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Editing 4D Movies with ImageJ Plugins

The goal is to edit a 4D movie to display only one or a few spots. An edited movie can be analyzed to quantify the fluorescence time courses.

Typical Workflow

This procedure assumes that you are starting with a deconvolved and bleach corrected movie in the form of a 4D TIFF hyperstack. The description is for a movie with three fluorescence channels plus a fourth channel for the cell images, but if there are fewer fluorescence channels, the procedure is essentially the same.

The custom plugins for 4D movies are in the IJ_Plugins folder. Detailed instructions for using these plugins are provided in later sections of this document.

1. Run the "Make Montage Series" plugin, and choose the 4D TIFF hyperstack. The result will be a scaled montage with adjusted gray values for the cells. In addition, the blue channel color will be adjusted to make the signal easier to see. Save this montage series.

2. Run the "Montage Series to Hyperstack" plugin. The result will be a 4D hyperstack that includes the adjustments described above. Save this hyperstack.

3. Run the "Project Hyperstack" plugin. The result will be an average projected composite TIFF movie showing all of fluorescence signals. Save this original movie.

4. View the original movie, and identify one or more structures that can potentially be followed cleanly in all three fluorescence channels. Note the positions and times at which those structures are present.

5. Run the "Edit Montage Series" plugin. Choose the previously saved montage series, and create a copy for editing.

6. Edit each fluorescence channel of this montage series so that it displays only the structure of interest in all three channels. Erase all of the extraneous fluorescence signals. Save this edited montage series.

7. If additional structures of interest were identified, repeat steps #5 and #6, creating an additional edited montage series for each structure.

8. Run the "Montage Series to Hyperstack" plugin for each edited montage series. Save these edited hyperstacks.

9. To quantify the fluorescence signals from each isolated structure, run the "Analyze Edited Movie" plugin for the appropriate edited hyperstack. Save the results.

10. If you wish to show more than one isolated structure in a combined edited movie, run the "Merge Two Hyperstacks" plugin and merge the first two edited hyperstacks.

The same plugin can be run again to merge in additional edited hyperstacks. Save the merged hyperstack, which can be projected to make the edited movie.

11. If you wish to show the original movie above the edited movie, run the "Merge Hyperstacks" plugin again, but this time choose the option of placing the original hyperstack above the edited one.

12. Once you have a fully processed hyperstack, run the "Project Hyperstack" plugin. Save the resulting final movie.

13. If you wish to make changes in how the fluorescence signals were edited, go back and edit the appropriate montage series, then regenerate the final movie.

14. As described below, use ImageJ to adjust the display parameters for the final movie if desired, and then generate an MP4 version of the final movie.

Plugin: Make Montage Series

1. Run the plugin, and choose a 4D 8-bit TIFF hyperstack from a confocal. This data set should already be deconvolved, bleach corrected, and cropped if desired.

2. Choose a scale factor for magnifying the individual slice images. The default value is 4.0, but you can choose another number as long as the montage will fit on the screen.

3. Choose the slices that will be used to create the montage. You can omit slices that have no fluorescence data. (Alternatively, such slices can be removed ahead of time by editing the hyperstack in ImageJ.)

4. Allow the montage series to be created. The plugin cannot be aborted during scaling, but can be aborted using Esc during assembly of the montage series.

5. Save the montage series, which will serve as the "original" montage series during the editing phase.

Plugin: Edit Montage Series

1. Run the plugin, and open the original montage series.

2. The first time you work with a given montage series, choose the option "Create New Montage" to duplicate the original montage series.

   If you have already begun editing the montage series, use "Open Existing Montage", and choose the edited montage series.

   In either case, the edited version of the montage series will be displayed on top of the original version.

