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Hydrostatic skeletons and muscular hydrostats

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1. Introduction

1.1 Description of hydrostatic skeletal support

A hydrostatic skeleton is a fluid mechanism that provides a means by which contractile elements may be antagonized (1). Hydrostatic skeletons occur in a remarkable variety of organisms with examples not only from invertebrates but also from vertebrates. A hydrostatic skeleton is typically considered to include a liquid-filled cavity surrounded by a muscular wall reinforced with connective tissue fibres. This form of hydrostatic skeleton is seen, for example, in cnidarian polyps, holothuroid echinoderms, echinoderm water vascular systems, many molluscan bodies and organs and annelid, sipunculid, nemertean, and nematode worms (for reviews see refs. 1–7). Comparison of a variety of hydrostatic skeletal support systems shows that the extent and volume of the liquid-filled cavity is variable. In particular, recent work has identified a number of hydrostatic skeletons, termed muscular hydrostats, that consist of a tightly packed three-dimensional array for muscle fibres (8, 9). Examples of muscular hydrostats include the arms and tentacles, fins, suckers and mantles of cephalopod molluscs, a variety of molluscan structures, the tongues of many mammals and lizards, and the trunk of the elephant.

1.2 Arrangement of muscle fibres and movement in hydrostatic skeletons

The basic arrangement of muscle fibres in vermiform hydrostatic skeletons typically includes both circular and longitudinal muscle fibres. The hydrostatic skeleton provides a means by which these two muscle fibre orientations can antagonize one another, producing a variety of movements including elongation, shortening, and bending. The function of the hydrostatic skeletal support system relies on the fact that the enclosed liquid-filled cavity, typically a coelom, is constant in volume. Any decrease in one dimension must therefore result in an increase in another. Thus, to create elongation of the body,

the circular muscles contract, decreasing the diameter and thereby increasing the length. Shortening of the body involves contraction of the longitudinal musculature. As the body shortens, its diameter must increase, with the resulting restoration of resting length in the circular muscles. The longitudinal and circular muscles are therefore antagonists.

In addition to the basic circular and longitudinal muscle arrangements, other muscle fibre arrangements are observed and allow additional types of movement. Four general categories of movement are possible: elongation, shortening, bending, and torsion. Elongation requires muscle fibres that are arranged such that their contraction decreases the diameter of the body or organ. In addition to the circular muscle fibres discussed above, two other muscle fibre arrangements create elongation: transverse muscle and radial muscle. Transverse muscle fibres extend across the diameter in parallel sheets and are seen, for example, in mammalian tongues, and the arms and tentacles of octopus and squid (8, 10). Radial muscle fibres radiate from the central axis in planes perpendicular to the long axis. Examples of radial muscle arrangements are observed in the elephant trunk and in the tentacles of the chambered nautilus (8, 11).

Shortening occurs as a result of contraction of longitudinal musculature and provides a means by which the muscle responsible for elongation may be antagonized. The relation between the diameter and length of a constant volume hydrostatic system allows amplification of the displacement or the force produced by the musculature responsible for elongation and shortening. This amplification is analogous to that produced in hardened skeletal elements in which joints and lever arms provide for leverage. For example, in a hydrostatic body or organ that is initially elongate—that is, one with a high length/diameter ratio—a relatively small decrease in diameter results in a large increase in length (see refs. 8, 10, and 12 for details). This means that the displacement and velocity generated by the musculature responsible for elongation is amplified. This amplification is significant, for example, in hydrostatic organs that are rapidly elongated such as the tentacles of squid, or in organs that are protruded over long distances such as the tongues of lizards and snakes (8, 9). Conversely, if the cylinder has a low length/diameter ratio, then a relatively small decrease in length creates a large increase in diameter. In this case force rather than displacement is amplified. This type of amplification may be of importance, for example, in anchoring individual segments of burrowing metameric worms. In the cases described above, the mechanical advantage of the antagonistic musculature is opposite; that is, if the hydrostatic skeletal system provides for amplification of *displacement* by a group of muscles, then the *force* of the antagonists will be amplified.

Bending movements require contraction of longitudinal muscle along one side of the body or organ. The bending moment is greatest if the longitudinal muscle is peripherally arranged—that is, located as far from the central axis

as possible. In order for this longitudinal muscle contraction to create bending, some component of the body or organ must resist the longitudinal compressional force that would otherwise cause shortening. In some hydrostatic skeletons, connective tissue fibres provide resistance to longitudinal compression (see below) while in others, muscle provides the resistance. Since any decrease in length due to a longitudinal compressional force must result in an increase in diameter of a hydrostatic system, muscles arranged to control diameter (circular, transverse, and radial) can provide the resistance to longitudinal compression required for bending (8). Note that these are the same muscle arrangements that produce elongation. In the case of bending, however, these muscles operate synergistically with the longitudinal muscles rather than antagonistically.

The final category of movement in hydrostatic skeletons is torsion or twisting around the long axis. The muscles responsible for this movement are arranged in helical layers around the body or organ. For torsion in either direction to be possible, both right- and left-handed helical muscle layers must be present. The torsional moment of these muscle layers is maximized if the layers are located toward the outer surface, as far from the central axis as possible. Indeed, the helical musculature that has been observed in hydrostatic skeletons typically wraps the remainder of the more central musculature. The fibre angle (the angle that a helical fibre makes with the long axis of the structure) of the helical muscles affects the forces that are exerted on the hydrostatic system as the helical contracts. If the helical muscles are arranged at a fibre angle of 54°44′, then their contraction will generate a torsional force without affecting the length or diameter of the cylinder. If, however, the fibre angle of the helical muscles is greater than 54°44′, then their contraction will create both a torsional force and a force that will tend to decrease the diameter and thereby increase the length of the cylinder. If the fibre angle is less than 54°44′, contraction of helical muscles will create both a torsional force and one that tends to shorten the cylinder. Helically-arranged muscle has been observed in the arms and tentacles of cephalopods, in some lizard tongues, and in the elephant trunk (8, 10, 11).

1.3 The role of connective tissue fibres in movement and changes in shape

Connective tissue fibres often play a crucial role in the determination of the range and type of movements possible in hydrostatic skeletons. The most prevalent arrangement of connective tissue fibres in hydrostatic skeletons is that of the crossed-fibre helical fibre array in which sheets of connective tissue fibres are arranged in both right- and left-handed helixes, wrapping the hydrostatic body or organ. The mechanical implications of these relatively inextensible fibres have been analysed for a number of vermiform hydrostatic

skeletons. The fibre angle of the connective tissue fibres plays a crucial role in determining the range of shape change that is possible. This has been explored with a geometrical model of a cylinder wrapped with a single constant length helical fibre. The model compares the enclosed volume as a function of the fibre angle (angle that the fibre makes with the long axis of the cylinder). As the fibre angle approaches 0° and 90° the volume of the cylinder approaches zero. A maximum volume occurs between these two extremes at a fibre angle of 55°44′. This model and various elaborations of it have been applied to the analysis of a number of hydrostatic skeletal support systems including, for example, nemertean, turbellarian, and nematode worms and the tube feet of echinoderms (13–17). These studies have demonstrated the importance of the crossed fibre array in controlling the range of shape possible in these systems.

