

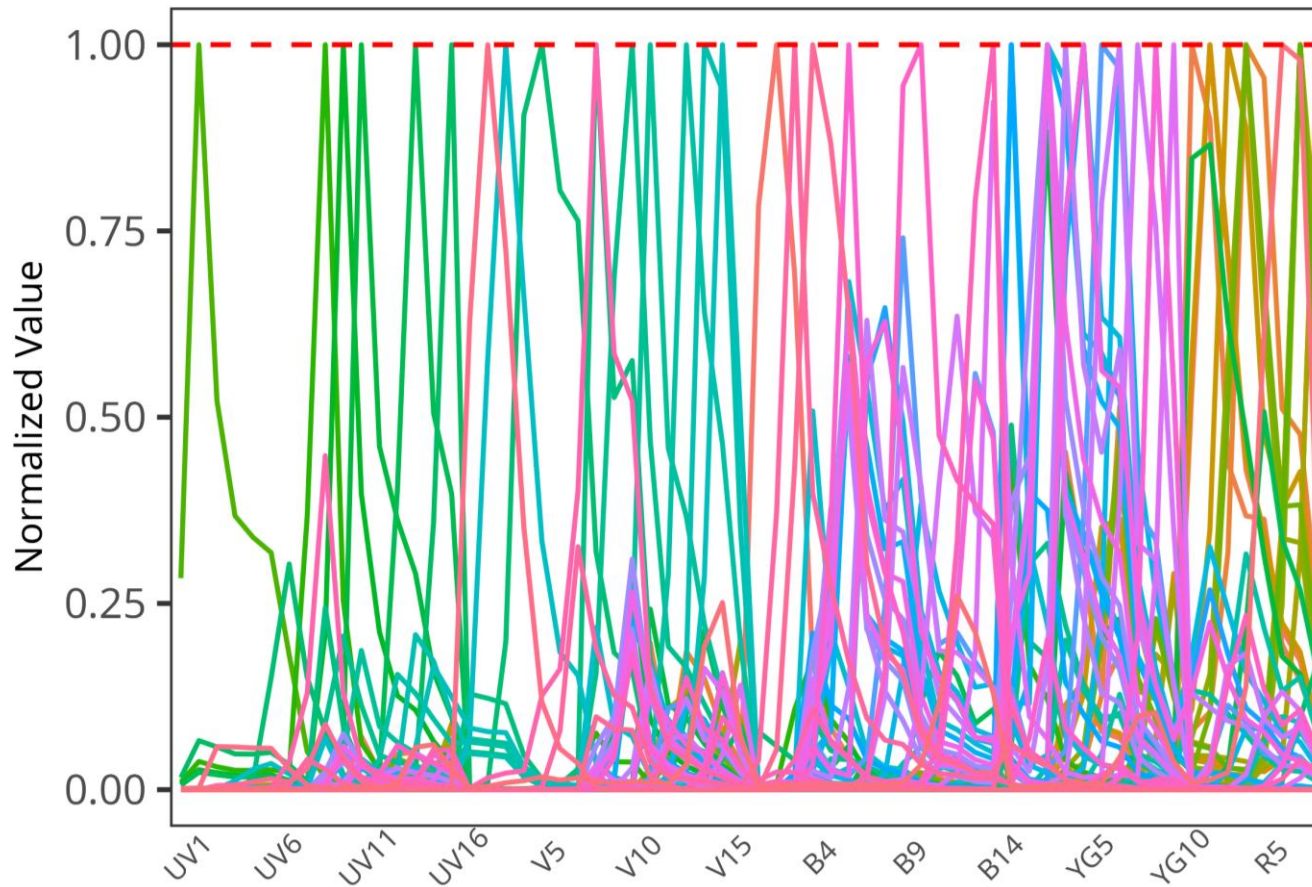
**“Well, how bright does it need to be?”:
Investigating the interplay of fluorescent
signature and brightness in single-color
unmixing controls.**

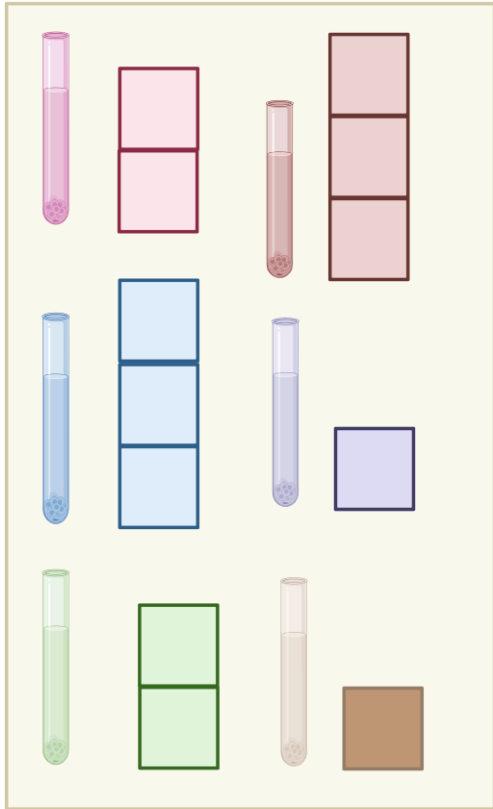
David Rach, Kirsten E. Lyke and Cristiana Cairo

University of Maryland, Baltimore

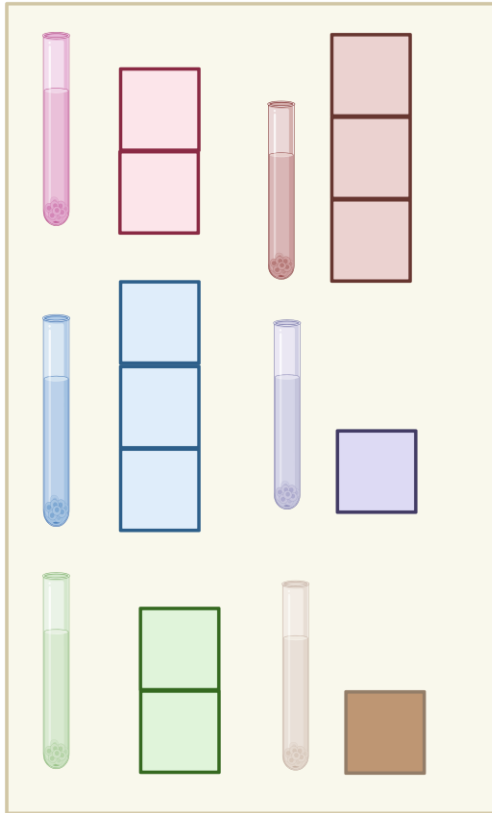
June 02, 2025

Spectral Flow Cytometry

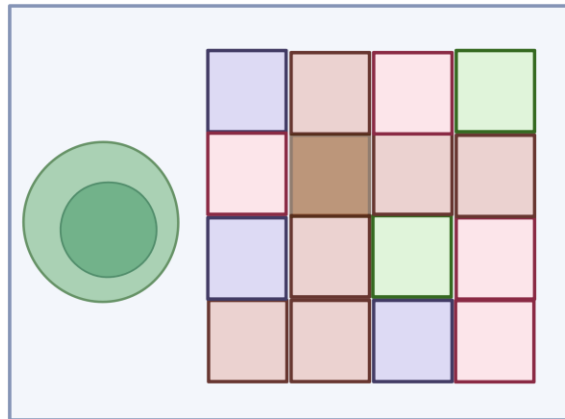




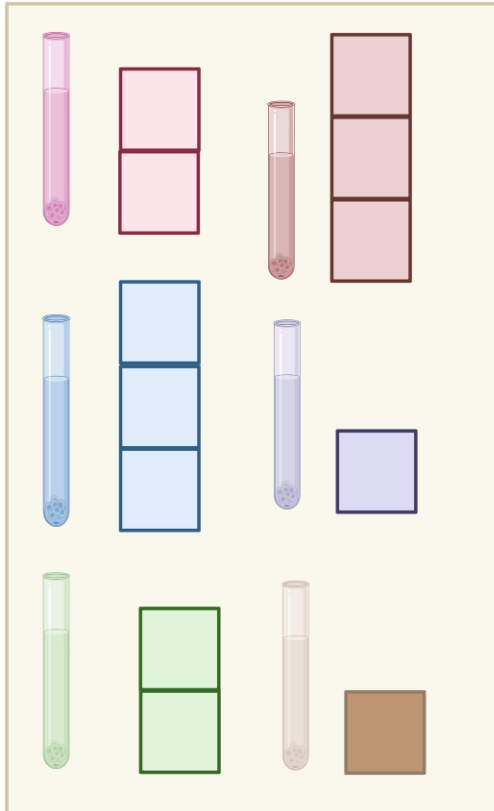
Unmixing Controls
(Single Color and
Unstained)



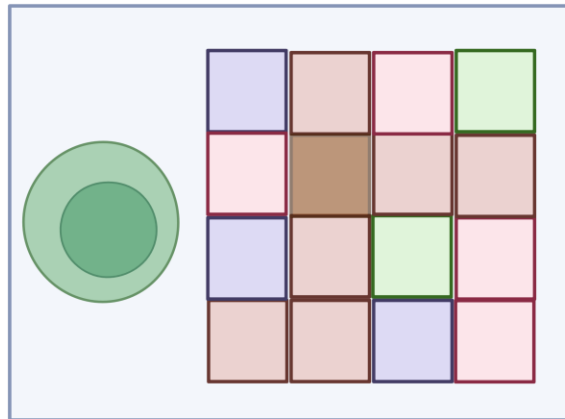
Unmixing Controls
(Single Color and
Unstained)



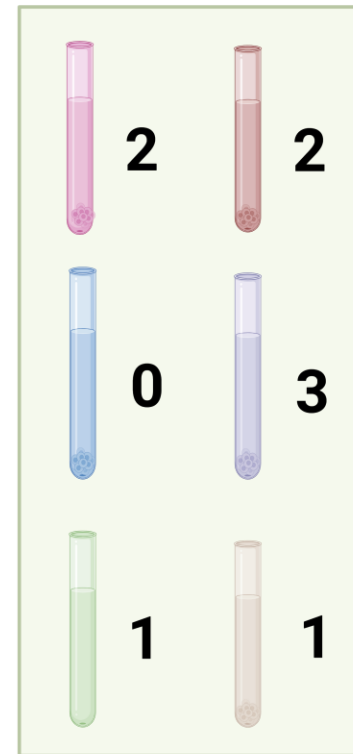
Single cell from
full-stained sample



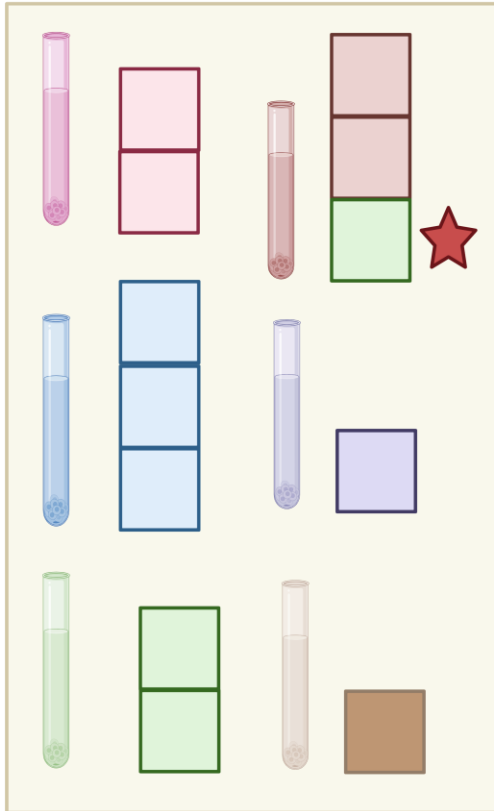
Unmixing Controls
(Single Color and
Unstained)



