Spectral Reference Adjuster and Autofluorescence Finder

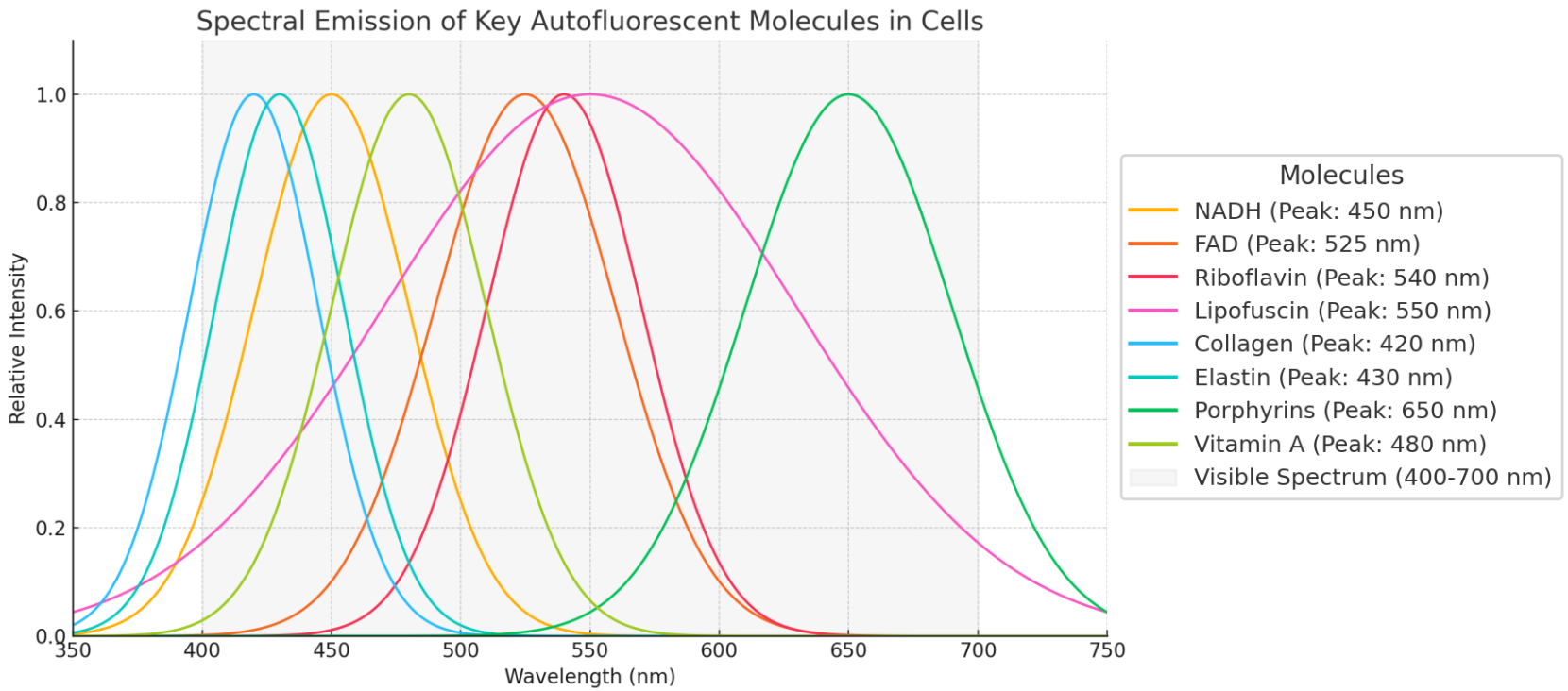
Sony Spectral Technology

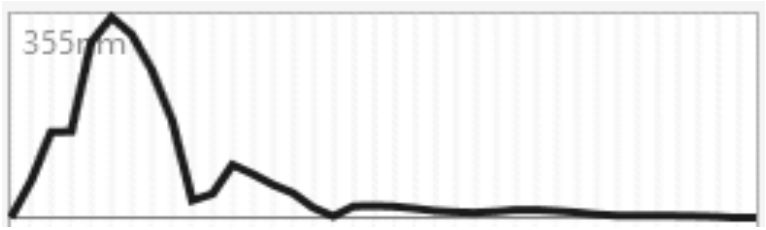
Deeper Insights to Empower Discovery Research

Agenda

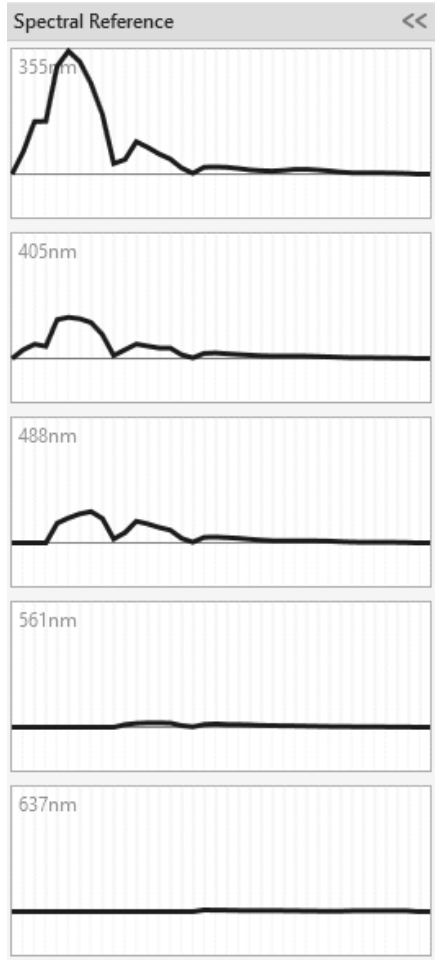
- Autofluorescence Workflows
- Spectral Reference Adjuster
- Titration and Validation
- Biological Controls

What is Autofluorescence (AF)?





Spectral reference for unstained lymphocytes

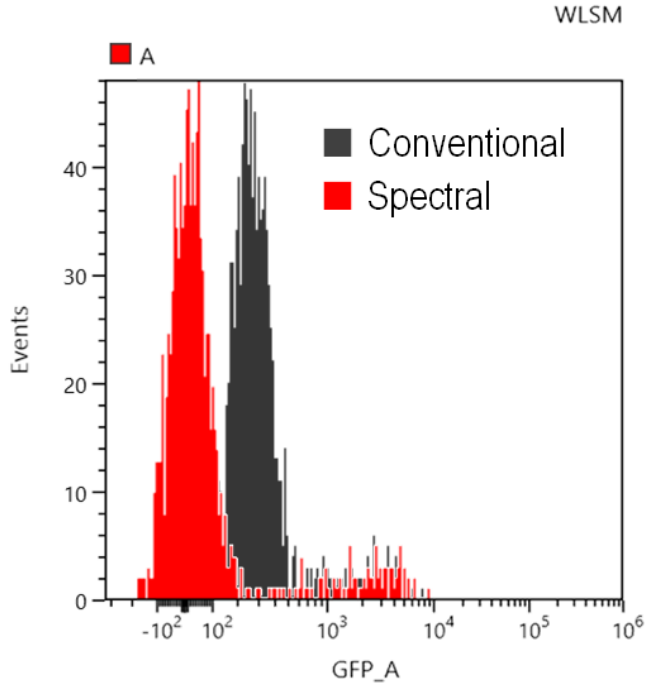


Autofluorescence is Affected by Different Factors

- Varies in spectral emission across different cell types
- Changes with treatments (e.g., fixation, permeabilization)
- Maintains similar shape but differs in intensity
- Affected by biological conditions (e.g., senescence, activation, differentiation)

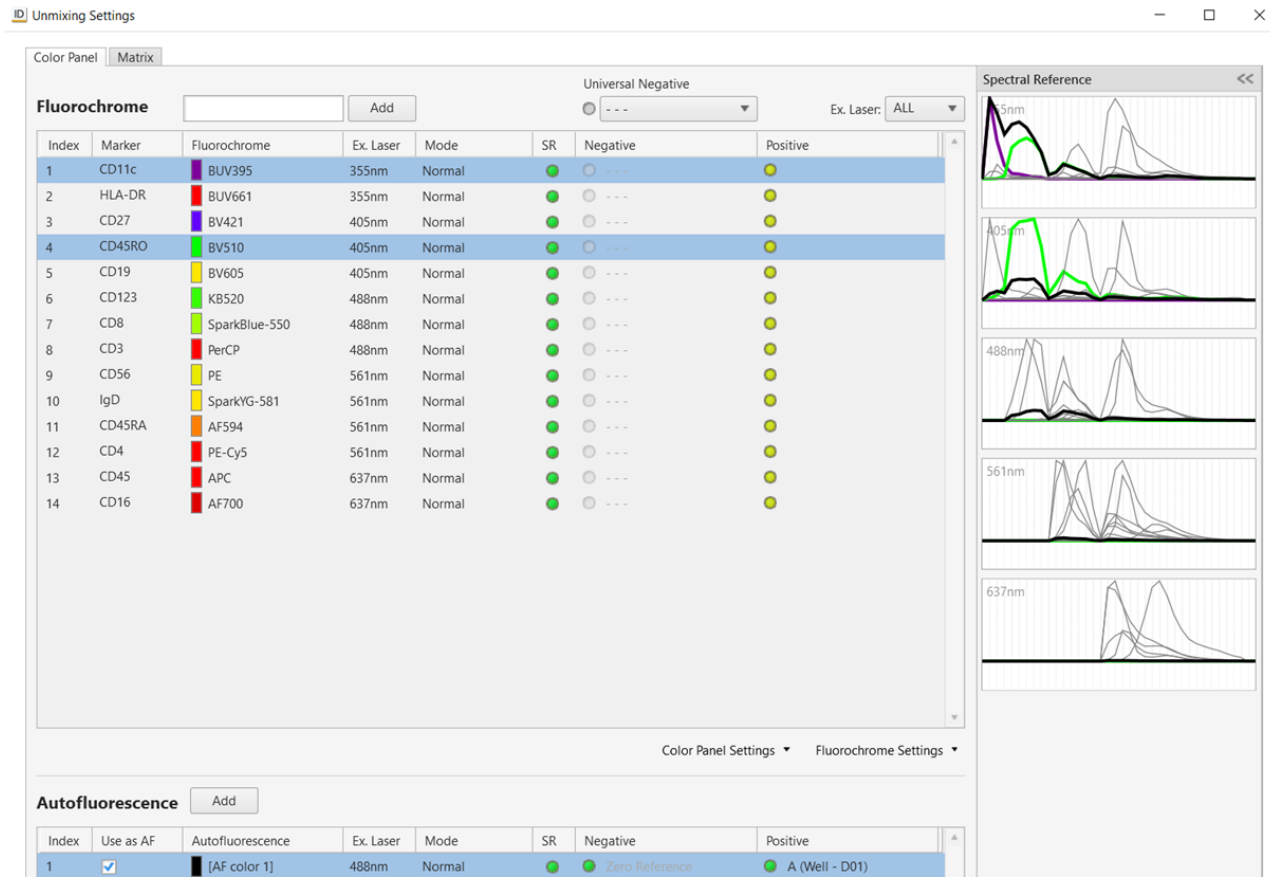
Unmixing Autofluorescence (AF) for Resolving Dim Signals

- Autofluorescence is always included with each fluorochrome intensity measurement. The higher the cellular AF, the greater the error.
- Spectral unmixing calculates the AF intensity cell by cell, removing just the right amount of signal from the other colors.
- Autofluorescence can be treated as another color in the un-mixing math. Cellular AF is then attributed to the AF parameter and not to other colors.

