

Thomas Frisk¹
Susanna Rydholm²
Helene Andersson¹
Göran Stemme¹
Hjalmar Brismar²

¹Microsystems Laboratory,
School of Electrical Engineering,
Royal Institute of Technology,
Stockholm, Sweden

²Cell Physics Laboratory,
School of Engineering Sciences,
Royal Institute of Technology,
Stockholm, Sweden

A concept for miniaturized 3-D cell culture using an extracellular matrix gel

This paper presents a novel method to embed, anchor, and cultivate cells in a controlled 3-D flow-through microenvironment. This is realized using an etched silicon pillar flow chamber filled with extracellular matrix (ECM) gel mixed with cells. At 4°C, while in liquid form, ECM gel is mixed with cells and injected into the chamber. Raising the temperature to 37°C results in a gel, with cells embedded. The silicon pillars both stabilize and increase the surface to volume ratio of the gel. During polymerization the gel shrinks, thus creating channels, which enables perfusion through the chip. The pillars increase the mechanical stability of the gel permitting high surface flow rates without surface modifications. Within the structure cells were still viable and proliferating after 6 days of cultivation. Our method thus makes it possible to perform medium- to long-term cultivation of cells in a controlled 3-D environment. This concept opens possibilities to perform studies of cells in a more physiological environment compared to traditional 2-D cultures on flat substrates.

Keywords: Embedded cells / Extracellular matrix / Microfluidics / Three-dimensional cell culture
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1 Introduction

Cells in tissues are organized in well-defined 3-D structures. However, most cell physiological studies are still performed on 2-D cell cultures or on single dissociated cells, environments that are far from representative for the cell in the *in vivo* situation. Microsystem technology offers the possibility to position cells in small structures and to achieve controlled microflows to maintain an environment closer to the *in vivo* situation [1, 2]. This paper presents a novel method to embed and anchor, culture and study cells in 3-D using an extracellular matrix (ECM) gel in a micromachined silicon fluidic structure.

Cells in tissues do not only connect to each other, but also to an extracellular support structure, the ECM. The ECM gives tissues much of their mechanical properties and helps to maintain the 3-D architecture. Further, the ECM is important for control of cell proliferation, motility, and migration [3]. Many scaffold proteins within the ECM and their constituent motifs have been identified during the past two decades [4, 5]. Those molecular cues are responsible for tissue organization during development and help to maintain the cellular organization within the

adult tissue [4, 6, 7]. It is thus important also for cells in culture to connect both to an ECM as well as to other cells in order to maintain functionality as similar as possible to their *in vivo* function. *In vivo*, cells produce the ECM themselves, in sufficient amounts to connect to other cells and cellular structures.

Cell adhesion to a 3-D matrix differs from 2-D adhesion in terms of structure, localization, and function. These different adhesion properties result in altered content of cellular components such as integrins, paxillin, and other cytoskeletal constituents [8–11]. Further, properties such as cell shape, mitosis, and cell ECM production are altered as a response to culture microenvironment [12]. These differences lead to different functionality of cells cultured in 3-D and thus different responses to treatments. For example, responses of cancer cells to treatment coupled to integrins are altered during 3-D cell growth [13]. Also, cancer cell migration, which is an important property in metastasis formation, is different in 3-D cultures compared to traditional 2-D cultures [14, 15].

As illustrated above, it is of relevance to perform studies on cells cultured in 3-D matrices and adapted to a 3-D environment. So far 3-D cultivation has been expensive and cumbersome using traditional cell culture methods, in, e.g., bioreactors. Previously described work in this field has been performed on standard glass substrates, in petridishes [16] and in molded poly(dimethylsiloxane) (PDMS) channels.

Correspondence: Dr. Thomas Frisk, Kungliga Tekniska Högskolan, SE-100 44 Stockholm, Sweden
E-mail: Thomas.Frisk@s3.kth.se
Fax: +46-810-0858

Abbreviations: ECM, extracellular matrix; PDMS, poly(dimethylsiloxane)

The purpose of this study was to facilitate and improve the possibility to perform single cell physiological studies of cells adapted to a 3-D, and thus more *in vivo* like, environment. The concept we propose bridges the gap between traditional organ-tissue 3-D cultures and conventional substrate based 2-D cell cultures.

To achieve this purpose, we have focused our study on a technique for filling a specially designed microstructure with an ECM-cell mixture. We have studied the ECM polymerization, buffer perfusion in the system, the mechanical stability of the gel, and the influence of pillars in the cavity for mechanical stability of the gel and finally viability of the cells.

By embedding cells in ECM we create an attractive cellular environment mimicking the *in vivo* situation, at least to some extent. The phase-change of ECM, polymerization, from liquid to gel [16] enables easy handling when setting up a cell experiment. After mixing, injecting, and incubating, the cells are prepared for different liquid-based treatments, and microscopic studies of cellular responses.

