Review Article

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Multilayer-based lab-on-a-chip systems for perfused cell-based assays

Abstract: A novel integrated technology chain of laser-microstructured multilayer foils for fast, flexible, and low-cost manufacturing of lab-on-a-chip devices especially for complex cell and tissue culture applications, which provides pulsatile fluid flow within physiological ranges at low media-to-cells ratio, was developed and established. Initially the microfluidic system is constructively divided into individual layers, which are formed by separate foils or plates. Based on the functional boundary conditions and the necessary properties of each layer, their corresponding foils and plates are chosen. In the third step, the foils and plates are laser microstructured and functionalized from both sides. In the fourth and last manufacturing step, the multiple plates and foils are joined using different bonding techniques like adhesive bonding, welding, etc. This multilayer technology together with pneumatically driven micropumps and valves permits the manufacturing of fluidic structures and perfusion systems, which spread out above multiple planes. Based on the established lab-on-a-chip platform for perfused cell-based assays, a multilayer microfluidic system with two parallel connected cell culture chambers was successfully implemented.

Keywords: cell culture; lab-on-a-chip; laser microstructuring; micropump; microsystems; perfusion.

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1 Introduction and motivation

The miniaturization, rapid prototyping, and automation of lab-on-a-chip technology play, nowadays, a very important role. The lab-on-a-chip technology is successfully implemented not only for environmental analysis and medical diagnostics but also as replacement of animals used for the testing of substances in the pharmaceutical and cosmetic industries [1–4].

For that purpose, the Fraunhofer IWS and the Technical University of Berlin developed a lab-on-a-chip platform for perfused cell-based assays during the last years, which includes different micropumps [5], valves, channels, reservoirs, and customized cell culture sections. This technology is already implemented for the characterization of different human cell cultures and organoids, like skin [6], liver [6, 7], endothelium [8], and hair follicle [9]. Furthermore, the platform offers interfaces for the integration of scaffolds and artificial blood vessels, for example, hollow fibers [10].

Heretofore, casted polydimethylsiloxane (PDMS) flow cells constitute the core of the lab-on-a-chip platform in which the microfluidic structures are basically embedded in one plane. For an effective testing of substances, the platform has to be further developed in order to integrate more fluidic circular flows in different layers (bloodstream systems, digestive systems, urinary system) [3].

For the integration of more fluidic circular flows by keeping the overall dimensions as well as the required size and shape of the cell culture compartments, an increase of fluidic layers is necessary [11].

2 Microstructuring of synthetic material

For the microstructuring of thermoplastics, there are extensive established procedures. The range comprises direct

methods like micromilling [12] and laser ablation [13–18] as well as indirect methods like casting [6–10, 19, 20] and hot embossing [21]. Among the indirect methods, the principal disadvantage for the structuring is the necessity of specific molding tools, which makes the modification of the structures difficult; moreover, the complexity and price for prototyping is increased. Because of that, the direct processing of the substrate by means of micromilling or laser ablation offers a great potential for rapid prototyping.

By means of laser ablation, it is possible to obtain smaller and more precise structures. The laser machining enables not only the modification of the surface topography but also targeted chemical modification of it [22]. Consequently, it is possible to directly produce the microchannels, microstructures, and fluidic contacts as well as the modification of the substrate characteristics [13, 14]. The microstructuring with laser of poly(methyl methacrylate) (PMMA) [15, 16], polycarbonate (PC) [17], polystyrene (PS), and cyclic-olefin-polymer (COP) [18] is already well characterized. Besides direct cutting, it is also possible to structure the molding tools with the laser [23].

3 Manufacturing technology for multilayer-based lab-on-chip systems

A novel integrated technology chain of laser-microstructured multilayer foils for fast, flexible, and low-cost manufacturing of lab-on-a-chip devices was developed and established. Initially, the microfluidic system is constructively divided into individual layers, which are formed by separate foils or plates. Based on the functional boundary conditions and the necessary properties of each layer (hydrophilic, hydrophobic, transparent, permeable, porous, etc.), their corresponding foils and plates are chosen. In the third step, the foils and plates are laser microstructured and functionalized from both sides. In the last manufacturing step, the fourth one, the multiple plates and foils are joined using different bonding techniques like adherence, thermal or plasma bonding, or welding (hot plate welding, hot gas welding, ultrasonic welding, beam welding, or friction welding). The multilayer technology permits the implementation of pneumatically driven micropumps and valves (see Figure 1). The connecting plate acts as linkage between the lab-on-a-chip device and external pneumatic and fluidic controllers. It accommodates seven air pressure fittings and four inserts for media exchange and later integration of organ equivalents.

