Published in final edited form as:

Methods. 2014 March 15; 66(2): 180–187. doi:10.1016/j.ymeth.2013.07.011.

Single Color FRET based Measurements of Conformational Changes of Proteins Resulting from Translocation inside Cells

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Abstract

Translocation of proteins to different parts of the cell is necessary for many cellular mechanisms as a means for regulation and a variety of other functions. Identifying how these proteins undergo conformational changes or interact with various partners during these events is critical to understanding how these mechanisms are executed. A protocol is presented that identifies conformational changes in a protein that occur during translocation while overcoming challenges in extracting distance information in very different environments of a living cell. Only two samples are required to be prepared and are observed with one optical setup. Live-cell FRET imaging has been applied to identify conformational changes between two native cysteines in Bax, a member of the Bcl-2 family of proteins that regulates apoptosis. Bax exists in the cytosol and translocates to the mitochondria outer membrane upon apoptosis induction. The distance, r , between the two native cysteines in the cytosolic structure of Bax necessitates the use of a FRET donor-accepter pair with $R_0 \sim r$ as the most sensitive probe for identifying structural changes at these positions. Alexa Fluor 546 and Dabcyl, a dark acceptor, were used as FRET pairs - resulting in single color intensity variations of Alexa-546 as a measure of FRET efficiency. An internal reference, conjugated to Bax, was employed to normalize changes in fluorescence intensity of Alexa Fluor 546 due to inherent inhomogeneities in the living cell. This correction allowed the true FRET effects to be measured with increased precision during translocation. Normalization of intensities to the internal reference identified a FRET efficiency of 0.45 ± 0.14 in the cytosol and 0.11 ± 0.20 in the mitochondria. The procedure for the conjugation of the internal reference and FRET probes as well as the data analysis is presented.

Keywords

FRET; Live-Cell Imaging; Microinjection; Translocation; Apoptosis; Conformational Changes

1. Introduction

The translocation of critical proteins from one compartment of the cell to another is utilized in many regulatory mechanisms, including apoptosis. In this important biological function, possible interactions between various translocating proteins and organelles are critical to its

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execution [1]. One powerful technique that is very useful in studying these translocation events is live-cell FRET imaging. The ability to extract distance information below the diffraction limit (< 200 nm) between a fluorescent donor and acceptor by measuring their relative intensities of fluorescent emission can provide remarkable detail at the molecular level of the biological processes in a cell [2]. However, one obstacle that must be overcome in utilizing this technique is to attribute changes in a fluorescent signal only to FRET rather than other factors inherent in the cellular environment.

Previous work has shown that factors that give rise to inhomogeneity in the cytosol can affect the intensity of fluorescent particles [3]. Changes in the density of the particles that crowd the cytosol can affect the excitation pathway of light used to excite fluorescent species, which can decrease the intensity. Molecular crowding can scatter photons, also affecting the intensity. If these effects on a fluorescent particle of interest are not taken into account, the observation of FRET can be systematically affected and consequently bias the interpretation.

To account for these inhomogeneities, ratiometric techniques have been employed to identify differences in cellular location, eliminating false positives in identification of changes in a signal in response to translocation events. For examples, the correlation between cellular morphology and changes in Ca^{2+} sensor's intensities has been used to measure responses to changes in Ca^{2+} flux [4]. In another study the effect of the regions in the vicinity of the plasma membrane on the fluorescent reporters of a biosensor needed to be determined before the response of the biosensor for phosphoinositides could be interpreted, [5]. Similarly, to interpret the cAMP activity of a protein kinase at the nucleus, the change in fluorescence on the reporter for cAMP activity in the particular environment needed to be determined [6].

In this study, a protocol is presented to determine the change in FRET efficiency in real time during the process of translocation of the protein Bax (Figure 1) from the cytosol to the outer-mitochondrial membrane, which is an early step in the onset of apoptosis. Steps taken in this protocol seek to minimize the number of control samples to be used to correct for spectral bleedthrough and environmental effects that occur when detecting fluorescent probes in two inherently inhomogeneous cellular locations. This is achieved by accurately obtaining the true effect of a non-fluorescent FRET acceptor (Dabcyl) by referencing changes in the signal of the fluorescent donor of the FRET pair (Alexa Fluor 546) to an internal fluorescent reference (Alexa Fluor 633). First, a sample without the FRET acceptor (containing only Alexa Fluor 546 and 633) is allowed to undergo translocation to quantify changes in the FRET donor. Next, another sample with the non-fluorescent FRET acceptor (containing Dabcyl, Alexa Fluor 546 and 633) undergoes the same process. As a result, any changes or detection of FRET can be readily observed by comparing the detected signals from both samples. The quantification in this protocol uses only two samples and one optical setup, which allows for additional flexibility in studying a variety of biological systems.

