**Foci Counting Instructions­­­­**

**The application can read in either ND2 or TIFF files as input. If it is TIFF files, it is assumed that the files are 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 of the nucleus channel can be performed automatically (by StarDist) or by providing pre-defined labeled masks. If providing labeled masks (TIFF format), follow the naming convention described below. Note: when providing labeled masks, input images must be only one FOV per image. An input ND2 file with multiple FOVs is not supported for this option.**

**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.

Segmentation Method: *LabeledMask*

To match up the mask file with the file used for measurements, the name of the mask file have the same name as the measurement file, optionally with the suffix \_mask.tif or \_MASK.tif. If mask files are in a different folder than the measurement files (or the measurement files are ND2 format), then it is not required to add the suffix.

Example:

Measurement file name: *myfile.tif (or myfile.nd2)*

Mask file name: myfile\_mask.tif , myfile\_MASK.tif, or myfile.tif (if masks files are in a different folder or the same folder with nd2 files)

Filtering Options

*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 when StarDist segmentation is used.*

**Volume Parameters**

Nuclear volume is measured by obtaining the automatic threshold value (Otsu, yen, etc.) for the DAPI image at a “home” z level (approximate cell center) and applying this value at each z level. The measurement of volume for each nucleus is restricted to the (StarDist) segmented nucleus region at each z-level. To ensure that the complete volume is measured at each z-level, the labeled mask from StarDist is dilated with a disk of radius equal to number of pixels from the user input (“Expand ROI px”). Set to 0 for no dilation. Once threshold is applied at a z-level, holes are filled on objects in the mask image prior to area measurement. Additionally, to remove nuclei that may be too far above or below the center of the imaging plane, a check is performed such that any nuclei where the area at the highest/lowest z-levels is found to be larger than a provided cutoff will be marked as “invalid” in the output table.

Volume Threshold Method: threshold method for obtaining the threshold at the “home” z level that will be used throughout all z-levels for measuring area.

Z microns per voxel: micron distance between z levels

XY microns per voxel: microns per pixel (x y resolution)

Home z level: the home z-level to measure the threshold value (set to the z-level that best matches cell center slice)

Expand ROI px: Structuring element size for binary dilation of nucleus mask image (see method description above)

Min z level: If the area measured at z-levels below this z-level is greater than Max px valid, then the nucleus will be marked as INVALID\* in the output table. Indicates nuclei that may be too far from the center of the imaging plane to obtain an accurate volume measurement.

Max z level: If the area measured at z-levels above this z-level is greater than Max px valid, then the nucleus will be marked as INVALID\* in the output table. Indicates nuclei that may be too far from the center of the imaging plane to obtain an accurate volume measurement.

Max px valid: Pixel count above this value for nuclei at low/high z-levels will result in the nuclei marked as INVALID\* for volume measurement in the output table.

*\* Note: no nuclei are removed, a column in the output table indicates the VALID/INVALID setting for the volume measurement for each nucleus. The user must manually remove these if desired.*

**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
* Volume px
* Volume microns
* Volume valid ROI
* Total Intensity (Intensity channel)
* Background intensity (Intensity channel)
* CTCF (Intensity channel)
* Total Intensity (Foci Channel)
* Background Intensity (Foci Channel)
* CTCF (Foci Channel)
* Foci count

(Some fields may not be present given user settings and the input format of the image file)

*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.

*volume (folder):* Folder with 3d images of the nucleus channel where the nucleus region at each z-level used for volume measurement is outlined in red. Use these images to evaluate the quality of volume approximation and help with choosing the Volume Threshold Method.