FRET measurement between YFP and CFP

EYFP and ECFP function as a donor-acceptor pair for fluorescence resonance energy transfer (FRET), in which excitation of the donor (cyan) molecule leads to emission from the acceptor (yellow) molecule, provided that the proteins are close enough for energy transfer to occur. FRET can therefore be used to monitor direct protein-protein interaction between EYFP and ECFP fusion proteins in living or fixed cells (for review see Pollock and Heim, 1999)

Pollok, B.A., and R. Heim. 1999. Using GFP in FRET-based applications. Trends Cell Biol. 9:57-60.

The EYFP and ECYP vectors are sold by clonetech as part of their 'living colors' collection, and you can visit their website to obtain ordering information and download vector maps (www.clonetech.com/gfp).

Measurement of FRET by this particular approach requires a microscope that allows independent control of emission and excitation filters. We purchase our filter sets from Chroma (www.chroma.com). If you do not have this independent filter control, you can try another type of FRET measurement, donor photobleaching (see below).

Procedure

Controls (determine YFP and CFP crosstalk into FRET channel)

1. Cells expressing YFP construct only (measure YFP_{ex}/YFP_{em}, CFP_{ex}/CFP_{em}, CFP_{ex}/YFP_{em})
   In this case there is a real YFP signal but no CFP signal. Any signal in the FRET channel (CFP_{ex}/YFP_{em}) is therefore due to crosstalk of the YFP signal into this channel. This crosstalk (measured as a ratio of the CFP/YFP signal to the YFP/YFP signal) will be subtracted from the FRET signal measured in experimental cells. In our system it is usually 20-30%. Call this value 'a'.
2. Cells expressing CFP construct only (measure YFP_{ex}/YFP_{em}, CFP_{ex}/CFP_{em}, CFP_{ex}/YFP_{em})
   In this case there is a real CFP signal but no YFP signal. Any signal in the FRET channel (CFP_{ex}/YFP_{em}) is therefore due to crosstalk of the CFP signal into this channel. This crosstalk (measured as a ratio of the CFP/YFP signal to the CFP/CFP signal) will be subtracted from the FRET signal measured in experimental cells. In our system it is usually 50-70%. Call this value 'b'.

