

Supplemental Material

A) User Quick Guide B) Supplemental Information for Using STAFF

A simple automated method for continuous fieldwise measurement of microvascular hemodynamics

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Spatial Temporal Analysis of Fieldwise Flow (STAFF)

A) User Quick Guide

The STAFF macro automatically measures microvascular flow velocities across entire microscope fields. Results are provided in the form of a segment velocities report in csv format (for analysis in spreadsheet programs), and a stack of color coded velocity maps (for visual analysis).

Before you start

Required Software:

- FIJI ImageJ <https://imagej.net/Fiji/Downloads>

STAFF macro files <http://githubXXXX> (Link will be identified following publication)

- STAFF.ijm Flow analysis macro
- STAFF_Dir.jar Modified Directionality plugin (an executable jar file)
- STAFF_Loader.ijm Script to autoload STAFF macro each time FIJI is started

IMPORTANT: All development and testing were done using FIJI ImageJ (64 bit) – we cannot promise performance in other versions of ImageJ.

Required Input Files and Directories:

- Epifluorescence or transmitted light time lapse movie in Tif stack (mTif) or AVI (uncompressed) format.

IMPORTANT: FIJI will open most native microscope formats using the included Bioformats. If necessary, convert your movie by opening in FIJI then saving as mTif or AVI (uncompressed).

- Skeleton file for the blood vessel network in the movie in png or tif format.
- Create a project directory named for the movie file name.
- Within the project directory, create an input file directory.

IMPORTANT: Do not include spaces in the file or directory names.

Place both the movie and skeleton in the input file directory.

Note - Sample input files (MOVIE.tif and SKELETON.tif) can be downloaded from the same location as the STAFF macro files.

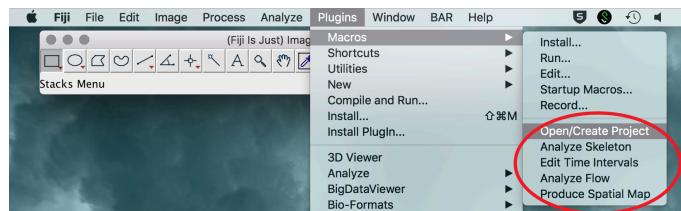
Required information:

- Spatial resolution (microns/pixel) - Sample Data: 0.65 microns/pixel
- Movie speed (frames per second) – Sample Data: 100 frames/second (fps)
- Shortest segment to be measured – (recommend 15-20 microns)
- Fastest speed to be measured – (recommend 2000 microns per second)

IMPORTANT: To verify these parameters, open movie file in FIJI, check spatial and temporal calibration (Image>Properties). MAKE SURE THESE VALUES ARE CORRECT.

Install the STAFF macro files

- In your FIJI folder: Open the plugins folder. Inside the plugins folder, open the Macros folder. Copy STAFF.ijm into this Macros folder.
Fiji\plugins\Macros\STAFF.ijm
- In your FIJI folder: Open the plugins folder. Copy STAFF_Dir.jar into this plugins folder.
Fiji\plugins\STAFF_Dir.jar
- In your FIJI folder: Open the macros folder. Inside this macros folder, open the AutoRun folder. Copy STAFF_Loader.ijm into this AutoRun folder.
Fiji\macros\AutoRun\STAFF_Loader.ijm
- Start FIJI ImageJ
- Verify installation - Select Plugins>Macros
Look for 5 macros shown in Supplemental Figure 1.



Supplemental Figure 1. Verifying installation of the STAFF macro

IMPORTANT: Multiple folders within the FIJI ImageJ folder have similar filenames (capitalized, not capitalized). Be certain that you have navigated to the correct folders when installing STAFF. Also note that we have found that Staff_Dir.ijm is sometimes removed by FIJI updates on Macintosh computer systems.

Running the STAFF macro

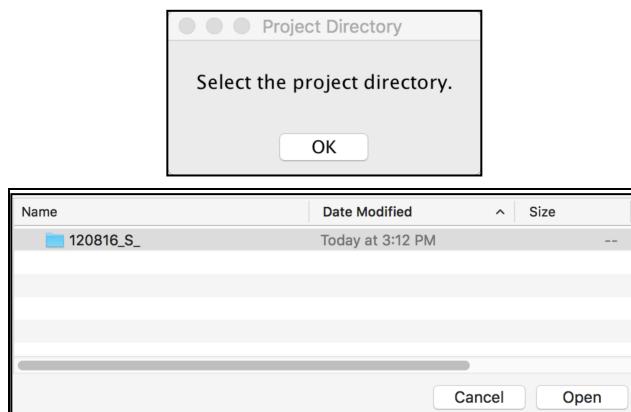
1) Open-Create Project (Supplemental Figures 2-6)

- Select Plugins>Macros>Open-Create Project

Follow prompts to create the configuration file. If you have run an analysis in this folder previously, you will edit or complete the pre-existing configuration file.

- **SELECT PROJECT FOLDERS AND INPUT FILES**

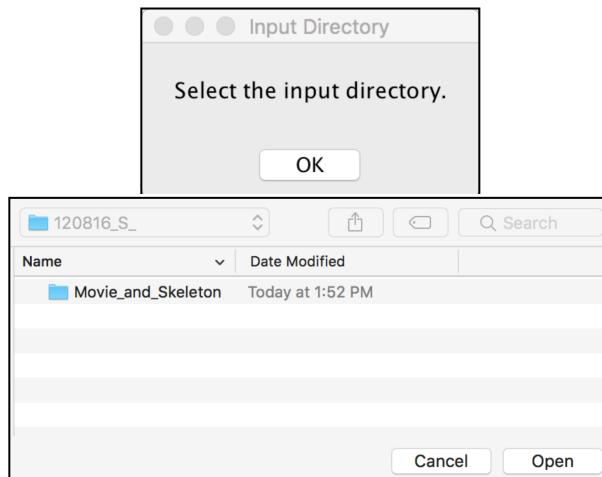
Follow prompts to navigate to and select Project Directory, Input File folder and input files (movie and skeleton).



