Supporting Information

Improved chemical-genetic fluorescent markers for live cell microscopy

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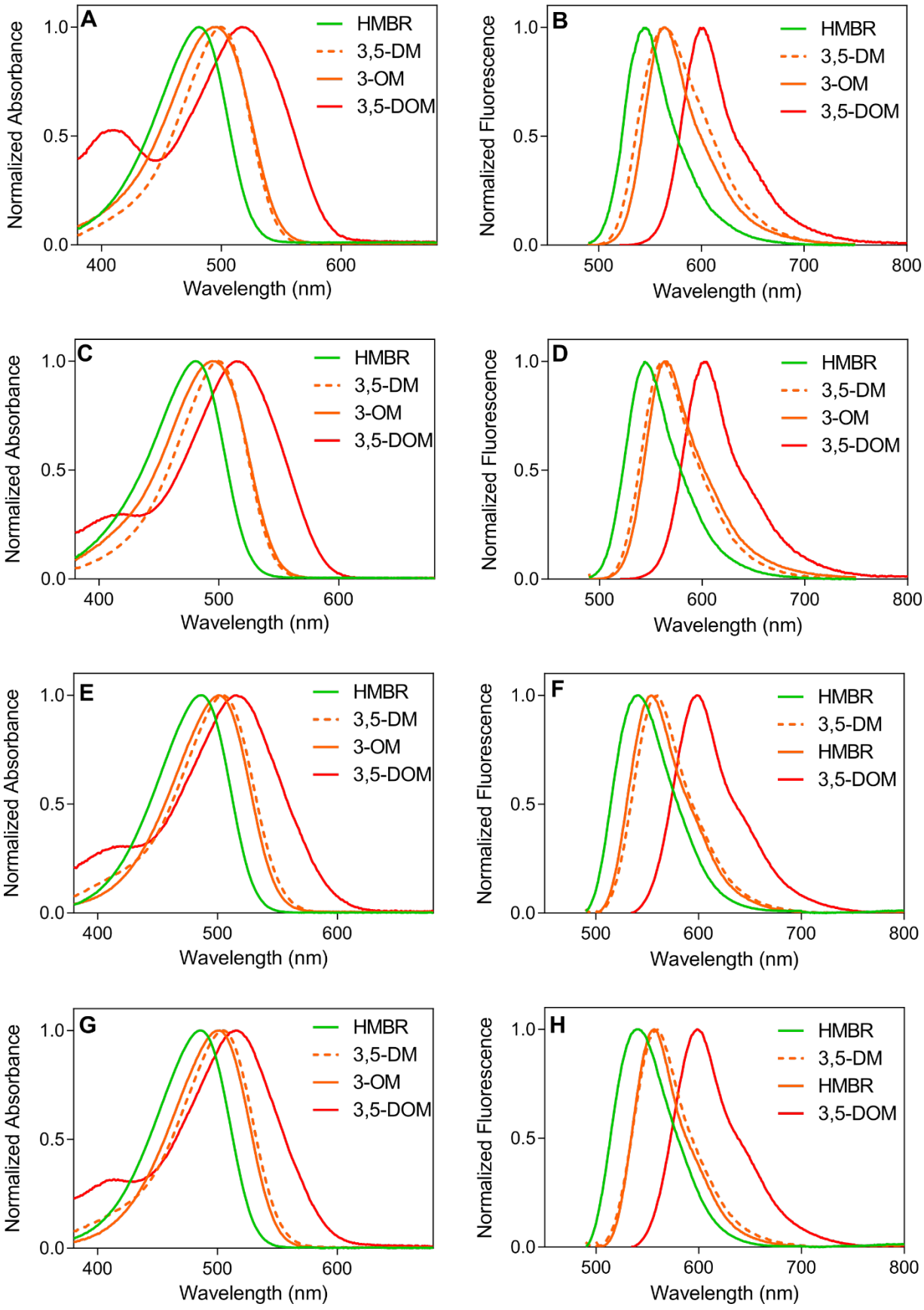
Content


