LIMNOLOGY
and
OCEANOGRAPHY: METHODS

Limnol. Oceanogr.: Methods 7, 2009, 673–681
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A novel method for imaging internal growth patterns in marine mollusks: A fluorescence case study on the aragonitic shell of the marine bivalve *Arctica islandica* (Linnaeus)

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Abstract

In this article, we explore the use of fluorescence spectroscopy to image growth patterns in the marine bivalve *Arctica islandica* (L.). The method presented here does not require any chemical treatment of the polished shell section and yields results comparable to acetate peels of acid-etched shell sections and Mutvei-treated shell sections. Further, our results indicate that the annual growth lines in *A. islandica* fluoresce in the blue light spectrum (450–490 nm), thus an ultraviolet source (mercury lamp) is not required. The reflected light entering the digital camera was filtered (510–540 nm) and later enhanced to emphasize the annual growth patterns. The fluorescence of annual growth lines was consistent among the four animals used in this study. Additionally, we measured growth increments in the umbo section of one *A. islandica* shell using both the traditional acetate method and fluorescence imaging. The two sets of measurements were highly correlated ($r = 0.97$; $P < 0.0001$). We suggest that the fluorescence imaging method presented here is a viable option for increment identification and measurement in this key marine archive. It is likely that the methods demonstrated here for *A. islandica* can easily be used/modified for other bivalve (mollusk) taxa. Fluorescence microscopy permits rapid analysis of shell growth patterns with minimal pretreatment and offers an objective method of determination of annual growth increments and lines.

Introduction

The motivation to develop improved techniques to document and visualize growth patterns in bivalve shells originates from their great utility as environmental recorders (Richardson 2001). Bivalves are distributed globally, inhabit a wide variety of environments and water depths, and their fossilized shells are abundant and widely available through geologic time (e.g., Krantz et al. 1987). Bivalves are often well represented in archaeological sites, providing a powerful means for understanding past environments and cultures (e.g., Jones et al. 2005; Sandweiss et al. 2001). A number of bivalves are extremely long-lived, with lifetimes of many decades or even centuries, some of which include freshwater pearl mussels (Schöne et al. 2004), geoduck clams (Strom et al. 2004), ocean quahogs (Schöne et al. 2005a; Wanamaker et al. 2008a), and deepwater oysters (Wisshak et al. 2009), making them ideal candidates for environmental studies. Further, bivalves deposit growth bands/lines in their shells with a tidal to annual periodicity (Clark 1976; Jones 1980; Richardson 1989), therefore paleo-environmental reconstructions can be temporally constrained with subseasonal to annual resolution. It has been shown that environmental conditions such as seawater temperature (Strom et al. 2004), air temperature (Schöne et al. 2004), productivity dynamics (Witbaard et al. 2003; Wanamaker et al. 2009), and dominant modes of atmospheric (Schöne et al. 2003) and oceanic circulation (Ambrose et al. 2006) may be reconstructed from bivalve growth records using sclerochronological methods.

Sclerochronology is the broad study of the chemical composition and physical structure of accretionary hard tissues of organisms (e.g., brachiopods, mollusks, corals, coralline algae,

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Acknowledgments
This research is funded by EU Millennium (European Climate of the Last Millennium; Project no. 017008) and resulted from an *Arctica islandica* workshop held at the Gregynog Conference Centre, Wales in 2007. JDS acknowledges a Royal Society-Leverhulme Trust Senior Research Fellowship. We are grateful for helpful suggestions from three anonymous reviewers.

