Continuous throughput long-term observation of single molecule FRET without immobilization

Swati Tyagi1, Virginia VanDelinder1, Niccolo’ Banterle, Gustavo Fuertes, Sigrid Milles, Morgane Agez, Edward A. Lemke

Supplementary Material and Methods
The upper panel shows cartoons for confocal spectroscopy, TIRF, and SWIFT microscopy. In confocal spectroscopy (left image) a laser spot is focused into the solution. The concentration of single molecules is about 100 pM, so that single molecules stochastically traverse through the confocal volume, which gives rise to short, millisecond long bursts that are detected on a confocal spectrometer. In TIRF microscopy (middle panel), molecules are immobilized to the surface, which is illuminated with an evanescent wave by coupling the laser into the objective at the critical angle where total internal reflection at the coverslip/water surface will occur. The surface is PEGylated for high biocompatibility. The high optical sectioning power of the evanescent wave (< 200 nm) gives rise to superb contrast. In some cases, a confocal spot is used to scan the surface. In SWIFT microscopy (right panel) biomolecules are confined to the TIRF field of the objective using a PDMS channel of <100 nm depth that is fully PEGylated for biocompatibility.

The middle panel shows a SWIFT device mounted on a 100x oil immersion objective lens of a custom-built microscope set up. The device is attached to four pieces of tubing to connect it with sample, buffer, Air/N2 source, and an outlet.

The lower panel shows a schematic cartoon of SWIFT device operation.
To achieve TIR and the concomitant high signal to noise, light incident at the interface between media with different indices of refraction, $n_1$ and $n_2$, must approach at an angle greater than the critical angle, $\theta_c = \sin^{-1}(n_2/n_1)$. Both PDMS and water have a smaller index of refraction than glass (1.41, 1.33, and 1.518, respectively). To avoid exciting a substantial amount of PDMS, we developed an illumination scheme in which light is incident at an angle $\theta_{c,PDMS} \geq 68.2$, so TIR occurs in both the PDMS and water. Crucially, under this condition the light does not propagate through the PDMS, eliminating background from autofluorescence in the PDMS above the nanochannels, as the evanescent field decays exponentially in the second medium $I(z) = I_0 e^{-z/d}$, yielding a characteristic depth $d = \lambda/4\pi(n_1^2 \sin^2 \theta - n_2^2)^{1/2}$. For 660 nm light incident at $\theta = 68.5$, $d_{PDMS} = 640$ nm while $d_{water} = 107$ nm as shown below. The effect of using EPI vs TIRF illumination to SNR can also be appreciated by comparing Supplementary Video 1 vs Video 2. Overall, adjusting the angle to get TIR in PDMS vs water is very small and lies within the adjustment tolerance of the coupling angle of most commercial microscope. As shown in Supplementary Video 9, TIRFM illumination and single molecule observation can also be achieved on SWIFT using a standard commercial TIRF microscope from Olympus.
The mixer implemented on the SWIFT device was designed as previously introduced by Gambin et al., containing a mixing and a rapid deceleration region. In the mixing region, the sample stream is sandwiched between two streams from the buffer inlets. Shown in upper panel of the figure is a finite element simulation performed in COMSOL multiphysics for generating the resulting concentration profile. In SWIFT we are interested in the sample stream that is formed near the coverslip, hence the effective velocity near the surface of coverslip is 1/11th the average velocity in the channel. As the velocity in the bottom 100 nm is 100 µm/s (average velocity measured was 1.1 mm/s) in the 40 µm long mixing channel, we estimate a dead time of ~400 ms. This is much faster than the most common way of sample changing on TIRF, which is usually achieved via simple flow chambers where solution exchange takes seconds. The lower panel shows the fluorescent micrograph of the SWIFT device filled with a dye solution to visualize the working principle of the mixer.
Supplementary Figure 4: Distribution of track lengths in different colors and SNR

2,400 bp dsDNA labeled with either Alexa 488, Alexa 594 or Alexa 647 was imaged in SWIFT device for 10 min and then analyzed through a custom particle tracking program. The upper panel of the figure shows the distribution of length of traces for a total of ~30,000 single molecules tracked in 15 min of measurement with N₂ or air in control channel. The average SNR each dye channel is >10, with 12.5 in Alexa647 channel, 10.5 in Alexa594 channel, and 9.3 in Alexa488 channel.