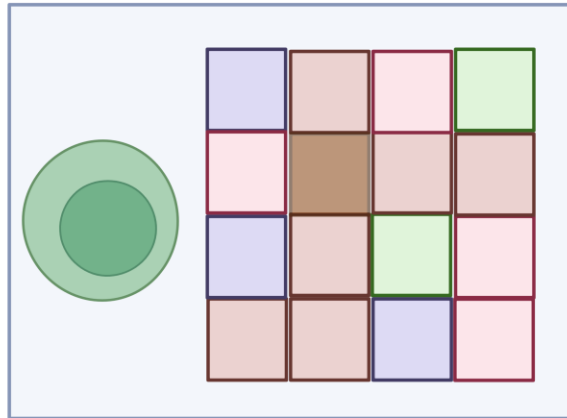
Single cell from
full-stained sample



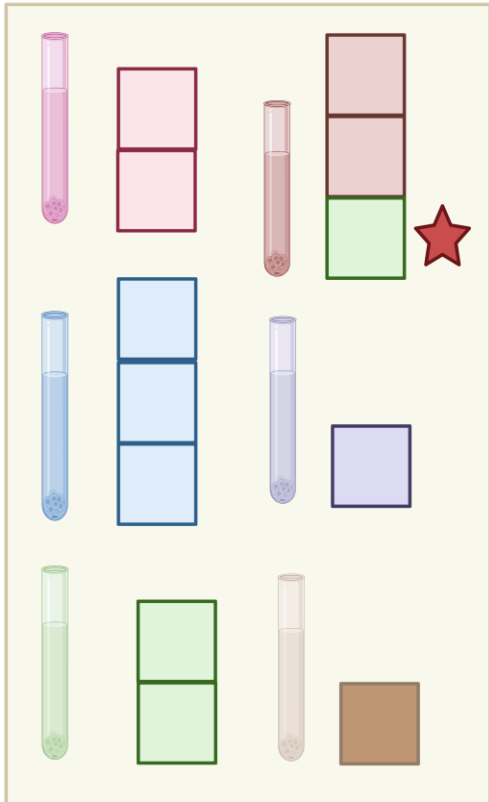
What fluorophores
are present, and
roughly how much



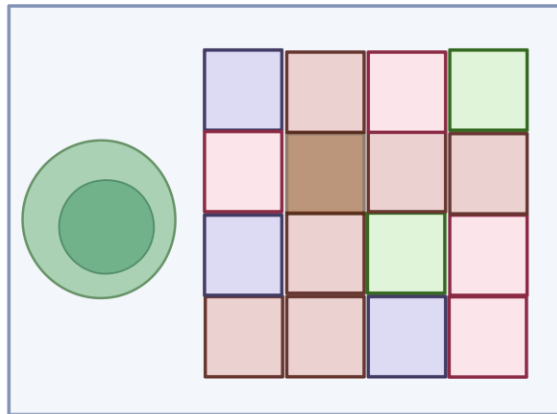
Unmixing Controls
(Single Color and
Unstained)



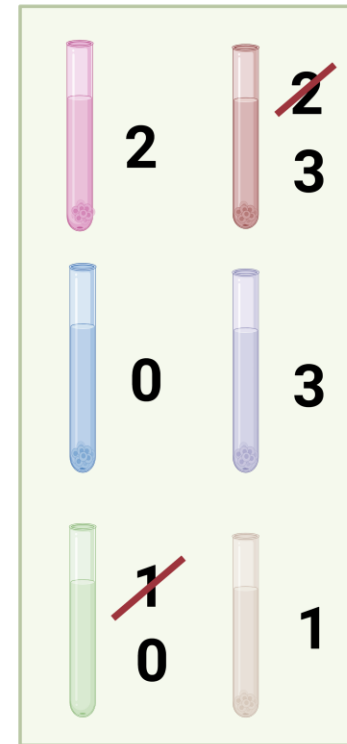
Single cell from
full-stained sample



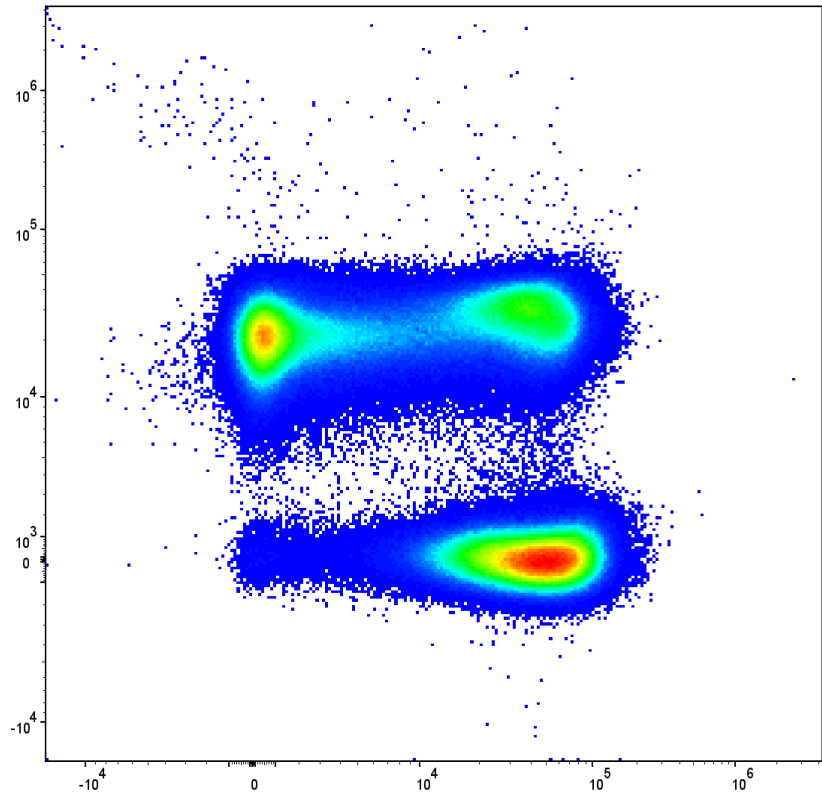
Unmixing Controls
(Single Color and
Unstained)

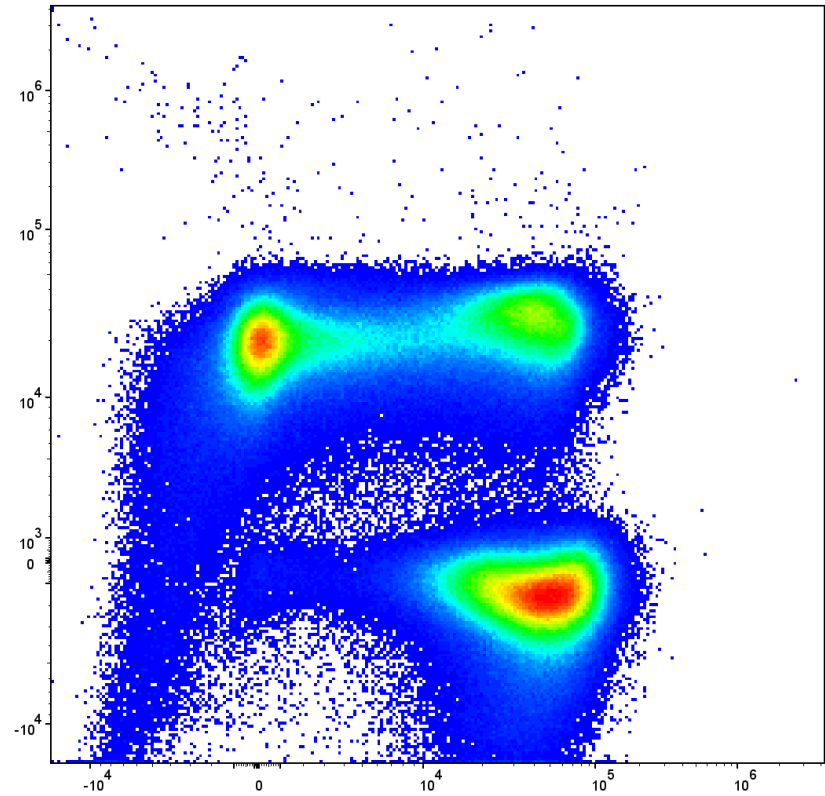
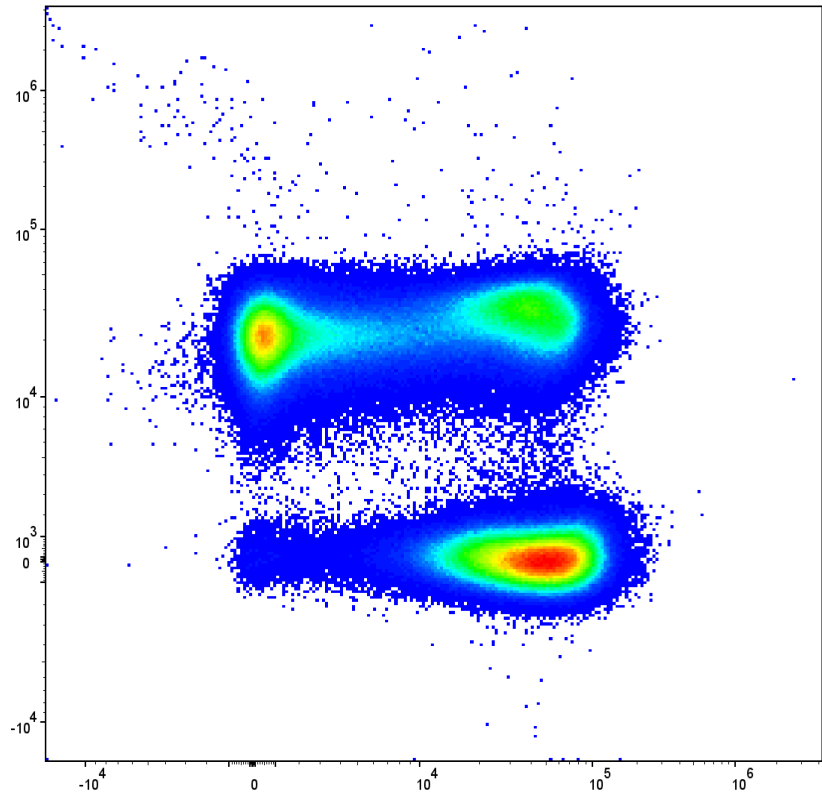


