FRETTY

User guide for calculating FRET using spectral unmixing with the ImageJ plugin 'FRETTY'

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Reference: Quantitative Förster resonance energy transfer efficiency measurements using simultaneous spectral unmixing of excitation and emission spectra; S Mustafa, J Hannagan, P Rigby, K Pfleger, B Corry; Journal of Biomedical Optics, 18, 026024, 2013. Open access online.

Installation

1. Download FRETTY_plugin.jar to the plugins folder of ImageJ (or subfolder thereof)
2. Newer versions of ImageJ require the Jama plugin to be installed. Please download the Jama jar file directly from here and copy it into the plugins folder of ImageJ (or subfolder thereof)
3. Restart ImageJ to add FRETTY to the Plugins menu

Fretty 3D deconvolution

This tool is designed to calculate FRET efficiency from a series of microscope images collected with a spectral detector and a range of excitation wavelengths using a 3D spectral unmixing algorithm. This algorithm allows for bleed through of the donor emission into the acceptor emission channels and direct excitation of the acceptor with the donor excitation to be directly accounted for. The concept is similar to the 2D algorithm described below, except now instead of each spectrum referring to the emission spectrum collected at a particular excitation wavelength, 3D spectra are used which contain the emission intensity for a range of both excitation and emission wavelengths.

To collect data you need to collect the emission spectra using a spectral detector for a number of excitation wavelengths. The stacks used for analysis are ordered such that all the emission wavelengths for one excitation are found in sequence before moving to the next excitation wavelength. For example if you had a stack with 40 slices corresponding to 4 excitation wavelengths, the first 10 slices would be the emission wavelengths at the first excitation wavelength. The next 10 slices would be the emission wavelengths for the second excitation wavelength and so forth.

How to use Fretty 3D deconvolution

Steps 1, 2 and 3 only need to be followed to set up the analysis tool. Once these have been completed you can analyse as many samples as you like by following steps 4 and 5

1. Enter the analysis parameters at the top of the interface:
   a) *Excitation wavelengths*: the number of different excitation wavelengths used. The number of emission wavelengths per excitation will be automatically determined from the number of slices in each stack.
   b) *Donor quantum yield*: the quantum yield of the donor fluorophore
   c) *Acceptor quantum yield*: the quantum yield of the acceptor fluorophore
   d) *Pixel intensity threshold*: A FRET image is only created for pixels for which at least one slice in the stack has a value above this threshold. Not required for ROI FRET analysis
   e) *Correct for instrument spectral response*: If you wish to correct for the different response of the detector to different wavelengths then you can tick the check box. Many PMT detectors do not
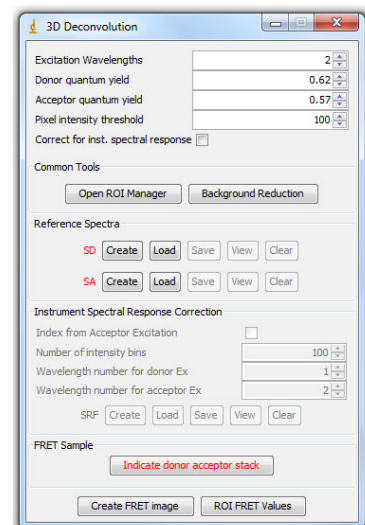
respond linearly to the intensity of incident light, and this may differ at different wavelengths. Checking this feature enables a correction to be applied to account for this.

2. Create or load reference spectra
Red in the spectrum name indicates no spectrum loaded, green indicates spectrum is loaded, grey indicates spectrum is not required
To create reference spectra:
   a) Load either the data from the donor only sample (for **SD**) or acceptor only sample (for **SA**) into ImageJ and create a stack ordered as described above.
   For example use File -> Import -> Image sequence in the main ImageJ window
   b) Conduct background reduction.
   To do this, draw background region of interest (ROI) -> press background reduction

   c) Create reference spectrum by
      i. Open ROI manager
      ii. For each cell of interest in the image, draw foreground ROI using the tools in the main ImageJ window and add to ROI manager using the 'add' button
      iii. Press 'create' in the 2D Deconvolution window to make the reference spectrum . This adds together the values for all currents ROIs to create a reference spectrum.

   d) Use optional save and view to store or look at spectrum data. Saving the spectra is very useful to expedite the analysis of future FRET images.

