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Fluorescence resonance energy transfer microscopy as demonstrated by measuring the activation of the serine/threonine kinase Akt

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Abstract

This protocol describes procedures for performing fluorescence resonance energy transfer (FRET) microscopy analysis by three different methods: acceptor photobleaching, sensitized emission and spectral imaging. We also discuss anisotropy and fluorescence lifetime imaging microscopy-based FRET techniques. By using the specific example of the FRET probe Akind (Akt indicator), which is a version of Akt modified such that FRET occurs when the probe is activated by phosphorylation, indicating Akt activation. The protocol provides a detailed step-by-step description of sample preparation, image acquisition and analysis, including control samples, image corrections and the generation of quantitative FRET/CFP ratio images for both sensitized emission and spectral imaging. The sample preparation takes 2 d, equipment setup takes 2–3 h and image acquisition and analysis take 6–8 h.

INTRODUCTION

Advanced light microscopy techniques are now providing researchers with the tools necessary for measuring protein-protein interactions and protein activation temporally and spatially across the cell or within small organisms. On a larger scale, multicolor microscopy allows one to image the dynamics of organelles, vesicles and entire cells along with the ability to observe changes on a subcellular, cellular and organismal level. Quantitative light microscopy techniques, including FRET, provide the tools necessary to begin to dissect submicroscopic molecular interactions within cells and organisms with high spatial and temporal resolution. In fact, FRET-based biosensors are now available to measure the activity of many highly dynamic biological molecules in living samples^{1–3}. In this protocol, we present procedures for FRET microscopy that we have used to show that the expression of the adaptor protein APPL1 leads to a marked decrease in the amount of active Akt, particularly at the cell edge⁴. As an example, to illustrate the procedure, we compare the intramolecular Akind FRET probe and the nonactivatable mutant (3A-Akind), in which the FRET activity is markedly reduced.

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AUTHOR CONTRIBUTIONS C.M.B. and J.A.B. developed and implemented the sensitized emission protocol, prepared the majority of the figures and drafted the manuscript. C.M.B. developed and implemented the spectral imaging protocol. J.A.B. performed all of the image analysis. D.J.W. contributed to the writing of the manuscript and provided substantial scientific feedback. B.R. participated in writing the manuscript, collected data and designed the FRET-FLIM introductory figures.

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FRET

FRET is also called Förster resonance energy transfer after the German scientist Theodor Förster, who originally described the phenomenon^{5–7}. The FRET process involves a transfer of energy from one fluorophore in the excited state to a second fluorophore through a nonradiative transfer of energy (i.e., no light is given off)⁸. FRET experiments with fluorescent proteins (FPs) rely on measuring the amount of acceptor protein emission (e.g., Venus, a variant of yellow FP, YFP) after excitation of the donor (e.g., cyan FP, CFP)⁹. In this case, after excitation of CFP, the emission light will be cyan; however, if energy is transferred to an acceptor protein, such as Venus, then yellow fluorescence will also be observed (Fig. 1), even though the yellow protein was not directly excited by a laser or lamp. For efficient FRET to occur, there must be a substantial overlap between the donor fluorescence emission spectra and the acceptor fluorescence excitation (or absorption) spectra (Fig. 1a)^{10,11}. FRET measurements are often termed ‘molecular rulers’ because FRET is only efficient when two fluorophores are within 2–10 nm of one another (Fig. 1b). In fact, the efficiency of energy transfer is highly dependent on the distance between the two proteins, as it is inversely related to this distance to the sixth power (<http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fret/fretintro.html>).

FRET probes

One of the most efficient pairs of cyan and yellow FPs are CyPet and YPet¹²; however, protein constructs with many variants of cyan and yellow proteins are typically used. With the development of brighter and more photostable red FPs, the use of EGFP and red FPs such as monomeric Cherry (mCherry) is becoming more common¹³. Several reviews discuss the currently available FP constructs^{14–22}. FRET is often used to examine interactions between two distinct protein binding partners through intermolecular FRET, with one binding partner containing the donor and the other partner containing the acceptor. Differential expression of the donor and acceptor FPs in each cell must be taken into account, as variable protein levels can alter the FRET results^{23,24}. Alternatively, FRET can be used as a tool to observe conformational changes in protein structure after activation. Conformational changes can be the result of post-translational modifications such as phosphorylation or other events including protein or membrane binding. These changes in conformation can be exploited in the construction of intramolecular FRET probes, in which both the donor and acceptor fluorophores are present within the same molecule (Fig. 2). Under basal conditions, the two fluorophores are far enough from each other that no FRET occurs (Fig. 2a). However, after activation, conformational changes within the protein bring the two fluorophores close together and FRET occurs (Fig. 2b). Intramolecular FRET biosensor probes circumvent some of the issues of variable expression levels, as the donor and acceptor FPs are expressed together in a one-to-one ratio as part of the same molecule. These probes can be designed to be very sensitive to conformational changes in a protein after ligand binding, phosphorylation, enzymatic cleavage^{22,25–29} and even in response to molecular force or binding of metals, such as zinc, which results in a gain or loss of FRET (Table 1)^{22,25–31}.

Here the intramolecular Akind FRET probe is used to detect Akt activation in cells. Akind is composed of the Akt pleckstrin homology domain at its N terminus, followed by Venus FP, then the Akt catalytic domain and finally CFP at the C terminus (Fig. 2)³⁰. Akt is recruited to the plasma membrane through its pleckstrin homology domain, where it binds to phosphatidylinositol (3,4,5)-trisphosphate (PIP₃) (ref. 31). It is subsequently activated by phosphorylation at two conserved residues, Thr308 and Ser473, within its catalytic domain³². This activation causes a conformational change, which brings the donor and acceptor molecules within the Akind probe into close proximity, thereby allowing FRET to occur (Fig. 2b).

Techniques for measuring FRET in microscopy

There are five basic techniques that can be used in FRET microscopy; each technique will be introduced here (for reviews see refs. 23,33–39). For simplicity, we will discuss FRET in terms of CFP as the donor fluorophore and Venus FP as the acceptor fluorophore. However, any suitable donor and acceptor fluorophore pair can be substituted.

Acceptor photobleaching

Acceptor photobleaching is the first FRET technique that should be applied to all experimental systems. It can quickly verify the presence of FRET before one proceeds to optimizing other FRET measurements. It is notable that this technique is most applicable to fixed cells or tissues or for live-cell experiments in which unbleached acceptor molecules do not quickly diffuse back into the bleached region. If cells are live and proteins move quickly, the entire cell can be bleached and compared with a nearby cell in the same field of view that was not bleached. However, care must be taken, as phototoxicity can be high for such an experiment, in which case using fixed cells may be more relevant than working with compromised living samples. The principle of acceptor photobleaching is that in the absence of the acceptor no FRET can occur. Therefore, there is a resulting increase in the direct emission, i.e., intensity of the donor signal following photobleaching of the acceptor⁴⁰. CFP images are taken before and after photobleaching of the Venus FPs within a region of interest (ROI; Fig. 3a). The images are corrected for background intensities and field nonuniformity and the CFP fluorescence intensity is quantified. We found that quantification showed a significant increase in the CFP intensity after Venus bleaching when compared with a control region where Venus was not bleached, confirming a FRET signal (Fig. 3b). Note that after photobleaching the CFP intensity increase can be small, and thus can often be difficult to measure or visualize by eye (Fig. 3a). This is expected, as the FRET efficiency with FPs is 10–40% at best and the human eye cannot visualize such small intensity differences. Acceptor photobleaching should also be conducted on a negative FRET control to verify that no increase in donor fluorescence is observed (Table 2).

If no increase in donor intensity is seen with the FRET samples after acceptor photobleaching, see the 'FRET pitfalls' section for possible reasons. Photobleaching on a confocal laser scanning microscope (CLSM) is relatively straightforward and most software platforms provide a simple interface to bleach an ROI. On a wide-field microscope, the fluorescence field diaphragm can be closed and used for localized photobleaching. In fact, gradual acceptor photobleaching can be used to measure FRET efficiencies using a wide-field platform⁴¹. It is important that acceptor photobleaching conditions are optimized to ensure that the donor is not also being bleached.

Sensitized emission

The sensitized emission FRET technique is based on measuring FRET from a series of sample and control images (Table 2). Images of the donor and the FRET signal are collected and corrected for background intensity, noise and intensity contributions that do not arise directly from the FRET signal. The resultant images are then presented as a ratio image of FRET/donor, and thus another name for this technique is ratio-based FRET. As the FRET signal goes up, the donor signal goes down and the ratio of FRET/donor will increase.

