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The pros and cons of using micro-computed tomography in gross and micro-anatomical assessments of polychaetous annelids

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Abstract

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The use of micro-CT scanners in the study of anatomy and functional morphology of marine invertebrates is becoming more common. The advantages and disadvantages of this methodology for the study of the internal anatomy of polychaetes are discussed. Soft-bodied invertebrates such as polychaetes pose some specific problems. It can be difficult to gain sufficient contrast between different types of tissues to be able to image them with X-rays. A range of stains can help enhance the contrast between tissues. In this study we investigate a number of stains, concentrating on those considered reversible. The advantages of such stains in the study of museum specimens and the resulting possibilities for large-scale comparative morphology studies are outlined.

Keywords

Polychaeta, internal anatomy, micro-CT, staining methods

Introduction

Phylogenetic studies of polychaetous annelids in recent years have mainly used molecular approaches (e.g. Struck et al., 2011; Wiklund et al., 2008; Zrzavý, et al., 2009). Less numerous but of equal importance have been those studies that have used recent methodological advances in morphological techniques, such as confocal microscopy to study nerves and muscles systems (e.g. Mao, 2007; Orrahage, 1990; Zanol et al., 2011; see reviews in Lanzavecchia et al., 1988; Purschke, 1988; 2005; Saulnier-Michel, 1992; Tzetlin and Purschke, 2005; Tzetlin and Zhadan, 2009). It may be argued that further progress in understanding the phylogeny of polychaetes and other taxa requires the pace of morphological work to quicken to match the rapidity of molecular investigations. Anatomical studies are more intensive in terms of the time needed, skills required and techniques involved. Undertaking large-scale anatomical studies can be a daunting task, not least of which is access to the necessary comparative

material. And yet "…it is the history of morphological change that we wish to explain…" (Raff et al., 1989, quoted in Nielsen, 2012).

The development and increasing availability of micro-computed tomography (micro-CT) scanners holds great promise in supporting structural and functional anatomical analyses (e.g. Golding et al., 2007; Li et al., 2008). CT scans have been used in medical fields for many years and their ability to produce 3-D renderings of many features is well known and documented (e.g. Udupa and Herman, 2000). Their use in anatomical studies of non-human subjects is increasing and there have been several studies focused on polychaetes. For example, Dinley et al., (2009) showed how the method could be used in functional anatomical studies, while Faulwetter et al. (2013) have shown how the rendered micro-CT images provide detailed taxonomic results.

There is no doubt that this is a maturing technology but what is perhaps the most exciting aspect of using micro-CT is that images of internal structures can be obtained without damage to the specimen. The technology provides the opportunity to undertake large-scale studies in a relatively short timescale and using museum collections not normally amenable to conventional anatomical studies. Nevertheless, because the technology is still emerging, questions need to be asked as to the efficacy of the approach, what it can and, as importantly, what it cannot as yet visualise, and from a curator's perspective that the method is safe to use on specimens.

In this paper we will: 1) evaluate micro-CT as a method for the study of internal anatomy of polychaetes; 2) assess the pros and cons of the various approaches that are possible using this methodology; and, 3) by way of example, present some preliminary results based on a study of the internal anatomy of the pharyngeal apparatus of 'errant' polychaetes (sensu Struck et al., 2011) re-examining the seminal work of Dales (1962). This study complements that of Faulwetter et al. (2013), focussing on the use of micro-CT for internal anatomical studies.

Material and methods

CT technology

Two different micro-CT scanners have been used to scan the polychaetes in this study. 1) Nikon metrology HMX ST 225 at the Imaging and Analysis Centre, Natural History Museum (NHM). The HMX ST 225 uses either a tungsten, molybdenum, silver or copper target and has a 4 megapixel (2000x2000 pixel) detector panel. The highest possible resolution is 5μm/pixel. The scanner can produce X-ray energies of up to 225kV and 200μA. 3,142 projections are taken over a 360° rotation and subsequently reconstructed with CT Pro software (Nikon Metrology, Tring, UK), which uses a modified Feldkamp's back-projection algorithm.

2) SkyScan 1172 microtomograph at the Hellenic Centre for Marine Research uses a tungsten source and is equipped with an 11 megapixel CCD camera (4000×2672 pixel). The highest possible resolution is 0.8 μm/pixel. Specimens were scanned at a voltage of 60 kV with a flux of 167μA without filter and scans were performed for a full rotation of 360°. Images were acquired at highest camera resolution. The projection images were subsequently reconstructed into a sequence of cross sections with the NRecon software (Bruker/SkyScan, Kontich, Belgium) which uses a modified Feldkamp's back-projection algorithm. These cross-sections were reconstructed from the full set of projection images (360°), other reconstruction parameters were chosen individually for each sample.

Three-dimensional models were created, from the tomographic datasets, and manipulated using the Drishti software suite (http://code.google.com/p/drishti-2) Limaye and VG-Studio Max 2.1 (Volume Graphics GmbH, Heidelberg, Germany). Drishti is recommended for the manipulation of this type of dataset. Drishti operates by loading a stack of 'back-projected' images (cross-sections of the sample) from the scan then converting it into 3-D volumetric data. This image is composed of voxels (3-D pixels) that are individually assigned a grayscale value, which represents the x-ray absorption at that point.