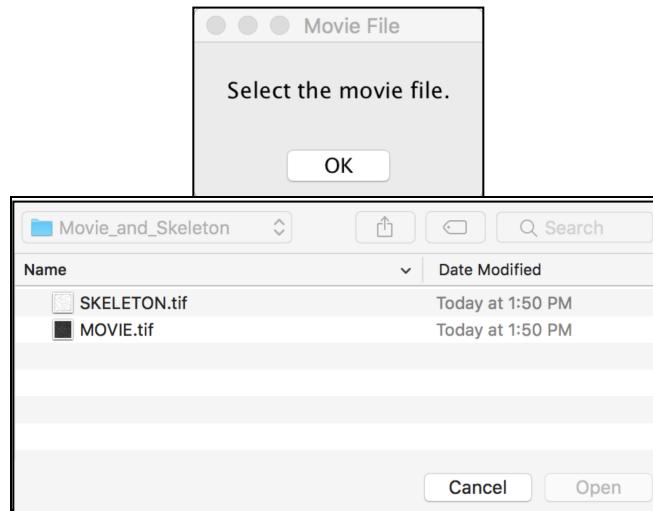
Supplemental Figure 2. Select the Project Directory and Open.



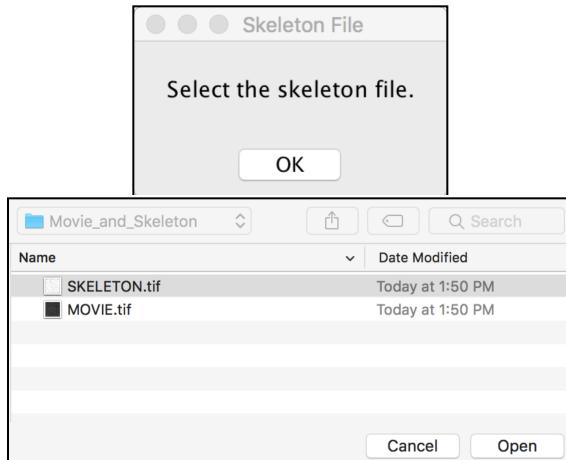
Supplemental Figure 3. Create a Configuration file.



Supplemental Figure 4. Select the Input Directory folder and Open.



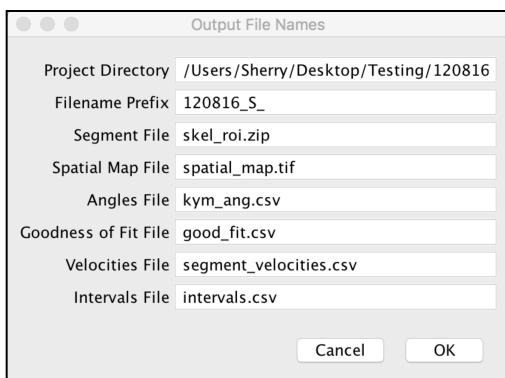
Supplemental Figure 5. Select the Movie File and Open.



Supplemental Figure 6. Select the Skeleton File and Open.

- **NAME the OUTPUT FILES (Supplemental Figure 7)**

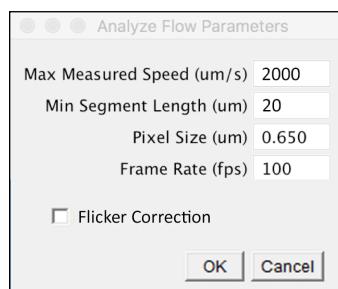
Program will autopopulate file names. The Project Directory name is used as a prefix when files are saved. All name fields can be edited.



Supplemental Figure 7. Output File Name Menu.

- **DEFINE ANALYSIS PARAMETERS (Supplemental Figure 8)**

For shortest segment length and max speeds measured and mapped, use suggested values to begin. Find spatial and temporal scale information in image file metadata and/or your experimental notes. **Pixel size and frame rate are initially set to zero and require user input.** To use Flicker Correction, check box.

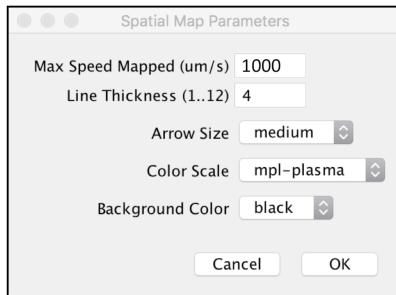


Supplemental Figure 8. Analyze Flow Parameters menu.

IMPORTANT: When time-lapse data has background flicker, the resulting kymographs have periodic horizontal banding which produces a single bin peak in directionality values at zero degrees that will result in impossibly high velocity measurements (See discussion in “Supplemental information for using STAFF”). If you think your data contains flicker, check the “Flicker Correction” box. This choice engages a modified version of the directionality plugin in the STAFF macro files that ignores the zero degree values. Since even the slowest segments show non-zero velocity measurements, this has no effect on velocity measurements.

- **SELECT SPATIAL MAP OUTPUT PARAMETERS** (*Supplemental Figure 9*)

Select parameters for best visualization of your data.



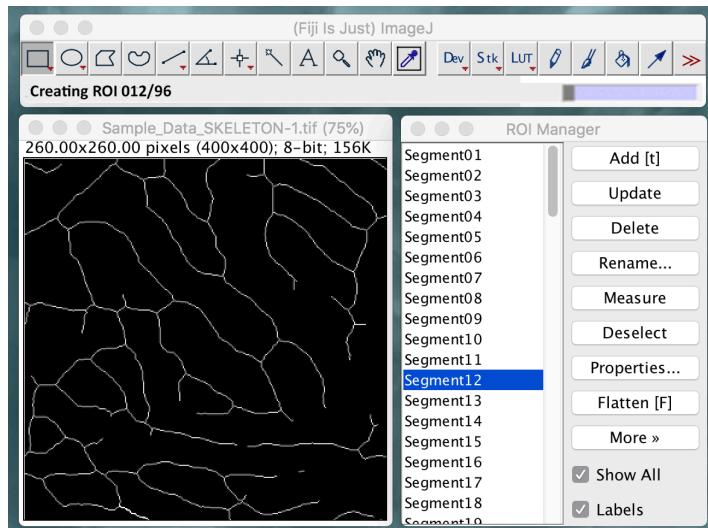
Supplemental Figure 9. Spatial Map Parameter Menu.