A
B
C
D
E
F
G
H
HMBR
3,5-DM
3-OM
3,5-DOM
Normalized Absorbance
Normalized Fluorescence
Wavelength (nm)
0.0
0.5
1.0
400
500
600
700
800

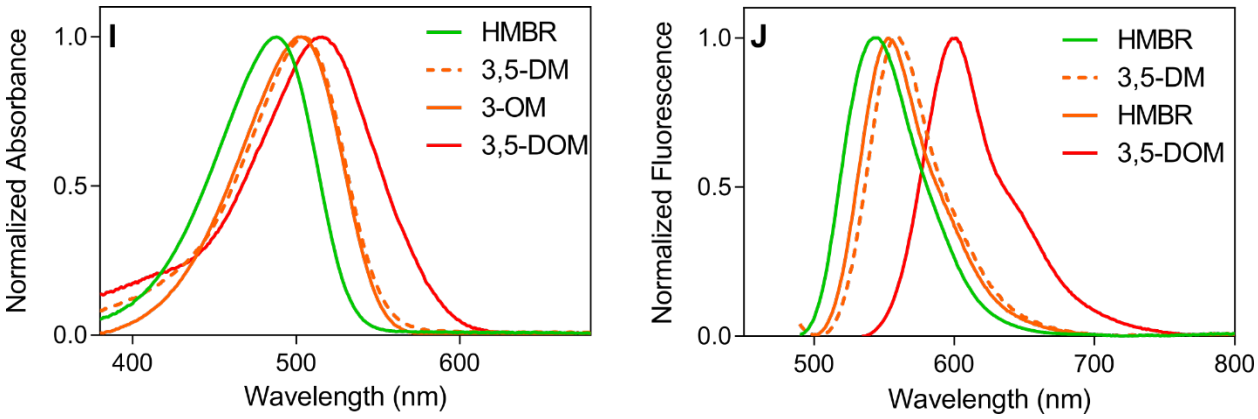


Figure S1. Absorbance and fluorescence spectra of FAST^{V122I} (A, B), FAST^{V107I,V122I} (C, D), mutant1 (E, F), mutant 2 (G, H), mutant3 (I, J). Fluorogen concentration was 3 μM and FAST mutant concentration was 40 μM. Spectra were recorded in pH 7.4 PBS at 25°C.

