techniques are available for the polymeric LoCs production and they can be classified in replication and direct structuration techniques. The former requires the fabrication of a mould, whose geometry depends on the target application, replication and assembling of the entire device. Among all, hot embossing, injection moulding and soft lithography are the most promising replication methods to fabricate polymeric microdevices at low costs and in high quantity. Despite the effort to improve their flexibility due to the presence of the mould, replica technologies are rather inflexible when it comes to rapid prototyping or optimizing the design of novel microfluidic tools. Technologies based on direct micro structuring of the substrate remain much more convenient during the design of a new device. In particular, the flexibility of ultrafast laser technology enables rapid prototyping and high precision micromachining of LoC devices with complex microfluidic channel networks, without the need of expensive masks and facilities, as required by the lithographic process. Furthermore, the ability of femtosecond (fs) laser pulses to produce "cold" ablation of the irradiated volume, thus avoiding debris and recast layers without restriction of the substrate materials, makes this technology particularly suitable for micro- and sub-microfluidic device fabrication, albeit with higher costs compared to other traditional techniques, such as mechanical micro milling which is more convenient for larger features.

The objective of my research was to design, fabricate and validate prototypes of polymeric lab on chips by smart procedures based on femtosecond laser technology and hot embossing for different biological applications. Before the laser microfabrication, in order to avoid a time-consuming trial and error process, we defined an accurate statistical Design of Experiment procedure to estimate the influence of the laser repetition rate, pulse energy, scanning speed, and hatch distance on the fs-laser micro milled depth of the microstructures. Found the model describing the relationship between the response variable depth and the main laser parameters, as the outcome of the research, two different polymeric LoCs have been fabricated: (1) a complete device for capturing circulating tumour cell and (2) a LoC platform for neuronal cell culturing. As part of the fabrication of LoCs a new simple and low-cost bonding technology has also been established.

The three-year period of my research activity has been divided as 1.5 years at home university *Università degli Studi di Bari*, 1 year at industrial partner *STMicroelectronics* (Lecce) and 6 months at *University of Strathclyde* (Glasgow).

Besides the research activities, during this three-year period I have focused on my training, with participation in courses (Tab 1) and schools.

Course	Professor	Period	Hours	CFU	Final test
Management and knowledge of European research model and promotion of research results	D'Orazio	June	16	2	Attestato frequenza
How to prepare a technical speech in English	White	April-May	16	2	Oral presentation
Fundamentals in advanced programming using C++ programming language	Cafagna	June-July	22	2	Final test
Interpolation Methods e techniques for Experimental Data Analysis	Pompili	September-October	20	2	Final test
Introduction to parallel Computing and GPU Programming using CUDA	Pantaleo	June	16	2	Final test
Fluidodinamica computazionale	Pascazio Giuseppe	September-October	40	4	Final test
Optical sensors and spectroscopic tecniques	Spagnolo/ Patimisco	June-July	20	2	Final test
Total				16	

Tab 1

2. Research activity

During my PhD, I developed two different polymeric LOCs:

1. a chip for tumour cell capturing exploiting fs-laser micro machining;

2. a chip for neuronal culturing exploiting both fs-laser micromachining and hot embossing technique.

In particular, I fabricated and characterized the device (1) in Bari and Lecce, respectively. The device (2) was fully developed in Glasgow.

In the following, the materials, micro fabrication and characterization of the devices are briefly summarized.

2.1 Materials

The device 1 was fabricated by fs-laser milling on PMMA samples (Vistacryl CQ, Vista Optics Ltd) of thickness <5 mm, with optical-quality (measured surface roughness Ra<5nm) and without any pre-treatment. Among

all the polymeric materials, PMMA has been selected for its specific characteristics such as rigidity, transparency in the visible light spectral range, biocompatibility and good environmental stability, which make it a valid and cheaper alternative to glass for the fabrication of microfluidic devices.
For microchannel functionalization we used Aptes (Sigma Aldrich) 5% in Ethanol, Glutaraldehyde (Sigma Aldrich) 0.05% in water and antibodies Anti-Epithelial Cell Adhesion Molecule (EpCAM) Mouse monoclonal (Sigma Aldrich). Anti-EpCAM antibody, specifically recognizes human EpCAM expressed at the surface of epithelial cells and is not reactive with normal or neoplastic non-epithelial cells.
OECM-1 Human Oral Squamous Carcinoma cell line (purchased by SCC/Sigma-Aldrich) is suitable for studies of cancer cell signaling, epithelial-mesenchymal transition (EMT), metastasis, invasion, and cancer cell stemness. Jurkat cell lines (purchased by ATCC) are human blood (leukemic T-cell lymphoblast)-derived cells.

