EXPERIMENTAL SYSTEMS FOR TEACHING, LEARNING & RESEARCH

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SOPHIA COLLEGE (AUTONOMOUS), MUMBAI

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We also acknowledge the grants from various sources including DST, DBT, BRNS, LTMT, UGC and RUSA for enabling purchase of equipment, chemicals and cultures over the years. All collaborators who have helped establish and standardize the various systems are warmly acknowledged.

Foreword

It is my pleasure to write a Foreword for the book "Experimental Systems for Teaching, Learning & Research". I have known the authors for more than two decades through Dr. Medha Rajadhyaksha, a longtime friend and a collaborator during my initial years in the National Centre for Cell Science (NCCS), Pune. Over the years, I have seen their passion for teaching, enthusiasm for improvising teaching methodologies and introducing novelty in research projects. Their efforts have improved the quality of graduate and post-graduate students passing out from the Department of Life Sciences of Sophia College. The present book is their effort to bring out a compilation of experimental systems that have wide applications in teaching and research in biological sciences. The experimental systems are thoughtfully chosen and complimented with workable protocols. The chapters are presented in modules with each of the experimental systems as independent "stand alone" units. The book covers experimental systems such as slime mould, hydra, soil nematodes, fruitfly, zebrafish, chick embryo and mammalian cell culture. Additionally, the compilation includes hydroponics to study nutrient availability and Stroop Test as a simple tool for analysis of information processing in the human brain. The experimental systems described will stimulate the thought process of students to conceptualize research projects and to address intriguing questions in diverse fields of biological sciences. To make it interesting, the chapters begin with a historical background of the system and highlight the potential of the systems in research towards understanding biochemistry, developmental biology, neurobiology and behaviour. Each of the chapters has a comprehensive introduction to the experimental system providing adequate information to understand the scientific concepts of the system. One of the striking features of the book is that the protocols for experiments are standardized and validated to ensure that the experiments are workable in college laboratories equipped with limited facilities. The book will not only strengthen the scientific concepts but will also give students the joy of having hands-on-experience of performing experiments, translating the observations to findings, learning to analyse the results and drawing logical conclusions. With experience, the young minds will learn to value the limitations of systems and get motivated to ponder over better and newer strategies for research. This will not only enhance the understanding of scientific concepts but will also give students the joy of having hands-on-experience of performing ęxperiments, translating the observations to findings, learning to analyse the results and drawing logical conclusions. With experience, the young minds will

learn to value the limitations of systems and get motivated to ponder over better and newer strategies for research methodologies. The protocols in the manual are well defined and written in a simple language, making the experimental systems "user-friendly".

The National Research Foundation (NRF) was recently established with the goal of promoting and mentoring scientific research in higher educational institutions in the country. This move has been a blessing for the academic community as it aims to enhance scientific research in the country by building better infrastructure, knowledge enterprise, and innovation potential. In this context, the book presents a compilation of experimental systems with diverse applications that can be set up in colleges and university laboratories with reasonable financial support. Furthermore, as the NRF will provide a platform for multi-disciplinary and multi-institutional collaborative research, it will offer an opportunity for colleges to form "clusters" with each of the participating institutions setting up specific experimental systems and making them accessible to other organizations.

I hope this book will prove beneficial for students as well as for teachers.

I wish the authors great success in their future endeavors.

Padma Shastry

Former Scientist, National Centre for Cell Science (NCCS), Pune

Preface

The understanding of biological concepts is facilitated by hands-on experimentation. The purpose of laboratory practicals in the curriculum aims at just that. This compilation of experimental systems furthers the aim by bringing to you workable protocols that can be used in teaching and research. We present here experiments involving commonly used plant and animal model systems that have been applied to address interesting questions in Biology over the years.

The chapters are presented as modules. Each module consists of one experimental model system. It begins with a brief historical, followed by the possible applications for which the system can be used. A few selected experiments and assays which can be easily performed in the laboratory are explained, with representative/expected results for each. At the end of the module, suggestions for open-ended projects are provided, which may be of help to give meaningful direction to the protocols given. In general, the protocols are directed at understanding biology and biochemistry, development, behaviour and neurobiology. References for further reading have been included.

The equipment required to maintain these model systems and perform the experiments are simple and generally available in most laboratories. Further, with restrictions on the use of animals for research, appropriate alternative systems could be used to obtain insights into the actual process in the animal, even if to a limited extent and though not quite substitutable.

All the protocols have been successfully standardized in the under-graduate and post-graduate laboratories at Sophia College (Autonomous), Mumbai, and used to generate publishable results.

With the introduction of National Education Policy-2020, which mandates research at under-graduate and post-graduate level, we hope that this effort will help to bring excitement in teaching and in learning. This compilation is an evolving one and we hope more contributors will be added to it over time.

Contents

Dictyostelium discoideum (Slime mould)

Since the discovery of *Dictyostelium discoideum* by Raper in 1935, its fascinating biology has made it a popular and productive model system for studying the molecular basis of cell and developmental biology. *Dictyostelium* grows by the mitotic division of single cells that feed by phagocytosis on bacteria, or by macropinocytosis on simple axenic liquid medium, making it possible to grow many cells for analysis.

S.B. Williums *et al*, Towards a molecular understanding of human diseases using *Dictyostelium discoideum*, 2006.

Dictyostelium discoideum (Slime mould) - the amazing social amoeba

Scherezad Kootar and H. Ramachandran

Abstract

The soil dwelling, social amoeba *Dictyostelium discoideum* was discovered in the year 1935 by Kenneth Raper. At present, many strains of this organism are used as a popular research model. The original strain called NC4 was found to grow and establish in the presence of bacteria *E.coli* and *K.aerogenes*. Its unique developmental stages include a unicellular stage in nutrient rich medium and multicellular stage by aggregation during starvation. Thus, an organized multicellular phase is generated due to morphogenesis and cell differentiation. These developmental features have encouraged biologists to explore *Dictyostelium* as a model for understanding cell motility and chemotaxis. The completed genome sequencing of this free-living *Dictyostelium* amoebae under the genome project of AX4 strain has further turned this into an attractive multicellular experimental model system.

The chemotaxis phenomenon exhibited during differentiation in *Dictyostelium* has prompted scientists to undertake studies related to neutrophil chemotaxis. *Dictyostelium* has been used to study the role of actin-associated proteins during malignancy. *Dictyostelium* is now a popular pharmacological model to understand the mechanism of drug action and drug sensitive pathways.

Introduction

Dictyostelium discoideum, is a free-living organism also known as social amoeba or cellular slime mould. It is a soil microorganism feeding on organic material most often on bacteria. Its unique feature being a unicellular organism capable of developing into a multicellular organism. Due to its economical and easy culturing method and possibility of long-term storage of amoebae and spores, *Dictyostelium* has now become a popular model for the study of a variety of fundamental biological processes like phagocytosis, cell migration, cell signaling and differentiation. The *Dictyostelium* genome has been completely sequenced.

During periods of sufficient nutrients, *Dictyostelium* lives as free-living unicellular amoebae. Whenever its natural food source supply is exhausted *Dictyostelium* cells start a developmental program to make spores, enabling them to survive unfavourable conditions. At this stage cAMP, a chemoattractant, serves as a signal to attract 10,000-100,000 amoebae in the immediate vicinity to respond and aggregate. The aggregation leads to the formation of a multicellular body. This multicellular mound, then forms a tipped mound, later the tip extends and forms a finger or a slug. Eventually, the finger or slug contracts and the anterior tip begins to rise to form a fruiting body. During the final culmination, a simple dichotomous decision is followed to form a stalk cell or a spore cell. The cells that differentiate into vacuolated stalk cells support spore heads containing spore cells. Almost 80% of the cells differentiate as pre-spore cells which later develop as spore cells. Meanwhile the remaining 20% differentiate as pre-stalk cells. During the process of differentiation, the stalk cells undergo programmed cell death by forming enlarged vacuoles and die as the pre-spore cells are lifted high above the substrate. *Dictyostelium* cell death is described to be a vacuolar cell death/ developmental autophagy/autophagic cell death (ACD). Here, autophagy enables the organism to undergo degradation, reuse its own

biomolecules and mobilize the same for development. This event with all its similarities is highly conserved in mammalian cells.

The spores of the mature fruiting body germinate once the food source becomes available. The life cycle from cells without food to the final structure of the fruiting body takes around 24 hours. The conditions which are very essential for the culmination of this stage of life cycle are temperature which is around 24°C and a pH of around 6 - 6.4.

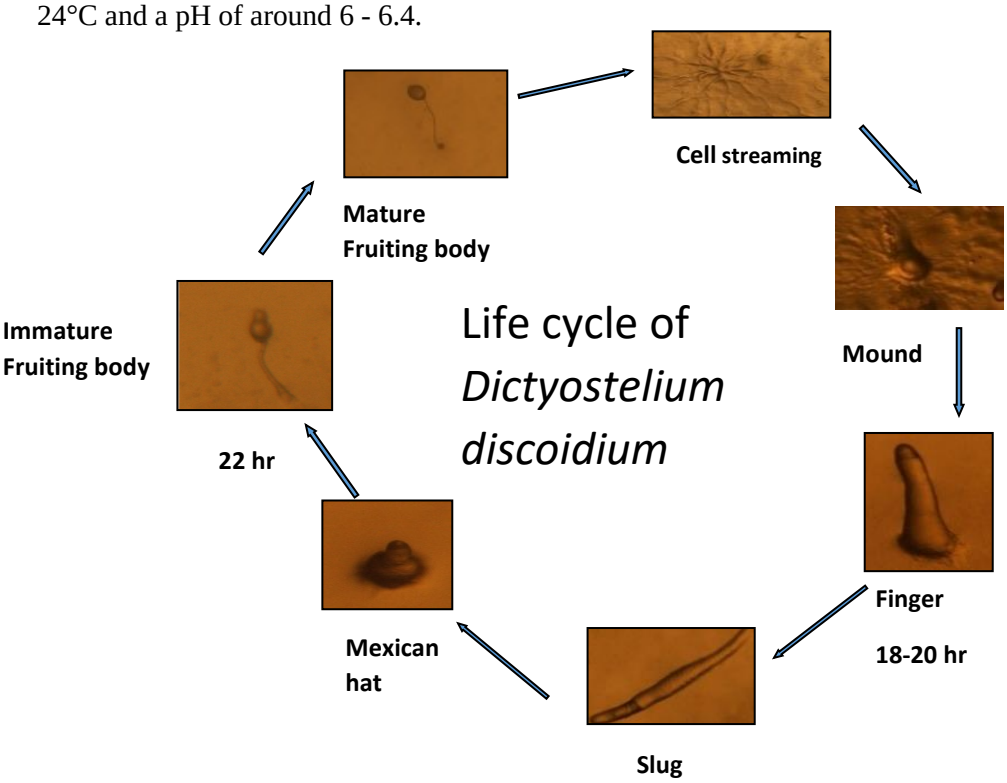

***Figure** 1. Life Cycle of Dictyostelium discoidium*

Methodology

Culturing of *Dictyostelium*.

Dictyostelium can proliferate in association with bacteria or it can survive in a nutrient rich liquid medium (axenic or AX strains). A constant temperature of 22°C is ideal for its growth when cultured on bacteria, with a doubling time of 4 hours. A reduction in growth takes place at 25°C.

Culturing of NC4 (wild type) strain on solid media using phosphate buffer plate

i. Take 200 µl of KK2 buffer in a microfuge tube and add *Klebsiella aerogenes* (culturing protocol given below) from a nutrient agar plate with the help of a nichrome loop. Please note bacteria are still alive but cannot grow in phosphate buffer thereby serving only as a food source for *Dictyostelium*

ii. Pick up the spore heads (around 2-3) (approximately 2 - 4 x 10^5 cells) with the tip of a micropipette from one plate and add them to the solution and triturate.

iii. Spread the suspension onto a phosphate buffer agar plate (2%) and allow it to dry. Invert the plates and incubate, covered in a black box at 24°C.
iv. To achieve uniform development fresh KK2 plates are highly recommended. Please ensure that the plates are not too dry nor are they too wet.
v. *Dictyostelium* cells developing on agar plates are suitable for microscopic observations, for example, to observe and record development stages.
vi. Note the observations till the formation of fruiting bodies (completed after 24 hours).

For calculating cell density, a 50 µl aliquot sample counted in a haemocytometer is ideal. An exponential growth phase (less than 4 x 10^6 cells / ml) is recommended for culturing *Dictyostelium* cells.

For growing food bacteria *Klebsiella aerogenes*: Sussman's / Standard (SM) medium with (pH 6.4) is used and SM/5 plates for inoculating *Dictyostelium* spores with bacteria to help get a large number of cells. pH is adjusted to 6.4 and medium autoclaved at 15 p.s.i for 10 minutes.

Culturing of bacteria *Klebsiella* aerogenes is done in SM Broth and on SM Agar plates to achieve mat growth.

i. Inoculate 10-20 µl of *Klebsiella* from the glycerol stock into Sussman's broth (around 2 ml) into a sterile glass test tube and leave overnight in an incubator at 37°C.

ii. Spread 300 µl of the broth onto the SM Agar plate. The plates must be left dried and inverted for overnight incubation at 37°C.

Preparation and use of *Dictyostelium* for long term stock preparation

A culture can be started by using spores of a particular strain. Stocks of either spores or cells of NC4 are kept in a microfuge tube and stored in a -20°C or -80°C freezer. This stock of spores/cells is made in 25% glycerol solution (i.e. 250 µl of 100% autoclaved glycerol) and 750 µl of spores/cells in KK2 buffer.

Cultures must be initiated from a permanent stock every 2-4 weeks. In order to avoid contamination (due to yeasts, bacteria or fungi) sterile hood is highly recommended. At temperature 21-23° growth is optimal and at temperature above 25°C it is inhibited.

Please note other *Dictyostelium* culturing technique options are available at dictybase.org

Harvesting of cells

Amoebae of the *Dictyostelium* feed on *Klebsiella aerogenes* until the supply of its food source gets depleted. Hence it is important to harvest the cells before they proceed to the developmental stages. The bacterial clearing in the plate indicates it is time to harvest the cells. This can done by flooding the plate with 3-4 ml of phosphate buffer. This buffer harvested cells are transferred to a 50 ml tube along with 40 ml of buffer solution and centrifuged at 500g at room temperature. The differential centrifugation helps in the separation of bacteria and amoebae. Now the cloudy supernatant can be discarded and cells resuspended using 45ml of phosphate buffer. Please note repeated washing with phosphate buffer will ensure complete removal of bacteria. It is extremely important to keep cell suspension on ice during these procedures.

Experimental Protocols

Neutral red staining of NC4 cells:

Neutral Red (NR) is a vital dye which acts as a pH indicator. When in acidic medium (pH < 6.8) NR turns red while in basic medium (pH > 8.0) it is yellow in colour.

There are two populations of cells in *Dictyostelium*: spore and stalk cells. NR staining can distinguish the spore and stalk cell populations by specifically staining autophagic vacuoles in the pre-stalk cells. Initially, all cells take up stain but the pre-stalk cells are the ones who retain it and hence these cells are stained throughout development, giving the distribution of pre-stalk cells in the development cycle.

Procedure:

i. $1x10^7$ cells (free from nutrients) are mixed with 200 µl phosphate buffer and plated on phosphate buffer agar plate. The plates are now incubated at 22°C. After 16-18 hours the slugs are obtained which can be used for experimental purposes.

ii. Cells of *Dictyostelium* ($1x10^7$) are treated with 0.005% neutral red prepared in sterile water for 30 seconds and the volume of buffer increased to 12 ml and centrifuged at 2300 rpm at 4°C

iii. The suspension is decanted and the cell pellet triturated in a fresh buffer. The above procedure is repeated three times.

iv. The neutral red treated cells are finally resuspended in 200 µl of buffer and plated on phosphate agar plates.

v. Plates are observed at different time points to see different developmental stages.

Spore Formation Efficiency test (S.F.E.)

In this assay the number of cells forming spores are calculated after *Dictyostelium* completes its developmental pathway. The controls under ideal conditions should result in 70-80% S.F.E. This suggests that there is no disturbance in the developmental cycle. This assay is a good parameter to check the number of spores formed after treatment. The number of cells which remain after treatment is subtracted from the total number of cells plated. This enables us to conclude how many cells were not involved in the formation of the fruiting body after exposure to the drug.

Procedure:

i. Once the fruiting bodies are formed, they can be harvested with cold phosphate buffer following which the number of spores are counted using a Haemocytometer.

ii. The total undifferentiated cells are subtracted from the total number of cells plated.
iii. The formula given below calculates the spore formation efficiency (SFE).

Formula

Spore formation efficiency =Number of spores counted x 100

(Total number of cells plated – number of cells undifferentiated)

Spore viability test:

This assay is used to check the number of cells which have differentiated into viable spores. It is carried out after a drug or inhibitor treatment. NP40, which is a detergent, is used to kill the cells and allow only the spores to germinate to form plaques. Under ideal conditions 100% spore viability is expected if the spores are healthy.

Procedure:

i. After the spore formation efficiency is calculated, the spores can be diluted using a phosphate buffer so that there are 10 spores present in the phosphate buffer.
ii. To this suspension of spores an equal quantity of 0.2% NP40 is added. The suspension is then incubated at 45°C for half an hour.
iii. After incubation, equal volume of *Klebsiella* suspension is added.
iv. This suspension, after mixing, is plated on phosphate buffer agar, left to dry and kept inverted at 24°C.
v. Plaques are observed after 48 hours and counted.
vi. The number of plaques, corresponding to the number of viable spores, is scored after 4-5 days of incubation at 22°C.

Suggested projects:

1. *Dictyostelium* genome has been completely sequenced and human genes associated with neuropathology are found in *Dictyostelium*. Due to similarities in the cellular processes between humans and *Dictyostelium*, it serves as a good model to study aberrant proteins. For example, treatment of *Dictyostelium* with chemicals / drugs brings about phenotypic changes leading to the formation of aberrant fruiting bodies, which can be easily studied.

2. *Dictyostelium a*s an ideal model system for the study of developmental toxicity thereby leading to understanding of mammalian toxicity.

Preparation of solutions

Preparation of SM media: (Please note SM5 media is a 1:5 dilution of SM medium)

Glucose 1 gm,
Peptone 1 gm,
Yeast extract 0.1 gm,
Magnesium Sulphate 0.1 gm,
KH2PO4 0.225 gm,
K2HPO4 0.066 gm,
Agar 2 gms, D/W 100 ml.

Phosphate buffer or KK_2 buffer (pH 6.4)

KH2PO4	0.225gms
K2HPO4	0.066gms
D/W	100 ml

For phosphate agar plates:

Add 2 grams of agar in 100 ml of phosphate buffer and use after autoclaving.

Suggested reading :

Dictybase is a useful website for all important resources including teaching protocols. http://dictybase.org/

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Hydroponics

Growing plants hydroponically means you never have to worry about the wellbeing of your plants. They are immersed in water and light just as they would be in the ocean and in the sunlight. No pesticides, no harmful chemicals, and super clean and crisp!

Hydroponics: A simple system of growing plants in the laboratory for potential use in plant research

Chinmoyee Vatsyayan

Abstract

Hydroponics is the science and art of growing crops in liquid nutrient solution without the use of soil. Hydroponics systems also includes use of other substrates like cocopeat, perlite, vermiculite etc. It finds application in those cases where root mass is required for extraction of secondary metabolites. As individual salts are used to prepare the nutrient medium, it is possible to have a composition suitable for a growth of a particular crop. The hydroponic system allows the study and understanding the effect of presence or absence of a particular element on plant growth. It can be used to understand how plants respond to biotic and abiotic factors under controlled nutrition.

Hershey (2008) said, "Growing plants in solution culture is often easier than soil culture because there is no need for dirty soil, there are no soil-borne diseases or pests, irrigation is less frequent in solution culture than in soil culture, solution culture irrigation can be easily automated, roots are visible, and the root zone environment is easily monitored and controlled."

Technique of hydroponics has been widely used in plant research and a wide variety of systems have been designed to study various aspects of plant science. A simple protocol is presented here which can be utilized in the laboratories for addressing many plant related questions.

Introduction

Hydroponics is the science and art of growing plants without soil. Plants take up nutrients from soil, but in hydroponics, the required nutrients are added in water in the right proportion depending on the crop's nutrition requirement, based on the crop age.

Hydroponics is used commercially for growing tomatoes, capsicum, cucumbers and other vegetables, especially exotic vegetables like lettuces, kale, celery, basil, arugula, Swiss Chard, baby spinach, blueberries etc. But, theoretically it is possible to grow any crop hydroponically.

The major advantages of growing crops in hydroponics is that water requirement is just 10% as compared to field grown crops. The crops can be grown in desert, saline & alkaline lands and other areas which are not suitable for agriculture use. The yield is more and produce quality better in hydroponics.

As, the crops are commercially grown under controlled climate, mainly in green houses, the crops are generally free of insecticides and pesticides. As the crops are not grown in soil, these are free of *E.coli* and *Salmonella*, which are generally found in soil grown crops, especially salad greens, which are eaten raw.

Hydroponic technology is not new and has been used for a long time. The Hanging Gardens of Babylon is one of the first examples of hydroponics. Growing money plant at home in a water bottle is also an example of hydroponics.

The pH range at which plants take up various elements differs and it is found that a nutrient solution pH between 5.5 and 6.5 is most suitable for uptake of all the nutrients. Plants need, N, P, K, Ca, Fe, S, B, Mg, Mn, Cu, Mo, Co, Zn etc. for the optimum growth and all these must be provided to the plants in the right proportion.

Some of the reasons why hydroponics is being adopted around the world for food production are the following:

1. As crops are grown under controlled climate, there is no need to apply fungicides and pesticides, and the produce is pesticide free. In case there is some infestations, it can easily be controlled using safe organic pesticides.

2. Savings of 90% water as compared to traditional agriculture as there is no runoff and no water pollution.

3. Total control of plant nutrition by supplying different nutrient composition depending on the crop stage.

4. Better crop quality and increased shelf life of produce.

Protocols:

Growing the plants in Hydroponic system

The plants are grown in the nutrient solution called Hoagland's solution (Appendix 1), which contains all the essential macro and micronutrients. (Hoagland, D.R. & Snyder, W.C.,1933)

A modified electrophoresis set-up is used, where a transparent plastic tray is filled with Hoagland's solution and a transparent thick plastic sheet is placed on top of it. The sheet has about thirty holes to form a template in order to place a filter paper with thirty holes superimposed on it. The two ends of the filter paper are made to dip in the Hoagland's solution to have a continuous flow by capillary action and keep the germinating seeds in contact with the Hoagland solution (Figure 1).

Process for germinating the seeds:

i. Take a clean 9 cm diameter petri dish.

ii. Put 3 layers of paper towel (Kitchen towel) or 1 germination paper on it.

iii. Moisten the paper with DM/RO water.

iv. Place about 20-25 soaked seeds, equally spaced in the petri dish.

v. Press the seeds lightly to the paper towel.

vi. Keep the petri dish in the dark for 48-72 hours.

vii. As soon as the seeds germinate take it out and expose to light (at least 25000 Lux).

viii. Conduct germination % experiment in petri dish.

ix. Use healthy seedlings for further growth experiments.

Take healthy germinated seeds/seedlings and carry out further growth using the above setup. The setup is chosen so that the growth of both root and shoot of the plants are visible. These should be kept under sufficient light (25000-30000 lux) and well-ventilated space for the optimum growth of the plants. This method makes the harvesting of the plant easier without causing any damage to the root and shoot.

Growing the plants, after being placed in Hoagland's Solution:

i. After the seeds germinate, and begin to show development of leaf, remove them from the petri dishes and move to the Hoagland's solution.

ii. Take thick plastic sheets and burn/cut through them 30 holes at regular interval, of diameter 0.25 inch or less depending on the size of the seedling.

iii. Cut 6 inches long slits, about 1.5 inch from either end of the plastic sheet.

iv. Place on the plastic sheet a double layered sheet of coarse filter paper, thereby superimposing on the plastic sheet in the area in which the holes were made.

v. Fit the paper evenly over the plastic sheet and the make same holes, mirroring those in the plastic.

vi. Meanwhile, a transparent plastic tray, containing 1.8 liter of Hoagland's solution is kept ready, with three small clean wooden sticks resting equidistantly along its length.

vii. Place the plastic sheet with the filter paper over the tray and then gently place the germinated seeds/ seedlings over the holes ensuring that the roots are safely inside the solution and that the sticks are keeping the sheet from touching the water.

viii. The extra filter paper on either side of the sheet act as wicks, in the solution, allowing it to slowly rise and saturate the filter paper.

