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Micro segmented-flow in biochemical and cell-based assays

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1. ABSTRACT

Micro-segmented flow (e.g. in microfluidic channels, capillaries or a length of tubing) has become a promising technique in modern biology. Compared to conventional formats such as microtiter plates, sample volumes can be reduced about 1000-fold, thus allowing a massive reduction of assay costs and the use of samples available in low quantities, only (e.g. primary cells). Furthermore, assays can be highly parallelized and performed at superb spatio-temporal resolution. Here, we review the state-of-the-art in micro-segmented flow as applied in biochemical, cell- and multicellular organisms-based assays. We discuss likely future applications such as single cell / single organism proteomics and transcriptomics and point out the specific advantages and limitations compared to emulsion-based (droplet-based) approaches.

2. INTRODUCTION

2.1. Segmented-flow microfluidics as an alternative to conventional screening formats

The growing demand for miniaturization is driven by the need of more efficient, time- and cost-effective products. Advances in miniaturization technologies have had dramatic impacts on our lives. Indeed, radios, music-player devices, computers, and telephones that were once bulky devices, now fit in the palm of a hand. Furthermore, miniaturized electronic devices are within everything, from telephones to cars; and even within the human body (e.g. pacemakers).

In contrast, miniaturization in the field of chemistry and biology has been less dramatic: biochemical assays may now be performed in volumes of a few microliters using microtiter plates, rather than in a few

milliliters when using test tubes. Consequently, the performance of high-throughput screenings using microtiter plates has provided benefits for research and diagnostics. Most conventional assays are currently being done in 96-well plates, where assay volumes are ~100 microliters and the throughput is up to 20,000 assays per day. However, other microplate formats of higher density have also been used, such as 384-well plates (1). This format allows working with assay volumes of ~10 microliters and a throughput of up to 100,000 assays per day. Further miniaturization of microtiter plates has also been shown when using 1536-, 3456- and 9600-well plate formats (2-4). Nonetheless, the adaptation of established assay systems to such miniaturized formats requires a careful examination of the feasibility with special attention to the pipetting error, reproducibility as well as sensitivity. Problems like cross contamination, formation of foam, evaporation and capillary action (causing "wicking" and bridging of liquid between wells) can cause problems at this scale (5, 6). Furthermore, typically no more than 10^6 assays can be run in a single screen due to practical considerations and cost / availability of reagents.

However, microfluidic systems have the potential to further miniaturize reactions down to pico- to nano-liter volumes, while overcoming the limitations of microtiter plate techniques. Microfluidics is a multidisciplinary field intersecting engineering, physics, chemistry and biotechnology (7). Microfluidic devices usually consist of networks of channels on the sub-millimeter scale where precise control and manipulation of fluids is enabled by electrokinetic or hydrodynamic pumping. In particular, when working with two-phase microfluidic systems reagents are compartmentalized into water drops or plugs (drops that completely fill the channel) of pico- to nano-liter volumes surrounded by immiscible oil. This way, thousands or even millions of independent assays or reactions can be carried out.

2.2. Segmented-flow and emulsions

In general, water drops within a continuous oil phase tend to fuse to decrease their surface per unit of volume, thereby decreasing the interfacial tension *("g",* gamma) of the system. The interfacial tension is caused by the attraction of various intermolecular forces between the molecules of two immiscible liquids. The molecules at the interface are in a higher state of energy than those within the liquid. Molecules within the liquid undergo attractive forces equal in all directions resulting in a net force of zero; however, the molecules at the interface experience attractive forces acting towards the liquid bulk of like molecules (the attractive forces from the neighboring liquid are weaker) resulting in a net force different to zero. Hence, *"g"* is the driving force to diminish the interfacial area, and therefore to decrease the number of molecules at the interface. On the macroscopic scale this ultimately results in the uncontrolled coalescence of drops, which can be avoided by the addition of surfactants adsorbing at the liquid-liquid interface and thus reducing the interfacial tension (8). However, for performing biological assays, full biocompatibility of the oil mixture (oil and surfactant) with the samples encapsulated in the drops is necessary. Since there are hardly any commercially available surfactants ensuring both, biocompatibility and sufficient stabilization of the droplets, they usually have to be custom synthesized, especially when using fluorinated oils and surfactants to prevent diffusion from hydrophobic compounds into the oil phase. Besides, when generating surfactant-stabilized drops cross-contamination based on mass transport between the drops can take place. When the concentration of surfactant is below the critical micelle concentration (CMC), all surfactant molecules are dissolved in the oil phase (unimers); however, for concentrations above the CMC (as typically used for the generation of droplets in microfluidic devices), additional surfactant molecules assemble into micelles (molecules associate placing their heads in a core and the tails in contact with oil) (9). These micelles can carry molecules dissolved in the water phase, bud from one droplet and fuse with the next one, thus generating cross contamination (10, 11). Furthermore, if the surfactant concentration is too high, an increased drop polydispersity or jetting (generation of a continuous aqueous stream instead of drops due to very low surface tension) can be observed during the production of drops. In contrast, when performing assays in plugs stably separated from one another during the whole experiment, the use of surfactant is not required for stabilizing the compartments. Stable separation can be achieved by permanently storing them in a cartridge such as a tubing or capillary with a diameter smaller than the plug size. Hence oil in between the plugs cannot drain and the plugs do not get in contact, even upon "segmented-flow" of the system. However if the fluid viscosities vary significantly, either between the carrier fluid and aqueous solutions in plugs, or only among the different plugs, coalescence might occur. To avoid this, stable separation can be ensured by separating the plugs by gas bubbles in addition to the carrier fluid, thus resulting in a three-phase liquid / liquid / gas system (12). Furthermore, when encapsulating plugs in capillaries larger than 1 mm inner diameter, the density difference between the fluids can lead to buoyancy forces, which eventually can also result in plug coalescence. Consequently several parameters have to be taken into account to ensure integrity of the plugs and robustness of the system.

