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Tiny Droplets for High-Throughput Cell-Based Assays

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Introduction

In chemistry and biology, assays have always been performed by manipulation of finite volumes of reagents in well-controlled environment. Indeed from the test tubes of the XIXth century to the microtiter plates of the pharmaceutical industries, reagents are brought together by dripping or pipetting one into another before being mixed and the evolution of sciences and technologies has pushed the assays to controlled containers and always smaller sizes. This quest of miniaturization is mainly governed by the need to perform increasing number of assays on biological objects which are sometimes impossible to obtain in large quantities (e.g. stem cells) or on chemical compounds produced by the pharmaceutical industries in small quantities for economic reasons. Nowadays, the technology used to assay – e.g. by the pharmaceutical industry – enables about one test per second to be performed on volumes of the order of 1 microliter.

Although this volume is relatively small – 1 microliter of water represents a droplet of about 1 mm diameter – it is huge when biological material is involved. As an example, considering cell cultures, with a typical density of about 10^7 cells per mL, an assay performed on 1 microliter represents the averaged response of 10^4 cells. Under those conditions, the presence of an individual with specific characteristics will be hidden in the statistical response of the population. Of course, by simple dilutions, one cell per microliter is achievable but the cell density becomes so low that the concentrations of molecules released by the single cell are very small. Alternatively the amount of reagents to be used for millions of tests of 1 microliter each becomes the bottleneck to perform the assays with reasonable costs. 1 microliter is also huge compared to the sizes at which biological reactions occur in nature. Indeed a bacterium cell such as *Escherichia Coli* has a typical size of about 1 to 2 microns which represents a volume of a few femtoliters. Therefore several orders of magnitude in volumes are available to miniaturize biological assays.

Standard technologies, such as microtiter plates have nearly reached the smallest sizes of samples they can handle. In order to miniaturize the assays, a further decrease of the size of the reactors in which reactions are performed is required. The vessels must keep their content independent from each other, and this content must be accessible to the operator on demand. Simple tasks have to be

performed, such as mixing of reagents or dilutions, aliquoting or detection and selection of specific variants encapsulated in the vessel.

The use of small droplets provides new ways to miniaturize assays. Indeed, water droplets – liquid structures with perfectly defined boundary - are ideal for compartmentalizing compounds. This well defined interface is the result of the cohesion of the fluid structure linked to the interactions of the fluid molecules [1]. Production and manipulation of droplets will require the ability to overcome on-demand the cohesion of the fluid structure. Droplets are produced from a bulk solution by the use of various forces (see for example the review [2]): gravity in the simple case of dripping, another interfacial force in the case of spotting or microcontact printing, inertia in the case of inkjet printing [3, 4], electrostatic force [5] in the case of electrospraying, shear forces in emulsion production [6]. Practically, droplet manipulation is also influenced by surface energies linked to the fluid cohesion. In the absence of other energies, the minimization of energy gives a single solution for a two phases system: one single large droplet. An ensemble of small liquid structures is not an equilibrium solution and has to be stabilized. This is achieved by separating the small structures spatially (e.g. for arrays spotted with a large distance between the droplets) or by the use of a physical barrier such as walls (e.g. the wells of a microtiter plate). A physical-chemical way to stabilize the system is to add surfactant molecules which prevents droplet coalescence. This approach enables foams (liquid / gas interfaces) and emulsion (liquid / liquid interfaces) stabilization [6]. An approach based on emulsions also permits to reduce evaporation. Evaporation of small liquid structures - sometimes as fast as the typical timescale of a biochemical assay [7] - modifies the concentration of the species and impacts the assay. By immersing the droplet in a saturated environment [7], evaporation can be reduced. Alternatively the immersion of the droplets in an immiscible phase, coupled to the use of surfactant molecules - the basis of emulsions [8, 6] - enables the production of independent micro-reactors which leads to the first attempts to miniaturize assays in micrometer-sized droplets [9]. Over the past few years the combination of reactions in droplets and controlled microfluidic droplets manipulation has provided new ways of performing quantitative assays at a high-throughput. Droplets of equal sizes are produced in series and manipulated on demand providing quantitative and miniaturized versions of the microtiter plate assays and enables the parallelization of the assay. A series of operations can be performed, just as they are on microtiter plate: splitting of liquid from

a reservoir in different compartments, mixing with another reagent, detection and selection of ‘hits’, namely organisms, small molecules or macromolecules with specific desired properties.

17.1 Droplet-based microfluidics

Droplet-based microfluidic systems enable the control of droplets in or on microfluidic chips through several systems. Among them, the most versatile and flexible solutions are digital microfluidics, a name originally given to the manipulation of droplet using electrowetting on dielectrics (EWOD) and droplet-based microfluidics in microchannels, which is the two phase flow variation of the standard and well established microfluidics technology [10, 11]. These two systems function at different level of operation.

17.1.1 EWOD and ‘digital microfluidics’: tools for high-content screening

Digital microfluidics is the main example of an array-based manipulation of droplets. Droplets are actuated on a chip that can be configured for different tasks almost in real time [12] (For a review, see also [13]). The technique proves to be very efficient for the manipulation of a relatively small number of droplets in parallel on a surface. The actuation mechanism is based on the local modulation of the wetting properties of a surface by the electrowetting effect [14, 15, 16]. Indeed droplets move in gradients of wettability towards the most wettable surface [17]. Here the microfluidic chip used is highly engineered with patterned electrodes, on top of which a dielectric insulating layer is deposited. An additional hydrophobic surface coating is usually added and counter electrodes are positioned to contact the droplet or a second plate closes the system (Figure 1 A). Finally the system is sealed to immerse the droplet in oil and reduce contact angle hysteresis. By patterning the surface with arrays of electrodes, the wetting properties of the surface are modulated in space and time in order to move droplets from one electrode patch to the other [18]. This technique enables a series of operations on droplets such as production of small droplet from a reservoir, splitting of droplets in two parts, merging of droplets and droplets actuation [18, 19] (Figure 1 B and C). Extensions or variants of this technique involve other means to manipulate droplets on planar surfaces, for example using dielectrophoresis [20], magnetic actuation [21] or

surface acoustic wave actuation [22]. In all of these cases, the system is interesting for the possibility to reconfigure the operation [23], to run complex multi-step assays or to repeat multiple operations on a few droplets – ideal for high-content screening - but fails to handle billions of droplets in parallel or at high-throughput.