While the solution structure of Bax has been solved [7] and a conformational change associated with a lipid environment has been detected [8], structural information after its translocation is not known, but some elements have been proposed [9, 10]. Bax conjugated with the FRET probes (Dabcyl and Alexa Fluor 546) and internal reference (Alexa Fluor 633) was delivered into MEF cells by microinjection and translocation was initiated by the addition of staurosporin (STS) to the cell culture media. By utilizing the internal reference, the uncertainty in the percent change in normalized intensity upon translocation has been reduced from 27–75% to 3%. This correction gave a reproducible determination of FRET efficiency before and after translocation of $45 \pm 14\%$ and $11 \pm 20\%$, respectively, over many cells. Accounting for changes in the fluorescent signal in different environments, sometimes

larger than changes due to intramolecular FRET, is absolutely required for meaningful interpretation of the data. The details in the implementation of this protocol to Bax are discussed below.

2. Determination of FRET efficiency using an internal reference

Bax, a 21 kDa protein (PDB: 1F16), has two native cysteines, Cys62 and Cys126, which have a distance of 26 Å between their C_β carbons (Figure 1). One of the FRET pairs that is sensitive to conformational changes between these two residues is Alexa Fluor 546 and Dabcyl, which have an R₀ of 29 Å [11], see *Section 4.1*. This particular FRET pair is compatible with our protocol because Dabcyl is a non-fluorescent acceptor, and therefore FRET is determined simply by changes in the fluorescence intensity of Alexa Fluor 546. The FRET efficiency is determined by measuring the fluorescent signal of Alexa Fluor 546 with (F_Q) and without (F) dabcyl and using equation 1. Distance information from the efficiency can be extracted by using equation 2 with value of R₀ defined above.

$$E = 1 - \frac{F_Q}{F} \quad (1)$$

$$E(r) = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6} \quad (2)$$

Alexa Fluor 633 was selected as an internal reference conjugated to Bax to measure the changes in fluorescence of Alexa Fluor 546 in a cell due to different environments. It does not undergo FRET with dabcyl (*Section 4.1*), and would not require additional FRET components in the analysis. As will be shown below, there is no spectral overlap between Alexa Fluor 633 and dabcyl, which is required for FRET. However, there is overlap between Alexa Fluor 546 and dabcyl, which gives rise to FRET.

To calculate FRET efficiency between Alexa Fluor 546 (A546) and Dabcyl (Dab) during translocation, while incorporating Alexa Fluor 633 (A633) as an internal reference, the intensities of A 546 in the protein with and without the conjugation of Dab, (F_{IQ}) and (F_I), respectively, would be normalized to the intensity of A633 (F_2) according to equation 3 and 4:

$$F' = \frac{F_I}{F_2} \quad (3)$$

$$F'_Q = \frac{F_{IQ}}{F_2} \quad (4)$$

Each of the F_2 terms in these equations refers to the internal reference on each of the samples with and without Dab, B-A546-Dab-A633 (Bax conjugated with A546, Dabcyl, and A633) and B-A546-A633 (Bax conjugated with A546 and A633), respectively. The normalized intensities, F' and F'_Q , in the presence and absence of Dab would be incorporated into the expression to determine FRET efficiency according to equation 5:

$$E' = 1 - \frac{F'_Q}{F'} \quad (5)$$

Since there is no contribution to FRET with Dab from A633, only one FRET efficiency needs to be calculated. Therefore, to determine the FRET efficiency before (*pre*) and after (*post*) translocation of Bax, the following equations are used:

$$E'_{pre} = 1 - \frac{F'_{Q,pre}}{F'_{pre}} \quad (6)$$

$$E'_{post} = 1 - \frac{F'_{Q,post}}{F'_{post}} \quad (7)$$

There is a possibility that there could be FRET between A546 and A633. However, since the ratio of intensities from these probes is used for calculating FRET efficiency, the FRET that could occur between them will not affect the measurement. Any FRET between A546 and A633 in the F'_Q sample will be cancelled out by dividing the F' ratio, where this same FRET will be present as well. Therefore, this division determines the FRET effects of dabcy1 without needing to know the absolute intensity of F_I exactly. In fact, since this intensity is sensitive to environmental factors, getting around this problem is the principle motivation for our protocol.

