DROPLET MICROFLUIDIC PLATFORM FOR CELL ELECTROFUSION

ROGIER SCHOEMAN

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Rogier Schoeman

11th of September, 2015

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DROPLET MICROFLUIDIC PLATFORM FOR CELL ELECTROFUSION

DISSERTATION

To obtain
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Chapter 1

Introductory chapter

In this chapter a short summary of how and why we started the project will be given.

Antibodies and hybridomas

Each year, the need for antibodies increases. This increase can for a small part be attributed to an increased use for research purposes but for the largest part to their strongly increasing use for diagnostic and therapeutic purposes. More and more therapies depend on the use of antibodies¹⁻⁶. The classical way of producing antibodies is by immunizing lab animals such as mice or rats and harvesting the antibodies they produce (See Figure 1). An alternative way is by using cell cultures, as the antibodies are secreted by plasma B cells. Unfortunately, plasma B cells do not survive long enough in cell culture to produce sufficient amounts of antibodies. Therefore the use of lab-animals is still unavoidable. However, the problem of the longevity of plasma B cells can be solved by fusing a plasma B cell with a myeloma cell, which is a cancer cell occurring in the blood⁷. This fusion creates a hybrid cell, hybridoma, which contains genetic material from the two different parental cells and therefore displays characteristics of both cells. In this case this means that it proliferates easily, as the myeloma cell, and is capable of secreting antibodies, as the plasma B cell.^{1,2,8-10} The newly formed hybridomas can thus easily be maintained in a cell culture for long periods of time while producing antibodies. The use of hybridoma cells has made the monoclonal antibodies more available for numerous purposes.^{7,11-13}

Figure 1. Schematic overview of classical hybridoma production. **A)** The animal is immunized. **B)** B-cells are isolated and mixed with myeloma cells. Cell fusion is induced to produce hybridomas. **C)** The mixture is cultured under conditions permitting only hybridoma survival. **D,E)** Single hybridomas are separated and screened for the desired antibody. **F)** The monoclonal antibodies are isolated. Adapted from SEMINAR TOPIC: MONOCLONAL ANTIBODIES PRESENTER: Dr. Ramakrishna J Junior resident (<http://www.authorstream.com/Presentation/aSGuest77764-708147-monoclonal-antibody-ppt/>)

Electrofusion

One of the critical steps in myeloma production is cell fusion. There are numerous methods to fuse cells, with as the most common ones the use of chemicals, viruses or electrical pulses. The latter method is called electrofusion and is considered to be the most efficient method for cell fusion^{8,9,14-16}. Conventionally, electrofusion takes place in a fusion chamber where cells are brought in close contact ('paired') by application of an electrical field and subsequently fused by an electric field. Unfortunately, the process in the fusion chamber cannot be controlled precisely, leading to random cell pairing that causes unwanted fusion products to emerge. Also, commercially available systems often have low fusion yields due to undefined or poor cell-cell contact^{14,15,17-21}. As a result these systems require large cell numbers to gain result. Moreover, the conventional fusion chambers utilize hundreds of microliters of cell suspension, making it practically impossible to fuse rare cell populations. Finally, because of the large distance between the electrodes in the fusion chamber, a high voltage is required to generate a sufficiently strong electrical field.

To overcome these limitations, microfluidic technology can be employed to develop microchips for cell electrofusion. When well designed, these microfluidic devices can provide a high control over cell pairing and the subsequent electrofusion process^{22,23}. Moreover, the small dimensions inherent to microfluidic technology enable the use of lower quantities of cells and media and allow working with lower voltages. Summarizing, the increasing need for (human) antibodies is a strong research driver to find a more efficient method for electrofusion of cells and subsequent generation of antibody-producing hybridoma cells, and microfluidic technology is a promising technique to address this problem.

History of the project

On the basis of the PhD project of dr. A. Valero at the BIOS/Lab on a Chip group, resulting in the thesis “Single cell electroporation on chip”(2006)²⁴⁻²⁶, the company ModiQuest B.V. approached prof. A. van den Berg to enquire if besides electroporation, electrofusion of cells on chip was possible and could contribute to current methods. As a result, preliminary research was conducted and a PhD project proposal was written. The goal of this PhD project was to develop a microfluidic device capable of electrofusion of plasma B and myeloma cells on chip. Dr. E.W.M. Kemna started this project, resulting in her thesis “Electrofusion of cells on chip” (2013). The thesis presented here is a follow-up of her work. In this thesis her work on cell electrofusion in picoliter droplets in a high throughput fashion is further developed. It is also demonstrated how the hybridoma cells can be further examined for functionality.

This PhD project is funded by European Research Council’s Seventh Framework Program as part of the ^elab4life grant. ^eLab4life focuses on the development of electrical lab-on-a-chip devices for health and life sciences. The project also received funding from NanoNextNL and Spinoza.

Thesis outline

To gain insight in the currently available microfluidic approaches for cell pairing and electrofusion, a literature review was performed, presented in **chapter two** of this thesis. The approach chosen was a high-throughput electrofusion process using

picoliter droplets. The different steps in designing the microfluidic chip needed for this process are treated in the following chapters. First cells need to be aligned and equally spaced in a microchannel to provide high single cell encapsulation yields in the picoliter droplets. The theory behind this process and the resulting chip design are treated in **chapter three**. In **chapter four** the development of the subsequent on-chip processing is reported. If the two different cell types are encapsulated in two different droplets, the next step needed is to pair and electro-coalesce the droplets, forming one large droplet containing the two different cell types. This droplet then is too large to subsequently electrofuse the cells, as the cells need to be in close contact. Therefore microfluidic functionality is added to shrink down the droplets in-flow to about 15% of their original volume, increasing the probability of the cells actually touching each other. **Chapter five** treats the in-droplet electroporation and cell fusion processes. Once the two different cell types are encapsulated in small droplets, the droplets for this purpose pass over six electrode pairs each creating an electric field inside the droplets sufficient to electroporate the cells and allow fusion. In **chapter six** all these different functionalities are integrated in a single microfluidic device which is then tested with HL60 cells. This chapter will thus conclude the thesis with the demonstration of an integrated functional microfluidic platform capable of electrofusing cells.

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Chapter 2

Review on microfluidic devices for electrofusionⁱ

Each year the need for monoclonal antibodies is increasing. The plasma B cells which are producing these antibodies have a very short lifespan, making antibodies expensive and sometimes unavailable. To increase the lifespan of these cells one can fuse a plasma B cell with a myeloma cell, resulting in an immortalized antibody-producing hybridoma cell. The cells can be fused chemically, virally and electrically. Moreover, to fuse cells they need to be in close contact with one another. Current methods lack the ability to control this cell pairing and therefore the yields of the currently used techniques are low. Lately, a lot of research in the field of microfluidics and microstructures has been performed, resulting in several platforms that facilitate and control cell pairing and by doing so increase cell fusion yield.

ⁱParts of this chapter have been adapted from Electrofusion of cells on chip (thesis E.W.M. Kemna)

Introduction to cell fusion

The need for the development of antibodies is increasing each year, not only for research but also for therapeutic and diagnostic purposes¹⁻⁶. The monoclonal antibodies that are used for these purposes are mainly produced by antibody secreting hybridomas^{7,8-14}. A hybridoma is a hybrid cell obtained by the fusion of two parent cells. The hybridoma contains intercellular genetic material from the parent cells, and also displays characteristics from both parent cells. Cell fusion can facilitate in the production of the hybridomas, and furthermore culturing and mediating methods are required to fuse the parental cells in an asexual manner to achieve hybridoma formation¹⁵.

In 1975^{16,17} Milstein and Kohler developed a technology based on the fusion of somatic cells, to create a B lymphocyte hybridoma. Mouse myeloma cells, which proliferate rapidly in vitro, were fused with activated mouse B lymphocytes, which have a short life span in vitro. The result of this fusion event was a hybridoma cell capable of synthesizing antibodies and able to proliferate rapidly in vitro. In other words the hybridoma cell contained both the desired qualities from the parental cells, enabling the hybridoma cell to secrete antibodies for longer periods of time. These two scientists were awarded the Nobel prize for their technology and opened the doors for many scientists to come up with new ideas to improve the cell fusion yield¹⁸.

The cell fusion is a multi-step process, that roughly proceeds as follows^{19,20}. The two parental cells have to be in close contact to achieve complete cell fusion on an external cue such as an applied electrical field. After fusion of the cell membranes, at first the fused cell still contains two separate nuclei, therefore nuclear fusion is what should follow. Unfortunately nuclear fusion is a random process, which cannot be influenced. Moreover, the cell has to recombine the two different sets of its parental DNA, which occurs 10-14 days after membrane fusion and can result in a functional hybridoma. To separate the functional hybridomas from the bulk, HAT medium can be used. The plasma B lymphocytes will die after three days and the HAT medium will kill the myelomas, enabling only the hybridomas to survive. After 10-14 days single hybridomas can be plated out in well plates after which an ELISA screening will determine which hybridomas are functional.

The first important step in cell fusion is thus cell pairing. In this chapter several microfluidic platforms that obtained high cell pairing efficiencies will be discussed in detail. The different cell fusion methods will also be discussed, however the focus will mostly be on electrofusion. High electrofusion yields can only be obtained by high one-to-one pairing efficiencies.

Efficiencies

In this chapter three types of efficiency will be discussed, which we will now define.

- *pairing efficiency*. The efficiency of pairing two different cells in a one-to-one fashion.
- *fusion efficiency or fusion yield*. The efficiency with which cells can be fused. In this paper it will be called fusion yield. This fusion yield depends tremendously on the pairing efficiency.
- *hybridoma development efficiency*. The efficiency to form hybridomas. Since this is a spontaneous process this strongly depends on the viability of the cells inside the device and speed at which they can be removed from the device and placed into a cell culture.

Cell fusion methods

Apart from electrofusion, chemical and virus-based methods and laser-induced methods have been carried out to induce cell fusion. Chemical fusion makes use of certain fusogens, such as polyethylene glycol (PEG)²¹⁻²³. PEG induces cell-cell contact and cell agglutination, which leads to cell fusion. The detailed mechanism underlying PEG-mediated cell fusion is not fully known. Nevertheless, studies indicate that within contacting membranes the PEG induces small perturbations in the lipid packing, which are necessary and most likely sufficient to promote membrane fusion^{24,25}. Virus-based fusion depends on the use of several fusogenic viruses, where the most known is the Epstein Barr virus^{26,27}. Also the mechanism of the virus-based fusion method is not fully understood. It can be found in literature that several cell surface proteins have emerged as candidate adhesion sites of fusion proteins²⁸. Amongst these cell surface proteins are members of the disintegrin and metalloproteinase (ADAM) gene family. Laser-induced fusion finally was developed using a focused laser beam²⁹.

Unfortunately, most of the methods mentioned have several limitations such as batch to batch variability and toxicity to cells. Furthermore, the methods have a low efficiency, in both functional hybridoma development and fusion. Fortunately, in the 70's cell fusion with the use of external electric fields was first reported and overcomes the limitations of the more conventional methods such as chemical and virus-based fusion^{16,17,30}. Electrofusion has multiple advantages, such as being an easy reproducible technique, being applicable to more cell types and resulting in a high post-fusion cell viability^{16,31}.

Electrofusion

The electrofusion process comprises three phases namely cell pairing, membrane electroporation and cell fusion. In its common application in larger volumes it proceeds as follows. By suspending the mixture of the two different parental cells into a weak conductive fusion medium, and applying a non-uniform alternating current field (AC) of moderate intensity (100-300 V/cm) and high-frequency (1-3 MHz), it is possible to pair the parental cells and bring them in close contact by dielectrophoresis (DEP)^{31,32}. This process is called pearl chain formation and is one of the most widely used and convenient methods to bring cells into close contact with one another. After the pearl chains of cells have been formed one or more short-duration (10-100 μ s) and high-strength (1-10 kV/cm) direct current (DC) pulses are applied to provoke pore formation in the zones where cells are touching. The electroporation of the membranes in the contact zones can lead to cell fusion if the electroporated cells stay in contact after the DC pulses have been applied, which again is made possible by DEP. The pores in the membranes will close and simultaneously reconstruct the cell membrane forming a hybridoma³³. The parameters for electrofusion are cell specific, and therefore depend on cell type and cell size²⁹.

Membrane fusion

After cell alignment and pairing, high intensity pulses of short periods of time need to be applied to induce electroporation. The cell membrane will break down when the electric voltage over the membrane (V_M) reaches its critical value V_C at room temperature³⁴⁻³⁶. In mammalian cells the threshold for electroporation is 300-350 mV.

An important parameter for electrofusion is the transition between reversible and irreversible breakdown. Irreversible breakdown of the cell membrane will ultimately lead to cell death, since the cell membrane is no longer capable of rearranging itself. An induced transmembrane potential around the reversible breakdown can result in electrofusion of cells^{8,37,38-40}. The V_M of an isolated spherical cell under the influence of an applied electric field, E_0 is given by^{38,41,42}:

$$V_m = 1.5 \cdot R \cdot E_0 \cdot \cos(\theta) \cdot (1 - e^{\frac{-t}{\tau_M}}) \quad (1)$$

Where R is the cell radius, θ is the angle between the normal to the membrane and the electric field vector, t is the time and τ_M is the time constant of membrane charging. When the pulse duration is 5-10 τ_M the membrane charging is completed and equation 1 simplifies to the simple equation:

$$V_M = 1.5 \cdot R \cdot E_0 \cdot \cos(\theta) \quad (2)$$

The induced membrane potential is thus proportional to the cell radius. Often, cells of different sizes are electrofused simultaneously. Large cells will then experience a higher voltage drop across the membrane than small cells and are more likely to be irreversibly damaged by the electrical treatment. Conversely, small cells experience a low voltage drop across the membrane, and they have less chance to be porated.

Electrofusion Buffer

Several parameters are important regarding the electrofusion buffer and have a strong influence on the electrofusion yield. The parameters are the conductivity, type of cations, certain additives and finally the osmolality of the buffer and will be discussed below.

Conductivity. DEP and pearl-chain formation usually have to be performed in a virtually nonconductive solution (conductivity less than $10^{-4} \text{ ohm}^{-1}\text{cm}^{-1}$), because the presence of electrolytes creates heating problems, which result in turbulence and disruption of the pearl-chain formation and the fusion process^{23,43}. Furthermore, low conducting

buffer promote the required positive DEP. The use of low conducting buffer has successfully been demonstrated by Steenbakkers et al. for bulk electrofusion of B-cells and myeloma cells.

Cations and additives. Pretreatment of cells with protease and the presence of divalent cations (Ca^{2+} and Mg^{2+}) are factors known to improve the fusion yield^{34,44}. Divalent cations have a stimulating effect on the viability of the electrofused cells. A concentration of around 0.1 mM of Ca^{2+} is optimal for generating hybridomas⁴⁵. A lower or higher concentration of these ions results in a dramatically decreased hybrid yield⁴⁶. This critical level of Ca^{2+} in the fusion buffer presumably originates from the interaction of this divalent cation with intracellular and membrane components after electroporation^{39,46}.

Osmolality. The osmolality of the fusion buffer is a critical parameter in electrofusion. The general opinion is that the fusion yield increases significantly if cells are pretreated briefly in hypotonic media and then returned to isotonic media for fusion or if fusion is carried out in hypotonic media⁴⁷. Extensive hypotonic preswelling of cells improves membrane contact due to smoothing of cell surfaces. Moreover, the dissolution of the cytoskeleton in hypotonically swollen cells and the increased mobility of membrane components, increases electrofusion yields⁴⁸.

In addition, hypotonic swelling enhances both membrane breakdown and cell alignment due the DEP force and induced membrane voltage scale linearly with cell volume (radius³) and radius, respectively. The optimal efficiency of hypotonic electrofusion, depends on several factors. The initial cell swelling can activate various volume-sensitive pathways in the cell membrane that mediate the efflux of cytoplasmatic ions and osmotically driven water loss, to restore normal cell volume. This process can abolish the beneficial effects of hypotonic swelling on electrofusion by restoring the cell size and initiating excessive leakage of cytoplasmatic ions⁴⁹. This process is known as regulatory volume decrease. By adding sorbitol, inositol or sucrose the osmolality of the fusion buffer can be adjusted.

Cell pairing by dielectrophoresis

When subjected to an electric field, dielectric particles such as cells, experience a range of electrokinetic forces, pressures and torques⁵⁰. An external electric field can

induce formation of pores in cell membranes, move cells by DEP and fuse membranes⁵¹. These effects are based on the electrostatic interaction of the induced cell dipole with the applied field.

The dielectric properties of cells are described by the permittivity ϵ and the conductivity σ . The permittivity is defined as the resistance of a material against the formation of an electric field in it. In other words, if a material has a high permittivity a high charge density is needed to create an electric field in that material. A material has a high permittivity when it can internally polarize as a result of the applied field and thereby partially cancel it. The conductivity is a measure for how strongly a given material allows the flow of electric current. In other words, a material has a high conductivity when the current density in response to an applied electrical field is high⁵².

It is found that the internal polarization of a material that causes its permittivity occurs with a certain delay (time response) because it needs reorganization in the material. At different frequencies of the applied field therefore a different permittivity is found.

The frequency-dependent permittivity of a material is called its dispersion behavior. When examining the frequency scale for biological materials, three distinct frequency regimes are observed. The α -dispersion, around a frequency of 10^2 Hz, is caused by the ionic diffusion effects at the outer cell membrane. At the range of $10^4 - 10^7$ Hz, the operation range of electrofusion, interfacial polarization (β -dispersion) is occurring. β -dispersion arises when the numerous intracellular lipid cell membrane structures can be charged and discharged. The γ -dispersion is seen around 10^9 Hz and is caused by dipolar re-orientation of water⁵².

