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Oil-sealed femtoliter fiber-optic arrays for single molecule analysis^{†,‡}

Huaibin Zhang, Shuai Nie[§], Candice M. Etson[§], Raymond M. Wang, and David R. Walt
Department of Chemistry, Tufts University, 62 Talbot Avenue, Medford, Massachusetts, USA.
Fax: +016176273443; Tel: +016176273470

David R. Walt: david.walt@tufts.edu

Abstract

This paper describes a novel method for fabricating and sealing high-density arrays of femtoliter reaction chambers. We chemically etch one end of a 2.3 mm diameter glass optical fiber bundle to create an array of microwells. We then use a contact printing method to selectively modify the surface of the material between microwells with a hydrophobic silane. This modification makes it possible to fill the wells with aqueous solution and then seal them with a droplet of oil, forming an array of isolated reaction chambers. Individual β -galactosidase molecules trapped in these reaction chambers convert a substrate into a fluorescent product that can be readily detected because a high local concentration of product is achieved. This binary readout can be used for ultra-sensitive measurements of enzyme concentration. We observed that the percentage of wells showing enzyme activity was linearly dependent on the concentration of soluble β -galactosidase in the picomolar range. A similar response was also observed for streptavidin- β -galactosidase captured by biotinylated beads. These arrays are also suitable for performing single-molecule kinetics studies on hundreds to thousands of enzyme molecules simultaneously. We observed a broad distribution of catalytic rates for individual β -galactosidase molecules trapped in the microwells, in agreement with previous studies using similar arrays that were mechanically sealed. We have further demonstrated that this femtoliter fiber-optic array can be integrated into a PDMS microfluidic channel system and sealed with oil on-chip, creating an easy to use and high-throughput device for single-molecule analysis.

Introduction

Single-molecule optical detection and imaging techniques have led to significant advances in our understanding of fundamental biochemical processes. Most traditional biochemical assays are performed on large ensembles of molecules, and yield information about the average behavior of the group. However, single-molecule studies of enzyme activity have revealed that there is a great deal of variation in the catalytic rates of ostensibly identical enzymes, suggesting that a given enzyme may have access to multiple active conformations.^{1–6} The catalytic rates of individual enzymes also fluctuate over time, and may be linked to conformational fluctuations.^{2,4–6} Single-molecule studies have also revealed the presence of sparsely populated intermediate states along a reaction pathway that

[†]Electronic supplementary information (ESI) available: Fluorescence micrograph showing on-chip sealing with mineral oil; histograms of the observed velocities of β -gal enzymes sealed with FC-70 oil and mineral oil; comparison of histograms of β -gal catalytic rates sealed by FC-70 and mineral oil. See DOI: 10.1039/c2lc21113k

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Correspondence to: David R. Walt, david.walt@tufts.edu.

[§]S. Nie and C. M. Etson contributed equally to this work.

are not observable in ensemble studies, and have facilitated ultra-sensitive detection of biologically relevant molecules. These latter studies have the potential to lead to great improvements in public health. For example, early stage detection of many types of cancer is associated with improved patient outcomes, but the serum concentration of proteins that reliably indicate the presence of disease is expected to be picomolar (10^{-12} M) or lower.⁷ Most immunoassays, the traditional detection method for disease-associated proteins, cannot measure such low concentrations. Digital immunoassays utilizing single-molecule detection approaches have pushed the theoretical limit of detection down by several orders of magnitude into the attomolar (10^{-18} M) range.⁸ Genomic changes such as chromosomal rearrangements,⁹ epigenetic changes,¹⁰ and single nucleotide polymorphisms (SNP)¹¹ as well as expression profile changes^{12,13} are also associated with malignant cell growth. Since the number of cells in an early stage tumor is vastly outnumbered by the number of normal cells, ultra-sensitive detection of these genomic changes is critical. Single-molecule detection methods have been essential to the development of the next-generation sequencing and genotyping technologies that make analysis of these changes possible.^{14,15} Clearly, the ability to detect and interrogate single molecules has practical applications.

The earliest single-molecule direct optical detection experiments were accomplished by minimizing the size of the excitation region, while maximizing the number of photons collected from each molecule.¹⁶ This approach is still widely used for single-molecule studies today, in techniques such as total internal reflection fluorescence (TIRF) microscopy,¹⁷ confocal microscopy,¹⁸ and two-photon excitation microscopy.^{19,20} However, these approaches generally require the labeled molecules to be immobilized, which can lead to artifacts in the observed intermolecular interactions and intramolecular fluctuations.²¹ Fluorescence fluctuation spectroscopy²⁰ can be used to analyze molecules freely diffusing in solution, but if the molecules of interest are part of a complex mixture, they must be very specifically labeled. These approaches all require expensive optical instruments, a high level of user technical expertise, and do not tend to have particularly high throughput. Such limitations make it difficult for these types of single-molecule imaging approaches to be widely applied for medical diagnostics and threat detection.

An alternative to minimizing the excitation region is confining the molecules to be studied into ultra-small volume containers for analysis, and using an indirect, amplifiable signal for detection, as pioneered by Rotman.²² A container with a volume of 1 nanoliter (10^{-9} L) filled with a 1 nM solution should contain 6×10^5 molecules of interest. As the container volume decreases, so should the number of molecules one would expect to find inside the container. Containers on the scale of the droplets selected for observation in the Rotman study, with a volume of about 1 picoliter (10^{-12} L), will contain 600 molecules at this concentration. When the container volume is further reduced to 1 femtoliter (10^{-15} L), the expected number of molecules falls below 1, to 0.6. This calculation does not, however, mean that in a large array of such containers, 60% will contain one molecule of interest and the rest of the containers will contain none. In this regime, the probability $P_\mu(x)$ of finding exactly x molecules in a single container is described by the Poisson distribution, $P_\mu(x) = e^{-\mu} \mu^x / x!$, where μ is the average number of molecules per container – in this case 0.6. Therefore, 55% of these containers will not contain any molecules of interest, 33% will contain 1 molecule, 10% will contain 2 molecules, and 2% will contain 3 molecules. If the average number of molecules of interest per container is further reduced to 0.05, 95% of containers will not contain a molecule, 5% will contain only one molecule, and less than 0.2% will contain more than one molecule.²³ A large array of such containers can therefore be used to simultaneously capture and isolate many individual molecules after being filled and sealed.

