

Measuring Fast and Slow Enzyme Kinetics in Stationary Droplets

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Supporting Information

ABSTRACT: We present a new microfluidic platform for the study of enzymtatic reactions using static droplets on demand. This allows us to monitor both fast and slow reactions with the same device and minute amounts of reagents. The droplets are produced and displaced using confinement gradients, which allows the experiments to be performed without having any mean flow of the external phase. Our device is used to produce six different pairs of drops, which are placed side by side in the same microfluidic chamber. A laser pulse is then used to trigger the fusion of each pair, thus initiating a chemcial reaction. Imaging is used to monitor the time evolution of enzymatic reactions. In the case of slow reactions, the reagents are completely mixed before any reaction is



detected. This allows us to use standard Michaelis—Menten theory to analyze the time evolution. In the case of fast reactions, the time evolution takes place through a reaction-diffusion process, for which we develop a model that incorporates enzymatic reactions in the reaction terms. The theoretical predictions from this model are then compared to experiments in order to provide measurements of the chemical kinetics. The approach of producing droplets through confinement gradients and analyzing reactions within stationary drops provides an ultralow consumption platform. The physical principles are simple and robust, which suggests that the platform can be automated to reach large throughput analyses of enzymes.

M easuring the rate of biochemical reactions is a critical step in the experimental characterization of dynamic biological processes. Steady-state kinetics are widely used to characterize enzymes and benchmark them by measuring Michaelis–Menten parameters.¹⁻⁴ The number of enzymes to be characterized is steadily rising, as more enzymes are discovered in functional metagenomic studies^{5,6} or by large scale sequencing efforts,⁷ followed by bioinformatic predictions. Databases that combine sequence, structure, and functional data will play a large role in recording and mapping molecular biodiversity and have to be supplied with steady-state data.⁸ Also, rational protein design⁹ and directed evolution¹⁰ create large numbers of mutants that have to be quantitatively characterized to assess the success of the protein engineering approach. Finally, mechanistic studies focus increasingly on the catalytic effects of interactions between networks of residues, requiring characterization of substantial numbers of mutants.^{11,12}

Practical limitations of such investigations include the time resolution, the sample consumption and the detection limit of the experimental technique in use, as sketched in Figure 1. Slow reactions are usually studied in titer plates, consuming typically $1-100 \ \mu$ L of sample per well and involving reaction times ranging from few tens of seconds up to few days. Fast and presteady state reactions are studied in stopped or quenched flow machines, in which the reagents are injected in a cuvette where turbulent flow ensures a good mixing within a few ms, which comes at the price of throughput as they allow for only one reaction to be studied at a time.

In the last two decades, microfluidic devices have shown much promise as analytical tools for chemical and biochemical reactions, as their micrometric size ensures drastically reduced sample consumption, especially when droplets are used to compartmentalize reagents into femto-to-nanoliter volumes.¹³ Fast chaotic mixing of the drop contents was achieved using winding channels, reducing the time resolution to a few ms at high injection rates.¹⁴ While the residence time of moving droplets on chip is limited, formats exist that hold droplets stationary for long-term observation (e.g., parking lots^{15,16} or dead-end channels¹⁷), in wide microchannels by placing obstacles on the path of droplets,¹⁸ or by etching pockets in the channel roof to anchor them.¹⁹ The sequence of droplets can be controlled in organized 2D arrays of droplets^{20,21} or by droplet-on-demand systems.^{22,23} Different microfluidic approaches have been developed to achieve the objectives of ultralow sample consumption per reaction, multiplexing of parallel reactions on chip or scan different reaction time scales.

Scaling down the sample size is not trivial, as the dominant physical processes governing flows at the micrometer scale differ greatly from macroscale flows: Interfacial effects and viscosity dominate in smaller formats, and mixing by turbulence is difficult to achieve. An alternative is to leverage the dominant phenomena and rely on diffusion of species, rather on active mixing, to allow the reactions to take place.²⁴ In the present

Received: September 19, 2015 Accepted: November 2, 2015



Figure 1. Approaches to measurement of kinetic data and their time scales. Currently, most kinetic studies are carried out in microtiter plates (often involving manual pipetting or robotic liquid handling systems). Sample consumption can be brought down 1 μ L with this system while the observed rections last at least 10 s. For faster reactions, stopped-flow machines are used with dead-times down to 1 ms and sample consumption of a 100 μ L per run. The past decade has brought microfluidic approaches to the fore. First, reaction-diffusion patterns were used to study fast chemical reaction using minute amounts of reagents, thereby bringing the sample consumption down to 10 μ L. Next, droplets were suggested as chemical vessels, either arrange along a train of drops for fast reactions, or trapped against obstacles for long-term observations. In both cases, drops allowed bringing the sample consumption down to 10 nL or smaller, depending on the drop size. This work introduces a setup for kinetic measurements in which slow and fast reactions can be monitored using minimal amounts of reagents, only one drop of each reagent.

