Extended Data

Orthogonal fluorescent chemogenetic reporters for multicolor imaging

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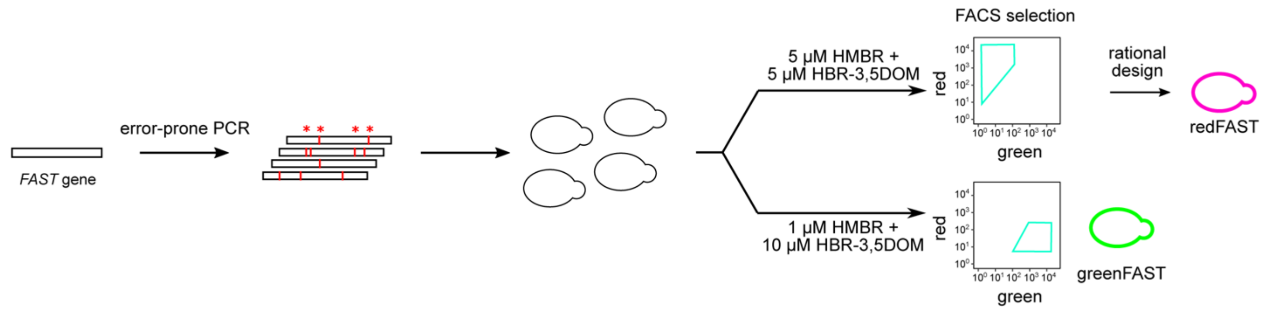
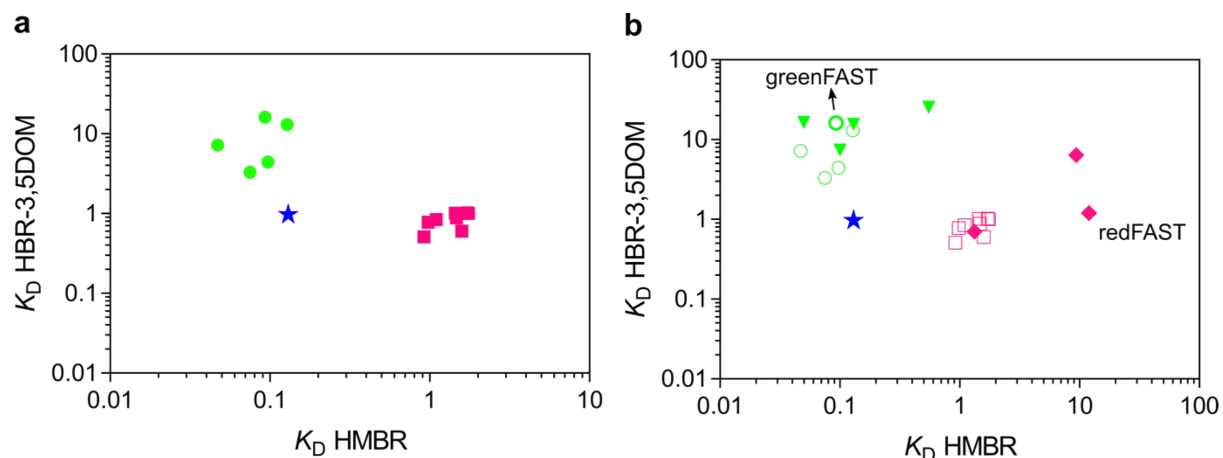
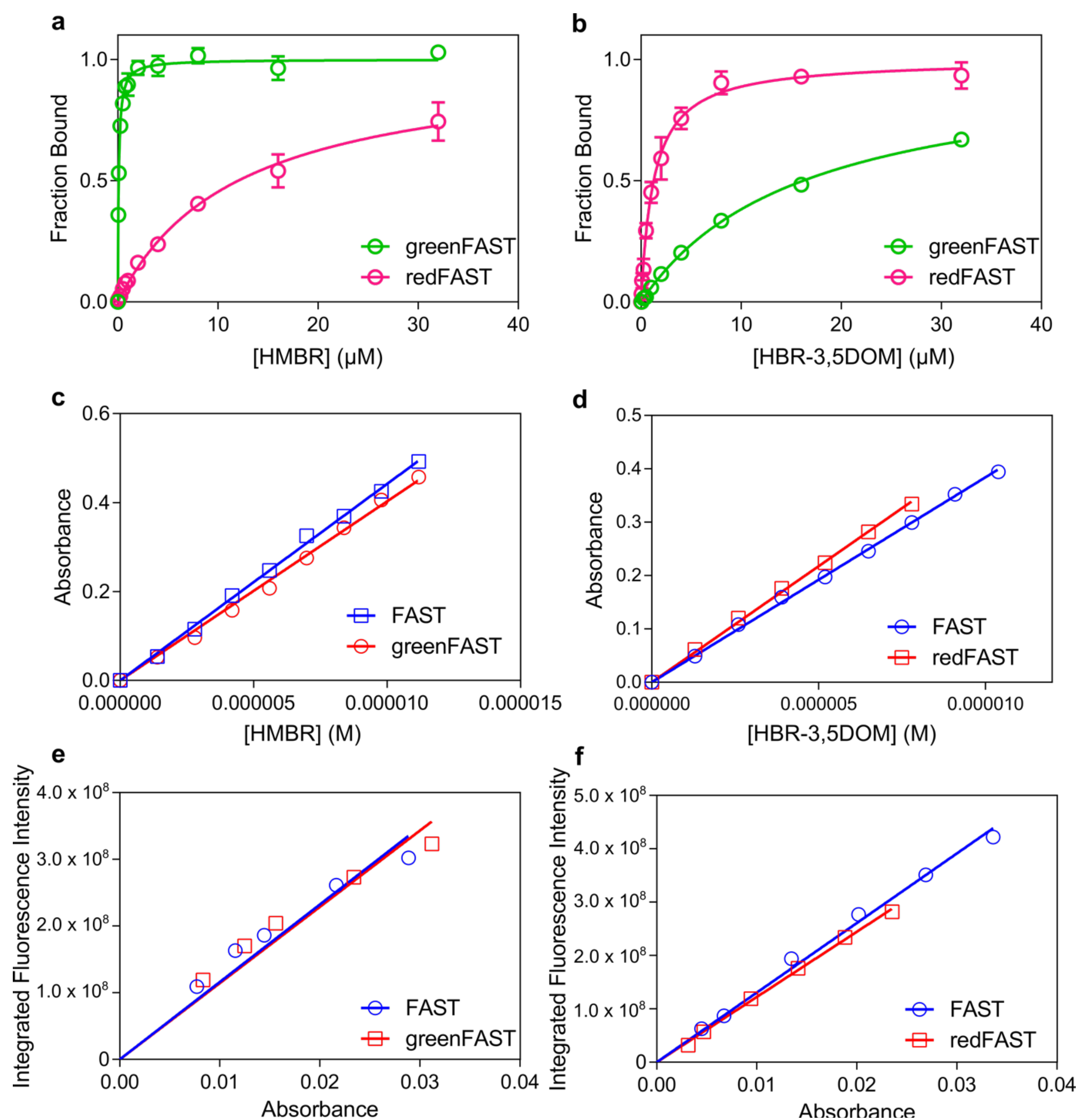


