Progress in Functional Neuroanatomy: Precise Automatic Geometric Reconstruction of Neuronal Morphology From Confocal Image Stacks

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Evers, J. F., S. Schmitt, M. Sibila, and C. Duch. Progress in functional neuroanatomy: precise automatic geometric reconstruction of neuronal morphology from confocal image stacks. J Neurophysiol 93: 2331–2342, 2005. First published November 10, 2004; doi: 10.1152/jn.00761.2004. Dendritic architecture provides the structural substrate for myriads of input and output synapses in the brain and for the integration of presynaptic inputs. Understanding mechanisms of evolution and development of neuronal shape and its respective function is thus a formidable problem in neuroscience. A fundamental prerequisite for finding answers is a precise quantitative analysis of neuronal structure in situ and in vivo. Therefore we have developed a tool set for automatic geometric reconstruction of neuronal architecture from stacks of confocal images. It provides exact midlines, diameters, surfaces, volumes, and branch point locations and allows analysis of labeled molecule distribution along neuronal surfaces as well as direct export into modeling software. We show the high accuracy of geometric reconstruction and the analysis of putative input synapse distribution throughout entire dendritic trees from in situ light microscopy preparations as a possible application. The binary version of the reconstruction module is downloadable at no cost.

INTRODUCTION

The morphology of a neuron’s dendritic tree is important for its computational characteristics (Euler and Denk 2001; Koch and Segev 2000; Mainen and Sejnowski 1996; Rall et al. 1992) and for an adequate spatial distribution of thousands of input and output synapses. Dendritic arborizations receive, integrate, filter, and process incoming synaptic information with complex spatio-temporal patterning to create structured information output that is transmitted to network partners. Therefore network and brain function are strongly dependent on neural architecture. The morphogenesis of dendritic trees is regulated by innate genetic factors, neuronal activity, and external molecular cues (Libersat and Duch 2004; McAllister 2000; Miller and Kaplan 2003; Wong and Gosh 2002) during developmental and experience-dependent plasticity (Spitzer 2002; Wong and Gosh 2000). Furthermore, alterations in neural morphology may occur during aging and neurodegenerative diseases (De Brabander et al. 1998; Uylings et al. 2000). Despite striking achievements during recent years, neuroscience is still far from having a comprehensive understanding of the computational function of dendritic shape (Gabiani et al. 2001; Koch and Segev 2000; Haussler et al. 2000; Kirchmar et al. 2002; Segev and London 2000; Single and Borst 1998; Stuart and Haussler 2001) and of the mechanisms underlying maturation, refinement, and aging of dendritic shape in the manifold types and subtypes of neurons in the brain (Cline 2001; Libersat and Duch 2004; Scharff 2000; Scott and Luo 2001; Wong and Gosh 2002). A commonly available tool is needed to create neuronal morphology databases (Van Pelt et al. 2001) and to quantitatively analyze changes in dendritic shape with high accuracy, as imaged during development or during learning in situ. Ideally, such a tool should provide precise three-dimensional reconstruction to determine the neuron’s length, diameter, surface, orientation, and branching pattern (Libersat and Duch 2004; Uylings and van Pelt 2002), as well as its sites of input and output synapses. Only with these measures in hand can a thorough geometric evaluation, as well as the construction of models for computational analysis, be performed.

Electron microscopy offers the highest precision of cellular morphology, but due to the method’s practical constraints, is only applicable to fixed tissue and is restricted to subvolumes of neurons. Confocal or two-photon microscopy offer excellent possibilities for monitoring the growth in situ and in vivo of fluorescent-stained neuronal structures at high resolution in three dimensions, while the neuron is in its natural environment.

Volume reconstructions, which provide surface and volume measures, are a commonly available method for the automatic reconstruction of neural morphology from confocal image stacks, but information about branching number, diameter, and length must still be determined. This, in principle, could be accomplished by a variety of methods developed to automatically extract midlines from binary volume definitions to build up a wire model of the neuronal tree that is equipped with diameters, i.e., a geometric reconstruction. Its precision, however, is limited by the quality of the volume reconstruction one starts with and usually suffers from various algorithmic constraints that make manual postprocessing necessary.

Due to the low accuracy and incomplete results of automatic procedures that are now available, geometric reconstructions are commonly done manually with programs such as Neurolucida (MicroBrightField) (Glaser and Glaser 1990) or Neurozoom (Neurome). The accuracy of manual reconstruction, however, is strongly dependent on individual data interpretation to estimate midlines and diameters of dendrites. In addition, it is extremely time consuming. Automation therefore is a better way to create morphology databases that quantitatively link physiological function and genetic composition to morphology.