work we demonstrate a new setup for measuring enzymatic steady state (Michaelis–Menten) kinetics and outline the formal treatment of the data emerging from this system. Reactions are initiated by fusion of adjacent droplets and reaction progress takes place via reaction and diffusion. As shown in Figure 2, the processes that must be modeled will depend



Figure 2. Timeline of the fusion and mixing of two drops: The two regimes for the analysis of a reaction with initially separated reagents. (a) Two equally sized droplets, one containing dye, are stored in our device using capillary traps. The trap is the goggle-like pattern where the height of the channel is slightly increased. After fusion, the trap still holds the merged droplet in place. The axis labeled x denotes the distance from the original interface between the two resting droplets. An x-value of zero refers to the position of the original interface. (b) Just after fusion, a strong flow takes place but the dye remains well separated from pure water. (c) After the flow has abated the dye diffuses from high to low concentrations. Reactions faster than the diffusion time of the front are therefore modeled using a reactiondiffusion model along the droplet. (d) Once the diffusion front has reach the edge of the drop, the merged drop becomes well-mixed. Reactions taking place on time scales much longer than the diffusion time can therefore be modeled using standard kinetics.

on the rate of the reaction compared with the rate at which the molecules diffuse within the droplets. Therefore, by adapting the analysis method to the type of reaction, the platform that we present here can be used to study both slow and very fast reactions without modification of the experimental protocols. Below we demonstrate the device and show how to obtain kinetics data for both the slow and fast reaction regimes.

EXPERIMENTAL SECTION

The microfluidic device has been described previously,²² where it was used to measure the evolution of a simple chemical reaction. It consists of a rectangular test section that is connected to nine microchannels for the different fluids, as shown in Figure 3a: one channel serves as an input for the



Figure 3. (a) Global design of the device, showing the central chamber connected to the nine microchannels: One input for the oil, seven inputs for the aqueous reagents, and one exit channel. (b) Topography of the rectangular test section $(4 \times 15 \text{ mm wide})$. Height modulations are etched into the ceiling to produce, transport, and anchor droplets. The arrows correspond to the different fluids connected to the device. (c) Color micrograph of six pairs of droplets paired with a common (light blue) sample. Each droplet has a diameter of nearly 500 μ m.

external oil phase, seven channels serve to introduce the aqueous reagents, and one channel is used as an exit. The ceiling of the test section is patterned with topographical features that determine the device functionality (Figure 3b for dimensions). These features allow the production, transport,

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and pairing of droplets of the aqueous reagents in a passive manner, once the chamber is filled with oil. These "anchors and rails"¹⁹ therefore allow the droplet operations to take place without relying on the flow of the external oil phase, which is at rest for most of the experimental procedure.

The droplets are first formed, at the junction between the inlet channels and the main chamber, by step emulsification.² Since the step to inlet height ratio determines the drop size,²⁶ the value of the height ratio is fixed at 0.5 for all of the reagent channels, in order to obtain drops of equal sizes (diameter \simeq 500 μ m, volume \simeq 30 nL). To propel a drop once it is formed, a V-shaped rail, with larger depth than the rest of the chamber. is placed directly in front of each junction. As a result, the droplet spontaneously moves toward the wider part of the groove as it experiences a gradual deconfinement, in order to lower its surface energy.²⁷ In this way, droplets of type B are produced and migrate until they meet the circular anchor where they become trapped. Droplets of type A, on the other hand, reach a flat region once produced. For this reason they need to be pushed by an oil flow to reach the bottom row of the array section. The device priming protocol is demonstrated in SI, movie 1. In some cases a laser is used to help guide a particular drop into a trap, as described previously.²⁰ While this involves some manual control currently, the anchors can be aligned diagonally in future versions of the device, in order to force the drops into them passively.

