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Supplemental information

Rapid and fully automated blood vasculature analysis in 3D light-sheet image volumes of different organs

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Supplementary Materials

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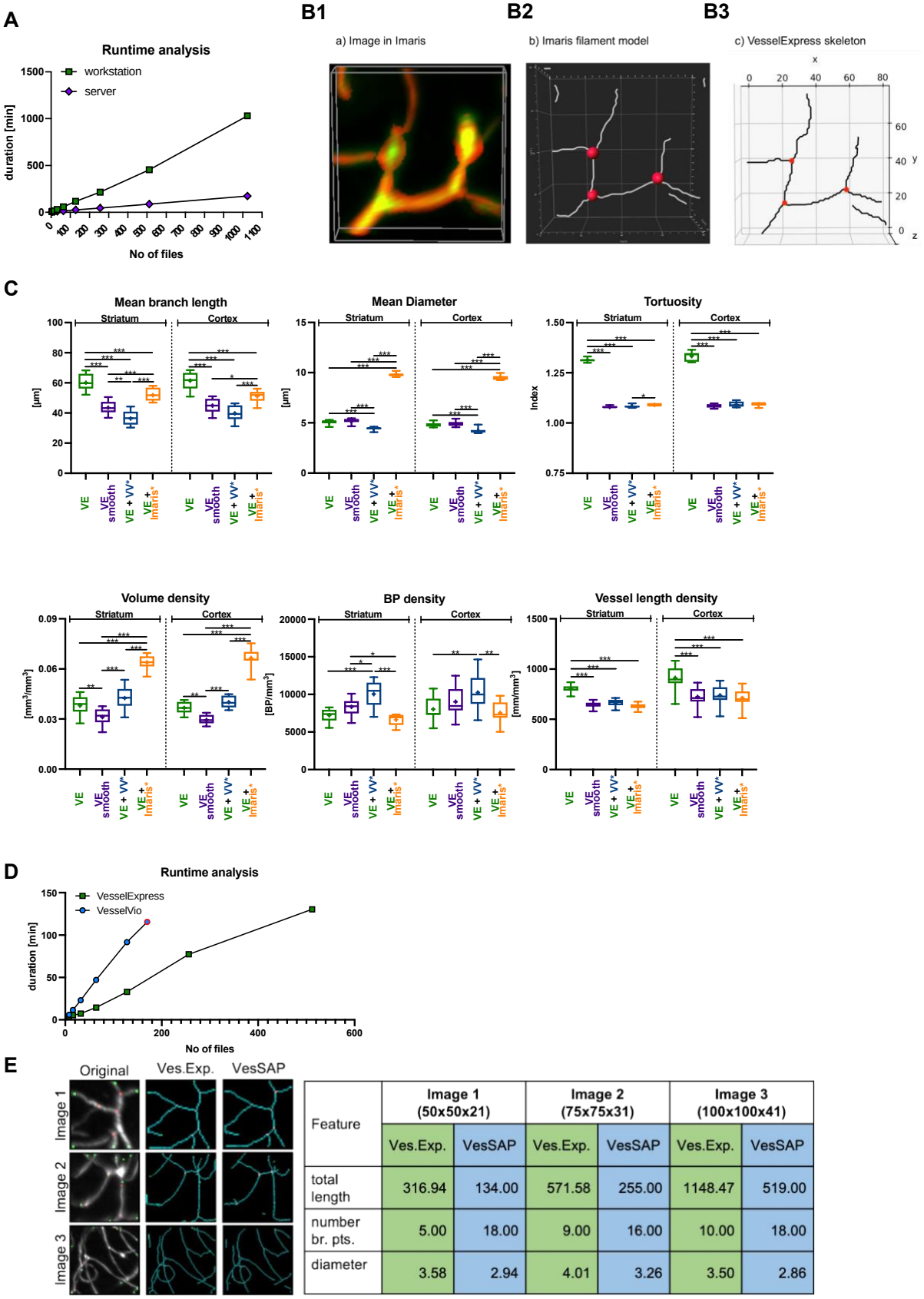
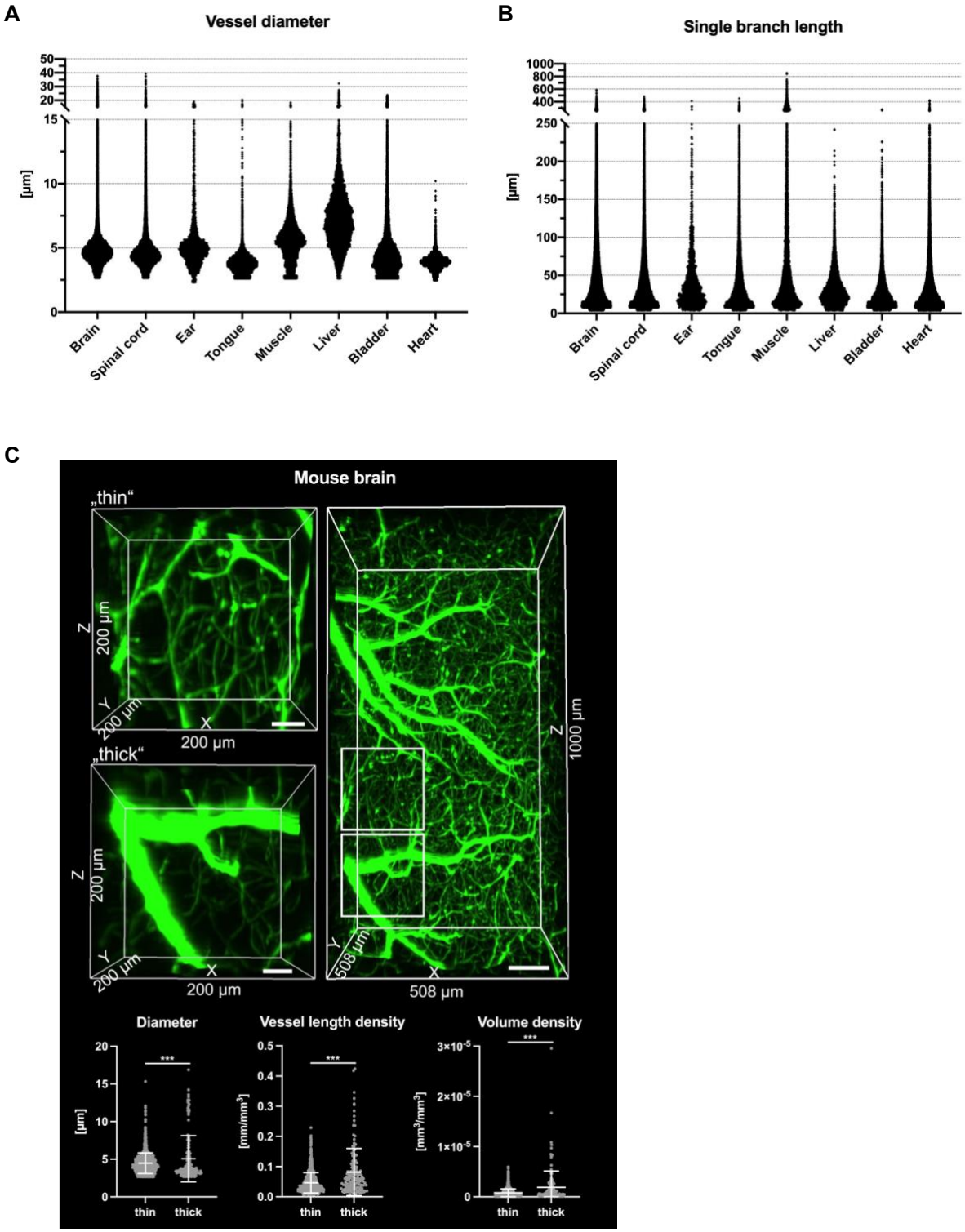
Supplemental Figure S1



