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Article

Fast Prototyping of Sensorized Cell Culture Chips and Microfluidic Systems with Ultrashort Laser Pulses

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Abstract: We developed a confined microfluidic cell culture system with a bottom plate made of a microscopic slide with planar platinum sensors for the measurement of acidification, oxygen consumption, and cell adhesion. The slides were commercial slides with indium tin oxide (ITO) plating or were prepared from platinum sputtering (100 nm) onto a 10-nm titanium adhesion layer. Direct processing of the sensor structures (approximately three minutes per chip) by an ultrashort pulse laser facilitated the production of the prototypes. pH-sensitive areas were produced by the sputtering of 60-nm Si_3N_4 through a simple mask made from a circuit board material. The system body and polydimethylsiloxane (PDMS) molding forms for the microfluidic structures were manufactured by micromilling using a printed circuit board (PCB) milling machine for circuit boards. The microfluidic structure was finally imprinted in PDMS. Our approach avoided the use of photolithographic techniques and enabled fast and cost-efficient prototyping of the systems. Alternatively, the direct production of metallic, ceramic or polymeric molding tools was tested. The use of ultrashort pulse lasers improved the precision of the structures and avoided any contact of the final structures with toxic chemicals and possible adverse effects for the cell culture in lab-on-a-chip systems.

Keywords: rapid prototyping; micro sensor chip; ITO; oxygen; pH; picosecond laser; cell monitoring system; top-down approach

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1. Introduction

The development of multifunctional microfluidic systems calls for the integration of sensors and actuators such as electrical sensors and heating or pump elements, all of which are routinely manufactured using well-established microelectronic fabrication techniques. Microfluidic components are also produced using photolithographic technology, especially in basic research [1]. Nevertheless, injection molding is unrivalled amongst the various polymer technologies used in the mass production of microfluidic components. Microinjection molding, hot embossing, or hot shaping are alternatives for low volume production or prototyping [2].

The materials used in biotechnological and medical applications must comply with certain requirements, e.g., biocompatibility or stability against heat sterilization. These requirements are challenging with respect to the physical and chemical properties, especially in lab-on-a-chip devices for long-term cell monitoring or for micro-reaction techniques in Micro Total Analysis Systems (μTAS) [3]. Unfortunately, glass, as the material of choice, requires special adaptations in the production methods [4]. For low volume production or prototyping, photolithography techniques are time-consuming and costly due to their high number of processing steps. Often, the outdated (4-inch) disk-wafer technology is used, which restricts the size and the degrees of freedom in the individual chip design [5].

To increase the frequency of prototype realization and for small batch series (a requirement in basic research), it is necessary to implement fast design cycles using flexible and fast production technologies. This strategy shortens development times and test cycles in the research-intensive field of microfluidic systems development. As a consequence, rapid prototyping techniques are gaining increasing importance because they help reduce costs through more efficient employment of human resources.

To our knowledge, lasers are routinely used in the production of precise circuit board structures as small as 50-μm wide [6,7], whereas the photolithographic technology dominates thin layer structuring, especially of platinum or ITO layers on silicon or glass [8,9]. With respect to the conventional long-pulse lasers, ultrashort pulse lasers improve the quality of ablation because a higher percentage of their pulse energy is employed in evaporation instead of melting processes [10,11]. This is another reason why the technology is gaining importance in the surface modification of thin layers [12] or in the industrial production of solar cells [13].

In some cases, laser techniques were used by other authors for the production of channels or recesses in microfluidic systems [4,14–16].

Here, we present a new prototyping technology for sensorized microfluidic cell-culture systems, which is based on a combination of different applications of an ultrashort pulse laser system. The system consists of a polycarbonate body with inlets and outlets for fluids, as well as a polycarbonate lid (*cf.* Section 2.4). The lid bears the microfluidic structure, which was imprinted in polydimethylsiloxane (PDMS). In the assembled system, the microfluidic structure is sealed by a microscopic glass slide, which bears the thin layer-sensor structures.

