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Examining Trichoptera Genitalia with Confocal Laser Scanning Microscopy

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Introduction

Confocal microscopy is a common tool that is based on the principles of fluorescent microscopy. It has been around for decades, but it has mainly been used by cellular biologists to view intracellular structures labeled with fluorescent dyes. Recently, confocal imaging has been used for examining internal morphology and the three-dimensional shape of the cuticle in Blattodea (*Periplaneta*, *Blaberus*; Larsen *et al*. 1997, Zill *et al.* 2000), and as well as muscle tissue and internal organs in Diptera (*Culex*, *Drosophila*; Klaus *et al*. 2003). This technique takes advantage of the fact that some types of insect tissue, including most cuticular structures, are autofluorescent under red (543 nm) laser excitation. The fluorescence is detected by the microscope and compiled into images by computer software. What makes confocal microscopy potentially useful for taxonomy and systematics is that it takes image slices through the specimen at user-defined intervals and depths. This "digital dissection" process assures that only the light that is in focus in any one optical plane is captured. Out-of-focus background noise, which often obscures images taken with conventional light microscopy, is eliminated. Each digital slice can be examined individually, or the slices can be compiled and rendered into a three-dimensional image that can be rotated about the X, Y or Z-axis. This allows the investigator to see a three-dimensional representation of the internal connections within a specimen without physically dissecting it.

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This technique is potentially useful for working with rare specimens, voucher specimens, type material, or other material that cannot be dissected practically or altered permanently.

In this report, we used an adult male specimen of *Polycentropus confusus* Hagen as a test species to measure the autofluorescence of the caddisfly genitalic structures, and to see if the three-dimensional images produced from confocal laser scanning microscopy would contain information useful to taxonomic and phylogenetic studies. Specifically, we wanted to know whether this technique can be used to view internal connections between membranous and sclerotized portions of the male phallus.

Methods

The protocols for mounting structures and taking images with the Carl Zeiss LSM 510 imaging software were adapted from Klaus *et al.* (2003; see reference for detailed imaging procedures).

Mounting: The cleared male genitalia of *Polycentropus confusus* were mounted between two glass cover slips in a solution of 1:17:17 (w:v:v) gelatin:glycerine:water (plastic cover slips cannot be used because they will melt during sample recovery). The mounted structures were allowed to dry for approximately 30 minutes until solidified. It was important to position the genitalia as close to the surface of one of the cover slips as possible to ensure they were in the working range of the objective. The farther away the structure is from the cover slip, the longer the distance the laser has to penetrate; this can decrease resolution (some useful tips: press the cover slips together only slightly -- too much pressure can flatten and/or damage the structures; using a label pen to circle the structures on the coverslips makes it easeir to find them under the microscope).

Imaging: The male genitalic structures were imaged with a Zeiss LSM 510 Confocal Microscope with either the 10X or 40X objective by light or red laser (543nm). To assess whether regions of the cuticle were autofluoresent, images using either the laser of white light were taken (Fig 1, Panels 1 & 2). Light and laser images were then digitally overlayed (Fig. 1, Panel 3). Next, a serial set of images through the Z-axis was collected (Fig. 2 A-D). The complete series of image slices was then used to render a three-dimensional image of the phallus (Fig. 3).

Specimen Recovery: To remove the genitalia from the gelatin solution the cover slip mount was placed in a glass dish with distilled water and heated for approximately 20 minutes (low heat). When the solution had melted enough for the cover slips to be separated, they were pulled apart carefully with forceps in order to avoid tearing the genitalia. After the top cover slip was removed the genitalia were con-

Editor's Remarks

It is my pleasure to bring to you the third issue of *Nectopsyche.* I apologize for the delay in bringing you this issue. I am pleased with the articles included herein contributed by CJ Geraci & Richard Laughlin on confocal microscopy, Paola Rueda on adult-larva association, and Ralph Holzenthal on the latest Neotropical publications. I am very thankful for their contributions.

