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MICRO-CT AS A METHOD TO VISUALIZE INTRAMOLLUSCAN STAGES OF DIGENEA

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X-ray micro-computed tomography (micro-CT) is a non-destructive method widely used for visualization of three-dimensional structures. Application of micro-CT for comparative morphology is limited due to low x-ray contrast of soft animal tissues, but staining can increase image quality for such specimens. We suggest that micro-CT may be used for rough visualization of branched sporocysts of Digenea within intact hosts, and tested this approach on sporocyst of *Leucochloridium paradoxum*. Two infected mollusks were treated following two different protocols. One specimen was scanned in ethanol; the other was dried before scanning. Anatomical features of the host were better visible on microtomographic sections of the dried specimen. Regardless of the sample preparation, full-grown and underdeveloped broodsacs of the sporocyst were visible, but we could not trace its central part. We suggest how the micro-CT protocol can be modified for better results on branched digenean sporocysts.

Keywords: micro-CT, phosphotungstic acid, critical point drying, Digenea, *Leucochloridium paradoxum*, sporocyst, metacercariae, Gastropoda, *Succinea putris*

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X-ray micro-computed tomography (micro-CT) is a non-destructive method applicable for visualization of three-dimensional structures, widely used in the studies on various free-living (e.g. Parapar et al., 2018; Marcondes Machado et al., 2019) and parasitic (e.g. Noever et al., 2016; Martín-Vega et al., 2018; O'Sullivan et al., 2018) invertebrates. However, the application of this method for comparative morphology is limited due to low x-ray contrast of soft animal tissues (Metscher, 2009). Staining with phosphotungstic acid (PTA) and IKI (1 % iodine metal + 2 % potassium iodide in water) with subsequent scanning in ethanol is the main tool to increase contrast and quality of micro-CT images for such specimens (Metscher, 2009). Staining could be combined with incubation in dimethyl sulfoxide (Marcondes Machado et al., 2019) or freeze-drying after staining (Noever et al., 2016) for better results.

In micro-CT studies of digeneans authors used basic protocols (Lee et al., 2007; Martín-Vega et al., 2018) and critical point drying after staining (Bulantová et al., 2016). This method was successfully adopted to determine the localization of metacercariae and juvenile adults within the second or definitive host organs (Lee et al., 2007; Bulantová et al., 2016; Martín-Vega et al., 2018). Nevertheless, all these works focused on the hermaphroditic generation of digeneans while parthenogenetic generations (sporocysts and rediae) have so far been neglected. In a few digenean taxa sporocysts have branched bodies with modular organization, where each module is different in terms of function and morphology (Galaktionov et al., 2014). This makes them quite hard to study with traditional approaches like dissecting from host tissues or histological sections with subsequent 3D reconstruction. Though histological techniques provide essential information on the internal structure and tissue organization, this method is time consuming and gives sections in one plane only. Micro-CT does not reveal the histological structure but it provides sections of three planes, it is relatively fast and easy, and may be used for rough visualization of branched digenean sporocysts within the host body and for estimation of branch numbers. Micro-CT also could be applied to determine the host/parasite volume ratio, as was demonstrated for Rhizocephala (Nagler et al., 2017). We tested this approach on sporocysts of *Leucochloridium paradoxum* Carus, 1835 (Brachylaimoidea: Leucochloridiidae). The sporocyst of this species is well-known for its colored broodsacs and extremely branched body composed of functionally and morphologically different regions: a central part, broodsacs with infective metacercariae, narrowed stalks connecting the broodsacs with the central part and underdeveloped broodsacs with developing metacercariae (Pojmanska, Machaj, 1991; Ataev et al., 2013; Ataev, Tokmakova, 2015). Our study is a first attempt to apply micro-CT for intramolluscan stages of Digenea.

