

The Segmentation of Microtubules in Electron Tomograms Using Amira

Stefanie Redemann, Britta Weber, Marit Möller, Jean-Marc Verbavatz, Anthony A. Hyman, Daniel Baum, Steffen Prohaska, and Thomas Müller-Reichert

Abstract

The development of automatic tools for the three-dimensional reconstruction of the microtubule cytoskeleton is crucial for large-scale analysis of mitotic spindles. Recently, we have published a method for the semiautomatic tracing of microtubules based on 3D template matching (Weber et al., J Struct Biol 178:129–138, 2012). Here, we give step-by-step instructions for the automatic tracing of microtubules emanating from centrosomes in the early mitotic *Caenorhabditis elegans* embryo. This approach, integrated in the visualization and data analysis software Amira, is applicable to tomographic data sets from other model systems.

Key words Microtubules, Segmentation, Automatic tracing, *C. elegans*, Electron tomography, Three-dimensional reconstruction

1 Introduction

During mitosis, cells form bipolar spindles to align the chromosomes on the metaphase plate and segregate them to the daughter cells during anaphase. Mitotic spindles are bipolar, self-organizing structures, composed of microtubules and numerous microtubule-interacting proteins, including microtubule polymerases, depolymerases, and stabilizers, and additional motor proteins.

Within the bipolar spindle, microtubules show a distinct organization. The minus ends are organized at microtubule organizing centers (MTOCs), such as spindle poles or centrosomes. The plus ends of microtubules radiate out from the MTOCs towards the cell cortex (astral microtubules) or the aligned chromosomes (kinetochore microtubules). In addition, some microtubules emanating from one of the MTOCs interact with microtubules from the opposite pole (interdigitating or interpolar microtubules).

The function of mitotic spindles is highly conserved across species and so are the proteins that form the spindle. However, the size, volume, shape, and dynamics of spindles vary dramatically. For example, the mitotic spindle in a one cell-stage embryo of the amphibian *Xenopus laevis* [1] has a volume approximately 50,000 times larger than that of the spindle in the budding yeast cell *Saccharomyces cerevisiae* [2]; and this diversity is mirrored in the different structural organization of these spindles [3]. Surprisingly, the dynamic properties of mitotic spindle assembly have been studied intensively by light microscopy, but the detailed three-dimensional architecture of kinetochore, interpolar, and astral microtubules has been described in only a very small number of model systems, namely, in budding yeast [4, 5], in the early *C. elegans* embryo [6], and in mammalian tissue culture cells [7–9].

Undoubtedly, such a 3D analysis of the microtubule cytoskeleton is essential for a deeper understanding of the mechanisms underlying spindle formation.

Here, we briefly describe the preparation of *C. elegans* early embryos for a reconstruction of the first mitotic spindle, followed by a detailed step-by-step description of microtubule tracing and modeling in tomograms using the Amira software.

Currently we use the EM PACT2+RTS high-pressure freezer (Leica Microsystems, Vienna, Austria) for correlative light and electron microscopy of single early *C. elegans* embryos [10–12]. The rapid transfer system (RTS) is used for a quick loading of embryos contained in cellulose capillary tubes, thus allowing a precise staging of embryos prior to cryo-immobilization and reducing the time window between light microscopic observation and preservation by high-pressure freezing. Electron tomography is then applied to study the three-dimensional organization of spindles and their components with a resolution of approximately 5–8 nm in 3D [13, 14].

We use the IMOD software package [15] to calculate tomograms and the Amira software for automatic tracing of microtubules [16, 17]. In Amira the obtained 3D models of traced microtubules can be edited. For example, microtubules can be segregated into different classes, for example depending on their origin or end morphology.

2 Materials

2.1 High-Pressure Freezing and Freeze Substitution

1. EM PACT2+RTS (Leica Microsystems, Vienna, Austria) (*see Note 1*).
2. Stereomicroscope with light source.
3. Inverted light microscope (phase contrast, DIC, epifluorescence).
4. Cellulose capillary tubes with an inner diameter of 200 μm (Spectrum, 23022 La Cadena Dr., Suite 100, Laguna Hills, CA 92653, USA, and Leica Microsystems, Vienna, Austria).

5. Micropipettor (0.5–1 μ l size) and gel loader tips.
6. Nail polish.
7. *C. elegans* strain of interest.
8. M9 buffer: 22 mM potassium phosphate monobasic (KH_2PO_4), 19 mM NH_4Cl , 48 mM sodium phosphate dibasic (Na_2HPO_4), 9 mM NaCl.
9. 20 % BSA (w/v) in M9 buffer (*see* **Note 2**).
10. Tools, for example, two syringe needles for cutting worms open to release the embryos, scalpels for cutting dialysis tubing, good forceps.
11. Microscope slides and high-precision cover slips.
12. Specimen holders for high-pressure freezer: 100 μ m deep membrane carriers [10].
13. Filter paper wedges for removing fluids.
14. EM-grade acetone.
15. Freeze substitution cocktail: 1 % OsO_4 + 0.1 % uranyl acetate in acetone [18].
16. Automated freeze-substitution device (Leica EM AFS2).
17. Epon/Araldite resin: 6.2 g Epon 812 substitute, 4.4 g Araldite, 12.2 g DDSA, and 0.55 ml DMP-30.
18. Teflon-coated glass slides for thin-layer embedding: Slides are coated with a Teflon[®] solution (MS-143V, Miller-Stephenson Chemical Co., Inc., Danbury, CT, USA).
19. “Dummy” blocks for remounting and fast glue.

