
A microfluidic toolbox for cell fusion

Flora W.Y. Chiu, Hakan Bagci, Amanda G. Fisher, Andrew J. deMello, Katherine S. Elvira

2016

Citation for this paper:

Chiu, F.W.Y., Bagci, H., Fisher, A.G., deMello, A.J. & Elvira, K.S. (2016). A microfluidic toolbox for cell fusion. *Journal of Chemical Technology & Biotechnology, 91*(1), 16-24. https://doi.org/10.1002/jctb.4803

A microfluidic toolbox for cell fusion

Flora Wing Yin Chiu,[1] Hakan Bagci,[2] Amanda G. Fisher,[2] Andrew J. deMello[1] and Katherine S. Elvira[1,]*

[1]Institute of Chemical and Bioengineering, Department of Chemistry and Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland

[2]Lymphocyte Development Group, MRC Clinical Sciences Centre, Imperial College, Hammersmith Hospital Campus, Du Cane Road, London W12 0NN, UK

*katherine.elvira@chem.ethz.ch

Abstract

Cellular fusion is a key process in many fields ranging from historical gene mapping studies and monoclonal antibody production, through to cell reprogramming. Traditional methodologies for cell fusion rely on the random pairing of different cell types and generally result in low and variable fusion efficiencies. These approaches become particularly limiting where substantial numbers of bespoke one-to-one fusions are required, for example for in-depth studies of nuclear reprogramming mechanisms. In recent years, microfluidic technologies have proven valuable in creating platforms where the manipulation of single cells is highly efficient, rapid and controllable. These technologies also allow the integration of different experimental steps and characterisation processes into a single platform. Although the application of microfluidic methodologies to cell fusion studies is promising, current technologies that rely on static trapping are limited both in terms of the overall number of fused cells produced and their experimental accessibility. Here we review some of the most exciting breakthroughs in core microfluidic technologies that will allow the creation of integrated platforms for controlled cell fusion at high throughput.

Keywords: droplet microfluidics, cell fusion, cell encapsulation, single-cell, high efficiency, cell sorting.

Introduction

Cellular fusion is an important part of the normal growth and development of organisms, ranging from yeast to humans.[1] The process occurs naturally, and the most prominent example of developmentally induced cell fusion is between an oocyte and a sperm cell, which gives rise to a fertilized egg and hence the generation of a new life.[2] The importance of cell fusion can also be exemplified by other biological processes such as the development of skeletal muscles (myoblast fusion), bones (osteoclast fusion) and placentae (trophoblast fusion).[3–5] Moreover, cell fusion plays a key role in innate immune responses, as macrophages can fuse to form multinucleated giant cells which can engulf and destroy pathogens.[6] Cell fusion also takes place in lower eukaryotes such as *Caenorhabditis elegans* (epidermal cell fusion) and *Drosophila melanogaster* (myoblast fusion).[1,7] On the other hand, failed or unregulated cell fusion is implicated in human diseases.[8] Despite the importance of these processes in living organisms, very little is known about the mechanism of cell fusion or the factors that control the fusion process.[9,10] Accordingly, much research has been directed towards the study of cellular membrane fusion and the cellular components and signalling factors involved, with fusion involving human cell types being of particular interest due to its therapeutic significance.[11]

In addition to cell fusion within physiological environments, cell fusion *in vitro* has also been reported. Fusion of cells of the same species or of different species results in the formation of homokaryons or heterokaryons respectively, where the nuclei of the fusion partners are included in a single cytoplasm but remain separate and stable over time (up to a few days, depending on cell type and culture conditions).[12,13] Growth and division processes can also occur in homo- or heterokaryons, where nuclei are subsequently fused to give proliferating hybrid cells that contain double the genome dose.[14] The possibility to fuse cells of distinct cell types has attracted much interest in molecular biology. For example, heterokaryons, in which nuclei of different cell types are contained in the same cytoplasm, serve as a valuable experimental system to study the control of gene expression and the impact of one genome on another**.**[12] In addition, fusing cells at different states of differentiation or at different stages of the cell cycle allows the study of genetic complementation and cellular dominance or differentiation plasticity.[15] Indeed, in recent times, cell fusion has become an experimental tool to induce nuclear reprogramming, a process by which the fate of a cell is altered.[14] Nuclear reprogramming falls into two broad categories: pluripotent reprogramming, in which the differentiated state of a cell can be reversed back to a pluripotent embryonic stem-like state, and lineage reprogramming, in which the differentiated state of a cell is directly switched into another.[16] For instance, it has been reported that the reprogramming of human B lymphocytes by mouse ES (mES) cells can be achieved by cell fusion *in vitro,* where the resultant reprogrammed B cells elicit the expression of a human ES-specific gene profile.[17] The development of nuclear reprogramming technology has led to great excitement in the scientific community regarding the potential use of reprogrammed cells to not only improve the understanding and treatment of diseases, but also in patient-specific cell replacement therapies.[18] Nevertheless, there is a need for a better mechanistic understanding of the reprogramming process. In particular, characterisation of the factors and regulators required for efficient derivation of induced pluripotent stem (iPS) and somatic stem cells and how they can subsequently be induced to differentiate towards the cell type of interest is critical.[19] In this respect, heterokaryons act as a useful tool to study nuclear reprogramming, because the effects of trans-acting factors specific for one cell type on altering the transcriptional programme of the partner can be investigated. It also allows examination of the earliest molecular events that occur in the nucleus during reprogramming that have until now been difficult to capture. Furthermore, interspecies heterokaryons have the additional benefit of allowing gene expression changes to be sensitively monitored on the basis of species-specific genetic differences, so that key events in successful reprogramming can be uncovered.[17,20]

Hybrid cells are also important tools for molecular biology. When cultured, the predominant growth of hybrid variants that have lost chromosomes derived from either one or both parental cell types becomes evident. Taking advantage of this, gene mapping[21] has been historically used to map specific phenotypes to gene products. Hybrids generated between tumour and normal somatic cells have also been widely used for malignancy studies.[22] Most importantly, the use of hybrid cells has led to the development of promising therapeutic applications[23], of which the production of hybridomas (hybrid cells between an immortalised cell and an antibody producing lymphocyte), and hence the generation of monoclonal antibodies (mAb) against an antigen of choice, is the most well-known. This technique was first introduced in the 1970s[24] and has been implemented extensively over the past few decades as a source of humanised monoclonal antibodies in targeted cancer therapies.[25] More recently developed cellular-based cancer vaccinations are another key application derived from hybrid cells. This technology is based on the fusion of dendritic cells and tumour cells, from which the hybrid cells can induce an anti-tumour specific immune response. Such an approach has been shown to be effective both *in vitro* and *in vivo*, and a plethora of clinical trials have been conducted.[26]