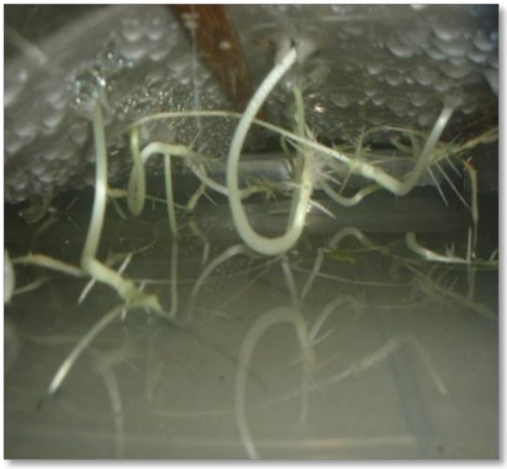
ix. The plants are left in this set-up to grow for the required number of days.

Figure 1: *A simple Laboratory Hydroponic Setup*

Cucumber Plants

Bitter Gourd – Germinating Stage

Lady finger – Day 3



Lady finger – Day 6

Study of possible Physical and Biochemical parameters of various stages of growing plants

The physical parameters chosen are percent germination, root length, shoot length, and moisture content. The proximate principles of food are proteins, carbohydrates and lipids besides vitamins and minerals. So, the biochemical parameters may be selected in as per the requirement of the study.

a) Proteins: The estimation of protein is an index for assessment of growth.
b) Maltose and Amylase: Starch is the food reserve in a plant, which in the presence of amylase breaks down to give maltose. Hence by estimation of both maltose and amylase, the effect on carbohydrate metabolism of the plant can be observed.
c) Vitamin C: It is a known antioxidant which may be responsible in imparting immunity and resistance to plants against diseases and pests.
d) Phosphorus: Besides being an essential nutrient, phosphorus is a vital component of both ATP (energy unit) and DNA (memory unit) of plants. It is essential for the general health and vigor of the plant.
e) Acid and Alkaline Phosphatase: These enzymes are involved in the metabolism of phosphorus and are responsible for conversion of inorganic phosphate to organic phosphate by hydrolysis of phosphate esters.
f) Estimation of Cation Exchange Capacity may reflect upon the growth and yield of plants.

1. Physical Parameters

a) Percent germination: This is measured by counting the number of germinated and non-germinated seeds.

b) Fresh weight: Take 10 germinated seeds and weigh immediately after harvesting.

c) Dry weight: Take the above 10 germinated seeds and keep them in an oven heated at 100^{o}C for 2 hours and weigh on cooling. The difference in weight is recorded.

d) Moisture percent is determined from the above readings using the formula:

e) $$\text{Moisture } \% = \frac{(\text{Fresh weight} - \text{Dry weight})}{\text{Fresh weight}} \times 100$$

2. Protocols for Biochemical parameters

All the biochemical parameters may be estimated using a plant extract. Weigh 1 g of the plant (Shoot/root/whole plant depending on the study) and macerate with 20 ml of chilled distilled water. Centrifuge at 3500 rpm for 15 minutes. The supernatant obtained is used as plant extract. Each ml of the extract contains 50 mg of the plant/seed. Only water-soluble components can be estimated in this extract.

2.1 Estimation of Proteins by Folin - Lowry Method (Adapted from Jayaraman J., 2005)

Principle: The tyrosine and the tryptophan aminoacids of the protein sample reduce the phosphomolybdic phosphotungstic compounds of the Folin Ciocalteau reagent. The peptide bonds of the protein also develop a coloured compound by the biuret reaction in the alkaline cupric tartrate.

Procedure:

i. Prepare dilutions of standard (0.2 mg/ml) protien solution with distilled water to make up a total volume of 1 ml.
ii. Prepare a control by adding distilled water instead of standard protein solution.
iii. To the above tubes add 5.0 ml alkaline solution and incubate the tubes at room temperature for 15 minutes.
iv. To all the above tubes add 0.5 ml Folin ciocalteau reagent and mix the tube contents.
v. Prepare the tube with plant extract to be estimated similarly using 1ml of extract instead of standard protein solution.
vi. Read the absorbance of all the tubes colorimetrically at 720 nm and then calculate the protein by plotting on a standard graph.

2.2 Estimation reducing sugar Maltose by DNSA Method [Adapted from Plummer D.T.,1998]

Principle: Maltose being a reducing sugar can reduce 3,5-Dinitro salicylic acid (DNSA) while being itself oxidized. The concentration of reduced DNSA is colorimetrically determined at 540 nm and the corresponding concentration of maltose required for the reaction can be obtained.

Procedure:

i. Make serial dilutions of standard (1 mg/ml) maltose solution such that final volume is 1 ml. Prepare a negative control using distilled water and unknown (plant extract) test tube containing 1 ml of plant extract.
ii. Add 0.5 ml of DNSA to all the above tubes
iii. Cover each test tube with a marble and keep the tubes in a boiling water bath for 5 minutes.
iv. Cool to room temperature. Add 5 ml of distilled water to each tube.
v. Read absorbance of the solutions at 540 nm.
vi. Plot a standard graph of maltose and plot the absorbance of the unknown to obtain the maltose content.

2.3 Estimation of amylase (in maltose units) (Adapted from Plummer D.T.,1998)

Principle: The reducing sugars of maltose produced by the action of beta amylases react with the 3, 5- dinitro salicylic acid and reduce it to a brown colored product 3-amino, 5-nitronitrosalicylic acid which can be estimated at 540 nm. From the amount of maltose produced, the amylase activity can be determined in maltose units.

Procedure:

i. Add 1ml of 2% starch in all test tubes except the water blank and sample controls i.e. substrate blank.
ii. Add 1 ml of acetate buffer and 1 ml of 0.5% NaCl in all tubes i.e. samples, controls, water blank and enzyme blank.
iii. Add 1ml of the enzyme/plant extract in the sample tubes and also in the control tubes.
iv. Keep all the tubes for incubation at 37^{o}C for 30 minutes.
v. Stop the enzyme activity by immediately adding 0.5 ml of 2N NaOH to each tube and mixing thoroughly.
vi. Take out 0.5 ml of the resultant reaction mixtures from all tubes into separate set of tubes containing 0.5 ml of DNSA reagent.
vii. Keep the tubes in boiling water bath for 5 minutes. After cooling add 2 ml of distilled water in all tubes.
viii. Measure the absorbance colorimetrically at 540 nm and compare the readings with that of a standard maltose solution.

2.4 Vitamin C by 2-6 di chlorophenol indophenol, DCPIP Method (Adapted from Sadasivam, S. and Manickam, A.,1992)

Principle: In this method, the ascorbic acid is estimated by titrating it with a dye 2-6 dichlorophenol indophenol (DCPIP) which is blue in acid solution. Vitamin C reduces the blue coloured dye to colourless leuco dye and it itself is oxidized to dehydroascorbic acid. The reaction is carried out in the acidic medium containing oxalic acid. The ascorbic acid gets oxidized to dehydroascorbic acid. Though the dye is a blue coloured compound, the end point is the appearance of pink colour. The dye is pink colour in acidic medium. Oxalic acid is used as the titrating medium.

Procedure:

i. Pipette out 5 ml of the working standard solution into a 100 ml of conical flask.
ii. Add 10 ml of 4% oxalic acid and titrate against the dye (V_1 ml). End point is the appearance of pink colour which persists for a few minutes. The amount of dye consumed is equivalent to the amount of ascorbic acid.
iii. Extract the sample (0.5 to 5 g depending on the sample) in 4% oxalic acid and make up to a known volume (100 ml) and centrifuge.
iv. Pipette out 5 ml of the sample, add 10 ml of 4% oxalic acid and titrate against the dye (V_2 ml).

Calculations:

$$\text{Amount of ascorbic acid mg/100 ml sample} = \frac{0.5 \text{ mg}}{\text{V1 ml}} \times \frac{\text{V2 ml}}{5 \text{ ml}} \times \frac{100 \text{ ml}}{\text{Wt. of sample}} \times 100$$

Suggestions / open ended projects

1. In this system, one may use a transparent plastic box with lid or just use floating thermocol sheet with holes. It is easier to buy this and make holes. Hot cork borer may be used to make holes in thermocol. The germinated seeds can be lightly covered with cotton wool or paper towel, so they don't fall through the holes. Using floating thermocol sheets would mean that the seed/seedlings are always wet and in touch with solution. In this system the media is to be intermittently aerated using fish tank air pump)

2. If it is a short time experiment, at the end of experiment, one can also check the residual volume, EC and pH of the solution.

3. The basic laboratory hydroponic setup system is versatile. It can be used for various types of studies on plants. To state a few possibilities:

i. Comparison of plants of same families at various stages of growth – morphological, biochemical.

ii. Comparison of plants of different families at various stages of growth - morphological, biochemical.

iii. Effect of various nutrients, plant growth promoters, plant hormones on the early stages of plant growth.
Screening of various plants for their possible yields.

Reagents and solutions:

Appendix 1

The composition of Hoagland solution (Hoagland D.R. & Snyder W.C., 1933):

Component	**Stock Solution**	**ml of Stock Solution in 1L**
Majors		
2M KNO_3	202 g/L	2.5
2M Ca $(NO_3)_2.4H_2O$	236 g/0.5L	2.5
Iron chelate	15 g/L	1.5
2M $MgSO_4.7H_2O$	493 g/L	1
1M NH_4NO_3	80 g/L	1
Minors		
H_3BO_3	2.86 g/L	1
$MnCl_2.4H_2O$	1.81 g/L	1
$ZnSO_4.7H_2O$	0.22 g/L	1
$CuSO_4$	0.051 g/L	1
$H_3MoO_4.H_2O$ or	0.09 g/L	1
$Na_2MoO_4.2H_2O$	0.12 g/L	1
1M KH_2PO_4 (pH to 6.0 with 3M KOH)	136 g/L	0.5

1. Make up stock solutions and store in separate bottles with appropriate label.
2. Add each component to 800 ml of deionized water then fill to 1 L.
3. After the solutions are mixed, it is ready to water the plants.

Adjust the pH of this solution to 5.8 using phosphoric acid. It would be a good idea to check EC of this solution.

Estimation of Proteins by Folin - Lowry Method (Adapted from Jayaraman J., 2005)

Preparation of reagents:

a) Sodium carbonate solution: 2% sodium carbonate in 0.1N sodium hydroxide

b) Copper sulphate – Sodium potassium tartrate solution: 0.5% copper sulphate solution in 1% Sodium potassium tartrate solution. Prepare fresh by mixing stock solutions

c) Alkaline solution: Mix 50 ml of (1) and 1 ml of (2) on the day of use.

d) Folin – Ciocalteau reagent: Dilute the commercial reagent with an equal volume of distilled water on the day of use.

e) Standard Bovine serum albumin (BSA) protein (0.2 mg/ml): Weigh 2 mg of the standard BSA and dilute to 10 ml with distilled water, of which take 4 ml and further dilute to 20 ml with distilled water.

Estimation reducing sugar Maltose by DNSA Method (Adapted from Plummer D.T.,1998)

Preparation of reagents:

a) Sodium potassium tartarate : Dissolve 300 g of this salt in about 500 ml of distilled water.

b) Sodium hydroxide 2N: Dissolve 80 g in distilled water and make up the volume to 1000 ml.

c) 3,5-Dinitro salicylic acid (DNSA): Dissolve 10 g of this reagent in 200 ml of reagent b.

d) Dinitrosalycylic acid reagent: Prepare this fresh by mixing solutions sr no.a & c and make upto 1000 ml with distilled water.

e) Stock maltose standard: 1g per liter(1mg/ml). Prepare 50 ml.

f) Working maltose standard solution: Dilute stock maltose standard four times with distilled water.

Estimation of amylase (in maltose units) (Adapted from Plummer D.T.,1998)

Preparation of reagents:

a) 2% starch: Dissolve 2 g starch in 100 ml of distilled water.

b) 0.5% Sodium chloride (NaCl): Dissolve 0.5 g NaCl in 100 ml of distilled water.

c) Sodium potassium tartarate : Dissolve 300 g of this salt in about 500 ml of distilled water.

d) Sodium hydroxide 2N: Dissolve 80 g in distilled water and make up the volume to 1000 ml.

e) 3,5 – Dinitro salicylic acid (DNSA): Dissolve 10 g of this reagent in 200 ml of reagent d.

f) Dinitro salicylic acid reagent: Prepare this fresh by mixing solutions sr.no.c & e and make upto 1000 ml with distilled water.

g) Acetate buffer pH 4.8:

i. 0.2M acetic acid: Dissolve 3 ml of acetic acid in 250 ml distilled water

ii. 0.2M sodium acetate: Dissolve 8.2 g of sodium acetate in 500 ml distilled water.

iii. Mix 150.8 ml of i and 200 ml of ii.

Vitamin C by 2-6 di chlorophenol indophenol, DCPIP Method (Adapted from Sadasivam, S. and Manickam, A.,1992)

Preparation of reagents:

a) Oxalic Acid (4%)

b) Dye Solution: Weigh 42mg sodium bicarbonate into a small volume of distilled water. Dissolve 52 mg 2,6-dichlorophenol indophenol in it and make up to 200 ml with distilled water.

c) Stock Standard Solution: Dissolve 100 mg ascorbic acid in 100 ml of 4% oxalic acid solution in a standard flask (1mg/ml).

d) Working Standard: Dilute 10 ml of stock solution to 100 ml with 4% oxalic acid. The concentration of working standard is 100 µg/ml.

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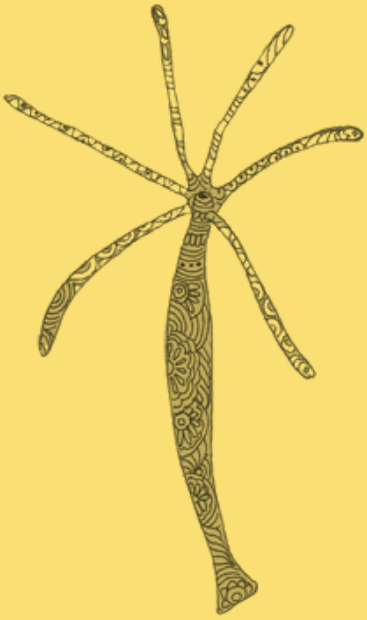

Hydra

The freshwater *Hydra* polyp emerged as a model system in 1741 when Abraham Trembley not only discovered its amazing regenerative potential, but also demonstrated that experimental manipulations pave the way to research in biology. Since then, *Hydra* flourished as a potent and fruitful model system to help answer questions linked to cell and developmental biology……. However, the Hydra model system is not restricted to cell and developmental biology; during the past 270 years it has also been heavily used to investigate the relationships between Hydra and its environment, opening new horizons concerning neurophysiology, innate immunity, ecosystems, ecotoxicology, symbiosis...

Brigitte Galliot, The Hydra Model System, 2012.

Hydra a multifaceted model in experimental biology

Sheryl Alphonso and H. Ramachandran

Abstract

Hydra is a freshwater Cnidarian polyp with a simple body plan which is two layered (diploblastic). *Hydra* can be easily collected from fresh water bodies as it prefers to inhabit well aerated slow flowing water. The transparent body of the organism and its mode of feeding can generate immense interest amongst students. *Hydra* can be easily maintained in dechlorinated water in well-aerated glass containers. Regular feeding with use of *Daphnia* or *Artemia*, avoiding fouling of water with excess feeding, is a requirement for its successful maintenance. The culture can also be maintained using the standard *Hydra* culture medium at 22 ± 1°C. This organism reproduces both asexually as well as sexually. *Hydra* polyps are well known as a model for regeneration studies. This invertebrate escapes senescence due to the vigorous activity of its three types of stem cells (epidermal, interstitial and endodermal) by self-renewal. The simple bi-layered body of *Hydra* enables it to quickly respond to chemicals in its immediate environment.

Introduction:

Hydra is a small aquatic organism found primarily in freshwater. It is used extensively to as a model organism to study ageing, cancer, regeneration and stem cell biology. *Hydra* has an advantage over *C. elegans* and *D. melanogaster* for studies on ageing as they can be used to study genes associated with human longevity. *Hydra* is useful in the study of cancer because of apparent lack of tumor formation.

The *Hydra* are very simple multicellular organisms that exist as polyps. The *Hydra* polyps attach to the floor of any body of water. The dimensions of the polyps (5-20 mm in length and 0.3-1.0 mm wide) are an approximation because the polyp is constantly contracting and extending. The simple body plan of *Hydra* comprises a cylindrical, tubular structure consisting of 2 germ layers – endoderm and ectoderm which are separated by an extracellular matrix. At one end of this central tube is the hypostome, a dome-like mound of cells with a mouth in the center. A whorl of tentacles (of varying number depending on species) encircles the hypostome. An adhesive basal disc with which the *Hydra* attaches to its substratum is at the bottom end of the cylindrical body tube. In the *Hydra* polyp, there are three cell lineages developing from their own stem cell population that replace the cells of the polyp body every 20 days.

Hydra colonies are very easy to maintain. The *Hydra* polyp has great regenerative capacity and is sometimes considered immortal. The cells of the polyps can be dissociated with ease, centrifuged and still reaggregate into intact organisms. This ability of the cells permits generation of Hydra cell lines. Cells from different *Hydra* lines can be reconstituted into polyps to produce transgenic animals easily. Further, different species of *Hydra* can be grafted onto each other. *Hydra* is, therefore, the model of choice to study of signaling and tissue dynamics in regeneration. Transgenic animals can also be created easily by microinjection of DNA into the embryo; the transgenic lines can then be maintained indefinitely once they are budding adults. As long as the *Hydra* are maintained in favorable conditions so as not to induce sexual reproduction, many clones of transgenic animals can be maintained with ease. When conditions are harsh, the *Hydra* polyp switches to sexual reproduction. The egg is fertilized whilst

still attached to the parent and subsequently breaks off and floats freely as an embryo. The embryo develops into a young polyp as it implants and matures into a budding polyp.

Ethical considerations:

As an approach to animal experimentation care must be taken to adhere to the 3 R's (replacment, refinement and reduction)

Easy culture maintenance:
Hydra can be maintained easily using fresh water medium.

***Figure1a**. Hydra budding*

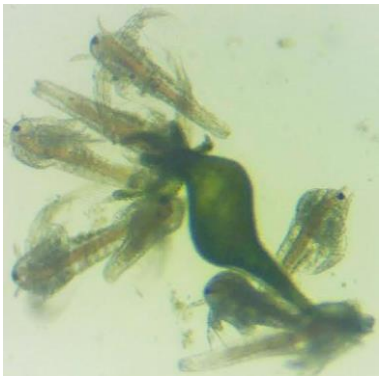

***Figure1b**. Hydra feeding.*

Hydra- prey interaction:

In *Hydra*, feeding response begins by the out-flowing of a chemical called glutathione from the injured prey. In the absence of a prey some of this behavior such as tentacle withdrawing and mouth opening can be produced by polyps that are exposed to reduced glutathione (GSH), suggesting that specific chemoreceptors mediate this response. The feeding response is interrupted by the removal of reduced GSH.

***Figure 2.**Prey paralyzed by nematocysts of the tentacles.*

Events involved in the feeding response.

Feeding activities in *Hydra*consist of a series of complex behaviors. This concise description was given by Josephson,1967. 1) The prey strikes the polyp, usually the outstretched tentacle and is attached there by the nematocyst discharge. 2) The tentacles contract and the prey are brought closer to the mouth.

3)The mouth opens and the prey is pushed inside the mouth and the mouth closes. The capture and engulfment of the prey can be enumerated as:

Nematocyst discharge- The first element in feeding behavior is the attachment of the prey to an outstretched tentacle by means of nematocyst discharge. The two types of nematocyst involved in prey capture, stenoteles and desmonemes, both discharge to the mechanical stimulation caused by the prey. The desmonemes coil around the prey, entrapping it while the stenoteles pierce its exoskeleton in a hair pin-like fashion releasing a neurotoxin and paralyzing the prey. The discharged nematocysts are released from the tentacle as the prey organism is enclosed by the mouth

Tentacular movements- When the prey is attached to the tentacle, there is a period before which there are observable movements of the tentacles. The tentacles usually contract at the proximal end at the point of contact with the prey. There are often bending or spiraling movements depending on the position of attachment. The tentacles bring the prey closer to the mouth and a concerted flexion of the other tentacles. Such flexions repeatedly occur and the mouth opens and the prey is ingested after which the mouth closes. The tentacle movement may happen as a result of mechanical stimulation from the prey and do not occur without nematocyst discharge. GSH is a specific chemical stimulator of the feeding response in *Hydra*. *Hydra* feeding activities were activated by GSH release when the prey is pierced by a discharged nematocyst.

Mouth Closure- The later stages of feeding behavior in *Hydra* consists of mouth creeping around the prey and closing. But once the gastrovascular cavity of the animal is filled, the *Hydra* does not attempt to ingest the captured prey.

Neurotransmitters involved in the Glutathione- induced feeding response.

In the vertebrate nervous system, g-aminobutyric acid (GABA) and glycine are the primary inhibitory amino acid neurotransmitters. GABA is a part of all regions of the CNS, while glycine is seen in the cerebrum, brainstem and spinal cord. In *Hydra*, GABA and glycine control the feeding behavior by responding to GSH. This information recommends that in *Hydra* numerous GABAergic loci exist in various body columns, perhaps adding to successive adjustment of the neuronal connections that controls functional and conducting systems.

Methodology:

1. Maintenance of stock culture

Polyps are cultured in glass crystallizing bowls containing dechlorinated water (DC) at a constant temperature of 22+1^{o} C with 12 hours of day and night cycle and are fed with freshly hatched *Artemia* nauplii daily to obtain a large number of polyps. Alternate day feeding pattern are also followed to maintain a fairly regular number. The medium is changed within five hours post feeding to avoid fungal and bacterial growth of dead and leftover Artemia. On days where *Hydra* are not fed, the medium is decanted and replaced with fresh medium.

Hydra can also be fed with other small crustaceans such as *Daphnia* and *Cyclops*.

Assay to induce feeding response in *Hydra*:

Construction of the assay.
Mature polyps are transferred into a single well of a 24-well plate. Fresh medium can be added to each well. 3.6 µM of freshly prepared glutathione is added into the well plate previously containing 500 µl of medium and the plates are placed under a Stereo dissection microscope and the cultures are pre-exposed to the microscope light for 5 minutes. (Nikon, MODEL CDSS230, Japan, 1x magnification).
A two- minute video recording of relaxed *Hydra* is taken before adding glutathione. 200 µl of freshly prepared glutathione is added and recordings are taken for the next five minutes.

Analysis.
Image J software can be used to analyze GSH induced contraction of the tentacles. Tentacle spread can be calculated as distance between the hypostome and the end of the tentacle. Average tentacle spread before and after induction of feeding response is now calculated over time as described by Kulkarni &Galande(2014).

Drug treatment (eg., using VPA)
Each *Hydra* is incubated in a separate well of a 24 well plate, with 1.5 ml of desired concentration of 0.1 mM/ 0.5mM VPA prepared in dechlorinated water for specific time periods, usually 3 to 24 hours (Berking, 1991).

Toxicity testing.
VPA treated *Hydra* isobserved under 1x of stereo dissection microscope. Morphology is compared with Wilby's scale as described (Quinn, Gagné, & Blaise, 2012; Zeeshan, 2017). Toxicity scores can be recorded for each Hydra based on the morphology of the polyp on a score ranging from 10 to 0 (here score 0 indicates disintegrated polyp).

Staining protocols:

1. Methylene blue staining (Burnett & Diehl, 1964)
Hydra is fixed in PFA for 30 minutes. Give 1X PBS washings (3 times) for 5 minutes each. Give a single wash with 1X PBST washing for 5 minutes. Incubate the polyps with 0.1 % Methylene blue at room temperature for 5- 10 minutes. Wash with water (3 times). Mount using 100 % glycerol and seal with nail polish. Store at 4 ^{o}C.