The miniaturization of the system is an added benefit as it results in small experiment volumes, requiring only small amounts of chemicals and low flow rates to maintain the environment.

2 Materials and methods

2.1 Micromachined silicon chamber

The microchamber consists of a microstructured silicon channel with pillars, inlet, and outlet (Fig. 1). It is encapsulated by a glass lid enabling microscopy of the con-

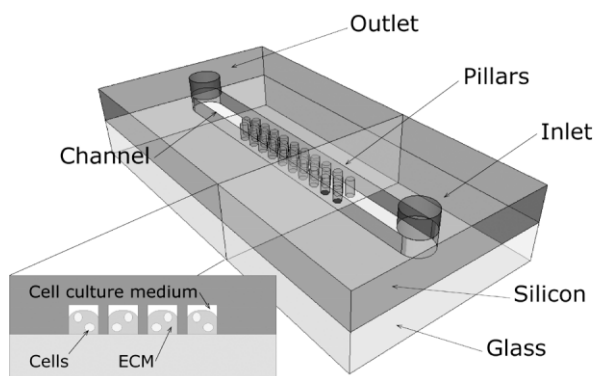


Figure 1. Conceptual image of chip for true 3-D cell culture in extracellular matrix. Typical dimensions: channel width 400 μm , channel depth 90 μm , pillar diameter 40 μm , spacing/pitch 20–70 μm . Cross-cut showing center of chip. Surface to volume ratio increase is approximately 3 times.

tents. The silicon structure (Si, p-type, 500 mm diameter wafer) was made with deep reactive ion etching (Surface Technology Systems, UK) after standard photoresist mask patterning, and the glass lid/encapsulation (Pyrex, 500 mm diameter wafer) of the channel was achieved through anodic bonding (Karl Süss, 400°C, 600 V). Three chamber types were used, with the following dimensions: chamber width = 400 μm , height = 90 μm , pillar diameter = 40 μm , and the distances between the pillars were 20, 35, and 70 μm , respectively (Fig. 2).

2.2 ECM

The ECM used here is a reconstituted basement membrane matrix, MATRIGEL (BD Biosciences, Bedford, MA, USA). The main part of its constituents are proteins such as laminin, collagen IV, heparan sulfate proteoglycans, entactin, and nidogen. It also contains growth factors, such as transforming growth factor- β (TGF- β), fibroblast growth factor, and tissue plasminogen activator [16]. The ECM is stored frozen at temperatures below -18°C . Thawing the ECM on ice results in a liquid that should be kept at 0–4°C to maintain its liquid properties

2.3 Cultivation of COS 7 cells

COS 7 cells, a cell line derived from fetal monkey kidney, was purchased from ECACC. The cells were cultured at 37°C in DMEM (Sigma) containing 10% fetal bovine serum (Gibco), 1.0% penicillin streptomycin (Sigma), and 1.0% L-glutamine (Sigma), and kept in an atmosphere with 5% CO_2 . After the cells had formed a confluent layer they were harvested by exposure to 0.25% trypsin (trypsin-EDTA, Gibco) for 5 min at 37°C.

2.4 Insertion of cells and ECM into the microchamber

MATRIGEL ECM was thawed on ice overnight in a refrigerator at 4°C. The cell-medium mixture containing 1, 2, or 4 million cells was centrifuged (5 min at 1700 rpm) creating a cell pellet. The cells were then placed in a refrigerator at 4°C for 10 min. After this, the medium was replaced with 200 μL ECM using a cooled pipette tip. The received concentrations were thus 5000, 10 000, and 20 000 cells/ μL , respectively. Cooled pipette tips were also used for the insertion of the cell-ECM mixture into cooled microchambers.

When the cells had been filled into the chamber, it was left in room temperature for 10 min to allow for a slow polymerization of the ECM. The chip was then incubated at

