# Photoconversion of YFP into a CFP-like species during acceptor photobleaching FRET experiments

**To the editor:** Fluorescence resonance energy transfer (FRET) is a useful technique to detect protein-protein interactions in cells[1,2]. Acceptor photobleaching approaches with CFP- and YFP-tagged proteins are simple and prevent problems associated with variable expression levels: when FRET occurs, the fluorescence of the CFP donor increases after bleaching the YFP acceptor chromophore, and this is recognized as a signature for FRET[2].

Surprisingly, we observed that cells expressing only YFP were generating increased cyan fluorescence after YFP photobleaching (**Fig. 1a**). This increase could be observed by exciting this fluorescent protein at 405 or 458nm, and it was only due to YFP, for the following reasons: (i) it did not appear in nontransfected cells (**Fig. 1a**), (ii) it appeared only in the subcellular compartment that contained the expressed YFP (**Supplementary Fig. 1** online), (iii) it was observed in all cell types tested (not shown), and (iv) when YFP was affinity-purified on sepharose beads and the beads imaged with a confocal microscope, YFP photobleaching resulted in increased cyan fluorescence (**Supplementary Fig. 2** online).

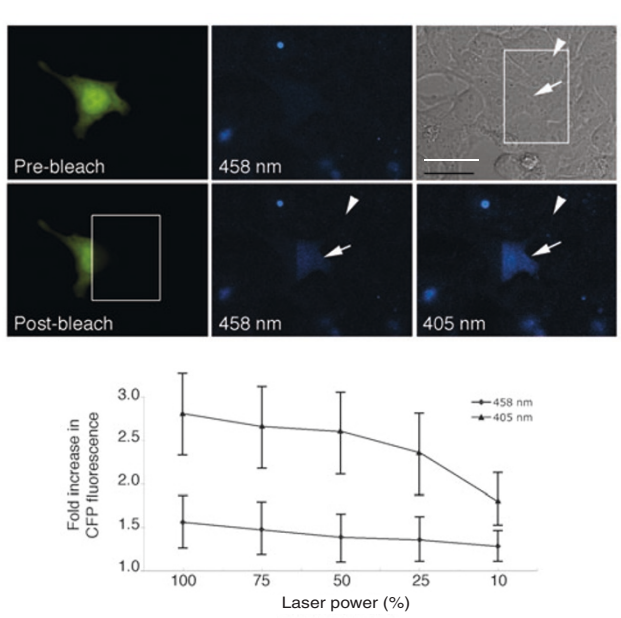


**Figure 1 |** YFP can yield false FRET signals with the acceptor photobleaching method. (**a**) Photoconversion of YFP into a CFP-like species in fixed cells. HeLa cells expressing YFP (the 10C GFP variant) were imaged as indicated in **Supplementary Methods** online. YFP was bleached at 514 nm in the area delineated by the box. Top, pre-bleach; bottom, post-bleach: Left, excitation at 514 nm and emission in the YFP channel. Middle, excitation at 458 nm and emission in the CFP channel. Bottom right, excitation at 405 nm and emission in the CFP channel. Upper right, phase-contrast image. Arrow, transfected cell showing YFP to CFP photoconversion. Arrowhead, untransfected but photobleached control cell. Bar, 25 microns. (**b**) Various laser powers can photoconvert YFP into a CFP-like species. YFP was affinity-purified on beads and imaged as described in **Supplementary Methods**. The increase in CFP emission (±s.d.) induced by YFP photobleaching is plotted as a function of the laser power used for bleaching (100% corresponds to a nominal output of 10mW at 514 nm). Bleaching time was adjusted to obtain 50% of YFP bleaching in each case.

We then bleached affinity-purified YFP at various laser powers (100% to 10%). The amount of YFP to CFP photoconversion decreased with diminishing power, but a substantial amount of CFP-like species was observed even with the lowest power tested (**Fig. 1b**). Next, we tested newer YFP variants with improved photophysical properties. We observed that photobleaching of Citrine[3] or Venus[4] also yielded an increased cyan fluorescence (**Supplementary Figs. 2** and **3** online).

Previous analyses have shown that eYFP yields detectable emission in the 400–500 nm range under particular conditions[5]. Our results extend these studies by showing that photobleaching of eYFP, Citrine or Venus, can generate species that have emission peaks similar to that of CFP. This emission is observed after excitation at 405 or 458 nm (**Supplementary Fig. 3**) thus preventing discrimination of CFP and photobleached YFP on the basis of their emission or excitation spectra. During photobleaching FRET experiments, any increase in the CFP channel could thus arise from either FRET, or YFP to CFP photoconversion.

The mechanism underlying this YFP to CFP photoconversion is unclear. YFP can be protonated on Tyr203 and this modifies its excitation spectrum[1,5]. Protonated YFP, however, is not excited at 458 nm, and it has an emission peak identical to that of unprotonated YFP[5], in contrast to photobleached YFP. Thus, YFP to CFP photoconversion is probably not related to Tyr203 protonation. YFP photobleaching also induces its decarboxylation on Glu222 (ref. 5). As light-induced decarboxylation of Glu222 occurs in wild-type GFP and modifies its spectral properties[6], it is plausible that this also induces YFP photoconversion into a CFP-like species.

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

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