Watkins, D. W., & Collinson, I. R. (Accepted/In press). Dynamics of Membrane Proteins Monitored by Single-Molecule Fluorescence Across Multiple Timescales. In V. Postis, & A. Goldman (Eds.), *Biophysics of Membrane Proteins : Methods and Protocols* (pp. 273-297). (Methods in Molecular Biology; Vol. 2168). Springer.
<https://doi.org/10.1007/978-1-0716-0724-4>

Peer reviewed version

Link to published version (if available):
[10.1007/978-1-0716-0724-4](https://doi.org/10.1007/978-1-0716-0724-4)

[Link to publication record in Explore Bristol Research](#)
PDF-document

This is the author accepted manuscript (AAM). The final published version (version of record) is available online via Springer at <https://www.springer.com/gp/book/9781071607237> . Please refer to any applicable terms of use of the publisher.

University of Bristol - Explore Bristol Research

General rights

This document is made available in accordance with publisher policies. Please cite only the published version using the reference above. Full terms of use are available:
<http://www.bristol.ac.uk/red/research-policy/pure/user-guides/ebr-terms/>

Dynamics of membrane proteins monitored by single-molecule fluorescence across multiple time scales

Tomas Fessl¹, Matthew A Watson^{2,3}, Daniel Watkins⁴, Marek Scholz¹, Joel Crossley^{2,5}, Tara Sabir⁵, Sheena E. Radford^{2,3}, Ian Collinson⁴, and Roman Tuma^{1,2,3}

¹ Faculty of Science, University of South Bohemia, Ceske Budejovice, Czech Republic

² Astbury Centre for Structural Molecular Biology, University of Leeds, Leeds, U.K.

³ School of Molecular and Cellular Biology, Faculty of Biological Sciences, University of Leeds, Leeds, U.K.

⁴ School of Biochemistry, University of Bristol, Bristol, U.K.

⁵ School of Clinical & Applied Sciences, Faculty of Health & Social Sciences, Leeds Beckett University, Leeds, U.K.

Abstract

Single-molecule techniques provide insights into the heterogeneity and dynamics of ensembles and enable the extraction of mechanistic information that is complementary to high-resolution structural techniques. Here, we describe the application of single molecule Förster resonance energy transfer to study the dynamics of integral membrane protein complexes on time scales spanning nanoseconds to minutes (10^{-9} to 10^2 s).

Key words: FRET, SecY, SecA, protein export, translocation, lipid, liposome

1. Introduction

Single-molecule techniques are now firmly established within the experimental portfolio of biophysics and mechanistic structural biology and more recently are being extensively applied to membrane proteins [1-3]. Complementary to ensemble techniques, such as NMR and X-ray crystallography, these techniques provide insight into the heterogeneity of the sample and allow the direct observation of sparsely populated intermediates. In particular, Förster resonance energy transfer (FRET) has emerged as a method of choice for monitoring conformational changes on the nanometre length scale [6]. In this method a pair of donor and acceptor dyes are attached at specific sites on the macromolecule within ca. 2-8 nm of each other. If the donor emission and acceptor absorption spectra overlap then there is a non-radiative (resonant) energy transfer from the donor to the acceptor, the efficiency of which decreases with the inter-dye separation distance and with a lesser dependence on the relative orientation of the two dyes [6]. Hence, to the first approximation FRET can be used as a “molecular ruler” and can be used to monitor conformational changes on the nanometer scale, e.g. domain motions, membrane channel opening dynamics and activation [7].

Here we describe FRET monitoring of conformational changes in the bacterial Sec translocon during the ATPase cycle of SecA. The translocon is responsible for the membrane transport of unfolded proteins into the periplasm or their insertion into the plasma membrane, depending on the identity of an N-terminal signal sequence of the substrate preprotein [8]. The minimal *E. coli* Sec translocon, which is employed in the studies described here, is composed of the

transmembrane SecYEG channel and a peripheral membrane protein SecA which is a RecA-like ATPase. The latter provides energy for translocation which is thought to proceed via a Brownian-ratchet mechanism [5,9].

Here we describe methodology for the analysis of conformational changes in membrane proteins using smFRET. First, we briefly describe protein preparation and dye labelling protocols. However, we refer readers to a recent review for a detailed discussion of dye attachment site selection that is based on structure and molecular dynamics [3]. The main focus of the present work is on time-resolved data collection and analysis strategies which give access to dynamics spanning eleven orders of magnitude (10^{-9} to 10^2 s). We start with the slow timescale (~100 ms to several minutes) which is accessible through imaging of surface-immobilized proteoliposomes using total internal reflection (TIRF) microscopy. Next, we describe how to take advantage of the slow diffusion of 100-200 nm proteoliposomes in order to access the millisecond and sub-millisecond dynamics using Alternating Laser Excitation (ALEX). Finally, we close with nanosecond lifetime measurements using pulsed excitation and time-correlated single photon counting (TCSPC).

2. Materials

Prepare all solutions using ultrapure water. Store all purification buffers at 4°C. Handling of all solutions involved in expression of SecYEG was performed under sterile conditions.

2.1 Expression of SecYEG

SecYEG expression vector: *E. coli* WT SecYEG was previously cloned downstream of an arabinose regulated promoter in a pBAD-HisA expression vector. Open reading frame codons encoding cysteine were mutated to encode serine using a standard PCR mutagenesis protocol.

Ampicillin (1000x): 100 mg/mL solution in water, passed through a 0.22 µm syringe filter under sterile conditions.

Petri dishes containing LB agar with 100 µg/mL ampicillin.

Competent *E. coli* C43 cells.

Preculture: 100 mL 2xYT broth prepared in a 250 mL conical flask and autoclaved.

Expression cultures: 3 x 2 L 2xYT broth prepared in 5 L conical flasks and autoclaved.

Arabinose: 20% solution in water, passed through a 0.22 µm syringe filter under sterile conditions.

2.2 Purification of SecYEG

Tris-buffered saline (TS; 10x): 0.02 M Tris-HCl (pH 8.0), 0.13 M NaCl and 10% glycerol.

n-Dodecyl β-D-maltoside (DDM): 10% solution in water. Prepare by adding DDM to water and stir gently as not to froth the solution.

Tris-buffered saline with glycerol (TSG): Prepare by diluting 100 mL TS buffer with 800 mL water and 100 mL glycerol.

TSG containing 0.03 M imidazole, 0.1% DDM and pH corrected to pH 8.0 (HisA).

TSG containing 1 M imidazole, 0.1% DDM and pH corrected to pH 8.0 (HisB).

25 mL FPLC column containing HiTrap resin charged with nickel.

TSG containing 0.02% DDM (SEC buffer).

TSG containing 0.02% DDM and 1 M NaCl (SEC wash buffer).

HiLoad 26/600 Superdex 200 pg SEC column followed by an FPLC column packed with 50 mL of Capto Q ion exchange resin

2.3 Labelling of SecYEG

Alexa Fluor 488 (AF488) maleimide: 10 mM solution in DMSO.

Alexa Fluor 594 (AF594) maleimide: 10 mM solution in water.

Superdex 200 Increase 10/300 GL FPLC column.

