Autofluorescence Finder Workflow Guide

The purpose of this tool is to find and gate on multiple Autofluorescence (AF) populations.

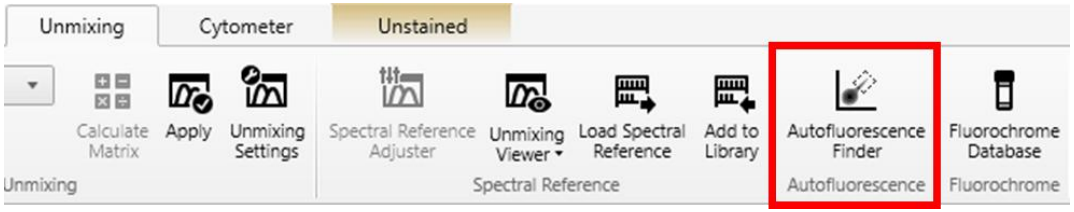
You will generally only need to use this tool on samples that require a tissue digestion protocol (lung, skin, brain, gut, liver, tumor), co-cultures, or patient samples where the patient has been treated with a drug that has its own AF signal. Each AF population will then have its own Spectral Reference (SR) that will be added to the Unmixing Matrix.

This guide will take you through how to use the tool and have multiple examples of different sample types.

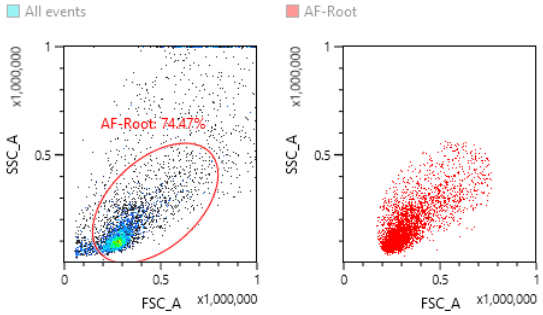
1. AF is calculated from unstained cells and has many different sources. **Unstained cells used to calculate AF should go through the exact same workflow as your fully stained samples. This includes any fixation/permeabilization, activation, drug treatment, etc.**

Above are examples of different fluorescent molecules that can contribute to AF

2. AF calculation should be performed after the recording of your sample but before you use the Unmixing Viewer to check unmixing accuracy. When recording unstained cells, **make sure that you record using the same voltage settings** as your fully stained samples. Start by recording about 50,000 cells in your scatter gate. This should be enough to see any rare AF populations, but you can also append more events.
   i. Navigate to the worksheet of your unstained cells.
   ii. Turn off unmixing
   iii. Go to the Unmixing tab at the top of the software and click on Autofluorescence Finder
   iv. Select “OK” on the popup