MATERIAL AND METHODS

Two specimens of amber snails, *Succinea putris* L., 1758, infected with *L. paradoxum* (referred here and onwards as S1 and S2) were collected at the Yuzno-Primorskij park of Saint-Petersburg in September 2017 and 2018. Sporocysts were visible through thin transparent shell of the hosts. Shells of mollusks were crushed and a small puncture was made in the mantle to provide a better infiltration by fixative and contrasting solutions. Samples remained intact without any dissection of the host. Infected mollusks were fixed in Zenker's solution with 100 % acetic acid (10:1). After 2 h fixation and 2 h rinsing in water, specimens were incubated in 70 % ethanol with iodine for 1 h and stored in 70 % ethanol for one week.

Contrasting protocol was generally adopted from studies of Metscher (2009) and Marcondes Machado et al. (2019). S1 was stained in 0.3 % ethanol solution of phosphotungstic acid (PTA) for 12 h, rinsed in 70 % ethanol for one hour to remove PTA, transferred to 96 % ethanol and stored in it for three days inside a 1.5 ml plastic tube. S2 was stained in mixture of 0.3 % PTA with 3 % dimethyl sulfoxide (DMSO) (10:1) for one week (Table 1). Next, S2 was gradually dehydrated through 70 %, 96 % ethanol, 96 % ethanol-acetone (3:1, 1:1, 1:3) and pure acetone (1 h in each liquid), and dried using a critical point dryer (CPD) (Hitachi HCP-2). Both samples were scanned using a microtomography scanner (Bruker SkyScan 1172). S1 was scanned twice in a 1.5 ml plastic tube filled with 96 % ethanol under slightly different scanning parameters (referred here and onwards as S1-1 and S1-2, Table 1). S2 was scanned once in a 1.5 ml plastic tube under a single set of scanning parameters (Table 1). Obtained data was processed using CTVox® and DataViewer® software packages (Bruker Micro-CT).

Table 1. Sample preparation and key parameters used for scannin

Sample	Contrasting	Dried in CPD	Acceleration voltage, kV	Source current, uA	Filter	Resolution, μm	Frame averaging for one scanning	Exposure, ms
S1-1	12 h in 0.3 % PTA	-	74	100	No	4.3	3	460
S1-2	12 h in 0.3 % PTA	-	74	108	No	2.5	5	470
S2	1 w in 0.3 % PTA with 3 % DMSO	+	58	161	Al 0.5 mm	3.03	3	1050

Sample rotation angle for all samples was 0.2 grade.

RESULTS AND DISCUSSION

In total we received three datasets, two for the first sample (S1–1, Fig. 1*A*; 2; S1–2, Fig. 1*B*; 3) and one for the second (S2, Fig. 4). S2 was overexposed, possibly due to extensive staining of the albumin gland by PTA (Fig. 4). Therefore, a microtomography scanner filter was applied to S2 during scanning to increase the contrast of other tissues (Table 1). We visualized all datasets with DataViewer® to generate three planes of microtomographic sections (Figs 2–4). Bright spots were observed in all datasets (Fig. 1–4), which are likely a scanning artifact. 3D reconstructions in CTVox® were performed only for S1–1 and S1–2 (Fig. 1). A 3D reconstruction for the S2 dataset is not shown here since the scanned wall of a plastic tube (Fig. 4) made it impossible to receive a high resolution image in CTVox®. This artifact possibly appeared due to the scanning parameters of S2, including filter.

3D reconstructions of both S1-1 (Fig. 1*A*) and S1-2 (Fig. 1*B*) give a general picture of the hosts morphology: head, foot, tentacles, lung and kidney are visible. Of the sporocyst, only broodsacs containing infective metacercariae could be clearly identified (Fig. 1). The resolution of S1-2 was higher than in S1–1, resulting in a better 3D reconstruction from S1–2 (Fig. 1*B*).

