Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy

Gerald W. Gordon,* Gail Berry,* Xiao Huan Liang,# Beth Levine,# and Brian Herman*#

*Laboratories for Cell Biology, Department of Cell Biology and Anatomy, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7090, and #Department of Medicine, Columbia University College of Physicians and Surgeons, New York, New York 10032 USA

ABSTRACT Fluorescence resonance energy transfer (FRET) is a technique used for quantifying the distance between two molecules conjugated to different fluorophores. By combining optical microscopy with FRET it is possible to obtain quantitative temporal and spatial information about the binding and interaction of proteins, lipids, enzymes, DNA, and RNA in vivo. In conjunction with the recent development of a variety of mutant green fluorescent proteins (mtGFPs), FRET microscopy provides the potential to measure the interaction of intracellular molecular species in intact living cells where the donor and acceptor fluorophores are actually part of the molecules themselves. However, steady-state FRET microscopy measurements can suffer from several sources of distortion, which need to be corrected. These include direct excitation of the acceptor at the donor excitation wavelengths and the dependence of FRET on the concentration of acceptor. We present a simple method for the analysis of FRET data obtained with standard filter sets in a fluorescence microscope. This method is corrected for cross talk (any detection of donor fluorescence with the acceptor emission filter and any detection of acceptor fluorescence with the donor emission filter), and for the dependence of FRET on the concentrations of the donor and acceptor. Measurements of the interaction of the proteins Bcl-2 and Beclin (a recently identified Bcl-2 interacting protein located on chromosome 17q21), are shown to document the accuracy of this approach for correction of donor and acceptor concentrations, and cross talk between the different filter units.

INTRODUCTION

Fluorescence resonance energy transfer (FRET) microscopy is a technique used for quantifying the distance between two different fluorophores (Clegg, 1996). FRET involves the transfer of energy from a fluorescent donor in its excited state to another excitable moiety, the acceptor, by a nonradiative dipole-dipole interaction (Lakowicz, 1983). FRET requires that 1) the donor be fluorescent and of sufficiently long lifetime, 2) the transfer not involve the actual resorption of light by the acceptor, 3) the donor molecule's fluorescence emission spectrum overlaps (to some extent) the excitation spectrum of the acceptor molecule, and 4) the distance between the donor and acceptor molecules is small (1–10 nm). The dependence of the energy transfer efficiency on the donor-acceptor separation provides the basis for the utility of this phenomenon in the study of cell component interactions. FRET does not require that the acceptor be fluorescent, but the methods of FRET measurement requiring three filter sets presented in this report do require that the acceptor be fluorescent and that the acceptor not quench the donor by any mechanism other than FRET.

In steady-state FRET microscopy FRET can be detected by exciting the labeled specimen with light of wavelengths corresponding to the excitation spectrum of the donor and detecting light emitted at the wavelengths corresponding to the emission spectrum of either the donor and/or the acceptor. When FRET occurs, the donor emission is decreased and the acceptor emission is increased (sensitized emission). Various methods have been used to measure FRET from the changes in donor and acceptor emission. Proper use of FRET measurements to characterize molecular interactions requires that corrections be made for 1) cross talk (the detection of donor fluorescence through the acceptor emission filter and the detection of acceptor fluorescence through the donor emission filter), 2) the situation that each of the measured fluorescence intensities consists of both FRET as well as non-FRET components, 3) the concentration of donor, and 4) the concentration of acceptor. This report presents a simple method to correct for each of these parameters. The method requires a minimum of spectral information and can be readily implemented on a microscope or in a fluorometer. Corrections for background fluorescence, autofluorescence, and photobleaching may also be required and are applied before using any of the methods discussed below.

Received for publication 5 December 1997 and in final form 11 February 1998.

Address reprint requests to Dr. Brian Herman, Department of Cell Biology and Anatomy, The University of North Carolina at Chapel Hill, CB #7090, 232 Taylor Hall, Chapel Hill, NC 27599-7090. Tel.: (919)-966-5507; Fax: (919)-966-1856; E-mail: bhgf@med.unc.edu.

0006-3495/98/05/2702/12 $2.00

METHODS

Cell preparations and microscopic measurement of FRET

COS cells co-transfected with Bcl-2 and flag epitope-tagged Beclin (Liang et al., 1997, submitted for publication) were used in the studies described in this paper. Beclin is a recently discovered coiled-coiled Bcl-2 interacting protein located on chromosome 17q21. Bcl-2 is an anti-apoptotic protein localized in the outer mitochondrial membrane whose activity is modulated by Beclin. The target proteins were labeled with donor and acceptor by

fluorochrome conjugated antibodies specific to the target proteins. Wild-type and mutant Flag epitope tagged Beclin were labeled with donor fluorochrome (FITC) and Bcl-2 was labeled with acceptor fluorophore (rhodamine). As a control for these studies we labeled the endoplasmic reticulum Ca^{2+}-ATPase (SERCA) with donor (FITC) and Bcl-2 with acceptor (rhodamine) in the same cell type.

FRET was detected by exciting the labeled specimen with light of wavelengths corresponding to the absorption spectrum of the donor and detecting light emitted at the wavelengths corresponding to the emission spectrum of the acceptor. FRET manifests itself by both quenching of donor fluorescence in the presence of acceptor and in sensitized emission of acceptor fluorescence. FRET microscopy was performed as previously described (Liang et al., 1993). The donor (FITC) filter set consisted of [excitation (ex) = 480–500 nm; dichroic mirror (dm) = 510 nm; emission (em) = 515–555 nm]. The acceptor (rhodamine) filter set consisted of [ex = 546/40 nm; dm = 580 nm; em = 590 nm long pass]. Images obtained with these two filter sets were used to directly quantify the intensities of each fluorophore. The FRET filter set consisted of [ex = 450–490 nm; dm = 580 nm; em = 580 long pass]. The signal recorded from this filter set is the FRET signal that arises from energy that has been transferred from FITC to rhodamine molecules. A background value was determined from a region in each image that did not have any cells. The background value was subtracted from the foreground value from a region within a cell. A mapping program written in-house was used to map fluorescent cells and quantify the intensity within each cell.

General equations for FRET

All the methods of FRET measurement discussed here use one or more of three filter sets, which are termed the Donor, FRET, and Acceptor filter sets. These filter sets are designed to isolate and maximize three signals: the donor fluorescence, the acceptor fluorescence due to FRET, and the directly excited acceptor fluorescence, respectively. The excitation filters for the donor filter set and the FRET filter set are either the same filter or two matched filters. The emission filters for the FRET and Acceptor filter sets are either the same filter or matched filters. Neutral density filters may be used to match the signals from the three filter sets to the dynamic range of the detector.

In previous publications describing analysis of FRET data authors have tended to use different symbols for the various fluorescence signals. Those symbols will be used when appropriate. In addition, a common set of symbols is defined which represents and replaces most of the previously used symbols and provides a common vocabulary for comparison of the various methods. Two- and three-letter symbols are defined to represent the signals using the type of filter set (Donor, FRET or Acceptor), the fluorochromes present in the sample (donor only, acceptor only, or both donor and acceptor) and the signal from either just the donor or acceptor when both are present in the sample. Each symbol starts with an uppercase letter representing the filter set, *D* for the Donor filter set, *F* for the FRET filter set, and *A* for the Acceptor filter set. The second letter is lowercase and indicates which fluorochromes are present in the specimen, *d* for donor only, *a* for acceptor only, and *f* for both donor and acceptor present (so FRET is possible). In the three-letter symbols, the third letter is lowercase and indicates the signal from only one of the fluorochromes when both fluorochromes are present. For example, if the third letter is *d*, this would indicate the donor component of the combined signal, while *a* would indicate the acceptor component of the combined signal. The donor- or acceptor-only signals represented by the three-letter symbols cannot be measured directly unless the acceptor is not present when donor is to be measured, and vice versa. (This is equivalent to the situation where there is no cross talk of that type.) A second type of three-letter symbol consists of a three-letter symbol of the first type with a bar over it and is used to indicate the signal that would exist if no FRET occurs. Tables 1–3 list each of the symbols used in the analysis of the FRET data along with the filter set, fluorochromes present, and an explanation of what is measured under each condition.