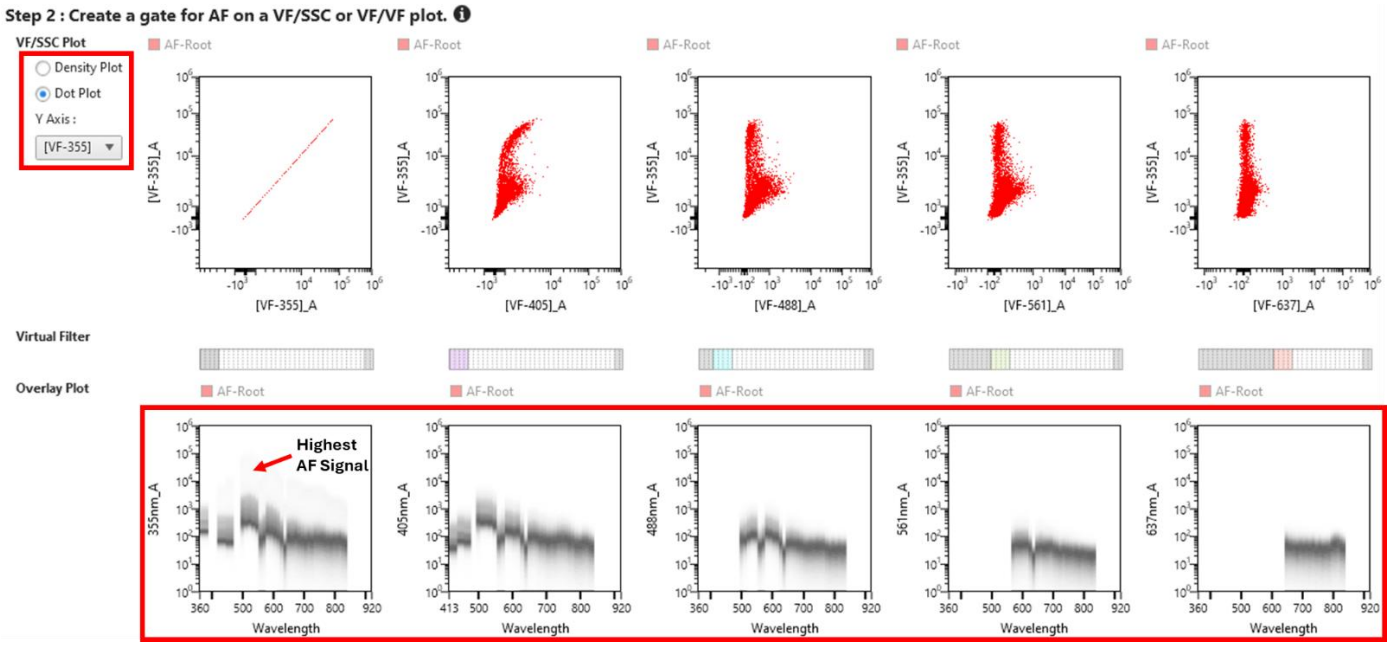


3. Draw a gate on your FSC vs SSC plot that approximately matches the position and size of the scatter gate you will use for analysis. This gate is called your AF-Root gate.
   You do not have to worry about the AF of any event not included in this gate. This includes debris, counting beads, and dead cells.

