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Life in a Bubble

Droplet Microfluidics—A Tool for Single-Cell Analysis

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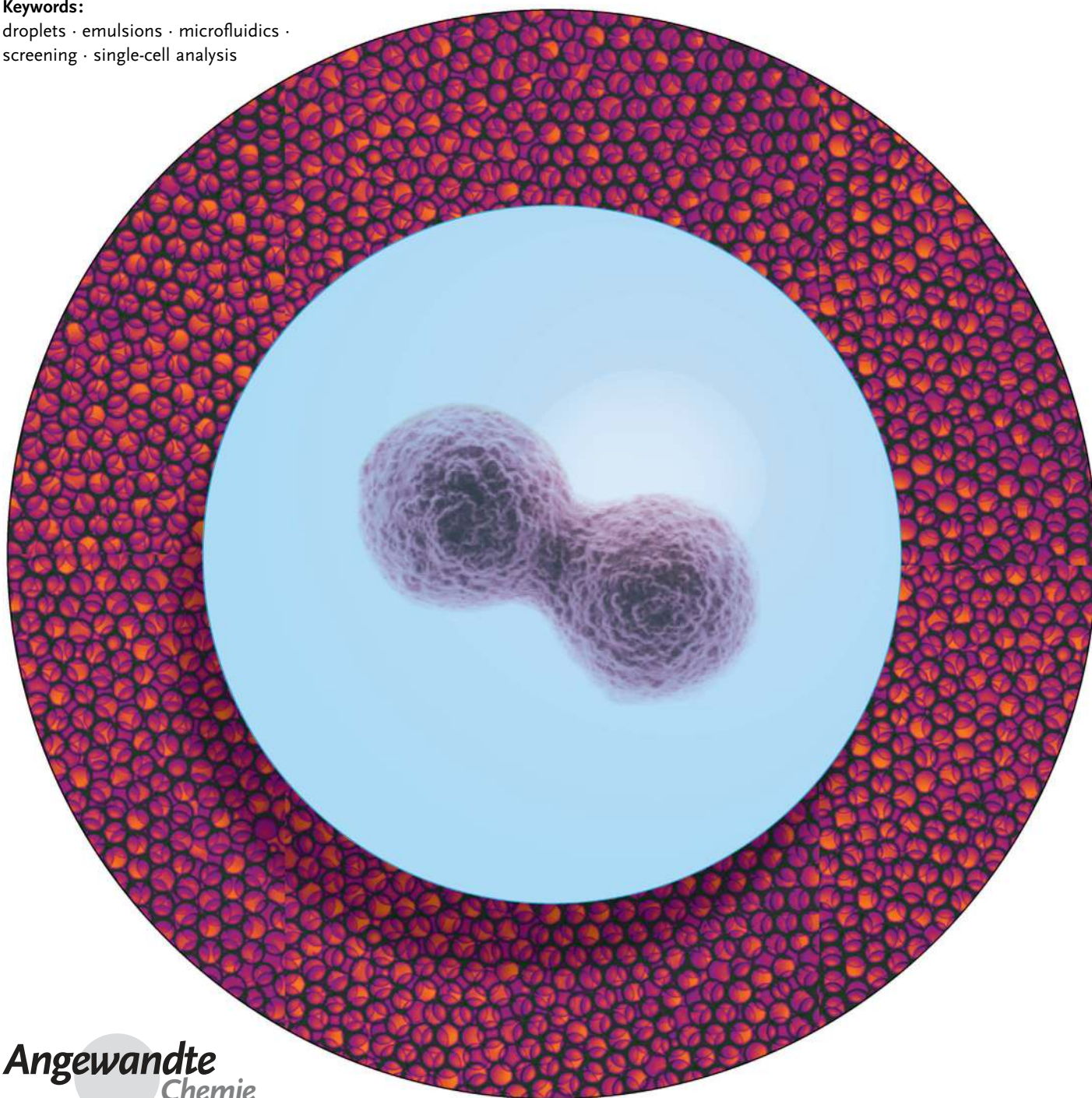
Droplet microfluidics allows the isolation of single cells and reagents in monodisperse picoliter liquid capsules and manipulations at a throughput of thousands of droplets per second. These qualities allow many of the challenges in single-cell analysis to be overcome. Monodispersity enables quantitative control of solute concentrations, while encapsulation in droplets provides an isolated compartment for the single cell and its immediate environment. The high throughput allows the processing and analysis of the tens of thousands to millions of cells that must be analyzed to accurately describe a heterogeneous cell population so as to find rare cell types or access sufficient biological space to find hits in a directed evolution experiment. The low volumes of the droplets make very large screens economically viable. This Review gives an overview of the current state of single-cell analysis involving droplet microfluidics and offers examples where droplet microfluidics can further biological understanding.

From the Contents

1. Introduction

The single cell is the fundamental component of life. The invention of the microscope and realization of the cellular makeup of life resulted in the similarities between cells as well as differences among them becoming evident. Cells have been categorized in terms of tissue origin as well as the characteristics of the cell itself and those of its secreted products. This stratification of the behavior of cell populations has helped pinpoint the cell types involved in disease[1] as well as to describe cell–cell interactions.[2] The use of high-throughput analytical molecular biology techniques have produced drafts of the genomes of a large number of organisms[3] and groups of organisms, such as the gut microbiome,[4] and allowed the study of the human proteome[5] as well as secretomes of organisms[6] and of cancers.[7]

As techniques have become available to study single cells, many examples of heterogeneities have been unveiled, even within isogenic cultures, in terms of size,[8] gene expression,[9] and growth characteristics.[10] Furthermore, the distributions of these traits in cell populations have, in a number of cases, been found to differ substantially from Gaussian distributions, with multimodal or other complex underlying distributions being evident (Figure 1). Despite this insight, many cell studies rely on averages of cell ensembles merely based on the assumption of an underlying normal distribution. An excellent report discussing single-cell and ensemble dynamics was published by the Lee research group in 2006.[11] To accurately describe and eventually elucidate the underlying causes of these heterogeneities requires the analysis of single cells in large enough numbers to correctly represent the population. Such methods are collectively termed high-throughput single-cell analysis techniques. The preeminent technique has been laser-based flow cytometry, which uses laser light to analyze the presence of fluorescent molecules and the light-scattering properties of single cells as they are moved single file past a detector at a rate of tens of thousands of cells per second. Flow cytometry is ideally suited for single time-point screening or population analysis of protein expression, typically by labeling with a fluorescent antibody or coexpression of fluorescent proteins. Some important single-cell analyses, such as the tracking of specific cells over time, the analysis of secreted products, and the analysis of isolated cells or clones, have however been beyond the purview of flow cytometry, to a large degree because of the lack of a robust compartmentalization of single cells by this technique.

Microfluidics deals with understanding the behavior and manipulation of fluids at the micrometer scale. In this field, a growing knowledge base and numerous techniques for fluid handling have been developed, with applications in the medical and biotechnology fields, as well as in materials science and chemistry. Research and development on single-cell analysis involving microfluidic techniques has increased significantly during the last couple of years. In 2011, a number of reviews on single-cell analysis by microfluidics were published,[12] one of which is partly dedicated to single-cell analysis in microfluidic droplets.[12d] A number of recent reviews on various aspects of biological analysis by droplet microfluidics have also been published.[13]

We believe that there are several reasons why microfluidics has emerged as an important enabling tool for single-cell analysis, including, for example, small reagent volumes, dynamic control of reagents, high-throughput, biocompatibility, and sensitivity. Single-cell applications in microfluidics chips include the polymerase chain reaction (PCR), culturing of cells, cytotoxicity, sorting, separation, clone formation, lysis, gene and protein expression, and antibody secretion studies. Most of the microfluidics systems used to study single-cell behavior employ some separation of the cells, by spatial separation on surfaces or by compartmentalization in solid arrays or in two-phase systems, such as droplets.

