Key Experiments in Practical Developmental Biology

Edited by
Manuel Marí-Beffa
Jennifer Knight

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KEY EXPERIMENTS IN PRACTICAL DEVELOPMENTAL BIOLOGY

This unique resource presents twenty-seven easy-to-follow laboratory exercises for use in student practical classes, all of which are classic experiments in developmental biology. These experiments have provided key insights into developmental questions, and many of them are described by the leaders in the field who carried out the original pioneering research. This book intends to bridge the gap between state-of-the-art experimental work and the laboratory classes taken at the undergraduate and postgraduate levels. All chapters follow the same logical format, taking the students from materials and methods, through results and discussion, so that they learn the underlying rationale and analysis employed in the research. Chapters also include teaching concepts, discussion of the degree of difficulty of each experiment, potential sources of failure, as well as the time required for each experiment to be carried out in a practical class with students. The book will be an invaluable resource for graduate students and instructors teaching practical developmental biology courses.

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This book is dedicated to our families

"…causes and effects are discoverable, not by reason but by experience,…"
(David Hume [1748] An Enquiry Concerning Human Understanding. Section IV. Part I.)

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Preface

Manuel Marí-Beffa

This handbook of laboratory exercises was first conceived at the Third Congress of the Spanish Society of Developmental Biology held in Málaga, Spain, in 2001. At the time, Professor Antonio García-Bellido suggested including collaborators from the United States and the rest of Europe to give the project a more international scope. The resulting book is a handbook intended to provide a bridge between top scientific researchers and practical laboratories taught at both the undergraduate and postgraduate level. Each chapter introduces a short, inexpensive, and, for the most part, straightforward laboratory project designed to be carried out by students in a standard lab environment. The book uses some of the most popular and best studied model organisms to examine the processes of development. Each chapter is written by specialists in the field describing, in most instances, original pioneering experiments that profoundly influenced the field. The book also demonstrates a historical bridge from classical embryological concepts, using Aristotle and Driesch's entelechia concept (Driesch, 1908) (Chapters 2 and 15) or morphogenetic gradient concept (i.e., Wolpert, 1969) (Chapters 1 and 16) to modern cellular, genetic, and molecular analyses of development such as homeotic genes (Chapters 11 and 20), compartmentalization (Chapter 14), or cell–cell interactions (Chapters 2, 13, and 22). In addition, the high-impact techniques of vertebrate cloning (Section IX) and embryonic stem cells (Section X), as well as the emerging discipline of evolution and development (Evo–Devo, Section XI), are also considered. Finally, although there is much still to learn in this field, Section XII is devoted to computational modelling in the search for a link between genotype and phenotype. During each laboratory exercise, it is our intent that the students imagine themselves working with these highly respected scientists, traveling the same road pioneered by the authors of each chapter.

The format of each chapter is intended to merge the format of standard scientific papers and practical laboratory protocols – a format inspired by texts with similar intent (Stern and Holland, 1993; Halton, Behnke, and Marshall, 2001). Each chapter also includes parts called "Alternative Exercises" and "Questions for Further Analysis" that will permit laboratory instructors or advisors to carry out an "inquiry-based" lab format as

supported by the National Research Council of the United States (NRC, 2000). With the guidance provided in each chapter, students can design and carry out their own, related experiments, potentially culminating in the writing of original papers. For most of the laboratory exercises described, the standard laboratory safety protocols maintained in all labs are sufficient; where necessary, more information is given about the controlled use of hazardous substances. IN GENERAL, CAUTIONS MUST BE TAKEN. MANY OF THE CHEMICALS USED IN THESE LABORATORY EXERCISES ARE HAZARDOUS. TO PREVENT EXPOSURE TO THESE CHEMICALS, YOU SHOULD WEAR GLOVES AND SAFETY GLASSES AND WORK WITH THE CHEMICALS IN A FUME HOOD. THIS IS PARTICULARLY IMPORTANT WHEN WORKING WITH SUBSTANCES LIKE PARAFORMALDEHYDE, GLUTARALDEHYDE, RETINOIC ACID, DEAB, DAB XYLENE, OR CHLORAL HYDRATE. MORE DETAILED INFORMATION ON PROPER HANDLING OF THESE CHEMICALS CAN BE OBTAINED FROM MATERIAL SAFETY DATA SHEETS (MSDS), WHICH ARE SUPPLIED BY THE CHEMICAL MANUFACTURERS. The animals used in each laboratory exercise can be obtained from the curators of many international stock centers around the world. In most countries, Home Office approvals are required so that appropriate responsibilities must be taken by receiving departments.

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Introduction

Jennifer Knight

Experiencing the process of scientific discovery is part of training to be a scientist. This book of laboratory exercises is designed to give students an opportunity to explore and carry out experiments that have each made significant contributions to the fields of Experimental Embryology and Developmental Biology over the past 100 years. It is our hope that students will experience the initial thrill of discovery as they learn how to do each experiment, analyze each outcome, and grasp the significance of each conclusion. However, science is not solely about the end discovery but also about the process. This process cannot be appreciated by reading textbooks or scientific journals alone. Rather, a budding scientist must experience firsthand the myriad pitfalls of each experiment. Despite the way this laboratory manual is designed (with step-by-step instructions to accomplish each experiment), students will encounter unforeseen problems in carrying out the experiments. If they are not already intimately familiar with experimental science, students will undoubtedly discover that this process demands a meticulous approach. Designing, setting up, and executing experiments cannot be accomplished in a haphazard way. For this reason, every student must keep a laboratory notebook, a task that many initially regard as "busy work." In fact, keeping careful record of everything one does in the laboratory is the only way to experience success. At the other end of this process is presenting a finished piece of work to the scientific community. Again, the only way to learn this aspect is to assemble data into a mock scientific "paper," ready for publication in a journal. If possible, verbally presenting the data to an audience is also a valuable learning experience. Below, we give some suggestions for these two important aspects of scientific discovery: keeping a laboratory notebook and writing a laboratory report.

KEEPING A LABORATORY NOTEBOOK

A laboratory notebook is a day-to-day record of plans, procedures, results and interpretations. When a scientist refers back to his/her notebook, the notes on procedures, pitfalls and outcomes should help him/her to easily repeat the experiment. In the scientific

community, a notebook is essential both for demonstrating integrity and for helping to keep track of each step of an experiment. In a student laboratory setting, a notebook can be just as useful, provided the student takes the time to make it so. A notebook must be bound, and the first two pages should be reserved for a Table of Contents that can be filled out as experiments are completed. When students begin an experiment, they should write down their thoughts on the experiments, questions they may have, and finally, their objective and hypothesis. Next, as students perform an experiment, the actual steps should be recorded, although often this can be done in an outline format rather than in great detail, since the procedures have already been described in the lab manual. It is most important that students make note of problems encountered during the experiment, or of deviations taken from the laboratory manual. These notes will help with trouble shooting if part of the experiment does not succeed. Next, students should record all of their observations and all of their data, both raw and calculated (graphs, tables, etc.). Finally, when the experiment is complete, the students should summarize their results, conclusions, and interpretations in the notebook, before moving on to the next experiment. It is essential that students realize that a laboratory notebook is a work in progress. It is only useful if it is used during the experiment to record the process.