Staining protocols

Stains such as phosphotungstic acid (PTA) and iodine are well established in micro-CT studies (see Metscher, 2009) and appear to have similar general properties. As part of a wider study on the use of micro-CT in the study of polychaete anatomy we reviewed the potential for existing histological stains to be developed for use in CT studies. Specifically, we were looking for stains known to highlight particular tissues and which also have the potential to increase the absorption of X-rays by those tissues, making them appear more opaque. The test determined how easy the protocols for staining were, the specificity of the stain in CT rendering and whether the process could be reversible, making them more amenable to use on museum specimens. In addition to Iodine and PTA, two traditional histological stains which stain specific tissues, were tested – silver stain (Golgi, 1873) and iron stain (Wigglesworth, 1952). The former stains nerve tissue while the latter highlights nucleic acids and proteins. As part of a Master's study project undertaken by one of the authors (RM) the efficacy of the various stains were assessed for a number of different staining and clearing regimes. Standard histological methods were used to assess stain penetration and using the results the timings and concentrations cited below were derived.

a) Silver stain. The Silver stain method is based on the method of Golgi (1873) but adapted as a bulk stain. Stain reversal is possible.

1) Specimens were stained in 3% aqueous potassium dichromate for up to seven days, and the solution replaced daily and kept in the dark. 2) Excess solution was removed and samples placed in a solution of 2% silver nitrate and stained for seven days; the solution was changed frequently until brown precipitate no longer appeared. The specimen will be red to black in colour. 3) Specimens were removed from the stain, rinsed with, and then stored in, 70% ethanol, ready to be scanned.

Stain removal. 1) Specimens were dehydrated, firstly in 90% ethanol for 24 hours then a further 24 hours in 100% ethanol. 2) Specimens were placed in a 1:1 solution of hexamethyldisilizane (HMDS) and 100% ethanol for 24 hours, then placed in 100% solution of HDMS for 24 hours. 3) Once the stain had disappeared the specimen was rehydrated in stages back to 70% ethanol.

Table 1. Comparison of traditional anatomical approaches, using the SEM and micro-CT.

	CT Approach	**Classical**
Serial section	Straightforward	Process well understood but histology can be complex
Dissection	Virtual–straightforward after training	Needs skill and manual dexterity
3-D Reconstruction	Easy–depending on equipment	Involved
Identification of anatomical feature/tissue	Difficult at times	Well established
Impact on specimen	Specimen available for further study	Specimen altered and in some cases destroyed
DNA impact	Limited depending on X-ray dosage	Compromised in some procedures
Resolution	Micron/submicron range	Thin sections can give high cell-level resolution but resolution in Z is generally compromised

HMDS should always be used in a fume cupboard and be handled with protective gloves and goggles.

b) Iron Stain. Wigglesworth (1952) developed the Iron stain to highlight and measure the abundance of nucleic acids and proteins. Exact timing will depend on specimen size. The method outlined below applies to large specimens (>3 cm in length), in this case a large nereidid.

1) Specimens were hydrated in stages to distilled water. 2) Placed in 0.25% solution of ammonium iron (III) sulphate (iron alum) for five minutes. 3) Rinsed gently with distilled water. 4) Placed in 10% solution of ammonium sulphide for 150 seconds (*this was carried out in a fume cupboard*). A black iron sulphide precipitate formed immediately. 5) Specimen were then blotted dry and transferred to 2% solution of potassium ferricyanide. A cloudy precipitate formed. The solution was changed until this no longer happened. 6) Specimens were left in final solution for 24 to 48 hours (changing the solution after 24 hours if longer staining was sought until specimens were a blue colour. 7) Finally the specimens were rinsed with and stored in 70% ethanol, ready to scan.

Stain removal. 1) Specimen placed in saturated solution of potassium oxalate for at least 48 hours until all the blue stain has been removed. The solution should be replaced every 24 hours. Stain removal can take up to one week on large specimens. 2) Specimen can then be dehydrated in stages back to 70% ethanol.

c) Iodine stain. Exact timing will depend on the size of specimen. These instructions are for a large nereidid. 1) Specimens were dehydrated to 100% ethanol, in two steps 80% then 100%, 24 hours per step. 2) They were then placed in I2E (stock solution of 1% metallic iodine in 96% alcohol) for 24 hours, ready for scanning.

Stain removal. 1) Specimens were placed in 90% ethanol for as long as it took to remove stain. As the stain comes out of the specimen The solution was replaced, at least every 24 hours as it became cloudy black, until the precipitate no longer formed2) Specimen was hydrated back to 70% ethanol in stages.

d) Phosphotungstic acid (PTA). The stock solution comprised 1% (w/v) phosphotungstic acid in water. The specimens were stained in a mixture of 30 ml 1% PTA solution and 70 ml absolute ethanol (0.3% solution).

1) Specimens were dehydrate to 70% ethanol (PTA in 70% ethanol keeps indefinitely). 2) They were stained for at least 2 hours but longer (e.g. overnight) was sometimes necessary depending on specimen size. 3) Specimens were then washed in 70% ethanol. Staining is stable for months. 4) Specimens were scanned in 70% – 100% ethanol.

This is an irreversible stain.

Enhancing contrast by use of (HMDS).

Hexamethyldisilizane (HDMS) removes water from tissues effectively increasing the clarity of boundaries between air and tissue which in turn enhances the contrast when scanning with X-rays. The use of HMDS emulates critical point drying and has therefore gained favour in scanning biological material using the SEM (Bray et al., 1993). However, the standard method (Oshel, 1997) has had to be adapted for polychaete specimens to be scanned using a micro-CT.

1) Specimens were dehydrated through ethanol series 70%, 80%, 90% to 100% with 24 hours in each. 2) Then transferred to 1:1 solution of 100% ethanol and HMDS for 24 hours. 3) Transferred to HMDS for at least 24 hours. 4) Specimen was removed from solution and air dried overnight in a fume cupboard. Specimen is then ready for scanning.

Rehydrating. 1) The procedure reversed the above starting with the 100% HMDS with at least 24 hours in each solution until the desired storage solution was reached.

