Analysis of Fluorescence From Algae Fossils of the Neoproterozoic Doushantuo Formation of China by Confocal Laser Scanning Microscope

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KEY WORDS algae fossil; confocal laser scanning microscope; fluorescence; three-dimensional image

ABSTRACT Chinese algae fossils can provide unique information about the evolution of the early life. Thin sections of Neoproterozoic algae fossils, from Guizhou, China, were studied by confocal laser scanning microscopy, and algae fossils were fluorescenced at different wavelengths when excited by laser light of 488 nm, 476 nm, and 568 nm wavelength. When illuminated by 488 nm laser light, images of the algae fossils were sharper and better defined than when illuminated by 476 nm and 568 nm laser light. The algae fossils fluoresce at a wide range of emission wavelengths. The three-dimensional images of the fluorescent algae fossils were compared with the transmission images taken by light microscope. We found that the fluorescence image of the confocal laser scanning microscope in a single optical section could pass for the transmission image taken by a light microscope. We collected images at different sample depths and made a three-dimensional reconstruction of the algae fossils. And on the basis of the reconstruction of the three-dimensional fluorescent images, we conclude that the two algae fossils in our present study are red algae. *Microsc. Res. Tech. 69:253–259, 2006.*

INTRODUCTION

The Neoproterozoic microfossils, from the Doushantuo Formation in Weng'an, Guizhou, China, very likely played a role in the evolution of early life. This preserved biota also includes the ancient animal egg fossils, algae fossils, and adult animal fossils (Chen at al., 2000, 2002, 2004; Xiao et al., 1998b, 2000). When sponge fossils with cellular structures and animal egg fossils were reportedly found in the Doushantuo Formation, many geologists and biologists came to know the importance of these biological remains (Li et al., 1998; Xiao et al., 1998b). In addition, small bilateral animal fossils and the lichen fossils have been found in the Doushantuo Formation (Chen et al., 2004; Yuan et al., 2005). The algae found as fossils, which included the red algae, the brown algae, the green algae, etc., were the dominant photosynthetic organisms in the Precambrian ocean (Yuan et al., 2002). Two kinds of preserved Doushantuo algae fossils in China have been found: one was the mineralized algae fossil and the other was a carbonaceous compression of the algae fossils (Yuan et al., 2002). Red algae, brown algae, and green algae have been found both in the carbonaceous compressions and in the mineralized fossils (Xiao et al., 1998a, 2002; Yuan et al., 2002). The algae fossils used from the Weng'an biota in this study were the mineralized fossils.

In the Weng'an biota, red algae are an important representative, and cells of differentiated tissues, including fibrous cortical tissue and cell wall, have been studied in detail (Zhang, 1989; Zhang and Yuan, 1992). The sexually reproductive structures of the Neoproterozoic red algae have also been reported (Zhang, 1997; Zhang and Yuan, 1995), and the brown algae and the green algae fossils have also been found in Weng'an Biota (Xue et al., 1995, 2001; Yuan et al., 2002).

In previous studies, transmitted light images and scanning electron microscope (SEM) images of the Neoproterozoic algae fossil were recorded (Xiao et al., 1998a, 2004; Yuan et al., 2002; Zhang, 1989; Zhang and Yuan, 1992). Fluorescenct images of the Neoproterozoic algae fossil have until now not been reported.

Confocal laser scanning microscope (CLSM) was conceptualized in 1953 and was later used for biological research, chemical analysis, and material testing. The CLSM detects structures by collecting light from a single focal plane of the sample and excluding light that is from a different focal plane. An image is collected for each specimen focal plane in the fossils. From this stack of digital serial optical sections of the specimen, a three-dimensional image is reconstructed (by Leica confocal software version 2.00).

Fluorescent fossils have been found by paleontologist and geologist, using a fluorescent microscope and a CLSM. During the investigation of the Eocene Messel oil shale in Germany, the algae layers were found by means of the fluorescent mode of the CLSM. In this work, the fluorescent character of algae was not the focus of the work (Nix and Feist-burkhardt, 2003). Fossil pollen and spores have been used by palynologists

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for studying the geological time scale, employing the fluorescent microscope (Yeloff and Hunt, 2005). Feistburkhardt and Pross (1998) have studied the Middle Jurassic dinoflagellate cyst marker species by using the CLSM.