A popup window will report when configuration selections are complete, click OK to save.
The configuration is then saved in the results folder as prefix_config.csv file.

When generating the spatial map, an image of the color scale chosen will open. The left (or bottom) end of the color scale corresponds to a flow velocity of zero microns per second. The right (or top) end of the color scale corresponds to the Max Speed Mapped. Save the color scale image in the same folder with the spatial map. Use the color scale when constructing figures using the spatial map.

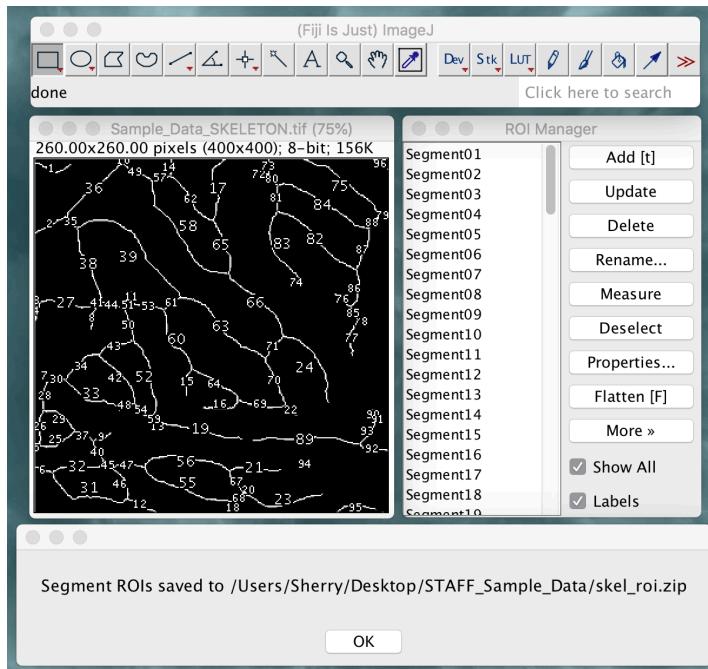
2) Analyze Skeleton (*Supplemental Figures 10 and 11*)

- Select Plugins>Macros>Analyze Skeleton
Skeleton file will open. ROI manager will open. Progress bar is active.



Supplemental Figure 10. Running Analyze Skeleton

When Analyze Skeleton is complete you will see the labeled skeleton and ROI manager.



Supplemental Figure 11. Analyze Skeleton analysis completed.

A popup window will report when skeleton analysis is complete; click okay.

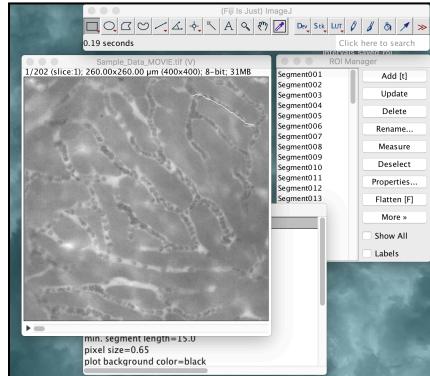
The analysis of the skeleton will be stored in the results folder as a zip file, Prefix_skel_roi.

3) Select Time Intervals (*Supplemental Figures 12 - 14*)

In this step, you will choose the intervals of time in which velocities will be measured throughout the field. At a minimum, this should be the interval between breaths, but can be shorter. However, for an initial run, you might want to evaluate your parameter choices using only 3 or 4 intervals, since it can take hours to run large datasets.

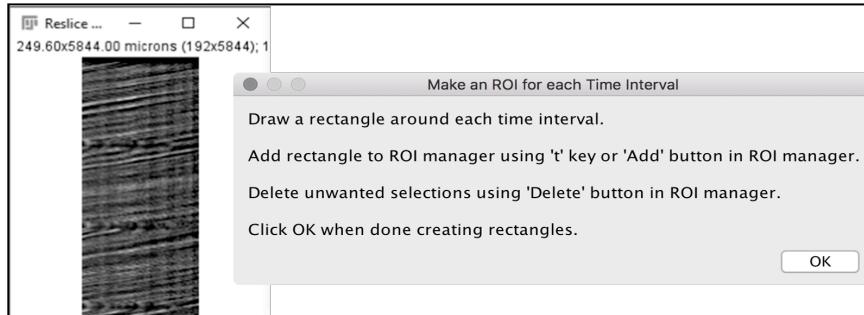
- Select Plugins/Macros>Select Time Intervals

The macro will generate a kymograph from a single segment over all time intervals.



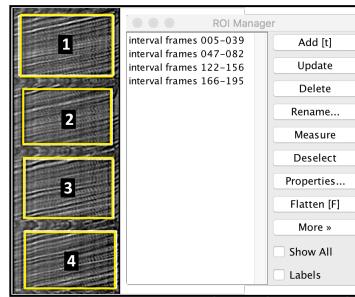
Supplemental Figure 12. Screen during generation of kymograph for selection of Time Intervals.

The kymograph from a particular segment and “Make an ROI for Each Time Interval” window will open.



Supplemental Figure 13. Time Interval selection kymograph and instructions for interval selection.

The Box tool is automatically activated. Use it to draw a box that incorporates the full length of the segment and the duration of time (box width and height, respectively). Then click the “Add” button. Repeat this for as many time intervals as you want to include.



Supplemental Figure 14. Selected intervals shown on interval selection kymograph and in ROI manager.

Click OK in the “Make an ROI for each interval” box when done.

A popup window will report that the intervals have been saved. Click okay.

