Biomed Microdevices (2007) 9:135–141
DOI 10.1007/s10544-006-9006-6

Screening cell surface receptors using micromosaic immunoassays

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Published online: 23 November 2006

Abstract This report presents a general method for screening cell surface receptors using so-called micromosaic immunoassays. This method employs a microfluidic chip having n ($n = 11$) independent flow paths to move cells over m ($m = 11$) lines of surface-patterned antibodies for screening individual cells in a parallel, combinatorial, fast and flexible manner. The antibodies are patterned as 30-μm-wide lines on a poly(dimethylsiloxane) layer used to seal the area of the chip in which screening is being monitored. Mouse hybridoma cells having CD44 cell surface receptors and anti-CD44 antibodies were used to establish a proof-of-concept for this method. Both the capture antibodies and the cells were fluorescently labelled to allow the position of the cells to be accurately tracked over the binding sites using an inverted fluorescence microscope. The chips and cells were maintained at a constant temperature between 20 to 37°C, and flow velocities of the cells over the capture areas were 100–280 μm s^{-1}, resulting in a ~0.1–0.3 s residency time of the cells on each of the eleven 30 × 30 μm s^2 capture areas. Binding of the cells appeared to be specific to the capture areas, with a yield of 30% when the assay was performed at a temperature of 37°C and with a slow flow velocity. We suggest that this proof-of-concept is broadly applicable to the screening of cells for medical/diagnostic purposes as well as for basic research on the interaction of cells with surfaces.

Keywords Microfluidic · Cell · Surface receptor · Screening · Micromosaic immunoassay

1 Introduction

Cellular assays are used to study how chemicals, biomolecules and factors such as electrical and mechanical stimuli, radiation, and heat affect the viability, metabolism, differentiation, cytoskeleton, motility, adhesion, receptor trafficking, and apoptosis processes of cells (Rowan et al., 2002). Cellular assays therefore are of fundamental importance in biology, medicine, pharmacology, and biotechnology. They are used both at a fundamental research level as well as for diagnostics. Whereas many assays focus on changes in the morphology, activity, or colour (due to staining) of cells, several assays aim at identifying cell membrane receptors to classify cells, detect diseases, or elucidate biological pathways (Mattanovich and Borth, 2006).

Cellular assays are also increasingly being used to test the safety of drugs and to identify potential drugs for cellular receptors. Cellular assays can require testing thousands of compounds at various concentrations, using individual compounds or a combination of compounds (Persidis, 1998). Clearly, these assays can benefit from miniaturization by decreasing the size and cost of the infrastructure and equipment needed (Li et al., 2003; Prokop et al., 2004). In addition, cells such as stem cells and cells from some mammalian cell lines are available in very limited quantities only.

Microfluidics are emerging as a powerful class of devices for performing miniaturized cellular assays (Wheeler et al., 2003; Pihl et al., 2005; Poulsen et al., 2005), patterning

Electronic Supplementary Material Supplementary material is available in the online version of this article at http://dx.doi.org/10.1007/s10544-006-9006-6.

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cells (Khademhosseini et al., 2005; Chiu et al., 2000), and controlling cellular environments (Takayama et al., 1999; Tourovskaia et al., 2005; Li Jeon et al., 2002). These devices can accommodate microliter and sub-microliter volumes of samples and reagents, can provide faster time to results than conventional technologies, can effect multiple assays in parallel or serial assays with high throughput, and sometimes are portable. We have developed a class of microfluidics in which liquids move under the action of capillary forces (Delamarche et al., 1997, 1998). These microfluidic chips are autonomous and do not need pumping elements. They can be used without peripherals for actuation. We call one independent flow path on such a microfluidic chip a capillary system (CS) (Juncker et al., 2002). CSs can be cloned and arrayed so as to move a number of solutions independently and run assays in parallel. Key to using these chips is the accurate patterning of receptors for analytes on the surface of a poly(dimethylsiloxane) (PDMS) elastomer, which reversibly seals sections of the CSs.

