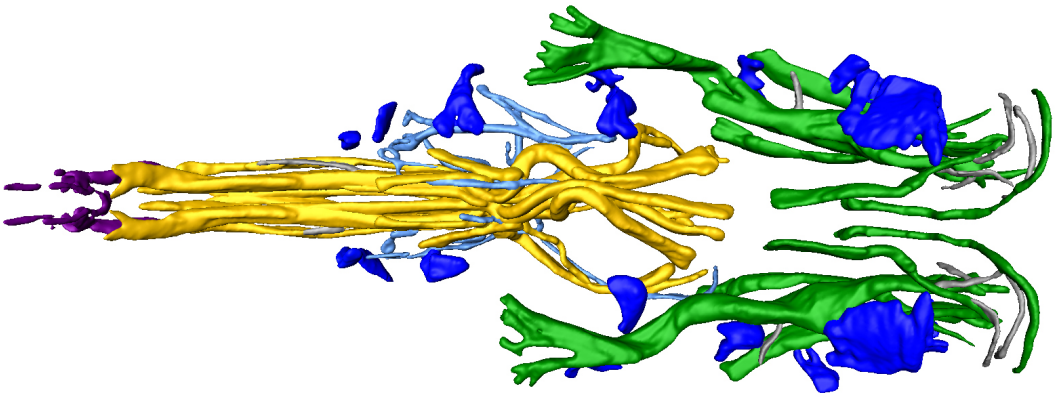
— Masterarbeit —

Three-dimensional reconstruction of the body musculature of *Testudinella clypeata* (Müller, 1786) (Rotifera: Floscullariacea)



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Abstract

In morphological research it is essential to know the internal structure of an animal. To have a clear conception e.g. of the musculature can also help in evolutional and systematic questions. The ways of representation of biological structures developed from simple drawings to sophisticated representations like CLSM images. In the last years, the three-dimensional reconstruction, due to increased computer power, gained more and more ground in biology and medicin. Up to now, there was no instruction how to get from a CLSM image stack to a 3D-model in rotifera research. To provide this is the aim of this work. It also contains a comparison of the body musculature with the findings of authors who worked with light micrsoscopes and CLSM images. The examined specimens, *Synchaeta spec.* and *Testudinella clypeata*, were prepaired according to standard procedures. The actin fibers in their muscles were stained and scanned with a CLSM. The resulting image stack was worked with Amira to get a three-dimensional data object. Finally, basic ways of a further work to create more abstract, ideal models of the muscles are given.

Introduction

Having a clear idea of the internal structure of an animal is very helpful in morphological and evolutional research. This study deals with the body musculature of a rotifera species. To know how the musculatur of different species is build up can e.g. possibly show systematic relations. Or the structure of a common ancestor could be deduced from two or more species. It also can contribute to improve the education of students.

The used animals were specimens of *Synchaeta spec.* and *Testudinella clypeata* (Müller, 1786). The specimen of *T. clypeata*, with which the most work was done, was compared with *T. patina*. “Synchaeta” is a composition of greek *σύν-* (with) and *η χαίτη* (hair, mane, here: bristle), and litteraly means “with bristles”. “Testudinella” litterally means “little turtle”, “patina” means “pan” or “dish/bowl”,

and “clypeata” means “having/wearing a shield”; all comming from Latin. According to Nogrady & Segers (2002) *Synchaeta* live truely planctonic in marine, brackish, and freshwater. The authors describe them as positive phototrophic, feeding on algae and often raptorial. According to Voigt & Koste (1978) the genus *Testudinella* consists of 34 species, living in the periphyton or on the surface of biogenic sediments. The habitat of *T. clypeata* is noted as brackish water and ponds at the beach. The figures given are: pH 7.7–9.1, 4.0–19.2°C, Cl 0.24–1.34 g/l.

Since the first microscope (virtually simple lenses adjustably fixed in a mounting), which invention is attributed to Antoni van Leeuwenhoek (1632 – 1723), drawings with pencils or pen and ink of the examined object were made. Even so the microscopes became increasingly powerful, the means of representation did not change notably in substance until the photography arose. Photographies depict more naturally and more exactly, but there is no, often useful and therefore desired, abstraction to the most important things. Modern microscopy together with computers provides even more precise results e.g. transmission electron microscopes (TEM) or confocal laser scanning microscopes (CLSM). Sørensen (2005) and Kotikova et al. (2006) conducted studies of the musculatur of rotifers (*Testudinella patina*) with CLSM. There, the musculature is stained with a marker substance which gives a signal when scanned. But up to now, all those images are only two-dimensional. At least in Sørensen (2005) there are pairs of stereo images which can be looked at with red/green 3D glasses to get a three-dimensional impression, but they still show only a single perspective. Today's computer programmes allow to create three-dimensional (data) representations by the use of image stacks. Of course, using those techniques is not novel in medicin and science, especially in biology, e.g. Neves et al. (2009), but up to now, rotifers were not yet examined as three-dimensional images.

Three-dimensional data objects are meshes consisting mostly of triangles. A tea pot as an example of a mesh is given in figure 1. The vertices of these triangles (nodes) are coordinates in a three-dimensional space, and are connected pairwise with edges to their neighbours. So, a mesh is nothing else than a graph, because a graph is a collection of nodes and edges.

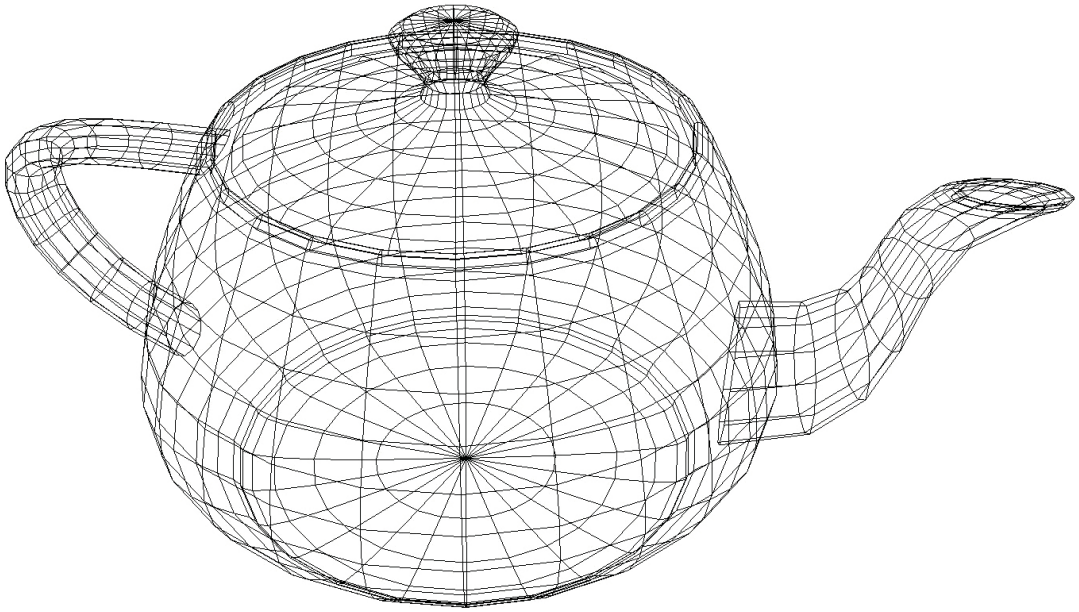

Figure 1: This teapot is an example of a three-dimensional object which is represented in computer programmes as a mesh grid.

Mathematically a graph G is a two-figure relation together with a set of nodes V. Two nodes v_1 and v_2 belong to the relation G, i.e. $(v_1, v_2) \in G$, if there is an edge between v_1 and v_2. That way, a graph G together with a set V is a set of pairs (v, w) with $v \in V$ and $w \in V$.

In computer science graphs mostly are stored as node lists and edge lists. The node list is a list of points in a three-dimensional space, where each node has a pointer (i.e. a number which functions as a name). The edge list only contains the pairs of pointers. There are two other possibilities: Each node has a list attached to it which contains its direct neighbours, and the so called adjacent matrix, a boolean matrix with zeroes, but ones in those cells where row and column number represent the node pointers of an edge. These internal representations have different properties of memory requirements and access times.

Since Amira imports an image stack, there is a certain distance between the individual images. The actual marking is done on the basis of those images, slice by slice. This way, the object initially is an accumulation of small cubes called voxels (i.e. volumetric + pixel). The object looks like made of LEGO bricks. There are several ways of how to get from this to a mesh. Generally, every cube which has at least one face with contact to the outside is involved in the computing. Protruding vertices of a cube are cut off leaving a triangular face. Which cubes are cut and how much is cut off depends on the position of the neighbouring cubes. The actual mesh rises by connecting the vertices or the centers of the surface voxels with edges to their neighbours. Rectangles are divided along their shorter diagonal to get two triangles.
Since the surface of a mesh in Amira is determined by the number and size of the triangles, it can be smoothed by reducing the number of triangles by merging neighbouring triangles. Which triangles are concerned is determined by the angle they have towards each other. This way, also the amount of data is reduced. But the less and the larger the triangles are, the coarser the surface is.

The goal of this work is to provide a description of the way how to get from an image stack obtained by a CLSM to a three-dimensional reconstruction of the musculatur of a rotifer. A comparison of the musculature of a *Testudinella clypeata* to earlier findings is included. And finally, an outlook is given how this data model and in particular individual muscles of it, separated from the others, could be worked further by simplifying the muscles until the abstraction shows the essencial shape. Then, it also is possible to apply an elaborate surface colour and an illumination of the scene presenting the muscles.
After the following description of the material and method, the results describe the way to the data object in text and figures and the muscles found. Finally, some problems with the methods, some striking features of the muscles, and possibilities to further work are discussed.

Material and Methods

In this study the body musculature of a rotifera was stained, scanned with a CLSM microscope, digitally reconstructed in 3D and compared with other CLSM and light microscope investigations. The attention was focused on the reconstruction to provide a novel method in rotifera research for systematic explorations by morphological traits using a newly created, ideal (abstructed) 3D data model.
The model organism should be a *Synchaeta spec.* The samples were collected in April 2010 in the ditch around the building of the EWE-Forschungszentrum für Energietechnologie, located on the campus of the University of Oldenburg, at 53° 9' 3.6" N, 8° 9' 58.9" E. This was done with a conical plancton net with a mesh size of 65 μm and a diameter of 20 cm. The net was dragged through the water just below the surface without touching the ground.
Under a light microscope specimens of the genus *Synchaeta* were separated with a pipette into a microscopy tray. The middle of the anterior part of the pipette was heated over a Bunsen burner and pulled at so that it tapered. There it was cut short to get a very narrow tip. Attached to the other end of the pipette was a flexible tube with a mouth piece which made it possible to draw in an animal together with only a very small quantity of water.
The following procedure is almost the same as that of Wilts et al. (2009). The animals were anaesthetised with CO_2 (water with soluted CO_2) for a few minutes to relax their muscles. This is necessary because otherwise the fixans would cause the muscles to contract so that they could not be examined well. The fixation with 4% paraformaldehyd at a temperature of 4℃ lasted for 1 h. It deadens the tissue by stopping the cell functions, but conserves its structure and condition. Thereupon the samples were rinsed repeatedly with PBS (i.e. phosphate buffered saline; pH 7.4, 0.1 mol/l). PBS increases the pH-level within the tissue again, since it was lowered by the paraformaldehyd during the fixation. It also removes the excessive fixans to prevent artefacts. Until the permeabilisation and staining the specimens were preserved at 4℃ in 0.1 M PBS, mixed with 0.05 g NaN_3 per 100 ml solution, to prevent microbial growth.

Finally, the animals were made permeable with 0.1% Triton-X-100, buffered in 0.1 M PBS, for circa 8 h. This process opens the membranes for the dyer by dissolving out the membran proteins. The staining lasted for 3 h and was done with 2 μl of a 38 μM methanolic TRITC-labelled phalloidin solution which was added to 100 μl 0.1% Triton-X-100, buffered in 0.1 M PBS. Phalloidin binds irreversible at f-actin in the muscles and thus serves as a marker.

Individual specimens were embedded in a drop of Citifluor (9:1 Citifluor to antibleaching solution) on object slides and covered with cover slips. To avoid compressing the animals, they were embedded within a reenforcer. Then, those which looked most promisingly under the light microscope were scanned with the CLSM at the Deutsches Zentrum für Marine Biodiversitätsforschung (DZMB) in Wilhelmshaven.

Since the CLSM images were not applicative because they were too vague, finally scans of a *Testudinella clypeata* were used for the reconstruction. The species was determined according to Remane (1929). The examined specimen as a light microscope image is given in figure 2.

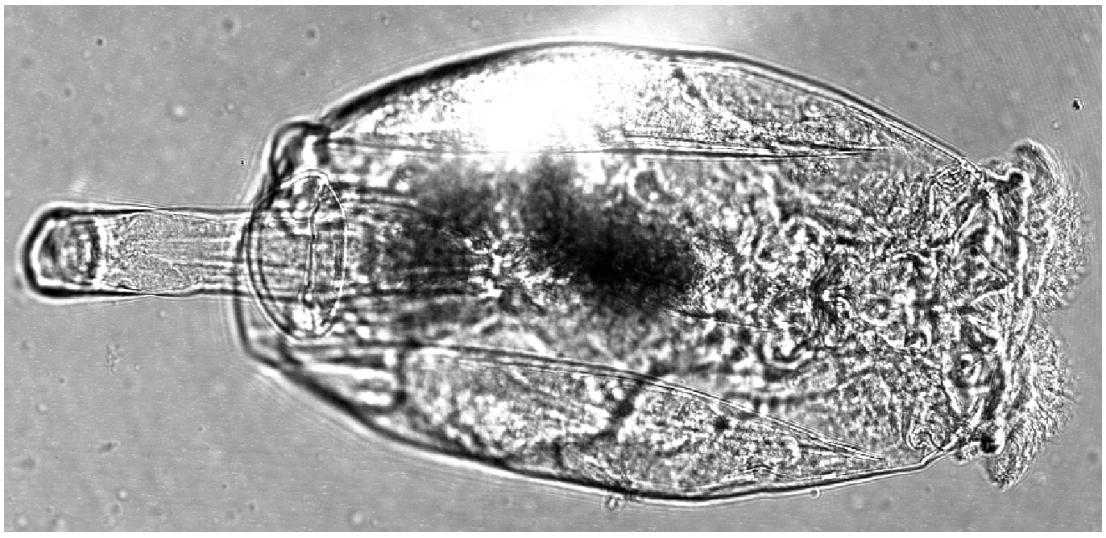

Figure 2: *Testudinella clypeata* under the light microscope, ventral view.

This sample was collected earlier in a salt meadow pool between the dike and the Jadebusen at Cäsiliengroden at 53° 29' 8.1" N, 8° 3' 22.8" E. It was done by dragging a plancton net with a mesh size of 75 μm and a diameter of 25 cm through the water. The treatment was the same as with the *Synchaeta* above.

The image stack from the CLSM was imported into Amira. Each muscle was labelled and finally all muscles together were rendered to a three-dimensional object. The procedure represented here is a further developement of an unpublished previous work by Reichl (2009).

For the three-dimensional reconstruction Amira, version 5.0.0, was used because it serves exactely this purpose and is often used in science and medicine. For the further work the programme Autodesk® 3ds Max® 2010 is intended. This programme makes it possible to pull at or press on any spot of the surface of an object, so that it can be arbitrarily deformed. More over, texture pattern can be applied to individual objects, the whole scene can be illuminated and even animation is possible. It also proved to be the most practical programme for the transition from Amira, because it does not make it necessary to use any transform programme which converts the export files of Amira in readable import files for an other programme. So, Autodesk® Maya® 2009 which was used in the first instance finally was not used due to such problems which finally lead to a failure in passing the Amira output into it.

It surely would have been an advantage to use *T. patina* instead of *T. clypeata* because Seehaus (1930), Sørensen (2005) and Kotikova et al (2006). also used *T. patina*. But there are no essential differences in the musculature expected and the used sample of *T. clypeata* was already available and acquiring a new sample would have taken too much time.

Results

This section explains in its first part how to get from an image stack to a three-dimensional reconstruction of the musculature. The second part is a description of the muscles which were found in this investigation. This coveres the corona retractors, the muscles of the foot, the dorso-ventral muscles and the visceral muscles. The mastax muscles were not looked into in this work.

The three-dimensional reconstruction

In the following it is explained at first how to read the CLSM data in and how to get the first impression of it as a three-dimensional object. Then follows how to mark the different muscles (labelling), which is by far the most time-consumig portion of the whole work. Thereupon the rendering and visualization is described. Finally comes the export of the data to use it in other programmes. Before the data can be read in with Amira, it has to be created. This is done with the CLSM. It is not described here how to do this. But the work with the CLSM ends with a *.lif-file which is a prerequisite for the following process.

Reading in

The first step in Amira is to load the image stack. This is done by clicking on Open Data... in the **Pool** (figure 3), or on File → Open Data... Ctrl+O. A dialog appears where the *.lif-file can be chosen (figure 4). Thereupon opens another window which gives information on the file to load (figure 5). It is not necessary to do anything here. Clicking OK closes this window and the loaded object appears in the **Pool** (figure 6).

