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TITLE:

Spatial Temporal Analysis of Fieldwise Flow in Microvasculature

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SUMMARY:

To quantify microvascular flow from high speed capillary flow image sequences, we developed STAFF (Spatial Temporal Analysis of Fieldwise Flow) software. Across the full image field and over time, STAFF evaluates flow velocities and generates a sequence of color-coded spatial maps for visualization and tabular output for quantitative analyses.

ABSTRACT:

Changes in blood flow velocity and distribution are vital in maintaining tissue and organ perfusion in response to varying cellular needs. Further, appearance of defects in microcirculation can be a primary indicator in the development of multiple pathologies. Advances in optical imaging have made intravital microscopy (IVM) a practical approach, permitting imaging at the cellular and subcellular level in live animals at high-speed over time. Yet, despite the importance of maintaining adequate tissue perfusion, spatial and temporal variability in capillary flow is seldom documented. In the standard approach, a small number of capillary segments are chosen for imaging over a limited time. To comprehensively quantify capillary flow in an unbiased way we developed Spatial Temporal Analysis of Fieldwise Flow (STAFF), a macro for FIJI open-source image analysis software. Using high-speed image sequences of full fields of blood flow within capillaries, STAFF produces images that represent motion over time called kymographs for every time interval for every vascular segment. From the kymographs STAFF calculates velocities from the distance that red blood cells move over time, and outputs the velocity data as a sequence of color-coded spatial maps for visualization and tabular output for quantitative analyses. In normal mouse livers, STAFF analyzes quantified profound differences in flow velocity between pericentral and periportal regions within lobules. Even more unexpected are the differences in flow velocity seen between sinusoids that are side by side and fluctuations seen within individual vascular segments over seconds. STAFF is a powerful new tool capable of providing novel insights by enabling measurement of the complex spatiotemporal dynamics of capillary flow.

INTRODUCTION:

The microvasculature plays a critical role in physiology, ensuring effective perfusion of tissues under changing conditions. Microvascular dysfunction is associated with myriad conditions including long-term cardiovascular morbidity and mortality, development of dementia, and disease of both liver and kidney and thus is a key factor of interest in a broad range of biomedical investigations[1–5]. While multiple techniques have been used to evaluate tissue perfusion, only intravital microscopy enables data collection at the temporal and spatial resolution necessary to characterize blood flow at the level of individual capillaries.

Microvascular flow can be visualized in fluorescence microscopy either by the movement of fluorescent microspheres or by the movement of red blood cells against the background of membrane-impermeant fluorescent markers (e.g., fluorescently-labeled dextran or albumin)[6, 7]. Microvascular flow can be imaged in superficial cell layers using widefield microscopy, or at depth using either confocal or multiphoton microscopy. However, capillary flow rates are such that the passage of red blood cells cannot generally be captured at speeds less than 60 frames per second. Since most laser scanning confocal and multiphoton microscopes require 1–5 s to scan a full image field, this speed can generally be accomplished only by limiting the field of view, sometimes to a single scan line[8]. The process of limiting measurements to selected capillary segments (1) has the potential to introduce selection bias and (2) makes it impossible to capture spatial and temporal heterogeneity in the rates of capillary blood flow. In contrast, images of capillary networks can be collected at speeds exceeding 100 fps using widefield digital microscopes equipped with scientific complementary metal oxide semiconductor (sCMOS) cameras[9, 10]. These inexpensive systems, common in typical biomedical laboratories make it possible to image microvascular flow across entire two-dimensional networks, essentially continuously. The problem then becomes one of finding an analysis approach that is capable of extracting meaningful quantitative data from the massive and complex image datasets generated by high-speed video microscopy.

To enable analysis of full-field flow data we have developed STAFF, a novel image analysis software that can continuously measure microvascular flow throughout entire microscope fields of image series collected at high speed[11]. The approach is compatible with a variety of different experimental systems and imaging modalities and the STAFF image analysis software is implemented as a macro toolset for the FIJI implementation of ImageJ[12]. The underlying principle used here to visualize microvascular flow is that first, some contrast must be provided to be able to image the red blood cells within capillaries. In our studies, contrast is provided by a bulk fluorescent probe that is excluded by the red blood cells. The velocity of flow can then be quantified from the displacement of the red blood cells that appear as a negative stain within the fluorescently labeled plasma in images collected at high speed from a living animal[8]. We then use STAFF to make plots of distance along each capillary segment over multiple intervals of time called kymographs, then detect the slopes present in the kymographs[13], and from those slopes calculate the rates of microvascular flow. The approach can be applied to images collected from any capillary bed that can be accessed for imaging. Here we describe the application of IVM and STAFF to studies of blood flow in the liver.

PROTOCOL:

All animal experiments were approved and conducted according to the Institutional Animal Care and Use Committee guidelines of Indiana University, and adhered to the NRC guide for the care and use of animals.

1. Surgical preparation for intravital microscopy

Note: This is not a survival surgery. Once section 1 “Surgical preparation for intravital microscopy” is begun, work cannot be paused until the completion of section 2 “Intravital microscopy”.

1.1. Acclimatize 9–10-week-old male C57BL/6 mice, for at least 4 days and fast for 16 h prior to studies.

1.2. Weigh the animal, sedate with 5% isoflurane and place on a heating pad to maintain body temperature. Use an oxygen flow rate of 1–2 L/min. Check reflexes by toe pinch. Monitor temperature using a rectal thermometer. Monitor heart rate and respiration visually.

1.3. When anesthesia is stable, shave the area for jugular cannulation placement and the area below the rib cage for the exposure of the liver.

1.4. For jugular cannulation, make a 1 cm left ventral incision 1–2 cm below the mouse’s jaw. Clear away all fat and fascia surrounding the jugular vein. Tie off the anterior end of the jugular using 06 suture string to prevent bleeding.

1.4.1. Make a tiny nick in the jugular vein and slide the cannula (30 G x ½ in, needle), needle holder and polyethylene tubing (0.011 in. x 0.024 in. attached to a Luer stub adapter and filled with 0.9% saline) in about 1 cm, and secure at the posterior end of the jugular using 0-6 suture string.

1.5. Using the jugular cannula deliver 70 kDa fluorescein dextran (to a dose of 30 mg/kg via injection of 0.1 mL of a 9 mg/mL solution in saline).

1.6. Expose the liver for imaging by making a 4–6 cm incision across the torso, 1–2 cm below the middle of the rib cage.

1.7. Place a wet (soaked in 0.9% saline) 2 in. x 2 in. gauze sponge below the left lateral liver lobe. Place tape on the periphery of the glass window of a 40 mm coverslip-bottomed dish and apply cyanoacrylate adhesive to the tape. Press the glass plate to the liver and using cotton tipped applicators press the gauze into the glue on the tape to minimize tissue motion for microscopy.

1.8. Move animal to the microscope stage. Add sterile 0.9% saline into the coverslip-bottomed dish to keep the liver moist throughout the imaging session. Maintain temperature at 36–37 °C via heating pads mounted on the stage, a heating pad placed over the animal and an objective heater.

2. Intravital microscopy

2.1. Perform intravital microscopy on an inverted epifluorescence microscope equipped with a video camera capable of high-speed image capture.

NOTE: Here, a Xenon arc lamp, fluorescein-specific excitation (480–500 nm) and emission (507–543 nm) filters, Plan Fluor 20X, N.A. 0.75 water immersion objective, and a high-speed sCMOS camera were used.

2.2. Select the area of interest for capillary blood flow analysis using minimal illumination.

2.3. Set the exposure time short enough for the camera to be able to acquire 100 fps. Set illumination level to clearly visualize red blood cell shadows and the topology of the vasculature while also avoiding phototoxicity and photobleaching of the fluorescent probe.

2.4. Collect images at a rate of 100 fps. Set the camera pixel resolution between ~0.65 and ~1.3 µm/pixel. Set frame size between 512 x 512 and 1024 x 1024 pixels. Set bit depth between 8 and 16 bits. Use the highest resolution, frame size and bit depth that still allows 100 fps image collection on the system.

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2.5. Save time series file(s) as a sequence of TIF files or as native camera/microscope format if Bio-Formats[14] in FIJI can open the native format files.

2.6. Image multiple areas over time. Maintain the mouse on the microscope stage for up to two hours. Euthanize the mouse at the end of imaging.