sclerosponges, otoliths, statoliths) and the temporal context in which they formed (e.g., *see* 1st International Sclerochronology Conference, 2007 Jul 17–21, St. Petersburg, Florida, U.S.A., http://conference.ifas.ufl.edu/sclerochronology/; also *see* Oschmann 2009). Sclerochronology can be considered the aquatic counterpart of dendrochronology. Often, sclerochronological techniques are used to develop a master chronostratigraphy within biogenic carbonates, which is then used as a template for growth and geochemical analysis (Weidman et al. 1994; Hendy et al. 2003; Delong et al. 2007; Halfar et al. 2008). It has been demonstrated that master shell chronologies (Witbaard et al. 1997; Marchitto et al. 2000; Scourse et al. 2006; Helama et al. 2006; 2007; Butler et al. 2009a) can be developed on a level consistent with dendrochronological studies. In particular, shell-based records from the very long-lived bivalve *Arctica islandica* can be absolutely dated like tree rings (Thompson et al. 1980; Jones 1980), enabling the generation of ultra-high-resolution and multicentennial paleoenvironmental records based on an absolute timescale (Butler et al. 2009a). These records have the potential to provide significant histories of marine environmental change, and to serve as a basis to document natural and anthropogenic change in key regions around the globe (Wanamaker et al. 2008a, 2008b). The ocean quahog (*A. islandica* L.), whose remarkable longevity has been found to exceed 400 years (Wanamaker et al. 2008a), is found along the continental shelves in the mid-to-high latitudes in the North Atlantic in water depths of <20 m to more than 200 m (Cargnelli et al. 1999). The usefulness of this proxy in ecosystem and ocean/climate studies is well established (e.g., Weidman and Jones 1993; Weidman et al. 1994; Marchitto et al. 2000; Witbaard et al. 2003; Schöne et al. 2003; 2005a; Scourse et al. 2006; Helama et al. 2007; Wanamaker et al. 2008a; 2008b; 2008c; Wanamaker et al. 2009; Butler et al. 2009a), and it is likely that *A. islandica* will be further established as a key marine archive from the North Atlantic (e.g., Schöne et al. 2005a; Wanamaker et al. 2008c) that will facilitate improved documentation and interpretation of recent and past environmental and climatic change.

With the exception of shell thin sections, which are often difficult and time consuming to prepare, imaging of growth patterns in bivalves generally require that a polished section of the shell surface be treated or etched with an acid to enhance the microshell structure (*see* Ropes 1984; Schöne et al. 2005b). In many cases, an acetate peel replica is made of the etched shell surface (Ropes 1984), placed on a microscope slide and then photographed. Additionally, if geochemical sampling (isotopes, minor and trace elements) is necessary, a second shell section would be required. More recently, treatment of biogenic carbonates with Mutvei's solution (a mixture of acetic acid, glutaraldehyde, and alcian blue; *see* Schöne et al. 2005b) has allowed the sectioned surface to be photographed without making an acetate peel. For many workers, the Mutvei method greatly improved their ability to visualize and interpret the growth record, but the acetate peel method and the Mutvei method both have some minor disadvantages. The acetate peel can introduce noise (blurring) unrelated to the growth record, or it may be unable to replicate adequately the physical structure of the increments. Treatment with Mutvei's solution is slightly destructive to the shell over time (especially if re-etching is required), and the shell surface scratches easily after it is etched. Although the Mutvei method has been very useful in highlighting growth lines/structures, it requires the use of glutaraldehyde (a respiratory toxin) to fix the organics in the shell structure. An ideal method of imaging biogenic growth structures would dispense with the need to treat the polished shell section with chemicals and create replicates of the surface using acetate peels.

Here, we image the annual growth increments in four specimens of the marine bivalve *Arctica islandica* (L.) using fluorescence microscopy. Fluorescence microscopy is a widely used technique in both geological and biological sciences (Isdale 1984; Scoffin et al. 1989; Baker et al. 1998, 1999; Proctor et al. 2000; Charman et al. 2001; Hendy et al. 2003). Typical components of a fluorescence microscope are the light source (typically xenon or mercury lamps), an excitation filter, a beam splitter, and an emission filter. The filters and beam splitter are chosen to match the spectral excitation and emission characteristics of the fluorescent material being analyzed. This fluorescent material may be intrinsic to the sample under analysis (e.g., natural organic matter preserved in geological materials such as stalagmites (Baker et al. 1993) and particulate organic matter in sedimentary deposits (Hart 1986),or it may be an artificially added fluorophore (e.g., fluorescent dyes or probes used to tag DNA; for example in fluorescent in-situ hybridization; Wilkinson 1999). In this article, we investigate the use of fluorescence microscopy as a suitable alternative to chemical-etching techniques as a method of imaging the internal growth patterns of polished shell sections of *A. islandica*.

Methods and procedures

The four *A. islandica* shells used in this study (WG060329, WG061254, WG061271, WG061290) were live-collected along the north Icelandic shelf in 2006 in 80 m water depth (*see* Wanamaker et al. 2008a for details), and prepared using methods outlined by Scourse et al. (2006), which are summarized below. The left-shell valves were embedded in resin (MetPrep; Kleer Set type FF) and the shells sectioned radially from the umbo to the shell margin (Fig. 1). The embedded left valve was sectioned through the center of the hinge tooth using a diamond saw. The cut surfaces were ground on progressively finer grades of silicon carbide paper (MetPrep; P120, P400, P1200) and polished with dilute diamond paste (Presi; 3 µm) on rotary magnetic pads (Presi; 200 mm, 3106 and 0307).