Results

General approach

Table 1 contrasts conventional anatomical methods employing such techniques as dissection and traditional histology with micro-CT. Both approaches have drawbacks. A conventional approach involves a range of techniques to produce data. Dinley (2013) demonstrated the range of approaches useful in functional anatomical studies of polychaetes. Such techniques range from the low resolution – gross anatomy provided by dissections – to ultra high-resolution derived from transmission electron microscopy. Reconstructing the structure of particular features or visualising the arrangements of internal anatomy is time consuming and can be compromised by artefacts caused by sample processing, for examples wrinkles and shrinkage in the sections can distort anatomical features and the resolution in thin sections might be very good in X and Y, but the sections are considerably thicker than this in conventional serial sections, so the data collected is not isotropic whereas computed voxels from micro-CT are isotropic. In addition to the range of skills required in developing this conventional anatomical 'pipeline', such studies also require the use of a number of specimens. Therefore, large-scale comparative studies are particularly challenging to undertake. Access to museum specimens, an obvious source of specimens from a broad range of species, is restricted because of damage resulting from destructive sampling, dissections and alteration of specimens in making serial sections.

By contrast, micro-CT scans and supporting software allow the researcher to perform many tasks virtually, such as dissection or sectioning in various planes as well as produce accurate 3-D rendering of anatomical features without any induced distortion. Using the micro-CT scanner overcomes many of the issues which restrict the use of specimens from museum collections and opens the way for relatively rapid yet detailed anatomical studies.

However, micro-CT also has challenges and problems. In this next section we will outline some of these issues and discuss the solutions or alternatives.

Issues and problems

X-ray transparency and anatomical imaging. Problems associated with trying to image soft-bodied invertebrates, such as polychaetes, stem from the fact that they absorb almost no X-rays, resulting in images with very little contrast. Whilst jaws and other hard structures such as chaetae can be visualised, other internal features such as nerves, muscles and blood vessels can be more challenging to discriminate.

With a low contrast image, internal anatomy may be difficult to describe or illustrate accurately. Unstained material poses particular problems as the images can be 'noisy' and lengthy manipulation with visualization software is needed to differentiate real structures from rendering artefacts (fig. 1). So it may be necessary to assess structures and features observed in micro-CT images by comparing them to classical anatomical studies in the initial stages.

There are approaches which can overcome, at least to some extent, the problem of X-ray transparency. Dependent on the scanner used, parameters (e.g. scanning time, filters, X-ray energy, wavelength) can be optimised to reduce noise and increases the contrast and so improve the final images.

Studies using the NHM micro-CT scanner on unstained polychaetes suggest that using a molybdenum target with exposure times of 354 ms and voltages of 110 kV at 200 μA and exposure times of 354 ms produces good quality images. Good images were obtained using the Skyscan 1172 with a tungsten target, 60kV / 167μm without filter (or with an aluminium filter if the specimen contains both hard and soft structures). In both cases the specimens were scanned in a sealed tube in air, and not immersed in liquid medium (a small reservoir of liquid at the bottom of the tube kept the specimens hydrated).

Resolution. Classical histological analyses making use of embedded and serially sectioned materials deliver high spatial resolution compared to many micro-CT scanners. It is possible to scan to relatively high resolutions using the micro-CT but this is dependent on the on the type of CT scanner employed. With the classical "cone beam" micro-CT scanner the spot size determines the maximum resolution possible and the geometry of the scanning system (origin of the X-rays; position of the sample; position and size of the detector panel; number and size of pixels in the panel) determine the maximum size of specimen that can be examined for any given spot size or resolution. An approximate guide is that the higher the resolution required, the smaller the area of the sample that can be scanned. Alternative micro-CT systems make use of X-ray focussing systems, lenses and detector panels derived from Synchrotron X-ray micro-CT technologies and these systems can overcome many of the limitations of the cone-beam scanners, but at increased cost and complexity.

Use of stains in anatomical studies. While stains described here increase the opacity of tissues, most are non-specific, unlike conventional histological stains which have a long history of study and many can be tissue or cell-type specific. Most stains currently employed in micro-CT analyses are used to enhance the bulk contrast rather than distinguishing between specific tissues. Thus it is often the case that distinctly different tissues appear to have the same or similar contrast in the resulting images. Also, bulk staining poses problems in that the stain has to be able to penetrate the specimen and still be of sufficient molecular weight to absorb effectively X-rays. Specimens stored in alcohol or dehydrated in various mediums have poorer permeability than fresh material. There are methods to 'relax' fixed tissue which increases the permeability of the cuticle but these have yet to be tested on polychaete specimens. Conventional histochemical stains are used on very thin sections of tissue so that penetration is not usually an issue.

Staining for specific tissues

Silver stain. Results indicate that the main drawback with Silver stain is that is does not penetrate effectively far within the tissues when used as a bulk stain. The stain is difficult to use and unstable in that it often does not stain but precipitates out of solution. Generally, whilst in some cases it has been shown to stain nerves, overall the resulting images are 'noisy' showing poor resolution (fig.1a). These results contrast with those of Butzloff (2011) for honey bees, where silver was used to good effect to stain a number of internal features. It is likely that the better results obtained are due to the chemistry of