2. Colchicine treatment (Campbell, 1976)
Hydra are incubated in 0.6% Colchicine for 8 hours. They are relaxed in $MgCl_2$. Silver staining of *Hydra* can be performed and then mounted in glycerol. This treatment needs to be done to relax the polyp and retain its expanded form for staining.

Silver staining (Carter, Hyland, Steele, & Collins, 2016)
Relax *Hydra* in 2.5% MgCl2 (1-2 mins)**.** Fix *Hydra* in 10% Formalin (1-5 minutes) and transfer to distilled water, 3 changes. Preheat a staining jar with 20% silver nitrate solution, 35 seconds in the microwave, add *Hydra*, and place in an incubator at 37°C for 15 minutes. Wash in distilled water, 3 changes each.
Mount the *Hydra* on to a slide and place the slides in ammonical silver solution, 10 minutes, in 37°C incubator. Place slides in Working Developer solution, 1 to 5 minutes, check under microscope. Wash in fresh ammonia water. Wash it in distilled water, 3 changes. Dehydrate, clear and mount.

3. Toluidine blue (Murugadas, Zeeshan, Thamaraiselvi, Ghaskadbi, & Akbarsha, 2016)
Hydra is fixed in 4 % Paraformaldehyde (PFA) overnight. After washes, *Hydra* are incubated with 0.05% Toluidine blue dissolved in 10mM Tris-Cl for 30 minutes. Washes of 90% and 100% alcohol are used to remove excess stain. Glycerol mounted *Hydra* now are viewed under 40X of microscope. (Carl Zeiss Axioscop 2, 40x magnification).

Suggested projects:

i. To study developmental biology: Morphogenesis, neurogenesis, sex reversal, regeneration and ageing, stem cells and its regulation.
ii. To study stress response, cell survival, cell death and autophagy, evolution of innate immunity, epigenetic regulation during development.
iii. To study behaviour: Assay to study feeding responses, evolution of symbiosis, light/ dark conditions and behavioural responses.
iv. For ecotoxicity related studies since they undergo graded changes in their body structure in response to lethal or sublethal concentrations of toxic compounds.

Chemical preparations:

A. Composition of 100X *Hydra* medium stock solution (MACS- Agharkar ResearchInstitute, Pune)

0.1 mM KCl
1.0mM NaCl
1.0m M MgSO4.7H2O
1.0mM CaCl2.2H2O
1.0 mM Tris base
(Adjust the pH to 8.0 using Tris-HCl)

Dissolve all the above chemicals in 50 ml of distilled water (previously heated to boil) except $MgSO_4$. To avoid precipitation adjust the pH and then make up the final volume to 100 ml. Prepare 1:100 dilution to obtain 1X HM.

B. Dechlorinated water
Tap water was kept in open containers (bottles or tubs covered with muslin cloth) for 48hours. This water is used to culture *Hydra*.

C. Preparation of *Artemia*

The *Artemia* cysts are hatched in a hatchery in 2% salt medium equipped with an aerator. Complete hatching requires 48 hours of aeration. To collect the *artemia* for feeding:

i. Switch off the aerator of the hatchery and collect the *Artemia* into a beaker. Allow them to settle for a few minutes.
ii. The beaker kept aside would have by now segregated into three layers: the upper shells, the middle *Artemia* (which are orange in colour) and all the unhatched *Artemia* would have settled at the bottom. Decant off the upper layer and only collect the hatched *Artermia* carefully.

iii. Wash the organisms thoroughly in RO water to remove all the salt. Feed approximately 20 *Artemia* per Hydra.

A. Behavioral assays:

1. Glutathione- Induced feeding Response.

Glutathione

Molecular weight= 307.3235grams/ Molar

a) Super stock solution (16269.5 µM) = 10 mg Glutathione in 10 ml dechlorinated water.

b) Stock solution (90 µM) = 55.318 µl of super stock in 10 ml of dechlorinated water.

c) Working solution (3.6 µM) = 1 ml of stock solution in 9 ml of dechlorinated water.

2. Valproic acid (for Ecotoxicity related research experiments)

1M= 144.211 g/ mol

1mM= 20 µl of 1M VPA in 20 ml of dechlorinated water.

0.1mM= 2 ml of 1mM VPA in 18 ml of dechlorinated water.

0.5mM= 10 ml of 1mM VPA in 10 ml of dechlorinated water.

B. Cellular assays:

1. Methylene blue staining.

10X PBS

8g NaCl

0.2g KCl

1.44g Na2HPO4

0.24g KH2PO4

Make up volume to 100 ml and adjust pH to 7.4 with Tris- Cl

1X PBS

Dilute the stock solution of 10X PBS 1:10 with D/W to prepare 1X solution.

PBT

0.1% (v/v) Tween 20 in 1X PBS

2. Silver staining of nerve cells.

a) Fixative- 10% Formalin

5 ml of Formalin in 45 ml of dechlorinated water.

b) 20% Silver nitrate

Silver nitrate 5g

Water 25ml

(Make fresh solution)

c) Ammonical silver solution

20% Silver nitrate 25ml

Ammonium hydroxide Add drop by drop till the precipitate dissolves.

d) Ammonia water

Water 25ml

Ammonium hydroxide 4 drops

e) Developer stock (Make fresh)
Formalin 10ml
Citric acid 0.25g
Nitric acid 1 drop
Water 50ml
f) Developer working
Water 25ml
Ammonium hydroxide 4 drops
Developer stock 4 drops

3. Removal of nerve cells and interstitial stem cells using Colchicine treatment.
0.6% Colchicine
0.6g Colchicine in 100 ml of dechlorinated water.

4**. Toluidine blue staining of Battery cell complex (BCC).**
a) 0.05% Toluidine blue
0.025g in 50 ml of 10mM Tris-Cl
b) 10mM Tris- Cl (pH 7.5)
Molecular weight= 157.60 grams/ Molar
0.0788g in 50 ml ddw (10mM)

We acknowledge the contribution from Dr. Anuttama Kulkarni during standardization of theseprotocols.

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Moina (Water flea)

Water fleas of the genus *Daphnia* have been a model system for hundreds of years and is among the best studied ecological model organisms to date. *Daphnia* are planktonic crustaceans with a cyclic parthenogenetic life-cycle. They have a nearly worldwide distribution, inhabiting standing fresh- and brackish water bodies, from small temporary pools to large lakes. Their predominantly asexual reproduction allows for the study of phenotypes excluding genetic variation, enabling us to separate genetic from non-genetic effects. *Daphnia* are often used in studies related to ecotoxicology, predator-induced defence, host–parasite interactions, phenotypic plasticity and, increasingly, in evolutionary genomics.

Dieter Ebert, *Daphnia* as a versatile model system in ecology and evolution 2022.

Moina (Water flea) – Adaptable system with multi-disciplinary approach

H. Ramachandran

Abstract

Daphnia and *Moina* are inhabitants of fresh water and both belong to the order Cladoceran and class Crustacea. *Moina* and *Daphnia* are commonly known as "water flea" and are well established aquatic crustaceans. During normal aquatic conditions *Daphnia* reproduces asexually by parthenogenesis and produces diploid eggs. Here, the eggs directly develop into larvae in the brood chamber and are released by the female. These offsprings are found to be exact genetic clones of the mother. During extreme environmental conditions and also during lack of food resources, meiosis leads to production of haploid eggs. Dormant eggs are formed post fertilization to enable them to overcome the extreme conditions.

The important factors for the popularity of this model system amongst biologists are its easy availability, simple maintenance (in chlorine free fresh water), rapid life cycle and low cost. As a primary consumer its food resource requirements are mere two drops of milk/*E.coli* suspension / dry yeast suspension culture in 100 ml of culture water.

In the current scenario of extensive contamination of our freshwater bodies (anthropogenic factors) *Daphnia/Moina* are considered to be ideal freshwater keystone species. Hence they have immense significance in the interdisciplinary studies of ecology, ecotoxicology and genomics. *Daphnia* as a crustacean is also becoming an emerging epigenetic model to study environmental factors inducing phenotypic differences in clones produced via parthenogenesis.

Introduction

Cladocerans are zooplankton that thrive in freshwater conditions. They are commonly called "water flea". They have an uncalcified shell carapace covering their body. They have body appendages used for feeding, respiration and swimming. For almost 150 years the freshwater microcrustacean, *Daphnia* has been studied due to its extreme sensitivity to aquatic conditions. They are often used to monitor water quality so that only safe water is released into the environment by industry and water treatment plants.

Males are smaller than females and sex determination is dependent on environmental cues. A distinct phenotype appears which includes helmets (cranial extensions) and neck teeth formation in response to predators (polyphenisms). Since clonal lines are exactly similar, it is an excellent candidate for study of epigenetics.

Based on environmental cues, these transparent crustaceans are cyclic parthenogens producing two types of eggs, diploid parthenogenetic eggs and haploid sexual eggs. A lack of food supply favors sexual reproduction. In female *Daphnia* egg development is direct and the young ones are released by ventral flexion of the abdomen.

Both, stressed and unstressed *Daphnia* are used as model systems for the study of both evolutionary and developmental biology. *Daphnia pulex* although is the only crustacean with its complete genome sequenced.

The tiny crustacean *Moina* belonging to the family of Moinidae inhabit freshwater and is the dominant genus found in Mumbai (Figure 1). It has both asexual and sexual phases. Females start reproducing asexually at 4 to 7 days of age and the broods can be observed within the transparent body. During adverse conditions parthenogenetic females produce haploid eggs which develop into males. Thus, it favours sexual reproduction. The fertilized eggs (resting eggs) are encased in a carapace called ephippium, which gets finally released when the female undergoes molting. Hence, sexual reproduction contributes to genetic diversity in comparison to parthenogenesis.

Moina populations vary widely in density within a growing season. Their stock culture can be maintained in a tank of 1 liter capacity with a density of 5 individuals/ml. Water temperatures of 26°C to 30°C and a light regime of 12 hours light and 12 hours of darkness should be maintained for a successful culture. 1 ml suspension of *E.coli*/Baker Yeast or 1 gm of poultry droppings, cattle dung, fish/cattle feed can also be a source of food for every day culture maintenance. The food source consumed will give a slight coloration to its gut, visible through its transparent body. The initial food suspension may give a cloudy appearance to the water which becomes transparent once the water flea have consumed the food sources. Excessive cloudiness of the water indicates its poor quality and it is time to start a fresh culture.

As an established model system for ecotoxicological studies, the heart rate recordings can be a very important parameter for undergraduate studies. The mean heart rate for *Moina* at 20^{o}C has been recorded to be around 200 beats/min. Heart is located dorsally. It has an open circulation, with oxy-Haemoglobin (Hb) increasing in response to low oxygen conditions giving a reddish tinge to the animal.

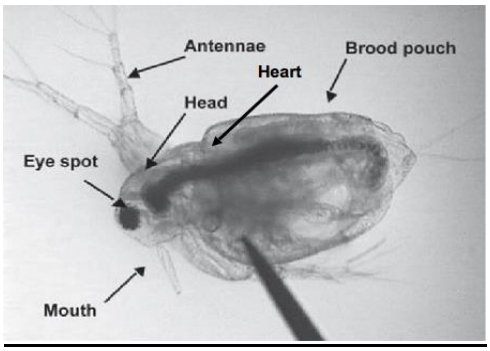

***Figure** 1 Moina*

http://cfb.unh.edu/cfbkey/html/Organisms/CCladocera/
FMoinidae/G*Moina*/*Moina*_micrura/*Moina*micrura.html

Methodology:

i) *Moina* is immobilized in a droplet of pond water under a thin layer of cotton fibers taken on a cavity slide.

ii) It is immediately located under 10X power of a compound microscope.

iii) The water medium is maintained at room temperature. After a minute of acclimatization, the heart beat observations can be recorded.

iv) For the experiment *Moina* can be exposed to different concentrations of the toxicant in a small petri dish/slide ranging from one minute / 30 minutes exposure each from the lowest concentration to the highest concentration. At least 5 sets of recordings are recorded for each exposed concentration along with a control. Observations are similarly recorded from at least 5 different *Daphnia*/*Moina* for each concentration.

v) Use of a timer helps with counting the heart beat with a light microscope. Alternatively, a video recording of the organism can be taken and the heartbeat can be counted from this.

vi) Ethical handling of the organism: *Moina* are returned to the culture pond by gently dipping the cotton mesh first into the pond water sample and then returned into the main culture pond.

Caution: Please check the culture samples supplied to you since the sample may contain both *Daphnia* and *Moina*. The crustacean *Moina* very often outnumbers *Daphnia*.

Suggested projects:

i. Using parthenogenetically produced clones of water flea (with a complete lack of genetic variability) for monitoring epigenetic effects.
ii. To conduct correlation studies using the phenotypic assay systems (sexual reproduction, helmets, neck teeth, growth, and fertility) based on its environmental responses.
iii. Exposing water fleas to heavy metals/temperature variations/hypoxia (stressed conditions)/pharmaceutical drug metabolites and relating it to evolutionary and developmental biology.
iv. Ecotoxicological studies can be undertaken due to the extreme sensitivity of the water flea to aquatic temperature variations and pesticide pollution.
v. To study the potential impacts of ambient water temperature and pesticide pollution on myogenic heart of *Moina* and comparing it to the cardiovascular functions of vertebrates.

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Caenorhabditis elegans (Soil nematode)

This was from a student in China. His e-mail said: "Dear Dr. Sydney Brenner, I wish also to win a Nobel Prize. Please tell me how to do it." I have been considering the reply which will say something like this: First you must choose the right place for your work with generous sponsors to support you. Cambridge and the Medical Research Council will do. Then you need to discover the right animal to work on – a worm such as C. elegans for example. Next, choose excellent colleagues who are willing to join you in the hard work you will need to do. How about John Sulston and Robert Horvitz for a starter. You must also make sure that they can find other colleagues and students. Everybody will have to work hard. Finally, and most important of all, you must select a Nobel Committee which is enlightened and appreciative and has an excellent chairman with unquestioned discernment.

Sydney Brenner, Nobel Banquet Speech, December 10, 2002.

Caenorhabditis elegans (Soil nematode) - A simple yet versatile model system

Nabila Sorathia and Medha S. Rajadhyaksha

Abstract

Research initiatives in graduate programs are often limited by resources available. The soil worm *Caenorhabditis elegans* is an ideal system to work on under such circumstances. An eukaryote, with a short life cycle, transparent body and ease with which it can be maintained has made the worm a popular animal model and has been used by scientists to address complex questions in cell and molecular biology. It has also emerged as a system of choice to study animal behaviour, ecology and environmental sciences, toxicology and pharmacology. It is possible to set up quantitative assays that can be done with minimal equipment using *C. elegans*. With its complete genome deciphered and the developmental trajectories of all its neurons known, the worm lends itself to understanding mechanisms of action of many complex biological phenomena. Here we narrate the basics of worm biology that would help set up the system effectively and suggest some assays to initiate a student friendly research program.

Introduction

Caenorhabditis elegans is a free-living (non-parasitic and non-pathogenic), transparent nematode (roundworm). It is an unsegmented pseudocoelomate, about 1 mm in length, lives in temperate soil environments, and lacks respiratory and circulatory system.

In 1963, Sydney Brenner proposed *C. elegans* to be used as a model organism. It is the first multicellular organism to have its entire genome sequenced containing more than 20,000 protein-coding genes (~60–80% human homologs) and the only organism to have its connectome (neuronal "wiring diagram") completed. Therefore, *C. elegans* is extensively used as a model organism, to understand neurobiology, reproductive biology, development, cell lineage, aging and much more. It can be easily grown in laboratory conditions; it has a short life cycle of about 2-3 weeks. It has a very simple nervous system of just 302 neurons with all their functions known.

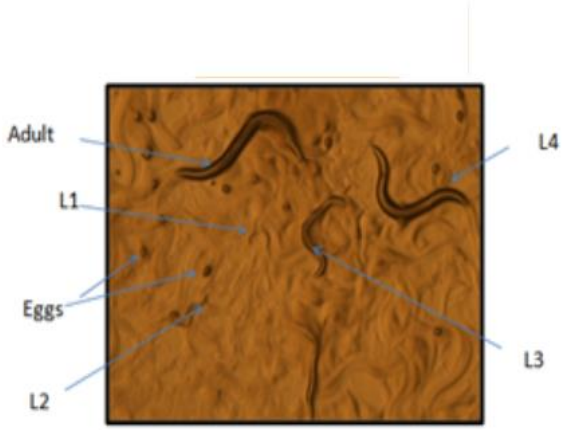


***Figure 1**. Image of various stages of development of C.elegans*

C. elegans has two sexes. There is a male sex (XO) and hermaphrodite (XX) which is self fertilizing. The two sexes can be distinguished with ease by the tail. The tail is simple and tapered in the

hermaphrodite but fan-shaped and specialized in the male. The genome of *C. elegans* has been sequenced and comprises six chromosomes (five autosomes and one sex chromosome).

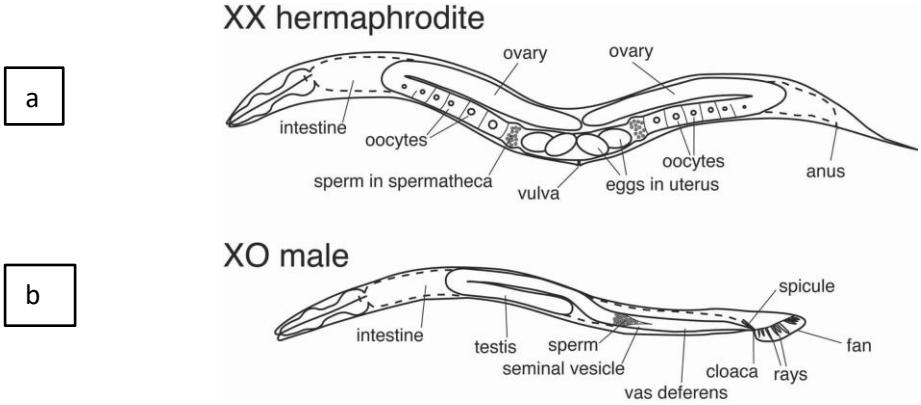


Figure 2 *Reproduction in C. elegans. (a) The hermaphrodite reproductive system (b) Male reproductive system.*

Life Cycle of the *C.elegans*: The oocytes and the sperms are produced in the hermaphrodite in the two gonadal arms (Fig. 2). In the hermaphrodite, the first 40 or so germ cells enter meiosis in each arm of the gonad. These cells develop into approximately 160 sperms which are stored in the spermatheca which is at the end of each oviduct. After this initial differentiation all subsequent germ cells differentiate as oocytes resulting in a switch of sex.

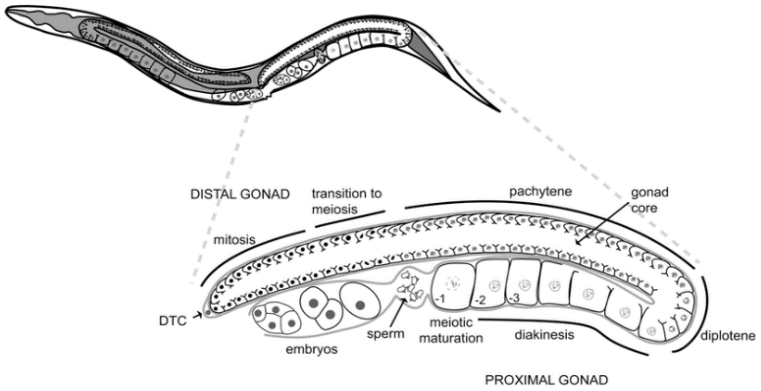


Figure 3*. Adult hermaphrodite gonad*

The oocytes pass through the spermatheca, are fertilized and enter the uterus. The fertilized eggs develop in the uterus and exit the uterus through a characteristic protrusion on the ventral side of the hermaphrodite called the vulva. The hermaphrodite can cross fertilize with the male. During this process the male slides its fan-shaped tail along the hermaphrodite's body surface and inserts specialized structures at the base of its tail into the hermaphrodite's vulva (Fig. 2b). Sperms from the male move through the vulva into the uterus of the hermaphrodite and migrate to the spermatheca. There the male sperms somehow gain an advantage over the resident hermaphrodite sperms so that for several hours after mating, the hermaphrodite produces almost exclusively outcross progeny.

Development from Zygote to Adult - Embryonic development begins in the hermaphrodite's uterus. A tough chitinous shell is secreted that encloses the around the fertilized egg. Figure 4 shows several stages of embryonic development that continues till the eggs are laid 2 hrs later.

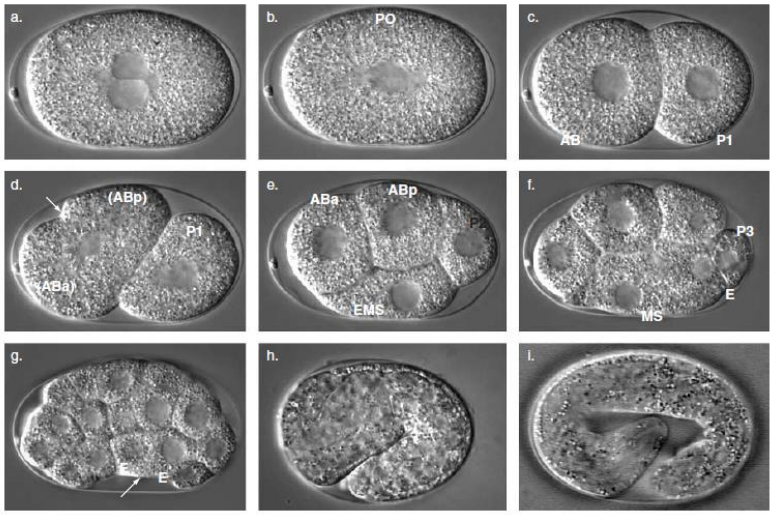


Figure 4. Stages of C. elegans development. *Embryonic development of C. elegans. Several of the earliest cell cleavages in the embryo are unequal; for example, the first mitotic division produces a larger AB daughter cell and a smaller P1 daughter cell (panel c). Gastrulation begins at the 28-cell stage as the two cells marked with a small E move into the embryo's interior (panel g). Later on, the embryo adopts a wormlike shape prior to hatching (panel i).*

Many of the early cleavages are unequal, producing daughter cells, called the blastomeres, of different sizes. Gastrulation, involving cell division and migration, leads to the formation of the three embryonic germ layers (endoderm, mesoderm, and ectoderm). Roughly 14 hours after fertilization, the first-stage juvenile, the L1 larva, hatches from the egg shell. The L1 larve are made of 558 cells and are about 250 mm long. Over the next 50 hours, three more larval stages appear L2, L3, and L4 by repeated molts. (Fig.5). A new cuticle, with different characteristics, is synthesized under the old cuticle at every molt before the old one is shed. The cells present at the hatching of the L1 larva do not undergo further cell divisions, although they may increase in size as the adult is formed. After the final L4-to-adult molt, the hermaphrodite germ line produces only oocytes. In the male in the last molt, the development of specialized mating structures in the tail is completed

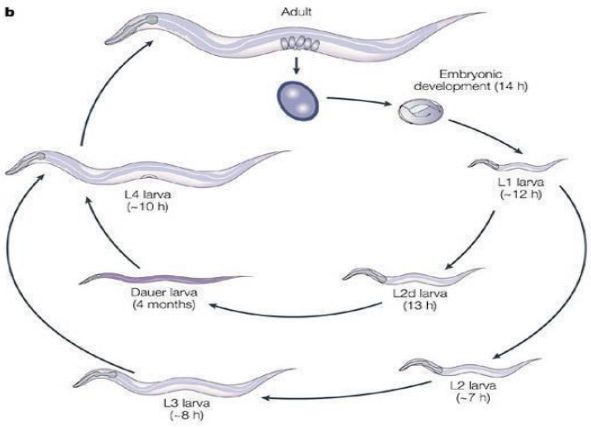


Figure 5*. Life cycle of C. elegans*

Interestingly, if conditions are crowded during early larval development, the L2 larva can molt to an alternative L3 form known as the dauer larva (German for long-lasting larva; Fig. 5). Dauer larvae do not feed. They move around rapidly at first and then become more dormant, although they are still able to respond to stimuli. Dauer larvae that do not experience excessive dehydration can survive for at least six months as they have a specialized cuticle that resists desiccation. Whenever food becomes available, they molt to L4 larvae and resume normal development Activation of the alternative dauer-larva pathway allows *C. elegans* to survive when conditions for reproduction are unfavorable and resume development later when conditions for reproduction improve.

