Preservation and Fluorescence of the Microfossils From Neoproterozoic Doushantuo Formation

HUIMEI CHI,[1]* MAN FENG,[2] ZHONGDANG XIAO,[1] AND ZUHONG LU[1,3]
[1]*State Key Laboratory of Bioelectronics, Southeast University, Nanjing 210096, China*
[2]*Nanjing Institute of Geology and Paleontology, CAS, Nanjing 210008, China*
[3]*Key Laboratory of Child Development and Learning Science, Southeast University, Nanjing 210096, China*

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ABSTRACT The phosphatized microfossils from Doushantuo Formation, Southeast China show us the biodiversity about 600 million years ago, which is a unique window for the evolution of the early life on earth. However, the process of phosphatic fossilization in detail still remains unknown. Here we report our study on the preservation state of the fossils by using confocal laser scanning microscopy. We found that fluorescent signal of the fossil could reflect the preservation state when compared with the transmission light microscopy. First, we found the fluorescent signal of the decayed cells of the fossil was weaker than that of the nondecayed part. Second, we found that the three-dimensional reconstruction of the fluorescent signals could help to judge the degree of mineralization of the fossil cells, compared with the observation by transmission light microscope. Third, we found that almost all of the fossil specimens we observed could fluoresce more or less when excited by laser light. Therefore, the fluorescent microscopy provides a useful method for the study of the preservation state of the phosphatic fossil cells. *Microsc. Res. Tech. 71:260–266, 2008.*

INTRODUCTION

The Doushantuo Formation, Guizhou, China is notable for the highly preserved multicellular animal and algae fossils, which is one of the oldest Lagerstätte found until now and presents a vivid biological world with diversification (Chen et al., 2000, 2002, 2004a, 2006; Li et al., 1998; Xiao et al., 1998a, 2000, 2004, 2007; Yuan et al., 2005; Zhang, 1989). Cyanobacteria, acritarchs, algae, and microscopic metazoans have all been described from Doushantuo phosphorites at Weng'an (Guizhou Province) and elsewhere in South China (Xiao et al., 1998b, 1999, 2000; Xue et al., 1995, 2001; Yin, 1999, 2001; Yin et al., 2001; Yuan and Hofmann, 1998; Yuan et al., 2002; Zhang, 1989; Zhang and Yuan, 1992; Zhou et al., 2001, 2002). The multicelluar algae were the main members of the Precambrian ocean about 600 MYA (Barfod et al., 2002), most of which were interpreted as red algae (the Rhodophyta) (Xiao et al., 1998a, 1999, 2004; Yuan et al., 2002; Zhang, 1989). Concomitant with the algae fossils, the multicelluar animal fossils were also reported by some researchers, including sponge fossils, Cnidairia fossils, and bilaterial fossils (Chen et al., 2000, 2002, 2004a; Li et al., 1998; Xiao et al., 2000).

However, there are still discussions about the interpretation of some fossils such as fossil embryos and small bilateral animal fossils (Bengtson and Budd, 2004; Chen et al., 2000, 2004a,b; Xiao et al., 2000). The microfossils have ever been interpreted as the preserved gastrulae of cnidarian and bilaterian metazoans could alternatively be interpreted as conventional algal cysts or egg cases modified by diagenetic processes (Xiao et al., 2000). The coelomic layers and sensory organs of small bilateral animals (Chen et al., 2004a) were considered as diagenetic origin by Bengtson and Budd (2004). Thus the discussions are focused on whether some of the structures interpreted are of biological origin or not. Then the preservation of the fossils became very important for the interpretation of the phosphatic fossils or even the phylogenetic evolution.

Encrustation and impregnation was considered the principle mode in phosphatization and calcification of animal soft tissue. Phosphatization appears to result from complex interactions between organic decay and mineral replication (Xiao et al., 1999). The decay-resistant tissues such as cuticles and xylem are inclined to preserve. However, the protoplasm of the cells seems difficult to be preserved. For the algae fossils from the Doushantuo Formation, Weng'an, China the cell wall was replicated by phosphatic minerals and preserved while the inside part was seldom preserved (Xiao et al., 1999, 2004; Yuan et al., 2002). The animal embryo from the Doushantuo Formation was reportedly to be preserved with a different way. The cell of the embryo was crystallized as a whole by phosphatic minerals without cell lumen (Chen et al., 2000, 2002). In three-dimensional embryos from acid residue the phosphatic crystals grow on both side of the envelope (Xiao et al., 1999), which help to keep the original shape of the envelope. The phosphatic coating was also found in the cell wall of algae fossils (Xiao et al., 1999,

