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## Active Flow Control and Dynamic Analysis in Droplet Microfluidics

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### Abstract

Droplet-based microfluidics has emerged as an important subfield within the microfluidic and general analytical communities. Indeed, several unique applications such as digital assay readout and single-cell sequencing now have commercial systems based on droplet microfluidics. Yet there remains room for this research area to grow. To date, the majority of analytical readouts are optical in nature, relatively few studies have integrated sample preparation, and passive means for droplet formation and manipulation have dominated the field. Analytical scientists continue to expand capabilities by developing droplet-compatible method adaptations, for example by interfacing to mass spectrometers or automating droplet sampling for temporally-resolved analysis. In this review, we highlight recently developed fluidic control techniques and unique integrations of analytical methodology with droplet microfluidics—focusing on automation and the connections to analog/digital domains—and we conclude by offering a perspective on current challenges and future applications.

### Keywords

microfluidics; droplets; programmable; dynamic analysis; analog and digital information

## INTRODUCTION

Nearly twenty years ago, informed by prior work on the stability of monodisperse emulsions (1, 2), an early foray into droplet microfluidics by Thorsen et al. (3) came about when the researchers realized the unique and predictable fluid physics within microfluidic channels (4) could be exploited to consistently form droplets between two immiscible fluids such as water and oil. Soon afterward, a number of studies revealed that a vast array of manipulations and novel analytical applications could be achieved with droplet microfluidics, mainly with aqueous-in-oil droplet formation. Among many other productive research groups, Ismagilov and coworkers suggested rapid dynamics could be explored (5), the Whitesides and Stone laboratories explored device dependent behavior (6), the Weitz group explored high-throughput analytical possibilities and multiple emulsions (2, 7), the Mathies laboratory investigated the use of valve-based automation in droplet microfluidics (8, 9), and Chiu and coworkers explored precise droplet analysis with fluorescence correlation spectroscopy (10). Generally, in these and other related studies, microfluidic

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devices have permitted the generation of precisely defined droplets which served as unique individual micro-reactors (usually pL or nL volume) that could be processed and analyzed downstream in operations such as delivery, merging, mixing, sorting, or analysis (11).

Researchers have leveraged these populations of typically monodisperse droplets to reveal several outstanding benefits (12). The extraordinarily small volumes not only save reagents, but also enable unique analyses on single cells or even single molecules (13, 14). With nearly identical droplets generated at high throughput (often kHz frequencies), scientists can explore biological systems and generate data sets at larger scales than previously accessible (15). Numerous biological and chemical applications were empowered by droplet microfluidics in recent years, such as single-cell genome sequencing (16), enzyme kinetics and inhibition (17), combinatorial synthesis and drug discovery (18), protein and nucleic acid quantification (19–21), and cellular secretion detection (22, 23). As such, there are quite a few review articles on droplet microfluidics, and we point the readers to a recent selection having varying perspectives: a thorough review on recent advances (24), a survey of analytical techniques (25), dynamic analysis with droplets (26, 27), passive and active formation (28), droplet tracking and barcoding (29), single-cell analysis (30), tissue engineering and analysis (31), synthetic biology (32), nucleic acid cytometry (33), and drug discovery within droplets (34).

To date, most studies in droplet microfluidics have remained reliant on optical readout of droplet contents (mainly with fluorescence) due to its simplicity, compatibility with microchannels, and the lack of interference from carrier fluids such as oils. Likewise, the majority of studies have also used passive fluidic features. More recently, several groups have expanded the use of active droplet formation and control using, for example, elastomeric valves, and other analytical steps such as bead-based cleanup and mass spectrometry have been integrated with droplet microfluidics. In this review, we focus on important recent developments in active or automated microfluidic flow control strategies for droplet formation and handling, and we highlight the connection to electronic concepts in digital and analog information with some example channel architectures and analytical applications.

## FLOW CONTROL IN DROPLET MICROFLUIDICS

### Passive Droplet Control

In a droplet-based two-phase flow microsystem, two immiscible fluids under flow or pressure regulation meet at a junction, resulting in segments of the one contained in the other that are transported downstream depending on viscous and interfacial forces (35, 36). To drive droplets in a preferred route at a desired speed, passive mechanisms can maintain this balance through regulation of channel geometry, hydrodynamic pressures, surface hydrophilicity/hydrophobicity, viscosity of the continuous phase, and interfacial tension between the two phases (7, 15, 37). As surface tension is highly relevant, surfactants play a significant role in modulating droplet properties (1, 28, 38, 39). Several droplet generation techniques have been developed so far, exploiting different device geometries that can passively regulate droplet size in various ways. In two-phase flow microfluidics, cross-flow, co-flow, and flow-focusing are three of the major generator types, named for how

the phases interact the interfaces (28, 36, 40). A number of reviews have covered passive droplet formation and maneuvering; for more detailed information, we direct readers to the thorough survey by Zhu and Wang (28) and to Dressier and coworkers' apt description of formation structures published in the 2017 issue (26).

### Active Droplet Control

In realizing the array of applications achieved through droplet microfluidics, precise control over the size, volume, and frequency of formed droplet is of utmost importance. While this can be accomplished passively without external actuation, active regulation provides more authority and precision, albeit at lower throughput and with additional input energy (15, 36). Device-to-device variations, ambient conditions, and surface chemistry fluctuations are example issues that cause droplet formation inconsistencies in passive systems. These issues can be largely negated by active mechanisms, which can be interfaced to a computer to give flexibly programmable droplet formation.

Active mechanisms execute different droplet operations by employing external controllers. Depending on the type of energy applied, these actuations can be classified as mechanical, electrical, thermal, or magnetic (41). Monolithic membrane valves are the most common mechanical means as they can precisely regulate fluidic flows and mediate droplet generation and other maneuvers (22, 23, 42–49). While a thin layer of polydimethylsiloxane (PDMS) is traditionally used to act as the valve gate, 3D printing technology has recently been introduced for rapid, cost-effective production of both normally-open and normally-closed valves that can be actuated at low operational pressures (50–52).

Alternating current (AC) electric fields are often applied externally or within microchannels with nearby electrodes to aid in destabilizing the fluidic interface and initiating droplet generation, merging, sorting, and more operations (53, 54). The Abate group described a clever approach making this method more accessible, where a concentrated salt solution in a microchannel replaced traditional metal electrodes and provided further customization of the electrode designs (49, 55). Alternatively, electrowetting is popular in controlling the dynamics of discrete droplets by tuning its ability to wet a planar surface, a phenomenon subject to an affiliated field of study known as digital microfluidics (56–59).

Magnetic and thermal transducers are other means of active droplet coordination. A permanent magnet or an electromagnet can be used to regulate the size of droplets forming off a ferromagnetic fluid (60, 61) depending on its point of application (62), magnetization (28), and strength of the magnetic field (61). Further, the viscosity and interfacial tension of most fluids decrease fairly linearly with increasing temperature (41), thus integrated heating can tune these properties to enable droplet operations such as thermally mediated breakup and formation (63).

Even though a larger number of off-chip controls may make the initial set-up cumbersome, the automation capability of these active methods can ensure both accuracy and precision, especially in experiments that require precise volume metering or have to be operated for a longer time. For instance, our laboratory has shown that phase-locked detection can permit up to 100-fold improvements in optical limits of detection using a pair of sample

and reference droplets, but to do so requires generation in an alternating pattern at very narrow bandwidth (49, 64–66). While passive mechanisms are still far superior for high throughput generation (28)—often one or two orders of magnitude faster compared to active methods (41)—analyses that demand quality over quantity require resorting to active means, as described herein.