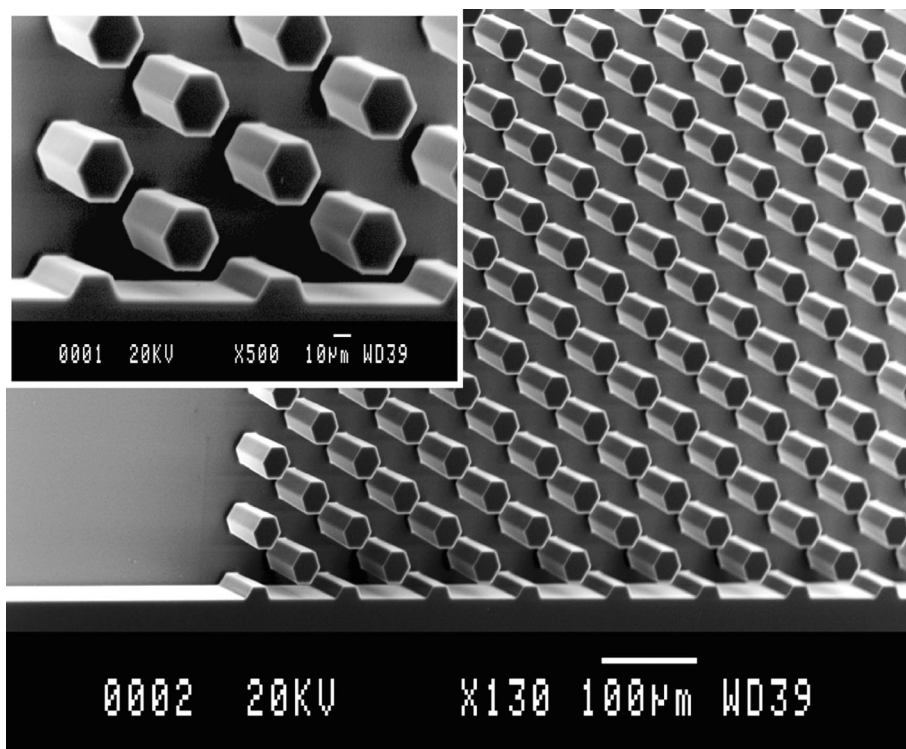


Figure 2. SEM picture of micro-machined silicon fluidic structure with insert on pillars. Pillar size $\phi \approx 40 \mu\text{m}$, channel inlet/outlet width $\approx 400 \mu\text{m}$ and inter-pillar pitch $\approx 70 \mu\text{m}$. Cavity depth, $90 \mu\text{m}$. Chamber volumes range from $1 \mu\text{L}$ to $1.5 \mu\text{L}$ (depending on inter-pillar pitch).

37°C for 30 min for final polymerization and after this cell culture medium was perfused. During cultivation the chip was placed in a petridish containing cell culture medium to allow for diffusion through the inlet and outlet ports into the chamber. Fresh cell culture medium with serum was also perfused through the chamber once or twice *per* day.

2.5 Staining of cells with calcein-AM

Both staining with the cell tracer dye calcein-AM (ex $495 \text{ nm/em } 515 \text{ nm}$, Molecular Probes) before and after insertion into the chamber was used. Prestaining of the cells ($1 \mu\text{M}$ calcein-AM for 30 min) was performed before centrifugation and mixing with the ECM. Staining of cells embedded in the chip was performed by insertion of $1 \mu\text{M}$ calcein-AM into the chamber and incubated at 37°C for 1 h to allow diffusion and homogenous distribution throughout the gel.

2.6 3-D imaging of ECM and flow in chip

To view the behavior of the ECM within the chamber, injected ECM was mixed with the fluorescent dye rhodamine (ex $560 \text{ nm/em } 580 \text{ nm}$, Molecular Probes). A confocal microscope (Zeiss, LSM 510 META) enabled high resolution 3-D imaging (Fig. 3a).

To view the perfusion over the ECM, water mixed with the fluorescent dye fluorescein (ex $480 \text{ nm/em } 520 \text{ nm}$, Molecular Probes) was used to fill the chip. Simultaneous imaging of the ECM and the liquid within the chamber was then possible, as the different dyes are easily spectrally separated in the microscope (Fig. 3b).

To evaluate exchange rates of the perfusion, a hydrostatic flow setup was used with water pressure at 5 kPa and flow of $20 \mu\text{L/min}$ (water and ECM stained as above). Consecutive switching between stained and unstained water during 600 s periods enabled intensity recording with the microscope. The scan rate was 1 scan in every 4 s with 1 s exposure *per* scan. A $500 \times 500 \mu\text{m}$ area was imaged by 512×512 pixels, resulting in an exposure time of $3.8 \mu\text{s}$ *per* pixel. With a measured laser power of $35 \mu\text{W}$, the total amount of energy received is 133 pJ *per* pixel and scan. During the exchange experiment 300 scans were made. The total experiment time was 1200 s.

The mechanical stability of ECM within the chip was also examined. MATRIGEL mixed with 1% fluorescent microspheres (Fluoresbrite PolyFluor 570, $1 \mu\text{m}$) was injected into the chip. Water was perfused at different flow rates and the gel integrity could be monitored by following the displacement of the gel embedded microspheres.