Feature	Image 1 (50x50x21)		Image 2 (75x75x31)		Image 3 (100x100x41)	
	Ves.Exp.	VesSAP	Ves.Exp.	VesSAP	Ves.Exp.	VesSAP
total length	316.94	134.00	571.58	255.00	1148.47	519.00
number br. pts.	5.00	18.00	9.00	16.00	10.00	18.00
diameter	3.58	2.94	4.01	3.26	3.50	2.86

Supplemental Fig. S1: **Performance of VesselExpress and comparison with other analysis tools, Related to Figure 2** (A) Duration of VesselExpress analysis was performed using increasing number of ROIs with an image dimension of 508 x 508 x 1000 µm. Every data point represents the doubling of previous image numbers starting with four. Analysis was performed either on a server (Intel® Xeon ® CPU E7-8890 v4, 2.20GHz, 96 cores, 1.97 TB RAM or a workstation (Intel® Xeon® W-1255 CPU, 3.30 GHz, 10 cores, 512 GB RAM). (B) Branching points identified by VesselExpress were visually overlaid and compared with branching points identified by the Imaris based workflow. (B1) Cropped region of a 3D LSFM mouse brain image visualized in Imaris. (B2) Filament model obtained from the Imaris based workflow with branching points in red. (B3) Skeletonization result of VesselExpress with branching points in red. (C) Analysis of blood vessels from brains of 12 weeks old healthy male mice in the striatum and cortex using VesselExpress (VE), VesselVio (VV) and Imaris shows that smoothing of Vessels results in 17.4% ± 1.3% smaller Vessel length density in VesselVio compared to VesselExpress. Furthermore, VesselVio detects considerably more branching points (38.5% ± 9.6%), resulting in shorter mean branch lengths (39.8% ±3.4%). Data are box plots with medians (line)/ means (plus) ± interquartile ranges (IQRs) with minimum and maximum data as whiskers *$p<0.05$, **$p<0.01$, ***$p<0.001$ analyzed by two-way Anova using Tukey´s multiple comparison test. The switchable smoothing step in VesselExpress (VE smooth) leads to results comparable to Imaris regarding vessel length and branching point density. Imaris yields systematically larger vessel diameters and therefore a higher volume density. ***Neither Imaris nor VesselVio provide segmentation methods for vessel segmentation, so that the segmentation provided by VesselExpress needed to be used to make comparison possible at all.** (D) runtime analysis between VesselExpress (without segmentation step but with B-spline smoothing of order 3) and VesselVio (includes B-spline smoothing) shows that VesselExpress is substantially faster than VesselVio. It is important to mention that the VesselVio encountered severe stability problems when dealing with more than 170 files (indicated with a red circle). A direct comparison with VesSap was not possible because the pretrained deep learning model provided by the VesSap authors yielded biologically wrong segmentations that were not usable for further downstream analysis. (E) Branching points (red) and terminal points (green) were annotated in 3 selected ROIs with different size (left). The identified branching points by VesselExpress (middle) and VesSAP (right) for the 3 ROIs are highlighted in the images. The images of VesselExpress also show the identified terminal points (green). VesSAP extracts 3 features (total length, number of branching points and mean diameter) which were compared with VesselExpress' output in the table. Since the VesSAP segmentation was not applicable to our data, the VesSAP feature extraction was applied to segmentations obtained from VesselExpress.