WRITING A LABORATORY REPORT

A laboratory report should follow the standard format for a scientific paper, described below.

- Abstract: a 4–6 sentence summary of the entire paper, including a brief statement of the hypothesis, the methods used, the outcome, and the relevance of the experiment.
- Introduction: a well-researched description of the topic addressed by the experiment. The introduction gives the reader the context of the experiment. This section should also restate the hypothesis and describe the predictions and goals of the experiment.
- Materials and Methods: a detailed section in which reagents and protocols are clearly described. Often, in a classroom setting, since these details are provided to students in the lab manual, instructors suggest a summary of the materials and methods used. It is still important to write in complete sentences and to accurately state how the experiment was carried out.
- Results: a description of the outcome of the experiments. This section of the paper includes only a description of the data and their presentation – figures, tables, and graphs – but does not discuss the interpretation of the findings.
- Discussion: an interpretation of the experimental data and how it compares to published information about this topic. In this section, students should discuss what their results mean, the implications or significance of these results, and how they might expand or clarify the results. Ultimately, it is important that students put their experiment into the context of other work on this topic.
- References: a detailed citation of each journal article used in writing the paper. There are many different possible formats for references. Students may choose a specific

format by consulting a journal and using that standard format for each article referenced (instructions for authors, often found at the beginning of journals, usually include instructions for referencing other journal articles).

By following the suggestions above, we hope that as instructors and students alike perform the experiments presented in this book, you will find yourselves engaged in and enticed by this exploration of Developmental Biology.

SECTION I. GRAFTINGS

1 Two developmental gradients control head formation in hydra

H. R. Bode

OBJECTIVE OF THE EXPERIMENT Two developmental gradients are involved in the axial patterning of the head and the body column of a hydra. One is a morphogenetic gradient of head activation [= head formation capacity], and the other is a gradient of head inhibition. The objective of the experiment is to demonstrate the presence of these two gradients in the body column of adult hydra using transplantation experiments.

DEGREE OF DIFFICULTY The experiments involve the isolation of a piece of the body column and transplantation to the body column of a second animal. Although this appears difficult at first sight, with a little practice, almost all students learn to carry out these grafts at the rate of 6–10 successful grafts/hour.

INTRODUCTION

In animals, the developmental processes associated with axial patterning occur during early stages of embryogenesis. One example involves the processes governing head formation at the anterior end and tail formation at the posterior end of the anterior–posterior axis. In hydra, a primitive metazoan, this type of axial patterning occurs not only during embryogenesis, but also in the adult. This is due to the tissue dynamics of an adult hydra.

As shown in Figure 1.1a, a hydra has the shape of a cylindrical shell. Along the single axis are the head, body column and foot. The head at the apical end consists of a mouth region, the hypostome, and beneath that the tentacle zone, from which tentacles emerge. The protrusions on the lower part of the body column are early [left] and advanced [right] stage buds, hydra's mode of asexual reproduction. The wall of the shell is composed of two epithelial layers, the ectoderm and endoderm, which extend throughout the animal. Among the epithelial cells (not shown in Figure 1.1a) are smaller cells such as neurons, secretory cells and nematocytes, the stinging cells of cnidaria.

The tissue dynamics is the following. The epithelial cells of both layers are continuously in the mitotic cycle (e.g. Bode, 1996). Yet, despite the ever-increasing number of

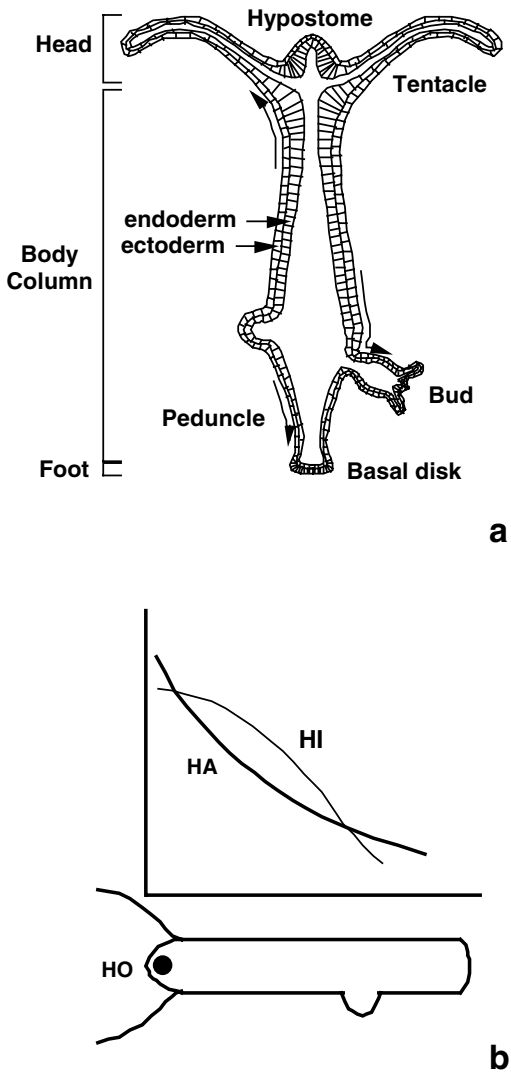


Figure 1.1. (a) Cross section of an adult hydra showing the three regions and the two tissue layers as well as two stages of bud formation. (b) Diagram of the developmental elements that control head formation. HO = head organizer; HA = head activation gradient; HI = head inhibition gradient. (a) is adapted from Amer. Zool., 41, 621–8 (2001).

epithelial cells, the animal remains constant in size. This occurs because the tissue of the upper body column is apically displaced onto the tentacles and eventually sloughed at the tentacle tips (Bode, 1996). Tissue of the lower body column is displaced down the body column and sloughed at the foot (Figure 1.1a). Tissue from the middle of the column is primarily displaced into developing buds, which eventually detach from the adult. Thus, the animal is in a steady state of production and loss of tissue.

As tissue is displaced apically, it is converted into head tissue, whereas tissue displaced basally becomes foot tissue. What are the axial patterning processes that control

the changes in the fate of these moving epithelial cells? A body of transplantation and regeneration experiments have provided insight into these processes (Browne, 1909; Wolpert, 1971; MacWilliams, 1983*a*, *b*; Bode and Bode, 1984). Bisection of the body column leads to the regeneration of a head at the apical end of the lower half. This indicates that body column tissue has the capacity to form a head. Transplantation experiments have shown that a head organizer region is located in the hypostome (Figure 1.1b) (Broun and Bode, 2002). This organizer transmits a signal, or morphogen, to the body column which sets up a gradient of head formation capacity, commonly referred to as the Head Activation Gradient (Figure 1.1b) (MacWilliams, 1983*a*). With this capacity, what prevents regions of body column tissue from forming heads? The head organizer also produces and transmits an inhibitor of head formation, which is also graded down the body column (Figure 1.1b), thereby preventing body column tissue from forming heads (Wolpert, 1971: MacWilliams, 1983*b*). These two gradients control the fate of the body column tissue as it is displaced apically. When the tissue reaches a point where [HA] > [HI], the body column tissue is converted into head tissue. This mechanism maintains the axial patterning at the upper end in the context of the tissue dynamics of the animal. These gradients and their behavior have been incorporated into a model that provides a useful overall view of axial patterning in hydra (Meinhardt, 1993; see Chapter 27).

