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Two-dimensional versus three-dimensional morphometry of monogenoidean sclerites

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Abstract

A new method of three-dimensional (3-D) analysis of sclerotised structures of monogenoids was performed by processing z-series images using 3D-Doctor. Z-series were obtained from Gomori's trichrome-stained specimens of marine and freshwater monogenoids under laser scanning confocal fluorescence microscopy. Measurements obtained from 3-D images were then compared with those from 2-D images taken from both flattened and unflattened specimens. Data comparison demonstrated that 3-D morphometry allowed avoidance of over-estimation due to deformation and the reduction of errors associated with different spatial orientations. Moreover, study of 3-D images permitted observation of morphological details that are not detectable in 2-D representations.

Keywords: Three-dimensional morphometry; Laser scanning confocal fluorescence microscopy; Monogenoidea; Monogenean; *Kuhnia scombri*; *Haliotrema curvipenis*; *Dactylogyrus extensus*

1. Introduction

During the past two decades, the technology of computer-assisted image analysis has developed rapidly, providing biologists with powerful new tools of investigation. Its broad application in science has led to the development of a wide variety of image–data acquisition, treatment and quantification techniques (Müller, 2002), with morphometry receiving major benefits from the technology. The development of these techniques has allowed scientists the ability to overcome limitations on precision associated with obtaining two-dimensional (2-D) measurements of 3-D objects. Two-dimensional measurements are generally obtained from a single plane surface, which, for microscopic structures, is usually represented by the focal plane of an optical microscope, drawings obtained using a camera lucida or a photomicrograph (Minnich et al., 2003; Roff and Hopcroft, 1986).

Since the 1970s, SEM has been broadly applied in biology to morphological studies, but such techniques with limited depths of focus provide a false 3-D to micrographs and require the destruction of specimens to investigate structures (Justine, 1993; Shinn et al., 1993).

Medicine has contributed most to the rapid development of software dedicated to 3-D reconstruction, i.e., 3D-DOCTOR (Able Software Corporation, Lexington, MA 02420, USA), an advanced 3-D modeling, image processing and measurement software package used for magnetic resonance imaging (MRI), computer tomography (CT) scan and positron emission tomography (PET) for scientific and industrial imaging applications (Styner et al., 1999; Müller, 2002; Enciso et al., 2003). Recently, laser scanning confocal fluorescence microscopy (LSCFM) has been applied to 3-D reconstruction of fungi, invertebrate animals and mammalian cells (Dickson and Kolesik, 1999; Zill et al., 2000; Fritz and Turner, 2002; Ebara et al., 2002;

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Koehler et al., 2002; Klaus et al., 2002; Neves et al., 2005; Schawaroch et al., 2005; Sonnek et al., 2005). Galli et al. (2006) successfully used LSCFM to obtain 3-D images of the haptoral and male copulatory sclerites of members of the Class Monogenoidea (some authors refer to this class as the "Monogenea") stained in Gomori's trichrome (Humason, 1979). These sclerites are generally less than 50 μm long, are essential for taxonomic identification and are usually morphologically described and depicted as 2-D drawings obtained using a camera lucida and light microscope. Measurements are usually determined directly from specimens using a microscope equipped with an ocular or filar micrometer, from drawings, or less frequently, using a digitising system on photomicrographs (Ergens, 1969; Chisholm et al., 2001; Davidova et al., 2005). The problem with these methods rests with the fact that the sclerites of monogenoids do not normally lie within the visual plane of the microscope, thus requiring that specimens be exposed to moderate to heavy compression on the microscope slide to orient structures to the optical plane of the microscope prior to study (see Section 2 introduced by Malmberg, 1957; Ergens, 1969; Kritsky et al., 1978). Compression always results in the specimen being somewhat damaged or completely destroyed (squashed), inevitably producing both morphological artefact and metrical error. Moreover, such manipulations irreversibly compromise the natural relative and absolute positions of sclerites in the body, adding to morphometric error during analysis.

