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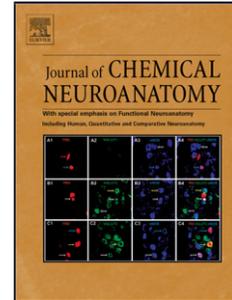
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TITLE: Evaluation of semi-automatic 3D reconstruction for studying geometry of dendritic spines

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HIGHLIGHTS

- -Spine geometry is an important indicator of synapse function.
- -The semi-automatic method is fast and reliable candidate for measuring spine geometry.
- -Results from the semi-automatic and the manual classification of spines are comparable.

ABSTRACT

Background: Spine geometry is considered to reflect synapse function. An accurate and fast method for 3D reconstruction of spines is considered a valuable tool for the purpose of studying spine geometry. Currently, most studies employ manual or automatic reconstruction methods, which still suffer from either poor accuracy or extreme time-consumption. The semi-automatic reconstruction method has previously been described as a time-economic and accurate tool for spine number counting. The purpose of this study is to further validate the semi-automatic method with regards to spine geometry investigation, by comparing it with the manual method as well as with the automatic method.

Methods: In this study, dendritic trees of six pyramidal neurons that belong to layers II/III of mouse frontal cortex are stained using the Golgi method. Thereafter, spines from 42 dendritic branches are 3D reconstructed by manual, semi-automatic and automatic methods using Imaris software. Spine features, including spine volume, spine area, spine length and spine neck length, and the relative distribution of classified stubby, mushroom and thin spines are compared between the semi-automatic method and the two other methods.

Results: Results from the semi-automatic and the manual reconstruction methods are in line with respect to all measured spine geometric features as well as spine classes. However, significant difference has been detected between the two methods and the automatic method in spine length, spine neck length and spine volume. Compared to the manual method, both the semi-automatic and the automatic methods have significantly reduced the spine reconstruction time.

Conclusion: These findings suggest that the semi-automatic method may represent both a time-economic and reliable option for the purpose of studying spine geometry.

ABBREVIATIONS

2D: two dimensional, 3D: three dimensional, 4D: four dimensional, NA: numerical aperture, RGB: red green blue.

KEY WORDS

Golgi staining , Semi-automatic method , Spine reconstruction.

I. INTRODUCTION

Dendritic spines are small protrusions that are crucial for neuronal connectivity. To meet their function, spines are distributed along the dendritic tree, where they occupy the post-synaptic part in the majority of the excitatory synapses (Hering and Sheng, 2001). Even though spines have a high degree of diversity, they have previously been categorized in four groups: i.e. filopodia, mushroom, thin, and stubby spines, according to their geometric and functional properties (Peters and Kaiserman-Abramof, 1970). During development, and in response to several diseases, spines experience a high degree of plasticity in number, but also in geometry; e.g. volume, length, diameter (Fiala *et al.*, 2002; Harms and Dunaevsky, 2007). Spine geometry is of special importance as it reflects the spine motility, and therefore the spine function (Noguchi J, 2005). More specifically, features like spine volume, spine area and spine length are suggested to be correlated with both number and release probability of vesicles in the synapse (Nimchinsky *et al.*, 2002). The early evidence for the functional role of spines in neuronal circuits has stimulated the development of several methods for both visualization and reconstruction of neurons. In this regard, the Golgi staining method is one of the first methods that have been applied to visualize neurons (Camello, 1873). Yet, it is still widely usable, especially in quantitative studies of spines. Additionally, important progress has been achieved regarding the reconstruction of neuronal morphology. However, spine reconstruction is still facing many unsolved challenges. Spine rendering is limited, not only by their small size and shape diversity, but also by the inadequate tracing sensitivity of the available software packages for 3D reconstruction. These software packages are implementing manual, semi-automatic, and automatic methods in order to 3D reconstruct spines, in addition to the recent 4D reconstruction method that allows to trace spines along the temporal dimension. The automatic reconstruction method lacks in many cases accuracy, and therefore manual reconstruction is still employed by the majority of studies, despite that it is labor intensive and time-consuming (Donohue and Ascoli, 2011). The semi-automatic method has therefore gained interest, and has been suggested as an alternative solution that is time saving and minimizes the manual input. This method has generated results from spine density estimation, that are comparable with the manual method (Orlowski and Bjarkam, 2012). However, there is still a lack of knowledge regarding the validity of the semi-automatic reconstruction for measuring the different features of spine geometry, which requires more complex image processing.

