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TITLE:

Single Molecule Fluorescence Energy Transfer Study of Ribosome Protein Synthesis

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SUMMARY:

Single molecule fluorescence energy transfer is a method that tracks the tRNA dynamics during ribosomal protein synthesis. By tracking individual ribosomes, inhomogeneous populations are identified, which shed light on mechanisms. This method can be used to track biological conformational changes in general to reveal dynamic-function relationships in many other complexed biosystems. Single molecule methods can observe non-rate limiting steps and low-populated key intermediates, which are not accessible by conventional ensemble methods due to the average effect.

ABSTRACT:

The ribosome is a large ribonucleoprotein complex that assembles proteins processively along mRNA templates. The diameter of the ribosome is approximately 20 nm to accommodate large tRNA substrates at the A-, P- and E-sites. Consequently, the ribosome dynamics are naturally de-phased quickly. Single molecule method can detect each ribosome separately and distinguish inhomogeneous populations, which is essential to reveal the complicated mechanisms of multi-component systems. We report the details of a smFRET method based on the Nikon Ti2 inverted microscope to probe the ribosome dynamics between the ribosomal protein L27 and tRNAs. The L27 is labeled at its unique Cys 53 position and reconstituted into a ribosome that is engineered to lack L27. The tRNA is labeled at its elbow region. As the tRNA moves to different locations inside the ribosome during the elongation cycle, such as pre- and post- translocation, the FRET efficiencies and dynamics exhibit differences, which have suggested multiple subpopulations. These subpopulations are not detectable by ensemble methods. The TIRF-based smFRET microscope is built on a manual or motorized inverted microscope, with home-built laser illumination. The ribosome samples are purified by ultracentrifugation, loaded into a home-built multi-channel sample cell and then illuminated via an evanescent laser field. The reflection laser spot can be used to achieve feedback control of perfect focus. The fluorescence signals are separated by a motorized filter-turret and collected by two digital CMOS cameras. The intensities are retrieved via the NIS-Elements software.

INTRODUCTION:

The ribosome is a ø 20 nm large ribonucleoprotein complex of a large (50S) and a small (30S) subunit. It assembles long peptides along the mRNA template processively and cooperatively. The ribosome 30S binds to the fMet-tRNA^{fMet} and mRNA to start protein synthesis, and the 50S

then joins to form the 70S initiation complex. The tRNAs bring amino acids to the ribosome at the A-site (aminoacyl- tRNA binding site), while the elongated peptidyl chain is held at the P-site (peptidyl- tRNA binding site). In the pre-translocation complex, the peptidyl chain is transferred to the tRNA at the A-site with one amino acid added. Meanwhile, the P-site tRNA is deacylated. Then, the A-, P- tRNAs move to the P-, E- sites to form the post-translocation complex, in which the E-site represents the tRNA exit site. In this state, the peptidyl-tRNA moves back to the P-site. The elongation cycle continues between the pre- and post-conformations while the ribosome translocates on the mRNA, one codon at a time[1]. The ribosome is highly coordinative of different functional sites to make this process efficient and accurate, such as inter-subunit ratcheting[2], tRNA hybridization fluctuations[3], GTPase activations[4], L1 stalk opening-closing[5], etc. Consequently, ribosomes quickly de-phase because every molecule moves at its own pace. The conventional methods can only deduce apparent average parameters, but low-populated or short-lived species will be masked in the average effect[6]. Single molecule method can break this limitation by detecting each ribosome individually, then identify different species via statistical reconstruction[7]. Different labeling sites have been implemented to probe ribosome dynamics, such as the interactions between tRNA-tRNA[8], EF-G-L11[9], L1-tRNA[10], etc. In addition, by labeling the large and small subunits, respectively, inter-subunit ratcheting kinetics and coordination with factors are observed[11,12]. Meanwhile, the smFRET method has broad applications in other central biological processes, and multi-color FRET methods are emerging[13].

Previously a novel ribosome FRET pair was developed[14,15]. The recombinant ribosomal protein L27 has been expressed, purified, and labeled, and incorporated back into the ribosome. This protein interacted with the tRNAs at a close distance and helped stabilize the P-site tRNA in the post-translocation complex. When tRNA moved from the A- to the P-site, the distance between this protein and the tRNA is shortened, which can be distinguished by the smFRET signal. Multiple ribosome subpopulations have been identified using statistical methods and mutagenesis, and spontaneous exchange of these populations in the pre- but not post- translocation complex suggests the ribosome is more flexible before moving on the mRNA, and more rigid during decoding[16–18]. These variations are essential to the ribosome function. Here, the protocol describes the details of ribosome/tRNA-labeling, their incorporation in the ribosome, smFRET sample preparation, and data acquisition/analysis[19].

PROTOCOL:

1. Preparation of labeled ribosome and tRNA for FRET detection

1.1. Isolate ribosome without L27 from *E. coli* strain IW312 according to standard protocols[20,21]. Extract the regular ribosome from *E. coli* strain MRE600.

1.2. Clone the L27's rpmA gene with C-terminal His-tag into pET-21b (+) plasmid, which is transformed and expressed in BL21(DE3)pLysS cells[15]. Purify the protein via a prepacked sepharose column.

1.3. Labeling of L27

1.3.1. Incubate 20–100 μM of purified L27 in 100 μL with 2–10-fold excess of TCEP (Tris-2-carboxyethyl-phosphine) at room temperature (RT) for 30 min. Buffer exchange the solution into PBS buffer (10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 2.7 mM KCl; 0.137 M NaCl), using a nap-5 column. Add 10-fold excess Cy5-maleimide mono-reactive dye in DMSO into the solution and incubate at RT in the dark for 2 h.

1.3.2. Load the labeled protein solution (500 μL) mentioned above on a Nap-5 column to remove the excess dye. Ensure that the protein elutes in the first colored band, followed by the free dye elute in the second band, respectively. Collect the eluted protein in 1 mL of TAM_{10} buffer (20 mM tris-HCl (pH 7.5), 10 mM Mg $(OAc)_2$, 30 mM NH_4Cl, 70 mM KCl, 0.5 mM EDTA, and 7 mM BME (2-mercaptoethanol)).

1.4. Reconstitute the ribosome with L27. Mix the labeled L27 protein with an equal amount of IW312 ribosome in TAM_{10} buffer and incubate at 37 °C for 1 h. Remove the free protein by sucrose cushion ultracentrifugation (100,000 x *g*, overnight) to allow the ribosome to form a blue-colored pellet at the bottom of the tube.

