# Chapter 4

# Enzymatic Reactions in Femtoliter Droplets

### 4.1 On-chip Generation and Manipulation of Water Droplets

#### 4.1.1 Introduction

The development of micro- and nanofluidics makes it possible to achieve an exquisite control of flow at microscale. It is thus natural to explore the possibility of putting the biomimetic compartments into microfluidic devices and to study their property individually under precisely defined conditions. The first step is the generation of the desired microreactors on demand. Second, we would like to process the information in each of them with high throughput and selectivity.

A lot of effort has been made to develop methods to generate phospholipid vesicles on chip [30, 155]. More attention has been paid to the application of two-phase immiscible system in microfluidics. Air bubbles in water, water-in-oil, or oil-in-water droplets have been the focus of a lot of groups recently (for reviews, see references [129] and [20]). With the help of suitable surfactants, micron-sized compartments can be generated and stabilized in microfluidic devices. Due to the discrete distribution of the objects of interest, this area is named "digital microfluidics." The advantage of digital microfluidics includes the high uniformity of objects' size distribution, the precise control of the microenvironment, the possibility of multiplexing and parallelization, and the small amount of reagent consumption.

In the context of biochemical investigation, water-in-oil droplets is of special attention. It is



Figure 4.1. The formation of water (red) droplets in oil (green) in a microfluidic channel. Top view is shown above and the cross section of the channels could be rectangular or rounded.

straightforward to generate these droplets in a microfluidic channel. The simplest design is a Tjunction where aqueous solution is injected into the flowing carrier oil which is immiscible with water. Figure 4.1 illustrates a typical T-junction for droplet generation. There are, however, a few issues to be concerned with when we wish to incorporate them into microfluidic devices as biomimetic reactors:

- The size of water droplets is still larger than typical prokaryotic cells or compartments in eukaryotic cells.
- The high flow rate in a microfluidic channel makes prolonged observation on the same droplet impossible if flow is not stopped; the generation of droplets is not decoupled from other functions of the device.

The main obstacle to the optimization of digital microfluidic devices is the lack of realistic physical models with predictive power. Two-phase flow involves the nonlinear interaction of surface tension and shear force. This is further coupled with the mass transfer of bulk phases and surfactants. The resulting coupled nonlinear partial differential equation can only be tackled by numerical methods [21, 22, 94]. Take droplet generation as an example, to model the process in full detail, one has to resolve the fine structure of the breakup point between the forming droplet and the bulk phase. This region becomes so small that the usual continuum approach to the problem is fundamentally

defective [141]. One essentially is confronted with a problem with vastly different scales.

Qualitative models, however, may still prove helpful in some cases. Talyor [156] suggested that the size of the droplet be determined by a dimensionless number reflecting the relative strength of shear stress with respective to surface tension. It is defined as

$$Ca = \mu \epsilon r / \gamma,$$

where  $\mu$  is the viscosity of the carrier fluid (oil),  $\epsilon$  is the shear rate in the channel, r is the radius of the droplet and  $\gamma$  is the surface tension between the two phases, that is, oil and water. Assuming that the droplets form as the result of balanced surface tension and shear force, Ca should be O(1), implying

$$r = \gamma/\mu\epsilon$$

This relation makes physical sense at least qualitatively. When the carrier fluid is more viscous, shear force is larger with the same shear rate, and the droplet has to assume a smaller radius to generate a comparable Laplace pressure. Alternatively, with a smaller surface tension, it is easier to generate smaller droplets because the penalty due to increased surface area is not so significant. Another model, provided by Whitesides and Stone, pays special attention to the geometric constraints inherent in a T-junction. To be specific, the periodic blocking of the oil flow by the aqueous streams generated a slightly higher positive backpressure in the oil channel, which swept the aqueous plug down the main channel, allowing the process to be repeated [22, 36].

With the help of available physical models and intuition, people have experimented with various clever designs to overcome the above-mentioned obstacles. For example, in order for the droplet size to reach micron or even submicron regime, a recycling channel was included to accumulate those tiny satellite droplets generated as a by-product [154]. In another study, a docking mechanism was developed to store droplets at specific positions for further prolonged treatment [124].

Here we report our result in the development of an integrated microfluidic platform with the ability of stable generation and simultaneous observation of selected micron-sized droplets.



Figure 4.2. An integrated platform for digital microfluidics. For details, see text.

#### 4.1.2 An Integrated Platform for Digital Microfluidics

Figure 4.2 shows the setup of our microfluidic workstation. A piece of PDMS device was fixed onto a microscope stage for epifluorescence and bright-field observation with 100X NA 1.3 objective. To feed fluids into the device, compressed nitrogen with pressure controlled by three different regulators (R) was injected into Teflon jars (J) containing aqueous solution (white or orange J) or oil (green J). Fluids entered the device through Tygon tubing and 23-gauge stainless needle. A critical function of the device is the ability of stopping the movement of droplet for prolonged observation. This was achieved by applying a pressure of 40 psi, managed by a solenoid valve, to the control port. The aqueous droplets usually contained fluorophores excited by a mercury–xenon lamp for fluorescence microscopy. After passing through a set of ND filters and excitation filter, the excitation light was deflected by a dichroic mirror (D) and then collimated by the objective onto the sample, and the resulting fluorescence was collected by the same objective, cleaned by emission filter, and finally recorded by a cooled CCD camera (Cool Snap). For measurements of higher time resolution, a photon-counting module (PCM) was employed to measure the photon flux due to emission. A



Figure 4.3. Layout of microfluidic devices.

mechanical shutter (S) was added between ND filter and light source to reduce photobleaching of fluorophores. The whole system was controlled by two computers (PC1 and PC2). PC1 was responsible for the collection and analysis of photon-counting data, and PC2 was mainly devoted to the control of Cool Snap. Both PCs had their own mechanism to operate the shutter via SC Lambda controller (L) and solenoid valve via a home-built relay board (B) (for details, see section 4.1.3).

The layout of the device is rendered in figure 4.3. Two aqueous inlets introduced reactants into the oil phase to form mother plugs. Reaction mixture was mixed in the chaotic mixer before daughter droplets were splitted from mother plugs at the second junction. Pneumatic pressure could stop the movement of daughter droplets by sealing the side channel at the control button. Other parts of the device was not affected by this action, so new droplets were generated in the side channel once the control button was released again.

#### 4.1.3 Instrument Control and Data Acquisition

All the electronic components in figure 4.2 were controlled by home-built softwares with graphical user interface (GUI) written in either Matlab 2007b or Labview 8.5. Matlab is a scripting language widely used in physics and engineering for its extensive computation library. It has sufficient support for GUI construction, but users have to modify the code to suit their needs. As such, a basic understanding of object-oriented programming is required. The learning curve, however, is not as steep as it seems. Furthermore, it is easy to incorporate complicated data processing routines into a new program. Labview, in contrast, offers a programing environment without the need of code writing. Labview is specially designed for instrument control and there is a huge library of device drivers for end users. However, one has to call Matlab or other languages for involved computational tasks.

The control signal widely used in our study was variable or binary DC voltage. We used a USB analog output board (USB-3103, Measurement Computing, Norton, MA) to generate required voltage ranging from 0 to 10 V with 16-bit resolution. Not all instruments had an ideal interface ready to take the signal source, so some basic electronic shop techniques were applied to build some simple circuit boxes.

Metamorph was used to save the images captured by Cool Snap. Data from the photon-counting module was acquired by a digital counter on a PCI-6014 multifunction data acquisition board (National Instruments, Austin, TX) which is controlled by Labview.

The following subsections give the details of the instrument control and data acquisition systems.

#### 4.1.3.1 Pressure Regulator

A precise control of the gas pressure applied to the solution jars is essential to the establishment of balanced flow and fine tuning of droplet size. One Marshal-Bellofram pressure regulator (Series 2000) was dedicated to each fluid jar to achieve independent control of the flow rates in different inlets. 60-psi nitrogen gas was supplied to the regulator, and the output gas pressure was controlled by DC voltage ranging from 0 to 10 V with a resolution of less than 0.5 mV. The voltage was conveniently generated by USB-3103 according to user's specification. A Matlab program was written with a

	White	
Update	Orange Orange	Valve Activation Voltage
	Green Green	

Figure 4.4. The user interface of the Matlab program to control pressure regulators.

user interface as in figure 4.4. In our system, there were three color-coded inlets: white, orange, and green. The white and orange inlets were aqueous channels, and the latter was usually dedicated to the one with fluorescent solutions. The green inlet was reserved for oil phase. To use the program, one first click the "Initialize AO" button to establish a connection between the computer and USB-3103. After typing desired voltages into the corresponding boxes, clicking "update" would generate the voltage signal and induce flow. A button "rezero" stopped all the flow by resetting the voltages to zero. A more advanced version of the control program is shown in figure 4.5. In addition to live monitoring of output voltages, a slow update function was implemented to reduce the pressure "shock" when big changes of voltage had to be made. This was achieved by specifying the step size and total duration for the voltage update.

#### 4.1.3.2 Photon Counting Module

The output of photon-counting module is a transistor-transistor logic (TTL) pulse train with  $\sim 10$  ns width, which can be recorded by a digital counter on a PCI-6014 multifunction data acquisition board. The number of photons arriving during a specified time period equals the difference of two counter readings at the beginning and the end of the period. A Labview program (figure 4.6) was

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Figure 4.5. The user interface of a more advanced Matlab program to control pressure regulators.

written to set the bin time and length of acquisition.

#### 4.1.3.3 Mechanical Shutter

The mechanical shutter was managed by a smart shutter controller which talked to both Metamorph and general TTL signal source. In the first case, the opening and closing of the shutter could be synchronized with the exposure of CCD array on Cool Snap. In the latter, any TTL pulse could be used to trigger the shutter. This is useful for coordinating the reading of the counter and the status of the shutter.

#### 4.1.3.4 Solenoid Valve

The activation of control valve was realized by opening the solenoid valve between the device and the pressure source. Because the available voltage source did not have enough current to activate the solenoid, a relay circuit, under the control of available voltage output, was built to supply the required current.

The relay circuit is detailed in figure 4.7. The solenoid valve needs 12 VDC and at least 250 mA to activate, and this can be provided by a commercial power adapter (ELPAC-WM113). Unfortunately, no switch is provided with the power source, so a Reed relay was inserted between the solenoid and the power source to control the application of voltage. The available relay module (W171-DIP-7)

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Figure 4.6. Labview program to control the counter.

needs an input voltage of 5 V and at least 10 mA which is bigger than what is available from either USB-3103 or TTL output signal. To amplify the current, a transistor (2N3904) was employed. Because  $h_{FE} > 100$  for the transistor, when 5 V signal is applied to the base, a base current of 0.42 mA (= 5 V/12 k $\Omega$ ) generates a collector current of 42 mA, sufficient to close the Reed relay and activate the solenoid. To buffer the sudden change of magnetic field in Reed relay and solenoid, two diodes (1N4001) were added in appropriate places to protect the transistor and external IC board (for example, USB-3103). Additional resistors were also added to attenuate the current flowing through the solenoid. Alternatively, a Darlington pair may be used as a switch to control the application of 12 VDC power supply. This design (figure 4.8) is conceptually simpler than the one in figure 4.7. In either way, a low current 5 V signal could generate the required 12 V source with a current high enough to open the solenoid valve.

