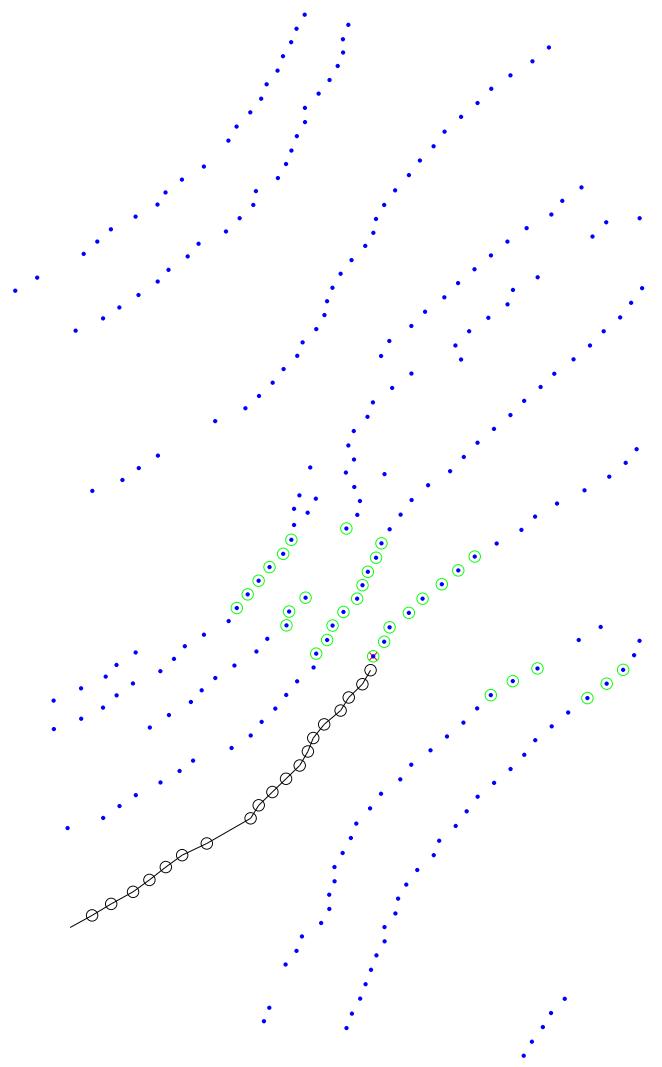


Code Instructions

Analyzing *in vivo* footage of fluorescently stained cells



Contents

1 Preliminaries	1
2 Pipeline overview	1
3 Detailed list of all analysis steps	1

1 Preliminaries

This document is associated with the publication *Impact of Red Blood Cell Rigidity on in vivo Flow Dynamics and Lingering in Bifurcations* by Rashidi et al. 2025 and contains a description and instruction set to use the MATLAB™ implementation developed and employed for the analysis of in vivo microscopic fluorescent footage of cells and blood flow. Documentation and code is available at <https://github.com/FelixMaurer/RBCsInBifurcations>.

2 Pipeline overview

The code consists of 15 main MATLAB scripts, F0, ..., F14 as well as a number of source scripts and functions they make use of. The code is designed to be executed in order of the index i in the name $F_i_\dots.m$. If the pipeline is supposed to be used as is, without changes, there are a number of prerequisites regarding date file formats and the folder structure.

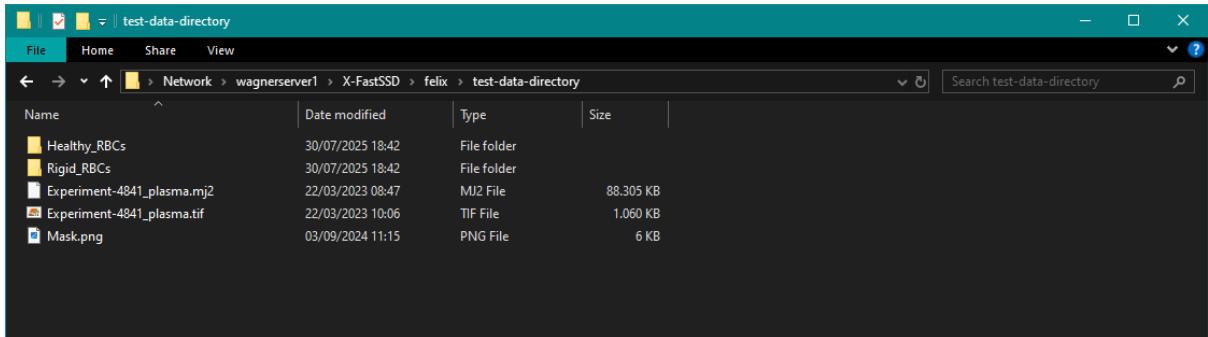
Script File	Description
Establish required file structure and generate plasma image.	
F0_compute_nir_detect_peaks.m	Calculate the NIR intensity map of all videos and detect peaks in all videos. The user should control the peak detection for optimal yield of cells with lowest noise.
F1_save_peak_images.m	Saves all detected peaks into images. For different cell types in different colors.
Manual alignment of peak images to plasma image.	
F2_extract_and_check_alignment.m	Overlays peak images with the plasma image as a visual feedback to check if the alignment is correct.
F3_geometry_extraction.m	Extracts the geometrical features of the mask. This includes vessel walls and axes and the bifurcation area boundaries.
F4_lucas_kanade_flow_estimation.m	Estimates flow from optical flow in the plasma footage.
F5_lucas_kanade_flow_evaluation.m	Evaluates the optical flow results.
F6_full_network_tracking.m	First coarse tracking of RBC trajectories for bulk flow estimation.
F7_full_network_tracking_evaluation.m	Evaluation of the bulk flow from coarse tracking.
F8_combined_network_flow_estimation.m	Combines both flow methods, F4 to F7, to obtain speed and angle maps.
F9_trajectory_tracing.m	Predictive tracing of trajectories based on flow estimations.
F10_lingering_analysis.m	Speed statistics of accumulated bifurcation data.
F11_lingering_bar_plots.m	Bar plots to check and compare parameter distributions.
F12_mode_changes.m	Investigates mode changes and speed pdfs from kde.
F13_mode_changes_results.m	Creates figure showing results of interaction fractions.
F14_apex_distance_analysis.m	Analyzes apex distances between cell groups.

Table 1: List of pipeline script files and their descriptions.

