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High density Resolution Synchrotron Radiation based X-Ray Microtomography (SR μ CT) for quantitative 3D-Morphometrics in Zoological Sciences

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ABSTRACT

Zoological sciences widely rely on morphological data to reconstruct and understand body structures of animals. The best suitable methods like tomography allow for a direct representation of 3D-structures. In recent years, synchrotron radiation based x-ray microtomography (SR μ CT) placed high resolutions to the disposal of morphologists. With the development of highly brilliant and collimated third generation synchrotron sources, phase contrast SR μ CT became widely available. A number of scientific contributions stressed the superiority of phase contrast over absorption contrast. However, here we demonstrate the power of high density resolution methods based on absorption-contrast SR μ CT for quantitative 3D-measurements of tissues and other delicate bio-structures in zoological sciences. We used beamline BW2 at DORIS III (DESY, Hamburg, Germany) to perform microtomography on tissue and mineral skeletons of marine sponges (Porifera) which were shock frozen and/or fixed in a glutamate osmium tetroxide solution, followed by critical point drying. High density resolution tomographic reconstructions allowed running quantitative 3D-image analyses in Matlab and ImageJ. By applying contrast and shape rule based algorithms we semi-automatically extracted and measured sponge body structures like mineral spicules, elements of the canal system or tissue structures. This lead to a better understanding of sponge biology: from skeleton functional morphology and internal water flow regimes to body contractility. Our high density resolution based quantitative approach can be applied to a wide variety of biological structures. However, two prerequisites apply: (1) maximum density resolution is necessary; (2) edge effects as seen for example in phase outline contrast SR μ CT must not be present. As a consequence, to allow biological sciences to fully exploit the power of SR μ CT further increase of density resolution in absorption contrast methods is desirable.

Keywords: High Density Resolution Absorption Microtomography, Synchrotron Radiation; Second Generation Storage Ring; Volumetrics, 3D-Particle Recognition, Animal Tissue, Mineral Scleres, Sponges, Porifera.

1. INTRODUCTION

1.1. 3D-Reconstruction in Zoological Sciences

Zoological sciences greatly rely on the understanding of morphology. The Greek root of this term is $\mu\omicron\rho\phi\acute{\eta}$, $\mu\omicron\rho\phi\acute{\eta}$ meaning 'shape' or 'form', which in a strictest sense refers to three-dimensionality. Independent of the zoological discipline, three-dimensionality plays an important role since all aspects of life take place in a spatial context. This applies to all levels from molecule to organisms or even ecosystems. As a consequence, from the beginning of zoological sciences, it was tried to grasp and depict animals, organs, tissues, cells and later molecules in 3D. The zoological literature is full of 3D-reconstructions displayed as habitus drawings and anatomic block diagrams. Traditionally, these structures were studied by dissection for macroscopic structures like whole animals or organs and serial sectioning for microscopic structures like small animals, tissue and cellular structures. These 3D representations are generated

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indirectly and usually require destruction of the sample. Only since x-ray based computer tomography methodologies are available, direct 3D-reconstruction is possible [1, 2]. Three-dimensional computer based reconstructions of biological objects are increasingly used, irrespectively of the method used for the creation of the dataset, which might be based on histological serial sections [e.g. 3] or x-ray microtomography [e.g. 4].

SR- μ CT is a relatively new valuable tool for qualitative and quantitative investigation in morphology, which supplement the existing methods, like histology, cLSM, scanning electron microscopy (SEM), all allowing to display the sample in 3D to a certain degree.

SR- μ CT uses monochromatic x-rays, to create a radiogram of the sample on a fluorescent screen, which is imaged by a digital CCD-camera. Controlled rotation of the sample in precisely defined steps creates a set of radiograms, which is subsequently transformed to a 3D-dataset using filtered backprojection algorithms [5-7]. X-ray based microscopic and microtomographic techniques method is mainly used for material sciences, but synchrotron based x-rays have recently shown its potency for medical and biological questions [4, 8, 9]. Although the resolution for μ CT is lower than for cLSM, the main advantage is the possibility to visualize objects, which are not opaque for visible light and much thicker. The μ CT setup allows for the investigation of samples with diameters up to several mm, whereas even under optimal conditions, the laser beam of the cLSM will not penetrate deeper into the sample than 300 – 400 μ m, depending on the working distance of the optics used [e.g. 10]. In case of poriferans, scanning depth might even become more limited due to optical distortions by the siliceous or calcareous spicules (own unpublished observations). While fairly detailed 3D-reconstructions are possible from histological serial sections [3, 11], SR- μ CT has some major advantages, especially based on the fact, that all structures remain in their natural position, whereas histological sections have to be correctly aligned manually, with additional computative compensations for distortions due to slide preparations, which makes quantitative morphometrics difficult. This topic is discussed in detail in previous publications on microtomography [4, 12].

