High-Contrast Imaging of Fluorescent Protein FRET by Fluorescence Polarization Microscopy

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ABSTRACT Detection of Förster resonance energy transfer (FRET) between fluorescent protein labeled targets is a valuable strategy for measurement of protein-protein interactions and other intracellular processes. Despite the utility of FRET, widespread application of this technique to biological problems and high-throughput screening has been limited by low-contrast measurement strategies that rely on the detection of sensitized emission or photodestruction of the sample. Here we report a FRET detection strategy based on detecting depolarized sensitized emission. In the absence of FRET, we show that fluorescence emission from a donor fluorescent protein is highly polarized. Depolarization of fluorescence emission is observed only in the presence of energy transfer. A simple detection strategy was adapted for fluorescence microscopy using both laser scanning and wide-field approaches. This approach is able to distinguish FRET between linked and unlinked Cerulean and Venus fluorescent proteins in living cells with a larger dynamic range than other approaches.

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Measurement of intracellular processes and protein-protein interactions in living cells relies extensively on green fluorescent protein-based bioassays. Many of these assays incorporate measurement of Förster resonance energy transfer (FRET) between cyan (CFP) and yellow fluorescent proteins (YFP) (1). Given the inherent sensitivity of FRET measurements to distance and orientation (2, 3), the changes in energy transfer are difficult to measure between FRET partners with broadly overlapping spectra, such as fluorescent proteins. Cross-talk excitation of the donor YFPs also leads to ''false positive'' indication of FRET, and serves as a barrier for adaptation of CFP:YFP FRET-based assays to high-throughput assays. Although numerous corrective algorithms and methods have been developed to handle this problem (4), these methods are generally difficult to apply and can introduce additional error into the measurement of FRET. These methods also require extensive controls, which gives rise to additional data-handling and storage issues when adapted to a high-throughput approach. Thus, an optimal solution would require collection of a minimal set of images and corrective processing to determine the presence of FRET.

To devise a simple method for detecting CFP:YFP FRET and to eliminate the observance of false positive results stemming from cross-talk excitation of YFP, we have taken advantage of the large size and slow rotational diffusion of fluorescent proteins. Fluorescence emission from fluorescent proteins is highly polarized, as indicated by steady-state anisotropy values of $\sim$0.296 for the monomeric variant of Cerulean (mCerulean) (5–7) (Fig. 1). Sensitized emission from an acceptor fluorescent protein (mVenus) (8) conjoined to mCerulean is depolarized ($r = 0.145$ at 525 nm). Because anisotropy is typically measured to an accuracy of $>$0.001 in cuvettes, and 0.01 in a microscope, this approach offers a dynamic range from 15 to $>$150. In comparison, fluorescence lifetime imaging of the same FRET pair shows changes of $\sim$0.6 ns with resolution of 0.1 ns for a dynamic range of $<$10.

Sensitized emission is highly depolarized because the orientation of emitted light is not wholly constrained by the excitation polarization. Thus, sensitive and high-contrast isolation of sensitized emission is achieved. This approach has the particular advantage of eliminating false positives, because systematic artifacts, such as cross-talk excitation of the FRET acceptor, results in increased polarization. This is consistent with our observation that the greatest contrast in polarization occurs at wavelengths that have been optimized for exclusive donor excitation. For single-photon excitation, wavelengths $<$425 nm work best, whereas 800 nm is optimal for two-photon excitation of the Cerulean:Venus FRET pair.

We were able to adapt fluorescence polarization microscopy (9) for measurement of FRET using either a wide-field or laser scanning approach. As a cautionary note, adaptation of fluorescence microscopes for polarized light microscopy is not always straightforward due to the impact of existing optics on fluorescence polarization. Vertically polarized excitation of the donor was used in combination with capture of both horizontal (*VH*) and vertical polarizations (*VV*).