In real experiments, the valve was controlled by any of the three voltage sources for different purposes. The simplest scenario was opening the valve from USB-3103 manually from the graphical user interface in figure 4.4 or 4.5. This was useful for device testing when timing was not important. Alternatively, the TTL-out pulse from shutter controller was used to open the solenoid and thus



Figure 4.7. Relay circuit to control the opening of solenoid valve.



Figure 4.8. Darlington pair to control the opening of solenoid valve.

stop the flow in side channel. In this way, the valve was synchronized with the image acquisition. This was normally used when a time series of fluorescence images was taken to monitor the enzyme kinetics in static droplets. Note that the pressure in the control valve was preserved for about 30 s even after the solenoid valve was closed, so the sequential opening and closing of the solenoid due to the multiple activation of shutter did not change the pressure in control valve significantly over time. Finally, the valve could be controlled by the result of photon counting. The control valve was closed once the photon number was above a certain threshold, which signified the arrival of a droplet in the detection volume. Of course, because of the delay of a couple tens of milliseconds in the activation of solenoid valve, there was little chance to capture the same droplet that was responsible for the threshold crossing. But a tunable delay could be intentionally inserted after the threshold-crossing event such that when the flow stopped, there was a significant chance for a droplet to land at the focal volume. This was achievable due to the regular spacing and stable speed of the droplet train. Indeed, as illustrated in figure 4.9, automatic closing of control valve using the threshold-crossing mechanism guaranteed that in most cases, droplets landed reproducibly at the same position (inside the white circle) after the flow is stopped. Figure 4.10 shows the Labview interface to realize this control mechanism.

# 4.2 Shear-driven Redistribution of Surfactant Affects Enzyme Activity in Well-mixed Femtoliter Droplets<sup>\*</sup>

#### Abstract

We developed a microfluidic platform for splitting well-mixed, femtoliter droplets from larger waterin-oil plugs, where the sizes of the daughter droplets were not limited by channel width. These droplets were separated from mother plugs at a microfabricated T-junction, which enabled the study of how increased confinement affected enzyme kinetics in droplets 4–10 µm in diameter. Initial rates

<sup>\*</sup>This section is adapted from Y. Liu, S.-Y. Jung, and C. P. Collier, Anal. Chem., 81:4922–4928, 2009. Copyright 2009 American Chemical Society.



Figure 4.9. Using threshold-crossing mechanism to automatic stop the flow guaranteed that in most cases (13 out of 20 shown here) droplets landed at roughly the same position. This was important to increase data acquisition efficiency when PMT, with a confined detection volume, was used as the detector.

for enzyme catalysis in the mother plugs and the largest daughter droplets were close to the average bulk rate, whereas the rates in smaller droplets decreased linearly with increasing surface-to-volume ratio. Rates in the smallest droplets decreased by a factor of four compared with the bulk rate. Traditional methods for detecting nonspecific adsorption at the water-oil interface were unable to detect evidence of enzyme adsorption, including pendant drop tensiometry, laser scanning confocal microscopy of drops containing labeled proteins in microemulsions, and epifluorescence microscopy of plugs and drops generated on chip. We propose that the slowing of enzyme reaction kinetics in the smaller droplets was the result of increased adsorption and inactivation of enzymes at the water-oil interface arising from transient interfacial shear stresses imparted on the daughter droplets as they split from the mother plugs and passed through the constricted opening of the T-junction. Such stresses modulate the interfacial area and density of surfactant molecules that can passivate the interface. Bright-field images of the splitting processes at the junction indicate that these stresses scaled with increasing surface-to-volume ratios of the droplets but were relatively insensitive to the average flow rate of plugs upstream of the junction.



Figure 4.10. Labview program to control the valve closing by fluorescence signal.

#### 4.2.1 Introduction

Advances in nanotechnology offer opportunities for discovering and characterizing new chemical and biochemical phenomena in ultrasmall, biomimetic systems containing only a few reacting molecules [15, 16, 115, 131]. Many biochemical transformations in living systems occur in highly nonideal, crowded, and confined cellular nanoenvironments, which can significantly affect thermodynamic and kinetic properties [99, 121]. Numerous methods have been reported for carrying out chemical and biochemical reactions in compartmentalized containers as model systems [63, 68, 70, 81, 112, 113, 153]. However, nonspecific adsorption and inactivation of enzymes at surfaces and interfaces at small scales, as the surface-to-volume (S/V) ratio becomes very high [77], can significantly degrade the ability to obtain accurate measurements of kinetic rates.

Water-in-oil plug formation in microfluidic devices has become an attractive and well-established method for the compartmentalization of aqueous reactions that avoids the inherent surface properties of polydimethylsiloxane (PDMS) and other solid materials [129]. Many reports have been published on the formation and rapid mixing of monodisperse water-in-oil plugs formed at the intersection of two or more aqueous streams with an immiscible oil stream (for a review, see reference [129]). As

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is the case for compartments defined in solid substrates, minimization of nonspecific adsorption of biomolecules at the aqueous–oil interface requires careful choice of passivating molecules, typically polyethylene glycol (PEG)-based phospholipids or surfactants. Control of nonspecific protein adsorption at the water–oil interface has been demonstrated by Roach et al. in microliter drops with pendant drop tensiometry, and nanoliter water-in-oil plugs in a microfluidic device by fluorescence microscopy and measurements of enzyme kinetics [114].

The sizes of water-in-oil plugs scale with microchannel dimensions, typically in the range 10–  $100 \ \mu m$ . We have designed a microfluidic device, shown schematically in figure 4.11 that included a second T-junction downstream of a mixing stage, which allowed smaller daughter droplets  $(\leq 10 \,\mu\text{m} \text{ in diameter})$  with well-mixed, homogeneous concentration profiles, to be split off from the larger water-in-oil mother plugs. Without a change in the geometry of the second T-junction [84] or implementing complicated pressure control schemes at both inlets and outlets [94, 170], daughter droplet volumes could be continuously and reproducibly controlled over more than 2 orders of magnitude by adjusting the backing pressures at the oil and aqueous inlets. The resulting daughter droplet size distributions for all diameters generated at the second T-junction had coefficients of variation (COV) that were less than 3%, indicating they were monodisperse and configurable. The nascent daughter droplets were then trapped in the side channel of the junction by closing a control valve. This device was used to measure the effects of daughter droplet volume on the reaction rates of confined  $\beta$ -galactosidase enzymes ( $\beta$ -Gal) at the same concentration using a fluorogenic assay involving the hydrolysis of nonfluorescent substrate molecules to yield fluorescent resorufin [112, 113]. The assay was carried out with the same concentration of enzyme and substrate as in the bulk, for droplets ranging from 4 to 10  $\mu$ m in diameter, which corresponds to volumes of 34–523 fL (10<sup>-15</sup> L).

Based on measurements from drop tensiometry and from reaction rates for  $\beta$ -Gal in larger daughter drops formed in our device (9–10 µm in diameter), which were close to the average bulk rate, we found that nonspecific adsorption of  $\beta$ -Gal enzymes was suppressed at the water–oil interface with the inclusion of a PEG-based surfactant, consistent with reports by Roach et al. [114]. These findings were corroborated by epifluorescence microscopy of drops and plugs containing fluorescently



**Figure 4.11.** (a) Schematic of the microfluidic device used to produce monodisperse, homogeneous water-in-oil droplets with diameters that could be precisely controlled from 2 to 10  $\mu$ m. (b) Detail of the T-junction used to generate daughter droplets from mother plugs and the control valve used to trap them in the side channel. The main channel was 30  $\mu$ m wide and 25  $\mu$ m high with a rectangular profile. The two aqueous inlets were each 20  $\mu$ m wide and 25  $\mu$ m high. The side channel was rounded to 30  $\mu$ m wide and 12  $\mu$ m high, except for the control button (60  $\mu$ m wide and 10  $\mu$ m high) and the T-junction opening (15  $\mu$ m wide and 5  $\mu$ m high).

labeled  $\beta$ -Gal enzymes and PEG surfactant generated in the microfluidic device and laser scanning confocal microscopy images of droplets in microemulsions. However, we found a linear decrease in the initial reaction rate for enzyme catalysis from smaller daughter droplets split off from mother plugs at the second T-junction in the device. The reduction scaled with decreasing droplet diameter, from 10  $\mu$ m, where the average initial rate was only slightly lower than the bulk rate, to 4  $\mu$ m, which had an initial rate that was four times slower than that in the bulk.

In this article, we propose a mechanism for this decrease in initial reaction rates for confined  $\beta$ -Gal enzymes with decreasing daughter droplet size. A key assumption in our analysis is that nonspecific adsorption of  $\beta$ -Gal enzymes at the water-oil interface results in a reduction of the enzyme's catalytic efficiency, by affecting either  $K_{\rm M}$  or  $k_{\rm cat}$  in the Michaelis-Menten (MM) mechanism or both. This assumption is supported by an extensive series of control experiments which demonstrated that the decrease in measured reaction rate did not result from substrate depletion, product inhibition, or optical effects in the confined volumes. Nor was it the result of instabilities due to pressure fluctuations in the device or uncertainties in determining droplet diameters, which were measured with high accuracy and precision. We believe that effective passivation against enzyme adsorption at the interface initially present in the larger mother plugs in the main channel of the microfluidic device decreased during the formation of the smaller daughter droplets in the side channel. This decrease in passivation effectiveness at the interface was the primary cause for the observed decrease in enzyme kinetics in the smaller droplets. Standard methods for characterizing passivation against nonspecific adsorption of biomolecules at surfaces and interfaces such as tensiometry and fluorescence imaging with labeled proteins failed to capture this effect.

We present evidence for loss of enzyme activity during daughter droplet formation in the microfabricated T-junction due to shear-induced changes in interfacial area and, hence, density of surface active molecules, including both PEG surfactant molecules and  $\beta$ -Gal enzymes. This evidence is consistent with reports in the literature characterizing shear-induced effects on surfactant interface distributions during droplet deformation and breakup in multiphase flows at low Reynolds numbers [22, 102, 142]. In particular, our proposed mechanism is similar to that published by Cate and co-workers for describing mixing of crowded biological solutions in water-in-oil plugs, based on interfacial shear stresses generated by microfabricated protrusions of the channel walls in their device [82].

Our results indicate that methods used for forming ultrasmall volumes could have significant effects on kinetics. Traditional methods for characterizing protein adsorption at liquid-liquid interfaces may show negligible adsorption in nanoliter volumes containing thousands of enzymes typically encountered in microfluidics devices, but fail to detect protein adsorption in smaller-volume daughter droplets that results from S/V scaling and mechanical manipulation of the water-oil interface during droplet splitting. These effects will become more prominent as the number of biomolecules at a given concentration in the confined space becomes small. Quantitatively accounting for these effects will be important in characterizing new chemical and biochemical kinetic phenomena in confined nanoenvironments [76, 118].

