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Three-Color Confocal Förster (or fluorescence) Resonance Energy Transfer Microscopy: Quantitative Analysis of Protein Interactions in the Nucleation of Actin Filaments in Live Cells

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Abstract

Experiments using live cell 3-color Förster (or fluorescence) resonance energy transfer (FRET) microscopy and corresponding in vitro biochemical reconstitution of the same proteins were conducted to evaluate actin filament nucleation. A novel application of 3-color FRET data is demonstrated, extending the analysis beyond the customary energy-transfer efficiency (E%) calculations. MDCK cells were transfected for coexpression of Teal-N-WASP/Venus-IQGAP1/mRFP1-Rac1, Teal-N-WASP/Venus-IQGAP1/ mRFP1-Cdc42, CFP-Rac1/Venus-IQGAP1/mCherry-actin, or CFP-Cdc42/Venus-IQGAP1/ mCherry-actin, and with single-label equivalents for spectral bleedthrough correction. Using confirmed E% as an entry point, fluorescence levels and related ratios were correlated at discrete accumulating levels at cell peripheries. Rising ratios of CFP-Rac1: Venus-IQGAP1 were correlated with lower overall actin fluorescence, whereas the CFP-Cdc42:Venus-IQGAP1 ratio correlated with increased actin fluorescence at low ratios, but was neutral at higher ratios. The new FRET analyses also indicated that rising levels of mRFP1-Cdc42 or mRFP1-Rac1, respectively, promoted or suppressed the association of Teal-N-WASP with Venus-IQGAP1. These 3-color FRET assays further support our in vitro results about the role of IQGAP1, Rac1, and Cdc42 in actin nucleation, and the differential impact of Rac1 and Cdc42 on the association of N-WASP with IQGAP1. In addition, this study emphasizes the power of 3-color FRET as a systems biology strategy for simultaneous evaluation of multiple interacting proteins in individual live cells. © 2015 International Society for Advancement of Cytometry

• Key terms

Key terms: FRET: Förster (or fluorescence) resonance energy transfer; E%: energytransfer efficiency; quantitative FRET analysis; actin nucleation; N-WASP; Rac1; Cdc42; IQGAP1; ROI: region of interest; PFRET: processed FRET or spectral bleedthroughcorrected FRET

THE nucleation of branched actin filament networks can be accomplished by coordinated activities of IQGAP1, N-WASP, Rac1, and Cdc42. Actin and these three proteins were investigated in different combinations, both in vitro using purified proteins and by live cell 3-color FRET imaging up to the level of energy-transfer efficiencies (*E*%) (1). The triple expressions included CFP-Rac1/Venus-IQGAP1/mCherry-actin, CFP-Cdc42/Venus-IQGAP1/mCherry-actin, Teal-N-WASP/Venus-IQGAP1/mRFP1-Rac1, or Teal-N-WASP/Venus-IQGAP1/mRFP1-Cdc42, and equivalent empty vector controls (CFP-Venus-mCherry and Teal-Venus-mRFP1). In spite of their 90% of homology, our in vitro results suggested differential effects of small G-proteins Rac1 and Cdc42 in the actin nucleation process. Most notably, Cdc42 stabilized binding of IQGAP1 to N-WASP, whereas Rac1 had the opposite effect.

Förster (or fluorescence) resonance energy transfer (FRET), when converted to *E*%, represents a powerful tool to investigate and quantify protein–protein interactions and molecular colocalization within distances of \sim 1–10 nm. This article assumes that the reader is familiar with the concept of FRET, as documented by an increasing number of applications and publications, mainly in the context of 2-color FRET (2–4). Quantitative analysis of 2-color FRET data requires additional processing steps to deal with spectral bleedthrough (SBT) corrections applying different algorithms—largely automated by software (5–7). 3-Color FRET increases the complexity of correction, but adds greatly to the utility of assaying three potential live FRET interactions in one experiment, instead of three separate tests with two labeled proteins each in multiple individual experiments (8).

