Chapter 9

Microsurgical Operations on the Giant Ciliate *Stentor coeruleus*

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Introduction

By demonstration and video tapes in which you are looking down the microscope tube at microsurgical operations in progress, you are prepared for your own practice of microsurgery on this ciliate. *Stentor coeruleus* has proved to be microsurgically the most operable of all cells. Any number of cells and cell parts can be grafted in any arrangement, posing new challenges to the morphogenetic capabilities of this unicellular organism. The functional nucleus (macronucleus) can be removed or transplanted, increased or decreased to investigate the relationships between nucleus and cytoplasm.

The expectation is that the study of morphogenesis in a single-celled animal, by avoiding the complexities of multicellular interactions, will lead us sooner to an understanding of organic form development, the least understood of all aspects of organisms.

Aside from the preparations, the operations require very little time; in fact, if unsuccessful at once, it is better to start over afresh. And within a day or less the living specimens will show how they respond to what stentors have never been confronted with before in nature. Because *S. coeruleus* is unicellular and is self-pigmented with a pattern of blue-green surface stripes, staining and sectioning are not needed to see how the animals respond or what they will do.

The first period will be used in preparing special micropipettes for handling the stentors individually and in drawing out glass needles for cutting and grafting. The second period of half a day is to practice operations. Mechanical micromanipulators are not used because they are slow, expensive, and do not permit both coarse and fine movements in succession.

For this technique, each student will be provided with a stereoscopic dissecting scope, embryological (spot) lamp, depression slides, and moist chamber for keeping specimens in these slides.

Instructor's Materials

Tools

First we will prepare unbreakable micropipettes for picking up stentors individually one by one. Snip off about 3 inches of fine plastic catheter tubing (obtainable from surgical suppliers) and hold it across a small gas burner to soften (Fig. 9.1.A). The degree of heating is critical. If too cool the tubing breaks when pulled, and if too hot it melts and collapses. One can expect to spoil a dozen pipettes before one gets the knack. When good tubes are drawn they are trimmed off to proper length and diameter. About 1½ inches of narrow bum-rubber tubing is plugged with a piece of glass rod at one end and slipped to make a bulb which will not be over-responsive to pressure of the

