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Strengths and Limitations of X-Ray Microtomography (*μ*CT) of Minute Metazoans Shown with Tardigrada

Stärken und Grenzen der Röntgen-Mikro-Computer-Tomographie (*μ*CT) von sehr kleinen Metazoen am Beispiel von Tardigraden

Thomas van de Kamp, Marcus Zuber, Tilo Baumbach & Hartmut Greven

Zusammenfassung: Röntgen-Mikro-Computertomographie (μCT) ist eine auch in der vergleichenden Morphologie seit längerem etablierte Methode, die den Vorteil hat, nicht invasiv zu sein und die Voraussetzungen dafür schafft, relativ rasch 3D-Schnittbilder (Tomogramme) des untersuchten Organismus zu erzeugen. Allerdings ist das Auflösungsvermögen der meisten kommerziell erhältlichen Geräte auf mehrere Mikrometer begrenzt. Wir haben daher Bärtierchen (*Macrobiotus hufelandi*, Tardigrada; Länge 300 bis 400 μm) mittels Synchrotron-basierter Hochdurchsatz-Mikro-Computertomographie (SR-μCT) gemessen, die eine sehr schnelle Aufnahmezeit, höhere Auflösung und zusätzlichen Phasenkontrast bietet, und haben einige sichtbare morphologische Details mit lichtmikroskopischen Aufnahmen (Differentialinterferenzkontrast (DIK)) von Bärtierchen verglichen. Im Hinblick auf die Auflösung ist das partiell invasive DIK-Verfahren der nicht-invasiven Mikrotomographie überlegen. Vor allem die für die Bestimmung von Bärtierchen wichtigen Details (Schlundkopfeinlagerungen, Krallenbau etc.) waren mit dem von uns verwendeten SR-μCT-Aufbau nicht aufzulösen. Aufgrund der Tatsache, dass größere morphologische Details im 3D-Bild durchaus sichtbar sind, sind Volumenbestimmungen, z.B. vom Darm, und Zählungen, z.B. der Speicherzellen in der Leibeshöhle, möglich. Insgesamt sind aber übliche kommerziell erhältliche μCT-Geräte und die meisten SR-μCT-Aufbauten nicht geeignet, bei so kleinen Organismen die ansonsten unbestrittenen Vorteile der μCT befriedigend zu nutzen.

Schlüsselwörter: Tardigrada, Synchroton-Röntgen-Mikro-Computertomographie (SR-μCT), Differentialinterferenzkontrast (DIK)

Summary: X-ray microtomography (μCT) is an established technique for morphologists, which does not need sophisticated preparations, does not destroy the object under study and allows visualizing them in full 3D. However, resolution of the conventional devices is limited to several microns. We therefore present herein 3D images of water bears (*Macrobiotus hufelandi*, Tardigrada; length 300 to 400 μm), made with a synchrotron-based high-throughput X-ray microtomography setup (SR-μCT) characterised by a very short recording time, that reaches a higher resolution and phase contrast, and compare some of the visualized details with microphotos using differential interference contrast (DIC). With regard to the resolution of these details, the (partially destructive) DIC procedure proved superior to the non-destructive microtomography. Especially details important for determination (placoids in the pharyngeal bulb, claws etc.) cannot be resolved with SR-μCT. Due to the fact that major organs can be visualized, volume measurements, e.g. of the intestine, and counting, e.g. of cavitary cells in the body cavity, are possible. Altogether, the devices currently used including most SR-μCT setups do not allow to benefit from the otherwise undisputed strengths of μCT when studying such small organisms.

Keywords: Tardigrada, Synchroton X-ray microtomography (SR-μCT), differential interference contrast (DIC)