1.5. Label the $tRNA^{Phe}$ according to the previously published report[22]. First, incubate the tRNA (10 A_{260} in 1 mL of water) with 100 μL of $NaBH_4$ (100 mM stock, adding dropwise) at 0 °C for 1 h. Free the tRNAs from unreacted $NaBH_4$ via three-time ethanol precipitation by adding 1/10 volume of 3 M KAc (pH 5.0) and 2.5x volume of ethanol. Incubate the reduced tRNA with 1 μL of Cy3-NHS dye solution (1 mg in 10 μL of DMSO) in a minimum volume (~20 μL) of 0.1 M sodium formate (pH 3.7). After 2 h of incubation at 37 °C, separate the tRNA from the unreacted dye via a small G25 Sephadex column, and then remove the free dye via two-time ethanol precipitation.

2. Preparation of the ribosome complexes

2.1. Express and purify the His-tagged proteins of IF1, IF2, IF3, EF-G, EF-Tu, EF-Ts, and tRNA synthetases using standard methods[15].

2.2. Prepare the initiation mix by adding the following ingredients in TAM_{10} buffer at 37 °C for 15 min: 1 μM of the labeled ribosome; 1.5 μM each of IF1, IF2, and IF3; 2 μM biotinylated mRNA (coding MF for the first two amino acids); 4 μM of charged fMet-$tRNA^{fMet}$; and 1 mM GTP.

2.3. Prepare the EF-Tu_G mix by mixing the following ingredients in TAM_{10} buffer at 37 °C for 15 min: 4 μM EF-Tu, 0.4 μM EF-Ts, 2 μM EF-G, 4 mM GTP, 4 mM PEP, and 0.02 mg/mL of Pyruvate Kinase.

2.4. Prepare the EF-Tu_NoG mix similar to step 2.3 without EF-G.

2.5. Prepare the Phe mix by mixing the following ingredients in nuclease-free water at 37 °C for 15 min: 100 mM Tris (pH 7.5), 20 mM MgAc2, 1 mM EDTA, 4 mM ATP, 7 mM BME, 2 mM Phenylalanine-specific synthetase, 2 A260/mL of labeled $tRNA^{Phe}$, and 50 mM phenylalanine.

2.6. Prepare the pre-translocation complex (PRE) by mixing the initiation, EF-Tu_NoG, and Phe mixes in the ratio of 1:2:2, at 37 °C for 2 min. After incubation, purify the PRE by 1.1 M sucrose cushion ultracentrifugation overnight at 100,000 x *g*.

2.7. Prepare the post-translocation complex (POST) by mixing the initiation, EF-Tu_WG, and Phe mixes in the ratio of 1:2:2, at 37 °C for 10 min. After incubation, purify the POST by 1.1 M sucrose cushion ultracentrifugation overnight at 100,000 x *g*.

3. Preparation of sample slides

3.1. Clean the microscope glass slides (75 mm x 25 mm) containing six pairs of holes (1 mm in diameter). Each pair forms an inlet-outlet of the sample chamber. Ensure that the distance between each inlet-outlet hole is 13 mm and the center-to-center distance between two channels is 6 mm. Place six slides in a glass crucible.

3.2. Fill the crucible with acetone and sonicate them for 5 min. Decant the acetone and rinse the slides three times with ultrapure water. Fill the crucible again with water and 1 mL of 10 M KOH. Sonicate for 20 min.

3.3. Rinse the slides three times with ultrapure water. Fill the crucible again with ethanol and sonicate for 5 min. Completely decant the solvent. Let the slides dry in the fume hood.

3.4. Clean the microscope glass coverslips (#1.5 thickness, 24 x 40 mm). Perform the cleaning steps as described in steps 3.1–3.3.

3.5. Bake the cleaned slides and coverslips at 300 °C for 3 h. Keep them in the furnace overnight to cool.

3.6. Coat the coverslip with aminosilane. In the crucible containing coverslips, pour in methanol mix (100 mL of methanol, 5 mL of water, 0.5 mL of HAc, and 1 mL of aminosilane). Warm the crucible in a water bath for 10 min, sonicate it for 10 min, and then warm the crucible in the water bath for another 10 min. Decant the methanol mixture, rinse the coverslip well in three crucibles of clean water. Purge the surfaces with a dry nitrogen stream through a needle.

3.7. Coat the coverslips with biotin-PEG in a laminar clean hood.

3.7.1. To make the PEG solution, use 98 mg of PEG and 2–3 mg of biotin-PEG in 70 mL of 100 mM $NaHCO_3$ solution.

3.7.2. Lay one coverslip flat on a surface. Carefully drop 60 mL of PEG solution on the top edge. Then, lay another coverslip on top of it, letting the capillary effect spread the solution between the two coverslips. Ensure no bubbles are formed.

3.7.3. Repeat step 3.7.2 for two more pairs of coverslips. Coat six pieces of coverslips with biotin. If more coverslips are needed, adjust the amount of PEG and Biotin PEG accordingly to make more PEG solution.

3.7.4. Store these coverslips in an empty tip-box filled with water and incubate for 3 h in the dark.

3.7.5. After 3 h, separate the coverslips, rinse with water in three consecutive crucibles and purge with a dry nitrogen stream. Place the coated coverslips on a ceramic rack. Mark the surface with coating.

3.8. Assemble the sample chambers[19].

3.8.1. Pull sharp pipette tips through the holes on the glass slides until they fit tight. Scrape the sharp ends off until it is completely flat to the glass surface. Cut the other end of the tip to be approximately 5 mm for sample loading.

3.8.2. Cut a double-faced tape with the channel pattern on it (25 x 40 mm).

3.8.3. Stick the double-faced tape onto the glass slides on the flat side.

3.8.4. Stick the coated coverslip onto the double-faced tape. Press on it to make a tight seal of the sample chamber. Make sure the coated side is facing inward.

4. Single molecule FRET imaging

4.1. Turn on the computer.

4.2. Turn on the microscope switch.

4.3. Start the laser control interface and turn on the laser. Push the enable button on the laser control box to warm up the laser.

4.4. Turn on the cameras.

4.5. Start the microscope-associated software program. Click the upper shutter **Off** and click the lamp **On**.

4.6. Prepare the TAM_{10}_WT buffer by adding 10 mL of 5% tween20 into 1 mL of TAM_{10} buffer.

4.7. Prepare the deoxy solution by weighing 3 mg of glucose oxidase in a small microcentrifuge tube. Add 45 mL of catalase. Vortex gently to dissolve the solid. Spin at 20,000 x *g* in a microcentrifuge for 1 min. Take the supernatant.

4.8. Prepare 30% glucose solution in water and 200 mM Trolox solution in DMSO.

4.9. Prepare 0.5 mg/mL of streptavidin solution in nuclease-free water.

4.10. Add one drop of imaging oil to the TIRF objective.

4.11. Put the sample chambers on the objective.

4.12. Fill the chamber with 10 mL of streptavidin solution. Wait for 1 min.

4.13. Wash the chamber with 30 mL of TAM10_WT buffer and collect the run-through solution with a folded filter paper. Wait for 1 min.