The polycarbonate body was produced using a standard printed circuit board (PCB) micromilling machine. Here, this machine was also used to produce the PDMS molding tools for imprinting the microfluidic structures into the PDMS lid. More precise, high temperature-stable molding tools, which are needed for hot embossing, injection molding or hot shaping, could be produced from ceramic wrought materials (Keralpor99, Kerafol GmbH, Eschenbach, Germany). Direct laser structuring is an

alternative to the common photolithographic production of PDMS molding tools, e.g., from SU-8 or silicon [1,17]. In our system, the microfluidic structure was confined by a microscopic glass slide on the bottom. The slide carried amperometric Clark oxygen sensors, potentiometric pH and impedimetric cell adhesion sensors interdigitated electrode structures (IDES) that were originally developed with photolithographic techniques and redesigned for laser structuring after testing [18].

Our new technology circumvents the use of clean rooms and toxic or harmful chemicals, thereby shortening manufacturing times. This technology allows for the use of a wide variety of wafer formats. Here, we present sensorized chips that were produced from cheap microscopic glass slides. Together with a microfluidic PDMS structure, they allow for long-term (days to weeks) cell culture and on-line registration of sensor readouts.

2. Experimental Section

2.1. Laser Structuring of Platinum and ITO Layers

Microscope slides (NK72.1, Carl Roth, Karlsruhe, Germany) were cleaned with aqueous 10% Tickopur W77 cleaning solution (BANDELIN electronic GmbH & Co. KG, Berlin, Germany) in an ultrasonic bath and dried in a pressurised air stream. The slides were sputtered with 130 nm of platinum on a 15-nm titanium undercoating using a magnetron sputter system (Ardenne LA-320S, VON ARDENNE GmbH, Dresden, Germany). The chips were structured by ablation with a picosecond laser (TruMicro 5X50, TRUMPF Laser- und Systemtechnik GmbH, Ditzingen, Germany) using a high-precision micromachining system (GL.5, GFH GmbH, Deggendorf, Germany). The laser could be switched between three wavelengths, 1030 nm (fundamental frequency), 515 nm (second harmonic frequency), and 343 nm (third harmonic frequency). The positioning precision of the system was better than 1 µm. The structure designs were drawn in AutoCAD 2010 (Autdesk, Inc., San Rafael, CA, USA) and directly exported into the laser control system as DXF-files [19]. For chip structuring, platinum or ITO (CEC020, Präzisions Glas & Optik GmbH, Iserlohn, Germany) were ablated from the microscope slide at a wavelength of 343 nm with a pulse frequency of 400 kHz with a pulse energy of 1.25 µJ, and an effective spot diameter of 10 µm. At 1000 mm/s driving, the pulse spot overlapped approximately 80%. To ensure the microscopic transparency of the chip areas without sensor structures, platinum areas were ablated in bidirectional stripes of 8-µm distance. This ablation step could be omitted in ITO structuring (Figure 1). For both materials, the sensor structures were outlined in a final step at 100 mm/s driving, a pulse frequency of 40 kHz and a pulse energy of 1.25 µJ. To compensate for the size of the laser spot, the outlines of the structures were enlarged by 6 µm in the AutoCAD files.

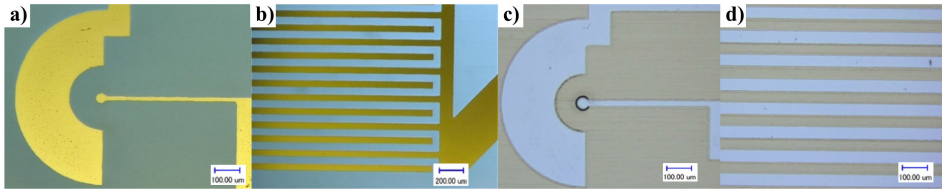

Figure 1. Platinum (**a**,**b**) and indium tin oxide (ITO) (**c**,**d**) structures of the oxygen sensors with a circular working electrode spot and semi-circular counter electrodes (**a**,**c**), as well as interdigitated electrode structures (IDES) with a50-µm pitch (**b**,**d**). The on-chip-sensor connectors were insulated in a following processing step.

2.2. Laser Structuring of Silicon Nitride (Si_3N_4)

After structuring, the chips were cleaned again, as described above, and sputtered with a 200-nm Si_3N_4 passivation layer (300 s at 400 W in high frequency mode). For a thicker passivation layer, another chip batch was passivated with 1000 nm of Si_3N_4 (GeSiM mbH, Grosserkmannsdorf, Germany) using a PE-CVD process. Si_3N_4 was chosen because of its chemical, electrical and optical characteristics, as well as its biocompatibility. A second laser-processing step was executed to create windows in the passivation layer for the external chip connectors and the sensors. Si_3N_4 layers of both thicknesses were ablated at 1000 mm/s driving, a pulse frequency of 200 kHz and a pulse spot overlap of approximately 70%. To remove the Si_3N_4 passivation layer only and not the platinum layers, a maximum pulse energy of 1.0 µJ was used. To produce pH sensors in a final step, a 60-nm Si_3N_4 layer was sputtered through four openings in a PCB, which was used as a cheap and simple mask.

