Guide: Quantification of the Zebrafish Brain Vasculature

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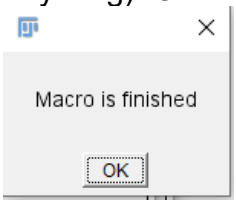
Contents

(1) Workflow with Graphical User Interface (GUI)

Steps can be done individually or all at once; see below for individual step requirements, input and output details.

What you need:

- ***How to open "Cranial Vascular Analysis":*** *Download "EKugler_GUI.ijm". Click Plugins > Macros > Edit > Open "EKugler_GUI.ijm" > hit run (the following user interface should come up)*
- MorphoLibJ plugin needed (https://imagej.net/MorphoLibJ)
- **Important** – do not have more than 9 samples in one folder, as this can cause issues with selection of correct ROIs.
- Close all other windows (ROI manager, results, other open images).
- Once you start the individual steps - let the computer do it's job (don't click anything). Once it's done with the respective step(s) it will tell you:



- Data should be on a harddrive.
- ***Computer specs:*** *Lightsheet Data are rather big; RAM higher than 8GB recommended*
- ***Time****: depends on computer specs, but general guideline:*

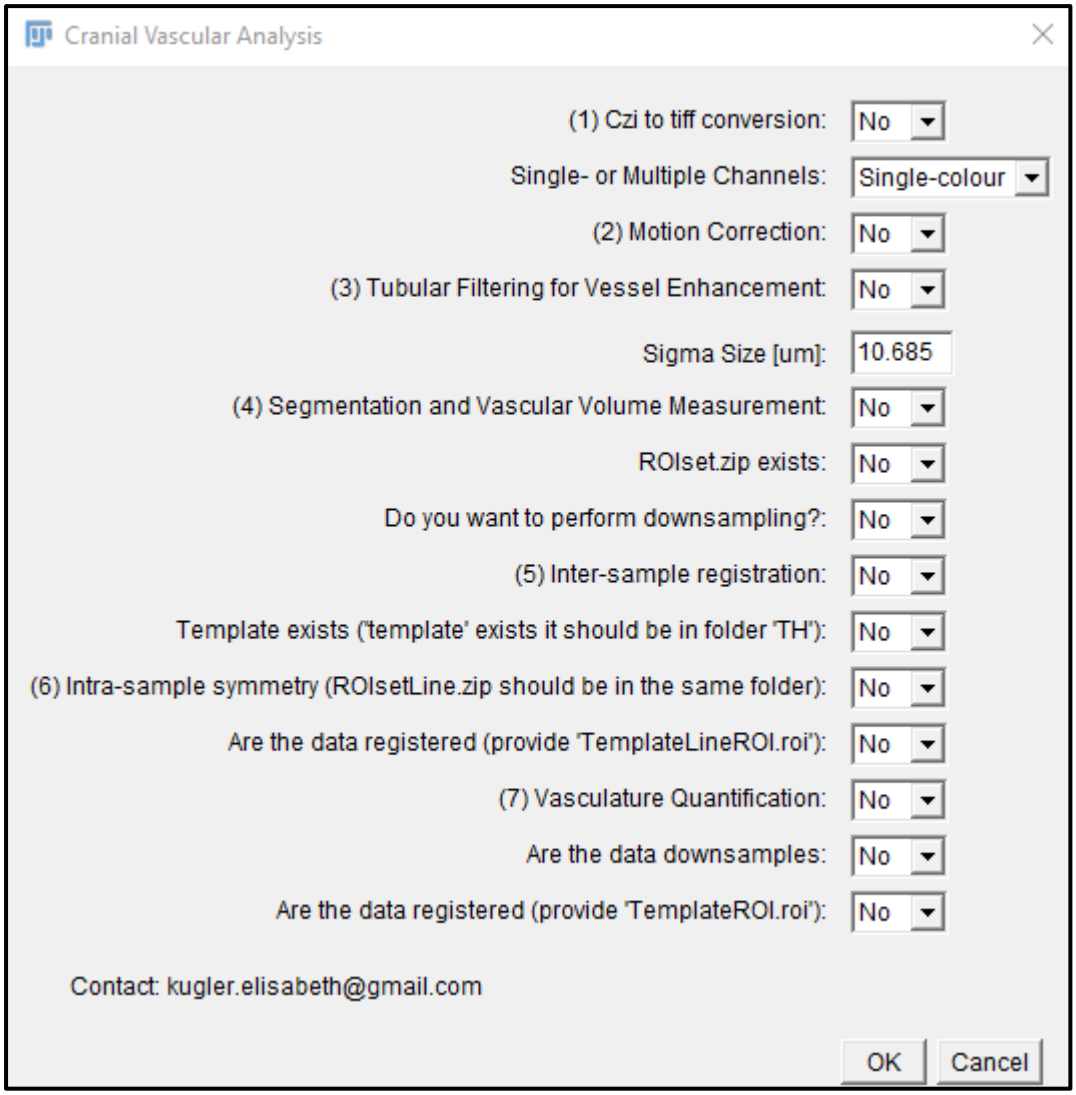
czi to tiff conversion	*<10min per image*
Motion Correction	*~15min per image*
Vascular Enhancement	*~45min per image*
Segmentation, Volume Quantification, Density, and Vascular Surface Quantification	*<5min per image*
Intra-sample Symmetry	*~15min per image*
Inter-sample Registration	*~20min per image*
Quantification of parameters	*~5min*