MATERIALS AND METHODS

In this section the equipment and materials required for carrying out transplantation experiments are described using a procedure developed by Rubin and Bode (1982). The culture of hydra and the source of specific pieces of equipment or materials are presented in the Appendix.

EQUIPMENT AND MATERIALS

Per student

Dissecting microscope with 10 × oculars and, optimally, variable magnification of 1–4 ×.

Pasteur pipette with rubber bulb (Fisher Scientific).

Two pairs of fine-tipped forceps (Fine Science Tools) to handle pieces of fish line and "sleeves."

Scalpel (Fine Science Tools). An ordinary razor blade will work equally well.

Medium-sized [60-mm diameter] plastic or glass petri dishes (Fisher Scientific).

Per practical group. If available, an 18°C incubator with a light that can be set with a timer so that it is on a cycle for 12 h on and 12 h off. If an incubator is not available, experimental samples can be left in the lab if the temperature is in the 15–25°C range.

Biological material. One-day–starved adult hydra (see Appendix) without buds. Two adult hydra are needed for each graft: one is the donor, and the other is the host. Determine how many grafts will be made and obtain twice that number of adult animals.

For each transplantation choose two adults that are the same size. Thus, 250–300 hydra are necessary for all four experiments [see Appendix on Maintenance of a Hydra Culture].

REAGENTS

$CaCl_2$	$NaHCO_3$
$MgCl_2$	KNO_3
$MgSO_4$	Glutathione

PREVIOUS TASKS FOR STAFF

Preparation of fish lines and "sleeves"

1–1.5 cm pieces of fish line [8 lb; diameter = 0.3 mm; local store for fishing supplies]. Using a scalpel and forceps, cut 1–1.5 cm long pieces of fish line.

"Sleeves": 2–3 mm pieces of polyethylene tubing (VWR Scientific). [As the ends should be pointed, cut the fish line at a 45 degree angle. Cut as many as are needed for an experiment. For the "sleeves," cut two for each piece of fish line. Make the cuts perpendicular to the axis of the tubing.]

Maintenance of hydra culture. During this and the previous experiment the hydra must be maintained under standard conditions (see Appendix).

Solutions

Hydra medium: 1.0 mM $CaCl_2$, 1.5 mM $NaHCO_3$, 0.1 mM $MgCl_2$, 0.08 mM $MgSO_4$, and 0.03 mM KNO_3; pH 8.0.

1 mM glutathione (Sigma) in hydra medium.

TRANSPLANTATION PROCEDURE

An individual transplantation, or graft, involves the following:

Isolation of a ring of body column tissue (Figure 1.2: Step A). Usually the ring of tissue isolated consists of 1/6 – 1/8 of the body column. Figure 1.2 shows a body column divided into 8 regions. To isolate a region do the following: Place a hydra in a medium-sized petri dish containing hydra medium, and let it stretch out. Determine the location of a region to be isolated. For example, for the 3-region, let the animal stretch out and estimate the location of the middle of the body column. Then estimate the location of the point half way between the middle and the top of the body column [where the tentacles emerge]. This location would be the top of the 3-region. With a pair of forceps in one hand, cradle the hydra. Using the scalpel in the other hand, gently bisect the animal at the apical end of the region you intend to isolate. Let the contracted animal extend, and bisect once more at the point below the apical end which will result in a ring of tissue approximating 1/8 of the length of the body column.

Thread the ring of tissue onto a piece of fish line (Figure 1.2: Step B). When grafting a ring of tissue into a host, it is important that the basal end of the isolated ring be

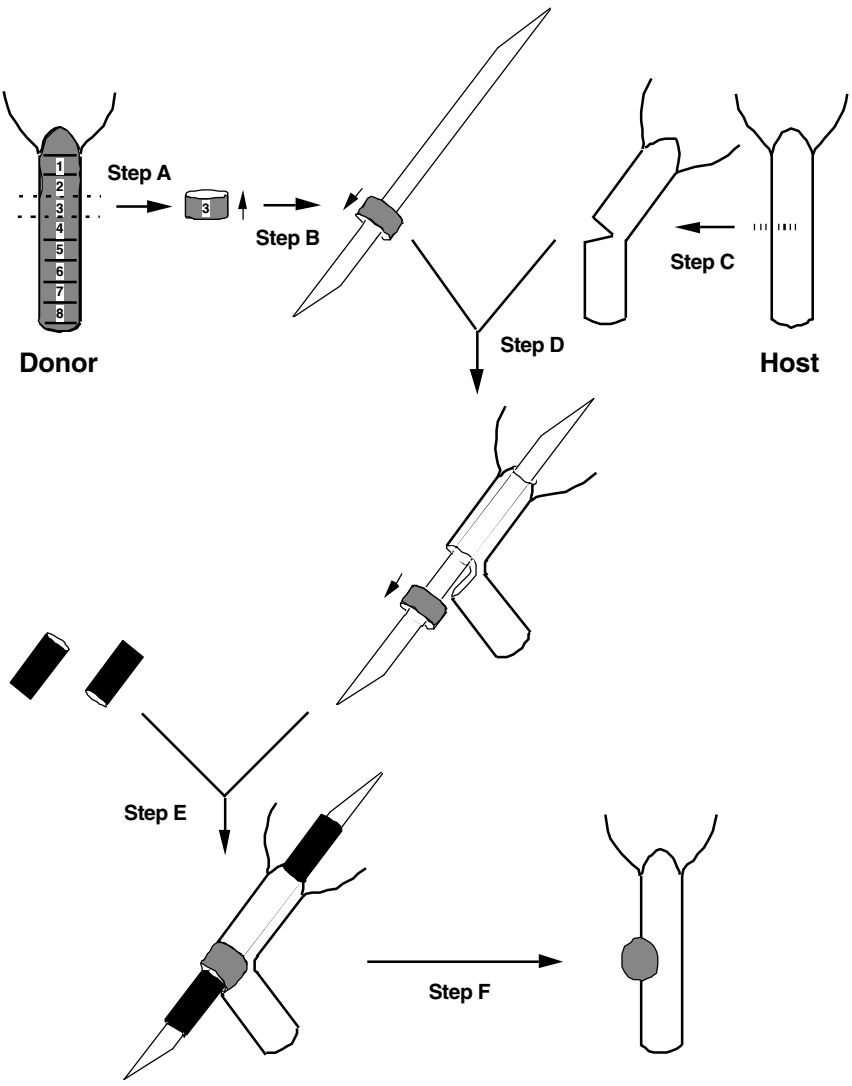


Figure 1.2. Detailed procedure for transplanting a ring of tissue from the body column of a donor hydra to the body column of a host hydra. The six steps for the procedure are described in the text.

brought into contact with the host. To ensure that this happens be certain that the ring of tissue is threaded onto the fish line in the appropriate orientation (as indicated by the arrow in Figure 1.2: Steps A and B). Using two pairs of forceps, gently cradle the ring with one pair, and holding a piece of fish line with the second pair, gently slide the piece of fish line through the ring. Make sure that the apical end of the ring of tissue is facing the end of the fish line. Slide the ring along the fish line until it is about 3–4 mm from the end.