As an example, *Df* is the signal with the Donor filter set and both fluorochromes present. $Df = Dfd + Dfa$ where *Dfd* is the donor signal and *Dfa* is the acceptor signal (i.e., *Dfa* represents the acceptor fluorescence with the Donor filter set with both fluorochromes present). $Dfd = \overline{Dfd} - FRET1$ where $\overline{Dfd}$ is the donor signal that would have existed if no FRET occurred (as if no acceptor were present) and *FRET1* is the loss of donor signal due to FRET (because acceptor was in fact present). *Dfa* will be zero if the wavelengths of emission of the acceptor do not overlap the wavelengths of transmission of the emission filter of the Donor filter set, or if the excitation spectrum of the acceptor does not overlap the wavelengths of transmission of the excitation filter of the Donor filter set. Expressing *Df*, *Ff*, and *Af* as their donor and acceptor components yields Eqs. 1.

$$Df = Dfd + Dfa \tag{1a}$$

$$Ff = Ffd + Ffa \tag{1b}$$

$$Af = Afd + Afa \tag{1c}$$

Ffd is nonzero only if the donor emission spectrum overlaps the wavelengths of transmission of the acceptor emission filter. *Afd* is nonzero if the donor is excited and the Acceptor filter set detects its emission. Thus, the terms *Dfa*, *Ffd*, and *Afd* are all due to cross talk; any one of these terms will be zero if its particular type of cross talk is not present.

In addition to these symbols, we also define a set of two-letter symbols for signals arising from specimens containing either only donor or only acceptor fluorochrome. *Dd*, *Fd*, and *Ad* represent signals obtained when only donor is present, while *Da*, *Fa*, and *Aa* signify signals obtained when only acceptor is present. These six measured values characterize the fluorophores' excitation and emission spectra including cross talk (*Fd*, *Ad*, *Fa*, and *Da* represent cross talk) and characterize the filter sets by providing the signal with each filter set from the same specimen. For example, *Ffd*/*Dfd* is the ratio of two quantities that are not directly measurable. However, this quantity can be measured as the ratio *Fd*/*Dd* = *Ffd*/*Dfd*. *Ffd*/*Dfd* is the ratio of the donor signals obtained with the FRET and Donor filter sets from a specimen with both donor and acceptor present. *Fd*/*Dd* is also the ratio of the donor signals with the FRET and Donor filter sets from a specimen with only donor present. The FRET that may occur in the specimen with both donor and acceptor does not affect the *Ffd*/*Dfd* ratio because the same fractional loss due to FRET occurs with both filter sets. Rearranging the equation yields *Ffd* = *Dfd* (*Fd*/*Dd*). By using similar logic *Dfa* = *Ffa* (*Da*/*Fa*), and *Afd* = *Dfd* (*Ad*/*Dd*). In the latter case the emission filter is the same between the two filter sets. Substituting these three relationships into Eqs. 1 yields Eqs. 2.

$$Df = Dfd + Ffa(Da/Fa) \tag{2a}$$

$$Ff = Dfd(Fd/Dd) + Ffa \tag{2b}$$

$$Af = Dfd(Ad/Dd) + Afa \tag{2c}$$

Each of the six terms on the right sides of Eqs. 2 can be expressed as two components: a FRET component and a non-FRET component. As in the example above, *Dfd* can be expressed as the donor emission that would occur in the absence of FRET, minus the loss of donor emission due to FRET ($Dfd = \overline{Dfd} - FRET1$). *Dfd* originates in the equation for the Donor filter set, Eq. 2 a. In Eq. 2, b and c, the donor contributions are also expressed in terms of *Dfd*. *Afa* originates in the equation for the acceptor filter set. The next step is to express the acceptor contributions in Eq. 2, a and b in terms of *Afa*. *Afa* has two components, the component representing the signal as if no FRET occurred, $\overline{Afa}$, and the component due to FRET, temporarily termed *AfaFRET*. *Ffa* has two components, the component representing the signal as if no FRET occurred, $\overline{Ffa}$, which equals $\overline{Afa}$ (*Fa*/*Aa*) (by logic similar to the logic above), and the component due to FRET, which is expressed as $G \cdot FRET1$ (the value *G* will be explained below). $G \cdot FRET1$ is expressed in terms of the loss of donor signal due to FRET (*FRET1*). The acceptor FRET signals in the other two equations will be expressed in terms of $G \cdot FRET1$. *Dfa* has two components, the component representing the signal as if no FRET occurred, $\overline{Dfa} = \overline{Ffa}$ (*Da*/*Fa*) = $\overline{Afa}$ (*Da*/*Aa*) by substitution and the component due to FRET = $G \cdot FRET1$ (*Da*/*Fa*) expressed in terms of the component due to FRET of

TABLE 1 Two-letter symbols and their interpretation

Two-Letter Symbol	Filter Set	Fluorochromes Present	Meaning
Dd	Donor	donor	The signal from a donor-only specimen using the donor filter cube
Fd	FRET	donor	The signal from a donor-only specimen using the FRET filter set
Ad	Acceptor	donor	The signal from a donor-only specimen using the Acceptor filter set
Da	Donor	acceptor	The signal from an acceptor-only specimen using the donor filter cube
Fa	FRET	acceptor	The signal from an acceptor-only specimen using the FRET filter set
Aa	Acceptor	acceptor	The signal from an acceptor-only specimen using the Acceptor filter set
Df	Donor	donor and acceptor	The signal from an acceptor-only specimen using the donor filter cube
Ff	FRET	donor and acceptor	The signal from an acceptor-only specimen using the FRET filter set
Af	Acceptor	donor and acceptor	The signal from an acceptor-only specimen using the Acceptor filter set
Dd′	Donor	donor	The same as *Dd* but with acceptor concentration proportional to donor concentration
Fd′	FRET	donor	The same as *Fd* but with acceptor concentration proportional to donor concentration
Ad′	Acceptor	donor	The same as *Ad* but with acceptor concentration proportional to donor concentration
Da′	Donor	acceptor	The same as *Da* but with acceptor concentration proportional to donor concentration
Fa′	FRET	acceptor	The same as *Fa* but with acceptor concentration proportional to donor concentration
Aa′	Acceptor	acceptor	The same as *Aa* but with acceptor concentration proportional to donor concentration

TABLE 2 Three-letter symbols and their interpretation

Three-Letter Symbol	Filter Set	Fluorochromes Present	Signal from which Fluorochrome	Meaning
Dfd	Donor	donor and acceptor	donor	Refers to only the donor signal when both donor and acceptor are present
Dfa	Donor	donor and acceptor	acceptor	Refers to only the acceptor signal when both donor and acceptor are present
Ffd	FRET	donor and acceptor	donor	Refers to only the donor signal when both donor and acceptor are present
Ffa	FRET	donor and acceptor	acceptor	Refers to only the acceptor signal when both donor and acceptor are present
Afd	Acceptor	donor and acceptor	donor	Refers to only the donor signal when both donor and acceptor are present
Afa	Acceptor	donor and acceptor	acceptor	Refers to only the acceptor signal when both donor and acceptor are present
$\overline{Dfd}$	Donor	donor and acceptor	donor	Refers to the donor signal that would have been if no acceptor were present and therefore no FRET occurred
$\overline{Afa}$	Acceptor	donor and acceptor	acceptor	Refers to the acceptor signal that would have been if no donor were present and therefore no FRET occurred

Ffa (which is $G \cdot FRET1$). In order to express *AfaFRET* in terms of $G \cdot FRET1$ it is important to note that the difference between the signals is the excitation filter used and that the FRET signal is proportional to the excitation of the donor (and not the direct excitation of the acceptor). The ratio of measured values that relates the excitation of the donor with the FRET and Acceptor filter sets is *Ad*/*Fd*. Therefore, $AfaFRET = G \cdot FRET1$ (*Ad*/*Fd*). This logic is slightly different from the logic above since a component of the acceptor signal depends on the donor excitation. Equations 3–6 summarize these results.

$$Dfd = \overline{Dfd} - FRET1 \tag{3}$$

TABLE 3 FRET symbols and their interpretation

Other Symbols	Meaning
FRET1	Loss of donor signal due to FRET using Donor filter set in the method using three-filter sets
FRETN	Normalized measure of FRET equal to $FRET1/(\overline{Dfd} \cdot \overline{Afa})$
FRET2	Equal to $FRET1/\overline{Dfd}$
FRET3	Equal to $FRET1/(\overline{Dfd} \cdot \overline{Afa})$, which equals *FRETN*
FRET4	Loss of donor signal due to FRET using Donor filter set in the method using two-filter sets
G	Factor relating the loss of donor emission due to FRET in the Donor filter set to the gain of acceptor emission due to FRET in the FRET filter set

$$Afa = \overline{Afa} + G \cdot FRET1(Ad/Fd) \tag{4}$$

$$Ffa = \overline{Afa}(Fa/Aa) + G \cdot FRET1 \tag{5}$$

$$Dfa = \overline{Afa}(Da/Aa) + G \cdot FRET1(Da/Fa) \tag{6}$$

Substituting Eqs. 3–6 into Eqs. 2 yields Eqs. 7.