For all steps: select correct input folder (data need to be in a folder); output folder will be created automatically.

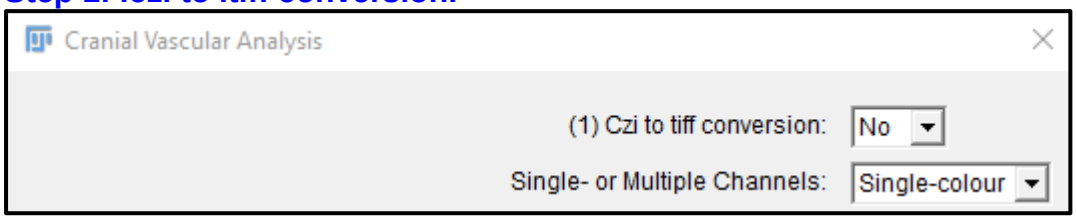
1.1. GUI

a. *"Plugins" > "Macros" > "Run"*
ZFVascQuant_GUI.ijm

c. Select the steps you want to perform (information in the following sections)
d. Klick "OK"
e. Select input folder



Step 1: .czi to .tiff conversion.

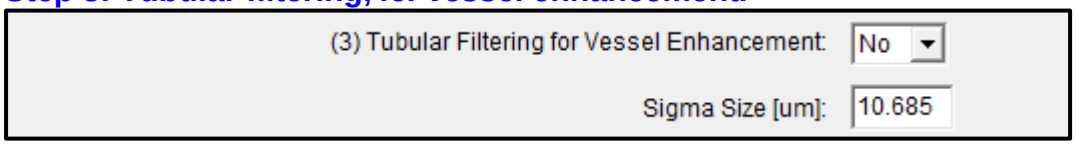


Purpose	converts .czi files to .tiff format
Options	single-colour or dual-colour
Input	Prompts input folder selection (raw .czi data)
Output	folders "VascTiff" and "NonVascTiff" in the input folder containing .tiffs and MIPs

Step 2: Motion Correction.

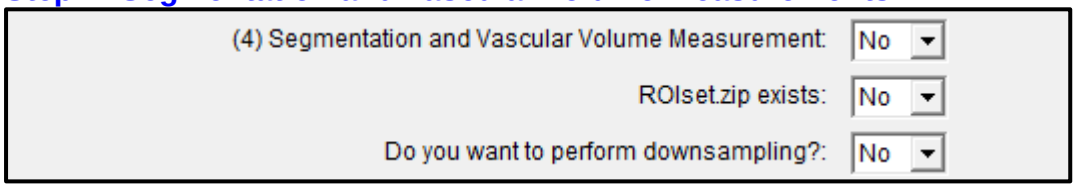
Purpose correct for motions occurred during image acquisition (ie heartbeat, gravity and muscle twitches)
Input Prompts input folder selection (.tiff data)
Output will create a folder "SIFT" inside the input folder containing .tiffs and MIPs

Step 3: Tubular filtering, *ie.* vessel enhancement.



