Analytical and Bioanalytical Chemistry
https://doi.org/10.1007/s00216-019-02294-z

REVIEW

Droplet-based optofluidic systems for measuring enzyme kinetics

David Hess [1] · Tianjin Yang [1,2] · Stavros Stavrakis [1]

Received: 30 July 2019 / Revised: 15 October 2019 / Accepted: 19 November 2019

Abstract

The study of enzyme kinetics is of high significance in understanding metabolic networks in living cells and using enzymes in industrial applications. To gain insight into the catalytic mechanisms of enzymes, it is necessary to screen an enormous number of reaction conditions, a process that is typically laborious, time-consuming, and costly when using conventional measurement techniques. In recent times, droplet-based microfluidic systems have proved themselves to be of great utility in large-scale biological experimentation, since they consume a minimal sample, operate at high analytical throughput, are characterized by efficient mass and heat transfer, and offer high levels of integration and automation. The primary goal of this review is the introduction of novel microfluidic tools and detection methods for use in high-throughput and sensitive analysis of enzyme kinetics. The first part of this review focuses on introducing basic concepts of enzyme kinetics and describing most common microfluidic approaches, with a particular focus on segmented flow. Herein, the key advantages include accurate control over the flow behavior, efficient mass and heat transfer, multiplexing, and high-level integration with detection modalities. The second part describes the current state-of-the-art platforms for high-throughput and sensitive analysis of enzyme kinetics. In addition to our categorization of recent advances in measuring enzyme kinetics, we have endeavored to critically assess the limitations of each of these detection approaches and propose strategies to improve measurements in droplet-based microfluidics.

Keywords Optofluidics · Droplet-based microfluidics · Enzyme kinetics · Fluorescence detection · Label-free detection

Introduction

Enzymes are proteins that catalyze biochemical reactions and are essential for nearly all the metabolic processes that occur in living cells [1, 2]. Due to their high specificity, enzymes also find application in a number of industrial processes such as the synthesis of antibiotics, the production of paper, or brewing [3–5]. In addition, enzymes play an important role in many genetic diseases, making them an active research topic in the health and life sciences [6, 7]. Accordingly, the study of the kinetics, structure, and function of enzymes as well as their relationship to each other is of widespread importance.

Published in the topical collection *Advances in Direct Optical Detection* with guest editors Antje J. Baeumner, Günter Gauglitz, and Jiri Homola.

✉ Stavros Stavrakis
stavros.stavrakis@chem.ethz.ch

[1] Institute for Chemical and Bioengineering, ETH Zürich, Vladimir Prelog Weg 1, 8093 Zürich, Switzerland

[2] Present address: Department of Biochemistry, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland

While experimental data concerning the structure of enzymes is typically obtained by three well-established methods, namely X-ray crystallography, NMR, and cryogenic electron microscopy, numerous alternative techniques can be used to study the kinetics of enzymes [8–10]. Such methods are necessary due to the great diversity of substrates and products involved in enzymatic reactions, the need to screen a variety of reaction conditions, and the varying timescales on which these reactions take place [11]. The ability to "solve" complete reaction mechanisms and determine the reaction coefficients of all involved steps often requires both kinetic and structural data obtained from different experiments [12, 13]. Modern enzymology is therefore an interdisciplinary field where the combination of results from various scientific disciplines is often needed to provide deep insight.

One such discipline that shows great potential is microfluidics. Microfluidic systems employ geometrical structures such as channels and chambers to constrain fluids to a small, typically submillimeter length scale [14]. This spatially restricted environment influences the physical behavior of the fluid, with viscous forces becoming more important when compared to inertial forces. Additionally, mass and heat transfer is dominated by diffusion rather than convention

Published online: 18 December 2019

[14–16]. Microfluidic technology is a powerful tool that facilitates a variety of complex biological experiments and allows for the development of highly integrated and fully automated systems [17, 18]. Expensive and scarce biological samples, such as enzymes, cells, or DNA, can be handled with ease, while consuming extremely low volumes [17, 19, 20].

A relatively new but potentially transformative tool for studying enzyme kinetics is droplet-based microfluidics (DM). These systems leverage immiscibility between input fluid streams to generate thousands of distinct reaction compartments each second using only minute amounts of reagents [21, 22]. This feature engenders significant application in ultra-high-throughput experimentation, where enormous numbers of reactions are screened in short periods of time. Amongst the biggest challenges facing the use of such technologies are the establishment of methods capable of creating user-desired reaction conditions and appropriate strategies for tracking and probing individual (sub-nL volume) droplets in a rapid and sensitive manner. The magnitude of recent literature on microfluidics addresses its broad applicability on developing strategies for high-throughput screening platforms.

The general aim of this manuscript is to critically review novel microfluidic tools and detection methods for measuring enzyme kinetics on short- to long-term timescales. After a brief introduction into the basic aspects of enzyme kinetics, we discuss current microfluidic techniques for enzyme analysis and DM research with emphasis on unit operations and their advantages in the study of enzyme kinetics. The second part of this review focuses on the basic operations (fluidic and detections) commonly performed when studying enzyme kinetics in droplets and provides a comprehensive list of available platforms that use DM for measuring enzyme kinetics.

Enzyme kinetics: basic theory

Enzyme catalysis

Enzyme kinetics describes the study of chemical reactions catalyzed by enzymes. The primary goal in such studies is to extract the involved reaction rates and investigate the effects of varying reaction conditions. As enzymes are essential for nearly all processes occurring in living cells, knowledge about rate coefficients is of great importance for understanding metabolic networks and their regulation [1, 2, 23]. In addition, enzyme kinetics is of particular interest in pharmaceutical science as many drugs act on enzymes and/or regulate their activity [6, 7]. Due to their high specificity, enzymes also find significant application in industry. Here, enzyme kinetics plays an important role when trying to improve the enzyme properties such as temperature stability, activity, or solvent tolerance [24, 25]. Common and potentially powerful approaches for altering the properties of enzymes include directed evolution and rational design [26, 27].

Enzymes are predominantly globular proteins that act on target molecules, called substrates. Initially, substrate molecules interact with an enzyme's binding site, before being transformed into a product (or products) at the active site [28]. This transformation typically involves several steps. It is generally agreed that the induced fit model best describes the binding interaction, suggesting a dynamic adaptation of both enzyme and substrate leading from an initially weakly bonded stage to a firmly bonded one [29, 30]. Enzymes, as with other catalysts, do not alter the thermodynamic equilibrium between substrates and products, but rather accelerate reactions by lowering the energy of the transition state and thus the required Gibbs energy of activation ($\Delta^{\ddagger}G^{\ominus}$) (Fig. 1a) [31, 32]. By varying accessible reaction conditions and investigating their influence on the observed kinetics, it is often possible to identify rate-limiting steps within complex reaction schemes. This aids the identification of potential catalytic mechanisms that are involved in the reduction of the transition state energy [33–35].

Steady-state and pre-steady-state kinetics

One of the oldest and best-known models for studying enzyme kinetics was introduced in 1913 by Leonor Michaelis and Maud Menten [36]. The reaction scheme (1) involves a substrate, S, binding to an enzyme, E, forming an enzyme–substrate complex, ES, in a reversible manner. Finally, the enzyme catalyzes the formation of the product, P.

$$E + S \underset{k_{\mathrm{off}}}{\overset{k_{\mathrm{on}}}{\rightleftharpoons}} ES \xrightarrow{k_{\mathrm{cat}}} E + P \tag{1}$$

The Michaelis–Menten equation (2) can be derived assuming the quasi-steady-state condition $d[ES]/dt = 0$, where v denotes the reaction rate while V_{max} and K_{M} are the maximum achievable reaction rate at saturation and the Michaelis constant, respectively [37].

$$v = \frac{d[P]}{dt} = \frac{v_{\mathrm{max}}\ [S]}{K_{\mathrm{M}} + [S]};\quad [S]_0 \gg K_{\mathrm{M}} + [E]_0 \tag{2}$$

Although the Michaelis–Menten equation is a simplistic model that only describes certain, one-substrate enzymatic reactions, Michaelis–Menten-type kinetics is still an important tool. The constants $v_{\mathrm{max}} = k_{\mathrm{cat}}[E]_0$ and $K_{\mathrm{M}} = (k_{\mathrm{off}} + k_{\mathrm{cat}})/k_{\mathrm{on}}$ can either be extracted by fitting the Michaelis–Menten equation to measurements of the initial reaction velocity for different substrate concentrations or by a full-time course analysis (Fig. 1b) [13]. In contrast to Michaelis–Menten kinetics, pre-

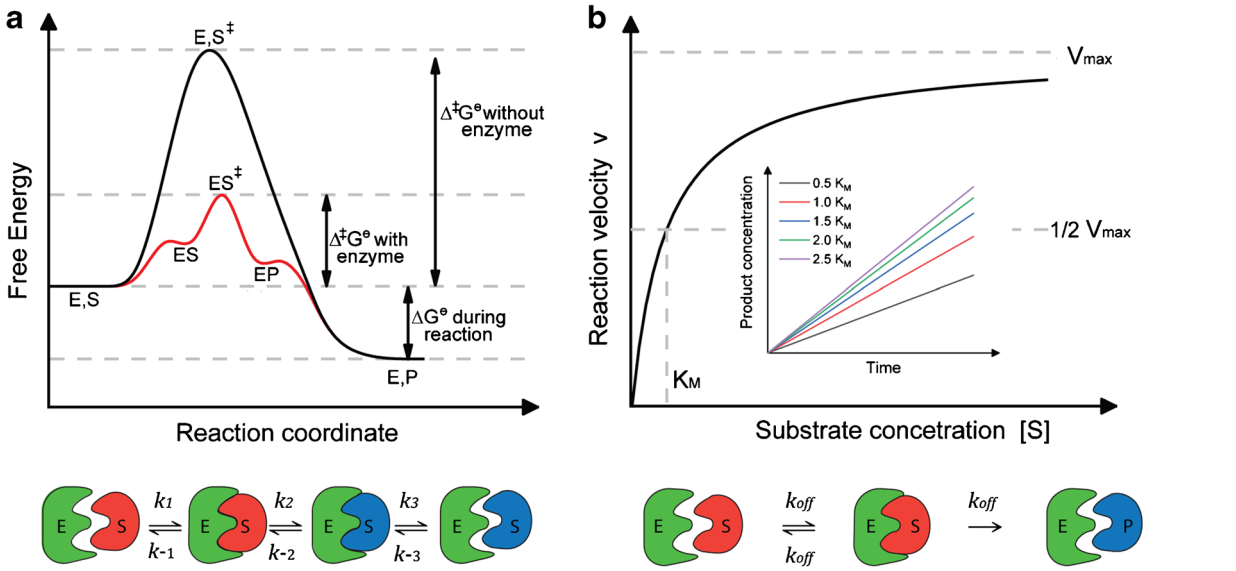

Fig. 1 Enzyme catalysis. **a** An exemplary free energy diagram of an enzymatic reaction. Compared to the uncatalyzed reaction (black), the catalyzed pathway (red) exhibits a lower Gibbs energy of activation ($\Delta^{\ddagger}G^{\ominus}$), while the thermodynamic equilibrium stays unchanged ($\Delta G^{\ominus}$). (*E*, enzyme; *S*, substrate; *P*, product; *ES*, enzyme–substrate complex; *EP*, enzyme–product complex; ‡, transition state). **b** Michaelis–Menten plot. The reaction velocity is half its maximum value v_{max} at a substrate concentration equal to the Michaelis constant K_M and asymptotically approaches v_{max} with increasing substrate concentration. The inset shows steady-state measurements for different substrate concentrations

steady-state kinetic analysis is concerned with the initial, transient phase of the reaction before steady-state concentrations of enzyme–substrate intermediates are reached [38]. Such experiments, which look at a single catalytic turnover, can provide insight into reaction mechanisms and are often used to investigate reaction rates which are inaccessible when using steady-state kinetics alone [13, 39, 40].

Estimation of rate constants for enzymatic reactions

One of the primary goals of enzyme kinetics is the extraction and estimation of rate constants. This is normally achieved by fitting experimental data to an appropriate model. Historically, most approaches have focused on selecting specific reaction conditions that result in data which can be fitted analytically [13]. However, in recent years, computer simulations have become increasingly important and useful, allowing for the numerical integration of the rate equations and regression analysis to fit data [13, 41, 42]. This advance allows for a direct derivation of the intrinsic rate constants from a reaction scheme. Furthermore, such an approach does not rely on any mechanistic assumptions apart from the law of mass action [13, 43]. In this context, a particularly useful aspect of computer simulations is the ability to perform global analysis, where different datasets from experimental measurements are evaluated simultaneously to extract a statistically rigorous and rich information [42]. These new ways of analyzing experimental data have direct implications on expedient experimental design. Indeed, they favor the measurement of full-time course kinetics that are difficult to approximate by analytical solutions but often contain a wealth of condensed information about the reaction coefficients.