As 3-color FRET simultaneously establishes the presence of three labeled proteins spatially and temporarily—implying direct interaction—and fluorescence serves as a proxy for molecular quantities and fluorescence ratios as relative concentrations, we sought to gain more insights by correlating fluorescence levels of the proteins engaged in 3-color FRET. Ideally, cellular investigations with labeled components should take into account the unlabeled endogenous species, but so far, we have not employed any strategies to suppress the expression of the native protein equivalents of the fluorescent fusion proteins discussed here. Furthermore, we selected only pixels where FRET occurs between all three labeled protein pairs, except between actin and Rac1 or Cdc42, where none is expected. The FRET event and minimum *E*% was used as the entry condition for fluorescence correlations (1).

Our previous study separated cell periphery sites (lamellipodia) from cell-cell boundaries, because of the possibility that actin filament nucleation mechanisms differ in those two locations (1). Here, we concentrate exclusively on the data from the cell periphery to demonstrate further the utility of the assay. In another related, but different 3-color-labeled protein study (Teal-N-WASP/Venus-IQGAP1/mCherry-actin), we compared the occurrence of FRET among all three labels (as in this article) with pixels where only two of the three were interacting—an additional analysis opportunity afforded by 3-color FRET (9).

MATERIALS AND METHODS

Transgene Expression

Triple-labeled MDCK cells, including those expressing control fluorescent proteins (CFP-Venus-mCherry or Teal-Venus-mRFP1), were transfected using Lipofectamine 2000 immediately after being plated and were imaged 24 h later. This transfection protocol was also used to drive the expression of each fluorescent fusion protein individually to facilitate SBT correction with our proprietary PFRET (processed FRET) software (copyright, University of Virginia), which functions as an ImageJ plugin. In all cases, the cells were plated onto 25-mm round coverslips in six-well dishes (35 mm diameter per well) using 4 μ g of total DNA per well. DNA amounts for each vector in triple-label transfections

were as follows: CFP/Venus/mCherry (as either fluorescent unfused or fusion proteins), 1 μ g CFP + 2 μ g Venus + 1 μ g mCherry; Teal/Venus/mRFP1 (as either fluorescent unfused or fusion proteins), 1.25 μ g Teal + 1.25 μ g Venus + 1 μ g mRFP1. Most other pertinent materials and methods, including the descriptions of the fluorescent fusion proteins, have been described in thorough detail previously (1).

3-Color Confocal FRET Microscopy

The 3-color FRET method (8) and PFRET software were employed throughout this study. As shown in Figure 1, the following multistep process was used for image acquisition and analysis; the examples of related images are shown in Figure 2. Each fluorescent fusion protein experiment shown in the figures was performed a minimum of three times, and representative results from one such experiment are shown. The empty vector control data were obtained from one experiment.

Step 1: Collection of six images for each field of view of a triple-labeled specimen and corresponding single-labeled specimens, using microscopy settings optimized for each fluorophore. An unlabeled specimen is also imaged at these settings for background noise subtraction. Starting with the lowest, wavelength, the three fluorophores are named F1, F2, and F3, their corresponding excitation wavelengths Ex1, Ex2, Ex3, and emission as Em1, Em2, and Em3. We verified the absence of any back-bleedthrough, that is, Ex2 did not excite F1 and Ex3 did not excite F1 or F2; this allowed us to collect the images at Ex1 in three channels (Em1-3), at Ex2 in two channels (Em2-3), and at Ex3 in one channel (Em3). As we were imaging live cells, we chose a line scan, where at one wavelength at a time (2.5 ms/line) a line is scanned at a total of 7.5 ms for the three wavelengths, so as to capture protein kinetics with minor time delay. A frame scan would complete three consecutive scans by each laser and return to any particular line in the image with greater delay. In either case, the FRET signal would not be affected as the emissions in the donor and FRET channel are captured simultaneously at donor excitation. We also did not observe any pixel shifts between the images of the three excitation wavelengths. All images were taken on a Leica TCS SP5 X confocal microscope equipped with a temperature-controlled stage (10). Argon laser lines at 458 nm were used for F1 and 514 nm for F2, whereas F3 was excited at 581 nm with a tunable white light laser, all at 400 Hz of scan speed. For each excitation wavelength, laser power was controlled through acousto-optical tunable filters. The three photo multiplier tubes emission channels were set at 468-515 nm (Em1) for F1, 525-585 nm (Em2) for F2, and 595-750 nm (Em3) for F3 using an acousto-optical beamsplitter. The images were acquired as 8-bit TIFF files of 512 \times 512 or 1,024 \times 1,024 pixels using a 60×1.2 NA water-immersion objective.

