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Review

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Recent Advances in Droplet Microfluidics

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Microfluidic platforms have changed the paradigm of biochemical experimentation over the past 3 decades.[1,2] Prominent within this technology set are droplet-based microfluidic systems,[3] in which passive microfluidic structures are used to rapidly generate and manipulate subnanoliter volume droplets within microchannel environments.[4,5] Droplets are formed in a continuous and robust fashion through the extrusion and shearing of two mutually immiscible phases in a microchannel,[6] with droplet volumes being precisely controlled through the variation of flow rate ratios and channel dimensions. The rapid production (and analysis) of droplets allows for exceptionally high-throughput experimentation and data acquisition, and configurable channel designs, coupled with on-demand control architectures, engender a range of robust manipulations, such as reagent dosing, droplet fusion, droplet splitting, washing, payload heating, incubation, content dilution, and droplet sorting.[7,8] Accordingly, and unsurprisingly, droplet-based microfluidic systems have become an indispensable and embedded tool within contemporary chemical and biological science.

In the 2 decades since its emergence,[9,10] droplet-based microfluidics has undergone two distinct stages of development. In its first decade, as an emerging technology set, most activity focused on the establishment of robust functional components for droplet manipulations,[11−14] the development of methods for controllable compartmentalization,[15] and the application of these systems to simple chemical and biological problems.[16] In its second decade, the focus very much shifted to the exploration of timely and complex applications in the fields of biology, chemistry, and material science,[17−20] with concomitant advances in detection methods able to probe small volumes at high speed.[21,22] As a result of all these developments, it is fair to say that droplet-based microfluidic technologies have extended the frontiers of high-throughput experimentation, providing a step change in experimental efficiency, where extensive and high-quality data sets can be rapidly assembled from a minimal amount of starting material or sample. The field has seemingly completed the transition from "breadth" to "depth", with high impact studies increasingly focusing on a small number of key applications in the biological sciences,[23,24] as will be discussed. Indeed, an increasing number of high impact studies present droplet-based technologies not as the core innovation but rather as a basic tool that facilitates novel science. Both facts provide strong indications that the field has reached an advanced level of maturity and is delivering on its early promise.

In the current commentary, we attempt to summarize some of the most successful and exciting applications of droplet microfluidics over the past 2 years. Although we are unable to highlight all such innovations or guarantee the future impact of these technologies, we hope this review will provide an informative account of recent progress, stimulate enthusiasm for the abundant potential of droplets, and inspire the next generations of "dropleteers" to push the boundaries of the technology beyond what we now consider possible.

UNDERSTANDING MICRODROPLETS

Before presenting some of the most interesting recent applications of droplet-based microfluidic systems, it is instructive to review the most important functional roles that droplets fulfill as experimental tools. Indeed, the rapid acceptance and uptake of droplet-based microfluidic systems has been driven in large part by a number of unique features that can be leveraged and exploited by end-users. Understanding these unique features allows the user to make smart and informed choices about when and where microfluidically

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produced droplets provide an advantage. In the current discussion, we focus our attention on three primary roles or functions that droplets play in contemporary chemistry and biology.

Sample Partitioning. As described in detail elsewhere, droplets may be created using a range of microfluidic tools. Such partitioning processes enable the rapid generation of nanoliter- or picoliter-volume droplets, with the sheer number of droplets and high generation rates lending the technique enormous utility in high-throughput experimentation.[25,26] Indeed, an essential feature of modern molecular and biomolecular science is the increasing size of associated data sets, which almost always necessitate the adoption of high-throughput techniques. Key examples in this regard include high-throughput drug screening and high-throughput gene sequencing.[27,28] The term "high-throughput" in both applications refers to the ability to perform large number of parallel experiments, but each raises different concerns. In the former, the user cares about the number of compounds that can be screened per unit time, while in the latter the total number of DNA sequences that can be read in a single experimental is of utmost concern. Nevertheless, there is a strong common need to reduce experimental cost, since the amount of data collected in both situations is astronomical, with any reduction in experimental costs having a significant impact. Accordingly, the smart use of droplets as a route to sample segmentation is a powerful means of both reducing experimental costs and enhancing the analytical efficiency when assaying volume-limited samples.

It is perhaps surprising that present-day high-throughput screening (HTS) platforms continue to leverage microplates (e.g., 96-, 384-, or 1536-well plates). Based on a principle of "arrangement" and "combination", automated robotic platforms systematically perform basic fluidic operations, with high sensitivity detection methods and computer-based data processing completing the analytical process. Candidate compounds with high activity to one or more targets can be screened from a large number of compound libraries. Such extensive and expensive bench-based instrumentation still represents the main route for the discovery of lead compounds in the drug discovery process.[29,30] Fortunately, with the advent of machine learning and artificial intelligence (as well as significant increases in the computing power), the enormous amount of data processing may not remain a bottleneck in the future. Indeed, it is likely that the biggest constraints will return to the efficiency and cost of experiment-based data acquisition. With this in mind, the substitution of microwells with microdroplets is quite simply revolutionary, providing a massive (over 4 orders of magnitude) leap in throughput, with concurrent and dramatic reductions in the cost per experiment and time per experiment. Predictably, the development of droplet-enabled HTS platforms has continued apace.[31−34] That said, it should be noted that there still exists a considerable gap between droplet-based microfluidic systems and microplates in terms of operational flexibility, sample tracking, and the number of accessible screening reagents. Put simply, reduced component footprints and rapid sample transport in droplet-based platforms necessitate more sophisticated control and analysis architecture. In this regard, microfluidic technologies based on pneumatic valves or digital microfluidic principles possess some advantage, but as these are outside the scope of this review we direct interested readers to appropriate commentaries elsewhere.[35−39]

Today, the application and utility of high-throughput DNA sequencing (next generation sequencing) techniques are diverse, with experimental throughput and read cost improving at startling rates.[40] Typically, high-throughput sequencing methods involve reading fluorescence signals generated by millions of parallel short chain DNA synthesis reactions fixed to a substrate surface to obtain long and accurate sequence reads. That said, most approaches cannot effectively determine the source of the measured DNA, a highly problematic limitation in single-cell sequencing. Due to the need to gather and interpret information relating to dynamic biological changes, such as tissue differentiation and gene variation, the demand for methods able to sequence single cells has increased dramatically.[41] Technically, it makes sense to extract and sequence single-cell genes separately, but this is impractical in terms of both cost and time. In this regard, recent advances in droplet-based technologies for the preparation of single-cell sequencing samples have achieved great technical and commercial success.[42] These methods typically involve the insertion of unique barcodes into thousands of single cells contained within droplets. In this way, after sequencing of all cells, DNA reads from an individual cell can be distinguished and registered via bioinformatics. In many ways, this single development is the most important and successful application of droplet-based microfluidics since droplet digital PCR and has been rapidly adopted in developmental, physiological, and disease research over the past 5 years.

Building Blocks and Templates. Besides their important function in partitioning samples, droplets themselves constitute an interesting structure: the microsphere. In this regard, microfluidic tools provide a fast, simple, and effective way of making microspheres with diameters ranging from hundreds of nanometers to hundreds of micrometers. Over the past decade, a large variety of microsphere structures have been assembled using microfluidic systems.[43] Common choices for the dispersed phase include thermosensitive hydrogels and chemically- or photopolymerizable monomers,[44−46] whose low viscosities allow them to be conveyed through microfluidic channels to droplet generators, after which they are physically or chemically "activated" to form cured or semicured microspheres. It must be remembered that droplets often require surfactants[15] (commonly added to the continuous phase) to stabilize them through spontaneous accumulation at the droplet−carrier phase interface. A variety of alternative materials have been shown to self-assemble at the droplet interface, including inorganic nanoparticles, organic polymer monomers, phospholipids, and protein−polymer conjugates.[47−50] Additionally, the intelligent manipulation of laminar flows allows fabrication of more complex structures,[51] such as Janus particles and double emulsions.[52−54] The ability to finely control droplet structure and the nature of the droplet−bulk interface through the use of different surfactants[55] has allowed microspheres obtained via microfluidic routes to find application as both building blocks of larger structures and as templates. We speculate that the approach will continue to have significant utility in 3D cell culture, artificial cell production, and 3D bioprinting as we will highlight subsequently.

Microreactors. The synthesis of molecules and materials in flow defines a paradigm shift when compared to traditional flask-based chemical methods. Although flow-based platforms necessitate the use of pumps, tubing, junctions and control architecture, the general approach opens up a range of

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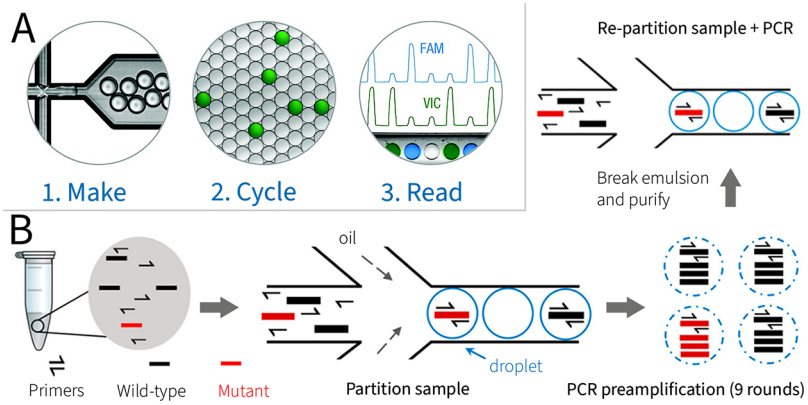

Figure 1. (A) ddPCR workflow. The diluted DNA samples and oil are first loaded into a microfluidic flow-focusing structure to form droplets that either contain a copy of DNA or not. End-point PCR amplification is then performed on these droplets in a conventional thermal cycler. Finally, droplets are loaded into a two-color detector for reading. Reproduced from Hindson, B. J.; Ness, K. D.; Masquelier, D. A.; Belgrader, P.; Heredia, N. J.; Makarewicz, A. J.; Bright, I. J.; Lucero, M. Y.; Hiddessen, A. L.; Legler, T. C.; Kitano, T. K.; Hodel, M. R.; Petersen, J. F.; Wyatt, P. W.; Steenblock, E. R.; Shah, P. H.; Bousse, L. J.; Troup, C. B.; Mellen, J. C.; Wittmann, D. K.; Erndt, N. G.; Cauley, T. H.; Koehler, R. T.; So, A. P.; Dube, S.; Rose, K. A.; Montesclaros, L.; Wang, S.; Stumbo, D. P.; Hodges, S. P.; Romine, S.; Milanovich, F. P.; White, H. E.; Regan, J. F.; Karlin-Neumann, G. A.; Hindson, C. M.; Saxonov, S.; Colston, B. W. *Anal. Chem.* **2011**, *83*, 8604−8610 (ref 67). Copyright 2011 American Chemical Society. (B) Experimental workflow for MED-Amp including nine rounds of PCR preamplification. Reproduced from Pratt, E. D.; Cowan, R. W.; Manning, S. L.; Qiao, E.; Cameron, H.; Schradle, K.; Simeone, D. M.; Zhen, D. B. *Anal. Chem.* **2019**, *91*, 7516−7523 (ref 80). Copyright 2019 American Chemical Society.