*Correspondence to: Dr. Huimei Chi, State Key Laboratory of Bioelectronics, Southeast University, Nanjing 210096, China. E-mail: hmchi@seu.edu.cn

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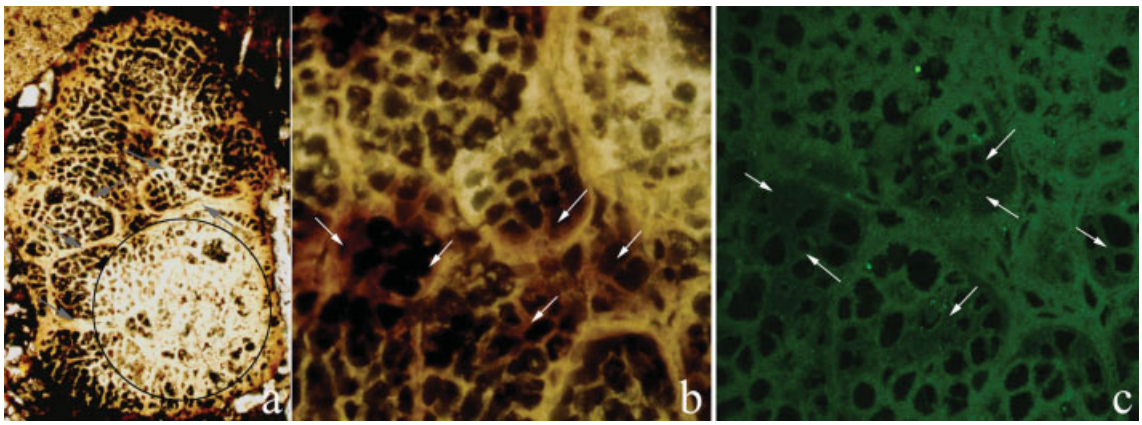

Fig. 1. The decayed parts of the fossil alga taken by transmission light microscope and scanned by confocal laser scanning microscope. (**a**) a red alga (see Chi et al., 2006); (**b**) the enlargement of the center part of the alga in (a); (**c**) the 3D fluorescent reconstruction of the same part with (b) by confocal laser scanning microscope. The decayed parts of alga were shown in (b) and (c) by arrows.

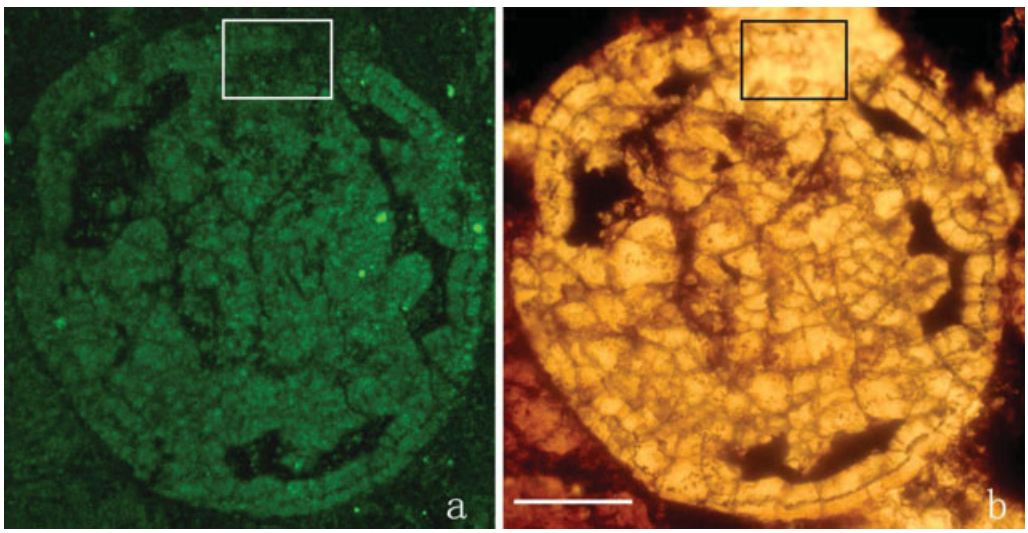

Fig. 2. A possible animal embryo. (**a**) the 3D fluorescent reconstruction of the animal embryo; (**b**) the transmission light image of the animal embryo. Scale bar in (b) represents 50 μm. The rectangle in (a) and (b) indicated the decayed part of the embryo. Structure in (b) was blurred and the cystallization was not as good as the other part. In Figure 1a we could not see the fluorescent signal of this part.