Structure–function relationship of enzymes

Understanding the structure–function relationship of enzymes is one of the most critical topics in contemporary enzymology [44, 45]. Enzyme kinetic data are not able to directly provide information about this relationship, but studies investigating the influence of temperature and pressure on reaction rates can be used to reconstruct free energy landscapes and their transition states [46, 47]. By combining free energy landscape information with structural data, valuable insights into catalytic mechanisms can be garnered. In this respect, in silico modeling has become a powerful tool for evaluating structure–function relationships, with significant progress being made in recent years [46, 48]. Indeed, in the future, it is almost certain that only interdisciplinary approaches will lead to a deeper understanding of the detailed catalytic mechanisms of many enzymes. Figure 2 presents a generic workflow suited to the uncovering of the details of enzyme catalytic mechanisms.

Microfluidic approaches for measuring enzyme kinetics

There are numerous methods available to the experimentalists to measure enzyme kinetics. Common approaches include continuous assays, typically using spectroscopic detection techniques [49, 50] as well as discontinuous assays based on

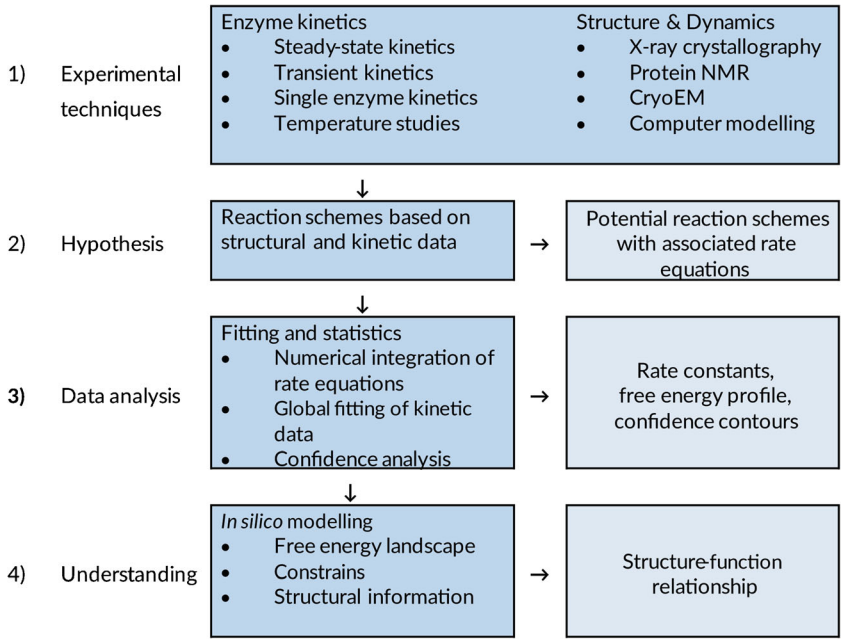

Fig. 2 Enzymology workflow. The scheme shows possible steps involved in investigating enzymatic reactions

radiometry [51], mass spectroscopy [52], chromatography [53], and capillary electrophoreses [54]. Spectroscopic measurements can often be performed using standard laboratory equipment and provide fast and reliable results, especially for absorbance and fluorescence-based assays. That said, they often require the synthesis of labeled enzymes or specially designed substrates that are converted to detectable products during the reaction. Nevertheless, the current discussion will exclusively focus on optical techniques due to their applicability, sensitivity, and non-invasive nature.

In general, the nature of the reaction under study will determine which detection technique is most appropriate. For example, when screening large numbers of distinct reaction conditions, 96- and 1536-well plates and fluorescence plate readers are popular choices [55]. Conversely, when investigating rapid kinetics, it is essential to initiate the reaction as promptly as possible. Here, popular techniques include stopped- and quenched-flow analyses, which allow for fast mixing and short dead times on millisecond timescales (Fig. 3) [56, 57]. Furthermore, there exist more specialized methods such as flash photolysis [58], temperature or pressure jump [59, 60], and pump-probe configurations [61] to measure even faster processes. In addition, of these conventional methods, recent years have seen the introduction of a variety of novel microfluidic techniques for the measurement of enzyme kinetics. Figure 4 illustrates some of the most interesting and powerful approaches of these techniques. All these methods are characterized by their need for only minute amounts of sample per experiment and their ability to leverage the atypical behavior of fluids on the microscale. Microfluidic research is concerned with the behavior and control of fluids that are geometrically constrained within environments having internal dimensions (hydrodynamic diameters) on a scale of microns [14]. Two of the most important consequences of downsizing the spatial dimensions of an experimental system are an increasing influence of viscous rather than inertial forces and the enhanced role of diffusion (over convection) in mass and heat transfer [14–16].

DM systems are well suited for high-throughput experimentation applications since each droplet can act as an individual reactor (Fig. 4a) [19]. In addition, many problems associated with single-phase flow, such as the interaction of analytes with the channel walls and dispersion, can be avoided by using segmented flow [19, 62]. Flow-focusing schemes can be used for extremely fast mixing of reagents (on the order of a few microseconds) making them particularly useful for probing rapid reactions (Fig. 4b) [63]. Continuous-flow mixers make use of special geometries (such as groves in the channel surface) to efficiently mix solutions under laminar flow conditions (Fig. 4c**)** [64, 65]. Valve-based microfluidic systems allow for the implementation of more complex and user-defined fluidic operations and as such can be viewed as a microfluidic equivalent to a well plate with extra functionalities (Fig. 4d**)** [66]. Finally, femtoliter arrays can be used to study the kinetics of single enzymes (Fig. 4e**)** [67]. In this manner, enzymatic reactions miniaturized to a droplet volume enable detection of their kinetics at extremely low concentrations. The enzymatic assays transferred to this microenvironment often target single-molecule detection [68]. Table 1 provides a summary of the

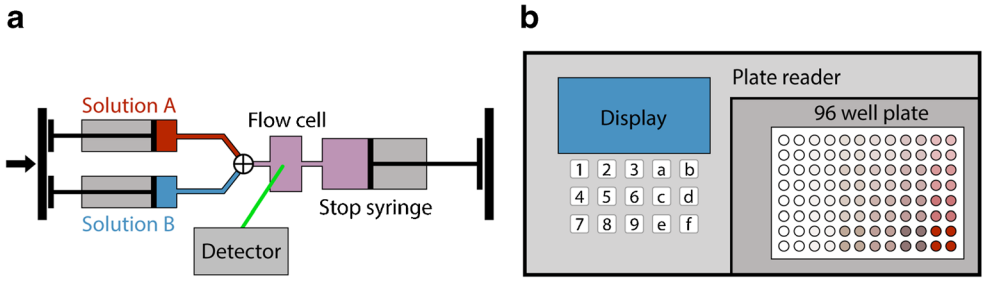

Fig. 3 Popular techniques for measuring enzyme kinetics. **a** Stopped-flow apparatus. Here, reagent solutions are rapidly mixed prior to introduction into a reaction cell. Optical interrogation of the reaction starts as soon as a stop syringe terminates the flow. **b** A plate reader. Here, a number of different reactions can be assayed in parallel using microtiter plates containing between 96 and 1536 wells

introduced microfluidic methods, with advantages, disadvantages, and their scope of application. Except for a few specialized applications, DM achieves superior performance for investigating a wide range of enzymatic reactions.

Selection of an appropriate method for a specific enzymatic reaction is the first step when planning a kinetic experiment. For the reasons discussed above, we will focus our discussion exclusively on DM tools for studying enzyme kinetics.

Droplet-based microfluidics

Droplet generation and properties of water-in-oil emulsions

In recent years, DM systems have proved themselves to be of great utility in chemical and biological experimentation [15, 69–71]. In simple terms, DM systems generate, manipulate, and process isolated droplets contained within an immiscible carrier fluid. Significantly, these platforms allow for the

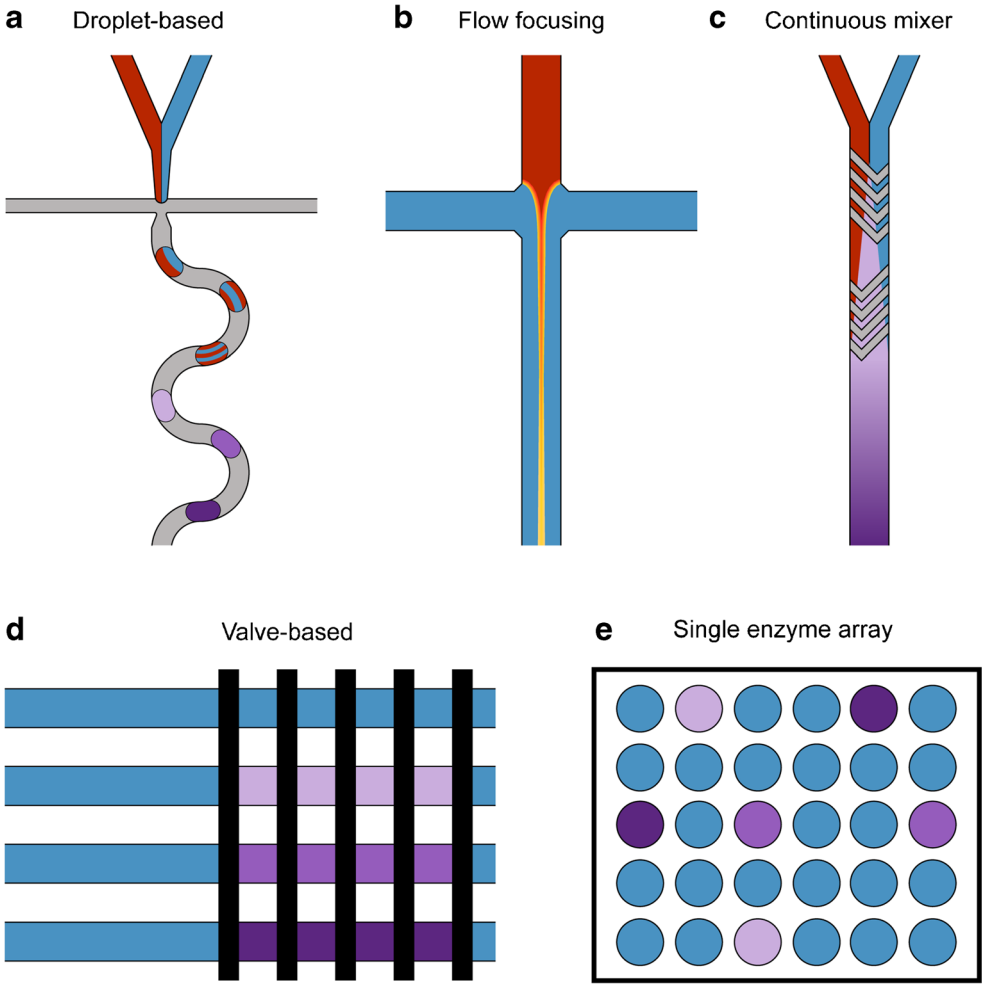

Fig. 4 Approaches for measuring enzyme kinetics using microfluidics. **a** In droplet-based microfluidic systems, reactions take place within picoliter–nanoliter-volume droplets. **b** Flow-focusing reactors make use of sheath flows to control and reduce the width of a central stream where a reaction occurs. **c** Continuous-flow mixers allow for controlled and efficient mixing by leveraging phenomena such as lamination and chaotic advection. For such "in-flow" methods, a variety of reaction timescales is accessible through control of the detector location. **d** Valve-based microfluidic systems can be used to perform complex fluid handling operations and process reactions in a batch manner. **e** Single enzyme arrays incorporate small chambers typically on the femtoliter scale, and provide for a digital approach to enzyme kinetic measurements

production of monodisperse droplets at rates in excess of tens of kilohertz and provide for independent control over each droplet in terms of its size, location, and chemical payload [72]. Critically, the use of droplets in complex chemical and biological experiments is enhanced by the ease of performing various integrated unit operations in an automated manner. Such operations include droplet generation, merging/fusion [73], sorting [27], splitting [74], dilution [75], storage [76], and sampling [77].