   Previously saved reference spectra can be loaded using the 'load' buttons without having to open or indicate stacks

3. Calculate spectral response correction (SRF) (optional)
This is only necessary if you are trying to normalise the donor and acceptor emission intensities to correct for the different repose of the detector to different wavelength as described in step 1. The process works by examining all the pixels in an image stack (or set of multiple image stacks) in an image that is expected to have the same FRET efficiency throughout. It then works out a numerical correction as a function of pixel brightness that will yield the same FRET efficiency for all the pixels.

   a) *Index from acceptor excitation*: this determines whether the pixel brightness is determined from a channel with donor excitation or acceptor excitation. We suggest ticking this box if the donor to acceptor ratio is known to be the same in the sample used to calculate the SRF as in the FRET sample being analysed. If this is not true or not known leaving this blank.

   b) *Number of intensity bins*: choose the number of intensity bins to use in the calculation. This refers to the number of bins into which to divide the intensity range in the image. The exact value is not critical, but something in the range of 50-100 works well for 16 bit images, a smaller number can be used for 8 bit images. If the value is too large you will get a warning when you try to create the SRF. In that case we suggest that you reduce the number of intensity bins and try again.

   c) Set the wavelength numbers. *Wavelength number for donor excitation* is used to say which excitation wavelength best excites the donor and *Wavelength number for acceptor excitation* is used to say which excitation wavelength best excites the acceptor

   d) Load one or more image stacks ordered as described above, corresponding to images that you expect to have the same FRET efficiency for all non-background parts of the image. Conduct background reduction on each. The more image stacks used here the better the correction

function is likely to be as there will be more pixels within each intensity range.

e) Create the spectral response function by pressing the 'create' button. The reference spectra must have already been loaded before you do this!

f) The function can be viewed, or saved for future use. Saving the IRF is very useful to expedite the analysis of future FRET images.

g) Previously saved IRF can be loaded using the 'load' button without having to load or indicate stacks

4. Load images for analysis
a) Load the data from the mixed sample (FRET sample containing both donor and acceptor) into ImageJ and create a stack ordered as described above.
b) Conduct background reduction.
c) Make this FRET stack the active window (bring it the front) by clicking on it if it is not already at the front
d) Press 'Indicate donor acceptor stack' button to classify this stack

5. FRET analysis

To create a FRET image, in which each pixel is replaced by the FRET efficiency value at that position, click 'create FRET image'

To calculate the FRET efficiency in a region of interest (ROI) using the average intensities in the region, first open the ROI manager, add as many ROIs as you like to the ROI manager, and then click 'ROI FRET values'

Example videos are available demonstrating the use of the 3D Deconvolution plugin

Fretty 2D deconvolution

This tool is designed to calculate FRET efficiency from a series of microscope images collected with a spectral detector using a 2D spectral unmixing algorithm. This algorithm allows for bleed through of the donor emission into the acceptor emission channels to be directly accounted for, improving the accuracy of the FRET calculation. A new image can be created in which each pixel contains the FRET efficiency value at that position, or the FRET efficiency can be calculated in a region of interest in the image. The direct excitation of the acceptor using the donor excitation wavelength can be corrected for if desired using the cross excitation function.

Before using this plugin, consider using the 3Dconvolution plugin. It is easier and accounts for cross excitation directly rather than requiring an addition correction.

For this tool to work a few simple guidelines must be followed when collecting the microscope data.

1. Two excitation wavelengths are required, one designed to primarily excite the donor fluorophore and one to primarily excite the acceptor.
2. The emission data should be collected for the same number and range of wavelengths in each case
3. All images, ie for donor only sample, acceptor only sample and mixed sample, should be taken with the same microscope settings (eg laser power, detector gain, pinhole etc)
4. One set of reference spectra can be used for many mixed samples, provided the same microscope settings were used for collection
5. When conducting cross excitation correction
   a. Acceptor only sample should be imaged using both donor and acceptor excitation without moving the field of view
   b. The mixed sample (containing both donor and acceptor) should be imaged using both donor and acceptor excitation without moving the field of view

How to use Fretty 2D Deconvolution

Steps 1, 2 and 3 only need to be followed to set up the analysis tool. Once these have been completed you can analyse as many samples as you like by following steps 4 and 5

1. Enter the analysis parameters at the top of the interface:
   a) *Cross excitation correction*: check this if you want to account for direct excitation of the acceptor fluorophores when using donor excitation wavelength. Requires additional reference spectrum (SAA) and acceptor excitation stack
   b) *Use IRF in FRET Calculations*: Check this if you want to use an 'instrument response function' (IRF) either when calculating the cross excitation correction or calculating FRET efficiency. The IRF is necessary is the detector does not respond linearly to the intensity of incident light. Experience has shown this may be necessary for PMT detectors.
   c) *Wavelengths per sample*: the number of emission wavelengths in each sample, should correspond to the number of images in each stack described below. This should be the same for all the reference spectra and the excitation stacks
   d) *Donor quantum yield*: the quantum yield of the donor fluorophore
   e) *Acceptor quantum yield*: the quantum yield of the acceptor fluorophore
   f) *Pixel intensity threshold*: A FRET image is only created for pixels for which at least one slice in the stack has a value above this

threshold. Not required for ROI FRET analysis

2. Create or Load the required reference spectra

Red in the spectrum name indicates no spectrum loaded, green indicates spectrum is loaded, grey indicates spectrum is not required

