Supporting Information

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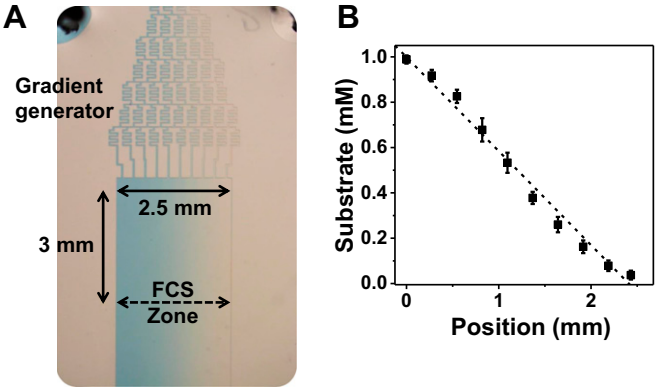

Fig. S1. Microfluidic device used to combine constant enzyme concentration with linear substrate gradient. (*A*) To demonstrate experimentally the predicted steady-state concentration gradient for these device dimensions and flow rate (10 min after entry at rate 50 μL/h), methylene blue dye solution (left) and distilled water (right) were injected. The images were obtained using a stereomicroscope (Olympus SZX16) and intensity in the obtained images was quantified (Fiji ImageJ software). (*B*) These line-scan data at ∼3.0 mm down the channel show the predicted near-linear concentration gradient. Solid symbols are the average of five line scans after 35 × 5 binning.

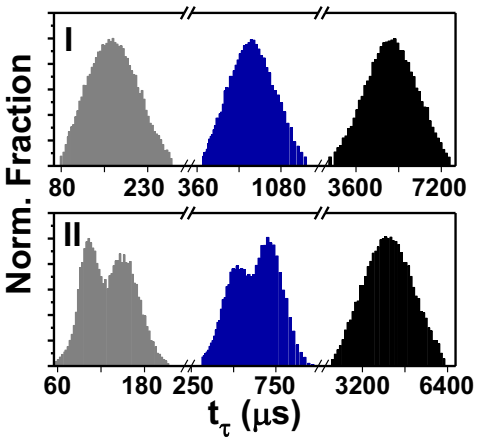

Fig. S2. Transit time distribution of urease in viscous solution. In the presence of 20% Ficoll added as crowding agent to raise the viscosity, transit time distribution t_τ is shown with w for urease without substrate (*I*, *Top*) and with 1 mM substrate (*II*, *Bottom*). The beam waist is $w = 50$ nm, 100 nm, and 250 nm from left to right. Data were analyzed with bin size of 0.5 ms. Compared with Fig. 3*C* the motion is about 10-fold slower.

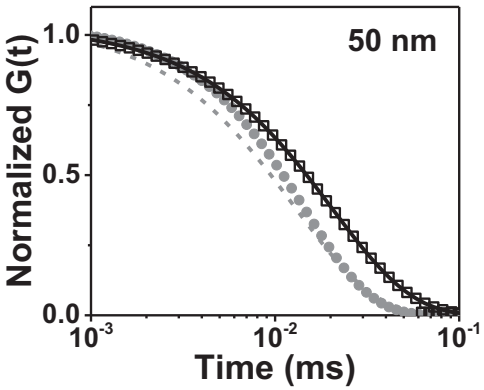

Fig. S3. Tracer dyes mixed with active enzyme also display enhanced mobility. Shown is normalized $G(t)$ for fluorescent tracer (Alexa 488, 10 nM) mixed with urease (10 nM) in buffer without substrate (open symbols) and with 1 mM urea (solid symbols). The STED beam waist is $w = 50$ nm. Lines show fit to single-component passive diffusion.

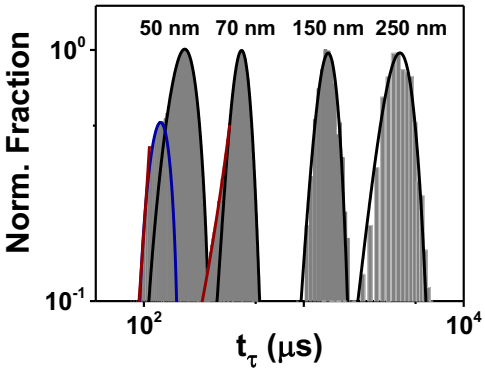

Fig. S4. On log–log scales, the transit time distribution of urease at substrate concentration 0.1 mM. Bimodal distribution of the transit times is evident starting at $w = 70$ nm. The black line is Gaussian fitting of the slow component. The blue line is Gaussian fitting of the fast component. The red line is an exponential fit.

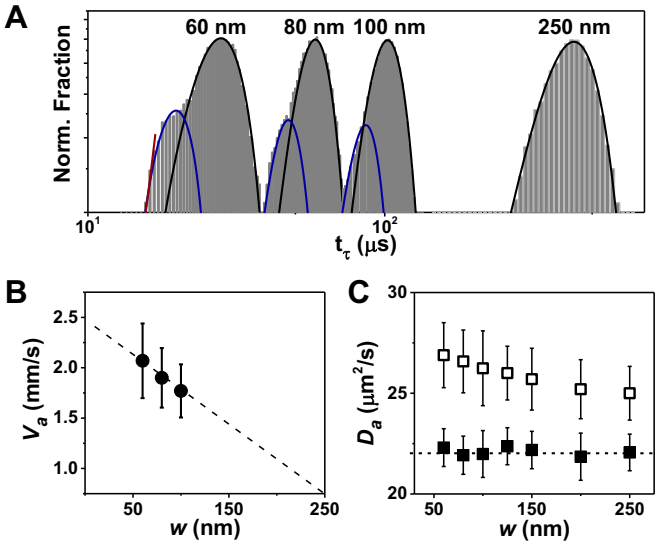

Fig. S5. For acetylcholinesterase the fast and slow components of transit time distribution are quantified for 10 nM enzyme with 0.2 mM substrate. (*A*) Transit time distribution is plotted against time on log–log scales with beam waist $w = 60$ nm, 80 nm, 100 nm, and 250 nm from left to right. The black line is Gaussian fitting of the slow component. The blue line is Gaussian fitting of the fast component. The red line is an exponential fit. (*B*) Apparent ballistic speed (v_a) plotted against w. (*C*) Apparent diffusion coefficient (D_a) for the slow component of the bimodal distribution ($w \leq 100$ nm) or the peak unimodal time, plotted against w without substrate (solid symbols) and with substrate (open symbols).