Published in final edited form as:

Nano Lett. 2012 June 13; 12(6): 3273–3278. doi:10.1021/nl301341m.

Nanochannel Based Single Molecule Recycling

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Abstract

We present a method for measuring the fluorescence from a single molecule hundreds of times without surface immobilization. The approach is based on the use of electroosmosis to repeatedly drive a single target molecule in a fused silica nanochannel through a stationary laser focus. Single molecule fluorescence detected during the transit time through the laser focus is used to repeatedly reverse the electrical potential controlling the flow direction. Our method does not rely on continuous observation and therefore is less susceptible to fluorescence blinking than existing fluorescence-based trapping schemes. The variation in the turnaround times can be used to measure the diffusion coefficient on a single molecule level. We demonstrate the ability to recycle both proteins and DNA in nanochannels and show that the procedure can be combined with single-pair Förster energy transfer. Nanochannel-based single molecule recycling holds promise for studying conformational dynamics on the *same* single molecule in solution and without surface tethering.

Keywords

single molecule detection; nanofluidics; trapping; FRET; electroosmosis

While fluorescence spectroscopy of molecules in solutions provides precise measurements of population averages of relevant parameters, such measurements are generally not capable of providing insight into heterogeneity of molecular populations, fluctuations in molecular behavior, or the detailed kinetics of molecular transitions. The problems associated with ensemble averaging can be circumvented using single molecule fluorescence.^{1–5} Single molecule experiments can be performed using molecules immobilized on surfaces,^{6–8} or diffusing in solution.^{9,10} Immobilization generally involves modification of the molecules being studied to allow them to be attached to a surface. Both the modifications and the proximity to a surface can alter properties of the molecules being studied.¹¹ On the other hand, single molecule analyses of freely diffusing molecules are subject to limitations in the number of photons that can be collected that are imposed by Brownian motion of molecules diffusing through the laser focus.¹² A constant drift velocity combined with hydrodynamic

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Supporting Information Available

Additional information is provided on (1) fluorescent labeling of OxlT proteins, (2) single molecule FRET, lifetime, and diffusion measurements, (3) data acquisition and measurement techniques, and (4) fabrication of nanofluidic channels. This material is available free of charge via the Internet at <http://pubs.acs.org>.

focusing,^{13,14} or mechanical confinement in channels or nanopipettes,¹⁵ can reduce variations in photon counts by providing more constant transit times through the laser focus. However, these passive approaches still only allow observation times on the order of a few milliseconds. Therefore, it is important to develop immobilization-free methods that remove surface-specific artifacts and that increase measurement time compared to single molecule diffusion experiments.

Trapping molecules inside lipid vesicles that are tethered to a surface provides a method for localizing minimally modified molecules in the laser focus of a microscope,¹⁶ however this approach results in high effective concentrations of the target held in close proximity to a lipid bilayer.¹⁷ While trapping in vesicles has provided useful single molecule analyses of some soluble proteins,¹⁸ it complicates experiments requiring rapid changes of solution conditions and has limited applicability for other proteins and systems involving surfactants, that would strongly interact with or disrupt membranes. This limitation can be overcome by methods based on active tracking and trapping, such as the anti-Brownian electrokinetic (ABEL) trap, which uses electrokinetic feedback to cancel Brownian diffusion, allowing individual fluorophores and covalently labeled proteins to be confined in liquid environments.^{19–21} However, the trapping times are limited by irreversible photobleaching and photoblinking. Since photobleaching increases with laser power and the on and off-times of photoblinking are known to be power-law distributed,^{22,23} the total trapping time can only be extended with a sampling protocol that does not require the molecule under investigation to be continuously illuminated. Furthermore, even at low concentrations of molecules there remains a significant probability that two molecules will enter the focus. For reliable detection of single molecules it is critical that a fluorescent detection technique be able distinguish situations in which more than one molecule enters a trap.

The method introduced here is similar to an ABEL trap, but it restricts the motion of a molecule to only one dimension by use of microfabricated nanochannels.^{24,25} This configuration allows the trapping of single molecules without the need of continuous observation, which considerably reduces the photobleaching rate and increases the overall trapping time. Furthermore, a one dimensional scheme is favorable from a device engineering standpoint since it provides a smaller device footprint and easier parallelization, which is of importance for high throughput lab-on-a-chip implementation.