Sensitized emission is probably the most commonly used form of FRET microscopy, as it can be conducted on a standard wide-field microscope, making it affordable. Because the image collection is fast, it is also ideal for live-cell microscopy. For wide-field sensitized emission FRET microscopy, three fluorescence cubes are required: a CFP cube, a Venus cube and a FRET cube (containing a CFP excitation filter, a dichroic mirror for CFP excitation and a Venus emission filter). If excitation and emission filter wheels are available,

then two filter cubes are required, one with a dichroic mirror for CFP excitation and one with a dichroic mirror for Venus excitation. The excitation and emission filters within the filter wheels are then rotated into place as needed.

In the protocol presented here, the images were collected using a CLSM. In this case, the excitation laser lines are varied to excite either CFP or Venus, and the emission characteristics are adjusted for collecting either CFP or Venus fluorescence emission. The sensitized emission FRET ratio image calculations are the same regardless of whether a wide-field microscope or a CLSM is used to collect the images.

Sensitized emission FRET controls

To properly calculate the FRET/CFP ratio images, control samples and control images are needed. Below is a description of the control samples that are required and/or recommended (see Table 2 for a summary of required and recommended samples for the various FRET techniques). CFP, Venus and FRET images should be collected using the same instrument settings for control and experimental samples. A 1- to 2-h laser warm-up period is required to ensure laser power stability during imaging⁴².

- i. *Unlabeled cells.* Images of the CFP, Venus and FRET signals from unlabeled, untransfected cells should be acquired. This will provide a measure of cellular autofluorescence. There is no need to correct for autofluorescence if it is <5% intensity relative to the specific CFP, Venus or FRET signal. If autofluorescence is more than 5%, it is best to correct for it using the spectral imaging protocol in PROCEDURE Steps 68–75. This sample is required.
- ii. *Cytosolic CFP-expressing cells.* The CFP and Venus emission spectra overlap, and thus some of the CFP emission will be detected in the FRET image channel (emission cross talk), giving a false positive FRET signal. Cells expressing only cytosolic CFP are used to measure the extent of the CFP emission cross talk and to calculate a correction factor that is used to remove the CFP signal within the FRET images. This correction factor is specific for the exact instrument settings. It is best to use a cytosolic protein for cross talk controls, as clustering into cellular structures can lead to FRET artifacts from high local FP concentrations. This sample is required.
- iii. *Cytosolic Venus-expressing cells.* The Venus FP is often directly excited by the CFP excitation light source (excitation cross talk), resulting in a signal in the FRET image channel that is not a direct result of FRET between the donor and acceptor. Cells expressing only cytosolic Venus are imaged to correct for excitation cross talk and to calculate a correction factor for the Venus signal in the FRET image. Again, this correction factor is specific to the exact instrument settings. It is also dependent on the excitation source used for CFP. For example, the excitation cross talk will be more substantial for the 458-nm laser excitation of CFP than for the 405-nm excitation (Fig. 4). This sample is required.
- iv. *Unlinked CFP- and Venus-expressing cells.* Cells expressing both the unlinked donor and acceptor are used as a negative control. Images from this sample are put through the image processing and analysis steps and no FRET signal should be observed. It is best to use proteins that do not localize into clusters or organelles. This sample is recommended but not required.
- v. *Linked CFP- and Venus-expressing cells.* Cells expressing CFP and Venus attached by a short linker (5–7 aa) act as a positive FRET control, because the two FPs will be within 10 nm of one another⁴³. Images from this sample are put through the image processing and analysis steps and the resulting FRET image should show a

high FRET signal. Plasmids for different positive FRET probes are available from AddGene (<http://www.addgene.org>). This sample is recommended but not required.

- vi. *Biological controls.* We recommend using biological controls that will show no or low FRET and high FRET.

Sensitized emission image processing and analysis

To calculate the FRET/CFP ratio image, it is necessary to go through a number of image processing steps. The details of the steps will be shown within the protocol, but the overall concepts for the corrections will be presented here. As with any quantitative image processing, all of the images must first be corrected for any field nonuniformity in the sample excitation and for any background intensity within the images⁴⁴. In our case, the illumination field is very uniform, because we use a CLSM with a $\times 63/1.4$ numerical aperture (NA) Plan Apo oil immersion lens and an image zoom factor of 2. Therefore, no correction is necessary. The back-ground correction is done by measuring the average intensity of an ROI in the image where there are no cells and subtracting this intensity from each pixel gray value within the image. All images ($FRET_{Raw}$, Venus and CFP) need to be corrected for back-ground and field nonuniformity (if necessary) before applying any calculations.

Correction factors for the Venus direct excitation cross talk (A) and the CFP emission cross talk (B) also need to be calculated. After this, the corrected FRET image ($FRET_{Corr}$) can be calculated on the basis of equation (1).

$$FRET_{Corr} = FRET_{Raw} - A * Venus - B * CFP \quad (1)$$

$FRET_{Raw}$ is the original FRET image collected on the microscope. A is the percentage of Venus excitation cross talk in the FRET image calculated from the control Venus sample. $Venus$ is the image of Venus expression in the sample after direct excitation by the 514-nm laser line. B is the percentage of CFP emission cross talk in the FRET channel calculated from the control CFP sample. CFP is the image of the sample collected in the CFP channel. The ratio image is then calculated from equation (2):

$$\frac{FRET}{CFP} = \left[\frac{\frac{FRET_{Corr} \times 1,000}{I_{Max}(FRET_{Corr})}}{\frac{CFP \times 1,000}{I_{Max}(CFP)}} \right] \times 1,000 \quad (2)$$

The numerator is essentially the normalized $FRET_{Corr}$ image and the denominator is the normalized CFP image. I_{Max} is the maximum gray value in the $FRET_{Corr}$ (numerator) or CFP image (denominator), which is calculated after image processing with a medium filter. This is done to avoid I_{Max} simply corresponding to a noisy pixel within the image. Images are based on integer values, and thus the factor of 1,000 is included to avoid fractional pixel intensities in the resultant ratio image. Alternatively, a floating-point image could be used.

Small changes in FRET are highlighted by the ratio image, because as the amount of FRET signal increases (increasing numerator) the amount of CFP signal correspondingly decreases (decreasing denominator). Maximal FRET efficiencies for FPs are typically on the order of 10–40% and the Förster distance (distance at which FRET efficiency is 50% of maximal) is ~5 nm (ref. 45), mainly because of the physical size and protection that the β -barrel structure within the FPs offers the fluorophore⁴⁶.

The ratio image for intramolecular FRET is often expressed without correcting the FRET image for cross talk. In principle, this is acceptable because the expression level of the donor

and acceptor is always 1:1. However, if the cross talk is substantial, the changes in FRET efficiency across and between samples can be minimized and possibly undetectable. Therefore, correcting the images using equations (1) and (2) can make subtle changes in FRET efficiency more readily apparent. In light of this, we recommend using the corrected $FRET_{Corr}$ image even for intramolecular FRET probes, especially when working with spectrally similar dyes such as CFP and Venus, which show considerable cross talk.

Spectral imaging

Spectral imaging can be used in combination with sensitized emission. Rather than collecting separate CFP and FRET images using band-pass filters and correcting them for CFP emission cross talk, the entire fluorescence emission spectrum can be collected (Fig. 5a; <http://zeiss-campus.magnet.fsu.edu/articles/spectralimaging/introduction.html>)^{47–50}. It is crucial to measure control spectra for CFP alone, Venus alone and for cellular autofluorescence from high signal-to-noise (S/N) ratio images (Fig. 5b). These spectral shapes are used in the unmixing process, during which an algorithm calculates what proportion of the signal in each image pixel is from autofluorescence, CFP and Venus (FRET) emission. After unmixing, a new image stack with three images, one for each of these signals, is generated (Fig. 5c).

The CFP (CFP_{Spec}) and FRET ($FRET_{Spec}$) images can then be used to calculate the FRET/CFP ratio image using equation (2) and substituting $FRET_{Corr}$ with $FRET_{Spec}$ and CFP with CFP_{Spec} . The spectral images still need to be corrected for nonuniform illumination, background intensity and Venus excitation cross talk (equation (1)) before the ratio image can be calculated. Corrections for CFP emission cross talk are no longer a factor, as the CFP signal is separated from the FRET image through the spectral unmixing process.