2.4 Vesicle preparation and SecYEG reconstitution into liposomes

E. coli polar lipids (chloroform solution, 25 mg/ml) from Avanti Polar Lipids, Inc.

E. coli biotinylated lipids (chloroform solution, 25 mg/ml) from Avanti Polar Lipids, Inc.

lipid suspension buffer: 50 mM NaCl, 10 mM Tris-HCl pH 8

Ultrapure compressed nitrogen or argon gas

Heat block

kDa MWCO dialyser tube

2.5 L of **TKM** dialysis buffer: 10 mM KCl, 10 mM Tris-HCl pH 7.5, 1 mM MgCl₂

Lipid extruder assembly and accessories (Avanti Polar Lipids)

100 nm pore membrane for the extruder (Avanti Polar Lipids)

2.5 Glass cover slip preparation and PL immobilization for TIRF microscopy

Round 0.17 mm thick microscope cover slips (Menzel Glaser)

Resealable microscope chamber (ASI imaging)

1M Sodium or potassium hydroxide

Methanol

APTES aminosilane (Sigma)

CH₃O-PEG-NHS ester and biotin-CONH-PEG-NHS ester (RAPP polymere)

0.1 M Sodium carbonate (NaHCO₃) solution

neutravidin

2.6 GODCAT photoprotection system for TIRF imaging

glucose oxidase 100 nM

catalase (1.5 μM)

D-glucose (56 μM)

ATP regeneration system

pyruvate kinase (700 U/mL)

phosphoenol pyruvate (2 mM)

lactate dehydrogenase (1000U/ mL)

NADH (0.2 mM)

Methods

3.1 Expression of SecYEG

A typical protocol for expression of double cysteine mutant SecY which was engineered from a cysteine-free variant [10,11] is described. Selection of the cysteine position is beyond the scope of this protocol (see **Note 1**).

1. Transform pBAD-SecYEG Δ Cys into *E. coli* C43 cells using a standard transformation protocol. Once complete, plate 50 μ L of the cells onto and LB agar petri dish containing 100 μ g/mL ampicillin and spread evenly. Incubate overnight at 37°C with shaking at 200 RPM and place at 4°C the following morning.
2. Prepare a sterile 100 mL 2xYT preculture containing 100 μ g/mL ampicillin in a 250 mL conical flask and inoculate it with a stab of a single colony from the transformed cells. Incubate overnight 37°C with shaking at 200 RPM. Prewarm 3x sterile 2L cultures of 2xYT broth in 5 L conical flasks containing 100 μ g/mL ampicillin at 37°C.
3. The following morning, pour 20 mL of the preculture into each expression culture. Place the cultures in a shaking incubator at 37°C and 200 RPM.
4. Monitor OD_{600 nm} of the cultures using a spectrophotometer until they reach 0.8, at which point add 20 mL of 20% arabinose to each culture, to give a final concentration of 0.2%.
5. After three hours of SecYEG expression, harvest the cells from the cultures by centrifugation at 5000 xg for 20 mins.
6. Resuspend the cell pellets in 50 mL TSG buffer, decant the mixture into two 50 mL falcon tubes and place in a -20°C freezer.

3.2 Purification of SecYEG

1. Defrost the resuspended cells at room temperature.
2. Lyse the cells using a cell disruptor. Wash the cell disruptor thoroughly with 50% methanol followed by TSG buffer, then pass the cells through the cell disruptor twice to ensure efficient cell lysis.
3. Pellet the insoluble fraction of the lysed cells by ultracentrifugation. Place the sample in a Ti45 tube in a chilled Ti45 rotor and centrifuge at 38000 RPM for 45 minutes.
4. While the sample is centrifuging, begin preparing chromatography systems for purification. For nickel affinity purification, wash an Äkta FPLC system with HisA and HisB buffer. Attach a 25 mL FPLC column containing HiTrap resin charged with nickel and wash it with 10 column volumes of HisB buffer, followed by two column volumes of HisA buffer. Also wash the sample pump with HisA buffer. For size exclusion chromatography, wash an Äkta FPLC system with SEC buffer and SEC wash buffer. Attach a HiLoad 26/600 Superdex 200 pg SEC column followed by an FPLC column packed with 50 mL of Capto Q ion exchange resin. Wash the columns with 100 mL of SEC wash buffer followed by 450 mL of SEC buffer.
5. On completion of centrifugation, decant the supernatant and rinse the pellet thoroughly with water. Resuspend the pellet in 42.5 mL of TSG buffer and homogenise thoroughly using a glass dounce homogeniser. Decant the sample back into a Ti45 tube and add 7.5 mL of 10% DDM, to a final concentration of 1.5%. Rock the sample gently for 1 hour at 4°C until the solubilisation process is complete.
6. Ultracentrifuge the sample again as described above, this time keeping the supernatant and discarding the pellet.
7. Load the supernatant onto the nickel chromatography column prepared in step 4.
8. Equilibrate the column with approximately 10 column volumes of HisA buffer.
9. Elute bound SecYEG from the column with 30% HisB buffer. Collect approximately 15 mL of sample.

10. Load the eluents into a superloop attached to the Äkta chromatography system prepared in step 4 for size exclusion chromatography. Set the flow rate to 2.6 mL/min of SEC buffer and release the contents of the superloop onto the column.
11. Measure absorbance at 280 nm. The retention time of SecYEG is approximately 190 mL. Collect 3 mL fractions. Typically, the elution profile will be a large peak (SecYEG) followed by a small shoulder (dissociated SecE). When selecting fractions to pool, be careful to avoid picking those that contain dissociated SecE.
12. Concentrate the sample in a 50 kDa molecular weight cut-off centrifugal concentrator at 4000 xg and 4°C until the sample is reduced to approximately 200 µL. Determine the concentration of SecYEG using the 280 nm extinction coefficient of 139000 M⁻¹cm⁻¹. Divide the sample into 10 µL aliquots, snap freeze and store at -80°C.

3.3 Labelling of SecYEG

1. Prepare a 50 µL solution of 50 µM SecYEG on ice.
2. For labelling single cysteine residues with a single type of fluorescent probe, add 5 µL of fluorescent probe functionalised with a maleimide group to the SecYEG solution to a final concentration of 1 mM. For shotgun labelling, mix 5 µL of one fluorescent probe with another fluorescent probe thoroughly, then mix the 10 µL probe mixture with the SecYEG sample, to give a final concentration of 1 mM of each probe. Incubate the sample on ice for 45 minutes.
3. In the mean time, attach a Superdex 200 Increase 10/300 GL FPLC column to an Äkta FPLC system and equilibrate it with 50 mL of SEC buffer, flowing at 0.5 mL/min. Attach a 500 µL sample loading loop to the injection port and wash it with 5 mL SEC buffer.
4. Dilute the labelled SecYEG sample to 500 µL and load it into the sample loading loop. Set the flow rate to 0.5 mL/min of SEC buffer and release the contents of the loop onto the column.
5. Monitor 280 nm and the wavelengths corresponding to the absorbance maxima of the fluorescent probes used, the extinction coefficients of which can be used to determine the labelling efficiency.
6. The retention volume of SecYEG is approximately 12 mL. Collect the entire homogeneous protein peak, place it in a 50 kDa centrifugal concentrator and centrifuge at 4000 xg and 4°C until the sample is reduced to approximately 100 µL. Snap freeze the sample and store at -80°C.