Electric fields exert forces on mobile and surface-bound charges and the charges accumulate at the interface of the dielectrics. The free motion of charge depends on the conductivity (ability of the material to conduct an electrical current to move through the material), while the charge redistribution in a limited space is determined by its permittivity⁷. A cell can thus become polarized due to the restricted motion of ions imposed by the plasma membrane. The induced dipole will be a function of the above-mentioned dielectric properties of the cell and the medium, as well as the frequency of the AC electric field.

DEP is the motion of dielectric particles, in this case mammalian cells, within a non-uniform AC or DC electric field. When an AC field is applied, the cell polarization behavior and the resulting induced dipole will be a function of the AC frequency as described above. The interaction of the induced dipole with a non-uniform electric field generates a DEP force (F_{DEP}), which is proportional to the cell volume (R^3), the field gradient ∇E_0 and the real part of the Clausius-Mossotti (CM) factor, $K(\omega)$. F_{DEP} is given by⁵³⁻⁵⁵:

$$F_{DEP} = 2 \cdot \pi \cdot R^3 \cdot \epsilon_m \cdot \nabla E_0^2 \cdot \text{Re} \cdot [K(\omega)] \quad (3)$$

where ϵ_m is the dielectric permittivity of the medium surrounding the cell, R the cell radius, E_0 the electric field strength, ω the angular frequency of the electric field and $K(\omega)$ the complex CM equation. The Clausius-Mossotti factor describes the relationship between the dielectric constants of the cell and medium^{53,56}. In the case of mammalian cells, this relationship is quite complicated, because the dispersion is a function of the frequency as described above. To account for this, the cell is generally represented by a shell model as depicted in figure 1 (from reference 57).

Figure 1. Schematic representation of the simplification of a cell (left) to a sphere with complex permittivity ϵ_p^* . The equations describing the complex permittivity in this model are given in the text. Figure from Pethig 2010.

From this shell model the Clausius-Mossotti factor is defined as⁵⁷

$$K(\omega) = \frac{\epsilon_p^* - \epsilon_m^*}{\epsilon_p^* + 2 \cdot \epsilon_m^*} \quad (4)$$

where the complex cell permittivity ϵ_p^* equals

$$\epsilon_p^* = \epsilon_{mem}^* \cdot \frac{\gamma^3 + 2 \cdot \left(\frac{\epsilon_{cyt}^* - \epsilon_{mem}^*}{\epsilon_{cyt}^* + 2 \cdot \epsilon_{mem}^*} \right)}{\gamma^3 - \left(\frac{\epsilon_{cyt}^* - \epsilon_{mem}^*}{\epsilon_{cyt}^* + 2 \cdot \epsilon_{mem}^*} \right)} \quad (5)$$

with

$$\gamma = \frac{R}{R-d} \quad (6)$$

Here R is the cell radius and d the membrane thickness. Equation 5 shows that the complex permittivity of the cell (ϵ_p^*) is a function of the complex permittivity of the cell membrane ϵ_{mem}^* ($\epsilon_{mem}^* = \epsilon_{mem} + \frac{\sigma_{mem}}{[j\omega]}$) and the complex permittivity of the cell interior ϵ_{cyt}^* ($\epsilon_{cyt}^* = \epsilon_{cyt} + \frac{\sigma_{cyt}}{[j\omega]}$) where j is $\sqrt{-1}$. The complex permittivity of the medium, $\epsilon_m^* = \epsilon_m + \frac{\sigma_m}{(j\omega)}$.⁷⁴⁻⁷⁶

Concluding the above, the absolute value of the DEP force on a particle depends on ∇E_0^2 , as well as on the real part of $K(\omega)$, $Re[K(\omega)]$, which is the in-phase component of the particle's effective polarizability. (The imaginary part of $K(\omega)$ relates to the electro rotational force⁵⁸.) Examining the expression, the real part of $K(\omega)$ is bounded by limits $-\frac{1}{2} < Re[K(\omega)] < 1$ and varies with the frequency of the applied field and the conductivity of the medium.

Figure 2. A graph of a schematic spectrum of the real part of the Clausius-Mossotti factor for a cell in an electrolyte with different suspending medium conductivities. Adapted from ref.⁵⁸

Figure 2 shows this dependence of the CM factor on the frequency, which is due to the fact that the internal compartments of the cell contribute differently to the polarizability, each with their unique kinetics. At low frequencies ($<100\text{kHz}$), the cell appears insulating and therefore less polarizable than the typical ionic media ($>0.16\text{ S/m}$). Here it will experience negative DEP ($\text{Re}[K(\omega)] < 0$), which means that it aligns against the field and is repelled from regions of high electric field, see figure 3.

Figure 3. A schematic drawing of a polarizable cell subjected to an AC field. The interaction between dipolar charges with the local electric field produce a force, when the cell is polarized. The force is greater at side facing the point than that on side of the plane, because of the inhomogeneous nature of the electric field. Adapted from *Electrofusion of cells on chip* (thesis E.W.M. Kemna)

However, when a very low conductivity media would be used (~ 0.0016 S/m), positive DEP will still occur ($\text{Re}[K(\omega)] > 0$). With positive DEP the cells align towards the region of the highest electric field, see figure 3. At higher frequencies (~ 1 -100 MHz), the field will bridge the membrane and the CM factor will compare the permittivity of the cytoplasm and the media, resulting in negative DEP at higher solution conductivities and positive DEP at low solution conductivities^{50,58-60}. Finally, the application of high frequencies (> 1 GHz) will result in negative DEP, likely due to cytoplasmic proteins that impart a net permittivity lower than the surrounding medium⁶⁴. In typical electrofusion experiments, the frequencies used for DEP are 1-100 MHz and a low conductivity fusion buffer is used, so that positive DEP is generated which favors close cell-cell contact^{30,61,62}.

Cell pairing by chemical methods

As described above, the standard for cell fusion in microfluidic systems is electrofusion, which requires AC and DC generators, for cell pairing and cell fusion respectively. Having to use multiple instruments can be costly and difficult to operate. Therefore, several groups have used a chemical method for cell pairing, and by doing

so only one DC source is required⁶³. This method can be based on the use of various chemicals like lectin-couplet and biotin-streptavidin and has high heterogeneous cell pairing efficiency. Biotin-streptavidin chemical linking is based on the biomolecular interaction between the two molecules^{64,65}. One half of the cell population was treated by streptavidin coating after biotinylation and the other half was labeled with Sulfo-NHS-LC-biotin, resulting in the conjugation mode of cell-biotin–streptavidin–biotin-cell. After mixing and incubation about 50–55% cell population was conjugated by this chemically linked protocol. Moreover, 30% of the sample resulted in one-to-one cell-pairs. The chemical approach thus has the advantages of a high cell pairing efficiency, no cell membrane damage and simplicity of instrumentation.

Unfortunately, this chemical conjugation method of cell pairing also has a few disadvantages. The process requires a pre-modification treatment of each cell sample prior to the cell pairing. Moreover, this chemical pre-treatment leads to potential damage to the cells with consequences for cell viability, due to the modification of the membrane structure of the cell sample. Also, due to the random cell contact the cell pairing between one cell of type 1 and two or more cells of type 2 (or vice versa) cannot be avoided by this method, especially within a mixture of cells with significantly different diameters is used, as is the case in pairing of myeloma and plasma B cells and will lead to low one-to-one cell pairing.^{60,61,66}

Microelectrode-assisted cell pairing by DEP

Microelectrode-assisted cell pairing is based on the earlier explained concept of DEP. In this approach microelectrodes are presented in microchannels, through which a cell suspension is offered. By applying a non-uniform alternating current field between these microelectrodes a positive DEP force is generated. The DEP force makes the cells stick to the electrodes, where the electric field strength is higher (Figure 4 from reference 75). After the first cells are stuck on the electrodes, a second cell suspension is offered in the microchannel causing the formation of pearl chains between the electrodes or simply a single contact pairing with the cells that were already present. Next, one or more DC pulses can be applied to induce electroporation and subsequently membrane fusion. Several groups have made different designs based on this method, with reported high cell trapping percentages and fusion yields.

Nevertheless, the cell pairing is still somewhat random and multiple cells can still be in close contact with one another. Therefore, one-to-one fusion yields remain low⁶⁷⁻⁷⁸.

Figure 4. On the left a schematic drawing of the electrodes on the microfluidic chip. In the middle a clear image of cell pairing on the electrodes (dashed red circles). Moreover, cells also get stuck in between the electrodes (blue dashed circles). On the right a fluorescent image of cell alignment on the microfluidic chip⁷⁵.

Microelectrode-assisted cell pairing by DEP with a separate fusion chamber

Electrofusion of individually selected cells in DEP field chambers was recently shown by Kirschbaum et al.⁷⁹ Here, a microfluidic platform is presented that is capable of single-cell electrofusion and also allows high control over the cells both before and after fusion (Figure 5 from reference 79). In two different channels the parental cells are presented in cell culture medium. With use of a combination of nDEP electrodes the cells can be redirected towards the fusion chamber. Subsequently another channel is used to offer the (non-physiological) electrofusion buffer to the cell fusion chamber. This will enable cell electrofusion as well as limit the time in which the cells are actually in the non-physiological electrofusion buffer. For cell pairing and fusion, dielectrophoresis and AC voltage pulses were employed respectively and the cells are held into place by DEP electrodes. Each cell has been characterized and selected before they were paired, fused and released from the fluidic system for subsequent analysis and cultivation. Fusion yields of more than 30% for individual pairs of mouse myeloma and B cell blasts were obtained. Also the proliferating ability of the hybrid cells 3 days after fusion was confirmed.

Figure 5. On top left a schematic drawing of the total chip design. On the top right a schematic drawing of the functionality of the design. On the bottom a time lapse of two cells being fused in the fusion chamber⁷⁹.

Cell pairing by local field enhancement due to microstructures

As we described above, microelectrodes can be used to create local field gradients to induce a positive DEP force for cell pairing. However, also insulating microstructures can be used to modify the spatial distribution of the electric field, and by doing so produce a DEP force to pair cells with high efficiency (Figure 6 from reference 82)⁸⁰⁻⁸³. In general this method is called iDEP (insulator dielectrophoresis). In these papers microstructures are mostly fabricated out of a dielectric material and located in a microfluidic channel at a specific position causing the electric field to be concentrated at certain positions in the channel. Once a cell suspension is flown through the

microfluidic channel, the positive DEP force at these positions compels the cells to stay fixed at the location with the highest field gradient.

In one example, a microfluidic chip was developed consisting of a pair of parallel electrodes and a fusion chamber, which was divided by an insulated microstructure with a small orifice. The electric field gradient was strongest in the small orifice in the microstructure, locally creating a positive DEP-induced cell trap. After the two different cells were loaded into the microfluidic chip, they could be trapped and fused. Unfortunately, the throughput of this system was clearly a problem⁸¹. For this reason another microfluidic was fabricated out of a glass substrate and contained two opposite electrodes and a fusion chamber. In the middle of the fusion chamber a microstructure with several micro orifices was present. Like the previous design this microfluidic chip has two different inlets to introduce the two different cell suspensions. Once the first cell suspension was loaded into the chip, the chip was slightly tilted to create a flow under hydrostatic pressure to push the cells into the traps. Next the AC field was applied for iDEP trapping and the chip was tilted to the other side to introduce the second cell suspension. Once both cell types were in position, the DC pulses were applied to fuse the cells. Pairing efficiencies can reach up to 95%. Although this chip showed a great pairing improvement compared with the traditional pairing method, the throughput is not very high due to the limitation in the micro-orifice integration, which is unsuitable for massive parallelism⁸⁰. To solve this problem another microfluidic device was developed, which consisted of a polyimide chip with 618 micro orifices, which was placed between indium tin oxide (ITO)-coated glass electrodes, and two PDMS sheet spacers with a circular opening conform the microfluidic device. The cells were loaded similarly to the previous microfluidic chip, except that this time the device needed to be flipped over completely in order to get successful trap loading. A stirring bar is added to the top side of the device to prevent the second cell type from staying attached to the electrode. In addition, this also disrupts possible pearl chain formation and distributes the cells evenly over all the cell traps. This device shows one-to-one pairing efficiencies up to 80%⁸³. Unfortunately, controlling the intensity of the AC field to keep the first cell type in the orifice without damaging the cell by excessive DEP force is very difficult and complex.

Figure 6. In the top left a schematic drawing of the microfluidic chip design. On the bottom left a close-up of the cell trap. On the right a time laps of two cells being fused and capable of leaving the trap⁸².

Another method was reported in which a microfluidic device was employed that uses DEP force to push cells towards micro-pits. The microfluidic device can improve the cell pairing between cytoplasts and the donor cells and automate the nuclear transfer procedure. There is no DEP force inside the micro-pits. The micro-pits have the dimensions of the first cell type, after which the second cell type is pushed directly above it by the DEP force. Subsequently the cells can be fused⁶⁵. The limitation of this device is the fixed size of the micro-pit, which limits cell trapping because cells may vary considerably in size. For example, if the cells are too small compared to the micro-pit, two or more cells of the same cell type might get trapped in the pit reducing the efficiency of one-to-one cell pairing. Moreover, if the cells are too big they will not fit in the micro-pit and the possibility of forming one-to-one pairs decreases drastically. Nevertheless, this method has been used and adapted by several groups, but cells have to be screened on size before being introduced in the device, which can be costly and labor intensive⁸⁴⁻⁸⁶.

Hydrodynamic cell pairing with microstructures

Recently a few studies have been published concerning cell pairing by flow control using microstructures in a microfluidic chip. The microstructures are formed into micro-traps, and cell pairing is achieved by trapping the two different cells in the traps by flow control only. The traps are designed in such a way that if the cells enter from one direction they are being trapped in a smaller trap, after which the flow is reversed and the cells are trapped in a larger trap (Figure 7 from reference 85). Subsequently a second cell population is flushed through the microfluidic chip which is also caught in the larger trap. Resulting in one-to-one pairing efficiencies up to 70%. Moreover, this system is capable of high throughput since there are 6000 traps on the microfluidic device. Also by varying the trap size, the microfluidic chip can be used for several cell types. Additionally, the system is easy to operate⁸⁷.

Figure 7. On the top a schematic drawing of the functionality of the microfluidic chip. In the middle a fluorescent image of actual chip loading. On the bottom a close-up of the chip design⁸⁵. Middle scale bar is 50 μ m and the scale bar at the bottom is 20 μ m.

Another study revealed even higher pairing efficiencies, up to 80%⁸⁸. This design also had traps of different size, although in this microfluidic chip the flow did need to be reversed, due to a clever design where two traps were placed on top each other, a small one on top of a larger one (Figure 8 from reference 88). A suspension containing the smaller plasma B cells was flushed into the chip first and due to the flow became caught in the smaller traps. Then a suspension containing the larger NS-1 cells was flushed through the trap array and the NS-1 got caught in the larger traps. This microfluidic chip design can be used for variety of cell types, by changing the size and possibly the shape of the cell traps and the distance between the traps. Moreover, the throughput of the device is determined by the number of traps on the microfluidic chip. In this chip significant attention must be paid to minimize the frictional force on the cell membrane because of its potential damage to the cells during the cell pairing process.

Figure 8. On the top left a SEM picture of the trap array. On the top right a close-up of a few rows of traps. On the bottom left a fluorescent image of a time-lapse of cells being fused in the trap. On the bottom right a close-up of a single trap⁸⁸.

These studies both have great potential for high efficiency cell pairing followed by electrofusion. Unfortunately, though these platforms are very easy to fabricate the alignment with possible fusion electrodes has proven to be very difficult.

Cell pairing with microdroplets.

Encapsulating cells in microdroplets is also a plausible method to pair cells prior to electrofusion. It has been demonstrated that by passing the droplets containing two different cells over six electrode pairs, it is possible to electrofuse the cells in droplets, with a fusion yield of 5%. The established yield is comparable to the yield of both

commercially available methods^{26,89,90} as well as on-chip methods^{44,79,87,88}. The large advantage of this method is that it is very high throughput, because of the sequential nature of the process and because the droplets can be generated at 1 kHz. However, to compete with existing methods, the electrofusion yield must be further improved. Several realistic opportunities for this follow from an analysis of the process. First, cell-cell contact can be enhanced geometrically (e.g. by placing a serpentine channel structure in front of the DC electric field to enhance cell movement,⁹¹) fluidically (e.g. by implementing a droplet shrinkage module on chip to increase cell-cell contact in a smaller droplet⁹²) or electrically (e.g. by applying an AC electric field to provide a DEP force to improve cell alignment and cell-cell contact^{55,93}). In addition, a 3D configuration of the poration electrodes can be used to generate a homogeneous electric field along pairing axis of the cells, giving higher fusion yield. Figure 9 shows an example from this thesis of this method.

Figure 9. On the left the schematic overview is shown with colored inserts of the different functionalities of the chip as presented in this thesis. In green, cell encapsulation in droplets. In red, droplets containing two cells passing the electrode array, consisting of six electrode pairs, all capable of giving a pulse of certain preset strength for a duration depending on the flow rate. On the left an overview of an electrofusion result. The images show the fluorescent, bright field and overlay images of HL60 cells in a droplet. The cells inside the droplets contain two nuclei with different colors and a rearranged cell membrane. The droplet size is 50 pL. Six pulses of 1.2kV/cm were applied.

Conclusion

In conclusion, the key to high yield cell fusion is heterogeneous cell pairing. In the microfluidic devices discussed in this chapter, cell pairing has been carried out electrically, with or without the aid of microstructures, chemically, hydrodynamically via cell traps, or via droplets. In table 1 an overview of the different methods is presented with for all these methods the positive and negative aspects.

	High throughput	viability	yield	controllability	Easy to operate	Size dependency	Easy to fabricate
DEP electrodes ⁶⁶⁻⁷⁸	-	-	±	-	±	±	+
Fusion chamber ⁷⁹	-	+	+	+	±	±	+
DEP structures ⁸⁰⁻⁸⁶	±	±	±	+	+	±	±
Micro traps ^{87,88}	+	±	+	+	±	±	-
Droplets	+	±	+	±	-	+	±

Table 1. Scores of different methods.