Graft the ring of tissue to the host (Figure 1.2: Steps C and D). Place an adult hydra, which will serve as the host, into the petri dish with the ring of tissue and let

it stretch out. Using the scalpel make a cut perpendicular to the body axis that extends about 1/2 way through the body column (Figure 1.2: Step C). For all the experiments described below the location of where the cut will be made will be indicated in terms of the body length [BL]. Thus, when grafting into a location that is 75% of the distance down the body column from the head, the location will be identified as "75% BL."

After cutting, a gap will appear (Figure 1.2: Step C). Using two pairs of forceps, cradle the host with one pair and slide the fish line, holding the ring of tissue into the cut, up through the gastric cavity, and out the mouth (Figure 1.2: Step D). When reaching the mouth, gently push, and the animal will open its mouth. Then slide the ring of tissue along the fish line so that it is in firm contact with the cut edges of the host.

Thread "sleeves" onto the ends of the fish line (Figure 1.2: Step E). It is important to keep the ring of tissue firmly in place as well to keep the animal from moving along the fish line. To do this, pieces of polyethylene tubing, referred to as sleeves, are threaded onto the two ends and brought into contact with the ring of tissue and the head respectively (Figure 1.2: Step E). The 2–3 mm pieces of polyethylene tubing are the "sleeves." With one pair of forceps hold the fish line extending out of the mouth. Use the second pair of forceps to slide a sleeve onto the piece of fish line extending from the ring of tissue, and use it to push the ring of tissue so that it is firmly in contact with the host tissue. Repeat this step with a second sleeve so that it is firmly in contact with the hypostome. Do not push so hard that the tissue folds.

Healing of the graft and removal of the fish line (Figure 1.2: Step F). With a pair of forceps gently transfer the graft to another medium-sized petri dish containing hydra medium. It is not a problem if the graft and fish line float on the surface. When all the grafts for a sample have been completed and transferred to this dish, place the dish [as is, or on a tray] in the 18°C incubator, or on the lab bench at 15–25°C.

The cut edges of the ring of tissue and the host will fuse together and heal within 1–2 h. At any time thereafter, remove the sleeves from each graft. Do this by holding one end of the fish line firmly with a pair of forceps, and gently removing the sleeve from the opposite end. Repeat this step for the second sleeve. Then, firmly holding the end of the fish line protruding from the mouth with one pair of forceps, place the other pair of forceps so that it gently cradles the fish line extending from the mouth. Now, slowly pull the fish line through the mouth until it is free of the host animal and the grafted ring of tissue. Or, gently push the animal down the fish line until the animal and the fish line are separated.

Examination of the grafts. Once the sleeves and fish line have been removed from all the grafts in the sample, the grafts should be incubated at 18°C. Thereafter, the grafts should be examined daily to determine the fate of the grafted ring of tissue.

OUTLINE OF THE EXPERIMENTS

Two pairs of experiments can be carried out to demonstrate the presence of the head activation and head inhibition gradients in hydra.

A. A HEAD ACTIVATION GRADIENT IN THE BODY COLUMN

These simple experiments demonstrate that tissue of the body column has the capacity to form a head and that this property, termed head activation, is graded down the body column.

1. Tissue of the body column has head formation capacity. Head formation capacity can be shown simply by bisecting an animal in the middle of the body column and letting the lower half regenerate a head at its apical end (Figure 1.3a).

PROCEDURE

1. From the stock culture pick out 10 1-day-starved adults of similar size.
2. Place them in a 60-mm petri dish containing about 10 ml hydra medium so that the dish is half full with medium.
3. Using a scalpel, bisect each animal in the middle of the body column resulting in an upper half with a head, and a lower half with a foot.
4. Using a Pasteur pipette with a bulb, remove the lower halves, and transfer to a second 60-mm petri dish with hydra medium.
5. Incubate the dish at 18°C.

DATA RECORDING. The head of a hydra consists of two parts (see Figure 1.1a): The dome-shaped upper half is the hypostome, which contains the mouth. The lower half is the tentacle zone from which a ring of tentacles emerge. Head regeneration will occur as follows (Figure 1.3a): Following bisection, the wound at the apical end of the lower half heals over. At an early stage, a ring of small protrusions, or tentacle bumps, forms below the apical cap. Subsequently, the bumps grow into short tentacles, and later into long tentacles. As the tentacles are forming, the mouth is developing in the hypostome. A fully formed mouth will open widely in response to glutathione treatment, which provides an easy way to assay the formation of the mouth. The analysis of head regeneration should be carried out in the following steps:

- Examine each of the 10 regenerating lower halves daily for 4–5 days with respect to tentacle formation and mouth formation using a dissecting microscope. When the daily analysis is complete, return the samples to the incubator.
- Determine the extent of tentacle formation. Start this examination on the day after decapitation, and carry out every 1–2 days until the end of the experiment.
  (a) Number of regenerates with a healed apical cap.
  (b) Number of regenerates with a ring of small protrusions, or tentacle bumps, which form a ring at the base of the apical cap.

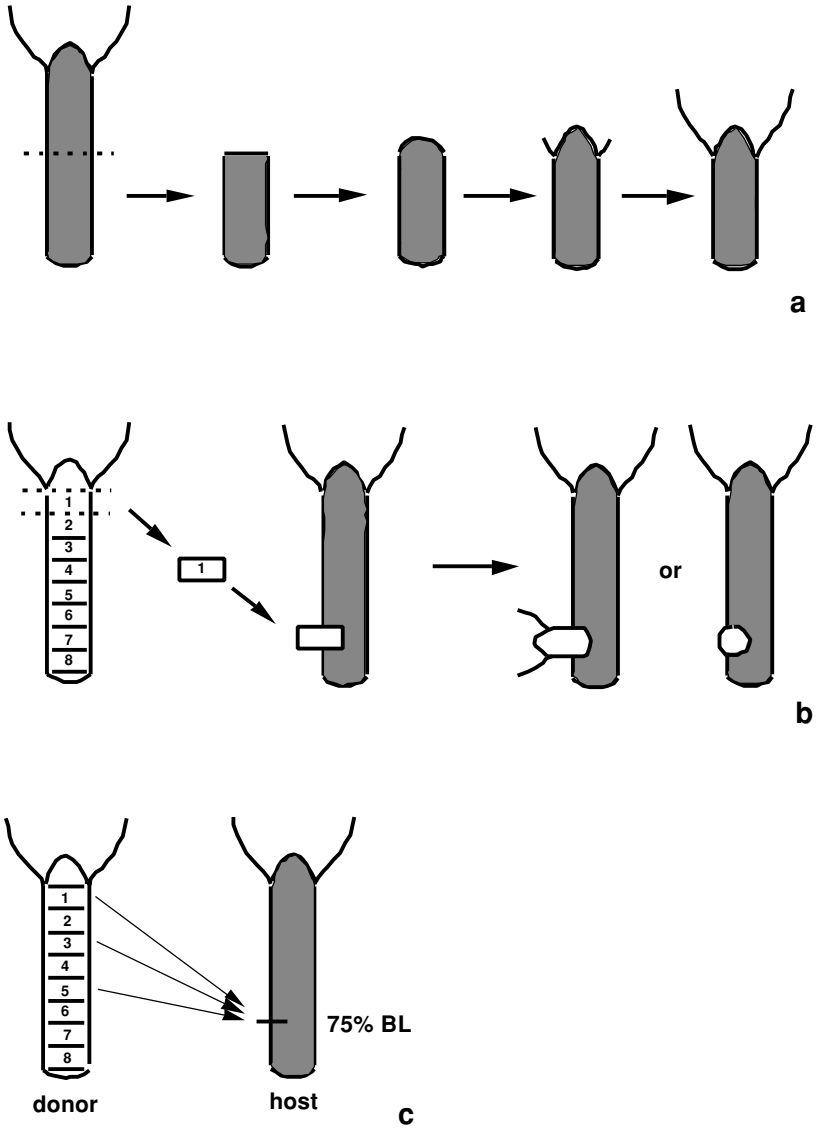


Figure 1.3. Two experiments demonstrating (a) head activation and (c) the head activation gradient. (b) Illustrates the transplantation procedure.

