1 Overview

ZlineDetection is a program written in MATLAB that quantifies z-line architecture in 2D images of α-actinin stained cardiac tissues. It has the option to calculate and report the following metrics, which are selected from a user-friendly graphical user interface (GUI) (Fig. 2):

- Z-line fraction
- Continuous z-line length
- Z-line Orientational Order Parameter (OOP)
- Actin OOP

ZlineDetection can also combine and summarize these features for a coverslip, and can compare between coverslip and conditions.

1.1 System Requirements

ZlineDetection has been developed and tested on Matlab 2018b on Windows 10. The required Matlab version and Toolboxes:

- MATLAB Version $\geq$ 9.5
- Image Processing Toolbox Version 10.3
- Statistics and Machine Learning Toolbox Version 11.4

2 Retrieve ZlineDetection from Github

1. Navigate to the Cardiovascular-Modeling-Laboratory Organization account and select the "ZlineDetection" repository.
   `https://github.com/Cardiovascular-Modeling-Laboratory/ZlineDetection`
2. Select **"Download ZIP"** from the **"Clone or Download"** dropdown menu.
3. Locate and unzip the zipped "ZlineDetection" file on your computer.

3 Prepare Data for Analysis

It is recommend that all fields of view (FOV) for a single coverslip should be in the same folder and each coverslip should have its own folder (Fig. 1). In order to use actin to guide segmentation, FOVs stained for actin and α-actinin should be contained within the same folder and have similar file names with the only major distinction being the name of the stain (Fig. 1).

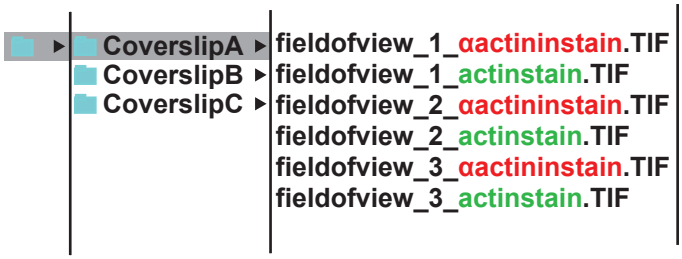


Fig 1. File organization for ZlineDetection. Each coverslip (A,B,C) has its own directory and within a coverslip directory each field of view is labeled by number and stain. Coverslip A has three FOV stained for actin and three FOV stained for α-actinin

4 Run ZlineDetection

1. Open MATLAB.
2. Navigate to the location of ZlineDetection, by browsing for the directory.
3. Once you have opened the directory containing ZlineDetection, type ZlineDetection in the command line and press enter. A window titled **Z-line Detection & Analysis** will open (Fig. 2).

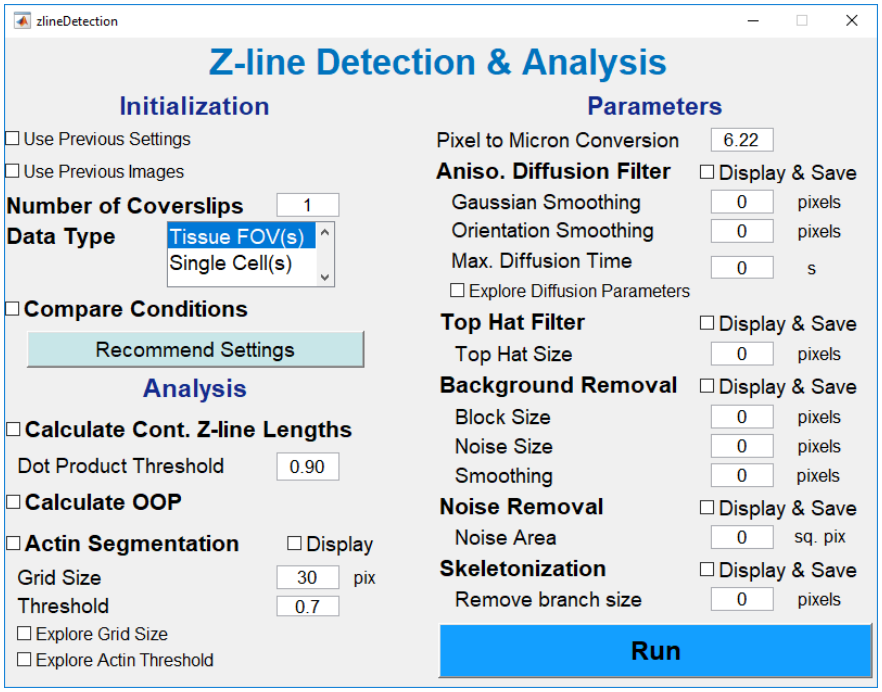


Fig 2. GUI for ZlineDetection in MATLAB.

4.1 Initialize Input Data

1. Under the heading **Initialization**, enter the number of coverslips you would like to run next to the text box **Number of Coverslips**.
2. In the drop down menu next to **Data Type** select either Tissue FOV(s) or Single Cell(s).
3. If you would like to analyze multiple coverslips that have different conditions (i.e. treated vs. control), check the box next to **Compare Conditions**.
4. By checking Use Previous Settings and/or Use Previous Images, you will be able to load and use the parameter and analysis settings chosen previously and/or the images you have previously analyzed.