The lower panel shows the oxygen concentration profile in the device from a finite element simulation corresponding to Supplementary Video 3.
Supplementary Figure 5: FCS data

Fluorescently labeled Nup153FG, 201 bp dsDNA, 2400 bp dsDNA, hTG2, Holliday Junction and nucleosomes at a concentration of 10 nM were subjected to routine fluorescence correlation spectroscopy analysis. The figure shows the obtained correlation curves (symbols) plus fits (lines) to recover the mean diffusion time and diffusion coefficient. Results from the fits are listed in Supplementary Table S1.
The above image shows a spatial distribution map of FRET efficiencies calculated from a 4 min data set of 2,400 bp dsDNA labeled with Alexa 488 and Alexa 594 at 12 bp apart that was used to generate the data in Figure 2. For all molecules used in the analysis the FRET efficiency is calculated and plotted at the image position where the molecule was actually localized. It can be seen that across the whole image area the same FRET efficiency is recovered, showing that neither the identity of the molecules nor in which channel it flowed influences the FRET measurements. The color legend goes from FRET=0 to FRET=1.
FRET labeled 2,400 bp DNA was subject to standard confocal based smFRET spectroscopy and SWIFT. In confocal spectroscopy, single molecules are observed by lowering the concentration to 50 pM, so that the confocal volume contains maximally one particle at a given time (i.e. less than 0.1 in average).6 Particles pass randomly through the volume by diffusion. In SWIFT microscopy, particles flow through the channels. As long as the spacing between particles is sufficiently large, several particles can be observed in a single channel. On a simple setup with two 512 by 512 pixel² chip cameras for two color channels to record FRET, we can on average record 7 channels out of the 24 channels. For a static system, the width of the $E_{\text{FRET}}$ population indicates the noise in the data. As explained in Supplementary Note 3, TIRF microscopy using EMCCD camera based detection suffers from many more noise sources then photon counting based detection using APDs. For the same number of detected photons, TIRFM and thus also SWIFT has naturally a lower SNR then confocal. SNR and other sources of heterogeneity all
contribute to the width of a smFRET histogram.\textsuperscript{4,7,8} The width of the FRET peak thus represents the overall quality of the data.

To compare the throughput of SWIFTM and confocal, the following approach was taken. Long data sets (shown in upper panel of the figure below) leading to very smooth histogram were first acquired in confocal and SWIFT. Then the photon threshold of confocal was adjusted such that the fitted width of the two smooth histograms was identical. This ensures that we compare data that has equal data quality, i.e. equal SNR/resolution, and is not dependent on the various differences between confocal and SWIFTM in hardware, working principle and data analysis. The smooth data sets both shown above have the same $E_{\text{FRET}}$ width of 0.085. Given these hardware and analysis settings, we tested how many events are acquired by both methods in a given time. The differences in throughput are dramatically visible from the plots shown above in the lower panel, which show 3 minute data acquisition for both methods, where around 10,000 events are collected in SWIFTM (also shown in Figure 2), leading to a smooth histogram, while only around 450 events are collected in confocal. In conclusion, over one order of magnitude more events could be detected with the SWIFT method while maintaining the same SNR and same total time of measurement.

Note that the low FRET state, the so called “Zero” peak in smFRET, arises from inactive or absent acceptor. Besides imperfect sample labelling, the photophysical reasons leading to inactive acceptor are a well-known phenomenon.\textsuperscript{9} Those reasons can be particular pronounced in confocal measurements, due to the very high laser power (typically at the limit of the linear regime), such that as many photons as possible can be collected during the short dwell time of the molecule in the confocal volume. Such effects can account for the slightly different ratio between Zero and FRET peak in the two measurements.\textsuperscript{9}

Increasing the laser power shifts the ratio between FRET peak and Zero peak towards the Zero peak, while due to the more collected photons (from the higher laser power), the number of events observed in the same time increases. For a conservative estimate (here and in Figure 2c) we only compared the total number of detected events with each other, i.e. the sum of Zero peak and FRET events.

Plots are color coded for frequency of occurrence, while the top and right histograms are maximum projections of the data along the vertical “S” axis and along the horizontal “$E_{\text{FRET}}$” axis, respectively. Red lines in the $E_{\text{FRET}}$ projections are the result from a Gaussian fit.

For confocal smFRET recordings we used custom built equipment that is described in Supplementary Note 2.\textsuperscript{10} The emission optics in confocal spectroscopy was as similar as possible to the ones used in SWIFT (same custom trichroic and emission filters).
Analog to Figure 3c, we show here three additional exemplar time-dependent intensity and FRET efficiency plots for individual molecules of hTG2 measured in pH4 buffer in a and for pH 7 in b, respectively. Also shown are the corresponding FRET histograms (as in Figure 3b, but this time shown separately in a and b). Green and orange color denotes D and A respectively.