Polished sections of the four *A. islandica* shells were analyzed episcopically using a Zeiss Axiotech fluorescence microscope fitted with a Q imaging Micropublisher 3.3RTV camera (CCD color; 3.3 million pixels; 2048 × 1536) and three filter sets; Zeiss

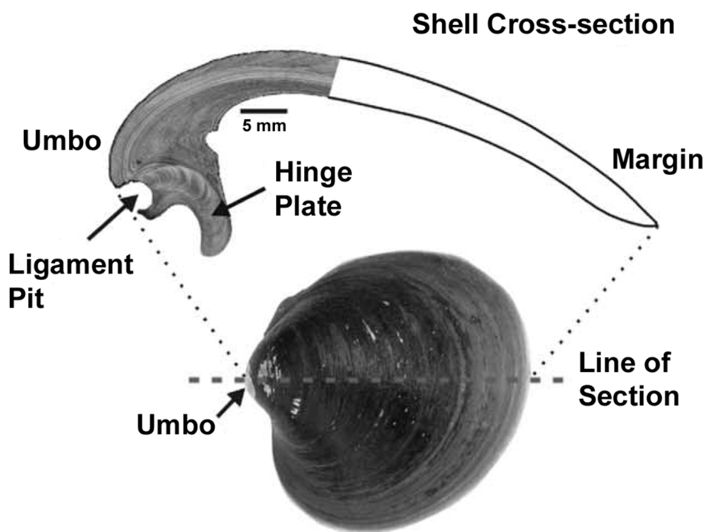

Fig. 1. Idealized line of section and an *A. islandica* shell cross section noting the major features (modified from Scourse et al. 2006)

sets 01 (ultraviolet; excitation 360-370 nm, emission >397 nm), 02 (blue-violet; excitation 320-380 nm, emission >420 nm), and 09 (blue; excitation 450-490 nm, emission >515 nm). These sets allow excitation of the sample with ultraviolet, blue-violet, and blue wavelengths respectively, with beam splitters permitting the viewing of any emitted fluorescence in the visible wavelengths. Images were taken using Image Pro Plus(c) using exposure times of 3 s (blue), 200 ms (blue-violet), and 2 s (ultraviolet), and subsequently contrast-enhanced using Image Pro Plus.

To compare traditional techniques to the procedure outlined here, we used methods of preparing and imaging acetate peel replicates. One shell (WG061290) was etched in a 0.1 M HCl solution for 3 min. An acetate peel replica (Agar Scientific; 35 µm) of the polished and etched surface was prepared and examined in transmitted light using a Leica Laborlux S microscope. Following the production of the acetate peel replica, annual growth increment widths were measured and recorded along the hinge plate in the umbo. Images of the incremental record in the sectioned hinge plates were captured using a digital camera (Soft Imaging Systems; Colorview) and the increment widths measured using the software analySIS(r) 3.2. Additionally, one shell (WG061254) was reground and polished to compare the method presented here with the Mutvei method. The shell was etched in Mutvei's solution using procedures outlined by Schöne et al. (2005a) for 15 min at room temperature.

Assessment

The ontogenetic age of the animals used in this study were 37 (WG061290; shell height = 53.6 mm), 45 (WG060329; shell height = 66.0 mm), 52 (WG061254; shell height = 67.2 mm), and 68 (WG061271; shell height = 62.0 mm) years old. We used the aragonitic hinge plate in the umbo region to construct the growth records. Annual growth lines were easier to visualize in the hinge region (Fig. 1) than on the outer shell surface, the latter showing greater variability in background fluorescence and more complex fluorescence adjacent to the growth increments. For clarity, we define an annual increment as the entire growth between two growth lines, which are the relatively thin and prominent growth cessation marks (*see* Fig. 2).

A comparison between the three filter sets showed that although the *A. islandica* hinge sections were the most fluorescent (= shortest exposure time) under blue-violet excitation, the growth lines were most prominent under blue excitation, suggesting a change in the composition of the organic matrix between the growth lines and the increments. This is illustrated in Fig. 2 (bottom panel), where the blue light excitation (450–490 nm) produces the clearest image of the annual growth increments and fluorescence lines (shell WG061254). To ensure that color imaging did not influence the fluorescence properties using the three filter sets, we processed each image in monochrome (Fig. 2). The results clearly indicate that the color imaging does not influence the readability of the annual growth lines. Further, the use of differential interference contrast and cross polarized light filters using the same microscope did not improve the visualization of the annual growth increments/lines in another shell sample (WG061271) (Fig. 3).

