

## IO and VSD Signal Processor

Release candidate version 1.0 8 (build c95)

Plugin for ImageJ<sup>1</sup> for analysis of Intrinsic Optical signal (IOS) or voltage sensitive dye (VSD) fluorescence signal.

This plugin loads a selection of TIFF files recorded in IOS or VSD experiments to quantify temporal and spatial distribution of evoked activity in neuronal tissue or *in vivo* as described in this paper and previously<sup>2</sup>.

Evoked IO signals are defined as the fraction of light reflection change that is specific to stimulation. Evoked VSD signals are defined similarly, for changes of fluorescence. As VSD signal may be recorded with higher time resolution and recordings may be subject to rundown by bleaching and other effects, we suggest normalizing the data to control recordings without stimulation.

The plugin is set up for evoked recordings in the somatosensory cortex *in vivo* but may be changed by the user for other subjects or specimens. Recordings should be stored as a series of multi-frame images in TIFF files named “*hi#.tif*” and “*lo#.tif*” within one folder, where the “#” stands for a natural number = 1 without leading zero. These file names may be changed in the “settings” dialog of the plugin. In the default setting, file series “*hi#.tif*” are thought to store recordings with forelimb stimulation, series named “*lo#.tif*” store recordings with hindlimb stimulation. If data should be normalized to control recorded without stimulation, a third series of images in TIFF files named “*no#.tif*” should be provided. Changing constants in the JAVA code allows different file names and a higher number of series. This option will be included in the user dialog in future releases of this plugin. By default, user settings are globally stored and recalled with each plugin run in a file, i.e. “C:\Windows\vsdiomap.ini” in a Windows<sup>3</sup> system. This allows the user to establish and keep settings for their particular needs. Optionally, settings can be kept separately for different users (“*personal*”) or different installations (“*local*”).

The plugin can be stopped at almost any time by pressing the [Esc] button or closing the active window of average stack in case it is displayed (which is optional).

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<sup>1</sup>National Institutes of Health, Bethesda, MD; available from <http://rsb.info.nih.gov/ij>

<sup>2</sup> Zhang, S. & Murphy, T. H. (2007) *PLoS Biol* **5**, e119.

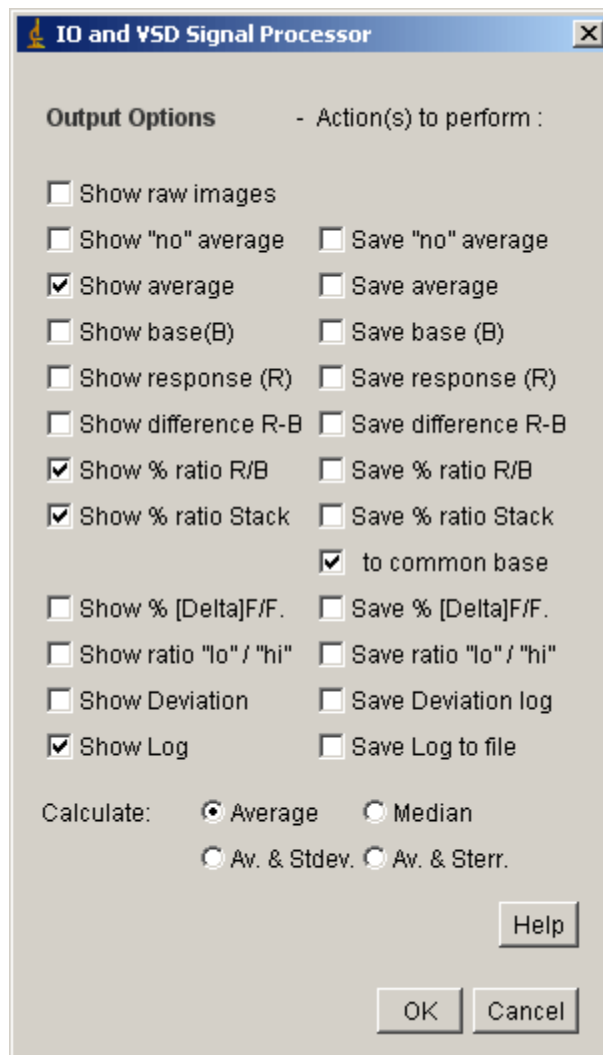
<sup>3</sup> Trademark of Microsoft Inc.