3.4 Preparation of proteoliposomes containing single labelled SecYEG

Since the Sec complex is purified in micelles it needs to be reconstituted into vesicles containing appropriate native lipids, which in this case are E. coli polar lipids. Then the vesicles are filtered to the desired size (extruded) and either diluted for confocal microscopy or further immobilized onto a derivatised cover slip surface for TIRF. The procedures are time consuming and thus is split over two days.

Day one

1. Remove a single aliquot of lipid in CHCl₃ (400 µL, 25 µg/µL) from the freezer and place in a fume hood to evaporate, if available, use a stream of nitrogen or argon to expedite the evaporation. (If surface immobilisation is desired, supplement aliquot with 4µL of biotinylated lipid prior to evaporation)
2. Turn heat block on and set to 50°C and place the extruder block on top

3. Once fully evaporated resuspend the lipids in 1 mL of lipid suspension buffer that has been heated in the heat block to 50 °C, yielding 1 mL of 10 µg/µL lipid suspension
4. Vortex for ~ 3 mins to assist suspension – the suspension of lipid should be turbid (very milky)
5. Decant into a 3.5 kDa MWCO dialyser tube and place the tube in the float and into a beaker and dialyze overnight against 2.5 L of TKM buffer while gentle stirring.

Day two

6. Recover the dialysed lipid and place in an Eppendorf tube – this will keep in the fridge for up to 4 days.
7. Assemble extrusion chamber according to the instruction manual (*see Note 2*)
 - Place two Whatman filters (supplied with the extruder) on the top of one of the PTFE cylinders ensuring rubber seal is in place
 - Wet the filters with a drop of milliQ water
 - Place a 100 nm track-etched membrane on top of the droplet and observe the sealing by capillary action around the rubber o-rings
 - Place another 2 filter papers on top of the membrane
 - Carefully drop the PTFE cylinder with filters and membranes on, into the deep half of the metal chamber and place the second PTFE cylinder with rubber seal in place on top with the seal facing down
 - Place the small PTFE ring onto the PTFE stack and screw the other metal half of the chamber down until just tight (*see Note 3*).
 - Place the chamber in the extruder block
 - Check the tightness of the metal needle mounts on the syringes and carefully and insert the stub-needled syringes into the holes in either side of the PTFE stack. There should be minimal resistance against insertion if the needles are lined up correctly (*see Note 4*).
8. When ready to extrude, place 950 µL of the TKM buffer (note that a different buffer is used for the extrusion step of the PLs for immobilization and TIRF measurements - TKM is further supplemented with TROLOX 1 mM and cysteamine 5 mM) in an eppendorf and heat it to temperature for 5 mins at 50 °C.
9. Take one aliquot of the labelled protein from the -80 °C freezer and dilute to 1.5 µM using 1 TKM –this solution will keep for the rest of the week at 4 °C.
10. Add 1 µL of the 1.5 µM protein to 49 µL of the 10µg/µL lipid and mix by pipetting, then place in the fridge or keep on ice and limit exposure to light.
11. After the buffer has come to temperature, add 100 µL to the lipid protein mixture and mix with the rest of the buffer, minimise wastage by ‘washing’ the tube that contained the lipid and protein with 100 µL of the lipid-protein-buffer mixture and returning it to the combined mixture, yielding ~ 1 mL of 1.5 nM protein, 440 µM lipid solution (approximate Lipid-to-protein molar ratio of 290000:1 which ought to yield mostly singly occupied or empty proteoliposomes for SecYEG and 100 nm vesicles, *see Note 5*).
12. Remove one of the syringes from the extruder and, working quickly, remove the plunger from the syringe body, then, with your thumb blocking the needle and holding the cylinder at an angle, use a 1 mL Gilson pipette to transfer the lipid protein mixture into the syringe.
13. Carefully put the plunger back into the end of the cylinder, turn the syringe so that the needle is pointing upwards and depress the plunger such that the air in the back of the cylinder is compressed, this will encourage the buffer to creep around the bubble and allow it to rise easily to the top of the syringe. Once it is at the top, relax the pressure

you are applying then remove your thumb from the needle, a small amount of liquid will likely escape along with the trapped air.

14. Slowly press the plunger to expel remaining air from the top of the cylinder and once more check the tightness of the needle mount on the syringe end.
15. Return the syringe to the PTFE block.
16. Take the extruder housing from the heat block and again working quickly, holding the back of the empty syringe, slowly push the plunger of the filled syringe so that the mixture is transferred to the second syringe. (*see Note 6*)
17. Transfer the lipid back and forth another twelve times i.e. thirteen transfers in total such that the lipid is now in the second syringe.
18. Transfer the extruded proteoliposomes (PL) solution to a clean Eppendorf. During the day that you are using the PL sample it should be kept in the fridge or on ice while limiting light exposure.
19. PLs are now ready for confocal ALEX experiments and a typical sample containing a pre-assembled SecYEG in PLs and SecA would be prepared as follows:
 - 12 μ L PL preparation
 - 1 μ L of 20 mM AMPPNP (to facilitate SecA binding)
 - 2 μ L of 500 μ M (i.e. 1 nmole) SecA
 - 6 μ L of 133 μ M (i.e. ~800 pmoles) substrate pre-protein (such as proSpy or proOmpA) in TKM buffer

This yields single-molecule concentration (<100 pM) of labelled SecYEG in PLs, with excess unlabelled substrate (760 nM) and SecA (1 μ M) that are kept above their respective nanomolar dissociation constants.

3.5 Cover slip surface preparation and derivatisation with PLs

Day one

Protocol is adapted from [12] and an alternative protocol can be also found in [4].

1. Clean coverslips thoroughly 30 min sonication in acetone (do not include this step if using plastic sample chambers with fused or glued cover slip)
2. Rinse with ultrapure fresh milliQ using clean wash bottle
3. Sonicate for 10 min in 1M KOH or NaOH
4. Rinse with milliQ using wash bottle followed by immersion in milliQ to ensure complete removal of residual base
5. Rinse with methanol (MeOH) using wash bottle followed by immersion in MeOH and place in fresh MeOH in a 200 mL beaker while you prepare an aminosilane solution
6. For the aminosilane solution mix use:
 - i. 100 mL MeOH
 - ii. 5 mL glacial acetic acid
 - iii. 1 mL APTES
7. Clean a conical flask by filling it with MeOH and sonicating for 30 min
8. Add the acetic acid to the MeOH in the clean conical flask and mix by swirling
9. Add the APTES and mix by swirling
10. Pour the MeOH off the coverslips and cover with the fresh APTES solution
11. Place foil over the beaker and allow to react for 10 minutes
12. Sonicate for 1 minute
13. Stand for 10 minutes
14. Pour off the aminosilane solution

15. Rinse with MeOH using wash bottle followed by immersion in MeOH then drip dry over paper towels while you prepare the PEGylation solution or using N₂ if using immediately
16. For the PEGylation solution:
 - i. weigh 8 mg of CH₃O-PEG-NHS ester and 0.2 mg of biotin-CONH-PEG-NHS ester (into an Eppendorf per each coverslip to be prepared – batches of 10 are a good idea so make 80 mg and 2 mg.
 - ii. prepare a solution of 0.1 M NaHCO₃ and freeze in aliquots of 640 µL (enough for 10 coverslips/sample chambers)
 - iii. at time of use thaw one aliquot and dissolve weighed NHS esters in it
 - iv. shake to solubilise
17. Put 75 µL of the PEGylation solution on each coverslip
18. Place a second clean coverslip on each droplet to minimise evaporation and limit contact with air
19. Place the coverslips on the tray of an empty pipette tip box containing ~ 100 mL of milliQ water underneath (to prevent drying, do not submerge the coverslips), close the lid and leave to functionalise overnight (*see Note 7*). A further enclosure in the form of a polystyrene box will prevent drying in arid conditions.