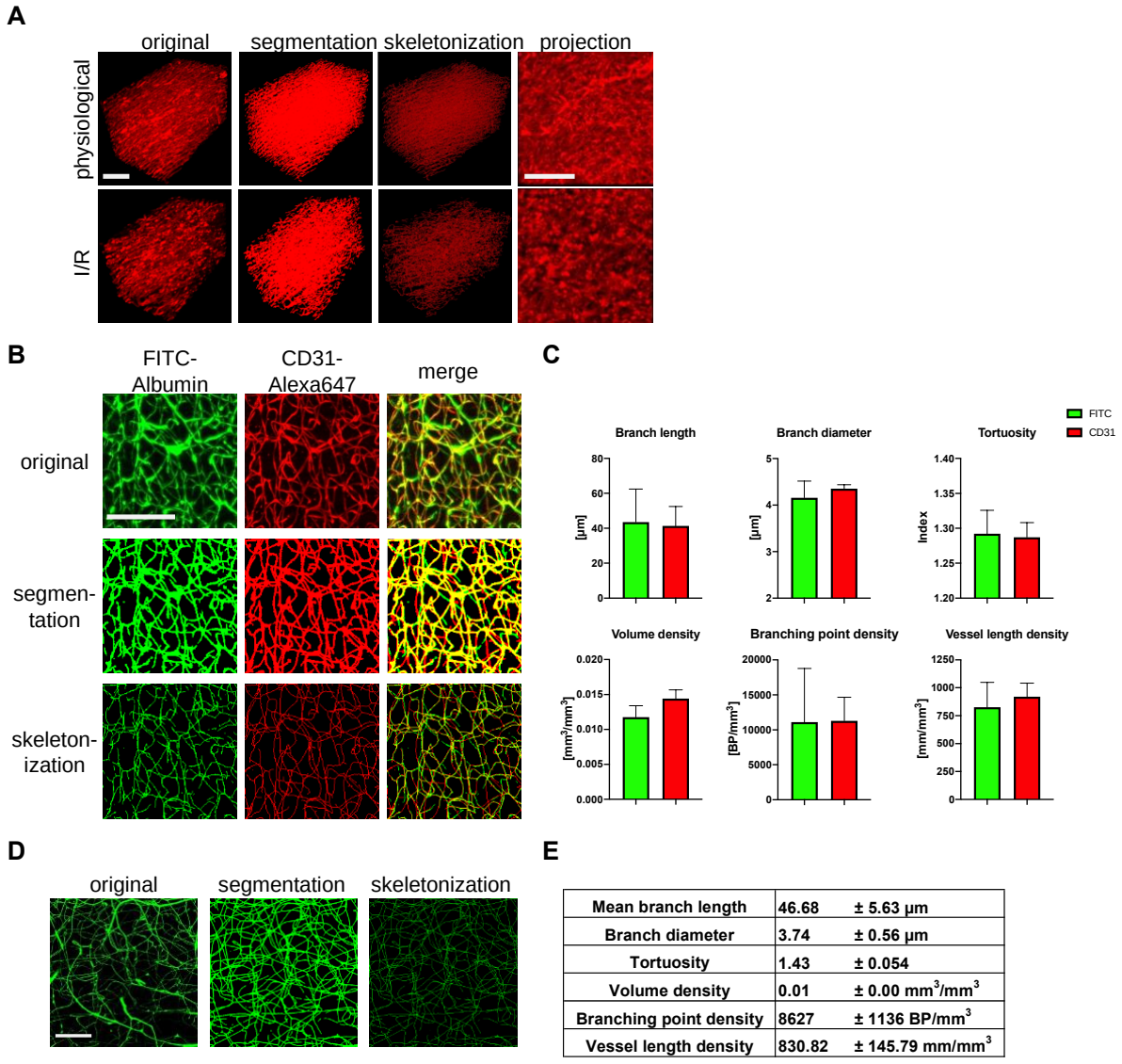
Supplemental Figure S2



Supplemental Fig. S2. Processing of a high variability of vessel diameters and lengths by VesselExpress. Related to Figure 4. (A and B) Dot plots representing single diameters (A) or vessel length (B) of all individual vessels in the respective organs. (C) VesselExpress performance was tested on heterogeneous vessel diameters. Vessel reconstruction in two regions of interest (RoIs) of mouse brain tissue. The first RoI (upper left) is clearly identified as containing thin vessels compared to the much thicker vessels in