Purpose Vessel enhancement
Options scale size (optimized for the cranial vasculature in zebrafish see https://link.springer.com/chapter/10.1007/978-3-030-39343-4_23)
Input Prompts input folder selection (.tiff data)
Output will create a folder "TF" inside the input folder containing .tiffs and MIPs

Step 4: Segmentation and vascular volume measurements.



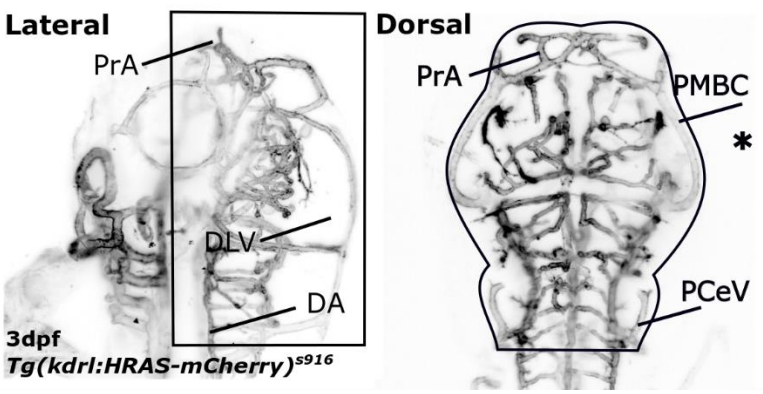
Purpose
- Segmentation of enhanced data
- Will quantify volume, surface, and density

Options
"Yes" – will prompt to ask in which directory (**recommended**)
"No" – you will get the option to
(a) Cancel.
(b) Stop after segmentation.

For (a):
- ***important*** Select images in the order they are in the folder
- draw ROI with Freehand Selection tool
- Edit > Selection > Add to Manager (can be saved as ROIset)
- Once all ROIs are drawn click "**ok**" in the pop-up window

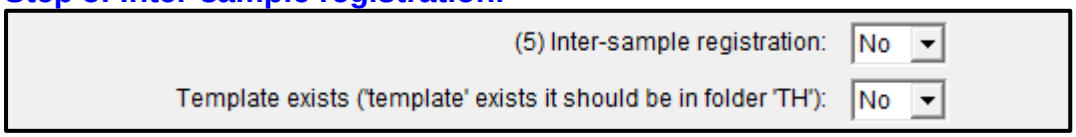
ROI selection ***important*** select images in the order they are in the folder

- Open MIP
- Draw ROI with Freehand Selection tool
- Edit > Selection > Add to Manager > Save > ROI_imageTitle
- Open next MIP > draw next ROI > Add [t] > Save > ROI_imageTitle
- Select all ROIs (click individually and hold "ctrl" > Save > save as "RoiSet" in folder with enhanced images)
- Definition of cranial vascular volume ROI: https://www.mdpi.com/2313-433X/5/1/14



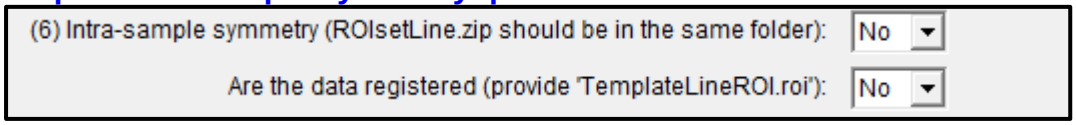
Input	Prompts input folder selection (enhanced data (TF folder (VascTiff or SIFT folder)))
Output	• folders “TH” in the input folder containing .tiffs and MIPs • folder “Edges” contains vascular surface images • will create a file “***VascVolResults***” inside the input folder containing results of vascular volume, vascular density and vascular surface

Step 5: Inter-sample registration.