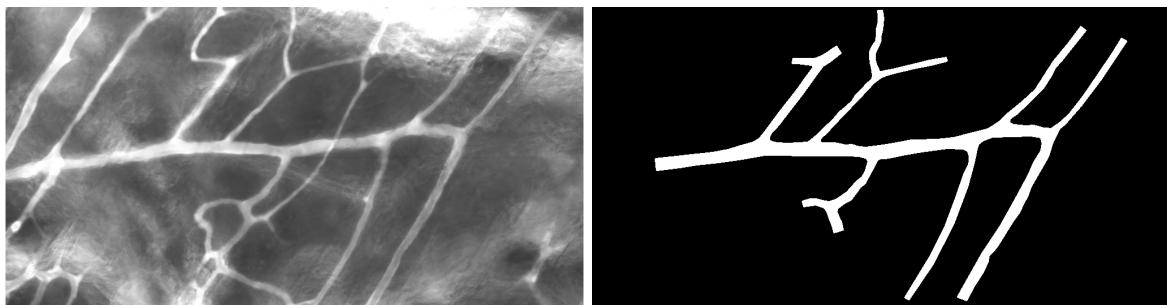
3 Detailed list of all analysis steps

Step 0: Requirements

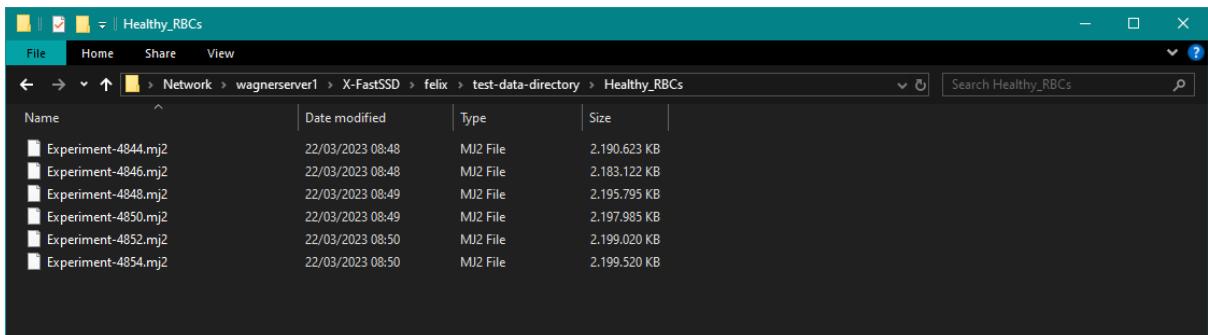
Take care of the folder structure and prepare a plasma image and binary mask.



The root directory should contain the folders for different cell types and directly the plasma recording. An average or maximum intensity image of the plasma video should be provided, by default as a .tif file, and a hand drawn mask should be prepared from this plasma image as Mask.png.



Plasma intensity image and a hand drawn mask. The mask should be white inside of vessels of interest and black in all other regions. Ends of vessels should be cutoff sharply perpendicular to the vessel axis.



Cell type folders should contain all recordings from the same position, i.e. geometry, in the color channel of the specific stain emission.

Step 1: Calculate NIR map and detect peaks

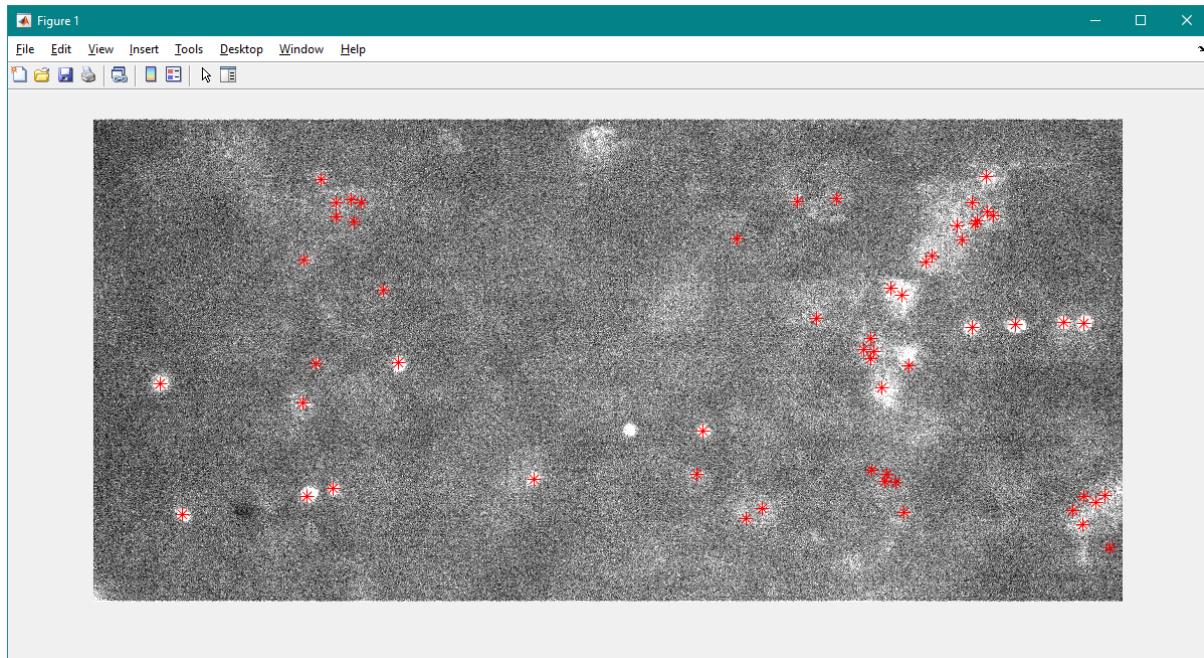
Execute script `F0_compute_nir_detect_peaks.m`. The detected peaks will be drawn onto each frame on screen. Choose an optimum between maximum cell yield and minimum non-cell detections from noise. The main parameters that might be adjusted to do so are the filter size and the noise threshold.

```
thres = 56; % noise threshold, too small--> too much noise, too high--> less cells
filt = ones(6,6); % similar to the cell size in pixel (diameter)
```

The filter size should be chosen close to the approximate cell diameter in pixels and the noise threshold choice depends on the given video. The output consists of .mat files with suffix

`_peaks.mat`. They contain a struct each of size $1 \times$ frame number and with the field `peak`, s.t. `allpoints(i).peak` contains all n peaks from frame i in the format `nx2 double`.

```
allpoints =
1×2250 struct array with fields:
    peak
```



Video still of cell signal with all detected peaks. Peaks might include cells as well as noise.

Healthy_RBCs			
Name	Date modified	Type	Size
Experiment-4844.mj2	22/03/2023 08:48	MJ2 File	2,190,623 KB
Experiment-4844_peaks.mat	31/07/2025 10:19	Microsoft Access ...	90 KB
Experiment-4846.mj2	22/03/2023 08:48	MJ2 File	2,183,122 KB
Experiment-4846_peaks.mat	31/07/2025 10:23	Microsoft Access ...	70 KB
Experiment-4848.mj2	22/03/2023 08:49	MJ2 File	2,195,795 KB
Experiment-4848_peaks.mat	31/07/2025 10:26	Microsoft Access ...	73 KB
Experiment-4850.mj2	22/03/2023 08:49	MJ2 File	2,197,985 KB
Experiment-4850_peaks.mat	31/07/2025 10:31	Microsoft Access ...	106 KB
Experiment-4852.mj2	22/03/2023 08:50	MJ2 File	2,199,020 KB

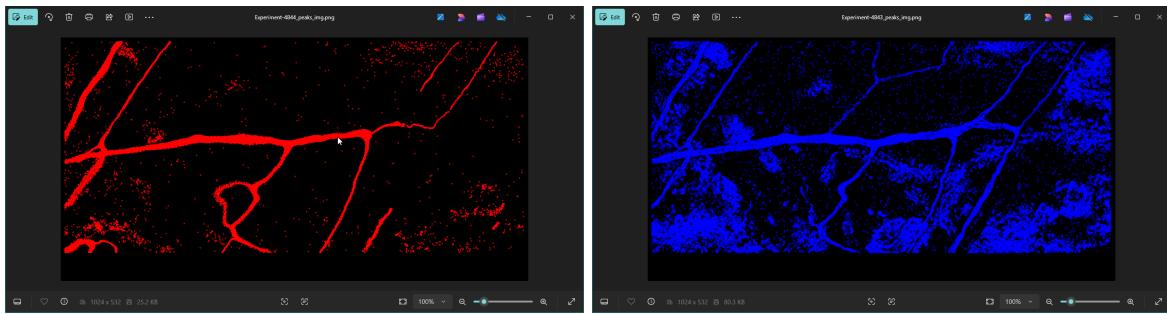
The output are `*_peaks.mat` files containing all peaks for in all frames of each video.