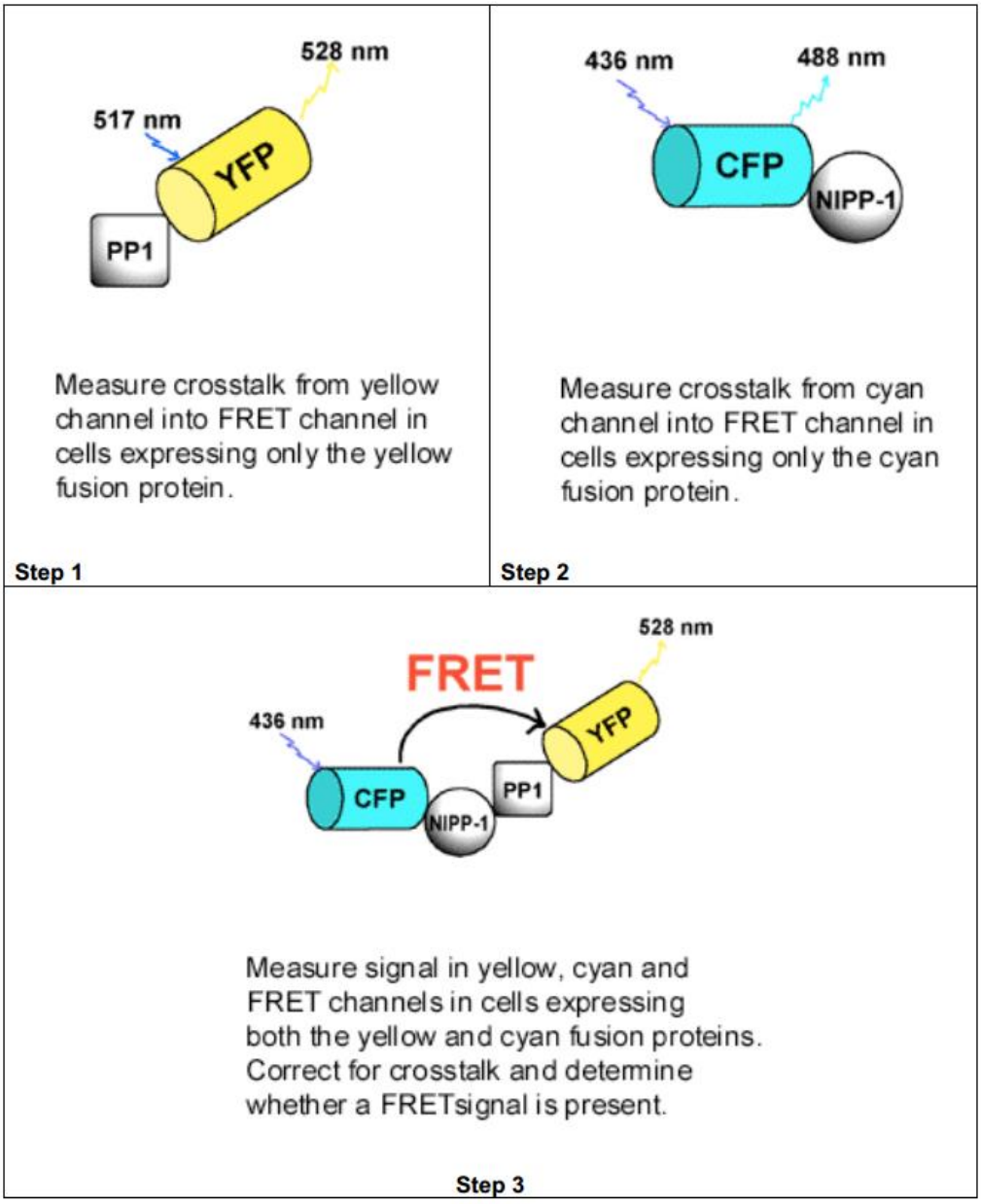
Experiment

3. Cells expressing both contracts (measure YFP_{ex}/YFP_{em}, CFP_{ex}/CFP_{em}, CFP_{ex}/YFP_{em})
   Net FRET= FRET signal – (a*YFP signal) – (b*CFP signal)
   In this case there are both YFP and CFP signals in the cell, and any signal measured in the FRET channel must have the appropriate percentages of these two signals subtracted from it. This can be done on a pixel by pixel basis at specific cellular substructures.

   Points to remember:

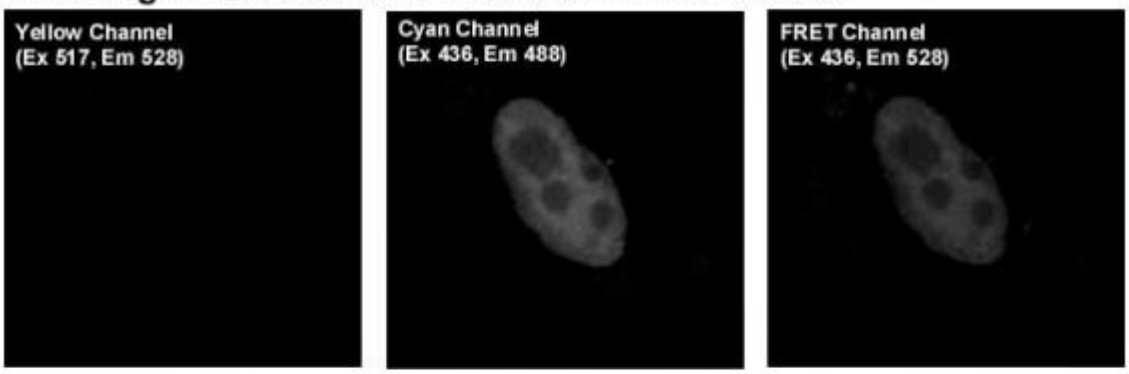
You want the same exposure time for all 3 channels. Therefore, it's important to find cells expressing the constructs in the appropriate relative levels. We find it works best if the YFP signal is equal to or slightly higher than the equivalent CFP signal for the same exposure time.

