Red blood cell velocity measurement - Fiji plugin

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October 18, 2024

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1 Setup

Put .jar file into the directory Fiji.app/plugins/ (on mac os, right click Fiji.app > Show content folder).

Restart Fiji.

Start plugin by going to the menu $Plugins > Red Blood Cell \ velocity > Red Blood Cell \ velocity$.

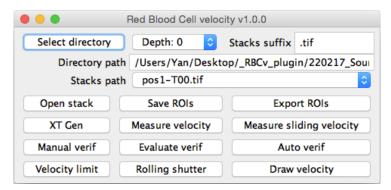


Figure 1 – Plugin's main window.

2 Files management

When starting the plugin, it will immediately prompt to choose a directory containing the source stacks. The stacks should be either directly in the directory or in subdirectories. The chosen directory can be changed with the button *Select directory*. Once a directory has been chosen, its path is written in the field *Directory path*. Manually modifying it won't do anything.

If the stacks are directly in the directory, choose *Depth: 0*. If they are in subdirectories, choose *Depth: 1*. In subsubdirectories, choose *Depth: 2*, and so on.

The stacks should all have the same filename ending. It could simply be the file extension if all files with these extension should be analyzed, or any suffix that could distinguish them from other files not to analyze, e.g. _preprocessed.tif. This ending must be entered in the field Stacks suffix.

Stacks path dropdown menu lists all the stacks detected with Stacks suffix with their path relative to the directory. If Depth: 0, it is directly the stacks filenames, whereas with a depth above 0, the subdirectories will also appear.

3 ROIs management

The first step is to select the stack on which ROIs will be drawn, then clicking on *Open stack*. It will open two stacks: the one selected and another one, composed of the middle image extracted from each stack listed. This will allow making ROIs that will be present in all stacks in order to measure velocities on every stacks. For easier visualisation, it can be interesting to do a max intensity projection of the stack to increase contrast (Image > Stacks > Z Project, choose Max Intensity then click OK).

ROIs can be drawn with line tools (Straight line, Segmented line or Freehand line) and added to the ROI Manager by clicking on the Add [t] button. Line width can be adjusted by double clicking on the line tool in the toolbar.

ROIs can be added to the ROI manager from any image, using t or clicking Add [t] in the ROI Manager, after drawing a region. By default, Segmented Line Tool is selected.

Click on Save ROIs to save all ROIs of the ROI Manager into a zip file located in a subdirectory called ROISets that will be placed in the same directory as the stack.

A group can be assigned to each ROI by using the 1/2/3/4 shortcuts or the menu items Plugins > Red Blood Cell Velocity > Shortcuts > RBCv SetGroup XXX. It is assigned to all selected ROIs in the ROI Manager, or to the current selection if none selected in the ROI Manager.

Click on *Export ROIs* to make a copy of the zip file for each stack. Use *Open stack* to verify the position of the ROIs on the other stacks, doing a *Save ROIs* to update the ROIs after eventual modifications. Make sure all the ROIs are drawn before doing the export (even if they are not on the current stack) to make sure the order and direction of the ROIs stays the same for every stack. Then only do modification of position, slight adjustment of shapes and evenly changing the group to discard if the ROI is not measurable anymore. Use ROI Manager > More > Translate to move all the ROIs from the ROI Manager at the same time and of the same amount if stacks alignment is not good enough.

3.1 Useful shortcuts

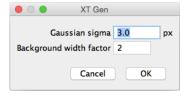
- a: Adds a vertex to current selection at the position of the cursor, linked to the closest vertex of the selection and the appropriate adjacent one.
 - d: Deletes the vertex from current selection that is closest to the position of the cursor.
- p: If the ROI is a straight line, segmented line or freehand line, it converts it to segmented line and places vertices every 20 pixels. It is particularly useful when the ROI was drawn using freehand line and it needs a slight modification of shape (which is not possible with freehand lines), as each vertex can be moved. Clicking several times on p can also help smoothing the polyline.
 - t: Adds current region to the ROI Manager.
- z: Toggles Show All with Labels. In the ROI Manager, click on More » Options, and make sure Associate "Show All" ROIs with slices is not ticked to see all the ROIs on any slice. When Show All and Labels are both ticked, selecting a ROI by clicking on its label in the image then modifying it will directly modify the one stored in the ROI Manager. Otherwise, select a ROI in the list, modify it, then click on Update button in the ROI Manager to propagate the modification to the ROI stored in the ROI Manager.
- 1/2/3/4: Changes the group of the currently selected ROI(s) from the ROI Manager (or the currently drawn ROI if none are selected in the ROI Manager) to Capillaries (yellow) / Arteries (red) / Veins (blue) / Discard (green) respectively.

