Technical Guide 3

Using Fluorescent Resonance Energy Transfer (FRET) Algorithms in MIPAV

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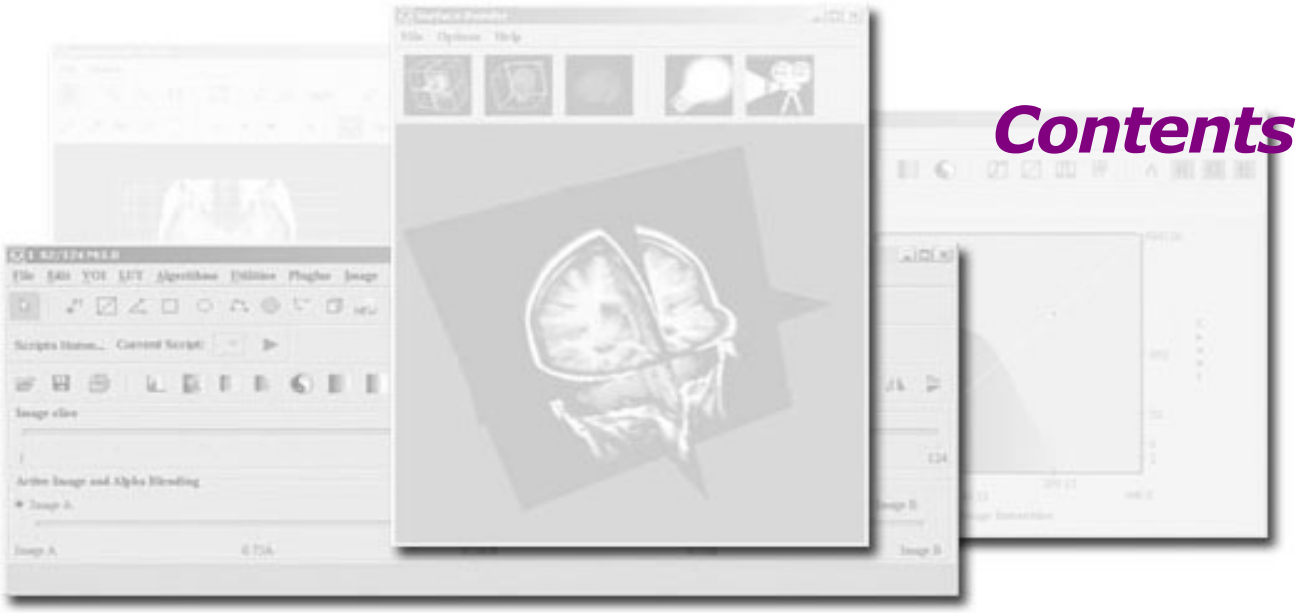

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Using Fluorescent Resonance Energy Transfer (FRET) Algorithms in MIPAV

This technical guide consolidates the information about the Fluorescent Resonance Energy Transfer (FRET) algorithms that are implemented in MIPAV. The following FRET commands appear in the Algorithms > Microscopy menu in the MIPAV main window:

- FRET—Acceptor Photobleaching algorithm (refer to “Microscopy: Fluorescence Resonance Energy Transfer (FRET)—Acceptor Photobleaching” on page 2)
- FRET Bleed Through and FRET Efficiency algorithms (“Microscopy: Fluorescent Resonance Energy Transfer (FRET) Bleed Through and Efficiency” on page 11)

Microscopy: Fluorescence Resonance Energy Transfer (FRET)—Acceptor Photobleaching

This algorithm uses acceptor photobleaching to compare the proximity of fluorescent-labeled molecules in two 2D images over distance:

- An image *before* acceptor photobleaching, also referred to as the prebleached image
- An image *after* acceptor photobleaching, which is the postbleached image

Background

FRET refers to the nonradiative transfer of energy from an excited state donor fluorescent molecule to a nearby acceptor fluorescent molecule. The energy transfer efficiency E, defined as the number of energy transfer events divided by the number of photons absorbed by the donor, is related to the distance R between the acceptor and donor by:

$$E = \frac{1}{\left[1 + \left(\frac{r}{Ro}\right)^6\right]}$$

Equation 1

where Ro, the Forster critical distance, is the distance at which $E = 0.5$.

We also have

$$E = 1 - \frac{FDA}{FD}$$

EQUATION 2

where FDA is the donor fluorescence in the presence of an acceptor and FD is the donor fluorescence in the absence of the acceptor.

The equivalent of an acceptor's absence can be created by photobleaching the acceptor. Thus, a method using equation 2 can be performed on a single sample by measuring the donor fluorescence before and after photobleaching the acceptor molecules.

Because FRET falls off as the sixth power of the distance between the donor and the acceptor, no FRET occurs for distances greater than 2*Ro*. Since *Ro* is on the order of 10 to 70 Angstroms, by performing FRET measurements it is possible to distinguish proteins that are merely nearby in the same compartment from those proteins that are interacting with each other.

To access the FRET algorithm, you first open the two images in question and then select Algorithm > Microscopy > FRET in the MIPAV window.

Since unbleached acceptors quench the donor fluorescence with FRET and bleached acceptors do not quench donor fluorescence, the postbleached image donor fluorescence is greater than the prebleached image donor fluorescence.

In the Fluorescence Resonance Energy Transfer dialog box, you may choose as an option to register the two images before running the FRET algorithm. During registration MIPAV registers the prebleached image to the postbleached image and uses correlation ratio as the default cost function. However, other cost functions available for use include least squares, normalized cross correlation, or normalized mutual information. After registration MIPAV runs FRET on the *registered* prebleached image rather than on the original prebleached image.

To run the algorithm, you must first delineate a VOI on the postbleached image. This requires that you select Add required donor fluorescence VOI in the dialog box and then return to the MIPAV window to choose an ellipse VOI, rectangle VOI, polyline VOI, or levelset VOI with which to draw the VOI. You can then, as an option, also identify a background VOI on the image by selecting Add optional background VOI and repeat the steps performed in drawing the required donor VOI. The VOIs must all be placed in the postbleached image, and the background region has a smaller average intensity than the donor region.

As an option, you can use both a signal normalization VOI and a background VOI. However, to use a signal normalization VOI, you must have a background VOI.

For color images, select the color corresponding to the donor fluorescence. For example, if the donor fluorescence is blue, select blue. if the donor fluorescence is red, select red.

Note: Only one color is used from a color image.

If a background VOI is present and no signal VOI is present, the average of the prebleached background is subtracted from the average of the prebleached donor region. The average of the postbleached background is subtracted is subtracted from the average of the postbleached donor region. Then, the energy transfer efficiency is calculated as (background subtracted postbleached donor intensity - background subtracted prebleached donor intensity)/background subtracted postbleached donor intensity.

Let b and s be the prebleached background and signal values, and let $b2$ and $s2$ be the postbleached background and signal values. Then, the following equation is used to linearly scale a donor value from the prebleached image into a donor value in the postbleached image range:

$$L = \left(\frac{(s2 - b2)}{(s1 - b1)}\right) \times P + \frac{(b2 \times s1 - b1 \times s2)}{(s1 - b1)}$$

where

L = Linearly scaled prebleached donor

P = Prebleached donor

Then

$$\text{Efficiency} = \frac{(\text{Postbleached donor} - \text{Linearly scaled prebleached donor})}{\text{Postbleached donor}}$$

IMAGE TYPES

You can apply this algorithm to two 2D images or one 2-slice 3D image.

NOTES

None.

REFERENCES

Refer to the following references for more information about the FRET algorithm.

Gordon, Gerald W., Gail Berry, Xiao Huan Liang, Beth Levine, and Brian Herman. "Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy." *Biophysical Journal* 74(May 1998):2702–2713.

Kenworthy, Anne K. "Imaging Protein-Protein Interactions Using Fluorescence Energy Transfer Microscopy." *Methods* 24(2001):289–296.

Applying the FRET algorithm

To use this algorithm, do the following:

1. Open two 2D images or one 2-slice 3D image that contain fluorescent-labeled components. The Load Bleached ROI message (Figure 1) appears.