The selections of Analysis Intervals will be stored in the results folder as Prefix_intervals.csv.

4) Analyze Flow

- Select Plugins>Macros>Analyze Flow

The “Analyze Flow Parameters” menu opens (Supplemental Figure 8), with values that you entered in the Open-Create Project step. Edit if needed then select OK.

The “Output File Names” menu opens (Supplemental Figure 7) with names you entered in the Open-Create Project step. Edit if needed, then select OK. If you need to change the filename prefix, Open-Create a new Project.

A menu opens that asks if you are ready to begin analysis. Select OK.

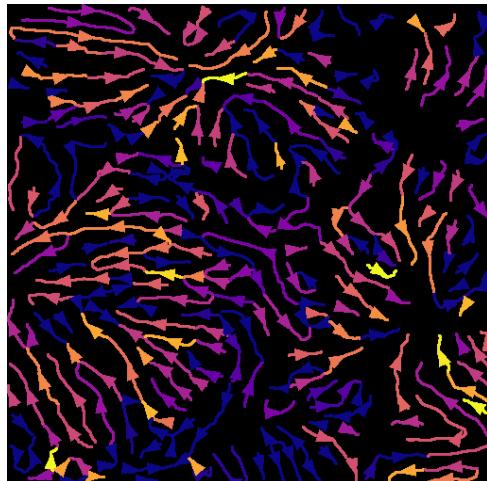
Analysis will begin. Depending on the speed of your processor, speed of your hard drive and the size of your dataset this step may take minutes (small dataset 512x512 pixels hundreds of frames) to hours (large dataset 1024x1024 pixels tens of thousands of frames). A popup will indicate when Flow Analysis is complete.

The results from Analyze Flow will be stored in the results folder as Prefix_segment_velocities.csv. This is the main results file that contains segment velocities for further analysis.

5) Produce Spatial Map (*Supplemental Figure 15*)

This step will generate a spatial map of flow velocities using color to indicate flow speed, which allows visualization of spatial and temporal variability. Data are output in the form of a Tif image stack with one image for each time interval.

- Select Plugins>Macros>Produce Spatial Map



Supplemental Figure 15. Single frame from a Spatial Map.

The “Spatial Map Parameters” window opens, with values that you entered in the Open>Create Project step (Supplemental Figure 2). Edit if needed, then select OK.

A popup window will report that the Spatial Map is created and saved. Click okay. The results from Produce Plot will be stored in the results folder as plot.tif.

6) Redo Analysis with Different Parameters

Upon inspecting your results, you may want to run STAFF with different parameters. If you want to save your previous analysis, then make a new output folder and start from step 1). If you do not want to save your previous analysis, then start over at the step where you want to make changes. For example, if your Time Intervals are fine, but you want to change the parameters for flow analysis, then start from step 4).

7) Redo analysis to eliminate artifacts induced by image flicker

As mentioned above, when time-lapse data has background flicker, the resulting kymographs have periodic horizontal banding which produces a single bin peak in directionality values at zero degrees (See discussion in “Supplemental information for using STAFF”). Since flicker may not be visually obvious, you should inspect the segment_velocities.csv file. If you find many cells labeled “OUT” (indicating outliers), you should rerun the analysis after checking the “Flicker Correction” box.

B) Supplemental Information for using STAFF

Additional details about the OUTPUT FILES

skel_roi.csv

The STAFF macro step Analyze Skeleton divides the skeleton into segments with endpoints (nodes) at ends and at branch points. Each line segment is then an ROI (region of interest) and the ROIs are stored in an ROI manager file (a zipped folder). If you re-run Analyze Skeleton on the exact same skeleton file, you will get the exact same set of ROIs numbered in the same way. If you change the skeleton file in any way the ROIs and numbering change.

To re-open the segment ROI file (a zipped folder), have an image open (this can be the original movie, a single frame from the movie, or the spatial map of velocities). Drag the zipped folder onto the ImageJ menu bar and it will open. Click show all and labels to visualize segments on the image.

To get segment lengths from the segment ROI file, click any segment in the list, press cntl-A to select all, then press Measure in the ROI menu. A results window will open. One of the columns measured is length. Save the results window.

intervals.csv

The STAFF macro step Edit Time Intervals creates this file. The intervals.csv file contains the start/stop points for temporal analysis periods that are selected by the user.

kym_ang.csv

This file contains the angles calculated from the kymographs using the directionality plugin. There is one kymograph generated for each line segment (ROI) for each interval.

segment_velocities.csv

This is the file used for subsequent quantitative analyses.

Cells for segments less than the minimum length value are filled with text – SHORT. Cells with velocity values greater than the maximum measured value are filled with text – OUT (for outlier). Velocity values have a sign (+/-) that designates direction of flow through that segment. Direction comes from which end of a given segment is the start/stop point. If your segment_velocities.csv has a lot of cells with outlier values, check to see that the minimum segment length to analyze value is at least 15-20 microns and re-run the analysis. If after increasing the minimum segment length to analyze, you still have many cells with outlier values, see the section on how to detect and correct for background flicker.

good_fit.csv

This file is the goodness of fit of a curve to the distribution of angles measured using directionality plugin from each kymograph. This occurs within the plugin. Although we do not currently use the goodness of fit measurement, poor goodness of fit (less than 0.8) can indicate the presence of: 1) hidden branch points within a segment; 2) change in velocity during measurement interval; 3) lamp or room light flicker introducing horizontal banding in kymographs.

Additional information about selecting ANALYZE FLOW PARAMETERS

Frames per second and Pixel size

This information should be found in the movie file metadata, but we strongly suggest a cross check with notes taken at the time the movie was acquired. Metadata information may not detect when a microscope objective has been moved to a non-standard position on the nosepiece, or when optivar magnification has been used.

Shortest segment

The shortest segment analyzed should be 15-20 microns or greater.

Max speed measured

Max speed measured for a given segment length = segment length (in microns) * fps.

For a segment 20 microns in length at 100 fps the max measured speed would be 2000 microns/sec.

