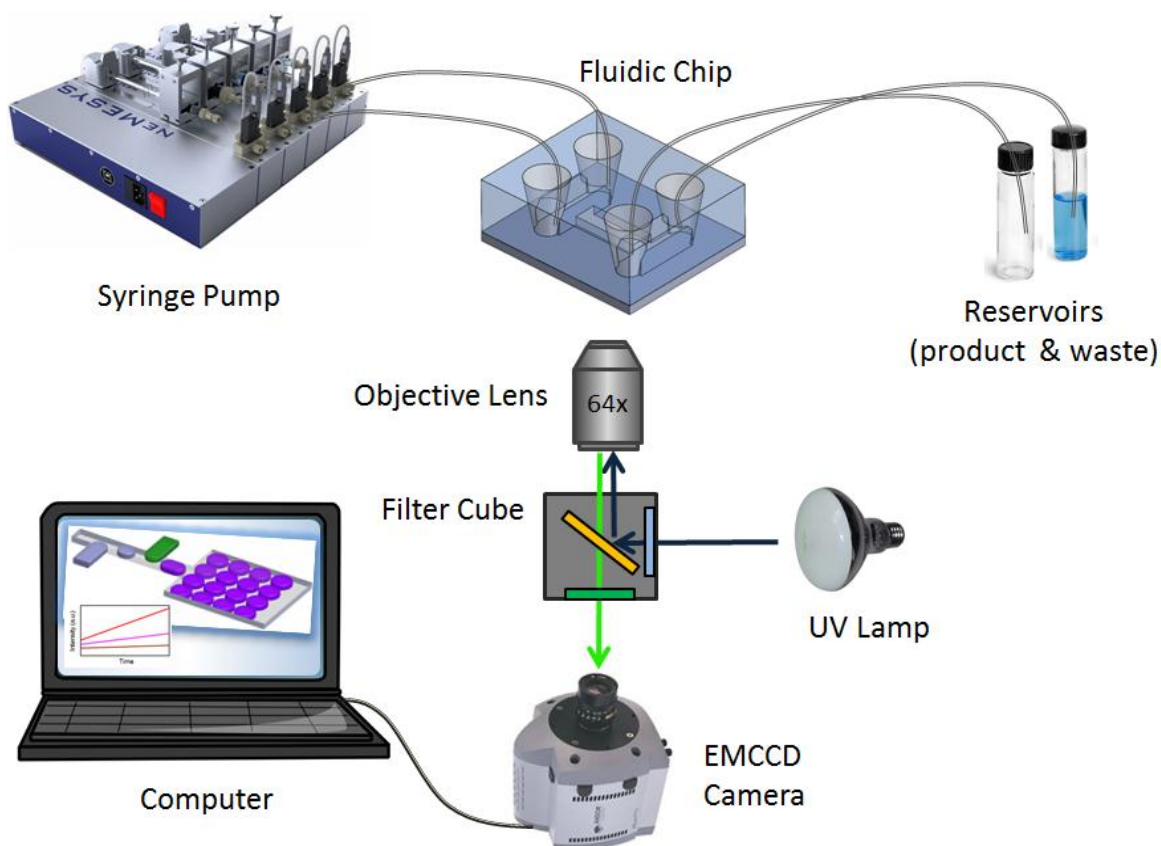


# Electronic Supporting Information

## for Single-enzyme Analysis in a Droplet-based Micro and Nanofluidic System

### 1. Fluidic and Optical Setup



**Fig.S1:** Illustration of the microfluidic chip mounted on the epifluorescence microscope and connected to the fluidic setup.

## 2. Calibration

Prior to the kinetic measurement of the enzymatic reaction, the fluorescence intensity of the product was calibrated to relate it to the concentration of the fluorescent product. To perform this calibration, a fluorescein solution in PBS buffer at pH 7 and silicone oil with 4 wt% Span 80 were loaded into the fluidic system to produce aqueous droplets in a continuous oil phase. The fluorescence intensities of the fluorescein solution were measured at different concentrations of fluorescein as depicted in Fig.6-4 showing that the fluorescence intensity is proportional to the concentration of fluorescein in the  $\mu\text{M}$  range. This calibration curve is used later to convert the measured increasing intensity to the increasing concentration of fluorescent product and hence the turnover number of the enzymatic reaction.

