STAR Protocols

Protocol

3D Quantification of Vascular-Like Structures in z Stack Confocal Images

Capturing 3D data for accurate quantification

A: Raw z-stack image
B: Deconvolution
C: Binarization
D: 3D reconstruction
E-F: Removal of small particles
G: Skeletonization
Output: Vessel Parameters

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HIGHLIGHTS

The 3D analysis of z stack confocal images is critical to obtain accurate data

Detailed step-by-step protocol for quantitative analysis of vascular-like structures

Tcl and Python scripts are included to facilitate automated image analysis

Optical slice microscopy is commonly used to characterize the morphometric features of 3D cellular cultures, such as *in vitro* vascularization. However, the quantitative analysis of those structures is often performed on a single 2D maximum intensity projection image, limiting the accuracy of data obtained from 3D cultures. Here, we present a protocol for the quantitative analysis of z stack images, utilizing Fiji, Amira, and WinFiber3D. This protocol facilitates the in-depth examination of vascular-like structures within 3D cell culture models.

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3D Quantification of Vascular-Like Structures in z Stack Confocal Images

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SUMMARY

Optical slice microscopy is commonly used to characterize the morphometric features of 3D cellular cultures, such as *in vitro* vascularization. However, the quantitative analysis of those structures is often performed on a single 2D maximum intensity projection image, limiting the accuracy of data obtained from 3D cultures. Here, we present a protocol for the quantitative analysis of z stack images, utilizing Fiji, Amira, and WinFiber3D. This protocol facilitates the in-depth examination of vascular-like structures within 3D cell culture models.
**For complete details on the use and execution of this protocol, please refer to Koch et al. (2020).**

BEFORE YOU BEGIN

Note: This protocol is an image analysis protocol. The presence of z stack confocal images is assumed. The presented steps were performed on a nd2 image sample data set with the following resolution: x/y resolution of 0.8123 px/μm; x/y size of 1,024 px or 1,260.6828 μm; x/y pixel size: 1.23 μm/px; total z-size of 452.5 μm with a step size of 2.5 μm per slice; z resolution of 0.4 px/μm; and voxel size x/y/z: 1.23 x 1.23 x 2.5 μm^3/voxel.

Other image data sets can be used and the relevant information input at various stages of the protocol. An example image and the output of the intermediate steps is provided so that the user can follow the steps (see Supplementary Material; available at https://data.mendeley.com/datasets/btrrwrmt7z/1).

To ensure correct quantification of structures, high quality 3D images need to be obtained. According to the Nyquist Theorem (Nyquist, 1928), the sample rate should be more than 2× of the size of the smallest features. Moreover, the numerical aperture of the objective determines the spatial
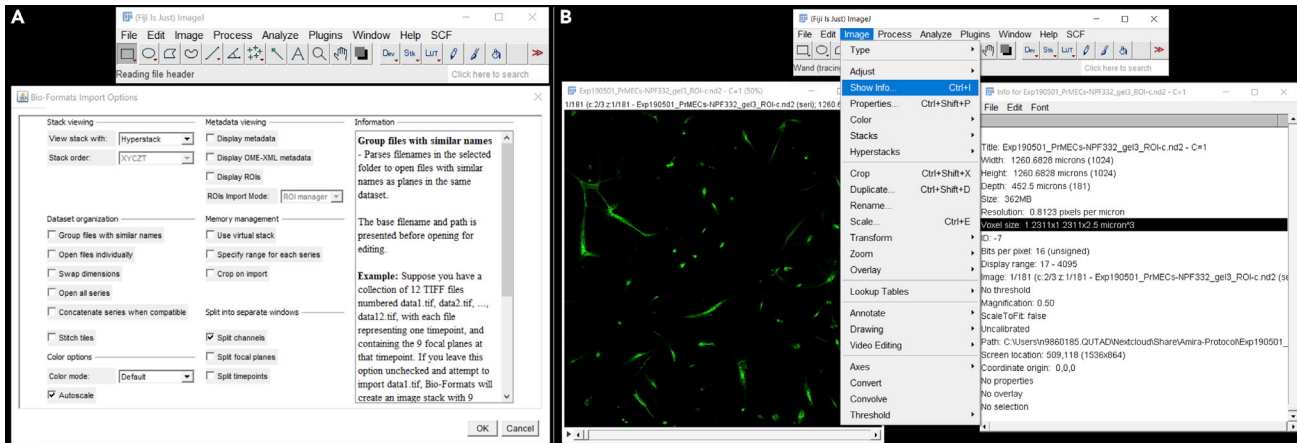
Figure 1. Convert Raw Image Data into Tiff Files Using Fiji
Screenshot of importing a nd2 file into Fiji (A) and viewing the voxel size and other metadata from the image file (B).

resolution with the smallest resolvable details being approximately (λ – emission wavelength). This protocol has been designed to minimize noise and artifacts in the raw image. However, an adequate signal-to-noise ratio is crucial for a close representation of the sample. While some noise in the lower slices can be accounted for (see Troubleshooting 1), a z stack with extensive background noise and/ or artifacts might not be suitable to use for quantification purposes. Further introductory information on image processing principles can be found in Soille (2003) and Russ and Neal (2016).

Ensure that imaging parameters used on the samples of interest are appropriately recorded. Information on how to access the meta data of the images used is described below.

> *Note:* We have provided here a step-by-step manual protocol for image analysis modified from Krishnan et al. (2008) and expanded upon. The protocol relies on Fiji, WinFiber3D, and Amira (see Key Resources Table). Amira is a proprietary software package (available from Thermo Fisher Scientific). In order to facilitate automated image analysis, Tcl and Python scripts were developed. See the Limitations section below. The timing information provided is based on 1 GB nd2-file processed on an Intel Core i7-6700 with 16.0 GB RAM machine running a Windows 10 (64-bit) operation system.

Convert Raw Image Data into Tiff Files

> ⏱ Timing: ~60 s per file with Fiji

Multi-dimensional microscopy images can be acquired on a variety of imaging platforms. The raw image data and the associated meta data are saved in proprietary file formats. These formats can be converted into an open standard, the Open Microscopy Environment (OME)-TIFF format, using the Bioformats software tool (Linkert et al., 2010). This tool is available for a variety of image analysis software, for example Fiji (Schindelin et al., 2012). To ensure the correct measurement of the structures, the voxel size needs to be provided in later steps to Amira. If the voxel size of the image was not noted down during image acquisition, it needs to be done in this step (Figure 1). Alternatively, additional software extensions can be purchased for Amira. The XBioFormats extension allows for loading a variety of vendor formats directly into Amira.

1. Open the microscopy image file in Fiji
    a. Either use "File" -> "Open" and choose the file;
    b. Or drag-and-drop the file into the Fiji window.

2. Read out the voxel size using "Image" -> "Show Info" or press Ctrl + I and note it down.
3. Use "File" -> "Save as" -> "Tiff…" to save the image as a tiff file.