17.1. 2 Droplet-based microfluidics: tools for high-throughput screening

Droplet-based microfluidics in microchannels is on the other hand the ideal tool for high-throughput screening. Over the past 15 years, a series of modules have been developed for droplet manipulation in microfluidic channels, at high-throughput (~1000 droplets per second, see Figure 2). The variety of materials in which these devices can be produced is extremely vast but the most widespread technique used is soft lithography which allows rapid prototyping [24] (See also Chapter 11 of the present book). Channels are easily produced in PDMS (PolyDiMethylSiloxane) using replica molding of a mould of a photoresist (for example SU8, microchem) deposited on a silicon wafers. Other materials such as PMMA (Poly(methyl metacrylate), glass or SU8 structures are also usable. Soft-lithography also enables the creation of complex three-dimensional structures and recent developments enabled the production of electrodes as microfluidic channels filled by a low melting temperature solder which improves the basic capabilities of the rapid prototyping method [25]. In these channels, droplet actuation is mainly predetermined by the flow of the droplets along those channels and includes droplet production and reinjection, droplet flow for incubation, droplet splitting, droplet pair fusion, droplet detection and droplet sorting. The droplets will flow in series along the channels and the same type of operation will be performed on each droplet. The integration of droplet-based microfluidic modules makes it a perfect tool to screen large libraries of variants containing small numbers of extraordinary variants [26, 27], typically interesting for high-throughput screening or directed evolution experiments. In the following we will present the progress on droplet manipulation in microchannels, usable for example for high-throughput applications. These modules can be split in two categories, passive modules and active modules. In passive modules, the flow of droplets is predetermined and controlled solely by the hydrodynamics. Three effects are involved: the mechanical pumping of the liquid, viscous dissipation and surface tension. Mechanical pumping is easily achieved using syringe pumps (controlled flow rate) or

pressure driven system (controlled pressure). A summary of additional pumping mechanisms can be found for example in [10, 22]. The interplay of channel geometry and the liquid physical characteristics (viscosity and surface tension of the fluid/fluid interface) controls the response of the fluid to the hydrodynamic forcing. In active modules, external forces such as, for example, electric or thermocapillary forces, are used to actuate single droplets or groups of droplets among the population of droplets. The combination of passive and active elements provides a high-level of control for the operator to process droplets at high-throughput (>1000 droplets per second).

17.2 Generating and manipulating droplets

17.2.1 Droplet production

Droplets are produced on-chip by co-flowing two immiscible fluids: the instability of the interface between the two fluids will lead to droplet production [28]. Three geometries are mainly used, T-junction [29], steps [30, 31] and Ψ -Junction (also known as ‘flow-focusing junction’) [32]. Already in simple co-flowing cylindrical geometries, several regimes of droplet production are observed depending on the flow conditions and the dynamic response of the interfaces to perturbations [28, 33, 34]. The so-called dripping regime leads to the most monodisperse droplet production with standard sizes of 10 to several hundreds of microns, at rates up to 10 kHz for the smallest droplets. In rectangular channels, the influence of geometry gives another level of complexity [35, 36] by the increased number of parameters (e.g. aspect ratio of the channels) that can be varied. Besides these geometrical parameters, droplet production is controlled by the interfacial properties of the liquid / liquid and liquid / solid interfaces [37]: single droplets [32] are easily produced but the design of microfluidic junction and the control of wetting properties also enables pairs of droplets [38, 39], multiple emulsions [40, 41, 42, 43] or foams [44] to be generated (Figure 2A). Finally the use of external electric fields gives an additional level of control on droplet production, either using the electrowetting effect [45] or electrospraying-based technique [46]. This use of electric field also enables the creation of charged droplets that can be further actuated in electric fields [47] and of droplets of sizes down to 1 micron [46] hardly accessible by other means. Additionally the use of surfactants for droplet stabilization enables collection of droplets, for example for incubation of biochemical reactions [27]. Microfluidic modules enable the production of stable and remarkably

monodisperse emulsions usable for specific applications but also for fundamental studies in emulsion sciences due to the perfect control on the emulsification process and the droplet characteristics (content, size, dispersity,...) [48, 49].

17.2.2 Droplet division

The flow of droplets at channel junctions enables droplets to be split apart. Such systems enable aliquots from a single mother droplet to be produced in a controlled way. The mechanism of breakup is linked to the destabilization of the interface induced by combined effect of the flow and the geometry of the channels [50, 51, 52] (Figure 2B). An additional level of control for this operation is achieved by the use of laser controlled breakup [53]. In this case, the volume of the droplets produced from the mother droplet can be actively tuned externally by laser power. This type of device will be ideal to aliquot droplets for example to make two types of assays on the same droplet and is also used to produce small droplets from a large mother droplet.

17.2.3 Droplet flow, droplet synchronization and droplet incubation

After production, droplets flow along the channels of the microfluidic chip. While in single phase flow the hydrodynamic resistance is simply given by Poiseuille's law [54], droplets modify this simple law [55, 56, 57, 58]: they are a source of pressure drop depending on their size and volume fraction in the oil phase which modifies the pressure distribution and therefore the flow of the continuous oil stream. This results in collective behaviour [59] or coupling between channels that influences droplet production by pressure feedback [38, 56, 60], direction [61] or synchronization [62, 63, 39, 31] (Figure 2C), which in turn provides additional control of droplets flow. On a practical viewpoint, the flow of droplets along channels is used for incubation of chemical or biochemical reactions [64, 65, 66, 67] and is perfectly compatible with high-throughput constraints: the flow of droplet reaches ~1000 droplets per second (Figure 2D). However, when the incubation requires the readout of complex information (e.g. kinetic data, growth rate measurement, imaging, spectroscopy...) this system is hardly usable. In this case, droplets are trapped and immobilized in the channels for measurement in wells [68] or by controlling the filling of channel [69]. These

systems are then hybrid between array-based systems and continuously flowing systems. Interestingly, besides these systems, other hybrid systems have been developed recently, coupling electrowetting-based droplet manipulation and droplet flow in microfluidic channels [70]. In the future, all of these systems should provide versatile and efficient droplet manipulation, both for high-throughput and high-content screening. They are complemented by active elements that enable direct and accurate manipulation of single droplets or groups of droplets.

17.2.3.1 Using valves

A first way to act on droplet flow is to modify the geometry of the channels during the flow. Although this looks like a surprising possibility, it is possible when the microfluidic channels are produced in an elastomeric material such as PDMS. Indeed by applying pressure in channels, the channel walls can be deformed which has been used for a long time already in valve-based systems [71, 72]. This method is now used to control the droplet production [73] or the orientation of groups of droplets [74]. Considering the time scale of actuation by this mechanism, linked to the mechanical response of the elastic material it cannot actuate single droplets at high-throughput (>1 kHz) but rather groups of droplets. This approach will enable a fine-tuning of the droplets behaviour by the operator and therefore increase the level of control given by the technology.

