# S2 Text. Details of *in vitro* image comparison

# (1) HaloTag-TMR molecules on glass surface

In vitro Experiment : HaloTag-TMR molecules were provided by Dr. Masahiro Ueda, laboratory for cell signaling dynamics, RIKEN QBiC. Data was taken by Dr. Satomi Matsuoka, laboratory for cell signaling dynamics, RIKEN QBiC. The molecules were distributed on glass surface, and observed using total internal reflection microscopy with 60X/1.40NA objective (Nikon). Fluorescent images of the HaloTag-TMR molecules are acquired with an EMCCD camera (iXon+, Andor). The images were obtained at a 30 msec exposure time.

**Particle model :** We constructed simple model of 100 stationary HaloTag tetramethyl rhodamine (TMR) molecules distributed on glass surface.

**Simulated imaging :** We simulated imaging the basal region of the particle model for the pecification and condition of the TIRFM simulation module shown in Table S2.1.

Beam flux density	$20, 30, 40, 50 \text{ W/cm}^2$
Beam wavelength	488nm
Refraction index	1.33 (glass), 1.27 (water)
Critical angle	$65.6^{\circ}$
Fluorophore	HaloTag TMR ligand (Abs. 555 nm/ Em. 585 nm)
Objective	$\times$ 60 / N.A. 1.40
Dichroic mirror	Semrok FF-562-Di03
Emission filter	Semrok FF-593-25/40
Linear conversion	$10^{-6}$
Tube lens	$\times$ 3.3
Optical magnification	$\times$ 198
Camera type	EMCCD (iXon+ Andor)
Image size	512  imes 512
Pixel size	$16 \ \mu \mathrm{m}$
QE	92~%
EM Gain	$\times 300$
Exposure time	30 msec
Readout noise	100 electrons
Full well	180,000 electrons
Dynamic range	71.1 dB
Excess noise	$\sqrt{2}$
A/D Converter	16-bit
Gain	11.1 electrons/count
Offset	100 counts
Optical background	1.0 photons/pixel

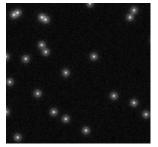
S2 Table S2.1: TIRFM specifications and condition to image the simple particle model of fluorescent molecules.

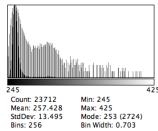
### Expected images

245

#### Simulated images

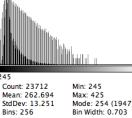
Beam flux density :  $20 \text{ W/cm}^2$ 





Max: 425 Mode: 253 (2724) Bin Width: 0.703







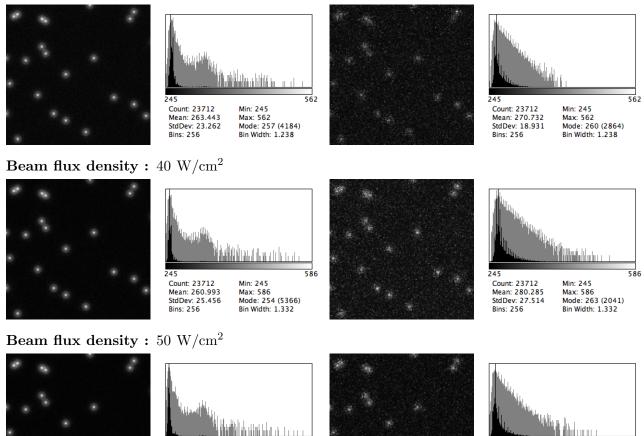
549

Min: 245

Count: 23712

Mean: 268.178

Beam flux density :  $30 \text{ W/cm}^2$ 



Count: 23712 Mean: 257.805 StdDev: 22.905 Bins: 256 Max: 549 Mode: 251 (7291) Bin Width: 1.188 Max: 549 Mode: 257 (2800) Bin Width: 1.188 StdDev: 21.148 Bins: 256 S2 Fig. S2.1: Comparison of *in vitro* images  $(156 \times 152 \text{ pixels})$  and intensity histograms. Log-scaled intensity histograms are shown in grey color.