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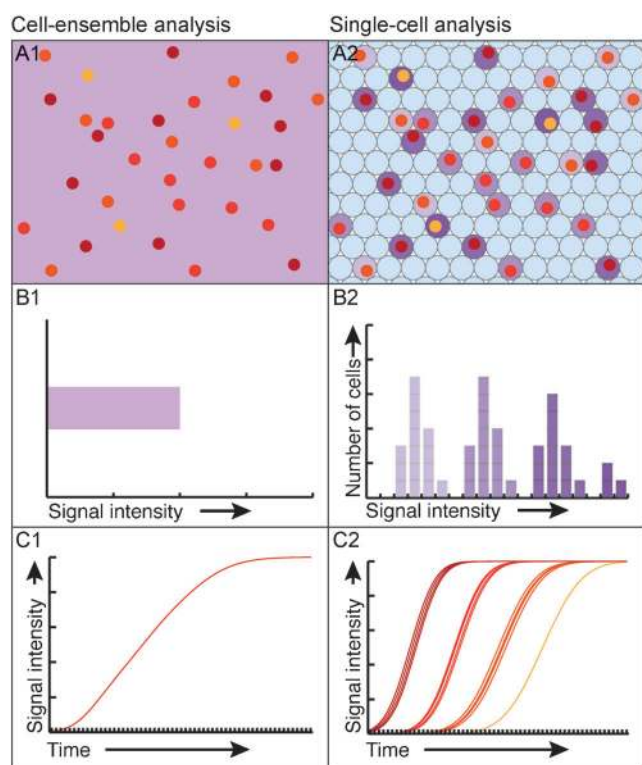E-mail: hakan.jonsson@biotech.kth.se

Figure 1. Schematic illustrations of the differences between ensemble analysis of cells (A1, B1, and C1) and single-cell analysis (A2, B2, and C2), which provide the motivation for assaying the cells in a population individually rather than as an ensemble. Single-cell analysis can characterize a bi- or multimodal distribution of a certain trait (B2) or dynamic (C2), while ensemble analysis of the same cells would yield the average of the trait (B1) or dynamic (C1), which in some cases, such as the hypothetical case described, does not describe the underlying population well. Rare cell types (such as those colored yellow) provide another motivation for single-cell analysis, as cells which only constitute a small minority of a population are easily masked by the majority of cells, even though their traits may differ substantially (Figure adapted from Ref. [11]).

1.1. Compartmentalization by Emulsions

Emulsions are colloids that result from the breakup of one liquid phase in another, typically in the presence of stabilizing surface-active agents (surfactants). An emulsion is a metasta-ble state which, with time, degrades by coalescence or Ostwald ripening.[14] However, emulsions can remain intact for more than a year. Emulsions can be produced by simply mixing an oil and aqueous phase together. These emulsions typically contain droplets ranging in size from hundreds of nanometers to tens of micrometers. The dispersity, defined as the standard deviation of the distribution of droplet diameters divided by the mean droplet diameter,[15] in these emulsions is high.

The use of emulsions to compartmentalize and study cell solutions has been employed by biologists since the 1950s, when Nossal and Lederberg generated water-in-oil droplets containing bacteria by spraying a bacterial solution into an oil film by using the submerged 100 µm wide tip of a micro-pipette.[16] This technique resulted in compartmentalization of single bacteria in polydisperse emulsion droplets. Later, a similar technique enabled the study of the production of antibodies from single cells[16] and some of the first experiments on single-molecule activity.[17] In these, β-galactosidase (β-gal) was detected and the activity of single enzymes characterized by encapsulating dilute suspensions of enzyme with a fluorogenic substrate. Interestingly, even in this early study, Rotman and Lederberg comment on the potential of these compartments for the study of single-molecule hetero-geneities. Later, researchers working on droplet microfluidics seized on the same potential to analyze heterogeneities not only in populations of molecules but also in cell populations. Despite the polydispersity of emulsions generated by macro-scale agitation, which result in individual compartments with a wide range of volumes, these compartments have been utilized for emulsion PCR (emPCR)[18] and BEAMing,[19] as used in many of the second generation DNA sequencing methods, as well as for directed evolution experiments.[20]

2. Droplet Microfluidics

This Review deals with high-throughput droplet micro-fluidics involving monodisperse aqueous droplets generated by a pressure-driven flow in a continuous oil phase where droplets are typically analyzed and manipulated at rates of over 1000 droplets per second. The droplets are generated and manipulated in microfluidic circuits in which the geom-etry of the circuit to a large extent defines the manipulation

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12178   www.angewandte.org

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Angew. Chem. Int. Ed. **2012**, *51*, 12176–12192

carried out. A number of other microfluidic techniques, notably electrowetting on dielectric (EWOD)[21] have at times also been referred to as droplet microfluidics. EWOD droplets are generally larger (micro- to nanoliter range) than the typical size used in droplet microfluidics and are manipulated on surfaces instead of in a fluid. In EWOD devices, droplets are addressed individually, which is not always the case in droplet microfluidics devices. Although these EWOD droplets have been used as vessels for single-cells analysis, they are beyond the scope of this Review because of their different characteristics in terms of drop size, drop stabilization, manipulation methods, and issues regarding biocompatibility.

2.1. Microfluidic Chips for Droplet Microfluidics

The microfluidics circuits used for droplet microfluidics are typically glass-polydimethylsiloxane (PDMS) devices fabricated by soft lithography,[22] although devices made from other materials such as fused silica,[23] thiolene resin,[24] poly(methyl methacrylate)[25] (PMMA), polystyrene[26] (PS), and fluorinated thermoplastic polymers[27] have also been demonstrated. The soft lithography process has been employed extensively and its uses and applications have been reviewed.[28] PDMS is inherently hydrophobic, but many techniques for surface modification have been developed[29] to afford PDMS devices with a wide range of channel coatings that have different wetting properties. Glass-PDMS devices for use with two-phase aqueous-in-fluorinated oil systems generally employ hydrophobic/fluorophilic coating schemes such as Aquapel (PPG Industries) flushing[30] or fluorosilane coating.[23] As some droplet manipulations require large electric fields close to the channels, methods to integrate electrodes in glass-PDMS devices[31] are often employed. An advantage of using PDMS devices is the materials permeability to O_2 and CO_2, since its porous structure allows gas bubbles to escape the channels and gas to be transported into and from cells within the channel. The porosity does, however, also allow the diffusion of other small molecules into the material.[32]

2.2. Droplet Generation

Highly monodisperse emulsions with a narrow distribution of droplet sizes were produced by a macroscale process analogous to fractionated crystallization[33] before dedicated droplet generation devices became available. This technique is, however, quite inefficient since it does not make use of the vast majority of the droplets produced. In 2000, techniques for generating monodisperse droplets by break-off from an aqueous jet exiting a capillary in a coflowing continuous phase containing a stabilizing surfactant were demonstrated.[34] In 2001, Thorsen et al. demonstrated a microfluidic device for the controlled generation of monodisperse (1–3% difference in diameter) aqueous droplets by the injection of water into a continuous oil phase in a T junction by using a pressure-driven flow.[35] These microfluidic approaches to

the generation of highly monodisperse droplets had the advantage, over earlier techniques, that droplets could be generated continuously. Many subsequent microfluidic devices for generating droplets have been demonstrated, and they generally exhibit droplet generation frequencies ranging between 0.1 and 10 kHz. Parallelization has yielded circuits for the generation of droplets that are capable of producing quantities as large as 1 liter of monodisperse 96.4 µm droplets per hour.[36] The three main strategies for continuous pressure-driven generation of droplets in the dripping regime are break-up in coflowing streams, cross-flowing streams in a T junction, and flow focusing[15] (Figure 2 A–C). Droplets are formed as a result of competing stresses. Surface tension acts to reduce the interfacial area while viscous stresses extend and drag the interfacing segment downstream. The two regimes of droplet generation in a two-phase system with a moderate difference in viscosity are commonly referred to as dripping and jetting or convective and absolute Rayleigh–Plateau instability. The droplets treated in this Review are, with few exceptions, generated in the former regime.