The challenge in implementing this technique is to make sure that the B-A546-Dab-A633 sample gives the same signal as the B-A546-A633 sample when there is no FRET with Dab. This ensures a proper measure of FRET on the efficiency curve, i.e., no FRET between A546 and Dab would truly give rise to a FRET efficiency of 0. This value would not be attained if the stoichiometry of the fluorescent probes on these two samples were different. Specific procedures along the synthesis of conjugated Bax are taken to eliminate species that would affect the determination of the FRET efficiency. The conjugation of the FRET probes and internal reference to Bax (B) with and without the quencher, B-A546-Dab-A633 and B-A546-A633, respectively, is described in the next section.

3. Methods and Procedures

3.1 Expression and purification of Bax

Wild-type Bax (B) was expressed and purified according to earlier procedures [7] with two modifications. First, BL21(DE3) cells transformed with pTY1B1-Bax were expressed in Luria-Bertani (LB) broth instead of isotopically enriched media. Second, after monoQ column purification, fractions containing Bax were further purified on a G-75 size-exclusion column to get rid of trace of high-molecular weight impurities.

3.2 Reaction of B to form B-A546

To conjugate A546 (AlexaFluor 546, Invitrogen) to Bax utilizing maleimide chemistry with one of the exposed cysteine residues, Bax was incubated in a pH 8.4 100mM Tris buffer with 100 mM NaCl, 1 mM TCEP at a concentration of 12 μ M (using $\epsilon_{280} = 36,105 \text{ cm}^{-1} \text{ M}^{-1}$, calculated from amino acid content) and allowed to react with 500 μ M A546 for 17 hours at 37 °C in the dark. The reaction was stopped by exchanging the buffer to pH 8.0 20

mM Tris and 100 mM NaCl using a PD-10 (GE Bioscience). A G-75 size-exclusion column, equilibrated with the same buffer, ensured complete removal of free dye. Unreacted Bax, B, was removed from reacted Bax, B-A546, on a monoQ column (Amersham) equilibrated in pH 8.0 20 mM Tris using a gradient of 1M NaCl shown in Figure 2A. The mixture was diluted 10× in running buffer before loading. Liquid chromatography-Mass Spectroscopy (LC-MS) confirmed an addition of one A546 probe to Bax after this step (observed mass – 22197.12, theoretical mass. – 22198.52). The remaining steps in the synthesis were carried out using this purified stock that contains no unlabeled Bax.

3.3 Reaction of B-A546 to form B-A546-Dab

A portion of the purified B-A546 stock was used to conjugate Dab (Anaspec) to the remaining buried cysteine utilizing maleimide chemistry. The pH was raised to 9.3 and additional NaCl was added to keep the salt concentration at 100 mM. The final B-A546 concentration was 5 μ M (using $\epsilon_{558} = 104,000 \text{ cm}^{-1} \text{ M}^{-1}$ as reported for the AlexaFluor 546 dye by Invitrogen). This mixture was allowed to react with 700 μ M of Dabcyl-C2 maleimide for 17 hours at 37 °C in the dark and was stopped by exchanging the buffer to pH 8.3 0.15 M KHCO₃ buffer using a PD-10 (GE Biosciences). LC-MS confirmed the conversion of B-A546 to B-A546-Dab by observing a single peak of 22626.85 (theoretical mass. – 22625.92). To confirm the location of the A546 and Dab probes, a portion of the reaction mixture was buffer exchanged to a pH 8.0 20 mM Tris buffer and enzymatic digestion using pepsin was performed at pH 1.9 1:20 enzyme to protein ratio at 37 °C in the dark. LC-MS experiment of fragments produced by the digestion identified the following fragments that contained Dab: 450.1881 (res 60–63: 450.1777), 819.51 (res 57–63: 819.4509), 1007.550 (res 55–63: 1007.5303), and 1045.583 (res 62–70: 1045.5590). These fragments confirm that Dab is on cysteine 62. The following fragment was detected that contained A546: 1100.6069 (res 122–131: 1100.6252). This confirms that A546 is on cysteine 126. While it may be possible that there is a population of conjugated protein with these probes in swapped positions and these peaks were not detected, this scenario would not change in the interpretation of FRET because the distance would still be the same. This assumes local mobilities of the probes themselves are not different at the two sites.

