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High-throughput Examination of FRET-detected Metal-Ion Response in Mammalian Cells

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Abstract

FRET-based genetically encoded metal-ion sensors are important tools for studying metal-ion dynamics in live cells. We present a time-resolved microfluidic flow cytometer capable of characterizing the FRET-based dynamic response of metal-ion sensors in mammalian cells, at a throughput of 15 cells/sec with a time window encompassing a few ms to a few seconds after mixing cells with exogenous ligands. We used the instrument to examine the cellular heterogeneity of Zn²⁺ and Ca²⁺ sensor FRET-response amplitudes, and demonstrate that the cluster maps of the Zn²⁺ sensor FRET changes resolve multiple subpopulations. We also measured the *in vivo* sensor response kinetics induced by changes in Zn²⁺ and Ca²⁺ concentrations. We observed a ~ 30 fold difference between the extracellular and intracellular sensors.

Genetically-encoded fluorescent biosensors have revolutionized the study of dynamic processes in live cells, and provided important information about the mechanisms of a vast array of cellular processes from Ca²⁺ homeostasis to kinase activity¹⁻³. Over 120 sensors which undergo a fluorescence response to diverse analytes are now available³. These FRET-based sensors have the potential to create time-dependent concentration or activity maps of ions, small ligands, or macromolecules in living cells. To meet the challenge of multidimensional visualization, the dynamic range and response kinetics of the biosensors are critical attributes, since they directly affect the sensor's spatial and temporal resolution. Furthermore, biological measurements are inherently heterogeneous with different levels of cell-to-cell variation⁴. The observed variability may arise from heterogeneity of the sensor molecules reporting the measurement, or from intrinsic biological variability. Characterizing the population-based heterogeneity is important: for the former it determines the sensor's sensitivity and resolving power; and for the latter, resolving the cell-to-cell variability is a critical step toward understanding the origin and mechanisms of biological noise.

A method capable of defining these properties with high-throughput and single cell resolution is essential for the characterization of sensors in cell populations or cell-based libraries, and to streamline further refinement of the fluorescent sensor toolbox. Available

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ASSOCIATED CONTENT

Supporting Information. Supporting figures, and material and methods. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Author Contributions

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bioanalytical methods are limited in their ability to perform this task. Fluorescence Activated Cell Sorting (FACS)⁵ is a powerful high-throughput method to screen or sort cells based on their fluorescence properties. However, the measured signals reflect single snapshots of cellular states, and thus provide little information about the dynamics or kinetics of a particular process. Automated fluorescence microscopy of single bacterial cells has been carried out in microfluidics arrays to study the gene expression dynamics of a library with 1400 strains⁶, yet this one-library-member-per-microfluidic-channel approach makes it difficult to extend the application to larger libraries or cell populations. Other methods such as stopped-flow spectroscopy⁷ can be used to measure the *in vitro* kinetics of a chemical reaction with sub-ms time resolution, but generally cannot permit measurement on living mammalian cells (with the exception of one hematopoietic murine cell line which can survive turbulent mixing in a topped flow mixer with 10 ms time resolution⁸) and with single-cell manipulation (whereas stopped flow would provide a population average), and hence are not suited for cell-based assays in a high-throughput fashion.