previously inaccessible opportunities.[56] The adoption of microfluidic reaction tools confers key advantages, including exquisite control of heat and mass transport, reduced sample/reagent volumes, and facile automation. Segmented-flow formats provide for a range of additional benefits, including extremely efficient mixing and reaction isolation, while overcoming the deleterious effects of Taylor dispersion and reactor fouling that plague single phase reactors. As noted, microfluidically prepared droplets are almost perfectly uniform in size, which enables quantitative analysis and comparison in a droplet-by-droplet fashion.[57] Moreover, the rapid mixing and heating of contents after droplet formation, coupled with the transparency of most microfluidic chips, means that reactions can be initiated and tracked down on the millisecond time regime,[58] defining an extremely powerful tool for studying reaction kinetics. Further, as droplets essentially behave as isolated reaction vessels, rapid variation droplet payloads, reaction temperatures and reaction times enables extensive and high-throughput reaction parameter scanning.[59] More generally, unit operations, such as mixing, fusing, dosing, and splitting, can be integrated to yield platforms able to perform complex reactive processes in an automated, brisk, and efficient manner.

■ ADVANCES AND APPLICATIONS

Rare Mutation Detection and ddPCR. Rare gene (mutation) detection has wide application in fields such as disease diagnosis, animal/plant genetics and microorganism monitoring.[60−63] The heart of such an analysis process is the elucidation of extremely low-frequency variants within a wild-type (WT) sequence pool. Despite the fact that gene sequencing remains the "gold standard" method for detecting rare mutations, its sensitivity is limited, with only mutations having a frequency of more than 20% being detectable.[64] High specificity methods, including quantitative PCR (qPCR) and digital PCR (dPCR), can also be used to detect rare mutations.[65,66] dPCR is of singular interest, since it does not require the use of standard curves (unlike qPCR), allowing absolute quantitation down to a single nucleic acid level, and with a rare gene detection limit as low as 0.001%.[67] In dPCR, a highly diluted DNA sample is distributed, together with a fluorescent reporter, into a large number of unique reaction vessels, with the requirement that each vessel contains either "one" or "zero" target molecules (Figure 1A). Such a single molecule amplification approach eliminates competitive reactions from the WT background, and after amplification, the number of mutant molecules can be simply extracted by counting the number of positive (fluorescent) vessels.

Droplet digital PCR (ddPCR) was the first commercially significant application of droplet-based microfluidics, and was adopted rapidly after its introduction.[67,68] It is robust and easy to operate and has a significantly higher throughput than microarray-based dPCR platforms. Put simply, ddPCR is now a mature and popular technology embedded in molecular biology laboratories, with current R&D efforts primarily focused on the establishment of higher throughput instruments and the certification of standardized probe kits, particularly in the field of liquid biopsies (e.g., circulating tumor DNA (ctDNA) detection[69] and noninvasive prenatal testing (NIPT)[70]). Indeed, in 2019, the U.S. Food and Drug Administration cleared the first ddPCR test for the accurate monitoring of treatment response in chronic myeloid leukemia.[71] In the meantime, academic and clinical researchers in diagnostics are continuing to expand the scope of application, enhancing sensitivity, and adapting protocols for further disease targets.[72−77]

Molecular detection of circulating tumor DNA is increasingly used as a tool for cancer surveillance and early detection in oncology.[78,79] However, for patients with low tumor burdens, ctDNA abundance is extremely low and masked by large amounts of wtDNA. In such situations, direct detection via ddPCR can be difficult, and thus the integration of preamplification strategies is desirable. In this regard, Pratt and co-workers developed the Multiplex Enrichment using Droplet Pre-Amplification (MED-Amp) method for the enrichment of ctDNA templates (Figure 1B).[80] In initial tests, the authors

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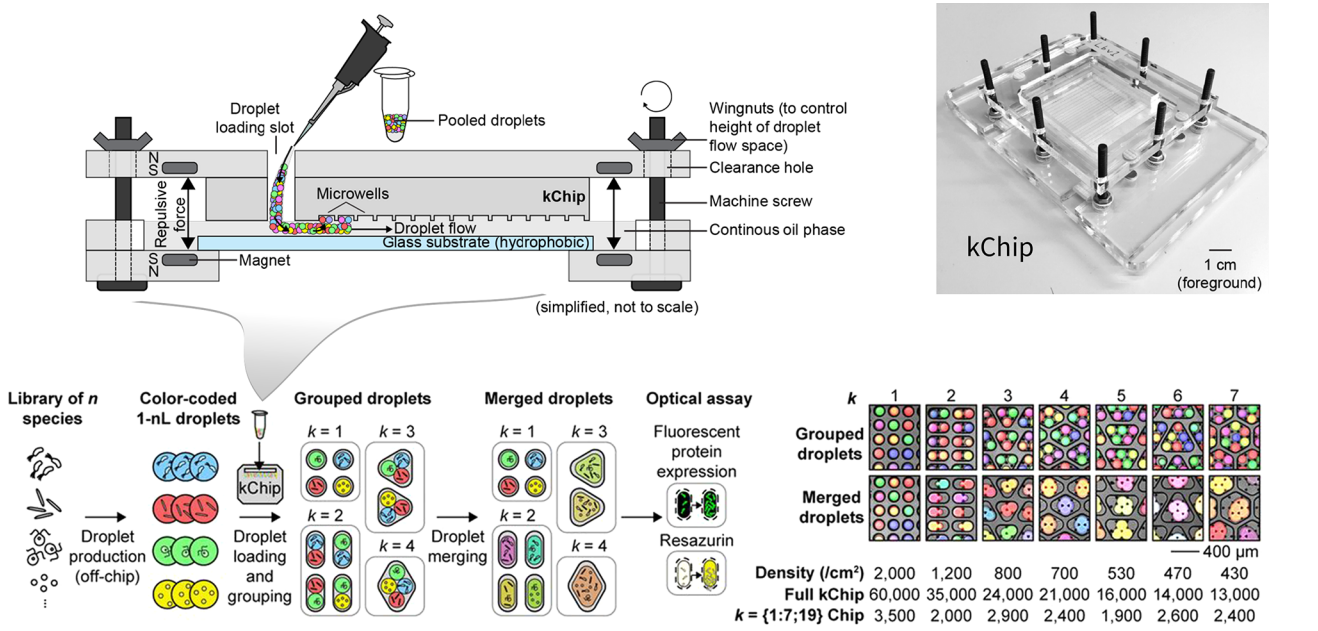

Figure 2. Massively parallel construction of microbial communities using the *kChip*. Each produced droplet contains a bespoke color code. Droplets are pooled and loaded onto the *kChip*, where they randomly group into microwells (grouped droplets) and then merge to form combinations (merged droplets). Reproduced with permission from *Proceedings of the National Academy of Sciences U.S.A.* Kehe, J.; Kulesa, A.; Ortiz, A.; Ackerman, C. M.; Thakku, S. G.; Sellers, D.; Kuehn, S.; Gore, J.; Friedman, J.; Blainey, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **2019**, *116*, 12804−12809 (ref 94).

performed nine rounds of high-fidelity polymerase preamplification in picoliter-volume droplets, with subsequent ddPCR detection of templates. Through such a strategy, mutation signals could be increased by over 50-fold, with successful detection of KRAS mutant ctDNA in plasma samples from metastatic pancreatic ductal adenocarcinoma patients; a significant improvement on previously reported data.[81,82]

In regard to noninvasive prenatal testing, the analysis of maternal peripheral blood can reduce the risk of miscarriage associated with invasive methods such as amniocentesis and chorionic villus sampling.[83] NIPT is recommended as the primary screening test and most sensitive screening option for fetal aneuploidy (i.e., trisomy 21, 18, and 13 and monosomy X).[84,85] Despite the fact that standard NIPT for the detection of autosomal recessive (AR) diseases is insufficient, recent studies have successfully extended NIPT to this class of genetic diseases through the help of ddPCR. For example, Hudecova and co-workers examined the possibility of combining ddPCR and (targeted) massively parallel sequencing to predict fetal inheritance of hemophilia.[86] Additionally, Chang et al. proposed an improved NIPT protocol and statistical model for AR mutation analysis.[87] Using this approach, fetal genotypes from all tested families with congenital sensorineural hearing loss were successfully predicted. NIPT has proven its prediction capabilities, and we predict that it will soon be extended to cover more detectable fetal abnormalities, with families able to access cheaper and more informative NIPT solutions with the help of ddPCR.