17.2.3.2 Droplet fusion, Coalescence and Electrocoalescence

When droplets are not stabilized by surfactants, droplet coalescence is straightforward. It has therefore been used to initiate chemical reactions at well defined locations [75]. On the contrary, when droplets are stabilized by surfactants, the mixing of the reagents initially encapsulated in the different droplets has to be induced by an external force. This forcing can be achieved using wetting patches [76]. In this case a first droplet spreading on an hydrophilic patch is coalesced with another following droplet or a group of several droplets. Alternatively, it has been shown recently that geometrical constraints inducing separation of the droplet after a collision promotes droplet fusion [48]. Using such systems will therefore enable fusion of droplet pairs (Figure 2E). However systems that can be externally controlled are more suited: the operator can induce externally and on-demand

the fusion of droplet pairs, for example to initiate the reactions only when the transient states linked to the starting or ending of an experiment are over. The use of electric fields is one of the most efficient ways to perform droplets coalescence. Electro-coalescence - based on the destabilization of the oil film between two water containers [77, 66, 78] - enables selective fusion of droplet pairs for example to initiate a chemical reaction between reagents present in the two droplets [79]. Although until now the electro-coalescence has been described only for large number of droplet pairs, it is possible to trigger the electric field on the detection of a specific droplet which could open the door to selective droplet fusion. Other systems have also been developed, for example using destabilization of the interface between two droplets by local heating by a laser focused in the channels [53]. To summarize, fusion of droplet pairs is an essential part for applications requiring several steps; the development of externally controlled modules for droplet fusion is a key to provide flexibility to the droplet-based microfluidic technology.

17.2.4 Droplet content detection and droplet sorting

When microfluidic modules are produced in transparent materials such as glass and PDMS, they are easily interfaced with optical systems which enable real-time measurement of droplet content as they flow in the channel. Measurement of droplet fluorescence or fluorescence of objects such as beads or cells encapsulated in the droplets can then be performed [26, 79, 80, 64, 81] in a similar manner as in flow cytometers (see e.g. [82]). More complex operation can also be performed such as Raman confocal microspectroscopy [83] on droplets. Finally single droplet can be sorted from the overall population [84, 47, 26, 27], as a function of the signal detection. Here again, several systems have been described. The principle is to actively control the flow of droplets at a junction by applying on demand a force larger than hydrodynamic forces. The most developed technique is the use of electric forces. Indeed electrodes are easily patterned on surfaces [84] or as microfluidic channels [25]. Droplets can be charged at production and a DC electric field applied across the electrodes will result in net force acting on the charged droplet [47]. However charged droplets are complex to handle because of repulsion during the flow and leakage of the charges in time which will restrict the use of this technique. Using AC field, it is possible to induce a net dielectrophoretic force on neutral droplets [84, 26] (Figure 2F). In this case, droplets are actuated at rates up to 2 kHz

at a sorting junction when the electric field is triggered on the detection of fluorescence intensities above a given threshold [27] and selection of single event becomes possible on populations of millions of droplets. Other systems have been developed for droplet sorting based on all-optic systems [53, 85]. Here local heating of the droplet interface will induce a force that can stop droplets in channels or force them at specific locations. In the future, these systems might provide new ways to obtain reconfigurable channel geometries which might improve the flexibility of the technology, maintaining the throughput of the assays.

In summary, droplet-based technology, based on the integration of modules of droplet manipulation, provides a flexible and versatile way to control droplets at a high-throughput and on small volumes. A large number of operations can be performed on droplets and the numerous applications of the technology drive efficiently the technological developments. The combination of technologies is the source of flexible droplet manipulation that provides tools both for high-content screening and high-throughput screening experiments. Finally, the use of these modules of droplet manipulation to perform biological and chemical assays can revolutionize assays performed in laboratories. In the following we will discuss the recent progress in the use of the modules described above, focusing on the applications of high-throughput manipulation of droplets.

17.2 In vitro compartmentalisation of biological reactions

In vitro compartmentalization (IVC) of biological reactions in microdroplets was developed initially for protein directed evolution purposes [9]. The basic idea is to perform billions of experiments by partitioning each experiment into a separate microscopic compartment: each compartment is a water microdroplet, containing all the ingredients for an experiment, which is separated from other microdroplets by an immiscible surrounding fluid phase (oil). This technique has been used to select a range of proteins [9, 86, 87, 88, 89, 90, 91, 92, 93] and RNAs [94, 95] for catalysis, and has also been used to select peptides and proteins for ligand binding [96, 97, 98, 99, 100] and for regulatory activity [101]. Recently direct analysis and sorting of water-in-oil-in-water double emulsions has been described for the directed evolution of *in vitro* expressed beta-galactosidases [92] or *in vivo* expressed thiolactonase (PON1) [93].

The combination of IVC and microfluidics has led to the development of droplet-based microfluidic systems that represents a powerful new paradigm in high-throughput screening (HTS) and directed evolution. First, the reduction of reagents volumes, thanks to the small size of the microreactors, greatly reduces expense of screening libraries containing millions of compounds. Second, combined to the potential high-throughput, the high level of precision for reaction and incubation times makes this technology ideal for a rapid, reproducible and quantitative readout of a particular process [102].

As mentioned earlier in this chapter, conventional methods for studying the effect of environmental stress or drugs on cells behaviour, implies the measurement of large number of cells in order to provide information over the population as a whole. However, in microdroplets even single-cells can be analysed while being at biologically relevant concentrations (due to the small volume of compartment) allowing quantitative biological studies on a single-cell basis for large populations [103, 104]. Droplet-based microfluidic represents a high-throughput phenotyping procedure, allowing, for example, the selection of cell-displayed proteins libraries, as well as libraries cloned in heterologous host for cytoplasmic expression, rate enhancement or efficient turnover. The restriction of product diffusion by compartmentalization affords a sensitive and general mode of detection. Moreover, a wide range of experimental conditions can be applied to the cells since they do not have to stay intact for selection (DNA could be recovered and characterized after selection).

An ideal platform [103] for single-cell analysis should allow for : (1) encapsulation of a predefined number of cells per compartment (with the option of encapsulating single cells being highly desirable); (2) incubation of the compartmentalized samples allowing efficient gas-exchange, nutrition, etc. ; (3) efficient read-out of the results of the experiments and/or recovery of the cells from the compartments in a way that does not abolish cell viability and (4) facile integration of functional components in biologically relevant platforms that should allow to manipulate the droplets (addition of reagents, fusion, division of droplets, sorting, etc.).

17.2.1 Cell compartmentalization in aqueous droplets

Microfluidic systems have been used to controllably compartmentalize both prokaryotic and

eukaryotic cells [102, 103, 81, 105, 106, 107] and even embryos of multicellular organisms [108] within aqueous droplets. Using microfluidic droplet generation modules (see above), the process of loading cells in drops is purely random and consequently, the number of cells per drops follows a Poisson distribution solely controlled by the cell density [103, 109]. Two main approaches have been described to overcome the inherent limitations linked to the variability of the number of cell per droplets due to the stochastic cell loading. The first one consists on passively sorting droplets containing single-cells from smaller empty droplets [110]. More recently self-organization of cells under flow has lead to an improved encapsulation [104]. In that system, the cells enter the drop generator with the frequency of drop formation which greatly increases the probability that only one cell is present whenever a drop is generated and thus minimize the number of droplets containing more than one cell or no cells at all.