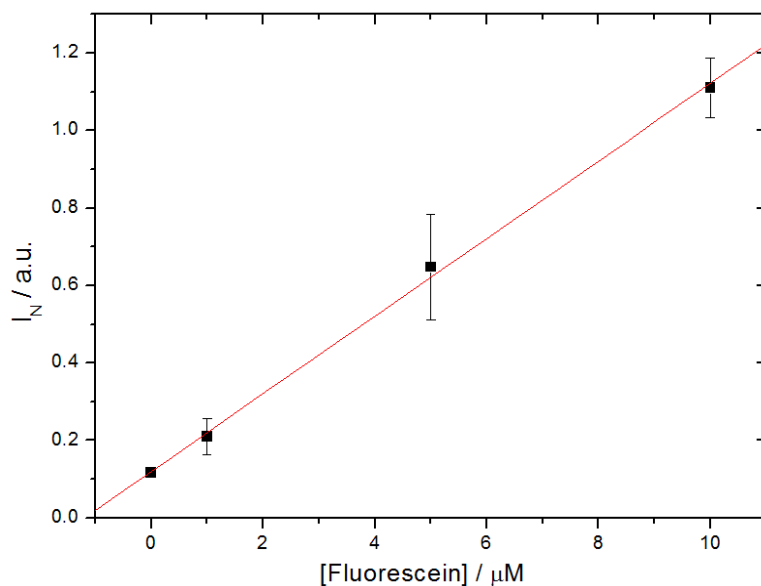
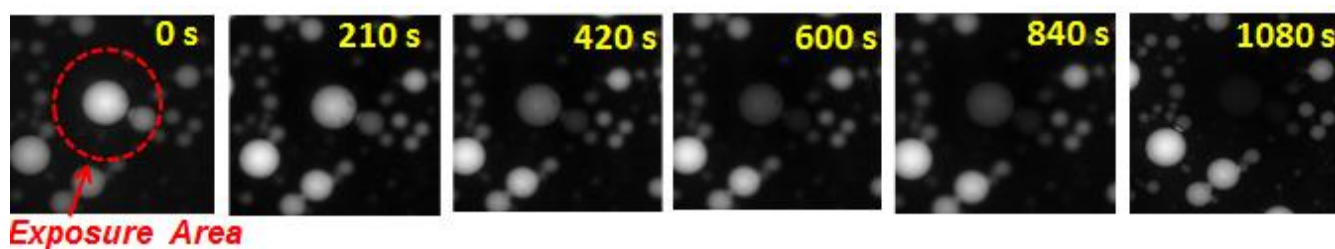


Fig.S2: A calibration curve of the fluorescence intensity against the concentration of the fluorescein at pH 7.

### 3. Photobleaching

One drawback of the fluorescein molecule is its relative high rate of photobleaching by which the fluorescent molecules lose their ability to fluoresce due to the photo-induced chemical destruction during the exposure to the excitation light in the presence of oxygen molecules.<sup>1</sup> Via this process, the fluorescence intensity reduces irreversibly leading to the misinterpretation of the amount of fluorescent molecules in the system. To avoid this problem, oxygen molecules should be removed from the system and anti-oxidant reagent should be added to the solution. Addition of the anti-oxidant reagent e.g. n-propyl gallate (n-PG) into a solution can diminish the photobleaching effect especially in a fluorescein solution.<sup>2-4</sup> Therefore, the photobleaching effect on the fluorescein molecules from our existing setup and the optimization of the amount of this anti-oxidant reagent added into the solution have to be studied. The water-in-oil emulsions were prepared from fluorescein solution (50 $\mu$ M fluorescein in PBS solution at pH 7) with or without n-PG as an aqueous phase and the silicone oil as a continuous phase. Then, the emulsion was placed between two glass substrates for the excitation by UV lamp and observation by a fluorescence microscope (DMI 5000M, Leica) equipped with mercury lamp and high-sensitivity camera (EMCCD, Andor Ixon, UK). Before mixing, each solution was degassed by placing into the desiccator to remove oxygen molecules in the solution. Firstly, no anti-oxidant reagent was added into the fluorescent solution. The light from UV lamp was exposed to one area (red circle in Fig.S3) and the fluorescence intensity was measured upon UV exposure (from 0 to 1080 sec) as can be seen in Fig.S3 and Fig.S4 *Left*.



**Fig.S3:** The decreasing fluorescence intensity due to the photobleaching effect on an aqueous droplet in oil without the addition of n-PG.