1.2. Synchrotron Radiation based X-Ray Microtomography: absorption versus phase contrast

During recent years several new techniques have been developed which are sensitive to the X-ray phase shift introduced by the specimen [13]. This behavior is described by the complex index of refraction, where the real part corresponds to the phase shift and the imaginary part belongs to the absorption. Several publications show the principle advantage of phase contrast against absorption contrast especially for sample consisting of low absorbing elements. Two different phase-contrast methods used for microtomography have to be distinguished. The phase outline microtomography is based on phase-enhanced projections which emphasize sharp changes in the refractive index. These projections can easily be obtained by varying the sample detector distance at third generation synchrotron radiation facilities. Phase contrast microtomography is based on phase projections which are calculated from a set of phase sensitive images. The instrumentation is therefore more sophisticated and often the overall phase shift of the sample has to be reduced by a liquid which is matched to the refractive index of the sample. The photon energy is chosen much higher than required for absorption contrast. Then, it is possible to map small changes in the refractive index [14]. Unfortunately, the sophisticated instrumentation is by now not available as a high throughput user experiment and only feasibility applications were done. Here we show that for samples consisting mainly of low absorbing elements, using the optimum photon energy for absorption contrast at the high flux synchrotron radiation source DORIS III at DESY allows for a user experiment resulting in high density resolution tomograms [15].

1.3. Sponge Morphology as a challenging Example for high density Resolution Absorption Contrast SR μ CT

Sponges (Porifera) are sessile, filter-feeding animals, mainly inhabiting the sea. In recent years interest in sponges rose, since members of the group contains a variety of interesting secondary metabolites, which could be used in pharmacology and biotechnology. Nevertheless, it became evident that a more detailed knowledge on the general biology of sponges is a prerequisite for successful cultivation. The skeletons consist of organic substances, like collagen and its derivative spongin, in conjunction with mineral sclera (spicules) in most groups of the phylum Porifera [16, 17]. Cellular growth and synthesis of skeletal elements are connected in sponges. The main function of the skeleton is to stabilize the bodies of sponges. On the other hand the skeletal elements possess a variety of other functions in contraction and locomotion of sponges. Since these processes are slow and inconspicuous they are not well investigated. Nevertheless, they are very important in the life of sponges, since they are linked to nutrition (filter feeding activity) and to response towards environmental changes. Although sponges possess a limited number of cell and tissue types, the complexity of their body structures have long impeded to apply quantitative 3D-morphometric analyses. Our research aims to understand the functional micromorphology of sponges, especially our model organisms *Tethya wilhelma* and

Fig. 1. Habitus (right) and schematic morphology (left) of the *Tethya* spp. investigated here as an example. Mineral skeletal elements in red/black, collagen layer in blue/ dark gray, tissue in gray, canals and lacunae in white. Abbreviations: cbs – collagen boundary sphere; cd – choanoderm (actively filtering tissue); co – cortex; ec – excurrent canal; exp – exopinacoderm (external epithelium); ext – body extension (filaments); lsc – lacunar system cavities; mas – megaster sphere; msb – megasclere bundles; msbt – megasclere bundle tip; tal – tylaster (microscleres) layer.

T. minuta (Porifera, Demospongiae, Hadromerida) [18-24], by applying synchrotron radiation based micro computed tomography SR- μ CT in combination with quantitative image analysis. The principal morphology of this species is schematically depicted (Fig. 1). The challenge of applying SR μ CT is the wide range of densities within the sponge, ranging from the high absorption of glass spicules over the moderate absorption of cells and extracellular matrix to the very low absorption of the canal system, when not embedded into paraffin or another matrix. In order to visualize all of the structures within one dataset and make them available for quantitative image analysis, high density resolution absorption contrast SR μ CT is necessary.

1.4. Aims and Motivation

In contrast to previous and forthcoming publications which focus on biological details [20-25], here we do not intent to provide new insights into sponge morphology. The present study aims at demonstrating the value of high density resolution absorption contrast in the context of volumetric measurements and particle recognition algorithm based providing arguments for the irreplaceable value of 2nd generation storage ring based SR μ CT applications for zoological sciences.