Methods for detecting and analyzing single molecules require large numbers of containers in order to achieve accurate and precise concentration measurements, and adequate sampling of population distributions. The variance of the Poisson distribution is equal to its mean, and the signal to noise ratio is therefore $N/\sqrt{N}$, where N is the average number of occupied wells. When N is small, the signal to noise ratio is small, and the inherent fluctuations of the measurement about its mean are on the order of the measurement itself. For a given rate of occupancy, increasing the number of containers observed increases the signal to noise ratio, thereby improving the accuracy of the measurement. Therefore, a digital assay requires a high-density array of ultra-small containers to ensure enough wells are occupied at low concentration. As discussed previously, the volumes of the containers must be uniform and small because the concentration at which less than one molecule is expected per container is dependent upon the volume of the containers.²³ Larger containers also require more signal amplification to achieve a detectable concentration of signal molecules. Ideally, the containers must also be closely packed to facilitate simultaneous observation of a large number of containers. Larger volume containers occupy more area and reduce the number of containers in the field of view. Furthermore, it is critical to have a way to effectively seal the containers to prevent evaporation, eliminate diffusion of the contents into bulk solution, and reduce transfer of product between containers.

If the captured molecule is an enzyme that acts on a fluorogenic substrate that is also in solution, enzyme activity will result in a fluorescent product that will reach a high concentration within sealed containers; the presence of this product can be detected by conventional fluorescence microscopy. Containers in which no enzyme is present will maintain background levels of fluorescence intensity. Instead of relying on the total amount of fluorescent product formed as is the case for bulk measurements, this approach has a digital readout, with the percentage of containers that show an increase in fluorescence intensity proportional to the concentration of the enzyme in solution. While the accuracy and detection limit of this method depend on the number of containers observed, quantification of enzyme concentrations in the femtomolar range has been achieved.²⁴

The unique qualities of fluid dynamics in small channels allow for precise manipulation of immiscible liquids.²⁵ For example, large quantities of monodisperse water droplets can be formed in an oil carrier stream, and can be transported, mixed with other reagent streams, and made to fuse with other droplets.^{26–28} These droplets can be used as miniature containers for a wide range of different types of reactions.^{29–31} Detergents are generally used to stabilize the droplets; as such, there have been challenges in the application of this technology to biological materials since many detergents denature proteins, or can be toxic to living cells.³² Microchannels and microfluidic control devices, as well as arrays of wells with femtoliter volumes can be made by micro-molding PDMS;³³ devices to rapidly mix reactants and to fill and seal the wells can also be fabricated by soft-lithography.³⁴ It is also possible to compartmentalize an aqueous solution into an array of microchambers connected by a microfluidic channel fabricated in PDMS, and then to seal isolated chambers by flowing oil through the channel.^{35,36} While these approaches are certainly innovative applications of microfluidics technology to single-molecule analysis, the resultant density of the arrays and the volume of the containers are still limited.

We have developed an approach to fabricating high-density arrays of femtoliter-sized reaction chambers using bundled optical fibers. Optical fibers are typically made with a core of pure silica surrounded by a cladding made of silica doped with germanium dioxide, which lowers the index of refraction. Light that is incident on the interface between the two types of glass at an angle less than the critical angle (θ_c), is refracted. At all angles of incidence greater than θ_c , the light is reflected at the interface. This phenomenon is called total internal reflection (TIR). Light that enters the fiber at an angle less than $90^\circ - \theta_c$ will exhibit TIR, and

will travel along the fiber with very little attenuation (Fig. 1a). The production of bundles of optical fibers results in an arrangement in which the position of each individual fiber at the end that collects light (proximal end) is identical to its position at the opposite end (distal end). This coherent arrangement means that optical fiber bundles can transmit position-specific image information from the proximal end to the distal end.³⁷ In addition to reducing the index of refraction, germanium dioxide doping also makes the optical fiber cladding more resistant to acid etching than the pure silica of the core. By subjecting the polished end of a fiber bundle to an acid bath, the differential etching rates of the two materials results in the creation of “wells”. The depth of the wells is uniform across the fiber bundle, and can be precisely controlled by manipulating the length of exposure to the acid.³⁸ In this way, we can prepare an optical fiber bundle with a highly packed and spatially ordered array of identical wells on the distal end (Fig. 1b). The wells can be sealed by pressing the etched end of the fiber onto a piece of silicone sheeting that functions as a gasket (Fig. 1c). We illuminate the fluorescent contents of the sealed wells by focusing the excitation light source on the proximal end of the fiber bundle; the fluorescence intensity of the contents of each well on the distal end of the fiber is carried, in register, back to the proximal end of the fiber. We typically use 2.3 mm outer diameter bundles containing 50,000 fibers with each fiber having a diameter of 4.5 μm (Fig. 1d),²⁴ which we etch to create 2.9 μm deep wells with a volume of 46 fL. Using a standard upright fluorescence microscope with a 10x objective and a charged-coupled device (CCD) camera, we can simultaneously interrogate the contents of approximately 10,000 wells.

The presence of an active enzyme molecule trapped in a well at the distal end with an excess of its fluorogenic substrate will be indicated by an increase in the fluorescence displayed at the same position on the proximal end of the fiber bundle (Fig. 1e).³⁹ The rate of catalysis for each individual enzyme molecule can also be determined from the rate of fluorescence intensity increase of the well (Fig. 1f),³ and the distribution of activity levels among a population of enzymes can be studied (Fig. 1g). This method is a powerful tool for the study of enzyme kinetics; it provides access to information that cannot be obtained from ensemble assays.^{3,39-41} Enzyme-catalyzed production of fluorescent molecules can also be used as a means to amplify the signal from single intermolecular binding events. Target molecules can be labeled with enzyme, and specifically captured within functionalized wells.³⁹ Micron-sized beads can also be functionalized to capture individual target molecules in solution, by antibody-antigen binding, nucleic acid hybridization, or any other specific and stable intermolecular interaction. Unbound molecules can be washed away, and bound molecules can be exposed to a secondary detection molecule that is coupled to an enzyme. These beads can be loaded into the femtoliter array, and the accumulation of fluorescent product can be detected in the sealed wells.⁴² While multi-step sandwich assays are often used in ELISA (enzyme-linked immunosorbent assay), with a digital ELISA approach, target molecules can be detected at sub-attomolar concentrations, with sub-femtomolar detection limits for serum proteins.^{43,44} At analyte concentrations that result in multiple enzymes captured on each bead, the number of enzymes per bead can be estimated from the fluorescence intensity increase, extending the upper limit of quantification for the assay to picomolar levels, and enabling concentration measurements with a dynamic range of four orders of magnitude.⁸