## ADVANCES IN FLUIDIC CONTROL AND INTERFACING

### Active Systems using Membrane Valves

The introduction of integrated membrane valving concepts in microfluidics (44, 45, 67) was followed by significant further development by the Quake (68, 69) and Mathies (44, 70) groups. Their work paved the way for a wide variety of unique and impactful microfluidic applications that are too numerous to adequately review here. Interestingly, for quite a few years, these types of valves were only sparingly used (9, 71–74) within the sub-field of droplet microfluidics, perhaps due to the ease with which droplets can be formed passively. Nonetheless, work in this area has continued, and several groups have recently demonstrated unique and powerful capabilities by combining membrane structures to facilitate droplet formation and various movements.

As shown in Figure 1a, the DeVoe group has recently demonstrated programmable integration of droplet generators, traps, and mergers with membrane valves and strategically placed bypass channels. Various actions were accomplished on-demand, such as trapping in a first-in first-out (FIFO) manner, analysis, selective ejection, merging, and sorting (47); and the device was applied to single cell trapping and screening. This group then used a microfluidic multiplexer and a novel, multi-depth droplet trap structure (Figure 1b) to demonstrate random-access trapping and release of arrays of picoliter droplets or cells (48). Using normally open valves and pumps for droplet formation and downstream analysis, our group has shown that the precise control of droplet frequencies at narrow bandwidths allows lock-in detection for significant reductions in optical detector noise (65, 66), in a device termed the  $\mu$ Chopper. By further combining this automation and  $\mu$ Chopper readout with salt water merging electrodes, a fully programmable device was validated for combinatorial analysis and on-chip immunoassays (Figure 1c). While a clear disadvantage of these systems is their low droplet frequencies ( $\sim 0.1 - 10$  Hz), one notable advantage of the programmability is the extraordinary consistency with which users can regulate droplet frequency, size (volume), merging ratios, trapping and retrieval positions, etc.

### Unique Droplet Junctions with Active Control

Typical droplet formation and handling structures are based on microchannel network designs with only two channels at each junction or node, such as the T-junction droplet generators shown in Figure 1a,c. Doonan and Bailey introduced a clever advancement in 2017, termed the “K-channel”, where multiple channels intersect at a single node interfaced to a nearby merging electrode and/or magnet (Figure 1d). The elegance of this design is that cross-channel flow can be coupled to droplet flow at the intersection point, enabling multiple versatile operations with a single design feature, such as reagent injection, fluid extraction (as shown in Figure 1d), droplet splitting, and magnetic bead capture. The K-channel is

categorized here as “active,” since it does require a precisely balanced flow rate in a separate liquid stream. Unlike other active structures, the K-channel operates successfully in the droplet frequency range of 200 – 500 Hz.

Despite the numerous advantages and potential applications of droplet microfluidics, a major limitation has been the lack of robust integration with solid-phase techniques. If these devices are to be successfully applied to solve many biochemical problems, the bottleneck of manual sample cleanup and extraction must be addressed. To attend to this need, the Bailey group recently developed another unique droplet junction, the coalesce-attract-resegment wash (CAR-Wash) design<sup>(76)</sup> (Figure 1e). The droplet CAR-Wash structure accepts three inputs with controlled flow (bead-containing droplets segmented in oil, a wash solution, and another oil stream for resegmenting), uses two active on-chip structures (salt water electrode and magnet), and outputs two streams with both aqueous and oil components (waste and resegmented droplets containing washed beads). This approach was useful at over 500 Hz droplet frequencies, it achieved 98% retention of beads, and it allowed over 100-fold dilution in the final droplets. The CAR-Wash design should enable users to integrate more sample preparation steps into on-chip droplet microfluidics workflows. One immediately obvious application could be to adapt multi-step, bead-based immunoassays into segmented flow streams.

### Novel Optical Interfacing

Photoinitiated reactions can also be achieved actively within droplets using light sources. For example, Choi et al. photo-polymerized monodisperse polyethylene glycol diacrylate (PEGDA) droplets on-chip using a UV light source integrated downstream of a cross-flow droplet generator<sup>(77)</sup>. Since PEGDA polymerization is strongly inhibited by oxygen molecules consuming photoinitiated free radicals, Oakey’s group introduced a stream of nitrogen gas that was able to purge the system, offering better control over microsphere size and uniformity<sup>(78)</sup>. Di Carlo and co-workers increased both scalability and throughput in microgel production using pH adjustments and an oil-soluble organic base<sup>(79)</sup>. The same group also used coordinated polymerization to fabricate “dropicles,” uniform particles that template aqueous-in-oil compartment formation<sup>(80)</sup>; these particles have significant potential due to their ability to form compartments without fluidic channels. By integrating an optical waveguide, mirror, droplet formation, and bead suspension hopper structures on-chip, Paegel and coworkers developed the hvSABR (Light-Induced and - Graduated High Throughput Screening After Bead Release) architecture<sup>(18)</sup> (Figure 2a). Accurate photochemical reaction dosing was possible with this system, and beads displaying photochemically cleavable pepstatin A were used for dose-response screening in an HIV-1 protease activity assay.

Optical detection is widely used for measuring droplet contents in microfluidics, with fluorescence most commonly employed. Absorbance detection has been applied, but the fundamental dependence on optical path length becomes limiting in micrometer channel dimensions. In fact, weak optical signals of any type require more elaborate optics or phase-locked detectors to efficiently improve sensitivity. Recognizing that droplet formation is essentially “chopping” the fluidic sample, our group exploited this inherent nature of

droplet formation as a microfluidic sample chopper—termed the  $\mu$ Chopper—an analog to the optical beam chopper (64). Absorbance detection limits were improved by more than 100-fold without modifying optics. Similarly, using continuous illumination while locking in the optical detector to the frequency and phase of droplet forming valves, we showed over 50-fold noise reduction in fluorescence (49, 65, 66) (Figure 2b). The uptake rate of free fat acids (FFA) in single 3T3-L1 adipocyte cells was quantified for the first time as  $3.5 \pm 0.2 \times 10^{-15}$  mol cell<sup>-1</sup>, and the mass detection limit of insulin using in-droplet homogeneous immunoassays was  $2 \times 10^{-16}$  mol. Since precise frequency regulation provides significant enhancements in this system, this device in particular—with 0.04 Hz bandwidth in droplet formation—highlights the fact that integration of computer-controlled valves with droplet microfluidics can be enabling to new analytical capabilities.

Another unique optical interface involves arrays of small lenses matched to the droplet detection spots. The Baret group showed that a micro-optical lens array (Figure 2c) could be used for massively parallel droplet detection, with throughputs of  $\sim 2000$  droplets s<sup>-1</sup> and per lens, and with over 625 measurement points (81). Later, the deMello group validated their version of a microlens array by analyzing 50 000 cells s<sup>-1</sup> (82). The same research group later used a counter-propagating lens/mirror system that enhanced signals by two orders of magnitude while also analyzing up to 40 000 droplets s<sup>-1</sup> (83). These microlens array systems are particularly powerful and efficient, since they can be microfabricated and matched to the dimensions of the microchannels.

### Oil Draining and Aqueous/Oil Separators

From injecting reagents to detecting analytes, droplets under operation require enough spacing in sequence to avoid undesired coalescence or content leakage, and to assist detection. Obviously, the oil carrier plays an important role here. While gas bubbles and liquid plugs have been used as spacers, these introduce more complexity (84). The reverse operation to spacing is oil drainage, which is often leveraged to help merge adjacent droplets. Lee et al. utilized a drainage channel with smaller dimensions than the main channel to draw off the oil spacers between droplets before electric field-based coalescence (85). The Bailey group recently applied two counter-flowing oil streams to help remove oil spacing in real time (86), a device named the counter-current continuous phase extraction (C<sup>3</sup>PE) module that was capable of processing at 40-200 Hz.

