

Detection of local Cdc42 activity using FRET

William B. Kiosses and Martin A. Schwartz

Fluorescence resonance energy transfer (FRET) assay for Cdc42.

The Cdc42 assay is based on a comparable assay developed by Hahn and colleagues [1, 2] for its close homolog Rac1. The Rac assay was subsequently used on several occasions by the Schwartz laboratory [3-5]. The assay is based on the principle that GFP-Cdc42 (or Rac) binds to its effector PAK only when the GTPase is in the GTP-bound state. When the effector region of PAK (the so-called p21 GTPase-binding domain or PBD) is labeled with Alexa546, Cdc42 activation therefore brings the two fluorophores into proximity, leading to increased fluorescence resonance energy transfer from GFP to Alexa546. Increases in FRET visualized in the microscope can therefore be treated as an indication of local Cdc42 activation.

Constructs

GFP-WTCdc42, activated GFP-V12Cdc42 and dominant negative GFP-N17Cdc42 were cloned in Clontech EGFP-C1 vector as described [6]. The acceptor fluorophore, Alexa546 was conjugated to the Rac effector domain from PAK (PBD) as described [2].

Introducing fluorophores into cells:

Cells can be transfected using either direct injection of cDNA into the nucleus [7], or by conventional transfection using Effectene reagent according to the manufacturer's instructions. For microinjections, cells are plated on coverslips coated with fibronectin (Fn) (10µg/ml) in medium containing serum (10% FCS in DMEM) in dishes that were prepared according to Kiosses et al., (1999) and placed in an open chamber on the microscope stage with atmospheric and temperature control. Cells are injected with cDNAs coding for GFP fusions at 50 µg/ml. If unlabeled dominant negatives are included to inhibit activation, these are present at 200 µg/ml. Cells are returned to the incubator for 2h after injection to allow protein expression. They are then microinjected into the cytoplasm with Alex-PBD at 50 µg/ml and used shortly afterward.

For transfections, 0.15 µg of cDNA is used per 60 mm dish. Alexa-PBD at 50 µg/ml can be introduced into the cytoplasm of adherent cells by microinjection into the cytoplasm. Alternatively, the entire population of cells can be shear loaded as described [8]. For this procedure, transfected cells are trypsinized and resuspended at $1-2 \times 10^6$ cells/ml in DMEM with 0.5% serum and 10 µg/ml Alexa-PBD. They are passed through a 30 ga. needle to induce loading, then replated onto FN-coated coverslips and used 2-4h later. Some practice is required to apply the correct force to the syringe so that cells are efficiently loaded without causing excessive damage, but once the technique is established 80-90% of the cells can be injected and viability is above 90% in our hands [9].

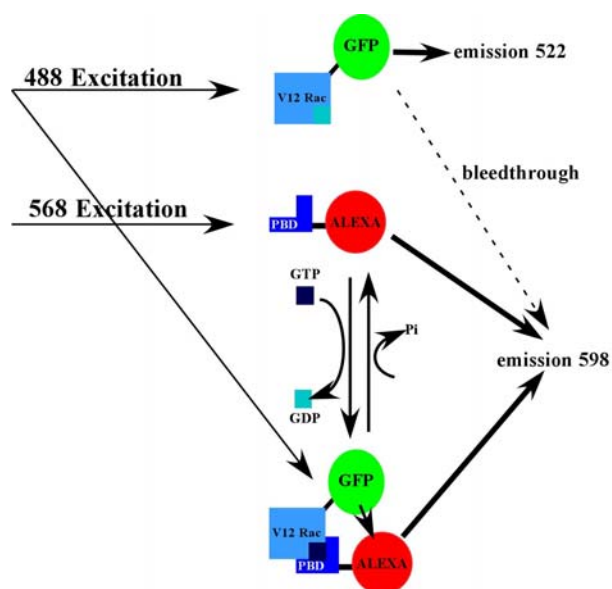
Image acquisition and Analysis

Filter Sets (BioRad)

Images were acquired using a BioRad 1024 Confocal Microscope where the filters and software for acquisition were modified to optimize detection of GFP, FRET and Alexa 546 images. The filters used for these experiments: (1) GFP filter set: excitation 488, emission 522, (2) Alexa 546 filter set: excitation 568 emission 598, (3) FRET filter set: excitation 488, emission 598. GFP, FRET and Alexa 546 images were acquired at the same plane and without any shift in the sample: thus, all images were

in registration at all times. It is often advantageous to open the pinhole to increase the signal intensity whenever precise z-axis resolution is not critical.

Bleed through and background corrections



All images should be background subtracted so that the signal in areas between cells averages to zero. Each of the individual fluorophores produces significant signal intensity using the 488 ex/598 em filter set designed to detect FRET (Figure 1). The GFP does so because it has some emission at 598 nm; the Alexa546 does so because it has some absorbance at 488 nm. These contributions are calculated from the GFP (488/522) and Alexa (568/598) images and subtracted from the apparent FRET image. To determine the correction factors, images are taken of cells expressing GFP Cdc42 only or Alexa PBD only. For GFP only, the FRET image will represent only bleed through from the 488 excitation to the 598 emission (FRET Filter set combination), for Alexa only, the bleed through of alexa 546 into FRET filter set. For both the GFP and Alexa control cells, 10

regions (of fixed pixel size, see figure 2) are randomly selected and the average fluorescent intensity per region compared to the exact same region using the FRET filter set and the filter set for the fluorophore itself (see green boxes in figure 2, for GFP control and red boxes for alexa control). A bleed through factor is computed by dividing the intensity through the FRET filter by that through the GFP or Alexa filter sets. We find it convenient to import this data into excel to construct a scatter plot of the results. The best-fit curve then represents the bleed through ratio. This value is used to subtract the bleed through from the raw FRET image to obtain a corrected FRET image according to the formula