First impression with Isosurface

Now the object should get a name. A left click on the object activates it and causes some orange and yellow buttons to appear on the top of the **Pool** which become important later. Those buttons are shortcuts for the most used functions. A following right click on the object opens a pop-up menu which allows to rename the object. The species name and the date of collecting are useful (figure 7 and 8).

To get an impression of the three-dimensional object an isosurface can be created: Clicking on the shortcut button Isosurface shows a new object in the **Pool**. The other way is right clicking on the object → Display ▶ → Isosurface. Below the **Pool** is the **Properties** window which shows the properties of the active object. There, the treshold should be set to about 50 and the option downsample ticked off (figure 9). The appearance of the property Average can be ignored. Pressing Apply induces the computer to generate a surface representation of the animal (figure 10).

The result with the *Synchaeta* was not usable because the staining had not only effect on muscles, but also on much other tissue. So, it was changed to an other record (*Testudinella clypeata*), as illustrated by the figures 11 and 12. The new object appears as a triple object in the **Pool**. This may be confusing, but it has effectively no meaning. The reason is a different way of scanning with the CLSM. The **Properties** and the isosurface of the *Testudinella* are given in the figures 13 and 14.

Labelling

The next step is the labelling. Amira provides an own editor for this. Clicking on the Segmentation Editor button above the **Pool** leads to it (figures 15 and 16). Also to be seen in figure 16 is the button New on the right of the Label Data. It has to be pressed to get a new albeit yet empty list of materials (figure 17). The **Pool** gets a new object: Testudinella090217.Labels (figure 18).

The New button right of Materials creates a new material in the list beneath. Its name and colour can be changed by right clicking on it and selecting the entry in the pop-up menu (figure 19). An other, more simple possibility is a double click on the actual name and the coloured square. By and by this list will get longer as one muscle after the other is marked in the windows on the left.

Each of these windows show the loaded data along the x-, y-, and z-axis respectively. The scale, contrast and colour of the content of these windows can be changed (figure 20). Within these windows left clicking and dragging the mouse marks an area, the mouse wheel scrolls through the image stack and pressing the wheel and dragging translates the image. To mark material a selection tool has

to be chosen. The most useful one is the brush. Often its size must be changed (figure 21). Every time an area of a muscle is marked (figure 22), it soon should be added to the material in the material list. It is added to that material which is selected in the list! Adding (or substracting) is done by clicking the ⊕ and ⊖ buttons under Selection (figure 23). The first of the four buttons under Display and Masking turns on a crosshair which often is useful to orient oneself within the three axis views (figure 24).

It is recomended to save the proceedings frequently: back in the **Pool** a left click on the objects (selecting) followed by a right click (pop-up menu) gives the possibility to do so. If one of the green objects is unsaved it has an asterisk (*) at the end of its name. Also the whole network should be saved by right clicking on an empty spot within the **Pool** which lets pop up a menu (figure 25).

Rendering

When a whole muscle is marked—or in between—the reconstruction can be rendered to have a look at the progress up to now. To do this the surface has to be generated. This is done by right clicking on Testudinella090217-labels.am → Compute ▶ → SurfaceGen. A new red object SurfaceGen and its properties appear (figure 26). Pressing Apply first opens a window which gives information about the input data and an advice (figure 27). It can be ignored by pressing Continue. The amount of data is reduced later. Then, computation takes place which results in a new object Testudinella090217-labels.surf.

Now the object should be simplified, i.e. the amount of data is reduced. This begins with pressing the simplifier button. The Surface line gives information on the object. In the next line (Simplify) the number of faces to be entered should be one half to three quater as great as in the above line (figure 28). The button Simplify now triggers computing again. So, the number of faces is reduced.

Next, the surface is to be smoothed: right clicking on the last object again → Compute▶ → SmoothSurface → setting iterations to 20 → Apply (figure 29). The new object Testudinella090217-labels.smooth.surf appears in the **Pool** (figure 30).

To make the surface visible the object must be right clicked on → Display ▶ → SurfaceView (figure 31). The reconstruction is displayed. The different parts (i.e. materials) have those colours specified in the labelling process (figure 32). To get a better overview of only a few or a single muscle the following has to be done: In the **Properties** of SurfaceView the Draw Style should be set to "shaded", Selection Mode to "Material", and the first pop-up menu of Materials to "Exterior" (figure 33). Different parts are toggled by selecting from the second pop-up menu and using the buttons Add, Remove, and Clear under Buffer. The surface of the object can be displayed smoother under Draw Style → more options → ✓Direct Normals. If there are holes in the muscles the white inner surface can have a disturbing effect on the whole representation. Since the holes can not be closed the impression can be improved by colouring the inner surface. This is done by ticking off "same" under Colors (figure 33) and finally (figure 34).

Export and Import

Is the newly created data to be worked with further programmes it has to be exported. This is done with a right click on Testudinella090217-labels.smooth.surf → compute → VRML-Export (figure 35). In the **Properties** of VRML-Export a path and a file name with the extension .wrl is to be chosen. Under Buffer there are buttons and a pop-up menu similar to the SurfaceView module. Here, all or only a few muscles can be chosen for the export (figure 36). Pressing Apply creates the *.wrl file.

This file can be imported in StudioMax by clicking on the greenish square with the StudioMax symbol in the upper left corner of the programme. A menu pops up where the entry Import allows to import (figure 37). A dialog opens where there is to select the file type .wrl and the file itself (figure 38). Thereupon, an other dialog with three options opens (figure 39). They are already ticked off and the OK button can be pressed. It can take a while until a large file is loaded (figure 40). There can be some artefacts in the imported scene. That are tiny bodies treated by StudioMax as normal object, so they can easily be deleted.

The procedure in figures

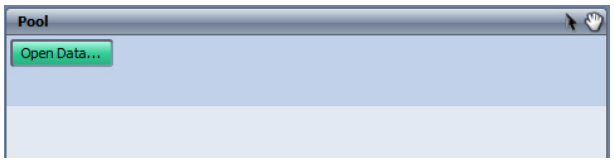

Figure 3: Press the button Open Data... to load the data.

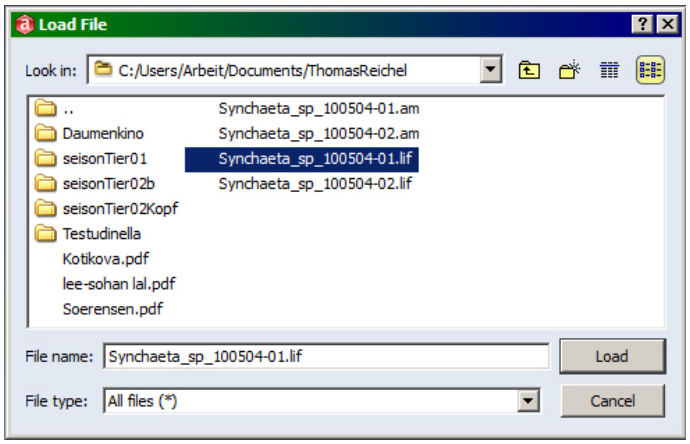

Figure 4: Choose the *.lif-file to open.

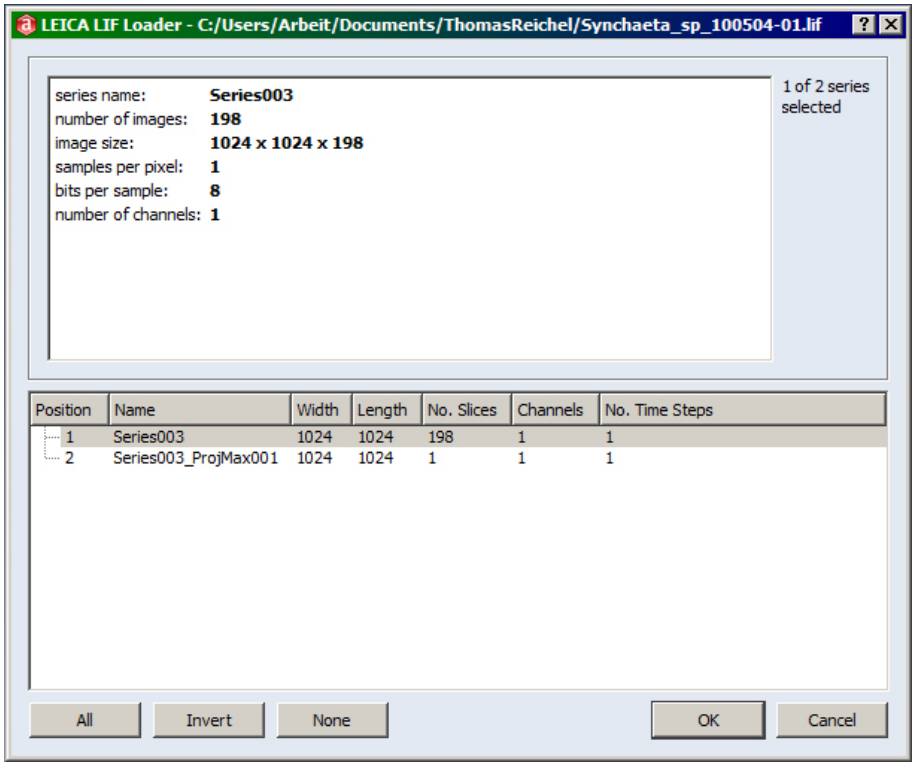

Figure 5: Information on the data to load. Nothing has to be done here aside from pressing OK.

Figure 6: The loaded file appears as an object in the **Pool**.

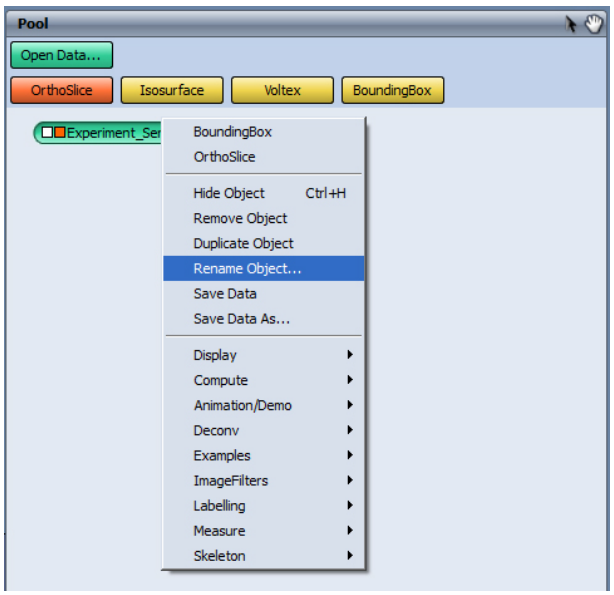

Figure 7: After a left (selecting) and a right click on the object a pop-up menu appears. The object can be renamed.

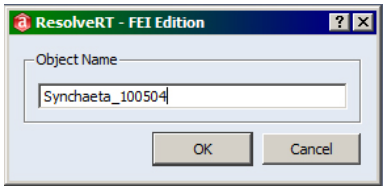

Figure 8: Type a new meaningful name, e.g. species and date.

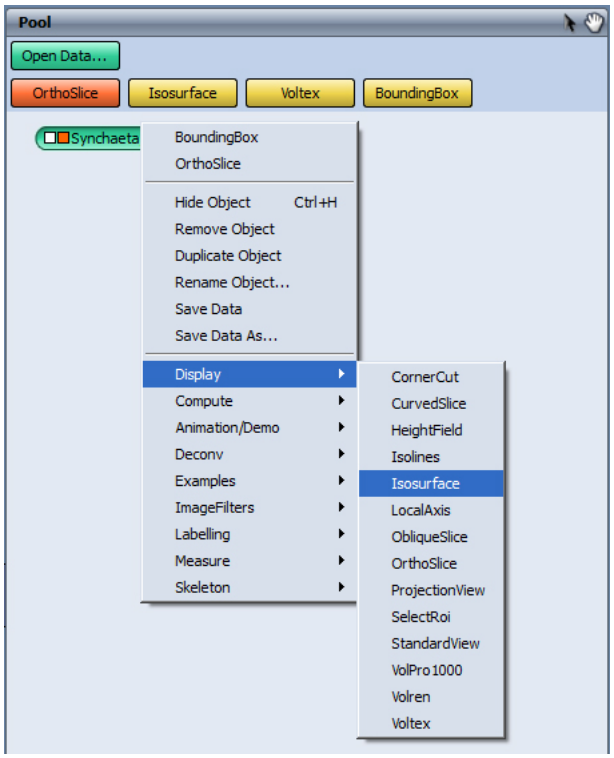

Figure 9: After a right click on the object choose Display and Isosurface from the pop-up menu or use the shortcuts above.

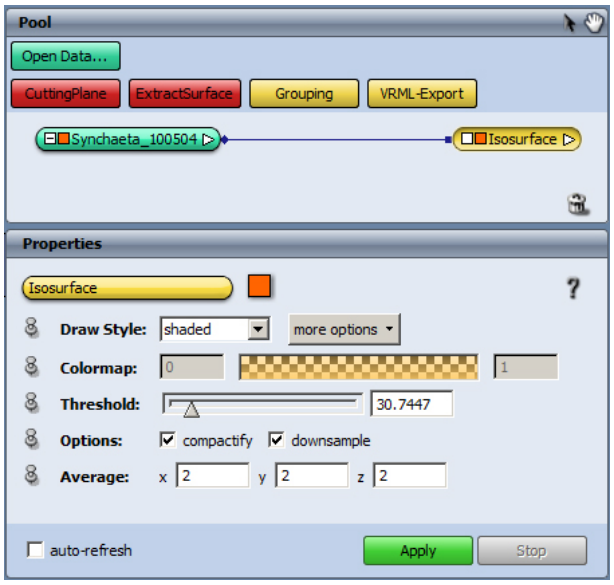

Figure 10: To get the isosurface set Threshold to about 30, tick off downsample in the Options line and press Apply.

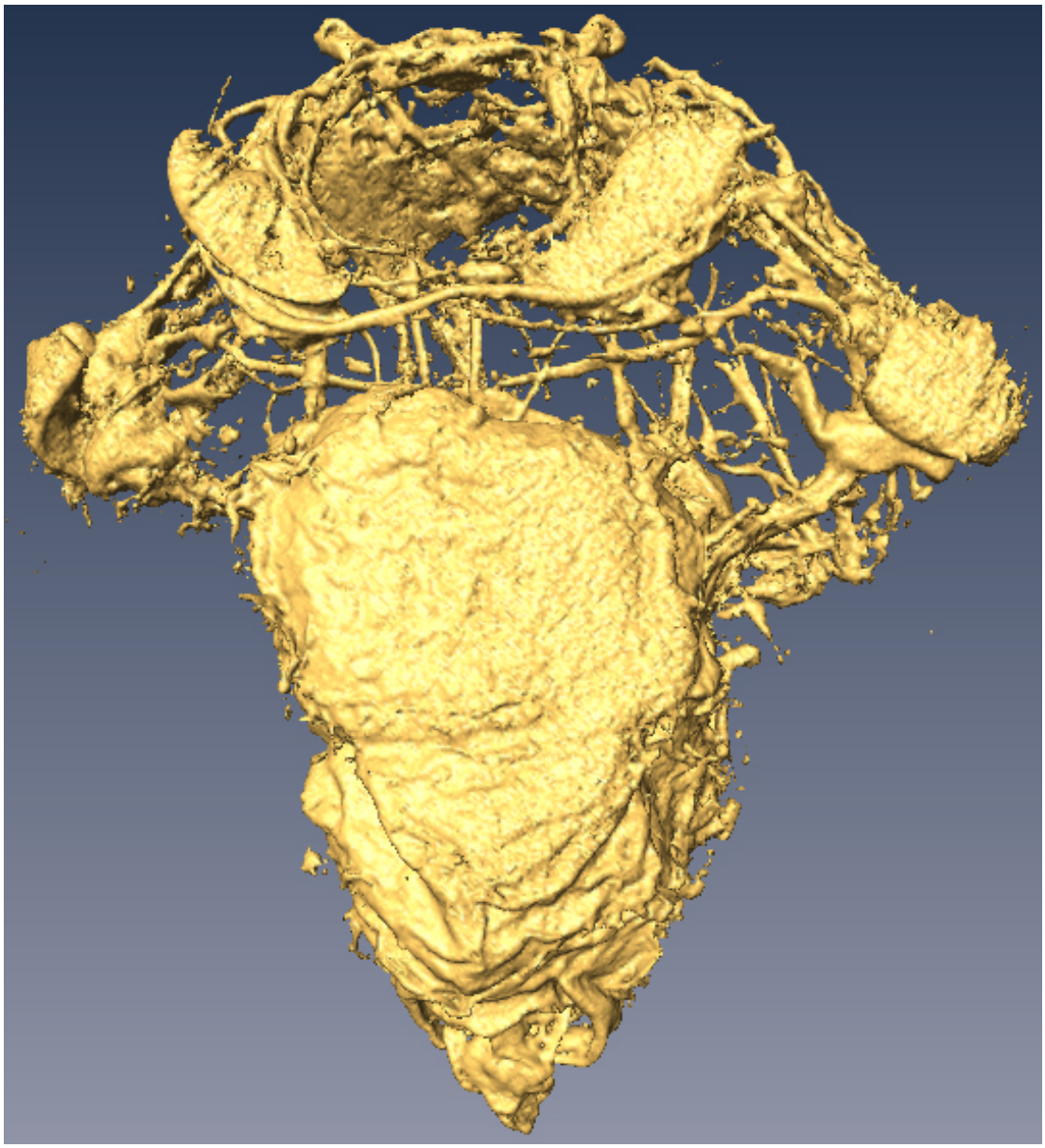

Figure 11: The isosurface of the *Synchaeta*.