Here we describe a time-resolved microfluidic flow cytometer (Fig. 1a, b, Fig. S1) to examine FRET changes, heterogeneity, and kinetics of biochemical reactions at the single cell level. Cellular reactions are initiated in individual cells using controlled fluid flow in microfluidics⁹⁻¹¹, and the reaction progress is probed with FRET sensors. Micron-scale laminar flow¹² and hydrodynamic focus techniques¹³ enable us to achieve rapid reaction initiation, accurate flow control, and high-throughput detection at the single cell level. A flow-control valve system (Fig. 1c) isolated the cell- and buffer-reservoirs and provided uninterrupted buffer switching. Pressure-driven streams of cells and buffers flow into the microfluidics device and meet at the entry junction (Fig. 1d). At the junction, the cells are rapidly exposed to chemicals carried in the buffer streamline, hydrodynamically focused to a single file, and subjected to fluorescence interrogation as they pass through the two laser beams separated by a delay line of various lengths. The detected signal is a train of Gaussian pulses, with the pulse width determined by the cell dwell time in the excitation beam. In order to pair-match the pulses from the same cell in this signal train (Fig. 2a), we adjusted the flow speed along the channel using a double hydrodynamic-focusing method (Fig. 1d) so that the pulse widths are different at the two measurement spots. Using this method which “labels” the signals at different measurement spots (Fig. 2b), we are able to extract the cell travel time (Fig. 2c, d), and match signal pairs within the signal train when a number of cells (n_{crowd}) are flowing inside the interrogation channel concurrently (Fig. 1b and Fig. 2a). The pair matching efficiency is $> 85\%$ in the worst case scenario ($n_{\text{crowd}} > 5$) (Fig. S2). The measurements are carried out in devices with delay line length ≈ 60 mm, except for the D3pD surface display sensors, for which shorter delay lines of 4 mm and 8 mm are also used to produce time intervals down to 1 ms. With precise flow control and flexible device design, the cell travel time can be tuned within a time window between 1 ms to 10 seconds, while maintaining the throughput of time-resolved single-cell FRET evaluation. Under our typical experimental conditions described in Supporting Information, the system is capable of measuring one-spot FRET at a throughput > 50 cells/s, or FRET change of individual cells at a throughput up to 15 cells/s.

We demonstrated the utility of this method for two applications: quantitative screening of cell-to-cell heterogeneity and measurement of ms time-resolved *in vivo* kinetics. For the first application, this method permits initiation of cellular reactions in a few ms, measurement of FRET ratio changes with single cell resolution, and analysis of FRET variability at the population level. For the second application, our instrument permits precise microfluidic flow control to measure the response kinetics of the sensors in their native cellular environment within time windows from 1 ms to 10 seconds. This time span is an important physiologically-relevant domain in which numerous biological reactions, e.g. protein folding and synaptic vesicle endocytosis^{14,15}, occur. However, it is a particularly difficult time

window for kinetics measurement of cellular processes using fluorescence microscopy, because the relatively slow macroscopic diffusion and mixing often occur at a comparable time scale, and hence could significantly interfere with the measured dynamics. Here we employ microfluidics techniques to reduce the diffusion-controlled mixing time to a few ms.

The diffusion time obeys a square relationship with distance. Hence with hydrodynamic focusing techniques¹³ in which the cell-containing stream is squeezed to a 10 μm “jet” by the two analyte-containing buffer solutions, the time for the molecules to reach cells can be significantly reduced. Fig. S3 shows the simulated Ca^{2+} concentration field as a 2D surface image and 1D cross section plots. The concentration profile along the cross-section demonstrates that Ca^{2+} rapidly penetrates the center of the hydrodynamic focus, reaching ~ 3 mM at ~ 2.5 ms after the entry junction. This concentration is ~ 1000 -fold higher than the minimum saturation point of the metal sensors, which have dissociation constants in the pM to μM range.

We measured the FRET response kinetics and response heterogeneity of HeLaS cells expressing intracellular Ca^{2+} and Zn^{2+} sensors. In particular, we employed genetically encoded Ca^{2+} sensors (D3cpV¹⁶, expressed in cytosol; and D3pd, displayed on the extracellular surface) and one Zn^{2+} sensor (ZapCY1¹⁷, expressed in cytosol). These sensors report the concentration change of intra- or extracellular metal ions by recruiting one (or a few) metal ion(s) to their metal-binding domains, inducing a conformational change leading to a higher FRET efficiency between the donor (CFP) and acceptor (YFP) (Fig. S4).

We first confirmed that metal binding reactions could be induced in microfluidics flow and observed as FRET changes. Prior to the microfluidics experiment, cells were pre-treated with metal chelators to deplete the sensors of the targeted metal ions (*e.g.* EGTA for Ca^{2+} or TPEN for Zn^{2+}), thus maintaining the sensors in a metal-free state with a minimum FRET ratio. In control experiments the cells showed no change in their ratiometric FRET values when mixed with metal-free buffer streams containing chelators (Fig. S5). In addition, fluorescent beads (unresponsive to metal-ion perturbation) are doped into the D3cpV cell population to serve as an internal calibration of the reaction. In the response measurement, buffer streams contain metal-ions, resulting in cell exposure within ms of the entry junction. As expected from the sensor design, the cells show a significant FRET increase after a time delay > 1 sec (Fig. 3a, c).

