SlideBook™ 4.2

Ratio and FRET Modules

for Windows 2000/XP

User's Manual

(Latest Revision 1.15.07)

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Contacting Intelligent Imaging Innovations, Inc

If you have any questions or experience any problems with SlideBook™™, please contact us by either phone or email. Our phone number is (303) 607-9429 and our email address is **support@intelligent-imaging.com**.

Manual Conventions

Typographic Conventions

Menu commands are written in bold and follow the order of menu navigation. For instance, choosing "Open" from the "File" menu is written as **File > Open**.

Dialog fields and other interface items are written in bold, as in **Initial Offset**.

References to other sections of the manual are underlined, as in Image Capture.

New terms are *italicized*.

1 Introduction to Ratio and FRET Modules

The Ratio and FRET Modules add functionality that simplifies a wide variety of quantitative techniques. Ratio imaging is a live cell imaging technique that measures pairs of wavelengths. Quantification of ratio pairs is relatively unaffected by issues such as photobleaching, uneven dye loading, and dye leakage. Thus, it is an excellent technique for performing quantitative measurements. Commonly, ratio imaging techniques employ ratiometric dyes that indicate ion concentration. Examples of such dyes are Fura-2 (for Ca^{2++}) and BCECF (for pH). Ratio imaging techniques may also be employed when performing live Fluorescence Resonance Energy Transfer (FRET) experiments (e.g. Two-Channel Corrected FRET).

Measurement of FRET allows you to determine whether or not two molecules are colocalized at a distance less than 10 angstroms, enabling measurements below the resolution of a light microscope. FRET is a distance-dependent interaction between the electronic excited states of two dye molecules where the excitation is transferred from a donor molecule to an acceptor molecule *without the emission of a photon.* FRET experiments may be performed on live or fixed cells. SlideBook™ supports two FRET methods: Sensitized Emission/Direct FRET (both Two-Channel Corrected and Three-Channel Corrected) and Acceptor Photobleaching FRET. For more information on FRET methods, please see Appendix A: FRET Introduction and Theory on page A-1.

The Ratio and FRET Modules add the following components to the SlideBook™ base package:

Ratio Module Tools

- Ratio system configuration
- Ratio indicator calibration guide
- Real-time ratio and concentration display during capture
- Post-acquisition ratio and concentration display
- Ratio and concentration data export

FRET Module Tools

- Channel bleed-through calculation
- Post-acquisition calculation of Sensitized Emission/Direct FRET (Herman equation)
- Post-acquisition calculation of Acceptor Photobleaching FRET

1.1 Ratio and FRET system configuration

Expanded filter definitions allow you to define two independent ratio channels (Ratio Module) and FRET acceptor, donor and transfer channels (FRET Module). Ratio channel definitions allow for real-time calculation of ratio values and subsequent viewing and quantification of ratio data.

1.2 Ratio Tools

1.2.1 Calibration Guide

SlideBook™ provides an easy-to-follow guide for calibrating a fluoroprobe such as Fura-2 using the technique outlined in Grynkiewicz et al (1985).

1.2.2 Real-time ratio and concentration display

During capture, SlideBook™ permits you to delineate multiple regions of interest (including a background) region and graph ratio or concentration change over time. Simultaneously, SlideBook™ provides a pseudocolored image of ratio information across the entire field.

1.2.3 Post-acquisition ratio and concentration display

All interactive SlideBook™ views (main view, three view, and tile view) allow you to display ratio or concentration information as a pseducolored image. Further, each view can display the computed ratio or concentration at the current cursor location.

1.2.4 Ratio and concentration data export

SlideBook™ can produce a tab-delimited text file that includes ratio and concentration data for multiple regions of interest across time.

1.3 FRET Tools

1.3.1 Channel bleed-through calculation

SlideBook™ allows you to characterize the bleed-through coefficients of your particular imaging system. Characterization of cross-talk between channels is necessary for making quantitative measurements of Three-Channel Corrected Direct FRET. This feature is discussed in the chapter titled Protocol for Sensitized Emission/Direct FRET.

1.3.2 Post-acquisition calculation of Sensitized Emission/Direct FRET

You may specify donor and acceptor channels, corresponding bleed-through coefficients, and a background region in order to automatically calculate direct FRET. These settings may be saved for each image. A complete protocol/tutorial for performing Direct FRET is provided.

1.3.3 Post-acquisition calculation of Acceptor Photobleaching FRET

You may specify donor and acceptor channels and a background region in order to automatically calculate photobleaching FRET. A complete protocol/tutorial for performing Acceptor Photobleaching FRET is provided.

2 Configuring your System for Ratio or FRET Imaging

2.1 Defining Ratio Channels

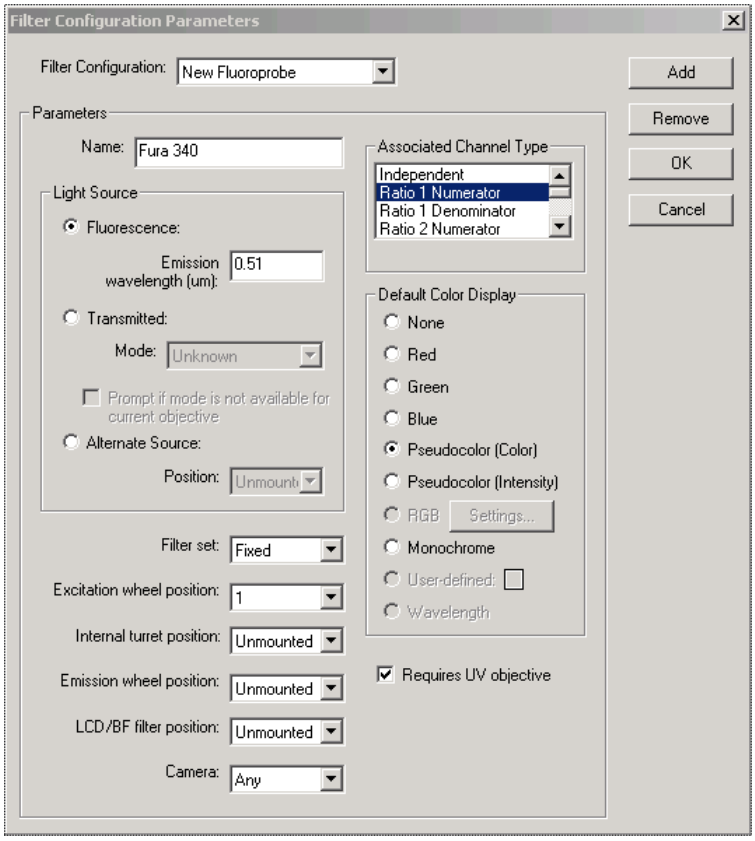
In order for SlideBook™ to be able to capture ratio images, the filter configurations must be set properly. One filter configuration should be defined as a **Ratio Numerator** and one filter configuration should be defined as a **Ratio Denominator**. These two filter configurations determine a *ratio channel*.

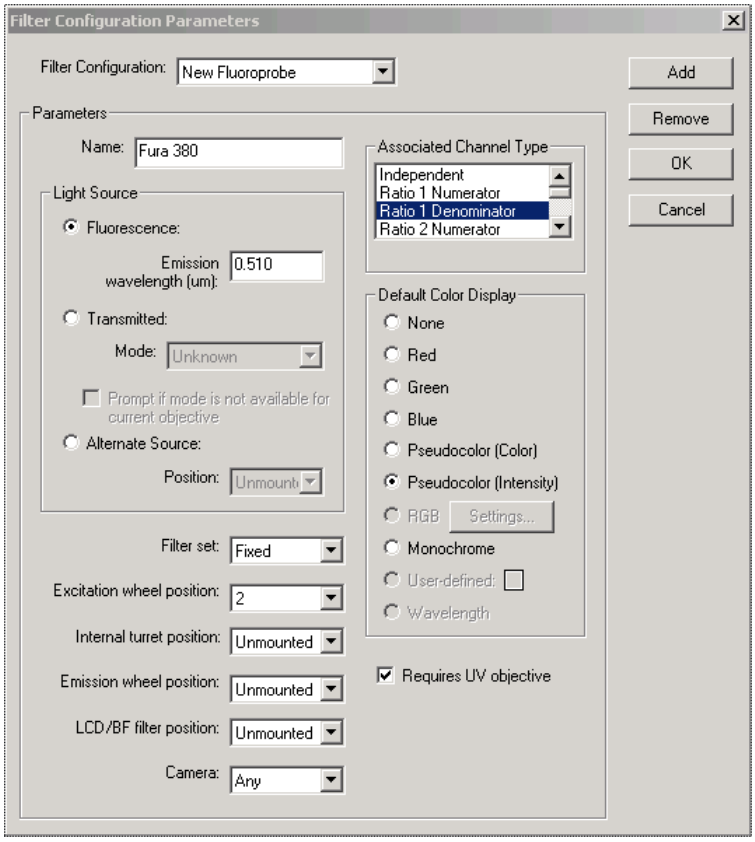
SlideBook™ lets you define two ratio channels: ratio channel 1 and ratio channel 2. If you wish to view ratio values real-time, you must set ratio channel definitions before initiating capture. You may also add ratio information post-capture as discussed in the section Adding and Viewing Ratio Channel Information on page 4-6. The following example shows how to set up a ratio channel for Fura-2.

NOTE: Ratio Channels may also used when performing Two-Channel Corrected FRET. See Appendix A for an introduction to Two-Channel Corrected FRET.

1. Select **Edit>Define Optics>Filter Configurations**. The Filter Configurations Parameters dialog box will appear.

2. Add a new channel by clicking **Add** and then entering all of the necessary parameters. A detailed explanation of all the settings is found in the SlideBook™ User's Manual, Chapter 4. Sample settings for Fura-2 are shown below.





NOTE: Make sure that the numerator and denominator configurations for a particular indicator are associated with the same ratio channel.

3. Close and restart SlideBook™ to update your preferences.

2.2 Defining FRET Channels

FRET channels will be defined using a procedure similar to that discussed above. Channel definitions for Direct FRET and Acceptor Photobleaching FRET will be discussed in this section.

2.2.1 Direct/Sensitized Emission FRET.

In order to perform Direct FRET, the appropriate filter definitions must exist. The common FRET pair, CFP and YFP, is used as an example. For three-channel corrected FRET, three filter definitions are necessary: CFP (donor), YFP (acceptor), and FRET. For two-channel corrected FRET, two filter definitions are necessary: *either* CFP or YFP, and FRET. CFP and YFP filter sets are configured to use the respective exciters and emitters. The FRET channel must use a CFP exciter and YFP emitter. The exact configuration of the filters will depend on your particular hardware configuration.

2.2.1.1 Three-Channel Corrected FRET

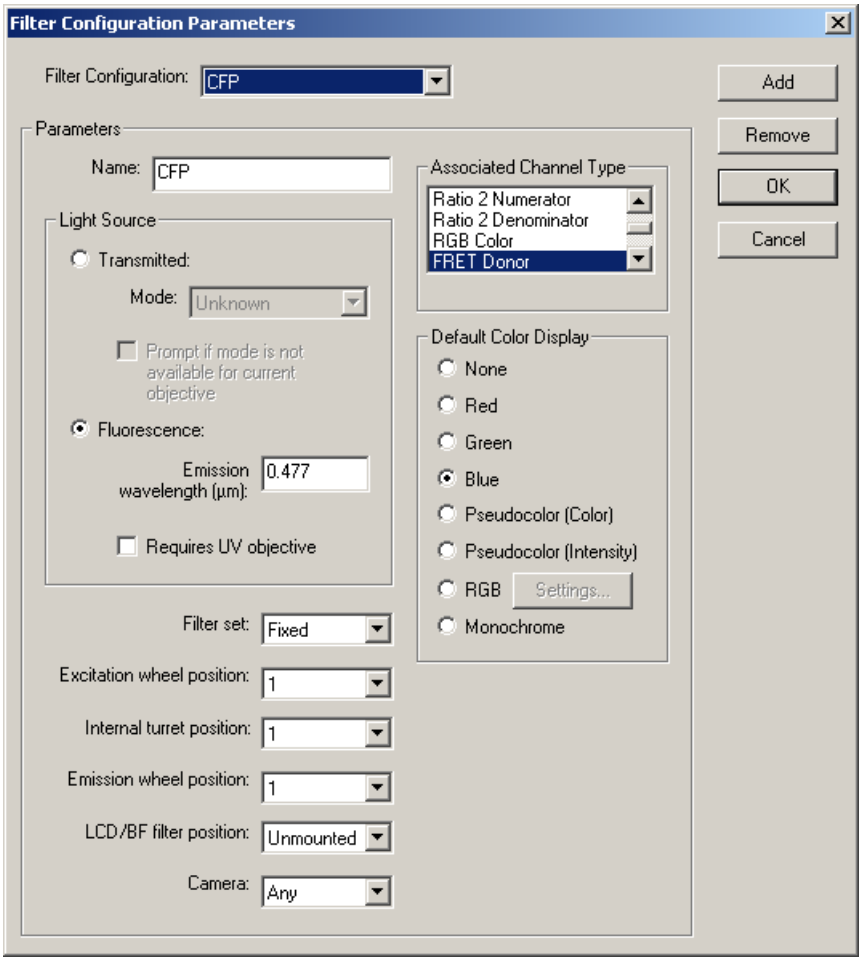
To add FRET filters for performing Three-Channel Corrected FRET:

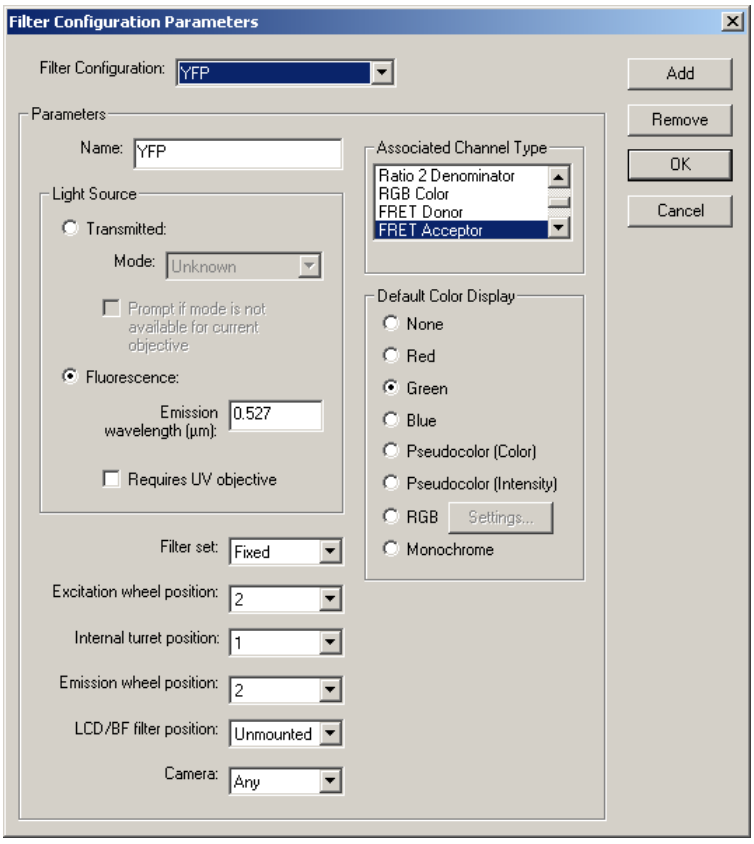
1. Select **Edit>Define Optics>Filter Configurations**. The Filter Configurations Parameters dialog box will appear.

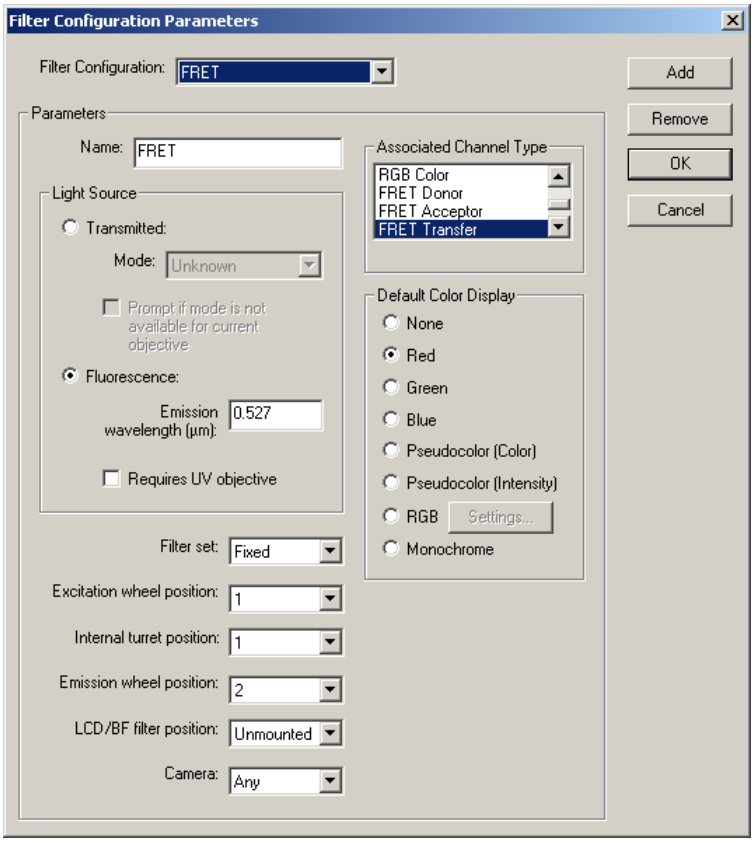
2. Add a new channel by clicking **Add** and then entering all of the necessary parameters. A detailed explanation of all the settings is found in the SlideBook™ User's Manual, Chapter 4. Sample settings for CFP, YFP, and FRET are shown below.

   This example shows typical settings when using a JP4 filter set, where the dichroic is in filter position 1, the CFP and YFP exciters are in filter wheel positions 1 and 2, respectively, and the CFP and YFP emitters are in filter wheel positions 1 and 2, respectively.

   NOTE: Make sure to select the appropriate **Associated Channel Type** (FRET Donor for CFP, FRET Acceptor for YFP, and FRET Transfer for FRET).




Filter Configuration Parameters
Filter Configuration: YFP
Add
Remove
OK
Cancel
Parameters
Name: YFP
Associated Channel Type
Ratio 2 Denominator
RGB Color
FRET Donor
FRET Acceptor
Light Source
Transmitted:
Mode: Unknown
Prompt if mode is not available for current objective
Fluorescence:
Emission wavelength (µm): 0.527
Requires UV objective
Default Color Display
None
Red
Green
Blue
Pseudocolor (Color)
Pseudocolor (Intensity)
RGB
Settings...
Monochrome
Filter set: Fixed
Excitation wheel position: 2
Internal turret position: 1
Emission wheel position: 2
LCD/BF filter position: Unmounted
Camera: Any


Filter Configuration Parameters
Filter Configuration: FRET
Add
Remove
OK
Cancel
Parameters
Name: FRET
Associated Channel Type
RGB Color
FRET Donor
FRET Acceptor
FRET Transfer
Light Source
Transmitted:
Mode: Unknown
Prompt if mode is not available for current objective
Fluorescence:
Emission wavelength (µm): 0.527
Requires UV objective
Default Color Display
None
Red
Green
Blue
Pseudocolor (Color)
Pseudocolor (Intensity)
RGB
Settings...
Monochrome
Filter set: Fixed
Excitation wheel position: 1
Internal turret position: 1
Emission wheel position: 2
LCD/BF filter position: Unmounted
Camera: Any

3. Close and restart SlideBook™ to update your preferences.

2.2.1.2 Two-Channel Corrected FRET

For Two-Channel Corrected FRET, define a ratio channel with the FRET channel set as the numerator, and the acceptor or donor channel set as the denominator (see page 2-1).

2.2.2 Acceptor Photobleaching FRET

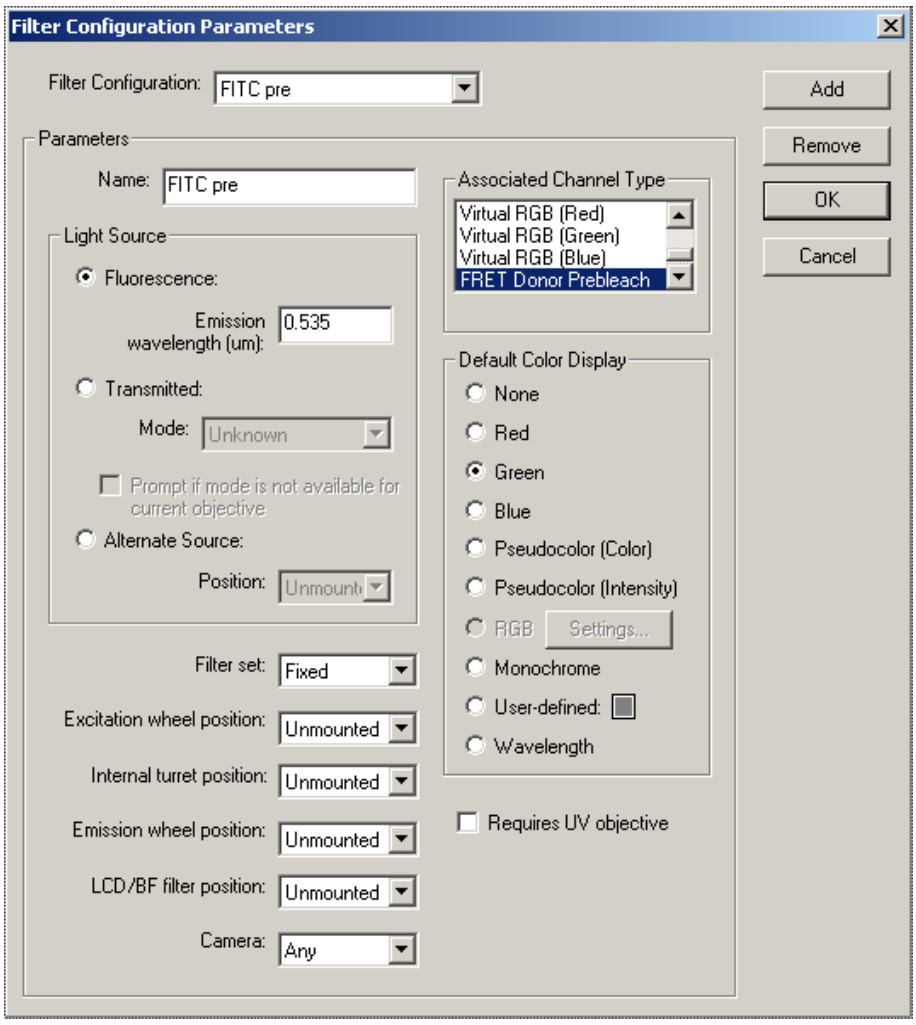
In order to perform acceptor photobleaching FRET, you must define five channels: "donor prebleach", "acceptor prebleach", "photobleach", "donor postbleach", and "acceptor postbleach". The common FRET pair, FITC (donor) and CY3 (acceptor), are used to demonstrate filter configurations. A full protocol for performing acceptor photobleaching FRET can be found in Protocol for Acceptor Photobleaching FRET on page 8-1.

