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The Cell Clinic: Closable Microvials for Single Cell Studies

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Abstract

We present the development of a cell clinic. This is a micromachined cavity, or microvial, that can be closed with a lid. The lid is activated by two polypyrrole/Au microactuators. Inside the microvials two Au electrodes have been placed in order to perform impedance studies on single or a small number of cells. We report on impedance measurements on *Xenopus laevis* melanophores. We could measure a change in the impedance upon cell spreading and identify intracellular events such as the aggregation of pigment granules. The electrical data is correlated to optical microscopy.

Keywords:

microactuators, polypyrrole, micromachining, melanophores, impedance measurements, microelectrodes

Introduction

The on-going revolution within biotechnology and biomedicine has led to an increasing research within genomics. After mapping of the genomic information, the real challenge is now to assess how the genomic information is translated into protein expression (proteomics) and cell metabolism (metabolomics). These studies require novel tools. Various tools are under development both within academy and industry. Microsystem technology (MST) might contribute with several such tools, as devices can be made with dimensions in the same order of magnitude as individual cells. MST could give devices with completely new functions, not available with classical methods.

Classical cell-biology studies carried out on petri-dish cell cultures may be not the most suitable methods to study single cells. They reveal responses that are statistical sums of a large population. The possibility to study individual responses such as on-off behaviour would be of great value [1]. By studying single cells averaging effects over large populations would be absent. Microbiologists are gaining interest in the individual study of single cells or single-cell organisms. The use of single cells for fast and specific medical diagnostic or pharmaceutical tests is an old idea, but has not been possible to realise yet due to the lack of microtools [2]. Recently some single cell devices have been presented. Lin *et al.* have developed a force transducer with which they could measure chemically induced tension in single myocytes [3]. Sager *et al.* have attached a MEMS fabricated lever/spring connected to an external probe [4]. By pulling the lever/spring they could measure the adhesion forces of bovine endothelial cells. Cell motility (speed and distance) under chemotactant stimulation has been studied in micromachined channels [5]. Using time lapse video microscopy individual cells were followed moving in the channels, which illustrates the potential of combining video microscopy and microtechnology for high resolution cell analysis.

Studying single cells could provide some complications. Cells can be extremely mobile, depending on the kind, and are able to crawl through holes and overcome obstacles with angles up to more than 310 degrees [2]. Neurons have been shown to escape from microwells even when hindered with a grillwork barrier [6]. This cell behaviour is to be expected since tissues are 3-D structures of cells and extracellular matrix proteins. A method to keep the cells contained in a vial would reduce the necessity to use immobilisation methods for mobile and non-adherent cells [7].

The containment of single cells is the first step towards single cell studies. Fuhr *et al.* have developed electric (AC) field cages by applying a high frequency AC voltage to a multipole electrode structure and demonstrated the possibility to capture a cell [8]. McConnell *et al.* have developed a *microphysiometer* [9]. This is a detector system that measured the cell responses of 10^4 - 10^6 cells simultaneously. They monitored the response of the cells to several chemical substances, such as ligands, by measuring the rate of proton secretion from the cells. A laminar flow over micromachined cavities was used to keep the cells in the cavity. This latter is not a real single cell application but a step in that direction.

Not only should containment or manipulation of (single) cells be achieved but also characterisation. Fluorescence probing, optical microscopy, and chemical analysis of metabolites are common methods to characterise cells. Impedance measurements might be an alternative. Impedance measurements are easily implemented not only on the macroscale but also on the microscale in "biochips". Impedance measurements have been widely used within cell biology for the characterisation of many cell types and bacteria, in set ups where the cells were allowed to adhere to the electrodes [10-12], in flow systems [13-15]), in systems where the total cell medium impedance was monitored as a function of cell metabolism [16], and even in a single cell set up [12]. The methods used include DC measurements as used in Coulter counters [17] that measure cell volume, and AC measurements with frequencies ranging from 1 Hz up to many GHz [10, 13, 18, 19].

However no standard has been agreed on for measurements, and the frequencies used seem to be somewhat arbitrarily chosen.

Design.

We have designed a *cell clinic* that could be a tool for the study of single or a small number of cells in a closed microenvironment (see Figure 1). Apart of being able to arrest a cell within a microenvironment without the need of immobilisations methods, the cell-clinic could have several other advantages. Signal amplification could be attained as the products secreted by the cell are contained, leading to a higher concentration, likewise for the depletion of metabolic substances. The cells can be easily shielded from the outer environment. For instance one could load a few cells in the vial, close the lid and rinse the surplus of cells from the chip. Individual cells can be easily tracked without the need of time lapse video microscopy.

We have chosen impedance measurements as it is an easy and fast measurement method and has been used extensively for cell characterisation. The microelectrodes can be implemented in the fabrication scheme without complicating the process significantly. However, other kinds of microfabricated sensors are possible, but require more complex fabrication (see discussion).