The main advantage of spectral imaging is that sensitivity is improved, as all of the light collected by the system can be detected. However, the emission light must be separated over many detector channels (24 in this case), and thus each image within the spectral data set can be noisy (Fig. 5a). The instrument settings may need to be modified (e.g., longer pixel dwell times, lower photomultiplier tube (PMT) gain, higher laser power, image or line averaging, smaller image size or region) in order to have adequate S/N in each emission channel when measuring the entire spectrum. Another advantage of spectral imaging is the ability to remove contributions from cellular autofluorescence, which can be high in certain cell types and small organisms. The main disadvantage of spectral imaging is that specialized confocal instrumentation is required. The system used here has a 32-array PMT detector, and thus all 24 images are collected simultaneously, allowing for rapid imaging and a single pass of the laser excitation. It should be noted that some spectral imaging confocal systems use slit-based scanning with one or two PMT detectors. Spectral imaging on these systems is slow, as only one or two spectral windows can be imaged at a time. In addition, the sample must be scanned multiple times for each spectral window, which increases photobleaching and phototoxicity, making these systems less ideal for live imaging.

Fluorescence anisotropy

Fluorescence anisotropy can be used to detect the rate of rotation of fluorophores⁵¹. High anisotropy indicates a slow rotation of a fluorophore, whereas low anisotropy indicates a fast rotation. If the fluorophore is excited with polarized light and the fluorophore rotates during the time that the molecule is in the excited state, then the difference between the polarity of the emission light and the excitation light will provide information as to how far the fluorophore rotated while the molecule was in the excited state⁵². The fluorophore within an FP is contained within a large β -barrel structure⁵³, and thus the molecule does not rotate

markedly during the few nanoseconds that the fluorophore is in the excited state⁵². However, if a fluorophore undergoes FRET, the polarity of the emission light will be dependent on the orientation of the acceptor fluorophore. Therefore, if the donor is excited and emits fluorescence directly, the polarity does not change, but if the donor gives its energy to an acceptor there is a change in polarity and a decrease in anisotropy. To perform fluorescence anisotropy-based FRET, the light source must be polarized such that fluorophores of a certain polarity (horizontal or vertical) are selectively excited. The lasers that are part of CLSMs are inherently polarized. For wide-field systems, a polarization filter is placed between the light source and the sample⁵². A variable polarizer is then placed in front of the detector and two images of the acceptor emission light are collected. The first image is of the donor emission light parallel to the excitation light ($I_{\parallel}$), and the second image is of the donor emission light perpendicular to the incident polarized light ($I_{\perp}$). The anisotropy (r) is calculated from equation (3). Lower anisotropy indicates higher FRET.

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad (3)$$

The main advantages of anisotropy-based FRET is that the measurements can be fast; in addition, it is relatively inexpensive to add polarizers to a system. This speed makes it ideal for live microscopy⁵⁴ or high-content screening applications⁵⁵. The main disadvantage is that it is not very sensitive to the FRET efficiency. Thus, it can give a qualitative FRET or no FRET result, but cannot be used to measure small changes in FRET from sample to sample or across a single cell.

Fluorescence lifetime imaging microscopy (FLIM)

The fluorescence lifetime of a molecule is the average time it spends in the excited state before giving off a fluorescence photon and returning to the ground state. This lifetime is highly dependent on the local environment surrounding the fluorophore and is sensitive to the refractive index of the medium⁵⁶, the pH⁵⁷, the presence of ions nearby or the presence of an acceptor molecule. In contrast to the fluorescence intensity, the lifetime is independent of the fluorophore concentration and is less affected by photobleaching.

FLIM is an imaging technique that generates an image based on the spatial distribution of the fluorophore lifetime in different locations across the sample rather than the intensity of the fluorophore^{58,59}. The fluorescence lifetime of a fluorophore depends on both radiative (i.e., fluorescence) and nonradiative processes, including quenching of the donor fluorescence by FRET⁸. Unlike sensitized emission, FLIM only requires the measurement of the lifetime of the donor fluorophore, and thus only images of the CFP fluorescence need to be collected.

Time-correlated single-photon counting (TCSPC) is commonly used to measure fluorescence lifetimes. The technique relies on a pulsed laser light source exciting the sample at a high frequency and a detector that measures the time taken for each photon to be emitted after the pulse. The total photon count, displayed in different time bins, creates a decay curve (Fig. 6a, green and red curves). This decay curve is also convoluted by the instrument response function (Fig. 6a, black curve), which must be measured and deconvoluted from the fluorophore decay curve (detailed methods can be found in Sun *et al.*⁶⁰ or Szabelski *et al.*⁶¹) before fitting the curve to measure the fluorescence lifetime. FLIM systems are typically coupled with a CLSM and will generate an image of the fluorescence lifetime for each pixel location (Fig. 6b). Spatial binning is usually needed to obtain enough photon counts to fit the lifetime decay curve for each pixel location.

When an acceptor molecule is present, FRET, which is a quenching process, will decrease the excited states of the donor fluorophore, thereby shortening the lifetime. For example, when EGFP is expressed alone, its lifetime is longer than when it is coupled to mCherry by a short amino acid linker. This change in lifetime is apparent in the TCSPC lifetime curves (Fig. 6a). The mCherry FP acts as an acceptor, FRET occurs, and the EGFP fluorescence life-time is significantly reduced (Fig. 6b). Care must be taken when performing FLIM experiments on fixed samples, as the local environment of the fluorophore can drastically change the fluorescence lifetime of the donor. In fact, when EGFP-expressing samples are mounted using either ProLong Gold or Fluoro-Gel mounting medium, the fluorescence lifetime decreases in the absence of the acceptor (Fig. 6c). Under these conditions, changes in the fluorescence lifetime while in the presence of an acceptor (mCherry) are completely masked (Fig. 6c), and the FRET efficiency is minimal at only ~5% (Fig. 6d). FLIM-FRET is quantified by calculating the energy transfer efficiency (E_{FRET}) from the lifetime information as follows:

$$E_{FRET} = 1 - \frac{\tau_{DA}}{\tau_D} \quad (4)$$

where τ_{DA} is the mean lifetime of the donor in the presence of acceptor and τ_D is the mean lifetime of the donor alone.

FLIM can also be performed using frequency-domain techniques in which the change in the frequency and the magnitude of the emission light, relative to the excitation light, reflect the amount of FRET occurring⁶². For a comparison of TCSPC and frequency-domain FLIM, see the work of Gratton *et al.*⁶³.

FRET pitfalls

Small intensity changes—It should be noted that even highly efficient intramolecular FRET probes designed with two FPs will only give rise to small FRET efficiencies of 10–40% (<http://micro.magnet.fsu.edu/primer/techniques/fluorescence/fret/fretintro.html>). These small intensity values can be challenging to measure using fluorescence microscopy. This is especially true for sensitized emission studies of spectrally similar probes (required for FRET), where contributions from excitation and emission cross talk can be considerably higher than FRET signals. Therefore, images must be collected with high S/N ratios, mathematical operations must be done carefully and instruments must be performing well. Instrument quality control is crucial⁴². For example, if laser powers are fluctuating on the scale of 10–20% during image acquisition and between successive images, then FRET signals may be lost.

False negatives—If two proteins or an intramolecular probe do not give rise to any FRET signal, there are several possible reasons—besides the fact that the proteins are not interacting or the protein is not changing conformation—for the absence of a FRET signal. It is possible that the two fluorophores are not close enough to interact, despite the proteins interacting or the probe changing conformation. It is also possible that the fluorophores are close together, but that their dipoles are not properly aligned relative to one another (Fig. 1c). If they are perpendicular to one another, then no energy transfer will occur. Finally, FRET may not occur because of quenching of fluorescence caused by the local environment (e.g., pH, high dye concentration, mounting medium)^{59,64}. Therefore, negative FRET results are not very informative.

False positives—Expression levels of FPs should be maintained at physiologically relevant levels to avoid biological changes due to overexpression. In addition, at high

protein concentrations within a localized area, FRET can occur between adjacent, noninteracting fluorophores, which can give rise to false positive FRET signals²³.

FRET techniques: advantages and shortcomings

Acceptor photobleaching is straightforward and should be done for all FRET experiments. The main downfall of acceptor photobleaching is that it is an irreversible endpoint assay.