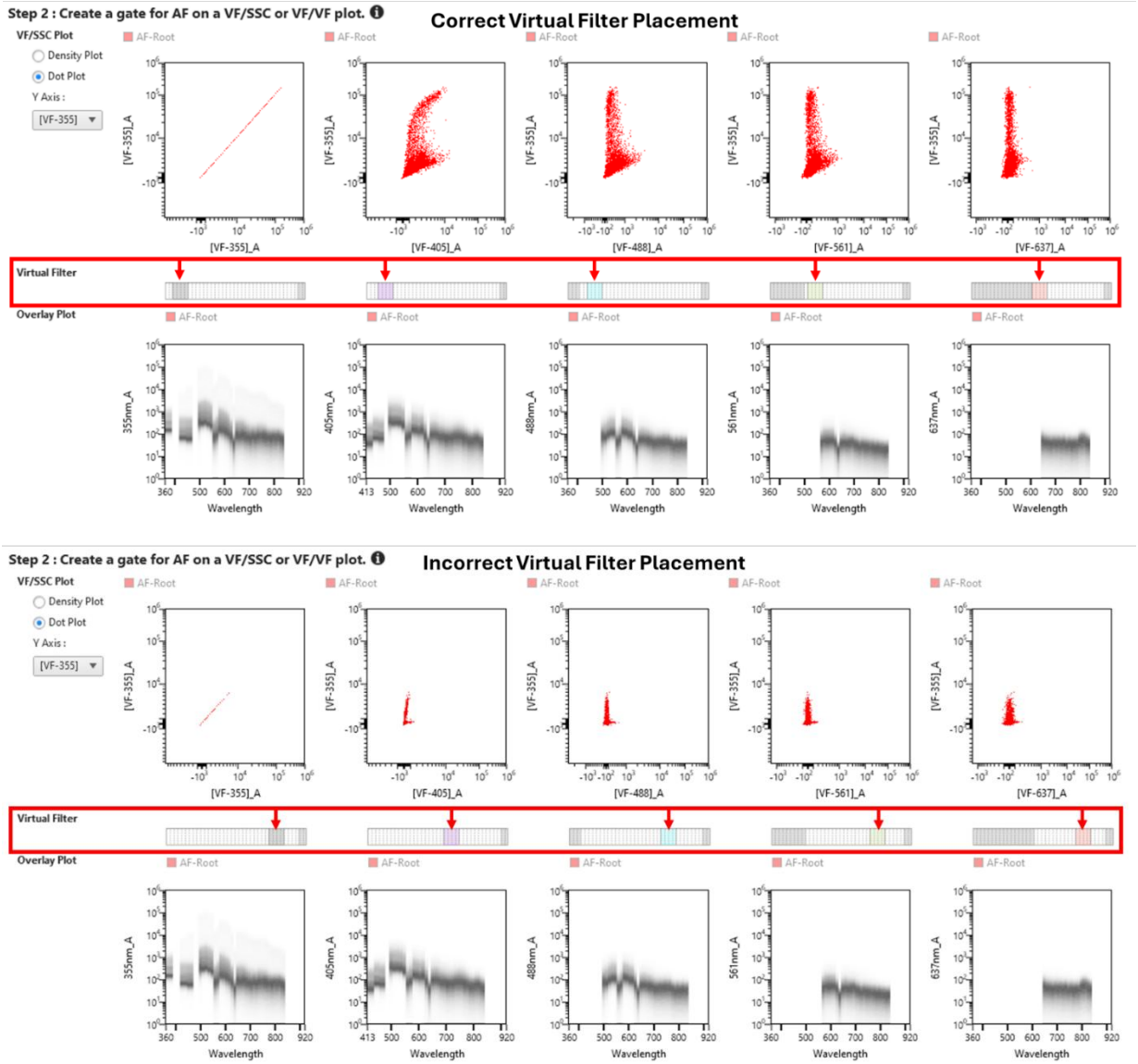


4. Under Step 2, select Dot Plot.

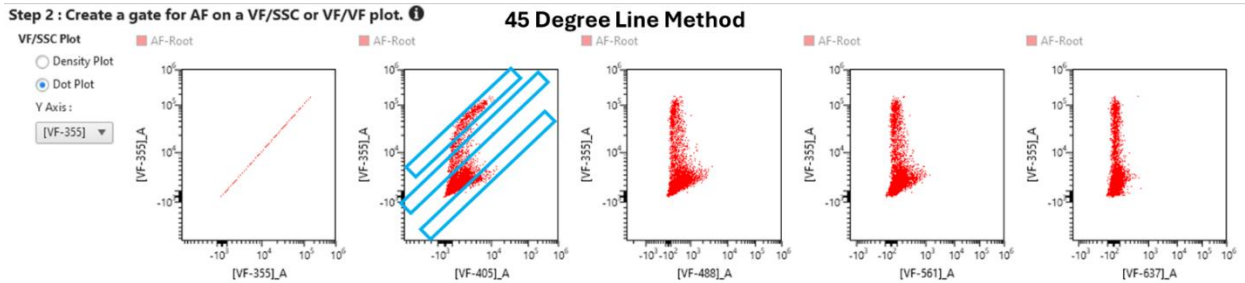
   The bottom row of Overlay plots in the AF Finder window show the raw spectral data from your unstained cells. Find the detector deck that has the brightest signal (it may look very faint on the Overlay plots) and display that detector deck on all of your y-axes by using the drop-down menu under Step 2.



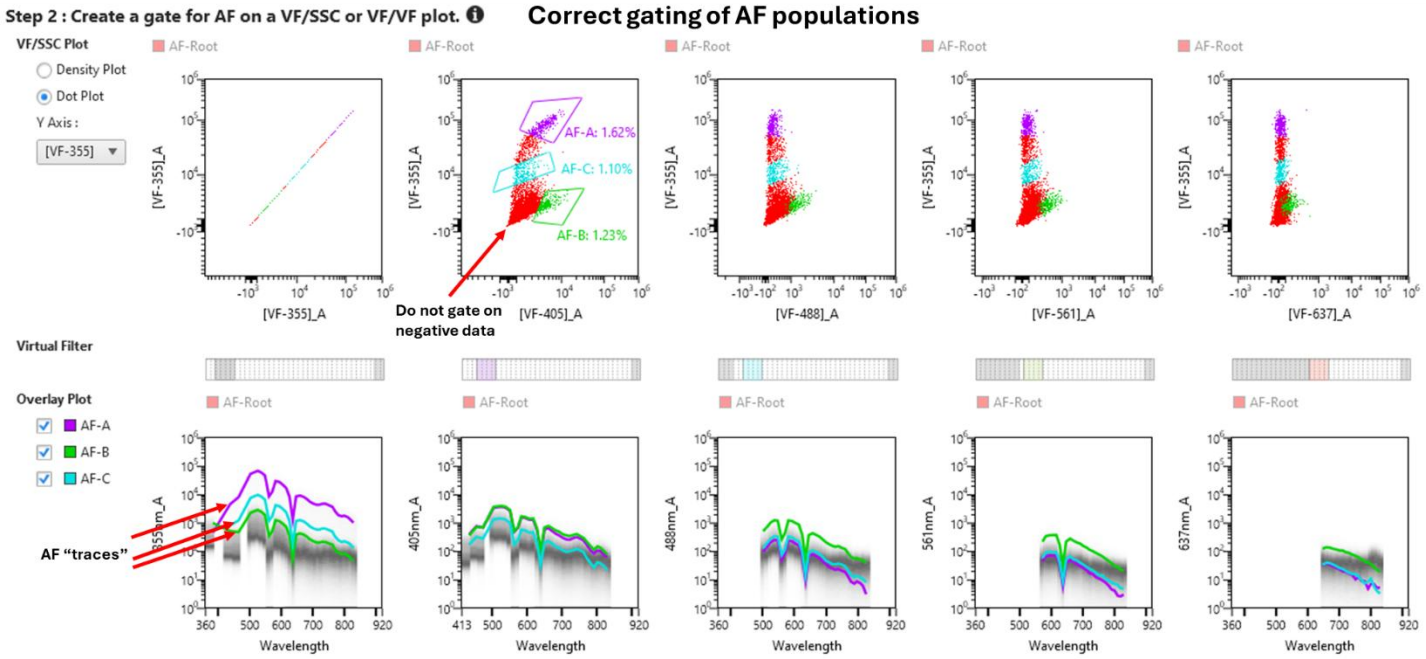
5. Virtual Filters (VF) are sliders that allow you to see data from a small range of detectors from each laser. Move the VF around to see where you have better separation of your AF signal.
   The pictures to the right show what it looks like when you have the VF in a good place and in a bad place on the same sample.
   Use the plots of raw spectral data at the bottom as a guide for where you will have more or less AF signal. You should also use the click-and-drag scaling to “pull out” the AF populations as much as you can.