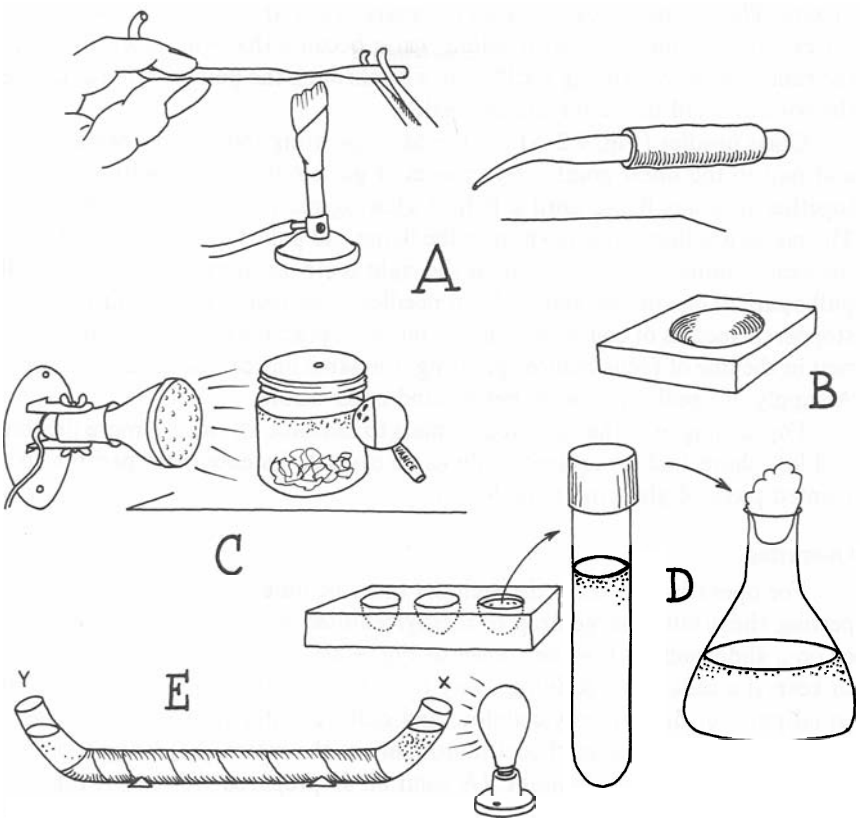

Figure 9.1. Equipment for culturing stentors.

A. To the right: micropipette with polyethylene tip, rubber tubing "bulb", and glass rod plug; as well as fine wire (bent) used when cleaning. To left: drawing out polyethylene catheter tubing over wing-top burner for pipette tips.

B. Glass block cell containing 1 ml in which specimens are clearly visible.

C. Culture in jar with hole punched in cap, examined briefly with spotlight and magnifying glass to follow development of a culture.

D. Development of clones. Single stentor first introduced into one cell of deep depression slide; transferred to test tube when multiplied to about 25 animals; transferred again from hundreds in the test-tube to a cotton-plugged Ehrlenmeyer flask. Filtered culture medium plus culture of food organisms used throughout.

E. Migration tube for obtaining clean stentors. Main body of half-inch diameter tube is covered with black plastic electrician's tape and filled with clean water. Concentrated *S. coeruleus* introduced at (x) will migrate away from lighted end and are recovered, clean at other end (y).

fingers. These pipettes can be used for years. They are flushed out with clear water after use, but never with boiling water because that will soften and bend the tube. Clean by twirling a stiff, fine wire through the points. Wipe and save the wire in a pill bottle for use as needed.

Glass needles (Fig. 9.2.A) are the best operating tools since they are stiff and pull to the finest points. Two pieces of glass rod are held with two ends together in a gas flame until a ball of glowing molten glass forms between. The pieces are then withdrawn from the flame and pulled quickly apart. Again, the exact temperature is crucial; at the right temperature the molten ball will pull apart to one or two short, sharp needles to be rested in upright one-hole stoppers. Needles of course will break, but with practice in locating the needle first in the line of focus before operating, the same one can be used for a week. A supply of needles prepared beforehand is advisable.

For turning over the operated animals to examine all sides, a more flexible and less sharp tool is required, such as an eyelash fastened with paraffin to a pointed piece of glass rod (Fig. 9.2A).

Operating

For operating on them the stentors must be quieted. This is done by pipetting them into a large drop of methylcellulose solution on a regular slide or on a slide such as the one shown in Fig. 9.2E, which has a fabric coating to keep the cells from skidding away from the needle. The solution is about equal parts of fluffy methylcellulose and culture water from a pond or well. In the beaker, wet the methocel thoroughly in the water, just bring to a boil (froth) and let stand overnight. (A solution so prepared will be provided to students). This solution is not a chemical anaesthetic, but is simply so viscous that the stentors are unable to push through it.

Two or three animals are placed in the drop so that if one misses on the first operation, others will be available. Place the slide on the stage of a dissecting scope with side-arm rests (or use books). I use a stereomicroscope without base or mirror because the instrument then stands low and the bench itself gives full support to the arms during delicate operations with the needle (Fig. 9.2C). Reflected light from a sharp spot or embryological lamp is best for seeing the animals in depth, instead of silhouetted against transmitted light. Adequate heat filters between light and object are needed in prolonged operations.

Begin by cutting a stentor in two, the simplest operation to demonstrate its regeneration. Pipette a large stentor into a big drop of the methocel solution on an ordinary slide. Then divide it with a transverse cut with a needle—transverse because most of the nucleus is located along the length of cell on one side, so nucleate fragments are assured.