Combinatorial Screening and Droplet Arrays. The adoption of droplets, instead of microwell plates, for drug screening experiments provides immediate and drastic reductions in reagent consumption, while dramatically increasing analytical throughput. However, these features do not guarantee that droplet-based technologies will seamlessly replace conventional approaches in high-throughput drug screening. Specifically, droplet-based approaches must solve or circumvent recognized difficulties associated with operational flexibility, sample registration and multiplexed reagent delivery, while delivering dramatic savings in both time and reagent consumption. In sequential screening schemes (where droplets are probed in a serial fashion), droplet-based microfluidic systems have proved their utility.[88] For example, droplet-based systems have recently been used to perform ultrahigh-throughput functional profiling of microbiota communities, providing a new and efficient tool for selecting antibiotics and probiotics and discovering mechanisms behind self-resistance.[89] A key element of high-throughput screening is the ability to accurately combine variable types and concentrations of test molecules in large numbers and to track them over extended time periods. Moreover, the synergistic effects of combination drugs have become increasingly important, resulting in a significant increase in both screening-space and the demands of the experimental workflow.[90] This demands advanced control over droplet dosing; however, accurate sequential addition of samples or reagents into droplets at a high volumetric flow rate remains a challenging task. To tackle this, droplet array technologies, have begun to provide a route toward high-throughput drug screening.[91−93] Such approaches allow for the precise positioning, manipulation, dosing, and storage of droplets (of variable payload) without external control, but existing systems can be relatively complex and costly to operate. However, recently higher throughput and simpler droplet microarray technologies have begun to emerge. The *kChip* droplet array platform, developed by Kehe and co-workers, uses color indexing and combinatorial droplet fusion to construct and quantitatively screen more than 10^5 synthetic microbial communities per day, without the need for liquid handling robots (Figure 2).[94] In subsequent work, the same authors applied a similar droplet array platform for drug combination screening.[95] Such progress

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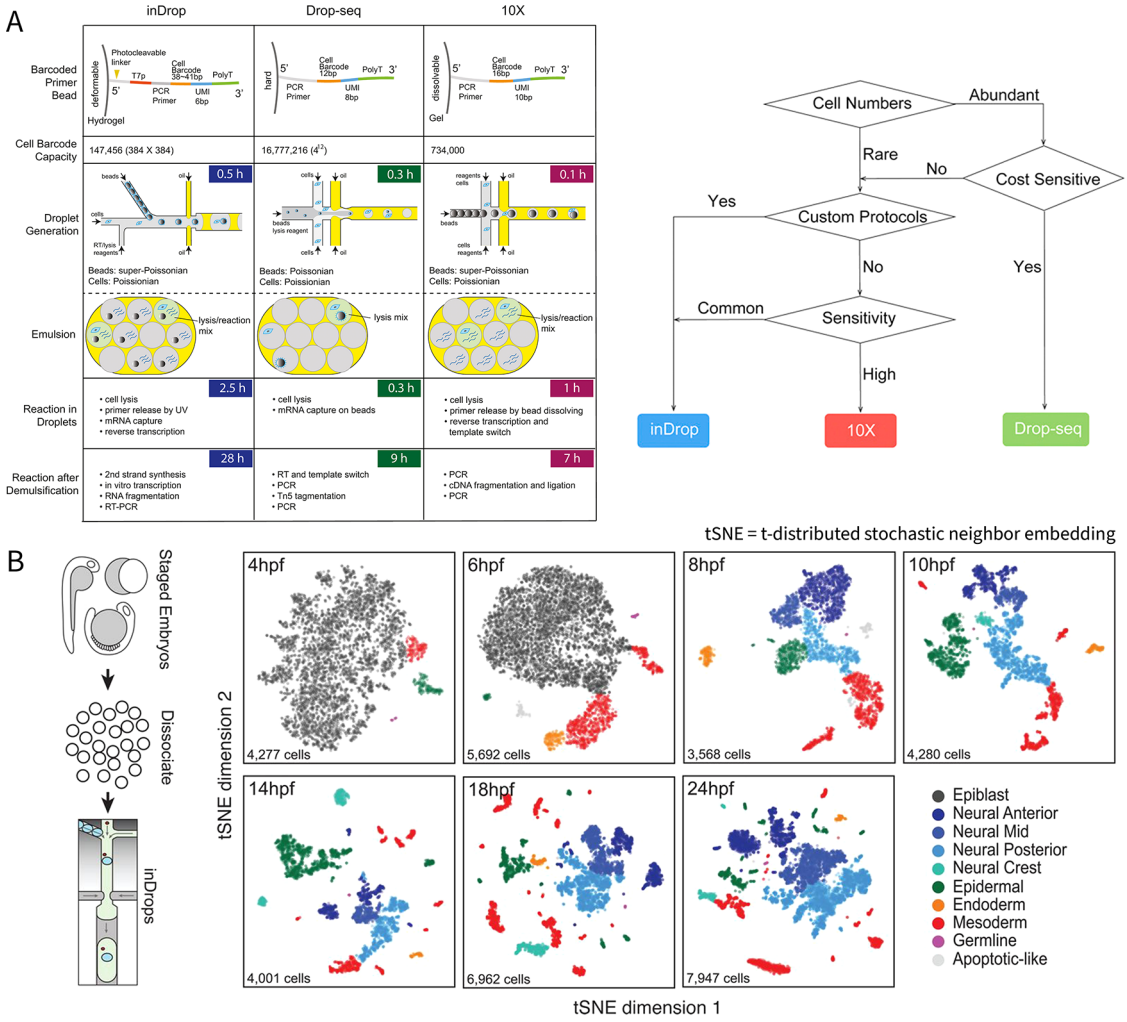

Figure 3. (A) Fluidic schematic and comparison of the experimental features associated with three scRNA-Seq systems and a flowchart summarizing the decision making process for each method. Reprinted from Zhang, X.; Li, T.; Liu, F.; Chen, Y.; Yao, J.; Li, Z.; Huang, Y.; Wang, J. *Mol. Cell* **2019**, *73*, 130−142.e5 (ref 42). Copyright 2019, with permission from Elsevier. (B) The generation of high-resolution gene expression maps of embryonic development using droplet-based scRNA-Seq. Tens of thousands of cells from zebrafish embryos during the first day of development were sequenced and mapped. Cells are colored by germ layer identities inferred from expressed marker genes. This work (ref 119), along with two other similar studies (ref 118 and ref 120), was highlighted on the cover of *Science* magazine (Vol. 360, Issue 6392) on the issue of mapping the vertebrate developmental landscape. Reprinted from Wagner, D. E.; Weinreb, C.; Collins, Z. M.; Briggs, J. A.; Megason, S. G.; Klein, A. M. *Science* **2018**, *360*, 981−987 (ref 119). Copyright 2018, with permission from American Association for the Advancement of Science.

is most encouraging, as it aims to fill the technological gap between droplet-based microfluidic systems and microplate-based approaches.

Massive Single-Cell RNA Sequencing. For decades, cell-biology studies have focused on the analysis of large populations of cells.[96,97] Such experiments provide a measurement of ensemble averages and yield information only on mean properties. In contrast, single cell measurements permit observation of large numbers of individual cells and examination of the interaction and behavior of heterogeneous populations in real time. Until recently, platforms for single cell analysis have been far less commonplace, partly due to the technological limitations associated with massively parallel experimentation, but more broadly due to the fact that the significance of individual cell analysis was not entirely apparent. Indeed, it was previously believed that individual cells within a "homogeneous" population were more or less identical and that cell-to-cell differences were of insignificance when compared to ensemble properties.[98] In recent years, rapid advances in fluorescence-activated cell sorting (FACS) and microfluidic and high-throughput sequencing technologies have enabled the rapid and facile separation and analysis of large numbers of individual cell populations. It is now well-recognized that populations of apparently identical cells are almost always composed of multiple subpopulations.[99] Accordingly, the fundamental significance of single cell experimentation lies in the assessment of cell heterogeneity, with batch cell studies yielding only averaged information that masks subtle differences and dynamic changes between cells. However, the onset of many diseases often starts with the production of a small number of variant cells. In this regard, single-cell RNA sequencing (scRNA-Seq) has recently emerged as one of the most powerful tools to reveal such cellular heterogeneity. Through high-resolution profiling of the cell transcriptome, cell type and gene expression status can be accurately portrayed.[100] Five years ago, the establishment of methods for preparing single-cell sequencing samples using droplet-based microfluidic tools catalyzed the large-scale

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application of single-cell RNA sequencing.[101−103] The integration of established microfluidic tools within the sequencing workflow provides substantial benefits with the respect to the capture of large numbers of single cells, the labeling and extraction of target RNA/DNA, and the amplification of low copy number targets. These features enable downstream sequencing to gather large amounts of data from many single cells in a parallel fashion. This is a transformative and game-changing improvement, increasing the number of cells sequenced at a time from a few hundred to tens of thousands or more.[104] This is significant, as a recent study indicated that the analysis of just 50 000 randomly selected cells allows the recapitulation of a great majority of biologically interpretable cell types from a 1.3 million cell database.[105] Droplet-based scRNA-Seq is unequivocally the most successful application of droplet microfluidics so far, both academically and commercially. Such a large-scale, unbiased, and highly sensitive method for revealing single cell gene expression patterns provides a diversity of new opportunities in characterizing the state of disease cells, identifying new immune biomarkers and revealing cell developmental trajectories and cell fates.[106−108]

At the current time, there are three core technical platforms for droplet-based high-throughput scRNA-Seq, i.e., the 10X Genomics, Drop-Seq, and InDrop instruments. Recently, Zhang et al. systematically compared the three platforms in terms of cell abundance, flexibility, sensitivity, accuracy, and cost (Figure 3A).[42] Additionally, Wang and co-workers have made a direct and quantitative comparison between the 10X Genomics platform and the plate-based Smart-Seq2 technology, which provides a valuable reference for researchers when selecting the appropriate platform for a given application.[109] Briefly, although gene coverage is less than the full-length sequencing of the Smart-Seq2 instrument, droplet-based RNA 3′ sequencing can assay more cells and thus has a particular advantage when detecting rare cell types and performing extensive cell classification and mapping. Indeed, in recent years researchers have been successful in classifying and characterizing cells from a specific organ, cancer, or immune system on a large scale, with much important science being uncovered.[110−117] Even more excitingly, scientists have for the first time mapped the process of cell differentiation during embryonic development, uncovering gene expression dynamics, and building the most complete embryogenic tree to date (Figure 3B).[118−120]

There is little doubt that large-scale RNA sequencing based on droplet processing of single cells has been a great success. Nevertheless, there is still scope for further improvement through process integration. For example, Habib and co-workers recently combined single-nucleus RNA sequencing with Drop-Seq, engendering large-scale single-cell RNA sequencing of tissue samples containing cells that are notoriously difficult to dissociate.[121] Additionally, the use of inertial-ordering technologies within the Drop-Seq platform has been shown to be useful in enhancing the probability that cells can be paired with appropriate barcodes.[122] Nan et al. have developed a droplet sorting system that physically removes empty or misloaded droplets, thereby minimizing noise and erroneous data interferences in final sequencing results.[123] In related work, Lun and co-workers developed a new statistical method to exclude sequencing noise data caused by empty droplets.[124] Furthermore, Wolock and colleagues have developed algorithms to remove mixed transcription data