In order to address this question, we have reconstructed spines belonging to a set of dendritic branches that are stained with the Golgi method, using automatic, semi-automatic and manual methods. We have used the commercially available Imaris software (Bitplane, Zurich, Switzerland), which has the advantage of detecting spines automatically, semi-automatically and manually. In doing so, we avoid the variability that may result from implementing different software packages.

II. MATERIAL AND METHODS

II.I. Animals

Six male C57BL/6N mice, bred in Taconic Artemis (Ejby, Denmark) were received at the age of 8 weeks. Mice were housed under standard conditions (12-h light/dark cycle, room temperature 22°C) with food and water ad libitum and environmental enrichment. The experiment was approved by the Danish Animal Experiments Inspectorate (2012-15-2934-00254).

II.II. Tissue preparation and Golgi staining

After one week of housing, mice were euthanized by intraperitoneal injection of 150 mg/kg pentobarbital sodium (Exagon vet, 60336/A). Coronal sections from the mouse frontal cortex were dissected using a cryostat (Microm HM 560) at 100 μm thickness and mounted on gelatin coated slides using Eukitt (03989, Sigma-Aldrich) mounting media, refractive index=1.51 and covered with coverslip #0 (Hounisen, Denmark), thickness 0.08-0.12 mm. Sections were stained with rapid Golgi kit (FD Neurotech) by following the manufacturer's instructions. All steps of cutting and staining were performed in darkness.

II.III. Z-stack of neurons

Image of the region of interest was primarily captured using a 4x lens, Figure 1A, on a light microscope (Olympus BX50, Olympus, Denmark) equipped with a motorized stage (Prior scientific), a microcator (ND281, Heidenhain, Germany) to measure the stage movements in the Z-direction, and VIS Software (newCAST version 4.4.5.0. Visiopharm, Denmark). Layers II–III of the frontal cortex from each hemisphere were identified according to (Van De Werd and Uylings, 2014). After delineating the region of interest, six neurons were sampled for 3D reconstruction using a 60x lens, NA=1.35, working distance 0.15 mm, with immersion oil (N5218800, Olympus, Japan) that has refractive index=1.51, and with a Z-navigator. One neuron per mouse was sampled according to specific criteria: a neuron should be located in the middle 30 μm of the Z-thickness with no or minimal breaks and it should be recognizable from the neighbor neurons. A Z-stack of images with 1 μm interval was acquired for each neuron using VIS Software "sample image" function. Every image in the acquired Z-stack is 1390x1038 pixels, with pixel size 235 nm.

II.IV. Dendrite tracing and spine reconstruction

Once the Z-stack was created, the process of neuron 3D reconstruction was initiated by processing the Z-stack with Imaris software (version 7.7.2). Image deconvolution was not applied, as deconvolution of pilot

branches did not result in significant enhancement of the image quality. In total, 42 dendritic branches from 6 neurons were selected for reconstruction with the automatic, semi-automatic and manual methods. To better identify the object of interest against the background, a global contrast threshold was set at 3.0. Dendritic branches were first traced with the “Filament Tracer” tool in Imaris surpass mode. Thereafter, spines were reconstructed with three different methods: manual, semi-automatic and automatic, separately. All the reconstruction work has been performed by the same experienced person. More variability is expected in case of inter-operator handling of the images. However, this variability can be minimized by training the operators before they start handling the images.