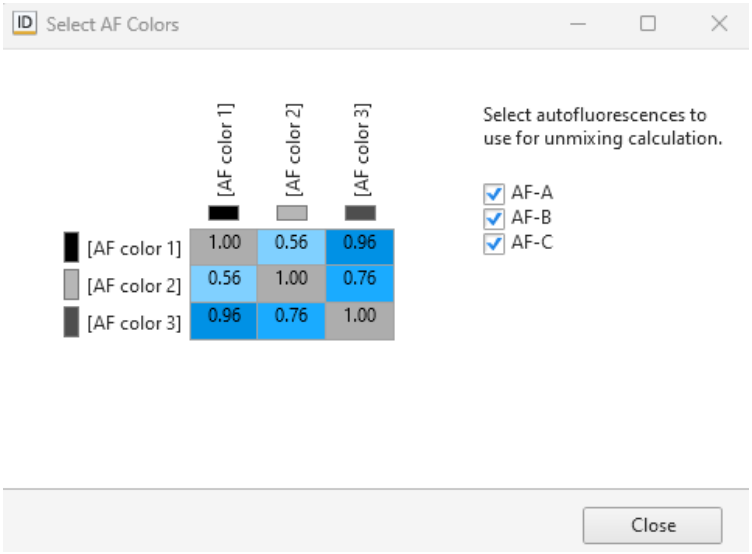
6. You do not have to gate on every single event that has AF signal. You also do not have to gate on negative events.
   A good way to tell if an AF population is different is by seeing if the populations fall along the same 45 degree line. If they do, they likely have the same AF signature but different intensities. If they fall along a different 45 degree line, they have different AF SRs.



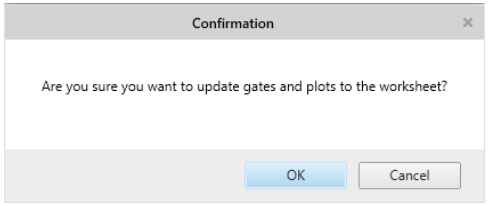
7. When you draw a gate to select an AF population, it will color those events on the other plots so you do not draw multiple gates on the same population. An average raw AF signal will also display for every selected AF population on the raw spectral graphs at the bottom of the window. You can place up to 8 different gates to select AF populations, but the vast majority of samples will not have more than 2-4 populations.