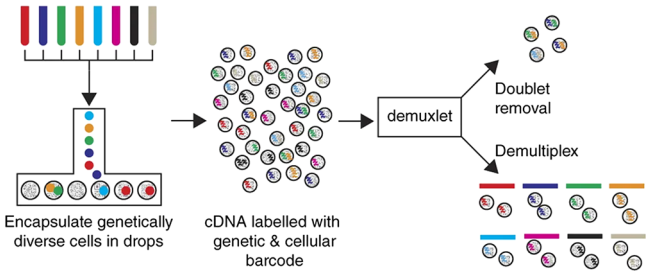

Figure 4. Demultiplexing cells from different species (genetically distinct) via the information on natural genetic variation. Demuxlet is a computational tool developed in this work. It harnesses natural genetic variation to determine the sample identity of each singlet (droplets containing a single cell) and detect doublets (droplets containing two cells). With the demuxlet enabled workflow, cells from unrelated individuals can be pooled and sequenced simultaneously. Reprinted from Kang, H. M.; Subramaniam, M.; Targ, S.; Nguyen, M.; Maliskova, L.; McCarthy, E.; Wan, E.; Wong, S.; Byrnes, L.; Lanata, C. M.; Gate, R. E.; Mostafavi, S.; Marson, A.; Zaitlen, N.; Criswell, L. A.; Ye, C. J. *Nat. Biotechnol.* **2018**, *36*, 89−94 (ref 126). Copyright 2017, with permission from Springer Nature.

from two or more cells receiving the same barcode.[125] Conversely, Kang et al. (Figure 4) and Shin et al. used genetic variation characteristics from different individuals or the addition of transient barcodes to achieve sequencing of multiple individuals in a single run. Such multiplexed methods are able to fully utilize the reading capacity of current high-throughput sequencing instruments, condense the number of sample preparations, and further reduce the cost of single-cell sequencing.[126,127]

3D Cell Culture and Cell-Laden Hydrogel Droplets. Cell culture models are commonly used to screen drug candidates.[128] However, there is significant doubt whether a 2D cell population accurately reflects the behavior of the 3D ensemble that would be found in a natural setting. Thus, there is strong interest in using 3D cell culture methods, which in contrast to traditional monolayer 2D methods, can simulate more realistic growth morphologies and cell environments (such as tissue-specific structures, intercellular connections and concentration gradients).[129] In contrast to animal models, 3D cell models are not only cheaper to establish but are also easier to control and standardize. Accordingly, 3D cell cultures are of great significance and hold wide application potential in disease modeling, drug screening, target identification and verification, toxicity testing, and safety evaluation. Current 3D cell culture methods are typically based on the use of spheroids and scaffolds.[130] Spheroids are spherical cellular aggregates and one of the most common and versatile way to culture cells in 3D. The spheroid-based strategy utilizes contained micro-spatial structures (e.g., hanging drops, or U- and V-shaped microwells with reduced surface adhesion) to allow seeded cells to grow and assemble into spheroid-like clusters within a confined environment.[131] Scaffolds utilize materials with appropriate porosity, permeability, mechanical properties, and surface chemistry to artificially generate a 3D network and simulate specific tissue microenvironments, so that cells can adhere and grow to form 3D structures, such as natural hydrogels or synthetic gels and fibers.[132]

In recent years, 3D culture systems (cell-laden microgels) that encapsulate individual or small numbers of cells in hydrogel droplets have attracted much attention.[133,134] Such an approach, which minimizes the scale of 3D cell culture, offers a

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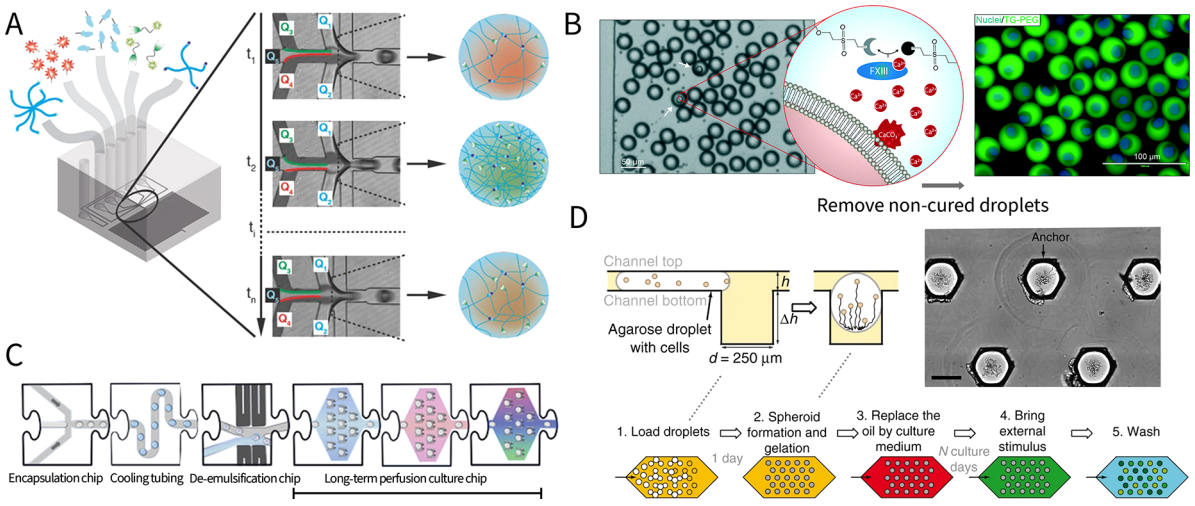

Figure 5. (A) Microfluidic programming of a compositional microgel profile. The fine-tuning of flow rates of the different inlets permits the generation of a continuous landscape of microgels with diverse physical and biochemical properties. Reproduced from Allazetta, S.; Negro, A.; Lutolf, M. P. *Macromol. Rapid Commun.* **2017**, *38*, 1700255 (ref 141). Copyright 2017, with permission from WILEY. (B) In the event that a cell is present in the droplet, HCl is used to dissolve $CaCO_3$ nanoparticles, leading to Ca^{2+}-induced activation of FXIII (fibrin stabilizing factor) and thus cell-laden microgel formation. Noncured droplets can be easily removed. Adapted from Lienemann, P. S.; Rossow, T.; Mao, A. S.; Vallmajo-Martin, Q.; Ehrbar, M.; Mooney, D. J. *Lab Chip* **2017**, *17*, 727−737 (ref 144), with permission of The Royal Society of Chemistry. (C) Schematic of a long-term perfusion culture platform involving cell-laden hydrogel beads. The steps include the encapsulation of single cells into hydrogel droplets, cooling and hydrogel formation, on-chip de-emulsification (to remove the oil phase and replace it with the aqueous phase), spatial immobilization of hydrogel beads, and long-term perfusion culture. Reproduced from Kleine-Brüggeney, H.; van Vliet, L. D.; Mulas, C.; Gielen, F.; Agley, C. C.; Silva, J. C. R.; Smith, A.; Chalut, K.; Hollfelder, F. *Small* **2019**, *15*, e1804576 (ref 153). Copyright 2018, with permission from WILEY. (D) An integrated long-term perfusion culture platform, where all component processes are performed within a single microfluidic device. Reprinted from Sart, S.; Tomasi, R. F.-X.; Amselem, G.; Baroud, C. N. *Nat. Commun.* **2017**, *8*, 469 (ref 154), under a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/).

number of advantages. First, the reduction in size increases the surface area-to-volume ratios, which in turn facilitates the diffusion and exchange of small molecules, and increases sensitivity in cell pharmacokinetic studies. Second, reduced compartment volumes and the control of unit size and cell numbers, are favorable when constructing consistent cell culture models on a large scale and are particularly suitable for HTS applications. Finally, cell-laden microgels are not only adept at evenly distributing cells but also at enhancing cell density. These features are critical in tissue engineering applications, where numerous studies have shown that there is a minimum initial cell seeding density needed to trigger cell growth and differentiation (typically in excess of 1 million cells per cm^3).[135−138] To meet such requirements, the microgel dimensions should be kept below 50 μm.[139] Typical cell-loaded microgel dimensions may be controlled between a few tens to a few hundred micrometers, a range that neatly falls within the operating range of microfluidic droplets and devices. As a result, microfluidic technologies are the most popular when making microgels loaded with single cells. Alternate methods, such as those based on (air-induced and electro-) sprays, although efficient with respect to throughput, yield unacceptably high microgel dimensions and dispersities.[140]

There have been some recent notable technological improvements for making cell-laden microgels using droplet-based microfluidic systems. For example, the automated multicomponent synthesis platform developed by Allazetta and co-workers enables the rapid production of microgel combinations of variable mechanical strength and biological activity (Figure 5A). In initial studies, the authors used fluorescent labels as proxies for the physical and chemical properties of the microgel, allowing the discovery of specific formulations through flow cytometry.[141] Kamperman et al. used a strategy of delayed cross-linking, curing droplets only after encapsulated cells have migrated toward the center of the microgel, to prevent cells from escaping and allowing them to be cultured for longer periods of time.[142] Further, Mao et al.[143] and Lienemann et al.[144] developed related strategies that cleverly sidestep the limits set by Poissonian loading (where only every 10−20 droplets typically contains a single cell, the rest are empty). By attaching a cross-linking precursor to the cells, the authors ensured that only cell-containing droplets can be cured to form microgels, allowing easy removal of noncured droplets (Figure 5B). Such an approach greatly increases the number of single-cell-laden microgels that can be pooled and allows sorting steps to be eliminated. This technique has since been applied to stem cell therapy studies, where the retention time of the stem cells *in vivo* was shown to increase by more than an order of magnitude after intravenous injection, due to the protection afforded by gel layers.[145] Other notable advances in this regard include air-assisted microgel generation,[146,147] parallel encapsulation,[148] protein-driven generation,[149] multicompartment creation,[150] the use of non-fluorinated oils,[151] and robotically automated platforms.[152]

An important advantage of microgel-based 3D cell cultures is the facility of on-chip culturing and monitoring under conventional microscopes. To this end, several integrated droplet-based 3D cell culture platforms have recently been reported. For example, Kleine-Brueggeney and co-workers presented a long-term perfusion culture platform for cell-laden hydrogel beads, integrating cell encapsulation, microgel formation, demulsification, microgel capture, and perfusion culture. The authors designed a butterfly shaped capture array that can hold 2000 microgels for perfusion culture and optical