The device 2 was fabricated exploiting hot embossing technique using a transparent PMMA sheet of thickness 5mm without any pre-treatment. The master mold for device 2 was fabricated by EpoxyAcast 690 from Smooth-On. The two parts A and B has been taken at a ratio 100 parts (A): 30 parts (B) by weight.
Part A contains: Oxirane,2,2'-((1-methylethylidene) bis (4,1-phenyleneoxymethylene)) bis-, homopolymer (CAS No. 25085-99-8).
Part B contains: Polyoxypropylenediamine (CAS No. 9046-10-0).
The epoxy resin master has been chosen because it is reusable, and it can withstand without any cracks up to a pressure load more than that of afford by silicon and SU8 Photoresist.
The fabricated PMMA devices were exposed to oxygen plasma (Pico A, Diener Electric, Germany) to render the surfaces hydrophilic and ethanol introduced via wells to prevent bubble formation in the channels. Ethanol was removed and poly-L-ornithine (PLO, Sigma) added to the top two wells and left for 3 hours to improve cell attachment. PLO was removed and channels were rinsed with medium, before filling all wells. Devices were stored in a humidified incubator (37°C/5% CO2) prior cell seeding.
Two different bonding technologies has been derived for PMMA microfluidic devices. One was Isopropanol assisted indirect thermal bonding and the other technique was by using ThermalSeal RTS pressure sensitive adhesive film.

2.2 Devices fabrication setup

2.2.1 Femtosecond laser micromachining

For the milling experiment, an ultrafast solid-state laser system (mod. *TruMicro Femto Ed*. From *Trumpf GmbH)* based on the chirped pulse amplification technique (CPA) and emitting at wavelength of 1030 nm was used. The laser source provides an almost diffraction limited beam (M^2<1.3) linearly polarized with pulse duration of 900 fs, maximum average power of 40 W, maximum pulse energy of 400 µJ, and repetition rate ranging from 100 kHz to 800 kHz. An external modulator allows reducing the repetition rate below 100 kHz and down to 1 kHz.
A quarter-wave-plate circularly polarizes the beam, and then a galvo-scan head (*IntelliSCANNse 14*, *SCAN-LAB, Puchheim, Germany*), equipped with a telecentric lens of 100 mm focal length, focuses and moves the beam onto the polymeric surface.

2.2.2 Hot embossing

Hot embossing was conducted on 5mm thickened PMMA sheet using an epoxy resin master as stamp. The hot embossing machine used was Specac Atlas Manual Hydraulic Press 15T customized with a capacity of 0.9 tonnes load (Figure 1). It was fitted with two metal platens (top and bottom) and these were connected to a temperature controller. The cooling was achieved by a chiller circulated cold water connected to the machine. The micropatterned epoxy resin master stamp and pre-cut PMMA substrate were hitched up into a set (Figure 2). The unpatterned open surfaces of this master-sample set were covered by

Polytetrafluoroethylene (PTFE) sheets to avoid the direct contact between master/sample with the metal platens. Prior to start hot embossing, the pressure release valve on the machine was firmly tightened and supplied water cooling to the machine. The master-sample set was placed in between the two metal platens with the covering of PTFE sheets. The top plate was screw down until it makes compressed contact with the master-sample set (Figure 3).