Protocols:

Maintenance of *C. elegans* in laboratory conditions

Preparation of NGM plates:

1. Weigh the components of nematode growth media (NGM). Autoclave it.
2. Let it cool to 55°-60° C.
3. Add 1 ml 1 M $CaCl_2$, 1 ml 1 M MgSO4and 25 ml 1 M KPO4buffer and 1 ml cholesterol (5 mg/ml in ethanol). Please note that the salt solutions and buffer need to be autoclaved. Do not autoclave cholesterol but prepare in sterile condition.
4. After adding the solutions to the media, swirl to mix well.
5. Under sterile conditions pour the media in sterile petri plates (can be oven/autoclave sterilized).
6. Keep the plates at room temperature for 1-2 days for detection of contamination. Please note: keep the plate upside down to avoid condensation which may lead to contamination.
7. The plates, stored in an air-tight container at room temperature, are usable for many weeks.

Maintenance of *E. coli OP50* (used as feed for *C. elegans*):

i. Prepare Luria broth. Pre-mixed Luria broth is also available in the market with the steps to prepare.
ii. Cotton-plug the flask and autoclave it. Allow it to cool. This can be stored for several weeks.
iii. Under aseptic conditions, using nichrome loop or toothpick, pick up a single colony from the streak plate and inoculate it in Luria broth.
iv. Allow the cultures to grow overnight at 37°C; then it is ready to use for seeding NGM plates.
v. For storing the culture for several months, streak plate the *E. coli OP50* culture and store it at 4°C.

Seeding of *E. coli*:

i. Under sterile condition, put approximately 100µl of *E. coli OP50* liquid culture to small or medium sized NGM plates and 200µl of *E. coli OP50* liquid culture to large sized NGM plates using a pipet to create a lawn. Keep the bacterial lawn in the center, do not spread it to the edges of the plate, it may cause the worms to crawl on the sides of the plate and die.
ii. Allow the *E. coli OP50* lawn to grow overnight at 37°C. Seeded plates can be stored in an air-tight container for 2-3 weeks.

Transferring /subculturing of worms: (Figure. 6)

i. Various methods are used for transferring worms from one petri plate to another.

ii. Easiest method is chunking; in this method a sterilized scalpel is used to cut a piece or chunk of agar from an old plate and transfer it to a fresh pre-seeded plate. Hundreds of worms will crawl out of the chunk and spread out onto the bacterial lawn of the new plate. It is a suitable method, when worms are to be subcultured from a starved plate.

iii. Another method is by using strips of sterilized filter paper (½ to ¼ inch wide and 2-3 inches long, autoclaved). The strip is gently placed on the petri plate, where it absorbs moisture and few worms. Then it is touched to a fresh NGM plate where the worms are to be placed. After use, discard the filter paper.

iv. For picking up a single worm, worm-picker can be used. A worm-picker is commercially available or it can be made by fixing 1-inch piece of 32 gauge platinum wire into the tip of a Pasture pipette. The tip of the wire can be flattened or can be bent to form a hook/loop. Firstly, identify the worm to be picked under a dissection microscope. Slowly, lower the tip of the wire and gently swipe at the side of the worm and lift it up. By putting a blob of *E. coli OP50* at the end of the picker, and gently touching the top of the chosen worm can also be helpful in lifting the worm. The worm will stick to the bacteria. Now for transferring the worm, slowly lower the tip of the worm picker and gently touch the surface of a fresh NGM plate; hold it there so that the worm crawls off the picker. A bit of experience is needed with a worm picker to avoid poking holes in the agar.

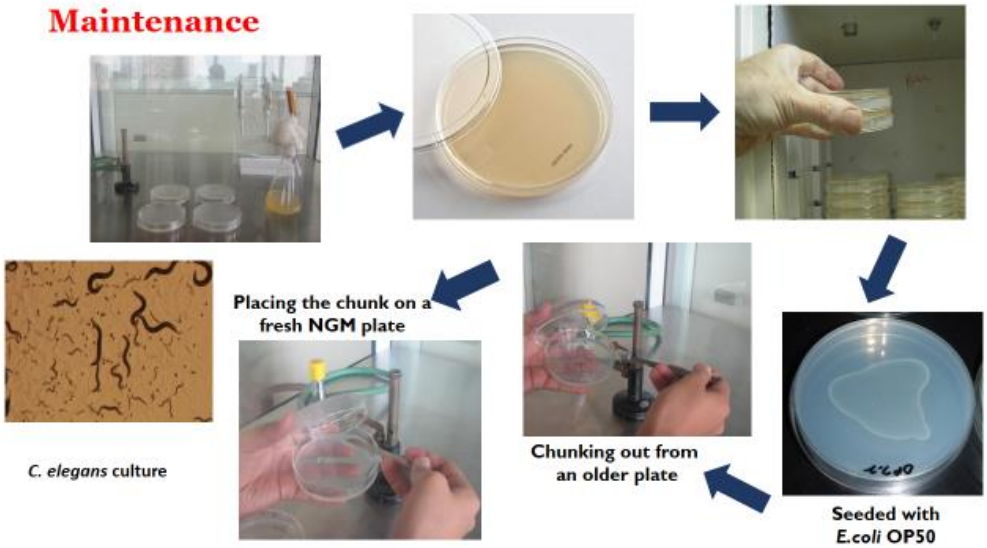
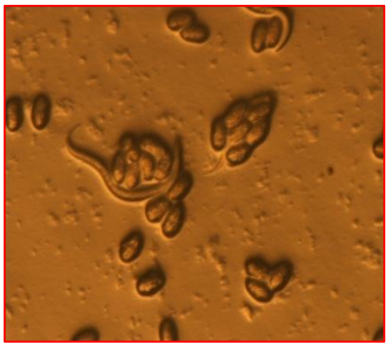


Figure 6. *Sub-culturing can be done every 2-3 days to prevent overcrowding and dauer formation due to limitation of food.*

Synchronization of worms

i. To obtain synchronized culture adult gravid worms are washed out of the NGM plates using M9 buffer in a microfuge tube.
ii. Allow the worms to settle down in the tube, then remove the supernatant.
iii. Add alkaline hypochlorite solution (1N sterile NaOH/NaClO 1:1); around 200 µl each, then make up the volume to 1 ml with sterile distilled water/M9 buffer.
iv. Triturate the pellet and centrifuge the tube at4000 rpm for 2 minutes.
v. Repeat the step iv twice;and finally the pellet obtained after bleaching is resuspended in fresh M9 and then placed on OP50- seededNGM plates and grown at 22º C until young adulthood.



Eggs obtained after bleaching

Freezing of worms:

Prepare Freezing Solution (1L)

i. When the plates are ready to freeze, they should have:
Little or no food (*just* starved)
Plenty of eggs, L1 and L2 worms (these are what will survive).
Worms with the correct phenotype.
No contamination (if contaminated, the worms will continue to eat and grow so there will be no stalling at the L1 stage)
ii. Add about 5 ml of M9 to each plate. Give each plate a swirl to loosen worms still stuck to the agar and then tilt plates so the liquid drains to one side of the plate.
iii. Using a pipette, collect the liquid (worms and M9) in a 15ml conical tube. Pellet the worms for about 1 minute at full speed in a clinical centrifuge.
iv. Aspirate as much supernatant as possible without disturbing the pellet.
v. Add about 10-15 ml of M9 and resuspend the pellet. Again, spin for 1 minute at full speed. Repeat step 3 and 4 till you get a clear solution with worms in it. Aspirate the supernatant.
vi. Add 3 mL of M9 and add an equal amount of freezing solution.
vii. Briefly agitate the vial to suspend the worms and aliquot the worm suspension into 6 cryovials, clearly labelled with the strain name, your initial and the date.
viii. The styrofoam container is useful for freezing worms. The insulation provides the slow decrease in temperature required for survival. Place the cryovials into the styrofoam, and cover with another. Secure with tape or rubber bands and label the outside for future reference.
ix. Store in your –70°C freezer space.
x. Test thaw the worms about 1 month later to ensure a successful freeze. A good freeze is when *at least* more than 10 worms survive. Pick several survivors on to a fresh plate to make sure they can produce progeny of the correct phenotype. Be sure to maintain the line while waiting for the results of the test thaw.

Thawing:

i. It is fine to pour all of the freezing liquid from a vial onto a plate when checking for survival.
ii. If a strain is being thawed for actual use the above step cannot be done. The vial should be thawed and the worms should be allowed to settle to the bottom. With a pipette, the worms should be

sucked up getting *as little liquid as possible*. Distribute the worms around the OP50 on a 6or 10cm plate. The worms cannot be centrifuged, because the glycerol will not allow a pellet to form.

The simple worm can now be used innovatively to answer complex questions !!

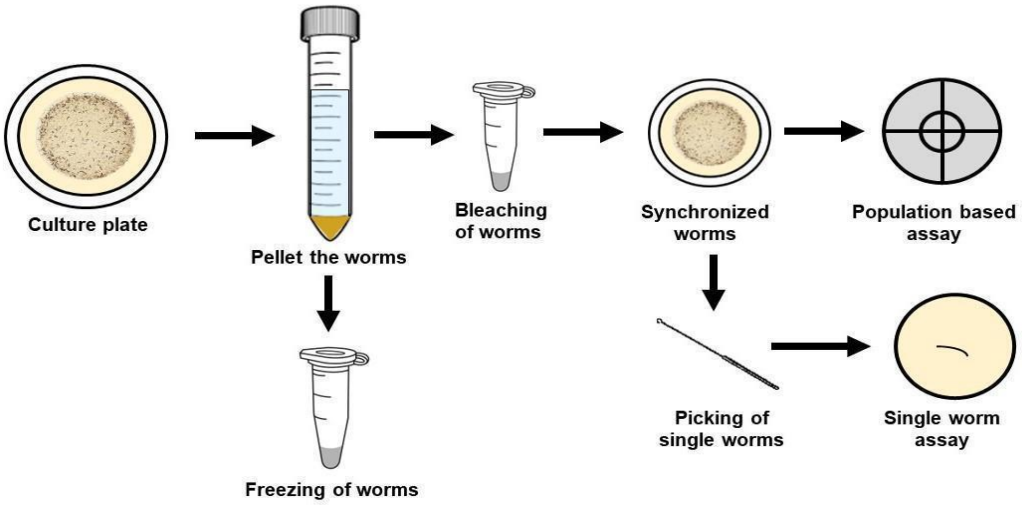


Figure 7. *Diagrammatic representation of steps for freezing and for Assays*

PROPOSED EXPERIMENTS
Behavioural Assays

1. Population based assays:
For population-based assays worms are bleached 3 days prior the assay to get a synchronized culture.

A. Olfactory assays

i. Prepare 2% Agar solution in distilled water, microwave it to obtain a clear solution.
*Prepare 10-20 ml extra solution than required since some of it might evaporate while heating.
ii. Add K_2PO_4 buffer, $MgSO_4$ solution and $CaCl_2$ solution to the agar solution (concentration of salts and buffer solution is similar to NGM media).
iii. Pour 8 ml of media in a 5 cm petri plate. (3 test+3 control). Allow it to solidify.
iv. Prepare the test solution by combining equal volumes of 1% butan-1-ol and 0.5 M sodium azide (an anaesthetic used to arrest the worms upon reaching a quadrant) and the control solution by combining distilled water with 0.5 M sodium azide. (Total Volume 1ml; it can be stored in fridge for future use)
v. Wash the worms using M9 buffer/distilled water and collect the pellet containing adult worms in a microfuge tube containing 200µl of M9 buffer/distilled water.Pipette 10µl of the worm solution from the pellet onto the origin (where the two lines intersect). Dab the area in the centre

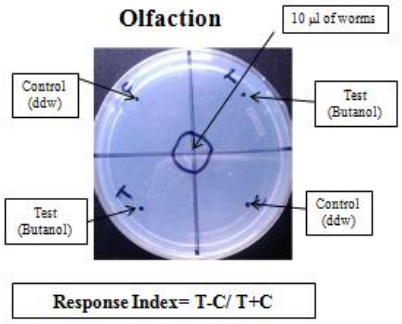
gently with a piece of tissue paper to remove excess M9 buffer so as to allow the worms to freely move on the plate.

vi. Immediately add 2 µl of the control solution onto the two "C" sites. Likewise, pipette the same amount of the test solution onto the two "T" sites. Replace the lids immediately.

vii. Place the worms in a dark place for 60 minutes at 22°C. (Avoid any form of disturbance)

viii. Count the number of worms present in each quadrant, avoid the worms present in the inner circle.

ix. Repeat steps 2 to 6 using the control solution in both the test and control quadrants. This will serve as the control plate. Three such plates should be made and run to serve as an appropriate control.



x. Calculate the chemotaxis response index (RI):

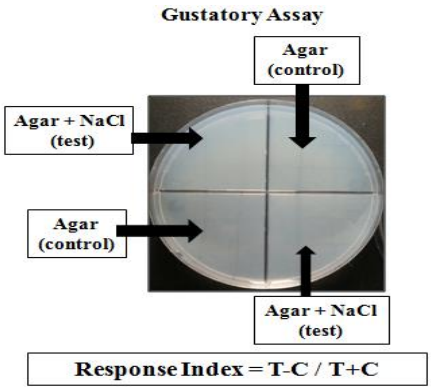
xi. R.I. = Number of worms in Test quadrant - Number of worms in Control quadrant
Number of worms in Test quadrant + Number of worms in Control quadrant

B. Gustatory assay

i. For gustatory assay four-quadrant plates are used.

ii. Prepare 2% Agar solution with and without 50 mM NaCl. Pour the two-diagonally opposite quadrant with 2% agar solution with NaCl and the remaining two with agar solution without NaCl. Allow it to solidify. Put a small amount of agar solution (without NaCl) at the centre (intersection region).

iii. Wash the worms using M9 buffer/distilled water and collect the pellet containing adult worms in a microfuge tube containing 200µl of M9 buffer/distilled water.



iv. Pipette 10µl of the worm solution from the pellet onto the centre. Dab the area in the centre gently with a piece of tissue paper to remove excess M9 buffer so as to allow the worms to freely move on the plate.

v. Place the worms in a dark place for 60 minutes at 22°C. (Avoid any form of disturbance).

vi. Count the number of worms present in each quadrant, avoid the worms present in the inner circle.

vii. Repeat steps 2 to 6 using the control solution in both the test and control quadrants. This will serve as the control plate. Three such plates should be made and run to serve as an appropriate control.

viii. Calculate the chemotaxis response index:
R.I. = Number of worms in Test quadrant - Number of worms in Control quadrant
Number of worms in Test quadrant + Number of worms in Control quadrant

2. Single worm assays:

A. Body Bend Frequency:

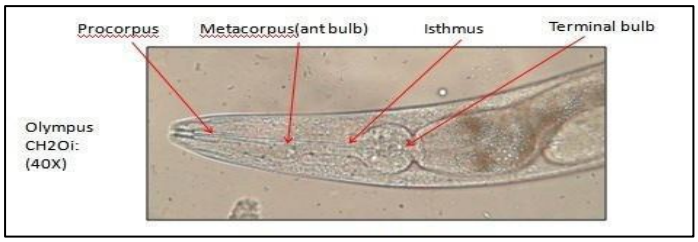
i. Pick up individual worms using a worm picker onto a fresh NGM plate and film for 20s-time interval using a camera attached to the microscope. You can also use an external camera.

ii. A body bend is defined as a change in the direction of propagation of the part of the worm corresponding to the posterior bulb of the pharynx along the y axis, assuming the worm was traveling along the x axis.

iii. Assays are performed on different independent days and in triplicates.

B. Pharyngeal pumping rate:

i. Feeding Behaviour was assessed by counting the number of times the terminal bulb of the pharynx contracts in 20s.



ii. 2. Individual worms are picked using a worm picker and placed on a fresh NGM placed seeded with OP50.

iii. 3. Worms are allowed to move for 10 mins at RT.

iv. 4. Plate is placed under a stereo-dissecting microscope and the worm is located at 11.25X. Video of the pharynx is recorded using an external camera.

v. 5. The rate of pharyngeal pumping is assessed by slowing down the video in VLC media player using application clicker.

Obtaining the worms

The worms may be obtained from preexisting cultures at Sophia College, Mumbai, or TIFR, Mumbai. In case it is not possible to do so a simple protocol can be used to isolate the worms from soil given below. The details can be found in the Worm Book (Check references)

***C. elegans* can be isolated from soil with ease :**

Spread about 1–2 grams of soil around the *E. coli* OP50 lawn of a standard NGM Petri dish, and moisten with about 1 ml M9 solution or water *C. elegans* are attracted and crawl out of the sample towards the bacterial lawn within minutes to hours. Pick individual worms of both sexes that have crawled out to a new plate to start a new culture. Be careful in identifying the worms!!

Proposed Open ended projects:

1. Metal toxicity studies can be carried out with end point observation on viability of worms, sensory activity (using gustatory and olfactory assays) and motor activity (using body bend and pharyngeal pumping assays).
2. Drug testing can be done to see the effect of drugs or drug combinations on behaviour of the worm.
3. Gut flora is known to alter behaviour. The worm can be grown on various bacterial lawns and its behavioural parameters can be monitored.
4. Effect of Environmental factors/pollutants/stressors can be assessed using the various assays.

Recipe to prepare reagents:

1. Nematode Growth Media (NGM contains 17 g Agar, 3 g NaCl, 2.5 g peptone and 975 ml double distilled water). Autoclave it. Let it cool to 55°-60° C. Add 1 ml 1 M $CaCl_2$, 1 ml 1M $MgSO_4$and 25 ml 1 M KPO4 buffer and 1 ml 5 mg/ml cholesterol in ethanol. Please note that the salt solutions and buffer need to be autoclave; do not autoclave cholesterol but prepare in sterile condition.
2. Luria broth:10 g Bacto-tryptone, 5 g Bacto-yeast, 5 g NaCl, H_2O to 1 liter, pH to 7.0using 1MNaOH.
3. M9 buffer: Dissolve 3 g of KH_2PO_4, 6 g of Na_2HPO_4, and 5 g of NaCl in 1 L of H_2O.
4. Freezing Solution (1L): 5.8g NaCl, 50mL 1M KH_2PO_4 (pH 6.0), 240mL glycerol, 710mL ddH_2O, Autoclave. Add 30µL 1M $MgSO_4$ per 100ml of solution.

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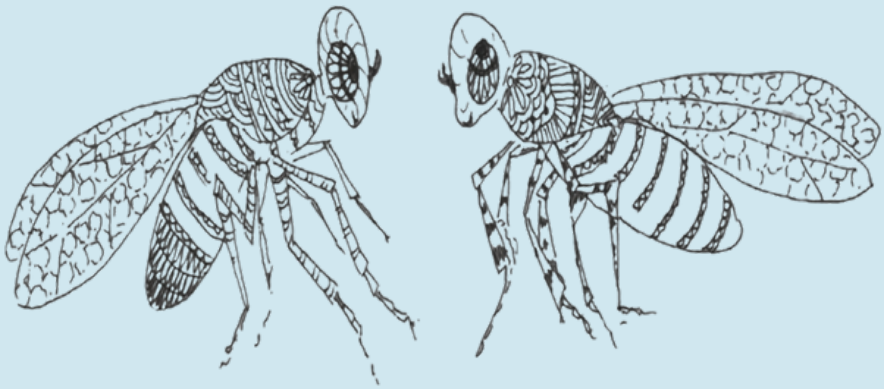

Drosophila melanogaster (Fruitfly)

….the small and totally harmless fruitfly, Drosophila....has revealed to us some of its innermost secrets and tricks for developing from a single celled egg to a complex living being of great beauty and harmony…

Christiane Nusslein-Volhard, Nobel Banquet Speech, December 10, 1995.

Drosophila melanogaster – Selected assays for Biology and Behaviour

Ojal D'Cunha, Saunri Dhodi Lobo and Hema Subramaniam

Abstract

Drosophila melanogaster or the humble fruit-fly, with its compact genome size, a short generation time of about 12 – 15 days and a lifespan of 60–80 days serves as an advantageous invertebrate model system to study various aspects of biology, right from developmental biology, genetics, immunology to neurobiology and evolution.

A few of the many experiments that have been tried in our laboratory using the fly to understand or answer questions posed for different Undergraduate and Postgraduate projects are presented here.

Introduction

Ever since its first cultivation in a laboratory at the Harvard University by Charles Woodworth in 1900-1901, the fruit fly has given researchers across the world an opportunity to understand biology at different levels of complexity. At Harvard, Woodworth suggested to his supervisor, William Castle, that *Drosophila* could be used for experiments in genetics. Castle's work inspired Thomas Hunt Morgan to use *Drosophila* to study genetic "linkage", explained in his book "Mechanism of Mendelian Heredity" in 1915. His discoveries led him to win the Nobel Prize in 1933.

Large populations of *Drosophila* can be easily maintained in any laboratory using minimum lab space. The maintenance of stock fly cultures can be effortlessly done on media containing readily available materials.

With reference to its genes, 75% of the disease-causing genes in humans have functional homologs in *Drosophila*. Many other diseases that do not have gene homologs in the fly can be mimicked artificially by gene introduction and manipulation and studied subsequently.

The Stages of the *Drosophila* life cycle

The fruit-fly *Drosophila* is a holometabolous organism. This means that its body structure changes completely as it matures from the worm-like larval form to the winged adult stage. The transient encased form in between is called the pupa. The young emergent adult is called the imago.

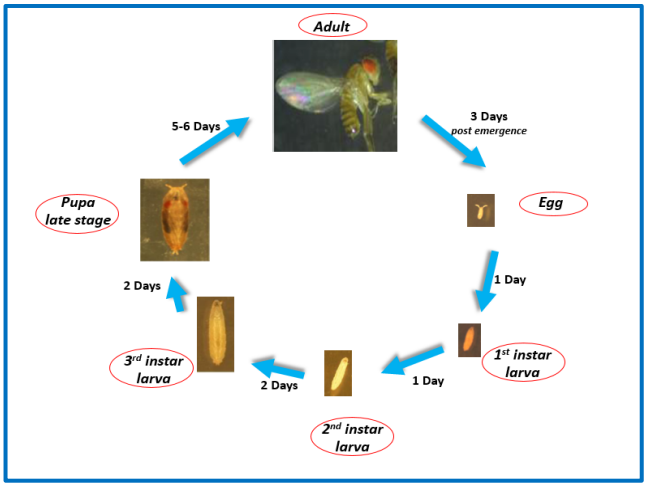

Figure 1: *The life cycle of Drosophila melanogaster at 25°±2° C. The sizes of the images are in proportion to each other.*

The entire cycle takes 12-15 days on an average, depending on the temperature, humidity and food availability.

Protocols

Medium preparation, Fly transfer, Maintenance and propagation of *Drosophila* culture

Medium preparation

The medium for maintenance and propagation of flies is prepared as given under the section on Reagents and solutions at the end of this module. The molten medium is poured into vials or bottles and allowed to solidify. Any moisture or vapour in the container is allowed to evaporate completely before transferring flies to be cultured in it.

Fly transfer

i) The prepared fresh medium bottle's / vial's cotton plug is uncorked.

ii) It is kept on a soft pad / sponge mat (usually called the "banger")

iii) The container with flies obtained for culturing is uncorked. Immediately that container is inverted on to the mouth of the prepared fresh medium bottle / vial so that both the containers' openings coincide. The two are held together as a set and tapped gently on the soft pad banger so that the flies from the top container enter the bottle below.

iv) The medium bottle is corked quickly so that the flies do not escape.

v) Maintain a log book of the transfers by numbering and labelling the bottles.

