Supporting Information

Ultra-Rapid Generation of Femtoliter Microfluidic Droplets for Single-Molecule-Counting Immunoassays

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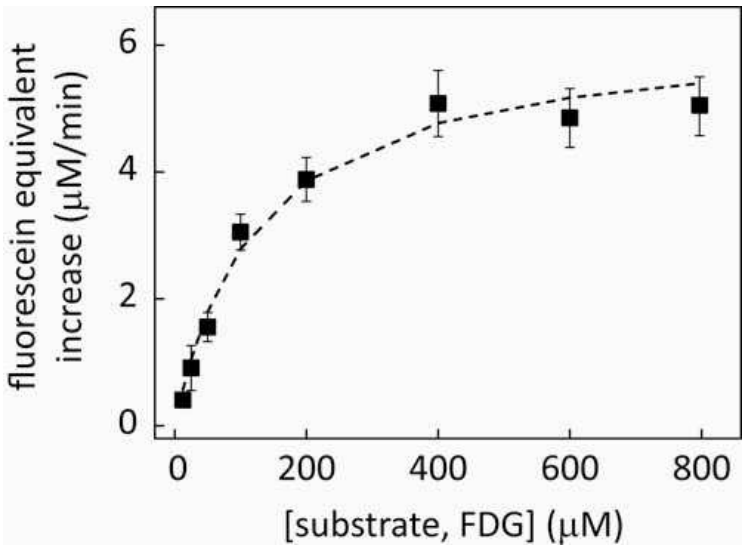
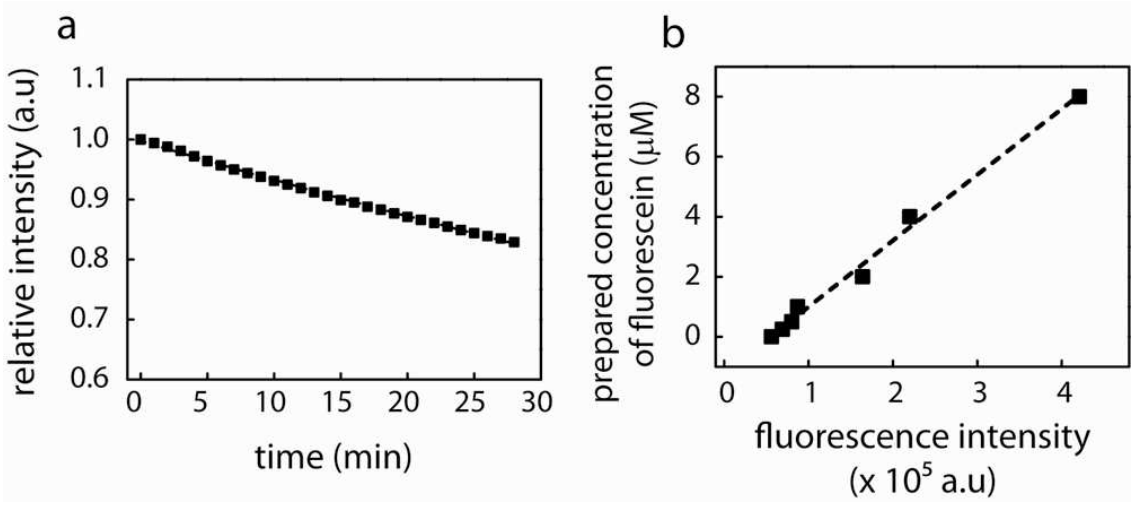
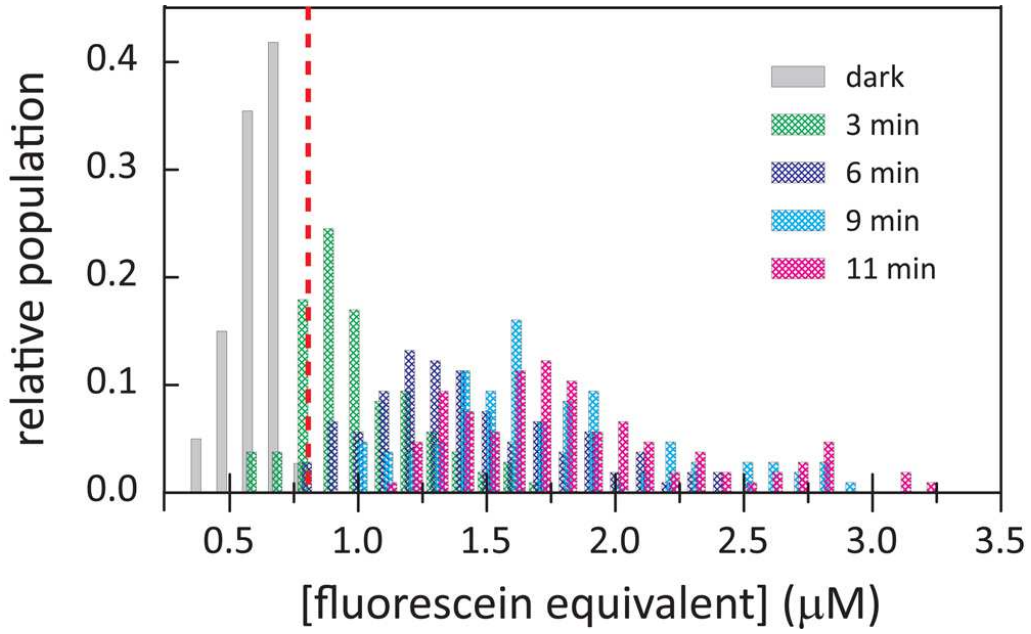
Supporting Figure 1. Kinetic saturation of fluorescence generation by β-galactosidase as a function of fluorescein di-β-D-galactopyranoside (FDG) concentration measured in bulk. The means and standard deviations (error bars) from three replicate measurements at each substrate concentration are plotted; the concentration of fluorescein cannot be directly calculated due to the unknown contribution of the partially-hydrolyzed product, fluorescein mono-β-D-galactopyranoside (FMG) to the observed fluorescence signal. The line connecting the data points is drawn merely to guide the eye. *Conditions:* [E] = 10 nM in buffer (PBS, pH 7.4). Fluorescence was measured at 525 nm (excitation at 490 nm).



