Droplet Microfluidic Flow Cytometer for Sorting on Transient Cellular Responses of Genetically-Encoded Sensors

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Supporting Information

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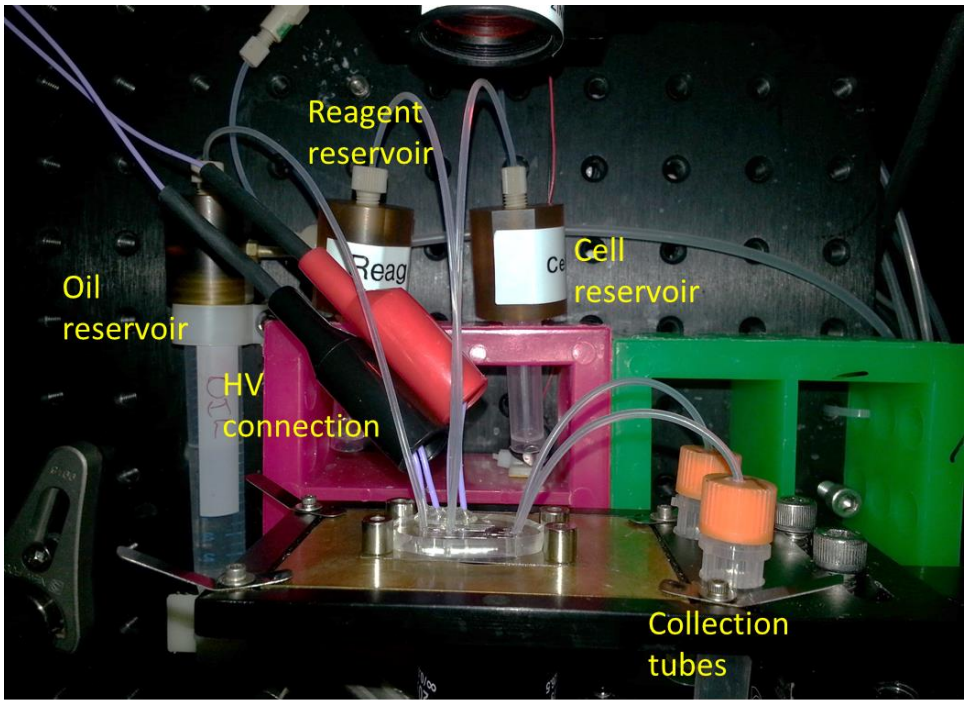

Figure S-1: Picture showing implementation of microfluidic device into the apparatus.