Day two

20. Collect coverslips and remove the second coverslip from the top of each functionalised coverslip (*see Note 7*)
21. Rinse the functionalised coverslips copiously with milliQ using a wash bottle
22. Allow to air-dry above paper towels and covered to prevent adhesion of dust
23. Store the functionalised slides in the fridge for up to 2 weeks (*see Note 8*)

3.6. Surface immobilization of proteoliposomes

1. use only PL containing biotinylated lipids
2. Wet the surface of the coverslip to be functionalised with the buffer for your experiment, ie. TKM. (*see Note 7*)
3. Remove the buffer and place a 150 µL drop of 0.2 mg/mL neutravidin on the PEG-derivatised surface of each coverslip (*see Note 7*) and allow to react for at least 10 minutes (this could be done overnight).
4. Rinse with experiment buffer, e.g. TKM.
5. Add 150 µL droplet of PLs to surface, again where the previous droplets were
6. Allow to react for 10 minutes (*see Note 9*).
7. Rinse with experiment buffer (*see Note 10*).
8. Mount into microscope chamber and add imaging buffer (*see Note 11*), e.g. TKM with additives (e.g. anti-photobleaching cocktail GODCAT, ATP and ATP regenerating system, oxygen scavenging system).

3.7. TIRF imaging (100 ms to 100 s time scale)

smFRET experiments were performed on a custom-built set-up in a standard inverted-stage configuration [13] equipped with alternating laser excitation [4]. The setup uses a micrometer precision motorized stage for changing field of view after photobleaching and consecutive data collection from fresh areas.

1. Setup the microscope in epifluorescence mode and minimal continuous laser power and high electron multiplication on camera (through camera control software or

micromanager 2.0 plug-in for imageJ). This will make focusing and selection of appropriate field of view easier.

2. Place the chamber on the microscope stage and focus onto a suitable area (*see Note 12*) using low continuous laser power (<1 mW prior to objective) to avoid photobleaching (*see Note 13*).
3. Turn on TIRF and optimize the evanescent field to be uniform (*see Note 14*).
4. Move away from the previously exposed area, close laser shutters, adjust power (*see Note 15*) and switch on alternating excitation (typically 100 ms on period which is followed with 100 ms off interval is used) and synchronous camera frame (100 ms per frame) collection in micromanager/camera control software.
5. Initiate the reaction, e.g. by adding ATP and mixing the chamber, open the shutters and collect data until the image fades due to photobleaching.
6. If steady state reaction is being monitored then more data can be successively collected from previously unexposed fields by systematically moving through the derivatised section of the coverslip while periodically checking focus and closing the shutters between data collection runs.

3.8. Confocal microscope ALEX data collection (10 μ s to 100 ms time scale)

smFRET experiments were performed on a custom-built ALEX setup as described [13].

1. Depending on the microscope setup it is important to check the power of both lasers (usually between 60 to 140 μ W at the objective) and their depth of modulation (at least 100:1 power ratio between open/on and closed/off state).
2. For slowly diffusing PLs alternating excitation period was set at 100 μ s with 40 μ s exposure for each laser followed by switching period of 10 μ s each.
3. For microsecond ALEX confocal experiments, proteoliposome samples should be prepared on the day of use and stored on ice or in the fridge when not in use.
4. Sample of 100 μ L (diluted to 50-100 pM of the Alexa Fluor 488 dye) in the presence or absence of substrates or inhibitor etc are added atop a coverslip set on the objective (*see Note 16*). A camera is used to monitor the distance of the focal plane from the coverslip and the objective height adjusted using a piezo-controller (PiezoSystems Jena) to 20 μ m above the surface of the coverslip (*see Note 17*).
5. smFRET bursts are captured in batches of 3 x 10 min files, with fresh sample prepared after every 3rd collection to counteract the issue of liposomes fusing and sedimenting/adhering to the cover slip. Typically, 1-2 hours of data collection time were required to capture enough bursts (~ 5000-10000) to perform robust statistical analyses.
6. Repeat data files (in sm file format for ALEX or ptu file format for nanosecond time resolved experiments) are then merged together and converted to photon-hdf5 format for further processing using FretBursts python package (*see section 3.11.*).

3.9. Nanosecond time resolved FRET setup and data collection

Time-resolved smFRET experiments were performed on a custom-built confocal epi-illuminated microscope in a standard inverted-stage configuration. Donor fluorophore molecules were excited with a 480 nm pulsed diode laser with fiber coupling optics (Advanced Laser Diode Systems, Germany) at a frequency of 40MHz and a power of ~100 μ W. Laser light is reflected from a dichroic beam splitter (Chroma Technology, USA) into a 100x 1.45 NA microscope objective (Nikon Instruments, USA) where it is focused onto the sample placed

on a standard 0.17 mm thick coverslip. Light emitted from the sample is collected by the same objective lens before being transmitted through a dichroic beam splitter. The emitted photons are then passed through a 100 μm pinhole before being spectrally separated into green and red channels by a long-pass dichroic mirror. Band pass filters further remove scattered light before it is focused onto single-photon avalanche diodes (MPD-100-CTB, MPD-050-CTB, Picoquant, Germany) with high timing resolution and low dark counts (<50 counts/s). Signals from the detectors are sent to a PCIe TCSPC card (TimeHarp 260 PICO Dual, Picoquant, Germany). A trigger signal from the laser driver was used as a synchronization signal for the data collection card (providing start point for nanotime, see data processing below).

Sample preparation of proteoliposomes for single-molecule confocal measurements followed the same procedure as described for the ALEX except the for changes in the laser setup already mentioned here. Both samples were measured in Tris-HCl (pH 7.5) 10mM, KCl 10mM and MgCl_2 1mM buffer.

3.10. Analysis of TIRF-FRET time trajectories of immobilized samples

There are several open source software packages available for processing of fluorescence time-traces of immobilized molecules. Some of them are based on the open Single-Molecule Dataset (SMD) form, created by Daniel Herschlag and Ruben Gonzalez to facilitate publication and exchange of data and analysis results obtained in single-molecule studies (<https://smdata.github.io/>). In our work, we use a well-documented platform of iSMS [14] because it offers faster performance and constitutes a robust platform allowing documenting processing protocols together with associated data sets, filter data based on photobleaching and fast implementation of all frequently used types of analysis (*see Note 18*).

