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Urodele (e.g., axolotl) embryos in the undergraduate laboratory class: an essay describing a multifaceted learning experience

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Introduction

During our one-semester Developmental Biology laboratory course for junior and senior level undergraduate students we experiment with a wide variety of living systems/embryos, including slime molds, sea urchins, *Drosophila*, *C. elegans*, chick embryos, and amphibian embryos. The two week session on amphibian (urodele) microsurgery draws special praise from students, for it provides one of the most *complete learning experiences* in our course. Among the various aspects of a well-rounded learning experience students encounter during the microsurgery exercise are the following:

Responsibility

Each student is given a small number of blastula stage embryos and access to incubators at various temperatures (e.g., 5°C to 25°C) at the start of this two week exercise. Students are responsible for nurturing the development of their own embryos up to stages which are appropriate for microsurgery.

Time management

Seldom can sufficient peace and quiet be obtained during the formal class meeting hours to permit the careful concentration required for successful surgical outcomes. As a university with an overpowering undergraduate social-life, our students must balance competing academic and social interests. Often, evenings and weekends provide the best opportunity for serious attempts at microsurgery. Thus, embryos must be timed by each student to match his or her work schedule.

Manipulation skills

Students prepare their own operating implements. Each is given a collection of Petri plates with agar bottoms, and we offer a demonstration on the preparation of micro needles (from Pasteur pipettes), ball tipped- micro probes, transfer pipettes, glass supports, etc. After our demonstrations (using golf ball-sized wax models) of alternative strategies to strip off tissue, to prop embryos up, to cut (but not too deeply) students are on their own. Some students acquire the necessary skills on their first or second try. A few will, regrettably, never master the requisite techniques. But each student is encouraged to develop a technique which "works for them".

Patience/discipline

For many undergraduate students, the goal of a (laboratory) course is to simply *hang in there, for in two weeks we are on to something else.* Not here. Since students are provided with adequate supplies of embryos (the embryos are constantly developing, mind you) and access to the laboratory at all hours, and above all, since the results are preserved in fixative and observed by an instructor , neither *delay* nor *escape* are possible. Often, even the most impatient of students will — after observing the success of classmates — exercise the requisite discipline, get into the proper state of mind, and "focus".

Perseverance

If at first you do not succeed, try and try again, is what we tell students who quickly get discouraged. Once the first successful surgical operation is recognized we often parade it around the classroom to illustrate that *yes, indeed,* ***we*** *are having success.* A sort of peer group awareness comes into play. As well, since many of the students are in the "pre-med" category, competitive drive often propels them to success.

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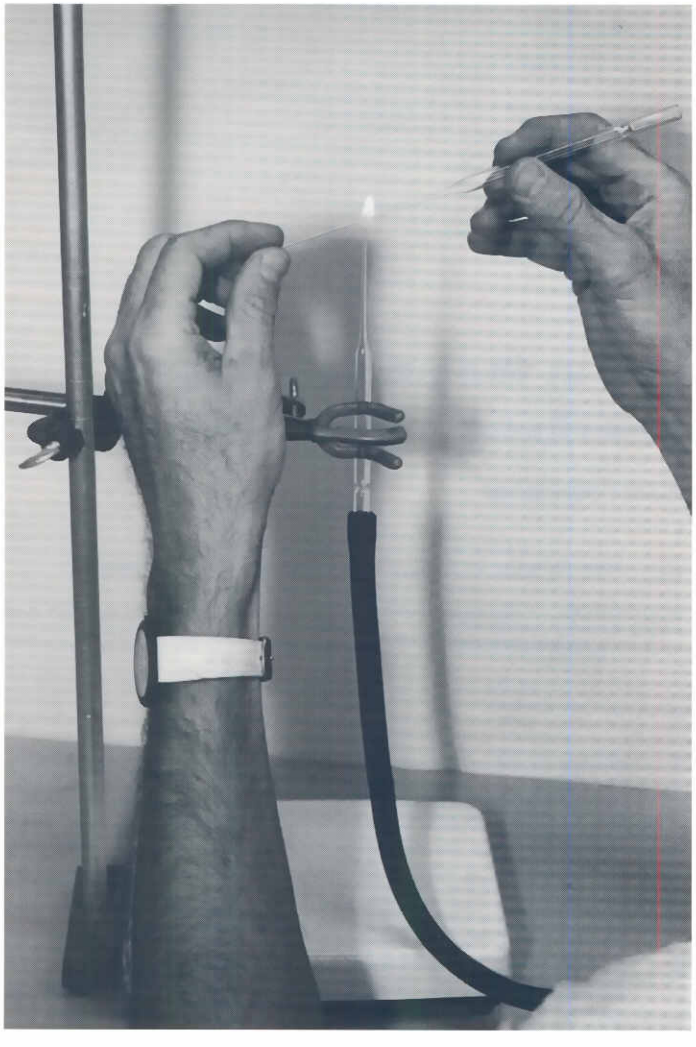