Despite its utility in a variety of applications, current methods for achieving cell fusion among cell populations *in vitro* are cumbersome and inefficient. They include the use of inactivated Sendai virus, polyethylene glycol (PEG), focused laser beams and electric pulses, of which PEG-mediated fusion and electrofusion represent the most commonly used techniques due to their relative simplicity.[27–31] Electrofusion is achieved through electroporation[32]: as cells are exposed to short pulses of a high-strength DC voltage, membrane reorganisation occurs, resulting in the formation of nanopores. Electroporation is reversible and pores on two cells under investigation must come into contact so that membrane connection can be induced. However, the use of excessive fields can result in cell rupture and lysis. Cell-to-cell connection facilitates cytoplasmic exchange between the two cells and eventually fusion between the pair. The mechanism of PEG-mediated fusion[33] is slightly different. The major effect of PEG is volume exclusion, which enables the formation of large areas of close membrane contact between cells. Subsequent removal of PEG and incubation of cells leads to the formation of small cytoplasmic bridges between cells, with the expansion of these cytoplasmic connections (promoted by cell swelling) resulting in fusion. The major drawback of such bulk cell fusion methods is that they rely on random initial cell-cell pairing, making it extremely difficult to fuse in a selective and controllable manner. Fusion efficiencies when using PEG as fusogen are also generally low. For example, using PEG to chemically fuse mES and human B cells typically yields between 10 and 15% viable heterokaryons.[17,34] Electrofusion has been shown to give higher efficiencies when compared to PEG treatment[35] (varying considerably with cell type) but the other drawbacks mentioned above remain unresolved. This prevents, for example, detailed mechanistic studies of fusion-mediated reprogramming, as screening of substantial numbers of heterokaryons fused in one-to-one ratio, is required. Additionally, for other applications such as hybridoma production and cell vaccine preparation, an efficient protocol is clearly needed. To this end, a more robust methodology that allows cell-to-cell fusion in high throughput and in a controlled manner is required.

Current microfluidic platforms for cell fusion

Microfluidic systems precisely control fluids that are geometrically constrained in sub-millimetre scale environments, and offer many advantages for cell manipulation such as the ability to use small quantities of samples and reagents, reduced analysis times and the possibility to conduct studies at the single cell level. Examples of reported applications include on-chip long-term cell culture[36], cell trapping[37], cell screening[38,39] and cell patterning.[40] Microfluidic systems for cell fusion have also been developed. In particular, much research has focused on the use of electrofusion to accomplish cell fusion due to the ease of microelectrode integration within a planar chip format and the ability to precisely manipulate electric fields, in both space and time, at a scale comparable to that of a biological cell. A recent review by Hu *et al.*[41] provides a detailed account of this class of microfluidic systems and therefore only key literature will be highlighted in here. In brief, most of these systems incorporate continuous fluid flows and consist of a microfluidic channel along which an array of microelectrodes is fabricated. These microelectrodes are designed such that the electric field is non-uniform within the channel, with higher field strengths at specific positions. For example, Hu *et al.*[42] used an array of protruding microelectrodes such that when an AC electric field is applied, cells flowing along a microfluidic channel are attracted to the side-wall surfaces of the protruding electrodes due to the higher field strength. This was then followed by cell alignment due to dielectrophoresis (**Figure 1a**). Cells can then be exposed to high direct current (DC) pulses, to induce (reversible) electroporation and ultimately cell fusion.[42] Generally speaking, the interplay between microelectrode geometry and electric field governs pairing efficiencies in this type of devices, which typically fall in the range of 40 to 70%.[41] The major disadvantage of using protruding microelectrode arrays is that cells can be trapped in areas between adjacent electrodes (indicated by white circles in **Figure 1a**). In these areas, electric field strength is lower, resulting in reduced fusion efficiencies. Moreover, pairing of cells is still a random process, where both homogenous and heterogeneous cell pairing can occur. Similar to bulk electrofusion methodologies, multi-cell fusion can occur in this type of microfluidic platforms and separation of fused cells from non-fused cells on-chip is not possible.

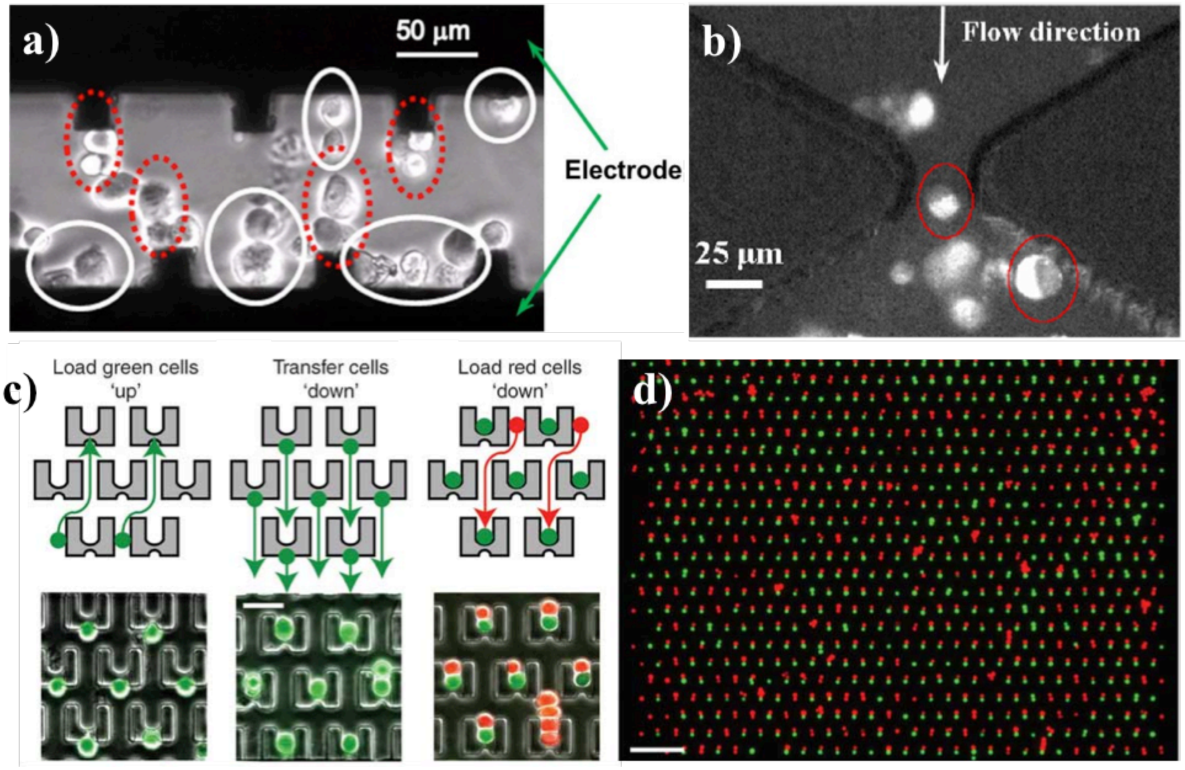

Figure 1. *Microfluidic platforms for cell fusion. a) Cell alignment in flow in a microchannel with an integrated microelectrode array such that electric field strength at sidewall surfaces of the protruding electrodes is higher than other positions of the channel. The red dotted circles show cell pairs aligning at the surfaces of protruding*

electrodes and white circles show cells trapped in between adjacent protruding electrodes.[42] *Reprinted with permission from Biomicrofluidics* ***5****, 034121 (2011). Copyright 2011, AIP Publishing LLC. b) Fluorescent image of fused cells created via the flow-through method proposed by Wang et al.* [43] *Cells are pre-conjugated based on biotin-streptavidin interactions and the red circles highlight fused cells where one cell is labelled with calcein AM and is one unlabelled. Reprinted with permission from Appl. Phys. Lett.* ***89****, 234102 (2006). Copyright 2006, AIP Publishing LLC. c) Three-step loading protocol to pair different cell types in weir-based cell traps (the scale bar is 50 μm) and d) overlay of red and green fluorescence images of cells after loading and pairing using traps shown in (c) (the scale bar is 200 μm).*[44] *Reprinted by permission from Macmillan Publishers Ltd: Nature Methods* ***6****, 147 - 152 (2009), copyright 2009.*