$$Df = \overline{Dfd} - FRET1 + \overline{Afa}(Da/Aa) + G \cdot FRET1(Da/Fa) \tag{7a}$$

$$Ff = (\overline{Dfd} - FRET1)(Fd/Dd) + \overline{Afa}(Fa/Aa) + G \cdot FRET1 \tag{7b}$$

$$Af = (\overline{Dfd} - FRET1)(Ad/Dd) + \overline{Afa} + G \cdot FRET1(Ad/Fd) \tag{7c}$$

In Eqs. 7, all the terms with ratio multipliers (*Da*/*Aa*, *Fd*/*Dd*, etc. are the ratio multipliers) are due to cross talk. None, some, or all of these cross talk terms may be zero for a given choice of fluorochromes and filters. However, the solution of this most general case can be processed to give the correct answer for any choice of fluorochromes and filters, whatever the cross talk situation.

G is the factor relating the loss of donor signal due to FRET with the Donor filter set to the increase in acceptor signal due to FRET with the FRET filter set.

$$G = \frac{QY_a}{QY_d}\frac{\phi_a}{\phi_d}\frac{T_F}{T_D}$$

where QY_a and QY_d are the quantum yields of the acceptor and donor, respectively, and ϕ_a is the fraction of the acceptor fluorescence transmitted by the acceptor emission filter. Similarly, ϕ_d is the fraction of the donor fluorescence transmitted by the donor emission filter. T_F and T_D are the fractional transmissions (or percent transmissions) of the neutral densities used in the two filter sets. The fraction of fluorescence transmitted is equal to the area under the product of the fluorescence emission spectrum, and the transmission spectrum of the emission filter divided by the area under the emission spectrum. The bit-mapped graphics program Adobe Photoshop was used to estimate the areas under the various curves by counting pixels with the histogram function.

Equations 7 contain three unknowns ($\overline{Dfd}$, *FRET1*, and $\overline{Afa}$), and whose solution is given by Eqs. 8.

$$\overline{Afa} = \frac{Af - (Ad/Fd)Ff}{1 - (Fa/Aa)(Ad/Fd)} \tag{8a}$$

$$FRET1 = \frac{Ff - (Fd/Dd)Df - \overline{Afa}[(Fa/Aa) - (Fd/Dd)(Da/Aa)]}{G[1 - (Da/Fa)(Fd/Dd)]} \tag{8b}$$

$$\overline{Dfd} = Df + FRET1[1 - G(Da/Aa)] - \overline{Afa}(Da/Aa) \tag{8c}$$

FRETN: A fully corrected measure of FRET

The measure of FRET normalized for the concentrations of donor and acceptor and derived from Eqs. 8 is termed *FRETN* and is given in Eq. 9.

$$FRETN = \frac{FRET1}{\overline{Dfd} \cdot \overline{Afa}} \propto \frac{[bound]}{[total\ d] \cdot [total\ a]} \tag{9}$$

Equation 9 also indicates the proportional relationship between *FRETN* and the concentrations of the interacting and noninteracting species. In Eq. 9, [*bound*] represents the concentration of interacting pairs of the donor labeled species and the acceptor labeled species, and [*total d*] and [*total a*] represent the total concentrations (interacting and noninteracting) of the donor and acceptor labeled species, respectively. *FRET1* is proportional to the FRET signal from the specimen, which in turn is proportional to the number of interacting pairs of donor and acceptor. $\overline{Dfd}$ is the donor signal that would take place if no FRET occurred and is therefore proportional to the total concentration of donor. $\overline{Afa}$ is the acceptor signal that would take place if no FRET occurred and is therefore proportional to the total concentration of acceptor. All three values, *FRET1*, $\overline{Dfd}$, and $\overline{Afa}$ are corrected for cross talk and have fully separated the FRET signal from the non-FRET signal. *FRETN* is a measure of FRET, which has the further correction that it is normalized for the donor concentration and acceptor concentration. *FRETN* is not equal to or proportional to the equilibrium constant, K_{eq}, but does look similar to K_{eq} for interaction between the donor labeled species and the acceptor labeled species (cf. Eq. 10).

$$K_{eq} = [bound]/([free\ d] \cdot [free\ a]) \tag{10}$$

where [*bound*] is as before, and [*free d*] and [*free a*] are the concentrations of the noninteracting donor labeled species and noninteracting acceptor labeled species, respectively. The relation between *FRETN* and K_{eq} is monotonic; that is, whenever K_{eq} increases so does *FRETN*. A sketch of the relation between *FRETN* and K_{eq} is shown in Fig. 1. The shape of the curve relating *FRETN* and K_{eq} is known but absolute values are not known. *FRETN* is a relative measure of K_{eq} even though the exact relation between the two is unknown. K_{eq} is the best measure of the interaction intensity of the donor labeled species with the acceptor labeled species. *FRETN* is not equal to or proportional to K_{eq}, but it is a relative measure of K_{eq}, and therefore of the interaction intensity. *FRETN* makes the best use of the data collected using the three filter sets described. Any better measure would require substantially more data.

With the proper choice of filters, the values of *Da* and *Ad* can be effectively zero for fluorescein and rhodamine as the donor and acceptor, respectively. In this case the calculation of *FRETN* is greatly simplified, as

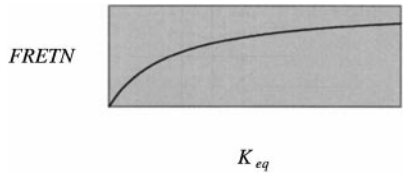

FIGURE 1 Relationship between *FRETN* and K_{eq}. See text for details.

shown in Eq. 11.

$$FRETN = \frac{Ff - Df(Fd/Dd) - Af(Fa/Aa)}{G \cdot Df \cdot Af} \tag{11}$$

In Eq. 11 G occurs only as a constant multiplier of *FRETN* and may be assigned an arbitrary value (e.g., $G = 1$) instead of being calculated or measured. If the two cross talk values, *Ad* and *Da*, are zero, then this formula for *FRETN* (Eq. 11) separates the FRET and non-FRET signal components, is corrected for the remaining cross talk, and is normalized for the concentrations of donor and acceptor. This is the same *FRETN* as in Eq. 9 but adjusted to account for the simpler cross talk situation, and is therefore a relative measure of the K_{eq} for the interaction as described above. The numerator of this formula for *FRETN* is the same as the corrected FRET value used in the MicroFRET method (Youvan et al., 1997). MicroFRET is not normalized for the concentration of either donor or acceptor.

Calculating *FRETN* under all cross talk situations

The first step in the calculation was to screen the data for aberrant data. The original intent of this step was to determine the occurrence of outliers. The screening method consisted of dividing the data into three groups according to which fluorochromes were present in the specimen: 1) group f if both donor and acceptor were present; 2) group d if only donor was present; and 3) group a if only acceptor was present. *FRETN* and *Ffa/Dfd* were calculated for all possible combinations of one specimen from each of the three groups. "Outliers" were identified by negative values for either *FRETN* or *Ffa/Dfd*. Each combination that produced a negative value of *FRETN* or *Ffa/Dfd* was flagged. If all of the combinations involving a particular specimen were flagged, that specimen was removed from its group and from further data analysis. If only some of the combinations involving a particular specimen were flagged, the specimen was taken as an ordinary outlier.

The second step was to calculate the required ratio multipliers for each of the remaining specimens in group d and then calculate the average across specimens of the ratio multipliers. For example, the ratio multipliers required from group d are *Ad*/*Fd* and *Fd*/*Dd*. If the denominator of a ratio for a particular specimen is zero, the entire ratio was set to zero. This is not meant to be a mathematical truth, but empirically it generates the correct solution of Eqs. 7 for the cross talk situation in which the value in the denominator would be zero. *Ad*/*Fd* and *Fd*/*Dd* were calculated for each specimen and then the average of *Ad*/*Fd* and the average of *Fd*/*Dd* were calculated across all of the donor group of specimens. Each of the ratio multipliers used to calculate *FRETN* is self-normalizing for concentration since the numerator and denominator are measurements of the same specimen. Thus, the use of these ratios removes the concentration-dependent variability from the calculation, which might result from variation in the concentration of donor in the donor-only specimen and variation in the concentration of acceptor in the acceptor-only specimen. This true reduction in noise is exploited by using the averages of the ratio multipliers instead of the averages of the measured values in the calculation of *FRETN*. The same process is applied to the specimens in group a to get averaged values of the required ratio multipliers *Fa*/*Aa*, *Da*/*Aa*, and *Da*/*Fa*.