In this article, we report that the Neoproterozoic algae fossils are fluorescent, and we have analyzed the changes of the fluorescence at different excitation wavelengths. We have also made 3D images of the fossil algae, using CLSM and compared these results with transmission light images.

MATERIALS AND METHODS

Samples Prepared

The samples included in the present study were collected from the Weng'an Biota, Doushantuo Formation, Guizhou, China, which has been estimated to be 580-million-years old (Condon et al., 2005). The geological setting was reported by Chen et al. (2002). The fossil rocks were cut into small square pieces and glued on the glass slide by epoxy resin. This rock section was polished until it became thin enough for observation in a light microscope in the transmission mode. Usually, the thickness of the fossils was about 50 μm.

Observation of the Thin Section of the Fossils

Transmission Light Observation by Light Microscopy. Using a Nikon E800 light microscope connected with a digital camera, we observed the polished rock sections and recorded digital images of the algae fossils.

Laser Confocal Scanning Microscope Observation. Using a LEICA TCS SP equipped with ArKr laser, which can emit light in 488 nm, 476 nm, and 568 nm, we observed the fossil specimens. In the reflection mode, the LEICA TCS achieves an *x*-/*y*-resolution of 0.18 μm and a corresponding *z*-resolution of better than 0.35 μm at 488 nm. (N.A. 1.32, glass-air interface, ideal environmental condition). Three-dimensional reconstructions of the algae fossils were made in different emission and excitation conditions, as described in the results of this article.

RESULTS AND DISCUSSION

We have observed that algae fossil fluorescence at different emission wavelengths when different excitation wavelengths illuminate the fossil specimens. The following were the results we got.

Quantitative Analysis of the Algae Fossil Images Scanned by CLSM at a Wavelength of 488 nm

The 488 nm wavelength laser source was chosen to illuminate the fossil specimens as shown in Figure 1. The emission wavelengths were divided into four parts, which were green, yellow, orange, red & infrared light. Using the 488 nm excitation wavelength, we chose to separate the fluorescence emission into four wavelength intervals when scanning the specimen. Subsequently, the specimen fluorescence emission was scanned by combining all four wavelength intervals. We could get images of the algae fossil by using the four separate wavelength intervals as well as combing all the wavelength intervals in one scan. Not surprisingly, the fluorescent images were different with each other and which means we could, in theory, get different biological information from each image scan.

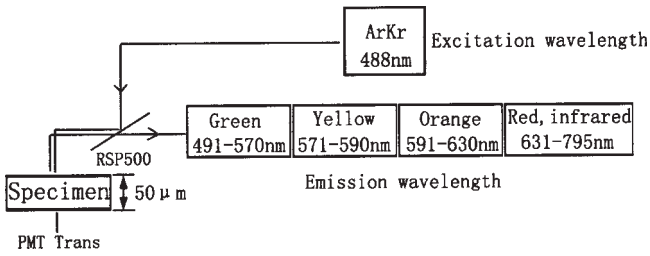


Fig. 1. Conditions used in the observation of the algae fossils by confocal laser scanning microscope (in 488 nm excitation wavelength, the emission spectrum was divided into four intervals: green, yellow, orange, red, and infrared light.).

Figure 2 shows the algae fossil image scanned at different emission wavelengths: image 2a, 491–570 nm; 2b, 571–590 nm; 2c, 591–630 nm; 2d, 631–795 nm; and 2e, 491–795 nm. We could almost record the whole algae fossil image in 2a, 2b, and 2c except that the intensity of the fluorescence emission was different. In image 2d the fluorescent signal was weaker than 2a, 2b, and 2c. From the graphs on the right of image 2a, 2b, 2c, 2d, and 2e, we know that the intensity of fluorescence of 2a, 2b, and 2c was similar and intensity of fluorescence of 2d was lower than the former three. Thus in red and infrared emission spectrum, the fluorescent signal is weaker than in any of the green, yellow, and orange emission spectrum. When the four fluorescent emission intervals were combined, the image in 2e was the result. In the graph beside 2e, the fluorescent intensity was higher than any of the four separated emission intervals; however, it was lower than the added intensity of the four separate intervals. Nonetheless, the quality of the image in 2e should be better than in any of the four separate wavelength interval images.

