SUMMER RESEARCH INTERNSHIP PROJECT

EVALUATION OF PHOTOTACTIC BEHAVIOUR IN PLANARIA

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CERTIFICATE

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INTRODUCTION:

Light sensing is known to have evolved in multiple ways and affect fitness over the course of evolution. The diversity among organisms in their ability to process information in incident light with specific behavioural outputs is fascinating and an area of valuable exploration. [1] [2]

Planarians are flatworms (phylum: Platyhelminthes) and are one of the simplest organisms with an organized nervous system. [3] The simple structure of the eye, their remarkable regenerative capacity and their structured behaviour to light input make planarians a suitable organism for interpreting the genetic mechanisms, understanding photoreceptors, and information processing in more complex organisms. [1]

The response and the cognitive process as a consequence to light stimuli and the altered response over the course of regeneration is being explored in planaria. Planaria are shown to be negatively photo-tactic (movement away from light). They also have a whole-body(extra-ocular) response apart from the processive eye-brain mediated (ocular) response. This has been shown by their UV avoidance post-decapitation. [1]

How organisms respond and process light is yet to be deciphered completely. Quantitative and detailed analysis of the light mediated behaviour in planarians will help us understand information processing in higher organisms.

OBJECTIVE:

To develop analysis methodologies to understand behaviour and light sensing capabilities of planarians.

REVIEW OF LITERATURE:

A. Planaria as a model organism:

Planarians are flatworms (phylum: Platyhelminthes) that live in freshwater and marine environments throughout the globe. Few are terrestrial and live in soil and humid areas.

Planarians have remarkable regenerative properties. A single worm can be cut into hundreds of pieces and each piece will grow back into a whole planarian. Regeneration happens very quickly; it takes a few days for a single worm to regrow its head. Cells at the location of the wound site proliferate to form a blastema that will differentiate into new tissues and regenerate the missing parts of the piece of the cut planaria. These adult stem cells are called neoblasts, and comprise 20% or more of the cells in the adult worm. They are the only proliferating cells in the worm, and they differentiate into progeny that replace older cells. In addition, existing tissue is remodelled to restore symmetry and proportion of the new planaria that forms from a piece of a cut-up organism. [3]

Neoblasts present throughout the body, are the only mitotically active cells in planarians, and their division progeny generate the approximately 40 different cell types found in the adult organism. [4]

Due to simple organization of their nervous system, recent studies have tried to understand and explore the architecture of their neuronal connectome and its functions. Several studies have also demonstrated the phototactic and chemotactic behaviour of planarians. [1][5][6]

A well -studied and suitable model organism for regeneration, stem cell research, and development of tissues such as the brain and germline and behavioural studies is the freshwater planarian ***Schmidtea mediterranea***.

1. *S. mediterranea* is a stable diploid possessing four pairs of chromosomes.
2. It has a relatively small genome (the haploid genome of *S. mediterranea* contains ~7 x 10^8 bp) making it relatively easy to manipulate and sequence the genome.
3. This species exists in two biotypes - one sexual, the other asexual - allowing for a comparison of both sexual and asexual reproduction and embryogenesis and regeneration.
4. Because of its robust regenerative capacity, it is possible to generate clonal lines that have limited polymorphisms in the population, thus facilitating gene isolation, and spatial and functional assays.
5. The complex anatomy of planarians is well represented in *S. mediterranea*, thus making it possible to identify tissue-specific markers and define and visualize all organ systems.
6. RNAi can be performed using dsRNA with ease in planaria. The mRNA levels can then can be checked by performing qPCR or in situ hybridization [10]. By introducing dsRNA technology into planarians, scientists have been able to transform *S. mediterranea* into a regeneration model system in which gene function can be analysed. RNAi based loss-of-function screens have been carried out and it is now possible to identify genes and genetic activities associated with regeneration, tissue homeostasis and behavioural phenotypes. [7]

The sequence of the *S. mediterranea* genome is available in both the National Centre for Biotechnology Information (NCBI) website or the *S. mediterranea* genome database (SmedGD). Alvarado, et al., introduced the SmedDB (*S. mediterranea* database) as a means of studying *S. mediterranea* at the molecular level. They have indicated that several planarian cDNAs display similarities with human genes that coded for proteins whose functions are unknown. These cDNA sequences were not found in the genomes of two commonly used model organisms, *C. elegans* and *D.*

melanogaster. This revealed that *S. mediterranea* could also prove to be important in studying genes that may be involved in pathways implicated in human diseases. [8]

Image Source: ***Schmidtea mediterranea.*** User: Alejandro64, From Wikipedia, the free encyclopedia