NOTE: The duration of thetime series will be based upon balancing the need to image for an interval of time that embraces the variability, while not affecting the viability of the tissue. The duration of the time series may also be dictated by file size considerations; a 1 min time series of 1024 x 1024 pixel 16-bit images collected at 100 fps will generate a file that is 12 GB in size. File size considerations may also dictate frame size and bit depth used. There are no explicit size limits within STAFF.

3. Define the vascular network using TrakEM2 in FIJI

NOTE: The protocol can be paused after saving work at any point in section 3.

3.1. Download and install FIJI from https://imagej.net/Fiji/Downloads.

NOTE: Version 1.51n of Fiji was used for this project, and can be downloaded from https://downloads.imagej.net/fiji/Life-Line/fiji-win64-20170530.zip. Note that on Windows, FIJI should be installed in the user space and not in Program Files.

3.2. Open the movie file in FIJI using **File** > **Open** or using **File** > **Import** > Image **sequence**. Select a single image from the stable images between respirations where the topology of the vascular network is easy to see. Select **Image** > **Duplicate** and duplicate the selected single image (not the stack) and save the image in a new folder. Do not include spaces in file or folder names.

3.3. Set up a new blank TrakEM2 project by selecting **File** > **New** > **TrakEM2 (blank)**. Select the new folder containing the single image from the movie as the project folder. The TrakEM2 windows will open.

3.4. Right-click in the main work area and select **Import** > **Import Image**. Navigate to the single image saved in step 3.3 and select it.

3.5. Right-click in the main work area again and select **Display** > **Autoresize canvas/Layer set**. Left-click in the main work window. The image will fill the work area.

3.6. To select the areas in the image that contain the vascular network, setup area list selection in TrakEM2. In the smaller TrakEM2 window (Template, Project Objects, Layers) right-click on “**• anything**”. Select **Add new child > area list**.

3.7. In the smaller TrakEM2 window, drag the "**Template** > **anything** > **• area list**" icon onto “**Project Objects** > **project**”. Under **Project Objects** “**Project** > **anything** > **• area list**” is now present.

3.8. In the main TrakEM2 window under the **Z space tab**, a bar labeled area list was created. Click to select it. In the main TrakEM2 window also select the **paintbrush tool**. Press **Shift** and roll the mouse wheel to select an appropriate size for the paintbrush, e.g., smaller than the diameter of the vasculature. To save the TrakEM2 setup press **Control + S** keys.

3.9. Paint the vascular network using the **paintbrush tool**. To erase hold down **Alt** while using the paintbrush tool. Do not include out-of-focus regions. Save often using **Control + S**.

3.10. When labeling of the vascular network is complete, right-click in the main TrakEM2 window and select **Export** > **AreaLists as labels** (tif). In the popup window select **Scale 100%** and **Export All area list**. Close TrakEM2 windows and choose **yes** for **Save project**.

3.11. The image of AreaLists will open and may appear as a blank black image. In the main FIJI Menu select **Image** > **Adjust** > **Brightness/Contrast**. In the B&C window press the **auto button** and the AreaList will become visible. In the main FIJI menu select **Image** > **Lookup Tables** > **Invert LUT**. Save this image of black labels on white background and close the image.

3.12. Open the labels file in FIJI. Select **Plugins** > **Skeleton** > **Skeletonize**. Save the skeletonized image as a tif. Use the skeleton.tif file as one of the input files needed to run the STAFF flow analysis. When saving the file, do not use spaces in file name.

3.13. Manually edit the skeleton file as needed, by placing a gap (using the **paint tool**) in the line drawing for example at locations where branches in the vasculature have not been captured because they go out of the image plane.

4. Prepare the movie sequence for STAFF analysis

4.1. Open the movie file in FIJI using **File** > **Open or File** > **Import** > **Image sequence**. Select a time period of the image sequence for flow analysis (can be seconds to minutes, hundreds to tens of thousands of frames) where tissue position has remained stable over time, except for during respiration.

4.2. Select **Image** > **Duplicate** and duplicate the selected portion of the sequence by typing in the numbers of the beginning and end frames. Check image spatial and temporal calibration under **Image** > **Properties** and correct if necessary. Save the image file as a TIF (or as an uncompressed AVI). This movie.tif is needed as an input file to run STAFF analysis. Do not include spaces in file name.

5. Install STAFF macros into FIJI

NOTE: (**Important**) Multiple folders within the FIJI folder subdirectories have similar file names, some capitalized, some not. Be certain that the correct folders are selected when installing STAFF.

5.1. Download the STAFF macros from https://github.com/icbm-iupui/STAFF.

5.2. To install, first open the FIJI folder.

5.2.1. On Windows find the folder where FIJI is installed in the user space, most commonly on the desktop. On MacOS find the FIJI icon in the Applications folder, right click and select **Open**.

5.3. In the 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.

5.4. In the FIJI folder, open the plugins folder. Copy STAFF_Dir.jar into this plugins folder: Fiji\plugins\STAFF_Dir.jar.

5.5. In the 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.

5.6. Start FIJI and verify macro installation. To verify installation, select **Plugins** > **Macros**. When correctly installed the dropdown menu will include: Open-Create Project, Analyze Skeleton, Edit Time Intervals, Analyze Flow and Produce Spatial Map.

5.7. If these commands are not present in the Macros menu, select **Plugins** > **Macros** > **Install**, open the Fiji\plugins\Macros folder, select the STAFF.ijm file, then click the **OK** button. The commands should now appear in the **Plugins** > **Macros** menu.

6. Quantifying vascular flow using STAFF

6.1. Create a new project.

6.1.1. Select **Plugins** > **Macros** > **Open-Create Project** and follow prompts to create or update a configuration file.

6.1.2. From the **Open-Create Project** menu, navigate to and select the Project Directory, Input File Folder and the input files Movie.tif and Skeleton.tif. Edit the auto-populated output file names if necessary.

6.1.3 Input values for shortest segment length (20 µm), max speed measured (2000 µm/s) and max speed mapped (1000 µm/s).

NOTE: The product of shortest segment length and frames per second gives the max flow speed that can theoretically be measured. The highest capillary flow speeds reported in literature are around 2000 µm/s[15]. In the liver, we observed that capillary flow speeds generally averaged around 300 µm/s and rarely exceeded 1000 µm/s.

6.1.4. Type in the values for pixel size and frame rate for the image sequence (from image metadata or experimental notes). These values require user input. Check the **Flicker Correction** box if the images have periodic background intensity flicker.

6.1.5. Select parameters for best visualization of the data. Select max speed mapped to include about 95% of the data so that the data is mapped across the full range of the color scale. STAFF maps high speed outliers to the high-speed end of the color scale.

6.2. Analyze the skeleton.

6.2.1. Select **Plugins** > **Macros** > **Analyze Skeleton**. The skeleton.tif file will open and the Region of Interest (ROI) Manager will open and run.

NOTE: Analyze Skeleton classifies each pixel by its number of neighbors as either at an endpoint, at a junction, or within a segment. For each segment an identifying number (ID number), a calibrated branch length and the spatial coordinates of the branch ends are recorded.

6.2.2. Wait for segment IDs to appear on the skeleton, the segment list to appear in the ROI manager, and a popup indicating that ROI Manager file for the skeleton is saved. Click **OK**.

6.2.3. Verify that between respiration movements, the skeleton remains over where red blood cells pass through the capillary (e.g., not the edge and not outside the capillary). Display the labeled segments as an overlay on the movie, by opening the movie file, then dragging the ROI zip file onto the open movie. Play the movie with the segments overlaid.

6.3. Select time intervals.

6.3.1. Select **Plugins** > **Macros** > **Select Time Intervals**. Wait while the macro generates a kymograph from a single segment over the total time for the movie. From this kymograph, the user selects time intervals for analysis. Use **Control** + **+** keys to increase the size of the kymograph if needed.

6.3.2. The rectangle selection tool is automatically activated. Draw a rectangle around each time interval. A good starting point for time interval length is the 1–2 s interval between respirations, which are apparent as horizontal blurred regions in the kymograph.

6.3.3. Click the **T** key or the **Add** button in the ROI manager to record time interval selections. Repeat for as many time intervals as desired. Make selections sequentially from the top (or left) to bottom (or right) of the kymograph.

6.3.4. Evaluate parameter choices (from Open-Create Project) by selecting a small number of time intervals (3 to 4) and completing analysis for just those intervals, since Analyze Flow (the next step) can take hours for large datasets.

6.3.5. Click **OK** in the **Make an ROI** for each time interval window when done. A popup window appears when the selected intervals have been saved in the **Project Folder**. Click **OK**.