We produced distinct mosaic growth records from a single shell section (WG061290), first using the fluorescence method described here, and then with the traditional acetate peel method. We then compared the normalized shell growth records to determine if the method described here would be suitable for cross-dating applications (*see* Helama et al. 2006), and hence growth chronology construction. We show that the quality of the "blue light" method is comparable with that of the acetate peel method (Fig. 4) (shell WG061290). After measuring the increment widths using mosaic images produced by both methods, and removing the ontogenetic growth trend with a 15-y cubic spline applied to the logged increment widths, we normalized the two series (Wanamaker et al. 2008a) for comparison. The corresponding normalized growth records demonstrate that the series (blue light and acetate peel) are strongly correlated ($r = 0.97$; $P < 0.0001$). This result indicates that the blue light fluorescence technique outlined here is suitable for cross-dating *A. islandica* growth records and for chronology construction. The slight discrepancy between the two growth series (Fig. 4) was primarily due to measuring differences. Additionally, we compared our technique with the Mutvei method for an *A. islandica* shell (WG061254) to assess the reproducibility of the blue light method (Fig. 5). Both methods produce prominent and easy to read annual increments and growth lines. Although the Mutvei-treated shell is slightly easier to read (better contrast), the blue-light image produces the same result as the Mutvei method without any chemical pretreatment.