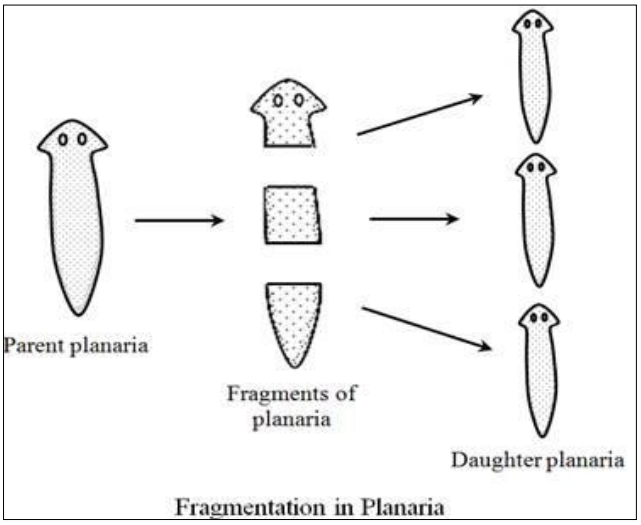


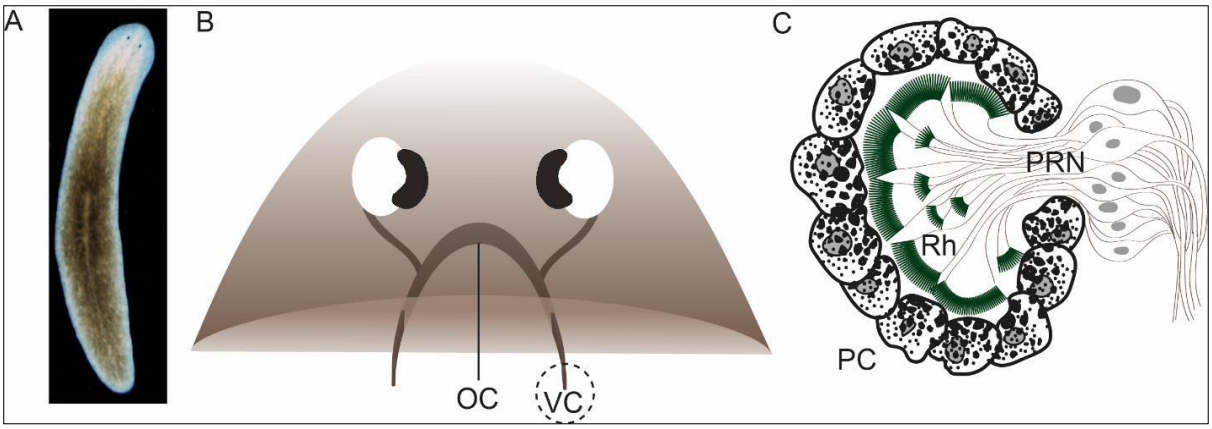
Image source: **Regeneration in planaria** https://school.gradeup.co/a-explain-the-process-of-regeneration-in-planaria-b-how-is-i-1njv58

B. Planarian eye structure:

Many planarian flatworms have basic eye features that are phylogenetically conserved such as pigmented cup structure, photoreceptor cells containing opsin, and a host of eye-specific developmental genes that are essential for eye formation. [2] [6] [9] Planaria with their two eye-spots linked to a bilobed, brain-like structure represents a true cerebral eye. [10][11]

S. mediterranea has prototypic rhabdomeric eyes located on the dorsal side of the body, with pigment cells and bipolar photoreceptor neurons (PRNs) [2][12][13]. Planarian eyes have been classified under low-resolution vision since they can detect minimum light and may be adept of the more advanced functions [2]. They also have the ability to regenerate their brain (dorsal ganglion) and eyes within days [14][15].

The dendrites of the planarian photoreceptors extend inside the optic cup and form a rhabdomeric structure where opsin accumulates. Opsins are a highly conserved class of G-protein coupled receptors that covalently bond to a chromophore forming the visual pigment rhodopsin. [9]



(A) Intact asexual planarian (*S. mediterranea*) as observed under a stereo zoom microscope. (B) Schematic image of the planarian visual network. Planarians form the optic chiasm (OC) and visual axons project into the visual centre (VC) for transmitting light information to the brain. (C) The components of the planarian photoreceptor. The multicellular pigment cup produced by apposition of several pigment cells (PC). The light-sensing photoreceptor neurons (PRN) and their rhabdomeres (Rh) project into the pigment cup.

Image source: AG LAB, [1]

C. Planarian light-sensing:

Planaria are shown to be negatively photo-tactic (movement away from light). They also have a whole-body(extra-ocular) response apart from the processive eye-brain mediated (ocular) response. These responses are confirmed by their UV avoidance post-decapitation.

The relationship and dynamics between two independent light-sensing networks is being explored. [1]

It has been displayed through FISH that planarians have only one major eye opsin. But a puzzling result is that, they are able to differentiate between 25nm wavelength changes. For instance, when provided a choice between equal intensities of red(625nm) and green(545nm), planarians consistently move away from green. But on increasing the intensity of red light, after a certain point the planarians reverse their behaviour and move away from red. Hence, it can be concluded that spectral differences are converted into effective intensities of the light distinguished depending

on the absorption spectrum of the photoreceptor. Consequently, a ‘behavioural action spectrum’ is generated through light intensity modulation in wavelength. [1]

Moreover, the knock-down of this primary opsin found in the eye inhibits the light-sensing through the visible spectrum in the worms. [1]

But unlike intact worms, headless worms cannot sense fine gradients. Also, the transition between extra-ocular to ocular response during the course of regeneration is being studied.[1]

APPROACH

I. To perform behavioural studies planarians and record their behaviour.

- **Negative phototaxis assay**
- **Response to UV**

II. Algorithm to track worm-movement

- **K means clustering to track worm centroids**
- **Analysis on worm tracks to extract parameters that report on light avoidance responses in planaria.**

I. Planarian behavioural studies

AIM:

To study the response of planarians to visible region and UV region of the electromagnetic spectrum.

