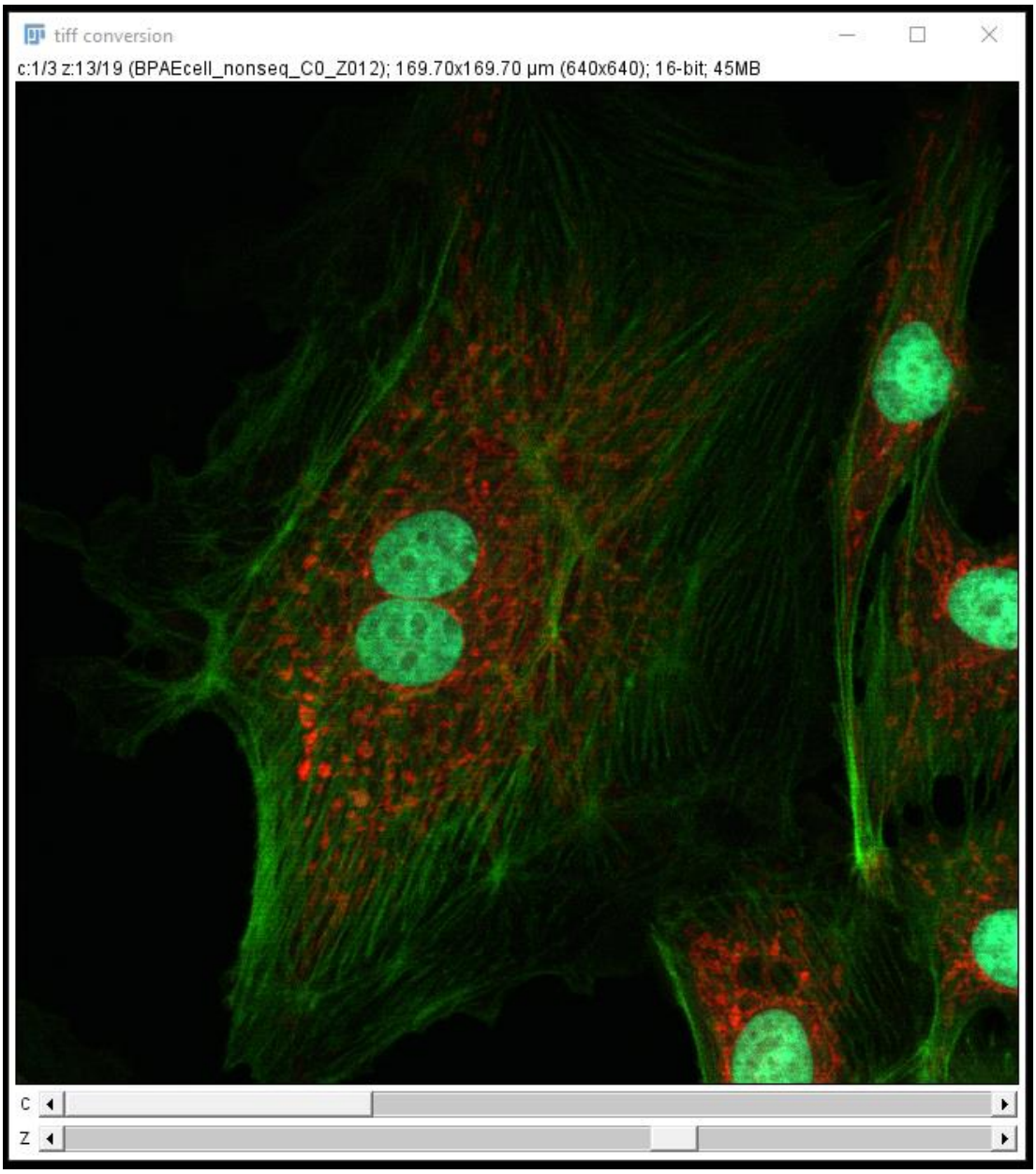
K-means Spectral Unmixing Plugin User Guide

Download Fiji.app(for spectral unmixing).zip from https://rochester.box.com/s/s1789ayo4m9uy1d6wrwvounrn1zoe1zq . (Contact Tristan_mcrae@urmc.rochester.edu if you need to be granted access).