⚠ CRITICAL: In the later steps, Amira does not read the metadata of tiff files. Therefore, the manual input of the correct voxel size is essential for the accurate output of the measurements. If the size of the image was not noted down during the acquisition, it can be read from the meta data in this step. In cases where the image size is unknown, the output of the last step must be read in pixels and not in metric units.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and Algorithms		
Fiji-ImageJ V1.52p	Schindelin et al., 2012	https://imagej.net/Fiji
Amira V2020.1	Stalling, Westerhoff and Hege, 2005	https://www.fei-software-center.com/forms/amira-trial/
WinFiber 3D V1.0	N/A	https://mrl.sci.utah.edu/software/winfiber3d/
Tcl and Python scripts for automated image analysis	This paper	https://github.com/theia-dev/acvq

## STEP-BY-STEP METHOD DETAILS
Correct Intensity Attenuation

🕐 Timing: ~30 s per file with Amira

In 3D microscopic images, the average intensity in lower slices (i.e., further away from the objective) can be decreased by light absorption in other slices. This intensity attenuation is corrected for using the CORRECT-Z-DROP module in Amira by fitting an exponential curve to the average intensities in each of the slices. The automatic mode uses with an automatic best fit of a and b, u specifies the slice number scaled between 0 and 1.

1. Start Amira and open the converted tiff file (Figure 2A). The Project view will open, and an ORTHO SLICE module will be attached automatically (Figure 2B).
   a. Either use the "Open Data" Button;
   b. or click on "File" -> "Open Data…" and select the image.

   Note: Here you enter the voxel size as obtained from the image metadata (Figure 1).

2. Attach the CORRECT-Z-DROP module by right-clicking on the input data (tiff file) (Figure 2C).
   a. Either using the search box;
   b. or click on "Image Processing" -> "Grayscale Transforms" -> "Correct Z Drop" and click "Create."
3. Click on the CORRECT-Z-DROP module to open the Preference view and adjust the settings (Figure 2D):
   a. The Automatic mode is ticked by default. This can be changed to a user-defined expression if appropriate.
4. Click on "Apply" to use the module (Figure 2E).
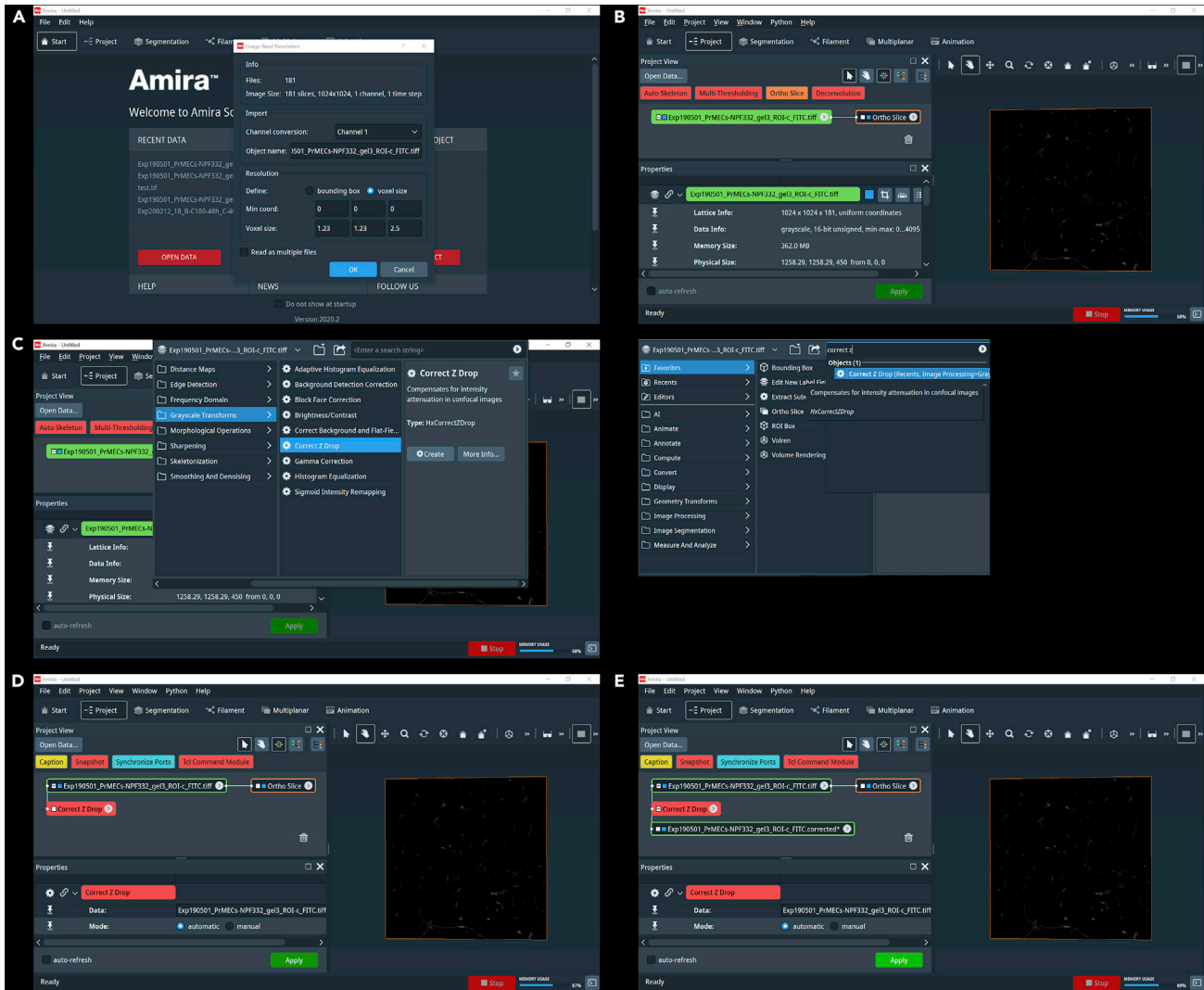
Figure 2. Open the Image File in Amira and Correct the Intensity Attenuation

Import the tiff file into Amira and provide the voxel size (A). The Project view opens automatically, and an ORTHO SLICE module is attached (B). Attach the CORRECT Z DROP module by right-clicking on the input data and either use the menu (left) or the search bar (right) (C). Adjust the settings in the properties window (D; lower left field). A new [filename].corrected object is created (E).

Noise Reduction and Smoothing

⏱ Timing: ~70 s per file with Amira

Before applying a threshold to the image to detect the edges of the features, the noise is reduced, and potential artifacts are removed. A median filter is applied to remove the "salt and pepper" noise from the image. This is followed by a Gaussian blur to smooth the boundaries of the features.