2.2 Electron Tomography

1. For electron tomography of semi-thick (200–400 nm) sections, we use a microscope operating at intermediate voltage (300 kV; TECNAI F30; FEI Company, Eindhoven, The Netherlands). Automated image acquisition programs are available from commercial vendors (FEI Company, Eindhoven, The Netherlands; Tietz Video and Image Processing systems (TVIPS), Gauting, Germany). In addition, several software packages are freely available (TOM, UCSF Tomography and SerialEM, Boulder Laboratory for 3D electron microscopy of cells).
2. Copper slot grids.
3. 0.7 % (w/v) Formvar in ethylene dichloride.
4. 2 % uranyl acetate in 70 % methanol.
5. Rinse solution of 70, 50, and 20 % methanol.
6. Reynold’s lead citrate.
7. 10 or 15 nm colloidal gold.

2.3 Three-Dimensional Reconstruction

1. We routinely use the IMOD software package (<http://bio3d.colorado.edu/imod>), which contains all of the programs needed for calculating tomograms [15]. The IMOD software package runs on multiple platforms, including Linux, Mac OSX, and Windows. The programs used for tomographic reconstruction are managed by a graphical user interface, eTomo. The eTomo interface guides users through the various steps of the process. This program can be run by command line; it contains windows for image display and slicer tool for rotating slices of image data and for modeling features of interest in the reconstruction.

2.4 Modeling

We currently use an extension to the filament editor of the visualization and data analysis software Amira [16] to automatically model and trace microtubules in our tomograms. This extension is freely available (*see Note 3*).

3 Methods

3.1 High-Pressure Freezing and Freeze Substitution

The combination of high-pressure freezing with freeze substitution has evolved into a routine technique to prepare *C. elegans* for electron tomography. In principle, either whole worms or isolated embryos can be cryo-immobilized within milliseconds [19, 20]. In general, not only *C. elegans* embryos are a suitable system for this method of high-pressure freezing but also basically any organism of a size below 200 μm .

3.1.1 Cryo-Immobilization of Isolated Embryos

1. Prepare a loading device for collecting isolated early embryos into cellulose capillary tubes by mounting a piece of tubing (approx. 2 cm) into a pipette tip and using nail polish to seal. Dry before using [11].
2. Cut worms open in a drop of M9 buffer on a microscope slide. Select an early embryo under the dissecting scope, and suck the selected embryo into the capillary tube.
3. Submerge the capillary into the buffer, and use the scalpel to cut off the region of the cellulose capillary containing the embryo. Shorten the tube to a size that will fit the specimen carrier. The ends of the tube should be crimped so that the tube is sealed and the embryo will not leak out. This can be achieved by using the blunt side of a scalpel.
4. Transfer the capillary from the microscope slide to a drop of M9 on a high-precision cover slip, and observe the early development of the embryo by DIC or fluorescence microscopy.
5. At an appropriate mitotic stage, transfer the cellulose capillary containing the embryo from the cover slip to a specimen carrier, prefilled with 20 % BSA in M9 buffer, freeze, and store in LN_2 .

**3.1.2 Freeze
Substitution, Embedding,
Ultramicrotomy, and
Screening of Serial
Sections**

1. Transfer samples to precooled (-90°C) cryovials containing the freeze substitution cocktail composed of 1 % OsO_4 plus 0.1 % uranyl acetate in acetone. Maintain temperature for 8–24 h at -90°C .
2. Warm samples to room temperature at a rate of $5^{\circ}\text{C}/\text{h}$.
3. Rinse samples in pure acetone and infiltrate with Epon/Araldite.
4. Process samples for thin-layer embedding, and remount selected specimens on dummy blocks for ultramicrotomy [19].
5. Cut ribbons of serial semi-thick sections (300–400 nm) through the embryos, and collect the ribbons of sections (serial sections) on Formvar-coated copper slot grids.
6. Stain sections with uranyl acetate, followed by Reynold's lead citrate.
7. Apply 10–15 nm gold fiducials to the samples by placing the slot grids on top of a drop of gold solution for 2 min, blotting excess fluid, turning the grid over, and repeating for the other side. Wash the slot grids briefly in a drop of water (*see Note 4*).
8. Examine semi-thick sections in a standard TEM operating at 100 or 120 kV to identify the sections containing the regions of interest. Map the features of interest through the serial sections by imaging at a low magnification.

**3.2 Acquisition
of Tomographic Data**

1. Image a tilt series of semi-thick sections in an intermediate-voltage (200–300 kV) EM equipped with a eucentric tilting stage. Collect serial tilted views of the section every degree over a $\pm 60^{\circ}$ or 70° range. If a full spindle should be acquired, montages have to be acquired instead of single frames. This can be done using the SerialEM software. To acquire a montage, go to “file” and “New montage.” A new window, the “Montage Control” box will appear, and the size of the montage, 1×1 , 2×2 , 3×3 , etc. has to be chosen to cover the region of interest. For a large-scale 3D reconstruction it is essential to cut serial sections. As an example, for a reconstruction of the first mitotic spindle in *C. elegans*, each section needs 12 single-tilt tomograms to cover the pole-to-pole area of the mitotic spindle in x - and y - and 20 consecutive sections to acquire the z -dimension of the spindle (*see Note 5*).
2. After the first tilt series has been acquired, rotate the grid 90° to image a second tilt series over a $\pm 60^{\circ}$ or 70° range.
3. Calculate a double-tilt tomogram using IMOD [21]. The calculated tomogram will be stored as the so-called .rec file. In order to use the automatic microtubule tracing in Amira it is essential to flatten the tomogram at the end of the reconstruction process and to trim off grey areas (*see Note 6*).