BACKGROUND:

S. mediterranea are negatively phototactic (movement away from light) when illuminated by any single wavelength in the range of 365 to 625 nm.

Photoreceptors in the head (eyes) region are found to be sensitive to visible and UV light. Photoreceptors along the body (trunk, tail) are found to be in-sensitive to visible light but respond strongly to UV light. [1]

MATERIALS:

75mm- 25mm glass slide, planaria media.

Electronic equipment

Light-emitting diodes (Roithner Lasertechnik) of 500nm (visible spectrum) & 395nm (UV region) wavelength, power source. Olympus SZX16 microscope, Olympus SZ61 microscope.

METHODS:

A. Planarian Maintenance

Media preparation:

The media containing 1X Montjuïch salts for *S. mediterranea* was prepared using the recipe published by Cebrià and Newmark (2005). The *S. mediterranea* were maintained submerged in 1X Montjuïch salt solution prepared in MilliQ double distilled water. The planarians were kept in a temperature-controlled incubator. The composition for the 1X Montjuïch salt solution is as shown in the table below. The pH of the solution was adjusted to 7 using Hydrochloric Acid.

Table 2. Planaria media composition

Reagent	Concentration
NaCl	1.6 mmol/L
$CaCl_2$	1.0 mmol/L
$MgSO_4$	1.0 mmol/L
$MgCl_2$	0.1 mmol/L
KCl	0.1 mmol/L
$NaHCO_3$	1.2 mmol/L

Cleaning:

The worms and the containers were cleaned regularly with only distilled water. The media was discarded, and fresh media was added regularly. The transfer of planarians from one container to another was done using Pasteur pipettes.

B. Negative phototaxis assay (Response to visible light)

33 *S.mediterranea* worms were taken in a petri-plate. Each worm was cut into three pieces. Head, trunk, tail. Therefore, in total 69 worm pieces were subject to the experiment.

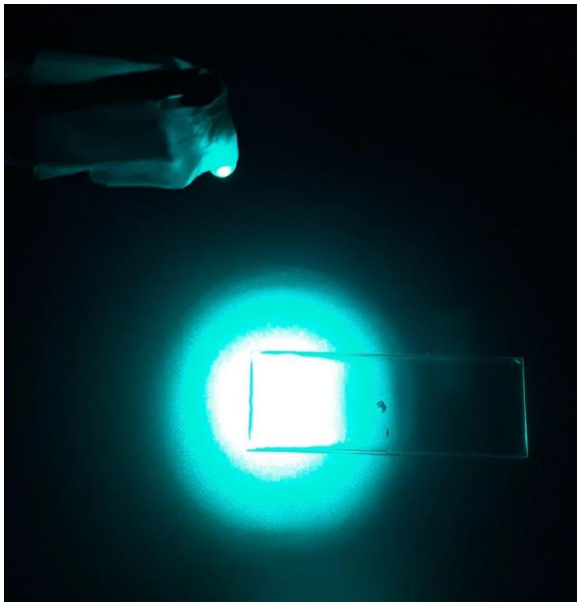


Image source: AG LAB- Negative phototaxis assay

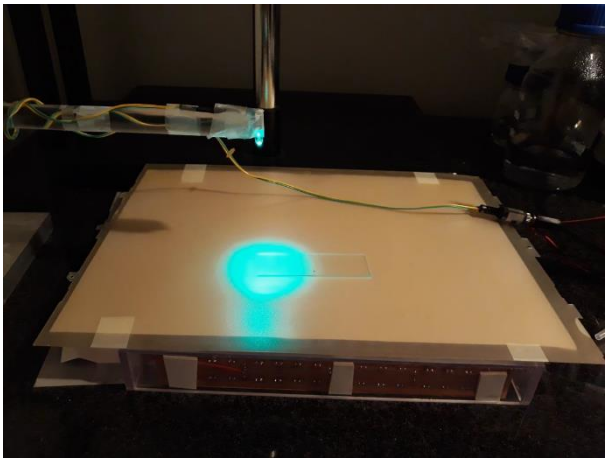
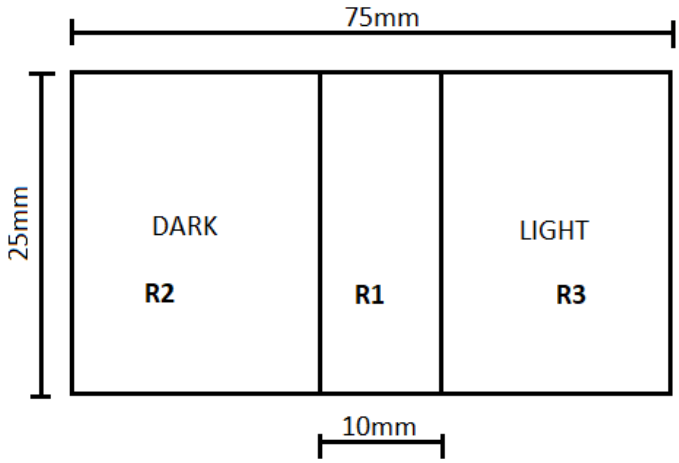