Microtomographic sections were more informative than 3D reconstructions (Figs 2–4). Anatomy of the snail was recognizable to different extents between the samples. On S1–1 the lung, kidney, digestive gland and its ducts were visible, but other organs were not (Fig. 2). On S1–2 sections, other parts of the digestive system including crop and parts of the intestine were visible, as well as the lung, kidney and urinary duct (Fig. 3). The only part of reproductive system that could be distinguished on S1–2 was the hermaphrodite gland (Fig. 3). Sections of the S2 dataset provided the most detailed overview of snail anatomy

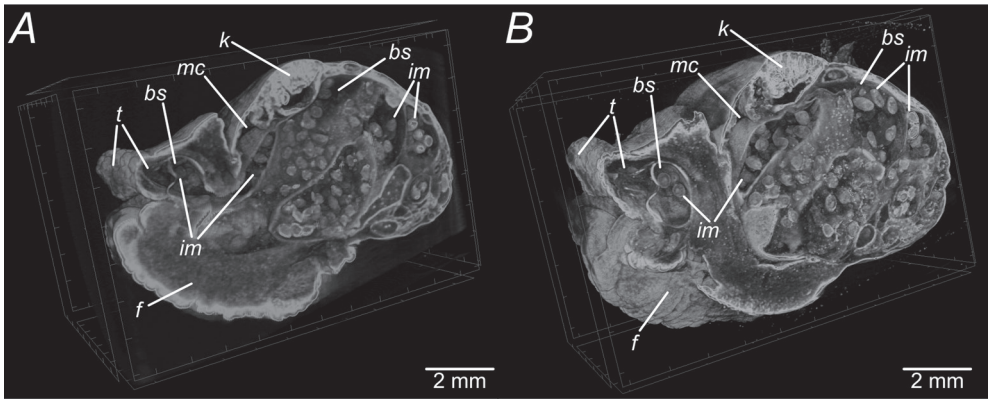

Figure 1. 3D reconstructions of datasets S1-1 (*A*) and S1-2 (*B*) made in CTVox®. Abbreviations: *bs* – broodsac, *f* – foot, *im* – infective metacercariae, *k* – kidney, *mc* – mantle cavity (lung), *t* – tentacle.

(Fig. 4), with kidney, urinary duct and lung clearly distinguishable. All parts of the digestive system were visible including the buccal cavity, crop, stomach, digestive gland with its ducts, whole intestine, and rectum. Besides the hermaphroditic gland many other organs of the reproductive system could be seen: the albumen gland, spermoviduct and distal parts of the reproductive ducts. However, the difference in reproductive systems of S1–2 and S2 might also be caused due to different degrees of reproductive system degradation caused by the sporocyst. The presence of a hermaphrodite gland in both samples is quite interesting since digeneans usually cause castration of their host via the disruption of the gonad (e.g. Lauckner, 1987; Tétreault et al., 2000).

