

Bi/BE 227 Winter 2018

Assignment #4

Comparing FCS and FRAP as methodologies for calculating diffusion

Schedule:

Jan 29: Assignment

Jan 29-Feb 14: Work on assignment

Feb 14: Student PowerPoint presentations.

Goals for this week

- A. Mount live transgenic fish embryos in low melting point agarose
- B. Fluorescence Correlation Spectroscopy (FCS).
- C. Fluorescent recovery after photobleaching (FRAP).
- D. Calculate diffusion coefficients using two different techniques
- E. Learn to use LSM 880 for experiments

Samples

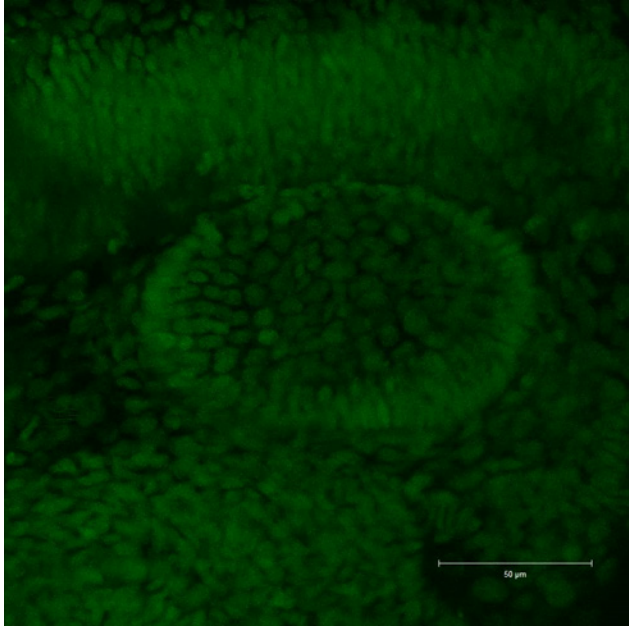
- A. Zebrafish transgenic embryos, ct94a (line #1011) which labels Synergistic Lethal with U5 snRNA 7 (Slu7), protein in nuclei tagged with citrine (YFP)
- B. Rhodamine 6G stock solution diluted 1:1,000 in H₂O or PBS for calibrating pinhole

INSTRUCTIONS:

A. Mounting Embryos in Low Melting Point (LMP) agarose

We will be mounting the zebrafish embryos for viewing on an UPRIGHT microscope. For the next assignment they will be mounted in a different chamber for the inverted confocals. For these experiments you will be using the following transgenic line of zebrafish: ct94a (line #1011) which labels Synergistic Lethal with U5 snRNA 7 (Slu7), a protein in nuclei tagged with citrine (YFP) (**Figure 1**). Ideally, we want the embryos to be at least 24 hours old but not more than 72 hours post fertilization (hpf). Coordinate with your group to reserve time on the LSM 880 in the imaging facility with the black box incubator (not the HHMI 880). Request embryos of the appropriate stage from Andres at least 4 days before you need them.

Figure (1) 30 hpf, Z-projection. Synergistic Lethal with U5 snRNA 7 (Slu7), Ubiquitously Expressed Nuclear Protein



A1. Sort embryos to find ones with fluorescent label

You will use a Fluorescent dissecting scope to select embryos that are fluorescently labeled. Anesthetize the embryos with Tricaine (10 drops of the stock solution into a 35 mm petri dish of embryos in egg water).

