Image data analysis statistics writeup

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A short document on the image analysis and statistics for multiphoton FRET

Using two-photon techniques for performing FRET-based stoichiometry requires including additional terms as compared to the one photon case. Namely, we cannot ignore the excitation of donor due to the acceptor excitation as well as the emission of acceptor into the donor emission channel. This has been detailed wonderfully in Meredith's thesis and I will not repeat it here. Instead, I will outline the image processing techniques that are used in the data analysis developed by Dan and Amar. But first, some definitions/reminders:

- $I_a = I\left(\lambda_{acc}^{ex}, \lambda_{acc}^{em}\right)$
- $I_d = I\left(\lambda_{don}^{ex}, \lambda_{don}^{em}\right)$
- $I_f = I\left(\lambda_{don}^{ex}, \lambda_{acc}^{em}\right)$
- $I_n = I\left(\lambda_{acc}^{ex}, \lambda_{don}^{em}\right)$

The basic algorithm is as follows:

- Use Donor only and FRET only cells
  - measure lifetimes at the SMART center. Get an average value of the FRET efficiency, E
- Donor only cells
  - pick out individual cells from each image, average I_d, I_n, I_f over each cell
  - calculate β and θ
  - repeat for each image
  - calculate average β and θ
  - 74 cells used for β
  - 66 cells used for θ

  Shown below is an example of how individual cells are thresholded (on the right) against the background and then each cell is individually selected (on the left, shown by the color coding) and calculations are performed on the signal from the entire cell
- Acceptor only cells
  - pick out individual cells from each image, average I_a, I_n, I_f over each cell
  - calculate α and η
  - repeat for each image
  - calculate average α and η
  - 28 cells used for α
  - 29 cells used for η
- Use FRET only cells
  - pick out individual cells, average I_a, I_d, I_f over each cell
  - use the average values of E, β, θ, α and η as calculated above

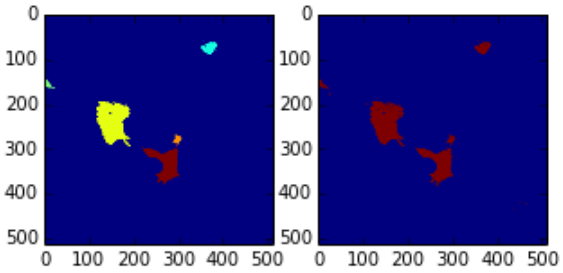

Figure 1: example of picking cells for β calculation

- calculate using

$$\gamma = \frac{E}{-1 + \frac{\eta(-\alpha+\theta)(-I_d\beta+I_f)}{\alpha(-\beta+\eta)(I_a\theta-I_f)}} \tag{1}$$

- repeat for each image
- calculate average γ
- 77 cells used for calculating γ

In the graph below, the histogram of γ is shown.

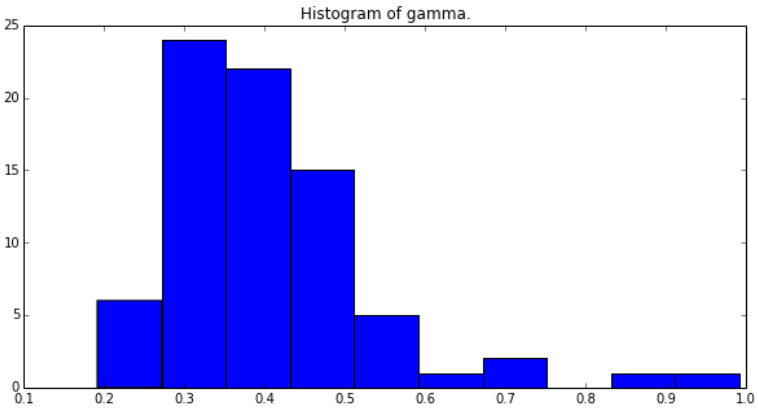

Figure 2: distribution of γ

- Use FRET only cells
  - pick out individual cells, average I_a, I_d, I_f over each cell
  - use the average values of E, β, θ, α, η and γ as calculated above
  - calculate using