All the discussed methods have been successful, yet all need a different expertise and show different qualities. The devices that make use of DEP force have proven to be very successful. Although they cannot reach high throughput they do show a reasonable efficiency and are easy to fabricate. Also the cells need to be in electrofusion buffer for a longer period of time, which can be harmful for the cells. Moreover, each cell type requires a different DEP force to stay in position or to be moved, which affects the controllability of the system and also might damage the membranes if there is an excessive force present on the cells.

The device from Kirschbaum, which combines microfluidics with the DEP force and a fusion chamber, is also not able to reach high throughput. However, it manages to keep the cells alive for a long time, because the cells only remain briefly in electrofusion (EF) buffer during the fusion process. Moreover, the device has a very high fusion yield, controllability, and an easy fabrication process. Also, cell size is not an issue, and the device can be used for other applications as well. However, operators of this device might need some training, and have to do some research to find the DEP parameters for different cell types.

The devices that make use of DEP force in combination with microstructures (often iDEP), show a high controllability and are easy to operate. Unfortunately, this device is difficult to fabricate. It is also cell size dependent and only one design is capable of reaching high throughput. Moreover, cell viability can be a problem. However, the device has shown reasonable yields.

The hydrodynamic microtrap structure devices on the other hand are high throughput, easy to control and reach high efficiencies. Also, if the device is well designed and operated cells can remain on chip for longer periods of time increasing cell viability and fusion yield. However, the devices are cell size dependent. Moreover, the devices are very hard to fabricate especially considering the alignment between the microstructures and the microelectrodes.

Microfluidic devices that use microdroplets to bring one-to-one cell pairs into close contact with each other are very high throughput, reach reasonable efficiencies, are not cell size dependent and can be used for various applications. However, the fabrication process and the ease to operate require expertise. This also influences the controllability of the device. Moreover, if the device is not operated fast enough, the cells will remain in EF-buffer for a long time which will decrease fusion yields drastically due to cell death⁹⁴.

In this review several microfluidic devices have been discussed and show great potential. In the nearby future a lab on a chip system might overcome all the problems that the currently used systems have. A complete system capable of cell pairing, electrofusion, continuous medium flow and finally hybrid cell selection and separation will not only increase cell viability but also the functional hybridoma production, which on its turn will lead to greater monoclonal antibody production.

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Chapter 3

High-yield cell ordering and deterministic cell-in-droplet encapsulation using Dean flow in a curved microchannelⁱ

In this chapter high-yield (77%) and high-speed (2700 cells/s) single cell droplet encapsulation is described using a Dean-coupled inertial ordering of cells in a simple curved continuous microchannel. By introducing the Dean force, the particles will order to one equilibrium position after a travelling distance of less than 1 cm. We use a planar curved microchannel structure in PDMS to spatially order two types of myeloid leukemic cells (HL60 and K562 cells), enabling deterministic single cell encapsulation in picoliter drops. An efficiency of up to 77% was reached, overcoming the limitations imposed by Poisson statistics for random cell loading, which yields only 37% of drops containing a single cell. Furthermore, we confirm that 90% of the cells remain viable. The simple planar structure and high throughput provided by this passive microfluidic approach makes it attractive for implementation in lab on a chip (LOC) devices for single cell applications using droplet-based platforms.

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Introduction

Droplet-based microfluidic systems have been used for various biotechnology research applications,¹⁻³ such as cell-based assays,⁴ polymerase chain reactions,⁵ proteome analysis⁶ and cell printing technologies,⁷ and have become a standard platform for high-throughput single-cell experimentation and analysis.⁸ Moreover, droplets facilitate high-throughput single cell analysis.⁹ Encapsulation of single cells within picoliter-sized droplets enable quantitative studies of large populations of single cells.^{10,11} The droplets serve as microvessels which entrap the secretion products of the individual cells and prevent mixing with other cells. In addition, the uptake of trace chemicals can be probed due to their depletion within the confined droplet. Using microfluidic devices, monodisperse droplets can be generated, merged and sorted at kilohertz rates, enabling high-throughput single cell screening.¹² However, the platform suffers from one fundamental limitation: the variability in the number of cells per droplet.¹³ Typically, cells are loaded into the droplets by diluting suspensions of the cells and randomly encapsulating them into the droplets. The distribution is thus dictated by Poisson statistics:

$$P(l; k) = \lambda^k \exp(-\lambda) / k! \quad (1)$$

where k is the number of cells in the droplet and λ is the average number of cells per droplet, which is adjusted by controlling the cell density.¹¹ Very low loading densities are required to minimize the number of drops that contain multiple cells. Hence, the majority of the droplets are empty and the efficiency of the single-cell encapsulation is drastically decreased.^{12,14} This inefficiency has stimulated the development of new methods to increase single-cell encapsulation efficiency, such as using a laser,¹⁵ hydrodynamic focusing and sorting¹⁶ as well as on demand impedance-based cell encapsulation.¹⁷ However, these approaches suffer from disadvantages, such as reduced throughput,¹⁵⁻¹⁷ the use of sophisticated optical equipment or extensive input from the user.¹⁵ Recently, Edd et al.¹⁰ demonstrated efficient single cell encapsulation by ordering the cells using inertial migration in a 6 cm straight channel. However, this approach suffers from a lack of robustness and a difficulty towards implementation in microfluidic LOC devices. We overcome these drawbacks with a fast, easy and novel

approach to deterministically encapsulate single cells within droplets using inertial ordering in a curved microchannel. The curvature introduces a second force, the Dean force, which causes the particles to focus into a single equilibrium position. The device shows a wider dynamic range with regards to the particle size and number of equilibrium positions. Moreover, it possesses a small footprint, making it easy to implement in microfluidic LOC devices. Curved microchannels also show faster cell ordering than straight channels.¹⁸ Inertial ordering of particles in curved microchannels has been used in sorting and filtration applications^{19–22} but not for single-cell encapsulation. The microfluidic chip consists of a curved microchannel which combines inertial ordering with Dean forces to evenly space the cells. By matching the periodicity of the cell flow with the droplet generation, we increase the efficiency of single-cell encapsulation. This deterministic encapsulation method reduces the number of empty and multi-cell droplets while increasing the number of droplets which contain a single cell.

Theoretical background

Fluid flowing through a straight rectangular microchannel experiences a primary Poiseuille flow which has a parabolic velocity profile. This parabolic velocity results in a fluidic shear gradient-induced inertial lift force (F_{IL}). This causes the particles in the flow to move away from the centerline. However, as the particles move towards the walls of the microchannel, a wall induced inertial lift force (F_{WL}) pushes the particles away from the wall, due to the asymmetric wake induced around the particles. This causes F_{WL} to increase as the particles approach the wall. As F_{IL} and F_{WL} are opposing lift forces, which vary in magnitude across the cross-section of the microchannel, the particles are forced to occupy positions where the two forces cancel. Hence, in a straight rectangular microchannel, there are up to four equilibrium positions present,^{23–25} depending on the channel dimensions and volume fraction.^{10,26} In a continuous curved rectangular microchannel, there is a secondary flow due to the difference between the downstream velocity of the fluid in the center and that near the wall. Thus, fluid elements have a larger inertia in the center of the microchannel than near the walls. This produces a pressure gradient in the radial direction of the microchannel, as the fluid tends to flow outward around the curve, leading to the

formation of two counter rotating vortices at the top and bottom halves of the microchannel. The magnitude of these secondary flows is quantified by the Dean number.^{19,22}

$$De = Re \cdot \sqrt{\frac{D_h}{2 \cdot R}} \quad (2)$$

in which R is the radius of the curvature (m) of the path of the channel, D_h is $2 \times w \times h / (w + h)$, in which w (m) and h (m) are the width and height of the channel, and Re is the flow Reynolds number, $Re = U_f \cdot D_h / (\mu/\rho)$. U_f is the flow velocity of the fluid (m/s), μ is the viscosity of the fluid (kg/ (m s) and ρ is the density (kg/m³). The secondary flows, called Dean flows (U_D , m/s), are a flow with a range of lateral velocities which are dependent on the position in the cross-section of the channel. The average velocity can be calculated by an expression formulated by Ookawara et al.²⁷ at a given De number^{28,29}

$$U_D = 1.8 \cdot 10^{-4} \cdot De^{1.63} \quad (3)$$

The Dean flows are capable of applying a drag force on the particles present in the fluid. The maximum value of this drag force (F_D) can be estimated by Stokes drag:^{27,28}

$$F_D = 3 \cdot \pi \cdot \mu \cdot U_D \cdot a_p = 5.4 \cdot 10^{-4} \cdot \pi \cdot \mu \cdot De^{1.63} \cdot a_p \quad (4)$$

in which μ is the fluid viscosity (kg / (m s)), and a_p is the particle diameter (m). The radius of the curvature of the microchannel must be minimized to create a large Dean number, and hence a large Dean force, thereby causing the particles to equilibrate faster. Depending on the particle size, F_D constrains them to follow the path of the Dean flows, causing them to circulate either at the top or bottom half of the channel. Moreover, in addition to the F_D , another force is present in the channel acting on the particles. This force is the net lift force (F_L), which is a combination of the shear-gradient-induced inertial lift force (F_{IL}), and the wall-induced lift force (F_{WL}).^{19,22,28}

$$F_L = \frac{2 \cdot \rho \cdot U_f^2 \cdot C_L \cdot a_p^4}{D_h^2} \quad (5)$$

in which ρ is the fluid density (kg/m^3) and C_L is the lift coefficient, which is a function of the particle position across the cross-section of the microchannel and has an average value of 0.5.^{25,28} Fig. 1 schematically illustrates the Dean flows, F_D and F_L as well as their directions at the particles. The balance between F_L and F_D determines the preferred location of the particles in the microchannels with a curved geometry. The additional force, F_D , does not create particle ordering, but instead acts in a different direction than F_L , reducing the number of equilibrium positions. Depending on the relative magnitude of F_D and F_L acting on a particle, focusing (dominant lift) or mixing (dominant Dean flow) can occur.²² The particles will circulate in the Dean flows until they have all reached an equilibrium position. Additionally, in a curved microchannel, the two stable positions on the sides of the microchannel are biased, which reduces the number of particles collected at the top and bottom focusing points, and two focused lines of particles are observed.²⁴ However, preferential focusing of particles can only be achieved when the particle and channel dimensions meet the following criteria. The a_p/D_h ratio must be >0.07 .^{25,28,30} In addition, F_L dominates the particle behavior only when the particles Reynolds number, $Re_p = Re \cdot a_p^2/D_h^2$, is in the order of 1.²⁴ The channel length (L_D , m), required for all the particles to travel to an equilibrium position on the inside of the microchannel wall is given by Di Carlo et al.:²³

$$L_D = \frac{\pi \cdot \mu \cdot H^2}{\rho \cdot U_f \cdot a_p^2 \cdot L_C} \quad (6)$$

Figure 1. Schematic illustration of a continuous curved rectangular microchannel in which both the Dean force (F_D) and lift force (F_L) are present as well as the directions of their applied force on the particles, resulting in a single equilibrium position at the inner wall of the microchannel, where both forces operate in opposite directions.

in which H is the channel width in the direction of particle migration (m) and L_c is the matching lift coefficient, which is 0.05 for this formula. The length requirements should follow eqn. (6), with the accompanied restriction that $R_f = a_p^2 \cdot R/x^3 > 0.04$, (x is the smallest dimension of the channel), which is fulfilled with regards to our device.

Particle spacing

As stated, in a straight channel the number of equilibrium positions for the particles can be determined by adjusting the channel dimensions and the volume fraction of particles in the solution:²⁶

$$\frac{m \cdot a_p}{l} = \frac{6 \cdot \phi \cdot w \cdot h}{\pi \cdot a_p^2} \quad (7)$$

where m is the number of equilibrium positions present in the channel, a_p is the particle diameter (m), l is the longitudinal distance between two particles (m), ϕ is the volume fraction of particles in the solution, w is the width of the channel (m), and h is the height of the channel (m). The longitudinal distance, l , between the particles on

the same side of the channel does not depend on the volume fraction. By adjusting the flow rate of the fluid, the channel dimensions, the volume fraction and the particle diameter, it is possible to align the particles at a single equilibrium position, all with similar longitudinal distances. This enables the encapsulation of single particles within droplets in a straight channel. Moreover, it is likely that a similar relationship between Re_p and l pertains to a continuous curved rectangular microchannel. Similarly, there should be a relationship between the volume fraction and the number of equilibrium positions.

Materials and Methods

Microfluidic chip design and fabrication

The microfluidic chip consists of a 5-loop curved microchannel, with one inlet for the cell suspension and one inlet for the oil. The curved microchannel is 50 μm wide and 29 μm high, with 100 μm spacing between the two successive loops. The initial radius of the spiral is 1500 μm and the total length is 7.2 cm. Three different types of droplet generators were tested, referred to as type A–C. A silicon master design was drawn in Clewin (version 4.0.1) and fabricated using standard UV-lithography. SU-8 2–25 (Microchem, Berlin, Germany) was spun on the silicon master with a thickness of 29 μm . The chip was made in PDMS (Sylgard 184, Dow Corning, Midland, MI, USA). The curing and base agent were mixed at a ratio of 1 : 10 and degassed. PDMS was poured onto a silicon wafer, degassed, and cured at 60 °C for 24 h. After curing, in- and outlets were punched using a dispensing tip (Nordson EFD, Maastricht, the Netherlands, ID 1.36 mm and OD 1.65 mm). Subsequently, the PDMS was sealed to a microscope slide (VWR, Leuven, Belgium) using an oxygen plasma (Harrick PDC-001, NY, USA). After sealing, the chip was heated at 60 °C for a minimum of 30 min. Before use, Aquapel (Vulcavite, the Netherlands) was introduced into the channels to ensure hydrophobic channel walls.

Materials

Two types of myeloid leukemic cells were used: HL60 (human promyelocytic leukemic cells) and K562 (human erythromyeloblastoid leukemic cells). Both cell types were grown in an RPMI medium (Invitrogen, Grand Island, NY, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), 100 U ml^{-1} penicillin (Invitrogen), 100

$\mu\text{g ml}^{-1}$ streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen) (= RPMI+ medium). Cell cultures were sustained in a 5% CO₂ humidified atmosphere at 37 °C. Cell cultures were split every 3–4 days at a ratio of 1 : 10. In the experiments, the cells were suspended at the desired volume fraction, ranging from 1 to 2.2%, in an RPMI+ medium prior to use. Polystyrene beads (Polysciences Inc, Germany) with a diameter of 10 μm were used for the control experiments. For the continuous phase, FC-40 (3M, St. Paul, MN, USA) was used with a 1% (w/w) oil-phase surfactant.

Experimental set-up

Cell suspensions and oil were separately introduced into the microfluidic chip using two 3 ml plastic syringes (BD, Breda, the Netherlands), and were connected to the two inlets with PEEK tubing (Vici, Schenk, Switzerland, ID 0.5 mm and OD 1.59 mm). PEEK tubing was also connected to the outlet of the device and routed into a collection tube. The flow was driven at a constant volume rate by a syringe pump (neMESYS dosing units, Cetoni GmbH, Germany). A magnetic stirrer bar was inserted into the cell suspension containing the syringe to prevent the suspension from settling during the injection. The flow rate of the continuous phase was 30–43 $\mu\text{L min}^{-1}$ and the aqueous flow rate, containing the cells, was set to 10–20 $\mu\text{L min}^{-1}$. The microfluidic chip was mounted onto the X–Y–Z translation stage of an inverted wide fluorescence microscope (Leica DM IRM, Leica Microsystems, Wetzlar, GmbH, Germany). In addition, a computer-controlled high-speed camera (Photron SA-3, West Wycombe, United Kingdom) was mounted onto the microscope for image recording, using the accompanied Photron software (Photron Fastcam Viewer). Illumination was supplied by a fiber optic illuminator (Leica KL 1500 LCD).

Image acquisition and analysis

Videos and images of the cell ordering in the curved microchannel and subsequent cell encapsulation were captured using the high-speed camera, at a frame rate of 3000–18181 frames per second and a shutter time of 5–30 μs . All the videos and images captured were analyzed with the image-processing program ImageJ (National Institute of Health, MD, USA). To determine the encapsulation efficiency, the number of droplets containing 0, 1, or more cells was divided by the total number of droplets generated.

Results and discussion

First, we characterized the relevant forces present in the microfluidic chip which cause the cell ordering.

Characterization of the microfluidic chip

The microfluidic chip consists of 5 loops with a total length of 7.2 cm, followed by an encapsulation part (Fig. 2). Using eqn. (2)–(5), the microfluidic chip was characterized for its cell ordering capacity.

Figure 2. Schematic drawing of the microfluidic chip consisting of a curved microchannel followed by an encapsulation part. The pictures are of the cell ordering and subsequent encapsulation. Scale bars are 50 μm .