A few short test runs will help you select the maximum speed to measure. To check whether you have chosen a reasonable max speed measured, in a short test run, set the max measured speed higher than is likely for capillary flow (\geq 2000 microns per sec), run the analysis and then (using your favorite spreadsheet or statistics program) make a histogram of the absolute values of segment_velocities.csv.. Examine the distribution. In the example below, the theoretical max speed to measure was 2000 microns/sec with the histogram revealing that about 99% of the data fell below 1000 microns/sec. Alternately, examine a Tukey outlier boxplot of the values.

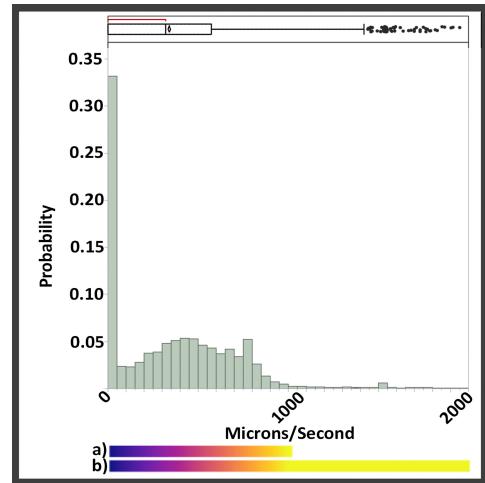
Additional information about how to SELECT SPATIAL MAP OUPUT PARAMETERS

Max speed spatially mapped (*Supplemental Figure 16*)

Selection of maximum speed mapped is also based on the distribution of your segment_velocities.csv data. To examine the distribution of your data, use your favorite statistical analysis software and make a histogram of the absolute values of segment_velocities.csv. In the example here, about 99% of the data had a max speed of less than 1000 microns/second, making 1000 microns/second a reasonable choice for Max Speed plotted. The Max speed measured was 2000 microns/second.

What is the relationship between the color map, the segment velocities and the quantities Max Plot Speed and Max Measured Speed in the Plots? Select maximum Spatially Mapped speed so that the bulk of the data is spread along the color scale, allowing better visualization of the range of velocities. The remaining relatively small number of very high speed segments are plotted using the color that represents the highest speed on the color scale.

Color scale a) is the standard Plasma color scale here set to represent 0 to 1000 microns/second. Color scale b) shows the same Plasma color scale but with the color assigned to the highest value extended to include the range from the maximum plot value to the maximum measured value. This approach is used in STAFF, and best shows the distribution of values in the dataset, while high values are not lost from the spatial mapping.



Supplemental Figure 16. Select color-mapping parameters to best visualize your data.

IMPORTANT: When visually comparing multiple datasets, select same Max speed spatially mapped for all.

Spatial Map LUT

Mpl-Inferno, Mpl-Magma, Mpl-Plasma and Mpl-Viridis are all perceptually uniform sequential colormaps with equal steps in data perceived as equal steps in the color space. Further, sequential colormaps have dark to bright mapping and can be converted to greyscale.

Physics is a popular very colorful colormap.

Select the colormap that provides best visualization of your data.

Spatial Map background color

If you plan to render your velocity sequence as a volume, choose black background. Black is rendered as transparent in most volume rendering programs. If you plan to view your velocity sequence as a movie or as single images, choose the background that best highlights your data.

Spatial Map line thickness

Thickness values possible are 1-12. Thickness = 4 works well in a 512 x 512 pixel image. Thickness = 6 works well in a 1024 x 1024 pixel image.

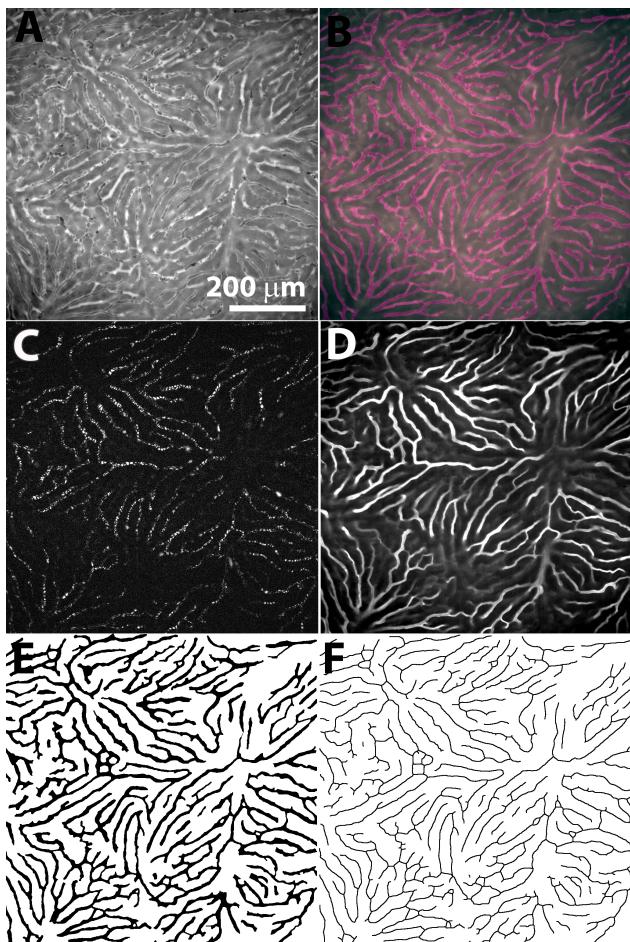
Spatial Map Arrow size

Arrow size values possible are small, medium, large. Medium works well in a 512 x 512 pixel image. Large works well in a 1024 x 1024 pixel image. Arrow direction reflects flow direction. Arrows are not plotted for segments with stopped flow (less than 1 $\mu\text{m/sec}$).

Additional Information about Analyze Skeleton and generating skeleton files.