Image source: Negative phototaxis assay set up



1. The worms were allowed to rest in a dark room for 1 hour.
2. Planaria media was added on a glass slide.
3. A 500 nm LED light was shone on the glass slide such that half of the slide was illuminated and the other half was dark.
4. The 1 cm region in the middle of the slide is R1, the dark region is R2 and the illuminated region on is R3.
5. Each piece was dropped in the 1 cm region in the middle of the slide and its position was observed and recorded for a total of 2 minutes. 3 pieces were placed at the centre at a time, throughout the experiment. This was repeated for all the pieces.
6. Discrimination index (DI) was calculated using the formula:

DI = (Number of worms in the dark region - Number of worms in light region)/Total number of worms

C. Response to UV

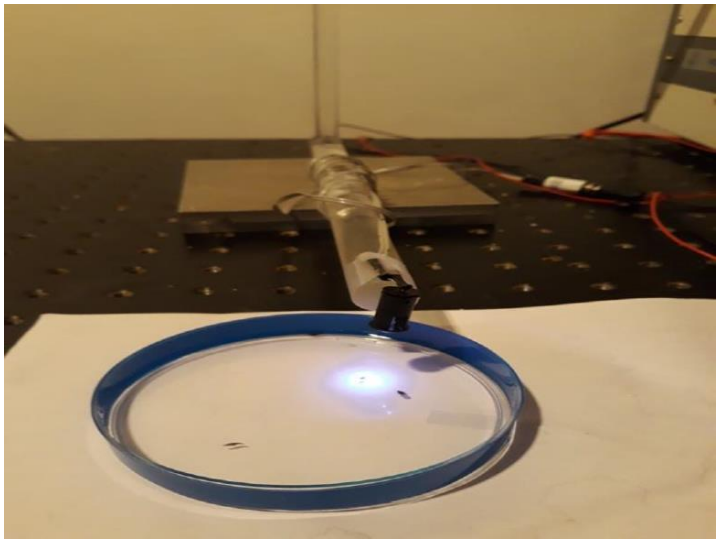


Image source: AG Lab, Response to UV

1. The worms were allowed to rest in a dark room for 1 hour.
2. Worm pieces were placed on a glass slide at a time.
3. A 1 cm diameter 395 nm LED light was shone on the worm pieces.

OBSERVATIONS:

Negative phototaxis assay

Few pieces moved to the left, few to the right and few remained in the centre.

No- of worms = 33.

Total number of pieces = 69 (Worms approximately cut into 3 parts- head, trunk, tail)

No. of pieces in R1 = 39

No. of pieces in R2 = 27

No. of pieces in R3 = 3

DI = (No. of worms in R2- No. of worms in R3)/ Total number of worms

DI = (27 - 3)/69

DI = 0.348

Response to UV:

All the pieces respond to UV light and move away from it.

RESULTS & DISCUSSIONS:

-In the negative phototactic assay, if we consider the whole worm with the head, we would ideally observe a DI~1, since planarians move away from light. The visible light sensitive structures; the eyes are present only in the head region and hence we observe a third of the total pieces to move away from the light, which is evident from the calculated DI. (DI = 0.348)

-The head, trunk and tail region are sensitive to UV, since all pieces of worms show a reflex-like avoidance to UV light.

CONCLUSIONS:

Behaviour analysis in planarians can help in our understanding of the light sensing capabilities of organisms.

II. Algorithm to track movement of worms

AIM:

To track worm movements, plot their trajectories over time, speed, discrimination index, location on slide regions, head direction.

ALGORITHM USED:

K-means clustering

BACKGROUND:

K-means clustering is an unsupervised learning algorithm that aims to partition n observations into k clusters in which each observation belongs to the cluster with the nearest mean distance. [16]

1. A given data set is classified into fixed number of clusters, say ‘c’, fixed a priori. Let X = {x1, x2, x3,,……..,xn} be the set of data points and V = {v1, v2,…….,vc} be the set of centres.
2. The ‘c’ cluster centroids are initialised randomly in the first step.
3. Each point belonging to the data set is associated to the nearest cluster centroid.
4. At this point, ‘c’ new centroids are re-calculated as the barycentre of the clusters resulting from the previous step.

$$v_i = (1/c_i) \sum_{j=1}^{c_i} x_i$$

where, ‘ci’ represents the number of data points in the ith cluster.

5. After ‘c’ new centroids are found, a new binding has to be done between the same data set points and the nearest new centre. Hence a loop is generated.
6. As a result of this loop, the ‘c’ centres change their location step by step until no more changes are done or in other words centres do not move any more.