Figure 1. Load Bleached ROI message

2. Click Yes. MIPAV loads the images along with the bleached region of interest (ROI).
3. Select the prebleached image (refer to "Selecting Image Slices Quickly" below).
4. Select Algorithms > Microscopy > FRET in the MIPAV window. The FRET dialog box (Figure 2) appears.

Selecting Image Slices Quickly

If the images or images slices are in the same file, you can quickly jump from one image or slice to another by using  , the Decrements image slice icon, or  , the Increments image slice icon.

5 Select the postbleached image.

6 Select—for color images—the fluorescence color in the Color selection panel.

Note: The Color selection panel does not appear in the dialog box if the images are grayscale or black and white. Also, the number of colors that you can select in the Color selection panel depends on the number of colors in the images. If, for example, only red and green appear in the images, then those are the only colors listed. If three colors are present, then red, green, and blue appear.

Prebleached image	Specifies the image *before* acceptor photobleaching.
Postbleached image	An image *after* acceptor photobleaching.
Add required donor fluorescence VOI	Adds a mandatory VOI to the postbleached image.
Add optional background VOI	Adds an optional background VOI on the postbleached image.
Add optional signal normalization VOI	Requires that a background VOI be used. Adds an optional signal normalization VOI on the postbleached image.
Color selection (only appears for color images)	Select one of the available colors—red, green, or blue—that corresponds to the fluorescence-labeled component being quantified in the images. By default, red is selected. Note that only colors that appear in the images are listed in this panel.
Registration before FRET`	Registers the prebleached image to the postbleached image. The default is no registration. Selecting this check box enables the Cost function list.
Cost function	Specifies the registration cost function. You may select correlation ratio (the default), least squares, normalized cross correlation, or normalized mutual information. To use this item, you must first select Registration before FRET.
Create registration image	Creates the registered image in a separate image window. To use this check box, you must first select Registration before FRET. By default, this check box is selected when registration is selected.
OK	Applies the algorithm according to the specifications in this dialog box.
Cancel	Disregards any changes that you made in the dialog box and closes this dialog box.
Help	Displays online help for this dialog box.

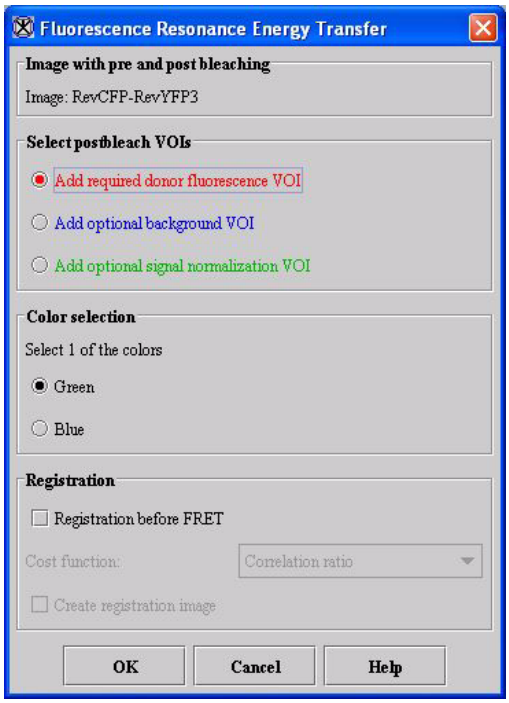

Figure 2. Fluorescence Resonance Energy Transfer (FRET) dialog box

7 Select Add required donor fluorescence VOI.

8 Go to the MIPAV window, and select one of these icons from the VOI toolbar:

- Draw rectangle VOI 
- Draw ellipse VOI 
- Draw polygon/polyline VOI 
- Draw levelset VOI 

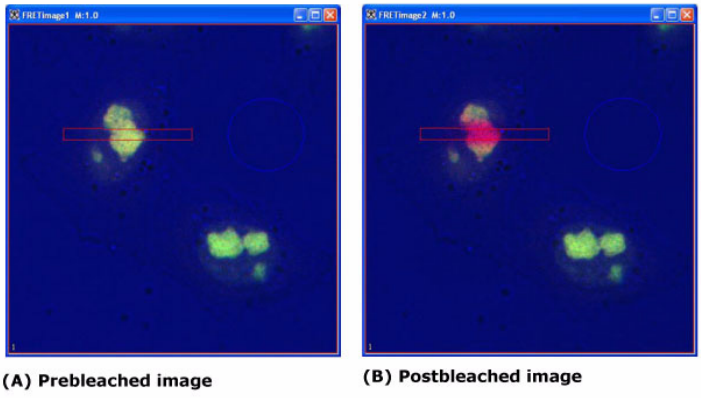

Figure 3. Example of (A) prebleached image and (B) postbleached image after FRET algorithm was applied

9 Draw the VOI on the postbleached image. The VOI appears in red.

Note: In drawing these VOIs, you do not need to first select the New VOI icon.

10 Go to step 12, or, if you wish, create an optional background VOI using the following directions:

a Select Add optional background VOI in the Select postbleach VOIs panel in the dialog box.

b Go to the MIPAV window and select  ,  ,  , or  to draw a rectangle, ellipsoidal, polygon/polyline, or levelset VOI on the image. A blue VOI appears on the postbleached image.

Background region: The background region should not contain any structures and should be darker than the donor region.

11 Go to the next step, or, if you wish, create an optional signal normalization VOI.

a Select Add optional signal normalization VOI in the Select postbleach VOIs panel in the dialog box.

b Go to the MIPAV window and select  ,  ,  , or  to draw a rectangle, ellipsoidal, polygon/polyline, or levelset VOI on the image. A green VOI appears on the postbleached image.

Note: To use an optional signal normalization VOI, you must create a background VOI.

12 Register the images as an option by doing the following:

a Select Registration before FRET.

b Select a cost function in the Cost function list. The default cost function is correlation ratio, but you can select least squares, normalized cross correlation, or normalized mutual information.

c Select or clear Create registration image. Since registration is far more time consuming than calculating FRET, if you selected the Registration before FRET check box, the Create registration image check box is selected by default.

13 Click OK. The algorithm begins to run, and a progress bar appears momentarily with the status.

When the algorithm finishes running, the progress bar disappears. The data appears on the Data page in the Output window (Figure 4).

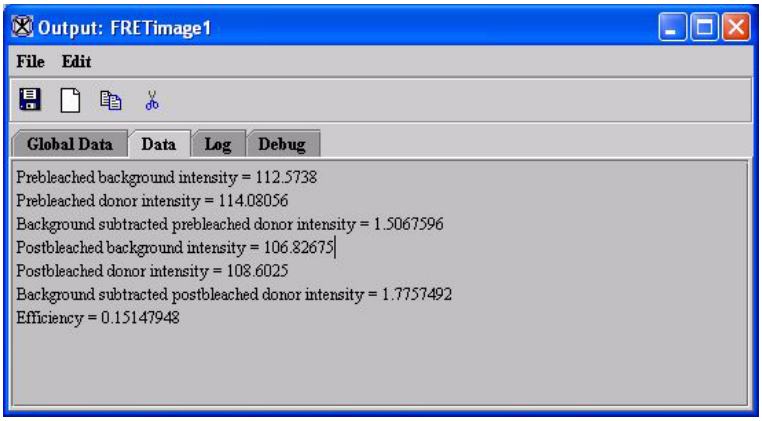

Figure 4. FRET data in the Data page of the Output window

Note: If you chose to register the images, the registered image appears in a separate image window, and the FRET data appears on the Data page in the Output window.

Microscopy: Fluorescent Resonance Energy Transfer (FRET) Bleed Through and Efficiency

This section provides information on and discusses how to use the following two FRET algorithms:

- FRET Bleed Through algorithm
- FRET Efficiency algorithm

Used consecutively, the FRET Bleed Through algorithm uses two sets of three 2D images and the FRET Efficiency algorithm uses one set of three 2D images to measure effects dependent on the proximity of fluorescent-labeled molecules.