In this review, we focus solely on these plug-based systems and disregard any applications that require off-chip incubation (e.g. in macroscopic reservoirs such as test tubes) of the samples in form of surfactant-stabilized emulsions. Readers interested in emulsions are pointed to other reviews (13-15).

2.3. Generation of plugs

When using a microfluidic chip, plugs can be created either by flow-focusing two immiscible liquids or using a T-junction (16, 17). In the flow-focusing configuration (Figure 1A), the continuous phase symmetrically shears the dispersed phase into plugs; and in the T-junction configuration (Figure 1B), the continuous phase perpendicularly intersects the dispersed phase resulting in the generation of plugs. An array of plugs containing different concentrations of a certain reagent can be easily generated by on-chip dilution of the aqueous reactant phase upstream of the plug formation. Simply by

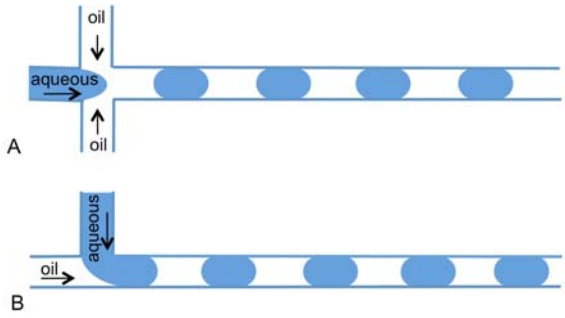

Figure 1. Generation of plugs using a microfluidic chip. Plugs can be generated either using flow-focusing (A) or T-junction (B) configurations.

changing the relative flow rates of the reagent and the dilution buffer, a wide range of concentrations can be generated, particularly when using automated pumps (e.g. syringe pumps) to allow precise control and manipulation of fluids.

However, the generation of plugs containing different reagents (instead of just different concentrations of the same reagent) is significantly more difficult, even though it is of major interest for biological screens. Ismagilov and co-workers showed the generation of chemically-distinct plugs by immersing the end of the target tubing into the sample solutions and an oil reservoir in an alternating fashion (either manually or by an X-Y-Z micromanipulator) (Figure 2A), while aspirating the desired volumes using a syringe-pump (18-20). However, the throughput of this approach is quite limited and washing steps can hardly be implemented. In an alternative system developed by Viovy and co-workers, all samples of a microtiter plate were covered with two additional fluid layers (oil and washing buffer), before aspirating them sequentially into the target tubing (21) (Figure 2B). In this system, cross-contamination between plugs was negligible, even in highly sensitive PCR assays as carried out by the authors. Nonetheless, on-chip manipulations of the plugs such as their splitting into small-volume subunits (*e.g.* to generate replicates), or the addition of further compounds to each of them (*e.g.* by droplet fusion) were not demonstrated. Additionally, the applied microtiter plates required special surface treatments as well as covering them with two additional liquids. Hence compound libraries in standard formats could not be processed. Recently, a platform that integrates the generation, incubation and on-chip manipulation of chemically-distinct plugs in an automated manner has been developed by our group (22) (Figure 2C). In this approach, a commercially available HPLC autosampler was used for loading a compound library from ordinary microtiter plates into a length of tubing in form of aqueous plugs separated by immiscible oil. Starting from standard microtiter plates, the integrated platform allowed the automated generation of chemically-distinct plugs, as well as the addition of further compounds (such as enzymes and fluorogenic substrates) to each individual one by passive drop-plug fusion.

Taken together, these approaches show that even chemically-distinct sample libraries can be generated and screened. However, it should be mentioned that the throughput of these systems is still significantly below that of conventional high-throughput systems (~60 seconds per sample), as the reagents have to be loaded sequentially (a parallel loading of hundreds or thousands of different reagents would require the same number of sample inlets and tubing and hence seems impossible). Furthermore, the sample loading generates additional dead volume (e.g. the volume of the inlet tubing) thus increasing the reagent consumption as well as the consumables costs.

2.4. Detection systems

Any assay carried out in a segmented flow system requires a readout module downstream of the plug generator. In the easiest case, the readout can be carried out by eye using a microscope integrated into the microfluidic platform. Alternatively, spectroscopic detection systems have been established and turned out to be very powerful, as they are very rapid (up to several thousand samples can be analyzed per second) and sensitive. Typically, these systems are based on absorbance (23), epifluorescence (24), fluorescence polarization (25), fluorescence lifetime imaging (26) or even Raman scattering (27): A light source (e.g. a monochromatic laser) is focused onto the channel and the transmitted or emitted light is diverted to a photomultiplier tube (using specific filters and / or polarizers) in which the light intensity is converted quantitatively into a voltage signal. More sophisticated readout systems have been established, too: For example, Huck and coworkers developed a system in which the contents of individual droplets can be applied directly to a mass spectrometry module (28). However, the sensitivity of this approach is relatively low (a detection limit of 500 micromolar protein was observed using 2 nanoliter droplets) thus restricting the application range. Low sensitivity (10 – 30 millimolar sample concentrations using 1 microliter plugs) was also observed for an NMR-based readout module developed by Kautz and coworkers in which the samples were flushed directly through a microcoil (29). Nonetheless this work nicely demonstrates that even highly sophisticated detection systems can be integrated into segmented flow platforms.

Obviously there is a certain tradeoff between information content, detection limit and sample throughput: While fluorescence analyses can be performed rapidly at high sensitivity (even single molecule measurements have been demonstrated successfully; (30)), more complex readouts can be integrated at the price of lower sensitivity and throughput. Taken together, this opens the way for a number of exciting applications and assays, as exemplified in the third and fourth part of this review.