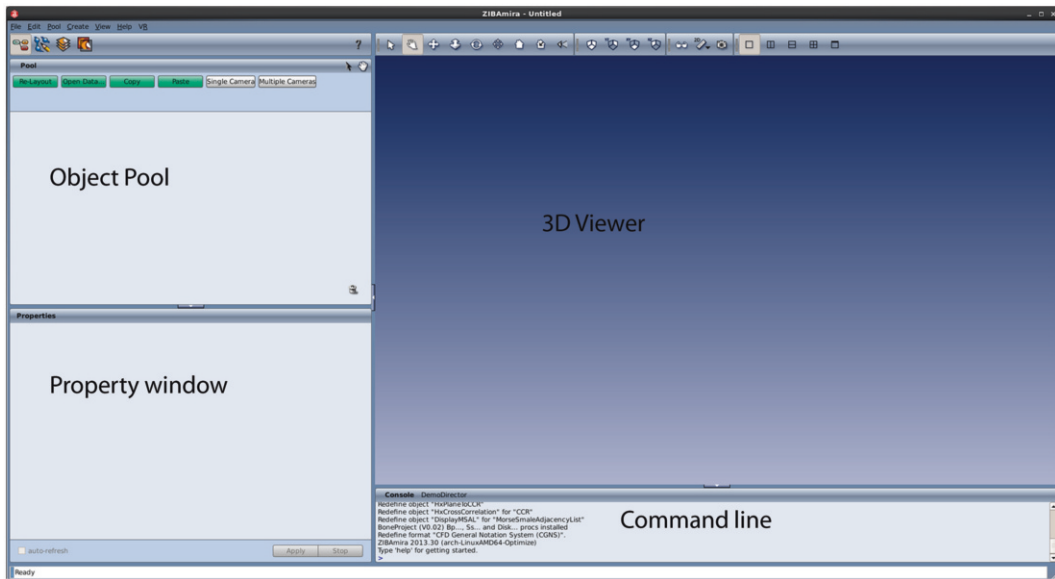


Fig. 1 Screenshot of the Amira interface. The interface of Amira can be divided into four major windows: the object pool, in which all the working files will be located; the property window, where values and parameters for different tasks can be adjusted; the 3D-viewer, which can display either the tomogram or any 3D model; and the command line

3.3 Automatic Tracing and Modeling of Spindle Microtubules

1. Open Amira, and open the reconstructed tomogram. This can be done by either choosing “File” and “open” from the taskbar or dragging and dropping the *.rec* file, which needs to be flattened and trimmed in IMOD, into the *object pool* (Fig. 1). Depending on the data size, a pop-up menu will appear. Always choose “Read complete volume into memory” from the buttons.
2. The first step for automatically tracing microtubules is a computation of normalized cross correlation of the tomogram with an idealized cylinder, resulting in a correlation and vector field. Clicking the right mouse inside of the *object pool* will open up a window with a menu. Choose *Create*, then *MicrotubuleTracing*, and then *CylinderTemplateMatchingCUDA* from the drag-down menu (Fig. 2). Adjust the settings in the properties windows by changing the *blocksize* to X:256 Y:256 and Z:256. Click apply. Computation is performed on a graphics card using the CUDA API [22]. You need an NVIDIA graphics card. For a $2k \times 2k \times 200$ tomogram, computation takes approximately 6 h on a GeForce 400 series.
3. The successful computation will generate a *correlation field* and a *vector field*, which will show up in the *object pool*. Note: Always save those files as Amira has no autosave function. For automatic microtubule tracing right click into the *object pool*. Choose *Create*, then *MicrotubuleTracing*, and finally



Fig. 2 CylinderTemplateMatchingCUDA. After the tomogram is loaded into the object pool, a right click inside the object pool opens up a drag-down menu. There the user first has to choose “MicrotubuleTracing” followed by “CylinderTemplateMatchingCUDA” to start the segmentation. Settings like the blocksize can be adjusted in the properties window