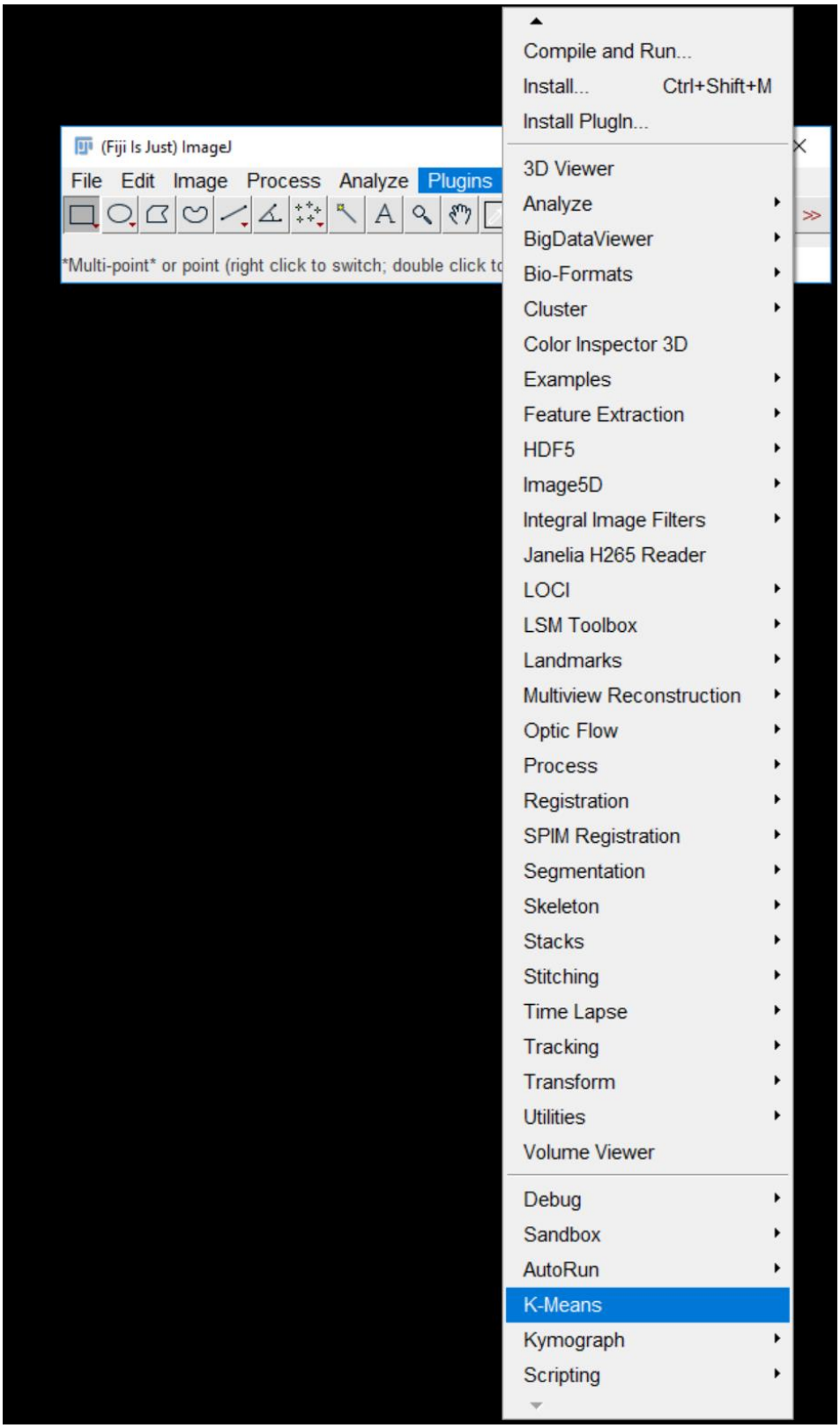
Unzip Fiji.app(for spectral unmixing) on your computer.

Open ImageJ by navigating into the Fiji.app(for spectral unmixing) folder and double clicking ImageJ-win64.exe.

