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## Z-Loc 4: The Standardized 4 dpf Zebrafish (*Danio rerio*) Larval Light/Dark Locomotion Protocol

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**Protocol status:** Working

**We use this protocol and it's working**

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**Protocol Integer ID:** 103639

**Keywords:** Zebrafish, Light/dark transition test, Standardised testing, Locomotor activity, 3Rs of animal research

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## Abstract

Zebrafish (*Danio rerio*) are a dynamic model organism, the larval stage ( $\leq 4$  days post-fertilization (dpf)) of which offers a unique avenue for high-throughput *in vivo* investigation in a vertebrate that aligns strongly with the 3Rs of *in vivo* research. The light/dark locomotor assay is widely employed for studying larval behavior; however, there remains a lack of experimental consistency. Here, we provide step-by-step experimental and analytical protocols for a standardized behavioral assay that exploits locomotion differences during light/dark phases. -

For details on the use and execution of this protocol, please refer to Hillman (2024).

## Image Attribution

Figures and images were created using [Biorender.com](#).

## Materials

The materials required for the protocol presented here can be found in **Table 1**.

Material	Source	Identifier
8-Channel Multi-Channel Pipette	FisherbrandTM EliteTM	11835772
DMSO	Sigma-Aldrich	5.89569
46-well plates	CorningTM Co starTM	Corning 3548
96-well plates	CorningTM Co starTM	CorningTM 35 48
P1000 Pipette	GilsonTM	F144059M
Zanscript	OSF	<a href="https://osf.io/k7y2c/">https://osf.io/k7y2c/</a>
Custom R script	OSF	<a href="https://osf.io/k7y2c/">https://osf.io/k7y2c/</a>
R Studio	Postit Software	<a href="https://posit.co/download/r-studio-desktop/">https://posit.co/download/r-studio-desktop/</a>
Benchtop Incubator	Online Reptile Shop	<a href="https://www.onlinereptileshop.co.uk/lucky-reptile-herp-nursery-ii-incubator.html">https://www.onlinereptileshop.co.uk/lucky-reptile-herp-nursery-ii-incubator.html</a>
Aqua-Sed	Vertark(R)	<a href="https://www.zmsystems.co.uk/new-aqua-sed-anaesthetic-treatment-250ml-410-p.asp">https://www.zmsystems.co.uk/new-aqua-sed-anaesthetic-treatment-250ml-410-p.asp</a>
Zebrafish: Strain AB	University of Portsmouth Fish Facility	N/A
250 mL Petri dish	Sigma-Aldrich	P5981
P1000 Pipette Tips	Brand(R)	Z740105
P200 Pipette Tips	Brand(R)	Z740105
Zantiks MWP	Zantiks	<a href="http://zantiks.com">zantiks.com</a>

**Table 1:** The materials and reagents required for this protocol.

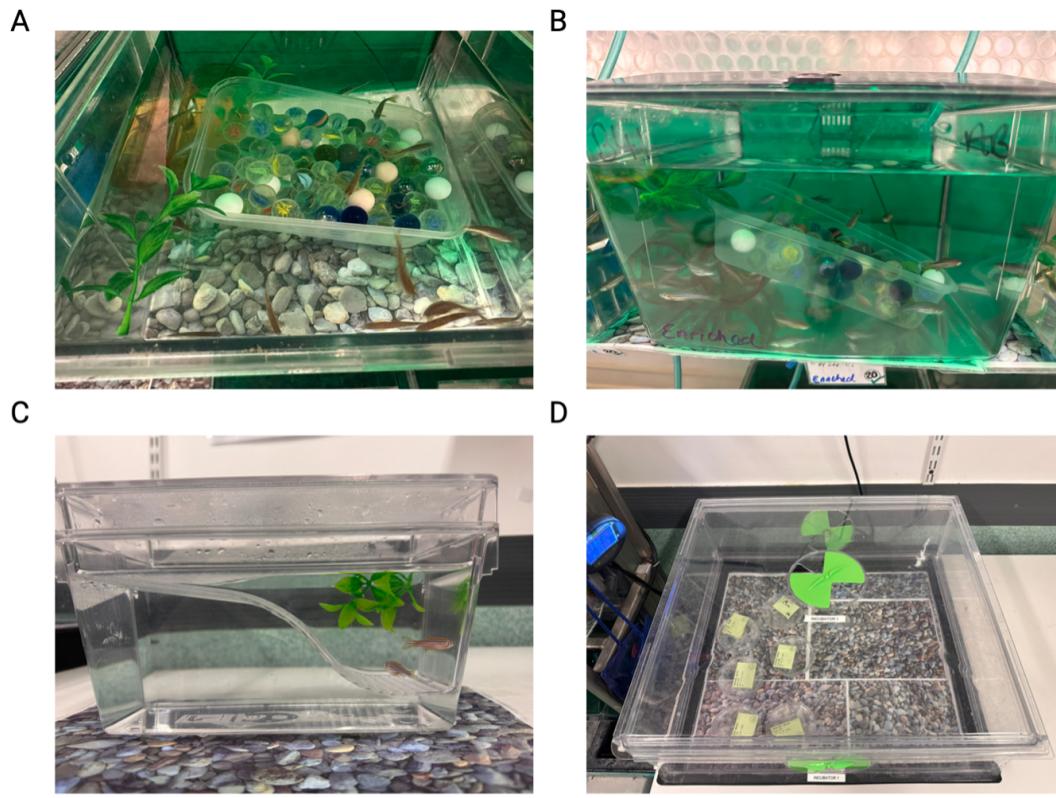
## Before start

### Breeding and Rearing of Zebrafish

#### Timing: 4 days

This section describes the method for breeding and rearing the zebrafish larvae in preparation for the light/dark behavioral assay. 5 days prior to running the larval behavioral assay the adult zebrafish (6-12 months) should be prepared for breeding.

