Supplementary data

Video-rate confocal microscopy for single-molecule imaging in live cells and super-resolution fluorescence imaging

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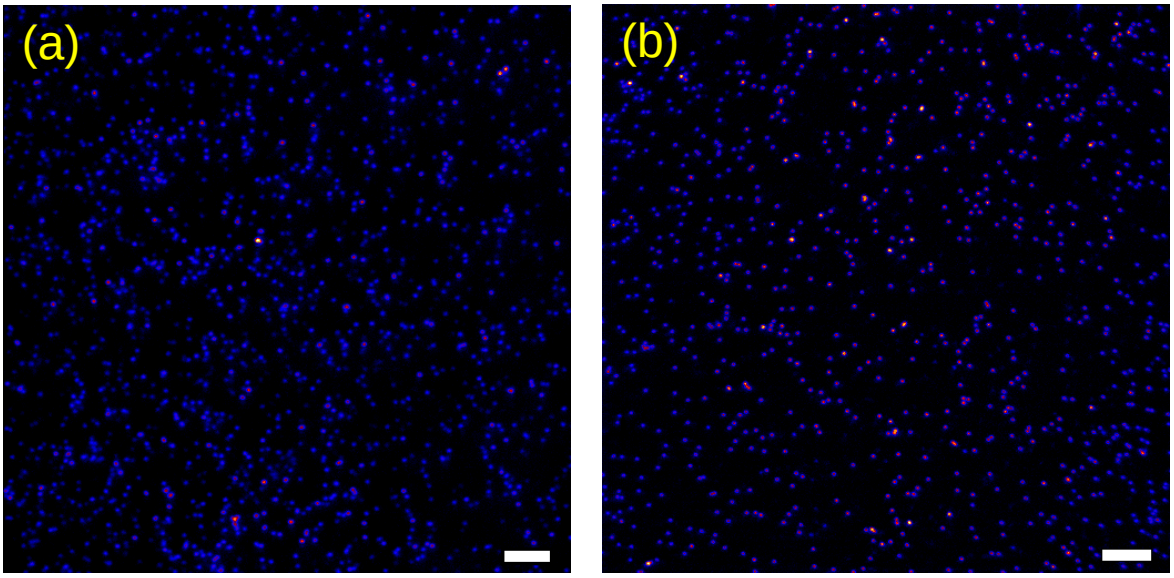
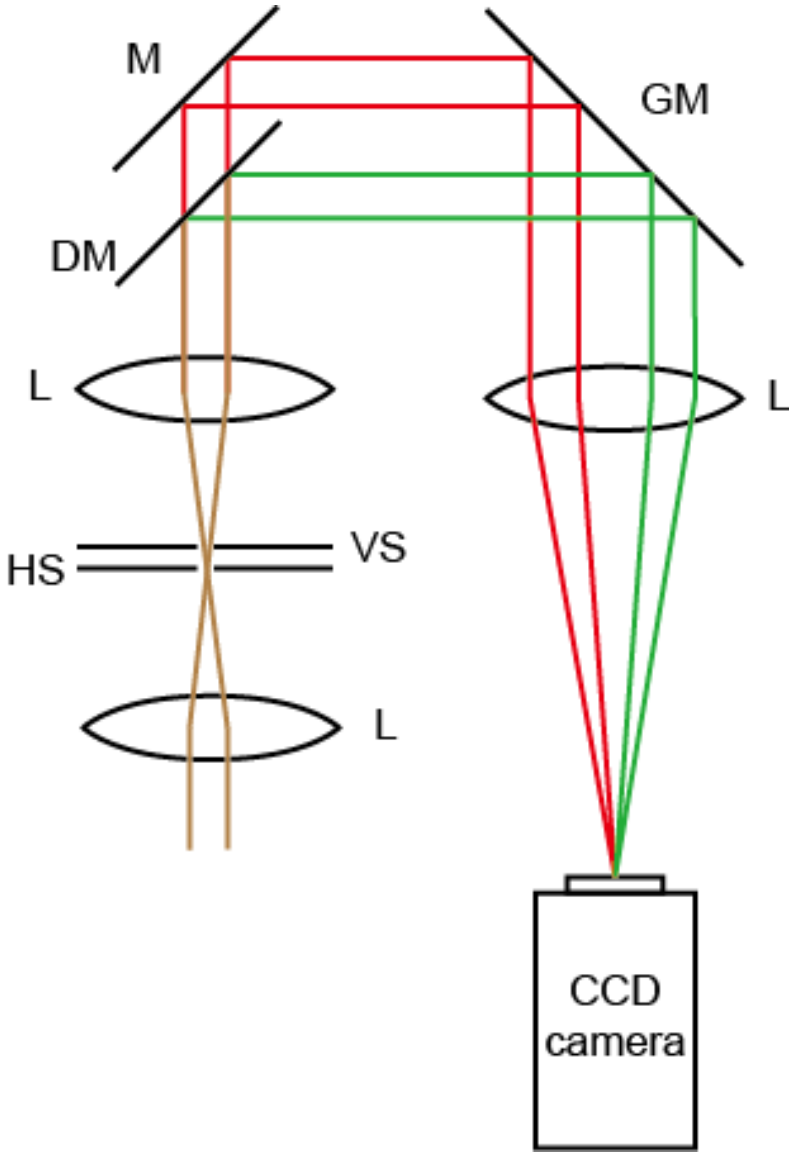
Content:

Fig. S1| Comparison of HILO and line-scan confocal microscopes in the absence of free dye



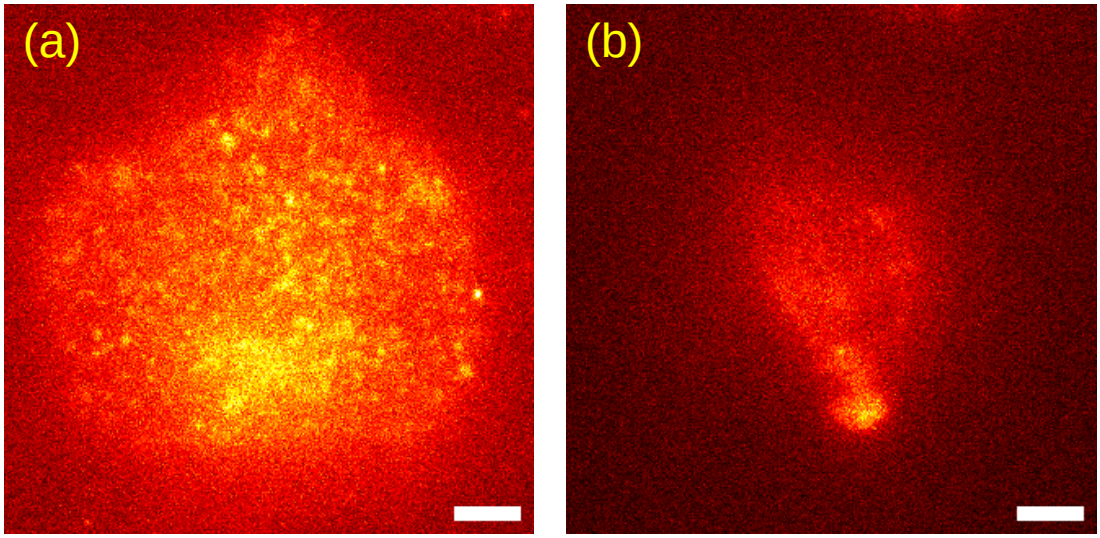
The images of Cy3 molecule immobilized on the surface in the absence of free dye obtained by HILO (a) and line scan confocal microscopy (b). To make the images, 10 frames with 100-ms exposure time were averaged and background fluorescence was subtracted. Scale bars: 3 um.

Fig. S2 | Schematic diagram of detection part for single-molecule FRET experiment