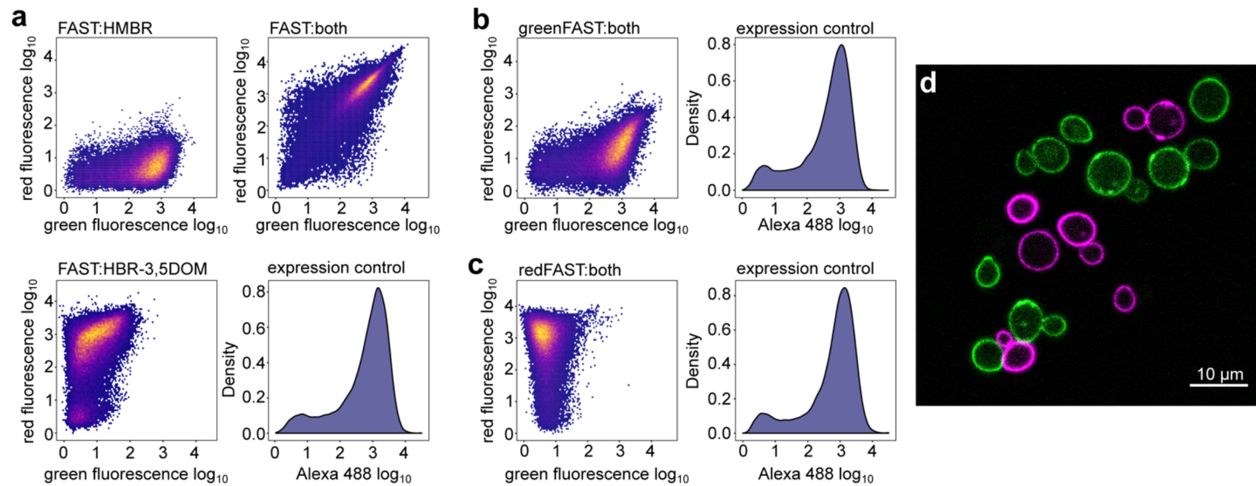
Extended Data Figure 1. Selection and design strategy for spectrally orthogonal FAST systems. A yeast displayed library of FAST variants was screened in presence of both HMBR and HBR-3,5DOM in order to identify redFAST and greenFAST.