Fig. 1. Micro burner for preparing tools for microsurgery. *Coverslips can be crushed by wrapping them in a paper towel and squeezing them. Then they are soaked in 95% ethanol, and "flamed" as they are used. They can be gripped with forceps and used as blades for cutting, or they can be employed as "props/bridges/weights" to hold embryos or tissue pieces in place while healing occurs. Short (e.g., 4 mm) pieces glass rod can be bent into "L" shapes with the micro burner (Fig. 2), and used for similar purposes.*

Ingenuity/initiative

Since *no single* recipe for success is ever offered, students are encouraged to be creative. Often, novel ways of propping up embryos for parabiosis are, for example, developed. Likewise, interesting surgical tools (e.g., hooked glass needles) are frequently invented.

Cooperation

Our courses are structured using a "cooperative learning" format. Although students do many of the laboratory manipulations individually, once per week all students meet for a discussion hour. Divided into teams of six, they work problem sets and thereby get to know one another, and above all, develop communication bridges, which are useful in the practical laboratory exercises such as surgery. *Sharing is everything*, is our motto. Thus, students are encouraged to look over each other's shoulder to improve techniques.

Observation skills

Recognizing the appropriate stage for extirpation of a piece of neural fold, or determining *how deep to cut is not too deep* enhance the student's observational powers.

Accomplishment

Surviving embryos are fixed, examined, and scored by a laboratory instructor. Since most of the students achieve success with at least one of the three operations (extirpation/transplantation/parabiosis), and many succeed with all three, a temporary sense of relief, as well as a more lasting sense of "I actually did it — all by myself" permeates the laboratory. This is, in our opinion, good. As practising scientists are aware, the single most lasting reward for achievement in science is the sense of personal satisfaction obtained from completing a successful (original) exercise. For virtually all of these students, microsurgery is indeed an "original" exercise.

The protocols

Three surgical operations are performed: extirpation, transplantation, and parabiosis. The procedures and descriptions are based on a short series of exercises in Johnson and Volpe's *Patterns and Experiments in Developmental Biology* laboratory manual. This manual is now in its second edition (Leland G. Johnson; Wm. C. Brown Publishers, 1995 - ISBN 0-697-12303-0) and pp. 51-58 contain illustrations and nice explanations of amphibian microsurgery.

In addition, the appendix contains recipes for the various operating solutions. Our experience has led us to conclude that the choice of salt solutions for operating is not critical. Johnson and Volpe's protocols call for Barth and Barth's solution which of course works well. Other common solutions, such as 100% Steinberg's solution for operating, the same solution containing additional calcium/magnesium (2x) for healing, and 20% Steinberg's for further development work equally well, and are used in our class laboratory since we have concentrated (100x) stock solutions of them readily available from our research laboratories.

Readers lacking access to a copy of this book should request from the authors a Xerox copy of the relevant pages.

The intellectual content

Why are we doing this"? , some students will ask. They might furthermore exclaim: *"After all, aren't we in the golden era of biology, where isolating genes, creating transgenic animals, and knocking out gene functions, are* ***de rigor***"?