Single cell from
full-stained sample

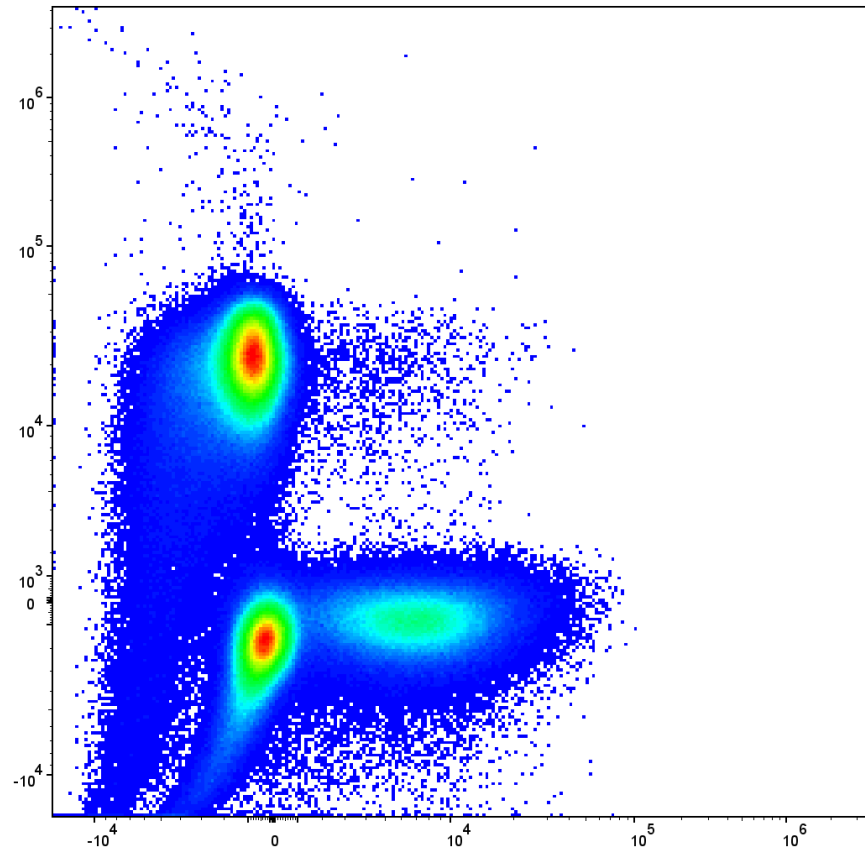


What fluorophores
are present, and
roughly how much

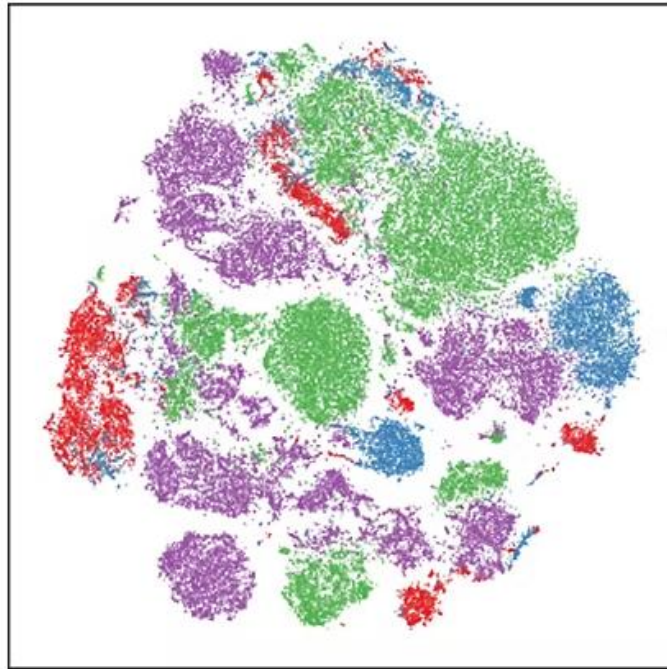




Even when following best practices,
we still sometimes end up here

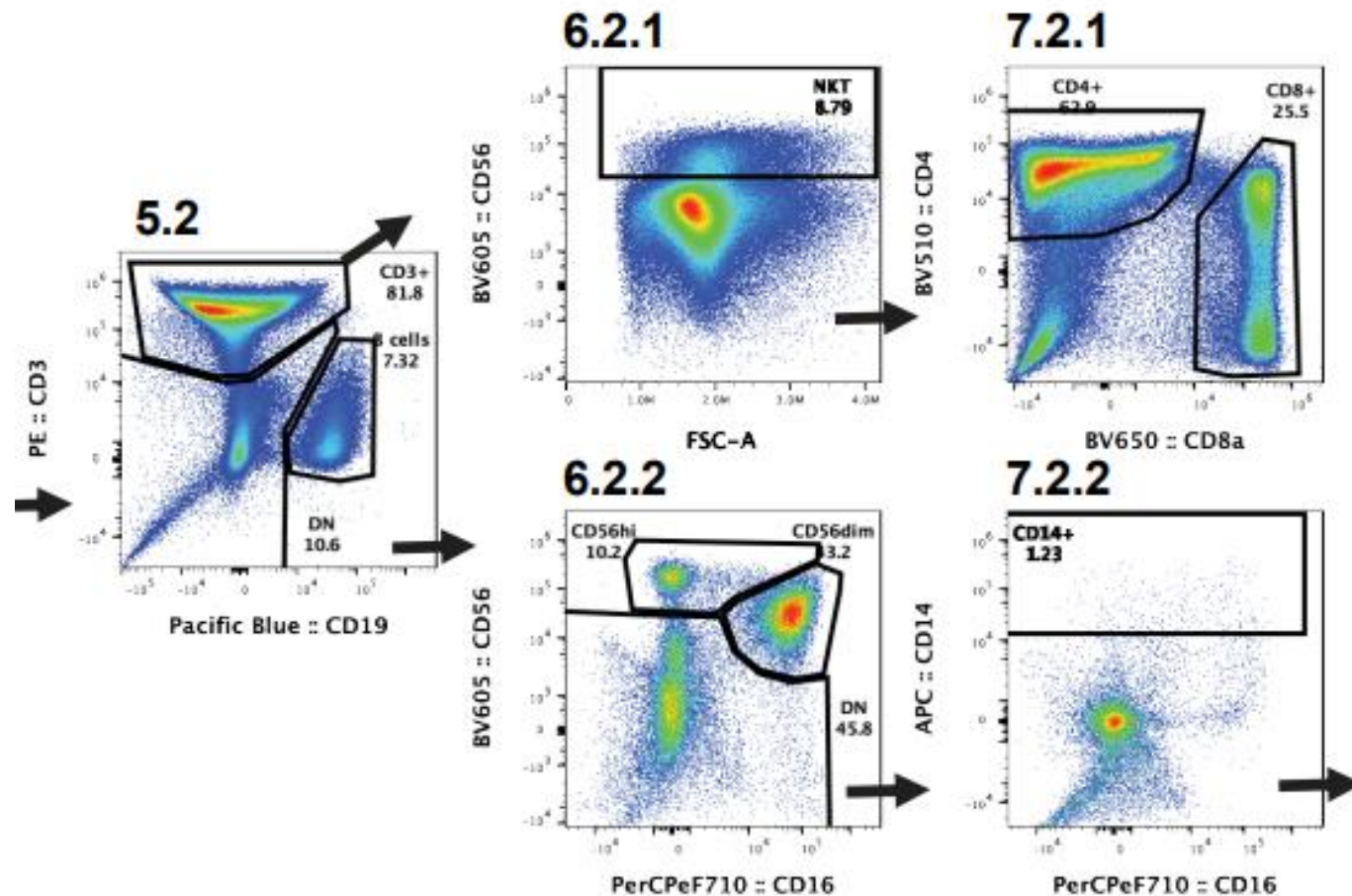


Unsupervised analysis

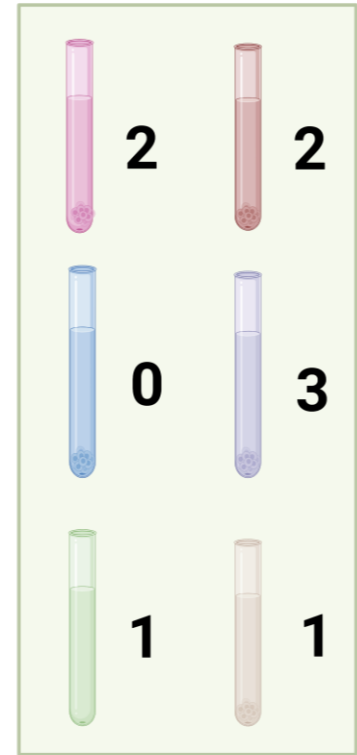


- expt2
- expt3
- expt4
- expt5

“We discovered this novel cluster”

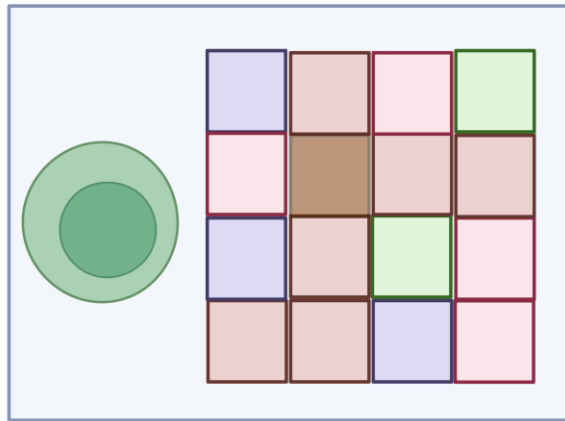


Previous efforts at quality control



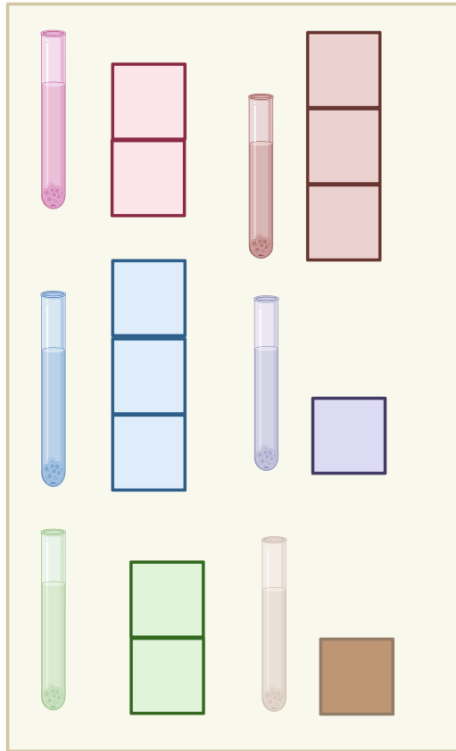
Unmixed
full-stained
sample

Previous efforts at quality control



Raw
full-stained
sample

But what about our unmixing controls?