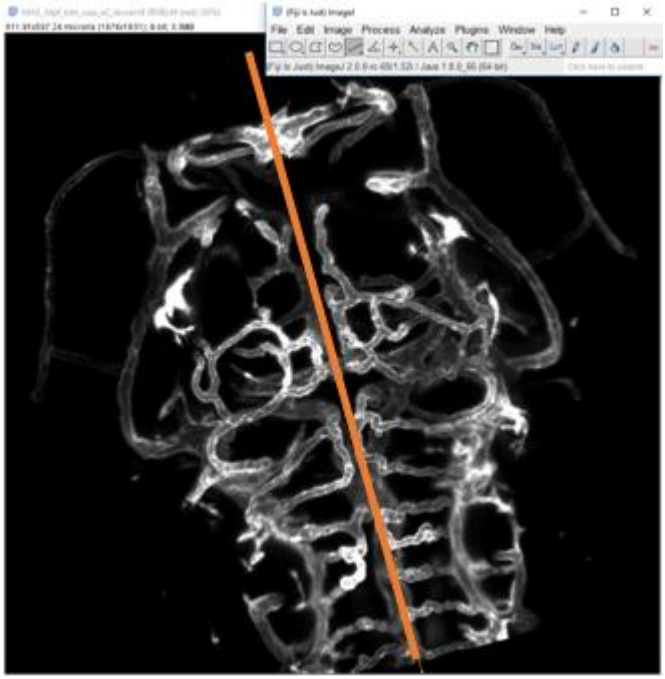
Purpose	Brings embryos into one spatial coordinate system
Options	“Yes”- provide “template” in TH folder (eg. copy+paste, rename) “No” - you will be prompted to select one sample as template after the input folder selection
Template	**(a)** sample orientation along common image axis (anterior-posterior along image y-axis, coronal plane along image z-axis and x-axis), **(b)** all common vessels visualized in image, **(c)** no obvious abnormalities.
Input	Prompts input folder selection (.tiff data (TH)); Data need to be segmented
Output	• will create a folder “Reg” inside the input folder containing .tiffs and MIPs • will create files of similarity quantification (Dice, Jaccard and Total Overlap) before and after registration (respective to the template)

Step 6: Intra-sample symmetry quantification.



Purpose	Quantifies intra-sample left-right-(a)symmetry
Input	Prompts input folder selection (.tiff data (TH))
RoiSetLine	• Create “***RoiSetLine.zip***” before starting in the folder with images. This is needed to rotate the image and bring the fish anterior-posterior axis into alignment with the image y-axis. This is done as follows *****important: have to be in order (ie 1,2,3)*****

- "*Drag and drop"* MIP (original, pre-processed or segmented) into Fiji
- Select Line ROI tool
- Draw along from posterior to anterior!

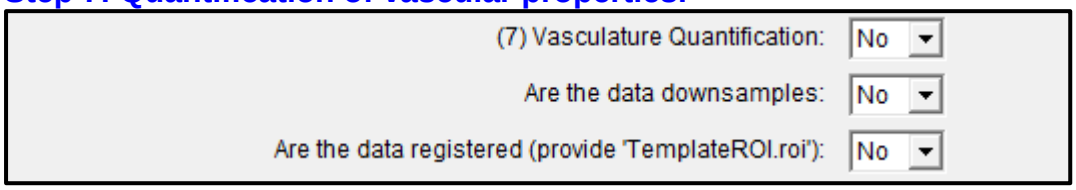


- "*Add*" to ROI manager
- Repeat for all images
- When all ROIs are drawn - select all ROIs and "save" as "*RoiSetLine*"

Output

- will create a folder "***Sym***" inside the input folder containing .***tiffs*** and ***MIPs*** of rotated images
- will create a folder "***LRVol***" inside the input folder containing .***tiffs*** and ***MIPs*** of the left and right vasculature
- will create a file "***IntraSampleSymmetryResults***" in the input folder containing L and R vascular voxel, vascular volume and skeleton voxel
- will create files of similarity quantification (Dice, Jaccard and Total Overlap) between L and R in folder "***LRVol***"

Step 7: Quantification of vascular properties.



Purpose Quantification of network length, skeleton properties (eg. number of junctions), and average diameter/radius

Options ***** important ***** data need to be down-sampled

"Yes" – data are down-sampled:

- will ask for input folder

"No" – data are not down-sampled:

- will ask for “RoiSet.zip” (copy “RoiSet.zip” into “TH” folder)
- Data will be down-sampled by factor 3.75
- Down-sampled data will be saved in “512x512” folder

Input Segmented data
(option “No” -> TH folder;
option “Yes” -> from down-sampled folder (512x512))

Output

- folder “analysis” containing .tiffs and MIPs
- will create file “DiametersAndNetworkLength”
- will create file “Skeleton Stats”