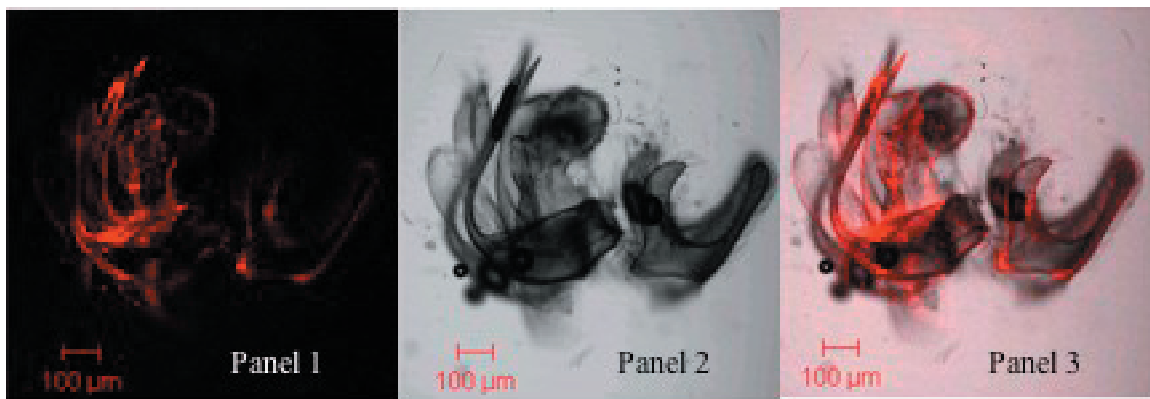

Figure 1. *Polycentropus confusus*, tergum X and genitalia of male. Panel 1: autofluorescence under the red laser (543 nm); Panel 2: bright field image; Panel 3: red fluorescence overlaid onto brightfield image (Carl Zeiss Microscope LSM 510 software, 10X objective).

tained in a drop of media on the bottom cover slip. The bottom cover slip was put back into the dish of distilled water and returned to the heat for approximately another 20 minutes (enough time to dissolve the media). Finally, the genitalia were removed from the cover slip and placed in the distilled water for an additional 30 minutes to fully dissolve the remaining gelatin. (Times are approximate, and may vary depending on the temperature used and the size of the mounted structures.)

Observations and Conclusions

Polycentropus confusus: Carl Zeiss LSM 510 imaging software allowed us to view each optical slice taken at the specified Z-axis interval and overlay the fluorescence onto the light image (Figure 1). This showed any structures that were not fluorescent. It is not fully understood why some some structures do not fluoresce while others do. All portions of the male genitalia of *P. confusus* were fluorescent. Figure 2 shows representative slices of the specimen at different Z-axis coordinates. We found the limit for the z-stack was ~150 um, with a minimum interval size of ~0.5 um. Viewing the individual image slices was useful for understanding connections in any one plane in the z-stack (Figure 2). The three-dimensional rendering was most useful for viewing internal structures, and the invagination of the endothecal membrane with its endothecal spines. Deleting the proximal slices exposed the invaginated endothecal membrane and setae, which could then be rotated about each axis to see where the membrane attached to a sclerotized tube (Figure 3).

Additional observations: We surveyed various other taxa to test their levels of autofluorescence. Muscle tissue in the femur of a baetid mayfly, the peritrophic matrix of a simuliid larva, and the exocuticle of a tardigrade all fluoresced brightly. However, hardened and darkened cuticle was not fluorescent, as has been reported previously (Zill et al. 2000). For example, Chironomidae larval mouthparts were fluorescent except for the mandibles, and the head capsule of *Micrasema rickeri* Ross & Unzicker did not fluoresce. We also found that setae and other hollow structures fluoresced brightly, but solid structures did not.

Conclusions: Confocal microscopy is useful for viewing arthropod cuticle and muscle tissue, but does not work well with darkened or solid cuticular structures. Confocal images could be used in addition to illustrations to show diagnostic characters whose three-dimensional shape is important. The internal connections between muscle and cuticle that are shown in the three-dimensional images can be used to help assess homology for phylogenetic studies. The most important factor in producing high quality images is properly mounting the structures between the cover slips. Damage to the structures may occur if care is not taken during the sample recovery process, or if the structures are compressed too much between the cover slips.