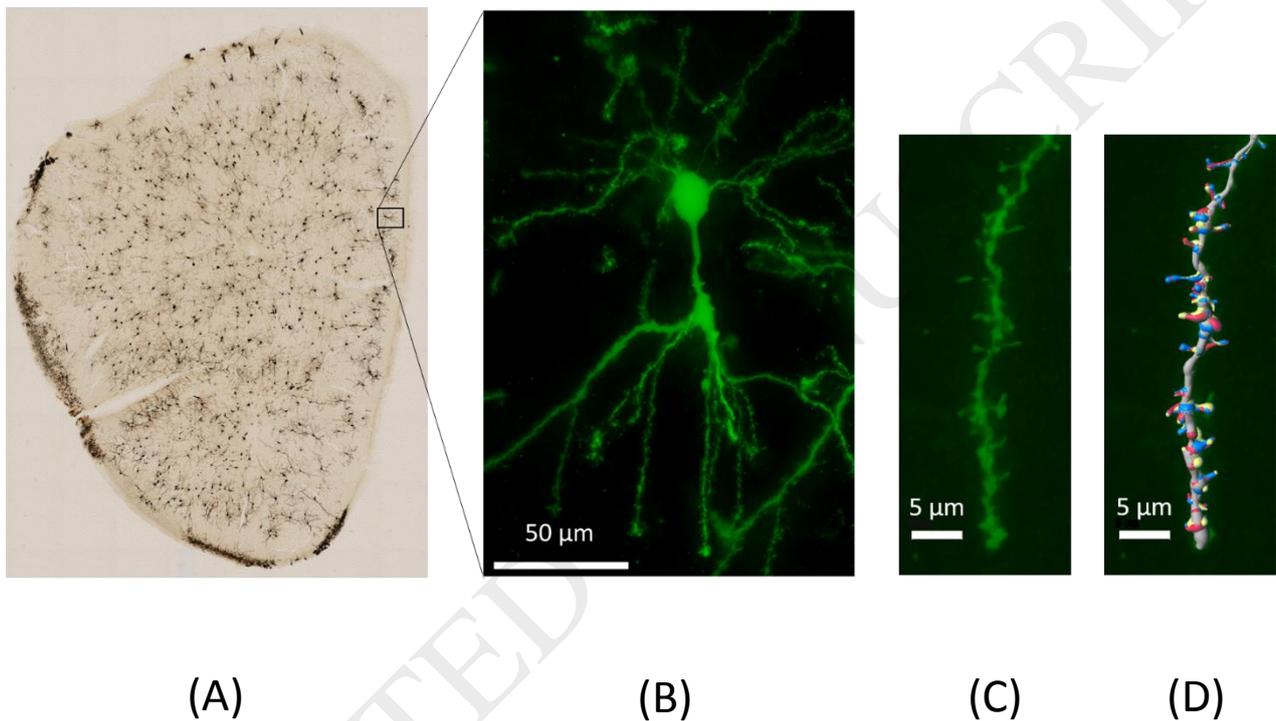


Figure 1: (A) Mouse hemisphere stained with the Golgi Cox method. (B) Pyramidal neuron from layers II/III of frontal cortex after Z-stack conversion to single channel image. (C) Dendritic branch selected for 3D reconstruction. (D) Spines are reconstructed with Imaris manual (red), semi-automatic (yellow), and automatic (blue) methods.

In order to start the reconstruction, a three channel image should first be converted to a single channel image (green) by applying “image processing”, “contrast change” and “invert” on the source image, Figure 1B.

The single channel image is thereafter processed by “new filament” tool. For all three reconstruction methods, the soma diameter and the thinnest dendrite diameter are measured from Imaris “slice” view, and the corresponding values are set to identify the dendrite start and end points, respectively.

To reconstruct a dendritic branch, seed points are automatically generated by Autopath algorithm along the dendritic tree. Thereafter, a local contrast-based threshold is selected to keep only the seed points that delineate the dendritic branch. The seed points will finally connect to each other, and the dendritic branch diameter will be calculated by the algorithm.

The process of spine reconstruction starts after building all the dendritic branches, Figure 1C-D. A spine is defined by the same criteria for all three methods: A protrusion that has a connectivity to the parent dendritic branch and has a minimum head width $\geq 0.4 \mu\text{m}$ and maximum length $\leq 4 \mu\text{m}$.

The automatic reconstruction of spines requires no manual selection of the spine start or end point. Instead, the minimum and maximum spine length are set, and a set of seed points are automatically generated along the dendritic branch. The seed points that do not represent spines are excluded by setting the “seed points threshold” at an appropriate value. Imaris then automatically connects each of the remaining seed points to the corresponding spine start point on the dendritic branch.