(2) Individual Macros

2.1. .czi to .tiff conversion and automatic MIP creation

a. Create folder for .tiff files in the folder with .czi files
b. "Plugins" > "Macros" > "Run"
   i. single-colour: ***EKugler_cziToTiffConversion.ijm***
   ii. multi-colour: ***EKugler_MultiColourcziToTiffConversion.ijm***
c. Select input folder

2.2. Motion Correction

a. Create folder for motion corrected files in the folder with original files
b. "Plugins" > "Macros" > "Run"
EKugler_MotionCorrectionSIFT.ijm
c. Select input folder
d. Select output folder

2.3. Vascular Enhancement

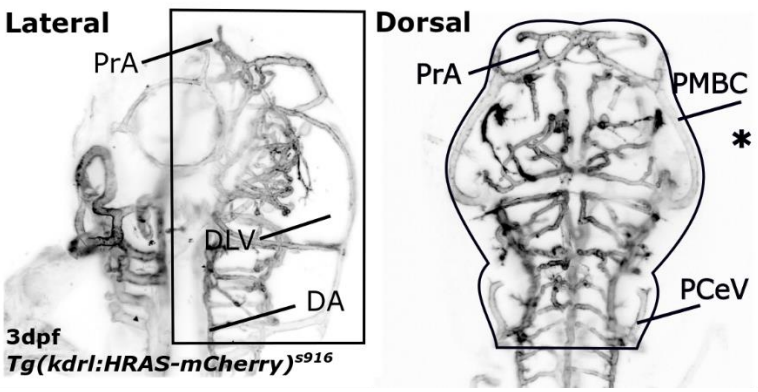
b. Create folder for enhanced files in the folder with original files
c. "Plugins" > "Macros" > "Run"
EKugler_VascularEnhancement.ijm
f. Select input folder
g. Select output folder

2.4. Segmentation and Vascular Volume Measurement

a. Create folder for enhanced files in the folder with original files
b. Create ROIs:
- Open MIP
- Draw ROI with Freehand Selection tool
- Edit > Selection > Add to Manager > Save > ROI_imageTitle
- Open next MIP > draw next ROI > Add [t] > Save > ROI_imageTitle
- Select all ROIs (click individually and hold "ctrl" > Save > save as "RoiSet" in folder with enhanced images)

c. "Plugins" > "Macros" > "Run"
make sure ROI manager etc is closed
d. Select input folder
e. Select output folder
f. will create a folder "***TH***" inside the input folder containing .***tiffs*** and ***MIPs*** of segmented images
g. will create folder "***Edges***" inside the input folder containing .***tiffs*** and ***MIPs*** of vascular edges
h. will create a file "***VascVolResults***" inside the input folder containing results of vascular volume, vascular density and vascular surface

Definition of cranial vascular volume ROI: https://www.mdpi.com/2313-433X/5/1/14



2.5. Automatic Inter-sample Registration

important needs a file called template!!!
Rigid registration used to automatically register images.

important MorphoLibJ plugin needed (https://imagej.net/MorphoLibJ)

important first image/template and following images (moving images) have to be the same age. (ie select one template for each age!)
"Plugins" > *"Macros"* > *"Run"* >
EKugler_AutomaticRigidInterSampleRegistration.ijm
Output folder will be called "Reg".

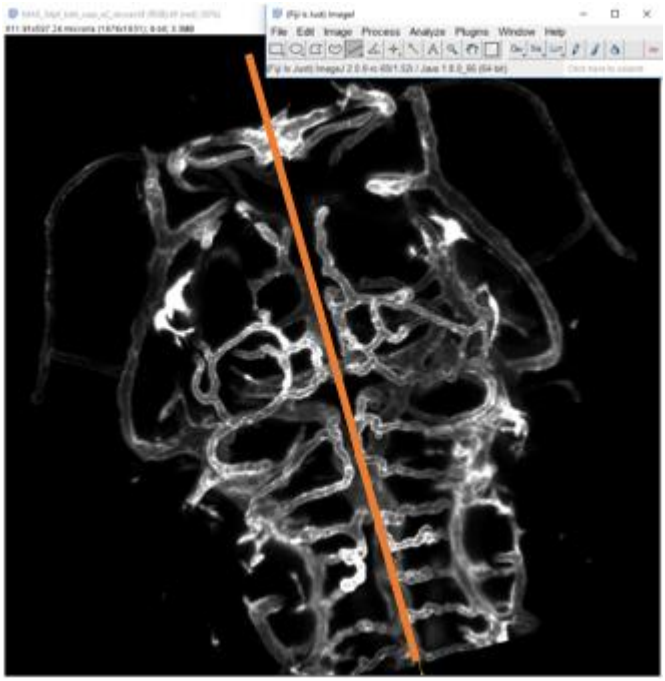
2.6. Intra-sample Symmetry

To compare left and right vascular symmetry. To be applied to pre-processed and segmented images.