The third step is to use the values of the averaged ratio multipliers to calculate *FRETN* for each specimen in the FRET group and calculate the mean and standard deviation of *FRETN*. A software program developed in-house carried out the entire three-step calculation. The same calculation is applied to the data from the suspected interacting specimen and the noninteracting control. The resulting *FRETN* values were compared by means of a Welch's *t*-test to evaluate the significance of the difference between the experimental and control specimens. If a noninteracting control were not available, it would be possible to assume that the mean of *FRETN* for the noninteracting control (if it existed) is zero and compare the suspected interacting data to the zero control with a *t*-test. However, it should be noted that incorrect conclusions might be reached if a real noninteracting control would in fact have significant FRET.

Other means of calculating *FRETN*

There are two alternative derivations for *FRETN* that produce the same values for *FRETN* but which are (at least superficially) different from the solution of Eqs. 7. The first of the alternative ways is an extension of formulation used to develop the FCET method (Trón et al., 1984). The second of the alternative ways is presented for completeness.

The first of the alternative methods for calculating *FRETN* is derived from Eqs. 7 by substituting $\overline{Dfd} \cdot FRET2$ for *FRET1* in all three equations yielding Eqs. 12. This produces an explicit recognition of the dependence of the FRET signal on the concentration of donor.

$$Df = \overline{Dfd} - \overline{Dfd} \cdot FRET2 + \overline{Afa}(Da/Aa) + G \cdot \overline{Dfd} \cdot FRET2(Da/Fa) \tag{12a}$$

$$Ff = (\overline{Dfd} - \overline{Dfd} \cdot FRET2)(Fd/Dd) + \overline{Afa}(Fa/Aa) + G \cdot \overline{Dfd} \cdot FRET2 \tag{12b}$$

$$Af = (\overline{Dfd} - \overline{Dfd} \cdot FRET2)(Ad/Dd) + \overline{Afa} + G \cdot \overline{Dfd} \cdot FRET2(Ad/Fd) \tag{12c}$$

The solution of equations 12 is given in Eqs. 13.

$$\overline{Afa} = \frac{Af - (Ad/Fd)Ff}{1 - [(Ad/Fd)(Fa/Aa)]} \tag{13a}$$

$$FRET2 = \frac{Ff - (Fd/Dd)Df - \overline{Afa}[(Fa/Aa) - (Fd/Dd)(Da/Aa)]}{Ff[1 - (Da/Fa)G] - Df[(Fd/Dd) - G] - \overline{Afa}[(Fa/Aa) - (Fd/Dd)(Da/Aa)]} \tag{13b}$$

FRET2 is already normalized for the concentration of donor so *FRETN* is given by Eq. 14.

$$FRETN = FRET2/\overline{Afa} \tag{14}$$

The second of the alternative means of calculating *FRETN* is derived from Eqs. 7 by substituting $\overline{Dfd} \cdot \overline{Afa} \cdot FRET3$ for *FRET1* in all three equations producing Eqs. 15. This produces an explicit recognition of the dependence of the FRET signal on the concentrations of donor and acceptor.

$$Df = \overline{Dfd} - \overline{Dfd} \cdot \overline{Afa} \cdot FRET3 + \overline{Afa}(Da/Aa) + G \cdot \overline{Dfa} \cdot \overline{Afa} \cdot FRET3(Da/Fa) \tag{15a}$$

$$Ff = (\overline{Dfd} - \overline{Dfd} \cdot \overline{Afa} \cdot FRET3)(Fd/Dd) + \overline{Afa}(Fa/Aa) + G \cdot \overline{Dfd} \cdot \overline{Afa} \cdot FRET3 \tag{15b}$$

$$Af = (\overline{Dfd} - \overline{Dfd} \cdot \overline{Afa} \cdot FRET3)(Ad/Dd) + \overline{Afa} + G \cdot \overline{Dfd} \cdot \overline{Afa} \cdot FRET3(Ad/Fd) \tag{15c}$$

FRET3 is already normalized for the concentrations of both donor and acceptor and is therefore equal to *FRETN* as expressed in Eq. 16. Solving Eqs. 15 for *FRET3* produces the same formula for *FRETN* as the solution of Eqs. 12. The solution of Eqs. 7 produces the same values for *FRETN* as produced by Eqs. 12 and 15, although the formula for *FRETN* is (at least superficially) not the same. Equation 16 expresses the equality of the

numerical values of *FRETN* calculated via *FRET1*, *FRET2*, and *FRET3*.

$$FRETN = FRET3 = FRET2/\overline{Afa} = FRET1/(\overline{Dfd} \cdot \overline{Afa}) \quad (16)$$

RESULTS

The interaction of the anti-apoptotic protein Bcl-2 with Beclin, a candidate novel tumor suppressor gene, was examined using FRET (Liang et al., 1993; submitted for publication 1997). The donor and acceptor were fluorescein and rhodamine, respectively.

Cos cells co-transfected with pSG5/Flag-Beclin and pSG5/Bcl-2 were labeled with donor (fluorescein conjugated) anti-flag and acceptor (rhodamine conjugated) anti-Bcl-2 antibodies, respectively. As a negative control, cells were labeled with donor (fluorescein conjugated) antibody against the endoplasmic reticulum Ca^{2+}-ATPase (SERCA) and acceptor (rhodamine conjugated) anti-Bcl-2 antibody. As shown in Table 4, there is a statistically significant difference in the FRET signal (*FRETN*) between Bcl-2 and Beclin versus Bcl-2 and SERCA. *FRETN* was also significantly greater in the case of Bcl-2 and Beclin versus Bcl-2 and mutant Beclin (Table 5). In these studies, the donor excitation filter was a 490/20 nm bandpass and the donor emission filter was a 515–555 nm bandpass. The acceptor excitation filter was a 546/40 nm bandpass, and the emission filter was a 590 nm longpass. The ratio of the quantum yields of the acceptor to donor was 0.25 (Haugland, 1996). The fractional transmissions of the donor and acceptor emission filters were 0.38 and 0.40, respectively. The percent transmissions of the FRET and Donor filter set neutral density filters were 100 and 3.2, respectively, for the experimental and the SERCA negative control ($G = 7.42$) and 20 and 1, respectively, for the mutant Beclin negative control ($G = 4.75$).

In Table 4, the *p* value of the *t*-test of the significance of the difference in *FRETN* between the Bcl-2 and Beclin versus Bcl-2 and SERCA is not very sensitive to the value of *G* over a range of 0.1 to 10.0 times the true value (data not shown). Therefore, using rough estimates of the quantum yields and the fractional transmissions of the emission filters should not be a problem. (*G* is the factor relating the loss of donor signal due to FRET with the Donor filter set to the increase in acceptor signal due to FRET with the FRET filter set.)

Other methods to measure FRET using three filter sets

Recently, Youvan et al. (1997) published a method for analyzing FRET that uses three filter sets and is termed MicroFRET. The FRET value calculated using this method is termed "corrected FRET" and is represented by F^c. The formula for the calculation of FRET using the MicroFRET method, in notation of the present report, is shown in Eq. 17.

$$F^c = Ff - Df(Fd/Dd) - Af(Fa/Aa) \quad (17)$$

F^c does separate the FRET signal from the non-FRET signal if *Ad* and *Da* are effectively zero. However, if *Ad* and *Da* are not zero, then F^c does not correct for the resulting cross talk and the FRET versus non-FRET signals are not separated. *Ad* and *Da* can often be made effectively zero by appropriate choice of fluorochromes and filter sets. F^c can be normalized for the concentration of donor by using F^c/Df, which is approximately normalized for the concentration of donor. Similarly $F^c/(Df \cdot Af)$ would be approximately normalized for the concentrations of both donor and acceptor (see Tables 1 and 2). Other authors have successfully used different three filter set methods of measuring FRET (e.g., Mittler et al., 1991; Liang et al., 1993; Szöllösi et al., 1987; Trón et al., 1984).