4.2 Analysis Options

ZlineDetection has the following analysis options found under the **Analysis** heading:

- Check **Calculate Cont. Z-line Lengths** to plot and calculate the length of continuous z-lines. Enter the dot product threshold next to the text Dot Product Threshold (See Section Section 4.2.1 for more information on parameter selection).
- Check **Calculate OOP** to report the OOP of the z-lines.
- Check **Actin Segmentation** to perform actin guided segmentation and calculate the z-line fraction. Enter the grid size and threshold next to the text Grid Size and Threshold, respectively (See Section Section 4.2.1 for more information on parameter selection). Actin stained images are required to perform actin guided segmentation.

Note: Pushing the button **Recommend Settings** will auto-fill recommended analysis options and parameters, overwriting any other settings (Fig. 3).

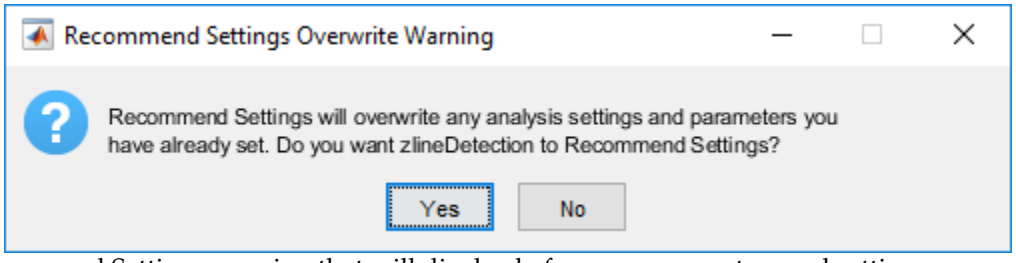


Fig 3. Recommend Settings warning that will display before any parameters and settings are overwritten.

4.2.1 Analysis Parameters

- **Continuous Z-line Detection and Measurements** (**Calculate Cont. Z-line Lengths**)
  - Dot product threshold : Maximum angle between pixels to be considered parallel and therefore continuous (Default: 0.9; <~25°)
- **Actin Guided Segmentation of Off-Target α-actinin Staining** (**Actin Segmentation**)
  - Threshold : Minimum angle between α-actinin and local actin orientation for pixels to be considered perpendicular (Default: 0.7; >~45°).
  - Grid size : Size of local actin orientation (Default: 30 pixels = ~5 µm)

4.3 Parameter Selection

Pushing the button **Recommend Settings** will auto-fill recommended analysis options and parameters, overwriting any other settings (Fig. 3).
Parameters were chosen for images with the resolution (Pixel to Micron Conversion) 6.22 µm/ pixel.

The following image analysis parameters can be set under the heading **Parameters** .

- **Coherence Enhancing Anisotropic Diffusion Filtering** (**Aniso. Diffusion Filter**)
  - Gaussian smoothing : Standard deviation of Gaussian smoothing before calculation of the image Hessian (Default: 1 pixels).
  - Orientation smoothing : Standard deviation of Gaussian smoothing of the image Hessian (Default: 1.4 pixel).

  - Max. diffusion time : Total diffusion time; determines the number of iterations of anisotropic diffusion filtering (Default: 1.5 s; 11 iterations).

- **Top-Hat Filtering** (**Top Hat Filter**)

  - Top hat size : Radius of the flat disk-shaped structuring element used for the top hat filter (Default: 3 pixels = ~0.5 µm).

- **Background Removal**

  - Block size : Size of blocks to break image into (Default: 15 pixels = ~2.5 µm).
  - Noise size : Size of blocks considered noise in the condensed image (Default: 10 pixels = ~25 µm).
  - Smoothing : Standard deviation of Gaussian smoothing to perform on image before calculation the magnitude (Default: 1 pixel).

- **Binarization** (**Noise Removal**)

  - Noise area : Size of small objects to be removed in the binarized image (Default: 8 pixels^2 = 0.2 µm^2).

- **Skeletonization**

  - Remove branch size : Minimum branch size to be included in analysis (Default: 4 pixels).

After starting the analysis, if you are performing actin guided segmentation, you will be asked for parameters for computation of the actin orientation vectors (Fig. 4).

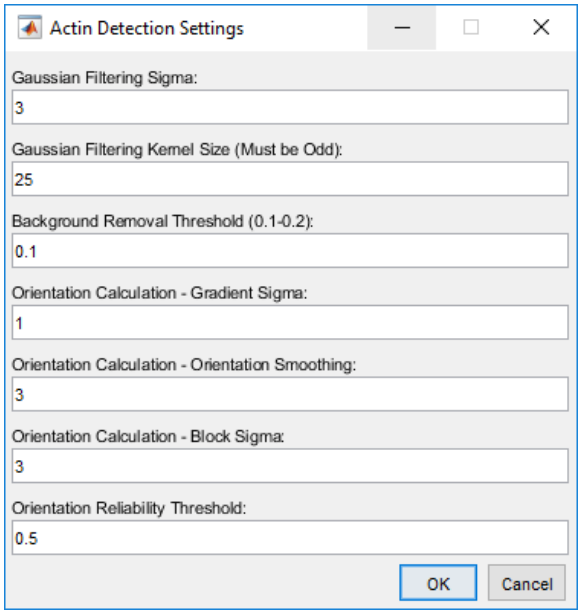


Fig 4. Actin Detection Settings.