4.14. Dilute the ribosome complexes (PRE or POST) to 10–50 nM concentration with TAM_{10}_WT buffer.

4.15. Load 20 mL of the ribosome sample into the channel. Wait for 2 min.

4.16. Make the imaging buffer (50 mL of TAM_{10} buffer plus 0.5 mL each of deoxy, glucose, and Trolox solutions). Mix the buffer well.

4.17. Flush the chamber with 30 mL of the imaging buffer.

4.18. Set the camera to acquire 100 ms/frame, 16 bits, 4 x 4 binning.

4.19. Click the upper shutter **On** and the lamp **Off**.

4.20. Start camera acquisition. Spread the images into three windows representing two individual cameras and the overlay.

4.21. Click on **Auto-focus.** Fine-tune to find the best focus.

4.22. Click on a method. Set the field points, file storage, and the loop number.

4.23. Click on **Run**.

NOTE: A new window appears with real-time imaging. The progress of the time series and field of view series are shown at the top of the window.

4.24. Once the image acquisition is complete, close the popup window.

4.25. Open the ND file (acquired file).

4.26. Click on **ROI**/**simple ROI editor**. Choose the option **Circle**.

4.27. Select the ROI on the image. Click on **Finish** when it is complete.

4.28. Click on **Measurement/Time Measurement** to show the data either as a plot or as a spreadsheet.

4.29. Open the **Export** tab. Set the export parameters.

4.30. Click on **Export** to save the intensities.

REPRESENTATIVE RESULTS:

The smFRET had the ribosome labeled at the middle position of tRNA traffic, to distinguish the tRNA translocation from the A- to the P-site (**Figure 1**)[15]. The distance from the L27 labeling residue to the A- or P-site tRNA is 52 or 61 Å, respectively, corresponding to FRET efficiency of 0.47 and 0.65. After the image collection, fluorescence intensities from the donor and acceptor channels were retrieved and plotted as time lapses (**Figure 1**). FRET efficiency was calculated by the formula $I_{acceptor}/(I_{acceptor}+I_{donor})$. A homemade program detected the single-step bleaching of the donor/acceptor and fitted the data before the bleaching points to avoid calculation of FRET from noises. After donor bleaching, both traces approached the baseline because no excitation can occur directly on the acceptor (**Figure 2A**). After acceptor bleaching, the donor intensity increased because fewer reaction pathways dissipate the excitation energy (**Figures 2B,C**).

It was found that the individual ribosomes exhibit different fluctuation, such as in the examples shown in **Figure 2**. These fluctuations are due to the wobbling motion of the tRNAs, which causes distance fluctuations to the L27[17,18]. In **Figure 2**, the dotted lines are the original data, and solid lines are the fitted data from the program. The fitted traces do not change the raw data reading but truncate the time-lapse traces after bleaching. The blue traces are the calculated FRET efficiencies. By tracking the exact same ribosomes after 5 min incubation at RT, it was found that one type of dynamics can switch to another[23]. Because these signals report on tRNA motions dictated by the surrounding ribosome, similar FRET efficiencies were grouped into various ribosome subpopulations.

Figure 3 shows very diverse subpopulations in the PRE complex. There are approximately 70% (640/951) of fluctuating species and 30% (311/951) of non-fluctuating species. These two categories can be further grouped into finer subpopulations. On the other hand, **Figure 4** shows the opposite dynamics distribution. In POST, 65% of subpopulations are non-fluctuating, and the majority of them exhibited high FRET efficiency. These results indicate that the ribosome is more flexible in the PRE state than the POST state, which corroborates a cryo-EM study that concluded that the peptidyl chain at the P-site locks the ribosome dynamics in the POST state. However, in the PRE state, the peptidyl chain is transferred to the A-site, unlocking the ribosome and promoting[5]. The results supported the structure study conclusion under more physiological conditions. The unlocked state is correlated with the ratcheting motion between the 30S and 50S, and the locked state is correlated with the un-ratcheted conformation.

The subpopulation sorting method reveals the ribosome conformation upon inhibition by the antibiotic viomycin, as shown in **Figure 5**[14]. The overall FRET efficiency histogram shows a major peak at 0.47, the classic state, without sorting. In this state, the ribosome is locked and un-ratcheted. However, sorting the subpopulations has revealed that 60% of the population is fluctuating as the unlocked PRE complex. Therefore, viomycin has trapped the ribosome at the ratcheted state. The results of this study contradicted an x-ray structure result at the time of publishing (2010) but were supported recently by another structure study (2020).

FIGURE AND TABLE LEGENDS:

Figure 1: The labeling position of the Cy3/Cy5 on the ribosome and the tRNA. The distances between the labeling residue of L27 to the A- and P-site tRNA are shown (the red and blue stars show the approximate labeling locations of the Cy5 and Cy3, respectively). One representative time-lapse trace of fluorescence intensities from the CY3/Cy5 FRET pair is shown. A program detects bleaching points on donor/acceptor traces and truncates the trace before that point. This figure has been modified from Altuntop, M. E. et al.[15].

Figure 2: The typical ribosome traces were classified by their different dynamics. (**A**) A non-fluctuating ribosome trace. (**B**) A fluctuating ribosome trace that only samples FRET values lower than 0.6. (**C**) A fluctuating ribosome trace that samples FRET value higher than 0.6. The legends are shown in the plot. The fluorescence intensities of donor and acceptor are green and magenta, respectively. FRET values are calculated as $I_{acceptor}/(I_{acceptor}+I_{donor})$ and plotted separately in blue. The original data are displayed in dotted lines, and data truncated before the bleaching points are displayed in solid lines. This figure has been modified from Altuntop, M. E. et al.[15].

Figure 3: FRET efficiency histograms of the Pre-complex. The top-tier plots show the FRET histogram of the total ribosomes. The second-tier plots show the FRET histograms of the ribosomes separated into fluctuating (F) and non-fluctuating (NF) groups. The third-tier plots show the FRET histograms of the ribosomes further separated into groups of fluctuations that were above or below a FRET value of 0.6. Similar criteria were applied to the NF ribosomes to separate them into stable FRET states below or above 0.6 (NF-low, NF-high). The fourth-tier plots display the representative traces for each subpopulation. This figure has been modified from Altuntop, M. E. et al.[15].

Figure 4: FRET efficiency histograms of POST-Complex. The arrangement and grouping are similar to Figure 3. Contrary to Figure 3, the NF-High population is the majority. This figure has been modified from Altuntop, M. E. et al.[15].

Figure 5: Histograms of the PRE complex in the presence of 100 µM viomycin. The arrangement and grouping are similar to Figure 3. This figure has been modified from Ly, C. T. et al.[14].

