Introduction:

This tutorial describes and explains the steps necessary in analyzing Sensitized-Emission Fluorescence Resonance Energy Transfer (FRET) images taken on a wide field microscope system. Both single time point and time-lapse series methods are covered. The concepts can also be applied to many other types of quantitative analysis of microscopy images. ImageJ macros and plugins referenced here can be downloaded from the ImageJ web site http://rsb.info.nih.gov/ij/ or the MSL webpage http://www.med.unc.edu/microscopy/. An ImageJ macro for analyzing single-chain FRET molecules is available here as well as documentation on how to use the macro.

The Digital Image:

A digital FRET image is just a bunch of numbers: It is so many picture elements (pixels) across by so many pixels down. Each pixel is a number. If a pixel has been digitized to 8-bits (2^8) it will be a number between 0 and 255; 12-bits (2^12) will be 0 to 4095; 16-bits (2^16) 0 to 65535. You get the idea. Each number represents a specific shade of gray with 0 being pure black and the maximum value being pure white.

What’s In the Numbers?

The number in each pixel represents the amount of light the camera detected at that x, y location. There are a number of things that influence this light in addition to the specimen. Sorting these out and removing or correcting them must be done in order to get a true FRET image. The trouble-makers are: Camera Noise, Uneven Illumination, Focus Drift, Non-Specific Background, Photobleaching and Spectral Bleed-through.

From the outset, I should say that, when images are being acquired, you should take pains to avoid “saturated” pixels (pixels with the maximum value) in the important parts of your images. You don’t really know what the real value should be in a saturated pixel, so keep the exposure time short enough to keep all the important pixels on scale.

Fixing the Numbers:
Let’s take these trouble-makers one at a time and describe what the problem is, what the fix is, and what problems the fix itself may cause.

**Camera Dark Noise –**

**PROBLEM:** The light detector of a digital camera is an array of pixels. When no light is shining on the array the pixels still have a small number of photo-electrons in them due to thermal noise and other stuff. So an image from the camera is not completely black, each pixel presenting a small signal that varies over time. This camera noise is still present when the camera is illuminated. All that this noise does is make an image slightly brighter and noisier than it otherwise would be. Confocal microscopes do not have this problem.

**SOLUTION:** The fix for this is first to take a series of images with all the optics in place for the Donor image acquisition, but with the lamp shutter closed, and a second series with the FRET optics in place but with the lamp shutter closed. Then, an averaged image is made from each of these series and it is subtracted from each corresponding image series. **Caveat:** Stray light can enter the camera through the microscope’s eyepieces and objective lens during acquisition of the dark series, thus the room should be dark and the eyepieces covered.

**TROUBLE:** Simple image averaging can be problematic if there is a chance that the added up images wind up having saturated pixels. However, the pixel values in the camera noise image should be so low that this is unlikely, especially if averaging about 20 images and digitizing to 12 or 16 bits. (An ImageJ plug-in for averaging a stack of 16 bit images, StackAverage_16, is available on the MSL web page).

**Uneven Illumination –**

This non-uniformity has many causes. It can be minimized, but not eliminated. Confocal systems do not have this problem.

**PROBLEM:** In wide field imaging where a lamp (such as a Hg arc lamp) is utilized, the illumination is not uniformly bright across the field of view. Typically the center is brighter than the edges and the variations are not symmetrical. Thus, a cell in the center would appear brighter than an identically stained cell at the edge. Under this condition, it would be impossible to compare one cell to another or possibly even one part of a cell to some other part of the same cell.

**SOLUTION:** The problem can be fixed by dividing the experimental image by an image of a uniformly stained specimen made using the same lighting conditions. This correction image is called a flat field or blank field or shade correction image. By doing this, pixels in the brighter areas of the experimental image will be divided by brighter pixels in the flat field image and pixels in the darker areas will be
divided by darker pixels in the flat field image thus leveling the uneven contribution from the illumination. The actual equation used can be slightly more involved. The equation used by the ImageJ plug-in Shading Corrector for instance is \( I = I \ast (M/BF) \) where \( I \) is the pixel in the image being corrected, \( M \) is the mean of the entire flat field image and BF is the pixel in the flat field image.

**Caveat:** The flat field specimen should be completely uniform and should not photobleach. There are very few such flat field specimens. One is a saturated solution of fluorescein but this will be very bright and will require the use of neutral density filters to get the exposure into a proper range.