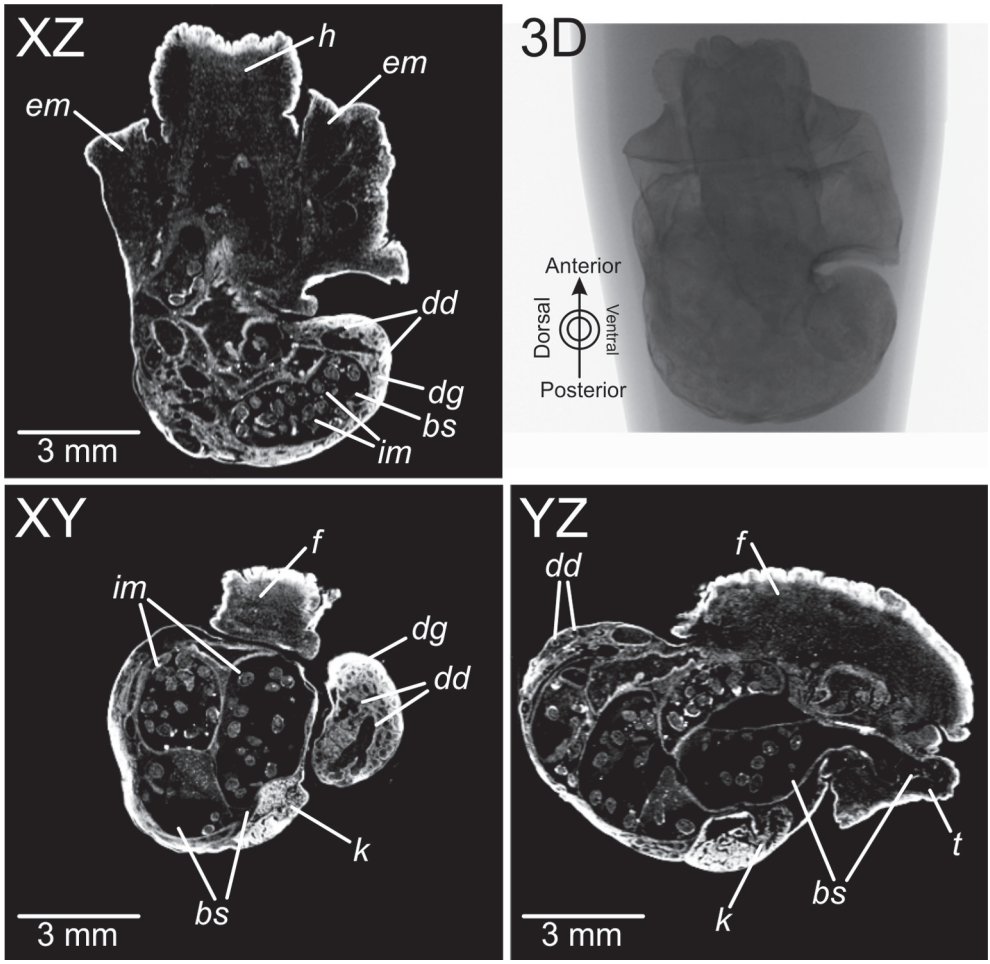

Figure 2. Microtomographic sections (dataset S1-1) visualized with DataViewer®. Abbreviations: *bs* – broodsac, *dd* – ducts of digestive gland, *dg* – digestive gland, *em* – edge of mantle, *f* – foot, *h* – head, *im* – infective metacercariae, *k* – kidney, *t* – tentacle.