17.2.2 Incubation and cell viability in droplets

Cell-based assays generally require the read-out of individual samples after an incubation step (to screen the phenotype of individual cell within an heterogeneous population) which implies the need for efficient and biocompatible storage conditions. Perfluorocarbon oils as continuous phase are perfectly suited for high-throughput cell-based assays. Such oils are compatible with PDMS devices, immiscible with water and transparent (allowing optical readout). Moreover most organic molecules are not soluble in fluorinated oil which limits phase partitioning of the compounds. Individual cells are in their own sterile microenvironment and remain healthy and viable given the remarkable solubility of respiratory gases in the perfluorocarbon carrier fluid [111]. Indeed such fluids can dissolve more than 20 times the amount of O₂ and 3 times the amount of CO₂ than water and have shown to facilitate respiratory gas delivery to both prokaryotic and eukaryotic cells in culture [65].

Plugs (droplets confined by the microfluidic channel without wetting the walls [112]) provide a simple method for manipulating samples with no dispersion or losses to interfaces [113]. They are suitable to create large compartments which have been reported as preferential for long-term assays since the cells proliferate during the assay [103]. Systems have been described where such droplets are generated within a microfluidic chip and afterwards flushed into a teflon capillary tube for cultivation [105, 103] or open-reservoir [103, 27]. A droplet-based microfluidic platform

has been described that allows the creation of miniaturized reaction vessels in which both adherent (HEK293T) and non-adherent cells (JURKART) can survive for several days [103]. In this study, incubation of the microcompartments in gas-permeable PTFE (Poly-Tetra-Fluoro-Ethylene) tubing allows for cell survival for several days when glass capillaries and vinyl tubing resulted in cell death within 24h. The authors also described a full life cycle of an encapsulated multicellular organism (*C. elegans*) [103].

When using surfactant-stabilized droplets, besides the continuous phase, the biocompatibility of the surfactant molecules is key. Contrary to mineral or organic oils, a few amphiphilic molecules are commercially available for an aqueous – perfluorinated oil interface. By the modification of the hydrophilic head (the part in contact with the biological samples) of a commercially available PFPE-based surfactants (poly(perfluoropropylene glycol)-carboxylates sold as Krytox by Dupont) new surfactants have been synthesized [103, 65] (see Figure 3). According to these works, while ionic surfactants seem to mediate cell-lysis, surfactants bearing PolyEthylene Glycol (PEG) [65] or di-morpholino phosphate groups [103] hydrophilic head groups have been shown to exhibit high biocompatibility (did not affect membrane integrity, allowed cell proliferation and recovery of alive cells after growth). These surfactants were used for *In vitro* translation of plasmid DNA encoding *E. coli* beta-galactosidase (Lac Z) as well as growth of encapsulated yeast cells [65], human cells (both adherent and non-adherent) [103] or mammalian hybridoma cells [109] in culture media. Cells remain mobile in the droplet, and do not adhere at the interface of the droplet. Moreover, simple procedures allowing the recovery of cells from droplets that have been stabilized with surfactants have been described without impact on cell viability [103].

In conclusion, using appropriate surfactant, oil and incubation procedures, cell growth in droplets is mainly limited by gas-exchange, lack of nutrition or accumulation of toxic metabolites, as in classical cell culture.

17.2.3 Cell-based assays and cell manipulation

Besides cell survival, cell-based protein expression in microfluidic generated droplets, have been demonstrated. The expression of Yellow Fluorescent Protein (YFP) in individual *E. coli* cells has

been analysed in microfluidic generated droplets with simultaneous measurement of droplet size and cell occupancy [81]. Such system should allow high-throughput protein expression to be performed and related quantification of the expressed proteins in a highly uniform and reproducible manner.

A challenging area of droplet-based microfluidic is the high-throughput phenotyping by enzymatic markers. To date, majority of screens for enzymatic activities rely on high-throughput screening (HTS) using chromogenic or fluorogenic substrates. However, in screening experiments – of either colonies on agar plates or individual clones in microtitre plate wells - typically 10^3 - 10^4 clones and rarely more than 10^5 clones can be screened, even using sophisticated automated systems [93]. One way of greatly accelerating HTS is to use fluorescence-activated cell sorting (FACS), which can routinely sort $>10^7$ clones per hour, and has a series of other advantageous features [114]. FACS has already proven a highly successful technique to select proteins (notably antibodies) with high binding affinities [115, 116, 117, 118, 119, 120, 121, 122]. In addition, FACS has significant potential to select for catalysis [93, 123], however, so far, this approach has only been possible when the diffusion of product out of the cell can be restricted [124], or the product can be captured on the surface of the cell [125, 126], or onto microbeads [90]. Such limitations could be overcome by double-emulsions (water-in-oil-in-water) screening [92, 93]. However, droplet-based microfluidic systems generate much less polydisperse droplets which facilitates quantitative analysis of concentration changes in the droplets and thus more stringent and efficient screening. Moreover, droplets can be steered, additional reagents added by droplet fusion. Interfacing with analytical techniques then allows simultaneous measurements of droplet size and fluorescence with higher precision than FACS [81]. Microfluidic procedures allowing fluorescence analysis of individual compartments (containing single-cell) subsequent to an incubation period have been described recently [103] as well as sorting of cells either based on the cell fluorescence [26] or on their enzymatic activity in droplets [27].

Another interesting feature of droplet-based procedure will be the tracking of individual cells over time to study phenotypic variations among cell population. A droplet parking device called ‘dropspots’, consisting on a simple microfluidic system that use an array of well-defined chambers to immobilize thousands of femtoliter to picoliter-scale aqueous droplets suspended in an inert carrier oil, has been recently described [69] (see Figure 4). The droplets can be stored, individually monitored, and then recovered and ultimately even sorted. Single yeast cells growth has been

monitored within droplets of water in perfluorocarbon oil parked in the ‘dropspots’. In the same study, the authors monitored enzyme levels in a population of single-cell using a fluorogenic assay. Such device can be used to study dynamic behaviour of libraries of individuals allowing, for example, the monitoring of the heterogeneity in individual gene expression.