Automatic method

Step1: Automatic seed points generation (Autopath mode).
Step2: Threshold-dependent spine building .

Semi-automatic method

Step1: Automatic computation of spine path (Autopath mode).
Step2: Manual tracing of the computed spine path.

Manual method

Step1: Manual identification of spine path (Autodepth mode).
Step2: Manual tracing of the identified spine path.

Figure 2: Summary of the two major steps for automatic, semi-automatic and manual spine reconstruction. For the semi-automatic spine reconstruction, the spine starting point is automatically generated by Autopath-based algorithm. However, a manual tracing of the spine path until the center of the spine head

is still required. With regards to the manual reconstruction of the spines, it requires a manual selection of the spine start and end points, and tracing of each spine between these two points in Autodepth mode. Figure 2 briefly describes the two major steps of spine reconstruction with each of the three reconstruction methods. More details about the exact steps for each method are available here (<http://www.bitplane.com/>).

Spines that are not detected with all the three methods, e.g. spines that are hidden beneath the dendritic segment, are not included in this study. The compared geometric features include spine volume, spine area, spine length and spine neck length.

II.V. Classification of spines

Imaris (XTension), a MATLAB (R2014a) dependent extension, is employed to determine the differences in spine morphology and to classify them under one of three distinguished classes: stubby, mushroom, and thin. Each spine is tested by three criteria, Table 1, so no spine can be classified under two different classes.

II.VI. Time consumption for reconstruction of spines

After the dendritic reconstruction was completed, the time used for spine tracing was recorded for each of the three reconstruction methods, separately. All reconstruction work, for both spines and dendrites was performed using a Fujitsu computer with an Intel® Xeon® 3.60 GHz processor and 64.0 GB RAM. Recorded time for spine reconstruction from both the semi-automatic and the automatic methods is shown as time consumed per branch.

STATISTICAL ANALYSIS

IBM SPSS Statistics 22.0 and GraphPad Prism 7.0 packages were used for the statistical analysis.

Kolmogorov-Smirnov test was used to compare the cumulative frequency distribution for each of the spine features between the three methods. For comparing the percentage of each of the three spine classes, McNemar test followed by Bonferroni multiple comparison test was applied. To compare the time consumed for the spine reconstruction, one-way ANOVA was applied. Results are considered significant when P value < 0.05 .

Table 1: Criteria for spine classification

Spine class	Criteria
Stubby	Spine length < 0.5 μm
Mushroom	Spine length < 1.5 μm and spine head width > spine neck width*2
Thin	Spine head width \geq spine neck width

III. RESULTS

III.I. Features of spine geometry

Data of dendritic spines belonging to six pyramidal neurons were collected and their geometric features were analyzed. Four features of spine geometry, namely spine length, spine neck length, spine volume, and spine area are compared between the three reconstruction methods.

For all the investigated features, no significant difference is detected between the semi-automatic method and the manual method. Spines that are reconstructed with the semi-automatic method show, however, a significant difference compared with the automatic method, at spine length ($p= 0.0092$) and spine area ($p= 0.0051$), Figure 3A and 3D. Another significant difference is detected at spine volume ($p= 0.023$), Figure 3C.

The comparison between the automatic method and the manual method also detects similar differences at spine length ($p= 0.0083$), spine area ($p= 0.0045$) and spine volume ($p= 0.012$), Figure 3A, 3D, and 3C, in addition to spine neck length ($p= 0.031$), Figure 3B.

III.II. Spine classes

The reconstructed spines have been classified into stubby, mushroom or thin according to the specified criteria for each class, as described in Table 1. Filopodia-like spines are excluded from this study as they are occasionally difficult to distinguish from the small dendritic segments. Relative distribution of spine classes are compared between the three methods, McNemar test ($p < 0.001$) and results are presented as percentage of the total spine number.

