SOP Test 1 - Measuring Locomotion in Larval Zebrafish

(See also the plug-ins: SOP Test 1b and 1c)

1.0 Purpose:

1.1 The purpose of this standard operating procedure (SOP) is to measure the locomotion of 6-day old larval zebrafish. Several parameters can be extracted from the initial Videotrack of larval locomotion. These parameters are described in detail below.

2.0 Scope:

2.1 This protocol is suitable for individuals who have been trained in zebrafish handling and care.

2.2. Any queries, comments or suggestions, either relating to this SOP in general, or to a specific problem encountered during the procedure should be addressed to the head of the AMATrace behaviour platform, Dr. Laure Bally-Cuif.

2.3. Any deviation from this protocol should be addressed to the head of the AMATrace behaviour platform, Dr. Laure Bally-Cuif.

2.4. All zebrafish should be kept, propagated and handled in accordance with the institutional guidelines on animal safety. Please also keep in mind the principle of replacement, refinement and reduction.

3.0. Safety Requirements

3.1. General laboratory safety procedures should be followed, which include: no eating, no drinking and no applying of cosmetics in the work area. Laboratory gloves must be worn at all times in the work area, unless the protocol specifically notes otherwise.

4.0. Associated Documents:

5.0 Notes:

5.1. This protocol is designed to compare the locomotion of 6-day old larvae that have been raised under similar conditions. Density in the Petri dish and age will play a significant role in modifying the expression of this behaviour. Ideally, larvae should be grown in small homogenous groups of about 25-30 animals from the single-cell stage onwards.

5.2. Maintain adult breeding zebrafish on a 14 hour light / 10 hour dark cycle at 28^0C according to standard protocols. Collect eggs from either group matings or pair-wise matings of wild-type fish. Remove any debris from the eggs and culture them in Petri dishes containing embryo medium for 6

days at 28^{o}C. Larvae will hatch from their chorion on day 2 or day 3. The discarded chorions can be removed using a plastic transfer pipette. The embryo medium should be replaced each day and any sick or dead larvae removed.

5.3. Environmental factors can play a significant role in changing the behaviour of larval fish. Behaviour should be recorded in a silent behavioural room with minimal experimenter disturbance. Lighting, temperature and time of day should be kept constant during testing.

6.0 Quality Control:

6.1. Larvae should be measured in clean 12-well plates. Plates can be reused if they are washed and thoroughly dried in between each experiment.

6.2. Larvae need to be raised in groups of a defined number (20-25 larvae in a group) from the single-cell stage onwards.

7.0 Equipment:

7.1. The locomotion setup contains three main parts:

a) A computer recording system that contains VideoTrack software from ViewPoint S.A.

b) A standard large plastic fish tank (30 x 30 x 50 cm) filled with 20L of water. A plastic model predator is made by filling a 15ml Falcon tube with dark blue modelling clay. The model is suspended from 5ml plastic pipette which is balanced across the top the tank. A thin metal wire is attached to the middle of the model, allowing it to be suspended in the middle of the water column at one end.

c) A Zebrabox (from ViewPoint S.A.) that is connected to the core computer. Each Zebrabox allows the measurement of 12 fish. Several boxes can be connected to each other to allow high-throughput measurement of larval behaviour.

8.0. Supplies:

Larval zebrafish for analysis, 20-25 for each genotype or treatment group.

(Optional) drugs or chemicals to modify behaviour

Embryo medium to fill the 12-well plate. A ten liter 60x stock solution can be prepared by adding 172g NaCl, 7.6g KCl, 29g $CaCl_2$ and 49g $MgSO_4$ to 10L sterile water. Add 160ml of the 60x stock solution to make ten liters of 1x embryo medium. Embryo medium can be stored for one week at room temperature.

Small 2ml plastic transfer pipettes for moving larval zebrafish between Petri dishes and 12-well plates.

20ml plastic graduated pipettes for filling each well of the 12-well plate with embryo medium.

9.0. Procedure:

9.1. Larval fish are raised to 6 days in groups of 25. Two hours before testing, the larvae are moved to the behaviour room to allow them to acclimatise to the ambient room temperature.