**DETAILS:** The flat field images are produced by making a series of images of a uniformly stained specimen using the same imaging conditions as for the experimental specimen – both Donor and FRET conditions should have an image set. The exposure should be adjusted to give pixel values just above the camera dark noise level in the darkest parts of the image. Next, averaged images are generated from these series. These then are the flat field or shade correction images.

This solution sounds reasonable, but there can be problems:

Let’s start with averaging a set of images.

**TROUBLE:** The most common way of averaging is to add up the images to create a sum image and divide the sum image by the number of images. A problem occurs if a pixel in the sum image reaches the maximum value for the image bit depth before the last image is added. The real value of that pixel becomes unknown. For example if a pixel is at 32767 in 16-bits the maximum value is reached after only two images are added together. If 20 images are averaged, that pixel would end up being much darker than it should be in the final averaged image.

**SOLUTION:** Keeping the pixel intensity of the darker parts of the flat field images just above the camera noise level will minimize this problem. Most image acquisition software has a method for monitoring pixel intensity during acquisition.

A different averaging method that minimizes this problem is Kalman averaging. In this method, two images are added together and the result divided by 2. Then another image is added to the result and the new result is divided by 2. This continues for the number of images to be averaged. This method tends to weight the average toward the later images, but if there is little or no photobleaching of the uniform standard during image acquisition, then this method is fine. A 16-bit Kalman averaging plug-in is available on the MSL web-page.

Now let’s take on what happens when you divide one image by another.

**TROUBLE:** Image division is used to make the flat field correction (and to do the final FRET calculation). In so doing, it is very likely that a pixel of smaller value will be divided by a pixel of a larger value giving a fractional result. In integer arithmetic in a computer this fractional value would be set to 0. One method of dealing with
this is to add an offset to the numerator images to insure that no results are just fractions. What this offset should be can vary and there is a chance that the addition of the offset could saturate some pixel values.

**SOLUTION:** The best way to do image division in a computer is to use 32-bit floating-point arithmetic. When an integer image is converted to 32-bit floating-point in a computer, the values become signed decimal values. Division can be done to 32-bit precision, which is greater than the bit depth of most cameras. The ImageJ plug-in Shading Corrector uses this method.

**TROUBLE:** In ImageJ, in a straight conversion of a 32-bit floating-point image to an integer image, any 32-bit value greater than 1.0 will be converted to 1 thus all precision will be lost. This conversion requires mapping fractional and possibly negative values back into an unsigned integer scale.

**SOLUTION:** Once calculations are finished using 32-bit floating-point arithmetic, pixels comprising an image can be linearly mapped into the decimal range between 0 and 1 using ImageJ’s Process/Enhance Contrast function. This range will be correctly converted to 16-bit or 8-bit unsigned integer values by ImageJ’s Image/Type function. Any image processing program that does calculations in 32-bits must deal with this conversion.

**TROUBLE:** When pixel values are below the average background value, an incorrect pixel value could be created by the division. For example say in a dark part of an image pair one pixel (in the numerator image) was much above background do to noise while its counterpart (in the denominator image) was in the lower part of the noise range. That pixel in the resulting ratio image would show a false positive signal.

Say the low value is 1 and the high value is 6, the result of the division is 6 and the difference in the values is 6. On the other hand if the low value was 10, to get the same result the upper value would be 60 and the difference between them is 50. It is less likely that an incorrect value will result form the division when the numbers are large compared to when they are small because noise is a much larger component of small values than it is of large values and noise is a random variable.

**SOLUTION:** Picking a threshold below which all values are set to 0 is a workable fix for this problem. This can be done by masking.

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Focus Drift –

**PROBLEM:** If you are making a time-lapse series of images you hope that the microscope remains in focus for the entire time. But no! Chances are that the focus will change over time due to more factors than I care to name.
**SOLUTION:** The only real solution is to use an active focus correction system that checks and corrects the focus at every time point and every location that is being imaged. Some systems use an autofocus that adjusts focus based on some image parameter such as contrast. These systems are generally quite slow and can be confused by junk in the field of view that is not in the plane of the cells. Other systems adjust focus based on keeping the distance between the objective lens and the coverglass constant. These systems are quite fast and accurate.

**Non-Specific Background –**

**PROBLEM:** The specimen, the microscope, the environment can introduce an amount of non-specific background light from many sources. This background light will make the image brighter than it should be.

**SOLUTION:** Non-specific Background is removed by subtracting the average value of an area of the image in which there is no specimen. This is repeated for each image in a series of images. This procedure is done after the image series has been corrected for uneven illumination and camera noise.