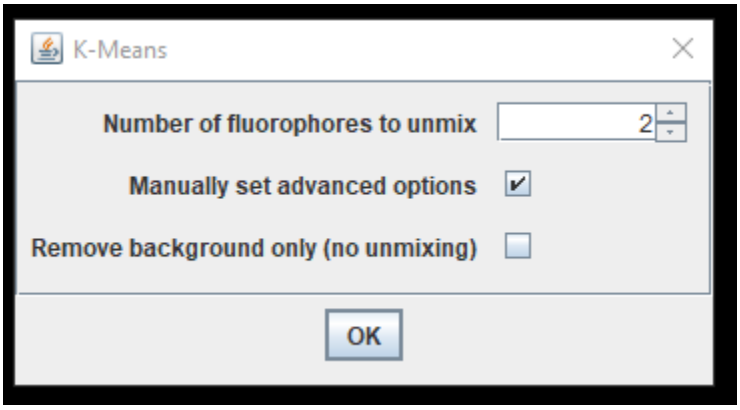
Open the Image you want to unmix



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:



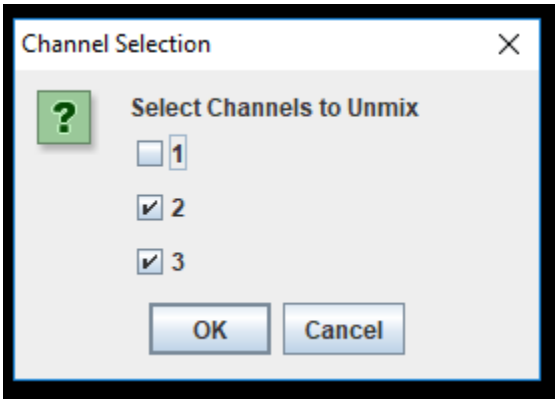
Number of fluorophores to unmix – The number of channels the algorithm will create for your fluorophores. An additional channel is added behind the scenes. This is the "*k*" in *k*-means. A good starting point is to select every channel for unmixing and ask it to unmix however many fluorophores were present during imaging. 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. 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 successful, this will result in two or more clusters that represent the same fluorophore but no clusters that represent multiple fluorophores.

Manually set advanced options – Selecting this will give you a new pop-up with more choices. If this is not selected, default values will be used for all advanced options.

Remove background only (no unmixing) - uses *k*-means to label each pixel as being background or not. It then removes the background pixels and leaves the rest of the pixels untouched. It does not require any selection of options.

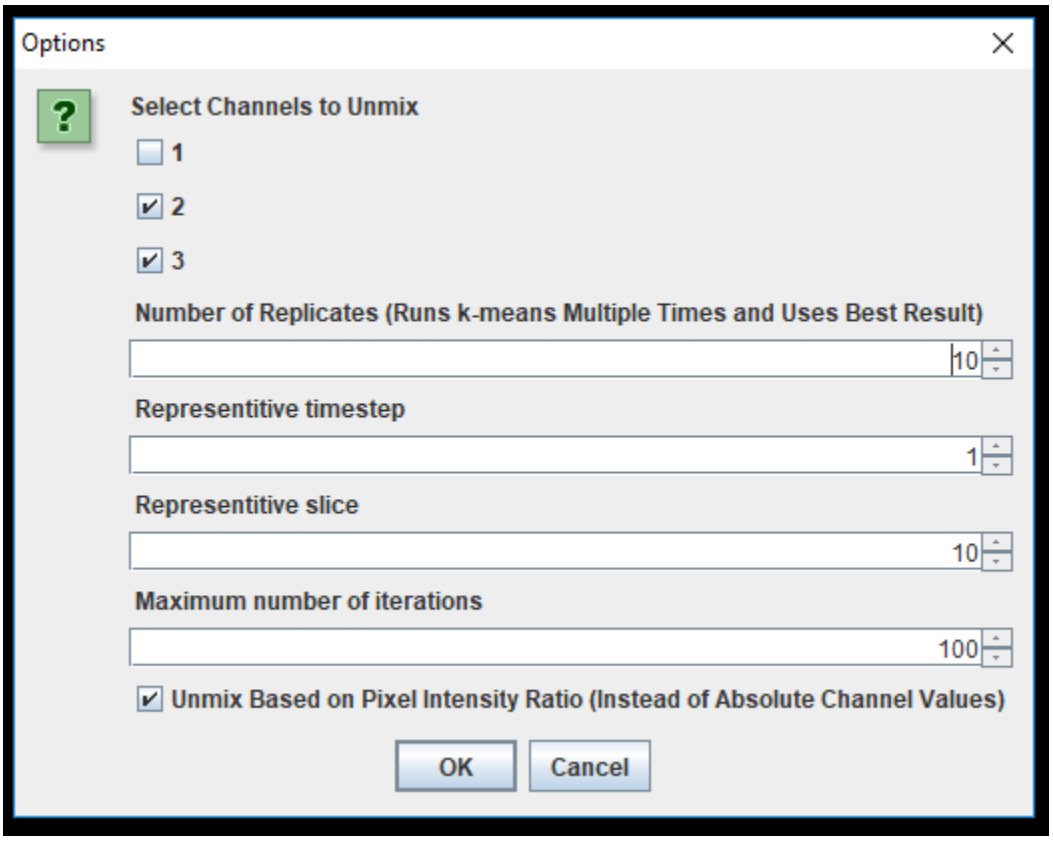
After making these selections, click ok. Depending on whether you chose to manually select advanced options, you will get one of two popups next.