Figures: The next few pages include diagrams and data describing FRET measurements that we've made using this approach. This work has been published as:
"Dynamic targeting of protein phosphatase 1 within the nuceli of living mammalian cells"; Trinkle-Mulcahy., L.,Sleeman, J. and Lamond, A.I. (2001) J.Cell Sci. 114:4219-4228.

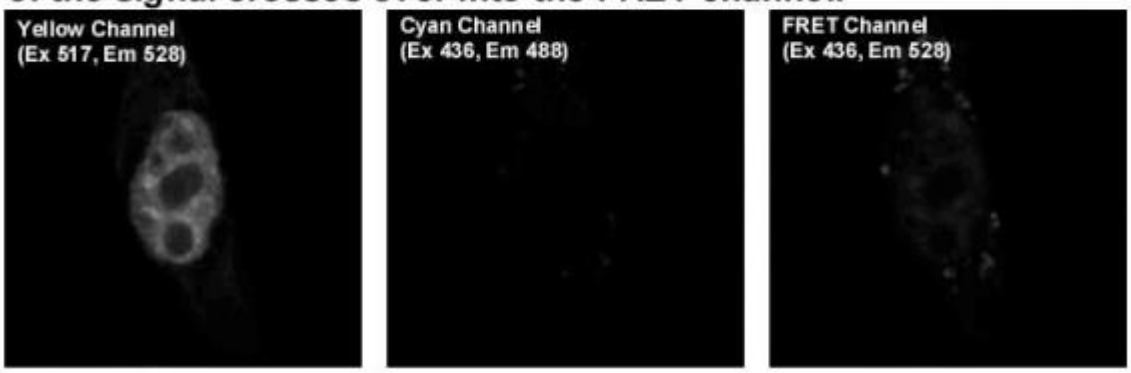


Example of crosstalk from CYP AND FYP constructs. All images are scaled the same.

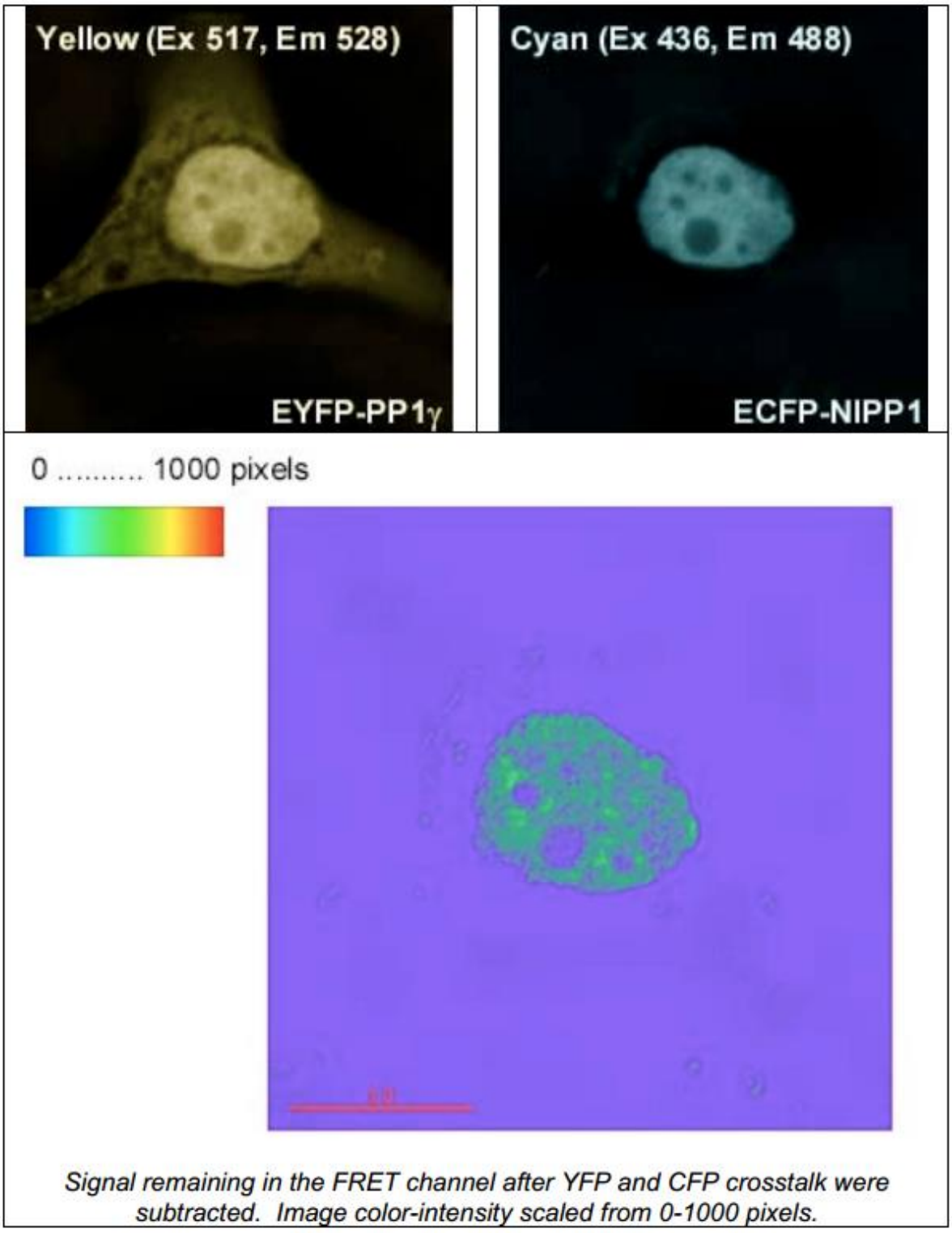
ECFP-NIPP1 expressed alone. As shown below, approximately 70% of the signal crosses over into the FRET channel.