important MorphoLibJ plugin needed (https://imagej.net/MorphoLibJ)

*****important*****

- During image acquisition - make sure embryos are not left-right tilted (z-axis). If they are - register to non-tilted sample before quantifying L-R symmetry.
- Create "*OutputFolder*" before starting. (in this folder all your output will be saved; logic could for example be *"../tiff/TF/TH/outputfolder"*)
- Create "*RoiSetLine.zip*" before starting in the folder with images.
  This is needed to rotate the image and bring the fish anterior-posterior axis into alignment with the image y-axis.
  This is done as follows
  *****important: have to be in order (ie 1,2,3)*****
  - "*Drag and drop"* MIP (original, pre-processed or segmented) into Fiji
  - Select Line ROI tool
  - Draw along anterior posterior



    - "*Add*" to ROI manager
    - Repeat for all images
    - When all ROIs are drawn - select all ROIs and "save" as "*RoiSetLine*" in the folder where the images which will be processed are saved (logic could for example be *"../tiff/TF/TH"*)
- *"Plugins" > "Macros" > "Run"*
  make sure ROI manager and results table are closed
- Select input folder
- Select output folder
- Macro will prompt you to draw another line ROI after images were rotated. This line ROI will be used to split L and R vol.
    - *****important: have to be in order (ie 1,2,3)*****
    - Draw line ROI and "add" to ROI manager
    - Close images
    - Click "ok"
- **IntraSampleSymmetryResults.csv** with left and right vascular volume and skeleton voxels will be created in input folder (VascVox = number of black voxels; VascVol = vascular volume in um3)
- Individual files for LR similarity quantification (Jaccard Index, Dice Coefficient and Total Overlap; see https://imagej.net/MorphoLibJ) will be created in **LRVol** folder

2.7. Down-sampling

- Create folder for enhanced files in the folder with original files
- *"Plugins" > "Macros" > "Run"*
    - ***EKugler_Downsampling.ijm***
- Select input folder
- Select output folder

2.8. Vascular Quantification

- Create folder for enhanced files in the folder with original files

- *“Plugins” > “Macros” > “Run”*
    - ***EKugler_DiameterSkel.ijm***
- Select input folder
- Select output folder

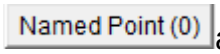
(3) Inter-sample Registration using Anatomical Landmarks

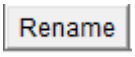
To be applied to pre-processed and segmented images.
Based on https://imagej.net/Name_Landmarks_and_Register

important first image/template and following images (moving images) have to be the same age. (ie select one template for each age!)

important if you want to measure sample similarities (before and after registration) - MorphoLibJ plugin needed (https://imagej.net/MorphoLibJ)

First Image // template (which will be template - make sure this one is aligned in x,y, and z):
Open segmented images and select "*Plugins > Landmarks > Name Landmarks and Register*"

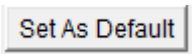
- select landmarks (see figure) using single point tool 
- 

after selecting first point
- 

to rename point eg right ACeV
- 

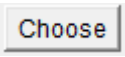
and repeat this for all points from the figure (left PrA, right PrA, left ACeV to PrA, right ACeV to PrA, left PCS to MtA, right PCS to MtA, left ACeV, right ACeV, left PCeV to PHBC, right PCeV to PHBC, MCeV to DLV)
- 

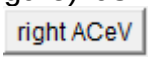
to save these landmarks
- 

this file in it's file location AND 

(so the computer knows this is your template)

Images to register // moving images (these will be registered to the first/template image):

- Landmark names and template should be saved from the above (if there is nothing, something went wrong)
- Make sure the **correct template** is chosen; otherwise 

- "*Plugins > Landmarks > Name Landmarks and Register*"
- select landmarks (see figure) using single point tool and select respective anatomical landmark; eg 