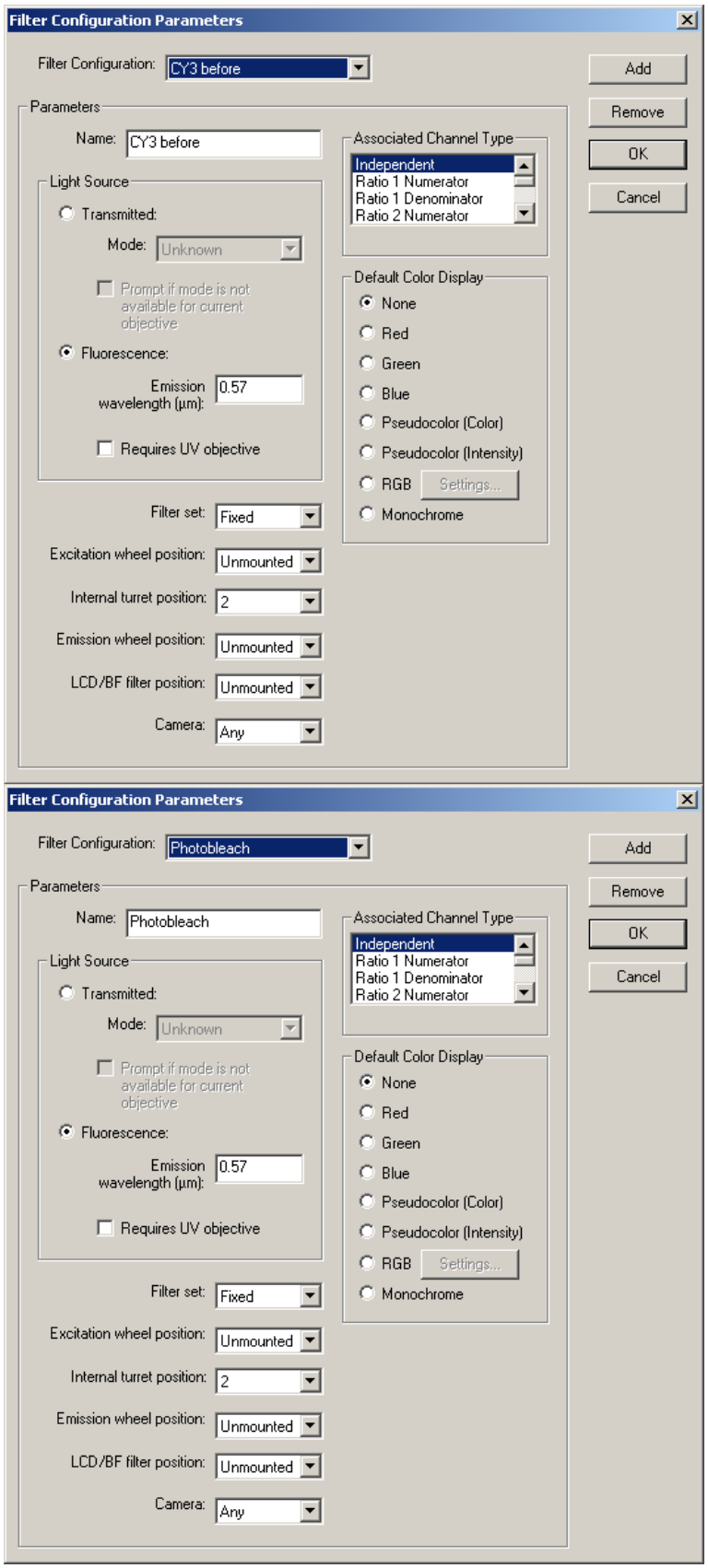
1. Select **Edit>Define Optics>Filter Configurations**. The Filter Configurations Parameters dialog box will appear.

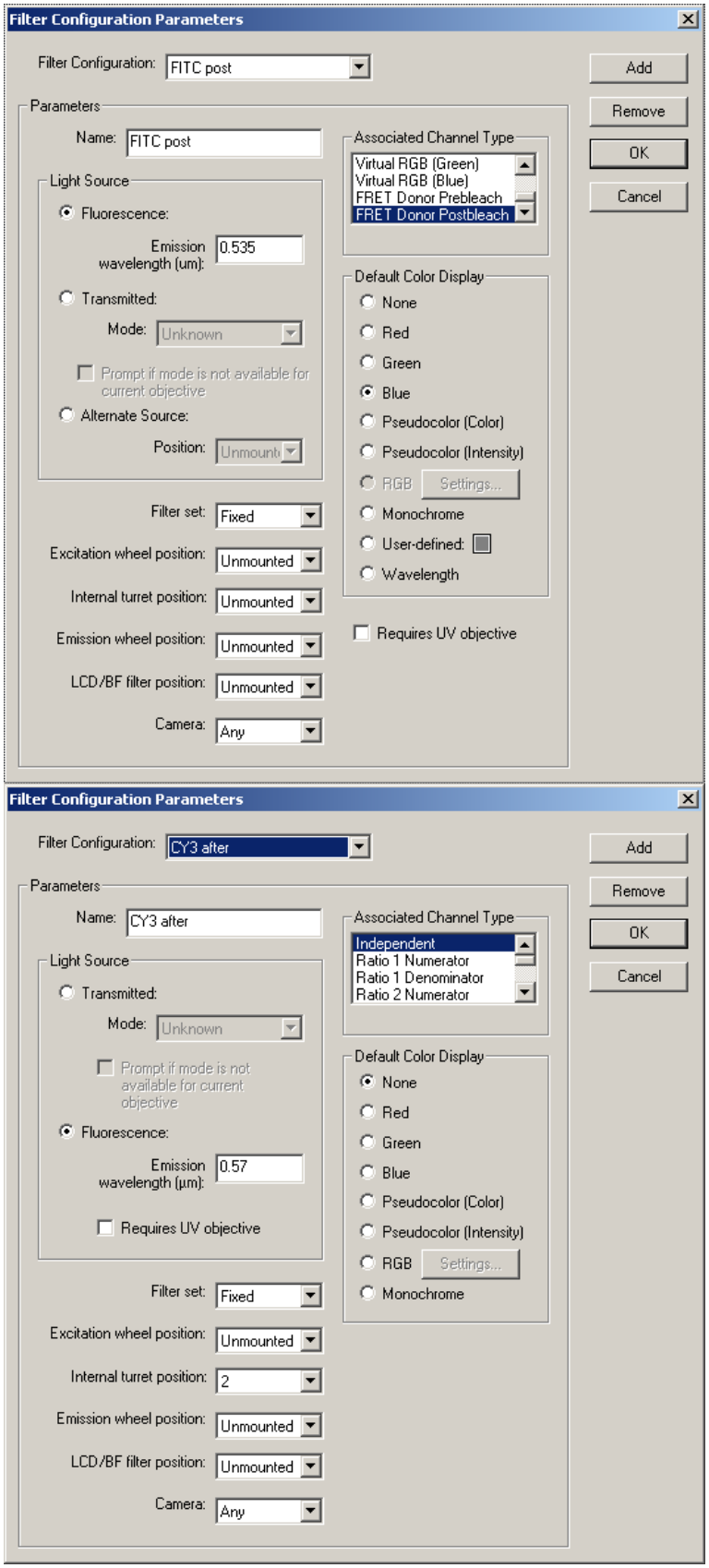
2. Add a new channel by clicking **Add** and then entering all of the necessary parameters. A detailed explanation of all the settings in the dialog box is found in the SlideBook™ User's Manual, Chapter 4. Sample dialog boxes for the five required filter definitions are shown on the following pages.

   The following example shows typical settings when using filter cubes. Note that the Photobleach channel is the same filter cube as the Acceptor (CY3) channels.

3. Close and restart SlideBook™ to update your preferences.


Filter Configuration Parameters
Filter Configuration:
FITC pre
Add
Remove
OK
Cancel
Parameters
Name:
FITC pre
Light Source
Fluorescence:
Emission wavelength (um):
0.535
Transmitted:
Mode:
Unknown
Prompt if mode is not available for current objective
Alternate Source:
Position:
Unmount
Associated Channel Type
Virtual RGB (Red)
Virtual RGB (Green)
Virtual RGB (Blue)
FRET Donor Prebleach
Default Color Display
None
Red
Green
Blue
Pseudocolor (Color)
Pseudocolor (Intensity)
RGB
Settings...
Monochrome
User-defined:
Wavelength
Filter set:
Fixed
Excitation wheel position:
Unmounted
Internal turret position:
Unmounted
Emission wheel position:
Unmounted
LCD/BF filter position:
Unmounted
Camera:
Any
Requires UV objective


Filter Configuration Parameters
Filter Configuration: CY3 before
Add
Remove
OK
Cancel
Parameters
Name: CY3 before
Light Source
Transmitted:
Mode: Unknown
Prompt if mode is not available for current objective
Fluorescence:
Emission wavelength (µm): 0.57
Requires UV objective
Associated Channel Type
Independent
Ratio 1 Numerator
Ratio 1 Denominator
Ratio 2 Numerator
Default Color Display
None
Red
Green
Blue
Pseudocolor (Color)
Pseudocolor (Intensity)
RGB
Settings...
Monochrome
Filter set: Fixed
Excitation wheel position: Unmounted
Internal turret position: 2
Emission wheel position: Unmounted
LCD/BF filter position: Unmounted
Camera: Any
Filter Configuration Parameters
Filter Configuration: Photobleach
Add
Remove
OK
Cancel
Parameters
Name: Photobleach
Light Source
Transmitted:
Mode: Unknown
Prompt if mode is not available for current objective
Fluorescence:
Emission wavelength (µm): 0.57
Requires UV objective
Associated Channel Type
Independent
Ratio 1 Numerator
Ratio 1 Denominator
Ratio 2 Numerator
Default Color Display
None
Red
Green
Blue
Pseudocolor (Color)
Pseudocolor (Intensity)
RGB
Settings...
Monochrome
Filter set: Fixed
Excitation wheel position: Unmounted
Internal turret position: 2
Emission wheel position: Unmounted
LCD/BF filter position: Unmounted
Camera: Any


Filter Configuration Parameters
Filter Configuration: FITC post
Add
Remove
OK
Cancel
Parameters
Name: FITC post
Associated Channel Type
Virtual RGB (Green)
Virtual RGB (Blue)
FRET Donor Prebleach
FRET Donor Postbleach
Light Source
Fluorescence:
Emission wavelength (um): 0.535
Transmitted:
Mode: Unknown
Prompt if mode is not available for current objective
Alternate Source:
Position: Unmount
Default Color Display
None
Red
Green
Blue
Pseudocolor (Color)
Pseudocolor (Intensity)
RGB
Settings...
Monochrome
User-defined:
Wavelength
Filter set: Fixed
Excitation wheel position: Unmounted
Internal turret position: Unmounted
Emission wheel position: Unmounted
LCD/BF filter position: Unmounted
Camera: Any
Requires UV objective
Filter Configuration Parameters
Filter Configuration: CY3 after
Add
Remove
OK
Cancel
Parameters
Name: CY3 after
Associated Channel Type
Independent
Ratio 1 Numerator
Ratio 1 Denominator
Ratio 2 Numerator
Light Source
Transmitted:
Mode: Unknown
Prompt if mode is not available for current objective
Fluorescence:
Emission wavelength (µm): 0.57
Requires UV objective
Default Color Display
None
Red
Green
Blue
Pseudocolor (Color)
Pseudocolor (Intensity)
RGB
Settings...
Monochrome
Filter set: Fixed
Excitation wheel position: Unmounted
Internal turret position: 2
Emission wheel position: Unmounted
LCD/BF filter position: Unmounted
Camera: Any

.

3 Real-time Ratio Display

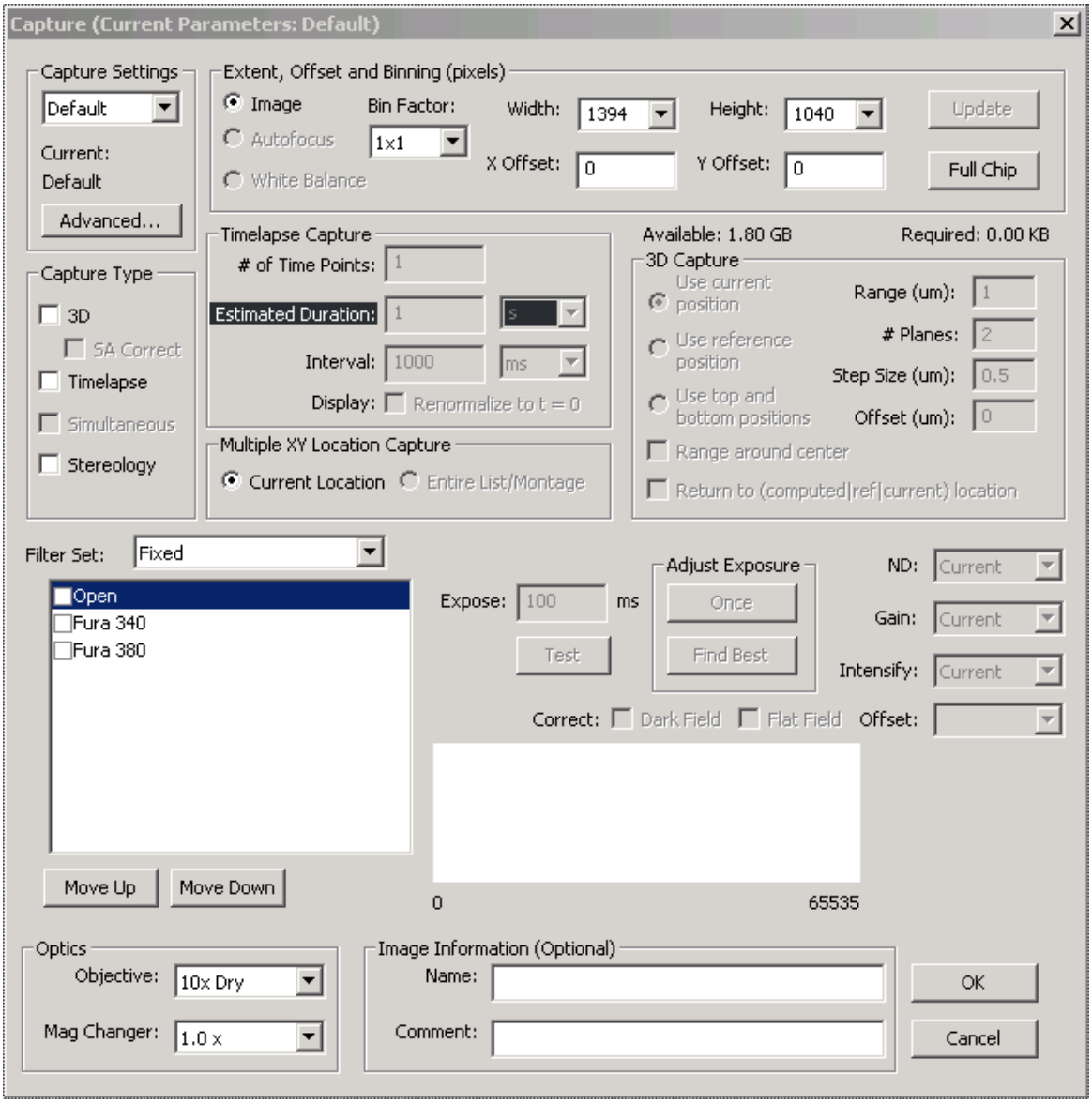
In this chapter, you will learn how to set parameters for capture and interactively view your data as it is captured.

3.1 Setting Capture Parameters for Time-lapse Ratio Imaging

Ratio capture works much like other image capture paradigms in SlideBook™. For a more detailed explanation of image capture, please see the SlideBook™ 4.2 User's Manual, Chapters 6 and 7. To capture a new time-lapse ratio image:

1. Bring your sample into view and focus in the Focus Window (Chapter 5, User's Manual).

2. Select **Image > Capture New Image…**. The Capture Dialog box will appear.



3. Select the **Time Lapse** check box and specify the time interval between collections as well as the total number of time points. Once you select Time Lapse, you will activate other options in the capture window.

   Note that currently, the time interval tells SlideBook™ how many milliseconds to wait after completing the capture of one time point before initiating capture of the next time point.

4. Select the appropriate checkboxes for live graphing:

   - **Renormalize to first time point** –uses the minimum and maximum pixel intensity values for the first time point in a timelapse capture series to determine the renormalization values of all subsequent time points. This

option only affects the image display during capture and will not affect the captured image

5. Specifiy a ratio capture by clicking on the checkboxes for both the ratio numerator and denominator channels and testing the exposure length for each.

   It is easy to collect other data (such as a GFP or DIC image) during ratio capture. Simply select additional channels to capture as in normal time lapse imaging. You may also capture a channel (such as DIC) at a slower rate than your ratio data by selecting the Periodic tab found in the Capture Preferences dialog box (use the **Advanced** button in the Capture Preferences section to access this dialog box). Please see Changing the Channel Capture Frequency in Chapter 7 of the SlideBook™ User's Manual.

6. Once you have captured a test image, you may use the ROI tools to draw regions to monitor during capture. To learn more about ROI tools, please see Creating ROIs and Graphs to Monitor Regions of Interest in Chapter 7 of the SlideBook™ User's Manual.

7. If you would like to record events, such as reagent addition, set up Notes using the **Advanced** button as discussed in the SlideBook™ User's Manual, Chapter 7 Creating Notes.

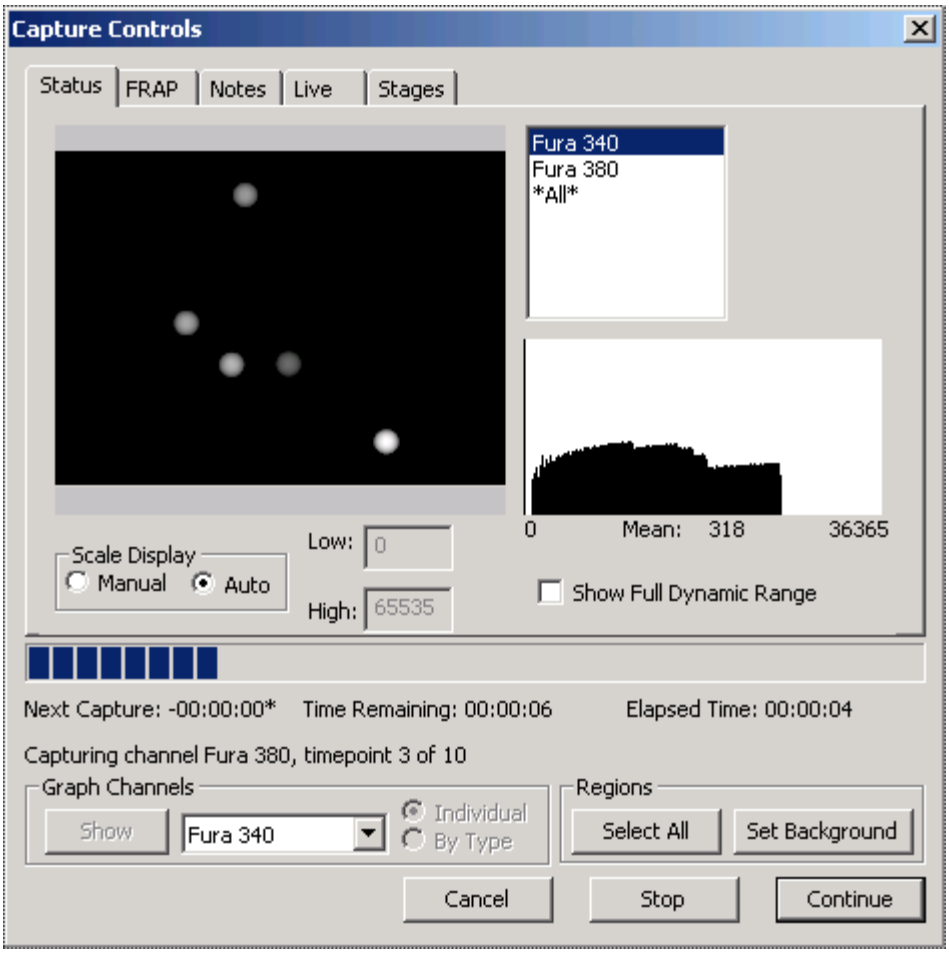
8. Select **OK** when you are ready to begin capture.

NOTE: It is often advantageous to use binning for time lapse ratio capture. Not only will this permit better temporal resolution, but it will also dramatically decrease the total size of the collected data.

3.2 Live Display

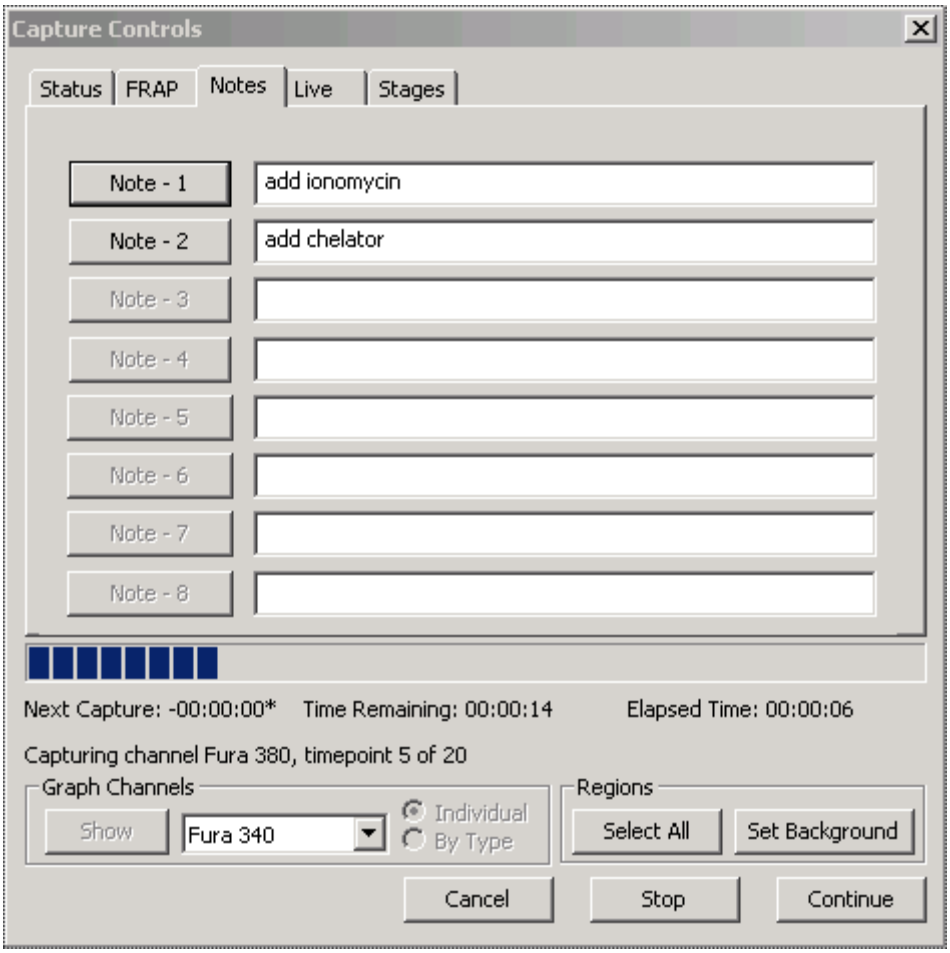
During capture, SlideBook™ can display a pseduocolored image of ratio values for each time point as well as update a graph of ratios for multiple regions of interest.