EYFP-U1A expressed alone. As shown below, approximately 20% of the signal crosses over into the FRET channel.

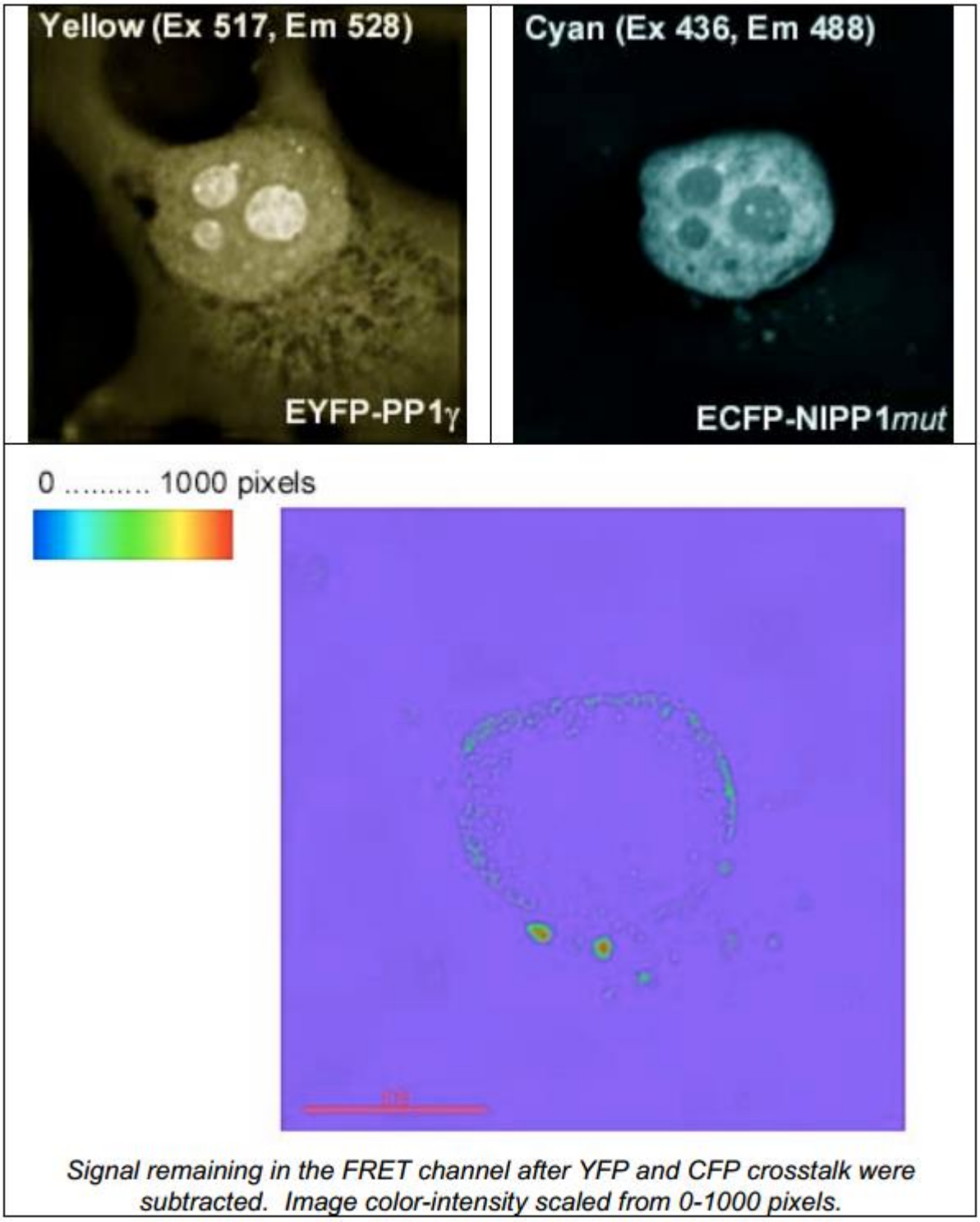


Example of a real FRET measurement. Cells are expressing both YFP-PP1g and CFP-NIPP1, and the NIPP1 has