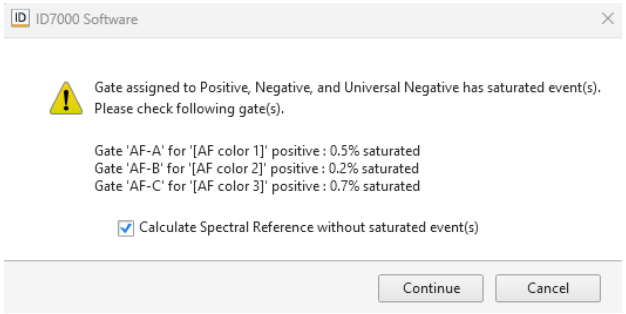


8. When you are done gating your AF populations, click “Select AF Colors”. This will calculate Spectral References for each AF population and display a similarity matrix. You can use these similarity scores to exclude any identical populations. *You may need to adjust your gates and then repeat this step.*



9. When you are done selecting your AF populations, select “OK” at the bottom right of the window. SRs for each selected AF population will automatically be calculated and added to the Unmixing Matrix. When prompted, click “OK” to update gates and plots to the worksheet and make sure to exclude any Saturated Events from the calculation of the SRs.





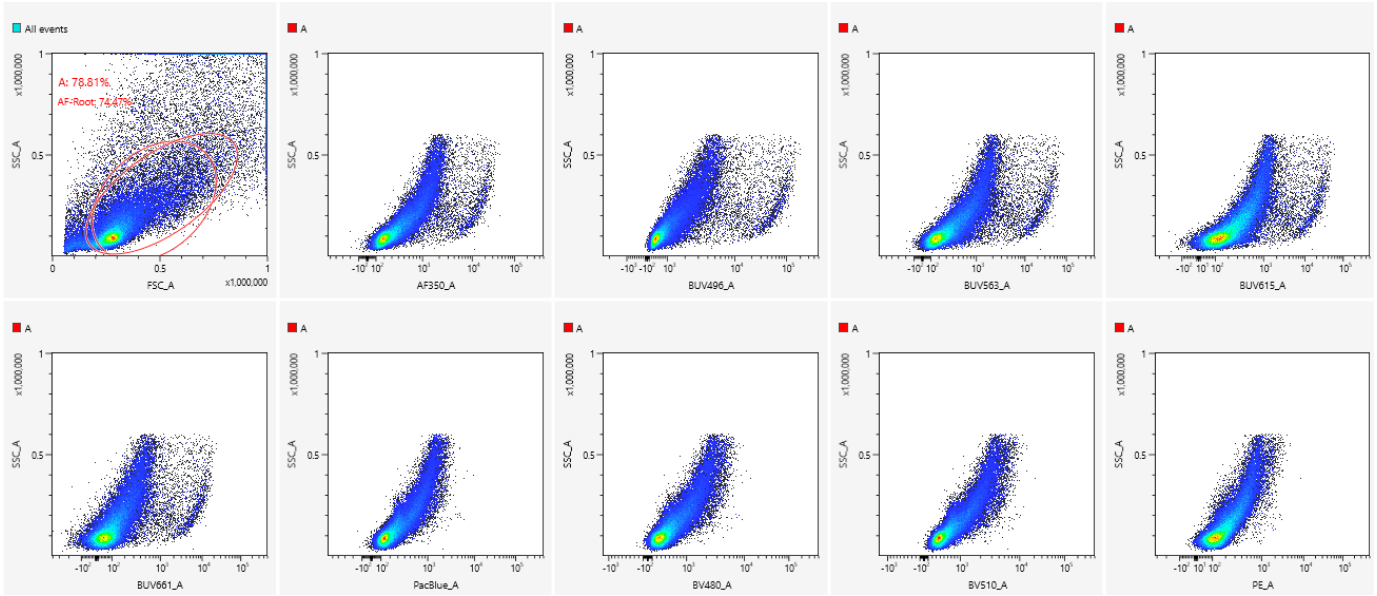
How to Check Autofluorescence Extraction

This section of the guide will take you through how to check if you have done your Autofluorescence Extraction properly. There are two ways that you can do the extraction incorrectly:

A. Too few AF populations.
    - You miss gating one or more AF populations and there is still AF signal that you are not accounting for.
B. Too many AF populations
    - You gate on the same population multiple times or on populations that are too similar. Your data will spread out if you do this.