### Getting started: Output Options

When the plugin is first run, users are asked to select procedures to set *Output Options* that are to be performed with this plugin. Results can be displayed on screen or saved as a file in the input folder. In detail, options are:

- Show raw image* - displays images on screen while data is read and will be kept open. Use this option with caution for large file series.
- Show/Save 'no' average* - displays/saves average of the control images (without stimulation) if applicable. In default setting, these files are named "*no##.tif*". This option is invisible if there are no control images.
- Show/Save average* - displays/saves averages of the images recorded with stimulation. (Default name setting is "*lo##.tif*" / "*hi##.tif*" for files subject to be averaged.
- Show/Save base (B)* - displays/saves "*baseline*" projection of the average for internal control frames recorded *before* stimulation.
- Show/Save response (R)* - displays/saves "*response*" projection of the average for internal frames recorded *during* and *after* stimulation.
- Show/Save difference (R-B)* - displays/saves the difference between "*response*" and "*baseline*".
- Show/Save % ratio (R/B)* - displays/saves the percent ratio of "*response*" and "*baseline*", multiplied by the factor of 100. Use this action as default to generate static maps for evoked cortical activity
- Show/Save % stack* - displays/saves a stack of percent ratios "*response*" / "*baseline*", in which each slice represents the response and baseline response for one trial. This option is thought be used for identifying outliers. It will yield one stack for each file series containing one slice for each file processed per file series. Use the "*common base[line]*" option to compare the response component only
- Show/Save [Delta]F/F<sub>0</sub>* - displays/saves the percent normalized difference to "*baseline*", divided by "*baseline*". Use this action as default to generate multi frame images (videos) to determine temporal changes in activity during and after stimulation.
- Show/Save Ratio 'lo'/'hi'* - displays/saves the ratio of the results, e.g. if default file naming preset is used, for forelimb ("*hi*") and hindlimb ("*lo*") stimulation for the calculations of their ratio of "*response*" and "*baseline*". This calculation yields a combined image for both results, in which the response to hindlimb stimulation will appear inverted. We suggest using this option only in combination with one of the other options above, as negative values (which might occur i.e. by inhibition in VSD fluorescence recordings) cannot be distinguished from positive results for the other form of stimulation. This option is disabled if less than two groups of file names are defined, control file group not counted. Use "Preferences" to change file name settings (Main dialog.)

- Show/Save Deviation log* - displays in a log window /saves to text file for each image stack the average percent deviation from mean of all “vicinal” files (with same file name except the indices). This value should be high for image stacks with abnormal data (i.e. due to artifacts or events of spontaneous activity etc). It’s suggested to visually check files that stand out with high deviation for presence of artifacts and to exclude these files form calculation in an additional run if applicable (by selecting them in the “Ignore List” of the main dialog).
- Show/Save log* - displays in a log window /saves to text file detailed information on the settings chosen for this particular plugin run. If applicable, text files will be saved together with results in the same folder used for data input. This option may be found helpful for keeping track of setting used for each calculation. It would be even more helpful for the case of data recovery after a hard disk failure.
- Calculation Mode* - set how the plugin should proceed with multiple input files. Default calculation is the average. Other options may run slower and require more memory. Be careful with using Median, Standard Deviation, or Standard Error calculation at 32-bit systems in combination with a large number of large files! Also please be reminded that results of Median calculation may be used for overview but not for scientific data analysis according rules of good scientific practice.