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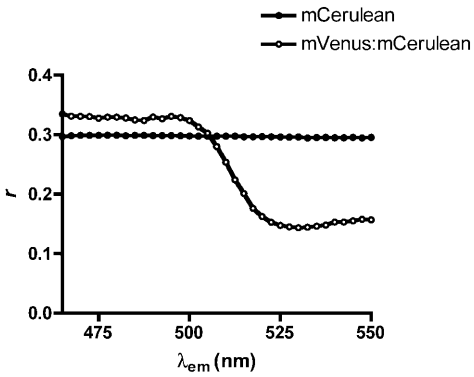

FIGURE 1 Steady-state anisotropy (*r*) was calculated from fluorescence emission spectra (425 nm excitation) for solutions containing the FRET donor, mCerulean (●) and a fusion protein containing the FRET acceptor, mVenus, linked to mCerulean (○). FRET between mCerulean and mVenus results in increased mCerulean (475 nm) anisotropy from a decreased fluorescence lifetime, and depolarized sensitized emission from mVenus (525 nm). Analysis of recombinantly generated proteins was performed as previously described (6).

Anisotropy (r) images were then calculated using the equation:

$$r = \frac{VV - gVH}{VV + 2gVH}$$

where the g corrects for polarization bias in the instrument (9, 10).

Wide-field and laser scanning fluorescence polarization microscopy was used to image COS7 cells expressing mCerulean, cotransfected mCerulean and mVenus, or a linked mVenus:mCerulean FRET pairing (Fig. 2). The polarization of sensitized emission was examined (520–550 nm for wide field; 515–540 nm for laser scanning) under conditions of vertically polarized donor excitation (425–445 nm for wide field; 800 nm for laser scanning two-photon excitation). Bleed-through emission of the donor mCerulean fluorescence is highly polarized, and thus gives a high anisotropy value using both approaches. Anisotropy values from two-photon excitation is slightly higher and results from more highly polarized excitation that is inherent to two-photon absorption. Cotransfection of mCerulean with the mVenus acceptor did not affect fluorescence polarization. Even under conditions of direct excitation of the mVenus, the fluorescence would remain highly polarized. We observe a reduction in fluorescence anisotropy only in the presence of FRET from linked mVenus and mCerulean.

Measurement of changes in fluorescence anisotropy has been previously used to quantify changes in homotransfer, but its application to the measurement of heterotransfer has been limited to a general approach with more complicated corrections (4, 11). Thus, measurement of polarized sensitized emission has been overlooked as a simple and high-contrast method for determining the presence of FRET. This approach works best when donor fluorescence is highly polarized, as is the case for FRET between fluorescent proteins. In addition, this method of FRET detection is particularly resistant to errors that produce false positives in other assays, including changes in acceptor concentration and inner filtration effects. Therefore, this method is particularly well suited for adaptation for high-throughput screening.

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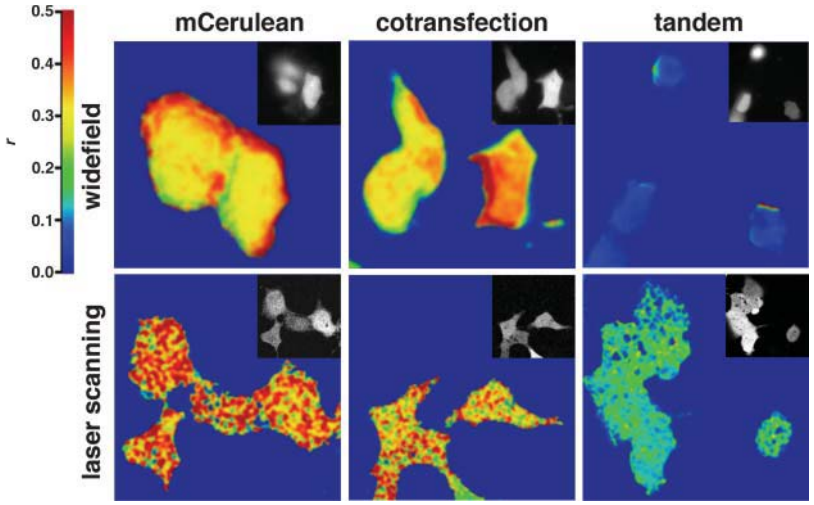

FIGURE 2 Reduction in anisotropy occurs only during FRET. COS7 cells expressing mCerulean, unlinked mVenus and mCerulean, or a linked mVenus:mCerulean fusion protein (*tandem*) were imaged using polarized wide-field excitation or two-photon laser scanning microscopy. Anisotropy images (pseudocolored) were calculated from sensitized emission images taken at parallel (*inset*) and perpendicular polarizations. In wide-field images, ratiometric processing of regions with very low signal introduced high anisotropy values at the cellular edges.

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