A platform combining optical trapping and microfluidic based droplet generation has been developed for single target cell or subcellular structures (mitochondria) analysis in picoliter or femtoliter aqueous droplets [107]. In this study, rapid laser photolysis has been performed on the cells upon encapsulation: the cells are frozen in the state in which they are at the time of photolysis and the lysate is encapsulated within the small volume of the droplet. A fluorogenic assay allowing the detection of enzymatic activity has then been performed on intracellular beta-galactosidases within single lysed cells. The key advantage of such lysis over bulk lysis is the confinement of the single-cells content using droplets. This is an important aspect of high-throughput approaches where the copy number present in the cell is low.

17.3 Towards integrated platforms for cell-based assays

While controlled manipulation of droplets has been extensively demonstrated, and individual operations (biological material encapsulation, droplet fusion or splitting, droplet sorting, etc.) often been described (see above), much work still remains in integrating these different manipulations into a single platform able to address specific biological questions. The works reported in this section consist on the development of platforms integrating different modules, which constitute proof-of-principles of the pertinence of droplet-based microfluidic for cell-based analysis.

A plug-based platform for rapid detecting and drug susceptibility screening of bacteria in samples, including complex biological matrices without pre-incubation has been described recently [127]. When for conventional bacteria cultures the clinicians have to perform incubation of a sample to increase the concentration of bacteria to a detectable level, thanks to the confinement of single bacterial cells in nanoliters plugs, the authors were able to eliminate such pre-incubation step and consequently reduce the time required to detect bacteria. More specifically, the authors were able to perform the antibiogram (chart of antibiotic sensitivity) of a methicillin-resistant *Staphylococcus aureus* (MRSA) to many antibiotics in a single experiment and to measure the Minimal Inhibitory

Concentration (MIC) of a drug (Cefutixin) against this strain. By permitting to perform multiple tests in parallel, such procedure could allow rapid and effective patient-specific treatment of bacterial infections with a significantly decreased detection time from 1-4 days to few hours (3 to 7.5h).

A droplet-based microfluidic method was developed for the detection and analysis of cell-surface protein biomarkers on individual human cells using enzymatic amplification [128]. Such biomarkers have already proven to be useful diagnostic indicators of disease state and clinical outcome [128]. When commonly used approaches (FACS analysis of cell labelled with fluorescent-dye-coupled antibodies) can only detect highly or moderately expressed biomarkers (several hundreds to thousands proteins per cell), the authors are using enzyme-based amplification techniques leading to the detection of low-abundance biomarkers. In addition, by incorporating a basic droplet optical labelling, they paved the way to perform high-throughput sensitive analysis on several cell samples.

The electroporation of single-cell within microfluidic system has been described [129]. In this system, the cell containing droplets are flowing between a pair of microelectrodes with a constant voltage established between them. The oil phase being non-conductive, each flowing droplet experiments a field intensity variation that is equivalent to a pulse while the two electrodes are connected by the droplet resulting in electroporation of the cells contained in the droplets. Plasmids allowing enhanced Green Fluorescent Protein (eGFP) expression have been successfully delivered into Chinese Hamster Ovary (CHO) cells. Such technique could lead to droplet-based High-throughput functional genomics studies.

Recently, a novel droplet-based microfluidic system capable of sorting bacterial cells, based upon their enzymatic activity was developed [27]. This system has been called FADS for Fluorescent-Activated Droplet Sorting (Figure 5). The sorting module that have been developed exploits an asymmetric sorting junction and the dielectrophoretic effect to displace specific droplets, containing the bacterial cells, from a flowing stream into a collection channel. The false positive error rate has been determined to be less than 1 in 10^4 analyzed droplets. To validate the platform, two *Escherichia coli* strains have been used: one strain expressing an active beta-galactosidase and the other expressing an inactive variant. By encapsulating mixtures of cells with a fluorogenic beta-galactosidase substrate (Fluorescein-di-beta-galactopyranoside, FDG) in a water-in-perfluorocarbon

emulsion and sorting the resulting droplets based upon fluorescence, the population have been successfully enriched for active cells with enrichment factors being function of the cell density. The enrichment is here limited by the co-encapsulation of positive and negative cells in the droplet and only positive cells can be recovered for sufficiently low cell dilutions. Throughput was ~400 droplets per second, meaning that 1,000,000 variants can be screened (and selected) in 0.7–7 hours, depending on the number of cells per droplet. Moreover, it has been demonstrated that active cells were recovered from the sorting procedure. In addition, this system has allowed the successfully recovery of bacterial colonies from single sorted droplets which makes possible the recovery of extremely rare events from, for example, large enzyme libraries.

17.4 Conclusions

Droplet-based microfluidics has led to the development of systems that represent a powerful new paradigm in high-throughput screening (HTS) and directed evolution where the individual assays are compartmentalized in microdroplet microreactors.

The first advantage of microfluidic is the flexibility of the technology: numerous modules have been developed to make highly uniform droplets, fuse droplet pairs, mix their contents, incubate droplets, split droplets, detect their fluorescence and sort desired ‘hits’ according to their fluorescent signals. All of these modules function at the kilohertz regime on droplet volumes ranging from 1 pL to several nL. Such progress in sub-nanoliter droplet manipulation allows for a level of control of picoliter scale biochemical assays that was hitherto impossible. In addition, the reduction of volume of reagent due to their small size greatly reduces expenses of screening libraries containing millions of compounds.

The second advantage of microfluidic devices, and especially droplet-based microfluidic devices, is that they are perfectly well suited for handling biological materials. Microfluidic systems have been used to controllably compartmentalize both prokaryotic and eukaryotic cells and even embryos of multicellular organisms within aqueous droplets; cell viability and proliferation in droplets as well as protein expression in droplets have been demonstrated using surfactants designed for these purpose and gas permeable systems. Practically, subnanoliter droplets enable statistical studies of single cell rather than population analysis. Thus droplet-based procedure will enable the

tracking of individual cells to study phenotypic variations among cell population.

In addition, droplets can be steered, additional reagents added by droplet fusion. Interfacing with analytical techniques then allows simultaneous measurements of droplet size and fluorescence with higher precision than FACS. Contrary to FACS, the range of selectable activities is not limited to products remaining in the cell or at the surface of the cells: the activity of molecules secreted by the cells can be assayed and there is no restriction to non-diffusing product. This technology should open the way for quantitative cell-based screening than can use 10^3 to 10^9 smaller assay volumes and around 1000 fold higher throughput than conventional microtiter plate assays. In addition, thanks to the small volume of the microdroplets, the expense of screening libraries containing millions of compounds will be greatly reduced.

The pertinence of droplet-based microfluidics for high-throughput and high-content quantitative cell screening has definitely been proven. Future works will consist in the design of integrated platforms able to address specific biological questions in many fields including single-cell analysis, cell populations dynamic probing, drug screening, directed evolution, gene sequencing or functional genomic. Moreover, thanks to the flexibility and versatility of design and processing of microfluidic devices, they will probably become an essential part of laboratory equipment and procedures.

