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.

In compliance with data protection regulations, you may request that we remove your personal registration details at any time. (Remove my information/details). Please contact the publication office if you have any questions.