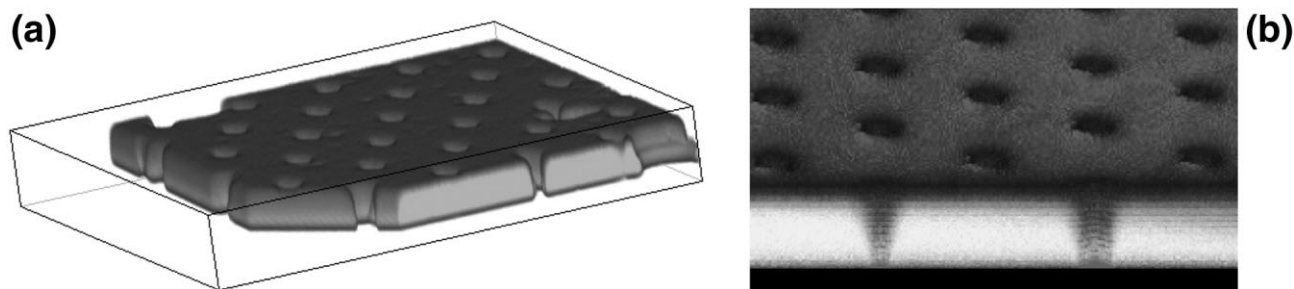


Figure 3. (a) ECM stained with fluorescein, 3D-imaged with confocal microscopy. Note the conical shaped shrinkage of the ECM around the pillars. (b) 3-D fluorescent confocal image of rhodamine-stained ECM (light grey) and fluorescein-stained flow (dark grey) inside the chamber.

The contraction of ECM was measured under a microscope after injection of MATRIGEL during 30 min incubation at 37°C in four identical chips with well-defined silicon-glass channels.

3 Results and discussion

This is a first study of 3-D cell culturing in microfluidic silicon structures designed to enable relevant biological studies in specific and controllable microenvironments.

3.1 ECM integrity and secondary flow in chamber

Filling of the chamber with ECM was performed with pipettes. By pipetting a well-controlled volume (*i.e.*, the cavity volume) of ECM it was possible to fill the cham-

ber without blocking the inlet or outlet channels and to minimize waste. With this procedure there were no problems with air bubbles remaining in the chamber. During polymerization the volume of the ECM decreases approximately 30%, creating flow paths for further flow through the chamber (Fig. 4). These flow paths are essential for the supply of cell culture medium during cultivation.

Figure 3a shows a 3-D confocal image of rhodamine-stained ECM within the chip after initial incubation and polymerization. In Fig. 3b both ECM (light grey) and perfusate (dark grey) were recorded. Here it can be seen that the ECM shrinkage has a conical shape close to the pillars, resulting in an increased surface to volume ratio and a more porous structure of the ECM gel. A porous structure is beneficial as it increases the exchange between cells and perfusate, and thereby improves the control of the milieu inside the chip.

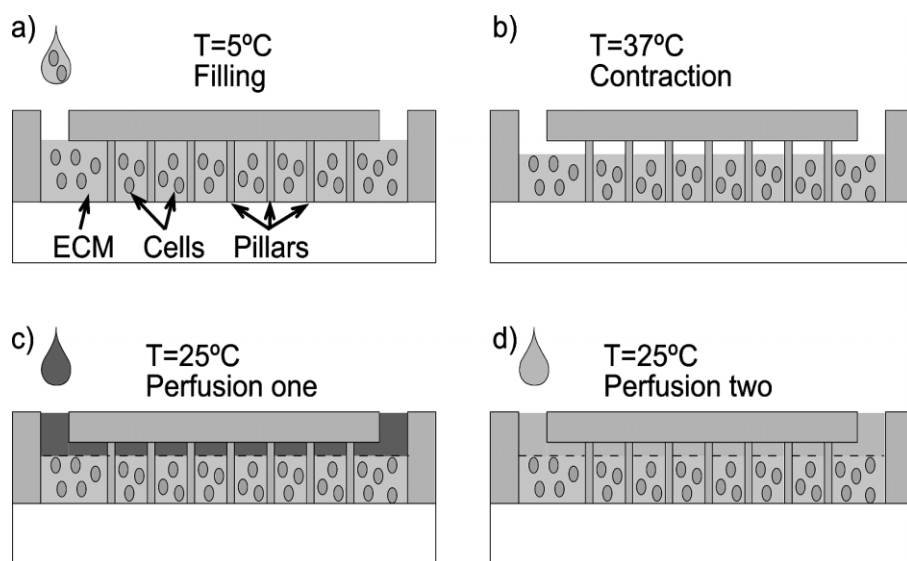


Figure 4. (a) Axial cross section of microchamber filled with ECM and cells at 5°C. (b) ECM shrinkage during polymerization at 37°C creates channels that enables cell treatment through perfusion. (c) and (d) Schematic illustration of consecutive perfusions.