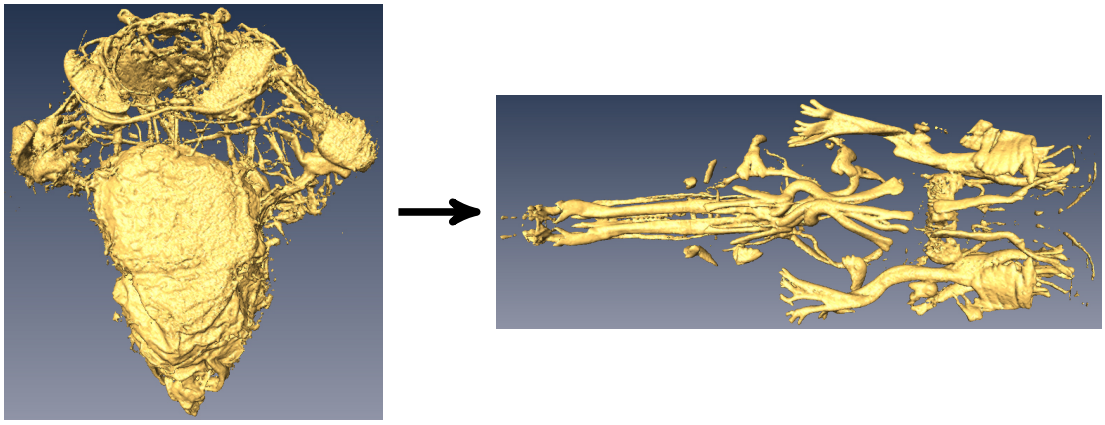

Figure 12: Since the staining of the *Synchaeta* was not usable there is a change to an other sample, a *Testudinella*.

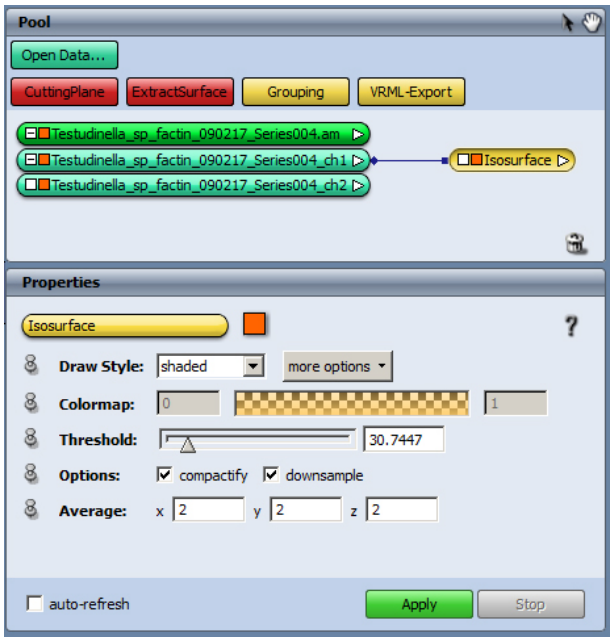

Figure 13: The properties of the isosurface of the *Testudinella*.

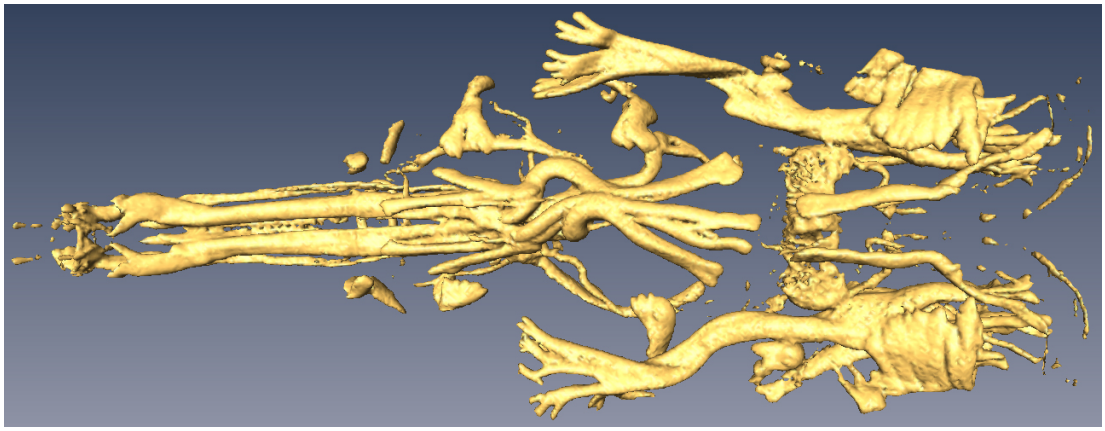

Figure 14: The isosurface of the *Testudinella*.

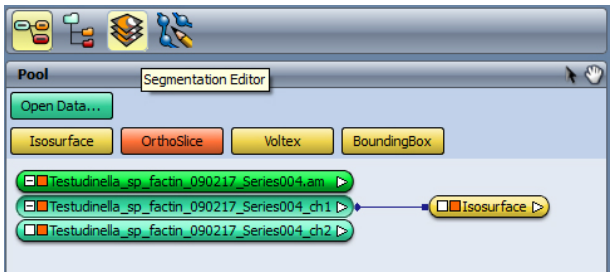

Figure 15: Pressing the Segmentation button leads to...

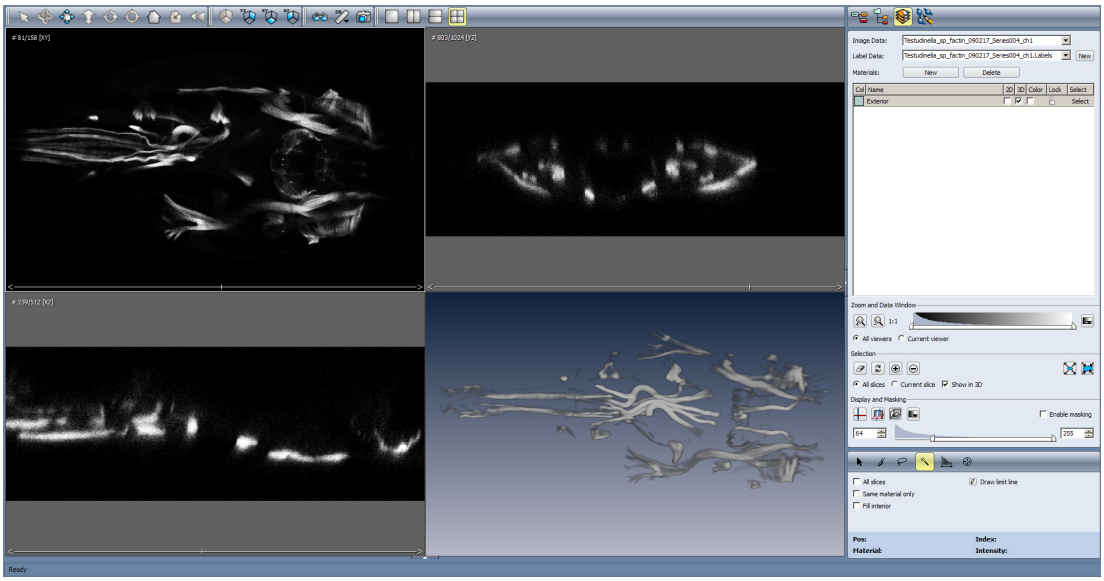

Figure 16: ...the segmentation editor. Click on New left of Label Data to set up a new list of materials and to display the four views on the left. Within these windows left clicking and dragging the mouse marks an area, the mouse wheel scrolls through the slices and pressing the mouse wheel and dragging translates the slide.

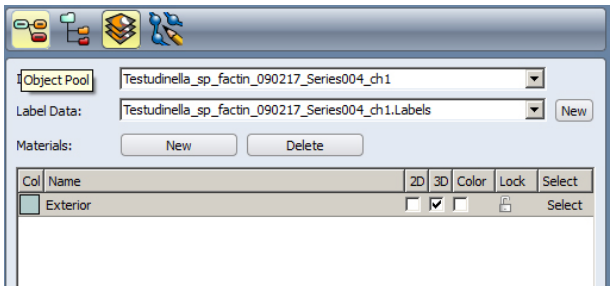

Figure 17: Pressing the Object Pool button switches back to the **Pool**.

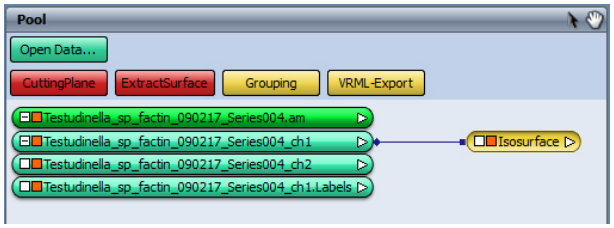

Figure 18: A new label object is in the **Pool**.

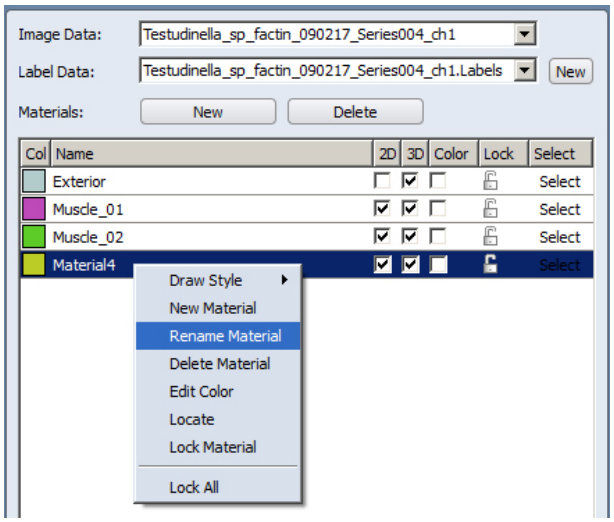

Figure 19: Create a new material by pressing the New button next to Materials. Select and right click on it to change its name an colour.

Figure 20: The scaling, contrast and colour of the three axis views can be changed here.

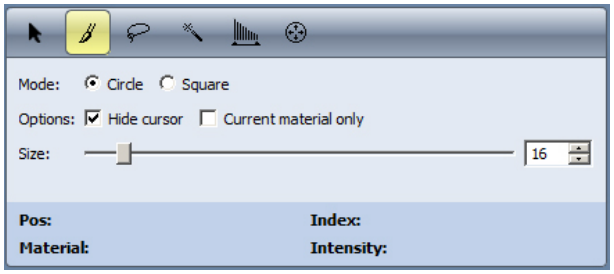

Figure 21: To mark an area a selection tool is needed. The brush is the most important one. Often its size has to be changed.

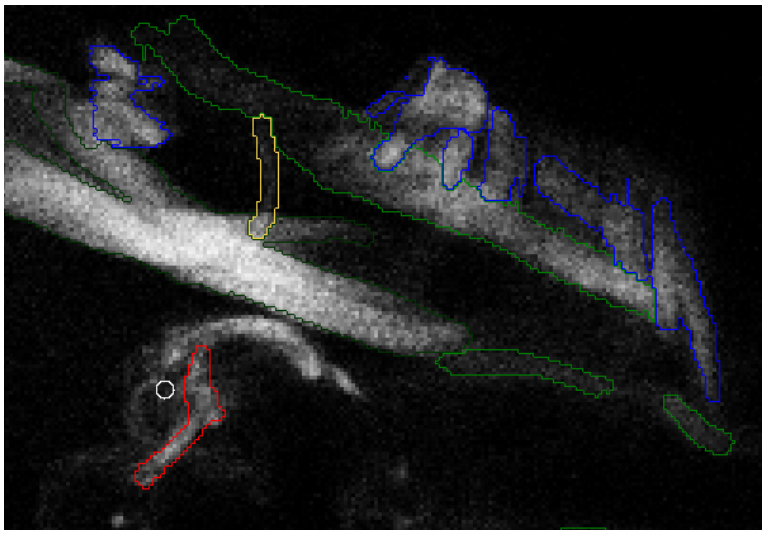

Figure 22: This red hemmed area is marked and can be added to the selected material. The areas with other colours are already assigned to other materials.

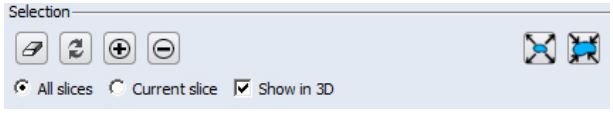

Figure 23: Adding to and substracting from a selected material is done with the plus and minus buttons.

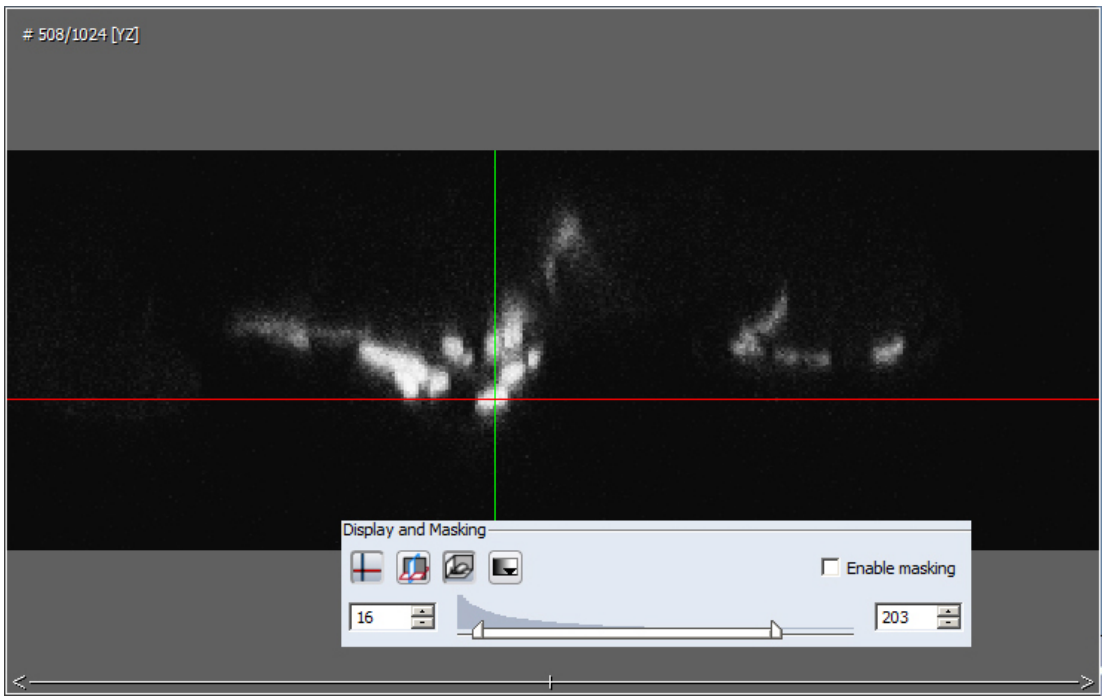

Figure 24: The crosshair, available by pressing the left of the four buttons, helps to orient oneself.

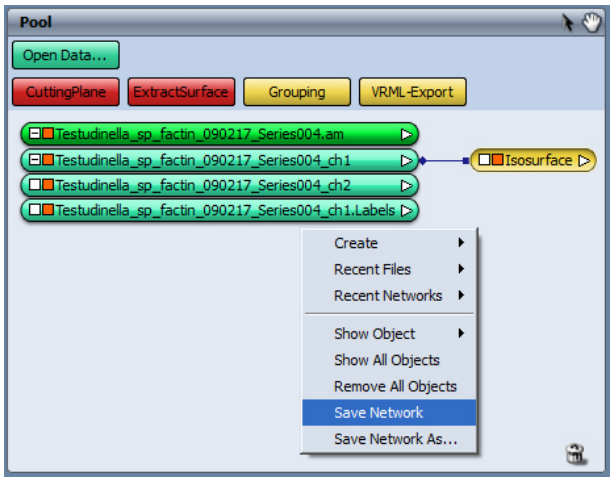

Figure 25: Save the unsaved objects (indicated by an * at the end of the name) and the whole network by a right click which invokes the pop-up menu.