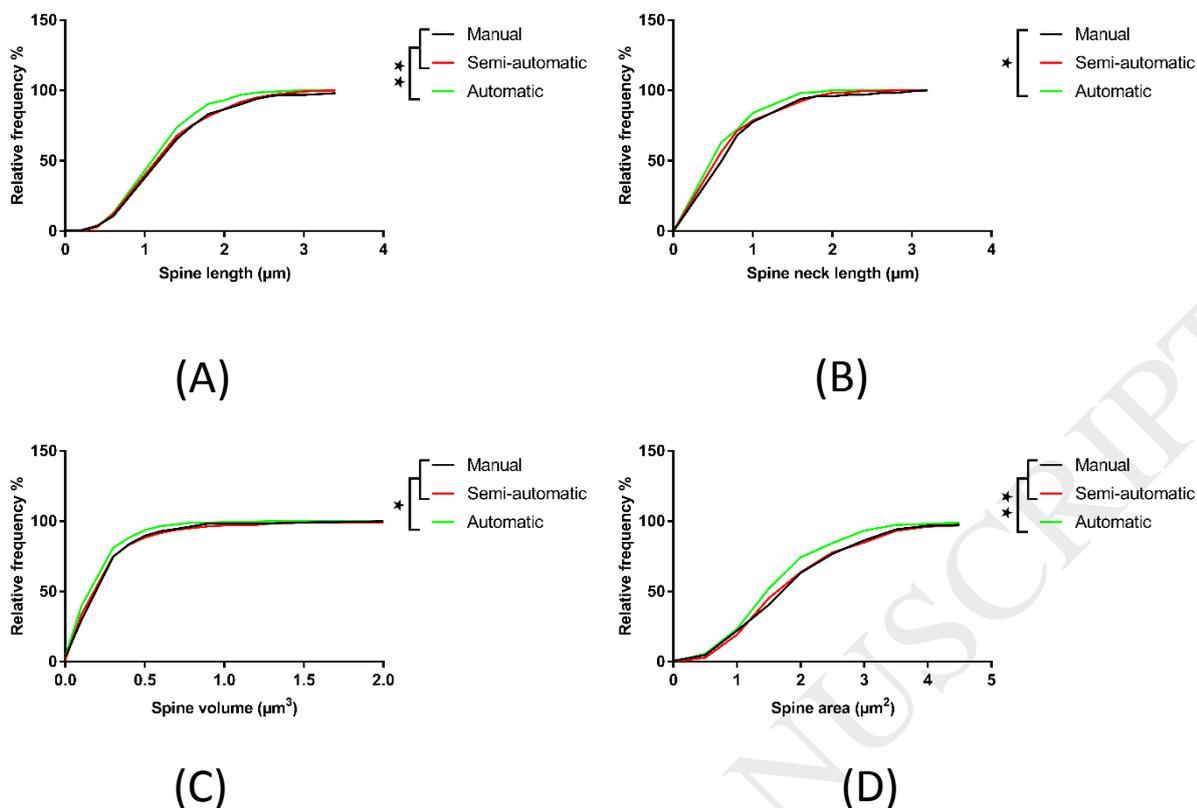


Figure 3: Spines from 42 dendritic branches of six pyramidal neurons are analyzed. Four spine geometry parameters: (A) spine length, (B) spine neck length, (C) spine volume, and (D) spine area are investigated after 3D reconstruction with manual, semi-automatic and automatic methods. Data are analyzed using Kolmogorov-Smirnov test. Results are considered significant when $p < 0.05$. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

Results show that the relative distribution of mushroom and thin spines from the manual method, is significantly different from the relative distribution of the corresponding spine classes from the automatic method (Bonferroni test, mushroom: $p = 0.028$, thin: $p = 0.019$), Figure 4A. Similarly, comparison of the relative distribution of mushroom spines between the semi-automatic and the automatic methods also shows a significant difference (Bonferroni test, $p = 0.032$), Figure 4A. However, no significant difference has been detected for the relative distribution of stubby spines among the three methods. Furthermore, comparison of the relative distribution of the three spine classes between the semi-automatic method and the manual method shows no significant difference, Figure 4A.

