LIGHT FIELD LC-POLSCOPE: THE NOVEL NON-INVASIVE AND LABEL-FREE IMAGING TECHNIQUE TO MEASURE 3-D BIREFRINGENCE OF THE JUVENILE CLAMSHELL

Tran Thi Mai[1], Rudolf Oldenbourg[2]

[1] *The College of Engineering and Computer Science, VinUniversity, Hanoi*
[2] *Marine Biological Laboratory, Massachusetts, USA*

SUMMARY

LC-PolScope is well known as the non-invasive and label-free imaging technique which can generate 2-D retardance map in a short time at a single-pixel resolution and high accuracy. Recently, the new imaging technique called Light Field imaging illustrates the ability to capture the 3-D structure of the isotropic sample. Taking advantage of both imaging techniques, by combining the exited LC-PolScope with light field imaging, the Light Field LC–PolScope can capture the inclination angle away from the focus plane of the microscope. In this report, we present a new non-invasive and label-free imaging technique to measure the three-dimensional birefringence of *Mercenaria mercenaria* hard clamshell using the new microscopy technique. Our work simulates the first success in generating the 3-D map at the lateral resolution of 2 μm and angular resolution of 9^o. This map provides structural insights into the early development of the clamshell.

Keywords: Calcite crystal, Clamshell, LC-PolScope, Light Field, Microscope, Uniaxial crystal.

INTRODUCTION

The LC- PolScope is a type of quantitative polarized light microscope that was developed at the Marine Biological Laboratory (MBL), Massachusetts, USA. It has been used extensively in many laboratories due to its speed, high sensitivity, and accuracy (Oldenbourg, Mei, 1995). Although it has significant progress in measuring and analysis of anisotropy materials, however, this microscope and other polarized light microscopes have a limitation in access to the three-dimensional anisotropy. To overcome this problem, Oldenbourg's group introduced the new imaging technique, which is called Light Field LC-PolScope (Oldenbourg, 2008; Tran, Oldenbourg, 2018). This novel microscope was built based on the LC-PolScope and exchanged the regular camera by the light field camera. The light field camera includes one microlens- array which can capture not only the positions but also the directions of the incoming light rays (Levoy *et al*., 2006). From a single snapshot of light field image, it can computationally generate the different respective views and optical sections (Broxton *et al*., 2013).

In the biology of bivalves and for studying environmental effects like ocean acidification on shell formation, it is important to identify the larval species and to differentiate normal shells from abnormal shells at the early stages of life. Calcium carbonate crystals in shells have the form of calcite, aragonite, or vaterite. These crystals are nucleated and grow on an organic matrix (Watabe, Wilbur, 1960). Since the matrix and nucleation mechanisms control the orientations of the crystals, each species has its birefringence patterns that reflect the orientations of the crystals in the organic matrix (Thompson *et al*., 2012; Tiwari, Gallager, 2003). Besides, environmental conditions affect the calcification and growth of shells, even causing the death of the whole organism (Tiwari, Gallager, 2003). Hence, measuring the optical properties of the shell can help identify species and detect abnormalities in larval development.

The shells of bivalve mollusks like the hard clamshell are composed of calcium carbonate crystals in the form of calcite or aragonite. As a consequence, they exhibit strong birefringence. Juvenile shells of M. mercenaria, 2 to 4 days after fertilization, resemble a bent, thin, crystalline sheet, in which the orientation of the optic axis of the underlying crystal structure varies systematically with the position in the sheet (Tiwari, Gallager, 2003). Hence, these juvenile shells represent a birefringent structure that is well suited to use the polarized light imaging, like LC-PolScope. The two-dimensional birefringence was well studied using LC-PolScope (Tiwari, Gallager, 2003). However, the three-dimensional birefringence is not able to assess directly by LC–PolScope and other current polarized light microscopes. The 3-D birefringence of hard clamshell can only be revealed in a sequence of orthoscopic and conoscopic observations that are combined through laborious analysis steps with only qualitative results (Hartshorne, Stuart, 1969). In this report, we present a new microscopy technique that can solve this problem.

