A MICROFLUIDIC ARRAY WITH CELLULAR VALVING FOR CO-CULTURING SINGLE CELL COUPLES

J.-P. Frimat¹, M. Becker¹, Y.-Y. Chiang¹, D. Janasek¹, J.G. Hengstler², J. Franzke¹, and J. West¹

¹ ISAS – e.V., Otto-Hahn-Str. 6b, 44227 Dortmund, GERMANY
² IfADo, Ardeystr. 67, D-44139 Dortmund, GERMANY

ABSTRACT
We present a highly parallel microfluidic approach for contacting single cell pairs. The approach combines a differential fluidic resistance trapping method with a novel cellular valving principle for homotypic and heterotypic single cell co-culturing. Optimal geometries were identified for efficient cell arraying (>98%) and cell coupling (~70%). The single cell co-cultures were in close proximity for the formation of connexon structures and the study of contact modes of communication. Beyond this application, the research validates the concept of using the natural behaviour of cells for mechanical operations within microengineered environments.

KEYWORDS: Microfluidic Arrays, Single Cell, Co-Culture, Plasma Stencilling, Juxtacrine Signaling

INTRODUCTION
The orchestrated behaviour of mammalian cells and tissues is achieved by signaling over a range of distances. The most direct communication mode, juxtacrine signaling, involves physical contact between neighbouring cells. Contact modes of communication enable the highly localized coordination of a wide variety of fundamental biological processes, including embryogenesis, tissue regeneration, stem cell differentiation, immune cell activation and cancer biology. Far from being fully understood, cell–cell interactions are a major topic of research, with the modern onus on isolating single cell responses from those of the assembled population.

To facilitate single cell–cell contact investigations new tools with unprecedented spatial control are required. Microfabricated systems, with cellular and sub-cellular length scale features, are well equipped for the task. Sequential cell patterning can be used for coupling cellular populations. Alternatively, microfluidics can be used for arraying single cells. These hydrodynamic arraying systems are based on differential fluidic resistances, where fluidic streamlines transport single cells into individual traps. Once loaded the cell body diverts the streamlines to exclude subsequent cells [1]. Taking this principle further Tan and Takeuchi have developed a linear particle trap array with each trap sequentially connected using a superimposed serpentine microchannel [2]. Importantly, particles are arrayed with a perfect yield, making it ideally suited to support research with precious cells, such as those harvested from biopsy samples. In this contribution we introduce a cellular valving principle for use with a Tan and Takeuchi microfluidic architecture for highly efficient and reliable single cell coupling [3].

CONCEPT
The single cell co-culturing method is based on a cellular valving concept: Cells in suspension have a spherical morphology, and following initial adhesion assume a flattened morphology. Positioning a viable cell at an aperture within a microfluidic path can therefore be used to produce a living valve. This principle can be used within a differential fluidic resistance circuit for coupling single cells. We have opted to adapt the Tan and Takeuchi serpentine microfluidic arraying system, with the difference being the use of mirrored traps. The series of apertures produce the linear path. Illustrated in Fig. 1(A), the serpentine flow path has a higher fluidic resistance than the linear path \( (R_2 > R_1) \), producing a higher linear flow rate \( Q_1 \) than the serpentine flow rate \( Q_2 \) (1):

\[
\frac{Q_1}{Q_2} = \left( \frac{C_2(\alpha_2)}{C_1(\alpha_1)} \right) \left( \frac{L_2}{L_1} \right) \left( \frac{W_2 + H}{W_1 + H} \right)^2 \left( \frac{W_1}{W_2} \right)^3 > 1
\]

where \( W_1 \) is the aperture width, \( W_2 \) is the channel width, \( L_1 \) is the aperture length, \( L_2 \) is the length of each U-bend channel segment, and \( C(\alpha) \) are constants defined by the aspect ratio \( (0 < \alpha < 1) \), and derived from the Darcy friction factor and the Reynolds number. The aperture \( C_1(\alpha_1) \) value is 78.8 and the microchannel \( C_2(\alpha_2) \) value is 57.9 [2].