Table 1 shows how the flow rate (Q_f) and the channel dimensions influence the Reynolds number (Re), the Reynolds particle number (Re_p), the Dean number (De), and the apparent Dean (F_D) and lift forces (F_L) using $13 \pm 1.6 \mu\text{m}$ HL60 cells ($n = 25$) or $13.8 \pm 2.2 \mu\text{m}$ K562 cells ($n = 25$). The Dean forces acting on the $13 \mu\text{m}$ cells at the three flow rates are less than the corresponding lift forces. Thus, the lift forces dominate, which predicts that ordering will occur and the cells will focus along the one equilibrium position at the inner wall, as illustrated in Fig. 1.

width · height	50 · 29 (μm)		
Q_f ($\mu\text{L} \cdot \text{min}^{-1}$)	10	15	20
Re	4.2	6.3	8.4
Re_p	0.53	0.79	1.06
De	0.46	0.7	0.93
F_L (N)	$5.6 \cdot 10^{-10}$	$1.3 \cdot 10^{-9}$	$2.2 \cdot 10^{-9}$
F_D (N)	$6.4 \cdot 10^{-12}$	$1.2 \cdot 10^{-11}$	$2 \cdot 10^{-11}$

Table 1. Summary of the Dean force (FD), lift force (FL) and Rep acting on 13 mm cells for varying Dean numbers

Cell ordering

Cell ordering was observed in the continuous curved microchannel, when the flow rates approached $15 \mu\text{L min}^{-1}$ or higher. The observed cell ordering is classified as two types of self-organization. Firstly, the cells were focused in one streamline along the inner wall (Fig. 2). Secondly, the cells were arranged in an array that alternated not in the z-direction¹⁸ but instead in the x-y direction (Fig. 3). These self-organization patterns shared three distinctive characteristics for cell ordering in straight microchannels:^{10,18} (1) the cells moved as a group in the direction of the flow, (2) the cells were separated from their nearest neighbor by a uniform spacing and (3) the cells were always found near a channel wall. Cell ordering was observed in the second loop in the direction of the flow (longitudinal) after $9400 \mu\text{m}$, corresponding to 67 ms, when flowed at a rate of $15 \mu\text{L min}^{-1}$, as shown in Fig. 2. Thus, curved microchannels cause faster ordering towards the predicted equilibrium position than straight

microchannels.¹⁸ The observed channel length required for ordering (~ 0.9 cm) is in the order of the calculated length (~ 0.6 cm) using eqn. (6). The shorter channel length required for ordering, offers a critical advantage because fluidic resistance decreases and hence, decreases the pressure and power required to drive the flow. In fact, it seems that fluidic resistance is the limiting factor for increasing the throughput of ordering because high pressure leads to leakage at the fluid inlets.¹⁸ Therefore, our system is more robust for single cell encapsulation when compared to straight channels devices. Additionally, channel clogging never occurred in the required first cm of the device, making this system more robust for particle ordering when compared to the necessary several cms in a straight channel. The longitudinal distance, between adjacent cells on the same side of the channel (Fig. 3, insert L1), at flow rates of $15 \mu\text{L min}^{-1}$ was $52 \pm 6 \mu\text{m}$. This flow rate corresponded to velocities of $14 \pm 0.4 \text{ cm s}^{-1}$. Furthermore, Fig. 4 shows that when the flow rate increased from 15 to $50 \mu\text{L min}^{-1}$ (Rep = 0.8–2.7), the longitudinal distance was not significantly influenced. However, this is mainly due to the large standard deviations in the longitudinal distance between the cells. The longitudinal distance for the cells varied from 39 to $52 \mu\text{m}$, corresponding to ~ 4 times the cell diameter. This observation has not been previously reported for a continuously symmetrically curved microchannel.²⁴ For comparison, we also determined the longitudinal distance (L1) of $10 \mu\text{m}$ beads for different flow rates. The average longitudinal distance between the beads was lower than that between adjacent cells, as shown in Fig. 4.

Figure 3. Double equilibrium position of the HL60 cells observed in the focal plane, in the second loop of the curved microchannel. Insert shows the longitudinal distance measurement, L1, of the cells at the single equilibrium position at the inner wall and L2 for the longitudinal distance of the cells in the alternating pattern. Scale bar is $50 \mu\text{m}$.

However this decrease is not significant. Moreover, the standard deviation of the distance between the beads was lower than that between the cells. One possible explanation is that the coefficient of variation (CV) for both HL60 and K562 cells is higher ($n = 25$, CV 13% and 16%) compared to that of the beads ($n = 25$, CV 10%). In addition, cell ordering into an alternating pattern across the channel was observed in the focal plane of the second loop at $15 \mu\text{L min}^{-1}$. Hence, the longitudinal distance between the particles was reduced to $28.3 \pm 3.3 \mu\text{m}$ (Fig. 3, insert L2). Here, the velocity is similar to that used to obtain the one-equilibrium position. The microfluidic device was capable of ordering particles with dimensions down to $6 \mu\text{m}$, demonstrating the dynamic range, which has not been seen in straight channels.^{10,18} Moreover, no difference in the ordering capability between the two different cell types was observed. One of the key factors causing the double-equilibrium position in the continuous curved microchannel was the volume fraction of the suspended cells. When the volume fraction reached 2.2%, an increasing number of double-equilibrium positions was observed, suggesting a similar relationship for curved microchannels as well as straight microchannels, as described in eqn. (7). In conclusion, the volume fraction provides increased control with regards to a single or double equilibrium position, in contrast to straight channels where there is always a minimum of two equilibrium positions. Furthermore, the longitudinal spacing (L1) between the particles remains unaffected when changing the volume fraction between 1.7–2.2%. Additionally, due to particle interaction, increasing the volume fraction above 2.2% will decrease the distance between the particles. However, no ordering occurs at that point. Moreover, increasing the flow rate from 15 to $50 \mu\text{L min}^{-1}$ did not influence the double equilibrium position. Finally, it has been suggested by Di Carlo et al.^{24,25} that an inertial force ratio, $R_f = a_p^2 * R / x^3$, is useful for predicting particle behavior. Here, R_f describes the order of magnitude scaling between FL and FD. It was proposed that the equilibrium positions can be modified by the secondary flow at an intermediate $R_f > 0.04$ ($R_f = 9.6$). This could lead to interesting new ordering modes, as shown here, for a continuous curved microchannel. However, we still lack a complete explanation as to why this geometry, in combination with specific volume fractions of cells, caused the single equilibrium position of the cells in continuous curved microchannels to shift to two equilibrium positions.

Figure 4. Longitudinal distance between cells (HL60) at different flow rates and 10 μm beads (error bars represents 1 SD) ($n = 25$).

Overall, the single equilibrium position is most stable and prominent during the experiments when using the appropriate volume fraction of cells (1.7%) and flow rate ($15 \mu\text{L min}^{-1}$).

Cell encapsulation

The curved microchannel presented here generates a cell train with a precise longitudinal spacing. This is critical for controlling the loading of single cells into the droplets. To demonstrate this, we analyzed the use of three different flow-focusing geometries (Table 2) for emulsifying concentrated suspensions of HL60 or K562 cells immediately after they traversed the curved microchannel. The longitudinal distance and the corresponding velocity at one equilibrium position resulted in a cell frequency of $2700 \text{ cells s}^{-1}$. Type A, a regular junction, showed no droplet formation at the flow rates of interest. The second design, type B, generated droplets at 18 kHz or higher, which was too high compared to the cell frequency ($2700 \text{ cells s}^{-1}$). Type C generated 73–99 pL droplets between 2.1–2.75 kHz. Hence, encapsulation experiments were performed with this type. To ensure that the droplets contained only a single cell, we

adjusted the oil flow to generate droplets with a frequency comparable to, or higher than, the frequency of the cells. Thus, the oil flow rate was set at $43 \mu\text{L min}^{-1}$. This generated 73 pL droplets at 2.7 kHz. We compared the cell encapsulation efficiency with that determined by a Poisson distribution in Fig. 5. The percentage of droplets containing single cells is significantly increased using the curved microchannel for cell ordering. The efficiency of encapsulating single cells approaches 80% and the percentage of droplets containing multiple cells or no cells remains very low. A Poisson distribution yields a similar percentage of droplets containing multiple cells but far more empty droplets. The efficiency of producing droplets with single cells using a Poisson distribution is around 30%.

	Frequency (kHz)	Volume (pL)
No droplet formation	x
>18	<24
2.1-2.75	73-99

Table 2. Overview of the droplet generator type A–C and the corresponding values of the droplet frequency (kHz) and volume (pL) observed for the flow rate range of interest (cells $15 \mu\text{L min}^{-1}$; oil $30\text{--}43 \mu\text{L min}^{-1}$). Scale bars are $50 \mu\text{m}$

Figure 5. Deterministic cell encapsulation showing cell ordering vs. Poisson distributed cell encapsulation

When using the curved microchannel this efficiency is almost three times higher. The cell volume fraction is an important factor for controlling the efficiency of single-cell encapsulation. Long empty spaces between the cells are undesirable, requiring values greater than 1.5% in this device. By contrast, when the volume fraction is greater than 2%, cell ordering is disturbed due to cell–cell interactions and double equilibrium positions. This can be compensated to some extent by adjusting the droplet frequency. The experimentally determined volume fraction corresponds to $\lambda = 1.1$, which may explain the percentage of droplets containing two cells. However, decreasing λ results in an increased percentage of empty droplets. Finally, the viability of the encapsulated cells was examined. The cells were stained with calcein AM and propidium iodide to determine cell viability and membrane integrity after encapsulation. The results showed that 92% of the cells retained their membrane integrity, compared with 97% in the control samples (data not shown). The device presented here is able to provide

up to 80% of the droplets containing only a single cell. Furthermore, when using the curved microchannel the ratio between the droplets containing one and two cells is 8 : 1. To establish this ratio with a Poisson distribution, the cell suspension would have to be diluted to a λ of 0.25, which would lead to less than 20% of the droplets containing single cells, a decrease of nearly a factor of four. However, due to the large standard deviation in the longitudinal spacing between the cells, it is not possible to obtain a 100% encapsulation efficiency of single cells using this approach. Future work will focus on real deterministic single cell encapsulation within droplets, such as coupling this device to an integrated impedance analyser, which might result in 100% efficiency.

Concluding remarks

In this chapter, we discuss a microfluidic device with a curved microchannel which is capable of cell ordering based on Dean forces. Calculations were performed to characterize and understand the forces influencing the cell ordering in the curved microchannel. Cell ordering was studied experimentally and two equilibrium positions were observed after travelling the curved microchannel for less than 1 cm. Subsequently, single cell encapsulation was successfully demonstrated by achieving a single cell encapsulation efficiency approaching 80%, whilst retaining a low volume of multiple and empty droplets. Furthermore, cell viability was maintained. This microfluidic chip can perform efficient encapsulation of single cells on a relatively small device footprint ($\sim 0.4 \text{ cm}^2$), which offers easy integration into droplet-based microfluidic LOCs having low sample availability. Moreover, the short ordering distance decreases the fluidic resistance and thus lowers the required pressure and power to drive the flow, which minimizes costs. Additionally, the microfluidic design is versatile and capable of ordering particles over a wide dynamic range (6–13 μm). This device is particularly suitable for use with small sample volumes. Thus, it can contribute to a variety of biomedical or manufacturing applications that involve rapid, low-cost, continuous encapsulation of single cells.

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Chapter 4

High-throughput deterministic single cell encapsulation and droplet pairing, fusion and shrinkage in a single microfluidic deviceⁱ

In this chapter, we present a microfluidic device capable of successive high-yield single cell encapsulation in droplets, with additional droplet pairing, fusion and shrinkage. Deterministic single cell encapsulation is realized using Dean-coupled inertial ordering of cells in a Yin-Yang shaped curved microchannel using a double T-junction, with a frequency over 2000 Hz, followed by controlled droplet pairing with a 100% success rate. Subsequently, droplet fusion is realized using electrical actuation resulting in electrocoalescence of two droplets, each containing a single HL60 cell, with 95% efficiency. Finally, volume reduction of the fused droplet up to 75% is achieved by a triple pitchfork structure. This droplet volume reduction is necessary to obtain close cell-cell membrane contact necessary for final cell-electrofusion, leading to hybridoma formation, which is the ultimate aim of this research and which will be reported in the following chapters.

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Introduction

Recently droplet microfluidics has evolved as a powerful platform for a large variety of applications^{[1] [2] [3]} Amongst these are cell-based assays,^[4] polymerase chain reactions^[5] and proteome analysis.^[6] In particular, droplet microfluidics has become a standard high-throughput platform for single cell experimentation and analysis.^[7] Monodispersed picoliter-sized droplets can be generated, merged and sorted at kilohertz rates. Combining this with single cell encapsulation offers the possibility for quantitative studies of large populations of cells.^{[8] [9] [10]} Here, we present an integrated microfluidic device that combines a variety of functions, with the ultimate aim to realize a system for high-throughput hybridoma formation. Such hybridomas, defined as fusion products of myeloma cells and B-cells, potentially have the capability to proliferate and produce specific antibodies. In order to generate hybridomas in a high-throughput fashion and with high-efficiency, a microfluidic system with several properties is needed. The microfluidic system should contain high-speed single cell droplet encapsulation, droplet pairing, droplet electrofusion and droplet shrinkage, followed by cell-cell in droplet electrofusion and hybridoma selection. Apart from these last two functions, we describe here all functions involved.

In the previous chapter, we showed deterministic single cell encapsulation in a rectangular curved microchannel with a yield up to 77%.^[11] In this chapter, we present similar encapsulation yields, by using a Yin-Yang shaped alternating curved rectangular microchannel. The Yin-Yang shape acts as an elongated funnel. The slow decrease in the microchannel width not only prevents the cells from clogging the microchannel, it also contributes to the homogeneity of the cell suspension and stable equilibrium position. Moreover, instead of using a full spiral as in our previous design, we now use alternating curves for faster cell alignment at lower flow rates. This enables us to reduce the footprint of our platform, and moreover to put more functions onto our platform, due to a reduction of pressure buildup in the microchannel. Figure 1 shows a schematic overview of this platform, in our case, to perform cell fusion in a droplet. However, this platform is not constricted to cell fusion and is applicable for various other cell-based assays such as single cell analysis and differentiation assays.^[3]

Figure 1. Schematic representation of the microfluidic design, in which the Yin-Yang shaped alternating curved microchannels are shown. A. Overview of the double T-junction with single cell encapsulation in picoliter droplets and automatic droplet pairing. B. Electrocoalescence of the paired droplets, due to the electric field produced by the electrode pair on either side of the microchannel. C. Droplet shrinkage caused by one to three pitchforks structures in a row, of which one is shown. D. Cell fusion due to an electrode pair across the microchannel.

In the center of figure 1, the design of the entire platform with all its components is shown. Clearly visible is the previously mentioned Yin-Yang shaped and alternatingly curved microchannel, followed by a double T-junction, which is responsible for the single cell encapsulation, and droplet pairing, represented schematically in figure 1A. This double T-junction is based on previous work of Frenz et al.^{[12] [13]}. The double T-junction consists of three microchannels and two hydrodynamically coupled nozzles (figure 2). Once a droplet is formed in the first nozzle (N1), the discontinuous phase in microchannel 1 blocks the continuous phase from the central microchannel (microchannel 2).

Figure 2. Schematic and real image of the double T-junction. The numbers 1, 2 and 3 represent the different inlets, 1 and 3 function as cell inlets and 2 functions as the oil inlet. N1 and N2 are the two nozzles at which the droplets are formed.

At the same time, the continuous phase is flowing through the second nozzle (N2), leading to a pressure buildup of the discontinuous phase in microchannel 3, which causes droplet formation at N2. The continuous phase flows through the first nozzle,

once the droplet in the first nozzle is released, which also applies for the second nozzle. Hence, the alternating flow of the continuous phase enables droplet pairing. Next, the paired droplets pass a pair of sunken platinum on-chip electrodes, placed diagonally on either side of the channel, to initiate the electrocoalescence of droplets (figure 1B). If the droplets are in close proximity of each other, the induced dipoles of both droplets align, which leads to an attractive Coulomb force between the two droplets causing them to coalesce. Electrocoalescence was first demonstrated by Chabert et al.^[14] and has found multiple applications in microfluidics over the past years^{[13] [15]}.

Subsequently, the droplets pass three pitchfork structures that are placed perpendicular to the microchannel (figure 1C). It is shown that T-junctions^[16] or side channels^[17] can be used to split a droplet. In our device, the side channels have a pitchfork structure, with the same length as the remainder of the microchannel and half the width of the main microchannel. As a consequence, part of the droplet is split causing the volume of the droplet to reduce. One key element in our device, which differs from general splitting geometries, is the placement of small pillars at the inlet of the pitchfork in order to prevent cells from entering the pitchfork. Generally, the pitchfork structure can either reduce or increase the volume of the fused droplet with cells. Increasing the volume can be achieved by applying a liquid flow, hence adding to the volume of the droplet. For electrofusion of cells in droplets, the cells need to be in close contact. Therefore, the droplet volume ought to be reduced. Figure 1D represents the final step and our application of the platform, namely cell fusion. In a continuously curved microchannel, several forces act on the cells, compelling them to find equilibrium positions.^{[18] [19]} Moreover, in a continuously curved microchannel an additional force, the Dean force (F_D), obligates the cells to move to a single equilibrium position on the inside of the curve.^[20] The magnitude of the F_D is determined by the dimensionless Dean number (De), the viscosity of the fluid (μ) and the particle size (a_p).^[21]

$$F_D = 5.4 \times 10^{-4} \cdot \pi \cdot \mu \cdot De^{1.63} \cdot a_p \quad (1)$$

According to equation 2, the Dean number is determined by the dimensionless Reynolds number (Re), the channel dimensions (D_h) and the radius of the curve (R).^[22]

$$De = Re \cdot \sqrt{\frac{D_h}{2 \cdot R}} \quad (2)$$

By reducing the radius of the curve, the F_D will increase. Additionally, by alternating small curves with larger curves, a drastic increase of the F_D in the small curves is achieved, compelling cells to reach their single equilibrium position faster and at lower flow rates.

