FEB 15, 2024

DOI:
dx.doi.org/10.17504/protocols.io.eq2lyjkwqlx9/v1

Protocol Citation: Amber Yanas 2024. Multi-parameter confocal TCSPC spectroscopy analysis. **protocols.io** https://dx.doi.org/10.17504/protocols.io.eq2lyjkwqlx9/v1

Created: Feb 14, 2024

Last Modified: Feb 15, 2024

PROTOCOL integer ID: 95223

Multi-parameter confocal TCSPC spectroscopy analysis

Amber Yanas[1]

[1]University of Pennsylvania

Amber Yanas

ABSTRACT

The following outlines the analysis of data generated by multi-parameter confocal time-correlated single photon counting (TCSPC) microscopy and spectroscopy (MicroTime-200; PicoQuant, GmbH).

Funders Acknowledgement:
Yale E. Goldman
Grant ID: R35GM118139

Conversion of .ptu to .hdf5

1 PQ_To_hdf5_All.ipynb

1.1 Use program: PQ_To_hdf5_All.ipynb

1.2 Enter pathname and run all to convert files from .ptu to .hdf5

1.3 A new folder will be created with these hdf5 files

a. Hint – convert to hdf5 in bulk to save time

Processing .hdf5 files for burst analysis and FRET

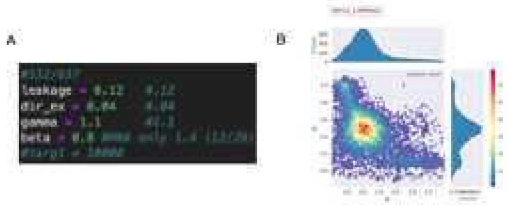
2 FRET_Anal_Y6_Vb.ipynb

2.1 Use program: FRET_Anal_Y6_Vb.ipynb

2.2 Enter pathname and run RNA only sample

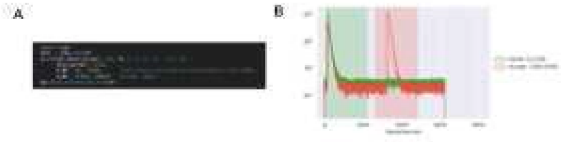
2.3 Check you are using the appropriate correction factors, usually beta is the only factor that varies from experiment to experiment. Make sure beta is set so that the double labeled population median is at 0.5.

2.4



Example 2D histogram
(Left) Example of the parameters used for leakage, direct excitation, gamma, and beta. (Right) Example scatter plot produced when running the FRETBursts program. The beta factor has been set so that the double labeled bursts are clustered with a median of 0.5.

2.5 Make sure the time ranges are appropriate for your experiment and cover the decay fully.



Example decay curves
(Left) Example of the area of code to edit to ensure decay curves are completely covered. (Right) Decay curves with the time ranges covering the complete decay.

2.6 Enter pathname and run +protein sample

Make sure beta is set so that the double labeled population median is at 0.5.

2.7 Separate files into individual folders:

Example of data separation for easier analysis.

a. Anisotropy – Acceptor only, donor only, and double labeled
b. FRET efficiency folder (“efficiency” for short)
b. Stoichiometry (of each species)

FRET Efficiency Proportion Analysis

3 FRET Efficiency Proportion Analysis

3.1 In the “FRET Efficiency” folder you created, go to the individual FRET Efficiency .csv file

3.2 Add up the number of bursts that had a FRET Efficiency above and below 0.5 from the “E” column in the .csv file

a. =IF(F5454>0.5,1,0)
b. This will assign 0 below 0.5 and 1 above 0.5

3.3 Sum the number of bursts that had a FRET efficiency above 0.5

a. =SUM(N2:N5454)
b. This will be the numerator for your high FRET efficiency bursts

3.4 Determine the number of bursts (sum the number of rows of data)

a. =ROWS(N2:N5454)
b. This is your denominator

3.5 Determine the proportion of bursts above 0.5 – this is proportion high FRET
a. Sum of bursts above 0.5 / sum of total bursts

3.6 Determine the proportion of bursts below 0.5 – this is proportion low FRET
a. Sum of bursts below 0.5 / sum of total bursts

Burst Analysis

4 Burst Analysis

4.1 The output will be called “BurstSearch.txt.”

4.2 Copy the entire text file contents to an excel sheet

4.3 Copy the bursts for acceptor only, double labeled, and donor only

4.4 Determine the proportion of these bursts by taking the quantity of these individual burst populations over the quantity of bursts in the RNA only sample

4.5 Normalize the RNA only sample to the maximum annealing proportion of 0.75 (for the RNA used in Yanas PNAS 2014 paper).

Processing files for Anisotropy

5 Anisotr_DDX2b_fig.py

5.1 In Spyder - Use program: Anisotr_DDX2b_fig.py

5.2 Enter pathname (make sure you separate the files into AO, DO, DL)

5.3 Determine the t shift, G, and time period that fits the decay curve of the RNA only sample:

a. The G value should bring the decay curve to zero

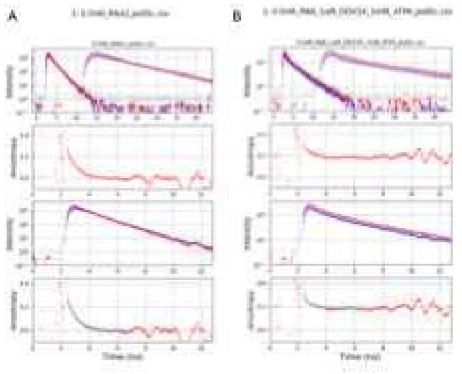
b. The t shift should align the beginning of the horizontal and perpendicular anisotropy decays.

c. The time period should begin when the data is less sparse in the decay curve and end when the curve has flatlined

5.4 Use the “G” “e” “T” commands to set these parameters.

a. For example: type “G”, enter, then “1” (as your G value), enter. You have now set the G factor. Repeat for the other parameters.

5.5



Example time resolved fluorescence anisotropy curves.
(Left) RNA only curve for a double labeled RNA. The anisotropy decay should come completely to zero. All parameters have been set correctly for this trace. (Right) RNA and protein curve showing high anisotropy. The parameters were set with the RNA only sample and used for this sample.

5.6 Apply these values to the +protein and +protein/ATP samples

5.7 Stop program and enter pathname for +protein samples

a. Use the “G” “e” “T” commands to set these parameters.

5.8 The values will be saved to SavedData.csv file

5.9 Determine B/B0 by using the anisotropy of the tightly bound protein only sample as B0 and using the B value column as B. Plot these values over time to observe anisotropy changes.

Processing .hdf5 files for FCCS

6

FCS_AnalY4_All_B.ipynb

PlotResults1.py

PlotResults1_SPADS.py

6.1 Use program: FCS_AnalY4_All_B.ipynb

6.2 Enter pathname and run all

6.3 Use beta value determined in the burst analysis program

6.4 Check each file has a good fit

6.5 In Spyder use program: PlotResults1.py

6.6 Enter pathname and run files

6.7 Extract file names, tau values, and N values

6.8 Copy and paste this output to excel

6.9 Split these cells into individual cells

6.10 Copy and paste transpose to sort data into vertical columns

6.11 Convert tau values to diffusion coefficients with the following equations:

$$\omega^2_{cross\ correlation} = \frac{(\omega^2 + \omega^2)}{2}$$

$$D[k] = \frac{\omega_0^2}{4\tau_{Diffusion}[k]}$$