Note: You must first run the FRET Bleed Through algorithm twice: once on acceptor-dyed images and once on donor-dyed images. Using the results achieved from running this algorithm, you then use the FRET Efficiency algorithm to process images that were dyed with both the donor and acceptor dyes to obtain the FRET efficiency.

Background

Fluorescent resonance energy transfer (FRET) refers to the nonradiative transfer of energy from an excited fluorochrome, called a *donor*, to a nearby fluorescent molecule, called an *acceptor*. The FRET technique measures the fluorescence signals of the donor, the acceptor, and the FRET signal. If FRET occurs, the donor channel signal is quenched and the acceptor channel signal is sensitized or increased.

The energy transfer efficiency E, defined as the number of energy transfer events divided by the number of photons absorbed by the donor, is related to the distance R between the acceptor and donor by:

$$E = \frac{1}{\left[1 + \left(\frac{r}{Ro}\right)^6\right]}$$

EQUATION 3

where R_0, the Forster critical distance, is the distance at which $E = 0.5$.

Because FRET falls off as the sixth power of the distance between the donor and the acceptor, no FRET occurs for distances greater than $2R0$. Since $R0$ is on the order of 10 to 70 Angstroms, by performing FRET measurements it is possible to distinguish proteins that are merely nearby in the same compartment from those proteins that are interacting with each other.

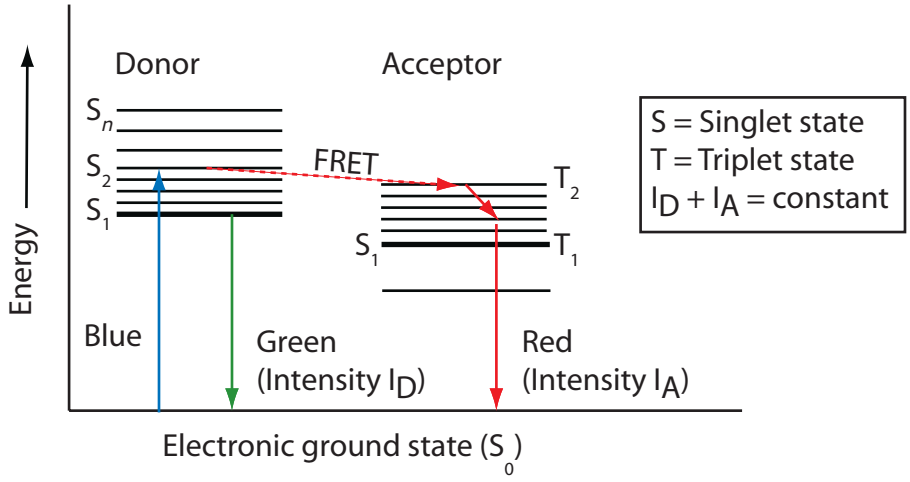

Figure 5. Jablonski diagram for FRET method

In acceptor photobleaching, since $I_D + I_A$ = constant, if the acceptor is eliminated by photobleaching, then I_A goes to zero and I_D increases to constant.

Measuring FRET using the sensitized emission method requires three sets of 2D images. You must first run the FRET Bleed Through algorithm once on three donor dye only images and once on three acceptor dye only images. You then run the FRET Efficiency algorithm on three images with both donor and acceptor dyes.

These images are:

- Image donor and/or acceptor dyes taken with a donor filter
- Image with donor and/or acceptor dyes taken with a FRET filter
- Image with donor and/or acceptor dyes taken with an acceptor filter

To obtain FRET efficiency, you need, therefore, a set of nine images. Table 1 on page 13 and Figure 6 on page 14 list and show the images that are used as examples in this discussion.

Table 1. Setup of nine example images

To obtain the FRET efficiency, you need to have a complete set of nine images modeled after the images listed here.

Algorithm	Dye used on images	Image name*	Filter used	Excitement wavelength		Emission wavelength	
				Donor	Acceptor	Donor	Acceptor
FRET Bleed Through	Acceptor	R3_543F.tif (Source)	Acceptor 1 (FP1)		✓		✓
		R3_488F.tif	FRET	✓			✓
		R3_488Y.tif	Donor 2 (PF2)	✓		✓	
	Donor	Y5_543F.tif	FP2		✓		✓
		Y5_488F.tif	FRET	✓			✓
		Y5_488Y.tif (Source)	FP1	✓		✓	
FRET Efficiency	Acceptor and donor	YR4_543F.tif (Source)	Acceptor fluorescence photobleaching (AFP)		✓		✓
		YR4_488F.tif	FRET	✓			✓
		YR4_488Y.tif	Donor fluorescence photobleaching (DFP)	✓		✓	

*Refer to Figure 6 on page 14 to see these example images. The naming convention for these images is the following:

R = Red (acceptor dye)
Y = Yellow (donor dye)
F = Filter for detecting acceptor signal
Y after number = Filter for detecting donor signal
488 = Excitation wavelength for donor dye
543 = Excitation wavelength for acceptor dye

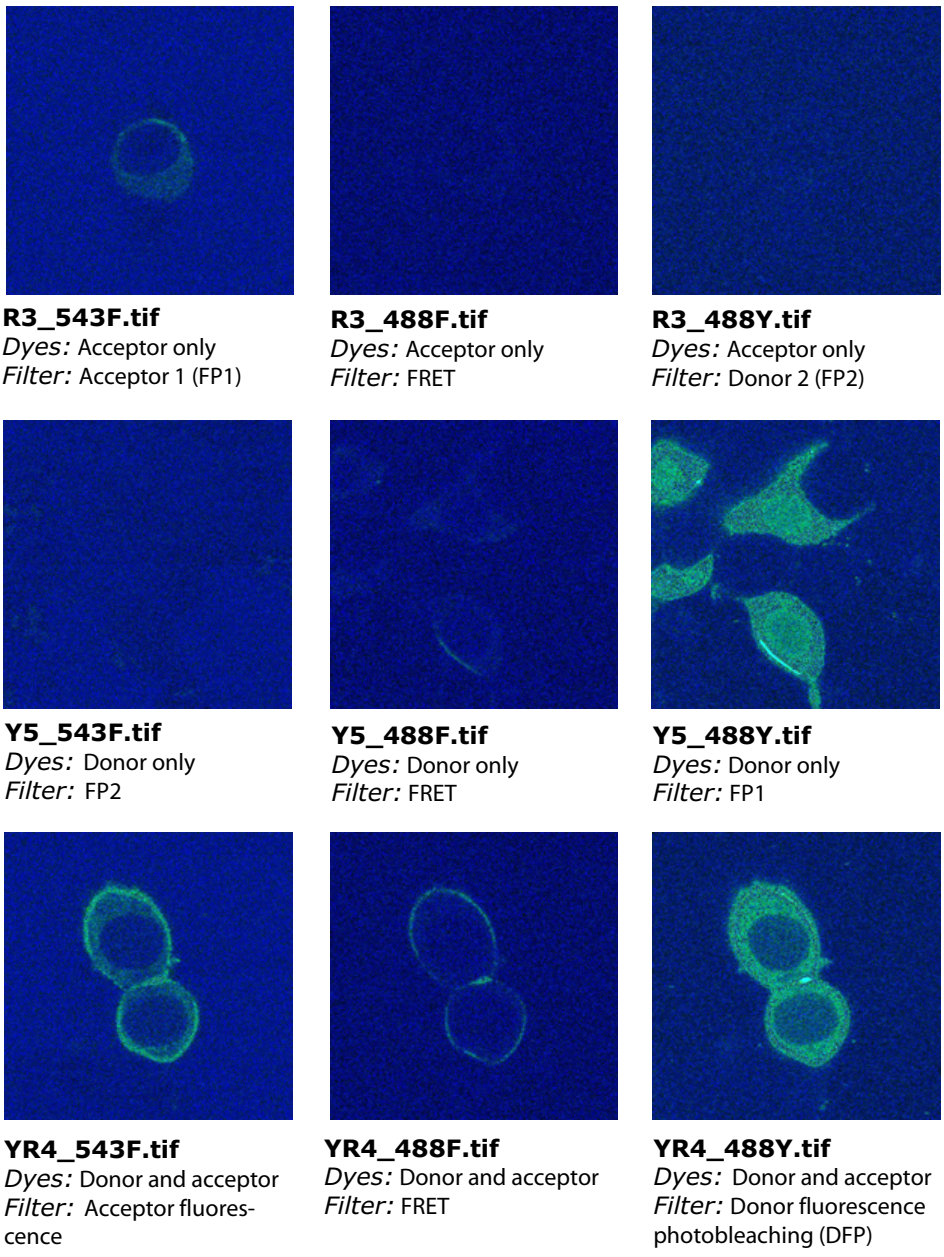

Figure 6. Nine example images used to calculate FRET efficiency

FRET Bleed Through Algorithm

The FRET Bleed Through algorithm requires the six types of images listed in Table 2. You need to run the algorithm twice: Once on the set of three images with acceptor dye only (the first three images listed in the table); and once on the set of three images with donor dye only (the last three images listed in the table).