5. Open the Console in Amira.
    a. Either by clicking "Window" -> "Consoles";
    b. or by clicking on the console button on the lower right corner of the Amira window;
    c. or with the shortcut *Ctrl + Alt + A.*
6. Type: create "HxImageFilters" and press the enter key.
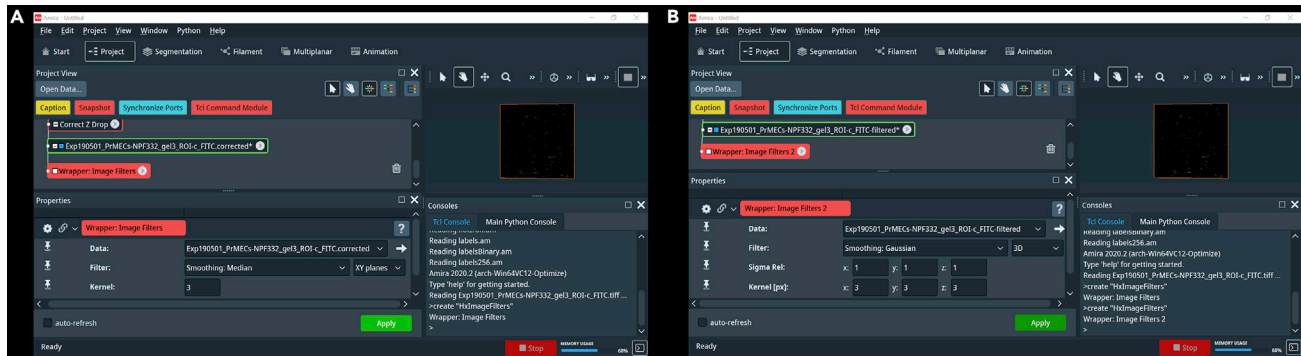
Figure 3. Noise Reduction and Smoothing in Amira

Use the console to create the IMAGE FILTERS module (A, lower right panel), select a median filter and adjust the settings (A, lower left panel). Repeat the steps and select a Gaussian smoothing (B).

    a. A module called WRAPPER: IMAGE FILTERS will be created in the Project view.

7. Click on the WRAPPER: IMAGE FILTERS module to open the Preference view and adjust the settings (Figure 3A):

    a. Select the [filename].corrected object as the Data Source.

    b. Filter: Smoothing: Median; 3D.

    c. The Kernel size determines the size of the pixel mask, meaning the area to be sampled when calculating the median. Here, a kernel size of 3, equating to a 3 × 3 × 3 pixel mask in 3D is applied.

8. Click on "Apply" and the filtered object will be created in the Project View.

9. Repeat step 6 to create a second WRAPPER: IMAGE FILTERS module for the Gaussian blur.

10. Click on the WRAPPER: IMAGE FILTERS 2 module to open the Preference view and adjust the settings (Figure 3B):

    a. Select the [filename]-filtered object as the Data Source.

    b. Filter: Smoothing: Gaussian; 3D.

    c. A Kernel size of 3 × 3 × 3 is applied for next neighbor detection. The "Sigma Rel" field allows the adjustment of the width of the Gauss function relative to the kernel size. Set the sigma to 1 in x, y, and z and observe the outcome to ensure all key objects are visible without artifacts. Adjust if necessary.

Note: There are two Consoles in Amira, for Tcl and for Python commands. We used the Tcl Console.

⚠ CRITICAL: For both filters, the kernel size is chosen to cover immediate neighbors. The relative sigma might be adjusted for images with varying noise levels. The outcome can be visualized in Amira by attaching an ORTHO SLICE module: "Display" -> "Ortho Slice" or by attaching an IMAGE ORTHO PROJECTIONS module: "Display" -> "Image Ortho Projections" to view a 2D projection of the image stack.

Deconvolution

⏱ Timing: ~180 s per file with Amira

Deconvolution removes the out-of-focus light present in 3D images. The light diffraction can be characterized by a point spread function (PSF) which is utilized to correct for the distortions and reverse the effects of the out-of-focus light. The PSF can be generated for an experimental set up using a fluorescent bead or created theoretically using known algorithms. We utilize the Amira
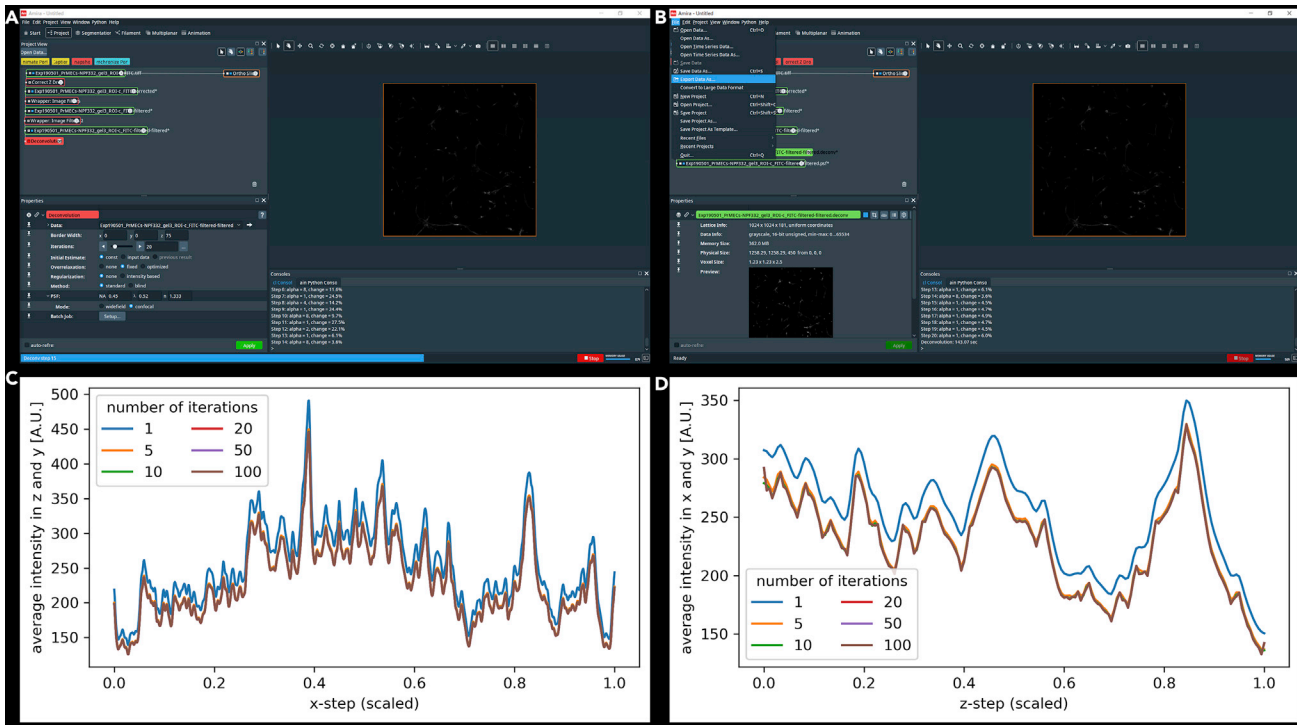
Figure 4. Deconvolution Using Amira

Attach the DECONVOLUTION module and adjust the settings in the Properties view (A). After applying the deconvolution algorithm, export the [filename].deconv file as a tiff file (B). The number of iterations effects the outcome of the deconvolution on the y/z axis (C) and the x/y axis (D).

DECONVOLUTION module to compute the PSF and apply a non-blind iterative maximum-likelihood deconvolution algorithm to the data.

