**FRAP protocol**

**Sam Hulbert (swh12@duke.edu)**

**4/24/14**

1. Prepare gold/DNA bullets for biolistic transfection.
2. Prepare hippocampal slice cultures from P5 pups (be sure to change media the day after and then every other day).
3. Biolistically transfect slice cultures with fluorescently tagged DNA of interest using gene gun and bullets approximately 1 week after dissection. Continue to change media every other day.
4. Check for successful transfection on a fluorescent microscope after 3-5 days. Reserve time on a core confocal microscope (e.g. Leica SP8) via the Duke LMCF website.
5. When you’re ready to image, use a needle to poke holes around the circumference of the filters containing the hippocampus slices. Use a glue gun to affix filters onto 60mm plates. Apply ~5mL of warmed slice culture media to the filter/plate.
6. On the Leica SP8: use “Neurons FRAP” settings file or manually change settings to the following parameters:
	1. Argon Laser ON @80% power, all other lasers OFF
	2. PMT 2 detector ON @ 498-580nm, all other detectors OFF
	3. Resolution: 1024x256, Speed: 1800 Hz, Zoom: 7.5x, Pinhole: 5.0 A.U.
	4. Time Course:
		1. Prebleach for 5 frames, 1 sec each (488 @ 1.5%)
		2. Bleach for 20 frames, 150 ms each (488 @ 100%)
		3. Postbleach 1 for 150 frames, 200 ms each (488 @ 1.5%)
		4. Postbleach 2 for 80 frames, 500 ms each (488 @ 1.5%)
		5. Postbleach 3 for 200 frames, 1 sec each (488 @ 1.5%)
7. Select a spine to bleach that is isolated, in close proximity to at least one other isolated spine (need an unbleached spine to use as a control during image analysis), and is bright but doesn’t have saturated pixels (adjust gain accordingly).
8. Create a circular ROI approximate 20um x 20um to encapsulate your spine. The ROI should be about twice as large as your spine. Run experiment. Make sure your ROI is the same size every time.
9. Save your data, transfer to your personal computer.
10. Import your images to (FIJI is Just) Image J for analysis. Work with one set of 5 images (Prebleach, Bleach, Postbleach 1, Post bleach 2, Postbleach 3) at a time.
11. Select your “Bleach Series” image and then go to Analyze 🡪 Tools 🡪 ROI Manager on the FIJI menu. Click on the oval shape on the FIJI menu. Create an ROI that matches the bleaching area exactly. Then click “add” on the ROI manager 3 times. Rename the ROIs “bleach,” “no bleach,” and “background.” Select “show all.”
12. Move “no bleach” and “background” to different locations. Click “update.” Click “More” and then save your ROI set (this way you don’t have to create these ROIs again for every set of images – you’ll just have to move them to appropriate locations).
13. Close out of the bleach series image. On the FIJI menu, go to Image 🡪 Stacks 🡪 Tools 🡪 Concatenate. Click “ok.”
14. Go back to your ROI Manager and select “Show All.” Move “no bleach” to a good control spine (similar intensity, size, etc. to bleached spine in the prebleach series). Click “update.”
15. Go to Analyze 🡪 Set Measurements. Select “Mean Gray Value” and nothing else.
16. Select all three ROIs (in the left hand column) and then click “more” and then “multi measure.”
17. Copy and paste this raw data into an excel spreadsheet. Repeat this process for all of your spines.
18. After you have all of your raw data, calculate the prebleach average (average of first five frames) for all of your spines (bleached and control) in excel.
19. Then calculate the background subtracted intensities for all frames and for the prebleach averages. (Raw “bleach” and “no bleach” data minus raw “background” data).
20. Calculate the photobleaching factor for all postbleach frames (6-435). This is the background subtracted intensities for “no bleach” spines divided by the background subtracted prebleach averages for those spines.
21. Calculate the photobleach corrected intensities for your bleached spines. This is the background subtracted intensities divided by the corresponding photobleaching factors.
22. Calculate percent recovery for bleached spines: (each frame’s photobleaching corrected intensity – starting [frame 6] photobleaching corrected intensity) / (background subtracted prebleach average – starting [frame 6] photobleaching corrected intensity).