In addition to crossed fibre helical arrays, other arrangements of connective tissue fibres have also been shown to serve important roles in hydrostatic skeletal support systems. In the mantle and fins of squid, connective tissue fibres are observed to be embedded in the tightly packed musculature of these structures, with the connective tissue fibres at an angle to the muscle fibres. These intermuscular connective tissue fibres control changes of shape, provide for muscular antagonism, and may serve in elastic energy storage, increasing the efficiency of movement (18–23).

1.4 Analysis of hydrostatic skeletal support

In hydrostatic support systems, the arrangement of the muscle and the connective tissue fibres determines the types of movement, range of movement, and changes of shape that are possible. Features such as connective tissue fibre orientation and three-dimensional course of muscle bundles have significant impact on mechanics and function. Thus, analysis of hydrostatic skeletal support systems requires a detailed analysis of the morphology of the musculature and connective tissues at a microscopical level. Much of this chapter will be concerned with the techniques used in morphological analysis at this level. Further analysis of hydrostatic skeletons requires direct documentation of the sequence and duration of muscle activity. The most convenient method is that of electromyography, reviewed in this volume by Gans (Chapter 8). Special electrode techniques are often required for electromyography of hydrostatic skeletons and are described below. The force produced by contractile activity of the musculature is transmitted as changes in pressure within hydrostatic skeletons and thus, techniques for measuring pressure of hydrostatic skeletons are important and are described below. Analysis of movement is also required and Chapter 3 in this volume, by Biewener and Full, provides details of kinematic analysis. A convenient method for kinematic analysis of aquatic animals is also described below; it is of particular use in the study of hydrostatic skeletons.

2. Morphological analysis

2.1 Introduction

Because the form of many hydrostatic skeletal systems is relatively poorly studied, one of the first steps in their analysis is a complete and detailed morphological study. This analysis involves a mixture of classical and recent morphological techniques. Although a technique such as paraffin histology might be assumed to have little relevance in today's world of DNA technology, it actually provides critical data concerning the microanatomy of hydrostatic skeletal support systems. Furthermore, today's students are in fact more likely to be familiar with modern molecular biological techniques than with morphological techniques. Therefore considerable attention will be devoted to the relative advantages and disadvantages of various morphological techniques, and to details concerning their use. The focus of this discussion will be on techniques useful for the analysis of structure at the light microscopic level. Electron microscopical analysis of hydrostatic skeletons is a more specialized application and a detailed description of the technique is beyond the scope of this chapter. Excellent descriptions of electron microscopical techniques are available (24).

The most useful techniques for morphological analysis of hydrostatic skeletons at the light microscopic level are sections of tissue embedded in paraffin or in glycol methacrylate (GMA) plastic. The choice of technique depends on both the specimen and the level of analysis. Tissue embedded in GMA plastic is subject to greatly reduced distortion and artefact (relative to paraffin) and can be sectioned at 0.5–3.0 μm, providing morphological detail that may rival low power electronmicroscopical analysis. Nevertheless, paraffin histology still serves an important role in morphological analysis. Most hydrostatic skeletal support systems are relatively large. In order to accurately evaluate the morphological components, it is typically necessary to section serially the structure in three mutually perpendicular planes. This is often the only way to obtain a grazing section and therefore observe the critically important connective tissue fibre components of the structure. Because paraffin sections are typically made at 5–10 μm, fewer sections are required in order to section through an entire structure, and thus there are fewer sections to mount and stain and fewer slides to mount coverslips on. For considerations of general microanatomy and tissue component arrangement, the additional resolution provided by plastic is unnecessary. Furthermore, paraffin forms serial ribbons with ease. Ribbons greatly facilitate the production of serial sections; successive sections are aligned relative to one another, making it a simple matter to affix them in the proper order on microscope slides. The resulting linear alignment also aids in observing the sequence of sections on the microscope. Although there are techniques that allow the production of serial ribbons with glycol methacrylate, in my experience it is much more difficult to obtain ribbons

with plastic. Assembling individual sections in a complete series on slides is a difficult, time-consuming, and painstaking process.

In addition to sectioning considerations, paraffin offers some distinct advantages in terms of staining of tissue components. A vast array of staining techniques are available for paraffin sectioned material. Those provided below are several of the ones that the author has found to be particularly useful. In particular, brilliant and beautiful staining techniques are available that show dramatic differentiation between various tissue components, especially between connective and muscle tissue. These stains are invaluable for an initial morphological analysis. Some of the techniques originally developed for paraffin can be adapted for staining of glycol methacrylate plastic sections but the results are often unpredictable.

After the microanatomy and arrangement of tissue components of the entire structure or body has been examined with paraffin sections, additional morphological detail in specific areas can be provided by GMA sections. Distortion and shrinkage is minimal in GMA embedded material, in part because heat is not required during processing of the tissue. In addition, the thinner sections obtained with the harder plastic embedding material allow much higher resolution of specimen detail (25, 26). Low-melting-point polyester wax (27) can also be used to reduce distortion and shrinkage due to heat but it is often difficult to adhere polyester wax sections to slides. Coating the sections, once on the slide, by dipping in a 0.1% solution of pyroxylin in 50% ether/50% ethyl alcohol helps to prevent them from floating off in the solutions but increases staining times considerably. In general, it is best to perform the initial morphological study with serial paraffin sections and then to use GMA sections for additional resolution of morphological details. This approach allows one to exploit the advantages of each technique.

Frozen sections of unfixed material are sometimes useful in providing an initial morphological survey of connective tissues, particularly if being viewed with polarized light microscopy. Nevertheless, a complete analysis with serial sections is usually required in order to analyse all tissue components and it is therefore more convenient to bypass frozen sections and begin with serial paraffin sections.

In the procedures that follow, several general considerations should be borne in mind. Most of the steps in processing tissue for plastic or paraffin embedding rely on diffusion. Difficulties experienced in these methods can usually be traced to lack of sufficient infiltration of the tissue by one component or another. Thus, one should always attempt to minimize the size and maximize the surface to volume ratio of the specimen. Often, it is possible to trim a specimen into a 'slab' rather than a cube. This strategy provides a full-size block face but greatly reduces diffusion distances. In addition, it is useful to augment diffusion with convection, typically by placing the vial of solution with tissue in a tissue rotator. In situations where rotating the specimen is impractical, one should consider the specific gravities of the component to be

removed from the tissue compared to that of the bathing solution and suspend the tissue accordingly. For instance, if the component to be removed is more dense than the bathing solution, the exchange will be augmented if the tissue is suspended in the bathing solution rather than resting on the bottom of the vial.

2.2 Fixation

The choice of fixative depends on the intended embedding procedure. With the advent of preparative techniques for electron microscopy, a variety of fixative procedures have been developed that provide remarkable preservation and minimize distortion, shrinkage, and artefact. These procedures, however, are generally applied to small blocks of tissue and thus, penetration of the tissue by the fixative is less of an issue. For the relatively large blocks of tissue that must be fixed for the examination of the morphology and microanatomy of hydrostatic skeletons by paraffin embedding, the more classical and simple fixatives seem to be superior because many are excellent in terms of penetration of the tissue. In addition, many of the fixatives developed for electron microscopy include glutaraldehyde as a component and glutaraldehyde often interferes with the staining procedures used for paraffin embedded tissue. For paraffin embedding, considerations of fixative osmolality seem to be less important, because the distortions and shrinkage induced as a result of the heat during the embedding procedures are more significant than distortions due to osmolality differences. For embedding in glycol methacrylate, the fixatives developed for electron microscopy are superior. These give excellent preservation with little distortion and since the tissue blocks are generally smaller, their reduced penetration is less of a problem. Adjustments to fixative osmolality may improve preservation. Below are several fixative recipes for paraffin and glycol methacrylate embedding.