DISCUSSION:

SmFRET is sensitive to background signals. First, it is necessary to coat the sample chamber with 0.05% tween and then be added concurrently with the ribosome solution to block non-specific binding of the ribosome to the surface. To see fluorescence from the acceptor Cy5 emission, the

oxygen scavenger cocktail (deoxy, glucose, and Trolox solutions) is essential. Without this solution, the bleaching is too fast in the acceptor channel to obtain useful information. Another critical step for ribosome experiments, specifically, is the PEG coating on the glass surface. The ribosome activity is sensitive to the surface environment; therefore, long brushing polymers are essential to shield unfavorable surface effects.

If the sample stage is too far from the focus, the auto-focusing system will not work. If this happens, turn off the auto-focus and manually adjust the objective position while observing the reflected laser spot. This is one advantage of a home-built total internal reflection illumination because the laser spot is visible. When the objective is near focus, the incident and reflected laser spots should be side by side, and the reflecting spot moves with the adjustment of the objective position. When these two spots are close, the auto-focus will work again.

One limitation of smFRET is the very low concentration range. Only up to 50 nM of fluorescently labeled substrates can be loaded without causing inhibiting background noise. Although surface-bound samples can require long-time acquisition on the same molecule, the time-resolution is limited to the ms range, while diffusion-based confocal smFRET can reach dynamics of the ms range[24]. Another limitation of the FRET method is the precise calculation of the distance from FRET efficiency. Due to different dye linkers and environments, the Forster distance varies from lab to lab. Therefore, comparing absolute distance can be problematic[25]. Although FRET efficiency change reveals the translocation mechanism, a more direct strategy was developed to measure the exact coverage of ribosome on the mRNA[26,27].

Nevertheless, using FRET values as relative references to distinguish inhomogeneous populations within one experimental setting is a powerful method to reveal mechanism and dynamics one molecule at a time, which is not accessible with conventional methods. Furthermore, multi-color FRET and the combination of FRET with an optical trap will reveal more orchestrated ribosome dynamics in the future[28]. These developments are providing unprecedented sensitivity (displacement of Å distance) and new parameters (such as forces of pico-Newton magnitude) that are not achievable with existing methods[28].

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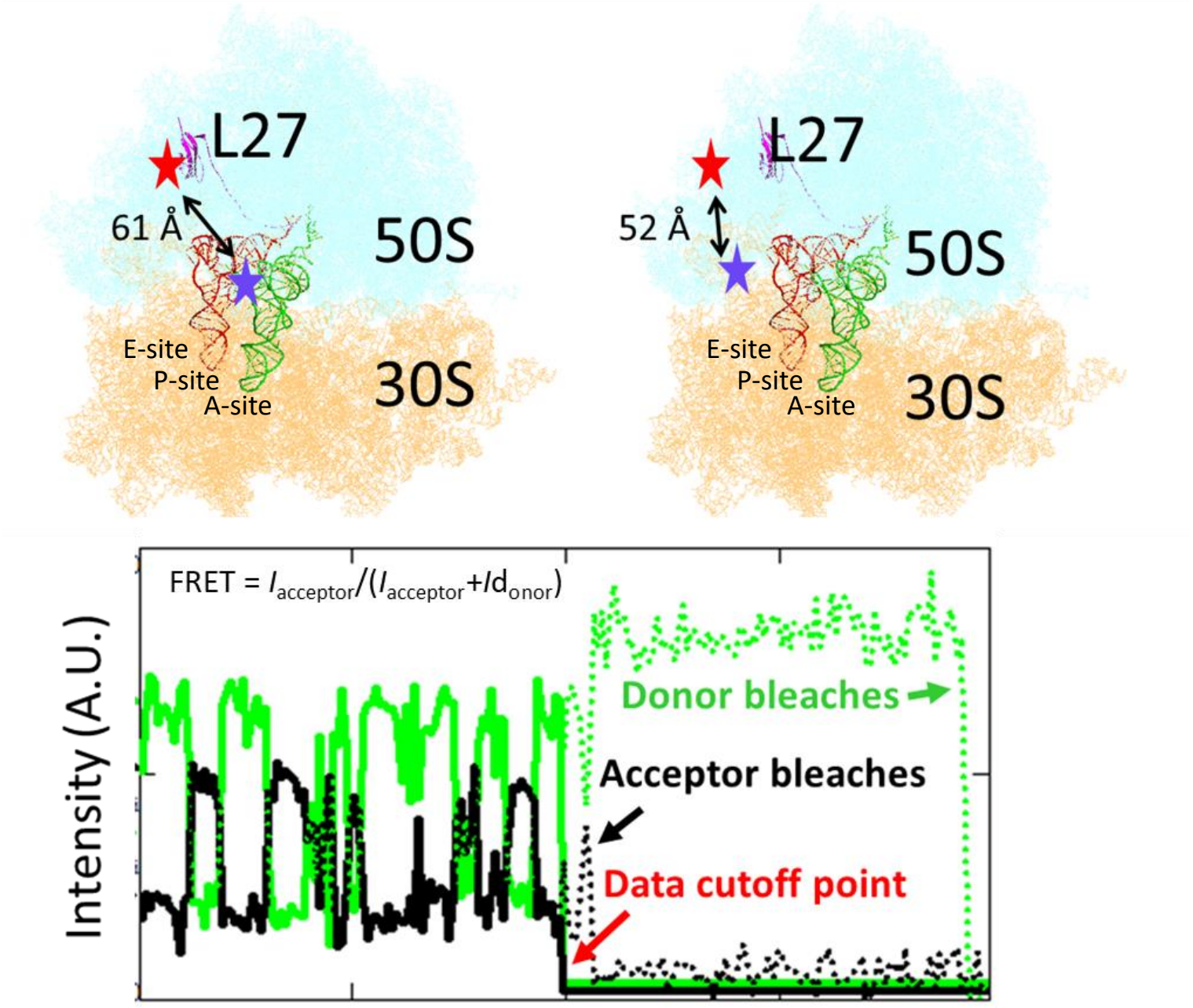
DISCLOSURES:
Y. Wang declares no conflicts of interest.