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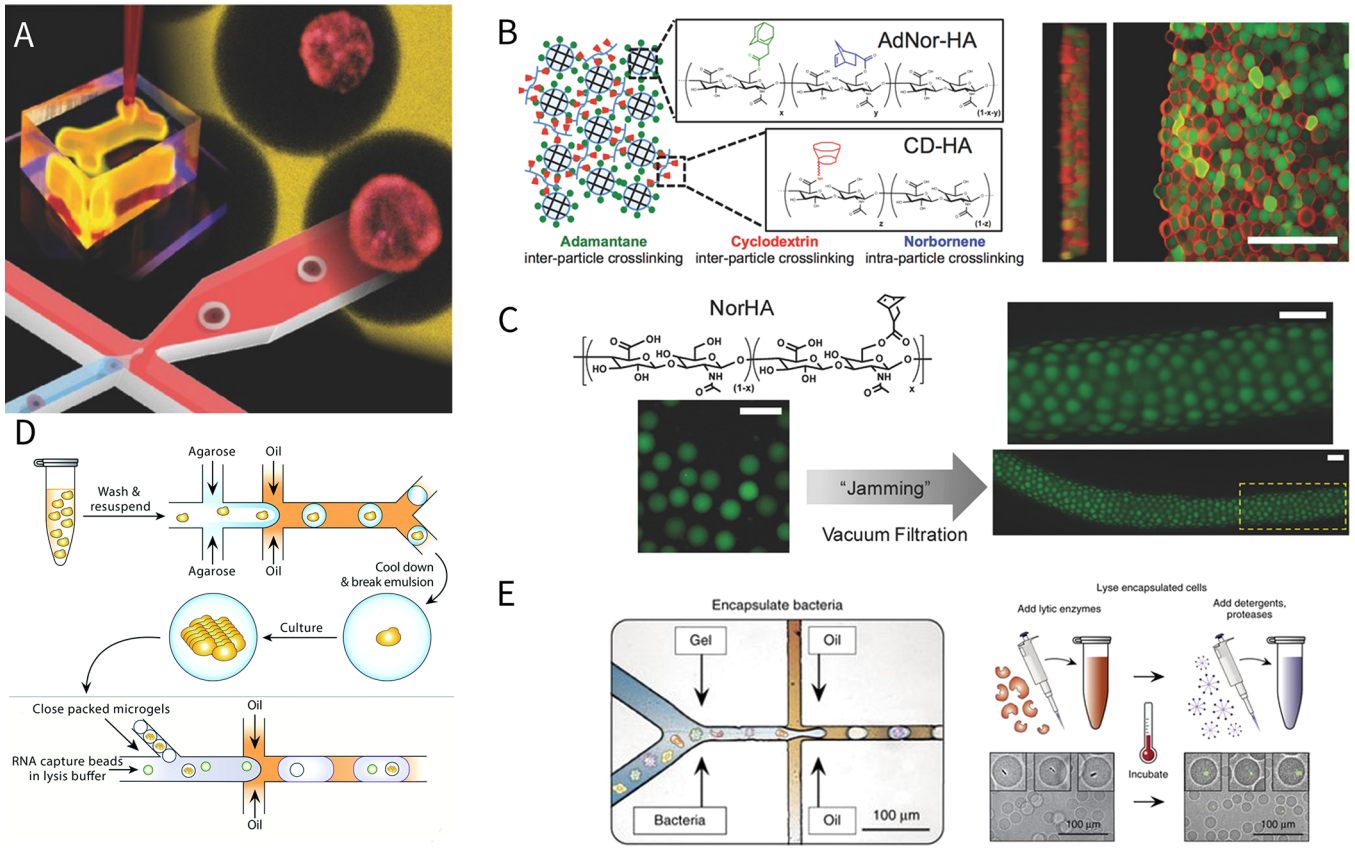

Figure 6. (A) Modular bioinks based on single cell microgels within distinct injectable prepolymers. Reproduced from Kamperman, T.; Henke, S.; van den Berg, A.; Shin, S. R.; Tamayol, A.; Khademhosseini, A.; Karperien, M.; Leijten, J. *Adv. Healthcare Mater.* **2017**, *6*, 1600913 (ref 139). Copyright 2016, with permission from WILEY. (B) Bioink composed of granular hydrogels, where the close packing of hydrogels is accomplished by a chemical guest−host reaction. The formation is accomplished via the mixing of cross-linked adamantane (green) and norbornene (blue) modified hyaluronic acid (AdNor-HA) microgels and cyclodextrin (red) modified HA (CD-HA). Reproduced from Mealy, J. E.; Chung, J. J.; Jeong, H.-H.; Issadore, D.; Lee, D.; Atluri, P.; Burdick, J. A. *Adv. Mater.* **2018**, *30*, 1705912 (ref 157). Copyright 2016, with permission from WILEY. (C) Bioink composed of granular hydrogels, where the close packing of hydrogels is accomplished by a physical compaction. The suspended microgels fabricated from NorHA that are jammed through vacuum filtration into a solid that can be extruded from a syringe. Reproduced from Highley, C. B.; Song, K. H.; Daly, A. C.; Burdick, J. A. *Adv. Sci.* **2019**, *6*, 1801076 (ref 158), under a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/). (D) Colony-containing agarose microgels can be generated by encapsulating single yeast cells into agarose microdroplets, followed by microgel recovery and colony formation within the microgel. Reproduced from Liu, L.; Dalal, C. K.; Heineike, B. M.; Abate, A. R. *Lab Chip* **2019**, *19*, 1838−1849 (ref 169), with permission of The Royal Society of Chemistry. (E) Agarose microdroplets allow for single-cell genome purification through a series of enzymatic and detergent lysis steps simply in bulk. Reproduced from Lan, F.; Demaree, B.; Ahmed, N.; Abate, A. R. *Nat. Biotechnol.* **2017**, *35*, 640−646 (ref 168). Copyright 2017, with permission from Springer Nature.

observation (Figure 5C).[153] In contrast, Sart et al. developed an integrated microfluidic platform, where all the steps can be performed on a single device (Figure 5D).[154] Here, the authors used rails to guide cell-encapsulating droplets directly into capture wells, cooling and solidifying the droplets after 3D spheroid formation. Due to hydrogel swelling, cells could be firmly locked within wells, and laminar flows were then used to perfuse drug molecules to user-defined regions. It is noteworthy that the authors demonstrated that gels can be melted by local light-induced heating, allowing the release of cells for downstream processing. Subsequently, the same group proposed a new trap design that allows different drugs to be applied to a single cell cluster.[155]

Further Uses of Gel Droplets. Microfluidically produced gel droplets are of significant general interest, since gel-based materials impart a porous structure and mechanical strength to droplets. Accordingly, and in addition to their use in 3D cell culture, gel droplet microfluidics has also found additional purpose. For example, droplet-based microfluidic tools have been used for 3D bioprinting in tissue engineering and regenerative medicine studies, with applications including the fabrication of tissue models for drug testing, tissue scaffolds and local drug implants.[156] For example, Kamperman and co-workers produced a bioink based on single-cell laden microgels (Figure 6A), where bovine chondrocytes or human bone marrow mesenchymal stem cells were initially packaged within microfluidic droplets to form gel particles, with flow cytometry then being used to enrich gel particles containing cells. Next, obtained microgels were mixed with a variety of additives to form modular bioinks. The authors emphasize that the diameter of such microgels should be below 50 μm to ensure a high cell density for the bioink, which is important in controlling the biological activity of the tissue.[139] To form an extrudable bioink, the disperse microdroplets formed by a microfluidic approach must be subsequently bound together. To this end, Mealy and co-workers recently reported the use of guest−host interparticle cross-linking to form bioinks with excellent strength and fluidity (Figure 6B).[157] The authors

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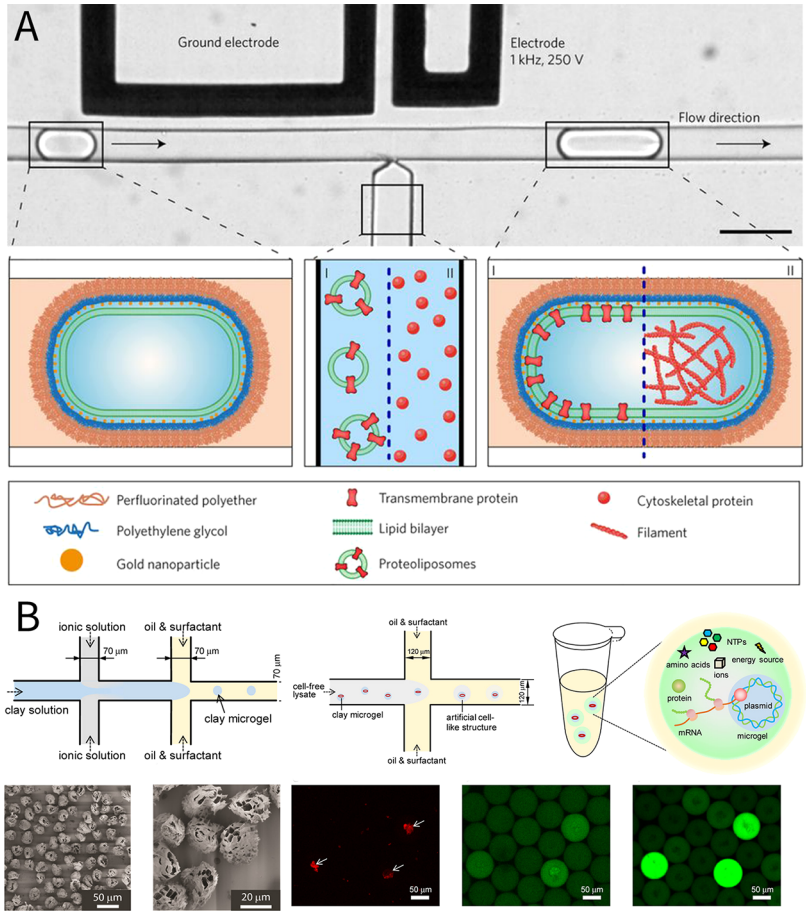

Figure 7. (A) Process for incorporating transmembrane and cytoskeletal proteins into dsGUVs (droplet-stabilized giant unilamellar vesicles) by pico-injection technology. Reproduced from Weiss, M.; Frohnmayer, J. P.; Benk, L. T.; Haller, B.; Janiesch, J.-W.; Heitkamp, T.; Börsch, M.; Lira, R. B.; Dimova, R.; Lipowsky, R.; Bodenschatz, E.; Baret, J.-C.; Vidakovic-Koch, T.; Sundmacher, K.; Platzman, I.; Spatz, J. P. *Nat. Mater.* **2018**, *17*, 89−96 (ref 175). Copyright 2017, with permission from Springer Nature. (B) The microfluidic formation of clay hydrogel and clay microgel-based cell-like structures. Reproduced from Jiao, Y.; Liu, Y.; Luo, D.; Huck, W. T. S.; Yang, D. *ACS Appl. Mater. Interfaces* **2018**, *10*, 29308−29313 (ref 176). Copyright 2018, American Chemical Society.