The spectra refer to those obtained in each of the following cases:

SDD: Donor only sample with donor excitation

SAD: Acceptor only sample with donor excitation

SAA: Acceptor only sample with acceptor excitation. Only required when using cross excitation correction

To create reference spectra follow the steps below:

For each reference spectra

i.   Open image stack   (for example use File -> Import -> Image sequence in the main ImageJ window)
ii. Draw background ROI -> press background reduction
iii. Open ROI manager
iv. For each cell of interest in the image, draw foreground ROI using the tools in the main ImageJ window and add to ROI manager using the 'add' button
v.   Press 'create' in the 2D Deconvolution window to make the reference spectrum . This adds together the values for all currents ROIs to create a reference spectrum.
vi. Use optional save and view to store or look at spectrum data. Saving the spectra is very useful to expedite the analysis of future FRET images.

Note that SDA and SAA spectra should be created using images of the exact same sample and the same ROIs

Previously saved reference spectra can be loaded using the 'load' buttons without having to open or indicate stacks

3. Create or load instrument response function (IRF) (optional)

If you want to use the IRF then follow the steps below, otherwise you move directly to step 4

To create an instrument response function:

a) Choose the number of intensity bins to use in the calculation. This refers to the number of bins into which to divide the intensity range in the SAD and SAA stacks. The exact value is not critical, but something in the range of 50-100 works well for 16 bit images. If the value is too large you will get a warning when you try to create the IRF.
b) Make the SAD stack the active window (bring it the front) by clicking on it. This is the same stack used for creating the SAD spectrum in step 2 above.
    SAD: Acceptor only sample with donor excitation
    SAA: Acceptor only sample with acceptor excitation.
c) Press 'Indicate SAD stack' button to classify this stack
d) Make the SAA stack the active window (bring it the front) by clicking on it. This is the same stack used for creating the SAA spectrum in step 2 above.
    SAA: Acceptor only sample with acceptor excitation.
e) Press 'Indicate SAD stack' button to classify this stack
f) Once both stacks have been indicated and the buttons are green, press 'create' to make an IRF
g) Use optional save and view to store or look at the IRF data. Saving the IRF is very useful to expedite the analysis of future FRET images.

Previously saved IRF can be loaded using the 'load' button without having to load or indicate stacks

If you wish to correct for the spectral response of the detector (for example if the detector does not respond the same in the donor and acceptor emission channels) then you can tick the check box '*Normalise D:A intensities with SRF*'. In this case you should
i.   Create the SRF using the 3D deconvolution plugin described above
ii.   Tick the checkbox '*Normalise D:A intensities with SRF*'
iii.   Use the 'load' button to load the previously saved SRF.

4. Load images for analysis
   a) Load in donor excitation stack (for example use File -> Import -> Image sequence in the main ImageJ window)

   b) Conduct background reduction (Draw background ROI -> press background reduction)

   c) If using cross excitation correction
       a. Load in acceptor excitation stack
       b. conduct background reduction

   d) Make donor excitation stack the active window (bring it the front) by clicking on it
   e) Press 'Indicate donor excitation stack' button to classify this stack

   f) If using cross excitation correction
       a. Make acceptor excitation stack the active window (bring it the front) by clicking on it
       b. Press 'Indicate acceptor excitation stack' button to classify this stack

5. FRET analysis

To create a FRET image, in which each pixel is replaced by the FRET efficiency value at that position, click 'create FRET image'

To calculate the FRET efficiency in a region of interest using the average intensities in the region, click 'ROI FRET values'

Glossary:

Stack: The stacks are always containing a series of images recorded at different emission wavelengths, ie from a spectral detector

Reference spectrum: Spectrum from donor only or acceptor only images, that will be used to analayse many FRET samples

SDD: Reference spectrum from a donor only sample with donor excitation
SAD: Reference spectrum from an acceptor only sample with donor excitation
SAA: Reference spectrum from an acceptor only sample with acceptor excitation. Only required when using cross excitation correction
Donor excitation stack: Sample for FRET analysis (ie containing both donor and acceptor) obtained with donor excitation

Acceptor excitation stack: Sample for FRET analysis (ie containing both donor and acceptor) obtained with acceptor excitation

ROI: Region of interest

Background reduction: Used to remove the background signal. For each slice in the stack, subtracts the average intensity value of the current region of interest from all pixels in the image.