K-means Spectral Unmixing Plugin User Guide

Download K-means-spectral-unmixing-fiji.jar from https://rochester.app.box.com/folder/60387154437 or https://github.com/tristan-mcrae-rochester/Multiphoton-Image-Analysis/tree/master/Spectral%20Unmixing/Code .

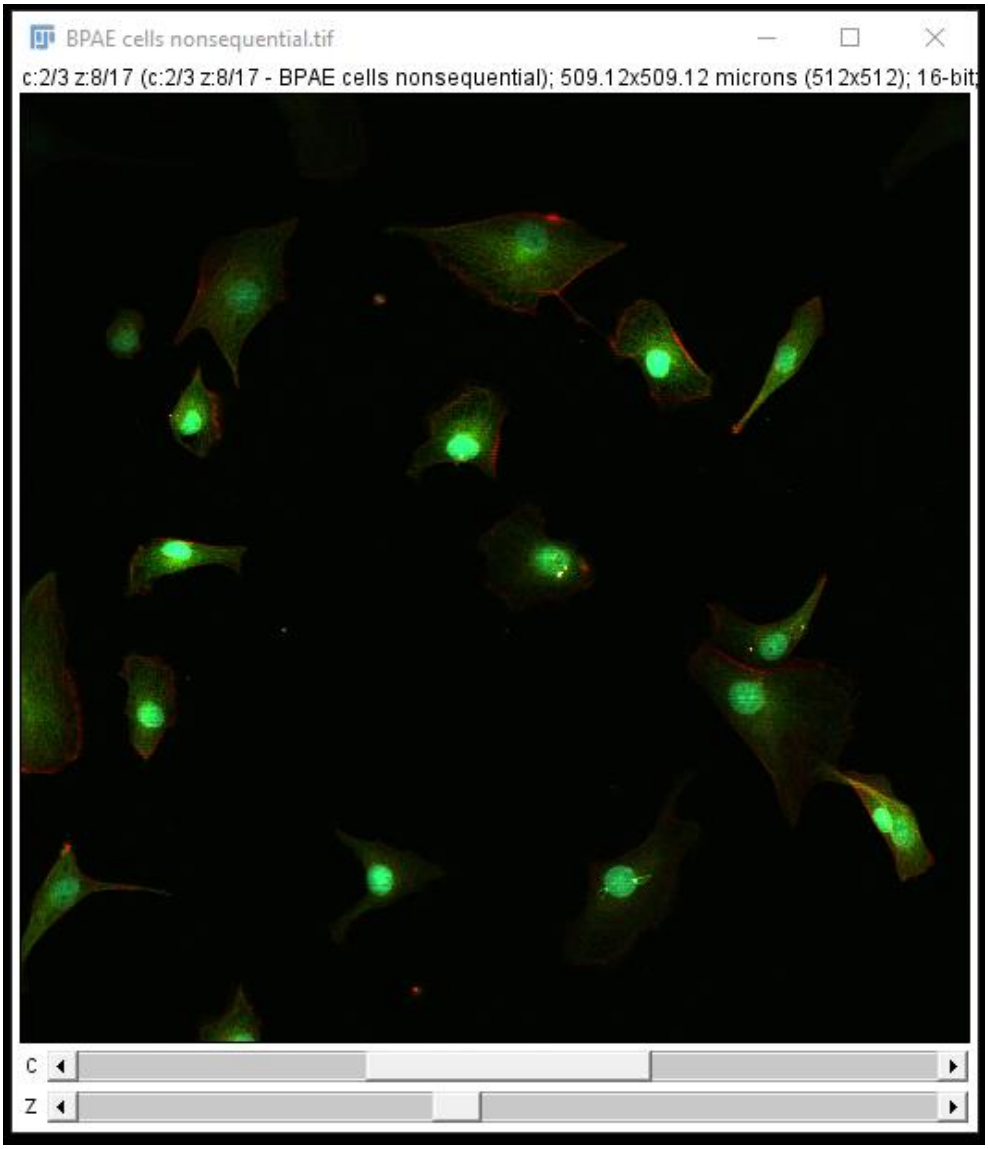
Install FIJI if you haven't already. FIJI is a "batteries included" version of ImageJ that is friendlier for plugin use. It can be downloaded from https://imagej.net/Fiji#Downloads .

In the folder where your FIJI installation is saved, navigate into the *jars* folder.