6.4. Analyze flow.

6.4.1. Select **Plugins** > **Macros** > **Analyze Flow** and the **Analyze Flow Parameters** dialogue box opens and displays the values entered in the Open-Create Project step. Edit if needed then click **OK**.

6.4.2. The **Output File Names** dialogue box opens and displays the names entered in the Open-Create Project step. Edit if needed then click **OK**.

6.4.3. A dialogue box opens asking if the user is ready to begin analysis. Click **OK** and the analysis will begin. Wait for a dialog box that opens to indicate when Flow Analysis is complete.

NOTE: Time needed depends on processor speed and size of the dataset.

6.4.5. Confirm that results files have been stored as .csv format spreadsheet files in the Project Folder.

NOTE: Output files include: *segment_velocities.csv* contains velocity values that have positive and negative values indicating flow direction. Cells representing segments less than the minimum length contain the text SHORT. Cells with velocity values greater than the maximum measured speed contain the text OUT (for outlier). *kym_ang.csv* contains kymograph angles measured by the Directionality plugin in FIJI. *good_fit.csv* contains goodness of fit of a curve to the distribution of angles measured from each kymograph. Poor goodness of fit (< 0.8) can indicate hidden branch points in a segment, change in velocity during the time interval, lamp or room light flicker.

6.4.6. Check to verify that segment_velocities.csv contains few OUT values. If there are a large number of OUT values, verify that the minimum segment length to analyze is not less than 15–20 µm, then rerun **Analyze Flow**. If the new segment_velocities.csv still contains a large proportion of OUT values, then check the **flicker correction** box, then rerun **Analyze Flow**.

6.5. Produce spatial maps.

6.5.1. Select **Plugins** > **Macros** > **Produce Spatial Map** and the **Spatial Map Parameters** window will open, displaying the values entered in the Open-Create Project step. Edit if needed, or to continue select **OK**.

6.5.2. Watch as a temporal sequence of spatial maps of flow velocities is generated with color indicating flow speed. The output is in the form of a .tif image stack with one image for each time interval. Scroll through the .tif stack to visualize spatial and temporal variation in flow. Save the stack as an AVI file (uncompressed for maximum portability) to share as a movie.

7. Quantitative analysis using STAFF output

7.1. Open the segment_velocities.csv file in a spreadsheet or statistical analysis program. These values have positive or negative sign that indicates direction of flow relative to the start/stop points of that segment (recorded in the Segment ROI file).

7.2. Make a new spreadsheet page containing the absolute values of all the velocity values. Use these values to calculate overall average flow velocity, average flow velocity for all vascular segments over time, flow velocity for each vascular segment over time, and make histograms of velocity distributions.

7.3. Calculate values around specific morphological regions, or other regions of interest by first finding the specific relevant segments in the labeled skeleton and then performing analyses on these selected segments.

REPRESENTATIVE RESULTS:

STAFF analysis generates a complete census of microvascular velocities across entire microscope fields over periods of time extending from seconds to minutes. Representative results are presented in **Figure 1**, **Figure 2**, **Figure 3**, and **Figure 4**. **Figure 1** shows an example of a time series of the microvascular network in the liver of a mouse, the generation of the skeletonized image that is used to define the axis of microvascular flow, and the STAFF-generated map of individual vascular segments identified for quantification. STAFF then uses the skeletonized image to break the microvascular network down into individual segments, then generates images of kymographs for each segment. These images are provided to the user, along with tools to identify the time intervals to be used for kymograph analysis (**Figure 2**). STAFF then uses the skeleton, and the user-supplied time intervals to break the kymograph of each segment into individual segment-time intervals. STAFF then identifies the predominant angle in the kymograph from each segment-time interval and provides velocity measurements as .csv data files (**Figure 3**) and in the form of stacks of color-coded velocity map images (**Figure 4**). In order to support exploration of the data analysis pipeline, STAFF also provides .csv files containing all kymograph angle measurements and goodness-of-fit values.

FIGURE AND TABLE LEGENDS:

Figure 1: Generating the vascular skeleton. (**A**) Original image of a single frame from time series images collected from the liver of a living mouse. Bar = 100 µm. (**B**) Image shown in Panel A with central veins (CV) and portal triads (PT) indicated, to identify main directions of sinusoid flow. (**C**) Image from Panel A with overlay of TrakEM2 segmentation of sinusoids. (**D**) Binary image of TrakEM2 segmentation. (**E**) Skeletonization of TrakEM2 segmentation. (**F**) STAFF output image of individual vascular segments with labels.

Figure 2: Kymograph analysis. (**A**) Magnified image of vascular segments shown in **Figure 1F**. (**B**) Typical kymograph from one segment collected over two inter-respiratory time intervals. Periods of respiration are noted with arrows. (**C**) Kymographs for segments 240, 252 and 254 from panel A.

Figure 3: Velocity data analysis. (**A, B**) Tables of velocity measurements of four segments over 19 time intervals expressed either as velocity with direction (**A**) or as absolute velocity (**B**). (**C**) Histogram showing distribution of absolute velocity values across the entire field over the entire 20 s time period. (**D**) Graph of the velocities of the three segments whose kymographs are shown in **Figure 2C** over the entire 20 s period.

Figure 4: Velocity map. (**A**) STAFF output image of the color coded velocity map for a single time interval for the field shown in **Figure 1**. (**B**) Composite of velocity maps for all time intervals presented as a 3D volume.

DISCUSSION:

There are multiple critical steps in this protocol. First, minimization of motion during intravital imaging of the liver is essential for generating movies that are usable for capillary flow analysis using STAFF. Due to the proximity of the diaphragm, short periods of respiration-induced motion occur, with the secured liver returning to its initial position after each breath. Securing the surgically exposed liver against the coverslip-bottomed dish using gauze, then imaging from below using an inverted microscope serves to immobilize the organ between respirations[16–19]. Second, an image acquisition speed of 100 fps is strongly recommended because the speed of flow that can be measured is a function of the speed of image acquisition and the minimum length of the segments in which flow is measured[11]. Third, producing a high-quality skeleton, the line drawing representation of the vascular network, is the next critical step in obtaining capillary flow velocities using STAFF. Skeleton line segments should lie near the midpoint of the vasculature between respirations over the time course being analyzed and vascular branching out of the image plane should be identified. The locations of these hidden branches along a vascular segment can be inferred by viewing the movie, by examining the kymograph or examining the velocity values over time for that segment. Viewing the movie, these vascular segments are seen either as having bidirectional flow, emanating away from or converging towards the location of the unseen branch, or as having an abrupt change in flow speed at that point along the vascular segment. The location of hidden branch points can be identified in kymographs as the location of a change in angle that is produced by the change in flow direction or velocity. In the spreadsheet, vascular segments with hidden branch locations may spuriously change direction (sign) or change between the values of the two contributing segments. If the time series includes too many branch points that are out of the image plane, STAFF analysis should be repeated after manually editing the skeleton, by placing a gap (using the paint tool) in the line drawing at the locations of the missed branch points.

We uncovered a common issue in image acquisition that typically goes unnoticed but had a strong effect on measuring flow velocities using STAFF. In image acquisition, instability or “flicker” in the intensity of the epi-illumination lamp light source or from area lighting in the microscope room can occur. From either source, light/dark banding over time with the period of the flicker occurs at zero angle in the kymographs. If the zero angle peak is the major peak, then even if the angle produced by motion of red blood corpuscles through the vessel where flow speed is being measured is obvious to the human eye, the directionality plugin fits a curve to the zero peak and reports a value very near zero degrees, resulting in velocity measurements that are unfeasibly high. Even if the zero-angle peak is not the major peak, it will influence the directionality curve fit such that the velocity reported is shifted toward a higher value. To address this problem, STAFF provides a “Flicker correction” option that ignores peak angles occurring at 0°. This modification eliminated the effects of lamp flicker without affecting velocity quantifications.

The main limitation of obtaining flow velocities using widefield microscopy is that image acquisition is restricted to thin preparations such as mesenteric vasculature, or the zebrafish intersegmental vessels or the superficial layers of organs such as liver or kidney that can be exteriorized.

A significant advantage of using STAFF over existing methods of flow quantification is that it enables rapid and unbiased detection of spatial and temporal patterns of vascular flow. Collection and analysis of microvascular flow data using raster scanning systems are generally limited to measurements of single user selected capillaries at a time[20–23]. Methods exist to extract flow velocities across fields[24–26], however none of these approaches support analysis across entire fields and all are labor intensive. Using STAFF, analysis of every capillary segment over time across the entire image field of a dataset with tens of thousands of images can be accomplished within a day. Manual analysis of even a single dataset of full-field vascular flow would take months to years. Thus, manual analysis is impractical even for characterization of normal flow and clearly does not permit comparison of multiple treatment groups.