III.III. Time consumption

Comparing the time consumption during spine reconstruction showed important differences between the three methods, one-way ANOVA ($Df = 41$, $F = 23.2$, $p < 0.001$). Reconstruction of spines with the semi-automatic method consumed on average (16.2 ± 0.9) minutes /per branch, comparing with (22.5 ± 1.6)

minutes/per branch for the manual method, (Tukey's post-hoc test, $p = 0.00092$), and (12.5 ± 1.6) minutes/per branch for the automatic method, (Tukey's post-hoc test, $p = 0.00012$), Figure 4B.

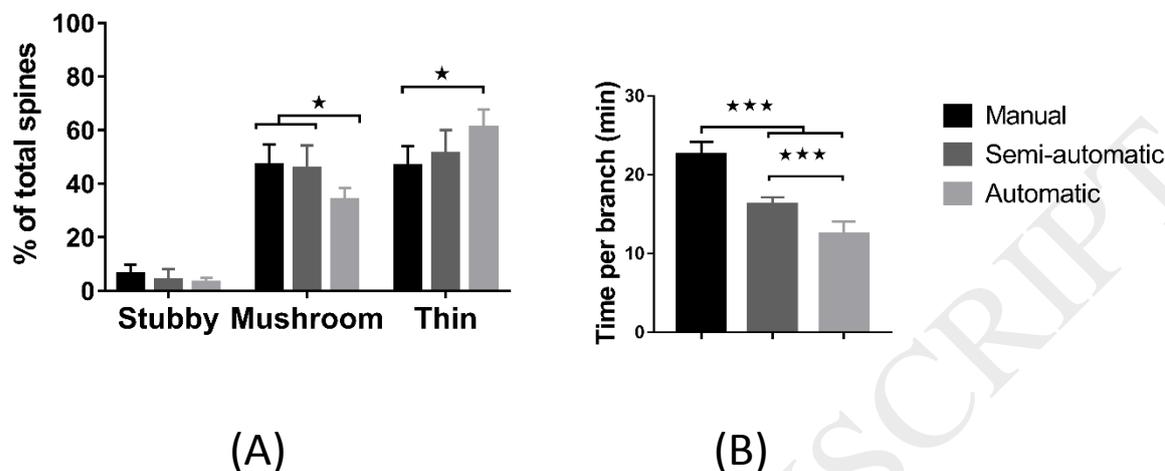


Figure 4: (A) 3D reconstructed spines from 42 dendritic branches are classified with Imaris (XTension) into three classes: stubby, mushroom and thin. Percentage of each spine class is compared between the three reconstruction methods using McNemar test.

(B) Time used to reconstruct spines, represented as average number of minutes per branch is compared between the automatic, the semi-automatic, and the manual methods using one-way ANOVA.

Data are represented as Mean \pm SD. Results are considered significant when $p < 0.05$. (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

IV. DISCUSSION

This study investigates the validity of the semi-automatic 3D reconstruction for measuring spine geometry. Reconstructed spines belong to Golgi stained pyramidal neurons. Our study, in line with the literature, demonstrates that the semi-automatic reconstruction is less time-consuming than the manual reconstruction, and it shows better accuracy than the automatic reconstruction (Myatt *et al.*, 2012). Studies that have previously investigated the validity of the semi-automatic reconstruction were limited to quantification of spine number (Orlowski and Bjarkam, 2012), and detection of neurites (Meijering *et al.*, 2004; Myatt *et al.*, 2012). There is a lack of knowledge regarding the validity of semi-automatic reconstruction for the purpose of studying the complex spine morphology. Quantification of spine geometry is a valuable method to understand the role of spine shape in information processing at the synaptic level (Nimchinsky *et al.*, 2002). Both spine length and spine neck length are known to play a critical role in the interaction between the spine head and the parent dendrite by controlling the Ca^{2+} traffic between the two sides (Korkotian and Segal, 2001; Noguchi J, 2005). Furthermore, area and size of spines

are suggested to be proportional to both the number of post synaptic receptors and pre-synaptically docked vesicles (Schikorski and Stevens, 1997).

A method that can balance between the accuracy of manual reconstruction and the objectivity of automatic reconstruction will be valuable for studying spine geometry. Studying spine morphology using electron microscopy has previously shown that the majority of spine parameters are within the submicron range (Arellano *et al.*, 2007). Therefore, the quality of spine images is critical for a reliable reconstruction of the spines. During image acquisition, several factors should be optimized in order to minimize the spherical aberration that degrades the spatial resolution. The refractive indices of the immersion oil and the mounting media should be very similar. In addition to that, the working distance and the numerical aperture of the objective lens are among the important factors that can significantly affect the image quality.