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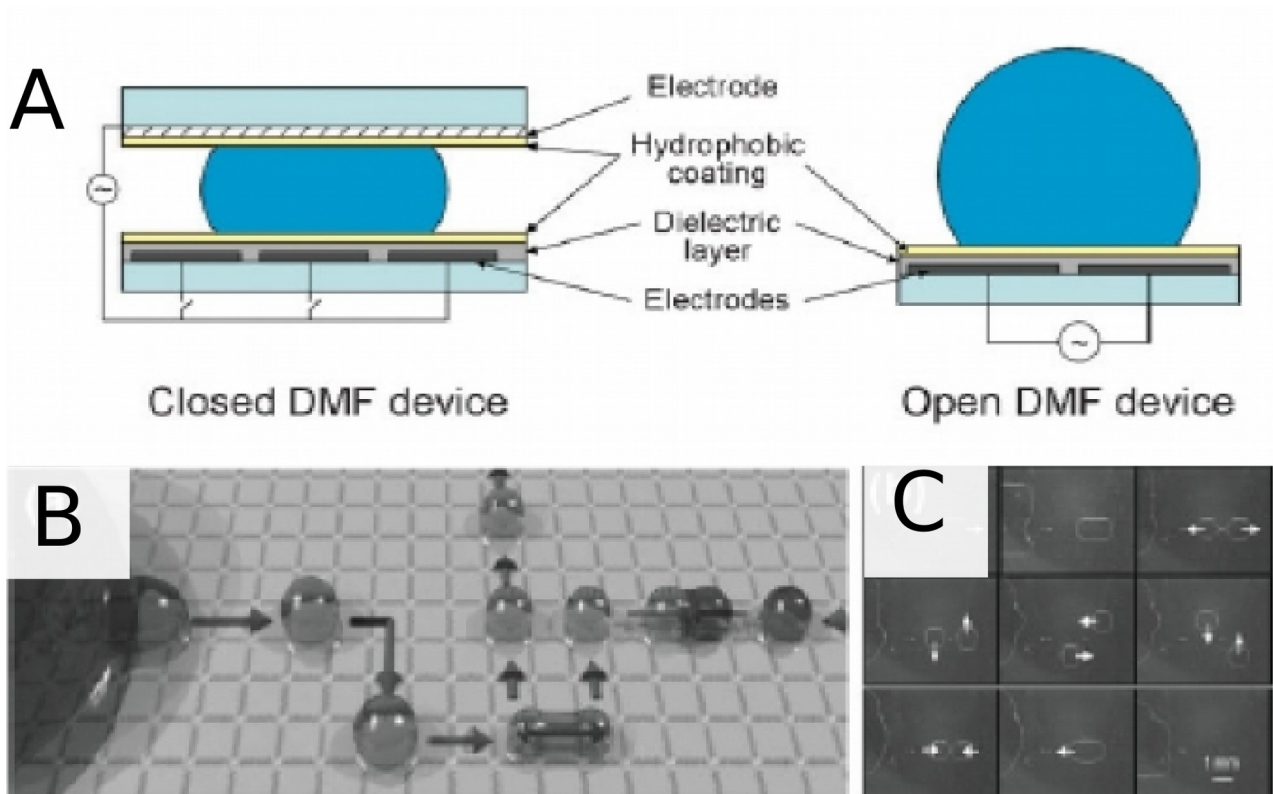


Figure 1: Digital Microfluidic Systems (DMF) for the manipulation of droplets.

Droplets are actuated on open or closed surfaces patterned with electrodes (Panel A). Using electrowetting on dielectric, droplets are produced, actuated, splitted, and fused on a surface patterned with electrodes (Panels B and C) in an array-based system. Figure reprinted from [13].

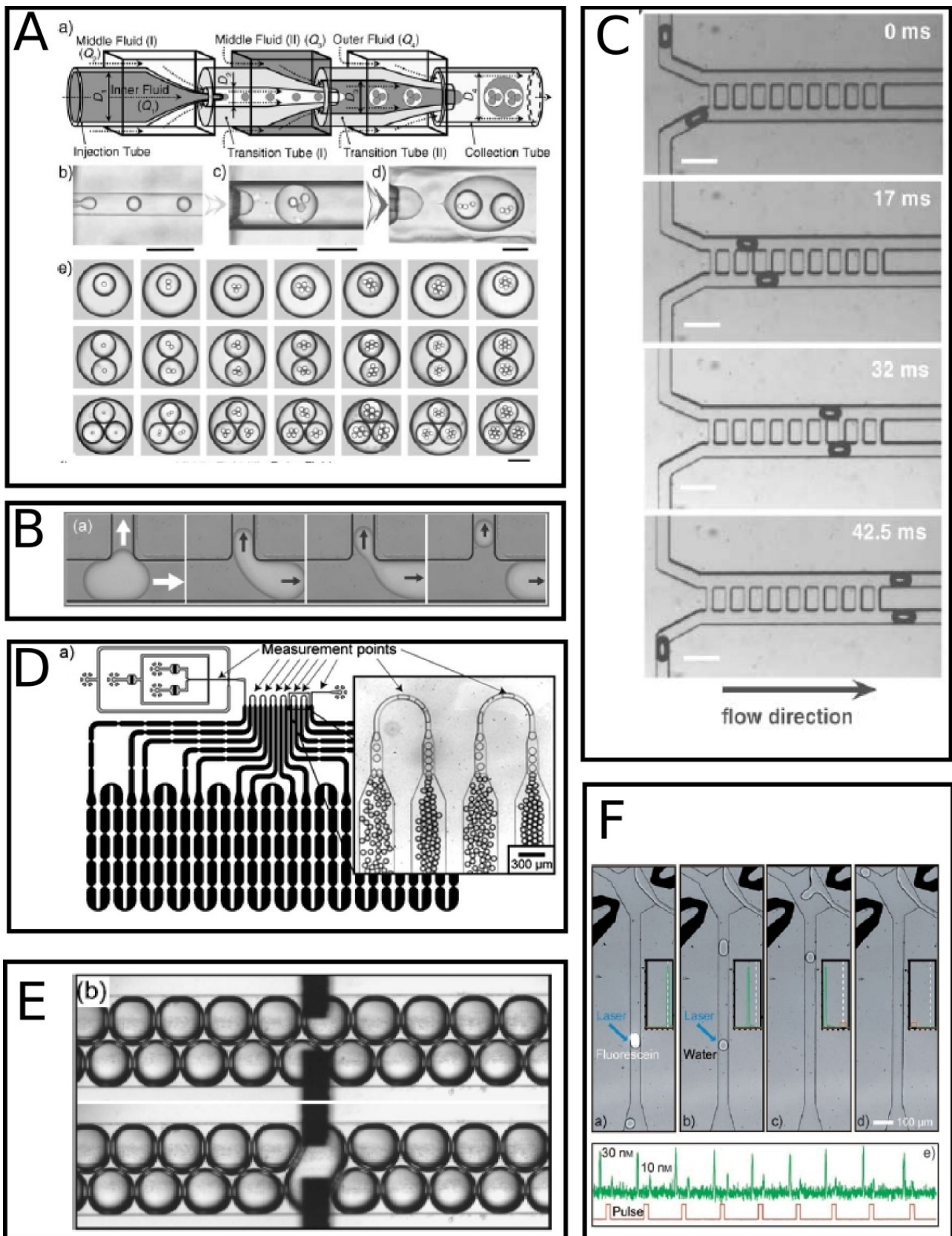


Figure 2: Manipulation of droplets in microfluidic channels.