$$F_t = F_a - (GB_1 + AB_2)$$

Where F_t = true FRET

F_a = apparent FRET

G = GFP intensity

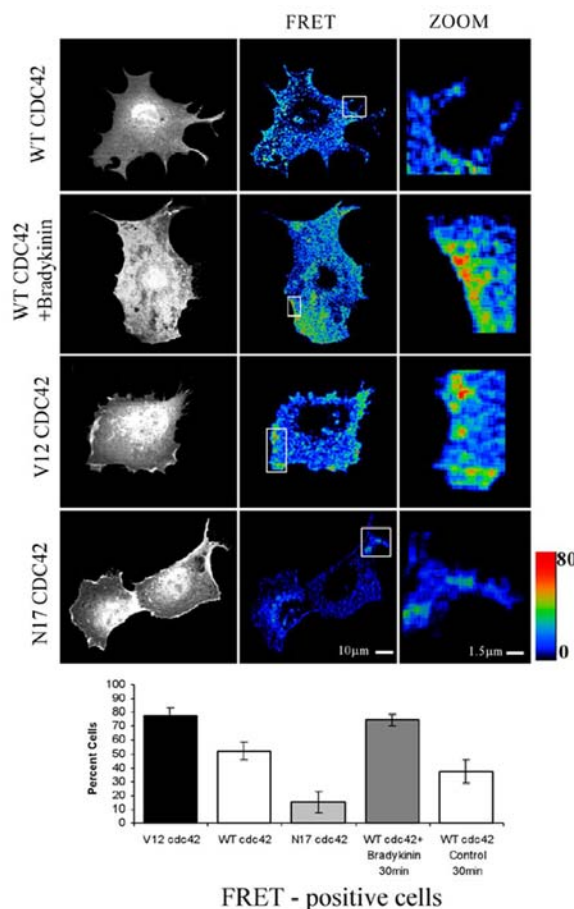
B_1 = bleedthrough factor for GFP

A = Alexa intensity

B_2 = bleedthrough factor for Alexa

The background was subtracted for each image before obtaining the best fit curve by obtaining a random sample of zones that are devoid of cells for each filter set combination. Details are outlined in the

CDC 42 FRET in NIH 3T3 Fibroblasts



description of the Rac assay in Chamberlain et al., 2000; and Kraynov et al., 2000.

Corrected FRET Images

In our labs, FRET corrections are done using a macro written using the ISEE software running on a UNIX workstation. The original and corrected FRET images are 8 bit, 512X512 and are displayed using a pseudocolor scale from 0-256 (see Figure 3). In practice, however, the corrected FRET images seldom use the entire range. In our hands, they typically have an intensity range of 0-125, with variations depending on the experimental conditions. Thus, results are displayed on a pseudocolor scale from red to violet. The scale used for display can be adjusted according to the experimental data as long as consistent scales are used for comparison. We commonly use a fluorescence intensity scale where blue was closest to 0 and red to 125. Baseline signals are usually around 25-40. Signals that are clearly positive were generally between 60 and 125 and appeared in the green-yellow to red range (see corrected FRET image in Figure 3).

Experimental Verification

In the experiments presented here, cells were injected into the nuclei with WT Cdc42 GFP, V12 Cdc42 GFP, WT Cdc42 GFP + Zizimin CAAX, N17 Cdc42 GFP (all at 50 µg/ml, except N17 Cdc42 GFP which was at 200µg/ml. Cells were maintained at 37°C incubator for 2 h, then GFP positive cells were microinjected into the cytoplasm with Alexa PBD (50µg/ml). After the second injection, cells were placed back into the 37°C incubator for 30 min then fixed in 2% formaldehyde for 15 min. For bradykinin experiments, cells were plated on uncoated coverslips in the presence of 10% serum, allowed to spread for 2-3 hrs then serum starved overnight in 0.2% Calf Serum. They were then injected as described above, incubated 30 min and treated with bradykinin (100ng/ml) for 30 min prior to fixation. Coverslips were rinsed in PBS 3 times and mounted onto glass slides using immunomount (ICN) and viewed using a BioRad 1024 Laser Scanning microscope.

Results

Results with NIH 3T3 cells are shown in figure 2. In these experiments, FRET signals varied from 0-80. Cells expressing N17Cdc42 showed low signals that remained in the background range (<30). Unstimulated cells with WT Cdc42 showed higher signals with regions of higher FRET intensity around the nucleus that sometimes went as high as 70, but over most of the cells FRET was in the range of 25-45. V12Cdc42 or WT Cdc42 expressed with the nucleotide exchange factor zizimin show strong regions of high FRET (50-80). Cells expressing WT Cdc42 treated with bradykinin show regions of high activation (50-80 range) though these were smaller than with zizimin or V12Cdc42. We noted that in cells treated with bradykinin these high zones were separated by regions of lower FRET that were completely negative (<30), whereas lower regions of cells with V12Cdc42 or WT Cdc42+zizimin tended to be moderate (40-50). We also noted that activation was visible over relatively broad regions rather than narrowly focused at cell edges, though it was often polarized toward one side of the cell.

Conclusions

These data verify that the Cdc42 is sufficiently sensitive to detect activation by physiological stimuli. Its sensitivity is similar to the cognate Rac assay. The signal-to-noise ratio is only about 2, which is lower than one might hope for. However, the data are sufficiently consistent that FRET signals in the range of 60-80 appear to be reliable indicators of local Cdc42 activation.

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