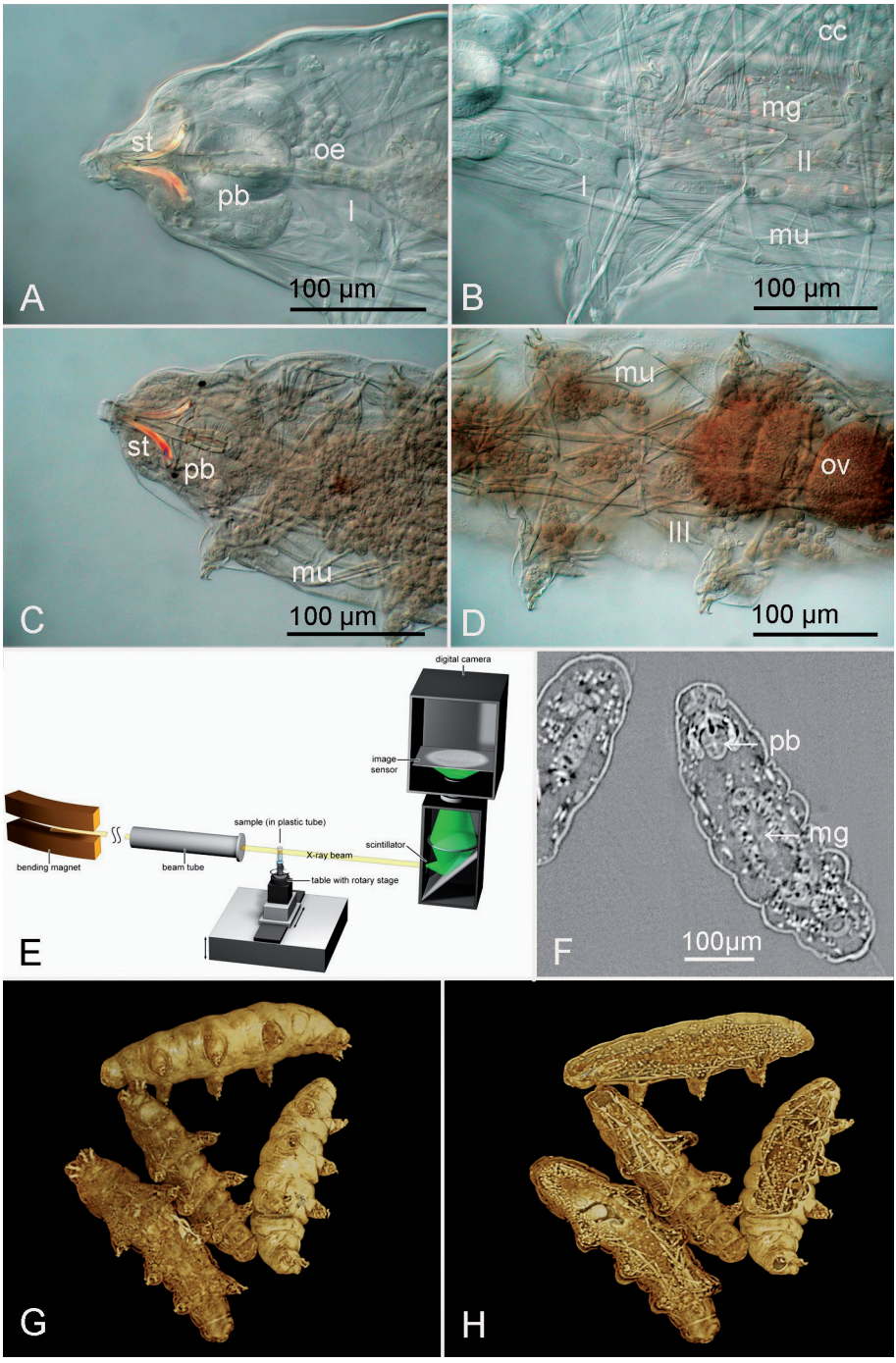
1. Introduction

X-ray microtomography (μCT) has been established as an important non-destructive tool allowing three dimensional imaging even of millimeter sized organisms (summarized by Mizutani & Suzuki 2012) such as mites, springtails etc. (e.g. Schmelzle et al. 2015; Blanke et al. 2015). However, due to the limited resolution (down to approx. 1 μm effective pixel/voxel size in conventional laboratory X-ray sources), imaging of minute metazoans is still unsatisfactory, especially when compared to destructive (scanning electron microscopy, transmission electron microscopy) or largely destructive techniques (light microscopy, e.g. Nomarski optics, phase contrast). On the other hand, apart from the fact that the objects remain intact, there are some other advantages such as little or no sample preparation, repeated examination, if necessary, and, depending on pretreatment, subsequent application of further (destructive) techniques as well. In addition, the technique allows to determine volumes of organs (e.g. van de Kamp et al. 2015) and to count structures (e.g. Hieber et al. 2016). Conventional μCT scanning procedures may be improved by dedicated staining protocols (Metscher 2009; Enders et al. 2017) and by application of grating-based phase contrast (Birnbacher et al. 2016).
The intensity of synchrotron-based X-rays is several orders of magnitudes higher than from laboratory X-ray tubes. The small size, its small divergence and the micrometer focusing allow investigating even small samples with high spatial resolution and additional contrast options (e.g. propagation-based phase contrast).
To show strengths and limitations of a current synchrotron-based high-throughput X-ray microtomography setup, we compare some morphological details visualized with this technique in tardigrades, minute Ecdysoza with affinities to Arthropoda (see Greven 2011), with some light microscopy images (Nomarski DIC).

2. Material and methods

We used specimens of *Macrobiotus hufelandi* collected in the field. Animals were extracted from a moss cushion, and examined and photographed under a light microscope either in tap water before fixation or after fixation with boiling 70-80% ethanol using differential interference contrast (Olympus, Vanox TAH-2 with a digital camera (Olympus C-3030-Z). Other fixed specimens were stored in ethanol in 2 ml Eppendorf vessels. Some of them were treated with 0.1% osmiumtetroxide to enhance X-ray absorption and hence the contrast of the tomograms (Metscher 2009).