Step 2: Establishment of the average background noise for each channel and excitation wavelength by generating several random regions of interest (ROIs) on the unlabeled specimen's images. The PFRET software subtracts this average



Step 1: collect raw data (the 12 indicated images)

Step 2: Remove background noise from all images

Step 3: Manually isolate cell-peripheral and cell-cell boundary regions using the Venus excitation/Venus emission triple-label images

Step 4: Select Regions of Interest (ROIs) using Step 3 images



Step 5: Run PFRET software to

- Remove spectral bleed-through based on single-label controls
- Only select pixels where FRET occurs, as defined by software choices
- Calculate E% and distance, produce PFRET, E% and distance images
- Provide a detailed data pool by ROI, covering all fluorescence parameters

Figure 1. Image acquisition, processing and analysis steps. *Step 1.* Raw images are generated for triple-label, single-label control (for SBT correction), and unlabeled specimens (for background noise correction) at identical imaging settings. Sequential excitation with one wavelength at a time in the line-scan mode captures donor, acceptor, and uncorrected (for SBT) FRET in the triple-label and reference fluo-rescence and SBT levels in the single labels. *Step 2.* The PFRET ImageJ plugin removes average background noise identified in unlabeled specimens, specific for each emission channel. *Step 3.* In ImageJ, the IQGAP1 reference images for ROIs are manually modified to show only cell-peripheral or cell-cell boundary areas. *Step 4.* The automatic ROI selection plugin allows to specify ROI size (here 3 × 3 pixels) and a lower threshold for the average gray-level units in the ROI (here 10) and applies this to the IQGAP1 images. These one-dimensional regional selections are applied to all images during the analysis; only those meeting all thresholds including the requirement of all FRET pairs having to interact (note exception in the text). Approximately, 12% of original ROIs meet all thresholds. *Step 5.* Based on pixel-by-pixel processing, the PFRET software will correct for SBT, generate *E*%, fluorophore distance, SBT-corrected images, and 26 data categories for each ROI meeting processing and threshold specifications. We consider the PFRET algorithm to be a most exacting approach to correct nonlinear SBT, taking into account Förster distances, fluorophore QYs, and the ability to isolate discrete populations relevant to investigating biological questions, such as the requirement for all fluorophores having to interact to be included in the data analysis. Note: This figure has been published previously (1), it was modified to include additional information, and is reproduced here with the publisher's permission. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.

channel-specific value from all images and later will generate background-corrected images.

Step 3: Manual isolation of cell-peripheral and cell-cell boundary regions of the Venus-IQGAP1 triple-label images and using those as a template for 3×3 pixel ROI selection for Step 4.

Step 4: Figure 1 shows an automatic ROI selection plugin (part of PFRET software) where variable ROI size and lower threshold are specified to meet selection criteria. These ROIs will be processed in the next steps below and only those that meet the criteria/thresholds of ALL three fluorophores will be included in the final data, the most important one being the



Figure 2. First row: Following the steps of the imaging protocol, using one of the Teal-N-WASP/Venus-IQGAP1/mRFP1-Rac1 images as an example, the images represent each of the fluorophores excited with their specific wavelength and emitted in an optimal nanometer range (see details in Materials and Methods section). They were background-noise corrected. ROIs meeting thresholds are shown in this example from an inset. Second row: Single-label control images used for SBT correction. Third row: At the same time images of the first row are taken, the uncorrected FRET images are produced, still containing background noise and SBT. Fourth row: After processing all images with the PFRET software, inter alia, the all-important corrected FRET (PFRET) images are generated forming the basis of all the data for *E*%, distance, unquenched donor, and SBT correction. The much lower intensity of the PFRET images compared with their uncorrected versions, highlighting the importance of SBT correction for quantitative evaluations should be noted. All of the charts are based on the ROIs applied to the images. Note: Some of the individual panels in this figure have been published previously (1) and are reproduced here with the publisher's permission. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

requirement of FRET interactions between all three (note exception). In this manuscript, only cell-peripheral data are presented.