3. Use the keyboard commands listed below to work with the original and edited montage series. You can also use the sliders at the bottom of the window. It is recommended to use these controls whenever possible rather than the standard ImageJ menu commands and image adjustment dialogs.

   a) Use Tab to switch between the original and edited montage series windows. These two windows should stay synchronized with respect to channel, time point, and display parameters.

   b) Use the Left Arrow and Right Arrow keys to move between time points. You can also drag the lower slider.

   c) Use Opt together with the Left Arrow and Right Arrow keys to switch between channels. You can also drag the upper slider.

   d) Use the Up Arrow and Down Arrow keys to increase or decrease the display brightness of the currently selected channel.

      Use Opt together with the Up Arrow and Down Arrow keys to jump to either the default brightness, or the maximum or minimum brightness, depending on the current brightness setting of the currently selected channel.

   e) Use the R, G, and B keys (uppercase or lowercase) to toggle visibility of the red, green, and blue channels, respectively. Use the Y key to toggle visibility of the gray channel showing the cells.

   f) With the edited montage series visible, use the Z key to replace the edited contents of the currently displayed channel and time point with the original contents.

      Note that Z is not a generic Undo key. This replacement function is irreversible.

   g) Use the A key to select the entire image.

   h) Use the E key to erase all of the fluorescence in the selected channel going forward or back in time. You will be prompted to choose the direction.

i) To delete everything except the desired spot, first select the spot in each of the relevant slices by drawing regions of interest (ROIs). A fast method for drawing ROIs is described below in (j).

You can use Shift to add pixels to an existing ROI, or Opt to trim pixels from an existing ROI.

When you are satisfied with the ROI, press the Delete key. For the currently selected channel, the nonselected pixels outside the ROI will be deleted.

If you make a mistake, reverse the procedure by pressing Z.

If you want to delete the selected pixels inside the ROI, use Opt together with the Delete key. If nothing is selected, the entire image will be deleted.

j) To automatically trace a spot in all of the relevant slices, do the following. First, select the spot by drawing a crude ROI. Make sure the appropriate channel is selected. Then press Return. The spot will be outlined in the original slice and in the adjacent slices.

If you change the brightness using the Up Arrow and Down Arrow keys, and then repeat the automatic tracing, the borders will expand or contract accordingly.

If the automatic tracing captures too much or too little, you can add to the selection using the Shift key or remove part of the selection using the Opt key.

4. Periodically save the edited montage series. The S key will work for this purpose. You can return to the edited file later if you wish to make additional edits.

Plugin: Montage Series to Hyperstack

1. Open an edited montage series.

2. Run the plugin. Choose the desired time points to generate an edited hyperstack.

3. This edited hyperstack can be merged with other hyperstacks as described below, or it can be projected to make a 4D movie. In addition, it can be quantified using the Analyzed Edited Movie plugin described below.

Plugin: Analyze Edited Movie

1. Open a hyperstack generated from an edited montage series.

2. Run the plugin. You will need to enter the time interval between Z-stacks.

3. The results will be displayed in a window.

   "Red", "Green", and "Blue" are the total signals from the Z-stacks at each of the indicated time points.

   "Red Integrated", "Green Integrated", and "Blue Integrated" are the time-dependent numerical integrals of the signals.

4. If you click on this window and then save, the result will be a CSV file that can be opened by Excel. You can then plot the data to show the primary or integrated fluorescence signals over time.

Plugin: Merge Two Hyperstacks

1. Run this plugin, and choose two hyperstacks generated by the “Montage Series to Hyperstack” plugin.

2. If you want to merge two edited hyperstacks to show two isolated structures in the same movie, choose the default “Merge fluorescence signals” option.

   If desired, the resulting hyperstack can be used as the input for another iteration to merge in data for a third isolated structure, and so on.

3. If you want to show the original movie about the edited movie, choose the “Place first above second” option.

   First choose the hyperstack corresponding to the original movie. Then choose the hyperstack corresponding to the edited movie, which may show two or more isolated structures as described in step #2.

Plugin: Project Hyperstack

1. Open a hyperstack that was either generated directly by the "Montage Series to Hyperstack" plugin, or further processed using the "Merge Two Hyperstacks" plugin.