the second RoI (lower left). This unambiguous visual difference is reflected in the VesselExpress-based

quantification of vessel diameters, vessel length density, and volume density, all of which differ in a statistically highly significant manner.

Supplemental Figure S3



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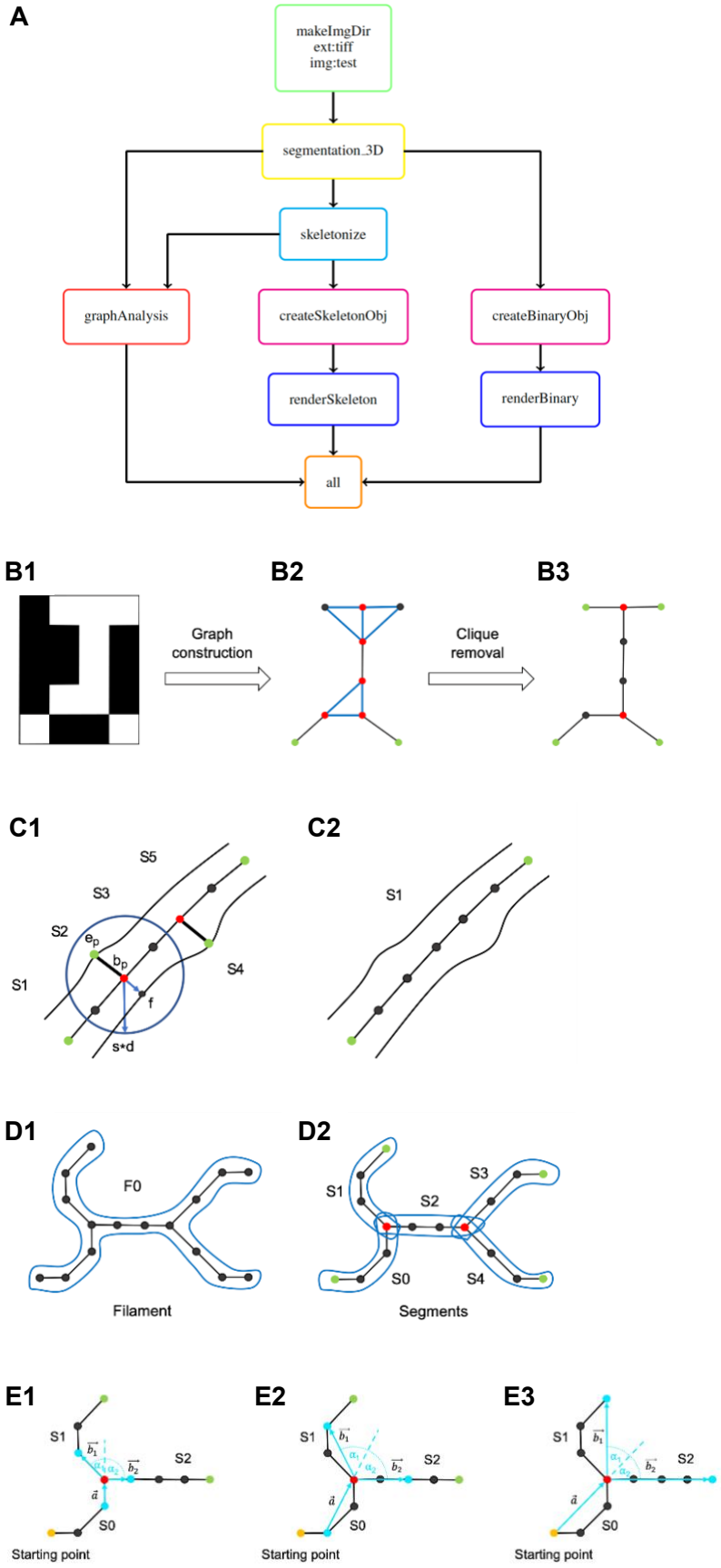
Mean branch length	46.68	± 5.63 µm
Branch diameter	3.74	± 0.56 µm
Tortuosity	1.43	± 0.054
Volume density	0.01	± 0.00 mm^3/mm^3
Branching point density	8627	± 1136 BP/mm^3
Vessel length density	830.82	± 145.79 mm/mm^3