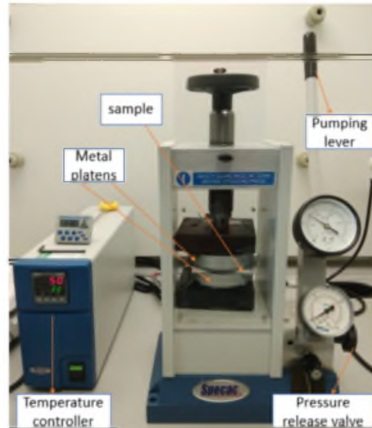


Figure 1: Hydraulic press machine for hot embossing

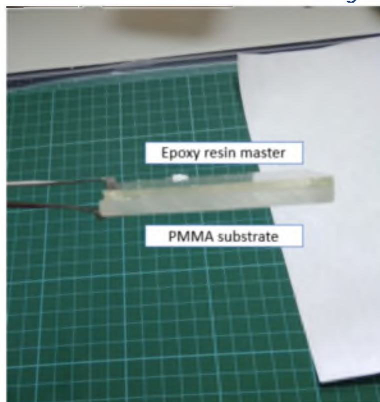


Figure 2: Hitched up epoxy resin master and PMMA substrate

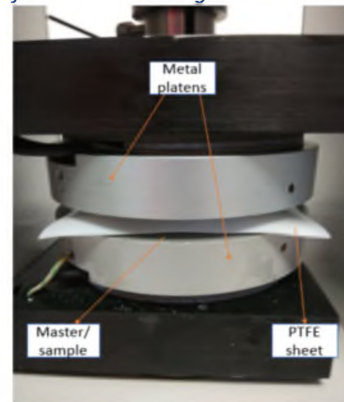


Figure 3: Compressed set of master-sample in between metal platens

2.2.3 Bonding

A cheap, simple, fast and deformation free method for bonding of two PMMA substrates was developed here. This method is based on depositing isopropyl alcohol (IPA) {((CH_3) $_2$CHOH)} using spin coater at the coating speed of 2000 RPM on the flat bottom substrate and capping it with micropatterned PMMA substrate. The excess solvent was eliminated through blotting paper.

The micro patterned PMMA slice and another plane PMMA slice of were cleaned with IPA by shaking it inside a petri dish and dried by using nitrogen (N_2) gas prior to bonding. The PMMA cannot be dissolved in IPA at room temperature because of the difference in the solubility parameter of PMMA with IPA. The solubility parameter of PMMA is 20$(Jcm^{-2})^{1/2}$ and of IPA is 23.4 $(Jcm^{-2})^{1/2}$. Few drops of hot IPA (70°C) were spin coated onto the plane PMMA slice and capped it with the micropatterned PMMA slice. Both PMMA slices were stacked together with a plastic clamp (Figure 4). The Plastic clamp provided a low pressure onto the stacked PMMA slices would decline the deformation of the chip after bonding. An oven was preheated to a temperature 60˚C below the glass transition temperature of PMMA. The pre-treated sample was put into the preheated oven for 20 minutes. At this temperature and pressure the PMMA monomers at the interface were got cross linked by solvent assistance of isopropyl acid.

Figure 4: Stacked PMMA slabs with plastic clamp

2.3 Design of experiment (DoE) approach for optimizing laser parameters

The design of experiment (DoE) approach can offer a methodical way to quickly determine the laser process settings limiting the use of resources. Here, an accurate DoE procedure to estimate the influence of the laser repetition rate, pulse energy, scanning speed, and hatch distance on the fs-laser micro milling process of PMMA specimens in terms of depth of removed material (Dh) has been defined. A predictive model describing the relationship between the response variable depth and the main laser parameters is defined and then validated.

Several process parameters contribute to the final surface quality and *depth* of a laser milled part. In particular, the laser pulse energy (E_p), the repetition rate (*R.R.*), the scanning speed (*s*) and the hatch distance (*d*), are relevant parameters that have been investigated in this section.

- pulse energy is the whole optical energy contained in a pulse. The output average power of the laser can be calculated by multiplying the pulse energy by the pulse frequency;
- repetition rate or pulsing frequency is the number of pulses per second emitted by the laser;
- scanning speed is the velocity with which the laser beam travels on the material surface;
- hatch distance is the distance between two consecutive scanning line.

Each laser milling test was performed superimposing two perpendicular scanning patterns, illustrated in Figure 5, in order to obtain uniform ablation all over the milled area.