#### 4.2.2 Experimental Section

 $\beta$ -Gal was purchased from Calbiochem (Gibbstown, NJ), and resorufin- $\beta$ -D-galactopyranoside (RGP) was obtained from Molecular Probes (Eugene, OR). Alexa568 labeled  $\beta$ -Gal (Alexa568- $\beta$ -Gal) was obtained using the Protein Labeling Kit (Molecular Probes, Carlsbad, CA). Resorufin was purchased from Sigma-Aldrich (St. Louis, MO). These were used without further purification for making aqueous solutions with Millipore 18 M $\Omega$  water (Billerica, MA) or 100 mM phosphate buffered saline (PBS) containing 1 mM MgCl<sub>2</sub> at pH 7.2. Soybean oil (Sigma-Aldrich) used in this experiment was purified of surface-active contaminants (mainly monoglycerides) by gravity filtration through a column packed with a 1:1 mixture of fluorisil and silica gel (100–200 mesh, Sigma-Aldrich) until the equilibrium interfacial tension at the oil–water interface matched that of reported value for purified soybean oil in the literature (31 mN/m) [34]. 4-Nonylphenyl-polyethylene glycol (NP-PEG) surfactant (Sigma-Aldrich) was diluted to 0.1% (v/v) in the purified soybean oil. We had originally tried both silicone oil and perfluorinated oil. We found that the PDMS in our microfluidic devices was swelled by silicone oil. This caused changes in flow rate over time, which made control of flow rate extremely difficult. When perfluorinated oil was used, daughter droplets were not formed at the T-junction reproducibly, due to the higher interfacial tension between the perfluorinated oil and aqueous buffer. These effects had been seen before for on-demand generation of femtoliter droplets by shear [87]. Purified soybean oil with NP-PEG surfactant worked the best in terms of both inertness to PDMS and stability of daughter droplet formation.

The microfluidic devices were fabricated using multilayer soft lithographic techniques (see supporting information for details). Three Teflon jars (Big Science, Huntersville, NC) containing purified soybean oil or aqueous solutions (one jar for enzyme solution, and one for substrate) were connected in parallel to the appropriate inlets on the PDMS device through Tygon (Small Parts, Miramar, FL) and 23-gauge stainless-steel tubing (Technical Innovations, Brazoria, TX). Backing pressures (compressed N<sub>2</sub>) for the aqueous and oil reservoirs, ranging from 10 to 25 psi, were controlled by high-precision voltage-to-pressure transducers (Marsh Bellofram, Newell, WV) which forced fluids from the jars into the main flow channel. A Matlab program (Mathworks, Natick, MA) precisely controlled the voltages applied to the transducers, routed through an analog output board (16 bit resolution, 0–10 V range, USB-3103, Measurement Computing, Norton, MA). The backing pressures at the different inlets were optimized independently to achieve stable formation of mother plugs in the main channel and the generation of daughter droplets of desired diameter in the side channel. The control valve reservoir and an osmotic bath in the control layer were filled with 18 MΩ water to prevent water pervaporation from the droplets [53]. The control valve was deflected downward by compressed  $N_2$  (30 psi) to seal the side channel and stop the movement of daughter droplets on demand in the field of view of an inverted optical microscope (Eclipse TE 300, Nikon Instruments, Melville, NY), operating in epifluorescence mode with a  $100 \times$  oil-immersion objective (NA 1.4). Bright-field or fluorescence images were captured with a CoolSNAP-HQ CCD camera (Roper Scientific, Tucson, AZ) and analyzed with Metamorph software (Universal Imaging Corp., Downing Town, PA), ImageJ (National Institutes of Health) and custom-made Matlab scripts. A 200 W mercury– xenon arc lamp (Ushio, Japan) acted as the light source in combination with appropriate filter sets (G-2E/C, Nikon Instruments) for the detection of resorufin fluorescence. Photobleaching of resorufin was minimized by utilizing neutral density filter sets (ND8 and ND4) and synchronizing the CCD camera with a Lambda SC smart shutter (Sutter Instrument Company, Novato, CA). Included in the supporting information are detailed descriptions of experiments to characterize the performance of our device for the stable generation and manipulation of well-mixed, monodisperse sub-10  $\mu$ m water-in-oil droplets, calibration of fluorescence intensities as functions of substrate concentration and droplet diameter, and traditional assays employed for measuring nonspecific interfacial adsorption such as pendant drop tensiometry and laser scanning confocal microscopy of labeled enzymes in microemulsions.

#### 4.2.3 Results and Discussion

#### Stable generation and trapping of droplets

The device design is similar to those reported by other groups for producing monodisperse, wellmixed water-in-oil plugs [114], with the addition of a second microfabricated T-junction downstream from the plug formation region and the mixing stage to generate smaller daughter droplets from mother plugs. The incorporation of a control valve in the side channel stopped daughter droplets on demand within the field of view of an inverted epifluorescence microscope. The overall scheme of the microfluidic device is depicted in figure 4.11. Two aqueous inlets (rectangular, 20  $\mu$ m wide  $\times$  25  $\mu$ m high) introduced enzyme and substrate streams independently into the main channel (rectangular, 30  $\mu$ m wide  $\times$  25  $\mu$ m high), where they formed mother plugs entrained in the oil phase. At the capillary numbers associated with fluid flow in the main channel in our device (0.06), the dynamics associated with plug formation is best described by Stone, Whitesides and co-workers [36], and is composed of periodic blocking of the oil flow by the aqueous streams. Each blocking event generated a slightly higher positive backpressure in the oil channel, which swept the aqueous plug down the main channel, allowing the process to be repeated. Once steady-state conditions were reached, the plug formation frequency and plug volumes were stable for extended times (20–30 min without any adjustments). At this point, minor increases of the backing pressure at the oil inlet resulted in slightly shorter aqueous plugs formed at moderately higher flow rates.

After formation at the oil–aqueous inlets, the mother plugs entered a winding mixing stage, which mixed the contents of the plugs by chaotic advection [114]. This process was facilitated by microfabricated "cavities" along the mixing channel periphery [82]. Fluorescent images of mother plugs formed by mixing Alexa568- $\beta$ -Gal and buffer solution indicated that mixing was complete within 300 ms. The supporting information includes descriptions of several tests of plug formation stability against pressure fluctuations in the device, as well as careful calibration of the relative rates of injection of the two aqueous streams (enzyme and substrate) into the main channel to ensure that there were always 1:1 mixtures in the mother plugs.

Usually, aqueous plugs formed by entrainment in an immiscible oil phase have widths and heights that match the cross-sectional dimensions of the microchannel and larger lengths [170]. Further reduction of the plug size by increasing shear force is limited by the dimensions of the aqueous inlets at the intersection with the main channel. In addition, reducing the interfacial tension with surfactant tends to destabilize plug formation, resulting in random break up of the plugs [173]. This occurs when the amount of surfactant in the oil lowers the interfacial tension to the point where the capillary number of the system,  $Ca = \mu v/\gamma$ , where  $\mu$  is viscosity, v is the average velocity, and  $\gamma$ is the interfacial tension, switches from a "squeezing," pressure-dominated regime ( $Ca < \sim 10^{-2}$ ), necessary for efficient plug formation, to a "dripping," shear-dominated regime ( $Ca > \sim 10^{-2}$ ), where the aqueous streams no longer periodically block the oil inlet channel sufficiently to form isolated plugs [36]. To form a monodisperse distribution of smaller droplets with tunable sizes, we incorporated a microfabricated T-junction (rounded, 30  $\mu$ m wide and 12  $\mu$ m high except at the control button and the T-junction) immediately after the mixing stage, as shown in figure 4.11(b).

Break up processes of monodisperse plugs or droplets in microfluidic flows have been the subject of extensive theoretical and experimental studies [20, 84, 94, 102, 142, 163]. In general, the volume of daughter droplets produced from a microfabricated junction will be dependent on the relative flow resistance of the smaller side channel compared with that of the main channel [84]. The available literature describes only junctions with uniform height. In our design, the height at the opening of the side channel (about 5  $\mu$ m at the junction) was considerably less than that of the main channel (25  $\mu$ m), effectively increasing the flow resistance of the side channel without decreasing its width (30  $\mu$ m). The resulting daughter drops had diameters unconstrained by the side channel width (about 15  $\mu$ m at the T-junction). Droplets ranging in diameter from 2 to 10  $\mu$ m were formed reproducibly by increasing the oil flow pressure in the main channel relative to fixed aqueous inlet backing pressures, which corresponds to a change of droplet volume over 2 orders of magnitude. All COV values of the droplet size distributions for our device were less than 3% (supporting information).

The stream of daughter droplets in this device could be stopped instantaneously and trapped in place by applying hydraulic pressure to the control valve (see figure 4.11(b)), which blocked the flow in the side channel without perturbing that in the main channel. In order for the control valve to close the side channel at reasonable pressure (30 psi), the channel was widened to 60 µm at the control point to decrease the local aspect ratio. Negligible vibration of the daughter droplets was observed in the process, in contrast to what has been reported using syringe pumps and sample injection valves [7]. Subsequent daughter droplets could be trapped and interrogated by releasing the valve and closing it again after a short recovery period. Multiple rounds of observation were performed in this manner to collect data from numerous experiments. Trapped droplets were further protected from shrinkage due to water pervaporation by introducing an osmotic bath in the control layer on top of the side channel [53]. The control valve adjacent to the osmotic bath also used water as its working fluid. Prolonged observation on the same droplet could be performed without ambiguity arising from droplet movement, shrinkage, or merging (supporting information).

#### Initial enzymatic reaction rates decrease with decreasing droplet size

Hydrolysis of the RGP substrate by  $\beta$ -Gal releases fluorescent resorufin, with galactose as a byproduct. Enzymatic activity can be followed by monitoring the increase of fluorescence in the droplets as a function of time, due to the accumulation of resorufin in the droplets [112, 113]. Values of  $K_{\rm M} = 128.5 \,\mu\text{M}$  and  $V_{\rm max} = 0.248 \,\mu\text{M/s}$  for the reaction were determined from a Lineweaver–Burk plot of the bulk rate data for  $\beta$ -Gal (0.1  $\mu$ g/mL) as a function of substrate concentration from 4.86 to 471  $\mu$ M (supporting information).

Figure 4.12 shows the initial steady-state kinetics for the  $\beta$ -Gal reaction carried out in droplets ranging in diameter from 4.25 to 9.75 µm, in comparison to the average rate from bulk stopped-flow measurements (solid line). The nominal enzyme concentration used for all experiments, both in the droplets and in the bulk, was 1.85 nM (1.0 µg/mL), and the initial concentration of the RGP substrate was 25 µM. Triplicate measurements of the time-dependent product concentrations as functions of time were used to generate the mean and standard deviation for each data point in figure 4.12(a). The error bars for each trace correspond to  $\pm$  one standard deviation from the mean. Although there were differences in individual reaction rate trajectories from similar-sized droplets in figure 4.12(a) (e.g., the average initial rate from the 9.0 µm droplets was greater than that from 9.5 µm droplets), the trend was the reaction velocity tracked the bulk rate initially and then slowed down significantly after a few seconds. It is important to stress that these fluctuations were not due to pressure fluctuations in the device or uncertainty associated with the measurement of droplet sizes, which were characterized with high precision (supporting information) but came from another source. Enzyme catalysis, in general, appeared to be much slower in smaller droplets.