Measurement of FRET using two filter sets

In addition to measurement of FRET using the three-filter set/three specimen approach described above, it is possible to measure FRET using only two filter sets. The use of only two-filter sets effectively means that only two unknowns can be determined. Since there are three unknowns in Eqs. 7, it would seem that a method using two-filter sets would result in less accurate and less stringent determination of FRET than the three-filter sets/three specimen method. In fact, this is the case when the concentration of donor and acceptor are not correlated. However, if the concentration of

TABLE 4 Calculation of FRET between interacting (Bcl-2 and Beclin) and noninteracting spatially distinct (Bcl-2 and SERCA) proteins

Measure of FRET	*FRETN*	$FRET1/\overline{Dfd}$	*FRET1*	F^c	F^c/Df	$FRET4/\overline{Dfd}$	*Ffa/Dfd*	*Ff/Df*	*Ff*
Number of filter sets interacting*	3	3	3	3	3	2	2	2	1
Mean	0.000578	0.0564	4.68	33.8	0.432	0.0513	0.601	1.21	92.1
SD	0.000262	0.0173	1.95	14.1	0.137	0.0227	0.188	0.18	36.5
Noninteracting sets#									
Mean	0.0000639	0.0051	0.330	2.18	0.032	0.0100	0.183	0.236	16.53
SD	0.0000390	0.0036	0.238	1.67	0.023	0.0142	0.112	0.094	7.55
t-test *p* value	0.0021	0.0002	0.0011	0.0010	0.0003	0.0021	0.0008	<0.0001	0.0017

*$n = 7$.
#$n = 10$.

TABLE 5 Calculation of FRET between interacting (Bcl-2 and Beclin) and noninteracting spatially identical (Bcl-2 and mutant Beclin) proteins

Measure of FRET	*FRETN*	*FRET1/$\overline{Dfd}$*	*FRET1*	F^c	F^c/Df	*FRET4/$\overline{Dfd}$*	*Ffa/Dfd*	*Ff/Df*	*Ff*
Number of filter sets interacting*	3	3	3	3	3	2	2	2	1
Mean	0.000578	0.0564	4.68	33.8	0.432	0.0513	0.601	1.21	92.1
SD	0.000262	0.0173	1.95	14.1	0.137	0.0227	0.188	0.18	36.5
Noninteracting sets#									
Mean	0.000189	0.0296	4.97	21.6	0.135	0.0272	0.194	0.680	92.8
SD	0.000151	0.0268	6.19	27.3	0.128	0.0271	0.141	0.123	57.2
t-test *p* value	0.0068	0.0150	0.8787	0.2051	0.0006	0.0531	0.0007	<0.0001	0.9738

*$n = 7$.
#$n = 13$.

donor and acceptor are correlated, calculation of FRET using two-filter sets can produce equivalent results to measurement of FRET with three-filter sets.

The simplest measure of FRET using two-filter sets is *Ff*/*Df*, which does not separate FRET from the non-FRET signal, nor does it correct for cross talk. It does, however, make a partial normalization for the concentration of donor. The normalization is partial due to the lack of full correction for cross talk and lack of separation of the FRET and non-FRET signals of *Df*. (*Ff*/*Af* is an analogous measure using the Acceptor filter set in place of the Donor filter set.)

A method for calculating FRET using two-filter sets and which does correct for cross talk, but does not separate FRET and non-FRET signals, is as follows. Note that in Eqs. 2, a and b there are two unknowns, *Dfd* and *Ffa*. Solving for *Dfd* and *Ffa* and then taking their ratio, *Ffa/Dfd* yields Eq. 18.

$$\frac{Ffa}{Dfd} = \frac{Ff - (Fd/Dd)Df}{Df - (Da/Fa)Ff} \tag{18}$$

This result is corrected for cross talk and is applicable even if one or more of the cross talk terms (*Fd*, *Da*, and *Fa*) is zero. In the case where $Fa = 0$, it is necessary to set $Da/Fa = 0$ to obtain the correct solution. While this measure of FRET is now corrected for cross talk and has improved normalization for the concentration of donor (*Dfd* is a better measure of donor concentration than *Df*), it is not normalized for the concentration of acceptor nor does it separate the FRET and non-FRET signals. *Ff*/*Df* and *Ffa*/*Dfd* both detect FRET with a very high significance (low *p* values) in Tables 4–6, implying that in these data a) the correction for cross talk is not required, b) the separation of FRET and non-FRET signals is not required, and c) normalization for acceptor concentration is not required. Other data may require methods that provide these corrections.

Any method using two-filter sets to calculate FRET must make an assumption about the missing data, which would have been provided by the third filter set. *Ff*/*Df* and *Ffa/Dfd* are missing data about the concentration of acceptor. The two-filter set method therefore makes a tacit assumption about the acceptor concentration. However, it is possible to make an explicit assumption about the missing data, and, if the assumption is valid, such a two-filter set method would be optimal. When using Donor and FRET filter sets, one possible explicit assumption about the acceptor concentration is that it is always a constant, *S*, times the donor concentration. If donor-only and acceptor-only specimens can be prepared that have the ratio of the acceptor concentration to donor concentration equal to *S*, then measuring those specimens with two-filter sets produces values *Dd′*, *Da′*, *Fd′*, and *Fa′* where the two-letter symbols are as before and the prime indicates that the measurements were made with the defined relation between acceptor and donor concentrations. In practice, it is unlikely that *S* will be known; however, it would not be necessary to know *S* if specimen preparation were consistent, such that any variations in donor and acceptor concentrations only caused changes in their respective signals, which were smaller than

TABLE 6 Calculation of FRET between interacting (Bcl-2 and Beclin) and noninteracting spatially identical (Bcl-2 and mutant Beclin) proteins: effect of removal of outlier data points

Measure of FRET	*FRETN*	*FRET1/$\overline{Dfd}$*	*FRET1*	F^c	F^c/Df	*FRET4/$\overline{Dfd}$*	*Ffa/Dfd*	*Ff/Df*	*Ff*
Number of filter sets interacting*	3	3	3	3	3	2	2	2	1
Mean	0.00063	0.0640	4.59	33.2	0.491	0.0623	0.691	1.21	80.6
SD	0.00021	0.0095	2.26	16.4	0.078	0.0126	0.107	0.10	36.7
Noninteracting sets#									
Mean	0.000189	0.0296	4.97	21.6	0.135	0.0272	0.194	0.680	92.8
SD	0.000151	0.0268	6.19	27.3	0.128	0.0271	0.141	0.123	57.2
t-test *p* value	0.0078	0.0011	0.1907	0.2927	<0.0001	0.0020	<0.0001	<0.0001	0.6037

*$n = 5$.
#$n = 13$.

the FRET signal to be measured. Empirically this condition may be common, since methods using two-filter sets have been used successfully with a tacit assumption about the missing data. Rewriting Eqs. 7, a and b with the primed symbols and at the same time replacing *FRET1* with *FRET4* to indicate a different analysis method yields Eqs. 19.

$$Df = \overline{Dfd} - FRET4 + \overline{Afa}(Da'/Fa') + G \cdot FRET4(Da'/Fa') \tag{19a}$$

$$Ff = (\overline{Dfd} - FRET4)(Fd'/Dd') + \overline{Afa}(Fa'/Aa') + G \cdot FRET4 \tag{19b}$$

In Eq. 19 a the term $\overline{Afa}(Da'/Aa')$ may be replaced by $\overline{Dfd}(Da'/Dd')$ because the acceptor signal is assumed to be proportional to the donor signal with the same filter set. The proportionality constant for the Donor filter set is Da'/Dd'. Similarly, in Eq. 19 b, the term $\overline{Afa}(Fa'/Aa')$ may be replaced by $\overline{Dfd}(Fd'/Dd')(Fa'/Fd')$, which equals $\overline{Dfd}(Fa'/Dd')$. Making these substitutions in Eqs. 19 yields Eqs. 20.

$$Df = \overline{Dfd} - FRET4 + \overline{Dfd}(Da'/Dd') + G \cdot FRET4(Da'/Fa') \tag{20a}$$

$$Ff = (\overline{Dfd} - FRET4)(Fd'/Dd') + \overline{Dfd}(Fa'/Dd') + G \cdot FRET4 \tag{20b}$$

Equations 20 are two equations with two unknowns, $\overline{Dfd}$ and *FRET4*. Solving for the unknowns yields Eqs. 21.