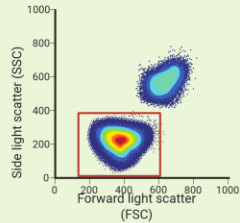
Unmixing Controls
(Single Color and
Unstained)

Luciérnaga



UNIVERSITY of MARYLAND
SCHOOL OF MEDICINE

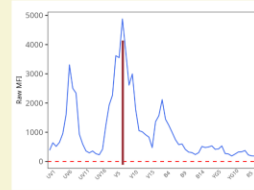
Traditional Workflow



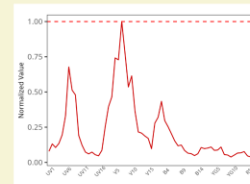
Gate the
Population



Subtract Out
Autofluorescence



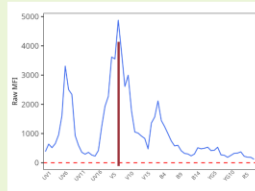
Identify detector
with brightest
MFI



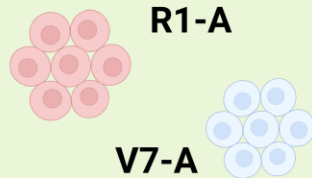
Normalize fluorescent
signature



Luciernaga Workflow



Identify detector
with brightest
MFI for each cell



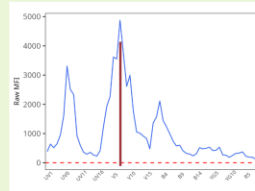
Group cells with
shared peak
detectors



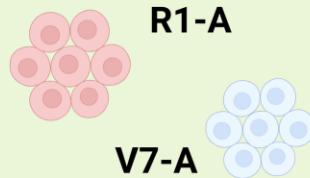
Subtract Out
Autofluorescence



Luciernaga Workflow



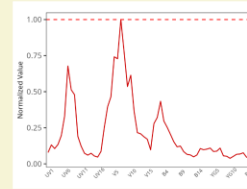
Identify detector with brightest MFI for each cell



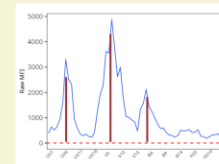
Group cells with shared peak detectors



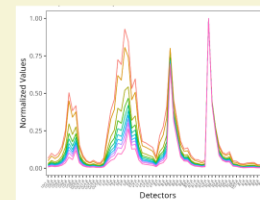
Subtract Out Autofluorescence



Normalize individual cell fluorescent signatures

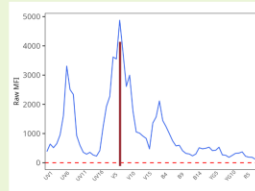


Identify peaks using local maxima

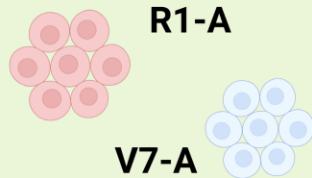


Group cells with shared peaks

Luciernaga Workflow



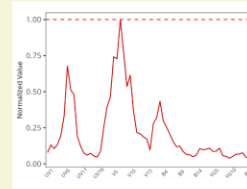
Identify detector with brightest MFI for each cell



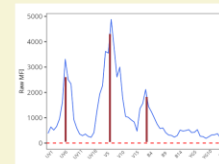
Group cells with shared peak detectors



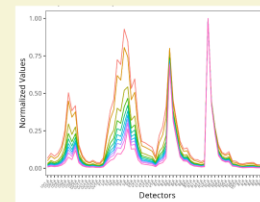
Subtract Out Autofluorescence



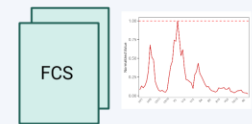
Normalize individual cell fluorescent signatures



Identify peaks using local maxima

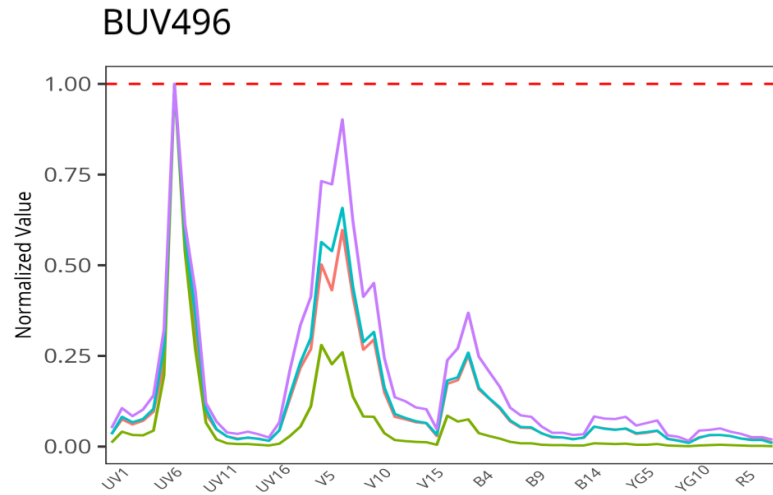


Group cells with shared peaks

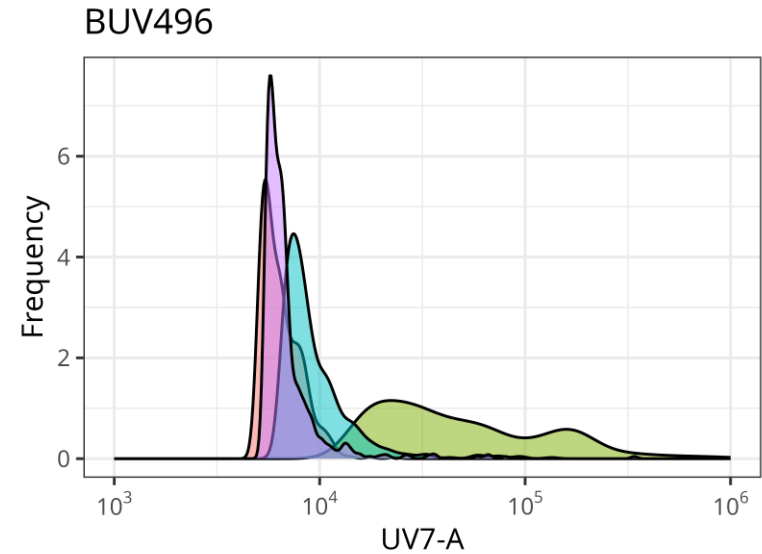
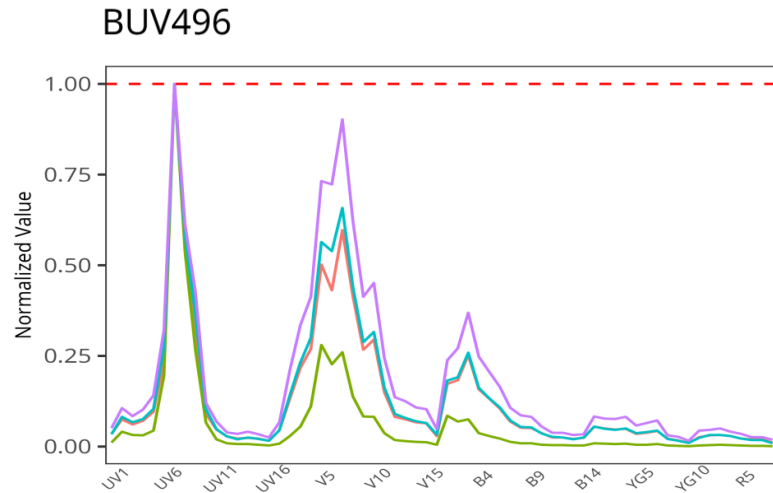