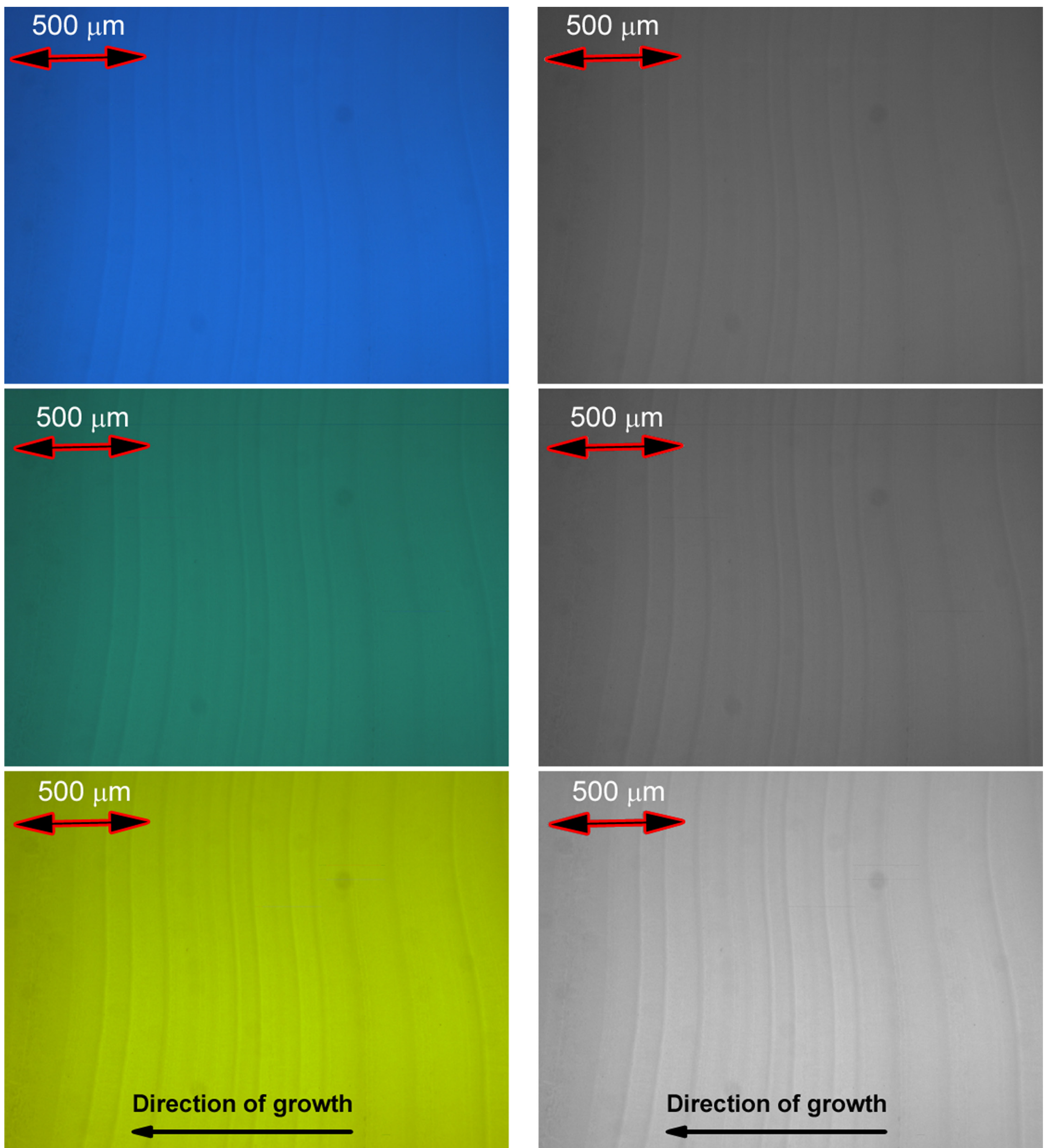

Fig. 2. Fluorescence results from the same region of the hinge plate of an *A. islandica* shell (WG061254) using ultraviolet (top left), blue violet (middle left), and blue (bottom left) filter sets. Additionally, each image is shown in monochrome (ultraviolet [top right], blue violet [middle right], and blue [bottom right]) to demonstrate that color imaging does not affect the fluorescence results or the interpretation of the annual growth lines. Annual growth lines are clearly evident in both the color and monochrome processed images using the blue light filter set (bottom).

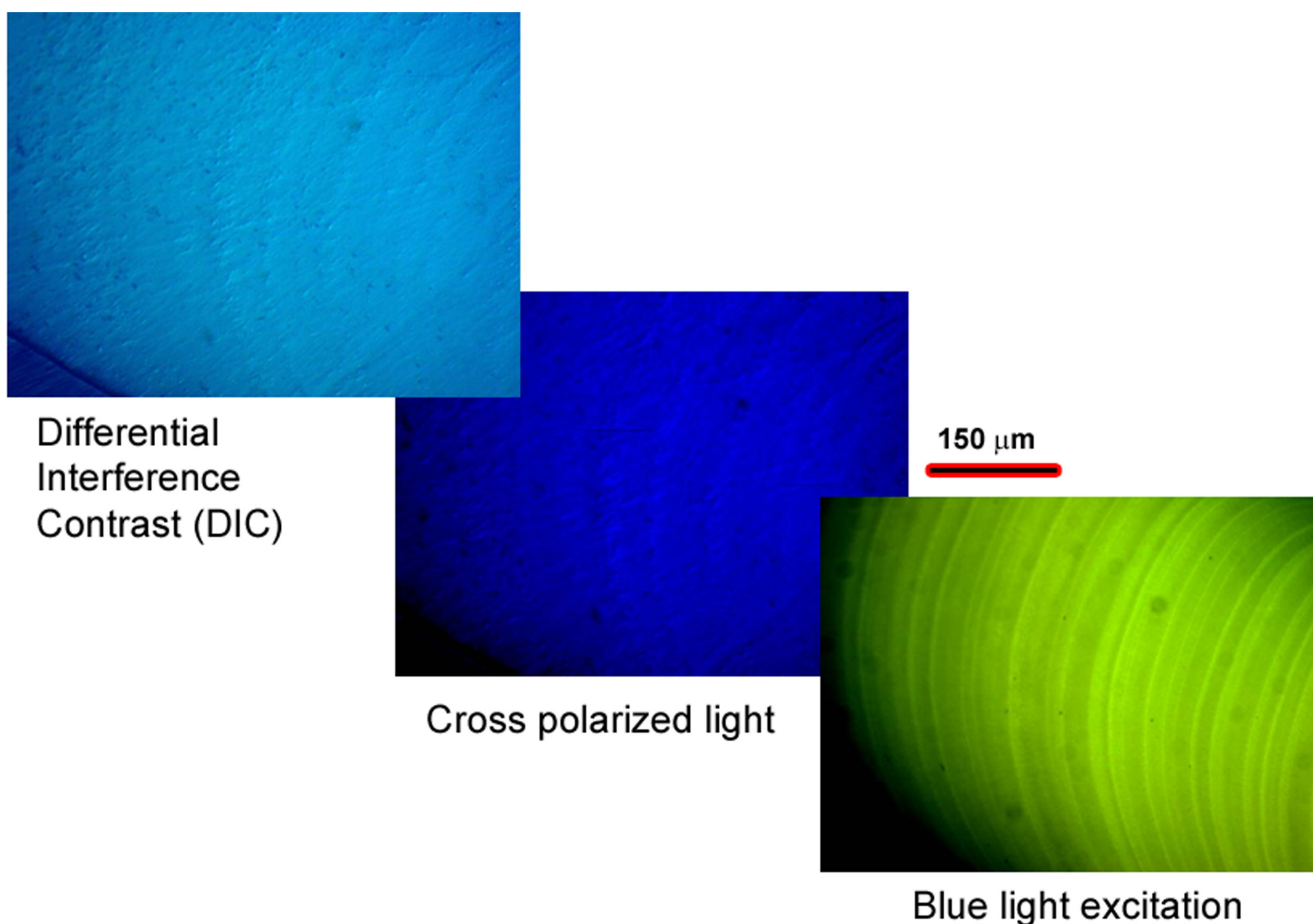

Fig. 3. Fluorescence results from the same region of the hinge plate of an *A. islandica* shell (WG061271) using differential interface contrast (left top), cross polarized light (middle), and blue light (right bottom) filter sets. Note that the scale bar is the same for each image; direction of growth is from right to left in each of the images.