(c) Number of regenerates with short tentacles.
(d) Number of regenerates with full-length tentacles.

➤ Determine if a mouth has formed. Start this assay when tentacle bumps have appeared, and carry out every 1–2 days until the end of the experiment.

(a) Add 100 μl 1 mM of glutathione to the 10 ml of hydra medium in the dish.
(b) With the dissecting microscope, observe the animals 5 and 10 minutes later.
(c) Determine the number of hydra that open their mouths.

(d) Determine the extent of opening: small, medium, or wide. The extent of mouth opening indicates the extent of completion of mouth formation.
(e) When analysis is complete remove the hydra medium containing glutathione, rinse with hydra medium, and then add 10 ml of fresh hydra medium.

2. The head formation capacity, or head activation, is graded down the body column. Another way to demonstrate that tissue of the body column has head formation capacity is the following. Isolate a piece of the body column from a donor animal and transplant it to a lower location on the body column of a second, or host, animal (Figure 1.3b). For example (Figure 1.3b), when the 1-region of a donor is transplanted to an axial level of 75% BL in many samples, it will form a second axis with a head at the apical end (see Figure 1.3b).

To examine the distribution of head activation along the body column, one can carry out this transplantation experiment using regions from successively more basal parts of the body column (see Figure 1.3c). By determining the fraction of transplants of each kind that form a second axis with a head, the distribution of head activation along the body column can be determined. For this experiment, compare the fraction of transplants of the 1-region, 3-region and 5-region that form second axes. The procedure for carrying out the transplants is described in Materials and Methods.

Carry out 15–20 grafts for each of the following type of transplantation:

(a) Transplant the 1-region of the donor into a host at 75% BL.
(b) Transplant the 3-region of the donor into a host at 75% BL.
(c) Transplant the 5-region of the donor into a host at 75% BL.

DATA RECORDING. Examine each animal in each set of transplants daily and determine the following:

(a) Number of samples with a normally developing head using the criteria for tentacle formation in the head regeneration experiment described in the previous section.
(b) Number of samples developing an attempted head. Such transplants form a single tentacle at the apical end of the transplant.
(c) Number of samples forming a foot. The apical end of the developing transplant will form a blunt end that is sticky. The stickiness can be tested by touching the end with a pair of forceps and seeing if the forceps remain attached to the tissue.
(d) Number of samples forming neither a head nor a foot. The apical end of the transplant remains round and smooth.
(e) The sets of transplants should be examined every 1–2 days until no further changes take place in the type of result formed. For example, some transplants may form a head later than others.

B. A HEAD INHIBITION GRADIENT IN THE BODY COLUMN

Head inhibition is produced by the head organizer in the hypostome and transported to the body column, where it prevents body column tissue from forming heads. The existence of head inhibition and its axial distribution can be demonstrated with the following experiments.

1. Absence of the head reduces head inhibition in the body column. If head inhibition is produced in the head and transmitted to the body column, then removal of the head should reduce the level of head inhibition in the body column. The experiment providing evidence for this statement, as shown in Figure 1.4a, is a variation of the second experiment in the previous section.

Carry out 15–20 grafts for each of the following types of transplantation:

(a) Experimental: decapitate the host and transplant the 1-region of the donor into this host at 50% BL.
(b) Control: transplant the 1-region of the donor into a normal host at 50% BL.

DATA DECORDING. Examine each animal in each set daily using the same criteria as described in the experiment for the distribution of head activation (see previous section, The Head Formation Capacity, or Head Activation, Is Graded Down the Body Column).

2. Head inhibition is graded down the body column. To examine the distribution of head inhibition along the body column, carry out an experiment similar to the one described above for the head activation gradient, as shown in Figure 1.4b. Again, the overall transplantation procedure is as described in Materials and Methods.

The graded distribution of the head inhibition gradient can be demonstrated by grafting 1-regions of donors to different locations (25% BL, 50% BL, and 75% BL) in a host.

Carry out 15–20 grafts for each of the following type of transplantation:

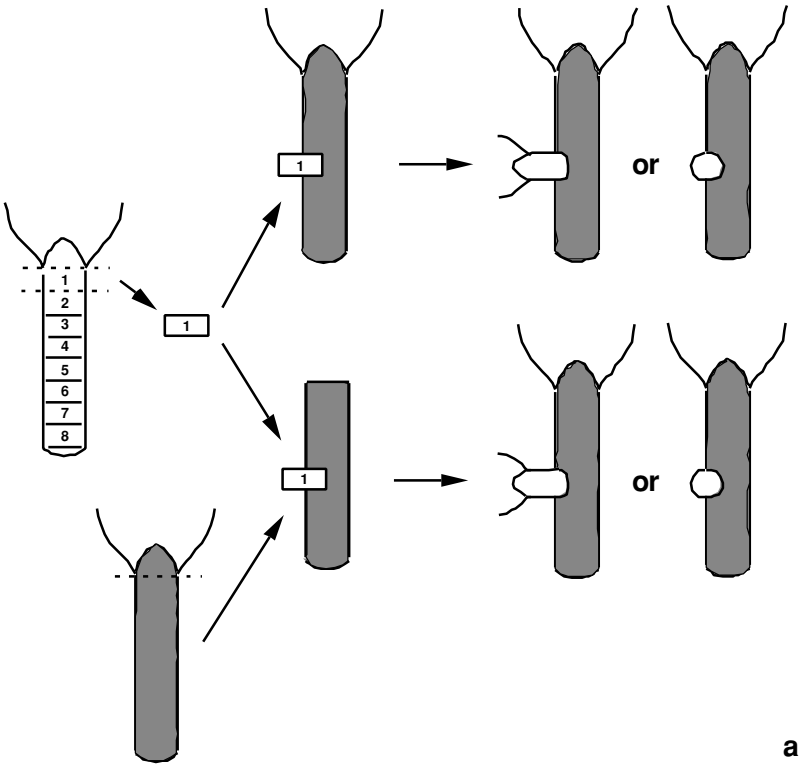
(a) Transplant the 1-region of the donor to 25% BL of the host.
(b) Transplant the 1-region of the donor to 50% BL of the host.
(c) Transplant the 1-region of the donor to 75% BL of the host.

DATA RECORDING. Examine each animal in each set daily using the same criteria as described in the experiment for the distribution of head activation (see previous section, The Head Formation Capacity, or Head Activation, Is Graded Down the Body Column).