- 

to save these landmarks

Registration:

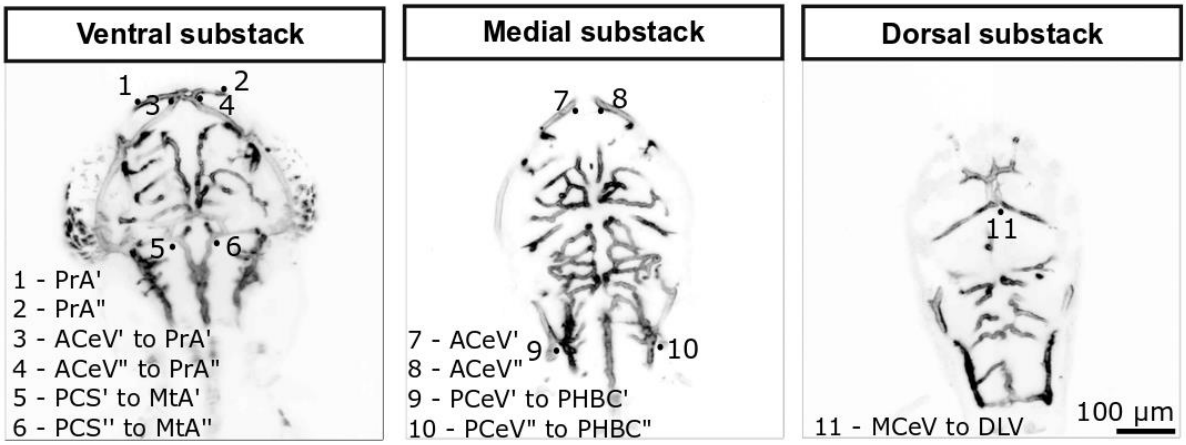
- make sure you select 

(and have the right template selected)
- 

Save file:

- Image > colour > split channels
- Select green channel > Edit > invert (yes, whole stack)
- File > Save as …

Anatomical landmarks:

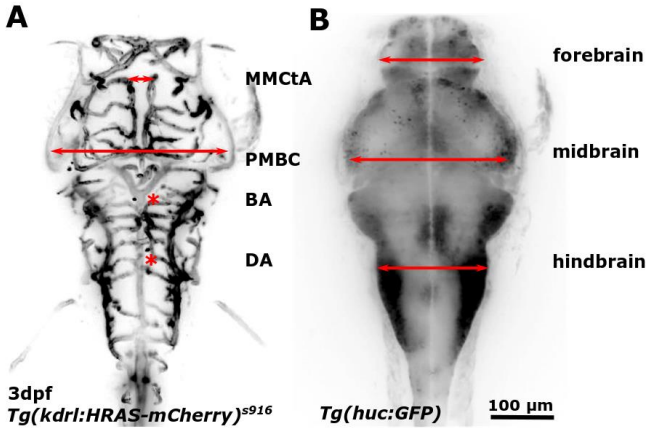


(4) Guide to Manual Measurements

3.1. Growth measurements

Open 3D stack in Fiji. Use line ROI.

- **Primordial midbrain channel** (PMBC) width: measure distance posterior to eye (Fig. A).
- **Basal artery** (BA) diameter: measure diameter about 50um before splitting into PCS (Fig. A).
- Measurements of **brain growth** (Fig. B):
  - Forebrain
  - Midbrain
  - Hindbrain
- **ISV diameter**: diameter of 3 ISVs at cloaca; consider if you want to measure aISV and vISV differences
- **DA diameter**

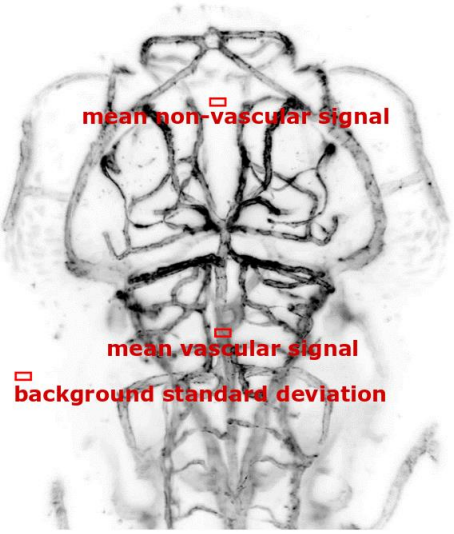


3.2. Contrast-to-Noise Ratio (CNR)

Select rectangle ROI (in 3D stack; position as indicated in image; 5um long; crossing basal artery width)
Analyse > Histogram [h] > *Mean vascular value*
Live > move to non-vascular region (same plane but without vessels) > *mean non-vascular signal*
Move to background region (same plane but outside embryo) > *background standard deviation*