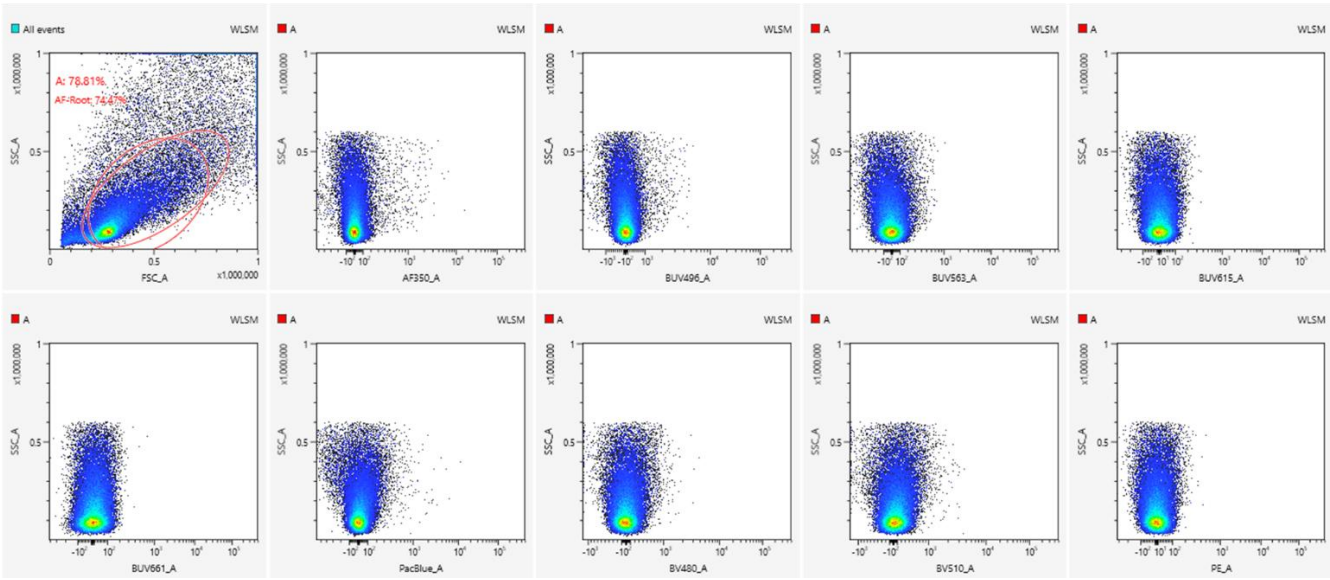
1. Right-click on the tube/well position of your unstained cells and Change Worksheet Mode to Individual. Then open Unmixing Settings.

2. See which of your fluorochrome SRs are most similar to your AF SRs and make plots of SSC-A vs. these fluorochromes off of your scatter gate as shown below.
   You do not have to look at every single fluorochrome in your panel.