Step 5: The automatic processing phase of the PFRET software (8). The software contains many options and thresholds described below to isolate data populations relevant to

biological processes (such as morphology) and reject marginal or outlier values. Its most important feature is the correction of the frequently nonlinear SBT. This is achieved by imaging single-label donor and acceptor specimens at identical settings and recording their emissions at donor excitation in the FRET channel. This is followed by matching single- and doublelabel absorption intensities within narrow ranges and subtracting the bleedthrough ratios of the single labels at donor excitation from the double label-pixel-by-pixel. In addition, several variables are manually entered before processing: fluorophore quantum yields (QYs), detector quantum efficiencies, FRET-pair specific Förster distances, plus many threshold choices on fluorescence levels. Although QYs of fluorophores conjugated to proteins frequently differ from the pure fluorophore, as a matter of practical application, published QYvalues are used. The PFRET software normalizes the differences between the QY of each FRET pair to achieve more comparable intensity levels and D:A ratios; the calculation of E% is not affected. Once these parameter values have been entered, the PFRET software calculates uncorrected FRET, PFRET, E%, SBT percent, and donor: acceptor ratios. The thresholds are applied to each pixel in the previously generated ROIs of the images and all thresholds must be met for the pixel to be included in the analysis. Typically, the first processing run is executed at default without any threshold restrictions. In the final processing step, we set the PFRET and E% threshold at 5 to eliminate borderline FRET events. The only exception was made for the G-protein-actin random FRET occurrence, where no biological interaction is expected, which was set at zero; not having done so, would have eliminated a large amount of valid FRET data between Rac1/Cdc42-IQGAP and IQGAP1-actin in shared ROI locations. The PFRET software output consists of images (background-subtracted-, E%-, fluorophore distance-, SBT images) and data. The data cover a complete account of the SBT ratios at fluorescence ranges and spreadsheet files of all ROIs in each image detailing 26 data categories for each ROI: ROI number; coordinates on the image; number of pixels meeting inclusionary/exclusionary thresholds (e.g., here, FRET interaction between all three fluorophores must take place before the pixel is included, exception noted above); quenched fluorescence of fluorophore1 and 2; raw/uncorrected FRET between fluorophores 1-2, 1-3, 2-3; unquenched fluorophore (uF) 1 and 2 and fluorescence of fluorophore 3; PFRET (corrected for SBT-PFRET) between fluorophores 1-2, 1-3, and 2-3; ratios of uF1:uF2, uF1:uF3, and uF2:uF3; E% of uF1-2, uF1-3, and uF2-3; FRET distance for uF1-2, uF1-3, and uF2-3; and the percent of SBT correction (SBT%) for uF1-2, uF1-3, and uF2-3. The separation of ROIs into subpopulations, charts, and statistics was performed using Excel spreadsheet software (Microsoft).

RESULTS

We initially used the 3-color FRET assay in live cells to evaluate the physiological significance of the following in vitro reconstitution results obtained using purified proteins: (a) the small G-proteins, Rac1 or Cdc42, cooperatively stimulate actin polymerization with IQGAP1, albeit with distinct kinetics; and (b) in dose-dependent manners, Rac1 suppresses and Cdc42 stimulates binding of IQGAP1 to N-WASP (1). Having established FRET interaction between each pair of the three labeled moieties, using the same basic experimental strategy (Figs. 1 and 2), we now correlate rising fluorescence of one protein with the fluorescence level of the second (in the presence of the third) and by extension, whether the changing ratio of two proteins affects the third. As the in vitro data showed dose dependency, a single correlation coefficient for the whole data set would miss a tipping or saturation point based on fluorescence. We consequently used a stepwise increasing series of correlation coefficients in accumulating cohorts. All charts and correlations also show the empty vector equivalents.