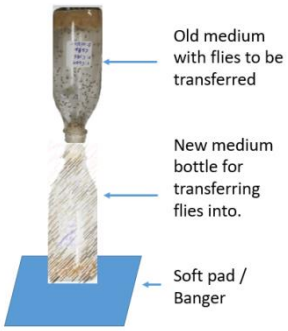

Figure 2: *Transferring flies*

Maintenance and propagation of the fly culture

i) The fly cultures are allowed to remain in the bottle / vial for about 12 – 15 days, depending on their rate of growth. They would have laid several batches of eggs by then, and larvae and pupae would have appeared.

ii) The flies are then transferred into fresh bottles / vials by the same procedure as explained above. This is necessary as medium depletion and/or overcrowding could cause stress to the flies.

Generally, there should not be more than about 40 flies in the bottle or more than about 20 flies in the vial.

Harvesting of larvae / fly

I. Harvesting of individual larvae

Individual larvae are harvested using a soft tip such as a paint brush. Any adult flies in the bottles are temporarily transferred into another bottle without medium. Wet the tip of a soft paint brush with water and gently lift off the larva you require from the bottle / vial. Keep it in a watch-glass / petri plate containing Phosphate Buffered Saline or Insect Ringer's solution, until use.

II. Bulk Harvesting of larvae

i) All adults (flies) are transferred into a fresh medium-containing bottle.

ii) 50 ml of 30% sucrose is added to the bottle with larvae and let sit for 10 minutes. The sucrose solution is dense, so the larvae will float to the top. If particles of medium are loosened, they will settle since they are denser than the Sucrose solution.

(Alternatively, the bottle with larvae can be rinsed with 20 – 30 ml Insect Ringer's solution to collect the larvae, poured into a plastic wire-mesh tea-strainer, and re-suspended in 5 ml Insect Ringer's solution. A 50 ml measuring cylinder can be filled to just below brim with 30% Sucrose solution and let sit for 10 minutes.)

iii) Collect larvae using a smooth edged broad-tipped pipette and place into the plastic wire-mesh tea-strainer.

iv) Wash larvae in the tea-strainer twice with Insect Ringer's solution. Keep the larvae moist in Insect Ringer's solution in a petri plate till use.

III. Bulk and individual Harvesting of flies

Flies can be harvested from the bottle / vial by anaesthetizing them by any of the following methods:

i) Etherisation ii) Cold inactivation iii) Mild CO_2 anaesthetization

Etherisation: The glass bottle / vial containing the flies is briefly exposed to 2-3 drops of ether placed on a fresh cotton cork for about 30 seconds to 1 minute. The immobilized flies are then removed by inverting the uncorked bottle and tapping on to a clean dry petri dish. The required flies are selected. The excess flies are returned to their bottle before they revive.

Cold inactivation: The glass bottle / vial containing the flies is placed in a refrigerator at 4°C for 10-15 minutes. The flies are temporarily immobilized by the cold known as "freeze coma". The immobilized flies are then removed by inverting the uncorked bottle and tapping on to a clean dry petri dish and the required flies are selected. The excess flies are returned to their bottle before they revive.

Mild CO_2 anaesthetization: This requires a CO_2 cylinder with a tubing set up for exposing the flies to the gas in an enclosed chamber. The procedure is similar to the etherisation protocol.

The first two methods are more commonly used.

Caution:

Do not over-expose the flies to chemical or cold treatment as it may cause death / defects. All the above processes must be done quickly to avoid the revived flies from flying away!

Identification of larval stages and obtaining larvae/adult flies at the required stage ("Staging")

Identification of larval stages

The eggs are 0.5 mm in size and found on the surface of the medium. They are easily visible along the edges of the medium in the bottle.

The first, second and third instar larvae have characteristics that can easily identify each stage.

The first instar larvae are 1 mm, found burrowing into the medium.

The second instar larvae are 2 mm, found crawling on the surface or lower regions of the wall of the bottle.

The third instar larvae range from 3-4 mm. Early 3rd instars remain on the middle regions of the wall of the bottle. Late 3rd instars climb to the upper regions of the wall of the bottle, and start darkening as they enter the pupal stage.

The pupae attach themselves to the topmost part of the bottle wall, often on the cotton cork also.

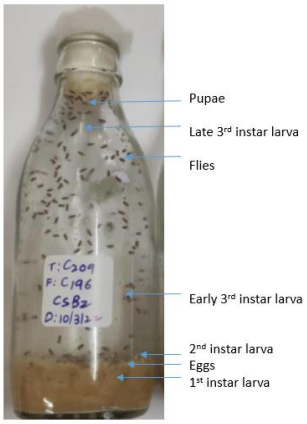

Figure 3: *The culturing of Drosophila melanogaster*

Identification of male and female flies

The adult male and female *Drosophila* are easily distinguishable – the "melanogaster" or black belly applies to the male. Other differences are males are smaller and bear bristles ("sex-comb") on their forelegs.

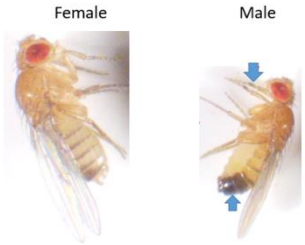

Figure 4: *Sexual dimorphism in Drosophila melanogaster – Males are smaller in size, have a sex-comb on their forelimbs (top arrow) and black abdomen tip (bottom arrow)*

Staging of larvae

To obtain selective stages of larvae in bulk, it is possible to synchronize the culture so that all or most of the individuals are at the same stage. For this, we ensure that the gravid female adult is allowed to lay eggs on the medium during a limited period of time, about 1-2 hours. The female is then transferred out of the bottle. Thus all hatched larvae will be of the same age. Allowing for the desired number of days according to the stage one wants for the experiment, one would be able to obtain the desired stage of larvae.

Staging of adult flies

To obtain adult flies of a particular age in bulk, clear / transfer out all the adults in the culture bottle, allowing only larvae and pupae to remain. Wait for the next group of adults to eclose from this bottle. Now transfer these imagos on the day of their eclosion into a fresh culture bottle with medium. This is day 1 of these adults. Label date of eclosion on this bottle. Fresh imagos can thus be harvested regularly from the same original bottle containing larvae and pupae. Allow for the required number of days and use the individuals as per the required age. (Males and females can be segregated if required, by anaesthetization.)

Behavioural Assays

Behavioural assays help understand a deficiency, mutation, or the effect of a drug at the functional level of the organism. Here, we explain a few selected simple-to-perform assays for larvae as well as for the adult flies.

Larval olfactory assay

The fruit fly offers multiple advantages for olfactory studies. At the level of gene homology, flies and mammals share surprisingly large numbers of olfactory gene orthologs. It thus offers the possibility of relating analyses from the genetic and molecular levels to olfactory behaviour, extending into learning and memory studies.

Drosophila larvae provide an added advantage over adult flies in such studies because they have nearly 2 orders of magnitude fewer receptor neurons -*Drosophila* larvae possess only 21 pairs of olfactory (and -80 pairs of gustatory) receptor neurons, compared with the 1300 pairs of olfactory (and -650 pairs of gustatory) receptor neurons in the adult fly, thus providing a cleaner molecular profile for analysis.

The larvae are sensitive to a wide variety of volatile chemicals.

The olfactory responses are measured by following its chemotactic movements on a petri plate. Several variants of this test have been employed. In the commonly used versions of the experiment, diluted odorant is placed near the edge of the dish and the diluent alone is placed in a diametrically opposite position.

Assay Protocol

Preparing the set-up for assay

i. Larvae are harvested from a bottle according to the bulk harvesting protocol above. Keep the larvae moistened in Insect Ringer's solution.

ii. Prepare 20 mL of 2 % agar and pour it into a 9 cm petri plate (either glass or plastic).

iii. Prepare paper discs by cutting or punching out 0.5 cm diameter Whatmann #1 filter paper..

iv. Place the discs in diametrically opposite positions on the agar in the plate.

v. Mark the center of the dish with a circle of 1 cm diameter, flanked by a pair of parallel lines running perpendicular to the filter paper axis, on the under-side of the plate. (See Figure 4)

vi. Rinse the harvested larvae by placing them in a tea strainer and collect them as a bunch or ball.

vii. Using a soft brush lift the larvae bunch (approximately 30-40 larvae) and place it within the center circle in the agar plate.

viii. Place 20 µL of D/W carefully on both filter paper discs, ensuring that no liquid flows over onto the agar.

Running the blank assay

To ensure that there is no bias in the set-up so as to obtain an even distribution of the crawling larvae when no cue exists, the blank assay is run.

ix. Close the petri dish immediately, cover the closed plate with a dark carton and start the timer.

x. After 2 minutes, remove the carton, open the plate and immediately count the number of larvae that have crossed the perpendicular lines on each side.

Running the assay for an odorant test sample

xi. Repeat steps i – vii stated above.

xii. In step viii, place 20 µL of D/W on one disc and 20 µL of the odorant sample (test solution) on the other disc. (Label below the plate "C" (control) at the D/W end and "S" (sample) at the sample end.)

xiii. The above steps ix – x are repeated to get readings.

xiv. At least three such sets are to be performed. Mean and Standard deviation are calculated.

Note: Only larvae that are distributed towards each end, C and S are counted. Those that are in the neutral zone (between the parallel lines) are not counted.

The Olfactory Response Index (O.R.I.) is calculated using the following formula:

$$\frac{S - C}{S + C}$$

Where *S* is the number of larvae in the sample zone and *C* is the number of larvae in the control (D/W) zone

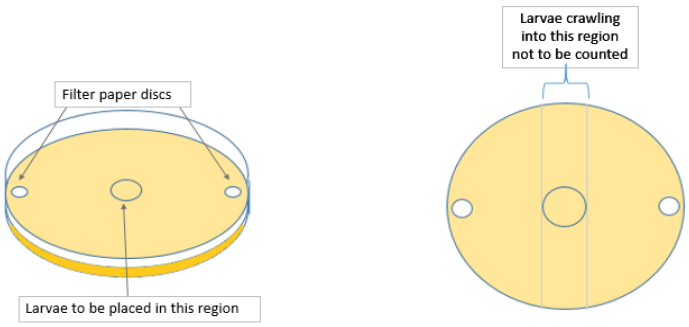

Figure 5: *The set-up for larval plate olfactory assay*

The response index ranges from +1 for a complete attractant through 0 for a neutral/no stimulus to –1 for a complete repellent.

The results obtained can be plotted on a graph as shown below

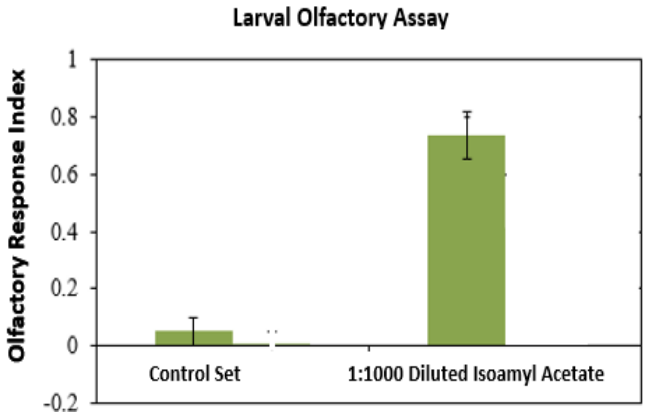

Figure 6: *Representative results obtained for Ethyl acetate (diluted 1:1000) by larval plate olfactory assay*

<u>Adult Locomotory Behaviour Assay (Climbing Assay)</u>

The behavior of an organism reflects its physiological condition. *Drosophila* displays several inherent behavioral responses that can be used as a test system. These assays range from the simple vertical locomotion (climbing) to complex assays like courtship.

Drosophila have a natural tendency to walk up the containers they are placed in. This is used as a measure of its "normalcy". Several methods to assess this have been commonly in use – it is possible to measure the time taken for the fly to reach a predefined position along the vertical surface, or the distance climbed in a given time. Flies with better motor abilities will be able to climb greater distances. Any impairment in this ability can be quantitated.

<u>Assay Protocol</u> (modified from Zografos et al, 2016)

<u>Preparing the set-up for assay</u>

i) A cylindrical tube with a diameter of 1.5 cm and 30 cm in height is used.

ii) Two marks, one at 9 cm and the other at 18 cm height are made with a glass marking pen. The bottom of the tube is corked with cotton.

iii) The apparatus is fixed upright using a burette clamp.

iv) 10 flies are isolated from the stock culture bottle according to the fly isolation protocol explained above, and introduced into the assay tube from the top opening. It is then sealed with a cotton plug

vi) A camera can be set up for recording the assay, if required. This is optional.

Running the assay

vii) The tube is tapped on a banger pad to give an even start to all flies as they all fall to the base at the same time before they start climbing.

viii) The timer is started at the time of tap.

ix) The activity of the flies is recorded for a total of two minutes using a camera.

x) The number of flies that climb past the marks is counted every 10 seconds.

xi) At least three such sets are to be performed. Mean and Standard deviation are calculated.

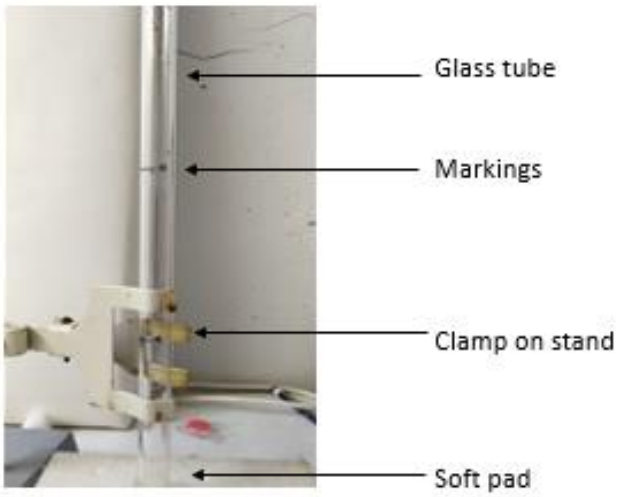

Figure 7: *The climbing assay set-up*

The results obtained can be represented as follows:

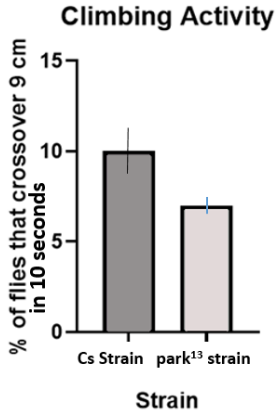

Figure 8: *Representative results of Drosophila Climbing Assay for two strains, Cs (Normal) and park^{13} (defective motor function) Note: Results for the 18 cm mark would also yield results representable in another such set of graphs.*

Adult Phototaxis Behaviour Assay (Visual Competence Assay)

This assay is based on the visual competence of the fly to differentiate light from dark and its natural preference for light. *Drosophila* with visual defects would not be able to do so. The extent of the defect can be quantitated thus. Genetic abnormalities, effect of a toxin or efficacy of therapeutic drugs could be checked by testing the phototactic index.

Assay Protocol (modified from Arya et al, 2010)

Preparing the set-up for assay

i. A "Y shaped" tube with a diameter of 1.5 cm for both arms and stem is used. The arms are 20 cms long and the stem of the Y is 10 cms. (This can be fabricated by glassware manufacturers.)
ii. One of the arms of the Y tube are sheathed in black paper. The mouths of both the arms of the "Y" are corked with cotton.
iii. A burette clamp is set up ready to hold the apparatus.
iv. 10 flies are isolated from the stock culture bottle according to the fly isolation protocol explained above, and introduced into the stem (bottom) of the Y tube and corked with cotton.

Running the assay

v. The tube is tapped on a banger pad to give an even start to all flies as they all fall to the base at the same time before they start climbing.
vi. The timer is started at the time of tap and at the end of 30 seconds, the number of flies in the two arms are noted.
vii. At least three such sets are to be performed. Mean and Standard deviation are calculated.

The standard formula is used for the calculation.

If L indicates the number of individuals preferring light and D indicates the number preferring dark,

$$\frac{L - D}{L + D}$$

The formula indicates the fraction of flies that preferred the light over dark.

If the calculated value tends to +1, the fly population prefers light as a visual cue. If it tends to -1, the fly population prefers the dark; a value of 0 indicates no preference to either.

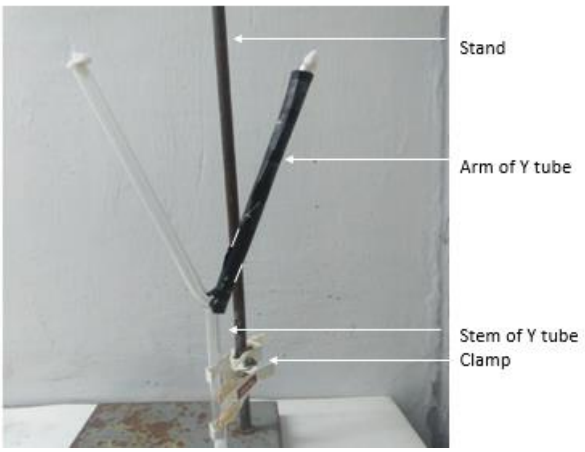

Figure 9: *The phototaxis assay set-up*

The results obtained can be represented as given below:

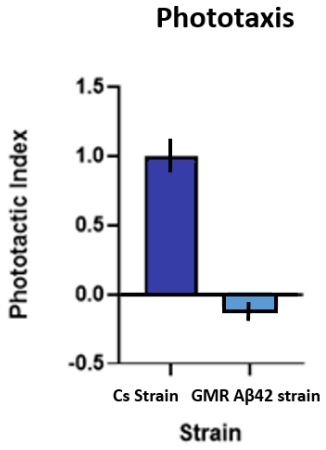

Figure 10: *Representative results of Drosophila Phototaxis Assay for two strains, Cs (Normal) and GMR Aβ42 (defective vision)*

The negative response index observed is indicative of light being a repellant for the GMR Aβ42 strain.

All above assays can easily be performed in any laboratory.

We hope these experiments will encourage you to pursue research.

Suggested projects

Each of the above protocols can be used in projects to assay

- Comparison of males and females behaviour in a population
- Assaying natural extracts as therapeutics and treatments
- Assaying effect of environmental toxins
- Toxicity assays by LC50/survival or longevity of fly lifespan
- Age related degeneration
- Evolutionary selection pressures
- Developmental defects

Reagents and solutions:

Medium preparation

Materials	Quantity
Maize powder	83 g
Dextrose	50 g
Sucrose	25 g
Agar agar	18 g
Yeast extract	21 g
Distilled water	1000 ml
Mix well without causing froth Pour into 2000 ml conical flask. Cork with cotton plug, cover with paper, tie with string to secure the paper cover Autoclave at 120°C (at 15 p.s.i) for 20 mins. Cool slightly and Add	
Orthophosphoric acid	0.6 ml
Propionic acid	0.4 ml
0.175ml Methyl paraben / ml 10% alcohol	1 ml
Swirl to mix well Pour into washed, clean cotton-plugged autoclaved glass bottles or vials as required 25 ml in bottles or 5-8 ml in vials	

To minimize errors note the following precautions

i) Avoid vigorous shaking to prevent frothing of the medium

ii) Mix by swirling so that lumps do not remain in medium

iii) After autoclaving, allow the medium to cool slightly before adding the last three items in the table.

iv) After the medium is poured into the glass bottles / vials, it will solidify. Let it remain for about 2 days to allow the moist vapour to dry before using it.

Insect Ringer's solution preparation

- 7.5 g NaCl
- 3.5 g KCl
- 0.21g CaCl

Dissolved in 1000 ml D/W

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For Larval plate Assay

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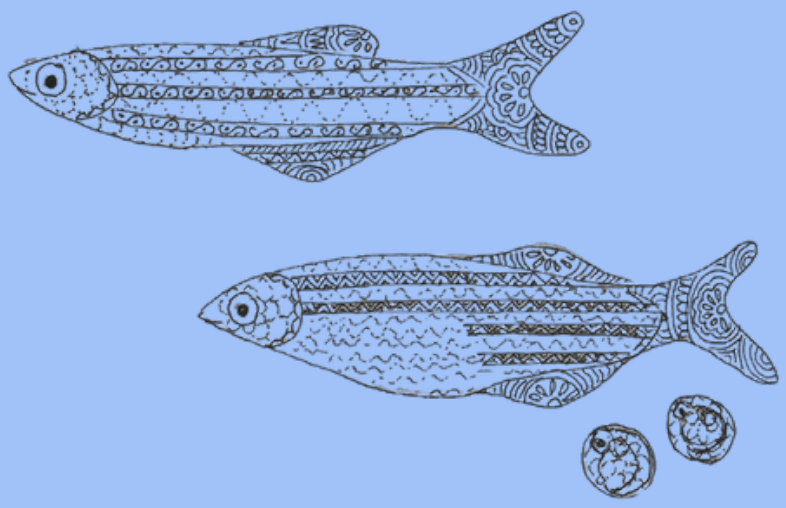
Good general sources

Drosophila: A Laboratory Handbook. By Michael Ashburner,. (1989) Cold Spring Harbour Laboratory. 1331 pages ISBN 0 87969 321 5.

Fly protocols https://www.flyrnai.org/tools/protocols/web/

Janelia support https://www.janelia.org/support-team/drosophila-resources



Danio rerio (Zebrafish)

Zebrafish has become a popular animal model for biomedical research. The number of publications per year on zebrafish as a model for biomedical research has been significantly increasing in recent years. One reason that zebrafish are an important biomedical model is because zebrafish embryos are transparent and they develop outside of the uterus. This unique developmental process allows scientists to study the details of development starting from fertilization and continuing throughout development. Innovation and development of molecular techniques in the later 20th century allowed zebrafish to be used as a model organism in almost all aspects of biology throughout the world.

Tsegay Teame, The use of zebrafish (*Danio rerio*) as biomedical models, 2019

Danio rerio (Zebrafish) – An easily accessible vertebrate model system

Iqra Khatri and Yasmin Khan

Abstract

Zebrafish is a small aquarium fish that is easily available in the pet shops. It is indigenous to the Ganges and therefore does not require any special conditions for maintenance. It is the only vertebrate model system that can be used for minor projects even in a college set up. The other advantages of using zebrafish, especially in its early developmental stages, is that these organisms undergo external fertilization, the eggs are large and transparent and hence very easy to visualize. A single breeding pair can give up to 200 - 300 fertilized embryos. As this is a favoured model in many labs, its genome has been sequenced (it shares 70% homology with humans) and several mutant and transgenic strains are available. Most of the organs in zebrafish develop within the first 4 days after fertilization and share several similarities to the human organs. These attributes make zebrafish an easy and attractive model system.

(https://www.zebrafishfilm.org/ is a short award-winning documentary film titled 'Zebrafish - Practically People')

Introduction

Danio rerio or as we like to call it zebrafish, is a tiny beautiful fish (around 5-7 cm) that has been used as a model system in the field of life sciences for numerous reasons. Zebrafish is a native of the Ganges River and was originally identified from there. Its use as a vertebrate model system was first undertaken by George Streisinger at the University of Oregon in the 1970s, and it is through his and other labs in Oregon, Tubingen and Boston that currently this is one of the most extensively used vertebrate model system, especially for developmental studies. Its importance lies in the fact that it combines the best features of all the model systems. It is a vertebrate like frogs and mice, and also has the advantage of a short life cycle, very simple maintenance, and easy availability of large numbers of transparent embryos. Like *Drosophila* and *C. elegans* it amends itself to genetic screens which have identified large numbers of mutants and transgenic lines.

Zebrafish is an experimental model that has genomic and molecular similarities to higher vertebrates including humans. Since the number of embryos obtained by a healthy female fish at a time is at least 150-200, mass study on developing embryos becomes possible. It leads to less errors as the sample size is high. Zebrafish embryos are completely transparent during their earlier days of development. This enables us to study development from a single cell stage with just the help of a simple microscope. After 24 hours we can easily remove the chorion of the embryos for easy visualization. The embryos naturally hatch at around 48 hours post fertilization. At this point one can easily visualize the beating heart and the circulatory system. Time lapse analyses on migrating pigmented cells and cells of the lateral line can also be studied. The embryos start swimming only after 48 hours, which makes image capturing also easy in the early stages.

Life Cycle of zebrafish

During breeding a pair of zebrafish will lay 200 -300 transparent eggs. The exact time of laying can be controlled by separating the males and females overnight and removing the partition at the required time. The embryos develop rapidly along a well-defined embryological staging series that permits observation of embryonic development from fertilization to maturation.