As a control to make sure there is no quenching from Dab on A633, the sample B-Dab-Dab was made to eventually conjugate A633 to see this by substituting unlabeled B for B-A546 in the synthesis described above. LC-MS analysis of the pepsin digestion of this sample identified the following fragments, which contain both cysteines, as containing Dab: 819.4686 (res 57–63: 819.4509), 1007.5473 (res 55–63: 1007.5303), 1045.5764 (res 62–70: 1045.5590), 1100.6448 (res 122–131: 1100.6252), and 901.5118 (res 125–132: 901.4926).

3.4 Reactions of B-A546 and B-A546-Dab to B-A546-A633 and B-A546-Dab-A633

The remaining portion of the purified stock of B-A546 from section 3.2 was buffer exchanged into pH 8.3 0.15 M KHCO₃ buffer using a PD-10 column. This sample and the B-A546-Dab sample from the previous section were concentrated to 5–10 μ M using Millipore centrifugal concentrators at 10,000 Da MWCO. These solutions were allowed to react with 500 μ M AlexaFluor 633 succidimidy-ester (Invitrogen) for 17 hours at 4 °C in the dark. The reaction was stopped by buffer-exchanging these reaction mixtures into a pH 8.0 20 mM Tris buffer using a PD-10 column. These solutions were loaded on a monoQ column as described in section 3.2 and the separation of B-A546-A633 and B-A546-Dab-A633 from B-A546 and B-A546-Dab are shown in Figure 2B. The fractions were collected and concentrated with Millipore centrifuge concentrators and washed once with pH 8.0 20 mM Tris. Residual NaCl is present in the solution. Intact masses of B-A546-A633 and B-A546-Dab-A633 detected by LC-MS, 23103.3 (theo. – 23102.0) and 23496.0 (theo. – 23493.4) verified conjugation of the A633 probe. The final concentration of these probes

was determined by using $\epsilon_{633} = 94,000 \text{ cm}^{-1} \text{ M}^{-1}$ reported for the AlexaFluor 633 dye by Invitrogen. A summary of the steps in the synthesis is provided in Figure 2C. From 900 μL of 20 μM B, the final yield of these conjugated proteins is 100 μL of 1 μM . However, as discussed in section 3.6, this is sufficient protein for microinjection.

3.5 Steady State Fluorescence measurements

To verify that B-A546-A633 and B-A546-Dab-A633 were sensitive to conformational changes in Bax, the fluorescence spectra of these species, F' and F'_Q , were measured in 0 and 8M guanidinium chloride (GdnHCl) at pH 8.0. In addition, the effect of Dab on A546 and A633 were determined by comparing the spectra of samples B-A546 and B-A546 Dab, termed BF_1 and BF_1Q , respectively, and the spectra of B-A633 and B-Dab-Dab-A633, termed BF_2 and $BQQF_2$, respectively, in 0 and 8 M GdnHCl. Fluorescence spectra were collected on a QuantaMaster Fluorimeter (PTI, London, Ontario). Excitation wavelengths were 543 or 600 nm as indicated. Concentrations of Bax were between 50–100 nM.

3.6 Cell Culture and Microinjection

MEF cells were cultured in modified Eagle Medium (DMEM) containing 10% FBS and 1% pen-strep stock and grown at 37 °C in a humidified 5% CO₂ incubator. When they became confluent, they were passaged using a 5% solution of trypsin and reconstituted in phenol-free DMEM (Opti-MEM), 10% FBS and 1% pen-strep media. The solution of MEF cells in suspension was diluted 1/8 and plated in MatTek 35 mm glass bottom no. 1.5 dishes coated with fibronectin and allowed to grow overnight before they were microinjected. Coating the glass bottom was achieved by incubating a solution of fibronectin (Millipore) diluted 20× from a 1 mg/ml stock in PBS and incubated at 37 °C for 4 hours. Before plating the cells, excess fibronectin was rinsed off with PBS.