The ease of STAFF quantification of spatiotemporal patterning of vascular flow provides the opportunity for future users to link vascular morphological observations to effects on capillary flow velocity patterning. By defining the relationship between the vascular morphology measures of vessel diameters and network topology to flow velocity patterning we may then be able to predict flow patterns from vascular morphology. Similarly, correlating vascular flow patterning and vascular morphology with events such as timing, localization and extent of immune cell infiltration, tissue damage from toxicant exposure or disease, or status of intracellular transport, would not only give us a better understanding of flow patterning and cellular function in health and disease, but would also provide a framework for identifying particularly harmful pathophysiological scenarios.

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The authors report no competing interests.

REFERENCES:

1. Shen, J. et al. Subclinical Vascular Dysfunction Associated with Metabolic Syndrome in African Americans and Whites. *The Journal of clinical Endocrinology and Metabolism*. **100** (11),

4231–9, doi: 10.1210/JC.2014-4344 (2015).

2. Sherman, I.A., Pappas, S.C., Fisher, M.M. Hepatic microvascular changes associated with development of liver fibrosis and cirrhosis. *American Journal of Physiology-Heart and Circulatory Physiology*. **258** (2), H460–H465, doi: 10.1152/ajpheart.1990.258.2.H460 (1990).
3. Zafrani, L., Ince, C. Microcirculation in Acute and Chronic Kidney Diseases. *American Journal of Kidney Diseases*. **66** (6), 1083–1094, doi: 10.1053/J.AJKD.2015.06.019 (2015).
4. Nielsen, R.B. et al. Capillary dysfunction is associated with symptom severity and neurodegeneration in Alzheimer's disease. *Alzheimer's & Dementia*. **13** (10), 1143–1153, doi: 10.1016/J.JALZ.2017.02.007 (2017).
5. Houben, A.J.H.M., Martens, R.J.H., Stehouwer, C.D.A. Assessing Microvascular Function in Humans from a Chronic Disease Perspective. *Journal of the American Society of Nephrology*. **28** (12), 3461–3472, doi: 10.1681/ASN.2017020157 (2017).
6. Clemens, M., Zhang, J. Regulation of Sinusoidal Perfusion: In Vivo Methodology and Control by Endothelins. *Seminars in Liver Disease*. **19** (04), 383–396, doi: 10.1055/s-2007-1007127 (1999).
7. Uhlmann, S., Uhlmann, D., Spiegel, H.U. Evaluation of hepatic microcirculation by in vivo microscopy. *Journal of investigative surgery : the official journal of the Academy of Surgical Research*. **12** (4), 179–93 (1999).
8. Dunn, K.W., Sutton, T.A., Sandoval, R.M. Live-animal imaging of renal function by multiphoton microscopy. *Current protocols in cytometry*. **Chapter 12**, Unit12.9, doi: 10.1002/0471142956.cy1209s62 (2012).
9. von Diezmann, A., Shechtman, Y., Moerner, W.E. Three-Dimensional Localization of Single Molecules for Super-Resolution Imaging and Single-Particle Tracking. *Chemical Reviews*. **117** (11), 7244–7275, doi: 10.1021/acs.chemrev.6b00629 (2017).
10. Huang, Z.-L. et al. Localization-based super-resolution microscopy with an sCMOS camera. *Optics Express*. **19** (20), 19156, doi: 10.1364/OE.19.019156 (2011).
11. Clendenon, S.G. et al. A simple automated method for continuous fieldwise measurement of microvascular hemodynamics. *Microvascular Research*. **123**, 7–13, doi: 10.1016/j.mvr.2018.11.010 (2019).
12. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods*. **9** (7), 676–682, doi: nmeth.2019 [pii]10.1038/nmeth.2019 (2012).
13. Liu, Z.-Q. Scale space approach to directional analysis of images. *Applied Optics*. **30** (11), 1369, doi: 10.1364/AO.30.001369 (1991).
14. Linkert, M. et al. Metadata matters: access to image data in the real world. *The Journal of cell biology*. **189** (5), 777–82, doi: 10.1083/jcb.201004104 (2010).
15. Sherman, I.A., Pappas, S.C., Fisher, M.M. Hepatic microvascular changes associated with development of liver fibrosis and cirrhosis. *American Journal of Physiology-Heart and Circulatory Physiology*. **258** (2), H460–H465, doi: 10.1152/ajpheart.1990.258.2.H460 (1990).
16. Babbey, C.M. et al. Quantitative intravital microscopy of hepatic transport. *IntraVital*. **1** (1), 44–53, doi: 10.4161/intv.21296 (2012).
17. Dunn, K.W., Ryan, J.C. Using quantitative intravital multiphoton microscopy to dissect hepatic transport in rats. *Methods*. **128**, 40–51, doi: 10.1016/j.ymeth.2017.04.015 (2017).
18. Ryan, J., et al. Intravital Multiphoton Microscopy with Fluorescent Bile Salts in Rats as an In Vivo Biomarker for Hepatobiliary Transport Inhibition. *Drug metabolism and disposition: the biological fate of chemicals*. **46** (5), 704–718, doi: 10.1124/dmd.117.079277 (2018).

19. Sandoval, R.M., Molitoris, B.A. Quantifying Glomerular Permeability of Fluorescent Macromolecules Using 2-Photon Microscopy in Munich Wistar Rats. *Journal of Visualized Experiments*. (74), 2–7, doi: 10.3791/50052 (2013).

20. Chhatbar, P.Y., Kara, P. Improved blood velocity measurements with a hybrid image filtering and iterative Radon transform algorithm. *Frontiers in Neuroscience*. **7**, 106, doi: 10.3389/fnins.2013.00106 (2013).

21. Drew, P.J., Blinder, P., Cauwenberghs, G., Shih, A.Y., Kleinfeld, D. Rapid determination of particle velocity from space-time images using the Radon transform. *Journal of computational neuroscience*. **29** (1–2), 5–11, doi: 10.1007/s10827-009-0159-1 (2010).

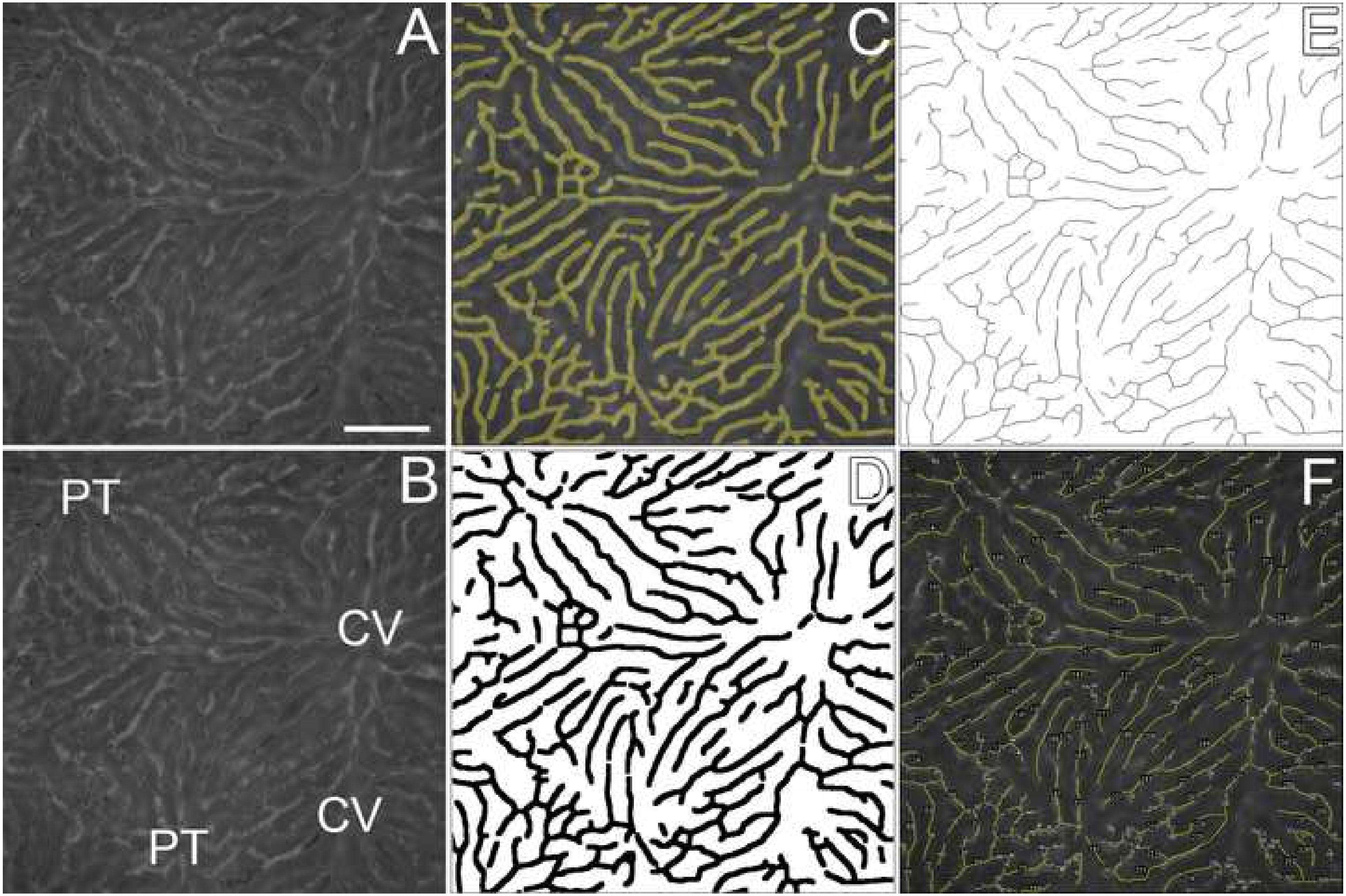
22. Kleinfeld, D., Mitra, P.P., Helmchen, F., Denk, W. Fluctuations and stimulus-induced changes in blood flow observed in individual capillaries in layers 2 through 4 of rat neocortex. *Proceedings of the National Academy of Sciences of the United States of America*. **95** (26), 15741–6, at <http://www.ncbi.nlm.nih.gov/pubmed/9861040> (1998).