1. Breeding is performed through the addition of substrate (typically marbles) to the home tank and/or pair breeding. For marbling, the night before embryo collection place a container of marbles into the tank of adult fish. The following day, at 1100 - mid-day remove the container and collect the embryos (**Figure 1A and B**). For pair breeding, select either one male and one female, or two females and one male, or two males and one female and place into a small tank with a middle divider and a slope. Split the sexes the night before and the next morning remove the divider when the lights in the facility turn on and allow 30-mins for breeding before returning to their home tank and collecting the embryos (**Figure 1C**).



**Figure 1: The breeding and rearing set-ups for generating the embryos for the experiment.** A and B show the substrate breeding, C is an example of pair-breeding and D is the rearing incubator the larvae are placed in following breeding.

2. Transfer ~80 0 hour post-fertilization (hpf) embryos into labelled 250 mL Petri dishes, filled with system water (or the water you use in your lab) and place the dishes into a temperature controlled incubator with a standard 14h:10h light/dark cycle (**Figure 1D**). Each morning post breeding perform a complete water change of the Petri dishes and remove any dead/unfertilized larvae.

### Note

We recommend keeping enrichment (e.g., pebble sheets and plants) in all tanks and incubators (**Figure 1**).

## Uploading Tracking Script to Behavioral Unit

### Timing: 5-mins

This section describes the setup and use of the **zantiks** (Zantiks Ltd., Cambridge, UK) automated tracking script for running the light/dark assay using MWP behavioural units.

3. Download the tracking script from the attached file  ZANTIKS script.docx 18KB or from the [OSF](#) linked to this protocol.
4. Connect to the Zantiks Wi-Fi specific for your unit(s)
5. Open the zanscript section and upload the downloaded script

**CRITICAL:** If you are using a different automated system ensure the tracking settings are identical (record in 1 second time-bins, 5-minute light (350 lx) to dark transitions repeated three times for a total of 30-minutes. Output as movement per mm.)

## Habituation to New Environment

30m

- At 8:30 am, transfer the Petri dishes of 4 dpf larvae from the fish facility holding room to the experimental room and place in a benchtop incubator set at  28 °C for  00:30:00 of acclimating in the light.

### Note

If behavior takes place in the same room as the rearing room there is not need to acclimate (skip step 1)

- Immediately after starting habituation, switch on the **Zantiks** MWP behavioral unit(s) and allow the temperature to get to  28 °C .

10m

### Note

Check the heating times of the automated system you are using to ensure adequate time to reach experimental temperature.

## Plating Larvae (Timing: 8-mins/plate)

8m

- Using either a wide bore pipette tip, or a standard p1000 with the tip cut at approximately 2 mm from the end, transfer  225 µL of facility water and a randomly selected larvae into each well of a 48-well plate.

## Acclimation to the Zantiks MWP Behavioural Unit

30m

- Transfer the full well plate with the lid on into the behavioral unit with the lights on for  00:30:00 of untracked acclimation

30m

### Note

**CRITICAL:** The front of the unit must be covered to prevent any external light entering and impacting the responses

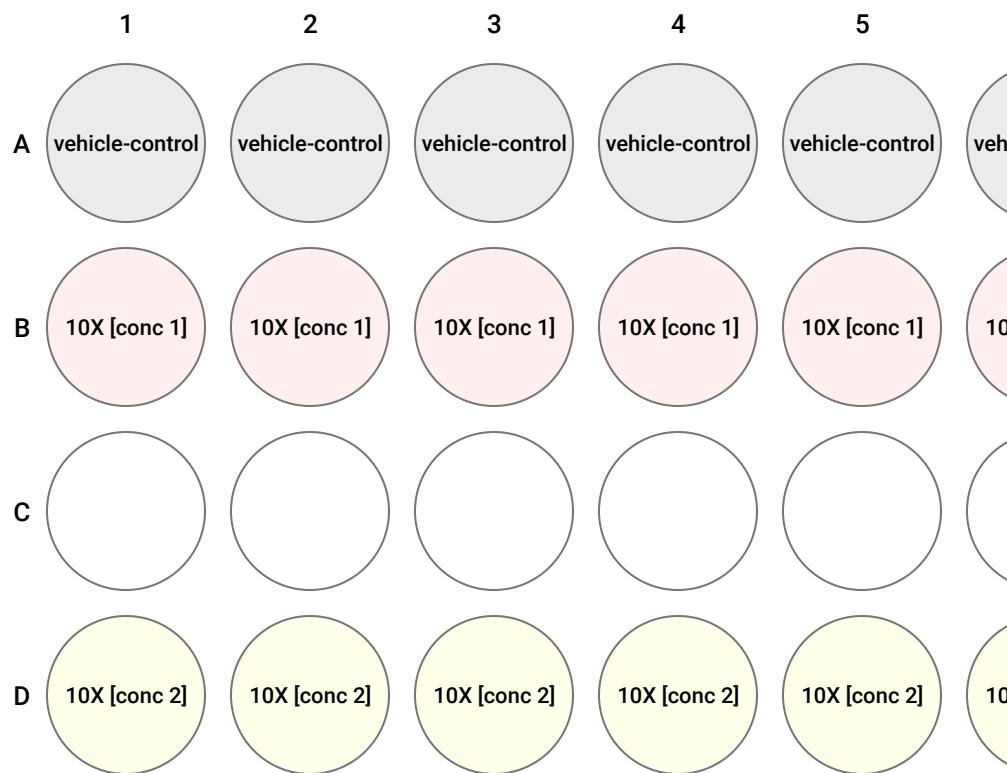
## Baseline Recording and Chemical Exposure Preparation