549

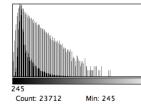
Min: 245

### Simulated images

### Actual images

Beam flux density :  $20 \text{ W/cm}^2$ 





Mean: 262.694 Max: 425 StdDev: 13.251 Mode: 254 (1947) Bins: 256 Bin Width: 0.703

425

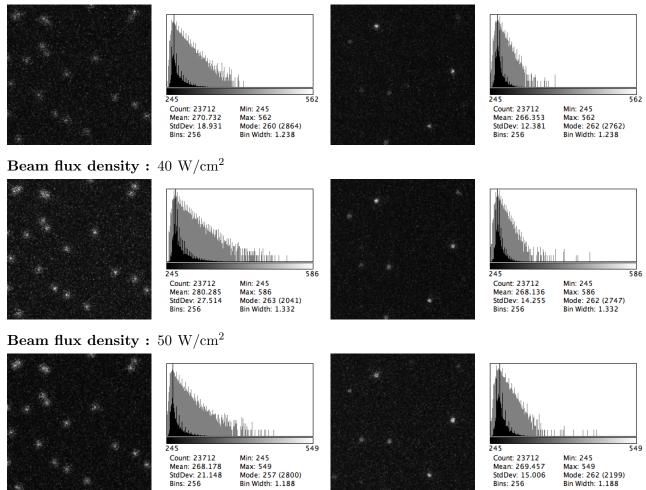


245

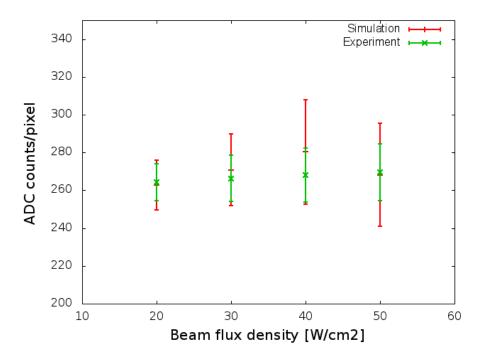
Count: 23712 Mean: 264.224 StdDev: 9.680 Bins: 256

425 Min: 245 Max: 425 Mode: 259 (1737) Bin Width: 0.703

Beam flux density :  $30 \text{ W/cm}^2$ 



S2 Fig. S2.2: Comparison of *in vitro* images  $(156 \times 152 \text{ pixels})$  and intensity histograms. Log-scaled intensity histograms are shown in grey color.



S2 Fig. S2.3: Linearity is shown for various beam flux density. Experiment (green) and simulation (red).

## (2) HaloTag-TMR molecules in aqueous solution

In vitro **Experiment :** HaloTag-TMR molecules were provided by Dr. Masahiro Ueda, laboratory for cell signaling dynamics, RIKEN QBiC. Data was taken by Dr. Satomi Matsuoka, laboratory for cell signaling dynamics, RIKEN QBiC. 5 nM concentration of HaloTag-TMR molecules in aqueous solution were observed using a laser scanning confocal microscope (A1; Nikon, Japan) with 60X/1.40NA objective (Nikon). Images of the HaloTag-TMR molecules were obtained at a time resolution of 1 sec.

**Particle model :** We constructed the particle model of 19,656 HaloTag-TMR molecules fast diffusing with 100  $\mu$ m<sup>2</sup>/sec and distributed in 30 × 30 × 6  $\mu$ m<sup>3</sup> box of aqueous solutions.

**Simulated imaging :** We simulated imaging the middle region of the particle model for the specification and condition of the LSCM simulation module shown in Table S2.2.

Beam flux	5, 10, 30, 50, 100 $\mu W$
Beam wavelength	512 nm
Beam waist	400 nm (Assumed)
Fluorophore	HaloTag TMR ligand (Abs. 555 nm/ Em. 585 nm)
Objective	$\times$ 60 / N.A. 1.49
Scan lens	× 1
Pinhole	57.6 $\mu m$ diameter (2 A.U)
Optical magnification	$\times$ 60
Linear conversion	$10^{-6}$
Scan time	$0.95 \ \mu sec/pixel$
Pixel length	207.16  nm/pixel
Image size	$1024 \times 1024$
PMT mode	Photon-counting
A/D Converter	12-bit
Gain	1.025  electrons/count
Offset	100 counts
Readout noise	0  counts/sec
Excess noise	N/A
Optical background	0 photons

S2 Table S2.2: LSCM specifications and condition to image the simple particle model of fluorescent molecules.

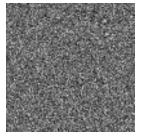
### Expected images

1424

Count: 10000 Mean: 2671.716 StdDev: 379.721 Bins: 256

### Simulated images

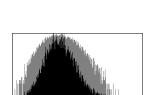
**Beam flux :**  $5 \mu W$ 



Beam flux :  $10 \ \mu W$ 



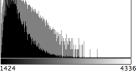
Beam flux :  $30 \ \mu W$ 



Min: 1424 Max: 4336 Mode: 2755 (144) Bin Width: 11.375

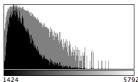
Count: 10000 Mean: 3031.948 StdDev: 534.074 Bins: 256 Min: 1424 Max: 5792 Mode: 2511 (144) Bin Width: 17.062