Assays based on this approach can be two-dimensional and combinatorial. A first microfluidic chip with n CSs can be used to pattern n types of capture antibodies (Abs) on PDMS as parallel lines. A second chip having m CSs can be used to bring m samples over the surface-immobilized capture Abs. Ligand-receptor interactions between the capture Abs and their antigens from solution result in a maximum of $n \times m$ binding interactions, which can be displayed as a "micromosaic" of fluorescence signals if fluorescently-tagged detection Abs are used to reveal where antigens are captured on PDMS (Bernard et al., 2001). Similar approaches for the combinatorial screening of clinical analytes (Rowe et al., 1999a,b; Kanda et al., 2004), and single-stranded DNA analytes have been developed (Nelson et al., 2001). A matrix of surface binding interactions can also be used to detect flowing antigens with a serial dilution strategy (Jiang et al., 2003).

In this paper, we explore how the concept of micromosaic immunoassays (Bernard et al., 2001) can be applied to the screening of cells. Several reasons motivate this work. First, micromosaic immunoassays are efficient. One microliter of sample or less is sufficient to screen eight or more types of analyte. The short paths for diffusion of analytes in microchannels can greatly accelerate diffusion-limited steps. The flow of solutions in microfluidics is typically laminar and makes rinsing steps short and effective. A fluorescent sandwich immunoassay having ~10 steps can be done in less than 10 min for detecting analytes at a concentration level of ~1 mg mL^{-1} or in ~45 min for analytes at concentrations down to 1 pM (Wolf et al., 2004; Cesaro-Tadic et al., 2004). This method suggests that cells can be screened rapidly and using small volumes of sample. Second, using microfluidic chips the PDMS substrate for the assay can be patterned in a flexible manner prior to analyzing samples (Delamarche et al., 2005). The accurate patterning of capture Abs on a PDMS surface limits the depletion of analytes from a small volume of sample and facilitates the reading of a large number (up to several hundreds) of fluorescent signals. The PDMS layer can be readily removed from the microfluidic chip at the end of the assay and the signals analyzed using a fluorescence scanner or microscope. With respect to cellular assays, it should then be possible to localize the interaction of cells with surface-bound receptors accurately. Third, the possibility to conduct assays and calibrate them simultaneously using reference samples may yield a better consistency and smaller intra-assay variation than assays do that are done as independent experiments. Similarly, it should be possible to screen different types of cells in parallel using a micromosaic format. In the remainder of this paper, we first review how a micromosaic immunoassay for screening cells can be set up and then provide a proof-of-concept by analyzing the trajectories of individual cells passing in microchannels over surface-immobilized antibodies.

2 Experimental

2.1 Chemicals and cells

Chemicals were purchased from Sigma-Aldrich or Fluka (Buchs, Switzerland) unless otherwise stated. Cells from the mouse hybridoma cell line PK136 were used for this work. Briefly, these cells secrete mouse IgG2a monoclonal antibody, which reacts with mouse natural killer cells. These hybridomas were obtained by fusion between SP2 myeloma cells and spleen cells (C3H × BALB/c) immunized with mouse spleen cells (Koo and Peppard, 1984; Kung et al., 2001). The cells were cultured and prepared using phosphate-buffered saline with pH 7.4 (PBS), bovine serum albumin (BSA), Tween 20, fluorescein diacetate, dimethylsulfoxide (DMSO), Iscove's Modified Dulbecco's Medium (Gibco, Invitrogen, Switzerland), foetal calf serum, normal mouse serum, hyaluronidase, and propidium iodine. The cells were cultured with 5% atmospheric CO_2 at 37°C in Iscove's Modified Dulbecco's Medium, which was supplemented with 10% foetal calf serum, in a cell incubator.

