Fluorescence Correlation Spectroscopy (FCS)

Home work for TD Preparation

A- In vivo TP preparation = Scientific Paper Analysis. (*Nucl. Acids Res.-2016-Guiziou-nar_gkw624.*).

Quantification of the absolute number of GFP molecules expressed per living cell.

Libraries of promoters will first be engineered in *E. coli* to explore a large range of gene expression levels during SynBio practical courses. Then, Quantification of GFP molecules expressed in these libraries will be performed by cytometry and Fluorescence Fluctuation Scanning microscopy using 2 Photons Excitation coupled with Number & Brightness analysis.

Exercises

From paper study:

1/ Sample Preparation: Write precise protocol for sample preparation (Growth media, OD, Bacteria Immobilization)

2/ Data Acquisition: Write a Data acquisition protocol (laser wavelength, power, Image size, laser dwell time, Number of images)

3/ Data Analysis: Explain N&B Analysis theory. Write on an application of your choice (excel or other) a script to obtain the absolute number of molecules /cell from N&B data. Explain.

Fluorescence Correlation Spectroscopy (FCS)

TD

A- Review and discussion around homework on Scientific Paper Analysis.

(*Nucl. Acids Res.-2016-Guiziou-nar_gkw624.*)**.**

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1/ Sample Preparation: Write precise protocol for sample preparation (Growth media, OD, Bacteria Immobilization)

2/ Data Acquisition: Write a Data acquisition protocol (laser wavelength, power, Image size, laser dwell time, Number of images)

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Data analysis Simulation with PaTrack Software:

test tif files: Rho_02-920 nm_6mW_40 usec_50 frames_Ch2.Tif & m_04-920 nm_6mW_40 usec_50 frames_Ch2.Tif

1-1: Calculate the average true brightness e (or ε).

1-2: If the fluorescence Intensity background is = 1; calculate the corrected brightness and the corrected Number of molecule/cell.

B- Diffusion Coefficient Calculus Exercises.

1-1: Calculate theoretical Diffusion Coefficient for Alexa 488 and GFP in water.

1-2: Look for in publication a measured (experimental) Diffusion coefficient of EGP in cytoplasmic of a cell (Eukaryotic and/or Prokaryotic). Calculate the apparent “crowdedness” of the cytoplasm.

1-3: Write autocorrelation function as function of ω_0, z_0,τ and τ_D. What happens when $\tau = \tau_D$

1-4: Calculate the Diffusion Coefficient for a monomeric and a dimeric form of protein (monomer= 100 KDa). If you have a perfect mixture of Monomer & Dimer in your tube, can you observe and quantify both species in a FCS experiment.

Fluorescence Fluctuation Spectroscopy
Laboratory Practical work

DAY 1: Microscope alignment - Acquisition software presentation - In vitro point FCS experiments.

1 / Microscope configuration and alignment

- Draw a schematic representation of the FCS microscope setup used. Briefly describe and explain the specifications of this microscope.

- Proceed to the excitation alignment. Proceed to the emission alignment. Describe the protocols and procedures used for these 2 alignments.

2 / Calibration of the size of the excitation beam.

- Use a solution of 60 nM Fluoresceine in Tris pH 9. 780 nm and 10 mW. Run a point FCS experiment with appropriate parameters. Fit the autocorrelation function with 3D Gaussian. What is the value of W0 and Z0? Calculate the excitation volume?

3 / Acquire *in vitro* FCS data

- 6 unknown solutions must be characterized by measuring their Diffusion Coefficients and Concentrations.
- Explain the choice of parameters used for the point FCS experiments.
- Comment the results obtained.

4 / Simulations Point FCS data using SimFCS software.

4-1. Exercise 1: Run simulations of single species Point FCS on SimFCS software:

Support material will be available.

4-1-1: Calculate the concentration of particle in the simulation box. ($1\mu m^3 = 1$ fL $= 10^{-15}$ L).

If you change beam waist or any others simulation parameters (diffusion coefficients-Concentration-Brightness- Sample frequencies). Comment your observations.

4-1-2: Fit autocorrelation function obtained in the simulation to different radial waists. Comment results obtained.

4-1-3: Fit autocorrelation function to different waist ratios (w_0/w_z). Comment on the sensitivity of FCS to the z dimension.

4-2. Exercise 2: Run simulations of 2 different species Point FCS on SimFCS software:

4-2-1: Calculate the concentration and diffusion coefficient of each species particle.

If you change beam waist and / or any others simulation parameters (diffusion coefficients-Concentration-Brightness- Sample frequencies). Comment your observations.

4-3. **Exercise 3: Run simulations of CrossCorrelation (2 colors) Point FCS on SimFCS software:**

4-3-1: Calculate the concentration and brightness of each species particle.

We know that our microscope shows 10% of crosstalk. Is it possible to calculate the concentration of each species? Comments.

5 / *In vivo* experiments Preparation for Day 2.

DAY 2:

Quantification of the absolute number of GFP molecules expressed per living cell.

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1 / Acquire images on live Bacteria expressing GFP in order to perform N&B experiments:

1- Sample preparation
2- Data acquisition
3- Data Analysis
4- Write a report as a scientific paper: Introduction, Materials and Methods, Results, Discussion.