The Capture Status dialog box will appear and display the current single channel image in the Status tab. The Image Window will display a pseudocolored ratio image.



You may also select a channel and the **Show** button which allows you to display data (background-subtracted if you have chosen a background region) for all channels for a single region of interest, or for one channel (including the ratio channel) for all regions of interest.

NOTE: In all display views, you can mark events using the **Notes** buttons you created when setting capture parameters. The buttons are displayed on the **Notes** tab of the Capture Status dialog.



3.2.1 Selecting Regions of Interest

You may select regions of interest for graphing either before capture has started or during capture. ROI tools may be used on test images generated before capture, or on the live image display during capture please see Creating ROIs and Graphs to Monitor Regions of Interest in Chapter 7 of the SlideBook™ User's Manual. If you choose a background region, the average value of this region will be subtracted from the average values in the other regions of interest before ratio computation. The background region will also be stored in the Ratio Settings dialog box (see next Chapter).

3.2.2 Graph Display

To display the ratio graph during capture, simply select the channel that you wish to graph from the drop-down list, then press the **Show** button in the Graph Channels section of the Capture Status dialog. Repeat for any additional channels you wish to display, or simply select the channels from the channel menus on the graph. **Note that you will only be able to select channels of the same type, e.g., independent or ratio, from the channel menus.** Thus, you must have at least two graphs open to display 340, 380, and a ratio.

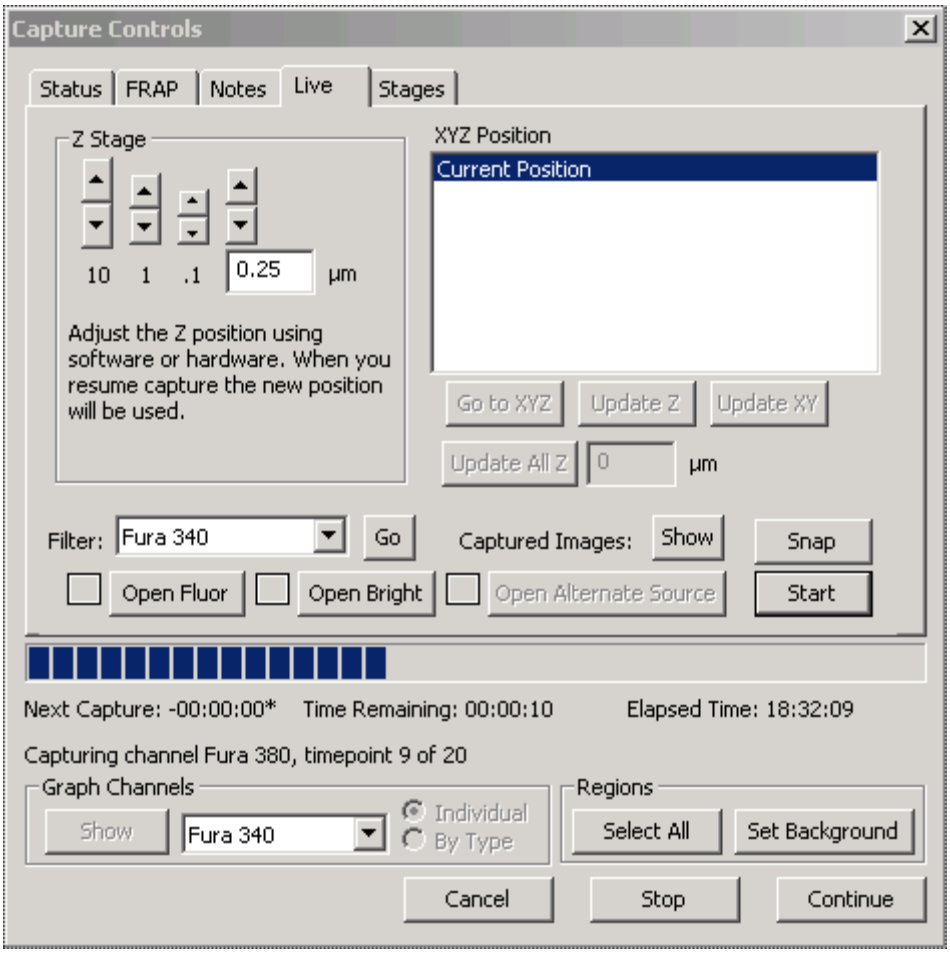
Use the Zoom In and Zoom Out tools to explore your data. At any time, you may reset the data to view full range using the **Reset** buttons. Once your capture is finished, your graph will remain open for viewing.

To recreate this graph post-capture, on an open view, display the ROIs (**View>Annotations>ROIs**), and then select **View>Graph...**.

3.2.3 Focusing During Capture

You may adjust the focus of your image during capture. To do so, go to the **Live** tab. The **Live** view works similarly to the Focus Window.

NOTE: Before using the **Live** tab, you must first select **Pause**. Once you are finished focusing, you must select **Continue** to resume capture.



The window has the following features:

- **Start/Stop –** begins/ends the semi-live camera readout
- **Open Fluor/Close Fluor** - opens and closes the fluorescent shutter
- **Open Bright/Close Bright** – opens and closes the transmitted light shutter
- **Filter** – moves motorized filters into position when you select **Go**.
- **Z Stage –** allows you to refocus your sample using precise movements.

To use this window, press **Start**, open the desired shutter, select the appropriate filter, and focus your sample. When you are finished, close the shutter, press **Stop**, and then **Continue**.

4 Post-Acquisition Display and Settings

Ratio data is generally best displayed using **Pseudocolor** settings. This will be the default setting if you define filters used for ratio imaging as discussed in Chapter 2 on page 2-1. We will now explore display of ratio data using the example slide, **ratiosample.sld.** This slide is included in the file **RatioFRET.zip**. If you are a new user, please complete the Quick Tour tutorial in the SlideBook™ User's Manual before beginning this tutorial.

4.1 Viewing Ratio Data in Pseudocolor

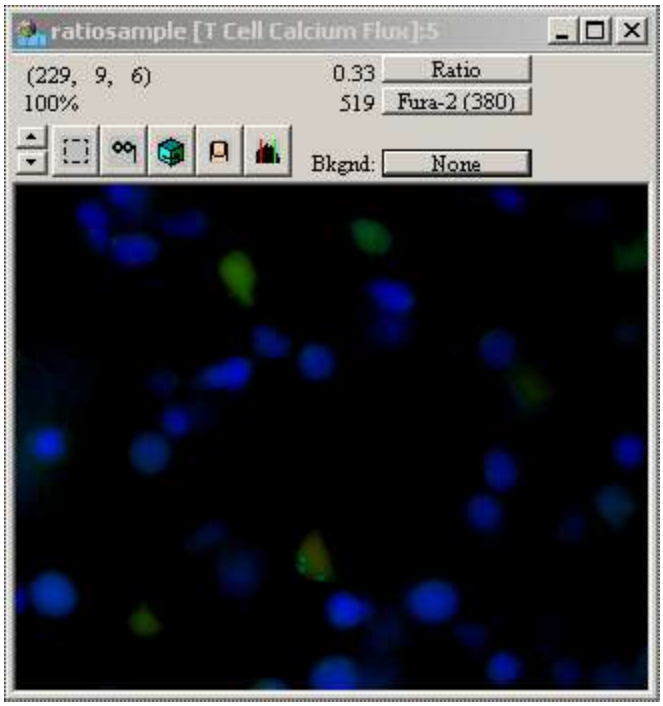
To begin this demonstration:

1. Open SlideBook™ and select **File>Open Slide**.

2. Navigate to **ratiosample.sld** and select **Open**.

3. Double-click on the "T Cell Calcium Flux" image to generate a main view. The following image will appear.

   Please see Chapter 8 of the SlideBook™ User's Manual for further information on data views.



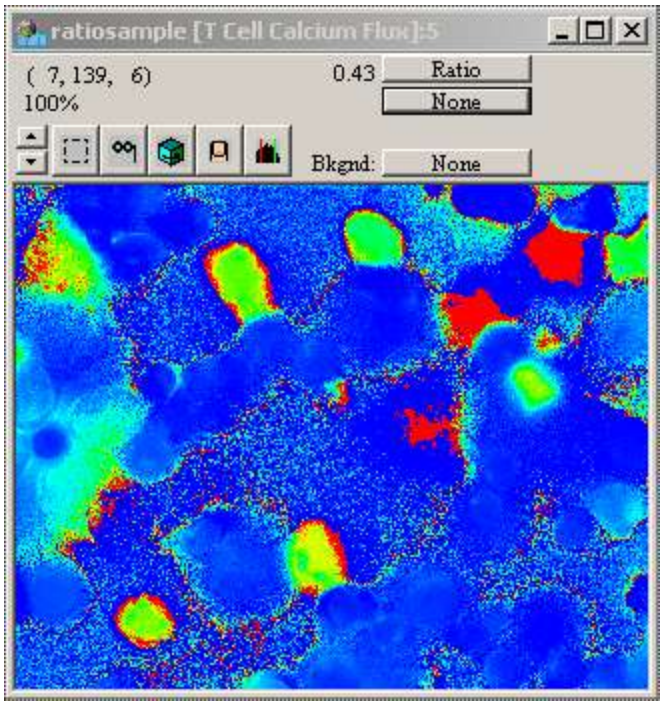
(T-cell image courtesy of Dr. Jackson Egen, University of California, Berkeley.)

Note that the image is displayed in pseudocolor, with the **Ratio** channel appearing in the first channel menu. The ratio channel name will always be called **Ratio** (or **Ratio 1 and Ratio 2** if two ratios are defined and captured). Also note that SlideBook™ stores both the numerator and denominator time-lapse image. The ratio values are computed when the image is opened and are not stored with the image.

NOTE: If you have not defined your ratio channels as discussed in Chapter 2, you may view your data in Pseudocolor by selecting **View > Pseudocolor**.

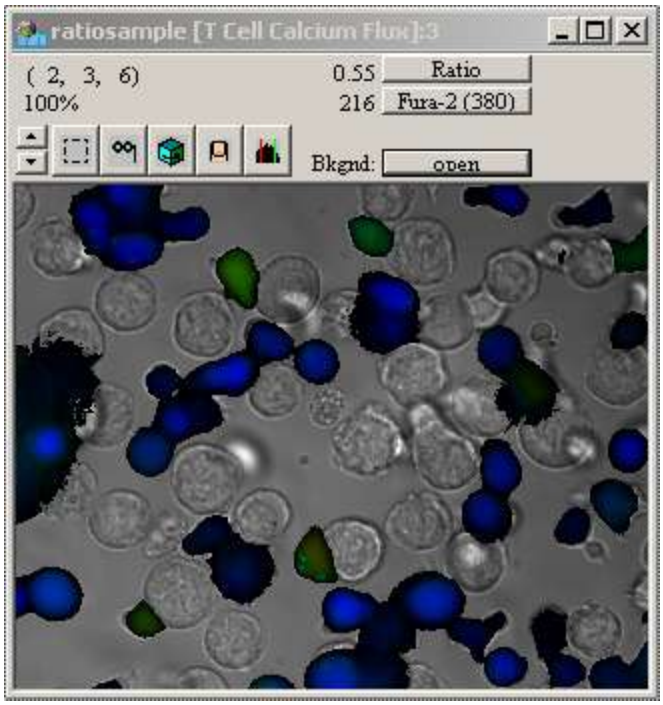
In the pseudocolor view mode, there are two channel selections. The first channel selects the hue of the display and should be set to the **Ratio** channel. The second channel selects what determines the intensity, or brightness, of the displayed colors. If it is set to either the numerator or denominator channel, the pseudocolored display will be scaled by the selected fluorescence intensity, such as in the example above. Thus, in the example above, an area with a high pixel intensity for the ratio channel and a low pixel intensity on denominator channel will appear as dim red. An area with a low pixel intensity for the ratio channel and a high pixel intensity on the denominator channel will appear as bright blue. This helps distinguish areas with real data from background. This is often desirable as computed ratio values for regions of the image that are just slightly above background can vary greatly. Please see Chapter 4 Default Color Display in the SlideBook™ User's Manual for further discussion of the pseudocolor view.

4. Set the second channel menu selection to **None**. The image is now displayed as a range of blue (low ratio) to red (high ratio). The brightness/dimness of the hue is not scaled.



5. Note the large amount of noise in this image and return the second menu to **Fura-2 (380)**.

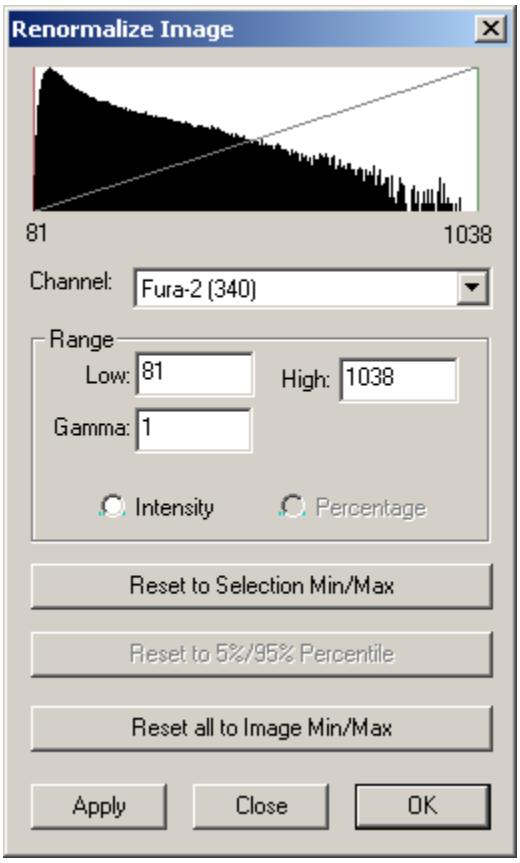
6. Set the **Bkgnd** drop down menu to Open. A DIC image is now displayed along with the ratio data. For further instructions on displaying a background image please see Chapter 8 Displaying a DIC image as Background of an RGB/pseudocolor image in the SlideBook™ User's Manual.



7. Blend the DIC image with the fluorescence image by selecting **View>Blend Background.**

8. Return the background channel to **None**.

9. Open the renormalization dialog box using the  icon on the tool bar. The following dialog box will appear:



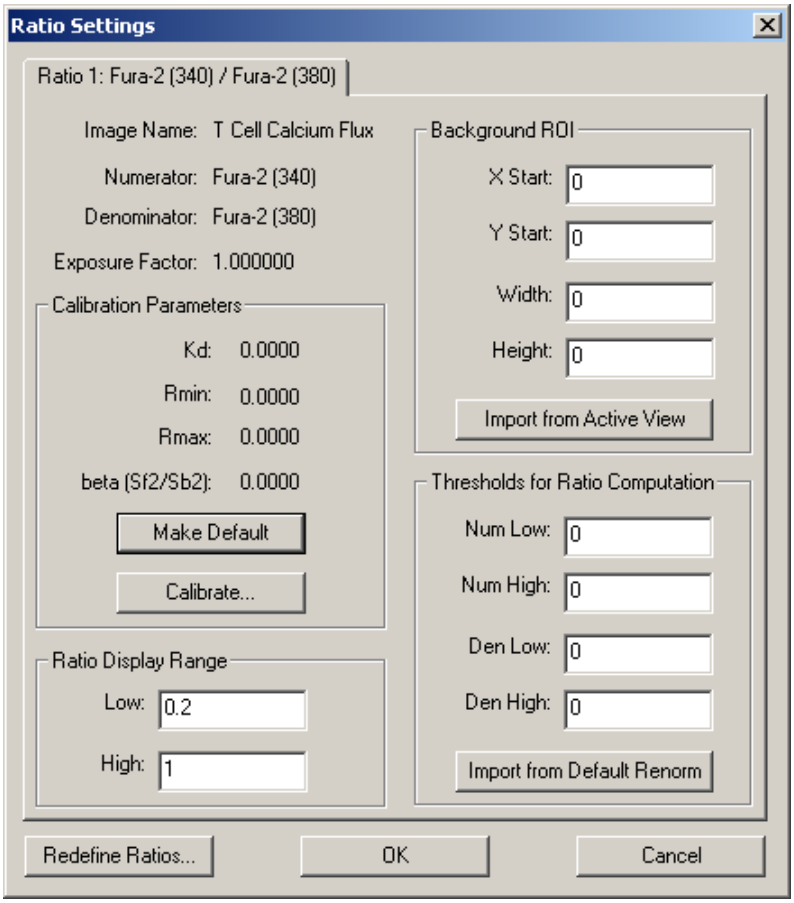
10. Move the red bar back and forth. Note that the display does not change. Fura-2 (340) is not a displayed channel in this image.

11. Select **Fura-2 (380)** from the channel drop-down menu and move the red bar back and forth. Note that the hue, or color, of the display does not change. Note also that the intensity of the color becomes dim as you move the red bar to the right and becomes bright as you move the red bar to the left.

    Note that the hues that are displayed cannot be changed using the renormalization dialog box, only the intensity of color may be changed. To change the hues that are displayed, you must alter the **Ratio Settings**, as discussed in the next section.

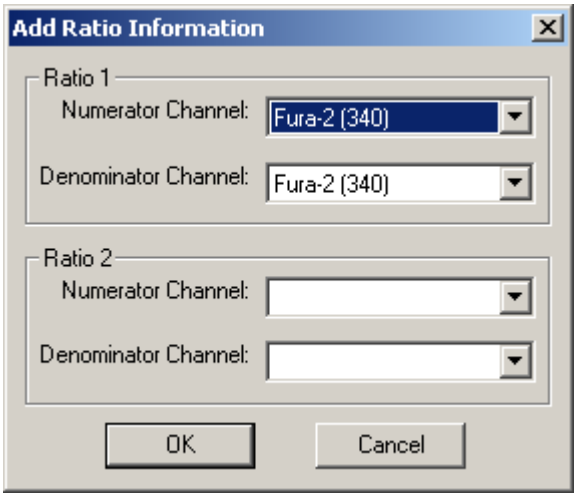
4.2 Ratio Settings

SlideBook™ lets you adjust, if necessary, many of the settings that go into both post-acquisition display as well as analysis. These settings are accessed in the Ratio Settings dialog box. A tutorial follows the explanation of this dialog box.



4.2.1 Adding and Viewing Ratio Channel Information

The numerator and denominator are displayed for a given ratio channel, as well as the *exposure factor*, which is the ratio of exposure times for the numerator and denominator wavelengths. The channels used for calculating the ratio can be edited by selecting **Redefine Ratios…**, which brings up the **Add Ratio Information** dialog box.



4.2.2 Calibration Parameters

The Ratio Calibration Guide may be used to calibrate or recalibrate an image by selecting **Calibrate…** The Ratio Calibration Guide is discussed in Chapter 5.

4.2.3 Ratio Display Range

You may select the range of ratio values that are displayed in any view of the ratio image. Any ratios below the **Low** value are displayed as blue, and any ratios above the **High** value are displayed as red. This operation will not affect the underlying data.

4.2.4 Background ROI

You may select a background region that will be used to subtract background intensities from the numerator and denominator channel prior to calculating ratio and concentration values.

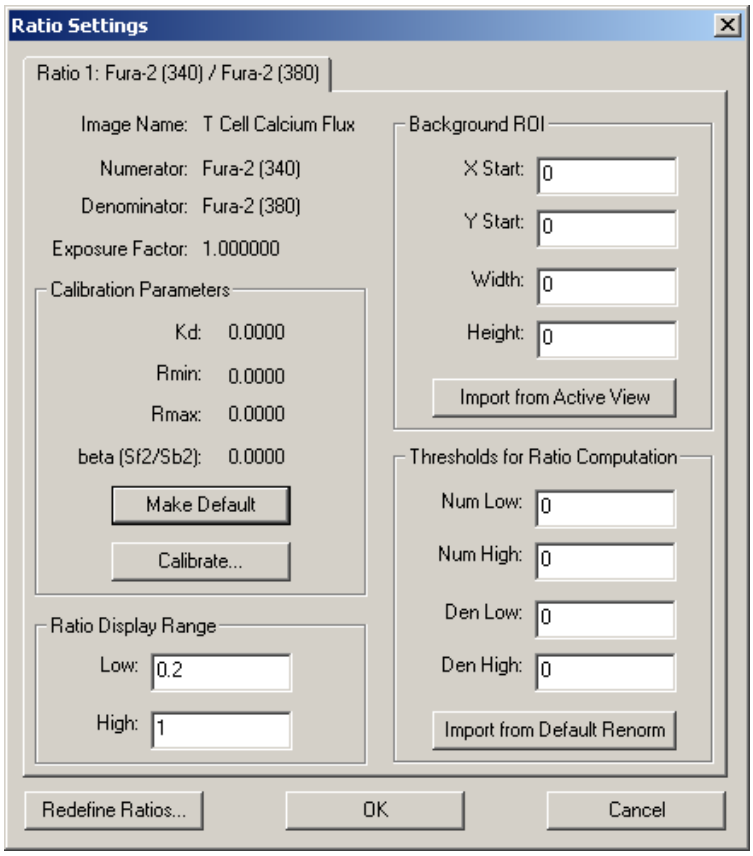
4.2.5 Thresholds for Ratio Computation

This feature is important when generating statistical data. These thresholds allow you to compute ratios for relevant data. This feature allows you to use simple masks to define ROI's, but only compute ratio data for objects that are within the desired intensity range. This allows you to "qualify" pixels that should be used for ratio computation. Only pixels that fall into the range for **both** the numerator and denominator values will be considered when statistics are calculated.