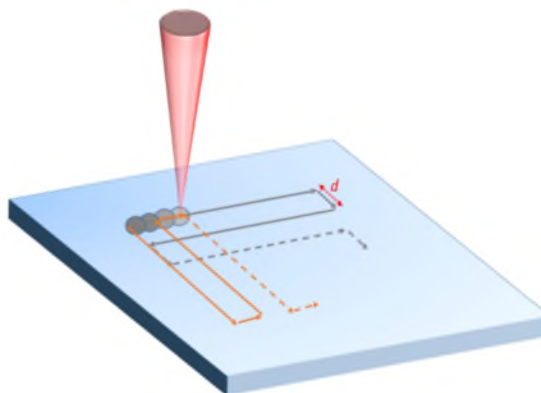


Figure 5: A schematic of the laser scanning pattern employed during milling of PMMA specimens; d is the distance between two consecutive scanning lines

Each test has been carried out on a target area of 1-mm x 5-mm. After fs-laser milling, the samples were washed with ultrapure water in an ultrasound bath for 15 min, in order to remove the processing residues.

A two-level full factorial design with resolution V has been defined so that main effects are unconfounded with 3-factor interactions. Two replicas, as defined by the Power Analysis, based on blocking on the day, have been set to evaluate the influence of the day as possible nuisance factor. The Factorial design indicates that, in the range investigated, the pulse energy and repetition rate are the main factors affecting the depth. A regression predictive model has been identified and validated in 3 points out of the factorial plan. All the depth values of the trials fall within the prediction interval (PI) and one of them fall within the confidence interval (CI), which is more restricted than the PI, proving the good prediction capability of the regression model.

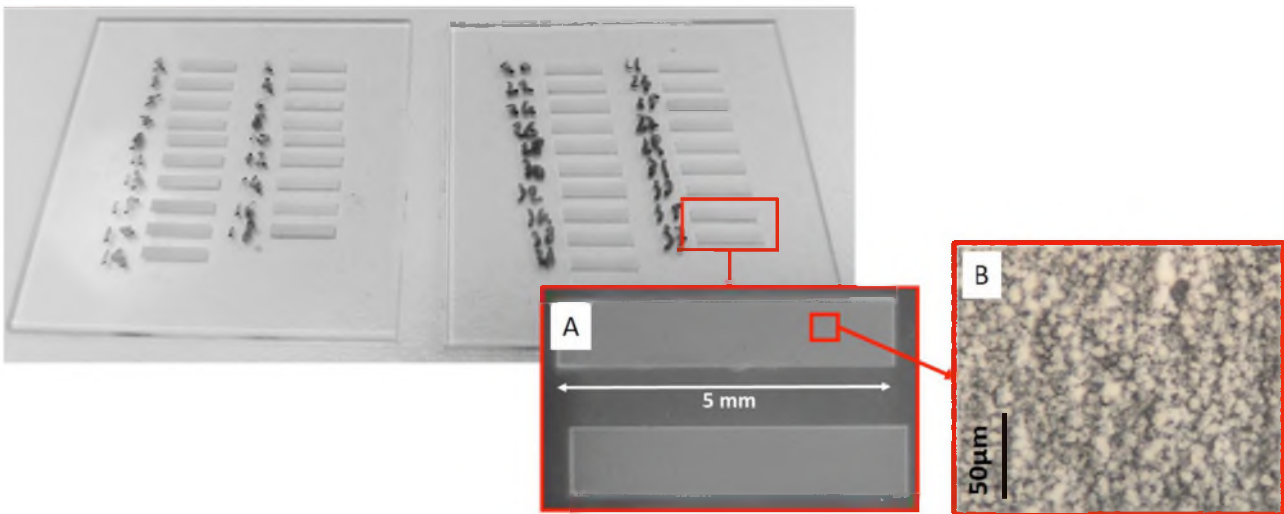


Figure 6: Image of the PMMA plates with the 38 pocket micromachined at different processing conditions. In the insets the optical image of 2 micromachined areas (A) and a further magnification of one of these (B).

2.4 Fabrication of the device to capture circulating tumor cells (CTC)

A new smart procedure for the microfabrication of PMMA LoC for tumor cells capturing has been developed by exploiting fs-laser technology, mechanical micro milling and solvent assisted thermal bonding. A serpentine microchannel was fabricated with a square cross section of 100 µm per side and a total length of 180 mm to increase the active path and therefore the probability of capturing cells. Two PMMA substrates were used in order to minimize the dimensions of the assembled device and to gain in transparency. The upper substrate (Figure 7a) was machined separately on the two faces. On the lower face the serpentine-shaped channel was fabricated by fs-laser ablation and gently removing the material layer-by-layer until reaching the depth designed with micrometric precision. The layer-by-layer milling procedure was performed superimposing two perpendicular scanning patterns. The lateral distance between two parallel scanning lines was 5 µm. A repetition rate of R.R. 50 kHz, a pulse energy of 12 µJ, and a scan speed 40 mm/s were selected thanks to the statistical study by DoE approach.

