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

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Potential competing interests: The author(s) declared that no potential competing interests exist.

Summary:

smFRET (Single-molecule Förster resonance energy transfer) imaging is a powerful technique to observe dynamics or kinetics of biomolecules. But, limited number of detectable photons due to unavoidable photobleach of fluorescent dyes limits measurement length, lowers signal-to-noise ratio, and so on. In this paper, Vermeer *et al.* proposed a new smFRET measurement technique named "DyeCycling", which is aimed to avoid photobleach problem. In DyeCycling, dyes are labeled on the target molecules with weak interaction instead of covalent bonding. It keeps labeled dyes fresh by continually exchanging them with ones in solution.

The same concept is now widely used in PAINT (points accumulation for imaging in nanoscale topography) or similar techniques, which have been often used for superresolution imaging. In that sense, DyeCycling is not very new conceptually and technically. Although it may be new to employ that concept for smFRET imaging experiments, there must be the reason why people didn't try it. Intermittent measurement with undetectable time windows is unfavorable for dynamics measurement because it causes significant chances to miss important moments, like state transitions. In order to compensate for that disadvantage, careful consideration of experimental conditions and analytical methods is required, but it is not described in this manuscript at all. The details are discussed below.

In other points, the manuscript is well organized. The authors considered some different methods and provide options for temporarily labeling dyes. Simulation and experiment seem to be reasonably executed.

In summary, this manuscript may be publishable as an introduction of a new concept of measurement. But, if the authors would like to establish DyeCycling as a useful measurement method seriously, more careful consideration will be required.

Discussion:

As mentioned above, the authors do not show usefulness of DyeCycling sufficiently. Although they mention currently known shortcomings in the section 4.2, there are much more essential points that should be discussed but were not mentioned.

As the authors also discussed, if ergodicity is assumed, intermittent detection is no more advantageous than parallel detection of short trajectories, which can be achieved by common smFRET imaging.

Therefore, as the authors mentioned "ergodicity breaking" many times, DyeCycling may be useful in measurement of non-ergodic dynamics. But if ergodicity cannot be assumed, interpretation of data will be more difficult, making it much more

important to accurately measure FRET trajectories and hence to continuously detect signals. It is one of advantages of the typical smFRET imaging technique to realize such measurement. But, in DyeCycling, it is inevitable to miss events (state transitions) in undetected time periods because both detection intermittency and biomolecular kinetics are stochastic. The readers of this paper cannot be sure if DyeCycling has an ability to correctly infer kinetics or not.
The authors should address the following two points to convince readers of efficiency of DyeCycling method. Although they may not be included in this paper, significance of the paper will be diminished.

1.
In DyeCycling experiment, there are many adjustable parameters, i.e. rates of association/dissociation/photobleach, depending on the labeling method and kinds of dyes. It is practically crucial to choose appropriate parameters. So, the authors should show how to determine the optimum parameter set.
How detectable/undetectable time periods appear highly depends on those parameters. For example, in order to reduce influence of photobleached dyes, the dye-exchanging rate must be much higher than the photobleach rate. But, high exchanging rate will cause frequent undetectable time windows, which increases chance to miss important events. The success of measurements also depends on balance between dyes' rates and kinetics of the sample (number of states, transition paths and rates, and so on). Important events must be efficiently detected within detectable time periods.
The authors can evaluate and should show statistical characteristics of detection and efficiency of inferring kinetics, analytically or by simple simulations. For example, fraction of detectable time in total measurement time, mean length of detectable/undetectable time windows, frequency of switching detectable/undetectable period (should be inverse of mean temporal lengths) can be calculated as functions of 6 rates (association/dissociation/photobleach rates for 2 dyes). The authors can show what they think are requirements of those values for efficient experiments. Especially, the lower bound of detectable fraction must be an important criterion. In addition, by assuming kinetics of the sample (number of states, transition paths and rates, etc), efficiency to detect events (percentage of detected events) can be evaluated. From those values, required number of measurements to correctly infer kinetics will be also estimated. (For example, if it requires millions of hour-long trajectories, it may be practically useless as a measurement technique.)
Based on those results, the authors will be able to provide a guide to find the optimum parameters. When dyes to be used are determined, what kind of kinetics can be correctly observed? If target kinetics (number of states, transitions paths and rates, etc) is assumed, how can dyes be chosen appropriately? (Of course, kinetics of target molecules may be unknown in prior to experiments. It is the purpose of experiment to know it. But, one must determine parameters to conduct experiments.) Can there be any appropriate parameter sets, in the first place?
The authors introduced example targets in the sections 5.1, 5.2 and 5.3. The authors can show what kind of kinetics the authors assume for each of them, and discuss which labeling methods and dyes should be chosen, as examples.

2.
As mentioned above, DyeCycling data necessarily contains missing events due to stochasticity of detection intermittency and of sample kinetics. It is not obvious how to interpret such data. It may become even more difficult when ergodicity is not assumed. The authors have to discuss the principle and specific methods for analyzing such data.

As examples, the authors can demonstrate how transition rates can be estimated from simulated data (Fig. 2) and experimental data (Fig. 4).

Data analysis method, for example, something like the hidden Markov model, may have to be developed. That has to consider undetected events and reproduce the whole kinetics model. Performance of the analysis method can be statistically evaluated using simulated data or simple control experiments.