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We have developed a complete and novel reconstruction tool set for creating precise geometric reconstructions as well as surface and volume reconstructions. The entire reconstruction process is fully automatic for confocal image stacks of well-stained dendritic trees as complex as those of cerebellar Purkinje cells. For even more complex dendritic trees, or for image stacks of inferior quality, the reconstruction procedure is semi-automatic. The user can manually define branch point hierarchy, and midlines and diameters of interconnecting segments are automatically determined to obtain a skeleton reconstruction. For surface reconstructions at single voxel precision, the skeleton reconstruction is used to extract localized intensity values for image segmentation to cope with staining gradients and intensity fading caused by bleaching or absorption. Furthermore, we present a method for assessing the concentration of labeled markers around the neuron’s surface or within its cytoplasm to determine the distribution of labeled molecules along neuronal processes. Finally, an export routine is included to write compartment model description files from geometric reconstructions in Genesis and Neuron format for computational analysis.

This tool set offers completely new approaches to address the development, refinement, and function of neuronal architecture. To speed up database establishment and common use, it is equipped with a graphical editor to allow extensive interaction; it incorporates into the commercially available visualization software, Amira. The binary version of the reconstruction module is downloadable at no cost, and its basic features are briefly documented (www.neurobiologie.fu-berlin.de/Evers.html). It requires Amira version 3.0 or 3.1 and is ready to install.

METHODS

Intracellular staining, immunocytochemistry, and image acquisition of multiple label specimens

Intracellular recording and staining of Manduca motoneuron 5 was done as described in Duch and Mentel (2004). Special care was taken to avoid tissue shrinking (Bucher et al. 2000). A Leica TCS SP2 laser scanning microscope equipped with three different laser lines was used to acquire image stacks of triple-labeled preparations. All images were obtained in simultaneous acquisition mode, i.e., the emitted fluorescence light of synchronously excited fluorophores was divided by an acoustic-optical beam splitter to be detected by separate photomultipliers. Thus no image misalignment occurred due to error in scan mirror positioning. Images were further processed with Amira 3.1 (TGS) and arranged into figures with Adobe Illustrator 10 (Adobe Systems).

Correction for chromatic aberration

To correct for chromatic aberration of the optical path, a neuron was filled with biotin and double-labeled with Cy3- and Cy5-coupled streptavidin (Jackson Immunochemicals).

An image stack was obtained as described above. The misalignment between images of the same structure was measured and corrected for in subsequent image stacks of identically treated specimens (see Wouterlood et al. 1998).

Programming

All programming was done in C++, making use of Amira 3.1 (TGS) and OpenInventor (SGI) libraries for image data handling and visualization. The software was compiled for Microsoft Windows 2000 using Microsoft Visual Studio 6.0, IRIX 6.5 (SGI) using MIP-Spro Compiler Version 7.41, and Suse Linux 9.0 using gcc3.3.1.

Reconstruction algorithms

The algorithms are described in detail in a parallel, theoretical paper (Schmitt et al. 2004). However, the basic principle of fitting reconstructions to confocal image data are briefly described below. The neuron’s branchpoint hierarchy is set by the user. Then the interconnecting segments are automatically reconstructed as cylinders. This is realized by adapting the active contour model by Kass et al. (1988). Thereby, the precision of the skeleton of interconnecting link segments is controlled by two criteria: first, the skeleton has to be smooth, avoiding wiggles, which are due to imaging noise. This is enforced mathematically by equipping the segment with spring properties and calculating its reset force, a value which describes the segments’ smoothness (smoothness measure). Second, the centerline has to lie close to the middle of bright voxels (medialness), guaranteeing that the precise morphology of the neuron is reconstructed. This medialness is evaluated algorithmically and, in turn, consists of two components: First, voxels with relatively higher gray value should lie inside the neuron’s circular cross-section, whereas relatively lower gray values should lie outside. Second, the change of staining intensity, i.e., the gradient of image intensities should be maximal at the circumference of the circular cross-section. Both criteria for medialness are dependent on the local staining distribution rather than on the absolute value of staining intensity. Both the smoothness measure and medialness measure are evaluated for every cylinder, giving a numerical expression of reconstruction quality, called energy in the following. By iteratively evaluating the summed energy of the segment (i.e., the energy functional) and adjusting its midline and diameters to minimize its energy, the reconstructed segment is fitted to the confocal image data.

Precise surface reconstructions are obtained by initially calculating a three-dimensional distance map of float values with the voxel resolution of the original image data, on which every voxel holds the distance to the nearest cylinder of the skeleton reconstruction. Negative values mark the inside of the structure, and the zero-crossing marks the structure’s boundary. The distance map is subsequently deformed to best fit the image intensity distribution, loosening the restriction to cylindrical cross-sections. For this, we deploy the geometric active contour method (Caselles et al. 1997), which allows introducing several weighing criteria to judge reconstruction quality on a voxel by voxel basis. These criteria namely are staining intensity at every single compartment of the cylindrical reconstruction, local staining gradients, and a user-defined surface smoothness. The distance map is iteratively optimized adjusting the distance values of every voxel to maximize reconstruction quality.

The distance map can be threshold-segmented to obtain a binary volume definition. Subsequent surface reconstruction can be generated, for example, by application of standard techniques (e.g., generalized marching cube algorithm), yielding a triangulated surface reconstruction.