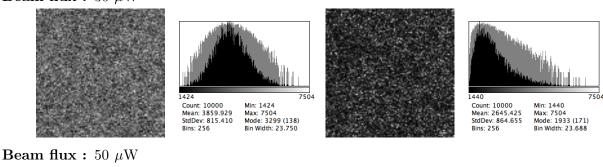
Count: 10000 Mean: 1834.849 StdDev: 308.203 Bins: 256





Count: 10000 Mean: 2125.906 StdDev: 521.600 Bins: 256

Min: 1424 Max: 5792 Mode: 1783 (202) Bin Width: 17.062



1440 Count: 10000 Mean: 2916.609 StdDev: 980.506 Bins: 256 424 857 8576 Count: 10000 Mean: 3971.393 StdDev: 859.793 Bins: 256 Min: 1424 Max: 8576 Mode: 3921 (149) Bin Width: 27.938 Min: 1440 Max: 8576 Mode: 2286 (180) Bin Width: 27.875

Beam flux : 100  $\mu W$ 9936 9936 1424 440 Count: 10000 Mean: 3031.219 StdDev: 1017.047 Bins: 256 Count: 10000 Mean: 4852.043 StdDev: 1103.834 Min: 1424 Max: 9936 Mode: 4829 (145) Bin Width: 33.250 Min: 1440 Max: 9936 Mode: 2001 (201) Bin Width: 33.188 Bins: 256

S2 Fig. S2.4: Comparison of *in vitro* images  $(100 \times 100 \text{ pixels})$  and intensity histograms. Log-scaled intensity histograms are shown in grey color. The PMT dark current has not been simulated yet.

### Simulated images

#### Actual images

424

Count: 10000 Mean: 1786.453 StdDev: 344.051 Bins: 256

iliinidad o

Min: 1424 Max: 4336 Mode: 1584 (1317) Bin Width: 11.375

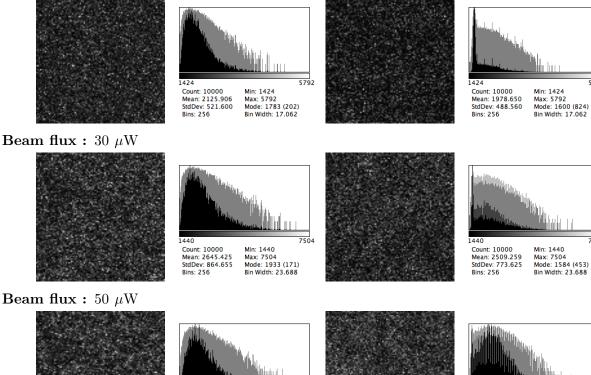
4336

5792

7504

Beam flux :  $5 \mu W$ Min: 1424 Max: 4336 Mode: 1740 (224) Bin Width: 11.375 Count: 10000 Mean: 1834.849 StdDev: 308.203 Bins: 256 Beam flux :  $10 \ \mu W$ 1424

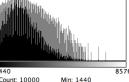
Beam flux :  $30 \ \mu W$ 



4336

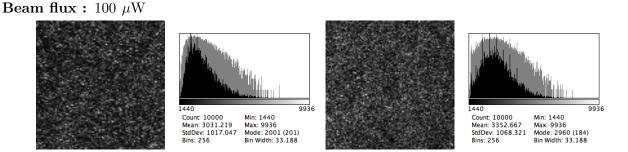
857 Count: 10000 Mean: 2916.609 StdDev: 980.506 Bins: 256 Min: 1440 Max: 8576 Mode: 2286 (180) Bin Width: 27.875

40

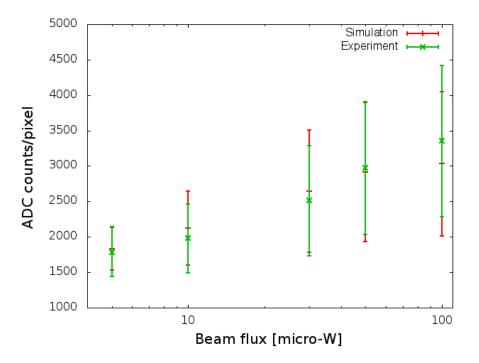


1440 Count: 10000 Mean: 2970.752 StdDev: 941.219 Bins: 256

Min: 1440 Max: 8576 Mode: 2480 (167) Bin Width: 27.875



S2 Fig. S2.5: Comparison of *in vitro* images  $(100 \times 100 \text{ pixels})$  and intensity histograms. Log-scaled intensity histograms are shown in grey color. The PMT dark current has not been simulated yet.



S2 Fig. S2.6: Linearity is shown for various beam flux. Experiment (green) and simulation (red).