Droplet generators are often coupled with various analytical tools to detect, screen or investigate. Some schemes require droplets to be completely separated from their oil carrier prior to analysis to ensure maximum throughput and minimum interference (87, 88). Many separation methods involve transferring the droplets from their oil carrier to an aqueous stream—in a parallel flow of the two—with the aid of a modified hydrophilic surface or an applied electric field, as in the CAR-Wash structure (Figure 1e) (76). Fidalgo et al. (87) and the Kennedy group (88, 89) have used similar aqueous/oil separators, which will be discussed in detail later. Some other separation techniques have drained the oil while retaining droplets by employing geometric obstacles on the flow path (90), creating hydrophobic side channels (91, 92), and introducing an oil-absorbing membrane on an extraction interface to couple to electrophoresis (93).



## FLUIDIC ANALOG-TO-DIGITAL OPERATIONS USING DROPLETS

Readers with a background in analytical chemistry, in particular, may quickly draw comparisons between droplet microfluidics devices and the flow injection analysis (FIA) systems developed in the 1970s (94, 95). While this analogy is sound, an important distinction of droplet microfluidics is the ability to sample from a fluid stream then essentially shut down diffusion and dispersion (5, 96) between adjacently sampled droplets. Where diffusion and dispersion are expected in FIA and are folded into the data analysis, droplet microfluidics minimizes longitudinal dispersion after segmentation, where mixing can only occur within each individual droplet through chaotic advection. Separated droplet segments are effectively chemically segregated from each other, unless some components are able to partition into the oil phase (97). As long as oil partitioning is regulated or is negligible at the experimental time scales, this spatial separation of chemical and biochemical components opens new potential applications in analytical and bioanalytical chemistry, where analogies to digital information processing are often more appropriate (22, 23, 27). In this section, we discuss the implications of droplet segmentation in the temporal analysis and control of fluidic streams and accompanying analyte signals. Moving forward, we refer to devices that continuously sample a fluidic stream and segment it into droplets as microfluidic analog-to-digital converters ( $\mu$ ADC) (Figure 3a); conversely, devices that start with segmented flow and generate well-controlled patterns in the output continuous flow stream are referred to as microfluidic digital-to-analog converters ( $\mu$ DAC) (Figure 3b).

### Microfluidic Analog-to-Digital Conversion ( $\mu$ ADC)

Some of the earliest research in this microfluidics sub-field recognized the power of droplet-based segmentation in preserving temporal dynamics of the system under study (5). One classic example is a device termed the “chemistrode” (98) (Figure 3c). This device used segmented flow streams as both the input and the output to an external system such as cells on a glass surface. The pioneering work proved a dynamically varying system could be fluidically sampled at short time scales then analyzed by immunoassays, fluorescence readout, and mass spectrometry (MS). Similarly, early work by Easley et al. (99) showed that continuous droplet sampling could be used for secretion sampling from pancreatic islets with 1.9-second resolution, revealing two major classes of oscillations in secreted zinc (periods at ~20–40 s and ~5–10 min). In other concurrent work by Kennedy’s group, the team coupled microdialysis sampling with droplet segmentation to preserve temporal resolution, and droplets were coalesced into a continuous stream and fed into a microchip electrophoresis device to evaluate glutamate and aspartate release in the striatum of living, anesthetized rats (100).

These systems—introduced just over a decade ago—can be fundamentally viewed as microfluidic analog-to-digital converters ( $\mu$ ADC) (Figure 3a). A system of interest that varies dynamically over time can be sampled with nanoliter or picoliter volume droplets, and the aqueous-in-oil segmentation will prevent diffusion and dispersion from compromising the temporal record within the fluid. Importantly, the fluidic sampling resolution,  $t_s$ , will ultimately define the achievable measurement resolution downstream, and this is particularly true if the measurement requires mixing with assay reagents prior to readout. By

coupling this type of  $\mu$ ADC system to the actively controlled merging structures shown in Figure 1a,c,d temporal resolution can be preserved by mixing with assay reagent droplets downstream of the sampling site (49), and essentially unlimited numbers of operations (albeit with dilution) could be carried out on the droplets downstream without detriment to  $\Delta t$ . Thus, by treating each sampled droplet as a snapshot in time of the chemical and biochemical information emerging from the system of interest, we have a fluidic system analogous to ADC digital circuitry. Conversely, with continuous flow much of the temporal information from system can be lost to diffusion and dispersion in the sampling channel, and any subsequent operations will further compromise and increase  $\Delta t$  (Figure 3a, top trace).

Two examples of  $\mu$ ADC fluidic architectures are shown in Figure 3, namely the chemistode (98) (Figure 3c) developed by the Ismagilov group and the valve-automated  $\mu$ ADC developed by our group for tissue secretion sampling and downstream quantitative analysis (22, 23) (Figure 3d). As shown in Figure 3d, automated valve-controlled segmented flow allows the time difference between droplets ( $\Delta t$ ) to be precisely regulated, and the fluidically digitized time record can be stored downstream in an incubation channel (inset image) akin to digital data storage on a hard disk.

### Applications of $\mu$ ADCs

Novel chemical and biochemical information has been uncovered through the application of such  $\mu$ ADC systems (Figure 4). In one example, a microdialysis based neural probe  $\mu$ ADC system developed by Petit-Pierre and coworkers (101) combined a microfluidic droplet generator with recording and stimulation electrodes. By positioning the droplet generator within 170  $\mu$ m of the sampling inlet, the resolution ( $\Delta t$ ) was only a few seconds (Figure 4a), and the engineered probe (top images) was validated for translating from an analog stream to digital fluid segments at high temporal resolution (bottom data). This system was later applied to monitor neurochemical dynamics in rat brains *in vivo* at a resolution of 50 seconds (102). The Kennedy group has also made measurements *in vivo* by combining a microfabricated push-pull probe integrated with droplet-based sampling (103) (Figure 4b). The small probe size permitted 1000-fold improvement in spatial resolution, and droplet-based segmentation closely downstream allowed 6 second temporal sampling resolution ( $\Delta t$ ) at low perfusion rates. Impressively, they also coupled the system to nanoelectrospray ionization mass spectrometry (nESI-MS) for simultaneous determination of glutamine, glutamate,  $\gamma$ -aminobutyric acid, and acetylcholine, revealing rapid *in vivo* neurochemical release times of 15 seconds in the brain of live rats following potassium stimulation. Figure 4b shows SEM images of the probe, with fluidic  $\mu$ ADC test data on the top right and *in vivo* data at the bottom right, both showing high resolution glutamine quantification by MS. Nightingale et al. recently demonstrated an impressive *in vivo* application with their wearable  $\mu$ ADC device. Droplet sampling was integrated into this wearable sensor to quantify glucose and lactate directly in dermal tissue (104) (Figure 4c).