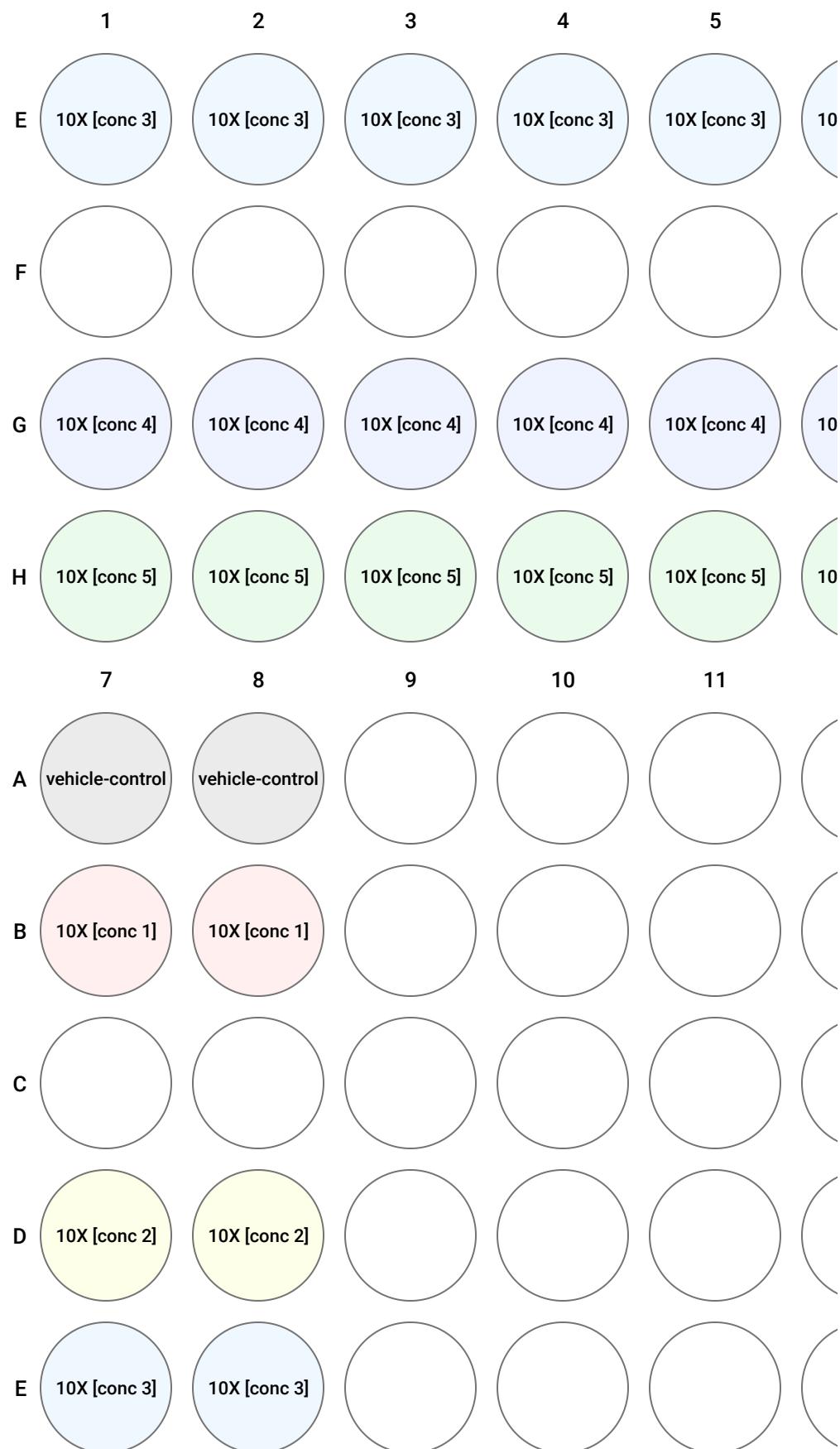
30m

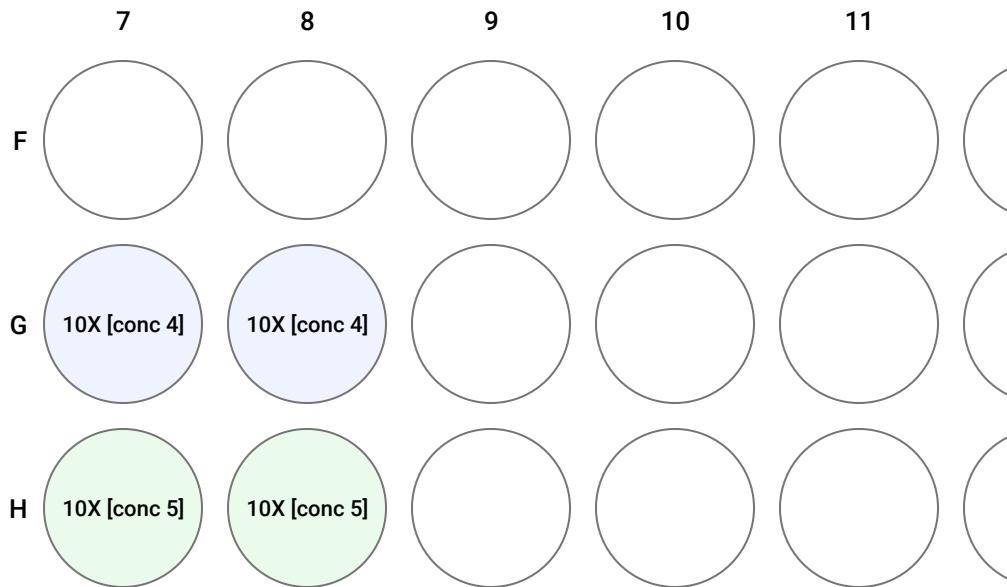
- 5 Run a baseline recording using the Zanscript tracking script (see before start: **Uploading Tracking Script to Behavioral Unit**). 30m
- 6 Immediately after starting the baseline recording, prepare your 10X solutions for drug exposure in a 96-well plate (**Figure 2 and Table 1**). 20m

### Note

NOTE:  $\ddot{\text{v}} 25 \mu\text{L}$  of the 10X concentration will be added to the larvae, therefore ensure a volume of at least  $\ddot{\text{v}} 30 \mu\text{L}$  is in the well of the 96-well plate. All drug exposures are performed using the same vehicle to ensure consistency throughout. We use dimethyl sulfoxide (DMSO) at a final concentration of  $[\text{M}] 0.5 \% (\text{v/v})$ , which is safe in larvae (Hoyberghs et al., 2021). **Table 1** provides a detailed summary of the requirements for a final 10X exposure volume of  $\ddot{\text{v}} 500 \mu\text{L}$ , which is enough to expose 16 larvae (2 well plates).







**Figure 2: Example 96-well plate setup with increasing 10X concentrations from A to H.** The vehicle control should also be a 10X concentration (e.g., for a final concentration of 0.5% DMSO, 5% DMSO should be made up in the 96-well plate). Rows C and F are kept empty.

Desired exposure concentration ( $\mu\text{M}$ )	10X concentration ( $\mu\text{M}$ )	Required stock concentration (mM)	Volumes