With this multilayer approach, it is possible to arrange microfluidic systems in several layers (see Figure 2). Thus, it is also possible to implement several fluidic circular flows (bloodstream systems, digestive systems, urinary systems) in different layers [3].

As a result of this, an enhancement of the functionality per chip area is achieved.

Arising from the use of foils and plates with different characteristics, it is possible, for example, to specifically control the wetting, to implement functions like capillary-stop-valves, barriers, or even targeted cell colonization. Furthermore, it is possible to integrate thin-layer electrodes to the foils and plates, what allows the operation of electric and electro-mechanics sensors and actuators.

Fluid can be pumped through the microfluidic devices by an integrated peristaltic pump consisting of three pumping chambers in a row. Each is actuated one after another by applying pressure or vacuum. As shown in Figure 3, the first and the last chambers are designed as valves. In phase 1, the pump chamber is actuated with closed inlet and opened outlet, which results in a main pump pulse. Afterwards, both valve states are switched, which causes a smaller pulse. Phase 3 represents the filling state of the pump chamber. As the outlet valve is closed, no fluid movement can be observed in pumping direction. In the last phase, both valves are switched again, which results in a backflow because fluid fills the outlet valve chamber.

4 Multilayer demonstrator

Based on the configuration for long-term cultivation and substance testing of human liver and skin tissue co-cultures [6–8], developed together with the TU-Berlin, a multilayer demonstrator was conceptualized and successfully

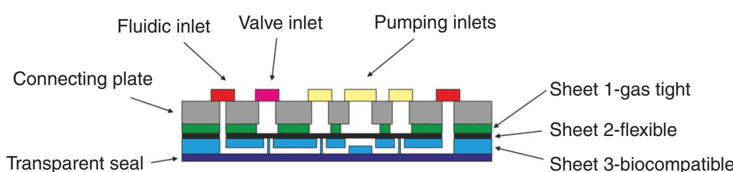

Figure 1 Scheme of a multilayer lab-on-a-chip system with integrated micropumps and valves.

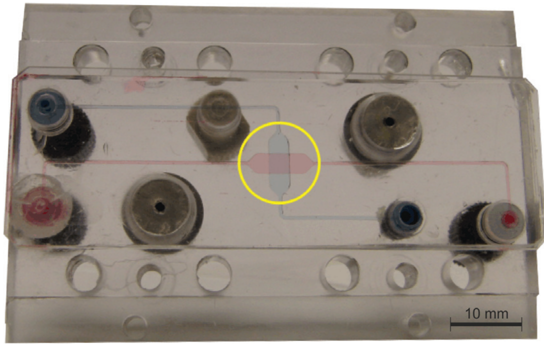

Figure 2 Prototype with intersecting channels manufactured in different layers (overall dimension 76×50 mm²). The channels were filled with two different dye solutions (red and blue).

realized. It includes a closed loop (see Figure 4), consisting of a reservoir (blue), a three-point peristaltic pump (orange) as well as two parallel-connected chambers for cell cultures (red), each of them equipped with inlet and outlet valves (yellow) together with microstructures for defined incident flow.

Besides the implementation of cell culture experiments, it is also possible, with the help of the demonstrator, to characterize the distribution of the volumetric flow rate of the pump of both parallel-connected chambers by means of selective activating the inlet and outlet valves. As seen in Figure 5, the multilayer demonstrator consists of a stack of three laser-structured polymer foils (2–4). The stack is finalized with a connecting plate on one side (1) and with a polymer or glass plate on the other side (5).

Based on the particular functional constrains of the layers, the materials shown in Table 1 were chosen.