The similar stream-wise distance induced by the Stokes flow,^[23] causes the cells to pass the double T-junction at a fixed frequency.^[24] By adjusting the flow rate of the continuous phase in channel 2, the droplet generation frequency is matched to the cell passing frequency, enabling the cells to be deterministically encapsulated.^[25]

The ultimate goal of our platform is to achieve cell fusion in droplets, schematically represented in figure 1D, especially the fusion of a B-cell with a myeloma cell. The fusion of these two cell types results in the formation hybridomas, which allow us to attain an unlimited antibody production.^[26] However, this platform can be used for several other purposes, since it is possible to combine different cell types but also cells with media of various substances to perform single cell experiments. Moreover, as mentioned above the pitchfork structures are capable of reducing droplet volume, but also able to add to the droplet volume. With this in mind, one can imagine that it would be possible to add drugs to a droplet for drug-screening and cell differentiation, making this platform ideal for multiple single cell experiments in a high-throughput fashion.

Materials and methods

Chip design

The microfluidic chip consists of an elongated funnel, that continues into one full loop, followed by three semi loops (figure 1 center). The dimensions of the microchannel loop are 27.1 μm high by 50 μm wide, and are spaced 100 μm apart from each other. The initial radius of the loops is 1600 μm and increases over microchannel length up to 2000 μm . The total microchannel length towards the double T-junction is 2.5 cm. After

the double T-junction, the microchannel remains 27.1 μm deep, however the width increases to 120 μm . Platinum electrodes are placed diagonally on either side of the microchannel, 1000 μm after the double T-junction, with a distance of 50 to 100 μm to the microchannel. After droplet fusion, the microchannel width slowly decreases from 120 μm to 25 μm . At this narrow section of the microchannel, the droplets encounter three perpendicular 25 μm wide microchannels. These microchannels together form the pitchfork structure that is used for droplet shrinkage. In order to prevent cells inside the droplet to be removed from the droplet during this shrinkage, little pillars of 10 μm width are placed in the center of each of the three microchannels. Directly after the first shrinkage module, the microchannel width increases to 50 μm where the second shrinkage module is placed, only to be slowly downsized to 25 μm for the third shrinkage module. Next, the microchannel dimensions will remain 27.1 μm deep but the width increases to 50 μm .

Chip fabrication

The silicon master was fabricated using standard UV-lithography, after the design was drawn in Clewin (version 4.0.1). SU-8 (Microchem, Berlin, Germany) was spun on the silicon master with a thickness of 27.1 μm . The chip was made in PDMS (Sylgard 184, Dow Corning, Midland, MI, USA). The curing and base agent were mixed at a ratio of 1:10 and degassed afterwards. PDMS was poured onto the silicon wafer, degassed, and cured at 60°C for at least 6 hours. After curing, the in- and outlets were punctured, using a dispensing tip (Nordson EFD, Maastricht, the Netherlands, ID 1.36 μm and OD 1.65 μm).

The 120 μm sunken platinum electrodes were sputtered on a glass wafer using standard sputtering technique. The glass wafer was diced into individual chips. The glass chip was bonded with UV-glue onto a PCB. The electrode pads on both the PCB and the glass chip were connected via a double wirebond, which was then sealed off with a drop of two component glue (Heisel). Onto the PCB, a plug was soldered, to make the connection with the pulse generator (Agilent 33250A, Amstelveen, The Netherlands) and amplifier (Trek model 2210, Medina NY, USA). An AC signal of 1 to 3 kHz and 2 to 4 V_{pp} , was generated by the pulse generator. This generated signal was fed to the amplifier, giving rise to a 100 times amplified signal.

The microfluidic PDMS chip was sealed to the glass/PCB electrode chip with the use of oxygen plasma (Harrick PDC-001, NY, USA). The chip was heated at 60°C for a minimum of 30 minutes after sealing. To ensure hydrophobic microchannel walls, Aquapel (Vulcavite, the Netherlands) was introduced before use.

Materials

HL60 (human promyelocytic leukemic) cells were grown in RPMI medium (Invitrogen, Grand Island, NY, USA), supplemented with 10% (v/v) fetal bovine serum (FBS; Invitrogen), 100 IU·ml⁻¹ penicillin (Invitrogen), 100 µg·ml⁻¹ streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen) (=RPMI⁺ medium). Cell cultures were sustained in a 5% CO₂ humidified atmosphere at 37°C. Cell cultures were split every 3–4 days at a ratio of 1:10. Prior to the experiments, the cells were counted, to establish the correct volume fraction of 0.8 to 2.2% in RPMI⁺ medium with or without a blue dye (Coomassie[®], SimplyBlue[™] SafeStain, Life technologies[™]). The blue dye was used to distinguish the two droplets formed at the double T-junction, and also to visualize the mixing of the two liquids after droplet coalescence. These cell containing solutions were used as the discontinuous phase. For the continuous phase, HFE 7500 (3M[™] NOVEC[™]) was used with 1% (w/w) oil-phase surfactant (kind donation of Sperling).

Experimental set-up

The HL60 cell suspensions (with or without the blue dye) were separately introduced into the microfluidic chip using two 1 mL glass syringes (Hamilton, Bonaduz, Switzerland). The oil suspension was introduced into the microfluidic chip using a 5 mL glass syringe (Hamilton). All three syringes were connected to their matching inlets with PEEK tubing (Vici, Schenkion, Switzerland, ID 0.5 mm and OD 1.59 mm). Pipette tips were placed in the outlets of the chip and used as a waste bin for the pitchfork outlets or as a collection vessel for the main outlet. The fluid collected at the pitchfork structures was discarded after the experiments. However, the fluid containing the fused droplets was captured and subjected to a live/dead staining to determine the number and viability of the cells present in the fused droplets. The flow was driven at a constant volume rate by a syringe pump (neMESYS dosing units, Cetoni GmbH, Germany). A magnetic stirrer bar was inserted into the cell containing syringes, to prevent the suspension from settling during the injection. The discontinuous phase

flow rates, containing the cell suspensions, were set at $5\text{--}10\ \mu\text{L}\cdot\text{min}^{-1}$, the flow rate of the continuous phase varied (specified in the results). The microfluidic chip was mounted onto the X–Y–Z translation stage of an inverted wide fluorescence microscope (Leica DM IRM, Leica Microsystems, Wetzlar, GmbH, Germany). Additionally, a computer-controlled high-speed camera (Photron SA-3, West Wycombe, United Kingdom) was mounted onto the microscope for image recording, using the accompanied Photron software (Photron Fastcam Viewer). Illumination was supplied by a Leica lamp 12V/100W in combination with a condenser.

Image acquisition and analysis

Images and videos were captured using the high-speed camera, at a frame rate of 3000 to 5000 frames per second and a shutter time of $5\ \mu\text{s}$. The captured images and videos were analyzed with the image-processing program ImageJ (National Institute of Health, MD, USA). To determine the encapsulation efficiency, the number of droplets containing 0, 1, or more cells was divided by the total number of droplets generated. The same method was used to calculate the droplet fusion efficiency. In order to determine the shrinkage percentage, the size of the droplet after passing the three pitchfork structures was divided by the size of the same droplet before it passed this shrinkage module.

Results

The Yin-Yang shaped microchannel caused a homogeneous cell distribution throughout the microchannel, gradually compelling the cells into their equilibrium position after 0.7 cm at a flow rate between 5 to $10\ \mu\text{L}\cdot\text{min}^{-1}$. This was faster compared to previous results described in chapter 1 using lower flow rates. Moreover, by adjusting the droplet formation frequency to the cell passing frequency, single cells were encapsulated in droplets. This matched frequency was 1 to 3 kHz per junction at a flow rate of the discontinuous phase between 5 and $10\ \mu\text{L}\cdot\text{min}^{-1}$ and a flow rate of the continuous phase between 70 and $120\ \mu\text{L}\cdot\text{min}^{-1}$. Figure 3 shows the percentage of single cell containing droplets, empty droplets and multicell droplets, comparing our device to the Poisson distribution.

Figure 3. Deterministic cell encapsulation in our device vs. the Poisson distribution.

It is clearly visible that in our device the percentage of empty droplets was greatly reduced and the percentage for single cell encapsulation doubled. For the double T-junction to produce stable paired droplets, the ratio between the discontinuous phase and the continuous phase had to be between 1:7 to 1:10. At this ratio-range, the success yield of droplet pairs was 100%. The droplet pairs need to be in close contact to promote droplet fusion. Therefore, the droplets from nozzle 2 were bigger than the droplets from nozzle 1 (figure 2). In our device, bigger droplets travel faster through the main microchannel than the smaller ones, enabling the droplets from nozzle 2 to catch up with the droplets from nozzle 1, hence forming pairs. However, to retain single cell encapsulation at nozzle 2, the cell passing frequency at nozzle 2 had to be reduced, which was accomplished by decreasing the cell volume fraction. The volume fraction in microchannel 1 was 2% and in microchannel 3 1.7%.

After single cell encapsulation and droplet pairing, droplet fusion was analyzed. Due to a slight diagonal position of the droplet pairs, the gradient of the electric field produced by the electrodes must be perpendicular to the tangent of the two droplets. This explains our electrode configuration. Once the droplet pair passes this electrode configuration, shown with dashed lines in figure 4, the droplets electrocoalesce. 95%

successful droplet fusion was achieved at an AC signal between 2 to 4 V_{pp} at a frequency between 1 to 3 kHz, which was amplified 100 times. Moreover, as in microchannel 1 a blue dye was added, the mixing of the two liquids upon the moment of fusion was clearly. Figure 2 and 4 show a slighter darker droplet in microchannel 1 and a lighter droplet in microchannel 3.

Figure 4. Time-scale recording of single cell encapsulation, droplet pairing and droplet fusion. Dashed lines represent the position of the electrodes. The flow rate for the discontinuous phase with HL60 cells and the blue dye in microchannel 1 was $7 \mu\text{L}\cdot\text{min}^{-1}$, in microchannel 3 with HL60 cells and without blue dye $8 \mu\text{L}\cdot\text{min}^{-1}$. The flow rate of the continuous phase was $55 \mu\text{L}\cdot\text{min}^{-1}$. The droplet generation frequencies was 1000 Hz per nozzle.

Next, the volume of the droplet was reduced in order to bring the cells in the droplets in close contact to make this platform suitable for future cell fusion. To reduce the

volume of the droplet, one to three pitchfork structures were used. With one pitchfork, the reduction was 40% (figure 5A), whereas this reduction was further increased by adding two more pitchforks to a volume reduction of 75% (figure 5B). Due to the pillars, the cells remained in the droplets, however in figure 5B, cells do tend to get forced into the pitchfork, whereas in figure 5A this did not occur at all.

Figure 5. Droplet shrinkage. A. One pitchfork. The cumulative flow rate was $75 \mu\text{L}\cdot\text{min}^{-1}$, the volume reduction 40%. B. Three pitchforks, the cumulative flow rate was $48 \mu\text{L}\cdot\text{min}^{-1}$, the volume reduction 75%.

Finally, the viability of the encapsulated cells was examined. The cells were stained with calcein AM and propidium iodide to determine cell viability and membrane integrity after encapsulation. The results showed that 92% of the cells retained their membrane integrity, compared with 97% in the control samples (figure 6). Moreover, 40% of the droplets contained two cells, which is in accordance with the calculated percentages of the individual functionalities of the platform.

Figure 6. Screenshots of cells in droplet array of two individual experiments. Cells were stained with calcein AM and propidium iodide to determine cell viability and membrane integrity after encapsulation, droplet fusion and droplet shrinkage. The amount of droplets containing two cells is in accordance of the calculated percentages of the individual functionalities of the platform.

Discussion

In this chapter, we present one single integrated microfluidic device, which is capable of high-yield deterministic cell encapsulation, droplet pairing, droplet fusion and droplet shrinkage, all in a high-throughput fashion. Due to the introduction of alternating curves in our design, cells were forced quicker into single equilibrium positions at lower flow rates. These lower flow rates also led to a lower pressure buildup in the microchannels, allowing the introduction of other functionalities onto

our platform. The percentage of single encapsulated cells was far above the Poisson distribution, however, not higher as the results previously published in our group as mentioned in chapter 1.^[11] The lower encapsulation percentage was caused by the double T-junction and the flow rates at which it was most stable. The ratio of the discontinuous and continuous phase, which was necessary to produce stable droplets of fixed size per junction, was between 1:7 to 1:10, leading to a high droplet generation frequency (6 kHz for the combined T-junctions). In order to meet these high droplet generation frequencies and preserve single cell encapsulation, the number of cells passing both T-junctions must be increased, otherwise the number of empty droplets will increase drastically. An increase in cell concentration results in an increase in double equilibrium positions, causing the droplet generation frequencies required for single cell encapsulation to go up even more²⁷. Moreover, an increase in volume fraction also led to aggregate formation and hence channel clogging. Additionally, a high volume fraction can disturb the inertial and Dean flows, revealing an absence of equilibrium positions. The use of accutase could reduce the number of aggregates, which would lead to even higher percentages of single cell encapsulation. To enable droplet fusion, the droplets need to be in close contact when they pass the electrodes. Therefore, one droplet is made slightly bigger than the other one. The bigger droplet will then catch up with the smaller one. Here, we used a difference in flow rate between the two discontinuous phases to accomplish varying droplet sizes. Another option could be to introduce a variation in the nozzle width of the two T-junctions, leading to different droplet sizes. However, close contact between droplet pairs can also be realized via the introduction of a small widening in the microchannel. In the widened section of the microchannel, the flow rate of the liquid is much lower than in the rest of the microchannel, causing the first droplet to slow down drastically which leads the next droplet to collide with the first one. By placing an electrode pair perpendicular to the tangent of the two droplets at that exact position would make electrocoalescence more successful, approaching 100%.

Although the volume reduction of the droplets was almost sufficient to result in the close cell pairing which would lead to high cell fusion efficiencies inside the droplets, a minor change to the design could already improve this even more. Now the

microchannel is narrowed down at the section where the pitchfork structures reach the main microchannel to impose continuous contact between the droplet and the pitchfork. Unfortunately, narrowing leads to channel clogging and more importantly to unstable droplets, because in the narrow section the droplets are forced into an elongated shape, which is unstable compared to their round stable shape. Due to this instability, larger sections are broken off from the main droplet, forming tiny droplets. It is more difficult to reduce the volume of the tiny droplets. Additionally, the narrowing imposes a short but strong increase in flow rate, reducing the contact time of the droplet with the pitchfork. Another disadvantage of the channel clogging caused by the narrowing, is the partial or total blocking of one or more pitchfork structures, which not only leads to the reduction in shrinkage but also to the disturbance of the overall pressure buildup in the platform. Both effects ultimately cause a severe fluctuation in the homogeneity of the droplet size, which is visible in figure 6. In conclusion, we have presented a multifunctional integrated microdroplet platform capable of encapsulating single cells in droplets, pairing the droplets, fusing the droplets and shrinking the droplets, all in a high throughput ($1500 \text{ droplets} \cdot \text{s}^{-1}$) fashion. The overall yield of the system in delivering droplets containing two cells is 40%, but we anticipate that with further optimization, mainly of the encapsulation modules we will be able to increase this. The next step is to use this platform to achieve cell fusion inside a droplet, and thus create a platform for high-yield, high-throughput hybridoma formation. Apart from this, the demonstrated integrated functionalities will enable a large variety of other cell biology applications combining single cell control with high-throughput operation.

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Chapter 5

Electrofusion of single cells in picoliter dropletsⁱ

In this chapter, we describe a microfluidic chip which enables electrofusion of cells in microdroplets, resulting in exchange of nuclear components. *To our knowledge this is the first time that the crucial intermediate step for controlled hybridoma formation, electrofusion of two cells, has been achieved inside a microdroplet.* We use a microfluidic device consisting of a microchannel structure in PDMS bonded to a glass substrate through which droplets with two cells are transported. An array of recessed platinum electrode pairs is used for electrofusion, applying six pulses of 1 ms at a constant membrane voltage of about 450 mV. This results in electrofusion of these cells with a fusion yield of around 5%. The method we present has the potential to operate in high-throughput (up to 500 cells sec⁻¹). The obtained results prove that microdroplet technology is a promising platform for efficient high-throughput electrofusion of individual cell-pairs, and has the potential to compete with the existing conventional methods. Besides, this platform is not restricted to cell fusion but is also applicable to various other cell-based assays such as single cell analysis and differentiation assays.

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Introduction

Currently, the demand for antibodies is increasing every year. They are used for research, as well as for diagnostic and therapeutic purposes^{1,2} such as to treat different types of cancer, Alzheimer's disease, Ebola, rheumatoid arthritis and multiple sclerosis³⁻⁶. All of these therapies have proven to be very promising. The production of antibodies primarily relies on the creation of antibody-secreting hybridomas^{2,7} that are obtained via cell fusion of antibody-producing B cells and immortal myeloma cells. B cells have a short lifespan in vitro and hence produce antibodies only briefly. Myeloma cells on the other hand proliferate rapidly in vitro due to their cancer characteristics. By fusing these two cells, a hybridoma can be formed capable of producing antibodies and able to proliferate rapidly in vitro, securing the prolonged production of antibodies.

For complete cell fusion, the cell membranes have to be in close contact. The newly formed fused cell contains two separate nuclei, and for this reason nuclear fusion will be the next focus. While membrane fusion can be induced by several methods, unfortunately nuclear fusion is a random process which can hardly be influenced. Nuclear fusion usually takes place within 1-2 weeks after the membrane fusion. The newly formed cell has to recombine the two sets of DNA, present in the two nuclei, which results in the hybrid cell (hybridoma). This newly formed hybridoma contains genetic material from both parental cells and will display the characteristics from both cells^{8,9}.