The throughput of the single cell-based FRET change measurement is dictated by the efficiency of the pair matching technique and n_{crowd} , and is variable from 15 cells/second for short time windows (< 0.5 second), to ~ 1 cell/second for time windows longer than 5 seconds. Statistical information, such as the average value, standard deviation, or skewness (asymmetry of the probability distribution) of the FRET response for thousands of cells can be obtained within minutes. Compared to the coefficient of variation ($\text{CV}_{\text{beads}} = 0.07 \pm 0.02$) of the doped beads, the CV of the cells is larger ($\text{CV}_{\text{cells}} > 0.1$), indicating there is inherent diversity of sensor expression and FRET ratio in live cells. It has previously been observed that genetically-encoded sensors incorporated into living cells demonstrate cell-by-cell variability¹⁸. Heterogeneity also depends upon the metal-binding state of the sensors. As shown in Figure 3b and observed as a general trend, FRET at spot 2 (Ca^{2+} or Zn^{2+} -bound and high FRET ratio) shows a larger CV compared to that at spot 1 (Ca^{2+} or Zn^{2+} -free and low FRET ratio). This observation suggests that the FRET efficiency at the metal-bound “active” state is probably more sensitive to small cell-to-cell variations in sensor molecules (*e.g.* conformational heterogeneity, post translational modifications, thermodynamic noise, *etc.*), or cellular properties (*e.g.* cell size, stage, metal buffering capacity, *etc.*).

The two genetically encoded sensors investigated here exhibit distinctly different heterogeneity profiles. In contrast to the roughly unimodal distribution of D3cpV FRET, the ZapCY1 FRET measurement resolves four subpopulations with differences either in the static FRET value or dynamic FRET response (Fig. 3c, d). Statistical analysis of the Zn^{2+} response of 3,358 cells from various measurements consistently shows that subpopulation P₂ ($35.2\% \pm 3.1\%$) demonstrates the expected Zn^{2+} -induced response, while the other three subpopulations (P₁ ($4.8\% \pm 1.5\%$), P₃ ($20.2\% \pm 2.6\%$), and P₄ ($39.8\% \pm 2.2\%$)) show negligible or weak Zn^{2+} response (Fig. 3d). Within distinct subpopulations there is also variability in distribution. The three “unresponsive” subpopulations show low, high, and medium baseline FRET values, suggesting that some of this heterogeneity may arise from proteolysis or sensor instability in the cellular milieu. For example, P₄ exhibits low CFP fluorescence leading to high FRET ratios and P₁ has low YFP fluorescence leading to a low FRET ratio. Importantly, this heterogeneity had not been identified in single-cell microscopy experiments, likely due to the comparative low throughput of microscopy and subjective nature of selecting “responsive cells”; to acquire a data-set equivalent to that presented in Fig. 3d would take at least 35 hrs on a standard fluorescence microscope, assuming a generous estimate of 10 transfected cells in a field of view and ~ 1 hr per calibration experiment.

The population-based measurements clearly show that the distribution functions describing the FRET response may take diverse forms, either as a unimodal FRET distribution or as a multimodal distribution with distinct subpopulations. Furthermore, different FRET variability is associated with each sensor state (baseline and active) and with each subpopulation (p1-p4). Our data reveal that two FRET sensors with similar molecular designs give rise to significantly different heterogeneity profiles. The ability to identify and define this heterogeneity has important consequences for sensor design and use.

Recent developments in biosensor design have led to significant improvements in sensitivity of FRET-based sensors^{17,19,20}, however, the characterization and optimization of their *in vivo* kinetic properties has lagged. Well characterized, fast sensor response kinetics are critical for cellular dynamics studies such as measurements of neural activity^{21,22}. The optimal sensor should faithfully report the kinetics of the biological signal; a slow responding sensor could distort or even miss a transient signal. Capitalizing on the benefit of microfluidics flow control and time-resolved FRET measurements, we expand this microfluidics method to investigate the kinetics of sensors within the cellular environment. Kinetics is measured as the average FRET of the normal metal-ion responding population and plotted as a function of cell travel time. The time-resolved response of genetically encoded Ca^{2+} and Zn^{2+} sensors in HeLa cells (Fig. 4 and Fig. S6) range from ~20 ms (extracellularly displayed sensor) to several seconds (intracellularly expressed sensor).