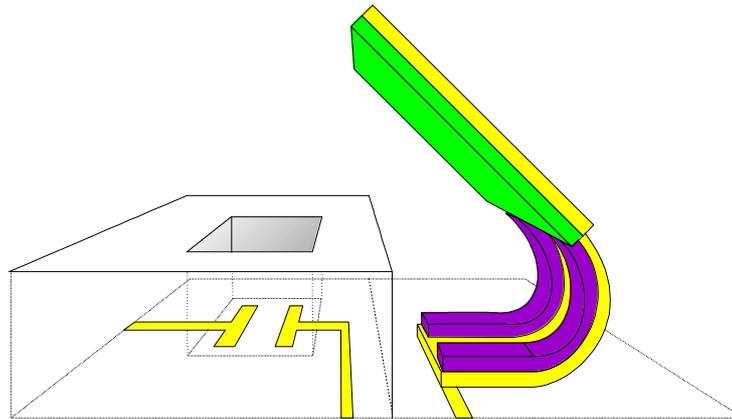


Figure 1 A schematic drawing of the second generation cell clinic. It consisted of a microvial (100 μm by 100 μm wide and 20 μm deep) defined in a layer of SU-8 that can be closed with a lid (green/yellow) activated by two PPy hinges (purple/yellow). On the bottom of the clinic, sensors have been placed, in this case two Au electrodes for impedance measurements.

We have made two designs of the microvials. In the first generation of microvials the vials were defined in the substrate. The vials were defined either in a Si substrate resulting in the typically pyramid shaped cavities [20] or in a glass substrate (Figure 5). In the second generation, we wanted to add sensors inside the microvial. Since micromachining is still quite limited to two-dimensional surfaces, it is easier to microfabricate the sensor electrodes on a flat surface, and define the cavities in a thick layer on top of the substrate. Novel thick film resists such as SU-8 (Microlithography Chemical Corp., Newton, MA, USA) that can be patterned using standard mask aligners without the need of X-ray lithography have made this possible. However, it is possible to make wires in Si holes and vias using electrodepositable photoresist [21]. A sketch of the cell clinic is shown in Figure 1. Since microscopy is needed for the visible study of the cells we have used transparent materials for both substrate and vial layer.

The microvial was $100 \times 100 \mu\text{m}^2$ and 10 μm (design 1) or 20 μm (design 2) deep. The vial could be closed by a lid of the size $150 \times 150 \mu\text{m}^2$. To activate the lid two PPy microactuators were used as active hinges. These microactuators were made of the conjugated or conducting polymer polypyrrole (PPy). Under electrochemical activation in a liquid electrolyte the polymer shrinks and swells. The main mechanism of this volume change is the insertion and de-insertion of counter or dopant ions from the electrolyte into

the polymer film and vice versa [22, 23]. When this volume-changing layer is combined with a passive layer like Au, an actuator or bending bilayer, like a metal bilayer, is created. These bilayer structures are the PPy microactuators and have among others been used to lift plates attached to the microactuators and activate a microrobot [24-27].

In the second design, two electrodes ($20 \times 80 \mu\text{m}^2$) have been placed on the bottom. In the first design four arrays (each with a different length of the hinges) of ten microvials were put on a chip. In the second design two arrays (with two different hinge lengths) of ten microvials were put on a single chip.

Fabrication

The cell clinic chips were fabricated using standard photolithography and micromachining procedures. For more details on the fabrication and design issues of PPy/Au microactuators we refer to [25, 27]. Here we report only the fabrication of the second generation of cell clinics. The first generation microvials were quite similar. In these devices the lids were made of a photopatternable plastic called benzocyclobutene (photoBCB) and the vials were made by wet chemically etching $10 \mu\text{m}$ deep cavities in the glass substrate (1.1 mm thick D263 from Schott-DESAG, Grünenplan, Germany).

Instead of the usual Si substrate, we used 0.4 mm thick glass D263 or 0.5 mm thick Pyrex wafers (Corning 7740, Corning, NY, USA), because it is both transparent and insulating for fabricating the second generation cell clinics. Figure 2 shows a schematic drawing of the process steps. After cleaning the substrate with RCA1 (5 parts H_2O , 1 part H_2O_2 , and 1 part NH_3), 40 \AA Cr and 200 \AA Au were thermally evaporated. The Cr layer is necessary as an adhesion promoter, because Au adheres poorly to the glass substrate. The Au layer was evaporated to prevent oxidation of the Cr and to improve the adhesion to the second Au layer. Standard photolithographic techniques were used to wet chemically etch windows in the Cr/Au layers. These windows are necessary for the releasing step in which a method called differential adhesion was used instead of a sacrificial layer [28]. Following this a $1000\text{-}1800 \text{ \AA}$ Au layer was deposited by thermal evaporation. The Au layer was used both as a structural layer in the PPy/Au bilayer and as a current collector for the redox reaction that drives the PPy microactuators.

Next, we etched the electrodes for the impedance measurements inside the microvials. We only etched the electrodes around the vials and not the complete electrical layout, because we intended to keep the Au layer intact for as long as possible in order to be able to grow the PPy for the actuators on the whole wafer simultaneously.

On the Au, SU8-5 was deposited and patterned. This plastic layer served as the rigid part of the lid. After the photopolymerisation we descummed the SU8 using Reactive Ion Etching (RIE) (250 W , 40 sccm CF_4/O_2 and 80 sccm O_2 at 50 mTorr [29]). The resulting SU8 layer was $4 \mu\text{m}$ thick.

After defining the electrodes and the lid, we made the microvials in a layer of SU8-10. The sample was then plasma etched in a RIE for about 4 minutes to descum the gold layer, resulting in $20 \mu\text{m}$ deep microvials.

