

Determination of living cell characteristics and behavior using biophotonic methods

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ABSTRACT

This paper describes the development of methods for the determination of the characteristics and the behavior of living neural cells. A technology which is used is the deep ultraviolet (DUV) modification of methylmethacrylate polymers which leads to a new surface chemistry affecting the selective absorption of proteins and the adhesion of living cells *in vitro*. The bi-functionality of the modified polymer chips supporting waveguides and cell anchorage capabilities at the same time provides the opportunity to monitor protein adsorption, cell attachment and spreading processes by evanescent-field techniques. This allows the defined spatial control of a cell / surface interaction and leads to a combination of desired biological and optical properties of the polymer. Among them are the high sensitivity of cultured mammalian cells to, for example, environmental changes and special features of integrated optical waveguides like their online compatibility, minuteness and robustness. The scientific fields, biology and optics, meet at the polymer surface becoming a cell culture substrate together with an optical waveguide by the application of special patterning and fabrication technologies. In addition to the already mentioned fabrication and immobilization technology, the technique proposed also offers the possibility of being able to couple to microstamping processes and to also incorporate electrical measurements on individual cells. Thus, by extending this method and coupling it to the DUV technique described above the possibility is given of being able to simultaneously optically and electrically interrogate individual cellular processes with spatial resolution.

Keywords: polymer, waveguide, DUV lithography, biophotonics, cells

1. INTRODUCTION

Integrated micro- and nanosystems for the determination of the characteristics and the behavior of living cells have attracted a lot of attention lately. For decades technical limitations have forced biologists to record the behavior not of individual proteins in single or few cells, but of populations of proteins in thousands or even millions of cells. Given the complex nature of the cellular mechanisms, the result was an average idealized description of cellular behavior. To understand these complex cellular mechanisms, it is necessary to determine how individual parts are integrated in space and time to form complex cellular functions and to measure multiple variables of living cells in real time. Integrated biophotonic devices are amongst the most promising systems for fulfilling this task.

The paper focuses on the development of a platform which is able to culture and pattern live single cells for a sufficient period of time, enables the integration of optical evanescent field polymer based photonic devices, and provides means to integrate electronic readout possibilities. This is realized by a combination of engineering nanofabrication and biological methods forming a cross discipline approach.

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2. STATE OF THE ART

Over the past few years, there has been an increasing demand and interest both scientifically and technologically for small and portable analytical tools, the so called Lab-on-Chip systems, for present and future applications in areas such as medicine, food, environment and public security [1, 2]. The ultimate goal has been to integrate as many analytical functions as possible into a single device and it has been undertaken by universities, research institutes and companies all over the world [3]. At the heart of this effort is the non-invasive analysis of isolated cell populations or even single cells. There is a great need to understand the basic cellular mechanisms and yield information unaffected from average measurements. In addition, several cellular signaling pathways act in a timescale of few seconds and there is a need for real time monitoring with similar time resolution.

Microfluidic chips are ideal platforms for housing and handling small numbers of living cells [4]. A large variety of microfluidic systems is available for cell analysis. The cell handling procedure in microfluidic chips follows cell sampling, cell trapping and sorting, cell treatment and cell analysis. Cell trapping and immobilization at predetermined places inside fluidic channels and keeping the cells alive for a long period of time is of fundamental importance.

Fluorescence from designed sensors and probes is the major information channel for obtaining information from living cells to different functional stimuli [5]. There are two major approaches for introduction of these sensors and probes without impairing the cell viability - by using cell-permeable dyes for operation in cytoplasm and labeling cell organelles and by using cell-impermeable dyes for labeling the cell membrane. This membrane contains a variety of receptor and transporter proteins that modulate the cellular function and serve for chemical and electrochemical communication between the cells. Different metabolites and drugs change the surface properties of this membrane.