A2. Mount embryos in LMP agarose

Use the #1.5 cover slip we provide to mount the embryos on. You will use a glass pipette with the green pipette Aid to suck up the embryos and drop them in the LMP agarose which is kept in glass tubes in a heating block at ~43°C. Do not transfer any of the water the embryo is in, let the embryo float down into the agarose. Then empty the pipette of any liquid and suck up the zebrafish in the LMP agarose, making sure to suck up some agarose before sucking up the fish. YOU HAVE TO MOVE RELATIVELY QUICKLY HERE. Drop the animal on the coverslip and make sure it is as close to the coverslip as possible. Plop drops of agar on top to help push the fish down. You can use a blunt probe to push it down or orient it very gently. If it breaks you have to start over. You have to do all this before the agar hardens as once it hardens you will kill the fish by poking it. You will use the 3 mm iSpacer to create a well to fill with egg water with anesthetic. Then place a slide on top and flip it over for imaging.

B. Fluorescence Correlation Spectroscopy (FCS)

There is a detailed guide for doing FCS using the Zeiss Zen software on the class website. Here we will discuss the protocol for doing FCS on live zebrafish embryos more generally. For all experiments set the incubator to 28°C so that all your measurements are at the same temperature. For all these experiments you will use the water C-Apo 40x with an NA of 1.2 and a 220 micron working distance.

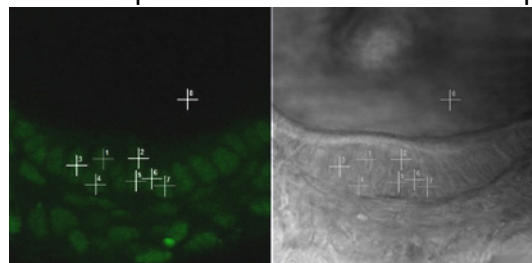
B1. Align the pinhole using the diluted solution of Rhodamine 6G.

You only have to do the alignment once per day. Make sure you set the pinhole for 1 AU. You will do this as demonstrated by Andres. In the FCS guide, the pinhole alignment is described on pages 12-13.

B2. Collect FCS measures on your zebrafish embryo.

Target the nuclei of your cell of interest (Figure 2). The nuclei of cells in the inner ear, epidermis and neurons are all good targets. You should do multiple cell types to see if the protein diffusion varies. You should also look at the same cell type in embryos at different stages to see how that affects diffusion. The workflow is as follows: In the Acquisition tab of the Zen software you will snap a picture of the cells you are interested in imaging. You will use the second detector (CHS1), 514 laser line and the emission “filter” for Citrine. Note the laser power you use to collect this image. Andres will show you this in the demo. You need this image to help you choose your positions. You may need to zoom in to make sure your nuclei are large enough in the field of view for you to target. Then go to the FCS tab and set up your acquisition parameter (page 14 of the FCS guide). You will use the following parameters: Measure Time is 5 seconds, Repetitions is 5. For laser power make it 1/10th to 1/5th of the power you used for the image acquisition. **MAKE SURE THE PINHOLE IS SET TO 1 AU.** Again, remember that you will be using the 514 laser line. You check the Positions button so you can choose multiple points for your experiments. The number of positions you choose is subjective but I would say 1 to 5 is fine. **BEFORE STARTING THE FCS EXPERIMENT MAKE SURE THE BLACK BOX IS COMPLETELY CLOSED AND THE ROOM LIGHTS ARE ALL OFF.** Under the FCS tab is the Start Experiment button (page 18 FCS Guide). After the FCS experiment, go to the Acquisition tab and snap another picture. If there is any movement from the picture you took before the FCS experiment you will have to discard this experiment and try again. Choose fewer positions. When you have a good experiment save the before and after images (export full resolution image window as jpeg or tif so you can save it with the crosshairs over the picture). There is a trick to saving the FCS file. **WHEN YOU GO TO SAVE IT AS AN FCS FILE YOU HAVE TO GO TO THE DROPDOWN BELOW THE FILE TYPE AND CHOOSE SAVE RAW DATA.** Unfortunately, the Zen software never saves this by default.

Figure (2) 48 hpf ct94a zebrafish imaged in ventral part of inner ear. 7 positions were imaged in this example. You should choose fewer positions.



B3. Analyzing your FCS data.