0	N/A	N/A	25 $\mu\text{L}$ of DMSO + 475 $\mu\text{L}$ embryo medium
0.5	5	0.1	25 $\mu\text{L}$ of 0.1 mM stock + 475 $\mu\text{L}$ embryo medium
5	50	1	2.5 $\mu\text{L}$ of 10 mM stock + 22.5 $\mu\text{L}$ DMSO + 475 $\mu\text{L}$ embryo medium
50	500	10	25 $\mu\text{L}$ of 10 mM stock + 475 $\mu\text{L}$ embryo medium

Desired exposure concentration ( $\mu\text{M}$ )	10X concentration ( $\mu\text{M}$ )	Required stock concentration (mM)	Volumes
500	5000	100	2.5 $\mu\text{L}$ of 1M stock + 2.5 $\mu\text{L}$ DMSO + 475 $\mu\text{L}$ embryo medium
500	50,000	1000	25 $\mu\text{L}$ of 1M stock + 475 $\mu\text{L}$ embryo medium

**Table 1: Example of the pharmacological calculations required for dosing 4 dpf larvae.** This ensures a final volume of 500  $\mu\text{L}$  which is sufficient for dosing  $n = 16$  larvae with a final vehicle concentration of 0.5% for all exposure concentrations.

## Dosing the Larvae and Recording Exposure Response

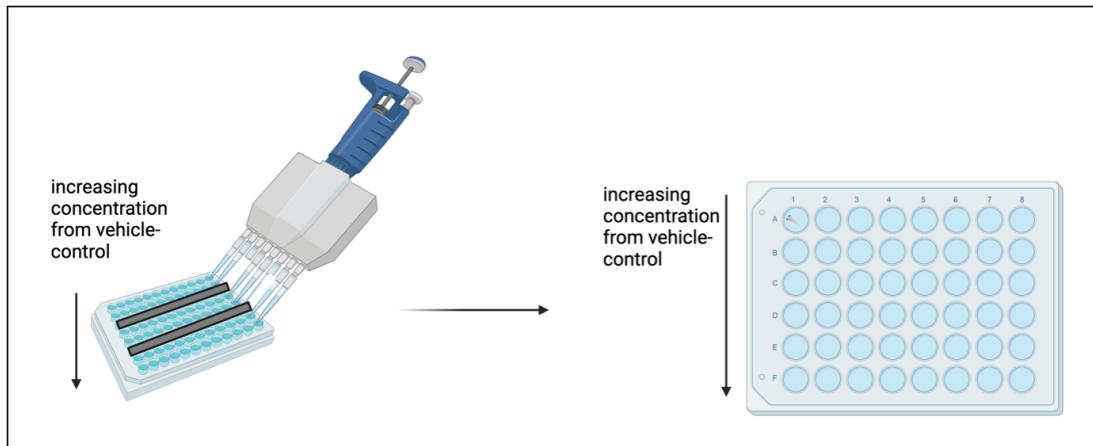
3h

- 7 Immediately after the baseline recording finishes remove the plate from the behavioral unit.

