**Acquiring arbitrary line scans**

1. Open scanimage (2016 or later)
2. In Frame Scan mode (Config>Scan Type>Frame Scan), navigate until the desired blood vessel is in view and well-positioned on the focal plane
3. (Optional, for snapshot-based vessel diameter measurement) In Frame Scan mode, grab a single frame
4. Check "Enable MROI"
5. Enable Line Scan mode (Config>Scan Type>Line Scan)
6. Click "Edit ROIs" to open ROI group editor window
7. Delete any existing lines/pauses (panel on the right)
8. Delete any existing image snapshot layers (panel on the bottom)
9. Uncheck and check Live Image, then select your vessel channel (e.g. CH4)
10. Click "Add ROI", and set function to "Line"
11. Add the line to the image window, and move the end points to desired position on the vessel
    1. Convention: Draw the line in the direction of larger branch to smaller branch – this way, arteries will have a negative slope (and S-shape curve during “focus”) and veins will have a positive slope (and Z-shape curve during “focus”)
12. Add a pause (pauses allow time for the scan to position itself for the next line)
13. Edit times for the line and pause (estimate that at max speed, the scanner can span the full frame width in ~0.5 ms)
14. (Optional, for line scan-based vessel diameter measurement) add another line crossing the vessel sideways, with the line being ~3x the width of the vessel. Add another pause. Edit the times for the new line and pause. (The snapshot-based method is more recommended – M.I.)
15. Grab the desired # of frames (e.g. a 30 s acquisition at 0.5 ms/line = 60,000 frames)
16. Verify that the following files were saved from the line scan:
    1. ...meta.txt
    2. ...pmt.dat
    3. …scnnr.dat
    4. ….tif (optional – for snapshot-based vessel diameter measurement)
17. Keep all these files together in a single folder, and proceed to analysis

**Analyzing arbitrary line scans**

1. Download code from the Blood-flow repository (https://github.com/sn-lab/Blood-flow)
2. Add paths to MATLAB (Home>Set Path>Add with subfolders>[Blood-flow folder]>Save)
3. Download and add paths for Pixel-To-Micron (https://github.com/sn-lab/Pixel-to-Micron)
4. Run ArbitraryLinescanPreprocess()
   1. Adjust settings:
      1. SETUP: Microscope setup used to acquire the Line Scan
      2. OBJECTIVE: Objective used (must match exactly to one listed in the Pixel-To-Micron conversion factors spreadsheet)
      3. VESSEL CHANNEL: Imaging channel with vasculature label, only including saved channels (e.g. if channel 4 is the vessel channel but you only saved channels [2,3,4], your vessel label is in the 3rd channel)
      4. VESSEL WIDTH (SNAPSHOT): whether to use a single-frame snapshot to estimate the diameter of the vessel being scanned
      5. VESSEL WIDTH (LINESCAN): whether to use a Line Scan drawn across the vessel to estimate the diameter of the vessel being scanned
      6. NEAR LINE MICRONS: The maximum distance away (in microns) the actual scanner position can be from a drawn Line Scan ROI to be included in that line scan data (the scan position can sometimes turn too far away from the line, especially at the ends of the line, which for small vessels can mean that the scan moves off the vessel entirely)
      7. NEAR DIAMETER MICRONS: Like above, but for line scans used to estimate vessel diameter (since diameter line ROI is drawn orthogonal to the vessel orientation, the scan position doesn’t need to as close to the line ROI to still be scanning/crossing the vessel)
   2. Navigate to an arbitrary line scan data folder, select the …pmt.dat file
   3. (Optional, for snapshot-based vessel diameter estimation) Navigate to and select the single frame snapshot associated with the line scan. If there is only one .tif file in the same folder as the rest of the line scan data, this will be assumed to be the snapshot.
   4. Line Position window: identify which ROI(s) you want to use for blood flow measurements, or for vessel diameter measurements
   5. Scan position window: No action needed. Drawings of the line ROIs and the logged/interpolated scanner positions, positions included for each line scan data, and median fluorescence across the line scan. Use these images to evaluate that the scan path was correct.
   6. (Optional, for vessel diameter estimation) Set vessel width window: drag the horizontal lines to the top and bottom of the vessel to estimate vessel diameter (measurements can be snapshot-based, line scan-based, or both).
   7. Results window: results of the line scan, including measurements need to continue with the blood-flow velocity pipeline (e.g. microns/pixel, ms/line). These results are also saved in a .mat file in the source data folder.
5. Run extractVelTiffShared()
   1. Navigate to the .tif file created from ArbitraryLinescanPreprocess (filename starts with “AL1”, containing line scan data linearized to the blood-flow ROI)
   2. Follow the instructions in “share\_instructions\_2012-03-08.pdf”
6. Run view\_velocities\_save\_data()
   1. Manually select velocity range to exclude outliers/bad data

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