3. BIOCHEMICAL ASSAYS

3.1. Protein crystallization

Segmented-flow microfluidics have become a highly useful tool for protein crystallization. Particularly the crystallization of membrane proteins is considered challenging due to limited sample availability, stability and solubility (detergents have to be added to keep the proteins

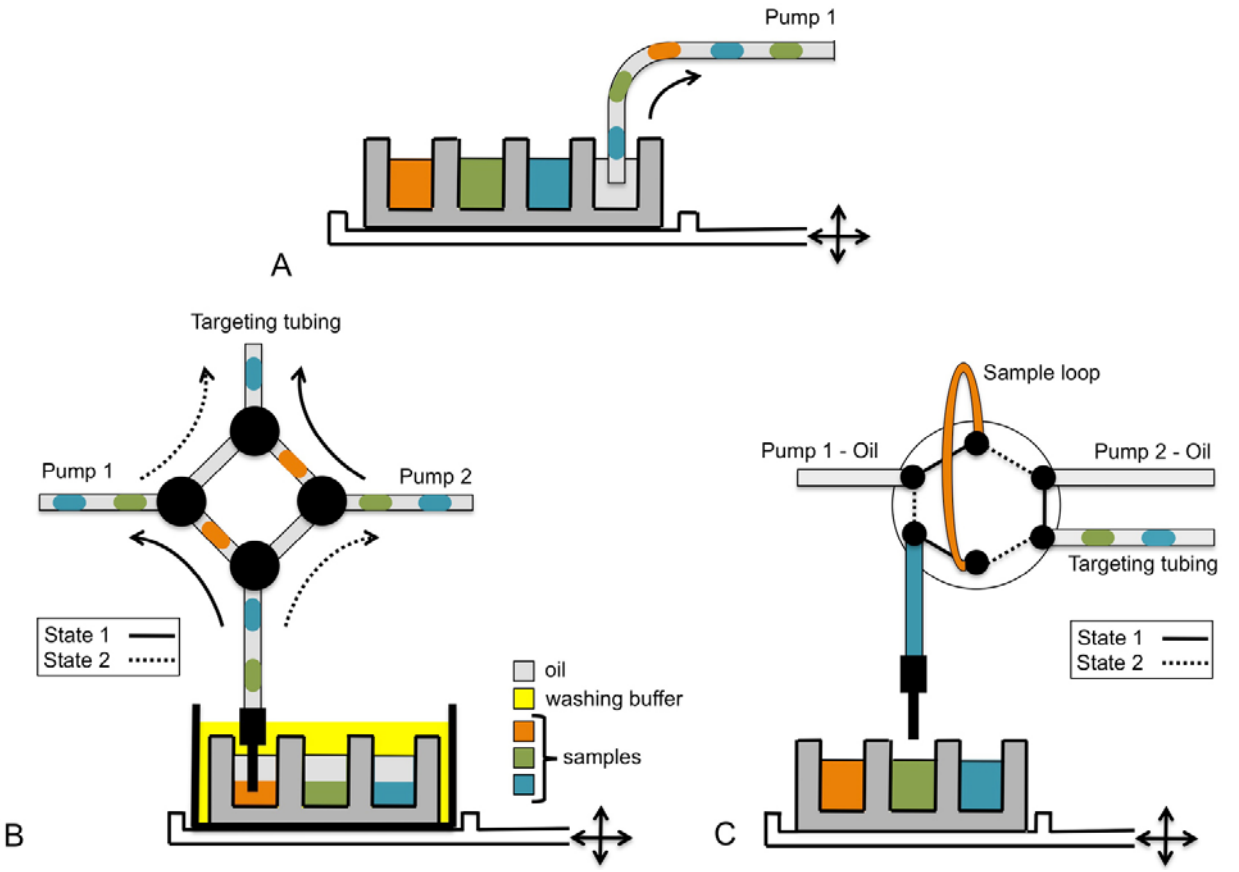

Figure 2. Different approaches for the automated generation of chemically-distinct plugs. (A) Alternating aspiration of aqueous samples and oil spacers. An X-Y-Z micromanipulator is used to move the tip of the target tubing while a computer-controlled syringe pump sequentially withdraws the desired volume (20). (B) Aspiration of aqueous samples covered by an additional oil layer. The generation of plugs consists of different steps: first, a certain aqueous volume is aspirated from a well in a microtiter plate; then, the tip is lifted and aspirates oil from the same well; subsequently, the tip moves through the cleaning buffer; and finally into the next well. A two-state loop with two synchronized syringe pumps controls the fluidic pathway. In state 1 pump 1 sequentially forms samples from the aspirating tip, while pump 2 continuously pushes the plugs formed in state 2 of the previous cycle into the target tubing. In state 2 pump 2 sequentially forms the plugs, while pump 1 pushes the plugs formed in state 1 of the previous cycle into the target tubing. The fluorinated oil is immobilized in between the two aqueous layers due to a special hydrophobic / fluorophilic treatment of the wells (21). (C) Aspiration / insertion of aqueous samples into a continuous stream of oil. An HPLC autosampler is used to generate the plugs (22). In state 1 the needle tip is immersed into a well and an aqueous sample is aspirated into the sample loop by pump 1. Pump 2 is continuously injecting oil into the target tubing, thus generating an oil plug. As the next step the valve switches to state 2, and hence pump 2 purges the liquid from the sample loop into the target tubing, thus generating an aqueous plug.

in solution). Hence there is a need for screening an extensive range of conditions for successful crystal nucleation and growth. The degree of protein crystallization depends on several factors, such as protein purity, pH, protein and detergent concentration, temperature and precipitants. For that purpose, miniaturization and parallelization of the procedures is highly desired. Ismagilov and coworkers have used nanoliter plugs for screening and optimizing the crystallization of purified membrane proteins. Here, an array of plugs containing different precipitants was brought into contact with an orthogonal co-flow of two continuous aqueous streams, one containing a buffer (to regulate the pH) and the other a membrane protein (substrate) (Figure 3). Starting with a protein solution of only 10 microliter in volume, 1300 crystallization trials were set up in just 20 minutes by changing the relative flows of each liquid (31). Using the same approach, Ismagilov and coworkers were also able identifying simultaneously conditions that can induce both, nucleation and ordered growth of crystals (32).