**TROUBLE:** Problems arise in time series because the background may not be uniform over time. For example, in a time series a fluorescent object could drift into the background area chosen for correction. Thus each image of a series should be checked before a background region is chosen.

**Image Registration –**

**PROBLEM:** FRET requires at least two filter sets. If the optics in the filter sets are not perfectly aligned with one another there can be a shift in the position of objects in one image relative to its counterpart. This is obviously not good if the images are to be divided into one another on a pixel-to-pixel basis. Strange edge artifacts that stretch off in the direction of the image shift will appear around cells.

**SOLUTION:** In microscopy, the most common type of misregistration is a shift in the x or y direction or in both x and y. This type of distortion is called Translation. The next most common type of misregistration adds a rotation to the x and y translation and is called Rigid Body distortion. There are other types of distortion such as change of scale and warping that typically do not occur in microscopy. Translation and Rigid Body distortion can be manually corrected by using software that lets the user nudge the source image around relative to the target image. As you can imagine, this is quite slow and tedious. Better to use software that is designed for this purpose and that works automatically.

The ImageJ plug-in TurboReg offers several choices for correcting image misregistration. It can be used as a standalone program or it can be called from
another ImageJ plug-in or macro. The ImageJ macro Biosensor FRET uses this plug-in to register two time-lapse series of images. Other software packages, such as MatLab also offer image registration methods.

Photobleaching –

PROBLEM: A fluorescent specimen loses intensity as it is being exposed to excitation light. This can be minimized by good technique but not eliminated. In a time series where the images are compared to one another, the loss of intensity makes comparisons difficult.

SOLUTION: This problem can be minimized by using neutral density filters to limit the excitation intensity. Photobleaching is less with lower light intensity and longer exposure time than the other way round. Mathematically, loss of intensity is compensated by multiplying each image by an appropriate correction factor. This “normalization” is done by correcting all images to the first image in the series.

TROUBLE: The Donor and FRET signals may photobleach at different rates and the rate of photobleaching is not linear over time but roughly follows a double exponential curve. (6)

SOLUTION: The differential bleaching rate can be compensated by using the ratio image (FRET / Donor) to calculate the correction factor. This image should be made from the cells only, excluding everything else in the image. Thus a mask must be made to retain the cell data while setting the rest of the image to 0. Then the calculation (FRETmean n/Donor mean n) / (FRETmean 1/Donor mean 1) is used to establish the photobleach curve. Next a double exponential curve is fit to this data and 1/curve value is calculated as the correction value for each image. Finally each image of the raw ratio image set of FRET / Donor is multiplied by its corresponding correction factor to finish the photobleach correction. (This correction is contained in the ImageJ macro Biosensor FRET.)

Spectral bleed-through –

PROBLEM: In sensitized emission FRET there is always some amount of light in the FRET channel that is not due to energy transfer between the donor and the acceptor. Donor emission bleeding through the acceptor filter into the FRET channel and excitation of the acceptor by the donor excitation are the main culprits.

The spectrum in figure 1 shows the excitation (dashed) and emission (solid) spectra of CFP and YFP. You can see that the CFP emission (solid blue) extends into the YFP emission (solid red). Suppose you have some CFP only labeled cells. If you use CFP excitation and look at the cells through a YFP emission filter you can see this bleed-through. No filter set can completely eliminate it. When there is FRET between CFP
and YFP, the amount of CFP emission is greatly reduced because the energy transfer between the CFP excited state and the YFP molecule is non-radiative. But, there is always some CFP emission even when there is FRET. So we must correct for the CFP emission bleed-through into the FRET channel. How to do this? There are several approaches and the one presented here is called ratio imaging.

![Figure 1. Excitation (dashed) and emission (solid) curves for CFP (blue) and YFP (red) showing the bleed-through of CFP emission into YFP emission and YFP excitation by CFP excitation.](image)

**SOLUTION:** Let’s define the “CFP filter set” as excitation of CFP and detection of CFP, the “YFP filter set” as excitation of YFP and detection of YFP, and the “FRET filter set” as excitation of CFP and detection of YFP.

Say you have a CFP only sample. You look around using your standard CFP filter set and see lots of cells of different brightness. Next you measure the brightness of a bunch of these cells each one with your standard CFP filter set and also with your FRET filter set to get the bleed-through. You will find that the ratio of FRET / CFP is nearly constant no matter how bright or dim a cell is. This is because the percentage of bleed-through is really a function of the filters, microscope optics, and camera system. Let’s call this the “SBT donor ratio”.

\[
\text{SBT donor} = \frac{\text{FRET}}{\text{CFP}}
\]

Now if we have a positive FRET sample, we should be able to measure the amount of real FRET by calculating this ratio in the FRET sample and measuring how much it differs from the SBT ratio of the CFP sample. *1

But wait! There is the second bleed-through problem: excitation of YFP by the CFP excitation. This will contribute extra YFP signal to the FRET channel.

