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MICROTOMOGRAPHY IN MORPHOLOGICAL STUDIES OF SMALL INVERTEBRATES

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Abstract. Despite great technological progress scientists still are not capable of ascertaining how many species are there on Earth. Systematic studies are not only time-consuming, but sometimes also significantly impeded by constraints of available equipment. One of the methods for morphology evaluation, which is gradually more often used for taxonomical research is micro-computed tomography. It's great spatial resolution and ability to gather volumetric data during single acquisition without sectioning specimen are properties especially useful in evaluation of small invertebrates. Nondestructive nature of micro-CT gives possibility to combine it with other imaging techniques even for single specimen. Moreover, in case of rare organisms studies it allows to collect full structural data without fracturing their bodies. Application of proper staining, exposure parameters or specific sample preparation significantly improves quality of performed studies. The following article presents summary of current trends and possibilities of microtomography in morphology studies of small invertebrates.

Key words: micro-CT, invertebrate, X-ray, morphology, volumetric imaging

INTRODUCTION

Thanks to discovery of invisible Röntgen radiation for over hundred years we can look inside objects and organisms without breaching their structure. Specialist apparatuses to emit X-rays were designed as well as methods allowing to detect such radiation. Since early 1970s, when sir Godfrey Newbold Hounsfield built first computed tomograph, to radiography entered the third dimension [Hounsfield 1977]. From that time manufacturers of X-ray tomographical equipment compete in improving this imaging method. On the one hand they are constantly optimizing radiation dose, which makes procedures safer, on the other, engineers are increasing resolution and quality of acquired scans. For human body imaging satisfying size of voxel – the smallest element of space in reconstructed 3D data, oscillate around few millimeters. In scientific research however, like in biological studies, it is possible or even necessary to decrease voxel size to micro-metric scale. Hence high resolution scanning techniques we could subdivide to medical high-resolution computed tomography (HRCT), offering thickness of single slice from half to two milometers, and micro-tomography (occurring under many names and shortcuts like: micro-CT, CMT, XMT or μCT), where spatial resolution descends even to nanometers, interchangeably called then nanotomography. Second of mentioned methods is subject of this article in which authors presented possibilities of visualizing small organisms using available equipment.

MICROTOMOGRAPHY – TECHNIQUE OVERVIEW

Basis for creating images in any radiographic technique is detection of X-ray electromagnetic radiation on analog or digital detector. Photons emitted from artificial source pass through examined object, where they undergo attenuation caused by absorption and scattering. Amount of radiation passing through object depends on atomic number of elements composing it. Heavier elements characterize with greater absorption, which is why lead is most commonly used to build protective shielding against ionizing radiation [Engineering compendium… 2011].

Currently in most cases gathering of information from X-ray photon beam take place on digital detectors, which had substituted photosensitive films and allowed to design volumetric methods of imaging thanks to mathematical operations on digital data sets. Each component of detector gathers different portion of radiation beam. This portion depends on attenuation which X-rays undergo during the way from the source. At the end it makes possible to create two-dimensional map of photons distribution which remained from primary beam with addition of scatter radiation [Hsieh 2003]. This distribution map usually is presented in grayscale and its spatial resolution depends on the size of single element of detector. To recreate examined object in three dimensions it is necessary to perform a whole sequence of such images, simultaneously rotating lamp-detector set around fixed axis, with target at the center. In micro-CT it is easier and most often attained by placing specimen on rotating table. It is important to properly stabilize examined object, because every change in position, even of its parts, influence the quality of whole reconstruction. Moving appendages of organisms such as antennae or legs should be immobilized before scanning [Stock 2008]. Specimen should not loosely float submerged in liquid.

Difficulty that appears in biological radiographic imaging is relatively small differences in absorption of soft tissues. One way to manage it is to apply phase contrast technique which can be achieved with usage of synchrotron radiation. This way however will not be discussed in present text, because it requires particle accelerator, which occupy large space in opposite of desktop microtomographs that can easily fit in even small laboratory. The other way to manage poor differentiation of tissues is to use chemical contrasting. This method can be implemented for any type of radiographic device. There are already quite a number of solutions containing heavy elements like iodine or gadolinium that could penetrate biological tissues enhancing their radiographic contrast [Metscher 2009, Descamps *et al*. 2014]. Similar effect provide chemical drying. Smaller amount of water in organism results in greater differentiation of soft tissues. Effect of contrasting will bring better results in the smallest specimens where noise from scattered X-rays influence images in greater degree.

