= fluorescence of naturally occurring substances, e.g., NAD(P)H, Collagen, Flavin

- Excitable mostly by 355nm > 350nm > 405nm > 488nm lasers and insignificantly by 561nm + 637nm

- Contributes to background noise in the approximate range 400 − 600nm in most mammalian cells, affecting fluorescent probes emitting in a similar range.

Examples of fluorochromes emitting on similar wavelenght as the autofluorescence
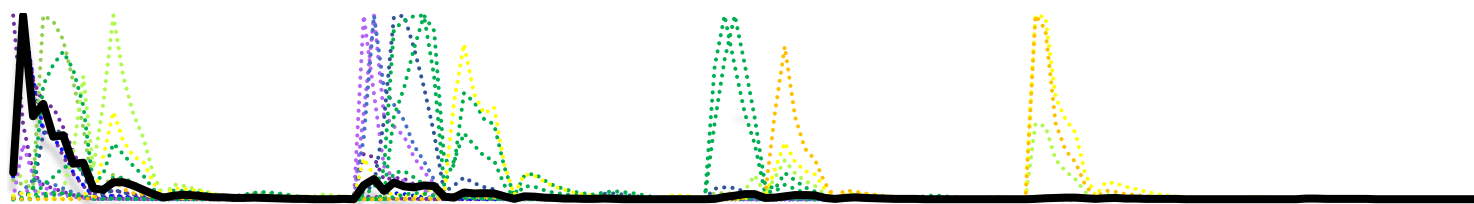
.... BUV395	.... AF350	.... Zombie-UV	.... Live Dead Blue	.... BUV496	.... BUV563
.... BV421	.... eFluor450	.... PacBlue	.... BV480	.... BV510	.... PacOrange
.... BV570	.... FITC	.... GFP	.... PE	——— AF color	

- For AF unmixing you will need an unstained sample treated the same way as your fully stained sample (fixed/non-fixed, activated/non-activated, fresh/thawed…) as **different treatments can change AF significantly**.

1. Use the Autofluorescence Finder

The AF Finder enables you to identify the cells of your interest by creating AF-root gate on the scatter plots; make sure to include only the cells which will be investigated in your panel. For example, if your panel is only about T cells, you do not need to unmix the AF of granulocytes:

Step 1 : On a scatter plot, create a gate on the population for which you wish to determine presence of AF. ⓘ
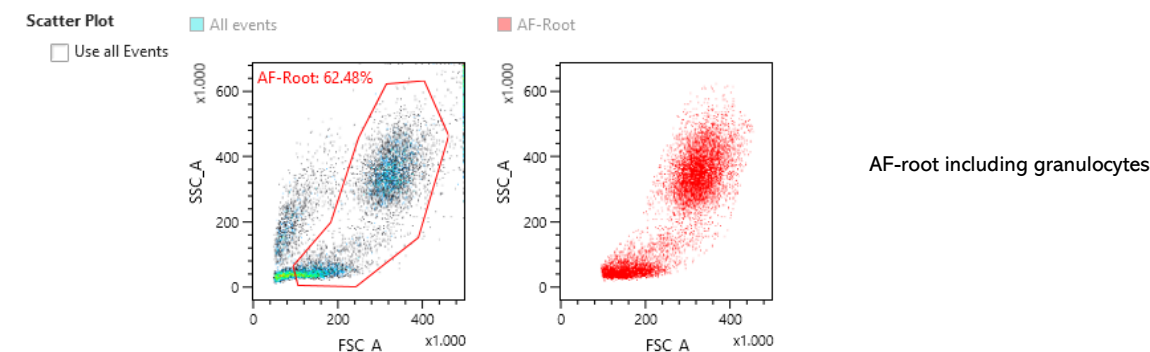