Cell trapping and flattening modulate the value of \( R_1 \) while \( R_2 \) remains constant. The single cell coupling process is as follows: A first cell is transported within the linear bulk flow and trapped at the first aperture. The cell occludes the linear path, increasing the local fluidic resistance \( (R_1 > R_2) \) and diverting the streamlines. A subsequent cell is diverted into the serpentine pathway for trapping at the second aperture \( (R_2 > R_1) \), see Fig. 1(B)). The process enables the serial arraying of single cells. The cells adhere and flatten, acting as a valve in the open state to restore the \( Q_1 > Q_2 \) condition \( (R_2 > R_1) \), see Fig. 1(C)). The flow is reversed and contains a second cell type for the serial arraying, as before, of single cells within the unoccupied traps (see Fig. 1(D)). The two cell types are in close proximity and can contact one another through the aperture connecting the mirrored traps. In this manner both heterotypic and homotypic single cell co-cultures can be established, with the array format providing the means to couple hundreds of single cell pairs for high throughput screening and the observation of rare events.
Figure 1: The cellular valving principle for single cell coupling. The linear path, through the apertures between the mirrored cell traps, has a lower fluidic resistance \( R_1 \) than the serpentine path \( R_2 \) (A). Loading of a spherical single cell (purple) reverses the fluidic resistance ratio, acting to divert subsequent cells \( (R_1 > R_2) \) for sequential \( (R_2 > R_1) \) single cell arraying (B). Viable cells adhere and flatten (lilac), restoring the \( Q_1 > Q_2 \) condition (i.e. \( R_2 > R_1 \)) (C). Flow reversal is used for introducing a second spherical cell type and sequential single cell coupling \( (R_1 > R_2; R_2 > R_1) \), D).

Figure 2: A single microstructured cell tap (A), a series of 6 traps (B) and an entire microfluidic circuit (C).

EXPERIMENTAL

The dimensions of the microfluidic circuit are defined by the dimensions of the cell types under investigation. Human SW480 epithelial cells \((14.6 \mu m, SD \pm 3.0)\) were used in this study. To accommodate these cells, traps with diameters (and typical trap/cell diameter ratios) of 15 \( \mu m \) (x1.0), 22 \( \mu m \) (x1.5), 30 \( \mu m \) (x2.0) and 37 \( \mu m \) (x2.5) were designed. The serpentine channel design had a width of 40 \( \mu m \), with U-bend segment lengths of 300, 500, 700 or 900 \( \mu m \) used to provide different flow ratios \( (Q_1/Q_2 = 0.6 \text{ to } 1.8) \). The aperture was 5 \( \mu m \) in length and 6 \( \mu m \) in width. Channel bifurcations were used to define 8 parallel analysis channels each containing 25 trap pairs (totaling 200 per device). The microfluidic system was prepared by replica moulding in poly(dimethylsiloxane) (PDMS, Sylgard 184, Dow Corning) using a 28-\( \mu m \)-high SU-8 master fabricated by standard photolithographic methods (see Fig. 2).

Human SW480 epithelial cells were purchased from DSMZ (Germany). Cells were cultured under standard conditions and harvested using accutase (PAA, Germany), a protease and collagenolytic mixture that retains the structural and functional integrity of the cells’ surface proteins. The cells were introduced into the microfluidic system and continuously perfused with media by gravity feed during incubation at 37°C in a 6% CO₂ atmosphere. The single cell coupling protocol involved loading cells at \( \sim 50 \mu m/s \) for 30 minutes, followed by incubation for 6 hours for cell adhesion and flattening. The pipette tips were then exchanged and the gravity-driven flow was reversed for loading the second cell type. Single cell co-cultures were incubated with continuous media perfusion at \( \sim 5 \mu m/s \) for periods up to 5 days.