3.2. Screening of active catalysts using genetic algorithms

A technically similar platform for discovering new homogeneous catalysts using genetic algorithms (GAs) has been developed, recently. GAs can generate solutions to problems involving the optimization of several parameters in parallel by exploiting principles of natural evolution, such as inheritance, mutation, selection, and crossover (33, 34). Since GAs require many reactions for

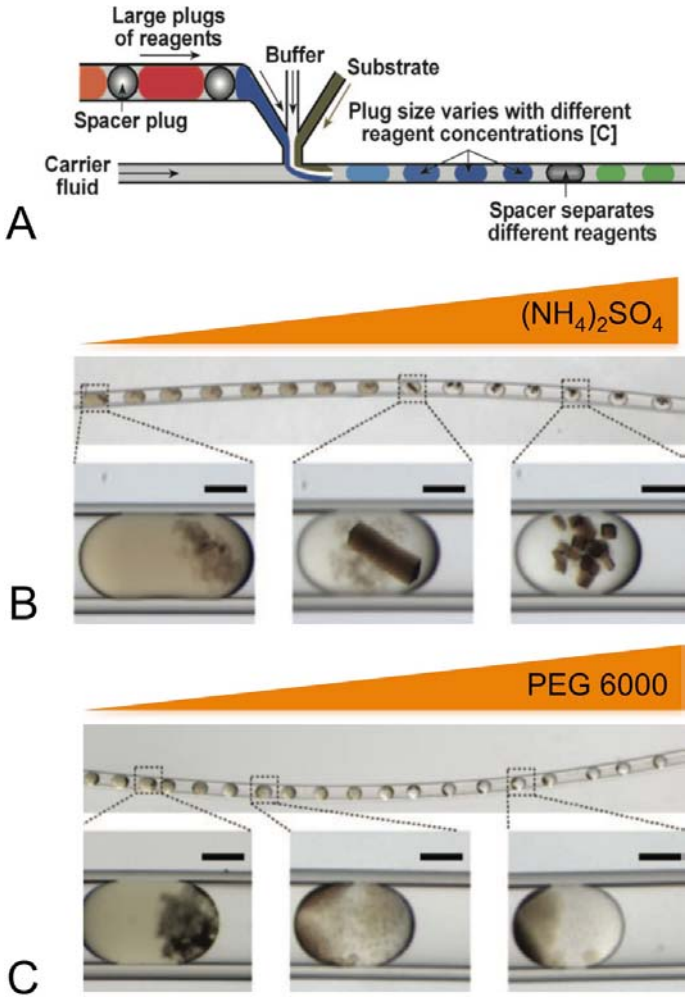

Figure 3. Microfluidic approach for screening protein crystallization conditions. (A) A pre-formed array of plugs containing different precipitation reagents is fused with two streams containing a buffer and a substrate before being segmented by a stream of fluorinated oil (31) - Copyright (2006) National Academy of Sciences, U.S.A. (B) Microphotographs of two different parts of the same Teflon capillary show how on one hand, as the concentration of one precipitant, $(NH_4)_2SO_4$, increases, a transition is seen from slight precipitation to large, and then from single crystals to many small ones. (C) On the other hand, as the concentration of another precipitant, PEG 6000, increases, a transition from precipitation to phase separation is seen [Provided by Nina Ponomarenko and James R. Norris (University of Chicago)]. Scale bar = 100 micrometer.

each generation, parallelization of these reactions in plug-based microfluidics seems highly desirable, especially since the miniaturization also results in reduced costs and waste, as well as improved safety (for reactions involving hazardous compounds). As a model system the oxidation of methane by molecular oxygen has been used (35). Here, 192 plugs of microliter volumes, containing mixtures of the catalysts, potential cocatalysts and ligands were generated and spaced out by additional indicator plugs (one on each side of a given catalyst plug) within a length of tubing. The tubing was subsequently placed inside a stainless steel reactor containing a mixture of methane and oxygen, and after pressurization the reactor was heated to initiate the reaction. Upon the formation of methanol due to the presence of a plug containing an active catalyst, the methanol diffused into the neighboring indicator plug, where it mediated a change in color (from yellow to purple, due to the oxidation of methanol by chromic acid contained within the indicator plug). Ismagilov and coworkers performed this experiment over eight generations and adjusted the composition of the samples according to the fitness of the active catalysts in the previous generation. In particular, they mimicked mating and genetic recombination via crossover, mutation and migration. Thereby, the selection pressure was increased resulting in the abolishment of unnecessary components. After eight generations of GA-guided evolution, Pt(II) was selected as a catalyst, polyoxometalate was selected as a cocatalyst, and iron turned out to be compatible with all stages of the catalytic cycle, while no ligand species significantly enhanced the catalytic activity.