Optical waveguides are the basic and the most important optical elements that must be integrated into Lab-on-a-Chip Microsystems [6]. So far, there have been many bioanalytical systems that use optical waveguides as their main analytical tool [7, 8]. The most common methods for waveguide fabrication are conventional deposition techniques like chemical vapor deposition, flame hydrolysis deposition and ion exchange on glass substrates. However, polymers are more favorable substrates for biophotonic devices due to the biocompatibility, the fabrication flexibility they offer and their low cost [9]. Polymers also have the advantage of acting as sensitive layers which is the prerequisite for the optimization of chemical and biochemical sensors [10].

Consequently, the fabrication of optical waveguides on polymeric substrates has the potential to solve a major integration problem.

The basic principle used in this paper for realizing polymer waveguides by deep UV modification of the polymer substrate has first been presented in 1996 by Frank et al. [11]. The refractive index can be increased in methacrylate polymers by deep UV irradiation. This change is large enough to fabricate single mode waveguides.

Various concepts for biosensors based on evanescent field sensing [12] have been demonstrated [13, 14, 15]. Recently it has been shown that living mammalian cell adhesion and spreading can be monitored online and quantitatively via the interaction of the cells with the evanescent electromagnetic field present at the surface of an optical waveguide [16]. The idea to study living cells in combination with optical waveguides has also been used in context with optical waveguide lightmode spectroscopy (OWLS) and confocal laser scanning microscopy (CLSM) which provides information about the shape of the cells at the surface [17]. This allows for the correlation between the cell-shape information from CLSM and the cell-surface interaction measurements from OWLS.

The evanescent field in planar waveguide structures reaches into the cell usually not more than 300 nm. Using a reverse symmetry waveguide presented in [18], where an evanescent field of about 1 μm is obtained, one can potentially penetrate 1 μm into the cell body. The reverse symmetry waveguide uses a cladding material with a higher refractive index than the substrate (here nanoporous silicon), thus enabling the optical mode field in the waveguide to turn around and penetrate into the cladding compared to a conventional waveguide design, where the optical mode field decays gradually into the direction of the substrate.

Living cells have been attached to optical fibers for realizing biosensors, e.g. [19]. The fiber is immersed in an aqueous media appropriate for cell viability. The response of the cells to small quantities of toxicant can be monitored spectroscopically [20].

Living cells on top of waveguides in a microfluidic chip can potentially give valuable information about cell attachment, spreading and proliferation on solid surfaces. In order to grow living cells on waveguides, the ability to specifically

pattern cells has to be given. Up to now the surface chemistry controlled cell patterning was performed predominantly on the basis of self assembled monolayers of substituted thiols on coinage metals [21]. However, polymeric substrates are not suitable or need special pretreatments like metal deposition or hydroxylation in order to be structured by that method. Although different polymers are the abundant material for cell culture substrates, some investigators have used polymers for patterned cell cultures [22, 23]. Detrait et al. [22] used conventional photoresist technologies to create patterns of oxygen plasma treated regions on polystyrene. Although this multi step protocol is rather time consuming, it allows a higher lateral resolution of the plasma treatment as compared to removable masks like metal grids for electron microscopy. Matsuda et al. [23] used the photoreactive copolymer poly(N,N-dimethylacrylamide-co-3-azidostyrene) which was spread uniformly on tissue culture polystyrene and partially immobilized on the substrate by UV irradiation. Subsequently, the copolymer in masked regions was removed with water. Kaibara et al. [24] used a mask assisted vapor deposition of carbon on segmented polyurethane and polystyrene to control the adhesion of bovine aortic endothelial cells. Alternative methods used poly(dimethylsiloxane) (PDMS) stamps [25] to directly stamp poly-L-lysine patterns onto silicon or glass surfaces although the method was not tried on polymeric surfaces.

The combinations of optical and biological active materials have not yet reached significant economic relevance. The implementation of living organisms is currently restricted to a low level, for instance, sensors for waste water testing based on luminescent bacteria. These systems show already the advantages of biophotonics, but are less complex than the projected concept which uses immobilized cells. K. Marx et al. [26] showed by using quartz micro gravimetry (QCM), relevant changes of the cytoskeleton of endothelial cells influenced by nocodazol in the nMol range. However QCM requires elaborate driver- and data acquisition electronics compared to integrated waveguide based biophotonic sensors proposed in this paper.