### Note

Keep a steady hand whilst moving the plate to minimize handling stress.

- 8 Using a multi-channel pipette, add  25  $\mu\text{L}$  of the 10X dilutions from the 96-well plate to the corresponding well of the 48-well plate (**Figure 3**).



**Figure 3:** Schematic of the dosing process using a multi-channel pipette with prepared drug solution from a 96-well plate to the larvae in the 48-well plate.

#### Note

**NOTE:** the third and sixth channels of an 8-channel pipette are removed to match with the 96-well plate setup.

**Critical:** This step must be completed as quick as possible to ensure all fish are exposed at the same time for accurate behavioral recording.

- 9 Once all the wells have been exposed, return the well plate with the lid on to the behavioral unit and restart the light/dark tracking script for a 0-30-min post-exposure recording.

#### Note

**OPTIONAL:** Following the 0-30-min recording, leave the fish untracked in the light for  00:30:00 and re-run the 30-min light/dark tracking script for a 60-90-min recording.

**Critical:** If you are running an extra 60-90-min recording the light untracked period is essential to prevent habituation to the light/dark transitions (Hillman et al., 2024).

**NOTE:** This process can be continued for as long as the experimenter requires.

- 10 Once complete, remove the fish and dispose of as appropriate.

## Note

**NOTE:** We use 2-phenoxyethanol (Aqua-Sed<sup>(R)</sup> Anesthetic by Vertak, Winchester, UK).

- 11 Download all the datafiles generated by the tracking script for analysis.

## Analytical Process

45m

- 12 Save and rename the baseline recordings as 'baseline\_x'.xlsx where x is an increasing number from 1 depending how many runs were performed.
- 13 Create a new Microsoft Excel document for the exposed analysis with the concentration in row 1 and the 1800 sec movement data in the following rows.
- 14 Open a new R project and upload the custom R script  R script for analysis.R 52KB or download from the [OSF](#) file.
- 15 Change the group sizes to the same sizes used within your experiment in lines 38, 299, 532, 715, 736 and 835.

```
>#Calculate movement per minute for each fish (each column)
>num_fish <- ncol(data_subset)
>group_sizes <- c(16, 16, 16, 16, 16, 16) #Group sizes INPUT YOUR
SAMPLE SIZES FROM EXPERIMENT HERE
```

**NOTE:** We strongly encourage a sample size of  $n = 16$  per concentration for preliminary power calculations.

- 16 Change the file path to your own file path where the saved datafiles are in lines 137 and 483.

```
># Process dataset
>dataset_1_date <- process_dataset("INPUT YOUR FILE PATH HERE FOR
THE EXPOSED DATASET")
```

- 17 Change the file path to your own file path where the saved baseline files are for lines 245, 248, 478 and 481.

```
># Process baseline_1 dataset  
>baseline_1_data <- process_baseline("baseline_1", "INPUT YOUR  
FILE PATH HERE FOR THE EXPOSED DATASET.xlsx")  
  
># Process baseline_2 dataset  
>baseline_2_data <- process_baseline("baseline_2", "INPUT YOUR  
FILE PATH HERE FOR THE EXPOSED DATASET.xlsx")
```

**NOTE:** If this is a preliminary run you will have two baselines. Alternatively, if you have a large sample size and numerous different baselines, add more lines.

- 18 Change the lines for calculating the mean and standard error of the mean (SEM) for producing the line graphs in lines 359-364 and 376-381).

```
># Initialize an empty dataframe to store the means  
>mean_df <- data.frame(Time= 1:30)  
  
># Calculate the means for each group, ignoring NA values  
>mean_df$Group_1 <- apply(dataset_normalised_minute_analysis[,  
1:16], 1, mean, na.rm = TRUE)  
>mean_df$Group_2 <- apply(dataset_normalised_minute_analysis[,  
17:32], 1, mean, na.rm = TRUE)  
>mean_df$Group_3 <- apply(dataset_normalised_minute_analysis[,  
33:48], 1, mean, na.rm = TRUE)  
>mean_df$Group_4 <- apply(dataset_normalised_minute_analysis[,  
49:64], 1, mean, na.rm = TRUE)  
>mean_df$Group_5 <- apply(dataset_normalised_minute_analysis[,  
65:80], 1, mean, na.rm = TRUE)  
>mean_df$Group_6 <- apply(dataset_normalised_minute_analysis[,  
81:96], 1, mean, na.rm = TRUE)  
  
># Print the resulting dataframe  
>print(mean_df)  
  
>#####  
  
>#Initialize an empty dataframe to store the SEM values  
>sem_df <- data.frame(Time = 1:30)  
  
># Calculate the SEM for each group, ignoring NA values  
>sem_df$Group_1 <- apply(dataset_normalised_minute_analysis[,  
1:16], 1, function(x) sd(x, na.rm = TRUE /sqrt(sum(!is.na(x)))))  
>sem_df$Group_1 <- apply(dataset_normalised_minute_analysis[,  
17:32], 1, function(x) sd(x, na.rm = TRUE /sqrt(sum(!is.na(x)))))  
>sem_df$Group_1 <- apply(dataset_normalised_minute_analysis[,  
33:48], 1, function(x) sd(x, na.rm = TRUE /sqrt(sum(!is.na(x)))))  
>sem_df$Group_1 <- apply(dataset_normalised_minute_analysis[,  
49:64], 1, function(x) sd(x, na.rm = TRUE /sqrt(sum(!is.na(x)))))  
>sem_df$Group_1 <- apply(dataset_normalised_minute_analysis[,  
65:80], 1, function(x) sd(x, na.rm = TRUE /sqrt(sum(!is.na(x)))))  
>sem_df$Group_1 <- apply(dataset_normalised_minute_analysis[,  
81:96], 1, function(x) sd(x, na.rm = TRUE /sqrt(sum(!is.na(x)))))
```