2004; Yuan et al., 2002). However, the preservation process of the phosphatic fossils on molecular level still remains unknown. Some researchers also try to disclose the fossilization process through experiments to simulate the environment of fossilization (Martin et al., 2005; Raff et al., 2006). They found that the animal embryos within fertilization envelope have high preservation potential and preserved as fossils without size bias.

Transmission light microscopy was the commonly used method by paleontologists when they studied fossils in thin-section. The other method such as fluorescent microscopy has ever been used when paleontologist and geologist studied fossils (Feist-burkhardt and Pross, 1998; Nix and Feist-burkhardt, 2003; Yeloff and Hunt, 2005). Recently, synchrotron X-ray tomographic microscopy was also involved into the study of the three-dimensional preserved fossil embryos (Chen et al., 2006; Donoghue et al., 2006). This method could help to observe the inner structure of the stereo fossil embryos. And micro CT was also used by paleontologist to observe the stereo fossil embryos (Hagadorn et al., 2006). However, the optical microscopy is still dominant in the observation of thin-section of fossils. When studied the structure of the fossils cells of algae and animal embryos by confocal laser scanning microscopy we found that the fossil cells could fluoresce when excited by laser light and have a steady fluorescent emission. Through 3D reconstruction of the fossil cells we get the image which could pass for the image that was taken by transmission light microscope (Chi et al., 2006). However, no one pay more attention on the rela-

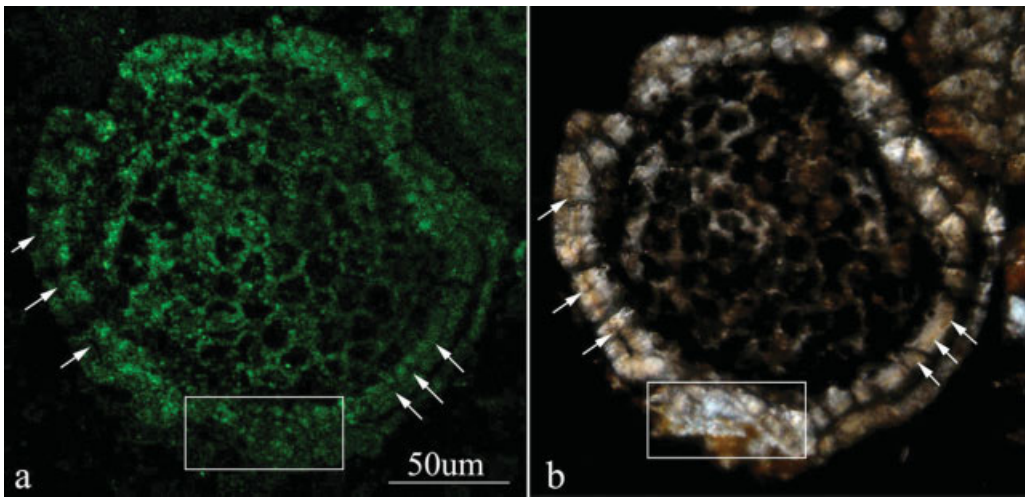

Fig. 3. An alga fossil with crystallized layers outside. (**a**) The 3D fluorescent reconstruction of the alga; (**b**) the transmission light image of the alga. Arrows indicated organic layer.