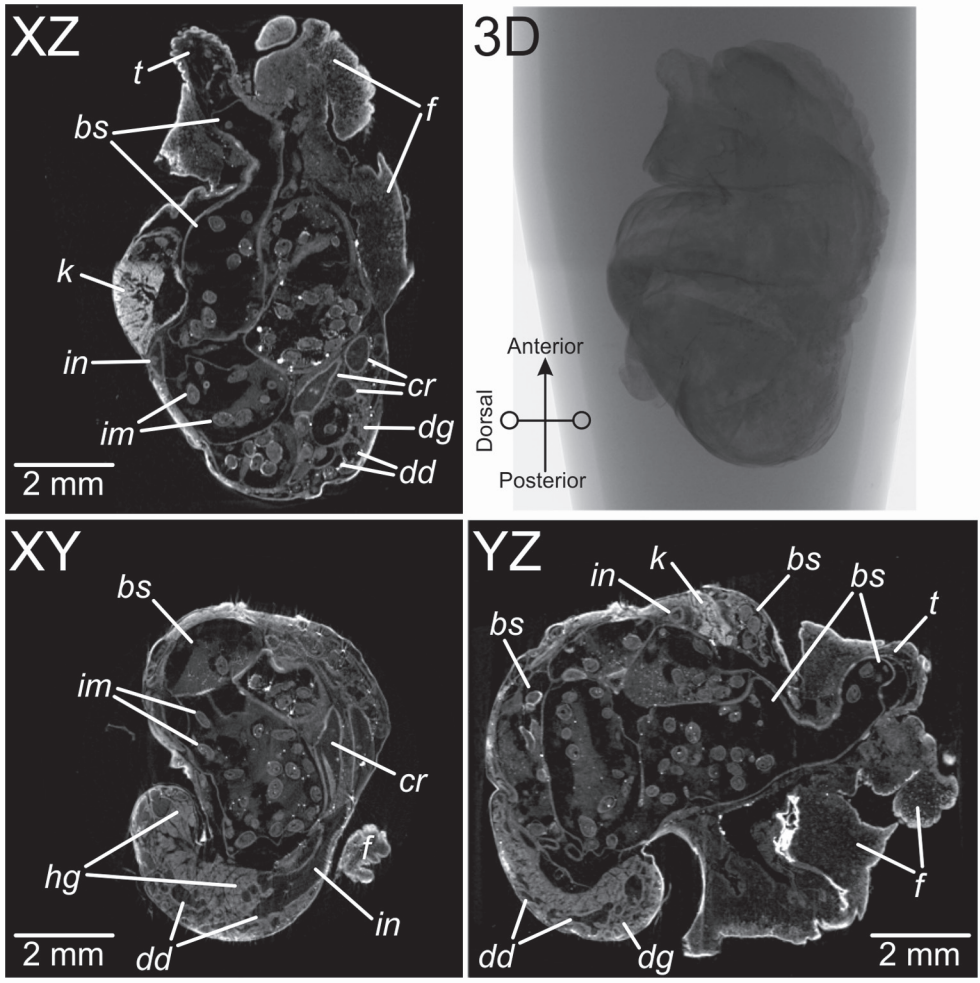

Figure 3. Microtomographic sections (dataset S1-2) visualized with DataViewer®. Abbreviations: *bs* – broodsac, *cr* – crop, *dd* – ducts of digestive gland, *dg* – digestive gland, *f* – foot, *hg* – hermaphrodite gland, *im* – infective metacercariae, *in* – intestine, *k* – kidney, *t* – tentacle.