Precise control over the droplet formation process is essential in any chemical or biological experiment, with the ability to control droplet volume, generation frequency, interface stability, and solute retention being paramount. Droplets can be formed via passive or active mechanisms [83]. Put simply, passive strategies leverage geometrical variations of microfluidic structures to transform arbitrary volumes of fluid into defined sub-nanoliter droplets at kilohertz to megahertz rates [84]. Conversely, active strategies require an external energy input for droplet formation and manipulation. Although active methods provide for an enhanced degree of user control, it is noted that such methods typically produce droplets at low generation frequencies and require the use of complex control equipment [85]. For this reason, the following discussion will exclusively focus on the use of passive mechanisms.

Figure 5 illustrates various kinds of droplet generators and highlights fundamental differences between pressure-driven segmented and continuous flows. The most common configurations for the generation of droplets involve flow focusing [86], co-flow [87], step emulsification [88], and cross-flow (T-junction) [89] (Fig. 5a). Flow-focusing and T-junction geometries are the most popular approaches for creating microfluidic droplets. In a T-junction geometry, an aqueous solution is discretized into droplets by shear forces induced by a perpendicular flowing oil stream. In the case of a flow-focusing geometry, water flows in the middle channel and is enveloped by two oil streams coming from either side. The two liquid phases are then forced through a small orifice, and droplets are formed by viscous stress exerted on the inner phase by the surrounding oil flow. While a T-junction and flow focusing are the simplest geometries for the microfluidic formation of droplets, they can be successfully used for producing a variety of different droplet sizes with uniform size distribution. Moreover, both geometries are easy to fabricate and can operate at a high droplet generation rate. A co-flow droplet generator consists of a 3D flow-focusing geometry, mainly used for the formation of double emulsions [90]. The primary disadvantage of this geometry is that it requires the lithographic fabrication of a microfluidic device with the inner capillary to be smaller than, and placed within, the outer capillary. For certain applications that require the formation of droplets smaller than the downstream channel dimensions, the T-junction geometry is not suitable. While the flow focusing remains the best geometry to produce sub-micron microfluidic droplets, when operating in the tip-streaming regime, it suffers from polydispersity and formation of satellite droplets.

Since the typical size of a microfluidic channel ranges between tens and hundreds of microns, Reynolds numbers are almost always very small, with fluid flow being laminar (Fig. 5b). Under such conditions, mixing of fluid streams is mediated solely by diffusion, with the extent of mixing being defined by channel dimensions, the mean diffusion coefficient, and the average residence time. In practice, this means that when two or more fluid streams are flowing simultaneously within a channel, each of the streams maintains its own flow pattern, with mixing occurring via molecular diffusion across fluid interfaces. For pressure-driven flow with a parabolic profile, the dispersion of a solute traveling in a laminar-flow microfluidic channel along the major axis of the channel is described by Taylor dispersion (Fig. 5c). Converting a continuous stream into discrete droplets is a plausible way to prevent dispersion induced by the parabolic velocity profile. When a droplet is moving through a straight microchannel, two

Table 1 Microfluidics for studying enzyme kinetics

Method	Advantages	Disadvantages	Scope of application
Droplet-based microfluidics	No dispersion High throughput No contact with channel walls Low sample consumption Fast mixing	Complex fluidic system	High-throughput screening [78] Fast enzymatic reactions [62]
Flow-focusing reactors	Fast mixing (μs) Low sample consumption	Dispersion Low throughput	Protein folding [79, 80] Binding reactions [81]
Continuous-flow mixers	Fast mixing	Dispersion High sample consumption	Fast enzymatic reactions [65]
Valve-based microfluidics	Precise metering Complex fluidic operations	Low-throughput cross contamination	Complex reactions [66]
Single-enzyme arrays	Information on the single-enzyme level	High surface-to-volume ratio	Stochastic processes [82] Enzyme heterogeneity [67]

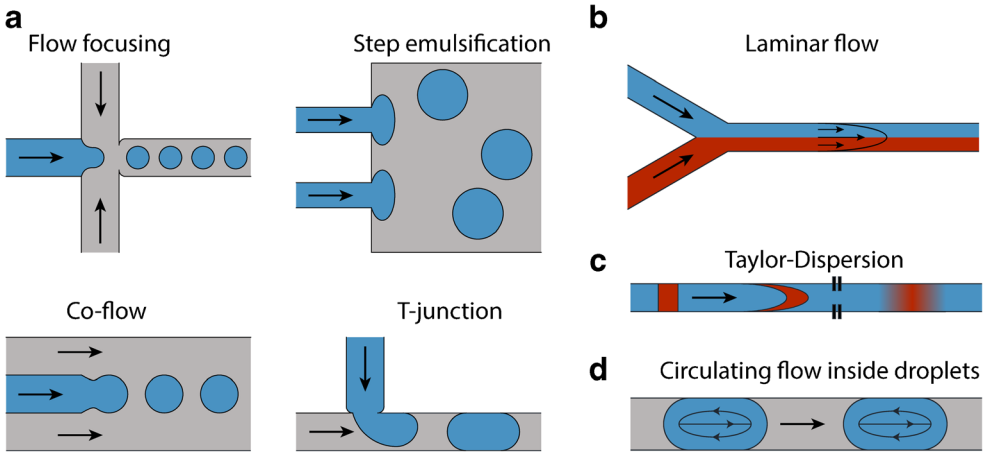

Fig. 5 Droplet generation modes. In **a**, the four most popular droplet generator geometries, namely flow focusing, step emulsification, co-flow, and T-junction are shown. **b** In pressure-driven continuous flows, a parabolic flow profile across the channel and laminar flow are observed, with diffusion and **c** parabolic flow giving rise to a higher effective diffusivity of solutes, a phenomenon called Taylor dispersion. **d** In contrast, segmented flows prevent dispersion of the discontinuous phase and induce circulating flows within droplets

vortices are induced and mixing occurs via the circulating flows due to the difference in the direction of the fluid motion relative to the channel walls (Fig. 5d).

Droplets can be formed using a wide variety of continuous and discrete phase combinations, but when studying enzymatic reactions, the discrete phase is almost always aqueous. Primary criteria used in choosing the continuous phase are biocompatibility, gas permeability, viscosity, surface tension, and retention of solutes inside the microdroplets. That said, fluorinated oils, mineral oils, or silicone oils are normally used [62, 91, 92]. Amongst these, low-viscosity fluorinated oils are the best choice for many biological assays. Such oils are chemically inert and exhibit high gas permeabilities. Additionally, the solubility of most organic chemicals is extremely low in fluorinated oils, which aids solute retention in the dispersed phase [93]. To prevent coalescence of droplets and maintain a stabilized emulsion, it is necessary to add a surfactant to the continuous phase [94, 95]. Ideally, surfactants stabilize the liquid–liquid interface and reduce the crosstalk between droplets without lowering the interfacial tension [94]. Indeed, when using DM systems for high-throughput screening, it is essential to prevent coalescence and crosstalk between the droplets on the timescale of the experimental process. Overall, selection of appropriate parameters for the droplet generation is a crucial aspect of DM experimentation, with the search for new and improved surfactants being an ongoing quest [94].

Optical detection methods

The advantages of reducing sample/reagent consumption and rapid heat and mass transfer are key in defining the utility of microfluidic systems in biological experimentation. However, system miniaturization presents some significant challenges that need to be considered and addressed. Put simply, although system downscaling leads to substantial improvements in analytical performance, these gains are in many ways offset by a reduction in the number of molecules which must be detected to report the chemical or biological state of the system. For DM systems, where droplet volumes are typically no bigger than a few tens of picoliters and droplet velocities are high, detection is a significant issue faced in almost all experiments.

Due to the relatively small number of molecules that reside within a picoliter-volume droplet, and the reduced optical path lengths that are characteristic of microfluidic environments, it is critical to choose a detection technique which can sensitively and accurately extract the required chemical and biological information at small numbers of molecules. Unsurprisingly, an enormous variety of fluorescence-based detectors have been used in DM experiments. These commonly involve the use of camera-based approaches or single-point analysis based on photomultiplier tube detection. For example, wide-field fluorescence imaging can be used to record the fluorescent intensities from thousands of droplets in a simultaneous fashion [96]. By imaging droplet arrays, large-scale (millions) measurements of enzyme activities of single cells can be achieved in a direct manner [97]. Indeed, it should be noted that wide-field fluorescence imaging systems have already been used to detect one million droplets in a single image to quantify DNA in droplet-based PCR with a high dynamic range [98]. A key limitation of fluorescence imaging in quantitative analysis relates to the frame rate of a CCD camera typically being much lower than the frequency of droplet generation. Accordingly, image-based fluorescence detection is ill-suited to applications where individual droplets need to be analyzed individually in flow and on short timescales. Single-point fluorescence detection schemes solve this problem by employing a photodetector. The popularity of single-point fluorescence detection owes much to its facile integration with DM formats [99, 100], exquisite sensitivity and low

limits of detection (down to the single-molecule level) [101, 102], high information content, and ability to operate on ultrashort timescales [103, 104]. Additionally, single-point detectors have been used in microfluidic fluorescence-activated droplet-sorting platforms for single-cell sorting at kilohertz rates [105], low-abundance biomarker detection [106], and protein evolution [107].

Despite the utility of fluorescence-based techniques, they are not without their problems. Commonly used fluorophores suffer from photobleaching and are often only suitable for end-point detection schemes. Conversely, real-time binding measurements require extended exposure of fluorophores to the excitation of a light beam. Moreover, the efficient use of fluorescence-based detection at low (sub-nM) analyte concentrations requires the use of fluorophores having large fluorescence quantum efficiencies. Although the number of synthetic fluorophores available to the experimentalist is vast, and almost any biological, chemical, or physical process can be monitored through the addition of a fluorescent moiety, this feature always presents an unnatural intrusion. In this respect, it is clearly desirable to have alternative detection strategies that provide rapid and sensitive detection of analyte molecules in a label-free manner.

UV–visible absorption spectroscopy is widely used in chemical analysis for the quantitative determination of molecular species. Its operational and instrumental simplicity has made it extremely popular for a diversity of macro- and microscale applications [108]. Moreover, when compared to fluorescence-based techniques, absorbance spectroscopy is less susceptible to bleaching and saturation, due to the higher photostability of non-fluorescent analytes. However, the performance and application of absorbance spectroscopy within microfluidic systems are severely compromised by reduced optical pathlengths, which directly affect both sensitivity and concentration detection limits [109]. This issue is especially problematic when probing picoliter-volume droplets moving at high linear velocities through microfluidic channels. To date, two main approaches have been used to overcome pathlength issues in DM systems. The first is to use single-pass extended pathlength techniques, which rely on modifying the fluidic path to stretch the droplet and increase the optical pathlength, e.g., through the use of Z-shaped channel structure (Fig. 6a) [110]. Using such an approach, a detection limit of 400 nM for fluorescein in water was achieved at a droplet frequency of 65 Hz. Such approaches typically access concentration detection limits in the high micromolar range. In contrast, multi-pass and cavity-enhanced techniques increase the effective optical pathlength by directing the optical beam multiple times through the analyte solution, yielding detection limits in the high nanomolar range, but at the expense of infrastructural complexity and large sample volumes [111, 112]. To address this significant limitation, Maceiczyk et al. used differential detection photothermal interferometry (DDPI) for single-point absorbance measurements in sub-picoliter-volume droplets in excess of 1 kHz with a detection limit of 1.4 μmol/L for erythrosin B (Fig. 6b) [113].

Droplet-based systems for measuring enzyme kinetics

DM systems are of enormous value in the study of enzymes for two general reasons. First, they are perfectly suited to high-throughput screening of a variety of reaction conditions due to their ability to create huge numbers of discrete droplets with controllable payloads, and second, rapid reactions can be probed with precision, due to ultra-fast reagent mixing via chaotic advection [62]. Droplets moving through a microfluidic channel unite and have the advantages of batch reactors (such as the absence of residence time distributions) with the benefits of flow reactors (such as the ability to continuously change reaction conditions). Moreover, the space-to-time relationship of moving droplets allows for image-based detection schemes with different positions in the image corresponding to different reaction/incubation times. This means that the integration time for signal acquisition is decoupled from the achievable time resolution of the measurement, which makes it possible to average the signal from a great number of droplets passing a detection point to improve the sensitivity. Figure 7 shows a schematic of an enzymatic reaction occurring within a droplet. A droplet provides a microenvironment, isolated from the channel walls and other droplets by the surrounding continuous phase. This, in principle, makes droplets almost perfect microreactors, but often, retention of solutes within each droplet is not complete. Accordingly, the choice of an appropriate fluidic system, where the solutes are retained inside the droplets, is of great importance.