2. MATERIAL AND METHODS

2.1. Biological Samples and Sample Preparation

For the tomography of the whole sponge, a small specimen was detached, immediately shock frozen in liquid nitrogen and subsequently fixed by freeze-substitution in an Leica AFS (Leica Bensheim, Germany) in methanol + 1% OsO₄ + 2.5% glutaraldehyde + 2.5% distilled water [modified after 26] at -80°C for 68.5 h, subsequently warmed to 0°C at a constant rate of 40°C · h⁻¹. The sample was washed two times in methanol and three times in dimethoxyphenol (DMP). The sample was transferred to approximately 25 ml DMP into a dish, which was partly covered by a glass slide, and allowed to dry slowly inside an extraction hood over night. Alternatively, samples for SR μ CT and SEM histology were directly fixed in a ice cold cocktail of 1% OsO₄ + 2.0% glutaraldehyde + 10% succrose in 0.45 M sodium acetate buffer at pH 6.4 [27]. After 30 min. cocktail was changed and fixation continued on ice over night. Samples were rinsed in sea water and distilled water for 3x 10 min. respectively, dehydrated by a graded series of ethanol or acetone (50, 60, 70, 80, 96, 100%). For the analysis of skeletal parts of the sponge, a specimen was detached from the substrate, immediately transferred to 80% Ethanol, dehydrated by a graded series of ethanol (80, 96, 100%) and embedded in paraffin, using standard protocols [28]. The paraffin block was subsequently trimmed to a cube with a final size of approximately 2 x 1 x 1 μ m, using a new razor blade. Direct fixation in 80% ethanol did not affect the state of contraction of the sponge, as could be judged visually.

2.2. Synchrotron Radiation based X-Ray Microtomography

The SR μ CT measurements were performed at the beamline BW2 at the storage ring DORIS III (HASYLAB, Hamburg) using the standard set-up for micro tomography in absorption contrast mode. The photon energy was chosen to 11.0 keV and 14.5 keV and the spatial resolution determined by means of the modulation transfer function corresponded to 3.91 μ m at a pixel size of 1.42 μ m. The samples were glued to the high-precision rotation stage of the experimental setup. The tomography scan was carried out by rotating the samples by 180 degrees and taking 721 projections. For the reconstruction of the 3D dataset the filtered back projection algorithm was used. As the samples exceeded the vertical beam size, it was necessary to move them vertically and scan them in several heights. After the reconstruction the 3D tomograms, each with 1536 x 1024 pixels, could be easily combined. The volumetric rendering of the 3D dataset was performed using VGStudioMax (VolumeGrafics GmbH, Heidelberg, Germany).

2.3. 3D-Reconstruction and Visualization

Using histograms and visual inspection of the 4byte float μ CT-slice raw data, μ CT-image raw data we converted it to 8-bit using histogram scaling setting which allowed for best representation of the investigated structures and their respective density values. 8-bit μ CT-image data was kept and processed as uncompressed Tiff or Bitmap stacks. For measurements and visualizations purposes, subsets were created using ImageJ [29, 30], which included image manipulation like clipping, inversion, manual artifact correction, filtering, thresholding, conversion to 2-bit, binning and resizing. All image processing steps were logged. The original datasets were not altered at any time. 3D-visualization of the complete skeleton and of single spicules was performed from reconstructed image stacks using the rendering software VGStudio 1.2 and VGStudioMax 2.0, applying ray tracing rendering algorithm 'Scatter HQ', and ImageJ/VolumeJ [30, 31], applying ray tracing rendering algorithm and classifier 'gradient + no index'. A gray value threshold was used to discriminate between skeletal structures and background signal resulting from paraffin.