Our laboratory has applied an automated, valve-based  $\mu$ ADC (Figure 3d) for sampling and quantification of secretions from endocrine tissue explants. Building from earlier work with a passive sampler (99), the first application of our automated  $\mu$ ADC was to murine pancreatic islet tissue (22). Small-volume, droplet compatible homogeneous immunoassays



were developed for insulin (105), and these reagents were mixed upstream of droplet formation using on-chip pumps (Figure 4d). Because we used the  $\mu$ Chopper concept (49, 64–66), highly sensitive insulin quantification was accomplished by simple optical measurements on-chip with a standard fluorescence microscope. This device revealed insulin secretion oscillations upon glucose stimulation (upper right data, Figure 4d), with timing that matched with calcium oscillations, and rapid insulin bursts in the hundreds of attomole range were observed quantitatively for the first time. The device was recently updated to include more assay reagent channels, and a coupled enzyme assay for glycerol was integrated (23) and used to study the differences between adipocyte spheroids (cultured cells) and *ex vivo* adipose tissue from mice. High-resolution data ( $\tau = 3.48$  seconds) on glycerol secretion (lower data in Figure 4d) showed that rapid lipolytic oscillations were present in adipose tissue but not in the cell spheroids. These previously unreported glycerol oscillations occurred at frequencies of 0.2 to 2.0  $\text{min}^{-1}$ , with low-level bursts ( $\sim 50$  fmol) released in basal conditions and larger bursts ( $\sim 300$  fmol) during stimulation. The application showed that high-resolution, quantitative sampling using our  $\mu$ ADC device enabled unique biological information concerning cellular connectivity to be observed. This analytical framework should be well-suited for future studies of dynamic oscillatory function in various other tissues.

### Microfluidic Digital-to-Analog Conversion ( $\mu$ DAC)

Using the reverse operation, devices that begin with a segmented flow stream, i.e. a digital input, then separate the aqueous and oil phases into individual streams (analog output) can be viewed as microfluidic digital-to-analog converters ( $\mu$ DAC) (Figure 3b). In this way, users can essentially program a dynamically varying signal into a segmented stream of nanoliter or picoliter volume droplets, and the  $\mu$ DAC will output a dynamically varying aqueous stream with precise changes defined by the input droplets with high temporal resolution (also defined as  $\tau$ ).

Many of the early works discussed above concerning droplet mergers or aqueous/oil separators, while not explicitly described this way in the manuscripts, fit the description of  $\mu$ DACs. Angelescu et al. showed that a passive aqueous/oil separator could be made using the native substrate (PDMS) surface chemistry and channel aspect ratios (91). The oil stream easily flowed downward (Figure 3e) through narrow channels, while the aqueous stream was driven forward, providing  $\mu$ DAC operation. This group also used oil-wet fluoropolymer membranes to instead retrieve the oil phase. A similar oil draining method was used by Ostromohov et al. (92), who created the highest resolution  $\mu$ DAC to our knowledge ( $\tau = 0.56$  seconds; 1.8 Hz) (see Figure 5b). Using dielectrophoresis (DEP) with high AC voltages applied to on-chip electrodes, Fidalgo et al. (87) demonstrated that a stream of segmented droplets could be coalesced with a continuous aqueous stream and later coupled to mass spectrometry (MS) analysis (Figure 3f). Also using surface chemistry and oil/droplet wetting behavior to their advantage, the Kennedy group separated oil and aqueous solution at a T-junction (88) (Figure 3g). In this device, a segmented flow input was converted to continuous streams of oil and aqueous solution, thereby functioning as a  $\mu$ DAC continuously injecting samples into a microchip capillary electrophoresis (MCE) channel. They later designed a modified device that interfaced droplet inputs to both MCE and gel

electrophoresis on-chip (106), studying protein-protein interactions and enzyme samples at 10 seconds per sample, and over 1000 separations were done before device reconditioning was necessary. One can imagine a variety of possibilities in applying these types of fluidic architectures ( $\mu$ DAC devices), and several example applications are discussed in detail below.

### Applications of $\mu$ DACs

A number of unique applications have been accomplished using the aforementioned devices (Figure 5). In one example, using the  $\mu$ DAC device concept shown in Figure 3g, Guetschow et al. (88) sampled a library of potential enzyme activity modulators from multiwell plates, segmented these into 8 nL aqueous-in-oil droplets (digital information), and introduced the droplets into a MCE device capable of sub-second separation times. Figure 5a shows their excellent example of  $\mu$ DAC operation, where a digital input (black trace, top) is translated to an analog output (red trace, top). Driven by the digital droplet input, this device output a continuous stream of sub-second mobility shift assays of protein kinase A (PKA) activity, and 96 samples were analyzed in only 12 minutes with minimal sample input. The middle data set shows ten rapid MCE electropherograms and the response to increasing rhodamine (R) in subsequent droplets, while the bottom data set shows electrophoresis peak areas of rhodamine (black), substrate (red), and product (blue) during analysis of 12 samples (2 controls, 10 test compounds). This example highlights the power of combining droplet-based microfluidics with rapid MCE, where the analytical benefits of rapid analysis, very low sample consumption, and high throughput screening are combined into one integrated device.

Ostromohov, Bercovici, and Kaigala (92) used their high resolution  $\mu$ DAC device ( $t = 0.56$  seconds) to deliver sequential reagents with minimal dispersion to a surface. Figure 5b shows fluorescence images of the device in operation (top), where digitized segmented flow is translated to a continuous aqueous stream using a wettability filter. One application of the device was to modify the fluorescence emission of surface-immobilized green fluorescent protein (GFP) by rapid pH changes in the  $\mu$ DAC-delivered buffer (Figure 5b, lower). The Inglis group developed a similar needle-like  $\mu$ DAC device to translate segmented flow through hydrophilic capillaries, resulting in a continuous stream of solution to a surface at a chemical signal response time of  $\sim 3$  seconds (108). This research group has published a recent review of literature on dynamic transport of chemical signals using droplets, which the readers may find especially informative and complementary to this review (27).

Finally, we chose to highlight one of the most powerful analytical applications of droplet-based microfluidics to date, mass activated droplet sorting (MADS) (107). This device integrates multiple droplet-handling features, of which a  $\mu$ DAC is merely one. As shown in Figure 5c, a previously formed emulsion of droplet-encapsulated library components is reinjected into the device, then a splitter breaks each droplet into two daughter droplets at 4:1 volume ratio. The larger daughter droplets are diverted to an exit leading to an electrospray ionization mass spectrometry (ESI-MS) interface; this is the  $\mu$ DAC part of the device since the ESI-MS requires continuous aqueous input. Meanwhile, the sequence of smaller daughter droplets is fed into a delay line to wait for MS data. Lastly, a DEP-

based sorting module is used to sort the smaller droplets based on the MS signal from its corresponding larger droplet. The MADS technique is able to use MS to sort thousands of droplets at  $0.7 \text{ samples s}^{-1}$  ( $\sim 15\,000$  samples in 6 hours). The method was ultimately used to directly detect a substrate of a transaminase, 1-(imidazo[2,1-b]thiazol-6-yl)propan-2-amine (ATA Substrate) for screening the activity of the enzyme via *in vitro* expression within the droplets. Since the ATA Substrate and the products are not distinguishable optically without chromatography, this MADS device represents an important new analytical tool in droplet microfluidics. Due to its label-free nature, this method should greatly expand the applicability of droplet sorting in the future.

## CONSIDERATIONS, CHALLENGES, AND FUTURE POSSIBILITIES

In this final section, we explore several of the considerations and questions that are important for those using or planning to use droplet-based microfluidics for dynamic analysis or control. We also provide a perspective on the current challenges, important aspects, and future possibilities in this area.