General workflow

The main application of DM is high-throughput experimentation. Apart from rapid and controlled heat and mass transfer and the reduced reagent consumption inherent to most microfluidic systems, there are a range of additional benefits associated with using segmented flows when screening a large number of reactions [16]. First, highly monodisperse droplets can be generated readily at kilohertz frequencies, where every single droplet can be viewed as a discrete reactor moving at a constant velocity [83]. In addition, the volume of such droplets is small, typically between femtoliters and nanoliters, and there is no contact between the droplet and channel walls, preventing contamination and other unfavorable interactions [83]. Finally, the sequential and automated use of established unit operations greatly facilitates the implementation of complex analytical procedures. Almost all experiments involving

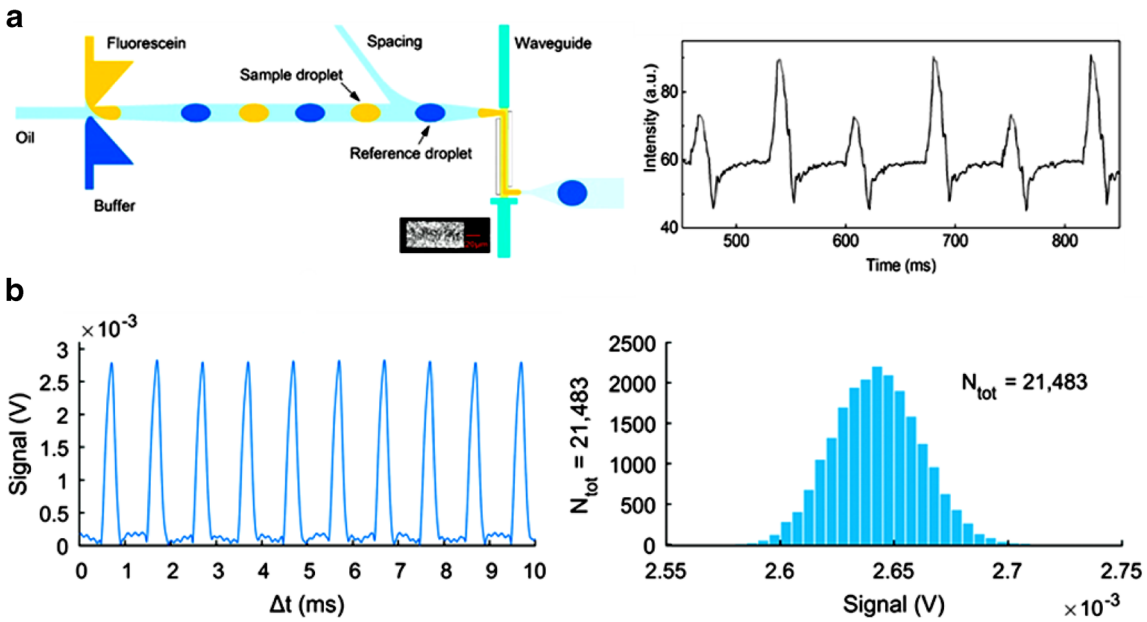

Fig. 6 Absorbance-based detection in droplet-based microfluidic platforms. **a** Schematic of the optofluidic device for single-droplet absorbance analysis (left panel). An alternating droplet train of "sample" and "reference" droplets is formed at a double T-junction and motivated towards the droplet detection region, which is flanked by the input and output waveguide channels. Right panel: transmitted light time traces of a single droplet collected from the side view of the output waveguide [110]. Reproduced with permission from the American Chemical Society. **b** Time trace of the photothermal signal (right panel) and histogram of individual droplet signals (left panel) for 120 fL droplets containing 40 μmol L^{-1} erythrosin B. The droplets propagate at a frequency of about 1 kHz [113]. Reproduced with permission from the Royal Society of Chemistry

droplets require the performance of additional manipulations subsequent to droplet generation. In this respect, a variety of functional components for droplet manipulations have been documented in literature. These include tools for droplet mixing [62], splitting [74], merging [73], incubation [114], reinjection [115], and dosing [19].

Certain basic operations are commonly performed when studying enzyme kinetics in droplets. These are typically reagent encapsulation into a droplet, reagent mixing, incubation, and detection. Additional operations are then used on a case-by-case basis to create concentration gradients, multiplex reactions, or barcode droplets. This general workflow is now summarized, with an explanation of the involved operations and possible implementations. Table 2 presents a brief summary of the operations discussed below. Methods for creating concentration gradients, reagent delivery, mixing, and detection are schematically illustrated in Fig. 8.

DM systems are ideal for a range of high-throughput screening experiments. To make full use of their many advantageous features, it is essential to find ways of creating different reaction conditions by varying parameters such as temperature, substrate concentration, pH, and concentration of enzyme inhibitors. The

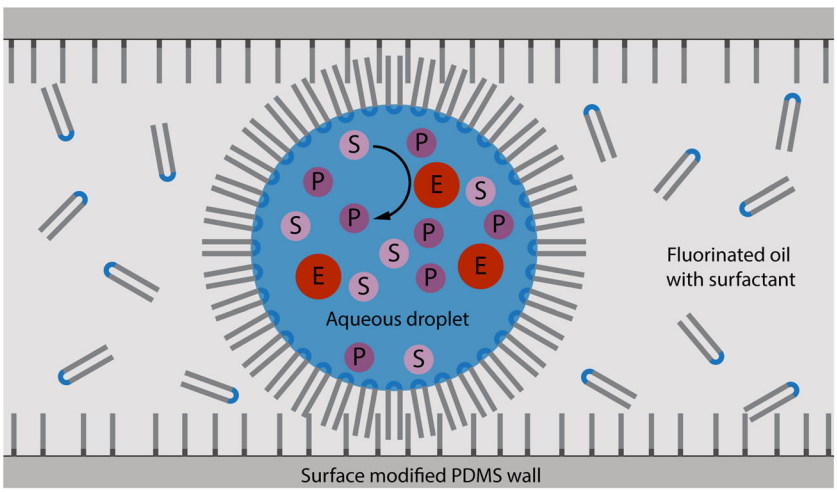

Fig. 7 Enzymatic reaction occurring inside the droplet. The continuous phase consists of a fluorinated oil containing a surfactant, which acts to stabilize the droplet interface. Channel walls are modified in a way that favors wetting by the continuous phase and not the discrete phase

reaction temperature is normally hard to control at the single-droplet level, but fast heat transfer in microfluidics makes it possible to rapidly and precisely control temperature on a global level between different experiments. In contrast, a vast range of different reaction conditions (within a single segmented flow) can be created via concentration gradient or multiplexing techniques (Fig. 8a). Concentration gradients are most easily created in a continuous flow prior to droplet formation, with the variation of relative flow rates between different fluid inputs over time being achieved by the control of pumps. Unfortunately, this method only allows for relatively small dilution factors due to instabilities and fluctuations at low volumetric flow rates [62, 96]. Other approaches depend on the diffusion of solutes between adjacent laminar flow streams, followed by division of the flow into several parallel channels [116], or by the repeated splitting and recombination of two or more flows in tree-shaped networks, where the relative flow rates in the different channels are controlled by the corresponding hydrodynamic resistances [117]. Such methods are capable of forming complex concentration gradients with excellent control and stability in parallel channels. However, the common need to parallelize droplet formation limits the number of accessible concentrations per experiment [116, 118]. An additional and elegant way to create concentration gradients of several orders of magnitude relies on Taylor dispersion, where a small volume of analyte is injected into a flowing stream of buffer [19]. Dispersion takes place due to a combination of the parabolic flow profile and diffusion of the analyte, leading to an evolving concentration gradient, which can then be segmented into droplets. Finally, it is also possible to create concentration gradients subsequent to the droplet formation process by the repetitive merging, mixing, and re-splitting of small delivery droplets with a static sample droplet, whereby a sequence of droplets with a well-defined differences in sample concentrations are created [75].

Multiplexing reaction conditions is an efficient way to extend the accessible parameter space (Fig. 8b). The easiest way to deliver reagents is during the actual process of droplet formation [62, 96]. Subsequently, it is possible to inject liquids from external channels into passing droplets in a controlled manner using an electric field to destabilize the interfaces temporally [119, 120]. This process is commonly termed "pico-injection." The later technique requires the addition of electrodes close to the microchannels, thus complexifying the microfabrication and experimental setup, but allows the precise control of the coalescence at very high frequencies since the electric field can be actively turned on and off. As an example, picoinjectors [121] can be used to add a picoliter volume to chosen droplets at a kilohertz rate. The electrical field ruptures the film separating the droplet and the reagent, allowing the reagent to be injected with great precision. Alternatively, merging of preformed droplets using both passive and active approaches can be used to meter reagents in a precise and rapid manner [122]. Controlled coalescence of droplets is an important method to perform reactions in droplets especially under the condition that two isolated droplet reactors containing intermediates from previous reaction steps need to be mixed for continuing to the next step reaction. Passive methods rely on geometrical or physico-chemical induction. Active methods provide higher fusion efficiencies at the expense of more complex equipment. Active fusion, for example, can be achieved through electrical coalescence by flowing a pair of droplets through a region of high electric field which destabilizes the interphase of the droplets, causing merging [123]. A combinatorial approach to produce different reaction conditions could, for example, involve the fusion of droplets or involve the use of pico-injectors and preformed droplet libraries [119]. Since the droplet sequence is generally lost during the process of multiplexing, it becomes necessary to use barcodes to identify

Table 2 Workflow for measuring enzymatic reactions in droplets

Step	Operation	Methods
(1)	Concentration gradient	On-chip dilution by changing relative flow rates [62, 96]
		Taylor dispersion of reagents prior to encapsulation [19]
		Flow distribution and diffusion [116, 118]
(2)	Reagent encapsulation	Droplet formation [62, 96]
		Picoinjector [119, 120]
		Droplet fusion [122]
(2b)	Droplet indexing/barcoding	Barcodes (e.g., fluorescent dyes) [96, 119]
		Secondary indexing droplets [126]
(3)	Mixing	Channel geometry inducing chaotic advection [62, 96, 125]
(4)	Incubation	Sequential in channel or using incubation chambers [96, 119]
		Static in an array or off-chip [97, 105]
(5)	Detection	Single-point approaches [19, 119]
		Image-based approaches [62, 96]

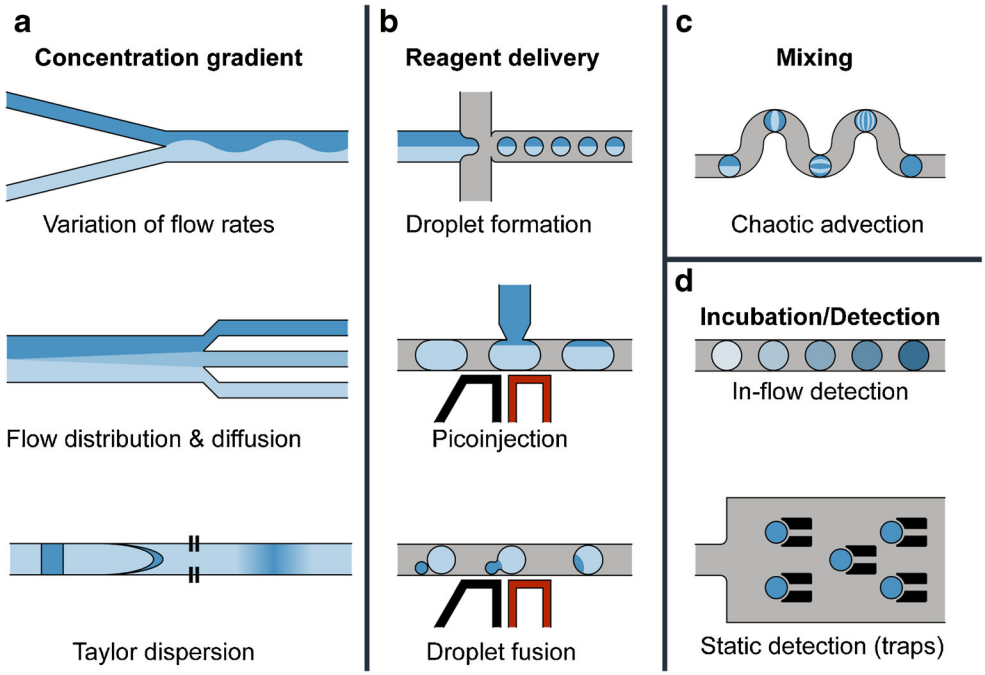

Fig. 8 Strategies for measuring enzyme kinetics in droplets. **a** Concentration gradients can be created by varying the relative flow rates between two streams (62), exploiting the diffusion between adjacent laminar streams followed by distribution of the flow into parallel channels (117) or using Taylor dispersion of samples injected into a pressure-driven continuous flow (19). **b** The easiest way to deliver reagents into droplets is during their formation (62,96). Afterwards, it is possible to add reagents by picoinjection (122) or the fusion of droplets (124), whereby electric fields are applied in both cases to temporarily destabilize the interfaces of the droplets. **c** The most common approach to mix reagents inside droplets is by chaotic advection, induced by the motion of droplets through serpentine channels (125). **d** Incubation of droplets on-chip can either be performed in flow (1, 115) or static by using traps to capture the droplets (128)

the different reaction environments within each droplet. Here, simultaneous "reading" of the barcode and detection of the enzymatic reaction is essential to maintain analytical throughput. The most common strategy for barcoding droplets relies on fluorescent dyes, e.g., by initially adding them to a solution containing a reagent, which usually guarantees that the concentrations of reagent and barcoding dye stay proportional during the multiplexing [96, 119].