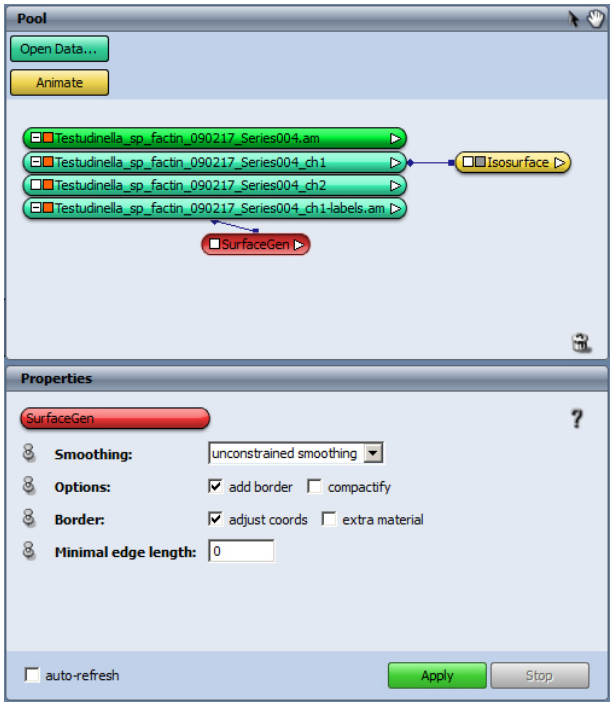

Figure 26: After a right click on the object choose Compute and SurfaceGen from the pop-up menu and press Apply.

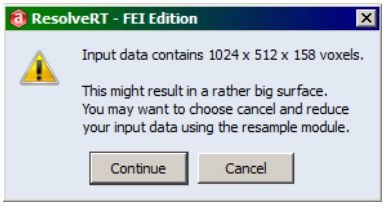

Figure 27: Information on the data and advice. It can be ignored by pressing Continue.

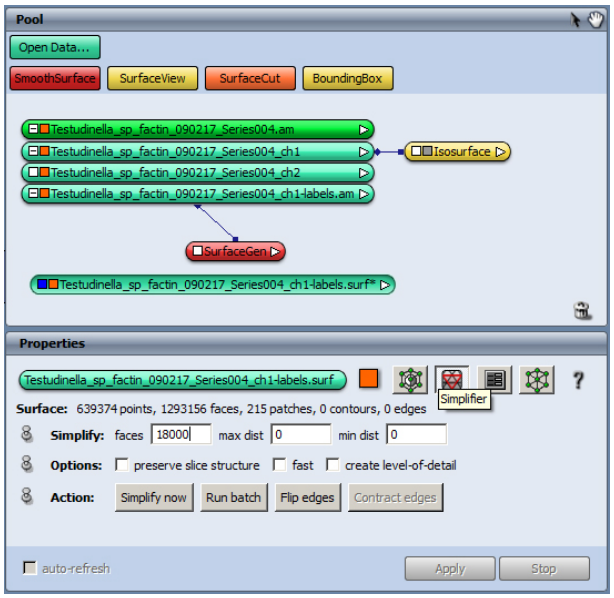

Figure 28: Activate the new object and invoke the simplifier. The Surface line shows the number of faces. It should be reduced to three quarter to the half in the Simplify line. Press Simplify now thereafter.

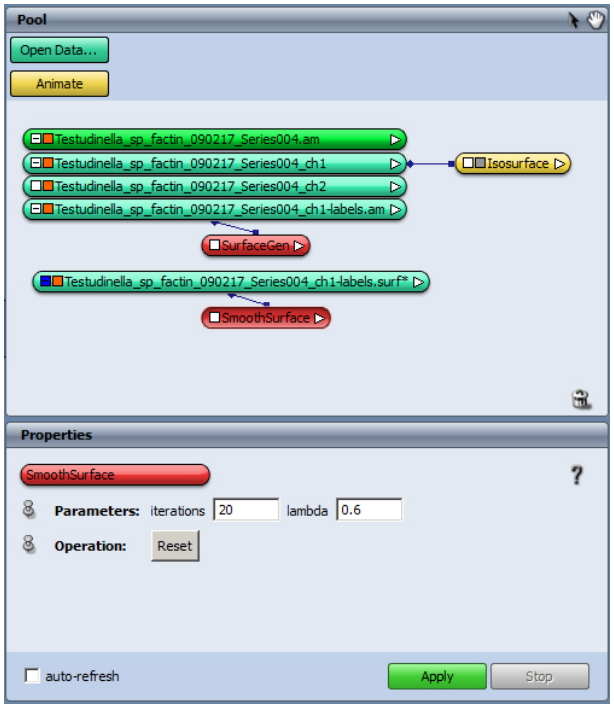

Figure 29: After a right click on the object choose Compute and SmoothSurface from the pop-up menu, set iterations to 20 and press Apply.

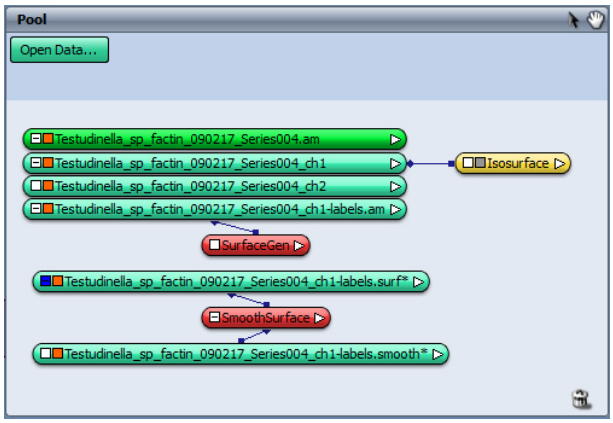

Figure 30: A new object appears in the **Pool**.

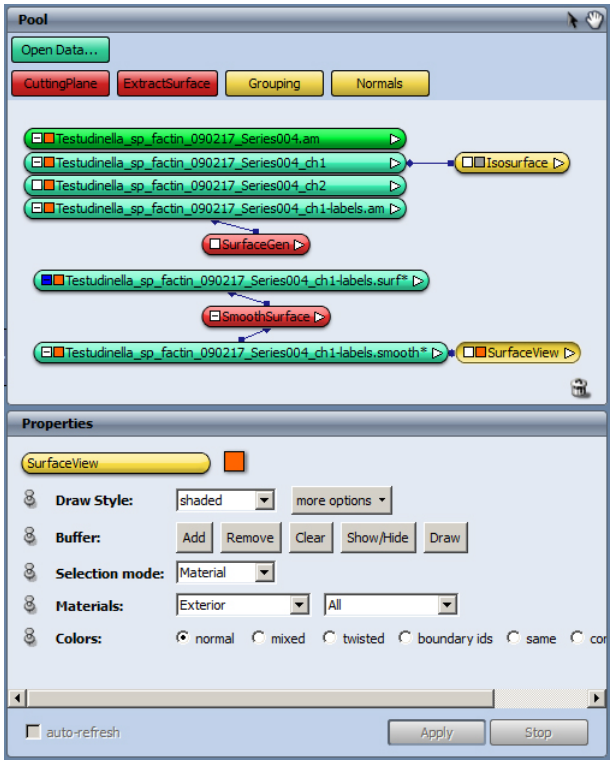

Figure 31: After a right click on the new object choose Display and SurfaceView from the pop-up menu.

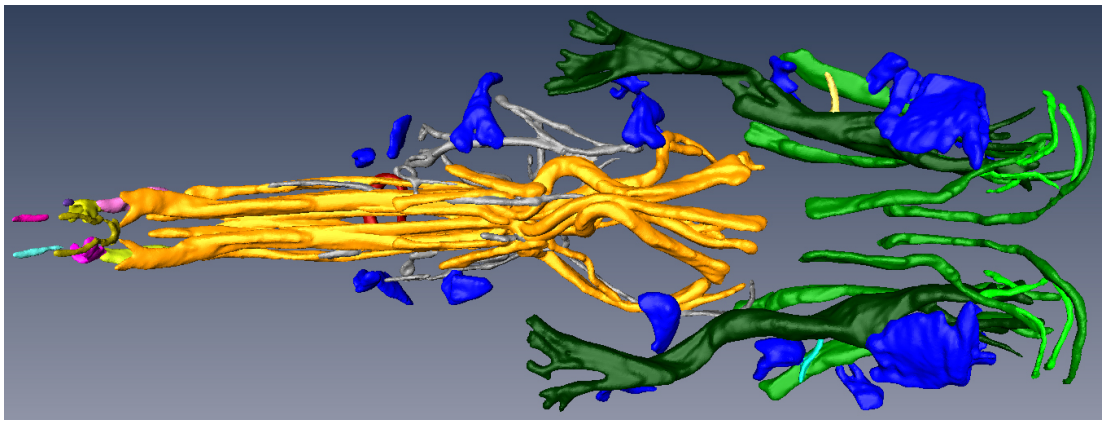

Figure 32: All marked areas which were assigned to a material are displayed.

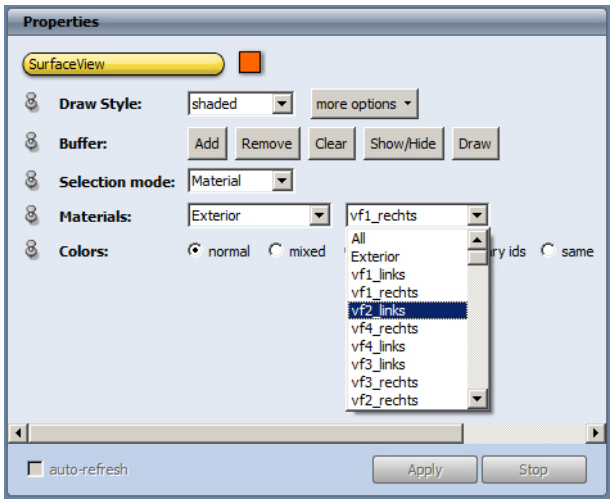

Figure 33: To display only a few muscles these are chosen from the second pop-up menu under Materials and added or removed with the buttons under Buffer.

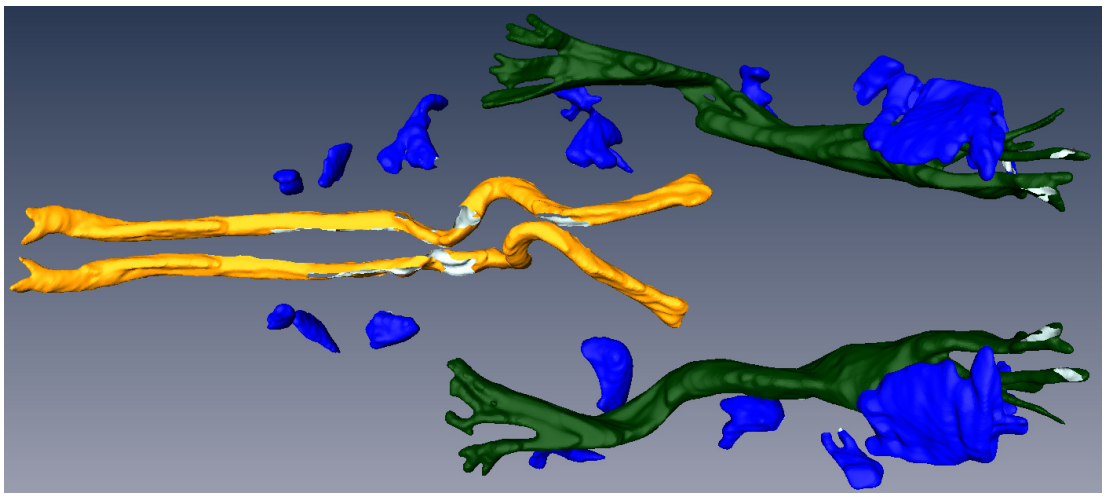

Figure 34: An example for displaying only a few muscles.

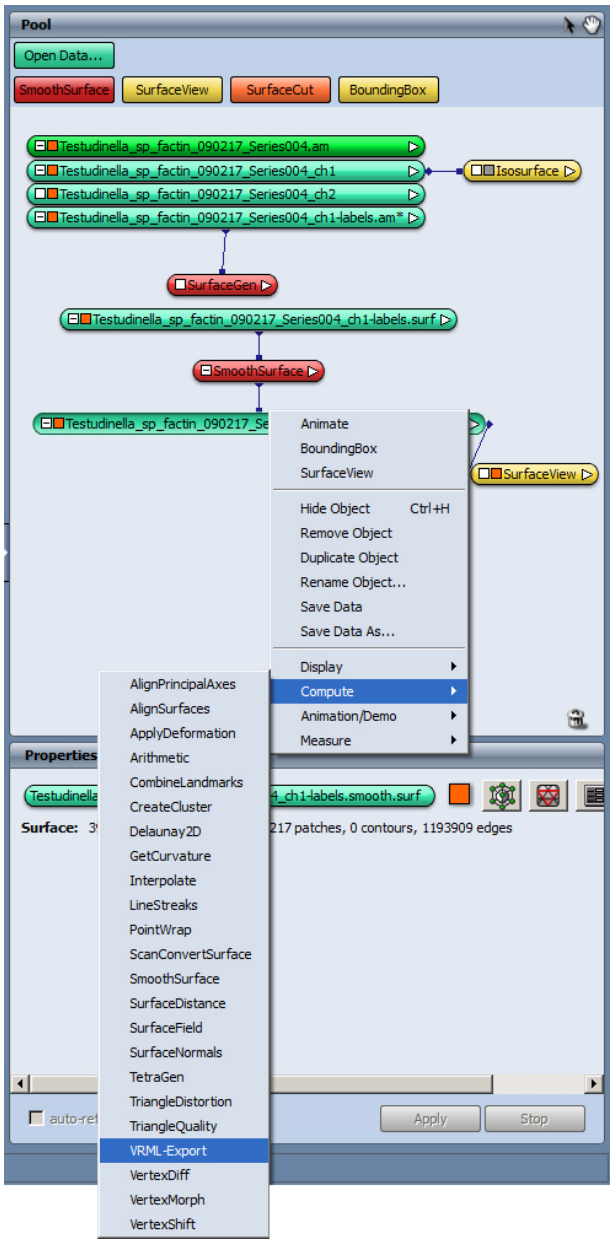

Figure 35: A right click on the *-labels.smooth.surf object and selecting Compute and VRML-Export creates an export module in the **Pool**.

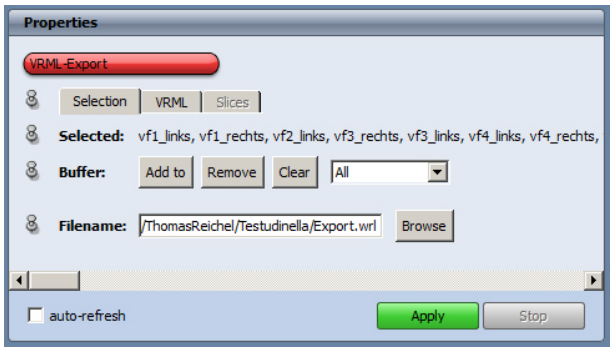

Figure 36: In the **Properties** of the VRML-Export module muscles can be selected and added or removed and a path and a file name with the ending .wrl has to be chosen. Pressing Apply creates the file.

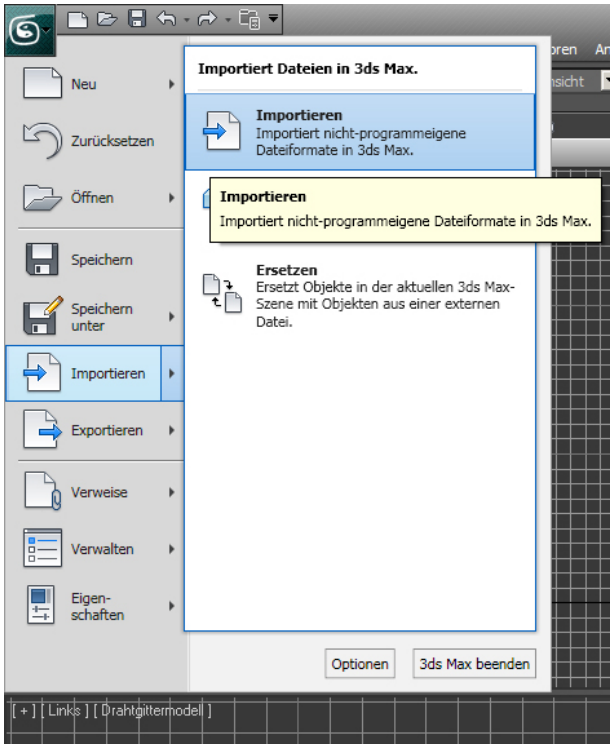

Figure 37: Selecting Import initializes the import in StudioMax.