2.3. Laser Structuring of Molding Tools by Laser Ablation

In an attempt to overcome the limitations of the stereo-lithographic (material, aspect ratio) and micro-milling techniques (precision, surface roughness), the laser system was used to create new molding tools for microfluidics. A wrought aluminum oxide ceramic chip was laser processed in the same system with at a wavelength of 1030 nm, a pulse energy of 125 µJ, 2000 mm/s driving and a pulse rate of 200 kHz. A structure depth of 240 µm was reached after 20 repetitions.

2.4. Cell Culture System and Microfluidics Fabrication

Figure 2 presents the cell culture system consisting of a body with fluidic connectors and a lid with a central PDMS membrane. An outside ring and inner spacer structures confined the microfluidic cell culture volume. All of the polycarbonate parts, *i.e.*, the system's body, lid and the PDMS molding tools for the microfluidic structures, were produced by a PCB micromilling machine (CCD 2, BUNGARD Elektronik, Windeck, Germany). The body and lid were designed in AutoCad and milled in an 8-mm polycarbonate plate, whereas the molding tools were milled in polymethyl methacrylate (PMMA) because of its better grindability with smaller milling tools (<500 µm in diameter). For the larger fluidic body structures of the cell culture system, Teflon® could also be used for the molding tools. Because of its low surface adhesion, Teflon® has advantages in the separation of the PDMS parts from the casting molds, though it is delicate to mill with small tools.

To produce the PDMS parts, PDMS monomer (Sylgard 184, Dow Corning Inc. Midland, MI, USA) was degassed, injected into the molding tools and cured for 4 h at 70 °C. After removing the molded parts, the bottom side of the PDMS body was manually coated with a very thin layer of uncured PDMS before the body was fixed to the chip under pressure and cured for a second time. For improved PDMS adhesion, the polycarbonate parts and the chip surfaces were coated with a bonding agent (GRUN G790, Wacker Chemie AG, München, Germany) before PDMS injection. After curing the PDMS, the microfluidic connectors were glued to the body. The connectors were shortened syringe needles fixed with their plastic connectors in cross-shaped notches of the body (Figure 2). The notches hindered rotation of the syringe heads and facilitated the attachment of Luer lock connectors. The removable lid enabled access to the culturing volume and the integration of 3D scaffolds.

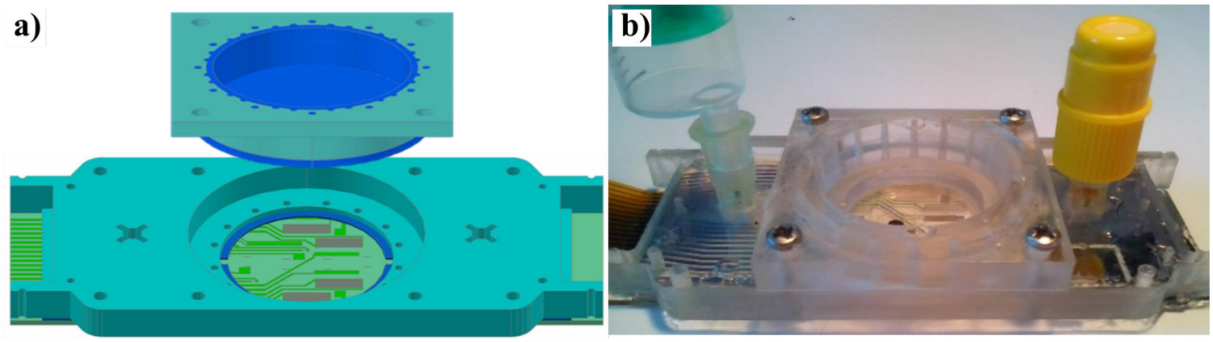

Figure 2. Computer-aided design of the microfluidic system (**a**) with the body (bottom) and lid (top) milled in polycarbonate (light turquoise). The blue polydimethylsiloxane (PDMS)-imprinted parts (gasket with fluidic channels in the body and PDMS membrane in the lid) confine the fluidic volume. The laser-structured chip (green) is glued to the lower PDMS surface. (**b**) The assembled cell culture system with a flexible printed circuit board connector (lower left), as well as a syringe and syringe-injection cap on the left and right inlets, respectively.