Several droplet generators have been applied in series to produce droplets within droplets of alternating phases, for example, water–oil–water (w/o/w) emulsions.[37] Modules for the generation of droplets have also been pressure-coupled by connecting channels between two generating circuits to synchronize the antiphase generation of droplets.[38]
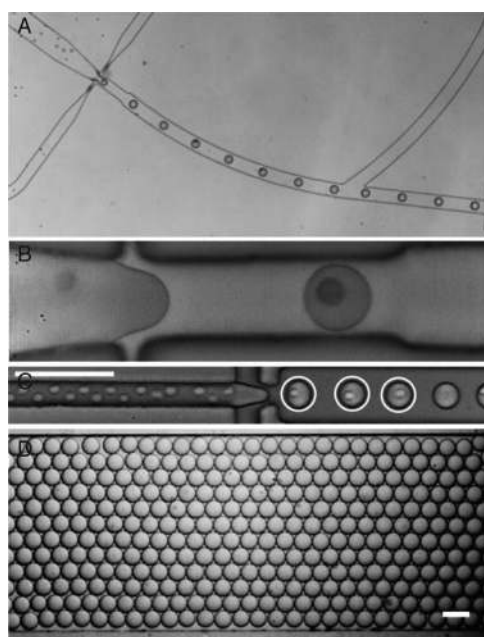
Figure 2. Micrographs depicting the generation of monodisperse droplets by flow focusing in droplet microfluidics devices. Overview (A) and magnification (B) of the encapsulation of randomly distributed cells[115] to yield a Poisson distribution of droplets containing U937 cells. C) The use of a narrow channel with the dimensions shown but larger in the dimension normal to the picture provides an ordering of cells. This enables the encapsulation of a significantly larger ratio of cells than does random encapsulation.[43] D) Constrained monolayers of droplets ordered in a crystalline pattern as a result of their size homogeneity.[30b] (Images reprinted with permission (A) and (B) from Wiley-WCH Verlag GmbH & Co KGaA, and (C) and (D) The Royal Society of Chemistry, 2008.)

Noncontinuous approaches for the generation of discrete droplets "on-demand" by electric[39] or pressure pulses[40] as well as laser-controlled droplet formation[41] have been demonstrated. These methods enable the ad hoc generation of single droplets with a high volume accuracy but at considerably lower rates than continuous generation. These "on-demand" methods are highly suited for single-cell analysis of preselected cell subpopulations or when the analysis of large numbers of cells is not required.

2.3. Single-Cell Encapsulation

Cells from a multitude of sources, ranging from human patient samples to cell lines and bacteria, have been encapsulated at high throughput, mainly by flow focusing (Figure 2 A,B), for culture and analysis in droplets generated in microfluidic circuitry. Encapsulating cells delivered to the droplet-generation nozzle at random is a process which yields a resulting population of droplets with a Poisson distributed cell occupancy.[42] This means that it is not possible to achieve uniform or even predominantly single-cell occupancy in droplets based on the standard circuits employed for droplet generation. Depending on how large a subset of droplets occupied by more the one cell can be tolerated in a certain application, the cell concentration can be tuned to achieve the Poisson distribution sought, for example, 90, 9, and 1 % or 40, 30, and 30 % occupancy of 0.1 and >1 cell per droplet. Methods to circumvent a Poisson distributed cell occupancy of droplets have been developed for high-throughput injection,[43] and depend on inertial effects in narrow channels (Figure 2 C). In the most reported studies, the throughput enabled by droplet microfluidics render the Poisson-distributed cell occupancy a non-essential issue and to some extent the droplets without cells can be useful as internal controls in the development of assays. Dielectrophoretic droplet sorting after encapsulation would be one potential method to circumvent the issue of differential cell occupancy for droplets containing cells that can be detected fluorescently or otherwise. Various hydrodynamic strategies to generate or separate single-cell/clone droplets from droplets not containing any cells have also been developed. Such methods include cell-triggered droplet generation and separation by size[44] or separation of droplets containing single clones by deterministic lateral displacement after cell-induced droplet shrinking.[45] Achieving a large population of homogeneous singly populated droplets would, for example, improve studies on cell–cell interactions and assays where microbeads are used to capture protein or DNA from a single cell. The Poisson distribution of cell occupancy is most acutely a problem for applications where two or more different single objects, such as cells or particles, are required to be present in each droplet.

2.4. Droplet Stability, Biocompatibility, and Leakage

Microdroplet emulsions are by definition metastable colloids which are stabilized by surfactants. Surfactants are not essential for the generation of droplets, but droplets not stabilized by surfactants will coalesce upon contact after formation.[13a] Metastability indicates that the droplets have a limited lifetime before coalescence. This lifetime can, however, vary from sub-millisecond to years depending on the stabilizing characteristics of the surfactant in the particular two-phase system and the physical conditions surrounding the emulsion. Two important characteristics of an oil and aqueous-phase surfactant system are described by the critical micellar concentration (CMC) and the interfacial tension. The CMC describes the amount of free surfactant soluble in the continuous phase, and the interfacial tension, γ_{CMC}, the degree to which the surfactant has organized the interface between the two immiscible phases, thereby stabilizing the droplet. General requirements for droplet stabilizations in the system are a CMC value in the 100 mM–10 μM range and a γ_{CMC} value of <20 mN m^{-1}.[46] A recent review by Baret[47] provides an in-depth account of the role of surfactants in droplet microfluidic systems.

The oils used for water-in-oil emulsions are generally hydrocarbon and fluorocarbon oils. Droplets in hydrocarbon oils, such as hexadecane or so-called mineral oil, are most commonly stabilized by commercially available surfactants, typically Span80[48] or Abil EM.[49] Fluorocarbon oils have advantages over hydrocarbon oils in terms of superior oxygen transport properties and most importantly immiscibility with organic as well as aqueous solvents. The immiscibility of fluorous liquids in these solvents renders them effectively a "fifth phase" in addition to gas, aqueous, organic, and solid phases.[50] Microemulsions in fluorocarbon oils, typically using Fluorinert FC-40, FC-77 (3M), or similar oils as the continuous oil phase, have been shown to be stabilized by perfluoropolyether (PFPE) based surfactants such as Krytox (DuPont)[30b] or pseudosurfactants such as perfluorooctanol.[51] Krytox consists of a perfluoropolyether (PFPE) tail and a carboxylic head group. Substituting the carboxylic head group with different nonpolar hydrophilic head groups has been investigated by Clausell-Tormos et al. for improved biocompatibility.[52] Several different surfactant head groups—polyethylene glycol (PEG), the ammonium salt of carboxy-PFPE, dimorpholino phosphate (DMP) PFPE and poly-L-lysine (pLL) PFPE—were tested by incubating HEK293T cells on top of an oil layer containing the surfactant. The ammonium salt and pLL-PFPE were found to cause cell lysis, while DMP-PFPE and PEG-PFPE performed well. Of these two, PEG-PFPE has been most widely characterized and used. To optimize the PEG-PFPE surfactant, Holtze et al.[30b] produced a number of PEG-PFPE diblock amphiphilic fluorosurfactants from PEG and PFPE chains of different lengths. The combination of 600 g mol^{-1} PEG with 6000 g mol^{-1} PFPE proved optimal in terms of balancing the characteristics of droplet formation and long-term droplet stability for off-chip storage. This surfactant was also shown to be biocompatible with a number of different biological uses, such as PCR and culturing of cells.

Recently, a number of novel fluorosurfactants resulting from combinations of PFPE or perfluoroalkyl (PFA) tails with carbohydrate, crown ether, and hexaethylene head groups have been synthesized and tested for the stabilization of organic solvent droplets; particularly promising results

12180   www.angewandte.org

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Angew. Chem. Int. Ed. **2012**, *51*, 12176–12192

were found for the two latter head groups.[46,53] In particular, PFPE-hexaethylene glycol stabilized aqueous as well as acetonitrile droplets.

Given the large surface to volume ratio of microdroplets, surface interactions at the oil/water interface are of great importance in selecting the oil/surfactant system so as to interfere minimally with the cells and biologically active molecules contained in the droplet. Certain combinations of fluorinated oils and (ionic) fluorosurfactants have been demonstrated to result in nonspecific protein adsorption at the interface.[54] Protein adsorption at the droplet surface may have detrimental effects where functional proteins are involved, such as in enzyme assays or screening.

A final consideration in selecting oil/surfactant systems is the possibility to transport compounds in the dispersed phase or of the bulk component of the dispersed phase itself across the droplet interface and into the bulk continuous phase. While droplets may in many cases, for simplicity, be viewed as closed compartments, this is not generally true. The transport of small molecules from the aqueous to the oil phase[55] as well as between droplets of different solute concentrations[56] has been demonstrated to occur. Therefore, the selection of an oil/surfactant system compatible with the droplet contents and the intended application are necessary for successful encapsulation, particularly in single-cell applications. The rate of transport of modified coumarins between droplets through the continuous fluorinated oil phase was shown to increase with decreasing hydrophilicity of the coumarins.[56] Also worth noting is that even molecules which are immiscible and insoluble in fluorinated oils can undergo micellar transport through the oil phase.