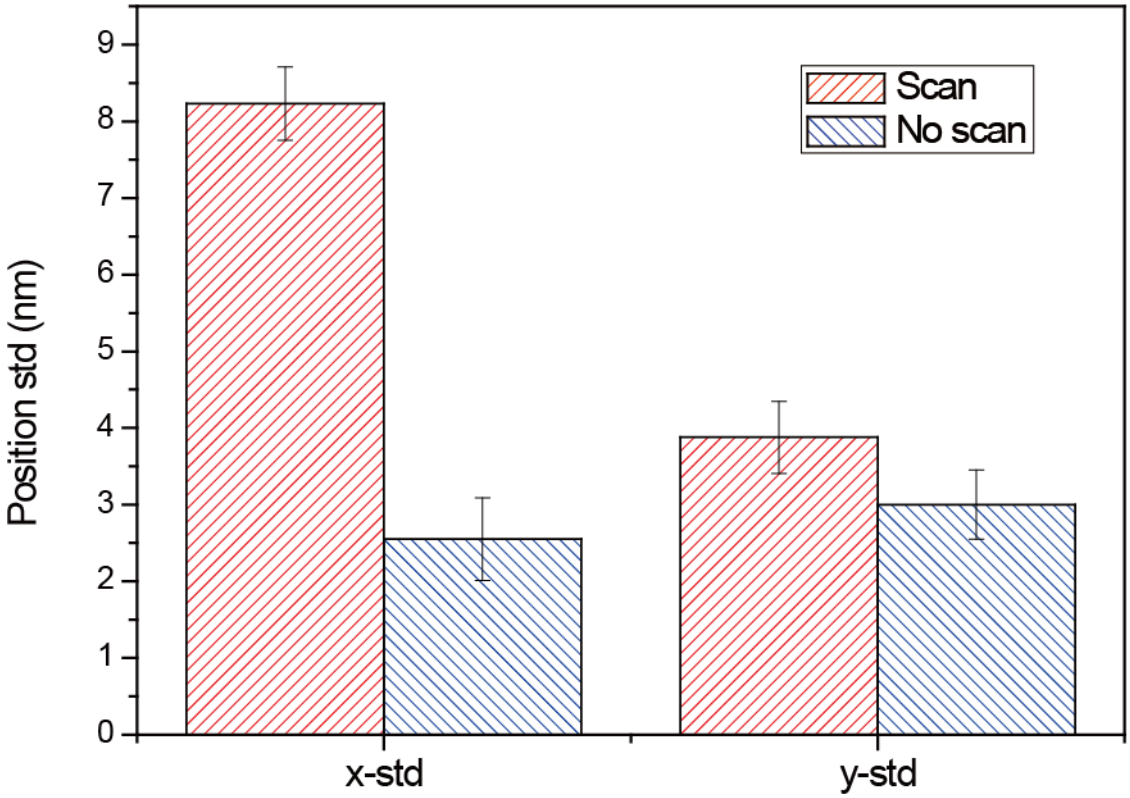
For FRET measurement, fluorescence of Cy3 and Cy5 should be separated. To do this, additional dichroic mirror (DM; 640dcxr, Chroma) was placed in front of mirror (M). Both of Cy3 and Cy5 signal were reflected and scanned by galvanometric scanning mirror (GM) and focused on the CCD camera. To fill with Cy3 and Cy5 signal on the CCD camera, the width of fluorescence signal was reduced to half of camera imaging area by adjustable vertical slit (VS; M-SV-0.5, Newport) placed just after the horizontal slit (HS; for rejection of background). Green line : Cy3; Red line : Cy5, Brown line : combined fluorescence.

Fig. S3| Imaging of *D discoideum* cAMP receptors via HILO microscopy



Images at the basal (a) and apical (b) surfaces obtained via HILO microscopy. Exposure time : 50 ms. Scale bars: 3 μm.