4 Velocity computation

4.1 XT Gen

Clicking this button will start the process of converting the line ROIs into their profile versus time images. First, parameters have to be set.

- Gaussian sigma (px): size of the gaussian filter applied to the image before extracting ROI's profiles. Useful to remove fiber cores from images taken with a fiberscope (if images were not preprocessed to correct that).



- Background width factor: if above 1, a second profile larger than the initial one will be computed to remove the background surrounding the vessel. E.g. for a value of 2, the second profile will have a radius twice bigger. Then the part of the larger profile that is outside the thinner one will be subtracted to the thinner one to give the final profile.

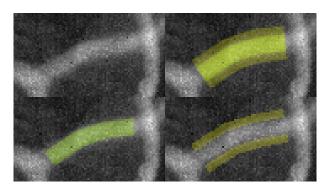


Figure 2 – Illustration for background width factor of 2. Top left: the vessel. Bottom left: the ROI traced. Bottom right: the background that will be subtracted. Top right: the ROI traced with its background.

For each ROI of each stack, the profile is computed for each image by averaging over the width. The profile are then concatenated to create an image with the time as x axis and the profile on y axis. Those images are saved in the subdirectory XT.

Additionally, if a ROI is partially out off the image or in a zero/nan valued area of the image (as can be the case after inter stack alignment), the part of the XT image that is zero valued is removed and the position of the crop is saved in a text file in the subdirectory XTcropParms. That file is used by the rolling shutter adjustment procedure but is of no interest to the user.

4.2 Measure velocity

Measure velocity parameters

Clicking this button will start the process of measuring velocities. First, parameters have to be set.

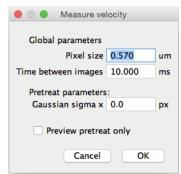
Global parameters:

Pixel size (μm): size in the sample corresponding to one pixel of the raw image.

Time between images (ms): time between the beginnings of two consecutive images (inverse of imaging frequency).

Pretreat parameters:

Gaussian sigma x (px): if above 0, performs a gaussian blur of the XT images with given value for sigma. Useful to get rid of high frequency fluctuations of light intensity, which would otherwise result in vertical stripes increasing chances of detecting a wrong angle. Note that it will also decrease sharpness of streaks.



Preview pretreat only: display the result of the preprocess on the first ROI of currently selected stack, without doing the following computations.

Processing

First a gaussian blur is performed on the image if a strictly positive sigma was chosen.

Second, a 3*3 horizontal Sobel filter is convolved with the image. Kernel:
$$\begin{bmatrix} 1 & 0 & -1 \\ 2 & 0 & -2 \\ 1 & 0 & -1 \end{bmatrix}.$$

Radon transform is computed on the resulting image from 0 to 179 degrees with a one degree step.

The variance is computed for each angle and its maximum is selected. A second order polynomial fit is done to refine the position based on the maximum and its two neighbors, giving θ_{fit} .

The radon transform is computed again on the same image, but from $\theta_{fit} - 1$ to $\theta_{fit} + 1$, with a 0.01 degree step.

The variance is computed for each angle and its maximum is selected. A second order polynomial fit is done to refine the position based on the maximum and its two neighbors, giving θ_f .

The resulting angle θ_f is used to determine the velocity using the formula: $v = \frac{px_size*\nu}{tan(\theta_f)}$, where ν is the imaging frequency.

Full width half max (FWHM) and separability (sep) are computed based on the result of the variance of the first radon transform: $sep = \frac{var(\theta_{max})}{mean(var(\theta))}$; $FWHM = \theta_1 - \theta_2$, where θ_1 and θ_2 are the first values for which $var(\theta) = var(\theta_{max})/2$, starting from θ_{max} and going to the left and to the right respectively.

A correlation coefficient (correl) is determined using the angle θ_f . To do so, the XT image is rotated by this angle in order to have the streaks vertical. The average horizontal line is computed and the correlation between this line and each horizontal line is computed. The average of these correlation gives the final correlation coefficient.