REFERENCES:
1. Ramakrishnan, V. What we have learned from ribosome structures. *Biochemical Society Transactions*. **36** (Pt 4), 567–574 (2008).
2. Frank, J., Agrawal, R. K. A ratchet-like inter-subunit reorganization of the ribosome during translocation. *Nature*. **406** (6793), 318–322 (2000).
3. Moazed, D., Noller, H. F. Intermediate states in the movement of transfer RNA in the

ribosome. *Nature*. **342** (6246), 142–148 (1989).
4. Schmeing, T. et al. The crystal structure of the ribosome bound to EF-Tu and aminoacyl-tRNA. *Science*. **326** (5953), 688–694 (2009).
5. Valle, M. et al. Locking and unlocking of ribosomal motions. *Cell*. **114** (1), 123–134 (2003).
6. Gromadski, K., B, Rodnina, M. V. Kinetic determinants of high-fidelity tRNA discrimination on the ribosome. *Molecular Cell*. **13** (2), 191–200 (2004).
7. Blanchard, S. et al. tRNA dynamics on the ribosome during translation. *Proceedings of the National Academy of Sciences of the United States of America*. **101** (35), 12893–12898 (2004).
8. Blanchard, S. et al. tRNA selection and kinetic proofreading in translation. *Nature Structural and Molecular Biology*. **11** (10), 1008–1014 (2004).
9. Wang, Y. et al. Single-molecule structural dynamics of ef-g-ribosome interaction during translocation. *Biochemistry*. **46** (38), 10767–10775 (2007).
10. Cornish, P. et al. Following the movement of the L1 stalk between three functional states in single ribosomes. *Proceedings of the National Academy of Sciences of the United States of America*. **106** (8), 2571–2576 (2009).
11. Cornish, P. et al. Spontaneous intersubunit rotation in single ribosomes. *Molecular Cell*. **30** (5), 578–588 (2008).
12. Chen, J. et al. Coordinated conformational and compositional dynamics drive ribosome translocation. *Nature Structural and Molecular Biology*. **20** (6), 718–727 (2013).
13. Feng, X. A., Poyton, M. F., Ha, T. Multicolor single-molecule FRET for DNA and RNA processes. *Current Opinion in Structural Biology*. **70**, 26–33 (2021).
14. Ly, C. T., Altuntop, M. E., Wang, Y. Single-molecule study of viomycin's inhibition mechanism on ribosome translocation. *Biochemistry*. **49** (45), 9732–9738 (2010).
15. Altuntop, M. E., Ly, C., T, Wang, Y. Single-molecule study of ribosome hierarchic dynamics at the peptidyl transferase center. *Biophysical Journal*. **99** (9), 3002–3009 (2010).
16. Wang, Y., Xiao, M., Li, Y. Heterogeneity of single molecule FRET signals reveals multiple active ribosome subpopulations. *Proteins*. **82** (1), 1–9 (2014).
17. Xiao, M., Wang, Y. L27-tRNA interaction revealed by mutagenesis and pH titration. *Biophysical Chemistry*. **167**, 8–15 (2012).
18. Wang, Y., Xiao, M. Role of the ribosomal protein L27 revealed by single-molecule FRET study. *Protein Science*. **21** (11), 1696–1704 (2012).
19. Lin, R., Wang, Y. Automated smFRET microscope for the quantification of label-free DNA oligos. *Biomedical Optics Express*. **10** (2), 682–693 (2019).
20. Moazed, D. et al. Interconversion of active and inactive 30 S ribosomal subunits is accompanied by a conformational change in the decoding region of 16 S rRNA. *Journal of Molecular Biology*. **191** (3), 483–493 (1986).
21. Wower, I. K., Wower, J., Zimmermann, R. A. Ribosomal protein L27 participates in both 50 S subunit assembly and the peptidyl transferase reaction. *Journal of Biological Chemistry*. **273** (31), 19847–19852 (1998).
22. Pan, D., Qin, H., Cooperman, B. S. Synthesis and functional activity of tRNAs labeled with fluorescent hydrazides in the D-loop. *RNA*. **15** (2), 346–354 (2009).
23. Yang, H., Xiao, M., Wang, Y. A novel data-driven algorithm to reveal and track the ribosome heterogeneity in single molecule studies. *Biophysical Chemistry*. **199**, 39–45 (2015).
24. Metskas, L. A., Rhoades, E. Single-molecule FRET of intrinsically disordered proteins.

Annual Review of Physical Chemistry. **71**, 391–414 (2020).
25. Hellenkamp, B. et al. Precision and accuracy of single-molecule FRET measurements-a multi-laboratory benchmark study. *Nature Methods*. **15** (9), 669–676 (2018).
26. Tsai, T. et al. High-Efficiency "-1" and "-2" Ribosomal Frameshiftings Revealed by Force Spectroscopy. *ACS Chemical Biology*. **12** (6), 1629–1635 (2017).
27. Yin, H., Xu, S. & Wang, Y. Dual DNA rulers to study the mechanism of ribosome translocation with single-nucleotide resolution. *Journal of Visualized Experiments: JoVE*. **149**, 59918 (2019).
28. Desai, V. et al. Co-temporal force and fluorescence measurements reveal a ribosomal gear shift mechanism of translation regulation by structured mRNAs. *Molecular Cell*. **75** (5), 1007–1019.e1005 (2019).

Figure



Figure

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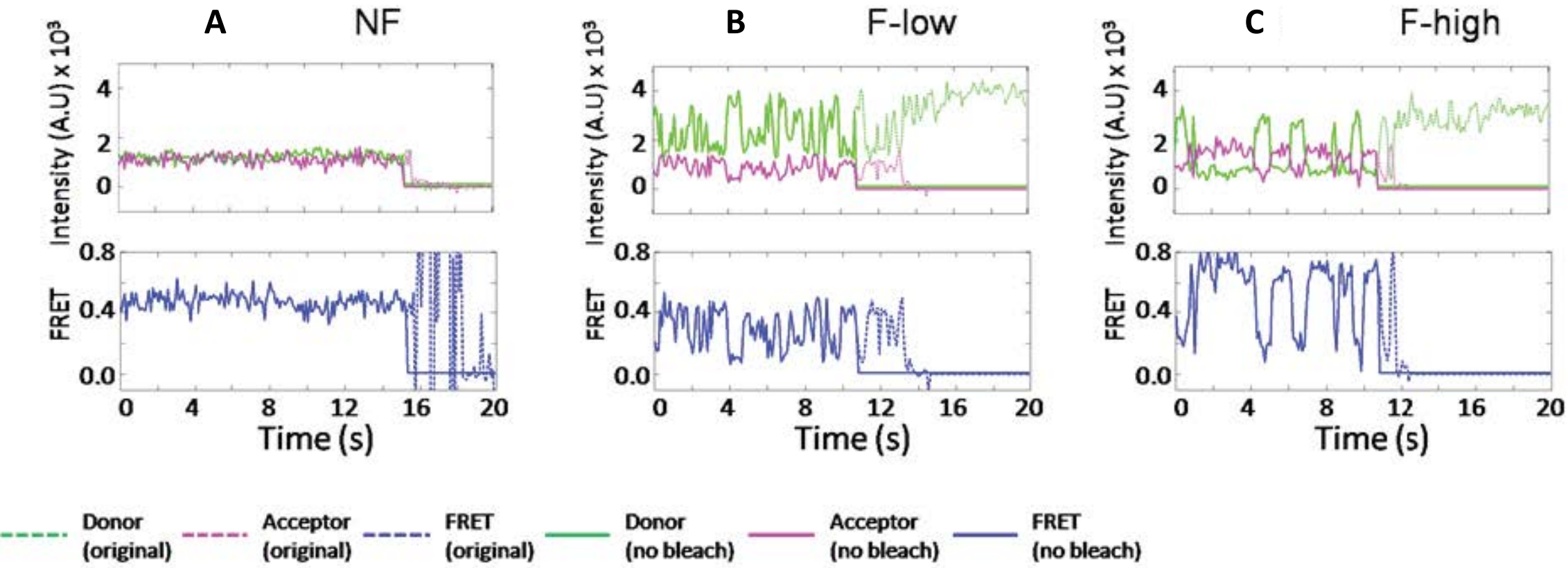