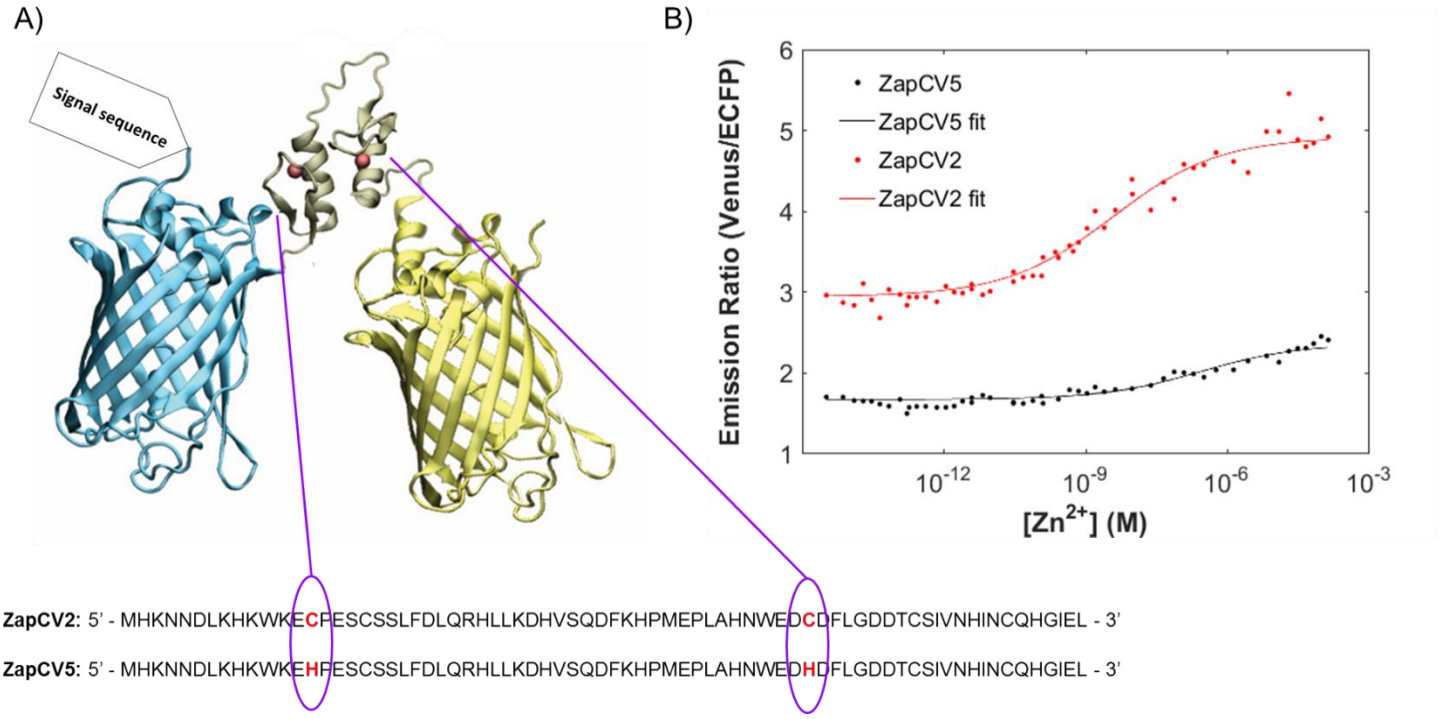

Figure S-2: A) A cartoon of a representative FRET-based Zn^{2+} sensor used in this work. The structure from N-terminus to C-terminus consists of a localization signal sequence, ECFP (blue), Zap1 Zn^{2+} binding domain (green), Venus (yellow). Zn^{2+} ions are shown as red spheres. Sequences of the Zn^{2+} binding domains of ZapCV2 and ZapCV5 are shown below with the differing residues bolded and highlighted in red. B) In vitro titration curves showing the binding affinities and Hill coefficients for ZapCV2 and ZapCV5. Experimental details shown below.

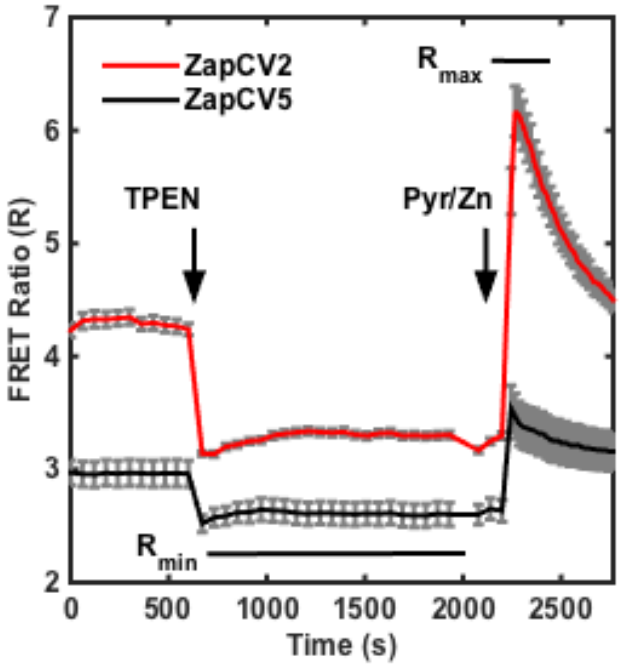

Figure S-3. Calibration plot with raw FRET ratios for data in Figure 1.

Sensor construction and characterization

Design of ZapCV2 and ZapCV5 Zn^{2+} sensors

ZapCV sensors are modified based on previously characterized ZapCY2 sensors.[1] The FRET acceptor (citrine fluorescent protein, YFP) in ZapCY2 was replaced by circularly permuted (at amino acid 173 position) Venus fluorescent protein, which generated ZapCV2 (YFP changed to Venus). The lower affinity ZapCV5 was created by quick change mutagenesis of the Zn^{2+} binding domain (C586H and C623H). The cytosolic NES-ZapCV sensors include an N-terminal nuclear export signal (MLQLPPLERLTL). These sensors were cloned in the pcDNA3.1 and PiggyBac Transposon vector backbone for mammalian cell expression and in the pBAD vector backbone for bacterial expression.

