**1. Plasmid construction**

1.1. Generate two-color fusion probes. Use a Clontech N1 mammalian cell expression vector in which mCherry1 12 and PA-mCherry1 13 have been inserted with the restriction sites *AgeI* and *BsrGI.*

1.2. Order custom oligo-nucleotides to amplify the monomeric variants of eGFP and PA-eGFP containing the A206K mutation, i.e. mEGFP and PA-mEGFP 14 without a stop codon as a *SalI-BamHI* fragment. Use the N-terminal primer 5’-AAT TAA CAG TCG ACG ATG GTG AGC AAG GGC GAG G 3’ and the C-terminal primer 5’-AAT ATA TGG ATC CCG CTT GTA CAG CTC GTC CAT GC 3’ and insert this *SalI-BamHI* fragment into the multiple cloning site of the Clontech N1 vector. This will create the five amino acid linker RNPPV between the green and red fluorescent protein.

**2. Cell culture and transfection**

2.3. Grow cells of a standard cell line in 8-well LabTek chambers with #1.0 cover glass for fluorescence live-cell microscopy.

2.4. Transfect cells 24 h after plating using Fugene 6 (Roche) per distributer’s protocol with the GFP—Cherry, PA-GFP—Cherry, and GFP—PA-Cherry chimeras.

2.5. Image cells after a total of 20 h post transfection to allow for protein expression, folding and maturation.

**3. Imaging and photoactivation**

3.1. Image cells in a humidified and heated environmental chamber at 37 degrees Celsius. To buffer the cell media at physiological pH and render it CO2-independent, add 20 mM HEPES; or use CO2 gas set to 5% flow.

3.2. First, image cells expressing the GFP—Cherry construct. Set parameters that define time-integrated laser intensity per pixel in a confocal image, i.e. pixel dwell time in microseconds, acousto-optical tunable filter (AOTF) transmission in percent, and digital zoom. Using a 60x objective and a digital zoom of 3x allows imaging of a cell in its entirety while providing sufficient magnification. Set pixel dwell time to 2-4 μs and AOTF transmission for the 488-nm and 561-nm laser such that images show a good signal-to-noise ratio without any bleaching and no pixels indicating fluorescence intensity saturation.

3.3. Image with set laser power, AOTF transmission, pixel dwell time and digital zoom 15-20 cells expressing GFP—Cherry.

3.4. Then, image with the same set laser power, pixel dwell time, AOTF transmission and digital zoom cells expressing GFP—PA-Cherry and PA-GFP—Cherry. Search for expressing cells in the green channel or red channel, respectively. Avoid long exposure of the cells during the search for expressing cells in order to not bleach the fluorescent proteins.

3.5. Set up a mini-time series with one pre-activation image and three post-activation images. The post-activation images will help identify potential transient dark states due to the exposure to UV-light.

3.6. To determine photoactivation efficiency, i.e. the fraction of PA-FPs that is switched on to be fluorescent, in the specific photoactivation experiments that you have already established, apply the photoactivation settings you have been using to 15-20 cells that are expressing the internal rulers. If you are beginning to set up photoactivation experiments, find here a few different settings based on our experimental experience with PA-GFP and PA-Cherry. Modify as needed for your own experiments.

3.6.1. For instantaneous photoactivation of PA-GFP and PA-Cherry using a confocal laser scanning microscope, apply 90 μW of 405-nm laser light in 3 or 5 iterations, respectively. With our microscopic set-up, using a 38% AOTM transmission and a 2 μs pixel dwell time, we measured this 405-nm laser power in line-scan mode at the objective lens. With these settings, about 8% and 16% of the PA-GFP and PA-Cherry expressed can be photoactivated to be fluorescent17.

3.6.2. If a higher fraction of photoactivated PA-GFP and PA-Cherry fluorophores is advantageous e.g. to achieve a higher signal-to-noise ratio, and photoactivation does not have to be immediate, apply 40 μW of 405-nm laser light with a 2 μs pixel dwell time and 6% AOTF transmission for 450 iterations. Then, photoactivation will take up to 4 min as opposed to only 1-2 seconds, but photoactivation efficiency for PA-GFP will be 29% instead of 8%, allowing for a higher signal-to-noise ratio.

3.7. If a Mosaic Digital Illumination System that contains micro mirror arrays in a spatial light modulator is available, 405-nm laser light can be used for widefield-photoactivation. This allows for efficient photoactivation within milliseconds. With 1.6 mW laser power as measured at the objective lens and an exposure time of 250 ms, 29% of PA-GFP can be photoactivated to be fluorescent.