### Questions and Considerations

First, a user must determine if high resolution sampling or stimulation are needed for the intended application. Using the electronics analogy, needless oversampling of data in an ADC can be compared to wasteful oversampling of droplets with a  $\mu$ ADC, which could overuse precious samples such as enzymes or biosensing reagents. In other words, if a dynamically varying system (Figure 3a) oscillates with an average period of  $\sim 1$  hour, there is no need to sample droplets from the system every second. On the other hand, if a system of interest is varying much more rapidly than droplets can be sampled, fluidic aliasing will occur, and the measurements may not be interpretable. Here, the user must update the droplet-based  $\mu$ ADC for sampling at least twice the frequency of the fastest changes in the observed system (Nyquist rate). This concept extends to  $\mu$ DAC devices as well, such that the temporal resolution of stimulation should be carefully chosen depending on the size and timing of the system to be stimulated.

The scale of system under observation should also be carefully matched to the microfluidic droplet generator. For example, if the adipose tissue explants ( $< 1 \text{ mm}$ ) studied in Figure 4d were much larger, say  $1 \text{ cm}$  in diameter, glycerol secretions would take place over too large of an area causing important dynamic information to be lost before droplets are even formed. Likewise, high resolution chemical or biochemical patterns emitted from a  $\mu$ DAC should be scaled to the appropriate volume to adequately stimulate or modify a system of interest.

Oil partitioning is another important issue. Analytes or stimulants of interest, their interacting partners, optical labels, etc. should be carefully evaluated for their potential to partition into the chosen carrier oil (97). This partitioning study should usually be carried out within the microdevice, since aqueous/oil surface area-to-volume ratios are very high in this setting. As long as the time scale of analyte partitioning into oil is negligible compared to the experimental time scale, the system should be functional. In fact, with the MADS device recently developed by the Kennedy group (107), the team's original plan was to measure the ketone product of the transaminase reaction, however the ketone was observed to transfer

rapidly (with respect to the experimental time scale) between droplets, confounding the droplet sorting downstream. The team chose to analyze substrate depletion instead, since their MS readout was highly flexible. If this type of solution is not accessible, it may be helpful to change the oil or surfactant used in the carrier phase. The worst case scenario would be that the analyte is simply not measurable using droplet microfluidics, at least with resolution at the relevant time scales.

### Value of Active Control

In this review, we chose to focus on actively controlled devices for an important reason. To reach their true potential, high resolution  $\mu$ ADC or  $\mu$ DAC devices should operate at a very narrow bandwidth around the intended frequency, as in the electronic counterparts. We contend that programmable, active regulation of the droplet frequency and phase is the most appropriate way to achieve narrow bandwidth. We have already shown the utility of active control with the  $\mu$ Chopper concept (Figure 2b) (64–66), and others have provided similar evidence (15, 36). Passive control can generate droplets at very high frequencies (tens of kHz), but the bandwidth can be broadened by device variations, environmental changes, and surface chemistry fluctuations.

### Challenges and Future Possibilities

While certainly advantageous, most active droplet regulation methods mentioned in this review remain much slower (0.1 – 10 Hz) than their passive counterparts (1 – 10 kHz). Thus, there is a clear need for faster approaches to droplet formation and maneuvering while maintaining narrow bandwidth. One possible solution would be to fabricate on-chip valves with more rigid materials, and recent 3D-printed valves may help address the challenge (50–52). Alternatively, off-chip controls that operate through continuous feedback from measurements of droplet frequency may suffice.

Efforts to interface with more analytical techniques should also continue (25). Optical readout by fluorescence has been most popular, but there are still opportunities to interface rapid-readout spectrometers to interrogate droplet streams. As exemplified by the new MADS technique, the droplet fluidics/MS combination can be particularly powerful (107), and we hope to see more MS experts embrace droplet microfluidics as an compatible, enabling technology. Likewise, the droplet CAR-Wash design (76) opens up new possibilities in high-throughput, continuous bead-based sample preparation in droplets, which is an area that needs more attention.

## CONCLUSIONS

The benefits of dynamic analysis with droplet-based microfluidics was summarized nicely by Feng, Shirani, and Inglis (27) who remarked, “A real-time analytical chemistry lab...to fit inside the brain of a mouse does not exist. However, we can achieve similar analytical aims if we can digitize the liquid environment from precise locations...at precise times...” Indeed, droplet microfluidics has provided the opportunity to do just that. In this review, we have compiled the most recent and relevant research in the context of temporal analysis with segmented flow, and we have focused on actively regulated systems. Viewing fluidic

signal transformations in the context provided ( $\mu$ ADC and  $\mu$ DAC), we contend that the high precision and programmable timing provided by active regulation is most ideally suited to push the field forward. Akin to electronic signal collection, maintaining narrow bandwidth in the sampling frequency is key, and this will allow improvements in noise reduction and also open the possibility of spectral mixing and de-mixing of input droplet “signals.” Finally, while it is important to understanding analogous concepts in electronics, the sheer abundance and complexity of chemical and biochemical information may lead to entirely new concepts in information processing.

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## LIST OF ACRONYMS

<b><math>\mu</math>ADC</b>	Microfluidic analog-to-digital converter
<b><math>\mu</math>Chopper</b>	Microfluidic sample chopper
<b><math>\mu</math>DAC</b>	Microfluidic digital-to-analog converter
<b>droplet CAR-Wash</b>	dfd
<b>DEP</b>	dielectrophoresis
<b>ESI-MS</b>	electrospray ionization mass spectrometry
<b>FIFO</b>	first-in first-out
<b>hvSABR</b>	Light-induced and -graduated high throughput screening after bead release
<b>MADS</b>	Mass-activated droplet sorting
<b>MCE</b>	Microchip capillary electrophoresis
<b>PDMS</b>	polydimethylsiloxane
<b>PEGDA</b>	polyethylene glycol diacrylate

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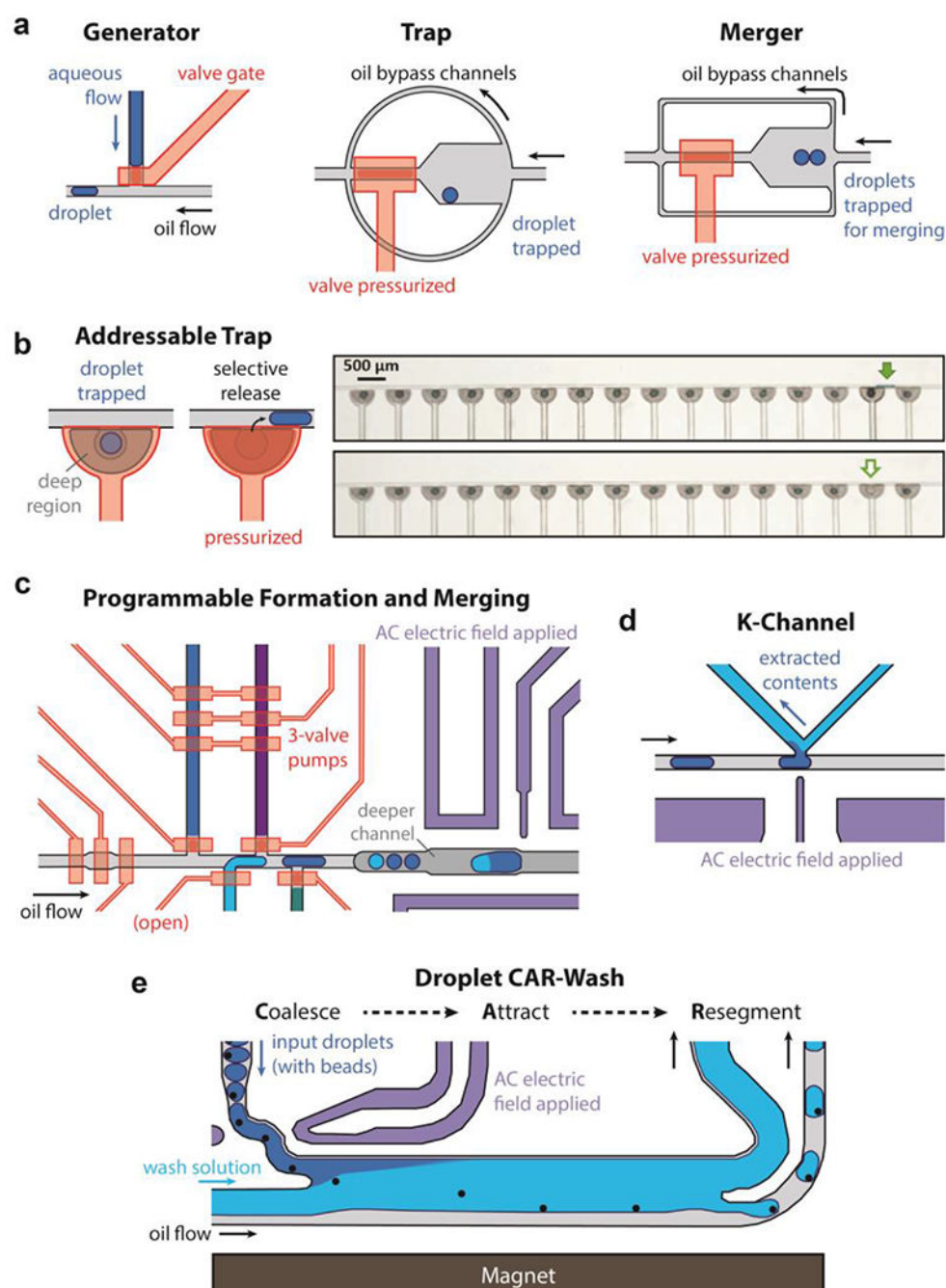