To truly improve the efficiency of cell fusion, both the mechanism of initiation of membrane fusion as well as control over how cells are brought into contact with each other and paired, are critical. At the same time, undesirable fusion events, such as those between the same cell type or multi-cell fusion, must be avoided or removed from final samples. Cell pairing by chemical methods or microstructures have been proposed to improve fusion yield. For example, Wang *et al.* reported a flow-through method in which cells are introduced into a narrow microfluidic channel (designed to contain no more than three cells across the channel), followed by the application of a continuous, DC voltage to initiate fusion, using electrodes integrated on-chip (**Figure 1b**).[43] Using this system, fusion of Chinese hamster ovary cells was demonstrated. Cells were conjugated based on biotin-streptavidin interaction before being subjected to an electric field. Depending on the electric field strength, the number of pulses applied and their duration, about 40% of the total number of cells loaded in device were fused and remained viable. Despite showing an improved efficiency when compared to conventional bulk methods, this approach lacks the ability to controllably pair cells, and thus the overall fusion yield is still low. Skelley *et al.* proposed the use of weir-based cell traps arrayed within a microfluidic channel (**Figure 1c and 1d**).[44] A key advantage of this method is that cell pairing relies solely on passive hydrodynamics, thus obviating the need for label-modified cells. Additionally, cell pairs are held close in contact in the traps, which is a prerequisite for successful cell fusion. Both electrofusion and PEG-mediated fusion can be accommodated in this system using mES cells, mouse embryonic fibroblasts (mEFs), myeloma cells, B cells and NINH3T3 fibroblasts to give rise to hybrid cells. Cell pairing efficiencies of up to 70% were demonstrated with overall fusion efficiencies significantly higher than conventional bulk protocols or commercial fusion chambers. However, the percentage of fused cells recovered from the device post-fusion and their viability were not reported. Reprogramming of mEFs *via* fusion with mES cells[44] and pair-wise interaction studies of mouse lymphocytes at a single-cell level[45] have also been performed using this system. Using a very similar cell-trapping microdevice, but implementing a deformability-based approach (use of high flow rates caused cells to deform and were hence squeezed through a constriction into each cell trap) to capture and pair cells, Dura *et al.* reported pairing and electrofusion efficiencies of up to 80% and 95% respectively, and an overall yield (fusion between correctly paired cells) of 56%.[46] This system also has the potential to fuse more than two cell partners. However, the exposure of cells to hypoosmolar buffer would be necessary to facilitate fusion of cells with a large difference in cell size, and since different cell types vary in their responses to deformation, excessive hydrodynamic forces induced within the device could impair cell viability. Overall, the application of these trap systems although promising, is limited as only a few thousand traps can be included in a single device.

Another interesting approach to perform cell fusion on-chip involves the use of micro-orifices to create an electric field constriction. The idea of field constriction using a micro-orifice for cell fusion was first proposed by Masuda *et al.*[47] in 1989 and later adopted by Techaumnat *et al.*[48] to perform real-time observation of cell fusion. In this system, two parallel microfluidic channels are separated by an insulating barrier along which an orifice is created (**Figure 2a**). When an AC voltage is applied across the electrodes, the presence of the insulating barrier results in a concentration of electric field lines at the small orifice. Cells are therefore attracted to, and forced into contact with each other, at the orifice based on dielectrophoresis. Electroporation and subsequent cell fusion were then induced by further application of a pulsed voltage. Most importantly, under the applied electric field, only one-to-one cell fusion between the cell pair in the orifice was plausible, even when cell chains are formed near the orifice. To further improve fusion yield, Gel *et al.*[49] developed a device comprised of an array of micro-orifices (**Figure 2b**). By modifying the mould fabrication process, the orifice size could be tailored (ranging from 2-10 μm) to accommodate different cell types and sizes.[50] For instance, fusion of mouse fibroblasts using the device shown in **Figure 2b** resulted in a pairing efficiency of 95-100% and a fusion efficiency of over 95%. Nevertheless, the throughput of this type of devices is generally low due to the limited number of orifices that can be created along the channel. At the upper limit, Kimura *et al.*[51] fabricated a micro-orifice array sheet that could accommodate up to 6×10^3 micro-orifices in a two-dimensional arrangement (**Figure 2c and 2d**). Using this device, fusion yield was reported to be about 80%. Despite the high fusion yield, the throughput of this method is still relatively low. Furthermore, operation of the device is rather complex, as pairing of cells relies on not only the dielectrophoretic force, but also on cell sedimentation. In other words, the device had to be flipped over whilst keeping the voltage on (to keep cells in the upper chamber trapped at the orifices), in order to allow cells from the lower chamber to sediment and reach orifices for pairing and fusion (**Figure 2e**).