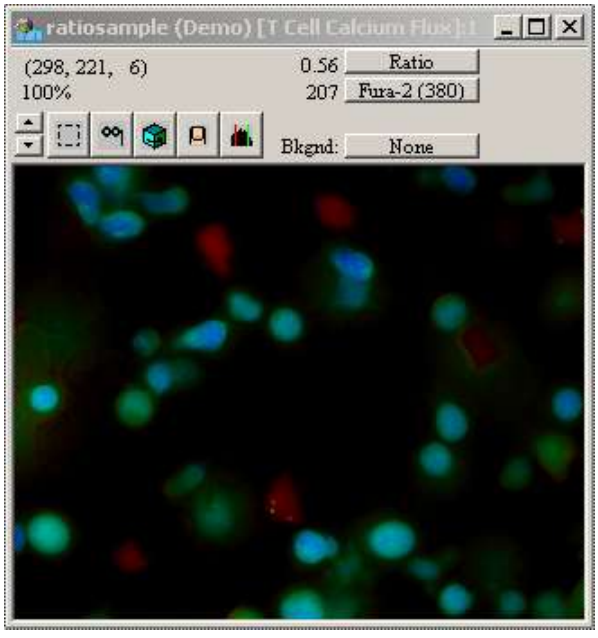
4.2.6 Using Ratio Settings

We will now continue the Ratio Tutorial.

1. Make sure a view of the T Cell image is active and then choose **Image > Ratio Settings…**. The following dialog box will appear.



2. Now change the Ratio Display Range **High** edit field to 0.5 and click **OK**. Note that the display hues have changed and more cells are red.



3. Bring up the Ratio Settings dialog box again by selecting **Image>Ratio Settings..**

4. Change the Ratio Display Range **High** edit field to 2.0 and click **OK**. Note that display hues are all blue/green.

5. Open the Ratio Settings dialog box again and return the Ratio Display Range High edit field to 1.0 and click **OK**.

6. Activate the main view of the image and draw a box using the marquee tool in a region that has no signal.

7. Open the Ratio Settings dialog box and click on the **Import from Active View** button in the Background ROI section of the Ratio Settings dialog box. The ROI dimensions and location will automatically be entered into the data entry fields.

8. Next, select **Import from Default Renorm** in the Threshold for Ratio Computation section of the dialog box. The renormalization settings that you have selected for the **Fura-2 (380)** channel will be displayed. Note that the **Fura-2 (340)** settings will be set to the min and max of the channel, as it is not a channel that is displayed in the view.

9. Select **File>Save Slide** to save your new settings.

In the next chapter, you will learn how these ratio settings affect ratio statistics.

5 Ratio Calibration

Ratio channels can be calibrated so that ratio values correspond to concentrations. SlideBook™ supports two different methods of calibration. The first is a solution-based *in vitro* method that may be performed pre- or post-acquisition. This calibration may be applied to multiple experiments. The second is an intracellular calibration that is performed post-acquistion for each experiment. This method performs calibration within a single cell and then applies those values to the entire data set. With either method, a ratio calibration guide assists you in determining three important numbers for correctly calibrated concentration display:

- **R_{min}**, the minimum ratio value (e.g., Fura-2 with no free Ca^{++} ions present)
- **R_{max}**, the maximum ratio value (e.g., Fura-2 in an ion-saturated solution)
- **beta**, the ratio of the denominators of the minimum and maximum conditions

The ratio calibration guide is also where you specify three other parameters:

- **K_d**, the dissociation constant of the fluoroprobe
- **R_{low}**, the ratio value below which a view displays the color corresponding to the minimum value
- **R_{high}**, the ratio value above which a view displays the color corresponding to the maximum value

From these parameters, calcium concentration can be calculated from the following equation:

$$[Ca++] = Kd * (R - Rmin)/(Rmax - R) * Beta$$

5.1 Solution-based in vitro Calibration

This type of calibration requires you to capture images of buffer solutions. When imaging buffer solutions, you may wish to add a small amount of non-fluorescent polystyrene beads to aid in obtaining focus. Alternatively, you may mark or scratch the surface of the coverslip or dish used for imaging. Example buffer solutions for calibrating calcium concentrations when using Fura-2 may be found in Appendix B: Buffers for Ratio Imaging.

NOTE: Do not attempt calibration using an AM ester derivative of an intracellular probe. AM ester derivatives have little to no response to ion concentration differences. You must use a salt form of your fluorophore for calibration.

5.1.1 Background Image

The first ratio image that you must collect (consisting of images of both the numerator and denominator channels) is a background image. This is usually an image (or average of a set of images) of the experimental setup with a buffer solution that has no fluoroprobe present. Prior to starting the **Ratio Calibration Guide**, you should place buffer solution on the stage

and adjust any microscope parameters in the **Focus Window** (please see the SlideBook™ User's Manual, Chapter 5 for instructions on using the Focus Window).

5.1.2 Ion-Free image

The second ratio image that you must collect is an ion-free image. This is usually an image (or average of a set of images) of the experimental setup with a buffer solution that has fluoroprobe present. The concentration of fluoroprobe should be approximately equal to the concentration of fluoroprobe present in loaded cells.

5.1.3 Ion Saturated image

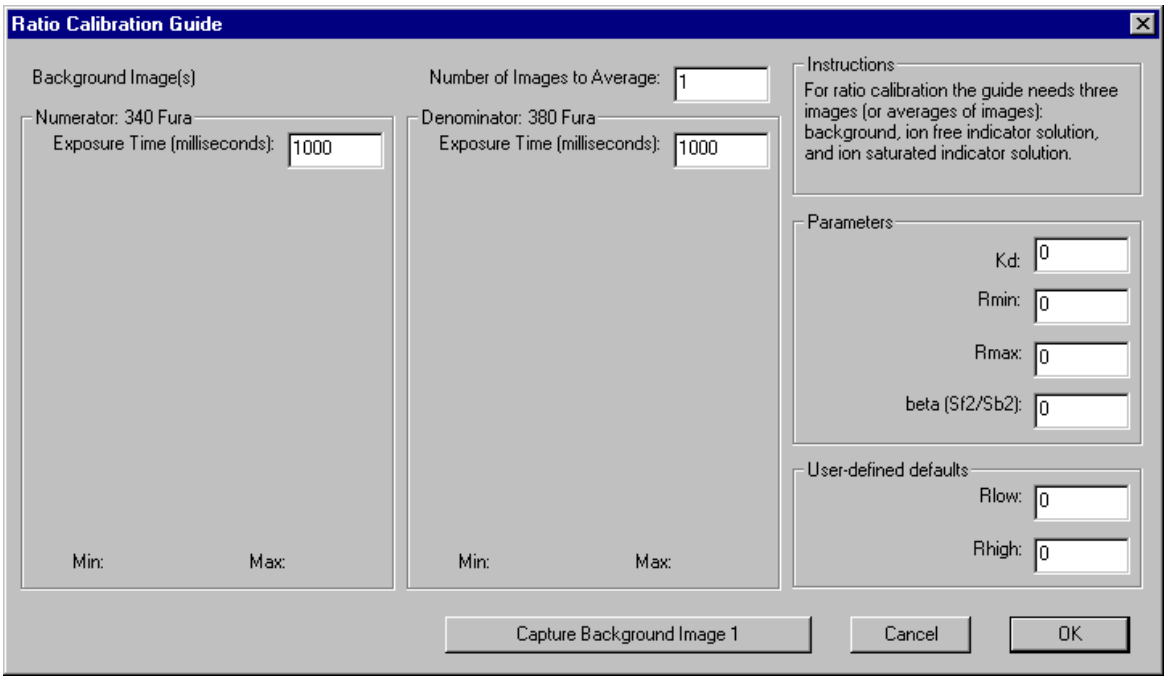
The third and final ratio image that you must collect is an ion saturated image. For this image, you will need a ion saturated buffer solution. The buffer should contain the same concentration of fluoroprobe as the buffer used to capture the ion-free images.

5.1.4 Generating Ratio Calibration Data

To generate your calibration data:

1. Place your background buffer solution in your experimental setup and bring your sample into view and focus (Please see the SlideBook™ User's Manual, Chapter 5).

2. Bring up the ratio calibration guide by selecting **Edit > Setup Guides > Ratio Guide**. Alternatively, you may bring up the calibration guide after acquiring a ratio image by selecting **Image>Ratio Settings** and selecting **Calibrate…**



3. Enter the number of images that you wish to average in the data entry field. In general, 3 images are sufficient.

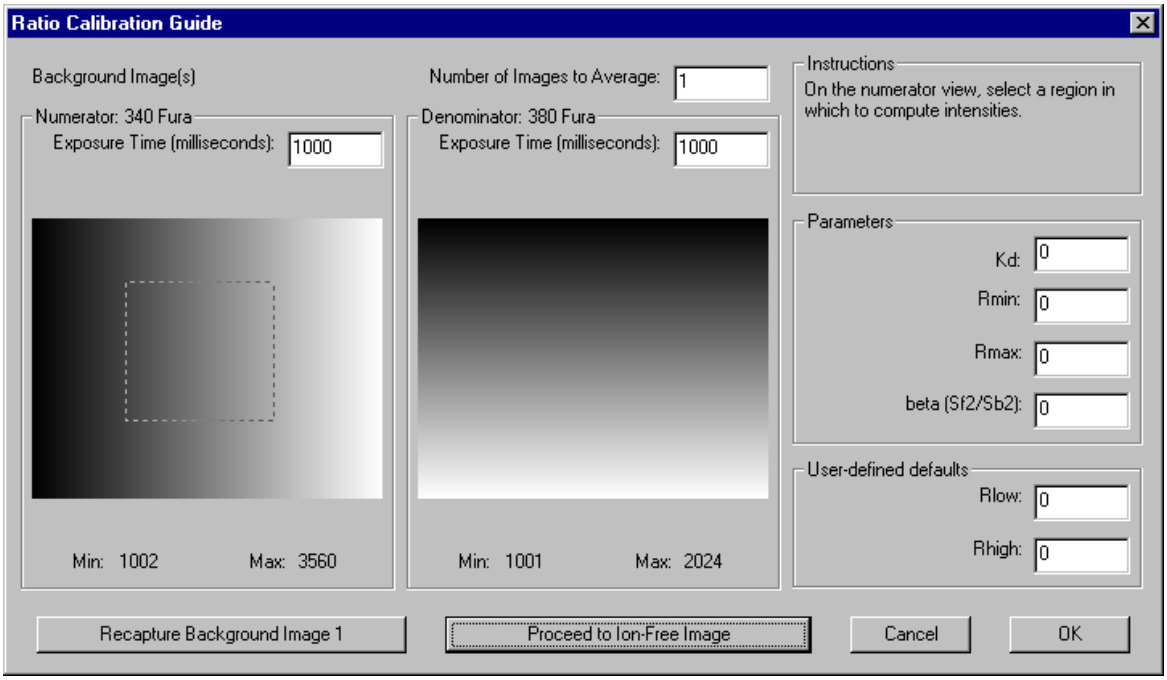
4. Enter the exposure time in milliseconds for both the numerator and denominator in the data entry fields.

   Note that you should aim for an exposure length that yields only moderate intensity values, since there is no fluoroprobe present. Otherwise, the subsequent capture of ion-free and ion-saturated images will certainly exceed the dynamic range of the camera.

5. Generate a test image by selecting **Capture Background Image 1** and observe the resulting intensity values reported beneath the image. You may recapture the image if the maximum intensity is too high by selecting **Recapture Background Image 1**.

6. After capturing the first background ratio image, select a region of interest by clicking and dragging on the numerator image view in order to determine the area over which the average background intensity (as well as ion-free and ion-saturated intensities) will be determined.

   In general, try to select the largest region possible, as it means the ratio calibration values will be computed from a larger sample. However, if there are obvious anomalies in the field, the region of interest selection permits you to exclude them.



7. Continue collecting background images by selecting **Capture Background Image**.

8. When you have finished collecting the background image or images, click on **Proceed to Ion-Free Image**.

9. Place your ion-free buffer solution (with fluoroprobe) in your experimental setup and bring your sample into view and focus using the focus window.

10. Select **Capture Ion-Free Image**. The region of interest that you selected in the background image collection will be used for these captures.

11. Continue collecting ion-free images by selecting **Capture Ion-Free Image**.

12. When you have finished collecting the ion-free image or images, click on **Proceed to Ion-Saturated Image**.

13. Place your ion-saturated buffer solution (with fluoroprobe) in your experimental setup and bring your sample into view and focus.

14. Select **Capture Ion Saturated Image**. The region of interest that you selected in the background image collection will be used for these captures.

15. Continue collecting ion-free images by selecting **Capture Ion Saturated Image**.

16. When you have finished collecting the image or images, click on **Compute Parameters**.

    The guide will automatically compute the $\mathbf{R_{min}}$, $\mathbf{R_{max}}$, and **beta** values (see definitions on page 5-1). For the exact mathematical equations used to compute these parameters, please see Appendix B: Buffers For Ratio Calibration.

17. Enter the dissociation constant, **Kd**, for your fluorophore. This value is specific for your fluorophore. A Kd calculator for Fura-2 is available at the Molecular Probes website http://probes.invitrogen.com/resources/calc/kd.html. Please enter the Kd value in units of nM concentration.

18. Enter the $\mathbf{R_{low}}$ and $\mathbf{R_{high}}$ values (see definitions on page 2-8).

    In general, you should set $\mathbf{R_{low}}$ and $\mathbf{R_{high}}$ to be equal to $\mathbf{R_{min}}$ and $\mathbf{R_{max}}$ initially. After running experiments, you may wish to revise the $\mathbf{R_{low}}$ and $\mathbf{R_{high}}$ values to reflect the actual range observed. You may access these values post-capture by selecting **Image>Ratio Settings..**

19. Click **OK** to exit the Ratio Calibration Guide.

    Your ratio calibration data will be used to generate concentration data for ratio images.

5.2 Intracellular Calibration

As described above, SlideBook™ provides an *in vitro* calibration tool for Ca^{++} imaging using fura-2. However, often cellular physiology interacts with fura-2 such that the dye's behavior inside a cell does not match the dye's behavior in calibration solutions. For more accurate measurement of absolute [Ca^{++}], it is best to measure the range of ratios observed *in vivo*, or within the cells themselves.

The intracellular calibration guide in SlideBook™ 4.2 allows you to select representative regions in a time series and use them as the basis for determining the maximum and minimum ratios that are observed within cells.

Typically, intracellular Ca^{++} calibration is done as an "end-step" in a live cell experiment. After observing meaningful Ca^{++} dynamics during an experiment, a calcium ionophore, such as ionomycin, is introduced into a cellular buffer solution that has a high free [Ca^{++}]. This raises the intracellular [Ca^{++}] dramatically and after the cytosol reaches equilibrium will allow us to measure a maximum intracellular ratio (R_{max}) as the 340 nm excitation signal will be at its highest and the 380 nm excitation signal will be at its lowest.

After that, a calcium chelator, such as EGTA, is added to drop the intracellular [Ca^{++}]. Again, after equilibrium, we can measure a minimum ratio (R_{min}). A typical experimental protocol is outlined below:

- Perform experiment
- Once the desired effect is observed, add solution so that final concentration in media is 1 μM ionomycin and 1.8 mM [Ca^{++}] and wait for ratio to stabilize using live ratio display. This will yield a maximal Fura-2 ratio.
- Add solution of 1 μM ionomycin and 2 mM EGTA and wait for ratio to stabilize. This will yield a minimal Fura-2 ratio.

This is a guideline, you may need to experiment to find out what concentration of calcium and EGTA is appropriate for your cells. The Rmax and Rmin from the intracellular calibration must be higher and lower than that which you observe during your experiment. If this is not the case, you must adjust the calibration solutions.

A tutorial for intracellular ratio calibration follows. Please use **IntracellularCal.sld** for the tutorial.

5.2.1 Selecting a background ROI and an ROI within a cell

1. Open a Main View of sample 1.

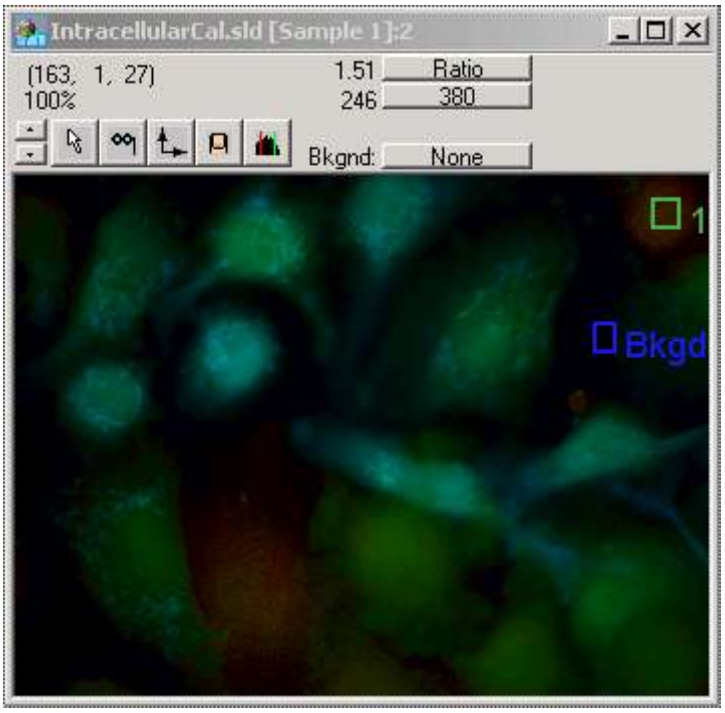
2. Draw a region in an area with no signal using the square ROI tool.

3. Select the region by clicking on it, then right click and select **Set as Background**.

It is a good idea to scroll through time and make sure that no debris or floating cells enter this region during some later time point, as the background level is evaluated for each time point. If you want to change the background ROI, simply select the ROI and click and drag it to a different spot or delete it and redraw.

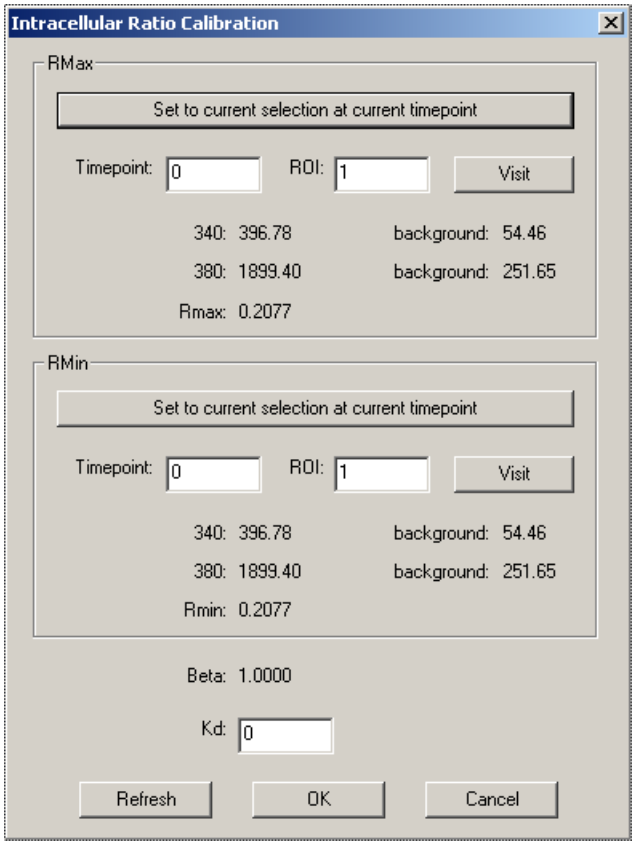
NOTE: The size of an ROI for calibration purposes does not need to be large. Even a small ROI such as the background ROI pictured above contains over 100 pixels, which is plenty to remove the effects of signal noise in determining a reliable background level.

4. Draw an ROI within a representative cell. It may be helpful to scroll through time and find a cell whose max ratio is among the highest in the field of cells.



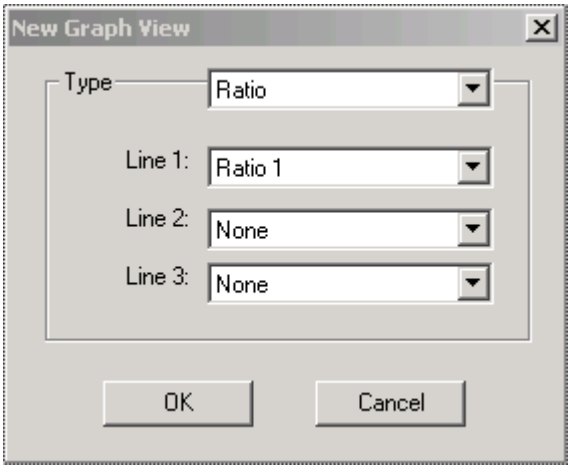
5.2.2 Finding R_{max} and R_{min}

1. Select **Image>Ratio>Intracellular Calibration…** to open the intracellular calibration dialog box.

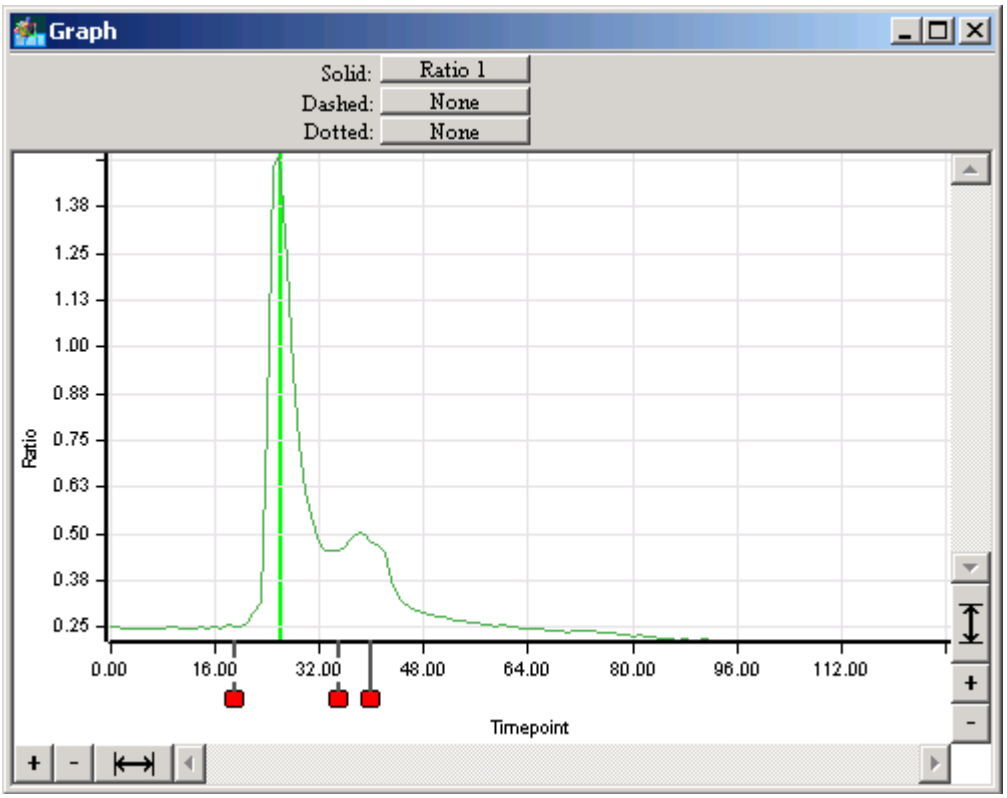


Initially, the dialog box shows the computed R_{max} and R_{min} based on the first timepoint and the first ROI.