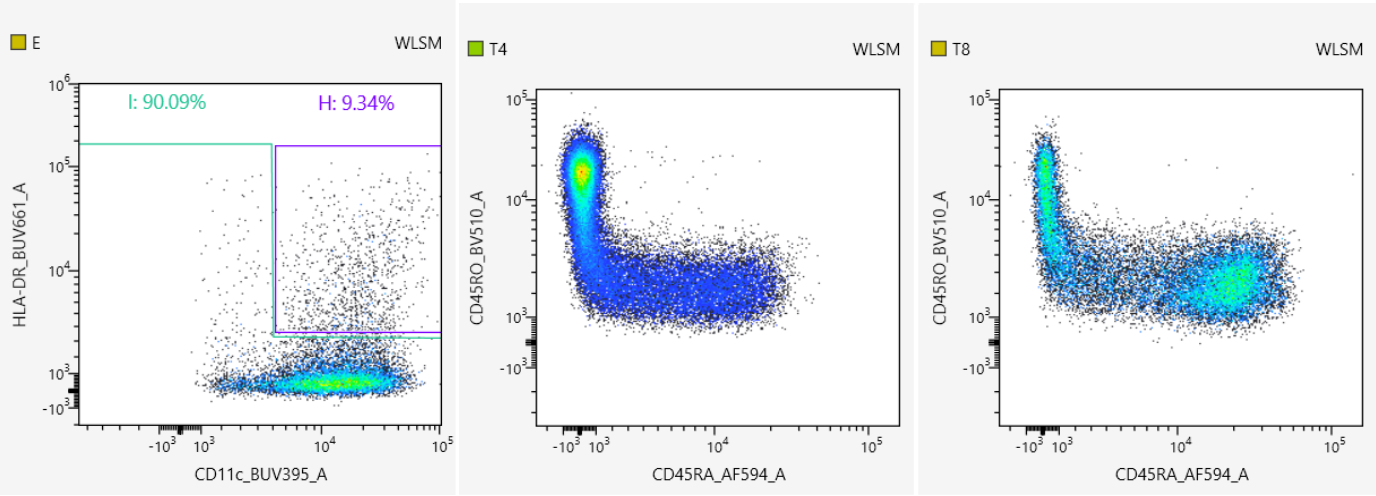


Autofluorescence Considerations

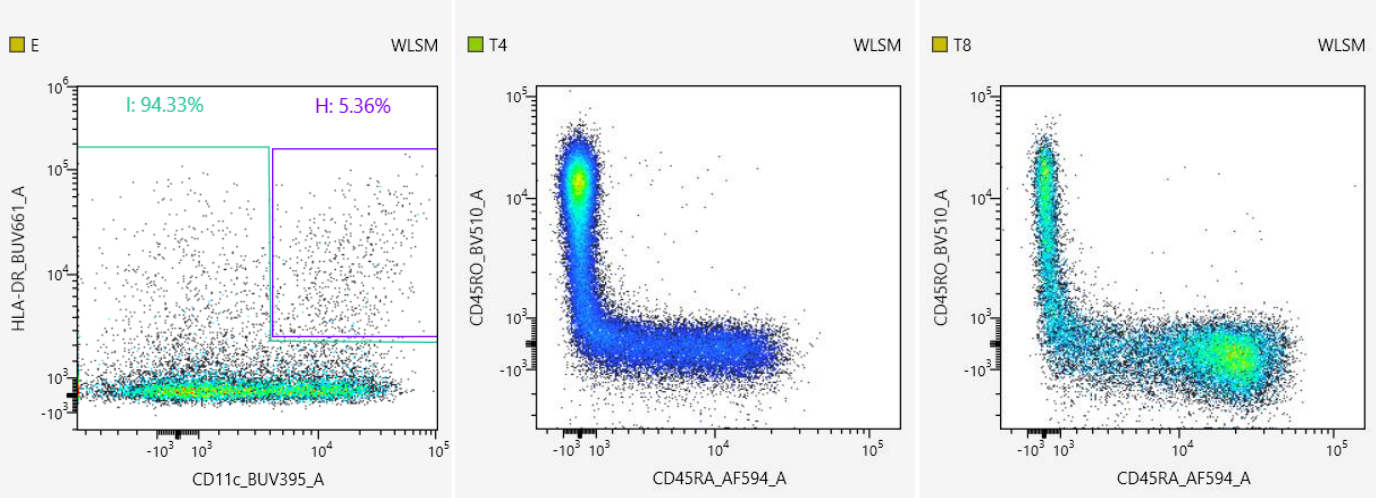
- The Autofluorescence of your sample can be a useful thing to know before building your panel
- Will spread the negative population in colors that it is similar to, reducing the resolution
- For PBMCs, AF is usually most similar to BUV496 and BV510
- For tissue digests (tumor, liver, brain, skin, lung, and gut) the multiple AF populations may look different and may be similar to unexpected fluorochromes



Autofluorescence Effects on Resolution

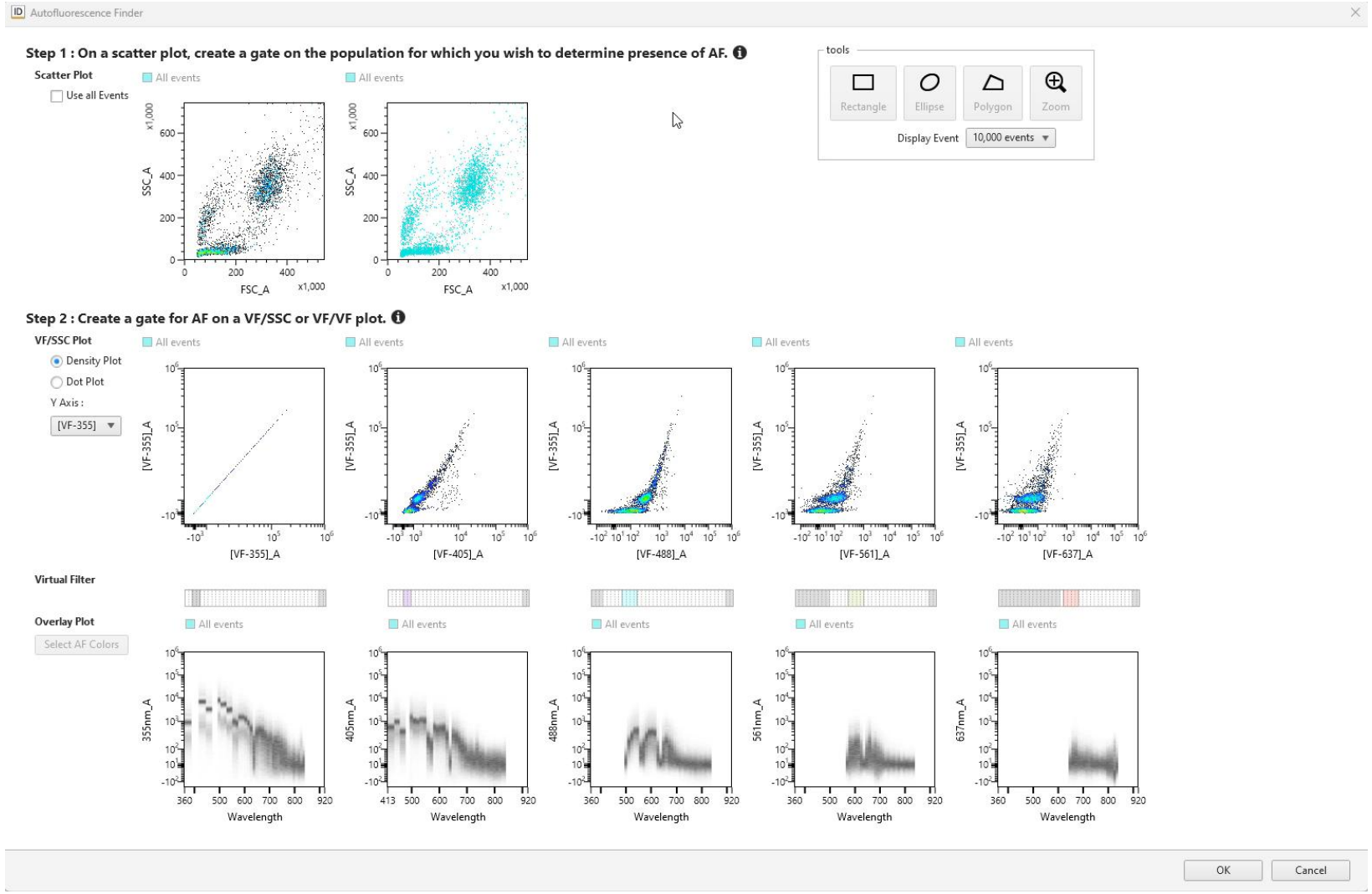


Autofluorescence NOT included in Unmixing



Autofluorescence IS included in Unmixing

Autofluorescence Finder



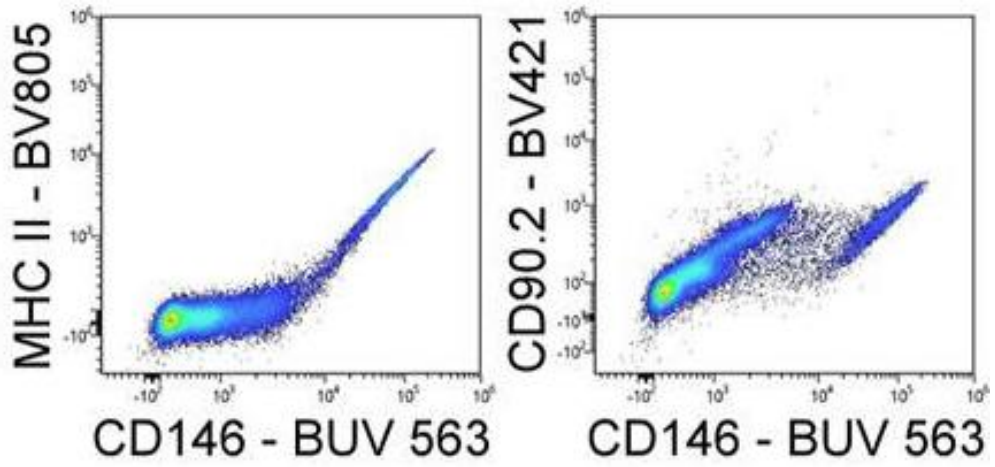
Multiple Autofluorescence Effects on Resolution

Front Bioeng Biotechnol. 2022 Mar 15:10:827987. doi: 10.3389/fbioe.2022.827987. eCollection 2022.