2.5. Complementary Formats for Cell Compartmentalization

In addition to microdroplets, a number of complementary highly parallel or high-throughput microfluidic technologies have been developed concurrently. To one extent or another, these are versions of microarrays. They include miniaturized compartments machined or etched into solid or polymer surfaces with densities of 10^6 compartments per device.[57] Microwell plates are particularly well-suited for single-cell or clone assays[58] of adherent cells as they provide a surface on which these cells can grow. The microwell plate devices generally require automated microscopy to enable high-throughput operation.

Another example of arraylike devices is large-scale integrated (LSI) elastomeric fluidic systems, where compartmentalization is controlled by valves.[59] These devices have been employed for many different large-scale experiments, such as the recent sequencing of the human whole genome by using single-molecule techniques.[60] This type of device provides a great deal of control and has many potential applications, although design complexity limits the maximum number of assays.

An intermediate between droplets and solid-compartment arrays are the so-called "SlipChip" devices, which combine two microfabricated compartments or channel surfaces that are slid across one another, thereby transporting

liquid plugs from one set of compartments or channels to another set in parallel.[61] These formats share many of the benefits of miniaturized compartmentalization in terms of increased speed and sensitivity with decreased reagent use.

3. Cells in Droplets

The ability to study single cells is necessary to elucidate many cellular and subcellular processes, and to probe the heterogeneity of a cell population. A droplet can provide a well-defined, controlled, addressable, and rapidly transportable compartment for the culture of cells. The picoliter compartment of the droplet has the benefits of having a size on the same scale as the cell and of coupling the single cell with its individual environment.

3.1. Cell Culture in Droplets

In vitro cell culture, in particular of mammalian cells, requires that the compartmentalization platform satisfies a number of conditions, such as the supply of nutrients from a growth medium, the supply of respiratory gas, as well as the removal of any toxic factors produced by the cell before the concentration reaches a growth-limiting or cell-death-inducing level. The standard culture methods using Petri dishes and culture flasks provide all these, but do not compartmentalize clones or individual cells. Droplets have been demonstrated to provide the necessary conditions to retain viability in several different cell types, such as bacteria,[42,62] yeast,[30b] hybridoma,[63] adherent mammalian,[64] adherent insect,[65] and even human cells[52,66] (Figure 3). For example, single cells of the human cell lines Jurkat (non-adherent) and HEK293T (adherent) have been demonstrated to remain viable at over 80% for 3 days.[52] Adherent insect cells have also been encapsulated.[65] These adherent cells were cultured on solid supports to better resemble the conditions under which they normally grow. Even entire nematodes (C. elegans)[52] and zebra fish (D. rerio)[67] have been encapsulated in stable droplets with sizes approaching a microliter. Proliferation in droplets has been demonstrated for bacterial (E. coli[68] and S. aureus[69]), microalgae[70] (C. reinhardtii, C. vulgaris, D. tertiolecta), and yeast (S. cerevisiae)[71] cells. For these cell types, proliferation rates of the encapsulated cells have quite closely resembled bulk proliferation rates. The proliferation of the encapsulated cells, however, ceased after a certain period of time, presumably because of a lack of nutrients or a build-up of toxic factors. Hybridoma cells, although they do not proliferate, have been demonstrated to secrete antibodies at similar rates as in bulk culture in analyses performed by collecting droplets, allowing them to coalesce, and then analyzing their contents for antibodies.[63]

Mammalian cells, however, have proliferated only very slowly or not significantly in the studies published.[52] Creating the conditions that allow mammalian cells to proliferate in droplets at normal rates presents a challenge, which has not been answered in full as of yet. Until this challenge is met, the question of the extent to which cells in droplets differ from
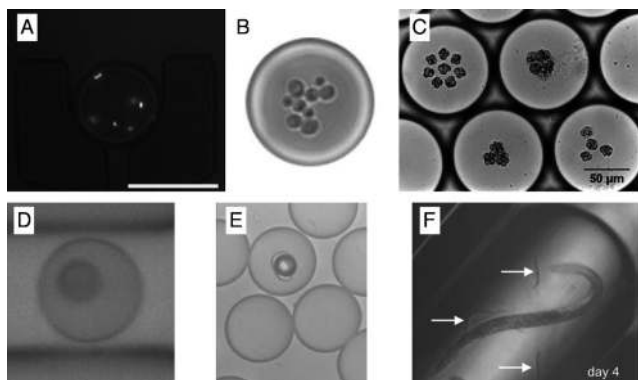
Figure 3. Many different cell types have been encapsulated and demonstrated to retain their viability in droplets. A) *E. coli* cells expressing RFP.[97d] B) Droplet-cultured *S. cerevisiae* clones proliferating in droplets.[30b] C) The green microalgae *C. reinhardtii*.[70] D) Human monocytic cell line U937.[115] E) Adherent insect cells *B. mori* growing on a bead surface.[65] F) Two generations of *C. elegans* cells cultured in droplets. The arrows indicate the second generation of droplet-cultured worms.[52] (Reprinted with permission from: (A), (B), and (C), The Royal Society of Chemistry 2009, 2008, and 2011, (D) Wiley-WCH Verlag GmbH & Co KGaA 2009, (E) and (F), Elsevier 2010 and Chemistry & Biology 2008.)

cells in standard culture will remain open. A crucial characteristic of the fluorinated oils for the long-term culture of aerobically respiring cells is their ability to efficiently transport oxygen and carbon dioxide.[72]

The most stringent evaluation to date of cell viability after encapsulation was carried out by Brouzes et al., who developed and applied a fluorescence-based intradroplet live/dead assay to human U937 cells and demonstrated a viability of over 80% over a period of 4 days (Figure 7 D2).[66] The same study does, however, describe cell death on the order of 15% of injected cells during or immediately following droplet generation. This was attributed to shear stress during injection and/or interactions with the fluorinated oils or surfactants.

3.2. Cell Retrieval, Freezing, and Lysis in Droplets

The retrieval of cells from droplets is most commonly performed by adding destabilizing chemical agents to the oil phase[52] and agitating the emulsion. A more controlled method involving electronically steering droplets to form a continuous phase[73] may prove a more elegant and potentially less cytotoxic method, although it has to date only been demonstrated for the retrieval of encapsulated microbeads. Freezing is one standard method to store cells or samples for extended periods of time. Thawing encapsulated mouse B cells that had been frozen while encapsulated resulted in them retaining their viability.[74]

One reason to compartmentalize single cells is to enable the manipulation of isolated cells individually in a controlled environment without affecting other cells. Individual mouse mast cells have, for example, been lysed in droplets by laser heating/photolysis.[75] Lysis of cells within a droplet allows for the controlled release of the cell contents into the picoliter

container provided by the droplet. This allows the cell contents to be delivered to downstream analysis by, for example, mass spectrometry and RNA or DNA sequencing.

3.3. Transfection, Transduction, and Transformation in Droplets

The transfer of exogenous DNA or RNA to a eukaryotic cell by transfection or to a bacterial cell by simple uptake of DNA, transformation, or by virus-mediated transduction are necessary to express foreign proteins, mediate RNA silencing, and many other common molecular biology techniques. The transfection of foreign plasmids to yeast[76] as well as Chinese hamster ovary (CHO) cells[77] has been successfully performed in droplets by electroporation, which momentarily perforates the cell membrane to allow passage of the plasmids into the intracellular compartment. CHO cells have also been chemically transfected while encapsulated.[78] The picoliter volume of the droplets allows for large concentrations of plasmid to be present in the immediate vicinity of the electroporated cell, thus potentially improving the efficiency while ensuring that a cell is only subjected to the gene present in the droplet. The transfection in microdroplets each containing different clones of a plasmid is an attractive method for generating cell-based diversity libraries.[79] The delivery of genetic material by bacteriophages while co-encapsulated with their host bacteria has also been demonstrated.[80]

4. Droplet Manipulations and Analysis

One of the main benefits of droplet microfluidics is the automation of droplet manipulations that is made possible by the technique. Droplet fusion, the addition of material through picoinjection, droplet splitting, incubation, and active as well as passive droplet sorting are some of the main modules of the droplet microfluidics system. These modules are combined into circuits tailored for a particular bioassay. The combination of these building blocks into increasingly complex circuits is not without complications, and this is why in some cases assays are carried out in batches, with the manipulations decoupled. Thus far, droplet microfluidics circuits or circuit workflows are typically fabricated and iteratively optimized for specific applications, although some consensus about the circuit designs for the most common unit operations (e.g. droplet generation, droplet reinjection, in-channel incubation, and droplet sorting) have evolved.