9.2. Setting up the Zebralab program (necessary for all three protocols)

9.2.1. Switch on computer and launch the "Tracking with Zebrabox" function within the ViewPoint application manager.

9.2.2. Select "New Protocol" from the File menu. A Protocol Parameters setup menu with four tabs will open automatically. Start with the locations tab. Specify the number of locations to record from (corresponding to the number of fish to analyze: 12 larvae per plate for locomotion or startle; 6 larvae per plate for thigmotaxis) by double clicking on each location name (e.g. c1, c2, c3 up to c6 / c12).

9.2.3. Switch to the second tab, Time. Specify an experiment duration of 5-minutes. Choose an integration period of 60 seconds. Each integration period will represent a data point in the final Excel results sheet. Thus an integration of 60 seconds will produce 5 data points in a 5-minute experiment.

9.2.4. Switch to the Start tab, and make sure that the "All locations at a time with background" option is selected. Switch to the Options tab and check that the Numeriscope box is ticked so that an AVI film of the behaviour will be recorded. Click on OK to exit the Protocol Parameters setup menu.

9.2.5. The Videotrack program has two main screens. The Full Screen mode shows a view of the 12-well plate, whereas the Tile screen shows each of the 12 locations as a Thumbnail. Toggle between the two screens by selecting "Full Screen" or "Tile" in the View menu. Alternatively, double-click on any of the 12 Thumbnails in the Tile screen to select that area and enter the View screen.

9.2.6. Toggle the action menu on the right-hand side by clicking on the Draw Areas button in the action bar. This button switches between the Draw Areas menu and the Detection Threshold menu. Start with drawing locations, one for each well (from now on referred to as "arena") of the twelve well plate. Switch to the Tile view and double click on Thumbnail 1. Click on the Circle Tool in the areas list, and using the mouse cursor draw a circle the size of the arena, starting in the centre of the arena. Make sure that this circle is specified as area 1 in the Area Number list. Areas can be modified by pressing the Select button and then clicking on the green line delineating the area. Active areas are shown as green lines, and inactive areas are shown as red or blue lines. Areas can be resized and moved with the mouse cursor. To resize an area, click and hold one of the small squares which are distributed around the outline. Areas can also be copied, pasted or deleted by choosing the appropriate button.

9.2.7. Toggle back to the Tile menu, double-click on the icon for area 2 and repeat this process for the 11 remaining areas.

9.2.8. Specify the size of the 12-well plate by clicking on the Draw Scale button and drawing a line across the image of the plate using the mouse cursor (click and hold the mouse button). Enter a reference length (in our case this is 120mm for the 12-well plate) and unit of length (choose mm from the dropdown list).

9.2.9. Move back to the Tracking menu (by clicking on the Draw Areas button) and set the movement threshold (Table 1). Small movements between 0 and 10 mm/second will be depicted as a green trace during the experiment. Large movements over 10 mm/second will be depicted as a red trace.

9.2.10. Set the background level for each larva separately. Choose an area and make sure that the Detection Threshold menu is visible on the right-hand side of the Full Screen view (toggle with the Draw Areas button). Select "Black" in the Animal Color menu and a detection threshold of 12. Click on the Background button and make sure that the camera detects the animal easily, with no disturbances from the background. Repeat this procedure for each area.

9.2.11. Save the protocol for future locomotion experiments (File > Save as). This protocol will form the basis of all the experiments described in this chapter.

9.2.12. To start the experiment, select Execute in the Experiment menu. Choose a file name and click on the Save button. Click on the Background button (this will reset the background in all areas and is unavoidable) and the Start button. A counter will display the amount of time elapsed during the experiment. At the end of the experiment, select Stop from the Experiment menu at the top of the screen. A pop up box will appear showing an overview of the results. Close the box by clicking on the OK button. An excel sheet will have appeared in the folder where you saved the experiment and is now ready for analysis.

9.3. Preparing 6-well or 12-well plates for analysis

9.3.1. Larval fish must first be placed in a 6-well (thigmotaxis) or 12-well (locomotion and startle) plate before the experiment begins. Use a 10ml sterile plastic pipette to place 2ml of embryo medium into each arena of a 12-well plate (or 4ml medium into each arena of a 6-well plate). Using a plastic transfer pipette, gently deposit a larva in each of the wells. If the volume of embryo medium significantly exceeds 2ml, remove the excess with the transfer pipette.