Supporting Figure 2. (a) Correction of fluorescence intensities for photobleaching. The photobleaching rate was determined by encapsulating 2 μM of fluorescein in the 32-fL droplets stored in the device, and measuring the fluorescence intensity decay over 30 minutes with an exposure time of 1 sec every minute. An exponential fit of the graph yielded a photobleaching rate (κ) of 0.0067 (1/min). The corrected total intensities were obtained using the equation, $I_c(t) = I_c(t-1) + \{1+\kappa\} \times \{I_m(t) - I_m(t-1)\}$ where $I_c(t)$ and $I_m(t)$ are the fluorescence intensities after and before the correction for photobleaching, respectively, at a time *t*. (b) Calibration curve of fluorescein. FDG (500 μM) was incubated with β-galactosidase (0.2 unit/ml) overnight to prepare 500 μM of fluorescein. The fluorescein was diluted to different concentrations (0, 0.25, 0.5, 1.0, 2.0, 8.0 μM) in PBS buffer containing 0.1% v/v Tween-20. Femtodroplets (32 fL) of the solution were formed and transferred to the storage compartment of the device, where their fluorescence intensities were measured. Total intensity is an integral of fluorescence of droplets above their background. The squares are measured from stored femtodroplets having known concentrations of fluorescein. The dashed line is a corresponding fit curve.



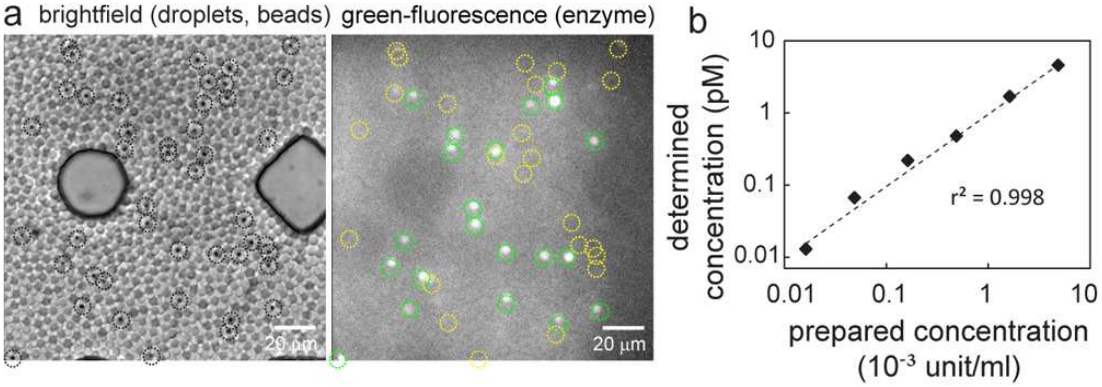
Supporting Figure 3. A histogram of femtodroplets *vs.* the fluorescein formation as a function of time