Enumerate and export out cells that share signature as .fcs files

What I normally see

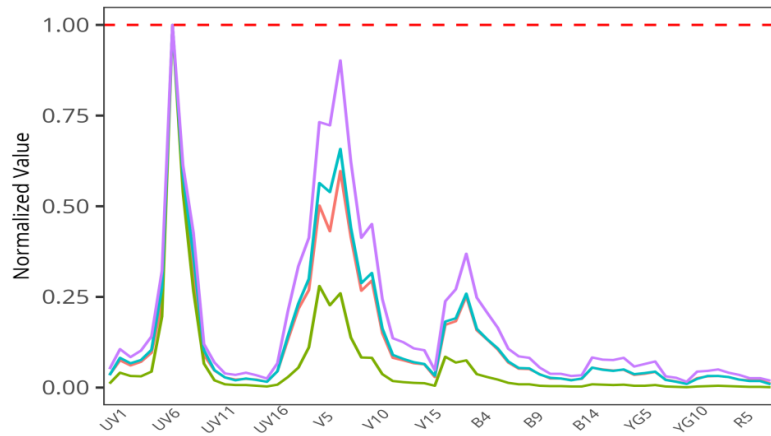


What I normally see

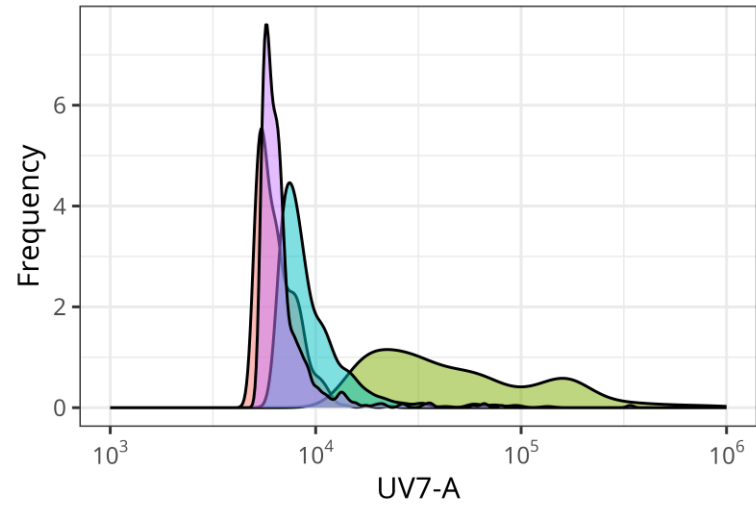


What I normally see

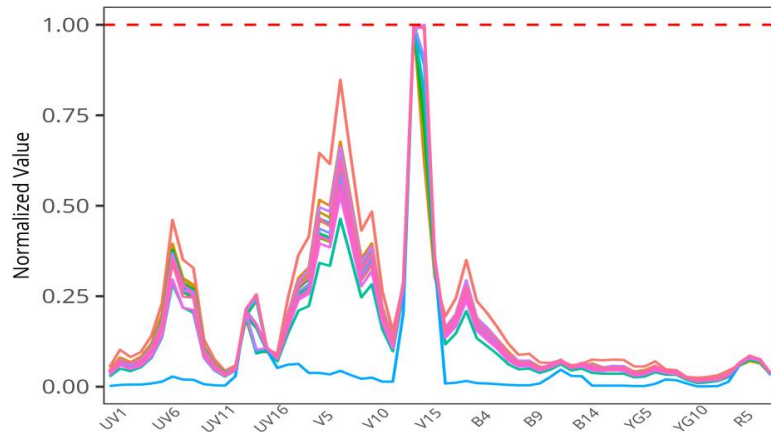
BUV496



BUV496

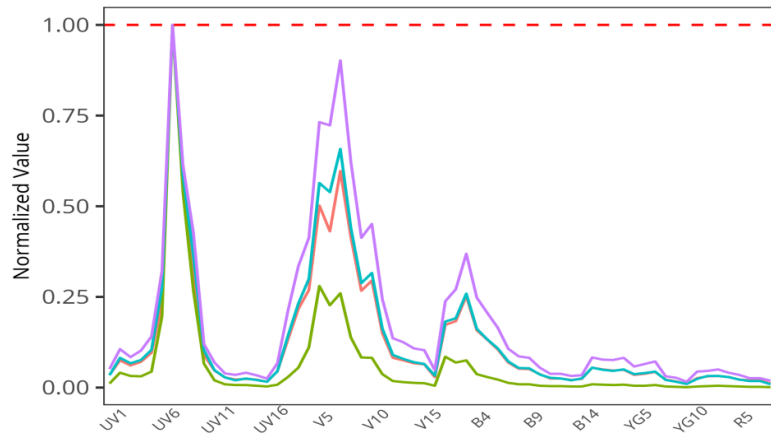


BV750

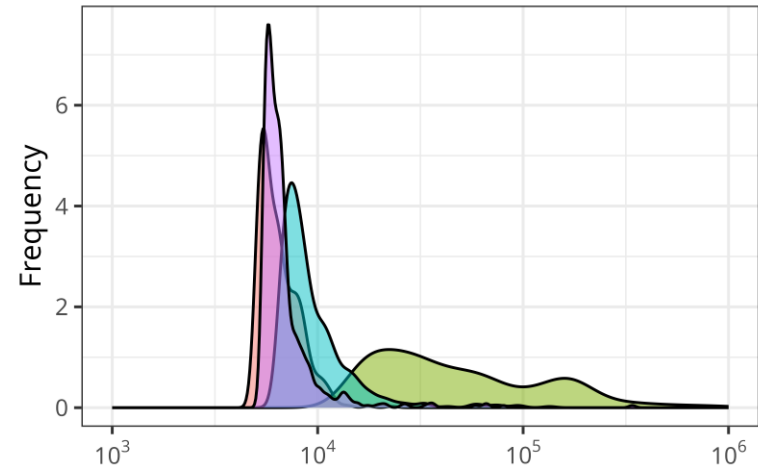


What I normally see

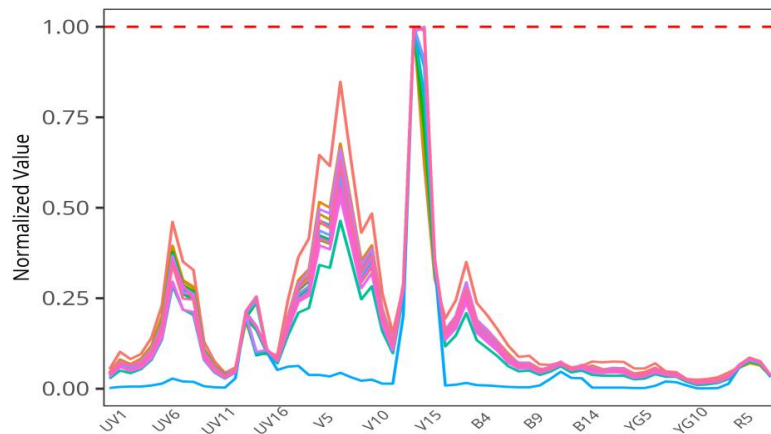
BUV496



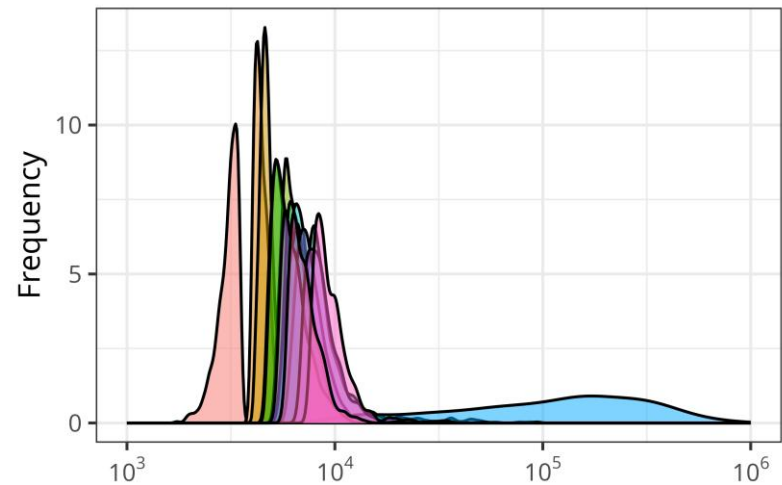
BUV496



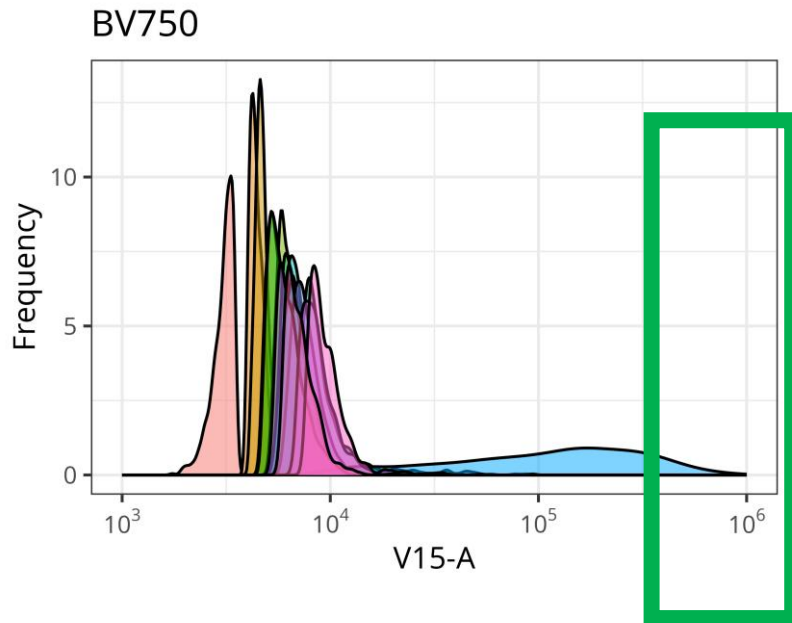
BV750



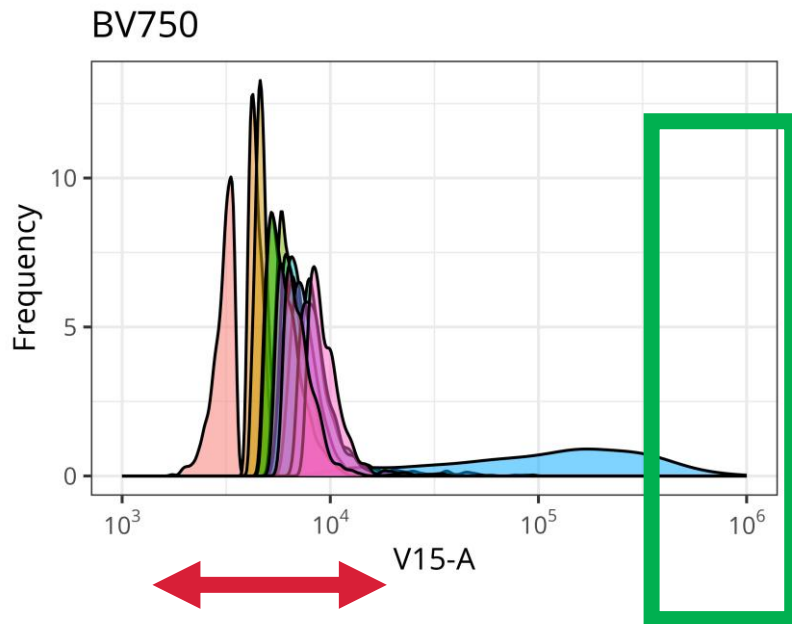
BV750



V15-A

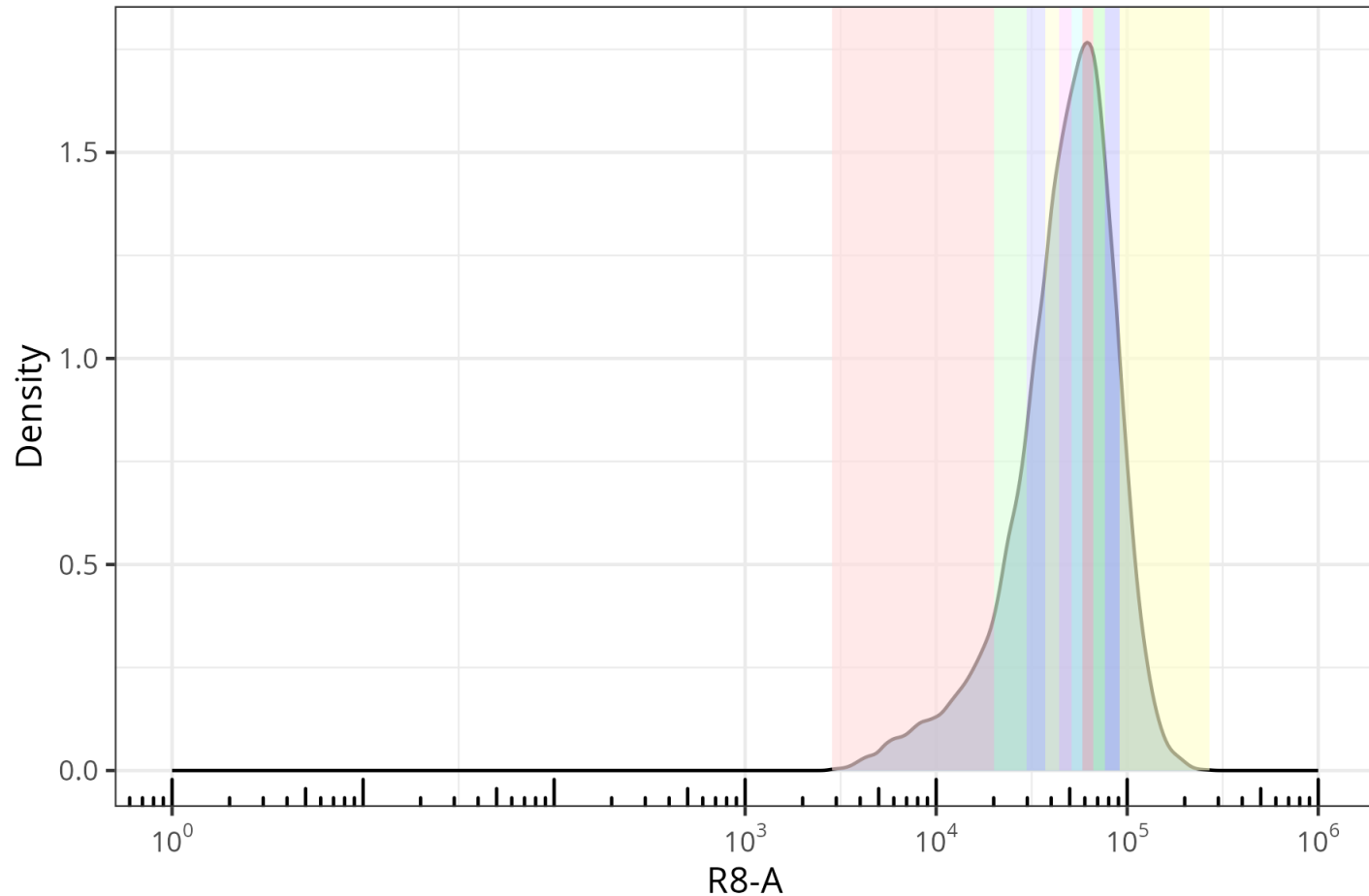


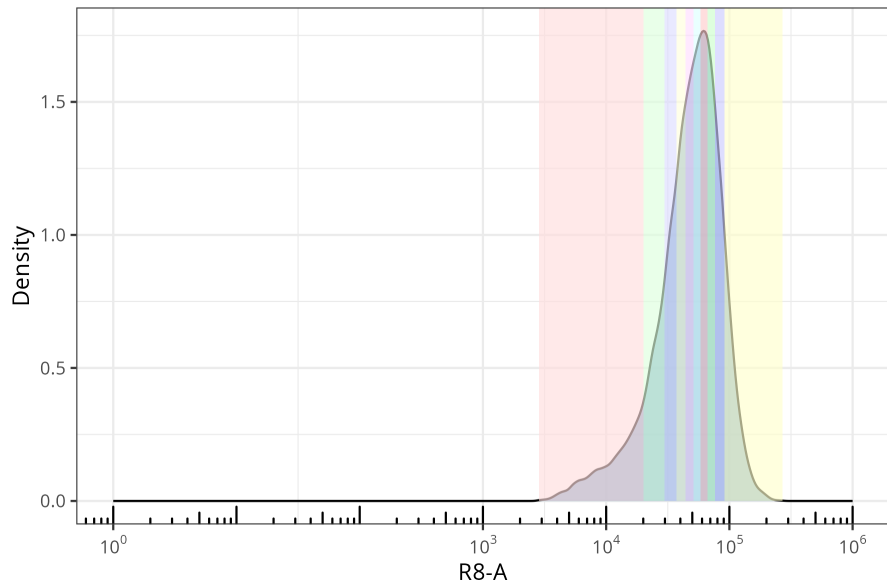
Cells with lower
MFI have more
variant signatures