3.2. Enzyme kinetics

Microfluidic devices facilitate mixing reagents

on-chip at very high temporal resolution (down to sub-millisecond timescales). This has been exploited to monitor enzyme kinetics, e.g. by recording fluorescence signals based on the generation of a fluorescent product. Ismagilov and coworkers recorded the kinetics of RNAseA by injecting the enzyme, a dilution buffer and a fluorogenic substrate through separate inlets immediately upstream of the plug generator (36). Subsequently, the plugs were flushed through a delay line allowing measurements to be carried out at different time points (at different positions within the delay line). Compared to conventional stopped flow methods this setup did not have any "dead time" since all reagents were mixed in a fully transparent device and could be monitored at any step of the whole procedure. Hence even the kinetics of mixing (the limiting step for very fast kinetics) could be quantified. Alternatively to performing the readout at different positions of a delay line, samples can also be collected in a length of tubing and reinjected into the microfluidic device at different time points. This enables monitoring a reaction over a longer period of time, as the incubation itself is carried out in absence of any flow within big reservoirs (microliter volumes). We have recently demonstrated that this approach can be used for the automated screening of enzyme inhibitors (starting from microtiter plates) and obtained IC_{50} data which was in very good agreement with literature and control experiments carried out in conventional plate readers (22).

3.3. Analysis of cell lysates using capillary electrophoresis

Segmented-flow microfluidics have not only been used for screening puposes, but as well for separation applications. Currently, for separating complex protein mixtures, 2D polyacrylamide gel electrophoresis is one of the most widely used techniques. Although, when automated analysis of a bigger range of biomolecules is required, other column-based 2D separation techniques based on liquid chromatography (LC) and / or capillary electrophoresis (CE) are used. However, coupling two different separation steps is very challenging for small sample sizes. To overcome this problem, deMello and coworkers have developed a plug-based microfluidic platform, which allows tiny sample amounts to be transferred between two orthogonal separation dimensions in a valve-free system (37). In the first module of the integrated device, analytes separated by LC were flushed from the LC capillary into a T-junction where an oil phase sheared the aqueous solution (containing the analytes) into plugs (Figure 4A). In a second module, the plugs were sequentially fused with a continuous aqueous phase (CE separation buffer) and subsequently applied to electrophoresis, thus allowing separation of the analyte mixture in the second dimension. As a proof of principle, the authors performed a 2D separation of crude yeast lysate. The first dimension (LC) was done in a gradient manner, using increasing acetonitrile concentrations. Afterwards, the protein-containing effluent was sheared into 260 droplets in 40 minutes, and subsequently a few selected droplets were applied to separation in the second dimension (CE) (Figure 4B). Overall, the technique allowed the analysis of nanoliter to femtoliter volumes without diminishing resolution in any single dimension.

4. CELL-BASED ASSAYS

The use of segmented-flow based techniques has not been restricted to biochemical screens and experiments mimicking the behavior of genetic populations. Instead, the technology has been exploited in many experiments involving living cells, too. Particularly the possibility of performing single cell / single organism assays has turned out to be of major interest and will certainly find further applications in future research.

4.1. Encapsulation of cells and multicellular organisms

One of the first cell-based applications of segmented flow systems was the encapsulation and clonal expansion of single GFP-expressing bacteria in plugs, as demonstrated by Koehler and coworkers. Using a microfluidic device and automated syringe pumps this was achieved at a rate of 30 Hz and the resulting plugs were subsequently incubated in a length of Teflon tubing. After several days of incubation, cell growth was confirmed by fluorescent measurements directly in the tubing and by determining the cell number in a counting chamber after different incubation times (38). The same group, one year later, demonstrated the encapsulation and growth of different microorganisms from an unknown natural sample in segmented microcompartments, which is of major interest for metagenomics applications (39, 40). Subsequent to filtration a suspension of soil was diluted on-chip with media in order to obtain single cells per plug. After 7 days of incubation within Teflon tubing, the plugs were individually flushed into different cultivation dishes, and thereby cell growth undergone in each plug could be studied separately by eye when counting the number of colonies on the dishes (41). In 2005, Chiu and coworkers encapsulated mouse mast cells in plugs for measuring protein expression on the single-cell level. Here, plugs were generated using a T-junction configuration in which the oil phase was controlled with a syringe pump and the aqueous phase was manually pressure pulse driven (using a syringe). As a model system, the authors performed an enzymatic assay on-chip: cells expressing beta-galactosidase were encapsulated together with a fluorogenic substrate. Subsequently they were lysed on-chip by a laser-beam to ensure mixing of the intracellular enzyme with the substrate, resulting in the generation of a fluorescent product as an indicator for the expression level of the enzyme (42). Using this microfluidic approach indeed allowed detecting enzyme activities in individual cells or even individual cellular compartments (such as mitochondria).

In another study, Koehler and coworkers demonstrated that segmented flow applications could be expanded to single organism assays. In particular, they analyzed the embryonic development of zebrafish (*Danio rerio*) within plugs (Figure 5). For this purpose, they encapsulated eggs by alternately aspirating aqueous samples and immiscible oil using a syringe-pump. After 3 days of incubation, embryos hatched from eggs. However, to minimize wetting of the channel walls by the plugs