Normally, mixing of delivered reagents is used to initiate an enzymatic reaction (Fig. 8c). Mixing inside droplets can be achieved using channel geometries which lead to chaotic advection, e.g., winding channels [124]. In this manner, the fluid inside the droplet undergoes stretching, folding, and reorientation to achieve fast mixing even in sub-millisecond timescale under certain conditions. Accordingly, the efficiency and robustness of the mixing process have a direct influence on the time resolution of the measurement. Typically, mixing in picoliter-volume droplets is a diffusion-controlled process, characterized most easily using the Péclet number. An elegant and popular approach to increase mixing efficiencies and reduce mixing times involves the use of a serpentine channel geometry to introduce chaotic advection and exponentially reduce the diffusion distances for mixing as a function of distance (or time) traveled [62, 96, 125].\

As soon as a reaction is initiated, the incubation can begin (Fig. 8d). During incubation, maintenance of the droplet sequence (and hence identity) typically requires the use of narrow channels, whose width does not significantly exceed the diameter of the droplets. This leads to significant backpressures in longer channels, which ultimately limits accessible observation times. The use of wide channels or chambers extends the accessible reaction time; however, this almost always means that the droplet sequence is randomized, resulting in finite residence time distributions. Bespoke chambers, separated by narrow channels, which induce a redistribution of droplets upon passage, have been used to minimize the dispersion [114], and for slow reactions, it is possible to separate the process of droplet generation and incubation into two consecutive operations. This enables droplet incubation off-chip or droplet trapping within static arrays on-chip [92, 97]. After off-chip incubation, they can be reinjected for further analysis

The detection and analysis of an enzymatic reaction occur during or after droplet incubation (Fig. 8d). Methods for measuring reaction progress can be broadly divided into image-based, single-point, and end-point detection schemes. Image-based approaches have the advantage of providing simultaneous access to different time points of the reaction (through analysis of multiple droplets) but normally require the use of epifluorescence microscopy [62, 96]. Recently, Maillot et al. used a streak camera to extract time-resolved fluorescence information from droplets and investigate the biomolecular

out-of-equilibrium reaction between rapidly mixed bovine serum albumin and Paten blue V [127]. They found evidence of a heterogeneous structural relaxation of the formed protein–dye complex over a 120-ms timescale. In contrast, single-point detection schemes require sequential measurements (at different spatial locations) to access temporal information, but allow for the adoption of a wider range of detection techniques, such as laser-induced fluorescence, fluorescence lifetime spectroscopy, fluorescence polarization spectroscopy, absorption spectroscopy, electrochemical detection, and Raman spectroscopy [77].

Finally, there exist analysis techniques that only allow for endpoint analysis such as capillary electrophoresis [128]. This method provides quantitative information regarding complex analytical environments within droplets; however, it allows only for a single detection per droplet. Accordingly, it is clear that all the detection strategies discussed above have specific advantages and disadvantages. Recently, Ouimet et al. presented a new microfluidic approach for introducing droplets to chip electrophoresis by removing the carrier phase based on its density with a throughput of about 10 s/sample [129]. The utility of this device was demonstrated for protein–protein interactions and enzymatic assays. Nevertheless, it should be noted that the majority of research incorporating DM for the measurement of enzyme kinetics relies on time-integrated fluorescence measurements due to excellent sensitivity and precision, straightforward implementation, and compatibility with high-throughput screening approaches. That said, the primary drawback associated with the use of such methods is need for fluorescently labeled enzymes or special fluorogenic substrates.

State of the art

In this section, an extended discussion of prior studies that have utilized DM for the study of enzyme kinetics is presented. Despite the compelling advantages of DM, currently, there is a surprising lack of appropriate platforms for measuring enzyme kinetics in high-throughput.

Song et al. [62] were the first to report a successful DM system for measuring enzymatic reactions in droplets. In this study, epifluorescence imaging was used to extract kinetic data from droplets, with millisecond timescale reaction kinetics being accessible through consideration of mixing with their kinetic models. To ensure adequate sensitivity, the authors used long exposure times (in the range of seconds) to acquire images constructed from the average signal of many hundreds of homogeneous (identical) droplets. This approach is well suited for the detection of rapid reactions, but has little utility for high-throughput applications, since detection relies on droplet ensemble measurements. However, most kinetic experiments in DM have relied on averaging signals over large numbers of droplets and are thus poorly suited to resolving dynamics at the single-droplet level. To address this issue, Hess et al. presented a novel imaging method based on stroboscopic illumination to characterize enzyme–inhibitor reaction kinetics by tracking individual, rapidly moving droplets that contained varying concentrations of substrate [96]. Fradet et al. developed a droplet-based microfluidic platform to measure the kinetics of β-d-glucosidase using static droplets on demand. Briefly, droplets containing enzyme and colorimetric substrate (4-nitrophenyl β-D-glucopyranoside) were fused and the accumulation of the product was detected by an optical microscope [130].This method allows monitoring of both fast and slow reactions with the same device using minute amounts of reagents.

Compartmentalized multistep enzyme cascades can be generated by using enzymes as the functional and structural element of the membrane. Production of water-in-oil emulsions, stabilized by enzyme conjugates called proteinosomes, can be transferred to a microfluidic platform, allowing for high-throughput processing [131]. The generation of proteinosomes exhibiting a two-enzyme cascade was demonstrated with membrane-bound poly (N-isopropylacrylamide)-GOx encapsulating horseradish peroxidase. Importantly, the resulting proteinosomes had superior monodispersity and higher enzymatic activity when compared to droplets produced using bulk methods.

Unlike conventional wide-field imaging of a homogeneous set of droplets in kinetic analysis, (which provide average values by integrating images over many seconds), single-point detection schemes can probe reaction kinetics with single-droplet resolution [132, 133], providing quantitative and accurate information relating to droplet heterogeneity. In a related study, Hassan et al. [134] developed a multi-detector approach, where enzymatic reactions in droplets can be probed at different positions along a microfluidic channel. Here, an absorption-based detection scheme was successful in realizing single-droplet resolution. Such an approach has the advantage that all detection components can be implemented at low cost, but suffers from a relatively low throughput and poor concentration limits of detection (Fig. 9b). A single-point detection absorbance approach for measuring enzymatic kinetics was demonstrated by Mao et al. by integrating optical fibers within a droplet-based microfluidic platform [135]. The reaction kinetics was measured by changing the reaction time through the adjustment of the droplet velocity inside the microchannel. As mentioned previously, DDPI is a relatively new tool for fast and sensitive single-point absorbance measurements in picoliter- and femtoliter-volume droplets with limits of detection as low as 0.1 μM [113]. This method was successfully applied to extract the Michaelis constant for the reaction of β-galactosidase and chlorophenol-red-β-D-galactopyranoside. However, apart from this study, there has not been any report of high-throughput photothermal absorbance spectroscopy studies in microfluidics droplets.

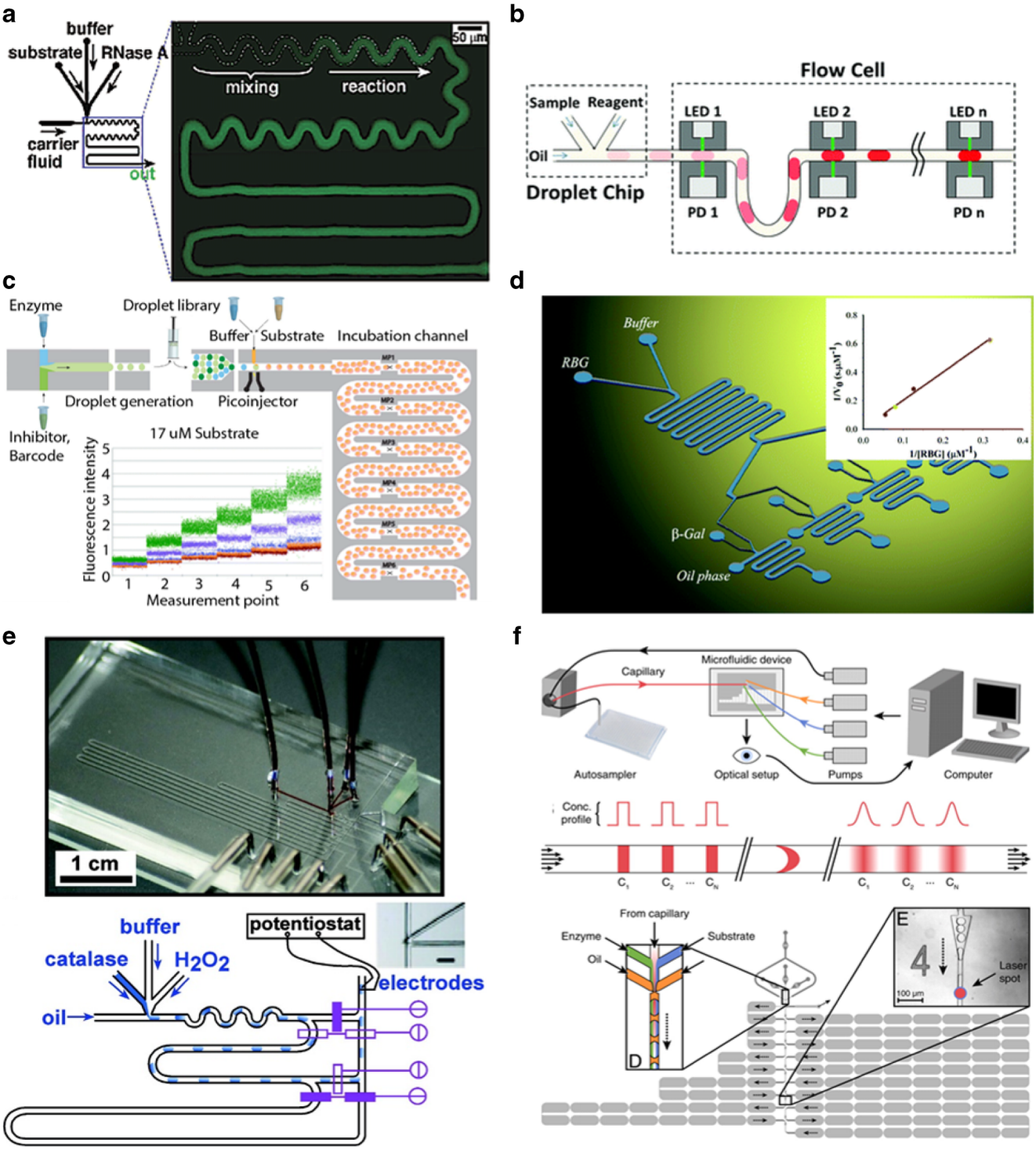

Fig. 9 Droplet-based microfluidic systems for the assessment of enzyme kinetics. **a** Enzymatic millisecond kinetics using epifluorescence imaging (62). Reproduced with permission from the American Chemical Society. **b** Absorbance-based multipoint measurement in microdroplets (137). Reproduced with permission from the Royal Society of Chemistry. **c** Using picoinjection on a barcoded droplet library to multiplex inhibitor and substrate concentration (120). Reproduced with permission from the Royal Society of Chemistry. **d** Applying flow distribution and diffusion to create a substrate concentration gradient followed by parallelized droplet generation (117). Reproduced with permission from the American Chemical Society. **e** Valve-based control of incubation time combined with amperometric detection (145). Reproduced with permission from the American Chemical Society. **f** Dose-response screening using Taylor dispersion to create inhibitor concentration gradient (19). Reproduced with permission from the National Academy of Sciences of the USA

This can be attributed to several problems that arise when carrying out thermal lens spectroscopy in such environments. First, at typical droplet generation frequencies, droplets spend less than a millisecond in the excitation laser volume. Accordingly, the measurement bandwidth must be large enough to capture the flowing droplet at a short transit time. Second, the passing droplets can introduce significant noise at frequencies up to tens of kilohertz. Finally, even if the refractive indices of continuous and discrete phases are matched, the passing droplet will introduce a significant phase shift of the probe laser beam. This phase shift interferes with noise cancelation in interferometric methods such as DDPI or differential interference contrast thermal lens microscopy (DIC-TLM) [136]. In the context of label-free detection, Polshin et al. demonstrated the integration of microfluidics with FT-IR microscopy for real-time monitoring of the enzymatic oxidation of glucose by glucose oxidase [137].