$$FRET4 = \frac{Ff - [(Fd' + Fa')/(Dd' + Da')]Df}{[G - (Fd'/Dd')] + [(Fd' + Fa')/(Dd' + Da')[1 - G(Da'/Fa')]} \tag{21a}$$

$$\overline{Dfd} = \frac{Df + FRET4[1 - G(Da'/Fa')]}{1 + (Da'/Dd')} \tag{21b}$$

Under these conditions, the optimal measure of FRET with two-filter sets is then $FRET4/\overline{Dfd}$, which is corrected for cross talk, for the separation of the FRET and non-FRET signals and is normalized for the concentration of the donor under the assumption that the acceptor concentration is a constant proportion of the donor concentration.

$$\frac{FRET4}{\overline{Dfd}} = \frac{FRET4[1 + (Da'/Dd')]}{Df + FRET4[1 - G(Da'/Fa')]} \tag{22}$$

In this measure of FRET there are ratio multipliers that are not self-normalizing in the sense that the numerator and denominator refer to different specimens. However, given the assumption that the donor and acceptor concentrations are correlated, the donor signal may at least partially normalize the acceptor signal. By choice of fluorochromes and filters it is often the case that *Da* is zero, and in that case the result simplifies to Eqs. 23 and 24.

$$FRET4 = \frac{Ff - [(Fd' + Fa')/Dd']Df}{[G - (Fd'/Dd')] + [(Fd' + Fa')/Dd']} \tag{23a}$$

$$\overline{Dfd} = Df + FRET4 \tag{23b}$$

$$FRET4/\overline{Dfd} = FRET4/(Df + FRET4) \tag{24}$$

In Eq. 24, $FRET4/\overline{Dfd}$ is the same as in Eq. 22, but adjusted for the simplified cross talk situation. In this case *G* is not a simple multiplier of the measure of FRET and therefore must be determined before calculating *FRET4*. This is an interesting contrast to the method using three-filter sets in which when *Da* and *Ad* were taken as zero, the value of *G* could be assigned arbitrarily. In Table 5, where the more stringent negative control was used, $FRET4/\overline{Dfd}$ failed to detect the FRET signal ($p = 0.053 > 0.05$). The analog to $\overline{FRET4}/\overline{Dfd}$ in the method using three-filter sets is $FRET1/\overline{Dfd}$, which did detect FRET but which also showed a very large increase in *p* value over the case with the less stringent negative control (Tables 4 and 5).

Methods to calculate FRET using one-filter set

If only one filter set is to be used for measurement of FRET, it makes sense to choose the FRET filter set since it is designed to be the most sensitive to the FRET signal. The value *Ff* could be used with no attempt at correction. *Ff* could be partially normalized by dividing by *Fd* or ($Fd \cdot Fa$) (see Tables 4–6 for examples). In the study represented in Table 4, even *Ff*, the uncorrected measurement using a single filter set, detected a difference in FRET between the interacting versus noninteracting donor and acceptor molecules. One-filter set methods have been used successfully (e.g., Erickson and Cerione, 1991; Shapiro and McCarty, 1990; Matayoshi et al., 1990).

Interaction-sensitive versus single-distance model for FRET

All of the methods described above are designed to measure FRET between molecules that are free to diffuse independently of each other and interact according to their specific K_{eq} for interaction. This is called the interaction-sensitive model because interaction between the donor labeled molecules and the acceptor labeled molecules is reflected in an increase in FRET (Lakowicz, 1983). In the interaction-sensitive model, the donor and acceptor do not bind directly to each other but are attached to two distinct molecules. Distinct molecules interact and bring the donor and acceptor closer together. Therefore, it is possible that the donor and acceptor are not at a fixed distance apart from one another, even in an interacting pair of molecules, primarily due to the flexibility of the intervening molecular structure. Another condition of the interaction-sensitive model is that, as observed

above, the donor labeled species and the acceptor labeled species will not in general have the same concentration.

In addition to the interaction-sensitive model, there is a single-distance model in which the donor and acceptor occur only in covalently bound pairs (Lakowicz, 1983). In the single-distance model, the distance between paired donor and acceptor molecules is assumed to be fixed within a given pair, the fixed distance is assumed to be the same for all pairs, and the concentration of donor is equal to the concentration of acceptor. Because there are fewer unknowns, the same measured data can better characterize the FRET signal.

The most popular equation used to measure distance with FRET (Clegg, 1996) is:

$$E = 1 - \frac{F_{DA}}{F_D} = \frac{1}{1 + (R/R_0)^6} \tag{25}$$

where E is the efficiency of FRET (defined as number of energy transfer events divided by the number of photons absorbed by the donor), R_0 (Förster critical distance) is the distance at which E is 0.5, R is the distance between donor and acceptor, and F_{DA} and F_D are the donor fluorescence in the presence and absence of acceptor, respectively. F_{DA} and F_D are normalized for their respective concentrations of donor. R_0 must be determined in a separate experiment and requires knowledge of the spatial orientation of the donor and acceptor dipoles.

Equations 8, a and b can be used to calculate accurate values of the efficiency of energy transfer and the distance between the covalently bound donor and acceptor using the single-distance model. All three filter sets must be used, even though results from only two of the three parts of the solution are used. To calculate $1 - (F_{DA}/F_D)$ note that F_{DA} is analogous to Dfd and F_D is analogous to $\overline{Dfd}$. Also note that F_{DA} and F_D in the original formulation refer to different specimens, but Dfd and $\overline{Dfd}$ refer to the same specimen. Therefore, the ratio of Dfd and $\overline{Dfd}$ is already normalized for the concentration of donor, so $(F_{DA}/F_D) = (Dfd/\overline{Dfd})$. The values $\overline{Dfd}$ and Dfd are corrected for cross talk, for the separation of the FRET and non-FRET signals and their ratio is normalized for donor concentration since they refer to the same specimen and the same filter set. Therefore:

$$1 - (F_{DA}/F_D) = 1 - (Dfd/\overline{Dfd}) \tag{26}$$

$$1 - (F_{DA}/F_D) = 1 - (\overline{Dfd} - FRET1)/\overline{Dfd} \tag{27}$$

$$1 - (F_{DA}/F_D) = (FRET1/\overline{Dfd}) \tag{28}$$

Therefore, given R_0, E and R can be calculated from Eq. 29.

$$E = \frac{1}{1 + (R/R_0)^6} = \frac{FRET1}{\overline{Dfd}} \tag{29}$$

Since in the single-distance model the donor and acceptor concentrations are equal, the model can be formulated having only two unknowns and can therefore be analyzed with a system of two equations based on two filter sets (in this case the Donor and FRET filter sets will be used). Additional information is required for the two filter set method: the filter sets must also be used to measure donor-only and acceptor-only specimens which have known concentrations of donor and acceptor (or at least the ratio of concentrations must be known). Equations 23a and b, which express a two-filter set method for the interaction sensitive model, apply also in the present case of the single-distance model. The ratio, S, of acceptor concentration to donor concentration is known to be one in the single-distance model. Thus, the same solution applies and $E = FRET4/\overline{Dfd}$.

These measures, $FRET1/\overline{Dfd}$ and $FRET4/\overline{Dfd}$, allow calculation of correct values of E and R in the single-distance model and are useful measures of FRET in the interaction-sensitive model when the concentrations of donor and acceptor are correlated. E and R are not well defined in the interaction-sensitive model.

DISCUSSION

We present methods to measure FRET, which can be used to detect the interaction between two distinct proteins inside single cells. There were a number of reasons for undertaking the analysis described in this paper. First, there is increasing interest in the detection of interactions between intracellular molecular species and FRET provides a powerful technique for achieving this goal. Second, a number of donor/acceptor pairs suffer significant cross talk. For example, fluorescein and rhodamine are a commonly used donor/acceptor pair and many of the commonly available fluorescein filter sets will excite rhodamine and allow its detection. This type of error in FRET measurement has not been accounted for in the flow cytometry energy transfer (FCET) method (Trón et al., 1984). Third, accurate measurement of FRET should not only correct for cross talk, but also normalize for the dependence of FRET on the concentration of the donor and acceptor. Acceptor concentration normalization is not done in the FCET method. Fourth, the use of FRET for the detection of interactions between fluorescently labeled molecules should use a minimal amount of spectral information so that the method can be readily implemented using a fluorescence microscope or other fluorometer.