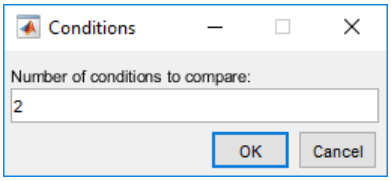
- **Actin Detection Settings**

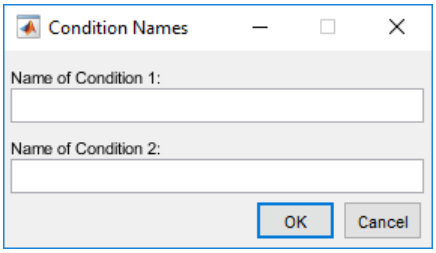
  - Gaussian Filtering Sigma: Standard deviation of Gaussian smoothing to perform on actin image (Default: 3 pixels).

- Gaussian Filtering Kernel Size (Must be Odd): Size of Gaussian filter kernel to perform on actin image (Default: 25 pixels).
- Orientation Calculation - Gradient Sigma: Sigma of the derivative of Gaussian used to compute image gradients (Default: 1 pixel).
- Orientation Calculation - Orientation Smoothing: Sigma of the derivative of Gaussian used to smooth the final orientation vector field (Default: 3 pixels).
- Orientation Calculation - Block Sigma: Sigma of the Gaussian weighting use to sum the gradient moments (Default: 3 pixels).
- Orientation Reliability Threshold: Minimum reliability of actin orientation vectors (Default: 0.5).

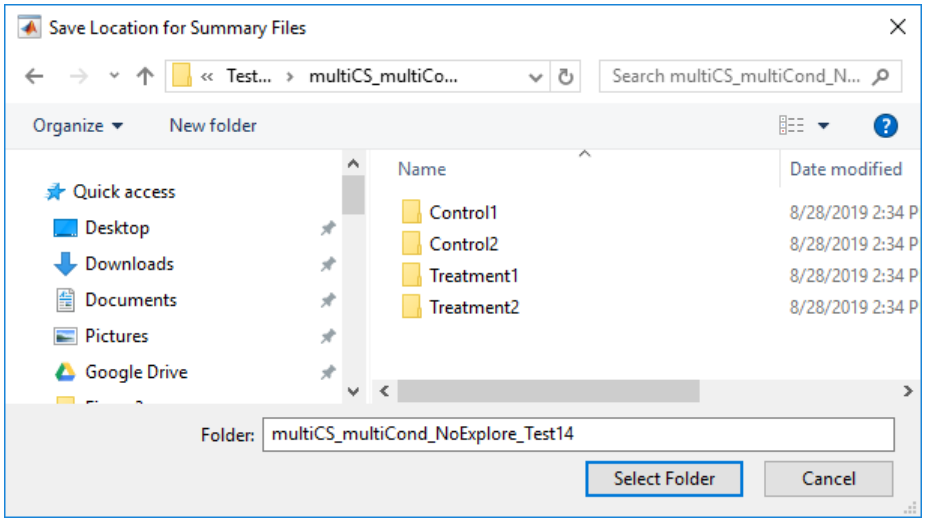
4.4 Start Analysis and Select Images

1. Push **Run** to start the analysis.
2. If you selected **Compare Conditions** you will be asked for the number of conditions, and the name of the conditions in popup dialog boxes.

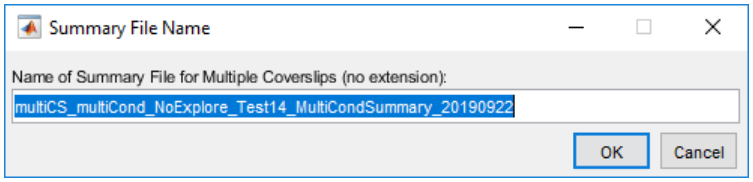




3. You will be prompted for a "base directory." This is where your coverslips are located, and where you would like to save summary files.



4. You will then be prompted for the name of your summary file.



5. After entering the name of your summary file, you will be asked to choose the images stained for z-lines (and then actin if selected) for each coverslip.

6. If you provided images stained for both actin and z-lines, you will be asked if the files are matched correctly.

7. After selecting all the z-line images (and actin if applicable) for a coverslip, you will be asked to select the condition for the current coverslip if you checked **Compare Conditions**. The name of the coverslip selected with output on the MATLAB command line. You will be asked to approve the selection, or re-select (and re-label) the coverslip.

After all of the data has been selected, each field of view for each coverslip will be analyzed without the need for user input.

5 Results of Analysis

5.1 Each Field of View

- For each field of view, a new folder will be created with the same name of that field of view (Fig. 5). This folder will contain a `OrientationAnalysis.mat` file with the results of the analysis (Table 1).
- If you did continuous z-line length analysis, a histogram of continuous z-line lengths and the plotted continuous z-lines (.fig and .tif) will also be contained in this folder.
- (**Single Cells Only**) If "Single Cell(s)" was selected, a summary pdf will be saved for each image (Fig. 6) in a folder "SingleCell_RESULTS_YYYYMMDD" in the directory chosen to store summary files.