Synchrotron X-ray microtomography (SR-μCT) of the tardigrades within the Eppendorf vessels was performed at the UFO

Fig. 1: A-D *Macrobiotus hufelandi* (DIC). **A, B** Live specimen; front part (A). Ventral ganglia (I, II) and muscles (B). **C, D** Fixed specimen, front part (C). Ventral ganglion II and ovary (D). For abbreviations see p. 156. **E** Setup of an imaging beamline at a synchrotron. X-rays travelling from left to right pass through the sample mounted on a rotary stage. While the sample is rotating, a scintillator crystal converts X-rays to visible light that is subsequently recorded by a digital camera. **F** Virtual slice of the tomographic volume. **G, H** Volume rendering of a sample with several tardigrades (in H digitally cut specimens).
Abb. 1: A-D *Macrobiotus hufelandi* (DIC). **A, B** Lebendes Exemplar, Vorderkörper (A). Bauchganglien (I, II) und Muskeln (B). **C, D** Fixiertes Exemplar, Vorderkörper (C). Bauchganglion II und Ovar (D). Abkürzungen s. S 156. **E** Synchroton-Röntgen.Mikrotomographie-Aufbau. Röntgenstrahlen kommen von links und durchdringen die Probe, die über einen Goniometerkopf mit einem Rotationsmotor verbunden ist. Während sich die Probe dreht, wandelt ein Szintillatorkristall die



Röntgenstrahlen in sichtbares Licht um, das von einer Digitalkamera aufgezeichnet wird. **F** Virtueller Schnitt des tomographischen Volumens. **G, H** Volumen-Rendering einer Probe mit mehreren Tardigraden (in H digital angeschnitten).

imaging station of the KIT Light Source. A parallel polychromatic X-ray beam was spectrally filtered by 0.2 mm Al to obtain a peak at about 15 keV. With 80 frames per second, 3,000 radiographic projections were acquired, resulting in a scan duration of about 37.5 seconds. The detector consisted of a thin, plan-parallel lutetium aluminum garnet single crystal scintillator doped with cerium (LuAG:Ce), optically coupled via a Nikon Nikkor 85/1.4 photo-lens to a pco.dimax camera with a pixel matrix of 2008x2008 pixels (Fig. 1 E). The magnification of the optical system was adjusted to yield an effective X-ray pixel size of 1.22 µm (dos Santos Rolo et al. 2014). Tomographic reconstruction was performed with the GPU-accelerated filtered back projection algorithm implemented in the software framework UFO (Vogelgesang et al. 2012). Volume renderings were created with Drishti 2.5.1 (Limaye 2012).

Abbreviations: cc = cavitary cells; mg = midgut; mu = muscles; oe = oesophagus; ov = ovary; pb = pharyngeal bulb, st = stylet; vm = vas Malpighii; I- III = the first III ventral ganglia (from altogether 4).