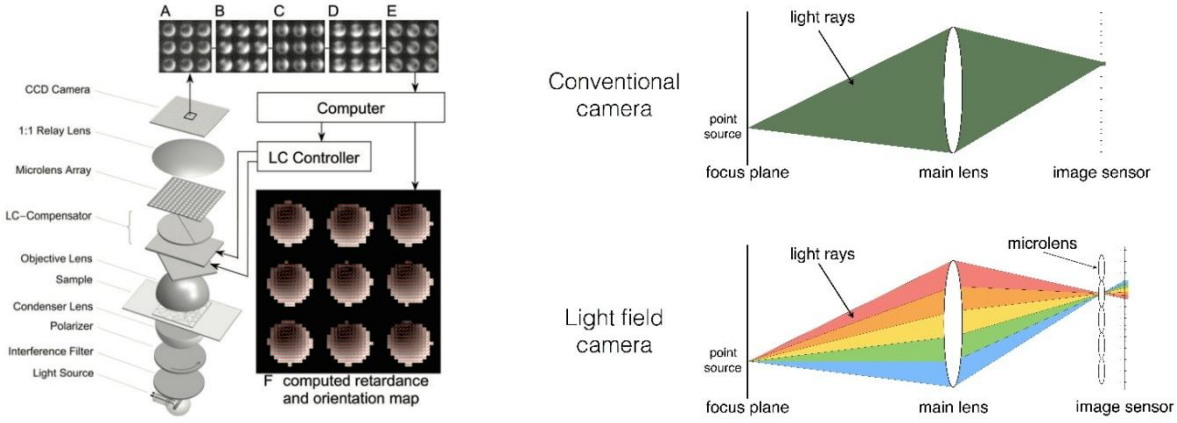
SAMPLE AND METHOD

Sample preparation

The three-day clamshells were fixed with 2% formalin (Fisher Scientific, USA). Then the batches were transferred to 10%, 30%, 50%, and 70% Ethanol, consecutively, rinsed with deionized water ten times, and soaked for six hours in 8.25% sodium hypochlorite (NaClO) bleach solution to remove organic materials. Finally, the larval shells were washed ten times in deionized water and stored in 70% Ethanol. For microscopic observations, a small number of shells, which had collected in the bottom precipitate, was transferred onto a coverslip and left to dry completely. Then the dried shells were immersed in oil (n_{medium}= 1.52) and sandwiched between a microscope slide and a coverslip and sealed with nail polish.

Microscope setup

The Light Field LC-PolScope was described in Refs (Oldenbourg, 2008; Tran, Oldenbourg, 2018). In this experiment, we used the microscope setup shown in **Fig.1A**. The optical parts include the light source, interference bandpass filter, left circular polarizer, a condenser lens, sample, objective lens, universal compensator, a 1:1 relay lens, and light field sensor. In this schematic of the Light Field LC-PolScope, the illumination light is nearly circularly polarized light. The traditional compensator is replaced by two electro–optical modulators, which together with a linear polarizer, are optically bonded and called the universal compensator. With the presence of the universal compensator, the incoming light can be set at any polarizations without changing the mechanical setup of the microscope. In the light field sensor (**Fig. 1B**), there's a microlens array inserted at the image plane of the main lens, which located at a distance of the focal plane 2.5 mm behind the microlens array. Each pixel on the CCD stores positional and directional information of the light rays. Moreover, because of the microlens array at the image plane, the image captured at the CCD are multiple conoscopic images at the different areas on the sample. To have a better explanation of the light field image, there is needed one more computational work to produce different angular views and optical sections (Broxton *et al*., 2013).



(A) **(B)**

Figure 1. (A) Schematic of Light Field LC-PolScope, including the optical setup, image acquisition, and processing components. Complete analysis of the polarized light field requires 5 raw images, corresponding to five settings of the LC-compensator. Based on the raw intensity images, the computer calculates a retardance and orientation map, shown here as a composite image with red lines indicating the slow axis orientation (Reprinted from Oldenbourg, 2008); (B) Simplification of the regular and light field sensor

RESULTS AND DISCUSSION

Establish the formula to calculate the retardance of the thick sample

The **Figure 2A** shows the 3-D shape of the clamshell which was reconstructed from the series of experimental focal images of the clamshell. However, the shape of the clamshell does not reveal any crystalline information inside the shell. To have a better idea how the crystals within the clamshell look like and align, we soaked the shells in the artificial seawater for three days. This work will induce epitaxial growth of calcium carbonate crystals on the surface of the shell. **Fig. 2B** shows the SEM of the treated shell. Clearly, the shape of the shell proves that the crystals are aragonite. The optic axis of the crystal is along the length of the crystal and nearly perpendicular with the surface of the shell. This result is in agreement with the other work (Tiwari, Gallager, 2003). As a consequence, we define the light rays and microscope coordinates as the **Figure 3**. In order to demonstrate our new imaging method, first, we establish the formula to calculate the accumulated retardance along a light ray.