11. Create the DECONVOLUTION module by right-clicking on the filtered-filtered object.
    a. Either using the search box;
    b. or "Image Processing" -> "Frequency Domain" -> "Deconvolution" and click "Create."
12. Click on the DECONVOLUTION module to open the Preference view and adjust the settings (Figure 4A):
    a. The first set of settings determines the parameters for the deconvolution algorithm. To reduce boundary artifacts at the top and bottom (z plane), we chose a border width of 0 in x, 0 in y, and 75 in z with 20 iterations. A constant initial estimate, fixed overrelaxation and no intensity-based regularization and a standard (non-blind) deconvolution method will work for most images. Further information on those settings can be found in the Amira help.
    b. The parameters for the point spread function are derived from the numerical aperture of the objective (NA, 0.45), the wavelength of the transmitted light (π, 0.52; an Alexa-488 fluorophore was used in the sample data) and the refractive index of the specimen (n, 1.333; the hydrogel has a similar refractive index as water). The mode was set to confocal. Adjust the settings to be relevant for your image. Alternatively, use the EXTRACT POINT SPREAD FUNCTION to obtain a point spread function from an imaged bead.
13. Two new objects are created, the point spread function ([filename].psf) and the deconvoluted image ([filename].deconv).
14. Export the deconvoluted image by clicking on the [filename].deconv object in the Project view and choose "File" -> "Export Data As." Change the file type to "3D Tiff (*.tif)" (Figure 4B).
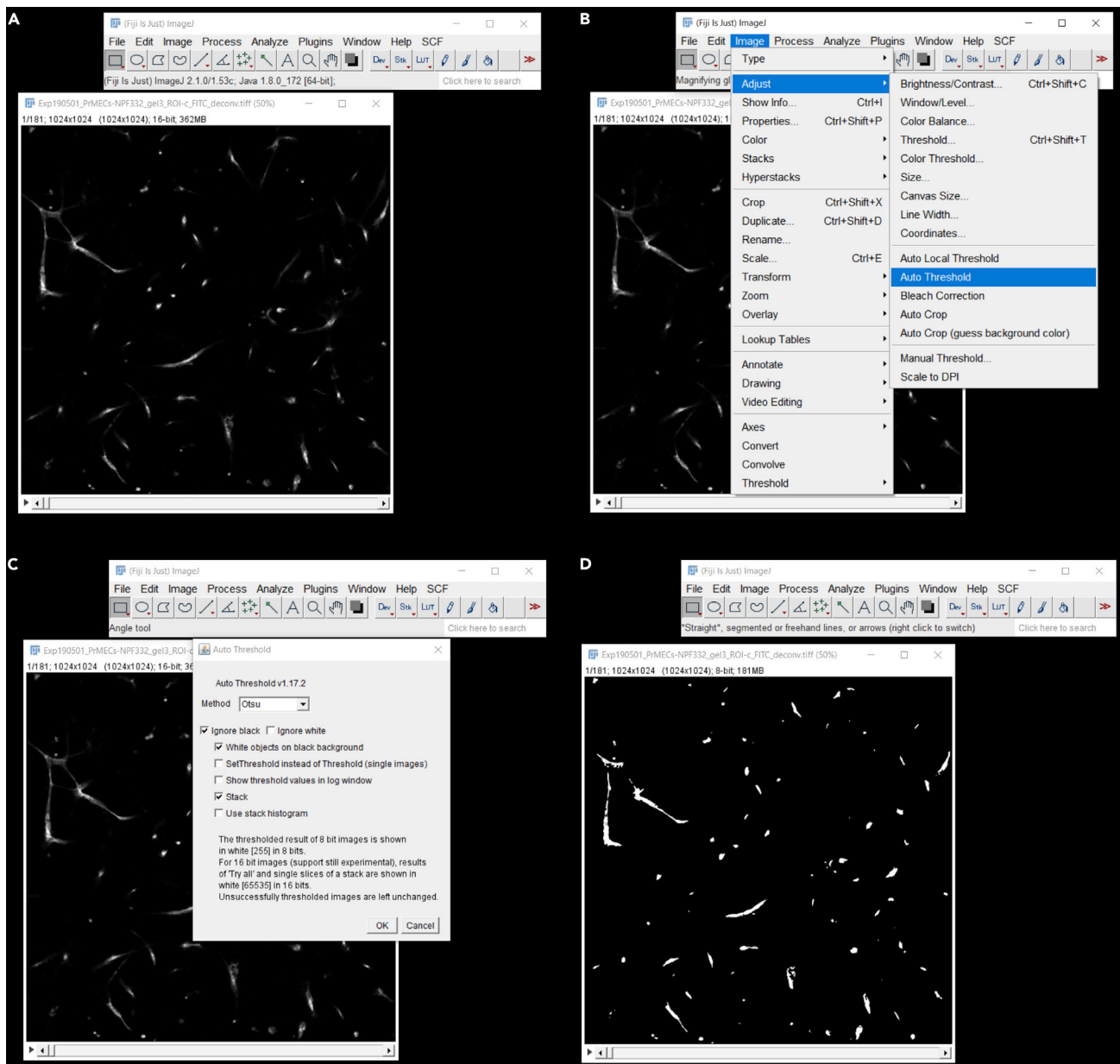
Figure 5. Binarization of the Image in Fiji
Open the deconvoluted images (A) and open the Auto Threshold module (B). Set the properties in the settings window (C) and apply the module. A binarized (black and white) image is the result (D).

> *Note:* The border width setting reduces potential deconvolution artifacts at the boundaries of the image. For our sample data set, we observed artifacts in z but not in x, and y. For performance reasons, it is advisable to choose the border width such that the sum of the size of the input data set and the border width value results in a power of two. For the example image, the border width in x, y was chosen to be 0 as 0 + 1024 = 1024 equals to 2^{10}, and in z 75 + 181 = 265 equals to 2^{8}.