Sensors expressed in different cellular locations are likely to exhibit different reaction kinetics. For extracellular membrane displayed sensors (Figure 4a), the measured rate constant is largely determined by the intrinsic on/off rates of the sensors and the applied metal-ion concentration. For intracellular expressed sensors (Figure 4c), the observed kinetics is further limited by the metal-ion transportation across the plasma membrane. To examine this effect, we expressed sensors at different cellular locations, and compared their response rate under identical experimental conditions. The observed rate constant of D3pd (a surface displayed Ca^{2+} sensor) is $k_{\text{obs}} \approx 43 \pm 6 \text{ s}^{-1}$ (Figure 4b). In contrast, D3cpV intracellular Ca^{2+} sensor of similar construct shows response kinetics ~30-fold slower ($k \approx 1.4 \pm 0.2 \text{ s}^{-1}$) (Fig. 4d). The different time scales indicate that metal ion transport across the cell membrane is likely the rate-limiting step for activation of the intracellular sensor.

Altogether we present a new approach for time-resolved single cell FRET measurement that is flexible and high-throughput. This method represents a time-resolved generalization of flow cytometry by adding the time dimension to single-cell FRET measurements. This system is capable of controlling and perturbing the extracellular environment, and optically probing the kinetics of cellular reactions. We resolved significantly different heterogeneity profiles associated with different genetically-encoded sensors, and reported sensor kinetics in different cellular locations, revealing both inherent kinetics of sensors and the kinetics of ion transport across the membrane. These measurements would not have been possible using traditional fluorescence microscopy, flow cytometry, or stopped flow spectroscopy. Our method can be readily extended to large-scale kinetics-based screening or measurement of other optically-labeled molecular interactions or reactions in live cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Experimental setup of the high-throughput microfluidics platform for dynamic FRET measurement in living cells. **(a)** Excitation/detection scheme for measuring FRET in microfluidics. **(b)** Representative channel design of a microfluidics device (delay line length ~ 60 mm). A number of cells (depicted as red dots, size not to scale) may be flowing inside the interrogation channel concurrently. **(c)** Flow-control valve system. **(d)** Image of on-device microfluidics flow. The arrows show the positions of the cell-buffer entry junction and the fluorescence measurement spots.

Figure 2.

Data analysis and pair-matching algorithm. **(a)** Signal train generated by fluorescent cells traversing through the excitation laser beams. **(b)** Distribution of pulse widths (standard deviation of Gaussian fitting) at the two measurement spots; the different widths are due to the different flow speed generated by the double hydrodynamic focusing method. **(c, d)** The inter-pulse interval histogram is extracted from the pulse train by assuming a trial n_{crowd} and counting the time intervals falling in different time bins. Cell travel time is obtained from the histogram. (c) Short travel time at high pressure. (d) Long travel time at low pressure.

Figure 3.

Ca^{2+} and Zn^{2+} -sensor responses in microfluidics. **(a)** Ca^{2+} response of HeLa cells expressing D3cpV Ca^{2+} sensor at $T = 1.29 \pm 0.06$ s, triggered by Ca^{2+} -loaded buffer containing 30 mM Ca^{2+} and 49 μM ionomycin. (●) spot 1, (●) spot 2. **(b)** FRET cluster map (FRET at spot2 vs. FRET at spot1) of D3cpV-HeLa cells and beads. **(c)** Zn^{2+} response of HeLa cells expressing ZapCY1 Zn^{2+} sensor at $T = 3.43 \pm 0.14$ s, triggered by Zn^{2+} -loaded buffer containing 4 mM Zn^{2+} and 70 μM pyrithione. (●) spot 1, (●) spot 2. **(d)** FRET cluster map resolves four subpopulations (p1-p4).

Figure 4. Time-resolved kinetics of sensors in HeLa cells. **(a)** Extracellular-sensor (D3pd) activation. **(b)** D3pd shows an observed rate of $k_{obs}=43 \pm 6 \text{ s}^{-1}$ upon exposure to 30 mM Ca^{2+} . **(c)** Intracellular-sensor (D3cpv) activation. **(d)** D3cpv shows an observed response rate of $k_{obs}=1.4 \pm 0.2 \text{ s}^{-1}$ upon exposure to 30 mM Ca^{2+} and 49 μM ionomycin. (●) experimental data, (—) exponential curve fitting.