Although the currently used cell fusion methods are expensive, time consuming and low efficient, cell fusion still plays a central role in biotechnology, with applications including determination of the genetic make-up of organisms and cloning of mammal¹⁰. Most often, cell fusion plays a crucial role in the generation of monoclonal antibody-secreting hybridomas¹¹⁻¹⁸. Here we present a new, low cost, cell fusion method that is able to fuse cells with high efficiency and in a high-throughput fashion. Here, to get the two different cell types in close confined space and electrofuse them, we make use of a microfluidic droplet platform. The platform takes some functionalities from the platforms developed in the previous chapters, and aims at demonstrating in-droplet electrofusion of cells. Droplet-based microfluidics for biological experiments has received increasing interest in recent years, for several

reasons^{19,20}. Firstly, it enables the generation of monodisperse reaction vessels at high frequencies (kHz). Secondly, the encapsulation possibilities are enormous, ranging from bacteria to multicellular organisms²¹, enabling a wide range of biological assays that can be performed in a high-throughput manner. Thirdly, the droplets can be manipulated by (electro)coalescence, splitting and sorting²²⁻²⁴. Our aim is to develop a high-throughput microfluidic system to create functional hybridomas with high efficiency for antibody production in a controlled fashion²⁵. The current method for hybridoma formation relies on random cell pairing in large fusion vessels²⁶, resulting in both a low fusion efficiency and very low hybridoma generation efficiency. Our droplet platform enables hybridoma generation and selection, and has the potential of becoming a continuous, high-throughput method.

The platform discussed in this chapter consists of two essential elements, i.e., encapsulation of cells in droplets and cell electrofusion in the droplets (Fig. 1). From the wide range of available components described in literature²⁷⁻⁴¹, we have selected the appropriate ones and modified and optimized their design for integration in our electrofusion platform, in order to assess the feasibility of electrofusion of cells in a droplet^{25,27}. Parts of the platform and their functioning were described in detail in the preceding chapters.

Figure 1. Microfluidic platform for cell electrofusion in droplets. At the top a schematic overview is shown with colored inserts of the different functionalities of the chip. In green, cell encapsulation in droplets. In red, droplets containing two cells passing the electrode array, consisting of six electrode pairs, all capable of giving a pulse of certain preset strength for a duration depending on the flow rate.

To achieve electrofusion of cells in a droplet, one first needs to gain insight in the transient changes in the electrical potential distribution inside a cell-loaded droplet when the droplet moves over an electrode pair. To this aim, the electrical potential distribution was calculated using an equivalent electrical circuit model and the calculated values were used to interpret the electroporation experiments performed⁴²⁻⁴⁴. Since our focus is on the electrofusion of cells in droplets using downstream placed electrode pairs, droplets containing two differently stained HL60 cells were generated. Due to the fluorescent staining, the process of electrofusion can then be assessed in detail at the single cell level. As we will show in the results section, we demonstrate for the first time successful electrofusion of cells in droplets. This can be a first step towards functional hybridoma creation on chip, which can be performed by combining this platform with picoinjectors to secure cell viability inside the droplets⁴⁵, and a microfluidic platform capable of screening functional hybridomas⁴⁶. Moreover, the

presented platform is not restricted to cell fusion and is applicable for various other cell-based assays such as single cell analysis and differentiation assays.

Methods

Chip design

The complete design consists out of three essential components (Fig. 1). Firstly, encapsulation of cells in droplets (green square), using a flow-focusing geometry. Secondly, cell electrofusion in a droplet (red square). Thirdly, the droplets containing the cells pass six electrode pairs, providing the droplet with sufficient electric field strength to induce electrofusion. The channel dimensions are 50x25 μm (blue structures in Fig. 1) and the electrode distance (yellow structures in Fig. 1) and electrode width are 20 μm .

Chip fabrication

A silicon master design was drawn in Clewin (version 4.0.1) and fabricated using standard UV-lithography. SU-8 (Microchem) was spun on the silicon master with a thickness of 25 μm . The chip was made in polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning). Curing and base agent were mixed at a ratio of 1:10 and degassed. PDMS was poured onto the silicon wafer, degassed, and cured at 60 °C for 24 hours. After curing, in- and outlets were punched using a dispensing tip (Nordson EFD, inner $\varnothing$ 1.36 mm and outer $\varnothing$ 1.65 mm). Subsequently, the PDMS was sealed to a printed circuit board (VWR) using an oxygen plasma (Harrick PDC-001). After sealing, the chip was placed at 60 °C for a minimum of 30 minutes. Before use, 10mM FDTs in HFE7500⁴⁷ was introduced into the channels to ensure the hydrophobicity of the channel walls.

The recessed platinum electrodes were sputtered on a glass wafer using a standard sputtering technique and the glass wafer was diced into individual chips. The glass chip was bonded with UV-glue onto a printed circuit board (PCB). The electrode pads on both the PCB and the glass chip were connected via a double wirebond, which was sealed off with a drop of two component glue (Heisel). Onto the PCB, a plug was soldered, to make the electrical connection with the pulse generator (Agilent 33250A). For electroporation and electrofusion in droplets, a DC voltage was applied, varying from 2 to 3 V.

Cell membrane voltage model

To calculate the possibility that electrofusion will occur, an equivalent electrical circuit model was built, which can be found in Fig. 2e. The circuit can be simplified to two coupled pairs of resistances and capacitances each with their characteristic charging RC time constants. The resistances change during movement of the cells over the electrodes, leading to a continuously changing charging of the capacitances. For the

resistor voltage across such an RC circuit we have $V_R(t) = V_0 \cdot e^{-t/RC}$

Here is V_0 the applied voltage, R and C the resistance and capacitance values. The RC time constant equals the product of the resistance R and capacitance C. It was assumed that the electrode directly contacts the electrofusion buffer, and that the capacitance of the electrofusion buffer/electrode interface was $20 \mu\text{F}/\text{cm}^2$. For the cell membranes the capacitance of a surface with a thickness of 5 nm and a relative permittivity of 2 was taken.⁴⁸ The conductance of the electrofusion buffer was 0.009 S/m and of the cell cytoplasm was 60 S/m and were used to calculate the resistances. The electrode distance and width were set at 25 μm and 20 μm respectively, similar to the dimensions in our microfluidic device. In our model the droplet encapsulated two HL60 cells with a diameter of 13 μm . The first electrode had a potential of 3 V, while the other electrode was set to ground potential (0 V). For the velocity of the droplet through the microchannel the measured value of 28 mm/s was used.

In our model $t = 0$ was taken at the moment that the droplet was fully in contact with the first electrode and started to move over the second electrode. All these parameters were included in the equivalent electrical circuit model as shown in Fig. 2e. The transient behavior of the circuit has been numerically approximated using a MATLAB script, the resulting membrane voltage over time can be seen in Fig. 2f.

Materials

HL60 (human promyelocytic leukemic) cells were grown in RPMI medium (Invitrogen), supplemented with 20 % (v/v) fetal bovine serum (FBS; Invitrogen), 100 IU ml^{-1} penicillin (Invitrogen), $100 \mu\text{g ml}^{-1}$ streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen) (=RPMI+ medium). Cell cultures were sustained in a 5 % CO_2 humidified atmosphere at 37 °C. Cell cultures were split every 3 - 4 days at a ratio of 1:10. Prior to

the experiments, the cells were counted, to establish the correct volume fraction of 1.0 % in electrofusion buffer (EF buffer). EF buffer is an isoosmolar low conducting buffer¹⁴, consisting of deionized water, supplemented with 280 mM inositol (Sigma), 0.1 mM calcium acetate (Sigma), 0.5 mM magnesium acetate (Sigma) and 1 mM L-histidine (Sigma). The conductivity was adjusted, using deionized water, to 0.009 S m^{-1} . The cell containing solutions were used as the dispersed phase. For the continuous phase, HFE 7500 (3MTM NOVECTM) was used, with a 1 % (wt/wt) oil-phase biocompatible fluorosurfactant. This surfactant was a kind donation of dr. Ralph Sperling (Harvard).

Experimental set-up

The HL60 cell suspension and the oil suspension were introduced into the microfluidic chip using 1 mL plastic syringes (Braun) with a needle (BD Microlance). All syringes were connected to their matching inlets with TYGON tubing (inner $\varnothing$ 0.02 inch and outer $\varnothing$ 0.06 inch). Pipette tips were placed in the outlets of the chip and used as a waste container or as a collection vessel for the main outlet.

The flow was driven at a constant volume rate by a syringe pump (neMESYS dosing units). A magnetic stirrer bar was inserted into the cell containing syringes, to prevent the suspension from settling during the injection. The dispersed phase flow rates, containing the cell suspensions, were set at $0.2 - 0.25 \mu\text{L min}^{-1}$, the flow rate of the continuous phase at $1 - 2 \mu\text{L min}^{-1}$. The microfluidic chip was mounted onto the X–Y–Z translation stage of an inverted wide fluorescence microscope (Leica DM IRM, Leica Microsystems). Additionally, a computer-controlled high-speed camera (Photron SA-3) was mounted onto the microscope for image recording, using the accompanied Photron software (Photron Fastcam Viewer). Illumination was supplied by a Leica lamp 12V/100W in combination with a condenser.

For the electrofusion experiments, various nuclear dyes (blue (NucBlue), yellow/orange [$5 \mu\text{M}$] (Cyto82), and membrane fluorescent dyes (green [$5 \mu\text{M}$] (Vybrant green), were used, as specified in the result section. All fluorescent stains were purchased from Invitrogen. Cells were stained according to the provided protocol. After the electroporation and electrofusion experiments, the droplets were collected and loaded on a microscope slide with single shallow depression (Corning). This allowed for the formation of a monolayer which could be scanned and analyzed.

Image acquisition and analysis

Images and videos were captured using the high-speed camera, at a frame rate of 3000 frames per second and a shutter time of 50 μ s. The captured images and videos were analyzed with the image-processing program ImageJ (National Institute of Health). Images of the fluorescently labeled cells in droplets were captured using a Life Technologies EVOS FL microscope with DAPI, RFP and GFP filter cubes.

Results - Modelling of the electrical field

In order to successfully realize electrofusion of cells inside a droplet, a sufficiently high voltage across the cell membranes of the paired cells is essential. Therefore, the transient changes in the electrical potential inside a droplet were calculated using an equivalent electrical circuit model. Since planar electrodes are used, the cells will often not have an optimal orientation with respect to the applied electrical field (Fig. 2a-c). The ideal cell orientation for electrofusion is when the plane of the contacting cell membranes lies perpendicular to the electric field (Fig. 2d). This best-case situation has been considered for the electrical circuit model (Fig. 2e). Even if the cells do not rearrange their membranes straightaway, they will endure electroporation in the membrane area perpendicular to the field. Due to cell movement inside the droplet, both electroporated regions of the cell membranes might touch, with possible membrane rearrangement as a result.

Figure 2. Simulation of electric field in a droplet. (a) A snapshot from a high-speed recording, reflecting situation 2(b). The white bar A-A indicates the plane in which the field lines of the electric field between the electrodes propagate. (b, c, d) Schematic representations of different situations in which the cells can align in a droplet, of which (d) is the ideal situation.. (e) Cell membrane voltage model of the electric field in a droplet passing one electrode pair. In this side view, one of the electrodes has a voltage of 3V and the other is grounded. Both electrodes are placed at the bottom. (f) The induced electric field in the membrane is strong enough ($500 \text{ V}/5 \text{ nm}$) to result in electrofusion.

The transient behavior was numerically approximated using MATLAB, and the resulting potential over a single cell membrane in time can be seen in Fig. 2f, where $t = 0$ is the moment the droplet has reached the second electrode. Direct contact between

electrofusion buffer and electrode surface is assumed. The time constant to charge the double layer at the electrode interface is found to be about 4 ms and the time constant for charging the cell membrane about 1 μ s. Thus the cell membrane is charged instantaneously with respect to the double layer membrane. The time to cover the electrode using the experimental droplet velocity is 0.71 ms. During this period the membrane voltage is approximately constant between 550 and 400 mV. When the second electrode is fully covered, the membrane voltage drops exponentially. These voltages are equivalent to an electric field in the cell membrane of 1 MV/cm, which is sufficient for membrane electroporation^{48,49} and therefore theoretically sufficient to enable subsequent electrofusion of the two encapsulated cells^{50,51}. The results from this model are in accordance with the results from the electroporation experiments, where the application of 6 pulses with the previous parameters showed successful electroporation. To establish viability, cells were stained with calcein AM before the experiments. Calcein AM is a green cell dye that is only visible if the cells are alive. 95% of the cells were positive (green) after the experiment with or without the applying the voltage.

Discussion of the experimental procedure and setup

Besides the magnitude of the electric field, as described above, various other factors play a role for successful electrofusion to occur. A crucial factor is the droplet traveling speed. As seen in the preceding section, the droplet speed and the electrode width determine the length of the applied fusion pulse. To increase the pulse length, the droplet speed can be decreased by reducing the net flow rate or widening the channel to slow down the droplet. To reduce the pulse length, the droplet speed can be increased by increasing the net flow rate. However, to generate droplets that are as small and stable as possible, the flow rate range is set²⁵. Our preferred droplet volume lies between $\sim$ 18 pL (defined by the diameter of two encapsulated HL60 cells of $d=13$ μ m) while in practice at the optimal flow rate for droplet formation, the droplets have a volume of 35 pL. The electrode width can also be adjusted, but should not be more than the droplet length, as the droplet must be in contact with both electrodes to create the electric field over the encapsulated cells. Additionally, previous results

showed that applying multiple pulses (hence employing multiple electrode pairs) resulted in higher fusion efficiencies⁵².

To generate the droplets for the cell electrofusion experiments, we used a flow-focusing geometry²⁷. The cell volume fraction was 1.0%, resulting in 15% of the droplets containing two HL60 cells. HL60 cells were used because they have dimensions similar to B cells and myeloma cells, and also because they consist almost completely out of a nucleus which facilitates visualization of electrofusion with nuclear cell staining. Finally, HL60 cells are easier to maintain in a cell culture. The settings of the performed electrofusion experiments were an applied voltage of 3 V and a droplet velocity of 28 mm/s.

To accurately assess cell electrofusion in a droplet, we used different fluorescent (nuclear and membrane) dyes, which enabled us to monitor and analyze the process in detail. To visualize electrofusion, which is defined as two fused HL60 cells with one rearranged cell membrane containing two nuclei, half of the HL60 cells were stained with a green membrane dye and a blue nuclear dye, and the other half with a red nuclear dye. After the experiment, droplets containing cells with a rearranged green membrane encapsulating both a blue and a red nucleus were observed (Fig. 3). Simply the observation of two nuclei in a single cell is insufficient, since at the start of the electrofusion experiments, the HL60 cells were in the exponential growth phase, and thus the suspension also contained polynucleated cells (0.8 %; 4/492). Electrofusion could therefore only be concluded if there were two differently and clearly distinctively labeled nuclei.

Several control experiments were performed. Before each electrofusion experiment, the two separately dyed cell populations were checked for successful staining under the microscope. Staining was found to be successful as, at the start of the experiment and in control experiments (no electric field applied), none of the cells contained a blue and a red nucleus with a green fluorescent membrane. Also, control experiments to check whether the cell dye transferred to other cells over time, were performed by removing the supernatant from the stained cells after thirty minutes and introducing it to a new cell population. Fluorescent images taken over time showed that no fluorescence was visible, ruling out cell dye transfer.

Figure 3. Overview of an electrofusion result. The images show the fluorescent, bright field and overlay images of HL60 cells in a droplet. The cells inside the droplets contain two nuclei with different colors and a rearranged cell membrane. The droplet size is 50 pL. Six pulses of 1.2kV/cm were applied.

Results and discussion

We found that six pulses of different lengths, ranging from 1 - 5 ms at an estimated cell membrane voltage of about 450 mV, could result in electrofusion. According to Poisson statistics with $\lambda = 1$, on average 15% of the droplets contain two cells. After counting on average 500 droplets per experiment ($n = 5$), of which 15% (75 droplets) contained two cells and qualified for possible electrofusion, 19 successful electrofusion events were found resulting in 5 % electrofusion efficiency. The established efficiency is comparable to the efficiency of both commercially available methods⁵³⁻⁵⁵ as well as on-chip methods^{52, 56-58}. To compete with existing methods, the electrofusion efficiency must be further improved, and there are several realistic opportunities for this. First, cell-cell contact can be enhanced geometrically (e.g., by placing a serpentine channel structure in front of the DC electric field to enhance cell movement⁵⁹ or implementing a droplet shrinkage module causing continuous cell-cell

contact²⁵) or electrically (e.g., by applying an AC electric field to improve cell alignment and cell-cell contact by DEP⁶⁰). In addition, a 3D electrode configuration will generate a homogeneous electric field along the channel wall, giving higher fusion efficiency. The overall conclusion can be that microdroplets are a promising platform for efficient high-throughput electrofusion of cells in droplets, which has the potential to compete with the existing conventional methods.

The functional elements demonstrated in this thesis can in potential be combined. In chapter 3 we have shown single cell encapsulation efficiencies up to 77%, droplet pairing and electrocoalescence up to 95%, and droplet shrinkage up to 75%. By combining this platform with the platform demonstrated in chapter 4 we expect to reach yields up to 50%, because a higher percentage of droplets will be filled with two different cell types, and droplets will be smaller enabling better cell-cell contact^{25,27}. Also the further coupling of this platform with picoinjectors to secure cell viability inside the droplets⁴⁵, and a microfluidic platform capable of screening functional hybridomas⁴⁶ is a possibility, and can be a major step towards functional hybridoma creation on chip. In chapter 6 such an integrated device will be presented. Finally, the use of the platform presented here is not restricted to cell fusion since it is applicable for various other cell-based assays such as single cell analysis and differentiation assays. In conclusion, we have shown for the first time electrofusion of cells in droplets, with exchange of nuclear components.