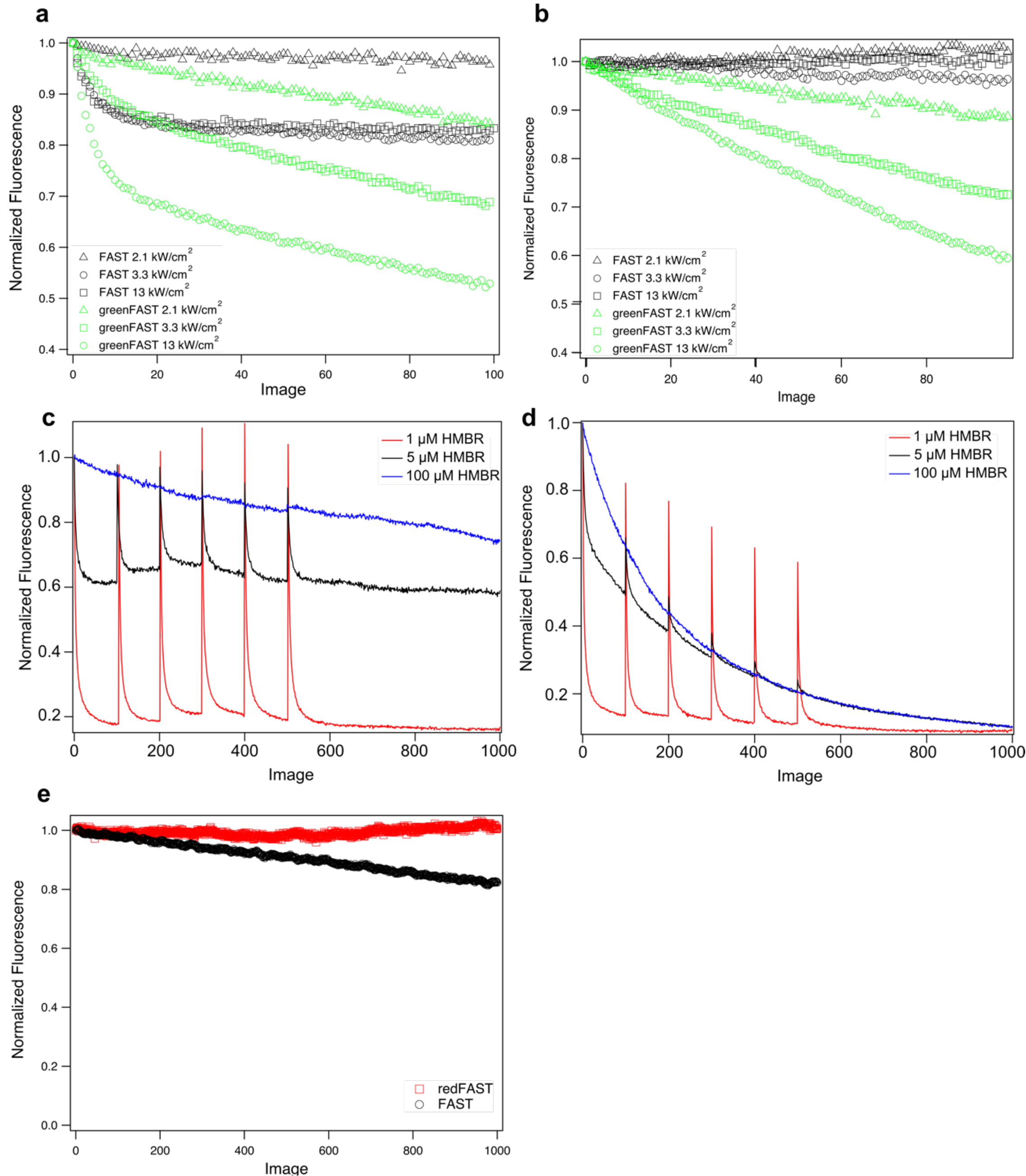
Extended Data Figure 2. Engineering of greenFAST and redFAST. Comparison of the K_Ds (in μM) for HMBR and HBR-3,5DOM of FAST (blue star) and clones selected from FACS (a) and variants constructed through rational design (b, filled markers). Green dots: greenFAST selection, magenta dots: redFAST selection.