Although the optical fiber microarray has proven to be a versatile platform for single-molecule detection and analysis, mechanical sealing of the microarray is not reproducible. This idiosyncrasy is not desirable for a technique that is meant to be accessible to a broad cross-section of researchers. Here we describe recent improvements to enhance the ease of use of the optical fiber bundle microarray, and to facilitate its integration into a microfluidic sample preparation system.

Results and discussion

In this work, we describe a novel approach to generating an array of sealed, femtoliter-sized reaction chambers. We fabricate an array of 50,000 microwells by selectively etching the core material on the tip of an optical fiber bundle. We then specifically modify the material between the wells with a hydrophobic silane, using a contact printing method. This modification makes it possible to seal an aqueous solution inside the wells using oil. We demonstrate that this platform can be used in single molecule analysis with the well-studied β -galactosidase/resorufin- β -D-galactopyranoside (β -gal/RDG) enzyme/substrate system. We demonstrate that oil sealing is compatible with our previously reported techniques for quantifying soluble enzyme as well as bead-immobilized enzyme molecules. We show that the oil-sealed femtoliter array can be used to characterize the kinetics of single enzyme molecules, and the results agree with previous work. We also demonstrate that integrating the femtoliter optical fiber array into a PDMS microfluidic channel system creates an easy to use, high-throughput, multi-use device for single-molecule analysis.

Surface modification of the fiber bundle

Controlling surface hydrophilicity of the optical fiber bundle is critical to oil sealing. The optical fiber bundle used in this study comprises 50,000 individual 4.5 μ m diameter hexagonally packed optical fibers with an overall array outer diameter of 2.3 mm. The pure silica core material is selectively etched using a 0.025 M HCl solution to form an array of 46 fL wells on the end of the bundle (Fig. 2a(i)).³⁷ After etching, the fiber bundle is hydrophilic, with a water contact angle of 50° (Fig. 2b). We use a contact printing method to modify the surface of the germanium dioxide doped silica cladding material with a hydrophobic silane such as n-octadecyltrichlorosilane (OTS), while keeping the inner surface of wells hydrophilic (Fig. 2a(ii)).⁴⁵ A freshly prepared solution of 2 mM OTS in anhydrous toluene was evenly spin coated onto a flat piece of PDMS. Freshly etched fiber bundles were mounted on a Z-axis stage, and were pressed perpendicularly onto the OTS coated PDMS surface. A self-assembled monolayer of silane modified the surface of the cladding material;^{46,47} the inner surface of wells was protected by the topography of the etched fiber (Fig. 2a(iii)). Controlling the stamping time is critical to achieving surface modification that provides good oil sealing. We found that 60 s of stamping results in a uniform and reproducible surface modification for sealing the array with mineral oil. A stamping time of 30 s is too short, as the oil sealing of the array was not uniform and the quality of the seal varied from fiber to fiber. A stamping time of 90 s, however, is too long, and resulted in arrays with wells that were more difficult to fill with aqueous solution, presumably because part of the inner surface had been silanized. After stamping, the fiber bundles were sonicated in methanol and toluene to remove unbound silane, and then baked at 80 °C to form a stable silanized surface. After modification with OTS, the fiber has a significantly higher water contact angle of 116° (Fig. 2c) than the freshly etched, unmodified fiber, indicating that the surface is significantly less hydrophilic. Silane-modified etched fibers can be kept in a tightly-sealed container at room temperature for several weeks without significant deterioration.

Off-chip oil sealing

We first established off-chip methods for achieving efficient loading and oil sealing of the surface-modified femtoliter optical fiber array. The wells of the array were filled by immersing the modified fiber in an aqueous solution containing a fluorescent dye or a mixture of enzyme and fluorogenic substrate (Fig. 2a(iv)). We monitor the filling of the wells *via* bright-field microscopy. As the microwells fill with water, there is a noticeable increase in the contrast between the wells and the fiber cladding in the bright-field because of the difference in the index of refraction of water and air. While it takes less than a minute

for water to fill all the wells on an unmodified fiber, it typically takes around 5 min for this process to complete when using an OTS-modified fiber bundle. This time can be minimized by priming the wells with aqueous buffer before immersing the bundle in the solution of interest. If the wells are already filled with water, it is only necessary to wait for diffusion to equilibrate the well contents with the overwhelmingly large volume of bulk solution. Brief ultrasonication of the fiber array in the priming buffer improves the efficiency of the priming step by decreasing the time it takes to fill all of the wells. When the fiber was removed from the aqueous solution, a drop of liquid usually remained at the end of the fiber (Fig. 2a(v)). In order to seal the solution in each well and isolate the wells from one another, all aqueous solution clinging to the surface surrounding the wells must be removed. We have found that shear force is an effective way to achieve complete removal of the excess solution from the fiber. A simple but efficient means to apply shear force is to briefly dip the fiber into a reservoir of mineral oil that is stirred by a magnetic stirring plate and stir bar (Fig. 2a(vi)). The speed of stirring was set to generate the fastest oil flow within the reservoir without creating stir bar instability or oil spilling. After removing the fiber from the oil, the tip of the fiber was covered by a drop of oil, which created a watertight seal of each individual microwell, effectively preventing evaporation (Fig. 2a(vii)). Water evaporation has presented a significant challenge in previous studies using microwells molded in or sealed by PDMS.^{36,33} It is well known that water vapor and many other gases can diffuse through PDMS.⁴⁸ Water loss is of critical concern in single-molecule studies given the small volume of individual wells and the requirement that the concentration of solutes in kinetic studies remain constant, except for the conversion of substrate to product by the enzyme. Previous studies have attempted to minimize water evaporation by applying a special coating to the device,³⁶ pre-saturating the PDMS with water vapor,³³ or limiting the experimental observation time. We found that aqueous solutions remain in the femtoliter wells of our optical fiber arrays for hours after sealing with oil without any obvious loss.