Analysis of the Fluorescence Emitted From the Alga Fossil Using Three Excitation Wavelengths

We collected fluorescence information about the fossil algae, using monochromatic 488 nm excitation light. The fossil algae fluorescence excited by 476 nm and 568 nm monochromatic light excitation was also recorded. Here the scanning work was also made when the two excitation wavelengths were chosen. The broadest emission spectrum was chosen when the fossil algae were excited with 476 nm, 568 nm, and 488 nm light, and a comparison between the fluorescent images was performed.

The Figure 3 fluorescent images were the result of the three excitation wavelengths. The graph of the fluorescence from the length of the fossil algae we know that illumination with 488 nm light, produces a stronger fluorescence signal than with 568 nm or 476 nm light. Therefore, 488 nm laser excitation and fluourescence emission from 491–795 nm produced a better fluorescent image than any other excitation condition and any other monitored emission wavelength intervals. The excitation and emission conditions aforementioned were chosen to make the three-dimensional CLSM images of the algae fossil specimens.

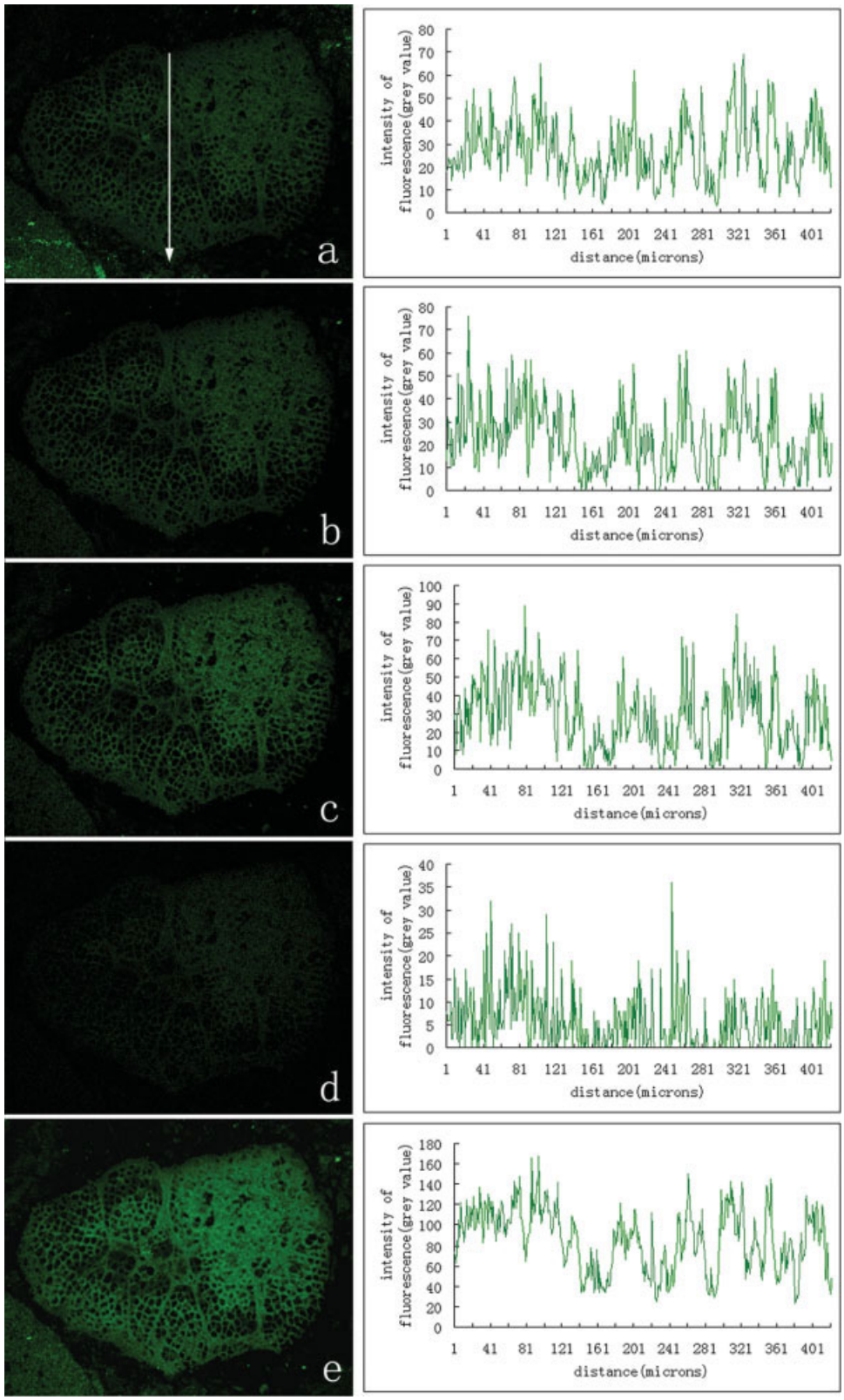


Fig. 2. Fluorescent images of the alga fossil in different emission wavelength in 488 nm excitation wavelength. **a**: The image scanned with 491–570 nm emission wavelength (green range of the visible spectrum); **b**: The image scanned with 571–590 nm emission wavelength (yellow range of the visible spectrum); **c**: The image scanned with 591–630 nm emission wavelength (orange range of the visible spectrum); **d**: The image scanned with 631–795 nm emission wavelength (red range of the visible spectrum and part of infrared spectrum); **e**: The image scanned with 491–795 nm emission wavelength. The graphs on the right of the corresponding images were the quantitative fluorescence analysis of the images along the arrows in image (a). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.].