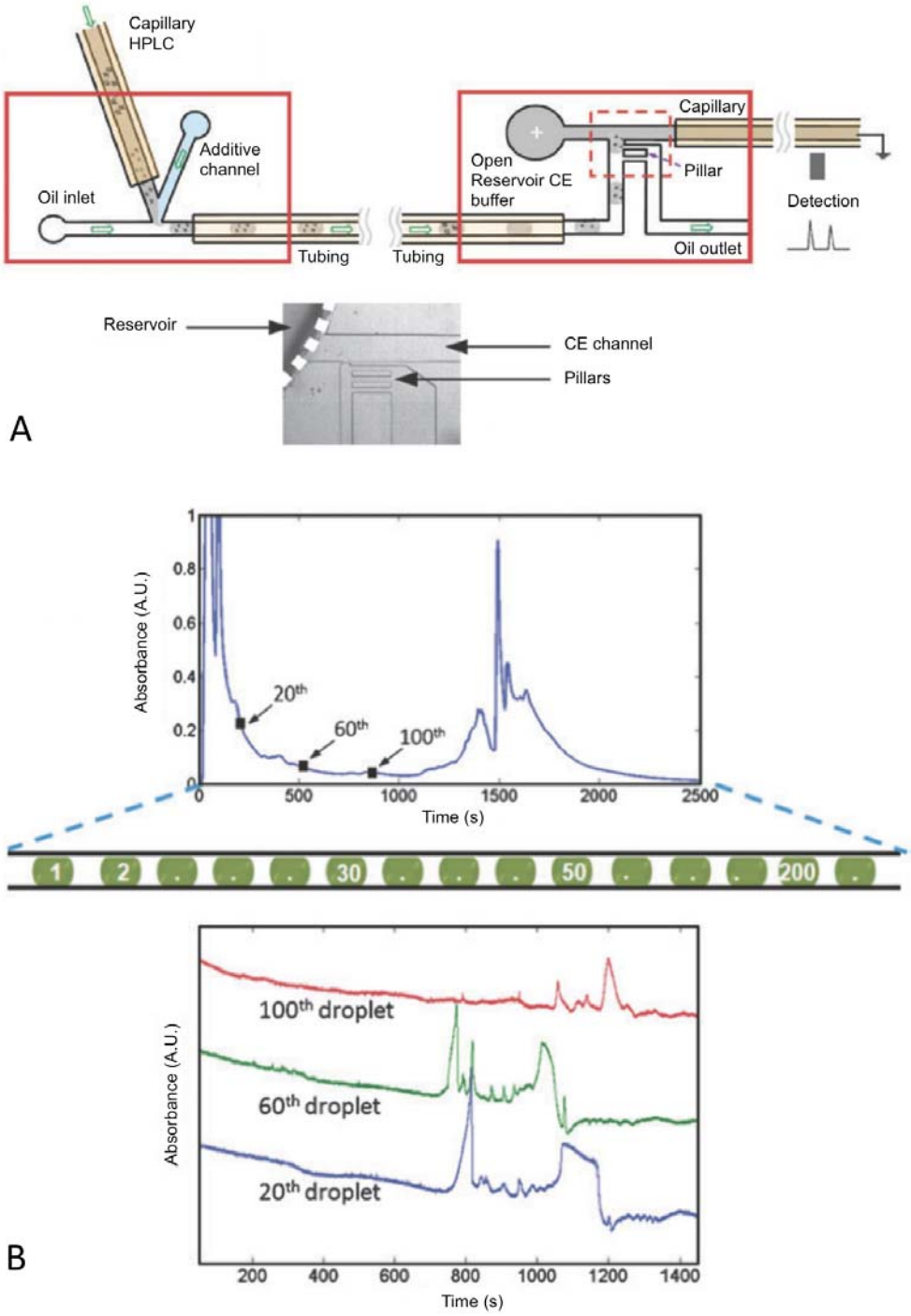

Figure 4. Microfluidic approach for 2D protein separation analysis. (A) First dimension LC separation and segmentation (left) and subsequent fusion of the resulting plugs to a continuous stream for initiating the second dimension CE separation (right). Prior to plug fusion, the oil is evacuated through pillars (bright-field image). (B) Chromatogram from a first dimension liquid capillary separation of yeast cell lysate (above) and from a second dimension CE separation of the selected plugs (below) (37). Reproduced by permission of The Royal Society of Chemistry.

(potentially causing plug breakoff), the applied oil contained a surfactant (sodium dodecyl sulphate (SDS)) which at high concentrations unfortunately led to negative impacts on the development of the embryo (43).

Nonetheless, unbiased embryonic development in plugs could ultimately be achieved: In 2008, further studies focusing on the cultivation of mammalian cells and multicellular organisms in plugs were carried out by our

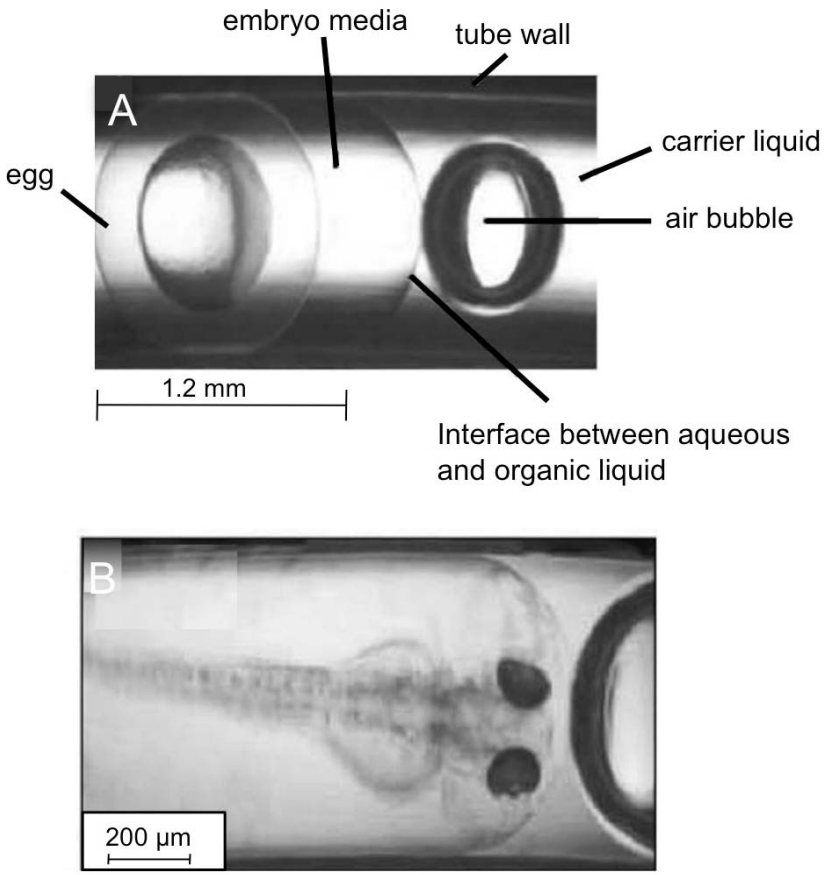

Figure 5. Zebrafish embryos encapsulated into aqueous plugs stored in Teflon tubing. (A) Individually encapsulated egg of *Danio rerio*. (B) Hatched embryo at an age of 75h (43). Reproduced by permission of The Royal Society of Chemistry.