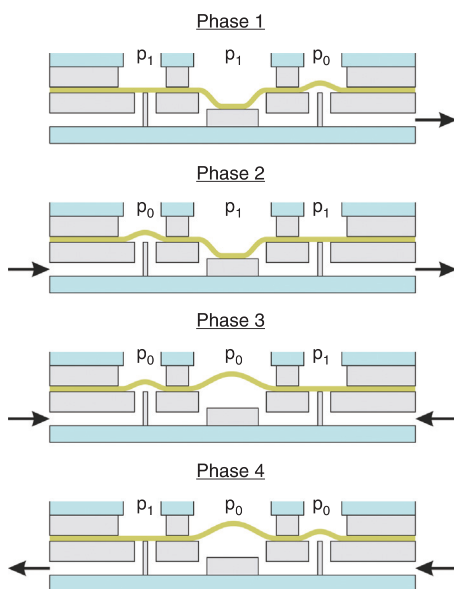

Figure 3 Valve and peristaltic pumping principle.

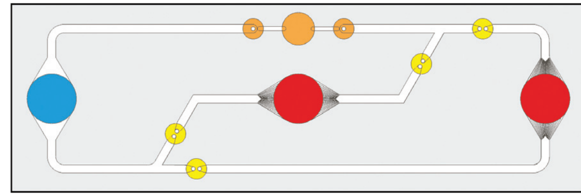

Figure 4 Functional layout of the demonstrator (overall dimension 76×26 mm²). Reservoir (blue), valves (yellow), three-point peristaltic pump (orange), chambers for cells cultures with defined microstructures for specific incident flow (red).

5 Laser structuring

The manufacturing of the filigree and polymer fluidic microstructures is done by laser-induced material ablation. In this process, the incident laser pulse energy causes the evaporation of the material in the surface resulting in microvoids. Therefore, the laser energy should be absorbed by the material to a great extent. This can be positively influenced based on the absorption spectrum of the material by choosing a suitable laser wavelength or taking advantage of nonlinear absorption effects, e.g., ablation with ultrashort laser pulses (multiphoton absorption). The utilization of ultrashort pulsed lasers is also meaningful, when thermal influences at the surface and substrate in the region of the microstructures has to be minimized. This is the case, for example, when structuring the here-mentioned polymer microfluidics with delicate structures in the area of the cell culture chambers; for that reason, a laser microstructuring device (microstruct Vario, 3D MICROMAC, Chemnitz, Germany) with a picoseconds laser (Fuego, Time-Bandwidth Products, Zurich, Switzerland) was chosen. The machining device is equipped with high-precision linear

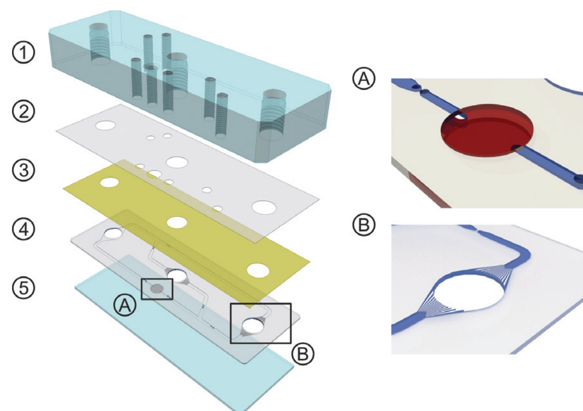

Figure 5 Exploded view of the multilayer-demonstrator system and details of (A) bilateral structured micropump (bottom structure blue, top red) and (B) the cell culture chamber (right).

Table 1 Construction of the multilayer demonstrator.

Layer	Material
1	Connecting plate, cyclic-olefin-copolymer, thickness depending on the application, machined → fluidic and pneumatic interface
2	Foil, cyclic-olefin-copolymer, 100 µm thick, laser-structured, optional adhesive coated
3	Foil, polyurethane thermoplastic elastomer, 25 µm thick, laser structured → pump and valve membranes
4	Foil, cyclic-olefin-copolymer, 250 µm thick, bilateral laser structured, optional adhesive coated
5	Foil or slide, cyclic-olefin-copolymer or glass, thickness depending on the application → channel sealing and optical interface

axes as well as a galvanometer scanning head and additional complex measurement devices for analysis of the resulting microstructures. With this technology, it is possible to generate and reproduce microstructures of approximately 5 µm.

