Photobleaching of YFP does not produce a CFP-like species that affects FRET measurements

To the editor: Fluorescence resonance energy transfer (FRET) efficiency between variants of fluorescent protein can be determined by photobleaching the acceptor molecule[1]. After complete acceptor bleaching, FRET efficiency equals the dequenched donor fluorescence divided by the total donor fluorescence. Recently, Valentin *et al.*[2] reported that photobleaching of yellow fluorescent protein (YFP), Citrine and Venus (commonly used acceptor fluorescent protein tags) produced a species with an emission peak similar to that of cyan fluorescent protein (CFP), a widely-used donor fluorescent protein. This result predicts that FRET efficiency determined by acceptor photobleaching will be overestimated. As this prediction potentially impacts the accuracy of numerous existing studies and raises concerns about the validity of the photobleaching method, we examined the effects of acceptor fluorescent protein photobleaching using several imaging modalities commonly used in FRET experiments.

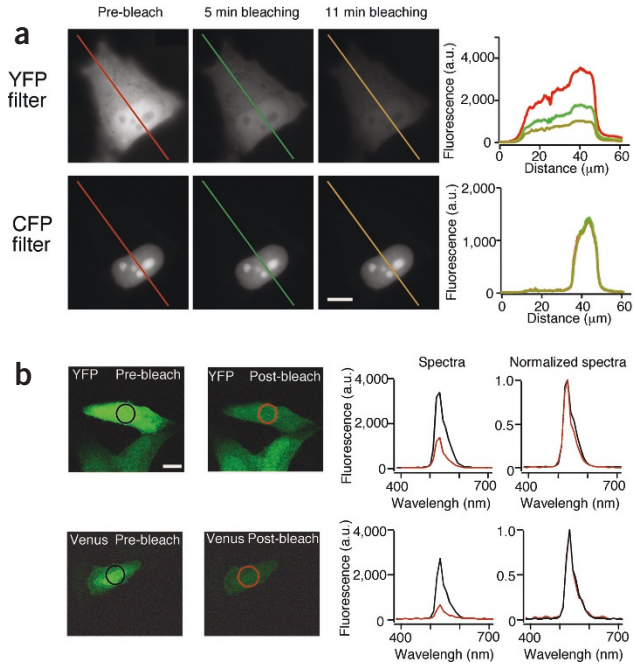

Figure 1 | Photobleaching of Venus and YFP does not produce a CFP-like species. (**a**) Images were acquired using conventional fluorescence microscopy (60× oil objective, numerical aperture (NA) 1.4). The lines in the images represent where the fluorescence intensity profiles were measured. (**b**) The emission spectral images were acquired using two-photon excitation at 940 nm as described in the text. Detailed procedures are described in **Supplementary Methods**. Scale bar, 10 μm.

We imaged HeLa cells coexpressing Venus and nuclear-localized CFP one day after transfection (**Fig. 1a; Supplementary Methods** online). Prolonged illumination through a YFP filter set (excitation: 500 ± 10 nm, emitter: 540 ± 15 nm, dichroic: 520LP) decreased the intensity of Venus fluorescence (YFP filter set) evenly throughout the cell. Conversely, the pattern and intensity of fluorescence detected through a CFP filter (excitation: 436 ± 10 nm, emitter: 480 ± 20 nm, dichroic: 455LP) remained unchanged. Additionally, fixation of cells expressing YFP or Venus with paraformaldehyde followed by photobleaching (50% decrease in YFP channel intensity) did not produce an increase in CFP channel fluorescence intensity (**Supplementary Fig. 1** online). We obtained similar results (**Supplementary Fig. 1**) using CFP excitation (excitation: 406 ± 7.5 nm) similar to that used by Valentin *et al.*[2].

We determined the emission spectra of either Venus or YFP before and after photobleaching (514 nm argon ion laser) of fixed HeLa cells expressing these proteins. Photobleaching of Venus or YFP (> 50%) did not alter the shape of the emission spectra acquired with an LSM510 confocal microscope using two-photon excitation between 750 and 940 nm (**Fig. 1b**, and **Supplementary Figs. 1** and **2** online). In particular, we observed no detectable increase in fluorescence intensity from 420–500 nm. This finding is in marked contrast to the result of Valentin *et al.*[2].

Our results demonstrate that photobleaching of YFP or Venus, under the conditions stated, does not produce CFP-like fluorescence, and thus FRET efficiency determination using acceptor photobleaching need not be encumbered by the reported phenomenon[2]. It is unclear why our results deviate from those of Valentin *et al.*[2] as experimental conditions were similar (but not identical).

Note: Supplementary information is available on the Nature Methods website.

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1. Kenworthy, A. K. *Methods* **24**, 289–296 (2001).
2. Valentin, G. *et al. Nature Methods* **2**, 801 (2005).

To the editor: Fluorescence resonance energy transfer (FRET) between cyan and yellow fluorescent protein (CFP and YFP) fusion proteins assessed as sensitized emission can be effectively controlled by measuring dequenching of CFP fluorescence after YFP photobleaching. Recently Valentin *et al.*[1] reported that photobleaching of YFP induced the formation of a fluorescent product excitable at 405 nm with an emission maximum similar to that of CFP. This could severely affect measurements of FRET between CFP and YFP fusion proteins based on donor dequenching after acceptor photobleaching. Therefore, we have tested whether photoconversion of YFP interferes with CFP-dequenching during