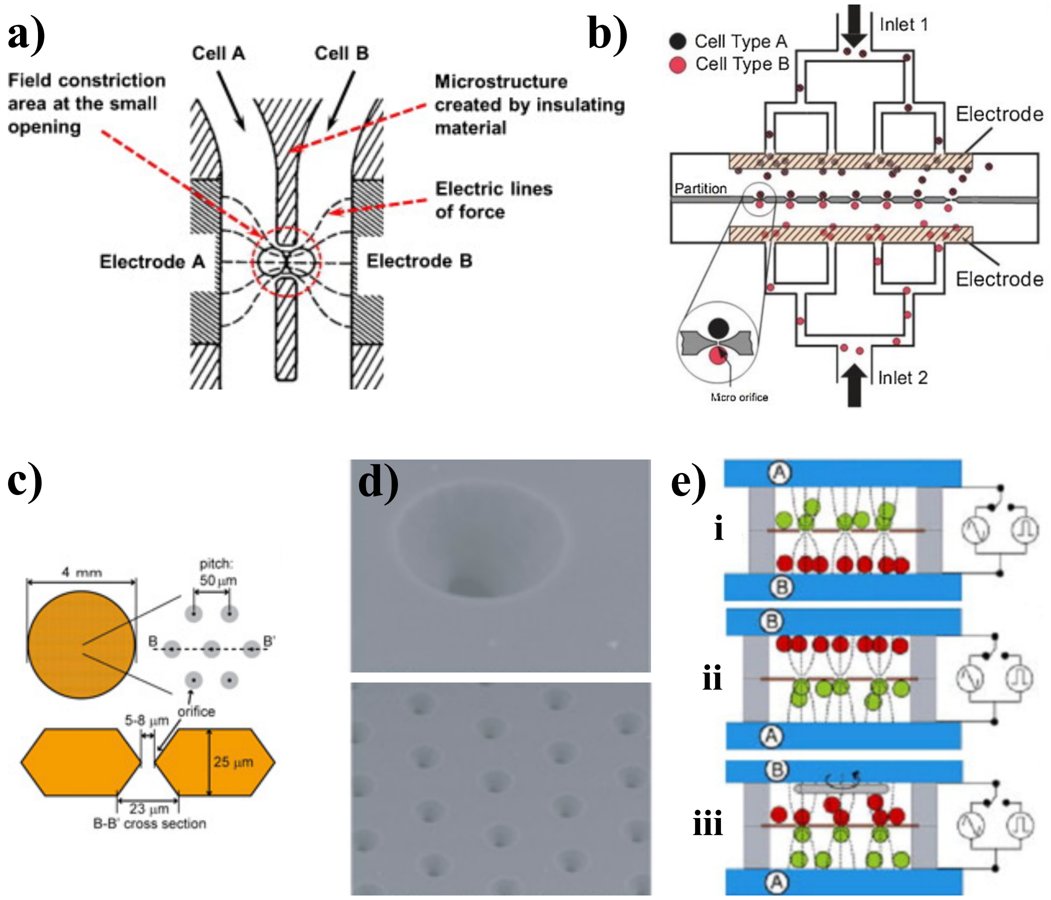

Figure 2. a) Cell pairing based on field constriction at a micro-orifice.[41,47] *Reproduced from Sens. Actuators B Chem.* ***178****, 63 (2013) with permission of Elsevier. b) Schematic of a microfluidic device containing an array of micro-orifices for cell fusion as proposed by Gel et al.*[49] *Reproduced from Biomicrofluidics* ***4****, 022808 (2010) with permission of AIP Publishing LLC. c) Structure of a micro-orifice array sheet and d) SEM images of the corresponding micro-orifices.*[51] *e) Schematic of the cell pairing process on the orifice sheet: (i) under applied electric field, cells in the top chamber (green circles) are attracted to orifices due to dielectrophoresis, (ii) the device is then flipped over whilst keeping the voltage on to allow (iii) cell pairing when cells originally in the lower chamber (red circles) fall into orifices via both sedimentation and dielectrophoresis.*[51] *Reproduced from Electrophoresis* ***32****, 2496 (2011) with permission of Wiley.*

Droplet-based microfluidic platforms for cell fusion

Droplet-based microfluidic technologies could offer the highest potential and versatility for single-cell studies. These platforms usually involve the generation of monodisperse aqueous droplets in a continuous oil phase, where each droplet effectively acts as an individual and isolated reaction chamber.[52] Droplets can be generated and manipulated at kHz rates, with each droplet having volumes between a few femtoliters and hundreds of nanoliters. Droplet contents can be varied, exchanged or sampled using a large variety of merging, splitting and sorting strategies,[53–57] and droplets significantly can support living cells and organisms for many days through the use of highly gas-permeable fluorinated oil phases and biocompatible surfactants.[56] Another key advantage of droplet microfluidic systems is the ease of integration of multiple experimental and analytical steps within a single platform. This makes them particularly suitable for single-cell studies, which usually involve complex workflows. For example, Cho *et al.*[58] describe the first fully integrated and high-throughput droplet-based microfluidic platform for the assessment of photodynamic therapy photosensitizer efficacy. This system enabled screening almost an order of magnitude faster than conventional methods. Perhaps the most interesting feature of the approach is its ability to gather multidimensional information in a rapid fashion; in this case the authors were able to measure the effect of oxygen saturation and dark toxicity in the same device, an operation not possible using traditional methods.

Not surprisingly, performing cell fusion in droplets has been attempted. Schoeman *et al.* reported a platform that allows parallel encapsulation of HL60 cells in two separate streams, pairing and merging of the droplets formed, and droplet shrinkage of merged droplets to promote cell contact for subsequent electrofusion. They achieved pairing and merging of droplets with efficiencies close to 100% and 95% respectively, and ultimately 40% of merged droplets were shown to contain exactly two HL60 cells. This platform was, however, only tested with one cell type, and, surprisingly, the authors did not perform the final step of cell fusion within the device.[59] Nevertheless, it is clear that the use of droplets potentially affords the high degree of control required for efficient cell fusion in a high-throughput manner. Furthermore, there are other microfluidic techniques that can be leveraged to overcome the current limitations of this platform. In the final section of this review we describe droplet-based microfluidic tools or building blocks that can be used to create an integrated platform for cell fusion studies with the aim of encouraging research in this field.

The first component required to enable the realisation of a fully integrated droplet-based[60] microfluidic platform for cell fusion is the encapsulation of cells within droplets. More specifically, the correct number of cells must be brought together in each droplet, which in most cases means one of each cell type. This can be achieved using two different approaches. The first involves single-cell encapsulation of each fusion partner separately, followed by droplet merging to bring the desired cells together inside the united droplet.[59] The second approach involves the co-encapsulation of the two cell types during droplet formation such that one cell of each type is delivered to each droplet.[61]

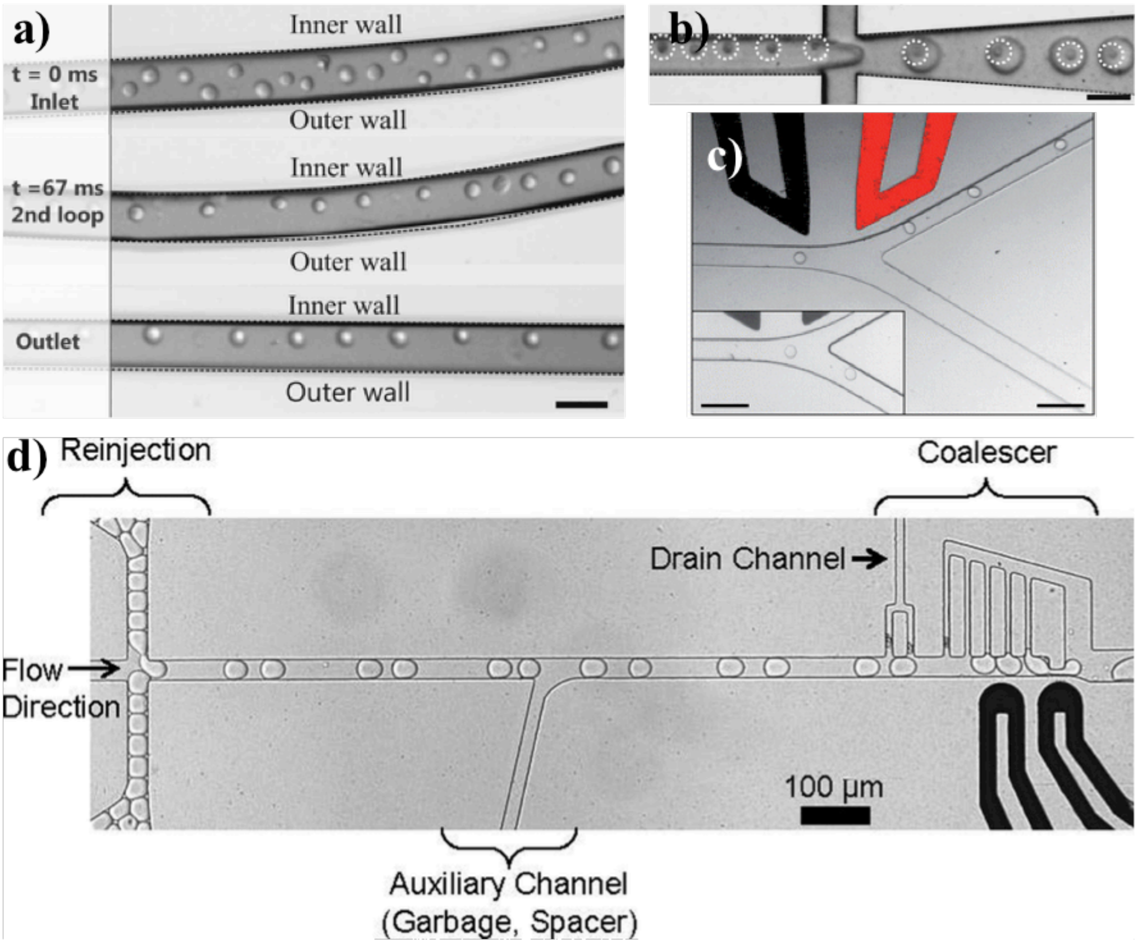

Figure 3. *Droplet-based microfluidic tools for cell fusion. a) and b) Dean-coupled inertial ordering in curved channels to allow high-efficiency single cell encapsulation in droplets.*[62] *Reproduced from Lab Chip,* ***12****, 2881 (2012) with permission of The Royal Society of Chemistry. c) Droplet sorting via dielectrophoresis. Main image shows sorting of droplets when an AC electric field is applied (droplets were deflected towards the upper channel). Inset image shows droplets flowing into the lower channel (due to hydraulic resistance in the absence of applied electric field).*[56] *Reproduced from Lab Chip,* ***9****, 1850 (2009) with permission of The Royal Society of Chemistry. d) Synchronisation of two populations of pre-formed droplets followed by electrocoalescence of the*

synchronised droplet pairs.[63] *Reproduced from Lab Chip,* ***14****, 509 (2014) with permission of The Royal Society of Chemistry.*