Staining density and localization extraction

Staining density distribution along neuronal projections can be calculated in two different ways. If membrane-associated proteins are under investigation, the mean intensity of the second channel image data around every triangle of a triangulated surface reconstruction is calculated within a predefined distance and stored for every triangle. For correlation analysis between the skeleton’s geometry and the staining localization, the skeleton is fragmented into truncated cones. Surface triangles are assigned to that cone-shaped compartment best approximating the triangle’s location and are stored for every node.
Thus staining distribution calculated for the surface reconstruction can be related to the neuron’s geometry.

If the localization of cytoplasmatic proteins should be analyzed, the mean staining density within the volume corresponding to a cone-shaped compartment of the skeleton reconstruction is extracted. The node’s volume is first confined to lie on the node’s side of the planes orthogonally intersecting the straight lines to its neighbor nodes in their center point, and second, the volume’s voxels must hold a negative value in the fitted distance map. The density values are stored for every node.

Exporting geometry data for analysis

Data about tree geometry can be exported as an ASCII table and include the following values for every branch segment: successive endings, segments, number of branchpoints, and tree length; length and mean radius of branch segment; tree distance; and air distance to tree origin.

Tags can be individually assigned to skeleton nodes. This enables us to define a selection of nodes for geometric analysis. If a geometric analysis is performed for selected nodes, the ASCII table will additionally include the following measures: length and mean radius of the branch segment the node is part of; the node’s tree distance and air distance to tree origin; distance to next higher branchpoint; radius of the node; and values stored for the node.

Importing three-dimensional reconstructions in nonproprietary format

An import and export filter for neuron morphology data in SWC-file format (Cannon et al. 1998) is included with the reconstruction module for Amira. This is a suggested format for on-line neuronal morphology databases, as used at http://www.cns.soton.ac.uk/~jchad/cellArchive/cellArchive.html. This file format is supported by CVAPP, a program for viewing and simple editing available from the above address, which also supports the import of Neurolucida reconstructions. Like this, it is possible to process Neurolucida reconstructions within Amira.

RESULTS

Here we present a comprehensive new reconstruction tool-set for measuring precise geometric parameters, describing the morphology of three-dimensional neuronal processes. The tool-set consists of four components.

1) A semiautomatic reconstruction procedure is realized by adapting the snake algorithm (Kass et al. 1988; Schmitt et al. 2004) to trace approximately tubular neuronal structures in confocal image stacks. User interaction is needed to define branch point hierarchy, whereby the algorithm automatically determines the midline and diameter of interconnecting link segments at a user-defined step size. The resulting tubular reconstruction of the neuronal tree, which we refer to as the neuron’s skeleton, contains precise information on length, diameter, orientation, and branching pattern.

2) To access exact surface and volumetric measures, we adapted the geodesic active contour algorithm (Caselles et al. 1997; Schmitt et al. 2004) to fit the idealized cylindrical shape of previously generated skeleton reconstruction to actual image data, resulting in precise boundary definition.

3) To accelerate the reconstruction process, we skeletonize threshold-segmented confocal image stacks with the TEASAR algorithm (Sato et al. 2000) to generate preliminary wire models, which then serve as an initialization for the above-mentioned tracing algorithm. This fully automates the reconstruction process in cases of simple branching (for instance Purkinje neurons) and well-stained neurons and significantly shortens the time needed to reconstruct neuronal trees with complex branching.

4) We present new methods for measuring staining density of second channel image data along surface definitions or within their respective volume. This enables relative geometric analysis of, for instance, protein location in close vicinity to the surface of the dendritic tree or within the cytoplasm along the axis of neuronal projections.

Tracing neuronal structures

To trace the run of a neuron’s processes in three-dimensional space from a stack of confocal image data, it was previously necessary to manually define midlines and diameters of all branches. This is extremely time consuming and depends on personal judgment. These drawbacks can be overcome as depicted in Fig. 1. As an example, a small part of a motoneuron dendritic field (shown as a volume rendering in Fig. 1A) is reconstructed mimicking a conventional manual procedure within Amira (Fig. 1B). Typically occurring inaccuracies are obvious. First, slight bends of neuronal processes often get ignored, which leads to shortcuts in these structures (see Fig. 1B2, oval). Second, strong tapers and sharp curves are represented by unrealistic abrupt changes in diameter and sharp angular bends (Fig. 1B, large arrows). Third, image contrast must be adjusted to make small, and therefore weakly stained, structures visible during reconstruction. The perceived diameter of all structures will then appear thicker, thus introducing further inaccuracy. As a consequence, the resulting reconstruction reflects only the coarse morphology, and an inordinate amount of time must be invested in manually describing tapers and curves with a small enough step size. Even a small step size cannot compensate for the restriction of manual reconstructions to the image plane of every optical section of confocal image data. This is expressed in a stair-like appearance of the midline’s course in a y-z aspect of the reconstruction, resulting in misestimation of length (Fig. 1, B, D, and E). This problem becomes strikingly obvious in a manual reconstruction of an entire dendritic tree (Fig. 1E). To overcome these limitations, we developed a semiautomatic reconstruction algorithm, only demanding user-provided information on interconnectivity. One method is to interactively define connected locations within the neuronal tree. The link segment is then traced automatically, producing a string of connected cylindrical compartments. Under the assumption of approximate cylindrical shape of the stained structures, the compartments’ axis and radii are fitted into the confocal image stack data by iteratively optimizing their values to achieve best image data congruency. The method does not depend on boundary definition by fixed threshold levels, but calculates intensity gradients to determine boundary information (see METHODS). Branchpoints are created, choosing already-reconstructed parts as starting or end points. Therefore to measure the geometry of a complete neuronal tree, only the location and connection pattern of branchpoints and endpoints must be defined manually. The algorithm automatically optimizes branchpoint location in three dimensions, but does not correct for user-defined branchpoints located outside the original staining. Branchpoints that are left out of the calculation will result in a straight reconstructed segment.
To show the advantages in the accuracy of this algorithm, we revised the manual reconstruction shown in Fig. 1B with our semiautomatic method. The resulting reconstruction (Fig. 1C) is a better fit to the confocal image data than the manual one and is independent of varying staining intensities. It precisely describes slight and sharp bends as well as continuous tapers (Fig. 1C, large arrows and 2), resulting in an exact geometrical reconstruction of neuronal processes. The center point of each compartment is not restricted to the image planes, but is adjusted to lie in the center point of its staining intensity distribution (see METHODS). Therefore the dependency of length accuracy on optical section thickness is strongly diminished.