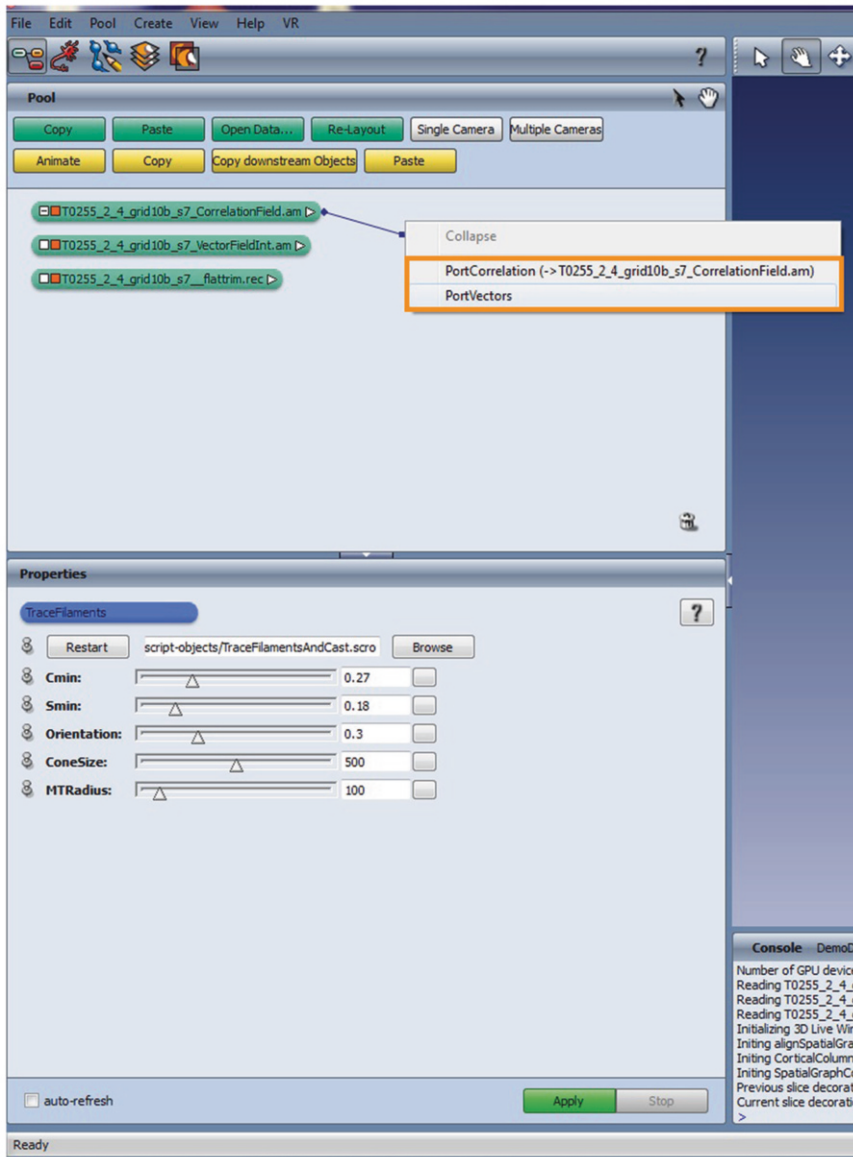


Fig. 3 *PortCorrelation* and *PortVector*. After a successful *CylinderTemplateMatchingCUDA* Amira will generate a vector field and a correlation field. For the automatic microtubule tracing those fields have to be linked to the corresponding ports, called *PortVectors* and *PortCorrelations*

TraceFilaments from the menu. A new box named *TraceFilaments* will appear in the *object pool*. Click on the little square on the left side of the box, and connect the port named *PortCorrelation* with the correlation field and the port named *PortVectors* with the vector field (Fig. 3). Click apply. This step is usually fast and will create another file in the *object*

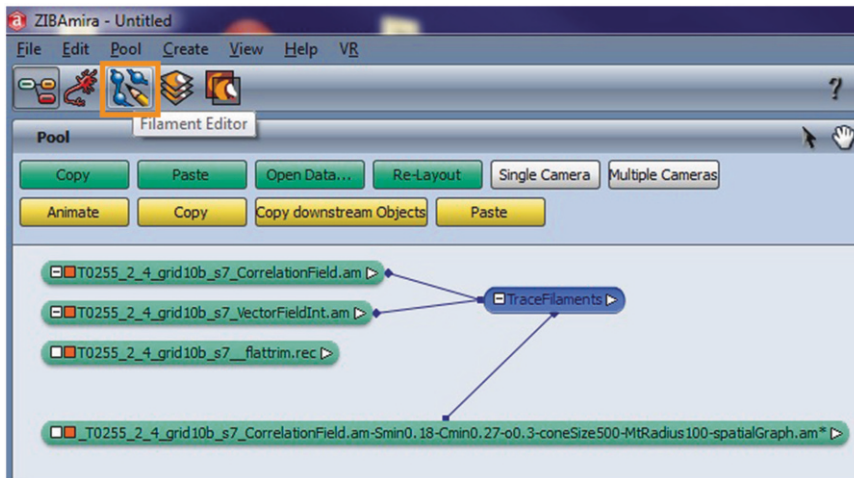


Fig. 4 Microtubule tracing. Successful microtubule tracing will generate the so-called *SpatialGraph* in the object pool, which subsequently can be opened and edited with the *filament editor*

pool, containing the traced centerlines. The lines are stored in a data structure called *SpatialGraph*.

For microtubules this *SpatialGraph* will only contain Edges, which represent microtubule centerlines and pairs of nodes that mark the endpoints of an edge.