Altogether, the device allows us to form six pairs of droplets in an array format. As shown on Figure 3c, the droplets from inlet A are all formed from the same solution and are paired with six droplets coming from six different solutions B1–B6. The physical principles behind the design are not limiting, and the number of pairs can, in principle, be increased indefinitely, since the interactions between droplets are very weak.¹⁹ However, the practical limitation is that each independent inlet must be connected to a different syringe, and the instrumentation around the microfluidic device can become overwhelming. Such issues can be solved, in principle, by using multiplexed control from pressure sources or preloaded devices, but we have not implemented such protocols at this stage.

SLOW REACTION: WELL-MIXED ANALYSIS

Before turning to the more complex case of fast reactions, as defined in Figure 2, we set out to show that slow reactions can be measured in our devices. For this we used the enzyme β -D-glucosidase from sweet almond and the model substrate, 4-nitrophenyl β -D-glucopyranoside, as a test case.²⁸ The reaction is modeled by a standard Michaelis–Menten formalism, in which enzyme E binds to the substrate S and then produces the product P,

$$E + S \underset{k_{\text{off}}}{\overset{k_{\text{on}}}{\rightleftharpoons}} E \cdot S \xrightarrow{k_{\text{cat}}} E + P$$
(1)

with k_{on} , k_{off} and k_{cat} being the rate constants for association, dissociation, and chemical catalysis, respectively.²⁹ We wish to measure the two constants k_{cat} and $K_{M} = (k_{cat} + k_{off})/k_{on}$ for our reaction.

Here the parallelized device provides a way to obtain six data points simultaneously on the same chip. As shown in Figure 4, six droplets containing the substrate at different concentrations $[S]_0$, are first produced from six different inlets to fill the top row of our array. Then, droplets with a fixed enzyme concentration $[E]_0$ are produced and pushed to the remaining sites



Figure 4. Reaction kinetics of β -D-glucosidase. (a) Steady state assay: the enzyme concentration is $[E]_0 = 5 \ \mu M$ and the substrate concentration increases from left to right $[S]_0 = 0-31$ mM. The gray levels are measured in the colored boxes. The scale bar is 500 μ m. (b) Time courses of the product concentration [P](t). The colored dots are extract from (a) and the solid lines are best fits with [P](t) = V_0t . (c) Variations of the initial reaction rate with the substrate concentration. The colored dots are experimental data points and the solid black line a best fit on k_{cat} and K_{M} . (d) Inhibition assay: the enzyme concentration is $[E]_0 = 5 \mu M$, the substrate concentration is $[E]_0 = 23 \text{ mM}$ and the inhibitor concentration is increased from left to right $[D]_0 = 0-1500$ nM. The gray levels are measured in the colored boxes. The scale bar is 500 μ m. (e) Time courses of the product concentration [P](t). The values for V_0 are extracted from (d) and the solid lines are best fits to the linear equation $[P](t) = V_0 t$. (f) The coloration indicates the corresponding concentrations. Variations of the initial reaction rate with the inhibition concentration. (f) The colored dots are experimental data points and the solid black line a best fit.

using an outer oil flow. This outer flow is maintained to mix the droplet contents once they have merged and, after a few minutes, a clear difference in light absorption between the drops is observed (Figure 4a).

For each of the merged droplets, a progress curve was generated by measuring the optical absorption during 10 min, and converting this signal to a concentration of the product *P*, as shown in Figure 4b. The dose response could thus be obtained by measuring the rate of the initial reaction V_0 with increasing concentrations of the substrate [S]₀. We observed that an increase in [S]₀ yielded a larger reaction rate (Figure 4c). $K_{\rm M}$ and $k_{\rm cat}$ were obtained after determination of initial rates and by fitting these rates to the classic Michaelis–Menten model, which provided the values $K_{\rm M} = 9.3 \pm 3.1$ mM and $k_{\rm cat} = 0.7 \pm 0.09$ s⁻¹. These were in good agreement with plate reader measurements ($K_{\rm M} = 9.0 \pm 1.0$ mM and $k_{\rm cat} = 0.50 \pm 0.02$ s⁻¹), as well as with previous experiments by Gielen et al.²⁸ who obtained $K_{\rm M} = 11.4 \pm 2$ mM and $k_{\rm cat} = 0.9 \pm 0.2$ s⁻¹.

In addition to measurement of the steady state parameters, $K_{\rm M}$ and $k_{\rm cat}$, we also addressed the determination of the inhibition constant $K_{\rm I}$ for the competitive inhibitor 1-deoxynojirimycin hydrochloride (denoted D), whose action is described by the following reaction:

$$DE + S \stackrel{k_{I}}{\rightleftharpoons} E + S + D \stackrel{k_{on}}{\underset{k_{off}}{\rightleftharpoons}} E \cdot S \stackrel{k_{cat}}{\longrightarrow} E + P$$
(2)

In this experiment, droplets from the enzyme solution at a fixed concentration (10 μ M) were produced and paired with droplets containing the substrate with increasing concentration of inhibitor [D]₀, while [S]₀ was kept constant ([S]₀ = 46 mM).