Supplemental Fig. S3. Analysis of vessels labeled with different methods or imaged using confocal microscopy. Related to Figure 4. (A) Representative images of healthy heart tissue (upper row) or infarcted heart tissue after 5 days of reperfusion (I/R; lower row). 2- or 3 Dimensional projections of original data as well as Frangi conversion and skeletonized images obtained from VesselExpress analysis are shown. Scale bars represent 100 µm. (B and C) VesselExpress analysis of striatal vessels was performed in brains of 12 weeks old healthy male mice labeled with FITC-albumin hydrogel and CD31-Alexa647 antibody. (B) Original, segmented or skeletonized images as maximum projections in FITC-albumin-labeled brain vessels and the corresponding region labeled with CD31-Alexa647 antibody. (C) VesselExpress analysis of indicated parameters of 0.258 mm3 images of n = 4 mouse brain regions labeled with FITC-albumin or CD31-Alexa647 antibody, respectively. Scale bar represents 100 µm (D and E) VesselExpress can be applied in images obtained with confocal microscope. (D) Maximum projections of confocal images of FITC-albumin-labeled striatal vessels from brains of 12 weeks old healthy male mice as original image, after segmentation and after skeletonization using VesselExpress. (E) Results of

indicated parameters obtained using VesselExpress in confocal images (n = 4). Scale bar represents 100 µm.

Supplemental Figure S4



Supplemental Fig. S4: VesselExpress workflow, Related to STAR Methods section VesselExpress pipeline and validation. (A) Visualization of Snakemake workflow for a 3D image as directed acyclic graph (DAG). Each node represents a rule of the Snakefile. First, a folder is created for the image to be processed (green) followed by the segmentation (yellow), which consists of 3 steps: pre-processing, core segmentation and post-processing. Next, the segmented image is skeletonized (light blue). This is followed by graph construction and analysis (red) which takes the binarized and skeletonized image as input. Optionally, the binarized and skeletonized images can be rendered. Therefore, the contours are first approximated via marching cubes (magenta) and then rendered in Blender (blue). In the rule, all (orange) output files are defined. (B) Graph construction from a skeletonized binary image. (B1) Binary skeleton image with white foreground and black background. (B2) Each foreground point is represented by a node in the graph. All neighboring nodes are connected via edges. This creates cliques (blue) and thus too many branching points (red) and not enough terminal points (green). (B3) After removing cliques, the graph contains the correct number of branching and terminal points. (C) Pruning of spurious branches. (C1) Branches with a length smaller than the distance of the branching point b_p to the nearest background point f multiplied by s are removed. (C2) The graph consists of only one segment after pruning. (D) Filament vs segments. (D1) A connected graph is called a filament (marked F0). (D2) The graph consists of 5 segments (numbered S0-S4). A segment is a branch between branching/terminal points. (E) Effect of different threshold values on the branching angles. The predecessor segments are determined starting from the starting point (yellow). The threshold value indicates the length from the branching point (red) to the point from which the vector is formed (blue). (E1) Threshold = 0. (E2) Threshold = 0.5. (E3) Threshold = 1.