$$\xi = \frac{E\gamma\left(I_d\eta - I_f\right)}{\left(-E+1\right)\left(-\beta+\eta\right)\left(-\frac{I_a\alpha\theta}{-\alpha+\theta} - \frac{I_d\beta\eta}{-\beta+\eta} + \frac{I_f(-\alpha\beta+\eta\theta)}{(-\alpha+\theta)(-\beta+\eta)}\right)} \tag{2}$$

  - repeat for each image
  - calculate average ξ
  - also 77 cells, because the same data set is used as γ

  In the graph below, the histogram of ξ is shown.
- Use FRET only cells (trivial case sanity check)
  - Individual cells have already been picked out above for γ and ξ calculation. Use the averaged I_a, I_d, I_f over each cell as above.
  - use all the above quantities to calculate fraction of donor f_d, and acceptor f_a

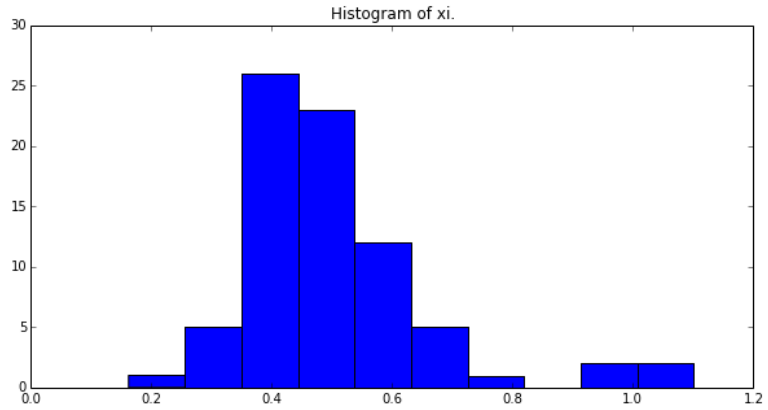

Figure 3: distribution of γ

- Use

$$f_a = \frac{\gamma}{E}\left(-1 + \frac{\eta\left(-\alpha+\theta\right)\left(-I_d\beta + I_f\right)}{\alpha\left(-\beta+\eta\right)\left(I_a\theta - I_f\right)}\right) \tag{3}$$

- Use

$$f_d = \frac{1}{E}\left(1 - \frac{I_d\eta - I_f}{\left(-\beta+\eta\right)\left(\frac{I_d\eta - I_f}{-\beta+\eta} + \frac{\xi}{\gamma}\left(-\frac{I_a\alpha\theta}{-\alpha+\theta} - \frac{I_d\beta\eta}{-\beta+\eta} + \frac{I_f(-\alpha\beta+\eta\theta)}{(-\alpha+\theta)(-\beta+\eta)}\right)\right)}\right) \tag{4}$$

- confirm that they average to one (trivial case because γ and ξ are calculated assuming f_a and f_d are one for FRET case samples

Shown below are the histograms of f_a and f_d for the FRET only case. The histograms should be centered about 1. Also included are the same numbers plotted f_a vs f_d. Ideally, this distribution should be a Gaussian distribution around the point (1,1) in this image.