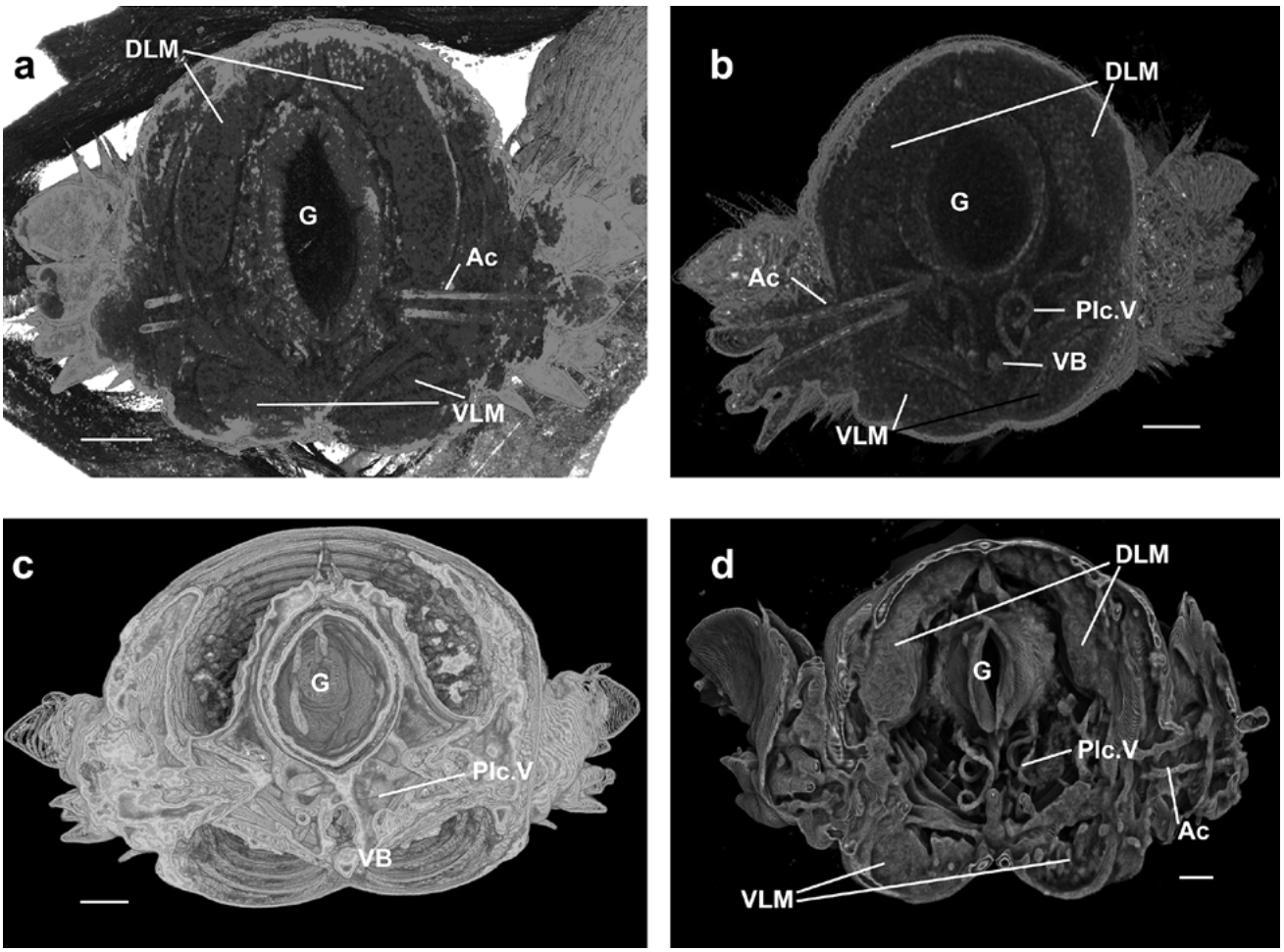

Figure 1. Transverse sections of *Hediste diversicolor* after treatment with reversible stains or drying agents. a) Silver stain, the gut and main muscle blocks can be seen but also showing paper material used to stabilise the specimen surrounding the central image (molybdenum target, 131 KV, 354 millisec exposure; b) iron stain, again gut and main muscles can be seen but also ventral blood vessels linking the central ventral blood vessel to the network surrounding the gut (molybdenum target, 131 KV, 500 millisec exposure); c) Iodine shows similar anatomical features as Iron stained material (molybdenum target, 130 KV, 320 millisec exposure); d) Hexamethyldisilizane (HDMS) image shows more clearly the internal anatomy including the ventral blood vessels (molybdenum target, 110 KV, 300 millisec exposure). Scale bar = 1.00 mm. Specimens were scanned using the Nikon metrology HMX ST 225 at the NHM. Abbreviations: Ac–internal paradpodial acicula; DLM–dorsal longitudinal muscle; G–gut; Plc-V–Plexus lateral connective blood vessels; VB–ventral blood vessel; VLM–ventral longitudinal muscles

chitin and silver but also due to action taken to improve the permeability and therefore uptake of silver. Chemically enhancing permeability through the epidermis is potentially also a useful area for future investigation for polychaetes.

Iron stain. Results of Iron staining showed more promise than the Silver stain. Surface features were clear and internal features generally showed greater contrast (fig.1b). Blood vessels were clearly identified in nereidids and arenicolids. This method is also reversible by placing the specimen in a saturated solution of potassium oxalate until the original blue stain disappears.

Iodine stain. Metscher (2009) described a range of methods using iodine to stain soft tissue. The ease of use and levels of contrast obtained have made this a popular method in micro-CT scanning. With polychaetes results are less consistent. For example, this stain works well with those species with well-developed muscle systems such as nereidids (fig. 1c) but is less successful with groups such as arenicolids where muscle systems are less concentrated. The method is also easily reversible by placing the specimen in 90% ethanol until the iodine is removed from the specimen.

PTA stain. Phosphotungstic acid worked very well on all studied specimens (fig.2). Muscles and the cuticle stained very well, a known feature of PTA, which binds preferentially to certain proteins (Quintarelli et al., 1973). However, PTA penetrates tissues slowly and is bound in high quantities, so staining can take several weeks for large specimens and the solution needs to be renewed frequently until the desired staining effect is achieved.