In the histogram the normalized numbers of femtodroplets enclosing a single molecule of enzyme are plotted versus the product (fluorescein) formation within droplets as a function of the incubation time (at 3, 6, 9, 11 minutes). The grey bar (dark) represents negative femtodroplets enclosing substrate only (FDG, 250 μM). The red dashed line is defined as three standard deviations above the mean of the background fluorescence. The plot shows that all positive femtodroplets are well above the threshold after incubation for 9 minutes. The enzyme concentration is 15×10^{-3} unit/mL (equivalent to 21 pM) and the number of analyzed positive femtodroplets is 146.

Supporting Experiment. Detection of individual biotinylated enzymes captured on streptavidin-coated beads

In order to validate the detection of single enzyme molecules in femtodroplets as a readout for a biomolecular binding event, we quantitated biotinylated β-galactosidase (at concentrations of 0.01-3 × 10^{-3} unit/mL) by capture with streptavidin-coated polystyrene beads (1-μm diameter, 1.2 × 10^{9} beads in 200 μL, equivalent to 10 pM). The beads (Polysciences) were mixed with biotinylated β-galactosidase (Vector, at concentrations of 0.015, 0.045, 0.15, 0.45, 1.5 and 4.5 × 10^{-3} unit/mL) in a final volume of 200 μL of PBS buffer containing 0.1% v/v Tween-20 and incubated for 1 hour at room temperature. The concentrations of the enzyme conjugate were chosen for each bead to be bound by either one molecule or no molecules. The beads were then recovered by centrifugation (12,100 × g for 6 min), the supernatant discarded and the particles resuspended with sonication for 3 min in PBS buffer containing 0.1% v/v Tween-20 (200 μL). This washing process was repeated six times before injection into the microfluidic device in order to ensure that unbound enzyme conjugates were removed. After binding and washing, the beads and substrate (FDG, 250 μM) were co-compartmentalized in femtodroplets and fluorescence images acquired after 10 minutes of incubation to detect the presence of single enzyme reporter molecules. Brightfield images were also taken to identify femtodroplets encapsulating beads. Since the concentration of the enzymatic reporter was lower than the bead concentration during anchoring of the enzyme to the beads, Poisson statistics dictate that most beads capture either a single enzyme reporter or none. As the bead concentration was known, the ratio of beads encapsulated in femtodroplets showing enzymatic reactivity to the total number of beads yields the concentration of the biotinylated enzyme. This proof-of-concept experiment showed the feasibility of using a bead-based femtodroplet assay to quantitate a low concentration of a target protein in an enzyme-linked binding assay.



Detection of individual biotinylated enzymes captured on streptavidin coated beads. (a) Brightfield (left) and green-fluorescence (right) images of droplets containing streptavidin-coated beads (1-μm diameter, 1.2 × 10^{9} beads in 200 μL, equivalent to 10 pM), following binding to biotinylated β-galactosidase (3 × 10^{-3} unit/ml), washing, encapsulation with substrate (FDG, 250 μM) and incubation for 10 minutes. Black circles in the brightfield image indicate beads encapsulated in femtodroplets; yellow and green circles in the fluorescence micrograph indicate bead-containing droplets that are positive and negative for enzymatic activity, respectively. (b) Plot of measured

concentrations of biotinylated enzyme captured on streptavidin-coated beads *vs*. prepared concentrations. The dotted line represents a linear fit. Molar concentrations were calculated from a Poisson distribution function as described in Figure 4b, except that the fraction of beads showing no enzymatic activity (*e.g*. the number ratio of yellow to black circles) and the known bead concentration (10 pM) were used instead of the inactive fraction and volume of the femtodroplets.