The skeleton image is a line drawing representation of the vascular network. Skeleton images for

complex vasculature can be generated using tools in FIJI (Supplemental Figure 17). Skeleton images for simple networks can be generated by hand as line drawings. Line segments should lie near the center of the vessel. Branch points (nodes) that are out of the image plane (hidden) should be marked by making a gap in the line.



Supplemental Figure 17. Skeletonization is creation of a line drawing representation of the vascular network. Skeletonization begins with segmenting the vascular network (A). Segmentations can be created manually using paint tools to trace the vasculature in a separate layer (1B), or using a semi-automated approach that begins with calculating difference images to highlight regions of flow (1C), then summing the difference images to create a segmented image of the vasculature (1D) that can be further refined using manual tools in TrakEM2 in FIJI ImageJ. Manual refinement is needed because difference images de-emphasize vasculature with low flow. The resulting segmentation is then converted to binary (E) and skeletonized (F) using FIJI. Hundreds of segments and branch points are visible in the vascular network (A) and in the corresponding skeleton (F). Some branch points are not visible within the plane of the image, but their location can be inferred by viewing the movies or at kymographs. Where there is a hidden branch point in a segment, a change in velocity is visible in the movie that is reflected as a change in slope in the kymograph. By making a gap in the segment corresponding to the location of the hidden branch point, each part is treated properly as a separate segment for analysis of flow velocities.

There may be translocation of the tissue being imaged over time. When this occurs, trim the movie sequence, keeping only the portion where that skeleton line segments lie at or near the midpoint of the vascular network. Short periods of motion that may occur during respiration (with the tissue returning to the previous position after the respiration) can be ignored as they can be skipped when selecting time intervals.

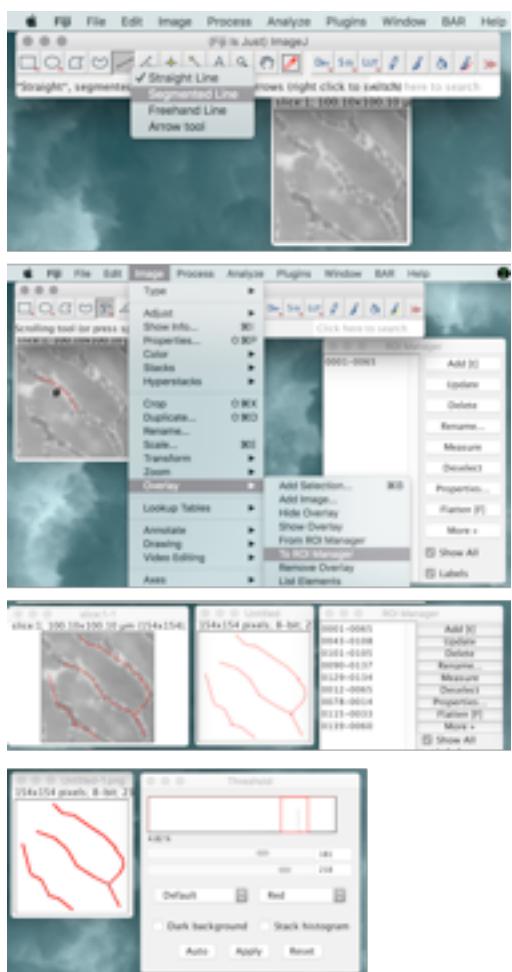
Be sure that your original image, (segmented image if made) and skeleton image are identical in size (example: all 512 x 512 pixel).

Hand editing of simple networks

Hand tracing may be the most expedient way to generate a line drawing of vasculature from a small field that is not very complex. There are multiple ways to do this. All start with a copy of a single frame image from your movie, during a period without motion.

1. The simplest approach within FIJI is to **draw directly on a copy of your single frame image using either the paint or pencil tool** in a contrasting color. While simple, this alters the underlying image.

When you have drawn your vascular network, select Image > Adjust Color Threshold and adjust sliders till the line segments are highlighted. Select Process > Binary > Make Binary, then Select Plugins > Skeleton > Skeletonize. Save as tif or png.



Supplemental Figure 18. FIJI ImageJ ROI Manager can be used to create a skeleton file.

2. **FIJI ImageJ ROI Manager** offers a simple non-destructive labeling approach by creating your line drawing as line segment overlays using the ROI manager (*Supplemental Figure 18*). Open your single frame image. Right-click on the line tool in the FIJI menu bar and select Segmented Line or Freehand Line. Draw a line segment over a vessel on your image. Select Image>Overlay>To ROI Manager and the segment will be recorded in the ROI Manager. Continue selecting segments. When done making line segments, select File>New>Image and create a blank image of the same size as your movie image. With the new blank image active, click the Show All box at the bottom of the ROI Manager. The segments will be transferred to the blank image. In the ROI Manager select Flatten. Select File>Save As> tif or png. Open the saved file and select Image >Type > 8 bit. Select Image > Adjust > Threshold and adjust sliders till the line segments are all highlighted. Select Process>Binary>Make Binary, then Select Plugins>Skeleton>Skeletonize. Save as tif or png.

3. **FIJI ImageJ TrakEM2 tools** (http://www.ini.uzh.ch/~acardona/trakem2_manual.html) enables work in a non-destructive transparent labels layer. TrakEM2 takes significant time to learn to use and is thus best used for larger more complicated networks that require iterative refinement over multiple sessions. A network that is initially generated as a difference image in FIJI can be imported as a labels layer into TrakEM and hand refined. When complete the labels layer of your line drawing or segmentation is exported as a tif (8 bit greyscale). Open the line drawing or vascular segmentation image in FIJI ImageJ. Go to Process>Binary>Make Binary, then go to Plugins>Skeleton>Skeletonize. Save as tif or png.

Additional information about Select Analysis Intervals

For selection of Analysis Intervals, the STAFF macro selects a single segment and creates a kymograph across all time points in the dataset. A kymograph is an x-t scan, where the intensity along a given line is plotted for all images of a stack. Motion of particles, in our case red blood cells (RBCs), along that line can then be tracked over time. RBC velocities can then be calculated ($\Delta x/\Delta t$) from that motion over time.