7. The algorithm aims at minimizing the objective function known as squared error function given by:

$$J(V) = \sum_{i=1}^{c} \sum_{j=1}^{c_i} (\|x_i - v_j\|)^2$$

Where '||xi - vj||' is the Euclidean distance between xi and vj.
'ci' is the number of data points in ith cluster.
'c' is the number of cluster centres.

Advantages:

1. Fast, robust and easier to understand.
2. Relatively efficient: O(tknd), where n is the no. objects, k is no. clusters, d is no. dimension of each object, and t is no. of iterations. Normally, k, t, d << n.
3. Gives best result when data set are distinct or well separated from each other.

Disadvantages:

1. The learning algorithm requires a prior specification of the number of cluster centres.
2. If there are two highly overlapping data then k-means may not be able to resolve them into two clusters.
3. The learning algorithm is not invariant to non-linear transformations i.e. with different representation of data we may get different results (eg: data represented in form of cartesian coordinates and polar coordinates will give different results).
4. Euclidean distance measures can unequally weigh underlying factors.
5. Applicable only when mean is defined i.e. fails for categorical data.
6. The learning algorithm provides the local optima of the squared error function.
7. Random initialization of the cluster centre may not result in the global optima.
8. Unable to handle noisy data and outliers. [17]

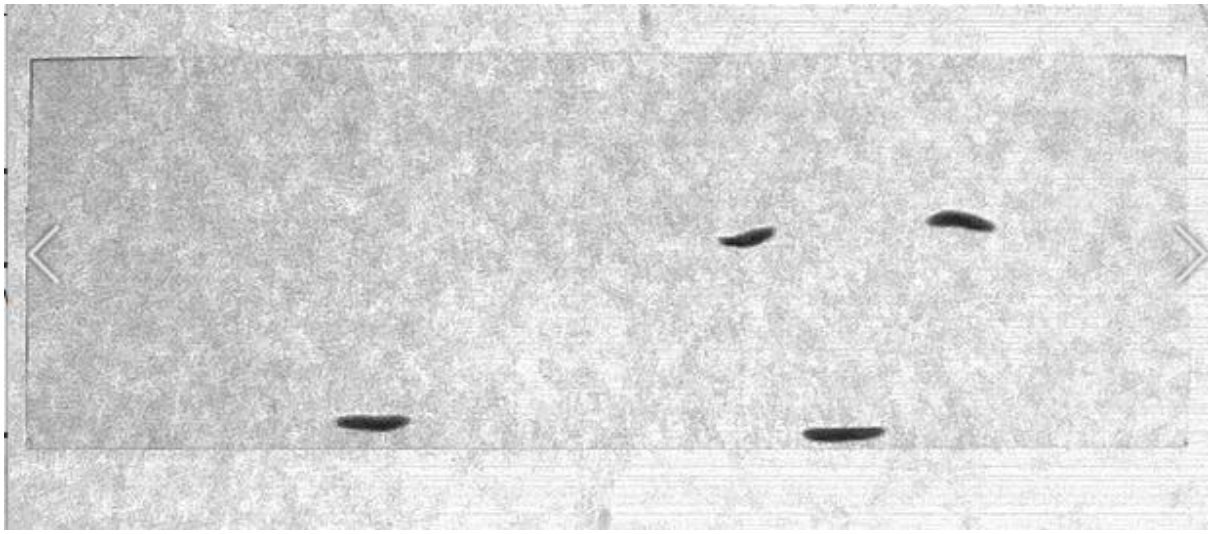


Image Source: AG Lab

METHOD:

1. MATLAB R-2018a was used for the analysis
2. Videos of the worm movements were recorded. Each frame of the video was processed separately.
3. The image in each frame was converted to binary values and the sensitivity adjusted. This ensured that black pixels were picked from the worm body (value 0) and the rest of the slide background corresponded to white (value 1).
4. The number of cluster centroids was initialised based on the number of worms in a given video.
5. MATLAB has inbuilt k-means algorithm which is fast and efficient.
6. K-means was run on the first frame during which the algorithm typically functioned, and converged in about 30-50 iterations. The centroids in the subsequent frames were processed by running k-means on each frame while initializing the cluster centroid obtained to the value obtained in the previous step.
7. The worm body pixels were taken as data points and assigned to clusters, i.e. each worm a cluster with centroid in each frame.
8. In this manner, the location of the centroid of every worm obtained over time on the glass slide.

RESULTS & DISCUSSIONS FROM ANALYSIS:

The following were plotted and measured using the videos on control worms. (No knockdown)

Given data:

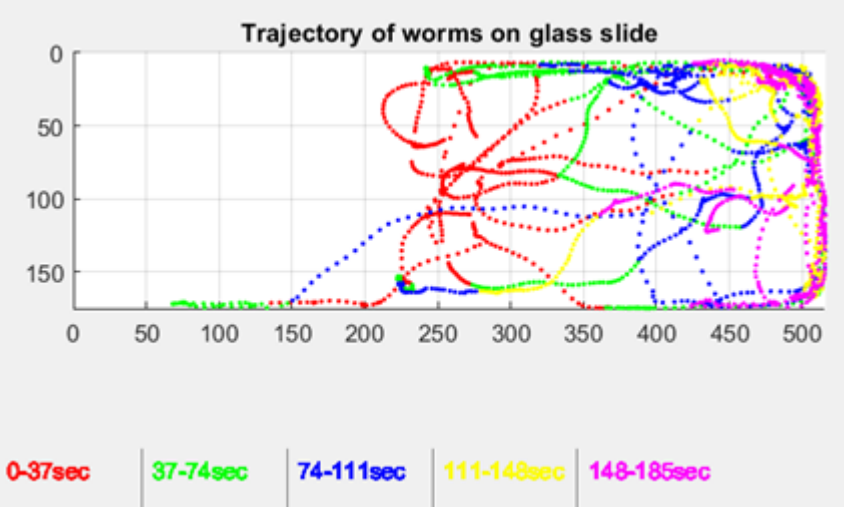
16 control worms were taken on a glass slide and subject to negative phototaxis assay as previously described. The light is concentrated on the left portion of the glass slide. The video for the below analysis was recorded for 185 seconds, with frame-rate of 5 frames/sec. (925 frames in total)

4 worms in each trial were taken. Therefore, 4 videos with 4 worms in each video were processed.

A. Trajectory of worms on the glass slide.

Each point corresponds to a worm's centroid at a particular time point.

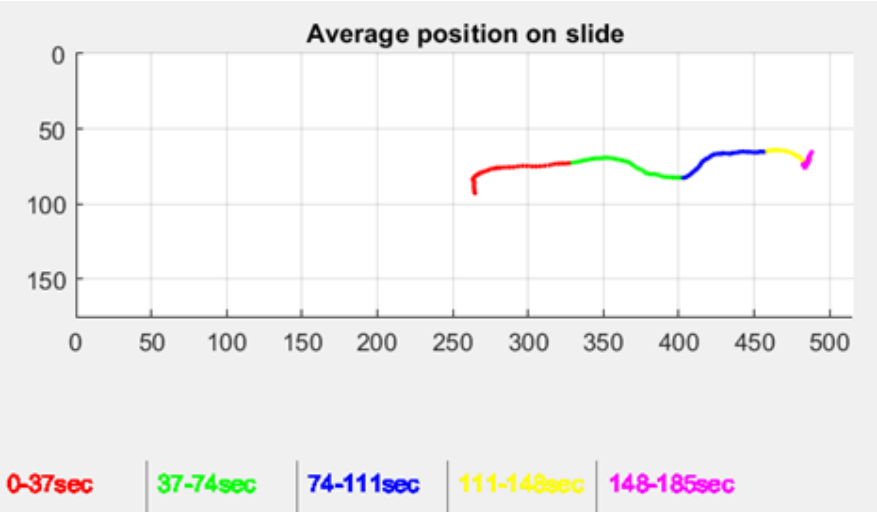
Observation: We observed that the worms are light-aversive, and move away from light (towards the right)



B. Average position of worms on a slide over- time

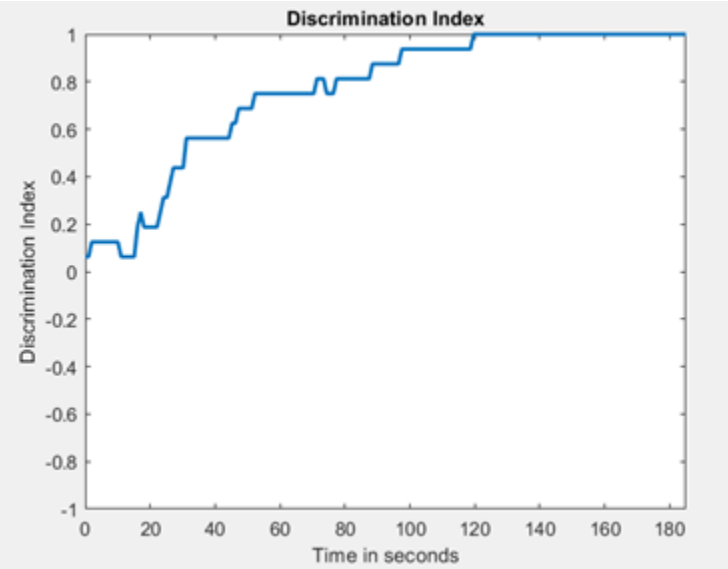
(Average centroid position of 16 worms in every frame is measured. The average point is plotted over-time)

Observation: The worms have a quick response initially & move away from the light.