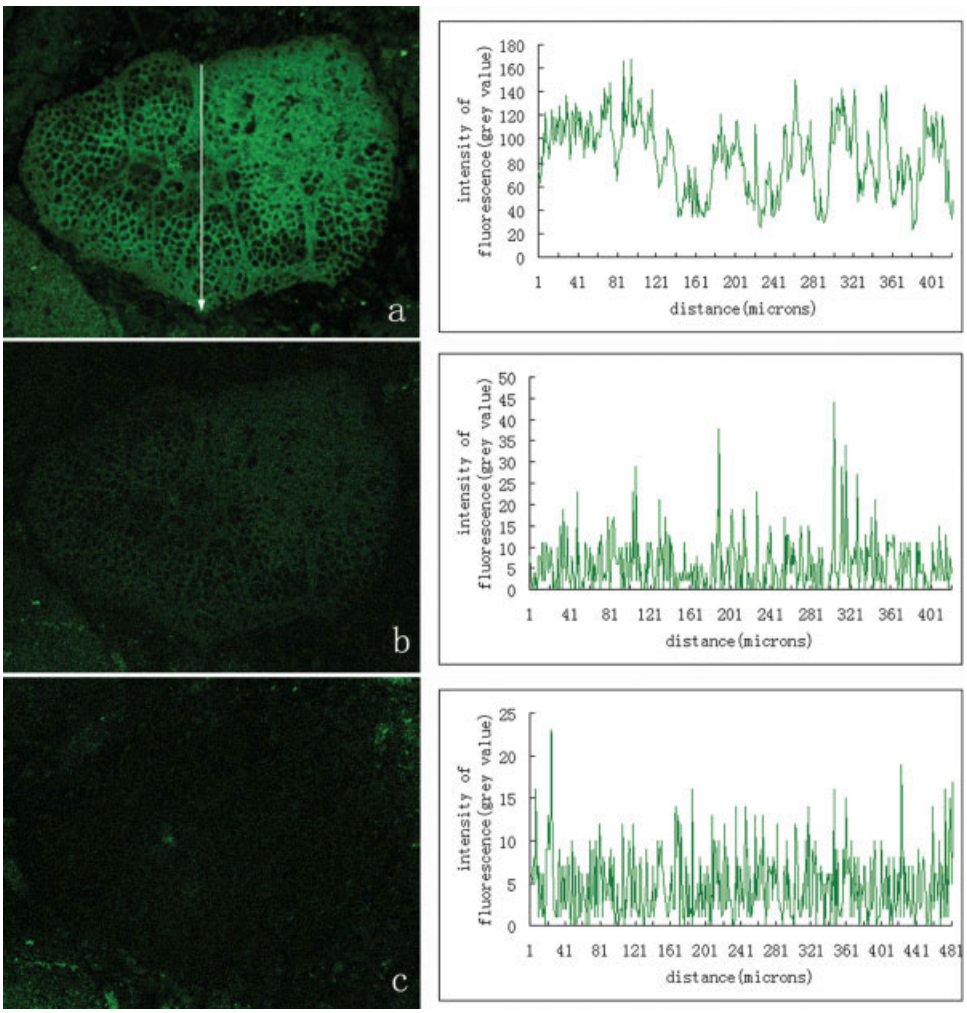


Fig. 3. Images are scanned by confocal laser scanning microscope in different excitation wavelength. **a**: The image scanned with 488 nm excitation wavelength; **b**: The image scanned with 476 nm excitation wavelength; **c**: The image scanned with 568 nm excitation wavelength. The graphs on the right of the images were quantitative analysis of the fluorescence of the corresponding images along the arrow in image (c). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.].

Transmitted Light Images and Three-Dimensional Images of Fossil Algae Scanned by CLSM

The CLSM can scan at different depths within the fossil specimen by excluding fluorescent light from outside of the plane of focus. Each plane of focus was used to reconstruct the three-dimensional image of the specimen. These different planes are shown in Figure 4 as a series of images from image 4a to image 4p. Each image was scanned by the CLSM from the top layer to the bottom layer of the algal fossil with the same focal depth for each plane. The fluorescence of the different focal planes began as a weak fluorescence to strong and to weak fluorescence again (from 4a to 4p in Fig. 4). And then all the images were synthesized automatically to a three-dimensional image about 50 μm in thickness as shown in Figure 5. In contrast, the transmission light image just shows the image of one plane at a time with interfering light scattering from planes above and below the focus image plane. The size of the algae fossil in Figure 5 was 500 μm in length and 300 μm in width. The size of the algae fossil in Figure 6 was 540 μm in length and 350 μm in width. The transmission light images taken are shown in Figure 5a and Figure 6a. Through comparison between transmission light images and fluorescent images, we knew that the fluorescent images express almost all the information that was caught by transmission light microscope so that the algae fossils could be represented completely by the three-dimensional fluorescent images as shown in Figure 5b and Figure 6b. The portions as arrows indicating have more structures in Figure 5b and Figure 6b than that in Figure 5a and Figure 6a. Therefore, the three-dimensional images of the algae fossils scanned by CLSM present more information than the images taken by light microscope.

The algae fossils involved in this study were found by Zhang et al. (1989, 1992) in their studies of multicellular thallophytes with differentiated tissues and cells from the same geological area. The alga fossil in Figure 5 could be *Paramecia incognata* and alga fossil in Figure 6 could be *Thallophyca phylloformis* (?), according to the comparisons of morphology between algae fossils. In Figure 5b (the three-dimensional image is scanned by CLSM), the fibrous cortical tissues were shown clearly extending to inner parts of the fossil. These structures have also been found by Zhang and Yuan (1992). However, in our study we could see them clearly and preserved completely in three-dimensional images, while observed by the transmission light