We examined the interaction of the anti-apoptotic protein Bcl-2 with a recently identified candidate tumor suppressor gene Beclin. Interactions between Bcl-2 and Beclin were examined because previous studies employing yeast-two-hybrid approaches have indicated a high degree of interaction of these proteins (Liang et al., 1997, submitted for publication). As controls, we examined the level of interaction assessed by FRET between Bcl-2 and a mutant Beclin protein which is known not to interact with Bcl-2, and between Bcl-2 and the endoplasmic reticulum Ca^{2+}-ATPase (SERCA). To our knowledge this is the first time that proteins constituting components of the apoptotic pathway have been examined at the single cell level and that proteins involved in the regulation of apoptosis have been shown to

interact with a protein in the tumorigenic pathways (Liang et al., 1997, submitted for publication). In previous work (Liang et al., 1993) has used FRET to detect intracellular molecular interactions using a different three-filter set method that normalizes for both donor and acceptor concentrations.

The data in Table 4 display the level of FRET calculated using the various methods described in this paper. All of the methods demonstrate a significant difference ($p < 0.05$) in the level of FRET between the suspected interacting proteins and the presumed noninteracting control proteins. We employed two different types of controls. The first was to determine the level of FRET in noninteracting proteins whose distribution was spatially distinct in cells (e.g., Bcl-2 is primarily localized to mitochondria and SERCA is primarily localized to the ER). The second was to determine the FRET between two presumed noninteracting proteins that are precisely co-localized in the cell (i.e., Bcl-2 and the mutant Beclin). These results are shown in Table 5. In the first set of controls, FRET is not anticipated due to the spatial segregation of donor and acceptor. In the second control, both proteins are primarily localized to the mitochondria and FRET might occur either due to diffusion or due to an interaction, which failed to produce the usual physiological response to the interaction. The second type of control is better than the first type in that it is much less likely to produce a false positive detection of FRET. In Table 5 only those measures of FRET having some degree of normalization for concentration of donor or for both donor and acceptor show a significant difference in FRET between the experimental and control. Therefore, in situations where the negative control may exhibit FRET and the donor and acceptor concentrations are variable, it is important to normalize the FRET data for the concentration of one or both the donor and acceptor. This will enhance the sensitivity of the FRET measurement to the interaction of the donor and acceptor.

There are two types of error in *FRETN* that cannot be corrected for using the three-filter set/three specimen method described in this manuscript. The first is that interaction of labeled with unlabeled molecules could occur. This interaction would not lead to FRET and would cause an error dependent on the ratios of labeled to unlabeled molecules. This error can be minimized by having a large excess of labeled over unlabeled species. In cases in which the labeled species are introduced by transfection of vectors with constitutive promoters, such large excess is likely. The second type of error results from FRET between any donor and acceptor that are not part of the same interacting pair. This case might arise if the donor and acceptor molecules moved close enough for FRET to occur in a transient fashion due to diffusion. It would be possible to estimate the size of the error due to FRET between noninteracting donor and acceptor if noninteracting species had the same spatial distribution as the interacting species under study. This error could be estimated measuring *FRETN* for a control specimen created with appropriately labeled noninteracting species.

When the values of *FRETN* for experimental and control specimens are compared, a difference between experimental and control in the ratio of total acceptor concentration to total donor concentration ([*total a*]/[*total d*]) is a possible source of error in judging the difference in K_{eq} between experimental and control. This possible error results from the fact that *FRETN* is not equal to or proportional to K_{eq} for interaction. *FRETN* is tabulated in Table 7 for various values of K_{eq} and [*total a*]/[*total d*]. The error of representation of K_{eq} by *FRETN* increases as [*total a*]/[*total d*] gets farther from 1. (The effect of [*total a*]/[*total d*] = x is the same as the effect of [*total a*]/[*total d*] = 1/x for any x.) The effect of [*total a*]/[*total d*] being farther from 1 becomes more pronounced as K_{eq} increases. Therefore the optimal sensitivity and reliability of the difference in *FRETN* as a measure of the difference in K_{eq} between experimental and control is achieved when experimental and control have [*total a*]/[*total d*] close to each other and close to 1. Table 8 contains the values of $(\overline{Afa \cdot T_d})/(\overline{Dfd \cdot T_a})$ for the data analyzed in this report where T_d and T_a are the percent transmissions of the neutral density filters in the Donor and Acceptor filter sets, respectively. $(\overline{Afa \cdot T_d})/(\overline{Dfd \cdot T_a})$ is proportional to [*total a*]/[*total d*] with a proportionality constant which is the same for experimental and control if no filters are changed in the filter sets, except for possibly the neutral density filters. Therefore, if the values of $(\overline{Afa \cdot T_d})/(\overline{Dfd \cdot T_a})$ for experimental and control are similar, then the values of [*total a*]/[*total d*] are also similar. No conclusion can be made as to whether the values of [*total a*]/[*total d*] are close to 1 without more information, namely the excitation intensity with each filter set and the excitation efficiencies of the donor and acceptor. In Table 8 the values of $(\overline{Afa \cdot T_d})/(\overline{Dfd \cdot T_a})$ (and therefore [*total a*]/[*total d*]) are indeed similar, increasing the confidence in the use of *FRETN* as a measure of K_{eq}. However, when the control has a low K_{eq} and the experimental has a high K_{eq}, as is the case of strong interaction versus weak or no interaction, then [*total a*]/[*total d*] may be quite different from 1 or quite different between experimental and control and still allow *FRETN* to correctly reflect the change in K_{eq}.

One puzzling finding that emerged from this study was that the *p* values in Table 4 become smaller when going from the condition where no normalization with respect to donor and/or acceptor concentration is performed compared

TABLE 7 ***FRETN*** **as a function of K_{eq} and [*total a*]/[*total d*]**

	[*total a*]/[*total d*]				
K_{eq}	1.0	1.4	2.0	10	100
0.01	0.010	0.010	0.010	0.009	0.005
0.1	0.084	0.081	0.078	0.049	0.009
1.0	0.382	0.342	0.293	0.090	0.010
10.0	0.730	0.605	0.458	0.099	0.010
100.0	0.905	0.698	0.495	0.100	0.010
Infinity	1.000	0.714	0.500	0.100	0.010

TABLE 8 $(\overline{Afa} \cdot T_d)/(\overline{Dfd} \cdot T_a)$ mean and SD for the data in this report

Protein Pair	$(\overline{Afa} \cdot T_d)/(\overline{Dfd} \cdot T_a)$ Mean	SD
Bcl-2, Beclin	1.387	0.503
Bcl-2, Beclin (outliers removed)	1.633	0.322
Bcl-2, SERCA	0.437	0.261
Bcl-2, mutant Beclin	1.538	0.972

to the condition where normalization for just the donor concentration is performed, versus the situation where the *p* values increase when going from the condition where normalization with respect to donor concentration is performed compared to normalization of both the donor and acceptor concentration. The purpose of normalization is to compensate for variability in the concentrations of the reactants. However, if the measurement error of the concentration exceeds the reduction in error expected after normalization, then normalization may actually increase the variability. It appears that normalizing the calculation of FRET for the donor concentration results in near-optimal measurement of FRET and subsequent normalization by the acceptor concentration increases the variability in the resulting *FRETN* value. This suggests that there is a correlation between the concentrations of donor and acceptor such that normalizing by both (with the present level of measurement error) does not improve the result. A corollary of this is that in such a case the method using two-filters sets and assuming that the acceptor concentration is a constant times the donor concentration, *FRET4*/$\overline{Dfd}$, would be expected to have *p* values as small or smaller than *FRETN*. This expectation is realized in Tables 4 and 6 where in the presumed interacting case the donor and acceptor concentrations are correlated (correlation coefficients 0.61 and 0.95, respectively). Table 6 uses the same data as in Table 4 except that two of the presumed interacting specimens have been omitted. The omitted specimens were outliers and their omission caused the correlation coefficient of the donor and acceptor concentrations to increase from 0.61 to 0.95. In Table 4, where there is lower correlation, *FRET4*/$\overline{Dfd}$ has the same *p* value as *FRETN*, but in Table 6, where there is higher correlation, *FRET4*/$\overline{Dfd}$ has a lower *p* value than *FRETN*. It is also possible to pose this argument in reverse: good performance of the methods using two-filter sets suggests that the donor and acceptor concentrations are correlated. In the data from Tables 4 and 5, $\overline{Dfd}$ and $\overline{Afa}$ have correlation coefficients of 0.61, 0.45, and 0.57 for Beclin with Bcl-2, mutant Beclin with Bcl-2, and Bcl-2 with SERCA, respectively. In situations where the donor and acceptor concentrations were less well correlated or not correlated at all, the *p* values would be smaller when normalization for both donor and acceptor is performed. Therefore it is worth considering whether a method using only two-filter sets and normalizing for only one concentration will detect the purported interaction in a given experimental system. A significant increase in FRET obtained using the two-filter set method may be due either to an increase in the equilibrium constant for interaction (true positive) or to a systematic difference between the experimental and the negative control in the concentration of donor or acceptor or both (false positive). If an argument can be made that such a false positive is improbable, then the result can be accepted. Demonstration of the lack of systematic difference between the experimental and the negative control with regard to the concentrations of donor and acceptor can be made via the three-filter set method generating *FRETN*, $\overline{Afa}$, and $\overline{Dfd}$. However, this is just what the use of the two-filter set method is trying to avoid. The validity of the two-filter set method could be verified by performing three-filter set measurements initially, and subsequently the two-filter set method could be used routinely. If the use of three-filter sets for a particular study results in a significant increase of *FRETN* of the experimental over the negative control, then this result should be accepted since the error in *FRETN* when the donor and acceptor concentrations are correlated increases the variability of *FRETN*, and therefore reduces the likelihood of false positives. If *FRETN* indicates no increase in FRET, but a measure of FRET normalized for only one concentration indicates an increase in FRET, the latter measure may be accepted if $\overline{Afa}$ and $\overline{Dfd}$ are correlated.

