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Supplementary Material for:

Heavy water: A Simple Solution to Increasing Brightness of Fluorescence Proteins in Superresolution Imaging

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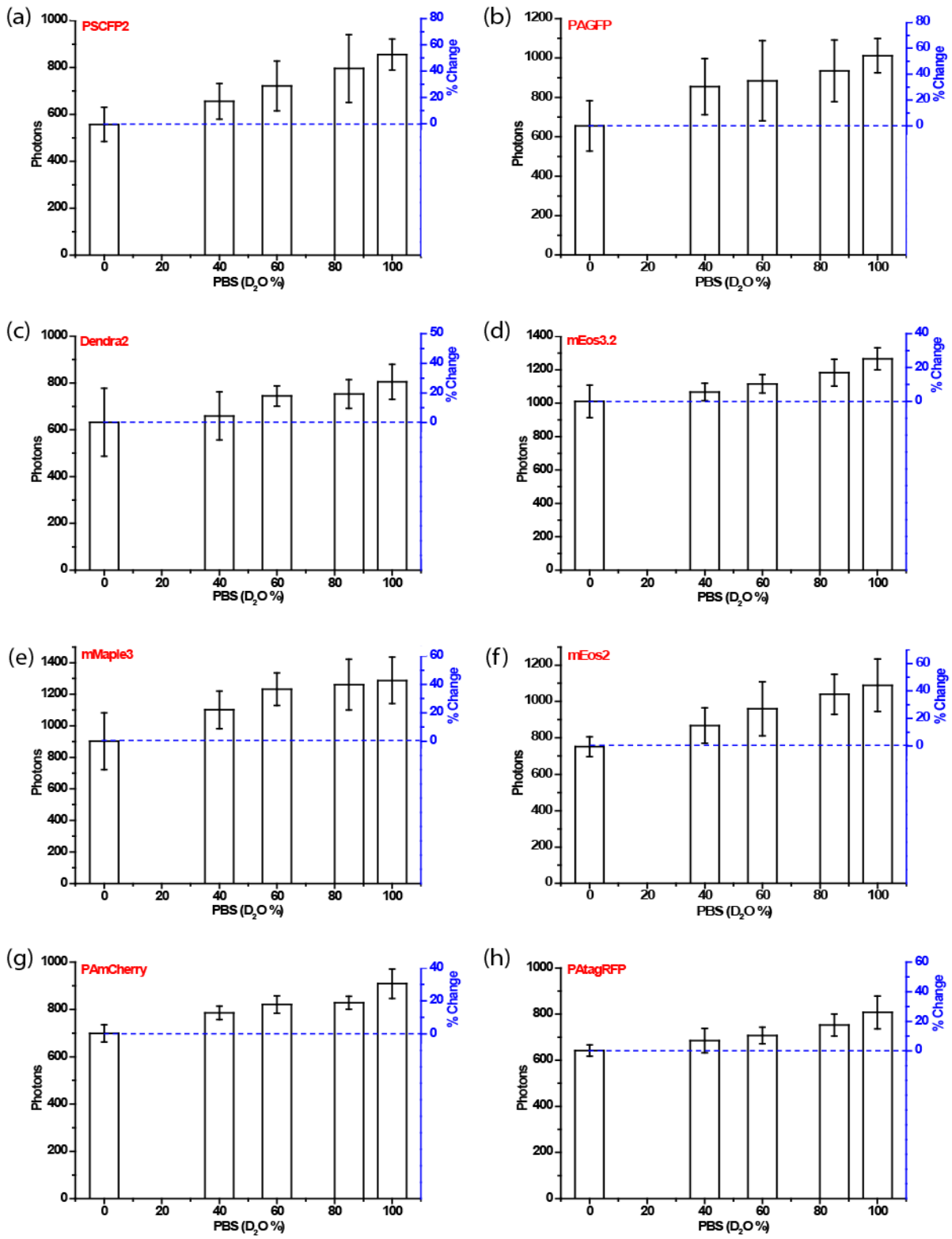
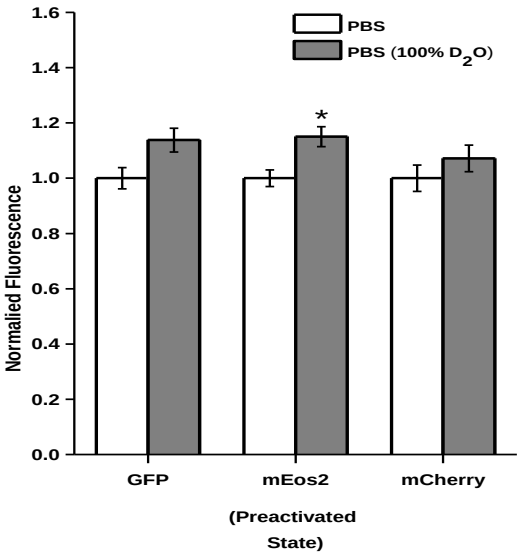
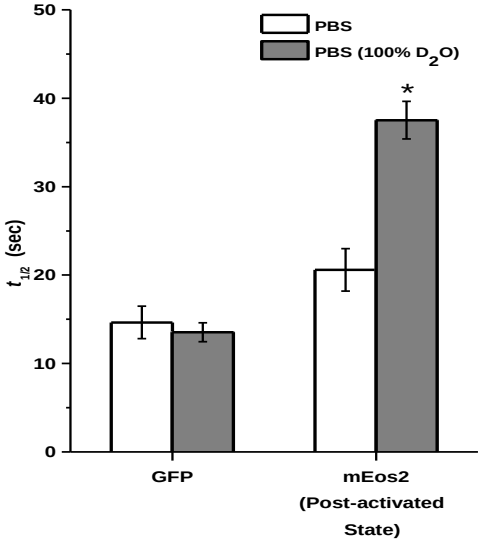
This SI in pdf format contains the following:



Supplementary Figure S1. Photon number of (a) PSCFP2, (b) PAGFP, (c) Dendra2, (d) mEos3.2, (e) mMaple3, (f) mEos2, (g) PAmCherry and (h) PAtagRFP in PBS buffer containing different fractions of D_2O (n = 10). Error bar denotes standard deviation.



Supplementary Figure S2. Normalized conventional fluorescence of GFP, mEos2 (preactivated green state) and mCherry in PBS & PBS (100 % D_2O) (n = 100). Error bars denote s.e.m. The increase of fluorescence intensity is small (< 10%).



Supplementary Figure S3. Photobleaching half-life ($t_{1/2}$) for GFP-tubulin and mEos2-tubulin, expressed in live S2 cells, in PBS & PBS (100 % D_2O). (n = 20). Error bar denotes s.e.m. Student's *t*-test $P < 0.01$.

Material and Methods

I. General

All chemicals were purchased from commercial sources and used without further purification. Deuterium oxide (D_2O) and sodium borohydride ($NaBH_4$) were purchased from Sigma-Aldrich. Paraformaldehyde (16 % solution) and glutaraldehyde (8 % solution) were obtained from Electron Microscopy Sciences (EMS). All oligonucleotides were purchased from Integrated DNA Technologies. The DNA for mMaple3 was synthesized from the published protein sequence, while plasmids for other photoactivatable fluorescent proteins were obtained from labs or companies where they were developed.

II. Cell culture

(a) Chinese Hamster Ovary cells, CHO-K1 cells

CHO-K1 cells were maintained in a 5 % CO_2 atmosphere at 37 ^oC in Dulbecco's Modified Eagle Medium containing phenol red (Gibco), supplemented with 10 % fetal bovine serum (FBS). Cells were passaged every 2–3 days or at ~80 % confluency. All transfections and experiments were performed using cells under 20 passages.

Prior to the transient transfection experiment, the CHO cells were passage and grown in an 8-well chambered cover-glass (Lab-Tek II). Transient transfections were carried out using *Trans*IT-CHO transfection kit (Mirus) following the company's provided protocol with plasmid containing the (PA-FP)-Tubulin. Cells were imaged 30 hrs after transfection.

(b) *Drosophila Melanogaster* Schneider 2 cells, S2 cells

S2 cells were maintained at room temperature in Sf-900 II SFM medium (Gibco), supplemented with 10 % FBS. Cells were passaged every week or at ~80 % confluency. All transfections and experiments were performed using cells under 20 passages.

Transient transfections were performed using Effectene Transfection Reagent (Qiagen) following the company's protocol with plasmid containing either mCherry-Tubulin or GFP-Tubulin in a 6-wells plate (Corning) for overnight. The following day, the transfected cells were seeded on Concanavalin A (Sigma-Aldrich) coated 8-well chambered cover-glass and the cells were allowed to adhere to the coverglass for overnight.

III. Fixation of S2 cell

S2 cells expressing FP-Tubulin were washed once with PBS. The cells were first fixed with 3 % paraformaldehyde + 0.1 % glutaraldehyde in PBS for 10mins, followed by a reduction with 0.1 % $NaBH_4$ in PBS solution for 7 mins. Finally, the cells were washed with PBS thrice.

IV. Imaging & Analysis

(a) STORM imaging

The imaging experiments were performed using a STORM microscope built from a Nikon Eclipse Ti-E inverted microscope. A 405 nm activation laser (OBIS 405, Coherent), 488 nm imaging laser (OBIS 488, Coherent) and a 561 nm imaging laser (Sapphire 561, Coherent) were aligned, expanded, and focused at the back focal plane of the UPlanSApo 1.4 NA 100x oil immersion objective (Olympus). Images were recorded with an electron multiplying CCD camera (iXon+ DU897E-C20-BV, Andor). The OBIS lasers were controlled directly by the computer whereas the Sapphire 561 nm laser was shuttered using an acoustic optical modular (Crystal Technology). A quadband dichroic mirror (ZT405/488/561/640rpc, Chroma) and a band-pass filter (ET525/50m, Chroma for 488 nm and ET595/50nm, Chroma for 561 nm) separated the fluorescence emission from the excitation light. The images were recorded at a frame rate of 60 Hz. The typical power for the lasers at the back port of the microscope was 20 mW for the 488 nm imaging laser, 40 mW for the 561 nm imaging laser and 1-500 μW for the 405 nm activation laser. During image acquisition, the axial drift of the microscope state was stabilized by a home-built focus stabilization system utilizing the reflection of an IR laser off the sample. Both data acquisition and analysis were performed using custom-written software.[1]