Step 2: Save peak images

The script `F1_save_peak_images.m` saves peak images to the drive in the same folder where each corresponding originating video is stored.

Healthy_RBCs			
Name	Date modified	Type	Size
Experiment-4844.mj2	22/03/2023 08:48	MJ2 File	2,190,623 KB
Experiment-4844_peaks.mat	31/07/2025 10:19	Microsoft Access ...	90 KB
Experiment-4844_peaks_img.png	31/07/2025 11:06	PNG File	26 KB
Experiment-4846.mj2	22/03/2023 08:48	MJ2 File	2,183,122 KB
Experiment-4846_peaks.mat	31/07/2025 10:23	Microsoft Access ...	70 KB
Experiment-4846_peaks_img.png	31/07/2025 11:06	PNG File	27 KB
Experiment-4848.mj2	22/03/2023 08:49	MJ2 File	2,195,795 KB
Experiment-4848_peaks.mat	31/07/2025 10:26	Microsoft Access ...	73 KB
Experiment-4848_peaks_img.png	31/07/2025 11:06	PNG File	29 KB

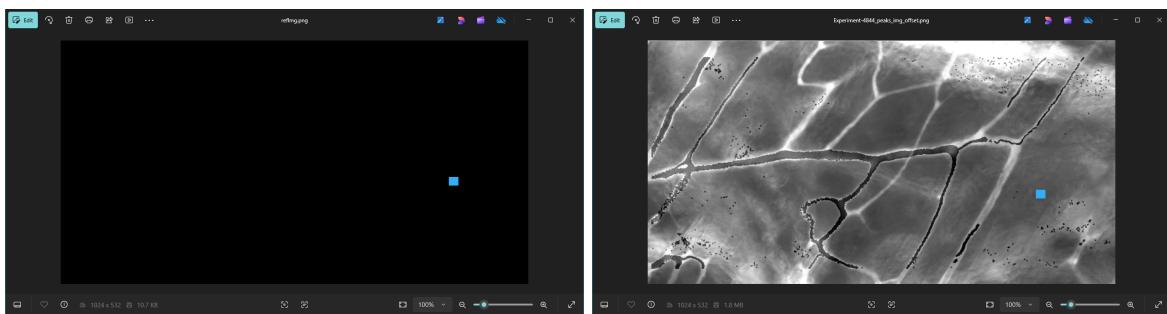
Output peak images with suffix `_peaks_img.png` in the folder containing the videos.



Output peak images showing an overlay of all peaks of all frames into one frame. Different colors for healthy (red) and rigid cells.

Step 3: Align peak images manually

For alignment, it is easiest to overlay the peak images with the plasma image. To do so, the layer mode or composite mode *difference* in a compositing software like GIMP® and Inkscape® might be used. That way, the peak image is subtracted from the plasma image and trajectories appear dark. Before moving the layer, a reference image should be created, by default containing a blue rectangle. The reference image should be duplicated and moved with the peak image until the trajectories coincide with the vessels. By default the resulting images should be saved with the suffix `_offset.png`.



Left, the reference image with a blue rectangle, right, overlay by difference composite mode of the peak image onto the plasma image. The blue rectangle was moved with the peak image to align dark appearing peaks with the vessels. The rectangle was thereby moved in the down-right direction.

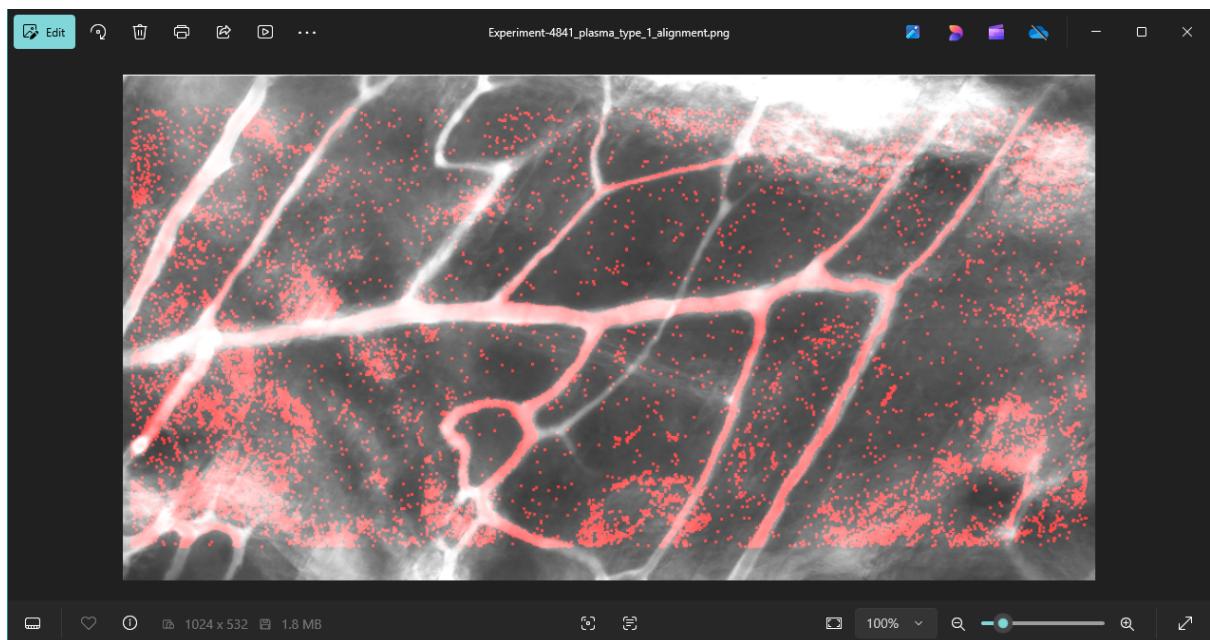
	Name	Date modified	Type	Size
■	experiment-4850_peaks.mat	31/07/2025 10:31	Microsoft Access ...	106 KB
■	Experiment-4850_peaks_img.png	31/07/2025 11:06	PNG File	34 KB
■	Experiment-4850_peaks_img_offset.png	22/03/2023 11:35	PNG File	1.906 KB
■	Experiment-4852.mj2	22/03/2023 08:50	MJ2 File	2.199.020 KB
■	Experiment-4852_peaks.mat	31/07/2025 10:35	Microsoft Access ...	97 KB
■	Experiment-4852_peaks_img.png	31/07/2025 11:06	PNG File	33 KB
■	Experiment-4852_peaks_img_offset.png	22/03/2023 11:37	PNG File	1.906 KB
■	Experiment-4854.mj2	22/03/2023 08:50	MJ2 File	2.199.520 KB
■	Experiment-4854_peaks.mat	31/07/2025 10:38	Microsoft Access ...	79 KB
■	Experiment-4854_peaks_img.png	31/07/2025 11:06	PNG File	28 KB
■	Experiment-4854_peaks_img_offset.png	22/03/2023 11:30	PNG File	1.887 KB
■	refImg.png	22/03/2023 11:49	PNG File	11 KB

25 items | 7 items selected 11,0 MB |

After this step there should exist a file `_offset.png` for each video and `refImg.png` for the folder of the given cell type.