Sensitized emission has the advantage of fast image collection, making it readily applicable to live-cell measurements. It is also affordable, because it can be implemented on a relatively inexpensive wide-field microscope equipped with the appropriate filter cubes. It is important to note that the FRET signal is weak compared with the cross talk contributions; therefore, it is essential to collect high-S/N-ratio images and to perform the image corrections carefully. This requires a large number of control samples and images for the extensive image processing steps. In this case, the resultant FRET images are noisy, as noise is introduced at each image processing step.

Anisotropy-based FRET measurements are very fast, making them the ideal choice for high-throughput microscopy applications involving FRET^{37,52,54}. Anisotropy is also affordable, only requiring a polarized light source and a variable polarizer before the camera-based detector. In general, anisotropy measurements are ideal for a qualitative, FRET or no FRET type of result, but do not provide highly accurate FRET efficiencies. That being said, if anisotropy is combined with FLIM, subtle changes in FRET efficiencies can be detected and a great deal of information can be ascertained about protein-protein interactions⁶⁵.

FLIM is very sensitive and is relatively independent of the acceptor and donor concentrations. It is also less sensitive to fluorophore bleaching, resulting in more accurate results over time. FLIM-FRET can also estimate the percentage of the interacting and noninteracting donor populations, a parameter that cannot be measured by most of the intensity-based approaches. However, the data acquisition for TCSPC FLIM is slow (minutes time scale), which makes it difficult to apply to living samples at physiological temperatures. FLIM requires expensive, highly specialized equipment and is more sensitive to many aspects of the sample environment. This includes the mounting medium, temperature and the local environment of the fluorophores.

Development of the protocol

This protocol provides information on how to perform FRET analysis using the Akind probe by acceptor photobleaching, sensitized emission and spectral imaging on a CLSM. We have previously used the sensitized emission technique to characterize the effects of the adaptor protein APPL1 on Akt activity¹. APPL1 expression in HT-1080 cells led to a substantial decrease in the total amount of active Akt, as determined by a reduction in Akind FRET signal. Furthermore, using line-scan analysis, we showed that APPL1 expression leads to a decrease in active Akt specifically at the cell edge, where Akt is typically activated. The protocol provides step-by-step details on the necessary control samples and the extensive image collection and corrections required for sensitized emission measurements. There is also a focus on the image-processing corrections and details on how to generate accurate ratio imaging results. Some protocols have been previously published on different aspects of FRET, including biosensors^{25,26,65,66}; single-molecule techniques⁶⁷; protein-protein interactions^{60,68,69}; measuring cytosolic metals such as zinc⁷⁰; and studies of DNA structure⁷¹ and kinetics⁷². However, to our knowledge, this is the first FRET protocol to provide step-by-step details on how to perform spectral imaging FRET. The conditions used in all of the protocols can be applied to both fixed and live cells. In particular, the CLSM settings used for sensitized emission are optimal for minimal sample damage (i.e.,

phototoxicity), which is necessary for live-cell applications. In fact, the main advantage of single-chain intramolecular FRET probes is that they can be used to measure dynamic changes in protein activation in living samples with high spatial resolution.

MATERIALS

REAGENTS

- Tissue culture dishes, 100 mm (Corning, cat. no. 430167)
- Tissue culture dishes, six-well (Corning, cat. no. 3516)
- Fibronectin (FN, Sigma, cat. no. F0895)
- Akind FRET probes were kindly provided by Michiyuki Matsuda (Kyoto University, Kyoto, Japan). See <http://www.lif.kyoto-u.ac.jp/labs/fret/e-phogemon/index.htm> for more information. Venus-C1 was a kind gift from Atsushi Miyawaki (Riken, Japan). pECFP-C1 was obtained from Clontech (discontinued, cat. no. 6076-1)
- HT-1080 cells (American Type Culture Collection, cat. no. CCL-121)
- Dulbecco's Modified Eagle Medium (DMEM; Invitrogen, cat. no. 11965-118)
- FBS (HyClone, cat. no. SH30396.03)
- Penicillin/streptomycin (Invitrogen, cat. no. 15140-122)
- Lipofectamine 2000 (Invitrogen, cat. no. 11668-019)
- Cover glass: 18 mm square, no. 1.5 (Fisher Scientific, cat. no. 12-541A)
- Microscope slides, Thermo Scientific, Gold Seal (Fisher Scientific, cat. no. 12-518-101)
- ProLong Gold mounting medium (Invitrogen, cat. no. P36934)
- Fluoro-Gel (Electron Microscope Sciences, cat. no. 17985-10)
- Paraformaldehyde (EM Grade, Electron Microscopy Sciences, cat. no. 19210)
! CAUTION This product causes severe eye irritation. It may cause irritation to the skin and respiratory tract.
- Sucrose (Sigma, cat. no. S5016-1KG)
- PBS (Invitrogen, cat. no. 14040-182)
- Opti-MEM reduced serum medium (Invitrogen, cat. no. 11058-021)
- Cargille immersion oil (Type DF) is used. Note: Cargille Labs has stopped the manufacture of the DF series of oils; the replacement oil is Type LDF (cat. no. 16241)
- Trypsin-EDTA
- Ethanol

EQUIPMENT

- Forceps
- Zeiss 710 CLSM with a Quasar 32 PMT array detector
- $\times 20/0.8$ NA objective lens

- $\times 63/1.4$ NA oil immersion objective lens
- Microcentrifuge tubes (Posi-Click 1.5 ml, Denville Scientific, cat. no. C2170)
- Kimwipes
- MetaMorph software (or equivalent)

REAGENT SETUP

Growth medium—‘Growth medium’ refers to DMEM supplemented with 10% (vol/vol) FBS and 1% (wt/vol) penicillin/streptomycin. The medium can be stored for up to 2 months at 4 °C.

EQUIPMENT SETUP

Zeiss 710 CLSM—Turn on the system and warm up the lasers for 1–2 h. A pixel image resolution of $1,024 \times 1,024$ is recommended, but a lower resolution can be used if high temporal sampling is required. Zoom 2 (pixel = $0.66 \mu\text{m}$ for an $\times 63$ lens), Zoom 1 (pixel = $0.415 \mu\text{m}$ for an $\times 20$ lens). A scan speed of 7 ($1.58 \mu\text{s}$ per pixel) is recommended, but the scan speed can be changed. A slower speed will give images with less noise, whereas faster speeds may be needed for higher temporal sampling. Do not leave the offset setting at zero or data clipping will occur⁷³.

PROCEDURE

Sample preparation ● TIMING 2 d

- 1| Passage the cells 1 d before transfection. To do this, remove growth medium from a 100-mm cell culture dish and wash HT-1080 cells once with 2 ml of PBS, swirling gently.
▲ CRITICAL STEP The growth medium must be removed, because the presence of serum will inhibit the enzymatic activity of trypsin.
- 2| Add 2 ml of 0.25% (wt/vol) trypsin-EDTA to the cells and incubate the mixture at 37 °C in the presence of 5% CO₂ for 2–3 min or until cells are no longer attached to the culture dish.
- 3| Add 4–6 ml of growth medium to the culture dish and mix well.
▲ CRITICAL STEP It is important to add at least 2 ml of growth medium to ensure neutralization of trypsin-EDTA.
- 4| Count the cells and transfer 450,000 cells into one well of a six-well tissue culture plate for each FP fusion transfection required. Allow the cells to grow overnight.
- 5| Transfect the cells with the FP-fusion constructs tabulated below using Lipofectamine 2000. Use 1.5 μg of each cDNA plasmid with a Lipofectamine 2000:cDNA ratio of 2.5:1. Refer to Table 2 for recommended samples for each FRET technique.