We report here the use of an active approach to extend the total observation duration of untethered single molecules to more than 10 seconds. The method is based on the use of electroosmosis to drive the transit of a molecule with a constant drift velocity through a laser focused onto a sub-micron channel. After passing through the laser focus, the molecule's direction of motion is reversed, sending it back through the focus. This process can be repeated many hundreds of times for the same dye molecule before photobleaching, or photoblinking occurs or until an "invader" molecule enters the trap. Since we have been able to collect 100–200 photons per pass through the laser focus, as many as tens of thousands photons can be collected from a single fluorescent molecule. The single molecule recycling (SMR) scheme is applicable to various single molecule fluorescence techniques, such as smFRET (single molecule FRET), fluorescence anisotropy, fluctuation analysis for determination of the diffusion constant, or Dexter transfer. Figure 1 illustrates the principle of SMR in the context of smFRET; which is used as a spectroscopic ruler that can monitor nanometer to Angstrom scale distance changes between a donor fluorophore and an acceptor fluorophore attached to a single molecule.²⁶

The SMR approach has allowed us to perform smFRET measurements of molecules of double stranded DNA 5' labeled with Atto 532 and Atto 647N (ATTO-TEC, Germany). The DNA molecules are allowed to traverse the focus of a 532 nm laser focused into a (~600 nm

wide and 400 nm deep) nanofluidic channel (Supporting Information). A uniform drift velocity of the dsDNA is achieved by applying a voltage across the channel with gold wires dipped into reservoirs as shown schematically in Figure 1. After fluorescence from a dsDNA molecule entering the focus is detected, a countdown timer is started. Following a controllable delay, the potential across the channel is reversed, sending the DNA molecule in the opposite direction back into the laser focus. This process is repeated until eventual photo-bleaching or photo-blinking of the molecule, until an invader molecule enters the SMR, or until the DNA escapes into the reservoir (see Figure 2).

Typical fluorescence time courses for SMR of 13 base pair dsDNA molecules are shown in Figure 2. Donor (Atto 532) emission is plotted on the positive y-axes (green) and acceptor (Atto 647N) emission is plotted on the negative y-axes (red). Panel A shows 144 recycling events over 3 seconds, achieved using a 6 ms delay between the time the molecule leaves the laser focus and the initiation of field reversal. Panel B shows a portion of the trace in Panel A displayed with an expanded time scale. Varying the length of the delay before field reversal makes it possible to collect fluorescence information from the dsDNA over different time intervals.

By adjusting the combination of laser power, focus size and electrode voltage, it is possible to independently vary the intensity of illumination of the sample and the number of photons collected per recycling event. The photon emission rate can be adjusted by the laser power whereas the observation time per passage can be controlled by the size of the laser focus and the flow speed. The data shown in Figure 2 used an electroosmotic potential of 80 V, an electrode separation of 1 cm, a laser power of 98 μ W, and a laser beam of wavelength $\lambda=532$ nm focused by an objective lens with numerical aperture NA=1.4. We slightly underfill the back aperture of the objective in order to enlarge the focal spot size; the full NA is used for fluorescence detection. With these settings we obtain roughly 100 photons per passage. The ability to manipulate flow rate and reversal times independently allows the experiment to be conducted over different total time periods, while maintaining useful photon counting statistics.

The motion of molecules undergoing SMR is subject to the relationship between the electroosmotic drift velocity and random Brownian diffusion, as described by the Fokker-Planck equation (Supporting Information),^{27,28} Figure 3 shows a distribution of the times between one molecule exiting the focus and the next consecutive occurrence of the same molecule entering the focus accompanied by a fit of a Gaussian centered at each peak. For a single molecule that is being recycled, the mean time between exits out of and arrivals into the laser focus is given by twice the turnaround time plus twice the software loop time (the peaks at $36.1 \pm .1$ ms and $65.43 \pm .06$ ms and with respective standard deviations 5.2 ± 0.2 ms and 8.2 ± 0.1 ms for the dsDNA15mers and dsDNA13mers respectively in Figure 3). The variance of the Gaussian may be used to calculate the diffusion coefficient for one single molecule, $D(x, t)$, whereas typical fluorescence correlation spectroscopy with flow measures the ensemble diffusion properties of molecules.²⁹ For these measurements, solving for D yields diffusion coefficients of $D \sim 0.98 \pm 0.06 \times 10^{-6}$ cm²/sec for the ds-DNA13mer sample and $D \sim 1.3 \pm 0.3 \times 10^{-6}$ cm²/sec for the dsDNA15mer sample. These values are reproduced independently with the diffusion time calculated with the established fluorescence correlation with flow equation (Supporting Information). They are also similar to previous experimentally measured diffusion constants of 1.2×10^{-6} cm²/sec and 1.4×10^{-6} cm²/sec for freely diffusing 14 and 12 bp DNAs, respectively.³⁰ Differences may be attributable to sequence-specific effects. This provides the potential for discriminating single molecules based on their hydrodynamic radii in addition to their spectroscopic signatures.