The investigated spine features in this study include spine area, spine volume, spine neck length and spine length. Our results show that the semi-automatic method generates measurements, that for the majority of spine features are comparable with the manual method. This has also been confirmed by the consistency between these two methods with regards to the relative distribution of stubby, mushroom and thin spines. In contrast, the automatic reconstruction method has resulted in significant differences for both the spine geometry features and the relative distribution of spine classes, as compared to both the manual method and the semi-automatic method. These results have been generated by implementing Imaris 7.7.2 software package to reconstruct the spines manually, semi-automatically, and automatically. Imaris employs MATLAB-dependent extension (Xtension) in order to classify spines after reconstruction.

It is of high importance to use the same software package for the purpose of comparing different reconstruction methods (Myatt *et al.*, 2012; Orłowski and Bjarkam, 2012). Implementing different packages may affect the validity of comparison results, especially if these packages assign different spine tracing algorithms. Furthermore, some of these software packages are limited to 2 dimensions (Meijering *et al.*, 2004), which is not suitable for studying spine morphology in 3D. For example, an Image J/Fiji plugin (Tarnok *et al.*, 2015) and 2dSpAn method (Basu *et al.*, 2016) have been developed to segment and quantify spine morphology. However, both packages are limited to 2D images.

Recently, MATLAB-based software package to analyze spines was released (Smirnov *et al.*, 2018). The package incorporates machine learning techniques to automatically identify spines, along with semi-automatic tool for spine labeling. The software shows high accuracy with regards to spine detection. However, the validity of this software for measuring spine geometry is still not known.

Until a few years ago, the majority of studies on neuronal morphology were dependent on the manual method, while few studies have employed the semi-automatic method (Donohue and Ascoli, 2011). This could be explained by the fact that not many studies have tried to prove the validity of the semi-automatic reconstruction method to study neuron morphology. However, there are also concerns regarding the reproducibility of the methods that require a manual input (Son *et al.*, 2010). In this respect, the automatic reconstruction method has been suggested as a tool that avoids the manual input, and therefore results in a more robust reconstruction (Risher *et al.*, 2014). However, the amount of false negative and false positive spines that are commonly generated during the automatic reconstruction is considered a serious drawback of this method. In our study, we have only included spines that have been successfully detected by all the three reconstruction methods. However, results from the automatic method are still significantly different from the other two methods for the majority of the spine investigated features. Different levels of manual input during the reconstruction of spines may, at least partially, explain the significant difference between the different methods. However, our study shows consistency in results from the semi-automatic method and the manual method. This could strengthen the validity of the semi-automatic method for quantifying spine geometry. The results also suggest that spine reconstruction with the semi-automatic method is significantly less time-consuming, compared with the manual method. As expected, the automatic reconstruction of spines is still the most time economic, as compared with both the manual and the semi-automatic reconstruction. Noteworthy, unlike the semi-automatic reconstruction, the automatic reconstruction of spines requires in most cases a post-processing step in order to gain better accuracy, and this step can be time-consuming (Myatt *et al.*, 2012).

In conclusion, our study shows that the semi-automatic method is an efficient tool for quantifying spine geometry with overall comparable results with the manual method. On the other hand, the automatic method is still the most time economic, but the results from this method are questionable, as they are significantly different both the semi-automatic and the manual methods.

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AUTHOR CONTRIBUTIONS

AA designed the study together with JN. AA collected the spine data and wrote the first draft of the manuscript. HC helped AA performing the statistical analysis. CS and JN helped editing the paper. All authors have approved the final article and they declare no conflicts of interest.

Declarations of interest: none

Ethical statement

I have read and have abided by the statement of ethical standards for manuscripts submitted to the Journal of Chemical Neuroanatomy. The experiment in this study was approved by the Danish Animal Experiments Inspectorate. All experimental procedures in this study were designed to be compatible with the guidelines EU Directive 2010/63/EU for animal experiment.

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