Past investigators have successfully applied methods using only two-filter sets (Wolf et al., 1992; Uster and Pagano, 1986; Adams et al., 1991; Herman and Fernandez, 1982). Data acquisition is faster with only two-filter sets and it is possible to use only a single excitation wavelength, which is convenient in confocal microscopy, flow cytometry, and plate-reading fluorometry. It should be relatively easy to accomplish FRET imaging using two filter sets with a confocal microscope or other spot-scanning microscope. These microscopes usually have two emission detectors so that the Donor filter set image is collected simultaneously with the FRET filter set image, and the two images will be in perfect register.

In summary, we present a simple method to calculate FRET from single cells expressing appropriate donor and acceptor molecules. Two models for calculation of FRET are presented. The interaction-sensitive model allows the detection of molecular interaction by the resulting increase in FRET. In the interaction-sensitive model, at least two-filter sets are required, and we present optimal methods for the use of either two- or three-filter sets. The methods detailed allow detection of FRET using donor and acceptor combinations that exhibit any possible type of cross talk. These methods make the fullest possible use of the input data and are flexible enough to handle all possible cross talk situations. There is no need to reject a donor and acceptor combination on the basis that a donor signal occurs in the Acceptor filter set or that an acceptor signal occurs in the Donor filter set. All that is needed is to use a method that corrects for the cross talk which occurs and collect more photons to get the same signal-to-noise ratio.

The most conservative and most general approach is to use three-filter sets and calculate *FRETN*. Under some circumstances another measure may be more sensitive, but *FRETN* will always be the least likely to produce a false positive. If measures other than *FRETN* are used, they should be normalized for either the concentration of donor or the concentration of acceptor, and their use should be justified by assessment of the correlation of the donor and acceptor concentrations and the cross talk situation. The single-distance model permits the absolute measurement of the distance between the covalently bound donor and acceptor. In the single-distance model, the two-filter set method and the three-filter set method should work equally well.

If data are collected using three filter sets, all of the measures of FRET using either two- or three-filter sets can be calculated and the most appropriate measure chosen to report. In cases where it is much more difficult to collect data using three filter sets rather than two, it makes sense to test whether a two filter set method is sensitive enough. In addition, when $\overline{Dfd}$ and $\overline{Afa}$ are correlated, FRET measurements can be made with either two- or three-filter sets; however, when using three filter sets and in the presence of significant measurement error, it is better to normalize for either the concentration of the donor or acceptor, but not both. For measurement of FRET using the single-distance model, use two- or three-filter sets and the methods presented here. Other methods of calculating FRET in the single-distance model have also been used (e.g., Clegg et al., 1992; Clegg, 1992; Rice et al., 1991).

This work was supported by Grants AGO7218, AG13797, and AG13637 from the National Institutes of Health, Grant BIR-9603428 from the National Science Foundation, and a grant from the North Carolina Biotechnology Center.

REFERENCES

Adams, S. R., A. T. Harootunian, Y. J. Buechler, S. S. Taylor, and R. Y. Tsien. 1991. Fluorescence ratio imaging of cyclic AMP in single cell. *Nature.* 349:694–697.

Clegg, R. M. 1992. Fluorescence resonance energy transfer and nucleic acids. *Methods Enzymol.* 211:353–389.

Clegg, R. M. 1996. Fluorescence resonance energy transfer. *In* Fluorescence Imaging Spectroscopy and Microscopy. X. F. Wang and B. Herman, editors. Wiley, New York. 179–252.

Clegg, R. M., A. I. H. Murchie, A. Zechel, C. Carlberg, S. Diekmann, and D. M. J. Lilley. 1992. Fluorescence resonance energy transfer analysis of the structure of the four-way DNA junction. *Biochemistry.* 31: 4846–4856.

Erickson, J. W., and R. A. Cerione. 1991. Resonance energy transfer as a direct monitor of GTP-binding protein-effector interactions: activated α-transducin binding to the cGMP phosphodiesterase in the bovine phototransduction cascade. *Biochemistry.* 30:7112–7118.

Haugland, R. P. 1996. Handbook of Fluorescent Probes and Research Chemicals, 6th Ed. Molecular Probes, Eugene, OR. 29.

Herman, B., and S. M. Fernandez. 1982. Dynamics and topographical distribution of surface glycoproteins during myoblast fusion: a resonance energy transfer study. *Biochemistry.* 21:3275–3283.

Lakowicz, J. R. 1983. Energy transfer. *In* Principles of Fluorescence Spectroscopy. Plenum, New York. 305–341.

Liang, X. H., M. Volkmann, R. Klein, B. Herman, and S. J. Lockett. 1993. Co-localization of the tumor suppressor protein p53 and human papillomavirus E6 protein in human cervical carcinoma cell lines. *Oncogene.* 8:2645–2652.

Matayoshi, E. D., G. T. Wang, G. A. Krafft, and J. Erickson. 1990. Novel fluorogenic substrates for assaying retroviral proteases by resonance energy transfer. *Science.* 147:954–958.

Mittler, R. S., B. M. Rankin, and P. A. Kiener. 1991. Physical associations between CD45 and CD4 or CD8 occur as late activation events in antigen receptor-stimulated human T cells. *J. Immunol.* 147:3434–3440.

Rice, K. G., P. Wu, L. Brand, and Y. C. Lee. 1991. Interterminal distance and flexibility of a triantennary glycopeptide as measured by resonance energy transfer. *Biochemistry.* 30:6646–6655.

Shapiro, A. B., and R. E. McCarty. 1990. Substrate binding-induced alteration of nucleotide binding site properties of chloroplast coupling factor 1. *J. Biol. Chem.* 265:4340–4347.

Szöllösi, J., S. Damjanovich, S. A. Mulhern, and L. Trón. 1987. Fluorescence energy transfer and membrane potential measurements monitor dynamic properties of cell membranes: a critical review. *Prog. Biophys. Molec. Biol.* 49:65–87.

Trón, L., J. Szöllösi, S. Damjanovich, S. H. Helliwell, D. J. Arndt-Jovin, and T. M. Jovin. 1984. Flow cytometric measurement of fluorescence resonance energy transfer on cell surfaces. Quantitative evaluation of the transfer efficiency on a cell-by-cell basis. *Biophys. J.* 45:939–946.

Uster, P. S., and R. E. Pagano. 1986. Resonance energy transfer microscopy: observations of membrane-bound fluorescent probes in model membranes and in living cells. *J. Cell Biol.* 103:1221–1234.

Wolf, D. E., A. P. Winiski, A. E. Ting, K. M. Bocian, and R. E. Pagano. 1992. Determination of the transbilayer distribution of fluorescent lipid analogues by nonradiative fluorescence energy transfer. *Biochemistry.* 31:2865–2873.

Youvan, D. C., W. J. Coleman, C. M. Silva, J. Petersen, E. J. Bylina, and M. M. Yang. 1997. Fluorescence imaging micro-spectrophotometer (FIMS). *Biotechnology et alia.* 1:1–16.