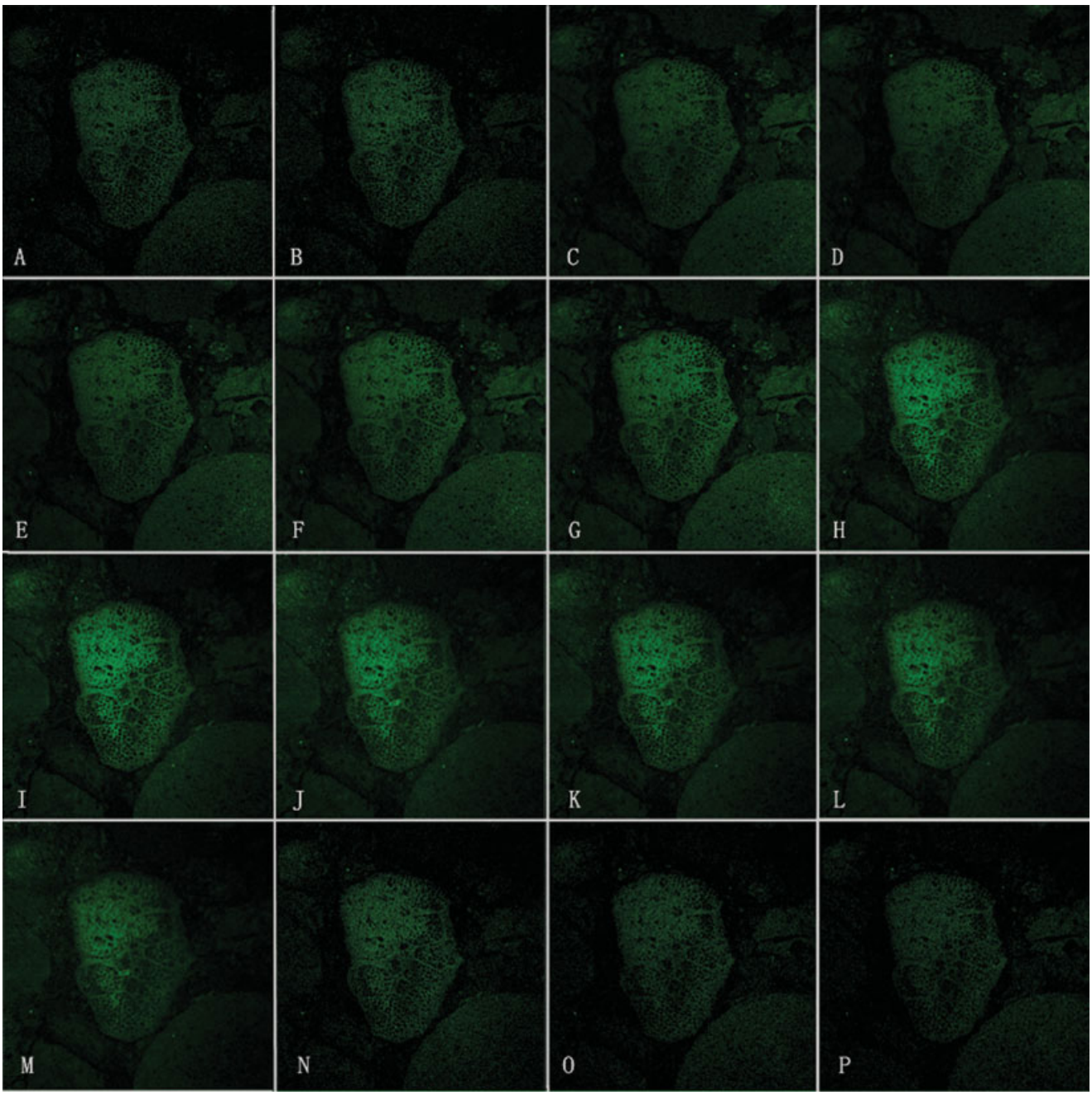
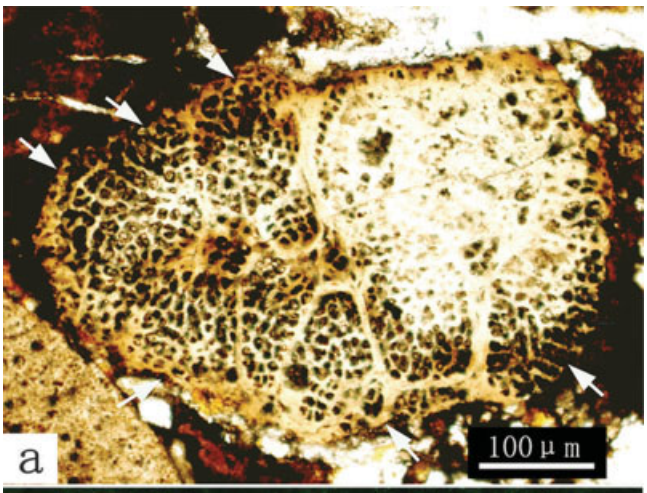
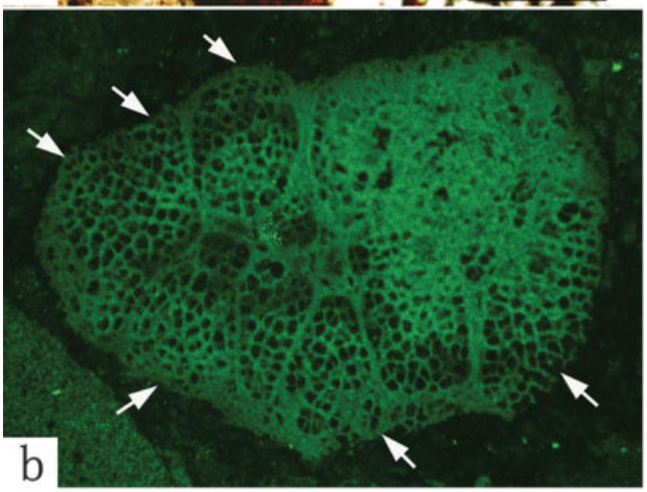