Fertilization in zebrafish is external. Fertilization is light sensitive. Female fish lay eggs into the water around her during the first exposure of light, where they are fertilized by the males. This fertilized egg then undergoes seven broad periods of embryogenesis--the zygote, cleavage, blastula, gastrula, segmentation, pharyngula, and hatching periods during the first three days after fertilization. The total generation time of zebrafish is 3 months. The zygote which is 0.7 mm in diameter at the time of fertilization (considered as zero hour time point) undergoes the first cleavage after 40 min. As the egg of zebrafish is telolecithal, discoidal meroblastic cleavage takes place where the calcium present at the time of fertilization contracts the actin cytoskeleton so as to stream non yolky cytoplasm toward the animal pole, segregating the blastodisc from the clearer yolk granule-rich vegetal cytoplasm. This is then followed by cleavage after every 15 min until it reaches 64 cell stage. Then the blastula period begins where the blastodisc begins to look ball-like, at the 128-cell stage, or eighth zygotic cell cycle, and until the time of onset of gastrulation. This stage is also marked by mid-blastula transition (MBT), the yolk syncytial layer (YSL) forms, and epiboly begins along with the initiation of gene transcription where the cell division slows down and cell movement becomes evident. Somites gradually start to form, the rudiments of the primary organs become visible, and the tail bud becomes more prominent resulting in the embryo elongation in the next stage, which is segmentation (10 - 24hr) period. By the time pharyngula stage starts, somites are fully developed giving rise to notochord, pigmentation and heart beat can also be observed. The period name focuses attention on the primordia of the pharyngeal arches, which later develops into jaws, operculum and the gills. The embryo hatches between 48 and 72 hpf. By this time pectoral fin is fully developed and the hatched larva has completed most of its morphogenesis, and it continues to grow rapidly, thus forming a fully formed adult by 3 months. The zebrafish has a life span of 5 years. Figure 1 depicts some developmental stages in the life cycle of zebrafish.

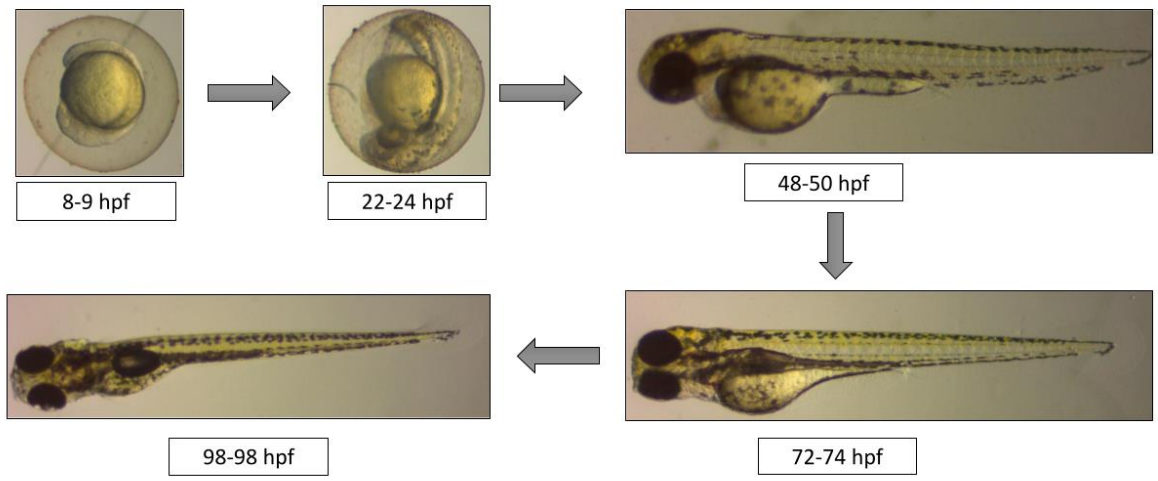


Figure 1. *Some stages in the life cycle of zebrafish*

Basic maintenance (In an aquarium and also using a fish facility)

Zebrafish can be easily maintained in aquarium tanks using RO water or dechlorinated tap water with aeration. The tanks need to be cleaned by siphoning water from the bottom of the tank to remove collected debris.

It is important to maintain water temperature at 28 – 29^{0}C with a light periodicity of about 13 hours daylight and 11 hours darkness. The fish need to be fed live feed for successful breeding. The ideal food is artemia (brine shrimp) that can be hatched in a saline environment. In the absence of artemia, *Chironomus* larvae serve as good alternatives. This is supplemented with regular dry feed both of which can be obtained from pet shops. The tanks can be cleaned using a scrubber to remove algae if collected on the surfaces. It is important to remember that detergent is extremely toxic to the fish and should be completely avoided. Dilute bleach may be used instead if required.

Figure 2a is a simple aquarium tank that can be purchased from a pet shop. Laboratories that use mutants and transgenic fish invest in a fish facility as shown below (Figure 2b).

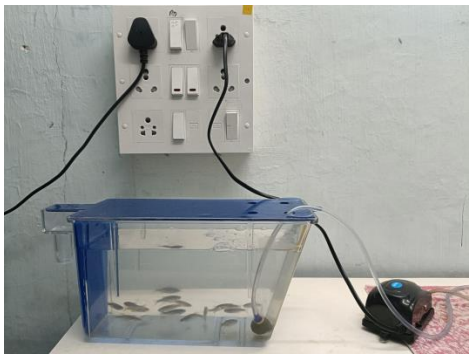


***Figure 2a**. An aquarium tank for maintenance of zebrafish*

***Figure 2b**. A basic unit for maintenance of zebrafish*

The facility keeps zebrafish in a circulating system that continuously filters and aerates the water to maintain the quality required for a healthy aquatic environment. The circulating system also helps to oxygenate the water and filter excess food and fish excreta. The excess water that overflows from the tank is collected and filtered through several stages until it is clean enough to re-enter the tanks. RO (Reverse Osmosis) water is used.

For zebrafish to breed regularly and produce healthy embryos, it is important to feed them a protein rich diet. In a laboratory setup, we usually feed them dry food (food size from 100 microns for larvae to 300/400 microns for adult fish) or live food (Artemia / brine shrimps) which have been kept to hatch over a period of 48 hours. Artemia hatch in a saline environment, so it's important to wash and transfer them in RO water before feeding to the fish.

Protocols

For Breeding

i. Adult healthy zebrafish can be kept for breeding every 15 days.

ii. The batch to be kept for breeding are fed with live feed in the morning and afternoon and again an hour before keeping them for breeding.

iii. The fish to be kept for breeding are transferred in breeding tanks in the evening (Approximately by 3.30-4 p.m.). Figure 3 is an image of a commercially available breeding chamber. A simple way to make a breeding tank is to use 2 plastic boxes that can stack on top of each other. The bottom layer of the box on top is removed by cutting the plastic. A mesh (such as from a mosquito net) is then trapped between the 2 boxes before stacking them over each other. The fish are kept in the upper tank and the eggs / embryos sink into the lower tank on laying and are easily retrieved the next day.

iv. The male and the female fish are kept in a particular ratio (usually 1:1). The male and the female fish are identified on the basis of their appearance. The females have a bloated belly whereas the males are slim and slender.

v. (If the embryos are to be obtained at a specific time then separators are used to keep the males and the females separated in breeding tanks. The egg release and fertilization will then happen as soon as the separators are removed).

Figure 3*. A commercially available breeding chamber*

Embryo Collection

i. Once the eggs are laid in the tanks, the fish are transferred back into their maintenance tanks.

ii. The lower tank is removed and water is filtered with the help of a sieve (a plastic tea strainer is ideal).

iii. All the embryos get collected in the sieve and then they are transferred in a petri dish containing egg medium.

iv. All the debris and dead embryos are removed from the plate using a dropper.

v. The plate is kept at 28ºC in an incubator until embryos reach 2-4 dpf (days post fertilization) stage.

Embryos are kept in an embryo media (E3) and can be transferred to RO water after 2-3 weeks.

Embryos and young larvae have stricter requirements and should be raised in egg water. Embryos removed from their chorions require additional calcium and should be maintained in embryo medium.

Some simple experiments that can be conducted using zebrafish embryos with minimal requirements are described below:

Toxicity assay

Zebrafish embryos lend themselves as model organisms to test for toxicity of various chemicals/drugs/pesticides etc. At the early stages the test chemical readily diffuses across the chorion if dissolved in the water. The transparent embryos permit easy visualization of the effect.

To study whether a particular compound causes developmental toxicity, one needs to first standardize the concentration of the compound which will not kill the embryos, but at the same time show some results.

We also need to determine the solubility of the compound we are interested in testing. For example, alcohol is soluble in water, and hence can be diluted in water. Some compounds (for example retinoic acid) may not be soluble in water, and will have to be diluted in an organic solvent like DMSO. We need to have a solvent control for our toxicity experiments as well as a normal control. This will ensure that the results observed are caused by the compound of interest.

Below is an example of testing the toxicity of alcohol on developing zebrafish embryos.

i. For Alcohol toxicity, after standardization of the concentrations of ethanol, 2% and 2.5% Ethanol solution (EtOH) was prepared in E3 medium.
ii. 8 embryos were kept in each of the 6 wells/petridishes as shown in the table below. Usually the embryos are exposed to the chemical at 4 – 6 hpf but this can be determined according to the chemical tested.

Control	1.5% EtOH	2.5 % EtOH
Control	1.5% EtOH	2.5 % EtOH

iii. After 24 hours the embryos can be dechorionated using forceps under a simple microscope.

iv. Parameters that can be recorded at this stage are :(Figure 4)

- Survival rate - measured as the number of embryos surviving at each time point
- Heartbeat per minute – At 24 hpf the heart is clearly visible and the heart beat can be readily counted per minute
- Edema in the heart region can be observed (one of the factors indicative of stress). The volume of the cardiac area can also be estimated using ImageJ software.
- Length of the embryo can be measured (using ImageJ software)
- Locomotion – At 48 hpf the larvae are unable to swim, however, if their caudal fin is gently touched with an eyelash brush the larvae show a darting movement. Defects in locomotion will not permit the larvae to demonstrate this movement.

4A - Control 4B - Treated with 2.5% Ethanol

Figure 4. *Images of zebrafish embryos taken at 72 hpf following treatment with Ethanol. Fig 4A is the control while Fig 4B is an embryo treated with 2.5% ethanol. Note the abnormality in body development and edema in the cardiac cavity.*

The embryos can be fixed in 4% paraformaldehyde for further staining at the stage we are interested in observing. One can stain the embryos to check for other developmental deformities. Some commonly used stains are given below.

i. The embryos were treated with Phalloidin stain. Fluorescent conjugated Phalloidin is a stain that allows for visualization of F- actin. This stain is used to see the gradation in muscular development in zebrafish embryos with respect to alcohol treatment.

ii. Acridine orange staining for dead cells. To detect degenerating cells, live cells were treated with Acridine orange and were viewed with fluorescence microscopy using the FITC filter set.

Cartilage and bone staining (Figure 5 and 6)

Alcian Blue Staining of Cartilage: Changes to cartilage and bone are definite indicators that there are developmental abnormalities. These are very easy to stain. Cartilage is observed from 3 dpf while bone is visible only after 8 dpf.

i. Embryos are anesthetized in 5 to 10% methyl ethane sulfonate (i.e., tricaine).
ii. They are fixed in 3.7% neutral buffered formaldehyde (10% formalin in phosphate buffered saline [PBS]) at room temperature for several hours to overnight. Care should be taken not to over fix the preparation.
iii. Embryos are washed in PBT (PBT: PBS, 0.1% Tween-20) for 3 to 5 times for at least 5 minutes per wash.
iv. They are transferred into a 0.1% solution of Alcian blue dissolved in 80% ethanol and 20% glacial acetic acid for at least 6 to 8 hours to overnight. (Do not overstain). Alcian Blue stain should be kept to settle overnight and the upper clear solution used for the staining.
v. Embryos are rinsed in ethanol and rehydrated gradually into PBS. Ethanol:PBT solutions of 70%:30%, 50%:50%, and 30%:70%, respectively are used.
vi. Pigmentation can be removed by bleaching the embryos in 3% hydrogen peroxide in 1% potassium hydroxide for 1 to 3 hours. The reaction should be monitored carefully.
vii. The embryos are then washed in PBS 2 to 3 times for 5 minutes per wash.
viii. Embryos are transferred into glycerol, gradually through a series of 30% and 50% dilutions in PBS, and stored in 90% glycerol.

Alizarin red staining of bones (can be performed on or after 8-10 dpf)

i. Embryos are anesthetized in 5 to 10% methyl ethane sulfonate (i.e., tricaine).
ii. They are fixed in 3.7% neutral buffered formaldehyde (10% formalin in phosphate buffered saline [PBS]) at room temperature for several hours to overnight. Care should be taken not to over fix the preparation.
iii. Embryos are washed in PBT (PBT: PBS, 0.1% Tween-20) for 3 to 5 times for at least 5 minutes per wash and then Alizarin red staining for bone visualization is performed. (A dual staining for both cartilage and bone can be done. Steps 1-7 are performed as per the above protocol for Alcian Blue staining followed by the following procedure for bone staining).
iv. The embryos are transferred into a 0.05% solution of Alizarin red dissolved in 1% KOH for several hours (2-4 hours).
v. The embryos are cleared in glycerol, gradually through a series of 30% and 50% dilutions in PBS. We were interested in studying the cranial development and hence the stained embryos were micro-dissected before observing under a stereo dissection microscope.

Note: Embryos stained with Alizarin red should not be stored in glycerol for long, as the stain tends to fade away.

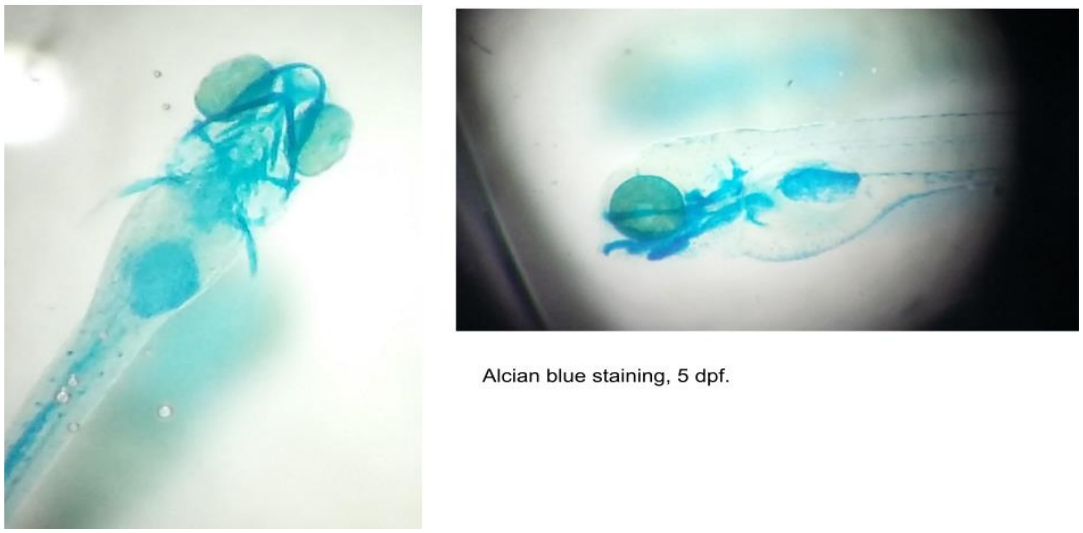


Figure 5*. Embryos at 5 dpf stained with Alcian blue to visualize the cartilage.*

Figure 6. *Zebrafish embryos stained with Alizarin red to visualize the bone. The age of the embryo is mentioned with the image. The last image is a dual staining of the cranium to observe both cartilage and bone.*

Use of zebrafish to undertake open-ended projects

- To study the effect of pesticides using the toxicity assay
- To test for teratogenic compounds like lithium (or other toxic metals)
- There are simple behavioural assays for adults that can also be done without requirement for sophisticated equipment.

Preparation of Medium and solutions

Egg water: Used for in vitro fertilization and raising young embryos.

Stock salts: 40 g “Instant Ocean” Sea Salt added to 1 L of distilled water

Egg water = 1.5 ml stock salts added to 1 L distilled water = 60 µg/ml final concentration.

Embryo medium: Don't confuse with the “egg water” above. Used in handling dechorionated embryos and storing young embryos in dishes. This is basically 10% Hank's with full strength calcium and magnesium.

Embryo medium

1.0 ml Hank's Stock #1

0.1 ml Hank's Stock #2

1.0 ml Hank's Stock #4

95.9 ml dd H_2O

1.0 ml Hank's Stock #5

1.0 ml fresh Hank's Stock #6

Use about 10 drops 1 M NaOH to pH 7.2

Hank's solutions: Hank's solutions can be made from stock solutions (kept refrigerated, they will last for several months). A premix of the salts can be stored in the refrigerator for several weeks. Sodium bicarbonate does not store well, so it is made up fresh each time Hank's solution is made.

Full Strength Hank's

0.137 M NaCl

5.4 mM KCl

0.25 mM Na_2HPO_4

0.44 mM KH_2PO_4

1.3 mM $CaCl_2$

1.0 mM $MgSO_4$

4.2 mM $NaHCO_3$

Hank's Stock Solutions

Stock #1 8.0 g NaCl 0.4 g KCl in 100 ml ddH_2O	Stock #2 0.358g Na_2HPO_4 Anhydrous 0.60 g KH_2PO_4 in 100 ml ddH_2O	Stock #4 0.72 g $CaCl_2$ in 50 ml H_2O	Stock #5 1.23g $MgSO_4.7H_2O$ in 50 ml ddH_2O	Stock #6 0.35 g $NaHCO_3$ 10.0 ml dd H_2O_2

Hank's Premix - Combine the following in order:

10.0 ml Solution #1

1.0 ml Solution #2

1.0 ml Solution #4

86.0 ml ddH_2O

1.0 ml Solution #5

Store Hank's Premix in the refrigerator along with the Hank's solutions.

Final Hank's

9.9 ml Hank's Premix

0.1 ml fresh Stock #6

References

An invaluable source of information on all zebrafish related studies is found in the following. THE ZEBRAFISH BOOK - A guide for the laboratory use of zebrafish *Danio (Brachydanio) rerio* by Monte Westerfield, Institute of Neuroscience, University of OregonIt can be accessed from the site zfin.org using the following link - http://zfin.org/zf_info/zfbook/zfbk.html

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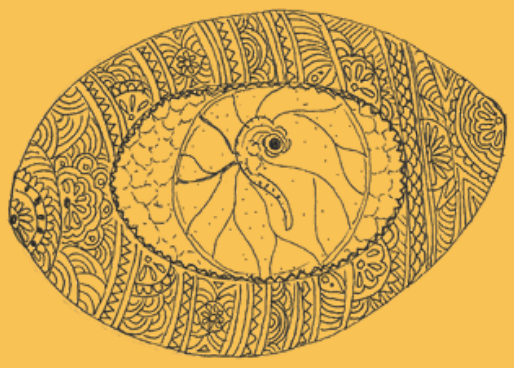

Gallus domesticus (Chick)

The chick embryo has a long and distinguished history as a major model system in developmental biology and has also contributed major concepts to immunology, genetics, virology, cancer and cell biology. Now, it has become even more powerful thanks to several new technologies: in vivo electroporation (allowing gain- and loss-of-function in vivo in a time- and space-controlled way), embryonic stem (ES) cells, novel methods for transgenesis, and the completion of the first draft of the sequence of its genome along with many new resources to access this information. In combination with classical techniques such as grafting and lineage tracing, the chicken is now one of the most versatile experimental systems available.

Claudio D. Stern, The Chick: A Great Model System Becomes Even Greater 2005.

Gallus domesticus (Chick) - An ever-emerging model system

H. Ramachandran

Abstract

Gallus domesticus (chick) embryo develops post fertilization as a single cell. The complex development process of the chick embryo can be easily visualized, accessed until late embryonic stages. The windowing of the egg shell makes it easily accessible for cell migration, differentiation and morphogenesis studies during its 21-day embryonic development.

The eggs of the domesticated chick are easily available from local poultry suppliers. Avian eggs are highly economical and embryo development can be achieved using simple laboratory equipment. Egg/lab incubators with temperature and humidity control is sufficient to set up the embryo culture.

The different modes of chick embryo culture options available makes it a popular model system both for teaching as well for the conduct of short-term research projects. The embryo can be isolated from the egg and successfully cultured in a glass bowl (shell-less culture). For this technique a basic aseptic condition with use of chick Ringer's solution is sufficient. The variations in development can be compared with established embryo staging nomenclature (Hamburger Hamilton series). The embryo developments can be recorded using simple light microscope and also by using time-lapse videos. After the first annotated chicken genome sequence was released in the year 2004, it has become a popular model for gene manipulation experiments.

Introduction:

Since time immemorial Hen's egg (*Gallus gallus domesticus*) has been observed and unboxed after short periods of incubation. A fairly good documentation of these embryos over a period of 200 years has helped us answer basic questions in embryology. Later the use of vital dyes helped us to follow cell movements during embryonic development. This exciting discovery was followed by photomicrography using 3-D enabled stereo microscopes and recordings with time-lapse microscopy.

The characteristic versatility of this avian egg is its embryonic development. Here, the development takes place within an egg that is incubated outside by the parent. The egg shell provides both vital calcium carbonate as well as protection. Thus, any early developmental stages can be easily accessed, visualized and even manipulated by simply creating a small hole in the eggshell.

Avian embryo development is accomplished in a short span of 21 days that finally leads to hatching. Thus, this system is easily adaptable, highly economical with minimal maintenance. Over the years this has become a popular model system for undergraduate teaching as well as for research.

A detailed embryonic staging system documented by Hamilton and Hamburg enable a researcher to identify the development stages easily. A complete chick genome sequencing in the year 2003 has further contributed to our scientific understanding of the human genome as well. Also, techniques such as in-ovo, ex-ovo cultures expand the research application to studies in different tissues of interest which otherwise are not easily accessible at the desired embryonic stage. This also includes cost-effectiveness with multitude of applications in cellular biology, cancer biology, genetics, immunology, virology, epigenetics and toxicity evaluation.

For an undergraduate student real-time visualization of development events can be an exciting experience. A 3D development is unimaginable for a student of biology after observing a tiny flat blastodisc on top of a yolk. Digital recording of heart beat, watching the embryo kicking around in amniotic fluid can generate great enthusiasm amongst young biology students. Culturing a chick embryo during undergraduate practical sessions will help young biologists to develop research skills like curiosity, logical thinking and creativity.

Working with chick embryos can also be economical since they are freely available as fertilized eggs (Day 0 at the time of laying of eggs). Day 0 is also indicative of temporary suspension of development until the incubation process begins. For classroom experimental purposes this temporary developmental halt can be prolonged by maintaining eggs around 10 to 15℃. The embryo development can be comfortably initiated by incubating eggs at 37℃. Once the embryo development is initiated, the embryo, along with yolk and embryonic membranes, is referred to as embryonated egg.

Basic Incubation :

The fertilized eggs are incubated at 37°C (98.6°F) in an airflow-thermal incubator. For example, if we need a three day old embryo we need 48 hours of incubation to be completed and the following day it can be used as Day 3 old embryos. Eggs need to be rotated manually at 180 degrees once during the day (please note automatic egg turner facility is available in egg incubators), control of humidity and temperature is extremely important. A small bowl of sterilized water is kept in the incubator for maintenance of humidity.

Isolation of embryo :

The egg is placed upright for 5 minutes to ensure the embryo is positioned to the top of the egg surface. Releasing the air (from air chamber) carefully by gently drilling a hole at the broad end of the egg (using arrow head or needle) will help in releasing the yolk from its immediate proximity to egg shell surface. Gently tap the lower side of the egg so that a clear visible crack appears. Now hold the egg closer to the glass bowl and gently open the egg shell beginning from the lower end. The egg contents are gently released into the bowl. Ensure that the edges of egg shell are not jagged else the yolk membrane will tear, with oozing of yolk. Once the contents are in the bowl separate the thick albumin with blunt/coarse forceps. In case the embryo remains on the lower surface of the yolk gently keep rolling the yolk with coarse forceps. Using a small scissor isolate the embryo by cutting a circle of yolk sac around the embryo. Now with the help of a spoon the embryo can be gently transferred into a bowl with fresh PBS. Under a dissection microscope, carefully remove the embryonic membranes. The excess yolk adhering to the embryo can be removed by gentle aspiration with PBS. Now the isolated blastoderm can be transferred to a fresh bowl containing phosphate buffered saline (PBS). Remove any yolk material attached to the embryo by flushing of the embryo with PBS. The transparent embryo is now ready for further processing.