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Chapter 6

Total microfluidic platform containing multiple functionalities to enable cell electrofusion

In this chapter we describe a microfluidic chip capable of pairing and electrofusing cells in microdroplets, with exchange of nuclear components, starting from two cell populations. The chip consists of a microchannel structure in PDMS bonded to a glass substrate. The device first encapsulates cells from two different populations in single droplets and then pairs and fuses the droplets. Subsequently the droplets with two cells are transported over an array of recessed platinum electrode pairs which is used for electrofusion, applying six pulses of ~ 1 ms duration creating an estimated membrane voltage of about 450 mV. Due to the integration of the different functionalities on a single chip the pairing efficiency and fusion yields are higher compared to the previously obtained results. The method we present furthermore has the potential to operate with high-throughput (up to $1000 \text{ cells sec}^{-1}$). The microdroplet technology thus offers a promising platform for efficient high-throughput electrofusion of individual cell-pairs, with the potential to compete with the existing conventional methods. Moreover, this platform is not restricted to cell fusion but is also applicable to various other cell-based assays such as single cell analysis and differentiation assays. In this chapter HL60 cells were used as model cells, which have an average diameter of $13 \mu\text{m}$ and thus are slightly bigger than the plasma B cells ($10 \mu\text{m}$) and slightly smaller than the myeloma cells ($15 \mu\text{m}$). Future research will aim at fusing myeloma and plasma B cells with the platform.

Introduction

Cell fusion is a multi-step process, that roughly proceeds as follows^{1,2}. The two parental cells are brought in close contact and an external cue such as an electrical field is applied to achieve complete cell fusion. After fusion of the cell membranes, firstly the fused cell still contains two separate nuclei and later nuclear fusion follows. Unfortunately nuclear fusion is a random process which cannot be influenced. Moreover, the cell has to recombine the two different sets of its parental DNA, which occurs 10-14 days after membrane fusion and can result in a functional hybridoma. In this thesis to bring the two parental cells into close contact, we individually encapsulate them in droplets. **In this chapter we integrate the elements of the preceding chapters in one single functional device** (see fig. 1). In chapter three we have shown a method for high yield single cell encapsulation. In this chapter, by combining this design with the Yin-Yang shaped elongated funnel from chapter four, we have increased the period of use due to less clogging. Moreover, directly after the spiral the microchannel is filled with circular microstructures. This feature is added to the design to improve the cell alignment even further. These circular microstructures are inertial focusing geometries with substantially larger dimensions that create a single stream microparticle train³. In short, single-stream focusing is achieved through inertial particle focusing, local secondary flow induced particle migration and vertical particle positioning steps. The design consists of a straight square channel ($H = 25 \text{ mm}$) that expands to a rectangular channel ($W = 150 \text{ mm}$) accommodating a series of 20 asymmetrically positioned cylindrical pillars ($D = 100 \text{ mm}$) that induce localized helical vortices. Focusing of initially randomly distributed microparticles into a single-stream is hereby achieved. The next step is a double T-junction, which is responsible for the single cell encapsulation, and droplet pairing. This double T-junction is based on previous work of Frenz et al., and was described in chapter four^{4,5}. After encapsulation, the paired droplets pass a pair of recessed platinum on-chip electrodes, placed diagonally on either side of the channel, to initiate droplet electrocoalescence. If the droplets are in close proximity of each other, the induced dipoles of both droplets align, which leads to an attractive Coulomb force between the two droplets causing

them to coalesce. Electrocoalescence was first demonstrated by Chabert et al.⁶ and multiple applications in microfluidics have been shown over the past years^{5,7}.

For electrofusion of cells in droplets, the cells need to be in close contact. Therefore, the droplet volume must be reduced. To cause this, the droplets pass six microchannels which are placed perpendicular to the main microchannel. It has been shown that such T-junctions⁸ or side channels⁹ can be used to split a droplet. One key element in our device, which differs from general splitting geometries, is the placement of small pillars at the inlet of the side channels in order to prevent cells from entering the side channels. This structure is similar to the structure used in previous chapters.

After the droplets have been shrunk down, the cells have a larger chance of being in close contact. The cells then pass over six electrode pairs as described in chapter five. Here the cells receive six electrical pulses designed to cause membrane electroporation. If the two cells touch after they have been electroporated their membranes can rearrange and form a fused cell. In a similar fashion as in chapter five we dyed the two different cell populations before the electrofusion experiments, to later observe fused cells and prove that our device is able to electrofuse cells.

Figure 1. Schematic drawing of the microfluidic chip design. In bleu the Yin-Yang shape gradually introduces the cells into the spiral-like channel ($l=3.2\text{cm}$, $r=1700\text{ }\mu\text{m}$, $w=50\text{ }\mu\text{m}$, $h=25\text{ }\mu\text{m}$) which is capable of ordering and encapsulating single cells with an efficiency of up to 77%. The device is based on the use of Dean force combined with inertial forces, resulting in fast processing (1.6 kHz). In red microstructures induce helical vortices for better cell alignment. In green the double T-junction enables alternating single cell encapsulation and droplet pairing. The two countersunk platinum electrodes (dashed triangles) induce droplet electrocoalescence. In purple the droplets are shrunk down to 15% of their original volume depending on the flow rates and the use of one or more shrinkage structures. Pillars prevent cell removal. In turquois, six pairs of recessed platinum electrodes producing a DC electric field of 1.5 kV/cm, with set flow rates resulting in 1 ms pulses enabling electrofusion of HL60 cells in droplets.

Methods

Chip functionality

The complete design has three essential functional elements (Fig. 1). Firstly, cells are encapsulated in droplets (green square) using two parallel flow-focusing geometries. Secondly, the droplets from the two droplet streams are paired, fused and shrunk. Thirdly, cell electrofusion in droplets (red square) is performed when the droplets with the cells pass six electrode pairs.

Chip design, material and fabrication

A silicon master design was drawn in Clewin (version 4.0.1) and fabricated using standard UV-lithography. The channel dimensions are $50\times 25\text{ }\mu\text{m}$ (blue structures in Fig. 1) and the electrode distance (yellow structures in Fig. 1) and the electrode width is $20\text{ }\mu\text{m}$. SU-8 (Microchem) was spun on the silicon master with a thickness of $25\text{ }\mu\text{m}$. The chip was made in polydimethylsiloxane (PDMS) (Sylgard 184, Dow Corning). Curing and base agent were mixed at a ratio of 1:10 and degassed. PDMS was poured onto the silicon wafer, degassed, and cured at $60\text{ }^{\circ}\text{C}$ for 24 hours. After curing, in- and outlets were punched using a dispensing tip (Nordson EFD, inner $\varnothing 1.36\text{ mm}$ and outer $\varnothing 1.65\text{ mm}$). Subsequently, the PDMS was sealed to a printed circuit board (VWR) using an

oxygen plasma (Harrick PDC-001). After sealing, the chip was placed at 60 °C for a minimum of 30 minutes. Before use, 10mM FOTS in HFE7500¹⁰ was introduced into the channels to ensure the hydrophobicity of the channel walls.

The recessed platinum electrodes were sputtered on a glass wafer using a standard sputtering technique and the glass wafer was diced into individual chips. The glass chip was bonded with UV-glue onto a printed circuit board (PCB). The electrode pads on both the PCB and the glass chip were connected via a double wire bond, which was sealed off with a drop of two component glue (Heisel). Onto the PCB, a plug was soldered, to make the electrical connection with the pulse generator (Agilent 33250A). For electroporation and electrofusion in droplets, a DC voltage of 3 V was applied.

Biological materials

HL60 (human promyelocytic leukemic) cells were grown in RPMI medium (Invitrogen), supplemented with 20 % (v/v) fetal bovine serum (FBS; Invitrogen), 100 IU ml⁻¹ penicillin (Invitrogen), 100 µg ml⁻¹ streptomycin (Invitrogen) and 2 mM L-glutamine (Invitrogen) (=RPMI+ medium). Cell cultures were sustained in a 5 % CO₂ humidified atmosphere at 37 °C. Cell cultures were split every 3 - 4 days at a ratio of 1:10. Prior to the experiments, the cells were counted, to establish the correct volume fraction of 1.0 % in electrofusion buffer (EF buffer). EF buffer is an isoosmolar low conducting buffer¹¹, consisting of deionized water, supplemented with 280 mM inositol (Sigma), 0.1 mM calcium acetate (Sigma), 0.5 mM magnesium acetate (Sigma) and 1 mM L-histidine (Sigma). The conductivity was adjusted, using deionized water, to 0.009 S m⁻¹. The cell containing solutions were used as the dispersed phase. For the continuous phase, HFE 7500 (3MTM NOVECTM) was used, with a 1 % (wt/wt) oil-phase biocompatible fluorosurfactant. This surfactant was a kind donation of dr. Ralph Sperling (Harvard).

Experimental set-up

The HL60 cell suspension and the oil suspension were introduced into the microfluidic chip using 1 mL plastic syringes (Braun) with a needle (BD Microlance). All syringes were connected to their matching inlets with TYGON tubing (inner Ø 0.02 inch and outer Ø 0.06 inch). Pipette tips were placed in the outlets of the chip and used as a waste container or as a collection vessel for the main outlet.

The flow was driven at a constant volume rate by a syringe pump (neMESYS dosing units). A magnetic stirrer bar was inserted into the cell containing syringes, to prevent the suspension from settling during the injection. The dispersed phase flow rates, containing the cell suspensions, were set at $0.2 - 0.25 \mu\text{L min}^{-1}$, the flow rate of the continuous phase at $1 - 2 \mu\text{L min}^{-1}$. The microfluidic chip was mounted onto the X–Y–Z translation stage of an inverted wide fluorescence microscope (Leica DM IRM, Leica Microsystems). Additionally, a computer-controlled high-speed camera (Photron SA-3) was mounted onto the microscope for image recording, using the accompanied Photron software (Photron Fastcam Viewer). Illumination was supplied by a Leica lamp 12V/100W in combination with a condenser.

For the electrofusion experiments, various nuclear dyes (blue (NucBlue), yellow/orange [$5 \mu\text{M}$] (Cyto82), and membrane fluorescent dyes (green [$5 \mu\text{M}$] (Vybrant green), were used, as specified in the result section. All fluorescent stains were purchased from Invitrogen. Cells were stained according to the provided protocol. After the electroporation and electrofusion experiments, the droplets were collected and loaded on a microscope slide with single shallow depression (Corning). This allowed for the formation of a monolayer which could be scanned and analyzed.

Image acquisition and analysis

Images and videos were captured using the high-speed camera, at a frame rate of 3000 frames per second and a shutter time of $50 \mu\text{s}$. The captured images and videos were analyzed with the image-processing program ImageJ (National Institute of Health). Images of the fluorescently labeled cells in droplets were captured using a Life Technologies EVOS FL microscope with DAPI, RFP and GFP filter cubes.

Results and discussion

The Yin-Yang and the pillar structure at the inlets improved the period of use due to less clogging, with respect to the devices used in chapter three and four. Moreover, the alternating curves described in chapter four have been replaced with continuous curved spiral like channels described in chapter three. Although they both proved to be functional, alternating curves got clogged up more often. Unfortunately, the circular microstructures were not very useful. Their functionality only proved to be

working at higher flow rates. If the cells were already aligned due to the spiral channel, the cells would stay in their equilibrium positions. However if they would enter the microstructure channel at multiple positions, the circular microstructures only compelled the cells to move into their single equilibrium positions at flow rates of around 30 $\mu\text{L}/\text{min}$ or higher. With these high flow rates however it was not possible to generate droplets. To generate droplets at this flow rate, the flow rate of the continuous phase needs to be at least five times higher which would lead to an enormous pressure buildup in the channels, causing them to deform and tear up the bonding resulting in leakage.

In chapter 3 we have shown single cell encapsulation efficiencies of up to 77%, droplet pairing and electrocoalescence up to 95%, and droplet shrinkage up to 75%. By combining the platform with the platform demonstrated in chapter 4 we expect to reach electrofusion yields of up to 50%, because a higher percentage of droplets will be filled with two different cell types, and droplets will be smaller enabling better cell-cell contact^{12,13}.

Concerning cell encapsulation efficiencies, with the design of this chapter we obtained similar results as in chapter 3. The cells aligned in so-called cell trains which are streams of cells all at the equilibrium position. However, these cell trains only had a certain length before they were being interrupted, which was caused by a lack of homogeneity of the cell suspension. Though the cell suspension in the syringe was stirred with a magnet to prevent inhomogeneities, the cell suspension can't be stirred in the tubing. Moreover, the cells seemed to accumulate at the chip inlet. By tapping the tubing or the syringe a burst of cells then was released. After entering the channel, these cells would align creating a cell train of a definite length. It was found to be extremely difficult to enable cell alignment simultaneously and for longer duration in both spiral channels and thus creating continuous cell trains.

The double T junction and the electrodes responsible for the electro coalescence which were already shown in chapter four obtained similar results. However, the pitchfork structures described in chapter four now were replaced by six individual shrinkage channels. The pitchfork structures caused satellite droplets to become pulled off from the main droplet at the first shrinkage channel and return to the main

channel via the third channel. These satellite droplets later electrocoalesced with the main droplet at the electrofusion electrodes. The six individual shrinkage channels did not give this problem and successfully reduced the droplet volume to 15% of its original volume.

In chapter five, we have shown for the first time continuous flow electrofusion of cells in droplets, with exchange of nuclear components. There six pulses of 1 ms at an estimated cell membrane voltage of about 450 mV resulted in electrofusion with an estimated electrofusion yield of 5 %. In this chapter, due to the difficulties encountered establishing simultaneous cell alignment in both spiral channels, we were not able to increase the electrofusion yield with respect to the results of chapter 5, and again obtained a fusion yield of 5%.

It must be remarked here that the current method used to screen the droplets for successful electrofusion is not sufficient, making it likely that the actual electrofusion yield is higher than the observed electrofusion yield because not every electrofusion event is accounted for. To establish the true electrofusion yield, more tests need therefore to be carried out. Also in future research, instead of using only HL60 cells, myelomas and plasma B cells should be used to form hybridomas, followed by testing for functionality by cultivation in HAT medium.

The established electrofusion yield of 5% is comparable to the yield of commercially available methods¹⁴⁻¹⁶ as well as on-chip methods¹⁷⁻²⁰. To strengthen the competition with these existing methods, the electrofusion yield must be further improved, for which there are several realistic opportunities. First, the cell-cell contact can be enhanced geometrically, e.g., by placing a serpentine channel structure in front of the DC electric field to enhance cell movement²¹ or by implementing a shrinkage module enabling continuous cell-cell contact,¹² or electrically, e.g., by applying an AC electric field to provide a DEP force to improve cell alignment and cell-cell contact²². In addition, a 3D electrode configuration will generate a homogeneous electric field along the channel wall, giving higher fusion efficiency.

Finally, once good electrofusion yield is obtained, coupling of this platform with picoinjectors to secure cell viability inside the droplets²³, and a microfluidic platform capable of screening functional hybridomas²⁴, can be a major step towards functional

hybridoma creation on chip. Moreover, this platform is not restricted to cell fusion and is applicable for various other cell-based assays such as single cell analysis and differentiation assays.

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Chapter 7

Summarizing chapter

This chapter is a short summary of everything that is discussed in all the previous chapters. It also provides some suggestions for further research and development

Summary

In this thesis a range of microfluidic devices is described and discussed, which finally result in an integrated design containing a number of functionalities that enables high-throughput continuous flow electrofusion of cells on chip. Electrofusion of cells on chip is important because it can lead to the production of functional hybridomas, which are the cell fusion product of two different parental cells, namely a plasma B cell and a myeloma cell. This plasma B cell is an antibody producing cell. These antibodies are required for research, diagnostics and several disease treatments. However these plasma B cells have a very short lifespan. A myeloma on the other hand is a cancerous version of the plasma B cell that proliferates rapidly. By combining these two, the daughter cell has characteristics from both cell types, in this case both the ability to produce antibodies and the ability to proliferate rapidly. These qualities make possible to maintain these hybridomas in a cell culture, creating a sort of antibody factory¹⁻⁸.

Of course we were not the first to come up with this idea and therefore a literature search has been carried out which forms chapter two. In this chapter first the fundamentals of the behavior of cells in an electric field are discussed. This is very important because most of the current state of the art techniques use electrical fields for cell fusion. First they often make use of dielectrophoresis⁹ either to push cells into a certain direction or to keep them in one spot prior to electrofusion, since the two different parental cells need to be brought into close contact with one another ('paired') to allow subsequent electrofusion. In this chapter also the electroporation of the cell membrane is thoroughly discussed^{10,11} to gain insight in the different conditions that the cells will need to be brought into for the highest electrofusion yield.

In chapter two furthermore the other state of the art fusion techniques are discussed and judged on their ability to pair cells, ease of operation, yields, etc.. Finally, a short introduction about the droplet platform for electrofusion is given¹²⁻¹⁶.

The droplet platform enables single cell encapsulation and by doing so creates the possibility to encapsulate the two different cell types in a single droplet by fusing two droplets each containing a single cell. The theory and technique behind single cell

encapsulation was clarified in chapter three. Based upon a spiral-shaped channel the cells are each hydrodynamically compelled into a single equilibrium position. It is demonstrated that if the cell passing frequency can be matched with the droplet generation frequency at the flow focus junction, single cell encapsulation efficiencies reach up to 77%¹⁷⁻¹⁹.