Extended Data Figure 3. Characterization of greenFAST and redFAST. (a,b) Affinities of greenFAST and redFAST for a) HMBR and b) HBR-3,5DOM. Mean of n = 3, represented as mean ± sem, protein concentration 100 nM. **(c,d)** Determination of molar absorptivity for c) greenFAST and d) redFAST with their cognate fluorogen by forward titration and standardization with FAST (protein concentration, 40 µM). **(e,f)** Determination of quantum yield for e) greenFAST and d) redFAST with their cognate fluorogen by reciprocal dilution using FAST:fluorogen as a standard (protein concentration, 40 µM).

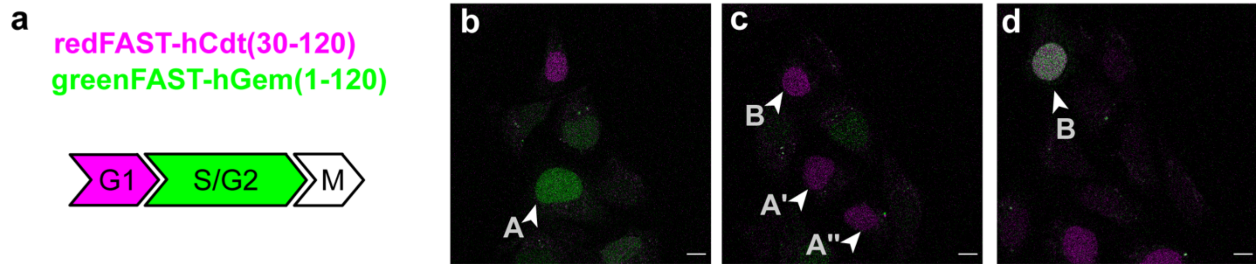


Extended Data Figure 4. Flow cytometry analysis. Yeast cells expressing FAST **(a)**, greenFAST **(b)** and greenFAST **(c)** were analyzed by flow cytometry in the presence of either only HMBR (5 μM) or HBR-3,5DOM (10 μM) or in the presence of both (5 μM HMBR and 10 μM HBR-3,5DOM). Efficient induction of protein expression was verified through independent labeling with an Alexa488-conjugated antibody**.** The analysis was done typically on about 110,000 cells**. Supplementary Figure 1** exemplifies the gating strategy used. **(d)** Representative confocal micrograph (n = 5 from 1 experiment) of a mixture of yeast cells expressing greenFAST (green) and redFAST (magenta) in the presence of 5 μM HMBR and 10 μM HBR-3,5DOM. Scale bar 10 μm.