In SMR, losses of molecules due to Brownian motion are minimized by the lateral confinement provided by the nanochannels, by using a channel length sufficient to minimize losses to the reservoir, and by using a drift velocity that is larger than the diffusion velocity. Losses of single molecules during recycling are mainly due to dye photophysics, such as photoblinking and photobleaching. Even when photobleaching is limited, organic dyes have been reported to follow power-law blinking distribution in both their on- and off-times, which we verify for on-time kinetics (Figure S3, supporting information).²²

Photon counting statistics during the short time during which a freely diffusing molecule remains in the laser focus impose a major limitation on the precision of conventional smFRET measurements of diffusing molecules in solution. SMR provides an improvement over free diffusion because the transit time distribution is more uniform, and because the introduction of electroosmosis and the control of the size of the focus allows us to collect single pass data with longer times spent inside the laser focus. Figure 4 demonstrates that the resolution in FRET measurements can be enhanced by repeated transits through the focus. In these measurements we have used samples of two different DNA lengths: 13 bp dsDNA, 15 bp dsDNA, and mixtures of the two. Energy transfer efficiency (ETE) values have been calculated by correcting for the finite detection efficiencies and quantum yields of donor and acceptor molecules ($\rho=1.56$, Supporting Information). Figure 4A shows a distribution of energy transfer efficiencies for dsDNA molecules that was created from *single passes* through the laser focus, as in traditional solution-based smFRET measurements. A distribution of a mixture of the two dsDNA's is shown in black. The mean energy transfer efficiency for the 13 bp dsDNA is ~ 0.5 whereas for the 15 bp dsDNA it is ~ 0.31 . The presence of the minor peaks in the distributions could reflect the existence of the donor in two different configurations (consistent with two lifetimes in the absence of the acceptor, *c.f.* Supporting Information). The acceptor (Atto 647N) exhibits only a single exponential lifetime curve in bulk fluorescence measurements. Figure 4B shows distributions similar to those in Figure 4A but for multiple passages per dsDNA. It is evident that single molecule recycling improves the resolution and the accuracy with which the two different dsDNA lengths can be distinguished. Additional structure in the ETE distributions is evidenced by the non-Gaussian shape of the distributions. A comparison of Figures 4A and 4B shows that the shoulders at high ETE values are preserved, indicating the presence of stable subpopulations of ETE values in samples consisting of one length of dsDNA.

The data presented in Figure 5 demonstrate that it is also possible to employ SMR for single molecule fluorescence measurements on proteins in the presence of surfactants. The trapped molecule in this case is purified oxalate transporter, OxIT, from the bacterium *Oxalobacter formigenes*, a member of the Major Facilitator Superfamily (Law et al., 2008). The experiments were conducted with an allele of OxIT from which the normal two cysteine residues were removed (C28G/C271A) and two cysteine residues were introduced (F18C/A307C). The two introduced cysteines were labeled with Cy3 and Cy5 dyes. OxIT was maintained in the presence of 0.1%(w/v) of the detergent dodecyl- β -D-maltoside and 10 mM potassium oxalate to maintain stability of the protein. The nanochannels were coated with 1 mg/ml polyL-Lysine to prevent OxIT adsorption to the channel surfaces.

Figure 5A shows a time-trace of donor and acceptor emission of a single OxIT that is being recycled in a nanochannel. The corresponding energy transfer efficiency (ETE) values are plotted in Figure 5B. The mean ETE is 0.315 and the standard deviation is $\sigma=0.086$. They are indicated in Figure 5B by horizontal lines. Since the variation in ETEs is of the magnitude expected for shot noise, these variations cannot be assigned to changes in the protein structure. These results demonstrate that it is possible to trap single proteins in the presence of detergent and to monitor FRET efficiencies for multiple sequential transits through the focus. Changes in the OxIT structure and the FRET efficiency can be initiated

by changing the substrate conditions (e.g. oxalate or malonate) while a single OxIT protein is being recycled.