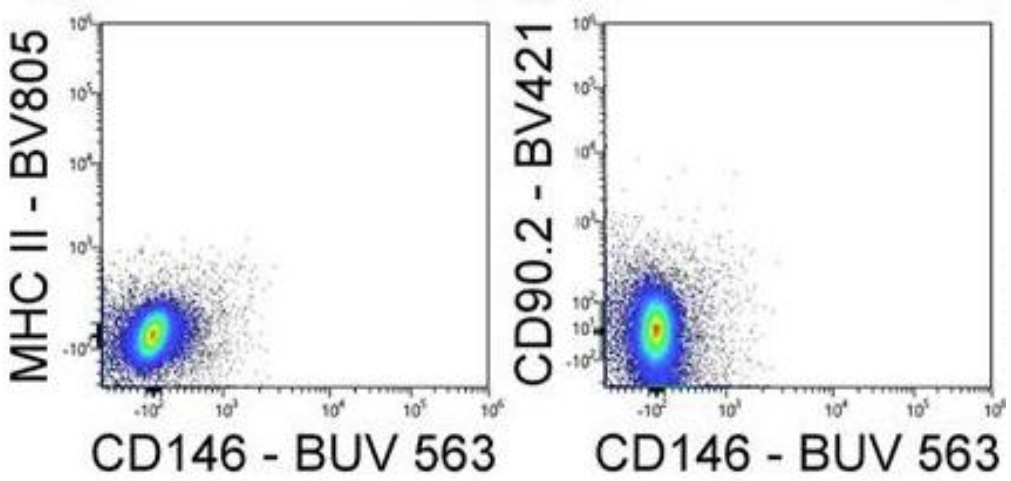
Using the Autofluorescence Finder on the Sony ID7000™ Spectral Cell Analyzer to Identify and Unmix Multiple Highly Autofluorescent Murine Lung Populations

Nicholas Wanner [1], Jerry Barnhart [2], Nicholas Apostolakis [1], Violetta Zlojutro [1], Kewal Asosingh [1 3]

Unstained cells showing AF



Unstained cells unmixed with AF



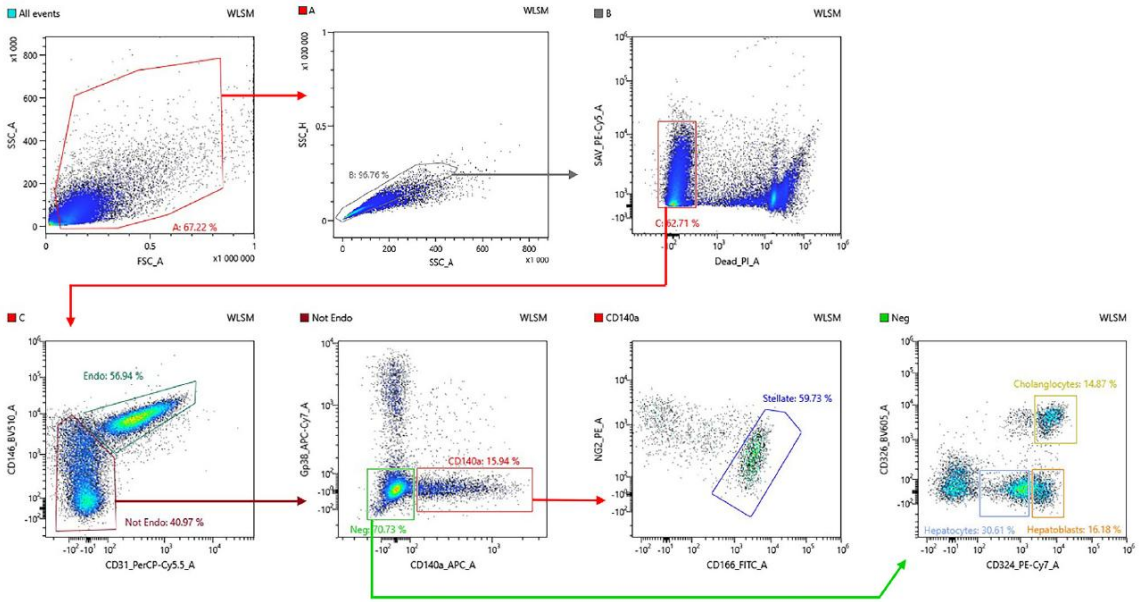
Autofluorescence can be used as a “marker”

- AF can differentiate type of cells and cell state

Cytometry A. 2022 Nov;101(11):960-969. doi: 10.1002/cyto.a.24567. Epub 2022 May 14.

Identification of fetal liver stroma in spectral cytometry using the parameter autofluorescence

Márcia Mesquita Peixoto [1 2 3 4 5 6], Francisca Soares-da-Silva [1 2 3], Sandrine Schmutz [7], Marie-Pierre Mailhe [1 2 3], Sophie Novault [7], Ana Cumano [1 2 3], Cedric Ait-Mansour [8]

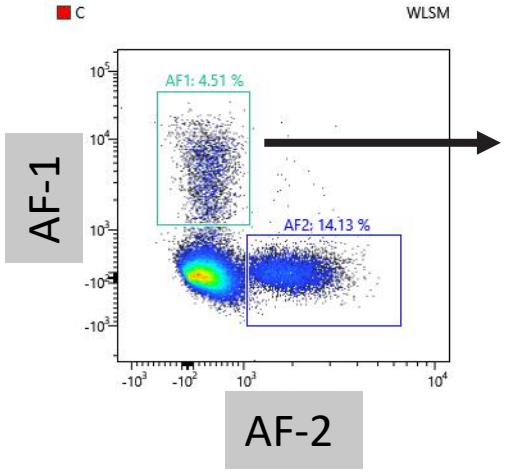


- Fetal liver is a highly autofluorescent tissue where hematopoiesis and hepatocyte differentiation occur.
- ID7000 was used to analyze non-hematopoietic stromal cells in FL (E14.5, E18.5, P3, P9).
- Two autofluorescence signals (AF-1, AF-2) were identified:
- Using autofluorescence as a parameter enables precise identification of FL stromal cells, overcoming limitations of conventional FCM.

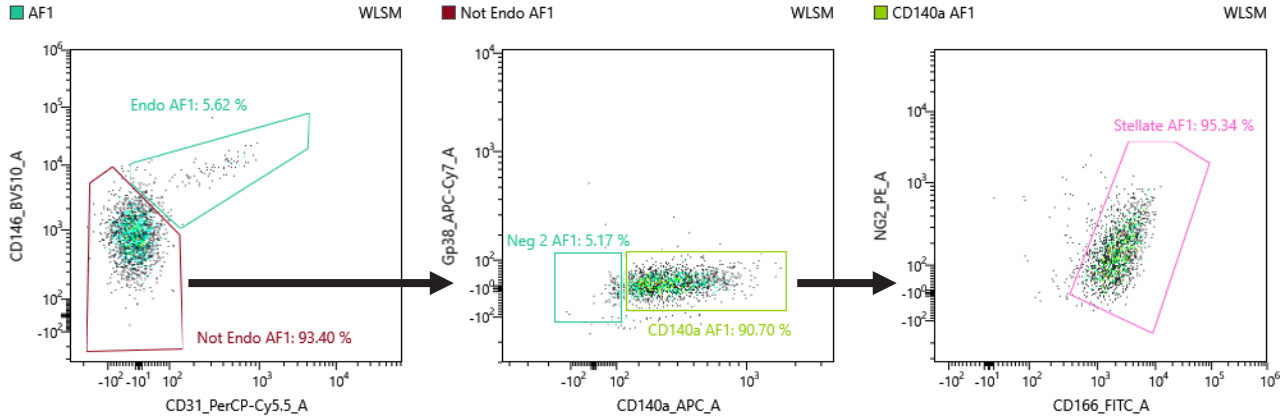
Autofluorescence can be used as a “marker”

- AF1 defines stellate cells (CD140a+, CD166+, Gp38–)