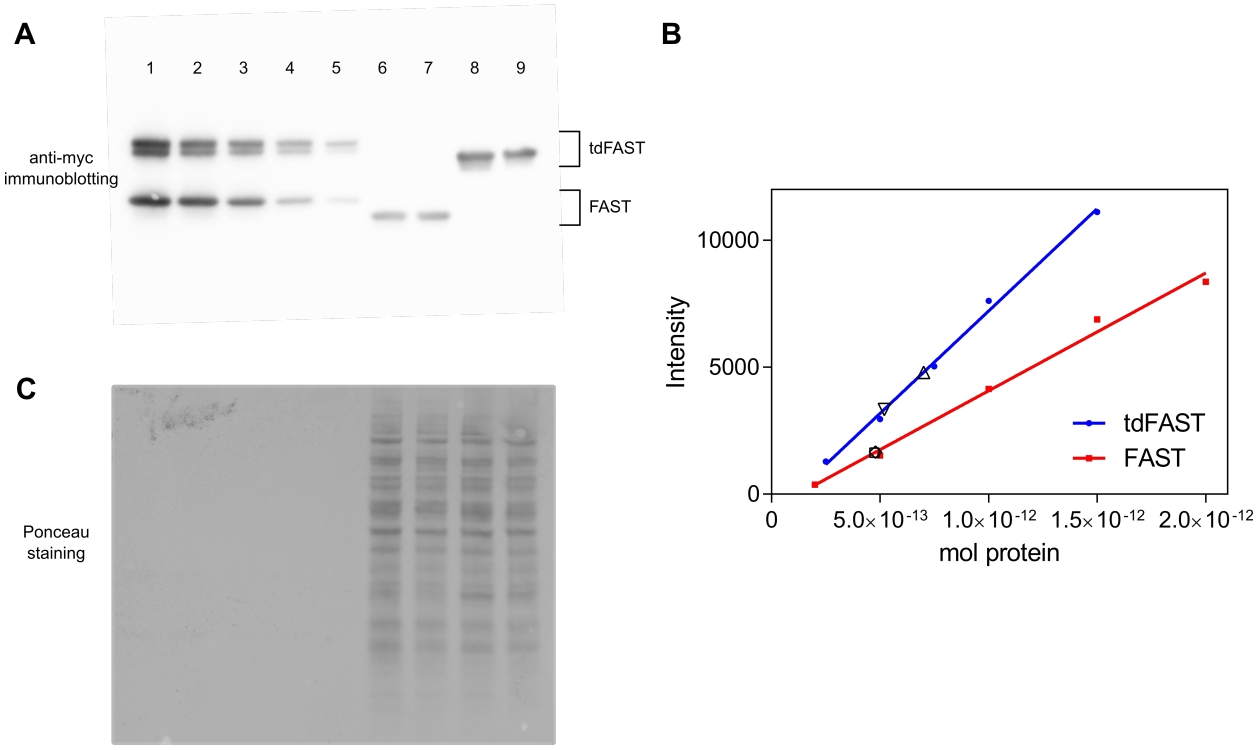


Figure S2. Quantification of protein concentration in microscopy samples. **(A)** Representative western. Lanes 1-5 are standards of Histag-tdFAST-myc and Histag-FAST-myc. The following four lanes (6-9) are microscopy samples: FAST-myc, iFAST-myc, td-iFAST-myc, and td-FAST-myc. Immunoblotting was performed using an anti-myc antibody. **(B)** Standard curves of intensity of chemiluminescence versus mol of protein for lanes 1-6. The quantity of protein from microscopy samples was interpolated from the standard curves for td-FAST (inverted triangle), td-iFAST (triangle), FAST (square), and iFAST (diamond) (note that square and diamond are overlaid in this example). **(C)** Ponceau staining of membrane before incubation with anti-myc antibody.

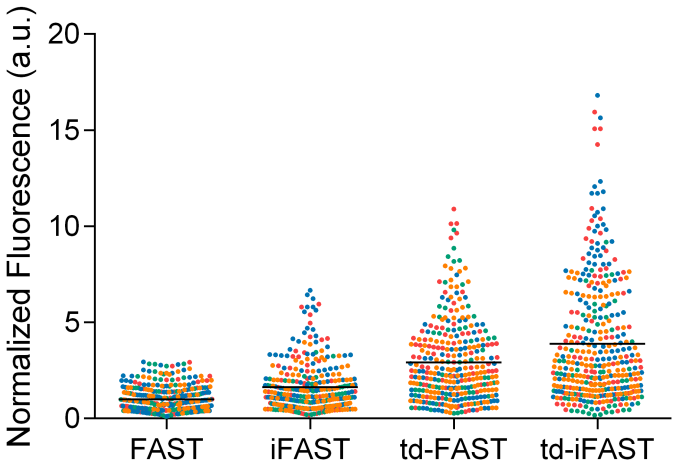


Figure S3. In-cell brightness of FAST, iFAST, td-FAST, and td-iFAST. Dot plot of all individual measurements of fluorescence intensity colored by experiment (n = 4 different experiments). Mean fluorescence is indicated by black line. Values were normalized by protein expression level (see Figure S2) and mean fluorescence of FAST was set to 1 for comparison.