The number of iterations determines the output and is closely linked to the computing time. For our sample data the changes between 5 and 100 iterations were minor (Figures 4C and 4D). We selected 20 as a good compromise for improvement versus computing time.
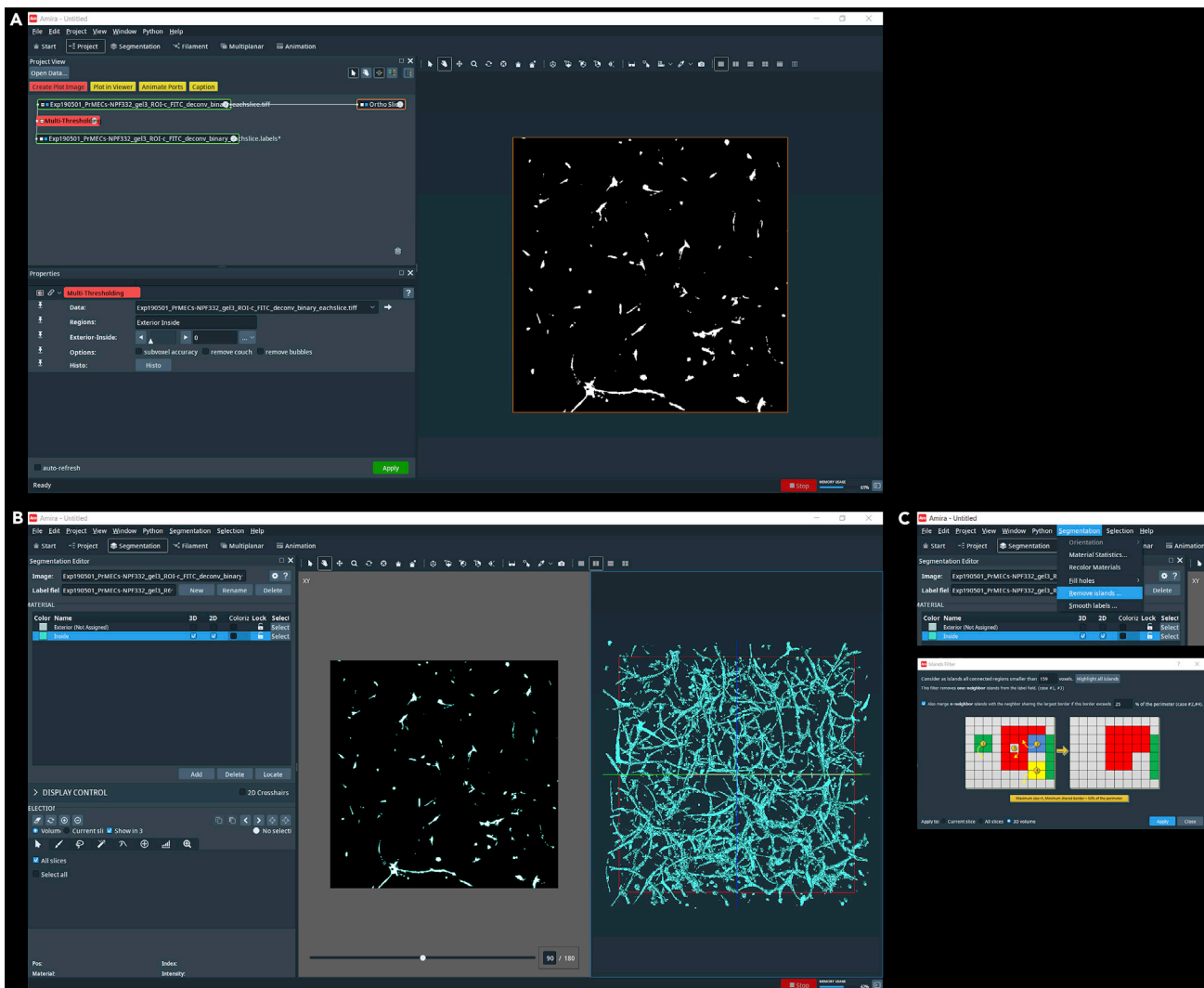
**Figure 6. Labeling and Removal of Small Fragments in Amira**

The MULTI-THRESHOLDING module is used to label the black (0) pixels as exterior (A). A 3D model can be rendered in the Segmentation View (B). Small particles are removed using the REMOVE ISLANDS function in Amira (C).

Binarization

⏱ Timing: ~30 s per file with Fiji

The image is binarized in Fiji using the Otsu method. The "Stack" option needs to be ticked in order to process all slices of one stack.

15. Open the deconvoluted tiff image in Fiji (Figure 5A).
    a. Either use "File" -> "Open" and select the file;
    b. or drag-and-drop the file into the Fiji window.
16. Press "Image" -> "Adjust" -> "Auto Threshold" (Figure 5B).
17. Select "Otsu" as the thresholding method and tick the following options: "Ignore black," "White objects on black background" and "Stack" (Figures 5C and 5D).
18. Save the binarized image "File" -> "Save as" -> "Tiff…."
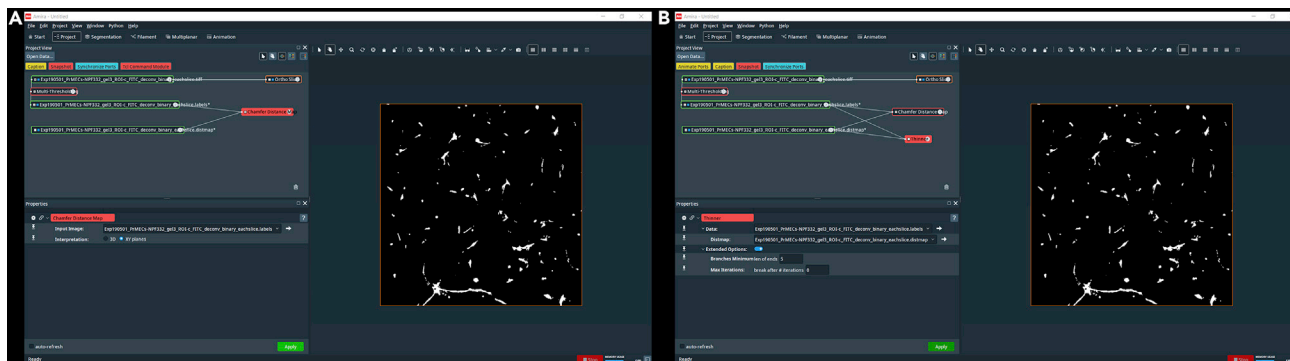
Figure 7. Distance Mapping and Thinning in Amira
Use a CHAMFER DISTANCE MAP module (A) and a THINNER module (B) to skeletonize the image.

Note: In images with a large intensity attenuation, the slices furthest away from the objective might be faint, even after intensity correction. That can lead to noise during binarization. See Troubleshooting 1. Use "Image" -> "Stacks" -> "Z Project" to visualize the outcome of the thresholding on a 2D projection. The thresholding method might be adjusted for various images. As an alternative to Fiji, the XImagePAQ extension for Amira includes an Auto Thresholding module which might be used instead.

Labeling and Removal of Small Fragments

⏱ Timing: ~45 s per file with Amira

Back in Amira, the multi-thresholding module is used to label the binary image for further processing in Amira. The exterior region is set to 0, so that all voxels with "1" will be labeled as interior.

To remove small fragments which are not of interest, the REMOVE ISLAND function is applied.