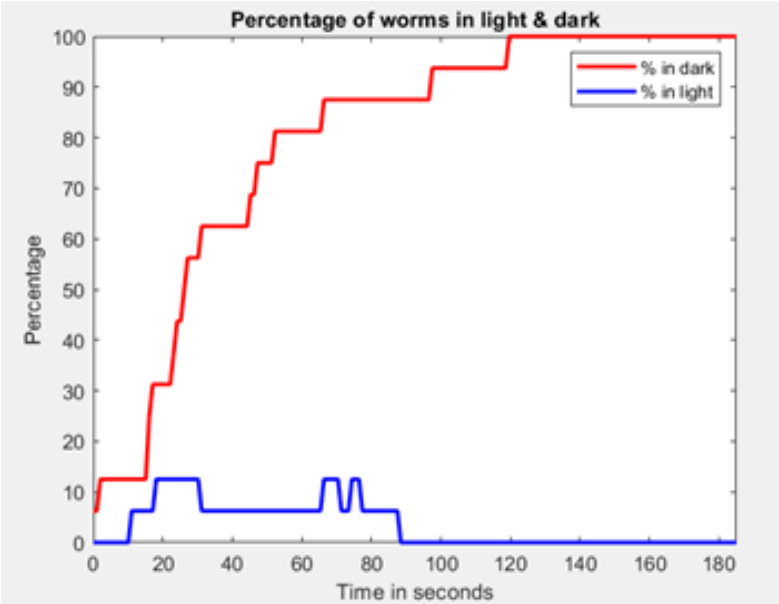


C. Discrimination Index (DI) over-time (DI- as described previously in behavioural studies)

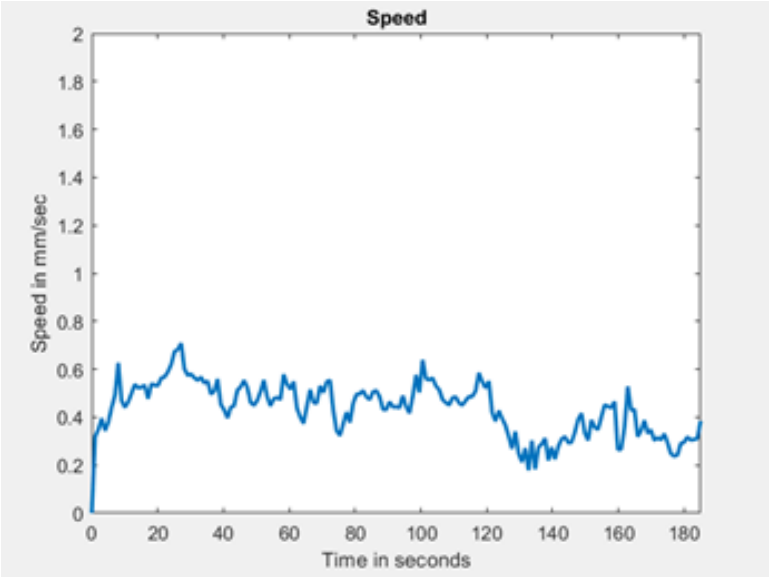
Observation: At around 120 seconds, all the worms are able to make a decision and move away from the light. (DI= 1).