We therefore precede the actual laboratory exercises with an explanation of some of the purposes for which extirpation/transplantation/parabiosis are carried out by contemporary researchers. These include — among others — the following:

Extirpation

Useful for learning about the cellular reprogramming which drives wound healing and tissue (and organ?) regeneration. A key experimental strategy employed by contemporary researchers involves removing tissue or appendages and monitoring the cellu-

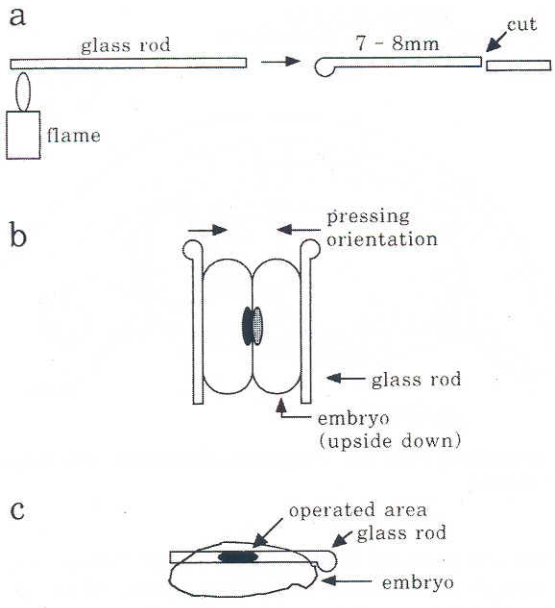

Fig. 2. Preparation (a) and use of glass micro rods for surgery. *Embryos for parabiosis* **(b)** *are tucked together by gently pressing the rods against the two embryos. Healing of grafted tissue is facilitated by pressure of rod* **(c)**. *Finally, it is often useful to melt the tip of a Pasteur pipette into a ball and while still hot, use it to burn a well into the agar surface of the operating dish for holding the embryo(s) during surgery/healing.*

lar and molecular events associated with the development of replacement components. The long term goal of such studies is to understand fundamental aspects of cell/tissue plasticity and to exploit that knowledge to design medical strategies for promoting tissue re-growth.

Transplantation

Useful for learning about cell and tissue fates. For example, the cues which establish cell migration pathways are not fully understood. One of the key experimental strategies involves transplanting migrating cells to novel locations in the embryo in order to determine the extent to which local environmental components (such as the extracellular glycoprotein matrix which coats cells) regulate pathway selection.

Parabiosis

Useful for establishing the general features of a newly discovered mutant gene. Several informative outcomes are possible when a mutant embryo which is destined to die due to the action of its altered gene is joined (via its circulatory system) to a wild type embryo: both members of the parabiotic pair survive, indicating that the mutant embryo lacks a circulating component which can be provided by the wild type embryo; both embryos die, indicating that the mutant embryo secretes a toxic component into the common circulatory system, and thereby blocks an essential function in both embryos; the mutant embryo dies, without interfering with development of the normal co-twin, indicating that it is unlikely that the mutant gene product enters the circulatory system.

The above *simplest explanation* scenarios serve as a stepping stone for further discussion and *enhanced intellectual pursuit* in the collaborative learning sessions held weekly. For example, the concept of *a gene product being necessary but not sufficient* can be explained and discussed, and the notion that *data which can be explained in a formal way may not make sense in an intuitive fashion* can be expounded upon.

Thus, those seemingly "old-fashioned" experimental manipulations provide an opportunity for a multi-faceted manipulative/observational/intellectual experience for the undergraduate student. For the professor, however, it might be a different story. Please see below.

The "information content vs process" conflict

Driving recent spectacular advances in developmental biology has been an increased emphasis on data collection. Conceptualizing sets of interrelationships, and attempts at formulating unifying theories are often viewed these days as less important than elucidating new facts about this or that phenomenon. Hence, the profound publicity associated with the race to isolate a novel gene, or the accolades associated with the publication of the 3-D structure of a "key" protein are easily understood.

Yet that data, and the *facts* which derive from it, comprise the domain of *history*. Science, in contrast, is best viewed as the domain of "evidence". That is, science specializes in explaining "why we believe this or that fact to be true". Thus, with extirpation/transplantation/parabiosis the teacher is presented with an excellent opportunity to discuss "evidence". For example, students might be asked "*how many grafts* need to be performed in order to prove a point"?; or "*how to score* a regenerated appendage which is only partially complete"?