Step 4: Check alignment with plasma image

Executing script `F2_extract_and_check_alignment.m` will extract from each peak image a single two-dimensional alignment vector, corresponding to the offset between the rectangles of peak image and reference image, and create a composite from all peak images from one cell type in one geometry.



Composite of all peak images of healthy RBCs to confirm proper alignment.

Healthy_RBCs			
File	Home	Share	View
←	→	↑	Network > wagnerserver1 > X-FastSSD > felix > test-data-directory > Healthy_RBCs
Name	Date modified	Type	Size
■ Experiment-4844.mj2	22/03/2023 08:48	MJ2 File	2.190.623 KB
■ Experiment-4844_peaks.mat	31/07/2025 10:19	Microsoft Access ...	90 KB
■ Experiment-4844_peaks_img.png	31/07/2025 11:06	PNG File	26 KB
■ Experiment-4844_peaks_img_offset.png	22/03/2023 11:32	PNG File	1.870 KB
■ Experiment-4844_peaks_img_offset_align...	31/07/2025 11:20	Microsoft Access ...	1 KB
■ Experiment-4846.mj2	22/03/2023 08:48	MJ2 File	2.183.122 KB
■ Experiment-4846_peaks.mat	31/07/2025 10:23	Microsoft Access ...	70 KB
■ Experiment-4846_peaks_img.png	31/07/2025 11:06	PNG File	27 KB
■ Experiment-4846_peaks_img_offset.png	22/03/2023 11:33	PNG File	1.879 KB

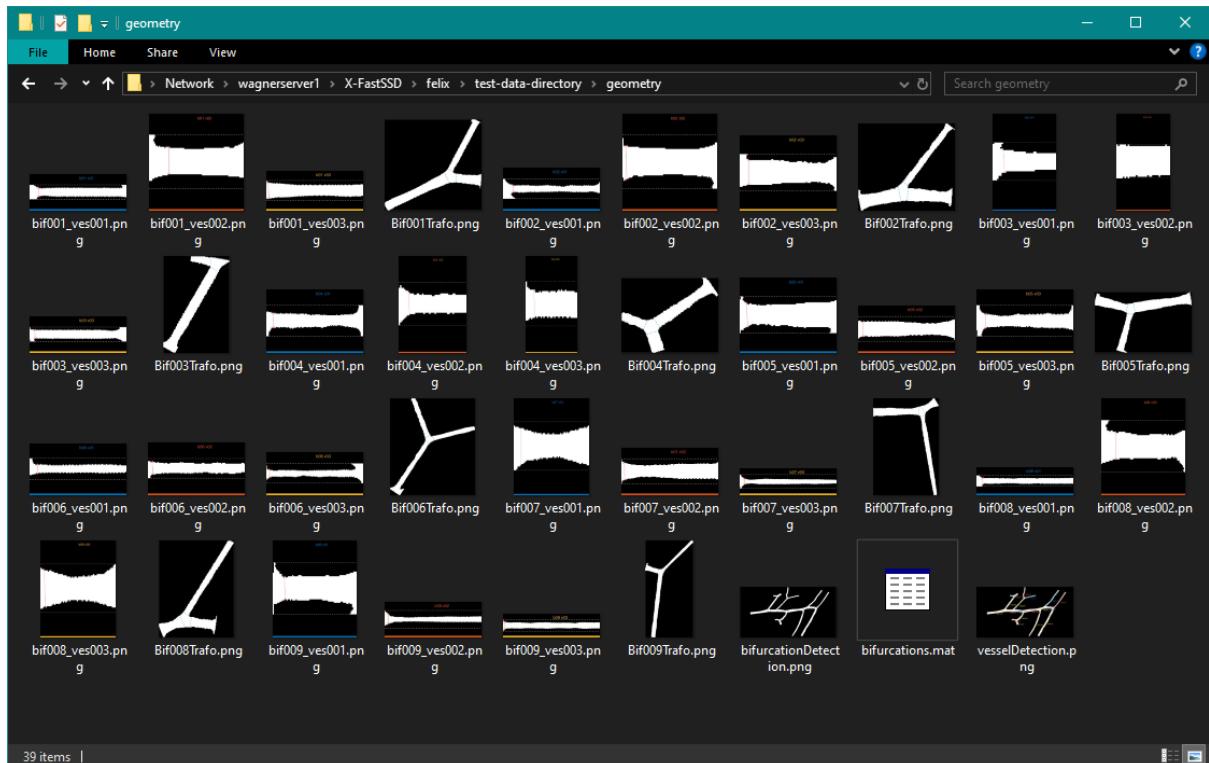
Output alignment vectors as `_alignVec.mat` files into the folder of originating videos.

Step 5: Extract vessel geometry

In this step, the geometrical features will be extracted by running `F3_geometry_extraction.m`. A mask needs to be drawn prior to this step as explained in [Step 0](#). Tweaking the results mainly involves applying filters to the mask, e.g. for smoothing against noisy or spiky edges. In terms of wrongful detections of bifurcation borders, tweaking the sampling parameters can help.

```
% sample image profiles  
fac = 6; dFac = 2;
```

Parameter `fac` is an initial sampling length and `dFac` an increment for the iteration. Starting, for example, with lower values for both might increase the resolution of details.



Output images in the geometry folder. It contains the detection of vessel center lines in `vesselDetection.png`, the detection of bifurcation centers and borders in `bifurcationDetection.png`, the transformed vessels `bif*_ves*.png`, and the transformed bifurcations with straightened vessels `Bif*Trafo*.png`.