Basic options:



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.

Advanced options:



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. Default is 100.

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. Default is 10.

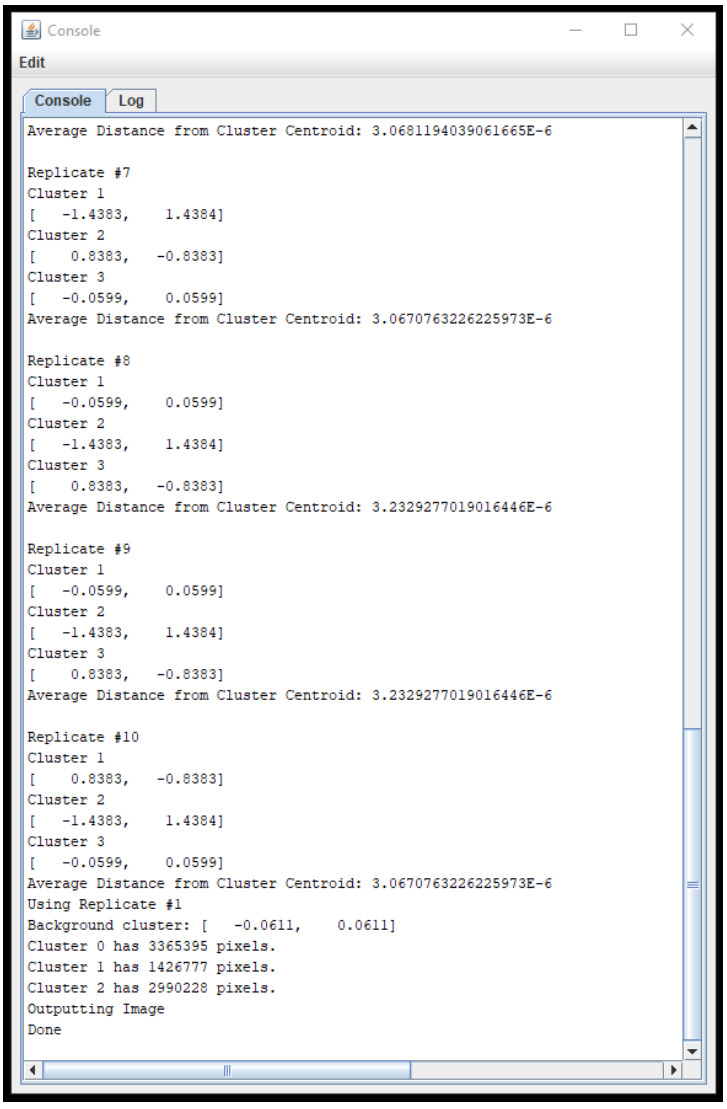
Representative Timestep for Clustering – In order to speed up clustering, clusters are defined based on a single 2D slice of your data and then applied to the rest of your data afterwards. Choose a timestep where you can see all of the different structures and colors. This number indexes from 1. If you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

Representative Slice for Clustering – Same as representative timestep but for an axial slice. Default is the middle slice.