In fact, when the initial reaction rate is plotted as a function of droplet size, a clear negative trend compared with the bulk rate with decreasing droplet size can be seen that is statistically significant. The mean and standard deviation of the initial enzymatic reaction rate as functions of droplet diameter from 1067 droplets are plotted in figure 4.12(b). The raw data from the 1067 measurements are included in the supporting information. The initial rates were determined within the first four second after forming the droplets. The initial reaction rate in bulk was  $0.36 \pm 0.06 \,\mu$ M/s (horizontal



Figure 4.12. Initial reaction rates in femtoliter droplets. (a) Resorufin product concentrations as functions of time for droplets with diameters from 4.25 to 9.75  $\mu$ m, compared with the bulk reaction. (b) Mean and standard deviations of the initial rate from 1067 droplets. Dashed and dotted horizontal lines represent the mean and  $\pm$  standard deviation of the initial bulk rate. (c) Subsets of data at two different backing pressures at the oil inlet show similar trends.

solid and dashed lines in figure 4.12(b)), determined from seven independent measurements taken over a 6-month period. The standard deviation in the bulk initial rate reflects the variation in enzyme activity under identical conditions from one lot to the next. (The same vendor had been used for all experiment.) Although there were significant fluctuations in initial reaction rates from the droplets in figure 4.12(a), the trend in figure 4.12(b) was a linear decrease in the average initial reaction rates with decreasing droplet size, from  $0.25 \pm 0.02 \,\mu$ M/s at the highest value (for 10  $\mu$ m diameter droplets) to  $0.10 \pm 0.05 \ \mu\text{M/s}$  at the lowest (for 4  $\mu\text{m}$  diameter droplets). The linear correlation coefficient for the regression of the droplet data (solid line in figure 4.12(b)) was 0.912. The *p*-value of the null hypothesis was less than  $10^{-10}$  using the Student's *t* test. The 95% confidence region of the fitted slope in figure 4.12(b) is  $0.029 \pm 0.004 \ \mu\text{M/(s·}\mu\text{m})$ .

We found very similar correlations between the initial reaction rate and droplet size, regardless of the average backing pressures used at the inlets. Figure 4.12(c) shows two subsets of the 1067 total measurements of initial reaction rate versus droplet size, taken at two different backing pressures at the oil inlet (68 droplets at 7.7 psi versus 158 droplets at 14.5 psi; the average backing pressures at the aqueous inlets were about 2 psi lower than the pressures at the oil inlets). Although we did not perform linear regressions on these subsets of measurements, they appear to follow a similar trend as the complete data set in figure 4.12(b).

This result was surprising, given the fact that laser scanning confocal microscopy and epifluorescence microscopy of droplets with labeled  $\beta$ -Gal enzymes, as well as tensiometry measurements, indicated negligible adsorption of  $\beta$ -Gal enzymes to the oil–water interface when 0.1% (v/v) NP-PEG surfactant was used, even for enzyme concentrations two orders of magnitude higher than that (1.85 nM) actually used in the experiments (supporting information). The supporting information includes descriptions of many control experiments performed to eliminate several other possible causes for the decreased reaction rates in smaller droplets. Also included in the supporting information is evidence that the loss of enzyme activity occurred during droplet splitting from the plugs at the second T-junction in the device, not upstream.

## Size-dependent interfacial shear redistributes surfactant at oil-water interface in daughter droplets

Detailed examination of the splitting process at the T-junction provides evidence in support of our hypothesis that droplet splitting affects the passivation of the water-oil interface against nonspecific binding and subsequent inactivation of  $\beta$ -Gal enzymes. Figure 4.13 includes bright-field images of daughter droplets being split off from mother plugs at the second T-junction, for two different droplet diameters: 6 µm (figure 4.13(a)-(d)) and 10 µm (figure 4.13(e)-(h)). All of the images were taken at

an average linear flow velocity in the main channel of 1.7 mm/s except for figure 4.13(c), which had an average flow velocity of 6.6 mm/s. The fact that two different flow rates (figure 4.13(c) versus (a), (b), and (d)) resulted in the same droplet diameter (6  $\mu$ m) is consistent with the correlation plot in figure 4.24 of the supporting information, which showed that droplet diameter was more dependent on the minor but well-controlled pressure difference of the oil inlet relative to the aqueous inlets at steady state than the overall magnitude of the backing pressure and, hence, flow rate in the main channel. This observation has been reported before for water-in-oil droplets in microfluidic devices [160].



Figure 4.13. Bright-field images of daughter droplets splitting off from mother plugs at the microfabricated T-junction connecting the main and side channels. Plugs are traveling from right to left in the main channel. (a)–(d), 6  $\mu$ m droplets; (e)–(h), 10  $\mu$ m droplets. The plug flow rate was 1.7 mm/s for all images except for (c), which was 6.6 mm/s. Scale bar is 10  $\mu$ m. Overlaid white crosses in (c) and (g) were used for graphical estimation of capillary numbers for budding droplets, which are included in Table 4.1.

It is this minor pressure difference at the intersection between the aqueous and oil phases that controls the length of the mother plug when it is formed, and, hence, the size of the resulting daughter droplet. The plug flow rate was not nearly as important in determining droplet diameter as plug length, which suggests that droplet diameter in our device was primarily controlled by the temporal overlap between a characteristic splitting-off time, determined by the nozzle geometry of the constriction of the T-junction as well as the viscoelastic properties of the fluidic interface, and the time it takes for the mother plug to travel across the opening. For the smaller 6 µm droplets, the initial budding event occurred closer to the end of the plug in figure 4.13(a), which limited the growth of the nascent daughter droplet before it was split off, compared with the 10  $\mu$ m droplet in figure 4.13(e). Apparently, the characteristic droplet splitting-off time and the plug transit time across the junction opening have similar relative scaling at higher and lower flow rates (as evidenced by the same droplet size for figure 4.13(c) as (a), (b) and (d)).

The constriction of fluid flow at the nozzlelike orifice of the junction (height  $5 \,\mu m$ ) generated local extensional and shear stresses responsible for the droplet budding and stretching from the mother plug seen in figure 4.13(a), (b), (e), and (f) [20, 84, 94, 102, 142, 163]. As a daughter droplet passes through the constriction and is deformed by the external flow field, the capillary pressure at the nose of the droplet will be less than that at the neck of the constriction, due to differences in radii of curvature. This pressure gradient results in an interface instability which allows an oil film to grow at the neck of the constriction, eventually splitting off the daughter droplet from the plug [102, 163]. The transient processes that occur during droplet splitting change the distribution of surface-active molecules at the interface, including NP-PEG surfactant, as well as  $\beta$ -Gal enzymes. The locally high fluid velocities and shear forces present at the neck of the constriction in the junction result in changes in the interfacial area of the droplet due to shape distortion effects such as dilation and contraction. These changes in interfacial area result in changes in surfactant density at the interface and, hence, passivation against nonspecific binding of enzymes. In addition, shear-induced convection of surfactants or enzymes to and on the interface can occur. The relative contributions of uniform dilution of surfactant at the interface due to droplet shape distortion and the formation of surfactant density gradients due to forced convection will depend on the relative contributions from local capillary numbers at the junction during droplet splitting and the surfactant Pélet number, which is the dimensionless ratio of surfactant convection to diffusion [102]. A similar mechanism was used by Cate and co-workers to describe the mixing of crowded viscous solutions in water-in-oil plugs, using microscopic bumps fabricated in the channel walls of their serpentine mixing stage to generate oscillating interfacial shear stresses in the plugs [82].

Although we were not able to directly measure the shear forces acting on a budding daughter droplet as it split off from its mother plug, we carried out a semiquantitative analysis based on

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mean aqueous	oil inlet	droplet	$Ca \approx$	Laplace	shear
inlet pressure (psi)	pressure (psi)	diameter (µm)	(L-B)/(L+B)	pressure (psi)	stress (kPa)
9.9	11.4	9.3	0.21	3.2	0.68
9.9	11.7	5.2	0.27	5.8	1.6
19.7	21.5	8.8	0.20	3.4	0.68
19.7	22.0	5.7	0.30	5.3	1.6

Table 4.1. Shear stresses for nascent daughter droplets at the T-junction

Taylor's theory of droplet deformation under shear stress [157]. A droplet subjected to shear stress by an external flow field will deviate from a spherical shape. The length (L) and breadth (B) of the deformed droplet can provide a measure of the capillary number  $Ca \equiv$  shear stress/Laplace pressure for a stationary droplet (i.e., not moving with the external flow). Specifically, Taylor derived a dimensionless parameter  $F \equiv (L - B)/(L + B)$ , which is equivalent to the capillary number for a stationary droplet. This condition should be approximately satisfied for a new droplet within the first few milliseconds as it splits from the mother plug, as seen in the images in figure 4.13. Included figure 4.13(c) and (g) are line segments corresponding to measurements of L and B overlaid on images of daughter droplets at the moment where they are almost completely split from their mother plugs. The images used to calculate  $F \equiv (L - B)/(L + B) \approx Ca$  for all the entries in Table 4.1 are included in the supporting information.

Table 4.1 summarizes the results of such a calculation for both large and small daughter droplets imaged under different backing pressures. The Laplace pressure was calculated from  $2\gamma/r$ , where  $\gamma$  is the interfacial tension at the oil-water interface in the presence of the 0.1% (v/v) NP-PEG surfactant (7.5 mN/m) and r is the radius of the imaged droplet. With an estimate for Ca and the Laplace pressure, the shear stress can be calculated.

The results in Table 4.1 show that smaller daughter droplets experienced higher shear stress during their formation, regardless of the magnitude of the average backing pressures applied at the inlets, consistent with the results shown in figure 4.12(c) and by others [160]. Because of the higher shear stress imparted on the smaller droplets as they were split off from their mother plugs, surfactant molecules initially uniformly distributed at the oil–water interface were perturbed, which led to a reduction in the passivation of the interface, and the adsorption and inactivation of enzymes.

Another possible mechanism that could explain the size-dependent reaction rates seen in droplets

would be the deformation or denaturation of  $\beta$ -Gal enzymes directly from shear stresses present in the aqueous interior of the droplets. We have not been able to find a quantitative analysis for shearinduced destabilization of  $\beta$ -Gal enzymes in the literature. However, for urease, a multimeric high molecular weight (480–545 kDa) enzyme similar to  $\beta$ -Gal in complexity, shear-induced decreases in enzyme kinetics were reported only for shear strain histories (shear rate multiplied by exposure time) greater than 10<sup>5</sup> (and only in the presence of propitious metal ions) [161, 162]. Such shear-induced decreases in urease activity were reversible. In our case, the maximum shear rate the enzymes could have experienced in the droplets can be estimated by dividing the shear stresses listed in Table 4.1 by the viscosity of water at 20 °C (0.1 cP) to give the range of shear rates:  $\sim 10^5-10^6 \text{ s}^{-1}$ . The maximum shear rate multiplied by the splitting-off time of daughter droplets from mother plugs when shear forces were present (2–10 ms, based on the flow rates of plugs in figure 4.13) result in, at most, shear strain histories of only 10<sup>3</sup>, two orders of magnitude less than that reported to deactivate urease. On the basis of these considerations, we believe it is more likely that irreversible adsorption at the interface is the predominant mechanism for enzyme deactivation.

The supporting information describes an experiment using soluble PEG molecules as crowding agents in the droplets to confirm an important prediction of our model: the size dependence of the reaction rate decrease would disappear, regardless of the strength of shear stress, if the interface could remain completely passivated during the splitting-off process.

#### 4.2.4 Conclusion

This article is the first report of a microfluidic device design for splitting off daughter droplets from mother water-in-oil plugs where the sizes of the droplets were not limited by the channel width of the device. This design has enabled the controlled initiation and interrogation of catalytic rates for confined enzymes in aqueous droplets down to 4  $\mu$ m in diameter. This has led to the discovery of shear-induced redistribution of surfactants and enzymes that occurred during the splitting process, which resulted in size-dependent inactivation of some of the initial enzyme population due to nonspecific adsorption. We expect to observe similar phenomena as long as small droplets experience high shear forces during their formation, regardless of the details of the device geometry.