**NOTE:** The example uses the sample size of 16 per concentration, change accordingly.

- 19 Change the labels for the line graph to match your tested concentration/exposure solutions (line 456).

```
>scale_color_manual(values = group_colors, labels = c("Change to  
match your concentrations")) +  
>theme_minimal() +  
>theme(...
```

- 20 Change the lines corresponding to the sample size for vehicle-controls in the swim phenotypes analysis (lines 672 and 675).

```
>#Select rows corresponding to control fish  
>control_fish_light_raw <- light_phase_results_raw[1:16, ]  
  
>#Select rows corresponding to control fish  
>control_fish_dark_raw <- dark_phase_results_raw[1:16, ]
```

**NOTE:** The example uses the sample size of 16 per concentration, change accordingly.

- 21 Change to match your concentrations/exposure solutions and the sample sizes for generating the dataframe to save the swim phenotype data (lines 712 and 715).

```
>#Define group names  
>group_names <- c("Change to your concentrations")  
  
>#Create a new column indicating the group for each row  
>group_column <- rep(group_names, c(16, 16, 16, 16, 16, 16))
```

- 22 Prepare to generate the heat maps by changing the group sizes and concentration labels accordingly (lines 736, 740, 748, 764, 835, 862).

```
>#Group sizes as per your requirement  
>group_sizes <- c(16, 16, 16, 16, 16, 16)  
  
>Exclude the control group  
>dark_phase_results_log10 <- dark_phase_results_log10 %>%  
    >filter(Group != "control")  
  
>#Calculate the average response for each concentration  
>average_responses_df <- dark_phase_results_log10 %>%  
    >group_by(Group)  
%>%  
    >summarise_all(mean, na.rm = TRUE)  
  
>#Define the desired order  
>desired_order <- c("Change to your concentrations")  
  
...  
  
>labRow = c("Change  
to your concentrations")
```

- 23 To create the radar charts, the concentrations must be changed and you must input your data from the files generated named "average\_responses\_df" and "average\_responses\_df\_light" (lines 928-33, 937, 972-78, 980). Below is an example of the dark phase data.

```
>#Sample data
>data <- data.frame(
  >  Group = c("Change to your concentrations"),
  >  total_movements = c(change to the data for dark total
  movements),
  >  zero_movenys = c(change to the data for dark zero
  movements),
  >  darting_movements = c(change to the data for dark
  darting movements),
  >  steady_swim_movements = c(change to the data for dark
  steady swim),
  >  slow_swim_movements =c(change to the data for dark
  slow swim)
>)

>#Reorder the levels of the Group factor
>data$Group <- factpr(data$Group, levels = c("Change to your
concentrations"))
```

- 24 To prepare the data for statistical analysis, the dataset has to be restructured, change this section depending on your sample sizes. (Lines 1033-1045).

```
>#Determine thegroup for each fish
>if(i <= 16) {
  >fish_group <- rep(1, num_timepoints)
>} else if (i <= 32) {
  >fish_group <- rep(2, num_timepoints)
>} else if (i <= 48) {
  >fish_group <- rep(3, num_timepoints)
>} else if (i <= 64) {
  >fish_group <- rep(4, num_timepoints)
>} else if (i <= 80) {
  >fish_group <- rep(5, num_timepoints)
>} else {
  >fish_group <- rep(6, num_timepoints)
>}
```

- 25 Input your sample sizes in the 1458 to calculate observed power.