Microinjection was performed with an Eppendorf Femtojet. Needles were prepared in house using a Shutter Instruments Co. Model P-97 puller. After a solution of protein was loaded onto a needle, microinjection was performed using a continuous flow of sample through the needle. The compensation pressure (p_c), which adjusted the continuous flow, was set between 18–25 hbar. The solution of conjugated Bax for microinjection was 75% PBS and 25% 20mM pH 8.0 Tris. A higher percentage of PBS in the injection buffer may also be used. The final protein concentration was 300 nM, as determined for a stock described in section 3.4. Similar results were observed with solutions as dilute as 30 nM protein. A lower percentage of microinjected cells undergoing translocation was observed if a higher concentration (> 300 nM) of protein was microinjected. Typically, 50–70% of injected cells underwent translocation. Microinjection was monitored using differential interference contrast visualization of the cells. Within a couple minutes after microinjection, STS was added from a 1.7 mM stock in DMSO to the cellular media at a final concentration of 3.6 M. The cells were then monitored for 1.5 hours and imaged every 15 min. The microinjected cells from this first image were treated as ‘preSTS’ cells. At the end of this time series, cells that underwent translocation in this final image were treated as ‘postSTS’ cells. Data was collected from microinjected cells from three separate dishes where each underwent STS-induced translocation. Per dish, 10–13 cells were microinjected and imaged.

3.7 Confocal Microscopy

Microinjected cells were imaged with a Zeiss LSM 5 Pascal inverted confocal microscope with a Plan-Neofluar 40×/1.3 Oil DIC objective. A confocal plane of 2.51 Airy units was used as a compromise between resolution and signal intensity. Microinjected cells were observed in images from two channels. One channel, F_1 , was the fluorescence emission from A546 detected using a band-pass filter of 560–615 nm after excitation with the 543 nm

laser line at 870 W. The other channel, F_2 , was the fluorescence from the A633 probe detected using a line-pass filter of >650 nm after excitation using the 633 nm laser line at 1.3 mW. Data was acquired for 100 s per pixel for a 1024×1024 resolution, 12-bit image. The total time to acquire an image was 4 minutes.

Cells were positively identified as facilitating translocation of microinjected Bax by observing a change in its cellular distribution from diffuse to punctate and a condensation of the nucleus. In a separate control experiment, conjugated Bax was microinjected into cells pre-stained with MitoTracker Green (Invitrogen) using standard protocols before STS-induced translocation. Co-localization of the MitoTracker Green signal and the microinjected protein confirmed translocation to the mitochondria. To observe the stained mitochondria, the dye was observed in a band-pass filter of 500–540 nm after excitation with the 488 nm laser line at 500 W.

3.8 Image Analysis

The intensities from the fluorescent probes in images were analyzed using ImageJ. The following steps were taken to extract this information to determine the FRET efficiency before and after translocation:

1. Cells were imaged less than five minutes after the addition of STS. The ones that eventually translocated within 1.5 hours were treated as ‘preSTS’ cells. Cells imaged after 1.5 hours that translocated were treated as ‘postSTS’ cells.
2. The images of the two channels were individually background subtracted. This was performed using the “background subtraction” feature on the plug-in PFRET [12] for each channel.
3. The background-subtracted images from each channel for a particular cell were stacked using ImageJ and an ROI was drawn around the cytosol. This way, the same ROI was used for each image of a channel. This ROI was different for every cell and was also different after a particular cell underwent translocation, reflecting their varying morphologies.
4. The total sum of the intensities within the ROI was calculated for each channel ($SUMF_1$, $SUMF_2$). These sums were used to determine F' and F_Q' for preSTS and postSTS cells using expressions 8, 9, 10 and 11.

$$F'_{pre} = \frac{SUMF_{1,pre}}{SUMF_{2,pre}} \quad (8)$$

$$F'_{post} = \frac{SUMF_{1,post}}{SUMF_{2,post}} \quad (9)$$

$$F'_{Q,pre} = \frac{SUMF_{1Q,pre}}{SUMF_{2,pre}} \quad (10)$$

$$F'_{Q,post} = \frac{SUMF_{1Q,post}}{SUMF_{2,post}} \quad (11)$$

The values from these expressions were used in expressions 6 and 7 to determine E_{pre}' and E_{post}' .

4. Results

4.1 Detection of Conformational Changes *in vitro*

4.1.1 Dabcyl can undergo FRET with F_1 but not F_2 —Figure 3A shows the spectral overlaps between the fluorescent probes and the non-fluorescent quencher. The absorbance of Dabcyl overlaps with the emission spectrum of A546 which allows for FRET to occur. However, there is no overlap with the emission spectrum of A633, which indicates there is no FRET between them. While the overlap of dabcyl with A546 is small, this is reflected in the relatively small value of R_0 compared to other FRET pairs, typically > 50 Å. This overlap is less than that between the emission of A546 and the excitation of A633. While there could be FRET between A546 and A633, as discussed in section 2, this does not affect the analysis.