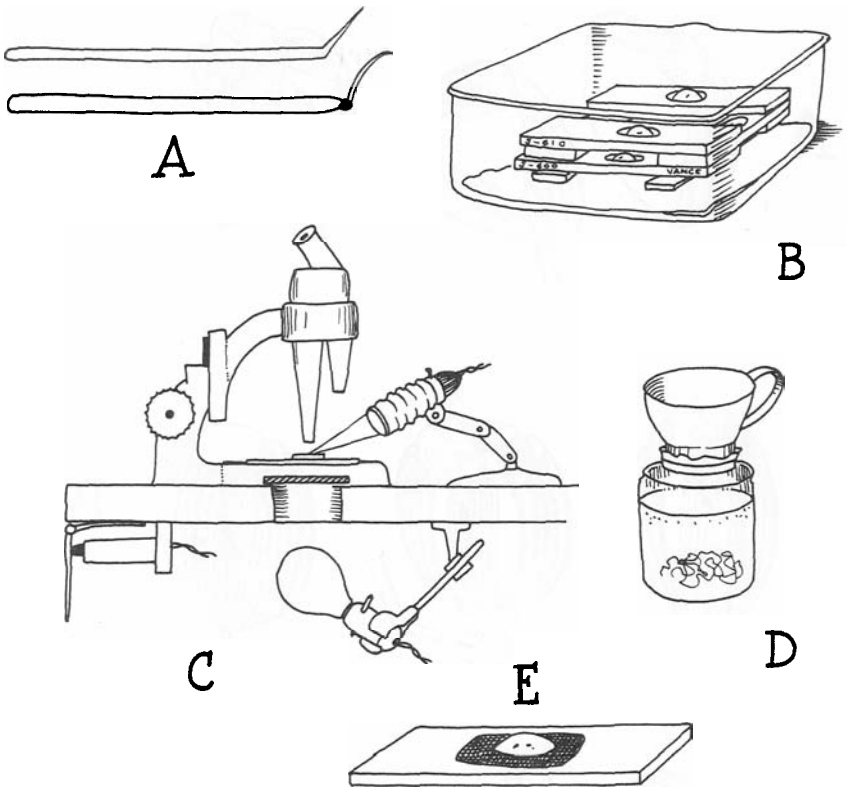

Figure 9.2. Equipment for operations on stentors.

A. Glass needle drawn from soft glass rod for cutting; eyelash fastened to handle for rolling over specimens to examine all sides when following operated animals.

B. Moist chamber, a plastic sandwich box with wet filter paper on the bottom and depression slides stacked on 2 bridges.

C. Bench for operating. As he bends over microscope, operator automatically presses hinge at edge which turns on spring-switch and embryological lamp. Bench top used for arm rests. Ordinary blue light below hole in bench is used in searching culture samples by transmitted light. Both sources of illumination have glass heat filters.

D. Canning funnel covered with drum-head of fine bolting silk and immersed in culture jar, for maintaining large fusion masses under optimum condition.

E. Operating slide to which a square of finely woven fabric is applied with melted paraffin carries large drop of methyl cellulose into which stentors have been introduced with the micropipette.