On the upper face the inlet/outlet drilled holes were micro-milled. The bottom layer was a flat and smooth PMMA substrate just used to seal the bottom of the channel (Figure 7a).

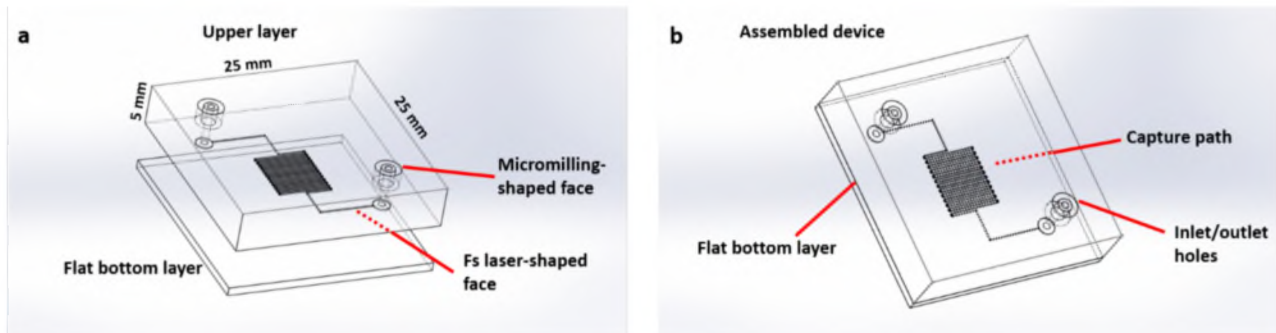


Figure 7: (a) modules of the PMMA device: the upper layer has been milled by both sides to obtain femtosecond pulse-shaped serpentine on the bottom and micro milling machine inlet/outlet holes on the top. A flat slice of PMMA sealed the serpentine channel; (b) architecture of the assembled device.

The inlet/outlets were obtained through a mechanical micro milling machine mounting a 400 µm tool, after careful alignment with the serpentine ends on the opposite face. The air-cooled milling was accomplished at feed rate of 100 mm/min and spindle speed of 20000 RPM according to Reichenbach. Inlet/Outlets were concentric cylinder holes that fit perfectly with the external and internal dimensions of the capillary, therefore making no additional glue or gaskets necessary for the watertight seal. From the center of these cylinders, channels of 600 µm in diameter and 5 mm in length have been realized, which allow the fluids injected from the opposite face to reach the serpentine.

Figure 8a shows a stereoscopic image of the micromachined serpentine. In Figure 8b a particular of the device is highlighted. The edge of the channel showed no cracks, burrs or recast layers that could hinder the sealing procedure. The bottom part of the channel had a roughness Ra of about 3 µm. This value was negligible compared to the channel height; therefore, it did not affect the fluidic transport of the cells.

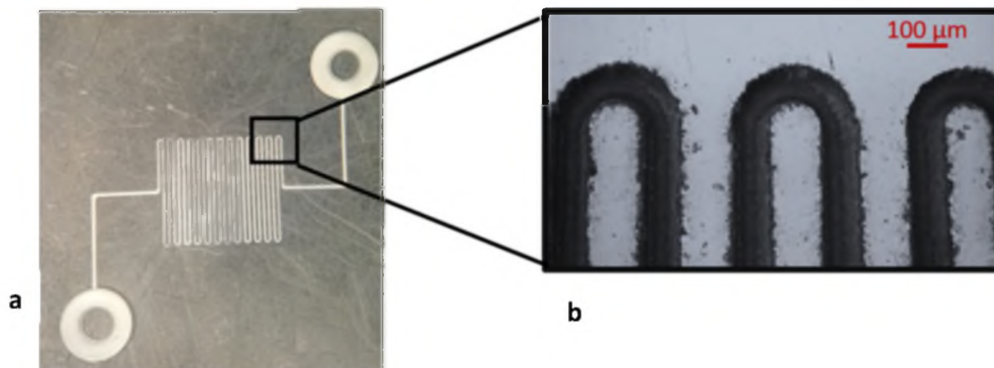


Figure 8: (a) Stereoscopic image of the laser micromachined sample. (b) A microscopic 3D detail of the serpentine channel (magnification 10x).

