Review of: "Can DyeCycling break the photobleaching limit in single-molecule FRET?"

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The manuscript, “Can DyeCycling break the photobleaching limit in single-molecule FRET?”, proposed a new labeling scheme, aiming to break the photo-bleaching limit of conventional single-molecule FRET experiments. The temporal bandwidth of single-molecule fluorescent experiments is limited by instrumental time resolution and labeling probe photo-bleaching lifetimes. The latter has forbidden us from using this method to probe critical slow biological dynamic processes. The authors have included a thorough review on the current status of using single-molecule FRET to probe biological macro-molecule conformational dynamics, putting an emphasis on the coverage of temporal bandwidth. The most important contribution of the current report is a proof-of-concept experiment on using a hybridizing DNA handles on a Halliday junction to realize reversible dye binding, where high concentration of unbleached dyes would come in and replenish the bleached and/or dissociated dyes. Under such condition, an intermittent single-molecule time trajectory on the same molecule last for over an hour. The labeling specificity was realized by nucleotide base pairing, to make sure the replaced FRET probes are still in pairs.

However, a number of critical elements for a successful “breaking of current bandwidth limit” are lacking from the current work. First and most importantly, a data analysis scheme for such intermittent characteristic. The author cited reference [44] for a data analysis algorithm which is not as sensitive to the length of the consisting trajectories. However, the SMACK method in Ref. [44] is still susceptible to blinking, which is by nature indistinguishable from bleaching and re-attachment as shown in this work. Using the proposed SMACK or other HMM to analyze the acquired Halliday junction data should be included in this proof-of-concept experiment, or at least on the set of Monte-Carlo simulated data. This can make sure the method is really feasible. Secondly, even if intermittent form of trajectories can be analyzed, a quantitative assessment of probe on and off frequency shall be determined and analyzed for application scheme selection. Standard HMM format of data analysis would inevitably capture the transition rate of this bleaching/dissociation. So, for choosing the suitable labeling strategy, it is significant to quantify the characteristic rates and design the proposed experiment away from such interferences. Finally, a comparison between the DyeCycling scheme and non-bleaching probes like Quantum Dots or nano-diamonds shall be discussed.

A number of interesting slow biological processes has been described at the end of the report. If we can really push the limit for a much wider observation bandwidth, we can make single-molecule fluorescent techniques much more powerful.