Since the goal of fixation is to preserve the tissue in a state that is as close as possible to that of living tissue, care must be taken to minimize post-mortem changes. It is thus necessary that the tissue be fixed as soon as possible after death, preferably in chilled fixative. Perfusion of the tissue with fixative is a help in many cases. In most cases narcotization is required. For marine animals, a 1:1 mixture of sea water and 7.5% $MgCl_2 \cdot 6H_2O$ is excellent. Other useful narcotization methods can be found in Pantin (28) and Humason (29). The comments above concerning diffusion and surface to volume ratio are of particular importance during fixation. As a general guide-line, a fixative volume to tissue volume ratio of 10:1 or greater is recommended.

A comment concerning formalin is necessary at this point. The term 'formalin' refers to a saturated solution of formaldehyde in water, typically 36–38% formaldehyde. Fixation protocols refer typically to concentrations of formalin rather than formaldehyde. When preparing fixatives, one simply considers formalin to be 100% and then makes the appropriate dilutions. If formalin is stored for extended periods, polymerization and oxidation of formaldehyde

occurs. The presence of a white precipitate suggests that the formalin will be less effective.

2.2.1 Buffered formalin

For initial morphological analysis, this simple fixative is an excellent choice. It works well with paraffin embedding and staining procedures. Substitute sea water for distilled water for marine specimens. If fixing marine animals in the field where simplicity of procedure is desirable, excellent results can be obtained with 10% formalin in sea water without the sodium phosphate.

The action of formalin is progressive, that is, fixation improves over time and the tissue can be left in fixative for extended periods. Fix for a minimum of 48 hours.

- formalin 100 ml
- distilled water (sea water, if marine) 900 ml
- sodium phosphate, monobasic ($NaH_2PO_4 \cdot H_2O$) 4.0 g
- sodium phosphate, dibasic (Na_2HPO_4) 6.5 g

2.2.2 Alcoholic Bouin's (28)

For tissues difficult to penetrate, for example dense muscular tissue, Bouin's fixative often works well. The shrinkage that occurs during fixation can be considerable and thus, it is useful only for paraffin embedded material. It cannot be used for organisms where preservation of calcareous inclusions is required because they are dissolved by the acid. Fix for 24 hours but remove soon after because tissue left in Bouin's tends to become over hardened. After fixation, transfer to 95% ethyl alcohol. Because picric acid can interfere with staining, the alcohol should be changed until most of the excess picric acid is removed.

- picric acid 1.0 g
- acetic acid 15 ml
- formalin 60 ml
- 80% ethyl alcohol 150 ml

2.2.3 Phosphate-buffered glutaraldehyde (30)

This is an excellent general fixative for use with GMA embedding procedures. Although the preservation is excellent and little distortion occurs, the fixative penetrates poorly. Tissue blocks should not be thicker than 1–2 mm. It is useful to maintain a 1.0 M solution of sodium chloride if you wish to modify the osmolality of the fixative. The 1.0 M solution can be diluted to the appropriate strength and used in place of the 0.34 M solution in the formula

below. Fix for 2–4 h. After fixation, rinse 15–30 min in a 1:1 solution of 0.4 M phosphate buffer and 0.6 M (7.01 g in 200 ml water) sodium chloride.

• 25% glutaraldehyde	5 ml
• 0.34 M sodium chloride (3.97 g in 100 ml)	20 ml
• 0.4 M Millonig's phosphate buffer (see below)	25 ml

Millonig's phosphate buffer (0.4 M):

• sodium phosphate (monobasic)	11.08 g
• sodium hydroxide	2.85 g
• distilled water to make	200.0 ml

2.3 Decalcification

Tissue with mineralized inclusions must be decalcified prior to dehydration and clearing. Although a variety of recipes for decalcifying solutions are available in histology texts, the decalcifying solutions sold commercially that include chelating agents are excellent and convenient. It is essential that the tissue be washed in several changes of water following decalcification. Following the wash, one can then proceed directly to the dehydration series.

2.4 Dehydration and clearing

Since water and paraffin are not miscible, the tissue must be completely dehydrated before it can be embedded. Additionally, since dehydration is most easily performed with ethyl alcohol, which is also not miscible with paraffin, an intermediate 'clearing' agent must be used that is miscible in both alcohol and paraffin. Poor histological results can most commonly be traced to a failure to completely dehydrate and then remove alcohol from the tissue. In the past, xylene was employed commonly as a clearing agent. In response to concerns about the health hazards associated with the use of xylene, non-toxic clearing agents have been developed. These clearing agents (for example, Histoclear, National Diagnostics, Somerville, NJ, USA; Hemo-De, Fisher Scientific Products, Pittsburg, Pennsylvania, USA) can be substituted for xylene with no change in procedure, seem to cause less hardening of the tissue, and provide excellent staining. Their use is highly recommended.

The amount of time required in each step of the dehydration and clearing process varies from tissue to tissue and must therefore be determined by experimentation. For tissue blocks 3–5 mm on a side, the author uses 1-hour steps of the following series as a starting point (all steps on tissue rotator): 30% ETOH, 50% ETOH, 70% ETOH, 95% ETOH, 100% ETOH (3 changes), 50/50 100% ETOH/clearing agent, clearing agent (2 changes). Tissue fixed in alcoholic Bouin's will be in 95% ETOH and can be started in the series at the 100% ETOH step. After the second clearing agent bath, transfer the tissue to

a vial filled with paraffin chips and clearing agent and allow the tissue to remain overnight before beginning the infiltration series in the oven the following day. These times can be shortened considerably in some instances.

Dehydration for embedding in glycol methacrylate plastic is usually performed in a graded ethyl alcohol series. Complete dehydration is unnecessary, however, since glycol methacrylate is miscible with water. No clearing agent is required. Shorter times are required than those listed for paraffin above, because the tissue blocks are typically smaller. As a starting point, 30-min steps of the following series are recommended: 30% ETOH, 50% ETOH, 70% ETOH, 95% ETOH. After the 95% ETOH step, the tissue can be transferred directly to unpolymerized GMA. See below. One can also eliminate the alcohol series and transfer the tissue directly to several changes of glycol methacrylate. This use of glycol methacrylate as the dehydrating agent is more expensive than dehydration in alcohol but yields superior results in some instances.

2.5 Infiltration and embedding

Successful embedding requires complete infiltration of the tissue with molten paraffin or unpolymerized GMA. In the case of paraffin embedding, complete removal of the clearing agent is necessary. This is accomplished by transferring the tissue through several baths of molten paraffin. As a starting point, three paraffin infiltration baths, 1 hour each should be tried. It is convenient to keep the paraffin baths arranged and labelled sequentially in the oven in order to ensure that the final infiltration bath contains as little clearing agent as possible. A vacuum oven is useful because reduced pressure is often required for complete infiltration of the tissue with paraffin.

The commercially available paraffin embedding media (for example, Paraplast Plus, Monoject Scientific, St Louis, Missouri, USA; Ameraffin, Baxter Healthcare, American Scientific Products Division, McGraw, Illinois, USA) work well and include additives that are claimed to aid sectioning and infiltration. Care should be taken in setting the paraffin oven because temperatures more than a few degrees above the melting point are said to reduce the effectiveness of the additives.