Then, the decreasing fluorescence intensity was plotted against the exposure time as shown in Fig.S4 *Left*. The fluorescence intensity decreased exponentially as a function of the exposure time corresponding to the following equation,

$$I = I_0 e^{-t/T} \quad \text{Eqn.(S-1)}$$

When  $I$  = Fluorescence Intensity

$I_0$  = Initial fluorescence intensity

$t$  = Exposure time

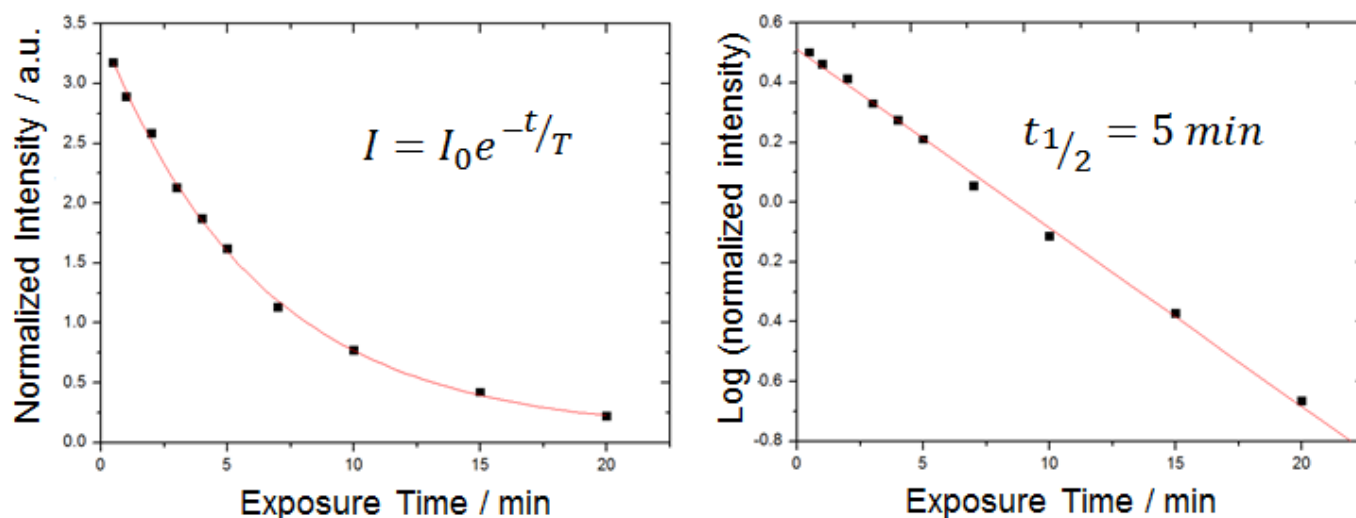
$T$  = Life time of a fluorescent molecule

From Eqn.(S-1),

$$\log(I) - \log(I_0) = \log(e^{-t/T}) \quad \text{Eqn.(S-2)}$$

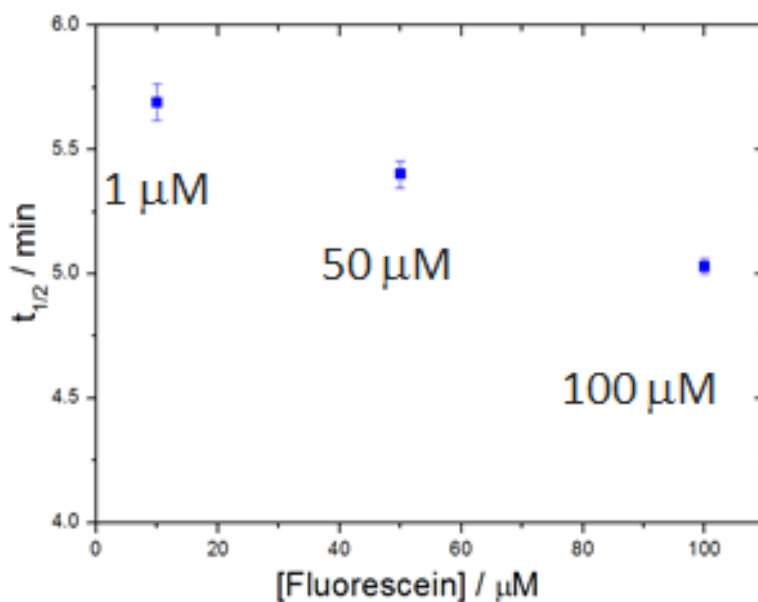
$$\log(I) - \log(I_0) \cong -0.434\left(\frac{t}{T}\right) \quad \text{Eqn.(S-3)}$$

A plot of the logarithm of the intensity against the exposure time (Fig.S4 *Right*) can thus determine the half time ( $t_{1/2}$ ) of this fluorescent molecule under the illumination conditions of our existing setup and is found to be 5 minutes.