2.4. Quantitative 3D-image analysis

Volumes and area measurements were performed in ImageJ on a reduced sub-stack for the whole sponge and on the original stack for control-volumes of $500 \cdot 500 \cdot 500 \mu\text{m}^3$. Eight control volumes respectively were chosen randomly to test for differences between cortex and choanoderm. We used modifications of previously described macros to perform area measurements on the slices of the stacks semi-automatically [18]. All measurements based on similar classifications as used for rendering. Three classifications were used for the volume measurements of the aquiferous system and the mineral skeleton and means were calculated, to minimize artifacts in form of over- and underestimation of fine structures, based on decisive classification errors. Surfaces of the aquiferous system were measured by thresholding the images according to the classification including the finest structures. The binary image was outlined automatically. The pixels of the outline were measured using a macro to allow automated image stack processing. Datasets were processed using spreadsheet programs. Volumes (V) and areas (A) were calculated as follows:

Equation 1: Volume calculation

$$V = \sum_{i=1}^n a_i \cdot s$$

Equation 2: Surface calculation

$$A = \sum_{i=1}^n l_i \cdot s$$

with n = number of slices; s = slice thickness; a_i = measured area in slice i; l_i = measured outline in slice i.

Proportions to the total sponge volume were calculated for the aquiferous system, mineral skeleton and poriferan tissue. Surface-to-volume ratios were calculated for several scenarios and compared to simple geometric forms. WinSTAT [32] was used to test data series for normality and for significance of differences between data series.

For *T. minuta* we developed a method to analyze the spicules automatically. No standard method for the analysis of 3D-image stacks is available, therefore all calculations were performed in Matlab (Version 7R14 with Image Processing

Toolbox, The Mathworks Inc.) using self programmed modules on the reconstructed image stacks, thresholded at a gray value of 205, which represents an absorption of 0.77 mm^{-1} .

For the analysis of megasters the following algorithm was applied: (i) group voxels according to 3D-chessboard neighborhood (each voxel has 26 possible neighboring voxels), (ii) use criteria for megaster recognition: number of voxels in particle ≥ 20 , volume of bounding box ≤ 2000 voxels, ratio of bounding box volume: volume of particle ≥ 8 , ratio of maximum: minimum bounding box diameter ≤ 1.6 ; 1 voxel = $3.5 \text{ }\mu\text{m}$.

Calculating an inner and an outer ellipsoid, respectively, approximated the megaster sphere boundaries. By applying a linear matrix inequality algorithm [33], the ellipsoids were fitted to all megasters, which had at least 3 neighbors within a distance of 15 voxels. For a graphical representation, the sphere was blotted into an image stack (outer ellipsoid $r_{\text{max}} = 407$ voxels, $r_{\text{min}} = 349$ voxels; inner ellipsoid $r_{\text{max}} = 255$ voxels, $r_{\text{min}} = 227$ voxels). For the graphic representation of the megaster interconnection network topology, connective lines were created to the three nearest neighbors (center to center), which were 3-dimensionally blotted using Matlab.

The macrosclere bundles were analyzed using the following algorithm: (i) eliminate megasters in image stacks by setting their voxel value to background color; (ii) exclude voxels with voxel distance to sponge center of < 180 from calculations. (iii) dilate non-background voxels by 3 (3D-chessboard); (iv) erode by 1 (3D-chessboard); (v) group neighboring voxels; (vi) erode by 2 (3D-chessboard); (vii) fit minimum volume ellipsoids with radii $r_1 \geq r_2 \geq r_3$ [33]; (viii) Criteria for valid bundles: (A) Number of non-boundary-points (3D-chessboard) > 2 , (B) for voxel number ≥ 501 and $\leq 150,000$: ellipsoid $r_{\text{max}} \geq 4 \cdot r_{\text{middle}}$, c. for voxel number $\geq 150,001$: ellipsoid $r_{\text{max}} \geq 2 \cdot r_{\text{middle}}$. Visual inspection revealed misclassifications, for which additional algorithms were applied: (a) one bundle of voxel size 292,140 and ellipsoid $r_{\text{max}} \geq 1.986 \cdot r_{\text{middle}}$ was accepted, (b) three bundles were interconnected by a reconstruction artifact, forming one large group, which was resolved by excluding voxels with voxel distance to sponge center of > 350 from calculations for this group, (c) eight groups consisted of bundles which split at distances from the center of > 180 voxels, for each of this group a part of the inner voxels, with distances between < 275 voxels and < 185 (depending on the group structure) was excluded from calculations, yielding in 19 valid bundles. For a graphical representation of the bundle vectors, cylinders were blotted into the same image stack as the megaster sphere ellipsoid. The cylinders were created by the following algorithm: (a) take center of bundle ellipsoid, (b) draw line through bundle ellipsoid center in direction of main axis of ellipsoid from minimum distance to center to point 500 voxels away from center, (c) draw cylinder of radius 15 around this line. In a second approach, convex covers were extracted, representing the outlines of the voxels related to each megasclere bundle, using a predefined Matlab operation. All Matlab programs performing the operations are available upon request. The statistical software SPSS (Version 11.5, SPSS Inc.) was used to classify the morphometric datasets.