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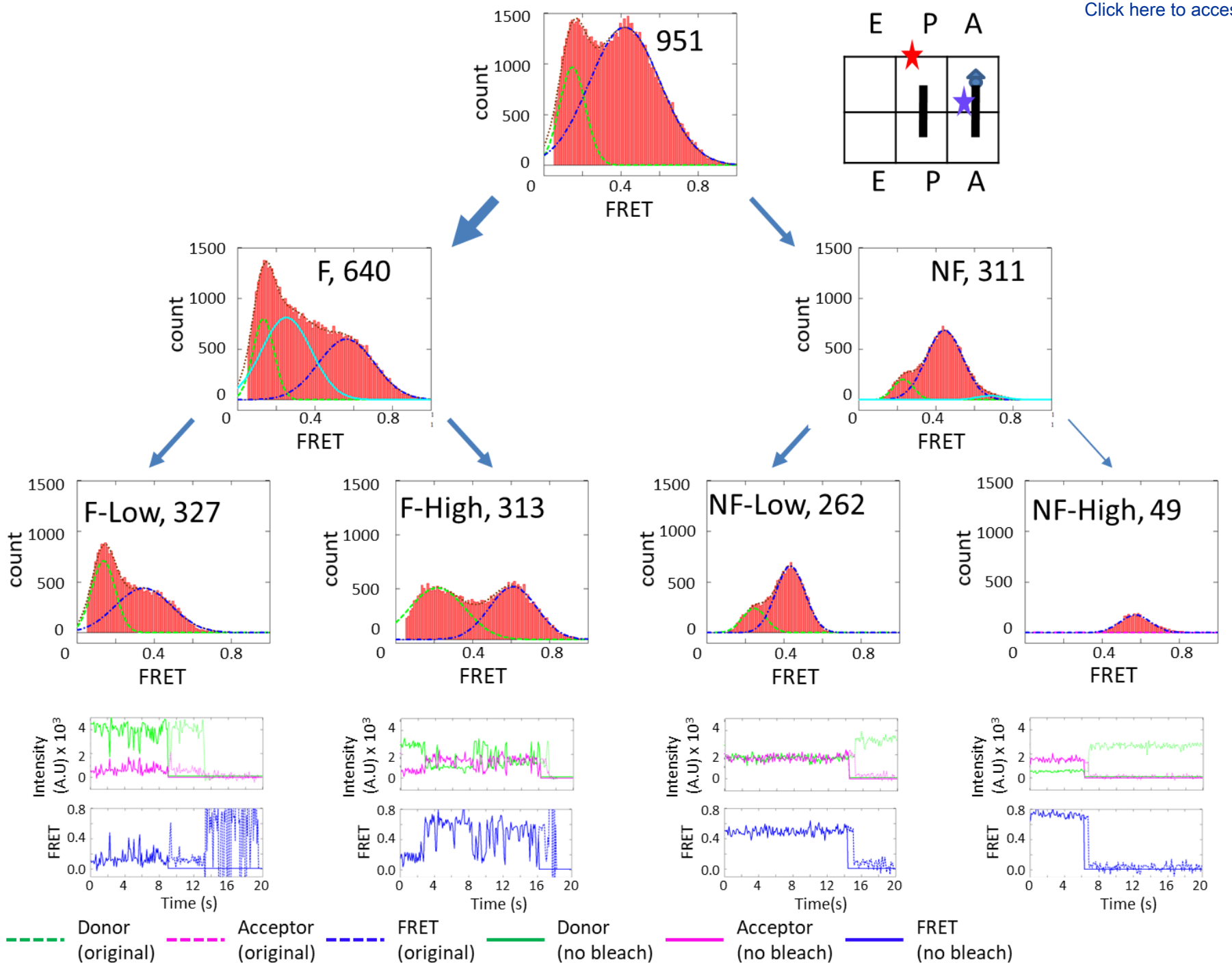