Once the single cells are encapsulated in droplets, two droplets both containing a different cell have to be paired and electro-coalesced to end up with a droplet containing both cells. The process to do this is explained in chapter four. The device that is reported in chapter four contains a double T-junction which is capable to encapsulate single cells and at the same time pair the different droplets.^{20,21} Once the paired droplets enter a main channel they pass by an electrode pair that causes the droplets to electro-coalesce^{22,23}. However, once the droplets have electro-coalesced, the two cells remain in a large droplet, reducing the chance of them to get into close contact ('paired') as is needed for electrofusion to be successful. Therefore, three pitchfork-shaped channels perpendicular to the main channel are used to reduce the droplet volume by draining the droplet volume based on hydrodynamic resistances^{24,25}. These pitchfork-shaped channels contain small pillars preventing the cells from being removed from the main droplet in the process.²⁶

The next step is actual cell electrofusion inside the picoliter droplets that contain the cell. This process is described and explained in chapter five. The design used in chapter five is a simple spiral-shaped channel ending up at a flow focus junction where the cells are encapsulated, followed by facilities for droplet pairing. Finally droplets were led over a sequence of six electrode pairs for electrofusion. The flow rates used during the experiments were too low to align the cells and therefore Poisson ($\lambda=1$) statistics determined the encapsulated cell numbers, resulting in 15% of all generated droplets containing two cells. Per experiment 500 droplets were screened, 15% of these droplets should contain two cells, which is a number of 75 droplets. During this study 5 experiments were carried out, enabling (5 x 75) 375 possible fusion events. In total 19 successful fusion events were found resulting in a 5% electrofusion efficiency. In this chapter an equivalent electrical circuit model was developed to describe the potential

over the cell membrane during passage of the cell-containing droplets over the electrofusion electrodes, in order to determine whether the applied electrode potentials were sufficient to create cell electrofusion in theory. In this particular case HL60 cells were used to demonstrate cell electrofusion. To establish successful electrofusion, the two different cell populations that were paired were dyed in different colors. One had a blue nuclear dye with a green membrane dye and the other had a red nuclear dye. In the droplets produced cells were found containing all three cell dyes which also showed a rearranged cell membrane, therefore proving that cell electrofusion inside picoliter droplets is possible²⁷⁻³⁰.

The next logical step is to incorporate all the previously described functionalities into one single device. Such a device is fully discussed in chapter 6. The integrated device contains the spiral-shaped channels from chapter three, and combines these with the Yin-Yang shaped inlets from chapter four. These are followed by a wider microchannel that contains microstructures inducing helical vortices that improve cell alignment even further. Once the cells are aligned, they are encapsulated with the use of the double T-junction discussed in chapter four. The double T-junction also enables the droplets to pair while subsequently two electrodes induce electro-coalescence. Instead of passing the large droplets over pitchfork structures as in the device of chapter four, the droplets now pass six single perpendicular side channels to reduce the droplet volume. Finally the droplets pass over a six electrode pairs to initiate cell electrofusion as discussed in chapter 6^{19,26}.

The current design is working and reached electrofusion efficiencies of 5%, therefore it is able to compete with existing techniques. However, there still exists lots of room for improvement, mostly in the cells alignment section of the device. In the structures used the cells align in so-called cell trains which are long streams of cells where all cells are equally spaced as they are at the hydrodynamic equilibrium position. However, these cell trains were found to only have a certain length before being interrupted, which was caused by a lack of homogeneity of the cell suspension. Though the cell suspension in the syringe was stirred with a magnet to prevent inhomogeneities, the cell suspension can't be stirred in the tubing. Moreover, the cells seemed to

accumulate at the chip inlet. By tapping the tubing or the syringe a burst of cells then was released. After entering the channel, these cells would align creating a cell train of a definite length. It was found to be extremely difficult to enable cell alignment simultaneously and for longer duration in both spiral channels and thus to create continuous cell trains in both cell channels.

The electrofusion part can also be improved. Cells at present often are not in contact before application of the electrical field. This contact can be improved by placing a serpentine channel structure in front of the DC electric field to enhance cell movement³¹ or by implementing a more efficient shrinkage module enabling continuous cell-cell contact,²⁶ or by electrical means, e.g. by applying an AC electric field to provide a DEP force to cause cell alignment and cell-cell contact³².

To improve electrofusion, a 3D electrode configuration can be used instead of the present 2D electrode configuration, as it will generate a homogeneous electric field along the channel wall, giving higher fusion efficiency.

Finally, once good electrofusion yield is obtained, coupling of this platform with picoinjectors to secure cell viability inside the droplets³³, and a microfluidic platform capable of screening functional hybridomas³⁴, can be a major step towards functional hybridoma creation on chip.

It should be stressed that the use of the present high-throughput platform is not at all restricted to cell fusion. The platform indeed is applicable for various other cell-based assays such as single cell analysis, cell transfection and cell differentiation assays. Instead of encapsulating cells in droplets, the platform can also be used to encapsulate cells in gel patches, creating a platform with still different properties.

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Journal articles

E. W. Kemna, R. M. Schoeman, F. Wolbers, I. Vermes, D. A. Weitz and A. van den Berg. High-yield cell ordering and deterministic cell-in-droplet encapsulation using Dean flow in a curved microchannel. *Lab Chip*, **12**, 2881-2887 (2012). (Chapter 3)

Schoeman, R.M. & Kemna, E.W.M. & Wolbers, F. & van den Berg, A. High-throughput deterministic single-cell encapsulation and droplet pairing, fusion, and shrinkage in a single microfluidic device. *Electrophoresis* **35**, 2-3, 385-392 (2014). (Chapter 4)

Rogier M. Schoeman, Wesley T. E. van den Beld, Evelien W.M. Kemna, Floor Wolbers, Jan C.T. Eijkel, Albert van den Berg. Electrofusion of single cells in picoliter droplets. Submitted to PNAS. (Chapter 5)

Rogier M. Schoeman, Evelien W.M. Kemna, Jan C.T. Eijkel, Albert van den Berg. Review on microfluidic devices for electrofusion. Manuscript in preparation. (Chapter 2)

Conference contributions

Oral

R.M. Schoeman, E.W.M. Kemna, F. Wolbers, A. van den Berg. Deterministic single cell encapsulation and droplet fusion, shrinkage and sorting in a single microfluidic device. NanoBioTech, November, 2012, Montreux, Switzerland.

R.M. Schoeman, E.W.M. Kemna, F. Wolbers, A. van den Berg. Deterministic single cell encapsulation, droplet pairing, fusion and shrinkage in a single microfluidic device. MicroNano Conference, December, 2012, Ede, The Netherlands.

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Poster

E.W.M. Kemna, R.M. Schoeman, L.I. Segerink, F. Wolbers, I. Vermes, A. van den Berg. Towards a high-throughput electrofusion platform using droplets: cell detection and deterministic encapsulation. 15th International Conference on Miniaturized Systems for Chemistry and Life Sciences, October 2-6, 2011, Seattle, Washington, USA

R.M. Schoeman, B. de Wagenaar, E.W.M. Kemna, F. Wolbers, I. Vermes, A. van den Berg. Lab-on-a-Chip platform for high-yield electrofusion in droplets. MicroNano Conference, December, 2013, Ede, The Netherlands

R.M. Schoeman, P.M. ter Braak, J. Bomer and A. van den Berg. Lab-on-a-Chip platform for high-yield electrofusion in droplets. NanoCity, October, 2014, Utrecht, The Netherlands.

R.M. Schoeman, P.M. ter Braak, J. Bomer and A. van den Berg. Lab-on-a-Chip platform for high-yield electrofusion in droplets. 18th International Conference on Miniaturized Systems for Chemistry and Life Sciences, October 26-30, 2014, San Antonio, Texas, USA

Lectures

R.M. Schoeman, E.W.M. Kemna, F. Wolbers, P.M. ter Braak, J. Bomer and A. van den Berg. Organen op Chip. Nederland Innoveert, Nationaal Techniekfestival, Februari, 2013, Eindhoven, The Netherlands.

Rogier M. Schoeman, Wesley T. E. van den Beld, Evelien W.M. Kemna, Floor Wolbers, Paul M. ter Braak, Johan Bomer, Jan C.T. Eijkel, Albert van den Berg. Lab-on-a-Chip platform for electrofusion of cells in droplets. University of Edinburgh, April, 2015, Edinburgh, Scotland.

Nederlandse samenvatting

In dit proefschrift worden een reeks microfluidische apparaten omschreven en besproken, met als resultaat een geïntegreerd ontwerp dat bestaat uit een aantal functionaliteiten die het mogelijk maken cellen te elektro-fuseren in een druppel met hoge continue doorstroom snelheid op chip. Het elektro-fuseren van cellen op chip is belangrijk, omdat dit kan leiden tot de productie van functionele hybridomas. Een hybridoma is het fusie product van twee verschillende ouder cellen, namelijk een plasma B cel en een myeloma cel. De plasma B cel is een antilichaam producerende cel. Deze antilichamen zijn nodig voor onderzoek, diagnostiek en verschillende behandelingstherapieën. Helaas hebben deze plasma B cellen maar een korte levensduur. Een myeloma cel daarentegen is een verkankerde variant van de plasma B cel, die een snelle celdeling ondervindt. Door deze twee cellen te combineren kan de dochtercel eigenschappen van de twee oudercellen krijgen, in dit geval een snelle celdeling en antilichaam productie. Deze kwaliteiten maken het mogelijk de hybridomas in een cel culture te houden en daardoor een soort antilichaam fabriek te creëren.

Natuurlijk waren we niet de eerste die op dit idee kwamen en daarom is er een literatuurstudie gedaan waarvan de resultaten te vinden zijn in hoofdstuk twee. In dit hoofdstuk worden eerst de basis van het gedrag van cellen in een elektrisch veld uitgelegd. Dit is erg belangrijk, omdat de meeste nieuwe technieken gebruik maken van elektrische velden om cellen te fuseren. Eerst maken ze vaak gebruik van dielektrophorese om cellen in een bepaalde richting te duwen of om ze op een bepaalde plaats vast te houden. Dit is nodig omdat de twee ouder cellen met elkaar in contact moeten zijn om ze te kunnen elektro-fuseren. In dit hoofdstuk wordt ook de elektroporatie van het celmembraan tot in detail besproken, om inzicht te krijgen in de verschillende condities waarin de cellen gebracht worden om een zo hoog mogelijk elektro-fusie rendement te krijgen.

In hoofdstuk twee worden verder de nieuwste technieken besproken en beoordeeld op hun vermogen cellen te paren, gebruiksgemak, rendementen, etc.. Als laatste wordt er een korte introductie gegeven over het druppel platform voor elektro-fusie.

Het druppel platform is in staat enkele cellen in te kapselen en daardoor ontstaat de mogelijkheid twee verschillende cel types in een enkele druppel te kapselen door twee druppels die allebei een ander cel type bevatten te fuseren. De theorie en techniek achter het inkapselen van enkele cellen in een druppel wordt uitgelegd in hoofdstuk drie. Het ontwerp van het platform bestaat uit een spiraal vormig kanaal dat door middel van hydrodynamische krachten de cellen een enkele evenwichtspositie dwingt. Er wordt aangetoond wanneer de passeerfrequentie van de cellen gelijk aan de druppelproductie frequentie bij de flow focus junctie, enkele cel inkapseling in een druppel een rendement behaald van 77%.

Als de enkele cellen ingekapseld zijn in de druppels, dan moeten de twee druppels die elk een ander cel type bevatten gepaard worden en vervolgens elektrisch samengevoegd worden zodat er een druppel ontstaat die de twee verschillende cellen bevat. Het proces hierachter wordt uitgelegd in hoofdstuk vier. Het apparaat wat beschreven wordt in hoofdstuk vier bevat een dubbele T-junctie die instaat is enkele cellen in druppels te kapselen en tegelijkertijd de verschillende druppels te paren. Als de gepaarde druppels het hoofdkanaal ingaan, dan passeren ze een elektrode paar dat ervoor zorgt dat de druppels elektrisch samengevoegd worden. Echter wanneer de druppels elektrisch zijn samengevoegd, dan bevinden de cellen zich in een grote druppel, waardoor de kans dat de cellen elkaar raken sterk verlaagd wordt wat wel nodig is voor cel elektro-fusie. Daarom zijn er drie hooivork vormige kanalen haaks op het hoofdkanaal geplaatst die ervoor zorgen dat het volume van de druppels verlaagd wordt door overtollig volume weg te zuigen. Deze techniek is gebaseerd op de verschillende hydrodynamische weerstanden in de kanalen. De hooivork vormige kanalen hebben kleine pilaren op hun raakvlak met het hoofdkanaal, waardoor voorkomen wordt dat de cellen uit de druppel verwijderd kunnen worden.

De volgende stap is het elektro-fuseren van cellen in een picoliter druppel. Dit proces wordt beschreven in hoofdstuk vijf. Het ontwerp wat in dit hoofdstuk wordt gebruikt is een eenvoudig spiraal vormig kanaal met aan het een einde een flow focus junctie waar de cellen ingekapseld worden, gevolgd door zes elektrode paren waarover de druppels geleid worden. Deze elektrode paren zijn verantwoordelijk voor de elektro-

fusie. De stroomsnelheden die gebruikt werden tijdens deze experimenten waren te laag om cellen in een evenwichtspositie te dwingen en daarom zijn Poisson ($\lambda=1$) statistieken gebruikt om het aantal ingekapselde cellen te bepalen. Dit resulteerde in 15% van alle geproduceerde druppels bevatte twee cellen. Per experiment werden er 500 druppels geanalyseerd, 15% van deze druppels bevatte twee cellen, dat zijn 75 druppels. Gedurende deze studie zijn er 5 experimenten uitgevoerd wat betekent dat (5×75) 375 mogelijke cel fusies hadden kunnen plaatsvinden. In totaal zijn 19 succesvolle cel fusies gevonden, wat resulteert in 5% elektro-fusie rendement. In dit hoofdstuk is een equivalent elektrisch circuit model ontwikkeld om het potentiaal over het celmembraan te beschrijven gedurende passage van cel bevattende druppel over een elektrode paar, om erachter te komen of in theorie het elektrode potentiaal over de elektrodes voldoende was om cel elektro-fusie te veroorzaken. In dit geval zijn HL60 cellen gebruikt om elektro-fusie aan te tonen. Om succesvolle elektro-fusie vast te stellen werden twee verschillende cel populaties, die later werden samengevoegd, gekleurd in verschillende kleuren. De eerste populatie had een blauwe kern kleuring en een groen membraan kleuring. De tweede populatie had alleen een rode kern kleuring. In de geproduceerde druppels werden vervolgens cellen aangetroffen die alle drie de kleuringen bevatten, waardoor bewezen is dat het mogelijk is cellen te elektro-fuseren in een picoliter druppel.

The volgende stap is het samenvoegen van alle eerder beschreven functionaliteiten samen te voegen in een enkel apparaat. Zo'n apparaat wordt beschreven in hoofdstuk zes. Het geïntegreerde apparaat bevat de spiraal vormige kanalen uit hoofdstuk drie en combineert deze met de Yin-Yang vormige ingangen van hoofdstuk vier. Vervolgens worden de kanalen breder en bevattende kanalen microstructuren die helische vortexen veroorzaken waardoor de cellen nog beter in hun evenwichtsposities geraken. Wanneer de cellen zich in hun evenwichtsposities bevinden worden ze ingekapseld door de dubbele T-junctie die besproken is in hoofdstuk vier. De dubbele T-junctie veroorzaakt ook druppel paring, waarna ze vervolgens elektrisch worden samengevoegd door de aanwezigheid van een elektrode paar. In plaats van drie hooivork vormige kanalen zoals in hoofdstuk vier, passeren de druppels nu zes haaks liggende zijkanalen om het druppel volume te reduceren. Wanneer uiteindelijk de

druppels over zes elektrode paren bewegen om cel elektro-fusie te initiëren, zoals besproken in hoofdstuk zes.

Het huidige ontwerp werkt en heft elektro-fusie rendementen van 5% behaald, daarom is het in staat te concurreren met bestaande technieken. Echter, er is nog veel ruimte voor verbetering, voornamelijk in het uitlijnen van de cellen. In de gebruikte kanalen worden de cellen uitgelijnd in zogenoemde cel-treintjes, wat lange rijen van cellen zijn waarbij de cellen zich allemaal op gelijke afstand van elkaar en in dezelfde hydrodynamische evenwichtspositie bevinden. Echter deze cel-treintjes kunnen maar een bepaalde lengte behalen voordat ze verstoord worden door een gebrek aan homogeniteit van de cel oplossing. Hoewel de cel oplossing in de injectiespuit continu geroerd werd met een magneet om inhomogeniteiten te voorkomen, kan de cel oplossing niet geroerd worden in de buisjes van de injectiespuit naar de chip ingang. Bovendien leken de cellen zich te verzamelen bij de chip ingang. Door op de buisjes of op de injectiespuit te tikken, kwamen cellen los en ontstonden cel-treintjes van verschillende lengte. Om continue cel-treintjes tegelijkertijd voor langere periodes in de twee kanalen te laten ontstaan werd als extreem moeilijk ondervonden

Het elektro-fusie gedeelte kan ook worden verbeterd. Nu zijn de cellen vaak nog niet in contact met elkaar wanneer ze zich in het elektrisch veld bevinden. Dit contact kan verbeterd worden door een serpentine kanaal structuur aan te brengen, waardoor cel beweging in de druppel wordt verhoogd. Door het volume van de druppels nog meer gereduceerd worden door de haaks geplaatste zijkanalen te optimaliseren, kan het cel contact ook verbeterd worden. Ook door een AC veld aan te brengen voor de elektro-fusie elektroden worden de cellen in de druppel door middel van dielektrophorese tegen elkaar aangedrukt.

Door 3D elektrode configuratie te gebruiken in plaats van een 2D elektrode configuratie, kan de elektro-fusie verbeterd worden doordat het elektrische veld meer homogeen is.

Tot slot, wanneer een goed elektro-fusie rendement is behaald, kan dit platform gekoppeld worden aan picoinjectors om de levensvatbaarheid van de cellen in een druppel te verhogen. Ook door het te koppelen aan een platform dat in staat is

hybridomas te analyseren, zou een grote stap voorwaarts zijn richting het creëren van functionele hybridomas op chip.

Bovendien is dit platform niet gelimiteerd tot cel fusie. Dit platform kan gebruikt worden voor talloze cell-based assays, zoals single cel analyse, cel transfection en cell differentiatie assays. In plaats van cellen druppels in te kapselen, kan dit platform ook gebruikt worden om cellen in gelen in te kapselen.

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