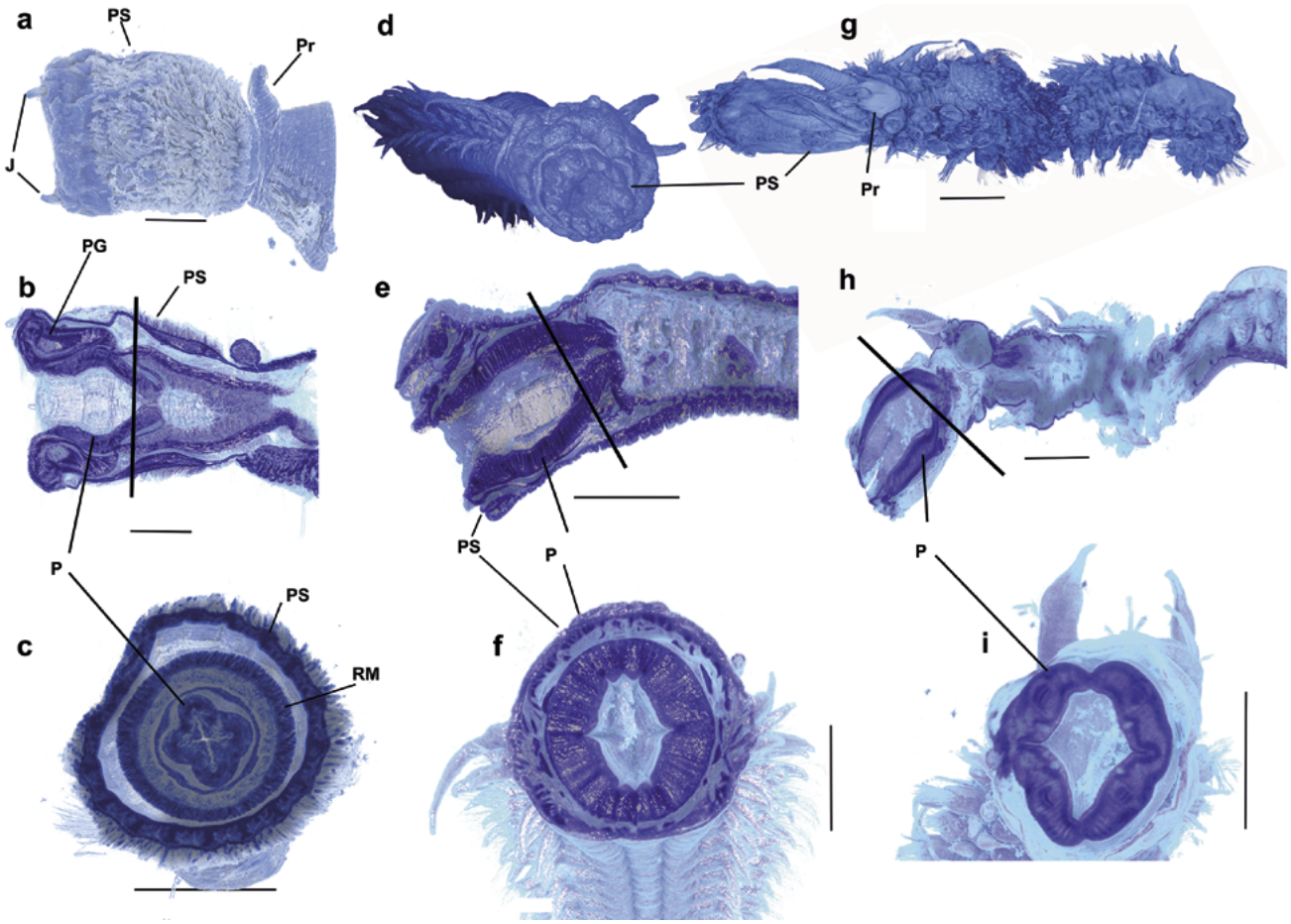

Figure 2. Pharyngeal anatomy of Glyceridae: *Glycera tesselata* (PTA-staining) (a–c); Pilargidae: *Sigambra parva* (d–f) and Polynoidae: *Lepidonotus clava* (g-i). *Glycera* a) surface morphology showing everted pharynx; b) longitudinal section through everted pharynx; c) transverse section of gut as indicated by line in b); scale bars = 0.5 mm. *Sigambra* d) surface morphology showing everted morphology; e) longitudinal section through the pharynx; f) transverse section through distal pharynx as indicated by the line in e); scale bars = 0.5 mm. *Lepidonotus* g) surface morphology showing everted pharynx; h) longitudinal section through pharynx; i) transverse section through distal pharynx as indicated by line in h); scale bar = 1.00 mm. P = pharynx. All three examples show a relatively short axial pharynx approximately as wide as long. The distal part of the pharynx is characterised by distinct muscle blocks which when contracted form a cruciform cross section. Specimens were scanned using the SkyScan 1172 microtomograph at HCMR at 60kV / 167μA, without a filter, no camera binning, full rotation of 360°, tungsten target. Abbreviations used: J–jaws; P–pharynx; PG–poison glands; Pr–prostomium; PS–proboscidian sheath; RM–ring muscle.

Creating greater contrast by drying

Protocols using Hexamethyldisilizane (HMDS) are gaining increased use in electron microscopy and micro-CT because of the greater clarity and contrast of the resulting data. HMDS effectively mimics the critical-point drying process, dehydrating the tissues and, as importantly, this drying process appears to be reversible with limited after effects on the specimen. Fig. 1d shows how effective HDMS can be. Fine scale internal anatomy such as the lateral connective blood vessels are clearly seen as are the dorsal and ventral blood vessels themselves. Muscular tissue is well differentiated and a reasonable degree of resolution is possible. However, internal tissue damage is possible, particularly tearing and ruptures, caused by differential drying during the dehydration process in HDMS. Specimens treated with HMDS become fragile and can be damaged if not handled carefully.

Further work needs to be undertaken to ensure that tissue damage either is not a problem or that a suitable protocol can be established to minimise these effects.