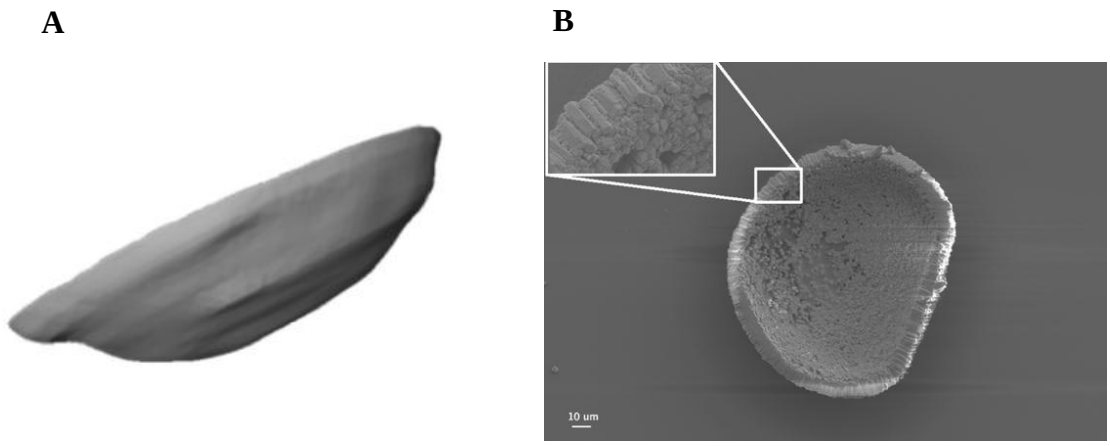


Figure 2. (A) The 3-D model of the clamshell was built from the optical sectioning images. (B) SEM image of the 3 day old treated clamshell

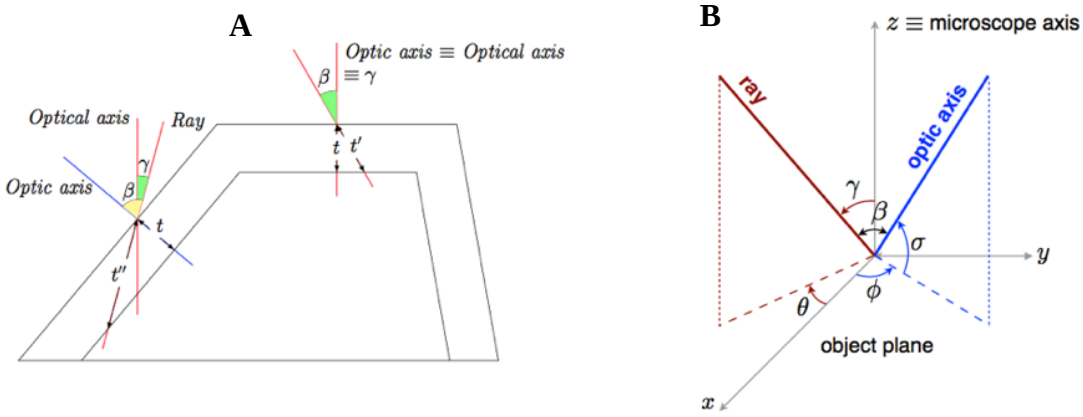


Figure 3. (A, B) The sketch of light rays and microscope coordinates. Optics axis orientation is defined by the inclination angle σ, azimuth angle ϕ. A light ray orientation is determined by the tilt angle γ and the azimuth angle θ. β is the angle between the ray and optic axis

Because in the light field camera, a pixel captures the retardance accumulated along a ray. The retardance imparted on a ray will depend on the physical path length (as t' and t" in **Fig.3A**) and the angle between ray direction and optic axis (Bon &Wolf).