3. POLYMER WAVEGUIDES

Two types of waveguides have successfully been developed and fabricated. Planar polymer waveguide based devices and ridge waveguide based devices have been realized by hot embossing. Embossing offers the direct combination of fluidic channels with photonic devices in one step compared to the planar type, where two steps are necessary for combining waveguides and fluidic channels.

3.1 Planar polymer waveguides

Deep UV-induced modification of the dielectric properties of polymers [27] is not only a useful technique for the realization of integrated optical circuits for telecommunication and sensor applications [28, 29], but has also been used for defined patterning and controlling of cell adhesion [30, 31].

The deep UV technique has several advantages with respect to common methods because only a single polymer layer is used, which serves as the substrate and waveguide as well and no further etching or development steps are required. In addition, the use of polymers as a biocompatible material, offers the combination with fluidics and biological substances. Photographs of fabricated basic components of photonic integrated circuits are shown in Fig. 1. The basic single mode waveguide has a graded index profile with an exponential decay. The waveguide width has to be fitted to the desired application in order to obtain single mode propagation of the light. In the case of biophotonic sensor applications in polymers, the wavelength region will be between 400 nm – 700 nm. In this wavelength region, the used polymer and also the fabricated devices have only 0.1 dB/cm loss which is ideal for the envisaged application. The width of the waveguide in this wavelength region is between 3 and 5 μm . The transfer of the design to the substrate is done by fabricating a quartz/chromium mask and using a deep UV exposure system. A mask aligner type EVG 620 with a cold mirror having a reflectance between 200 and 240 nm was used for fabricating the devices.

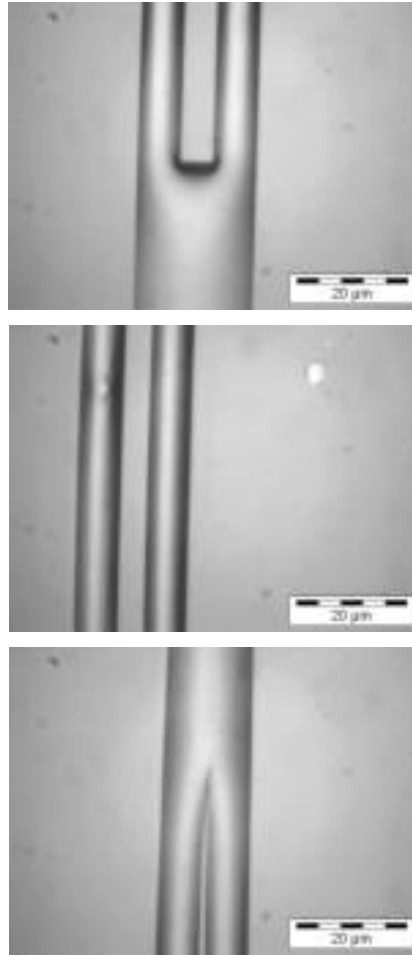


Fig. 1 Photograph of photonic integrated circuits, multimode interference coupler (top), directional coupler (center), Y-splitter (bottom).

UV-exposure of the polymer results in a modification of the surface behavior of the polymer substrate as well as in a change of the refractive index in a surface layer of a depth of about 5 μm to max. 10 μm . The polymer used for realizing photonic devices is commercially available polymethylmethacrylate (PMMA). Recently another polymer with higher glass transition temperature and higher thermal stability, alicyclic methacrylate, showed a similar behavior as the used PMMA and further investigation of the material is in progress [32].

3.2 Polymer waveguides made by hot embossing

The combination of deep UV-induced modification with ridge waveguide structures made by replication of different methylmethacrylate polymers is a promising approach. Optical waveguides made of hot embossed ridge waveguide structures of methylmethacrylate polymers require an additional deep UV flood exposure in order to partly change the refractive index of the polymer at the surface of the ridge waveguide.

