The Single-Molecule Centroid Localization Algorithm Improves the Accuracy of Fluorescence Binding Assays

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Supporting Information

ABSTRACT: Here, we demonstrate that the use of the single-molecule centroid localization algorithm can improve the accuracy of fluorescence binding assays. Two major artifacts in this type of assay, i.e., nonspecific binding events and optically overlapping receptors, can be detected and corrected during analysis. The effectiveness of our method was confirmed by measuring two weak biomolecular interactions, the interaction between the B1 domain of streptococcal protein G and immunoglobulin G and the interaction between double-stranded DNA and the Cas9−RNA complex with limited sequence matches. This analysis routine requires little modification to common experimental protocols, making it readily applicable to existing data and future experiments.

Biomolecules, such as proteins and nucleic acids, interact with each other inside cells during their various functions. It is often informative to determine the kinetic and equilibrium constants of these interactions. One can perform binding assays to determine these constants.1 When combined with single-molecule detection capability, binding assays can detect the events of single-biomolecule complex formation and dissociation.2,3 In a commonly used single-molecule assay format, one biomolecule of interest is immobilized on an imaging surface while its binding partner that is fluorescently labeled is introduced in solution. Figure 1a shows a representative frame from a single-molecule movie obtained from such an experiment (Video S1). Each fluorescent spot, which may represent an individual complex formed by two biomolecules, can be detected by single-molecule spot detection routines, and its fluorescence intensity can be extracted through a Gaussian-weighted summation method. The centermost pixel has the highest weight (1.00) in the total intensity calculation, while each of the surrounding pixels has a lower weight (<1.00). A typical Gaussian kernel is shown in Figure 1b. For the sake of simplicity, we will, in this paper, call the surface-immobilized molecules “receptors” and their binding partners in solution “ligands”. When this analysis is performed for every frame in a single-molecule movie, an intensity−time trajectory can be generated for each receptor (Figure 1c). From these trajectories, one can determine the dwell times of ligand-bound (high fluorescence) and unbound (low fluorescence) states.

A common artifact, however, in single-molecule fluorescence binding assays comes from nonspecific binding of ligands to the imaging surface. These nonspecific binding events occur randomly and uniformly on the surface unless passivation defects exist, which preferentially attract ligands. Nonspecific binding events can be partly rejected during analysis by including only those events that co-localize with fluorescently labeled receptors through multicolor labeling and imaging.4 However, for conventional wide-field microscopy, the diffraction-limited resolution is much larger than the size of a ligand−receptor complex (lateral resolution of ~260 nm at λon = 620 nm and objective NA = 1.2). Consequently, diffraction-limited co-localization analysis cannot reject nonspecific binding events that occur closer to a receptor than the resolution; the analyzed intensity−time trajectory of that receptor is therefore “contaminated” by these nonspecific binding events. In some severe cases, intensity−time trajectories obtained around passivation defects could contain mainly nonspecific binding events. Effective surface passivation can alleviate the issue of nonspecific binding [see the comparison between the PEG and dichlorodimethylsilane-Tween 20 (DT20) surfaces (Figure S1)] and is typically required in single-molecule fluorescence binding assays.5

In addition to nonspecific binding, insufficient optical resolution can lead to another artifact; i.e., when multiple receptors are located closer together than the resolution, specific binding events at these closely spaced receptors cannot be distinguished. We will define the “minimal detection area” as an area on the imaging surface that has a radius equal to the resolution. Quantitatively, apparent binding rate constant k,app is given by k,app = nk,un + AΨ,un, where n is the number of receptors within a minimal detection area, k,un is the specific binding rate constant of a single receptor, A is the size of a minimal detection area, and Ψ,un is the flux of nonspecific binding events per unit ligand concentration (thus Ψ,un has

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The stochastic nature of binding and dissociation ensures image sparsity for super-resolution imaging. When imaged at a resolution comparable to the size of ligand–receptor complexes, specific binding events at a single receptor appear within a small cluster (full width at half-maximum of ~30 nm), where successive binding and dissociation events happen repeatedly (Figure 1d, inside the circle). Random nonspecific binding events occur in a scattered fashion, mainly outside specific clusters (Figure 1d, outside the circle). Closely spaced receptors can also be resolved (Figure 1e). Single-molecule localization information thus allows us to correct the raw intensity–time trajectories to remove most measurement artifacts (Figure 1f). On the basis of this additional level of information from the single-molecule centroid localization algorithm, we have developed a workflow to extract the corrected intensity–time trajectories for single receptors as illustrated in Figure S2.

To test the effectiveness of the proposed method, we measured the kinetic and equilibrium constants of the interaction between the B1 domain of streptococcal protein G (pGB1) and rabbit immunoglobulin G (IgG). Because of its generally high binding affinities for IgG from many species, protein G has been used in a variety of immunoasays for the detection of antibodies. The full-length protein G (pG) is made of three almost identical domains (B1–B3) linked by peptide linkers. Previous studies have shown that pG has a picomolar affinity for IgG. However, for truncated pG with only two or one of the three repeat domains, their affinities are reduced by 1 or 4 orders of magnitude, respectively. We chose to measure the interaction between IgG and pG with only one repeat domain (pGB1), for it is known to be difficult to measure low-affinity interactions (K_D in the approximately micromolar range) with single-molecule methods.