AF-root including granulocytes

In the next step, the AF Finder enables you to investigate the fluorescence detected by each laser's detection deck. Use the Virtual Filter Sliders to choose the part of the spectrum you are looking at (355nm laser on the example):
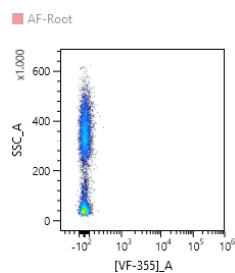
Virtual Filter Slider is set on the detectors catching the far-red part of the spectrum. There is no AF detected there, the signal is low.
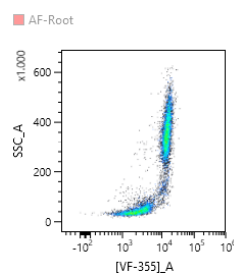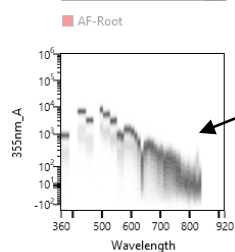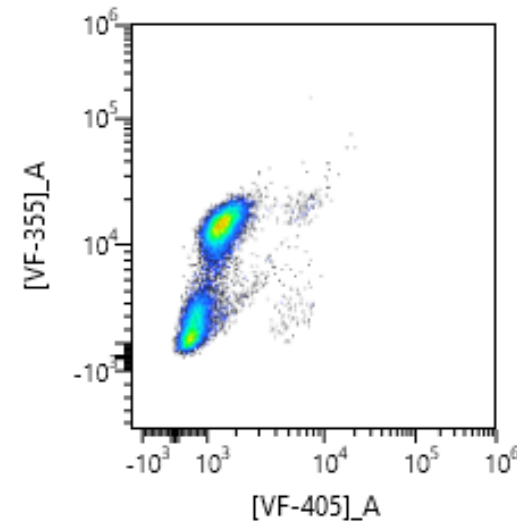

Virtual Filter Slider is set on the detectors catching ~420 – 500nm. In this case, the signal of AF is the brightest there.
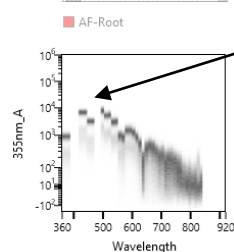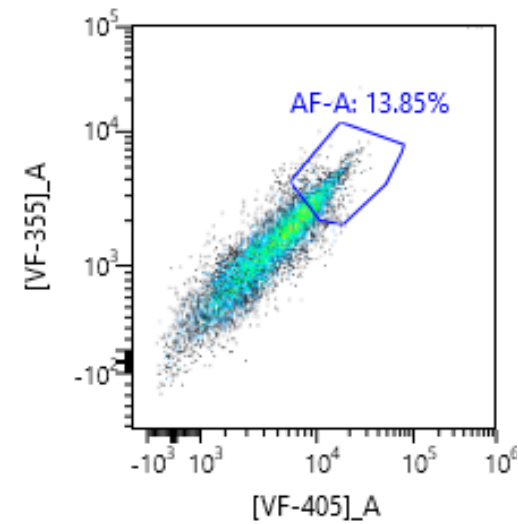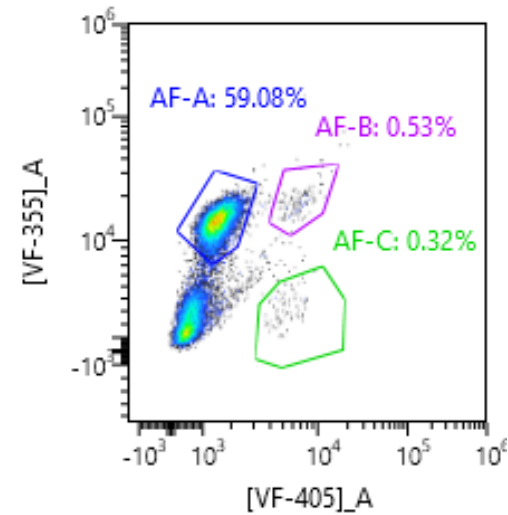
You can visualize the signal against SSC or against the signal detected by other laser's detection decks to get a better separation of AF populations. **Remember to rescale the biexponential settings for an optimal resolution!**


Create gates around the AF populations that you can resolve →

If the AF population looks more like a smear, you can simply gate on the most positive corner → all the cells on the diagonal will have the same color, only of a lower intensity.