Gel integrity at different flow rates was studied by mixing the gel with fluorescent beads. Gel displacement was assessed by following the movement of embedded beads. These tests showed that the gel remained undisturbed at a perfusion rate of 20 $\mu\text{L}/\text{min}$ and was minimally displaced at 25 $\mu\text{L}/\text{min}$. This perfusion corresponds to surface flow rates of 10 and 12 mm/s, respectively, which is twice as fast as in the previously reported system [17, 18], where the ECM was minimally displaced at 5 mm/s. These higher flow rates imply that the pillars help to anchor and reinforce the gel.

By switching between fluorescent and nonfluorescent perfusate, the characteristics of the perfusion was visualized. Figures 5a–c show the flow pattern over the ECM at three different time points during a transition from fluorescent to nonfluorescent perfusate. The time constant τ (10–90% rise time) for the transition from fluorescent to nonfluorescent flow was independent of position within the chamber and measured to 120 s at a flow rate of 20 $\mu\text{L}/\text{min}$ (Fig. 6). This is an unexpected long time considering the high flow rate, however fluorescein is accumulated in the ECM and the recording shows not only the flow characteristics in the perfusion but also the wash-out of ECM accumulated fluorescein.

After the wash-out the fluorescein perfusate was restored and the fluorescence intensity returned to the same level as before wash-out. This indicates that a complete exchange of fluids can take place in the chip. Thus cells embedded in the gel will be reached by fresh culture medium and products from the cellular metabolism can leave the system, also different stimulation schemes can be envisioned by adding substances to the perfusate. The differences in intensity among the different regions of interest in Fig. 6 are likely due to a slight variation of the thickness in the ECM layer, resulting in variations of the fluorescein liquid layer thickness.

This work focuses on establishing and maintaining a stable environment for medium- to long-term cell cultivation and analysis, rather than instant cellular responses to rapidly changing environments. Hence, designs to optimize the flow rate were not tested. For rapid responses to short-term chemical treatments of cells, traditional 2-D substrate based culture systems (e.g., petridishes, cover slips) are superior to our system, where diffusion through the ECM will slow down the reactions.

3.2 Cell insertion and viability

Cell-ECM mixture was inserted into the chamber with pipettes containing a controlled volume of the mixture. Incubation at 37°C results in polymerization of the ECM and embedment of the cells. By storing the chip immersed in cell-culture medium during cultivation, diffusion through the inlet and outlet ports can occur. This not only prevents the gel from drying, but will also enable the exchange of nutrients in the chip. In addition, fresh cell culture medium was perfused through the chamber once or twice *per* day to increase the exchange of nutrients and maintain the cell viability. This setup permits longer unattended culturing than with a procedure of constant perfusion through the inlet/outlet ports of a chip in a dry environment.

Addition of cells to ECM did not result in any observable change of viscosity. The distance between the pillars in the cavity did not influence the complete filling of the structure with cell-free ECM. However, with the cell-ECM mixture, clogging of cells at the inlet was observed when the distance between the pillars was 20 μm , *i.e.*, too narrow (Fig. 7a). Increasing the distance between the pillars (>35 μm), the cells nicely filled up the volume within the structure (Fig. 7b). To monitor the distribution, cells were

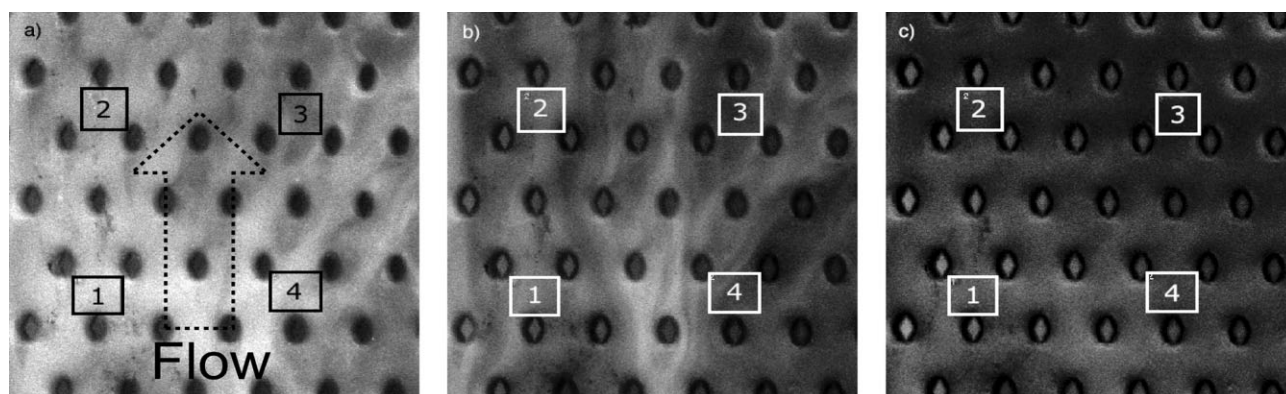


Figure 5. Fluorescent to non-fluorescent flow at (a) $t = 75$ s, (b) 120 s and (c) 360 s in an ECM-filled chamber. The loss of signal (*i.e.* darker image) indicates the fast substitution of liquid. Imaged in a time series using fluorescence microscopy.