23. Dasari, S., Weber, P., Makhloufi, C., Lopez, E., Forestier, C.-L. Intravital Microscopy Imaging of the Liver following Leishmania Infection: An Assessment of Hepatic Hemodynamics. *Journal of visualized experiments : JoVE*. (101), e52303, doi: 10.3791/52303 (2015).

24. Hoshikawa, R. et al. Dynamic Flow Velocity Mapping from Fluorescent Dye Transit Times in the Brain Surface Microcirculation of Anesthetized Rats and Mice. *Microcirculation (New York, N.Y. : 1994)*. **23** (6), 416–25, doi: 10.1111/micc.12285 (2016).

25. Sironi, L. et al. In vivo flow mapping in complex vessel networks by single image correlation. *Scientific reports*. **4** (1), 7341, doi: 10.1038/srep07341 (2014).

26. Kamoun, W.S. et al. Simultaneous measurement of RBC velocity, flux, hematocrit and shear rate in vascular networks. *Nature Methods*. **7** (8), 655–60, doi: 10.1038/nmeth.1475 (2010).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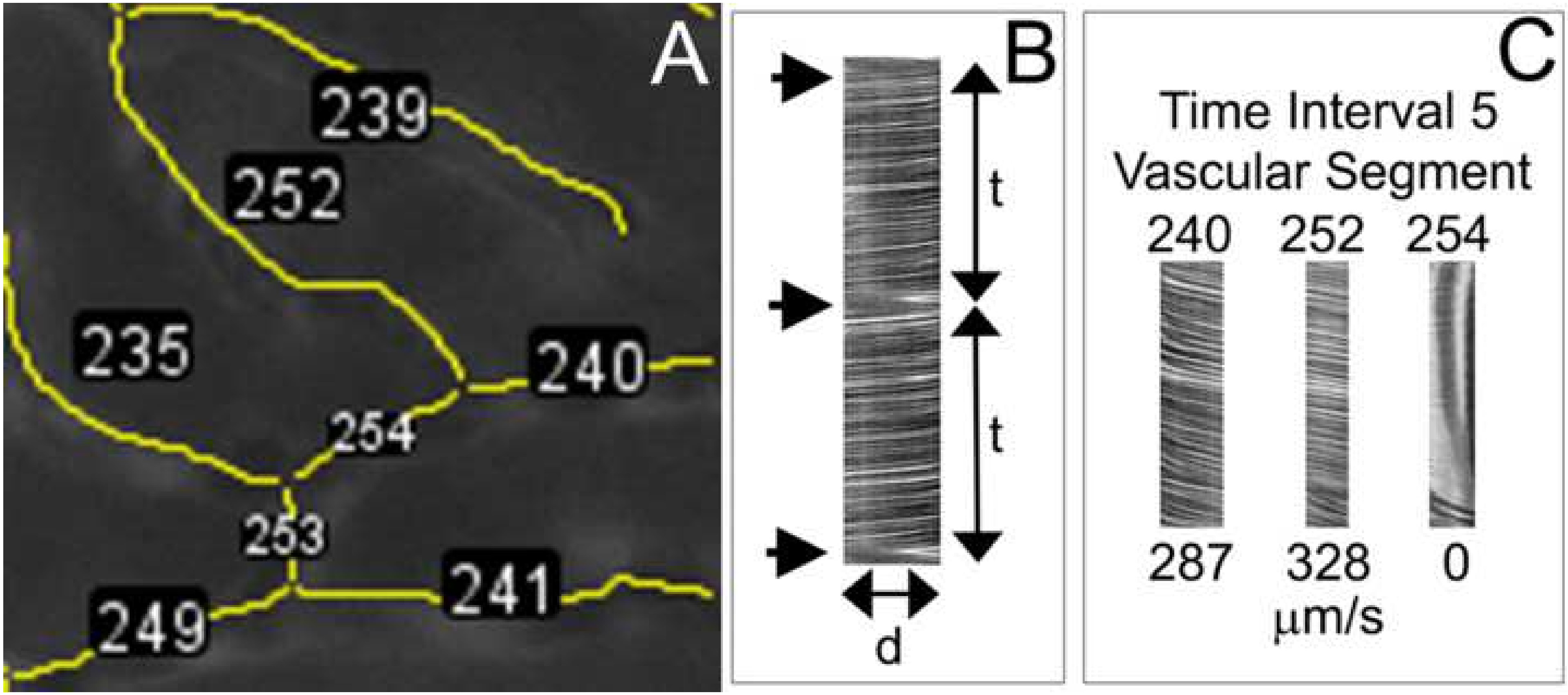
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Vascular Segment
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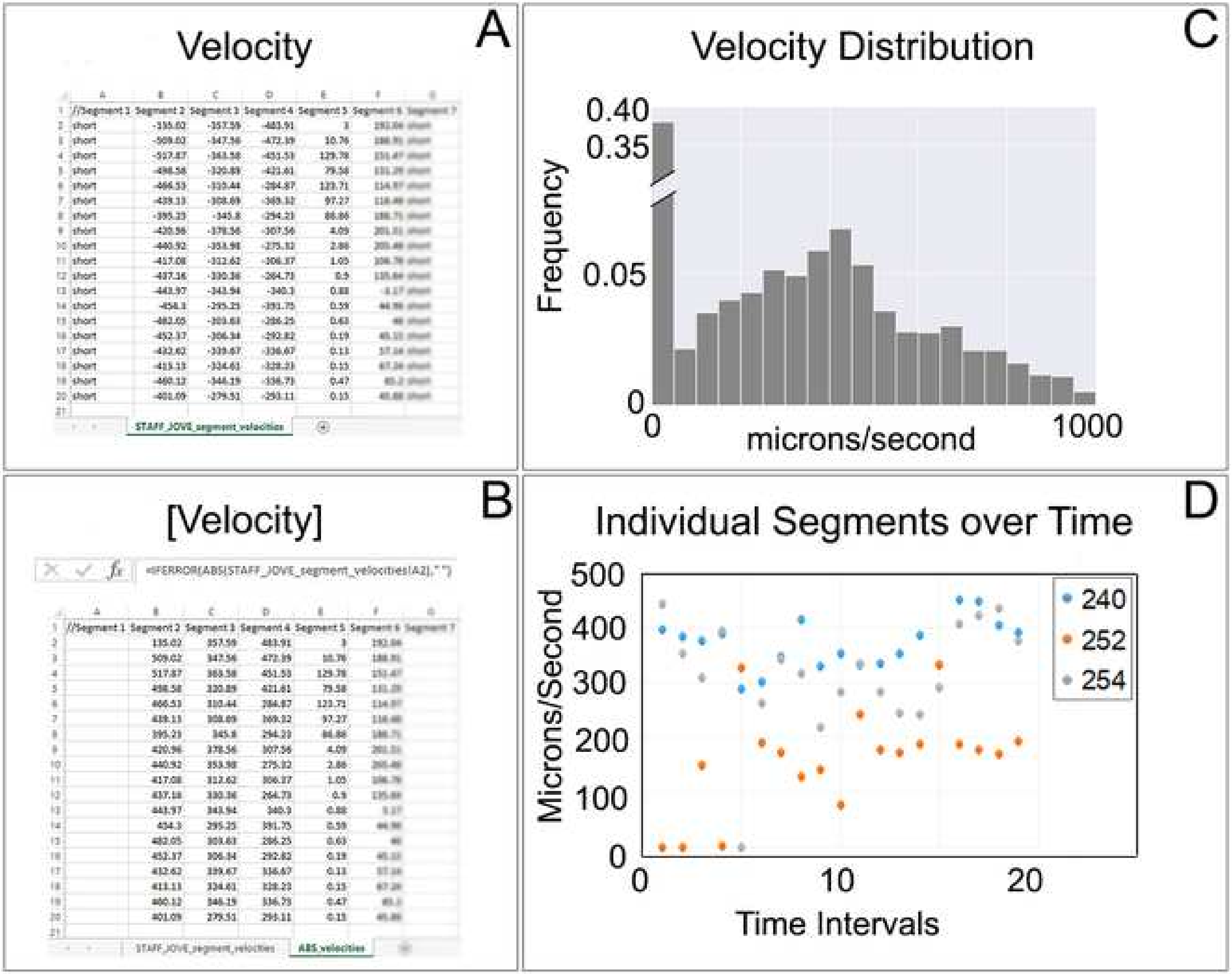
328
0
µm/s