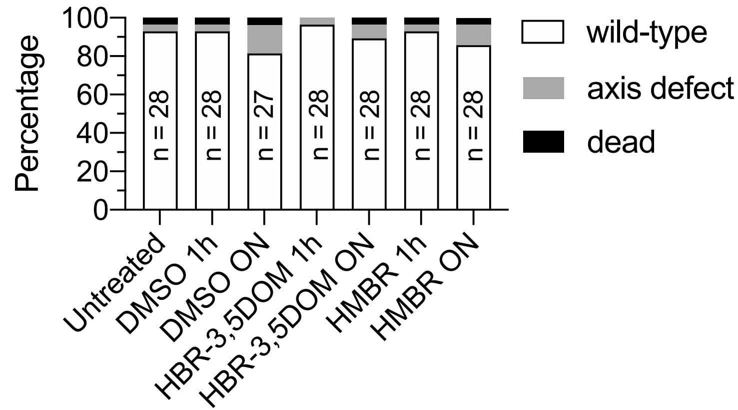


Extended Data Figure 5. Photostability measurements for greenFAST and redFAST. (a,b) comparison of greenFAST and FAST photostability in different illumination conditions. Both were expressed as H2B fusions in HEK293T and imaged with 10 μM HMBR. Images taken every a) 1 s and b) 10 s with 1.27 μs pixel dwell, excitation with 488 nm laser. **(c,d)** Comparison of c) FAST and d) greenFAST photostability as a function of fluorogen concentration at 13 kW/cm^2 for 488 nm laser, 1.27 μs pixel dwell, images taken every 1s. 100 images were acquired followed by 60 s in the dark before acquisition was restarted. **(e)** redFAST and FAST expressed in the

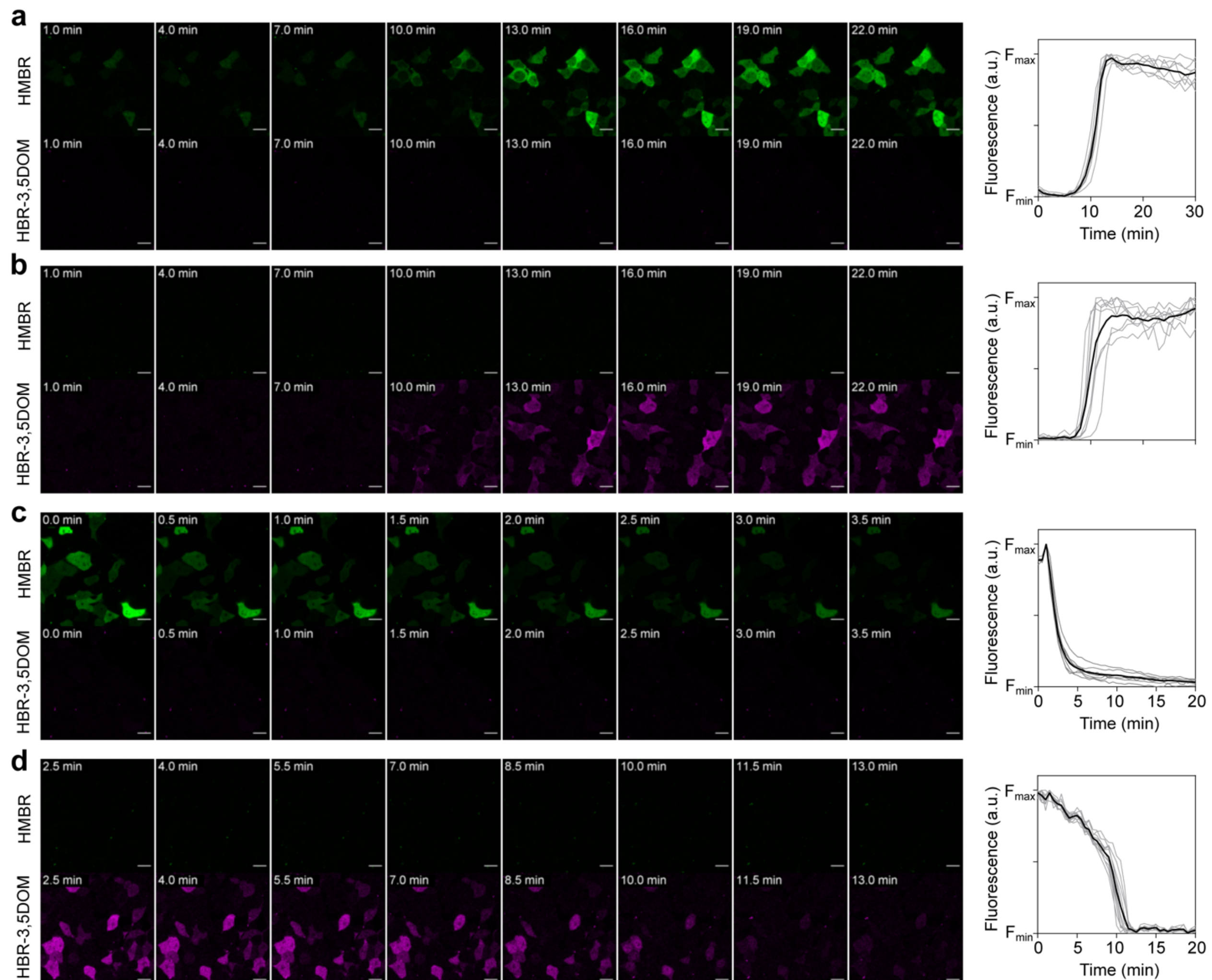
cytosol in HEK293T cells labeled with 10 μM HBR-3,5DOM were illuminated with 4.9 kW/cm^2 for 541 nm laser, 1.27 μs pixel dwell, images taken every 1 s.