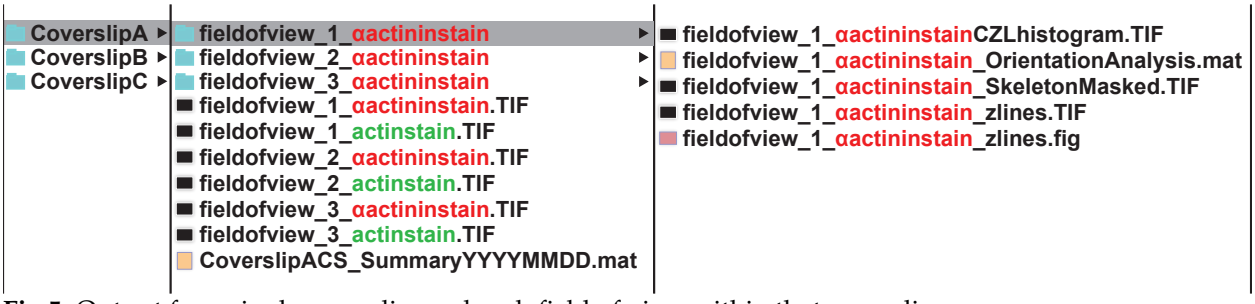


Fig 5. Output for a single coverslip, and each field of view within that coverslip.

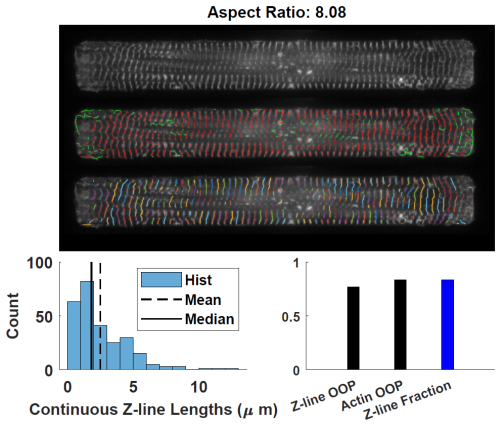


Fig 6. Representative single cell summary .pdf. Each summary pdf contains images of the z-lines (top), the actin segmented image (middle), and plotted continuous z-lines (bottom). Additionally, it has a histogram of continuous z-line lengths, the z-line OOP, actin OOP, and z-line fraction.

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5.2 Each Coverslip

The results of each coverslip will be contained in a .mat file with name, directory_name + CS_Summary + YYYYMMDD (Fig. 5, Table 2).

5.3 Coverslip Comparison

An excel spreadsheet (Table 4), along with a MATLAB .mat file (Table 3) will be created to summarize results when multiple coverslips are analyzed (Fig. 7). It will be located in the summary path you selected with the name you selected.

If you checked **Compare Conditions** , the coverslips for each condition will be compared and plotted. The plots will be stored in a folder with the name: your summary filename + _RESULTS.

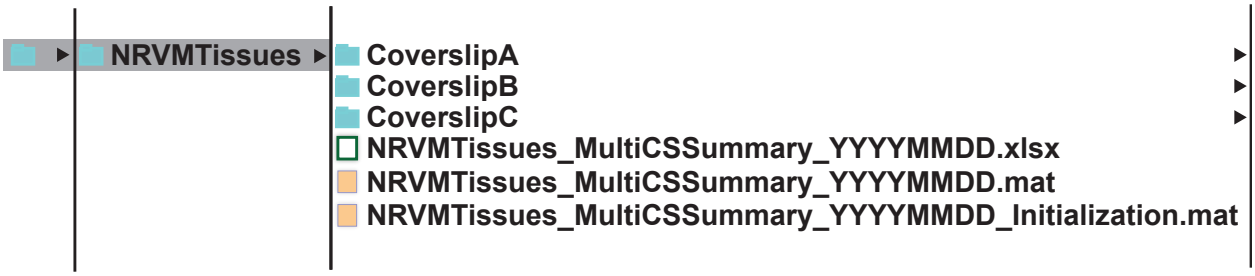

Fig 7. Output for multiple coverslips.

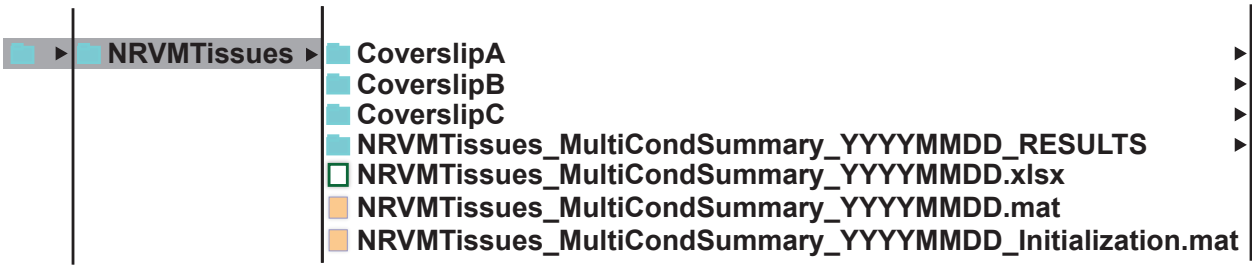

Fig 8. Output for multiple coverslips with conditions selected.