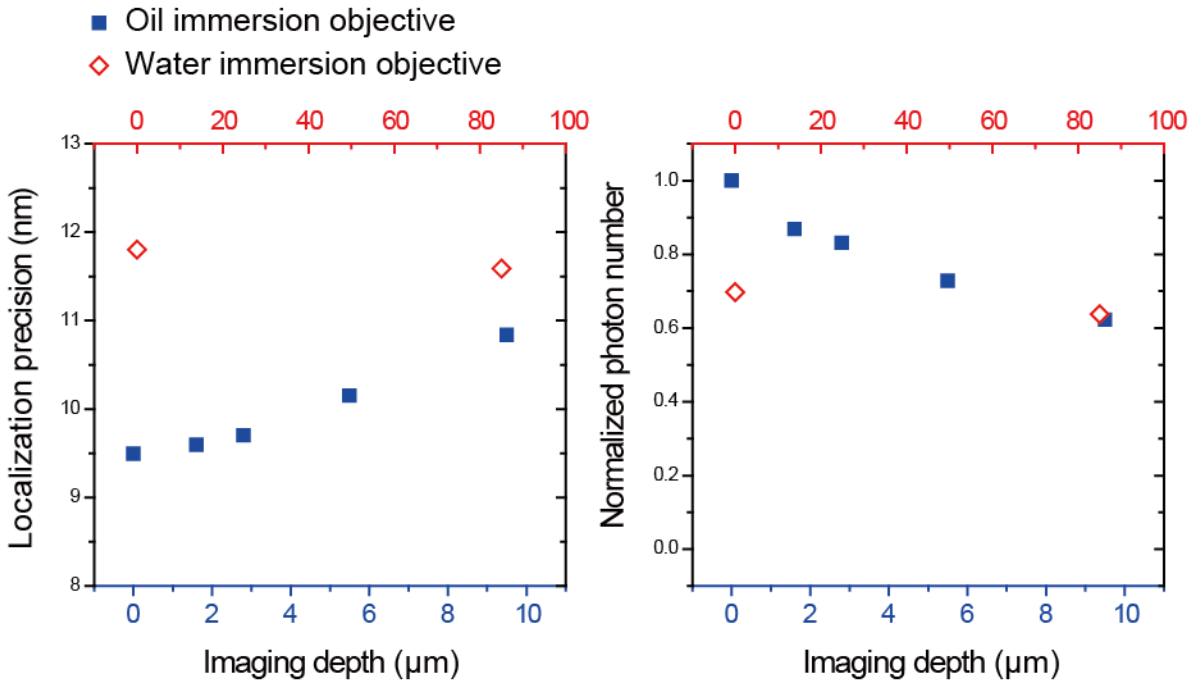
Fig. S4| Comparison of the localization errors of line-scan and TIRF microscopies



Fluorescent beads with 200-nm diameter were immobilized on a coverslip, and imaged via either line-scan confocal microscopy or TIRF microscopy (exposure time: 50 ms, confocal slit width: 40 μm). Five beads in a movie were localized during 800 frames, resulting in five position clusters. Each position cluster was fitted to 2-D Gaussian function, and resulting standard deviations from each fitting were averaged. Resulting graph was obtained from five movies. The localization error in the scanning direction (x-axis, 8.2 nm) was larger than that of TIRF microscopy (2.6 nm). The localization error in the axis vertical to the scanning direction (y-axis, 3.9 nm) was similar to that of TIRF microscopy (3.0 nm). By assuming that the total localization error was independently contributed by the scanning instability and

other factors ($\sigma_{tot} = \sqrt{\sigma_{OTIR}^2 + \sigma_{scan}^2}$), we estimated that the localization error was increased due to scanning by 7.8 nm in the scanning direction, and 2.5 nm in the non-scanning direction, respectively.

Fig. S5| Number of detected photons and localization errors at varying imaging depth



The localization precision, σ_{tot}, was obtained by adding the contributions of the standard localization error, σ, and the localization error due to scanning— $\sigma_{scan,x}$, and $\sigma_{scan,y}$–as follows.

$$\sigma_{tot} = \sqrt{\sigma^2 + \sigma_{scan,x}{}^2 + \sigma_{scan,y}{}^2}$$

The standard localization error, σ, is given by the following equation(1)

$$\sigma = \sqrt{(s^2 + a^2/12)/N + 8\pi s^4 b^2/a^2 N^2}$$

, where N = number of photons, S = standard deviation of point spread function, a = pixel size, b = background noise. For the estimation, we used N and S averaged from several hundred thousand molecules.

Supplementary Movie 1 | Diffusion motion of TMR-cAMP receptors of *Dictyostelium* at the basal surface imaged via line-scan confocal microscopy (exposure time: 50 ms, scale bar: 3μm)

Supplementary Movie 2 | Diffusion motion of TMR-cAMP receptors of *Dictyostelium* at the apical surface imaged via line-scan confocal microscopy (exposure time: 50 ms, scale bar: 3μm)

Supplementary Reference

31. Thompson, R. E., D. R. Larson, and W. W. Webb. 2002. Precise nanometer localization analysis for individual fluorescent probes. *Biophys J* 82:2775-2783.