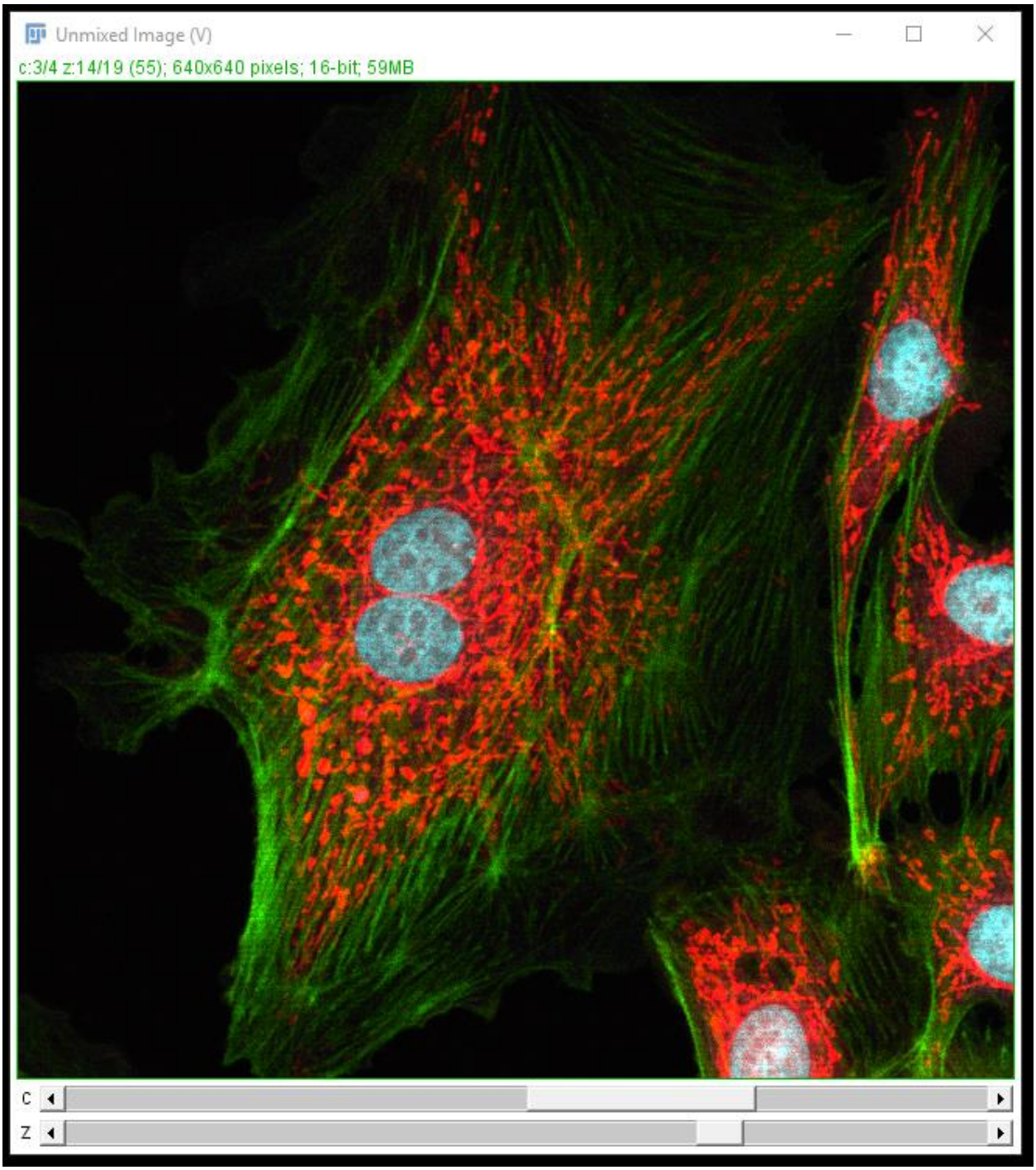
Cluster Based on Pixel Intensity Ratios – If checked, the algorithm will treat two pixels with the same ratio of channel intensities as being similar to one another. This is useful when overall intensity drops off with depth but the color stays the same. If pixels of different absolute intensity can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

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

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. (Note how the cell nuclei no longer show up in the green channel in this example.) 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>*.



Colocalization

This spectral unmixing method can also be used for detecting co-localization of fluorophores in an image. If you wish to use the spectral unmixing algorithm to create a separate channel for pixels where co-localization is occurring, add one “fluorophore” for each co-localization combination when asked how many fluorophores you want to unmix. For example, if you have two channels, - CH1 and CH2 - and wanted to separate pixels that are purely CH1, purely CH2 and a mix of CH1 and CH2, you would tell the algorithm to unmix three fluorophores. The co-localization of CH1 and CH2 will have its own spectral signature and be treated as a unique fluorophore. This will only work if there are instances in your image where CH1 and CH2 both exist apart from one another.