Figure03



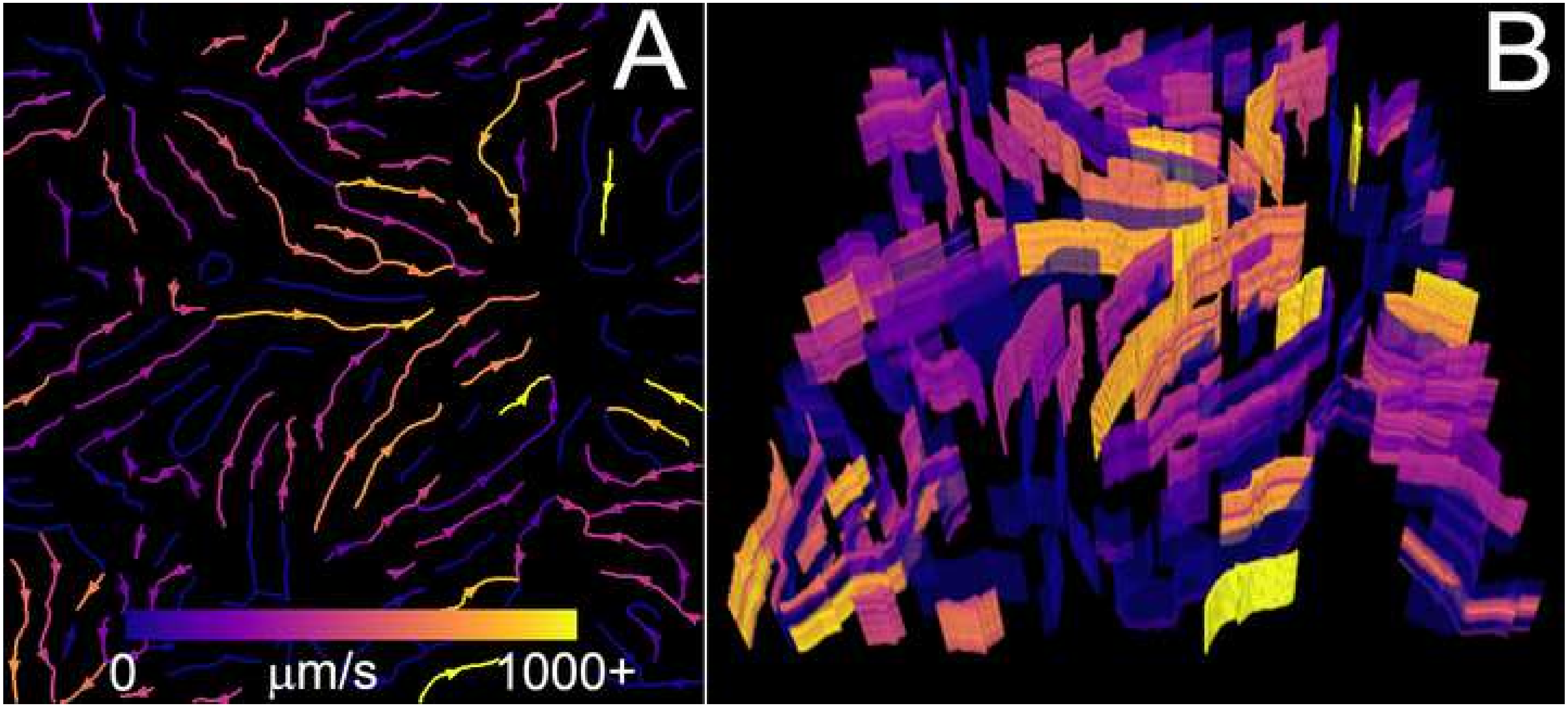
	A	B	C	D	E	F	G
1	//Segment 1	Segment 2	Segment 3	Segment 4	Segment 5	Segment 6	Segment 7
2	short	-135.02	-357.59	-483.91	3	192.04	short
3	short	-509.02	-347.56	-472.39	10.76	188.91	short
4	short	-517.87	-363.58	-451.53	129.78	151.47	short
5	short	-498.58	-320.89	-421.61	79.58	131.29	short
6	short	-466.53	-310.44	-284.87	123.71	114.97	short
7	short	-439.13	-308.69	-369.32	97.27	116.48	short
8	short	-395.23	-345.8	-294.23	88.86	186.71	short
9	short	-420.96	-378.56	-307.56	4.09	201.21	short
10	short	-440.92	-353.98	-275.32	2.86	205.48	short
11	short	-417.08	-312.62	-306.37	1.05	106.78	short
12	short	-437.16	-330.36	-264.73	0.9	135.64	short
13	short	-443.97	-343.94	-340.3	0.88	-3.17	short
14	short	-434.3	-295.25	-391.75	0.59	44.98	short
15	short	-482.05	-303.63	-286.25	0.63	48	short
16	short	-452.37	-306.34	-292.82	0.19	45.12	short
17	short	-432.62	-339.67	-336.67	0.13	57.04	short
18	short	-413.13	-324.61	-328.23	0.15	67.28	short
19	short	-460.12	-346.19	-336.71	0.47	85.2	short
20	short	-401.09	-279.51	-293.11	0.15	45.88	short
21							

STAFF_JOVE_segment_velocities

=IFERROR(ABS(STAFF_JOVE_segment_velocities!A2)," ")

	A	B	C	D	E	F	G
1	//Segment 1	Segment 2	Segment 3	Segment 4	Segment 5	Segment 6	Segment 7
2		135.02	357.59	483.91	3	192.04	
3		509.02	347.56	472.39	10.76	188.91	
4		517.87	363.58	451.53	129.78	151.47	
5		498.58	320.89	421.61	79.58	131.29	
6		466.53	310.44	284.87	123.71	114.97	
7		439.13	308.69	369.32	97.27	116.48	
8		395.23	345.8	294.23	88.86	186.71	
9		420.96	378.56	307.56	4.09	201.21	
10		440.92	353.98	275.32	2.86	205.48	
11		417.08	312.62	306.37	1.05	106.78	
12		437.16	330.36	264.73	0.9	135.64	
13		443.97	343.94	340.3	0.88	3.17	
14		434.3	295.25	391.75	0.59	44.98	
15		482.05	303.63	286.25	0.63	48	
16		452.37	306.34	292.82	0.19	45.12	
17		432.62	339.67	336.67	0.13	57.04	
18		413.13	324.61	328.23	0.15	67.28	
19		460.12	346.19	336.71	0.47	85.2	
20		401.09	279.51	293.11	0.15	45.88	
21							