Another interesting aspect of the fluorescence lines imaged in the hinge region of the *A. islandica* shell (WG061329) section is that each fluorescence line is followed by a dark line/shadow (Fig. 6). There appears to be an increase in fluorescence (and organics) just prior to the growth cessation period.

Discussion

Our results show that the annual growths lines in *A. islandica* fluoresce in the blue light spectrum (450–490 nm), so an ultraviolet source (mercury lamp) is not required and a traditional transmitted light microscope, fitted with the appropriate filters sets, can be used to conduct this work. Currently, the source of the intrinsic fluorescence of *A. islandica* shells is unknown; *A. islandica* is known to precipitate its aragonite shell within an organic polymer matrix, which likely includes chitin polysaccharides and proteins (e.g., Addadi et al. 2006; Cusack and Freer 2008; Dunca et al. 2009). Perez-Huerta et al. (2008) suggested that organic macromolecules associated with chitin polysaccharides and proteins in calcitic brachiopods (*Terebratalia transversa* and *Novocrania anomala*) are the likely cause of shell fluorescence in these species. The variability in the organic content within *A. islandica* growth increments has not been studied in any detail, and further work is required to establish the biogeochemical drivers of the fluorescence variability observed here. The observed increase in fluorescence (i.e., fluorescence lines) near the growth cessation mark (Fig. 6) is immediately followed by a dark line/shadow. This may indicate that during the period of slow growth (or during the growth cessation period) when the growth line is being deposited a dark line/shadow is formed that may contain less (fluorescence) organic material in the aragonite shell structure; this result warrants further study.

It has been demonstrated here that fluorescence microscopy produces images of comparable quality to