The microfluidic device has been tested to capture cancer cells from a mixture of normal and tumor cells. For this reason, in the last step Anti-EpCAM antibody has been immobilized, which is able to recognize human EpCAM, a membrane biomarker that is typically present on the surface of epithelial-type cancer cells. With a view to demonstrate the effectiveness of the device, a culture medium samples with two different population of cells (blood and tumor cells) have been spiked in order to simulate a complex real sample. In particular, Jurkat cells (blood derived cells) and OECM-1 Human Oral Squamous Carcinoma cell line (Epithelial-like cells from human oral cancer) were used. 5 ml suspensions of cells containing 10^6 cells/ml from Jurkat line and 10^4 cells/ml from OECM-1 line have been prepared separately. Cell suspensions were allowed to slowly flow through the serpentine microchannel with a flow rate of 8 µl/min. The low velocity of the fluid, the size of the channel and its serpentine shape have been optimized to maximize cell interactions with the internal walls, where antibodies fixed on the surface can recognize cells expressing the membrane epithelial antigen.

In the case of Jurkat cells, no or very few cells were identified in the channels after a washing step with PBS solution. Jurkat cells indeed did not express EpCAM antigen on the surface of cell membrane, thus they could not be recognized and fixed by antibodies immobilized on channel walls.

In the case of OECM-1 suspension of cells, instead, a high number was captured on the inner walls, adhering to the surface also after a PBS washing step. As demonstrated by Figure 9, cells are clearly visible through the transparent PMMA device, confirming the possibility of such a tool to be used as a diagnostic instrument to immobilize and discriminate cancer cells from normal blood cells.

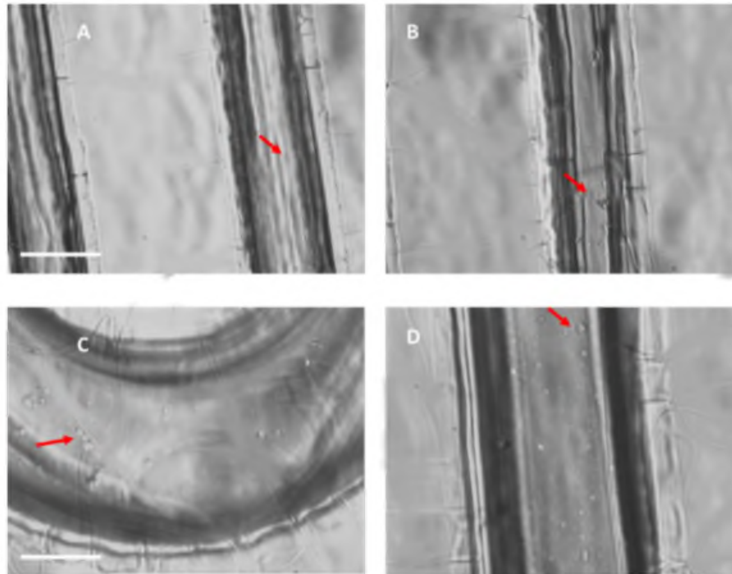


Figure 9: PMMA microchannel after flowing with OECM-1 cells and subsequent washing step with PBS. A high number of cells are captured on the inner surface of channel.

2.5 Fabrication of the device for neuronal cell culture

Neuroscience is the investigations on basic functions of the nervous system for understanding nervous system disorders and medical treatments. Recent developments in miniaturization based on microfabrication helps biologists to overcome the limitations of traditional cell culture methods. Soft lithography is a widely used method for creating micro and nanoscale structures using elastomeric elements and moulds by photolithography. Usually, the elastomeric polymer polydimethylsiloxane (PDMS) is the main component of BioMEMS platforms due to its optical transparency, thermal stability, low cost, biocompatibility, gas permeability and ease of fabrication. Here, the lab on chip platform for neuronal cell culturing was replaced with a rigid PMMA substrate and the device was fabricated exploiting different techniques, namely fs laser ablation and hot embossing. In both cases, the neuronal cell culture device was composed of fluidically isolated culture channels connected by a series of microchannels (Figure 10). This gives more control over the cellular microenvironment, with the ability to create distinct regions to mimic in vivo conditions.