4.1. Droplet Fusion and Picoinjection—Adding Media and Reagents

Droplet fusion is an essential function in droplet microfluidic systems that allows material to be added to an existing droplet. For single-cell applications, droplet addition can be used to deliver enzyme substrates, genetic material, or fresh cell medium as well as reaction-initiating, stimulating, or cell-

lysing reagents. Droplet fusion of surfactant-stabilized droplets requires two conditions to be satisfied. To fuse, droplets must be brought into contact and have their stabilizing surfactant layer disrupted. A number of strategies have been devised to destabilize the surfactant layer, including subjecting the droplets to a large electric field gradient,[81] charging them,[82] removing surfactant from the oil phase to cause a depopulation of the interface surfactant layer,[83] or by laser heating.[84] Water droplets in oil or fluorocarbon oil not stabilized by surfactants spontaneously fuse when brought into contact.[85]

Bringing droplets into contact to fuse is achieved either through chip structures or surfaces which impede the leading droplet, thus allowing the following droplet to catch up, through synchronized generation with[82] or without[38] electrical charge. Examples of channel designs for droplet catch-up include pillar channels (Figure 4A),[86] turns,[83] and channel expansions (Figure 4B).[66] Hydrophilic surface patch fusion (Figure 4C) has also been reported.[85b] The throughput characteristics of these methods vary between thousands of droplets per second for electrocoalescence and surfactant tuning to single droplets per second in the case of laser heating. One of the main benefits of electrically controlled fusion is that it allows the field to be turned on and off at will so that only certain droplet pairs in contact fuse, for example, based on droplet fluorescence. The electronic

control of fusion and the required decision-making electronics do, however, add to the complexity of the microfluidic system employed. A detailed understanding of the dynamics of droplet fusion without the influence of electric fields has been published by Bremond et al.[87] One somewhat surprising result of this study is that droplet fusion occurs, when two droplets, which have been in contact, are moving away from one another. A similar investigation revealed that the electrically controlled droplet fusion occurs when droplets sufficiently close together are exposed to an electric field of adequate strength.[88]

Recently, an alternative method for electric field controlled addition of a defined volume by direct injection into a passing droplet (Figure 4D) was reported by Abate et al.[89] In this method, the liquid to be added to the droplets are held at constant pressure in an adjoining channel. Injected droplets can pass by the liquid meniscus without coalescing to it, as all the oil/aqueous interfaces are stabilized by surfactant. Switching on an electric field produced by electrodes opposite to the meniscus causes the disruption of the surfactant-stabilized interface, and the liquid from the adjoining channel is injected into each droplet as it passes. Picoinjection may prove a powerful alternative to traditional fusion when the objective of fusion is to inject a droplet population with liquid from one or a few reservoirs rather than to fuse droplets from two droplet populations generated separately prior to fusion.
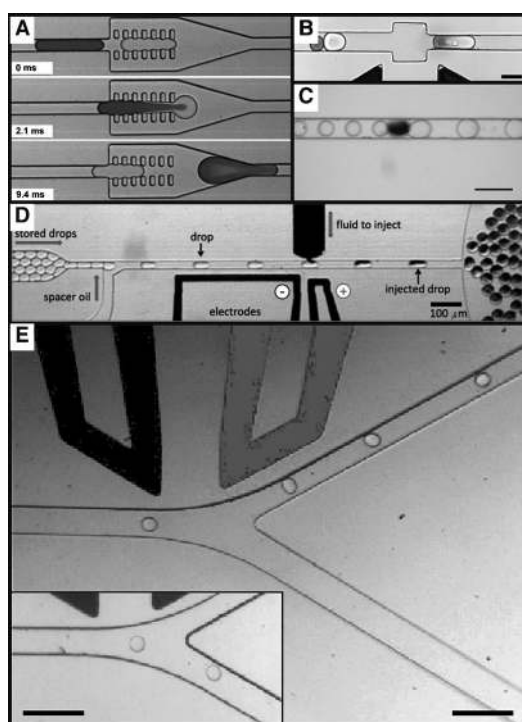

Figure 4. Microfluidic module for droplet fusion by A) passive pillar induced coalescence of surfactant-free plugs,[86] B) electrocoalescence of surfactant-stabilized droplets,[66] C) surface wetting, and D) inline droplet injection.[89] E) Droplet sorting by dielectrophoresis with the electric field on (main image) and the electric field off (inset).[68] (Reprinted with permission (A) and (E) The Royal Society of Chemistry 2008, (B) and (D) National Academy of Sciences USA 2009 and 2010, and (C) H. N. Joensson 2010.)

4.2. Droplet Sorting and Separation

The sorting or separation to retrieve a specific subset of droplets has a long list of applications in cell studies. The retrieval of a small subpopulation of cells for further analysis is a crucial step in cases of circulating tumor cells, hybridoma production, or drug screening. In droplet sorting, the different subsets must be distinguishable by a characteristic of the droplet. Active droplet sorting, involving computerized or ad hoc decision making, has generally made use of fluorescence analysis to discriminate between droplet subsets, while passive droplet separation, which makes use of selective channel geometries, has used droplet size as the discriminating characteristic.

Droplets generated by microfluidics have been actively sorted by charging droplets and sorting them in an electric field,[82] by dielectrophoresis,[90] and localized heating.[41] Dielectrophoretic sorting based on a fluorescent signal has been performed at rates of about 2000 droplets per second (Figure 4E).[68] This sorting technique relies on very steep electric field gradients, typically using AC fields of 30 kHz and 1 kV (peak to peak) with an electrode separation of < 100 μm. The application of the field forces droplets into the higher hydrodynamic resistance arm of a Y junction rather than allowing them to exit through the default, lower resistance, arm. Throughput is limited by shearing at the Y junction and the error rate increases drastically as the distance between droplets decreases.[68] The inclusion of bypass channels inaccessible to droplets at the Y fork improves the robustness of the dielectrophoretic sorting.[91] While cells (e.g. yeast cells[68]) have been recovered from

droplets and cultured after dielectrophoretic droplet sorting, no detailed studies of the subsequent cell viability has been published. However, the cell viability of human U937 cell line cells following electrically controlled droplet fusion, by using somewhat lower potentials, was reported to be $> 80\%$ following overnight incubation.[66] In comparison, cell sorting by fluorescence-activated cell sorters (FACS) has been reported at rates of over 70 000 cells per second,[92] but does not allow the sorting of cells with their surrounding environment (containing interacting cells or molecules or secreted molecules).

Sorting based on localized heating uses high-powered lasers, generally of > 100 mW, to induce localized heating of the droplets, which results in them being diverted. Sorting by localized heating is generally considered a lower throughput sorting method.

In contrast to active separation, passive separation does not employ active decision-making electronics to bring about separation, but instead employs chip structures and hydrodynamics to effect separation. Hydrodynamic devices have been demonstrated that separate droplets by size on the basis of a Rayleigh–Plateau instability of droplets generated by jetting,[44] by employing a Ψ-shaped forked channel and negative pressure outlets,[93] and by deterministic lateral displacement (DLD).[45] The passive nature of hydrodynamic separation makes these techniques amenable to parallelization. It is interesting to note in the context of single-cell analysis that in the cases of the Rayleigh–Plateau instability and DLD sorting, the droplet size depended on the presence of cells in the droplets. In the case of Rayleigh–Plateau instability, the presence of cells triggered a larger droplet to be produced, which could then be sorted hydrodynamically. In the DLD separation, droplets containing yeast cells were observed to shrink as the encapsulated cells grew and divided, while those not containing cells did not. Droplets could subsequently be sorted according to size on the basis of the cell content.

structures bring about in terms of incubation time are offset by concessions in timing exactness, that is, the localization, of droplets. This variability in the incubation time stems from droplets moving at different speeds at different positions in the channel cross-section because of the parabolic shape of the flow velocity function. The significant differences in speed between droplets at different positions in the channel in these devices may be alleviated to a large extent by reshuffling droplets through the use of constrictions along the length of the channel[95] or by splitting the channel into several daughter channels by branching. Despite dispersion effects, droplets from a 64% aqueous volume fraction flowing through channels several hundred micrometers in diameter have been successfully analyzed following incubation.[96] For incubation periods of hours to days, droplets are generally collected in vials or syringes for storage, and requires a very stable emulsion. In this case, the entire population is incubated as a batch and no information about the sequence in which the droplets were generated is retained. Syringe storage is closely associated with re-injection and the dispersal of droplets in an oil stream. The geometry of re-injection devices resembles the flow-focusing geometries used for droplet generation.