3.4 Editing of the Traced Microtubules

1. Load the .rec file, the vector, and the correlation field and the traced microtubules (*SpatialGraph*). After loading, start the *filament editor* by clicking on the icon marked in Fig. 4.
2. The *filament editor* is organized in several panels (Fig. 5), which are briefly described here. *MPR-viewer*: Displays an oblique slice through the tomogram. Additionally, any crossing of elements of the currently processed *SpatialGraph* with the current slice is rendered. Usually, this window is used for drawing and editing lines.
3D-viewer: The 3D-viewer displays the *SpatialGraph* containing the modeled microtubules in three-dimensional space. Along with the model, you can also display the orientation of the slice currently rendered in the MPR-viewer. You can configure the size of the MPR- and 3D-viewer by clicking on the separating bar and moving it.
Data management: This lets you choose, which tomogram and *SpatialGraph* is displayed in the viewers. In the first row, you can choose the tomogram; in the second row, the corresponding model of microtubules can be chosen; and in the third row the contrast for displaying the tomogram can be adjusted. The fourth row allows the configuration of the maximum intensity projection of the current slice.

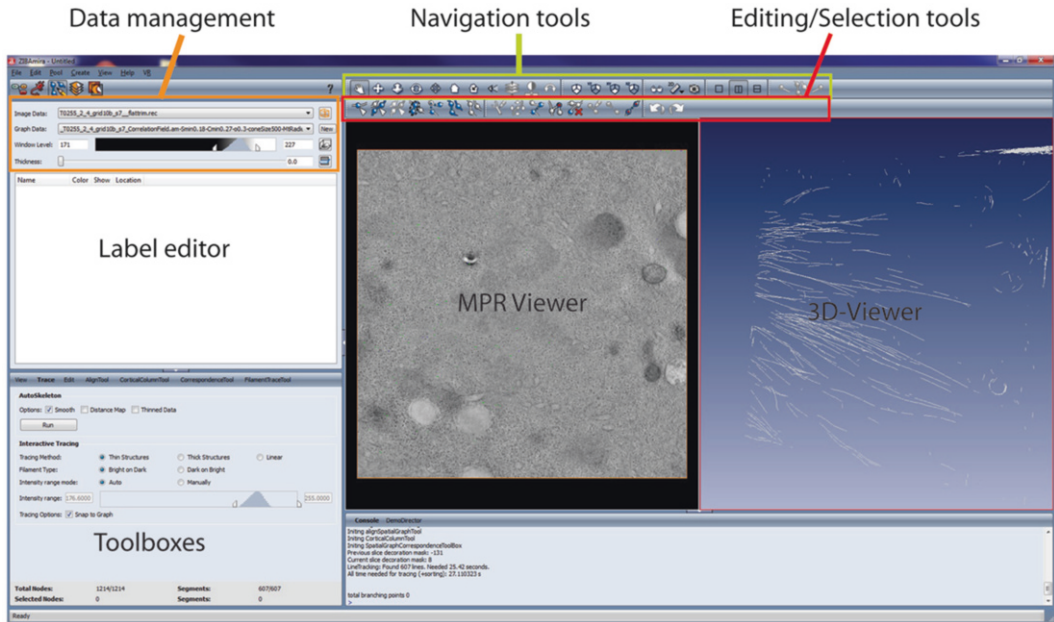


Fig. 5 Screenshot of the *filament editor*. The *filament editor* is organized into different panels, which are depicted here. It consists of a data management panel, where the working tomogram and corresponding *SpatialGraph* can be chosen; the *Label editor*, which is needed if microtubules will be annotated according to origin or end morphology; the Toolboxes, in which parameters can be adjusted; the MPR-viewer, which shows the tomogram; the 3D-viewer, in which the model is shown; and the navigation and editing/selection tools to edit the microtubule model

Label editor: Modeled edges and nodes can be labeled according to properties (for further description *see* below).

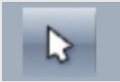




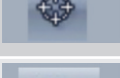

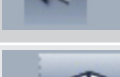
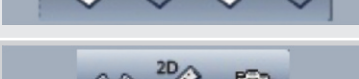
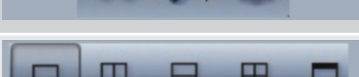
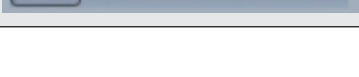
Tools: Tools offer the functionality to navigate and to edit a traced graph.

Toolbox: Toolboxes are used to configure tools and run algorithms.

3. You can navigate in your tomogram by changing the slice currently displayed in the MPR-viewer or in the 3D-viewer. The navigation tools are located in the upper right bar, and their availability and functionality depend on the currently selected viewer. Table 1 explains the most common tools used for navigation and their function in the two viewers. To use the tools, first select a viewer by clicking in the viewer. A red border marks the active viewer. Activate a tool by clicking on it.
4. Because the automatic tracing of microtubules can make mistakes, verifying the tracing is essential; one can manually edit nodes and edges. Occasionally, one can find microtubules in the MPR-viewer, which have not been detected by the tracing program. In this case, it is possible to manually trace the microtubules.