STAFF_JOVE_segment_velocities | ABS_velocities


A
B
0
µm/s
1000+

Name of Material/ Equipment	Company	Catalog Number
#5 forceps	Fine Science Tools	11251-20
C57BL/6 mice	Jackson Labs	
Cannula	Instech	BTPE-10
CMOS camera	Hamamatsu	C11440-42U30
Coverslip-bottomed dish	Electron Microscopy Sciences	
Dissecting scissors	Fine Science Tools	
Fiji ImageJ Image analysis software		
Fluorescein dextran	Thermo Fisher, Invitrogen	D1822
Gauze sponge	Fisher	22-415-504
Heating pad	Reptitherm	RH-4
Heating pad	Sunbeam	000732-500-000U
Inverted epifluorescence microscope	Nikon	
Isis Rodent electric shaver	Braun Aesculap	GT420
Isofluorane	Abbott GmbH	PZN4831850
Luer stub adapter	Fisher	14-826-19E
Micro scissors	Castro Viejo	
Microscope objective	Nikon	
Needle	Fisher	
Needle holder	Olsen-Hegar	
Objective heater	BioScience Tools	MTC-HLS-025
Rectal thermometer	Braintree Scientific, INC	TH-5A
STAFF macros		
Suture string	Harvard Bioscience	723288

Comments/Description
Dumont #5 Inox Forceps
male 9-12 weeks old
Polyethylene Tubing .011x.024in
4.0LT Scientific CMOS
WillCo Dish glass bottom GWST5040
https://fiji.sc/ ; https://downloads.imagej.net/fiji/Life-Line/fiji-win64-20170530.zip
Dextran, Fluorescein, 70,000 MW, Anionic, Lysine Fixable
2x2 inch Dukal sterile gauze sponges
between mouse and stage
over mouse
Nikon TiE inverted microscope
Catheter adapter
Plan Fluor 20x, NA 0.75 water immersion
30 Ga.x1/2"
Temperature controller with objective heater
Mouse Body Temperature monitoring
https://github.com/icbm-iupui/STAFF
silk black suture, 6-0, spool

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Institution:	Indiana University
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August 30, 2019

Alisha DSouza, Ph.D.
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We thank the editor and the reviewers for their helpful comments. They have improved the manuscript.

Sincerely yours,

Kenneth Dunn, Ph.D.
Professor of Medicine and Biochemistry
Director of the Indiana Center for Biological Microscopy

Editorial comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

• Textual Overlap: Significant portions show significant overlap with previously published work. Please re-write both abstracts to avoid this overlap.

Both Abstracts have been rewritten.

• Introduction:
1) Please expand your Introduction to include the following: The advantages over alternative techniques with applicable references to previous studies; Description of the context of the technique in the wider body of literature; Information that can help readers to determine if the method is appropriate for their application.

Text outlining advantages of the method, context within existing literature and when the method is appropriate has been clarified and added.

2) Please do not highlight contents in this section.

Highlighting in introduction has been removed.

3) Several statements are lacking appropriate literature references.

Appropriate literature references have been added.

• Protocol Language: Please ensure that all text in the protocol section is written in the imperative voice/tense as if you are telling someone how to do the technique (i.e. “Do this”, “Measure that” etc.) Any text that cannot be written in the imperative tense may be added as a “Note”, however, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Protocol language was changed to the imperative where possible and use of notes was reduced.

1) For example, the opening paragraph should be trimmed and made into a note or merged with the introduction.

Opening paragraph was shortened and merged with the Introduction.

2) Steps 6.1.3-6.1.7,6.2.3, 6.3.4., 6.4.4., 6.4.5 need to be rewritten in the imperative voice.

Indicated steps were rewritten in the imperative. Some were also merged.

• Protocol Detail: Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. Please add more specific details (e.g. button clicks for software actions, numerical values for settings, etc) to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) 1.2: What is the oxygen flow rate?
2) 1.3: mention how you test for depth of anesthesia, e.g., toe pinch?
3) 1.4: Mention surgical tools used.
4) 1.5: Via tail vein? Mention needle gauge.
5) 2.1: Mention excitation and emission filter wavelengths.

Requested information has been added to the protocol.

6) 6.1.3-6.1.7, 6.2.2. : Unclear what actions are to be performed here. Please mention button clicks and menu selections explicitly. These steps cannot be filed as written.

Indicated steps have been rewritten for clarification. Some were merged.

• Protocol Numbering: Please adjust the numbering of your protocol section to follow JoVE's instructions for authors, 1. should be followed by 1.1. and then 1.1.1. if necessary and all steps should be lined up at the left margin with no indentations. There must also be a one-line space between each protocol step.

Numbering has been corrected, indentations have been removed and spaces between steps have been added.

• Protocol Highlight: Please highlight ~2.5 pages or less of text (which includes headings and spaces) in yellow, to identify which steps should be visualized to tell the most cohesive story of your protocol steps. Please see JoVE's instructions for authors for more clarification. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

The amount of text highlighted has been reduced to include only those steps needed to visualize the sections "Define the vascular network using TrackEM2 in FIJI" and "Quantifying vascular flow using STAFF".

1) The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.

2) The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next.
3) Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length.
4) Notes cannot be filmed and should be excluded from highlighting.

Highlighting has been edited.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

The discussion covers the requested topics. The discussion has been edited for clarity.

• Figures: A scale bar on fig 1 will be useful

A scale bar has been added to Figure 1.

• Commercial Language: JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are Jackson Labs, Willco, (GWST5040, Warner Instruments, Reptitherm, Bioscience Tools, Sunbeam, Nikon Plan Fluor, Hamamatsu Orca Flash 4.0,
1) Please use MS Word's find function (Ctrl+F), to locate and replace all commercial sounding language in your manuscript with generic names that are not company-specific. All commercial products should be sufficiently referenced in the table of materials/reagents. You may use the generic term followed by "(see table of materials)" to draw the readers' attention to specific commercial names.

Commercial names have been removed from the body of the manuscript.

• Please define all abbreviations at first use.

Abbreviations have been defined at first use.

• If your figures and tables are original and not published previously or you have already obtained figure permissions, please ignore this comment. If you are re-using figures from a previous publication, you must obtain explicit permission to re-use the figure from the previous publisher (this can be in the form of a letter from an editor or a link to the editorial policies that allows you to re-publish the figure). Please upload the text of the re-print permission (may be copied and pasted from an email/website) as a Word document to the Editorial Manager site in the "Supplemental files (as requested by JoVE)" section. Please also cite the figure appropriately in the figure legend, i.e. "This figure has been modified from [citation]."

Figures and tables are original and not published previously.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Clendenon et al. present a manuscript to describe the use of a new tool called STAFF (Spatial temporal analysis of fieldwise flow) for the analysis of microcirculatory flow using epifluorescence based intravital microscopy. The authors present evidence of feasibility using hepatic sinusoidal microcirculatory beds of rodents under anesthesia.

General Comments:
The authors present a clear manuscript of importance to the field. Indeed, the authors should be commended for undertaking this important, yet very difficult task. This is a very significant contribution to the literature and to the tools researchers will have to analyze microcirculatory videos. As the authors well state, to date manual analysis continues to be required given that no reliable software exists to analyze these type of videos. Below, some I enumerate a few comments and suggestions to the authors.

Major Concerns:
1. The series of steps to set up TrakEM2 project (under 3. Define the vascular network using TrakEM2 in FIJI) are clear, however, either a video demonstrating how to set this up or a pdf as supplementary material providing screen shots of the steps will help clarify for the users. I am familiar with FIJI and did it myself, and still took me a while to get it done.

> We appreciate your effort and agree with your comment. TrakEM2 is not initially intuitive to use even for long time users of FIJI. Therefore we plan to include "Define the vascular network using TrakEM2 in FIJI" as part of the JoVE filmed video.

2. In the section of 'Intravital microscopy' in step 2.4 the authors state: 'save time series file(s) as native camera/microscope format or as sequence of tif files'. Will any native camera format will do? We have camera format coming from a Leica instrument, and this file type was not recognized using these steps. Files had to be converted to tiff to be able to use it. Can the authors discuss/clarify this further?

> We have edited the text to reflect that while most native camera formats are readable by FIJI using Bio-Formats (https://www.openmicroscopy.org/bio-formats/), not all are and in those cases a sequence of tif images can be used.