For time-series experiments, where several signals are to be recorded from each individual droplet, different droplet trapping strategies have been employed, such as the so-called "dropspots"[71] and similar methods,[97] wells,[98] and channel side compartments.[99] Of these, the elegant solution by Boukellal et al. stands out as it retains the sequence of droplets upon ejection while the others do not. Droplet layering has been suggested as a method to maximize the number of droplets being analyzed per area unit.[100] A collection of incubation methods is depicted in Figure 5. Droplet storage techniques involving gas-permeable chip materials, for example, PDMS, must take evaporation into consideration, particularly when conditions above room temperature are used, such as in the case of on-chip thermocycling PCR.

4.3. Droplet Incubation and Storage

Incubation plays an important role in most biological assays. There are several different approaches to the storage or incubation of droplets that are directed towards distinctive incubation scenarios. Exact timing on the timescale relevant to the assay and the precision in sample localization are the characteristics pertinent to selecting the incubation method. For incubation periods on the time scale of seconds (< 1 min), droplets can be incubated in-line in single file,[94] a method that allows exact timing on the millisecond scale and exact control of the droplet order. However, this method becomes unpractical for incubation over several minutes, as hydrodynamic resistance increases with channel length. Wider and deeper delay channels, which allow droplets to pass each other, have been used to allow for longer incubation times.[66] The gains that these wider and deeper

Figure 5. Droplet incubation in A) a narrow channel, B) a wider channel with droplet shuffling constrictions (adapted from Ref. [95]), C) an array with serial retrieval,[97c] D) "dropspot" array,[71] E) an oil-filled syringe, F) a static array held in place by flow,[97d] and G) an array of inverted wells.[98] (Reprinted with permission (C), (D), and (F) The Royal Society of Chemistry 2009, and (G) American Chemical Society 2009.)

12184 www.angewandte.org

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Angew. Chem. Int. Ed. **2012**, *51*, 12176–12192

4.4. Additional Droplet Manipulations

A large number of droplet manipulations have been developed in addition to fusion, sorting, and incubations. These include droplet splitting, which is a passive manipulation step where droplets are split into two daughter droplets of well-determined size.[48] The splitting of droplets can be used as a means of sampling a larger droplet or, in combination with droplet fusion, as a means of diluting the contents of the droplets.[101] This dilution approach has not been used to any great extent for droplets containing cells, but it may prove important for the long-term culture of cells as a means of adding nutrients and diluting toxic substances produced by the cells.

Another approach developed for droplet manipulation is the rapid mixing of droplet contents by chaotic advection in winding channels,[102] which results in complete mixing of the contents of the droplets on sub-millisecond timescales.[103] Mixing the contents of picoliter compartments has also been accomplished by rotating self-assembled chains of magnetic nanoparticles within the droplets.[104]

4.5. Droplet Detection and Analysis Methods

A large number of detection modalities have been demonstrated for the analysis of droplet contents. These are well-described in a recent review by Theberge et al.[13a] Light-based methods, in general, and fluorescence techniques, in particular, are predominant. These detection modalities include the sequential measurement of laser-induced fluorescence (LIF) intensity at high throughput (< 10 kHz) by photomultiplier tubes or parallel measurement by fluorescence microscopy imaging. The optics and detectors used in sequential LIF measurements are very similar to those used in flow cytometry,[105] with the addition of circular lenses to shape the illuminating laser to a line placed perpendicularly to the channel to evenly illuminate the entire droplet as it passes. The imaging of static droplets and LIF detection of moving droplets are complementary techniques suited to different applications. In general terms, LIF measurements can be coupled with droplet screening and allow for high time resolution of fast events, whereas continuous imaging of static arrays enables better resolution of a large number of compartments with different contents over a long time scale, but has thus far not been coupled with sorting. Several fluorescence techniques such as fluorescence polarization,[106] Förster resonance energy transfer (FRET),[107] and fluorescence correlation spectroscopy (FCS) have been incorporated to increase the sensitivity or to enable the entire assay to be performed on a chip.[97a, 108] Fluorescent microscopy imaging of droplet monolayers containing tens of thousands of droplets or more is often used as an alternative or complement to laser-induced fluorescence analysis. Simultaneous imaging of tens of thousands of droplets is used in droplet digital analysis (DDA). This technique, which is used to determine the variation in the copy number of genes[109] or to quantify rare mutations,[110] allows the concentration of a certain DNA fragment to be determined effectively simply by counting the number of molecules present in a compartmentalized solution. In this way, the lower limit of detection in a sample is determined only by the number of droplets analyzed. In DDA, the DNA fragments are diluted to a concentration corresponding to less than one molecule per droplet volume and encapsulated in droplets with PCR reagents and sequence-specific fluorescent probes activated by hybridization and polymerase action. After thermocycling, the droplets containing the specific DNA sequence sought are detected and counted on the basis of the emitted fluorescence. Up to three planes of droplets packed in a crystalline structure can be imaged; thus, the fluorescence from a million droplets can be imaged simultaneously.[111]

A number of nonfluorescence-based analytical chemistry methods, such as mass spectrometry (MS),[112] capillary electrophoresis (CE),[113] and Raman spectroscopy, have also been coupled with droplet microfluidics to allow analysis of biomolecules. Of these, CE and MS have been shown to allow the analysis of single droplets while Raman spectroscopy thus far has only yielded ensemble measurements on a large number of droplets.

5. Single-Cell Analysis in Droplets

5.1. High-Throughput Single-Cell Analysis

Following syringe collection, the analysis of droplets in series by, for example, transporting them past a laser for concurrent LIF analysis enables the high-throughput analysis of single cells and their encapsulated environment. One of the first uses of this approach was demonstrated in the analysis of GFP expression by encapsulated single cells.[42] The detection of cell fluorescence is similar to flow cytometry. A very common technique in flow cytometry involves detection of cells labeled with fluorescent antibodies. In this fashion, encapsulated human periosteal cells from patient tissue samples have also been analyzed with confocal fluorescence detection, which allowed the distinction of rare progenitor cells from a heterogeneous cell sample.[114] In this case, the cells were labeled with antibodies before encapsulation. Encapsulation in stable droplets does, however, provide benefits unavailable to flow cytometry, in that the droplet compartment can serve as a picoliter catchment vessel for secreted molecules, such as antibodies from hybridoma.[63] Secreted biomolecules are then available for analysis by droplet-based homogeneous assays, such as fluorescence spatial distribution analysis,[63] FP,[106] or FRET,[107] while maintaining the genotype–phenotype link to the cell. Flow cytometry has been limited to the study of high- and medium-abundant biomarkers,[8] mainly because of the fluorescent background of the cell. The compartmentalization of single cells in picoliter droplets provides a vessel where the fluorescent signal from a labeled antibody can be amplified by using an enzyme-linked antibody assay.[115] Human monocytic cells (U937) were analyzed by this type of assay for the low-abundant cell-surface protein biomarkers CCR5 and CD19. Although these are examples of protein detection in droplets, there has been some struggle in realizing straight-

forward antibody-based detection schemes in which droplets do not require prelabeling and washing of the cell sample before encapsulation. Perhaps the washing or dilution method described by Mary et al. brings the field one-step closer to automated antibody assays in droplets.[101a]

5.2. Enzyme Analysis of Single Cells

The analysis of enzymes compartmentalized in an emulsion is one of the earliest uses of droplet microfluidics, and was developed into a high-throughput format by Griffiths and Tawfik.[20, 116] A large number of reports on enzyme characterization[81c, 94, 117] have been published in which in vitro expressed or purified enzymes are analyzed. DDA analysis has also been realized for enzyme analysis.[118]

Cell-expressed enzymes have also been analyzed in droplets produced by microfluidics. Of particular importance are the cases where the protein is secreted or where the protein analysis is performed using an unbound extracellular reporter or signal molecule, such as in the case where an extracellular fluorogenic enzyme substrate is present in the droplet. Again, the droplet provides the compartment linking the genotype (within the cell) and the phenotype (generated fluorophores or secreted biomolecules). Cell-based expression of enzymes in droplets has been demonstrated and analyzed for alkaline phosphatase (AP)[119] as well as β-gal[68] expression in *E. coli*. Fluorogenic substrates were used in both cases. These substrates mimic the natural substrates of the enzymes, but include fluorophores that remain quenched while attached to the substrate molecule. Release of the fluorophore by the enzyme will, therefore, radically increase the fluorescence intensity. In the AP assay, cell-containing droplets were stored in inverted well structures and imaged repeatedly to acquire time series data that yielded enzyme kinetic data. Moreover, the *E. coli* cells used coexpressed a red fluorescent protein (mRFP1), thereby enabling normalization of the protein expression.