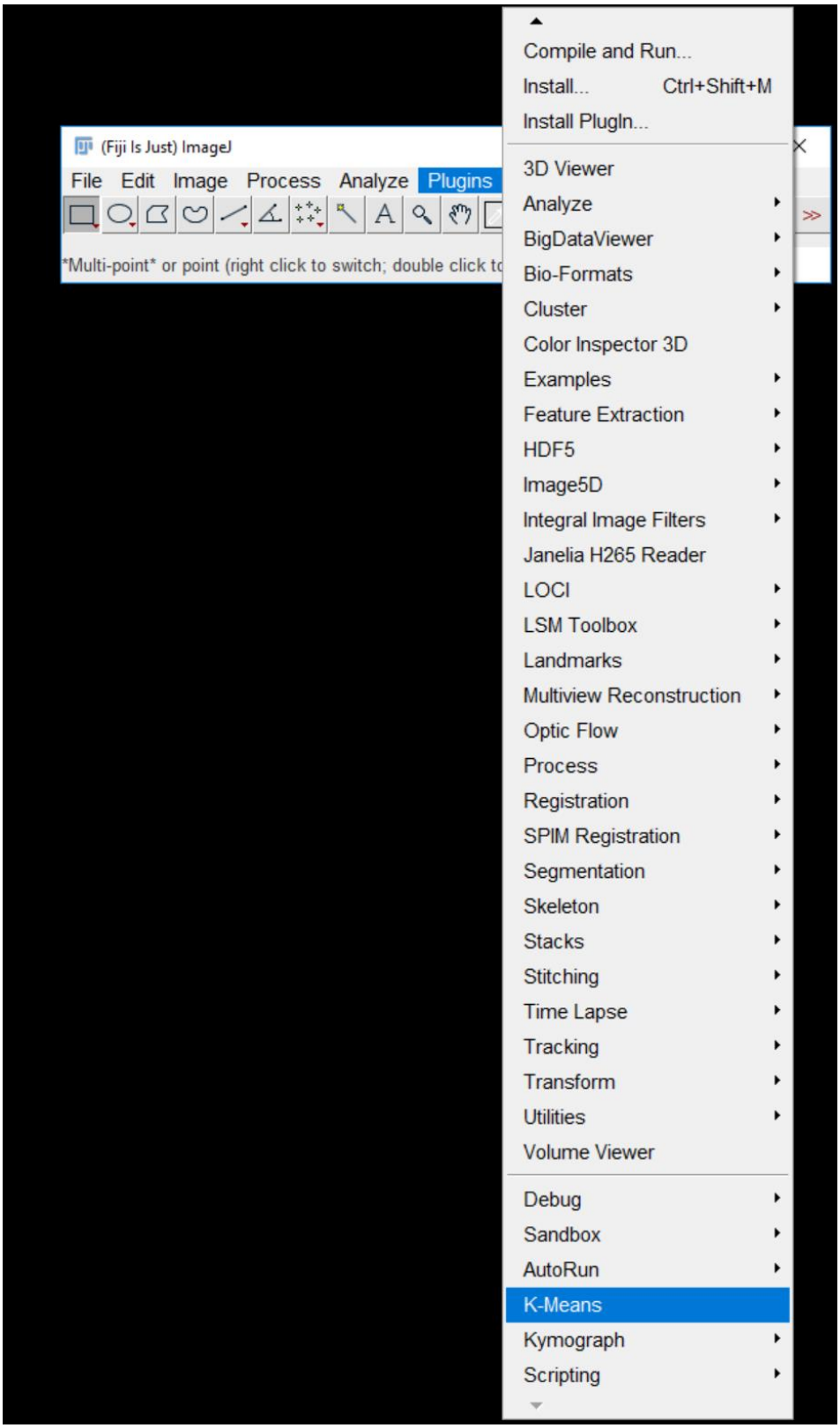
Move K-means-spectral-unmixing-imageJ.jar into the *jars* folder.

