Bi/BE 227 Winter 2018

Assignment #5

Live imaging II: zebrafish embryos

Schedule:
Feb 5: Assignment
Feb 5 to Feb 26: Work on assignment
Feb 26: Assignment due. Presentations 10:00 am

Goals for this week
   A. Comparison of Optical sectioning methods: Confocal versus Deconvolution.
   B. Time lapse analyses of fluorescently labeled zebrafish embryos

Samples
   A. Live Sox10 GFP transgenic zebrafish embryos, 24 to 36 hours post fertilization (hpf)

INSTRUCTIONS:

A. Comparison of Optical sectioning methods: Confocal versus Deconvolution
For this assignment we will be imaging living zebrafish embryos from the Sox10 GFP transgenic line which labels the developing neural crest (a migratory tissue in the early embryo that contributes to the peripheral nervous system, PNS, jaws and sensory organs among other tissues) (Carney et al., 2006). Expression in the living zebrafish embryo is shown in Figure 1 from the paper. We will be collecting 3D Z-stacks with the confocal, LSM 410 or 310. Before starting any 3D acquisition, read the section "*Considerations for Z-sectioning*" at the end of section B.

A1. Score and mount fluorescent embryos for 3D imaging on the confocal
Using the fluorescent Stereo dissecting microscope in the lab, select embryos that are fluorescently labeled for imaging. Anesthetize the embryos with MS 222 (tricaine), 6-9 drops of stock in a 120 mm petri dish of embryo water. Make sure embryos are out of their chorion, egg membrane, if they are not already. Chorions can be removed easily with sharp forceps (Dumont No. 5) by gently making a tear in the chorion and turning it upside down so that the embryo falls out. Once embryos stop moving, take them and place them in the Low Melting Point (LMP) agarose in the 1.5ml or glass tubes in the heating block (42 ° C). This is 1% LMP with MS 222 added. Try not to transfer too much liquid into the LMP agarose, let the embryo float down into the agarose by itself. With an empty pipette now suck up the embryo in agarose and place it into one of the wells of the 8 well slides. With a probe ***very carefully*** press the embryo down so it is lying against the coverslip. Here are the 8 chamber slides you will be using:

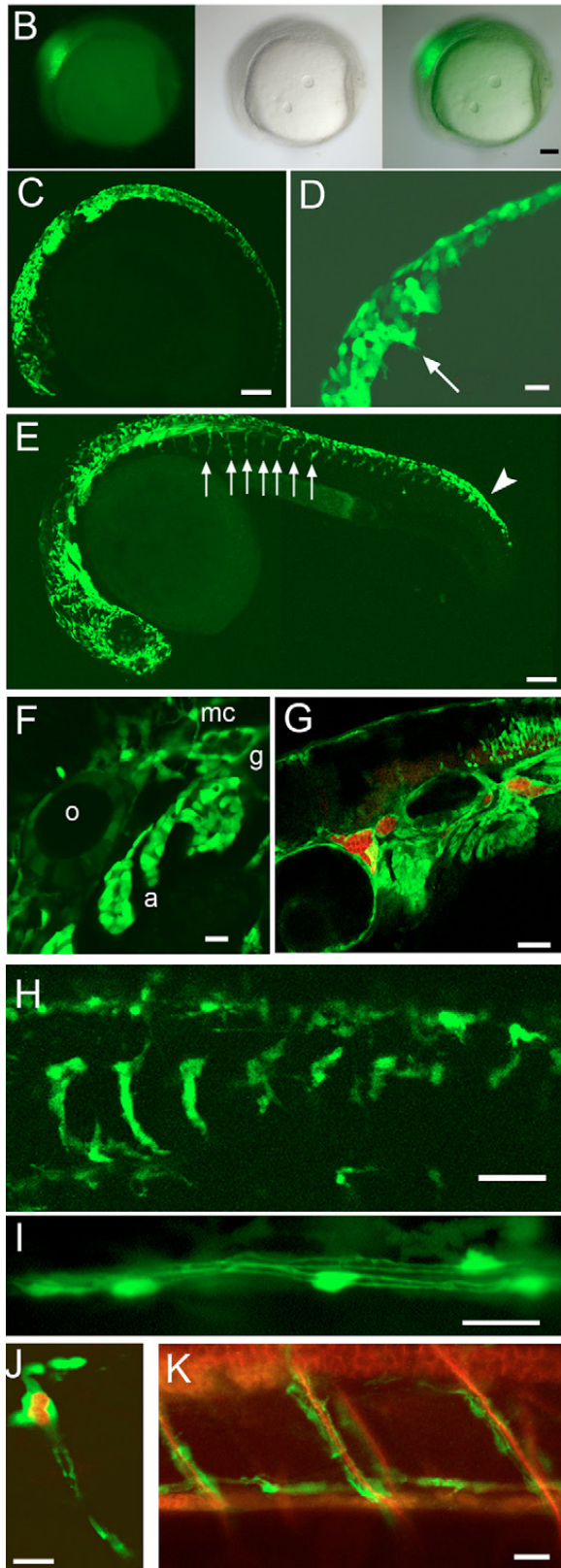| Nunc® Lab-Tek® II chambered coverglass 8 well	Electron Microscopy Sciences	70377-81	L.T. II Coverglass, 8 Chamber	2 trays	$245