The structuring of the foils and plates with overall dimensions of 26×76 mm^2 took place with a wavelength of 355 nm and a pulse duration of 10 ps. The microstructures, which were previously designed with the help of CAD software (Inventor, Autodesk Inc., San Rafael, CA, USA; microMMI, 3D MICROMAC, Chemnitz, Germany), are then filled with hatching lines by means of the device's control software. With a spot diameter of 15 µm and a distance of 5 µm between scanning lines, the required overlap is achieved [18]. In this way, by a repeating ablation cycle, it is possible to obtain a sinking of the ablated structures in the substrate's surface. As the optimization of the quality of the prototypes is more important than their manufacturing time, the pulse frequency and laser power, respectively, pulse energy, were lowered to a necessary minimum value. The parameters listed below were used for manufacturing the cyclic-olefin-copolymers (Topas 5013, TOPAS Advanced Polymers GmbH, Frankfurt am Main, Germany):

- Wavelength 355 nm
- Pulse length duration 10 ps
- Repetitions rate 66 kHz
- Middle average power 0.6 W.

The manufactured microfluidic structures satisfy all the requirements (geometry, transparency, etc.) needed for successful cell cultivation.

Figures 6 and 7 show a complete structured polymer foil as well as a detail magnification of the inflow structures sections of the cell culture chambers.

6 Fluidic characterization

Subsequent to the fabrication, a complete functionality test of the fluidic demonstrator (see Figure 5) was realized. Besides the leak proof of the system, the volume flow rates inside the system were characterized by means of micro-particle image velocimetry (µPIV) [8, 24] and pressure ratios by analyzing the deflection of the integrated membranes [25].

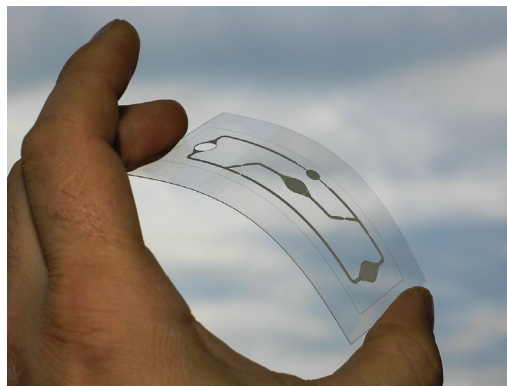

Figure 6 Laser-structured polymer sheet.

For the demonstrator, the following configuration was used in all experiments: 4 mm pump membrane diameter; 100 µm pump chamber height; 500 mbar pumping pressure; -750 mbar filling vacuum with pneumatic outlets throttled to 1.5 l min^{-1} air flow at 1500 mbar. The flow rate can be varied by adjusting of the pumping frequency. Using pump frequencies in the range of 0.84–4.2 Hz pulsatile flow rates with mean rates in the range of 1–5 µl s^{-1} could be realized.

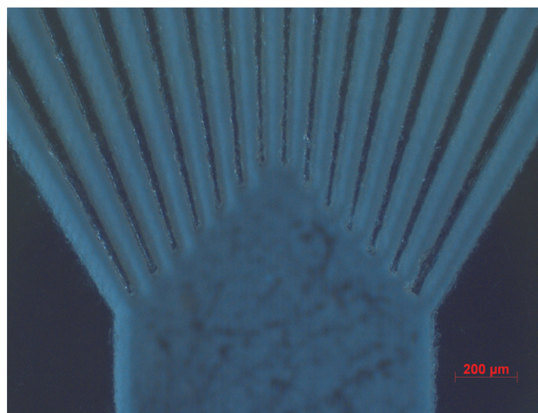

Figure 7 Detailed view of the laser-structured polymer sheet.

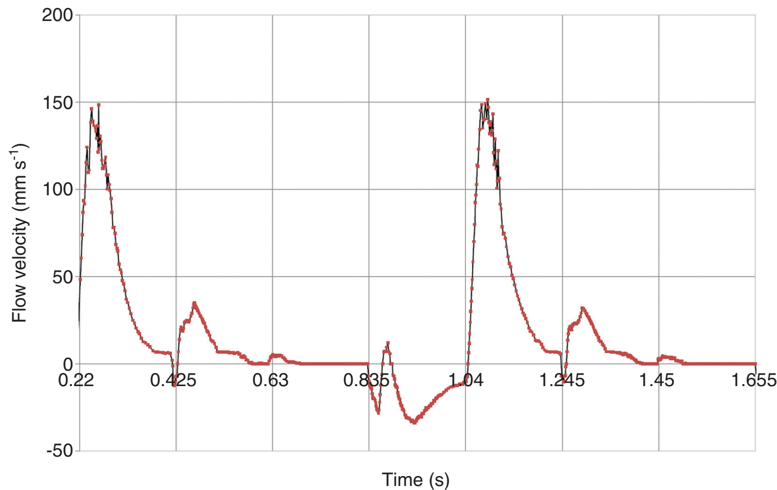

Figure 8 Pulsatile flow velocity at the outlet of the pump for a pump frequency of 1 Hz.