In vitro characterization of ZapCV2 and ZapCV5

For *in vitro* studies, the sensors were cloned into pBAD and expressed in Top-10 Escherichia coli with the addition of 0.2% arabinose. Sensor proteins were purified using Ni^{2+} ion affinity chromatography and the His tag was removed with TEV protease in 20mM Tris, 100 mM NaCl, pH8 buffer at room temperature overnight (sensor to TEV protease ratio was 10:1). The sensor was then concentrated with an Amicon centrifugal filter to 50-100 µM as determined by the absorbance at 516 nm using $\varepsilon = 77000\ M^{-1}\ cm^{-1}$.

Purified sensor was titrated with Zn^{2+} to determine the disassociation constants of Zn^{2+} from the sensors. Zn^{2+} titrations were performed in HEPES buffer (150 mM Hepes, 100 mM NaCl, and 10% glycerol, pH 7.4) with 1 µM sensor protein, treated for 5 minutes immediately prior to titration with 0.5 mM TCEP, and different concentration of free Zn^{2+} as described previously.[1] The in vitro fluorescence measurements used for Zn^{2+} titrations were made on a Tecan Safire-II fluorescence plate reader using the following parameters: excitation: 420 nm, emission: 435-649 nm, and the emission bandwidth was 10 nm. The apparent dissociation constant was determined using the fitting method described by Pomorski et al.[2] The intensities of the fluorescence emission at 481 nm were used as λ_1 and at 529 nm for λ_2. The ratios of these intensities were

plotted against the zinc concentration and fitted using one of the following equations depending on the ratio used (λ_1/λ_2 vs. λ_2/λ_1).

$$R_{\frac{2}{1}} = \frac{(I_{2b} \cdot Z^n) + (I_{2u} \cdot {K_d}^n)}{(I_{1b} \cdot Z^n) + (I_{1u} \cdot {K_d}^n)}$$

$$R_{\frac{1}{2}} = \frac{(I_{1b} \cdot Z^n) + (I_{1u} \cdot {K_d}^n)}{(I_{2b} \cdot Z^n) + (I_{2u} \cdot {K_d}^n)}$$

Where the $I_{2b,}$ $I_{2u,}$ $I_{1b,}$ I_{1u} are fixed using the average intensity at λ_1 or λ_2 at the five most or least concentrated Zn^{2+} conditions, Z is equal to the –log of the free zinc concentration, K_d is the disassociation constant, and n is the hill coefficient. The reported K_d's are the average of 2 or more titrations of the sensor.

Cellular imaging for in vivo calibration

Imaging experiments were performed on a Nikon Ti-E wide-field fluorescence microscope equipped with Nikon elements software, Ti-E perfect focus system, an iXon3 EMCCD camera (Andor), mercury arc lamp, and YFP FRET (434/16 excitation, 458 dichroic, 535/20 emission), CFP (434/16 excitation, 458 dichroic, 470/24 emission), and YFP (495/10 excitation, 515 dichroic, 535/20 emission) filter sets. External excitation and emission filter wheels were controlled by a Lambda 10-3 filter changer (Sutter Instruments), while dichroic mirrors were placed on cubes in the dichroic turret. Images were collected using a 60X oil objective (NA 1.40), 100 ms exposure time, EM gain 1 MHz at 16-bit readout mode with an EM gain multiplier of 100, and a neutral density filter with 12.5% light transmission.

For the calibration experiments shown in Figure 1, sensor constructs were transfected into HeLa cells with TransIT-LT1 (Mirus Bio). Cells were imaged 48 hours post-transfection. Cells were maintained at 37°C and 5% CO_2 in a LiveCell™ environment chamber (Pathology Devices) during the experiment. Cells were imaged in phosphate-free HEPES-buffered HBSS, pH = 7.4 to prevent zinc precipitation. Resting images were collected for 5 minutes, followed by treatment with 150 µM TPEN to collect R_{min} data until a stable signal was achieved. Cells were then washed with phosphate, calcium, and magnesium-free HEPES-buffered HBSS, pH = 7.4,