CFP-Rac1/Venus-IQGAP1/mCherry–Actin and CFP-Cdc42/Venus-IQGAP1/mCherry–Actin Combinations

Rac1 and Cdc42 do not interact directly with actin (11), confirmed by low-level random FRET. However, increasing fluorescence of both Rac1 and Cdc42 correlates with rising actin fluorescence (Fig. 3A) as indicated by in vitro results via other effectors-we will show one of them (N-WASP) in the next section. When the overall correlation coefficient is dissected into discrete accumulating cohorts (Fig. 3B), Rac1 quickly reaches a steady-state ending with a final coefficient of 0.28, with Cdc42 peaking at 0.43 to end with 0.33 for the total set. Another important regulator (IQGAP1) in this triple specimen provided a clue as to how the small G-proteins exert their differential effect. Correlating actin fluorescence as a function of rising Rac1:IQGAP1 or Cdc42:IQGAP1 ratios (Fig. 3C) revealed a progressive lowering of actin fluorescence as the Rac1:IQGAP1 ratio rose, whereas rising Cdc42:IQGAP1 ratios had a small, opposite impact at low initial ratios (Fig. 3D). T-tests comparing actin fluorescence data points in the Rac1 versus Cdc42 triple label showed a statistical difference of P = 9.2E - 124 and IQGAP1 P = 2.23E - 30; adding the empty vector data of CFP and mCherry, ANOVA produced P = 0 in both cases, with all the other parameters confirming a statistical difference.

Supporting Information figures show the details on mean fluorescence of each of the triple combinations and their mean ratios (Supporting Information Fig. S-1A-B); The percent frequency distribution of the meaningful G-protein:IQ-GAP1 ratios supports the finding that fewer fluorescence units associate with Rac1 than Cdc42; empty vector CFP:Venus ratios show a much narrower range (Supporting Information Fig. S-1C). Another approach to demonstrate the impact of the G-prot:IQGAP1 ratio on the G-protein's correlations to actin is to sort the data shown in Figure 2A into discrete cohorts and correlate each by the ratio (Supporting Information Figs. S-2A–2C).

Returning to the in vitro results, the imaging findings not only strongly support the dose-dependent effects of Rac1 and Cdc42, but also the role of IQGAP1, which 3-color FRET is able to correlate, all three proteins being in the same space and time.



Figure 3. Differential effects of Cdc42 versus Rac1 and Cdc42/Rac1:IQGAP1 Ratios on actin fluorescence. (**A**) Correlation of Rac1 (blue), Cdc42 (red) versus actin fluorescence data points—in the presence of IQGAP1—plus empty vector CFP (green) versus empty vector mCherry fluorescence—in the presence of Venus. (**B**) Gradually segmenting the changing correlation coefficients by accumulating Rac1, Cdc42, or CFP fluorescence to detect at which level change occurs. In (A) and (B), the narrow range of the empty vector control (green) and its virtually unchanged correlation coefficient over that range indicates no biological interrelationship; equally, rising levels of Rac1 (blue) maintain their correlation virtually unchanged with Cdc42 (red) increasing their correlation to a peak between 40 and 60 gray-level units, suggesting a modest concentration response. (**C**) To include IQGAP1 in the equation, the impact on actin fluorescence is charted by the rising ratio of Rac1:IQGAP1 (blue) and Cdc42: IQGAP1 (red), also including empty vector data (green). (**D**) At the lowest Cdc42:IQGAP1 (red) ratio, there is insignificant positive correlation, whereas increasing Rac1:IQGAP1 (blue) ratio reaches negative correlation peak at accumulation ratio of 0.1–0.4. This strengthens the idea that the impact on actin nucleation by the small G-proteins is modulated (or not, in the case of Cdc42) by their changing ratio inter alia to IQGAP1 (Supporting Information Figs. 1 and 2). [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