The importance of the distribution of surfactants at fluidic interfaces on multiphase fluid flows was first recognized years ago when the terminal velocity of an air bubble in water appeared to be a function of bubble volume [169]. Many authors have worked to characterize the effects that surfactants have on droplet motion and deformation in porous media, in fields such as oil recovery, pollution remediation, and in understanding the rheological properties of polymers [102]. In return, mechanical manipulations of droplet size and shape will change the distribution of surface active molecules at interfaces. These effects were exploited by Cate and co-workers to aid in the mixing of water-in-oil plugs crowded with large macromolecules [82]. However, the same phenomena can also result in driving initially active  $\beta$ -Gal enzymes in the aqueous interior of a droplet to the interface. The consequences for catalysis become proportionately more severe at higher S/V ratios and as the population of enzymes in the confined environment decreases (supporting information). At nanomolar concentrations, the total number of  $\beta$ -Gal enzymes decreases from thousands of molecules in the mother plugs, to hundreds of molecules in 10  $\mu$ m droplets, and to just tens of molecules in 4  $\mu$ m droplets. As the degree of confinement increases, S/V effects become increasingly important. A fully quantitative understanding of how these phenomena impact chemical and biochemical kinetics in confined systems would require real-time spatial or spectral imaging of the redistribution of surfactant and proteins as the droplets pass through the junction with laser scanning confocal or total internal reflection microscopy, in conjunction with numerical analyses of the time-dependent convection-diffusion equation relating droplet deformation with surfactant transport, which is beyond the scope of this work.

This knowledge will help in the choice of an appropriate combination of working fluid, device geometry, surfactant concentration, and flow conditions to reduce the effect of surfactant redistribution on the nonspecific adsorption of enzymes. It has been shown recently that either reducing shear force or increasing surfactant concentration or mobility [169] can decrease the extent of surfactant redistribution at the droplet interface. However, splitting-off daughter droplets from mother plugs is a multistep process in our device. The operating parameter space is large, and optimizing a critical parameter for one step can have negative consequences for a previous step. For example, increasing the surfactant concentration to better passivate the droplet interface tends to destabilize plug formation, as described above. By monitoring enzymatic reaction kinetics in femtoliter droplets formed in this manner, we were able to identify a new mechanism for enzyme adsorption and deactivation at ostensibly completely passivated interfaces, with important implications for interpreting kinetic data in microfluidics and nanofluidics systems.

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#### Supporting information

#### Mode fabrication

Triple-layer photolithography was used to fabricate a silicon master for fabricating PDMS devices by micromolding. Patterns containing the round control button for the control valve, the side channel, and the main channel were drawn in separate layers in AutoCAD 2004 and imaged at 20,000 dotper-inch resolution onto optical transparencies (CAD/Art Services, Bandon, OR). To make the control button, a piece of 2-propanol (IPA)-cleaned silicon wafer was spin coated with positive-tone SPR 220-7 photoresist (Shipley Company, L.L.C., Marlborough, MA) at 4000 rpm. After soft baking at 115 °C for 90 s, the resist was exposed to UV light under a photomask for 100 s at 14.9 mW/cm<sup>2</sup> on a Karl-Suss MA 6 mask aligner (Suss MicroTec AG, Garching, Germany). MIF-319 developer (Rohm & Haas Electronic Materials, Philadelphia, PA) was used to develop the pattern, followed by rinsing with 18 M $\Omega$  water. Overnight baking in an oven at 190 °C rounded the feature and stabilized the photoresist. A second layer of AZ 50 positive photoresist (AZ Electronic Materials USA Corp., Branchburg, NJ) was spin coated at 4000 rpm onto the wafer to make the side channel. The soft bake consisted of three steps: 2 min at 65 °C, 5 min at 115 °C, and 2 min at 65 °C. After aligning the side channel pattern with the control button, UV exposure at 14.9 mW/cm<sup>2</sup> for 50 s transferred the pattern from the mask to the wafer. A 4:1 (v/v) mixture of water and 2401 developer (Shipley) was used to develop the pattern. Hard baking at 200 °C rounded the side channel and stabilized the AZ 50 resist. For the third layer, corresponding to the main channel, SU8-2025 negative-tone photoresist was spin coated onto the wafer at 2500 rpm and soft baked at 65 °C for 3 min and 95 °C for 6 min. Following careful alignment of the photomask for the main channel and the side channel already on the master, the wafer was exposed to UV illumination for 20 s at 14.9 mW/cm<sup>2</sup>. Post exposure baking at 65 °C for 1 min and 95 °C for 6 min cross-linked the exposed resist; the remaining non-cross-linked residue was removed by immersing the wafer for 1 min in SU8 developer. The master was then hard baked at 150 °C for an additional 10 min. The mold for the control valve layer was made by single-layer photolithography using 30 µm thick SU8-2025 resist with the same baking, exposure and development protocol. All the cross-sectional profiles of the patterns were checked with a profilometer (XP2, AmBios Technology, Santa Cruz, CA).

#### **Device** fabrication

Multilayer soft lithography was used to make the three-layer PDMS device. The molds for both the flow and control layers were treated in trimethylchlorosilane (Sigma-Aldrich) vapor for 3 minutes to facilitate the separation of cured PDMS from the molds. 40 g PDMS prepolymer (RTV 615, GE Silicones, Wilton, CT) with 5:1 mass ratio of base to curing agent was thoroughly mixed for 1 min, defoamed for 3 min, and then poured into a Petri dish containing the control mold. Dissolved air in the mixture was further removed by vacuum in a desiccator. The PDMS was partially cured in an oven at 80 °C for 45 min. For the flow layer, PDMS prepolymer with 20:1 mass ratio of base and curing agent was mixed similarly and spin coated onto the flow mold at 2000 rpm. PDMS of the same composition was also used to spin coat at 8000 rpm an IPA-cleaned # 0 coverslip (Erie Scientific Company, Portsmouth, NH) to form the bottom layer. The flow and bottom layers were cured at 80 °C for 40 and 30 min, respectively. The control layer was peeled from its mold, drilled at

preset punch marks, and aligned with the flow layer. These two layers were bonded together at 80  $^{\circ}$ C for 90 min before being peeled away from the flow mold as a monolithic block. Inlet and outlet holes were drilled through the control layer to the main and side channels in the flow layer with 22-gauge blunt-edge needles, and the top two layers were bonded to the PDMS-coated coverslip permanently by heating overnight at 80  $^{\circ}$ C.

#### Inlet and mixing stability tests

It was critical that the relative rates of injection of the two aqueous streams (enzyme and substrate) into the main channel be balanced by tuning the pressure transducers such that there were always 1:1 mixtures in the mother plugs. This would ensure that the relative concentrations of enzyme and substrate were known accurately when comparing the enzymatic reaction rate as a function of droplet size with the bulk value at the same concentration of enzyme and substrate. We found that pressure fluctuations at the three-way intersection of the aqueous and the oil channels, due to changes in radii of curvature of the oil-aqueous interfaces of the plugs, resulted in fluctuations in the position of the boundary line separating the two aqueous streams at the intersection, and, hence, the relative concentrations of enzyme and substrate incorporated in each plug. These fluctuations, however, could be minimized by careful adjustments of the three pressure transducers, which have a guaranteed accuracy of 0.25% (0.1% typical). Figure 4.14 quantifies the stability of the interface between Alexa568- $\beta$ -Gal solution and nonfluorescent buffer solution at the intersection of the two aqueous channels by plotting the standard deviation of fluorescence intensity at each pixel from a set of 60 images taken at the inlet. If the interface was perfectly stationary, a peak of zero width would appear in figure 4.14. The width of peak in the center indicates that the error in the final reactant concentration due to the interface fluctuations is less than 10%. Uncertainties in the initial concentrations of enzyme and substrate of 10% would lead to errors in the estimation of enzymatic reaction rates in the Michaelis–Menten formulae of, at most, 5%.

As a further test, we injected fluorescently labeled enzymes into one or both of the aqueous inlets and quantified the concentration of labeled enzymes in daughter droplets relative to that



Figure 4.14. Standard deviation of intensity at interface between fluorescent and nonfluorescent streams at aqueous inlet of microfluidic device.

Table 4.2. Mixing tests in daughter droplets with fluorescently labeled  $\beta$ -Gal enzymes

Droplet	(1) Inject same concentration	(2) Inject $50/50$ mixture	(3) Mix directly
diameter $(\mu m)$	fluorescent enzymes in both inlets	in both inlets	on chip
5.00	$0.91 \pm 0.06$	0.5	$0.45 \pm 0.06$
5.25	$0.92\pm0.06$	0.5	$0.47 \pm 0.08$
8.00	$0.90\pm0.04$	0.5	$0.42\pm0.09$
8.50	$0.95\pm0.03$	0.5	$0.46\pm0.04$

in the aqueous inlet(s) for the following three protocols: (1) Inject same concentration fluorescent Alexa568- $\beta$ -Gal (13 µg/mL) into both aqueous inlets. (2) Mix 1:1 ratio Alexa568- $\beta$ -Gal (13 µg/mL) and reaction buffer first, then inject the mixture into both aqueous inlets. (3) Mix 1:1 ratio Alexa568- $\beta$ -Gal (13 µg/mL) and reaction buffer directly on chip. Table 4.2 shows that the maximum error in labeled enzyme concentration in the daughter droplets for protocol (3) compared with protocol (2) was about 16%, irrespective of droplet diameter, proof that the chip could produce homogeneously mixed daughter droplets throughout the size range from 4 to 10 µm, with reagent compositions identical to that from mixing in the bulk. Protocol (2) was taken as the standard and its intensity was normalized to be 0.5 because it was supposed to give a 1:1 mixing of enzyme and buffer solutions. Protocol (2) should be comparable to (3), which was the on-chip mixture and close to 0.5, within the experimental uncertainty. Solution (1) was enzyme stock only, giving a fluorescence signal about twice that of solution (2).

#### Plug formation stability

Flow rates of aqueous mother plugs and daughter droplets were determined by analyzing brightfield images from the CCD camera at a specific integration time (1–5 ms). The measured length of the blur (in micrometer) at the leading or trailing edge of a plug or droplet divided by the integration time yielded the linear flow velocity (in millimeter per second). Averaged results of triplet measurements were recorded. If necessary, flow velocity may be converted to volumetric flow rate (volume/time) using the channel dimensions (see caption to figure 4.11). The same setup also allowed for photon-counting experiments at milliseconds time resolution. These were undertaken to quantify the stability of steady-state plug formation by the device against pressure fluctuations. Fluorescence signal from the steady-state train of plugs containing  $0.5 \ \mu M$  resorufin was directed to the side port of the microscope where it was detected by a photon-counting module (PCM 942, Perkin-Elmer, Wiesbaden, Germany) and recorded by a digital counter on a NI PCI-6014 multifunction data acquisition board with two milliseconds bin time (National Instruments, Austin, TX). A Labview (National Instruments, Austin, TX) program was written to control the data acquisition. The stability of mother plug formation right before the T-junction was monitored in this way. Fig 4.15 shows the histogram of detected photon numbers. A superposition of a Poisson distribution, corresponding to the background, and a Gaussian distribution, corresponding to the fluorescence signal from plugs, was used to extract the mean and standard deviation of the signal distribution. A threshold, defined as the mean minus three times the standard deviation of the Gaussian distribution, differentiated the raw data points to be either due to background or fluorescence from the plugs [65].