Table 2. Six images used in applying the FRET Bleed Through algorithm

Sets of images	Example image*	Type of image
Acceptor only dyed images	R3_543F.tif	An acceptor only dyed image taken with an acceptor filter set
	R3_488F.tif	An acceptor only dyed image taken with a FRET filter set
	R3_488Y.tif	An acceptor only dyed image taken with a donor filter set
Donor only dyed images	Y5_543F.tif	An donor only dyed image taken with an acceptor filter set
	Y5_488F.tif	An donor only dyed image taken with a FRET filter set
	Y5_488Y.tif	A donor only dyed image taken with a donor filter set

*Refer to Figure 6 on page 14 to view these example images.

For both the acceptor dye only run and the donor dye only run, you need to create two or more VOI regions on the source image:

- **A background VOI**—The background region should have a smaller average intensity than the active region. Background VOIs appear in blue.
- **One or more active VOIs**—The area of the active regions are used for the bleed through calculations. Each different active VOI appears in a different nonblue color.

Caution: Do not use blue as an active VOI color.

You must create all of the VOIs on the source image, and the source image must be an image of the FP1 dye taken with a FP1 filter. That is, the source image must be either:

- An image with donor dye only taken with a donor fluorescent filter set
- An image with acceptor dye only taken with an acceptor fluorescent filter set

Note: Pixels that are saturated in any of the three images are excluded from all calculations.

Performing the Acceptor dye only run

Running the algorithm on images in which only the acceptor dye was used obtains the following values for each active VOI region:

- AFP (acceptor fluorescence photobleaching) to FRET bleed through value
- AFP to DFP (donor fluorescence photobleaching) bleed through value

The algorithm displays these values in the Output window.

Performing the Donor dye only run

Running the algorithm on a set of three images in which only the donor dye was used obtains the following values for each active VOI region, which are displayed in the Output window:

- DFP to FRET bleed through value
- DFP to AFP bleed through value

These values are displayed in the Output window.

Moving to FRET Efficiency

Once the four bleed through parameters (AFP to FRET bleed through, AFP to DFP bleed through value, DFP to FRET bleed through, and DFP to AFP bleed through) are obtained, you can then run the FRET Efficiency algorithm. You run the FRET Efficiency algorithm on the last set of three images in Figure 6 on page 14. This last set of images contain both donor and acceptor dyes.

Calculating the FRET and FP2 values

The FRET Bleed Through algorithm calculates the FRET and FP2 values using the following equations:

denom = mean(FP1 dye with FP1 filter active VOI) – mean(FP1 dye with FP1 filter background VOI)

FRET bleed-through = [mean(FP1 dye with FRET filter active VOI) – mean(FP1 dye with FP2 filter background VOI)]/denom

FP2 bleed-through = [mean(FP1 dye with FP2 filter active VOI) – mean(FP1 dye with FP2 filter background VOI)]/denom

Applying the FRET Bleed Through algorithm

The images used in the following instructions are the ones listed in Table 2 on page 15 and shown in the first two rows in Figure 6 on page 14.

Performing the acceptor dye only run

To use this algorithm, do the following:

1. Select File > Open to open the image file on which the acceptor dye and FP1 filter were used (example image: R3_543F.tif in Figure 6) (this image is the source image).
2. Select Algorithms > Microscopy > FRET Bleed Through (Figure 7).

   The FRET Bleed Through dialog box (Figure 9 on page 20) opens.

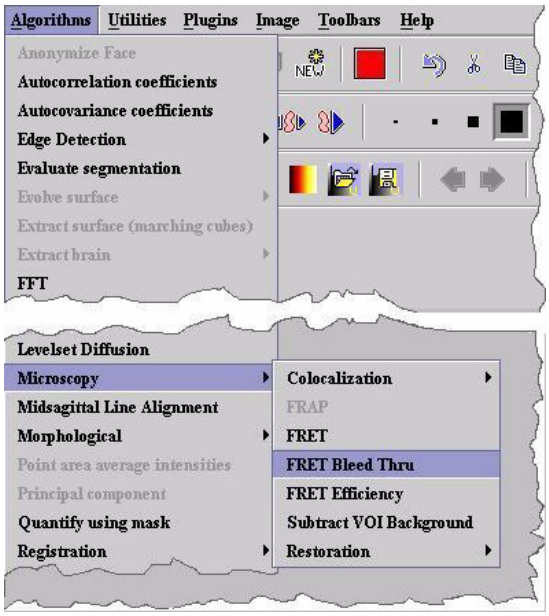

Figure 7. Algorithms > Microscopy > FRET Bleed Through command

Note: If images on which you're running the algorithm are in color, the FRET Bleed Through dialog box shown in Figure 8A appears. This dialog box includes a Channels group, which allows you to select the color channel on which to run the algorithm. If the images are in grayscale, the Channels group does not appear. Instead, the dialog box shown in Figure 8B opens.