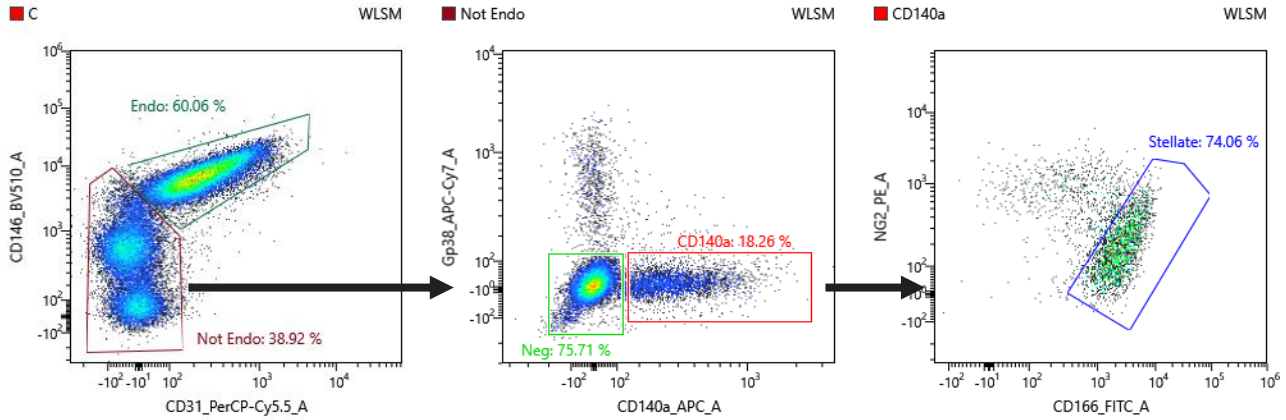
E14.5



Gated in AF1



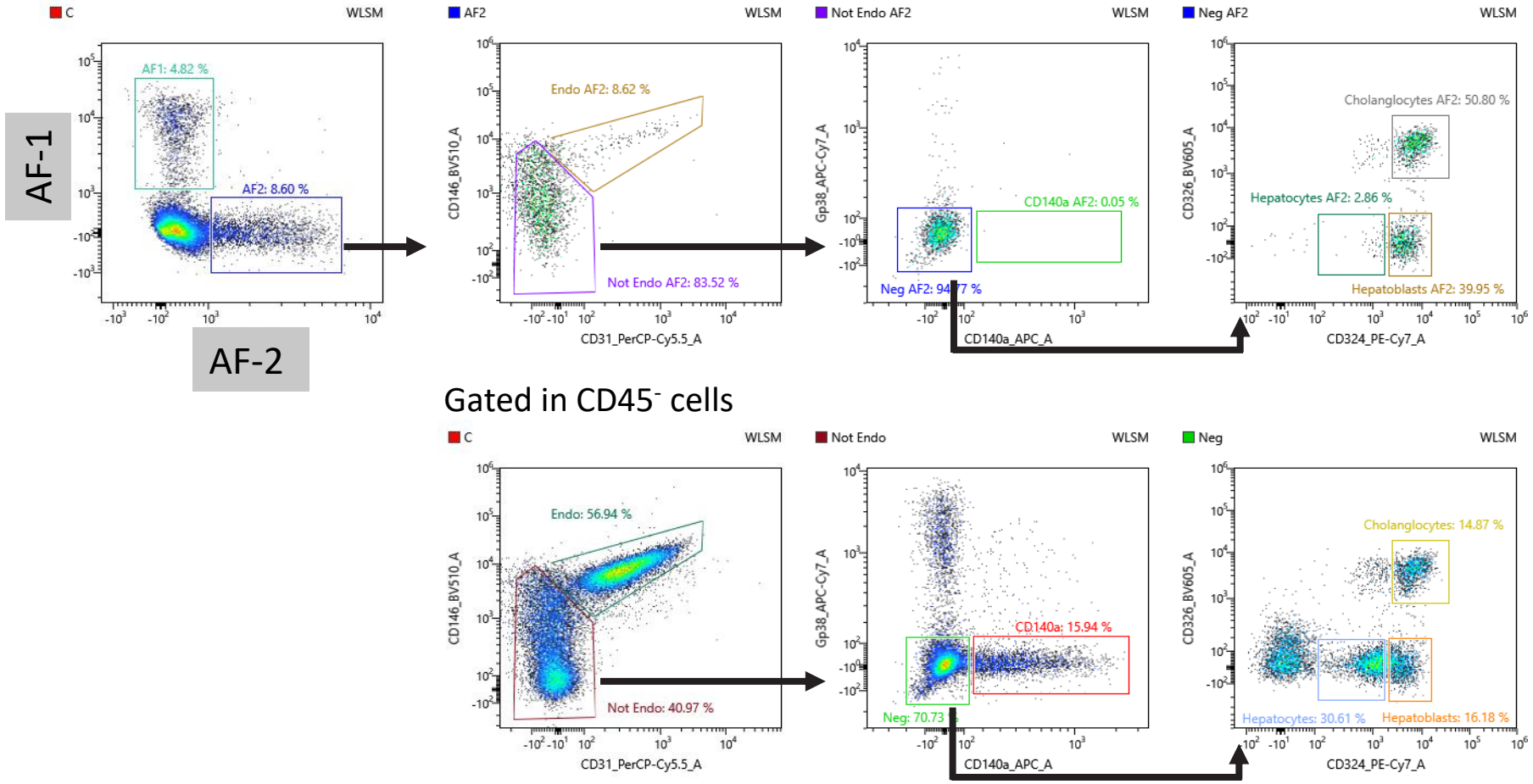
Gated in CD45^{-} cells



Autofluorescence can be used as a “marker”

- AF2 defines hepatoblasts: CD324^{high}, CD326– / cholangiocytes: CD324^{high}, CD326+).

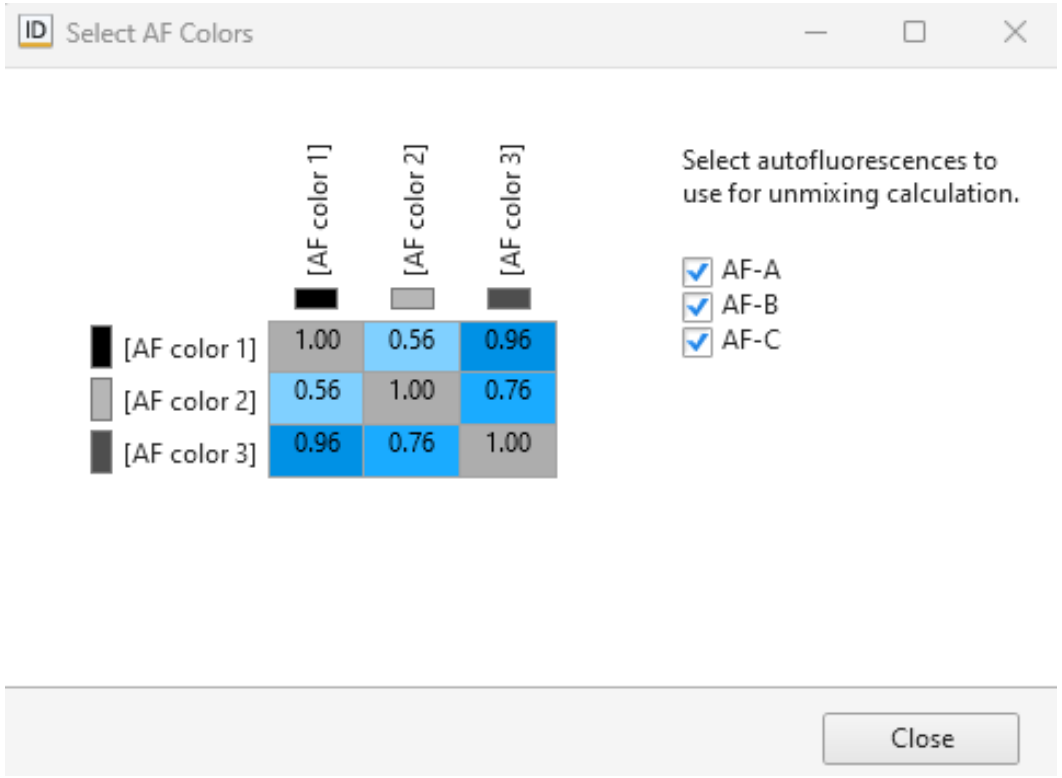
E18.5



Autofluorescence Management : Key Findings and Conclusions

- AF1 serves as a marker for stellate cell differentiation.
- AF2 helps distinguish hepatoblasts from hepatocytes at late stages.
- AF1 and AF2 spectral profiles remain stable post-cell sorting, confirming their cell-specific nature.
- These findings deepen our understanding of the fetal liver stromal niche and demonstrate the potential of autofluorescence as a tool for improving flow cytometry phenotyping in highly autofluorescent tissues.

New Software Feature! Similarity Matrix



Unique Software Feature - Unmixing Viewer and Spectral Reference Adjuster

Unmixing Viewer

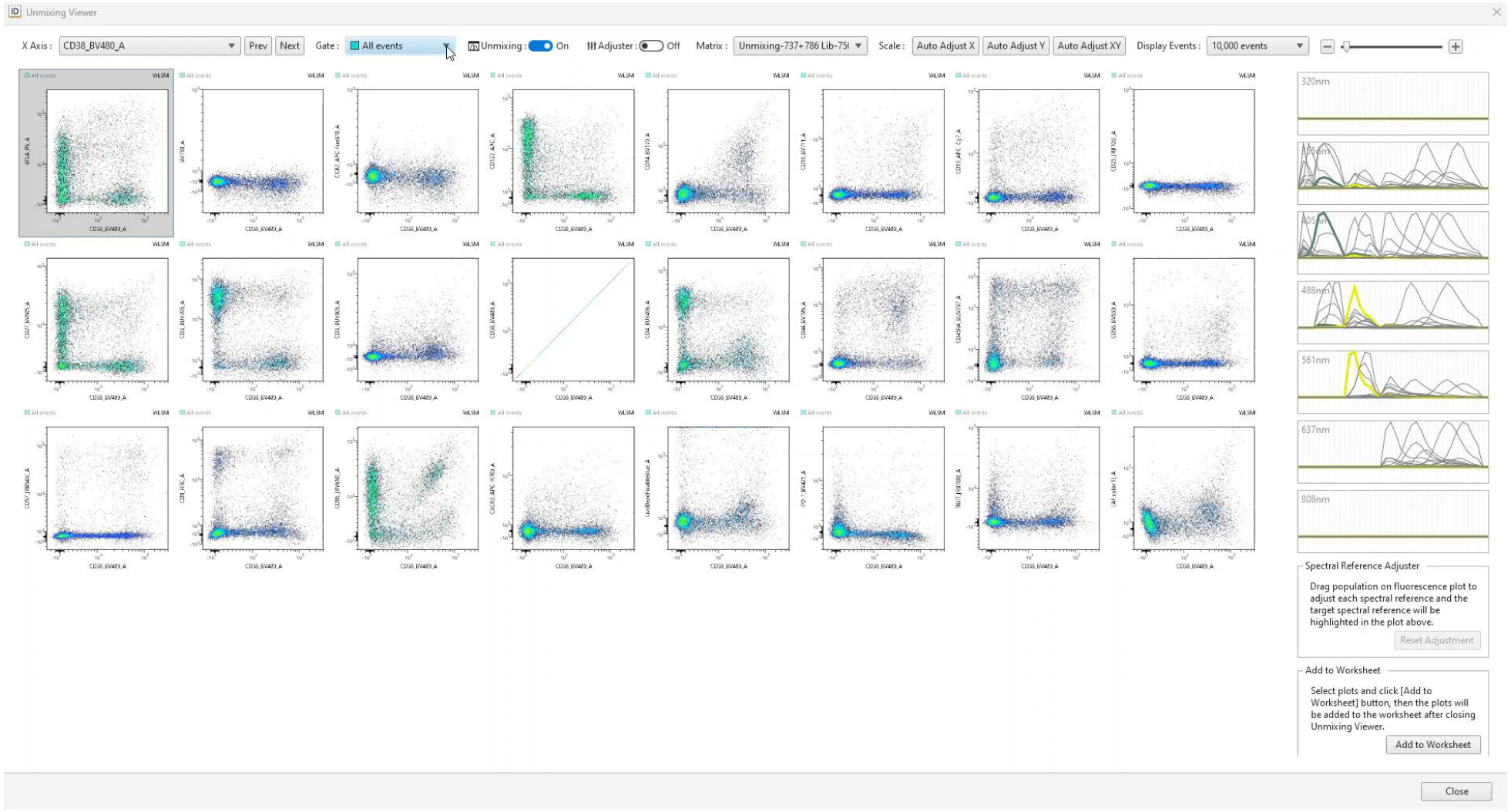
- Tool to verify and troubleshoot unmixed data
- Displays one-vs-all (1 × N) plots for any gated population
- Useful for identifying unexpected contributions from other fluorochromes
- Auto-scaling facilitates visualization and saves time

Spectral Reference Adjuster

- Used to adjust the spectral references when single stained controls don’t match the real sample
- Adjustments impact the **input model**, not the output data