2.5. Cell Culture

To verify the biocompatibility, a mouse osteoblast precursor cell line (MC3T3-E1) was used. These cells, which are a common model system for bone regeneration, were obtained from the German collection of microorganisms and cell culture (DMSZ GmbH, Braunschweig, Germany). The cells were cultured in an incubator at 37 °C with 95% humidity and 5% CO_2. The cells were grown to confluence in 50-mL cell culture flasks (25 cm^2; Greiner bio-one, Frickenhausen, Germany) in alpha medium (order No. F 0925) supplemented with 1% penicillin/streptomycin (stem solution: 100 U/mL penicillin and 100 µg/mL streptomycin) and 10% foetal bovine serum (all purchased from Biochrom AG, Berlin, Germany). After the cells grew to confluence, they were trypsinated in phosphate-buffered saline supplemented with 0.05% trypsin and 0.02% EDTA (PAN Biotech GmbH, Aidenbach, Germany) and were diluted before subculture or transfer to the microfluidic systems.

For cell culture in the microfluidic systems, carbonate-free alpha medium (P03-2510, Pan Biotech, Aidenbach, Germany) was supplemented as described above and buffered with 20 mM HEPES (Carl Roth, Karlsruhe, Germany). For cell seeding, 500 µL of the cell suspension with up to 500,000 cells/mL (corresponding to 50,000 cells per culture system) were injected by slow flushing of the system. Then, the system was closed with a medium-filled 6-mL syringe at the inlet and a syringe injection cap as a cannula injection port at the outlet (Figure 2). For medium exchange, an empty, open syringe with a 0.6-mm cannula was plugged into the injection port, and 500 µL of medium from the filled syringe was slowly flushed through the system.

3. Results and Discussion

3.1. Structuring of Platinum and ITO

For processing, the sputtered chips were aligned with the limit stop of the laser workbench. The determination of the optimal laser parameters (power, driving, pulse rate, and pulse spot overlap) for

structuring platinum or ITO layers required several pre-runs. Less than 3 min per chip were needed with the optimized laser process (chip area of 11.5 cm^2). The structures could be manufactured with widths less than 20 µm and minimal distances of 10 µm. The smallest on-chip structures were the working electrodes of the oxygen sensors with 40-µm diameter and a conducting path with 20-µm width.

The edge roughness of the structures was usually in the range of ±1.5 µm. We found that this result was less due to a problem of the beam guidance but primarily a consequence of the inhomogeneous ablation in the outer zones of the laser spot where the ablation depended on the trace curvature and the materials (Figure 3).

As observed in Figure 4, the distances between the IDES fingers were approximately 2 µm wider than expected, whereas their width was approximately 2 µm too small. This small mismatch between the design and manufactured structure resulted from an insufficient offset correction.

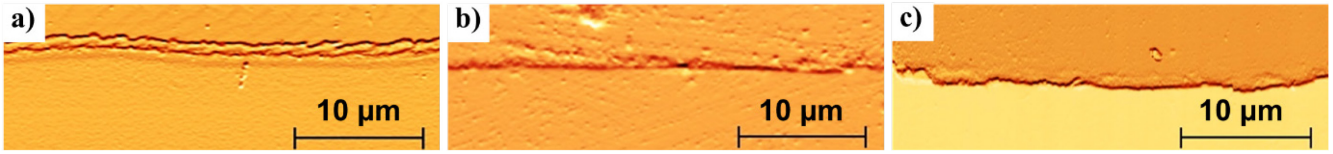

Figure 3. Edge quality of an IDES finger controlled by colour contrasted atomic force microscopic images. (**a**) ITO on glass; (**b**) platinum on glass; (**c**) photolithographically (photo mask) produced chip (GeSiM®).