We confirmed the integrity of sealing by performing a photobleaching experiment.²⁴ A solution of resorufin, the fluorescent product of β -gal-mediated hydrolysis of RDG, was sealed in the microwell array (Fig. 2d). A uniform fluorescence signal across all the wells suggests that the volume of solution in each microwell is well controlled, which is an important requirement for the quantitative analysis of single-molecule kinetics. A fraction of the fiber was exposed to high-intensity light through a field stop on the imaging system, forming a discrete region of bleached wells (dark area in Fig. 2e). The edge of the bleached pattern remained well-defined after 30 min (Fig. 2f), indicating that the sealing between microwells was tight and that there was not a significant degree of resorufin diffusion from one well to another as a result of incomplete isolation of wells from one another. The increased stability afforded by sealing the solution in glass microwells with oil offers a way to avoid water loss and paves the way for longer observation times of enzymes with slower catalytic rates.

The oil used for sealing the etched and surface-modified fiber can be removed and the microarray can be reused several times. An effective cleaning regimen includes wiping the surface with a lint-free swab followed by three 2 min periods of ultrasonication first in water, next in acetone, and a final time in water. A slightly increased fluorescence background of the regenerated fiber due to surface adsorption of dye can be eliminated by exposing the entire optical fiber bundle to high-intensity light for 10 min. We have reused a single fiber more than five times without significant deterioration of the signal to noise ratio. Reusability of microarrays is not only cost and time effective, but is also useful for experiments where fiber-to-fiber variation is a concern.

Digital determination of enzyme concentration

We have used β -gal mediated hydrolysis as a model enzyme system to verify the compatibility of oil sealing with digital enzyme concentration measurements. β -gal in concentrations ranging from 1.8 pM to 72 fM, together with 100 μ M RDG, were loaded into an optical fiber bundle microarray comprised of 50,000 wells, each with a volume of 46 fL. At these concentrations, the ratio of molecules to wells ranges from 1/20 to 1/500; therefore, each microwell will essentially contain either one or zero enzyme molecules, based on Poisson statistics.³⁹ The fluorescent product, resorufin, accumulates only in microwells that contain an enzyme molecule, leading to an elevated fluorescence intensity that can be easily distinguished from background. A binary readout of wells that display increased fluorescence and wells that do not fluoresce is used to count the number of enzyme molecules trapped within the wells of the microarray. The fraction of wells with enzyme activity was linearly dependent on the concentration of β -gal (Fig. 3a), similar to what has been observed in previous studies using mechanical sealing with a PDMS gasket,³⁹ thereby confirming the effectiveness of the sealing procedure.

We have also demonstrated the utility of microarray oil sealing for concentration measurements using a bead-based capture scheme. We captured the enzyme conjugate streptavidin- β -galactosidase (S β -gal) on 2.85 μ m diameter biotinylated beads. For each experiment, a fixed volume of beads at a constant concentration was mixed with a fixed volume of S β -gal at concentrations ranging from 440 fg/mL to 220 pg/mL. Excess unbound S β -gal was washed away, and the beads were loaded into surface-modified optical fiber microarrays by centrifugation. The wells in these microarrays had a larger volume than those used for solution phase enzyme detection because they were 4.4 μ m deep so that the beads could be completely contained inside the wells. Wells containing beads were identified by fluorescence detection of a Europium encoding dye physically trapped within the beads (Fig. 4a). The percent occupancy of the beads in the microarray is defined as $N_B/N_{tot} \times 100\%$, where N_B is the number of bead-containing wells and N_{tot} is the total number of wells; percent occupancy is usually greater than 50%. After bead loading, a solution of 100 μ M RDG was introduced into the wells and sealed with mineral oil as described above. The fluorescent product, resorufin, generated by S β -gal enzymes captured on the biotinylated beads was allowed to accumulate in the sealed wells for 10 min. Analysis of the fluorescent wells at the end of this time provides a binary readout for the presence or absence of an enzyme molecule captured on a bead in each occupied well (Fig. 4b). We calculated the fraction of beads with an S β -gal captured on the surface. Fig. 4c shows a log-log plot of the percentage of active beads and the bulk S β -gal concentration. The plot shows a linear response to the concentration of the conjugate. In these experiments, the ratio of the number of available enzyme molecules to the number of beads ranges from 50 to 0.1, calculated using the protein concentration provided by the manufacturer and an estimated molecular weight of 520 kDa for a 1:1 complex of streptavidin and β -gal, assuming the S β -gal is free from impurities and unconjugated streptavidin and β -gal. The actual ratio of captured enzyme molecules to the number of beads can be expected to be much lower than these values because of lower capture efficiencies and loss of enzyme activity over time. The protocol described here has not been optimized for maximum binding efficiency; the limit of detection could likely be improved by taking steps to prevent the non-specific adsorption of S β -gal to the beads, purifying the S β -gal to remove unconjugated streptavidin and β -gal, treating the fibers with a chelator to remove heavy metal ions present in the optical fiber glass that may catalyze the hydrolysis of RDG, as well as optimizing coupling buffer components, incubation time, and washing steps. Nonetheless, these preliminary results demonstrate oil sealing is compatible with bead-based analyte capture schemes. In a recent publication, fluorocarbon was used to seal paramagnetic beads trapped in plastic

microwells manufactured by injection molding. This study demonstrated that oil sealing is also compatible with a more complex, bead-based ELISA capture scheme.⁴⁹

