Methods 66 (2014) 180–187

Contents lists available at ScienceDirect

Methods

journal homepage: www.elsevier.com/locate/ymeth

Single color FRET based measurements of conformational changes of proteins resulting from translocation inside cells ☆

Robert F. Gahl [a], Ephrem Tekle [b], Nico Tjandra [a,*]

[a] Laboratory of Molecular Biophysics, Biochemistry and Biophysics Center, National Heart Lung and Blood Institute – National Institutes of Health, Bethesda, MD 20892, United States
[b] Laboratory of Biochemistry, Biochemistry and Biophysics Center, National Heart Lung and Blood Institute – National Institutes of Health, Bethesda, MD 20892, United States

ARTICLE INFO

Article history:
Available online 18 July 2013

Keywords:
FRET
Live-cell imaging
Microinjection
Translocation
Apoptosis
Conformational changes

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.

Published by Elsevier Inc.

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 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 example, 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

☆ This work was supported by the Intramural Research Programs of National Heart, Lung, and Blood Institute of the NIH.
* Corresponding author. Fax: +1 301 402 3405.
E-mail address: tjandran@nhlbi.nih.gov (N. Tjandra).

1046-2023/$ - see front matter Published by Elsevier Inc.
http://dx.doi.org/10.1016/j.ymeth.2013.07.011

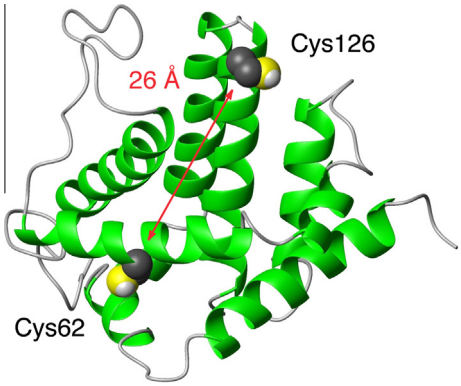

Fig. 1. Solution structure of Human Bax (PDB: 1F16). The C_α, C_β and sulfhydryl atoms of Cysteines 62 and 126 are shown. The distance between their C_β atoms is indicated.

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 (Fig. 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 inhomogenous 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 ± 14% and 11 ± 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 (Fig. 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 R_0 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 Eq. (1). Distance information from the efficiency can be extracted by using Eq. (2) with a value of R_0 defined above.

$$E = 1 - \frac{F_Q}{F} \tag{1}$$

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

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 A546 in the protein with and without the conjugation of Dab, (F_{1Q}) and (F_1), respectively, would be normalized to the intensity of A633 (F_2) according to Eqs. (3) and (4):

$$F' = \frac{F_1}{F_2} \tag{3}$$

$$F'_Q = \frac{F_{1Q}}{F_2} \tag{4}$$

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 Eq. (5):

$$E' = 1 - \frac{F'_Q}{F'} \tag{5}$$

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:

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

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

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 canceled out by dividing the F' ratio, where this same FRET will be present as well. Therefore, this division determines the FRET effects of Dabcyl without