SINGLE CELL ANALYSIS IN A MULTILAYER MICROFLUIDIC DEVICE: MONITORING OF DRUG-INDUCED GENE EXPRESSION

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ABSTRACT

In this paper a multilayer microfluidic device is presented to monitor gene expression profiles of individual cells that can be stimulated by tetracycline. This microdevice enables positioning and culturing of adherent human embryonic kidney cells (HEK 293) over long periods of time. By introduction of a second fluidic layer, separated by a porous membrane, the additional supply of stimulating reagents towards the cells becomes possible, without perturbing the constant, weak flow of growth media. This allows addressing and systematically analyzing cells in parallel with high temporal and spatial resolution and high sensitivity on the single cell level.

KEYWORDS: Multilayer microfluidic device, induced gene expression, single cell

INTRODUCTION

With the goal to investigate individual cells and reveal the origins of heterogeneities within cell populations, it is important to precisely control the microenvironment around cells, i.e., to keep it constant or create well-defined changes in its chemical composition. Most often, only a low amount of molecules induce already a cellular response, therefore it is crucial to handle small liquid volumes and low concentrations [1,2]. We report here on the development of an advanced microfluidic device by combining multilayer microfluidics with the concept of multilaminar streams (Figure 1). In the two-layer device it is possible to separate cell culturing and the supply of cell stimulating agents with high temporal and spatial resolution. A porous membrane separates the layers to allow diffusion of the stimulating agents to the cells. We extend the functionality of our former approaches [3-5] by implementing cell traps for fast and reliable mammalian cell positioning. In this device, up to 450 single cells can be investigated in parallel. Furthermore, semi-automatic analysis of high cell numbers is established.

To demonstrate the performance of the device, we study the expression of a mutant of the Green Fluorescent Protein (GFP) as a model gene of interest. The gene expression is controlled by tetracycline as external effector, which is supplied to adherent mammalian cells in various concentrations. The increase in fluorescence due to the expression of GFP is observed over 15 hours.

Figure 1: Sketch of the multilayer microfluidic device. The top channel is used for trapping of cells, the bottom channel for the supply of the soluble effector tetracycline. It is transported with high temporal and spatial resolution through a porous membrane below the traps that allows diffusion of tetracycline to the cells.

EXPERIMENTAL

The cell line HEK 293 (human embryonic kidney) is transfected with the T-Rex™ system. It consists of two plasmids: The first expresses tetracycline repressor proteins that form homodimers. Two of these homodimers can bind to two tetracycline operator sequences on the second plasmid and hence, the expression of the gene of interest (here: green fluorescent protein, GFP) on the second plasmid is inhibited. The target gene expression is induced by addition of tetracycline; it binds to the repressor homodimers and effects their conformational change, whereby the repressor is set free and gene expression is possible [6]. GFP expression is monitored online by time-lapse fluorescence microscopy for 15 hours on an inverted fluorescence microscope (Olympus IX81), equipped with a long-distance objective and an incubation chamber. Images are recorded every 10 minutes using the Olympus CellR 3.0.x software with a digital camera (Hamamatsu Orca ER, exposure times: 40 ms). Data are processed using the ImageJ 1.43g software (NIH, USA). Thereby, only single cells that show a positive response after 900 min are considered and the fluorescence in-
tensities are normalized to the initial fluorescence intensity, i.e., the autofluorescence of the cell. The cells are trapped in the top channel, while the bottom channel is filled with a continuous flow of tetracycline in buffer at different concentrations. Multilayer microchips made of poly(dimethylsiloxane) (PDMS) are prepared by standard processes described elsewhere [7]. Both channel structures are 20 µm in height; the bottom channel is 370 µm wide and the top channel has a width of 4830 µm at the crossing section. Microchannels and the porous polyester membrane (pore sizes 1.2 µm) are used without further treatment. The channels are connected via tubings to a pressure pump.

RESULTS AND DISCUSSION

Directly after chip assembly the channels are completely prefilled with Millipore water. Cells are introduced into the microdevice (1.5x10⁴ cells/ml, in DMEM low glucose media containing 10% fetal bovine serum (tetracycline negative), 1% MEM, 1% Penicillin-Streptomycin, 25 mM HEPES buffer and 1 µg/ml propidium iodide) and trapped at the microsized hurdle structures. After almost all traps are occupied, the cell suspension is washed out and a constant flow of medium (200 nl/min) is applied in the top channel. Staining tests confirmed that most of the cells (96%) sustain these flow rates over at least 15 hours.

Tetracycline is supplied in the bottom channel at a flow rate of 350 nl/min and in concentrations between 0.1 µg/ml and 10 µg/ml. Fluorescence imaging reveals the increase of intracellular GFP within 200 min after supply of tetracycline (Figure 2A). The histogram in figure 2B shows the distributions at three time points (300/600/900 min) for a tetracycline concentration of 0.5 µg/ml. Although the cells are treated equally, the increase of fluorescence intensity is very heterogeneous, and the heterogeneity increases further within time indicating that GFP is expressed in different levels in the cells. Frequently, a few extraordinary bright cells are observed. Without addition of tetracycline, no increase of fluorescence intensity can be observed.

![Figure 2: A) Increase of fluorescence intensity in individual cells over time. Embedded: 8 fluorescent cells (green, GFP fluorescence) and 1 dead cell (red) after 900 min (stained with propidium iodide). B) Histogram at three time points (300/600/900 min) exemplarily shown for a tetracycline concentration of 0.5 µg/ml. The distribution of fluorescence intensities broadens over time. C) Normalized fluorescence intensity for various tetracycline concentrations at three different times after induction of the gene expression.](image)

The average increase of fluorescence intensity of all observed cells is dependent on the supplied concentration of the effector (Figure 2C). The dependency indicates a saturated titration curve for concentration dependent cell responses, as theoretically expected. As long as the amount of effector molecules is below the level to set every repressor protein free that is bound to the inducible expression vector, the gene expression of GFP is still inducible and linearly dependent on the tetracycline concentration. Above an effector concentration (here: 10 µg/ml) no repressor protein can bind to the inducible expression vector due to the tetracycline-caused conformational change. In this case the GFP expression is continuously induced and the fluorescence intensity is dependent of the equilibration between protein expression and degradation. Tetracycline concentrations above 10 µg/ml result in delayed and reduced gene expression, most probably due to the harmful effect of high tetracycline concentrations.

Furthermore, the effect of transient supply of tetracycline is studied. For an exposure time of 40 minutes of tetracycline (1 µg/ml), the expression of GFP is monitored, again over 15 hours (Figure 3). In this experiment, the GFP fluorescence increases initially as expected. However, after 600 minutes only little further increase can be detected. This indicates that GFP expression is repressed, which is a result of increasing concentration of repressor proteins (expressed constantly by the first plasmid of the T-REx™ system) that finally bind to the tetracycline operator sequence on the second plasmid of T-REx™ expression system.
CONCLUSION

The results demonstrate the applicability of the multilayer microchip for single cell studies. In the experiments described herein we could monitor the expression of the green fluorescent protein in individual cells upon tetracycline supply over more than 15 hours. Further research is required to obtain detailed information on the mechanism and to elucidate the reason for the inhomogeneous expression of GFP in the cell population. We believe that the microfluidic platform will be of general use for applications in systems biology, pharmacy or biotechnology, in which the effect of perturbations on cells induced by small molecules should be investigated. The device is particularly useful for long-term investigations, where slow processes are observed, as well as for time-resolved stimulation, where the cells are exposed to only short pulses of a chemical inducer.

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