Table 1: Description of .mat file output for each field of view (OrientationAnalysis.mat).

Analysis Stage	Description	Variable Name	Special Options
Initialization	Location of z-line image + name of z-line image file	im_struct.im_location	
Initialization	Name of z-line image file	im_struct.im_name	
Initialization	Location of z-line image	im_struct.im_path	
Initialization	2D matrix of z-line image	im_struct.im	
Initialization	Location to save zlineDetection outputs	im_struct.save_path	
Initialization	Grayscale z-line image	im_struct.im_gray	
Diffusion Filtering	Diffusion filtered image	im_struct.im_anisodiffuse	
Orientation Calculation	Angle corresponding to orientation vectors in z-line image	im_struct.orientim	
Orientation Calculation	Angle corresponding to orientation vectors in z-line image before skeletonization	im_struct.noskel_orientim	
Orientation Calculation	Angle corresponding to orientation vectors in z-line image before actin guided segmentation	im_struct.noactinfilt_orientim	
Top Hat Filtering	Top hat filtered image (same dimensions as 2D image)	im_struct.im_tophat	
Background Removal	Logical size of the image where background is 0 and foreground is 1	im_struct.background	
Background Removal	Considered background in the iamge	im_struct.im_background	
Background Removal	Percentage of the image in the foreground	im_struct.per_rem	
Background Removal	Interpolated background of image	im_struct.surface_thresh	
Binarization	Binarized image	im_struct.im_binary	
Binarization	Binary image with small objects removed	im_struct.im_binaryclean	
Skeletonization	Skeletonized binary image	im_struct.skel_initial	
Skeletonization	Trimmed initial skeleton	im_struct.skel_trim	
Skeletonization	Skeleton after actin guided segmentation	im_struct.skel_final	
Skeletonization	Trimmed final skeleton	im_struct.skel_final_trimmed	
Actin Orientation Calculation	Actin filename and path	im_struct.actin_struct.filename	Actin Segmentation
Actin Orientation Calculation	Angle corresponding to orientation vectors in actin image	im_struct.actin_struct.actin_orientim	Actin Segmentation
Actin Orientation Calculation	Grayscale actin image	im_struct.actin_struct.actin_im	Actin Segmentation
Actin Orientation Calculation	Logical background image	im_struct.actin_struct.actin_background	Actin Segmentation
Actin Orientation Calculation	Smoothed actin image	im_struct.actin_struct.actin_smoothed	Actin Segmentation
Actin Orientation Calculation	Actin image with 0 mean and unit standard deviation	im_struct.actin_struct.actin_normalized	Actin Segmentation
Actin Orientation Calculation	Reliabilitiy of orientation vectors in actin image	im_struct.actin_struct.reliability	Actin Segmentation
Actin Guided Segmentation	Regions where z-lines are perpendicular to actin (1) or parallel (0)	im_struct.mask	Actin Segmentation

Actin Guided Segmentation	Dimensions of each actin grid	im_struct.actin_struct.dims	Actin Segmentation
Actin Guided Segmentation	OOP of each grid	im_struct.actin_struct.oop	Actin Segmentation
Actin Guided Segmentation	Director of each grid	im_struct.actin_struct.director	Actin Segmentation
Actin Guided Segmentation	Number of nonzero pixels and the total number of pixels in the actin image	im_struct.actin_struct.grid_info	Actin Segmentation
Actin Guided Segmentation	Matrix to visualize the director in the middle of each grid	im_struct.actin_struct.visualization_matrix	Actin Segmentation
Actin Guided Segmentation	Director of each grid	im_struct.actin_struct.director_matrix	Actin Segmentation
Actin Guided Segmentation	Dot product of z-line orientation matrix with actin orientation matrix	im_struct.dp	Actin Segmentation
Actin Guided Segmentation	Fraction of α-actinin skeleton removed due to actin filtering	im_struct.nonzlinefrac	Actin Segmentation
Actin Guided Segmentation	Fraction of α-actinin skeleton kept after actin filtering	im_struct.zlinefrac	Actin Segmentation
Continuous Z-line Length	Location of continuous z-lines	CZL_struct.zline_clusters	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Continuous z-line lengths in pixels	CZL_struct.distances_no_nan	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Continuous z-line lengths in μm	CZL_struct.distances_um	Calculate Cont. Z-line Lengths
Orientational Order Parameter	Actin Orientational Order Parameter	oop_struct.ACTINoop	Calculate OOP
Orientational Order Parameter	Actin director in degrees	oop_struct.ACTINdirectionAngle	Calculate OOP
Orientational Order Parameter	Actin director in vector form	oop_struct.ACTINdirector	Calculate OOP
Orientational Order Parameter	Director in degrees	oop_struct.directionAngle	Calculate OOP
Orientational Order Parameter	Director in vector form	oop_struct.director	Calculate OOP
Orientational Order Parameter	Orientational Order Parameter	oop_struct.oop	Calculate OOP

Table 2: Description of .mat file output for each coverslip.

