Improved Analysis for Determining Diffusion Coefficients from Short Single-Molecule Trajectories with Photoblinking

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**Supporting Information**

The nanocar data presented in the text represent an example of a 2-dimensional diffusing analyte that exhibits short trajectories and photoblinking. The MLE analysis is complicated by the fact that the sample is not monodisperse, and contains both moving and non-moving nanocars.

As an example of a simpler analyte, a similar analysis was performed on freely diffusing fluorescent beads. This analyte is monodisperse, does not photoblink, and trajectories are not short. Instead, the presence of 'off' frames exists due to out-of-focus motion.

*Total Internal Reflectance Fluorescence (TIRF) Wide Field Microscopy Setup*

Data was collected on a home-built TIRF wide field microscope, shown schematically in Figure S1. A 532 nm solid state laser (Coherent, Compass 315M-100SL) was used for excitation. The beam was passed through an acousto-optic modulator (IntraAction, 402AF1) controlled by frequency generators (Fluke, 271-U 115V) in a

master-slave setup to synchronize the excitation at the same frequency as collection rate. The beam was expanded prior to focusing at the edge of a 1.45 NA, 100x, oil-immersion objective (Carl-Zeiss, alpha Plan-Fluar) for through-the-objective TIRF microscopy. The TIRF excitation penetrated a 1/*e* depth of ~85 nm at an intensity of ~2 mW/cm$^2$. Emission was collected in an epi-fluorescence setup and was separated from excitation with a dichroic mirror (Chroma, z532/633rpc) and notch filter (Kaiser, HNPF-532.0-1.0) and further filtered with a bandpass filter (Chroma, ET585/65m). The signal was detected on an electron-multiplied charge coupled device (Andor, iXon 897) at an integration time of 10 ms; no electron multiplying gain was employed.

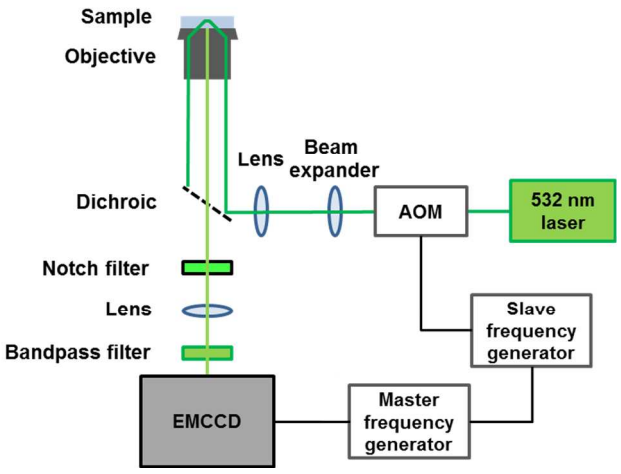


**Figure S1.** Schematic of wide field microscope, including acousto-optic modulator (AOM) and electron-multiplying charge coupled device (EMCCD) detector.

*Sample Preparation*

All solutions were prepared with HyPure Molecular Biology grade water (Thermo Scientific). No. 1 glass coverslips (VWR, 22 x 22 mm) were cleaned in a TL1 solution (4% (v/v) $H_2O_2$ (Fisher Scientific) and 13% (v/v) $NH_4OH$ (EMD Chemicals) at 80 °C for 1.5 min and then plasma cleaned in $O_2$ for 2 min (Harrick Plasma, PDC-32G).

Orange fluorescent FluoSpheres® beads (100 nm, max abs/em: 540/560 nm,

Molecular Probes) were diluted to 1:1000 in solutions of 20% (w/w) methyloxypolyethylene glycol 5000 propionic acid N-succinimidyl ester (Fluka Analytical). Solutions were pipetted into the well of a custom silicon template (Grace Bio-Labs, 43018M) placed on a clean coverslip.

*Experimental Particle Tracking and Analysis*

Acquired data was process using the same MATLAB code as the nanocar data. [Claytor, K.; Khatua, S.; Guerrero, J. M.; Tcherniak, A.; Tour, J. M.; Link, S. *J. Chem. Phys.* **2009**, *130*, 164710.] The out of focus drifting of the beads creates a behavior similar to photoblinking and was therefore modeled in analogy to what has been introduced in the main text. On average, a trajectory has six displacements and a 'photoblinking level' of 0.15. Trajectories were first analyzed by MLE (2). The estimated $\sigma^2$ is at least three orders smaller than $Ddt$, which means we should apply MLE (1) on the trajectories. The distribution of $D$ calculated by MLE (1) is shown in Figure S2. The single Gaussian distribution verified that a monodisperse diffusing analyte exhibiting intermittent 'off' frames, in this case due to motion out-of-focus, can be analyzed by our proposed method.

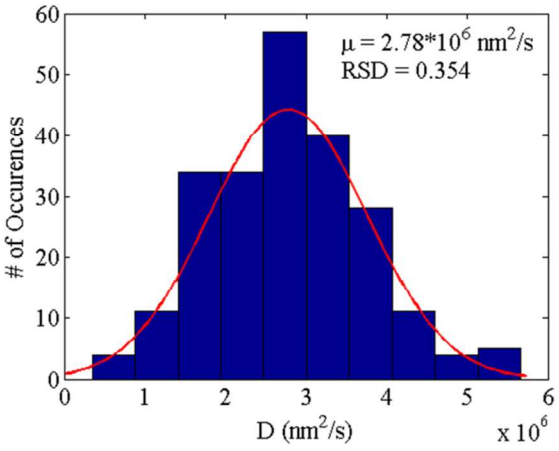


**Figure S2.** The distribution of $D$ obtained by applying MLE (1) to trajectories of

FluoSpheres® . The red line is the Gaussian fitting of the histogram. The mean value of *D* (μ) and the relative standard deviation (RSD) are shown in the figure.