Again, a clear difference between the drops was observed after a few minutes, as shown in Figure 4d. The resulting initial rates V_0 decreased as the inihibitor concentration increased (Figure 4e), which yielded an inhibitor concentration for a 50% reduction of activity IC₅₀ = 180 ± 58 nM and $K_{\rm I}$ = 51 ± 16 nM, consistent with a plate reader measurement (IC₅₀ = 240 ± 13 nM and $K_{\rm I}$ = 67 ± 4 nM) and previous measurements (IC₅₀ = 110 ± 40 μ M and $K_{\rm I}$ = 36 ± 13 nM).²⁸

■ FAST REACTION: REACTION-DIFFUSION ANALYSIS

In order to analyze fast reactions, we must revisit the Michaelis–Menten formalism in the context reaction-diffusion systems. We begin by writing the reaction-diffusion equations, following Ristenpart et al.,³⁰ then make simplifications that are relevant to our experiments in order to obtain an analytical approximation of the reaction evolution.

Michaelis–Menten Kinetics for Initially Separated Species. In traditional experimental conditions, the substrate and enzyme are well mixed, and the initial rate of the reaction V_0 is measured under conditions where (i) the substrate concentration exceeds the enzyme concentraiton ($[S]_0 \gg [E]_0$), (ii) the enzyme is not consumed by the reaction so that, at all times, $[E]_0 = [E] + [E \cdot S]$, and (iii) the intermediate complex forms as fast as it degrades so that, at all times, $[E][S] = K_M[E \cdot S]$. By then, writing the initial rate of the reaction as

$$V_{0} = \frac{k_{\text{cat}[\text{E}]_{0}[\text{S}]_{0}}}{K_{\text{M}} + [\text{S}]_{0}}$$
(3)

the two parameters (k_{cat}) and (K_M) are obtained from measurements of V_0 at different values of $[E]_0$ and $[S]_0$.

Conversely, when the reagents E and S are initially separated, they must diffuse toward the region where they will encounter each other and react. As a result, the overall progress of the reaction will be determined by an interplay between reaction and diffusion. This replaces the ordinary differential equations (ODEs) that describe the well-mixed system by partial differential equations (PDEs) that are both functions of time and space. In our formalism we will keep only one spatial dimension, which corresponds to the axis labeled x in Figure 2a. Indeed, the chemical species transport mostly takes place along this direction since it corresponds to the highest concentration gradients.

We can therefore write this reaction-diffusion system as a set of PDEs in time t and one spatial direction x:

$$\frac{\partial[\mathbf{E}]}{\partial t} = D_{\mathbf{E}} \frac{\partial^{2}[\mathbf{E}]}{\partial x^{2}} - k_{\mathrm{on}}[\mathbf{E}][\mathbf{S}] + k_{\mathrm{off}}[\mathbf{E}\cdot\mathbf{S}] + k_{\mathrm{cat}}[\mathbf{E}\cdot\mathbf{S}]$$
(4a)

$$\frac{\partial[S]}{\partial t} = D_{S} \frac{\partial^{2}[S]}{\partial x^{2}} - k_{on}[E][S] + k_{off}[E \cdot S]$$
(4b)

$$\frac{\partial[\mathbf{E}\cdot\mathbf{S}]}{\partial t} = D_{\mathbf{E}} \frac{\partial^{2}[\mathbf{E}\cdot\mathbf{S}]}{\partial x^{2}} + k_{\mathrm{on}}[\mathbf{E}][\mathbf{S}] - k_{\mathrm{off}}[\mathbf{E}\cdot\mathbf{S}] - k_{\mathrm{cat}}[\mathbf{E}\cdot\mathbf{S}]$$
(4c)

$$\frac{\partial[\mathbf{P}]}{\partial t} = D_{\mathrm{S}} \frac{\partial^{2}[\mathbf{P}]}{\partial x^{2}} + k_{\mathrm{cat}}[\mathbf{E} \cdot \mathbf{S}]$$
(4d)

with $D_{\rm E}$ and $D_{\rm S}$ the diffusion coefficients of E and S. Here we have distinguished the large molecules (E and E·S) from the small ones (S and P), and assumed a common diffusion coefficient for each type: $D_{\rm E}$ for both large species, and $D_{\rm S}$ for the

small. The size contrast between the enzyme molecules and their substrates and products justifies this simplification.