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**Figure 1.**

Recent advances in active droplet control. (a) Membrane valve structures for programmable generation, trapping, and merging of droplets and (b) addressable trapping and releasing of droplets or cells. Panel a adapted from reference (47) with permission from the Royal Society of Chemistry, copyright 2019; panel b adapted from reference (48) with permission from AIP Publishing, copyright 2020. (c) Pneumatic valve-based, on-chip pumps combined with salt water electrodes for programmable formation and merging of multiple droplet populations at well-controlled ratios. Adapted with permission from reference (49). (d)

The K-channel architecture enables multiple unique functions, such as selective extraction of contents (dark blue) into an aqueous stream (light blue) in real time. Adapted from reference (75) with permission from the American Chemical Society, copyright 2017. (e) The coalesce, attract, and resegmenting structure for bead washing (CAR-Wash) provides efficient, continuous washing of magnetic beads within droplets. Adapted from reference (76) with permission from the Royal Society of Chemistry, copyright 2019. Gray = oil, Blue colors = aqueous solutions, Red = pneumatic valves, Purple = salt water electrodes.

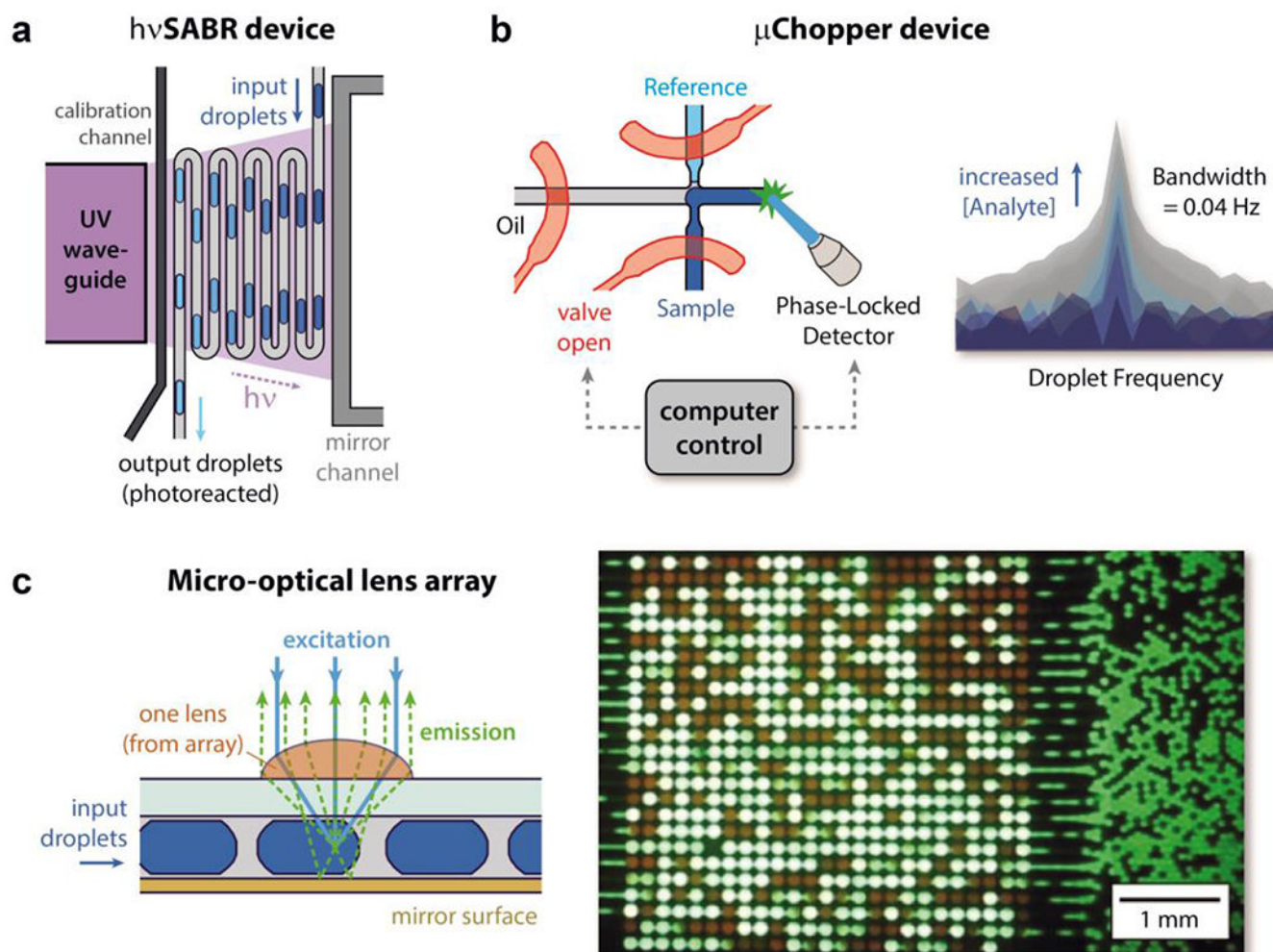
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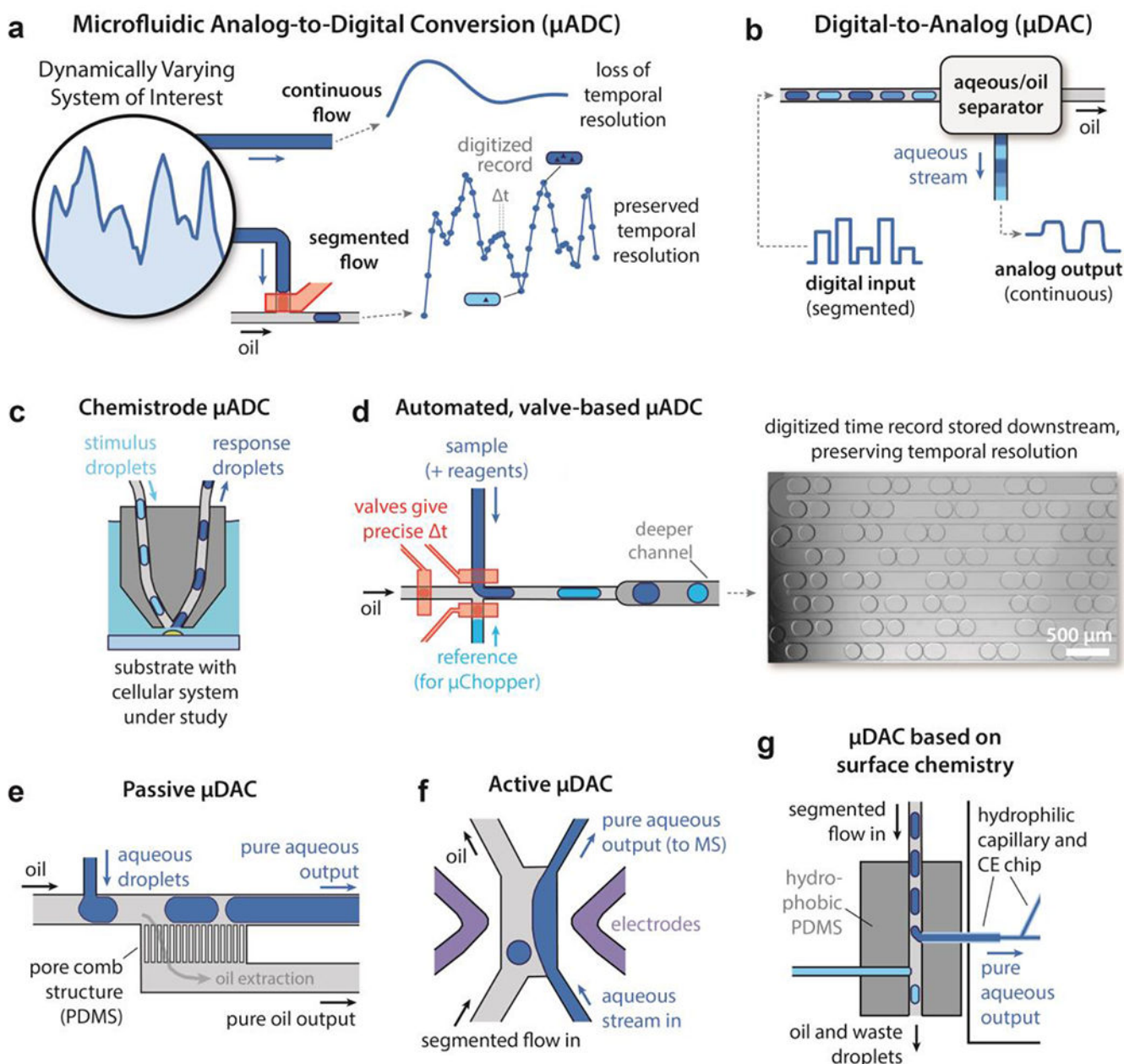
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**Figure 2.**