Step 6: Estimate optical flow

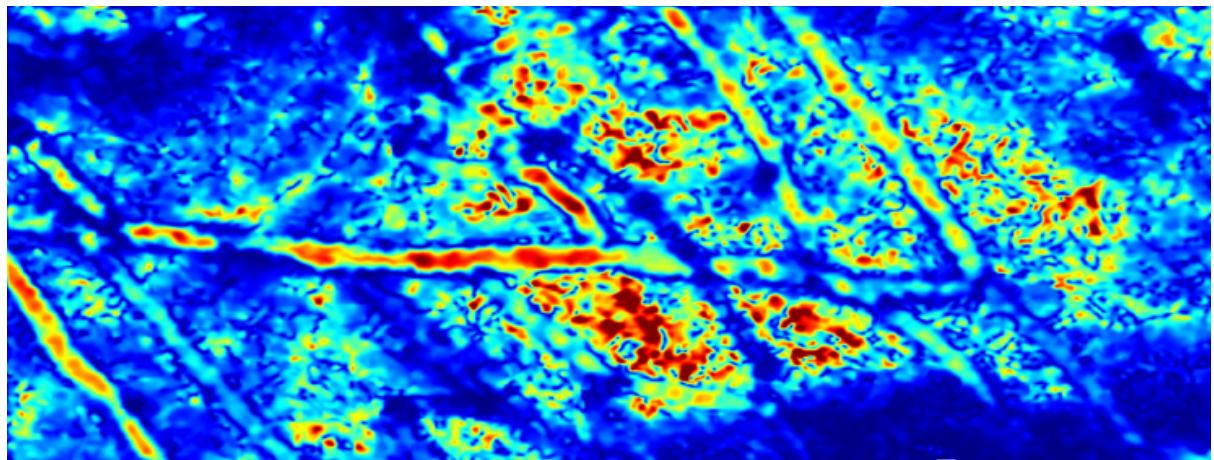
Execute script `F4_lucas_kanade_flow_estimation.m`. The scale, on which flow is computed is an important tweaking parameter.

```
resizeFac = 0.3;
```

The smaller `resizeFac`, the coarser the spatial averaging. The scale should be adjusted to a suitable value to the scale of vessels, low enough that noise does not contribute to the flow. The parameters of the optical flow model are

```
opticFlow = opticalFlowLK;  
opticFlow.reset;  
opticFlow.NoiseThreshold = 0.001;
```

and thus do not contain information about scale.

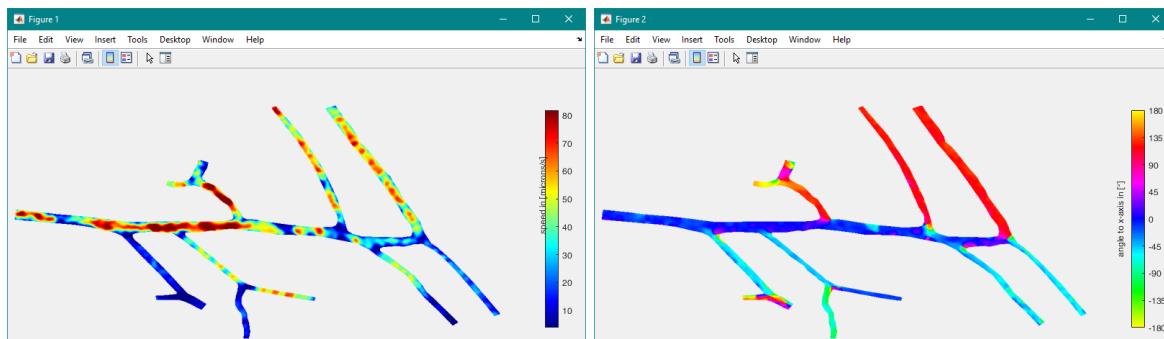


Raw optical flow output from the script.

Step 7: Evaluate optical flow

Execute script `F5_lucas_kanade_flow_evaluation.m` to evaluate the optical flow and to reduce it to the inside of vessels. The main parameters are regarding the level of smoothing or spatial averaging.

```
flowX = smoothdata(flowX,1,"gaussian",5);
flowX = smoothdata(flowX,2,"gaussian",5);
flowY = smoothdata(flowY,1,"gaussian",5);
flowY = smoothdata(flowY,2,"gaussian",5);
```



Left, estimated velocity magnitude, right estimated angles.

Step 8: Track RBCs coarsely

In script `F6_full_network_tracking.m` the detection points for RBCs are clustered into trajectories in space-time. A conversion of frame number to position in pixels is done by the factor `dist`.

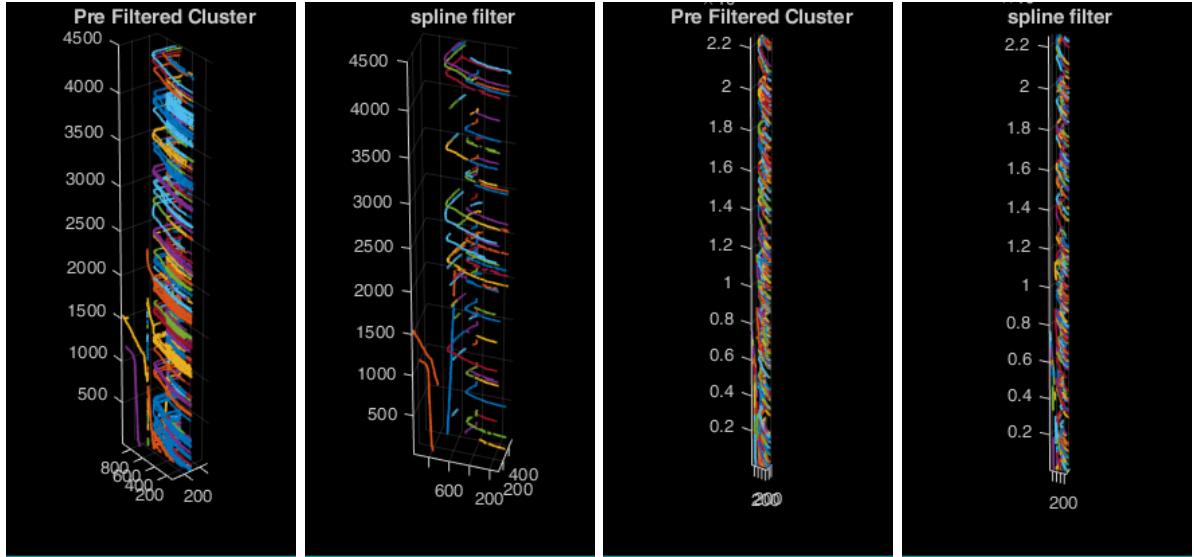
```
dist = 10; % increase to make curves steeper / increase angle, should be less than 45°
```

It can be adjusted to reach an optimum of separation into trajectories, and should be chosen roughly to make trajectories have an angle below 45° angle to the x - y -plane. The minimum distance inside of which points are still considered for clustering can be tweaked in addition.

```
for minDistance = 20
```

For finding suitable parameters for the given video, the test mode with a test ROI should be chosen.

```
% testing the code and settings
flag_test = true;
% optionally select one ROI to test
testROI = 1; % zero means take all ROIs
```

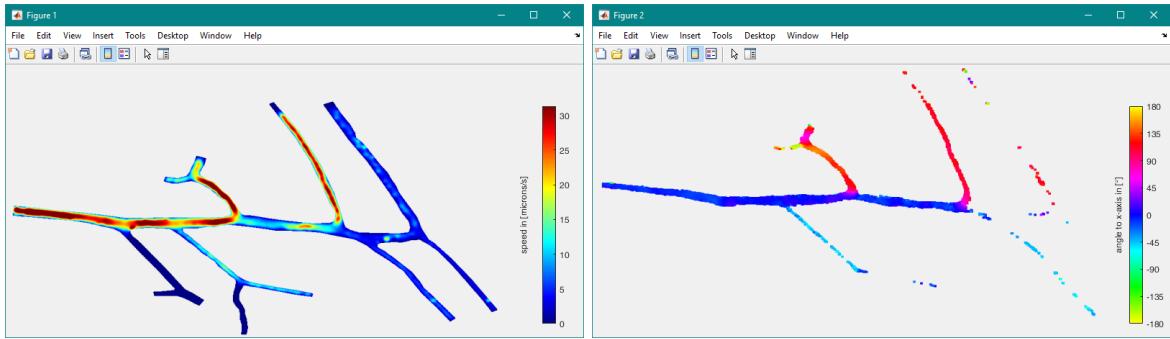


Using the test mode for parameter adjustment. From left to right: bad separation in the pre-filtered cluster, visible through the fact that multiple trajectories are grouped by the same color to a detected single trajectory. Second, The resulting trajectories after spline filtering. The number is insufficient. Third, pre-filtered cluster with different parameters and last, the corresponding spline filtered trajectories.