Droplets are produced as emulsions by coflow which also enables complex multiple emulsions to be controlled (Panel A [42]). The modules also enable droplet splitting (Panel B [51] 63), droplet synchronization (Panel C [63]51), droplet incubation (Panel D [67]), droplet fusion (Panel E [78]) and droplet detection and sorting (Panel F [26]).

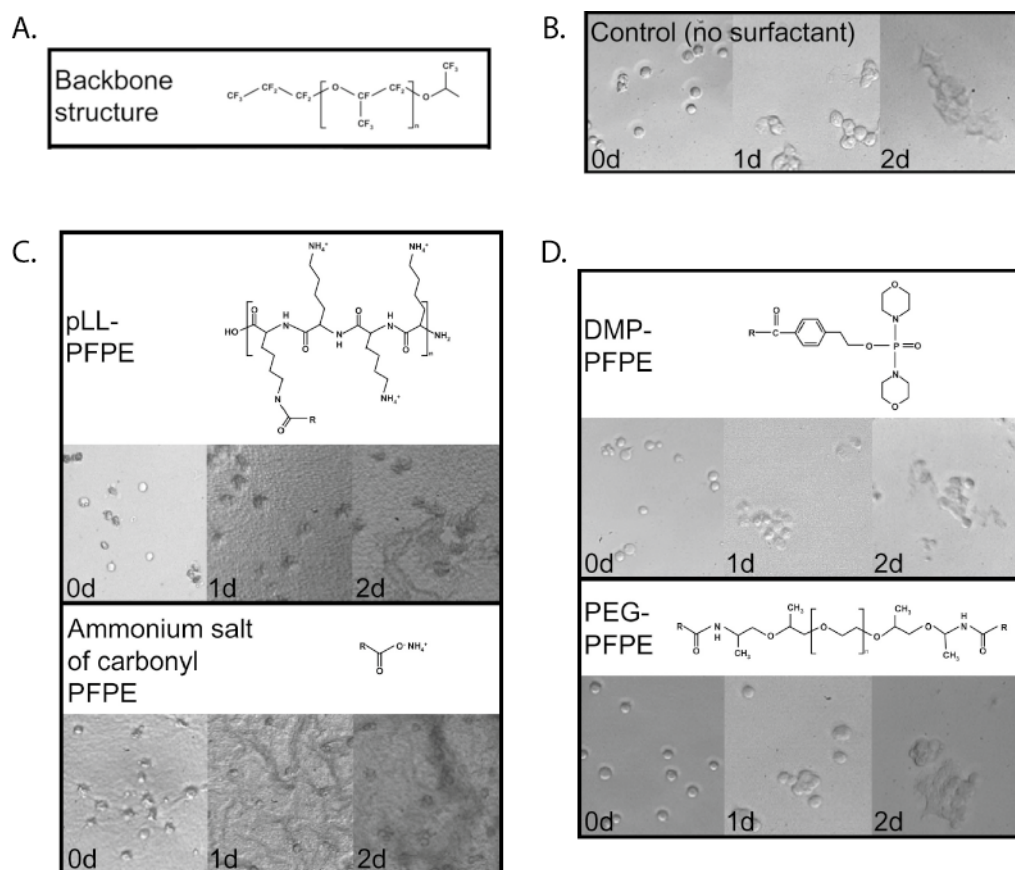


Figure 3: Examples of surfactants obtained by modification of the hydrophilic head of commercially available PFPE-based surfactants (poly(perfluoropropylene glycol)-carboxylates, Panel A) and their effect on long-term survival of eukaryotic cells (modified from [103]). For each surfactant, the chemical structure and the results of the biocompatibility assay (microscopical bright-field images) are shown. For the assay, HEK293T cells were incubated for 48h on a layer of perfluorinated FC40 oil in the presence or absence of the indicated surfactant (0.5% w/w). In the absence of any surfactant, the cells retained an intact morphology and even proliferated (control, Panel B), whereas the ammonium salt of carboxy-PFPE and poly-L-lysine-PFPE (PLL-PFPE) mediated cell lysis (Panel C). However, PolyEthylene Glycol-PFPE (PEG-PFPE) and dimorpholinophosphate-PFPE (DMP-PFPE) showed good biocompatibility, did not affect the integrity of the cellular membrane, and even allowed for cell proliferation (Panel D).