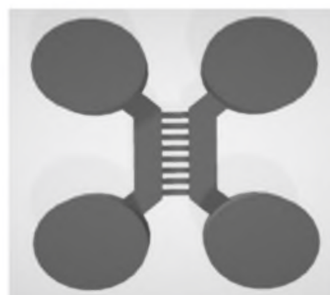


Figure 10: Schematic diagram of a microfluidic device for neuronal cell culture

2.5.1 Laser ablation:

The device was fabricated on a 3cmx3cmx1mm PMMA slab consists of 2 large culture channels connected by microchannel arrays (Figure 11) of 8μm deep and 10 μm wide. The laser parameter used for the fabrication are in the Tab.2

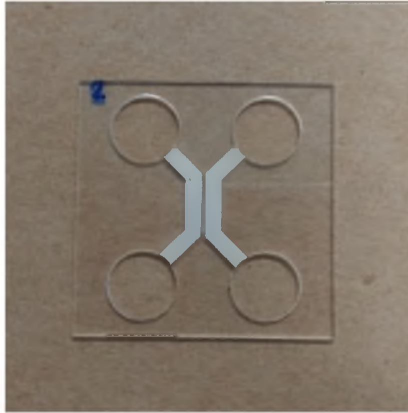


Figure 11: Laser ablated device

	Laser parameters	
	Microcahnnel array	Large culture channel
Frequency	0.625KHz	50KHz
Power	0.010W	0.6w
Laser scan speed	1mm/s	25mm/s
No.of loops	1	1

Tab.2: Laser parameters used for the fabrication of microfluidic device for neuronal cell culture

2.5.2 Hot embossing

A prior fabricated micropatterned epoxy resin master mold and plane PMMA substrate set were heated to different loading temperature L_T (from 130°C to 150°C) above PMMA glass transition temperature (T_g=120°C) and under various pressure loads (loading pressureL_P, 0.2 ton to 0.3 ton) for 15 minutes. After 15 minutes, the loading temperature has been decreased to room temperature (22°C) by maintaining the pressure load and leaving the system for 1 hour to reach room temperature. Once the master-sample set was cooled, pressure release valve was released and took the sample out from the machine. The imprinted PMMA substrate was demoulded from the epoxy resin master by hand (Figure 12). 4-mm inlet and outlet holes were drilled by a mechanical drill machine.