The purposes of this paper are three-fold: (i) to illustrate how z-series images of monogenoidean sclerites obtained from LSCFM can be processed with 3-D reconstruction and quantification software (3D-Doctor); (ii) to compare morphometric results obtained from 3-D morphometric analysis using LSCFM with those collected by traditional methods; and (iii) to demonstrate how movies obtained from LSCFM analysis can be integrated with original hand drawings of some intricate, complex sclerites of these helminths.

2. Materials and methods

Monogenoids were collected from marine and freshwater fish: *Kuhnia scombri* (Kuhn, 1829 in Sproston, 1945) from *Scomber scombrus* Linnaeus, 1758 (a marine fish from the Mediterranean Sea); *Haliotrema curvipenis* Paperna, 1972 from *Mulloidichthys vanicolensis* (Valenciennes, 1831 in Cuvier and Valenciennes, 1831) (a marine fish from the Red Sea); and *Dactylogyrus extensus* Mueller and VanCleave, 1932 from *Cyprinus carpio* Linnaeus, 1758 (a freshwater fish from Northern Italy). Comparison of measurements obtained from 2- and 3-D morphometric analyses were performed using specimens of *D. extensus*, while subjects for LSCFM studies included specimens of all three parasite species.

2.1. Processing specimens for confocal microscopy

Gill baskets of respective hosts were removed at the site of collection and placed in containers of hot (60 °C) 4–5% formalin to relax and fix the attached monogenoids. Fixed gills were placed in vials containing the respective fluid, labeled and stored until study. A formalin-fixed specimen(s) was subsequently removed from the gills or picked from the sediment using a fine probe and dissecting microscope and placed in NaOH (1 M solution) for 10 min before being transferred to a small droplet of Gomori's trichrome (Humason, 1979) located near the center of a small disposable Petri dish. After 1–2 min, the droplet containing the specimen(s) was flooded with absolute ethanol to stop absorption of stain. Destaining of the specimen(s) was accomplished by adding water to the dish to dilute the ethanol–stain mixture to about 50%. When the desired level of stain remained in the specimen, the helminth was removed with a fine probe and placed in absolute ethanol for about 1 min, after which it was transferred to beachwood creosote for clearing and mounting in euparal.

2.2. Confocal microscopy

LSCFM images of monogenoids were obtained by using a Leica TCS SP2 confocal microscope coupled to an inverted Leica DMIRE2 microscope equipped with a PL APO 63 × oil immersion objective (NA = 1.4). The sample was excited with the argon laser at 515 nm and fluorescence emission was collected through a band-pass filter between 525 nm and 730 nm. Images (8-bit) with 1024 × 1024 pixels per frame were obtained. Z-series were collected with a step size of 0.115 μm to maximize axial resolution of 3-D images.

2.3. Morphometric analysis

For 2- and 3-D morphometric analyses, 10 *D. extensus* were prepared according to the procedures described above, and another 10 specimens of *D. extensus* were collected live from the gills of *C. carpio* and flattened with coverslips on slides in ammonium picrate glycerine according to the procedures of Malmberg (1957). Eight linear measurements, illustrated in Fig. 1, were used to compare the methods of morphometric analysis. Specimens prepared under both methods (Gomori's trichrome and ammonium picrate) were observed with an optical microscope equipped with phase contrast and a calibrated micrometric lens to obtain the 2-D measurements of the haptoral and copulatory sclerites. Z-series in TIFF format were then collected from the 10 specimens stained with Gomori's trichrome using LSCFM and loaded onto 3D-Doctor software 4.0.061025 (Able Software Corporation). Voxel were calibrated using the TXT report file automatically generated by LCS. Image contrast and thresholds for segmentation were manually calibrated in order to maximize resolution and minimize loss of digital information. Three-dimensional surface models of each structure of interest were generated, and linear measurements were obtained with the dedicated tool in 3D-Doctor after appropriate rotation of the 3-D objects. A Principal Components Analysis (PCA) was conducted on data collected using the