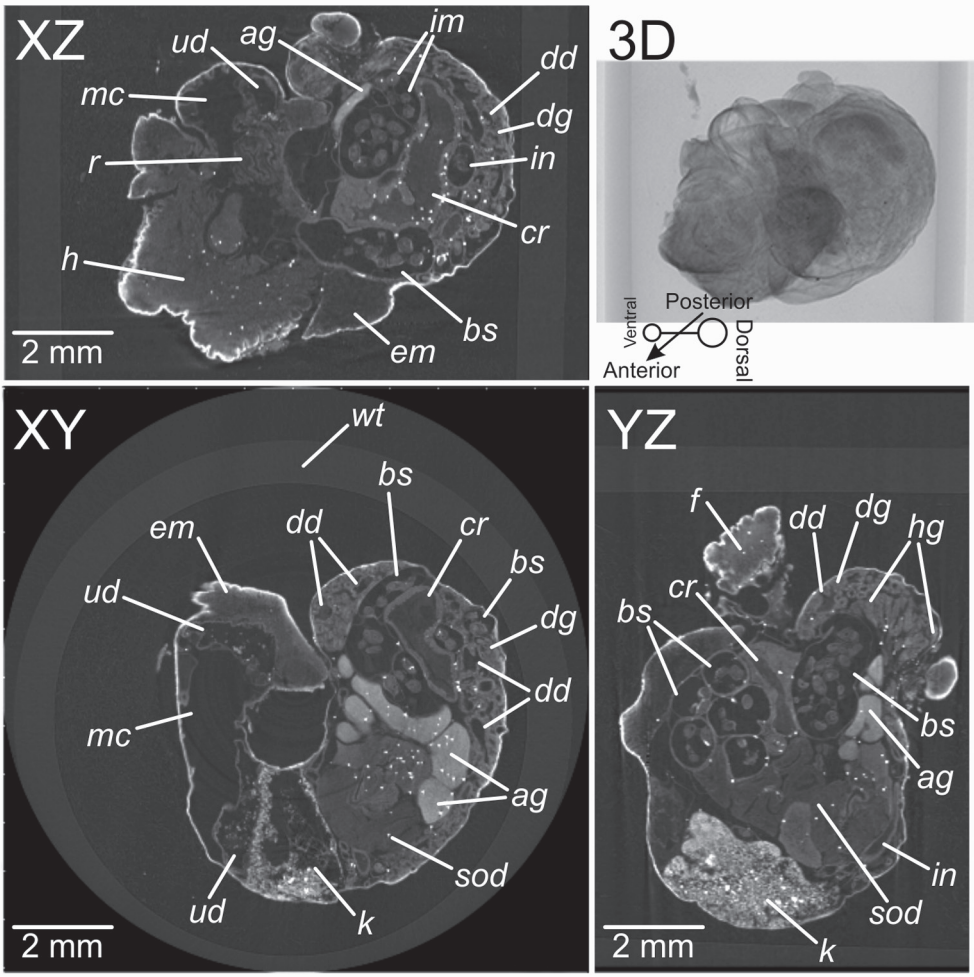

Figure 4. Microtomographic sections (dataset S2) visualized with DataViewer®. Abbreviations: *ag* – albumen gland, *bs* – broodsac, *cr* – crop, *dd* – ducts of digestive gland, *dg* – digestive gland, *em* – edge of mantle, *f* – foot, *h* – head, *hg* – hermaphrodite gland, *im* – infective metacercariae, *in* – intestine, *k* – kidney, *mc* – mantle cavity (lung), *r* – rectum, *sod* – spermoviduct, *ud* – urinary duct, *wt* – wall of plastic tube.

Broodsacs with infective metacercariae of *L. paradoxum* could be easily distinguished on sections from all three datasets (Figs 2–4) and some underdeveloped broodsacs were visible as well. Suckers and ceca of metacercariae could clearly be seen in S1–2 (Fig. 3) and S2 (Fig. 4) datasets but were hardly discernible on S1–1 (Fig. 2). Unfortunately, regardless of sample preparation, we could not trace the stalks of the broodsacs and the central part of the sporocyst.

The following advantages of the obtained micro-CT datasets can be outlined. Firstly, basic anatomical features of snails, including parts of digestive and reproductive systems, were easy to trace and locate. Secondly, localization of sporocyst broodsacs within the intact mollusk body can be readily studied. It is important to note that the protocol including drying of the sample provided the most contrast dataset with the highest resolution. However, the applied sample preparation and scanning parameters did not allow us to visualize the entire sporocyst. The central part of the sporocyst remained obscure possibly due to its low contrast with surrounding tissues of the host. Nevertheless, micro-CT seems to be applicable for studies on *L. paradoxum* sporocyst but changes in the protocol should be tested in future studies. The contrast of the sporocyst might be further increased by injection of the PTA or IKI into the sporocyst broodsac. Such technique was successfully applied by Nagler et al. (2017) for visualization of root system in two rhizocephalan species. A similar approach can be tested on other branched sporocysts of Digenea.

To conclude, micro-CT can be applied to study the localization of digenean sporocysts or rediae among the host organs, and in perspective – to make spatial reconstructions of branched sporocysts. However, this method provides no information on the tissue organization of the parasite comparing to the histological serial sections.

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КОМПЬЮТЕРНАЯ МИКРОТОМОГРАФИЯ КАК МЕТОД ВИЗУАЛИЗАЦИИ ПАРТЕНИТ ТРЕМАТОД ВНУТРИ МОЛЛЮСКА-ХОЗЯИНА

Г. А. Кремнев, Л. Ю. Крючкова, А. А. Миролюбов, В. А. Калашникова, Д. Ю. Крупенко

Ключевые слова: компьютерная микротомография, фосфорновольфрамовая кислота, сушка через критическую точку, трематоды, *Leucochloridium paradoxum*, спороциста, метацеркарии, Gastropoda, *Succinea putris*

Рентгеновская компьютерная микротомография позволяет визуализировать трехмерную структуру объектов без нарушения их целостности. Использование этого метода в сравнительной морфологии ограничено, поскольку мягкие ткани животных имеют низкую плотность и практически не ослабляют поток рентгеновских лучей. Для увеличения контрастности изображения такие образцы обрабатывают специальными красителями. Мы предположили, что компьютерная микротомография может быть использована для приблизительной визуализации разветвленных спороцист трематод в теле моллюска-хозяина, и попробовали протестировать этот метод на спороцисте *Leucochloridium paradoxum*. Два зараженных моллюска были обработаны в соответствии с двумя различными протоколами. Один образец был отсканирован в этиловом спирте, второй был предварительно высушен. Анатомические признаки хозяина лучше читались на микротомографических сечениях высушенного образца. Вне зависимости от примененной пробоподготовки, нам удалось различить только полностью сформированные и созревающие отростки спороцисты, содержащие зрелых и развивающихся метацеркарий, тогда как центральную часть спороцисты проследить не удалось. В данной работе мы предлагаем возможные изменения протокола для более точной визуализации спороцисты *L. paradoxum* с помощью компьютерной микротомографии.