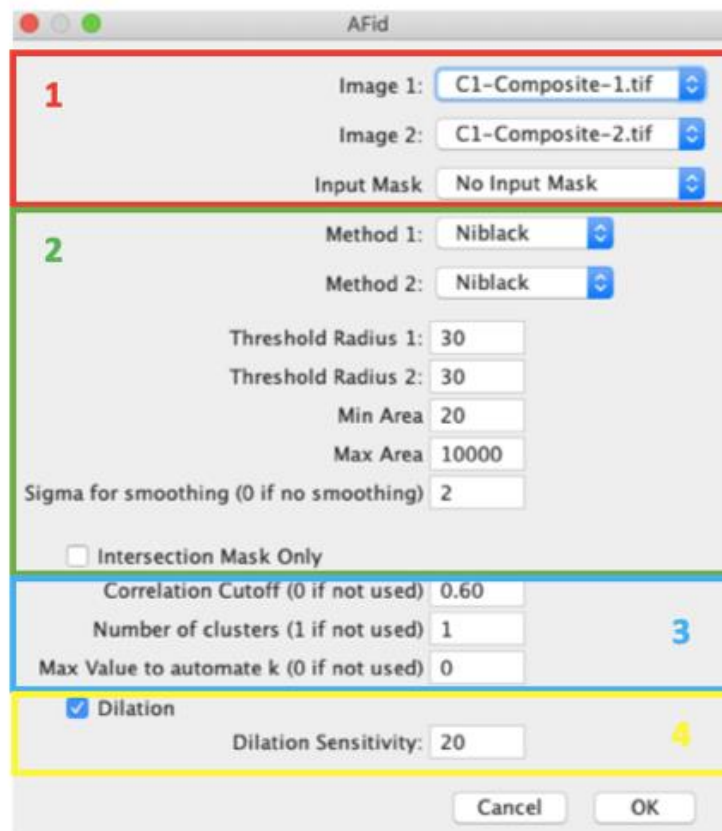# AFid User Guide for ImageJ

This plugin identifies autofluorescence from two-channel immunofluorescence images of tissue. The algorithm uses only two fluorescent channels, classifying ROIs as autofluorescent or real based on a correlation cut-off or through the *k*-means clustering algorithm using additional texture features.

Installation:
Download the .jar file from **ellispatrick/AFidImageJ**. To install onto ImageJ, copy the .jar file into the ImageJ/Plugins folder, or go to **Plugins>Install Plugin…**. After resetting ImageJ, this plugin can be accessed under **Plugins>AF Remover**.

Step 1: Read in Images
Here, users choose two image channels of an immunofluorescence image to run AFid under **Image 1** and **Image 2**.

Step 2: Creating an intersection mask
Here, an 'intersection mask' is generated, capturing both autofluorescence and any overlapping signal. The objects within this mask are then classified as autofluorescence and non-autofluorescence.

Under **Input Mask**, users can bypass the creation of an intersection mask, instead providing their own mask. Leaving this entry as 'No Input Mask' results in the creation of the intersection mask.

Under **Method 1** and **Method 2**, users can choose which of the built-in ImageJ Auto Local Threshold algorithms to threshold **Image 1** and **Image 2**, respectively.

Furthermore, users can set the threshold radii under **Threshold Radius 1** and **Threshold Radius 2**, respectively. Choosing larger threshold radii allows the full autofluorescent body to be obtained, but can result in longer runtime and smaller objects being missed.

Objects in the generated mask can be filtered based on object size, under **Min Area** and **Max Area**. Note that smaller objects can be misclassified as autofluorescence, possessing high pixel correlation values across the two image channels because of the low pixel number.

Finally, a Gaussian blur can be applied to the images, with the size of the filter being set under **Sigma for smoothing**. This allows more of the autofluorescent body to be captured. This image will also be used for glow removal. Setting the size filter to 0 results in no Gaussian blurring to be applied to the images.

Step 3: Autofluorescence Classification
Here, objects in the intersection mask are classified as autofluorescence or non-autofluorescence based on a correlation cut-off, the results of k-means clustering, or both. The pixel correlation of objects in the intersection mask across the two provided image channels are measured. Additional texture measurements are also made, namely the standard deviation and kurtosis measurements of the objects in the two channels, which serve as input parameters for k-means classification.

Under **Correlation Cutoff**, users can set a cut-off correlation value between 0 and 1. Objects above this cut-off will be classified as autofluorescence. If used in conjunction with k-means clustering, any objects above this cut-off within the identified autofluorescence cluster is classified as autofluorescence. Setting this parameter to 0 results in a correlation cut-off not being used.

Under **Number of clusters**, users may manually set the number of clusters required for k-means clustering. The greater the number of clusters, the greater the specificity for identifying autofluorescence, but the lower the sensitivity. Setting this parameter to 1 results in k-means clustering not being used, or that k will be estimated.

Under **Max value to automate k**, users can set the maximum cluster number for estimating k. An elbow test and the t-statistic are used to estimate an optimal value of k between 3 and the value set by the user. Setting this parameter to 0 results in the number of clusters not being estimated.

Step 4: Dilation
The full body of objects are not always captured by the thresholds used, and so further dilation is to be performed. This is performed by distributing expansion points within autofluorescent objects and drawing lines outwards to capture the boundary of the objects. A boundary is identified when the pixel intensity along the line starts to increase as defined by the blurred image.

The tick-box **Dilation** allows the user to select whether or not to run Glow Removal.

The parameter **Expansion Sensitivity** represents the number of steps taken by a tracing algorithm when dropping expansion points within an object. The lower this parameter, the closer these expansion points.