Towards addressing the challenge of producing microfluidic-based concentration gradients in a way that every droplet represents one unique reagent combination, Gielen et al. used a robotic compartment-on-demand (COD) platform for the automatic formation of droplets with different substrate concentrations coupled with absorbance-based detection [138]. A simple micro capillary technique was used to generate aqueous droplets by applying negative pressure on a tubing inserted into an open well filled with the sample (e.g., a titerplate). Precise control of the generated droplet content enabled the generation of large concentration gradients. Such concentration gradients were subsequently used to determine the steady-state kinetic parameters of haloalkane dehalogenase using 150 enzyme/substrate/inhibitor combinations in less than 5 min. This platform is cost-effective, easy to assemble using only a fraction (~ 100-fold less) of the reagents consumed in a microwell format. Droplet microfluidics with its isolated compartments provides a tool to perform many individual experiments in parallel. Wei et al. [139] developed a microfluidic dilution system capable of automatically generating a large and tunable range of concentration gradients (6 orders of magnitude) in 8-nL droplet arrays. The system was developed by combining a droplet robot platform with a unilateral dispersion approach. The droplet robot enables the automated screening of hundreds of samples by directly interfacing a commercial multiwell plate to the nanoliter droplet-array chip. The versatility of this system was validated by screening enzyme inhibitors from a library of 102 compounds leading to approximate 1600-fold reduction in enzyme consumption compared with multiwell plate-based assays. The increasing push towards higher density formats has led to the development of high-throughput/lower-volume assay formats. Towards this goal, Lee et al. developed a platform consisting of microvalves for droplet generation combined with a droplet storage array [140]. Within this array, high-resolution concentration gradients were obtained by merging binary concentrations of droplets. As a proof of concept, a dose-responsive inhibition reaction with β-galactosidase was performed. This system can serve as a general assay platform capable of high-throughput screening.

A successful method to multiplex enzyme kinetics measurements within droplet flows was demonstrated by Jambovane et al. [141] using a series of valve-based injections of reagents into droplets, followed by fluorescence imaging. The authors were able to determine K_M and k_{cat} for two metalloproteinases within a single experiment using only 10-pL-sized droplets. Additionally, Sjostrom et al. [119] used pico-injectors to add substrate to preformed droplets from a barcoded droplet library containing enzyme and inhibitor at varying concentrations. Here, detection relied on single-point measurements using laser-induced fluorescence (Fig. 9c). Other studies, notably those by Bui et al. [116] and Damean et al. [118], have used microfluidic devices that leverage flow splitting and diffusion to create a concentration gradient of a fluorogenic substrate in neighboring channels, followed by the addition of enzyme and parallel droplet generation. Both studies used epifluorescence imaging to quantify reaction kinetics in multiple channels simultaneously. While such an approach allows for stable and controllable concentration gradient formation, the ability to extend the system to larger numbers of reaction conditions is limited (Fig. 9d). Han and co-workers [142] introduced an elegant way to access different reaction times using an amperometry-based single-point detection method, where pneumatic valves are used to control the length of the incubation channel that droplets must transit until reaching the detection zone. This approach enables consecutive measurements after different incubation times using endpoint detection methods (Fig. 9e).

Kinetic analysis of large chemical compound libraries forms an essential part of early-stage drug discovery, and DM have been developed to enable such measurements. Due to fast droplet generation rates combined with reduced sample volumes, high-throughput screening of large chemical compound libraries is possible. Rather than generating dose-response curves by traditional methods (which are built from < 10 data points per compound), Miller and co-workers [19] have demonstrated the highest level of integration in a fully automated screening platform. In their system, an autosampler is used to inject possible inhibitors from a chemical library containing 704 compounds into a buffer stream. A concentration gradient (in excess of three orders of magnitude) is then created by Taylor dispersion and approximately 10,000 droplets per compound formed after the addition of enzyme and substrate. Droplets could be incubated in a delay channel, with laser-induced fluorescence detection taking place after an incubation time of 210 s. This is currently the only droplet-based platform for measuring enzyme kinetics in a fully unsupervised manner and the first demonstration of an application that goes beyond proof of principle (Fig. 9f).

Conclusions and future perspectives

In this review, we discussed general strategies for the use of DM in the study of enzyme kinetics. Although significant improvements have been made within the last decade, there is still room left for studies focused on developing strategies for high-throughput kinetic screening, improving the time resolution of kinetic measurements, and the implementation of novel optical detection schemes in the context of DM.

Herein, we have presented some of the most important advantages of DM systems for high-throughput screening and the measurement of fast reactions providing generalized workflows for measuring enzymatic reactions. This constitutes a niche application of DM since the potential to create and analyse enormous amounts of kinetic data, using DM combined with novel computational methods, is undoubted. The advantages of computer simulations to fit data using numerical integration of the rate equations, as well as the use of global fitting to extract maximal information regarding reaction constants, provide valuable tools to researchers for obtaining insights into complex reaction mechanisms. Since these approaches profit from the combination of multiple data sets recorded under different reaction conditions, new techniques capable of screening enzymatic reactions are of undoubted interest and importance. The biggest challenge for multiparametric screening experiments is to find new and efficient ways to create series of droplets with varying and well-defined payloads in an automated fashion. In addition, the use of microfluidic platforms instead of conventional analysis offers low sample consumption and high analytical throughput, which allow extensive sampling of reaction parameter space. By combining different unit operations with automation, it is possible to build complex, integrated systems for monitoring multi-step kinetic processes with real-time detection.

Mixing/mass and heat transfer effects taking place in droplet-based microfluidic systems are considered one of the most important features for monitoring fast reactions. To gain access to the transient phase of enzymatic reactions, which often provides valuable information about the catalytic mechanism, new microfluidic platforms with improved time resolution and sensitivity have to be developed. Additionally, due to rapid heat transfer (the large surface area-to-volume ratio of the droplets) and the millisecond mixing, temperature-jump microfluidic platforms combined with temporally and spatially resolved imaging represent a promising approach to monitor rapid biomolecular reactions at high temperatures. Temperature-controlled droplet-based microfluidic platforms can also provide temperature-dependent single turnover or steady-state kinetic measurements for extracting thermodynamic parameters for the involved catalytic steps of an enzymatic reaction.

While fluorescence-based detection schemes provide excellent sensitivities, they are generally limited to applications involving fluorescent probes. Absorbance-based measurements would provide access to a wider range of reactions, but unfortunately, few current methods allow the sensitive measurement of absorbance within rapidly moving droplets. To address this significant limitation, DDPI has been introduced as a new tool for fast and sensitive single-point absorbance measurements in picoliter- and femtoliter-volume droplets [113]. We envision a variety of future applications for DDPI in high-throughput experimentation, which is currently dominated by fluorescence-based approaches. DDPI can enhance the capabilities of microfluidic detection platforms in terms of higher sensitivity, and could prove to be crucial to establish absorbance as a broadly used detection method for microfluidic analytical applications such as performing digital absorbance detection of enzymatic assays in femtoliter droplets.

Finally, we have presented a comprehensive list of available platforms that use DM and optical detection methods for high-throughput and sensitive analysis of enzyme kinetics. Now that researchers have a complete set of DM and detection tools, it seems to be time to conduct high-throughput and sensitive analysis of enzyme kinetics that cannot be accomplished by using standard approaches. Moreover, the development of user-friendly and highly flexible optofluidic platforms for elucidating complex biocatalytic processes that are difficult to test with conventional methods could be of interest for a wide community of researchers working in various fields of biology including enzymology and protein dynamics.

Acknowledgments The authors would like to acknowledge support from ETH Zürich.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Abbreviations *DDPI*, Differential detection photothermal interferometry; *DM*, Droplet-based microfluidics

References

1. Wienkers LC, Rock B. Multienzyme kinetics and sequential metabolism. In: Nagar S, Argikar UA, Tweedie DJ, editors. Enzyme kinetics in drug metabolism: fundamentals and applications. Totowa, NJ: Humana Press; 2014. p. 93–118.
2. Noda-Garcia L, Liebermeister W, Tawfik DS. Metabolite–enzyme coevolution: from single enzymes to metabolic pathways and networks. Annu Rev Biochem. 2018;87(1):187–216.
3. Bajpai P. Application of enzymes in the pulp and paper industry. Biotechnol Prog. 1999;15(2):147–57.
4. van Donkelaar LHG, Mostert J, Zisopoulos FK, Boom RM, van der Goot A-J. The use of enzymes for beer brewing: thermodynamic comparison on resource use. Energy. 2016;115:519–27.