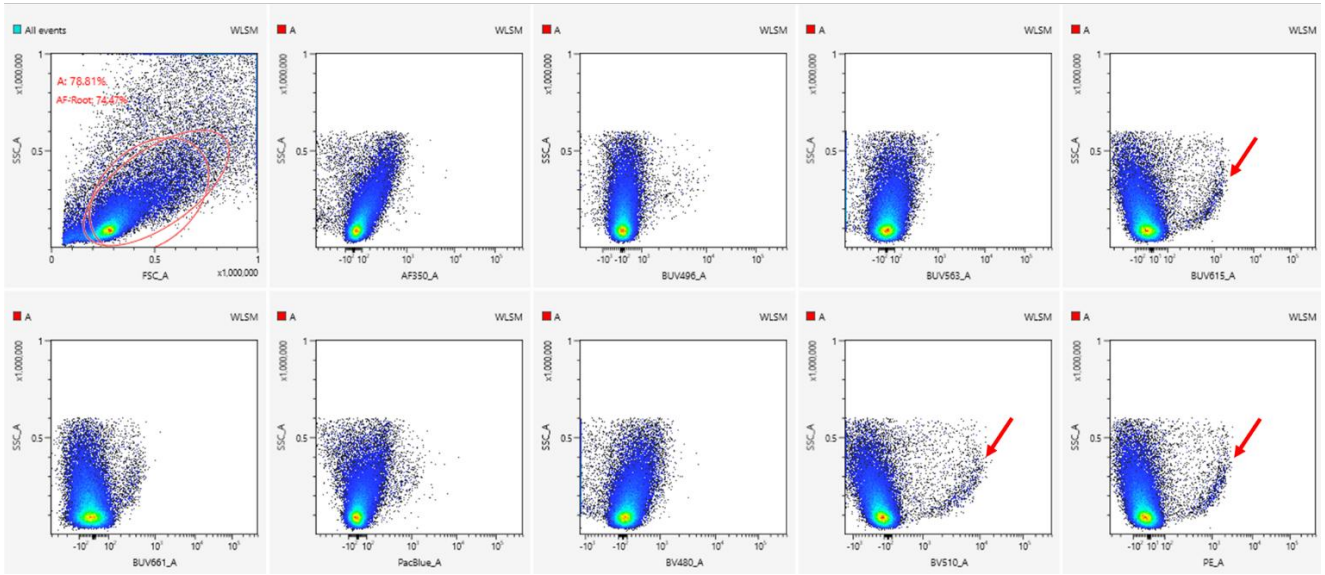


3. Turn on unmixing to check if you have missed any AF populations. See pictures to the right to see what it will look like on the plots you created in Step 2 if you have missed any AF populations or if you have performed AF extraction correctly. If you have missed any AF populations, open the AF Finder again and use the VF sliders to search for the missing population.

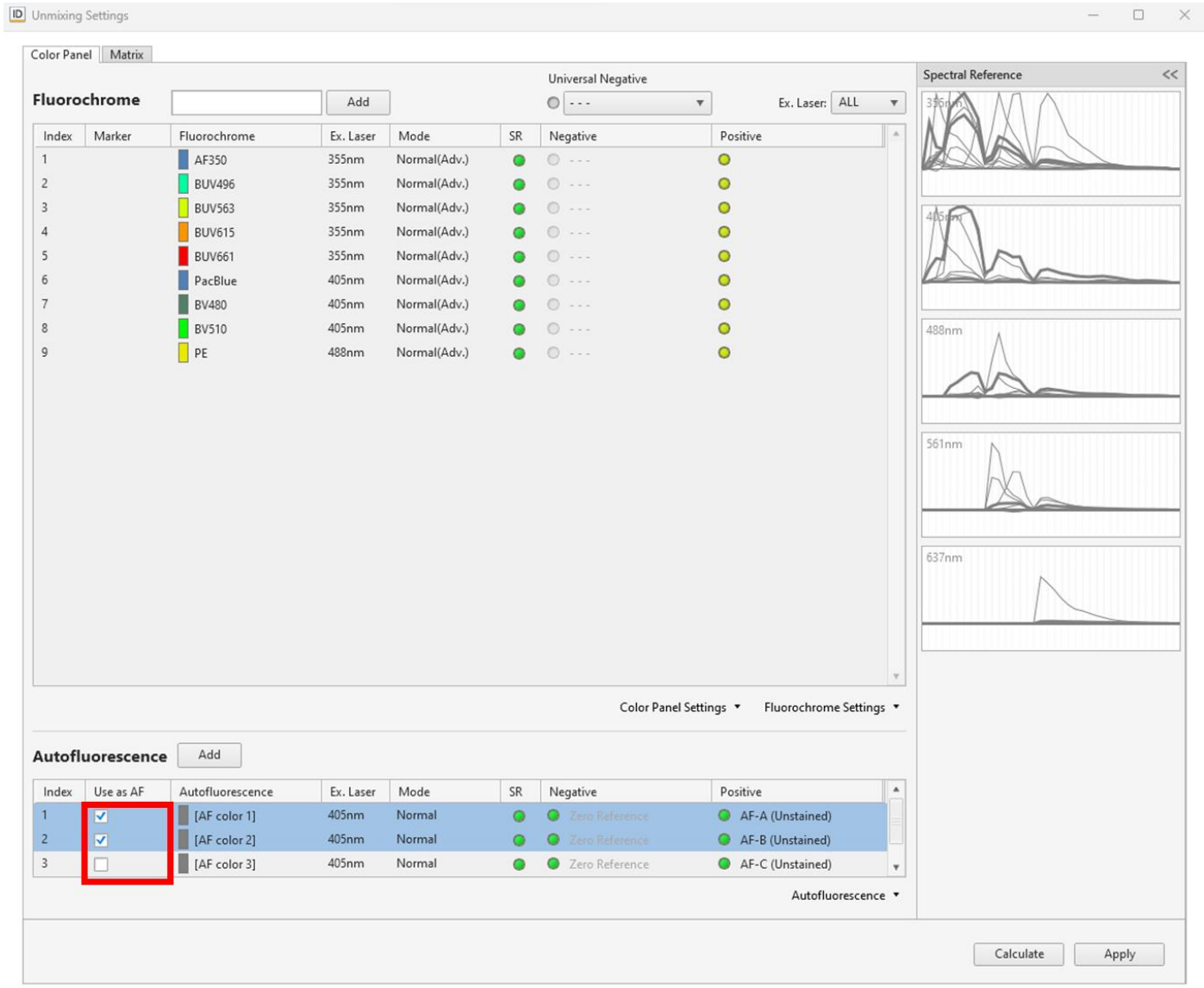
Correct AF Extraction



Incorrect AF Extraction – Missing AF Population(s)