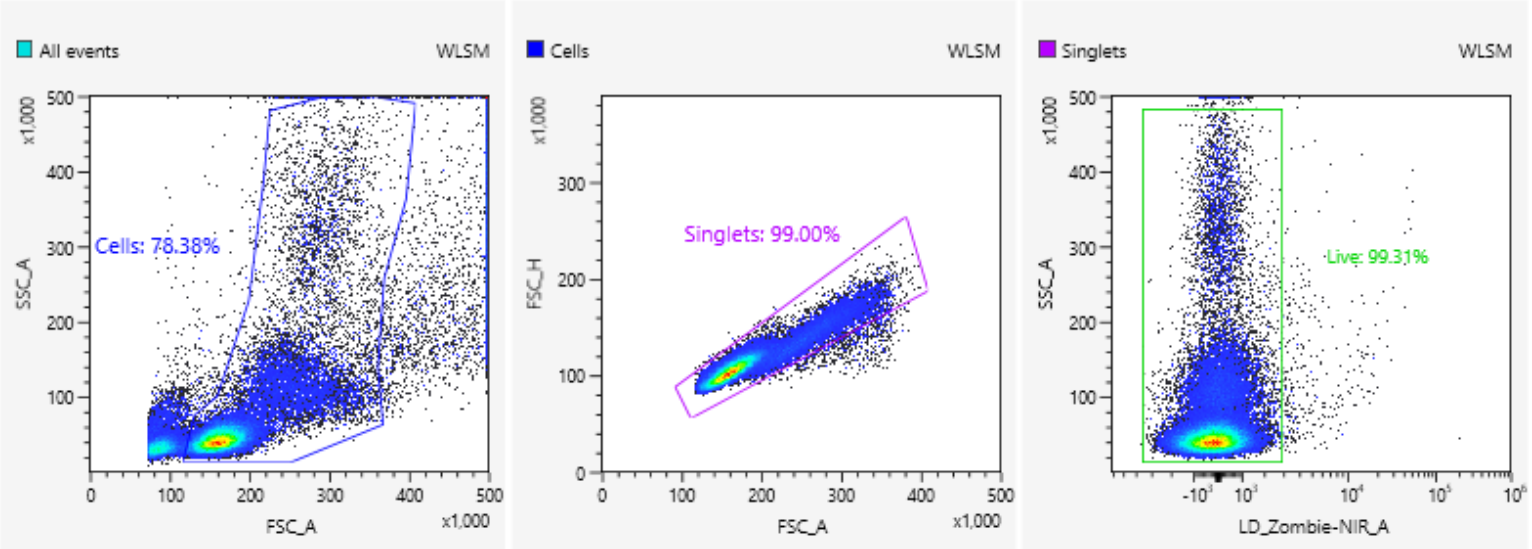
THIS IS NOT APPLYING COMPENSATION ON TOP OF UNMIXING. It re-runs the unmixing using an updated reference model.

Unmixing Viewer and Spectral Reference Adjuster

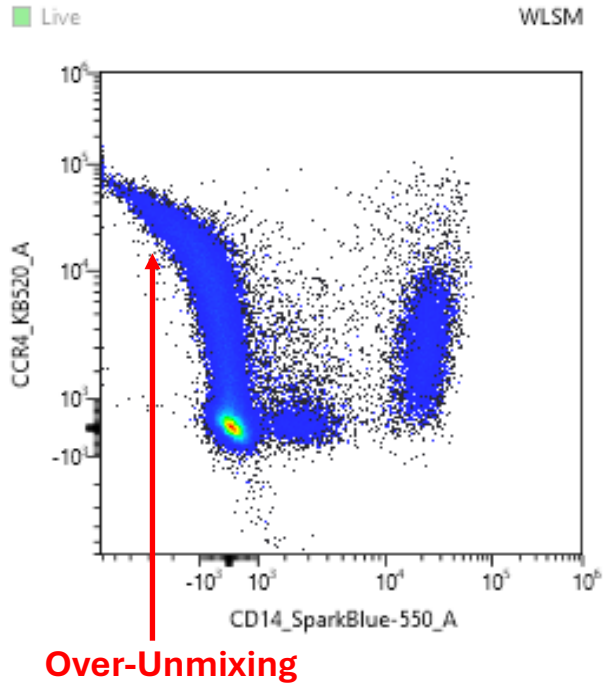


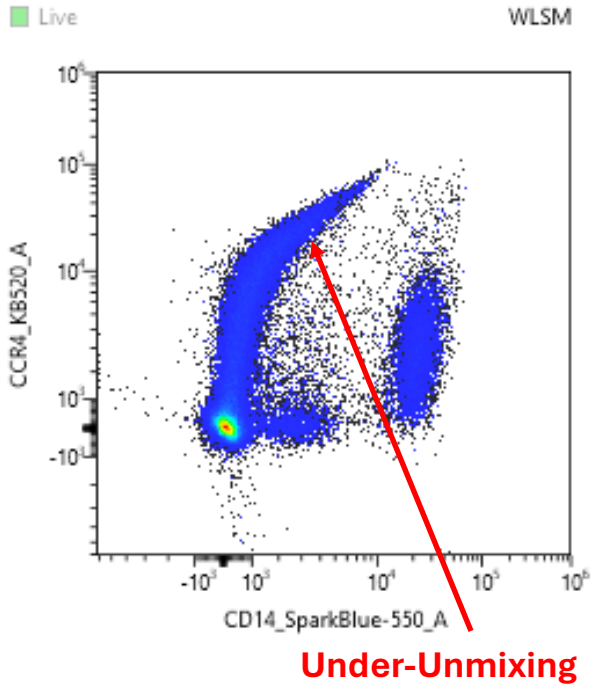
Spectral Reference Adjuster and Unmixing Viewer Workflow

1. All Spectral References, including Autofluorescence(s), should be calculated and unmixing turned on before checking for unmixing inaccuracies
2. Draw Scatter, Singlet, and Live gates before opening Unmixing Viewer
3. Start by viewing events only from the Singlet gate.



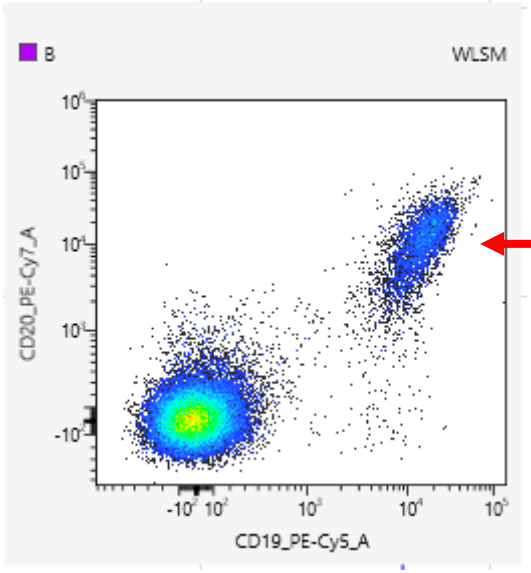
Over-Unmixing and Under-Unmixing Identification





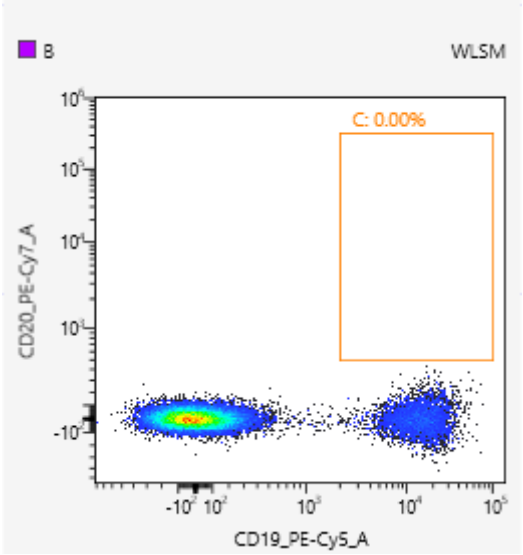
FMO Controls

- Full/Fluorescence Minus One
  - Used as a gating and unmixing control
  - Controls for effects of data spread
- FM2/FM3 controls
  - Streamlined approach for larger panels
  - Make sure that fluorochromes excluded do not overlap



Under-Unmixing or Coexpression?

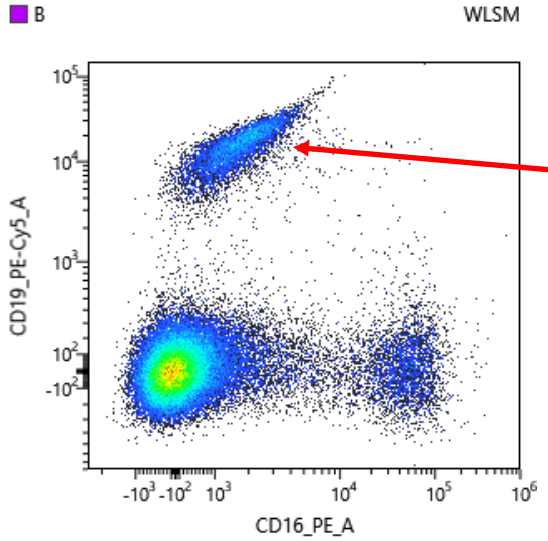
Where to Place the Gate?



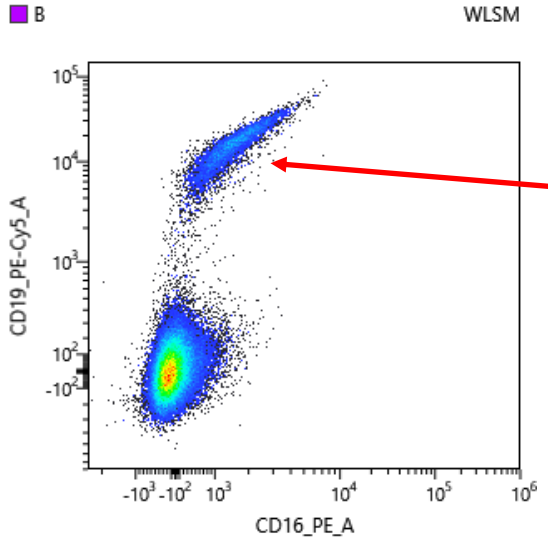
CD20 FMO Control shows accurate unmixing –> Coexpression

Location of single positive population on CD20 FMO gives most accurate gate placement for double positive population

FMO Control Pop Quiz!!!



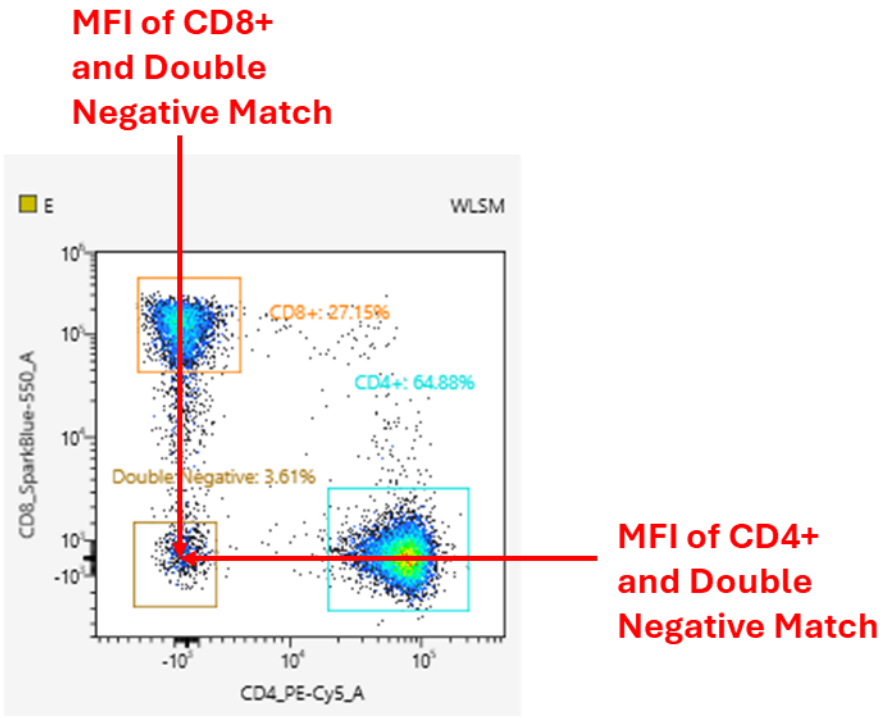
Under-Unmixing or Coexpression?