Teal-N-WASP/Venus-IQGAP1/mRFP1-Rac1 and Teal-N-WASP/Venus-IQGAP1/mRFP1-Cdc42

The main objective of the above triple combination was to analyze—by 3-color FRET—the in vitro results that Rac1 and Cdc42 modulated the binding of IQGAP1 to N-WASP in a dose-dependent manner, which in turn had a profound effect on actin nucleation. We interpreted the imaging data by targetting the effect of Rac1 and Cdc42 on *E*% between N-WASP and IQGAP1 and the ratio of N-WASP: IQGAP1, the former being an expression of distance between the two fluorophores, the latter to investigate differential ratios. Although overall, the effect on *E*% between N-WASP and IQGAP1 was independent of increasing amounts of Rac1 as expressed by fluorescence, Cdc42 had a negative effect, that is, diminishing *E*%s signaled an increase of the distance between N-WASP and IQGAP1 (Fig. 4A). When breaking down the correlation coefficients as described earlier into small steps, Rac had only a small effect at low fluorescence, negative to start; Cdc42 continued the negative trend, reaching a peak 60 gray-level units of fluorescence (Fig. 4B). These results support the notion of "dose dependency," here interpreted by the level of fluorescence. This fluorescence-dependent feature is repeated when correlating the small G-proteins with the N-WASP:IQGAP1 ratio. At low Rac1 and Cdc42 fluorescence levels, the impact on this ratio is small, but at increasing amounts their effects diverge in opposite directions, with Rac1 increasing the amount of N-WASP associating with IQGAP1 and Cdc42 increasing that amount modestly. Empty vector data on the



Figure 4. Differential effects of Cdc42 versus Rac1 on *E*% between the N-WASP-IQGAP1 and the N-WASP:IQGAP1 Ratio. (**A**) Correlation of Rac1 (blue), Cdc42 (red) fluorescence versus *E*% (an expression of distance) between N-WASP and IQGAP1. (**B**) Increasing levels of Rac1 (blue) have little effect on the correlation coefficient, whereas increasing Cdc42 (red) fluorescence levels show concentration-dependent negative correlation coefficients. Rac (blue) has only a modest effect on the distance (binding?) of N-WASP to IQGAP1 at very low fluorescence, whereas rising Cdc42 (red) correlates with increasing that distance (lowering *E*%). (**C**) The rising fluorescence of Rac1 (blue) and Cdc42 (red) correlated to the N-WASP:IQGAP1 ratio shows up again differences between the two small G-proteins. (**D**) Breaking down the coefficients by G-protein levels of fluorescence into discrete cohorts, Cdc42 (red) has a mildly positive effect of more N-WASP associating with IQGAP1 at lower fluorescence rises; this confirms our in vitro results. Empty vector control (green) data show different, random correlation features. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

effect of RFP on Teal-Venus show, in both cases, a different distribution.

Figures 5A and 5B correlate Rac 1 and Cdc42 with N-WASP (in the presence of IQGAP1), basically reflecting their effects already shown in Figures 4C-4D, with Cdc42stimulated increase in N-WASP also driving the increased ratio of N-WASP:IQGAP1. Figures 5C-5D reverse the correlations, juxtaposing IQGAP1 and N-WASP (in the presence of either Rac1 or Cdc42) showing similar trends. To capture the interrelationship among all three labeled proteins, Figures 5E-5F analyze the impact on N-WASP by the increasing ratios of IQGAP1:Rac1 and IQGAP1:Ccd42. Although the changes in the former show N-WASP's independence of that ratio, rising IQGAP1:Ccd42 creates a positive dependency up to a peak ratio of ~ 1.0 (correlation coefficient, ~ 0.4), declining thereafter. T-tests of the N-WASP populations in the presence of IQGAP1 and/or Rac1/Cdc42 show robust statistical differences at P = 3.1E - 107, IQGAP1 at P = 1.75E - 197, N-WAS-

P:IQGAP1 ratio at P = 4.76E - 08 and E% N-WASP-IQGAP1 at 3.6E - 37. Adding empty vector data to the statistical pool resulted in all cases in ANOVA-based *P*-values of zero.