subsequently tested another method, particle jamming, instead of cross-linking additives, again yielding a bioink with good fluidity and stability (Figure 6C).[158] Furthermore, Yu and co-workers used droplet-based microfluidic tools to make a new type of bioink composed of core−shell microspheres. In its initial state, such an ink has high fluidity and can be easily injected into a mold. Microspheres can then be rapidly gelated using a thermal trigger to form scaffolds with self-healing capabilities.[159] A second notable application of microfluidically produced gel droplets is as carriers for macromolecules. Here, the porous interior of gel beads can be chemically modified to increase a specific adsorption effect, allowing such beads to act as carriers of molecular cargoes. For example, high-throughput scRNA-Seq approaches have been assisted by adding molecular tags to gel beads.[101] Furthermore, high-throughput immunoassays can be performed using single cells by adding immune markers to gel beads.[160] The soft and elastic nature of gel beads is advantageous during their loading into droplets, as close-packed and regular ordering can be achieved in microfluidic channels prior to encapsulation.[161] For example, Kanai et al. recently proposed an interesting method for the preparation of monodisperse hybrid gel particles (with different morphologies) by modulating the microfluidic flow rate and temperature of a thermosensitive gel.[162] Finally, there has been recent interest in using droplet-based microfluidic systems to form agarose droplets.

Agarose is a thermosensitive gel with readily accessible melting and gelling points. Therefore, its droplets can be transformed repeatedly between a liquid and gel state through temperature modulation, allowing a range of liquid droplet and gel bead-based operations to be performed. For example, PCR can be performed in liquid-phase agarose droplets at elevated temperatures, then gelled by cooling to trap amplicons inside the bead for easy washing and collection.[163−165] Furthermore, this property allows encapsulation and culturing of cells in agarose droplets, followed by solidification and sorting via FACS.[166,167] Recently, Lan et al. demonstrated a smart application of agarose microdroplets, using them for complex material exchange during high-throughput single-cell genome sequencing (Figure 6E). This is challenging task when using "conventional" droplets since genomic DNA must be isolated from cellular material and processed through a series of enzymatic steps prior to sequencing.[168] Additionally, Liu and co-workers have used agarose droplets to culture yeast colonies from single cells, which were then subjected to gene expression profiling (Figure 6D).[169] We expect that the smart use of unusual materials, such as agarose, will more broadly expand the horizons of droplet-based microfluidics.

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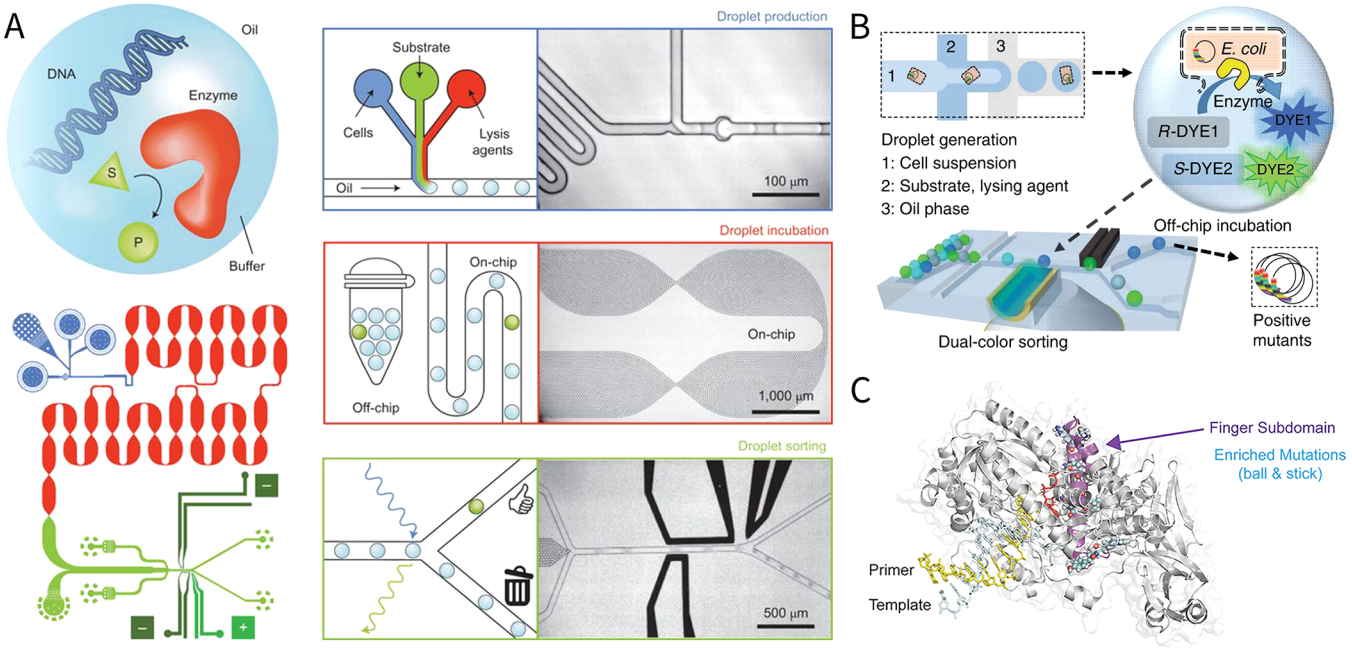

Figure 8. (A) Microfluidic platform for the directed evolution of aldolases. The process is divided into three steps (droplet formation, incubation, and sorting) which can be performed either on separated chips or an integrated chip. Reproduced from Obexer, R.; Godina, A.; Garrabou, X.; Mittl, P. R. E.; Baker, D.; Griffiths, A. D.; Hilvert, D. *Nat. Chem.* **2017**, *9*, 50−56 (ref 189). Copyright 2016, with permission from Springer Nature. (B) Schematic of DMDS (dual-channel microfluidic droplet screening) operation. Mutant enzyme-expressing single cells are encapsulated in water-in-oil droplets with two fluorogenic substrates and lysis buffer. After the droplets are incubated for a specified time, those droplets containing the desired mutants are enriched via fluorescence-activated droplet sorting. Reproduced from Ma, F.; Chung, M. T.; Yao, Y.; Nidetz, R.; Lee, L. M.; Liu, A. P.; Feng, Y.; Kurabayashi, K.; Yang, G.-Y. *Nat. Commun.* **2018**, *9*, 1030 (ref 191), under a Creative Commons Attribution 4.0 International License (https://creativecommons.org/licenses/by/4.0/). (C) Enrichment and mutational tolerance data identified 13 mutations that occur at 8 amino acid positions in the finger subdomain (magenta) of Kod DNA polymerase. Reproduced from Nikoomanzar, A.; Vallejo, D.; Chaput, J. C. *ACS Synth. Biol.* **2019**, *8*, 1421−1429 (ref 194). Copyright 2019, American Chemical Society.

Artificial Cells from the Bottom Up. Interest in the construction of artificial- or proto-cells continues to flourish and has been driven by research into the origin of life. Although cells are the building blocks of life, with modern biology clarifying many of their structural and biochemical pathways, their complexity is to a large extent beyond the understanding of contemporary science. Indeed, our observation of cells and the basic activities of life bring to mind the parable of the blind man and an elephant; although we have made many observations of specific aspects of cells, we are yet to fully understand the nature of the whole.[170] To aid in unravelling this complexity, researchers aim to construct *de novo* building blocks that mimic the basic structure and function of natural cells and recreate the characteristics of life *in vitro*. Such a "container first" strategy follows Gánti's abstract model of minimum life.[171] In his "Chemoton" model, Gánti argues that the simplest life form requires three subsystems; a chemical motor enabling the self-production of substances, a chemical boundary for spatial isolation of the self, and a chemical information system for self-replication.[172] Accordingly, a common approach for building artificial cells begins with the construction of an isolated container and is followed by the addition of capabilities for material production and information replication. In this regard, droplet-based microfluidic tools have significant potential utility in the construction of cell-sized containers and the convenient addition of functional substances, with four major functional operations being realized, namely, compartment formation, compartment and content manipulation, content analysis, and adaptation.[173]

Although we are still far from the ultimate goal of building a functional and "living" artificial cell, a number of meaningful advances have been reported over the past 2 years. Beyond the formation of spherical cell constructs, rod- and disklike containers (more in line with the natural shape of many cells) have been reported.[174] In addition, transmembrane proteins and cytoskeletal proteins have been added via picoinjection techniques into self-stabilizing artificial monolayer vesicles (Figure 7A).[175] Further, novel clay microgels have been used to immobilize plasmids, with a view to assessing cell-free protein synthesis (Figure 7B).[176] Using double emulsion methods, coacervate organelles can be formed within liposomes, where DNA transcription is performed.[177] We have also seen proteinosome-based artificial cells,[178] as well as hybrids that combine living and artificial cell constructs.[179] Such advances all suggest exciting applications of droplet-based microfluidics in artificial life research.

Biocatalyst Discovery, Characterization, and Evolution. The repurposing of natural biocatalysts spans human history, stretching from primitive developments in fermentation and brewing[180] to our present use of enzymes and organisms in molecular biology, food processing, and biofuel production.[181] Furthermore, the successful discovery and development of new and improved biocatalysts is seen as a vital step toward to a sustainable postpetroleum bioeconomy.[182] To realize meaningful impact, advanced abilities in unearthing new cells and enzymes, developing and refining known biocatalysts (for example, through directed evolution), and developing the reaction pathways and reactors are required.[183,184] Although ingenuity and creativity is of

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paramount importance, highly automated and high-throughput methods must be applied to the laborious workflows associated with the screening, characterization, and engineering of enzymes. Taking enzyme variant screening as a representative example, conventional approaches (based on agar or microwell plates) can sample approximately 10^4 variants over a period of several weeks. Robotic automation has increased this to nearly 10^6 variants over several days, but application of droplet-based microfluidic tools has had a far more dramatic effect, with over 10^8 variants being screened per day and a concurrent 10^6-fold reduction in sample/reagent volumes.[185,186] In this regard, it follows that the rapidity of droplet generation necessitates a sufficiently rapid means of droplet detection and analysis. This is most often achieved by combining fluorometric enzyme assays in droplets with fluorescence-activated droplet sorting (FADS).[32,187] FADS allows detection and capture of rare events with a throughput in the range of 1−2 kHz and has already established itself as a powerful technique in high-throughput studies of enzymes.