Traditional methods for single cell encapsulation use diluted cell samples and deliver cells to a droplet generation nozzle in a random fashion. This yields a population of droplets with a Poissonian-distributed cell occupancy[64], i.e.

$$P_{\lambda,k} = \lambda^k exp(-\lambda)/k!$$

Here $P_{\lambda,k}$ is the probability of a droplet containing k number of cells, with λ being the mean number of cells *per* droplet. Single cell encapsulation efficiencies in this case are generally low, for example, the probability of a droplet containing only one cell is limited to 36.8% when $\lambda = 1$.[61] However, to avoid droplets containing multiple cells, diluted samples are normally used. For instance, for $\lambda = 0.3$, the probability of droplets containing a single cell is around 22%, and while 74% of the droplets formed will be empty, only around 3.7% will contain two cells or more.[65] Co-encapsulation of cell pairs of two distinct cell types based on Poissonian statistics will occur with even lower efficiencies, with the probability of a droplet containing one cell of each type being limited to 13.5%.[61] However, the use of this method to encapsulate both fusion partners simultaneously into the same droplet does not require droplet paring and merging, and the handling of cells prior to fusion can hence be minimised. In light of the limits imposed by Poissonian statistics, various methods have been developed to increase encapsulation efficiencies beyond the predicted probabilities (for both the case of single cell encapsulation and co-encapsulation). In this respect, inertial microfluidic strategies have gained much popularity in recent years.[66] These passive methods make use of the inertial lift forces within narrow and high aspect ratio channels to focus (at specific cross-sectional positions in a microchannel) and/or order (regular cell-to-cell distances) cells prior to encapsulation.[67] Subsequently, by matching the periodicity of cell flow with droplet generation, cell encapsulation efficiencies can be significantly increased. Edd *et al.*[68] first demonstrated self-ordering of HL60 cells *via* inertial migration within a straight rectangular microchannel of just 6 cm in length. The system was tested using samples with a range of cell densities and resulted in much higher fractions of droplets with single-cell occupancy when compared to experiments without ordering. For example, with a cell sample of $\lambda = 0.5$, over 50% of droplets were shown to have only one cell, while droplets with multiple cells were kept below 5%. Employing the same ordering channel, Lagus *et al.*[61] generated ordered trains of two separate strains of *C. reinhardtii* cells and performed co-encapsulation. The reported co-encapsulation efficiency of one cell of each strain in a droplet showed a two-fold improvement compared to that based on Poissonian statistics. Kemna *et al.*[62] achieved ordering of HL60 and K562 cells within a curved channel with a single cell encapsulation efficiency of almost 80% (**Figure 3a and 3b**). Due to the use of curved channels, additional Dean forces are introduced, and in combination with the inertial forces, cells are focused and ordered efficiently.[69]

The platform proposed by Schoeman *et al.*[59] described previously also made use of Dean-coupled inertial ordering to enhance single-cell encapsulation efficiency. In this case, the efficiency of single cell encapsulation was close to 70%. That said, the primary drawback of inertial microfluidic strategies is the requirement for relatively high flow rates, which may compromise the survival rates of cells post-encapsulation. Also, for cell populations that have large intrinsic size variations, inter-cell ordering distances may vary considerably, making controlled single cell encapsulation challenging. Chabert *et al.*[70] reported another approach based purely on passive hydrodynamics to increase single cell encapsulation efficiencies. This system utilised cell-triggered Rayleigh-Plateau instabilities induced in a jet flow[71] to encapsulate cells and create monodisperse droplets. Resulting droplets underwent self-sorting on the basis of two hydrodynamic mechanisms: lateral shear-induced drift[72] and sterically driven dispersion, with up to 80% of the sorted droplets containing only one cell and with less than 1% of droplets being empty.

An alternative strategy to such passive methods is to actively sort droplets containing cells from empty droplets. Baret *et al.* developed the first system capable of sorting picoliter-sized droplets according to their fluorescent signature based on dielectrophoresis (**Figure 3c**).[56] To validate the system, mixtures of two different strains of *E. coli* cells (expressing either the reporter enzyme β-galactosidase or an inactive variant) were first emulsified with a fluorogenic substrate, followed by re-injection of the pre-formed droplets into the sorting system to separate the population of droplets containing the active variant, which were 100-fold more fluorescent upon hydrolysis with the fluorogenic substrate than the inactive variant. The sorting throughput was 300 droplets *per* second, with a false positive error rate of less than 1 out of 10^4 droplets. Mazutis *et al.*[65] integrated a similar sorting system into their droplet-based platform for binding assays. Depending on various factors including the flow rates of emulsion and fluorinated oil (for spacing droplets) flows and the duration of the sorting pulse, this type of sorter system can sort droplets at rates up to 2 kHz.[56,65,73] However, this is still much lower than commercially available fluorescence-activated cell sorters (FACS), which can sort at rates of up to 50 kHz[74] (although FACS sorts based on a continuous flow and cells are not compartmentalised individually like in the case of droplets). Recently, Sciambi *et al.*[57] described a sorter that can accurately sort 25 µm-sized droplets encapsulated with fluorescent beads at rates up to 30 kHz. This system was designed based on the aforementioned system, but with new microstructures called gap dividers being introduced to the device to reduce droplet splitting at the sorting junction as well as to minimise oil spacer flow rate, which are the major factors limiting throughput in previously reported sorters. This system is ten times faster than existing droplet-sorters and provides better enrichment of specific droplet subpopulations. Moreover, since the use of this type of sorting systems is independent of the cell type involved or cell size, they are more robust and reliable than passive methods for obtaining droplets encapsulating a targeted number of cells.

Once populations of single cells in droplets are achieved in the required numbers, there are two important and closely related microfluidic processes required for realising cell fusion, namely droplet synchronisation (pairing) and droplet merging (fusion). In other words, droplets containing cell fusion partners have to be brought into contact with each other and fused to create one entity. The most straightforward methods are passive and simply involve ensuring that the two populations of droplets flow in a sequential manner that causes pairing. Droplet fusion can then be realised when the synchronised flow is subjected to a merging architecture downstream. A range of strategies for producing paired droplet streams have been reported. For example, the use of combined T-junctions,[59,75,76] where synchronisation is achieved on the basis of hydrodynamic coupling and pressure equilibration between the two droplet flows has proved popular. A similar system integrated with a passive pressure oscillator was proposed by Hong *et al.*,[77] and Schoeman *et al.*[59] used a double T-junction to achieve synchronised formation of droplets containing HL60 cells from two separate streams. Step emulsification (the production of droplets occurs due to a step change in the height of the microchannel at the interface between two immiscible fluids) has also been used to achieve synchronisation.[78,79] Application of these systems for cell fusion is, however, limited unless it can be coupled with a droplet formation unit that allows high efficiency single cell encapsulation (for example cell ordering prior to encapsulation) or with a droplet sorter, since otherwise the subsequent droplet merging step will cause even lower cell pairing efficiencies. Another strategy is to decouple droplet formation from droplet synchronisation, and use a microfluidic platform that has the ability to synchronise two streams of pre-formed droplets. This provides much more flexibility and control of droplet populations. Lee *et al.*[63] demonstrated the synchronization of two different populations of pre-formed droplets *via* the use of opposing T-junctions, with merging of the synchronised droplets also shown in the same device (**Figure 3d**). The synchronisation error rate was 13% with the droplet merging yield approaching 85%. However, Dressler *et al.*[80] recently reported a different methodology to perform synchronisation also using pre-formed droplets, with error rates less than 0.2%. The only disadvantage of this system is that a large number of droplets are discarded during the synchronisation process, although future refinements could recycle discarded droplets to ensure complete and waste-free synchronisation of the two droplet populations. However, to date, most of these synchronisation methods have not been tested with cell-containing droplets.