Fig. 4. The images from different focus plane are scanned by confocal laser scanning microscope. The thickness is about 50 μm from (**A**) to (**P**) and the distance is average between each plane. Fluorescent signal is greatest in the middle part of the fossil and become weaker gradually along the depth.

microscope they were not clearly seen. And the cell walls shown in Figure 5b were preserved, integrated, and connected closely with one another. However, in Figure 5a (the transmission light image), parts of the fibrous tissues and cell walls could not be shown and it presented an alga fossil with poor preservation. In this transmission light image, we could not judge the position of some cells and contour among them. In Figure 6, it was also the comparison between the transmission light image and the fluorescent image of another fossil alga, *Thallophyca phylloformis* (?). In Figure 6b, the reconstruction of the alga fossil showed more integrated cell wall structures than the transmission light image of the fossil in Figure 6a.

Usually, the transmission light images of the algae fossils from thin sections are reported in most studies of Neoproterozoic algae fossils or even Neoproterozoic microfossils. The affinities of the algae fossils were interpreted after the morphological comparison was made with the modern algae. However, only a few





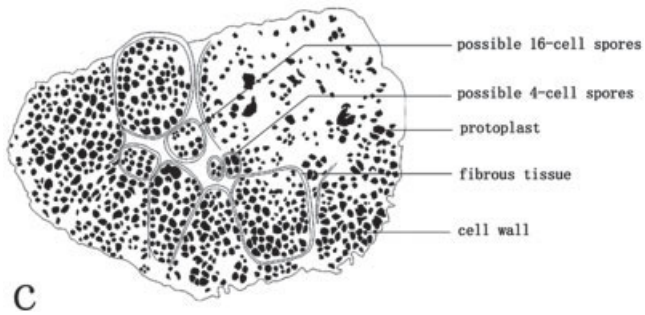
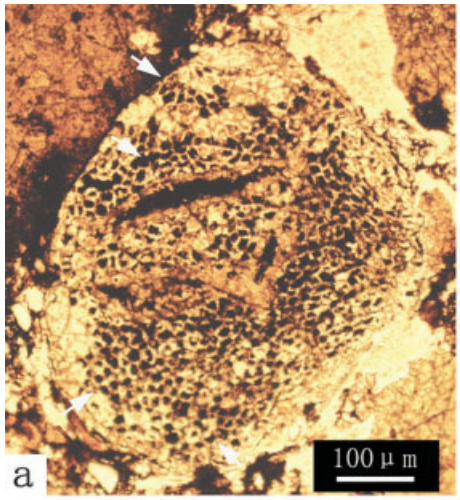
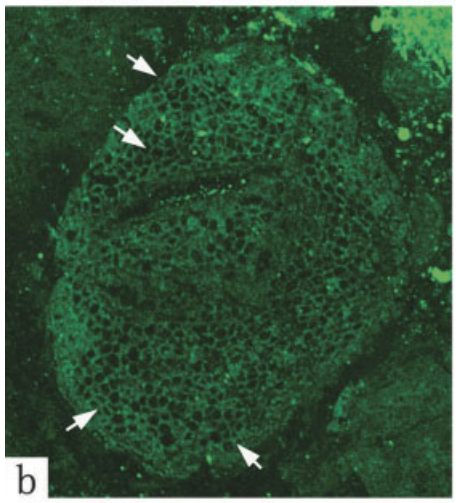