Unique interfaces of droplets with light sources. (a) Photoinitiated reaction management within droplets using integrated on-chip structures by Paegel and coworkers. Adapted from reference (18) with permission from the American Chemical Society, copyright 2016. (b) By locking in the detection and valving systems, our group has shown up to 100-fold noise reduction with the  $\mu$ Chopper device. Adapted from reference (65) with permission from the American Chemical Society, copyright 2017. (c) Baret and coworkers designed a micro-optical lens array for high throughput fluorescence emission measurements. Adapted from reference (81) with permission from the Royal Society of Chemistry, copyright 2013.

**Figure 3.**

Concepts and fluidic designs for microfluidic analog-to-digital ( $\mu$ ADC) and digital-to-analog conversion ( $\mu$ DAC) using droplets. **(a)** Concept of  $\mu$ ADC, where dynamic information lost in continuous flow systems is preserved at high temporal resolution ( $\Delta t$ ) using droplet microfluidics. **(b)** Concept of  $\mu$ DAC, where trains of input droplets can be coalesced and separated from oil to provide a high-resolution continuous aqueous stream with concentrations defined by the input droplet train. **(c)** Fluidic design of the chemistode device, also a  $\mu$ ADC. Adapted from reference (98) with permission from the National Academy of Sciences, copyright 2008. **(d)** Automated, computer-controlled valves give highly consistent  $\Delta t$ , and fluidic digitization can be stored downstream as digital data

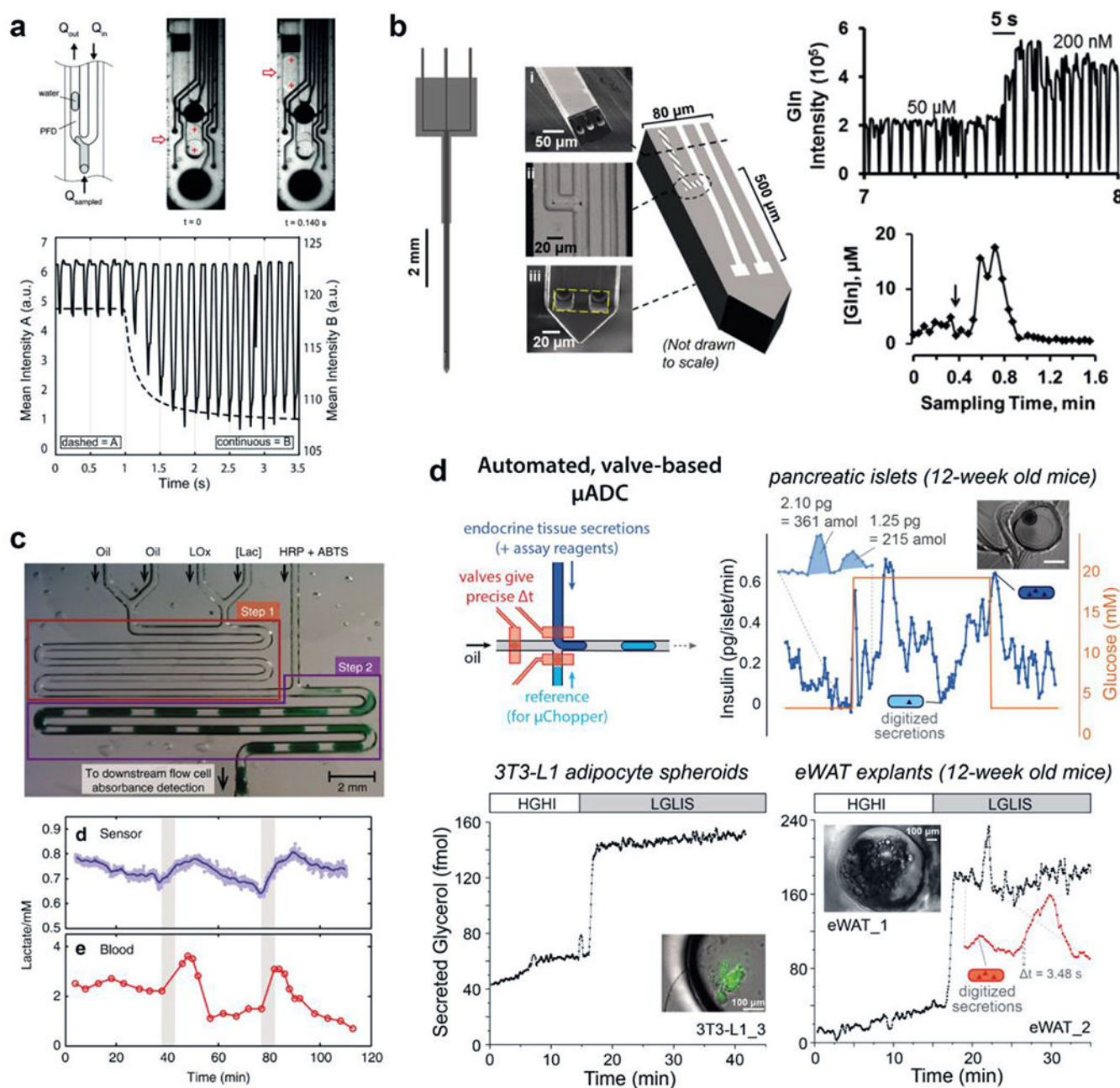
(inset image). Image obtained from reference (22) with permission from the Royal Society of Chemistry, copyright 2018. (e) Fluidic design of a passive aqueous/oil separator that converts digitized fluidics into a continuous stream to serve as a  $\mu$ DAC. Adapted from reference (91) with permission from the American Chemical Society, copyright 2010. (f) Active  $\mu$ DAC design based on dielectrophoretic merging of a droplet train with a flowing aqueous stream, interfaced with mass spectrometry (MS). Adapted from reference (87) with permission from the Wiley, copyright 2009. (g)  $\mu$ DAC using surface wettabilities to separate oil and aqueous streams. Adapted from reference (88) with permission from the American Chemical Society, copyright 2014.