1. Load raw data and detect positions of individual molecules in first 100 frames, if necessary correct for drift.
2. Split the two channels of each image align them and correct for background using integration masks.
3. Extract fluorescence count traces (donor and acceptor) and compute raw FRET efficiencies (E) and stoichiometries (S).
4. Automatically check for bleaching and blinking events.
5. To eliminate contributions from complexes with single type of dye or photobleached acceptor dye, consider only traces with S values between 0.25 and 0.75 for further analysis.
6. Select such molecules which show anti-correlation of intensity in donor and acceptor channels.
7. Use molecules showing bleaching to obtain correction factors, such as donor leakage, direct acceptor excitation and gamma factor [14].
8. Apply hidden Markov model using Viterbi algorithm to find steps in traces.
9. Extract corrected histograms of FRET values and dwell times (*see Note 19*).
10. Systematically explore dependence of FRET histograms and dwell times on conditions, e.g. ATP binding and hydrolysis or ligand/substrate binding.

3.11. Data analysis from freely diffusing samples - confocal microscopy

Single photons are detected by avalanche diodes in the donor and acceptor detection channels. Each individual photon collected is then tagged by the photon counting board with the

following information: time of arrival (macrotime), detection channel number (donor/acceptor), and, in the case of pulsed excitation, nanotime. The macrotime is the time elapsed from the beginning of the experiment and is measured in the units of clock periods (either 12.5ns / 80MHz or 25ns/40MHz) in our setup. The nanotime corresponds to the time difference between the photon arrival and the excitation laser pulse and is measured with time-resolution of 0.025ns in our setup.

When a fluorescent particle passes through the confocal volume, the rate of incoming emission photons increases significantly above the background noise level and the resulting bunch of photons arriving at this elevated rate is then referred to as a burst. The duration of each burst is limited by diffusion and the size of the confocal volume to a few milliseconds. It is generally longer for slowly diffusing objects, such as PLs, for which bursts as long as 10 ms are not uncommon.

Each experiment generates a large raw smFRET data file containing an array of timestamps and channel identifiers (donor/acceptor) in a format that depends on data collection software. Usually, these data files are first converted to the open hdf5 format [15] (<http://photon-hdf5.readthedocs.io/en/0.4/phdata.html>) using a Python package `phconvert` (<http://phconvert.readthedocs.io>) and then processed by a Python based, open source `FretBursts` package ([16], *see Note 20*). Principles of the data processing methods used to reveal dynamics of the membrane proteins based on single molecule FRET are described in [4]. Here we provide a brief example, “a cookbook” of analysis of microsecond ALEX data file using `FRETBursts` package. The code applies to Python version 3.6.3, `FRETBursts` version 0.6.5 and `phconvert` version 0.8.1.

Cookbook:

1. Download and install Anaconda Python distribution with Spyder.
2. Open Anaconda prompt and install `fretbursts` package and `phconvert` package by typing:

```
conda install fretbursts -c conda-forge
conda install -c conda-forge phconvert
```

3. Create a new script in Spyder and insert the following lines to import `fretbursts` package and other packages:

```
from fretbursts import *
import phconvert
import numpy as np
import matplotlib.pyplot as plt
```

4. Convert `.sm` file generated by the photon counting board software into hdf5 file format (*see Note 21*):

```
dic =
phconvert.loader.usalex_sm('datafile.sm', donor=0, accept
or=1,
alex_period=3200, alex_offset=0, alex_period_donor=(200, 1
500), alex_period_acceptor=(1700, 3000))
dic['description'] = 'Experiment description'
phconvert.hdf5.save_photon_hdf5(dic, overwrite=True)
```

5. Load the hdf5 file, which creates an object of `Data` class, a fundamental data container in `FRETBursts`:

```
d = loader.photon_hdf5('datafile.hdf5')
```

6. Plot the alternation histogram to check whether the alternation parameters passed to `phconvert.loader.usalex_sm` were correct:


```
bpl.plot_alternation_hist(d)
```
7. If the alternation histogram looks fine, i.e. if the donor and acceptor emission signals are located mainly in the donor and acceptor excitation periods, respectively, then apply the alternation period. Otherwise you can adjust the alternation parameters: e.g.:


```
d.add(D_ON=(200,1500),A_ON=(1800,3100)).
loader.alex_apply_period(d)
```
8. Calculate background rates by fitting an exponential into the tail of the interphoton-time histograms. The data trajectory is divided into time-windows of duration time (s) and background is calculated separately for each:


```
d.calc_bg(bg.exp_fit, time_s=30, tail_min_us='auto',
F_bg=1.7)
dplot(d, hist_bg, show_fit=True)
```
9. Set the correction parameters [17] and perform burst search (*see Note 22*). All photons are taken to calculate the rate at this stage but further selections are available (*see Note 23*) and FRET values for bursts and stoichiometry are calculated along the way (`compute_fret=True`) (*see Note 23*):


```
d.leakage, d.dir_ex, d.gamma = 0.05, 0.0, 1.0
d.burst_search(m=10, L=10, F=5, ph_sel=Ph_sel('all'),
compute_fret=True)
dplot(d, timetrace, binwidth=1e-3, scroll=True)
```
10. Burst selection can be performed according to various criteria and the selections can be chained. Here we select burst which contain number of photons (including AexAem photons: `ad_naa=True`) within the range from 30 to 1000 (small burst have too noisy FRET values and large bursts may not correspond to single molecule events):


```
ds = d.select_bursts(select_bursts.size, add_naa=True,
th1=30, th2=1000)
```
11. Then we further select only bursts with reasonable FRET values within the range from -0.2 to 1.2:


```
ds = ds.select_bursts(select_bursts.E, E1=-0.2, E2=1.2)
```
12. Plotting stoichiometry against FRET efficiency results in a 2D ES plot which reveals different FRET populations in the sample and clearly separates out the non-FRET species such as the donor-only and acceptor-only labelled molecules. FRET and stoichiometry values for all burst are contained in `ds.E[0]` and `ds.S[0]` arrays. When only a one spot experiment is performed then the index is equal to 0. The ES plot can be rendered either by the standard matplotlib plotting package or by using `alex_jointplot` function defined in `FRETBursts`:


```
plt.hexbin(ds.E[0],ds.S[0],gridsize=50, extent=(-
0.2,1.2,-0.2,1.2))
alex_jointplot(ds, kind='hex')
```
13. Data object `ds` has many attributes, which can be listed by typing `ds.__dict__`. Now the data is prepared for analysis of dynamics.

3.12 Analysis of dynamics on millisecond to microsecond time scale

The dynamics and conformational changes in the single molecules diffusing through the confocal volume can be studied by several methods. Burst Variance Analysis (BVA) enables

us to probe the dynamics on the timescale of the burst duration, *i.e.* typically in the range 0.1-10ms [18]. The BVA strategy sub-divides individual bursts into contiguous sub-bursts consisting of a fixed number of photons. FRET efficiency is then calculated for each sub-burst within the burst (appended to the list `E_sub` in the script below) and the variance of FRET within the burst is calculated from (σ^2 , stored in the list `E_sub_std`).