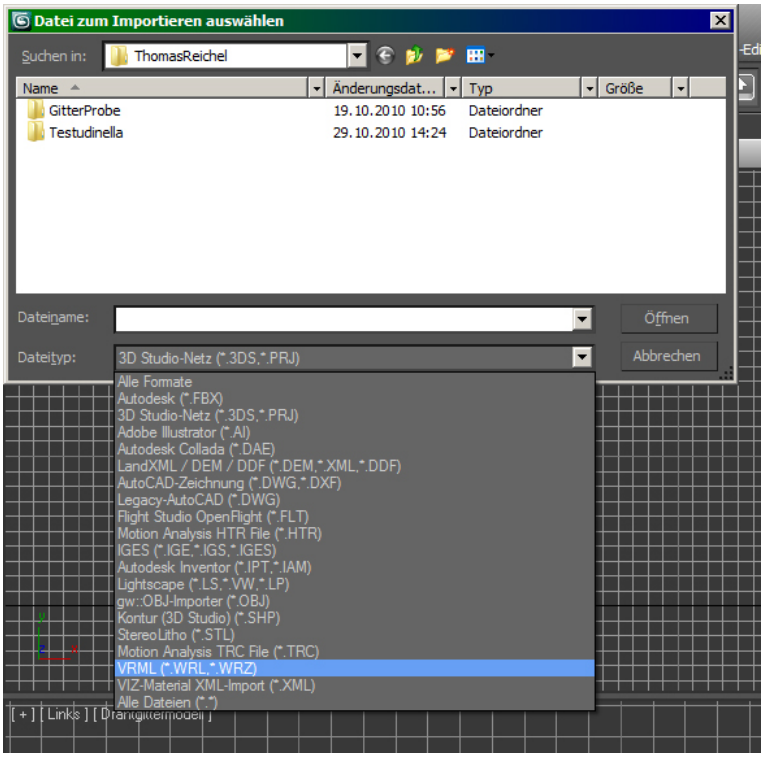

Figure 38: Selecting the file type .wrl displays the *.wrl files in the current directory.

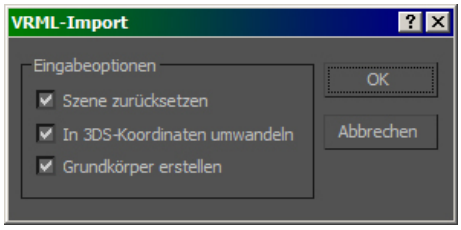

Figure 39: Those three options are already ticked off; pressing OK imports the data.

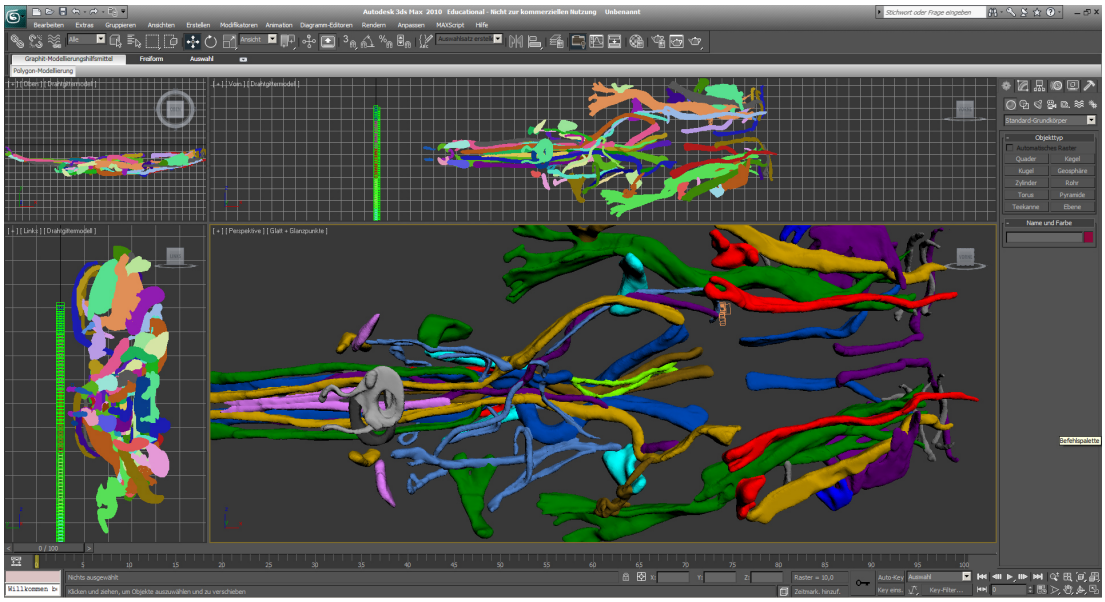

Figure 40: The whole object imported in StudioMax as a set of individual muscles.

The body musculature

In the following the musculature of the examined specimen is described. The musculature was divided in corona retractors, foot muscles, dorsolateral muscles, visceral musculature and toe muscles. For this examination the mastax was excluded, because the focus should be on the body musculature. The corona muscles were named after Sørensen's suggestion (Sørensen, 2005). The idea of the consecutive numbering of the foot muscles and the dorsoventral muscles traces back to Seehaus (1930). This is also the case with the term "dorsoventral muscle". The following figure 41 gives an overview of the musculature as it is shown in the filament editor. Here, all slices are projected in a single plane. The figures 42 and 43 actually are three-dimensional, although they only have the effect of a relief. In all three images the lorica can be seen as a contour.

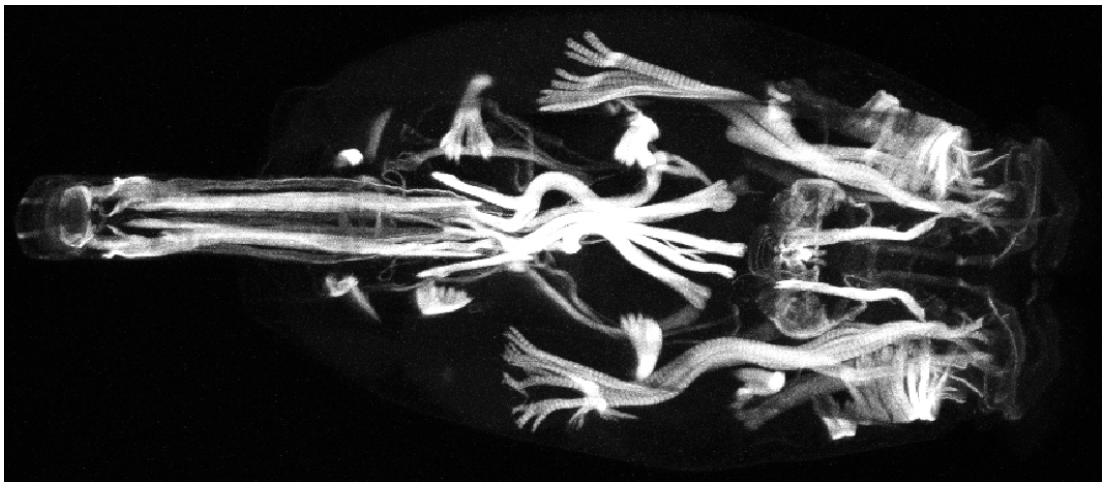

Figure 41: The whole musculature as Amira shows it at the beginning of the work projected in one image, dorsal view.

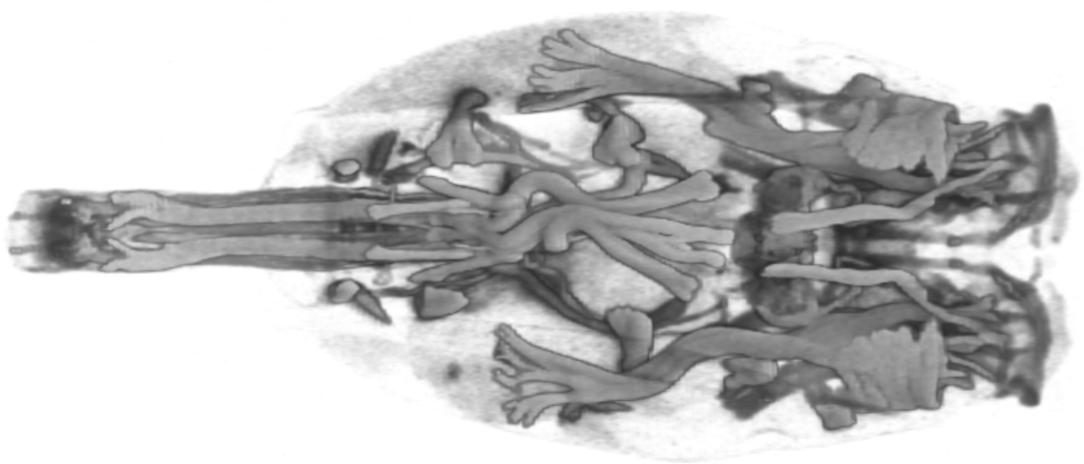

Figure 42: The musculature as Amira shows it in the labelling editor, ventral view.

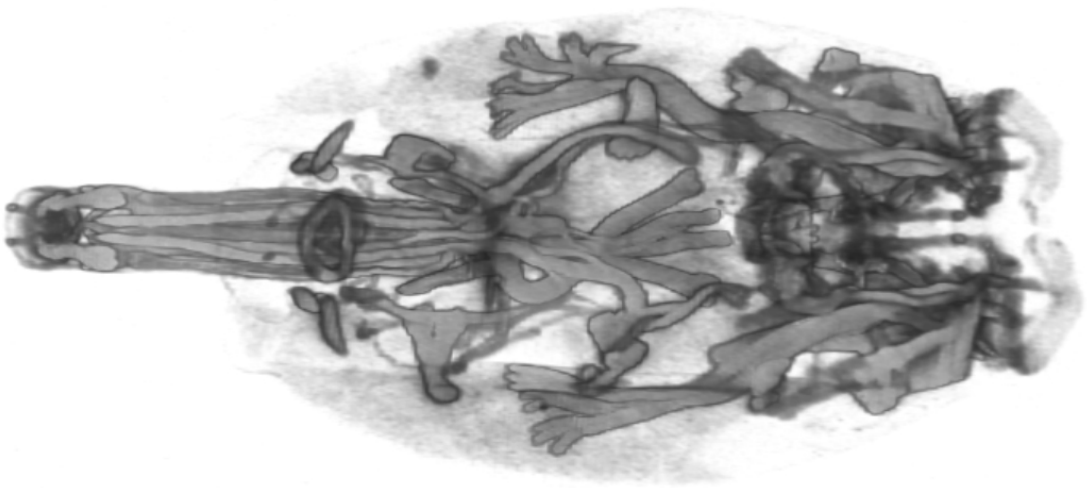

Figure 43: The musculature as Amira shows it in the labelling editor, dorsal view.

The corona retractors

As presented in figure 44 five pairs of corona retractors were found in this study. The most conspicious ones are the broad retractors. They insert posterior to the middle of the lorica and extend into the corona. At both ends they bifurcate several times. Outside the broad retractors and more to the dorsal side lie the lateral retractors. They insert laterally at the border line of the anterior and middle third of the length of the lorica and run to the end of the corona. Dorsally and partially more to the median of the broad retractors lie the dorsolateral retractors. They are a bit longer than the lateral retractos, but also end in the corona. The dorsomedial retractos lie rather closely adjoined to each other on the dorsal side, bend to the ventral side and extend in a bow within the corona outward (figure 45). They insert approximately at the anterior end of the mastax, thus being those corona retractors which insert most anteriorly. The ventromedial retractors on the ventral side also extend into the corona. They insert a bit anteriorly of the posterior end of the mastax. There were some thin muscles found in the corona the affiliation of which is undiscernible, because there were no connections to the thicker muscles recognized. Furthermore, there are two short muscles which seem to be adjusted to the broad retractors and extend laterally outward.

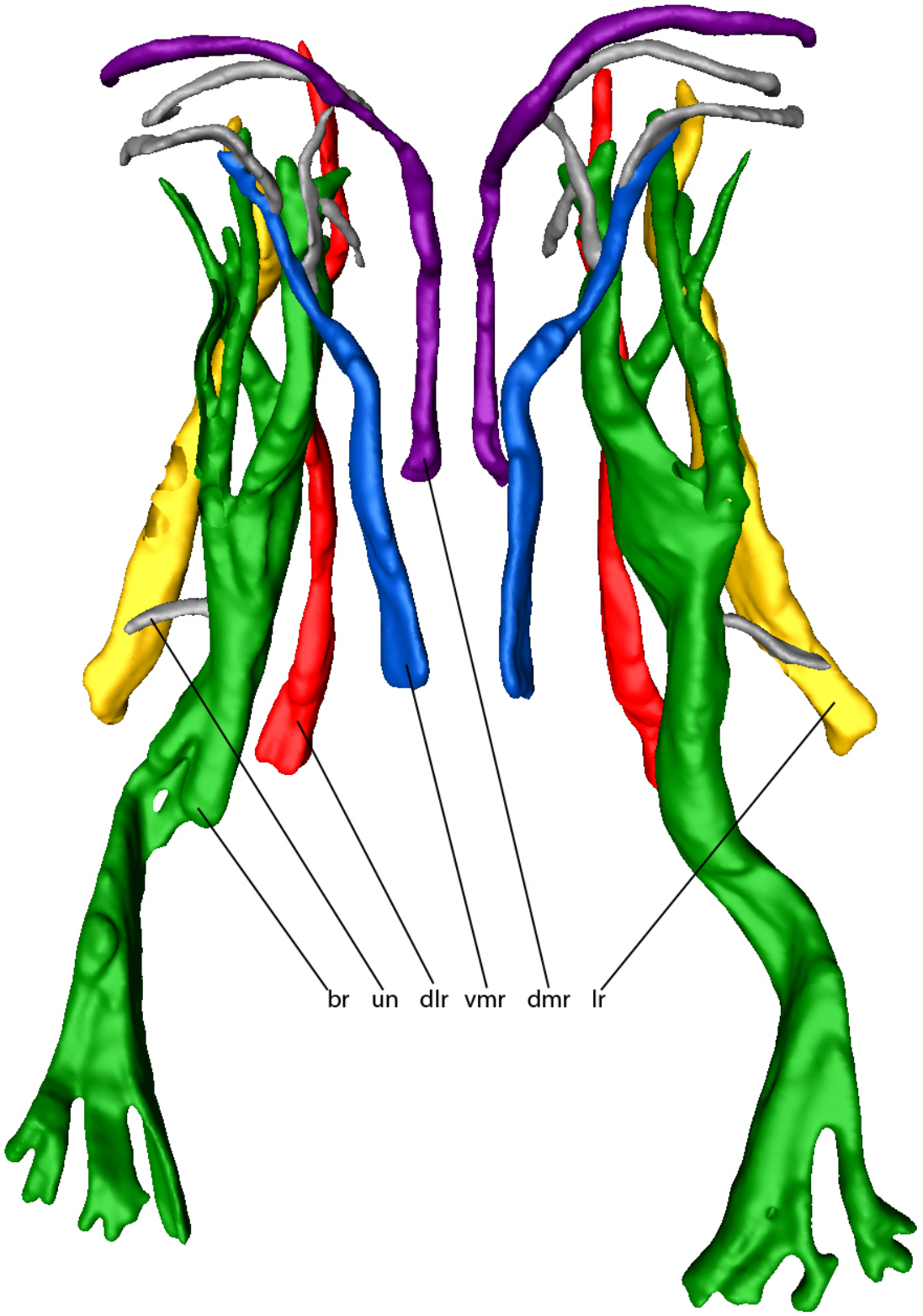

Figure 44: The corona retractor muscles, ventral view. The affiliation of the unnamed grey muscles is not clear. broad retractor (br), dorsolateral retractor (dlr), dorsomedial retractor (dmr), lateral retractor (lr), ventromedial retractor (vmr), unclear affiliation (un).

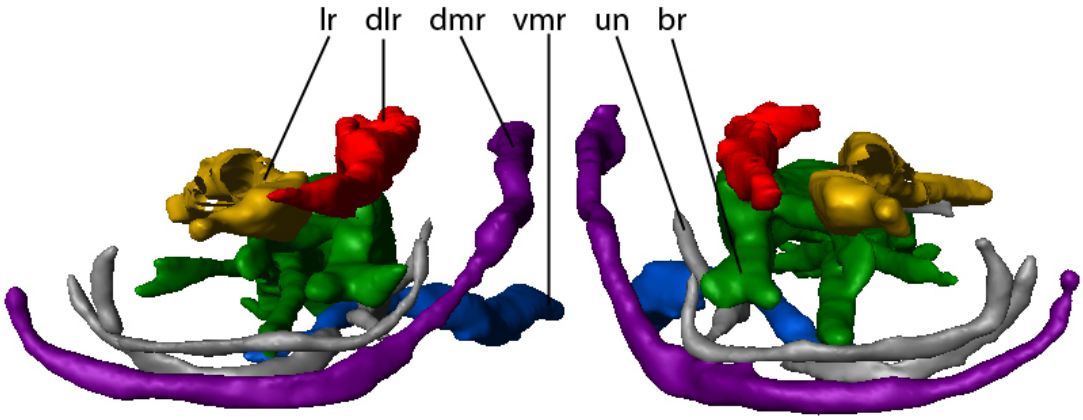

Figure 45: The corona retractor muscles, frontal view, ventral side at the bottom. The affiliation of the unnamed grey muscles is not clear. broad retractor (br), dorsolateral retractor (dlr), dorsomedial retractor (dmr), lateral retractor (lr), ventromedial retractor (vmr), unclear affiliation (un).

The foot muscles

The foot muscles are divided into two groups, i.e. the dorsal and the ventral muscles, and are numbered consecutively from lateral to medial.