Anti-CD44 monoclonal antibodies (BD Biosciences, San Jose, CA), either conjugated with FITC or Phycoerythrin, were used as capture antibodies for the microfluidic-based assays (see below) as well as for fluorescence-activated cell sorting (FACS). FACS (FACScan, BD Biosciences, San Jose, CA) was used to verify the presence of CD44 on the PK136 cells before performing microfluidic-based assays as follows. First, cells were taken from the cell culture and washed, resuspended in PBS at a concentration of 2.5×10^6 cells mL^{-1}, and 200 μL of the suspension was loaded into the wells of a 96 microtiter plate (Falcon), which corresponds to ~500,000 cells per well. The cell

aliquots were centrifuged, and their Fc receptors (a potential nonspecific cellular receptor for antibodies) blocked using 100 μL solution of mouse serum in PBS (1:50) for 15 min at room temperature. To label the CD44 cellular receptors fluorescently, the cells were centrifuged again and incubated in a 1:200 solution of antibody (anti-CD44-Phycoerythrin conjugate) in 0.1% normal mouse serum in PBS for 15 min at room temperature. The cells were centrifuged and resuspended in 200 μL of PBS twice. Immediately prior to FACS, propidium iodine was added to sort out nonviable (necrotic) cells. Viable cells were assessed using FACS scatter plots by gating on propidium iodine-negative populations.

Cells for the microfluidic-based assays were handled and stained as follows. A suspension of PK136 mouse hybridoma cells was taken from the cell culture and incubated for 1 h at 37°C with hyaluronidase (5000 IU per $\sim 5 \times 10^6$ cells mL^{-1}) to prevent hyaluronic acid-mediated cell aggregation (Miyake et al., 1990). To visualize the cells during the assays, 0.1 μL of a 5 mg mL^{-1} solution of fluorescein diacetate in DMSO was added to 1 mL of cell suspension. After 30 min of incubation at room temperature, the cells were washed three times with PBS, resuspended in a 2% solution of normal mouse serum in PBS, and used after a minimum resting time of 15 min.

2.2 PDMS and microfluidic chips

The prepolymers of PDMS (Sylgard-184, Dow Corning, Midland, MI) were mixed at a 10:1 ratio using a Dopag Mixer (Cham, Switzerland), dispensed on the bottom of a Falcon Petri dish, and cured for at least 24 h at 60°C. PDMS slabs of desired dimensions were then cut and used to prepare lines of capture Abs or simply to seal some areas of the chips during the assays.

The microfluidic chips were microfabricated using photolithography. A chrome mask was used to pattern a photoresist on 4" silicon wafers and to etch selectively 20 μm of silicon using a deep reactive ion etcher (STS ICP, Surface Technology Systems plc, Newport, U.K.). After removal of the photoresist, the wafers were coated with Ti (10 nm) and Au (150 nm), and then diced to yield individual chips. Each microfluidic chip had 11 independent CSs (Cesaro-Tadic et al., 2004). The top surface of each chip was microcontact-printed with octadecanethiol to form a hydrophobic coating (advancing contact angle with water of $\sim$115°). The chips were then immersed in a 10 mM ethanolic solution of thiolated poly(ethylene glycol) (ABCR, Karlsruhe, Germany) for 30 s to make the recessed areas of the chips wettable (advancing contact angle with water of $\sim$40°) and protein-repellent. Used chips were cleaned prior to reuse by immersion in a "Piranha" solution (H_2SO_4:H_2O_2, 3:1, CAUTION) (Comment), after which the chips were copiously rinsed with deionised water and dried under a stream of N_2.

3 Patterning of capture antibodies and screening assays

Capture Abs were patterned on PDMS by covering the microchannels of a chip with a $\sim$1-mm-thick slab of PDMS and filling the loading pads of the chip with 0.5 μL of a 0.1 mg mL^{-1} PBS solution of anti-CD44 Ab conjugated with FITC. After 2 min, most of the Ab aliquots had passed through the microchannels, which were flushed by adding (i) 0.5 μL of a 1% solution of BSA in PBS and (ii) 0.5 μL of PBS to the loading pads. Finally, the PDMS layer was removed manually from the chip, rinsed with deionised water and blown dry under a stream of N_2.