Kinetics of single enzyme molecules

We have also studied the kinetics of single β -gal molecules in oil-sealed femtoliter fiber-optic arrays. β -gal was mixed with substrate solution resulting in final concentrations of RDG and β -gal of 100 μ M and 1.8 pM, respectively. The enzyme-substrate mixture was immediately applied to a buffer-primed, surface-modified optical fiber microarray and sealed with oil as described above. The total time elapsed between combining the enzyme solution with the substrate solution and acquiring the first image was less than 2 min. We have attempted to minimize the lag time between enzyme-substrate combination and the initial time point of the experiment in order to limit the amount of fluorescent product generated prior to the acquisition of the first data point. When studying enzyme inhibition, it will be especially important to begin the experiment quickly so that the observed off-time for the inhibitor is as accurate as possible. While our current protocol requires that the microarray be loaded and sealed prior to mounting the fiber bundle on the microscope, with an improved experimental setup that permits loading and sealing of the microarray *in situ*, we believe that this time lag can be reduced to less than one minute. We observed the sealed microarray for a total of 10 min, acquiring fluorescent images once every 15 s. Wells that are occupied by an active β -gal molecule display an increase in fluorescence intensity over the course of the experiment. As shown in Fig. 5a–c, there is significant variation in the rate of fluorescence intensity increase among the active enzyme containing wells. We subtracted an averaged background trajectory from the fluorescence intensity trajectory for each well that showed activity and applied a recursive correction for product photobleaching. The slope of a linear fit to the resulting corrected fluorescence intensity trajectory for a given well (Fig. 5d) is converted into the turnover rate for the individual enzyme trapped in that well by applying a conversion factor for the number of product molecules required for a unit increase in intensity (see Experimental). Our current experimental set-up allows us to simultaneously observe approximately 10,000 wells. Given the estimated enzyme occupancy of 5% of the wells, this platform allows us to observe the kinetics of about 500 single enzyme molecules in a single experiment. Both the average single enzyme turnover rate of 350 s^{-1} observed in the presence of 100 μ M RDG substrate, and the shape of the distribution of the velocities of these enzymes (Fig. 5e) are similar to those observed in previous work using mechanical sealing with a PDMS gasket.³ This result illustrates that oil sealing of an array of femtoliter-sized wells in an optical fiber array is suitable for single-molecule enzyme kinetics studies.

On-chip oil sealing

Substituting oil sealing for the application of pressure to a solid gasket facilitates the integration of optical fiber microwell arrays into microfluidics-based sample preparation and delivery systems. We attempted on-chip oil sealing of etched, surface-modified optical fiber bundles to explore this possibility. The 2.3 mm outer diameter hexagonal fiber bundles were inserted into 2 mm circular holes punched through a PDMS block that forms the top of a flow channel. Since the diameter of the hole is smaller than the fiber bundle, the elastomeric PDMS forms a seal around the fiber bundle. We injected an aqueous buffer solution into the channel to prime the microarray wells before injecting a solution containing 1.8 pM β -gal combined with 100 μ M RDG substrate (Fig. 6a(i)). Shear force was applied by rapidly injecting mineral oil into the channel. The mineral oil displaced the aqueous solution in the channel, removed residual aqueous solution from the cladding surface, and sealed the microwells (Fig. 6a(ii)). As we observed with off-chip oil sealing, a small percentage of randomly distributed wells displayed an increase in fluorescence intensity indicating the presence of an enzyme molecule (Fig. S1, ESI[†]). We hoped that it would be possible to

regenerate the array after each experiment by injecting buffer, ethanol, and/or acetone into the channel to wash away the mineral oil. We found, however, that these steps were not sufficient to remove all the mineral oil from the fiber surface and the results of subsequent attempts to fill and seal the same array were usually poor. One possible solution to this problem would be to remove the optical fiber bundle from the device and regenerate the microarray using the off-chip cleaning procedure described above. On the other hand, the throughput and ease of use of this device would be improved with an on-chip array regeneration protocol. With this goal in mind, we investigated alternatives to mineral oil for array sealing.

We tested two commercially available fluorinated oils, Fluorinert® FC-40 and FC-70, both liquid mixtures of per-fluorinated aliphatic compounds produced by 3M. Both the average molecular weight and kinematic viscosity at 25 °C of FC-70 (820 g/mol and 12.0 mm²/s, respectively) are greater than those of FC-40 (650 g/mol and 2.2 mm²/s). We selectively modified the surface of the cladding material between the etched wells with the fluorinated silane, (heptadecafluoro-1,1,2,2-tetra-hydrodecyl) trimethoxysilane, using the contact printing method described above. We found that FC-70 effectively sealed the microwells when tested in the off-chip sealing protocol, but FC-40 failed to do so. We observed the enzyme kinetics of β -gal sealed off-chip in the femtoliter fiber-optic array using FC-70 fluorinated oil and duplicating all other experimental parameters detailed above for off-chip mineral oil sealing (Fig. S2(a), ESI[†]). In this experiment, the observed mean single enzyme turnover rate of 220 s⁻¹ is lower than that observed in experiments where mineral oil was used to seal the array. The reasons for this difference in observed mean turnover rate are still under investigation; however, the shapes of the two velocity distributions are similar to each other and to previously published data (Fig. S2(b), ESI[†]). Our on-chip array sealing procedure (described above) was successful with FC-70 fluorinated oil and we observed an increase in fluorescence intensity in a small percentage of wells, presumably due to the activity of single β -gal enzymes (Fig. 6b). As hoped, regeneration of the microarray after sealing with FC-70 fluorinated oil is possible without resorting to an off-chip cleaning procedure. At the conclusion of a single-molecule experiment, FC-70 can be removed from the fiber surface by injecting aqueous buffer through the channel continuously for 2 min (Fig. 6a(iii)). The array can then be reloaded and resealed; we were able repeat this load, seal, flush cycle multiple times without disassembling the device. There were no significant memory effects following the flushing procedure, as the pattern of active wells is different from cycle to cycle. This observation suggests that flushing with aqueous buffer results in complete exchange of microwell contents and that non-specific adsorption of enzyme to the walls of the microwells or any other part of the array is negligible.

Experimental

Materials

β -galactosidase (β -gal) from *Escherichia coli* (Grade VIII, lyophilized powder) was purchased from Sigma-Aldrich, and re-suspended in reaction buffer (10 mM phosphate buffered saline (PBS) with 1 mM MgCl₂, 0.05 mg/mL bovine serum albumin (BSA), and 0.05 mg/mL Tween-20) with 50% glycerol. Aliquots were flash frozen in liquid nitrogen and stored at -80 °C. Resorufin- β -D-galactopyranoside (RDG) was purchased from Life Technologies, dissolved in dimethyl sulfoxide (DMSO) at a concentration of 10 mM, and stored at -20 °C. Anhydrous toluene and n-octadecyltrichlorosilane (OTS) were purchased from Sigma-Aldrich, stored in a dessicator, and used within 3 months.