2.5. Histology

Immediately after detachment from the substrate, specimens were fixed, desilicified in 2% HF [34] and dehydrated as described above. Following a protocol of Weissenfels [34], we embedded samples into methacrylate, trimmed and sectioned it and took several semi-thin sections which were transferred on microscopic slides for imaging under DIC illumination using an Olympus BH-12 microscope in order to compare them with SR μCT slice images.

3. RESULTS

3.1. Structural Resolution Limits of high density Resolution Absorption contrast SR μCT

Using the standard setup for microtomography at beamline BW2, we were able to distinguish choanocyte chambers which represent multicellular units within the sponge tissue, imaged at a voxel size of $(1.42 \text{ }\mu\text{m})^3$. Developing choanocytes in the core region of the sponge clearly stand out against the collagenous matrix (figure 2A). Due to osmium staining, it was even possible to distinguish the cells from the chamber lumen. Single cells can be recognized as single spots. However, choanocytes are around 4-5 μm in diameter and thus close to the spatial resolution. Although the spatial resolution of SR μCT is lower than in light microscopy (figure 2B), it is high enough to reveal the tissue anatomy of the two main sponge functional units, the inner choanoderm core and the peripheral cortex with lacunar cavities. In the same images, the silica scleres are also clearly visualized, despite of their much higher x-ray absorption. Both, enhancing absorption of soft tissue structures using osmium tetroxide staining and minimizing the attenuation of the x-ray beam before it reaches the sample, led to a higher density resolution potential.

Fig. 2. Comparison of a SR μ CT slice image at effective pixel size of $(1.42 \mu\text{m})^2$ (A) and DIC light microscopy image of a histological semi-thin section (B) of two similar juvenile specimens (bud) of *T. wilhelma*. Both samples are osmiumtetroxide-stained. The collagen rich cortex (co) and cell rich developing choanoderm (dcd) are clearly distinguishable tissue types. Mineral scleres are visible as bright structures in the μ CT image: macrosclere bundles (msb), megasters (ma) and microscleres (tylasters, ta). The lacunar system cavities (lsc) stand out against the surrounding tissue. Even single cells are visible in the μ CT image: the choanocytes chambers (cc) display clear cellular staining with a darker central cavity inside (compare cc in B).

3.2. Density based Volumetrics and 3D-Reconstruction of delicate morphological Structures

The absence edge enhancement effects in absorption contrast SR μ CT images allowed us to perform density based segmentation of the three main body structures: skeleton, tissue and canal system. As a result, it was possible to quantify and visualize the spatial distribution of these structures over the sponge body (figure 3) and specify volumetric shares of these structures in distinguished regions of the sponge body (figure 4 B). The measurements reflected the characteristic morphology with volume share peaks for the canal system in the cortex and skeletal volume share peaks in the centre of the sponge and at the most peripheral parts, where the spicule-dominated body extensions show no canal system and only a very limited tissue volume around the spicules (figure 3A, B). The aquiferous or canal system volume shares are most variable, when volumetric measurements for the whole sponge are compared with specific ones for the cortex and the choanoderm (figure 4B).

The semi-automatic measurement of inner epithelial surfaces (defined as boundary layers between tissue and canal system) plus the external epithelial boundary allowed us to calculate surface-to-volume ratios for the whole sponge and for specific regions of the body (figure 4C). Based on our measurements we calculated a total surface-to-volume-ratio of $142.4 \text{ mm}^2 \text{ per mm}^3$ which is higher than typical surface-to-volume ratios of other surface-dominated biological structures (i.e. the human lung). The absorption-based segmentation allowed us to visualize all three body structures directly by volume rendering (figure 4A), without subsequent manual editing of surfaces. By applying a mask to cut out the external air-filled volumes, we created a virtual cast of the inner air-filled volumes of critical-point-dried samples. This cast represents the canal system as a virtual solid structure, which can be virtually sectioned like all other virtual representatives of real solid body structures.