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**Figure 4.**

Applications of microfluidic analog-to-digital conversion ( $\mu$ ADC). (a) Neural probe  $\mu$ ADC with device designs at top, and analog (dashed) to digital (continuous) data shown at bottom. Adapted from reference (101) with permission from the Royal Society of Chemistry, copyright 2016. (b) Combined microfabricated push-pull probe, droplet sampling, and nESI-MS analysis for multiplexed *in vivo* neurotransmitter quantification at  $t = 6$  seconds. Adapted from reference (103) with permission from the American Chemical Society, copyright 2018. (c) Wearable  $\mu$ ADC device for high resolution analysis of glucose and lactate directly in dermal tissue. Adapted from reference (104) with permission from Nature



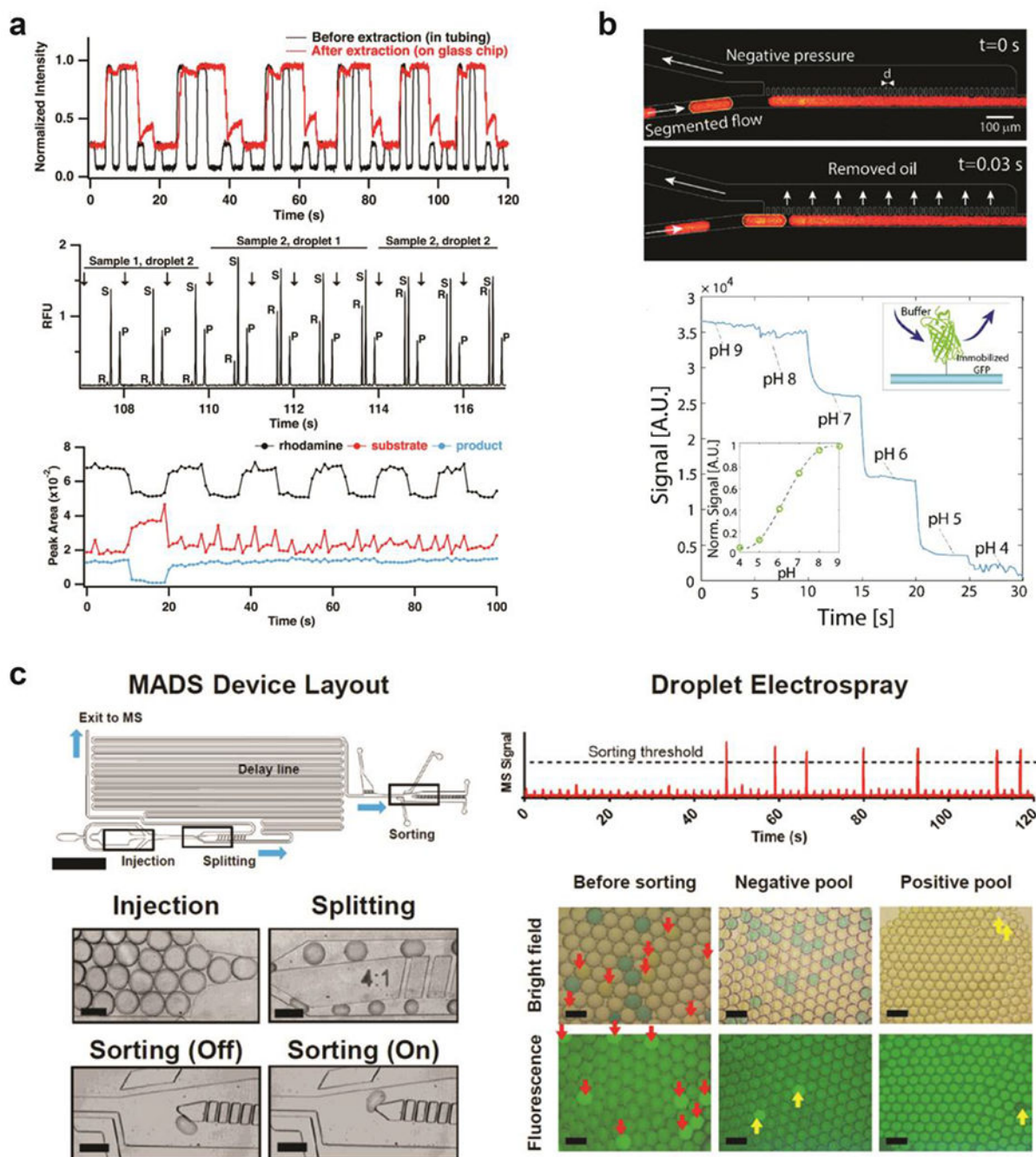
Research, copyright 2019. (*d*) Automated  $\mu$ ADC integrated with homogeneous insulin and glycerol assays to study pancreatic and adipose tissues, respectively. Unique biological information was observed in both classes of *ex vivo* tissue explants at  $t = 3.5$  seconds. Scale bars represent 100  $\mu$ m; HGHI = high glucose, high insulin; LGLIS = low glucose, low insulin, with isoproterenol. Adapted from references (22) and (23), with permission from the Royal Society of Chemistry, copyright 2018 and 2020.

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**Figure 5.**

Applications of microfluidic digital-to-analog conversion ( $\mu$ DAC) using droplets, (a) Interfacing a  $\mu$ DAC to inject a stream of nanoliter samples directly into sub-second mobility shift assays of PKA activity. Adapted from reference (88) with permission from the American Chemical Society, copyright 2014. (b) A high-resolution  $\mu$ DAC device ( $t = 0.56$  s) was validated by rapidly inducing changes to surface GFP fluorescence. Adapted from reference (92) with permission from the Royal Society of Chemistry, copyright 2016. (c) Mass activated droplet sorting (MADS) where an input droplet train was split and passed



to a  $\mu$ DAC for ESI-MS interfacing while simultaneously sorting using DEP. Adapted from reference (107) with permission from Wiley, copyright 2020.

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