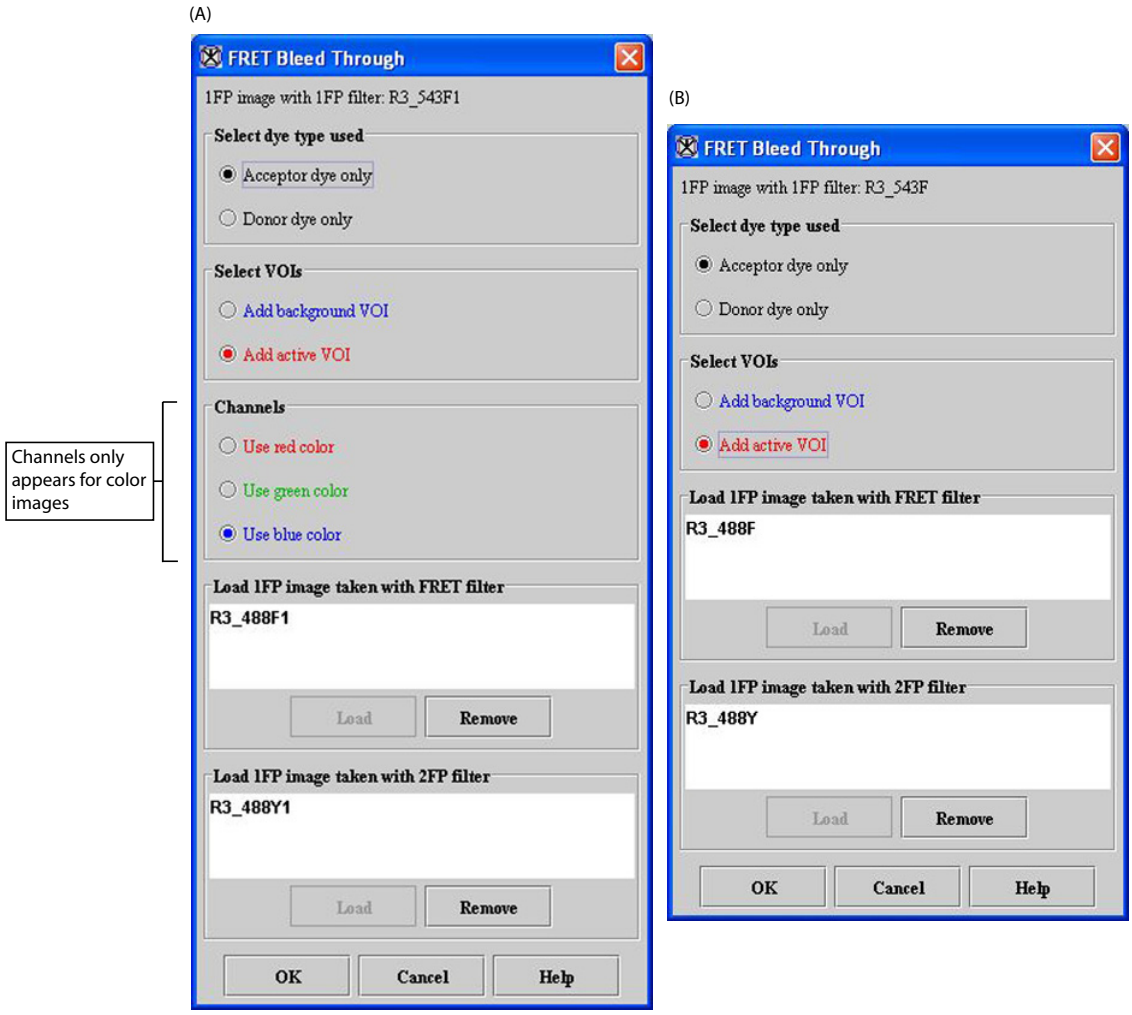

Figure 8. FRET Bleed Through dialog box for (A) color images and (B) grayscale images

3 Select Acceptor dye only in Select dye type used.

4 Select Add background VOI in Select VOIs.

5 Create a background VOI using the ellipse VOI, rectangle VOI, levelset VOI, or polyline VOI on the image in the background of the image. This VOI appears in blue (Figure 9).

6 Select Add active VOI in Select VOIs.

7 Create an active VOI using the ellipse VOI, rectangle VOI, levelset VOI, or polyline VOI in the foreground of the image, and adjust the contours of the VOI to eliminate any image background. This VOI appears in a *nonblue* color (Figure 9).

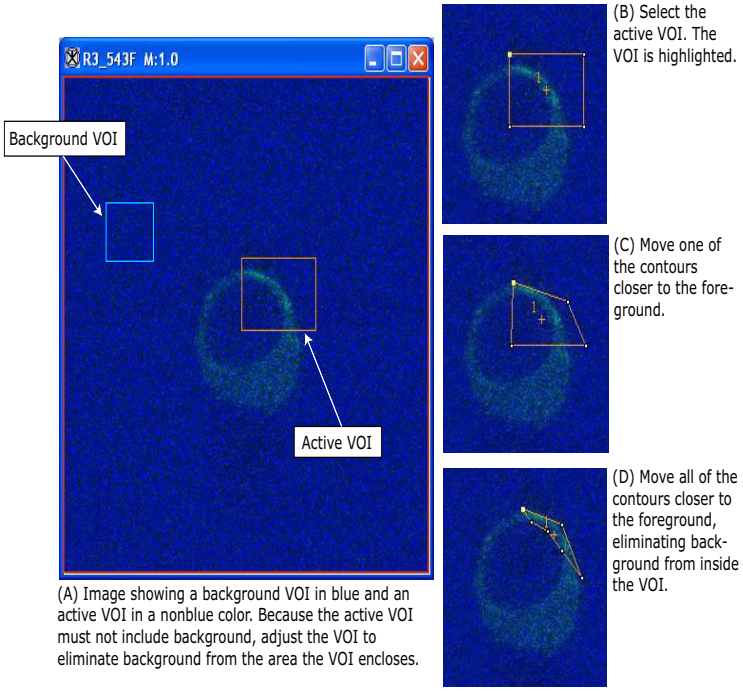

Figure 9. Acceptor only run: FP1 image with a background VOI in blue and an active VOI in orange (example image: R3_543F.tif)

8 Decide whether to create additional active VOIs.

If you decide against creating additional active VOIs, go to the next step. If you decide to create additional active VOIs, do the following:

a Select  , the New VOI icon.

b Create another active VOI using the ellipse VOI  , rectangle VOI  , levelset VOI  , or polyline VOI  , and adjust the contours of the VOI to eliminate any image background. This VOI appears in a nonblue color that is different from the color of the first active VOI.

Note: If you do not select  , the New VOI icon, MIPAV creates additional contours of the same color for the first active VOI and sums all areas within the contours of this VOI together.

9 Select Use red channel, Use green channel, or Use blue channel in Channels.

Remember: The Channels group only appears if the source image is in color.

10 Click Load under Load 1FP image taken with FRET filter box. The Open Image dialog box (Figure 10) appears.

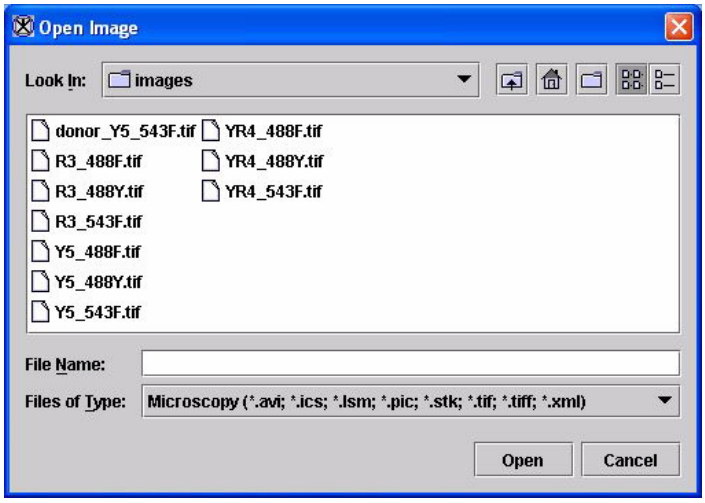

Figure 10. Open Image dialog box

11 Navigate to the directory where the image (example image: R3_488F.tif) is stored, and select the image. (Refer to Figure 6 on page 14 to see this image.)

12 Click Open. The name of the image appears in the Load 1FP image taken with FRET filter box (Figure 8 on page 19).

Note: The Remove button only becomes enabled when an image appears in the box (as it does in Figure 8 on page 19).

13 Click Load under the Load 1FP image taken with 2FP filter box. The Open Image dialog box (Figure 10) appears.

14 Navigate to the directory where the image (example image: R3_488Y.tif) (refer to Figure 6 on page 14 to see this image) is stored, and select the image.

15 Click Open. The name of the image appears in the Load 1FP image taken with 2FP filter box (refer to Figure 8 on page 19).

16 Click OK. The algorithm begins to run. When the algorithm finishes, it places the data in the Output window (Figure 11).

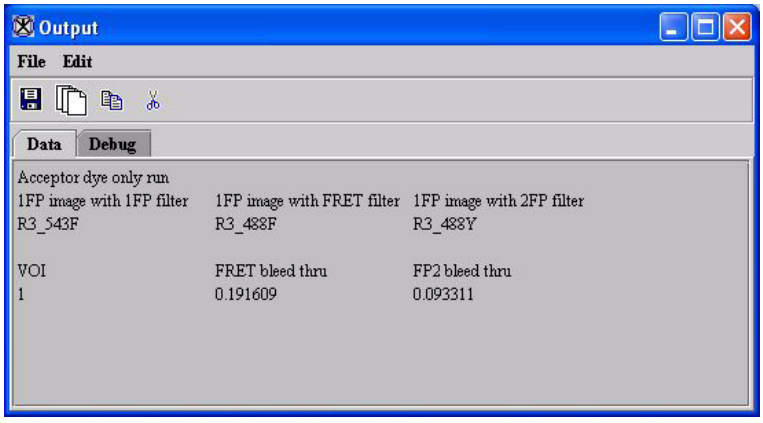

Figure 11. Results in the Output window from the Acceptor dye only run of the FRET Bleed Through algorithm

PERFORMING THE DONOR DYE ONLY RUN

1. Select File > Open to open the image file on which the donor dye and FP2 filter were used (example image: Y5_488Y.tif in Figure 6 on page 14) (this image is the source image).
2. Select Algorithms > Microscopy > FRET Bleed Through. The FRET Bleed Through dialog box (Figure 8 on page 19) opens.
3. Select Donor dye only in Select dye type used (Figure 12).