The good thing is that once you collect the data you can analyze it at any time afterwards. You will have 5 measurements for each point imaged. Ignore the first trace as this will have the bleaching of your fluorophore. Page 19 of the FCS Guide has suggestions on how to choose the right plots. Select by only checking the plots that are relatively level. This could be as many as

four or as few as 1. Copy them to a new FCS file and don't forget to check that you are saving the raw data when you save this new file with just the traces you want.

You will be using the Zen software for the initial part of the data analysis. You need to Fit a model to the data from which you will pull out the numbers you need to calculate the diffusion coefficient. You can create the model as described on pages 15-17 in the FCS guide. For the model we only need Triplet and Translation (page 16) and that the model has just one component (page 17). Save the current model configuration for future fitting. Fitting of the subset of data with the good traces that you saved above is done with this model. This is described on pages 20-21 of the FCS guide. Fit for the diffusion time, you can then translate to the diffusion coefficient in excel using the formula $\text{Diffusion Time} = \frac{w_r^2}{4D}$ where w_r is the beam waist and D is the diffusion coefficient. If you then decide to change the beam waist, excel will update the entire data set instead of having to refit. For the 40x lens you're using w_r should be 0.22. Again, you should only use 1 component with the diffusion and include blinking in the fitting formula which is found under the triplet option. Fix the blinking time to 625 microsec and fix the structural parameter to 5 (see model parameters on FCS guide page 21). Start your fit at ~200microsec (red line on plot on page 21 of the FCS guide) and then hit the Fit button to generate your numbers. You can also start at 100 microsec. I usually do multiple fits like 50, 100, 150 and 200 microseconds. Save the data in the table to a text file you can import into an excel spreadsheet (you only really need the Diffusion Time for the Average for each position but I save it all as a tab delimited text file) and then plug the diffusion time for just the average for each position into the formula above. The excel formula you can use for the diffusion coefficient in microns²/second is as follows:
=(((POWER(0.22,2))/4)/K5)*1000000

Where K5 = Component 1 Diffusion time [s]

Q1: *What are the diffusion coefficients you measure? Does it vary across cell types? Does it vary across ages?*

Q2: *How consistent are the diffusion values you measure across different cells and times?*

C. Fluorescent recovery after photobleaching (FRAP)

There is a detailed protocol for doing FRAP on the class website. This protocol is for Drosophila using the LSM 710 but the LSM 880 uses the exact same software. You should make sure to do the FRAP experiments during the same session and on the same cell types you are using for the FCS experiments. The FRAP experiments take more time so account for that when you are signing up for microscope time.

C1. Settings for FRAP Experiment

As for the FCS experiments make sure the incubator is set to 28°C so that all your measurements are at the same temperature. Again use the water C-Apo 40x with an NA of 1.2 and a 220 micron working distance not the 25x in the protocol. The embryos will be mounted as for FCS as you will probably be imaging the same embryo (but different cells). Everything you need will be in the Acquisition tab where you will check the Bleaching function which will check a bunch of things like region and if not Time series, check that as well. Here is a summary from the FRAP protocol

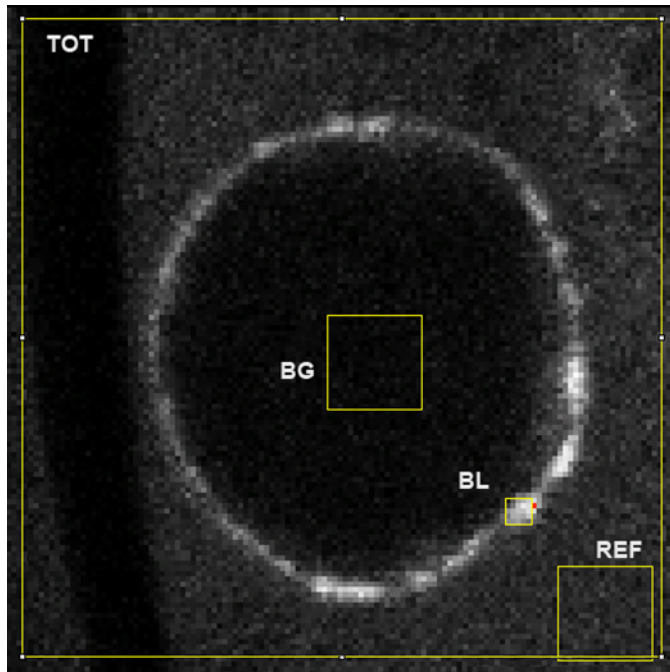
with some suggested modification for the zebrafish experiments but you will have to play with the parameters to see how long it takes for the fluorescence to recover.