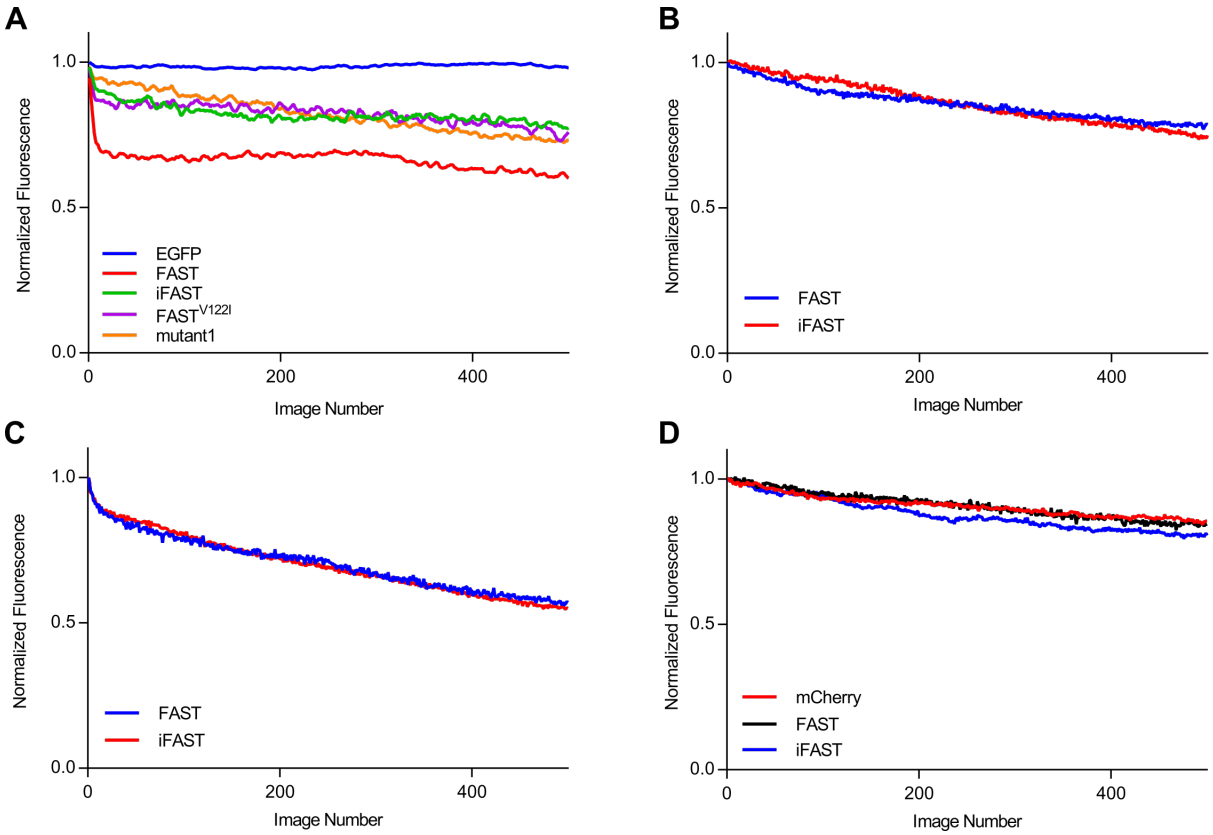


Figure S4. In-cell photobleaching of FAST and iFAST. (A) Comparison of photobleaching behavior between EGFP, FAST, iFAST, FAST^{V122I}, and mutant1 in the presence of 10 μM HMBR. **(B)** Comparison of photobleaching of FAST and iFAST in the presence of 10 μM HBR-3,5DM. **(C)** Comparison of photobleaching of FAST and iFAST in the presence of 10 μM HBR-3OM. **(D)** Comparison of photobleaching of mCherry, FAST and iFAST in the presence of 10 μM HBR-3,5DOM. **(A-D)** HEK 293T cells expressing the different FAST mutants were imaged using a scanning confocal microscope equipped with a 488 nm laser (with a power of 4.6 kW / cm^2 at the specimen plane) and a 541 nm laser (with a power of 2.7 kW / cm^2 at the specimen plane) at a frame rate of 1 image / 750 ms (pixel dwell time of 1.27 μs), n = 3 cells per curve.