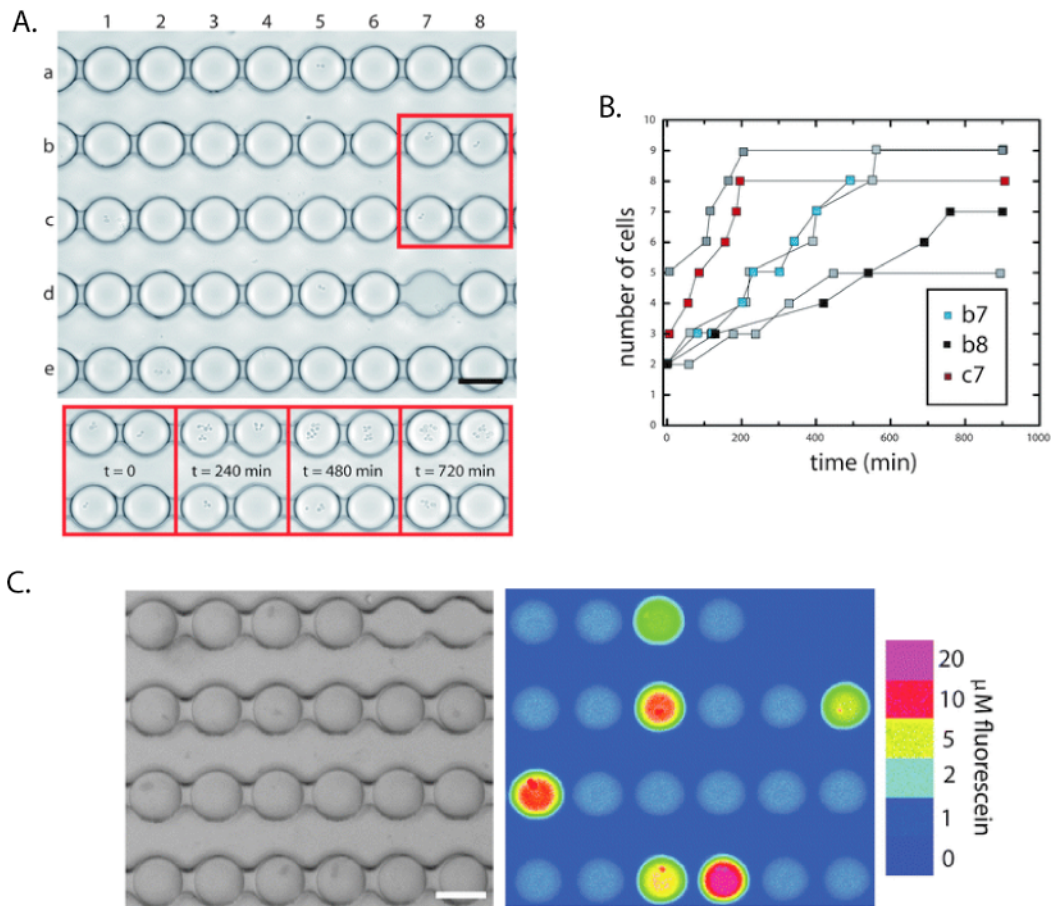


Figure 4: Droplet parking device for cell growth studies or cell-based assays (modified from [69]).

Panels A and B. Monitoring of growth rates of yeast cells in an array of chambers of a ‘dropspots’ device. Panel A: Bright field image of cells parking in the device at the beginning of the experiment (top) or at specific time over 12 hours for a sub-set of droplets (bottom). Panel B: Tracking of the number of cells in individual droplets over a 15 hours incubation period for 6 individual representative droplets. Colour plots represent droplets identified in the image (panel A, bottom). Panel C: Monitoring of beta-galactosidase activity of cells in droplets in an array of chambers of a Dropspots device. Left: Bright field image of the parked cells; Right: Colour map gradient of a fluorescence image at time = 45 min. Scales, 40 micrometers.

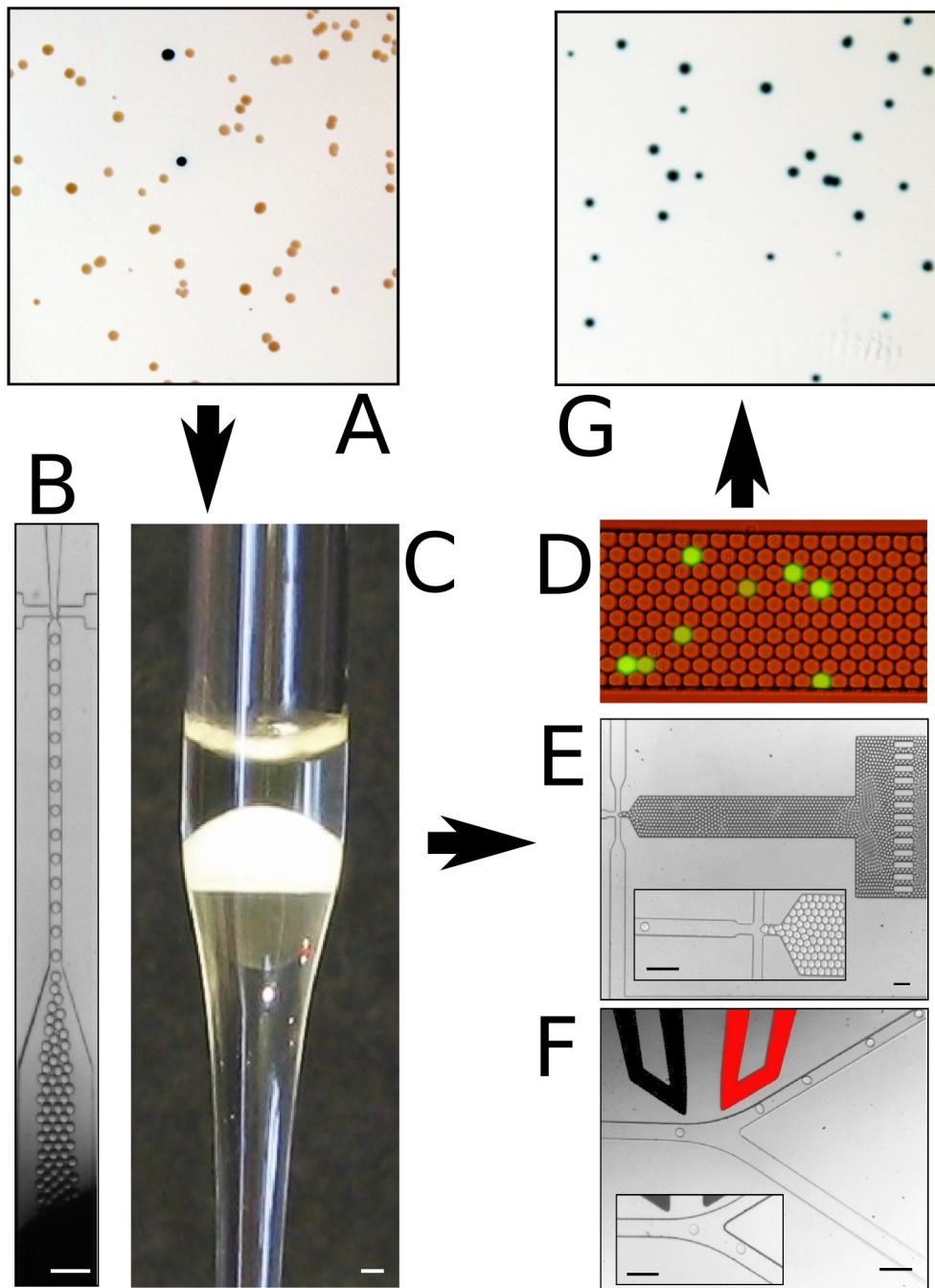


Figure 5: Microfluidic cell sorting based on enzymatic activities of the cells.

(Panel A) A mixture of beta-gal positive (blue) and negative (white) bacterial cell is encapsulated in droplets with a fluorogenic substrate (Panel B) and incubated in a Pasteur pipette to allow the enzymatic reaction to occur (Panel C). After incubation, the emulsion is reloaded in a microfluidic device. Picture obtained using a fluorescent microscope shows positive droplets (Panel D) in the pool of empty droplet containing either no cell or negative cells. The reinjection of the droplets in a sorter (Panel E) enables the selection of the fluorescent droplets using electric fields (Panel F). The cells in the droplets are recovered and plated back onto agar plates (Panel G) (adapted from [27])