Fig. 5. Transmission light image is taken by light microscope and three-dimensional fluorescent image is scanned by confocal laser scanning microscope. **a**: The transmission light image of alga fossil is taken by light microscope, the scale bar represented 100 μm; **b**: Three-dimensional image is scanned by confocal laser scanning microscope with 488 nm excitation wavelength and the emission wavelength was from 491 nm to 795 nm. The image was scanned in *xyz* mode. And the image scanned by confocal laser scanning microscope contained more detail information than the transmission light image taken by light microscope in the portions as arrows indicating. **c**: Rough drawing of the alga fossil in image 5(b). The black areas indicate protoplast that were not preserved. The white areas around the black areas were cell walls with good preservation. The alga cells were surrounded by fibrous tissue with good preservation.





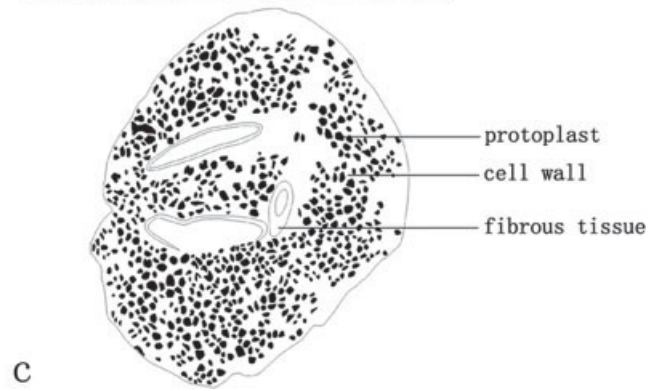


Fig. 6. Three-dimensional image of another alga fossil is scanned by confocal laser scanning microscope and transmission light image is taken by light microscope. **a**: Transmission light image of the alga fossil taken by light microscope; **b**: Three-dimensional image of the same alga scanned by confocal laser scanning microscope with 488 nm excitation wavelength and the emission wavelength was from 491 nm to 795 nm. The scale bar in image (a) represented 100 μm. And in the portions as arrows indicating the image scanned by confocal laser scanning microscope contained more detail information than the image taken by light microscope. **c**: Rough drawing of the alga fossil in image 6(b). The black areas indicate protoplast that were not preserved. The white areas around the black area were the cell walls with good preservation. Fibrous tissues were also preserved. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.].

microfossils with good preservation among hundreds have been found by light microscopy. Actually, the transmission light image only presents one plane of the fossil specimen and the other planes are not in focus and light from these planes can add noise and artifacts to the image.

The SEM has also been used in the study of algae fossils (Xiao et al., 1998b, 2004). SEM is only good for surface topological observation and for palaeontologists, it is mostly used for observation of surface structures of fossils from acid residues. In the study of algae fossils from Neoproterozoic Doushantuo Formation in Weng'an, Guizhou, China, Xiao et al. (1999), the SEM was used to observe the surface of thin-sectioned algae fossils.

CONCLUSION

In conclusion, the Neoproterozoic algae fossils in this study emit fluorescence when excited by our laser source. Using the 488 nm excitation wavelength, the definition of the fluorescent image of the fossil sample was better than when a 568 nm or when a 476 nm excitation wavelength was used. The emission wavelengths of the algae fossils occurred between 491 nm and 795 nm, and the image scanned by the laser was clearer than images taken with the transmission light microscope. Images scanned by CLSM also contained the similar structural information when the same sample plane was observed as the images taken with the transmission light microscope. With the CLSM, three-dimensional fluorescent images of the algae fossils were obtained, from a series of fluorescing image planes. On the basis of the comparison with algae fossils reported by other researchers, we suggest that the two algae fossils in the present study are red algae (Zhang, 1989; Zhang and Yuan, 1992) with a similar morphology.

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