An example of the uses of micro-CT in the study of polychaete anatomy

Dales' (1962) seminal paper laid out the fundamental gross anatomy of the polychaete pharynx and, whilst there have been a number of revisions of parts of this schema, a comprehensive review of this work has yet to take place. Using both micro-CT scanners, we have scanned the pharyngeal anatomy of a representative species from most families currently considered

to be part of the Aciculata clade (*sensu* Rouse and Pleijel, 2001). The basic gross morphology was assessed. A list of the species examined is given in the figure captions. Figs 2-4 indicate that there are significant differences in the overall proportions of the pharynx and associated structures. Fig. 2 illustrates what might be termed taxa with a short pharynx, i.e one where the length to breadth ration is 1:1 or 2:1. The relative proportions of the pharynx varies from being relatively short and approximately as wide as long in the glycerid, pilargid and polynoid. Fig. 3 shows taxa where the pharynx is much longer than broad i.e. >3:1. The hesionid and phyllodocid have long pharynxes while the nephtyid has an intermediate length. The pharynx among taxa shown in fig. 4 have different anatomical arrangements. In the syllid the basic pattern of a thin mucular tube (sensu Dales 1962) connecting to a thick muscular pharynx was not observed. The muscles of the buccal tube in the syllid are not well developed, and this region could be better described as a proboscidain tube leading to a muscular proventicle (sensu Tzetlin and Purschke, 2005).

Dales (1962) proposed that the muscular pharynx in *errant* taxa was used primarily to crush prey. A second character found in some families is the development of four sets of longitudinal muscle blocks in the distal part of the pharynx (Figs 2, 3) resulting in a cruciform cross-section. Dinley et al. (2009) showed this arrangement in Nephtyidae (*Nephtys hombergi,* fig. 3i), suggesting that it facilitated the crushing of ingested prey. Other families showing this arrangement are the Glyceridae (fig. 2c), Pilargidae (fig. 2f), and the scaleworm families Polynoidae (fig.2i), Sigalionidae (not shown here) and Aphroditidae (not shown here). It was absent in the Hesionidae (fig3c), Phyllodocidae (fig.3e), Syllidae and Nereididae specimens examined (fig. 4).