3.3. Density, Size and Shape based algorithms for 3D-Particle Recognition and 3D-Morphometrics

In order to obtain a conceptional model of the architecture of the elements of the skeleton of *T. minuta*, we created a geometrical model of the megasclere bundles and the megaster sphere, based on the data gained from particle recognition algorithms. The tylaster layer cannot be addressed in this way, since resolution limitations did not allow recognizing single tylasters by such algorithms. Extracted vectors of all 85 megasclere bundles were used as basic representatives of the bundles. To represent the megaster layer we calculated two ellipsoid boundaries, which included

Fig. 3 (left). **A & B.** Quantification of the same SR μ CT dataset displayed in Fig. 2A. Proportions [%] of sponge tissue, skeleton and aquiferous system on slices of $1.42 \mu\text{m}$ displayed in all three spatial directions (A) and as averages of all directions (xyz) with standard deviations for slices relative to the coordinate $x,y,z = 0,0,0$; arrows and lower case letters refer to C – N (slice images). **C – N.** Examples for $1.42 \mu\text{m}$ slices in grayscale (C – H) and segmented in color (I – N); xy-slices (C, I, D & J), xz-slices (E, K, F & L), yz-slices (G, M, H, & N); lower case letters and lines mark corresponding positions of orthogonal slides shown in C – N.

Fig. 4 (right). **A.** The complete morphology of *T. wilhelma*: yellow/ light gray, tissue; blue/ gray, aquiferous system; red/ dark gray: skeleton. Choanoderm (cd), cortex (cx), glue artifact (ga), excurrent canal (exc), exopinacoderm (exo), body extension (ext), interlacunar space (ils), lacunar system cavity (lsc). Scale bar: $1000 \mu\text{m}$. **B.** Quantification of tissue, aquiferous system and skeleton content of two distinct morphological compartments in comparison to the total sponge. **C.** Surface-to-volume rates of compartments in relation to different epithelias (pinacoderm). Figures modified from Nickel et al. [21].

98.5 % of all oxyasters. The geometrical model (Fig. 5) displays the simplistic principle on which the skeletal structure of *T. minuta* bases on: 85 radial megasclere, bundles originating from a common center, span a globular space, which is divided into two compartments by the megaster sphere, which is a relatively solid structure, due to the massive allocation of collagen between the asters and basally, underlying to the megaster sphere. The whole structure strongly resembles technical constructions, eventually referring to fundamental static standards, which apply for the sponge. By interconnecting each oxyaster center with the centers of the ten nearest neighbors, we created a megaster interrelation network, in order to analyse its topology (Fig. 6). The network topology confirms the baso-apical density gradient of the oxyasters, resulting in a massive basal half shell of the aster sphere, displaying a significantly increased density of oxyasters: $1,352 \text{ mm}^{-3}$, in comparison to 849 mm^{-3} for the apical part of the megaster sphere and $1,135 \text{ mm}^{-3}$ in average. However, there is no size-sorting phenomenon, since we found no correlation between oxyaster size and distance to neighboring oxyasters, neither for the basal half shell, nor for the apical part of the sphere (data not shown). In addition,

Fig. 5. Volume renderings of *T. minuta* skeleton and a segment of structural model I. A. Skeleton without outer Epithelium-micraster layer; msb – macrosclere bundles; mas – megaster sphere; ra – reconstruction artifact. B. Mathematically extracted structural model I. Scale bar: 500 μ m. Figure modified from Nickel et al [20]

Fig. 6. 3D-visualization of a segment of structural model II (A, B), displayed at two different angles with additional convex representations of the megascclere bundles (B), displaying the megaster interconnection network topology. Each of the megaster centers (blue dots/ black dots) is connected (red lines/ dark gray lines) to its three nearest neighbors, revealing the massive megaster sphere skeleton superstructure (mas) with canal system orifices (cso). Only few megasters fall outside the mas and are related to msb (bra). These few megasters can be discriminated into cortical bra (co-bra) and choanodermal bra (cd-bra). Figure modified from Nickel et al. [20].

local variations in megaster density across the megaster sphere are demonstrated. Numerous outlets for components of the canal system exist, appearing as extraordinary wide 'meshes' of the network or as areas of lower density of oxyaster interconnections.

The 254 oxyasters found to be displaced from the megaster sphere, form mainly radially interconnected distinct groups, both, the 172 cortical ones as well as the 82 choanodermal ones (Fig. 6). However, the cortical oxyasters tend to form groups of higher volumes and to be more scattered than the choanodermal ones, which built up conical network topologies, based in the megaster sphere, pointing towards the sponge center (Fig. 6). In most cases, both types are located directly in the neighborhood of megascclere bundles. We included the extracted convex boundaries of the megascclere bundles in some of the visualizations of the interrelation network topology, in order to demonstrate this correlation (Fig. 6).