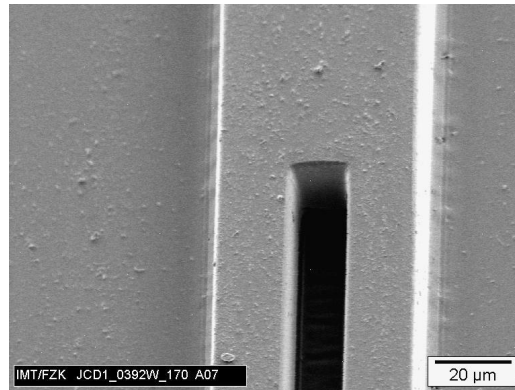


Fig. 2. Photograph of the output region of a MMI coupler fabricated by hot embossing.

An example of the output region of a fabricated 1x2 multimode interference coupler is shown in Fig. 2. The concept of a biosensing device consists of a multimode interference coupler (MMI), where one input waveguide is coupled to two output waveguides. The idea is to pattern neural cells on certain areas of the MMI where there is a maximum of the interference of the waveguide modes. This cell – mode field interaction changes the intensity of the output waveguides, which is fed to a detector, thus monitoring cell behavior. This idea has been used in optoelectronic devices for telecommunication systems, where a MMI with tunable output intensities is presented [33].

4. PHOTOCHEMICAL POLYMER SURFACE PATTERNING CONTROLLING CELL ADHESION

Patterned arrays of living mammalian cells are essential for studies of a variety of defined cell (co-)cultures, cell differentiation, and intercellular communication and for engineered tissues as highly functional bioartificial organs for therapy and drug research.

The surface modification was performed by an UV irradiation of polymer samples in air using a low pressure mercury lamp (Heraeus Noblelight, NNQ lamp, $\lambda = 185\text{nm}$, 15W) at 10cm distance for 30 minutes. For patterned exposure a chromium mask on quartz was placed in contact to the polymer surface (Fig. 3)

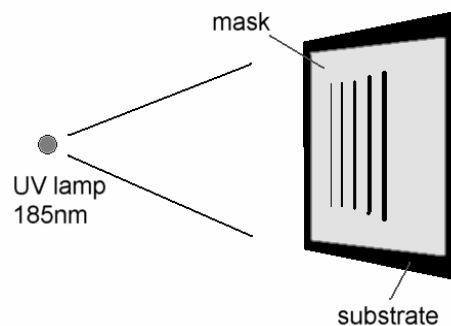


Fig. 3. UV patterning process.

UV irradiation induces:

1. The formation of peroxides at the polymer surface allows graft coupling. Peroxides decompose within a couple of days during storage.
2. Formation of carboxylic acids affecting cationic dye binding, wettability and surface energy contributions.

3. Surface ablation. Due to corrosion at longer irradiation times, a constant COOH surface density is obtained at $t > 60$ minutes. Carboxylic acids groups were found to be stable for shelf lives of several months.

Due to these changes in surface chemistry the adsorption of albumin (BSA) is hindered on irradiated as compared to unmodified regions (Fig. 4). In addition, the viscosity of the surface bound albumin layer on for example polystyrene (PS) ($\eta = 7.6 \pm 1.3 [\times 10^{-3} \text{ Ns/m}^2]$), being a cell repellent material in its native state, is lowered on irradiated surfaces (4.5 ± 0.4) [13-15]. Laminin or other proteins from foetal calf serum (FCS) are preferentially adsorbed on photo modified areas and serve as adhesion stimulus for different cells (hepatoma cell line HepG2, L929 fibroblasts and others).

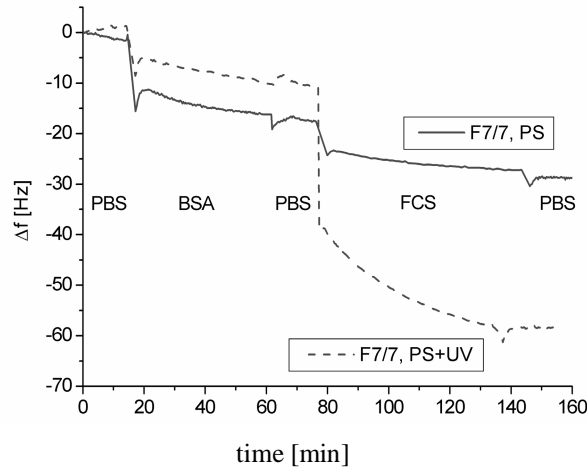


Fig. 4. Quartz crystal microbalance (QCM-D) recording of albumin (BSA) and serum protein (FCS) adsorption from buffered saline on native (solid line) and photomodified (dashed) polystyrene.