Teachers frequently, however, emphasize the "information content" associated with a discipline such as developmental biology. The most popular textbooks represent encyclopedia, and teachers often — in lecture courses — "recite" the equivalent of an abridged version of a verbal encyclopedia, week-in, week-out, desperately trying to make it to the marathon-like finish line at the end of the semester, but being certain to "cover all the most recent discoveries" before they actually cross that line.

Justification for such an approach to teaching is abundant: "if students don't learn all the terms (vocabulary) they won't be able to go on to the next higher-level course"; "if the facts are not understood, the phenomena they are associated with will not make sense"; and (often most importantly) "if I, as instructor, don't display a plethora of facts, students will not respect me as an authority figure in the classroom"!

The following two quotes further explain features of the "information content vs. process" conflict:

From p. 4 of vol. 7 (February, 1994) of The Howard Hughes Medical Institute Bulletin (on undergraduate education) the following statement is excerpted:

...argued in the meeting's keynote address that biology educa-

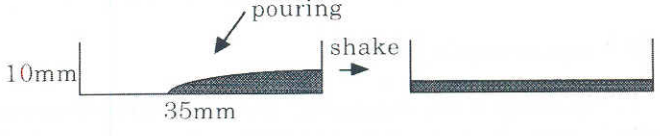

Fig. 3. Casting agar in shallow dishes. *Melted agar solution is poured into 35 mm x10 mm Petri dish to a depth of approx. 3 mm, to cover approx. 2/3 of the bottom of the dish. Then the dish is gently shaken to distribute the molten agar, before it begins to solidify.*

tors have known for decades that the traditional method of teaching the life sciences, with its heavy reliance on rote memorization, is inadequate. Recent critiques of science education reflect those from the 1940's and even earlier in calling for less emphasis on imparting facts and more on helping students master the scientific method.

From an anonymous source:

...scientists have a positive attitude toward problem solving. They consider a problem to be a challenge, an opportunity for new experiences, and enrichment of the repertoire of tools for thinking, a learning experience. With a positive attitude, a frustrated effort to identify a solution is deemed to be compensated for in great measure by the lessons that can be learned when no solution is found. Creative people view an obstacle in a problem-solving situation as a challenge, an intellectual and emotional adventure. Creative people do not run away from complex situations. They tolerate complexity, uncertainty, conflict, and dissonance. They enjoy new experiences. They are more active than passive, and they have capacity for producing results. They are doers. They seem to be in control. They radiate self-confidence.

The former statement emphasizes "evidence", which is what extirpation/transplantation/parabiosis are all about. The latter statement, which emphasizes "process", is revealing in our context: it is relatively easy to understand how performing those surgical exercises as an undergraduate would cultivate in a young student the character traits of a successful scientist.

The experimental material

The laboratory manual mentioned above employs anuran rather than urodele embryos as experimental material. That is likely because of the relative ease with which common laboratory frogs (e.g., *Xenopus*) can be spawned, or the availability of *Rana (pipiens)* from commercial supply houses. Urodeles, however, offer more important advantages for the undergraduate student. These include the following:

(1) large size of embryos (making tissue cutting easier for the beginner)
(2) slow developmental rate (so students can work slowly and methodically)
(3) wide temperature tolerance (for regulating developmental rate)
(4) gigantic neural folds (for convenient extirpation)

Thus, the success rate for undergraduate students is much higher for urodele (e.g., axolotl) embryos than for anuran embryos. There are, of course, some disadvantages to axolotl (i.e., urodele) embryos. These include the following:

(1) availability of embryos depends on access to large animal colonies
(2) embryos must be manually de-jellied (chemical reducing agents such as cysteine-HCl are not effective)
(3) the slow developmental rate mentioned above can prolong the laboratory exercise beyond the two weeks usually allotted

The Surgical Implements

As mentioned in the Introduction, students prepare their own surgical tools. Each student is provided with a pair of sharp watchmaker's forceps (necessary for removing the vitelline membrane) and supplies of glass Pasteur pipettes, small diameter (approx. 1.5 mm dia) glass rods, and glass microscope cover slips (for breaking into "knife blades"). Cutting needles and ball-tipped