From this kymograph the user selects Analysis Intervals as described in the User Quick Guide.

Additional information about Analyze Flow and how to detect and correct for background flicker.

If you confirm that background flicker is present in your data, select the Flicker Correction option at the define analysis parameters step in configuration setup. How to detect background flicker is explained in detail below.

During analysis, for each time interval for each individual segment the STAFF macro creates a kymograph. Then each individual kymograph undergoes directionality analysis using the Fourier components method. When using the STAFF macro these steps are performed internally and not displayed. Individual kymographs are not saved. Not displaying or saving the individual kymographs cuts down on time to run the analyses and disk space used. Analysis results are sorted and saved into csv files.

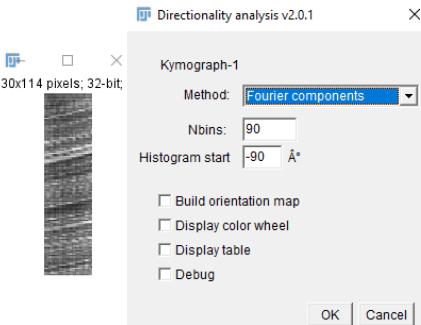
The segment_velocity.csv file contains the flow velocity results. Cells in the segment_velocity.csv file that contain the word SHORT, are segments that were shorter than the segment length analyzed. Cells that contain the word OUT (for outlier) contained values higher than the max speed measured. There should be very few cells containing OUT. If your segment velocities.csv file has a large number of OUT cells, and your max speed measured is reasonable (e.g. 1500-2000 $\mu\text{m/sec}$) then you may have a source of flicker (periodic change in light intensity). (Most of the outlier values generated from flicker are greater than 10-100,000 $\mu\text{m/sec}$.)

To identify whether flicker may be a problem in your data, examine the corresponding values to the cells containing OUT in the good_fit.csv file and the kym_ang.csv file. Corresponding values are easy to find. In segment_velocities.csv, good_fit.csv and in kym_ang.csv the column header contains the Segment ID number and the row number (minus 1 for the header row) is the Time Interval. If the goodness of fit is poor (<0.70) and the kymograph angle reported is near zero (-1 to +1), then your data may have background flicker.

To confirm that background flicker is present, generate kymographs from some of the spreadsheet cells containing OUT, with poor goodness of fit and kymograph angle near zero.

Open the movie in FIJI ImageJ

- Drag the skel_roi zipped folder onto the bottom part of the FIJI ImageJ main menu bar. The ROI manager will open and the labeled skeleton will be displayed on the movie. In the ROI manager select the segment ID number that you want to find. It will then change color in the image.
- Using the line selection tool, draw a line on that segment.
- Select Analyze>Multi-Kymograph>Multi-Kymograph. A kymograph that includes all time intervals will be generated for that segment.
- Open the intervals.csv file.
- Find the corresponding Time Interval and make a note of the first and last frame.
- In the kymograph that you just generated, use the box selection tool. First and last frame numbers from the Time Interval file are the start and stop coordinates for the area you should select in the kymograph.



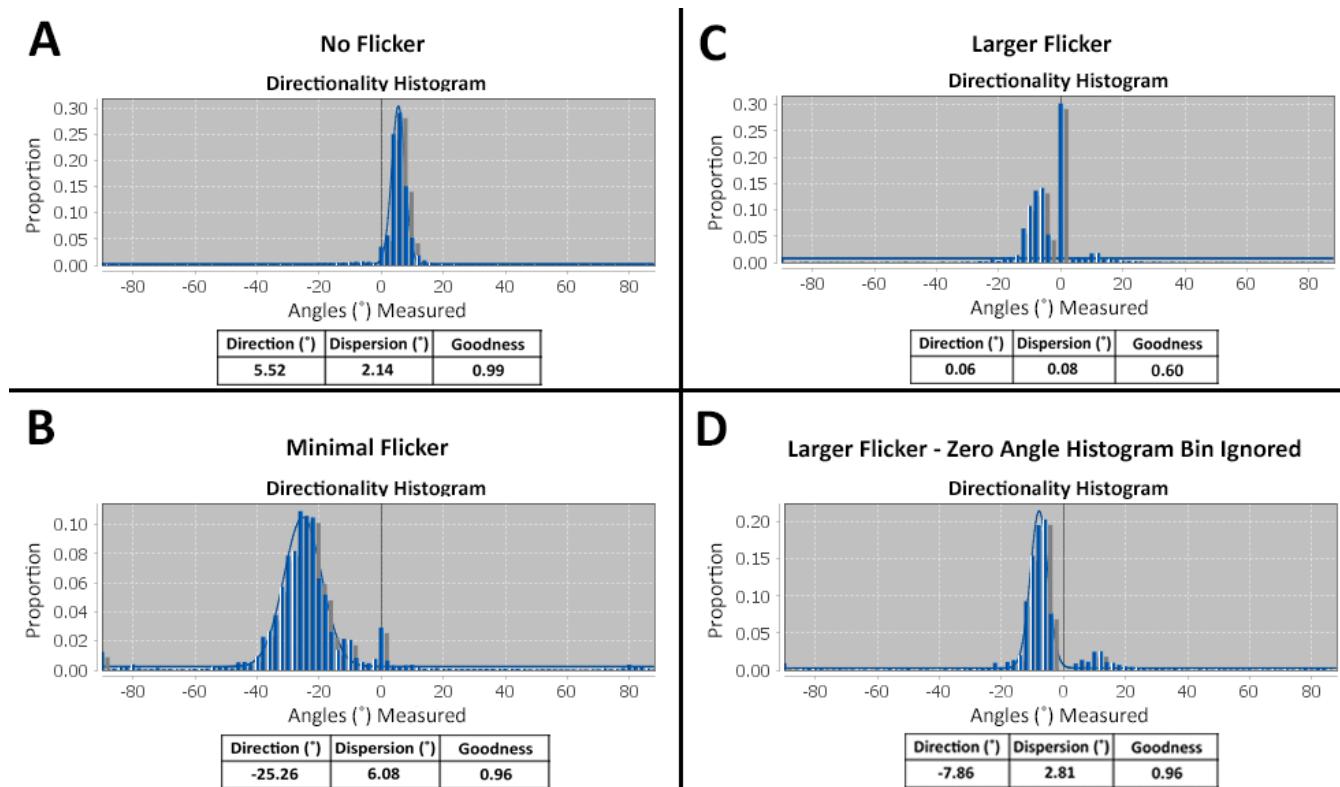
Supplemental Figure 19. Running Directionality on a single kymograph outside the

- Select **Image>Duplicate** to duplicate the kymograph for just that single Time Interval of that specific segment. Select **File>Save as>Tif** to save if desired. Give file a name that includes the image name, the segment ID and the time interval.