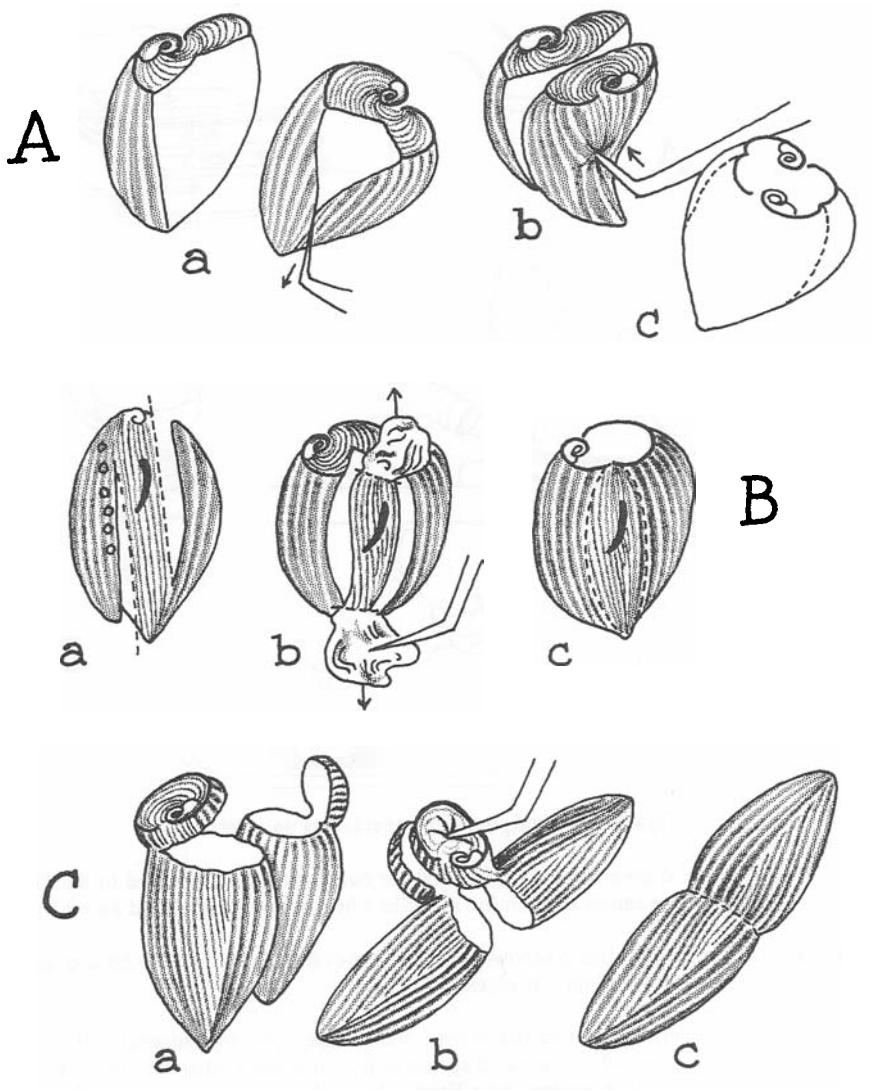

Figure 9.3. Grafting operations.

A. Producing a doublet. a: Two stentors are split down the back with sharp needle and opened wide to expose the endoplasms. b: With a blunt needle (broken tip) animals are oriented and one is pushed against the other, exposed endoplasms pressing together. c: Doublet stentor resulting.

B. Implanting a cell sector. a: Cuts made from both ends to isolate the primordium sector with or without nuclear nodes leaving cell remnants at each end. b: Host split open and graft put in place, either homopolar or heteropolar. Posterior remnant

Quickness and flexibility are essential in these operations. After gently moving the stentor into position the proper cut is made. With practice the needle can be precisely "located" under the microscope so that in time breakage becomes infrequent. After cutting at high magnification, the objectives are shifted to lower power and the specimen removed to a depression slide with a large drop of filtered culture medium to wash off the methylcellulose. Washed specimens are transferred into two large drops of filtered culture medium in a depression slide. The code number of the experiment can be written on the edge of the slide and the slides laid out or stacked in a moist chamber (Fig. 9.2B).

To complete themselves, the tail piece has to regenerate a head, and the head piece needs to regenerate a tailpole and holdfast. Both need to regain the normal proportions and shape (Morgan 1901). Do they? How quickly is a tail pole reconstructed? Can you find on the posterior piece the oral primordium which regenerates a new head or set of feeding organelles?

