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Preparation of Ascidians for Taxonomy and Molecular Analysis

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Relaxing and fixing ascidians for taxonomy
The ascidians should first be relaxed, then fixed in 10% seawater formalin buffered with sodium borate to help preserve spicules and color.

The formula for 1 liter of fixative:

- 100 ml of full strength formaldehyde (37%), 850 ml of seawater, and 50 ml of distilled water (or reverse osmosis water, or tap water if it does not have a lot of minerals in it; sometimes I get a precipitate with tap water). It is necessary to use the 50 ml of distilled water instead of all sea water so that the solution will not be hypertonic. [Ethanol is definitely NOT a good preservative for taxonomy, and ascidians should never be placed directly into ethanol, except a subsample for molecular analysis. Ethanol makes the tissues opaque and brittle, and it removes all color.]
- To this add 1 gram of sodium borate. Mix thoroughly before use. The borate is not very soluble, so it takes a while to dissolve; thus I make the solution a few hours or even a day ahead of when I want to use it.

Two methods for relaxing ascidians:

- An easy way to relax ascidians is with menthol

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[Recent research papers on *Didemnum*]

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crystals. You can carry a small vial of crystals in the field with you, place a few crystals in a ziploc bag with the sample in sea water and seal tightly. By the time you get back to the lab, the animals will usually be at least partially relaxed. If not, keep them in the bags for a bit longer, or proceed as follows.

- A second method for relaxing ascidians uses menthol in ethanol, a technique I learned from Don Abbott. Fill a small bottle (10-20 ml or so) with crystals of menthol. Then fill the bottle with 95% ethanol and shake to dissolve the menthol, which is much more soluble in ethanol than in water. Place the ascidian in a dish of seawater (or the menthol/seawater from your ziploc bag). Then add about 5 drops of the menthol/ethanol and QUICKLY cover the dish tightly (I use a small sheet of glass or plastic with a weight on top) to prevent evaporation of the menthol. Every 10 minutes or so add another few drops of the menthol/ethanol until when you insert a sharp probe into an open siphon there is absolutely no response. Use a hand lens or microscope to be sure about this! Relaxation may be achieved in as little as 10-15 minutes or so for some species, but may take several hours for others.

Fixing relaxed ascidians:

- Fill a dish with fresh seawater and have a jar of the 10% seawater/formalin fixative ready. Lift off the crystallized menthol that will be floating on the water surface (I put it on a paper, dry it and put it back into the bottle for re-use). Transfer the relaxed specimens to the dish of fresh seawater, rinse briefly to remove the extra menthol crystals (you may have to do this twice) and then quickly transfer to the jar of fixative and cover. If it's a large solitary specimen, hold it upside down to let the seawater drain out of the open siphons before immersing it into the fixative with the siphons pointing upward so that the animal will quickly fill with the fixative.
- If you won't be returning to the lab after collecting specimens, take the formalin, bottles, etc in the field with you. Leave the relaxing samples in the ziploc bags with menthol for several hours, then rinse in sea water or blot on paper towels and transfer to formalin. You can of course fix the ascidians directly in the 10% seawater formalin without relaxation but it makes identification especially of colonial species very difficult.

Fixing ascidians for molecular analysis and preservation of calcium carbonate spicules

Fixation in 95% ethanol and freezing:

- Before placing a specimen into 95% ethanol, if you

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Text in gray (italic) indicates the topic has not been addressed to date.

can, rinse in tap or distilled water to remove as much seawater as possible, because the seawater will cause precipitates to form in the ethanol. At the very least, blot on paper towels before placing in the ethanol. Do this in the field with fresh specimens. Don't use colonies that have been relaxing in menthol for several hours and may be half dead by the time you get to the lab.

- With colonial species, cut into small pieces before placing in the ethanol. With solitary species, remove the tunic and discard, and blot the body thoroughly (or rinse and then blot) to remove as much liquid as possible before placing it in ethanol. You can also dissect out the gonads and preserve only the gonads in ethanol. Store the specimens in a freezer as soon as you can.

Long-term storage of specimens

Opinions vary as to how to store ascidians long-term for museum vouchers. I and the Monniots in Paris leave them in the formalin forever, but even buffered formalin slowly becomes acidic and needs to be replaced periodically. Patricia Kott in Australia transfers specimens to 70% ethanol for permanent storage, after a minimum of 4 months' fixation in formalin.

Because ascidian taxonomy is very difficult, and many species, especially colonial ones, resemble one another morphologically, it is imperative that we all begin assembling a permanent subset of tissue for molecular analysis that is preserved directly into 95% ethanol and stored in a freezer. This will also be of tremendous value in helping to determine the point of origin of invasive non-indigenous species. Some new techniques are being developed to even be able to utilize dried and formalin-fixed museum specimens [Yue and Orban 2001. Rapid isolation of DNA from fresh and preserved fish scales for polymerase chain reaction. Marine Biotechnology 3 (2): 199-204. email orban@ima.org.sg] We must augment 19th century style taxonomy's total reliance on morphology with the molecular systematics of the 21st century. Museums, now the repositories of the world's species, should set up facilities for storage of companion vouchers for molecular analysis.

I agree with Patricia Kott that "no power on earth will maintain the living colour of ascidians after they are collected - so nothing can replace: (1) colour notes on living specimens before they are removed from the substrate; and/or (2) in situ photographs." To this, I would add notes on the general appearance of the living animal or colony. For example, the atrial languets of many aplousobranchs are highly contractile, and their short stubby appearance in preserved zooids bears little resemblance to their appearance in living zooids.

Larval morphology is of great importance in the identification of colonial forms; care should be taken to preserve brooded embryos and any swimming tadpoles.

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