Due to the use of paraffin embedding in pathology, a variety of embedding aids and supplies are available. The most useful are disposable plastic embedding boats and embedding cassettes and moulds. The disposable embedding boats are available in a variety of sizes, can be labelled with indelible markers and reduce time in trimming the solidified blocks. The embedding cassettes and moulds have all of the advantages of the disposable boats and, in addition, eliminate the step of mounting the block because the cassette can be clamped into the microtome directly. Mould release compounds are available that help to free the solidified block from the mould.

Once the tissue has been placed in the molten paraffin in the mould and

oriented properly, heated forceps are used to release all bubbles from the tissue. The paraffin should then be solidified quickly by placing the mould in a water bath. Blocks that solidify slowly in air do not section as well.

Successful GMA embedding requires complete infiltration of the tissue followed by complete polymerization of the GMA monomer. Three infiltration steps of 30 min duration on a tissue rotator are recommended. Two aspects of the polymerization are of particular importance: heat and oxygen. For most GMA formulations, oxygen inhibits the polymerization and must therefore be excluded during polymerization. In addition, the polymerization is exothermic and, in larger blocks, the excess heat produced can create distortions in the tissue similar to those produced by the heat of paraffin embedding. Butler (31) describes a simple chamber that can be purged of oxygen during polymerization and is easily constructed. The chamber incorporates an aluminium plate and water bath that serves to conduct heat away from the blocks during polymerization. As an alternative, Bennett *et al.* (25) recommend polymerization in an oil bath. In addition, embedding moulds and stubs that include heat sinks are available for GMA embedding. Recently, the author has obtained excellent results using a GMA formulation (Historesin, Reichert-Jung, Cambridge Instruments, Deerfield, IL, USA) that polymerizes in the presence of oxygen.

2.6 Sectioning

2.6.1 Tissue embedded in paraffin

With tissue that has been fixed and embedded carefully, serial paraffin sections of 5–10 μm are readily obtained. Humason (29) includes an excellent trouble-shooting chart for paraffin sectioning. If the tissue is embedded in a disposable mould, the mould is peeled away from the solidified block and the block is then mounted with molten paraffin on the appropriate microtome tab. With cassettes, the block is simply popped out of the mould. The block can then be trimmed with a razor blade. It is critical that the upper and lower sides of the block (that is, those parallel to the knife edge) be trimmed as close to parallel to one another as possible. If they are not trimmed parallel, ribbons of serial sections may be more difficult to obtain and the ribbons of sections will curve, making it difficult to mount as many sections on a slide and less convenient to study the series on the microscope. It is helpful to trim the block as a pyramid with the block face (plane of section) as a trapezoid (*Figure 1*). The trapezoidal shape aids in sectioning and provides a reference for the sequence of sections in a ribbon. The pyramid is useful because it allows retrimming of the block as sectioning proceeds, which aids in correcting non-parallel or damaged block sides.

The choice of knife for paraffin sectioning is somewhat subjective. A well-sharpened steel microtome knife will cut excellent sections, but honing and stroping a knife by hand is an art. Automated knife sharpeners work quite well and the disposable abrasive sheets (Thomas Scientific, Swedesboro, NJ,

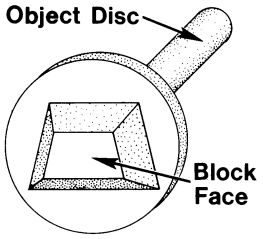

Figure 1. Diagram illustrating convenient method for trimming paraffin blocks. A block mounted on an object disc is shown. The object disc is clamped in the jaws of the microtome during sectioning. The block is trimmed as a pyramid with the block face (the plane of section) shaped as a trapezoid.

USA) that are now available reduce the mess and inconvenience of liquid honing compounds. A convenient alternative is a blade holder with disposable blades. Many of the disposable blades sold for the holders cut excellent sections and one simply replaces the blade when dulled or nicked. Glass Ralph knives (see below) can also be used for paraffin sectioning, although one must experiment with the profile of the edge to obtain good results.

Once a paraffin block has been sectioned, the ribbons of sections must be adhered to microscope slides. Care must be taken in cleaning the slides, otherwise the sections may float off during the staining procedures. Soaking the slides in acid alcohol (1% hydrochloric acid in 70% ethyl alcohol) does an excellent job of cleaning the glass surface. The clean, dry slide can then be coated with a thin layer of Mayer's albumen which is most conveniently obtained from commercial sources (Fisher Scientific Products, Pittsburg, Pennsylvania, USA). Paradoxically, the less albumen applied to the slide, the better the adherence of sections to the slides. Adhesion can also be provided by using 'subbed' slides (29), although the author has found albumen to be more suitable, and in preparing serial sections finds it most convenient to place water on the surface of the albumen-coated slide with an eye dropper and then to place the ribbon of sections on the drop of water. The slide can then be transferred to a hot-plate set at 40–50°C. The heat causes the sections to expand, removing much of the compression created during sectioning. Once the sections have expanded, excess water can be drained off the slides, and the sections can be precisely positioned with a brush or forceps. The slides are then allowed to dry on the hot-plate for approximately an hour. The heat is necessary for adhering the sections to the slides. The slides should be labelled clearly. Slides frosted on one end have been found to be most useful because they are easily labelled with pencil or India ink.

2.6.2 Tissue embedded in GMA

Sectioning of tissue embedded in GMA plastic requires a retracting microtome; that is, a microtome with a mechanism that moves the block to the side or

away from the knife on the return stroke. Several manufacturers sell retracting microtomes designed specifically for GMA sectioning. If a project will involve extensive GMA sectioning, purchase of one of the GMA microtomes is recommended. Alternatively, small GMA blocks can be sectioned on an ultramicrotome. This is often an inexpensive solution because ultramicrotomes that have reached the end of their useful life for ultramicrotomy will nevertheless do an excellent job of cutting the much thicker GMA sections.

Glass knives work well for sectioning GMA blocks. For block faces smaller than 6 mm, triangular glass knives (31) are excellent. In addition, these knives are usually readily produced because most electron microscopy facilities include mechanized knife breakers that automate the production of triangular glass knives. These knife breakers allow one to produce quickly glass knives that are of excellent quality for GMA sectioning. The mechanized knife breakers can also be used with thicker glass strips for use with block faces as wide as 9 mm. For even larger block faces, the best results are obtained using Ralph knives. Ralph knives can be broken by hand as described in Bennett *et al.* (25). Alternatively, a number of mechanized Ralph knife breakers are now available that provide for more convenient and consistent production of Ralph knives. The author has had little success in adapting steel knives or disposable steel blades to the sectioning of GMA blocks. Nevertheless, steel knives designed specifically for GMA are now available and may be worth investigating.

Sectioning of GMA is usually done with a dry knife. It is helpful to lift and hold the bottom edge of the section and to maintain slight tension on the section as it comes off of the knife edge. This procedure helps to reduce folding of the sections. The sections are then transferred to the surface of a water bath where they flatten. Folds in the sections can usually be avoided by dropping the section on the water surface carefully. Once the section is floating on the surface, folds can sometimes be removed by probing with an eyelash or brush. Flattened sections are picked up on cleaned slides by inserting the slide at an angle through the water surface underneath the section and lifting the slide. Before removing the slide from the water, the position of the sections can be adjusted with a brush. The slide is then dried on a hot plate at 60 °C. The sections adhere to clean slides without application of any adhesive (25, 26).