then cells were treated with 1 µM pyrithione and 50 µM $ZnCl_2$ to obtain R_{max} data. Resting and R_{min} images were collected every 1 minute, and R_{max} images were collected every 20 seconds. All data were analyzed in MATLAB (Mathworks). Images were background corrected by drawing a region of interest (ROI) in a blank area of image and subtracting the average fluorescence intensity of the background ROI from the average intensity of each cell. FRET ratios for each cell were calculated by dividing the background-corrected YFP FRET intensity by the background-corrected CFP intensity $((I_{cellular\ FRET} - I_{background\ FRET})/(I_{cellular\ CFP} - I_{background\ CFP}))$. This background subtraction and FRET response measurement is done in image post-processing, though it should, in theory, be possible to speed up the process of dynamic range determination by incorporating a real-time algorithm in the acquisition software.

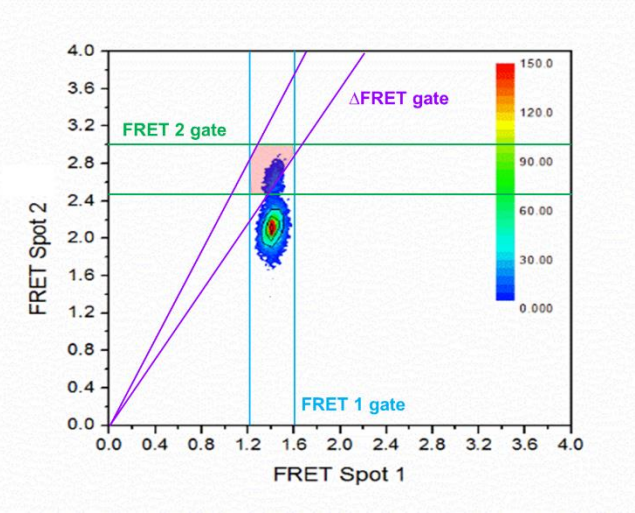

Figure S-4: Contour plot for the data shown in Figure 5.