Two types of living cells have been successfully immobilized and deep UV patterned in the form of straight lines (Fig. 5), fibroblasts (L-929) perpendicular to polymer waveguides, Fig. 5 (done at the Forschungszentrum Karlsruhe),

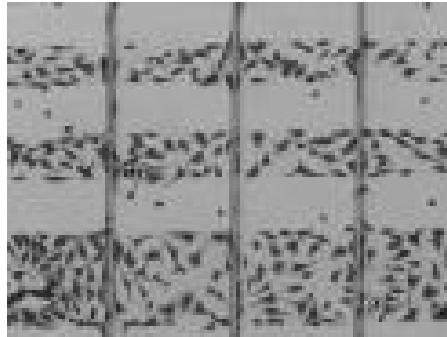


Fig. 5. Fibroblasts on waveguides.

and primary rat brain neural cells, Fig. 6 (Fischer 344 rat hippocampus) (done at the University of California Santa Cruz), grown on 5 μm lines with 25 μm islands every 100 μm . Additionally, primary rat brain neural cells were grown on 50 μm per side squares with 50 μm separating adjacent squares.

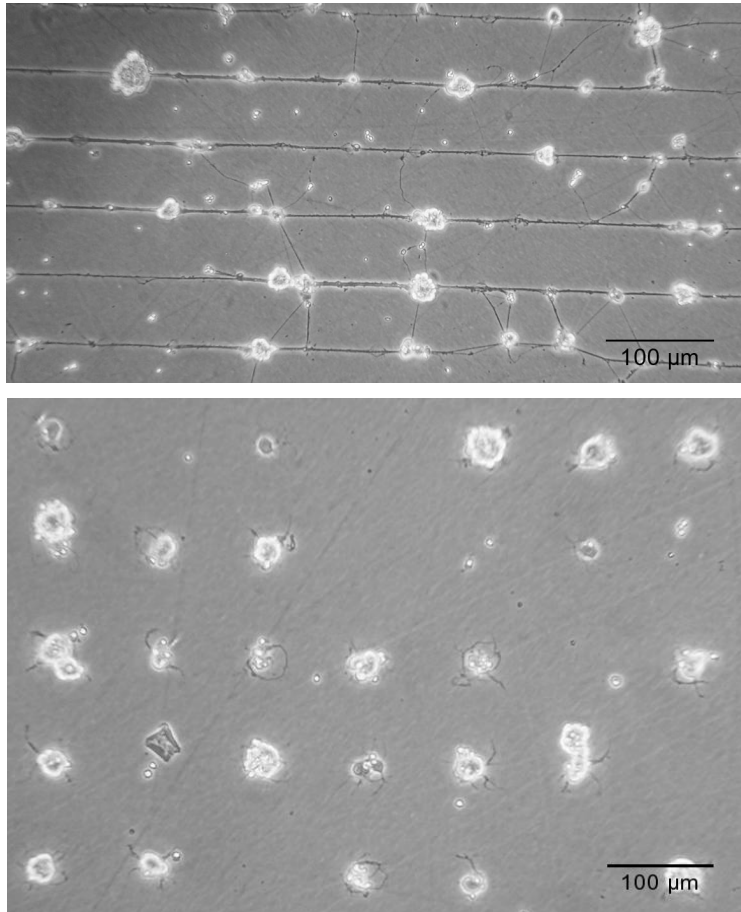


Fig. 6. Immobilized and patterned fibroblasts L-929 perpendicular to polymer waveguides (top) and primary rat brain neural cells (Fischer 344 rat hippocampus) with processes (center and bottom).