There were found four pairs of dorsal muscles as figure 46 displays. The second and fourth dorsal foot muscles insert laterally and shortly posterior to the mastax. The insertion of the first dorsal foot muscle is laterally attached to the second at a location where the foot muscles are very densely packed. Also in this location lie the insertion of the third muscle. The second muscle extends to the end of the foot, whereas the other dorsal foot muscles end short before it. There were found two further muscles, probably a matching pair, with unclear affiliation. They are located ventrally of the middle part of the first muscle.

Five pairs of ventral foot muscles were found in this study, which can be observed in figure 47. The first, second and fifth muscle insert, like the second and fourth dorsal muscles, shortly posterior to the mastax, but more medially, in particular the second and fifth muscle. The first—a thick pair—extends to the end of the foot. The second ends between the end of the foot and the dense area mentioned above. The fifth pair, which is very thin, seems to disappear in the left muscle of the first pair before the dense area. This dense area is also the origin of the third and fourth pair. The third pair lies laterally, inserts a bit farther anteriorly and runs in a narrow angle outward. The fourth pair lies ventrally of and parallel to the first. Both, the third and fourth pair are rather short.

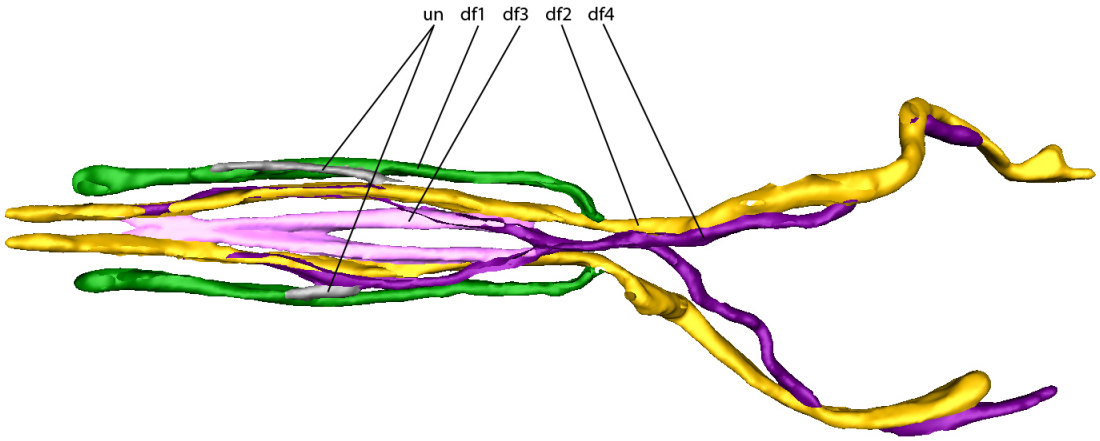

Figure 46: The dorsal foot muscles, ventral view. The abbreviation un stands for muscles of unclear affiliation.

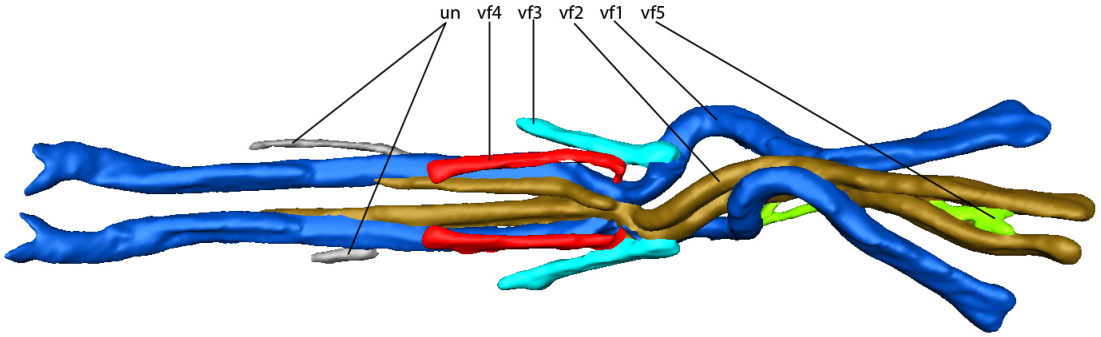

Figure 47: The ventral foot muscles, ventral view. The abbreviation un stands for muscles of unclear affiliation.

The toe muscles

There were also found some toe muscles. They are represented in figure 48. They comprise a pair of broad, flat muscles above the largest pair of ventral muscles, a pair of C-shaped muscles attached to those, two pairs of short, thin muscles at the extreme end and a muscle which is U-shaped. This one actually are two muscles which were not identifiable well enough.

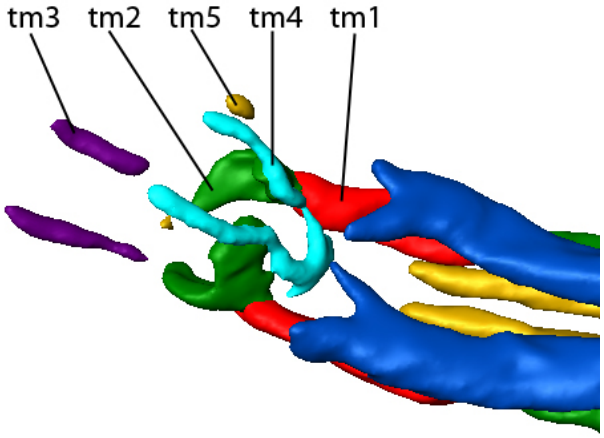

Figure 48: The muscles assumed to be toe muscles, ventro-lateral view. Also shown are some dorsal and ventral foot muscles.

The dorsoventral muscles

The eight dorsoventral muscles are numbered consecutively from anterior to posterior according to figure 49. The first three lie outside of the broad retractor. The first one is the biggest, consisting of several fibers, longer and thinner anteriorly getting shorter and thicker posteriorly. This muscle is C-shaped and bends around the broad retractor and the lateral corona retractor as detailed in figure 50. The second seems to be composed of two fibers and lies directely behind the first. The third dorsoventral muscle lies between the lateral corona retractor and the broad retractor next to the posterior end of the mastax. Definitely on the inner side of the broad retractor and after the insertions of the foot muscles lies the fourth muscle. The fifth muscle is located more posterior to the fourth, but it is not clear on which side of the broad retractor: On the left side of the examined animal it seems to lie outside the broad retractor, inside of it on the right side. This muscle possibly consists of two fibers. Also the sixth muscle

could be a composition of two fibers, but maybe it bifurcates. It lies posterior to the broad retractor and is curved almost similar to the first dorsoventral muscle. Short before the foot opening there are the seventh and eighth muscle. They are close to each other.

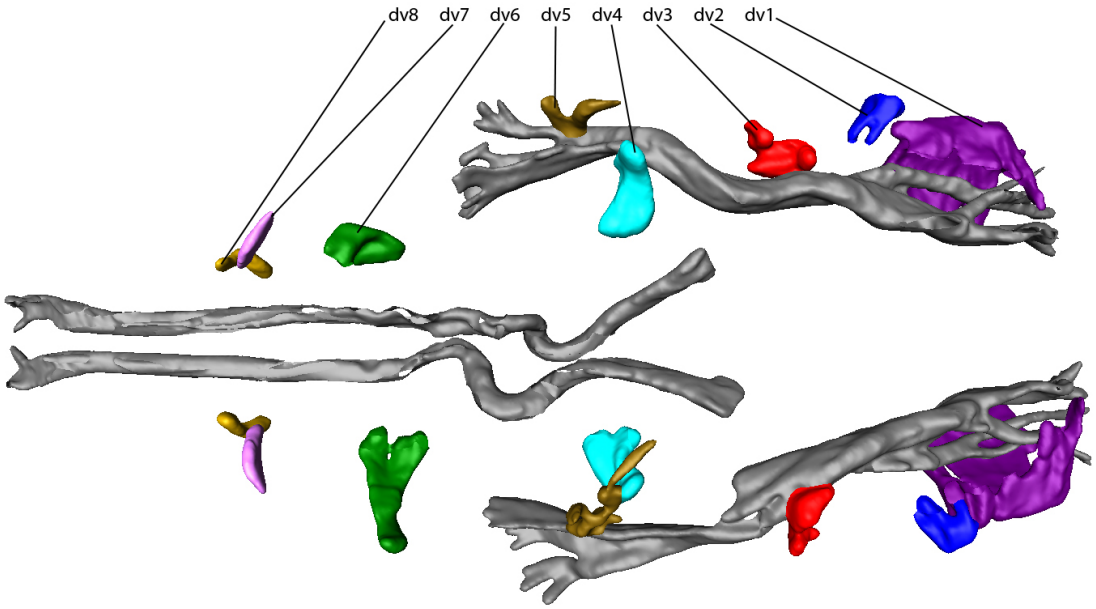

Figure 49: The dorsoventral muscles, dorsal view. The broad retractors and a large pair of the ventral foot muscles are given as a guidance.

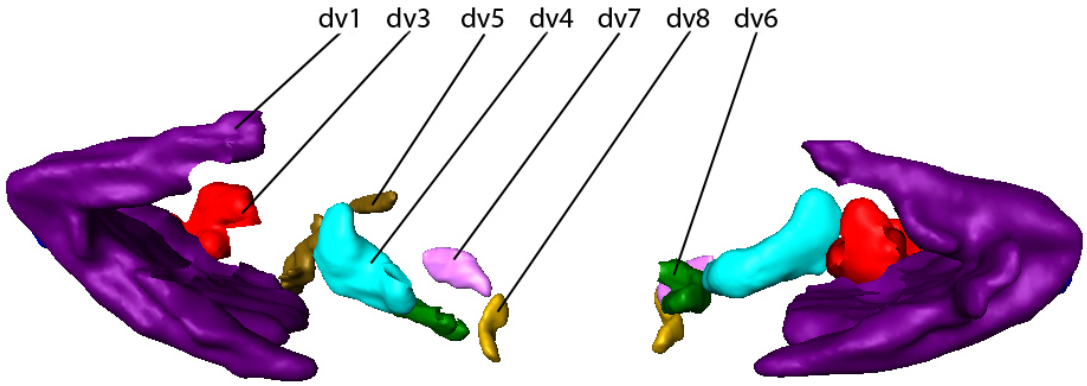

Figure 50: The dorsoventral muscles, frontal view, ventral side at the bottom. Clearly visible is the C-shape of the biggest dorsoventral muscle. The second muscles (dv2) are covered by the first ones (dv1).

The visceral muscles

Inspection of figure 51 indicates that visceral musculature was found in the surounding area of the dense area. Most of these muscles lie in the dorsal side of the body. There is one muscle on the left side which attracts attention. It runs parallel to the second and fourth dorsal foot muscles and extends beyond their insertion. An other muscle crosses the body from one side to the other. It is not quite transparent where the visceral muscles insert and where they run to, because they are very thin and were not stained well enough. They were not named.

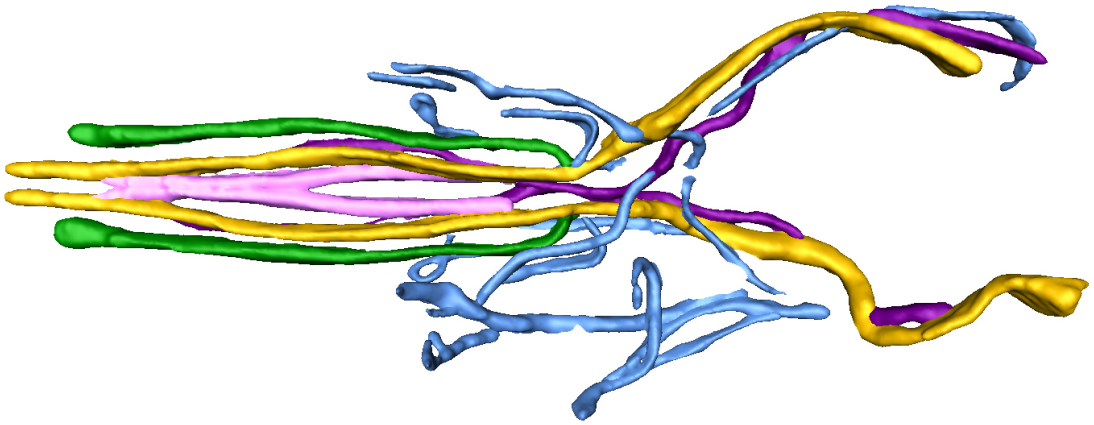

Figure 51: The visceral muscles, dorsal view. The dorsal foot muscles are given as a guidance.

The rings

Two torus-shaped structures were found on the dorsal side short before the end of the lorica (figure 52). That one lying more dorsally has two processes which extends posteriorly. It is not clear what those structures are.

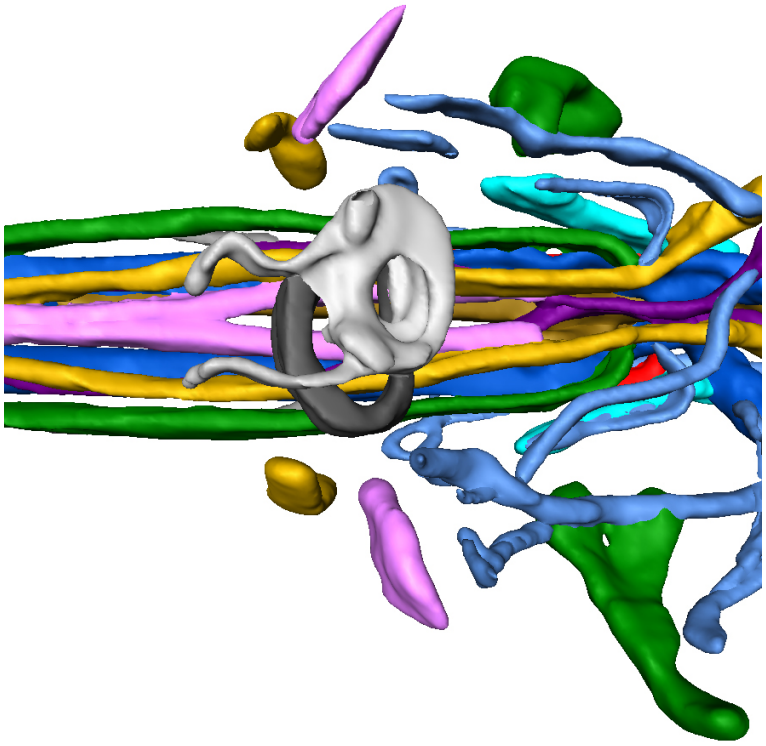

Figure 52: The rings, dorsal view.

Whole body musculature

Finally, an overview of the whole body musculature is given. The figures 53 and 54 provide it in dorsal and ventral view. Obviously matching muscles are pooled in groups visualized by different colours. Muscles of unclear affiliation remained in grey, the rings are omitted.

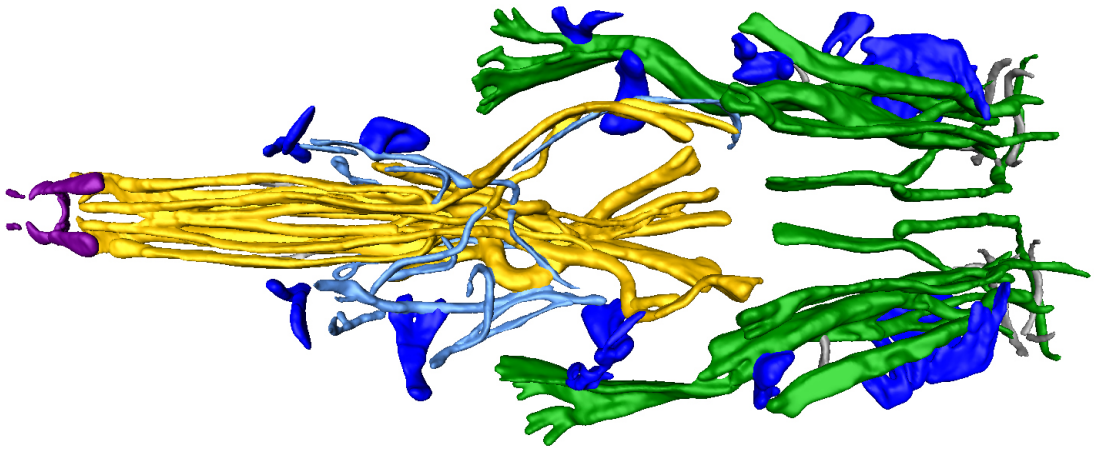

Figure 53: The whole body musculature, dorsal view. Different muscle groups have distinct colours, muscles of unclear affiliation are in grey.