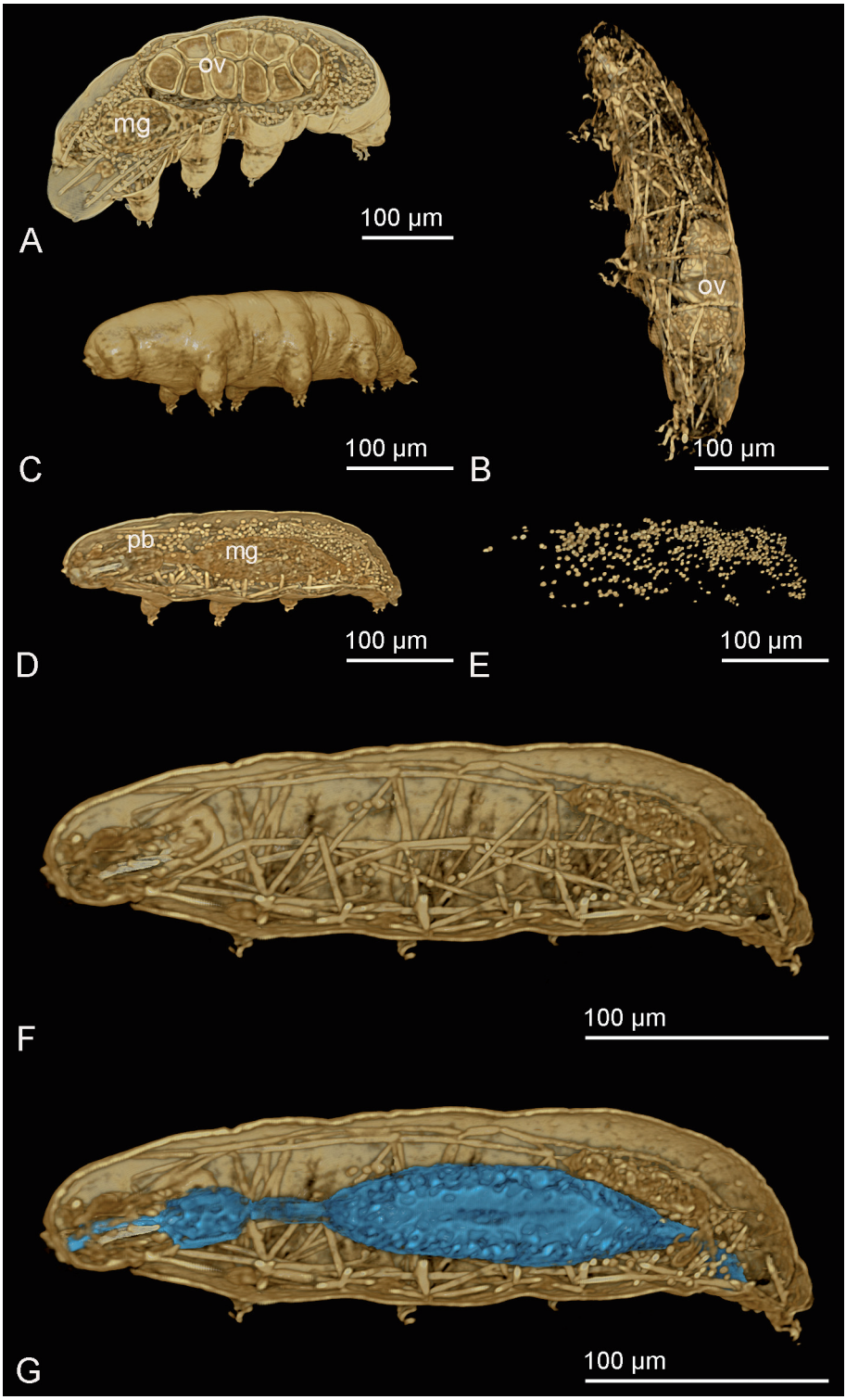
3. Results and discussion

The figures 1 A-C show some details of the organization of *Macrobiotus hufelandi* visualized with DIC, i.e. the front body with the prominent pharyngeal bulb and its specific cuticular macroplacoids, the stylets to puncture prey, the thin oesophagus (Fig. 1A) and the more voluminous midgut, muscles, ventral ganglia and cells floating freely in the body cavity (cavitary cells) that store nutrients (Fig. 1 B). In principle, the heat-fixed specimens showed the same structures, but tissues show some shrinkage (Fig. 1 C, D). The volume renderings of the tomograms (Fig. 1 G, H, Fig. 2 A-G), and their legends speak for themselves. All the structures labelled in figures 1 A-D can be identified, but are not satisfactorily resolved compared to the DIC images. Details of other structures, e.g. those important for the determination of tardigrades, such as claws, lamellae surrounding the mouth, macro- and microplacoids in the pharyngeal bulb etc. are not or are insufficiently resolved (not shown in detail). However, the spatial relationships of the identifiable organs can be better analyzed than in the light microscopical preparation that have been slightly squashed due to the small depth of field. Generally, however, it is possible to determine the volume of identified organs, by image segmentation and subsequent voxel (= volumetric pixel) counting (e.g. van de Kamp et al. 2015) and/or to count selected structures such as the cavitary cells (see Fig. 2 E), the number of which highly depends on the nutritional state of the animals as stated already in the older literature (Marcus 1929; see also Hyra et al. 2016).

In brief, currently most synchrotron X-ray microtomography (SR-µCT) setups and conventional µCT devices do not reach the resolution necessary to visualize finer details in very small metazoans such as tardigrades. Nevertheless, SR-µCT already reveals a more 'natural' insight in the 3D organisation, i.e. 3D images show spatial relationship of

Fig. 2: Examples of volume renderings. **A** Female carrying eggs. **B** Lateral view. Note muscles and the ovary. **C** Complete specimen. **D** Cut of C. **E** Storage cells isolated by volume segmentation. **F** Storage cells removed by masking the 3D volume to show anatomical details. **G** Ditto. Selective staining of the the intestine.