Table 1

Description of the navigation tools in the MPR- and 3D-viewer. This table shows a description of the most common navigation tools of the MPR-viewer and the 3D-viewer

Icon	Function in MPR-viewer	Function in 3D-viewer
	Rotate slice around the center of image (marked by a yellow cross)	Rotate scene around the center of volume
	Rotate slice around the center of image (marked by a yellow cross)	Rotate scene around the center of volume
	Translate image	Translate scene
	Zoom in and out	Zoom in and out
	Small rotation	Small rotation
	Local zoom	
	Return to initial view of image	Return to initial view of volume
		Switch to orthographic view
	Change the plane of view	
	Tools for measurements and snapshots	
	Arrangement of windows	

For this, open the *semiautomatic tracing toolbox* (see Fig. 6) and choose the proper fields in the menus correlation and vector field. The parameters need not be configured for tracing microtubules, except for the adjustment radius. Fill in the radius of a microtubule (10 nm). Note that the voxel size of the volume might be measured in Angstroms: in this case, the adjustment radius needs to be set to 100. The semiautomatic tracing is activated by pressing the button indicated in Table 2. The *filament editor* will try to

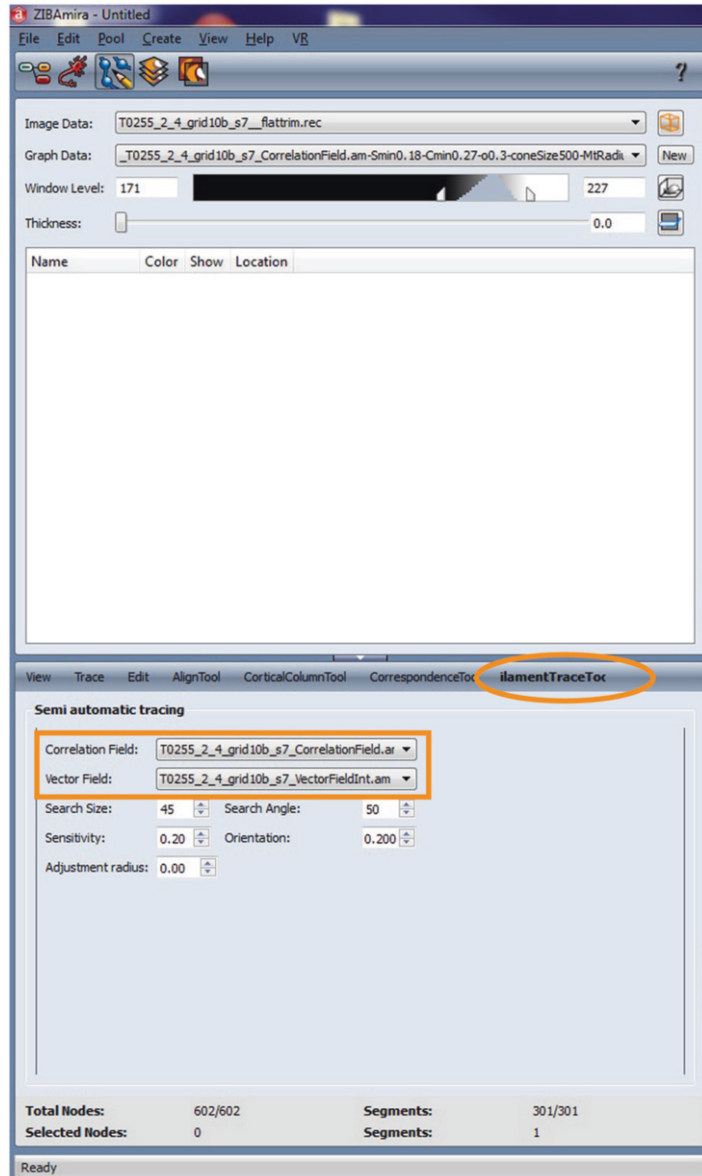


Fig. 6 Semiautomatic tracing toolbox. For a functional semiautomatic tracing of microtubules, which in the course of editing of the model is needed, the appropriate correlation and vector fields have to be inserted into the *Filament Trace Tool* in the toolbox

find a microtubule centerline whenever you click at the slice in the MPR-viewer (it will also do so even if you clicked somewhere where there is no microtubule).

5. To systematically check each line in a model for correctness, the *edge stepper tool* (Fig. 7) is helpful. This tool allows you to systematically step through all lines and show their embedding

Table 2

Description of the editing tools in the *filament editor*. This table shows a description of the most important editing tools of the *filament editor*, which can be used in the MPR-viewer and the 3D-viewer. Hotkeys for the different functions are also shown in this table

Icon	Function	Key
	Selected tool for picking and selecting a node or a segment (shift selects both)	e
	Draw a line around an area, and select everything within the area	q
	Selects all items in the spatial graph	a
	Deselects all items	c
	Semiautomatic tracing tool, start tracing or drawing a new segment	
	Connect tool, select two nodes/edges, and connect them	s
	Extract line, click on any point in the MPR viewer, and line is shortened or prolonged (+shift)	n
	Select a segment, go to the points that should be separated, then split	

in the volume. Stepping through the edges is triggered by increasing/decreasing the edge number in the *Segment* input field. The edge stepper will automatically focus the beginning of the edge in the MPR-viewer. To step through the edge points, you can increase/decrease the point number in the *Points* field. The slice in the MPR-viewer will automatically be adjusted to an optimal view of the currently selected edge point. When stepping through edges, the chosen edge will automatically be selected. When stepping through points, only the current point will be selected.