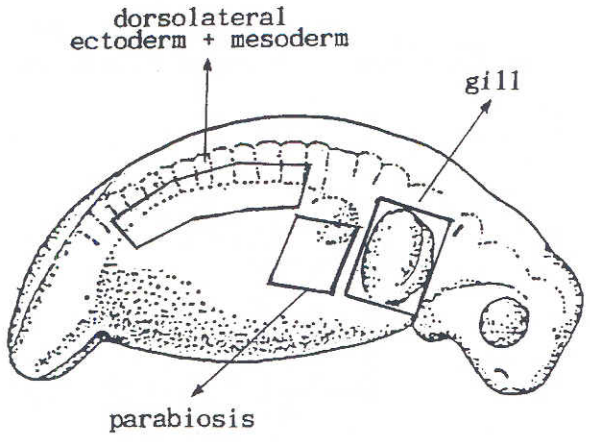

Fig. 4. Suggested areas for extirpation illustrated on a stage 26-28 axolotl embryo.

glass rods are prepared by heating on a micro burner. The micro burner is itself prepared from a Pasteur pipette. Its flame can be precisely adjusted and (contrary to intuition) the burner tip (pipette) never melts. A *micro burner* is indispensable for it allows beginning students to draw extra thin needles, without needing much practice. Figure 1 illustrates this inexpensive piece of equipment.

The Do's

Do have as many embryos available as possible. Since urodele (e.g., axolotl) development (from early cleavage onwards) can be virtually stopped by incubation at 5C, they can be maintained for several weeks if necessary. Some students will achieve success with 10 embryos, while others will require twice as many (or more).

Do use micro burners of the type illustrated in Figure 1, for students become encouraged to experiment with tool design. By using "ready-made" tungsten needles instead, students miss the opportunity to be creative with tool design (Fig. 2).

Do de-jelly urodele embryos (manually) while still in a spherical shape (i.e., before the completion of gastrulation), for afterwards (tailbud stage onwards) embryos are frequently damaged as they extrude from tears in the vitelline membrane.

Do include antibiotics in operating solutions. Infection is rampant in surgeries which lack adequate antibiotics. We routinely use 0.01% Penicillin, 0.01% Streptomycin, and 0.01% Gentamycin in our solutions.

Do hold back (preferably in a secret hiding place) extra embryos, in case of catastrophe (e.g., tray of surgeries is dropped on the floor; incubator overheats; etc.).

The Don'ts

Don't let students cut too deep (e.g., into the archenteron) for any of the surgeries. Large gashes which expose the archenteron usually do not heal properly.

Don't cast agar surfaces which are so deep that there remains too little space for air, once the dish is flooded with operating solution and covered with the Petri plate top. Embryos will suffocate, due to lack of oxygen, if the fluid — by capillary action — seals the Petri dish top to the sides of the bottom-half of the dish. Figure 3 illustrates a convenient method for casting agar-bottomed dishes.