x	**prepared concentration of biotinylated beta-galatosidase (10^{-3} unit/ml)**		0.015	0.045	0.15	0.45	1.5	4.5
a_1	**experiment 1**	number of beads with enzymatic avtivity	2	16	34	75	189	413
b_1		total number of beads considered	2103	1935	1727	1514	1332	1098
λ_1		average occupancy	0.00095	0.0083	0.020	0.051	0.15	0.472
c_1		determined concentration (pM)	0.010	0.083	0.20	0.51	1.53	4.72
a_2	**experiment 2**	number of beads with enzymatic avtivity	4	9	43	71	245	341
b_2		total number of beads considered	2217	1892	1683	1676	1430	942
λ_2		average occupancy	0.0018	0.0048	0.026	0.043	0.188	0.449
c_2		determined concentration (pM)	0.018	0.048	0.26	0.43	1.88	4.49
y	**determined concentration of biotinylated beta-galatosidase (pM)**		0.014	0.065	0.23	0.47	1.70	4.61
z	**fit concentration (pM)**		0.016	0.047	0.16	0.47	1.56	4.67

The ratio of bead-containing droplets showing no enzymatic activity to the total number of bead-containing droplets in measurements, $f(0) = (b_i - a_i)/b_i$, is inserted into the Poisson distribution function, $f(n) = \lambda^n \cdot e^{-\lambda}/n!$ where $n=0$, which provides an average occupancy of the enzymatic reporter per bead, $\lambda_i = -ln[f_i(0)] = -ln[1 - a_i/b_i]$. The average occupancy is multiplied by the bead concentration (1.2×10^9 beads in 200 μL, equivalent to 10 pM) to yield the determined molar concentration of the target molecules (c_i). The determined concentration of biotinylated β-galactosidase (y) is the mean of c_1 and c_2. The fit concentration is obtained from the linear fit to the equation, fit concentration (z) = 1.04 * prepared biotin-enzyme concentrations (x).

Supporting Table 1; Femtodroplet volumes and generation frequencies

oil flow rate (μL/hr)	water flow rate (μL/hr)	experiment			prediction	
		period (μs)	frequency (f_e, MHz)	drop volume (fL)	frequency (f_p, MHz)	drop volume (fL)
200	40	4.01	0.25	44.9	0.28	39.7
220	40	3.17	0.32	35.5	0.33	34.1
240	40	2.59	0.39	29.0	0.38	29.0
260	40	2.18	0.46	24.4	0.45	24.5
280	40	2.08	0.48	23.3	0.54	20.4
300	40	1.47	0.68	16.5	0.66	16.9
320	40	1.14	0.88	12.8	0.81	13.8
340	40	1.05	0.95	11.8	1.00	11.0
360	40	0.80	1.25	8.96	1.27	8.72
480	40	stable formation, not measurable			9.75	1.1

The generation frequencies of femtodroplets and their volumes are predicted using the equations below, which are derived according to a dripping-mode mechanism for droplet break-up. The droplet-formation frequency in this regime is given by $f = Q_w / V_{droplet} = 3Q_w / 4\pi r_{droplet}^3$ where f is the generation frequency, $r_{droplet}$ is the radius of the droplet, r_{jet} is the radius of the jet and Q_w is the flow rate of water. We assume that $r_{droplet} = \gamma \cdot r_{jet}$ with $\gamma \sim 2$,[1, 2] which is also consistent with what is shown in Supporting Movie 2. We can write r_{jet} and Q_o in a first approximation as $r_{jet} = a - Q_o / b$ where a and b are fitting parameters,[3] to give:

$$f = \frac{3Q_w}{\gamma^3 4\pi} \frac{1}{\left(a - Q_o / b\right)^3}.$$

The experimental data fit this equation with a coefficient of determination (r^2) of 0.99 between the measured and predicted frequencies, returning fitting parameters of a = 1.58 and b = 382. The predicted radius of the jet (r_{jet}) is 1.0 μm at an oil flow rate of 200 μL/hr, which is of the same order of magnitude as the value of 1.6 μm obtained by balancing the flow rates of oil and water in the nozzle (10 μm wide × 5 μm deep), without accounting for the flow profile across the channel. Although the droplet-formation frequency at the largest oil flow rate tested (480 μL/hr) could not be measured using our current experimental setup, very stable droplet generation was observed. The predicted frequency (f_p) and droplet volume under these conditions are 9.8 MHz and 1.1 fL respectively (shaded in yellow), given the values established above for the fitting parameters a and b. We note that this analysis is not intended to provide a complete physical description of the droplet-formation process, but rather to confirm that the measured droplet diameters are consistent with a dripping-mode-formation mechanism.