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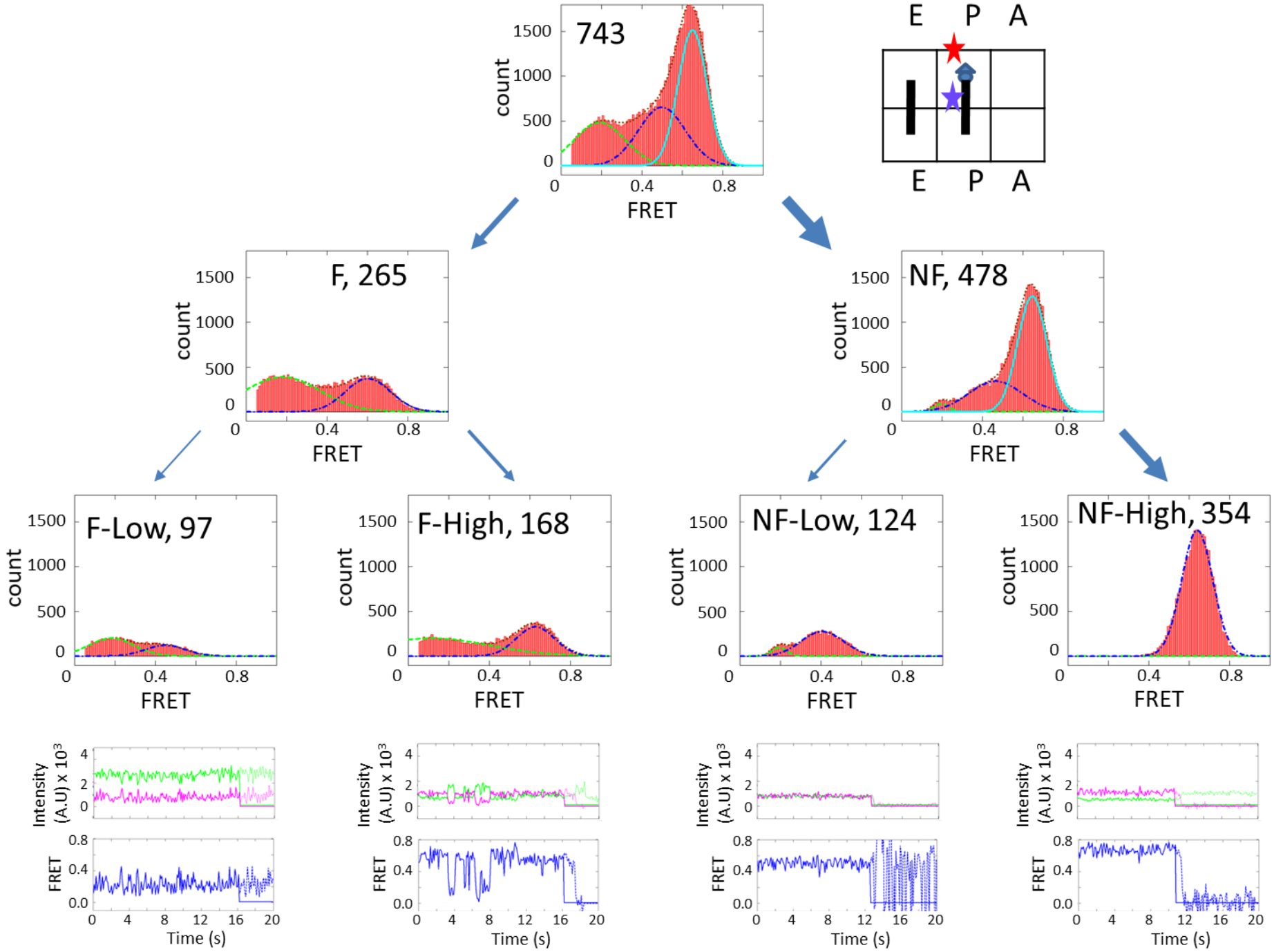
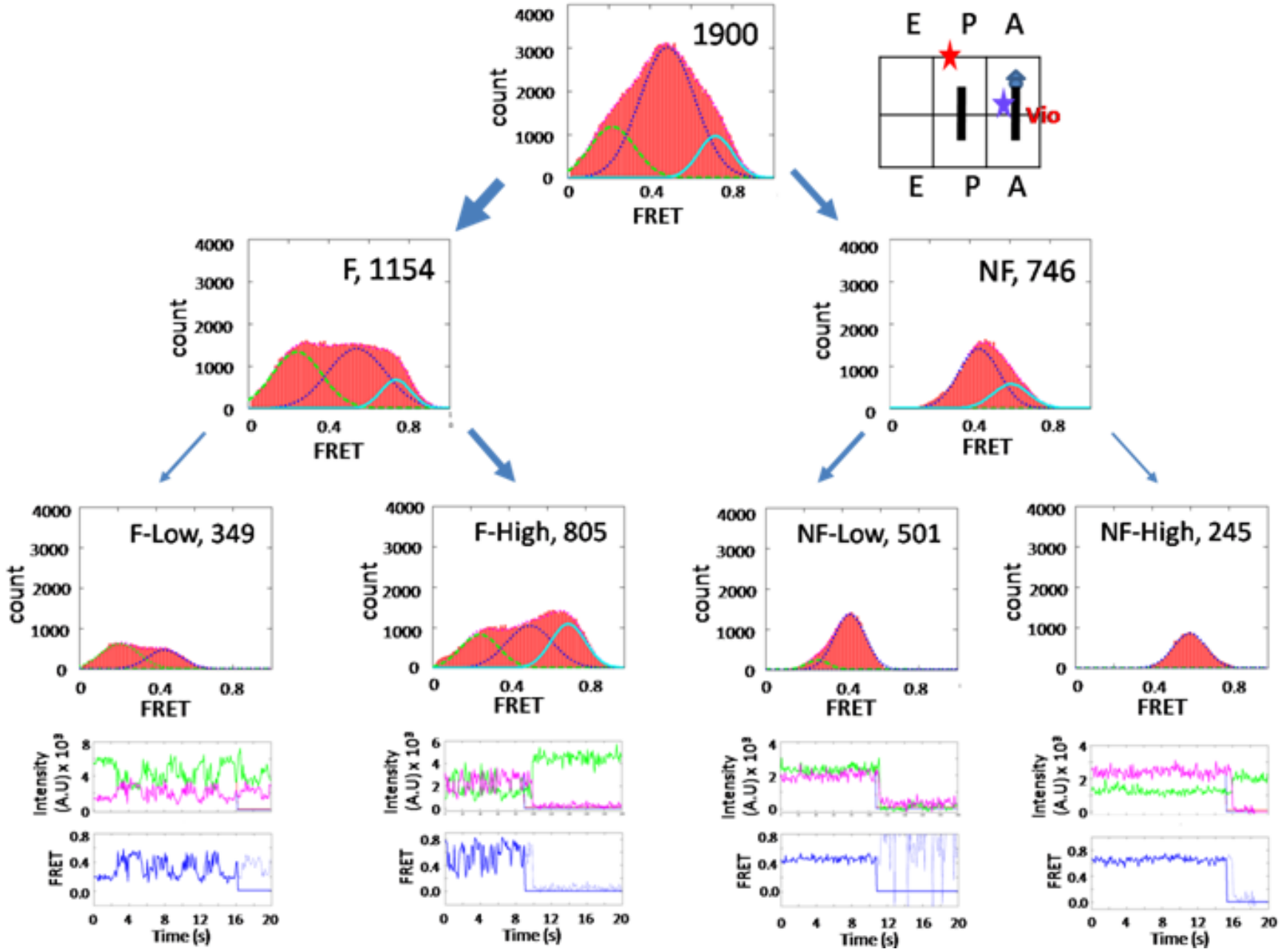


Figure



Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Aminosilane	Laysanbio	MPEG-SIL-5000	
Biotin-PEG	Laysanbio	Biotin-PEG-SVA-5000	
BL21(DE3)pLysS cells	Novagen	71403	
Catalase	millipore sigma	C3515	
CS150FNX Micro Ultracentrifuge	nuaire		
Cy3/C5-maleimide	ApexBio	A8138/A8140	
ECLIPSE Ti2 inverted microscope	Nikon		
EdgeGARD Laminar Flow Hood	Baker		
Glucose oxidase	millipore sigma	G2133	
Histrap HP column (Prepacked sepharose column)	Cytiva	17524701	
Microscope cover slip	VWR	48393-230	
Microscope glass slides	VWR	470235-792	
ORCA-Flash4.0 V3 camera	Hamamatsu		
PEG (5,000)	Laysanbio	MPEG-SVA-5000	
pET-21b (+) plasmid	Novagen	69741	
Sonicator	VWR	CPX-952-518R	
TCEP	Apexbio	B6055	
Trolox	millipore sigma	238813	

Dear Dr. Vineeta Bajaj,

I highly appreciate your comments as well those from the reviewers. I have attached the rebuttal to each point you have raised. Please let me know any more issues you may have. Thank you very much!