Analysis Stage	Description	Variable Name	Special Options
Initialization	Location of the coverslip	CS_results.zline_path	
Initialization	Name of all z-line fields of view in the coverslip	CS_results.zline_images	
Orientation Calculation	All z-line orientation vectors for the coverslip	CS_results.CS_angles	
Orientation Calculation	Number of z-line orientation vectors for the coverslip	CS_results.angle_count	Calculate OOP
Actin Orientation Calculation	All actin orientation vectors for the coverslip	CS_results.ACTINCS_angles	Actin Segmentation
Actin Orientation Calculation	OOP of actin orientation vectors for the coverslip	CS_results.ACTINCS_OOPs	Actin Segmentation
Actin Orientation Calculation	Director of actin orientation vectors for the coverslip	CS_results.ACTINCS_directors	Actin Segmentation
Actin Orientation Calculation	Number of actin orientation vectors for the coverslip	CS_results.ACTINangle_count	Actin Segmentation

Actin Guided Segmentation	Fraction of α-actinin skeleton removed due to actin filtering for the coverslip	CS_results.CS_nonzlinefrac	Actin Segmentation
Actin Guided Segmentation	Fraction of α-actinin skeleton kept after actin filtering for the coverslip	CS_results.CS_zlinefrac	Actin Segmentation
Actin Guided Segmentation	Actin threshold value(s) for the coverslip	CS_results.CS_thresholds	Actin Segmentation
Actin Guided Segmentation	Actin grid size(s) for the coverslip	CS_results.CS_gridsizes	Actin Segmentation
Continuous Z-line Length	All continuous z-line lengths for the coverslip	CS_results.CS_lengths	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Median continuous z-line length for the coverslip	CS_results.CS_medians	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Sum of the continuous z-line lengths for the coverslip	CS_results.CS_sums	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Mean continuous z-line length for the coverslip	CS_results.CS_means	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Skewness of continuous z-line lengths for the coverslip	CS_results.CS_skewness	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Kurtosis of continuous z-line lengths for the coverslip	CS_results.CS_kurtosis	Calculate Cont. Z-line Lengths
Orientational Order Parameter	OOP of z-line orientation vectors for the coverslip	CS_results.CS_OOPs	Calculate OOP
Orientational Order Parameter	Director of z-line orientation vectors for the coverslip	CS_results.CS_directors	Calculate OOP
Initialization	Location of the coverslip	FOV_results.zline_path	
Initialization	Name of all z-line fields of view in the coverslip	FOV_results.zline_images	
Orientation Calculation	The z-line orientation vectors for each field of view in a cell (size: 1 x number of FOV)	FOV_results.FOV_angles	
Orientation Calculation	Number of z-line orientation vectors for each field of view (size: 1 x number of FOV)	FOV_results.FOV_anglecount	
Actin Orientation Calculation	The actin orientation vectors for each field of view in a cell (size: 1 x number of FOV)	FOV_results.ACTINFOV_angles	Actin Segmentation
Actin Orientation Calculation	OOP of actin orientation vectors for each field of view (size: 1 x number of FOV)	FOV_results.ACTINFOV_OOPs	Actin Segmentation
Actin Orientation Calculation	Director of actin orientation vectors for the coverslip (size: 1 x number of FOV)	FOV_results.ACTINFOV_directors	Actin Segmentation
Actin Orientation Calculation	Number of actin orientation vectors for the coverslip (size: 1 x number of FOV)	FOV_results.ACTINFOV_anglecount	Actin Segmentation
Actin Guided Segmentation	Fraction of α-actinin skeleton removed due to actin filtering for each field of view (size: 1 x number of FOV)	FOV_results.FOV_nonzlinefrac	Actin Segmentation
Actin Guided Segmentation	Fraction of α-actinin skeleton kept after actin filtering for each field of view (size: 1 x number of FOV)	FOV_results.FOV_zlinefrac	Actin Segmentation
Actin Guided Segmentation	Number of pixels in the α-actinin skeleton for each field of view (size: 1 x number of FOV)	FOV_results.FOV_prefiltered	Actin Segmentation
Actin Guided Segmentation	Number of pixels in the z-line skeleton for each field of view (size: 1 x number of FOV)	FOV_results.FOV_postfiltered	Actin Segmentation

Actin Guided Segmentation	Actin threshold value(s) for each field of view (size: 1 x number of FOV)	FOV_results.FOV_thresholds	Actin Segmentation
Actin Guided Segmentation	Actin grid size(s) for each field of view (size: 1 x number of FOV)	FOV_results.FOV_grid_sizes	Actin Segmentation
Continuous Z-line Length	Continuous z-line lengths for each field of view (size: 1 x number of FOV)	FOV_results.FOV_lengths	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Median continuous z-line length for each field of view (size: 1 x number of FOV)	FOV_results.FOV_medians	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Sum of the continuous z-line lengths for each field of view (size: 1 x number of FOV)	FOV_results.FOV_sums	Calculate Cont. Z-line Lengths
Orientational Order Parameter	OOP of z-line orientation vectors for each field of view (size: 1 x number of FOV)	FOV_results.FOV_OOPs	Calculate OOP
Orientational Order Parameter	Director of z-line orientation vectors for the coverslip (size: 1 x number of FOV)	FOV_results.FOV_directors	Calculate OOP

Table 3: Description of .mat file for multiple coverslips and conditions.