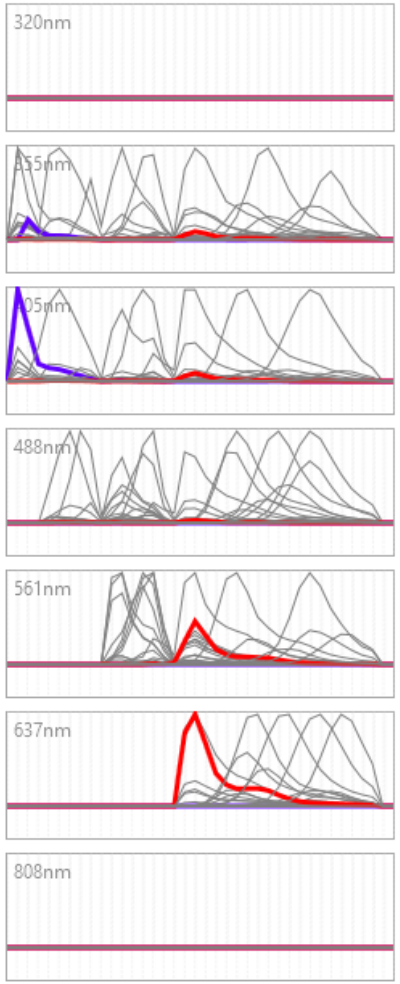
Under-Unmixing!

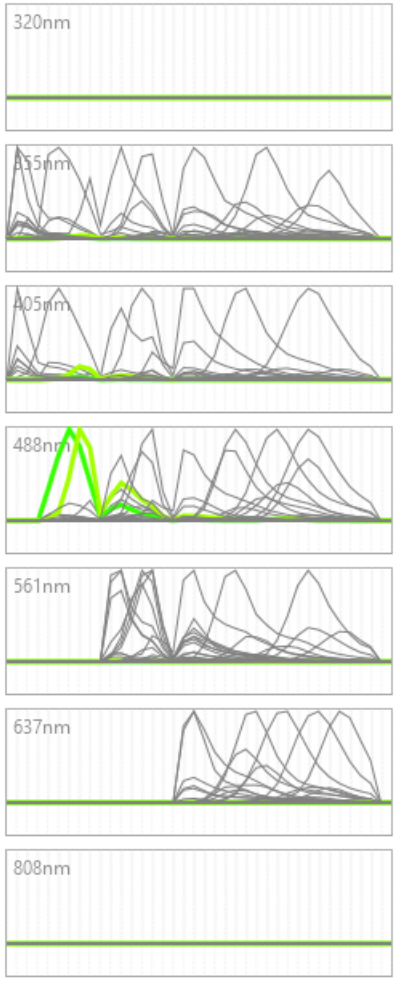
The single positive population is bent over towards the middle of the plot in the FMO

Rule #1: MFI of Single Positive Population in a Properly Unmixed Sample will match that of the Double Negative Population

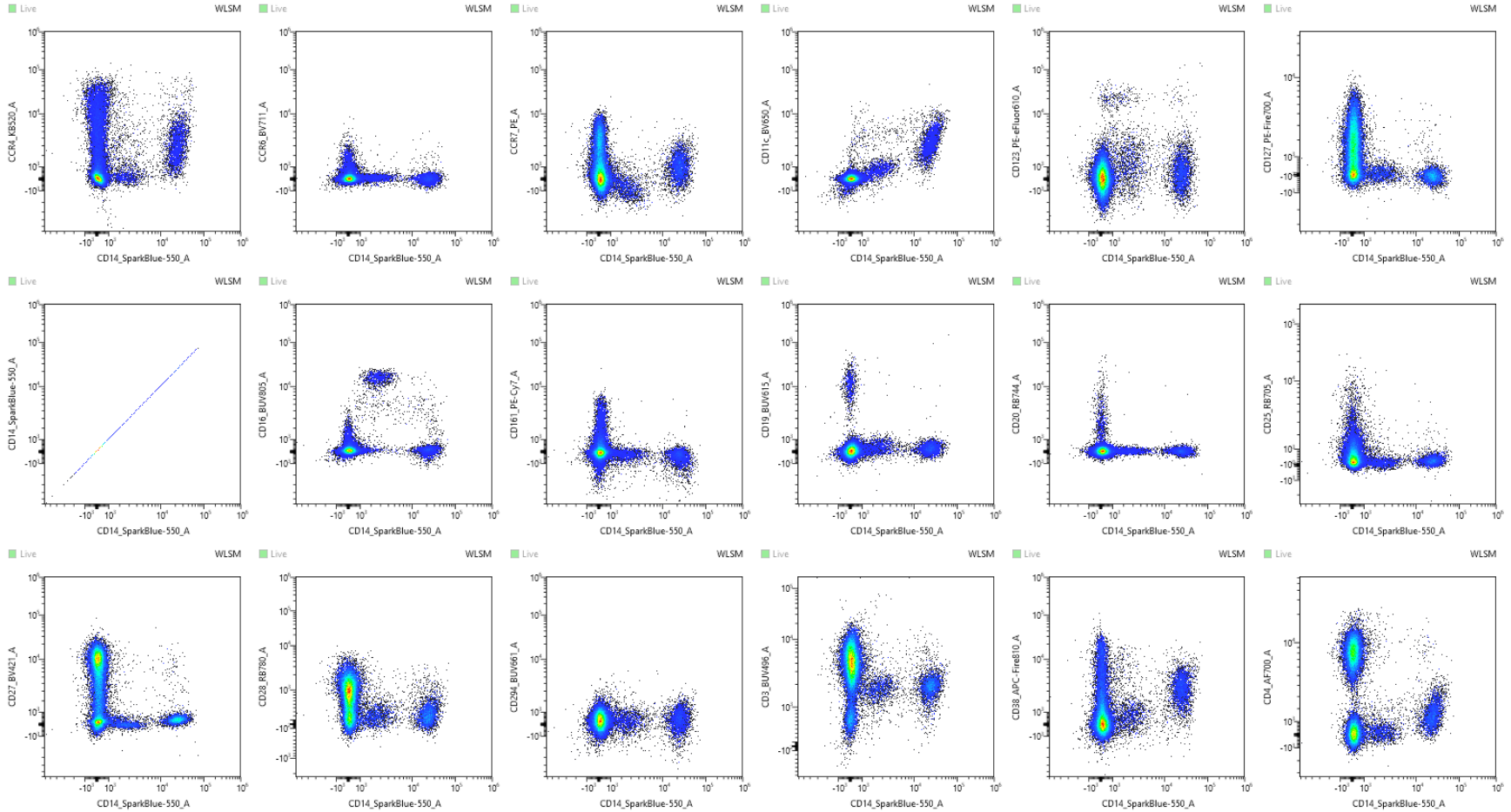


Rule #2: Only adjust Spectral References on plots where the two Spectral References overlap with each other

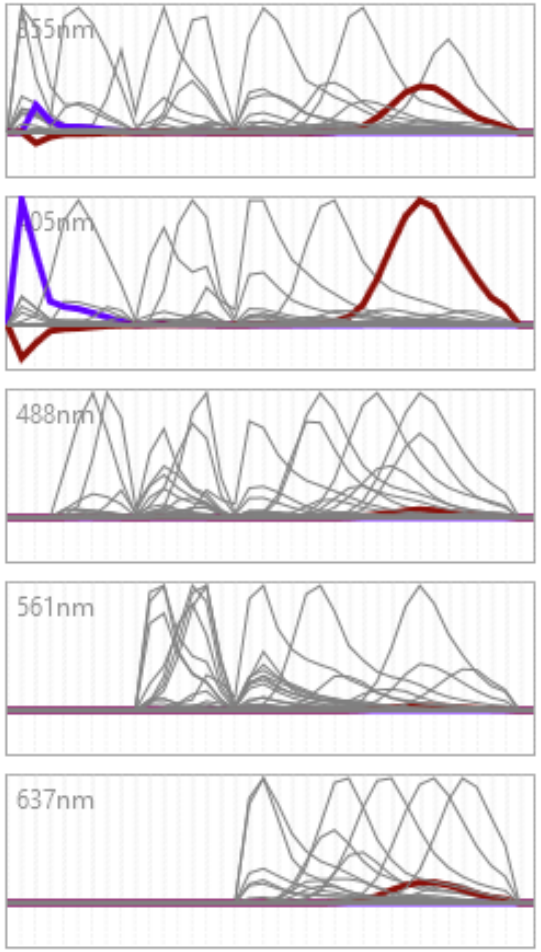




Rule #3: Perform Spectral References Adjustments on Single Positive Populations that completely show the unmixing inaccuracy

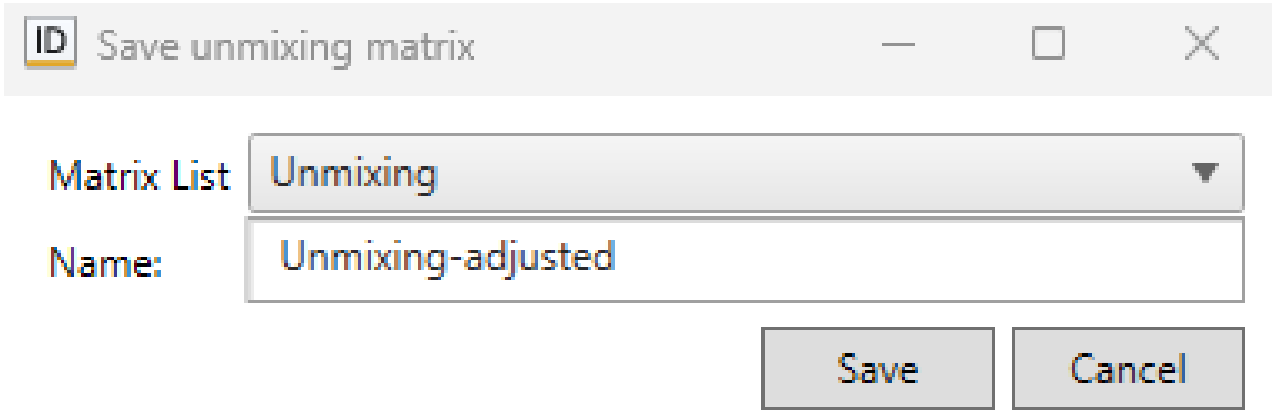


Rule #4: Do not over-adjust to make any of your Spectral References go below the baseline



- Use Ctrl+Z to go back to last adjustment. Can be used multiple times
- Use Reset Adjustment to go back to last saved position
- Spectral References going below baseline is usually a result of mis-identification of the correct plot to adjust on
- **Rerunning controls is always a good option!!!**