Step 9: Evaluate coarse tracking

Executing `F7_full_network_tracking_evaluation.m` will, similarly to the optical flow estimation, evaluate the coarse RBC tracking to obtain a flow field. Also here the smoothing stages have arguably to biggest influence on the estimation.

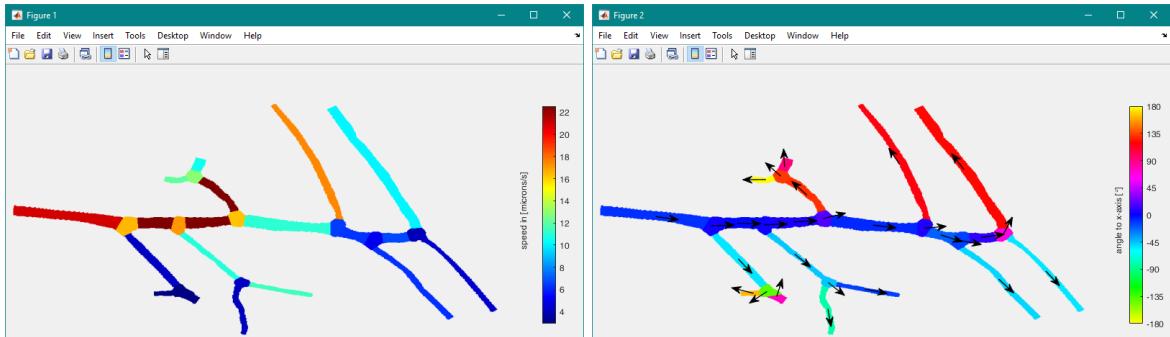
```
circX = smoothdata(circX,1,"gaussian",5);
circX = smoothdata(circX,2,"gaussian",5);
circY = smoothdata(circY,1,"gaussian",5);
circY = smoothdata(circY,2,"gaussian",5);
```



Left, estimated velocity magnitude, right estimated angles, from RBC trajectories. The angles are sparse because not at every point in this sampling a cell was detected. In contrast, the optical flow method estimates a dense flow field as shown before.

Step 10: Combine flow estimation methods

The script `F8_combined_network_flow_estimation.m` will combine both estimated flows to obtain average flows for each region.



Left, estimated velocity magnitude, right estimated angles, averaged over both flows and over the region. The arrows indicate the average flow direction in the given region.

geometry	31/07/2025 11:25	File folder
Healthy_RBCs	31/07/2025 11:28	File folder
Rigid_RBCs	31/07/2025 11:28	File folder
Experiment-4841_plasma.mj2	22/03/2023 08:47	MJ2 File
Experiment-4841_plasma.tif	22/03/2023 10:06	TIF File
Experiment-4841_plasma_flow_lk_scale.mat	31/07/2025 11:25	Microsoft Access ...
Experiment-4841_plasma_type_1_alignment.png	31/07/2025 11:20	PNG File
Experiment-4841_plasma_type_2_alignment.png	31/07/2025 11:20	PNG File
Mask.png	03/09/2024 11:15	PNG File
velocity_direction_LK_scale.mat	31/07/2025 12:49	Microsoft Access ...
velocity_direction_LK_scale.png	31/07/2025 12:49	PNG File
velocity_direction_traj.mat	31/07/2025 12:43	Microsoft Access ...
velocity_direction_traj.png	31/07/2025 12:44	PNG File
velocity_magnitude_LK_scale.mat	31/07/2025 12:49	Microsoft Access ...
velocity_magnitude_LK_scale.png	31/07/2025 12:49	PNG File
velocity_magnitude_traj.mat	31/07/2025 12:43	Microsoft Access ...
velocity_magnitude_traj.png	31/07/2025 12:43	PNG File

Output flow files including files from the Lucas-Kanade method as well as from the RBC trajectory tracking.

Step 11: Predict RBC trajectories

Execute script `F9_trajectory_tracing.m` for a predictive search and trajectory tracing. It comes with a test mode and a way to witness the tracing by setting `framePause` to a finite number.

```
% pause between plotting frames
framePause = inf; % [sec] set to inf for no plotting, good values > 0.5 s
% for testing, activates intermediate steps plotting
flag_test = false;
```

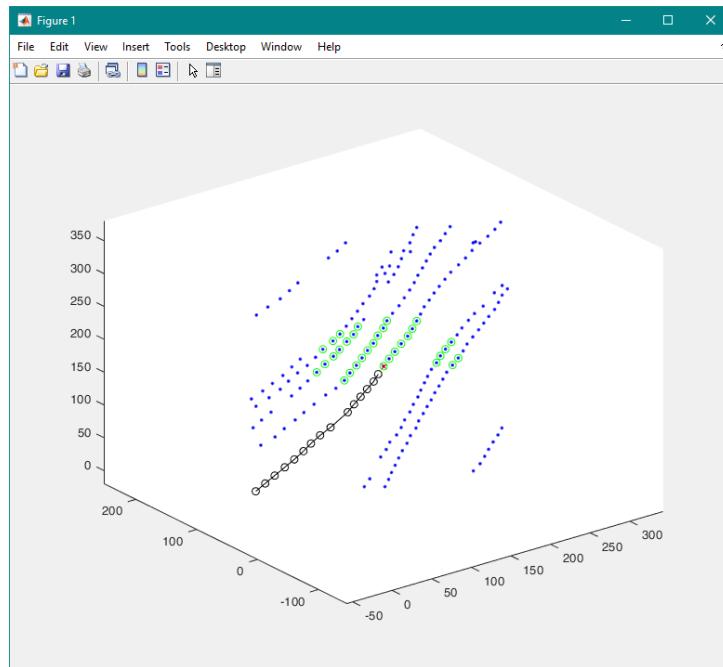
The tracing algorithm performs a coordinate transformation of the point cloud such that the inlet vessel of the given bifurcation is oriented parallel to the x -axis. A first point is chosen and a trajectory traced applying prediction weights to the neighborhood to each point at the front of the current trajectory. After completing a trace, i.e. if the probability for the entire neighborhood to belong to the trajectory is below a threshold, or there are no points at all left, the end point is the starting point of a backward tracing. The reason for the backward tracing is that starting points might not belong to trajectories while end points have a much higher probability. Also, the trajectories that intersect in space-time, can be separated using backward tracing. For tweaking, the global distance threshold

```
distanceThres = 80; % maximum distance between points
```