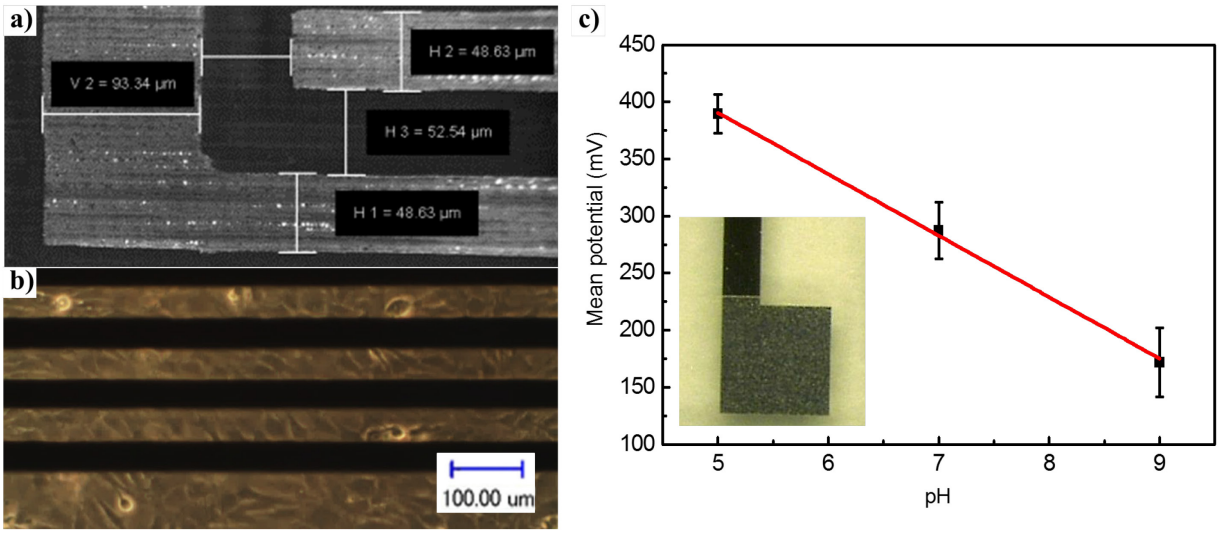

Figure 4. Images of a platinum IDES. (**a**) Scanning electron microscopy image with dimensions. (**b**) Confluent cells above the IDES. (**c**) Sensitivity test of a Si_3N_4 sensor layer on platinum (insert: pH sensor with 1.4 × 1.4 mm^2 electrode area). The data represent measurements with six chips in pH-buffered solutions with pH values of 5, 7, and 9. The straight line corresponds to a sensitivity of −53.8 ± 1.8 mV/pH.

Despite the ease of small offset corrections, small circular structures are more difficult to produce. The effective laser driving, *i.e.*, the linear distance between three pulse spots, is decreased in curved areas, which led to an increased pulse spot overlap, a stronger removal of material, and even some ablation of the glass carrier (see Figure 1c, oxygen working electrode). This problem will either be

solved in a preliminary approach by a modification of the laser parameters in the individual software files for curved structures or with the next update of the driver software of the manufacturer of the laser positioning system (GFH GmbH).

3.2. Structuring of Si_3N_4

The laser parameters for processing the Si_3N_4 passivation layer were confined to a very small window by the underlying platinum and ITO structures of the IDES and oxygen sensors or the peripheral chip contacts. Within this window, 200-nm layers of Si_3N_4 could be removed without removing the platinum layer [19]. The ablation efficiency depended on the underlying substrate. The ablation of Si_3N_4 from glass required higher pulse energy than that over platinum because the ablation thresholds for Si_3N_4 were reduced over glass. Nevertheless, for platinum, the process led to roughening of the surfaces due to the punctual ablation (see the IDES in Figure 4). Interestingly, the 200- and 1000-nm Si_3N_4 layers could be removed using the same laser parameters, sustaining the theory of the effects at the Si_3N_4-platinum or Si_3N_4-glass interfaces that influenced the ablation process [13].

To produce pH sensors, 60 nm of Si_3N_4 were deposited on the laser-manufactured platinum structures. The chips were tested for their stabilities and pH sensitivities by measurements in standard buffer solutions with pH values of 5, 7 and 9 against an Ag/AgCl microelectrode (Microelectrode Inc., Bedford, NH, USA). The chips provided reproducible potentials 10 s after medium exchange. They were kept in the solution at room temperature for up to 600 s to confirm the stability of their readouts. The potentials were linearly dependent on the pH in the range from 5 to 9 at a sensitivity of -53.8 ± 1.8 mV/pH. This sensitivity was comparable to the sensitivity of ISFETS with a Si_3N_4-gate passivation layer [20].