2.7 Staining

A tremendous variety of staining procedures are available that allow identification of various tissue and cellular components in paraffin embedded tissue (28, 29). For the study of hydrostatic skeletons and muscular hydrostats, howver, the most useful are those that clearly differentiate muscle and connective tissue. The routine stains used by pathologists (for example, Hema-

toxylin and Eosin), are of little use in this regard. K. Smith (Duke University) and I have tried a variety of stains and have found several excellent protocols that give beautiful results and provide clear differentiation of muscle and connective tissue. These protocols are highly recommended and are listed below.

Staining of paraffin sections typically involves aqueous stains. The paraffin must therefore be removed from the sections using the clearing agent (2 changes, 5 min each), the clearing agent must be removed with 100% ethyl alcohol (2 changes, 5 min each) and then finally, the alcohol must be removed and the sections rehydrated (95% ETOH, 70% ETOH, 50% ETOH, 30% ETOH, distilled water: 5 min each). Since examination of hydrostatic skeletons and muscular hydrostats requires serial sections, these steps and the staining process itself, involve many slides. It is thus most convenient to use $8 \times 10 \times 8$ cm glass staining trays with glass carriers that hold multiple slides. A number of slides can be processed quickly and variation in staining between slides is reduced.

Bennett *et al.* (25) provide protocols and information on staining of sections embedded in glycol methacrylate plastic. The most useful and convenient is Lee's methylene blue–basic fuchsin stain for GMA sections (*Protocol 1*). The thinner sections possible with GMA embedding appear to be much less intensely stained than thicker sections. One must therefore become accustomed to studying sections that show weaker staining effects (25).

2.8 Staining protocols

2.8.1 Milligan trichrome for paraffin sections, adapted from ref. 29

This is an excellent stain for hydrostatic skeletons because it clearly differentiates muscle (magenta) and collagen fibres (blue with aniline blue and green with fast green). Nuclei stain magenta and red blood cells stain orange to orange red.

Protocol 1. Preparation of stock solutions

1. Mordant

(Mix 3 parts of solution A with one part solution B below; use within 4 h)

Solution A, Mix:

- potassium dichromate (carcinogen-mix in hood) 3.0 g
- distilled water 100.0 ml

Solution B, mix:

- hydrochloric acid, concentrated 10.0 ml
- 95% ethyl alcohol 100.0 ml

2. Acid fuchsin, mix:
   - acid fuchsin (C.I. 42685) 0.1 g
   - distilled water 100.0 ml
3. Phosphomolybdic acid solution (1%), mix:
   - phosphomolybdic acid 2.0 g
   - distilled water 200.0 ml
4. Orange G, mix:
   - orange G (C.I. 16230) 2.0 g
   - 1% phosphomolybdic acid 100.0 ml
5. Aniline blue (fast green may bė substituted for aniline blue)
   Stock solution, mix:
   - aniline blue (C.I.42755) 10.0 g
   - 2% acetic acid (2 ml/98 ml distilled water) 100.0 ml

   Working solution, mix:
   - aniline blue stock 10.0 ml
   - distilled water 90.0 ml

Protocol 2. Staining procedure for Milligan trichrome stain

The following procedure includes staining times that the author has found to work well, but experimentation may be necessary depending on the tissue, fixative, etc. Consult Humason (29) for additional information.

1. Deparaffinize and transfer slides through 100% ethyl alcohol into 95% ethyl alcohol.
2. Mordant slides in potassium dichromate–hydrochloric acid solution for 5 min.
3. Rinse in distilled water.
4. Stain in acid fuchsin: 30 sec.
5. Rinse in distilled water.
6. Fix stain in phosphomolybdic acid solution: 2 min.
7. Stain in orange G: 30 sec.
8. Rinse in distilled water.
9. Treat with 1% aqueous acetic acid (1 ml/99 ml distilled water): 2 min.
10. Stain in aniline blue: 45–60 sec.
11. Treat with 1% acetic acid: 3 min.
12. Rinse in 70% ethyl alcohol. Transfer to 95% ethyl alcohol: 5 min.
13. Finish dehydration in absolute alcohol, 2 changes: 3 min each.
14. Clear and mount coverslip.

2.8.2 Picro-ponceau with haematoxylin for paraffin sections (29)

This is also an excellent, beautiful stain that clearly differentiates between muscle (yellow) and connective tissue fibres (red). Nuclear and nervous tissue differentiation is much greater with this stain than with Milligan's, as both stain dark brown to black. Elastic fibres, red blood cells, and epithelia stain yellow.

Protocol 3. Preparation of stock solutions for picro-ponceau with haematoxylin

1. Picro-ponceau stain, mix:
   - ponceau S (C.I. 27195), 1% aqueous — 10.0 ml
   - picric acid, saturated aqueous — 86.0 ml
   - acetic acid, 1% aqueous — 4.0 ml
2. Weigert iron hematoxylin
   (Mix solutions A and B below. The solution will turn black, and can be used for 1–2 weeks.)

   Solution A, mix:
   - ferric chloride ($FeCl_3 \cdot 6H_2O$) — 2.5 g
   - ferrous sulfate ($FeSO_4 \cdot 7H_2O$) — 4.5 g
   - hydrochloric acid, concentrated — 2.0 ml
   - distilled water — 298.0 ml

   Solution B, mix:
   - haematoxylin — 1.0 g
   - 95% ethyl alcohol — 100.0 ml

Protocol 4. Staining procedure for picro-ponceau and haematoxylin

1. Deparaffinize, transfer through alcohol and hydrate slides down to water.
2. Overstain in haematoxylin: 5–10 min.
3. Wash in running water until slides are deep blue: 5–10 min.
4. Stain in picro-ponceau: 3–5 min. Picro-ponceau acts as both a stain and as a destaining agent on haematoxylin. Its action should be monitored by rinsing the slides for a few seconds in distilled water and checking under the microscope. The nuclei should be sharp.
5. Dip several times in 70% ethyl alcohol.
6. Dehydrate in 95% ethyl alcohol, 2 changes, to remove excess picric acid.
7. Dehydrate in 100% ethyl alcohol, clear, and mount coverslip.

Protocol 5. Lee's methylene blue–basic fuchsin stain for GMA sections

1. Prepare the following stock solutions:
   (a) 4×10^{-3} M methylene blue
   - methylene blue (C.I. Basic Blue 9, C.I. No. 52015) 0.13 g
   - deionized water 100 ml

   (b) 4×10^{-3} M basic fuchsin
   - basic fuchsin (C.I. Basic Red 9, C.I. No. 42500) 0.13 g
   - deionized water 100 ml

   (c) 0.2 M phosphate buffer (pH 7.2–8)
   (Use protocol for Millonig's phosphate buffer; see section 2.2.3, phosphate buffered glutaraldehyde.)
2. Prepare staining solution by mixing:
   - 4×10^{-3} M methylene blue 12 ml
   - 4×10^{-3} M basic fuchsin 12 ml
   - 0.2 M phosphate buffer 21 ml
   - 95% ethyl alcohol 15 ml
   - Filter after mixing. Useful for 4–5 days.
3. Stain for 10–15 sec, then dip slide in distilled water briefly, dry with a blast of inert gas, and mount coverslip.