A typical result is shown in figure 4.16. From more than 4000 peaks, we calculated the peak width to be  $18 \pm 1$  ms and the duration between peaks to be  $13 \pm 1$  ms, which corresponded to a plug length of 95 µm and a head-to-tail plug spacing of 69 µm. These values were corroborated by plug length (100 µm) and plug spacing (63 µm) determined from bright-field images captured under the same flow conditions with the CCD camera. The standard deviation in peak width was smaller than the time resolution (2 ms) in figure 4.16, indicating that plug formation instability due to pressure fluctuations in the system was negligible.



**Figure 4.15.** The histogram of photon number from a train of plugs containing resorufin. The data (circles) are fitted (line) to a linear superposition of a Poisson and a Gaussian distribution with proper normalization.



Figure 4.16. Time traces of fluorescent plugs moving in the main channel. Raw photon counts (blue) are assigned as signal from plug or background according to a threshold based on the distribution of the raw data. The result from a peak identification algorithm (red) is shown for comparison. More than 4000 peaks in the full trace of 2 min could be identified without ambiguity. The first 1 s (a) and 0.2 s (b) of the raw data and peak identification are shown.

#### Droplet size distributions and optical calibration procedures

The optical calibration procedure to determine the concentration of resorufin product as a function of time, and, hence, the rate of reaction in different-sized daughter droplets, consisted of three steps:

(1) bright-field determination of the droplet diameter, (2) determination of the total fluorescence signal from the droplet, and (3) determination of the conversion factors relating resorufin concentration to fluorescence signal from different-sized droplets.

#### Determination of droplet diameter

Bright-field images of trapped stationary droplets, taken under Köhler illumination conditions, were used to determine droplet diameters according to the following procedure. A bright-field image of an individual droplet was first selected. A rectangular region of interest (ROI) was then drawn about the droplet. Each horizontal line in the ROI was analyzed with a custom made Matlab script to extract the distance between two intensity minima corresponding to a chord length of the droplet. The longest chord length was taken as the diameter of the droplet. The uncertainty in droplet diameter using this method was about one pixel, or  $0.25 \,\mu\text{m}$ . Figure 4.17 shows the relation between droplet diameter distribution and the backing pressure at the oil inlet. Size distributions are usually characterized by the coefficient of variation (COV = SD/m × 100%, where m and SD are the mean and standard deviation of the distribution, respectively). The COV values of the droplet size distributions for our device were less than 3% for all the data sets reported in figure 4.17 (seven to nine data points for each set).

#### Determination of total fluorescence signal $(I_{tot})$ from the droplet

The total fluorescence signal  $(I_{tot})$  from the droplet was calculated on the basis of its diameter, which was determined previously from the bright-field image. A rectangular ROI was drawn around a droplet in the fluorescence image. The location of a tentative droplet center was estimated from the image. The tentative droplet center was moved systematically over a user-specified range in order to maximize the integrated fluorescence signal over the area of a disk with the diameter determined in the previous step. The maximum of the integrated signal was used to define the true center of the droplet. Another region of interest with the same area was then drawn in the vicinity of the droplet to calculate the average background intensity. The maximum of the total signal from the droplet minus the total background signal for the same area defined  $I_{tot}$ .



Figure 4.17. Daughter droplet size distributions as functions of backing pressure (labeled in figure in unit of psi) at the oil inlet. The pressures at the two aqueous inlets were held constant at 16.22 and 16.66 psi. Droplets of desired size can be conveniently generated by tuning oil inlet pressure at fixed aqueous inlet pressures.

#### Conversion between $I_{tot}$ and fluorescent product (resorufin) concentration

 $I_{\rm tot}$  was a function of both droplet diameter and resorufin concentration. However, because the fluorescence intensity integrated over the circular area of a selected droplet with measured diameter showed a linear relationship with the concentration of resorufin, we were able to determine the resorufin concentration as a function of time in each droplet from a time-lapse series of images. This was used to calculate the rate of resorufin concentration increase with time due to enzymatic reactions in droplets. For droplets of the same diameter,  $I_{\rm tot}$  was proportional to concentration in  $\mu$ M was determined from the absorbance of resorufin at 571 nm, using a molar extinction coefficient of 58,000 M<sup>-1</sup> cm<sup>-1</sup> [95]. Solutions with resorufin concentrations ranging from 0 to 4  $\mu$ M were prepared by serial dilution from a standard solution at 100  $\mu$ M, and introduced in both aqueous channels of the microfluidic device to generate fluorescence and bright-field images of droplets of

different diameters. In addition to correlating the fluorescence intensity with droplet size, brightfield images of the droplets also ensured that the fluorescence images were focused properly. The average  $I_{tot}$  for droplets as a function of size could then be calculated. The absolute concentration of resorufin in the standard solution was determined with a UV/Vis spectrometer (Uvikon 933, Research Instruments International, San Diego, CA). The linear proportionality coefficients used in the calibration of the enzymatic rate data were  $I_{tot}/c$ , which varied for different-sized droplets.

#### Trapped droplet stability

Figure 4.18 compares bright-field images (a)–(c) and fluorescence images (d)–(f) of trapped droplets at different times; no changes in size or position of the droplets were detected after ten minutes. The slight difference between droplets in figure 4.18(b) and (c) was due to minor drift in the focus of the microscope objective for the boundary of the channel in the two images also appeared to change slightly. Prolonged observation on the same droplet could be performed without ambiguity arising from droplet movement, shrinkage, or merging. An alternative process to measure kinetics on a drop by drop basis would be to identify and track a specific moving droplet in real time for sufficiently long times to obtain reliable kinetic information from that droplet. We believe our method is far easier and more amenable for generating large data sets consisting of numerous measurements of different-sized droplets.

#### Bulk kinetics and Lineweaver–Burk plot

The kinetics of  $\beta$ -Gal–RGP reactions in bulk solution was monitored with a stopped-flow instrument (SX.18MV, Applied Photophysics, Surrey, UK) by measuring the fluorescence increase due to the enzymatic generation of resorufin. Figure 4.19 is a Lineweaver–Burk (LB) plot [19] from bulk reaction rate data for  $\beta$ -Gal (0.1 µg/mL or 1.85 nM), yielding values of K<sub>M</sub> = 128.5 µM and V<sub>max</sub> = 0.248 µM/s. The absolute concentration of RGP substrate for each data point in figure 4.19 was checked with the UV–Vis spectrometer ( $\epsilon = 18,000 \text{ M}^{-1} \text{ cm}^{-1}$  at 470 nm).



Figure 4.18. Droplet stability after being trapped in the side channel by the control valve. Bright-field [(a)-(c)] and fluorescence [(d)-(f)] images taken when control valve first closed [(a) and (d)], three minutes later [(b) and (e)] and 10 minutes later [(c) and (f)].



Figure 4.19. Lineweaver–Burk plot for  $\beta$ -Gal enzymes, determined from bulk stopped-flow fluorescence measurements.

#### Control experiments for kinetic data from droplets in figure 4.12

Many control experiments were performed to eliminate several other possible causes for the decreased reaction rates in smaller droplets observed in figure 4.12 of the manuscript. Consideration of figure 4.12(a) indicates that substrate depletion can be ruled out because the time at which the average bulk rate (0.36  $\mu$ M/sec) and the fastest droplet rate (from 9.5  $\mu$ m droplets) diverge, roughly two seconds, corresponds to, at most, the consumption of just 0.7  $\mu$ M substrate, or roughly three percent of the initial substrate concentration. Bulk absorbance measurements performed both before and after mixing of aqueous solutions containing 50  $\mu$ M RGP substrate with soybean oil containing NP-PEG surfactant did not show evidence for substrate incorporation in the oil. Inner-filter effects, due to absorption of emitted light from fluorescent resorufin molecules by other product molecules in the droplets, can be ruled out from the calibration curves of fluorescence intensity versus resorufin concentration, which were linear over the concentration range 0–4  $\mu$ M for all droplet sizes.

#### Product inhibition of $\beta$ -Gal by galactose

To test whether galactose, a by-product of the hydrolysis reaction of the RGP substrate by  $\beta$ -Gal, can act as a product inhibitor, we carried out a bulk assay of initial enzymatic reaction rates using 10 pM  $\beta$ -Gal and 87.5  $\mu$ M RGP in phosphate buffer, with increasing amounts of added galactose (up to 10 mM). The series of reaction rates was used to generate a dose-response curve and determine the IC<sub>50</sub> value for galactose inhibition of the reaction [19]. The dose-response plot in figure 4.20 suggests an IC<sub>50</sub> value for 50% galactose inhibition that is well above the maximum concentration of 10 mM used, orders of magnitude beyond what could have been generated by enzymatic hydrolysis of RGP in the microfluidic experiments.



Figure 4.20. Dose-response plot of enzyme initial rate as a function of galactose concentration. Enzyme concentration was 10 pM, and RGP concentration was 87.5  $\mu$ M, both in phosphate buffer. From this plot, the IC<sub>50</sub> value for galactose is greater than 10 mM.

Photoreactions involving singlet oxygen

Figure 4.21(a) and (b) are fluorescence plots of 100  $\mu$ M RGP substrate alone, and 100  $\mu$ M substrate with 200 nM resorufin, contained in 5  $\mu$ m diameter chambers (~100 fL) formed by micromolding in PDMS [68], under the same illumination conditions as the femtoliter droplet experiments carried out in the microfluidic devices as described in the paper. If there was autohydrolysis of RGP due to reaction with singlet oxygen species generated from the photobleaching of resorufin, we would expect to see significant differences in the relative increases in fluorescence in figure 4.21(b) versus (a) over the time course of five minutes.



Figure 4.21. Fluorescence increases over five minutes measured with CCD camera in 5  $\mu$ m diameter chambers defined in PDMS containing (a) RGP substrate only, and (b) a mixture of RGP and resorufin. Illumination conditions were the same as for the droplet experiments in the microfluidic devices.

#### Photobleaching

Figure 4.22 shows the decrease in resorufin fluorescence due to photobleaching from a 9.5  $\mu$ m drop (figure 4.22(a)) and from a 6  $\mu$ m drop (figure 4.22(b)), under the same illumination conditions as used during the experimental determination of enzymatic reaction rates. The fitted first-order decay constants ( $k_{\text{bleach}}$  was 0.015 s<sup>-1</sup> for 9.5  $\mu$ m droplets and 0.029 s<sup>-1</sup> for 6.0  $\mu$ m droplets) were incorporated as an additional decay channel in the Michaelis–Menten formulae,

$$\frac{dP(t)}{dt} = \frac{V_{\rm max}S(t)}{K_{\rm M} + S(t)} - k_{\rm bleach}P(t),$$

and then integrated numerically with an ODE solver (MathCAD) using the values of  $K_M$  and  $V_{max}$  determined from the bulk Lineweaver–Burk plot to simulate the net bulk product concentration as a function of time from competition between product formation due to enzymatic catalysis and loss of fluorescence due to photobleaching. Figure 4.22(c) and (d) show that the effects of photobleaching in the droplets on the reaction rate are not sufficient to account for the measured decreases in reaction rate for droplets of either size.



Figure 4.22. (a) and (b), Plots of fluorescence decay (dot) due to photobleaching in daughter droplets 9.5  $\mu$ m (a) and 6.5  $\mu$ m (b) in diameter were fitted with an exponential function (line) to get  $k_{\text{bleach}}$  reported in text; (c) and (d), Plots of simulated enzymatic reaction rates when first-order photobleaching decay constants were included in the bulk Michaelis–Menten mechanism, compared with data from the droplets.