3.3. Structuring Molding Tools by Laser Ablation

Laser manufacturing of molding tools from wrought aluminum oxide ceramic chips was fast and provided a very good surface quality (Figure 5). Per ablation scan, a depth of approximately 12 µm was obtained with 2000 mm/s driving and a repetition rate of 200 kHz. For our structures, the area-specific processing time of approximately 3600 mm^2/min per scan largely referred to the actual ablated area. A structure depth of 240 µm was reached after 20 repetitive scans, resulting in a 5 min processing time for the surface of the 30×30 mm^2 moulding tool (Figure 5a).

The moulding tool for the large sensor chip with a final depth of 465 µm was processed with modified parameters (Figure 5b). The driving and repetition rate were increased to 4000 mm/s and 400 kHz, respectively. This modification reduced the manufacturing time to approximately 35 min for 42 scans despite the larger surface of the tool.

The small tilts of the vertical structures were caused by optical effects in the ablating process (see the laser scanning microscope images in Figure 5). We believed that the resulting tilts in the fluidic channel walls would be advantageous in finally stripping the mold from the cast. If necessary, the tilts could be reduced by using special trepanning optics. Nevertheless, the smooth removal of the cured PDMS molds from the ceramic tools required the use of a mold release agent.

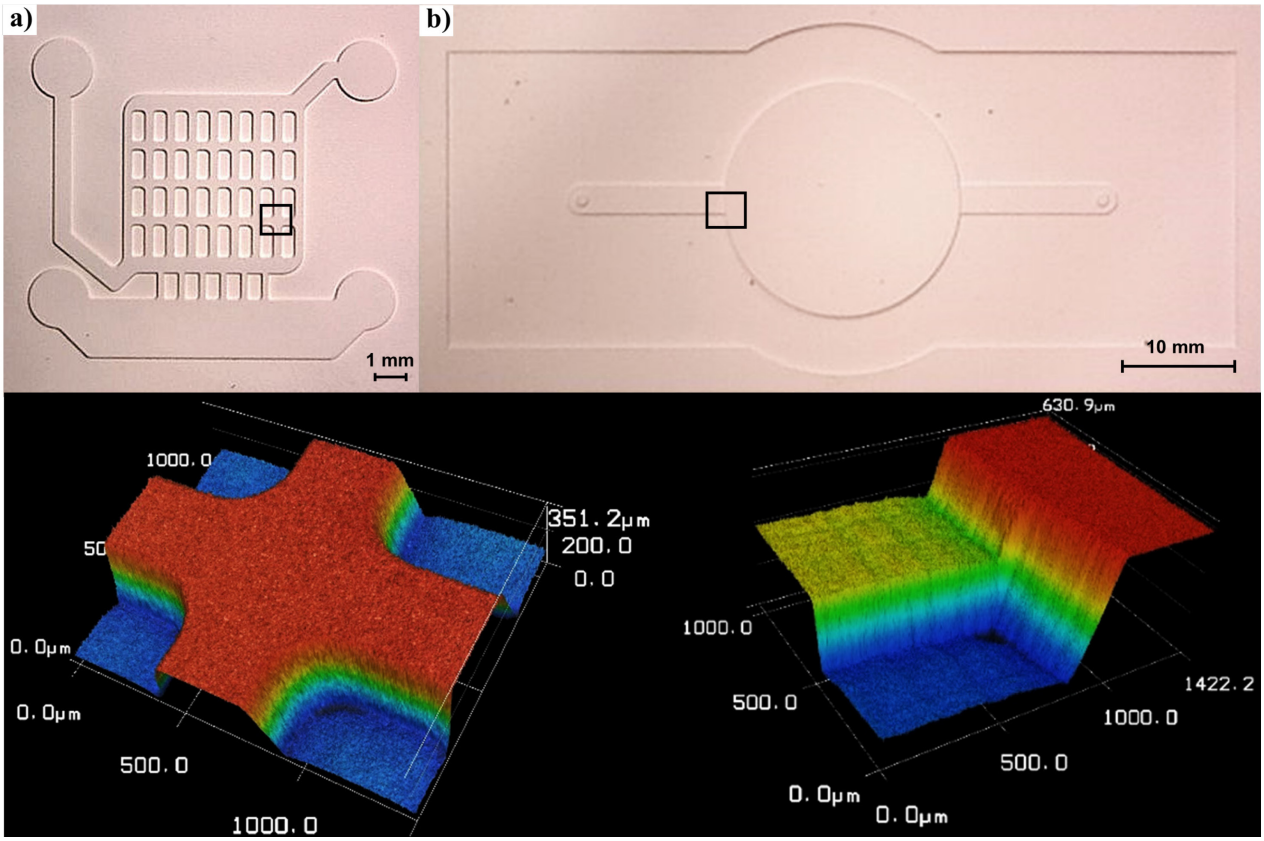

Figure 5. Two ceramic mold chips processed by laser ablation. Black squares mark the positions of the laser scanning microscope images below the mold images. (**a**) A 30 × 30 mm^2 chip for a microfluidic pattern with 240-µm channel height. (**b**) Larger structure (33 × 76 mm^2) for the cell culture system with heights of approximately 230 µm (horizontal inlet and outlet channels) and 465 µm (circular area). Please note that the measures given in the images for the vertical scaling refer to a larger measuring frame.

3.4. Cell culture

MC3T3-E1 cells showed good proliferation in the microfluidic cell culture systems, reaching confluence approximately three days after seeding. The cells adhered to the Si_3N_4, as well as the platinum surfaces without surface coatings, e.g., with poly-D-lysin or laminin [5,18], suggesting that the laser ablation-induced surface roughness may improve cell adhesion [21,22]. Interestingly, the aqueous cell culture medium evaporated through the PDMS lid, resulting in the formation of perturbing gas bubbles. Bubble formation was likely enhanced by the roughness of the cutting edges of the mold structures, which may have been transferred to the PDMS surfaces. In our system, the bubble formation could be suppressed by overlaying the lid of the cell culture system with deionized water, allowing for cell culture times longer than 10 days.