Construct label	Construct
a	Acceptor alone (Venus)—preferably cytosolic
b	Donor alone (CFP)—preferably cytosolic

Construct label	Construct
c	Acceptor and donor (Venus + CFP)—preferably cytosolic and not clustered into aggregates
d	Wild-type Akind FRET probe (Wt-Akind) or similar positive FRET probe. Could be a single chain protein with two fluorophores connected by a short amino acid linker
e	Dominant negative Akind FRET probe (3A-Akind) e.g., a single chain protein with two probes that do not interact or two labeled proteins that are in close proximity but do not interact

- 6| For each construct (a–e in the table above), dilute the cDNA into 250 µl of Opti-MEM in a microcentrifuge tube.
- 7| For each cDNA, dilute an appropriate amount of Lipofectamine 2000 (3.75 or 7.5 µl) into 250 µl of Opti-MEM in a second microcentrifuge tube.
- 8| Incubate the cDNA and the Lipofectamine 2000 separately for at least 5 min at 23 °C.
▲ CRITICAL STEP According to the manufacturer's instructions, proceed to the next step within 25 min.
- 9| Add the diluted Lipofectamine 2000 to the cDNA/Opti-MEM mixture and mix well by pipetting up and down several times. Incubate the mixture at 23 °C for at least 20 min.
▲ CRITICAL STEP According to the manufacturer's instructions, proceed to the next step within 6 h, as the constructs are not stable at room temperature (23 °C) longer than this.
- 10| Remove the growth medium from each well and wash the cells once with 2 ml of PBS, swirling gently.
- 11| Add 2 ml of DMEM + 10% (vol/vol) FBS (without antibiotics).
- 12| Add cDNA/Lipofectamine 2000 mixtures to each well and mix gently.
▲ CRITICAL STEP Add the mixtures gently at the side of each well such that the cells do not become dislodged from the surface of the culture dish.
- 13| Incubate the cells at 37 °C with 5% CO₂ for 24 h. The cDNA/Lipofectamine 2000 mixtures may be replaced with full growth medium after 4–6 h if desired.
- 14| Wash 18 mm square no. 1.5 coverslips once with 2 ml of 70% (vol/vol) ethanol.
- 15| Rinse the coverslips twice with 2 ml of PBS.
- 16| Place a coverslip into each well of six-well tissue culture dishes for each sample to be prepared.
- 17| Make a solution of 2.5 µg ml⁻¹ FN in PBS by adding 2.5 µl of 1 mg ml⁻¹ FN to each ml of PBS.
- 18| Add 2 ml of 2.5 µg ml⁻¹ FN in PBS to each well and incubate at 37 °C for 60 min or at 4 °C overnight.
- 19| Wash the FN-coated coverslips three times with 2 ml of PBS.
▲ CRITICAL STEP Add the PBS gently at the side of each well such that the FN layer is not dislodged from the surface of the coverslip.
- 20| Add 2 ml of growth medium into each well.

- 21| Resuspend the transfected cells as in Steps 1–3; however, for each well, use 0.5 ml of trypsin-EDTA in Step 2 and 1.5 ml of growth medium in Step 3.
- 22| Transfer 100–150 μ l of the transfected cell suspensions into each well containing an FN-coated coverslip.
- 23| Allow the cells to adhere by incubation at 37 °C with 5% CO₂ for at least 60 min.
- 24| Wash each coverslip once with 2 ml of PBS.
- 25| Fix the cells by adding 2 ml of 4% (wt/vol) paraformaldehyde PBS solution containing 0.12 M sucrose into each well and incubate the mixture at 23 °C for 15 min.
- 26| Wash each coverslip three times for 5 min with PBS and store them in 2 ml of PBS at 4 °C.
- **PAUSE POINT** Samples can be stored in the dark for up to a week in PBS before mounting.
- 27| Clean a microscope slide with 70% (vol/vol) ethanol for each sample.
- 28| Place a small drop of ProLong Gold onto the microscope slide.
- 29| Remove the coverslips from the wells one at a time using forceps. (A needle tip can also be used to lift the coverslip from the bottom of the 6-well plate.)
- 30| Wick away any excess PBS by touching a Kimwipe to a corner of the coverslip.
- 31| Place the coverslip at a 45° angle above the drop of mounting medium, and then slowly lower it onto the drop of mounting medium.
- 32| Remove any air bubbles in the mounting medium by lightly pressing on the coverslip with a cotton-tipped applicator, forcing the bubbles to the edges of the coverslip.
- 33| Place the samples in the dark overnight at room temperature in order to allow the ProLong Gold to cure. If samples are placed in the fridge, the ProLong Gold will not harden.
- **PAUSE POINT** Samples stored in the dark at 4 °C can last for weeks or even months.

Image acquisition ● **TIMING** 3–4 h

- 34| Put a high-resolution immersion objective in place on the microscope (e.g., $\times 63/1.4$) and apply immersion medium (water, oil or glycerol).
- 35| Place the sample with the coverslip side toward the objective.
- 36| Use bright-field imaging to focus on the cells within the sample.
- 37| Use epifluorescence to find cells that are expressing the protein of interest by eye.
- ▲ **CRITICAL STEP** Use neutral density filters to attenuate the excitation lamp intensity to a minimum in order to avoid fluorescence bleaching of the sample. You may need to adjust your eyes to darkness and turn off any room lighting. We routinely use <1% of the incident light. This is especially important when working with live samples.

- 38| Set the image acquisition parameters to Zoom 2, 12-bit images of $1,024 \times 1,024$ pixels, scan speed of 7, line averaging of 4, detector gain of 750, digital gain of 1, offset of 20, sequential scanning and pinhole to 2 Airy units.
- 39| *CFP image.* Set the light path to excite the sample with 2% power from the 405-nm laser (25-mW) and collect CFP emission from 454–568 nm. If you are using a cube-based system, choose band-pass filters appropriate for CFP excitation and emission. By using these settings, acquire an image of the CFP emission from a single cell expressing the Akind FRET probe.
- 40| *FRET image.* Keep all of the settings the same as above, except collect the FRET (Venus) emission from 516–621 nm. If you are using a cube-based system, choose band-pass filters appropriate for CFP excitation and Venus emission. Acquire an image of the FRET_{Raw} emission from the same cell as in Step 39.

? TROUBLESHOOTING

- 41| *Venus image.* Set the light path to excite the sample with 2% power from the 514-nm laser line (25-mW argon ion laser) and collect Venus emission from 516–621 nm. If you are using a cube-based system, choose band-pass filters appropriate for Venus excitation and emission. Acquire an image of the Venus emission based on direct excitation from the same cell as in Step 39.
- 42| Repeat Steps 39–41 for at least 25 individual cells.
- 43| *Venus excitation cross talk.* Image cells expressing cytosolic Venus FP alone twice: first by exciting the Venus fluorescence with the 514-nm laser line and second with the 405-nm laser, each time collecting images in the FRET_{Raw} channel (516–621 nm). Image a minimum of five individual cells in this way. Use these images to calculate the percentage of the Venus signal in the FRET_{Raw} channel, which is due to direct excitation of Venus by the CFP laser (Fig. 4a). If the instrument settings are kept constant, the amount of Venus excitation cross talk should not change. Therefore, this experiment does not need to be done each time sensitized emission is conducted, but should be verified periodically.

? TROUBLESHOOTING

- 44| *CFP emission cross talk.* Image cells expressing cytosolic CFP alone. Excite the CFP with the 405-nm laser and acquire images in both the CFP (CFP expression: 454–568 nm) and the FRET_{Raw} (516–621 nm) detection channels. Image a minimum of five individual cells under these conditions. Use these images to calculate the percentage of the CFP signal in the FRET_{Raw} channel, which is due to CFP emission at longer wavelengths that overlaps with the Venus emission spectra (Fig. 4b). As with the YFP cross talk, CFP emission cross talk should not change if instrument settings are kept constant, but this should be verified periodically.
- 45| *Negative control.* It is important to image cells expressing unlinked donor and acceptor fluorophores, which should not undergo FRET. Repeat Steps 39–41 for at least five cells expressing unlinked CFP and Venus.
- 46| *Positive control.* If possible, it is ideal to image a probe containing the two fluorophores connected with a small linker (5–7 aa). This validates the instrument settings and confirms that the image processing and analysis are giving the expected results. It also provides an estimate of the maximum FRET efficiency. If a positive control is available, repeat Steps 39–41 for at least five

cells expressing linked CFP and Venus. We did not perform control experiments on a positive control sample for CFP and Venus. We used biological controls as stated in Step 47.

- 47| *Biological control.* A dominant negative Akind mutant (3A-Akind) that cannot undergo the conformational changes necessary for FRET to occur was used as an important biological negative FRET control (inactive Akind has three key amino acid residues mutated to alanine: K179, T308 and S473). Repeat Steps 39–41 for at least 25 cells expressing dominant-negative 3A-Akind. Standard *t* test statistics can be used to compare FRET/CFP ratios between samples.
- 48| *Acceptor photobleaching.* One control that should be carried out for all FRET experiments is acceptor photobleaching. Image cells expressing the Akind probe as in Steps 39–41. Photobleach the Venus FP within an ROI on a cell using the 514-nm laser line (1–5 scans with 100% or lower power should be sufficient). Repeat Steps 39–41 to confirm that the photobleaching is successful and determine whether the CFP intensity increases after acceptor bleaching. Repeat this step for at least five cells. The intensity of CFP signal in the Venus-bleached ROI should increase (Fig. 3).