The retardance Δ imparted on a ray after passing through the shell:

$$\Delta = t' * (n_e - n_o) * sin^2\beta \quad [1]$$

According to Fig.3A: $t' = \frac{t}{cos\beta}$ then:

$$\Delta = \frac{t}{\cos\beta}(n_e - n_o)\sin^2\beta \quad [2]$$

$$\cos\beta = \cos\theta\cos\sigma\cos\phi\sin\gamma + \cos\gamma\sin\sigma + \cos\sigma\sin\gamma\sin\theta\sin\phi \quad [3]$$

where t is the thickness of the sheet, the term (n_e - n_o) $sin^2\beta$ refers to the increase in birefringence as the ray direction tilts away from the optic axis. Equation [3] was developed by the work of Oldenbourg (Oldenbourg, 2008). Ray directions in object space are described by the ray's tilt angle γ with respect to the microscope axis and its azimuth angle θ in the focus plane. The optic axis is defined by the inclination angle σ and azimuth angle φ, as shown in **Fig.3B**.

Analysis of optic axis and estimation of the thickness

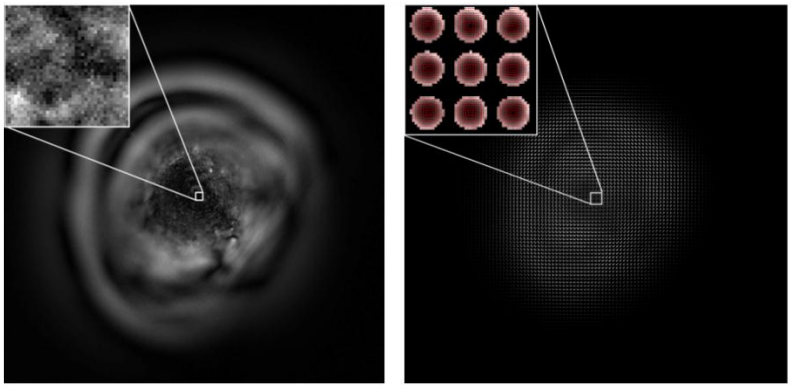
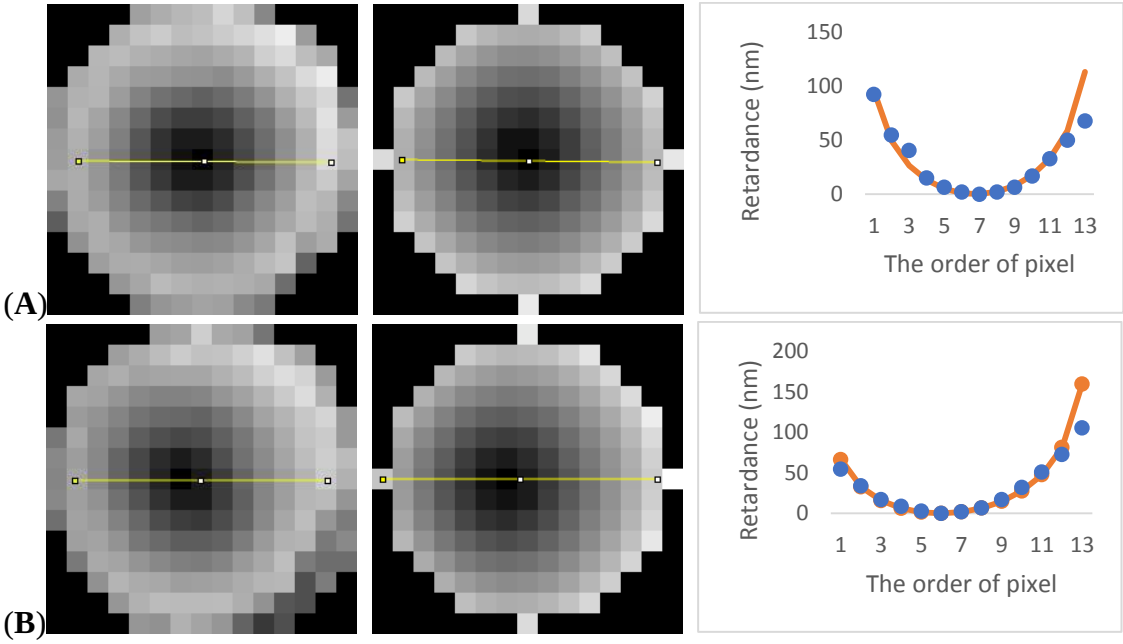


Figure 4. The retardance images of hard clamshell at the top plane taken with: (Left) Regular camera. The inset in the top corner does not provide any information about the orientations of the crystals. (Right) Light field camera. The inset in the top edge is a magnified aperture image projected by an array of 3×3 microlenses. Each aperture image is 13×13 pixel retardance image, including azimuth lines, of the aperture projected behind one microlens located near the center of the top focus plane of the shell