5. Volpato G, Rodrigues RC, Fernandez-Lafuente R. Use of enzymes in the production of semi-synthetic penicillins and cephalosporins: drawbacks and perspectives. Curr Med Chem. 2010;17(32):3855–73.
6. Andersson HC, Charrow J, Kaplan P, Mistry P, Pastores GM, Prakesh-Cheng A, et al. Individualization of long-term enzyme replacement therapy for Gaucher disease. Gen In Med. 2005;7(2):105–10.
7. Qiu WQ, Folstein MF. Insulin, insulin-degrading enzyme and amyloid-β peptide in Alzheimer s disease: review and hypothesis. Neurobiol Aging. 2006;27(2):190–8.
8. Shi Y. A glimpse of structural biology through x-ray crystallography. Cell. 2014;159(5):995–1014.
9. Fesik SW, Zuiderweg ER, Olejniczak ET, Gampe RT Jr. NMR methods for determining the structures of enzyme/inhibitor complexes as an aid in drug design. Biochem Pharmacol. 1990;40(1):161–7.
10. Vonck J, Mills DJ. Advances in high-resolution cryo-EM of oligomeric enzymes. Curr Opin Struct Biol. 2017;46:48–54.
11. Acker MG, Auld DS. Considerations for the design and reporting of enzyme assays in high-throughput screening applications. Perspect Sci. 2014;1(1):56–73.
12. Ishikawa K, Nakatani H, Katsuya Y, Fukazawa C. Kinetic and structural analysis of enzyme sliding on a substrate: multiple attack in β-amylase. Biochemistry. 2007;46(3):792–8.
13. Johnson KA. A century of enzyme kinetic analysis, 1913 to 2013. FEBS Lett. 2013;587(17):2753–66.
14. Sackmann EK, Fulton AL, Beebe DJ. The present and future role of microfluidics in biomedical research. Nature. 2014;507:181–9.
15. Elvira KS. i Solvas XC, Wootton RCR, deMello AJ. The past, present and potential for microfluidic reactor technology in chemical synthesis. Nat Chem. 2013;5:905–15.
16. de Mello AJ. Control and detection of chemical reactions in microfluidic systems. Nature. 2006;442:394–402.
17. Velve-Casquillas G, Le Berre M, Piel M, Tran PT. Microfluidic tools for cell biological research. Nano Today. 2010;5(1):28–47.
18. Easley CJ, Karlinsey JM, Bienvenue JM, Legendre LA, Roper MG, Feldman SH, et al. A fully integrated microfluidic genetic analysis system with sample-in–answer-out capability. Proc Natl Acad Sci U S A. 2006;103(51):19272–7.
19. Miller OJ, Harrak AE, Mangeat T, Baret J-C, Frenz L, Debs BE, et al. High-resolution dose–response screening using droplet-based microfluidics. Proc Natl Acad Sci U S A. 2012;109(2):378–83.
20. Kim S, De Jonghe J, Kulesa AB, Feldman D, Vatanen T, Bhattacharyya RP, et al. High-throughput automated microfluidic sample preparation for accurate microbial genomics. Nat Commun. 2017;8:13919–28.
21. Song H, Tice JD, Ismagilov RF. A microfluidic system for controlling reaction networks in time. Angew Chem Int Ed. 2003;42(7):768–72.
22. Shang L, Cheng Y, Zhao Y. Emerging droplet microfluidics. Chem Rev. 2017;117(12):7964–8040.
23. Hatzimanikatis V, Li C, Ionita JA, Broadbelt LJ. Metabolic networks: enzyme function and metabolite structure. Curr Opin Struct Biol. 2004;14(3):300–6.
24. Stepankova V, Bidmanova S, Koudelakova T, Prokop Z, Chaloupkova R, Damborsky J. Strategies for stabilization of enzymes in organic solvents. ACS Catal. 2013;3(12):2823–36.
25. Xu B-L, Dai M, Chen Y, Meng D, Wang Y, Fang N, et al. Improving the thermostability and activity of a thermophilic subtilase by incorporating structural elements of its psychrophilic counterpart. Appl Environ Microbiol. 2015;81(18):6302–13.
26. Kokkonen P, Koudelakova T, Chaloupkova R, Daniel L, Prokop Z, Damborsky J. Structure-function relationships and engineering of haloalkane dehalogenases. In: Rojo F, editor. Aerobic utilization of hydrocarbons, oils and lipids. Cham: Springer International Publishing; 2017. p. 1–21.
27. Gielen F, Hours R, Emond S, Fischlechner M, Schell U, Hollfelder F. Ultrahigh-throughput–directed enzyme evolution by absorbance-activated droplet sorting (AADS). Proc Natl Acad Sci U S A. 2016;113(47):E7383–E9.
28. Robinson PK. Enzymes: principles and biotechnological applications. Essays Biochem. 2015;59:1–41.
29. Koshland DE. The key–lock theory and the induced fit theory. Angew Chem Int Ed. 1995;33(23-24):2375–8.
30. Csermely P, Palotai R, Nussinov R. Induced fit, conformational selection and independent dynamic segments: an extended view of binding events. Trends Biochem Sci. 2010;35(10):539–46.
31. Gao J, Ma S, Major DT, Nam K, Pu J, Truhlar DG. Mechanisms and free energies of enzymatic reactions. Chem Rev. 2006;106(8):3188–209.
32. Schramm VL. Enzymatic transition state theory and transition state analogue design. J Biol Chem. 2007;282(39):28297–300.
33. Maenpuen S, Amornwatcharapong W, Krasatong P, Sucharitakul J, Palfey BA, Yuthavong Y, et al. Kinetic mechanism and the rate-limiting step of Plasmodium vivax serine hydroxymethyltransferase. J Biol Chem. 2015;290(13):8656–65.
34. Watt ED, Shimada H, Kovrigin EL, Loria JP. The mechanism of rate-limiting motions in enzyme function. Proc Natl Acad Sci U S A. 2007;104(29):11981–6.
35. Zhang D, Kovach IM, Sheehy JP. Locating the rate-determining step(s) for three-step hydrolase-catalyzed reactions with dynafit. Biochim Biophys Acta. 2008;1784(5):827–33.
36. Michaelis L. Menten M. Biochemistry. 1913:333–69.
37. Briggs GE, Haldane JBS. A note on the kinetics of enzyme action. Biochem J. 1925;19(2):338–9.
38. Begley TP, Johnson KA. Transient state enzyme kinetics. In: Begley TP, editor. Enzeyme kinetics: principles and methods. In Wiley Encyclopedia of Chemical Biology; 2008. p. 1–9.
39. Sassa A, Beard WA, Shock DD, Wilson SH. Steady-state, pre-steady-state, and single-turnover kinetic measurement for DNA glycosylase activity. J Visual Exper: JoVE. 2013;78:50695–702.
40. Prokop Z, Monincová M, Chaloupková R, Klvaňa M, Nagata Y, Janssen DB, et al. Catalytic mechanism of the haloalkane dehalogenase LinB from Sphingomonas paucimobilis UT26. J Biol Chem. 2003;278(46):45094–100.
41. Goličnik M. Exact and approximate solutions for the decades-old Michaelis-Menten equation: progress-curve analysis through integrated rate equations. Biochem Mol Biol Educ. 2011;39(2):117–25.
42. Johnson KA. Fitting enzyme kinetic data with KinTek Global Kinetic Explorer. Methods Enzymol. 2009;467:601–26.
43. Chen WW, Niepel M, Sorger PK. Classic and contemporary approaches to modeling biochemical reactions. Genes Dev. 2010;24(17):1861–75.
44. Kingsley LJ, Lill MA. Substrate tunnels in enzymes: structure-function relationships and computational methodology. Proteins. 2015;83(4):599–611.
45. Pegg SCH, Brown SD, Ojha S, Seffernick J, Meng EC, Morris JH, et al. Leveraging enzyme structure−function relationships for functional inference and experimental design: the structure−function linkage database. Biochemistry. 2006;45(8):2545–55.
46. Jones HBL, Wells SA, Prentice EJ, Kwok A, Liang LL, Arcus VL, et al. A complete thermodynamic analysis of enzyme turnover links the free energy landscape to enzyme catalysis. FEBS J. 2017;284(17):2829–42.
47. Benkovic SJ, Hammes GG, Hammes-Schiffer S. Free-energy landscape of enzyme catalysis. Biochemistry. 2008;47(11):3317–21.

48. Lonsdale R, Harvey JN, Mulholland AJ. A practical guide to modelling enzyme-catalysed reactions. Chem Soc Rev. 2012;41(8):3025–38.
49. Bisswanger H. Absorption and fluorescence spectroscopy. In: Bisswanger H, editor. Enzyme kinetics: principles and methods: Wiley-VCH Verlag GmbH & Co. KGaeA; 2017. p. 235–67.
50. Her C, Alonzo AP, Vang JY, Torres E, Krishnan VV. Real-time enzyme kinetics by quantitative NMR spectroscopy and determination of the Michaelis–Menten constant using the Lambert-W function. J Chem Educ. 2015;92(11):1943–8.
51. O'Brien JF, Forsman RW, Rohrbach MS. Spectrophotometric and radiometric assays of angiotensin-converting enzyme compared. Clin Chem. 1983;29(11):1990–1.
52. Ge X, Sirich TL, Beyer MK, Desaire H, Leary JA. A strategy for the determination of enzyme kinetics using electrospray ionization with an ion trap mass spectrometer. Anal Chem. 2001;73(21):5078–82.
53. Hsieh FY, Tong X, Wachs T, Ganem B, Henion J. Kinetic monitoring of enzymatic reactions in real time by quantitative high-performance liquid chromatography-mass spectrometry. Anal Biochem. 1995;229(1):20–5.
54. Nehme H, Nehme R, Lafite P, Routier S, Morin P. New development in in-capillary electrophoresis techniques for kinetic and inhibition study of enzymes. Anal Chim Acta. 2012;722:127–35.
55. Galgani F, Cadiou Y, Bocquene G. Routine determination of enzyme kinetics using plate reader. Biotechnol Bioeng. 1991;38(4):434–7.
56. Houston CT, Taylor WP, Widlanski TS, Reilly JP. Investigation of enzyme kinetics using quench−flow techniques with MALDI-TOF mass spectrometry. Anal Chem. 2000;72(14):3311–9.
57. Prokop Z, Monincová M, Chaloupkova R, Klvana M, Nagata YB, Janssen D, et al. Catalytic mechanism of the haloalkane dehalogenase LinB from Sphingomonas paucimobilis UT26. J Biol Chem. 2003;278(52):45094–100.
58. Hazzard JT, Marchesini A, Curir P, Tollin G. Direct measurement by laser flash photolysis of intramolecular electron transfer in the three-electron reduced form of ascorbate oxidase from zucchini. Biochim Biophys Acta. 1994;1208(1):166–70.
59. Font J, Torrent J, Ribó M, Laurents DV, Balny C, Vilanova M, et al. Pressure-jump-induced kinetics reveals a hydration dependent folding/unfolding mechanism of ribonuclease A. Biophys J. 2006;91(6):2264–74.
60. Phillips RS, Miles EW, McPhie P, Marchal S, Georges C, Dupont Y, et al. Pressure and temperature jump relaxation kinetics of the conformational change in Salmonella typhimurium tryptophan synthase l-serine complex: large activation compressibility and heat capacity changes demonstrate the contribution of solvation. J Am Chem Soc. 2008;130(41):13580–8.
61. Tosha T, Nomura T, Nishida T, Saeki N, Okubayashi K, Yamagiwa R, et al. Capturing an initial intermediate during the P450nor enzymatic reaction using time-resolved XFEL crystallography and caged-substrate. Nat Commun. 2017;8(1):1585–93.
62. Song H, Ismagilov RF. Millisecond kinetics on a microfluidic chip using nanoliters of reagents. J Am Chem Soc. 2003;125(47):14613–9.
63. Burke KS, Parul D, Reddish MJ, Dyer RB. A simple three-dimensional-focusing, continuous-flow mixer for the study of fast protein dynamics. Lab Chip. 2013;13(15):2912–21.
64. Kwak TJ, Nam YG, Najera MA, Lee SW, Strickler JR, Chang W-J. Convex grooves in staggered herringbone mixer improve mixing efficiency of laminar flow in microchannel. PLoS ONE. 2016;11(11):e0166068.
65. Buchegger W, Haller A, van den Driesche S, Kraft M, Lendl B, Vellekoop M. Studying enzymatic bioreactions in a millisecond microfluidic flow mixer. Biomicrofluidics. 2012;6(1):012803–9.
66. Rho HS, Hanke AT, Ottens M, Gardeniers H. Mapping of enzyme kinetics on a microfluidic device. PLoS ONE. 2016;11(4):e0153437.
67. Zhang H, Nie S, Etson CM, Wang RM, Walt DR. Oil-sealed femtoliter fiber-optic arrays for single molecule analysis(). Lab Chip. 2012;12(12):2229–39.
68. Guan ZC, Zou Y, Zhang MX, Lv JQ, Shen HL, Yang PY, et al. A highly parallel microfluidic droplet method enabling single-molecule counting for digital enzyme detection. Biomicrofluidics. 2014;8(1):014110 -1-13.
69. Dressler OJ, Maceiczyk RM, Chang S-I. deMello AJ. Droplet-based microfluidics: enabling impact on drug discovery. J Biomol Screen. 2013;19(4):483–96.
70. Brouzes E, Medkova M, Savenelli N, Marran D, Twardowski M, Hutchison JB, et al. Droplet microfluidic technology for single-cell high-throughput screening. Proc Natl Acad Sci. 2009;106(34):14195–200.
71. Shestopalov I, Tice JD, Ismagilov RF. Multi-step synthesis of nanoparticles performed on millisecond time scale in a microfluidic droplet-based system. Lab Chip. 2004;4(4):316–21.
72. Bardin D, Kendall MR, Dayton PA, Lee AP. Parallel generation of uniform fine droplets at hundreds of kilohertz in a flow-focusing module. Biomicrofluidics. 2013;7(3):034112.
73. Ahn K, Agresti J, Chong H, Marquez M, Weitz DA. Electrocoalescence of drops synchronized by size-dependent flow in microfluidic channels. Appl Phys Lett. 2006;88(26):264105.
74. Link DR, Anna SL, Weitz DA, Stone HA. Geometrically mediated breakup of drops in microfluidic devices. Phys Rev Lett. 2004;92(5):054503.
75. Niu X, Gielen F, Edel JB. deMello AJ. A microdroplet dilutor for high-throughput screening. Nat Chem. 2011;3:437–42.
76. Laval P, Lisai N, Salmon J-B, Joanicot M. A microfluidic device based on droplet storage for screening solubility diagrams. Lab Chip. 2007;7(7):829–34.
77. Zhu Y, Fang Q. Analytical detection techniques for droplet microfluidics—a review. Anal Chim Acta. 2013;787:24–35.
78. Gu S, Lu Y, Ding Y, Li L, Zhang F, Wu Q. Droplet-based microfluidics for dose–response assay of enzyme inhibitors by electrochemical method. Anal Chim Acta. 2013;796:68–74.
79. Hertzog DE, Ivorra B, Mohammadi B, Bakajin O, Santiago JG. Optimization of a microfluidic mixer for studying protein folding kinetics. Anal Chem. 2006;78(13):4299–306.
80. Ivorra B, Ferrández MR, Crespo M, Redondo JL, Ortigosa PM, Santiago JG, et al. Modelling and optimization applied to the design of fast hydrodynamic focusing microfluidic mixer for protein folding. J Math Ind. 2018;8(1):4.
81. Borgia MB, Borgia A, Best RB, Steward A, Nettels D, Wunderlich B, et al. Single-molecule fluorescence reveals sequence-specific misfolding in multidomain proteins. Nature. 2011;474(7353):662–5.
82. Gorris HH, Rissin DM, Walt DR. Stochastic inhibitor release and binding from single-enzyme molecules. Proc Natl Acad Sci U S A. 2007;104(45):17680–5.
83. Zhu P, Wang L. Passive and active droplet generation with microfluidics: a review. Lab Chip. 2017;17(1):34–75.
84. Teh S-Y, Lin R, Hung L-H, Lee AP. Droplet microfluidics. Lab Chip. 2008;8(2):198–220.
85. Chong ZZ, Tan SH, Gañán-Calvo AM, Tor SB, Loh NH, Nguyen N-T. Active droplet generation in microfluidics. Lab Chip. 2016;16(1):35–58.
86. Anna SL, Bontoux N, Stone HA. Formation of dispersions using "flow focusing" in microchannels. Appl Phys Lett. 2003;82(3):364–6.
87. Hong Y, Wang F. Flow rate effect on droplet control in a co-flowing microfluidic device. Microfluid Nanofluid. 2007;3(3):341–6.