In addition to the improved accuracy, the semiautomatic reconstruction process is much faster than manual approaches, because the number of points that have to be defined is considerably reduced (Fig. 1B, large arrows and 2). The step size in which the skeleton’s midline and diameter are determined can be adjusted to any value, limited only by the computational power of the computer, and not by the time invested by the experimenter. In contrast, when reducing the number of manually defined points to the number needed for the semiautomatic reconstruction, but not applying the new algorithm, the resulting reconstruction is greatly impaired (Fig. 1D). If the metric parameters of the manual reconstruction are compared with those of the precise semiautomatic reconstruction (C), the accuracy of the resulting manual reconstruction is strongly impaired. Resulting error of the manual reconstruction compared with the new semiautomatic method is depicted quantitatively in the table in A. To show the resulting difference for an entire dendritic tree, a manual reconstruction of the dendritic field of an insect motoneuron (E) and its revision with the semiautomatic method (F) are depicted in z-x-view in E and F. Selective enlargements of the distal ends in y-z-view show only the midlines. This shows that the manual reconstruction’s midline is restricted to optical sections of the confocal microscope, resulting in a stair-like appearance and false estimations of dendrite length and branch trajectory.

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described above (Fig. 1F). Diameter precision, smoothness of fit, midline definitions, and length calculation are all far better with the new automatic tool. This becomes especially apparent when comparing selective enlargements of the tip of the dendritic field in a y-z view (Fig. 1, E and F). The manual reconstruction shows a stair-like appearance compared with the smooth fit with the automatic method. Comparing the metric parameters of the reconstructions of the whole dendritic trees shown in Fig. 1, E and F, a deviation of the manual reconstruction as high as 7% in length, 7% in surface area, and 11% in volume is observed. In summary, compared with manual solutions, our methods save considerable amounts of time and provide higher accuracy with respect to finding the midlines and the diameters of the neuronal segments under investigation.

Boundary determination/distance map calculation/surface extraction

Because the cylindrical fit discussed above precisely describes the midline of neuronal structures, it only approximates the actual run of the surface of the neuron and does not give good boundary definition for nontubular structures. Exact surface representation of neuronal structures may be of fundamental importance, however, when exact volumetric measures are needed, and also for a proximity correlation between multiple structures acquired when considering multiple channel image data.

Most surface extraction methods rely on global threshold criteria, although confocal image stacks bear method-constrained data blurring and distortion described through the point spread function (PSF), as well as decreased fluorescence intensity with increased distance from the site of dye injection due to dye diffusion delay before sample fixation or live tissue image acquisition. The confocal image data can be corrected for the microscope and tissue-specific PSF by applying deconvolution algorithms, principally enabling threshold-based image segmentation to build volume and surface reconstructions. Dye concentration gradients, however, may significantly influence accuracy particularly in distant parts of the neuron. Thin processes with fluorescent signals that are close to background noise, staining discontinuities, and ruptures occurring through histological processing need manual editing, which is subject to individual perception. To show the importance of developing methods other than globally defined threshold values for boundary extraction, we show a skeleton reconstruction of an individual dendritic growth-cone obtained with the semiautomatic method superimposed onto a single optical section (Fig. 3). This leaves the user to add only a few leftover branches with staining intensity below threshold level (arrows) or to remove a few possible loops within the threshold segmented image data. The entire reconstruction of the Purkinje cell (Fig. 3, A and B) takes the experimenter <15 min of working time, including the creation of dendrograms (Fig. 3F) and the extraction of accurate values for the length, diameters, and numbers of all segments and their order and orientation in three-dimensional space.