For the active part of the hinges, PPy has been used. The polymer was electrochemically grown at the Au surface from a 0.1 M pyrrole, 0.1 M $\text{Na}^+ \text{DBS}^-$ (dodecyl benzene sulphonic acid, sodium salt) at a voltage of 0.55 V vs. Ag/AgCl . The sample was connected to the working electrode, and as the counter electrode a Au coated Si wafer was used. During the growth the large DBS^- anions were incorporated in the polymer film, by which the polypyrrole was doped. By covering the Au layer with resist and opening it only on the parts where the hinges would be defined, the PPy was automatically patterned. The resist is then removed using ethanol. The resulting thickness of the PPy layers was 0.8 to $1.7 \mu\text{m}$.

The last step was removing the excess gold to free the hinges and lids, and to pattern the leads and contact pads to the electrodes. This was done by covering those parts with photoresist and wet chemically etch the Au and Cr layers. The resist was removed by ethanol or acetone. Hereafter the devices could be operated.

The SU-8 layer that defined the microvials also insulated parts of the leads from the electrodes to the contact pads. The uncovered parts of those leads were insulated with epoxy (Loctite 401, Loctite Sweden AB, Gothenburg, Sweden). A nitrile O-ring, 3 mm inner diameter or a short piece of a plastic pipette tube was glued around the 20 microvials on the chip to form a miniature container. In this container the samples could be added.

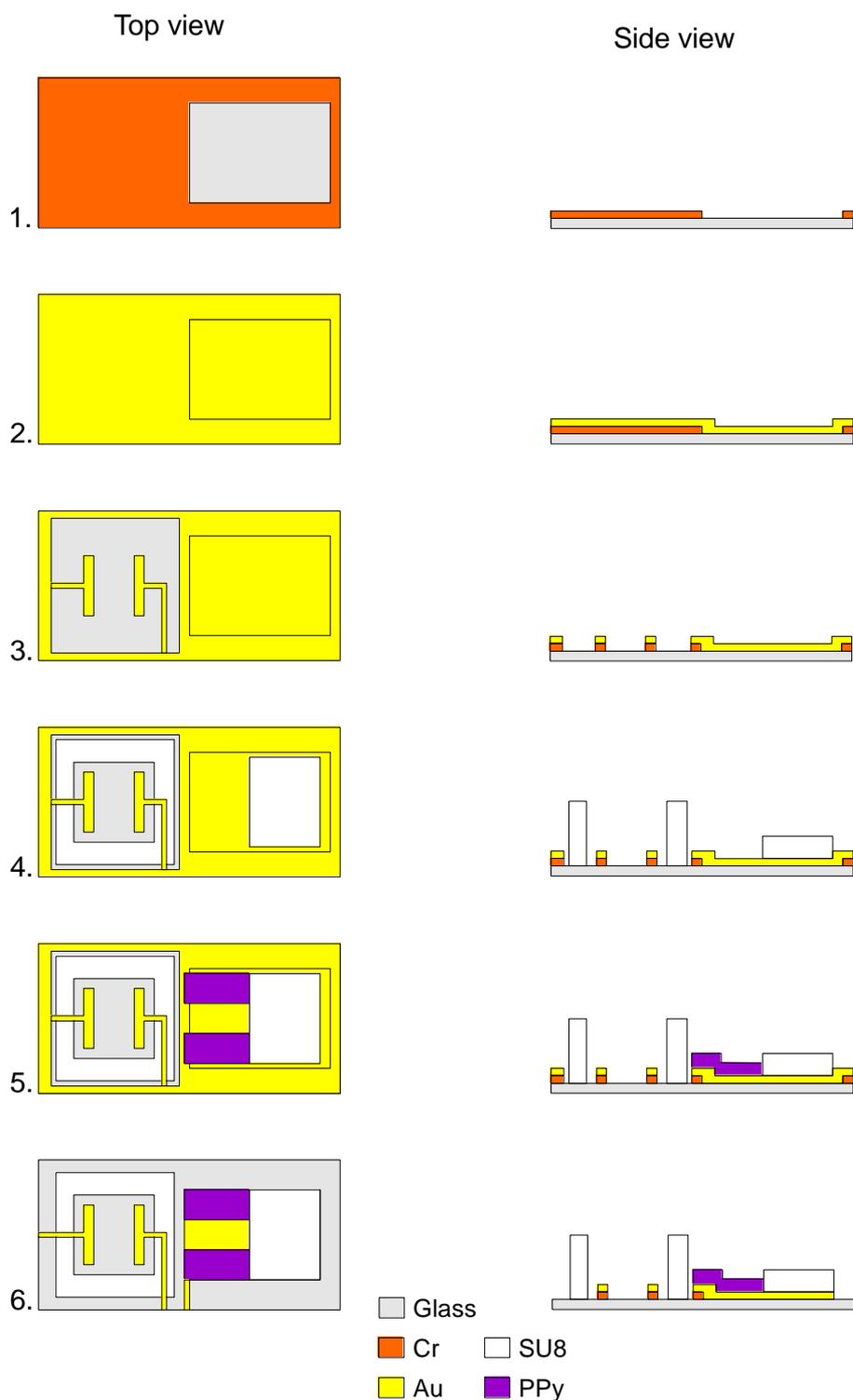


Figure 2 A schematic processing scheme for fabricating the microvials of the second generation. (1.) Deposition and patterning of Cr and Au adhesion layer. (2) Evaporation of the structural Au layer. (3) Etching of the Au electrodes and part of the wires. (4) Deposition and patterning of the SU-8 for the lid and microvials. (5) Electropolymerisation of the PPy. (6) Final Au and Cr etch to free the lids and pattern the rest of the wires with the contact pads.

