**Foci Counting Instructions**

**Segmentation Parameters**

Nucleus Channel: (when indicating the channel, *first channel is channel 0*)

Segmentation Method: *StarDist* should be chosen by default unless it does not work well, generally the *Threshold* method will give worse results unless the nuclei in the images are too different from those used to train the *StarDist* model

Rescale Factor: The images will be rescaled by this factor before running through *StarDist*. If *StarDist* does not correctly segment large nuclei, change this to rescale images down further. (Lower the value, e.g., 0.4 or 0.25). If only some of the images will have large nuclei, run these separately with a lower rescale factor, as rescaling too low unnecessarily will also result in suboptimal performance.

These parameters are only used if *Threshold* method for segmentation is chosen:

* CE px saturation: percentage of pixels to saturate (top and bottom) when enhancing contrast
* Smoothing radius: radius of structuring element for median filter (signal smoothing)
* Closing radius: radius of greyscale closing to fill erroneous segmentation holes/gaps (larger radius will result in more filling)
* WS seed distance: min distance in watershed seeds when separating touching nuclei by watershed method (larger distance will result in less over-segmentation but may also result in more touching nuclei being segmented as one)

The nucleus area/solidity cutoffs can also be adjusted *after* foci counting by running **Re-filter data**. Plots of nucleus area/solidity will be output to help the user choose the correct cutoffs, as needed. These cutoffs are more important when using the *Threshold* method for segmentation.

Nuclei min area (px)

Nuclei max area (px)

Nucleus min solidity: solidity defined as the ratio of pixels in the region to pixels of the convex hull image. It ranges from 0-1 and is generally in 0.90 or higher for correctly segmented nuclei.

***\*Note\*****: nuclei touching the borders are automatically removed by the segmentation algorithm.*

**Foci Parameters**

Foci Channel:(when indicating the channel, *first channel is channel 0*)

Foci Threshold Method: generally, FoCo will work best, but minimum and yen threshold methods can also be chosen. FoCo is a slightly modified version of this method: <https://bmcbioinformatics.biomedcentral.com/articles/10.1186/s12859-015-0816-5>

Intensity cutoff (FoCo): Adjust this intensity cutoff when FoCo finds too many (or too few) foci. It indicates the factor to multiply by the max pixel value for the image bit depth to get an absolute cut off for the max foci intensity. Any foci with max intensity less than this value will be ignored, e.g., for 12-bit images, an intensity cutoff of 0.5 will result in the removal of any foci with max intensity less than 4096 x 0.5 = 2048.

Foci max area (px): max area cutoff for counted foci, can also be adjusted after foci counting by running **Re-filter data**. Plots of nucleus area/solidity will be output to help the user choose the correct cutoffs, as needed.

**Run Buttons**

Count Foci: clicking this button will run the segmentation and foci counting. Results will be output in the output directory. *Please scroll to Output section for a description of the results.*

Re-filter data: clicking this button will filter the nuclei by the given area/solidity cutoffs and the any foci above max area will not be counted. The file “final\_results.txt” will be adjusted and re-saved (overwriting previous results). *In the file “results.txt” all data is stored so it’s possible to filter the data and then re-run the filter with different cutoffs (no data will be lost).*

**Output**

*nucleus\_data.txt*: a tab-delimited text file listing the following fields for each detected nucleus (not filtered):

* file
* fov
* nucleus\_label
* nucleus\_area
* nucleus\_solidity
* foci\_count

*foci\_data.txt:* a tab-delimited text file listing the following fields for each detected foci (not filtered):

* file
* fov
* nucleus\_label
* foci\_count *(total foci count in the nucleus)*
* foci\_area
* foci\_mean\_intensity

*final\_results.txt*: a tab-delimited text file of the same format as *nucleus\_data.txt*. This is a filtered data set using the nucleus area/solidity and foci max area parameters.

*Foci\_properties.png:* graphical output showing foci area distribution, and foci area vs. foci count per nucleus scatter plot, with current cutoff for max area labeled.

*Nucleus\_properties.png*: graphical output showing nucleus area and solidity distributions and area vs. solidity scatter plot, with current cutoffs labeled.

*segmentation (folder)*: Contains nucleus channel images with segmented nuclei outlined. Each nucleus is labeled with its id (label), area and solidity. *Use these images to evaluate quality of segmentation and help with choosing nucleus area/solidity cutoffs.*

*foci (folder)*: Folder with foci channel images with segmented nuclei outlined (green) and detected foci labeled in red. Use these images to evaluate quality of foci detection and help with choosing foci max area cutoff.