2. Graph the ratio data of the ROI by selecting **View>Graph…**and setting the dialog as follows:

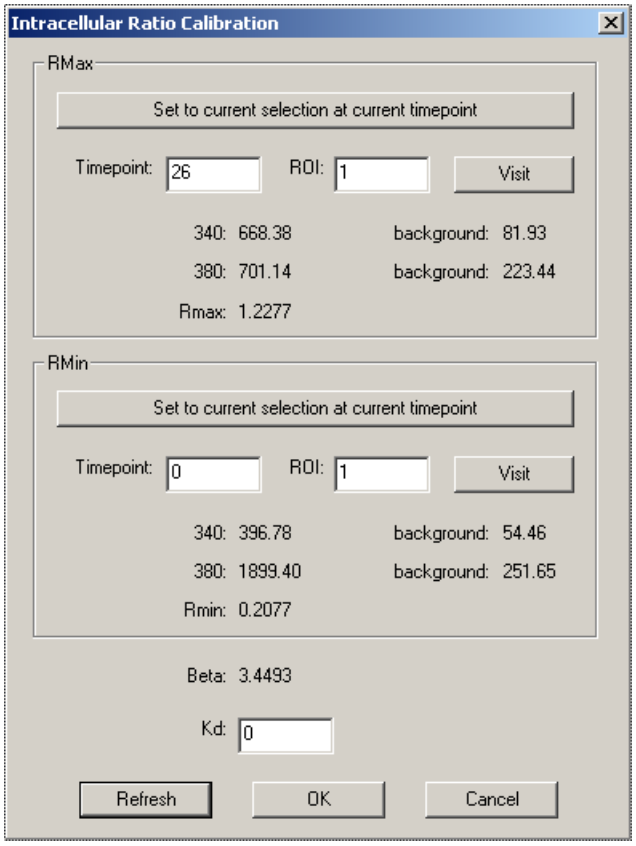


A graph similar to the following will appear.

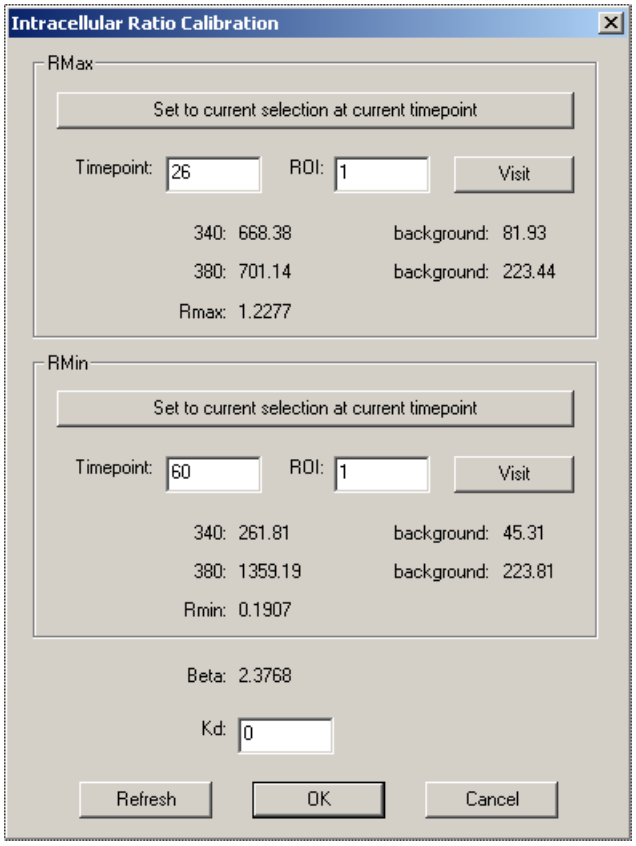


Simply click on the highest ratio value in the graph, and the main view will update to timepoint 26. The green line indicates the timepoint showing in your main view.

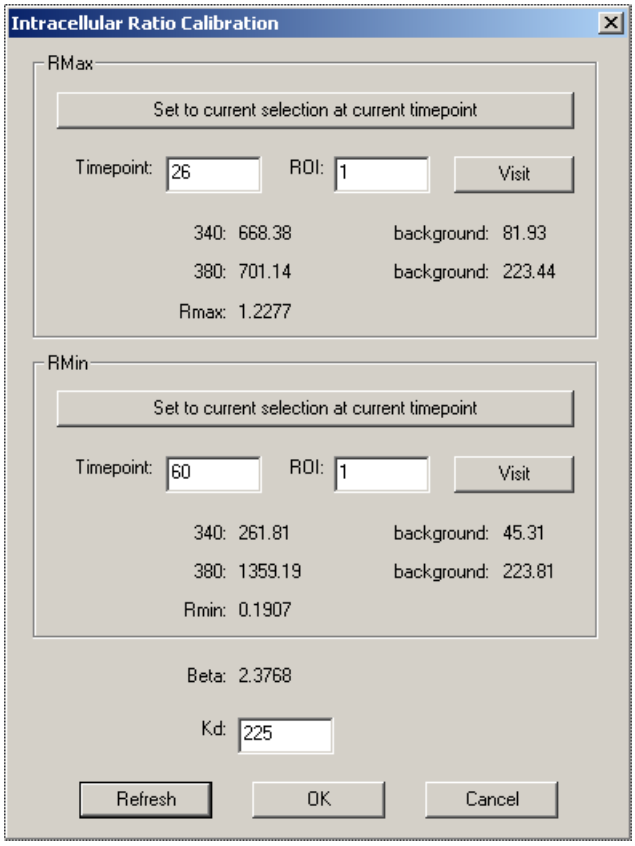
3. Either type in "26" into the **Timepoint** edit field for R_{max}, or if your main view is on timepoint 26, simply press **Set to current selection at current timepoint**. If your main view is not currently displaying timepoint 26, you may visit that timepoint by pressing **Visit** after entering the timepoint in the edit field.



4. Now repeat steps 2 and 3 for R_{min}. In this example, R_{min} is located between timepoints 50 and 65. After timepoint 65, the sample begins to photobleach.



5. Enter the dissociation constant, **Kd**, for your fluorophore. This value is specific for your fluorophore and biological system. A Kd calculator for Fura-2 is available at the Molecular Probes website http://probes.invitrogen.com/resources/calc/kd.html. Please enter the Kd value in units of nM concentration. In this example, we will use 225 nm.



6. Click **OK** to complete the calibration. Scroll over the main view and observe that calcium concentrations are displayed next to the ratio channel menu.

7. To set your display back to ratio calculations, enter 0 in the Kd field and click **OK**.

6 Ratio and Concentration Data Graphing and Export

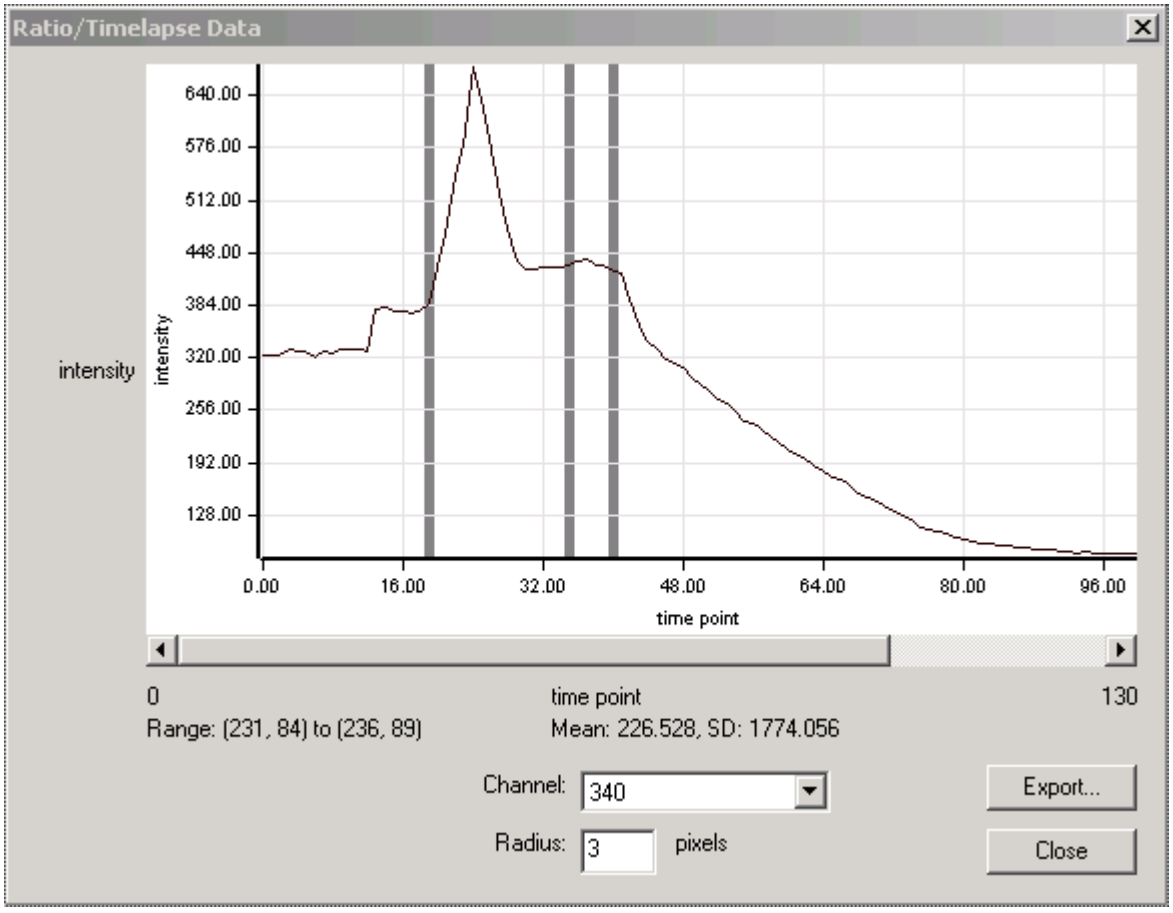
There are three ways to mark areas for graphing and exporting ratio data from an image: single region export from a marquee or point-and-click selection, multiple region export from ROIs, and multiple region export from a mask created manually. You may wish to use the **ratiosample.sld** file to experiment with data graphing and export.

6.1 Quick Graphing and Data Export

To rapidly generate ratio data:

1. Select a single cell region by selecting the Point Selection tool  .

2. Hold the CTRL key while clicking on a cell. A graph similar to the following will appear.



   You may select the data you wish to display using the drop-down channel menu. You may increase the radius to increase the area that is being graphed.

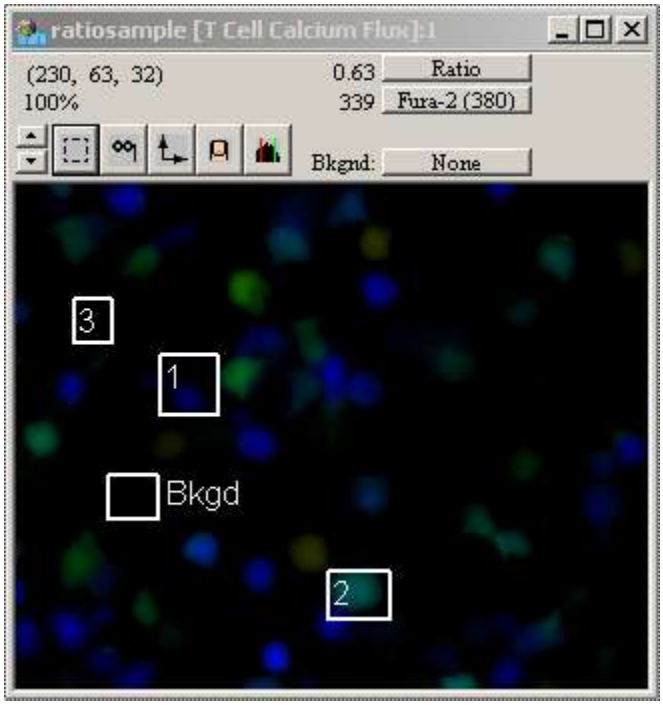
3. To export the data, press the **Export…** button.

4. Type in the desired name of the text file where your data will be written.

5. Open the data file using Excel. You may wish to repeat steps 2 and 3 for different fluorophores.

6.2 Multiple Region Graphing and Export from ROI selections

You may also export data from multiple regions that you selected during capture. You may also add or delete regions post-capture.



6.2.1 Working with ROIs

You may display regions that you chose during capture, add or delete selections, and select a background region.

- *Display ROIs* – select **View>Annotations>Regions**.
- *Create new ROIs* - use the ROI tools to draw regions (please see the SlideBook User's manual for a detailed description of ROI tools and usage).
- *Delete ROIs* – click on the ROI identification number, then right click and select Delete
- *Select Background Region* – click on the ROI to select it, then right click and select Set as Background.

6.2.2 Graphing and Exporting Data

To graph your ROI data, select **View>Graph..** and select the channel you wish to display. You may explore the graph as described on page 3-5. To export the ratio/concentration data, select **Image>Export>Ratio/Timelapse Data…**. The output should look similar to the following:

Ratio information present.
Numerator channel: Fura-2 (340)
  Exposure Length (ms): 25
Denominator channel: Fura-2 (380)
  Exposure Length (ms): 25
Exposure factor: 1.000000

Plane	Time	Elapsed (s)	Fura-2 (340)	Fura-2 (380)	open	Num Bkgnd	Den Bkgnd	Ratio	Qualified	Total
0	18:07:38	0	127.389	264.921	636.115	134	283	-	-	
1	18:08:40	62	156.293	349.773	92.584	184	468	-	-	
2	18:08:56	78	136.427	291.306	896.681	167	429	0.3125	3	529
3	18:09:12	94	145.836	355.204	1613.015	149	349	0.181287	124	529
4	18:09:28	110	191.009	589.49	1524.064	151	283	0.152749	307	529
5	18:09:44	126	313.609	1168.469	1563.009	155	311	0.18768	432	529
6	18:10:00	142	337.471	1274.902	1618.142	156	304	0.188592	471	529
7	18:10:15	157	344.798	1320.493	1641.541	149	261	0.186341	469	529
8	18:10:31	173	325.764	1232.783	1569.025	150	267	0.185284	449	529
9	18:10:47	189	312.181	1196.042	1535.664	154	280	0.176044	436	529
10	18:11:03	205	326.45	1255.057	1688.936	141	236	0.183691	455	529
11	18:11:19	221	340.648	1320.662	1622.681	137	227	0.187705	467	529
12	18:11:35	237	339.289	1343.817	1591.9	140	230	0.180893	454	529
13	18:11:51	253	334.665	1339.098	1494.662	139	232	0.178979	437	529
14	18:12:06	268	297.382	1140.826	1522.478	129	205	0.182955	376	529
15	18:12:22	284	264.548	1009.849	1507.147	125	192	0.176145	329	529
16	18:12:38	300	246.754	920.529	1521.391	125	192	0.174486	308	529

Along with the ratio settings, SlideBook™ writes out for each time point the following fields:

- **Plane** – plane number
- **Time** - time of day that the plane was captured
- **Elapsed (s) or (ms)** - elapsed time in seconds or milliseconds
- **Numerator channel [Fura-2 (340)]** - average numerator intensities for the region of interest at the given time point; value is not background subtracted
- **Denominator channel [Fura-2 (380)]** – average denominator intensities for the region of interest at the given time point; value is not background subtracted
- **Num Bkgnd** or **Den Bkgd** - average numerator and denominator intensities for the background region at that time point
- **Ratio** - computed background-subtracted ratio for qualified pixels
- **Qualified** - number of voxels within the region whose values satisfied all threshold conditions (see Thresholds for Ratio Computation on p. 4-7 to set conditions); only qualified pixels are used for computing ratio values

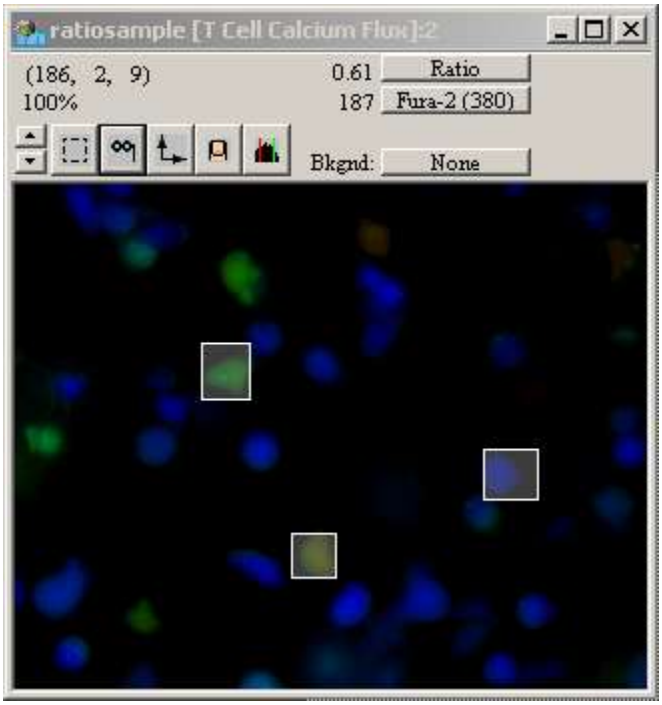
- **Total** - total number of voxels within the region
- **Total concentration** – computed concentration if calibration data is present (See Chapter 3)

Note: The exact mathematical equations used for computing Ratio data can be found in Appendix D: Ratio Data Export Calculations.

6.3 Multiple Region Export from a Mask

You may also export data from multiple regions by creating masks. Masks are flexible and can be copied to multiple images in a slide. Please see Chapter 9 of the SlideBook™ User's Manual for a complete discussion of masks. To perform multiple region export from a main view of your image:

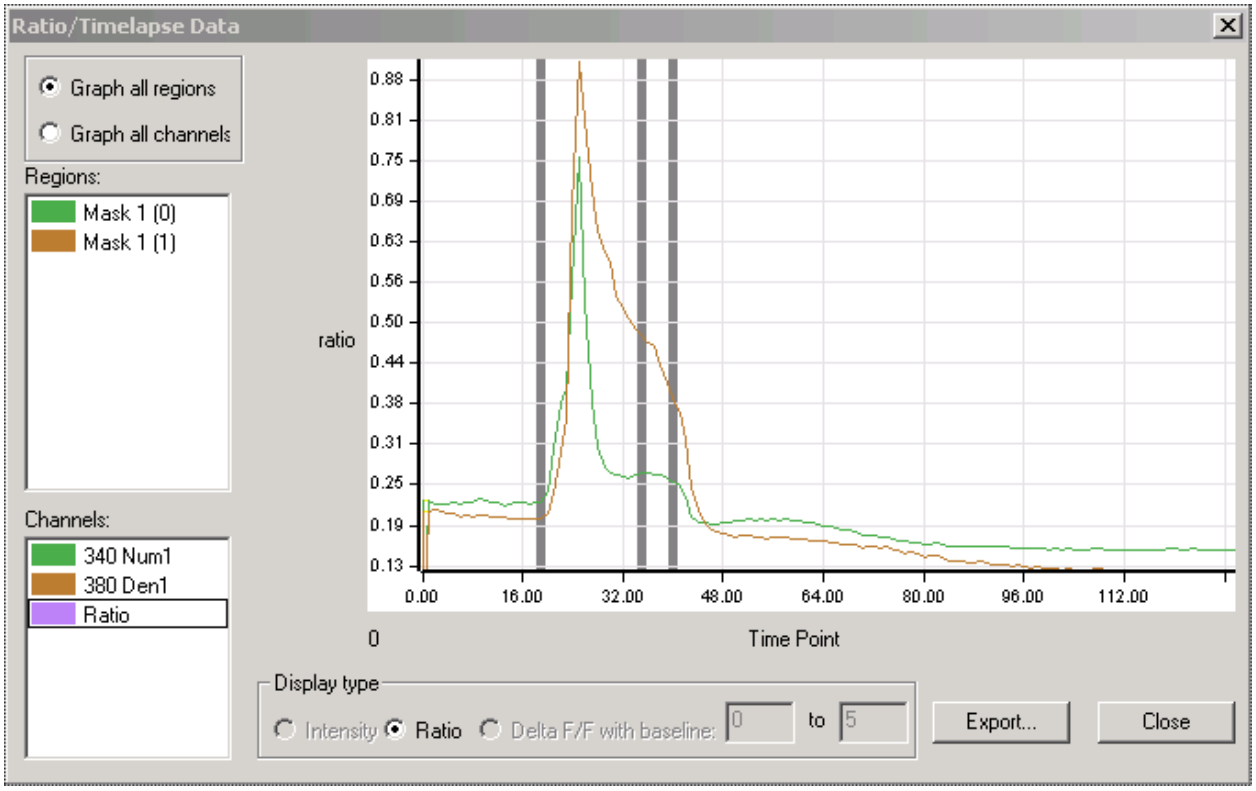
1. Create a mask by choosing **Mask>Create** and draw your regions using mask drawing tools. Please see Chapter 9 of the SlideBook™ User's Manual if you are unfamiliar with mask drawing tools.



2. Extend the selection to all planes by choosing **Mask > Copy This Plane To All**.

3. Divide the mask into objects so that SlideBook™ can individually find and number each region by choosing **Mask > Define Objects…**. and selecting **OK**.