3.8. Image with any set photoactivation parameters 15-20 cells expressing PA-GFP—Cherry and GFP—PA-Cherry, respectively.

**4. Image analysis and algorithm for ratiometric intensity-based quantification of photoactivation efficiency**

4.1. Image analysis can be done with the open source image processing platforms ImageJ or Fiji. Determine the background fluorescence intensity in non-transfected cells in the green (B1) and red (B2) channel. Avoid perinuclear or any areas showing increased auto-fluorescence.

4.2. To determine the fluorescence intensity in a transfected cell, outline the cell body with the freehand selections tool. Again, avoid perinuclear or any other areas showing auto-fluorescence.

4.3. Subtract the background from the measured fluorescence intensity in each channel.

IG = IGreen\_measured – B1

IR = IRed\_measured – B2

4.4. Use the GFP—Cherry construct to calculate the red-to-green ratio (RtoGr) and correct for donor-quenching due to fluorescence resonance energy transfer (FRET). The FRET efficiency E was determined to be 0.3 in previous experiments for the GFP—Cherry construct using the same amino acid linker between the two fluorophores 18.

RtoGr = (IRed\_measured – B2) / (IGreen\_measured – B1)

RtoGrcorr = RtoGr \* (1 – E)

4.4.1 Caveat: In this intensity- based approach, donor quenching for mEGFP and PA-mGFP may be different since the rate of FRET (kET) and the Foerster distance (R0) depend upon the quantum yield of the donor which have not been characterized for mEGFP and PA-mGFP.

4.5. Use the GFP—PA-Cherry construct to assess the fraction of photoactivated PA-Cherry. Determine the expected fluorescence intensity of PA-Cherry by multiplying the measured unquenched green fluorescence intensity of the GFP—PA-Cherry construct *prior* to photoactivation with the corrected red-to-green-ratio (RtoGrcorr).

IRed\_expected = (IGreen\_measured – B1) \* RtoGrcorr

4.5.1. Caveat: As indicated above, the molecular brightness of the always-on FP and the photoactivatable FP may be different. The molecular brightness (MB) is the product of quantum yield (QY), extinction coefficient (EC) and percent absorbance at the given excitation wavelength relative to the absorbance peak. For Cherry 12 and PA-Cherry 13, respective values of QY and EC have been published. The percent absorbance at the given excitation wavelength of 543 nm relative to absorbance peak is 0.5 and 0.7, respectively.

4.5.1.1. MBCherry = 0.22 \* 72,000 \* 0.5 = 7,920

MBPA-Cherry = 0.46 \* 18,000 \* 0.7 = 5,796

Thus, the lower molecular brightness of PA-Cherry compared to Cherry can be taken into account by dividing IRed\_expected by 1.37.

4.5.1.2. However, it is unknown under which photoactivation conditions the published molecular brightness of PA-Cherry has been determined. This is important, since we show here that the mode of photoactivation changes the measured fraction of photoactivated PA-FPs. Furthermore, for the monomeric versions comprising the A206K mutation. i.e. mEGFP and PA-mEGFP, no molecular brightness has been published.

4.5.1.3. In this ratiometric intensity-based approach, the molecular brightness of the PA-FPs and the always-on FP counterparts in a first approximation have been considered identical. We decided on this approach, since (i) for some FPs no molecular brightness has been reported, and (ii) it is thus far unclear in how far different modes of photoactivation may affect the molecular brightness of the PA-FPs reported in the literature. Furthermore, (iii) for a comparative analysis the knowledge of the molecular brightness is not necessary, it is only needed for the intensity-based determination of the absolute fraction of photoactivated PA-FPs which can be calculated as shown above.

4.6. Calculate the PA-Cherry photoactivation efficiency as a fraction of the measured red fluorescence intensity *after* photoactivation and the expected fluorescence intensity in the red channel.

(FPA-Cherry) = (IRed\_measured – B2) / IRed\_expected

4.7. Use the PA-GFP—Cherry construct to assess the fraction of photoactivated PA-GFP. Determine the expected fluorescence intensity of PA-GFP by dividing the measured red fluorescence intensity of the PA-GFP—Cherry construct *prior* to photoactivation by the red-to-green-ratio (RtoGr). Here, the RtoGr does not need to be corrected for donor quenching, because GFP and PA-GFP are subject to donor quenching to the same amount.

IGreen\_expected = (IRed\_measured – B2) / RtoGr

4.8. Calculate the PA-GFP photoactivation efficiency as a fraction of the measured green fluorescence intensity *after* photoactivation and the expected fluorescence intensity in the green channel.

(FPA-GFP) = (IGreen\_measured – B1) / IGreen\_expected