All data was generated after applying a threshold of 200 photons. The standard deviation of the FRET histograms (also in Fig. 3b main text) for pH4 and pH7 is 0.125 and 0.124 respectively, which is only 1.1 fold more than the predicted value from Supplementary Note 3. Hence no substantial contribution from conformational changes/dynamics of hTG2 is detected under these experimental conditions.
Supplementary Figure 9: Holliday junction FRET histogram and traces

Complementary to Figure 3c, the top panel shows the FRET histogram for the Holliday junction measured in the SWIFT device with 10 mM MgCl$_2$ and 50 mM MgCl$_2$ showing two FRET states with $E_{\text{FRET}} = 0.25$ and $E_{\text{FRET}} = 0.6$, respectively. The lower plots show the time dependent intensities of D, A and $E_{\text{FRET}}$ for two individual single molecules of Holliday junction measured with 50 mM MgCl$_2$.

Nature Methods: doi:10.1038/nmeth.2809
**Supplementary Table 1: MSD analysis results vs diffusion coefficient from FCS**

<table>
<thead>
<tr>
<th></th>
<th>$D_{SWIFT} [\mu m^2/s]$</th>
<th>$D_{FCS} [\mu m^2/s]$</th>
<th>$\Delta [%]$</th>
<th>$V_{SWIFT} [\mu m/s]$</th>
<th>MW [kDa]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Holliday junction</td>
<td>22</td>
<td>28</td>
<td>20</td>
<td>13</td>
<td>43</td>
</tr>
<tr>
<td>Nup153 FG</td>
<td>45</td>
<td>46</td>
<td>3</td>
<td>39</td>
<td>61</td>
</tr>
<tr>
<td>TG2</td>
<td>22</td>
<td>26</td>
<td>14</td>
<td>17</td>
<td>117</td>
</tr>
<tr>
<td>201 bp DNA</td>
<td>23</td>
<td>31</td>
<td>25</td>
<td>7</td>
<td>132</td>
</tr>
<tr>
<td>Nucleosomes</td>
<td>13</td>
<td>16</td>
<td>20</td>
<td>14</td>
<td>203</td>
</tr>
<tr>
<td>2400 bp DNA</td>
<td>5</td>
<td>5</td>
<td>4</td>
<td>4</td>
<td>1583</td>
</tr>
</tbody>
</table>

MSD analysis results and comparison of resulting diffusion coefficient ($D_{SWIFT}$) with diffusion coefficient measured by FCS ($D_{FCS}$ from **Supplementary Figure 5**). $D_{SWIFT}$ and the velocity $V_{SWIFT}$ were obtained fitting the MSD along the flow axis ($\rho_x$) shown in maintext **Fig. 2a** with the equation $\rho_x(t) = 2D_{SWIFT}t + V_{SWIFT}^2t^2$. $\Delta$ was calculated as the deviation between $D_{SWIFT}$ and $D_{FCS}$ and is given in percent. On average the difference was <15% between the two methods. We note that FCS itself has a limited accuracy for measuring exact diffusion coefficients.13
Supplementary Note 1:  Review of other non-immobilization techniques

FRET experiments of diffusing single molecules utilizing a confocal format were first demonstrated by Deniz et al. The amount of time molecules spent in the confocal volume is diffusion limited and every molecule is measured for a very short time, typically around 0.5-5 ms. Non-equilibrium and kinetic studies can also be performed using confocal spectroscopy by employing microfluidic technology. In conventional single molecule TIRF experiments, pioneered by Ha, molecules are immobilized to the surface of a coverslip to keep them within the TIR field, and the need to modify the biomolecule to achieve tethering is a major requirement of the technique. Multiple molecules can be imaged simultaneously in continuous recordings, with lengths of observation limited by photobleaching. These experiments are typically performed in a flow chamber made with double-sided tape or in microfluidic devices. One approach to study non-tethered molecules with TIRF is to use encapsulated vesicles tethered to the surface (e.g. reviewed in reference ). This technique only works with molecules that are amenable to the conditions used for encapsulation. By using porous vesicles to add the capability to introduce changes in the molecule environment, Okumus et al expanded this technique to non-equilibrium systems.