DISCUSSION

We previously confirmed and quantitatively evaluated 3color FRET interactions among IQGAP1, N-WASP, Cdc42, Rac1, and actin (1). Here, we extended the analysis on the assumption that in pixels exhibiting 3-color FRET, most, if not all, fluorophore-labeled species were interacting during the millisecond time scale of imaging. To increase the statistical probability, we analyzed several thousand data points. We also did not suppress endogenous protein species in this set of experiments although there are several strategies that can be employed to accomplish this in the future.

Although our 3-color FRET assay offers an exponential increase in exploring correlations between the three



Figure 5. Differential effects of Cdc42 versus Rac1, IQGAP1, and IQGAP1:Rac1 versus IQGAP1:Cdc42 ratios on N-WASP fluorescence. **(A, B)** Correlating the small G-proteins (Rac1-blue, Cdc42-red) with N-WASP (in the presence of IQGAP1), the correlation coefficients again show up their differences and opposite effects in a fluorescent-level dependent manner. **(C, D)** Equally, tracking the effect on N-WASP as a function of increasing IQGAP1 fluorescence in the presence of either Rac1 (blue) or Cdc42 (red) highlights the different influence of the small G-proteins. **(E, F)** The effect of Rac1 and Cdc42 is best demonstrated by correlating their ratios with IQGAP1 against N-WASP fluorescence. The IQGAP1:Rac1 (blue) ratio changes show N-WASP being independent on that ratio, whereas the opposite is true for IQGAP1:Cdc42 (red) rising ratios, which peak between 0.6 and 1.0. Empty vector (green) data distribution largely differs from labeled protein species. The response in terms of fluorescence as a surrogate measure of protein numbers of the important effector N-WASP is explored with respect to Cdc42/Rac1, IQGAP1, and the ratio of IQGAP1 and the small G-proteins. In each case, it is the presence of Cdc42 (red) that drives the increases, modulated by the ratio to IQGAP1. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

components as compared with 2-color FRET (9), we have concentrated on a limited number of objectives using fluorescence data to analyze protein interactions in live cells beyond the level of *E*%. What was already known, that is, the involvement of N-WASP, IQGAP1, Rac1, and Cdc42 in the nucleation of actin was further quantitated, by demonstrating that while both Rac1 and Cdc42 indirectly stimulate actin polymerization, they do so at differing degrees depending on their ratio with IQGAP1. The other important effector in actin nucleation, N-WASP, was also shown to be influenced by the levels of Rac1 and Cdc42, the specific nature of their ratio to IQGAP1 and thereby their apparent ability to affect the N-WASP:IQGAP1 ratio. In spite of their >90% of homology, Rac1 and Cdc42 seem to cause subtle, finely tuned changes on N-WASP, IQGAP1, and their ratios and indirectly actin, as part of the regulation of actin polymerization. Although suggested in our in vitro results, the 3-color FRET assay in live cells lends considerable support to the notion of the small G-proteins playing a differential role-in concert with other effectors-in actin nucleation. Future applications of this imaging assay could include labeled, constitutively active Rac1 or Cdc42 together with labeled actin, N-WASP or IQGAP1, to explore the effect of changing ratios of Rac1: Cdc42 on the third FRET partner.

There is a paucity of 3-color FRET publications in live cells as the technique is mostly applied to in vitro assays, examining molecules in solutions, mainly by spectroscopy, tracking conformational changes and the like (12-17). We believe that 3-color FRET offers promise for many other systems level, cell biological applications, where capturing simultaneous interactions among three components in the same cell provides insights not possible with 2-color FRET. Any live-cell FRET imaging-including 3-color FRET-has to consider a number of important variables which can make interpretation challenging. Transient interactions (as in this manuscript) lead to wider ranges of E%, E% being an expression of distance between fluorophores. This distance varies as donors and acceptors are in the process of approaching or departing from each other-within a 1-10 nm FRET distance-at the instance of imaging. Complex formations or tracking protein clusters during cellular trafficking produce a somewhat lower range of E%s, but still contain some of the same dynamics. Both need to consider inter- versus intramolecular FRET, particularly when fusion-labeled proteins are overexpressed, potentially increasing the number of non-FRET donors, depressing E% levels. Thresholding

donor:acceptor ratios and analyzing subpopulations offer the solutions to concentrate on specific areas of interest. Because of the heterogeneity of cells and their cellular functions, large number of data points need to be generated for robust conclusions. This in turn requires computational resources, modeling, and the like.