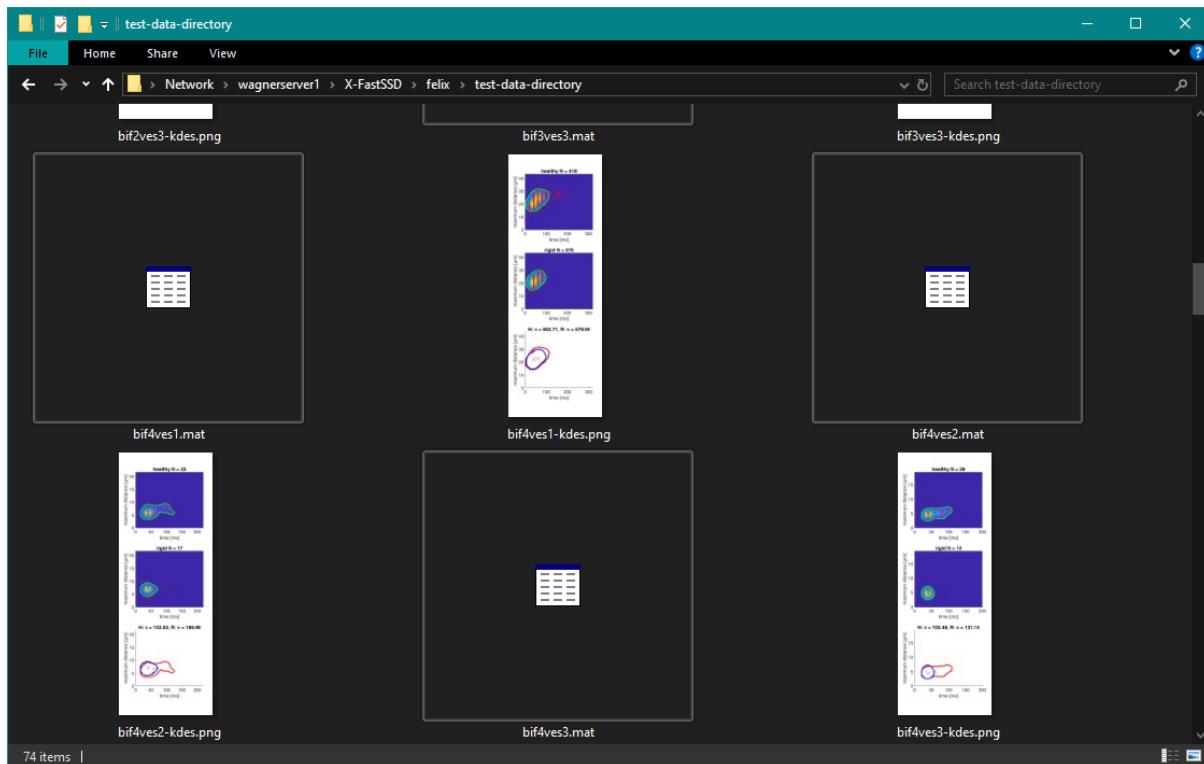
and the weighting in the probability scores can be changed.

```
% weighting by angle
if ~isempty(prevDir)
    cosTheta = zeros(1,size(nbDir,1));
    for nbIdx = 1:size(nbDir,1)
        cosTheta(nbIdx) = dot(prevDir,nbDir(nbIdx,:));
    end
    nbAngleScore = (cosTheta/2+0.5).^(2);
else
    nbAngleScore = ones(1,size(nbDir,1));
end
nbDistScore = 1./nbDists';
%% (5) compute distance and angle weighted average
avgVec = sum(nbVec.*repmat(nbDistScore',1,3).*repmat(nbAngleScore',1,3),1)./sum(nbDistScore.*nbAngleScore);
totalScore = nbDistScore.*nbAngleScore;
```

The output also contains kernel density estimations of the two-dimensional probability density function of maximum distances traveled versus time spent in each region, in the beginning of a vessel as an exit of a bifurcation and the end of a vessel as an inlet. The script outputs files `bif*ves*.mat` containing all data that was so far computed for the given bifurcation including masks, velocities and statistics.



Trajectory tracing as viewed with a finite `framePause`. Blue points are all detected points in the viewing window. Black connected circles mark traced points belonging to the current trajectory. The neighborhood indicated by green circles are candidates for the next point to be added to the trajectory. The choice probability is determined by weights depending on the flow estimation and the previous points of the trajectory.



Kernel density estimated pdfs of distance versus time for different vessels.

Screenshot of a Windows File Explorer window showing the contents of a directory named "test-data-directory". The directory path is: Network > wagnerserver1 > X-FastSSD > felix > test-data-directory.

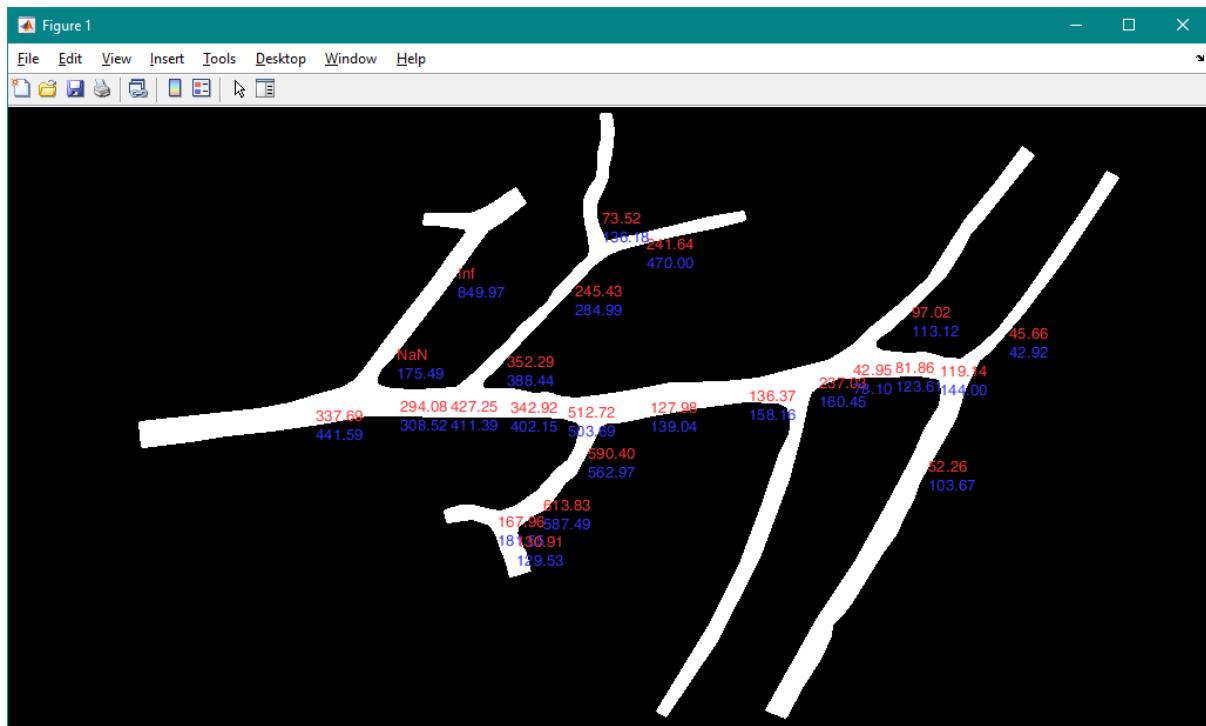
Name	Date modified	Type	Size
geometry	31/07/2025 11:25	File folder	
Healthy_RBCs	31/07/2025 11:28	File folder	
Rigid_RBCs	31/07/2025 11:28	File folder	
AngleMap.mat	31/07/2025 13:03	Microsoft Access ...	50 KB
bif1ves1.mat	31/07/2025 13:28	Microsoft Access ...	19.884 KB
bif1ves1-kdes.png	31/07/2025 13:28	PNG File	200 KB
bif1ves2.mat	31/07/2025 13:28	Microsoft Access ...	19.942 KB
bif1ves2-kdes.png	31/07/2025 13:28	PNG File	233 KB
bif1ves3.mat	31/07/2025 13:28	Microsoft Access ...	20.089 KB
bif1ves3-kdes.png	31/07/2025 13:28	PNG File	263 KB
bif2ves1.mat	31/07/2025 13:28	Microsoft Access ...	20.176 KB
bif2ves1-kdes.png	31/07/2025 13:28	PNG File	219 KB
bif2ves2.mat	31/07/2025 13:28	Microsoft Access ...	20.337 KB
bif2ves2-kdes.png	31/07/2025 13:28	PNG File	264 KB
bif2ves3.mat	31/07/2025 13:28	Microsoft Access ...	20.467 KB
bif2ves3-kdes.png	31/07/2025 13:28	PNG File	307 KB
bif3ves1.mat	31/07/2025 13:28	Microsoft Access ...	19.810 KB
bif3ves1-kdes.png	31/07/2025 13:28	PNG File	169 KB
bif4ves1.mat	31/07/2025 13:29	Microsoft Access ...	20.028 KB
bif4ves1-kdes.png	31/07/2025 13:29	PNG File	262 KB
bif4ves2.mat	31/07/2025 13:29	Microsoft Access ...	19.981 KB
bif4ves2-kdes.png	31/07/2025 13:29	PNG File	231 KB
bif4ves3.mat	31/07/2025 13:29	Microsoft Access ...	19.955 KB
bif4ves3-kdes.png	31/07/2025 13:29	PNG File	208 KB
bif5ves1.mat	31/07/2025 13:29	Microsoft Access ...	20.314 KB
bif5ves1-kdes.png	31/07/2025 13:29	PNG File	244 KB
bif5ves2.mat	31/07/2025 13:30	Microsoft Access ...	20.339 KB