3. Microscopy

3.1 Brightfield microscopy

Simple brightfield microscopy is most useful for the initial examination of both paraffin and plastic sections. Oculars with high field number (for example, 23–25) are particularly convenient because they provide a wide field of view and thus allow a more rapid survey of the sections. Contrast enhancing methods such as phase-contrast and differential inference contrast may be helpful in some instances.

3.2 Polarized light microscopy

Polarized light microscopy is of particular use in the examination of hydrostatic skeletons. One of the most important aspects in the analysis of hydrostatic skeletons concerns the arrangement and orientation of connective tissue fibres. The connective tissue fibers are generally collagen. Because of the alignment of the collagen molecules, the fibres are birefringent and are therefore amenable to analysis with polarized light. Indeed, in many instances, it is extremely difficult to visualize the crossed fibre arrays of hydrostatic skeletons without polarized light microscopy.

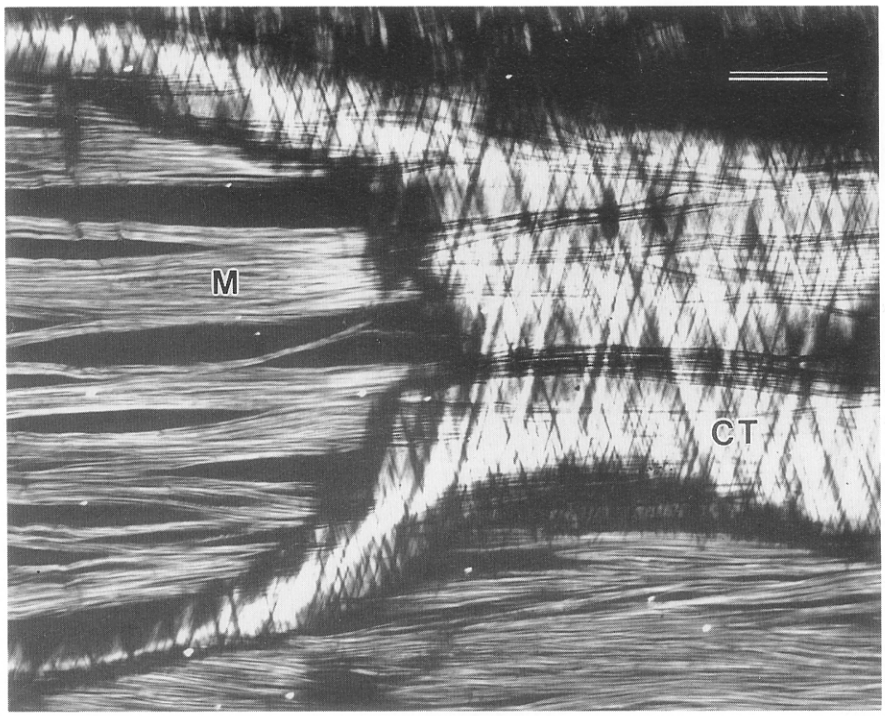

Figure 2. Photomicrograph of 7 μm frontal paraffin section of the arm of the squid, *Sepioteuthis sepioidea*, viewed with polarized light microscopy. With polarized light, the arrangement of the birefringent connective tissue fibres (CT) is apparent. The muscle fibres in the plane of the section (M) are also birefringent. For additional detail concerning the morphology, see ref. 10. The scale bar length equals 100 μm.

Polarized light microscopy is useful in the analysis of fibre arrays primarily in terms of visualizing the fibres, documenting their arrangement and measuring fibre angles (*Figure 2*). Birefringence can sometimes be used to distinguish between collagen fibres and rubber-like protein 'elastic' fibres, which are isotropic (19, 33). Crystallographic techniques such as measurement of the sign of the birefringence or obtaining crystal interference figures are unnecessary. The equipment required is therefore much less elaborate and expensive. While a polarizing microscope with a rotating stage, strain-free objectives, and high-quality polarizer and analyser is ideal, a good quality brightfield microscope can be adapted for simple polarized light microscopy. A polarizing filter, or even a piece of polarizing film, can be placed in a holder in the microscope condenser. A second polarizing filter serves as the analyser and can be inserted above the microscope nosepiece. The polarizing filter is then rotated in the condenser slot until the field is as dark as possible. If the stage is not rotatable, the slide holder can be removed and the slide itself can

then be rotated on top of the stage. A first-order red filter is sometimes helpful in visualizing fibre arrangements but is not absolutely necessary.

Polarized light microscopy may be used on specimens stained according to the procedures outlined above, but it is often useful to deparaffinize several slides from the series, mount coverslips without staining and use the unstained section for polarized light analysis. For glycol methacrylate sections, a coverslip is simply mounted on the unstained section.

4. Analysis of sections

Once serial sections through an object have been obtained, the sections must be interpreted with respect to the three-dimensional arrangement of the structure. As discussed by Elias (34), misinterpretation of three-dimensional structures from the observation of sections is common in the literature. The recommendation above of obtaining serial sections in three mutually perpendicular planes is of particular importance in this regard. The perpendicular section planes are a tremendous aid in interpreting the three-dimensional arrangement of the structure because they allow one to check predictions based on observation in one plane with the observations from another plane. For complex structures, this process of checking predictions of a particular interpretation in other section planes requires considerable patience and time.

4.1 Computer-assisted three-dimensional reconstruction

Computer-assisted three-dimensional reconstruction of serial sections is often useful in the interpretation of complex morphology. Several three-dimensional reconstruction software packages are now available for microcomputers and the equipment required is therefore relatively inexpensive. The system used in my laboratory (PC3D, Jandel Scientific, Corte Madera, California, USA) has proved to be extremely useful, in particular for students learning morphological techniques. Individual sections are traced using a microscope equipped with a camera lucida and the tracings are then digitized. The software allows the different objects in the sections to be categorized and takes into account the magnification and distance between sections. Once all of the sections in a series have been digitized, the software then stacks the sections in three-dimensional space and displays a reconstruction that can be rotated in any direction. The reconstruction can include or exclude any of the categorized objects, they can be displayed in a variety of colours, and they can be reconstructed with or without hidden line elimination. See *Figure 3* and Kier and Smith (35) for examples. In addition, it is possible to plot two images of the object that differ in rotation and view the plots as a stereo pair. The resulting three-dimensional image is often extremely useful in resolving questions about the precise shape, arrangement, and location of objects in the