The spectral signature of the gated populations will be highlighted on the spectral plots below. Here, you can compare how similar/different they are to each other and decide on whether you need to include them all or not. If the lines only differ in the intensity but otherwise copy each other's patterns, it means they are of the same color, and you should include only the brighter one. **If you are unsure, it is best to keep all the gated AF populations for now – you will eliminate the ones you do not need in the next step.** When you are happy with your selection, you can confirm and continue to the next step
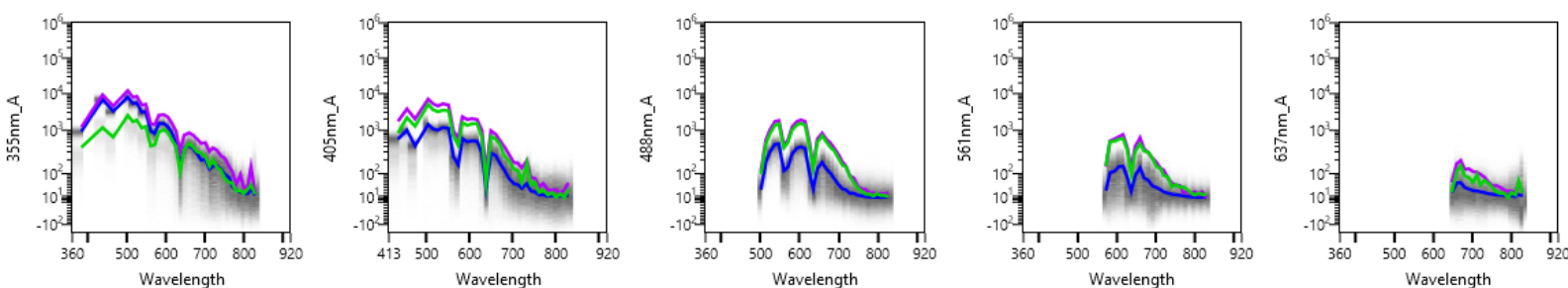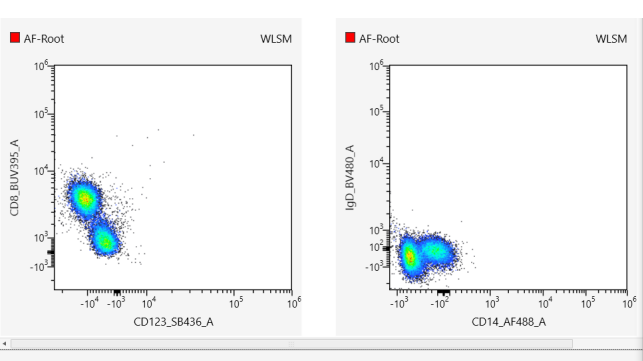
2. Evaluation of the AF unmixing

Back in the analysis part of the software, there are a couple of tools to investigate your AF unmixing and compare different AF populations.

a) Have a look at your unstained control with and without unmixing, including the AF – open some plots with the colors which get the most AF background noise, in this example BUV395, SB436, BV480 and AF488 were selected:
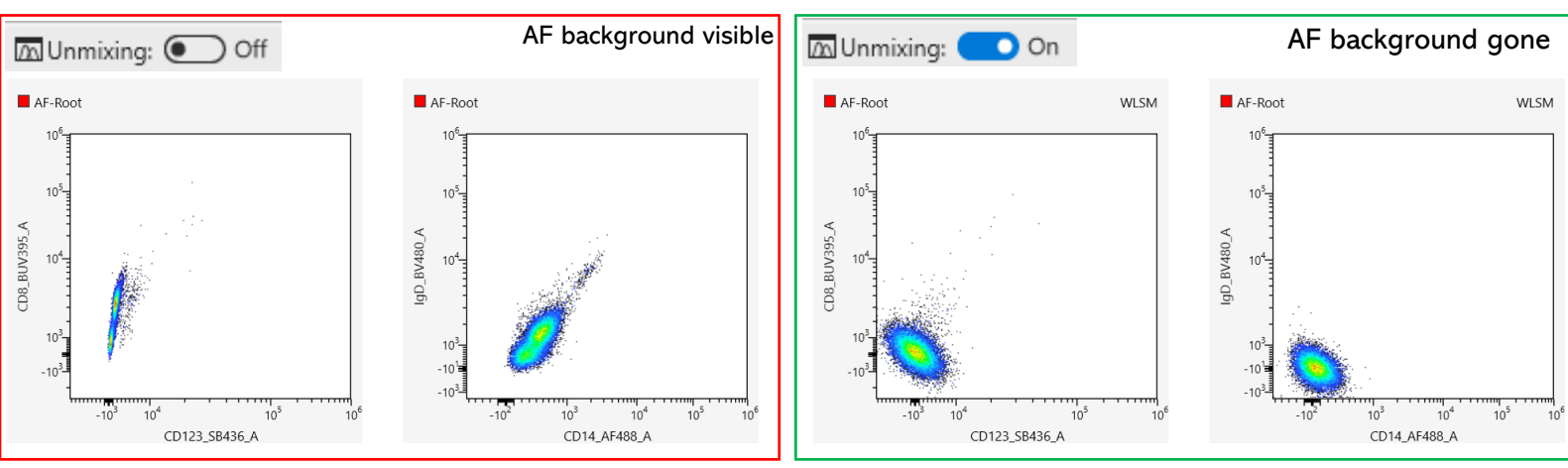
b) The new AF parameters are now listed in the Unmixing Settings. If you created multiple AF populations, here you can exclude/include them from the unmixing to see what it does to your data – to verify if all of them are needed and helpful.
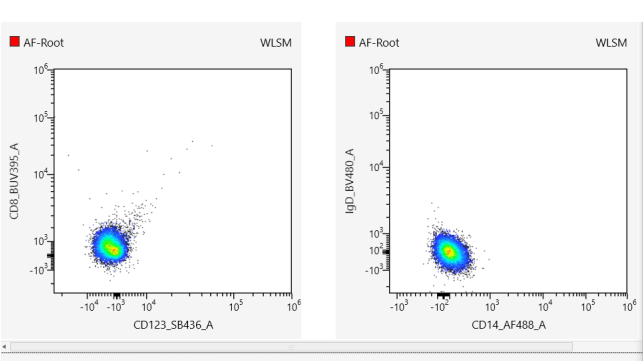
In this example, we have 3 AF populations. We will leave the unmixing ON **and first exclude all of them by unticking, then include the AF colors one by one**