   If desired, you can examine this hyperstack to see if there are slices at the top or bottom that contain either no fluorescence throughout the time course, or extraneous background fluorescence.

2. Run the "Project Hyperstack" plugin. When prompted, choose whether to project all of the slices, or to remove undesired slices from the top or bottom of the stack.

   Make sure that "Average intensity" is chosen for the projection, and that all time frames will be projected.

Adjusting and Converting the Final Movie

1. The result of the "Project Hyperstack" plugin should be a movie that is easy to view and appropriately scaled. However, you can adjust the output for each channel as follows.

    a) Choose Image > Adjust > Brightness/Contrast. In the projected movie, use the "c" slider to choose the channel (red, green, blue, or gray) that will be adjusted.

    b) Adjust the "Maximum" slider as desired to set the output of a given fluorescence channel.

    Alternatively, click "Auto" and then drag the "Minimum" slider back to "0".

    Alternatively, click "Set" and specify the values manually. This approach is particularly useful if you wish to have identical display values for two movies, e.g., an original and an edited movie.

    c) Another way to adjust a channel is by choosing Process > Enhance Contrast. A value of 0.1% saturated pixels will give the same result as the "Project Hyperstack" plugin. You can vary this number as desired.

2. To add a time stamp, choose Image > Stacks > Label. Use the format 00:00, with the appropriate time interval in seconds.

    To place the label in the lower left corner, set the Y location to be 1 less than the Y value of the lowest row of pixels.

    To place the label in the lower right corner, also set the X location to be 55 less than the X value of the rightmost column of pixels.

3. To make a movie for publication, choose File > Save As > AVI. For the compression, choose PNG. A frame rate of 10 fps is typically suitable.

    Convert the AVI file to MP4 format at https://video.online-convert.com/convert-to-mp4.

Plugin: Average Traces

1. Collect multiple CSV trace files generated by quantifying complete maturation events using the Analyze Edited Movie plugin. The recommended number is approximately 10 maturation events. All of the events need to have the same interval between Z-stacks.

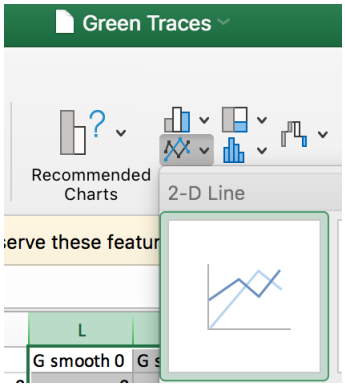
   Place all of the CSV trace files in a folder, which can also contain other non-CSV items, including subfolders.

2. Run the "Average Traces" plugin. Choose any of the CSV trace files from the folder.

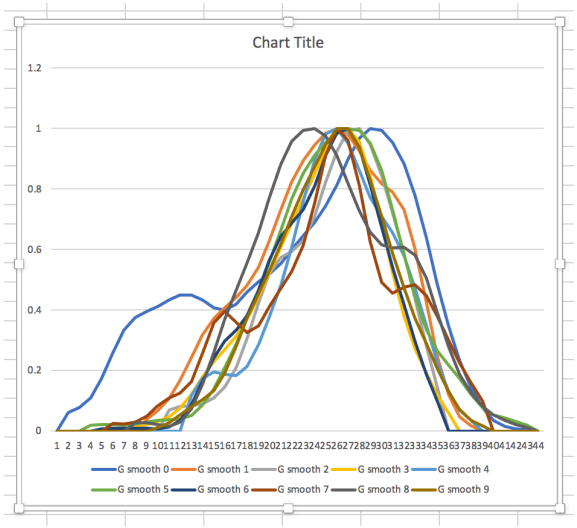
3. The results will be a series of windows, which can be saved as CSV files to a subfolder within the folder of CSV trace files.

4. Open the Green Traces.csv file in Excel. Click to select the column labeled "Green smooth 0", then Shift-click on the final column of smoothed green traces.