MICROTOMOGRAPHY – APPLICATION IN SMALL ANIMALS IMAGING

It's estimated that currently on Earth lives between five and ten millions eucariotic species, those estimations however are so uncertain that could be easily extended to values of 1–100 millions [May 2010]. Scientific attempts to calculate number species indicates, that it exist ~8.7 million of species, which of only around 1.25 millions are earnestly described and cataloged [Mora *et al*. 2011]. Hi-tech molecular methods allows to rapidly sequence genome, which helps to identify new species with remarkable efficiency. Nevertheless interpretation of genetic data is still needed and description of morphology performed by scientist should be included. Unfortunately, less and less resources both human and financial are accessible for systematic and taxonomic research [de Carvalho *et al*. 2007]. It results in great amount of formally undescribed species separated only by DNA analysis, therefore every improvement in description and cataloging process is exceptionally valuable. It also pertain to imaging methods necessary for visualizing diagnostic body parts and for comparative analysis with other organisms.

Especially problematic is describing microscopic invertebrates, from several micrometers to few millimeters, which often differ only by small details. One of the reasons causing poor recognition of smallest invertebrates are inaccuracies in descriptions in currently known species. Many of earlier descriptions are insufficient to identify animal, often redescription is needed. Thus, analysis of diagnostic attributes should be widened, to avoid necessity of redescribing in future, when amount of described forms will be much greater. For many groups of invertebrates there are still not elaborated good methods of conserving species, which would allow to preserve important diagnostic features. More to that, permanence of created preparations is generally mild. That situation is very burdensome in studies of gastrotrichs (*Gastrotricha*), where studies are performed only on living material because of uselessness of preserved material from the taxonomy and ecology point of view. Under the effect of fixatives these animals shrink, and their body structures becomes hard to recognize [Nesteruk 2007]. For that reason, in that cases intravital measurements plays particular importance. Documentation include drawings, photographs and measurements. Published photographs not always accurately reflect true image of examined object structure. Drawings, on the other hand, are burdened with observer subjectivity, therefore intravital measurements can help objectify conducted studies. Observations in diagnostic process for submillimeter invertebrates, on base which taxonomic analysis is performed, are usually done with optical microscopes. This method don't allow to determine measurement error of characteristics fluctuations in acquired images of organisms. Although in this research aspect were made many new discoveries and improvements for older ones, still each of the methods have its own restrictions. One of recent promising methods, which have great resolution and can visualize inner structure of entire specimen in three dimensional space is computed microtomography. Table 1 shows comparison of main aspects, important for morphological studies, of currently most popular imaging methods.

When with the end of 20th century computed tomography achieved submillimeter resolution it took little time to find its way to morphological studies. This technology, inter alia, had changed the way phylogenetic is practiced and enabled additional tools to describe new species [Michalik and Ramírez 2013] or redescribe existing due to new data [Parapar and Hutchings 2014]. Greatest advantages of micro-CT are: good spatial resolution, volume imaging and nondestructive nature.

Table 1. Comparison of popular methods for morphology visualization. μCT – X-ray microtomography, μMRI – micro magnetic resonance imaging, SEM – scanning electron microscopy, TEM – transmissive electron microscopy, RLM – reflected light microscopy, TLM – transmitted light microscopy

Specification	μCT	μMRI	SEM	TEM	RLM	TLM
Spatial resolution	12–50 μm	10–100 μm	~5 nm	~5 nm	<200 nm	<200 nm
Intravital imaging	+	+			+	+
Surface imaging	+	+	+		+	
Volumetric imaging	+	+				
Transmissive imaging	+			+		+
Nondestructive imaging	+	+	+/- in some cases		+	

Main goal of every microscopic technique is to make visible what's too small to see with bare eyes. So it is crucial to map specimen or chosen structures in detail great enough to use it for morphological studies. With microtomography it's possible to examine objects as small as few hundred micrometers and virtually reconstruct it with incredible resolution. But that's not the greatest advantage of method. Probably the most important thing about micro-CT is that single scan acquire all volumetric data from selected 3D space. Figure 1 shows quick scans of spider pedipalp, without any staining or special preparation. It is extracted from tomography of whole animal and reconstructed in basic views that are offered by free software.