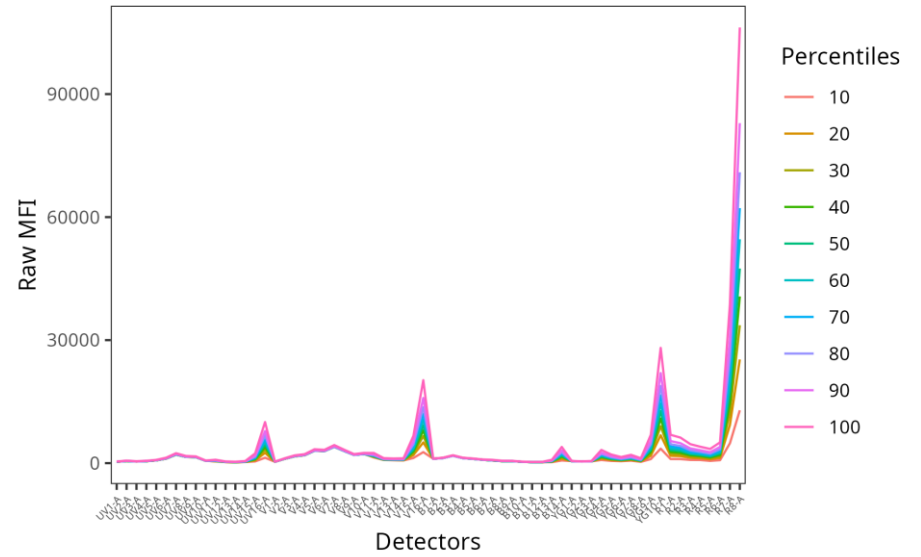
Cells with lower
MFI have more
variant signatures

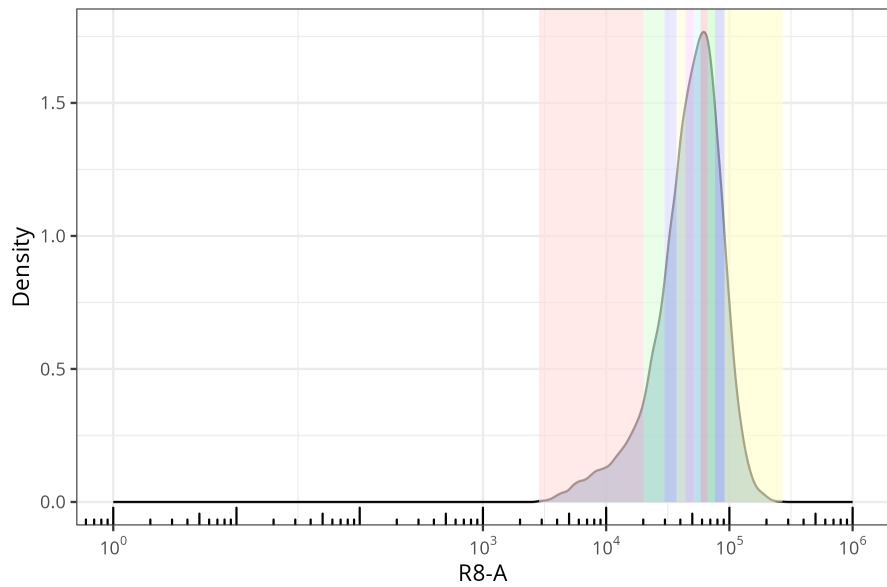
Split positive cells in 10 percentile increments



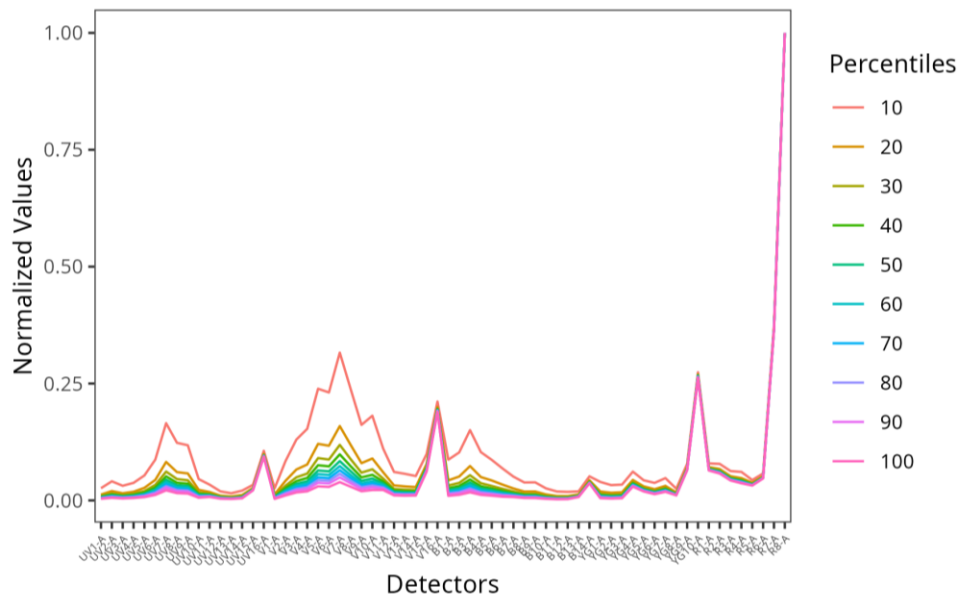
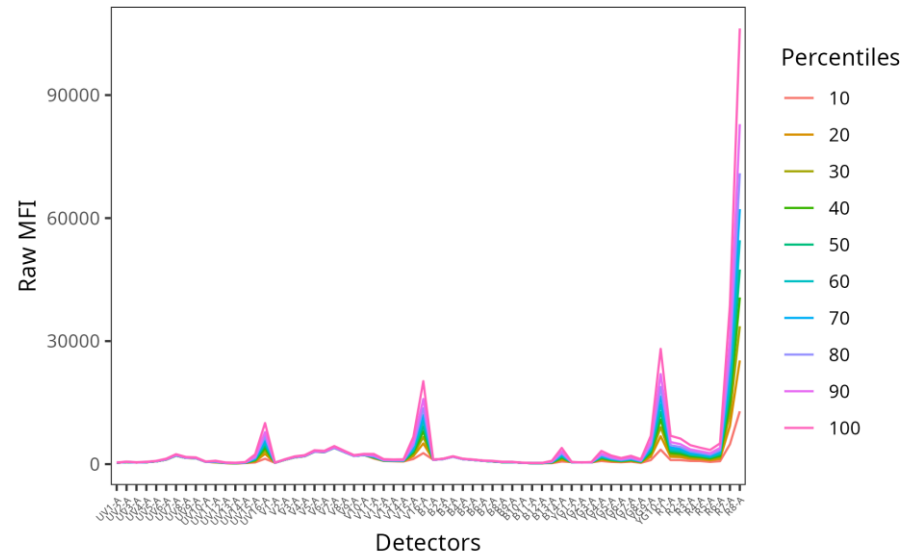


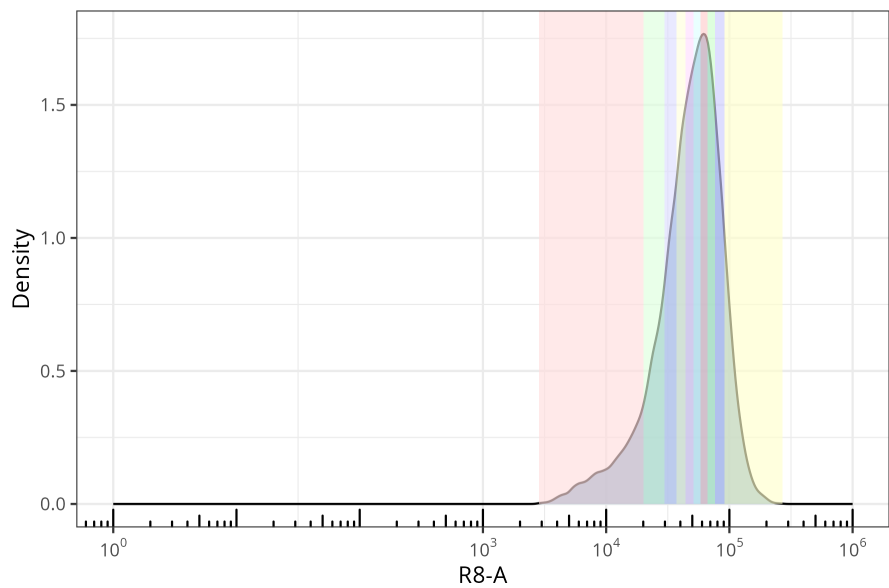
APC-Fire 810



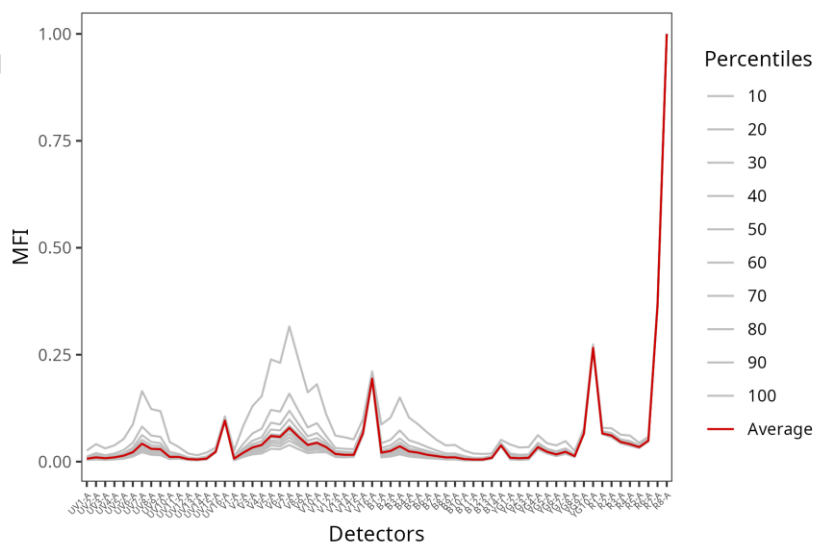
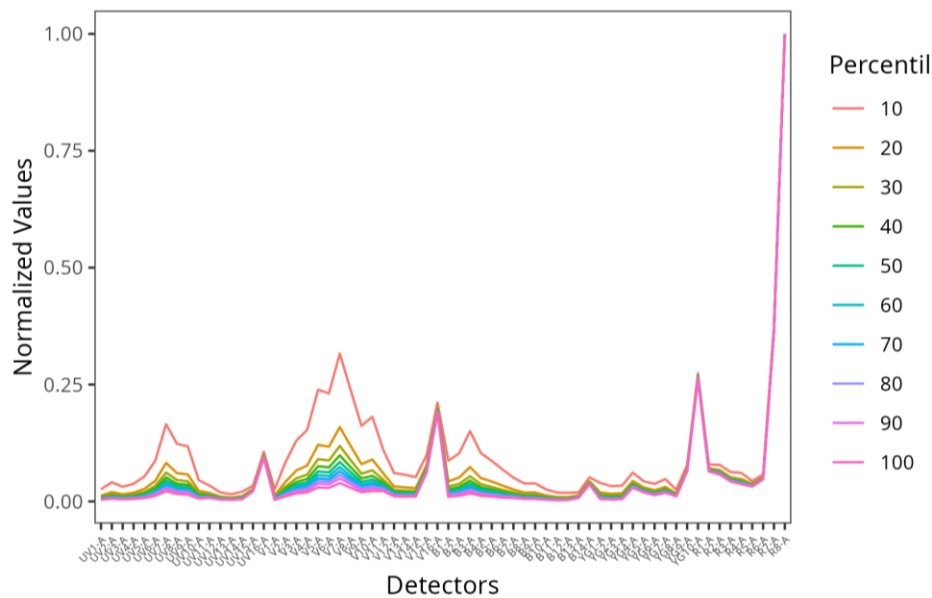
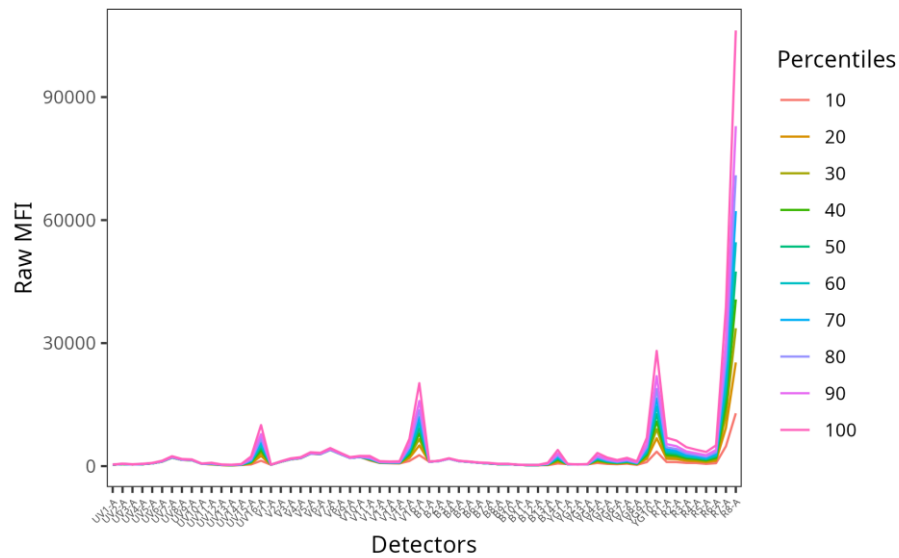