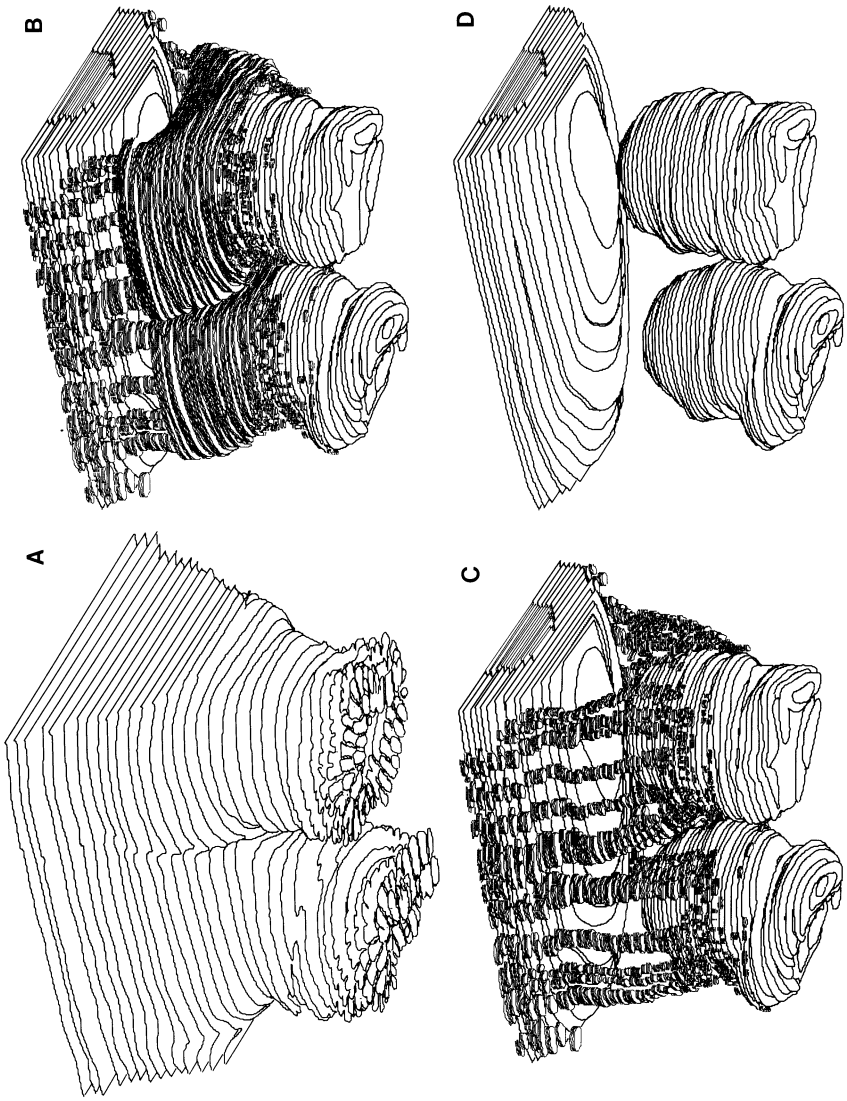

Figure 3. Computer-assisted three-dimensional reconstructions of a portion of the arm of the octopus *Eledone*. In the reconstructions labelled A–D, components of the arm are sequentially removed in order to reveal the components underneath. For colour examples of these reconstructions and additional detail concerning the morphology, see ref. 35.

structure. See Westbrook *et al.* (36) for examples of stereo pairs produced in this manner.

4.2 Fibre angle measurement

The measurement of fibre angles is often a necessity in the analysis of hydrostatic skeletons and is one of the reasons for obtaining serial sections. Measurements of fibre angle can be made easily on a microscope with a rotating stage equipped with a goniometer and ocular cross-hairs. One simply aligns the fibre of interest with one of the cross-hairs, notes the reading, and then rotates the stage until the cross-hair is aligned with a morphologically relevant line of reference; for instance, the longitudinal axis of a given structure. Often, the line of reference of the structure being studied is not easily defined, and it is more accurate to bisect the included angle between the right- and left-handed fibres. A variety of software packages for microcomputers with digitizing tablets are available that are also useful for making angular measurements. A tracing of the fibres can be made on a microscope equipped with a camera lucida and the tracing can then be digitized.

5. Functional analysis

5.1 Introduction

In addition to morphological analysis, research involving functional and experimental analyses of hydrostatic skeletons is required. To date, relatively few experimental studies have been performed that serve to test the predictions of the biomechanical analyses of hydrostatic and muscular-hydrostatic skeletal support. In the following sections, some of the techniques that are useful in experimental analyses of these systems have been outlined with the hope that further work in this area will be encouraged.

5.2 Pressure measurement

The measurement of pressure is often critical to the understanding of hydrostatic skeletal support. A variety of pressure transducers are available commercially, many of which are remarkably small, sensitive and require only limited displacement of fluid. The transducers fall into several general categories:

- remote transducers that are connected via tubing (usually polyethylene) to a catheter implanted in the animal
- flush-mounted transducers that measure the pressure applied to a surface
- catheter tip transducers with the pressure-sensing diaphragm located at the tip of a catheter.

5.2.1 Remote transducers

The remote transducers are convenient to use because a variety of catheter bores and types can be used. Nevertheless, they are subject to movement

artefacts due to vibrations of the tubing connecting the transducer to the catheter and to reduced amplitude and frequency response due to compliance of the tubing. For this reason, a remote transducer, and any other pressure transducer for that matter, should be not only calibrated, but its dynamic response should be determined. The dynamic response is most easily characterized using a 'pop test' in which one monitors the free resonant vibrations produced by a pressure transient applied at the end of the catheter. See Chapter 10 (Section 5.1) and Gabe (37) for details. An additional consideration in the use of remote transducers concerns potential blocking of the bore of the catheter with tissue debris. This is a particular problem in the study of muscular hydrostatic systems because fluid-filled cavities are not available for monitoring of pressure. A provision can be made for flushing of saline through the catheter. Alternatively a 'wick catheter' may be employed. A wick catheter is made by inserting a small piece of suture into the bore of the catheter (38). The suture prevents tissue from entering the bore of the catheter but allows pressure to be transmitted.

5.2.2 Flush-mounted transducers

Flush-mounted pressure transducers do not suffer from many of the movement artefact and frequency response problems of remote transducers because the sensing face of the transducer is exposed directly to the fluid under pressure. In addition, relatively little displacement of the sensing diaphragm occurs during measurement. Many of the flush-mounted transducers are miniaturized and could possibly be implanted in an animal.

5.2.3 Catheter tip transducers

The catheter tip pressure transducers also avoid some of the problems of remote transducers because the sensing face of the transducer is located on the tip of catheter (for example, Mikro-Tip Catheter Transducer, Millar Instruments, Houston, Texas, USA). They are convenient to use because a catheter placement needle can be used to insert the transducer and surgery is often not required. In addition, they are sealed and are thus convenient to use on experiments with marine organisms who in general seem to have remarkable aim when it comes to covering electronic devices with sea water!

5.3 Electromyography

Electromyography provides an important experimental approach to the testing of predictions concerning the pattern and timing of muscle activity. Electromyography using fine-wire bipolar electrodes has been used extensively in the study of vertebrate musculoskeletal systems. Chapter 8 in this volume by Gans provides details of electrode design, amplification, and recording techniques.

Electromyography has not been used extensively, however, in the analysis of the biomechanics of hydrostatic skeletal support. In part this reflects some

unique difficulties associated with recording from the musculature of hydrostatic skeletons. For example, in a study of the fin musculature of the cuttlefish *Sepia officinalis* (22), the bipolar fine-wire electrodes used commonly for electromyographic recordings were tried initially but the thinness and flexibility of the fins made electrode placement difficult and unreliable. An alternative technique using pairs of individually implanted tungsten fine-wire electrodes was developed. This technique allows precise and controlled electrode placement in thin muscle layers, as in the cuttlefish fin. Since similar difficulties with bipolar fine-wire electrodes may be encountered in future work applying electromyography to the study of hydrostatic skeletons, the technique is described below.