→ AF background is improved

→ AF background is gone

No more improvement, only creates more spread → the 3rd AF parameter is unnecessary and can be removed! (Right click the AF parameter in the Unmixing Settings → Remove Fluorochrome)

3. Spectral Overlays for a deeper understanding

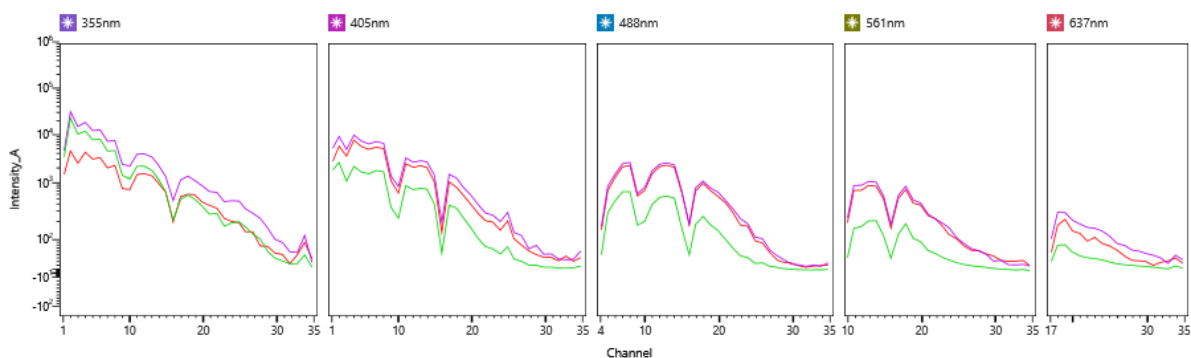If necessary or desired, we can use more advanced tools to compare and investigate AF populations in depth.

Using the same example, we will now overlay the 3 original AF parameters in Spectral Overlay, where you can compare them in more detail than the AF Finder offers:

		Gate	Sample	Sample Group	Index	Plate	Experiment	Events	
☑	▬	AF-A	Well - A01	Sample Group - 1	A01	24 Tube Rack (5mL)	23c CSL	54	
☑	▬	AF-B	Well - A01	Sample Group - 1	A01	24 Tube Rack (5mL)	23c CSL	119	
☑	▬	AF-C	Well - A01	Sample Group - 1	A01	24 Tube Rack (5mL)	23c CSL	13,451	

You can export the Spectral Overlay data into .csv file and further process in Excel. The csv will contain mean per fluorescent channel per each overlayed population:

Experiment	Plate	Sample Group	Sample	Row	Column	Gate	Events	Pulse Type	Data Type	355nm_CH1	355nm_CH2	355nm_CH3	355nm_CH4	355nm_CH5	355nm_CH6
AF	24 Tube Rack (5mL)	Sample Group - 1	Well - A01	A	1	AF-A	54	Area	Mean	1.426.97	4.427.00	2.444.22	4.170.34	2.984.68	3.189.83
AF	24 Tube Rack (5mL)	Sample Group - 1	Well - A01	A	1	AF-B	119	Area	Mean	4.476.96	31.559.38	14.959.00	18.416.17	12.186.29	12.675.11
AF	24 Tube Rack (5mL)	Sample Group - 1	Well - A01	A	1	AF-C	13451	Area	Mean	3.303.21	22.752.55	10.266.18	11.826.91	7.740.73	7.958.95

An example of comparing the AF populations is calculating the correlation coefficient (=CORREL(array1,array2)):

- Correlation = 1 → the variables are in a perfect positive correlation (as variable x increases, variable y increases as well)

- Correlation = 0 → indicates no correlation

- Correlation = -1 → a perfect negative correlation

AF-A/AF-B 0.75

AF-A/AF-C 0.53

AF-B/AF-C 0.96

4. Checking the Unmixing

The unmixing quality control using the **Unmixing Viewer and Adjuster** should be performed **after the correct AF parameters are added to the unmixing.** The AF unmixing will correct the minor unmixing issues which the AF background would introduce. Without adding them first, you might end up overcorrecting something which would be fixed by the AF unmixing.