As shown in Fig. 3 for a cultured astrocyte (Fig. 3, D and E) and the central projection patterns of a locust hair receptor cell (Fig. 3F), this combination of methods fully automates the reconstruction process of many different cell types, as long as uniform staining intensity can be achieved experimentally. In the case of complex neurons with largely differing staining intensities, only clearly articulated parts of the neuronal tree can be reconstructed fully automatically (compare the high complexity of the dendritic field of the insect motoneuron in

Complete automation by skeletonization of threshold segmented image data

As described above, fixed-level threshold segmented surface reconstructions are limited in their accuracy. However, if applied to relatively large and well-stained structures, they give a good preliminary approximation of the actual neuronal shape. Here, we use the TEASAR algorithm (implementation into Amira kindly provided by Steffen Prohaska) to extract an approximated centerline tree from prior segmented confocal image stacks, equipped with diameters. This tree is subsequently used as initialization for the previously described semiautomatic method. The interplay between both algorithms produces a skeleton, which is precisely adapted to the image data without further user interaction, not impairing the reconstruction's accuracy.

This fully automatic reconstruction approach is shown for the dendritic field of a Purkinje cell, a cultured rat astrocyte, and for the axonal arborization of an insect sensory neuron (wind sensitive accessory hair of Locusta migratoria). As the TEASAR algorithm relies on strict hierarchical organization of the segmented image data, the threshold level for all cases was adjusted to include the maximum of the arborizations while avoiding artificial formation of loops within the tree. In cells labeled as evenly as the Purkinje neuron depicted in Fig. 3A (projection view of all optical sections into one focal plane), most arborizations are included in the resulting fully automatic geometric reconstruction (Fig. 3B). This leaves the user to add only a few leftover branches with staining intensity below threshold level (arrows) or to remove a few possible loops within the threshold segmented image data. The entire reconstruction of the Purkinje cell (Fig. 3, A and B) takes the experimenter <15 min of working time, including the creation of dendrograms (Fig. 3F) and the extraction of accurate values for the length, diameters, and numbers of all segments and their order and orientation in three-dimensional space.

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To avoid the formation of loops within the volume reconstruction, the threshold value for image segmentation then must be chosen moderately, excluding fine arborizations from the full automatic reconstruction and producing unconnected fragments of the neuronal tree as a consequence. However, this still saves the user a large amount of time, as unconnected parts can be reconnected manually and only fine structures are left over to be traced interactively applying the semiautomatic method described above (see Fig. 1).

**Evaluation of the geometric relation between multiple channel data**

Neuronal function and development are strongly influenced by both molecules that are in close spatial relationship to the dendritic surface, such as cell surface proteins, growth factors, and synaptic receptor molecules, and by molecules that occur compartmentalized in the cytoplasm, such as kinases along the neuron’s projection axes. Therefore many questions rely on an analysis of the proximity and localization relation between fluorescently labeled structures in multiple channel image data. Ideally, the boundaries of each stained profile would be defined with a precision that allows distance measurement between surfaces in the submicron range. This is now possible for the dendritic surface by local threshold extraction, as shown in Fig. 2. However, if this is to be done for immunocytochemically labeled molecules that are diffusely distributed throughout the neuropil, boundary definition by threshold segmentation is usually not applicable because of the antigen concentration-dependent variation of emitted light intensity.

**FIG. 2.** From a skeleton with fitted diameters to an exact surface. Semiautomatic cylindrical reconstruction is insensitive to local staining intensities, because it evaluates the steepest slope of the intensity gradient to determine the structures boundary. This problem is shown by superimposing a skeleton reconstruction onto a single optical section, in which the contrast is optimized according to 2 different criteria: 1st, to make thin neuronal processes visible (A), and 2nd, optimized for thicker structures (B). Perceived diameters depend strongly on the contrast setting as indicated by the white arrowheads in A and B. This problem is overcome by using an algorithm to extract local thresholds. Skeleton reconstruction is used to initialize a distance map that is fitted to the actual staining distribution (C). Negative values mark inside of structures, and 0 crossing indicates the structure’s boundary. Arrowheads indicate sites where conventional global threshold-based boundary extraction would fail to produce correct results for either one of the neuronal processes, depending on the chosen threshold value. Resulting distance map can be converted into a binary volume definition by selecting voxels with distance values ≤0 (D). Resulting surface overcomes limitation to strictly tubular topology and takes nontubular neuronal shapes into account (D), thus representing a precise surface definition.
With this in mind, we suggest a novel evaluation method for measuring the second and third channel staining density around surface areas. This is done by calculating the mean staining intensity around every triangle of a triangulated surface in a user-defined distance in three-dimensional space. Consequently, a neuronal surface is searched for its spatial relation-
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A

B

C

D

E

F

compartment radii at synapse location
distance of synapse location to tree origin

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ship to labeled proteins by evaluating their staining intensity in a user-defined surrounding subvolume. This staining density can then be mapped onto the surface for visualizing not only the distribution but also the staining intensity of labeled antigens along neuronal processes.