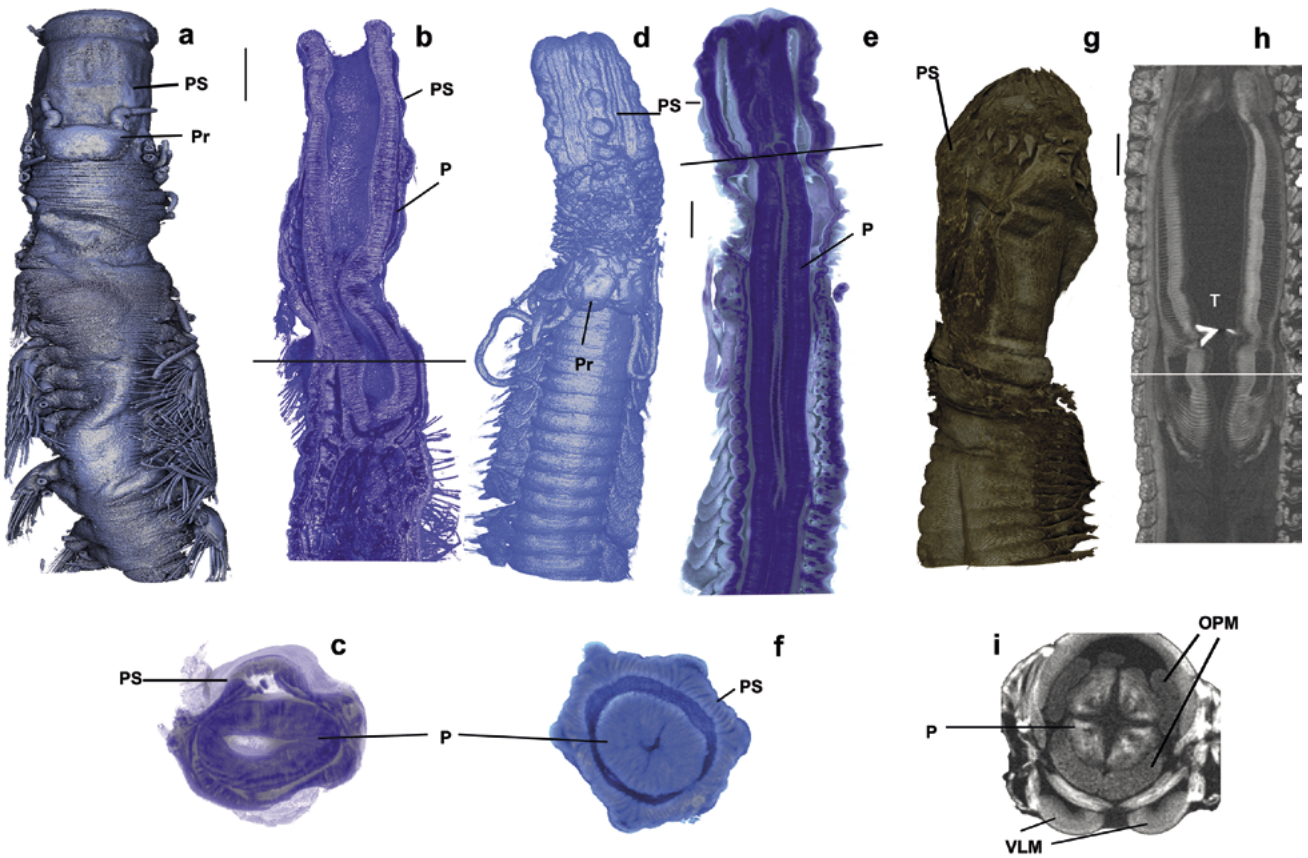

Figure 3 Pharyngeal anatomy of Hesionidae: *Hesiospina similis* (a-c, PTA staining); Phyllodocidae: *Phyllodoce lineata* (d-f, PTA staining) and Nephtyidae *Nephtys hombergi* (g, Iron stain, h-i, unstained). *Hesiospina* a) surface morphology showing everted pharynx; b) section through everted pharynx; c) TS showing distal pharynx as indicated by line in b. *Phyllodoce* d) surface morphology showing everted pharynx; e) section through pharynx; f) TS showing distal pharynx as indicated by line in e. Scale bars = 0.5 mm. *Nephtys* images from three different individuals g) surface morphology showing everted pharynx; h) section showing pharynx but not everted; i) TS of distal pharynx indicated by line in h. Scale bar = 1.00 mm *Hesiospina* and *Phyllodoce* have long thin pharynges while *Nepthys* has a medium lengthed phaynx. Only *Nepthys* shows the cruciform muscle arrangement in the distal pharynx, in the others the muscles do not appear to form these discrete blocks. Images 3a–f were produced using the SkyScan 1172 microtomograph at HCMR at 60kV / 167μA, without a filter, no camera binning, full rotation of 360°, tungsten target. Images 3g–i were produced using the Nikon metrology HMX ST 225 at the NHM (60 KV, 2 sec exposure, molybdenum target.) Abbreviations used: OPM–outer pharyngeal muscles; P–pharynx; Pr–prostomium; PS–proboscidian sheath; T–teeth; VLM–ventral longitudinal muscle.

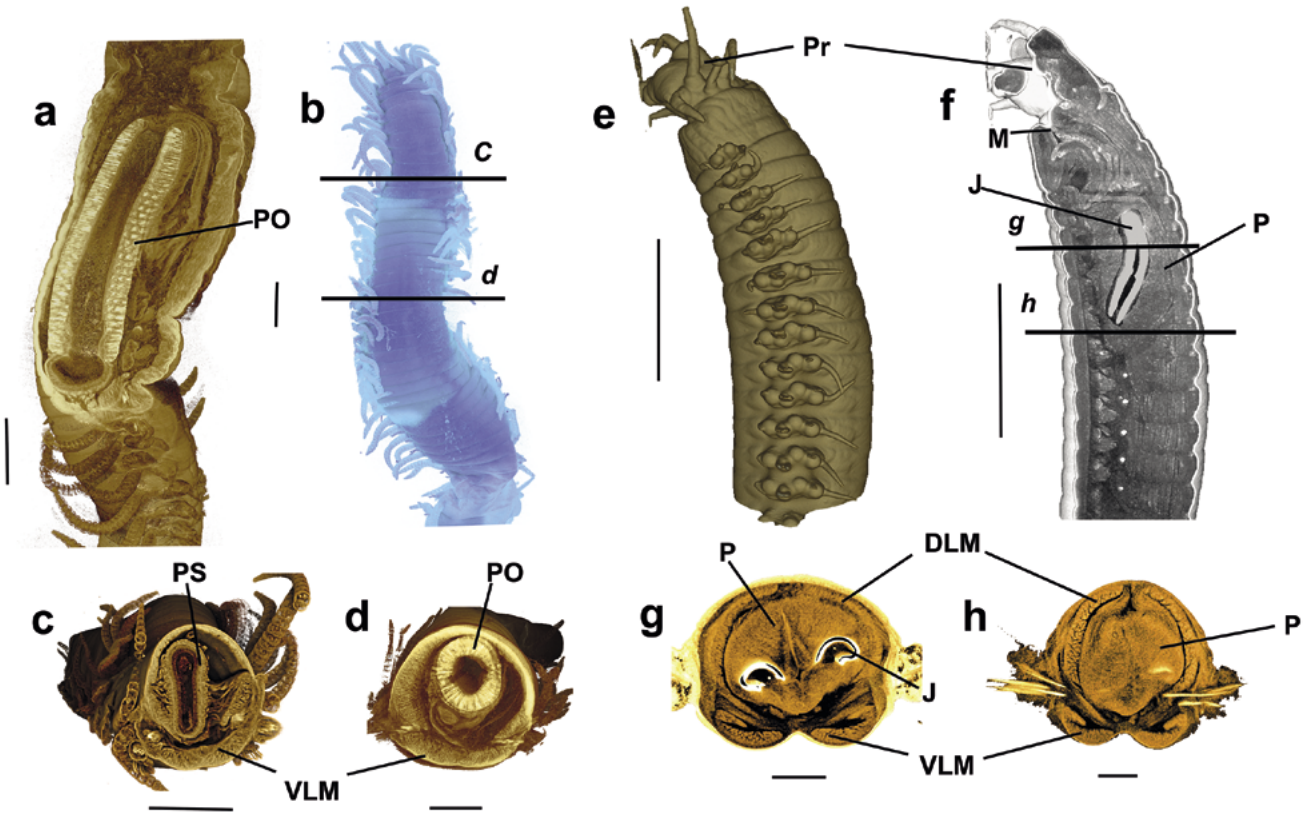

Figure 4. Pharyngeal anatomy of Syllidae: *Syllis gracilis* (a-c, PTA stained) and *Hediste diversicolor* (d-h,). *Syllis* a) section through body showing the proventriculus; b) surface morphology, lines *c* where transverse section c image taken, line *d* where transverse section d image taken; c) TS showing pharyngeal tube; d) TS showing proventricle. Scale bars = 0.5 mm. *Hediste* e) surface morphology; f) section through pharynx, lines g and *h* where transverse section images taken; g) TS through anterior pharynx at level of jaws; h) TS through distal pharynx. TS through pharynx indicates that the pharynx is not symmetrical, particularly in the distal part. Scale bars e, f = 5.00 mm, g,h = 1.00 mm. Images 1a–d were produced using the SkyScan 1172 microtomograph at HCMR at 60kV / 167μA, without a filter, no camera binning, full rotation of 360°, tungsten target. Images i-h were produced using the Nikon metrology HMX ST 225 at the NHM (60 KV, 2 sec exposure, molybdenum target). Abbreviations used:; DLM–dorsal longitudinal muscles; J–jaws; M–mouth; P–pharynx; Pr–prostomium; PO–proventricle; PS–proboscidian sheath; VLM–ventral longitudinal muscles.