In Figure 1, you can see that the CFP excitation curve (blue dash) just overlaps the YFP excitation curve (red dash). What to do?

Say you have a YFP only sample with many cells of differing intensity as seen through your standard YFP filter set. If you measure intensities and make ratios as before only this time using FRET filter set value / YFP filter set value you will again
get a nearly constant value no matter how bright or dim the cell is. Call this value the “SBT acceptor ratio”.

\[
\text{SBT acceptor} = \frac{\text{FRET}}{\text{YFP}}
\]

Now you can combine this with the SBT donor ratio and fully correct the FRET signal.

What we need now is a proper equation for making the correction. This has been discussed at length in the literature (1, 2, 3, 4, 5). The following consensus has emerged:

\[
\text{FRET} = \text{Intensity FRET} - (\text{SBT donor} \times \text{Intensity donor}) - (\text{SBT acceptor} \times \text{Intensity acceptor})
\]

Finally, this equation will give different FRET values for cells that have different CFP and YFP expression levels. This may not be desirable when comparing one cell to another within an experiment or when comparing different experiments. Several methods are available for normalizing the FRET value: against the acceptor intensity, the donor intensity, or both (3). Thus the above equation becomes:

\[
\text{FRET} = \frac{\text{Intensity FRET} - (\text{SBT donor} \times \text{Intensity donor}) - (\text{SBT acceptor} \times \text{Intensity acceptor})}{N}
\]

Here \(N\) is the normalization method and can be 1) the acceptor intensity, 2) the donor intensity, 3) donor intensity \(\times\) acceptor intensity, 4) square root (donor intensity \(\times\) acceptor intensity). The more robust of these is method 4. Method 1 does not account for variations in the donor intensity, method 2 does not account for variations in the acceptor intensity, method 3 works well only when the donor and acceptor intensities are about the same.

Keep in mind that these calculations are made between a donor and the corresponding FRET image on a pixel-to-pixel basis.

The ImageJ plug-in Pix-FRET by Feige et.al. calculates FERT by any of these methods on a pixel-to-pixel basis.

**EXCEPTION:** There is one situation in which the bleed-through might be ignored and that is when the concentration of CFP to YFP is known to be exactly equal as in intermolecular FRET where the donor and acceptor fluorophores are attached to the
same molecule. You have seen that the proportion of SBT in the FRET signal is nearly constant regardless of the concentration of CFP and YFP (it is dependant on the imaging system) whereas the absolute amount of SBT in the FRET signal will vary with the concentration of CFP and YFP. In intermolecular FRET, where donor and acceptor are at 1:1 concentration, FRET can be determined simply by the acceptor / donor ratio because here the absolute value of SBT, that normally varies with donor and acceptor concentration, is also constant.

*1 The SBT ratio is nearly linear for wide field FRET using scientific grade digital cameras. It has been shown that it may not be linear for confocal systems using PMTs. In that case the SBT ratios can be fit to an exponential curve to improve accuracy. – See the PIX-FRET plug-in and its documentation. (5)

**Conclusion:**

FRET is a very powerful tool, but it does require proper image acquisition, correction and processing. I hope the above tutorial and the accompanying ImageJ plug-ins and macros will help those who are just getting started.

1) YOUVAN, D.C, ET.AL. 1997. CALIBRATION OF FLUORESCENCE RESONANCE ENERGY TRANSFER IN MICROSCOPY USING GENETICALLY ENGINEERED GFP DERIVATIVES ON NICKEL CHELATING BEADS. BIOTECHNOLOGY. 3:1-18


3) XIA, Z., LIU, Y. 2001, RELIABLE AND GLOBAL MEASUREMENT OF FLUORESCENCE RESONANCE ENERGY TRANSFER USING FLUORESCENCE MICROSCOPES. BIOPHYSICAL JOURNAL 81, 2395-2402

4) SCHAUFFLE, F., DEMARCO, I., DAY, R. 2005: FRET IMAGING IN THE WIDE-FIELD MICROSCOPE, IN MOLECULAR IMAGING FRET MICROSCOPY AND SPECTROSCOPY ED PERIASAMY, A. DAY, R. OXFORD UNIVERSITY PRESS 2005
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