4. Conclusions and Outlook

The use of micromachined bodies and molding tools produced by the well-established PDMS technology in combination with ultrashort pulse laser micromachining of the sensor chips enabled fast prototyping of sensorized microfluidic cell culture systems without any photolithographic processing

steps. We could produce thin-layer structures on glass chips with resolutions of approximately 10 μm. The use of this system reduced the fabrication times because the ablation of larger chip areas for microscopy could be spared. The optical transparency of the ITO substrates improved the microscopic analysis of the cell culture.

Fresh pH-sensitive Si_3N_4 layers were finally sputtered on the laser-machined structures. The layers showed a very good sensitivity of −53.8 ± 1.8 mV/pH when compared with ISFETs with Si_3N_4 gate passivation layers [23].

Our cell culture systems could be vapor sterilized and easily modified by remolding the PDMS structures of their lids. MC3T3-E1 cells could be cultured longer than 10 days with good cell adhesion to the laser-manufactured chip surfaces even without further surface modifications. The gas permeability of the PDMS lid ensured 100% air saturation in the culture medium, which may be desirable for the cells but could cause bubble formation. Smoother molds produced, for example, by laser processing, would likely reduce this problem in future microfluidic designs.

Ultrashort pulse laser techniques could also be used to produce casting molds for PDMS molding or even hot embossing of PMMA. This would require robust, temperature-stable casting molds manufactured, for example, from aluminium oxide ceramics. Our prototypes were produced in 3 to 35 min with a resolution of approximately 10 μm in the horizontal direction and approximately 13 μm in the vertical direction. This resolution outperformed that of the currently available stereo-lithographic 3D printers.

For the current system, a machine hour costs approximately €220, resulting in processing costs of €5 per ITO chip in a 60-chip batch. This system also allowed for the production of holes or recesses for advanced microfluidic designs (not shown). The maximum driving of our system of up to 4000 mm/s will be improved in the next generation of ablation systems.

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Author Contributions

Sebastian M. Bonk designed all experiments with the microfluidic cell culture system. The laser experiments were performed by Paul Oldorf. Jan Gimsa, Werner Baumann, and Rigo Peters contributed knowledge, devices, materials, and reagents. Sebastian M. Bonk provided a manuscript draft. He was financed by grants to Jan Gimsa who finalized the manuscript.

Conflicts of Interest

The authors declare no conflict of interest.

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