group (44). In a first place, cell viability assays of the encapsulated cells were performed during two weeks of incubation, demonstrating for the very first time that mammalian cells can proliferate within aqueous microcompartments. Furthermore, the cultivation and proliferation of *C. elegans* within plugs (Figure 6) was shown, too. In particular, eggs of the nematode C. *elegans* were encapsulated into plugs and subsequently monitored by time-lapse imaging. After 2 days of incubation, hatched worms in the L2-L3 larvae stage could be observed, followed by the growth of adult worms and the birth of the next generation after 4 days. Once again it turned out that biocompatibility of the surfactant is of major importance for the cultivation of cells in aqueous microcompartments: While ionic surfactants lysed the cell membranes rapidly, some non-ionic fluorosurfactants (e.g. perfluorooctanol) could be identified that did not interfere with cell viability. Furthermore, only when storing the samples in gas permeable PTFE tubing and ensuring sufficient supply of nutrition (by using relatively big sample volumes of 660 nanoliters and keeping the cell density low) cell survival could be guaranteed. Taken together, these results clearly indicate that certain precautions have to be taken into account when using plugs as individual cell culture vessels. However, doing so, the technology opens the way for many interesting applications such as single cell / single organism assays (revealing population variances) or screens based on cells that are available in small quantities, only (primary cell, stem cells, etc.).

4.2. Cell-based assays at superb spatio-temporal resolution

The compartmentalization of cells and molecules into aqueous microcompartments is also of major interest for studies focusing on high spatio-temporal resolution. One example is the monitoring of cell-responses upon their exposure to small molecules or proteins (e.g. stimuli or inhibitors). For this purpose, Ismagilov and coworkers developed a device termed chemistrode (20), in which small molecules and / or proteins compartmentalized individually within plugs are flushed through an open-ended V-shaped microfluidic channel, in which they are brought into contact with a substrate (such as a cell or tissue) by coalescence (Figure 7). In particular, the substrate is placed on a hydrophobic (wet) layer below the V-shaped channel in order to stimulate it by each incoming plug (coalescing with the wet surface). Molecules released by the cells in response to the stimulation are trapped inside the continuously moving plugs and can be analyzed further downstream, using techniques such as fluorescence microscopy, fluorescence correlation spectroscopy or MALDI-MS. In order to demonstrate the advantages of the

Figure 6. Growth of the nematode *C. elegans* within 660 nanoliter plugs. Eggs (black arrow) were encapsulated at room temperature, and bright-field microscopic images were taken after 0, 2, and 4 days. White arrows show larvae of the second generation of encapsulated worms (44) - Copyright (2008) by Elsevier. White bar = 100 micrometer.