? TROUBLESHOOTING

Image processing ● TIMING 3–4 h

- 49| *Correct all images for field nonuniformity and background intensity (Steps 49 and 50).* In our case, there was no need to correct for field nonuniformity, because we used an $\times 63$ oil-immersion lens at Zoom 2 on a CLSM. However, this must be confirmed, and if the field is not uniform the field uniformity should be measured and the images corrected^{42,44}.
- 50| The following steps are discussed for the MetaMorph software, but they can be performed on any image processing software platform, including freeware such as ImageJ and Fiji. Perform background subtraction on all of the images before any other calculations are done. Open the images in MetaMorph and select ‘Process → Background and Shading Correction’. In the dialog box, select the image name in the drop box for ‘Source Image’. Under ‘Operation’ select ‘Statistical correction’ and under ‘Parameters’ select ‘Average’. Draw a region in an area of the image that does not include any portion of a cell, and then hit ‘Apply’ in the dialog box. Repeat this for all images. In other image processing platforms, measure the average intensity of an ROI that does not contain any cells. Subtract this average intensity from each pixel intensity value in the image. Use background-subtracted images for ALL subsequent steps.
- ▲ **CRITICAL STEP** If background and field nonuniformity corrections are not done, quantitative data will be inaccurate.
- 51| *Correction factor calculations (Steps 51–55).* Open images of cells expressing cytosolic Venus alone in MetaMorph. The first image is of Venus excited by the 405-nm laser (Venus₄₀₅). The second image is of Venus excited by the 514-nm laser line (Venus₅₁₄).
- ▲ **CRITICAL STEP** Some versions of MetaMorph do not support the Zeiss file format and require conversion of *.lsm files into the *.tif format. Open the *.lsm stacks in Fiji (<http://fiji.sc/wiki/index.php/Fiji>), duplicate each layer individually and save them using the *.tif file format.

- 52| Draw an ROI within a Venus₅₁₄ cell. Select 'Measure → Show Region Statistics' and record the average gray level. Copy the region and paste it into the Venus₄₀₅ image of the same cell. Select 'Measure → Show Region Statistics' and record the average gray level. Repeat this step for at least four additional cells.
- 53| Calculate the correction factor for each cell by dividing the average gray level in the Venus₄₀₅ image by the average in the Venus₅₁₄ image. Average the five values to determine value of *A*, to be used in equation (1) for the FRET image correction.

? TROUBLESHOOTING

- 54| Open images of cells expressing cytosolic CFP alone in MetaMorph. The first image is of CFP in the CFP detection channel (CFP₄₀₅). The second image is of CFP emission in the FRET_{Raw} image channel (CFP_{FRET}).
- 55| Repeat Steps 52 and 53 and calculate the correction factor from intensity CFP_{FRET}/intensity CFP₄₀₅. Average the five values to determine the value of *B*, to be used in equation (1) for the correction of CFP emission cross talk in the FRET image.

? TROUBLESHOOTING

- 56| *Creating FRET images (Steps 56–60).* For each cell, open the following images in MetaMorph: CFP (excited by the 405-nm laser), Venus (excited by the 514-nm laser line) and FRET_{Raw} (FRET_{Raw} image channel with 405-nm excitation). Ensure they have all been corrected for background (Fig. 7a) and shading correction.
- 57| Select 'Apps → FRET' and in the 'FRET' dialog box under the 'Setup' tab, select 'Component' under 'Source' and select 'Sensitized Emission' under 'FRET Method'. In the drop boxes, select the following: 'Donor' = CFP image, 'Acceptor' = Venus image, 'Raw FRET' = FRET_{Raw} image.
- 58| Under the 'Image Correction' tab in the 'FRET' dialog box, select the following: 'Background Subtraction' = 'None', 'Constant A' = value calculated above (we calculated 0.042 or 4.2%), 'Constant B' = value calculated above (we calculated 0.184 or 18.4%). This dialog box will basically do the calculation as stated in equation (1).
- 59| Click 'Apply' and save the resulting FRET_{Corr} image (Fig. 7a). Repeat Steps 56–58 for all of the image sets.
- 60| To speed up image processing, it is possible to put all of the CFP, Venus and FRET_{Raw} images into three individual MetaMorph stacks. In this case, under the 'Setup' tab of the 'FRET' dialog box, select 'All Planes' next to each of the image names. After clicking 'Apply', a stack of FRET_{Corr} images will be generated. In other software programs, simply perform the operations necessary to calculate the FRET_{Corr} image using equation (1).
- 61| *Creating FRET/CFP ratio images (Steps 61–67).* Open the CFP and FRET_{Corr} images in MetaMorph (Fig. 7b).
- 62| Median-filter each image with a 3-by-3-pixel kernel under 'Process → Basic Filters'. Determine the maximum gray-level value in each median-filtered image by selecting 'Measure → Show Region Statistics' and select 'Entire Image' under 'Measure'. Record the maximum gray-level value for each image.

▲ CRITICAL STEP Note that for cells that have autofluorescent vesicles, instead of selecting 'Entire Image' under 'Measure', select 'Active Region'. Next, draw a region in the cell that contains your highest-intensity areas, not including the autofluorescent vesicles. Record the maximum gray value for this region. The maximum intensity value should never be the maximum gray value for the bit depth of the image (e.g., 255 for an 8-bit image, 4,095 for a 12-bit image). If this is the case, the detector was saturated during image collection, data clipping has occurred and the image is not quantitative. Detector offset (sometimes called dark current or black level), laser powers, and PMT gain or voltage should be adjusted during image acquisition to ensure that no image pixels read zero intensity units or maximum intensity⁷³.

- 63| Take the CFP image, multiply it by 1,000 and divide by the maximum intensity determined above. In MetaMorph, do the following: select 'Process → Arithmetic' and select: 'Source image 1' = CFP image, 'Source 2' = 'Constant value', 'Bit depth' under 'Result' = '16', 'Operation' = 'Divide', under 'Constant values' 'Numerator' = '1,000', 'Denominator' = the maximum gray level in the CFP image determined above. Click 'Apply' and the resulting image will be named 'Divide'; see the normalized CFP image in Figure 7b.
- 64| Repeat the previous step using the FRET_{Corr} image for the same cell and using the maximum gray level value for that FRET_{Corr} image. The resulting image will be named 'Divide-2'; see the normalized FRET image in Figure 7b. Note that the FRET_{Raw} image has a much lower intensity than the CFP image. However, after the normalization step the two images have similar intensity values between 0 and 1,000.
- 65| Create a FRET_{Corr}/CFP ratio image by first selecting the following: 'Source image 1' = 'Divide-2', 'Source 2' = 'Image' and choose 'Divide' in the drop box. All other selections in the dialog box should remain the same as in Step 63, except for 'Denominator' under 'Constant values'; in this case, enter '1'. Hit 'Apply' and save the resulting FRET_{Corr}/CFP ratio image. To reduce the shot noise from the confocal images and the excess noise added from the processing steps, select 'Process → Basic Filters' and under 'Operation' select 'Median'. For 'Parameters', enter '3' for 'Filter width' and 'Filter height', and then enter '1' for 'Sub-sample ratio'. Hit 'Apply' and a resultant smoothed image will be generated (Fig. 7c). The filter will be a 3-by-3-pixel or a 9-pixel kernel. A smaller kernel of 2 by 2 pixels will result in less smoothing and a larger filter in more filtering. In this application, with the image resolution that we have, 3 by 3 pixels is the ideal filter size.
- 66| Repeat Steps 61–65 for all of the CFP and FRET_{Corr} images created above.
- 67| To quantify the average FRET_{Corr}/CFP ratio for each cell, first select 'Regions → Regions Tools → Trace Region' and draw a region outlining a cell of interest. Next, select 'Measure → Show Region Statistics' and select 'Active Region' under 'Measure'. Record the average gray-level value for each cell. Avoid areas with high autofluorescence. Repeat this for all images. Rather than taking average values for ROIs, images can simply be displayed; however, cell-to-cell and sample-to-sample differences may be difficult to visualize. Spatial variations across the cell, such as from the central regions to the leading edge, can be visualized using intensity data averaged over a line or rectangular ROI across the cell⁴.