4. To display a graph, select **Image>Statistics>Ratio/Timelapse Data…**. A graph similar to the following will appear.



You may choose to display all regions for a single channel or all channels for a single region using the radio buttons.

5. To export the data, press the **Export…** button. (To export the data without first viewing the graph, select **Image > Export > Ratio/Timelapse Data…**.)

6. Type in the desired name of the text file where your data will be written.

7. Open the data file using Excel. The output should look as shown above in the section Graphing and Exporting Data. The object identity will be shown in parentheses.

7 Protocol for Direct FRET/Sensitized Emission FRET

Direct FRET/Sensitized Emission FRET can be performed with SlideBook™ 4.2 as shown in the following protocol. This example protocol uses the common FRET pair, CFP and YFP. You must first configure your filter definitions as discussed in Defining FRET Channels for Direct/Sensitized Emission FRET on page 2-2. This protocol specifically describes *Three-Channel Corrected FRET*. Two-Channel Corrected FRET is a technique used when the donor and acceptor are present in a fixed concentration ratio (e.g. Cameleon imaging). Two-Channel Corrected FRET can be viewed and analyzed as a ratio of FRET intensity to donor or acceptor intensity using the ratio tools discussed in Chapters 4 and 5. For further explanation on the theory and calculations for direct FRET, please see Appendix A: FRET Introduction and Theory on page A-1.

This protocol includes procedures for:

- Capturing Images
- Performing Post-Capture Analysis
- Viewing Direct FRET Data
- Obtaining FRETc Statistics

7.1 Capturing Images

In order to perform direct FRET, you must be able to calculate bleedthrough coefficients, or cross-talk, for your particular filter sets. In order to calculate bleedthrough coefficients, two control samples must be imaged: one that contains donor only, and one that contains acceptor only.

7.1.1 Control Images

1. Mount the donor sample on the stage and open the focus window in SlideBook™™. See Chapter 5 of the SlideBook™ User's Manual for instructions on using the Focus Window.

2. Select the CFP filter set, and bring the sample into focus.

3. Close the fluorescent shutter and then close the focus window.

4. Open the Capture Window. (See Chapter 6 of the SlideBook™ User's Manual for instructions on using the Capture Window).

5. Set the CFP exposure time by selecting the CFP checkbox and then pressing **Test** and **Once**. See Chapter 6 for further instructions on setting exposure times.

6. Set the FRET exposure time by selecting the FRET checkbox and manually entering the same exposure time from step 5. It is important that the exposure times of both fluors are equal to each other.

7. Click **OK** to capture your image.

8. Repeat steps 1-7 with a sample containing only YFP, capturing with the YFP and FRET channels.

7.1.2 Experimental Sample

Now bring your experimental sample into view and focus in the focus window. Use the FRET filter when focusing your sample. Then, image your sample(s), capturing the CFP, YFP, and FRET channels. Make sure that all exposure times are equal to each other. If you are performing a live FRET experiment, you may wish to take advantage of the live graphing features that are discussed above in Chapter 4.

7.2 Post Capture Analysis Tutorial

Next, you will perform background subtraction, calculate bleed-through coefficients, and generate the corrected FRET signal. You may follow this protocol step-by-step using the example file **directFRETsample4-1.sld**.

7.2.1 Performing Bleedthrough Calculations

Bleed-through is calculated using the two control images.

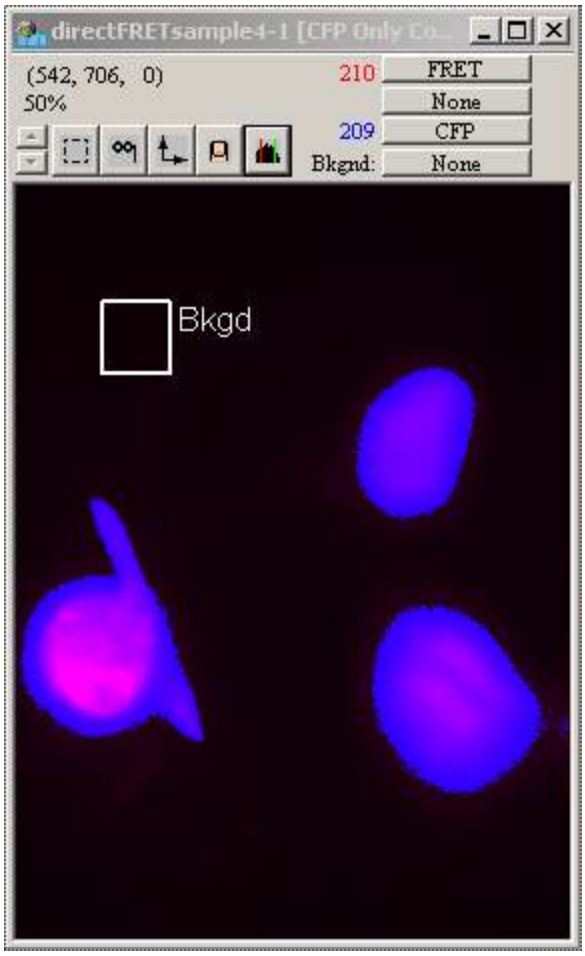
1. Open the sample slide by selecting **File>Open Slide** and navigating to **directFRETsample4-1.sld**

2. Open a main view of the CFP only control by double-clicking on the thumbnail.

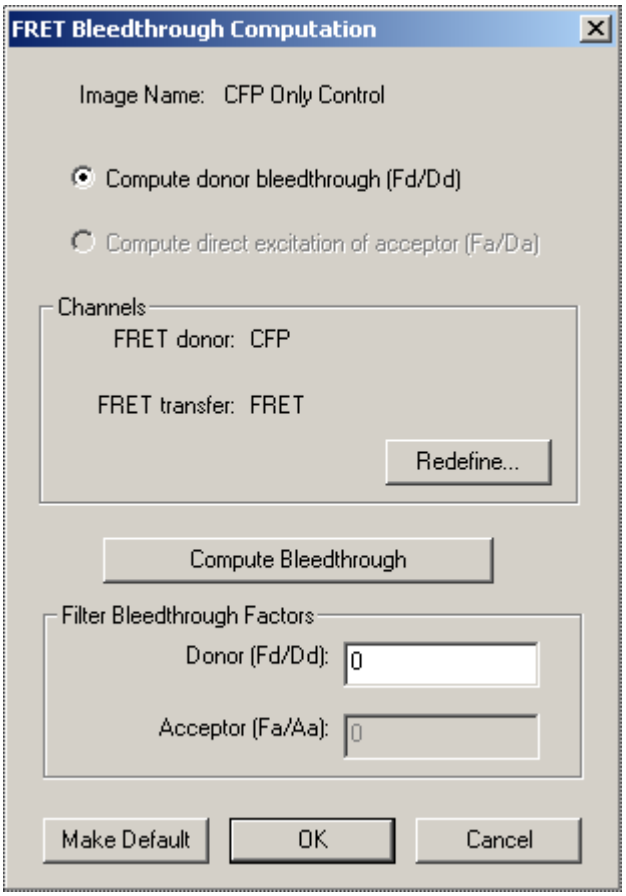
3. Next, go to **Mask>Segment**. Move the red bar on the histogram to the right to segment your image. You should select a significant amount of sample to increase the accuracy of the bleed-through calculation. For this example, a low value near 240 can be used.

4. After segmentation is complete, draw a region in an area with no signal using an ROI tool. A region will appear.

5. Select the region by clicking on it (the number will turn white), then right-click on the region and select “Set as background”.

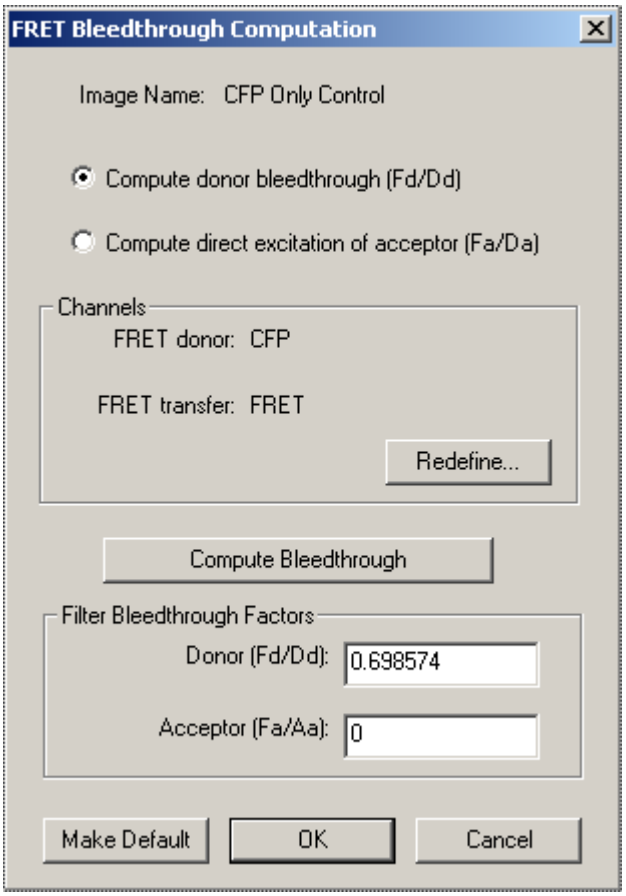


6. Select **Image > FRET > Compute FRET Bleedthrough ...**. The following dialog will appear.



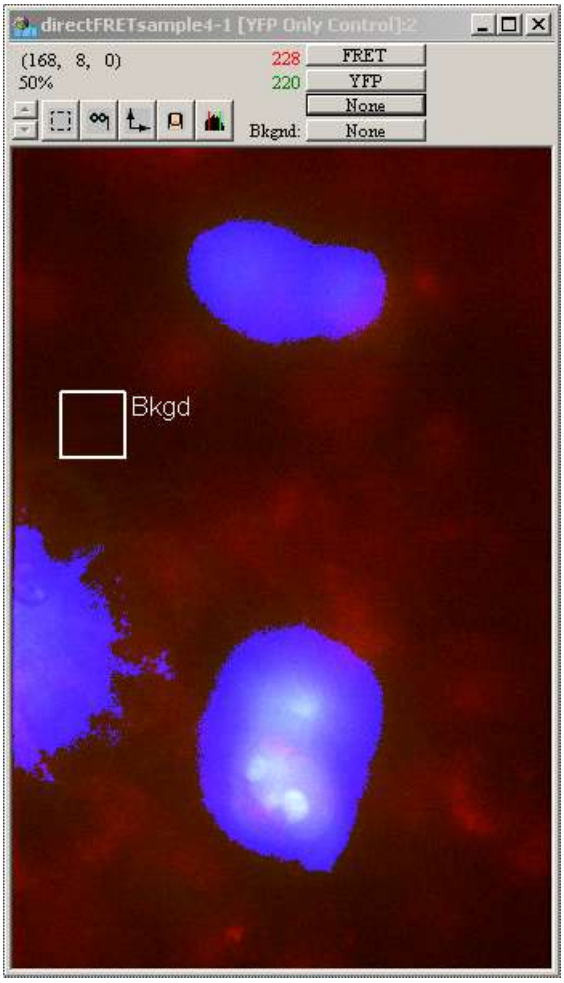
NOTE: If the channel selection is not appropriate, you may redefine the channels as necessary.

7. Select **Compute Bleedthrough** to calculate the donor bleedthrough. Your dialog will look similar to the one below.

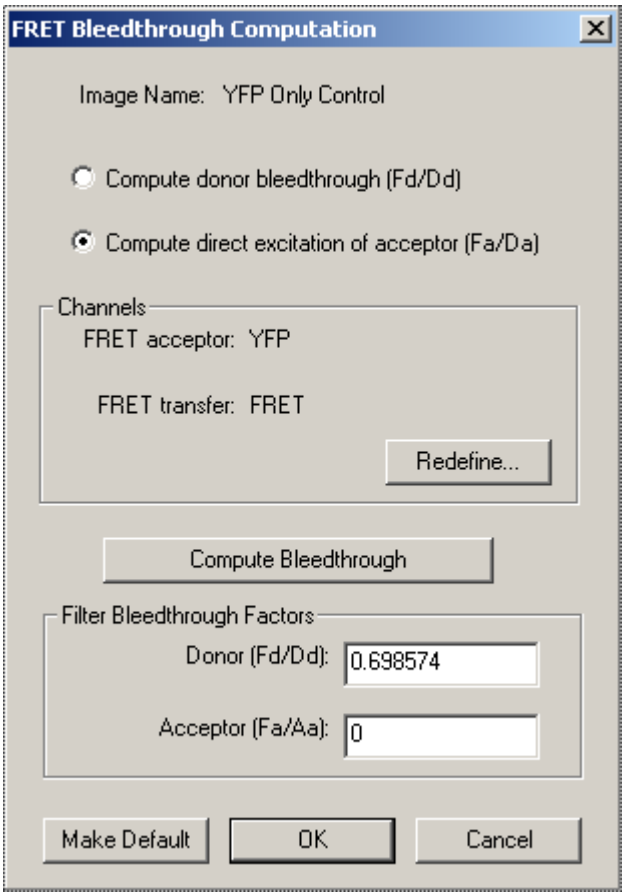


8. Select **Make Default** to use this bleedthrough coefficient for subsequent calculations and then click **OK** to close the dialog box.

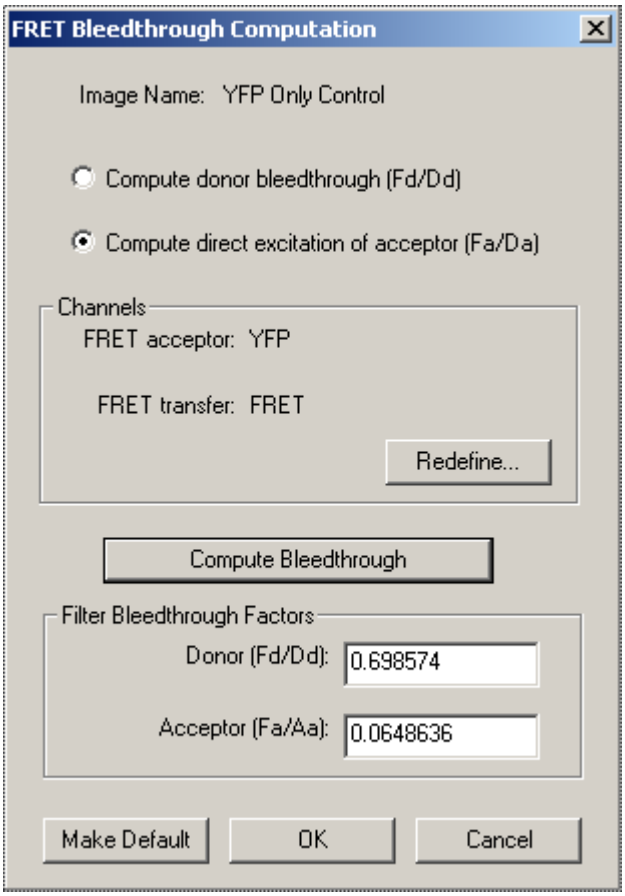
9. Open a main view of the YFP only sample, and create a mask and select a background region as you did for the CFP only sample. Create the mask using **Mask > Segment** and choose the YFP channel as the basis for segmentation. A low value of about 300 is appropriate.



10. Select **Image > FRET > Compute FRET Bleedthrough ….** The dialog will appear as follows.



11. Select **Compute Bleedthrough** to compute the direct excitation of acceptor.



12. Select **Make Default** to retain the bleedthrough values you have generated.

Now, you will determine FRET settings and generate a corrected FRET, or FRETc, channel for viewing your experimental sample images.

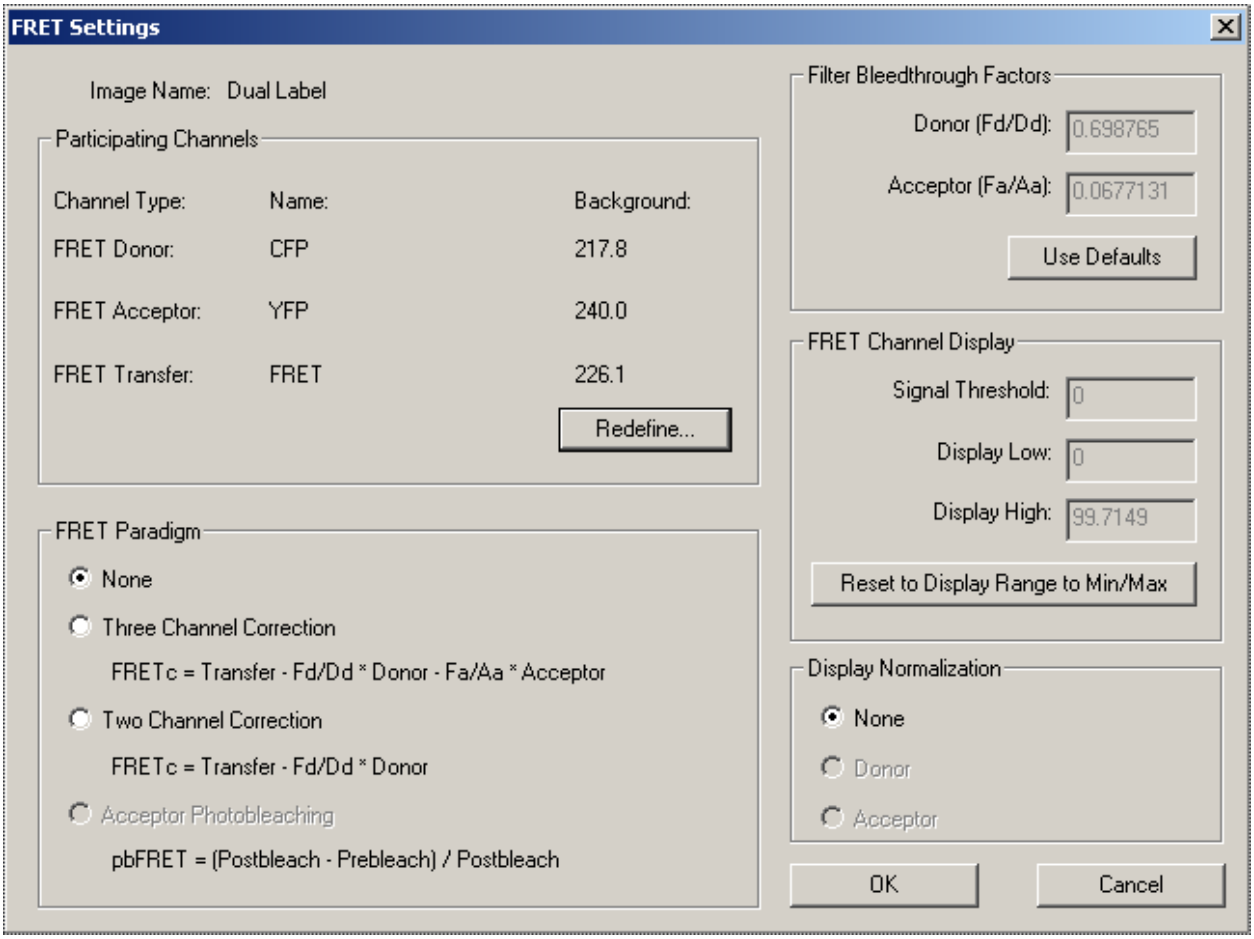
7.2.2 Using FRET Settings

FRET settings may be used to select the type of FRET calculation you wish to perform.

1. Open a Main View of the Dual Label image.

2. Select a background region as you did when calculating bleedthrough coefficients.

3. Go to **Image > FRET > FRET Settings...** The FRET Settings dialog box will appear.



4. Select the **Three Channel Correction** radio button for this example. You will be asked whether or not you would like to import default bleedthrough settings.

5. Select **Yes**, and the bleedthrough coefficients will be imported. You may have one set of bleedthrough coefficients set as default at any time. If you do not wish to use defaults, simply type in the bleedthrough coefficients in the edit fields.

   Once the bleedthrough coefficients are imported, a new channel called FRETc will be created. The **Display Range** will update to list the low and high intensity values of the new channel. If you wish to change the values, enter the new values in the edit fields for **Low** and **High**.
   The corrected FRET values for your sample are calculated using equation (4) described in Appendix A: FRET Introduction and Theory on page A-1.

6. If desired, you may choose to normalize your corrected FRET channel with either the donor or acceptor intensity. This will create a new channel called **<FRETc/D>** or **<FRETc/A>**. For this exercise, choose **Donor** and enter 50, 0 and 0.2 in the **Signal Threshold**, **Display Low** and **Display High** edit fields, respectively.

- **Signal Threshold** – the intensity above which FRETc and normalized FRETc values will be calculated. This threshold is background subtracted. Therefore, if you select a value of 50, any pixel that is above 50 after background subtraction will be used for calculating FRETc.
- **Display Low** – the value that corresponds to black in RGB display or blue in pseudocolor display. See next section for further explanation of pseudocolor display.
- **Display High** – the value that corresponds to saturated color in RGB display or red in pseudocolor display.

NOTE: The new channels **<FRETc>**, **<FRETc/D>**, or **<FRETc/A>** are created for viewing purposes only. To obtain meaningful transfer efficiency (E%) data, three channel FRET (FRETc) must be normalized for either donor or acceptor concentration using masks statistics (see Obtaining FRETc Statistics on page 7-12).

7. Click **OK** to close the dialog box.

8. Repeat steps 1-7 for the Mutant image.

7.3 Viewing FRET Data

It is very easy to view your corrected FRET data with SlideBook™'s pseudocolored main views. Further explanations and an example for viewing data in pseudocolor can be found above in Viewing Ratio Data in Pseudocolor on page 4-1.