The standard deviation evolution (sd_evol) following a binning is computed as the standard deviation of XT image after a binning of 2*2 divided by the standard deviation of the XT image. The idea behind this measure is that binning an homogeneous image where all the variations come from noise from pixel to pixel will greatly reduce the standard deviation, while binning an image where there are bigger regions of different contrast will not affect much the standard deviation. In the case of pretreated XT image, if there are no streaks, the measure is expected to be lower than if there are streaks.

If group value is 3: discard, no measure is done and all the values are set to NaN.

Output table

● ● ResultsVelocity.csv											
File	ROI	yPx_um	xPx_ms	length	velocity	angle	sep	FWHM	correl	sd_evol	group
pos1-T00.tif	1	0.570000	10	217	759.252887	4.293358	53.969970	2.187172	0.707690	0.573771	0
pos1-T01.tif	1	0.570000	10	217	719.859942	4.527352	38.489417	2.787779	0.611839	0.595514	0
pos1-T02.tif	1	0.570000	10	217	668.719156	4.871977	39.559839	2.497530	0.651064	0.657720	0
pos1-T03.tif	1	0.570000	10	217	409.140168	7.931201	22.969583	2.950476	0.461475	0.621833	0
pos1-T04.tif	1	0.570000	10	217	480.803055	6.760954	36.777706	2.518660	0.652239	0.699130	0
pos1-T05.tif	1	0.570000	10	217	2127.371744	1.534794	11.210674	10.069673	0.322085	0.543296	0
pos1-T06.tif	1	0.570000	10	213	9178.405189	0.355815	16.245539	5.643455	0.499054	0.682588	0
pos1-T07.tif	1	0.570000	10	217	1114.365701	2.928137	41.137337	2.496474	0.605711	0.543464	0
pos1-T08.tif	1	0.570000	10	217	1332.399339	2.449618	29.186904	2.814170	0.617298	0.570142	0
pos1-T09.tif	1	0.570000	10	217	995.233245	3.277921	51.691704	2.090171	0.707862	0.544958	0
pos1-T10.tif	1	0.570000	10	217	834.922791	3.905511	51.178790	2.109293	0.708752	0.608654	0

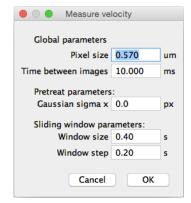
Figure 3 – Measure velocity output table. File: name of the file for current line. ROI: id of the ROI for current file. yPx_um and xPx_ms: resolution of the px for this XT image. length: height of the XT image. velocity: initial value based on angle and resolution. angle: angle measured on XT image, in degrees. sep, FWHM, correl and sd_evol: metrics measured for automatic verification. group: ROI's group (0: capillaries, 1: arteries, 2: veins, 3: discard)

4.3 Measure sliding velocity

Clicking this button will start the process of measuring velocities with a sliding window. Refer to the *Measure velocity* section, as only the new parameters will be discussed here. Instead of doing the radon transform on the whole XT image, it will be done on subparts of the image. Each subpart will correspond to a restriction of the measure to a certain time window (effectively cropping XT image by selecting a limited part of the horizontal time axis).

There are two new parameters: Sliding window parameters. Window size (s) defines the temporal length of the subparts on the XT image.

 $Window\ step\ (s)$ defines the temporal shift between two measures.



This method allows measuring velocities on shorter time scale to better reflect fast changes of velocity. The drawback is that the time window for the measure being smaller, there will be less streaks in the image and so the resulting measure has a higher chance of being incorrect. Another point is that as there will be multiple measures for each stack, it would be intractable to do a manual verification and so it has not been implemented.

5 Verifications

Manual verification can be used either to verify the whole dataset and be the final verification, or to generate a ground truth on a subpart of the dataset to optimize criterion with Evaluate verif and finally do an automatic verification on the whole dataset with Auto verif.

5.1 Manual verif

Clicking this button will start the process of manual verification of the computed angles. It will first open the *Verification* frame and the resultsRadon.csv table. Upon verification, three new columns will be added and progressively filled: *Verified*, *velocity_ok* and *angle_ok*.



Figure 4 – Verification frame.

For each ROI, a montage of all its XT images is displayed, with blue stripes indicating the computed angle (column *angle* in the table). If the angle value is NaN (not a number, happens if group value is 3: discard), a blue cross appears.

If one angle seem incorrect and the correct angle is visible in the image, draw a line with the correct angle in that sub-image and click on the *Update angle [u]* button (shortcut: *u*). *Verified* is set to 1, *angle_ok* is set to the angle of the line and *velocity_ok* is set to the corresponding velocity. Also, red stripes are overlaid onto the images to show the new angle.