After a train of single cell-containing droplets has been synchronised in the required sequence, droplet merging, or fusion, must be induced to bring the cells into contact. In most cases, water-in-oil emulsions are stabilised with the use of a surfactant and simply inducing a collision between droplets will not result in merging. To ensure merging, two droplets must be brought into close contact and the surfactant layer covering the droplets must be destabilised. There are multiple methods to induce destabilisation, such as electrical impulses (*i.e.* electrocoalescence),[63,81,82] addition of a poor solvent for the surfactant,[83] laser heating,[84] or through the use of different concentrations of surfactant.[85] Perhaps the most challenging part of droplet merging is to bring the droplets into close enough contact for a sufficient period time. This is usually achieved using microstructures on-chip to slow down the leading droplet and allow the following droplet to catch up within the synchronised stream. Examples of reported geometries for this purpose include widening channels,[86] pillar structures[63,87] and zig-zag structures.[85] A more vigorous method to bring droplet together involves the use of on-chip electrodes to attract and deform the lead droplet within the microchannel and allow the continuous phase to flow around the trapped droplet. As the second droplet approaches, merging occurs and the resultant merged droplet is directed along the channel due to pressure upstream.[81] The use of microfluidic tools such as these should enable populations of single cells to be paired in the required order and merged so that they are contained in a single droplet, prior to fusion of the paired cells. To date, no induced fusion of cells within droplets has been reported in the literature, although of the methods for cell fusion described previously, both PEG-mediated and electrofusion of cells have been shown in microfluidic (non-droplet) formats.

The integration of cell fusion in droplet-based microfluidic systems will lead to the generation of large numbers of individual heterokaryons that are necessary for more detailed analysis, for example in the case of pluripotent reprogramming. These platforms could be used to assess reprogramming success and failure over time and to examine how transcription factor binding to somatic DNA initiates reprogramming (using for example chromatin immunoprecipitation methods). To enable these studies, reporters of successful reprogramming will need to be engineered within somatic cells (for example fibroblasts carrying Oct4 promoter driven GFP) to allow real-time tracking of events in heterokaryons. In addition, high-throughput cell fusion platforms will allow the application of drug and RNAi-based screens for determining factors that enhance or prevent cellular reprogramming. Overall, it is clear that the components of a droplet-based microfluidic platform for high-efficiency cell fusion are ready and waiting for integration and application to fundamental biological questions.

Conclusions

We hope that this review highlights various wide-ranging microfluidic tools that are available for use in cell fusion studies. The process of single cell encapsulation in droplets, sample enrichment and droplet synchronisation and merging are highly applicable to cell fusion studies and the use of microfluidic technologies to perform and study cell fusion is an emerging and exciting area of research. The inherent ability of such systems to exert high levels of control over single cells whilst enabling the manipulation of thousands of cells *per* second is likely to impact cell fusion studies in a major way, allowing the pairing and fusion of distinct cell types in a controllable, on-demand and high throughput fashion. Such technologies will undeniably prove useful for in-depth biological studies of a variety of cell-based systems.

References

1. Ogle BM, Cascalho M, and Platt JL. Biological implications of cell fusion. *Nat Rev Mol Cell Biol*. **6**:567–75 (2005).
2. Wassarman PM, Jovine L, Qi H, Williams Z, Darie C, and Litscher ES. Recent aspects of mammalian fertilization research. *Mol Cell Endocrinol*. **234**:95–103 (2005).
3. Horsley V, and Pavlath GK. Forming a Multinucleated Cell: Molecules That Regulate Myoblast Fusion. *Cells Tissues Organs*. **176**:67–78 (2004).
4. Pötgens AJG, Schmitz U, Bose P, Versmold A, Kaufmann P, and Frank H-G. Mechanisms of Syncytial Fusion: A Review. *Placenta*. **23, Supplement A**:S107–13 (2002).
5. Vaananen HK, Zhao H, Mulari M, and Halleen JM. The cell biology of osteoclast function. *J Cell Sci*. **113**:377–81 (2000).
6. Vignery A. Osteoclasts and giant cells: macrophage–macrophage fusion mechanism. *Int J Exp Pathol*. **81**:291–304 (2000).
7. Mohler WA, Shemer G, del Campo JJ, Valansi C, Opoku-Serebuoh E, Scranton V, Assaf N, White JG, and Podbilewicz B. The Type I Membrane Protein EFF-1 Is Essential for Developmental Cell Fusion. *Dev Cell*. **2**:355–62 (2002).
8. Dittmar T, and Zänker KS, editors. Cell Fusion in Health and Disease. Dordrecht: Springer Netherlands; (2011).
9. Chen EH, and Olson EN. Unveiling the Mechanisms of Cell-Cell Fusion. *Science*. **308**:369–73 (2005).
10. Sapir A, Avinoam O, Podbilewicz B, and Chernomordik LV. Viral and Developmental Cell Fusion Mechanisms: Conservation and Divergence. *Dev Cell*. **14**:11–21 (2008).
11. Shinn-Thomas JH, and Mohler WA. Chapter five - New Insights into the Mechanisms and Roles of Cell–Cell Fusion. In: Jeon KW, editor. International Review of Cell and Molecular Biology. Academic Press; p. 149–209 (2011).
12. Kennett RH. Cell fusion. *Methods Enzymol*. **58**:345–59 (1979).

13. Piccolo FM, Pereira CF, Cantone I, Brown K, Tsubouchi T, Soza-Ried J, Merkenschlager M, and Fisher AG. Using heterokaryons to understand pluripotency and reprogramming. *Philos Trans R Soc B Biol Sci*. **366**:2260–5 (2011).

14. Yamanaka S, and Blau HM. Nuclear reprogramming to a pluripotent state by three approaches. *Nature*. **465**:704–12 (2010).

15. Davidson RL. Control of the differentiated state in somatic cell hybrids. *Symp Soc Dev Biol Soc Dev Biol Symp*. **31**:295–328 (1973).

16. Sanges D, Lluis F, and Cosma MP. Cell-Fusion-Mediated Reprogramming: Pluripotency or Transdifferentiation? Implications for Regenerative Medicine. In: Dittmar DT, Zänker DKS, editors. Cell Fusion in Health and Disease. Springer Netherlands; p. 137–59 (2011).

17. Pereira CF, Terranova R, Ryan NK, Santos J, Morris KJ, Cui W, Merkenschlager M, and Fisher AG. Heterokaryon-Based Reprogramming of Human B Lymphocytes for Pluripotency Requires Oct4 but Not Sox2. Roopenian DC, editor. *PLoS Genet*. **4**:e1000170 (2008).