Rule #5: Save your Spectral Reference Adjustments as a different Unmixing Matrix



Spectral Reference Adjuster and Unmixing Viewer Workflow

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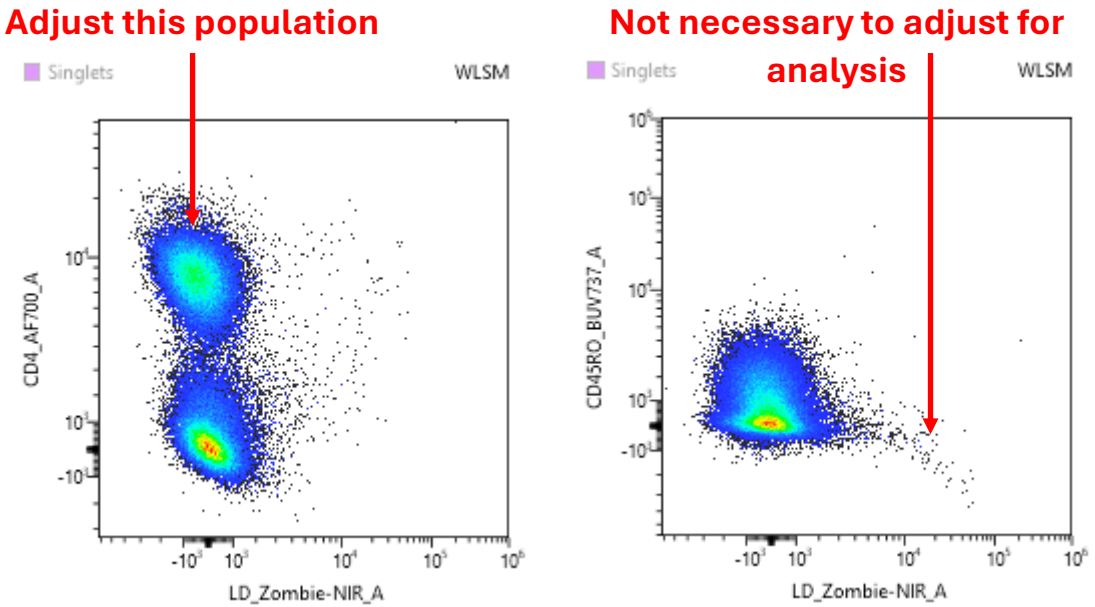
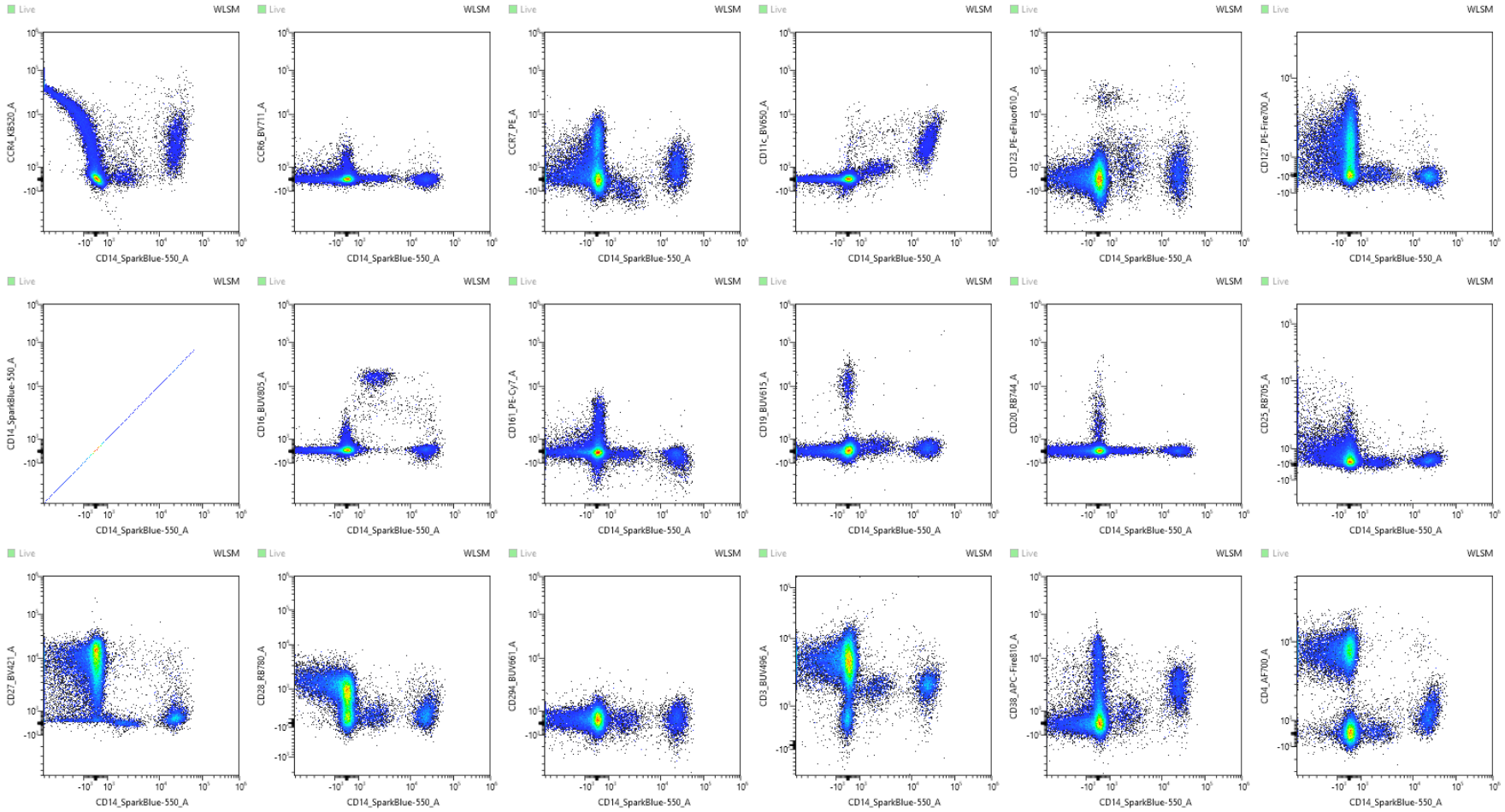
2. Draw Scatter, Singlet, and Live gates before opening Unmixing Viewer
3. Start by viewing events only from the Singlet gate.
4. **Display Live/Dead on all x axes and adjust what is Live and Dead.**



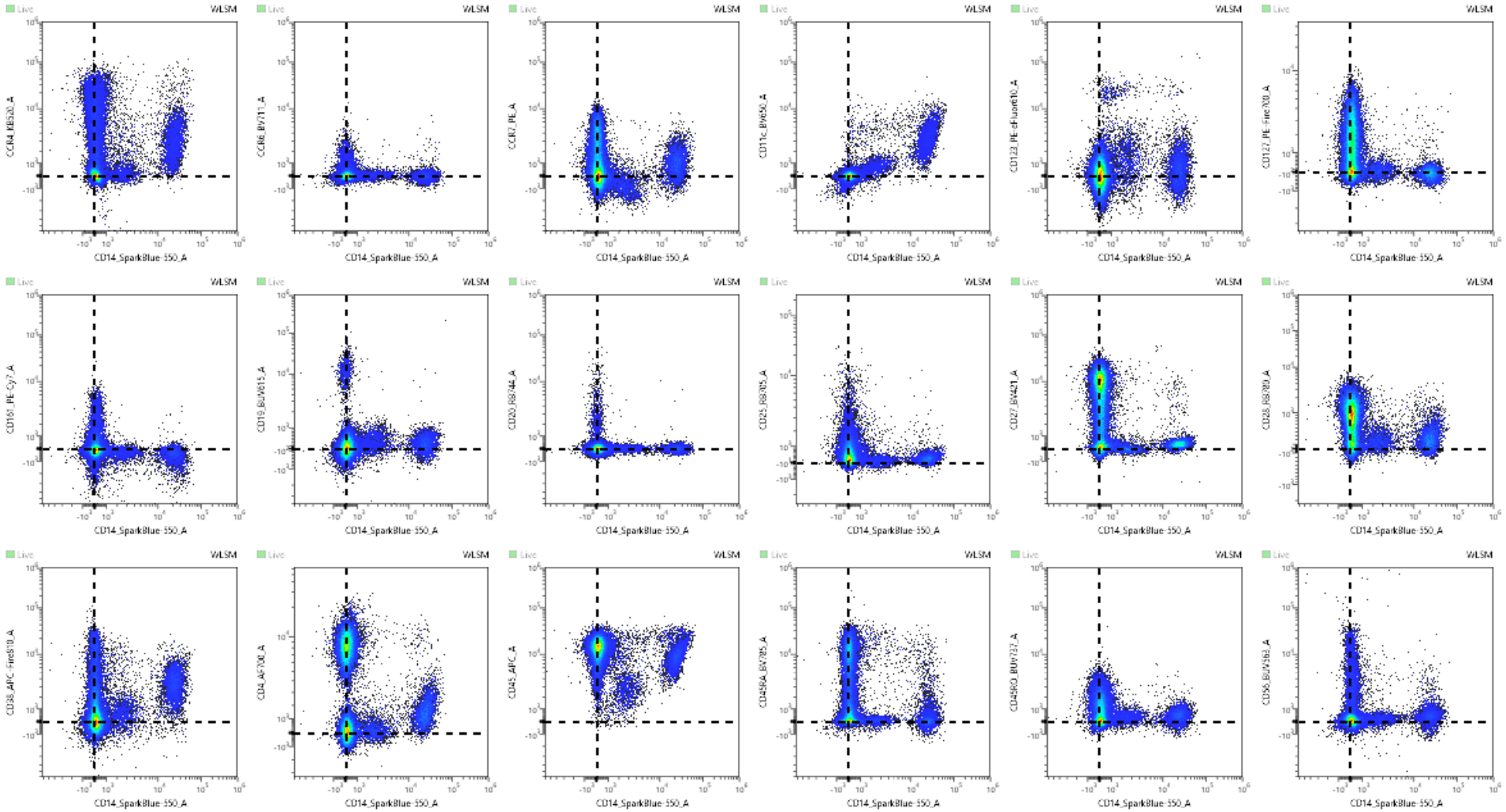
Spectral Reference Adjuster and Unmixing Viewer Workflow

1. All Spectral References, including Autofluorescence(s), should be calculated and unmixing turned on before checking for unmixing inaccuracies
2. Draw Scatter, Singlet, and Live gates before opening Unmixing Viewer
3. Start by viewing events only from the Singlet gate.
4. Display Live/Dead on all x axes and adjust what is Live and Dead.
5. Change display population to Live gate.
6. Cycle through one parameter at a time to look for any unmixing inaccuracies. *Make sure to follow all the rules for unbiased adjustments!!!*
7. Once adjustments or rerunning controls (and subsequent rechecking of unmixing) is complete, perform analysis in ID software or export fcs files.