Next the PDMS layer was brought into contact with a second microfluidic chip so as to expose the lines of capture Abs orthogonally to the microchannels of the second chip. A slab of PDMS was used to seal the capillary pumps . The chip was then placed on a thermally conductive adhesive, which covered a thermoelectric element. The cellular assays proceeded by filling the loading pads of the chip with 0.5 μL of a 1% BSA solution in PBS and 0.5 μL of a 10^7 cells mL^{-1} suspension of PK136 hybridomas. During the filling of the capillary pumps, air trapped in the pumps slowly diffused through PDMS, which resulted in a slow and homogenous flow rate of solution in all microchannels. After dispensing the cells, two cover glass slides separated by a 1-mm-thick PDMS frame were quickly placed over the chip and its thermoelectric element. The thermoelectric support with the chip and glass slides were turned upside down and placed on the stage of an inverted fluorescence microscope (Axiovert 100, Zeiss, Switzerland). A thermo-regulator (TC2812, Minco, Niederuzwil, Switzerland) with pulse-width modulation and thermo sensor was used to regulate the temperature of the thermoelectric element and chip from 20 to 37°C. A digital camera (Coolpix E990, Nikon) was used to record the trajectory of cells flowing over the region in which the capture Abs are located. The focal plane of the microscope was set to the lines of capture Abs. The videos showing the trajectories of the cells were analyzed after the assay using the software Image Pro and its object-tracking functions.

4 Results and discussion

The influence of the environmental parameters (e.g. culture medium, pH, temperature, shear stress) on the metabolism of living cells and the tendency of cells to sediment require the adaptation of micromosaic immunoassays to the particular needs of living cells. The setup used for screening cells using CSs is displayed in Fig. 1. It consists of a microfluidic chip of silicon, covered with a thin layer of Au and self-assembled monolayers, and has two separate blocks of PDMS. One block serves as the substrate for the assay: it is patterned with lines of capture Abs and placed on the region in which the

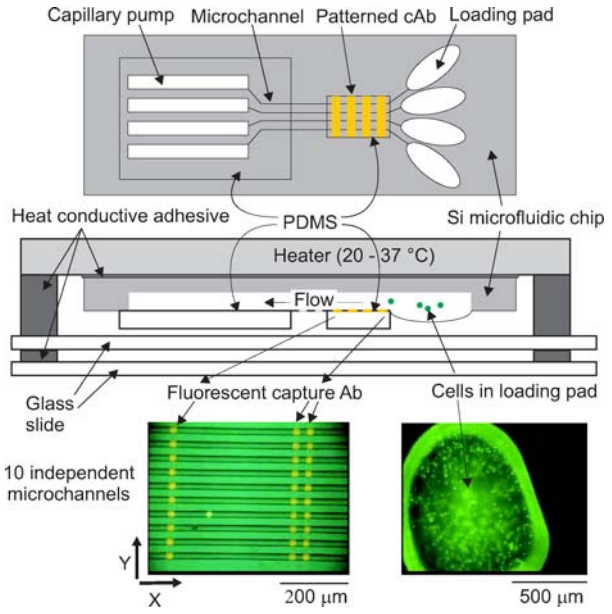

Fig. 1 Experimental setup for a micromosaic immunoassay screening of cells. Aliquots of cell suspensions are placed in the loading pads of independent capillary systems, from where they fill the microchannels and capillary pumps of the capillary systems. As the cells cross lines of capture Abs, which are patterned on a sealing PDMS layer, capture of some cells may occur. A heater, two glass slides, and conductive foil spacers form a chamber inside which the microfluidic chip and the cells are maintained at a constant temperature; moreover evaporation of water from the loading pads is negligible. The trajectory of the cells in the region of the capture sites is monitored in the X- and Y-directions using an inverted fluorescence microscope and recorded as a video for analysis. The fluorescence microscope images show (left) three lines of capture Abs (orange) and a cell (green) moving in one microchannel and (right) ∼300 nL of cell suspension in one loading pad