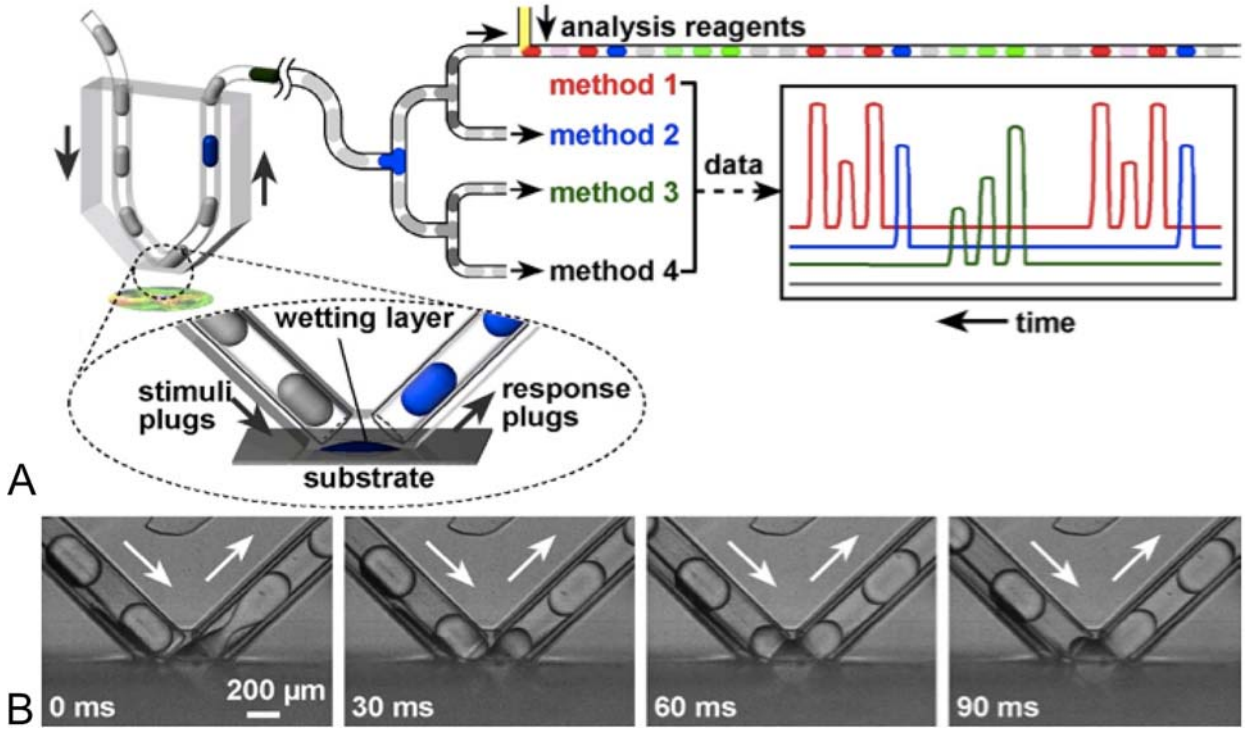

Figure 7. Cell-based assays at high spatio-temporal resolution using the chemistrode setup. (A) Different stimuli such as small molecules or proteins are encapsulated into plugs, which are flushed through an open-ended V-shaped microchannel. At the intersection the plugs get in contact (coalesce) with a substrate such as a cell embedded in a wet layer. Molecules released by the cells (in response to the stimulus) are captured in the corresponding plug at very high temporal resolution (millisecond scale). Subsequently, the plugs are split to enable different readouts in parallel (e.g. based on spectroscopy or MALDI-MS). (B) Time-lapse bright-field images (side view) of plug-substrate coalescence followed by the formation of a response plug (20). Copyright (2008) by National Academy of Sciences, U.S.A.

setup, the authors focused on a model system in the context of diabetes. Mouse islets of Langerhans cells were stimulated with plugs containing different concentrations of glucose (from 2 millimolar (resting effect) to 14 millimolar (stimulating effect)); and the response, based on an increase of intracellular [Ca^{2+}], was successfully monitored for individual cells, by measuring the fluorescence intensity of fluo-4 (a reporter for the intracellular Ca^{2+} concentration). Using a segmented flow setup not only allowed performing single cell assays, but as well enabled capturing molecules released by individual cells at millisecond resolution. This clearly points out the conceptual advantages of the approach.

5. DISCUSSION AND OUTLOOK

Compared to conventional macroscopic equipment, micro-segmented flow systems offer a number

of benefits for biological experimentation: The miniaturization not only enables massive cost savings and increased throughput, but as well allows addressing biological questions at unpreceded spatio-temporal resolution. Even though this is also true for emulsion-based (droplet-based) approaches which allow even higher sample numbers due to almost unlimited off-chip storage capacities, the methodologies described here have some specific advantages: When permanently storing all samples in a holding cartridge such as a length of tubing or a microfluidic channel, the addition of stabilizing surfactants is not absolutely required. This not only allows avoiding biocompatibility problems, but as well eliminates any micellar transport, potentially causing cross-contamination. Furthermore, applications such as the chemistrode (in which the plugs fuse with a substrate embedded into an aqueous layer on a microscopic slide) would be hardly feasible when using surfactant-stabilized droplets. Segmented-flow also provides a high flexibility in terms of the sample sizes: While very big droplets (diameter > 500 micromiter) become extremely unstable when stored in large reservoirs (even in presence of stabilizing surfactants) and tend to coalesce, their storage in capillaries or microfluidic channels is easily possible; optionally by gaining further stability through the use of an additional gas phase (12). Large plugs are of major importance for studies involving whole organisms or long-term cultivation of cells (in which case sufficient supply of nutrients has to be guaranteed). Furthermore, the sequential storage of aqueous microcompartments facilitates tracking each sample over time (e.g. by flushing the whole train of plugs several times across the readout unit; (22)), without the need for any bar coding of the sample identity (e.g. optical encoding using fluorescence dyes).

The segmented-flow applications discussed in this review constitute a comprehensive toolbox of microfluidic modules which can probably be recombined for further exciting applications: For instance, the upstream integration of a cell encapsulation device into a 2D protein separation platform as described by deMello and coworkers could ultimately pave the way for protein purification on the single cell- or at least single organism-level (as the detection limit is most likely the crucial factor). This could, for example, enable first steps into the direction of proteomics or even "structuromics" (when combined with a microfluidic platform for protein crystallization) of entire strain or mutant collections.

Finally, plug-based systems seem to offer an interesting starting point for single-cell whole transcriptome assays. Single-cell analysis is a powerful approach for understanding changes in gene expression within an isogenic cell population and hence for the identification of epigenetic factors. Current bulk techniques for gene expression analysis only measure the average value of a large number of cells, thus not taking into account the heterogeneity of a cell population, which is of particular interest for cancer cells. Microarrays for example, require at least 10^5 cells (more than 1 microgram of total RNA) for obtaining reliable results (45). Therefore, techniques allowing the amplification of mRNA from single cells, e.g. by performing RT-PCR in tiny sample volumes (resulting in high template concentrations) are of big interest. Recently, Potier and coworkers have developed a microfluidic platform for performing single cell whole transcriptome assays (46). Here, they successfully used valve-based nanoliter reactors for the mRNA amplification. However, it is obvious that segmented flow systems could take these approaches to an entirely new level in terms of throughput (the current platform allows no parallelization and hence RT-PCR of only one cell at a time).

Since all these applications do not require any off-chip incubation steps and involve relatively large volumes of the microcompartments (nanoliter scale; the efficiency of some steps of the RT-PCR even decreases when the concentrations of the cellular contents become too high), they seem to be perfectly suited for segmented-flow approaches. Taken together, there are many more exciting applications on the horizon and we are looking forward to see the deliverables of the coming years.

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Abbreviations: CMC: critical micelle concentration; GAs: genetic algorithms; SDS: sodium dodecyl sulphate; RT-PCR: reverse transcription-polymerase chain reaction; PTFE: polytetrafluoroethylene; LC: liquid chromatography; CE: capillary electrophoresis; HPLC: high performance liquid chromatography

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