**Fig. 1**

Output Options

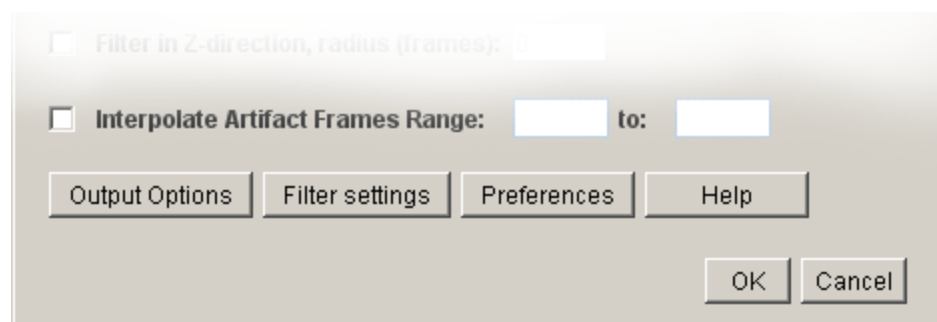
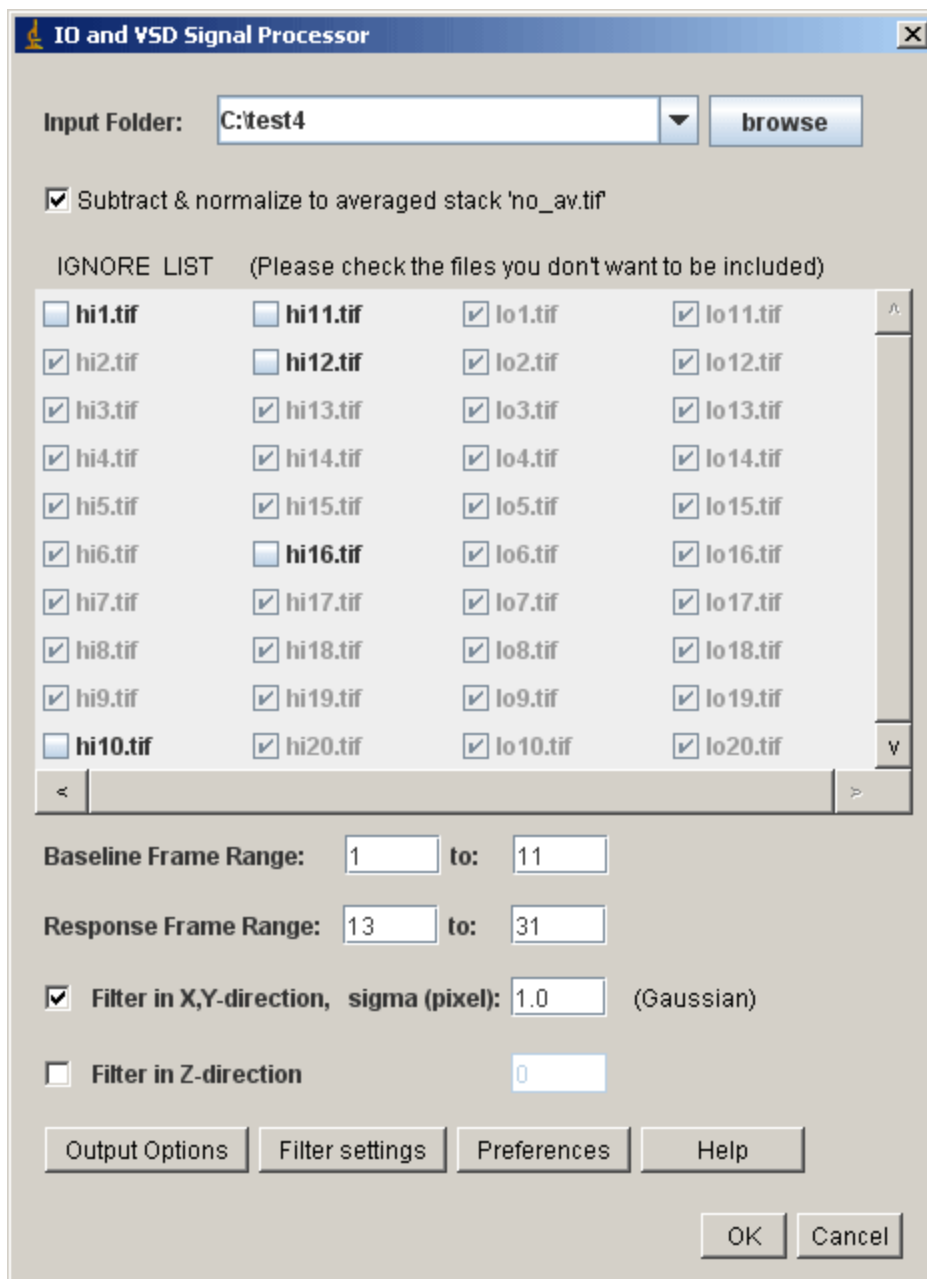
## The main dialog

The main dialog is displayed with every run of the plugin. It serves to select the input folder and to modify further settings. Pressing “OK” starts the calculation.