Due to the pressing need to observe single molecules for long times without tethering, a few fascinating approaches have already been presented in the literature: Hollars et al. used a TIRF setup with a 500 nm deep channel fabricated in glass. The manufacturing procedure of such devices requires rather sophisticated glass etching technology, and the channels are not shallow enough to fully utilize the potential of confining to molecules to a high resolution TIRF/evanescent field. Furthermore, the high resistance of the small channels requires voltage driven forces to load the device. As suited to their application –colocalization of DNA probes bound to DNA targets– no particle tracking was necessary, only still frames, so time traces were not recorded. Kinoshita et al. used glass capillaries with a laser introduced into the capillary coaxially to study the folding dynamics of Cytochrome C labeled with a single Alexa532 dye by looking at variations in intensity due to quenching. This format enabled them to observe molecules for about 0.1 s with a rather low SNR. A recent improvement to this setup utilized a spherical mirror and a feedback controlled flow rate increased the SNR and the observation time several seconds. However, this method only observes a single molecule at a time, limiting its throughput and requires a rather complex excitation and microscope scheme (feedback, spherical mirror etc.).

Cohen et al. demonstrated the use of an Anti-Brownian Electrokinetic (ABEL) trap to suppress Brownian motion and observe e.g. GroEL. This format has since been expanded to trap single fluorophores and study other biomolecules including the photosynthetic antenna protein Allophycocyanin and the binding of RecA to DNA. This technology comes at the cost of a complex setup and feedback system and intrinsically has a low throughput due to the observation of only one molecule at a time.

Shon et al. recently introduced an array of Dimples (450 nm diameter, 200 nm deep glass cavities sealed by a PDMS membrane) to observe double labeled DNA for extended times in order to study statistical effects of occupancy on steady-state fluctuations. The Dimple fabrication requires more complex fabrication procedures and the measurement buffer contained several stabilizers to accommodate the measurement conditions. They achieved high SNR in the red channel green channels. However, due to the time required to seal the dimples, only 2000 dimples per hour were observed with occupancy of dimples Poisson distributed.

In contrast to the aforementioned techniques, which require rather complicated manufacturing procedures, the CLIC technique introduced by Leslie et al., which uses a convex lens to confine freely diffusing molecules in a TIR field, is simple to realize. The technology is, however, limited to studies at equilibrium as entry of molecules into the space between the lens and coverslip cannot be controlled.
but depends solely on diffusion. The CLIC technique was previously used e.g. to image lipid vesicles containing a small number of proteorhodopsin singly labeled with Atto647N.⁴⁰
Supplementary Note 2: Confocal FCS and smFRET setup and analysis

Fluorescence correlation spectroscopy measurements were performed on a custom built confocal microscopy setup with a pinhole of 100 µm. Fluorescence was split 1:1 onto two detectors, two single-photon avalanche diodes (τ-SPADs, Picoquant, Berlin, Germany) in the red and two micro photon counting devices (MPDs, Picoquant, Berlin, Germany) in the green, which were then cross correlated. The confocal volume was calibrated with Atto655 in the red (diffusion coefficient of 4.26*10^-6 cm²/s) and Alexa488 in the green (diffusion coefficient 4.35*10^-6 cm²/s).

\[ f(\tau) = \frac{1}{N} \times \frac{1}{1 + (\tau_{\text{diff}})^{-1}} \times \frac{1}{1 + \left( \frac{\tau}{\tau_{\text{diff}} \cdot SP^2} \right)} \]

where \( N \) denotes the number of molecules, \( \tau_{\text{diff}} \) the diffusion time, and \( SP \) the structure parameter of the confocal volume. The \( SP \) was determined through the calibration measurement and afterwards fixed to that value. With knowledge of the \( SP \), \( \tau_{\text{diff}} \) and the diffusion coefficient \( D \) are directly related to each other through

\[ D = \frac{\omega_{xy}^2}{4\tau_{\text{diff}}} \quad \text{and} \quad SP = \frac{\omega_z}{\omega_{xy}} \]

where \( \omega_{xy} \) and \( \omega_z \) denote the radial and axial dimensions of the confocal volume.

smFRET experiments were performed on a custom built multiparameter (MP) spectrometer centered around an Olympus IX81 microscope (Hamburg, Germany) equipped with a high numerical aperture water objective (60×, 1.2 NA). A laser diode (LDH 485, Picoquant, Berlin, Germany) and a white light laser (SuperK Extreme, NKT Photonics, Denmark) filtered through a z572/15 excitation filter (Chroma, Olching, Germany), which were pulsed alternately at 80 MHz total, were used to excite freely diffusing labeled proteins with linearly polarized light. Fluorescence originating from single molecules was first spatially filtered by a 100 µm pinhole and then split into parallel and perpendicular polarization directions before separation into green donor (D) and red acceptor (A) fluorescence light. D and A fluorescence was detected with MPDs for the green and τ-SPADs for the orange. Acquired data were subjected to multiparameter fluorescence analysis. Fluorescence time trace data were binned to 5 ms. For FRET analysis, a threshold of 75 photons was applied over \( I_D + I_A \).