Operation of the microvials

After the last fabrication step, the lids and hinges were etched free, but were still attached to the surface. The Au under the polypyrrole served as current collector and working electrode. For the mechanical testing, we operated the devices completely submersed in a 0.1 M $\text{Na}^+ \text{DBS}^-$ aqueous electrolyte, which we use as a standard solution for the

microactuators. Here, we used a Au wire as the counter electrode (CE), and a Ag/AgCl electrode (Bioanalytical Systems, USA) as the reference electrode (RE). When we operated the device for cell measurements, we filled the miniature container with 15-40 μL conditioned cell culture medium (CCFM) as an electrolyte. We used a Au coated probe needle (Karl Süss, München, Germany) as the CE and a Ag wire as the quasi RE. Both were inserted into the miniature container. The microactuators were controlled with a potentiostat (Autolab PGStat10, Ecochemie, Utrecht, The Netherlands). The voltage applied varied from 0.2 V to -1.0 V vs. Ag/AgCl. At the negative potential PPy is reduced, cations diffuse into the PPy to compensate the negative charge, the material swells, and the microactuators straighten, thus lifting the lids. At the positive potential the reverse occurs. The PPy is oxidized and the cations leave the polymer layer. The layer shrinks, thus the actuators bend, closing the microvials.

After a few cycles the structures were released from the substrate due to internal forces of the bilayers peeling of the gold from the bare silicon. On the small areas where there still was chromium between the gold and silicon the gold adhered and these sites served as the anchors for the hinges.

Impedance set-up

The impedance measurements were performed using a second potentiostat equipped with a Frequency Response Analysis module (Autolab PGStat20 + FRA2, Ecochemie, Utrecht, The Netherlands). Two tungsten probes were used to contact the electrodes. We measured only at a pair of electrodes in one microvial at the time. We performed both frequency scans, and time measurements. When measuring the impedance over time three frequencies were used 200 Hz, 1000 Hz, and 10 kHz. Cells can be considered as a sphere of conducting cytoplasm surrounded by a non-conducting membrane. They can be represented as a capacitance, the membrane, in series with a resistor, the cytoplasm. The membrane capacitance is $\sim 1\mu\text{F}/\text{cm}^2$, depending on the cell type [12, 15]. However a membrane capacitance $1\text{nF}/\text{cm}^2$ has been reported for red blood cells [30]. At lower frequencies the cells are electrically insulated and information about the bulk electrolyte can be obtained. At higher frequencies the cell membrane is short-circuited and intracellular effects are probed. Likewise the electrode interface is probed at higher frequencies [10]. By measuring at three frequencies simultaneously, we measure both medium and cellular impedance. The impedance was sampled every 90s by applying a sinusoidal potential ($V_{p-p}=40\text{mV}$). The data was collected and analysed in the FRA software supplied with the potentiostat. Throughout the measurements images were taken with a CCD camera (Sanyo, Japan) connected to a long working distance microscope (Nikon, Japan). Pictures were taken with a framegrabber that was connected from the CCD camera via a digital VCR (DSR-20P, Sony, Japan).

Results and discussion

Microvials

After a few initial bending cycles during which the microactuators have to “exercise”, full range of bending was achieved. The lids could move 180° and close the vials, but a tight seal has not yet been accomplished. The opening and closing was completely reversible and could be repeated for about a hundred cycles until mechanical failure occurred; the PPy and Au layers delaminated.

The second generation microvials were not only operated in a NaDBS solution, but also in CCFM cell culture medium. The actuators were able to move in the culture medium and behaved the same way as actuators operated in NaDBS solution. Figure 3 shows the cyclic voltammogram (CV) of the tenth scan of 40 PPy microactuators in CCFM, with a Au probe as the CE and a Ag wire as a quasi RE. The scan rate was $100\text{mV}/\text{s}$. The CVs looked well and were similar to other CVs of PPy(DBS) run in NaDBS. The oxidation and reduction peaks could be clearly distinguished, but as a quasi RE had been used no

statement could be made about the absolute positions of the peaks. The achieved currents were of the same order of magnitude as currents obtained when using aqueous NaDBS solution.

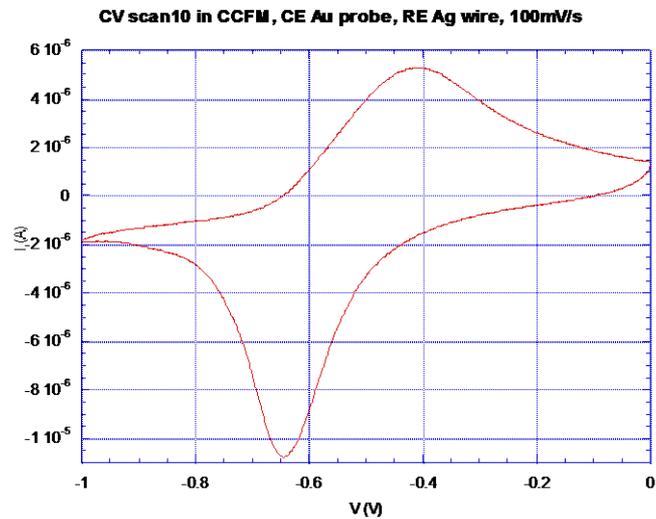


Figure 3 The cyclic voltammogram of the tenth scan of the PPy 40 microactuators that form the hinges of the microvials. The microactuators were activated in the cell culture medium CCFM with a Au probe as the CE and a Ag wire as the quasi RE at a scan rate of 100mV/s.