Fig. 1. Generation and characterisation of *sox10*:*egfp* transgenic fish. (**B-H**) GFP in germline *sox10:eGFP* transgenic fish is seen in premigratory and migratory NCCs, in time and manner recapitulating the expression of *sox10*. Premigratory cranial NC at 11 hpf (B; fluorescence (left panel), DIC (middle) and combined (right)) and 17 hpf (C). (D) Cranial NCCs at 15 hpf to show resolution of fine cell morphology. (E) Premigratory (arrowhead) and migrating NCCs in head and on medial pathway in anterior trunk (arrows) at 24 hpf. (F) At 24 hpf, cranial GFP-expressing cells include migrating NC (mc), NC in branchial arches (a), glial precursors surrounding the cranial ganglia (g), and otic epithelium (o). (G) Double immunofluorescence imaging of GFP (green) and neurons (Hu, red) at 48 hpf. There is continued expression in otic epithelium and arches. (H) Tail of 40 hpf embryo to show nascent DRG and migrating NCCs. (**I-K**) GFP expression persists in peripheral glia, including DRG satellite glia (J), Schwann cells [posterior lateral line (I) and spinal nerves (J,K)], here at 5 dpf. (J) Double immunofluorescence demonstrates complementary expression of GFP (green) and Hu (red) in DRGs. (K) GFP+ Schwann cells are intimately associated with spinal nerve axons (DM-GRASP, red). All are confocal images except (B) and are of live embryos except (G), (J) and (K). In this and all subsequent figures, embryos are shown in lateral view unless stated otherwise. Scale bars: 100 _m in B,C,E; 20 _m in D,F,I,J,K; 50 _m in G,H.

A2. 3D Imaging of Zebrafish embryos
Try and image the trunk neural crest cells for this exercise (20x or 40x are probably best). Using the 488 nm laser line, acquire two Z-stacks of the labeled zebrafish with 2 different pinhole size settings (wide open, and 1 Airy unit), adjusting the intensity, exposure time, brightness, and contrast such as to fill the whole dynamic range. Make sure your Z- step size is optimal, a step size determined by the lens' section thickness at the appropriate airy unit (see below for examples). For the wide-open pinhole at the very least use the same step size used with the pinhole. However deconvolution benefits from over-sampling so you should probably collect more Z-sections, hence a smaller step size. Remember to record your X-scale, Y-scale, and Z-interval values.

A3. Compare optical sections from the confocal to the deconvolved images
For the Z-stack collected with the wide open pinhole, effectively a wide field image, you will process the data with the Huygens deconvolution software from SVI in the Biological Imaging Facility. For the Z-stack collected with the confocal pinhole in place, visualize the sample in 3D using the Zeiss software or FIJI (Image J) software.

Q1: How do the two 3D data sets compare (confocal versus deconvolved images)?

Q2: Which image is better? Are there any artifacts? Which 3D data set took longer to generate?

B. Time lapse analyses of fluorescently labeled zebrafish embryos
As in Part A we will be imaging living zebrafish embryos from the Sox10 GFP transgenic line which labels the developing neural crest. The goal is to do an overnight time-lapse experiment with each time point being a Z-stack and generate a movie of the migrating neural crest.

B1. Acquire Z-stacks over time of migrating neural crest cells.
Embryos will be sorted and mounted into a well in the 8 well dish as described in Part A. Mount multiple embryos so you have a selection to choose from (preferably 1 per well, not more than 2 per well). You can image either the cranial neural crest cells (such as those forming the jaws and gills) or the trunk neural crest cells as above. For this exercise you want to use a dry objective (20x is probably best). For these Z-stacks use the pinhole at 1 or 2 airy units. You want to keep the laser power relatively low, if you can, so open the pinhole up rather than upping the laser power (again use the 488 nm laser line). You will under sample for these Z-stacks, try and keep the number of sections relatively low so each time point will not take very long to collect. Time interval should be either 5, 10 or 15 minutes (depending how long it takes to collect your Z-stack). Set up a time lapse overnight. For the XYZT data set, generate a projection of each Z-stack, creating one image per time point and then generate a movie from the single projection at each time point using the Zeiss software or FIJI (Image J) software.