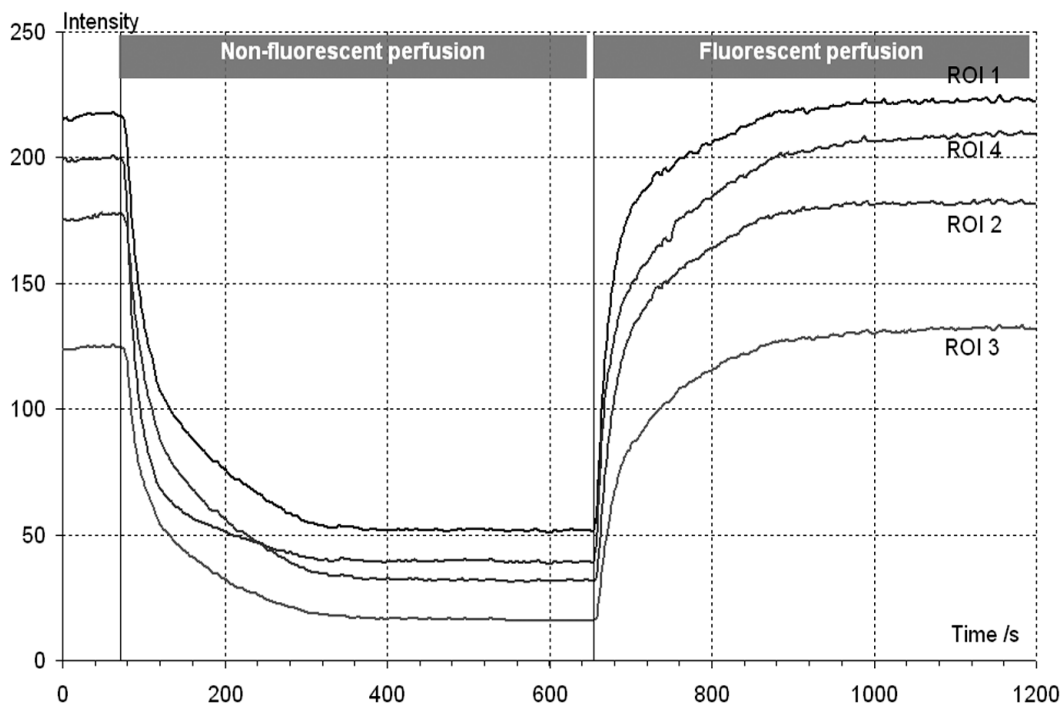


Figure 6. Exchange rate between fluorescent and non-fluorescent flow in ECM-filled channel. Time constant $\tau = 120$ s (rise time 10 to 90% or fall time 90 to 10% of final value) for the switch-over. The scan rate was 1 scan in every 4 s with 1 s exposure per scan. A $500 \times 500 \mu\text{m}$ area was imaged by 512×512 pixels, resulting in an exposure time of $3.8 \mu\text{s}$ per pixel. With a measured laser power of $35 \mu\text{W}$ the total amount of energy received is 133 pJ per pixel and scan. During the exchange experiment 300 scans were made. The total experiment time was 1200 s.