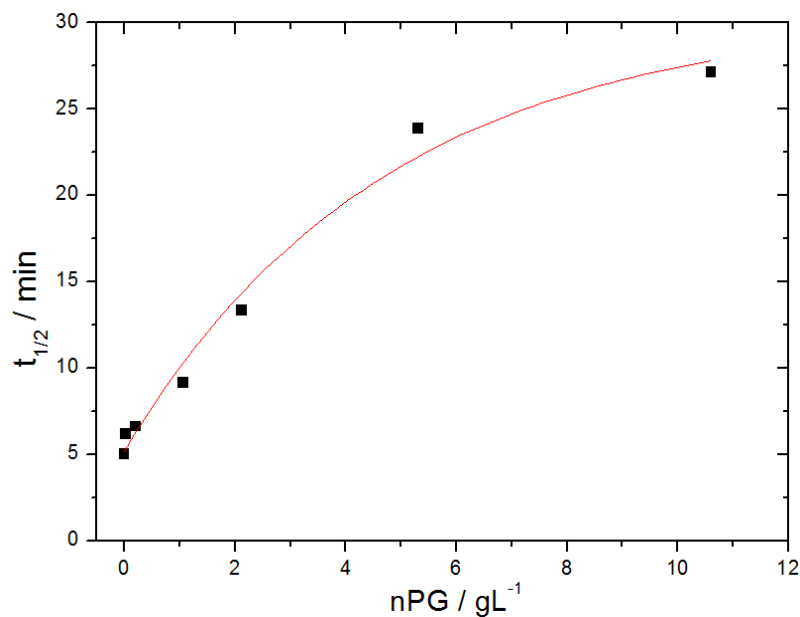
**Fig.S4:** The exponential decay of the fluorescence intensity under illumination by a UV-lamp from our setup (*Left*). The half time ( $t_{1/2}$ ) of this fluorophore can be determined from the plot of the logarithm of the intensity against the exposure time (*Right*).

In addition, we performed the same experiment with other concentrations of fluorescein solution and found, interestingly, that when the concentration of fluorescein was lower, the half time ( $t_{1/2}$ ) became relatively higher as illustrated in Fig.S5. We speculate that this might be the self-quenching effect by which the fluorescence intensity reduced due to the interaction between fluorescent molecules and another fluorescent molecule or other species molecules in the environment.



**Fig.S5:** The life time ( $t_{1/2}$ ) of the fluorescein solution (pH 7) at different fluorescein concentrations.

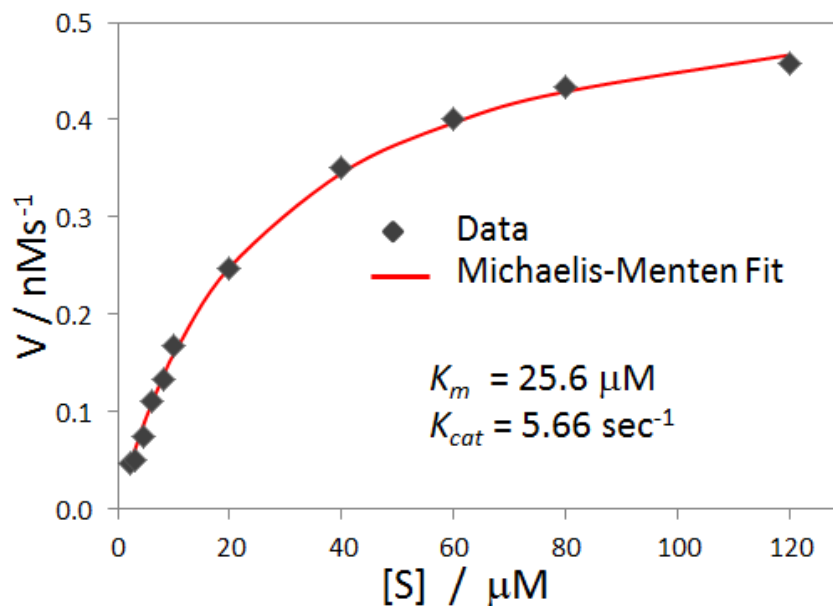
Next, the amount of the anti-oxidant reagent added into the fluorescein solution was optimized. A solution of n-PG was prepared by dissolving n-PG powder in pure glycerol at a concentration of 50 g/L, and then diluted with an equal volume of PBS buffer (pH 7). All chemicals in these experiments were purchased from Sigma Aldrich, The Netherlands. The reduction in the rate of photobleaching was then plotted against the added concentration of n-PG as shown in Fig.S6.