Options to set are:

- Input Folder* -name of the folder that contains imaging data files.  
The same folder is also used to save the results as image files and logs as text files if applicable. The folder may be either set by pasting its name into the text folder or by pressing the “*browse*” button, which allows the user to select files in a different folder. Select only files that match the criteria given in the folder to use for data input/output, e.g. “*lo#.tif*”, “*hi#.tif*”, or “*no#.tif*” if the default presetting for file names is used. Press “Preferences” Button to change file name presetting.
- Subtract & normalize to control...* -Check this box for data normalization. This usually applies to processing evoked VSD imaging data.
- Ignore List* -After having chosen a certain folder, users may select single files that ought to be excluded from the data analysis. This applies if single trials have false results due to artifact. Only found files can be selected. The ignore list should provide this way information about files found in the selected folder. Note that file names of control files are printed with italic letters. Use arrow buttons at right and bottom border to scroll between pages in case the range of present index numbers is larger than the number of displayed Checkboxes in Ignore List.
- Baseline Frame Range* -Set minimum and maximum frame number of the range that should be considered as “*baseline*” before stimulation. Note that the frames are counted starting with 1.
- Response Frame Range* -Set minimum and maximum frame number of the range that should be considered as “*response*” during and after stimulation. Frame number shouldn’t entered here should not exceed the number of available frames per stack.
- Filter in X,Y direction* -This option allows the user to set and define a lateral *Gaussian* or Mean filter to reduce noise from images before calculation. It can be applied to the averaged stacks, to the “*baseline*” and “*response*” projections or specifically to the data used for  $\Delta F/F_0$  calculation (se above).

- Filter in Z direction* -The second optional filter allows averaging in temporal direction (here referred to as Z direction) among frames. If data are used to normalize to control (i.e. for VSD fluorescence recordings), we suggest to apply the same filter settings to recordings done both with simulation (e.g. “*hi#.tif*” and “*lo#.tif*” for default file name presetting) and without (e.g. “*no#.tif*”) in order to avoid artifacts in the time course of the resulting  $\Delta F/F_0$  image stacks.
- Interpolate artifact frames range* - Replace voxels in frames by interpolating the voxels of the frames of the surrounding frames. This optional feature is thought to be used to blank an intrinsic artifact. If applicable, enter the range of frames to be replaced. Note that this feature is displayed only if enabled in the “Preferences” dialog (press button).
- Output Options (Button)* -Dialog to define what calculation this plugin should perform and to define the calculation method.
- Filter Settings (Button)* -Dialog to define filter parameters, i.e. to choose between *Gaussian* and *Mean* filtering and to enable/disable filtering in Z direction.
- Preferences (Button)* -Dialog to set operational settings for the plugin run. This includes layout options, preferences saving behavior, and the name mask of files to look up.



**Fig. 2**

The main dialog (in two different layout options)

## Filter settings

To reduce noise from in analyzed images, data can be filtered before calculation of ratios. Use the Button “Filter Setting” in the main dialog to change options:

*Treatment of NaN and Infinity values* - define how to mask values of pixels that have invalid value (i.e. NaN or Infinity). This applies to 32-bit data only. Default option is to preferably 'ignore' those voxels in a stack. Alternatively replace these pixels in each frame by an average of surrounding pixels values. Choose “interpolate” in case input files of different trials have different amplitude.

*Filter in X,Y direction* -Set radius (in number of pixels similar as in the user interface of ImageJ. In case of *Gaussian* filtering, you may set the  $\sigma$  value here.