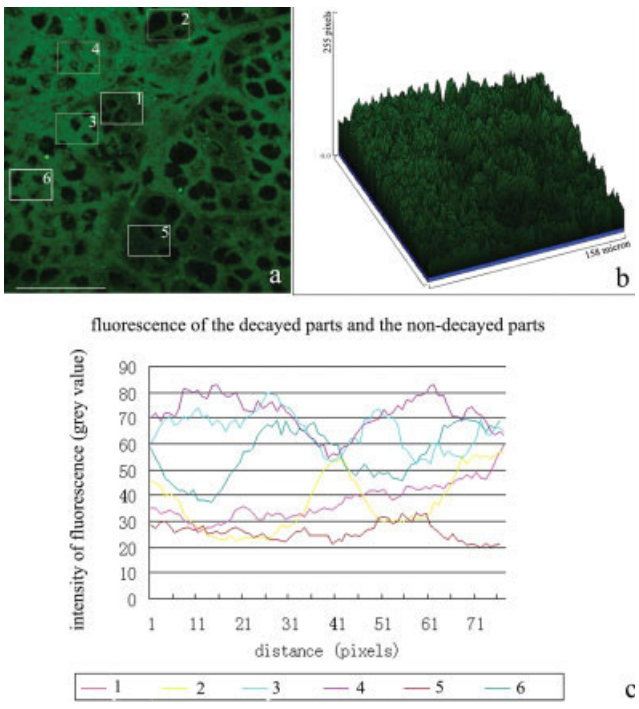

Fig. 4. The quantitative fluorescence analysis of the decayed part and the non-decayed part of fossil alga in (**a**) the 3D fluorescent reconstruction of the center part of the alga in Figure 1a; (**b**) surface plot of (a); (c) the quantitative fluorescence analysis of the selected parts, including decayed parts 1, 2, and 5 and nondecayed parts 3, 4, and 6. The scale bar in (a) represents 50 μm.

tionship between the preservation state and the fluorescent signals.

Here in this manuscript we report our observation about the relationship of the preservation state of microfossils and the fluorescence of the fossils.

MATERIALS AND METHODS

The samples included in the present study were collected from the Doushantuo Formation, Guizhou, China, which has been estimated to be 632–551 or 600 million years ago (Barfod et al., 2002; Condon et al., 2005). Preparation of thin-section was described in Chi et al. (2006).

RESULTS AND DISCUSSION

The Fluorescence of the Decayed Part of the Fossils

Transmission light microscopy is a commonly used method by paleontologists when the thin-section is involved. The multicellular algae and animals 600 million years ago were also reportedly found by this method in thin-section of fossils (Li et al., 1998; Xiao et al., 1998a; Zhang, 1989). However, when we need more information about the mineralization of the fossils we found that we still need the help of the other method like the confocal laser scanning microscopy. In Figure 1 we could see the comparison between the transmission light microscopy and the confocal laser scanning microscopy when observed the preservation state of the fossil cells. Figure 1a show the published possible red alga (Chi et al., 2006) and the image was taken by transmission light microscope. From Figure 1c we could see some of the fossil cells collapse indicated by arrows. However, in Figure 1b we could only see the color of corresponding cells indicated by arrows was brown, which was different from the other cells. We could not see distinct decayed structure in Figure 1b. From Figure 1c, the 3D fluorescent reconstruction by confocal laser scanning microscope, we know which part was decayed and which part was not decayed. Therefore, the fluorescent reconstruction could help to differentiate the decayed part of the fossil cells.

From Figure 1 we also know mineralization of the decayed cells was different from that of the nondecayed cells. The fibrous structure indicated by arrows in