Reversible electroosmosis can be used to send individual molecules (DNA, OxIT) back-and-forth through a laser beam focused into a nanofluidic channel. We were able to cycle a single molecule for as long as 15 seconds, allowing a substantial increase in the precision of smFRET determinations compared with what would be possible by single-pass detection of freely diffusing molecules. Single molecule recycling (SMR) is a “true” single molecule technique, in the sense that it is able to establish a distribution for an individual molecule. This distribution can then be compared with that for other single molecules. This approach is expected to be particularly useful for studying fluctuations and conformational heterogeneity of biomolecules or polymers that may be sensitive to tethering systems and proximity to surfaces. It is also well-suited to examination of proteins in the presence of detergents. It provides the capability to determine precise smFRET determinations that can be followed over relatively long time scales relevant to understanding conformational changes underlying protein folding, catalysis, and other biosynthetic and functional mechanisms. Real-time data processing can be implemented to determine the diffusion coefficient of single molecules for potential applications such as in DNA sorting. Simultaneous hydrodynamic and smFRET measurements allow diffusion coefficient variations to be correlated with changes in ETE due to conformational changes. The independent measurement of hydrodynamic size in addition to spectral information from different fluorescence channels can increase the number of observable signals in colocalization measurements if different sized molecules are being studied. This technique is also scalable because an array of nanofluidic channels on the same microfluidic device can be used to track many molecules at the same time. The illumination could come from multiple confocal spots focused in different nanochannels or from a line focused by a cylindrical lens across the nanochannel array. The flexibility of this approach will also allow it to be integrated into present and future microfluidic or nanofluidic devices.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Zachary J. Lapin for help with editing the manuscript and Zhenzhen Zia and Di-Cody Kang for providing purified OxIT mutants. This work was supported in part by NIH (Grants 1R21AI085543-01A1 and GM24195) and by a Provost's Multidisciplinary Award by the University of Rochester. The nanochannels were fabricated at the Cornell NanoScale Facility, a member of the National Nanotechnology Infrastructure Network, which is supported by NSF (Grant ECS-0335765).

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Figure 1.

Schematic of Single Molecule Recycling. Donor-acceptor labeled dsDNA are transported along a nanofluidic channel (cross-section $\sim 600\text{nm} \times 400\text{nm}$) by use of electroosmotic flow. A controller reverses the flow in the nanochannel after detecting a fluorescence burst, causing the molecule to pass the excitation focus again. This procedure is repeated and makes it possible to sample the same molecule over and over again at periodic times that can be adjusted by the flow speed and the parameters of the flow controller. BS: Beam splitter; F: Filter; NA: Numerical aperture. Inset (top left): SEM image of fabricated nanochannels.

Figure 2.

Single Molecule Recycling of dsDNA13mers. Single dsDNA13mers are captured with the single molecule recycler and measured at the seconds time scale. Donor curves are plotted in green and acceptor curves are plotted in red. For clarity, acceptor time traces are plotted towards negative values. A: Recycling of the same dsDNA13mer 144 times in ~3 s with a 6 ms delay in reversal of electroosmosis after the molecule exits the focus. B: Same data as in panel A, plotted with an expanded time scale demonstrating the optical shutter effect.

Figure 3.

Time Between Consecutive Passes. Distribution of times between one molecule exiting the focus and the next consecutive molecule entering the focus. The peaks at $36.1 \pm .1$ ms and $65.43 \pm .06$ ms with respective standard deviations 5.2 ± 0.2 ms and 8.2 ± 0.1 ms correspond to twice the turnaround times of 15 s and 30 s plus twice the software loop time ≈ 2.9 ms for experiments with dsDNA15mers (circles) and dsDNA13mers (triangles) respectively. The widths of the Gaussians are the result of the uncertainty in position due to diffusion. The Gaussians are superimposed on exponential distributions, which describe the times between arrivals of *different* molecules into the trap.

Figure 4.

Single Molecule Recycling combined with spFRET. Distributions of energy transfer efficiency (ETE) for dsDNA molecules of two different lengths (13 base pairs and 15 base pairs). Panel A: Distributions resulting from measuring different dsDNA only once (standard diffusion-based experiment). 65030 transits are used for the dsDNA13mer distribution, 120435 transits for the dsDNA15mer distribution, and 148830 transits for the mixture. Panel B: Same as in Panel A but sampling each dsDNA molecule at least nine times. Single molecule recycling improves the resolution and accuracy. 10789 transits are used for the dsDNA13mer distribution, 22875 transits for the dsDNA15mer distribution, and 4341 transits for the mixture. The distributions were created using the function, `ksdensity`, in MATLAB with a width of (A) 0.015 and (B–D) 0.02.

Figure 5.

Repeated FRET measurements on a single OxIT membrane protein. Panel A: Fluorescence time trace of donor (green) and acceptor (red). Panel B: Corresponding energy transfer efficiency (ETE). The mean ETE is 0.315 (solid horizontal line) and the standard deviation is 0.086 (dashed horizontal lines). The variations in ETE are the result of shot noise. The inset shows the homology model of OxIT with the donor and acceptor sites (red features).