Figure 4a and b show two framegrabbed images of the microvials of the first generation. Each chip consisted of four arrays of 10 microvials on a Si substrate with different hinge lengths (50 μm , 100 μm , 150 μm , and 200 μm). As can be seen all the microvials could be opened and closed, except for the forth row with the 50 μm long hinges. These were too short and could not be closed. Instead, the lids remained standing 90° vertical to the surface. A close up of the opened and closed microvials (100 μm hinge length) is shown in Figure 4c and d.

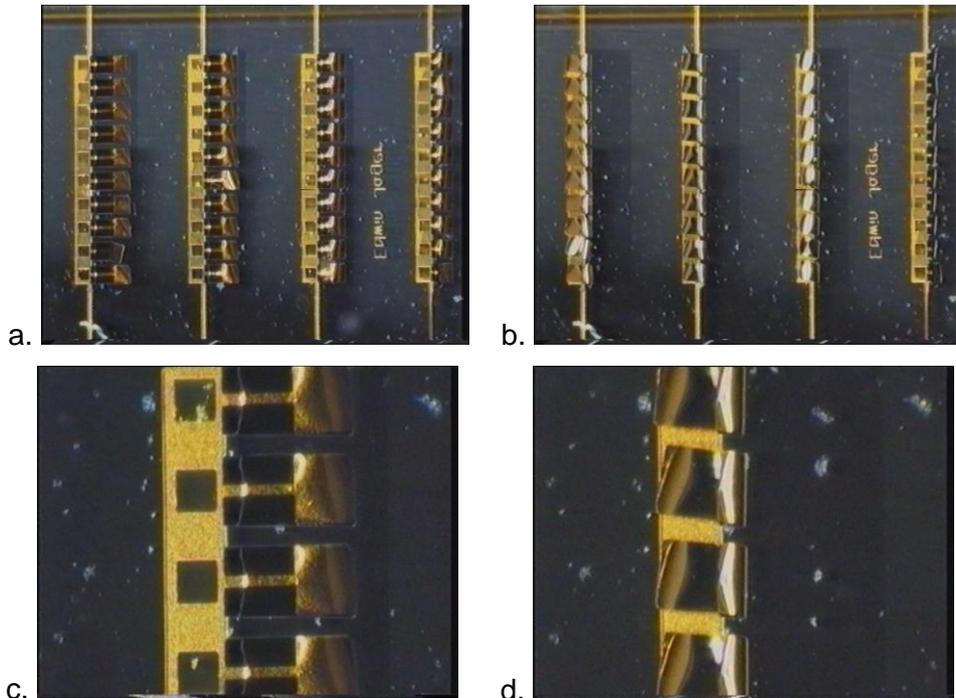


Figure 4 Four framegrabbed images the microvials of the first generation on a Si substrate. (a) Four arrays of 10 microvials with different hinge lengths (from left to right 200 μm , 150 μm , 100 μm , and 50 μm) that are opened. (b) The microvials are closed, except for the forth row with 50 μm long hinges. The hinges were too short and the lids stood 90° vertical to the surface. Four microvials with 100 μm hinges at a higher magnification both opened (c) and closed (d).

Not only Si but also glass (D263) was used as a substrate for the first generation microvials. These 10 μm deep vials were wet chemically etched. In order to demonstrate the possibility for single cell studies some of the vials were manually loaded with glass beads of 100 μm in diameter. Figure 5 shows two framegrabbed images of two microvials, where one of the microvials is loaded with a single glass bead. The lid could not be closed because the glass bead exceeded the vial dimensions.

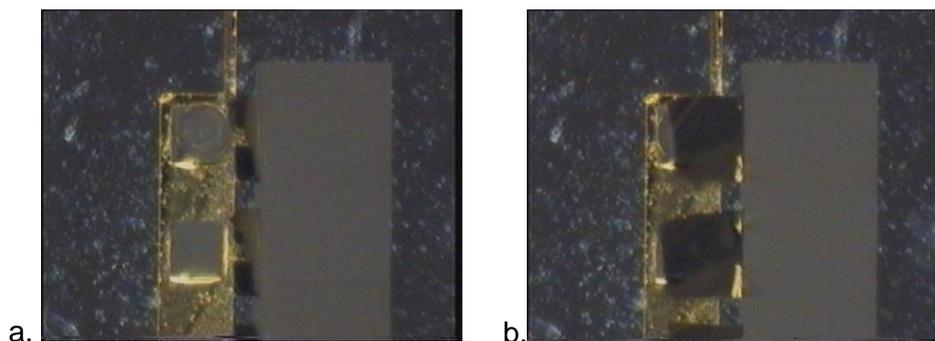


Figure 5 Two microvials made on a glass substrate (D263) with 10 μm deep etched vials (100 μm by 100 μm). The top vial was loaded with a 100 μm diameter glass bead. (a) Opened and (b) closed. As the glass bead was too large for the vial the lid could not close the vial.

Although the second design was only slightly altered with respect to the first, we experienced some problems with the release of the lids after fabrication. The cause of this is under investigation. The lids that did release moved similar to the lids in the first design. Figure 6 shows a picture of an array of microvials that could be opened and closed. As both the substrate and the SU8 are transparent the microvial contours are difficult to distinguish. Each microvial has one pair of Au electrodes that are clearly visible.