19. Open the binarized image in Amira (see step 1 for details).
20. Attach the MULTI-THRESHOLDING module by right-clicking on the binarized input object.
    a. Either using the search box;
    b. or "Image Segmentation" -> "Multi-Thresholding" and click "Create."
21. Click on the MULTI-THRESHOLDING module to open the Preference view and adjust the settings (Figure 6A):
    a. Set the Exterior-Inside value to 0. Thereby all pixels with value 0 (black) are labeled as exterior. Click "Apply" and a [filename].labels object is created.
22. Switch to the Segmentation view (Figure 6B).
    a. Select the binarized input image as Image source and the [filename].labels object as Label field.
    b. Switch on the two-viewer panel.
    c. Check the box at Inside 3D to view a 3D model of the data.
23. "Segmentation" -> "Remove Islands" (Figure 6C).
    a. Select a size filter (in voxel) in the dialog box. A pictogram explains the neighbor merging function. The "Highlight all islands" button can be used to preview the islands in the Segmentation viewer. Click "Apply" and close the dialog box when finished. For our sample image, we used 159 voxel (600 $\mu m3$; 600 μm^3/1.23 ×.1.23 × 2.5 μm^3/voxel) as a cut-off and merging if the border exceeded 25% of the perimeter, applied to 3D.
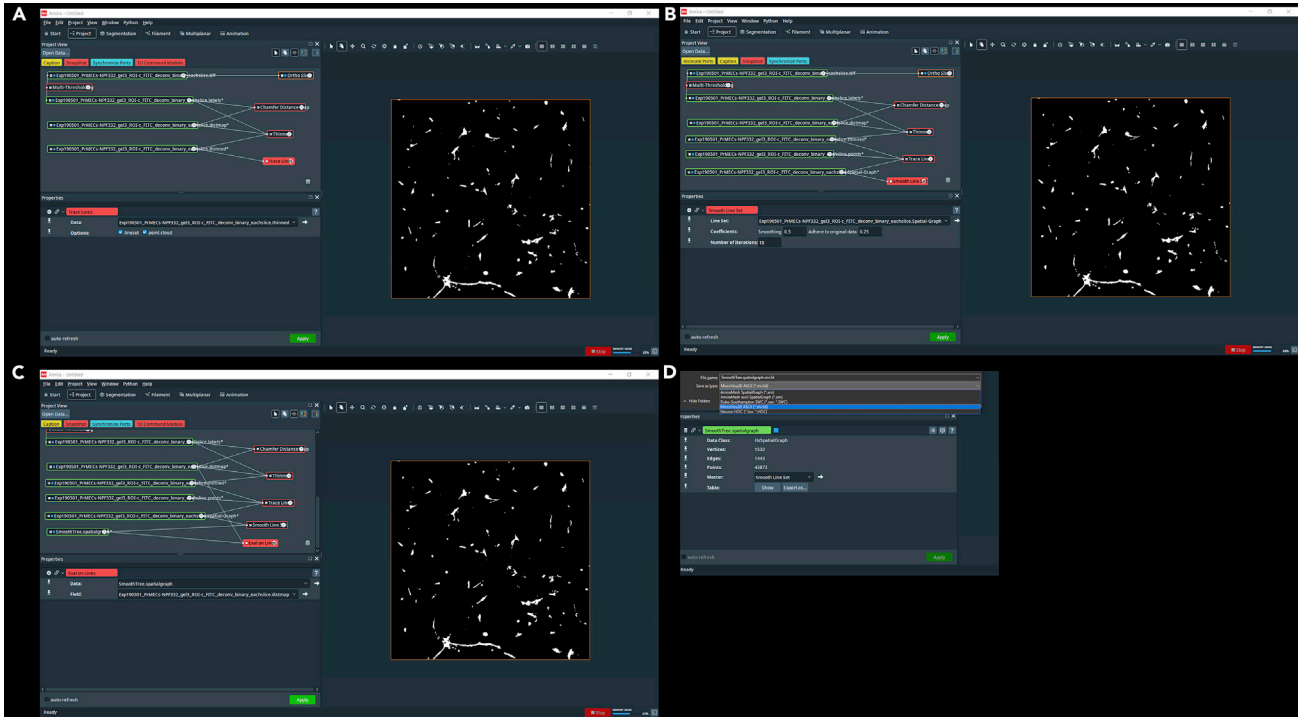
Figure 8. Convert to Spatial Graph and Compute Thickness in Amira

Attach a TRACE LINES module to convert the thinned image into a Spatial Graph object (A) and use the SMOOTH LINE SET module (B). Calculate the thickness information with the EVAL ON LINES module (C) and export the spatial graph object as an .mv3d file (D).

> *Note:* Rendering the 3D model and highlighting islands requires a high-end graphics card and might not be feasible on all machines. Also, the manual removal of fragments in this view is possible.

The size of the "islands" has to be provided in voxel.

Distance Mapping and Thinning

> ⏱ Timing: ~20 s per file with Amira

The distance mapping step labels each pixel with the distances to its boundary pixels to determine the separation of points. The THINNER module takes the segmented objects and removes voxel by voxel until a skeleton of connected voxels remains.

24. Switch back to the Projects view and right click on the [filename].labels object and right click to attach a CHAMFER DISTANCE MAP module by:
    a. either using the search box;
    b. or "Image Processing" -> "Distance Maps" -> "Chamfer Distance Map" and click "Create."
25. Click on the CHAMFER DISTANCE MAP module to open the Preference view and adjust the settings (Figure 7A):
    a. Set the interpretation to XY planes.
26. Select the labels object and right click to attach a THINNER module by:
    a. either using the search box;
    b. or "Image Processing" -> "Skeletonization" -> "Thinner" and click "Create."
27. Click on the THINNER module to open the Preference view and adjust the settings (Figure 7B):
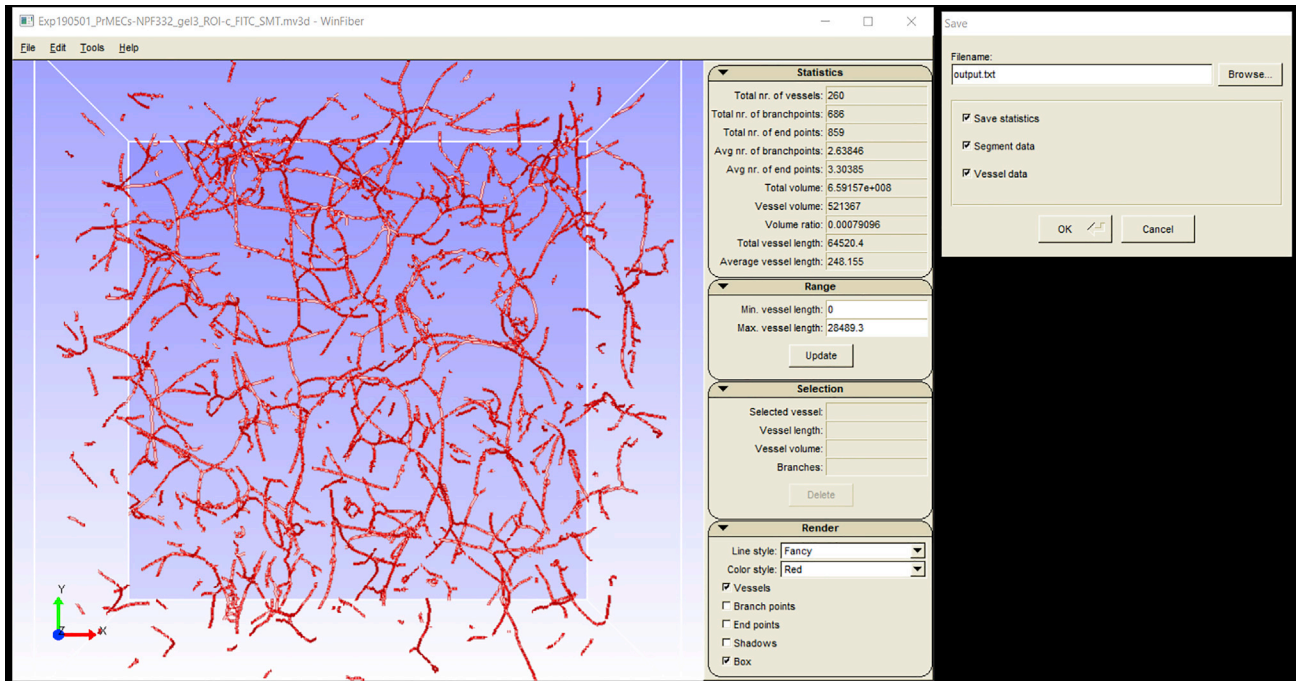
Figure 9. The WinFiber3D Workspace (Left Side) and the Save File Dialog (Right Side)

a. Select the [filename].labels object as data source and the [filename].distmap object as Distmap source.

b. The len of ends parameter (extended options) controls the length of unconnected ends (in voxel) and thereby control's the sensitivity to generate branch points. Select a size according to your data set and the structures of interest. For the sample data, we applied a size of 5 voxel.