MATERIALS AND METHODS

General FAST is a variant of the photoactive yellow protein (PYP) containing the mutations C69G, Y94W, T95M, F96I, D97P, Y98T, Q99S, M100R, T101G. HMBR, HBR-3,5DM, HBR-3OM, and HBR-3,5-DOM were prepared as previously described[1,2].

Molecular Biology The point mutations were inserted in FAST via site-directed mutagenesis using complementary primers and Gibson assembly using the previously reported pAG87. The tandem construct was synthesized by Eurofins and subcloned into plasmids for bacterial expression using *Nhe* I and *Xho* I restriction sites. Mutations were subsequently introduced sequentially in the same manner as for FAST. The genes for mutants (1-3) were synthesized by Eurofins and subcloned into plasmids for bacterial expression by Gibson assembly. All proteins were then subcloned into plasmids for mammalian expression by Gibson assembly.

Protein expression and purification Expression vectors were transformed in Rosetta (DE3) pLysS *E. coli* (New England Biolabs). Cells were grown at 37°C in LB medium complemented with 50 µg/ml kanamycin and 34 µg/ml chloramphenicol to OD_{600nm} 0.6. Expression was induced for 4 h by adding isopropyl β-D-1-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. Cells were harvested by centrifugation (4,000 × g for 20 min at 4°C) and frozen. The cell pellet was resuspended in lysis buffer (phosphate buffer 50 mM, NaCl 150 mM, $MgCl_2$ 2.5 mM, protease inhibitor, DNase, pH 7.4) and sonicated (5 min at 20 % of amplitude, 3 sec on, 1 sec off). The lysate was incubated for 2 h at 4 °C to allow DNA digestion by DNase. Cellular fragments were removed by centrifugation (9200 × g for 1h at 4°C). The supernatant was incubated overnight at 4°C under gentle agitation with Ni-NTA agarose beads in phosphate buffered saline (PBS) (sodium phosphate 50 mM, NaCl 150 mM, pH 7.4) complemented with 10 mM imidazole. Beads were washed with 20 volumes of PBS containing 20 mM imidazole, and with 5 volumes of PBS complemented with 40 mM imidazole. His-tagged proteins were eluted with 5 volumes of PBS complemented with 0.5 M imidazole. The buffer was exchanged to PBS (50 mM phosphate, 150 mM NaCl, pH 7.4) using PD-10 desalting columns.

Physico-chemical Measurements Thermodynamic dissociation constants were determined with a Spark 10M plate reader (Tecan) and fit in Prism 6 to a one-site specific

binding model. Steady state UV-Vis absorption spectra were recorded using a Cary 300 UV-Vis spectrometer (Agilent Technologies), equipped with a Versa20 Peltier-based temperature-controlled cuvette chamber (Quantum Northwest) and fluorescence data were recorded using a LPS 220 spectrofluorometer (PTI, Monmouth Junction, NJ), equipped with a TLC50TM Legacy/PTI Peltier-based temperature-controlled cuvette chamber (Quantum Northwest). The quantum yields of fluorescence (Φ_F) for the variant:fluorogen complexes were determined using different concentrations of fluorogen and a constant concentration of FAST variant (40 μM), such that the complex formed was always ~100%. Complex absorbance was kept below 0.05 and plotted as a function of the integrated emission intensity according to the equation:

$$\Phi_F^S = \Phi_F^R \cdot \frac{I_S}{I_R} \cdot \frac{Abs_R}{Abs_S} \cdot \left(\frac{n^S}{n^R}\right)^2$$

where *S* and *R* are the sample and reference, respectively, *I* is the integrated intensity of fluorescence emission, *Abs* is the absorbance at the excitation wavelength and *n* is the refractive index of each solution. FAST:HMBR was used as a reference.