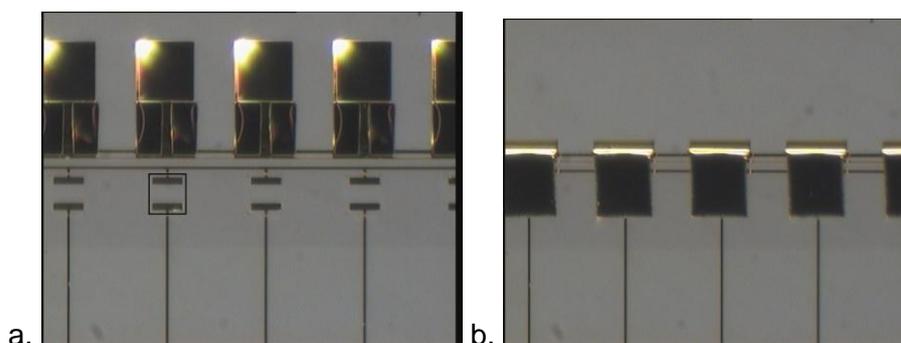


Figure 6 Two pictures of four cell clinics of the second design, (a) opened and (b) closed. The microvials were defined in a 20 μm thick layer of thick film photoresist (SU-8) on a glass substrate, making the device transparent and therefore accessible for microscopy. The contours of the microvials are difficult to distinguish therefore the contour of the second microvial is marked with a black line. The electrodes and contact wires are clearly visible.

Impedance measurements

To evaluate the system we measured the impedance of NaCl aqueous solutions using physiologically relevant salt concentrations (0.1, 0.2, and 0.3 M) over a frequency range of 100 Hz to 50kHz. Using a pipette the solutions were added to the miniature container and the impedance was measured using an electrode pair of one of the twenty microvials. The samples were measured at increasing salt concentrations and in between the

measurements the container was thoroughly washed using deionised water. Figure 7 shows the real part of the impedance. The imaginary part showed similar behaviour. As expected a decreasing impedance was seen with increasing salt concentration. This is due to the increase of bulk conductivity of the solution with increasing salt concentration.

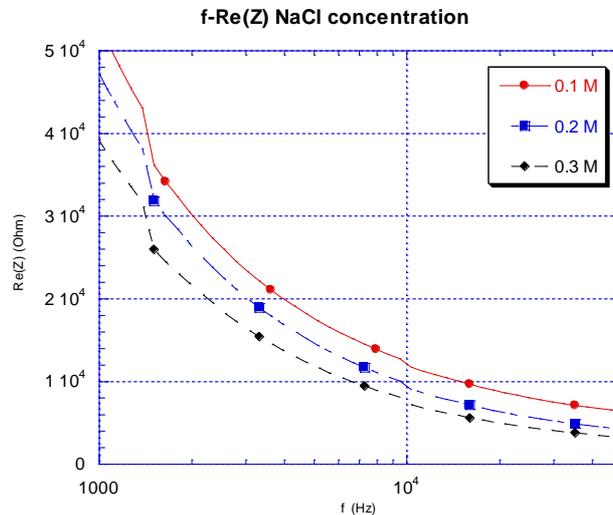


Figure 7 The real part of the impedance as measured on three aqueous NaCl solutions (0.1 M, 0.2 M, 0.3 M) at frequencies of 100 Hz to 50 kHz.

Cell type

For the cell studies we used frog melanophores (*Xenopus laevis*). Melanophores are a subgroup of chromatophores, colour changing pigment cells. Chromatophores are present in many amphibia, reptiles, and fish, and are used to change the colour of the skin. The colour change is caused by redistribution of pigment granules, melanosomes, within the cell [31, 32]. The melanophores are brown-black in the dispersed state and transparent when the melanosomes are aggregated. The movement of the pigment granules can be induced by submitting the cell to external stimuli, like different hormones, causing either a dispersion or aggregation of the granules. The granules move along the microtubules within the cell. The aggregation takes about 45 minutes. Aggregation of the granules can also be induced by chemotoxins. We have used the marine toxin latrunculin B that inhibits actin polymerisation and disrupts microfilament mediated processes. We have chosen this cell line because the cells can be cultured at room temperature, are relatively large, and the colour change is well visible under the microscope.

Biocompatibility.

Data on the biocompatibility of SU-8 when culturing cells on microsystems containing SU-8 is sparse. Therefore, we tested our microvials using *in-vitro* cell studies [33]. We did not see any negative responses of these cells from the SU-8 on the time scale of the experiments, which was 24h. The melanophores spread out well. They attached to the SU-8 as well as to the rest of the surface. Also these experiments showed that the dimensions of the vials and electrodes were well chosen for this cell type. We noticed that the cells seemed to prefer the 3D structure of the SU-8. This behaviour has been observed on other 3D microstructured surfaces, such as microchannels for directed neural growth [34]. For more details on the biocompatibility we refer to [33].

Impedance Studies on Melanophores

Before the measurement, the chip was exposed to an O₂ plasma for 30 s to turn the surface hydrophilic. Without this O₂ plasma etch the culture medium would not wet the

microvials. In fact, the miniature container was coated with poly-L-Lysine before the plasma treatments in order to increase the cell adhesion. However, we believe that the poly-L-lysine disappeared during the plasma treatment.