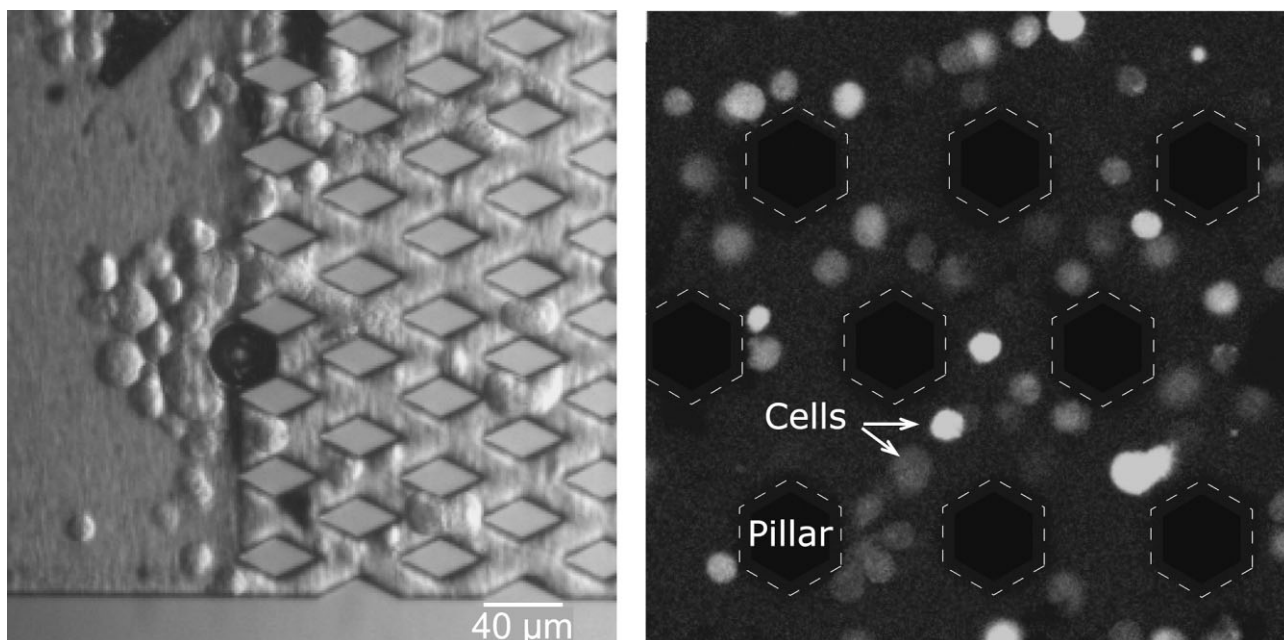


Figure 7. (a) Too narrow paths (approximately $20 \mu\text{m}$) between pillars results in cell clogging. (b) Prestained cells (20 000 cells per μL ECM) in chip. Cells shown in grey, black areas are pillars, dark grey areas ECM.

stained with calcein-AM before insertion into the chip. The distribution was found to be even throughout the entire chamber.

Three cell concentrations of 5000, 10 000, and 20 000 cells/ μL were tested in order to evaluate suitable densities with respect to growth space, clogging, distribution, and visibility. The two higher concentrations resulted in relatively densely packed cells with little space in between. To enable single cell identification and ease measurements of proliferation the lower concentration was used in the majority of the subsequent experiments.

Cell viability within the chamber was examined by staining the cells with the fluorescent dye calcein-AM, a commonly used dye for cell tracking. The dye is cell permeable and nonfluorescent extracellularly. When the AM-group is cleaved by esterases in the cell the dye becomes fluorescent and also cell impermeable, *i.e.*, it is trapped in the cell. Thus only viable cells will be positively stained (Fig. 8).

Cells were injected into the chamber at a concentration of 5000 cells/ μL . After 3 days the cell density was ranging from 7700 to 10 400 cells/ μL , with a mean value of 8900 cells/ μL (three separate recordings). This corresponds to a mean cell density increase of 78%, showing that the cells do proliferate and function within our system and that medium- to long-term cultivation is possible.

The proposed use of this system is for single cell analysis of cells adapted to a 3-D heterogen *in vivo* like environment. We have thus focused on a culture time of 3 days rather than to optimize the time of culture, as this time should be enough for the cells to adapt to the environment. However, for some samples, the culture was continued for up to 6 days, and at that point the cells were still viable and proliferating.

4 Concluding remarks

We show that by using ECM embedding of cells in a specially designed silicon-glass microfluidic chamber, it is possible to achieve a controlled 3-D environment for cell assays.