***In-ovo* culture :**

In-ovo culture is an ideal method for screening of drugs, angiogenic factors, biosensors etc. This is possible by gently exposing the vasculature of the chorioallantoic membrane just underneath the egg shell. After waiting for 5 minutes, the blastoderm comes on to the surface. For windowing experiments, the chick eggs are placed on a stretched Cello tape of 3 inches. Using a needle/arrow head gently make a small pore at the broad end of the egg. This will bring the blastoderm away from the immediate shell surface. Mark a one-inch square with a marker pen on the tape. Using a small angular scissor make a

tiny opening on the shell by turning the scissor tip clockwise and anticlockwise. The egg shell can be windowed by cutting with a scissor held absolutely horizontal onto the surface. One can also use a blade with rough edge. Keep one corner of the egg shell window intact so it can be put back or sealed once again with a cello tape. The embryo can now be kept back into the incubator with window facing upwards (Figure 1).

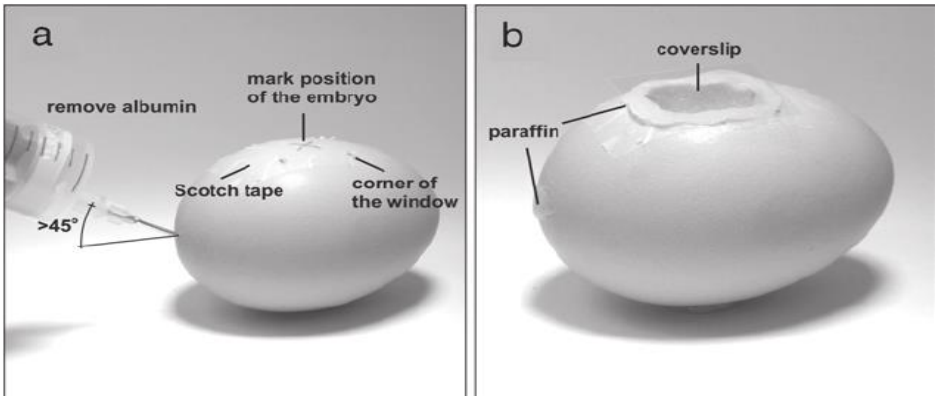

Figure 1*Windowing method (Esther T Stoaeckli, (2008)*

***Ex-ovo* culture:**

Ex-ovo culture is when an avian embryo is cultured outside a natural egg shell. Disinfect the egg shell surface with 70% alcohol. Crack the egg and carefully transfer the egg contents. Here a Styrofoam cup or a glass bowl (sterile) is lined with plastic wrap (surrogate egg shell) which holds the embryo. A lid is then placed over the container and the embryo is kept in the incubator with required humidity (Figure 2).

Shell-less culture in a glass bowl

After disinfecting the egg surface with 70% alcohol it is placed in an incubator for 10 minutes. This helps the embryo to move to the upper surface of the yolk. A sterile bowl is first taken and albumin from an unfertilised egg is added as a layer before the transfer of the embryo. This extra added albumin acts both as a shock absorber and prevents desiccation of the developing embryo. After cracking the lower side of the egg, gently transfer the egg contents into a sterile bowl (4 cm. diameter). Cover the bowl with a glass lid which just about snuggly fits onto the glass bowl. A minimal space under the glass lid permits carbon dioxide to escape out. Return this culture set up back to the incubator. To observe and record the embryonic developments do not keep the culture set up for more than 5 minutes outside the incubator. Ensure all glassware and dissectors are sterile and embryo transfer in a laminar air flow unit is recommended (Figure 3).

Avian eggs and ethical regulations:

Currently avian embryos are used extensively both in teaching and research at Institutions of higher education. As in any other experiments involving animals, ethical aspects must be considered during use of chick embryos (embryonated) and emerging hatchlings (if any). The 3Rs (replacement, refinement and reduction of animals in research) must be emphasized. Unnecessary repetitions, and wastages must be strictly avoided. Sensitizing animal ethical issues in biology teaching institutions is very important in this direction.

Poultry eggs may get contaminated with *Salmonella* bacteria hence washing your hands before leaving your lab is highly recommended.

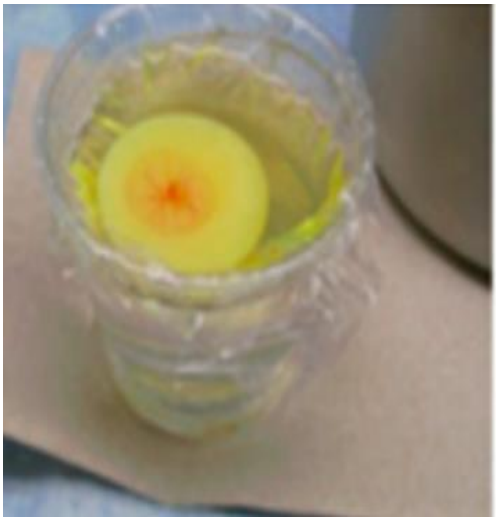

Figure 2 *Ex-ovo culture (Hammock method)* ***Figure 3*** *Shell-less culture (J. Jadhav,2015)*

(J T Butcher,2010)

Basic protocol for whole mount :

The embryos can be isolated, processed for temporary staining or permanent chick embryo slides can be procured and observed too. The embryo stages can be identified using Hamilton Hamburger staging method (Figure 5).

The following stages - 18 hour, 33 hour, 48 hour and 72 hour embryos are recommended for whole mount preparation.

The egg shell can be cracked by gently tapping on the surface of the table. The entire egg can be slowly introduced into a bowl containing chick Ringer's solution. This allows the Ringer's saline to flow into the egg. Now gently pull the egg shell halves and discard the shell. Now the yolk and albumin with blastoderm on its surface will be visible.

Observe the following as you carefully remove egg shell : Egg shell pores, egg shell membrane immediately underneath the shell, air space at the broad end of the shell, yolk and the thick and thin albumin. (Figure 4).

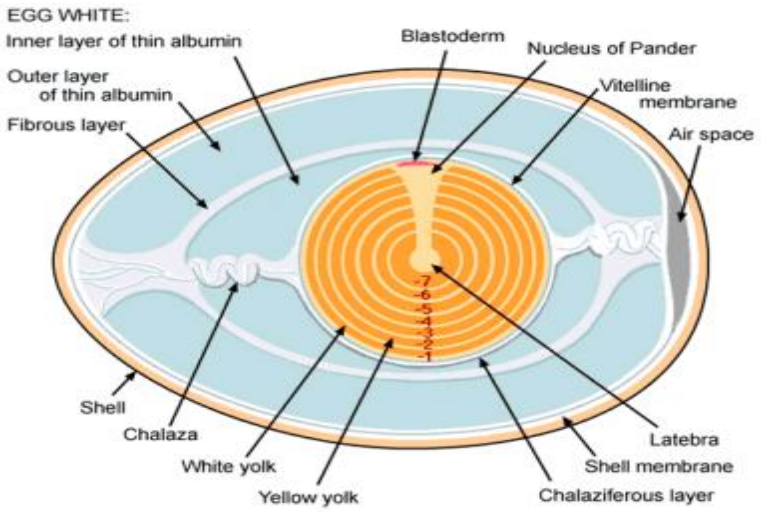

Figure 4 *adapted from (A.O. Adegbenjo,2020)*

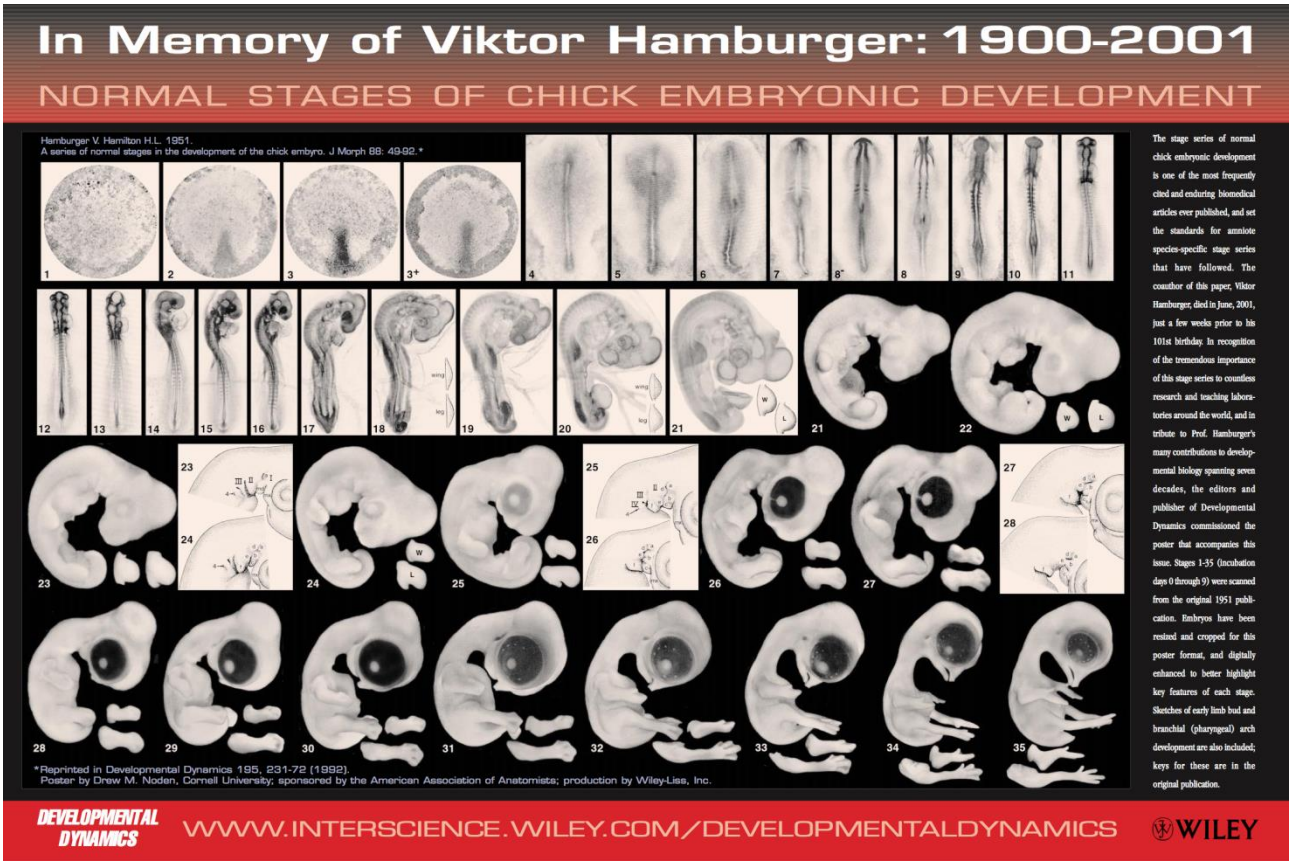

Figure 5 Staging of chick embryonic development

https://www.swarthmore.edu/NatSci/sgilber1/DB_lab/Chick/hamburger.pdf

Methodology for isolation of blastoderm:

i. The yolk can be moved around in the Ringers by gently grabbing the strand (chalaza) attached to the yolk. Locate the blastoderm on the surface of the yolk.
ii. By grabbing the chalaza the blastoderm can be cut and separated out from the yolk membrane.
iii. One can drop a few drops of the stain to cover the blastoderm and the entire yolk using a dropper.
iv. Gently lift the blastoderm and place in a petri dish containing Ringer's solution. Check if any yolk is adhering to blastoderm by gently moving it in Ringer's solution.
v. Now we can gently drag the blastoderm onto a microscope slide and observe under the Dissection/light microscope.
vi. You can also observe the blastoderm under the stereomicroscope by withdrawing excess saline carefully using a dropper.

Sketch a neat diagram, identify and describe the functional role of the following :

24 hour embryo: Zona pellucida and zona opaca, neural crest, neural groove, primitive streak, somites.

48 hour embryo : Fore, mid and hind brain, eye, ear, spinal cord, heart, somites, tail bud.

72 hours: Brain, eyes, wing bud, leg buds, tail bud, heart, vitelline artery and vitelline vein, somites.

Localisation of Cytochrome c oxidase in chick embryo.

Cytochromes are proteins that strongly absorb light in visible range, due to the presence of prosthetic group. Cytochrome a and b are present in the inner mitochondrial membrane while cytochrome c have electrostatic interactions with the outer surface of the inner membrane.

Electron transport chain (ETC) is a sequence of electron carriers most of which are the integral proteins with prosthetic groups (Heme or iron) capable of accepting or donating electrons. ETC consists of five complexes; complex (I, II, III, IV and V). Complex IV of ETC shows presence of cytochrome c oxidase enzyme, which is a transmembrane protein complex. The activity of cytochrome c oxidase depends upon the metabolic state and therefore on the rate of development. It is an iron containing mitochondrial enzyme which is a part of the ETC. Hence it acts as an index of the rate of development. An important feature of this enzyme is that it is synthesized during development and not prior to it. The intensity of localisation is different in various parts of the embryo i.e. differential development.

Cytochrome c oxidase is localized histochemically by the NAD reaction (Napthal Diamine Reaction). Azo dye coupling takes place and the resultant dye produced is blue in color (indophenol blue) which turns brown on oxidation. It is a mitochondrial enzyme which reflects the oxygen consumption of the embryo. Neural tube shows increased activity as more and more structures appear and develop, the enzyme activity hence increases. (Brown/dark brown color indicates cytochrome c oxidase activity).

Methodology:

i. Isolate a chick embryo (48-96 hrs) from an embryonated egg.
ii. The egg surface can be sterilized with 70% alcohol The egg is placed horizontally on the lab bench for few minutes to ensure blastoderm moves to the surface of the yolk.
iii. With a gentle cracking of the egg shell the contents can be emptied into a bowl containing Avian ringer's solution.

iv. Blastoderm is neatly cut, extra embryonic membranes removed, and dragged on to a glass slide and air dried for 2 minutes.
v. The slide is introduced into the incubation mixture and kept in a incubator at 37^{o}C degrees for 5-10 minutes.
vi. The incubation mixture is drained of and the slide is now flooded with 5% Cobalt acetate. A single rinse is given in D/W and mounted in glycerine or avian saline.
vii. The areas of enzyme activity localised can be recorded and interpreted.

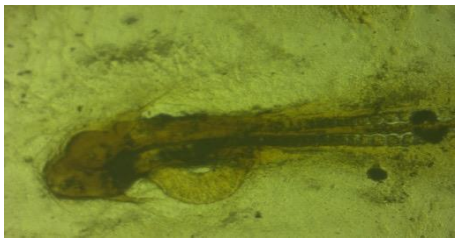

Figure 6 *Intense cytochrome activity noticed near the somite region.*

Neutral red vital staining and demonstration of interdigital cell death

During limb development cell death is a important event which enables in sculpting of the limb. This selective process is genetically programmed where in cells between the digit cartilage undergo apoptosis/programmed cell death. Using a Neutral red stain helps us in identifying these areas since the dye is preferentially absorbed and endocytosed by viable cells. This further is internalized inside the lysosome. The staining is thus indicative of lysosomal activity and cell integrity.

Figure 7 *The necrotic zone in chick embryo stage HH 22. and HH stage 32, adapted from (Juan, A. Montero ,(2021)*

Using a vital stain Neutral Red, Hinchliffe & Ede (1973) the chick embryos (HH stage 32) can be isolated, embryonic limbs dissected free and is incubated in Neutral Red in 1/10−5 g mL−1 PBS at 37°C. The embryos can then be observed under dissecting microscope.

Whole mount embryo skeletal staining: (Figure 8 and 9)

Embryos are fixed with 99% ethanol for 48 hours, followed by removal of internal organs.

It is then treated with acetone for 24 hours and incubated with skeletal staining solution. Alcian blue stain at 37°C for 1-3 days. Destain in 70% Alcohol and incubate in 95% Ethanol overnight.

In order to pre-clear the tissue the 95% Alcohol is removed and placed in 1% KOH for one hour at room temperature.

The KOH solution can now be replaced with Alizarin Red stain and incubated for 3-4 hours.

The KOH solution can be now be replaced 50% Glycerol : 50% KOH.

For long term storage the sample can be stored in 100% Glycerol.

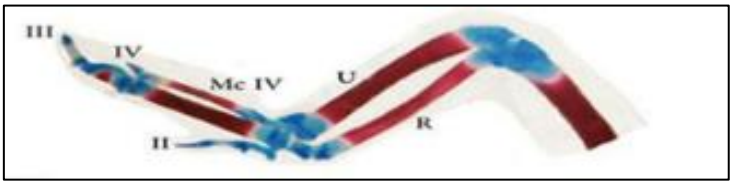

Figure 8 *Alizarin Red staining (Marwa Nabil Atallah, 2012)*

Alcian blue stains cartilage and Alizarin red stains ossified bones in the developing chick embryos. The embryos are now washed with water, placed in 1% KOH in 20% glycerol and 0.01% KOH in 20% glycerol (clearing solution). Stained embryos can be stored in 50% glycerol till observations are recorded.

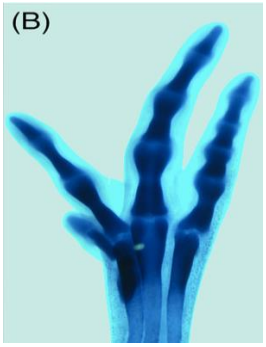

Figure 9 *Alcian blue staining HH Chick embryo stage 35 adapted from (Juan, A. Montero ,(2021)*

Suggested projects :

i. Chick as a model system for the study of pharmaceutical metabolites in the environment.
ii. Chick as a model system for environmental contaminants (for example bisphenol A) (BPA).
iii. Effect of stimulants like Caffeine/Nicotine on heart rate.
iv. Effect of metal ions/alcohol on embryonic brain development.
v. Chick as a model system to understand mechanobiology of skeletogenesis
vi. Food dyes and embryonic skeletal development.

Preparation of solutions :

Chick Ringer's solution :

NaCl 9gm, KCl 0.42g, CaCl2.H2O 0.16g dissolve in 1 liter D/W and maintain pH to 7.0.

Phosphate buffered saline (PBS): NaCl 4 gm, KCl 0.1 gm, KH2PO4 0.12gm,NaH2PO4 0.72gm, D/W 500 ml. Adjust pH to 7.2 to 7.4.

Preparation of reagents for Cytochrome c oxidase staining

i. Prepare Incubation mixture as follows:
   A. N – phenyl p-phenylenediamine - 20mg,
   B. 1 –hydroxy – 2 – naphthoic acid - 20mg,
   Tris HCl buffer (pH 7.5 – 9.6) - 50ml
   Ethanol - 0.2 ml,
   Dissolve the A and B in 0.2 ml of Ethanol and add the buffer, filter if necessary.

ii. Preparation of Fixative: 5% cobalt acetate, 10% formalin
iii. Miscellaneous requirements: Distilled Water, Alcohol, Slides, Cover slips, dissecting microscope, dropper, petridish, Avian saline (0.9%).

Preparation of Neutral red solution

5 g Neutral red powder
1.53 g sodium acetate (anhydrous)
0.6 mL acetic acid (glacial)
Prepare in 500 mL H2O. Stir overnight and filter.
Neutral red solution is light-sensitive and should be protected from light.

Preparation of Alcian blue stain

Alcian blue stain: 0.03 % (w/v)
80 % EtOH
20 % (glacial) acetic acid.
To make a 200 mL solution, weigh 0.06 g of Alcian blue place in a beaker, add 160 mL of 100 % EtOH and 40 mL of 100 % glacial acetic acid.
Alizarin red stain: 0.005 % (w/v) in 1 % (w/v) KOH

Suggested readings :

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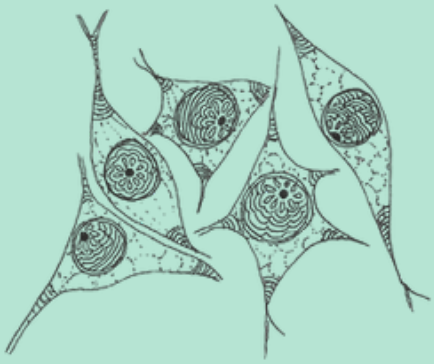
HH chick embryo stage:

https://www.swarthmore.edu/NatSci/sgilber1/DB_lab/Chick/chick_stage.html



In vitro research system (Cell culture)

Cell culture is a little like gardening. You sit and you look at cells, and then you see something and say, 'You know, that doesn't look right'.

Siddhartha Mukherjee, The song of the cell, 2022.

In vitro research system (Cell culture) - An alternative to animal model systems

Sonali Dhaval and Yasmin Khan

Abstract

Tissue culture uses isolated cells instead of the whole organism as a model. In the current scenario where there is a lot of resistance to the use of animals for experiments due to ethical reasons, tissue culture serves as a convenient alternative. The advantages are that it offers an isolated well controlled system for investigations. The cells could be of an identical lineage or they could be a mixture of more than one cell type. Most cells can be cultured in vitro using complex or defined media, and that ensures complete homogeneity from batch to batch. Though some sophisticated equipment such as a laminar air flow and CO_2 incubator are required for doing large numbers and long term cultures, we have described protocols that do not need these for short term cultures. Tissue culture serves as an excellent model for preliminary studies and to understand mechanisms; however, it is difficult to extrapolate results to an organism, which is a limitation of this system.

Introduction

The idea that cells can be maintained in a physiological state even after the organism is dead was shown way back as 1907 when Ross Harrison demonstrated the growth of a nerve fibre in frog embryos. However, it was in the 1950 to 60s that cell cultures were used in large numbers and at industrial scale. This progress was a consequence of some important discoveries. One was the maintenance of aseptic conditions as suggested by Carrel in 1913, that allowed survival of cultured cells over long periods of time; later enhanced by addition of antibiotics. The other milestone was the use of enzymes like trypsin that helped in isolation of single cells from tissues and also from the surface of culture flasks for repeated subculturing. Later, various defined media were used that made it easier for culture of different types of cells. In 1949, Enders was able to make the polio virus vaccine using human embryonic cells and it became the first commercial viral vaccine to be prepared using tissue culture. In 1953 the first cell line (HeLa) was established from human cervical carcinoma and this allowed for the use of standardized cells across the world.

Cell cultures can be used for a variety of applications. They serve as model systems for understanding basic cell biology and biochemistry, to study interaction of disease-causing organisms with their host cells or to elucidate the effect of drugs on cells. Transformed cells (or cell lines) are used in cancer studies. Cell cultures are excellent as a first screen for toxicity testing. Cell lines have been used extensively for growth of viruses for vaccine production (ex., polio, rabies, hepatitis B and many others) and also for making genetically engineered proteins such as monoclonal antibodies, hormones and insulin. With increased pressure to reduce the use of animals in experiments due to ethical reasons, cell lines serve as an ideal tool for primary screening of large number of potential chemicals to check for efficacy of a new drug and its safety for humans.

Experiments can be performed using primary cell culture isolated from donor organisms or established culture deposited in banks. Primary cultures are isolated from living organisms which contain the different types of cell population in tissue; therefore, it is important to isolate the correct cell type. The characteristic features of primary cell cultures are that they closely mimic the in vivo genetic characteristics and thus give opportunity to perform functional assays. However, they are difficult to isolate and have a short life span.

An alternative option is to use an established cell line. Bioresource Centers, such as the ATCC (American Type Culture Collection), or NCCS (National Centre for Cell Science in Pune) provide characterized models of various types of cell lines which are used routinely in research centers. Cell lines are usually used in preclinical research for testing of drugs, in cancer research, and in studies on gene function. Selection of the best cell culture technique in the area of cancer research may allow better understanding of cancer biology, and hence aid in finding new treatment strategies.