18. Sullivan S, and Eggan K. The potential of cell fusion for human therapy. *Stem Cell Rev*. **2**:341–9 (2006).

19. Soza-Ried J, and Fisher AG. Reprogramming somatic cells towards pluripotency by cellular fusion. *Curr Opin Genet Dev*. **22**:459–65 (2012).

20. Bhutani N, Brady JJ, Damian M, Sacco A, Corbel SY, and Blau HM. Reprogramming towards pluripotency requires AID-dependent DNA demethylation. *Nature*. **463**:1042–7 (2010).

21. Kelsell DP, and Spurr NK. Gene mapping using somatic cell hybrids. Gene Isolation and Mapping Protocols. Springer; p. 45–52 (1997).

22. Miller FR, Mohamed AN, and McEachern D. Production of a More Aggressive Tumor Cell Variant by Spontaneous Fusion of Two Mouse Tumor Subpopulations. *Cancer Res*. **49**:4316–21 (1989).

23. Kandušer M, and Ušaj M. Cell electrofusion: past and future perspectives for antibody production and cancer cell vaccines. *Expert Opin Drug Deliv*. **11**:1885–98 (2014).

24. Köhler G, and Milstein C. Continuous cultures of fused cells secreting antibody of predefined specificity. *Nature*. **256**:495–7 (1975).

25. Scott AM, Wolchok JD, and Old LJ. Antibody therapy of cancer. *Nat Rev Cancer*. **12**:278–87 (2012).

26. Strioga MM, Felzmann T, Powell Jr. DJ, Ostapenko V, Dobrovolskiene NT, Matuskova M, Michalek J, and Schijns VEJC. Therapeutic Dendritic Cell-Based Cancer Vaccines: The State of the Art. *Crit Rev Immunol*. **33**:489–547 (2013).

27. Harris H, Miller OJ, Klein G, Worst P, and Tachibana T. Suppression of Malignancy by Cell Fusion. *Nature*. **223**:363–8 (1969).

28. Pontecorvo G. Production of mammalian somatic cell hybrids by means of polyethylene glycol treatment. *Somatic Cell Genet*. **1**:397–400 (1975).

29. Miller RA, and Ruddle FH. Pluripotent teratocarcinoma-thymus somatic cell hybrids. *Cell*. **9**:45–55 (1976).

30. Teissie J, Knutson VP, Tsong TY, and Lane MD. Electric Pulse-Induced Fusion of 3T3 Cells in Monolayer Culture. *Science*. **216**:537–8 (1982).

31. Long MA, and Rossi FMV. Targeted Cell Fusion Facilitates Stable Heterokaryon Generation In Vitro and In Vivo. *PLoS ONE*. **6**:e26381 (2011).

32. Zimmermann U. Electric field-mediated fusion and related electrical phenomena. *Biochim Biophys Acta BBA - Rev Biomembr*. **694**:227–77 (1982).

33. Knutton S. Studies of membrane fusion. III. Fusion of erythrocytes with polyethylene glycol. *J Cell Sci*. **36**:61–72 (1979).

34. Pereira CF, and Fisher AG. Heterokaryon-Based Reprogramming for Pluripotency. In: Bhatia M, Elefanty AG, Fisher SJ, Patient R, Schlaeger T, Snyder EY, editors. Current Protocols in Stem Cell Biology. Hoboken, NJ, USA: John Wiley & Sons, Inc.; (2009).

35. Hui SW, and Stenger DA. [16] Electrofusion of cells: Hybridoma production by electrofusion and polyethylene glycol. In: Nejat Duzgunees, editor. Methods in Enzymology [Internet]. Academic Press; p. 212–27 (1993).

36. Halldorsson S, Lucumi E, Gómez-Sjöberg R, and Fleming RMT. Advantages and challenges of microfluidic cell culture in polydimethylsiloxane devices. *Biosens Bioelectron*. **63**:218–31 (2015).

37. Yun H, Kim K, and Lee WG. Cell manipulation in microfluidics. *Biofabrication*. **5**:022001 (2013).

38. Brouzes E, Medkova M, Savenelli N, Marran D, Twardowski M, Hutchison JB, Rothberg JM, Link DR, Perrimon N, and Samuels ML. Droplet microfluidic technology for single-cell high-throughput screening. *Proc Natl Acad Sci U S A*. **106**:14195–200 (2009).

39. Clausell-Tormos J, Lieber D, Baret J-C, El-Harrak A, Miller OJ, Frenz L, Blouwolff J, Humphry KJ, Köster S, and Duan H. Droplet-Based Microfluidic Platforms for the Encapsulation and Screening of Mammalian Cells and Multicellular Organisms. *Chem Biol*. **15**:427–37 (2008).

40. Mu X, Zheng W, Sun J, Zhang W, and Jiang X. Microfluidics for manipulating cells. *Small*. **9**:9–21 (2013).

41. Hu N, Yang J, Joo SW, Banerjee AN, and Qian S. Cell electrofusion in microfluidic devices: A review. *Sens Actuators B Chem*. **178**:63–85 (2013).

42. Hu N, Yang J, Qian S, Joo SW, and Zheng X. A cell electrofusion microfluidic device integrated with 3D thin-film microelectrode arrays. *Biomicrofluidics*. **5**:034121–034121 – 12 (2011).

43. Wang J, and Lu C. Microfluidic cell fusion under continuous direct current voltage. *Appl Phys Lett*. **89**:234102 (2006).

44. Skelley AM, Kirak O, Suh H, Jaenisch R, and Voldman J. Microfluidic control of cell pairing and fusion. *Nat Methods*. **6**:147–52 (2009).

45. Dura B, Dougan SK, Barisa M, Hoehl MM, Lo CT, Ploegh HL, and Voldman J. Profiling lymphocyte interactions at the single-cell level by microfluidic cell pairing. *Nat Commun*. **6**: (2015).

46. Dura B, Liu Y, and Voldman J. Deformability-based microfluidic cell pairing and fusion. *Lab Chip*. **14**:2783 (2014).

47. Masuda S, Washizu M, and Nanba T. Novel method of cell fusion in field constriction area in fluid integration circuit. *IEEE Trans Ind Appl*. **25**:732–7 (1989).

48. Techaumnat B, Tsuda K, Kurosawa O, Murat G, Oana H, and Washizu M. High-yield electrofusion of biological cells based on field tailoring by microfabricated structures. *IET Nanobiotechnol*. **2**:93 (2008).

49. Gel M, Kimura Y, Kurosawa O, Oana H, Kotera H, and Washizu M. Dielectrophoretic cell trapping and parallel one-to-one fusion based on field constriction created by a micro-orifice array. *Biomicrofluidics*. **4**:022808 (2010).

50. Gel M, Suzuki S, Kimura Y, Kurosawa O, Techaumnat B, Oana H, and Washizu M. Microorifice-Based High-Yield Cell Fusion on Microfluidic Chip: Electrofusion of Selected Pairs and Fusant Viability. *IEEE Trans NanoBioscience*. **8**:300–5 (2009).

51. Kimura Y, Gel M, Techaumnat B, Oana H, Kotera H, and Washizu M. Dielectrophoresis-assisted massively parallel cell pairing and fusion based on field constriction created by a micro-orifice array sheet. *ELECTROPHORESIS*. **32**:2496–501 (2011).