If one angle seem incorrect and the correct angle seem impossible to determine, draw a line in that sub-image and click on the *Remove angle [r]* button (shortcut: r). *Verified* is set to -1, $angle_ok$ and $velocity_ok$ are set to NaN (Not a Number). Also, a red cross is overlaid onto the image to indicate the measure was discarded.

If all the remaining angles seem correct, click on the Validate ROI [y] button (shortcut: y). For all the lines for which Verified is still 0, it is set to 2 and the values of angle and velocity are copied into angle_ok and velocity_ok. One exception when the group value is 3: discard, the Verified value is set to -1, while angle_ok and velocity_ok are set to NaN.

If there are too many missing angles and the region should be removed altogether, click on the $Discard\ ROI\ [n]$ button (shortcut: n). For all the lines Verified is set to -2 and the values of $angle\ ok$ and $velocity\ ok$ are set to NaN.

When the velocity is null, it creates horizontal stripes on the XT image, but as the preprocessing of those images is designed to suppress this kind of stripes (which are often caused by inhomogeneous intensity along the vessel, or patterning of the image through a multicore fiber), it can be impossible to measure it. In case of doubt for a specific sub-image, click on *Open stack* to inspect the original stack or on *Open raw XT* to inspect XT image without preprocessing. With *Virtual stack* enabled, stacks are read on the fly and not fully loaded in memory. With *Autoclose* enabled, stacks and raw XT images are automatically closed when using *Open stack* or *Open raw XT* buttons again, or moving to next ROI with *Validate ROI* or *Discard ROI*.

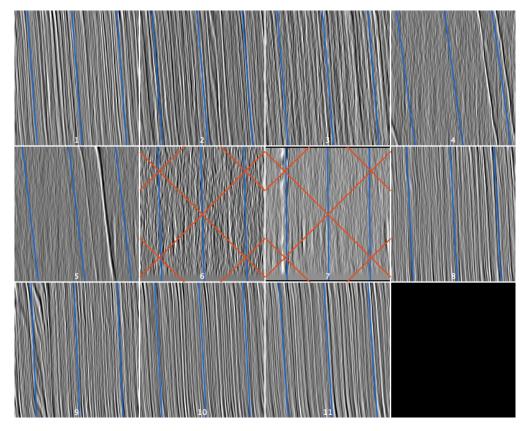


Figure 5 – Sample image showing all XT images for a given ROI with computed angles drawn in blue, updated angles in red. Crossing means measure is discarded.

5.2 Evaluate verif

Clicking this button will start the process of evaluating various parameters against the ground truth given by *Manual verif* to find the limits that better separate good measures (accurate detection of streak slant) and bad measure (inaccurate detection of streak slant or no streak to detect).

Verification metrics

Metric 1: Metric 2:

Cancel

First, two metrics need to be selected among these choices:

- sep: the separability of the variances of the radon transforms, which is defined as $sep = \frac{var(\theta_{max})}{mean(var(\theta))}$. Higher values for good measures.
- FWHM: the full width half max of the variances of the radon transforms. Lower values for good measures.
- correl: the mean of the correlations between each line and the average line in the direction orthogonal to the angle found on the pretreated XT image. Higher values for good measures.
- sd_evol : the evolution of the standard deviation of the pretreated XT image undergoing a 2*2 binning. Higher values for good measures.

Matthews correlation coefficient (MCC) is computed for each pair of metric 1, metric 2, ranging from the minimum value to the maximum value with an adaptative step (the power of

10 that gives between 31 and 317 steps).

An image is created with the local maxima highlighted with a multipoint selection (if more than one). Images are calibrated to give the coordinates in the metrics spaces.

In parallel, a plot is displayed (Figure 6) in the two metrics spaces, showing the different classes in different colors (green: the angle was correctly detected, blue: the angle was incorrectly detected, red: there was no angle to detect). In practice, the goal is to separate as best the green class from the rest. Blue class is indicative and is considered the same as red in the computation of the MCC. Legend gives the coordinates of the maxima found and the corresponding MCC score. Two black segments show the separation in the parameters space.