We now proceed to graft two stentors together. One stentor is split pole to pole and opened out flat on the slide with the exposed endoplasm uppermost (Fig. 9.3A). Then another is cut likewise, and it is pushed at once onto the first, thrusting the two naked endoplasms together. A slightly blunt needle will accomplish this without impaling the cells. In a moment adhesion will occur, and it will spread so that all endoplasm (unpigmented) is enclosed. But the organisms are not yet fully healed together and can be shifted on one another for whatever orientation is desired, e.g. homopolar or heteropolar. If homopolar, a doublet may be produced, a 2–mouthed stentor which can reproduce a culture of doublets.

Other operations are possible. Fig. 9.3B shows how a strip bearing an oral primordium is grafted into the split-open "back" of another stentor. Notice that the dangling pieces of the first stentor are impaled with the glass needle to orient the strip on the host cell and to start its adhesion there. In Fig. 9.3C is shown how two stentors are grafted end-to-end heteropolar. The two heads are cut into "flip-tops" and pressed together for a melding which spreads to the major parts. Afterwards these heads are excised.

Macronuclear transplantations and enucleations are done on starved animals with few food vacuoles to obscure the nuclear nodes. Also, dark-field

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Figure 9.3.—*Continued*

pushed into slit to fuse, then each remainder pulled as indicated to orient graft as fusion extends to it. Then cell remnants excised. c: Graft in place; in this case its anlage will be caused to resorb by the non-differentiating host.

C. Head-to-head telobiotic. a: Heads of two stentors cut but left attached to cell bodies. b: Underparts of heads, with exposed endoplasms thrust together, then excised as fusion spreads to the main bodies. c: Resulting telobiotic.

illumination shows up the nuclear chain. This is accomplished by placing the specimen slide on a dark-paper background and illuminating with the spot lamp from the side. As the specimen is first cut open the nodes will be even more clearly visible. All or most of the nuclear nodes can be removed or, conversely, several strips bearing most of the nuclear chains can be fused to greatly increase the proportion of nuclear material. Pieces of the nodal chain can scarcely be removed without a coating of endoplasm and by this they may be grafted into enucleated stentors of the same or even different species. In this way compatibilities and incompatibilities among species-different cytoplasms and nuclei can be investigated. (Fig. 9.4).

The points to remember are that these operations *can* be done because they have been done, and that with prolonged practice manipulative skills are gradually developed. On the first try, you would not expect to repair your watch, yet there are many watch-repairers who trained for the task.

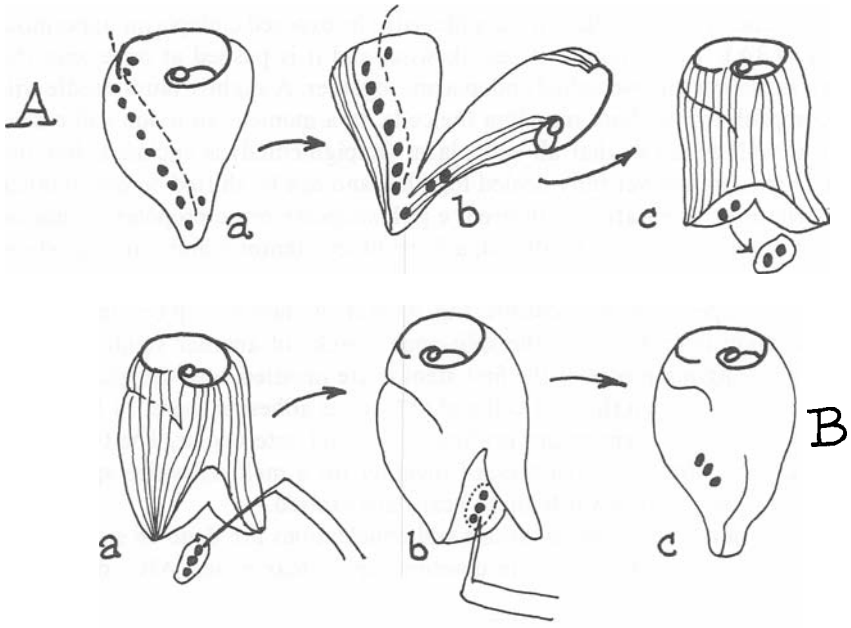

Figure 9.4. Enucleation and renucleation.