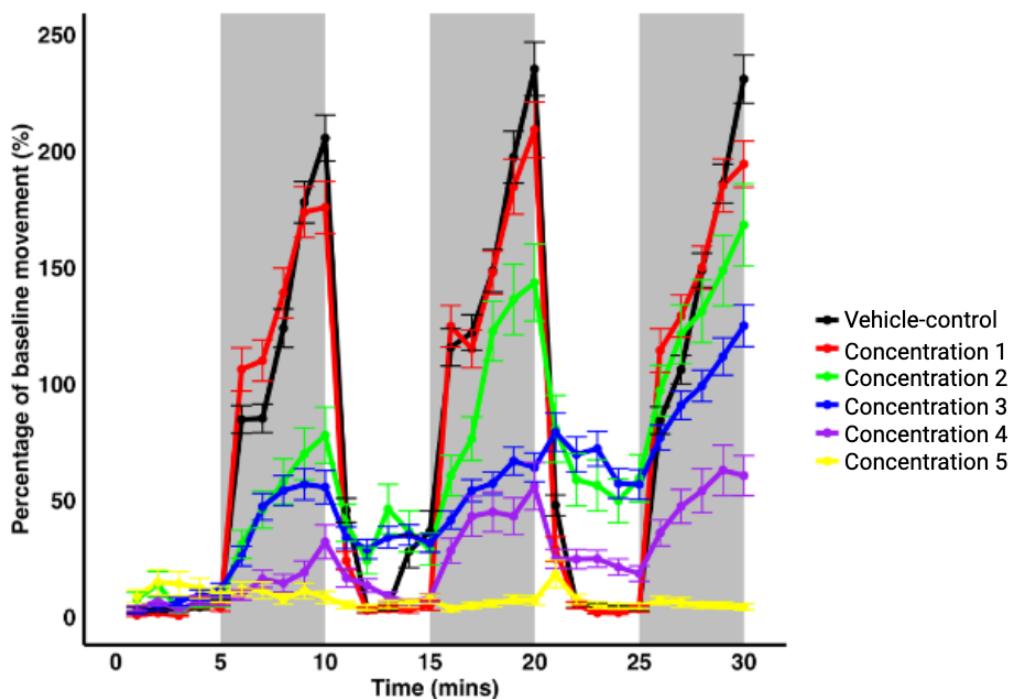
```
># Example sample size per group  
> n <- 16
```

## Expected Outcomes

26

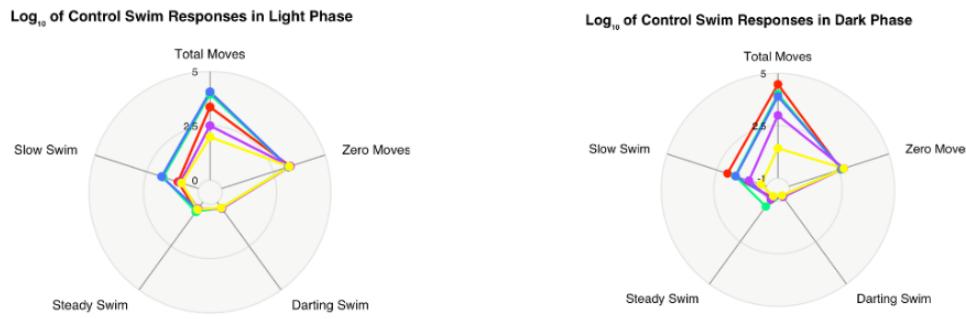
## Expected result

The experiment will produce a baseline and post-exposure recording with distance travelled in mm per second per fish as the outcome over a 1800 second period. These files can then be used in the custom R script linked to this protocol to produce a range of findings. The overall locomotion for each exposure concentration over a 30-minute period is visualized with a line graph (**Figure 4**).



**Figure 4: The minute-analysis of the larval light/dark assay.** This demonstrates an example of the expected line graph produced following exposure to 6 different concentrations. The traditional light/dark response can be seen with vehicle-control.

In addition, the script will compute the different swim phenotypes that the fish display during exposure compared to vehicle-controls (total movements, zero movements, darting movements, steady swim movements and slow swim movements). These findings are visualized with radar charts and/or heat maps (**Figure 5**).



**Figure 5: The light phase and dark phase radar charts of the different calculated swim phenotypes.** This shows an example of the potential radar charts that can be produced using the custom R script. The heat maps show the same data and are included in the code but are not shown here.

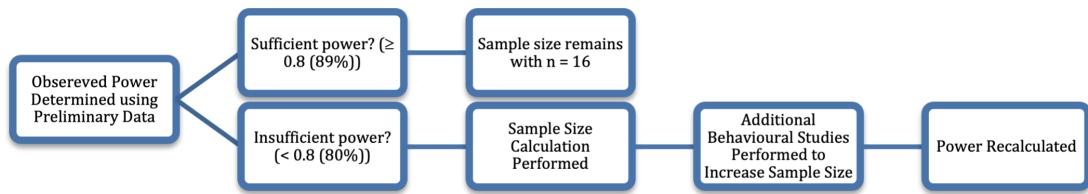
For further information regarding the types of movements and what can be interpreted from the swim phenotypes see the [paper](#) linked to this protocol.

## Quantification and Statistical Analysis

1h

- 27 We perform a normalization of the data by calculating the percentage of movement per minute per fish to the overall baseline response. This step is essential to ensure reproducible and robust responses (Hillman et al., 2024). Tracking errors are common and are eliminated using a regularly used method for outlier identification and removal (median absolute deviation) (Leys et al., 2013).

An important step in the statistical analysis is the calculation of observed power and sample size. We have provided a flow chart for how to deal with 4 dpf larval behavior sample size and power analyses (**Figure 6**). A preliminary behavioral experiment must be conducted with a sample size of  $n = 16$  per concentration. The R script can be run and observed power calculated for dark phase responses. Where power is below 0.8 (80%), sample size is calculated. If the observed power is above or equal to 0.8 (80%) then the sample size can remain as  $n = 16$ .