[†]Electronic supplementary information (ESI) available: Fluorescence micrograph showing on-chip sealing with mineral oil; histograms of the observed velocities of β -gal enzymes sealed with FC-70 oil and mineral oil; comparison of histograms of β -gal catalytic rates sealed by FC-70 and mineral oil. See DOI: 10.1039/c2lc21113k

(Heptadecafluoro-1,1,2,2-tetrahydrode-cyl) trimethoxysilane was purchased from Gelest (Morrisville, PA) and stored in a dessicator at 4 °C. Sterile-filtered mineral oil (M5310) was purchased from Sigma-Aldrich. Fluorinert® FC-70 (F9880) and Fluorinert® FC-40 (F9755) were manufactured by 3M (St. Paul, MN) and purchased from Sigma-Aldrich. All reagents were used without further purification.

Imaging system

All experiments were observed under 10x magnification using an Olympus BX-51 upright epifluorescence microscope equipped with a short arc mercury lamp, a filter cube for resorufin ($\lambda_{\text{Excitation}} = 560\text{--}580\text{ nm}$, $\lambda_{\text{Emission}} = 590\text{--}650\text{ nm}$, #41034, Chroma, Bellows Falls, VT), a computer-controlled mechanical shutter (Uniblitz, Vicent Associates, Rochester, NY), and a CCD camera (ORCA-ER, Hamamatsu, Bridgewater, NJ). All images were acquired with IPLab software (BD Biosciences, Rockville, MD) at an exposure time of 1 s.

Femtoliter array preparation and surface modification

Optical fiber bundles containing ~50,000 individual 4.5 μm diameter optical fibers were purchased from Schott North America Inc. (Southbridge, MA). The bundles were cut to lengths of 6 cm for off-chip sealing and 1.5 cm for on-chip sealing, and polished on both ends using a MultiPrep fiber polisher and a series of lapping films ranging from 30 μm to 0.05 μm grit (both produced by Allied High Tech Products, Rancho Dominguez, CA). The polished bundles were etched in 0.025 M HCl solution with stirring for 115 s to create 2.9 μm deep wells for solution phase enzyme experiments or 175 s to create 4.4 μm deep wells for bead-based capture experiments. The etched fiber bundles were sonicated for 1 min in ultrapure water and dried with a stream of filtered air. A freshly prepared solution of 2 mM OTS in anhydrous toluene was spin-coated onto a 2 mm-thick, glass-supported polydimethylsiloxane (PDMS) sheet at 1,000 rpm for 20 s, and then dried with a stream of filtered air. The etched fibers were mounted on a custom-built z-axis stage, brought into contact with the OTS-coated PDMS, and held in place with light pressure normal to the surface for 60 s. OTS modified fibers were sonicated for 1 min in methanol, then for 1 min in toluene to remove loosely adsorbed OTS. The fibers were dried, baked at 80 °C for 20 min, and stored in a sealed glass container at room temperature. The static water contact angle at the fiber surface was measured using a Ramé-hart goniometer and analyzed using Ramé-hart DROPimage software.

Solution phase enzyme experiments

RDG and β -gal were added to reaction buffer (10 mM PBS with 1 mM MgCl_2 , 0.05 mg/mL BSA, and 0.05 mg/mL Tween-20), immediately before the start of each single molecule experiment. The surface-modified, etched fiber bundle was sonicated in 500 μL of the reaction buffer for 1 min to pre-fill all the wells with aqueous solution. The tip of the fiber bundle was quickly wiped with a lint-free swab to remove excess buffer, then immediately dipped into the enzyme/substrate solution for 30 s. The fiber bundle was quickly transferred into a reservoir of oil for 5 s. The oil was constantly stirred with an IKA stirring plate (1,800 rpm maximum speed) and stir bar; the flowing oil removed excess aqueous solution from the fiber bundle tip, and sealed the wells. The stirring plate speed setting was adjusted manually for maximum flow velocity without stir bar instability or oil spilling over the sides of the reservoir; the appropriate speed varied with the viscosity of the oil in use. The fiber was removed from the oil and immediately mounted on custom-built holder for imaging. For digital determination of enzyme concentration, the reaction was allowed to proceed for 5 min, and then a fluorescence image taken after 5 min of reaction was used to identify active wells. For kinetic studies, a time-lapse series of fluorescence images were taken, one every 15 s for 10 min.

Bead-based enzyme quantification

60 μL of COMPELTM magnetic, carboxyl modified 2.85 μm diameter beads (UMC3N/10111, Bangs Laboratories, Fishers, IN) were washed with 600 μL PBS three times and then three times in a mixture of tetrahydrofuran (THF) and methanol (1: 3, v/v). The beads were then incubated in 600 μL THF and methanol (1: 3, v/v) containing 0.075 M Europium (III) thenoyltrifluoroacetate trihydrate (Acros Organics, Morris Plains, NJ) for 24 h at room temperature. Unincorporated dye molecules were removed by washing the beads with 600 μL PBS 12 times. 2 μg of Europium encoded beads were activated with 15.8 mg/mL ethyl(dimethylaminopropyl) carbodiimide (EDC) and 24.4 mg/mL *N*-hydroxysulfosuccinimide (sulfo-NHS) in 1 mL 2-(*N*-morpholino)ethanesulfonic acid (MES) buffer and followed by three MES buffer wash. Activated beads were incubated in 500 μL PBS containing 1 mg/mL biotinylated BSA for 4 h, then incubated in StartingBlock (TBS) blocking buffer (Thermo Scientific) for one hour to reduce nonspecific adsorption of protein to the beads. Functionalized beads were stored in StartingBlock (TBS) blocking buffer at 4 $^{\circ}\text{C}$ until use. 25 μL of bead solution, containing approximately 1.5×10^6 beads, was added to 100 μL of PBS with 1% BSA containing varying concentrations of β -gal, and incubated for 1 h. Beads were then washed with 500 μL of wash buffer (PBS with 0.1% BSA and 0.1% Tween-20) six times. After the washing steps, beads were re-suspended in 50 μL of wash buffer. 10 μL of the final bead solution was pipetted onto the top of an etched and modified fiber bundle. The fiber bundle was then centrifuged at 1,200x g for 15 min to help drive the beads into the wells. The bead loaded fiber bundle array was dipped into reaction buffer containing 100 μM RDG for 30 s, and then sealed with oil as described above before imaging.