Next, examine the distribution of the directions of the angles in the kymographs that correspond to cell identified as having a problem with flicker (Supplemental Figure 19):

- With kymograph image open, Select **Analyze>Directionality**, then choose OK.
- Use the Fourier components method.

The output of the ImageJ Directionality plugin includes a histogram of the measured angles in the kymograph. To this histogram of measured angles a line is fitted to the highest peak and the location of the peak of the fitted line is the angle reported by the Directionality plugin. By looking at the Directionality Histogram, we can confirm whether a dataset has a peak at zero degrees from background flicker (Supplemental Figure 20).



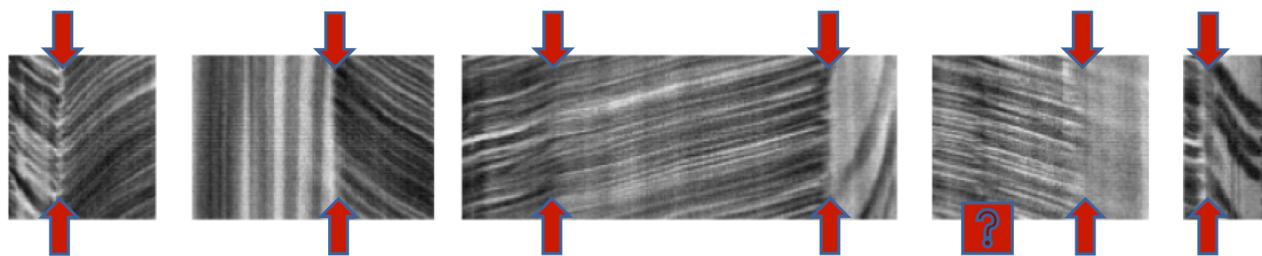
Supplemental Figure 20. Background intensity flicker can be confirmed by examining Directionality Histograms. A) Kymograph angles from movie data with no background flicker have a roughly Gaussian distribution and high goodness of fit. B) Kymograph angles from movie data with minimal background flicker have a small additional peak in the zero degree bin. Since Directionality fits to the highest peak, the output in this case is not affected. C) Kymograph angles from movie data with a larger amount of flicker can have a large additional peak in the zero degree bin. When this peak is the highest peak, the angle reported is near zero, and the calculated speed is tens to hundreds of thousands of microns/second. D) Same data as in C, but with Flicker Correction selected so that our altered Directionality plugin is used during analysis. With the zero bin data ignored, the angle reported is correct. The additional secondary peak that becomes visible in D when the zero bin is ignored is due to the presence of a hidden branch point (see text below). Goodness is the goodness of fit of the line to the histogram dis-

tribution. Amount is the amount of the data that falls under the peak. Dispersion is a measurement of how much spread there is of the data around the peak.

Other reasons for poor goodness of fit values

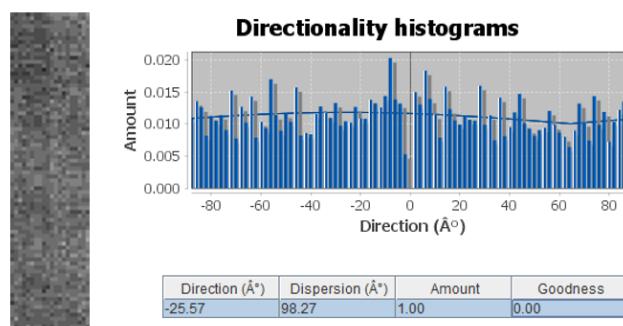
In a cell that contains a velocity value that falls within the normal range of values, poor goodness of fit can indicate the presence of multiple peaks in the directionality histogram. Multiple peaks can come from temporal speed changes within a segment or from a hidden branch point within a segment.

Hidden branch points can be easily identified by looking at the flow movie and identifying a change in flow along the segment, or by looking at the kymograph, which will contain a distinct change in angle at the location of the hidden branch point (Supplemental Figure 21). If hidden branch points are identified, make a gap in the skeleton at that location re-run “Analyze Skeleton” then re-run Analyze Flow and Produce Spatial Map.



Supplemental Figure 21. Hidden branch points can be easily identified in kymographs. Red arrows show the locations in the kymographs along the length of the segment where change in angle (which is produced by a change in flow velocity) indicates a hidden branch point.

Poor goodness of fit can also indicate that there is insufficient signal in the original image for directionality to identify a distinct angle from the kymograph. To assess whether an image sequence at a given fps and laser intensity is of sufficient quality, examine the spreadsheet of velocity values and the corresponding goodness of fit. While flow velocities do change over time, changes normally persist for a few time periods and very few fluctuate widely, rapidly and continuously. If for a given fps and laser intensity most segment velocity values fluctuate and have very poor goodness of fit, then examine corresponding directionality histograms. If the software cannot reliably discern the directionality from the kymograph, the histogram will show a random distribution of single spikes rather than the normal bell shaped distributions and have a very low goodness of fit. In this case it may be necessary to reacquire image data using either longer exposure times or higher levels of laser intensity.



Supplemental Figure 22. Kymograph and corresponding directionality histogram from an image with insufficient signal. Note low goodness of fit.