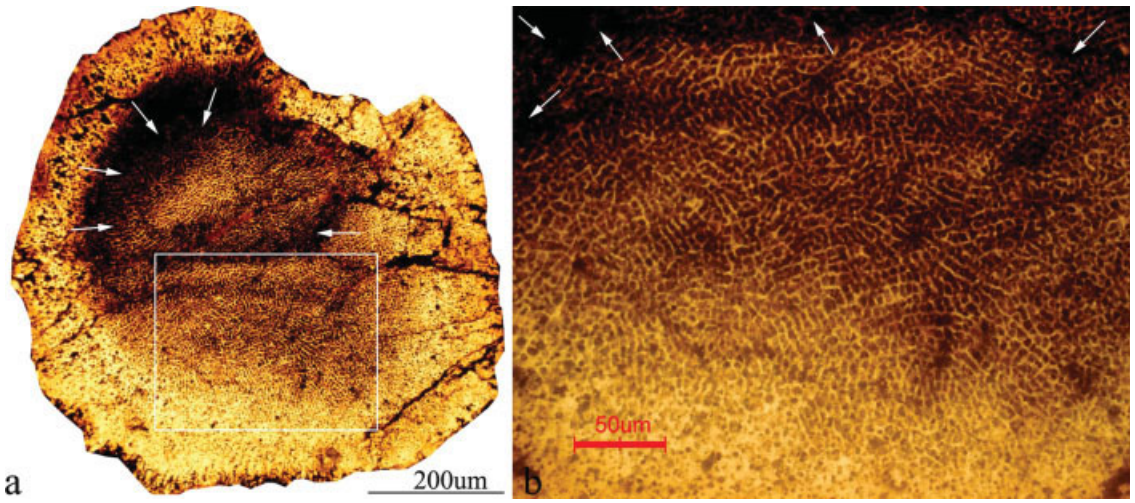

Fig. 5. The alga fossil of *Wengania globosa*. (**a**) the alga image taken by transmission light microscope; (**b**) enlargement of (a) in rectangle part.

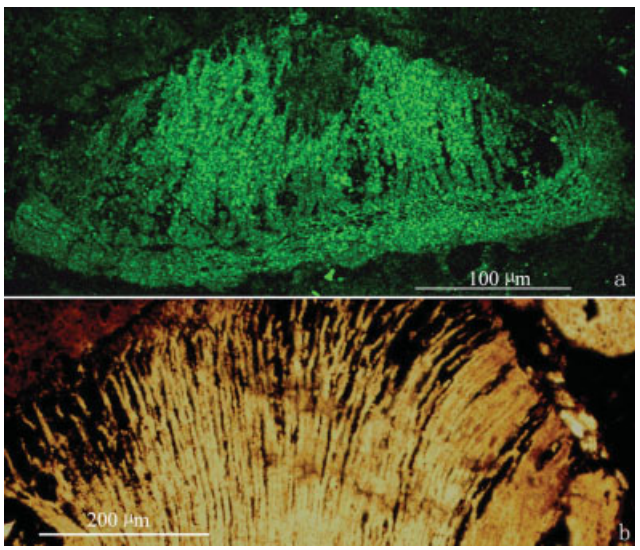

Fig. 6. The cell fountain structure of the alga fossil. (**a**) The 3D fluorescent reconstruction of the fountain-like structure of the alga; (**b**) the transmission light microscopy of the cell fountain structure of the alga fossil.

Figure 1a was crystallized very well most probably because there were more interspaces inside it than the other cells. Thus the minerals surrounding could be easy to enter these structure and finish the replication process. Thus under transmission light microscope we could see these parts with bright color and under confocal laser scanning microscope we could see obvious fluorescence. And the cells nearby the fiber were crystallized rapidly and meanwhile the inner cells continue the decay process until the mineralization process finished.

The decay event also occurred in the outer part of the fossil indicating by ellipse in Figure 1a. However, the decay with big area in ellipse produced enough interspace for minerals from surrounding to finish the replication process and crystallized quite well. Thus the light from this decayed part was stronger relatively.

Figure 2 indicated a possible fossil embryo with two crystallized layers. We could see one part indicated by rectangle was not as well-preserved by mineralization as the other part. In transmission light image (Fig. 2b) we could see the blurred part in rectangle was not crystallized as the other part so that we estimated this part was decayed. The 3D fluorescent reconstruction of the embryo indicated that this part has not distinct fluorescent signal in Figure 2a. Through the observation of the alga and possible embryo in Figures 1 and 2 and the other fossils from this geological area we speculate that decayed part of the fossils could preserve its amorphous shape, which fluoresced weaker relatively than that of the nondecayed part in the same condition when excited by laser light.

Because there was no fibrous structure outside the animal cell was easier to be replicated by minerals in surrounding. Thus the whole of the embryo was crystallized rapidly before the decay process began in such an embryo with small size (Fig. 2). Under confocal laser scanning microscope we could also get the whole fluorescent image of the embryo. However, we still have no evidence to judge whether the outer layer and some inner part of the embryo was diagenetic origin or not.