**Fig.S6:** The half time ( $t_{1/2}$ ) of the fluorescent molecule increases proportionally with the concentration of added n-propyl gallate.

From this experiment it is clear that the addition of n-PG to the fluorescein solution can reduce the photobleaching effect. The higher the amount of n-PG, the smaller the photobleaching effect in the solution. However, when the amount of n-PG was higher than 5 g/L, the photobleaching effect was found to be only marginally further reduced. Thus, the optimal amount of n-PG in our solution was found to be around 5 g/L.

#### 4. Enzyme Activity determined by a Fluorescence Spectrometer



**Fig.S7:** The experimental result of the enzymatic reaction from a fluorescence spectrometer and the Michaelis-Menten fitting curve.

In general, the enzymatic activity obeys the Michaelis-Menten equation (see e.g. J.M.Berg, J.L.Tymoczko and L. Stryer, Biochemistry, 5<sup>th</sup> edition, W.H. Freeman, New York 2002)

$$v = \frac{V_{\max} [S]}{K_m + [S]}$$

Where

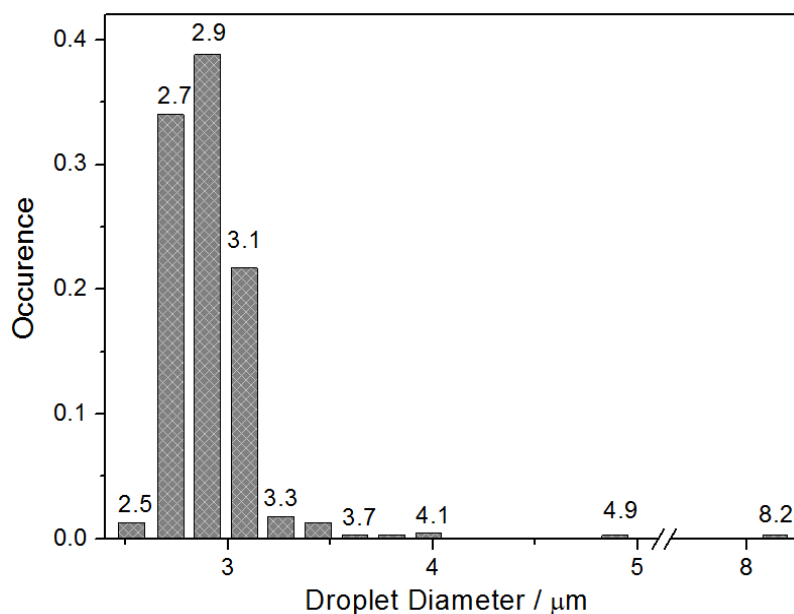
$v$	=	production rate of (fluorescent) product,
$V_{\max}$	=	maximal production rate,
$K_m$	=	Michaelis-Menten constant,
$[S]$	=	concentration of substrate.

At low concentration of substrate, the reaction is first order in  $[S]$ , but at high concentration of substrate, the reaction is zero order in  $[S]$ . In our experiment,  $[S]$  is much higher than  $K_m$ , so that the obtained production rate of is equal to the maximal rate and not disturbed by the changing concentration of the substrate during the enzymatic reaction. Michaelis-Menten kinetics furthermore gives that  $V_{\max} = k_{cat}[E]$ , where  $k_{cat}$  is the turnover number and  $[E]$  the enzyme concentration. This implies that the production

rate we measure is linearly proportional to the enzyme concentration. Since the product is fluorescent and the fluorescence intensity is linearly related to the concentration of the fluorescent product [see part 2 in ESI], the rate of fluorescent intensity increase can be used to determine  $V_{\max}$ . From the values of  $V_{\max}$  in the different droplets, we can derive the enzyme concentrations (the number enzyme molecules in the droplet volumes) as well as the turnover number via  $V_{\max} = k_{cat}[E]$ .



## 5. Size Distribution of generated droplets at minute 1335<sup>th</sup>



**Fig.S8:** The size distribution of the generated droplets after incubation for 1335 minutes.

### References

1. L. L. Song, E. J. Hennink, I. T. Young and H. J. Tanke, *Biophys J*, 1995, **68**, 2588-2600.
2. H. Giloh and J. W. Sedat, *Science*, 1982, **217**, 1252-1255.
3. A. K. Gaigalas, L. Wang, K. D. Cole and E. Humphries, *J Phys Chem A*, 2004, **108**, 4378-4384.
4. M. Battaglia, D. Pozzi, S. Grimaldi and T. Parasassi, *Biotech Histochem*, 1994, **69**, 152-156.