1. An example of a minimal code implementing the BVA method for sub-bursts of 5 photons:

```
n = 5
E_bursts, E_sub_std = [], []
for b in ds.mburst[0]:
    E_sub = []
    mask_Dex_burst = ds.D_ex[0][b.istart:b.istop]
    photon_indexes =
np.arange(b.istart,b.istop)[mask_Dex_burst]
    for i in np.arange(0,len(photon_indexes)-n+1,n):
        indexes_sub = photon_indexes[i:i+n]
        Aem_count = ds.A_em[0][indexes_sub].sum()
        E_sub.append(Aem_count/float(n)) #append FRET of
sub-burst
    E_bursts.append(np.mean(E_sub))
    E_sub_std.append(np.std(E_sub))
plt.figure('BVA')
plt.scatter(E_bursts,E_sub_std,alpha=0.15)
x = np.arange(0,1,0.001)
plt.plot(x,np.sqrt(x*(1-x)/n),'r-') #predicted variance
plt.xlabel('E')
plt.ylabel('std.dev(E_sub)')
```

2. After running the script values of σ against FRET for each burst are displayed in a scatter plot (Figure 1A). When variance of sub-bursts is larger than the shot-noise-limited predicted variance for specific FRET regions, then it indicates the presence of state interconversion on the given burst timescale (see **Note 24**).

A related method consisting in observation of variations in donor and acceptor local photon rates within the bursts is photon density analysis using FRET-2CDE method [19]. Each burst is attributed a FRET-2CDE estimator value and this value is plotted against FRET (Figure 1B). FRET-2CDE estimator values significantly larger than the theoretically predicted value for static population in a certain FRET region indicate presence of dynamics in the corresponding population. The implementation of FRET-2CDE method in FRETbursts is explained in detail on the documentation web page of the FRETburst project <http://fretbursts.readthedocs.io/en/latest/>.

Another method capable of revealing dynamics on longer timescales (typically 5-200ms) is Recurrence Analysis of Single Particles (RASP) [20]. Highly diluted diffusing particles are likely to revisit the confocal volume within a short time, while entry of another particle in the same time frame has low probability. RASP method cycles through bursts and searches for pairs of bursts whose time separation δt falls within certain time limits (δt_{limits}). The FRET value of the first burst in the pair is denoted E1 (E0 in the script) and the second one E2 (E1 in the script). These pairs of bursts are then filtered according to the δt and plotted into a two-dimensional histogram (Figure 2) which represents frequencies of transitions between E1 and E2 states within a given time window. Any off-diagonal densities (i.e. $E1 \neq E2$) within such a plot indicate a change of state within the set time window. A change in time can be

Figure 2: RASP analysis of plug motion in SecYEG translocon [4]. (A) Two-dimensional FRET efficiency contour plots (transition density plots) were obtained from bursts collected for SecYMKEG:SecA:SecB:pOA (proOmpA 100 aa) in the presence of 0.1 mM ATP using the confocal setup. The events were classified according to initial FRET (E1) and the burst recurrence FRET (E2) observed after the indicated time delay. Data shown as Probability Density Function (PDF) contour plots with scale on the right. Compare this dynamic movement of the plug with lateral gate which remains static on the millisecond time scale *c.f.* Figure 1C. (B) RASP analysis was performed with the initial state interval of 0.4 (± 0.1). The RASP PDF shows a rapid decreasing closed state population (red) and concomitantly increasing open state population (blue), see colour coding legend. (C) A two state approximation of interconversion between states in panel B yields exponential kinetics and associated rate constants.

visualized by plotting the 2D histograms for increasing δt time (Figure 2A). The 2D histogram

can be cut along the y-axis to obtain a 1D histogram, which allows to observe how certain FRET states convert into states with different FRET values over time (Figure 2B) from which kinetic information on millisecond time scale can be obtained (Figure 2C).

1. A minimalistic example of RASP analysis implementation using FRETBursts:

```
dt_limits = (0,0.1)
bursts = ds.mburst[0]
E = ds.E[0] # FRET in individual bursts
bu_center_times = ds.clk_p*(bursts.stop +
bursts.start)/2.
E0E1dt = [] #List of RASP pairs
for i,b in enumerate(bursts):
    j = i+1
    while j<bursts.size:
        dt = bu_center_times[j]-bu_center_times[i]
        #time separation
        if dt_limits[0] <= dt < dt_limits[1]:
            E0E1dt.append([E[i],E[j],dt])
        elif dt >= dt_limits[1]:
            break
        j+=1

E0E1dt = np.array(E0E1dt)
E0,E1,dt = E0E1dt[:,0],E0E1dt[:,1],E0E1dt[:,2]
mask_dt = (0<=dt)*(dt<0.020)
plt.figure('RASP_2D_histogram')
plt.hexbin(E0[mask_dt],E1[mask_dt],gridsize=50) #2D RASP
histogram
plt.xlabel('E0'); plt.ylabel('E1')
mask_E0 = (0.4<=E0)*(E0<0.6)
plt.figure('RASP_1D_hist')
bin_edges = np.arange(-0.2,1.2,0.05)
plt.hist(E1[mask_E0],bins=bin_edges,histtype='step',density=False)
```

3.13 Analysis of dynamics on nanosecond time scale

Data was analysed using the FRETBursts python package in a similar fashion as illustrated above but using custom built scripts for the burstwise lifetime fitting.

1. A burst search with a minimum threshold of 4 times the background signal of ~2Hz and ~1.3KHz in the donor and acceptor channels respectively and a minimum burst size of 80 photons was used to select signal from single-molecules.
2. Donor only molecules were removed using a minimum acceptor burst size of 20 photons (this is necessary in the absence of alternating laser excitation).
3. Single-molecule fluorescence decays were analysed using a maximum likelihood method as described in [21].
4. FRET efficiency is computed from raw intensities after applying corrections for quantum yield, sensitivity and detection efficiency.

5. FRET efficiency and lifetime for individual bursts are collated in a two dimensional histogram $\tau_D - E$.
6. In a static case there should be linear anti-correlation between FRET efficiency and fluorescent lifetime of the donor molecule (τ_D) (data fall on solid red line in Figure 3A). The line starts at FRET efficiency zero for molecules far from the acceptor (i.e. donor only $\tau_{D(0)}=4.1$ ns, i.e. similar to that of free dye in solution).
7. Departure from the anti-correlation indicates population of dynamic states on nanosecond time as a result of dynamic interconversion between two fluorescent lifetimes [22] (Figure 3B, dashed red line).