Nevertheless, we could know the diagenetic part of the similar two-layer globular specimen indicated in Figure 3. From Figure 3 we know it could be some kind of alga fossil because there was the cell-wall structure preserved inside the crystallized layer. We could also see a thin layer inside the crystallized layers indicated by arrows with brown color in Figure 3b. We considered it was the original organic outer layer of the alga. And it was preserved between two crystallized coatings. Because the outer coating form a relatively close

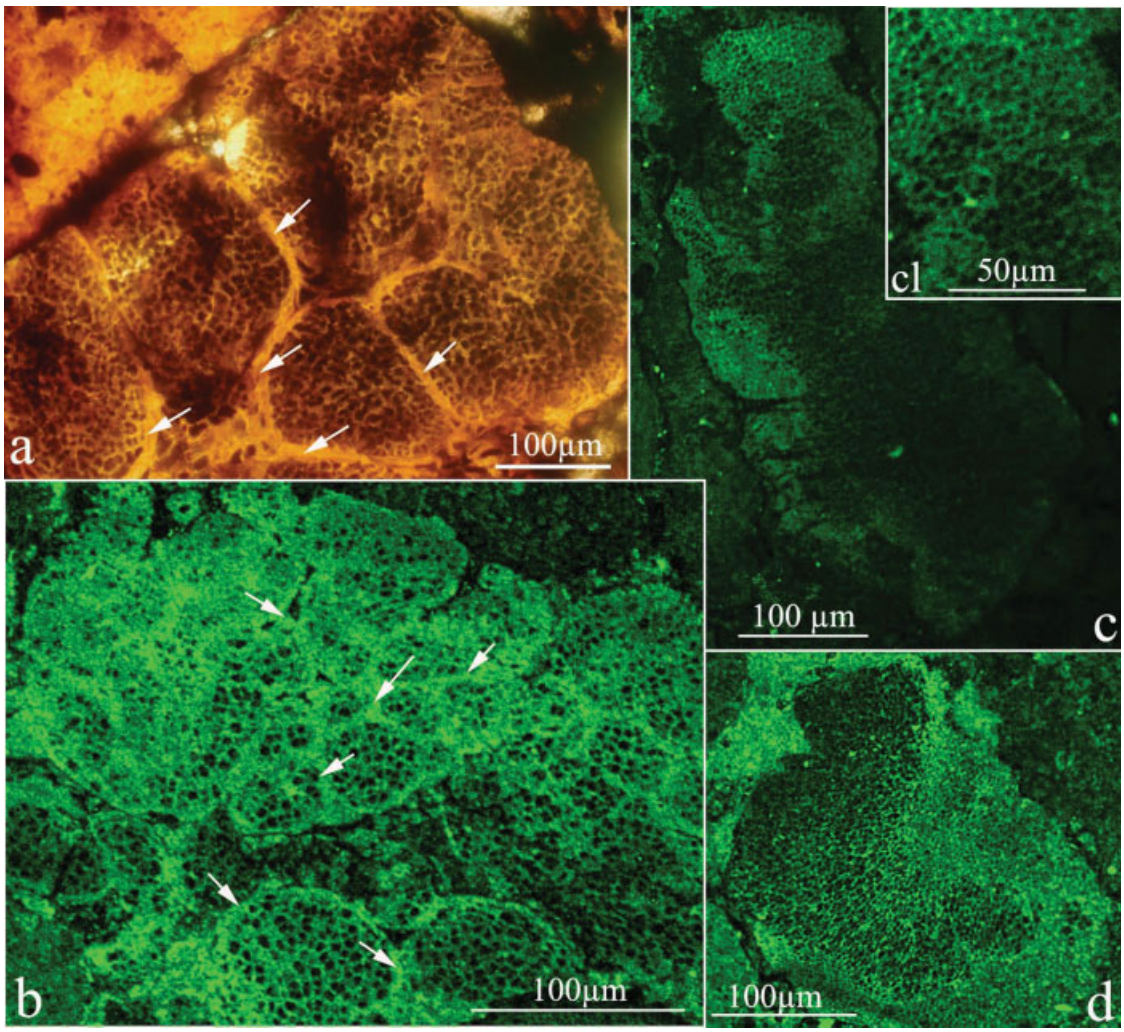

Fig. 7. The 3D reconstruction of the fossil algae by confocal laser scanning microscope. (**a**) Berry-like clusters interpreted as carposporangia and carpospores by Xiao et al. (2004) taken by transmission light microscope; (**b**) the 3D reconstruction of alga fossil in (a) by confocal laser scanning microscope; (**c**) the 3D reconstruction of the alga fossil by confocal laser scanning microscope; (**d**) the 3D reconstruction of the alga fossil by confocal laser scanning microscope.

globular, the inside part was not occupied by minerals completely. From the part in rectangle in Figures 3a and 3b we could also know this part was the result of second diagenesis because we could know from the fluorescent image in Figure 3a this part existed on its own and not belonged to the two crystallized layers.