APC-Fire 810

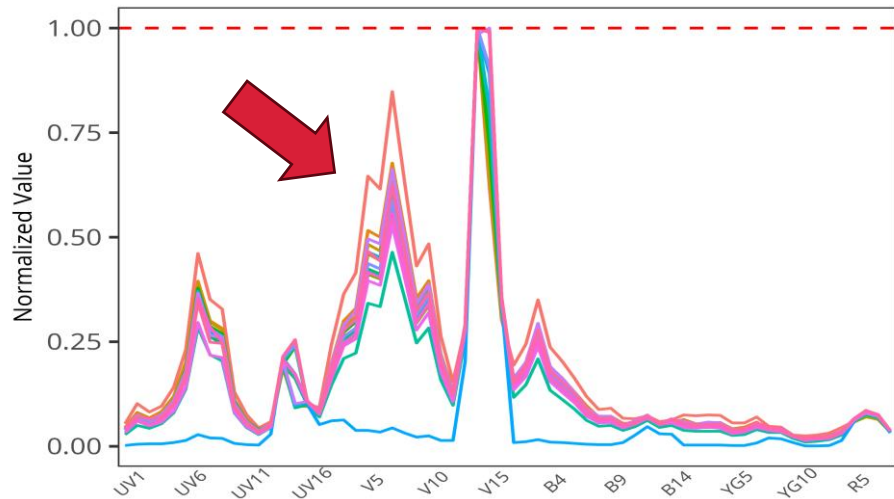
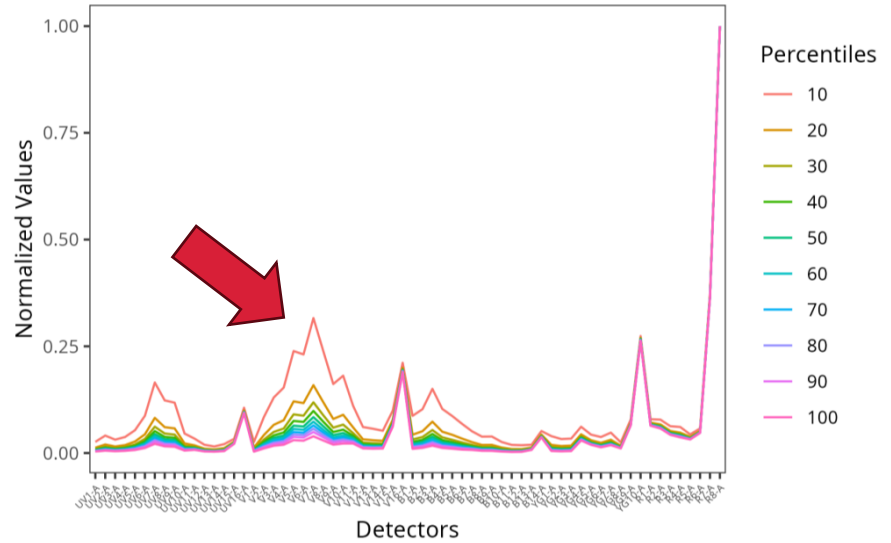
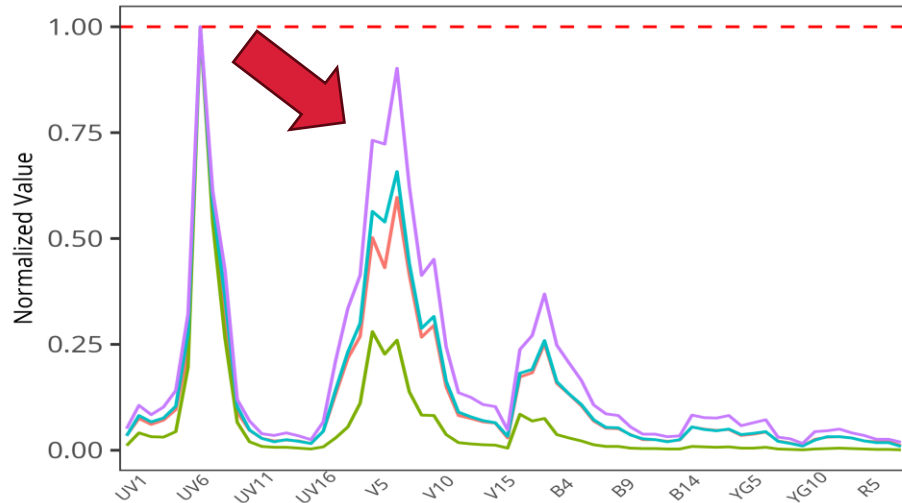




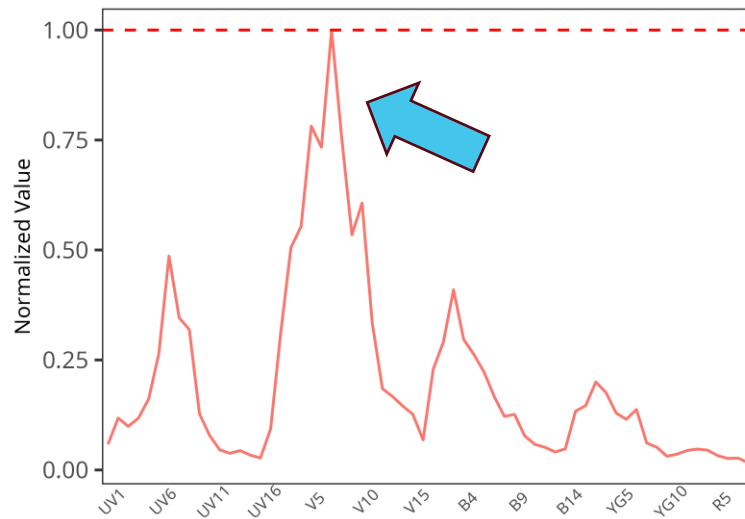
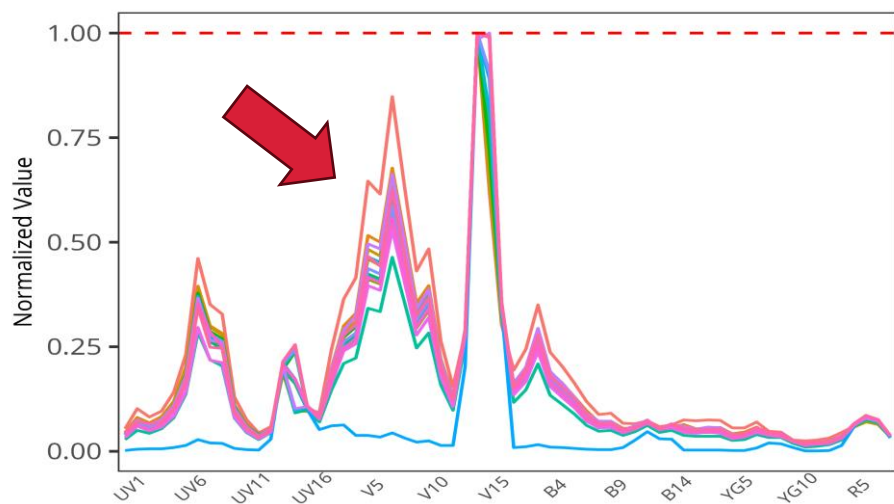
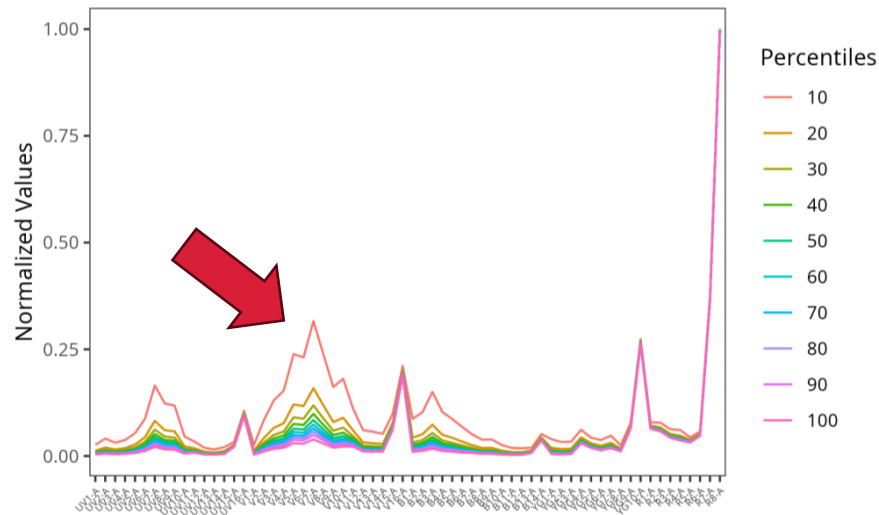
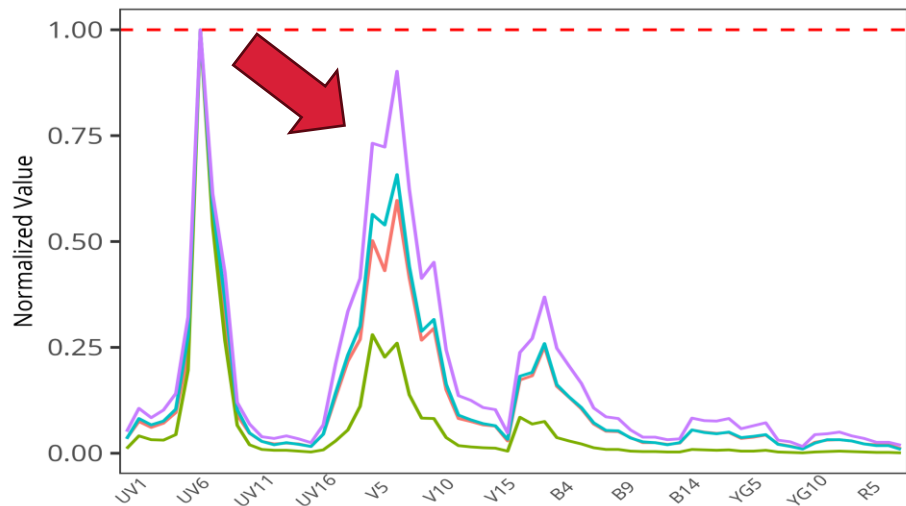
APC-Fire 810