If the critical distance between both labels of the double staining is at about the same magnitude as the resolution of the recorded images, the surface has to be defined with great precision. In this case, the skeleton reconstruction and consecutive surface generation described above are pivotal, particularly if synaptic proteins or surface molecules are under investigation. For instance, considering the relation of presynaptic structures to their postsynaptic membrane in the case of the Calyx of Held, the synaptic cleft is about 20 nm in size, and the vesicle pool extends to about 200–300 nm (Sätzler et al. 2002) from the active zone. This sums to a distance of 220–320 nm, which would be the critical distance if vesicle-associated proteins are to be analyzed. The scanning resolution of the confocal microscope is set to 100 × 100 × 300 nm, which exceeds the actual optical resolution, thus resulting in oversampling. This procedure counteracts partial volume effect and facilitates algorithmic structure recognition. However, the critical distance for evaluating staining distribution is a width of only a few voxels. To show the power of this evaluation method, we chose an intracellular staining of a dendritic growth-cone of an insect motoneuron (Manduca sexta, MN5) combined with an antibody staining against synaptotagmin (Fig. 4, A–C). This addresses the distribution of putative presynaptic terminals contacting the postsynaptic growth-cone.

Because of the density of immunoreactive synaptotagmin-positive profiles in the neuropil, the image information has to be excised around the dendritic surface for a better visualization of the relation between dendrites and the labeled proteins in the second channel image data. This is accomplished by a distance map, generated as described above. All image intensities further than 300 nm from the dendritic surface are set to zero, so that the volume rendering of the resulting data, displayed together with the surface reconstruction, allows comprehensive visualization (Fig. 4A). To also obtain quantification, the surface reconstruction is equipped with a color code representing the staining density within 300 nm (Fig. 4B). Warmer colors represent the higher staining intensities of synaptotagmin label located within 300 nm of the dendritic surface. Used in conjunction with the skeleton reconstruction of the dendrite, each patch of the surface reconstruction can be projected onto the individual cylindrical compartments. This enables analysis of geometric relation between staining distribution and the neuron’s morphology (Fig. 4C). This type of evaluation makes it possible to analyze entire dendritic trees for synaptic contact probability, at least under the assumption that a high staining intensity of a synaptically localized labeled protein might indicate a high likelihood of a putative synapse. Figure 4, D–F, shows how this type of analysis can be further extended by an additional third label. In this example, a triple-staining is used to address the question of how putative GABAergic synapses might be distributed throughout an entire dendritic tree. An intracellular staining of a motoneuron is combined with immunocytochemistry for synapsin-I (2nd channel) and for GABA (3rd channel). The motoneuron dendritic tree is reconstructed, and both immunolabels further than 300 nm from the dendritic surface are excised with the help of the distance map as described above. In Fig. 4D (top) a small part of the reconstruction of the dendritic tree is superimposed with the remaining immunolabels for GABA (left) and for synapsin-I (right) as false color views. In Fig. 4D (bottom) the mean staining intensities of the immunolabels within 300 nm distance of the reconstructed surface are visualized on the dendritic surface in false color code. Asterisks indicate co-localization of high staining intensities of GABA and of synapsin-I on the dendritic surface and arrows indicate sites on the surface that are in close relation to a high staining intensity of one label only. Calculating the correlation between both yields a color map that results from co-localization of both GABA and synapsin-I at high staining intensities within 300 nm of the dendritic surface (Fig. 4E). This offers a method of assessing the distribution of putative synapses of a specific transmitter throughout entire dendritic trees on the light microscopic level. As all staining intensity maps and correlation analysis can be projected onto the skeleton reconstruction, numerical data are available. As an example, we analyzed the occurrence of putative GABAergic synapses detected with this method through the entire dendritic tree of an insect motoneuron (Fig. 4F). The frequency of putative GABAergic synapses is plotted as a function of dendrite radius and as a function of the distance of the origin of the first order branch of the tree. As supplemental data, a movie can be downloaded, showing the geometric reconstruction of the complete dendritic tree and a close-up into the subsequently generated surface reconstruction. The surface reconstruction is then embedded into the first order branch of the tree.