We immobilized IgG on a DT20 imaging surface through a biotin–neutravidin linker and added pGB1 that was stoichiometrically labeled with Alexa 594 at a single cysteine residue (Methods) in solution (Figure 2a). Long single-molecule movies (30–40 min) were taken so that we could obtain enough binding and dissociation events (12–16 events on average) for each IgG molecule to calculate single-molecule kinetic constants. k_on and k_off were determined from intensity–time trajectories as the reciprocals of the average dwell times of the unbound and bound states, respectively, after removing artifacts based on localization information (Figure 2e and raw trajectories in Figure S3). The left panel of Figure 2b shows the scatter plot of the k_on and k_off values with each point representing an individual IgG molecule. The mean k_on, k_off, and K_D values were 0.73 ± 0.34 μM⁻¹ s⁻¹, 0.62 ± 0.29 s⁻¹, and 0.94 ± 0.45 μM, respectively. Previously measured k_on, k_off, and K_D values ranged from 0.14 to 0.29 μM⁻¹ s⁻¹, from 0.027 to 0.76 s⁻¹, and from 0.091 to 5.3 μM, respectively. All previous studies used Fc fragments of IgG molecules from various sources and clones, and their measurements were performed in ensemble except for those of ref 13. In general, our results are in the same range as those previously measured. The surface density of IgG molecules was 0.75 μm⁻², as determined by the number of detected clusters per imaging area (Figure 2b, right).

A histogram of the fluorescence intensity of single complexes (N = 198) showed a single peak when localization information was used in analysis (Figure 2c, top), suggesting that pGB1 and IgG predominantly formed 1:1 complexes at 10 nM pGB1. The wide distribution of intensity is likely due to the nonuniformity in the illumination pattern. In contrast, an additional low-
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Next, we tested the versatility of our method by measuring the interaction between double-stranded DNA (dsDNA) and the Cas9–RNA complex. The Cas9–RNA complex consists of the endonuclease Cas9 and a guide RNA (gRNA). Cas9–RNA complex binding requires a protospacer-adjacent motif (PAM) on the target strand of dsDNA, and the \( k_{\text{off}} \) of this interaction depends on the length of matched sequences between gRNA and DNA adjacent to PAM. When there is only a short match (<9 bp) adjacent to PAM, Cas9–RNA complex binding is transient.\(^{22}\) We used a DNA that has 4 bp PAM-proximal matches and 16 bp mismatches relative to the gRNA and a catalytically inactive mutant of Cas9.\(^{22}\) The gRNA was biotinylated and immobilized on a PEG imaging surface through a biotin–neutravidin linker, while the DNA labeled with Cy3 was added in solution (Figure 3a). The kinetic and equilibrium constants were determined with the same method described above, and the measured \( k_{\text{off}} \), \( k_{\text{on}} \), and \( K_D \) values were 2.3 ± 1.9 \( \mu \text{M}^{-1} \text{s}^{-1} \), 0.82 ± 0.66 \( \text{s}^{-1} \), and 0.48 ± 0.34 \( \mu \text{M} \), respectively (Figure 3b,c and raw trajectories in Figure S9), in agreement with those previously measured by single-molecule fluorescence resonance energy transfer (FRET) between a Cy3 (donor)-labeled DNA immobilized on the surface and a Cy5 (acceptor)-labeled Cas9–RNA complex in solution (\( k_{\text{off}} = 4-8 \times 10^6 \text{M}^{-1} \text{s}^{-1} \), and \( k_{\text{on}} = 0.8-4 \times 10^7 \text{s}^{-1} \)).\(^{21}\) In comparison, when the same single-molecule movies were analyzed without localization information, the measured \( k_{\text{off}} \), \( k_{\text{on}} \), and \( K_D \) values became 2.4 ± 1.5 \( \mu \text{M}^{-1} \text{s}^{-1} \), 0.91 ± 0.71 \( \text{s}^{-1} \), and 0.42 ± 0.26 \( \mu \text{M} \), respectively. We did not observe an improvement using localization information likely because the receptor density was not high, and the PEG surface was effective in preventing the nonspecific binding of DNA.

In summary, we demonstrated that the accuracy of fluorescence binding assays can be improved by using the single-molecule centroid localization algorithm. The two major artifacts in this type of assay, i.e., nonspecific binding events and optically overlapping receptors, can be detected and corrected during analysis. For instance, apparent binding rate constant \( k_{\text{on,app}} \) of the pGB1–IgG interaction on the DT20 surface is equal to \( n \times 0.73 \mu \text{M}^{-1} \text{s}^{-1} + A \times 0.16 \mu \text{M}^{-2} \mu \text{M}^{-1} \text{s}^{-1} \). With localization information, \( n \) is equal to 1. Also, because the minimal detection area is reduced after data acquisition through the single-molecule centroid localization algorithm, the \( A \Psi_{\text{on,app}} \) term is reduced at least 100 times compared to that of conventional analysis (and this is considering a moderate 10 times higher lateral resolution in the \( x \) and \( y \) directions). Besides, having high densities of receptors allows us to obtain the same amount of data with relatively small EMCCD regions. Therefore, we can image at a higher time resolution with a higher efficiency. This analysis routine requires little modification to common experimental protocols and could be performed synergistically with existing methods (e.g., multicolor co-localization analysis), making it readily applicable to existing data and future experiments.

**ASSOCIATED CONTENT**

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.biochem.7b01293.

Experimental details and supplementary figures (PDF)

Movie 1 (AVI)

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**Author Contributions**

B.H., W.C., and T.H. designed the project. B.H., Y.W., D.S., and J.H.K. performed the experiments. D.S. provided the Cy3-labeled dsDNA and biotinylated Cas9–RNA complexes. J.H.K. provided the Alexa 594-labeled protein G B1 domain sample. B.H., S.P., and K.Y.H. wrote the data analysis software and analyzed the data. B.H., W.C., and T.H. wrote the manuscript.

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Notes
The authors declare no competing financial interest.

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