The combination of these developed technologies of realizing waveguides and patterned surfaces for the immobilization of living cells, enable the realization of novel biophotonic devices not only for sensors, but also for the characterization and determination of the behavior of living cells.

5. ELECTRICAL READOUT USING MICROELECTRODE ARRAYS (MEA)

Electrical measurements have been successfully made using custom fabricated microelectrode arrays (MEA). The MEAs used have 20 μm diameter round electrodes arranged in clusters of five with a center-to-center distance of 60 μm . Clusters of five are separated by 3.5 mm and arranged in a 9x9 grid for a total of 405 electrodes. Carbon nanotubes are used as the electrode material and have an impedance of 1 $\text{M}\Omega$ at 1 kHz. Signals recorded from cells were sent to a bipolar amplifier with a gain of 1000. The amplifier output was then sent to a PC controlled data acquisition system.

Embryonic day 18 primary rat brain neural cells (Fischer 344 hippocampus) cultured for 14 days have been successfully interrogated using carbon nanotube MEAs. Cell substrates were coated with poly-L-lysine, prior to cell culture, to promote cell adhesion. Electrical signals measured from cultured cells ranged from 50-200 μV with 40 μV peak-to-peak noise (Fig. 7).

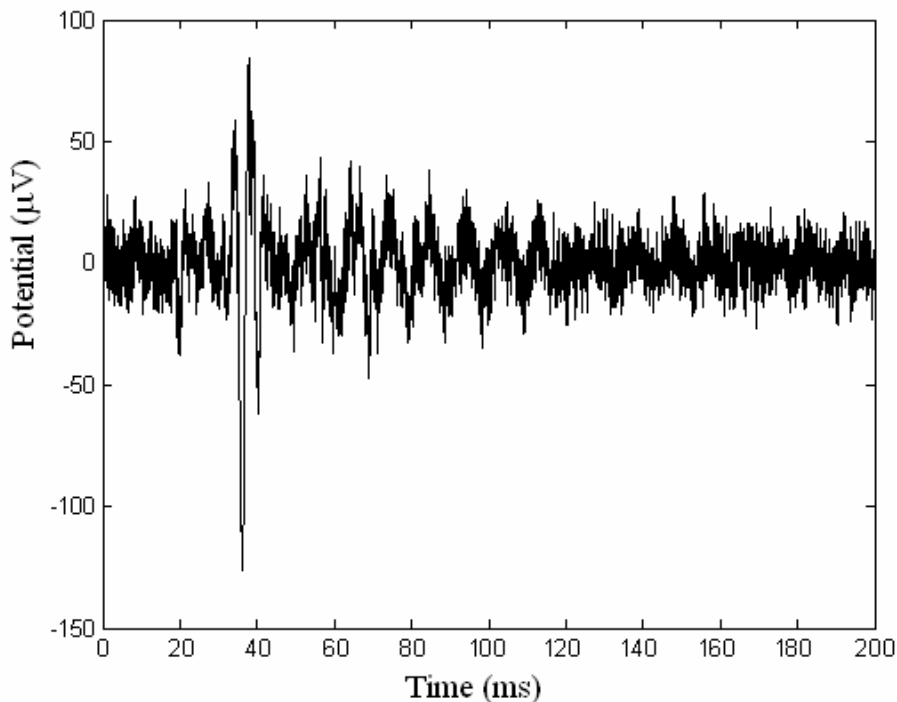


Fig. 7. Electrical recording showing a spike train recorded using a microelectrode array from primary rat neural cells (Fisher 344 rat hippocampus) after 14 days in culture.

By combining the developed technologies of polymer waveguides and patterned surfaces for cell immobilization with microelectrode arrays, novel biophotonic devices can be realized for both the optical and electrical probing of cells.

6. CONCLUSION

A technology platform has been established for the simultaneous realization of integrated photonic devices and immobilization of living cells including neural cells. Current research is focusing on the combination of this technology platform with microelectrode arrays to optically and electrically determine the characteristics and behavior of living cells.

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