Q3: Why do we want to keep the laser power low?

Q4: Generate a movie file of your time lapse (prefer avi files over quick time). How fast do the neural crest cells migrate?

Considerations for Z-sectioning

PLEASE BE CAREFUL: *Watch out for clearance of the objectives! It is very important that you do not plow the objective into the coverslips. Adjust the focus slowly and watch for and avoid contact with the coverslip. Be careful that your z-stack is not too large to cause the objective to slam into the coverslip toward the end of the stack.*

Choosing Top and bottom boundaries of a Z-stack. There are a couple ways to set this parameter:

The best Z-stacks are collected using optimal intervals. Sections spaced too far apart will leave gaps. Sections placed too close means oversampling and bleaching due the excessive illumination. These best intervals are ~50% of the thickness of the optical slice that you are collecting. Remember that the thickness of the optical slice is related to the NA of the objective and the size of the pinhole used. Below are listed the approximate thickness of optical sections collected with our objectives.

All @ pinhole = ~1 Airy unit

Objective: 40x Air (NA=0.75)
Optical section thickness = 1.7µm
interval for Z sectioning = 0.84 µm

Objective: 40x Oil (NA=1.3)
Optical section thickness = 0.8 µm Optimal
Optimal interval for Z sectioning = 0.41 µm

Objective: 63x Oil (NA=1.4)
Optical section thickness = 0.7 µm Optimal interval
for Z sectioning = 0.35 µm

Choosing Top and bottom boundaries of a Z-stack

There are few options to set this parameter:

1. Do it by eye. Using epifluorescence or a fast LSM scan - focus down and up. Note the Z-position at bottom on the parameter windows. Then focus up until you get slightly above the specimen region of interest and note that Z position. Leave the scope focus at that top position. Make sure that you designate your current section position to 1. Then figure the total size of the stack you want to collect and divide by the interval to get the number of sections, add 1 and put that information into the dialog box for number of sections. Then start the Z-stack collection and it should go from the top ->down to the bottom that you selected (one section at a time).

2. Software based boundary setting. Make sure the **Number of Sections** times the **z Interval** is a distance greater the sample you are imaging but less than the working distance of the

objective selected! Place the Current Section Pos. where you think it is approximately (this does not have to be precise). Click on the Z-scan radio button. The scope will do a fast vertical scan. Then it will display this vertical scan with the current focus position in a dotted line and the top and bottom (as shown in the Z section dialog box) as solid lines. The user can move the top and bottom boundaries accordingly.

NOTE: Be aware that the software isn't intelligent and it will change the interval size as you move the top and bottom boundaries. So you will have to go back and change the number of sections in order to get you desired interval.

There isn't a way to keep the interval parameters constant while changing the boundaries.

Final Note: The output from a Z-stack is going to be a series of images. Make sure you save the output to a folder or you will have a mess on your data disk.

Z Sectioning in Zeiss software

Z Interval: Step size in microns

Number of Sections: User defined based on sample size.

Current Section Pos: Take care to always make sure that you are at the beginning of your stack

Refractive Correction: N2/N1

(Example: Oil/Water =1.518/1.0= 1.518)

Destination:

Video Memory: internal video memory buffer, **do not use.**

File: Use this option

Screen: Display only one image on the image screen

Host Memory: Save into computer's RAM, must be saved through the File menu.

Z-Scan: Line Scan through your sample at the center of the image window. Users can define boundaries for z-sectioning through the use of this function. **NOTE** that adjusting the top and bottom boundaries will change the interval size; you'll have to readjust it.

Move to X: Single scan at first, middle, or last positions.

Ok: starts the image acquisition.

Assignment: You can work in groups. Only written presentation is required.

- Answer Qs and present select images/movies illustrating the topics and tasks covered in this assignment.
- Use the Zeiss confocal software or ImageJ/Fiji to visualize your data and select images. Explore the various ways to visualize and analyze your data, for example: single-plane view; z-projection (average, maximum intensity); orthogonal-plane views in xy, yz, or xz. Generate a movie file to turn in (avi preferred) from projections of Z-stacks at each time interval.
- Remember to annotate all of the presented images, with relevant imaging parameters, descriptions of the sample, scale bar, visual aids (eg arrows pointing to feature that you want to highlight), etc.
- Discuss any problems and successes you had during imaging.

REFERENCES

Carney, T.J., Dutton, K.A., Greenhill, E., Delfino-Machín, M., Dufourcq, P., Blader, P., Kelsh, R.N., 2006. A direct role for Sox10 in specification of neural crest-derived sensory neurons. Development 133, 4619-4630.