Mammalian cell culture HEK 293T cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with phenol red, Glutamax I, and 10% (vol/vol) fetal calf serum (FCS), at 37 °C in a 5% CO_2 atmosphere. For imaging, cells were seeded in μDish IBIDI (Biovalley) coated with poly-L-lysine. Cells were transiently transfected using Genejuice (Merck) according to the manufacturer's protocol for 24 hours prior to imaging.

Fluorescence microscopy Confocal micrographs were acquired on a Zeiss LSM 710 Laser Scanning Microscope equipped with a Plan Apochromat 63×/1.4 NA oil immersion objective. Cells were imaged in DMEM supplemented with 20 μM HBR-3OM for quantification. Images were collected as a series of z-stacks by defining the first and last image whereby positive intensity is detected so as to capture all in and out of focus light. The number of images varied on the thickness of the cells but the imaging interval between stacks was kept constant for all stacks. Stacks were analyzed by generating a sum intensity projection across the stack and the integrated density was measured for each cell for about 80-90 cells per sample. The experiment was repeated four times, with

a total number of cells analyzed of 373, 324, 374, and 329 for FAST, iFAST, td-iFAST, and td-FAST, respectively.

Photobleaching measurements for HMBR, HBR-3,5DM, and HBR-3OM were carried out at 10 μM fluorogen at 488 nm excitation (4.6 kW / cm^2, 1.27 μsec pixel dwell); EGFP was used as a control. HBR-3,5DOM and mCherry were excited at 541 nm (2.7 kW / cm^2, 1.27 μsec pixel dwell). Samples were imaged continuously for 500 images.

Western Blots Samples used for microscopy were washed twice with Dulbecco's Phosphate-Buffered Saline (DPBS) and lysed in 100 uL CellLytic (Sigma). The samples were then centrifuged for 5 minutes at 6,000 x *g* at 4 °C and the supernatant was used for further analysis. Total protein concentration was determined by BCA assay (Sigma) in parallel with purified protein samples. Samples were fixed to have the same amount of total protein and separated by SDS-PAGE (4-12% Bis-Tris NuPAGE). Proteins were transferred by semi-dry blotting to a nitrocellulose membrane (Amersham Protran 0.2 μm). Membranes were imaged with Ponceau-S (Sigma) to ensure homogenous transfer and loading and then incubated with blocking buffer (Tris Buffer Saline with 0.1% Tween-20 [TBS-T] + 5% dry milk) for 1 hr. Membranes were incubated overnight at 4 °C with anti-myc (Cell Signaling Technology) at 1:2000 in blocking buffer and then washed with TBS-T for 1 hour. The membranes were then stained with diluted (1:2500) secondary antibody, anti-rabbit (Cell Signaling Technology), in blocking buffer for one hour. Membranes were then washed for 1 hour in TBS-T. Antibody was visualized with BioRad ClarityMax reagent after 5 min incubation using a BioRad ChemiDoc MP. Blots were analyzed in ImageJ using the gel analyzer tool without background correction. Standard curves using purified FAST-myc and td-FAST-myc were used to interpolate the quantity of protein in the sample.

References

(1) Plamont, M.-A., Billon-Denis, E., Maurin, S., Gauron, C., Pimenta, F. M., Specht, C. G., Shi, J., Querard, J., Pan, B., Rossignol, J., Morellet, N., Volovitch, M., Lescop, E., Chen, Y., Triller, A., Vriz, S., Le Saux, T., Jullien, L., and Gautier, A. (2016) Small fluorescence-activating and absorption-shifting tag for tunable protein imaging in vivo. *Proc. Natl. Acad. Sci. U. S. A 113*, 497–502.

(2) Li, C., Plamont, M.-A., Sladitschek, H. L., Rodrigues, V., Aujard, I., Neveu, P., Le Saux, T., Jullien, L., and Gautier, A. (2017) Dynamic multicolor protein labeling in living cells. *Chem. Sci. 8*, 5598–5605.