13. Please ensure that there are no more than 2-3 actions per step.
Response: This has been fixed throughout the whole protocols.
14. For the imaging/analysis steps please include all button clicks.
Response: This has been added as steps 4.26-4.31.
15. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
Response: Table is removed.
16. There is a 10-page limit for the Protocol, but there is a 3-page limit for filmable content. Please highlight 3 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
Response: Three-pages or less were highlighted for video.
17. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
Response: representative results are in the order of the figures.
18. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. “This figure has been modified from [citation].”
Response: copy right permission from Biophysical Journal is still pending. Permission from biochemistry is obtained and attached. Proper citations were included in figure captions.
19. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.
Response: This is proofread for each figure.
20. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
Response: Paragraph 1.
b) Any modifications and troubleshooting of the technique
Response: Paragraph 2.
c) Any limitations of the technique
Response: Paragraph 3.
d) The significance with respect to existing methods
Response: Paragraph 4.

e) Any future applications of the technique
Response: Paragraph 4.
21. Please do not abbreviate the journal titles in the reference section.
Response: These have been corrected.
22. Please sort the materials table in alphabetical order.
Response: These have been corrected.

Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript outlines methodology for performing single molecule FRET measurements of individual mRNA-bound ribosomes performing protein synthesis .
Major Concerns:
(1) While a detailed and easy to follow protocol describing single molecule FRET studies of ribosome-dependent protein synthesis is a welcome addition to the field, I feel that the article in its current state is not ready for publication. I looked forward to reading about this methodology but unfortunately it appears the article has been assembled in a rush, and has not been properly proofread. There are far too many inexactitudes, misuse of words, lack of indefinite articles and typos through out the text. This leads to a general lack of clarity in the writing that makes it hard for the reader to follow, which is a shame. The lack of proofreading is best exemplified in the opening sentence of the Abstract which refers to "the ribosome is a large ribonucleoprotein complex that ensembles (?) proteins processively along mRNA templates". I assume this author means assembles, but this is not just a one off mistake. The large amounts of errors really interrupt the reader trying to understand the text.
I suggest that the text is re-drafted and properly proofread before it is re-submitted.
Response: The revision is re-drafted and edited by a native English speaker.
(2)The introduction would greatly benefit from a more precise description of protein synthesis in E.coli, such as defining the aminoacyl-site (A-site), peptidyl-site (P-site) and exit-site (Esite) sites or using the correct terminology such as deacylated tRNA rather than 'de-charge?' tRNA or phenylalanyl-tRNA synthetase rather than 'Phenylalanine specific synthetase'.
Response: These suggestions are implemented.
(3) The long methodology list would benefit from greater clarity. Perhaps by underlining and/or using capitals for section titles and having a separate buffer recipe section followed by the methods.
Response: Buffer recipes are attached in the supplementary files session.
(4) Greater emphasis in the Introduction and the Discussion should be given to the range of experimental questions this technique could be applied to, perhaps using different fluorescent donor/acceptor labelling sites etc.
Response: These suggestions were implemented. For example "Different labeling sites have been implemented to probe ribosome dynamics, such as the interactions between tRNA-tRNA, EF-G-L11, L1-tRNA, etc. In addition, by labeling the large and small subunits respectively, inter-subunit ratcheting kinetics and coordination with factors are observed. Meanwhile, smFRET method has broad applications in other central biological processes, and multi-color FRET methods are emerging."
(5)Improve the naming and clarity of Figures and respective legends to better guide the reader through the data. After all, the whole point of this methods paper is for this technique to be

reproduced by other labs.
Response: Figure captions were checked. Additional details were added to Figure 2.
Minor Concerns:
(1) A number of examples of incorrect use of units. Please use correct SI unit convention.
Response: These have been corrected.
(2) E, P and A site should be clearly located on the ribosome structure (panel A) Figure 1 (?). The quality of this could substantially improved.

Response: The labeling of A-, P-, and E-sites were added to Figure 1.
(3) Bacterial strains used in this methodology should be made available to readership (please provide details).
Response: L27-lack ribosome strain was provided in the original submission. The normal ribosome strain of MRE 600 was added in the revision.
Reviewer #2:
Manuscript Summary:
In this manuscript, the author has described a single molecular FRET protocol for tracking the tRNA dynamics during the translation process in vitro. With this method, the author found spontaneous exchange of various ribosome sub-populations in the pre- but not the posttranslocation
complex, thereby drawing a conclusion regarding the flexibility of ribosomes
during different stages of the translation process. The method and its application described in this manuscript is not novel. Overall, the description of the protocol is clear and comprehensive, which allows readers to follow up easily.
Major Concerns:
None
Minor Concerns:
1. Section 1.2.2, please indicate the protein concentration used here.

Response: This was corrected.
2. Section 1.2.3, Line 82, "The protein is eluted first and collected…" reads a bit weird, what does "first" mean here?
Response: we mean the first colored band on the column. This was clarified.
3. Section 2.1, Line 101, "hist-tagged" should be "His-tagged".
Response: This was corrected.
4. Section 4.7, Line 195, "weight" should be "weigh"
Response: This was corrected.
5. Representative Results part, Line 290-291, please add proper citations.
Responses: multiple citations were added in the revision at proper sites.
6. Line 302, a space is missing here.
Response: This was corrected.
7. The arrangement of panels in Figure 2 is strange. Better place panel a, b and c horizontally in a line.
Response: This was rearranged.
8. There are some typos in the manuscript. Please make a proofread carefully.
Response: The revision was extensively re-drafted and edited by native English speaker.
9. Please uniformize the tense and capitalization usage throughout the manuscript.

Response: This was corrected.

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Single-Molecule Study of Viomycin's Inhibition Mechanism on Ribosome Translocation

Author: Cindy T. Ly, Mediha E. Altuntop, Yuhong Wang

Publication: Biochemistry

Publisher: American Chemical Society

Date: Nov 1, 2010

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Single-Molecule Study of Ribosome Hierarchic Dynamics at the Peptidyl Transferase Center

Author: Mediha Esra Altuntop,Cindy Tu Ly,Yuhong Wang
Publication: Biophysical Journal
Publisher: Elsevier
Date: 3 November 2010

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May 24, 2021

Dear Dr. Amit Krishnan,

Thank you for your editing on my submission: JoVE62664R1. I have finished all your suggestions. I also did the following change:

1. Some part of the manuscript lost the symbol font. So I have replaced those “μ” with “micro” to be clear. Other parts the symbol units are fine.

2. I have loaded 6 figures but only discussed 5 of them. So I have removed the original Figure 3, renumbered the other figures to match the text. Sorry for this mistake.

Thank you for your excellent work. I am looking forward to hearing about the next step.

Sincerely,

Yuhong