Droplet-based enzyme screening for cell-based expression also has uses in a directed evolution context. Directed evolution seeks to harness the power of evolution in a laboratory. Starting from a known molecule, repeated rounds of diversification are iteratively employed and this is followed by selection of the desired trait (schematically described in Figure 6 A1–A7), for example, increased enzyme activity to improve the characteristics of the molecule. A recent report by Agresti et al. demonstrated the benefits of droplet-based systems for directed evolution by improving the activity of the horseradish peroxidase (HRP) enzyme, expressed on the surface of *S. cerevisiae*, 12-fold after two rounds of mutation.[96] The study, which used droplet generation, a 5 min wide-channel incubation, and sorting (Figure 6 B1–B5), compared this droplet microfluidic directed evolution scheme to the current state of the art automated screening of 384-well microtiter plates. The comparison showed a 1000-fold increase in speed and a million-fold decrease in cost for the droplet-based automated microtiter plate screening. Interestingly, the cost of
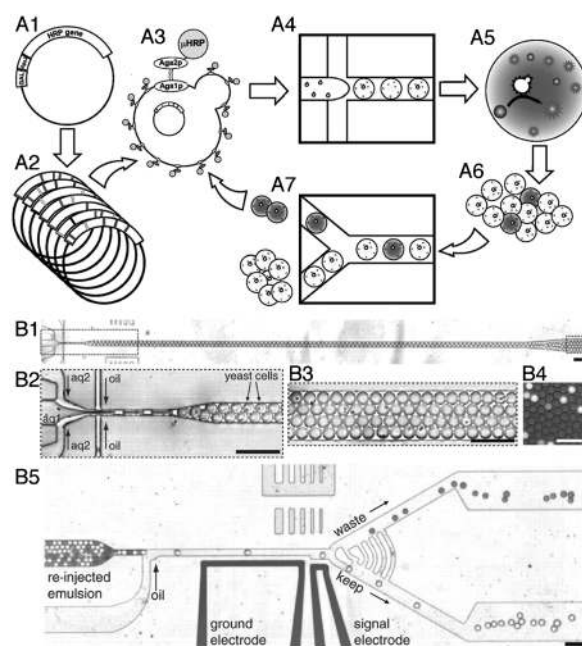

Figure 6. Directed evolution workflow for the improvement of the enzyme horseradish peroxidase (HRP) expressed in *S. Cerevisiae* reported by Agresti et al.[96] Starting from A1) a plasmid containing the HRP gene, A2) a library of mutated HRP plasmids is generated, and A3) expressed in *S. Cerevisiae*. A4) The yeast cells are encapsulated with a fluorogenic substrate for HRP and A5) incubated flowing in a tube to allow functional HRP to turn the nonfluorescent substrate into a fluorescent product. A6) At the end of the incubation, tubing droplets are fed into a sorting device where they are sorted on the basis of their fluorescence signal, with the best performing enzymes retained. The microfluidic device used in the assay. B1) A micrograph of the droplet generation device, B2) (magnification in B1) the encapsulation nozzle. B3) and B4) show droplets prior (by brightfield microscopy) to and following (by fluorescence microscopy) incubation, respectively. B5) An overview of the droplet re-injection and dielectrophoretic sorting area, which demonstrates the sorting of highly fluorescent droplets into the lower outlet arm of the device. (Reprinted with permission, National Academy of Sciences USA 2010).

pipette tips is the main cost in the microtiter plate screen, accounting for 2/3 of the estimated $15.81 million price tag.

5.3. Time-Resolved Single-Cell Analysis

Many of the assays described thus far rely on moving droplets past a laser and fluorescence detector for analysis. This approach yields data on the distribution of single-cell traits and enables screening, but unless each cell-containing droplet is labeled with a specific fluorescent "color code", these methods do not lend themselves well to the analysis of the same cell over an extended period of time. The trapping mechanisms described in Section 4.3, on the other hand, allows parallel monitoring (or continuous rastering) of time series, with the precise identity of each droplet maintained by the droplet position. These techniques enable analysis of a large number of cells with much higher time resolution. The choice between continuous monitoring and concurrent sequential analysis of droplets in the flow depends on the

12186 www.angewandte.org

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Angew. Chem. Int. Ed. **2012**, *51*, 12176–12192

intended application and combinations such as sorting for cells of interest followed by continuous monitoring are possible.

Time series analysis of a large number of droplets has found use in cell assays to study the interaction of phage with their target bacteria in "dropspots" arrays.[80a] In this study, droplets containing phage and single bacteria were imaged at a number of time points to determine the time before lysis for wild-type and modified phage under different conditions to elucidate the phage biophysics. Another time series analysis using the same device design and analysis method studied the heterogeneous growth rates of yeast cells and their expression of β-gal.[71] The ability to monitor the growth or protein expression of many isolated clones over time could be utilized to determine the distribution of responses to, for example, normal or drug perturbed environments.

A similar trapping method used single droplet capture wells to study the activity of AP expressed by single bacteria[119] in a large number of droplets in parallel at a high time scale resolution.[98] All these studies illustrate the variation in the behavior of single cells. Such variation is often accounted for by underlying stochastic processes.

5.4. Drug Screening in Droplets

A particularly challenging screening scenario among high-throughput biological screens is the droplet-based screening of drug candidates and formulations. Drug screens require the identification of the formulation inside each droplet, often long incubation times of cell-containing droplets, and potentially a wide range of molecules with varying chemical characteristics. Nonetheless, with the high cost of screening conventional automated microtiter plates (ca. $1 per well by one estimate[120]) and the growing role of biomolecular pharmaceuticals, a case can be made for the use of droplet microfluidics in early drug discovery. Conceptually, screening for biomolecular pharmaceutical compounds could be quite similar to the selection schemes already in use, albeit often with considerably longer incubation times and in many cases eukaryotic cell culture. Despite these challenges, model screens for cytotoxicity,[66] nuclear receptor activation,[65] antibiotic species specificity,[121] and enzyme inhibition[122] have been reported. In a recent study, Griffiths and co-workers coupled droplet generation to automated sampling to perform a fully automated screen of 704 chemical compounds for inhibition of the protein tyrosine phosphatase 1B. This approach produced dose–response curves with extremely high resolution by analyzing 10000 droplets for each compound.[123]