EXPECTED RESULTS AND DISCUSSION

The expected results from the two experiments are relatively straightforward. The first experiment of each set demonstrates the existence of the property, whereas the second experiment of each set demonstrates that the property is distributed as a gradient along the body column.

To determine if there are statistically significant differences between the percentage of transplants forming a second axis in, for example, the control and decapitated hosts in the experiment illustrating head inhibition, the following analysis can be carried out (e.g., Zar, 1974): Each student will carry out 15–20 grafts for each of the control and decapitated hosts. A percentage of each type of graft will form a second axis. Calculate the average value for the percentage +/− standard deviation for each of



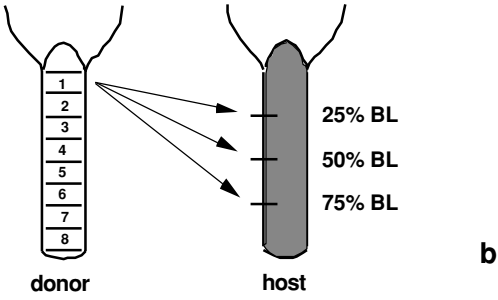


Figure 1.4. Two experiments demonstrating (a) head inhibition and (b) the head inhibition gradient.

the two types of grafts. If these values do not overlap, the difference is statistically significant.

DEMONSTRATION OF THE HEAD ACTIVATION PROPERTY

Bisection of the animal will result in the regeneration of a head at the apical end of the lower half. This indicates that the tissue of the body column is capable of head formation and contains head activation in some molecular form. Because the body column can be bisected anywhere along its length and the lower piece will regenerate a head, the head activation property is distributed all along the body column.

DEMONSTRATION OF THE DISTRIBUTION OF HEAD ACTIVATION

If the distribution of head activation is graded down the body column, one would expect to see the number of grafts forming a second axis with a head decreasing along the lower body column, the source of the isolated ring of tissue. That is, comparing the number of heads formed by each of the three regions, one would expect the 1-region to form more heads than the 3-region. In turn, the 3-region would form more than the 5-region.

DEMONSTRATION OF THE HEAD INHIBITION PROPERTY

Decapitation removes the source of head inhibition. Accordingly, one would expect a lower level of head inhibition in the body column and an increase in the proportion of grafts that form a second axis in the decapitated hosts compared to the grafts in the normal hosts. This is expected if one assumes that head inhibition decays rapidly so that the level is reduced. This is, in fact, the case as the half-life of head inhibition is 2–3 h (MacWilliams, 1983*b*).

DEMONSTRATION OF THE DISTRIBUTION OF HEAD INHIBITION

Here one would expect the reverse of the results in the experiment demonstrating the distribution of head activation. Assume that head inhibition is maximal at the upper end of the body column and graded down the body column. If so, one would expect to see more transplants that form a second axis with a head the farther down the body column the 1-region is transplanted into the body column. That is, the number of heads formed by the 1-region would be higher when transplanted to the 5-region compared to the 3-region. In turn, the number of heads would be higher when transplanted to the 3-region compared to the 1-region.

These results illustrate that the two developmental gradients play a major role in determining the pattern of structures formed along the axis of the body column in hydra. In the instance examined here, the two gradients – the morphogenetic gradient of head activation and the head inhibition gradient – control where a head is formed. Morphogenetic gradients also play a role in other animals, usually during very early stages of embryogenesis when the axes are being set up (see Chapter 19). Hydra is unusual in that the gradients are continuously active in the adult animal.

TIME REQUIRED FOR THE EXPERIMENTS

The execution of these experiments involves learning how to carry out the transplantation procedure. Usually a student will need 1–3 h to learn, become comfortable with, and then, successful with the grafting process. Thereafter, the student will usually be able to carry out 6–10 grafts/h.

To get enough data to obtain a clear result, it is necessary to carry out at least 10 grafts (preferably 15–20) for each type of transplantation. Then, the amount of time required for each experiment would be the following:

- Regeneration experiment demonstrating head activation in the body column. This experiment does not require much time. The manipulations, which are the bisecting

and handling of the animals, would take 15–20 min. Using a dissecting microscope to examine the extent of regeneration of each animal in a sample requires about 15–20 min/day.

- Grafting experiment illustrating head inhibition. As there are two types of grafts involved in this experiment, the total number of grafts would be 20 if 10/type are carried out, or 40 if 20/type are carried out. Assuming that one can do 6–10 grafts/h, then 2–3 h would be required for carrying out 20 grafts and 4–6 h for 40 grafts. Analysis of the grafts would most likely require 30–60 min/day.
- Grafting experiments demonstrating the head activation gradient or the head inhibition gradient require a similar amount of time. In both experiments there are three different types of grafts. Thus, the total number of grafts would be either 30 at 10 grafts/type of graft, or 60 at 20 grafts/type. This would require 3–5 h for 30 grafts, or double that for 60 grafts. Analysis of the grafts requires 30–60 min.

One way to reduce the time required for the experiments would be to divide the experiment among several students. For example, for the head inhibition experiment, students could work in groups of 4, each carrying out 5 control grafts and 5 experimental grafts. For the two experiments demonstrating the presence of the head activation and head inhibition gradients, groups of 6 students each doing 10 grafts of one type in an hour would provide the 60 grafts needed for acquiring a reasonable amount of data for each experiment.

POTENTIAL SOURCES OF FAILURE

As the only manipulations involved are the isolation and transplantation of a ring of tissue into a host, the only significant source of failure is a failure of the ring of tissue to graft onto and heal to the host. Practice usually takes care of this problem.

TEACHING CONCEPTS

The major concept illustrated with these experiments is that the pattern along the axis of an animal can be controlled by a morphogenetic gradient. When the morphogen concentration is above a threshold, such as for head formation, then the tissue becomes committed to forming a head. The inhibition gradient illustrates a second process common in embryogenesis and developing systems. Once a piece of tissue, or region of the embryo, has become committed to forming a particular cell type, or a structure, then an inhibitory mechanism, commonly referred to as lateral inhibition, is initiated to prevent that same cell type or structure from forming in the vicinity of the first one (see Chapter 22).

ALTERNATIVE EXERCISES

Two additional experiments can be carried out which extend the information gained from the experiments described above. They would also begin to provide insight into the molecular basis of the head activation gradient.