This system of equations is subject to initial conditions on all concentrations, which we impose by setting the initial distributions of E and S as two Heaviside step functions \mathcal{H} , that is, [S] = 1 for x < 0 and [S] = 0 for x > 0, while [E] = 0 for x < 0 (Figure 2). In addition, the initial conditions for intermediate complex $[E \cdot S]$ and the product [P] are taken as zero for all positions x. This gives the following initial conditions for the problem:

$$[S](x, 0) = [S]_0 \mathcal{H}(x \le 0), \ [E](x, 0) = [E]_0 \mathcal{H}(x \ge 0),$$
$$[E \cdot S](x, 0) = [P](x, 0) = 0$$
(5)

The mathematical description of the different concentration fields is finally closed by assuming no-flux boundary conditions at the edges of the droplet $x = \pm l$.

The system of eqs 4a–4d) is readily solved numerically using finite differences. Such a procedure, however, fails to provide physical insight into the underlying processes, in addition to being impractical for routine measurements. A complementary approach would be to derive an approximate analytical solution to the equations that reduces the analysis to a measurement of a single variable in time. This is what is done below by revisiting the assumptions that underly the standard Michaelis–Menten theory in a reaction-diffusion framework.

Analytical Solution. Comparing the experimental measurements with the theoretical model consists of comparing the profile of the reaction product as a function of space and time. This process is described by eq 4d, which is a diffusion equation for [P] with a source term that depends on $[E \cdot S]$. By estimating this source term we can therefore provide an analytical approximation of [P](x, t), which we will do below.

By applying the three Michaelis–Menten hypotheses at a local level, we can reduce the set of equations as follows:

(i) When [S]₀ ≫ [E]₀, we assume that the substrate distribution is nearly insensitive to the chemical reaction so that the reaction terms in the substrate mass balance (4b) can be neglected. Then, the substrate concentration field is solely given by its diffusion:

$$[S](x, t) = \frac{[S]_0}{2} \operatorname{erfc}\left(\frac{x}{\sqrt{4D_S t}}\right)$$
(6)

with erfc the complementary error function.

(ii) The overall conservation of the enzyme implies that the total amount of enzyme $[E]_{tot} = [E] + [E \cdot S]$ is constant. Indeed, summing eqs 4a and 4c yields a diffusion equation on E_{tot} whose solution is

$$[E]_{tot}(x, t) = [E](x, t) + [E \cdot S](x, t)$$
$$= \frac{[E]_0}{2} \operatorname{erfc}\left(-\frac{x}{\sqrt{4D_E t}}\right)$$
(7)

(iii) Finally, we assume that the steady state situation that governs the formation and degradation of the intermediate complex E·S applies at every instant and position. This translates into a local equilibrium between the concentrations of the enzyme, the substrate and the intermediate complex:

$$[\mathbf{E}](x,t)[\mathbf{S}](x,t) = K_{\mathbf{M}}[\mathbf{E}\cdot\mathbf{S}](x,t)$$
(8)

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Now combining eqs 7 and 8 yields the relation $[E \cdot S] = ([E]_{tot}[S])/(K_M + [S])$, which is the source term we were searching for in eq 4d. It is written in terms of two error functions (eqs 6 and 7) that correspond to two diffusing but nonreacting species. Putting all this together and using the shorthand notation $\xi_S = \operatorname{erfc}\left(\frac{x}{\sqrt{4D_S t}}\right)$ and $\xi_E = \operatorname{erfc}\left(-\frac{x}{\sqrt{4D_E t}}\right)$, the mass balance of eq 4d becomes

$$\frac{\partial[\mathbf{P}]}{\partial t} = D_{\mathrm{S}} \frac{\partial[\mathbf{P}]}{\partial x^{2}} + \frac{k_{\mathrm{cat}}[\mathbf{E}]_{0}}{2} \frac{\xi_{\mathrm{E}} \cdot \xi_{\mathrm{S}}}{\frac{2K_{\mathrm{M}}}{S_{0}} + \xi_{\mathrm{S}}}$$
(9)

Through these simplifications, the system of four coupled nonlinear equations (eqs 4a-4d) has been replaced by a single diffusion equation with a known source term (eq 9). The spatiotemporal dependence of this source term is limited to a dependence on a diffusive similarity variable $\eta = x/\sqrt{4D_S t}$ and on the ratio of the diffusion coefficients $\rho = (D_S/D_E)^{1/2}$. Its amplitude depends on the chemistry through the chemical constants k_{cat} and K_{M} .