microchannels of the CSs run parallel. The second PDMS block covers the capillary pumps of the CSs. It helps to maintain a reproducibly low flow rate in the microchannels when the solutions added to the loading pads progressively displace air from the CS and fill the capillary pumps. To prevent cell lyses due to fast cell flow in the microchannels, a small volume of buffer is added first to each loading pad so as to fill the microchannels up to approximately the inlet of the capillary pumps. During the assay, the air compressed by the filling liquid permeates through the PDMS, resulting in a slow flow of solution inside the microchannels. The microfluidic chip is placed on a heat-conductive adhesive and heating device. Once the solutions with cells are loaded on the chip, the chip and its heating element are placed upside down on the stage of an inverted fluorescence microscope. Two glass slides separated by ∼1-mm-thick slabs of PDMS insulate the chip from the microscope stage to prevent condensation of water in front of the microscope's optical path. The chip was maintained at a constant temperature between 20 and 37°C for the assays. Sedimentation of cells resulted in cells coming into contact with the capture sites. The fluorescence images in Fig. 1 show cells labelled with fluorescein (green) and capture sites on PDMS having antibodies labelled with R-phycoerythrin (orange).

For the proof-of-concept shown here, we selected mouse hybridoma cells having a CD44 cell surface receptor. These cells partly derive from white blood cells, and CD44 is involved in numerous cell-cell interactions important for lymphocyte activation, hematopoiesis, and tumour metastasis. In addition, the transmigration of blood vessel walls by white blood cells is a physiological process, which is mediated by rolling and tethering through receptor molecules in the cell membranes (Chen et al., 1997; Eniola et al., 2003). This cell-binding mechanism guides immunocompetent cells to inflammatory target areas. The receptor profile expressed and displayed by the endothelial cells provides specificity for subpopulations of white blood cells that can be differentiated by their membrane protein expression, that is their cluster of differentiation (CD) (Chen et al., 1997). Using a micromosaic immunoassay and specific antibodies, it should be possible to imitate the capture of a subpopulation of white blood cells on the surface of blood vessels to a certain extent: depending on the combination of antibody and receptor, rolling, tethering or firm binding of leukocytes to a surface may occur under a certain shear rate. In clinical medicine, the examination of white blood cell subpopulations is crucial for diagnosis and treatment of diseases, e.g. AIDS. A device capable of separating subpopulations would allow further examination of these cells or an isolated ex vivo treatment of these cells and their reinsertion into the blood stream. In current clinical practice, conventional FACS machines require incubating the cell sample to be examined with fluorescently labelled antibodies for at least half an hour.

The cellular assays consist in following the real-time trajectories of the cells in each microchannel along the main axis of the microchannel (X-coordinate) and orthogonal to the microchannel (Y-coordinate). Figure 2 shows the trajectory of cells in 11 independent microchannels with the microfluidic chip maintained at 20°C. First, ∼140 cells are tracked during 42 s and their Y-positions are analyzed, Fig. 2(a). Short segments in this graph correspond to cells entering and leaving the channels without noticeable interaction with the capture Abs. On average, the cells remain ∼2.5 s in the length of the microchannels that is visible in the field of view and their mean velocity in the microchannel is ∼280 μm s^{-1}. Note that the curvature of the segments reflects an optical artifact and not a curved trajectory of the cells in the microchannels. In addition, several cells bind to the capture areas and become immobile during this experiment. The graph in Fig. 2(b) shows the trajectory of selected cells along the main axis of the microchannels as a function of time. Cells flow along the microchannels at a steady speed until they become bound to one of the 11 capture zones they cross.

Figure 3 shows selected frames of a video in which the trajectory of cells over the capture areas was followed. This

