**Foci Counting Instructions**

**Segmentation Parameters**

Nucleus Channel: (when indicating the channel, *first channel is channel 1*)

Segmentation Method: *StarDist*

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.

*Filtering nuclei (in case of incorrect 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.

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 were more important when using a *Threshold* method for segmentation (this has been removed) but these properties can still be checked to filter out bad segmentation by StarDist (if needed).

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

**Foci Parameters**

Foci Channel:(when indicating the channel, *first channel is channel 1*)

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>

AdpM filter size (FoCo): (Adaptive median filter size) an adaptive median filter with a rectangle of s x s (s = size of filter) is applied to the foci image as a first step. Larger values of s will result in more smoothing of the image.

Opening radius (FoCo): (Morphological opening) a morphological opening with a disk of radius r (r = opening radius) is applied to the image to quantify background. This type of operation will remove small objects from the foreground of an image. Set the radius to approximate size of the minimal foci radius in pixels.

Intensity cutoff (FoCo): Adjust this intensity cutoff higher (lower) when FoCo finds too many (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 and foci area will be output to help the user choose the correct cutoffs, as needed.

**Intensity Channel:** (when indicating the channel, first channel is channel 1)

Set this to the channel of the image to quantify CTCF for each nucleus. If set to 0, no calculation will be performed.

CTCF = corrected total cell fluorescence

CTCF = total cell intensity – (background mean intensity x cell area)

Background mean intensity is calculated by taking the mean of the region of the image that is not included in any segmented nuclei.

**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 files “nucleus\_data.txt” and “foci\_data.txt” all data is stored so it’s possible to filter the data and then re-run the filter with higher/lower cutoffs and 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
* CTCF Intensity channel
* 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.