52. Joensson HN, and Andersson Svahn H. Droplet Microfluidics-A Tool for Single-Cell Analysis. *Angew Chem Int Ed*. **51**:12176–92 (2012).

53. Niu X, Gulati S, Edel JB, and deMello AJ. Pillar-induced droplet merging in microfluidic circuits. *Lab Chip*. **8**:1837–41 (2008).

54. Link DR, Anna SL, Weitz DA, and Stone HA. Geometrically Mediated Breakup of Drops in Microfluidic Devices. *Phys Rev Lett*. **92**:054503 (2004).

55. Abate AR, Hung T, Mary P, Agresti JJ, and Weitz DA. High-throughput injection with microfluidics using picoinjectors. *Proc Natl Acad Sci U S A*. **107**:19163–6 (2010).

56. Baret J-C, Miller OJ, Taly V, Ryckelynck M, El-Harrak A, Frenz L, Rick C, Samuels ML, Hutchison JB, Agresti JJ, Link DR, Weitz DA, and Griffiths AD. Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. *Lab Chip*. **9**:1850–8 (2009).

57. Sciambi A, and Abate AR. Accurate microfluidic sorting of droplets at 30 kHz. *Lab Chip*. (2014).

58. Cho S, Kang D-K, Sim S, Geier F, Kim J-Y, Niu X, Edel JB, Chang S-I, Wootton RCR, Elvira KS, and deMello AJ. Droplet-Based Microfluidic Platform for High-Throughput, Multi-Parameter Screening of Photosensitizer Activity. *Anal Chem*. **85**:8866–72 (2013).

59. Schoeman RM, Kemna EWM, Wolbers F, and van den Berg A. High-throughput deterministic single-cell encapsulation and droplet pairing, fusion, and shrinkage in a single microfluidic device: Microfluidics and Miniaturization. *ELECTROPHORESIS*. **35**:385–92 (2014).

60. Hudson SD. Wall migration and shear-induced diffusion of fluid droplets in emulsions. *Phys Fluids 1994-Present*. **15**:1106–13 (2003).

61. Lagus TP, and Edd JF. High-throughput co-encapsulation of self-ordered cell trains: cell pair interactions in microdroplets. *RSC Adv*. **3**:20512–22 (2013).

62. Kemna EWM, Schoeman RM, Wolbers F, Vermes I, Weitz DA, and van den Berg A. High-yield cell ordering and deterministic cell-in-droplet encapsulation using Dean flow in a curved microchannel. *Lab Chip*. **12**:2881 (2012).

63. Lee M, Collins JW, Aubrecht DM, Sperling RA, Solomon L, Ha J-W, Yi G-R, Weitz DA, and Manoharan VN. Synchronized reinjection and coalescence of droplets in microfluidics. *Lab Chip*. **14**:509–13 (2013).

64. Huebner A, Srisa-Art M, Holt D, Abell C, Hollfelder F, deMello AJ, and Edel JB. Quantitative detection of protein expression in single cells using droplet microfluidics. *Chem Commun*. 1218 (2007).

65. Mazutis L, Gilbert J, Ung WL, Weitz DA, Griffiths AD, and Heyman JA. Single-cell analysis and sorting using droplet-based microfluidics. *Nat Protoc*. **8**:870–91 (2013).

66. Carlo DD. Inertial microfluidics. *Lab Chip*. **9**:3038–46 (2009).

67. Carlo DD, Irimia D, Tompkins RG, and Toner M. Continuous inertial focusing, ordering, and separation of particles in microchannels. *Proc Natl Acad Sci*. **104**:18892–7 (2007).

68. Edd JF, Carlo DD, Humphry KJ, Köster S, Irimia D, Weitz DA, and Toner M. Controlled encapsulation of single-cells into monodisperse picolitre drops. *Lab Chip*. **8**:1262–4 (2008).

69. Amini H, Lee W, and Carlo DD. Inertial microfluidic physics. *Lab Chip*. **14**:2739–61 (2014).

70. Chabert M, and Viovy J-L. Microfluidic high-throughput encapsulation and hydrodynamic self-sorting of single cells. *Proc Natl Acad Sci*. **105**:3191–6 (2008).

71. Guillot P, Colin A, Utada AS, and Ajdari A. Stability of a Jet in Confined Pressure-Driven Biphasic Flows at Low Reynolds Numbers. *Phys Rev Lett*. **99**:104502 (2007).

72. Chan PC-H, and Leal LG. The motion of a deformable drop in a second-order fluid. *J Fluid Mech*. **92**:131–70 (1979).

73. Agresti JJ, Antipov E, Abate AR, Ahn K, Rowat AC, Baret J-C, Marquez M, Klibanov AM, Griffiths AD, and Weitz DA. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution. *Proc Natl Acad Sci U S A*. **107**:4004–9 (2010).

74. Eisenstein M. Cell sorting: Divide and conquer. *Nature*. **441**:1179–85 (2006).

75. Frenz L, Blouwolff J, Griffiths AD, and Baret J-C. Microfluidic Production of Droplet Pairs. *Langmuir*. **24**:12073–6 (2008).

76. Frenz L, Harrak A El, Pauly M, Bégin-Colin S, Griffiths AD, and Baret J-C. Droplet-Based Microreactors for the Synthesis of Magnetic Iron Oxide Nanoparticles. *Angew Chem Int Ed*. **47**:6817–20 (2008).

77. Hong J, Choi M, Edel JB, and deMello AJ. Passive self-synchronized two-droplet generation. *Lab Chip*. **10**:2702–9 (2010).

78. Chokkalingam V, Herminghaus S, and Seemann R. Self-synchronizing pairwise production of monodisperse droplets by microfluidic step emulsification. *Appl Phys Lett*. **93**:254101 (2008).

79. Dangla R, Fradet E, Lopez Y, and Baroud CN. The physical mechanisms of step emulsification. *J Phys Appl Phys*. **46**:114003 (2013).

80. Dressler O, Yang T, Chang S-I, Choo J, Wootton RCR, and deMello A. Continuous and low error-rate passive synchronization of pre-formed droplets. *RSC Adv*. (2015).

81. Zagnoni M, and Cooper JM. On-chip electrocoalescence of microdroplets as a function of voltage, frequency and droplet size. *Lab Chip*. **9**:2652–8 (2009).

82. Zagnoni M, Le Lain G, and Cooper JM. Electrocoalescence Mechanisms of Microdroplets Using Localized Electric Fields in Microfluidic Channels. *Langmuir*. **26**:14443–9 (2010).

83. Akartuna I, Aubrecht DM, Kodger TE, and Weitz DA. Chemically induced coalescence in droplet-based microfluidics. *Lab Chip*. **15**:1140–4 (2015).

84. Baroud CN, Vincent MR de S, and Delville J-P. An optical toolbox for total control of droplet microfluidics. *Lab Chip*. **7**:1029–33 (2007).

85. Mazutis L, Baret J-C, and Griffiths AD. A fast and efficient microfluidic system for highly selective one-to-one droplet fusion. *Lab Chip*. **9**:2665–72 (2009).

86. Liu K, Ding H, Chen Y, and Zhao X-Z. Droplet-based synthetic method using microflow focusing and droplet fusion. *Microfluid Nanofluidics*. **3**:239–43 (2006).

87. Yoon DH, Jamshaid A, Ito J, Nakahara A, Tanaka D, Akitsu T, Sekiguchi T, and Shoji S. Active microdroplet merging by hydrodynamic flow control using a pneumatic actuator-assisted pillar structure. *Lab Chip*. **14**:3050–5 (2014).