5.3.1 Individually implanted tungsten electrodes for electromyography

One end of a piece of tungsten wire (0.25 mm diameter) is etched in a saturated solution of sodium nitrite using electrical current to form a sharp tip (*Figure 4*). It is convenient to control the current applied to the tungsten wire using a variable transformer and a carbon rod suspended in the sodium nitrate solution to complete the circuit. The most durable electrode tips are formed using high current initially and then reducing the current during the final stages of the etching. The electrodes are then dipped in orthophosphoric acid for cleaning and removal of deposits formed during the etching process. The tungsten wire is cut approximately 3–4 mm from the tip and a tight loop is formed. The stripped end of 0.075 mm Teflon-coated annealed stainless-steel wire is then tied to the loop and soldered. Immersion in orthophosphoric acid aids in soldering. Because of the difficulty of achieving a good solder joint between the stainless and tungsten wire, the joint is also painted with silver paint to ensure electrical continuity before being encapsulated in a

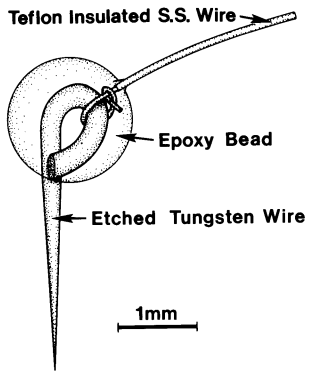

Figure 4. Diagram illustrating construction of one electrode of a pair of individually implanted tungsten electrodes for electromyography. Two such unipolar electrodes are implanted adjacently and are wired for bipolar recording. See text for details of fabrication.

small bead of epoxy resin. The electrode and attached wire is then dipped in insulator resin (3 coats) to ensure that both the stainless-steel wire at the solder joint and the tungsten electrode itself are insulated. The insulator resin is then removed from the tip of each electrode by scraping with a scalpel blade, forming a bared tip approximately 1 mm long. The wire leads of an electrode pair can be then glued together along their length and soldered to the appropriate connector.

The single electrodes of each bipolar electrode pair are then implanted individually. The bead of epoxy that encapsulates the connection between the stainless and tungsten wire provides a limit to the depth of implantation of the electrode. By varying the distance between the loop and the tip of the tungsten electrode, one can control precisely the depth of implantation of the electrode tip. The application of a thin layer of tissue adhesive (for example, Histoacryl, B. Braun Melsungen AG, Melsungen, W. Germany) over the electrodes, once inserted, helps to prevent accidental removal or changes in location of the electrode tips. It is also advisable to anchor the electrode leads to the animal so that any tension applied to the leads is borne by the anchor rather than by the electrodes themselves. A small plastic clamp capable of holding the leads without damaging the insulation is ideal.

Consult Chapter 8 for a discussion of considerations of preamplification, amplification, filtering, recording, digitization, and analysis of electromyographic signals.

5.4 Kinematics

Kinematic analysis is discussed in detail by Biewener and Full in Chapter 3 of this volume. The considerations of kinematic analysis of hydrostatic skeletons are similar to those discussed in Chapter 3. In many cases, kinematic data are collected on cine-film or video tape. Kinematic analysis using videotape or film is generally time-consuming and, as discussed by Biewener and Full, errors resulting from orientation and parallex complicate the analysis. Time can be saved with the use of a video motion analyser which provides a DC voltage that is proportional to the separation of two contrast boundaries on a horizontal line of a video image. See Chapters 10 and 11 and Gosline *et al.* (21) for a discussion of this approach. An alternative method for monitoring the movements of aquatic animals is described below that greatly reduces the time required for kinematic analysis and may also increase its accuracy and precision.

5.4.1 Electronic movement monitoring system for aquatic animals

The position of an animal or portion of an animal can be monitored by generating an electrical field in the tank and detecting the voltage measured by a sampling electrode at the location of interest on the animal. The circuit design for the system (*Figure 5*) was provided by Dr Douglas M. Neil,

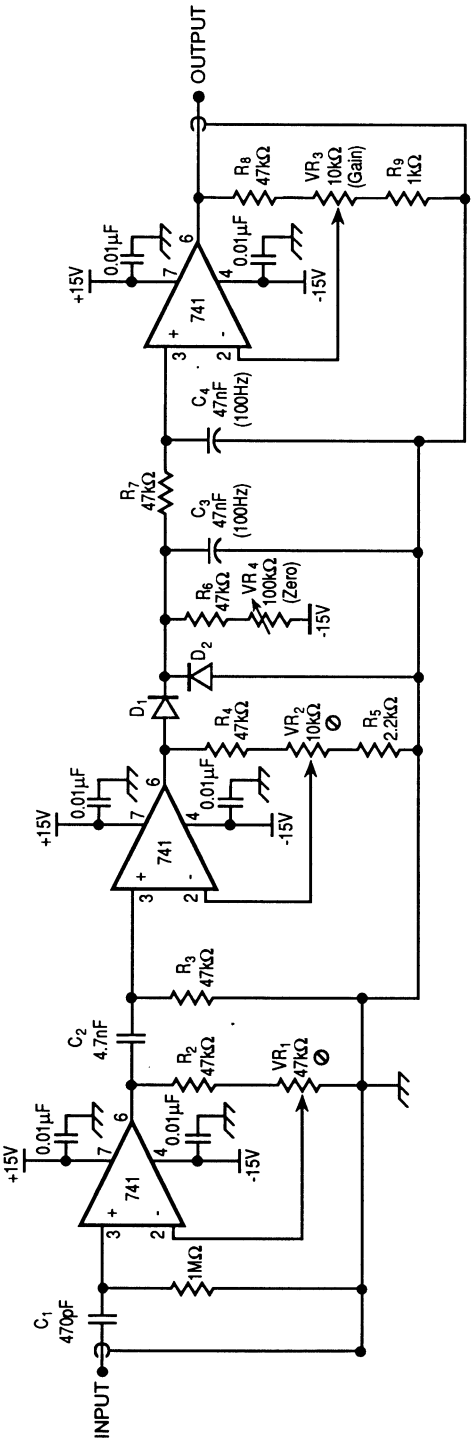

Figure 5. Schematic diagram of circuit used for monitoring movement in aquatic animals. See text for details.

Department of Zoology, Glasgow University, Scotland. The system is based on a circuit designed for the measurement of joint angles in arthropods (39) and was used to monitor the vertical position of the fin in a recent analysis of the muscular hydrostatic system of the fins of *Sepia officinalis* (22).

The movement monitor requires an experimental tank equipped with stainless steel plates on the bottom and at the surface of the water. The upper plate is connected to a function generator and a 40 kHz sine wave signal is supplied to it. The other plate is grounded. (For 30 cm × 25 cm plates separated by 12 cm in sea water, a 200 to 300 mV signal is required.) An electrode is made by simply removing 0.5–1.0 mm of insulation from 0.075 mm Teflon-coated annealed stainless-steel wire. The wire is then attached to the animal with tissue adhesive in such a way that the uninsulated end of the wire is located on the portion of the animal one wishes to monitor. The electrode is wired to the movement monitor circuit. The movement monitor rectifies, filters, and amplifies the input from the electrode and provides an output voltage that is proportional to the height of the electrode above the bottom of the tank. A number of electrodes and movement monitor circuits may be used in a single experimental tank and thus, the vertical position of several parts of the animal may be monitored simultaneously. The movement monitor signals can be recorded on separate channels simultaneous with other physiological recordings, providing direct correlation of movement with electromyograms, pressure recordings, etc.

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