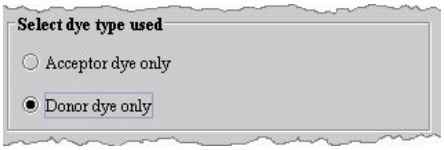

Figure 12. Donor dye only selection

4. Select Add background VOI in Select VOIs (Figure 13).

Figure 13. Add background VOI selection

5 Create a background VOI using the ellipse VOI, rectangle VOI, levelset VOI, or polyline VOI on the image in the background of the image. This VOI appears in blue (Figure 15).

6 Select Add active VOI in Select VOIs.

Figure 14. Add active VOI selection

7 Create an active VOI using the ellipse VOI, rectangle VOI, levelset VOI, or polyline VOI in the foreground of the image, and adjust the contours of the VOI to eliminate any image background. This VOI appears in a nonblue color (Figure 15).

8 Decide whether to create additional active VOIs.

If you decide against creating additional active VOIs, go to the next step. If you decide to create additional active VOIs, do the following:

a Select NEW, the New VOI icon.

b Create another active VOI using the ellipse VOI, rectangle VOI, levelset VOI, or polyline VOI, and adjust the contours of

the VOI to eliminate any image background. This VOI appears in a nonblue color that is different from the color of the first active VOI.

Note: If you do not select NEW, the New VOI icon, MIPAV creates additional contours of the same color for the first active VOI and sums all areas within the contours of this VOI together.

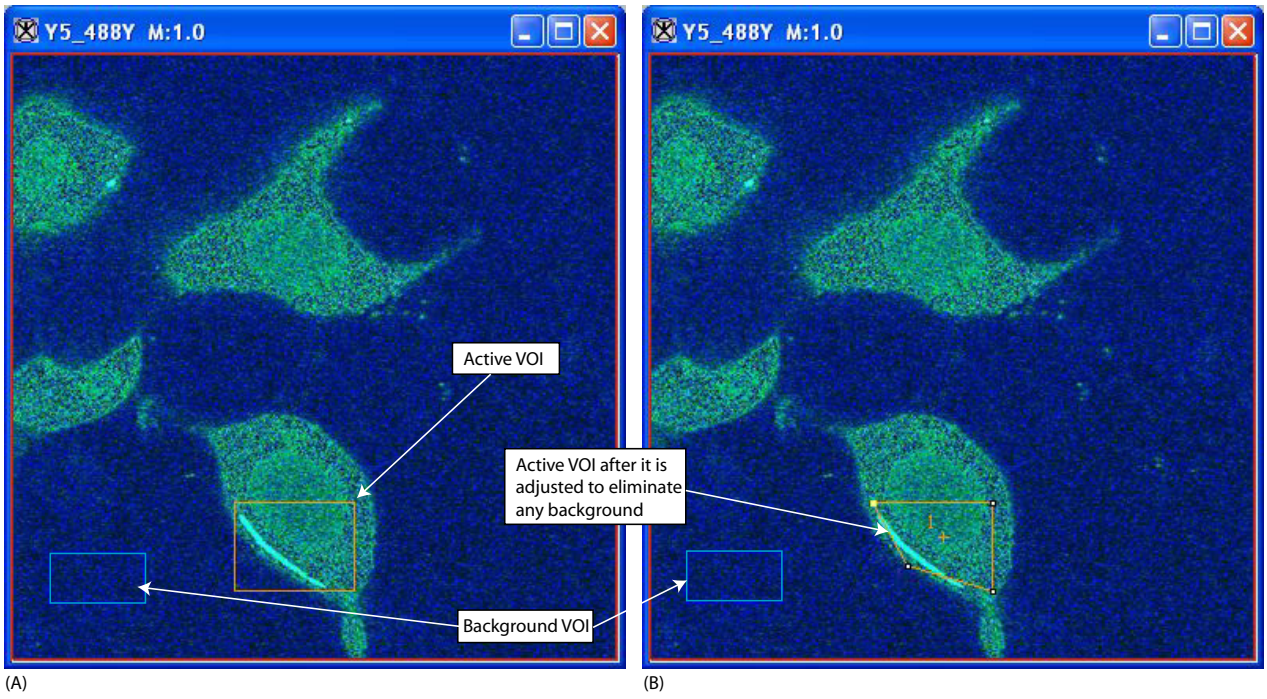

Figure 15. Donor only run: FP1 image with a background VOI in blue and an active VOI in orange (example image: Y5_488Y.tif)

9 Select Use red channel, Use green channel, or Use blue channel in Channels if the images are in color.

Remember: The Channels group only appears if the source image is in color.

10 Click Load under Load 1FP image taken with FRET filter box. The Open Image dialog box (Figure 16) appears.

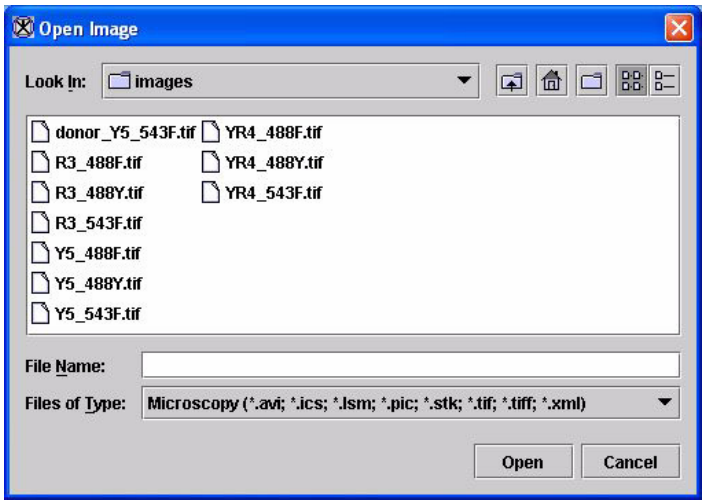

Figure 16. Open Image dialog box

11 Navigate to the directory where the image (example image: Y5_488F.tif) is stored, and select the image. (Refer to Figure 6 on page 14 to see this image.)

12 Click Open. The name of the image appears in the Load 1FP image taken with FRET filter box (Figure 17).

Note: The Remove button becomes enabled when an image appears in the box.

13 Click Load under the Load 1FP image taken with 2FP filter box. The Open Image dialog box (Figure 16) appears.

14 Navigate to the directory where the image (example image: Y5_543F.tif) is stored, and select the image. The name of the image appears in the Load 1FP image taken with 2FP filter box (Figure 17).

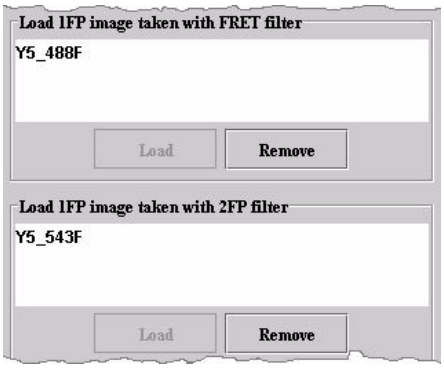

Figure 17. The loaded images in the FRET Bleed Through dialog box

15 Click OK. The algorithm begins to run. When the algorithm finishes, it places the data in the Output window (Figure 18).