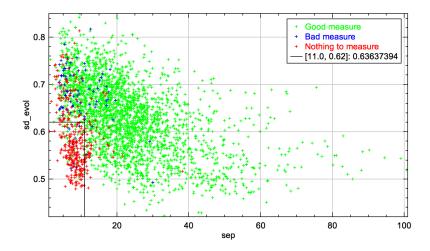


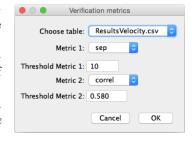
Figure 6 – Verification result. X axis: metric 1, Y axis: metric 2, black lines: separation of the two groups that maximizes the Matthews correlation coefficient.

5.3 Auto verif

Clicking this button will start the process of automatic verification of the computed angles. First, parameters have to be set.

First, choose on which table to operate (either ResultsVelocity.csv or ResultsVelocitySliding.csv, the respective results of *Measure velocity* and *Measure sliding velocity*).

Then choose the two metrics which will separate the results in two class and their threshold values, as determined with *Evaluate* verif.



The output results are respectively Results Velocity Auto.csv and Results Velocity Sliding Auto.csv for input tables Results Velocity.csv and Results Velocity Sliding.csv. Those new tables contain three new columns. Verified containing a 3 for assumed correct values and -3 for assumed incorrect values. velocity_ok containing the same value as velocity for correct values and NaN for incorrect values. angle_ok containing the same value as angle for correct values and NaN for incorrect values.

5.4 Velocity limit

In order for a velocity to be considered measurable, red blood cells should be visible in the ROI on at least two frames. In practice, the minimum angle is defined as $\theta_{min} = atan(\frac{2}{length(ROI)})$ and $|\theta| > \theta_{min}$.

Clicking this button will start the process of comparison between the angle found and the minimum measurable angle computed using the length of the ROI (the height of the XT image).



Choose table: ResultsVelocity.csv

Line time 10.00000

Y offset 0 Rolling shutter start line 0

Camera parameters

First, choose on which table to operate (it can be ResultsVelocity.csv, ResultsVelocityManual.csv, ResultsVelocityAuto.csv, ResultsVelocitySliding.csv or ResultsVelocitySlidingAuto.csv).

Then, for each angle value that is evaluated as not measurable, *Verified* is set to -4, *velocity_ok* and *angle_ok* are set to NaN.

5.5 Rolling shutter

With sCMOS camera, each line of the sensor is typically not exposing at the exact same time, as the rolling shutter of the camera moves from line to line to start and stop the exposure. It means that any ROI that begins and ends at a different position of the rolling shutter will need a slight adjustment of the velocity value.

Clicking this button will start that process. First, camera parameters have to be set.

- Choose table: choose on which table to operate (it can be ResultsVelocity.csv, ResultsVelocityManual.csv, ResultsVelocityAuto.csv, ResultsVelocitySliding.csv or ResultsVelocitySlidingAuto.csv).
- Line time: time for the rolling shutter to go from one line of the camera to the next.
- Y offset: the position of the first line of the image relative to the whole camera sensor array.
- Rolling shutter start line: starting edge of the rolling shutter. It can be the center of the camera sensor if there are two separate rolling shutters, or the top or bottom with one rolling shutter

As stated before, velocity is computed as $v = \frac{px_size*\nu}{tan(\theta)} = \frac{dl}{dt}$, where dl is the length of the ROI in μm and dt is the time it takes a red blood cell to go from the beginning to the end of the ROI. If there is a rolling shutter effect, dt becomes $dt + \epsilon_t$, with $\epsilon_t = camera_line_time*n_lines$. n_lines is the number of camera lines between the beginning and the end of the ROI. It is positive if the ROI follows the rolling shutter and negative if they go in opposite directions. Finally, we define $rs_coeff = \frac{dt}{dt+\epsilon t}$ and get the new formula $v = \frac{px_size*\nu}{tan(\theta)}*rs_coeff$. The column velocity_ok is updated with this new formula and the column rs_coeff contains the correction factor.