A. a: Incision to enucleate *coeruleus* without disturbing feeding organelles and with minimum loss of cytoplasm. b: After cell is laid open, margins with macronuclear nodes are excised or nodes teased out. c. As specimen heals together, posterior end is opened to cut out remaining nodes obscured by carbohydrate reserves.

B. In renucleation with nodes from same or a different species, enucleated host is split open when endoplasmic sac with nodes is available (a): the sac is broken against host wound, the endoplasms fusing. Nuclear nodes are then securely inside (c).

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APPENDIX A
Collecting Stentors

Stentors are most likely to be found in large, permanent ponds or lakes, but they also live in streams; and the outflows of sewage plants are not to be neglected. The collector may equip himself with a set of cream cans of two-quart capacity and a kitchen strainer fastened to a long handle. Keeping in mind that stentors are usually attached, one gathers into the can with minimum disturbance samples of submerged and floating vegetation, such as duckweed, *Spyrogyra* material and dead cat-tail leaves. More vegetation is then scooped up with the strainer and gently wrung out into the can until it is nearly filled. Attached and loosened stentors are likely to be gathered in this way, with sufficient natural medium to start a culture. Location of each sample as a possible source should be noted on the container so that one can return to a source year after year and find the same species of *Stentor.*

Returning to the laboratory, the lids are removed from the cans to expose them to the air. They are left to stand for half a day, but no longer. In this time the stentors will swim to the top. If allowed to stand longer an injurious putrefaction may set in, or worms and water fleas may take their toll, for stentors do not appear to be a dominant form like paramecia or hypotrichs, and persisting cultures are usually not obtained by simply letting the samples stand. After a few hours, stentors, if present, will be found near the surface where they are gathered by pipetting along the miniscus and agitating the floating vegetation and debris. A scraping action with the tip of the pipette when water is being sucked up will serve to loosen stentors which have become attached. This material is transferred to a caster dish or other shallow container and examined for stentors under low powers of a stereomicroscope. If stentors are found the whole sample container may then be rotated for gentle agitation and more samples poured out. A portion of the original sample is then passed through filter paper of medium porosity which will remove all large forms and pass only minute organisms on which stentors can feed, and this natural medium can then serve for the starting of cultures.

If stentors cannot be collected in the field they may be obtained in mixed culture from several biological supply companies.

The next step is to select stentors out of the sample dishes, leaving competitors and predators behind. For this purpose micropipettes are necessary. Sample dishes are searched and individual stentors picked up and transferred to glass block cells, one for each species desired. About 50 stentors of a kind should be isolated, if available, and the isolation dish should then be surveyed, this time to pipette out any contaminating organisms that may have been carried over with the stentors. Block cells or their equivalent are recommended because in them no organisms escapes from view (Fig. 9.1B).

APPENDIX B
Culturing Stentors

I shall now describe my method of setting up cultures, though this is not the only, nor possibly the best procedure. A half-pint, wide-mouthed jar is filled to a depth of about one inch with the filtered pond water. A large pinch of polyester "cotton" (supplied for aquarium filters or for filling quilts) is then pulled apart to form a loose mesh and dropped in. This material is a non-rotting substitute for pond vegetation. The isolated stentors are then washed into the jar with a squirt of filtered pond water. One drop of skimmed milk, one or two boiled wheat, or Bulgar (cracked wheat cereal), or rice grains or a fragment of a rabbit-food pellet is then added as a source of nutrients, producing a population of bacteria and tiny flagellates and other food organisms from the original pond water which passed through the filter paper. In this way as many seeding stentors as obtainable are returned to the same water from which they came. Only about 100 ml of starting culture is set up in order that the stentors may themselves possibly regulate the medium to their liking. Very little nutrient is at first added, in proportion to the few stentors present.