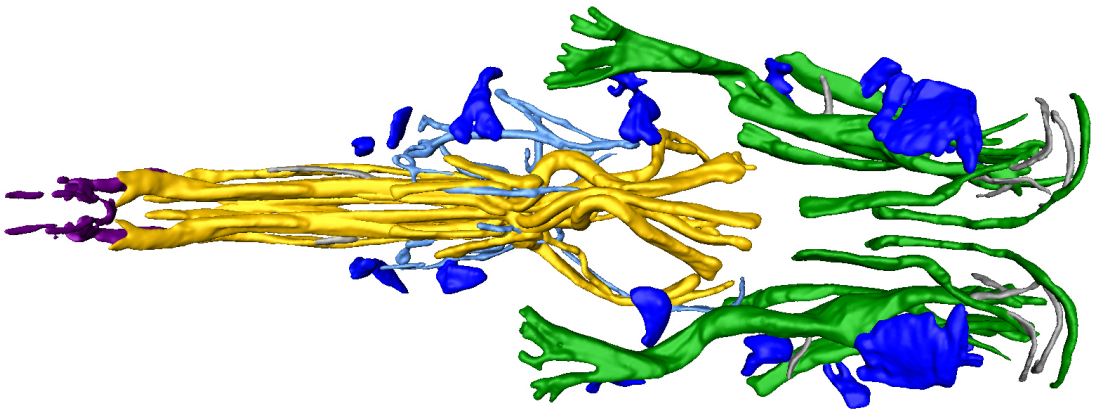

Figure 54: The whole body musculature, ventral view. Different muscle groups have distinct colours, muscles of unclear affiliation are in grey.

Discussion

This discussion covers some weaknesses of Amira, deals with the import of the data object in other programmes, gives an outlook on the intended future work and compares the reconstructed musculature to the findings of other authors.

Remarks on Amira

Amira offers many tools to work one's data with. In this work only a few were used and most tools or potentialities remained unnoted. Certainly, there is a potential not used, but it would take a lot of time to learn about it and to become familiarized with it. Since the labelling takes by far the most time of the work—up to several weeks—it were useful to know some tricks which allow to do the labelling more quickly. In this study solely the brush was used as a labelling tool. But the lasso with the freehand option, the magic wand together with the draw limit line option and the blow tool could save time. There is a pdf user guide for Amira, but it does not cover all functions and options, and the built-in help is not good because the type size is rather small and not changeable and the search function is crude.

It often is useful to change the name or the colour of a material. Although it is possible to do that, it is a bit too complicated to be convenient. The material has to be selected by pressing the button Select displayed in figure 55, then name and colour can be changend as usual. Thereafter the material has to be assigned to itself again by clicking on ⊕.

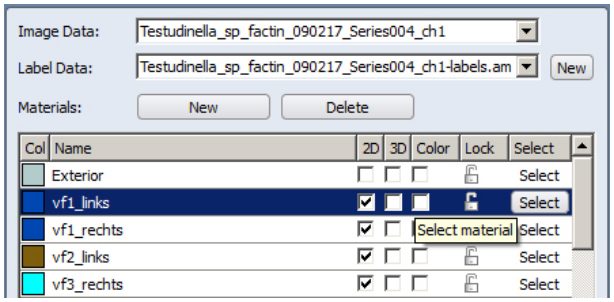

Figure 55: Selecting a material in the material list.

It also can be very useful to sort the materials in a certain logical order in the material list, e.g. all corona muscles, then all foot muscles, all unclear muscles

at the end and so on. But unfortunately Amira offers no possibility to do so. A solution could have been to open the file "...-labels.am" with a text editor and to sort the materials manually. Also the colours and names could be changed there. Figure 56 lists the first lines of that file in a text editor. This possibility was tested with the result that indeed the colour, name, and order of the materials can be changed so that these changes are shown in the material list in the label editor in Amira, but the complete information about the voxels (i.e. muscles) assigned to the materials was lost. Most likely this problem occurs if the "...-labels.am" file is saved with an application not being Amira. To test this, in the editor one digit of a colour was deleted and written again so that the editor recogniced a change in the file and gave the possibility to save the file. Although the file effectively was not changed, the complete material information mentioned above was lost.

```
00001 # AmiraMesh BINARY-LITTLE-ENDIAN 2.1
00002
00003
00004 define Lattice 1024 512 158
00005
00006 Parameters {
00007     Materials {
00008         Exterior {
00009             Id 1
00010         }
00011         vf1_links {
00012             Id 2,
00013             Color 0 0.28 0.7
00014         }
00015         vf1_rechts {
00016             Id 3,
00017             Color 0 0.28 0.7
00018         }
00019         vf2_links {
00020             Id 4,
00021             Color 0.498039 0.372533 0.0498039
00022         }
00023         vf3_rechts {
00024             Id 5,
00025             Color 0 1 1
00026         }
```

Figure 56: The materials as a text file opened in an editor.

Figure 57 illustrates some spots where there are holes in the surface of some muscles. The reason for this remains unclear, also how these holes can be prevented or at least closed. The object opened in Maya shows that there actually are no holes in the mesh, so it merely should be a display problem in Amira.
The figures 58 and 59 demonstrate holes where two muscles (here: broad retractor and dorsoventral muscle dv3) are attached to each other. If one muscle is not displayed a hole is to be seen in the other muscle. The reason for this is that the

object is only a surface—it is hollow, not solid. Wherever two materials touch each other Amira seems to assess the interface as redundant and excludes the information from the mesh to get one single, as small as possible surface area. Otherwise the interface information would have to be saved one (shared) or even two times for each muscle. Figure 60 gives an additional impression of such a nonexistent surface. It is a shot from inside the broad retractor towards posterior into the dorsoventral muscle dv3. The mesh grid was added to show more clearly where the interface could be. Since those holes cannot be filled with Amira, this has to be done with StudioMax.

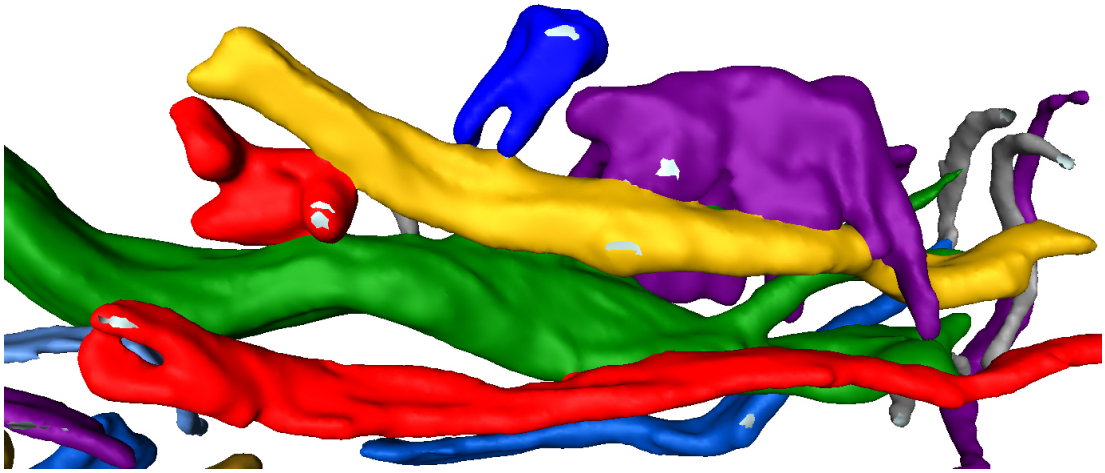

Figure 57: Holes in the surface of some muscles which allow to look inside the object.

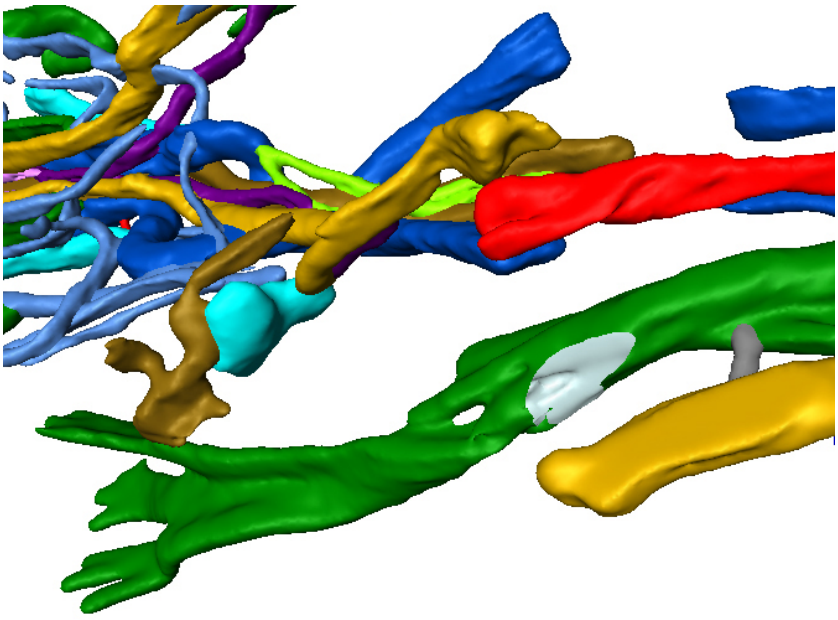

Figure 58: A hole in the broad retractor were it is attached to the dorsoventral muscle dv3, dorsal view.

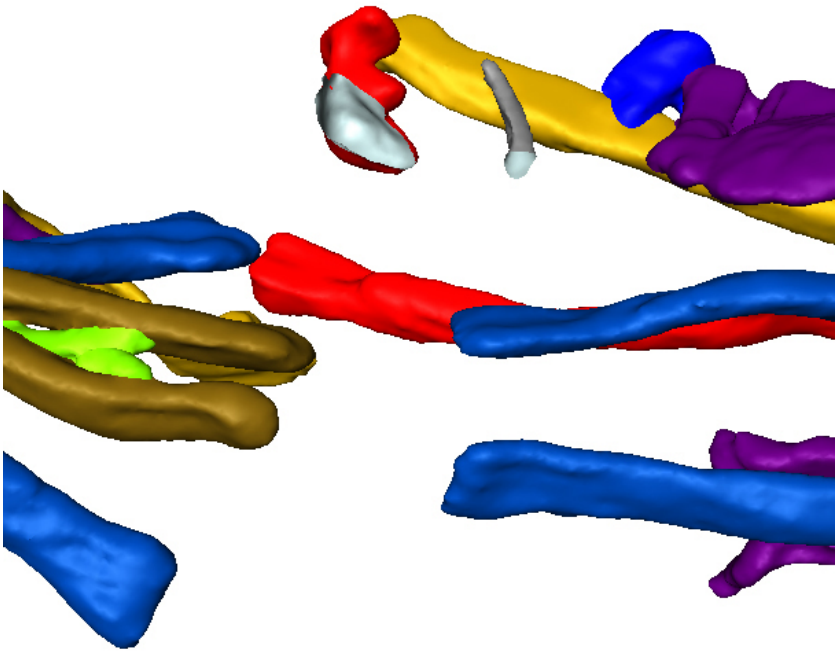

Figure 59: A hole in the dorsoventral muscle dv3 were it is attached to the broad retractor, ventral view.

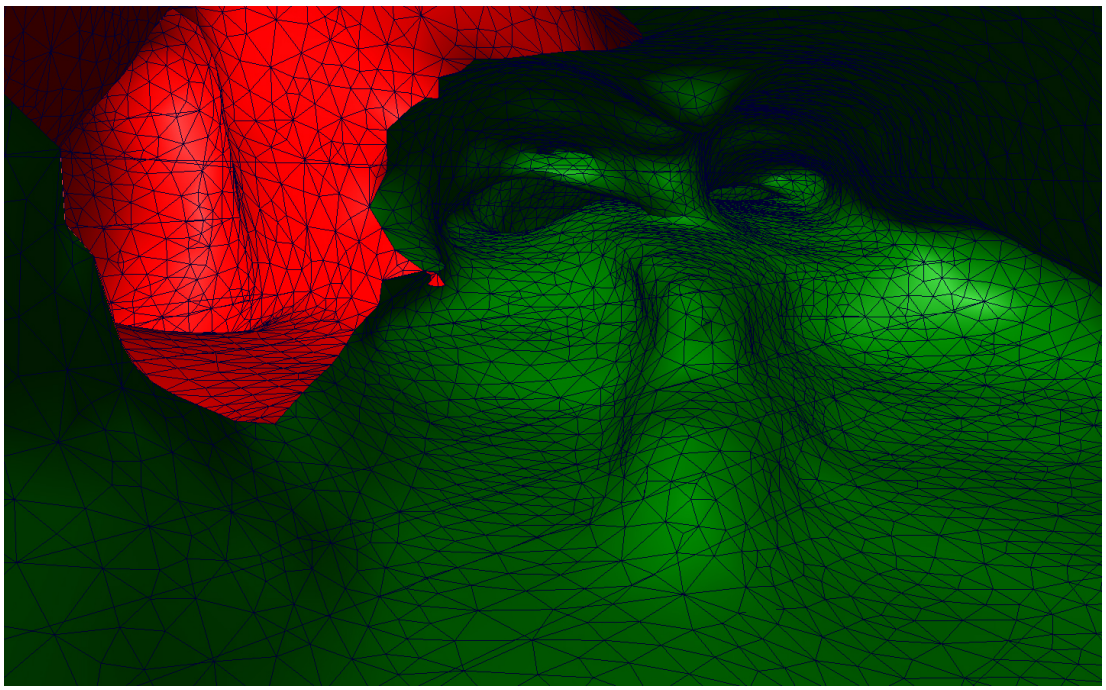

Figure 60: Frontal view from inside the broad retractor towards the dorsoventral muscle dv3 where there is no interface.

Outlook to a further processing

During the further processing it must be possible to work on (small groups of) single muscles. This way it is much easier to simplify the shape or to change the colour of a muscle.

In this work the further processing should be done with Maya. Since Maya cannot import the files Amira exports, a further programme was used to convert the files. This programme is called transform.exe and was written by Heiko Stark. When selected the single surface export option in Amira, after using transform.exe the object could be imported in Maya. But it still was one single surface without differenciation of the individual muscles which made the further processing impossible. When the individual material export option (as described in the results) was used, the data did not pass through transform.exe. It resulted in a file, which did not contain anything. This became clear by the file size of 1 kB. The opening with a text edior displayed only two comment lines of name and version of the file and finally Maya displayed nothing, although it imported without a warning or an error message. This problem was described to the author of transform.exe, whereupon he explained that his programme originally was meant to convert a certain other file type and was enhanced later by and by, but this particular problem with *.wrl files was simply not solved yet. It was also tested to use StudioMax as a transformer, but this way too, Maya imported without an error, but this time only a small cube was displayed. There may be a certain potential in this way, because there are many export options and only one of it (the default) was tried. Finally, the only way was the export from Amira and the direct import in StudioMax as described in the results.

When finally the reconstructed musculature shall be simplified or abstracted to an ideal model, the bilaterality can be taken advantage of, but not all species of rotifera are bilateral. Only one half must be reconstructed and then mirrored; and there are already two natural paragons from where to extract the essence of the shape of an individual muscle.

In the following, four different basic ways of abstracting the musculature with StudioMax are introduced briefly. The first idea is to begin with an empty scene,

but having the reconstruction anywhere in sight. Similar to the cubism, introduced in the art of painting by Picasso and Braque, an individual muscle is built up of a few fundamental geometric bodies like spheres, cylinders, cones and so on. Those bodies then are connected to each other and finally smoothed. An other idea is to put a body, e.g. a sphere, within a muscle and to pull and press at this sphere until it is a simplier model of the muscle. The third suggestion is similar. Here, the muscle itself is deformed until it has a more simple shape. The disadvantage is that soon there is no pattern any more to orient oneself to. In the last way one muscle of a pair is mirrored into its counterpart and then they are merged or united to a single body from which the average is taken somehow.

Comparison of the muscles to the findings of other authors

In this work the examined specimen was a *Testudinella clypeata*. Sørensen (2005), Kotikova et al. (2006), and Seehaus (1930) used the more common species *T. patina*. In Seehaus (1930) the author sometimes refers to *T. clypeata* especially in the context of the dorsoventral musculature. A comparison of these two species nevertheless is useful, because there are no differences in the body musculature expected which are essential since they belong to the same genus, either. That does not mean that it is impossible to find some differnces. In fact, there were found some differences in the musculature, for instance the number of foot muscles, but it also is open to question what the reasons for these are. Be it that there really are differnt numbers of muscles due to the fact that it is a differnt species or that the staining was not lucidly enough, i.e., a problem in the method. Only one single specimen was examined. Admittedly, this is too less to get reliable results for a comparison with the findings of other authors. In one specimen for instance a muscle can be contracted or is shifted a bit, resulting in a different appearance. An average over several individuals would level out those differences. But since the primary goal was to provide a guidance how to get a three-dimensional reconstruction it was sufficient to use one single specimen.