Finally, while most of the families examined showed a symmetrical or nearly symmetrical axial pharynx, Nereididae did not. (fig. 4h). There was a distinct asymmetry with the ventral muscle blocks more developed than the dorsal (also noted by Dales, 1962). This arrangement may be related to the orientation of the large jaws, a feature absent in most other families.

Analyses of the gross anatomy is subject of continuing study but it appears that the stomodeum and associated structures can produce more characters for phylogenetic studies than have been used the past.

Discussion

Micro-CT is an imaging tool *par excellence*. Table 2 summarises the advantages and disadvantages of using micro-CT in anatomical studies. The advantages centre around the ease of studying specimens without damaging them and the relative ease of interpreting resulting images. A range of techniques can be deployed to produce virtual dissections of key features and serial sections in any plane desired. The resulting files, both original image stacks and rendered images are standard image files and thus can be distributed without compatibility problems between researchers. CT rendering can also be embedded within PDFs (see Faulwetter et al. 2013 for an example), which enables readers to examine and interact with the images produced. It is also possible that rendered images of type specimens could be sent as virtual loans instead of delicate specimens.

Despite the apparent high capital costs (conventional cone-beam scanners range from US$80K to over US$400K depending on the features), scanners are actually comparable in price with highly specified traditional compound microscopes in the case of the cheaper scanners; while the more expensive scanners are comparable with scanning electron microscopes, thus bringing CT scanning within reach of many institutions. Questions regarding the resolution of the resultant images depend on the specimens and to some degree the techniques employed, particularly whether staining is used. However, technological advances in instrument design are resulting in greater resolution (Stock, 2012).

Table 2. Comparing the advantages and disadvantages of imaging with a micro-CT.

Pros
MicroCT is relatively quick to scan – 40 minutes to 12 hours (overnight)
Specimens are available for future study
Ease of reconstruction and investigation
Volumes created can be distributed and reanalysed easily
Images are easy to interpret and display – 2-D and 3-D
Using a range of techniques it is possible to use Types and rare specimens
Micro-CT scannere are becoming relatively inexpensive (less than the cost of a SEM)
Free analytical software exists (e.g. Drishti, Image J)
Cons
Lack of stains for specific tissues
Image volumes are large (>3+ GB)
Rendering the images is very time consuming depending on what you want to achieve
Storage and retrieval of large numbers of files
Data pipelines and IT infrastructure can be an issue
Technical support helps enormously in running and developing techniques

Attempts to develop stains to highlight specific tissues have mixed success and more development is needed – a topic which is of interest to other disciplines as well (Pauwels et al., 2013). Drying with HMDS appears to provide a useful procedure to enhance tissue contrast in soft-bodied invertebrates like polychaetes. However, there is some development still needed on the methodology to understand the risk posed by differential drying which can result in tissue damage.

Perhaps the most important considerations when embarking on micro-CT studies are the time and infrastructure required. The amount of time is dependent on two distinct aspects of the study. The first is the degree of detail and discrimination required, while the second depends on the IT infrastructure and support available. The first is driven by the scientific question and is mediated by factors such as the need for contrast enhancement, the resolution of the micro-CT scanner, the x-ray source, etc., as explained above. High resolution studies will require more effort in adjusting the initial parameters than those undertaken to look at gross anatomical features and can only be undertaken on small samples. Micro-CT studies can be considered as analysis-heavy. It is relatively quick to acquire the X-ray images needed to create the reconstruction but it then requires a reasonable investment of time to process the images into a coherent and recognisable result. While powerful software is available, some free, it nevertheless takes time to produce images of specific tissues or structures. Rendering of surface features and anatomy is easiest to undertake but generating pictures of internal anatomy can involve considerable manipulation of the rendered images to isolate and display specific features. The results are, however, considerably easier for third parties to interpret in resulting publications and the data files are available allow others to manipulate, explore and evaluate the data produced.

Consideration must also be given to data management when undertaking micro-CT studies. In laboratories with existing imaging capability such data pipelines will be well established but for individuals and newly established micro-CT systems, consideration must be given to the transfer, retrieval, manipulation and long-term storage of files. An image stack of X-rays is often gigabytes in size (depending on specimen size and how much of the specimen is imaged). Manipulating and analysing such files requires a powerful computer with considerable RAM (read-only memory) size and dedicated graphics card. Individual scientists need to consider how they will store original images and rendered results and will need access to a secure off-site server for long-term storage.

One aspect of the use of X-rays is their potentially damaging effect on genetic tissue. Given that X-rays are a core tool for human medicine, this suggests that use of micro-CT may have limited impact on genetic material. Trials using bird specimens did not find any discernible effects (Paredes et al., 2012) and tests on polychaete material also failed to show any major impact, at least for the 16S rRNA gene (Faulwetter et al., 2013). These results indicate that – at least with commonly used scanning parameters – there should be no impediment to using this approach on specimens in museums.

Conclusions

The current state-of-the-art suggests that the micro-CT is a particularly useful tool for anatomical studies, particularly for large-scale comparative projects. In conjunction with other methods, the micro-CT data are also useful in isolating specific areas or internal structures for further studies.

New instruments, software and processors mean that the technology is advancing and that increased use will advance

our understanding of the anatomy at increasingly higher resolutions. Real time functional anatomical analyses will also be possible. Thus, the potential for polychaete anatomical studies has never been so great, and three-dimensional imaging techniques such as micro-CT have the potential to give a strong boost to the discipline and pave the way for new discoveries.

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