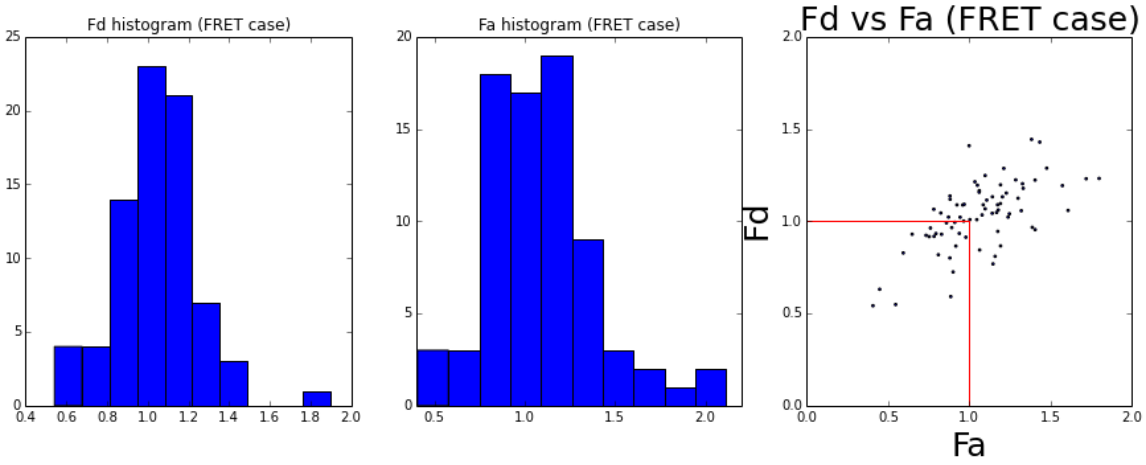

Figure 4: distribution of f_a, f_d and f_a vs f_d for FRET

- Use unlinked donor and acceptor cells (sanity check)
  - pick out individual cells, average I_a, I_d, I_f over each cell
  - use all the above quantities to calculate fraction of donor f_d, and acceptor f_a
  - confirm that they average to zero
  - Use same formulas as above for f_a and f_d
  - 45 cells used for calculating f_a and f_d

Shown below are the histograms of f_a and f_d for the donor and acceptor unlinked only case. The histograms should be centered about 0. Also included are the same numbers plotted f_a vs f_d. Ideally, this distribution should be a Gaussian distribution around the point (0,0) in this image.

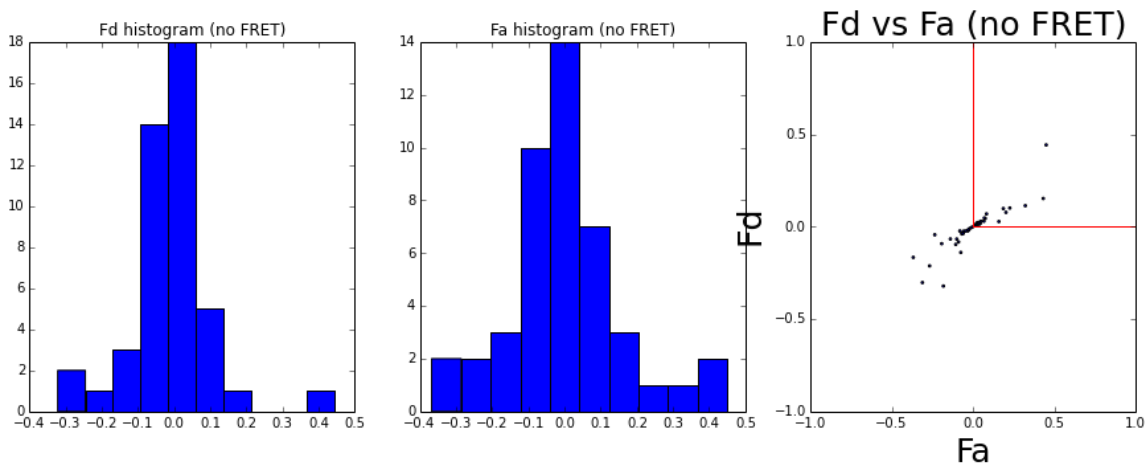

Figure 5: distribution of f_a, f_d and f_a vs f_d for no FRET

Here lies the worrying part. The distribution of f_a and f_d should not display significant covariance with respect to each other, since they should be random quantities. Is there an underlying relation that causes those numbers to naturally vary together because many of the numbers used for their calculations are the same? Would a completely random set of I_a, I_d and I_f still generate this covariance?