The retardance light field image is a matrix of conoscopic images (**Fig. 4B**). Each conoscopic image sampled one area in the sample, and each pixel behinds one microscopic image contains the 3-D directional information of the ray. The ray travels parallel to the optic axis; there is no split between ordinary and extraordinary rays. As a consequence, the retardance accumulated along that ray is equal to zero and appears as darkest spot. On the other hand, the 3-D directional information of the darkest spot within one conoscopic image reveals the 3-D orientation of the optic axis. We optimized the three parameters: inclination angle σ, azimuth angle ϕ, and the thickness of the shell t to best fit with the experimental data. **Figure 5** shows the three sets of experimental and simulated aperture images that correspond to three different focus planes or sets of (σ, ϕ, t). The profiles in the last column showed the accuracy of the optimized algorithm. **Fig. 5A** was taken at the top plane of the shell, which yields to (σ, ϕ, t) = (87^o, 145^o, 490 nm); **Fig. 5B** was taken at the lower distance of the shell, the result (σ, ϕ, t) = (81.15^o, 13^o, 480 nm) and the Fig. 5C illustrated (σ, ϕ, t) = (72.5^o, 15^o, 430 nm). The thickness of the shell measured at the different focus plane is slightly different. The closer to the top plane is, the thicker the shell is. In this case, the thickness of the shell is about 500 nm, which is consistent with other research (Tiwari, Gallager, 2003).



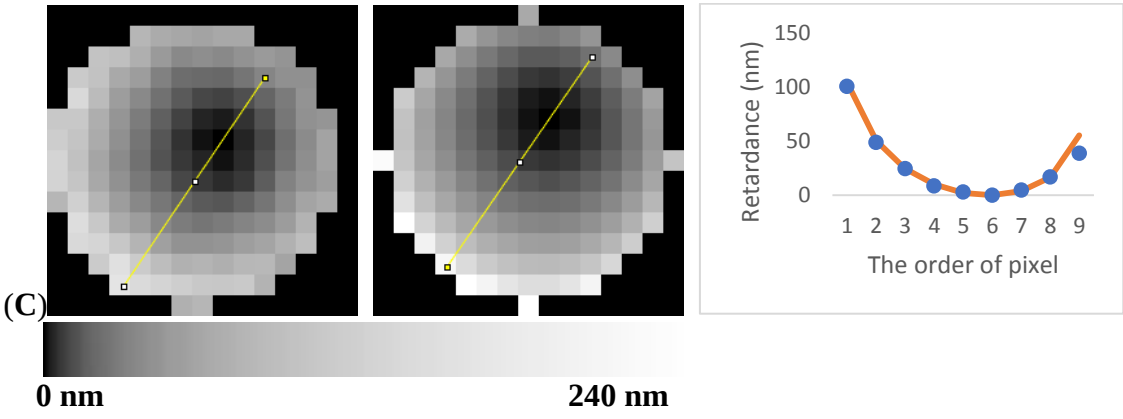


Figure 5. The experimental and simulated retardance conoscopic images and retardance profiles of yellow lines: (A) (σ, ϕ, t) = (87°, 145°, 490 nm); (B) (σ, ϕ, t) = (81.15°, 13°, 480 nm); (C) (σ, ϕ, t) = (72.5°, 15°, 430 nm). The blue dots represent for experimental retardance, the orange lines represent the simulated retardance values. The retardance ceiling for all four patterns is 240 nm and contrast enhanced

The 3-D birefringence maps of the hard clamshell were generated at a lateral resolution of 2μm and angular steps of 9° in terms of the azimuth and inclination angles of the optic axis. Our results also provide benchmark and analysis approaches that relate the optical properties of juvenile shells to their morphology that can be exploited for monitoring the health and development of bivalve aquacultures.

CONCLUSIONS

In this paper, we presented the results of the new approach with the Light Field LC-PolScope to generate an array of small conoscopic (aperture) images that characterize the birefringence of the hard clamshell. Analyzing those patterns reveals the 3-D optical properties and the thickness of the shell that can be related to its age. Polarized light field imaging illustrated a faster approach that doesn't require a change of the mechanical setup when acquiring the images. Our results indicate that by capturing a single light field image and combining it with comprehensive polarization analysis, we can measure three-dimensional optical anisotropy of the entire specimen. Our approach will facilitate the studies of shells in determining their morphology, including thickness, as a function of age, and in revealing abnormalities in shell development during environmental stresses. Further work will be needed for developing deconvolution algorithms that disentangle the superposition of polarization signals that stem from different optical sections in the specimen.