Directed evolution is a protein engineering method that enables the generation of bespoke enzymes by screening large libraries of mutants for members that exhibit desirable properties. Such libraries are normally created by random mutagenesis (e.g., error-prone PCR, DNA-shuffling, and saturation mutagenesis[188]) of a starting sequence. This starting sequence is typically derived by rational enzyme design, where knowledge of the enzyme structure has guided researchers to focus on varying amino acids in and around active sites. Screening is then performed through multiple iterations, where genes linked to improved enzyme properties are identified and used as the basis for a subsequent round of screening. This approach narrows down gene variants that possess desired enzyme characteristics, finally yielding an enzyme (or panel of enzymes) that have advanced properties when compared to their forbears. Accordingly, it is evident that directed evolution is a "numbers game", with the more mutants that can be screened enhancing the probability identifying extremely rare but highly beneficial mutations.

Recent developments have advanced droplet-based, high-throughput directed evolution in a number of aspects. A key study by Obexer and co-workers showed how droplet-based directed evolution can be used to create high-performance artificial enzymes, particularly when combined with computer-aided design (Figure 8A).[189] Specifically, the authors developed a novel microfluidic device, integrating droplet generation, incubation, and sorting within a single PDMS device able to sort between 10^6 and 10^7 library members per experiment. After six rounds of screening, mutants of a computationally designed and evolved artificial aldolase enzyme[190] exhibited up to 30-fold increases in activity. Beyond catalytic rates, there are various other enzymatic properties that can be optimized through directed evolution. For example, Ma et al. used a droplet screening platform to engineer the enantioselectivity of an esterase enzyme by employing two different enantiomers of the substrate (each labeled with a different fluorescent dye). These were analyzed using a dual-channel microfluidic droplet screening (DMDS) method to evolve the preference of the enzyme toward a specific isomer (Figure 8B).[191] The authors elegantly demonstrated the use of this technology on Profen drugs, whose (*S*)-enantiomers yield therapeutic benefits, while their (*R*)-enantiomers cause serious deleterious side effects. Over five rounds of directed evolution, a specific esterase variant (among a pool of 5 million) with a 700-fold enhancement of enantioselectivity for the desired (*S*)-Profen was identified.

Synthetic genetics concerns the creation of unnatural analogues of nucleic acids (artificial genetic polymers) and the development of artificial enzymes that can recognize them, with possible applications in exobiology, molecular medicine, and synthetic biology.[192] The creation and optimization of such enzymes is a task perfectly suited to droplet-based directed evolution, as recently demonstrated by Chaput and colleagues, who previously reported a droplet-based optical polymerase sorting (DrOPS) platform (for polymerase evolution) and used it to evolve a polymerase that works on artificial threose nucleic acid (TNA) with >99% template-copying fidelity.[193] The authors adapted the platform to incorporate FADS and reported the development of three additional fluorescent sensors for measuring activities of nucleic acid enzymes.[185] This refined system is able to screen fluorescent droplets at a rate of 3 kHz (approximately 10 million droplets per hour) and has the potential to make significant impact in synthetic genetic research. Subsequent studies have since reported the use of this high-throughput droplet method in advanced polymerase enzyme analysis, with the mapping of sequence−function relationships through a combination of droplet-based optical polymerase sorting and deep mutational scanning (Figure 8C).[194]

Droplet-based high-throughput screening is also particularly useful when screening natural enzymes for particular functionalities. For example, droplet-based screening has been used to study filamentous fungi that secrete large amounts of hydrolytic enzymes (such as amylases, cellulases, and proteases) and are thus a potent source of industrially relevant enzymes, particularly for the degradation of biomass. Beneyton and co-workers presented an elegant adaptation of droplet-based high-throughput screening for screening filamentous fungi, assaying a 10^4 clone UV-mutated library of *Aspergillus niger* for amylase activity using FADS.[195] Single fungal spores were encapsulated in droplets along with a fluorogenic substrate and then incubated for 24 h to germinate the fungi prior to analysis and sorting. One round of microfluidic screening allowed a 196-fold enrichment of active clones in only 90 min. Moreover, Girault et al. presented a droplet-based microfluidic device able to assay plankton, integrating the encapsulation, incubation, and enzymatic assay of single living phytoplankton cells in droplets.[196] Phytoplankton are unicellular organisms responsible for approximately 40% of inorganic carbon fixation on Earth. Accordingly, they play a significant role in global climate control, with the changing environment necessitating the development of a deep understanding of their function. By assaying the alkaline phosphatase activity of *Tetraselmis* sp. as a function of inorganic phosphorus concentration, Girault and colleagues showed that the enzyme kinetics are highly variable under identical environmental conditions, spanning 1 order of magnitude. These observations highlight once again the importance of single-cell experimentation in understanding variability within populations.

Although the emergence of FADS has and will continue to have an impact in droplet-based high-throughput screening of enzymes, a common downside is that screening is normally based on enzyme characteristics that can be assayed using fluorescence. In future, it will be desirable to expand this repertoire with other analytical techniques that can assay enzymes via the natural (unmodified) substrate. In this vein, a

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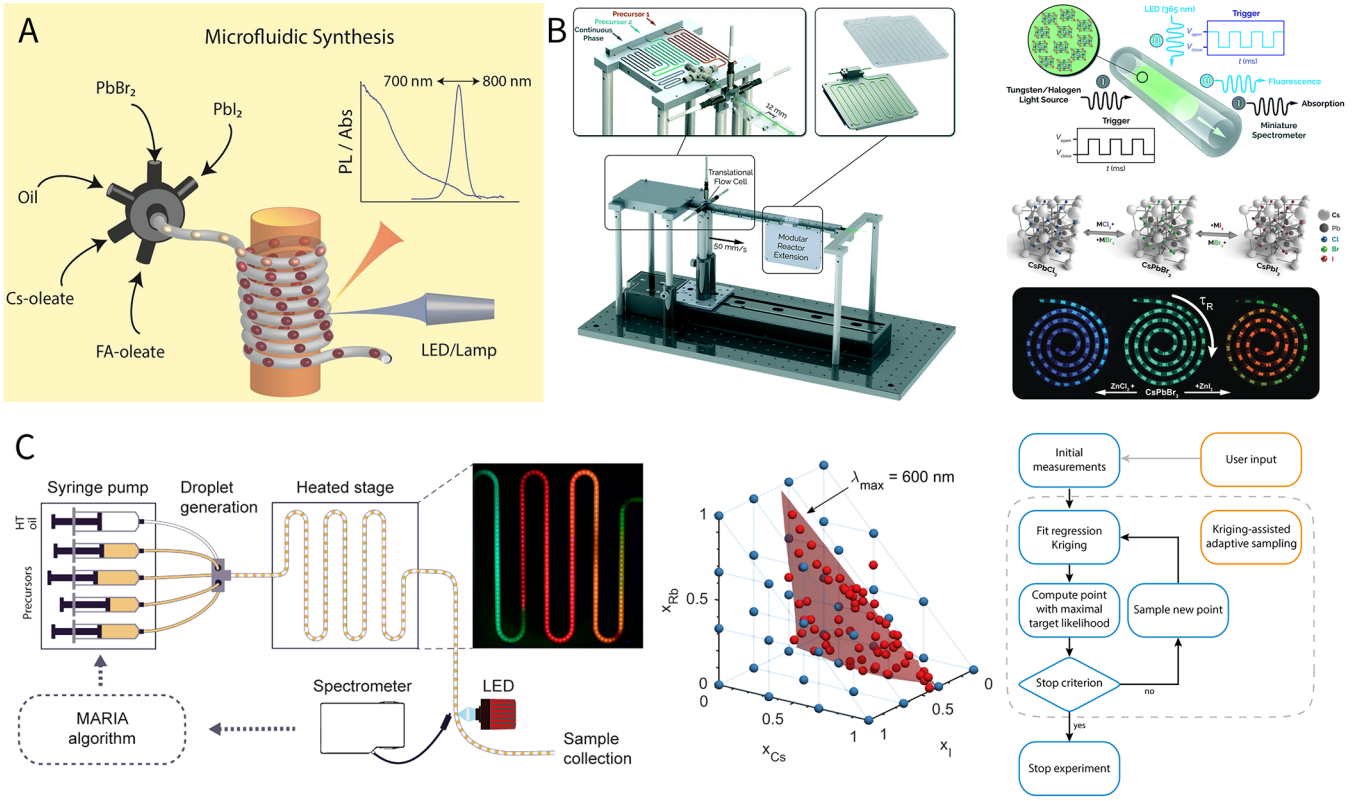

Figure 9. (A) Illustration of a segmented-flow reaction platform equipped with online PL (photoluminescence) and absorbance modules for the synthesis and real-time monitoring of $Cs_xFA_{1-x}PbX_3$ perovskite NCs. Reproduced from Lignos, I.; Morad, V.; Shynkarenko, Y.; Bernasconi, C.; Maceiczyk, R. M.; Protesescu, L.; Bertolotti, F.; Kumar, S.; Ochsenbein, S. T.; Masciocchi, N.; Guagliardi, A.; Shih, C.-J.; Bodnarchuk, M. I.; deMello, A. J.; Kovalenko, M. V. *ACS Nano* **2018**, *12*, 5504−5517 (ref 214). Copyright 2018 American Chemical Society. (B) Automated microfluidic platform for systematic studies of colloidal perovskite nanocrystals, illustrating how dual photoluminescence-absorption measurements are performed and the effects of anion exchange reactions. The left and upper right are reproduced from Epps, R. W.; Felton, K. C.; Coley, C. W.; Abolhasani, M. *Lab Chip* **2017**, *17*, 4040−4047 (ref 215), with permission of The Royal Society of Chemistry. The lower right is reproduced from Abdel-Latif, K.; Epps, R. W.; Kerr, C. B.; Papa, C. M.; Castellano, F. N.; Abolhasani, M. *Adv. Funct. Mater.* **2019**, *29*, 1900712 (ref 216). Copyright 2019, with permission from WILEY. (C) Multiparametric automated regression kriging interpolation and adaptive sampling for the targeted synthesis of LHP NCs. Reproduced from Bezinge, L.; Maceiczyk, R. M.; Lignos, I.; Kovalenko, M. V.; deMello, A. J. *ACS Appl. Mater. Interfaces* **2018**, *10*, 18869−18878 (ref 218). Copyright 2018 American Chemical Society.

number of recent studies aimed to expand FADS, with a notable focus on mass spectrometry[197−201] and absorbance-activated droplet sorting (AADS).[202] We are intrigued to see how the emergence of new and improved microfluidic-integrated analytical techniques will have an impact in the burgeoning field of biocatalyst discovery, characterization, and evolution.