3. In step 3.6, what does 'setup area list selection in TrakEM2' mean? In the toolset in TrakEM2 there are several 'area selection tools' available. Should users use a squared area, how broad should the selection be? Please see comment 2 which may offer an easy alternative to clarify these steps.

> We have clarified the text and we plan for these steps to be included in the JoVE video.

4. How accurate is the skeletonization of the vascular network using only one image to represent the entire time series? Is there a maximal permissible 'movement' of the image that would preclude flow analysis and make the skeletonization inaccurate? Along those same lines, what is the recommended duration of video clips to analyze?

> The accuracy of one image to represent the entire time series depends on the stability of that individual time series. The duration of a video clip to analyze also depends on the stability of that individual time series. Additionally, duration of video clip acquisition is limited by the time period that the animal can be maintained on the microscope stage. For imaging the exteriorized liver we limit this time period to under two hours because this is a period during which the animal's temperature and respiration can be reliably maintained. Keeping an organ preparation stable on the microscope stage is highly operator dependent and improves with

experience. We have used STAFF to analyze videos of up to 60,000 frames representing 10 minutes.

We routinely assess movement but have not formally described our assessment. We recognize that this is critical for the end user. Excluding the short periods of motion during respiration, as long as the skeletonization still overlies any part of the capillary that the red blood cells pass through (e.g. not the edge and not outside the capillary) then STAFF analysis works. This can be visually assessed by overlaying the skeleton on the movie and playing the movie. Step 6.2.4. now includes how to overlay the skeleton (in the form of labeled segments) on the movie and assess stability.

5. Can the authors comment on how this analytic tool handles and reports the normal time-dependent variability in velocity within individual microvessels? Furthermore, there are vessels that will have intermittent flow particularly if exposed to pathologic conditions like infections. How does the software analyze and report these variations in flow in time?

We generally select time intervals to encompass a single period between respirations (Section 6.3). For each time period, for each segment a velocity is calculated and the data is output as a spreadsheet. Velocity over time can be read across the velocity spreadsheet for each individual segment. Indeed, changes in flow and intermittent flow are observed over time within individual segments.

What is not captured are the changes in velocity that occur within a single time period. If a change in velocity occurs within a time period, the velocity that occurs over most of that time period is reported. This is because the Directionality plugin detects the directional banding within each kymograph, then plots all the angles by frequency and fits a curve to the highest peak in that plot and uses the value at the peak of that fitted curve to calculate distance/time for that time interval. Calculated velocity from such segments can appear to vary between two values over time and have poor goodness of fit values. At this time we think that reporting the predominant velocity that occurs within each time period is sufficient, given the vast increase overall in capillary flow data that we can now collect, analyze and visualize using STAFF.

6. In relation to the Kymographs generated by this analytical tool (Figure 2B), can the authors specify how much time are these Kymographs constructed in? In other words, what is 't' in figure 2B? Is this variable? Does it matter? The reason why this is important goes back to comment No. 7.

We selected the period between respirations as the unit of time for our analysis. The overall time period between respirations in our anesthetized mice varied between one and two seconds. It is variable insomuch as the period between respirations is slightly variable. In response to does it matter? Early in our development of STAFF we briefly examined dividing each respiratory period into two time periods and/or selecting a standard amount of time from each respiratory period and the results overall did not differ.

7. Can the authors clarify in the methods how is velocity calculated from these kymographs? Is the result reported an average of the time-distance relationship in time?

The Directionality plugin detects the directional banding within each kymograph, then plots all the angles by frequency and fits a curve to the highest peak in that plot and uses the value at the peak of that fitted curve to calculate distance/time for that time interval. We have clarified this in the manuscript.

8. Can the authors comment on whether this tool can be used with videos collected at lower speed (i.e. < 100 fps). Can this still tool be useful to assess changes between different biologic conditions or in time?

"The product of shortest segment length and frames per second gives the max flow speed that can theoretically be measured. The highest capillary flow speeds reported in literature are around 2000 microns/sec[2]. In liver we observed capillary flow speeds generally averaged around 300 microns/sec and rarely exceeded 1000 microns/sec."

Our image capture at 100 fps with the minimum vascular segment length set to 15 microns allows capture of flow speeds up to 1500 microns/second and at a minimum segment length of 20 microns allows capture of flow speeds up to 2000 microns/second. If we were to capture at 30 fps with minimum segment lengths of 15 and 20 microns it would allow capture of flow speeds up to 450 and 600 microns/second respectively. If you can be certain that your velocity distribution mostly falls within some lower range then it could work. You could assess this by looking at a histogram of your velocity values.

Yes, this tool can be used to assess changes in flow patterning and velocity between different biological conditions over time. In development we used this method to look at differences in blood flow in livers of mice treated with acetaminophen compared to untreated mice. We found profound changes in the spatial patterning of flow velocity and an increase in proportion of vascular segments with little to no flow while the average flow velocity was nearly unchanged. This could be applied similarly to other drug treatments or disease conditions.

9. Along the same lines, can this tool be used with other acquisition techniques other than epifluorescence (i.e. 2P-microscopy?).

The limitation to using confocal or multiphoton microscopy to generate image sequences for fullfield flow analysis is scan speed. Scan speed for a single 512x512 image in these systems is typically 1 second. Scan speed can be increased by decreasing the x,y dimensions of the image down as far as to a single line scan to achieve the speed, but then you have a single vessel segment or at best a small group of segments rather than most of a cross section of a lobule. Scan speed in some raster scanning systems can also be increased by using a resonant scanner, but the images produced tend to be noisy enough to cause difficulty with detection of the directional banding in kymographs.

To see if we could apply STAFF to other imaging modalities we have tested the approach using an old dataset of diffraction interference contrast (DIC) images of blood flow in the forming intersegmental vessels in zebrafish embryos. The contrast was sufficient to generate kymographs and calculate flow velocities. These images had however been collected at low frames per second so we did not capture data from segments with higher flow speeds.

Minor Concerns:

1. What type of video files can this software analyze?

Any video file that can be opened using Bio-Formats within FIJI ImageJ can be opened then be saved as tif or AVI and analyzed using STAFF. We have clarified this in the manuscript.

2. In that same section, in step 3.4 although its intuitive, could specify which image should be imported.

We have specified the image that should be imported.

Reviewer #2:

Manuscript Summary:

This manuscript from the Dunn lab describes a new macro for FIJI, called STAFF, that enables the analysis of data on microvascular flow acquired by intravtial microscopy. This method is very well-written and easy to follow by a broad audience.

I do not have any concern

Reviewer #3:

Manuscript Summary:

This manuscript demonstrates a computational pipeline for quantifying blood velocity for a whole field of vessels using high frame rate widefield microscopy and a novel software called STAFF. It should be of interest for those looking to automate quantitative analysis of blood flow.

Major Concerns:

No major concerns

Minor Concerns:

- The underlying principle for calculating flow is straightforward but could be better explained. The manuscript states "The underlying principle used by STAFF to quantify rates of microvascular flow is that the velocity of red blood cells in capillaries can be quantified from the angle formed by their shadows as they pass through capillaries labeled with a bulk fluorescent probe". Angle here refers to a kymograph with one axis being time and the other parallel to the direction of flow so slope is naturally unit length/time. Better just to say, it calculates the slope on a kymograph as defined above, slope is in the right units whereas angle is not.

-Further use of the word "shadow" is confusing in that context as it sounds like the authors are measuring the spatial angle of the shadow cast by an object, as with a sundial. Can simply say, that some contrast must be provided to visualize the blood. This could be positive staining of the blood cells or a negative stain by labeling the plasma. It of course is physically not a shadow (something blocking light) but rather the lack of fluorescent label within the blood cells.

> We thank you for these suggestions for alternate and clearer phrasing. We have made the suggested changes in the manuscript. (last paragraph of the Introduction)

-The authors use 1024x1024 16-bit images as example. My guess is that this is way overkill for xy resolution and bit depth. Can the authors give more typical guidance. The software presumably accepts images of different resolutions and bit depths.

> We have tested the software with images of 1024x1024, 800x800, and 512x512 pixels at 16, 12 and 8-bit depth. There are no explicit size limits within STAFF. We have added this information to the manuscript.

-The authors note that care must be taken to avoid artifacts caused by respiration which would shift the vessel outside the ROI. Presumably such artifacts could be automatically flagged by the software. Otherwise, is there a good way for the user to catch such errors?

> In the second half of our answer to Reviewer #1’s comment 4 we describe our approach to verifying the stability of an image sequence over time.