X-ray tomography imaging already found it's way to many aspects in field of morphological research. Not only for evaluation of full grown specimen but also for in vivo observation of spider eggs development [Babczyńska *et al*. 2014] or butterfly chrysalis metamorphosis [Lowe *et al*. 2013].

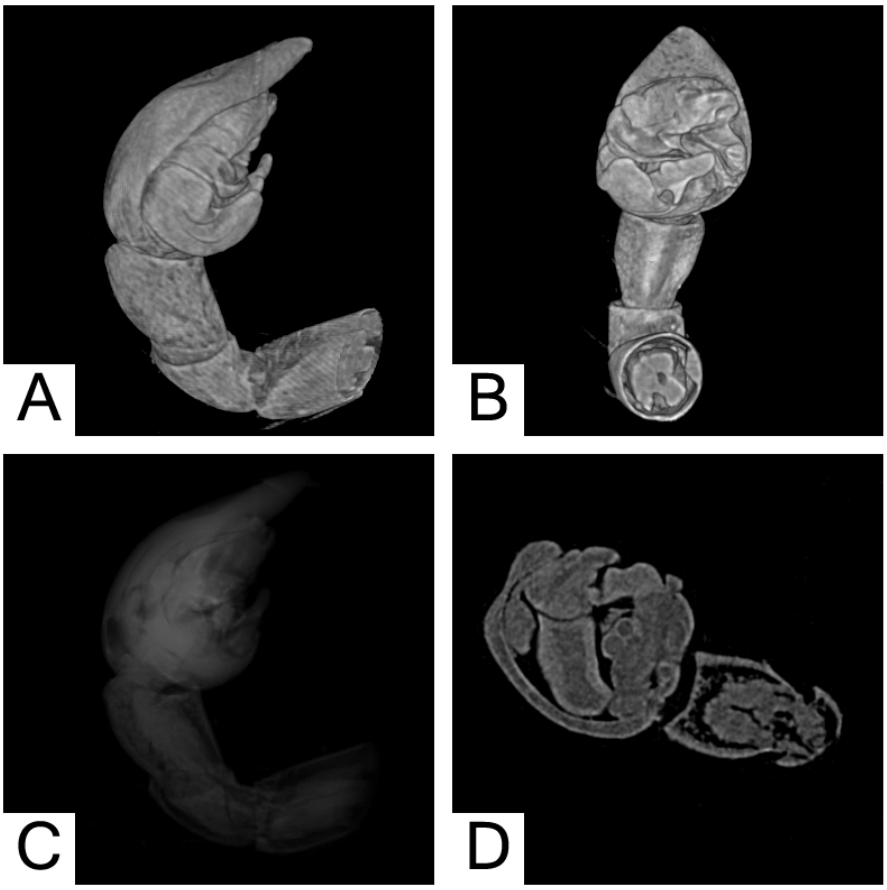

Fig. 1. Reconstruction of spider pedipalp (approx. 1 mm length), micro-CT without staining. A, B – volume rendering of surface, C – attenuation reconstruction, D – single slice

Without any staining it's possible for example to measure animal respiratory volume because air passages are naturally almost fully transparent for X-rays so they appear as black in contrast to gray soft tissues [Shaha *et al*. 2013]. With staining however these formerly almost homogenous tissues can be distinguished between each other. It applies for whole body but also for single organs [Ribi *et al*. 2008].

CONCLUSIONS

Microtomography is dynamically developing technique of volumetric imaging, which successfully can be used for detailed morphological studies of small invertebrates. Application of inexpensive contrast staining make possible to differentiate soft tissues, that normally are too homogenous to evaluate, and find many details of internal morphology. Received data could be freely processed with dedicated computer software allowing for surface, volume or density measurements of tissues and organs. Virtual segmentation can replace sectioning and manual or automatic coloring will help to aesthetically and understandable present findings. Different reconstruction methods like volume rendering, shaded surface display or maximum intensity projection can express different attributes of scanned specimen [Flohr *et al*. 2005].