Analysis Stage	Description	Variable Name	Special Options
Initialization	Name of all fields of view for all coverslips analyzed	zline_images	
Initialization	Location of all fields of view for all coverslips analyzed	zline_path	
Initialization	Name of each coverslip analyzed	name_CS	
Initialization	Condition number for each coverslip analyzed	cond	Compare Conditions
Initialization	Coverslip number	MultiCS_Data.MultiCS_CSID	
Initialization	Coverslip condition number	MultiCS_Data.MultiCS_CONDID	Compare Conditions
Initialization	Name of each coverslip	MultiCS_Data.name_CS	
Orientation Calculation	Orientation vectors for each coverslip	MultiCS_Data.MultiCS_orientim	
Orientation Calculation	Number of orientation vectors for each coverslip	MultiCS_Data.MultiCS_anglecount	
Actin Orientation Calculation	Actin orientation vectors for each coverslip	MultiCS_Data.MultiCS_ACTINorientim	Actin Segmentation
Actin Orientation Calculation	Number of actin orientation vectors for each coverslip	MultiCS_Data.MultiCS_ACTINanglecount	Actin Segmentation
Actin Guided Segmentation	Non z-line fraction for each coverslip	MultiCS_Data.MultiCS_nonzlinefrac	Actin Segmentation
Actin Guided Segmentation	Z-line fraction for each coverslip	MultiCS_Data.MultiCS_zlinefrac	Actin Segmentation
Actin Guided Segmentation	Actin grid size(s) for each coverslip	MultiCS_Data.MultiCS_grid_sizes	Actin Segmentation
Actin Guided Segmentation	Actin threshold(s) for each coverslip	MultiCS_Data.MultiCS_actin_threshs	Actin Segmentation
Continuous Z-line Length	Continuous z-line lengths for each coverslip	MultiCS_Data.MultiCS_lengths	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Median continuous z-line length for each coverslip	MultiCS_Data.MultiCS_medians	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Sum of the continuous z-line lengths for each coverslip	MultiCS_Data.MultiCS_sums	Calculate Cont. Z-line Lengths

Continuous Z-line Length	Mean continuous z-line length for each coverslip	MultiCS_Data.MultiCS_means	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Skewness of the continuous z-line lengths for each coverslip	MultiCS_Data.MultiCS_skewness	Calculate Cont. Z-line Lengths
Continuous Z-line Length	Kurtosis of the continuous z-line lengths for each coverslip	MultiCS_Data.MultiCS_kurtosis	Calculate Cont. Z-line Lengths
Orientational Order Parameter	OOP for each coverslip	MultiCS_Data.MultiCS_OOP	Calculate OOP
Orientational Order Parameter	Director of the orientation vectors for each coverslip	MultiCS_Data.MultiCS_directors	Calculate OOP
Orientational Order Parameter	Actin OOP for each coverslip	MultiCS_Data.MultiCS_ACTINOOP	Actin Segmentation
Orientational Order Parameter	Director of the actin orientation vectors for each coverslip	MultiCS_Data.MultiCS_ACTINdirectors	Actin Segmentation
Actin Guided Segmentation	Average z-line fraction for each condition	MultiCond.CondValues_MeanZline	Compare Conditions and Actin Segmentation
Actin Guided Segmentation	Standard deviation of the z-line fractions for each condition	MultiCond.CondValues_StdevZline	Compare Conditions and Actin Segmentation
Actin Guided Segmentation	Average actin OOP for each condition	MultiCond.CondValues_ACTINMeanOOP	Compare Conditions and Actin Segmentation
Actin Guided Segmentation	Standard deviation of the actin OOPs for each condition	MultiCond.CondValues_ACTINStdevOOP	Compare Conditions and Actin Segmentation
Continuous Z-line Length	Average median continuous z-line length for each condition	MultiCond.CondValues_MeanMedians	Compare Conditions and Calculate Cont. Z-line Lengths
Continuous Z-line Length	Standard deviation of the median continuous z-line length for each condition	MultiCond.CondValues_StdevMedians	Compare Conditions and Calculate Cont. Z-line Lengths
Continuous Z-line Length	Average sum continuous z-line length for each condition	MultiCond.CondValues_MeanSum	Compare Conditions and Calculate Cont. Z-line Lengths
Continuous Z-line Length	Standard deviation of the sum continuous z-line length for each condition	MultiCond.CondValues_StdevSum	Compare Conditions and Calculate Cont. Z-line Lengths
Continuous Z-line Length	Average mean continuous z-line length for each condition	MultiCond.CondValues_MeanMeans	Compare Conditions and Calculate Cont. Z-line Lengths
Continuous Z-line Length	Standard deviation of the mean continuous z-line length for each condition	MultiCond.CondValues_StdevMeans	Compare Conditions and Calculate Cont. Z-line Lengths