#### Loss of ions at the interface

Additional controls were performed using confocal laser scanning microscopy of water-in-oil microemulsions with droplet sizes in the same range as those generated in the microfluidic devices. Figure 4.23 shows that inclusion of Magnesium Green dye indicated negligible loss of  $Mg^{2+}$  ions in the aqueous droplet interior due to sequestration by PEG groups at the interface [24], which would negatively affect enzyme activity. Similarly, SNARF-5F 5-(and-6)-carboxylic acid (pKa 7.2) and resorufin (pKa 6.4) dyes indicated the pH did not change in the droplets from their values in the reaction buffer used to prepare the aqueous streams (pH = 7.2) due to loss of hydronium ions at the interface. 1 mg/ml aliquots of these indicators were stored in a  $-20 \,^{\circ}$ C freezer or 4  $^{\circ}$ C cold room. Before use, these aliquots were thawed and diluted in reaction buffer (100 mM phosphate buffer with 1 mM MgCl<sub>2</sub>, pH 7.2) to the appropriate concentration. 100 µL purified soybean oil was mixed and stirred with 50 µL buffer which contained the indicator dyes or fluorescent proteins.



**Figure 4.23.** Fluorescent indicators used to visualize distribution of ions in water–oil microemulsions containing 0.1% (v/v) NP-PEG: (a) Magnesium Green; (b) SNARF-5F. Scale bar: 20 µm.

# Correlation plots of enzyme kinetics, plug length and droplet diameter with backing pressure

Figure 4.24(a) is a plot of the average fluorescence intensity (background subtracted) versus inverse flow rate in the main channel due to the enzymatic production of resorufin in mother plugs, captured with the CCD camera just before a daughter droplet is splitting at the second T-junction. All experimental conditions, including the initial concentrations of enzyme and substrate, and the mixing and initiation of the reaction in the plugs, were the same as those used for the measurements of reaction rates in the daughter droplets described in the manuscript. The data can be grouped into three families classified by the average backing pressures applied at the aqueous inlets (approximately 20, 15, and 10 psi). Within each family, mother plugs and daughter droplets of different sizes were intentionally generated by fine tuning the backing pressure at the oil inlet relative to the fixed backing pressures used at the aqueous inlets. For example, in figure 4.24(a), the three data points within the "10 psi" ellipse correspond to oil backing pressures of 11.3, 11.5, and 11.6 psi. Tuning the oil backing pressure relative to the fixed aqueous pressures within each family (ellipse) also changed the flow rates of plugs and daughter droplets.

The reciprocal of the flow velocity in figure 4.24(a) multiplied by the distance the plugs travelled from the inlet region to the focal point of the microscope in the main channel (2.3 mm) is simply the reaction time (mixing time) for the  $\beta$ -Gal-catalyzed hydrolysis of RGP substrate to give product (roughly 300 to 900 ms, depending on flow velocity). Figure 4.24(b) and (c) are plots of the correlations between flow velocity and average mother plug length in the main channel and average daughter droplet diameter in the side channel, respectively, determined from bright-field images. Importantly, these plots show that the full range of mother plug lengths (roughly 70 to 90  $\mu$ m) and daughter droplet diameters (4 to 10  $\mu$ m) were observed in *each* family (ellipse), which indicates that the size of the plugs and droplets was determined primarily by the minor differences in backing pressures applied at the oil inlet relative to the two aqueous inlets at steady state, and was relatively independent of the magnitude of the average value of the backing pressures. This observation has been reported before for water-in-oil droplets in microfluidic devices [160].

These correlations in turn indicate that the fluorescence increases in figure 4.24(a) were far more dependent on the reaction time in the plugs before they encountered the second T-junction (which depended on the flow velocity through the mixer) than on the sizes of plugs (or the resulting daughter droplets after splitting at the junction). Although we were not able to convert fluorescence intensity in figure 4.24(a) to absolute resorufin concentrations directly due to the low signal-to-background ratio from the rapidly moving plugs, the fluorescence increase with transit time should be linearly proportional to the initial enzymatic reaction rate in the plugs, assuming that none of the nominal population of enzymes in the plugs was inactivated. Figure 4.24(a) shows that this rate, in fact, was relatively insensitive to changes in plug length, whereas the reaction rate in the daughter droplets slowed down as a function of their size by as a much as a factor of 4 compared with the bulk rate, as was shown in figure 4.12. We consider this to be strong evidence that the loss of enzyme activity occurred predominately during droplet splitting from the plugs at the second T-junction in the



Figure 4.24. (a) Average fluorescence intensity versus inverse flow rate from mother plugs in the main channel measured just before the splitting of daughter droplets. The data are grouped into three families, classified in the figure by the average backing pressure applied at the aqueous inlets. Plug lengths (b) and droplet diameters (c) versus flow rate, using the same classification scheme as for (a). The full range of mother plug lengths and daughter droplet diameters were possible in each family of average backing pressure.

device, and not upstream.

#### Estimation of shear stress based on the geometry of deformed droplet

See Table 4.3.

Testing role of interface with inclusion of aqueous PEG 600 as crowding agent in droplets

An important prediction of our model is that the size dependence of the reaction rate decrease in femtoliter droplets would disappear, regardless of the strength of shear stress at the oil–water interface, if the interface could be completely passivated by a high enough concentration of NP-PEG surfactant. However, we could not test this prediction directly by increasing the amount of NP-PEG (or other surface active molecules, such as blocking proteins like BSA) because increased concentrations

Shear stress (kPa)	0.68	1.6	0.68	1.6
Laplace pressure (kPa)	3.2	5.8	3.4	ى. ى
$\begin{array}{c} Ca \approx \\ \frac{L-B}{L+B} \end{array}$	0.21	0.27	0.20	0.30
Droplet image			R	
Droplet diameter (μm)	9.3 2	5.2	8.8	5.7
Oil inlet pressure (psi)	11.4	11.7	21.5	22.0
Mean aqueous inlet pressure (psi)	6.6		19.7	

Table 4.3. Determination of shear stresses for nascent daughter droplets at T-junction

resulted in premature breakup of mother plugs at the device inlets, as described earlier. Instead, we used high concentrations (up to 100 mg/mL) of soluble polyethylene glycol 600 (PEG 600) in the aqueous phase as a crowding agent to help passivate the interface against nonspecific adsorption of  $\beta$ -Gal enzymes. Although PEG 600 is not surface active, at the high concentrations used, we expect

the interface would be occupied primarily by PEG 600 molecules due to excluded volume effects and reduced diffusion coefficients [174]. In addition, PEG 600 can form extended hydrogen-bonded networks with the NP-PEG surfactant molecules already present at the interface [167].

It is well known that molecular crowding affects reaction rates, however, these effects should depend primarily on the concentration of crowders in the aqueous environment, not on the size of the reaction vessel (droplet) itself, and should therefore be the same (within experimental uncertainty) for large and small droplets, as well as the bulk, as long as the concentration of crowders is the same [99, 121]. The inclusion of crowding agent thus serves as a test for the importance of the oil–water interface in the size-dependent kinetics we observed (in figure 4.12) by limiting the importance of enzyme adsorption at the interface in affecting reaction rates. We first tested the efficiency of the mixing stage of the device for mixing the more viscous solutions containing up to 100 mg/mL PEG 600 using Alexa568- $\beta$ -Gal enzymes, and found the mixing efficiency to be comparable to that without the crowding agent included in the plugs.

Figure 4.25 shows that with crowding agent present, the reaction rates in the droplets were comparable to the bulk rate for all droplet sizes. All other experimental conditions were the same as for the data presented in figure 4.12. Although not attempting to characterize the mechanisms responsible for lowering enzyme activities with the addition of crowding agents, which have been the subject of many reports in the literature, here we note simply that the addition of the crowding agent has apparently masked the effects that the decrease in passivation at the interface with increasing S/V ratio of the droplets had on enzyme activity. Thus, this experiment serves as an additional control indicating that the decreases in initial enzymatic rates in femtoliter droplets with decreasing diameters seen in figure 4.12 (i.e., without crowding agent) were in fact due to progressive inactivation of enzymes resulting from nonspecific adsorption at the oil–water interface.



Figure 4.25. Initial enzyme reaction rate in large and small daughter droplets, and in the bulk, with inclusion of 100 mg/mL soluble PEG 600 as crowding agent at different backing pressures at the oil inlet.

## Comparison of droplet kinetics with traditional assays for detecting nonspecific adsorption

Control of nonspecific interfacial enzyme adsorption in water–oil systems has been demonstrated from interfacial tension measurements with surfactant in the oil phase and proteins in the aqueous phase, and in nanoliter water-in-oil plugs in a microfluidic device by fluorescence microscopy and measurements of enzyme kinetics [114]. Nonspecific adsorption of  $\beta$ -Gal enzymes without the inclusion of NP-PEG surfactants at the interface was readily apparent from fluorescence images of labeled proteins in emulsion droplets and in mother plugs generated in our microfluidic device, and from interfacial tension measurements using the pendant drop technique. However, there was no evidence for nonspecific adsorption using either fluorescence or tensiometry when 0.1% (v/v) NP-PEG surfactant was included in the oil, even for enzyme concentrations up to two to three orders of magnitude greater than those used in the droplet experiments on chip (1.85 nM). Initial reaction rates for  $\beta$ -Gal in larger daughter drops formed in our device (9–10 µm diameter) were close to the average bulk rate. Our observation of minimal change in enzyme activity due to interfacial adsorption in mother plugs and the largest daughter droplets from the average bulk rate is consistent with previous reports in the literature describing well-passivated aqueous-oil interfaces in microfluidic devices [114]. What, then, is different about the splitting-off process described in this paper for forming daughter droplets in the second T-junction from the more established assays for detecting nonspecific enzyme adsorption? How can we rationalize why the NP-PEG surfactant appears to passivate the oil-water interface adequately only for the larger size droplets and plugs?

First, for interfacial tension measurements using the pendant drop technique, drops were formed by slowly extruding aqueous solution into a quiescent oil phase, so there was little shear stress exerted on a growing drop due to the gradient of the flow field compared with the shear stress experienced by daughter droplets at the second T-junction in our microfluidic device.

Second, it is not possible to quantitatively compare the stresses present during the formation of the microemulsion droplets used in the laser scanning confocal images with the daughter droplets generated on chip. The emulsions were prepared by rapidly mixing buffer and oil in a micropipet tip, which likely involved turbulent conditions that were difficult to compare meaningfully with simple shear generated in the microfluidic device. However, assuming the same density of  $\beta$ -Gal enzymes in the aqueous interior as at the water-oil interface, on the basis of the homogeneous intensity profiles from laser scanning confocal images of emulsion droplets containing fluorescently labeled enzymes shown below, scaling the S/V ratio corresponding to decreasing daughter droplet diameters in our device results in a higher relative percentage of adsorbed enzymes, based purely on geometrical considerations (see below). This effect is inversely proportional to the cubic root of enzyme concentration in the drops, being ten times greater at nanomolar concentrations than at micromolar concentrations. The concentration of labeled  $\beta$ -Gal enzymes in the emulsion was  $1.06 \ \mu$ M, whereas for the droplet experiments in the microfluidic device the enzyme concentration was 1.85 nM. Because of the inherent amplification involved with the use of fluorogenic substrates in enzymatic reactions, monitoring kinetics in droplets was a more sensitive way to detect enzyme adsorption than confocal images of emulsions containing labeled proteins.