9.3.2. Larvae must be arranged in the plate and placed in the behavior room at least two hours before the experiment begins. This will ensure that the temperature is similar in both the testing room and the wells of the plate, as well as reducing the stressful effect of transporting the larvae.

9.4. Recording larval locomotion

9.4.1. Larval fish must first be arranged in a 12-well plate as described in section 3.3.

9.4.2. Place the 12-well plate containing larvae into the Zebrabox. Remove the lid from the plate and close the door. Be careful to place the 12-well plate in exactly the same orientation for each experiment.

9.4.3. Allow the larvae 5 minutes to recover from being placed into the Zebrabox.

9.4.4. Check that the protocol parameters are correct. Check that the "Numeriscope" function is activated and save the protocol with a name such as "locomotion.vtr". Start the experiment.

9.5. Data Analysis

9.5.1. The data should be analysed by using standard statistical tests. For data comparing two groups with a normal distribution, the Student's -test can be used. If more variables (such as drug or morpholino treatment) are included in the experiment, an analysis of variation (ANOVA) followed by an appropriate post hoc test should be used.

9.5.2. **Mean distance swum**

Use the "Sum" function in Excel to add together the distance swum in each of the integration periods (corresponding to 30 seconds in this experiment). Use the "Average" function to calculate the mean distance swum for each genotype or treatment group.

9.5.3. **Speed**

Speed can be calculated by dividing the total distance swam by the total recording time for each genotype or treatment group.

9.5.4. Plot the data as a histogram and use appropriate statistical tests to compare the different groups.

10.0 Supporting Information:

Norton, WHJ. Measuring larval zebrafish behavior: Locomotion, thigmotaxis and startle. In : Zebrafish Behavioral protocols, Part II ; Humana Press / Elsevier ; 2011 (in press).

11.0 History Review:

12.0 Emergency Procedures:

SOP Test 1b – Locomotion stability

SOP Test 1c - Locomotion impulsivity

1.0 Purpose

1.1 The aim of this standard operating procedure (SOP) is to replay locomotion data obtain following the Larval locomotion protocol to obtain new locomotion parameters. Steps 2 - 8 are similar to the ones described in SOP 1 (measuring larval locomotion).

4.0 Associated Documents

Measuring Locomotion in Larval Zebrafish (SOP Test 1)

9.0 Procedure

The data must be replayed using a protocol modified from the original and with a 3 seconds integration period to allow the analysis. To change the protocol and restart the experiment follow the steps below:

9.1 Setting up the Zebralab program

9.1.2 Change the protocol

–> *" parameter" > "protocol parameters" > second tab "time" > in integration period enter "3" secs > confirm the modification by clicking on "ok"*

9.1.2. Save the modified protocol

–> "file">"save as"

9.1.3. Restart the experiment; you must replay the .raw data file in the new protocol

–> *"raw data"> "replay" select your file of interest in .raw and then save with the new name*.

9.1.4. Results export; the results must be exported from .vtr to .xlsx extension in order to open them in excel and then sort the data.

–> *"results">" export" select your .vtr of interest and click on "ok".*

NB: The file will be saved in the folder where your original data are located.

9.2 Data analysis

In the Excel file, copy the columns "animal" "an" "end" "smldist" "lardist", and paste everything onto a new page. Select all and in the tab sort (data>sort), class your column in the following order: "an","animal","end". Use the "Sum" function in Excel to add together the distance swum (smldist"+ "lardist") per integration periods (corresponding to 3secs here) for all the animals.

9.2.1 Locomotion impulsivity

For the locomotion impulsivity analysis, you must plot the distance swum every three seconds during 5mn for individual animals, using the function graph on excel (linear representation). We counted as a peak all acceleration events where the fish travelled more than 5mm in less than 12s.

9.2.1 Locomotion stability

To see the locomotion stability we plotted the total distance swum after every three second-interval during a 120-second experiment for individual fish. When the fish locomotion is stable, the single curve obtained must be linear and represented by the equation f(x)=m.x where m (the slope of the linear equation) is the mean speed of the animal.