Open ImageJ

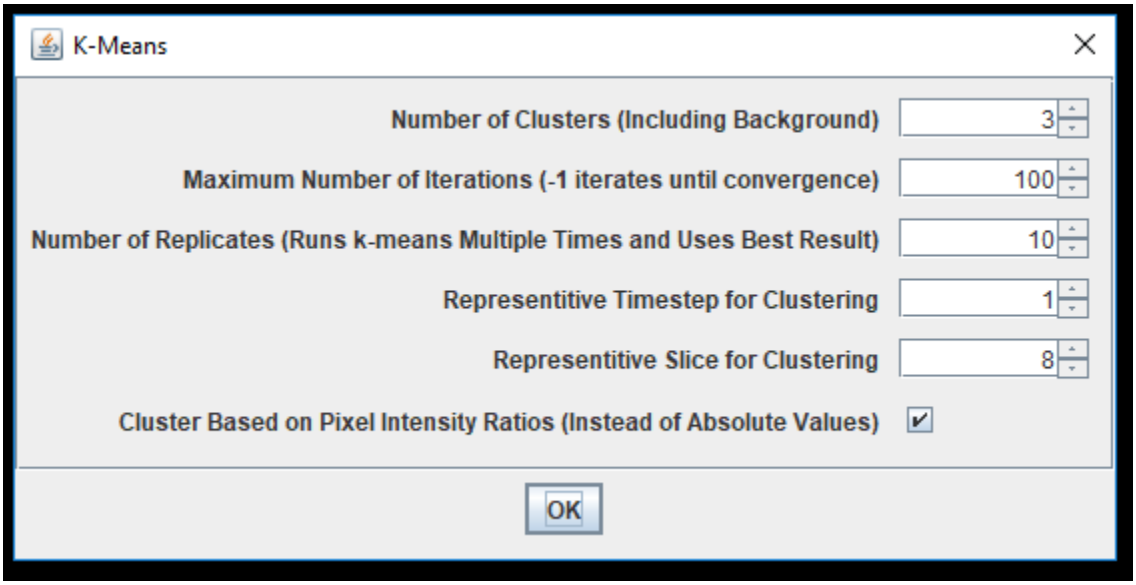
Open the Image you want to unmix



Start the spectral unmixing plugin by going to *Plugins -> K-means*. You may have to scroll down a bit to find it.



After selecting k-means, you will then be asked about a number of options:



Number of Clusters – The number of clusters the algorithm will create. This is the "*k*" in *k*-means. A good starting point is to select every channel for unmixing and ask it to unmix however many fluorophores were present during imaging +1 for a background cluster. If there are some channels that you already know only include one fluorophore, you can reduce the number of fluorophores you want to unmix by one. Note! This is different than previous versions where a background cluster was automatically added behind the scenes. If, after unmixing, it seems like there are still multiple fluorophores contained in some of the output channels, you can increase the number of fluorophores you ask it to unmix. This will have the effect of adding more clusters to k-means. If done successfully, this will result in two or more clusters that represent the same fluorophore but no clusters that represent multiple fluorophores.

Maximum Number of Iterations – K-means is an iterative algorithm and this option can cap the number of iterations in each replicate of k-means. A lower number cuts off the algorithm earlier which could potentially speed the algorithm up at the expense of quality. Setting this to -1 tells the algorithm to run until it converges.

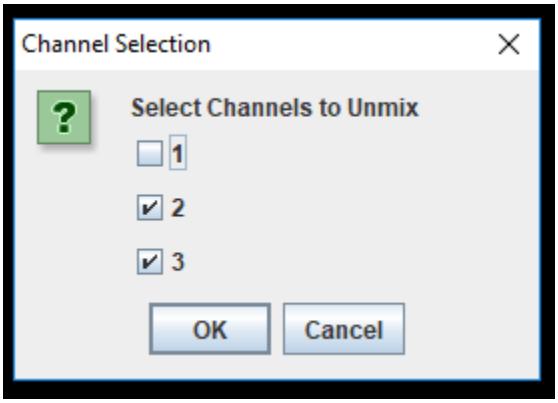
Number of replicates – Runs k-means multiple times and uses the best result. More replicates will improve clustering quality at the expense of taking longer. There tends to be diminishing returns with number of replicates. I recommend starting with something around 25 and changing it as needed for faster or higher quality results.

Representative Timestep for Clustering – In order to speed up clustering, clusters are defined based on a single 2D slice of your data and then applied to the rest of your data afterwards. Choose a timestep where you can see all of the different structures and colors. This number indexes from 1. If you only have one timestep, put 1 here.

Representative Slice for Clustering – Same as representative timestep but for an axial slice.