**Fig. 4.** Evaluation of labeled protein concentration along dendritic surfaces at submicron resolution. Analysis of the distribution of labeled putative presynaptic sites around solely postsynaptic dendritic structures (all putative synapses marked by anti-synaptotagmin, or only putative inhibitory synapses marked by anti-synaptotagmin and anti-GABA). A: precise surface reconstruction of a single growth-cone from an insect motoneuron (MN5 from *Manduca sexta*) as generated by local threshold fitting of the reconstructed skeleton. To neglect all labeled presynaptic proteins further than 300 nm from the growth-cone surface, a distance map around the precise surface reconstruction is used to delete all synaptotagmin label outside the 300-nm range. Resulting cut-out of the anti-synaptotagmin label is visualized as a volume rendering. B: to visualize and quantify the average molecule concentration around each patch of the reconstructed growth-cone surface, the mean anti-synaptotagmin label staining intensity is depicted in false color code on the surface reconstruction, with warmer colors representing high concentrations. For further quantification and/or export into modeling software, the surface reconstruction is projected back onto the individual cylindrical compartments of the geometric reconstruction (C). D: selective enlargement of a small part of a dendritic surface reconstruction and volume rendering of both, anti-GABA immunocytochemistry from a 2nd image channel (top left), and volume rendering of anti-synapsin immunostaining from a 3rd image channel (top right). Average molecule concentration around each patch of the reconstructed surface is depicted for each staining (anti-GABA, bottom left; anti-synapsin, bottom right) in false color code, with warm colors representing high concentrations. Asterisks indicate sites on the dendritic surface reconstruction that are in close proximity to high concentrations of both immunolabels, but arrows indicate close proximity to high concentrations of 1 label only. The co-localization index [A × B/(A + B)] is shown in E, with warmer colors representing high concentrations of both labels within 300 nm of the surface. F: example of an entire dendritic tree for which this type of analysis was conducted. Numerical data for the distribution of putative GABAergic presynaptic sites detected with this method throughout the entire dendritic tree with 1,900 branch points is plotted as a function of dendritic diameter (top diagram) and as a function of distance to the dendritic branch origin (see arrow).
original image data of the intracellular staining, Synapsin-I label, and GABA label consecutively and equipped with the color codes generated as described above. This movie allows a comprehensive judgment on the applicability of the method. Many other numerical analyses are possible; this is just to show the power of quantitatively evaluating proximity relations from multiple fluorescent labeled channels. In principle, the distribution of every molecule that gives a clear label in immunocytochemical detection can be analyzed. Depending on the scale, correction for chromatic aberration of the optical path may have to be applied before (Wouterlood et al. 1998).

To measure the distribution of labeled cytoplasmic molecules along neuronal arborizations, the staining intensities of labeled antigens in the interior of the cell surface must be evaluated with regard to their distribution along the neuron’s projection axis. To ensure correct definition of the cell’s interior, a precise definition of neuronal shape—irrespective of the idealized cylindrical shape of geometric reconstructions—is of equal importance as for surface-localized proteins. Therefore a distance map has to be computed as described above to calculate the mean staining intensity of the second channel image data within the volume of all skeleton nodes. The volume corresponding to a node is first confined to lie on the node’s side of the planes orthogonally intersecting the straight lines to its neighbors in their center point. Second, its voxels must hold a negative value in the distance map.

**Discussion**

We describe a novel and ready-to-use tool-set that enables fast and reproducible geometric reconstruction of fluorescent-labeled neuronal arbors from confocal or two-photon image stacks. As shown, the reconstruction accuracy is very high, and it is largely independent of the individual doing the reconstructing. In addition, we introduced a technique to analyze staining intensity distribution along neuronal projections with respect to their surface or cytoplasmatic localization using multiple channel image stacks. This method allows investigation of protein distribution and concentration along whole neuronal trees without time consuming immunoelectron microscopy (EM) studies. Due to limits in optical resolution, however, the method is restricted in its applicability. It has to be carefully tested for each type of analysis conducted (for example by acquiring test images of pressure injected fluorescently labeled beads) and cannot replace EM studies for many questions. If co-applied with the geometric reconstruction described above, metric correlation analysis with respect to the neuronal morphology is possible. Combined with the recent achievements in two-photon imaging and genetically expressed dyes, this method will allow to estimate the relative concentration variations of molecules along the surfaces of living and growing neurons to be determined at the optical resolution the imaging setup delivers. The key of this new approach is to statistically evaluate staining intensity distribution with respect to geometry. This may be particularly useful for evaluating morphogenetic gradients, as occurring for instance from guidance molecule distribution during CNS development.

Image stacks from well- and evenly stained neurons that are acquired at high scanning resolution without detector saturation can now be reconstructed within minutes, as shown for the examples of a Purkinje cell and the afferent projections of an insect mechanoreceptor neuron. Dendritic spine necks show distinct morphological characteristics and are usually too thin for automatic recognition, and thus must be reconstructed semiautomatically by defining their origins on the dendrite. Algorithms optimized to detect spine morphology have recently been published by other authors (Weaver et al. 2004) and may be combined with these methods.

The combination of user interaction and semiautomatic tools now allow precise reconstructions of even the most complex dendritic fields and fine dendritic filopodia, accomplished much more quickly than with conventional methods. This will be advantageous for both, single neuron analysis (life cell imaging, modeling, etc.) and the creation of neuronal morphology databases (see Complete automation by centerline extraction).

**Complete automation by centerline extraction**

Centerline extraction from binary volume definition is carried out with the TEASAR algorithm. This algorithm performs closest to our intuitive perception of the structure’s midline. Its disadvantage, however, is the dependency on a strict hierarchichal organization of the segmented image data. Deviation from hierarchical organization, for example, circular connections within the segmented image data, will occur if the threshold level for image segmentation is not chosen carefully or if the distance between two structures is below optical resolution. The TEASAR algorithm unpredictably chooses one way, regardless of the size of connection. In these cases, it would be favorable to achieve a centerline of all the connections and allow the user to delete those that are unwanted. In principle, this is possible by applying geometric thinning algorithms. These, however, are sensitive to nonsmooth surfaces, erroneously producing a high number of artificial branches. This removes the time benefit of automatic centerline extraction, because one has to manually correct each of these. Therefore the tool set can be further improved by developing skeletonization algorithms that also work dependably on nonhierarchal data. The programming of our tool set is strictly object-oriented, therefore allowing easy integration of other algorithms for further programming development.