Supporting Table 2; Determination of the molar concentration of β-galactosidase

x	**prepared concentration of beta-galactosidase (x 10^{-3} unit/ml)**		0.75	1.5	3.75	7.5	15	30
a_1	**experiment 1**	number of droplets with enzymatic avtivity	45	119	161	343	596	785
b_1		total number of droplets considered	1618	2080	1615	1783	1894	1593
λ_1		average occupancy	0.028	0.059	0.105	0.214	0.378	0.679
c_1		determined concentration (pM)	1.47	3.07	5.48	11.2	19.7	35.4
a_2	**experiment 2**	number of droplets with enzymatic avtivity	28	68	208	352	762	956
b_2		total number of droplets considered	1812	1789	1746	1825	1723	1645
λ_2		average occupancy	0.016	0.039	0.127	0.214	0.584	0.870
c_2		determined concentration (pM)	0.81	2.02	6.62	11.9	28.2	45.4
y	**determined concentration of beta-galactosidase (pM)**		1.14	2.55	6.05	11.5	24.0	40.4
z	**fit concentration (pM)**		1.06	2.11	5.28	10.6	21.1	42.2

The ratio of droplets showing no enzymatic activity to the total number of droplets in measurements, $f_i(0) = (b_i - a_i)/b_i$, is inserted into the Poisson distribution function, $f(n) = \lambda^n \cdot e^{-\lambda}/n!$ (n=0), where *n* describes the number of enzyme molecules in a droplet, yielding the average occupancy of the enzymatic reporter per droplet, $\lambda_i = -ln[f_i(0)] = -ln[1 - a_i/b_i]$. Since the droplet volume (32 fL) is known, the determined molar concentration of the target molecule (c_i) can be calculated. The determined concentration of β-galactosidase (y) is a mean of c_1 and c_2. The fit concentration is obtained from the linear fit to the equation, fit concentration (z) = 1.41 * prepared enzyme concentrations (x).

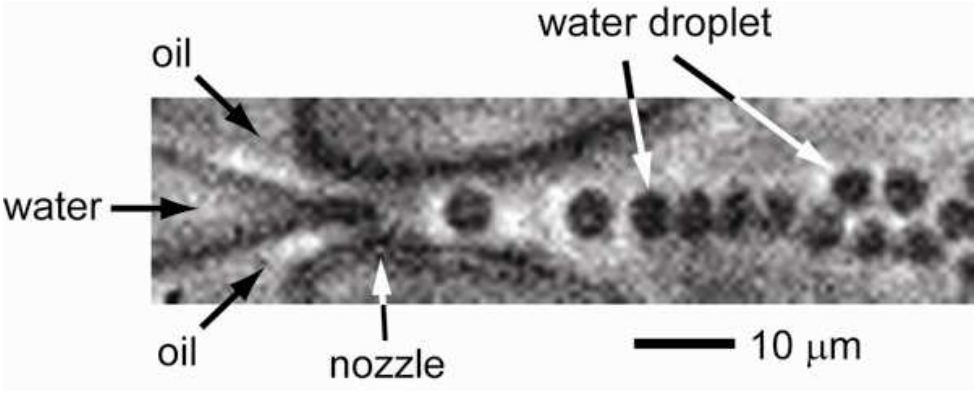
Supporting Table 3; Quantification of PSA

x	**prepared concentration of PSA (pg/ml)**		1.4	4.2	14	42	140
a_1	**experiment 1**	number of beads with enzymatic avtivity	8	24	43	120	272
b_1		total number of beads considered	1598	1770	1283	896	864
λ_1		average occupancy	0.005	0.014	0.034	0.144	0.378
c_1		determined concentration (pM)	0.05	0.14	0.34	1.44	3.78
a_2	**experiment 2**	number of beads with enzymatic avtivity	14	24	28	86	192
b_2		total number of beads considered	1716	1877	1218	872	642
λ_2		average occupancy	0.008	0.013	0.023	0.104	0.355
c_2		determined concentration (pM)	0.08	0.13	0.23	1.04	3.55
a_3	**experiment 3**	number of beads with enzymatic avtivity	10	14	37	132	224
b_3		total number of beads considered	1928	1622	1422	960	696
λ_3		average occupancy	0.005	0.009	0.026	0.148	0.388
c_3		determined concentration (pM)	0.05	0.09	0.26	1.48	3.88
a_4	**experiment 4**	number of beads with enzymatic avtivity	16	22	26		
b_4		total number of beads considered	1432	1438	1296		
λ_4		average occupancy	0.011	0.015	0.020		
c_4		determined concentration (pM)	0.11	0.15	0.20		
y	**determined concentration of PSA (pM)**		0.074	0.13	0.26	1.32	3.74
	standard deviation of determined concentration (pM)		0.029	0.029	0.059	0.24	0.17
z	**fit concentration (pM)**		0.038	0.11	0.38	1.13	3.78