1. Open a Main View of Dual Label.

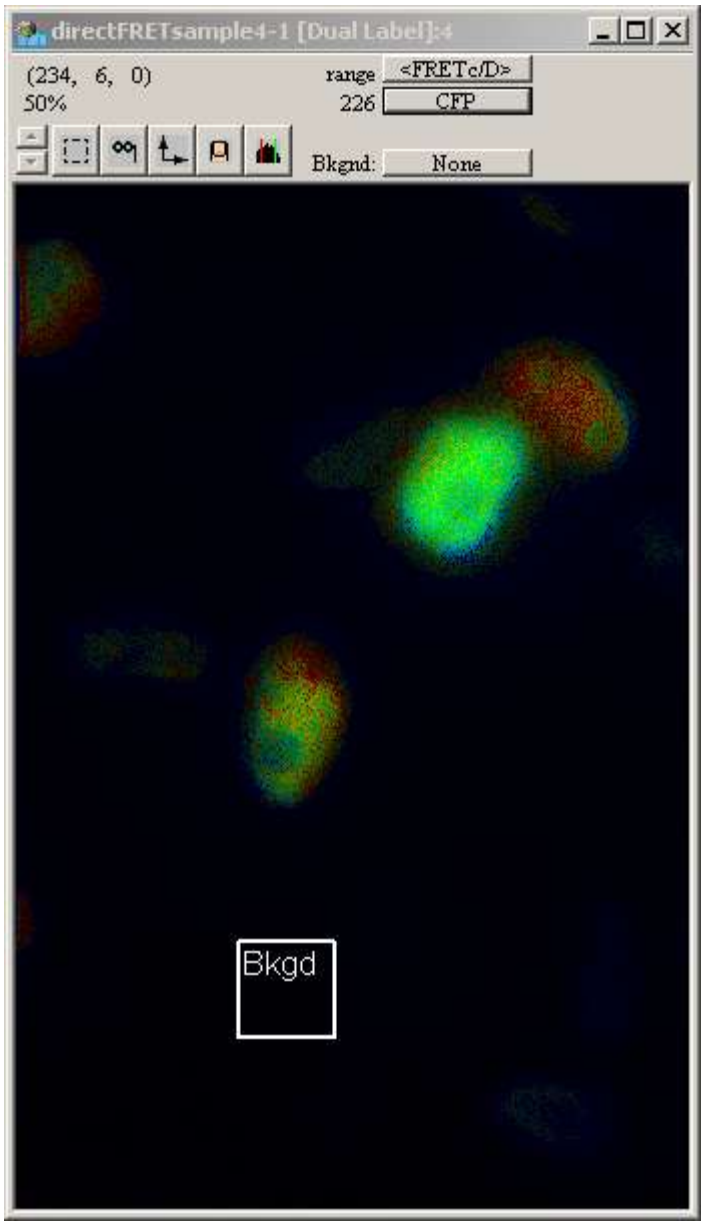
2. Go to **View > Pseudocolor**.

   Two channel menus will be available. The first channel will be displayed as a pseudocolored image ranging from saturated red to saturated blue, known as **Pseudocolor (Color)**.

3. Select **<FRETc/D>** from the Hue (top) drop-down channel menu.

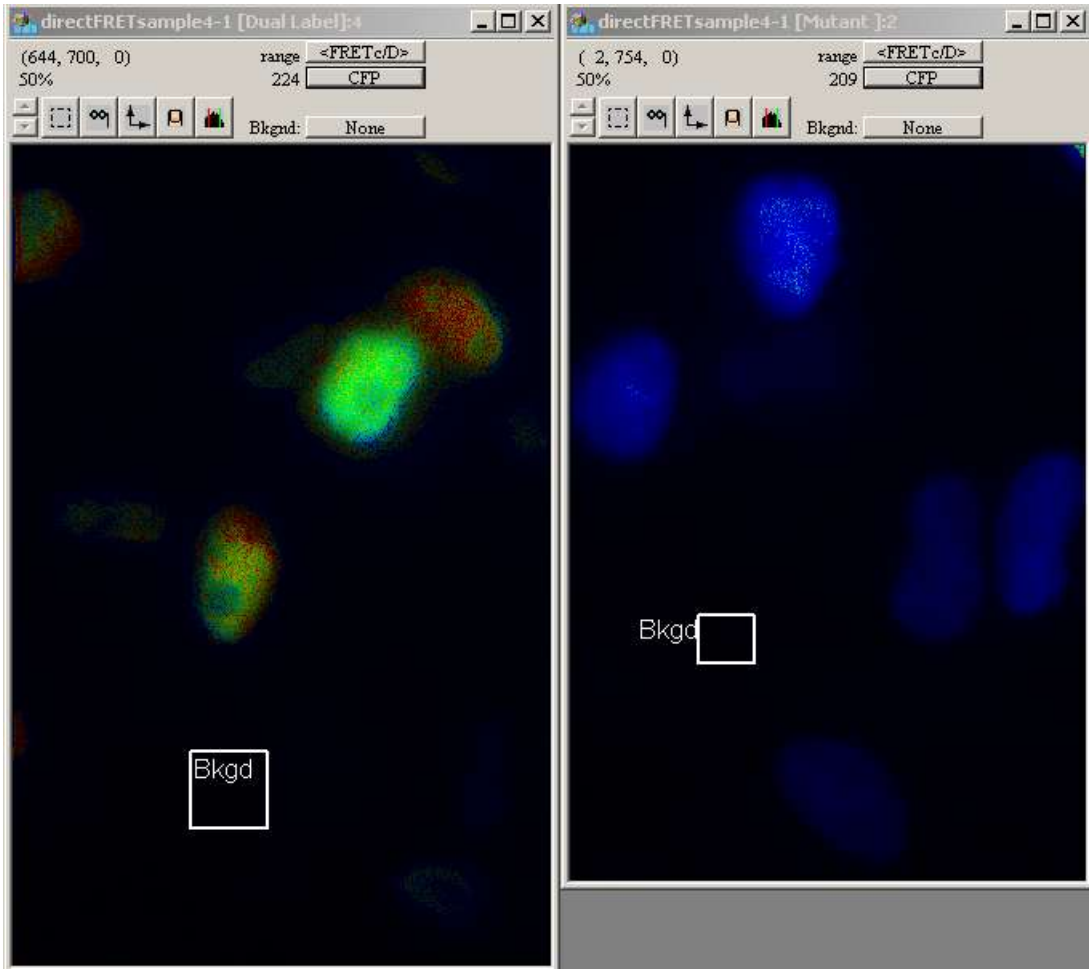
   The second channel menu will act to gate the pseudocolor image based on intensity. This second channel is called **Pseudocolor (intensity)**. Thus, an area with a high pixel intensity for the first channel and a low pixel intensity on the second channel will appear as dim red. An area with a low pixel intensity for the first channel and a high pixel intensity on the second channel will appear as bright blue. This helps distinguish areas with high intensity from background.

4. Select CFP from the second channel drop-down menu. Your image will appear similar to the following.



5. Repeat steps 1-4 for Mutant. In pseudocolor, red denotes high ratios and blue denotes low ratios. Therefore, red regions have higher normalized FRET relative to blue regions.

6. Compare the two images again. Decide which sample is a positive FRET control and which is a negative FRET control.



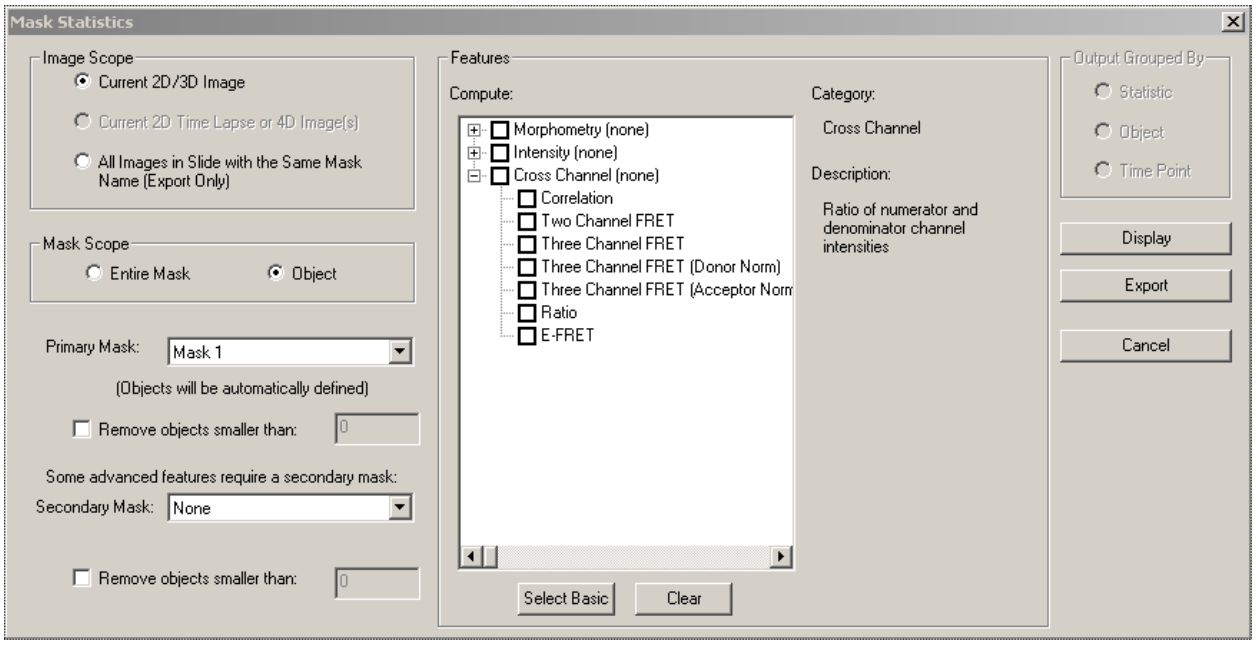
7.4 Obtaining FRETc Statistics

You can now generate FRETc information using masks and statistics.

1. Open a main view of your sample image.

2. Go to **Mask > Segment** and generate a mask on the regions you wish to measure FRETc (see Chapter 10 of the SlideBook™ User's Manual for instructions on masking). Alternatively, you may generate a mask manually.

3. Select **Mask > Statistics…**. Select the mask you have just created from the drop-down menu, and click on the **+** next to the **Cross Channel** category as shown below.



4. Select the Three Channel FRET statistic that you wish to calculate and click **Display** to display the results. You may eliminate objects below a certain size threshold by selecting the **Gate Objects by Size** checkbox.

    - **Two Channel FRET** – FRETc for two channel corrected direct FRET
    - **Three Channel FRET** – FRETc for three channel corrected direct FRET
    - **Three Channel FRET (Donor Norm)** – FRETc normalized by the donor channel for three channel corrected direct FRET
    - **Three Channel FRET (Acceptor Norm)** – FRETc normalized by the acceptor channel for three channel corrected direct FRET

5. After viewing your data, you may select **Export**. Your data will be saved as a .txt file. You may then open this using spreadsheet programs such as Excel.

8 Protocol for Acceptor Photobleaching FRET

Acceptor Photobleaching FRET can be performed with SlideBook™ 4.1 as shown in the following protocol. This example protocol uses the common FRET pair, Cy3 and FITC. For further explanation on the theory and calculations for Acceptor Photobleaching FRET, please see Appendix A: FRET Introduction and Theory on page A-1.
This protocol includes procedures for:

- Capturing Images
- Performing Post-Capture Analysis
- Viewing Photobleaching FRET Data
- Obtaining PB FRET Statistics

8.1 Capturing Images

In order to perform Acceptor Photobleaching FRET, you must first configure your filter definitions as discussed in Defining FRET Channels for Acceptor Photobleaching FRET on page 2-5. You will need to determine an appropriate photobleaching time before capturing your desired sample. To begin capture:

1. Mount your sample on the stage and bring it into view and focus using the Focus Window. See Chapter 5 of the SlideBook™ User's Manual for instructions on using the Focus Window.

2. Use the field diaphragm to create a small area of illumination on the sample. You will be using this small area to determine an appropriate photobleaching exposure time.

3. Select **Image>Capture..** The Capture dialog box will appear.

4. Now, you must determine an appropriate exposure time for photobleaching. Select the **Photobleach** channel and enter an approximate time for photobleaching.

5. Select **Test** to generate a test exposure. Increase the illumination area and observe the amount of photobleaching that has occurred via the oculars. Repeat the testing procedure on fresh areas of sample until an appropriate photobleaching exposure time is determined.

6. Use the Focus Window to move to an area of interest, and if desired open the field diaphragm so that entire sample area is illuminated.

7. In the Capture dialog box, determine exposure times for **CY3 pre** and **FITC pre** by generating test exposures (See Chapter 6 of the SlideBook™ User's Manual).

8. Select the following channels for capture:

   - **FITC pre** – using the exposure time determined above

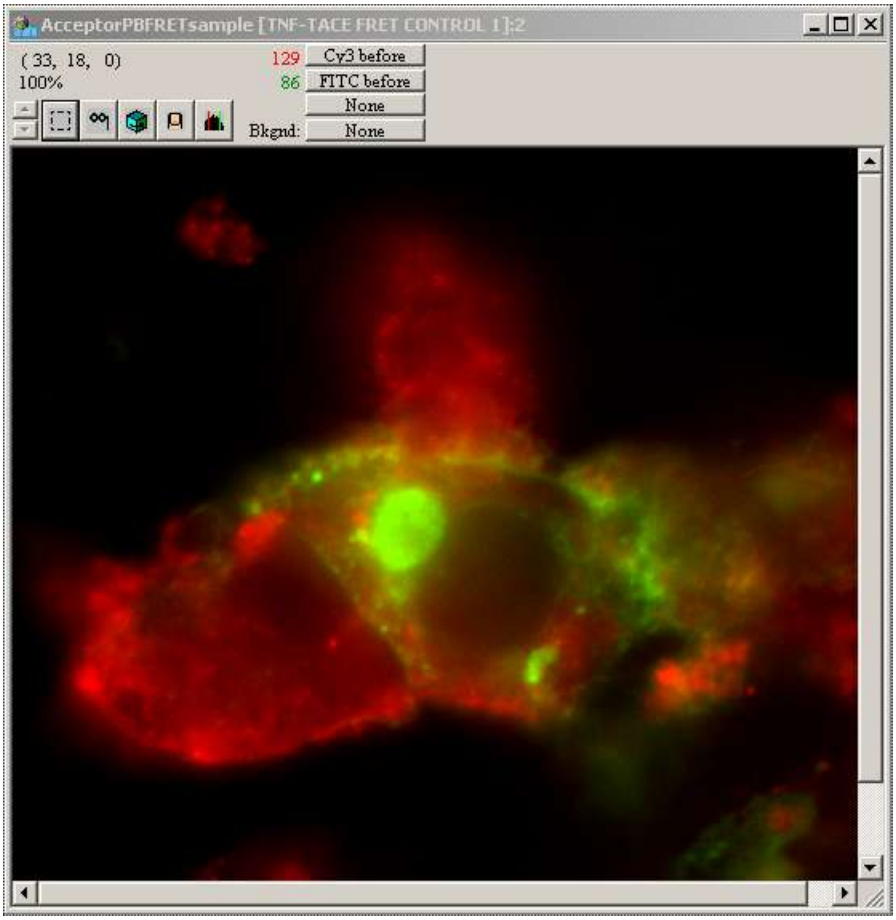
- **Cy3 pre** – using the exposure time determined above
- **Photobleach** – using the exposure time determined above
- **FITC post** – using the **FITC pre** exposure time
- **Cy3 post** – using the **Cy3 pre** exposure time

9. Set camera parameters and other settings and select **OK**. See Chapter 6 of the SlideBook™ User's Manual for a complete description of options in the Capture Dialog box.

   Five channels will be captured in succession. It is important that the channels are captured in the order shown in the bulleted section above. You may alter the channel order by selecting the **Move Up** or **Move Down** buttons located below the channel listbox.

8.2 Performing Post-Capture Analysis

1. Open a Main View of the image you wish to analyze.

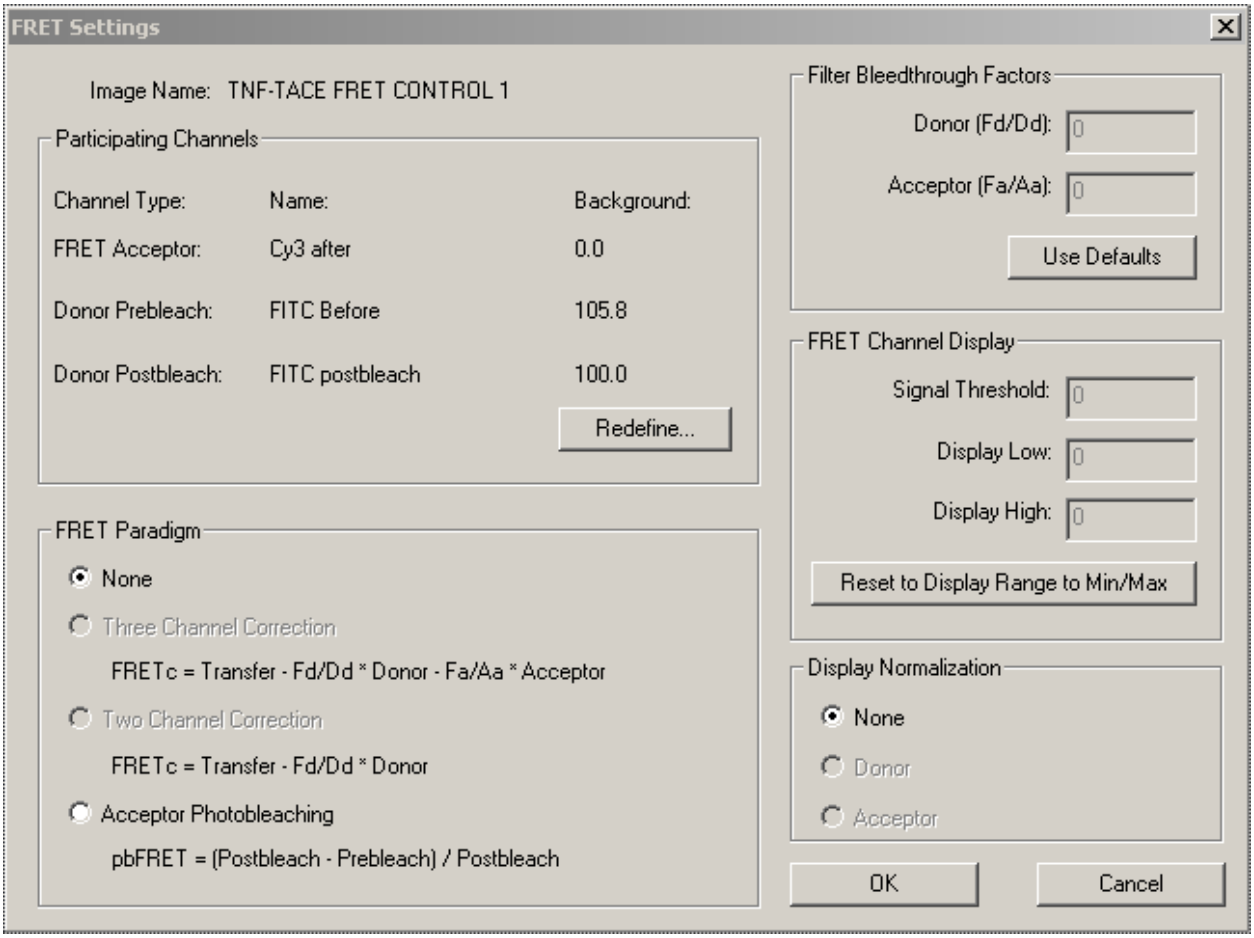


(Image courtesy of Dr. Luis Marrero, LSU Health Sciences Center.)

2. Use an ROI tool to mark a background area with no signal. A region will appear.

3. Select the region by clicking on it (the number will turn white), then right-click on the region and select "Set as background".

4. Select **Image>FRET> FRET Settings**. The following dialog box will appear.



2. Select **Acceptor Photobleaching** and then **OK**.

3. Return to the Main View of your image and select <pbFRET> from any of the channel menus to display and view your acceptor photobleaching FRET values.

4. To alter the display values in your image, alter the edit fields in the **FRET Channel Display** section of the dialog box.

    - **Signal Threshold** – the intensity above which pbFRET values will be calculated. This threshold is background subtracted. Therefore, if you

select a value of 50, any pixel that is above 50 after background subtraction will be used for calculating pbFRET.

- **Display Low** – the pbFRET value that corresponds to black in RGB display or blue in pseudocolor display. See next section for further explanation of pseudocolor display.
- **Display High** – the pbFRET value that corresponds to saturated color in RGB display or red in pseudocolor display.

8.3 Generating Photobleaching FRET Statistics

To obtain Photobleaching FRET statistics:

1. Create a mask using either segmentation or manual techniques. Please see Chapter 10 of the SlideBook™ User's Manual for information on creating masks.

2. Select **Mask>Statistics** and select **Photobleaching FRET** from **Cross-Channel** statistics.

3. Select **Display**. This will generate statistics on a region basis using the following formula:

   PB FRET = [(Donor Post) – (Donor Pre)] / (Donor Post)

   where the intensities in parentheses are first averaged for each region before performing division.

   NOTE: Confirm that the appropriate channels are being used for the calculation by checking the FRET settings dialog box (**Image>FRET>FRET Settings…**).

4. To export the data, select **Export**, enter a location for the data and select **Save**. You may now open your data file in Excel.

9 Selected References

Bolsover, S. R. *et al.* (1993) Ratio imaging measurement of intracellular calcium and pH. In *Electronic Light Microscopy* (New York: Wiley-Liss), 182-210.

Dunn, K. *et al.* (1994) Applications of ratio fluorescence microscopy in the study of cell physiology. *FASEB Journal* **8**: 573-582.

Grynkiewicz, G. *et al.* (1985) A new generation of Ca^{2+} indicators with greatly improved fluorescence properties. *Journal of Biological Chemistry* **260**: 3440-3450.

Moore, E. D. W. *et al.* (1990) Ca2+ imaging in single living cells: Theoretical and practical issues. *Cell Calcium* **11**: 157-179.

Sorkin, A. *et al.* (2000) Interaction of EGF receptor and Grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Current Biology* **10**: 1395-1398.

Appendix A: FRET Introduction and Theory

Flourescence Resonance Energy Transfer (FRET) is a distance-dependent interaction between the electronic excited states of two dye molecules where the excitation is transferred from a donor molecule to an acceptor molecule *without the emission of a photon.* More specifically, FRET is the radiationless transmission of energy from its site of absorption to the site of its utilization by resonance between chromophores (Figure 1).

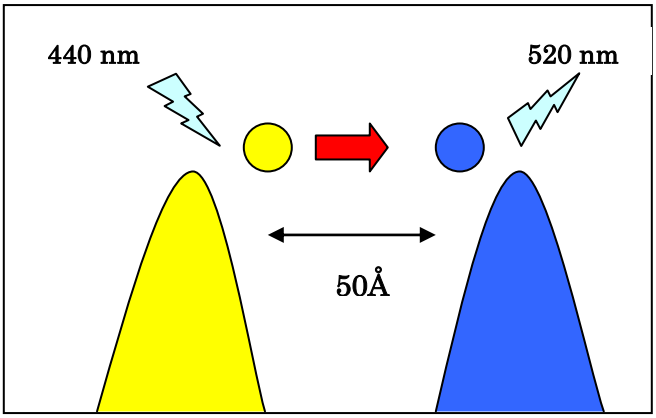


Figure 1

There are three types of FRET, two of which SlideBook™™ currently supports. The three types of FRET are:

- Acceptor Photobleaching Recovery
- Direct FRET/Sensitized Emission FRET
- Fluorescence Lifetime Measuring

SlideBook™™ supports Direct FRET and Acceptor Photobleaching Recovery and will support Fluorescence Lifetime Measuring in the near future.