Formulas:
(1) SNR (signal-to-noise ratio) = mean vascular signal / standard deviation of background

(2) CNR (contrast-to-noise ratio) = (mean vascular signal - mean non-vascular signal) / standard deviation of background



(5) References

4.1. Image analysis workflow and code:

(1) **Kugler, Chico, Armitage** (2018) *Image Analysis in Light Sheet Fluorescence Microscopy Images of Transgenic Zebrafish Vascular Development.* In Nixon M., Mahmoodi S., Zwiggelaar R. (eds) Medical Image Understanding and Analysis. MIUA 2018.; Springer, Cham, 2018; Vol. Communications in Computer and Information Science, vol 894, pp. 343–353.
https://link.springer.com/chapter/10.1007/978-3-319-95921-4_32

(2) **Kugler, Plant, Chico and Armitage** (2019), *Enhancement and Segmentation Workflow for the Developing Zebrafish Vasculature,* J. Imaging 2019, 5(1), 14; https://doi.org/10.3390/jimaging5010014

(3) **Kugler, Chico, and Armitage.** *Validating Segmentation of the Zebrafish Vasculature.* In Yalin Zheng, Bryan M. Williams, and Ke Chen, editors, Medical Image Understanding and Analysis, Communications in Computer and Information Science, pages 270–281, Cham, 2020. Springer International Publishing. ISBN 9783-030-39343-4. https://link.springer.com/chapter/10.1007/978-3-030-39343-4_23

4.2. Documentation/papers of other Plugins and mathematical justifications:

SIFT - Linear Stack Alignment:
https://imagej.net/Linear_Stack_Alignment_with_SIFT
Lowe, David G. (2004) Distinctive Image Features from Scale-Invariant Keypoints, International Journal of Computer Vision. 60 (2): 91–110. CiteSeerX 10.1.1.73.2924.

Sato Vessel Enhancement Filter:
https://www.longair.net/edinburgh/imagej/tubeness/
Sato, Nakajima, Atsumi, Koller, Gerig, Yoshida and Kikinis (1997) 3D multi-scale line filter for segmentation and visualization of curvilinear structures in medical images, International Conference on Computer Vision, Virtual Reality, and Robotics in Medicine, CVRMed 1997, MRCAS 1997: CVRMed-MRCAS'97 pp 213-222.

Otsu Thresholding: https://imagej.net/Auto_Threshold
N Otsu. A threshold selection method from gray-level histograms. Trans. Sys.Man., 9(1):62–66, 1979.

Vascular Surface: https://imagej.nih.gov/ij/docs/menus/process.html#find
Canny, J., A Computational Approach To Edge Detection, IEEE Transactions on Pattern Analysis and Machine Intelligence, 8(6):679–698, 1986.

3D skeletonization: https://imagej.net/Skeletonize3D
T. C. Lee, R. L. Kashyap, and C. N. Chu. Building Skeleton Models via 3-D Medial Surface Axis Thinning Algorithms. CVGIP: Graphical Models and Image Processing, 56(6):462–478, November 1994.

Euclidean Distance Map (EDM): https://imagej.net/Distance_Transform_3D
Gunilla Borgefors. On Digital Distance Transforms in Three Dimensions. Computer Vision and Image Understanding, 64(3):368–376, November 1996. ISSN 1077-3142. doi: 10.1006/cviu.1996.0065. URL http://www.sciencedirect.com/science/article/pii/S107731429690065X.

4.3. Vessel nomenclature

Isogai, Horiguchi, and Weinstein (2001) *The Vascular Anatomy of the Developing Zebrafish: An Atlas of Embryonic and Early Larval*, Development Developmental Biology 230, 278–301.
https://pdfs.semanticscholar.org/59e9/3cb024a2c570da5be958ceb5949c87bab3df.pdf