Third, intensity profiles from epifluorescence images of Alexa568- $\beta$ -Gal proteins in the daughter droplets generated on chip were homogeneous, with no evidence for interface adsorption, similar to the laser scanning confocal images of droplets in microemulsions. However, the decreased spatial resolution and increased contributions to the measured intensity in those images from the greater excitation volume compared with laser scanning confocal microscopy lowers the sensitivity for detecting gradients in fluorescent enzyme density across the drop.

#### Interfacial tension measurements from pendant drops

For interfacial tension measurements, a contact angle goniometer (Ramé-Hart Instruments, Netcong, NJ) with micrometer syringe (Barnant, Barrington, IL) was coupled to a microscope-eyepiece digital camera (EM-500M, Big Catch, Torrance, CA) to capture the images of the pendant drops. All glassware and blunt-tip metal needles (Hamilton, Reno, NV) were cleaned in warm (45–50 °C) RBS 35 detergent solution (Pierce, Rockford, IL) for two hours and rinsed with Millipore water thoroughly before drying at 80  $^{\circ}$ C overnight in an oven. Because the density of the aqueous solution was greater than that of soybean oil, aqueous pendant droplets were formed in the oil phase. A quartz cuvette (Hellma, Plainview, NY) containing 700 µL soybean oil, purified by passage through a fluorisil column, was placed on the 3-axis stage of the goniometer. The glass micrometer syringe, containing aqueous solution, with the luer-lock metal needle attached, was carefully lowered into the cuvette until the blunt tip of the needle was immersed a few millimeters into the oil. After focusing the camera on the tip of the needle,  $2-4 \,\mu\text{L}$  aqueous solution from the syringe was carefully extruded into the oil phase to form a droplet at the needle tip. Images were captured at 10 s intervals starting immediately after the formation of the droplet. These images were processed with ImageJ software and fitted with an axisymmetric drop shape analysis program to get a numerical solution for the interfacial tension from the Young–Laplace equation [13]. For each set of conditions (e.g., varying the concentration of NP-PEG surfactant in the oil phase), three to five identical measurements were made in order to obtain reliable statistics. The effective aspect ratio and magnification of the imaging setup was calibrated by imaging a 0.125 inch steel ball bearing affixed to the end of a blunt metal needle with epoxy and immersed in the oil-filled cuvette. The interfacial tension of aqueous pendant drops in purified oil including NP-PEG surfactant at the concentration used in the microfluidic experiments (0.1% v/v) was 7.5 mN/m. As a check, the surface tension from a droplet of 18 M $\Omega$  water in air was determined to be 72.8 mN/m, which agreed with literature [83].



Figure 4.26. Interfacial tension (IFT) measurements from pendant aqueous drops suspended in oil phase. IFT data without (a) and with (b) 0.1% (v/v) NP-PEG surfactant included in the oil.

Figure 4.26(a) and (b) are plots of the interfacial tension (IFT) from microliter aqueous pendant drops suspended in the oil phase, with and without NP-PEG surfactant, respectively. Even in the absence of NP-PEG in the oil phase (figure 4.26(a)), differences in the IFT values due to protein adsorption could not be detected beyond the measurement uncertainty (standard deviation) for droplets containing 1.0  $\mu$ g/mL  $\beta$ -Gal enzymes compared to droplets containing only reaction buffer. With 0.1% (v/v) NP-PEG included in the oil, the minimum protein concentration needed to detect a change in measured IFT was 100  $\mu$ g/mL, two orders of magnitude greater than that used for the on-chip experiments.

#### Laser scanning confocal microscopy images of microemulsions

To study nonspecific adsorption of enzymes at the water-oil interface,  $\beta$ -Gal was labeled by Alexa Fluor 568 using the Protein Labeling Kit (Molecular Probes, Carlsbad, CA). Microemulsions were formed by mixing in a micropipet tip aqueous buffer containing labeled proteins with purified soybean oil containing 0.1% (v/v) NP-PEG surfactant. The emulsion was delivered to a # 0 glass coverslip and images were taken on an Axiovert 100 M inverted microscope, configured for confocal laser scanning microscopy (LSM 5 PASCAL, Zeiss), and equipped with a Plan-Neofluar  $63 \times$  objective lens (1.25 NA). The pinhole was set to 120 µm (2.0 Airy units), and detector gains were set at 800 V for both red and green channels. The images were recorded as 16-bit tiff files and processed using the Metamorph version 7.0r2 (Universal Imaging Corp, Downington, PA) and ImageJ software.

Figuer 4.27(a) and (b) are confocal images of 20  $\mu$ m diameter droplets in water-in-oil microemulsions containing 570  $\mu$ g/mL (1.06  $\mu$ M) fluorescently labeled Alexa568- $\beta$ -Gal enzymes, without (a) and with (b) the presence of 0.1% (v/v) NP-PEG surfactant in the oil phase. Because the images in figure 4.27 were from individual z-slices, we assumed that there were negligible contributions to the measured intensities from voxels that were out of the focal plane. The lack of discernible fluorescence contrast at the periphery of the droplet in figure 4.27(b) when NP-PEG surfactant is present indicates effective passivation of the interface with NP-PEG.



Figure 4.27. Laser scanning confocal fluorescence images of water–oil microemulsions containing 1.06  $\mu$ M Alexa568- $\beta$ -Gal enzymes without (a) and with (b) 0.1% (v/v) NP-PEG surfactant in the oil phase. Droplet diameters are 20  $\mu$ m.

#### S/V scaling of enzyme densities in droplets

We used the confocal image of fluorescently labeled  $\beta$ -Gal enzymes in figure 4.27(b) as a starting point for estimating how increasing the S/V ratio in water-in-oil droplets would change the relative percentage of proteins nonspecifically adsorbed at an interface, assuming the density (mol/m<sup>2</sup>) of enzymes at the interface and in the interior of the droplet remained equal. This assumption places an upper bound for the number of Alexa568- $\beta$ -Gal enzymes that could be adsorbed at the interface relative to that in the aqueous interior and still result in a uniform line profile of fluorescence intensity observed in figure 4.27(b). The concentration of Alexa568- $\beta$ -Gal enzymes used was 1.06  $\mu$ M, which translates to 6.02 × 10<sup>20</sup> enzymes/m<sup>3</sup>. The equivalent territorial volume per enzyme is the inverse of this number, 1.66 × 10<sup>-21</sup> m<sup>3</sup>/enzyme, with radius 73.5 nm. The corresponding territorial area per enzyme at the interface is 1.70 × 10<sup>-14</sup> m<sup>2</sup>/enzyme, which results in a molar surface density ( $\Gamma$ ) at the interface of 9.80 × 10<sup>-11</sup> mol/m<sup>2</sup>. The number of enzymes at the interface in moles is this number multiplied by the surface area of the droplet (e.g., 4.19 × 10<sup>-10</sup> m<sup>2</sup> for the 20 µm diameter droplet used in figure 4.27(b)). Thus, the maximum percentage of enzymes at the interface (4.10 × 10<sup>-20</sup> moles) relative to the total (4.19 × 10<sup>-18</sup> moles), was slightly less than 1% for the droplet shown in figure 4.27(b).

By simple scaling of these numbers with an increasing S/V ratio, the relative percentage of interface-bound enzymes increases. Figure 4.28(a) plots this relative percentage as a function of increasing S/V ratio for four different nominal enzyme concentrations  $(10^{-6} \text{ to } 10^{-9} \text{ M})$ . The lowest S/V ratio for figure 4.28(a) and (b)  $(10^5 \text{ m}^2/\text{m}^3)$  corresponds to the 20 µm spherical drops imaged in figure 4.27, and roughly to the aqueous mother plugs in the main channel of the microfluidic device (30 µm width and height, with lengths varying from 70 to 90 µm, depending on flow rate). The highest value of the S/V ratio corresponds to 4 µm diameter daughter droplets generated on chip.

Increasing the S/V ratio by a factor of five results in increasing the relative percentage of interfacebound enzymes by a factor of five as well, for each concentration plotted in figure 4.28(a). However, a much higher proportion of the total enzyme population would be interface bound at nanomolar concentrations than at micromolar concentrations, for all S/V ratios. This is because the ratio of the number of interface molecules to the total number of molecules is equal to  $\frac{Sc^{2/3}}{Vc} = \frac{S}{V}c^{-1/3}$ , where S is the surface area of the interface, V is droplet volume, and c is concentration. Hence, the relative percentage of interface-bound enzymes increases by a factor of ten in going from micromolar to nanomolar concentrations. At nanomolar concentration, almost half of the total enzyme population for a 4.0 µm diameter droplet would be bound to the interface, when the enzymes in the interior and the interface area at the same density. in the aqueous interior of a daughter droplet, presumed to be the only enzymes that are catalytically active, decreases with increasing S/V ratio, assuming a nominal starting concentration of 1.85 nM (1  $\mu$ g/mL). For comparison, the bottom trace is the effective concentration of active enzymes that would result in the initial rate data presented in figure 4.12(b), estimated by solving for  $[E]_0$  from the Michaelis–Menten formulae,

$$[E]_0 \approx \left[\frac{K_{\rm M} + [S]_0}{[S]_0}\right] \frac{V}{k_{\rm cat}},$$

with  $K_{\rm M} = 128.5 \ \mu {\rm M}$  and  $k_{\rm cat} = 1341 \ {\rm s}^{-1}$ . V is the initial reaction rate data from figure 4.12(b), and the approximation  $[S](t) \approx [S]_0 = 25 \ \mu {\rm M}$  should be valid at early times. Comparison of the two traces indicates that S/V scaling alone does not fully explain the 4-fold loss of enzyme activity in daughter droplets compared with the bulk initial rate. It does, however, offer a partial explanation for why the larger droplet in figure 4.27(b), with a higher concentration of labeled enzymes, was not as sensitive an assay for protein adsorption at the interface than the reaction rate data from the smaller daughter droplets generated by shear in the T-junction of the microfluidic device.

#### Epifluorescence images of daughter droplets containing labeled enzymes

Figure 4.29 shows that the fluorescence intensity profiles from Alexa568- $\beta$ -Gal proteins in the daughter droplets were homogeneous, with no evidence for interface adsorption, similar to the laser scanning confocal image in figure 4.27(b). However, the decreased spatial resolution and increased contributions to the measured intensity in the image from the greater excitation volume with epi-fluorescence microscopy compared with laser scanning confocal microscopy lowers the sensitivity for detecting gradients in fluorescent enzyme density across the drop in figure 4.29.

#### Calculation of *p*-value using Student's t test in figure 4.12(b)

In figure 4.12(b), the reaction rate was fitted to a linear function of daughter droplet diameter using Matlab, and the p-value using Student's t test was obtained by the built-in Matlab function "regstat."



Figure 4.28. (a) Relative percentage of total enzyme population adsorbed at the interface, as a function of S/V ratio and concentration, assuming the same density of enzymes  $(mol/m^2)$  throughout droplet. (b) Effective concentrations of active enzymes left in aqueous interior of droplet after interface adsorption (1.85 nM nominal concentration), using S/V scaling (dashed line), and initial reaction rate data from figure 4.12 (solid line).



Figure 4.29. Epifluorescence microscope image (a) and line scan (b) of 5  $\mu$ m daughter droplets containing 13  $\mu$ g/mL Alexa568- $\beta$ -Gal enzymes in the side channel.

#### Raw data for figure 4.12(b)

Raw data for figure 4.12(b) is available free of charge via the Internet at http://pubs.acs.org.