Acknowledgment: *The author would like to acknowledge the Aqua-cultural Research Corporation (ARC hatchery) in Dennis, Massachusetts, the USA, for their generosity to provide the samples for this work.*

REFERENCE

Broxton M, Grosenick L, Yang S, Cohen N, Andalman A, Deisseroth K, Levoy M (2013). Wave optics theory and 3-D deconvolution for the light field microscope. *Optics Express* 21(21): 25418-25439.

Hartshorne NH, Stuart A (1969). *Practical optical crystallography*. Edward Arnold.

Levoy M, Ng R, Adams A, Footer M, Horowitz M (2006). Light field microscopy. In *ACM SIGGRAPH 2006 Papers*: 924-934.

Oldenbourg R, Mei G (1995). New polarized light microscope with precision universal compensator. *J Microscopy* 180(2): 1 40-147.

Oldenbourg R (2008). Polarized light field microscopy: an analytical method using a microlens array to simultaneously capture both conoscopic and orthoscopic views of birefringent objects. *J Microscopy* 231(3): 419-432.

Thompson C, Hare M, Gallager S (2012). Semi-automated image analysis for the identification of bivalve larvae from a Cape Cod estuary. *Limnology and Oceanography: Method* 10(7): 538-554.

Tiwari S, Gallager S (2003). Machine learning and multiscale methods in the identification of bivalve larvae. *ICCV*: 494-501.

Tran MT, Oldenbourg R (2018). Mapping birefringence in three dimensions using polarized light field microscopy: the case of the juvenile clamshell. *J Microscopy* 271(3), 315-324.

Watabe N, Wilbur KM (1960). Influence of the organic matrix on crystal type in molluscs. *Nature* 188(4747) 334.

KÍNH HIỂN VI TRƯỜNG ÁNH SÁNG LC-POLSCOPE: KỸ THUẬT MỚI CHỤP ẢNH KHÔNG PHÁ MẪU VÀ KHÔNG NHUỘM ĐỂ ĐO CƯỜNG ĐỘ LƯỠNG CHIẾT BA CHIỀU CỦA VỎ SÒ CỨNG

Trần Thị Mai[1], Rudolf Oldebourg[2]

[1] Viện Kỹ thuật và Khoa học Máy tính, Trường Đại học VinUni, Hà Nội
[2] Phòng Thí nghiệm Sinh học biển, Bang Massachusetts, Hoa Kỳ

TÓM TẮT

LC-PolScope nổi tiếng là kỹ thuật hình ảnh không phá mẫu và không nhuộm mẫu, có thể tạo bản đồ độ trễ 2-D trong một thời gian ngắn ở độ phân giải một pixel và độ chính xác cao. Gần đây, kỹ thuật hình ảnh mới gọi là hình ảnh Trường ánh sáng minh họa khả năng chụp cấu trúc 3 chiều của mẫu đẳng hướng. Tận dụng cả hai kỹ thuật hình ảnh, bằng cách kết hợp LC-PolScope với công nghệ chụp ảnh trường ánh sáng, kính hiển vi trường ánh sáng LC-PolScope có thể chụp được góc nghiêng ra khỏi mặt phẳng tiêu cự của kính hiển vi. Trong báo cáo này, chúng tôi trình bày một kỹ thuật chụp ảnh không phá mẫu và không nhuộm mẫu mới để đo độ lưỡng chiết ba chiều của vỏ sò cứng *Mercenaria mercenaria* bằng kỹ thuật kính hiển vi mới. Chúng tôi đã mô phỏng thành công đầu tiên trong việc tạo ra bản đồ 3 chiều ở độ phân giải mặt phẳng là 2 μm và độ phân giải góc là 9^o. Bản đồ này cung cấp những hiểu biết về cấu trúc trong sự phát triển ban đầu của vỏ sò.

Từ khóa: Kính hiển vi, LC-PolScope, tinh thể canxi, trường ánh sáng, tinh thể đơn hướng, vỏ sò.

Author for correspondence: Tel: 033 255 0300; Email: mai.tt@vinuni.edu.vn