28. Click on "Apply" and a [filename].thinned object is created.

Note: The CHAMFER DISTANCE MAP module can process the image slice by slice (Interpretation: XY planes) or as a whole in 3D (Interpretation: 3D) depending on the application. For our data set, we used the XY interpretation due to the higher resolution in the x/y plane.

Convert to Spatial Graph and Compute Thickness

⏱ Timing: ~30 s per file with Amira

The TRACE LINES module converts the thinned image into a Spatial Graph object in which the data is organized as a graph. Branch or endpoints are represented by the vertices of the graph (Nodes) which are connected by curved lines (Segments). This Spatial Graph is then smoothed using a SMOOTH LINE SET module. The EVAL ON LINES module connects to smoothed skeleton and the distance map to compute the thickness information of the skeleton.

29. Select the [filename].thinned object and right click to create a TRACE LINES module by:
    a. Either using the search box;
    b. or "Image Processing" -> "Skeletonization" -> "Trace Lines" and click "Create."
30. Click on the TRACE LINES module to open the Preference view and adjust the settings (Figure 8A):

```
output.txt
1  # STATISTICS
2  # ==========
3  # Range min           : 0
4  # Range max           : 28489.3
5  # Total vessels       : 260
6  # Total branch points : 686
7  # Total end points    : 859
8  # Avg branch points per vessel : 2.63846
9  # Avg end points per vessel : 3.30385
10 # Total volume of box  : 6.59157e+008
11 # Total vessel volume  : 521367
12 # Volume ratio         : 0.00079096
13 # Total vessel length  : 64520.4
14 # Average vessel length : 248.155
15 #
16 # SEGMENT DATA
17 # ID   angle(Pr(x))   angle(z)   angle(x)   length    avg. diam
18   0    63.435         74.7468    64.4398    29.5523   2.35741
19   1    36.6897        67.4396    42.2243    89.316    1.61636
20   2    56.7251        66.9832    59.6701    57.5651   4.5155
21   3    20.3764        63.8978    32.6675    81.1329   2.57929
22   4    12.9946        81.3379    15.5755    17.0011   1.30669
23   5    72.2554        66.9318    73.7156    74.2002   2.39173
24   6    35.2564        86.7245    35.3886    90.9144   2.21052
25   7    74.0541        90         74.0541    9.01025   1.23
26   8    85.2364        71.3462    85.4871    16.1718   1.53996
27   9    61.6992        62.6961    65.0847    186.688   2.91077
28   10   58.7363        60.8192    63.0564    98.7021   3.95235
29   11   56.31          80.4012    56.8431    57.6637   3.77929
30   12   13.2406        80.0975    16.4808    51.8007   2.89109
31   13   68.0782        58.1657    71.5075    142.884   3.14489
32   14   2.66299        81.9377    8.488      57.5032   3.23738
33   15   86.8202        88.3855    86.8214    182.963   2.37389
34   16   38.6598        86.3674    38.8035    82.3031   1.95276
35   17   47.2906        83.4463    47.6353    22.4296   1.25562
36   18   0              80.3866    9.6134     17.3475   2.35592
37   19   4.04448        87.6545    4.67438    126.104   2.48272
38   20   0              80.3866    9.6134     16.1033   2.08039
39   21   21.8014        52.9523    42.1791    36.5433   1.53016
40   22   61.8763        69.6516    63.7709    95.4632   3.24113
41   23   9.17355        87.8588    9.41804    208.562   2.16272
42   24   51.3402        75.1834    52.8486    52.0443   2.27069
```

```
output.txt
1493 1475  11.3097    90   11.3097   6.66328   1.27307
1494 1476  9.46224    90   9.46224   7.72708   1.23
1495 1477  29.7451    90   29.7451   10.1368   1.23
1496 1478  40.6014    90   40.6014   12.2037   1.4173
1497 1479  63.4349    90   63.4349   9.41157   1.29769
1498 1480  63.4349    90   63.4349   6.13482   1.29831
1499 1481  59.0361    90   59.0361   14.735    1.26726
1500 # SEGMENT END
1501 #
1502 # VESSEL DATA
1503 # ID   Length    Volume    branches
1504   0    80.5512   1057.77   3
1505   1    89.316    186.319   1
1506   2    28489.3   273175    655
1507   3    81.1329   554.152   1
1508   4    17.0011   23.1677   1
1509   5    90.9144   362.241   1
1510   6    9.01025   10.7062   1
1511   7    1133.53   11870.7   25
1512   8    186.688   1364.8    1
1513   9    142.884   1260      1
1514   10   325.651   2293.92   5
1515   11   22.4296   27.3142   1
1516   12   36.5433   70.5443   1
1517   13   208.562   823.618   1
1518   14   400.163   3638.38   3
1519   15   488.523   3834.72   11
1520   16   34.9835   41.5684   1
1521   17   22.7142   31.4557   1
1522   18   9.59371   13.2572   1
1523   19   20.4529   79.8538   1
1524   20   10.8397   21.7726   1
1525   21   240.64    2651.94   1
1526   22   578.925   5939.8    6
1527   23   237.279   1489.31   4
1528   24   13.9328   40.2016   1
1529   25   22.7927   66.6858   1
1530   26   143.276   1339.38   1
1531   27   16.1914   22.0809   1
1532   28   779.286   10213.2   13
1533   29   15.2251   23.0994   1
```

Figure 10. A Screenshot of the Expected Output Text File
The first lines present the overall statistics followed by the segment data (left side). Below the segment data, the vessel data can be found (right side).

    a. Tick "lineset" and "point cloud" to compute lines (vessels) and points (branch- and end-points).

31. Click on "Apply" and a [filename].Spatial-Graph object is created.

32. Select the [filename].Spatial-Graph object and right click to create a SMOOTH LINE SET module by:

    a. Either using the search box;

    b. or "Compute" -> "Smooth Line Set" and click "Create."

33. Click on the SMOOTH LINE SET module to open the Preference view and adjust the settings (Figure 8B):

    a. The smoothing coefficient controls the effect of adjacent points on the position of a point. Adhere to original data controls the effect of the initial position. For our sample data set we set a smoothing of 0.5 and adhere to data of 0.25.

34. Click on "Apply" and a "SmoothTree.Spatialgraph" object is created.

35. Select the "SmoothTree.Spatialgraph" object and right click to attach an EVAL ON LINES module by:

    a. Either using the search box;

    b. or "Compute" -> "Eval on Lines."

36. Click on the EVAL ON LINES module to open the Preference view and adjust the settings (Figure 8C):

    a. Data Source: SmoothTree.Spatialgraph.

    b. Field: [fielname].distmap object.