Tip: Identifying Over-Unmixing by looking for “Negative Data”

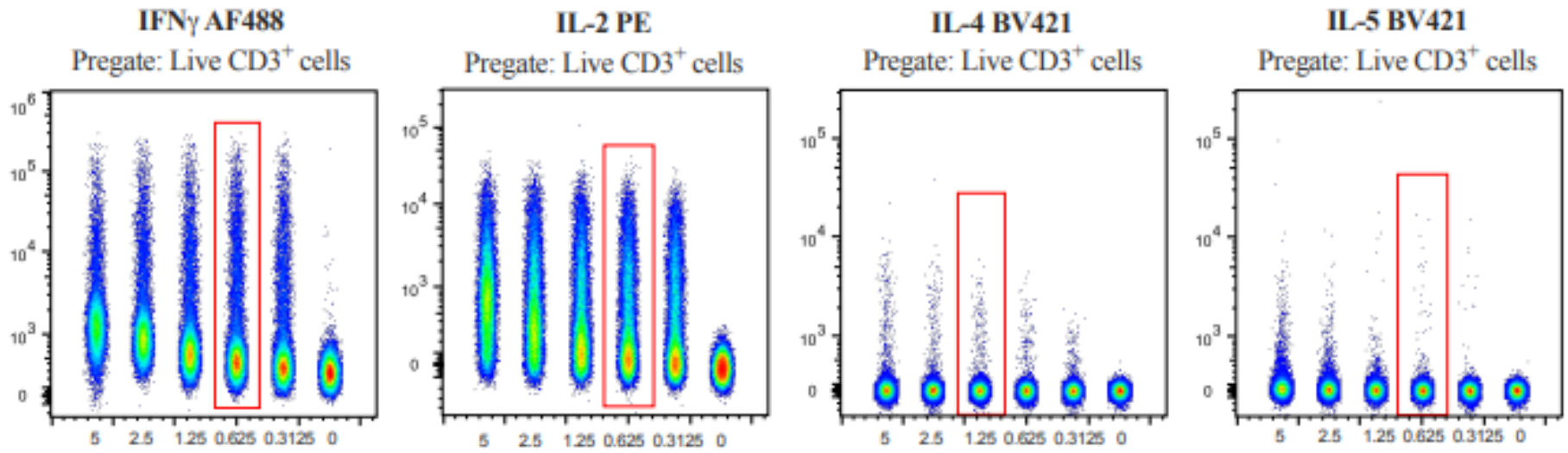


New Software Feature! Grid Lines to help with Rule #1



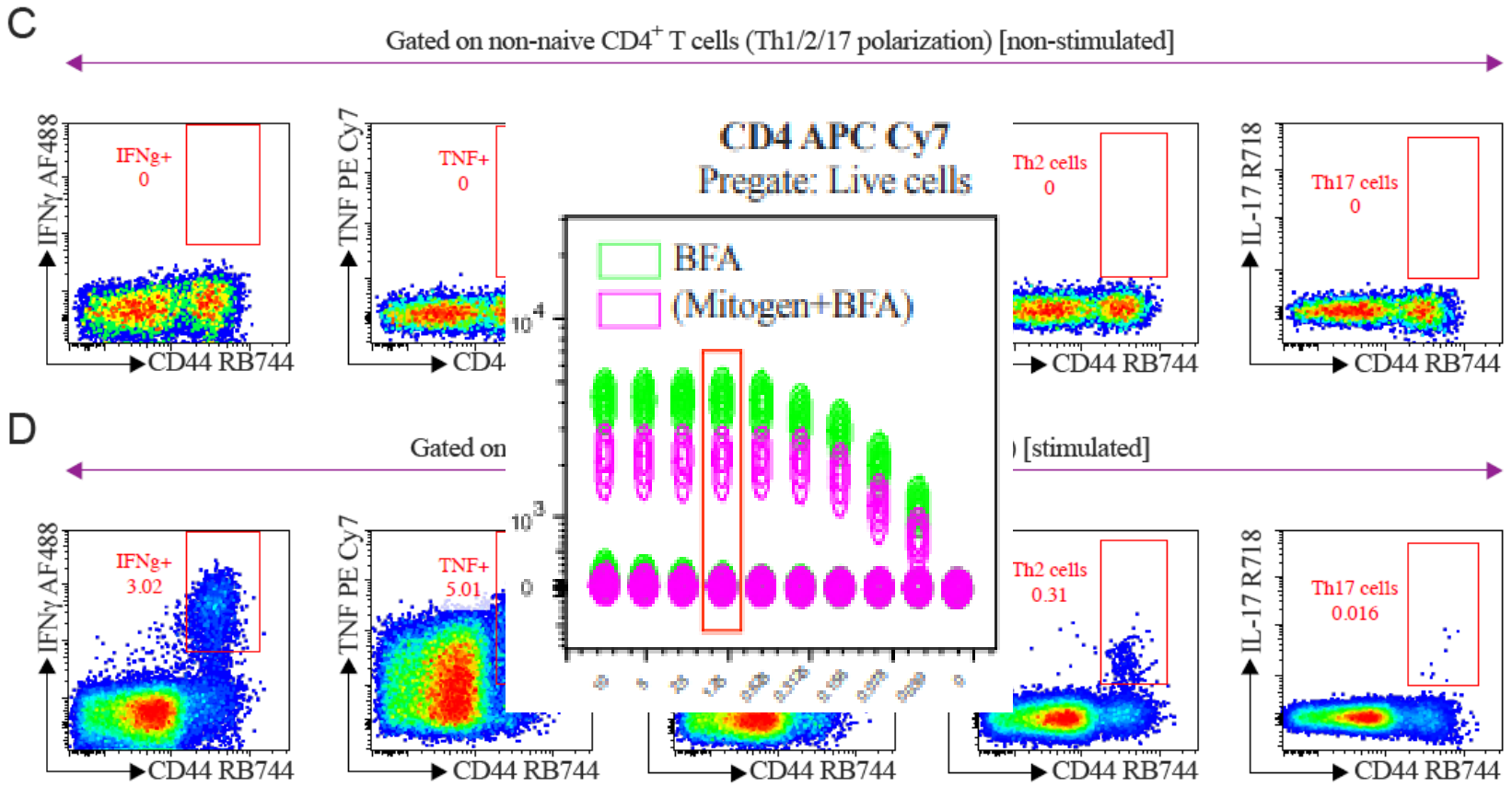
Titration

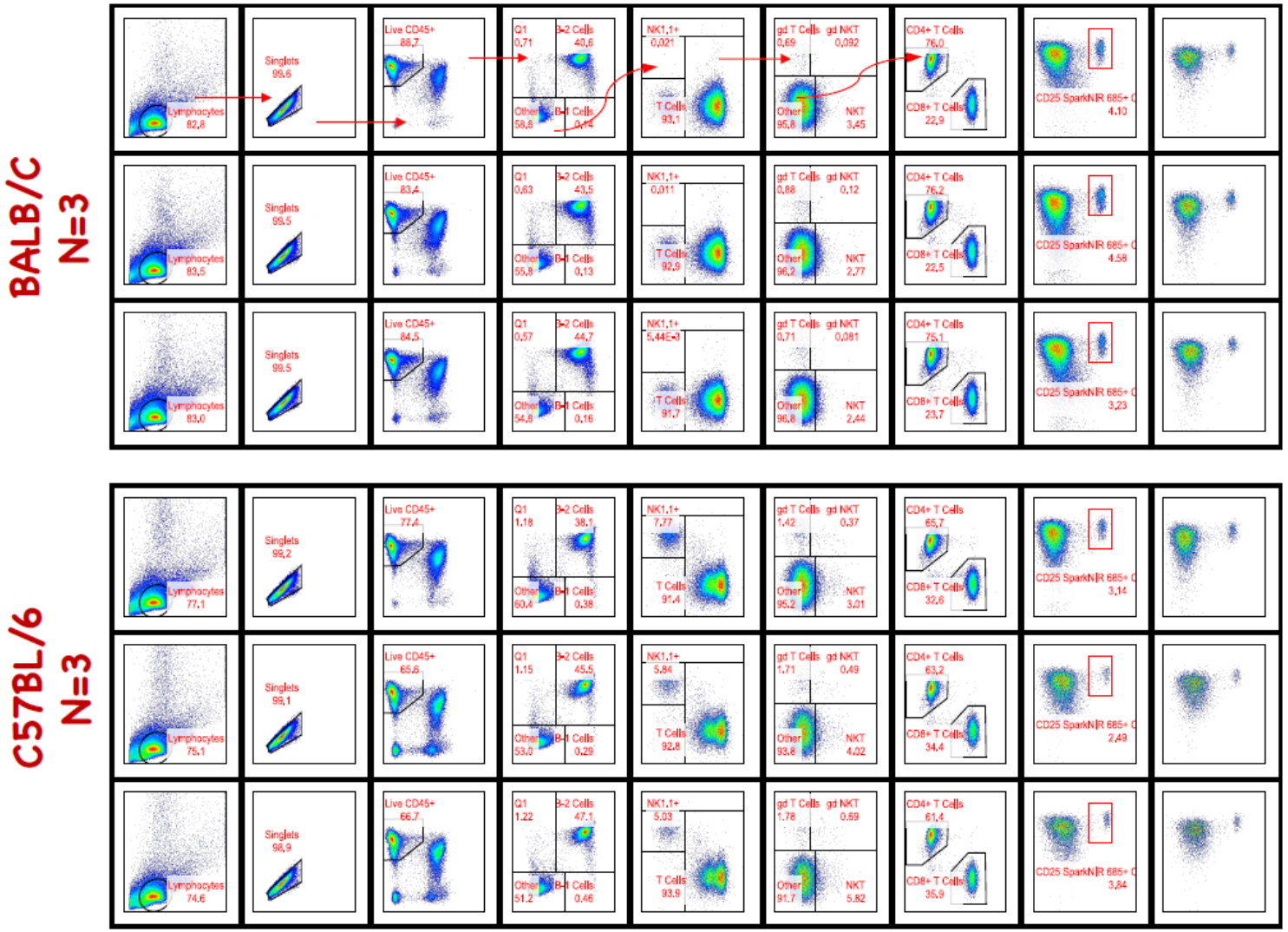
- Always titrate new antibodies
  - Titrations are sample and protocol specific
  - Most of the time, titrations are transferable between instruments
  - Combo titrations are possible but do not use overlapping fluorochromes



Titration and Validation Considerations

- **Driving expression for certain markers (e.g. activation markers)**
  - **CD3/CD28 beads, CytoStim, PMA-Ionomycin, Mitogen, LPS (old-school), co-culture with APCs, etc.**
  - **Brefeldin and Monensin are used to block the export of cytokines**
  - **Used for titration and validation/positive controls**
  - **Downregulation of surface markers – esp. CD3 and CD4**



Validation and Biological Controls

- Validation is key to building confidence in the data
  - Test on a variety of the samples that will be used during the study
  - Patient samples can have a lot of variability in expression levels
- Biological Controls build confidence in your conclusions