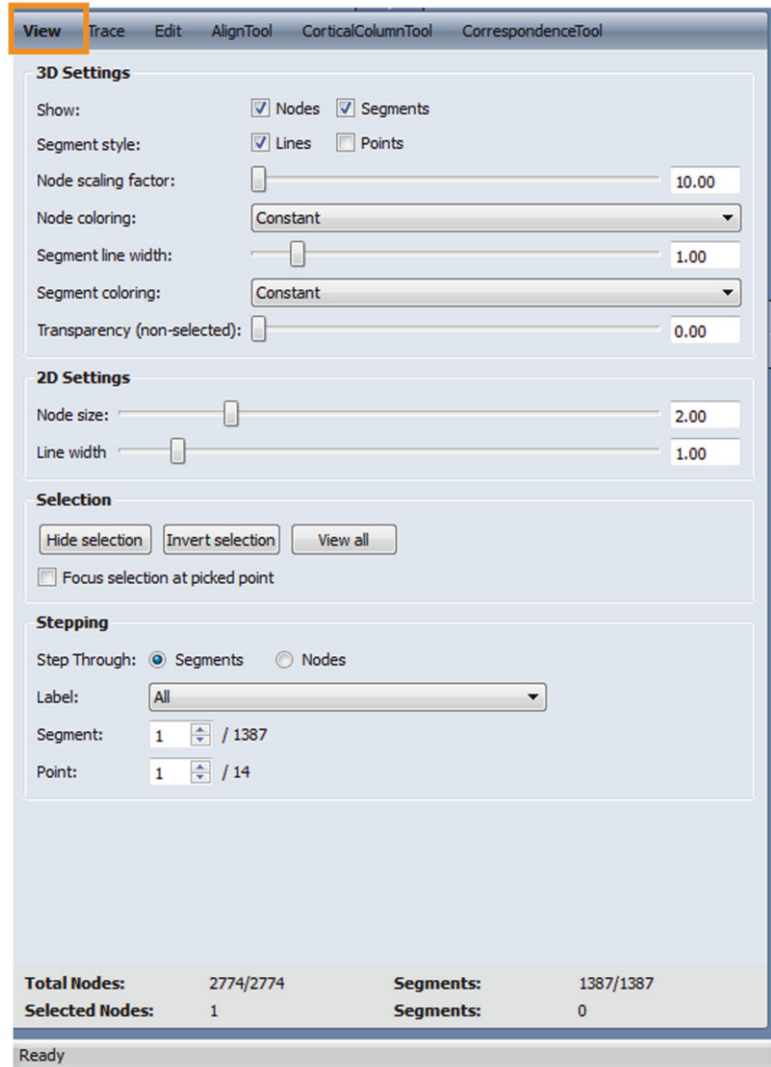


Fig. 7 The *edge stepper* tool. This tool allows the user to systematically step through all lines and show their embedding in the volume. Stepping through the edges is triggered by increasing/decreasing the edge number in the segment input field. To step through the edge points, the point number in the points field can be increased/decreased

6. Another important step is to check the correctness of traced lines and correct errors. To correct a traced microtubule, it needs to be selected first. To do so, push the select button (Table 2) and then click on the edge or the node of interest in the MPR-viewer. Clicking and pressing *shift* simultaneously selects nodes and edges of a microtubule. In the case that the traced line overshoots a microtubule end, you can jump from one end of the microtubule to the other with the keys *k* and *h*. Pressing *p* will remove the last endpoint. If the semiautomatic

tracing tool is active, you can click at a position on the edge where the microtubule ends and the microtubule are shortened up to this point. A falsely detected microtubule can be selected (*shift*+select to select edges and nodes) and then deleted by pressing *d*. If the traced line stops before the true microtubule end, you can select the end that needs to be lengthened. If the semiautomatic tracing tool is active, you can then click on a point where the microtubule continues and the microtubule will be continued. If you prefer not to use the automatic tracing, press *shift* and click on the point where the microtubule ends. The current endpoint will then be connected to the clicked point by a straight line. Occasionally, you will need to connect two edges to form a single microtubule. This can be done by pressing *ctrl* and selecting both microtubules. Then press the connect icon (Table 2).

7. You can assign attributes to edges and nodes of the *SpatialGraph*. For example, a microtubule end can be closed or open. You might also want to distinguish between different kinds of edges, since some might, for example, represent a filament and others the centrosome border. In the filament editor the attributes are called labels. A *SpatialGraph* can have three kinds of labels, NodeLabels, SegmentLabels, and GraphLabels, that can be assigned to nodes and segments. The labels are organized in label groups. A label group represents a property of an item, such as end type of microtubule or spindle part, and contains different labels such as plus, minus, or for the second case actin, microtubule, and centriole border. To create a label, right click in the *label editor* and select the kind of label group you want to create. Add labels by right clicking again on the label groups, and add labels as you wish. You can choose a color for each label by clicking on the color icon next to the name. The created labels will be saved together with the *SpatialGraph*. To assign a label, select the items you want to assign a label to. Right click in the label you want to assign in the label editor, and select assign selection. Leaves in the label tree can be assigned to selected items by the keys 1–9 (Fig. 8).