All of these advantages makes micro-CT more and more often present in taxonomical research as additional or even replacement technique. This method can be used to settle many issues concerning biology of smallest invertebrates. And so, until recently it was thought, that freshwater gastrotrichs (*Gastrotricha*) do not undergo full sexual reproduction and their only form of reproduction Is parthenogenesis. Life cycle of *Lepidodermella squamata* was studied by Hummon [1986]. Author stated that after parthenogenic phase of life packets of spermatozoa and egg cells shows on trunk. Kisielewska (unpublished data) revealed that in natural conditions, in two families *Chaetonotidae* and *Dasydytidae*, over 1/5 specimen have developed spermatozoa. At six representatives from different species she found sperm cells which location indicating that they came from partner. With so small body size of these animals their gametes and gonads are almost impossible to see. As well as observations of their digestive system and mouth parts requires better parameters of imaging. Great resolution of micro-CT, which can still be improved because of very short wavelength of X-ray radiation [Mokso *et al*. 2012], allows to examine structures and individual elements of these structures that are used by scientist for classification of organisms. Moreover, microtomography collects volumetric data, so anatomical structures are presented in natural position relative to each other in space.

Computed microtomography still has some technological restrictions, they are however systematically overcame. Spatial resolution is continually improved, achieving currently voxel size even under 1 μm. Development of new, more precise detectors brings better and better effects, and closing with ability to visualize tiniest structures to electron microscopy level will significantly simplify many of taxonomical studies [Nesteruk 2007].

Better differentiation of tissues achieved by implementation of proven solutions from medical imaging, where iodine-based contrast agents within last hundred years came in for common use. For biological imaging even more staining methods are available, osmium tetroxide or phosphotungstic acid are only examples, staining protocols can be freely adjusted for specific study. Great opportunities in tissue differentiation creates phase-contrast technique possible in synchrotron radiation tomography (SRμCT) [Momose *et al*. 1995]. At the moment, enormous area of synchrotron accelerators, where such radiation can be achieved, makes it inconvenient and hardly accessible for many scientists, unlike desktop microtomographs fitting on single table. Compact synchrotrons, that would be placed in laboratory room, could in close future gain importance and popularity in research and first such constructions already exists [Bech *et al*. 2008].

Pictorial documentation in natural sciences goes far before age of Carl Linnaeus drawings. Leonardo da Vinci is well known for his anatomical sketches, Ernst Haeckel [1904] created beautiful illustrations for his book *Kunstformen der Natur* and prehistoric cave paintings also represents some sort of record of wildlife observation. Invention of microscope allowed to create drawings of life invisible with naked eye, later inseparable addition for new species descriptions became photography. Currently there are so many different microscopic techniques, that every researcher can choose among them, depending on his needs and access to equipment. Three-dimensional microtomographic imaging not only facilitates specimen evaluation for the researcher, but allows to transfer data to other institutions for consultation. Providing processed and described images can be an excellent teaching tool by creating virtual databases containing multiple taxonomic groups [Berquist *et al*. 2012]. Combining Micro-CT with other techniques have potential to solve any morphological ambiguity.

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MIKROTOMOGRAFIA W BADANIACH MORFOLOGICZNYCH DROBNYCH BEZKRĘGOWCÓW

Streszczenie. Mimo wielkiego postępu technologicznego naukowcy nadal nie są w stanie stwierdzić, jak wiele gatunków organizmów znajduje się na Ziemi. Badania systematyczne są nie tylko czasochłonne, ale czasem znacząco utrudnione przez ograniczenia dostępnej aparatury. Jedną z metod oceny morfologii, która stopniowo coraz częściej jest wykorzystywana w badaniach taksonomicznych, jest komputerowa mikrotomografia rentgenowska. Jej duża zdolność rozdzielcza oraz możliwość obrazowania przestrzennego podczas pojedynczego skanu jest szczególnie przydatna w ocenie drobnych bezkręgowców. Nieniszczący charakter mikrotomografii daje możliwość jej połączenia z innymi technikami badawczymi dla pojedynczego osobnika. Również w przypadku rzadkich okazów pozwala na zebranie danych obrazowych bez naruszenia struktur ciała. Zastosowanie odpowiednich kontrastów, parametrów promieniowania lub odpowiedniej preparacji organizmów znacznie poprawia jakość badania. Niniejsza praca stanowi podsumowanie dotychczasowych badań wykorzystujących mikrotomografię w badaniach morfologicznych zwierząt bezkręgowych.

Słowa kluczowe: mikro-TK, bezkręgowce, promieniowanie rentgenowskie, morfologia, obrazowanie przestrzenne