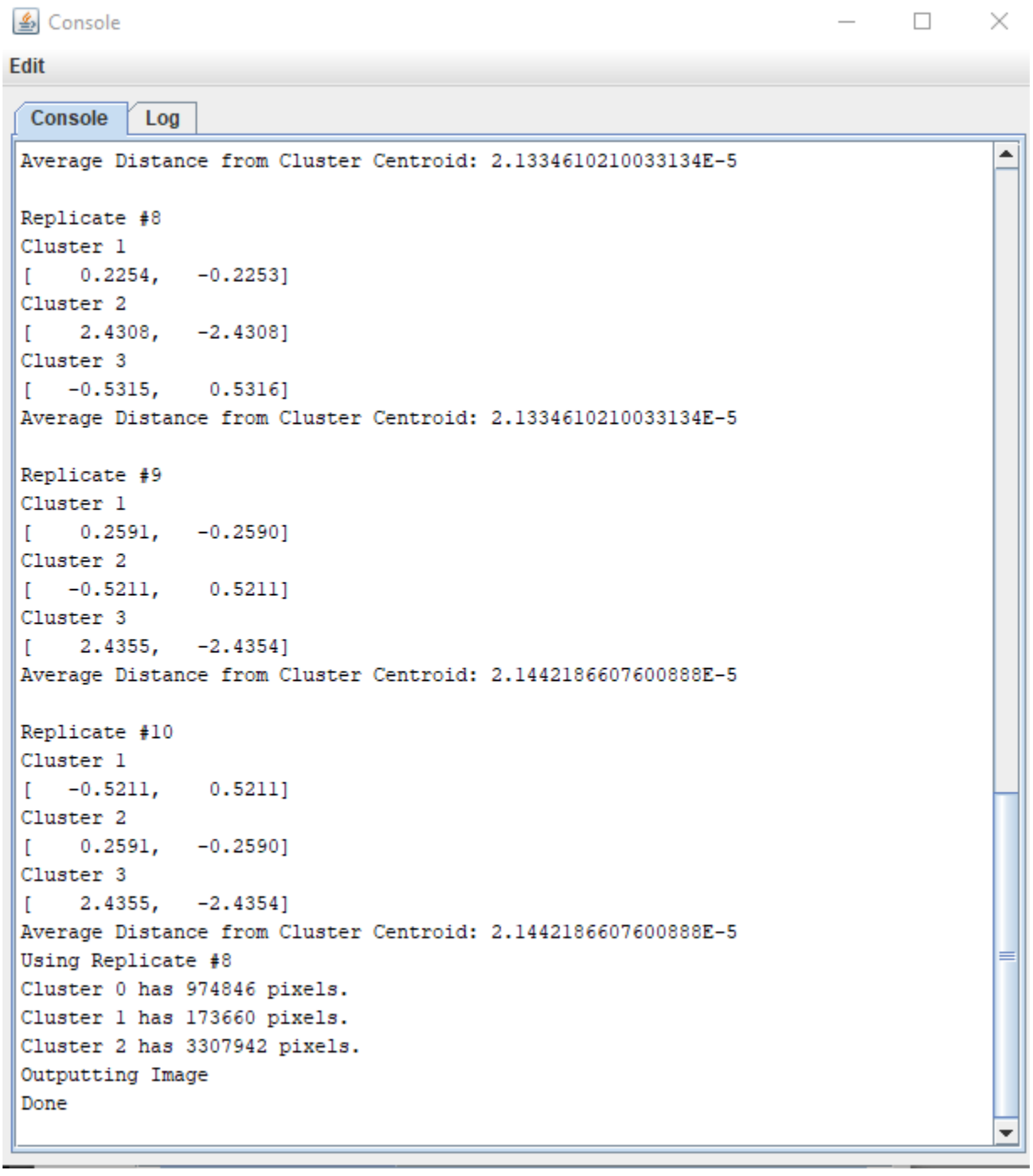
Cluster Based on Pixel Intensity Ratios – If checked, the algorithm will treat two pixels with the same ratio of channel intensities as being similar to one another. This is useful when overall intensity drops off with depth but the color stays the same. If pixels of different absolute intensity can represent different things in the image, leave this box unchecked.

Choose your desired settings and click “OK”. You will now be presented with one more popup asking which channels you would like to unmix.



Select which channels to unmix - Any channel whose box you check will be included in the unmixing and any unchecked channel will simply be preserved in the final image. If there are some channels that you know already only include one fluorophore, you can uncheck their box.

The progress of k-means will be displayed in a window which will pop up when you start the plugin.



When it is finished running, the unmixed image will pop up in ImageJ. The output image will have one channel for each cluster, including the background. Display colors will not necessarily align with the colors of the original image and can be modified with *Image -> Lookup Tables -> <desired color>*.