Continuous Z-line Length	Average skewness for the continuous z-line lengths for each condition	MultiCond.CondValues_MeanSkewness	Compare Conditions and Calculate Cont. Z-line Lengths
Continuous Z-line Length	Standard deviation of the skewness for the continuous z-line lengths for each condition	MultiCond.CondValues_StdevSkewness	Compare Conditions and Calculate Cont. Z-line Lengths
Continuous Z-line Length	Average kurtosis for the continuous z-line lengths for each condition	MultiCond.CondValues_MeanKurtosis	Compare Conditions and Calculate Cont. Z-line Lengths
Continuous Z-line Length	Standard deviation of the kurtosis for the continuous z-line lengths for each condition	MultiCond.CondValues_StdevKurtosis	Compare Conditions and Calculate Cont. Z-line Lengths
Orientational Order Parameter	Average OOP for each condition	MultiCond.CondValues_MeanOOP	Compare Conditions and Calculate OOP
Orientational Order Parameter	Standard deviation of the OOPs for each condition	MultiCond.CondValues_StdevOOP	Compare Conditions and Calculate OOP

Table 4: Description of excel file summary for multiple coverslips and conditions.

Column Position	Column Name	Description	Special Options
A	ConditionValue	Number of the condition	Compare Conditions
B	ConditionName	Name of the condition	Compare Conditions
C	CoverslipName	Name of the folder the coverslip is contained within	
D	DateAnalyzed_YYYYMMDD	Analysis date	
E	OOPzline	Orientation order parameter of z-line orientation vectors	Calculate OOP
F	OOPactin	Orientation order parameter of actin orientation vectors	Actin Segmentation
G	DirectorZline	Average direction of z-line orientation vectors	Calculate OOP
H	DirectorActin	Average direction of actin orientation vectors	Actin Segmentation
I	ZlineFraction	Fraction of α-actinin skeleton classified as z-lines by actin guided segmentaiton	Actin Segmentation
J	NonZlineFraction	Fraction of α-actinin skeleton classified as off-target α-actinin staining	Actin Segmentation
K	TotalZline	Number of z-line pixels	
L	TotalActin	Number of actin pixels	Actin Segmentation
M	MedianCZL	Median continuous z-line length	Calculate Cont. Z-line Lengths
N	MeanCZL	Mean continuous z-line length	Calculate Cont. Z-line Lengths
O	TotalCZL	Total (sum) continuous z-line length	Calculate Cont. Z-line Lengths

P	SkewnessCZL	Sample skewness of continuous z-line lengths	Calculate Cont. Z-line Lengths
Q	KurtosisCZL	Kurtosis (how outlier-prone a distribution is) of continuous z-line lengths	Calculate Cont. Z-line Lengths
R	GridSize	Grid size defining local actin orientation	Actin Segmentation
S	ActinThreshold	Minimum angle between α-actinin and local actin orientation for pixels to be considered perpendicular	Actin Segmentation
T	CoverslipPath	Full path of coverslip	

Table 5: Description of ZlineDetection settings, which is stored in each .mat file.

Analysis Stage	Description	Variable Name
Initialization	Pixel to micron conversion	settings.pix2um
Diffusion Filtering	Diffusion time step	settings.Options.dt
Diffusion Filtering	Standard deviation of Gaussian smoothing before calculation of the image Hessian	settings.Options.rho
Diffusion Filtering	Standard deviation of Gaussian smoothing of the image Hessian	settings.Options.sigma
Diffusion Filtering	Total diffusion time	settings.Options.T
Top Hat Filtering	Radius of the flat disk-shaped structuring element used for the top hat filter	settings.tophat_size
Background Removal	Size of blocks to break image into	settings.back_blksze
Background Removal	Size of blocks considered noise in the condensed image	settings.back_noisesze
Background Removal	Standard deviation of gaussian smoothing to perform on image	settings.back_sigma
Binarization	Size of small objects to be removed in the binarized image	settings.noise_area
Skeletonization	Minimum branch size to be included in analysis	settings.branch_size
Actin Orientation Calculation	Sigma of the Gaussian weighting used to sum the gradient moments	settings.actin_blocksigma
Actin Orientation Calculation	Sigma of the derivative of Gaussian used to compute image gradients	settings.actin_gradientsigma
Actin Orientation Calculation	Size of Gaussian filter kernel to perform on actin image	settings.actin_kernelsize
Actin Orientation Calculation	Sigma of the Gaussian used to smooth the final orientation vector field	settings.actin_orientsmoothsigma
Actin Orientation Calculation	Minimum reliability of actin orientation vectors	settings.actin_reliablethresh
Actin Orientation Calculation	Standard deviation of gaussian smoothing to perform on actin image	settings.actin_sigma
Actin Guided Segmentation	Minimum angle between α-actinin and local actin orientation for pixels to be considered perpendicular	settings.actin_thresh
Actin Guided Segmentation	Size of local actin orientation	settings.grid_size

Continuous Z-line Length	Maximum angle between pixels to be considered parallel and therefore continuous	settings.dp_threshold