On-chip oil sealing

A PDMS channel (2 mm $\times$ 5 mm $\times$ 50 μm , W $\times$ L $\times$ H) was fabricated using a previously described rapid prototyping method.⁵⁰ In brief, a master mold was produced by spin-coating SU-8 2025 (Microchem, Newton, MA) onto a 3" silicon wafer (Prime grade, <100>) at 2000 rpm for 30 s, followed by a soft-bake step (75 $^{\circ}\text{C}$ for 2 min, 105 $^{\circ}\text{C}$ for 5 min), UV exposure through a Mylar mask (10s, 20 mW/cm²), and a post-bake step (75 $^{\circ}\text{C}$ for 1 min, 105 $^{\circ}\text{C}$ for 3 min). Uncrosslinked polymer was removed using SU-8 developer (Microchem). The master mold was treated with methyltrichlorosilane (Sigma-Aldrich) vapor for 2 min, and then covered with PDMS prepolymer (10: 1 mixture of elastomer and curing agent, Sylgard 184, Dow Corning). The PDMS was de-gassed for 30 min, cured at 80 $^{\circ}\text{C}$ for 30 min, and peeled off of the master mold. A circular hole was punched through a PDMS channel using a 2 mm-diameter biopsy punch. The PDMS channel and a glass microscope slide were treated with air plasma for 2 min, placed in contact, and baked at 80 $^{\circ}\text{C}$ for 10 min to form a permanent bond. A silane-modified, etched optical fiber bundle was inserted carefully into the hole. The fiber was positioned and held perpendicular to the channel by a custom-built plastic holder. Reaction buffer was manually injected into the channel using a syringe and left undisturbed for 10 min to prime the microwells. The injection syringe was exchanged with one containing freshly prepared reaction buffer containing 1.8 pM β -gal and 100 μM RDG, taking care to avoid introducing any air bubbles into the channel. After 30 s incubation, the injection syringe was again exchanged to one containing mineral or fluorinated oil; oil was injected rapidly through the channel to replace the enzyme/substrate solution, remove residual aqueous solution from the cladding surface, and seal the microwells. When Fluorinert FC-70 was used as the sealing oil, a 2 min injection of reaction buffer effectively removed oil from the fiber, and the sealing process could be repeated several times.

Data analysis

Kinetics data were analyzed using a custom-designed Matlab algorithm to determine the locations of all wells. Each image in the time-lapse series was converted into a logical array where a “1” denotes a pixel with intensity above a threshold value, and a “0” denotes a pixel with intensity below the threshold. All of the logical arrays were summed and normalized to yield a single array representing the fraction of exposures for which the intensity of each pixel was above threshold. Each pixel was then assigned a score by integrating the values of pixels within a user-defined distance. For each well, there was typically one pixel with the highest value, and that pixel established the location of the region of interest that was used to determine the fluorescence intensity of the well. A region of interest was not assigned to wells for which there was not a single maximum, preventing redundant analysis. For each well, a trajectory of the average fluorescence intensity over the entire time-lapse series was calculated. The ratio of the final fluorescence intensity to the initial fluorescence intensity was used to determine which wells contained an enzyme molecule. Wells that did not contain an enzyme typically did not display more than a 10% net intensity increase over the observation time. The trajectories of all these empty wells were averaged, and subtracted from the trajectories of wells that displayed larger net intensity increases. We converted the trajectories from arbitrary units into a number of product molecules using a standard curve. The average intensity of wells containing fluorescent product was linearly dependent on the concentration of the product over the working range of the experiment. The slope of a linear fit to these data was used to determine the number of product molecules required for a unit increase in intensity. Product photobleaching occurred at a rate of 0.0075 per 1 s exposure when arrays were sealed with mineral oil, and 0.0040 per 1 s exposure when arrays were sealed with fluorinated oil. We accounted for product photobleaching by using a recursive correction algorithm. The catalytic rate for each individual enzyme molecule is given by the slope of a linear fit to the resulting corrected trajectory.

Conclusions and plans for future development

Oil sealing of femtoliter fiber-optic arrays has several advantages over mechanical sealing methods because it is a completely liquid-based method. Mechanical sealing methods require the user to apply and control pressure in excess of 20 psi perpendicular to the fiber axis over the entire time course of the experiment to obtain and maintain a tight seal. It is necessary to use a specially designed platform and holder to apply this force and prevent slipping of the fiber during an experiment without occluding the distal end. The approach angle of the PDMS gasket must also be controlled to ensure reproducible and uniform sealing. Since glass is a brittle material in which flaws easily propagate, the application of pressure can result in mechanical failure. Oil sealing does away with the need to apply large forces during an experiment. Replacing the PDMS gasket with oil also removes a conduit for water evaporation, resulting in a more stable platform for experiments requiring a higher operating temperature or a longer observation time. As we have shown, oil sealing facilitates direct integration of the femtoliter fiber-optic array into a microfluidic channel. There is vast potential for devices that utilize the high density of ultra-small wells and the convenient observation geometry of microarrays on the tip of an optical fiber bundle while exploiting the capabilities of microfluidics technology for the manipulation, preparation, delivery, and analysis of biological samples.⁵¹ Both digital detection schemes and single-molecule distribution characterization require large numbers of observations to make statistically significant conclusions, and fiber-optic femtoliter arrays are an extremely effective way to obtain a large number of simultaneous measurements. Also, a fiber-optic femtoliter array can be regenerated with a simple protocol and used multiple times in both off-chip and on-chip oil sealing regimes. This reusability is not only cost and time effective, but it is also useful for experiments where fiber-to-fiber variations are a concern. Finally, it is likely that oil sealing will prove to be a very robust method. Mechanical sealing requires an extremely

smooth sealing surface while oil sealing does not have such stringent requirements, resulting in a larger tolerance to fiber polishing and preparation protocols. Since the process of achieving good sealing will depend less on the expertise and experience of the operator, oil sealing is likely to result in a greater percentage of successful experiments.