In Figure 8, an example of measured flow velocity at the outlet of the pump is shown.

It was possible to realize the same fluidic boundary conditions in the multilayer system as in the already established PDMS-based lab-on-a-chip platform.

7 Sterilization, biocompatibility, and cell culture characteristics

Following the sterilizing capability, biocompatibility, and cell culture properties were characterized.

The developed multilayer systems can be cleaned and disinfected with ethanol and sterilized by means of autoclaving at 121°C.

Initial investigations of biocompatibility and cell culture properties on *Saccharomyces cerevisiae* cells with the protocol established at Fraunhofer IWS for automated vital staining and fluorescence-based characterization of eukaryotic cells [26, 27] were realized. It was possible to cultivate *S. cerevisiae* cells for several days within the developed multilayer system. The cell viability was verified by staining with Cell-tracker Green CMFDA (Invitrogen).

Furthermore, investigations with human umbilical vein endothelial cells (HUVEC) based on existing protocols [8, 28] were done. After coating the microfluidic with fibronectin; it was also possible to cultivate these cells for several days in the developed multilayer system, and they showed the same adhesion behavior as in the already established PDMS-based lab-on-a-chip platform.

Figure 9 exemplarily shows a microscopic phase-contrast image of a fibronectin-coated channel covered with HUVEC after 2 days of cultivation. The detail shows a fluorescence image of the same channel with CellTracker™ Red CMTPX (Life Technologies)-stained HUVEC [26].

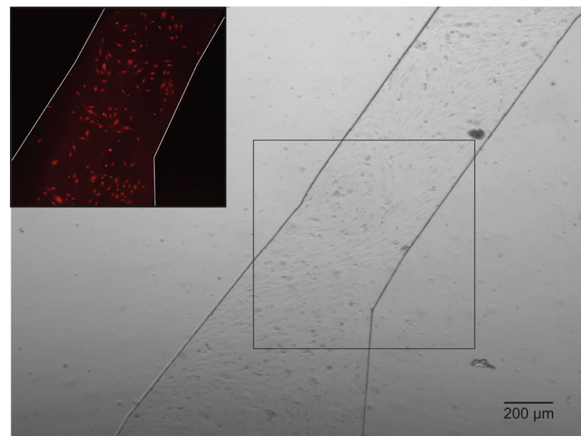

Figure 9 Microscopic phase contrast image of the fibronectin-coated channel covered with HUVEC after 2 days of cultivation; the detail shows a fluorescence image of the same channel with CellTracker™ Red CMTPX-stained HUVEC.

8 Conclusions and outlook

A novel integrated technology chain of laser-microstructured multilayer foils for fast, flexible, and low-cost manufacturing of lab-on-a-chip devices especially for complex cell and tissue culture applications, which provides pulsatile fluid flow within physiological ranges at low media-to-cells ratio, was developed and established.

This multilayer technology together with pneumatically driven micropumps and valves permits the manufacturing of fluidic structures and circular flow systems, which

spread out above multiple planes. Based on the established lab-on-a-chip platform for perfused cell-based assays, a multilayer microfluidic system with two parallel-connected cell culture chambers was successfully implemented.

It was possible to realize the same fluidic boundary conditions in the multilayer system as in the already established PDMS-based lab-on-a-chip platform.

The developed multilayer systems can be cleaned and disinfected with ethanol and sterilized by means of autoclaving by 121°C. After fibronectin coating, it was possible to cultivate HUVEC for several days, and they showed the same adhesion behavior like it was demonstrated for the PDMS-based lab-on-a-chip platform.

Subsequently, this multilayer technology has to be optimized, and additional application-specific lab-on-a-chip systems will be developed and characterized.

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