Free image analysis software: *Carl Zeiss LSM Image Browser* Version 3.5 available for free download from the Carl Zeiss web site (http://www.zeiss.com/)

Software for converting LSM files to QuickTime or .mpeg files: *ImageJ* (*Image Process-ing and Analysis in Java*) available for free download from the US National Institutes of Health web site (http://rsb.info.nih.gov/ij/)

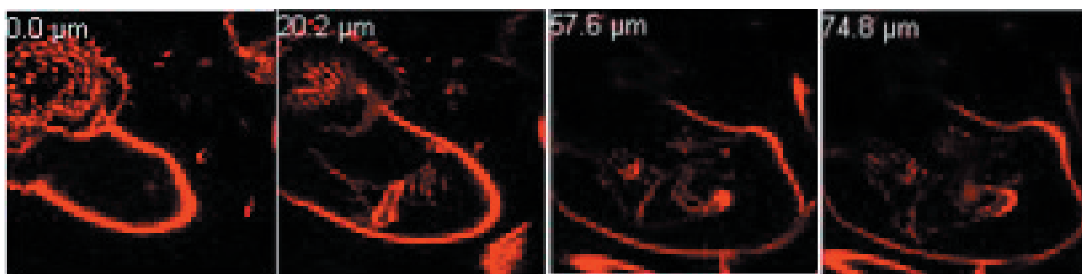

Figure 2. Digital slices of the right lateral view of the phallus of *Polycentropus confusus* taken at sample intervals (40X objective; z-stack range: 0.0 - 118.4 um, 1.6 um interval).

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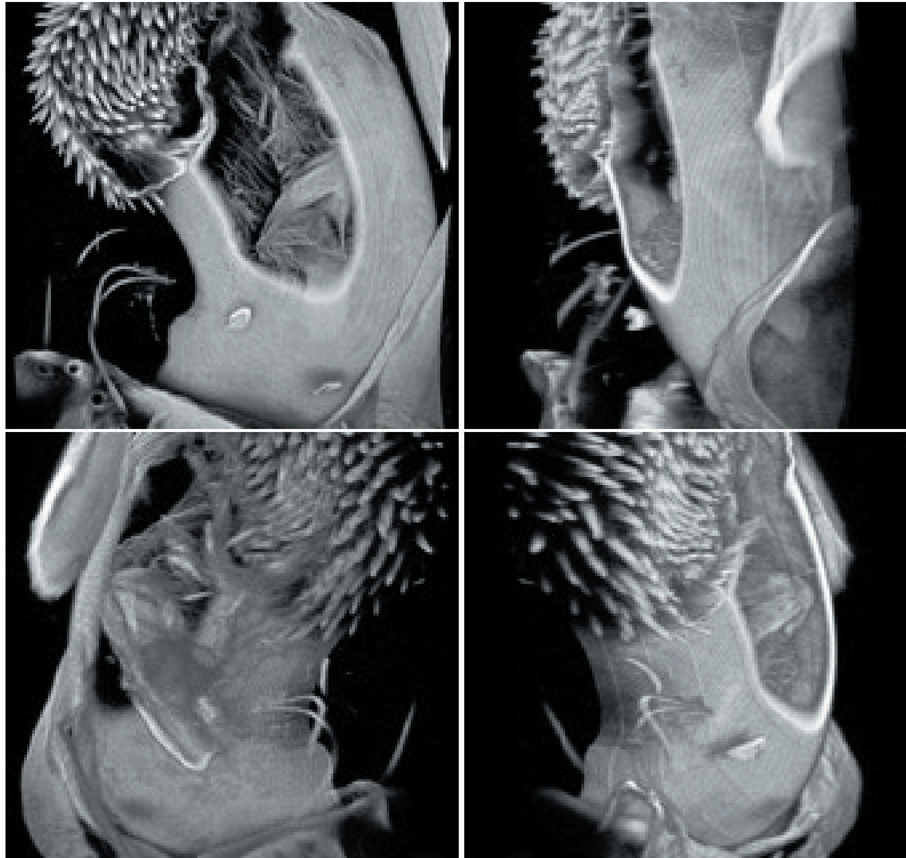

Figure 3. *Polycentropus confusus*, three-dimensional lateral view of right half of phallus and endothecal membrane rotated about the y axis; 40X objective.