bound to and retargeted PP1 to nuclear speckles (it is normally found in the nucleous):



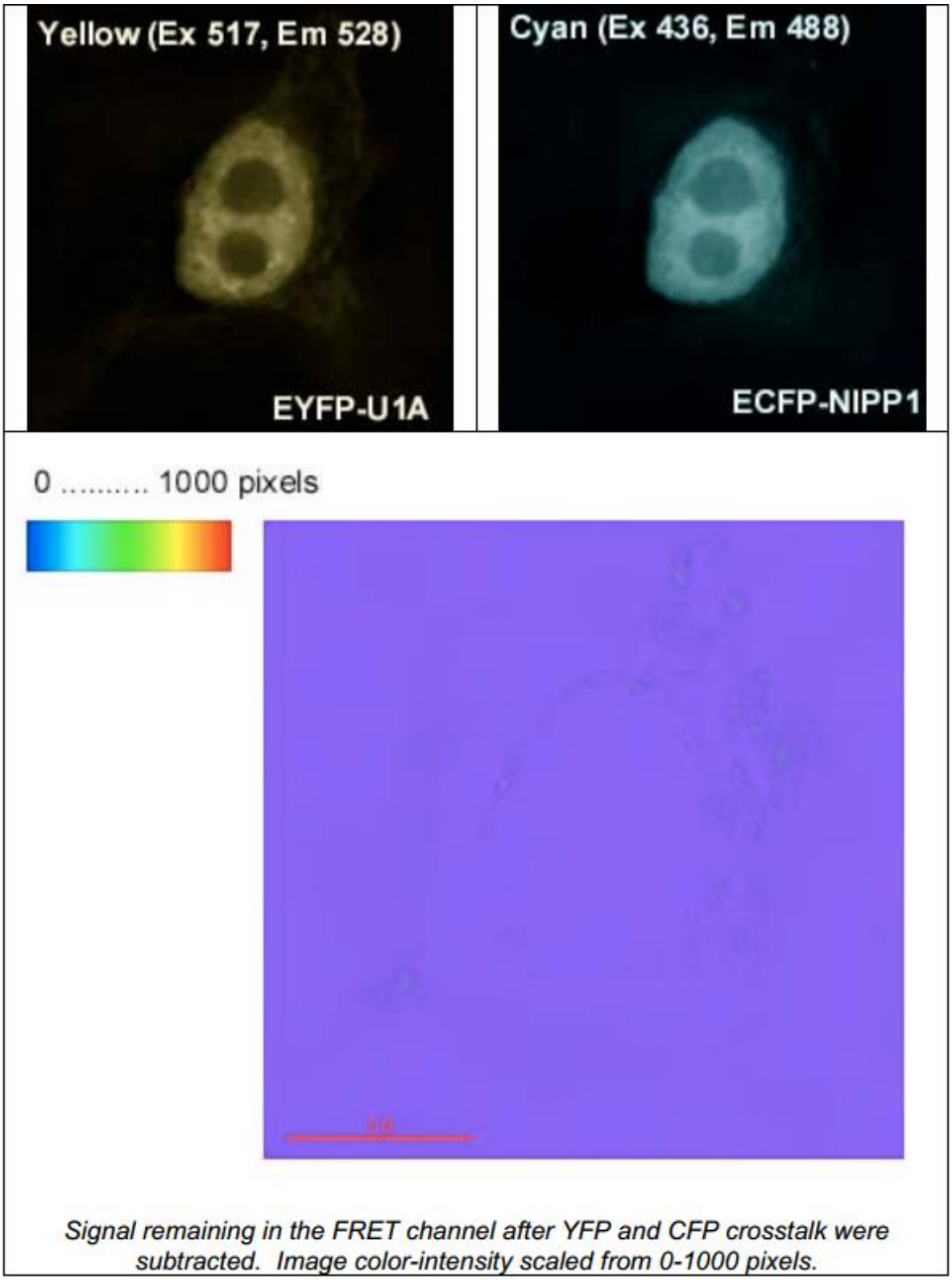
Signal remaining in the FRET channel after YFP and CFP crosstalk were subtracted. Image color-intensity scaled from 0-1000 pixels.