75 items |

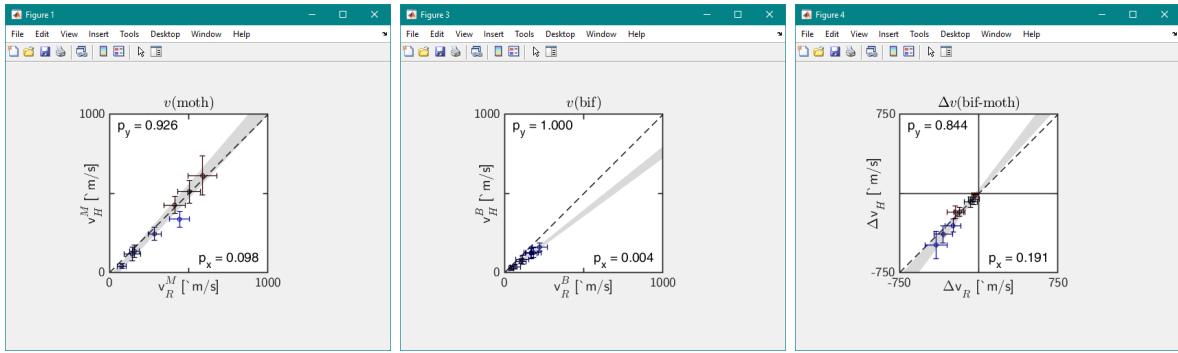
Output files from the tracing through bifurcations.

Step 12: Analyze lingering speeds

Execute script F10_lingering_analysis.m. The output is the speed in each vessel along with various statistical results. The sign ranked test is used to evaluate significance.



Speeds in all vessels. Insufficient data can lead to inf or nan values. These vessels are taken out of further statistics.



Example of statistical output. From left to right: speed in the mother, the speed in the bifurcation and the speed differences between mother and bifurcation. This is only for the bifurcations in one example geometry and does not necessarily reflect statistics over all geometries. The code can be used to combine statistics of multiple geometries.

Step 13: Plot lingering parameter bars

The script `F11_lingering_bar_plots.m` creates bar plots for the statistical comparison of quantities. An example would be the comparison of the lingering Peclet number. This is based on an unpaired t-test.

Step 14: Analyze mode changes

The script `F12_mode_changes.m` finds cells that move with a speed below a threshold for a certain duration. The threshold for speed `intThres` was chosen to be 30 % of the velocity in the vessel and may be adjusted to change this condition.

```
intThres = conVes(vesselIdx).HRBCVel/cBif.velScaleHRBC*0.3;
```

The duration threshold is given implicitly by a moving filter on the speed condition. Here the size of the moving window translates to 80 ms.

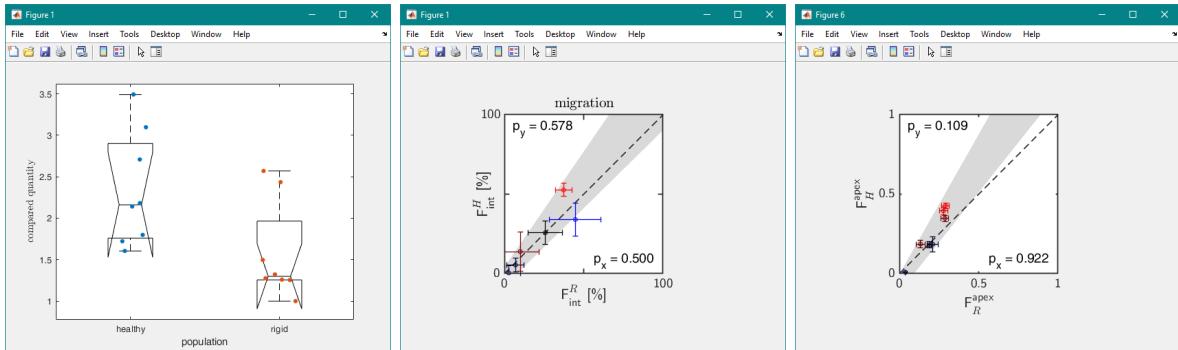
```
rolCond = logical(round(movmean(double(rolCond),4)));
```

Step 15: Plot mode change results

Execute script `F13_mode_changes_results.m` to show the results for the interaction fraction.

Step 16: Analyze apex distances

In `F14_apex_distance_analysis.m`, the number of cells with trajectory points closer than a threshold to the bifurcation apex is determined, and from that the fraction of cells in this proximity. The threshold might be set in microns in the parameter `maxDist`.



Example of statistical output from one geometry. Left, comparison of lingering between healthy and rigid cells, middle comparison of the fraction of interacting cells, right, comparison of the fraction of cells near the apex.