4. DISCUSSION

4.1. High Density Resolution Absorption Contrast SR μ CT allows almost cellular Resolution

The present combination of osmium tetroxide staining and an optimized microtomography set up using monochromatized synchrotron radiation allowed us to reach almost cellular resolution, visualizing defined multicellular structures within the sponges. This is not a question of spatial resolution, since it is mainly a function of sample size (roughly 1/1000 of its diameter), but a question of density resolution. Cells in their tissue context are embedded in an extracellular matrix, which is of highly similar density, physical properties and thus similar resulting x-ray absorptions. It has been suggested to overcome this limitation using phase contrast SR μ CT, stating that absorption contrast SR μ CT does not have the power to do so [12]. Here we demonstrated the contrary. Our main intention was to quantitatively image on the tissue level in order to distinguish fine canal structures from surrounding tissue. However, the present set up allows visualizing and quantifying sponge choanocyte chambers (Hammel et al., in preparation). However, it has been shown before that single cells can be resolved at HASYLAB beamline BW2 [35]. But the full density resolution capacity of absorption contrast SR μ CT in conjunction with maximum spatial resolution on tissue samples remains to be evaluated. Sophisticated labeling and staining methods (i.e. gold- or silver enhanced immuno-staining [36]), allowing to enhance the absorption of desired target structures, like certain cell types or extracellular matrix components, specifically will most likely further expand the possibilities of absorption contrast.

4.2. Absorption Contrast SR μ CT Images allows semiautomatic Volumetrics of delicate biological Structures

A clear advantage of absorption contrast over phase outline contrast is that all material or biological structures of different x-ray absorption are accessible to automated or semi-automated segmentation. Thus, quantitative image analysis based morphometric measurement methods, like volumetrics, can be applied. This is not the case on images which show strong edge-enhancement effects as seen in phase outline contrast [12]. Measurements on delicate and extremely complex 3D structures like the sponge canal system presented above would hardly be possible under phase outline contrast conditions, since extensive manual editing would be necessary to delimitate the structures. Such segmentation approaches by manual editing and outlining are widely used for less complicated biological structures in order to obtain iso-surface renderings (for methodological details of manual segmentation see e.g. [12]). However, even in the case of such biological structures of lower structural 3D-complexity (e.g. in insect morphology [37]), density aided semi-automated segmentation methods would greatly reduced the often biased manual editing. Such rule-based intelligent approaches have to date not been used to the maximum possible extent.

4.3. Density Contrast Data allows for more complex 3D-Particle Recognition

The same applies for even more complicated approaches of rule-based particle recognition. In the example given here, we applied particle recognition algorithms which make use of information within the 3D-datasets by means of x-ray absorption (image gray values), particle size and shape. As a result, we were able to allocate more than 16,000 single structural elements within one sponge and gather a variety of quantitative data on each single particle. This allowed for topology analyses as shown in figure 6 and deeper statistical analyses (not shown here, see [20] for details), which resulted in the understanding of the functional relation of these skeletal particles. Again, edge-enhancement effects as seen in phase outline contrast SR μ CT would have complicated the approach very much. In this case, particle recognition algorithms would have had to cope with the higher image complexity and it is uncertain, if such a data set could be computed sufficiently.

5. CONCLUSIONS

As the examples given above clearly demonstrate the power of high density resolution absorption SR μ CT is manifold. The present microtomography set-up at HASYLAB beamline BW2 provides a density resolution which allows distinguishing cells within the tissue context. This is achieved by staining methodologies (i.e. osmium tetroxide) which can be regarded as preliminary, since more sophisticated methodologies have only been tested to a limited amount. The advantage of absorption data over phase outline contrast data lies clearly within the homogeneity of the representation of similarly absorbing structures within the image, which allows the application of a wide range of quantitative image analysis tools. These quantitative tools are at present still underrepresented in their application upon zoological microtomography datasets, since zoological sciences are traditionally marked by visualization of data rather than in

depth measurements. In cases, where visualization is the only purpose, phase outline contrast SR μ CT might in deed be simpler to use. However, as long as the true limits of absorption contrast methods have not been evaluated and reached, it should not be presumed that phase outline contrast is superior per se as it has unfortunately repeatedly been stated in some publications. This does not withstand deeper, critical examination as we demonstrated here.

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