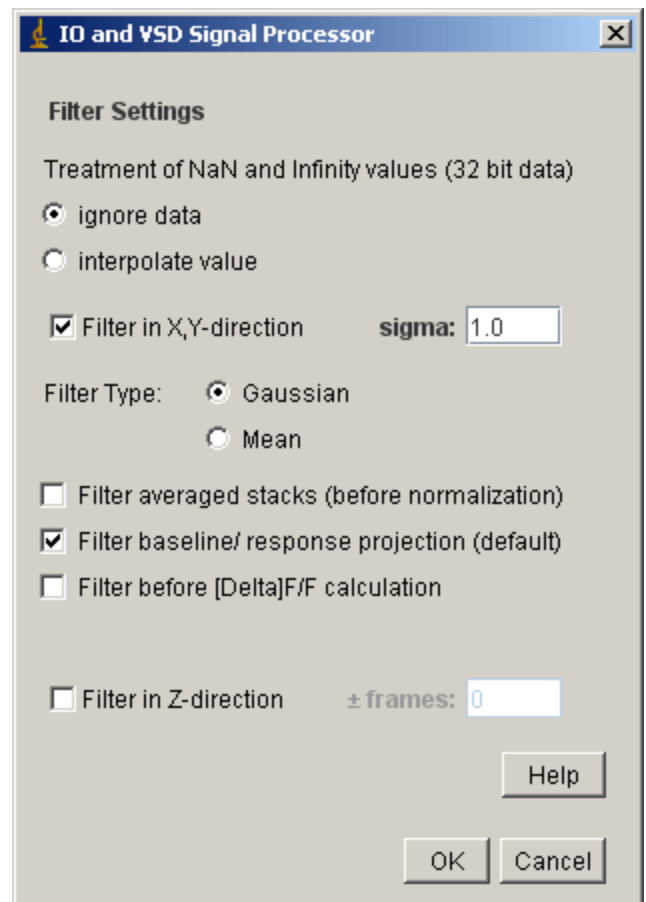
*Filter type* -Select the filer type (*Gaussian* vs. Mean filter)

*Filter averaged stacks* -Filter raw images before projection. This option may be useful to reduce noise in difference-ratio stacks.

*Filter baseline/response* -Filter projections of image stacks before calculating the ratio of them. Use this option to reduce noise in forms of single-frame results.

*Filter before  $DF/F_0$  calculation* -Additional filtering, to further reduce noise in difference-ratio stacks. This may be useful if the number of available trials is relatively small.

*Filter in Z-direction* -Set number of frames before and after each frame, except for the first and the last frames with a stack. This option may reduce noise in normalized ratio-of-difference stacks.



**Fig. 3**  
Filter settings

## Program Preferences

Program settings that usually aren't changed after first use of the plugin are found in the "Preferences" dialog, which is to be started by a button from the main dialog.

### Layout Preferences:

*Number of "Ignore" boxes* -Set number of boxes that are displayed simultaneously in the "Ignore List" (in the main dialog) for each of the two simultaneously displayed file series. If the number of available files (or the value of their indices) are larger than the number of checkboxes with the "Ignore List", you may use the arrow buttons at the edge of the "Ignore List" to browse them. Note that the plugin needs to be restarted for changes of this option to take effect.

*File name(s), "\$\$.tif"* -Preset names of the files series to be loaded by the plugin. Omit indices and file extension. You may separate file names by space or typical list separators such as comma, semicolon, etc. Default presetting is "no, hi, lo".

*Control file (series)* -Select file names to be used as control file series, if applicable. Data in this file series may be used to normalize the other data, i.e. for VSD recordings. Default setting is "no".

### Operational Preferences:

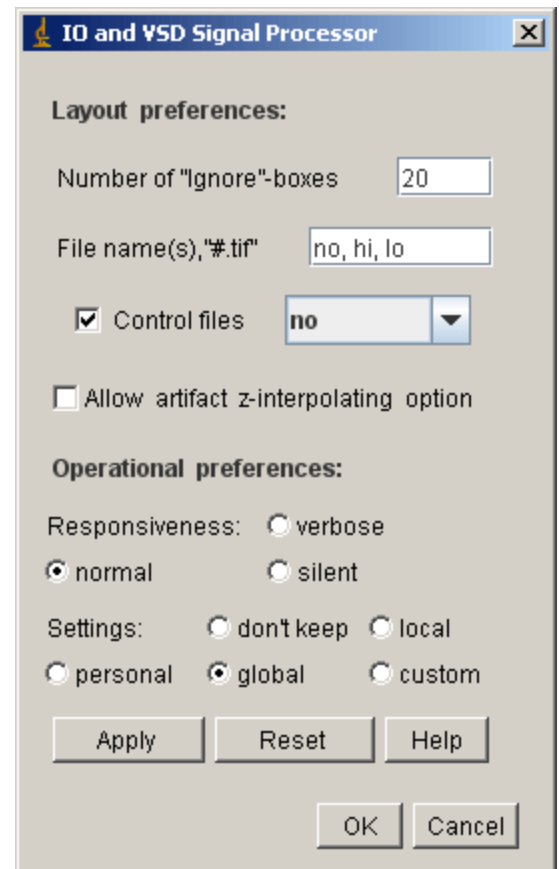
*Allow artifact z-interpolating option* - if enabled, the main dialog screen will be extended with an additional option that allows to define which frames should be replaced by voxels with value that is an interpolation of values in the frames before and after the range of frames to be interpolated in stack.

*Responsiveness* -Preset how the plugin should response to errors and unexpected events, which are "verbose" for a large number of messages and hints and "silent" for a minimum of messages.



- Settings (saving behavior)* - By default, this plugin keeps settings for future runs. In 'global' mode, settings are generally shared by all users of the computer. Administrator or advanced user privileges may be required for this mode. In 'personal' mode, settings are stored for all ImageJ installations for the current user. In 'local' mode, settings are stored for the current ImageJ installation (for all users). Some limitations in this functionality may apply for Macintosh<sup>4</sup> computers.
- Treatment of NaN and Infinity values* - with 32-bit input data, pixel with value *NaN* or *Infinity* would cause artifacts if neither ignored nor interpolated. Default option is 'ignore', which excludes these pixels from data (wherever possible.) Choose “*interpolate*” in case input files of different trials have different amplitude.
- Apply (button)* - Saves all current settings as preferences for next run. These include also settings done in the main dialog of the plugin (except if keeping settings is switched off) but do not apply to any selection of individual files to be ignored at /excluded from loading in “*Ignore List*”.
- Reset / Undo Reset (button)* -Clear all saved settings. This button resets the plugin to all default. Use this function only if required. For example, this may be applicable in cases of need to clean up an erroneous presetting. Button “*Undo Reset*” allows restoring it, if done in the same plugin run in that the “*Reset*” button was (mistakenly) pressed.

**Fig. 4**  
Preferences



<sup>4</sup> registered trademark of Apple Inc.

## Results / File output

If applicable, results are saved as new TIFF image files within the folder used for data input or are displayed in windows with headings as follows:

<i>lo_base.tif</i>	multi-trial baseline average
<i>lo_resp.tif</i>	multi-trial response average
<i>lo_av.tif</i>	frame by frame averaged stack for multiple trials
<i>lo_avn.tif</i>	average stack, normalized frame by frame to control without stimulation (i.e. in VSD recordings)
<i>lo_diff.tif</i>	difference of “ <i>lo_resp.tif</i> ” and “ <i>lo_base.tif</i> ”
<i>lo_div_%change.tif</i>	percent ratio of “ <i>lo_resp.tif</i> ” and “ <i>lo_base.tif</i> ”
<i>lo_deltaI_I%.tif</i>	percent $\Delta I / I_0$ for the averaged stack
<i>lo_deltaf_f%.tif</i>	percent $\Delta F / F_0$ for the averaged stack (normalized)
<i>lo_div_by_hi_32bit.tif</i>	ratio of ratio results for “ <i>lo</i> ” and “ <i>hi</i> ”
<i>log.txt</i>	text file with detailed information on the settings of the plugin run.
<i>data quality map.txt</i>	text file containing deviations of each file to identify (potentially artifact related) artifacts within the input data set

Analogous naming rules apply for the other files, depending on their name (e.g. “*hi\_base.tif*” and “*no\_av.tif*” for controls, if the default file naming is used). To avoid overwriting files created in previous plugin runs (as well as to keep unique window names), file names and window headings might possess a numerical index [#] before the file extension. This numerical index is the same for all file names and windows in each plugin run.

## Handling noisy raw data using the “Deviation Table”

The option “*Show/Save Deviation log*” (option menu) allows the user to display or to save to file information on how far the recorded data differ from the average of all images of the same files series. Instead of comparing the whole data set pixel by pixel, spatial averages of the center square 50 % area are compared frame by frame with the average value in an average stack. The plotted values shown in the deviation table are the average deviation for all frames to the average stack of the file series. It is expected that image stacks with results that are affected by artifacts or spontaneous cortical activity differ more from average than those that are affected to a lesser extent. The deviation table is designed to help users identify files that may contain false positive or negative findings and to exclude them if applicable. Outliers may be identified by their high deviation and by high rank value. Users can exclude single files from the data analysis by checking their name in the “*Ignore List*”, which is part of the main dialog of this plugin.

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