Figure 3B shows that FRET is observed between A546 and Dab in the sample B-A546-Dab when compared to B-A546 at 0 M GdnHCl. Under this condition, the structure of Bax is intact and A546 and Dab are close enough to experience FRET. As a result, the fluorescence intensity of A546, F_1 , is decreased in the presence of the non-fluorescent acceptor, Dabcyl. When these samples are placed in a buffer with 8 M GdnHCl, where the structure of Bax is no longer intact, no FRET is expected between A546 and Dab. The intensity of A546, F_1 , is the same in both samples, as if no FRET acceptor was present. To properly identify a change in FRET, the effects of the addition of GdnHCl to the buffer on A546 first needed to be determined. This was done by measuring the fluorescence spectra of B-A546, BF_1 , for each condition.

Figure 3C shows that there is no FRET between Dab and A633 in the samples B-Dab-Dab-A633 compared with B-A633. This is consistent with the lack in overlap between dabcyl excitation and the emission spectrum of A633. There is no difference between the intensity of F_2 with or without Dab in the folded or unfolded state of Bax. This makes A633 an effective internal reference to changes in A546 due to FRET with Dab. Just as in figure 3B, the fluorescence spectra in different conditions must be determined before an interpretation of FRET can be made. It appears that A633 behaves differently in 8M GdnHCl than A546. Since the structures of these probes are not the same, they could have different sensitivities to different environments.

4.1.2 Conformational changes quantified relative to Alexa Fluor 633—Figure 3D shows that under 0 M GdnHCl, where Bax maintains structure, FRET between A546 and Dab can be observed relative to A633 by comparing the spectrum of B-A546-Dab-A633, F_Q' , with the spectrum of B-A546-A633, F' . In contrast, no FRET is observed when 8 M GdnHCl is present in figure 3E also by comparing spectra F_Q' with F' . By capturing the effects observed in figure 3B, A633 can be used as an internal reference to changes in intensity to A546. In addition, B-A546-A633 can reflect changes in the intensity of F_1 , like the BF_1 spectrum in figure 3B, due to different solvent conditions before the contribution to the FRET acceptor in the sample B-A546-Dab-A633 can be interpreted. The identical spectra of F_Q' and F' under conditions where there is no FRET between A546 and Dab confirm that the stoichiometry of A546 and A633 are the same and that B-A546-A633 can be treated as a sample with no acceptor present.

4.2 Detection of Conformational Changes in cells

4.2.1 Verification of Biological Activity of Labeled Bax—Microinjected Bax transitioned from a diffuse to punctate cellular distribution upon addition of STS to the media. This shift in distribution was confirmed as STS-induced translocation to the mitochondria by observing co-localization with mitochondria stained with by MitoTracker

Green, shown in Figure 4A, similar to previous work [13]. In addition, nuclear condensation was observed, another feature of STS-induced translocation [14, 15], shown in Fig. 4B.

4.2.2 Quantification of fluorescence intensity during translocation—Figure 5A shows images of a microinjected cell before and after STS-induced translocation. The images from the F_1 and F_2 channels are overlaid. Figure 5B shows background-subtracted images from the F_1 and F_2 channels for a cell in the preSTS and postSTS state. Superimposed on these images are ROIs drawn in the cytosol of this cell over stacked images of the two channels. A single ROI is used for the preSTS state and another is redrawn for the postSTS state to account for changes in morphology. An ROI drawn over the entire cytosol gives a global picture of FRET effects, while an analysis of smaller ROIs selected in different areas of the cytosol can potentially identify subpopulations within the global picture that are affected by the biological environment, i.e. interactions with vesicles or other organelles. While these ROIs were determined manually, this protocol would be compatible with other algorithms to search for other environmental effects that would be of interest to study. The fact that only two samples need to be prepared with one set of optical settings allows for more freedom to analyze biological phenomena.