Spectral image acquisition ● TIMING 3–4 h

- 68| Use the same confocal settings as in Step 38. However, choose the lambda mode rather than the channel mode and set the detector gain to 700 nm and the 405-nm laser power to 5%. Set the system to collect the emission spectrum between 416 nm and 650 nm for autofluorescence, CFP emission and Venus emission.
- 69| Find suitable cells to image by repeating Steps 34–37 for each set of samples.
- 70| *Autofluorescence reference spectrum.* Focus on a sample of unlabeled cells. Perform a single scan of the sample in order to measure the spectrum of the cellular autofluorescence. Choose the unmixing tab on the side of the image. Choose an ROI on an autofluorescent cell. Choose ‘add spectrum to the database’ and name the file autofluorescence.
- 71| *CFP reference spectrum.* Repeat Step 70 for cells expressing cytosolic CFP alone. Choose an ROI that is in an area of the cell with low autofluorescence (e.g., no vesicles, not in the perinuclear region). Save this spectrum into the database as CFP.
- 72| *Venus reference spectrum.* Repeat Step 70 for cells expressing cytosolic Venus alone. However, the instrument settings need to be modified to directly excite Venus with the 514-nm laser line. Choose the 514-nm laser line at 5% power and put a dichroic mirror in the light path that will reflect 514-nm light. Change the spectral range to 516–650 nm. Choose an ROI in the cell with high Venus expression and save the spectrum to the database as Venus.
- 73| *Experimental spectra.* Place an experimental sample of labeled cells on the microscope and select a field of view for imaging. Perform a single spectral scan and save the lambda stack of images (Fig. 5a). Repeat this step for about 25 cells for each experimental sample.
- 74| After all of the spectral image stacks are collected, they can be unmixed to generate three images: autofluorescence, CFP_{Spec} and $FRET_{Spec}$. In the upper right corner of the ZEN software (Zeiss) desktop, choose the ‘processing’ tab. Choose ‘linear unmixing’. Choose ‘3 components’ and select the three spectra that were created for autofluorescence, CFP and Venus (Fig. 5b). With the appropriate image open on the ZEN software desktop, click the ‘Select’ button and then the ‘Apply’ button. The software will generate three new images and an overlay image in the display window (Fig. 5c). Save this file as the unmixed data.

? TROUBLESHOOTING

- 75| The ratio images for spectral imaging can be generated in the same manner as above. However, the corrections for CFP emission cross talk do not need to be done, as the CFP emission has been placed in the CFP image through the mathematical unmixing process. The corrections for Venus excitation cross talk do not have to be done, in this case, because our controls show that Venus is not substantially excited by the 405-nm laser (data not shown). Therefore, the $FRET/CFP$ ratio images can be created using equation (2), with $FRET_{Raw}$ substituted by $FRET_{Spec}$ and CFP substituted with CFP_{Spec} . If a 440-nm laser line is used, then the images of Venus directly excited with a 514-nm laser line and spectral images of Venus emission after excitation by the 440-nm laser will need to be generated to calculate the correction factor A needed for equation (1).

? TROUBLESHOOTING

Troubleshooting advice can be found in Table 3.

● TIMING

Equipment setup: 2–3 h

Steps 1–33, sample preparation: 2 d

Steps 34–48, image acquisition: 3–4 h

Steps 49–67, image processing: 3–4 h

Steps 68–75, spectral image acquisition: 3–4 h

ANTICIPATED RESULTS

Sensitized emission

FRET_{Corr}/CFP ratio images with the wild-type (WT)-Akind probe show a significantly higher intensity compared with FRET_{Corr}/CFP ratio images with the dominant negative 3A-Akind probe (Fig. 8a). Quantification of the results shows an approximately threefold increase in the FRET_{Corr}/CFP ratio for Akind relative to the dominant-negative 3A-Akind (Fig. 8b). In addition, our previous results using this protocol show that the FRET_{Corr}/CFP ratio of the WT-Akind probe is significantly reduced when coexpressed with APPL1 (ref. 4). This reduction in FRET is due to an APPL1-mediated decrease in the amount of active Akt in cells, especially at the cell edge.

Spectral imaging

Without calculating the ratio images, it is clear from the unmixed spectral imaging data that there is a relatively high FRET signal for the Akind probe, low FRET signal for the dominant negative 3A-Akind probe and no FRET for the CFP-Venus unlinked control (Fig. 9a). In addition, the CFP- and Venus-alone controls lack any FRET signal (data not shown) and the autofluorescence control shows that the spectral unmixing assigns autofluorescence to the appropriate channel (Fig. 9a). In fact, when you image cells expressing the Akind probe at $\times 20$, the untransfected, autofluorescent cells are not detected in the CFP and FRET images (Fig. 5c). It should be noted that if only cellular data are required, and not subcellular information, imaging at $\times 20$ with a high-NA lens (NA = 0.8) is a very efficient option. The $\times 20$ images should be collected with the same settings as stated in the protocol for the $\times 63$ lens aside from using a zoom setting of 1, a detector gain of 600 and a pinhole setting of 4 Airy units. Many cells can be imaged within each acquisition (Fig. 5c), thereby increasing sampling and reducing image acquisition and processing time. The ratio imaging analysis of the spectral results imaged at $\times 20$ (Fig. 9b) shows a very similar result to the $\times 63$ single cell data (Fig. 8b) with a higher FRET/CFP ratio for the Akind probe relative to the dominant-negative 3A-Akind.

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Figure 1.

Schematic diagrams depicting the three conditions that must be met for efficient FRET. (a) The energy of donor emission must be an energy that the acceptor can absorb. In other words, the emission spectrum from the donor fluorophore must overlap with the excitation spectrum of the acceptor fluorophore. (b) If the FRET donor and acceptor are more than 10 nm apart, then no FRET occurs and the donor emits fluorescence. If the donor and acceptor are within ~10 nm of one another, then energy transfer can occur from the donor (CFP) to the acceptor (Venus). (c) If the donor and acceptor fluorophore dipoles are perpendicular to one another, then the donor molecule will emit fluorescence. However, if the dipoles are parallel to each other, FRET will occur.

Figure 2. Schematic diagram of the intramolecular Akind FRET probe. This figure was adapted with permission from ref. 30. Akind consists of an Akt pleckstrin homology (PH) domain, followed by Venus FP, an Akt catalytic domain (CD) and finally CFP. **(a)** In its basal state, there is minimal FRET, as the CFP and Venus fluorophores are not within 10 nm of one another. Thus, if CFP is excited, cyan fluorescence will be emitted. **(b)** When Akind is recruited to the plasma membrane via an interaction between its PH domain and PIP₃, it is activated by two phosphorylation events (P). These phosphorylation events cause a conformational change in the Akind probe, bringing the CFP and Venus fluorophores in close-enough proximity to allow FRET to occur.

Figure 3.

Acceptor photobleaching within regions of interest (ROI). HT-1080 cells expressing Akind are shown. **(a)** Images of CFP and Venus FP before (left) and after photobleaching Venus with the 514-nm laser line (right). Red circles denote the ROI where Venus was bleached with the 514-nm laser line, and white circles represent the unbleached control region. **(b)** Quantification of the change in CFP fluorescent intensity after photobleaching in both an unbleached control region (CFP control region) and a region in which Venus has been photobleached with the 514-nm laser line (CFP bleached region). Error bars represent the s.e.m. for 16 cells (* $P=0.0022$). Scale bar, 10 μm . a.u., arbitrary units.

Figure 4.

Crucial excitation and emission cross talk corrections. **(a)** Excitation curves for CFP and Venus showing excitation cross talk (blue region) from the 405-, 440- or 458-nm laser light. In the example shown, when a 440-nm laser is used to excite CFP, a large fraction of the Venus fluorescence (Venus image, based on direct excitation of Venus with 514 nm light) is also excited directly, and the emission enters the FRET image channel (FRET image). Venus excitation cross talk can be corrected for by calculating the ratio between the Venus image when the fluorophore is directly excited (514 nm) and when the fluorophore is excited by the CFP incident light (440 nm) in control cells only expressing Venus. See equation (1) for more details. When performing FRET measurements, a direct image of Venus expression excited at 514 nm is used so that the proportion of Venus direct excitation cross talk can be subtracted from the FRET image. **(b)** CFP emission is highly overlapping with the emission of Venus, and thus some emission from the CFP fluorophore is transmitted and collected within the FRET image channel (blue region of curve). This emission cross talk will be extensive regardless of which light source is used to excite CFP. A ratio of the CFP signal in the CFP channel and the FRET channel from a sample of cells only expressing cytosolic CFP can be used to correct FRET images for emission cross talk using equation (1). Adapted from <http://www.microscopyu.com/articles/fluorescence/fret/fretintro.html> with permission.