Negative control for the previous FRET experiment. Cells are expressing both YFP-PP1g and a mutant form of CFP-NIPP1, which does not bind PP1 and therefore cannot retarget it to speckles:



Signal remaining in the FRET channel after YFP and CFP crosstalk were subtracted. Image color-intensity scaled from 0-1000 pixels.

Another negative control for the PP1-NIPP1 FRET experiment. Cells are expressing CFP-NIPP1 and YFP-U1A (both at speckles but do not interact).



Signal remaining in the FRET channel after YFP and CFP crosstalk were subtracted. Image color-intensity scaled from 0-1000 pixels.

Decay Curves (donor photobleaching)

This type of approach is based on the idea that the donor signal will decay more slowly in the presence of an acceptor molecule, due to the introduction of FRET of an additional deactivation pathway shortening the lifetime of the excited state. Decay curves are therefore measured for CFP constructs in the presence and absence of the YFP construct, and the kinetics of photobleaching compared for both conditions. A noticeable shift to slower decay kinetics in the presence of the YFP construct indicates that FRET is occurring between the two proteins.

All that is required to do this type of experiment is a microscope with the CFP filter set. The YFP signal can be measured at the end of the experiment simply to demonstrate that it was present and where it was localised.

Procedure

Control: CFP construct alone in a cell. Measure a decay curve for this construct by setting up a time course:

1 second exposure CFP/CFP (image 1)

5 second exposure CFP/CFP (bleach)

1 second exposure CFP/CFP (image 2)

5 second exposure CFP/CFP (bleach)

1 second exposure CFP/CFP (image 3)

5 second exposure CFP/CFP (bleach)

1 second exposure CFP/CFP (image 4)

5 second exposure CFP/CFP (bleach)

1 second exposure CFP/CFP (image 5)

5 second exposure CFP/CFP (bleach)

1 second exposure CFP/CFP (image 6)

5 second exposure CFP/CFP (bleach)

The exposure times shown here are only examples. The shorter exposure is the image that you'll be monitoring, while the longer exposure is the actual bleach.

Each image can be considered a time point, as it gives the intensity of the signal over a time course of photobleaching. For a chosen region you can then plot a decay curve of the CFP signal.

Experiment: Repeat this for cells which express both the CFP and YFP constructs and compare the decay curve in the presence of an acceptor molecule.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