**Variable Threshold User Instructions**

**Installation**

Requires FIJI: <https://imagej.net/Fiji/Downloads>

Copy the Variable\_Threshold.jar file into FIJI plugins folder.

Increase the memory of ImageJ to accommodate higher numbers of images/particles. Do this by starting up FIJI and selecting “Edit”, “Options”, “Memory and Thread” and entering a number into “Maximum Memory”, (leave some memory for your system and any applications that you need to keep open while using FIJI. 20000MB is an example starting value) After changing this option, exit FIJI.

Copy the Variable\_Threshold.jar file into FIJI plugins folder.

**Running the Plugin**

Start up FIJI

Import Image Sequence, if images are rgb, be sure to check the ‘convert to 8 bit grayscale’ checkbox.

Save this stack as a TIF. Filename must not have space, parentheses, etc

Go to Plugins, slide down and over to Variable Threshold

The first dialog box is a choice whether to do a Variable Threshold analysis on the stack (default), or to perform filter measurements on a previously processed stack. On first use, go with the default. (If the latter option is selected, a new dialog box appears where some options are presented (Save Results, Label collage particles, Label interval for collage particles). Following this panel, the user selects the .zip file containing the set of analyzed regions of interest (ROIs) from a previous analysis. Then the program skips to the filter section (below.))

**Variable Threshold analysis.**

First step is to create background using a median image. The median image selects the median value of each pixel from the range of source images. Thus, particles that appear in individual frames are effectively removed and a background image results.

There is a dialog box that allows you to set the range of slices used to calculate a median.

Also there is an item ScaleFactorLightPix that specifies the use of pixels brighter than the background for inclusion into the analyzed images. A value of 1.0 gives equal weight to light and dark pixels. A value of 0 will only include pixels darker than the background in the analyzed images. Consider starting with the default (0).

After calculating the background, and creating a background-subtracted image stack “subfiles,” the next dialog box “Variable Threshold Process” sets the image process parameters.

**Beginning Threshold:** Set to the lowest threshold value sufficient to capture all particles. May need to experiment with this parameter initially. Note one can mouse around an image from your stack and observe the grayscale value at that location in the bottom line of the FIJI control panel. Mousing over a particle and then a background area will give you an idea about what minimum threshold might work.

**MaxGray2ThreshFactors:** Converts the maximum intensity of particle (absolute value) into a tentative threshold value for that particle.

**Number of Thresholds:** Number of bins for thresholding particle

**Max Value for Reset Threshold**: , Upper limit for threshold; (assigned threshold value for a high contrast particle will not exceed this value.)

**Minimum Circularity:** , to be considered for inclusion in results

**Maximum Circularity:** , to be considered for inclusion in results;

**Minsize pixels:** , to be considered for inclusion in results

**Maxsize pixels:** , to be considered for inclusion in results

**InitialDilateErodeSteps:** , prior to variable thresh algorithm, (helps to reduce fragmentation effects )

**FinalDilateErodeSteps** if Do Hull not selected, will perform after variable thresh algorithm

**Label interval for collage particles**:,

**Save Results** , checkbox;

**Do Hull Analysis** , checkbox performs convex hull analysis after variable threshold , fills in arcs, completes joined fragments,

**Enhance Contrast collage particles** checkbox (displayed collage will have an auto contrast applied if checked)

**Label collage particles** , checkbox. will label every Nth particle (where N is specified above ‘Label interval’)

**Show Particles in Image Stack with black backgnd**, checkbox will leave open at the end of the run the image stack with identified particles and black background in each frame.

The final dialog box, “Set Measurements” , only appears if Save Results checkbox was checked. The available parameters are described under 30.7 “Set Measurements” at the following link

<https://imagej.nih.gov/ij/docs/guide/146-30.html>

A log file listing the settings used for the measurement will be saved as a text file. The name consists of a prefix (= name of the TIF image stack being analyzed) +”log”+ a sequential number

The results file is saved as a xls file

The name consists of a prefix (= name of the TIF image stack being analyzed) + a sequential number

These files are stored in the same folder as the saved TIF image stack (that you saved at the beginning of this process.)

A file containing the ROIs (regions of interest) is stored as a .zip file. This file is used when doing a subsequent analysis to filter measurements on a previously processed stack.

When the processing is complete, the plug-in will store an excel (xls) spreadsheet with parameter values for each particle. Also saved is a TIF collage with each particle referenced to the particle number on the spreadsheet. The filenames are generated automatically.

**Filtering Results**

A dialog box is presented providing the option to continue with filtering the results or ending the VT plugin. Based on the column headings that are in the results table, a set of options for filtering data will be presented in a dialog box. Check the parameters on which filtering will be performed. A second panel then allows setting the filter range for each parameter. If SaveResults was checked earlier, a new collage and spreadsheet file are saved with the filter ranges included in the filenames. A dialog box is presented providing the option to continue with performing another filter study or exiting the plugin. The filtering for the next cycle is based on the original set of ROIs (all particles) not the previous filtered results.

If the collage is re-opened in FIJI, the overlay with particle boundaries and numbers will be shown. If opened in other applications, the overlay may not be displayed. To join the overlay with the collage, use “Image” “Overlay” “Flatten” from the menu in ImageJ. Then save as TIF. Now the collage and overlay will be shown in other applications (though in FIJI, you will no longer be able to hide the overlay).

**Questions?**

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