Table 1 shows the decrease in uncertainty when an internal reference is used to quantify the intensity of F_1 during translocation. The percent change of signal is given by measuring the intensity per pixel of F_1 in a cell postSTS divided by the intensity per pixel of F_1 in the same cell preSTS. The normalized intensity of F_1 pre- and postSTS is the intensity of F_1 divided by the intensity of F_2 . The same set of cells is used to make this comparison. As is seen by the standard deviation of these values, normalization decreases the standard deviation of these intensities, providing a more precise determination of the intensity of F_1 before and after translocation. From these measurements, a precise change in the intensity of F_1 relative to F_2 is detected, which is not obvious without normalization. A similar change is observed when Q is present; however, there is a statistically significant difference in the change in normalized intensity. This difference can be interpreted as different effects from Q pre- and postSTS. The measurement of the effects of Q is presented in the next section.

4.2.3 Detection of conformational changes measured by FRET—Table 2 shows the calculation of the change in FRET efficiency, E' , between A546 and Dab before (preSTS) and after (postSTS) STS-induced translocation. The values for F' and F_Q' were collected from 16 and 17 individual cells, respectively, over three dishes each undergoing translocation as described in section 3.8. Just as was observed in Table 1, there is a large change in normalized intensity of F_1 upon entering the mitochondria. This change is larger than the observed change in FRET efficiency. The utilization of an internal standard made it possible to extract changes due to FRET rather than from other environmental effects on the fluorescence intensity.

The value of E_{pre}' , 0.45 ± 0.14 , is close to the middle of the FRET efficiency curve, which corresponds to R_0 for Alexa Fluor 546 and Dabcyl. This distance is close to the distance between the C_β atoms of Cys62 and Cys126 in the solution structure. However, upon insertion into the mitochondria, the FRET efficiency decreases to 0.11 ± 0.20 , which means that these two residues have become further apart.

5. Discussion

The corrections employed in this protocol enabled the detection of conformational changes in Bax as it translocated from the cytosol to the mitochondria. The precision in the determination of the effects of a non-fluorescent quencher, Dab, allowed for observation in the change of distances with Bax within different portions of the FRET efficiency curve of

Alexa Fluor 546 and Dabcyl. One requirement to make this assumption is that R_0 , the distance of 50% FRET between the two probes, remains constant before and after translocation. Two factors that can affect this characteristic distance are the quantum efficiency of the donor and the relative orientation between the two probes. Two environmental factors that can affect the quantum efficiency are pH and temperature [16]. In this experiment, the temperature does not drastically change during translocation. According to an earlier study [17] there is a temporary pH increase during the translocation, but returns to the cellular value when Bax is inserted into the mitochondria. The relative orientation and motion involving the two probes can affect the efficiency of energy transfer and as a result, R_0 . In a crowded environment, like the mitochondrial membrane, this may be a concern. However, in an earlier study [18] with muscle thin filaments, interwoven alpha-helical proteins, IEDANS and dabcyl were utilized to study FRET and only small deviations from R_0 were observed assuming isotropic motion of the two probes. Therefore, in this study, a constant value for R_0 is a reasonable assumption.

The utilization of this protocol is not restricted to a particular value of R_0 . Different probes can be utilized for studies in different systems. The only requirements for applicability are to choose a FRET pair with a non-fluorescent acceptor and have an internal standard that does not engage in FRET with this acceptor. There are also no restrictions on hardware, which makes this protocol very practical. The only major hurdle is finding a delivery system to observe FRET changes in cells in real time, but certain aspects of this can be overcome utilizing the flexibility of the probes chosen.

6. Conclusions

We have shown that utilizing a fluorescent internal reference can allow for the detection of intramolecular conformations in Bax in live cells in real time during a translocation event. This correction allows for the measurement of subtle changes in the intensity of Alexa Fluor 546, which is the result of FRET with the non-fluorescent acceptor, Dabcyl. The conformational change detected was an increase in distance between Cys126 and Cys62. The FRET system described in this protocol could be modified for other systems to identify conformational changes or other interactions resulting from translocation in the cell.

Acknowledgments

This work was supported by the Intramural Research Programs of National Heart, Lung, and Blood Institute of the NIH

The authors would like to thank Motoshi Suzuki for providing the plasmid for the expression of Bax and Ingo Thievsen for providing the MEF cells. We also acknowledge D.-Y. Lee of the Biochemistry Core Facility (NHLBI) and R.L. Levine for help with mass spectroscopy analysis and Grzegorz Piszczek of the Biophysics Core Facility (NHLBI) for the acquisition of fluorescence spectra.