D. Percentage of worms (Total of 16) in light and dark region over-time



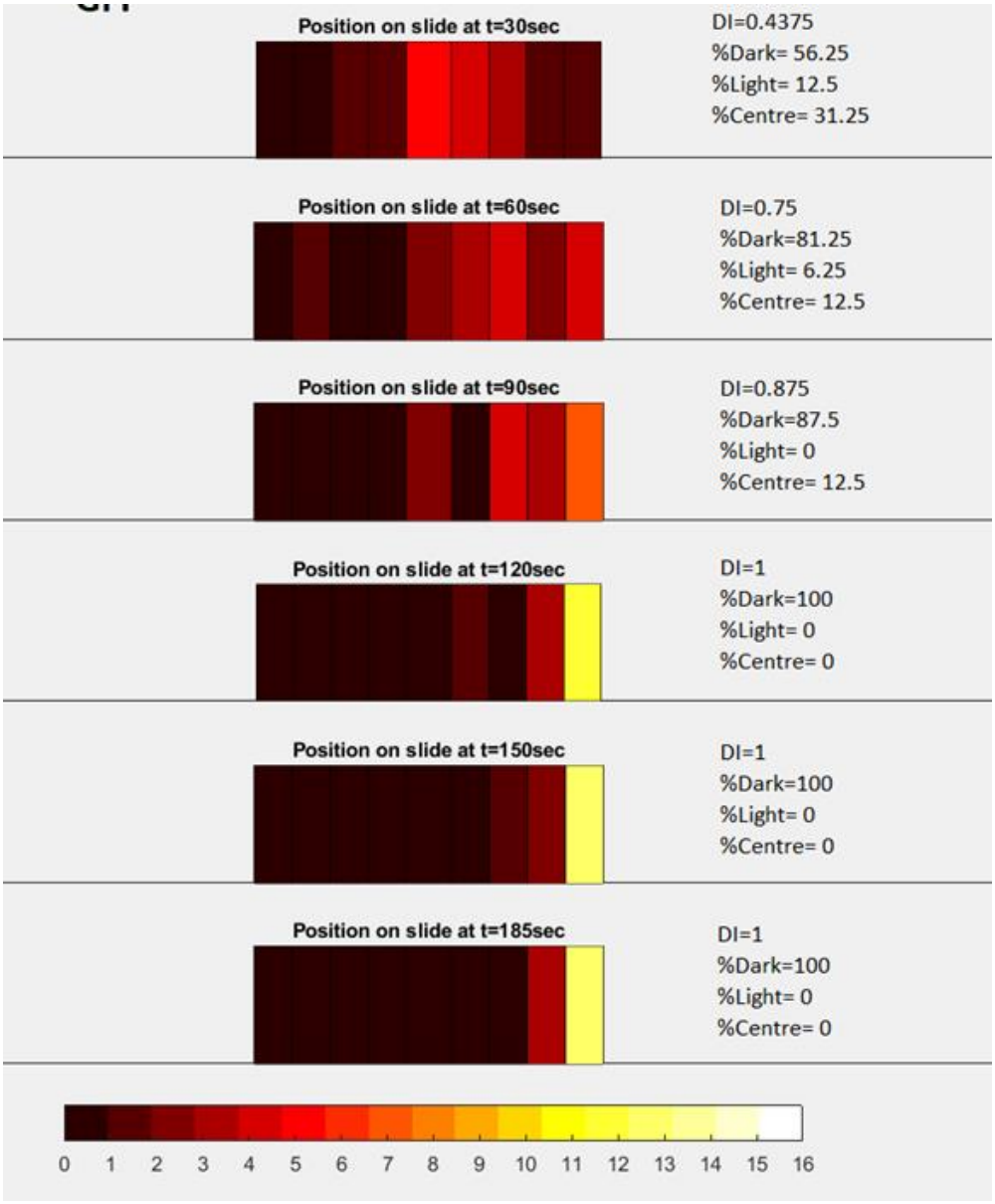
E. Average speed of 16 worms over time



F. Number of worms in a given slide region at different time points.

(Colour code for number of worms is represented below)

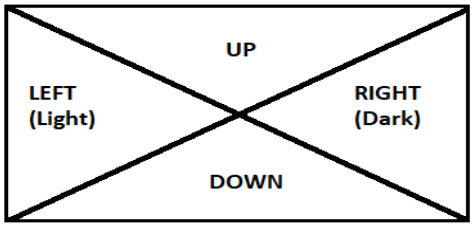
Observation: The worms move away the light. At around 120 seconds, all the worms are able to make a decision and move away from the light. (DI= 1)

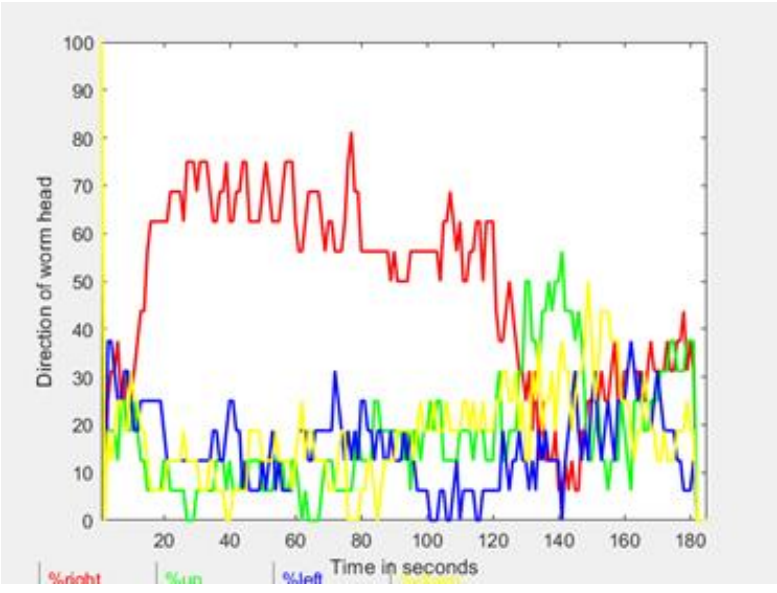


G. Average direction of worm-head movement over time.

The two farthest points from worm centroid are found. The decision of which point is a head and which point is a tail is made by looking at previous 2 frames and next 4 frames. Since the worm moves only in the forward direction (due to movement of cilia in one orientation) i.e. in the direction of head we can differentiate head from tail. Once the head of a worm is found, its direction is determined using the following figure as reference.

Observation: We observe that most worms swim towards the right (dark region) initially. Once in the dark region, the direction of worm head is random.





Limitations of the algorithm:

1. When the worms overlap at the edges of the slide, towards the end of the video, the centroids between worms may inter-change. If centroid locations at a given time-point is to be measured it may not matter. However, if there is a need to track a particular worm in a group of worms, centroid overlap may cause inter-change of paths.

2. Determining head and tail will fail if worms are stationary continuously for 6 frames. Also head and tail positions will be inaccurate when worms overlap at edges towards the end of the video.

CONCLUSIONS:

The algorithm runs efficiently in computing centroids. It can help in detailed analysis and studying behaviour post gene knock-down worms efficiently over time.

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ABBREVIATIONS:

1. *S. mediterranea/ Smed: Schmidtea mediterranea*
2. dsRNA- Double stranded Ribonucleic acid
3. RNAi- RNA interference
4. cDNA- Complementary DNA
5. DI: Discrimination Index
6. PRNs: Photoreceptor neurons
7. GD: Genome Database
8. qPCR: Quantitative PCR
9. bp: Base pairs
10. FISH: Fluorescent in situ hybridisation
11. UV: Ultra-violet

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CODES: