HIGH ACCURACY MEASUREMENTS OF NANOMETER-SCALE DISTANCES BETWEEN FLUOROPHORES AT THE SINGLE-MOLECULE LEVEL

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Understanding the spatial arrangement of macromolecules is crucial for discerning their molecular mechanisms. By labeling single molecules or complexes at defined sites with a pair of chromatically distinct fluorescent probes, it is possible to obtain static or dynamic distance information using fluorescence microscopy. Such methods have been described [1, 2] but are not widely used, likely due to implementation difficulties. We developed a reproducible, high-throughput procedure with associated software tools (distributed as μManager plugin and Python scripts) that can be employed on most TIRF microscopes.

Registration is accomplished using multi-colored beads as fiducial markers. Images of many positions containing beads are combined in a high density registration map. Use of a piecewise affine transform results in sub-nanometer registration errors over the whole field of view.

It was previously [1] shown that most distance measurements in the low nanometer range no longer follow a Gaussian distribution but are accurately modeled with a 2D probability density function (P2D). However, using both experimental data and Monte-Carlo simulations, we found that small changes in fitted variance lead to large changes in distance using the P2D fit. We overcame this problem by incorporating additional information, including knowledge of localization and registration errors, and are able to determine small distances with nanometer accuracy. To ensure high-throughput and consistent measurements we implemented all analysis tools as a μManager plugin.

The accuracy of our method was confirmed by two-color measurement of the head-to-head distance of rigor-bound homo-dimeric kinesin-1. We found 8.5 ± 0.3 nm, similar to the EM measured distance of 8.2 nm. Experimental results showed that many samples are highly heterogeneous. For instance, coiled-coil 1 of the Bicaudal-D protein had an apparent length of 19.8nm ± 15.9nm, contrasting with a length of 32nm predicted based on coiled-coil size. This difference is likely caused by bending of the protein. In contrast, we found coiled-coil 3 of Bicaudal-D to be 11.7 nm ± 8.2nm (coiled-coil length predicted to be 12nm), where the variation can be explained by flexibility of the labeling tags (HALO and SNAP). In summary, our methods facilitate high-throughput, nm-precision distance measurements of fluorescent molecules.

[1] Stirling Churchman, L., H. Flyvbjerg, and J.A. Spudich. 2006. A Non-Gaussian Distribution Quantifies Distances Measured with Fluorescence Localization Techniques. *Biophysical Journal*. 90:668–671.
[2] Pertsinidis, A., Y. Zhang, and S. Chu. 2010. Subnanometre single-molecule localization, registration and distance measurements. *Nature*. 466:647–651.