This motivates us to search for a general solution to eq 9 of the form $[P](x, t) = \mathcal{A}(t) \cdot p(\eta)$, where $p(\eta)$ has a self-similar shape that does not vary in time. The time-dependent amplitude $\mathcal{A}(t)$ is expected, from scaling analysis, to have the form $\mathcal{A}(t) = k_{cat}E_0t$. Injecting this form into eq 9 and writing $k = 2K_M/[S]_0$ yields the following equation for p:

$$p''(\eta) + 2\eta p'(\eta) - 4p(\eta) + 2\frac{\operatorname{erfc}(-\rho\eta)\operatorname{erfc}(\eta)}{k + \operatorname{erfc}(\eta)} = 0$$
(10)

subject to the boundary conditions $p(\pm \infty) = 0$. Equation 10 must be solved numerically, which yields a series of bell-shaped curves $p(\eta)$ that depend on $\rho = (D_S/D_E)^{1/2}$ and $k = 2K_M/[S]_0$. Focusing on the variations of p with k, we find that the shape of p is preserved but that its amplitude $p_m = \max(p)$ shows a strong dependence on k, as shown in Figure 5.



Figure 5. Simulations of *p* based on the analytical model of eq 10. These curves are obtained by solving eq 10. (a) Self-similar form *p* as a function of the similarity variable η , for varying KM/[S]₀. (b) Variations of the maximum of *p* as a function of KM/[S]₀.

The above mathematical analysis served to replace the PDE for [P] by an ODE for p. In practice, however, the measured concentration of the product in an actual experiment would depend on both \mathcal{A} and p, such that the overall amplitude of the measured signal would grow linearly in time with a growth rate as $V_0 = [E]_0 k_{cat} p_m$. In this sense, the plot of Figure 5 closely resembles a Michaelis–Menten curve for the well-mixed case: the growth rate V_0 increases linearly with [S]₀ for [S]₀ $\ll K_M$, before saturating at $[E]_0 k_{cat}$ as $[S]_0$ becomes larger than K_M .

COMPARING EXPERIMENT AND THEORY

The model described above can be compared with measurements in droplets, after switching to a fast enzymatic reaction that takes place faster than diffusion can mix the contents. Here we choose to study the hydrolysis of 4-methylumbelliferyl phosphate (4-MUP) catalyzed by alkaline phosphatase (AP) as a model for an enzymatic reaction well-known to be fast.^{31,32} The reaction product 4-methylumbelliferone (denoted *P*) was detected by fluorescence, using a standard epi-fluorescence setup and DAPI filters.

A basic experiment involving merging two droplets in our reaction chamber was set up: starting with one droplet containing the substrate 4-MUP (left, Figure 6a), the other droplet



Figure 6. Evolution of a fast enzymatic reaction: (a) A droplet containing a solution of MUP at 100 μ M (left-hand side) has been merged with a droplet containing a solution of AP at 25 nM (right-hand side). The fluorescence image is taken at t = 16 s. The scale bar is 500 μ m. (b) Profile of the product concentration [P] as a function of x at several time points. The solid lines are simulated from the RD system of eqs 4a-4d. h_p denotes the height of the concentration curve at each time step.

containing the enzyme AP (right). After merging, the fluorescent product P forms in the zone between the reservoirs of fresh reagents, so that a fluorescent strip emerges and widens along the merged droplet. The evolution of this strip is monitored along the axis labeled with the x coordinate, during 50 s, and translated to product concentration through an intensity calibration.

The spatiotemporal evolution thus measured is first fitted with the profiles obtained from a full simulation of the RD system of eqs 4a-4d. We find that the simulated profiles are in very good agreement with the measured profiles for the initial moments of the reaction (up to 6 s in this case, Figure 6b). However, this fitting procedure depends on four unknown parameters, K_{M} , k_{cav} , D_E , and D_S . It is therefore not expected to be very selective on the parameter values that are obtained. Nevertheless, the best fit between experiment and simulation is obtained for $K_M = 4 \ \mu M$, $k_{cat} = 85 \ s^{-1}$, in very good agreement with plate measurements for K_M ($K_M = 4 \ \mu M$ in plate), but overestimates k_{cat} by a factor of 8 ($k_{cat} = 11 \ s^{-1}$ in plate).