The Quantitative Analysis of the Fluorescence of the Decayed Part of Alga Fossil

To confirm our observation of fluorescence from the decayed part and the nondecayed part we made the quantitative fluorescent analysis of the fossil cells of alga in Figure 1. From the surface plot in Figure 4b we knew the fluorescent signal of the decayed parts was absolutely weaker than that of the nondecayed parts. Then we selected the typical decayed parts and nondecayed parts to make plot profile, indicating in Figure 4c. Through Figure 4c we could see clearly that intensity of the fluorescence of the decayed parts (1, 2, 5 in Fig. 3) was definitely lower than that of the nondecayed parts (3, 4, 6 in Fig. 3). This result indicated that the process of mineralization would affect the fluorescence of the fossil cells. And the 3D fluorescent reconstruction of the fossil cells by confocal laser scanning microscope could help us to judge the mineralization state of the fossil cells.

The Preservation and Fluorescence of the Other Algae Fossils We Selected

We observed more than hundreds of the algae fossil specimens and we found that almost all of the specimens we selected could fluoresce more or less when excited by laser light. We made three-dimensional reconstruction of the fossils and found that most of the specimens we selected could not form the image with the same information as the image taken by transmission light microscope. We could hardly get the whole

image of the fossil by confocal laser scanning microscope, especially the algae fossil like *Wengania globosa* indicated in Figure 5. We found the dark part under transmission light inside the globular fossil (Fig. 5) has weak fluorescent signal and the light part outside the fossil has stronger fluorescent signal relatively than the inner part. The same result occurred on alga fossil indicated in Figure 7c. Thus we know that the outer parts of these algae fossils with light color were crystallized pretty well. As we know that the decay event accompanied the mineralization of the fossil cells (Xiao et al., 1999), the outer layer of these fossils would be replicated first and then did the inner parts to some extent. After mineralization the outer layer would prevent the minerals in surrounding come into the inner parts. Thus decay event continued in the inner parts indicating by arrows in Figure 5 until the whole fossil was mineralized. However, the decayed inner part was not crystallized as well as the outer part so that the light illuminated from the inner part was weaker than the outer part.

Concerning the fluorescence of the other kind of algae such as the cell fountain structure in algae, it could emit stronger fluorescence relatively. We could observe the fluorescence in Figure 6a. From the transmission light microscope we could also observe that these cell fountain structures were crystallized quite well (Fig. 6b). Thus these structure could illuminate strongly and show light color under transmission light microscope (Fig. 6b). The cell fountain structure indicated in Figure 6 had enough interspace among every thread-like structure and thus the whole part was replicated and crystallized rapidly. Therefore, we considered that the rate of mineralization rested with the interspace among cells and whether the minerals outside could enter easily.

The other algae fossils indicating in Figures 7b and 7d could also fluoresce quite well. And the fibrous structure indicated by arrows in Figures 7a and 7b was crystallized first and then the inner cells were replicated and crystallized. Thus the decay process also proceeded in the inner cells until the mineralization process finished. Moreover the decay process occurred first outside the alga in Figure 7d so that the minerals in surrounding entered rapidly and all inner cells were crystallized without decay. Thus we could get the fluorescent image in Figure 7d.

CONCLUSION

It is the first time to observe the multicellular fossil specimens from Doushantuo Formation in details by confocal laser scanning microscope. And from the fluorescent reconstruction we know that the fluorescent signal is related with the mineralization state of the fossils. The decayed parts in the center of the fossil alga have lower fluorescent signals than that of the neighboring nondecayed parts, which indicate that crystallization process of decayed parts was not as good as the nondecayed parts in the same condition. Decay of a large number of cells resulted in rapid crystallization. The 3D fluorescent reconstruction of the fossil by confocal laser scanning microscope could reflect the decay state better than the image taken by transmission light microscope. And from our observation we found that almost all the fossils could fluoresce more or less under laser light. Therefore, confocal laser scanning microscope is a good method to study the preservation state of phosphatic fossils, compared with the transmission light microscope.

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