37. Click "Apply."
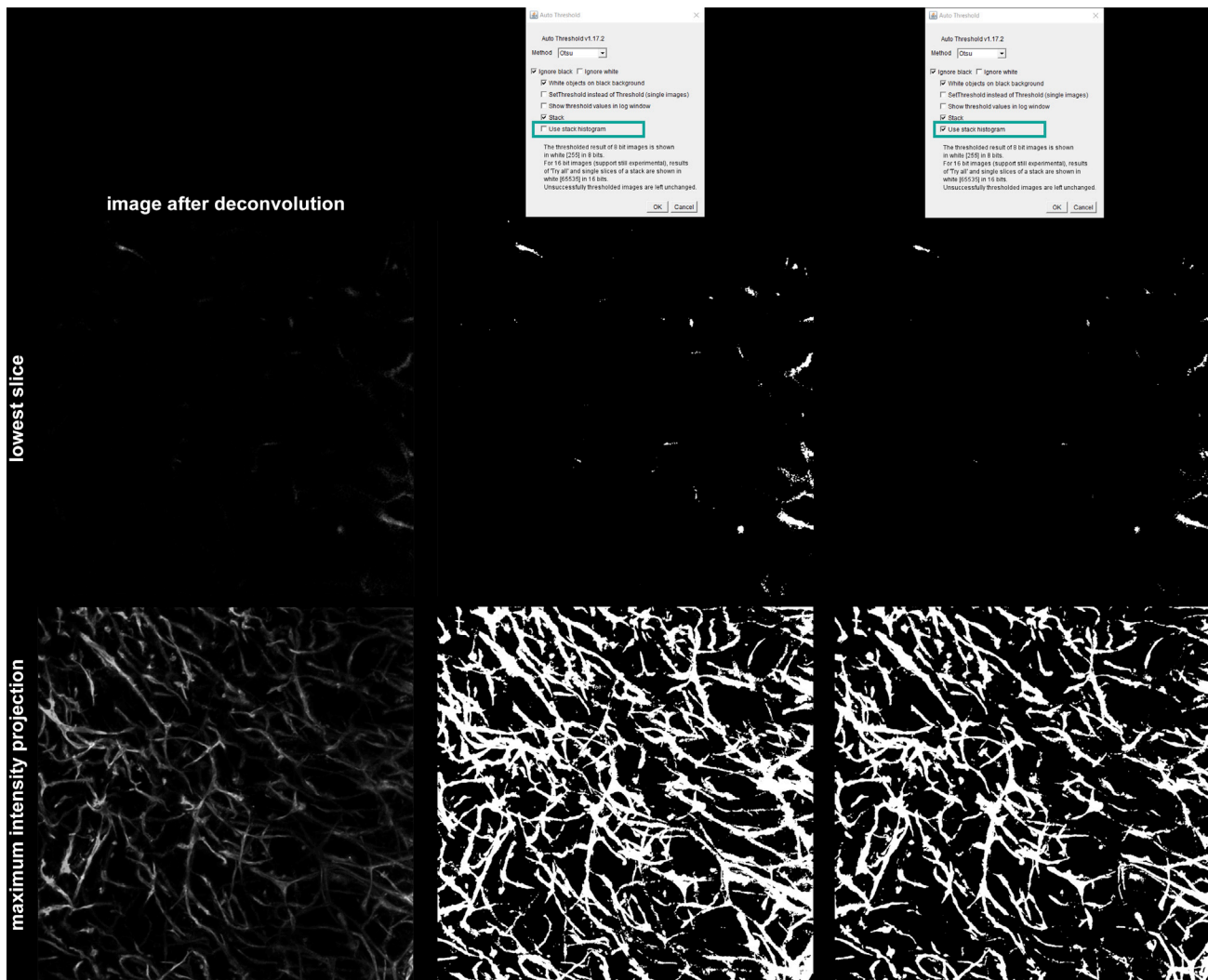
Figure 11. Troubleshooting the Binarization Step

The image after deconvolution is displayed in the left column. A few faint structures are present in the lowest slice. With the standard binarization method noise is introduced (middle column), so that the binary image appears fuzzy (middle column, bottom row). Using the histogram calculated for the complete z stack before thresholding (right column) solves the problem and generates a clear binary image (right column, bottom row).

38. Select the SmoothTree.Spatialgraph. Export the data "File" -> "Export Data as" and change the file type to microvisu3D ASCII (.mv3D) (Figure 8D).
    a. The mv3d file is a text file containing points specified by a x, y and z coordinate, and number (the ID of the segment this point belongs to).

    Optional: Go to the Filament View to view the output of the Spatial Graph object.

Measurements

⏲ Timing: ~30 s per file with WinFiber3D

WinFiber3D is used to visualize the spatial graph data and to export the measurements (Figure 9).

39. Open WinFiber3D and open the mv3D file by

  a. either "File" -> "Open";

  b. or *Ctrl + O.*

40. Export the measurements

  a. either "File" -> "Save";

  b. or *Ctrl* + S.

  c. The output file is saved as a tab delimited text file. However, the file extension (.txt) needs to be provided with the file name.

  d. Tick "Statistics" to save the measurements of the complete network.

  e. Tick "Segment data" to save the orientation data, length, and average diameter of each segment.

  f. Tick "Vessel data" to export the length, volume, and branch points information for each vessel.

Optional: Loops, segments which start and stop at the same point, can be removed with "Tools" -> "Delete Loops." The boxes on the right-hand side present the overall statistics as well as properties of a selected structure. Structures can be removed manually by selecting them with the mouse and click on "Delete". Various options for rendering the network are available below the selection box.

EXPECTED OUTCOMES

The output file is a text file containing measurements of the network (Figure 10). The following parameters are available for the network: total vessel number, total branch points, total end points, average branch points per vessel, average end points per vessel, total volume of box, total vessel volume, volume ratio, total vessel length, and average vessel length. Additionally, the orientation data, length, and average diameter can be determined for each segment. For each vessel, the length, volume, and number of branch points can be computed.

QUANTIFICATION AND STATISTICAL ANALYSIS

The .txt file can be imported in most of the commonly used data analysis software such as R, Python, or Excel. The user can select the parameters of interest for further comparison and statistical analysis.

LIMITATIONS

The image analysis protocol is based on manual handling, "point-and-click," and is therefore not feasible to use with large sample sizes. However, we have developed Tcl and Python scripts to facilitate the automated image analysis. https://github.com/theia-dev/acvq

TROUBLESHOOTING

Problem

If the signal intensity in lower slices (further away from the objective) decreases significantly, noise can arise during the binarization process (steps 15–18).

Potential Solution

By ticking the "Use stack histogram" option in Fiji, the histogram is calculated for the complete stack instead of for each slice. This reduces the noise in image with large intensity differences over the z axis. (Figure 11).

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Laura Bray (laura.bray@qut.edu.au).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

The developed Tcl and Python scripts to facilitate the automated image analysis are available at: https://github.com/theia-dev/acvq. The supplementary dataset is available at: https://data.mendeley.com/datasets/btrrwrmt7z/1

AUTHOR CONTRIBUTIONS

Conceptualization, C.W., E.M.D.-J.-P., and L.J.B.; Investigation, U.B., A.J., A.L., and J.B.; Writing – Original Draft, U.B., A.J., and L.J.B.; Writing – Review & Editing, U.B., A.J., A.L., J.B., C.W., E.M.D.-J.-P., and L.J.B.; Funding Acquisition, C.W., E.M.D.-J.-P., and L.J.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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