5.6 Final table

● ● ■ ResultsVelocityManual.csv														
File	ROI	yPx_um	xPx_ms	length	velocity	angle	sep	FWHM	correl	sd_evol	group	Verified	velocity_ok	angle_ok
pos1-T00.tif	1	0.570000	10	217	759.252887	4.293358	53.969970	2.187172	0.707690	0.573771	0	2	759.252887	4.293358
pos1-T01.tif	1	0.570000	10	217	719.859942	4.527352	38.489417	2.787779	0.611839	0.595514	0	2	719.859942	4.527352
pos1-T02.tif	1	0.570000	10	217	668.719156	4.871977	39.559839	2.497530	0.651064	0.657720	0	2	668.719156	4.871977
pos1-T03.tif	1	0.570000	10	217	409.140168	7.931201	22.969583	2.950476	0.461475	0.621833	0	2	409.140168	7.931201
pos1-T04.tif	1	0.570000	10	217	480.803055	6.760954	36.777706	2.518660	0.652239	0.699130	0	2	480.803055	6.760954
pos1-T05.tif	1	0.570000	10	217	2127.371744	1.534794	11.210674	10.069673	0.322085	0.543296	0	-1	NaN	NaN
pos1-T06.tif	1	0.570000	10	213	9178.405189	0.355815	16.245539	5.643455	0.499054	0.682588	0	-1	NaN	NaN
pos1-T07.tif	1	0.570000	10	217	1114.365701	2.928137	41.137337	2.496474	0.605711	0.543464	0	2	1114.365701	2.928137
pos1-T08.tif	1	0.570000	10	217	1332.399339	2.449618	29.186904	2.814170	0.617298	0.570142	0	2	1332.399339	2.449618
pos1-T09.tif	1	0.570000	10	217	995.233245	3.277921	51.691704	2.090171	0.707862	0.544958	0	2	995.233245	3.277921
pos1-T10.tif	1	0.570000	10	217	834.922791	3.905511	51.178790	2.109293	0.708752	0.608654	0	2	834.922791	3.905511

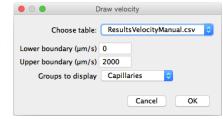
Figure 7 – Final table after verifications. Verified: value of the verification process, ranging from -4 to 3. 0: not verified. 1: manually corrected by tracing the slant. 2: manually validated. -1: manually discarded this time point. -2: manually discarded the whole ROI. 3: automatically validated. -3: automatically discarded by velocity limit criterion. velocity_ok: value of velocity after verifications. angle_ok: value of angle after verifications. rs_coeff: value of rolling shutter coefficient to apply to counteract the rolling shutter effect of the camera.

5.7 Draw velocity

This button creates a summary image displaying a velocity vector for each measure, where the arrow point the direction of the flow and the color indicate the velocity absolute value.

Clicking this button will start the process. First, parameters have to be set.

- Choose table: select on which table to operate, it can be ResultsVelocity.csv, ResultsVelocityManual.csv, ResultsVelocityAuto.csv, ResultsVelocitySliding.csv or ResultsVelocitySlidingAuto.csv. Only the existing tables will be displayed.



- Lower boundary $(\mu m/s)$ and Upper boundary $(\mu m/s)$: select the limits of the color Look Up Table.
- Groups to display: select which velocity vectors should be drawn, based on the groups assigned to the ROIs. It can be either Capillaries, Arteries, Veins, Arteries and veins or All three of them.

Resulting images are saved in the subdirectory VelocityArrows. Each image is named after the stack it originated from with a suffix that depends on the table chosen: _raw for ResultsVelocity.csv, _manual for ResultsVelocityManual.csv, _auto for ResultsVelocityAuto.csv, _sliding for ResultsVelocitySliding.csv and _sliding_auto for ResultsVelocitySlidingAuto.csv.

For sliding tables, the velocity displayed is the average of the valid velocity measures.

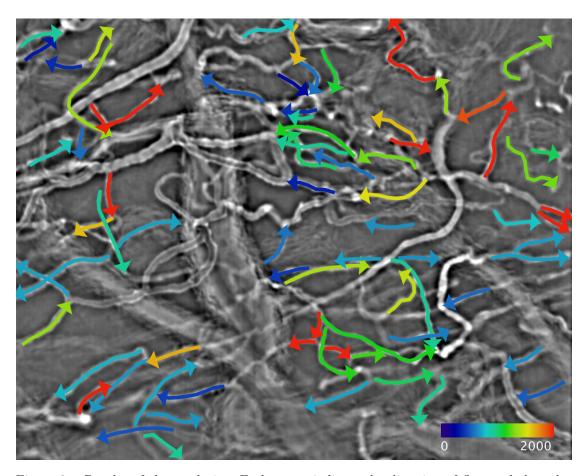


Figure 8 – Results of draw velocity. Each arrow indicate the direction of flow and the color indicates the absolute velocity. Vessels rejected at the verification step (Verified < 0) are not drawn.