**Applying reconstruction on time lapse images**

Time-lapse image stacks of living tissues must be analyzed to quantify growth dynamics of neurons in three-dimensional space. Slight movement of preparations and declining fluorescent signal intensities due to dye bleaching rule out comparing structures by their absolute coordinates or by fixed-threshold level surface reconstructions. With our reconstruction framework, however, it is possible to align a copy of a reconstruction obtained from an earlier image stack to subsequently acquired data, letting the algorithm automatically adjust it to interim movement in space. Only minor additions or deletions have to be done to reflect retraction or growth of arbors. Persisting branchpoints can be named individually as absolute landmarks, which enables comparison of multiple subsequent skeleton reconstructions. If additional proteins are marked with a different wavelength dye, whole cell analyses of growth dynamics can be correlated to protein distribution. These new possibilities of quantitative neuronal tree analysis may open new doors
for data analysis in modern in vivo and in situ imaging studies on living neurons.

Export into modeling software

Cylindrical models of neuronal projections are commonly used to simplify neuronal geometry and therefore speed up computational analysis of neuronal information processing without losing the fundamental neuronal computation characteristics (Gabbiani et al. 2001; Hausser et al. 2000; Koch and Segev 2000; Single and Borst 1998; Stuart and Hausser 2001). A major problem in building these cylindrical models for computational analysis is caused by parts of the neuronal tree that show a strong deviation from a cylindrical volume to surface ratio. In such a case, cylindrical compartments with nicely fitted diameters produce an insufficient approximation of the neuronal geometry for computational modeling. To overcome this limitation, the automatic generation of volume reconstructions can, in turn, be used to optimize the algorithmically built cylindrical model. Each voxel of the volume reconstruction is assigned to the nearest cylinder of the geometrical reconstruction, and thus can be used to optimize the cylinder’s geometry to best reflect either the surface or volume of the reconstructed shape or any calculated intermediate. The high accuracy of the geometric reconstruction allows optimization of multicompartment modeling, at least with regard to the geometric variables. To allow easy transition to modeling programs, we provide export filters to generate geometrically correct models for either Genesis or Neuron. For both modeling programs, their respective innate three-dimensional method is deployed to specify shape, orientation, and location in three dimensions. For Neuron, sections are generated for every tree segment located between two branchpoints or one branch and an endpoint. Every sampling point, as generated by the semi-automatic reconstruction algorithm, is used as a three-dimensional point in the section. The sections are connected to each other, preserving the neuron’s topology. For Genesis, compartment geometry information is created at every sampling point of the geometric reconstruction by defining a point identifier, its three-dimensional position and diameter, and the identifier of its preceding sampling point in the neuron’s tree hierarchy. Downsampling of the spatial resolution to optimize computation speed must be done elsewhere.

Neuronal model and morphology databases

Neuroanatomical databases of reconstructed neurons can help in understanding the role of morphological variations between cell types or between individual cells of the same type. Morphological alterations of neurons occur during development (Cline 2001; Libersat and Duch 2004; Wong and Gosh 2002), during postembryonic plasticity such as learning (Müller et al. 2002; Yuste and Bonhoefer 2001, 2004), but also during degenerative processes, such as aging (Uylings et al. 2000) or diseases (Arendt 2001). Therefore changes in neuronal shape have multiple causes and consequences. Accordingly, the patterns of morphological changes probably follow distinct rules. Such rules cannot be inferred from the structure of single neurons but require sampling of many neurons to extract general principles. Another major question is whether neuronal morphology falls in distinct classes or follows a continuum (Monyer and Markram 2004). Cluster analysis of neuronal shape is necessary to address this. Such problems require to pool geometric neuronal models generated by many different individuals in databases. In this regard, two things are crucial: standardization of file format and a convention about the reconstruction procedure to ensure the possibility of comparison. Our method offers the possibility to standardize not only the sampling density, but gives a high user independency in determining the center point and diameter of every compartment of the neuronal tree automatically. However, the method cannot fully account for the high variability in image data quality due to differing tissue processing or imaging setups.

The usefulness of a neuron morphology database is also dependent on the availability of physiological data recorded from each neuron. As it is not possible to achieve this if each contributor must produce labor-intensive reconstruction work, a high degree of automation is pivotal. Therefore our reconstruction framework will provide practical simplification for this approach. To permit direct integration of our reconstruction toolset into on-line databases for neuronal morphology, it is equipped with import and export filters for the currently used SWC file format. To speed up database buildup and common use by many scientists, it incorporates the commercially available visualization software Amira, and the binary version of the reconstruction module will be downloadable at no cost at http://www.neurobiologie.fu-berlin.de/Evers.html. Having such a tool set available to many neuroscientists may help in tackling new aspects of functional neuroanatomy with the goal of addressing the functional interplay between dendritic morphology and dendritic computation, a functional relationship that lies at the very basis of information processing in our brains.

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