Progress of the starting culture can then easily be followed by placing the jar briefly in front of a bright spotlight and examining with a magnifying glass (Fig. 9.1C). At the end of a week, if the stentors are multiplying, more nutrient is added, at first only a drop or two of skimmed milk, but only if the water has become clear. If turbid with uneaten flagellates and bacteria, the jar is let stand another week before nutrifying. Since milk is a complex mixture forming a nearly perfect food, it serves as a good basic nutrient and tonic medium for stentors and a variety of other protozoa, including of course the food organisms (Tartar 1950).

As the stentors increase in number more lake or other natural water which has been passed through a Millipore filter to remove all protozoa and their cysts is introduced from a stock jar, with a little more "cotton". Eventually the culture jar will be filled to the top and can be nutrified once a week with 5 or 6 drops of skimmed milk. (Cream content would form a film on top and exclude the air.) From the beginning the jar is covered with its original cap, in the center of which a hole is punched with a large nail or ice pick. The cap prevents contamination and evaporation, and the hole allows gaseous exchange (Fig. 9.1C).

Such cultures will remain in thriving condition for many months. If removal of detrimental cohabitants was unsatisfactory, or if hypotrichs, nematodes, etc. should later infest the culture, one has to begin again, treating the culture as if it were a pond sample and isolating stentors as before. A cardinal precaution is never to over-nutrify the culture so that a distinctly putrid condition arises. In the course of months the stentors may diminish in abundance in spite of the regular additions of milk. When this occurs it is assumed that the water should be changed. Since the stentors are mostly attached to the sides and the fibers, the whole jar can be gently emptied, or the "cotton" can be retained, and then immediately filled with filtered water. In the meantime the stentors have remained attached to the sides and are protected by a fluid film. In spite of some loss there will probably still be enough animals to handle the large amount of new water. One may want to add less milk now until the animals become plentiful. A continued source of food organisms will of course have been retained in the film adhering to the emptied jar. It is well to have three or four jars of the same stock. These can be developed by splitting the contents of one jar between two and refilling both jars to the top with filtered lake water, adding more "cotton" as needed.

These procedures may not appear elegant but they have served to maintain healthy stock animals in more than sufficient abundance for my microsurgical operations continuously for 8 years, during which not one of 10 stocks has died out.

Genetically more uniform material is assured by developing clones or cultures derived from a single individual. This is best done after a good culture of the wild stock is obtained, for one can then use filtered water from the culture itself as a starting medium and be sure of its optimal nature.

To obtain concentrated animals one can gently shake the flask cultures to loosen stentors attached to the sides and pour the contents into graduate cylinders. At first the oxygen will be uniform throughout, the stentors (at least *coeruleus*) will rapidly sink to the bottom in mass, and the overlying fluid can be decanted. If it is now desired to free these animals from most of the food organisms one may take advantage of the speed with which most races of *coeruleus* swim away from the light—or perhaps the reverse in the case of green *polymorphus* and *niger*. Whiteley introduces the concentrated animals at the lighted end of a large, horizontal, covered tube with both ends bent upward and filled with Millipore-filtered medium (Fig. 9.1E). Stentors soon migrate to the lighted end, leaving the slower bacteria and food organisms behind, and are promptly removed for study.

Stentors are to be provided with food organisms. Very likely stentors can accumulate and ingest bacteria, but the eating of larger organisms should be more efficient. The following organisms have been observed to be eaten and digested by stentors: *Colpidium; Blepharisma; Paramecium bursaria; Minoidium,* and other colorless flagellates; small rotifers; *Chilomonas; Halteria; Tetrahymena; Glaucoma; Gonium,* and other colored flagellates. For further details see Tartar (1961).