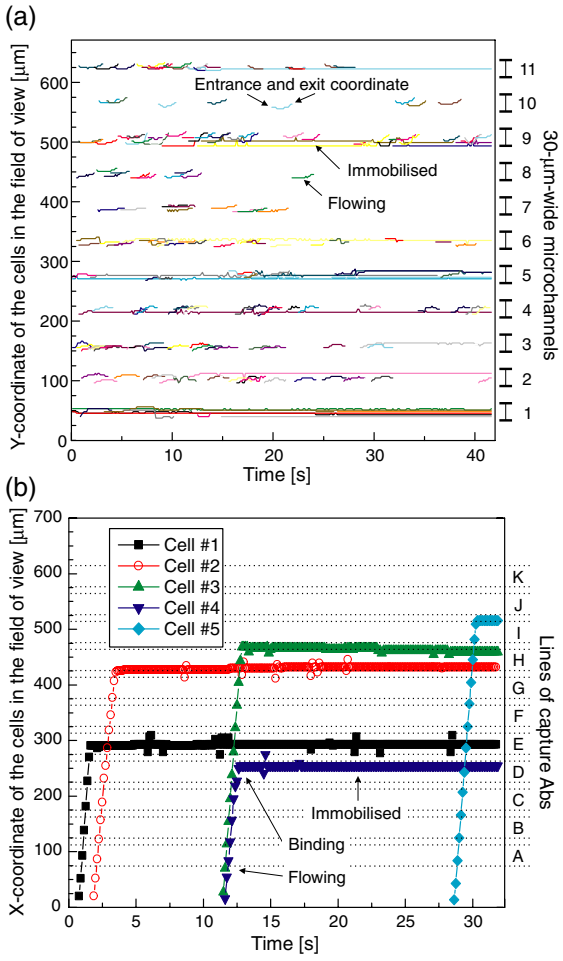

Fig. 2 Trajectories of PK136 hybridoma cells having CD44 surface receptors and flowing inside microchannels across patterned lines of anti-CD44 Abs. (a) Graph showing the lateral positions of the cells inside the microchannels as a function of time. The entrance and exit coordinates of one cell flowing through a microchannel without binding are marked. Following a binding event, cells acquire an invariant Y-coordinate. (b) Graph showing the longitudinal position of cells along the microchannels as a function of time. Cells flow over 11 areas having capture Abs. The trajectories of only a few binding cells are depicted for better clarity

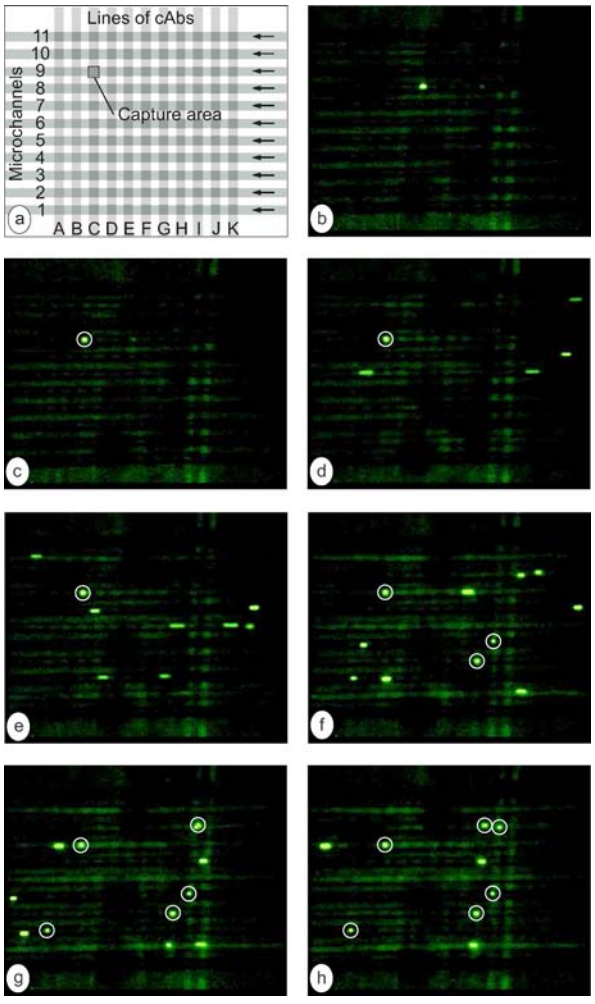

Fig. 3 Outline (a) and movie frames (b–h) illustrating a micromosaic immunoassay with cells as recorded using a fluorescence microscope and video camera. The cells are PK136 hybridomas, have CD44 surface receptors, and are labelled with a fluorescein dye (green). Anti-CD44 Abs labelled with fluorescein, which are patterned as lines on a PDMS layer used to seal the microchannels, form the capture areas. The cells flow from left to right inside 30-μm-wide microchannels. The cells binding to anti-CD44 Abs are circled. The video corresponding to this assay is provided as supplementary material