There is still room for improvement. The effect of different sealing oils on the kinetics of the enzymes under observation is still under investigation. The applicability of this sealing technique on other enzymes and biomolecules, and possible impact of nonspecific adsorption of proteins on the reusability of the fiber will be evaluated. We are also working to improve the integration of the optical fiber into the microfluidics device. We have observed some buffer leakage at the PDMS-fiber junction, which, if unresolved, has the potential to limit the ability to use this approach with multiple channels due to the high pressure differential required to drive liquid through the channels. However, the oil sealed femtoliter fiber-optic array also has many features that should make it attractive for both fundamental and applied research applications, including simplicity, robustness, and cost-effectiveness. It also offers some unique capabilities, such as long-term kinetic studies of slow enzymes, and more precise temperature control. Continued development of this platform is likely to lead to considerable advances in the fields of single-molecule detection and analysis.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Single molecule studies using the femtoliter optical fiber array

(a) Light propagates from one end of the fiber to the other *via* total internal reflection at the interface between the core and cladding of an individual optical fiber when the incident angle is greater than the critical angle (θ_c). (b) Three dimensional representation of an etched optical fiber bundle. Wells containing active enzyme (red) display increased fluorescence. Part of the cladding material has been peeled away to make visible the individual fibers. (c) A schematic of the femtoliter optical fiber array and mechanical sealing platform. The fiber optic bundle (i) is mounted, etched side down, in a custom-made fiber holder (ii) fixed in the stage of an upright fluorescence microscope. A drop of enzyme/substrate solution is placed on the PDMS gasket (iii), and the mechanical platform (iv) is raised to apply pressure to the distal end of the fiber, sealing the solution in the microarray. Enzyme activity is monitored through the proximal end of the fiber. (d) Image of the entire optical fiber bundle comprising ~50,000 individual 4.5 μm diameter, hexagonally-packed optical fibers. Individual fibers, and a fiduciary marker placed at the outer edge of the bundle are visible at higher magnification (inset). (e) A small section of the femtoliter microwell array after the enzyme/substrate solution has been mechanically sealed with a PDMS gasket. The accumulation of fluorescent product in wells containing an enzyme molecule makes them clearly distinguishable from the background. (f) Fluorescence trajectories of four representative active wells. The slope of these trajectories is used to calculate the catalytic rate of single enzyme molecules. (g) A histogram of the observed catalytic rate of hundreds of individual enzyme molecules. The highly distributed rate suggests the presence of different activities of individual β-gal molecules. Data presented in (d), (e) have been published in ref. 24, 39, and data presented in (f), (g) have been published in ref. 3.

Fig. 2. Fiber modification and oil sealing

(a) The core material (brown) of the optical fiber bundle is selectively etched to form an array of femtoliter-sized wells (i), and a contact printing method (ii) is used to selectively modify the surface of the cladding material (yellow) with a hydrophobic silane (blue) (iii). The modified fiber is dipped into an aqueous dye solution to fill wells (iv); a drop of excess solution remains on the fiber (v). When the fiber is dipped into a reservoir of oil, the excess solution can be removed by the shear force generated by stirring the oil (vi); the aqueous dye solution is sealed in the wells by a layer of oil (vii). (b) Photograph of a droplet of water applied to a freshly etched fiber; the contact angle is 50° ; (c) Photograph of a droplet applied to a fiber modified with OTS; the contact angle is 116° . (d–f) Fluorescence micrographs taken before (d), immediately after (e), and 30 min after (f) photobleaching a portion of the dye-loaded array with high-intensity light. The bleached pattern (dark area, e–f) remained sharp after 30 min, indicating the integrity of sealing between microwells. Contrast enhanced to increase visibility.

Fig. 3. Digital determination of β -gal concentration in solution

β -gal, free in solution, but trapped in a fraction of the microarray wells, catalyzes the generation of a fluorescent product, resorufin, from a fluorogenic substrate, RDG (inset). A plot of the percentage of active reaction wells as a function of the concentration of β -gal is well fitted by linear regression (red line) ($y = 0.18 + 1.78x$, adjusted $R^2 = 0.99$).

Experiments were performed in triplicate; error bars represent the standard deviation.

Fig. 4. Digital readout of streptavidin β-gal captured on biotinylated beads

(a) Fluorescence micrograph of Europium-encoded beads loaded into the femtoliter well array. (b) Fluorescence micrograph showing that ~20% of biotinylated beads are active after incubation with 44 pg/mL Sβ-gal. (c) The percentage of active beads is linearly dependent upon the concentration of Sβ-gal. Data (black squares) and linear regression fit (red line) ($y = 20.04 + 0.85x$, adjusted $R^2 = 0.99$) are displayed on a log-log scale plot. Experiments were performed at least in triplicate; error bars represent the standard deviation.

Fig. 5. Kinetics of single enzyme molecules

The activities of single β -gal molecules are obtained by comparing fluorescence micrographs taken at the beginning of the experiment (a), after 150 s (b), and after 300 s (c). Background subtracted, bleaching corrected trajectories of five representative wells are plotted in (d). Trajectories of wells containing enzyme (#1–4) are clearly different from the background and wells that do not contain enzyme (#5). Turnover rates are determined by linear regression fitting (solid lines) to the fluorescence trajectories after background subtraction and correction for photobleaching. (e) A histogram of the observed rates of a few hundred enzymes shows a broad distribution.

Fig. 6. On-chip oil sealing

(a) Schematic illustrating multiple cycle on-chip oil sealing of a femtoliter optical fiber array. A fluorinated silane-modified fiber array is integrated into a PDMS-based channel and primed by a buffer solution. Enzyme/substrate solution is injected to the channel (i); the injection of fluorinated oil (Fluorinert® FC-70) pushes the aqueous solution away from the fiber and seals the enzyme/substrate solution in the microwell array (ii); fluorescent product accumulates in the microwells containing an enzyme; the fiber can be regenerated and reused for a second round of the experiment after washing with buffer (iii). (b–d) Multiple experiments can be performed in this way with the same optical fiber array with no obvious memory effects.