Figure 5.

Spectral imaging of autofluorescence and the CFP-Venus Akind probe. **(a)** Spectral images of the Akind probe expressed in HT-1080 cells. The spectrum was measured from 421 nm to 645 nm in 10-nm increments using a $\times 20/0.8$ -NA objective lens and excitation from a 405-nm laser. Scale bar, 100 μ m. **(b)** Reference spectra that were collected for cellular autofluorescence (green line), CFP (cyan line) and Venus (yellow line) and used for spectral unmixing of lambda image stacks such as the one shown in **a**. **(c)** Unmixed images from **a** showing autofluorescent cells, with a subset expressing the Akind FRET probe. As

excitation was only from the 405-nm laser, the Venus image is a result of FRET from CFP to Venus fluorophores. Scale bar, 100 μm .

Figure 6.

FRET-FLIM analysis of cytosolic GFP and a FRET-positive GFP-mCherry probe, as well as the effects of the local fluorophore environment. **(a)** Representative TCSPC decay curves for cytosolic GFP (green) or the FRET-positive GFP-mCherry probe (red), together with the instrumental response function (IRF; black). Note the steeper slope, or shorter GFP lifetime, of GFP-mCherry, indicating that FRET is occurring. **(b)** FLIM images (spatial distribution of lifetime value) of GFP fluorescence lifetime in HEK-293 cells expressing cytosolic GFP or the FRET-positive GFP-mCherry probe. Yellow and red represent a long lifetime for cytosolic GFP, whereas green and blue show the reduced lifetime due to FRET between GFP and mCherry. Scale bar, 10 μm. **(c)** Mean lifetime values for cells expressing either cytosolic GFP or the FRET-positive GFP-mCherry are shown for the indicated environmental conditions: live cells in their appropriate growth medium (live), fixed cells mounted with PBS (fixed), ProLong Gold (ProLongGold) or Fluoro-Gel (Fluoro-Gel) mounting medium. **(d)** Respective FRET efficiencies for cells expressing the FRET-positive GFP-mCherry probe calculated using equation (4). Fixed samples have a similar lifetime and FRET efficiency compared with live samples, whereas the same parameters measured in samples mounted in ProLong Gold or Fluoro-Gel are strongly affected by the mounting procedure. Error bars represent the s.e.m. for 5–7 cells for each condition.

Figure 7.

Calculation of FRET/CFP ratio images. **(a)** Background-subtracted CFP, Venus and FRET_{Raw} images (first three images) are used with equation (1) (where Venus is the acceptor and CFP is the donor) to create a FRET_{Corr} image (far right image). **(b)** Upper images, the newly created FRET_{Corr} image and the CFP image from **a** are shown with a rainbow or pseudocolor-coding lookup table to emphasize subtle changes in intensity across the cells. These images are then normalized using the indicated equations to produce images with similar maximum intensity values (lower images). **(c)** The normalized images from **b** are then used to create a FRET/CFP ratio image by dividing the FRET_{Corr} image by the CFP image and multiplying the result by 1,000 (left panel). A median filter was used to reduce the image noise (right panel). Images are shown in pseudocolor coding. Scale bars, 10 μm .

Figure 8.

Comparison of the FRET ratio images for wild-type and mutant Akind probes. **(a)** Representative FRET/CFP ratio images collected with a $\times 63/1.4$ NA objective lens for HT-1080 cells expressing either wild-type Akind (WT-Akind) or a nonactivatable Akind mutant in which three key residues that have been mutated to alanine (3A-Akind). Scale bar, 10 μm . **(b)** Quantification of the average FRET/CFP ratio in HT-1080 cells expressing either WT-Akind or 3A-Akind. Error bars represent the s.e.m. for at least seven cells (* $P = 0.035$). The asterisk indicates a statistically significant difference compared with WT-Akind. FRET/CFP ratio images were calculated using equations (1) and (2).

Figure 9.

Spectral imaging FRET of WT-Akind and 3A-Akind. **(a)** Spectrally unmixed images of autofluorescence, CFP and Venus (FRET) for HT-1080 cells expressing either WT-Akind, 3A-Akind or unlinked CFP and Venus collected with an $\times 63/1.4$ -NA objective lens. Untransfected HT-1080 cells are also shown for comparison. Note that essentially the entire fluorescence signal is due to autofluorescence. Scale bar, 10 μm . **(b)** Quantification of the average FRET/CFP ratio from images collected at $\times 20/0.8$ NA in HT-1080 cells expressing either Wt-Akind or 3A-Akind. Error bars represent the s.e.m. for 10–21 cells ($P = 0.0014$). The asterisk (*) indicates a statistically significant difference compared with Wt-Akind. FRET/CFP ratio images were calculated using equation (2), where $FRET_{Corr} = FRET_{Spec}$ and $CFP = CFP_{Spec}$.

Table 1

Summary of other key FRET protocols.

Reference	Summary
Ai <i>et al.</i> ²²	Dual FRET pair imaging of caspase-3 activity in live cells
Brumbaugh <i>et al.</i> ²⁵	Constructing FRET sensors, which respond to post-translational modifications such as phosphorylation
Aoki <i>et al.</i> ²⁶	Intramolecular FRET biosensors of small GTPases imaged dynamically in live cells
Dezhurov <i>et al.</i> ²⁷	Using quantum dots in constructing FRET biosensors for oleic acid
Salonikidis <i>et al.</i> ²⁸	The effects of ion sensitivity on cAMP FRET biosensors
Newman <i>et al.</i> ²⁹	Extensive review of genetically encoded fluorescent biosensors appropriate for studying dynamics in live cells
Grashoff <i>et al.</i> ⁷⁴	Measuring tension across vinculin using a mechanosensitive FRET biosensor
Vinkenburg <i>et al.</i> ⁷⁰	Measuring cytosolic zinc concentration using eCALWY FRET biosensors

Table 2

Samples required for the various FRET microscopy methods.

Sample	Acceptor bleaching	Sensitized emission	Spectral	Fluorescence lifetime	Correction or measurement
Unlabeled cells	Yes	Yes	Yes	Yes	Autofluorescence
CFP alone	No	Yes	Yes	Yes	CFP emission cross talk, CFP spectra, determine CFP lifetime
Venus alone	No	Yes	Yes	No	Venus excitation cross talk, Venus spectra
Unlinked CFP and Venus	Yes	Recommended	Recommended	Recommended	Negative FRET control
Linked CFP and Venus	Recommended	Recommended	Recommended	Recommended	Positive FRET control, determine CFP FRET lifetime
CFP and Venus experimental samples	Yes	Yes	Yes	Yes	Sample of interest
CFP and Venus Positive biological control	Recommended	Recommended	Recommended	Recommended	Should show high FRET, short CFP lifetime
CFP and Venus Negative biological control	Recommended	Recommended	Recommended	Recommended	Should show low FRET, long CFP lifetime

Table 3

Troubleshooting table.

Step	Problem	Solution
40	Images are saturated	Reduce the laser power and/or the detector gain
	Images are noisy	Use or increase line (live samples) or frame (fixed samples) averaging, use slower scan speeds, increase the laser power and reduce the detector gain
43	No image of Venus in the FRET channel	If there is very little excitation of Venus by the CFP excitation laser, then correction for the Venus excitation cross talk may not be necessary
48	No increase in CFP signal in bleached ROI	It is possible that the Venus bleaching settings could also bleach the CFP fluorophore directly. Use a lower 514-nm laser power and repeat the acceptor photobleaching experiment. Many bleach scans at lower laser power may be required
53, 55	Highly variable <i>A</i> and <i>B</i> values	If the values of <i>A</i> and <i>B</i> are highly variable, test the laser stability ⁴² , and ensure there is not a lot of autofluorescence contribution in the ROI chosen for analysis. For example, avoid the perinuclear region of the cell or highly autofluorescent vesicles. More than five cells may need to be imaged to minimize the variability
74	Unmixed images are not well separated	Control spectra may not be of significant quality. Retake control spectra at higher a S/N ratio (i.e., higher laser power, lower detector gain)
	Unmixed images are noisy	Spectral images may need to be collected at a higher S/N ratio (i.e., slower scan speed, more line or frame averaging, higher laser power, lower detector gain). Remember that the signal is being split over 24 individual detectors