**Figure 6:** Flow chart depicting the steps taken when running a power analysis on the preliminary light/dark findings and sample size determination.

The first analysis performed is a linear mixed model (LMM) analysis with movement as the dependent variable and lights as the independent variable with fish ID as the random effect. This is done for group and time in place of lights as the independent variables. An example of the output is below:

```
>Linear mixed model fit by REML ['lmerMod']
>Formula: movement_percentage ~ Lights + (1| ID)
> Data: new_dataframe

>REML criterion at convergence: 26662.2

>Scaled residuals:
> MIN           10          Median
3Q           Max
>-1.9739      -0.6410    -0.1463      0.4106
6.8067

>Random effects:
>Groups       Name        Variance   Std.Dev.
>ID           (Intercept) 2247        47.4
>Residual                 5972        77.28
>Number of obs: 2294, groups: ID, 96

>Fixed effects:
>             Estimate   Std.Error   t value
>(Intercept) 47.56101   3.6814    12.92
>LightsDark   53.31557   1.02314   52.11

>Correlation of Fixed Effects:
>                   (Intr)
>LightsDark -0.337
```

Next, a full model is run (this includes all three of the independent variables of interest) and a null model, which is only including the dependent variable and the random effect. This allows for likelihood ratio tests to be performed with the null model and all the tested models. For example:

```

> Data: new_dataframe
>Models:
>null_model: movement_percentage ~ (1|ID)
>model_1: movement_percentage ~ Lights + (1|ID)
>
      npar      AIC      BIC      logLik
deviance      Chisq    Df   Pr(>Chisq)
>null_model  3       27221 27238 -13607          27215
>
>model_1      4       26680 26702 -13336          26672
543.11 1      <2.2e-16 ***

```

These calculations are performed with dark phase data as well.

A large amount of data can be obtained from these calculations. **Table 2** describes the different outcomes:

Outcome	Interpretation
REML convergence	Used to compare models, a lower value indicates a better model fit.
Scaled residuals	Provide an idea of the distribution of residuals (differences between observed and predicted values)
Random effects ID	Provide an overview of the variability in movement due to differences between the individual fish.
Fixed effects intercept values	Represent average movement response across the fish.
Npar	The number of parameters estimated in each model.
AIC (Akaike information criterion)	A measure of the relative quality of statistical models for a given set of data. Lower values indicate a better fit.
BIC (Bayesian information criterion)	A measure of the relative quality of statistical models for a given set of data. Lower values indicate a better fit.
LogLik	The Log-Likelihood of the model with higher values indicating a better fit.
Deviance	Represents the deviance of the model, a lower value indicates a better fit.
Chisq	Likelihood ratio test statistic to measure the fit of the model, a large value indicates an improved fit compared to the null model.
DF	degrees of freedom
p-value	Probability of observing a large chisq if the null hypothesis were true. Significant means the tested model is a better fit than the null model.

**Table 2:** The different statistical outcomes that you will obtain when running the R code and how to interpret them.

Next, linear regressions for the different tested concentrations in the dark phase are run with time as the independent variable. This allows investigation of the impact of time on dark phase response. For example:

```
>Call:
>lm(formula = movement_percentage ~ Time, data = group_data)

>Residuals
> MIN           1Q       Median
3Q           Max
>-163.11      -86.23      -16.07
    72.98      313.54

>Coefficients:
>                         Estimate        Std. error        t value
Pr(>|t|)
>(Intercept)   116.0597      5.793
20.034          <2E-16
>Time          1.7426      0.2963
5.881          4.81E-9

>Residual standard error: 106.1 on 1911 degrees of freedom
>Multiple R-squared: 0.01778, Adjusted R-squared: 0.01726
>F-statistic: 34.58, p-value: 4.81E-9
```

The findings suggest an increase in dark phase locomotion as time progresses (time estimate). Despite a significant positive effect, the low R-squared value suggests that time alone does not account for the variability in the movement.

The swim phenotypes are analyzed individually using ANOVAs comparing the group (concentration) locomotion in either light or dark conditions. For example:

	df	SumSq	MeanSq
F-value	Pr(>F)		
>Group	<b>7      244.8</b>	<b>34.97</b>	
<b>14.33</b>	<b>1.96e-13***</b>		
>Residuals	<b>120     292.9</b>	<b>2.44</b>	

The df represents the degrees of freedom and residuals refers to the degrees of freedom associated with the residuals of the model. The sum of squares represents the variability explained by the group (concentration) and the residuals represents unexplained variability after accounting for the group factor. The F-value is the test statistic for ANOVA's which assesses whether the mean differences among the groups are statistically significant. Here the output indicates a significant difference among the group factor in terms of the effect on total movement.

## Troubleshooting

### 28 Problem 1: Issues with tracking larvae go to step #5

Potential Solution: Tracking errors are typically seen with poor tracking settings on the MWP unit. The script we have provided should ensure this doesn't occur. However, there may be an issue with the asset set up on your unit. Each unit should have a uniquely set up asset matching to the 48-well plate you are using. **Zantiks** provide a step-by-step guide for adding your own assets to the MWP unit

## Protocol references

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