The division of the foot muscles in dorsal and ventral muscles was actually arbitrary, but followed Seehaus (1930) as far as possible. Sørensen (2005) and Seehaus (1930) found three dorsal and four ventral foot muscles. Kotikova et al (2006)

found two dorsal and three ventral foot muscles. In this study four dorsal and five ventral foot muscles were found. There is an additional muscle in the foot (see figures 46 and 47, abbreviated with un) which definitely belongs to the foot muscles. One pair (vf5, figure 46) possibly belongs to the visceral musculature. Figure 61 contains the above mentioned dense area which caused some trouble, because it was rather difficult to follow the (not always so obvious) course of a muscle through it. Additionally, in this area there are some muscles attached to others or have their insertion there. Sørensen (2005) found an S-shaped subterminal muscle in this area. Whereas Kotikova et al. (2006) did not and Seehaus (1930) did not mention something like that at all. In this examination there were no subterminal muscles found, neither. But, as evident from figure 62, there is something that looks like a fissure between two muscles located at the left vf1. Such a fissure was found at the right vf1, too (not displayed in a figure here). Both fissures occur right where both vf1 muscles segue from a straight course into an S-shape. It can be seen clearly that those two muscles lie in an S-shape. Maybe those muscles are simply especially relaxed. But it is open to question, if that really is the case, because such a relaxion has not happend to the other muscles. So, possibly there is a subterminal muscle after all.

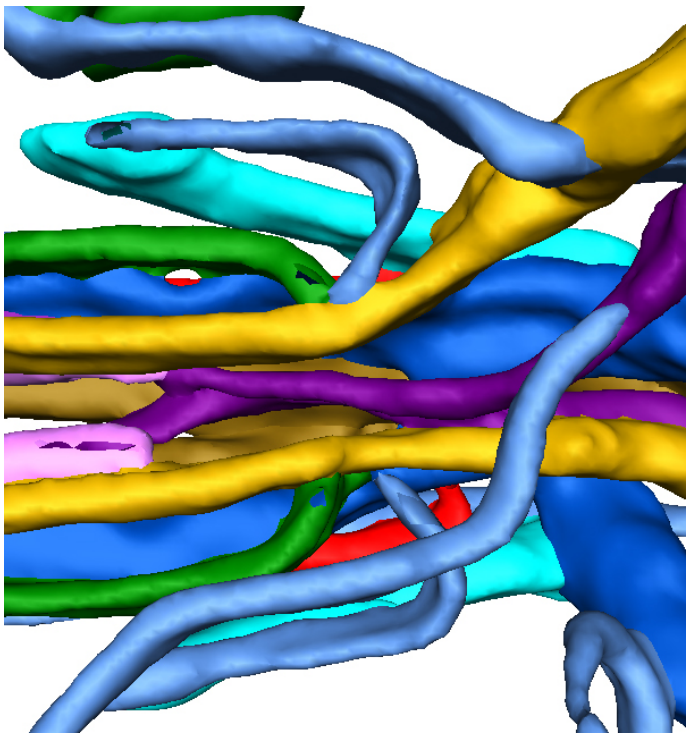

Figure 61: The dense area in the foot, dorsal view.

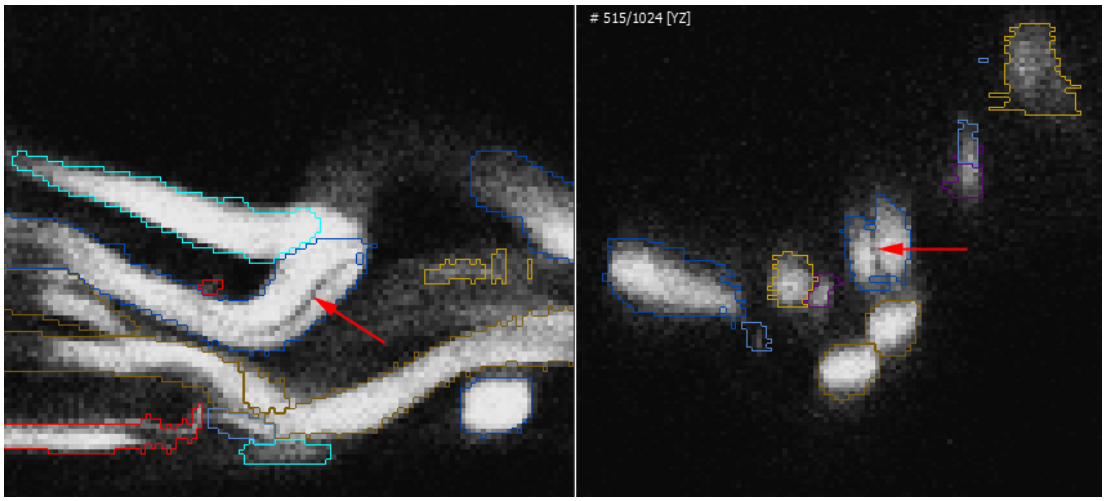

Figure 62: The left image, a transversal section, shows a fissure which is possibly a connection of the left foot muscle vf1 and a potential subterminal foot muscle. The right image, a frontal section, shows something like a gap in the right vf1 streaching over a short part of this muscle.

Aside from the fifth ventral foot muscle (vf5), maybe other muscles from those here classified as foot muscles, actually are visceral muscles e.g. the muscles vf3 and vf4 which are represented in the figures 46 and 47. The above mentioned conspicious visceral muscle in figure 51 probably crosses diagonally to the other side of the body; a piece in the middle of it is missing in the reconstruction. There is this other muscle also mentioned above which crosses the body. This one could be the pair partner to that one mentioned just now; its course seems to permit this opinion.

Seehaus (1930) did not describe the ventrolateral retractor (vlr) while Sørensen (2005) did so. In this work this muscle neither was found, but there is a structure merged with the broad retractor which could be the vlr. The figures 63 and 64 show these structure. It only is visible at the right side. But both broad retractors have inner bifurcations which are vertically rather thick. That, too, points to the existence of the vlr. According to Sørensen (2005) the broad retractor and the vlr lie one upon the other which blends with the thickness of that bifurcation found here.

The mastax retractors were not found, too. But here as well, structures were observed which could represent at least the insertions of the mastax retractors, as indicated in figure 65. They possibly lie so close next to the dorsolteral retractors (dlr) that they appear as one muscle or they were not stained enough.

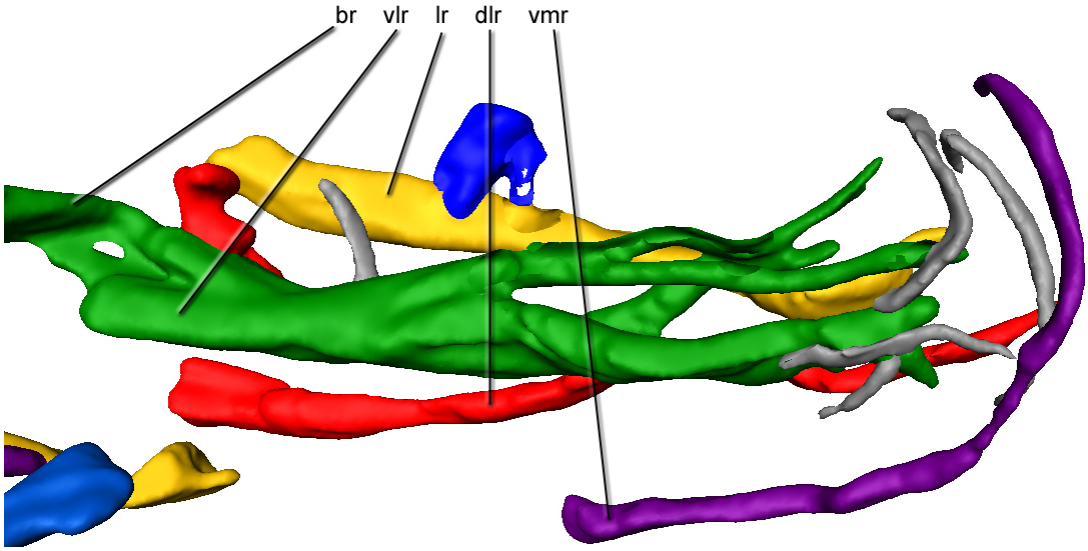

Figure 63: The broad retractor and the suspected position of the ventrolateral retractor, ventral view. broad retractor (br), dorsolateral retractor (dlr), dorsomedial retractor (dmr), lateral retractor (lr), ventrolateral retractor (vlr)

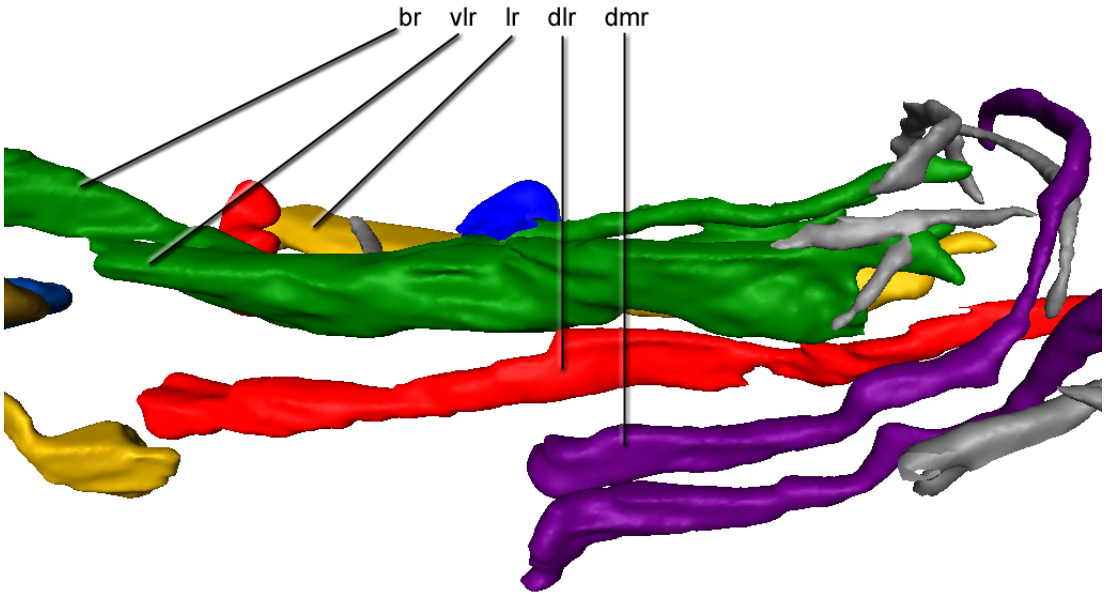

Figure 64: The broad retractor and the suspected position of the ventrolateral retractor, lateral view. broad retractor (br), dorsolateral retractor (dlr), dorsomedial retractor (dmr), lateral retractor (lr), ventrolateral retractor (vlr)

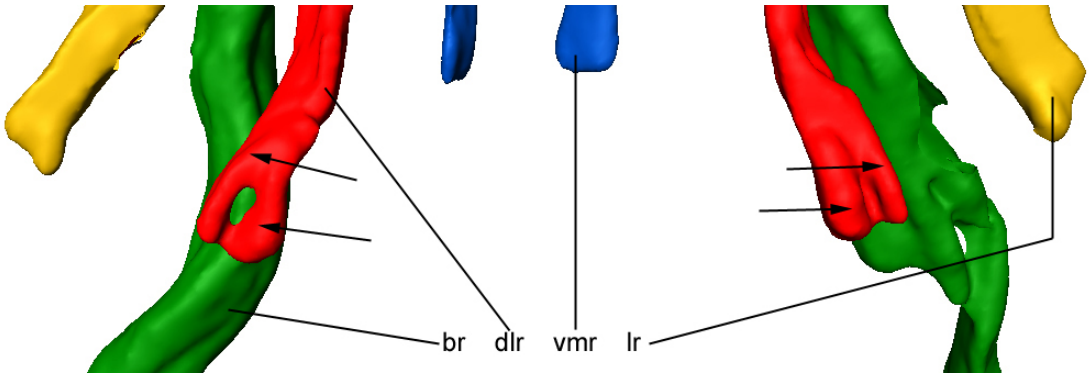

Figure 65: The arrows point to the possible insertions of the mastax retractors, dorsal view. broad retractor (br), dorsolateral retractor (dlr), lateral retractor (lr), ventromedial retractor (vmr)

Figure 66 provides a comparison of a drawing of a *Testudinella clypeata* in Seehaus (1930) and the reconstruction of this study. In the following the muscles named by Seehaus are written in italic and have indices. The dorsoventral muscle Seehaus called dv_{1+2} was not found here, instead of this an other muscle named dv2. The muscles dv_3 and dv1 are in accordance with each other. The muscles dv_4 and dv_5 are the same as dv3 and dv4, but there is a greater distance between these. Seehaus wrote that the muscles dv_6 and dv_7 are cleaved in two to four and dv_8 in two parts. Here, the counterparts would be the muscles dv5 and dv6, and dv7 plus dv8. In this study the muscles dv7 and dv8 do not look like one cleaved muscle, but like two separate muscles.

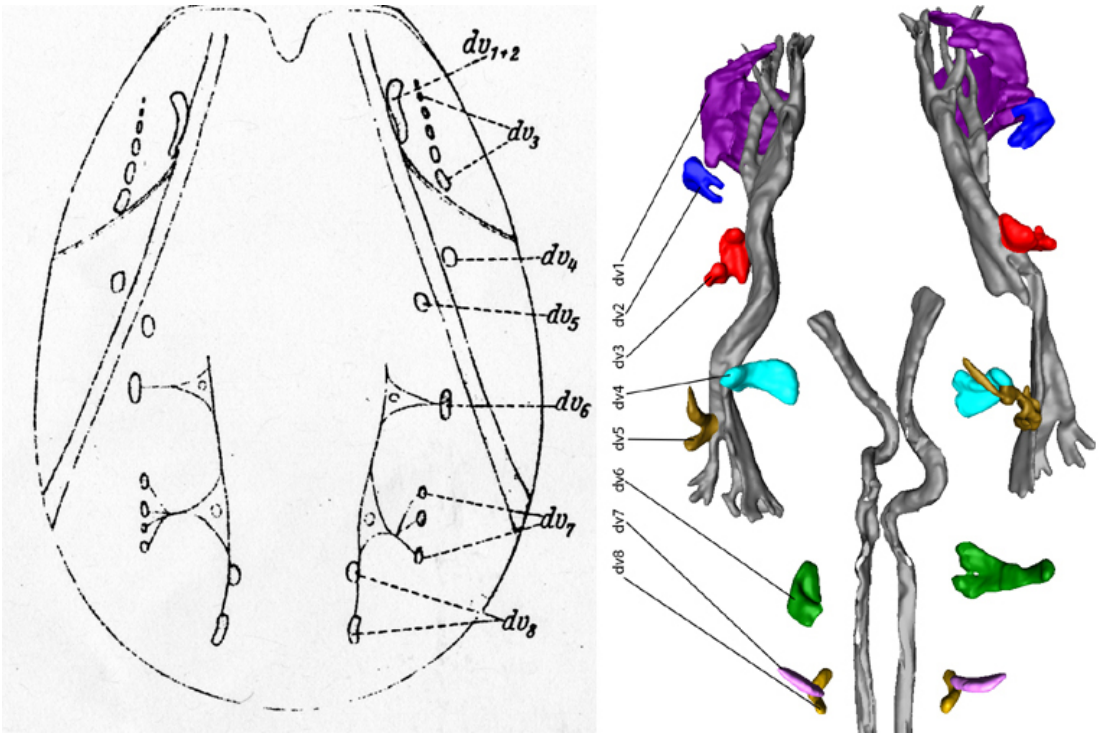

Figure 66: A comparision of Seehaus's finding and those gained in this study. Seehaus's drawing on the left side either represents a *Testudinella clypeata*.

Those two processes which run laterally from the broad retractors (see figure 44) could have something to do with the lateral antennae, but that is improbable because the antennae do not have muscles and the staining was targeted to muscles. Maybe those muscles serve, together with the dorsoventral muscles, as lorica contractos which press out the corona again after it was retracted.

The torus-shaped structures, visible in figure 52, could be related to the intestinum. The two processes could be the foot glands according to Seehaus (1930). There are no such rings in *T. patina*, so maybe these muscles are unique to *T. clypeata*. It also is improbable that they are artefacts, because the signal is quite distinct and looks like the other muscles. Here, the limits of light microscopy are reached. An examination of the ultrastructure with scanning electron microscopy could throw light on that matter.

Finally, an assessment of the procedure of the reconstruction shall be given. One positive point is that it is a further developement of the methods to examine rotifera. It not only uses the advanced means of CLSM images, but goes beyond this kind of representation, thus offering novel possibilities in rotifera research.

Also the steps to perform in Amira can be reproduced easily. The labelling is long-winded, but although the other suggested labelling tools could save some time, it always will be a time consuming venture, because the underlying algorithms ultimately do not more as to test the grey values of the neighbour voxels against a certain threshold. The intelligent human user can train his or her eyes in recognising what belongs to a material and what to an other or to the overexposed background, but a programme can not. But in some cases the user neither can differentiate what belongs to which material, if e.g. the muscles lay so closely next to each other that they appear as one single muscle or if the muscles (especially the thin ones) are not stained well enough.

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Appendix

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Hiermit versichere ich, dass ich diese Arbeit selbständig verfasst und keine anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Außerdem versichere ich, dass ich die allgemeinen Prinzipien wissenschaflicher Arbeit und Veröffentlichung, wie sie in den Leitlinien guter wissenschaftlicher Praxis der Carl von Ossietzky Universität Oldenburg festgelegt sind, befolgt habe.

Oldenburg, 24.11.2010