The next step is to verify that the analytical solution indeed agrees with the experimental data. For this, the self-similarity and the scaling of the analytical solution, predicted by eq 10, are first tested. We begin by verifying that the amplitude of the product concentration (h_p in Figure 6b) indeed increases linearly with time, for earlier times (Figure 7), which confirms



Figure 7. Reaction-diffusion experiments: (a) Product amplitude h_p as a function of time, for the experiment shown in Figure 6. The points are experimental and the solid line extracted from the definition of $V_0 = k_{cat}$ [E]₀ $p_{m\nu}$ as defined in the previous section. (b) Product amplitude, scaled by its maximum at each time step, vs the similarity variable η . Solid line is the solution of eq 10. The collapse of the experimental points confirms the self-similarity of the shape function. Here, $E_0 = 25$ nM and $S_0 = 100 \ \mu$ M. (c, d) Variations of the product amplitude initial rate V_0 with the substrate and enzyme initial concentrations, respectively. The black curves are obtained from simulations of the RD system. In all subfigures, the values of the kinetic and diffusion coefficients were obtained from a single fit of the experiment of Figure 6 by the RD model.

the expected scaling of the amplitude \mathcal{A} . Moreover, the selfsimilarity is also well confirmed, by plotting the profile of [P], normalized by h_p at every time step versus similarity variable η . The curves collapse onto a master curve, which corresponds to the time-independent shape function that characterizes a given experiment.

These measurements are repeated for several values of $[S]_0$ and $[E]_0$, and the value of the initial rate is plotted, as shown in Figure 7c,d. As expected for a standard Michaelis–Menten analysis, we find that the evolution of V_0 with the substrate initial concentration yields a saturation curve, while the dependence on the enzyme initial concentration shows a linear increase.

CONCLUSIONS

Here we present a format to perform on-demand reactions between the contents of two droplets. The stationary droplet format allows the observtaion of either fast or slow reactions in the same device, without any modifications of the experimental protocol. However, while the analysis of the slow reactions is straightforward and can follow the well established models for well-mixed vessels, the analysis of faster reactions must take into account the full reaction-diffusion problem. For simple reactions, we have previously shown that comparing the experiments with numerical solutions to the reaction-diffusion front could provide a very good estimate of the reaction rate.²² In the case of enzymatc reactions, however, one must obtain the Michaelis–Menten parameteres $K_{\rm M}$ and $k_{\rm cat}$ from different elements of the experiment/numerics confrontation.

In the slow reaction case, the measurements we obtain from the droplet format reproduce the values obtained using different techniques. The same is also true of the value of the binding constant $K_{\rm M}$ for the case of fast reactions. However, we observe a large deviation in the value of the catalysis constant in the droplets, compared with the microtiter plates. We attribute this to the fact that the water-oil interface may interfere with the reaction, particularly because AP has been shown to adsorb onto the water-oil interface.³³ Such an exchange between the interface and the bulk has also been shown to modify the kinetics of a reaction by reducing the system's entropy and providing different routes for the reaction to take place.³⁴ We therefore conjecture that similar phenomena are operating in this case. In spite of this potential artifact, the measurements that we obtain from our experiments remain semiquantitative and we expect that their precision can be improved, for example by screening the interfacial effects using bovine serum albumin³⁵ or other inert proteins.

Compared with other microfluidic techniques,^{14,30} the current approach requires a single droplet to perform a measurement. Most other droplet-based approaches rely on average measurements on very large number of droplets and coflow measurements require relatively large volumes. In contrast, the droplets in our devices are produced in small numbers by relying on the unique microchannel geometry for their production and transport. This implies that the total reagent consumption remains small. Indeed, the six parallel measurements of Figure 4 are performed using below 200 nL of enzyme solution and the device requires a few minutes to be loaded.

This reduction of the sample consumption and time to obtain measurements suggests that a large number of successive screens can be performed by an automated system. For example, a robot can be used to form successive aqueous plugs of enzyme in oil^{28,36,37} and lead them into the reaction chamber. There, the plugs will break into equally sized droplets by the action of the step junctions and can be transported into the anchors by an external flow. Once the drops are merged with their counter-drops and the reaction observed, they can be evacuated for the next plug to be introduced and analyzed.

Finally, the microfluidic platform is not limited to kinetic measurements. Other applications can include simple measurements to dose chemicals in an environmental or clinical sample, or even the production of well-controlled microbatches of compounds on demand. The robustness of the droplet manipulation by confinement gradients makes this type of approach well suited for industrialization and for field or bedside applications.