88. Ofner A, Moore DG, Rühs PA, Schwendimann P, Eggersdorfer M, Amstad E, et al. High-throughput step emulsification for the production of functional materials using a glass microfluidic device. Macromol Chem Phys. 2017;218(2):1600472.
89. Thorsen T, Roberts RW, Arnold FH, Quake SR. Dynamic pattern formation in a vesicle-generating microfluidic device. Phys Rev Lett. 2001;86(18):4163–6.
90. Utada AS, Lorenceau E, Link DR, Kaplan PD, Stone HA, Weitz DA. Monodisperse double emulsions generated from a microcapillary device. Science. 2005;308(5721):537–41.
91. Terekhov SS, Smirnov IV, Stepanova AV, Bobik TV, Mokrushina YA, Ponomarenko NA, et al. Microfluidic droplet platform for ultrahigh-throughput single-cell screening of biodiversity. Proc Natl Acad Sci U S A. 2017;114(10):2550–5.
92. Arayanarakool R, Shui L, Kengen S, Van den Berg A, Eijkel J. Single-enzyme analysis in a droplet-based micro- and nanofluidic system. Lab Chip. 2013;13(10):1955–62.
93. Baret J-C. Surfactants in droplet-based microfluidics. Lab Chip. 2012;12(3):422–33.
94. Gruner P, Riechers B, Chacòn Orellana LA, Brosseau Q, Maes F, Beneyton T, et al. Stabilisers for water-in-fluorinated-oil dispersions: key properties for microfluidic applications. Curr Opin Colloid Interface Sci. 2015;20(3):183–91.
95. Bibette J, Leal-Calderon F, Poulin P. Rep Pros Phys. 1999;62: 696–1033.
96. Hess D, Rane A. deMello AJ, Stavrakis S. High-throughput, quantitative enzyme kinetic analysis in microdroplets using stroboscopic epifluorescence imaging. Anal Chem. 2015;87(9):4965–72.
97. Schmitz CHJ, Rowat AC, Koster S, Weitz DA. Dropspots: a picoliter array in a microfluidic device. Lab Chip. 2009;9(1):44–9.
98. Hatch AC, Fisher JS, Tovar AR, Hsieh AT, Lin R, Pentoney SL, et al. 1-Million droplet array with wide-field fluorescence imaging for digital PCR. Lab Chip. 2011;11(22):3838–45.
99. Guo F, Lapsley MI, Nawaz AA, Zhao Y, Lin S-CS, Chen Y, et al. A droplet-based, optofluidic device for high-throughput, quantitative bioanalysis. Anal Chem. 2012;84(24):10745–9.
100. Dittrich PS, Jahnz M, Schwille P. A new embedded process for compartmentalized cell-free protein expression and on-line detection in microfluidic devices. ChemBioChem. 2005;6(5):811–4.
101. Jeffries GDM, Lorenz RM, Chiu DT. Ultrasensitive and high-throughput fluorescence analysis of droplet contents with orthogonal line confocal excitation. Anal Chem. 2010;82(23):9948–54.
102. Rane TD, Puleo CM, Liu KJ, Zhang Y, Lee AP, Wang TH. Counting single molecules in sub-nanolitre droplets. Lab Chip. 2010;10(2):161–4.
103. Srisa-Art M, de Mello AJ, Edel JB. High-Throughput DNA droplet assays using picoliter reactor volumes. Anal Chem. 2007;79(17):6682–9.
104. Srisa-Art M, de Mello AJ, Edel JB. High-efficiency singlee-molecule detection within trapped aqueous microdroplets. J Phys Chem B. 2010;114(48):15766–72.
105. Baret JC, Miller OJ, Taly V, Ryckelynck M, El-Harrak A, Frenz L, et al. Fluorescence-activated droplet sorting (FADS): efficient microfluidic cell sorting based on enzymatic activity. Lab Chip. 2009;9(13):1850–8.
106. Joensson HN, Samuels ML, Brouzes ER, Medkova M, Uhlen M, Link DR, et al. Detection and analysis of low-abundance cell-surface biomarkers using enzymatic amplification in microfluidic droplets. Angew Chem Int Ed. 2009;48(14):2518–21.
107. Agresti JJ, Antipov E, Abate AR, Ahn K, Rowat AC, Baret JC, et al. Ultrahigh-throughput screening in drop-based microfluidics for directed evolution (vol 170, pg 4004, 2010). Proc Natl Acad Sci U S A. 2010;107(9):4004–9.
108. Petersen NJ, Mogensen KB, Kutter JP. Performance of an in-plane detection cell with integrated waveguides for UV/Vis absorbance measurements on microfluidic separation devices. Electrophoresis. 2002;23(20):3528–36.
109. Deal KS, Easley CJ. Self-regulated, droplet-based sample chopper for microfluidic absorbance detection. Anal Chem. 2012;84(3): 1510–6.
110. Yang TJ, Stavrakis S. deMello A. A High-sensitivity, integrated absorbance and fluorescence detection scheme for probing picoliter-volume droplets in segmented flows. Anal Chem. 2017;89(23):12880–7.
111. Neil SRT, Rushworth CM, Vallance C, Mackenzie SR. Broadband cavity-enhanced absorption spectroscopy for real time, in situ spectral analysis of microfluidic droplets. Lab Chip. 2011;11(23):3953–5.
112. James D, Oag B, Rushworth CM, Lee JWL, Davies J, Cabral JT, et al. High-sensitivity online detection for microfluidics via cavity ringdown spectroscopy. RSC Adv. 2012;2(12):5376–84.
113. Maceiczyk RM, Hess D, Chiu FWY, Stavrakis S. deMello AJ. Differential detection photothermal spectroscopy: towards ultrafast and sensitive label-free detection in picoliter & femtoliter droplets. Lab Chip. 2017;17(21):3654–63.
114. Frenz L, Blank K, Brouzes E, Griffiths AD. Reliable microfluidic on-chip incubation of droplets in delay-lines. Lab Chip. 2009;9(10):1344–8.
115. Lee M, Collins JW, Aubrecht DM, Sperling RA, Solomon L, Ha JW, et al. Synchronized reinjection and coalescence of droplets in microfluidics. Lab Chip. 2014;14(3):509–13.
116. Bui M-PN, Li CA, Han KN, Choo J, Lee EK, Seong GH. Enzyme kinetic measurements using a droplet-based microfluidic system with a concentration gradient. Anal Chem. 2011;83(5):1603–8.
117. Wang X, Liu Z, Pang Y. Concentration gradient generation methods based on microfluidic systems. RSC Adv. 2017;7(48): 29966–84.
118. Damean N, Olguin LF, Hollfelder F, Abell C, Huck WTS. Simultaneous measurement of reactions in microdroplets filled by concentration gradients. Lab Chip. 2009;9(12):1707–13.
119. Sjostrom SL, Joensson HN, Svahn HA. Multiplex analysis of enzyme kinetics and inhibition by droplet microfluidics using picoinjectors. Lab Chip. 2013;13(9):1754–61.
120. Beneyton T, Thomas S, Griffiths AD, Nicaud J-M, Drevelle A, Rossignol T. Droplet-based microfluidic high-throughput screening of heterologous enzymes secreted by the yeast Yarrowia lipolytica. Microb Cell Factories. 2017;16(1):18.
121. Abate AR, Hung T, Mary P, Agresti JJ, Weitz DA. High-throughput injection with microfluidics using picoinjectors. Proc Natl Acad Sci U S A. 2010;107(45):19163–6.
122. Mazutis L, Baret J-C, Treacy P, Skhiri Y, Araghi AF, Ryckelynck M, et al. Multi-step microfluidic droplet processing: kinetic analysis of an in vitro translated enzyme. Lab Chip. 2009;9(20):2902–8.
123. Frenz L, El Harrak A, Pauly M, Bégin-Colin S, Griffiths AD, Baret J-C. Droplet-based microreactors for the synthesis of magnetic iron oxide nanoparticles. Angew Chem Int Ed. 2008;47(36): 6817–20.
124. Bringer MR, Gerdts CJ, Song H, Tice JD, Ismagilov RF. Microfluidic systems for chemical kinetics that rely on chaotic mixing in droplets. Philo trans Ser A, Mathem, phys, and engi sci. 2004;362(1818):1087–104.
125. Wang J, Wang J, Feng L, Lin T. Fluid mixing in droplet-based microfluidics with a serpentine microchannel. RSC Adv. 2015;5(126):104138–44.
126. Zheng B, Tice JD, Ismagilov RF. Formation of droplets of alternating composition in microfluidic channels and applications to indexing of concentrations in droplet-based assays. Anal Chem. 2004;76(17):4977–82.
127. Maillot S, Carvalho A, Vola J-P, Boudier C, Mély Y, Haacke S, et al. Out-of-equilibrium biomolecular interactions monitored by

picosecond fluorescence in microfluidic droplets. Lab Chip. 2014;14(10):1767–74.
128. Pei JA, Nie J, Kennedy RT. Parallel electrophoretic analysis of segmented samples on chip for high-throughput determination of enzyme activities. Anal Chem. 2010;82(22):9261–7.
129. Ouimet CM, D'Amico CI, Kennedy RT. Droplet sample introduction to microchip gel and zone electrophoresis for rapid analysis of protein-protein complexes and enzymatic reactions. Anal Bioanal Chem. 2019;411(23):6155–63.
130. Fradet E, Bayer C, Hollfelder F, Baroud CN. Measuring fast and slow enzyme kinetics in stationary droplets. Anal Chem. 2015;87(23):11915–22.
131. Ugrinic M, Zambrano A, Berger S, Mann S, Tang TYD, Demello A. Microfluidic formation of proteinosomes. Chem Commun. 2018;54(3):287–90.
132. Srisa-Art M, Dyson EC, Demello AJ, Edel JB. Monitoring of real-time streptavidin-biotin binding kinetics using droplet microfluidics. Anal Chem. 2008;80(18):7063–7.
133. Clausell-Tormos J, Griffiths AD, Merten CA. An automated two-phase microfluidic system for kinetic analyses and the screening of compound libraries. Lab Chip. 2010;10(10):1302–7.
134. S-u H, Nightingale AM, Niu X. Continuous measurement of enzymatic kinetics in droplet flow for point-of-care monitoring. Analyst. 2016;141(11):3266–73.
135. Mao ZM, Guo F, Xie YL, Zhao YH, Lapsley MI, Wang L, et al. Label-free measurements of reaction kinetics using a droplet-based optofluidic device. Jala-J Lab Autom. 2015;20(1):17–24.
136. Shimizu H, Mawatari K, Kitamori T. Sensitive determination of concentration of nonfluorescent species in an extended-nano channel by differential interference contrast thermal lens microscope. Anal Chem. 2010;82(17):7479–84.
137. Polshin E, Verbruggen B, Witters D, Sels B, De Vos D, Nicolai B, et al. Integration of microfluidics and FT-IR microscopy for label-free study of enzyme kinetics. Sensor Actuat B-Chem. 2014;196:175–82.
138. Gielen F, Buryska T, Van Vliet L, Butz M, Damborsky J, Prokop Z, et al. Interfacing microwells with nanoliter compartments: a sampler generating high-resolution concentration gradients for quantitative biochemical analyses in droplets. Anal Chem. 2015;87(1):624–32.
139. Wei Y, Zhu Y, Fang Q. Nanoliter quantitative high-throughput screening with large-scale tunable gradients based on a microfluidic droplet robot under unilateral dispersion mode. Anal Chem. 2019;91(8):4995–5003.
140. Lee B, Jin SH, Noh YM, Jeong SG, Jeong HH, Lee CS. Scalable static droplet array for biochemical assays based on concentration gradients. Sensor Actuat B-Chem. 2018;273:1572–8.
141. Jambovane S, Kim DJ, Duin EC, Kim S-K, Hong JW. Creation of stepwise concentration gradient in picoliter droplets for parallel reactions of matrix metalloproteinase II and IX. Anal Chem. 2011;83(9):3358–64.
142. Han Z, Li W, Huang Y, Zheng B. Measuring rapid enzymatic kinetics by electrochemical method in droplet-based microfluidic devices with pneumatic valves. Anal Chem. 2009;81(14):5840–5.