Don't permit embryos to contact the fluid/air interface in the

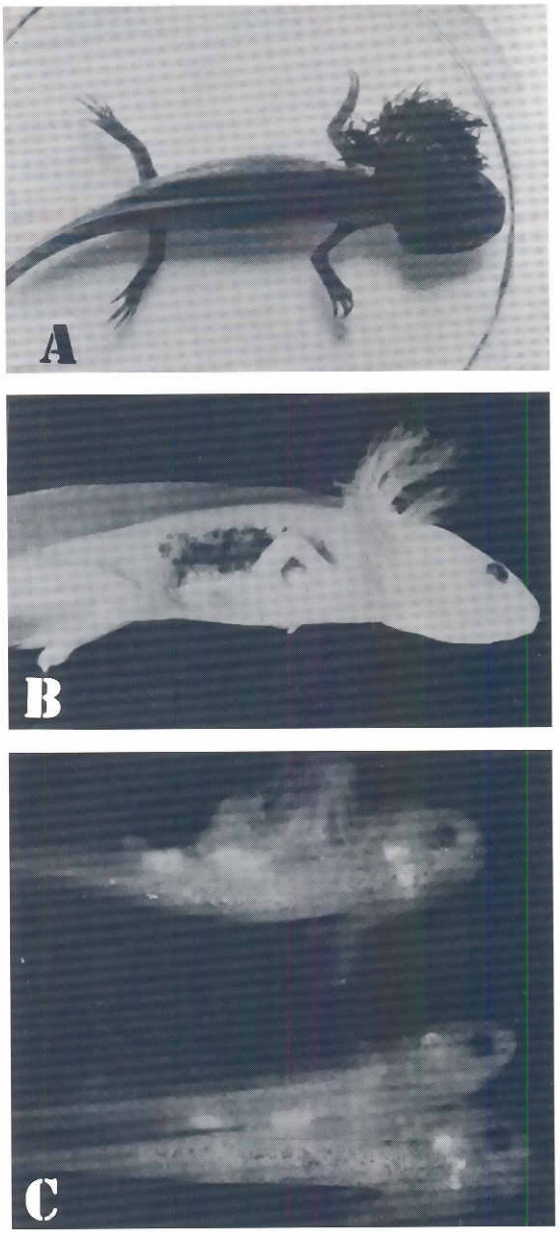

Fig. 5. Examples of results of surgical manipulations. (A) *Extirpation of gill primordium shown after five months. The right-side gill primordium was extirpated. During five months of subsequent growth and development it was not replaced (1.2x).* **(B)** *Transplantation of dorsolateral ectoderm plus mesoderm shown after 6 months. A tissue patch from a normal dark (D/D) embryo was transplanted to a white (d/d) host (1x).* **(C)** *Parabiosis between a normal and lethal mutant embryo shown two months after a cell-autonomous lethal mutant (l/l) embryo was attached to a normal partner embryo (top). There was no rescue of the mutant embryo. Instead, the mutant embryo degenerated and its tissue was absorbed by the normal partner. The bottom parabiont pair represents a control operation, since both partner embryos were wild type, and both developed normally (1.6x).*

operating dish. Surface tension sometimes causes an embryo from which its vitelline membrane has been removed to split open. This is especially the case for embryos which have already been cut.

Don't allow students to attempt surgical manipulations while standing up (e.g., at a standard height laboratory bench). Arm fatigue causes unsteadiness. Encourage students to sit on a stool (at a bench), or on a chair (at a standard height table).

Bon Voyage

The following three operations offer many possibilities for individual initiative on the part of the student. Below are offered some suggestions and comments. Figure 5 illustrates results.

Extirpation of various organ primordia

Figure 4 illustrates the position of various organ primordia which are easily extirpated by students. Two outcomes are, in principle, possible. Either the extirpated tissue is reconstituted by cells which surround the "removed area", or "wound healing" occurs without the development of a replacement organ. An advantage to performing these particular extirpations is the fact that the healed embryo retains its body form, and thus indirect effects on body morphogenesis such as distortion or twisting which might arise from removal of a neural fold or muscle mass are avoided.

Transplantation of neural folds

A successful result from this operation is most easily recognized when pigment markers are employed. For example, with axolotl embryos wild type and white (*d/d*) embryos can be employed. The neural fold area from the right side of a white embryo can be removed and discarded. Then a similar area is removed from a donor (e.g., dark) embryo. It should, however, include a slightly larger piece of tissue than the total area of the extirpation site so that should the edges of this donor tissue curl up before it is implanted, it will still fully fill-in the host's wound. This tissue patch is then put into place on the host. With a glass rod laid gently over the dark implanted tissue (see Fig. 2) healing is facilitated. Within 1-2 h (room temperature) the glass rod can be removed.

Parabiosis

First, a shallow groove large enough to accommodate two embryos side-by-side should be made in the agar of an operating dish. Then in that same dish surgically remove an area of ectoderm and subjacent mesoderm just posterior to the gill bulge (see Fig. 4) from both embryos. Be careful that the gill rudiment is not disturbed, for urodeles such as the axolotl require functional gills for development. Place the two embryos in the groove, ventral side up, and gently press them together with glass rods (see Fig. 2). It is important that the wound sites be closely pressed together, for if a gap in the flank tissue which is uncovered by epidermis develops, bacterial infection is likely to result.

The glass rods can be removed after healing (several hours), and the embryos should be sufficiently well healed to one another after 2-3 days to permit transfer to a larger bowl.