Cultures of cell lines can be carried out in two ways - adherent (e.g. fibroblasts) or suspension (e.g. cultures of lymphocytes). In adherent cell cultures, cells grow as a monolayer on the Petri dish or plastic flask surface. The advantage of cell lines is they are easy to maintain, by doing subculture, and have a low cost of maintenance compared to animal models. Unfortunately, cell lines also have disadvantages. They do not mimic characteristic structures of tissue. In these methods, cell –cell and cell – extracellular environments are not represented as they would be in the in vivo situation. These interactions are important for cell differentiation, proliferation, viability, expression of genes and proteins, responsiveness to stimuli, drug metabolism and other cellular functions.

Protocol

Culturing and maintenance of C6 glioma cell line (an adherent cell line)

The C6 glioma is a rat derived astrocytic cancer cell line (ATCC-CCL-107). C6 glioma cells are maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% foetal bovine serum (FBS), containing 1000 U/ml penicillin, 50 µg/ml streptomycin and 2µg/ml Forcan at 37°C in a humidified atmosphere containing 5% CO_2 (Freshney, 2005). (Fig 1). In the absence of a CO2 incubator, cultures can be maintained in a sealed desiccator with a beaker containing 1N HCl to which a spatula of $NaHCO_3$ is added prior to sealing the desiccator. The CO_2 gas that is released is sufficient for the cultures for a period of 24 hours as long as the desiccator is well sealed.

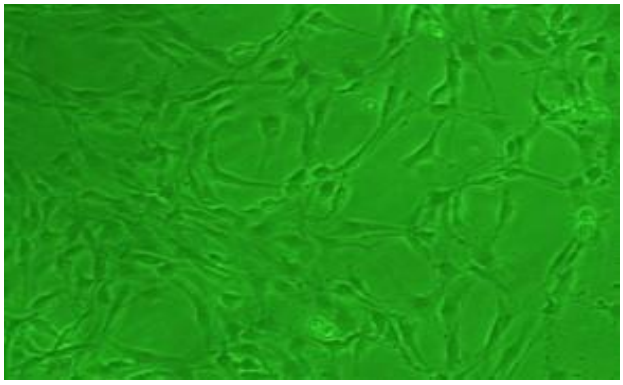


Figure 1*. Image of live C6 cells at a magnification x 100*

Method:

i. Sub- culturing is done once the cells reach 70-80% confluence.
ii. Previous medium is discarded followed by a wash with 1 X PBS.
iii. Adherent cells are removed by trypsinization (0.25% Trypsin) for 60 seconds and immediately deactivated by addition of 1ml of whole medium (DMEM with 10% FBS) The flask is washed with 1 ml of 1 X PBS to remove any leftover adherent cells.
iv. This suspension is centrifuged at 3000 r.p.m. for 3 minutes, after which the supernatant is discarded.

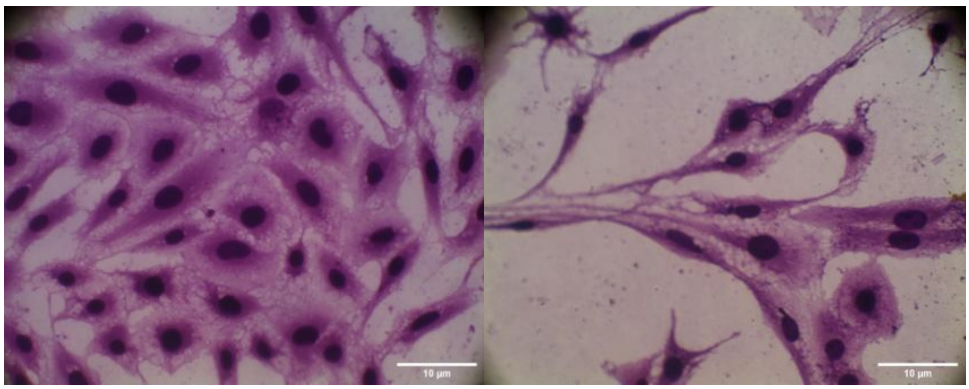
v. Pellet is broken and re- suspended in 1 ml of whole medium.
vi. Cell count is taken by using hemocytometer and desired cell density is seeded in a new flask.
vii. The remaining cells can be transferred to a cryo - vial and again centrifuged at 3000 r.p.m for 3 minutes. The supernatant is discarded. The pellet is broken and resuspended in 10% DMSO in FBS and immediately transferred to 4^0C for 30 min, then at -20^0C for 2 hrs. and finally stored in -80^0C for long term preservation. Cells can be maintained at this temperature for periods ranging from 6 to 9 months.

Experimental Protocols

Giemsa staining

It is a type of Romanowsky stain which contains both acidic and basic dyes having affinity to stain acidic and basic components of the cell respectively. It is a combination of two dyes, which is Methylene blue and Eosin. The acidic dye Eosin stains the cytoplasm of the cell and the basic dye Methylene blue stains the nucleus. (Freshney, 2005).

i. Coverslips kept in a petri dish are seeded with the 4000 cells/100µl and incubated at 37℃ for 24 hrs. in a CO_2 incubator.
ii. Old medium is discarded and the coverslips are flooded with fresh medium.
iii. Coverslips are washed with 1X PBS and cells fixed in 4% PFA for 45 minutes.
iv. Fixed coverslips are washed using 1X PBS and transferred into 1:1 (1X PBS: methanol) for 10-15 minutes.
v. After this, coverslips are incubated in 100% methanol for 10- 15 minutes.
vi. Coverslips are flooded with Giemsa stain for 5 minutes. The stain is then diluted by adding a few drops of DDW and kept for 20-25 minutes.
vii. Excess stain is removed by washing the coverslips with DDW and then air dried.
viii. Coverslips are mounted in DPX and observed under the microscope. (Fig 2)



2A 2B

Figure 2 *– Image of C6 cells stained with Geimsa (x 400). Fig 2A shows control cells while fig 2B has cells treated with LiCl, which causes alteration in their morphology*

Trypan blue Staining

Trypan blue stain is used to determine the number of viable cells present in the cell suspension. Live cells possess intact cell membrane that restrict certain dyes such as trypan blue, Propidium iodide and

Eosin from entering the cell. The number of live cell (unstained) and dead (blue) cell can be counted using a hemocytometer under the microscope.

i. Adherent cells are detached by using 1x trypsin and centrifuged at 3000 rpm for 5 min.
ii. Pellet is resuspended in 1ml 1X PBS.
iii. A mixture of 1:1 trypan blue:cell suspension is taken in a small microfuge tube and incubated for 2-3 min on RT.
iv. 10 µl stained cell suspension is loaded on the hemocytometer and observed under the microscope.
v. Viable (unstained) and dead (stained) cells are counted separately in the hemocytometer. (Fig 3)
vi. The percentage of the viable cells is calculated as follows.

$$\text{Viable Cell (\%)} = \frac{\text{Total number of viable cells in a given area}}{\text{Total number of cells in the same area}} \times 100$$

To count the total number of cells per ml of the suspension the following formula is used:

No. of cells / ml = Average no. of cells counted in 16 squares of the corner boxes x dilution factor x 10^4

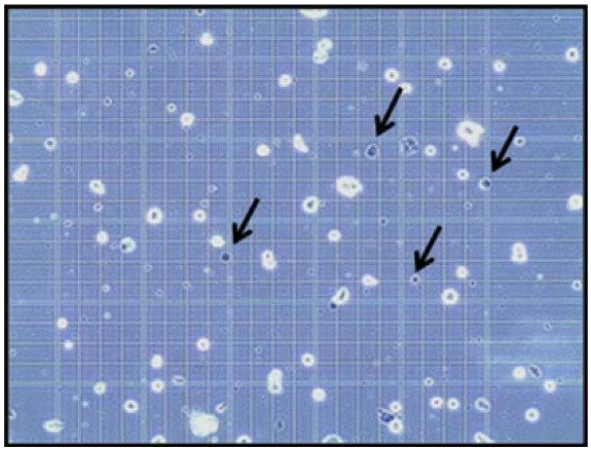


Figure 3*. Mixture of viable and dead cells in a hemocytometer. The cells indicated by arrows are some examples of dead cells turned blue after staining.*

Reagents:

For maintenance of cell cultures

1) Dulbecco's Modified Eagle Medium (DMEM)
2) Foetal Bovine Serum (FBS)
3) Phosphate Buffer saline (PBS)
4) 10x trypsin (2.5%)
5) DMSO (Dimethyl sulfoxide)

For Geimsa Staining

1) Whole medium (DMEM -10% FBS)
2) 1XPBS
3) 4% PFA (Paraformaldehyde)
4) 100% Methanol
5) 1X PBS : 100% Methanol (1 : 1)
6) Giemsa stain
7) Double distilled water (DDW)
8) DPX (Dibutylphthalate Polystyrene Xylene)

For Trypan Blue staining

1) Whole medium (DMEM -10% FBS)
2) 1XPBS
3) 0.4% Trypan blue
4) Hemocytometer slide

Some projects that can be undertaken using cell lines and these simple techniques

1) The effect of any chemical / drug / neurotransmitter can be studied using cell viability as a measure of toxicity.
2) Several chemicals may not be directly toxic to cells but may cause other alterations. Simple observation of morphology may indicate if the chemical has an abnormal effect on the cells.

Reference

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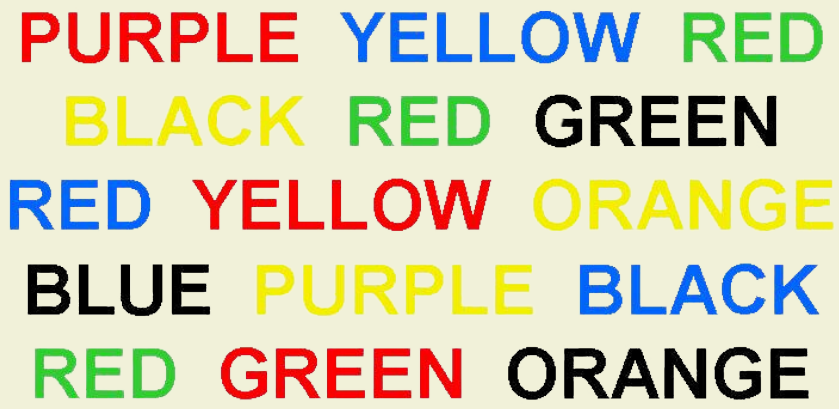


Stroop Test

These tests provide a unique basis (the interference value) for comparing the effectiveness of the two types of associations. Since the presence of the color stimuli caused no reliable increase over the normal time for reading words (D / PEd = 3.64) and the presence of word stimuli caused a considerable increase over the normal time for naming colors (4.35 standard deviation units) the associations that have been formed between the word stimuli and the reading response are evidently more effective than those that have been formed between the color stimuli and the naming response. Since these associations are products of training, and since the difference in their strength corresponds roughly to the difference in training in reading words and naming colors, it seems reasonable to conclude that the difference in speed in reading names of colors and in naming colors may be satisfactorily accounted for by the difference in training in the two activities.

J. Ridley Stroop, 1935

Stroop Test - Analysis of information processing in the human brain

Divya Sinha, H. Ramachandran

Abstract

Stroop effect is one of the well-known psychological experiments first described by John Ridley Stroop in 1935. This neuropsychological test helps us to assess how an individual is able to inhibit cognitive interference while processing a specific stimulus. The task employed in the basic Stroop test is to name the color in which a word has been printed. Thus, while giving response, the individual has to ignore the word but name the color correctly. Human beings exhibit both automatic as well as well controlled processing of information. Hence, we find it difficult to comply with the instruction to ignore reading the word. This is because reading a word is faster than naming the color. Color naming involves computation in our brain that requires selective attention.

Over the years several modifications in Stroop Test have been standardized for understanding the cognitive process, attention and information processing, learning and memory, language and associated cognitive skills. The task called Emotional Stroop is also being investigated to understand the processing of emotional information of a stimuli.

Introduction:

When you initially learnt to brush your teeth, you had to carefully observe where your brush moved to clean your teeth and whether any areas were left unclean. When you clean your teeth every day, you probably notice the first step but not the next. Everyday chores such as brushing your teeth, taking a bath, and tying your shoelaces no longer involve any experiencing or learning process; cognitive psychologists refer to this as automatized behaviour.

Cognitive psychologists often place observers in situations where an automatized reaction is in conflict with the desired behaviour in order to investigate the features of automatized behaviours. This enables researchers to investigate the underlying characteristics of automated actions by observing their influence on more easily measured behaviours. The Stroop test can be effectively used to investigate this neuro-cognitive process.

Stroop (1935) reported that when ink was used to make colour names that were different from the ink, observers were slower to correctly identify the colour of the ink. That is, when red ink was used to spell the word blue (Incongruent), spectators took longer to recognize it. This is an interesting discovery because observers are instructed to ignore the word names and merely record the colour of the ink. However, this appears to be a practically impossible assignment, as the name of the word appears to interfere with the observer's ability to describe the colour of the ink.

Thus congruent stimuli include color-words wherein the ink color and color name are the same. In the incongruent stimuli ink color and color name do not match. In Stroop test sometimes neutral stimuli are also used wherein color-words are all in black.

Example of congruent stimuli 

Example of incongruent stimuli 

Methodology

Conducting the Stroop test:

There are two gray boxes (Figure 1 and 2) that each contain a list of words in color.

The task is to name, as quickly as possible, the color of the letters of each word **correctly** (not to read the words).

***Figure** 1.Congruent Stroop condition (https://faculty.washington.edu/chudler/java/timesc.html)*

***Figure** 2 Incongruent Stroop condition (https://faculty.washington.edu/chudler/java/timesc.html)*

One popular explanation for the Stroop effect is that participants (particularly college undergraduates) have automated the reading process. As a result, regardless of the ink colour, the color names of the words are always processed very quickly. Identifying colors, on the other hand, is not a task that participants undertake frequently, and it is slower because it is not automated. The rapid and automatic processing of the colour name of the word conflicts with the reporting of the ink color. The Stroop task, and its several versions, is a popular technique in cognitive psychology for investigating how different types of actions interact.

*Printed sheets for Stroop test given at the end. Appendix 1 and 2.

A. Stroop test using Paper cards.

Materials and requirements:

- 5 sheets of cardstock with color words. Each page has the 20 sequences of five color words (red, blue, green, brown, purple) printed in pseudo-random order.

  (https://www.sciencebuddies.org/cdn/Files/3001/2/HumBeh_p027_StroopWords.pdf)
- Stopwatch

- Volunteers to take a simple color-naming test
- Informed Consent form (sample provided in Appendix 3)

Methodology:

i. A colour printout of a congruent sheet and an incongruent sheet should be ready for each volunteer.

ii. An observer will administer the test and will record the time taken by the volunteer to correctly complete each of the sheets.

iii. To prevent experimental bias, reverse the procedure and have half of the participants name the colors of the matching words first.

B. Computer based Stroop test using Coglab software:

https://coglab.cengage.com/labs/link_word.shtml

i. Log in and make sure that you can see the full area before you begin the task.

ii. Start a trial by pressing the *spacebar*.

iii. A fixation dot will appear in the middle of the window.

iv. A short time later (less than a second) a word (RED, GREEN, or BLUE) will appear on the screen, and the word will be printed in either **red**, **green**, or **blue** colour.

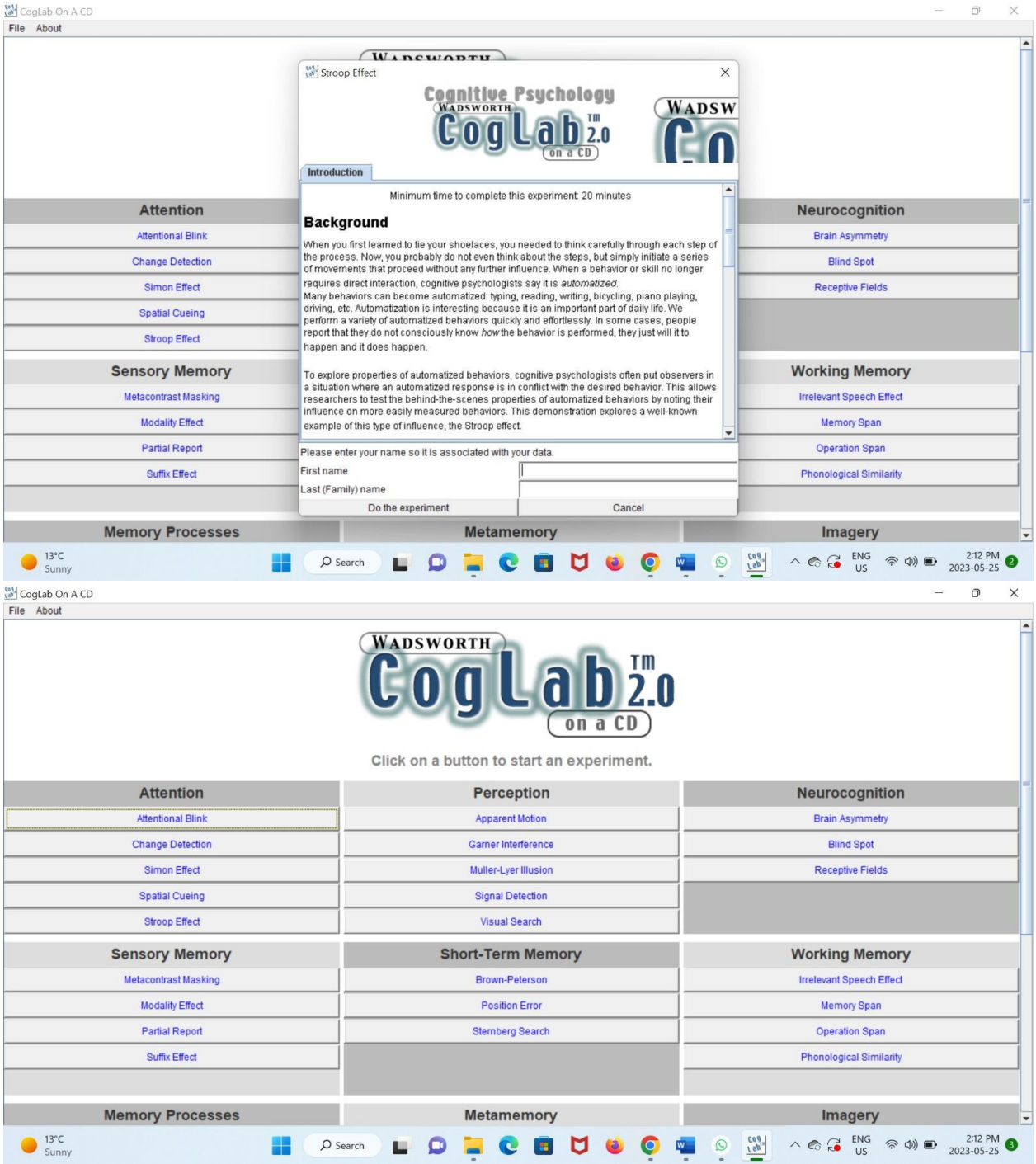
v. The task is to classify the colour of the font as quickly as possible, regardless of the actual word.

vi. After pressing a key to identify the font color, you will receive feedback on whether you were correct.

vii. If you were incorrect, the trial will be repeated later in the experiment.

viii. If you find you are making lots of mistakes, you should slow down or make certain you understood which key goes with which font color.

ix. There are at least 48 trials, 24 in which the font colors and word names are different (e.g., the word "RED" in **red** font color), and 24 in which the font colors and color names match (e.g., the word "RED" in **red** font color).



Observations:

a) Naming the colour will be far more challenging on the sheet with the non-matching colour words.

b) Note that the volunteers will want to read out the colour word rather than name the colour of the letters.

How to calculate results:

a) Calculate the average time it takes to name the colours for each word list.

b) Calculate each volunteer's time difference (i.e., non-matching word time minus the

matching word time).

c) The data can be computed for individuals or for groups of matched volunteers. The average difference for the group of volunteers is then computed.

c) Create bar graphs to illustrate your results.

Time taken to read the list (in seconds) recorded for Condition 1 and 2

Word list	Time taken in seconds
Condition 1 Congruent list	
Condition Incongruent list	

Suggestions for research:

a) You can use Coglab software, which includes a computer-based Stroop test that takes about 30 minutes to complete. Other softwares (OpenSesame, E prime, etc.) can be used for creating your own modified Stroop tests.

b) You can check the interference of music on the Stroop effect for a simple project on the Stroop effect. You can use different music to stimulate different emotions and see how it affects the Stroop Test.

c) Turn the words upside down or rotate them 90 degrees.

d) Compare between Stroop results for a person where the colour name is written in two languages, one in which they are proficient and another in which they are not proficient.

Suggested readings :

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- Cox, W. M., Fadardi, J. S., Pothos, E.(2006) The Addiction: Stroop Test – Theoretical Considerations and Procedural Recommendations, Psychological Bulletin, Vol. 132, No. 3, 443-476.doi 10.1037/0033-2909.132.3.443

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- MacLeod, C. (1997, March/April). Is your attention under your control? The diabolic Stroop effect. Psychological Science Agenda, pp. 6-7.

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- Williams, J. M. G., Mathews, A. & MacLeod, C. (1996). The emotional Stroop task and psychopathology. Psychological Bulletin, 120, 3-24

- https://www.sciencebuddies.org/science-fair-projects/project-ideas/HumBeh_p029/human-behavior/warped-words-stroop-effect

- https://faculty.washington.edu/chudler/java/timesc.html

- https://faculty.washington.edu/chudler/pdf/ministroop.pdf

Appendix 1

Stroop Effect: Color/Word Tests

Instructions: print on card stock and cut each page into horizontal strips. See the Science Buddies project What Conflicting Mental Tasks Reveal About Thinking: The Stroop Effect for complete information.

brown	green	purple	red	blue	red	blue	brown	green	purple
red	brown	purple	green	blue	green	brown	red	blue	purple
purple	red	brown	green	blue	red	brown	green	purple	blue
brown	red	blue	green	purple	brown	blue	purple	red	green
blue	purple	red	green	brown	green	purple	blue	brown	red
green	purple	brown	red	blue	brown	green	red	blue	purple
blue	red	purple	brown	green	green	brown	blue	red	purple
red	blue	purple	brown	green	brown	blue	green	purple	red
green	brown	purple	blue	red	green	brown	purple	red	blue
brown	red	green	blue	purple	blue	red	green	brown	purple

Appendix 2

Stroop Effect: Color/Word Tests

Instructions: print on card stock and cut each page into horizontal strips. See the Science Buddies project What Conflicting Mental Tasks Reveal About Thinking: The Stroop Effect for complete information.

green	purple	blue	brown	brown	green	green	purple	red	green
purple	red	brown	brown	red	blue	blue	blue	purple	red
brown	blue	red	purple	purple	brown	green	blue	red	green
blue	green	brown	red	green	purple	red	blue	purple	brown
brown	green	brown	brown	red	purple	brown	purple	purple	green
blue	blue	blue	blue	green	green	red	purple	red	red
brown	blue	red	purple	red	purple	red	red	purple	blue
brown	purple	green	green	blue	blue	green	brown	brown	green
purple	purple	red	green	purple	red	green	blue	blue	purple
blue	brown	brown	red	brown	brown	green	blue	red	green

Appendix 3

Informed consent (A sample format)

- I have been informed satisfactorily the intention behind the conduct of this experiment.
- I was informed that all information/data about me will remain anonymous and confidential.
- I am also aware that during the conduct of this experiment the participants are not harmed in any form.
- I am allowed to raise questions related to this experiment.
- I will be given a debriefing session at the end, and the outcome of my result will be shown and discussed.
- I am aware that as a participant I also have the right to withdraw from the experiment.

Name of the Participant : _____.
Date : _____.

Briefing and debriefing :

In a psychology experiment with human subjects, **Briefing** and **Debriefing statements** are given/or read.

During **Briefing** there is a friendly verbal interaction between the facilitator of the experiment and the participant. An overview of the experiment, the two lists of words and the mode of attempting them is explained. Participants are encouraged to raise queries (if any) regarding the conduct of the experiment. All participants are informed that responses shall be kept anonymous and will remain confidential. Following this the participants are asked to read the Consent Form and are requested to sign the form (if they agree to continue) or they have an option to withdraw.

After the conclusion of the Psychology experiment there is a **Debriefing** step where appropriate intention and important insights are verbally conveyed. Participants are encouraged to ask queries, and the outcome of the results are shown to the participant. Finally, the participants are thanked for their participation and cooperation.