LITERATURE CITED

- Wallrabe H, Cai Y, Sun Y, Periasamy A, Luzes R, Fang X, Kan HM, Cameron LC, Schafer DA, Bloom GS. IQGAP1 interactome analysis by in vitro reconstitution and live cell 3-color FRET microscopy. Cytoskeleton 2013;70:819–836.
- Jares-Erijman EA, Jovin TM. Imaging protein molecules using FRET and FLIM microscopy. Curr Opin Biotech 2003;16:19–27.
- Sun Y, Wallrabe H, Seo SA, Periasamy A. FRET microscopy in 2010: The legacy of Theodor Forster on the 100th anniversary of his birth. Chemphyschem 2011;12: 462–474.
- Wallrabe H, Periasamy A. Imaging protein molecules using FRET and FLIM microscopy. Curr Opin Biotechnol 2005;16:19–27.
- Berney C, Danuser G. FRET or no FRET: A quantitative comparison. Biophys J 2003; 84:3992–4010.
- Chen Y, Elangovan M, Periasamy A. FRET data analysis: The algorithm. In: Periasamy A, Day R, editors. Molecular Imaging: FRET Microscopy and Spectroscopy. New York: Oxford University Press; 2005. pp 126–145.
- Elangovan M, Wallrabe H, Chen Y, Day RN, Barroso M, Periasamy A. Characterization of one- and two-photon excitation fluorescence resonance energy transfer microscopy. Methods 2003;29:58–73.
- Sun Y, Wallrabe H, Booker CF, Day RN, Periasamy A. Three-color spectral FRET microscopy localizes three interacting proteins in living cells. Biophys J 2010;99: 1274–1283.
- Wallrabe H, Sun Y, Fang X, Periasamy A, Bloom G. Three-color FRET expands the ability to quantify the interactions of several proteins involved in actin filament nucleation. Proc SPIE 2012;8226:82260J.
- Sun Y, Booker CF, Kumari S, Day RN, Davidson M, Periasamy A. Characterization of an orange acceptor fluorescent protein for sensitized spectral fluorescence resonance energy transfer microscopy using a white-light laser. J Biomed Opt 2009;14: 054009.
- Ma L, Rohatgi R, Kirschner MW. The Arp2/3 complex mediates actin polymerization induced by the small GTP-binding protein Cdc42. Proc Natl Acad Sci USA 1998;95: 15362–15367.
- Duncan TM, Duser MG, Heitkamp T, McMillan DG, Börsch M. Regulatory conformational changes of the epsilon subunit in single FRET-labeled FF-ATP synthase. Proc Soc Photo Opt Instrum Eng 2014;8948:89481J.
- Gehne S, Flehr R, Altevogt A, Berg M, Bannwarth W, Kumke MU. Dye dynamics in three-color FRET samples. J Phys Chem B 2012;116:10798–10806.
- Lee S, Hohng S. An optical trap combined with three-color FRET. J Am Chem Soc 2013;135:18260–18263.
- Ratzke C, Nguyen MN, Mayer MP, Hugel T. From a ratchet mechanism to random fluctuations evolution of Hsp90's mechanochemical cycle. J Mol Biol 2012;423: 462–471.
- Voss S, Zhao L, Chen X, Gerhard F, Wu YW. Generation of an intramolecular threecolor fluorescence resonance energy transfer probe by site-specific protein labeling. J Pept Sci 2014;20:115–120.
- Zhao M, Huang R, Peng L. Quantitative multi-color FRET measurements by Fourier lifetime excitation-emission matrix spectroscopy. Opt Express 2012;20: 26806–26827.