MICROSCOPE settings:

We use Zen (black) software. The titles refer to default options or modules that can be activated for each experiment. As a complete novice, it took me 4-6hrs. to optimize the parameters for our needs. This was important because it is ideal to use the same parameters for all experiments. Here is what we chose to use:

- **Frame Size:** 256x256 pixels; 128 resolution (*For the fish you can use a larger frame size like 512x512 pixels*).
- **Zoom:** 10 (pixel size: 0.07 μ m) (*You won't need to zoom this much if you use a larger frame size, you also don't need such a small pixel size though there is nothing wrong with this size*)
- **Scan Speed:** 614.4ms (1.0 μ s pixel dwell time) (*I prefer a 2-3 μ s pixel dwell time for bleaching but this speed is fine. Faster might be useful here, see Regions below*)
- **Laser Power and PMT Gain:** For acquisition, we decided to set the laser power equal for every specimen that we tested, since a variable laser power setting for each specimen could introduce unwanted bias. Some transgenic proteins were more stable than others, so we did have to change the gain control on the microscope for each line we tested. The laser power we chose was 7.0; Gain was adjusted from about 600 to 850 depending on each line tested. (*whatever you used to snap the before and after pictures for your FCS experiments should work here. The laser power is percent*)
- **Time series:** 120 images (each scan represents 0.6144 seconds elapsed time) (*This is where you might find a big difference. You may need many more images*)
- **Bleach:** Start Bleach after 5 scans (at least one prebleach acquisition is required for analysis); Select Safe Bleach for GaAsP; Select lasers 488; 561; 633nm. (*Power of these lasers will be much higher than what you used to image. 30-100%? Also you can repeat the bleach scan. Here Alex just used one repetition but you can use any number to bleach the area. You will have to experiment here.*)
- **Regions:** Select a Region Of Interest (ROI) of desired area. We chose 7x7pixels, as this was a sub-micron bleaching area equal to approximately (0.5 μ m)², and could be bleached within the acquisition interval of 614ms, considering the pixel dwell time of 1.0 μ s. (*This is a nice small size for micro FRAP. Not a bad setting to start with. Just be consistent on the size of your ROI and remember the value for later analyses*)
- **Pinhole:** should be set at 1AU. Other attributes like Digital Offset or Gain were kept at default settings. (Yes!)
- **Incubation:** between 20-25°C. (*For fish we will want this at 28°C just as for the FCS experiment*)

Figure (3) Image from Fly FRAP Protocol showing Regions of Interest saved in ROI Manager controller. This was figure 2 from that protocol. BL: Bleach region that will test for recovery, BG: Background region used to correct for noise, REF: Reference region to show decay of fluorescence resulting from acquisition, TOT: Total signal intensity from the full frame. Substitutes for REF.



More details will be forthcoming on how to pull out the diffusion coefficient from the FRAP data. The protocol only shows you how to calculate mobile fraction.

Q3: *What are the diffusion coefficients you measure? Does it vary across cell types?*

Q4: *How consistent are the diffusion values you measure across different cells?*

Q5: *Did you get the same values you measured using FCS? If not, how did the diffusion coefficients differ? Which method did you think is better?*