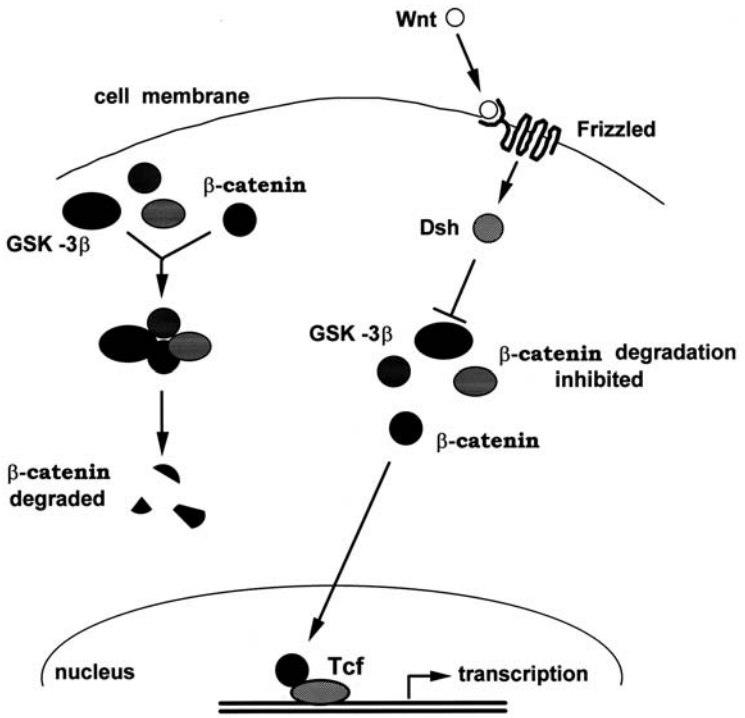


Figure 1.5. Effect of GSK-3β on β-catenin in the presence and absence of Wnt.

PROPOSED EXPERIMENTS

A major pathway that affects a number of developmental events, or processes, during early embryogenesis is the Wnt pathway (Cadigan and Nusse, 1997). On the outer surface of a cell, the pathway consists of Wnt, a signaling molecule, and Frizzled, a receptor for Wnt on the cell surface (Figure 1.5). Inside the cell, the pathway, for the sake of simplicity, consists of Disheveled, GSK-3β, β-catenin and Tcf. As shown in Figure 1.5, when Wnt is absent, GSK-3β causes the degradation of β-catenin. When Wnt is present, the activated form of Disheveled blocks GSK-3β. This in turn prevents the degradation of β-catenin. Then β-catenin coupled with Tcf enters the cell nucleus and acts as a transcription factor, stimulating the transcription of genes required for a specific developmental process. In hydra, *HyWnt*, and *HyTcf*, the hydra homologues of the *Wnt* and *Tcf* genes are expressed in the hypostome (Hobmayer et al., 2000), suggesting the Wnt pathway has a role in the formation and/or activity of the head organizer. LiCl is known to block the activity of GSK-3β (Phiel and Klein, 2001), thereby allowing β-catenin to enter the nucleus, and with Tcf initiate a new developmental process. If so, one might expect treatment of hydra with LiCl to result in the formation of head structures such as tentacles, or complete heads, along the body column; such results have been obtained (Hassel, Albert, and Holfheinz, 1993). Further information on the Wnt pathway can be obtained from http://www.stanford.edu/~rnusse.

The following pair of experiments illustrate this possibility.

Effect of 2 mM LiCl on the body column. Tentacles that form on the body column are called ectopic tentacles. To demonstrate that treatment with 2 mM LiCl will cause the formation of ectopic tentacles, the following experiment can be carried out. This experiment is ideally carried out with a strain of *Hydra vulgaris* or *Hydra littoralis*.

1. Treat 20 animals with 2 mM LiCl in hydra medium for 2 days and then return to hydra medium.
2. Examine animals every 1–2 days for 4–7 days and determine:
   (a) How soon after end of treatment do ectopic tentacles appear on the body column?
   (b) What fraction of the treated animals produce ectopic tentacles?
   (c) Where along the body column do the tentacles appear?

Effect of 2 mM LiCl on the head activation gradient. The formation of ectopic tentacles suggests that the head activation level has risen in the body column, surpassing the level of head inhibition, thereby permitting the formation of head structures. To directly determine if 2 mM LiCl affects the head activation gradient, a transplantation experiment of the type described in a previous section can be carried out as follows:

1. Treat 20 animals with 2 mM LiCl in hydra medium for 2 days.
2. Transplant the 4-region of a LiCl-treated animal to 50% BL of an untreated host using the usual transplantation experiment. Carry this out for all 20 animals.
3. As a control, transplant the 4-region of an untreated animal to 50% BL of an untreated host. Carry out this experiment for 20 animals.
4. Compare the number of animals in the treated and control samples that formed a second axis, or head.

Expected results. One would expect treatment with 2 mM LiCl to result in the formation of ectopic tentacles along the body column. Presumably this reflects a rise in head activation in the body column. If so, one would expect the fraction of transplants using LiCl-treated donors to form a higher fraction of 2nd axes than the controls. The first experiment has been done several times (e.g., Hassel et al., 1993). However, there are no published data concerning the second experiment.

QUESTIONS FOR FURTHER ANALYSIS

These three questions probe the nature and effects of the gradients a little further:

- What kind of grafting experiment would you carry out to demonstrate that the head produces the signal that sets up the head activation gradient?
- An early step during bud formation involves the initiation of head formation, or the formation of the head organizer. How would you show that the head inhibition gradient has a role in the initiation of bud formation?
- The head activation gradient is said to confer a polarity on the tissue in terms of a head forming at the upper end and a foot forming at the lower end of the body column. How would you demonstrate this polarity?

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APPENDIX: MAINTENANCE OF A HYDRA CULTURE

A total of 250–300 hydra will be needed to carry out all four experiments. The number of hydra needed per student will depend on the number of students as well as which experiments are selected. For practical purposes it is useful to obtain the hydra 3–4 weeks before they are used in experiments. In this way, the number can be increased to reach a level required for the class. When hydra are fed 3 times/week, the population size will double because of asexual reproduction by bud formation in 7–10 days. To obtain a faster doubling time, hydra can be fed 5 times a week. If experiments are to be carried out for a few weeks, it is worthwhile maintaining a culture containing enough hydra so that no more than 40% of them are used each week. With the indicated doubling time, this should permit maintenance of a steady-state culture of animals. In the following, the materials, equipment, and procedures for maintaining a hydra culture will be described. Any species of brown hydra is appropriate for these experiments. If available, a strain of *Hydra vulgaris* or *Hydra magnipapillata* is preferable as most of

the work on developmental gradients has been done with these species. *Hydra littoralis* is very closely related to these two species and will work equally well.

MATERIALS AND EQUIPMENT (PER CLASS OF STUDENTS)

50–200 hydra of a single species [*Hydra littoralis*: Carolina Biological Supply Co.].

Dishes for culturing hydra: 150-mm petri dishes (200 hydra/dish; Fisher Scientific), plastic containers, or glass baking dishes (1000 hydra/dish).

Hydra medium: 10 liters. Composition of hydra medium: 1.0 mM $CaCl_2$, 1.5 mM $NaHCO_3$, 0.1 mM $MgCl_2$, 0.08 mM $MgSO_4$, and 0.03 mM KNO_3, pH 7.5–8.0.

Pasteur pipettes and rubber bulbs for the pipettes (Fisher Scientific).

A one-liter glass bottle with rubber stopper and glass tubing for hatching brine shrimp cysts.

NaCl for hatching brine shrimp cysts: 40 g/liter (least expensive option is to obtain it from a supermarket or store for home aquarium supplies).

1000 × antibiotic stock solution: 2.5 g Penicillin (Sigma) + 2.5 g Streptomycin (Sigma) in 50 ml water.

1 can brine shrimp eggs: these are cysts (= desiccated fertilized eggs) of the brine shrimp, *Artemia salina* (Great Salt Lake Artemia Cysts; Sanders Brine Shrimp Co).

A one-liter beaker.

A light source (fluorescent light or incandescent light bulb).