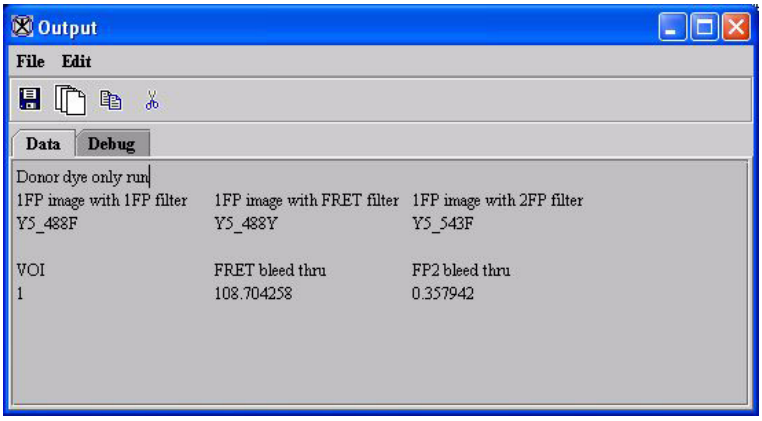

Figure 18. Results in the Output window from the Donor dye only run of the FRET Bleed Through algorithm

Select dye type used	**Acceptor dye only**—The dye used on images that are processed through the acceptor-dye run of the FRET Bleed Through algorithm. As a result of this run, the algorithm calculates the AFP to FRET bleed through value and the AFP to DFP bleed through value.
	Donor dye only—The dye used on images that are processed through the donor dye only run of the FRET Bleed Through algorithm. During this run, the algorithm calculates the DFP to FRET bleed through value and the DFP to AFP bleed through value.
Select VOIs	**Add background VOI**—Indicates a region in the image that is the background. Using the ellipse VOI, rectangle VOI, levelset VOI, or polyline VOI, you may create one background VOI. The background VOI appears in blue.
	Add active VOI—Indicates one or more regions in the image that are in the foreground. You may create the first active VOI using the ellipse, rectangle, levelset, or polyline VOI. To create more active VOIs, first select New VOI and then select ellipse, rectangle, levelset, or polyline VOI. Each active VOI appears in a different, nonblue color.
Channels	**Use red color**—Selects the red channel for the FRET analysis.
	Use green color—Selects the green channel for the FRET analysis.
	Use blue color—Selects the blue channel for the FRET analysis.
Load FP1 image taken with FRET filter	Lists the FRET filtered image that you selected after clicking Load.

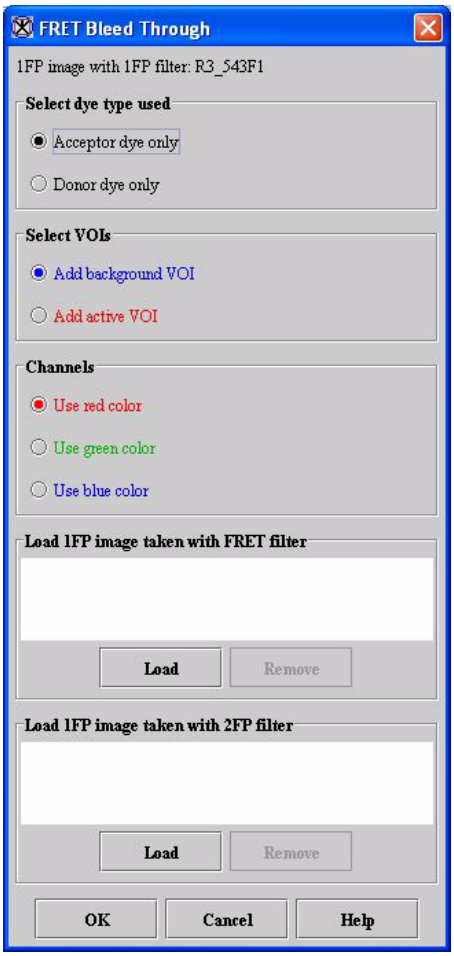

Figure 19. FRET Bleed Through dialog box

Load	Allows you to load an FP1 image that was taken with the FRET filter. Clicking this button causes the Open Image dialog box—from which you can select an image—to appear.
Remove	Removes the image that is selected in the Load FP1 image taken with FRET filter box.
Load FP1 image taken with FP2 filter	Lists the FP2 filtered image that you selected after clicking Load.
Load	Allows you to load an FP1 image that was taken with the FP2 filter. Clicking this button causes the Open Image dialog box—from which you can select an image—to appear.
Remove	Removes the image that is selected in the Load FP2 image taken with FP2 filter box.
OK	Applies the algorithm according to the specifications in this dialog box.
Cancel	Disregards any changes that you made in this dialog box and closes the dialog box.
Help	Displays online help for this dialog box.

Figure 19. FRET Bleed Through dialog box (continued)

FRET Efficiency algorithm

This FRET Efficiency algorithm uses the four bleed through parameters obtained from running the FRET Bleed Through algorithm. It requires a set of three images:

Sets of images	Example image*	Type of image
Donor and acceptor dyed images	YR4_543F.tif	An acceptor- and donor-dyed image taken with an acceptor filter set
	YR4_488F.tif	An acceptor- and donor-dyed image taken with a FRET filter set
	YR4_488Y.tif	An acceptor- and donor-dyed image taken with a donor filter set

*Refer to Figure 6 on page 14 to view these example images.

- Donor- and acceptor-dyed image taken with a donor fluorescence photobleaching (DFP) filter
- Donor- and acceptor-dyed image taken with a FRET filter

- Donor- and acceptor-dyed image taken with an acceptor fluorescence photobleaching (AFP) filter

The algorithm assumes the presence of two or more VOI regions on the source image:

- **A background VOI**—The background region has a smaller average intensity than the active region. Background VOIs appear in blue.
- **One or more active regions**—The area of the active regions, or VOIs, are used for the efficiency calculations. Each different active VOI appears in a different nonblue color.

Caution: Do not use blue as an active VOI color.

Note: Pixels with saturation values in any of the three images are excluded from all calculations.

For each active VOI region, this algorithm outputs the FRET efficiency and the adjusted donor and adjusted acceptor intensities.

Applying the FRET Efficiency algorithm

The images used in these instructions were the ones shown in Table 2 on page 15 and shown in the last row in Figure 6 on page 14. You only need to run the FRET Efficiency algorithm once on the images.

To use this algorithm, do the following:

1. Select File > Open to open the image file on which both the acceptor dye and donor dyes were used and DFP filter were used (example image: YR4_488Y.tif).
2. Select Algorithms > Microscopy > FRET Efficiency. The FRET Efficiency dialog box (Figure 20) opens.