experiment was done at 37°C to optimize the chances of binding of the cells. The fluorescently-labeled capture Abs appear faint as a result of photobleaching, whereas cells appear as bright green ~10–15 μm-wide spheres. The frames cover a timeframe of ~20 s during which the cells flowed with an average velocity of 150 ± 45 μm s^{-1} or equivalent flow rate of ~0.15 nL s^{-1}. This range of velocities is mainly due to the laminar flow profile, which transports cells positioned in the center of the microchannel faster than those positioned elsewhere. The average residence time of one cell above one 30 × 30 μm^2 capture area is 0.2 s. A basic analysis of the trajectories and binding of the cells using commercial vision software revealed that ~30% of the cells were captured on one of the 11 capture sites aligned in each microchannel. A more detailed analysis would require the identification of the three-dimensional position of the cells in the microchannels and the calculation of the specific velocity and residence time on the capture areas for each cell. Such an analysis could for instance use methods known from particle image velocimetry, which requires a high-speed imaging setup. Interestingly, the retardation of some cells passing over surface-immobilized receptors can yield insight into the protein expression of retarded cells (Fitzpatrick et al., 2006). This suggests that qualitative cellular assays can provide useful information without the need for full parameterization of the factors governing the interaction between the cells and the receptors.

The flow rate of the cell suspension may be varied using peripheral equipment having several heating/cooling elements placed in contact with the microfluidic chip (Zimmermann et al., 2005) to study the effect of hydrodynamic forces on the behavior of immobilized cells (Gallant et al., 2002). The direction of flow, for example, can be changed back and forth to obtain statistical information on the binding of one cell over one type of capture area. In addition, micromosaic immunoassays for screening cell surface receptors can be combined with an interrogation of the mechanical properties of cells (size and elasticity) to identify particular cells, such as cells that are infected with parasites (Shelby et al., 2003).

5 Conclusions

The proof-of-concept shown here can be further refined. First, many types of capture Abs may be used to screen for various types of cells or receptors simultaneously. This may require using cells that are labeled differently in a first step when developing the assay. Second, the geometries of the microchannels and capture sites can be varied to capture cells at particular locations. This could minimize or prevent the risk of collisions and interferences between flowing and surface-immobilized cells. Third, "advanced" capillary pumps can be designed (Zimmermann et al., 2006) so as to vary the flow conditions in the microchannels and to study the influence of the flow rate on the statistics of binding. In this paper, the emphasis was to miniaturize the assays and screen individual cells from microliter volumes of samples. Larger volumes of sample can of course also be used, and capture sites can be made much larger and continuous, for example. The setup, microscope, camera and chemicals used here are relatively standard and did not require any particular modification for the assays, except for holding the microfluidic chips on an element having a controlled temperature.

We believe that the work shown here is broadly applicable and provides a flexible and efficient method for screening cell surface receptors. This concept may be used for histocompatibility testing to screen human leukocyte antigens. It can also be used for blood typing and for verifying the presence of cells in routine applications in which samples are available in limited volumes. There is a number of diagnostics that require the analysis of the number and type of white blood cells, and the status of some diseases such as HIV can be monitored by counting CD4-T-cells, for example. Although we are mainly interested in developing simple research and diagnostic platforms based on capillary-driven microfluidics, actuation mechanisms may be added to the microfluidic chips used here for sorting cells (Fu et al., 1999; Huh et al., 2005). In general, multidimensional assays are needed when testing large numbers of cells and compounds to obtain statistically meaningful results. It is therefore desirable to have cell assays that can screen cell/compound interaction in parallel (i.e. multiplexed assays), a task at which micromosaic assays excel.

Acknowledgements M. W. and P. H. acknowledge financial support from the NCCR Nanoscale Science initiative and M. Z. from the Swiss Commission for Technology and Innovation. The PK136 hybridoma cells were generously provided by the pediatric immunology group of G. Holländer, University Hospital Basel. We are grateful to Prof. U. Eriksson and his group from the intensive care unit (University Hospital of Basel) for having given access to their laboratory infrastructure and for useful discussions. We thank W. Riess and P. Seidler for their continuous support of the work.

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