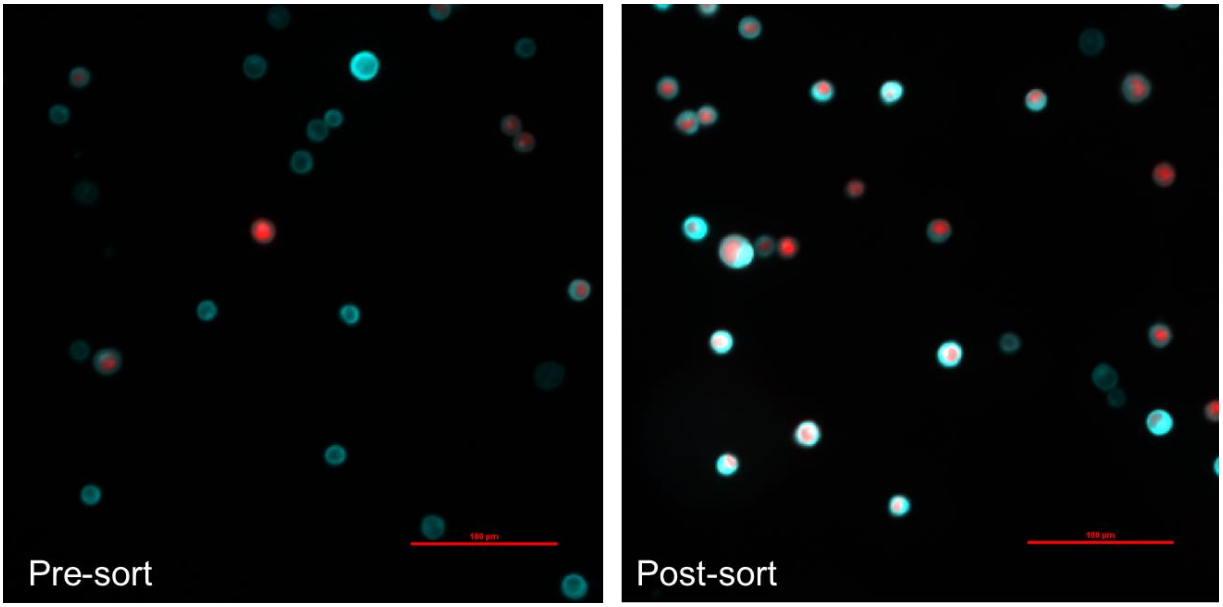

Figure S-5: Representative, pseudo-colored images using CFP (434/16 excitation, 458 dichroic, 470/24 emission) and mCherry channels (560/20 excitation, 585 LP, 610/50 emission) showing pre- and post-sort collections of cells used to collect data in Figure 5. Images were collected using a 20X air objective (NA 0.8), 100 ms exposure time for CFP and 200 ms for mCherry, EM gain 1 MHz at 16-bit readout mode with an EM gain multiplier of 100, and a neutral density filter with 12.5% light transmission. Cell expressing ZapCV2 are coexpressed with an NLS-mCherry. Scale bar = 100 uM.