Supplemental Table S1. Image dimensions and image volumes of ROIs used for VesselExpress analysis. Related to Figure 2, 3 and 4

Organ	Image dimensions (xyz [mm])	Image volume [mm^3]
Brain	508 x 508 x 1000 to 2030 x 2030 x 1000	0.258 to 4.121
Spinal cord	508 x 508 x 400	0.103
Ear	254 x 254 x 100	0.006
Tongue	305 x 305 x 200	0.017
Muscle	508 x 508 x 300	0.077
Liver	305 x 305 x 100	0.009
Bladder	305 x 305 x 200	0.019
Heart	253 x 253 x 555	0.036

Supplemental Table S2: **Validation of VesselExpress and VesselVio against a synthetic ground truth. Related to STAR methods.** The average deviation was determined as the mean of the absolute deviations of all tube images.

Image index	Vessel length					Vessel diameter				
		VesselExpress		VesselVio			VesselExpress		VesselVio	
	Target (µm)	Actual (µm)	Deviation (%)	Actual (µm)	Deviation (%)	Target (µm)	Actual (µm)	Deviation (%)	Actual (µm)	Deviation (%)
1	125	122.70	-1.84	122.67	-1.86	3	4.00	33.33	3.84	28.00
2	250	246.00	-1.60	246.00	-1.60	6	5.66	-5.67	5.82	-3.00
3	375	367.70	-1.95	367.67	-1.95	9	9.62	6.89	10.86	20.67
4	500	490.00	-2.00	490.00	-2.00	12	12.00	0.00	12.63	5.25
5	625	612.00	-2.08	611.67	-2.13	15	15.11	0.73	15.48	3.20
6	750	737.00	-1.73	735.00	-2.00	18	17.89	-0.61	17.94	-0.33
7	875	856.30	-2.14	856.00	-2.17	21	21.26	1.24	21.57	2.71
8	1000	983.00	-1.70	981.00	-1.90	24	24.33	1.37	24.86	3.58
9	1125	1101.70	-2.07	1101.67	-2.07	27	26.92	-0.30	27.30	1.11
10	1250	1235.00	-1.20	1233.05	-1.36	30	30.27	0.90	32.28	7.60

Supplemental Table S3: Feature comparison of VesselExpress to VesselVio, Imaris, ClearMap and VesSAP. Related to Figure 1. Three selected ROIs of different size were analyzed with VesselExpress and with the provided Python code for feature extraction of the VesSAP authors (Fig. S1E). Since the pretrained deep learning model provided by the VesSap authors yielded biologically wrong segmentations, we used the segmentation of VesselExpress in both cases for comparing the extracted features. While the features in VesSAP are directly extracted from the skeleton mask, features in VesselExpress are extracted from the graph which includes a clique-removal to prevent wrong annotations of branching points. The images in Fig. S1E show that VesSAP identifies multiple branching points where only a single branching point is expected.

	Vessel Express	**VesselVio**	**Imaris**	**ClearMap**	**VesSAP**
Open source	+	+	-	+	-
Parallel image processing	+	-	-	-	-
Uses Python version >2.7 (secure and extendable)	+	+	**o**	+	-
Does not require transfer learning or pre-trainig	+	+	-	-	-
Workflow management system	+	-	-	-	-
Container Virtualization (OS independent)	+	-	-	-	+
Includes stitching	-	-	+	+	-
Supports segmentation	+	-	+	+	+
End-to-end automatization (raw data to statistical output)	+	-	-	-	-
Validated on multiple organs	+	-	-	-	-
Validated on LSFM and confocal	+	+	+	-	+
Whole-organ and RoIs	-	+	-	-	+
Usable without programming skills	+	+	+	-	-
Maintenance in last 3 years	+	+	+	+	-
Number of statistical outputs	**≥5**	**≥5**	**≥5**	**(n.a.)**	**<5**
Requires only one staining	+	+	+	-	-
Supports solid fill staining	+	**(n.a.)**	+	-	-