

2. Protocols

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Handling and Fixation of *Crepidula fornicata* embryos

Rationale and background:

Basic techniques for handling embryos of *Crepidula fornicata* and other species of calyptraeid gastropods.

Protocol:

1. Embryos of *C. fornicata* and other calyptraeid gastropods are brooded within the pallial cavity of the adult, between the neck and the substratum. Each brood consists of multiple egg capsules that are attached to the substrate at a common point like a bouquet of balloons. The walls of the capsules are thin, and easily opened using fine watchmakers forceps that have been sharpened with a diamond sharpener. Embryos should be removed carefully from their capsules into a gelatin coated dish petri dish. Non-ciliated early embryos will stick to plastic dishes and even to glass pipettes, and so should be pre-coated with gelatin (see instructions below).
2. Older embryos and larvae should be relaxed prior to fixation, or they will retract into their shells and be useless for immunostaining or other morphological investigations. Embryos can be relaxed with a 1:1 mixture of 6.5% - 7.5% (weight/volume) $MgCl_2$ (dissolved in dH_2O) and filtered sea water. For best results, remove a small volume of sea water and replace over a period of 30 minutes to 1h until the ratio is 1:1. Good results have been obtained with a further relaxation adding three drops of chlorbutanol (chloretone) while swirling the petri dish every thirty seconds for 3 minutes. Relaxed embryos or larvae can then be transferred to a 1.5ml microcentrifuge tube for fixation. Squat glass 4ml scintillation vials also make good relaxation and fixation vessels (and can be viewed under a dissecting microscope to ensure that larvae are not lost during subsequent rinses).
3. Embryos can be fixed in 3.7% formaldehyde for 30-35 minutes at room temperature. Prior to adding fixative, remove as much sea water or relaxation medium as possible. Formaldehyde can be diluted in 0.22um filtered sea water (FSW) or 1X PBS. Embryos can be stored for short periods (up to a few weeks) in 1X PBS. For long term storage, embryos should be transferred into 100% methanol and kept at $-20^{\circ}C$ (to $-80^{\circ}C$).