A droplet-encapsulated single-cell screen for cytotoxicity (Figure 7) of a model compound library consisting of eight concentrations of the chemotherapeutic mitomycin C demonstrated the concept of drug screening in droplets on human cell line U937 cells.[66] In this study, the concentration library was emulsified with a fluorescent optical code and fused to droplets containing single cells. The encapsulated cells were incubated for 24 h, re-injected onto a second device, fused with droplets containing a positive and negative viability
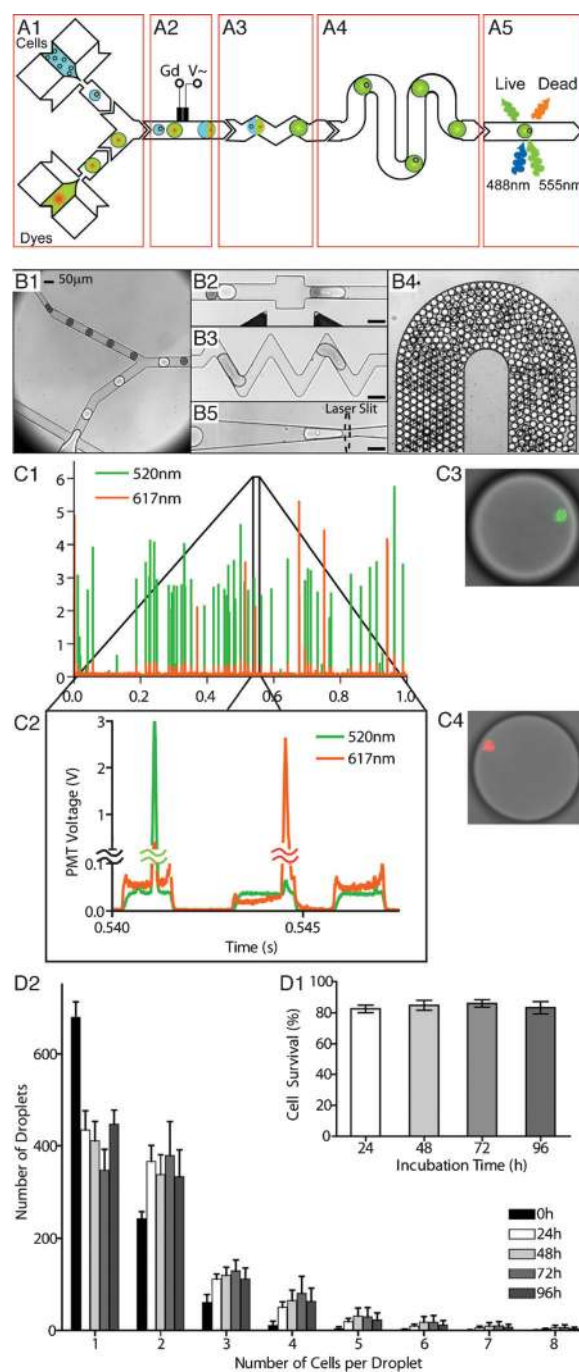

Figure 7. Assay employed by Brouzes et al. for the cytotoxicity screening of droplets.[66] A) schematic representation and B) with corresponding micrographs. 1) Dual droplet generation (where the cell nozzle was exchanged for a re-injection nozzle to determine the viability of cells following incubation). 2) Electronically controlled droplet fusion. 3) Mixing of droplet contents. 4) Incubation and finally 5) dual channel fluorescence detection. C1) Data trace showing the fluorescence detected in the green 520 nm (live cell signal) and 617 nm (dead cell signal) channels. C2) Magnification demonstrating the spatial distribution of the fluorescent signal from cells (narrow peaks) and the wider base of the droplets. C3) and C4) Showing false-colored live and dead cells, respectively, stained with Calcein AM and Sytox Orange. Cell viability during a 4 day droplet culture were determined to > 80% for all four days. D2) The Poisson distributed droplet cell numbers were determined. (Reprinted with permission National Academy of Science USA 2009.)

stain, incubated on-chip briefly, and analyzed for fluorescence. This yielded an IC_{50} curve for drug cytotoxicity following optical decoding. The results from the cytotoxicity study showed good correlation with those obtained by traditional techniques. Baret et al. reported the use of a quantitative gene reporter assay for nuclear receptor activation at different concentrations of the hormone 20-hydroxyecdysone in a droplet-based assay screening of adherent *Bombyx mori* cells.[65] This study demonstrates a concurrent analysis of thousands of single cells for 10 optically coded hormone concentrations. The single-cell data enables analysis of the heterogeneity, which is not available by, for example, microtiter plate assays. Another study aimed at modeling drug screening reported an inhibition screen of an enzyme (β-gal) in sub-microliter plugs generated at 0.33 Hz.[122] Plugs of each inhibitor concentration, automatically generated from a prepared 96 well plate, were split in a tree-shaped splitter and merged with the enzyme and a fluorogenic enzyme substrate (FDG). Following in-line incubation in teflon tubing, the plugs were assayed for fluorescence, and IC_{50} curves for 96 conditions were generated (14 contained inhibitor and 82 PBS control). Here, the 96 conditions were identified by a combination of serial position in the tube and a fluorescent label added to the first well in each row of the 96 well plate.

As these studies show, drug screening assays on cells can be performed in microfluidic droplets. In contrast to flow cytometry analysis, the screened compounds are added to compartmentalized single cells as opposed to a cell ensemble. This allows uncoupling of cell–cell interactions from the effect of the compound added. Further increased throughput will require identification of the contents of the droplets, by labeling, location, separate analysis, or a combination of these. The generation of large-scale droplet libraries is another challenge that is very relevant to drug screening and will in part determine whether droplet microfluidics can offer throughput advantages over automated compound screening in microtiter plates.

5.5. Single-Cell Genetic Analysis in Droplets

The encapsulation of the processing reactions and analysis of genetic materials confers benefits in terms of enabling the multiplexing and isolation of amplified genetic material stemming from a single DNA or RNA strand. This found realization in the so-called BEAMing protocol[124] and polydisperse emulsion PCR[18a] even before the use of monodisperse droplets generated by microfluidics had been put into practice. Given this background, it is not surprising that the processing and analysis of genetic material is one of the most explored areas of droplet microfluidics applications, with most variations of the PCR in droplets having been explored. PCR has been realized by stop flow,[23,125] continuous flow in serpentine channels across different temperature zones,[126] and batch collection of droplets compatible with standard thermocyclers[127] as well as isothermal PCR.[128] Most functional variations of PCR, such as reverse transcription PCR (RT-PCR),[23] quantitative PCR (qPCR) with

fluorescent detection,[126] and real-time PCR analysis[125] have been realized and applied to, for example, genetic analysis of tumor material through DDA[129] and multiplex PCR of samples from patients with spinal muscular atrophy.[130]

Genetic analysis of single cells requires the lysis of encapsulated cells for DNA extraction and subsequent DNA amplification and detection. Integration of these functions in the droplet format has been achieved by Novak et al. in a droplet/agarose bead hybrid assay generated by microfluidics.[131] In this protocol, droplets containing single cells and primer-functionalized beads in an agarose solution were generated and cooled to let the agarose form a gel. Subsequently, the beads were extracted from the oil and a detergent and proteinase enzyme cocktail was added to lyse the cells. The agarose gel droplets could then be extracted from the oil to rinse out lysis reagents, which might interfere with the PCR. The PCR mix was added and then the agarose droplets re-emulsified in oil by shaking and thermocycled to generate amplicon-labeled beads with genetic material from one cell per bead. By using a similar method, the same research group also analyzed *E. coli* samples for mutated pathogenic cells, and detected these cells against a background of 10^5 wild-type *E. coli* per pathogenic mutant.[132]

6. Conclusions

Single-cell analysis is one of the most compelling targets on which to focus the abilities of droplet microfluidics. Droplet microfluidics provides compartments on the same size scale as the cell. Furthermore, it has the ability to encapsulate and rapidly manipulate large numbers of cells along with their immediate environment in monodisperse compartments amenable to automation. Thus, droplet microfluidics is certainly in a position to play a significant role in elucidating the heterogeneities of cell populations and their underlying causes, finding the rare cells that average only a single cell per milliliter of blood, or sample a sufficient amount of prospective drug compounds or secreted enzyme variants to find those which are more effective than those currently available to us.

For a number of years, technical development had been the focus of the droplet microfluidics field; however, the focus has now clearly shifted to the application of these technical advances to the development of biological assays. Several of these assays are now at a point where their sensitivity—such as in the case of digital droplet analysis or detection of low-abundant biomarkers on single cells—and their throughput—such as for massively parallel PCR or droplet-based enzyme screening—rival or surpass the capabilities of standard methods—in some cases, such as in enzyme screening, by as much as a thousand fold. Looking ahead there are a number of opportunities, such as the investigation of isolated cell–cell interactions, as well as linking single-cell protein and genomic analyses, where droplet microfluidics has the potential to make additional scientific impact.

There are of course challenges with integration as the droplet microfluidics circuitry becomes more complex. Furthermore, as the field turns to clinical samples and longer

culturing periods, the demands on the droplet environment to provide a milieu which does not perturb the cellular characteristics under study will grow. Nonetheless, droplet microfluidics has the potential to support scientific progress in further analysis of the fundamental component of life—the cell.

Received: January 17, 2012

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