Forster resonance energy transfer was calculated via

\[ E_{\text{FRET}} = \frac{I_i}{I_D + I_A} \]

and stoichiometries using

\[ S = \frac{I_D + I_i}{I_D + I_A + I_{\text{ALEX}}} \]

\( E_{\text{FRET}} \) was corrected for leakage of the donor into the acceptor channel.
**Supplementary Note3:  Experimental SNR in confocal, TIRF and SWIFT**

We compared the FRET resolution achieved by SWIFT with the one obtainable by single molecule confocal spectroscopy and immobilized TIRF microscopy. For confocal microscopy the resolution (defined as the standard deviation $\sigma$ of the FRET histogram) is a function of the FRET efficiency ($E_{\text{FRET}}$) and the total number of photons acquired ($N$, see eq. 1 in reference 4). Ignoring thresholding (which has a strong impact only on $\sigma$ when the average $N$ of the single molecules is significantly smaller than the applied threshold), the standard deviation of the FRET measurement is:

$$\text{Equation 1} \quad \sigma_{\text{Conf theory}} = \sqrt{\frac{E(1-E)}{N+1}}$$

For TIRF microscopy the resolution is additionally affected by the noise introduced by the EMCCD camera and the background noise as given by Holden et al 5:

$$\text{Equation 2} \quad \sigma_{\text{TIRF theory}} = \text{SIM} \times \sqrt{f_G \left(\frac{E(1-E)}{N}\right) + \frac{4\pi}{a^2N^4}\left(D^2s_D^2b_D^2 + A^2s_A^2b_A^2\right)}$$

Where $E$ is the average FRET efficiency, $D$ and $A$ are respectively the average Donor and Acceptor signals in photons, $N$ is the total photon number, $a$ is the CCD camera pixel size, $s_D$ and $s_A$ are respectively the standard deviation of the donor and acceptor PSF, $b_D$ and $b_A$ the standard deviation of the donor and acceptor background, and $f_G$ a factor termed excess noise factor and accounts for the noise introduced by the EMCCD. SIM is a constant that has been determined by simulations by Holden et al. to be $\sim 1.33$ 5.

For the FRET efficiency of 0.65, i.e. the FRET efficiency measured for our 2,400 bp DNA (see plot below) we computed $\sigma_{\text{TIRF theory}}$ using the typical TIRF acquisition parameters (as used in reference 5):

$a = 94 \text{ nm}, s_D = 132 \text{ nm}, s_A = 150 \text{ nm}, b_D = 2.9 \text{ photons}, b_A = 2.9 \text{ photons}, f_G = \sqrt{2}$

For calculating $\sigma_{\text{SWIFT theory}}$, we used our experimentally determined settings, which are:

$a = 83 \text{ nm}, s_D = 82 \text{ nm}, s_A = 88 \text{ nm}, b_D = 7.52 \text{ photons}, b_A = 2.73 \text{ photons}, f_G = \sqrt{2}$

The table below lists the results obtained for our experimental determined values, and for the different theories in dependence of varying photon numbers $N$. 

17

Nature Methods: doi:10.1038/nmeth.2809
An immediate consequence of the additional terms in equation 2 vs equation 1 is that in order for confocal and TIRF to achieve the same resolution, a higher photon number needs to be collected in the TIRF acquisition scheme. SWIFT relies on a TIRF excitation scheme, and thus suffers from the same theoretical limitations as TIRF. For the case of 400 photons, the idealized theory thus predicts a $\sigma_{SWIFT}^{realistic} = 0.065$ using our experimental input parameters and a $\sigma_{TIRF}^{realistic} = 0.060$ for the values given in Holden et al 5.

To experimentally validate our resolution comparison, we evaluated the standard deviation of the FRET histogram of a 2,400bp dsDNAs labeled with Alexa488 and Alexa594 at 12bp distance with at a photon threshold of 400 photons, as shown in FRET histogram above. The $\sigma_{TIRF}^{realistic} = 0.060$ as obtained from idealized simulations done by Holden et al.5 (which should still be considered a maximal boundary), is only about 1.3 fold better then $\sigma_{SWIFT}^{measured} = 0.078$. This underlines that SWIFT realistically performs in a similar league as optimized ultra high resolution measurements in TIRFM.
Supplementary references