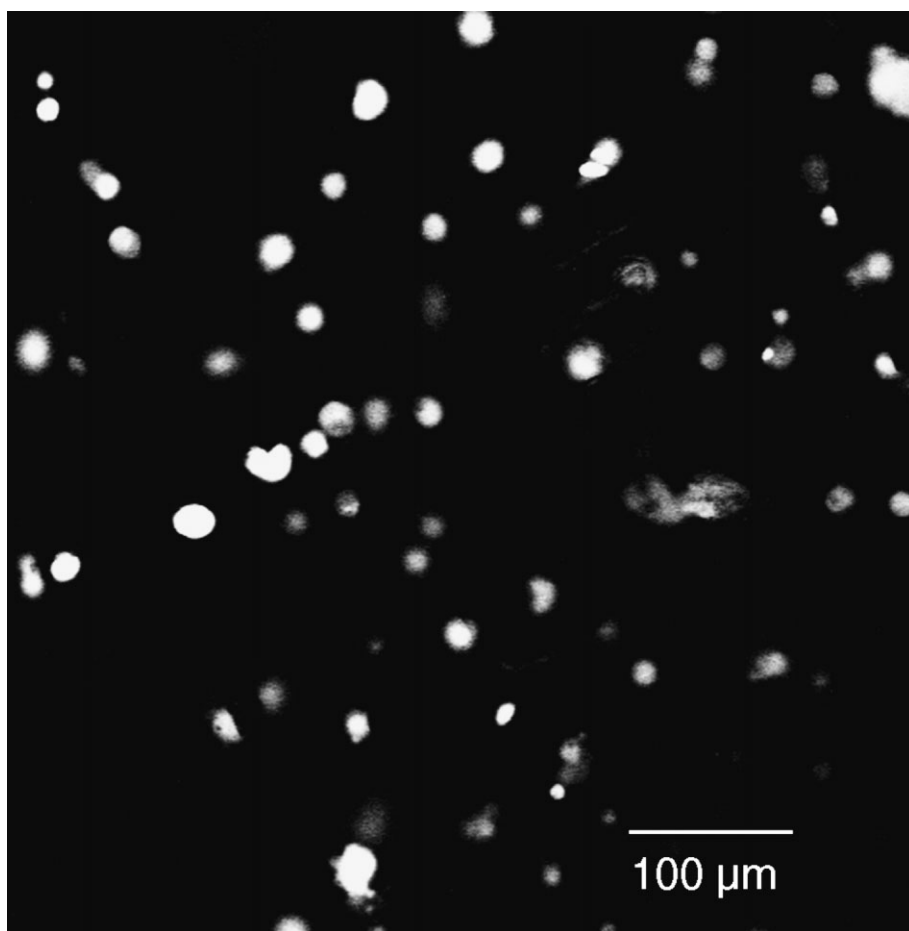


Figure 8. Cells incubated in a chip for 72 h, stained by perfusion of calcein-AM (1 μM) in physiological solution.

Cells were successfully embedded and anchored in a 3-D ECM gel within the chamber. This enabled both 3-D cultivation and studies of cellular responses to perfused solutions.

The microstructure design included pillars in the ECM-cell cavity. The pillars contribute with two important functions. By stabilizing and reinforcing the gel, higher surface flow rates can be used in the system than has been previously reported [18]. Furthermore, the pillars contribute by increasing the surface to volume ratio in the gel after polymerization-induced shrinkage, facilitating exchange between embedded cells and perfusate by reducing the diffusion length in ECM. This has importance in maintaining a controlled environment within the structure, *i.e.*, fresh cell culture medium will reach all the cells, and products from cellular metabolism will leave the system. Furthermore, this capacity of the structure has importance for the proposed use of the system in cell physiological studies where the response from cells after perfusion of different solutions will be studied.

Cells were alive and viable after up to 6 days of incubation within the chamber. Thus medium- to long-term cultivation and subsequent cell physiological studies will be possible. During cultivation the cell density increased in average 78% after 3 days of cultivation, showing that the cells are viable and proliferate in the chamber. Our concept for 3-D cell culture and analysis now opens up novel possibilities for relevant cell physiological studies in well-controlled 3-D chemical environments.

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5 References

- [1] Anderson, H., Berg, A. V. D., *Lab Chip* 2004, 4, 98–103.
- [2] Kane, R. S., Takayama, S., Ostuni, E., Ingberg, D. E., Whitesides, G. M., *Biomaterials* 1999, 20, 2363–2376.
- [3] Raines, E. E., *Int. J. Exp. Pathol.* 2000, 81, 173–182.
- [4] Holmes, T. C., *Trends Biotechnol.* 2002, 20, 16–21.
- [5] Hynes, R. O., *Trends Cell Biol.* 1999, 9, 33–37.
- [6] Tan, W., Desai, T. A., *Tissue Eng.* 2003, 9, 255–267.
- [7] Hynes, R. O., *Cell* 1992, 68, 303–322.
- [8] Cukierman, E., Pankov, R., Stevens, D. R., Yamada, K. M., *Science* 2001, 294, 1708–1712.
- [9] Jockusch, B. M., Bubeck, P., Giehl, K., Kroemker, M., Moschner, J., Rothkegel, M., Ruediger, M., *Annu. Rev. Cell Dev. Biol.* 1995, 11, 379–416.
- [10] Adams, J. C., *Cell Mol. Life Sci.* 2001, 58, 371–392.
- [11] Sastry, S. K., Barridje, K., *Exp. Cell Res.* 2000, 261, 25–31.
- [12] Gruber, H. E., Jr., Hanley, E. N., *BMC Musculoskeletal Disorders* 2000, 1, 1471–2474.
- [13] Weaver, V. M., *J. Cell Biol.* 1997, 137, 231–245.
- [14] Abbott, A., *Nature* 2003, 424, 870–872.
- [15] Wolf, K., *J. Cell Biol.* 2003, 160, 267–277.
- [16] Biosciences, B. D., *Product Literature–MATRIGEL* 2003, Vol. 1, 4–5.
- [17] Tan, W., Desai, T. A., *Biomaterials* 2004, 25, 1355–1364.
- [18] Tan, W., Desai, T. A., *Biomed. Microdev.* 2003, 5, 235–244.