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Highlights

We propose a protocol to detect conformational changes in cells using FRET

An internal reference is utilized to quantify FRET efficiency during translocation

Effects from changing microenvironments are taken into account

As Bax translocates to the mitochondria, a conformational change is detected

Figure 1.
Solution structure of Human Bax (PDB:1F16). The C, C and sulfhydryl atoms of Cysteines 62 and 126 are shown. The distance between their C atoms is indicated.

Figure 2.

Purification of products along the synthesis of FRET probes on Bax. (A) monoQ column separation of B, at 20 mL, and B-A546, at 26 mL, with the 1M NaCl gradient superimposed (black line) on the chromatogram. (B) monoQ column separation of B-A546-A633 (red line), at 27 mL, and B-A546-Dab-A633 (black line), at 27 mL, with the 1M NaCl gradient superimposed (black dotted line). An arrow indicates where B-A546 and B-A546-Dab would have eluted in this gradient. (C) Diagram of the synthesis for the two conjugated Bax species. In the first step, all B is removed to avoid any contamination in later reactions. The A546 in both final species comes from the same stock of B-A546.

Figure 3.

Observation of FRET with Dabcyl under different solvent conditions. (A) Spectra of the absorption of Dabcyl, and excitation and emission for A546 and A633 (B) By comparing the fluorescence spectra of B-A546 (black) and B-A546-Dab (red), FRET is observed between A546 and Dab at 0 M GdnHCl (solid lines), where Bax has its structure intact and is lost at 8 M GdnHCl (dotted lines). Excitation wavelength was 543 nm. (C) No FRET with A633 and Dab is observed whether or not Bax has its structure intact (solid lines) or not (dotted lines), according to the fluorescence spectra of B-Dab-Dab-A633 (red) and B-A633 (black). Excitation wavelength was 600 nm. (D) At 0 M GdnHCl, FRET between A546 and Dab can be observed in the sample B-A546-Dab-A633 relative to the signal from A633 when compared to B-A546-A633. Both spectra were normalized to the intensity at 641 nm, the maximum emission of A633 at this condition. Excitation wavelength was 543 nm. (E) Following normalization to 645 nm, the maximum emission of A633 at 8M GdnHCl, no FRET is detected between A546 and Dab at this condition. For this condition, the same stoichiometry between A546 and A633 is confirmed, and the sample B-A546-A633 represents the situation where not FRET acceptor is present.

Figure 4.

Confirmation of biological activity of microinjected Bax. (A) STS-induced translocation to the mitochondria was confirmed by observing co-localization with mitochondria stained with MitoTracker Green and microinjected Bax. Fluorescence from F₂ on microinjected Bax is shown. The F₂ channel is the emission from a line pass filter > 650 nm following excitation at 633 nm. (B) Nuclear condensation is also observed after translocation. The nucleus of the cells is outlined in blue.

Figure 5.

Images used to extract signal intensities to calculate FRET efficiencies. (A) Overlay of images from channels F_1 and F_2 of preSTS and postSTS cells. The F_1 channel is the emission from a band pass filter of 560–615 nm following excitation at 543 nm. The F_2 channel is the emission from a line pass filter > 650 nm following excitation at 633 nm. (B) ROI's shown in yellow drawn from stacked images of channels of preSTS and postSTS cells after background subtraction. Even though the same cell is observed, a different ROI is drawn after translocation to account for changes in morphology.

Table 1

Percent change fluorescent signal of F_I with and without normalization to the signal of F_2 , pre- and postSTS.

	With Normalization		Without Normalization
F' (16 cells)	28 ± 3^a %	F_I	100 ± 75^b %
F_Q' (17 cells)	37 ± 3 %	F_{IQ}	45 ± 27 %

^aPercent change in signal with normalization was determined by dividing the values obtained for F' or F_Q' in 'postSTS' cells by the value determined for 'preSTS' cells.

^bThe fluorescent signal for F_I or F_{IQ} without normalization was determined by summing the intensities of pixels within an ROI, divided by the per pixel area of the ROI.

Table 2

Normalized intensities of F_I with and without dabcyI, F_Q' and F' , respectively, used to calculate changes in FRET efficiency in equations 6 and 7 for preSTS and postSTS cells

	preSTS	postSTS
$F'(16 \text{ cells})$	1.9 ± 0.28^a	0.47 ± 0.08
$F_Q'(17 \text{ cells})$	1.1 ± 0.20	0.42 ± 0.06
E'	0.45 ± 0.14	0.11 ± 0.20

^aThese values were determined over many individual 'preSTS' and 'postSTS' cells as described in section 3.8.