The impedance measurements were performed with opened microvials. The measurements were initiated by establishing a base line: 40 μl CCFM was added to the miniature container and the impedance was measured for 1 h. Having a stable base line, 40 μl CCFM containing 2×10^4 cells was added. The cells were allowed 2.5 h to sediment, adhere, and spread on the surface. The cells spread and adhered on both the SU8 layer and in the microvials. At $t = 3.5$ h 10 μl CCFM was added to the miniature container. This extra CCFM addition was made to see whether the impedance change with latrunculin was caused by the injection of liquid or due to cellular response. At $t = 4.5$ h 10 μl latrunculin was added to a final concentration of $5 \mu\text{M}$. Experiments showed that evaporation of the sample aliquots was a problem. Therefore, to minimise the evaporation during the measurement the miniature container was closed with a small plastic cap after each liquid addition. Inside the cap a moist piece of filter paper was wrapped to keep the environment humid.

Figure 9 shows the impedance plot for the real (R) and imaginary (X) part of the impedance at 205 Hz (A.) and 10 kHz (B.). In this experiment 205 Hz was used instead of 200 Hz. The signal at 1 kHz was similar to 10 kHz, although at a lower impedance. The lower frequency should probe the surrounding extracellular liquid, while the higher frequency should study interface effects at the electrode and intracellular processes. Figure 8 shows framegrabber images taken at $t = 1$ h, when the melanophores were added, at $t = 3.5$, before the second CCFM was added, and at $t = 6$ h when the experiment was finished. There was no visible effect on the cells after addition of CCFM.

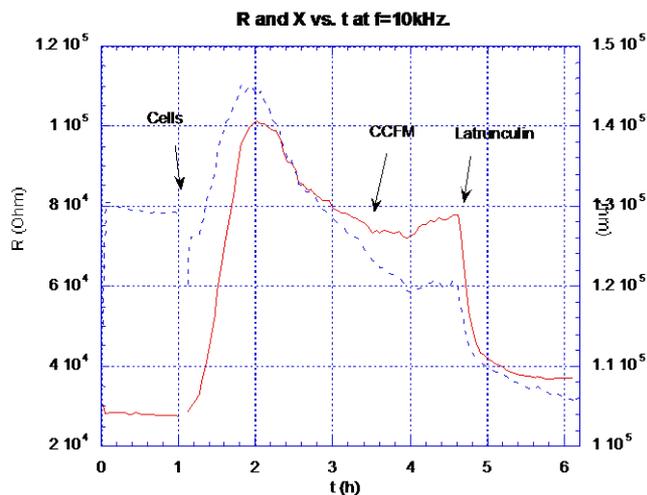
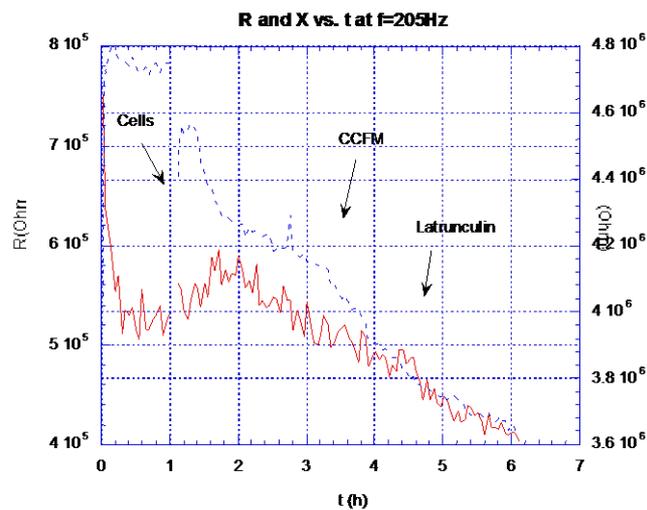


Figure 9 The impedance plot of both the real (R , unbroken line) and the imaginary (X , dotted line) part in the cell experiment. At $t=1h$ the melanophores were added, at $t=3.5h$ extra CCFM was added, and $t=4.5 h$ Latrunculin was added. (A.) $f=205 \text{ Hz}$ (B.) $f=10 \text{ kHz}$.

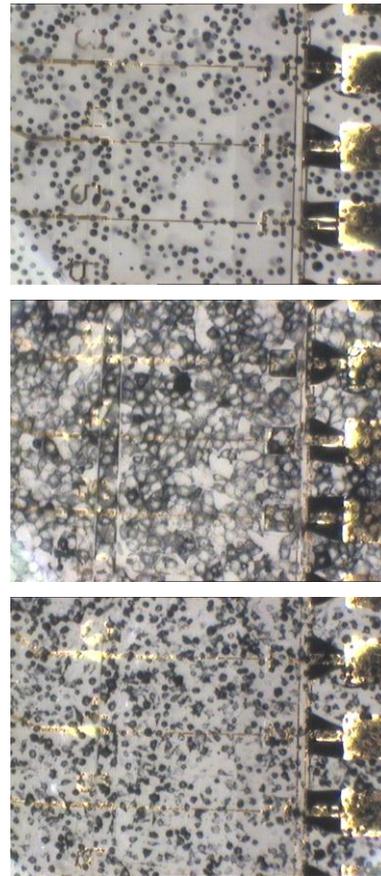


Figure 8 Photos of three opened cell clinics at different stages of the experiments. The impedance data is from the centre microvial. (A.) At $t=1h$ with freshly seeded frog melanophores (*Xenopus laevis* melanophores). The cells are still round and sediment down on to the substrate and into the vials. (B.) The same cell clinics at $t=3.5 h$, the melanophores have nicely spread out both on the substrate and in the cell clinics covering most of the electrode area. (C.) At $t=6 h$, after adding latrunculin the pigment granules have aggregated.