As stated above, FRET involves the non-radiative transfer of energy from a fluorophore in an excited state to a nearby acceptor fluorophore. In order for this transfer to take place, the donor and acceptor molecules must be within 10-50Å (Förster distance) and the emission spectra of the donor fluorophore must overlap the excitation spectra of the acceptor fluorophore. In principle, this overlap will allow the exchange of energy through a non-radiative dipole-dipole transfer. The energy transfer manifests itself by quenching the donor fluorescence in the presence of the acceptor and increased (sensitized) emission of the acceptor fluorescence. The figure on the next page illustrates the spectral overlap of the corresponding fluorphores.

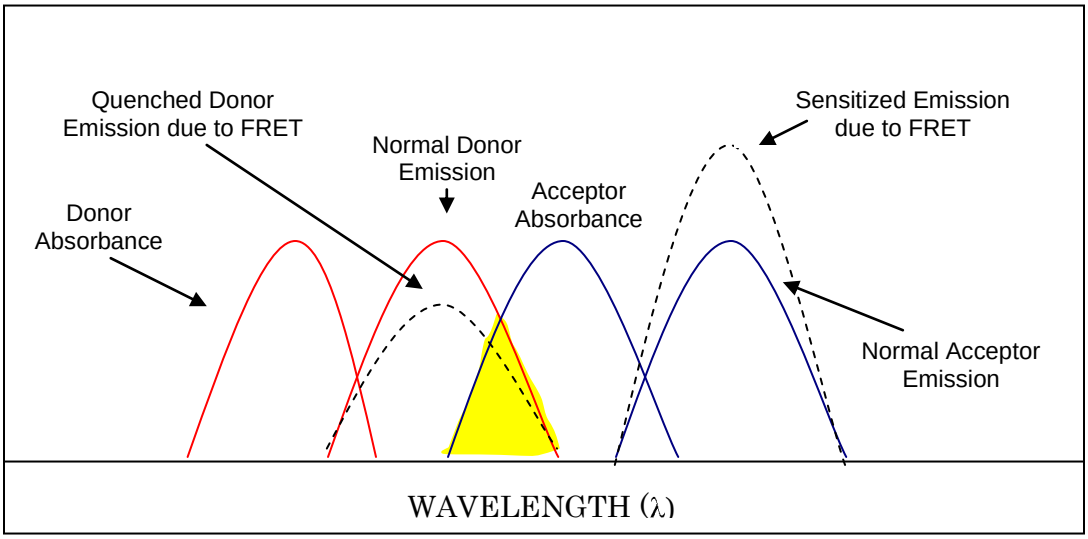


Figure 2

The distance dependent characteristics of FRET make it an excellent tool for investigating a variety of biological phenomena that produce changes in molecular proximity. Further, this distance dependency also allows FRET to distinguish between two fluorophores that are less than 200 nanometers apart.

Acceptor Photobleaching Recovery

The underlying principle of Acceptor Photobleaching Recovery takes advantage of statistical probabilities. Whenever a molecule is excited by a photon, there is a statistical probability of either of three things happening. These three things are:

A. The specimen under questions fluoresces and emits the exciter energy as the electrons drop back to ground state.
B. The specimen becomes photobleached
C. The specimen FRETs – or rather it loses (in the case of the donor) energy through a dipole transfer.

Knowing these three outcomes, it is possible to calculate FRET through a subtractive process. Prior to photobleaching, the defining equation for the donor is as follows:

$$A + B + C = 100\%_{\text{ before photobleaching}} \qquad (1)$$

Once the acceptor is photobleached, FRET cannot occur and C is set to 0. The equation is then reduced to:

$$A + B = 100\%_{\text{after photobleaching}} \qquad (2)$$

Subtracting equation 2 from equation 1, and assuming the probability of spontanteous photobleaching of the donor remains the same, yields:

$$A + C_{\text{before photobleaching}} = A_{\text{after photobleaching}} \quad (3)$$

Thus, after photobleaching, the amount of A is increased by C, the amount of FRET occurring prior to bleaching. The process of photobleaching, a destructive process, eliminates the possibility of FRET occurring in the acceptor. Therefore, if the donor cannot FRET to the acceptor then the acceptor emission is increased.

In practice, there are three steps to calculating FRET using Acceptor Photobleaching Recovery. These steps are

1. Capture an image of the donor
2. Bleach the acceptor
3. Capture an image of the donor

After taking these two images, FRET can be calculated by subtracting image one from image three.

Direct FRET/Sensitized Emission FRET

Direct FRET/Sensitized Emission FRET is a faster, and less intrusive method of FRET imaging than Acceptor Photobleaching FRET, at the cost of computational accuracy. As the name of the technique indicates, FRET is measured directly by imaging the sample through donor excitation and acceptor emission filters, or FRET filters. In theory, this would be the only image necessary if the donor excitation and acceptor emission spectra were perfectly separated. In practice, the spectra do overlap, and corrections for spectral bleedthrough must be performed. There are two techniques for calculating Direct FRET:

- **Two-Channel Corrected FRET** – applicable when donor and acceptor are present in a fixed, stoichiometric ratio (e.g. Cameleon imaging)

- **Three-Channel Corrected FRET** - separately corrects for donor bleedthrough and direct excitation of the acceptor, appropriate when donor and acceptor are not present in a fixed ratio

We will now discuss the details of Three-Channel Corrected FRET. A discussion of Two-Channel Corrected FRET follows.

Three-Channel Corrected FRET

For Three-Channel Correct FRET, three images are necessary:

8. Control containing donor only imaged with donor and FRET filters.
9. Control containing acceptor only imaged with acceptor and FRET filters.
10. Experimental sample containing both donor and acceptor imaged with donor, acceptor, and FRET filters.

For demonstration purposes, let us assume CFP is the donor and YFP is the acceptor. When CFP excites, a large portion will emit normally (corresponding to the CFP emission spectrum). If the CFP FRETs, a radiationless energy transfer will occur that causes YFP to emit. The CFP emission spectrum overlaps with the YFP emission spectrum, therefore some of the normal CFP emission will be visible through the YFP emission filter. This cross-talk confounds the signal due to FRET and must be corrected. The first image, the CFP control, is used to calculate the spectral bleedthrough of CFP emission visible through FRET filter set.

Additional corrections must be made for non-FRET signal due to the overlap of CFP and YFP excitation spectra.. This overlap causes normal YFP fluorescence to be visible through the FRET filter set. The second image, the YFP control, is used to calculate the spectral bleedthrough of normal YFP emission through the FRET filter set..

The third image is the raw FRET image. Corrected FRET, or $FRET_c$, is calculated with consideration of all three images. The following equation is used to subtract the non-FRET portions from the raw FRET signal:

$$FRET_c = FRET_{raw} - D_f/D_d[CFP] - D_f/D_a[YFP] \qquad (4)$$

where $FRET_{raw,}$, [CFP], and [YFP] are the signals visualized through the FRET, CFP, and YFP filter sets, respectively. The constants D_f/D_d and D_f/D_a are the transmissivity, or bleed-through, constants describing donor emission visible in FRET channel and direct excitation of acceptor, respectively [Sorkin, et al.]. These constants are calculated using images 1 and 2. Failure to account for these intensities in raw FRET will result in erroneous data.

Two-Channel Corrected FRET

For Two-Channel Corrected FRET, a single two-channel image of the sample is required. The two channels that must be captured are the donor (or acceptor) channel and the FRET channel.

Continuing with the CFP/YFP example above, if the concentration of CFP equals that of YFP, equation (4) reduces to:

$$FRET_c = FRET_{raw} + (D_f/D_a - D_f/D_d)[CFP] \qquad (5)$$

Dividing through by [CFP] yields:

$$FRET_c/[CFP] = FRET_{raw}/[CFP] + (D_f/D_a - D_f/D_d) \qquad (6)$$

Thus, the normalized, uncorrected values will differ from the normalized corrected values by a constant offset. Further, for two-channel FRET, if the donor and acceptor bleedthrough factors (D_f/D_d and D_f/D_a) are unknown, changes in normalized, corrected FRET, $FRET_{cN,}$ can still be measured:

$$\Delta FRET_{CN} = \Delta \{FRET_c/[CFP]\} = \Delta \{FRET_{raw}/[CFP]\} \qquad (7)$$

Therefore, simply measuring the ratio of the raw FRET channel to the donor channel allows for comparison of two-channel corrected FRET values in various regions of the experimental sample.

Appendix B: Buffers For Ratio Calibration

You may wish to purchase a Fura-2 Calcium Imaging Calibration Kit from Molecular Probes (Cat# F-6774). Sample buffer recipes are shown below. Rmin, Rmax and beta values will vary depending on your particular setup, but typical values for Fura-2 may be Rmin ~ 0.25, Rmax ~ 2.5 and beta ~ 5.

Background Buffer

10X stock solution

Dissolve in 90 ml H_2O:

8.95 g KCl (1.2 M)
0.29 g NaCl (50 mM)
0.1 g KH_2PO_4 (7.4 mM)
0.42 g $NaCO_3$ (50 mM)
4.77 g HEPES (200 mM)

Do not adjust pH

Bring volume to 100 ml with H_2O

For 1X solution dilute 1:9 in H_20, pH to 7.4

Ion-Free Buffer

Add 25-50 μM (final concentration) fluorophore (not AM ester derivative, must be salt form) to the Background Buffer. Note that this value may vary based on your fluorophore and loading conditions. You will want to approximate the final concentration of fluorophore in your loaded samples.

Ion-Saturated Buffer

10X stock solution $CaCl_2$

Dissolve in 90 ml H_2O:

0.22 g $CaCl_2$ (20mM)

Do not adjust pH

Bring to 1X using 1 part $CaCl_2$ 10X solution, 1 part 10X background buffer solution, 8 parts H_2O, pH to 7.4 and add 25-50 μM (final concentration) fluorophore.

Appendix C: Ratio Calibration Calculations

Terms

λ_1	Excitation wavelength used in ratio numerators
λ_2	Excitation wavelength used in ratio denominators
$B_{\lambda 1}(n; x, y)$	Intensity of numerator background image n at position x, y
$B_{\lambda 2}(n; x, y)$	Intensity of denominator background image n at position x, y
$I_{f\lambda 1}(n; x, y)$	Intensity of numerator ion-free image n at position x, y
$I_{f\lambda 2}(n; x, y)$	Intensity of denominator ion-free image n at position x, y
$I_{b\lambda 1}(n; x, y)$	Intensity of numerator ion-saturated image n at position x, y
$I_{b\lambda 2}(n; x, y)$	Intensity of denominator ion-saturated image n at position x, y
ROI	Region of interest: a subset of all possible x, y positions
$\|ROI\|$	The number of positions in the region of interest
γ	Correction factor: exposure time at λ_1 / exposure time at λ_2

Background

The background is computed from averaging intensities of N images 1…n at both λ1and λ2 over the region of interest:

$$\underline{b}_{\lambda 1} = 1/N \, \Sigma_n \, 1/|ROI| \, \Sigma_{ROI} \, B_{\lambda 1}(n; x, y)$$
$$\underline{b}_{\lambda 2} = 1/N \, \Sigma_n \, 1/|ROI| \, \Sigma_{ROI} \, B_{\lambda 2}(n; x, y)$$

R_{min}

$R_{min}(n)$ is computed for each set of images 1…n and the minimum is taken for the calibration value R_{min}:

$$\underline{i}_{f\lambda 1}(n) = 1/|ROI| \, \Sigma_{ROI} \, I_{f\lambda 1}(n; x, y)$$
$$\underline{i}_{f\lambda 2}(n) = 1/|ROI| \, \Sigma_{ROI} \, I_{f\lambda 2}(n; x, y)$$

$$R_{min}(n) = (\underline{i}_{f\lambda 1}(n) - \underline{b}_{\lambda 1}) / (\gamma \, (\underline{i}_{f\lambda 2}(n) - \underline{b}_{\lambda 2}))$$

$$R_{min} = \min_n R_{min}(n)$$

R_{max}

$R_{max}(n)$ is computed for each set of images 1…n and the maximum is taken for the calibration value R_{max}:

$$\underline{i}_{b\lambda 1}(n) = 1/|ROI| \, \Sigma_{ROI} \, I_{b\lambda 1}(n; x, y)$$
$$\underline{i}_{b\lambda 2}(n) = 1/|ROI| \, \Sigma_{ROI} \, I_{b\lambda 2}(n; x, y)$$

$$R_{max}(n) = (\underline{i}_{b\lambda 1}(n) - \underline{b}_{\lambda 1}) / (\gamma \, (\underline{i}_{b\lambda 2}(n) - \underline{b}_{\lambda 2}))$$

$$R_{max} = \max_n R_{max}(n)$$

Beta

β is computed as the quotient of average denominator intensity in the ion-free images over the average denominator intensity in the ion-saturated images:

$$\beta = (1/N \Sigma_n \underline{i}_{f\lambda 2}(n) - \underline{b}_{\lambda 2}) / (1/N \Sigma_n \underline{i}_{b\lambda 2}(n) - \underline{b}_{\lambda 2})$$

Appendix D: Ratio Data Export Calculations

Currently there are two ways of defining a ROI for post-acquisition analysis:

1) Using a selection cube for generating ratios (and concentrations) for a single rectangular region across all time points included in the selection
2) Using a mask (divided into submasks) for contiguous isolated user-delineated regions across all time points included in the mask

Each method uses the same ratio and concentration calculations.

Terms

λ_1	Excitation wavelength used in ratio numerators
λ_2	Excitation wavelength used in ratio denominators
$I_{\lambda 1}(t; x, y)$	Intensity of numerator image of time point t at position x, y
$I_{\lambda 2}(t; x, y)$	Intensity of denominator image of time point t at position x, y
ROI(n)	Region of interest n: a subset of all possible x, y positions
$\|ROI(n)\|$	The number of positions in the region of interest n
γ	Correction factor: exposure time at λ_1 / exposure time at λ_2
$\theta_{\lambda 1}$	Minimum threshold intensity for ratio numerator
$\theta_{\lambda 2}$	Minimum threshold intensity for ratio denominator
$\omega_{\lambda 1}$	Maximum threshold intensity for ratio numerator
$\omega_{\lambda 2}$	Maximum threshold intensity for ratio denominator

Background

The background is computed for each time point for both the numerator and denominator images by averaging intensities over the background region of interest ROI_b:

$$\underline{b}_{\lambda 1}(t) = 1/|ROI_b| \sum_{ROIb} I_{\lambda 1}(t; x, y)$$
$$\underline{b}_{\lambda 2}(t) = 1/|ROI_b| \sum_{ROIb} I_{\lambda 2}(t; x, y)$$

ROIs and Thresholding

Individual regions of interest for analysis are restricted by the minimum and maximum thresholds for each wavelength. This means that a delineated region of interest ROI(n; t) actually yields two regions, $ROI_{\lambda 1}(n; t)$ and $ROI_{\lambda 2}(n; t)$, which may not necessarily be identical:

$$ROI_{\lambda 1}(n; t) = \cup_{x,y} \text{ such that } (x, y) \in ROI(n; t) \wedge \theta_{\lambda 1} \le I_{\lambda 1}(t; x, y) \le \omega_{\lambda 1}$$
$$ROI_{\lambda 2}(n; t) = \cup_{x,y} \text{ such that } (x, y) \in ROI(n; t) \wedge \theta_{\lambda 2} \le I_{\lambda 2}(t; x, y) \le \omega_{\lambda 2}$$

Ratio Computation

The ratio r(n; t) is computed for ROI(n; t) by first computing average intensities for the numerator and denominator:

$$\underline{i}(n; t)_{\lambda 1}(n) = 1/|ROI_{\lambda 1}(n; t)| \sum_{ROI_{\lambda 1}(n; t)} I_{\lambda 1}(t; x, y)$$
$$\underline{i}(n; t)_{\lambda 2}(n) = 1/|ROI_{\lambda 2}(n; t)| \sum_{ROI_{\lambda 2}(n; t)} I_{\lambda 2}(t; x, y)$$

and then computing the background and exposure time corrected ratio:

$r(n; t) = (\underline{i}(n; t)_{\lambda 1}(n) - \underline{b}_{\lambda 1}(t)) / (\gamma(\underline{i}(n; t)_{\lambda 2}(n) - \underline{b}_{\lambda 2}(t)))$

Ratio Error Conditions

The following conditions will prevent r(n; t) from being computed:

$|ROI_{\lambda 1}(n; t)| = 0$
$|ROI_{\lambda 2}(n; t)| = 0$
$\underline{i}(n; t)_{\lambda 1}(n) < \underline{b}_{\lambda 1}(t)$
$\underline{i}(n; t)_{\lambda 2}(n) \leq \underline{b}_{\lambda 2}(t)$

Concentration Computation

The concentration c(n; t) is computed using the calibration parameters:

$c(n; t) = K_d \beta (r(n; t) - R_{min}) / (R_{max} - r(n; t))$

Concentration Error Conditions

The following conditions, in addition to those that prevent r(n; t) from being computed, will prevent c(n; t) from being computed:

$r(n; t) < R_{min}$
$r(n; t) \geq R_{max}$

Appendix E: Direct FRET Calculations

Terms

λ_D	FRET Donor emission wavelength
λ_A	FRET Acceptor emission wavelength
λ_F	FRET Transfer emission wavelength
F_d/D_d	Donor emission bleedthrough
F_a/A_a	Direct excitation of acceptor
$I_{\lambda D}(t; x, y)$	Intensity of image measured through FRET Donor filter set of time point t at position x,y
$I_{\lambda A}(t; x, y)$	Intensity of image measured through FRET Acceptor filter set of time point t at position x,y
$I_{\lambda F}(t; x, y)$	Intensity of image measured through FRET Acceptor filter set of time point t at position x,y
ROI(n)	Region of interest n: a subset of all possible x, y positions
$\|ROI_{(n)}\|$	The number of positions in the region of interest n

Background

The background is computed for each time point for FRET donor, acceptor, and transfer images by averaging intensities over the background region of interest ROI_b:

$\underline{b}_{\lambda D}(t) = 1/|ROI_b| \ \Sigma_{ROIb} \ I_{\lambda D}(t; x, y)$
$\underline{b}_{\lambda A}(t) = 1/|ROI_b| \ \Sigma_{ROIb} \ I_{\lambda A}(t; x, y)$
$\underline{b}_{\lambda F}(t) = 1/|ROI_b| \ \Sigma_{ROIb} \ I_{\lambda F}(t; x, y)$

Three Channel Corrected FRET - $FRET_c$ Computation

$FRET_c$ (n; t) is computed for ROI(n; t) by first computing average intensities for the donor, acceptor, and transfer channels:

$\underline{i}(n; t)_{\lambda D}(n) = 1/|ROI_{\lambda D}(n; t)| \ \Sigma_{ROI\lambda D}(n; t) \ I_{\lambda D}(t; x, y)$
$\underline{i}(n; t)_{\lambda A}(n) = 1/|ROI_{\lambda A}(n; t)| \ \Sigma_{ROI\lambda 2}(n; t) \ I_{\lambda A}(t; x, y)$
$\underline{i}(n; t)_{\lambda F}(n) = 1/|ROI_{\lambda F}(n; t)| \ \Sigma_{ROI\lambda F}(n; t) \ I_{\lambda F}(t; x, y)$

and then computing the background corrected FRETc:

$FRETc(n; t) = (\underline{i}(n; t)_{\lambda F}(n) - \underline{b}_{\lambda F}(t)) - F_d/D_d*(i(n;t)_{\lambda D}(n) - \underline{b}_{\lambda D}(t)) - F_a/A_a*(i(n;t)_{\lambda A}(n) - \underline{b}_{\lambda A}(t))$

Two Channel Corrected FRET – FRETc Computations

$FRET_c$ (n; t) is computed for ROI(n; t) by first computing average intensities for the donor and transfer channels:

$\underline{i}(n; t)_{\lambda D}(n) = 1/|\,ROI_{\lambda D}(n; t)\,| \;\Sigma_{ROI\lambda D}(n; t)\; I_{\lambda D}(t; x, y)$
$\underline{i}(n; t)_{\lambda F}(n) = 1/|\,ROI_{\lambda F}(n; t)\,| \;\Sigma_{ROI\lambda F}(n; t)\; I_{\lambda F}(t; x, y)$

and then computing the background corrected FRETc:

$FRETc(n; t) = (\underline{i}(n; t)_{\lambda F}(n) - \underline{b}_{\lambda F}(t)) - F_d/D_d * (i(n;t)_{\lambda D}(n) - \underline{b}_{\lambda D}(t))$

Appendix F: Photobleaching FRET Calculations

Terms

λ_{pre}	Donor emission wavelength measured before photobleaching
λ_{post}	Donor emission wavelength measured after photobleaching
$I_{\lambda pre}(t; x, y)$	Intensity of image measured through FRET Donor filter set of time point t at position x,y
$I_{\lambda post}(t; x, y)$	Intensity of image measured through FRET Acceptor filter set of time point t at position x,y
ROI(n)	Region of interest n: a subset of all possible x, y positions
$\|ROI_{(n)}\|$	The number of positions in the region of interest n

Background

The background is computed for prebleach and postbleach images by averaging intensities over the background region of interest ROI_b:

$\underline{b}_{\lambda pre} = 1/|ROI_b| \ \Sigma_{ROIb} \ I_{\lambda D}(x, y)$
$\underline{b}_{\lambda post} = 1/|ROI_b| \ \Sigma_{ROIb} \ I_{\lambda A}(x, y)$

Photobleach FRET - pbFRET Computation

pbFRET (n) is computed for ROI(n) by first computing average intensities for the prebleach and postbleach channels:

$\underline{i}(n; t)_{\lambda pre}(n) = 1/|ROI_{\lambda pre}(n; t)| \ \Sigma_{ROI\lambda pre}(n; t) \ I_{\lambda pre}(t; x, y)$
$\underline{i}(n; t)_{\lambda post}(n) = 1/|ROI_{\lambda post}(n; t)| \ \Sigma_{ROI\lambda 2}(n; t) \ I_{\lambda post}(t; x, y)$

and then computing the background corrected pbFRET:

$pbFRET(n) = [(\underline{i}(n)_{\lambda post}(n) - \underline{b}_{\lambda pre}) - (i(n)_{\lambda pre}(n) - \underline{b}_{\lambda post}(t))] / (\underline{i}(n)_{\lambda post}(n) - \underline{b}_{\lambda pre})$