Extended Data Figure 6. Cell cycle sensors based on orthogonal FASTs. (a-d) Representative micrograph (n = 8 from 3 experiments) of U2OS cells stably expressing a FUCCI cell cycle sensor. **(a)** Design of a mammalian cell cycle sensor. Tracking of individual cell cycles is possible through stable expression of redFAST-hCdt(30-120) and greenFAST-hGem(1-120). **(b-d)** Cell A can be tracked S/G2 (**b**, 40 mins) through division (**c**, 6 hrs 10 mins) while Cell B (**c**, top arrow) can be tracked through the G1-S transition (**d**, 19 hrs 50 mins). Images were taken every 5 mins. 5 μM HMBR and 10 μM HBR-3,5DOM. Scale bars 10 μm.



Extended Data Figure 7. Effects of the fluorogens on zebrafish embryogenesis. Zebrafish embryos were incubated with 5 μM fluorogen during 1 hour at 50% epiboly or overnight (o/n) from 50% epiboly to 24 hpf. The graph shows the percentage of embryos with no defect (white), axis defects (grey), or dead (black) at 48 hpf. Controls untreated and treated with DMSO only were performed. The number (n) of embryos used for each condition is indicated.



Extended Data Figure 8. Kinetics of association and dissociation of split-greenFAST and split-redFAST. (a,b) Representative micrographs (n = 3 from 3 experiments) of HEK293T cells co-expressing FK506-binding protein (FKBP) fused to CFAST11 and FKBP-rapamycin-binding domain of mammalian target of rapamycin (FRB) fused to either greenNFAST **(a)** or redNFAST **(b)** were labeled with both 5 μM HMBR and 10 μM HBR-3,5DOM, and imaged before and after addition of 500 nM rapamycin. The green channel shows HMBR fluorescence, while the magenta channel shows HBR-3,5DOM fluorescence. Graphs show the temporal evolution of the fluorescence intensity of individual cells (n = 7 and 8) after rapamycin addition. **(c,d)** Representative micrographs (n = 3 from 3 experiments) of HEK293T cells co-expressing FKBP fused to CFAST11 and FBBP fused to either greenNFAST **(c)** or redNFAST **(d)** treated with 100 nM AP1510 and labeled with both 5 μM HMBR and 10 μM HBR-3,5DOM. Cells were then imaged before and after the addition of 1.1 μM rapamycin. The green channel shows HMBR fluorescence, while the magenta channel shows HBR-3,5DOM fluorescence. Graphs show the temporal evolution of the fluorescence intensity of individual cells (n = 8 and 11) after rapamycin addition.