The ratio of bead-containing droplets showing no enzymatic activity to the total number of bead-containing droplets in measurements, $f(0)=(b_i-a_i)/b_i$, is inserted into the Poisson distribution function, $f(n)=\lambda^n \cdot e^{-\lambda}/n!$ where $n=0$. This provides the average occupancy of the enzymatic reporter per bead, $\lambda_i = -ln[f_i(0)] = -ln[1-a_i/b_i]$. The average occupancy is then multiplied by the bead concentration (1.2×10^9 beads in 200 µL, equivalent to 10 pM) to yield the determined molar concentration of the target molecule (c_i). The determined concentration of PSA (y) is a mean of c_1, c_2, c_3 and c_4. The fit concentration is obtained from the linear fit to the equation, fit concentration (z) = 0.027 * prepared PSA concentrations (x).

Supporting movie 1 (movie_1_drop form.avi)
Movie of a microfluidic device in operation, showing the fluid inlets, flow-focusing nozzle and flow channel. ~30-fL aqueous femtodroplets are shown forming at ~300 kHz in a continuous oil stream by hydrodynamic focusing at the nozzle, as illustrated in Figure 1a. All fluids are driven by syringe pumps, with oil introduced *via* the lower two inlet channels and the pre-mixed aqueous solution entering from the inlet channels at the top of the picture.

Supporting movie 2 (movie_2_drop form_time stamp.avi)
Time-stamped movie of droplet generation taken and analyzed using an ultra-high speed camera (V1610, Vision Research) and its operating software. It was taken with 0.9 Mfps. Droplets with a volume of 45 fL are shown forming at a frequency of around 123 kHz at a flow rate of 20 μL/hr of water and 200 μL/hr of oil.



Supporting movie 3 (movie_3_trap working.avi)
Trapping of femtodroplets in storage compartments and their subsequent release. When external pressure of ~50 psi is applied, each trap captures about 5 x 10^3 droplets; during this time - in which turnover of the fluorogenic substrate occurs – new femtodroplets continue to be generated but bypass the pressurised storage chamber. Upon removal of the external pressure, the trapped droplets are flushed out by the stream of newly-formed droplets. This process can be repeated every 10 seconds.

Supporting movie 4 (movie_4.avi)
Accumulation of fluoresence resulting from enzymatic turnover of FDG (250 μM) by single β-galactosidase molecules (bulk concentration 1.1 pM) encapsulated in femtodroplets, over a period of 10 minutes after trapping. Since the mean occupancy of femtodroplets is <1 enzyme molecule, only a fraction of the droplets become fluorescent.

Supporting movie 5 (movie_5.avi)
Accumulation of fluoresence resulting from enzymatic turnover of FDG (250 μM) by single β-galactosidase molecules (bulk concentration 5.3 pM) encapsulated in femtodroplets, over a period

of 10 minutes after trapping. Since the mean occupancy of femtodroplets is <1 enzyme molecule, only a fraction of the droplets become fluorescent.

Supporting movie 6 (movie_6.avi)
Accumulation of fluoresence resulting from enzymatic turnover of FDG (250 μM) by single β-galactosidase molecules (bulk concentration 21 pM) encapsulated in femtodroplets, over a period of 10 minutes after trapping. Since the mean occupancy of femtodroplets is <1 enzyme molecule, only a fraction of the droplets become fluorescent.

Supporting movie 7 (movie_7.avi)
Accumulation of fluoresence resulting from enzymatic turnover of FDG (250 μM) by single β-galactosidase molecules (bulk concentration 42 pM) encapsulated in femtodroplets, over a period of 10 minutes after trapping. Since the mean occupancy of femtodroplets is <1 enzyme molecule, only a fraction of the droplets become fluorescent.

References

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