A modified method for processing fluorescently marked sea cucumber ossicles

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Abstract

This communication presents a modification to the current method for processing fluorescently marked sea cucumber ossicles, used to identify hatchery-bred individuals from wild conspecifics in sea ranching, restocking and stock enhancement programs. Sea cucumbers are marked by immersing them in a fluorochrome solution, which is taken up by growing calcareous ossicles in the body wall. Mark detection is performed by digesting sea cucumber tissue samples from the outer body wall with bleach, allowing the remaining ossicles to be observed under an epifluorescence microscope. The bleach is removed by five freshwater exchanges using the current method, which is time consuming especially when large sample numbers are involved. Instead, one exchange of a solution of sodium thiosulfate was found to effectively neutralise bleach in samples, with no observed effect on processed samples. This modified method contributes time efficiencies to the development of sea cucumber marking, in addition to reducing the risk of sample loss and cross-contamination associated with multiple sample handling.

Introduction

The ability to tag or mark marine invertebrates is very useful for tag-recapture studies where parameters such as growth, movement and survival of individuals are being assessed. This is crucial in wild fishery interventions involving hatchery-bred animals because the ability to monitor and evaluate animal releases requires differentiation between wild and cultured individuals (Blankenship and Leber 1995).

The use of holothuroids in restocking, ranching and stock enhancement (Juinio-Meñez et al. 2013; Purcell 2012; Purcell and Blockmans 2009; Purcell and Simutoga 2008), has resulted in a need for suitable marking methods and techniques to identify marked individuals. An ideal marking method would be long lasting, visible in the field, inexpensive, and have no effect on growth and movement of the animal (Purcell et al. 2008). Most common tagging methods have been unsuccessful for sea cucumbers due to poor tag retention and animal stress (Conand 1990; Purcell et al. 2008; Purcell et al. 2006). Marking using passive integrated transponder (PIT) tags was recently found to successfully identify larger individuals but had lower efficacy in smaller animals (Gianasi et al. 2015), thus making it unsuitable for mass releases of small sea cucumbers, particularly where it is not necessary to identify individuals within the group. A novel technique for fluorochrome tagging of calcareous ossicles in the body wall was developed in 2006 (Purcell et al. 2006; Purcell and Blockmans 2009; Purcell and Simutoga 2008) and is currently the most suitable method for marking large numbers of small sea cucumbers. Fluorochrome marking has been used extensively in research involving cultured juvenile sandfish, *Holothuria scabra*, a commercial Indo-Pacific sea cucumber species (Conand 1990; Hamel et al. 2001). Animals are immersed in a fluorochrome solution, which is taken up by growing calcareous ossicles in the body wall. Marked sea cucumbers can then be released into marine areas and distinguished from wild conspecifics after recapture, although unfortunately not in the field. Marking sea cucumbers in this way also has applications for identifying individuals in experimental trials, particularly considering the availability of different fluorochromes that produce marked ossicles of different colours with the use of different optic filters in an epifluorescent microscope. Sea cucumbers can also be tagged with multiple fluorochromes (Purcell and Blockmans 2009), creating a double tag, further facilitating the use of multiple experimental treatments.

Marker detection is non-destructive, performed by processing very small samples taken from the ventral outer body wall. Samples are immersed in bleach ($NaClO_4$) to digest tissue, leaving the calcareous ossicles, and then rinsed five times with freshwater. Ossicles are then dried for examination under an epifluorescence microscope to determine the presence of fluorochrome exposure (Fig. 6 in Purcell 2012) (Table 1).

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After bleach digestion of sea cucumber tissue, performing five freshwater exchanges to remove bleach is time-consuming and fiddly, particularly when dealing with large numbers of samples, and provides opportunities for loss of ossicles at each exchange. Multiple samples handling also increases the risk of cross-contamination between samples. Because only a few marked ossicles in a sample are required to confirm the presence and colour of a fluorochrome tag, cross-contamination of ossicles between samples greatly reduces the reliability of results.

This communication reports on a modification to the original method (Purcell et al. 2006), detailed fully by Purcell (2012) and Purcell and Blockmans (2009), to improve efficiency and reduce the likelihood of sample loss and cross-contamination.

Material, method and results

The new method involves replacing the multiple freshwater rinses with one exchange of sodium thiosulfate ($Na_2S_2O_3$) solution. Sodium thiosulfate neutralises chlorine in water (DAFF 2008; McCauley and Scott 1960; OIE 2003) and is commonly used in hatcheries to remove bleach from water after use in sterilisation. The modified method is fully described and compared to the method detailed by Purcell (2012) and Purcell and Blockmans (2009) in Table 1. Explanatory technical points have been added.

As recommended by Purcell and Blockmans (2009), exposure to light was minimised using aluminium foil throughout the entire procedure to reduce photodegradation of fluorochrome. Pipettes should be thoroughly washed between each exchange, or a micropipette with a disposable tip should be used to avoid ossicle cross-contamination between samples.