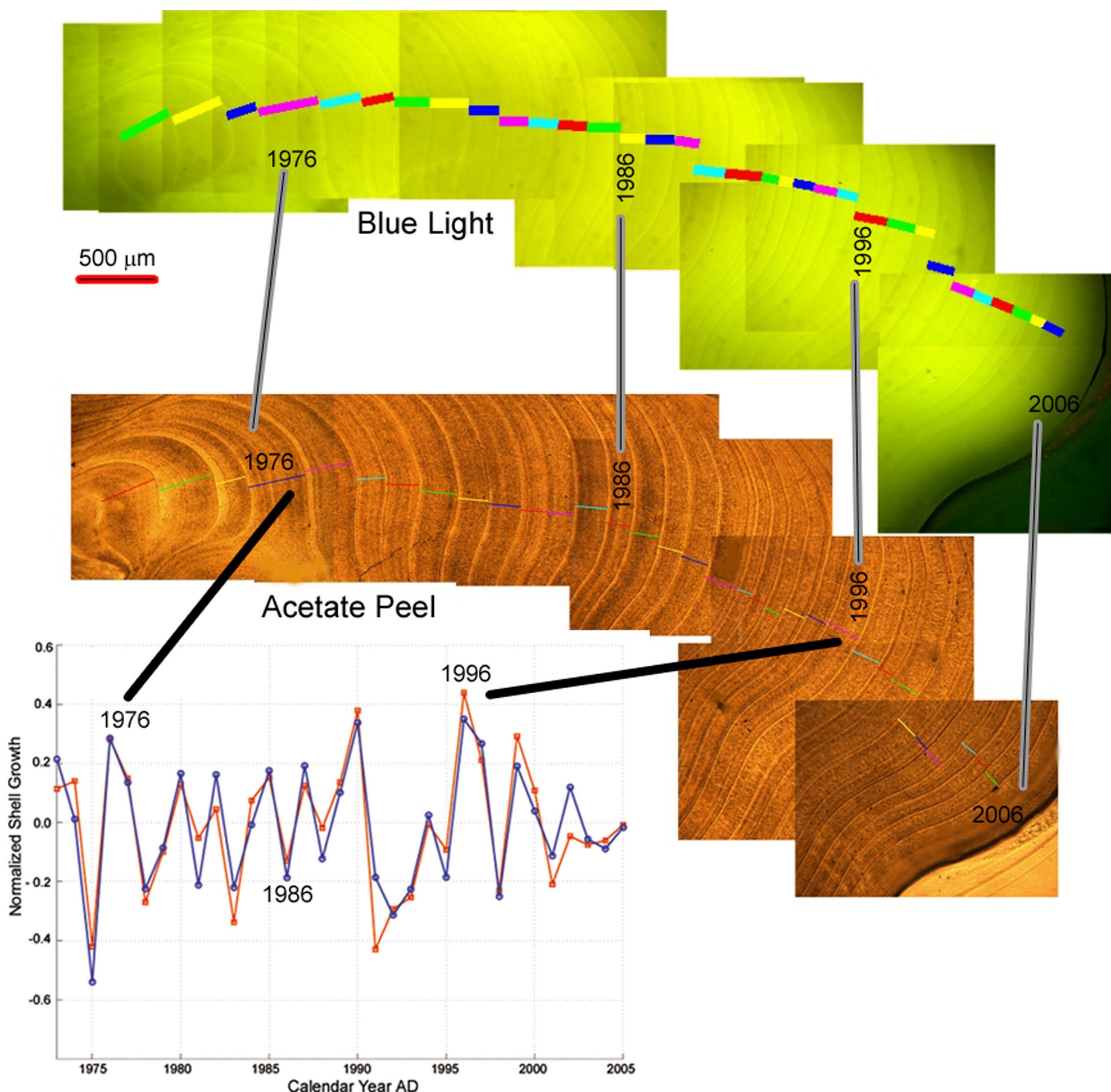

Fig. 4. Comparison between the "blue light" (top) and acetate peel (middle) methods for an *A. islandica* shell (WG061290). Each shell growth mosaic was measured independently and the corresponding normalized growth records are shown (bottom). The blue curve represents the blue light method, whereas the red curve is from the acetate peel technique. Highlighted years on both shell sections (1976, 1986, 1996, and 2006) are shown for comparison.

those observed in traditional acetate peels with the additional benefit that no chemical pretreatment is required prior to imaging. This method may increase shell throughput and increase the rate of chronology construction, as well as reducing the use of chemicals during the preparation process. Additionally, shell sections imaged in this manner can be used directly for geochemical studies, with no requirement to create further sections. The method

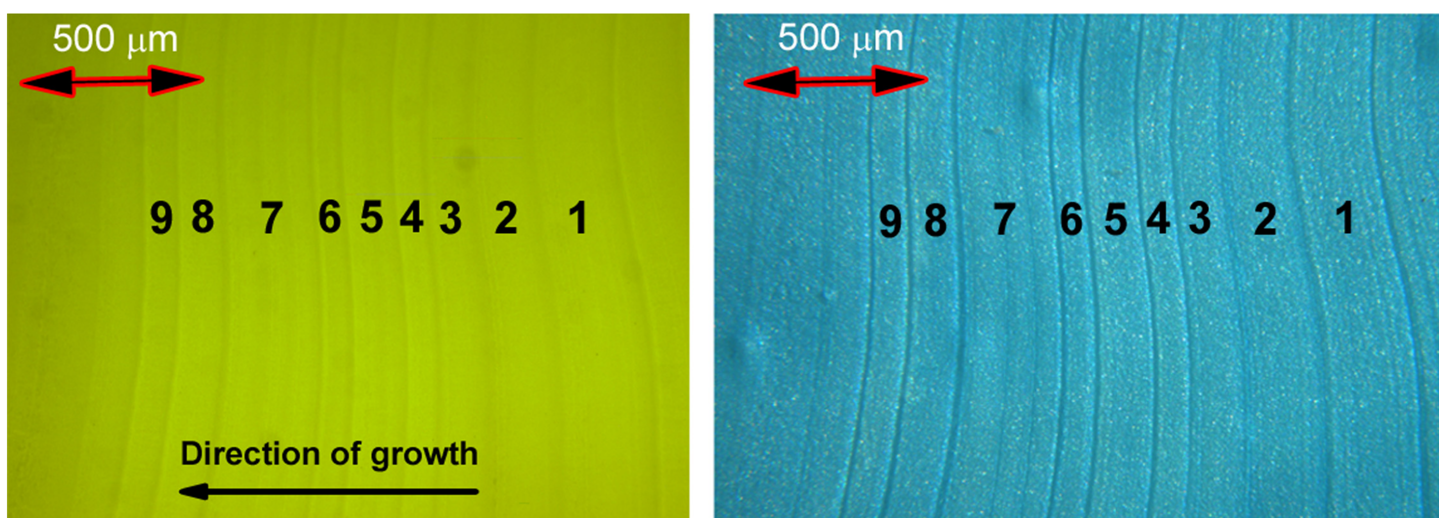

Fig. 5. Blue light fluorescence (left) and Mutvei-treated (right) results for an *A. islandica* shell (WG061254). We labeled nine complete increments (1-9) on both shell sections to show that each increment is clearly visible using both methods. The Mutvei-treated shell is slightly easier to read (better contrast), however the image captured using the blue light method also reproduces the same result without any chemical pretreatment of the shell.