METHODS

Device Fabrication. All devices were made of a PDMS block (Dow Corning Sylgard 184) sealed onto a glass slide by plasma bonding. Molds for PDMS were fabricated using dry film photoresist soft lithography techniques.³⁸ The multilayer masters were etched in stacks of Eternal Laminar negative films of thicknesses 15, 35, and 50 μ m, depending on the desired local thickness. Successive layers of photoresist were deposited using a PEAK Photo Laminator (PS320) at a temperature T = 100 °C and exposed to UV (Hamamatsu Lightningcure LC8) using a succession of masks that determine the features at each height. Once all layers were deposited and exposed, the complete device was developed by immersion in an aqueous

bath of carbonate potassium at 1% (w/w) to reveal the mold. The geometry was then verified using an optical profilometer (Zygo NewView 7100).

The mold was used to produce a PDMS channel. After plasma bonding on a glass slide, the internal channel surface was made hydrophobic using a dilute solution of 1H,1H,2H,2Hperfluorodecyltrichlorosilane (Sigma-Aldrich) in FC40 oil (3 M Fluorinert; 20 mL in 1 mL of FC40) for approximately 5 min. The channel was then rinsed with pure FC40 to remove the residue chemicals remaining in the bulk.

Device Operation. The top row of traps is first filled with a single drop of type B reagents which originate from different syringes outside the chip. To this end, the flow rate of the reagent solutions is fixed at 0.5 μ L/min, stopped once a drop has detached and then fixed to -1μ L/min in order to remove the thread from the step junction. This is performed using a Nemesys (Cetoni) syringe pump. Once the top row is occupied, six drops of the type A reagent are generated and pushed toward the bottom row of the array with an oil flow. As droplets are pushed toward the array section, a laser is focused on the water/ oil interface to help guide a given drop into a trap.²⁰ This leads to all six positions being occupied by six different pairs of drops as illustrated on Figure 3c with dyed colored droplets.

Adjacent droplets trapped in our device do not coalesce as their surface is covered with surfactant molecules. Nonetheless, a laser pulse on the droplet/droplet interface can merge two touching drops,³⁹ the fusion starting once the laser is removed. By doing so, droplets fusion can be triggered at will in our static array of paired drops.

Imaging and Optics. Imaging was performed using an inverted microscope (Nikon TE2000) equipped with epifluorescent illumination (Exfo X-cite 6210C). Two cameras were connected to the microscope side ports (Photron Fastcam 1024 PCI and Spot Insight). To manipulate droplets by laser heating, a 4f conjugate lens system was built to focus a 1480 nm continuous wave infrared laser source (Fitel Furukawa FOL1424) in the microscope focal plane.⁴⁰ Two galvanometric mirrors (Cambridge Technologies 6210H) placed on the laser path permitted beam positioning in the microchannels to be controlled by a mouse click using in-house Labview programs.

Determining IC₅₀ for the Inhibitor. The increase in absorbance was recorded for 10 min and the experiment was repeated three times. Conditions: $T = 25 \,^{\circ}C$, [PBS] = 50 mM, pH = 7. Enymes were obtained from Sigma-Aldrich. A value for $K_{\rm I}$ was extracted by fitting the normalized reaction rates $V_0/V_{\rm max}$ against the initial inhibitor concentration [I]₀ via the Cheng–Prusoff relation:

$$\frac{V_0}{V_0^{\text{max}}} = \frac{1}{1 + (D_0/\text{IC}_{50})} \quad \text{with} \quad \text{IC}_{50} = \left(1 + \frac{S_0}{K_M}\right) K_{\text{I}}$$
(11)

ASSOCIATED CONTENT

S Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.5b03567.

Supporting video (AVI).

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Notes

The authors declare the following competing financial interest(s): C.N.B. and E.F. are co-inventors on a patent filed by Ecole Polytechnique and the CNRS covering this technology.

ACKNOWLEDGMENTS

The authors acknowledge the help of Caroline Frot with microfabrication and useful discussions with Liisa van Vliet, Fabrice Gielen, Stéphanie Descroix, and Anais Ali Cherif. C.B. and F.H. are ERC Starting Investigators. The research leading to these results received funding from the European Research Council (ERC) Grant Agreement 278248 Multicell. C.D.B. was supported by the Cambridge european Trust and the BBSRC. We acknowledge funding from the EPSRC (Engineering and Physical Sciences Research Council).

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