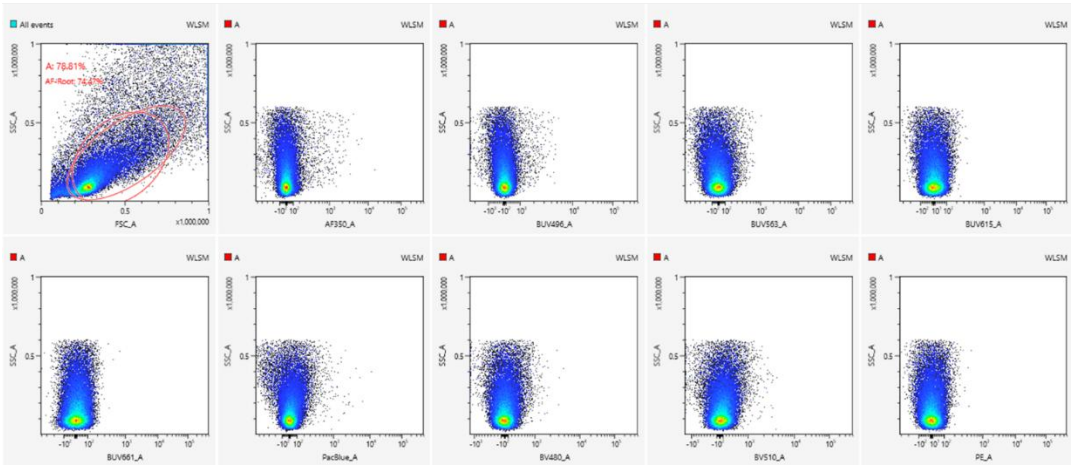
4. To check if you have selected too many AF populations or if some of your AF populations are too similar, use the checkboxes next to each AF population to turn them off one by one. Look at the plots you created in Step 10 to see if your data looks better or worse for each population excluded from the unmixing.
   Unchecking an AF population will instantaneously exclude it from the unmixing matrix.



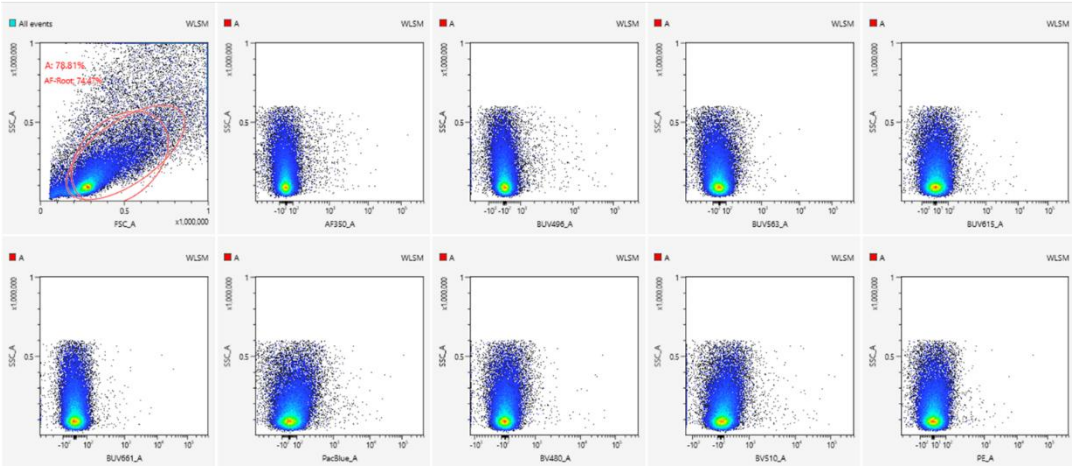
This is an example of having too many AF populations (this will spread out your negative population).

If you find that you have too many AF populations, find the combination that makes your data look the best and delete the extra population(s) by right-clicking on them and selecting “Remove Fluorochrome”.

Correct AF Extraction



Incorrect AF Extraction – too many AF populations



5. You are now ready to check your unmixing accuracy using the Unmixing Viewer and Spectral Reference Adjuster. Please use the Unmixing Viewer and Spectral Reference Adjuster Workflow Guide if you have questions.