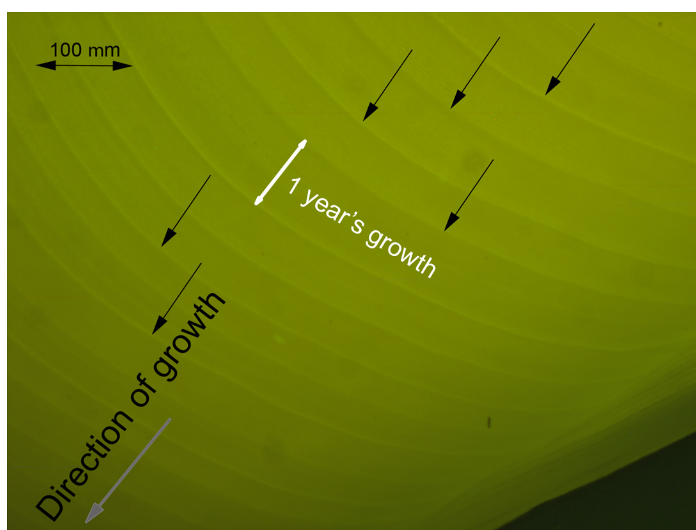

Fig. 6. Blue light fluorescence for an *Arctica islandica* shell (WG061329). A complete annual growth increment is shown in the middle by the white arrow. Some of the annual lines (here fluorescence lines) are shown by the black arrows. The direction of shell growth is noted with the gray arrow. Fluorescence lines are followed by a dark shadow/line during the "slow growth" or growth cessation period.

presented here may also be used in conjunction with other methods (thin sections, acetate peels, Mutvei solution) to objectively identify difficult banding patterns in biogenic carbonates (e.g., Butler et al. 2009b). Fluorescence microscopy produces images of comparable quality to traditional acetate peel techniques and the Mutvei method with the additional benefits of rapid analysis and minimal pretreatment.

Comments and recommendations

We present a novel and practical technique that uses fluorescent microscopy to image the internal growth patterns in the shell of the long-lived marine bivalve *Arctica islandica*. The polished and untreated aragonitic shell layer fluoresces under blue light (450–490 nm), revealing annual growth increments and fluorescent lines. We compared our fluorescence results with traditional acetate peel techniques and conclude that the blue light method is equivalent in quality. We have also demonstrated that the growth increments in the *A. islandica* specimen used here can be identified and measured using both blue light and acetate peel methods. An important benefit of this technique is that geochemical analyses can be performed on the same shell section that was imaged using blue light microscopy. Further, this method does not require an ultraviolet light source (xenon or mercury), which can be expensive and entail certain safety precautions. Although we did not test alternate light sources (e.g., tungsten, fluorescent) with the mercury lamp used in this article, future work using such light sources may prove beneficial.

Although the variability in the organic content within *A. islandica* growth increments has not been studied in much detail, we show that there is a prominent fluorescence from blue light excitation likely related to a change in the organic matrix in the aragonitic shell. Specifically, the observed increase in fluorescence near the growth cessation mark that is followed by a dark line/shadow merits further investigation (*see* Fig. 6). More work is required to establish the biogeochemical drivers of the fluorescence variability observed here in *A. islandica*. Additionally, because of the extraordinary longevity of *A. islandica*, this technique should be employed to determine if older specimens (>200 y) produce similar results as those presented here for the relatively young (<100 y) animals that we used. Future efforts should include fluorescence

work with additional mollusc taxa (and other biogenic carbonates) from different oceanographic settings, including sub-fossil material to assess any potential diagenetic effects, and to refine and extend the methods presented here. The usefulness of *A. islandica* as a proxy in ecosystem and ocean/climate studies is well established, and this new "blue light" technique may mitigate some methods-based constraints and further extend its utility in environmental studies around the North Atlantic.

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Submitted 16 April 2009
Revised 17 June 2009
Accepted 3 August 2009