Nanomaterials Chemistry. The benefits of droplet-based microfluidic reactors over both continuous-flow and flask-based approaches for nanomaterial synthesis are now without doubt.[203,204] The ability to regulate both heat and mass transfer on short time scales allows for rapid mixing of reagents and controlled particle nucleation and growth and ultimately the generation of high-quality materials with bespoke properties. Indeed, droplet-based microfluidic methods have been shown to yield inherently narrower size distributions and enhanced in-batch and batch-to-batch reproducibility, when compared to alternate formats.[205] Despite the maturity of the field, the last 2 years have seen some elegant demonstrations of how droplet-based reactors may aid in the synthesis of nanomaterials. For example, Niu and co-workers recently reported an advanced droplet microreactor for the synthesis of noble metal nanocrystals, demonstrating inline carrier oil−aqueous phase separation and product purification/concentration within a cross-flow filtration unit.[206] Such inline operations are critical in ensuring that droplet-based workflows are successful in replicating and improving more complex flask-based synthesis routes. In this regard, Sachdev et al. have shown how gold nanoparticles (spheres, platelets, and sheets) can be synthesized in a controllable fashion at the droplet−carrier fluid interface, whereas corresponding bulk reactions yield only spherical nanoparticles.[207] Moreover, Zhou and co-workers recently showed how the controlled-deformation of microdroplets may be used to make micromotors, consisting of polymer microparticles doped with magnetic and catalytic nanoparticles.[208]

Although the inherent advantages of droplet-based reaction systems for nanoparticle synthesis are without doubt, the basic fluidic approach is transformed through the inclusion of integrated analytics and control architecture. Here, real-time modulation of reaction parameters (including reagent concentrations, reaction time, and temperature) married with *in situ* product characterization (via optical spectroscopies) engenders systems able to perform multidimensional parameter scanning and reaction kinetic characterization with unparalleled accuracy and rapidity. An elegant demonstration of this concept in the recent literature concerns the synthesis of lead halide perovskite nanocrystals (LHP NCs), which have

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only recently emerged,[209] but have garnered considerable attention due to their utility in optoelectronic applications.[210] LHP NCs are well-suited to synthesis in microfluidic systems as their reaction kinetics are fast. This necessitates rapid mixing and temperature equilibration to ensure that product size distributions are as small as possible. Furthermore, LHP NCs do not (currently) require the synthesis of sequential crystalline shells, in contrast to traditional quantum dots, with reactions proceeding at relatively modest temperatures. Unsurprisingly, since the first report of LHP NC synthesis in droplets in 2017,[211] a number of important developments have been reported.[212] For example, Lignos and co-workers used a droplet-based reactor to target the synthesis of blue-emitting formamidinium mixed-halide LHP NCs, inspired by the fact that the controlled formation of blue-emitting perovskite nanocrystals is far less advanced than compared to their green- and red-emitting counterparts.[213] A simple PTFE tube reactor (in which reagents are mixed in droplets and conveyed around a copper heating rod) and an in-line photoluminescence detection system was used to perform extensive and rapid parametric scanning, the synthesis of products with tunable emission between 440 and 515 nm, and the elucidation of reaction boundaries defining nanoparticle and nanoplate formation. In an extension of this work, the authors subsequently targeted near-infrared-emitting LHP NCs, demonstrating the simultaneous use of photoluminescence and absorption spectroscopy to unveil reaction kinetics (Figure 9A).[214] In related studies, a modular microfluidic platform was also used for parameter screening and synthesis optimization of LHP NCs (Figure 9B).[215] Here, the use of a linear flow cell, which is optically accessible at fixed points along its length, allows the facile probing of the entire reaction time course. Simultaneous photoluminescence and absorption measurements from each reaction droplet were achieved by rapidly alternating two light sources linked to a single spectrometer. In an extension of this work, the platform was used to study the kinetics of anion exchange reactions of LHP NCs (Figure 9B),[216] illustrating the effects of ligand composition and halide salt source on the rate and degree of reaction. All these works clearly demonstrate the immense value of automated microfluidic platforms with integrated analytics, with respect to the study of nucleation, growth, and end point characteristics of nanoparticles, and the performance of rapid and multidimensional parameter scanning.

As an important aside, it should not be forgotten that the field of droplet-based microfluidics is primed for the use of smart algorithms to control reactive processes in real-time, given feedback from integrated analytics.[217] Such an approach allows for synthetic processes to be actively directed toward products with user-defined properties. A key example in this regard was reported by Bezinge and co-workers, who employed a Kriging algorithm to direct the synthesis of LHP NCs, given feedback from an inline photoluminescence spectrometer (Figure 9C).[218] The method, termed "multiparametric automated regression kriging interpolation and adaptive sampling" (MARIA), scans through an initial but short list of predefined reaction parameter sets that are coarsely dispersed through reaction parameter space (blue dots in Figure 9C). The algorithm then creates a model to calculate a "best guess" of parameters that will yield the target emission wavelength, assesses the best guess, measures the result, and refines again. The process is repeated for a fixed number of cycles or to a stop criterion and yields a list of distinct parameters sets that will produce nanocrystals with the target emission properties (red dots in Figure 9C). Techniques such as MARIA pave the way for advanced characterization and optimization of nanomaterial systems, for example, by exploring synthesis routes with complex blends of surface ligands, solvents, and precursors.

CHALLENGES AND THE FUTURE

There is little doubt that droplet-based microfluidic tools have profoundly changed the field of high-throughput biochemical experimentation, bringing simple and effective solutions to a range of challenging problems. Indeed, the technology set has now reached a level of maturity that, as previously noted, means that droplet-based components are best viewed as basic tools engendering novel science. That said, we feel that future technology developments should be directed downstream, with a view to helping end-users answer critical but unmet questions. From a user perspective, droplet-based microfluidics is one of a variety of available methods, strategies and technologies, where the trade-offs between effectiveness, cost and availability must be considered on a case-by-case basis. To reiterate, it is essential to appreciate the functions and advantages of droplet-based components, so as to ensure use in the most appropriate applications.

As a maturing technology, the challenges associated with droplet-based microfluidics are like elephants in the room, obviously apparent but often overlooked. Therefore, to conclude our review, we feel it is instructive and important to address directly the most important limitations of the platform, to both explain why droplet microfluidics is not always the correct solution to a problem and to bring to light to aspects that are worthy of attention. In doing so, we hope to provide an honest but impactful guide to help researchers in the field.

Droplets Are Imperfect. Microfluidic droplets are soft containers, more akin to a permeable cell membrane than a solid reaction vessel. This gives rise to some fundamental issues. First, droplets are not perfectly stable. Although a large variety of surfactants can be used to stabilize droplets over extended time scales, they cannot address all situations. Indeed, droplet payloads can be highly complex (e.g., cell lysates), such that they themselves destabilize droplet integrity. Second, droplets are never completely isolated. Studies have shown that there is almost always some material exchange between droplets.[219] This is a double-edged sword. Sometimes such a phenomenon can afford novel innovation[220] but often will result in unwanted cross-contamination, especially through the transport of small molecules. Third, in such low volume and confined environments, where exchange of substances is severely limited, cells will not necessarily act naturally. Fluorinated oils, which are gas-permeable and have low viscosity and cytotoxicity, have long been favored for cell-based experiments, but droplets in this environment are not ideal for long-term culture. Issue 3, for example, has a significant and negative effect on high-throughput experiments of paired immune responses between T cells and B cells.[221] In such a situation, the production of an immune response takes time and must be maintained in a state in which the cells are highly active. Solutions to such a problem are probably beyond the scope of the core microfluidics itself and will require the emergence of new materials or other revolutionary technologies.

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Chips Are Not Always Affordable. Microfluidic devices are consumables that are ideally only used once, to reduce the chance of cross-contamination, and to ensure optimal and reproducible fluid-handling performance. Although the raw materials (glasses and plastics) used to make microfluidic devices are, in themselves, not inherently expensive, structured microfluidic chips are rarely cheap. This simple fact limits, to some extent, the widespread use of chip-based systems. A key reason for such high fabrication costs stems from the fact that current end-use scenarios of microfluidics are highly variable. Therefore, it will be a long time before the price of a consumable microfluidic chip reaches the level of the 96-well plate. That said, having seen the huge success of droplet-based scRNA-Seq, we can envisage more commercial enterprises using droplet microfluidics in other applications, which will likely drive the modularization of microfluidic chips and the standardization of interfaces, in a manner similar to that which previously occurred in the semiconductor industry. We believe this to be an important direction for the development of the microfluidic industry and note that a good level of cooperation across academic and industrial boundaries will be vital. Finally, it is worth noting here that tubing-based microfluidic reactors, such as those used for the synthesis of nanomaterials, are often composed of readily available components and thus do not suffer from these chip-related restrictions, although are limited with respect to complexity of fluid-handling operations that may be performed.

Operations Are Not Necessarily Convenient. While the capabilities of droplet-based microfluidic systems bring advantage to many applications in the chemical and biological sciences, they are not suited to all situations, with the complexity of the some platforms outweighing any potential benefit. For example, use of droplet-based tools in point-of-care diagnostic devices may always struggle with regard to operational complexity and unit cost. Although there have been some interesting advances in manually propelled droplet generation methods,[222,223] we feel that the real power of droplet-based technologies lies in their application to high-content and high-throughput experiments. Indeed, we foresee that highly integrated, automated, and functional droplet microfluidic platforms for specialized use will be the more likely future development direction.

FINAL WORDS

To date, academic activities have defined much of the development of droplet-based technologies. Looking to the future, we believe that commercial opportunities will increasingly drive the field forward and toward timely biological and chemical problems. The contemporary advances discussed in the current review, and the level of maturity of the field as a whole demonstrate that droplet-based microfluidic technologies have delivered on much of their early potential. That said, the true impact, and limits to the potential in the field, remain to be uncovered, and we are excited to see how it will progress in the coming years.

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Notes

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