The localization precision of each localization was estimated based on a previously established formula for the expected variance (Mortensen et al.)[2]:

$$\sigma^2 = \frac{\sigma_a^2}{N}\left(\frac{16}{9} + \frac{8\pi\sigma_a^2 b^2}{Na^2}\right),$$

where $\sigma_a^2 = \sigma_{PSF}^2 + a^2/12$. σ_{PSF}^2 is the variance of the Point Spread Function in a two-dimensional Gaussian approximation, a^2 is the pixel area, N the number of photons and b^2 the expected number of background photons per pixel. More specifically, the localization precision is reported as the Full Width Half Maximum (FWHM), considering excess noise of the Electron Multiplying Charge Coupled Device (EMCCD):

$$LP_{FWHM} = 2\sqrt{2\ln 2}\sqrt{F_{EMCCD}\sigma^2},$$

where $F_{EMCCD} = 2$.

The mean localization precision in an image area was measured by a modified form of pair correlation analysis[3] utilizing the reversible blinking behavior of mEos2.[4] Specifically, we calculated the distances between each localization point and all localization points within the 15 frames following the frame where this point appeared. The *i*'th bin of the distance histogram was then normalized by $2i - 1$ to obtain the pair-correlation function. We fit the pair-correlation by a Gaussian peak centered at zero. The width of the peak reflects the uncertainty to localize the same mEos2 molecule when it is repetitively activated during the 15 frame time window. We note that this measurement may not yield an accurate absolute number for the localization precision, because those molecules not blinked during this time window will not contribute to the correlation peak. Nevertheless, it still provides an experiment-based, objective metric to compare the relative localization precision in the H_2O and D_2O cases.

(b) Wide-field fluorescence imaging

The imaging experiments were performed using an epi-fluorescence microscope built from a Nikon Eclipse Ti-E inverted microscope with a perfect-focusing system (PFS). Excitation light was provided by an X-Cite-XLED1 fluorescence illuminator (Lumen Dynamics) with emission filters around 485 nm (FF02-485/20) and 560 nm (FF01-560/25, Semrock), and images were recorded with an ORCA-Flash4.0 V2 sCMOS camera (Hamamatsu). A quad-edge dichroic beamsplitter (FF410/504/582/669-Di01, Semrock) was used to separate the fluorescence emission from the excitation light. Additionally, an emission filter (FF01-440/521/607/700, Semrock) was used. Images were recorded at a frame rate of 5 Hz using ~0.8 W/cm^2 of light around 485 nm or ~0.7 W/cm^2 of light around 560 nm. During image acquisition, the axial drift of the microscope state was stabilized by the PFS. The data acquisition was performed using custom-written software and the analysis was performed with ImageJ. For analysis, we used 2×2 pixel binning.

(c) Photobleaching kinetics

The photobleaching experiments were performed on the same set-up that was used for PALM imaging. Cells expressing GFP-tubulin were illuminated with ~13 mW/cm^2 of 488 nm, while cells expressing mEos2-tubulin were first activated with ~7 mW/cm^2 of 405 nm for 20 secs before illuminating with ~13 mW/cm^2 of 561 nm. Images were recorded at 5 Hz. Plot z-axis Profile function in ImageJ was used to trace the fluorescent intensities. An ROI with a 0.7 × 0.7 μm square region on a microtubule was applied and the data of 20 regions for a given experimental condition was analyzed using Origin 8. The half-life ($t_{1/2}$) was calculated using the equation $t_{1/2} = t1 \bullet \ln 2$, where t1 was obtained from fitting the data

curve with ExpDec1 function in Origin. The decay rate (k) was calculated using the equation $k = 1 / t_1$.

Reference:

1. Huang, B.; Wang, W.; Bates, M.; Zhuang, X. *Science* 2008, **319**, 810.
2. Mortensen, K. I.; Churchman, L. S.; Spudich, J. A.; Flyvbjerg, H. *Nat. Methods* 2010, **7**, 377.
3. Sengupta, P.; Jovanovic-Talisman, T.; Skoko, D.; Renz, M.; Veatch, S. L.; Lippincott-Schwartz, J. *Nat. Methods* 2011, **8**, 969.
4. Puchner, E. M.; Walter, J. M.; Kasper, R.; Huang, B.; Lim, W. A. *Proc. Nat. Acad. Soc. USA* 2013, **110**, 16015.