Samples can be processed in microwell trays as described, or in Eppendorf vials. Microwell trays have the benefit of ease of viewing under a microscope if an appropriate microscope is available, and the ossicles do not need to be transferred to microscope slides for observing fluorochromes marks. If trays are not an option, 2-ml Eppendorf vials may be used. This may also be preferable when only a small number of samples are to be processed. To reduce the need to thoroughly wash pipettes between samples, or consume multiple disposable pipettes, liquid can be decanted from vials at each stage rather than be removed by pipette. This process can cause an increased loss of ossicles, and so an increased sample size – as recommended by Purcell and Blockmans (2009) – is recommended (5–8 mm^2) when using vials. Ossicles processed in vials are transferred to glass slides using a micropipette with individual disposable tips, and dried before viewing under an epifluorescence microscope. Larger samples will also provide enough ossicles to easily view on a glass slide because they will be less concentrated than in microwell cells. Samples up to 5 mm^2 in smaller and 10 mm^2 in larger animals are not found to cause any bacterial infection, and heal

Table 1. Modified method for processing fluorescently tagged sea cucumber ossicles compared with original Purcell (2012) method.

Original method	Modified method
Take a 2.5–5 mm^2 sample from outer body wall on ventral side of sea cucumber and place in a cell of a microwell tray. Preserve with buffered alcohol.	Take a 2.5–5 mm^2 sample from outer body wall on ventral side of sea cucumber and place in a cell of a microwell tray. Preserve with buffered alcohol unless sample is to be processed immediately.
Remove alcohol.	Remove alcohol.
Add bleach and leave for 30 minutes to digest body wall tissue.	Add 12% bleach and leave for 30 minutes to digest body wall tissue. Allow ossicles to settle to the bottom of the cell.
Remove bleach.	Remove bleach using a pipette, leaving a maximum of 0.5 ml in cell, including settled ossicles.
Add fresh water and remove.	Add sodium thiosulfate (50g L^{-1}) and ensure sample is thoroughly mixed. Leave ossicles to settle and remove liquid with a pipette.
Add freshwater and remove.	Add freshwater, leave ossicles to settle and remove liquid with a pipette.
Add freshwater and remove.	Once dry, view under epifluorescence microscope.
Add freshwater and remove.	
Add freshwater and remove.	
Once dry, view under epifluorescence microscope.	

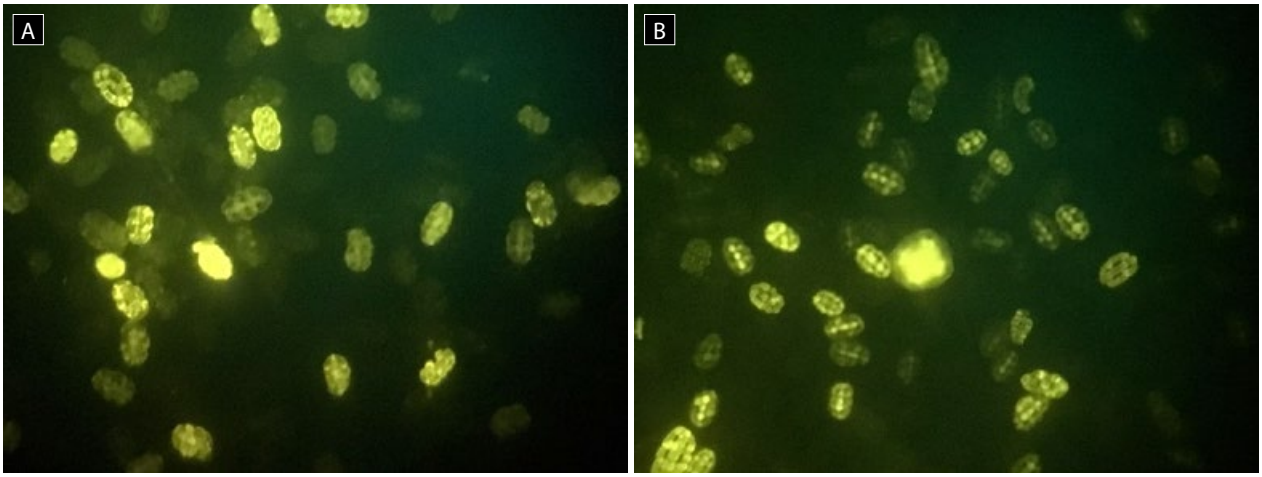

Figure 1. Sea cucumber *Holothuria scabra* ossicles marked with tetracycline and processed by original (A) and modified (B) methods, viewed through an epifluorescence microscope.

quickly in all cases (A. Birch unpublished data; C. Hair, pers. comm.)

The final freshwater exchange to remove the majority of the sodium thiosulfate solution is included in this modified method as a precaution. Further trials may show that this is not necessary, and should test the effect of removing this step on samples in the immediate and long term.

To assess any effects on the end result, the original method was used on samples collected from the same sea cucumbers alongside the modified method. There was no observable difference in the number or brightness of tagged ossicles produced from the two methods, when viewed under an epifluorescence microscope (see Fig. 1), and dried samples still fluoresced brightly after three years of storage (kept in the dark).

This revised method has been used to process ossicles from tagged *H. scabra* in experimental releases in the Northern Territory, Australia (A. Birch, unpublished data) using tetracycline and calcein. It has also been shown to be effective with calcein blue (C. Hair, pers. comm.). In addition to reducing the risk of sample loss and cross-contamination, the modified method contributes time efficiencies to the development of sea cucumber tagging. It is hoped that this will improve the ability to process large numbers of samples for sea cucumber tag-recapture projects.

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