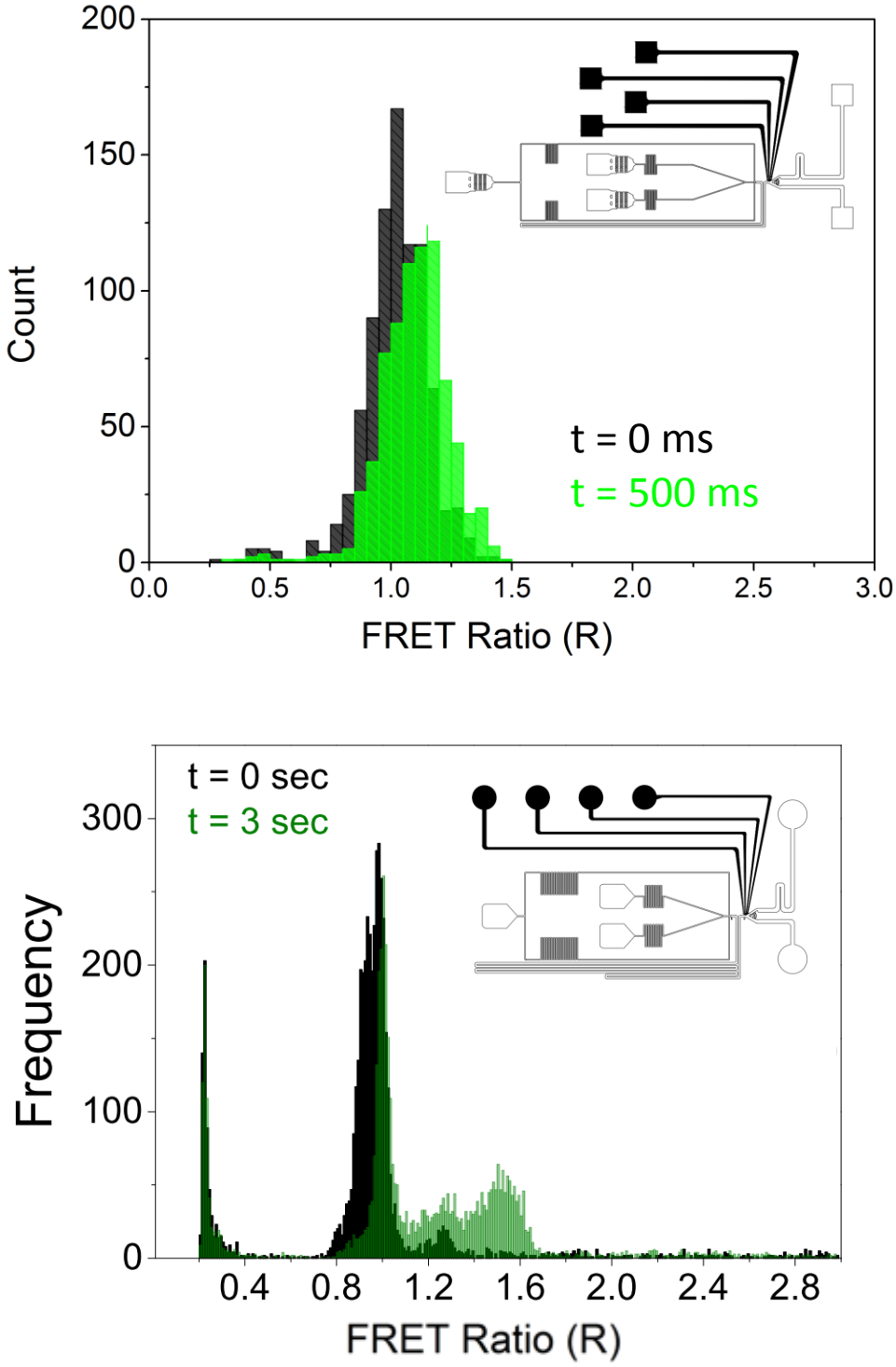

Figure S-6: Top: FRET histogram of a screening run of cells stably expressing NES-ZapCV2 with a time delay of 500 ms and delay length of 40 mm. Bottom: FRET histogram of a screening run of cells expressing a virally-transduced stable line of ZapCY1, a sensor used in a previous study, [3] at a time delay of 3 seconds and a delay length of 115 mm.

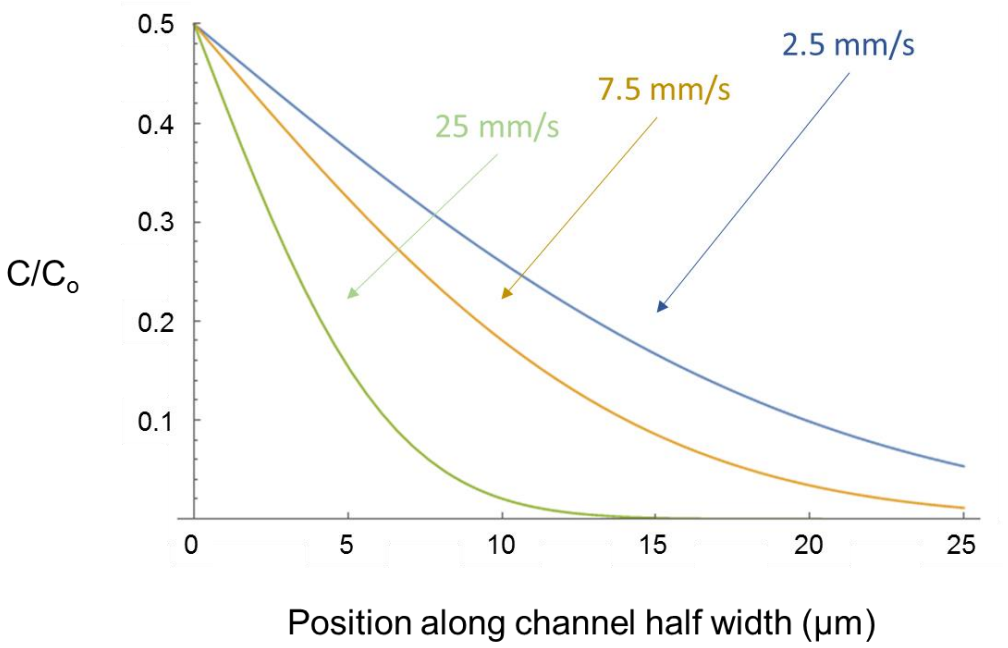

Figure S-7: A slice of the calculated concentration profile at x = 300 μm (where x is the distance along the axis of flow) after the mixing junction. The y-axis shows the concentration C, normalized by the input concentration C_o . The x-axis is the position along the width of the channel, beginning in the middle. Equation used is $\frac{C(x,y)}{C_o} = \frac{1}{2}\left(1 - erf\left(\frac{y\sqrt{U}}{\sqrt{4D_{eff}x}}\right)\right)$, where y is the distance perpendicular the axis of flow, U is the flow velocity, and D_{eff} is the effective diffusion constant (estimated at 1E-9 cm^2/s for the small ion/ionophore complex). Plotted at a series of flow velocities relevant to chip operation.

References

(1) Qin, Y.; Dittmer, P. J.; Park, J. G.; Jansen, K. B.; Palmer, A. E. *Proc. Natl. Acad. Sci. U. S. A.* **2011**, *108*, 7351.

(2) Pomorski, A.; Kochańczyk, T.; Miłoch, A.; Krężel, A. *Anal. Chem.* **2013**, *85*, 11479.

(3) Ma, H.; Gibson, E. A.; Dittmer, P. J.; Jimenez, R.; Palmer, A. E. *J. Am. Chem. Soc.* **2012**, *134*, 2488.