RESULTS AND DISCUSSION

Microfluidic arraying of SW480 epithelial cells was highly efficient (see Fig. 3(A)) and insensitive to variations in the size of the SW480 epithelial cells \((0 \text{ SD } \pm 3.0 \mu m)\). We examined flow ratios ranging from 0.6 to 1.8 and confirmed the \( Q_1/Q_2 > 1 \) requirement for effective microfluidic arraying [2]. When using flow ratios \( \leq 1.0 \) only \( \sim 25\% \) of traps were occupied, whereas flow ratios \( \geq 1.4 \) enabled efficient arraying \((\sim 98\%)\), with high \((\sim 85\%)\) single cell occupancy levels (see Fig. 3(B)). In comparison the trap size variable had minimal impact on the efficiency of cell arraying. To suppress cell migration and ensure sustained contact during co-culture a plasma stencilling method was used for patterning the cells [4]. Here, the plasma was routed along the linear path of least resistance, oxidizing it to create a cell adhesive pattern for confining the cells to the trap regions. Using this approach, 96% of cells remained within the traps during 48 hours of culture, whereas without plasma stencilling only 76% remained.

Cell viability is a fundamental pre-requisite for cellular valving. Viable cells can adhere to the substrate and flatten, acting as a living valve to restore the \( Q_1 > Q_2 \) condition and enable coupling of the second cell type using a reversed flow. To demonstrate heterotypic single coupling we have paired unlabelled cells with cells labelled with the fluorescent product of calcein AM metabolism. A series of six heterotypic single cell co-cultures are shown in Fig. 4(A). An additional experiment was undertaken to identify the formation of gap junctions by connexin 43 immunostaining (see Fig. 4(B,C)). The image was recorded following 24 hours of co-culture and serves to demonstrate that on-chip conditions are suitable for the maintenance of viable cells and the formation of contact structures necessary for communication. The effect of the trap size on cell coupling was also examined. As documented in Fig. 4(D), only the larger 30 and 37-\( \mu m \)-diameter traps could be used to achieve cell coupling rates in excess of 65%. The largest, 37-\( \mu m \)-diameter, traps enabled the highest levels of cell coupling with a rate of 70.0% (SD \( \pm 7.4 \)). The trap dimensions are approximately 2.5-fold larger than the spherical cell diameter. In comparison a coupling efficiency of only 27.8% (SD \( \pm 7.4 \)) was obtained with the
smallest, cell-sized (15-μm-diameter) traps. Our hypothesis is that the smaller traps have insufficient area for complete cellular flattening and valving to render the adjacent trap permissive for arraying of the second cell type.

Figure 3: Highly efficient single cell arraying (A). The flow ratio significantly impacted the efficiency of single cell arraying (B). Flow ratios ≤1.0 resulted in large numbers of empty traps (dark grey). Flow ratios ≥1.4 enabled efficient single cell (white) arraying, with few traps containing multiple cells (light grey). Figure 4: Heterotypic single cell co-culture (A). A single cell couple (B), and the same couple immunostained for connexin 43 (red, C). A trap:cell diameter ratio of ≥2.0 was required for efficient (~70%) cell coupling (D).

CONCLUSION
We have introduced and demonstrated a novel cellular valving concept for use within a differential resistance microfluidic circuit for efficient single cell coupling. The microfluidic conditions support the culture of viable cells and the formation of cell–cell contacts required for intercellular communication. The method is suitable for both homotypic and heterotypic single cell co-culture experiments, requires that one or more of the cell types is adherent and can be applied to differently sized cell type combinations. In summary, the microfluidic arraying technique has great potential to advance co-culture research at the level of a pair of single cells.

ACKNOWLEDGEMENTS
The research was financially supported by the German Research Foundation (DFG WE3737/3-1) and the Ministry of Innovation, Science, Research and Technology of the state of North Rhine-Westphalia.

REFERENCES

CONTACT
*J. West, west@isas.de