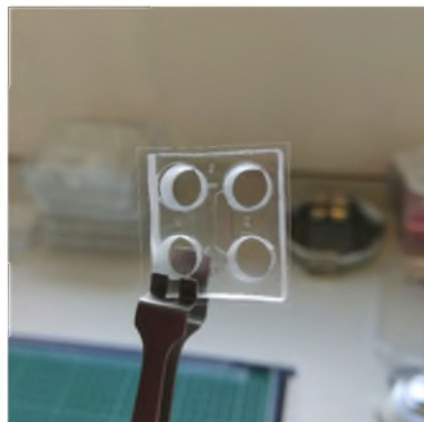


Figure 12: Hot embossed micropattern on a 5mm thickened PMMA substrate

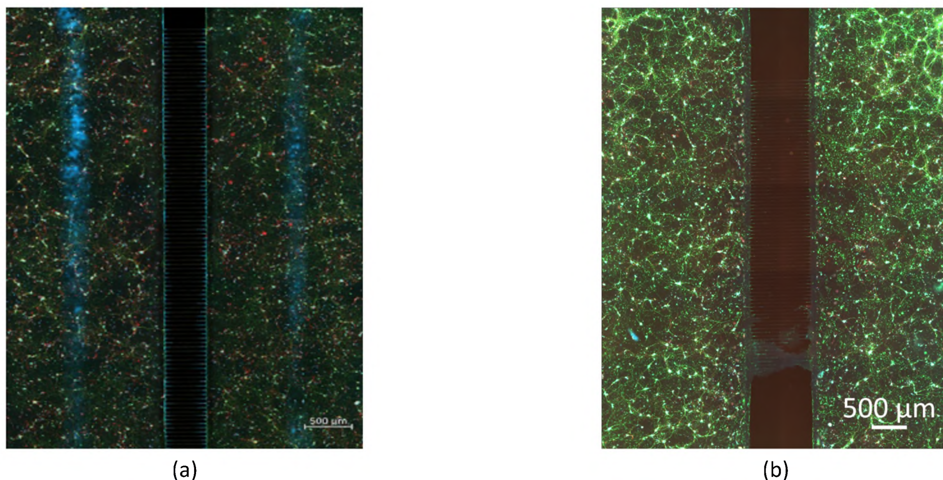
2.5.3 Primary hippocampal cell culture

1-2 days old Sprague Dawley rat pups were killed via cervical dislocation and the hippocampus dissected out. The tissue was chopped up and incubated in papain solution (1.5 mg/ml) followed by trituration in bovine serum albumin solution (10 mg/ml). The isolated cells were then spun down and the pellet resuspended in culture medium at a density of 3-4 x 10^6 cells/ml. This medium consisted of Neurobasal-A supplemented with L-glutamine (2 mM) and B27 (2% v/v). Cells were loaded into the culture chambers of devices by pipetting close to the entrance of the channel through the emptied wells and incubated for ~1 hour. Wells were filled with medium in a staggered process and media was refreshed every 2-3 days by removing half the well

volume and replacing with fresh media. Devices were incubated at 37°C/5% CO_2 and analysed after 12-14 days *in vitro* (DIV).

2.5.4 Immunocytochemistry

Immunocytochemical staining was performed on cultures. The cells were washed with PBS and permeabilised by applying 0.01-0.1% Triton-X 100 for 10 minutes. Cultures were then incubated with a blocking solution containing foetal bovine serum (5% v/v) and BSA (1% w/v) in PBS for 1 hour at room temperature. Primary antibodies were diluted in blocking solution (1:500) and incubated with the cultures at 4°C overnight. Antibodies used were for ßIII-tubulin (neuronal marker), synaptophysin (synaptic vesicle marker) and glial fibrillary acidic protein (GFAP, marker for astrocytes). Cells were then rinsed with PBS and incubated with complimentary fluorescently labelled secondary antibodies (diluted 1:200 in blocking solution) for 1 hour at room temperature. Finally, cells were rinsed with PBS and wells were filled with PBS prior to imaging.



(a) (b)

Figure 13: neuronal cell culturing images (A) laser ablated device and (B) hot embossed device

The presence of intracellular calcium on laser ablated device (Figure 13 a) suggests that viable cells on this platform and functional synaptic connectivity between culture chambers via neurites through microchannels have been realized. In the case of hot embossed device (Figure 13 b) βIII-tubulin, GFAP and DAPI staining were visible and realized the cell growth and connection forming between the cells.

Publications

1. "Prediction model of the depth of the femtosecond laser micro-milling of PMMA", Annalisa Volpe, Gianluca Trotta, Udith Krishnan, Antonio Ancona. Optics and Laser Technology 120 (2019) 105713. https://doi.org/10.1016/j.optlastec.2019.105713
2. "Smart procedure for the femtosecond laser-based fabrication of polymeric lab on a chip for tumor cells capturing", Annalisa Volpe, Udith Krishnan, Maria Serena Chiriaco, Elisabetta Primiceri, Giuseppe Maruccio, Antonio Ancona, Francesco Ferrara – Submitted in Engineering journal

Poster presentations

1. "Fs-laser based smart procedures for the fabrication of polymeric Lab on a Chip devices" – Science and Industry for environment, Health and Digital Society Technologies; Industrial PhD Day at Università degli Studi di Bari Aldo Moro – 26 June 2019

Conferences

1. 21st International Symposium on Laser Precision Microfabrication, 23-26 June 2020 – Dresden, Germany, "Femtosecond laser based smart procedures for the fabrication of polymeric lab on a chip devices"- Oral presentation

Summer schools

1. International School on Laser Micro/Nanostructuring and Surface Tribology 1-5 October 2018 – Bari, Italy. “Femtosecond laser micro-fabrication of polymeric lab-on-chip for advanced and mini-invasive diagnostics” – Oral presentation