Notes

1. Few aspects for selection of dyes and their attachment sites which need to be considered: (1) Possibility of interaction with membrane lipids and the protein (e.g. charge and hydrophobicity); This can be tackled by using available structures and simulations including the attached dyes as described recently [3] and using a longer linker between the dye and the attachment site; (2) high quantum yield and extinction coefficient, i.e. high brightness; (3) high dye photostability and the use of anti-photobleaching cocktails for long TIRF measurements. (4) triplet state and blinking suppression.
2. Detailed instructions for extruder use can be found on Avanti's website www.avanti.com.
3. Do not under or overtighten the extruder chamber!
4. Take care not to inadvertently insert the needle into the PTFE itself.
5. This is based on the assumption that a 100 nm diameter liposome contains ~80000 lipid molecules and that the lipid to protein ratio will reduce the probability of multiple occupancy. See [10] for more details.
6. Take care as the metal needle mounts can work themselves loose during the process, if this happens the solution escapes out into the extruder housing and you need to clean it and make a fresh sample!

7. It is important to track which surface of the glass is derivatized – only the surfaces exposed to PEG i.e. the inner sides within the cover slip “sandwich”, are modified. One way to keep track is to place coverslips in a tray with a mark or sign indicating directionality and use this device throughout the procedure.
8. Use clean and tightly closed container to prevent contamination.
9. 10 min is minimum for a surface adhesion and the reaction could be allowed to proceed for longer but do not exceed 1 hour to limit vesicle fusion.
10. Due to PL fusion it is recommended to use the slide within 1 hour after immobilization and change the sample chamber/slide frequently during experiment.
11. Due to adverse effects of oxygen (reactive singlet oxygen) on the emission characteristics of fluorescent species during laser irradiation it is customary to degas the buffer before use e.g. by cycles of vacuum and sonication.
12. Since TIRF microscopy relies on a high numerical aperture objective (e.g. Zeiss Plan Fluar, 100X, NA 1.4) make sure to use appropriate low-fluorescence immersion oil.
13. For the initial focusing it is good to find a field of view containing fused vesicles or a fluorescent aggregate. For data collection try to limit the number of such aggregates.
14. This is system specific, depending on way the evanescent field is created. In commercial automated setups this step may not be necessary. In manual setups TIRF conversion is usually achieved by changing the entry angle of the laser beam into the objective via a micropositioner. Thus, fine adjustment of this angle can be used to make the illumination uniform and centred within the camera field of view.
15. This should be as low as feasible for long observations (few mW entering the objective). However, for faster processes higher excitation power may be needed at the expense of observation duration.
16. If possible try to maintain temperature of the sample constant within a few degrees – this is particularly important when trying to extract kinetic information, e.g. rate constants, from the data.
17. The distance depends on the working distance of the objective. Note that commercial microscopes may have calibrated focusing system referenced to the position of the cover slip so there is no need to use camera for referencing.
18. Read detailed documentation and step-by-step tutorial on iSMS webpage: <http://inano.au.dk/about/research-groups/single-molecule-biophotonics-group-victoria-birkedal/software/documentation/>. There are other packages offering similar capabilities, such as TwoTone [23], VbFRET [24], ebFRET [25], Boba FRET [26], HaMMMy [27], QuB [28], SMART [29] and Ha [30]. It is also a common practice that laboratories write their own custom-built data processing software in Matlab [31] or Python [32]. For more detailed description see following review of publically available software see [33].
19. To construct histograms from TIRF data, we recommend using only the TIRF time traces which show transitions detectable by Hidden Markov Model algorithm implemented in iSMS [14], this means, that such complexes are responsive/active. This approach eliminates contribution from SecYEG complexes which are reconstituted with their cytoplasmic side facing the vesicle interior and thus inaccessible to SecA and the substrate. Corrected FRET values are then used to produce histograms and fit their populations. For further details of application on membrane proteins see [4].
20. See <https://opensmfs.github.io/FRETBursts/> to download the code (version 0.6.5) and <http://fretbursts.readthedocs.io> for the documentation and various application examples. Thorough inspection of the original paper and documentation is highly recommended. Our data

processing scripts, further application examples and various useful links are available at <http://makrokomplex.cz/data-processing>. Python scripts have been written and executed in Anaconda Python distribution using Spyder as IDE <https://anaconda.org/anaconda/python/>.

21. The laser alternation periods are expressed in the units of clock periods of the photon counting board. The parameters passed to the function are used to decide whether the photons belong to the donor or acceptor excitation period.

22. Bursts are regions of the photon stream where the local photon count rate (computed using m photons) is above a minimum threshold rate. We choose a rate threshold that is F times larger than the background rate. The common range for m is 5 to 15 and for F it is 4 to 9.

23. Example: by setting `ph_sel=Ph_sel(Dex='DAem')` only photons during donor excitation would be taken into account.

24. For some FRET intervals there are only few bursts and the average σ has a relatively large inaccuracy. Therefore, it is desirable to calculate a confidence interval for the average of σ at different FRET values. If the experimental average σ is out of the confidence interval at a certain FRET region, then this is a strong indication that there is a dynamics present in the corresponding population of molecules. For the details on the construction of confidence intervals please refer to [18] and [4].

Acknowledgements

This work was funded by the BBSRC: MW, TF, RT and SER (BB/N017307/1), DW and IC (BBSRC: BB/N015126/1 and BB/I008675/1), RT, SER (BB/I008675/1). Additional support was provided by the Wellcome Trust (104632) to IC and ERC ((FP7/2007-2013) / ERC grant agreement 32240) to SER. MS, TF and RT are supported from European Regional Development Fund-Project "Mechanisms and dynamics of macromolecular complexes: from single molecules to cells" (No. CZ.02.1.01/0.0/0.0/15_003/0000441). JC and TS are supported by a PhD studentship and capital equipment allocation from the School of Clinical and Applied Sciences, Leeds Beckett University.

References

1. Jefferson RE, Min D, Corin K, Wang JY, Bowie JU (2018) Applications of Single-Molecule Methods to Membrane Protein Folding Studies. *Journal of molecular biology* 430 (4):424-437. doi:10.1016/j.jmb.2017.05.021
2. Martinac B (2017) Single-molecule FRET studies of ion channels. *Prog Biophys Mol Biol* 130 (Pt B):192-197. doi:10.1016/j.pbiomolbio.2017.06.014
3. Harborne SPD, Strauss J, Turku A, Watson MA, Tuma R, Harris SA, Goldman A (2018) Defining Dynamics of Membrane-Bound Pyrophosphatases by Experimental and Computational Single-Molecule FRET. *Methods in enzymology* in the press
4. Fessl T, Watkins D, Oatley P, Allen WJ, Corey RA, Horne J, Baldwin SA, Radford SE, Collinson I, Tuma R (2018) Dynamic action of the Sec machinery during initiation, protein translocation and termination. *eLife* 7. doi:10.7554/eLife.35112
5. Allen WJ, Corey RA, Oatley P, Sessions RB, Baldwin SA, Radford SE, Tuma R, Collinson I (2016) Two-way communication between SecY and SecA suggests a Brownian ratchet mechanism for protein translocation. *eLife* 5:e15598. doi:10.7554/eLife.15598
6. Lakowicz JR (2006) *Principles of Fluorescence Spectroscopy*. 3 edn. Springer-Verlag, New York