Abb. 2: Beispiele für Volumen-Renderings. **A** Weibchen mit Eiern. **B** Lateralansicht. Man beachte die Muskeln und das Ovar. **C** Gesamtansicht. **D** Digital angeschnitten. **E** Mittels Segmentierung des Volumens isolierte Speicherzellen. **F** Die Entfernung der Speicherzellen erlaubt den Blick auf weitere anatomische Details. **G** Ditto. Selektive Färbung des Darmsystems.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organs (so far identified) more realistic and may allow volume determination and counting (not further pursued in this note).

X-ray microtomography is of special relevance for museums as it allows studying fixed and stored rare and unique material without destruction and this material may be studied again and again according to the technical progress (Joger 2018; van de Kamp et al. 2018). Concerning minute metazoans X-ray microtomography is not progressed to the point where it might replace destructive techniques, but technological developments (nano-CT, X-ray microscopy) started already enhancing the resolution substantially (see note added in proof). If so, a better preservation using more gentle (aqueous) fixatives might become relevant, but aqueous fixatives need careful dehydration of the animals before storing in ethanol, which involves (at least in tardigrades) a high risk of considerable shrinking. However, fixing and storing in fluids, is currently not the usual way to store tardigrades in collections.

Literature

Birnbacher, L., Willner, M., Velroyen, A., Marschner, M., Hipp, A., Meiser, J., Koch, F., Schröter, T., Kunka, D., Mohr., J., Pfeiffer, F., & Herzen, J. (2016): Experimental realisation of high-sensitivity laboratory X-ray grating-based phase-contrast computed tomography. Scientific Reports 6: 24022.

Blanke, A., Rühr, P.T., Mokso, R., Villanueva, P., Wilde, F., Stampanoni, M., Uesugi, K., Machida, R., & Misof, B. (2015): Structural mouthpart interaction evolved already in the earliest lineages of insects. Proceedings of the Royal Society B 282: 20151033.

dos Santos Rolo, T., Ershov, A., van de Kamp, T., & Baumbach, T. (2014): In vivo X-ray cine-tomography for tracking morphological dynamics. Proceedings of the National Academy of Sciences 111: 3921-3926.