On the addition of the cells the impedance at 10kHz increased sharply, then decreased slowly, and more or less stabilised. We contribute this to the adhesion and spreading of the melanophores. The spreading and adhesion can be seen at the photographs too. The increase of impedance upon cell adhesion and spreading has been seen by others [10, 12]. The addition of a second small volume of CCFM had only a small effect. The impedance seemed to stabilise some more and even slightly increase. When latrunculin was added, we can see a sharp decrease in the impedance. The pigment granules aggregated to the middle of the cells due to the latrunculin. The cells became transparent, as can be seen in Figure 8C.

At 205 Hz there was only a small increase of the impedance on cell addition, followed by a more or less continuous decrease of the impedance, independent of the addition of both the second CCFM and latrunculin. The initial increase of the impedance is probably due to the effect that the cells that can be regarded as electrically insulating at that frequency,

replacing the higher conducting liquid. The decrease could be caused by evaporation of the liquid (even though we tried to minimize this effect by placing a cap on the container) leading to a continuous increase of salt concentrations and thus decreasing impedance.

We attribute the decrease of the impedance at 10 kHz upon latrunculin stimulation to the intracellular aggregation of the pigment granules as can be confirmed by the photographs. The response time of the impedance drop is about 45 min, which is similar to the characteristic time for the pigment aggregation in experiments [35]. No effect of the latrunculin has been found in the low frequency signal that should probe the bulk liquid.

To further investigate the electrode processes we repeated the experiment, but with a dummy solution that contained no cells. No effects could be seen on adding the dummy solution or adding the second CCFM. When adding the latrunculin a sharp increase of the impedance was noticed, probably due to adsorption of the latrunculin to the Au electrodes fouling them (data not shown).

Although we could clearly detect the effect of pigment granules aggregation due to latrunculin stimulation in the electrical impedance data and correlate this with visual data, the question remains whether we measure the intracellular rearrangement of the granules or an extracellular effect due to the intracellular changes. After the experiments, the cells were fixed and the cell contours seemed not to have changed visibly, however this aspect is under investigation. There is great variation within the reported frequencies at which the cell membrane is short-circuited and intracellular effects can be measured. This frequency is probably dependent on the cell type and measurement/electrode set up. Sohn *et al.* reported measuring the DNA content of cells at a frequency of 1kHz [14], but others claim that frequencies >10kHz [13] and even >1MHz [15] are needed to interrogate intracellular processes. Cellular movement has been measured at frequencies of 1 kHz [12] and 4 kHz [10]. The impedance change measured by Giaever and Keese was partially attributed to a change in distance between the cells and the electrode due to micromotion of the cells. They also theoretically showed that a change of the cell shape influences the impedance. The melanophores used in our experiments could have altered their shape or separation on the electrode upon latrunculin stimulation, outside the visible detection. It has been observed that sometimes during the aggregation process of the melanophores, granule-free peripheral regions may become less adherent to the substrate [36].

Conclusions

We have demonstrated the fabrication of cell clinics using microfabrication. These cell clinics consisted of a microvial with volume of 200 pl with Au electrodes inside. The microvials could be opened and closed with a lid that is electrochemically activated by PPy/Au hinges. We have also shown that it is possible to use impedance measurements to characterise intracellular processes using the microvial electrodes. We could correlate the optically visible movement of pigment granules with a measured change in impedance. The cell clinic might be a potential tool to interrogate intracellular effects.

The base line measurements were quite stable and reproducible. The biological measurements varied more. We attribute this to the cell conditions. This could be seen in the cell spreading. When the cells spread out a good response signal could be measured [33].

The next step will be to perform such experiments in a closed microenvironment to exploit the advantages of such a microenvironment, which could be faster response times, more sensitive measurements, and selective choice to measure on individual or a small number of cells.

We have demonstrated some general possibilities of such devices for cell biological studies. Although easy to employ, electrical impedance data is a quite difficult to interpret biologically and an accurate model should be developed for the electrode interactions in

order to validate the results. However, modelling impedances is difficult and no agreement on the models exists. More specific sensor schemes, however, are well possible.

Here, we presented a microvial design containing two electrodes on the bottom of the microvials. Alternative sensor positions are possible. In addition, sensors could be put on the lid. When closing the lid the sensors on the bottom and lid would be automatically aligned. In the present design this option has already been taken into account. Between the two PPy hinges an area has been left open for wiring to future sensors on the lid. Another option is integrating the sensors in the sidewalls of the microvial.

In these experiments we have added the cells in a suspension and let them sediment to the cell-clinics. The reagents were pipetted on the chip. In order to get a more controlled insertion of cells in the microvials and easy liquid addition, one could use microfluidics to add and direct the cells and reagents to the vials [37]. The microfluidic circuit could also be used for drug testing and high throughput screening using the cells in the microvials as test objects. The loading of the cells into the microvials could also be done by microrobots that we have presented recently [26].

Flat shallow microvials could be used as a *push-clamp* device as an alternative to patch-clamp. Using cellular adhesive molecules on the lid a cell is adhered to the lid and pressed to the electrodes for impedance measurements. A completely different use for such microvials might be in "smart pills", implantable chips that can release drugs on command [38].

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