4 Summary and Outlook

In this chapter, we briefly described the cryo-immobilization of *C. elegans* embryos and further preparation for electron microscopy and tomography, followed by a more extensive introduction on how to use the automatic microtubule tracing tool in Amira. We have used Amira to successfully trace and model microtubules in tomograms of semi-thick sections of the *C. elegans* one-cell embryo (Fig. 9). A tool to stitch tomograms and models from several consecutive serial sections is currently developed to enable a

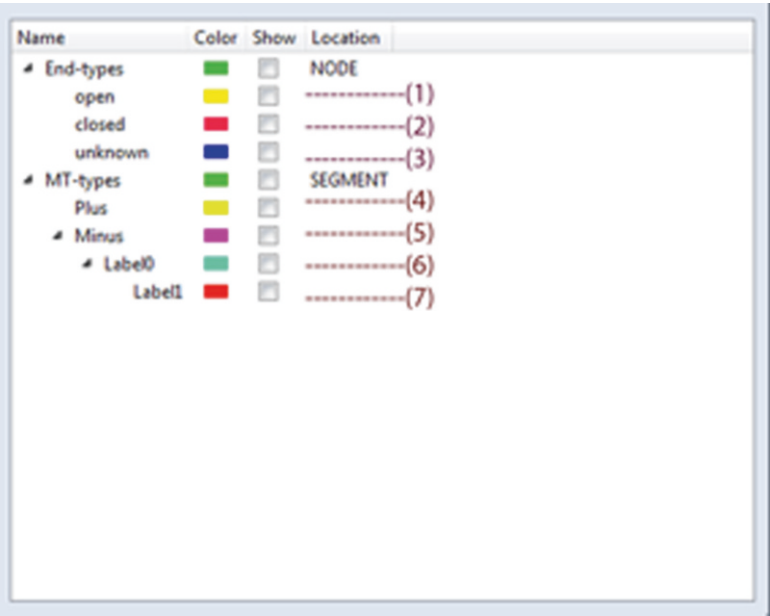


Fig. 8 *Label editor*. Labels (annotations) of microtubules can be added with the *label editor* by a right click into the *label editor*. Labels can be named individually. A color for each label can be chosen by clicking on the color icon next to the name. The created labels will be saved together with the *SpatialGraph*. Leaves in the label tree can be assigned to selected items by the keys 1–7

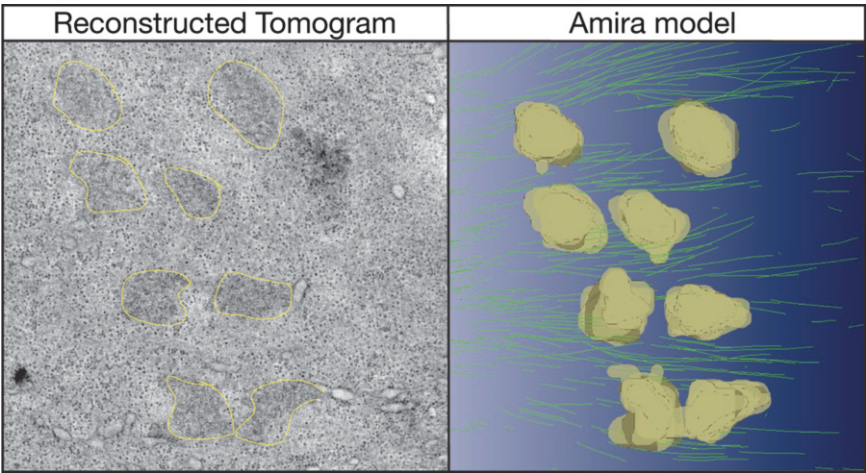


Fig. 9 Model of microtubules within a tomogram created with Amira. In the *left panel* one section of the reconstructed tomogram is shown; the *right panel* shows a snapshot of the microtubules (*green*) and chromosomes (*yellow*) created using the automatic microtubule tracing in Amira (Color figure online)

full reconstruction of larger volumes. This chapter can only be considered as an introduction on how to use the Amira program for microtubule tracing. Depending on the specific project and question asked, other operations and tools within the software package, including the filament editor, might be of more importance to other users. The Amira program offers a help file with several tutorials, which the users might find helpful.

5 Notes

1. The EM PACT2 + RTS (Leica) high-pressure freezer is a portable machine that can be easily moved to the microscope, where staging of the isolated embryo is performed. The RTS allows fast loading of the specimen into a preloaded high-pressure freezer under standardized conditions [10, 12].
2. The use of 20 % BSA gives reproducibly good freezing results, also for other samples such as *Drosophila* embryos and tissue culture cells [10]. However, we have also made good experiences with BSA concentrations as low as 10 %.
3. Information and help for SerialEM can be found here: <http://bio3d.colorado.edu/SerialEM/>. Information on how to use IMOD, as well as test data, can be obtained from <http://bio3d.colorado.edu/imod/>. IMOD as well as SerialEM are freely available. The packages needed for the described microtubule tracing and editing functionality can be obtained from <http://www.zib.de/en/visual/software/MicrotubuleTracing.html>. A free test license for Amira can be requested at the same site. In addition, test data described in Weber et al. [16] are provided at <http://publications.mpi-cbg.de/4629-data>.
4. It is advisable to put the gold on the sections and grids before they have been observed with an electron microscope. Exposure to the electron beam seems to severely affect the affinity of gold to the sections.
5. The Navigator window of SerialEM can be used to acquire several positions or sections automatically in one go. This can save a lot of time as the acquisition can be run overnight.
6. Prior to segmentation and modeling in Amira the reconstructed tomograms have to be flattened and trimmed to avoid problems with the microtubule tracing. The best way to trim a flattened tomogram is by command line using the command

newstack -sec zstart-zend inputfile outputfile
zstart, zend are a list of integer ranges

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