Greven, H. (2013): Tardigrada. Pp. 465-471 in: Spezielle Zoologie Teil 1: Einzeller und wirbellose Tiere (3. Auflage) (Westheide, W. & Rieger, G., eds). Springer Spektrum, Heidelberg.

Hieber, S.E., Bikis, C., Khimchenko, A., Schweighauser, G., Hench, J., Chicherova, N., & Müller, B. (2016): Tomographic brain imaging with nucleolar detail and automatic cell counting. Scientific Reports 6: 32156.

Hyra, M., Rost-Roszkowska, M.M., Student, S., Wlodarczyk, A., Deperas, M., Janelt, K., & Poprawa, I. (2016): Body cavity cells of Parachela during their active life. Zoological Journal of the Linnean Society 178: 878-887.

Joger, U. (2018): Research collections in Germany: modern trends in methods of sorting, preserving, and research. Pp. 17-28 in: Beck, L. (ed.): Zoological Collections of Germany. Springer, Cham.

Limaye, A. (2012): Drishti: a volume exploration and UFO: a scalable GPU-based image processing framework for on-line monitoring. Proceedings of HPCC-ICESS, 824-829 presentation tool. Proceedings of SPIE 8506, Developments in X-Ray Tomography VIII, 85060X.

Marcus, E. (1929): Tardigrada. Pp. 1-608 in: H.G. Bronn's Klassen und Ordnungen des Tierreichs 5, IV, 3. Akademische Verlagsgesellschaft, Leipzig.

Metscher, B.D. (2009): MicroCT for comparative morphology: simple staining methods allow high-contrast 3D imaging of diverse non-mineralized animal Tissues. BMC Physiology 9: 11.

Mizutani, R., & Suzuki, Y. (2011): X-ray microtomography in biology. Micron 43: 104-115.

Schmelzle, S., Norton, R.A., & Heethoff, M. (2015): Mechanics of the ptychoid defense mechanism in Ptyctima (Acari, Oribatida): one problem, two solutions. Zoologischer Anzeiger 254: 27-40.

van de Kamp, T., Cecilia, A., dos Santos Rolo, T., Vagovic, P., Baumbach, T., & Riedel, A. (2015): Comparative thorax morphology of death-feigning flightless cryptorhynchine weevils (Coleoptera: Curculionidae) based on 3D reconstructions. Arthropod Structure & Development 44: 509-523.

van de Kamp, T., Schwermann, A.H., dos Santos Rolo, T., Lösel, P.D., Engler, T., Etter, W., Faragó, T., Göttlicher, J., Heuveline, V., Kopmann, A., Mähler, B., Mörs, T., Odar,

J., Rust, J., Tan Jerome, N., Vogelgesang, M., Baumbach, T., & Krogmann, L. (2018): Parasitoid biology preserved in mineralized fossils. Nature Communications 9: 3325.

Vogelgesang, M., Chilingaryan, S., dos Santos Rolo, T., & Kopmann, A. (2012): UFO: a scalable GPU-based image processing framework for on-line monitoring. Proceedings of HPCC-ICESS: 824-829.

Dr. Thomas van de Kamp
Marcus Zuber,
Prof. Dr. Tilo Baumbach
Karlsruhe Institute of Technology (KIT),
Laboratory for Applications of Synchrotron Radiation (LAS) & Institute for Photon Science and Synchrotron Radiation (IPS)
Hermann-von-Helmholtz-Platz 1
D-76344 Eggenstein-Leopoldshafen
E-Mail: thomas.vandekamp@kit.edu

Prof. Dr. Hartmut Greven
Department Biologie der Heinrich-Heine-Universiät
Universitätsstr. 1
D-40229 Düsseldorf
E-Mail: grevenh@uni-duesseldorf.de

Note added in proof

During the 14th International Symposium on Tardigrada held in Kopenhagen Denmark this year Gross et al. (2018) presented impressive 3D images of the tardigrade *Hypsibius exemplaris* at voxel sizes of approx. 200 and 270 nm and a newly established nanocomputed tomography.

Gross, V., Müller, M., & Mayer, G. (2018): X-ray vision: 3D imaging of whole tardigrades using nanocomputed tomography. Abstract Book. 14th International Symposium on Tardigrada, Kopenhagen, p. 57.