5. Click "Insert" at the top of the Excel file, then choose the option to draw a line plot from the selected columns, which represent the full set of smoothed green traces:

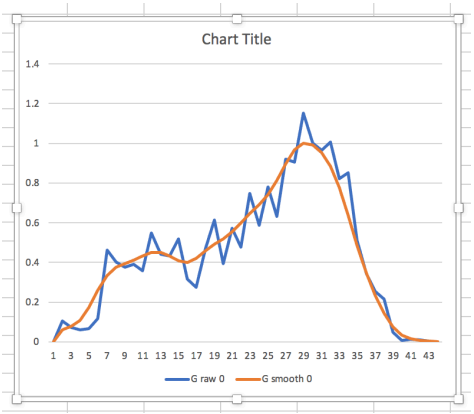


   The result will be a plot of the aligned smoothed green traces. It should look something like this:



6. The goal at this point is to determine whether there are any outlier traces that will skew the average results. In this example, “G smooth 0” is clearly an outlier.

   If an outlier is identified, select the “smooth” column for that outlier, and then Command-click to select the corresponding “raw” column. Plot this pair of traces:



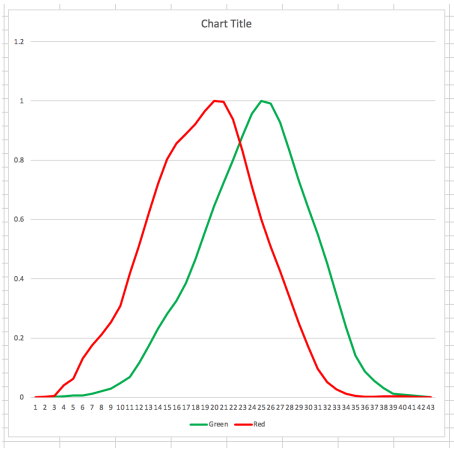
   Based on the shape of the raw trace, you will need to go back and determine which of the original CSV trace files is the culprit. Unfortunately, there is not a direct way to figure out which CSV trace file names correspond to the traces listed in the Excel file.

   Repeat the analysis to look for outliers using the Red Traces.csv and Blue Traces.csv files, if present.

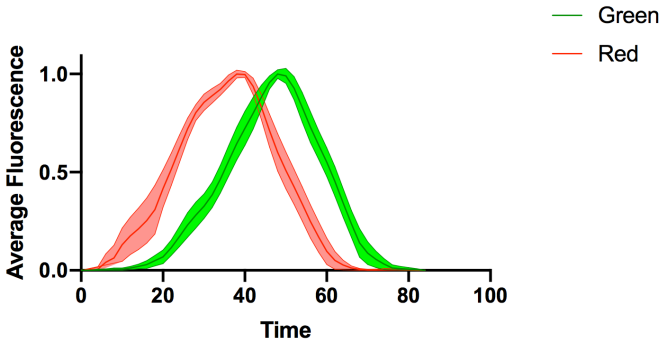
7. Remove any outlier CSV trace files from your folder. If CSV trace files were removed, run the “Average Traces” plugin again with the edited set of CSV trace files, and save the resulting windows to replace the originally saved versions.

   If you want to preserve plots of the aligned traces, use “Save As” to make copies of the open .csv files in Excel’s .xlsx format.

8. Open Averaged Traces.csv in Excel. Click on the “Green” column, then Command-click on the “Red” column if present and on the “Blue” column if present. Plot the averaged traces:



If the results are satisfactory, transfer the full averaged data set to Prism to make a plot with 95% confidence intervals:



9. The Offset Values.csv file includes estimates of the width in seconds of each averaged trace with SEM. If a red channel is present, the file also lists the estimated offsets with SEM values between the start and end points for the green (G) and red (R) averaged traces, and similarly if a blue (B) channel is also present:

Offset Values.csv

G Width	G SEM	R Width	R SEM	G-to-R Start	G-to-R Start SEM	G-to-R End	G-to-R End SEM
55.1	1.8	52.8	2.5	-7.5	1.6	-9.9	1.7

In this example, the negative values indicate that the red trace started and ended before the green trace.