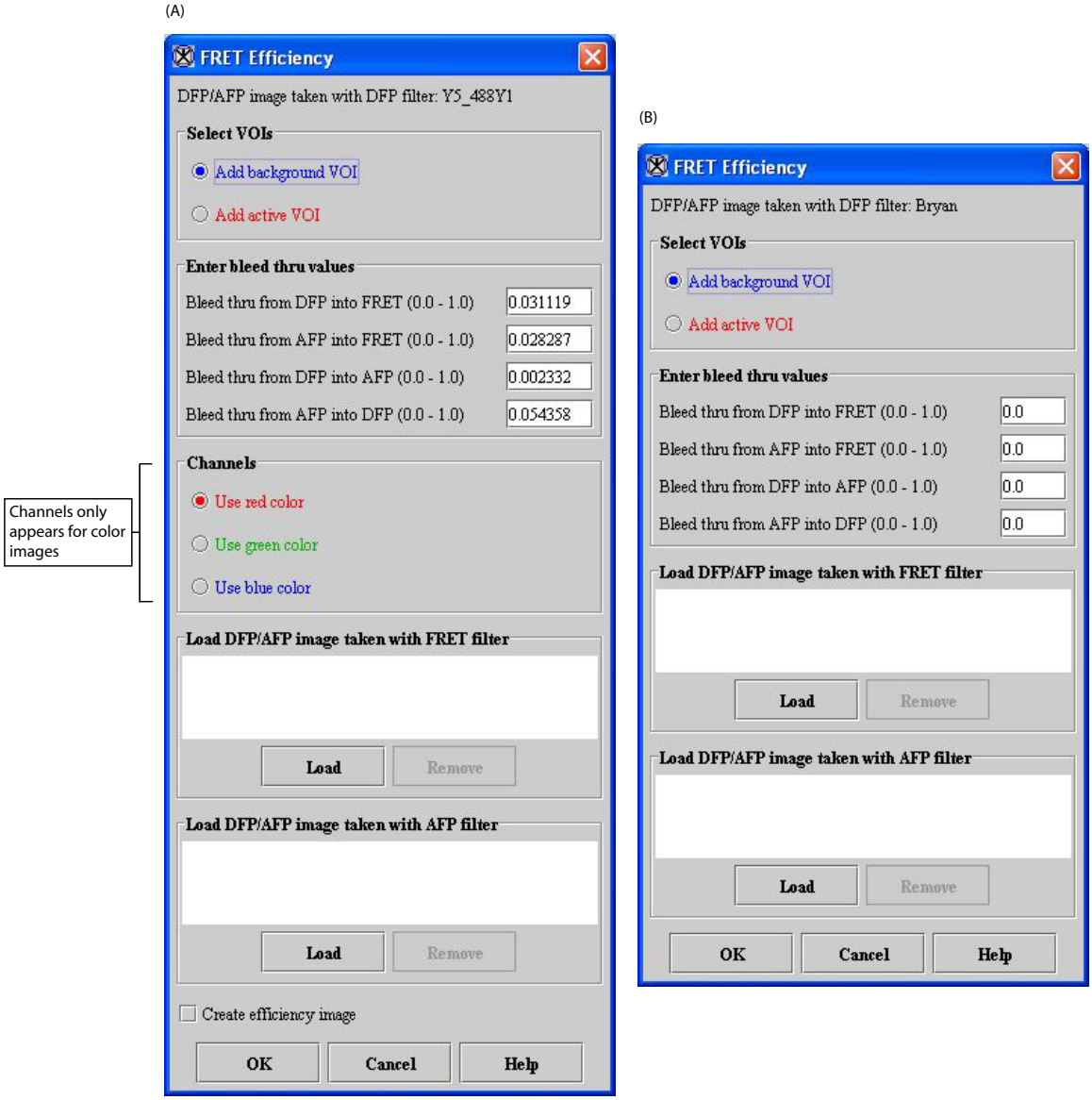

Figure 20. FRET Efficiency dialog box (A) color images and (B) grayscale images

3 Select Add background VOI in Select VOIs on the FRET Efficiency dialog box.

4 Create a background VOI on the image in the background of the image. This VOI appears in blue lines.

5 Select Add active VOI in Select VOIs on the FRET Efficiency dialog box.

6 Create an active VOI using the ellipse VOI  , rectangle VOI  , levelset VOI  , or polyline VOI  in the foreground of the image, and adjust the contours of the VOI to eliminate any image background. This VOI appears in a nonblue color (Figure 15).

7 Decide whether to create additional active VOIs.

If you decide against creating additional active VOIs, go to the next step. If you decide to create additional active VOIs, do the following:

a Select  , the New VOI icon.

b Create another VOI using the ellipse VOI  , rectangle VOI  , levelset VOI  , or polyline VOI  , and adjust the contours of the VOI to eliminate any image background. This VOI appears in a nonblue color that is different from the color of the first active VOI.

Note: If you do not select  , the New VOI icon, MIPAV creates additional contours of the same color for the first active VOI and sums all areas within the contours of this VOI together.

Note: MIPAV prefills the Enter bleed through values. However, if the correct bleed through values are not automatically entered, type them into the fields.

8 Click Load under Load DFP/AFP image taken with FRET filter box. The Open Image dialog box appears.

9 Navigate to the directory where the image (example image: YR4_488F.tif) is stored, and select the image.

10 Click Open. The name of the image appears in the Load DFP/AFP image taken with FRET filter box.

Note: The Remove button becomes enabled when an image appears in the box.

11 Click Load under the Load DFP/AFP image taken with AFP filter box. The Open Image dialog box appears.

12 Navigate to the directory where the image (example image: YR4_543F.tif) is stored, and select the image. The name of the image appears in the Load DFP/AFP image taken with AFP filter box.

13 Click OK. MIPAV places the data from this second part of the procedure in the Output window (Figure 11).

Select VOIS	**Add background VOI**—Indicates a region in the image that is the background, which has a smaller average intensity than the active region. Using the ellipse VOI, rectangle VOI, levelset VOI, or polyline VOI, you create a background VOI. The background VOI appears in blue. **Add active VOI**—Indicates one or more regions in the image that are in the foreground. You may create the first active VOI using the ellipse VOI, rectangle VOI, levelset VOI, or polyline VOI. To create more active VOIs, select New VOI. Each different active VOI appears in a different nonblue color.
Bleed through from DFP into FRET (0.0–1.0)	Indicates the bleed through value from DFP into FRET.
Bleed through from AFP into FRET (0.0–1.0)	Indicates the bleed through value from AFP into FRET.
Bleed through from DFP into AFP (0.0–1.0)	Indicates the bleed through value from DFP into AFP.
Bleed through from AFP into DFP (0.0–1.0)	Indicates the bleed through value from AFP into DFP.

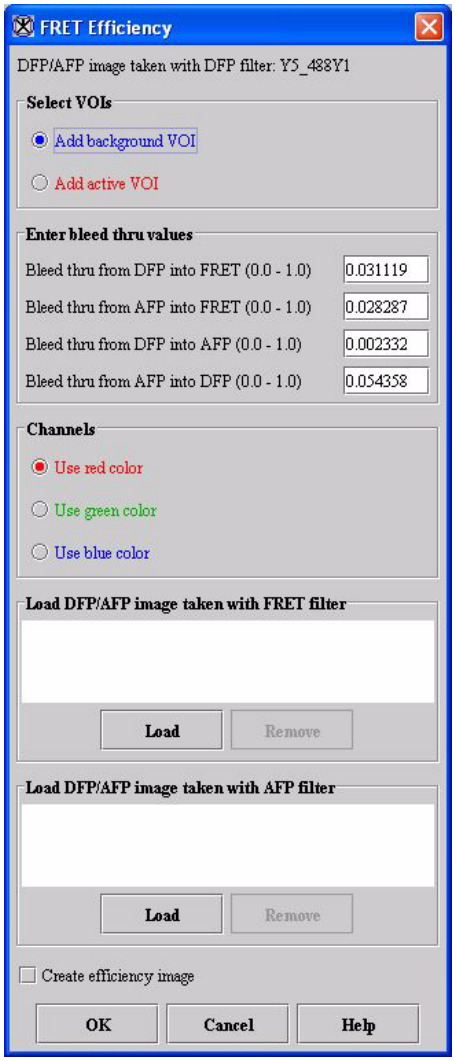

Figure 21. FRET Efficiency dialog box

Channels	**Use red color**—Selects the red channel.
	Use green color—Selects the green channel.
	Use blue color—Selects the blue channel.
Load DFP/AFP image taken with FRET filter	Lists the FRET filtered image that you selected after clicking Load.
Load	Allows you to load a DFP/AFP image that was taken with the FRET filter. Clicking this button causes the Open Image dialog box—from which you can select an image—to appear.
Remove	Removes the image that is selected in Load DFP/AFP image taken with FRET filter.
Load DFP/AFP image taken with AFP filter	Lists the AFP filtered image that you selected after clicking Load.
Load	Allows you to load a DFP/AFP image that was taken with the AFP filter. Clicking this button causes the Open Image dialog box—from which you can select an image—to appear.
Remove	Removes the image that is selected in Load DFP/AFP image taken with AFP filter.
OK	Applies the algorithm according to the specifications in this dialog box.
Cancel	Disregards any changes that you made in this dialog box and closes this dialog box.
Help	Displays online help for this dialog box.

Figure 21. FRET Efficiency dialog box (continued)

IMAGE TYPES

You apply both algorithms to three 2D images.

NOTES

Only one user-selected color can be used from a color image.

Both algorithms port the MATLAB routines provided by Dr. Stephen Lockett.

REFERENCES

Refer to the following references for more information about FRET.

Gordon, Gerald W., Gail Berry, Xiao Huan Liang, Beth Levine, and Brian Herman. "Quantitative Fluorescence Resonance Energy Transfer Measurements Using Fluorescence Microscopy." *Biophysical Journal* 74(May 1998):2702–2713.

Kenworthy, Anne K. "Imaging Protein-Protein Interactions Using Fluorescence Energy Transfer Microscopy." *Methods,* 24(2001):289–296.