Shrimp net: mesh attached to a circle of plastic (6–8 cm in diameter) attached to a plastic handle – similar to a net for catching butterflies, but smaller. Mesh should be fine enough to retain the hatched shrimp larvae (local store for fishing supplies or pet store).

Round glass bowl (~25 cm in diameter at top of bowl).

Container for hydra medium: 20 liters carboy with spigot (Fisher Scientific).

Fish tank air pump (this kind of pump is commonly used to bubble air into a small home aquarium, or fish tank, and is available in pet stores).

RAISING AND HANDLING HYDRA

In the laboratory, hydra are grown in any convenient transparent container with lids. These include 150-mm plastic petri dishes, plastic boxes, or glass baking dishes covered with a lid of available material. Plastic films such as Saran Wrap should not be used as they may be covered with a reagent or compound that dissolves in hydra medium and damages the animals.

Hydra medium. Hydra medium consists of a dilute salt solution (see Materials and Equipment for composition) made up in fresh water. Use tap water if it is free of high levels of compounds, such as chlorine, meant to reduce the level of micro-organisms. Otherwise, it is wise to use water that has undergone reverse osmosis, or is distilled. For convenience, it is useful to make up 5–20 liters of hydra medium at a time in a large plastic container with a spigot. Store at room temperature [15–25°C].

Handling of hydra. To transfer hydra from one dish to another, use a Pasteur pipette with a rubber bulb attached to the end. With such a pipette one can suck up one or

more hydra and some of the medium in one dish, and expel the animals into a second dish. If the hydra are floating in the medium simply use the pipette to suck them up and transfer them. If the animals are attached to the floor of the dish, one can detach them from the dish in three ways: (1) sucking them up directly; (2) expelling fluid at their feet forcing them to be released from the dish; (3) placing the tip of the pipette against the bottom of the dish next to the animal and gently pushing at the foot.

Growth conditions. Hydra are normally grown at 18°C in an incubator with light that is controlled by a timer. The light cycle consists of 12 h on and 12 h off. In case an incubator is not available, hydra can be grown in the laboratory as long as the temperature does not rise above 24–25°C. Above that temperature, the animals suffer, and exposure to 30°C leads to death within an hour. Exposure to lower temperatures [10–18°C] has no effect on the animals although they may grow more slowly at lower temperatures. The density of hydra per dish is optimally 1–2 animals/cm^2. If grown at double that density, it is difficult to keep the animals clean. Unclean animals become ill and damaged. When using 150-mm petri dishes, enough hydra medium should be used to fill to a depth of 10–12 mm.

FEEDING AND WASHING HYDRA

Hydra catch food with the nematocytes in their tentacles. When a piece of food, such as one or more shrimp larvae, bumps into a tentacle, nematocytes are discharged which capture and kill the larvae. Then the hydra moves the tentacle towards the hypostome, or mouth, and ingests the dead larvae.

Food for hydra. The simplest and most convenient form of food available for hydra is the hatched larvae, or nauplii, of the brine shrimp, *Artemia salina.* Embryos of *Artemia salina* in the form of stable dormant cysts are commercially available (see list of reagents). Once a can of cysts has been opened distribute the cysts to 50 ml or 100 ml plastic tubes with caps and store at 4°C. This will provide enough shrimp for several years without loss of viability.

Hatching of brine shrimp eggs. Dissolve 40 g NaCl in one liter of hydra medium in a one-liter glass bottle. To minimize bacterial growth, add 1 ml of the stock solution of antibiotics. Then add 25 ml of brine shrimp cysts. Firmly insert a rubber stopper containing two holes with a glass tube through one of the holes into the opening of the bottle. The glass tube should extend about 90% of the distance along the length of the bottle in the solution and several centimeters outside the bottle. Attach a rubber or plastic tube to the outer end of the tube and to a fish tank air pump. Let air bubble through the bottle for about 2 days at room temperature (15–25°C).

Collection of hatched shrimp. The hatched shrimp larvae will be bright orange while the unhatched cysts will be brown. To collect the larvae, pour the contents of the bottle into a beaker, and place the beaker on the lab bench. Then place a light source next to the bottom of the beaker. The shrimp larvae migrate towards light and will accumulate

near the light source. With a Pasteur pipette or regular pipette withdraw the larvae into the pipette. Transfer the larvae to a shrimp net with a mesh that is fine enough to retain the hatched larvae. Rinse the shrimp by holding the net under a faucet and letting water flow over the shrimp and through the net. Insert the net into a beaker containing hydra medium.

Feeding hydra. To feed animals, use a Pasteur pipette with a bulb to transfer hatched larvae to the dish containing hydra. Scatter the larvae around so that all hydra have a chance of catching larvae. The larvae will swim around until they run into the tentacles of the hydra and are captured. A good meal for a hydra amounts to 4–8 larvae. Larvae that manage to escape the hydra will swim around for less than an hour and die because they are salt water animals and cannot tolerate the freshwater environment of the hydra.

Washing hydra. Two to six hours after feeding, the animals are washed by pouring off the hydra medium and remaining shrimp into a round glass bowl. This also removes any food remains expelled by the hydra. To remove remaining larvae, rinse the hydra bowl with hydra medium and pour into the round bowl.

Most of the hydra will stick to the bottom of the culture dish, and are covered with a film of hydra medium. Add fresh hydra medium to the culture dish. Pouring the medium plus larvae into the glass bowl will most likely result in some hydra being carried along. Gently stir the medium in the bowl in a circular motion which brings all hydra into one place in the middle of the bottom of the bowl. Recover these animals with a Pasteur pipette, and return them to the culture dish.

2 Embryonic regulation and induction in sea urchin development

C. A. Ettensohn

OBJECTIVE OF THE EXPERIMENT Cell–cell interactions play an important role in the early patterning of animal embryos. Polarity inherent in the oocyte or established soon after fertilization entrains subsequent cell signaling events that subdivide the early embryo into distinct territories of gene expression and cell fate.

The objective of the experiments described in this chapter is to illustrate the role of cell–cell signaling in patterning early animal embryos. The sea urchin, a deuterostome that relies extensively on cell interactions to specify blastomere fates, is used as a model system. Two major experiments are described: (1) Analysis of the development of individual blastomeres isolated from early cleavage stage embryos, illustrating the phenomenon of regulative development. (2) Recombination of micromeres with animal blastomeres, illustrating the process of embryonic induction. In addition, a third experiment is described that involves the use of molecular markers (antibodies) to analyze cell fates, an approach that can be applied to either of the first two experiments.

DEGREE OF DIFFICULTY Experiment 1 requires students to collect sea urchin gametes, fertilize eggs, and dissociate embryos. All these skills can be learned relatively easily. Experiment 2 is moderately difficult as it involves micromanipulation, a technique that demands dexterity and patience. Both experiments require stereomicroscopes. The results of Experiments 1 and 2 can be assessed morphologically or by staining embryo whole mounts with antibodies that label specific tissue types (Experiment 3). The latter approach is technically straightforward but requires a compound microscope, preferably one equipped for epifluorescence.

INTRODUCTION

The earliest studies in experimental embryology involved splitting embryos into separate parts. W. Roux (1888) destroyed one blastomere of the 2-cell amphibian embryo