7. Calebiro D, Sungkaworn T (2018) Single-Molecule Imaging of GPCR Interactions. *Trends Pharmacol Sci* 39 (2):109-122. doi:10.1016/j.tips.2017.10.010
8. Collinson I, Corey RA, Allen WJ (2015) Channel crossing: how are proteins shipped across the bacterial plasma membrane? *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 370 (1679). doi:10.1098/rstb.2015.0025
9. Corey RA, Allen WJ, Collinson I (2016) Protein translocation: what's the problem? *Biochemical Society transactions* 44 (3):753-759. doi:10.1042/BST20160047
10. Deville K, Gold VA, Robson A, Whitehouse S, Sessions RB, Baldwin SA, Radford SE, Collinson I (2011) The oligomeric state and arrangement of the active bacterial translocon. *The Journal of biological chemistry* 286 (6):4659-4669. doi:10.1074/jbc.M110.175638
11. Gold VA, Duong F, Collinson I (2007) Structure and function of the bacterial Sec translocon. *Molecular membrane biology* 24 (5-6):387-394. doi:10.1080/09687680701416570
12. Chandradoss SD, Haagsma AC, Lee YK, Hwang JH, Nam JM, Joo C (2014) Surface passivation for single-molecule protein studies. *J Vis Exp* (86). doi:10.3791/50549
13. Sharma A, Leach RN, Gell C, Zhang N, Burrows PC, Shepherd DA, Wigneshweraraj S, Smith DA, Zhang X, Buck M, Stockley PG, Tuma R (2014) Domain movements of the enhancer-dependent sigma factor drive DNA delivery into the RNA polymerase active site: insights from single molecule studies. *Nucleic acids research* 42 (8):5177-5190. doi:10.1093/nar/gku146
14. Preus S, Noer SL, Hildebrandt LL, Gudnason D, Birkedal V (2015) iSMS: single-molecule FRET microscopy software. *Nature methods* 12 (7):593-594. doi:10.1038/nmeth.3435
15. Ingargiola A, Laurence T, Boutelle R, Weiss S, Michalet X (2016) Photon-HDF5: An Open File Format for Timestamp-Based Single-Molecule Fluorescence Experiments. *Biophysical journal* 110 (1):26-33. doi:10.1016/j.bpj.2015.11.013
16. Ingargiola A, Lerner E, Chung S, Weiss S, Michalet X (2016) FRETbursts: An Open Source Toolkit for Analysis of Freely-Diffusing Single-Molecule FRET. *PloS one* 11 (8):e0160716. doi:10.1371/journal.pone.0160716
17. Hohlbein J, Craggs TD, Cordes T (2014) Alternating-laser excitation: single-molecule FRET and beyond. *Chemical Society reviews* 43 (4):1156-1171. doi:10.1039/c3cs60233h
18. Torella JP, Holden SJ, Santoso Y, Hohlbein J, Kapanidis AN (2011) Identifying molecular dynamics in single-molecule FRET experiments with burst variance analysis. *Biophysical journal* 100 (6):1568-1577. doi:10.1016/j.bpj.2011.01.066
19. Tomov TE, Tsukanov R, Masoud R, Liber M, Plavner N, Nir E (2012) Disentangling subpopulations in single-molecule FRET and ALEX experiments with photon distribution analysis. *Biophysical journal* 102 (5):1163-1173. doi:10.1016/j.bpj.2011.11.4025
20. Hoffmann A, Nettel D, Clark J, Borgia A, Radford SE, Clarke J, Schuler B (2011) Quantifying heterogeneity and conformational dynamics from single molecule FRET of diffusing molecules: recurrence analysis of single particles (RASP). *Physical chemistry chemical physics : PCCP* 13 (5):1857-1871. doi:10.1039/c0cp01911a
21. Maus M, Cotlet M, Hofkens J, Gensch T, De Schryver FC, Schaffer J, Seidel CA (2001) An experimental comparison of the maximum likelihood estimation and nonlinear least-squares fluorescence lifetime analysis of single molecules. *Analytical chemistry* 73 (9):2078-2086
22. Kalinin S, Valeri A, Antonik M, Felekyan S, Seidel CA (2010) Detection of structural dynamics by FRET: a photon distribution and fluorescence lifetime analysis of systems with multiple states. *The journal of physical chemistry B* 114 (23):7983-7995. doi:10.1021/jp102156t
23. Holden SJ, Uphoff S, Hohlbein J, Yadin D, Le Reste L, Britton OJ, Kapanidis AN (2010) Defining the limits of single-molecule FRET resolution in TIRF microscopy. *Biophysical journal* 99 (9):3102-3111. doi:10.1016/j.bpj.2010.09.005
24. Bronson JE, Fei J, Hofman JM, Gonzalez RL, Jr., Wiggins CH (2009) Learning rates and states from biophysical time series: a Bayesian approach to model selection and single-molecule FRET data. *Biophysical journal* 97 (12):3196-3205. doi:10.1016/j.bpj.2009.09.031

25. van de Meent JW, Bronson JE, Wiggins CH, Gonzalez RL, Jr. (2014) Empirical Bayes methods enable advanced population-level analyses of single-molecule FRET experiments. *Biophysical journal* 106 (6):1327-1337. doi:10.1016/j.bpj.2013.12.055
26. Konig SL, Hadzic M, Fiorini E, Borner R, Kowerko D, Blanckenhorn WU, Sigel RK (2013) BOBA FRET: bootstrap-based analysis of single-molecule FRET data. *PloS one* 8 (12):e84157. doi:10.1371/journal.pone.0084157
27. McKinney SA, Joo C, Ha T (2006) Analysis of single-molecule FRET trajectories using hidden Markov modeling. *Biophysical journal* 91 (5):1941-1951. doi:10.1529/biophysj.106.082487
28. Qin F, Li L (2004) Model-based fitting of single-channel dwell-time distributions. *Biophysical journal* 87 (3):1657-1671. doi:10.1529/biophysj.103.037531
29. Greenfield M, Pavlichin DS, Mabuchi H, Herschlag D (2012) Single Molecule Analysis Research Tool (SMART): an integrated approach for analyzing single molecule data. *PloS one* 7 (2):e30024. doi:10.1371/journal.pone.0030024
30. Roy R, Hohng S, Ha T (2008) A practical guide to single-molecule FRET. *Nature methods* 5 (6):507-516. doi:10.1038/nmeth.1208
31. Li M, Jorgensen SK, McMillan DG, Krzeminski L, Daskalakis NN, Partanen RH, Tutkus M, Tuma R, Stamou D, Hatzakis NS, Jeuken LJ (2015) Single Enzyme Experiments Reveal a Long-Lifetime Proton Leak State in a Heme-Copper Oxidase. *Journal of the American Chemical Society* 137 (51):16055-16063. doi:10.1021/jacs.5b08798
32. Fessl T, Lilley DM (2013) Measurement of the change in twist at a helical junction in RNA using the orientation dependence of FRET. *Biophysical journal* 105 (9):2175-2181. doi:10.1016/j.bpj.2013.09.042
33. Blanco M, Walter NG (2010) Analysis of complex single-molecule FRET time trajectories. *Methods in enzymology* 472:153-178. doi:10.1016/S0076-6879(10)72011-5