**Foci Counting Instructions­­­­**

**The application can read in either ND2 or TIFF files. If it is a TIFF file, it is assumed that the file is 2d (one z level) or multiple z-levels and a single field of view. If it is an ND2 file, the format may contain multiple fields of view and z-levels. A maximum z-projection on the foci channel will be performed for measuring the foci, and an average z-projection will be performed for all channels CTCF measurement. If there are multiple z-levels, the DAPI (nucleus) channel will be used to measure nuclear volume.**

**Segmentation Parameters**

Nucleus Channel: when indicating the channel, *first channel is channel 1 (not 0*)

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 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.*

**Volume Parameters**

Volume Threshold Method

Z microns per voxel

XY microns per voxel

Home z level

Expand ROI px

Min z level

Max z level

Max px valid

**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. Set this to 0 for no smoothing. Foci finding works even without smoothing, but this can help if too many foci are identified.

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; this will result in the intensity of smaller objects lowering to background. To detect even small foci, this can be set to 0 to skip this step.

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**. Usually, foci area is 1 to a few pixels, as they are local maxima. However, some nuclei with very bright/large areas of intensity may contain a small number of very large foci. It is recommended to check for these and consider how they can affect the distribution of foci counts. CTCF may be a helpful secondary measurement if these types of nuclei are present in your data set.

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 an additional channel of the image that you would like 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 considered background after applying an Otsu threshold.*

**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 Name
* Field of View (number)
* Nucleus Label
* Nucleus Area
* Nucleus Solidity
* Total Intensity (Intensity channel)
* Background intensity (Intensity channel)
* CTCF (Intensity channel)
* Total Intensity (Foci Channel)
* Background Intensity (Foci Channel)
* CTCF (Foci Channel)
* Foci count

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

* File Name
* Field of View (number)
* Nucleus Label
* Foci Count *(total foci count in the nucleus)*
* Foci Area (this is the area of the local maxima; it is usually 1 or a few pixels)
* Foci Mean Intensity (foci are local maxima, so mean/max intensity of foci is equivalent)

*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.