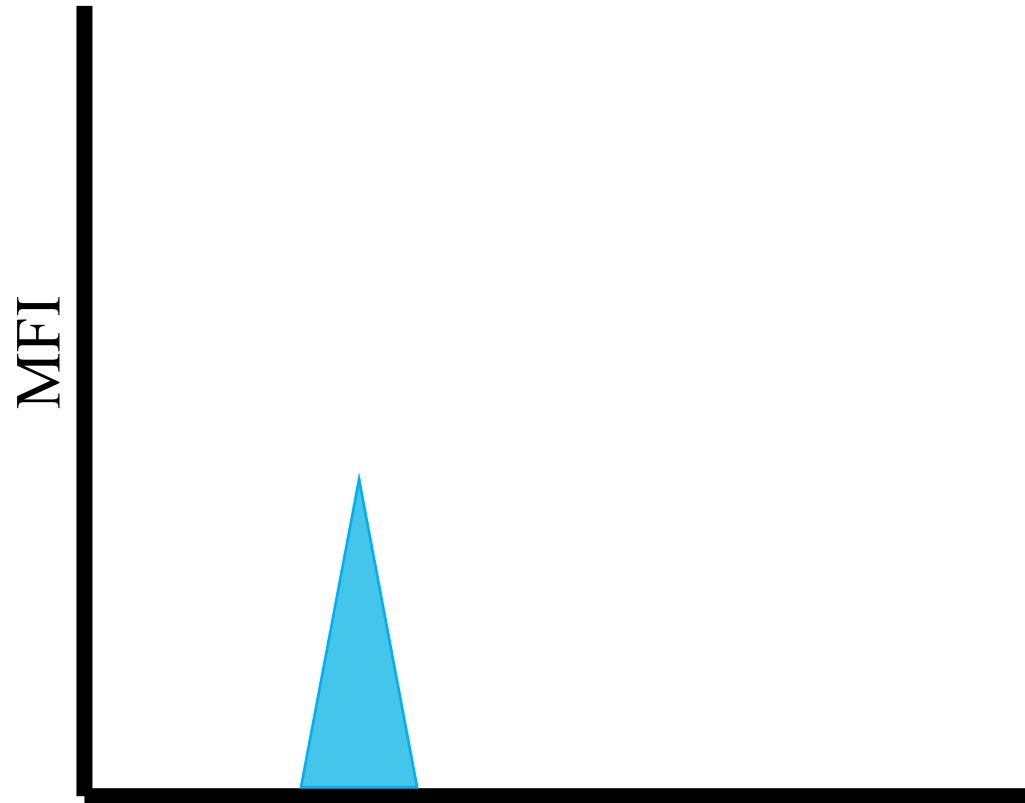
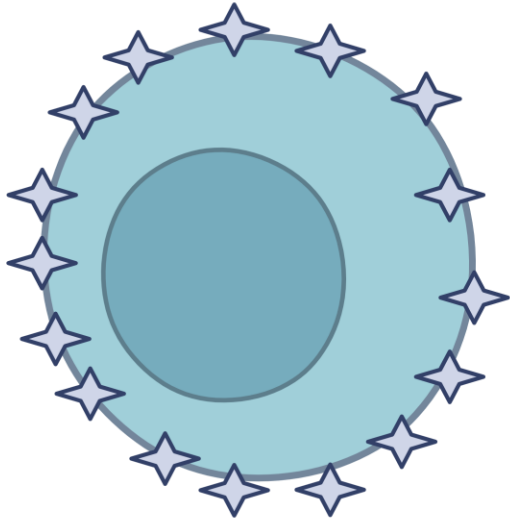
What do they have in common?



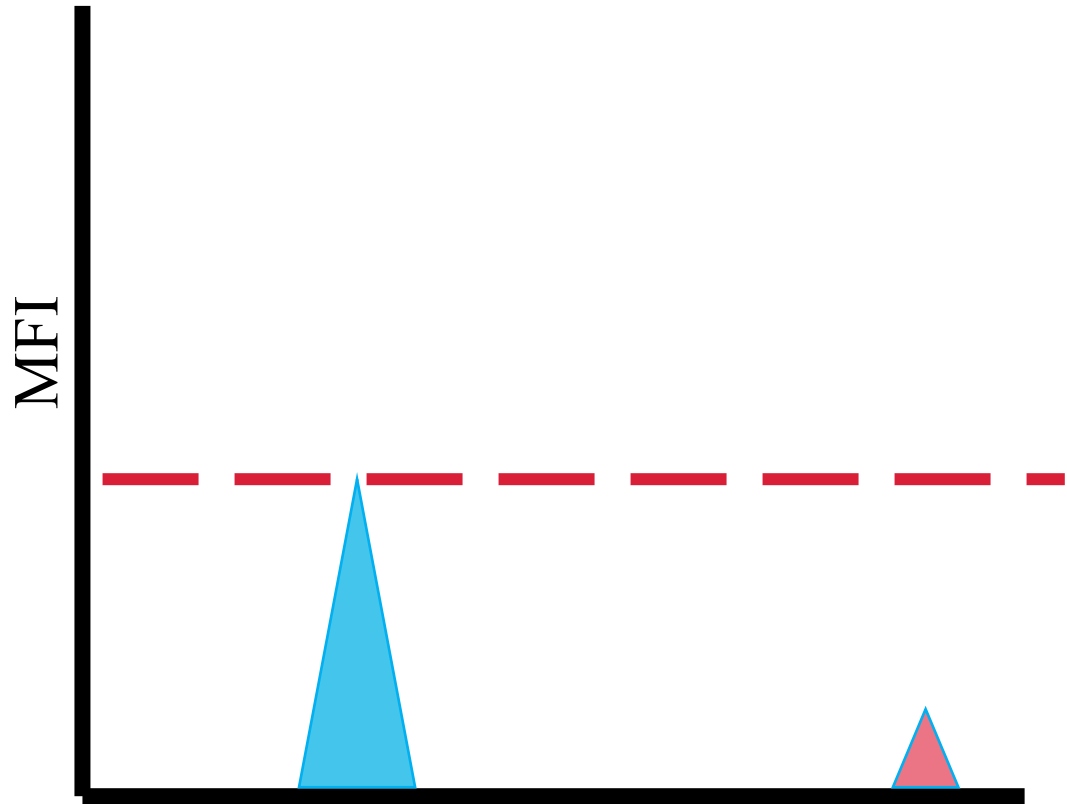
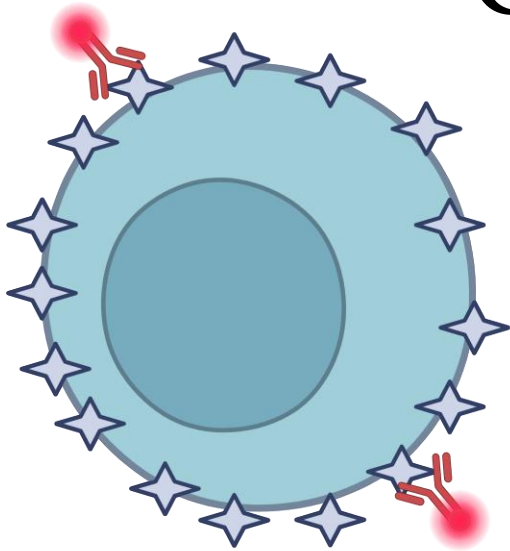
Autofluorescence!



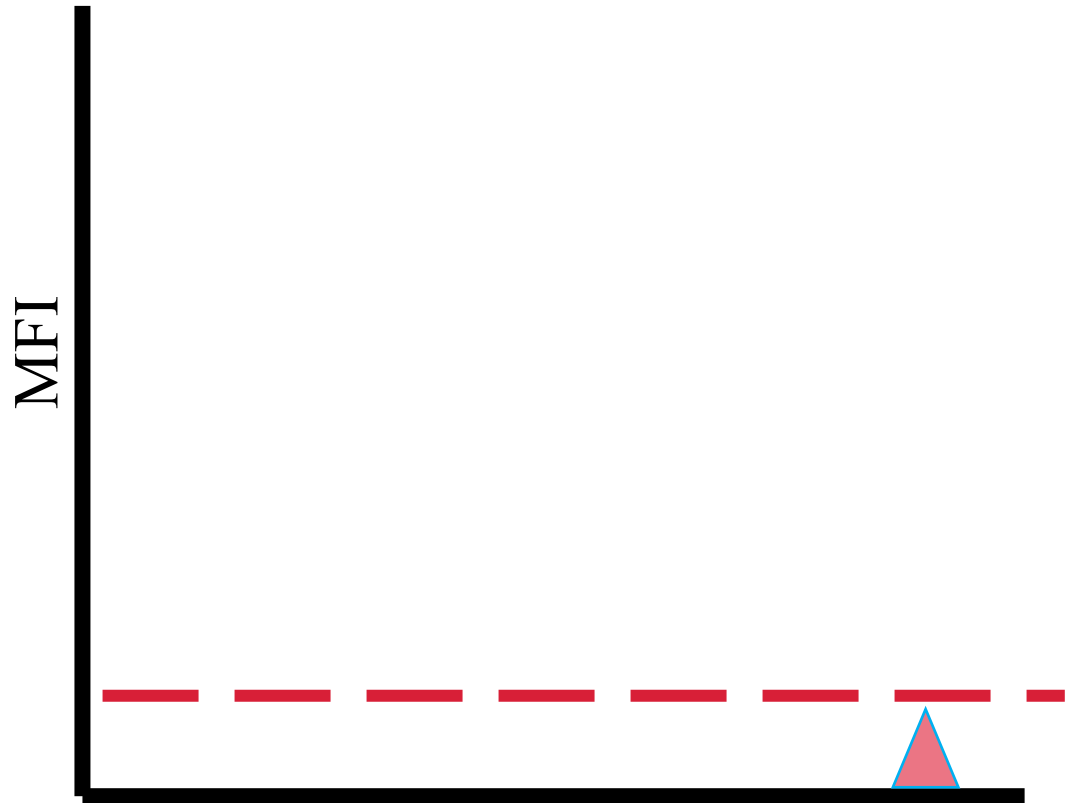
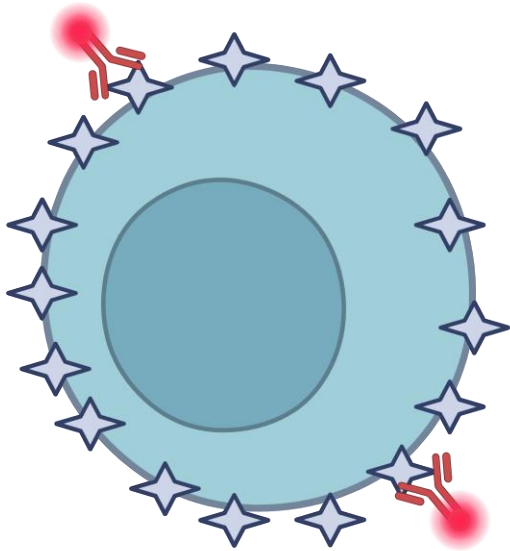
Conceptually



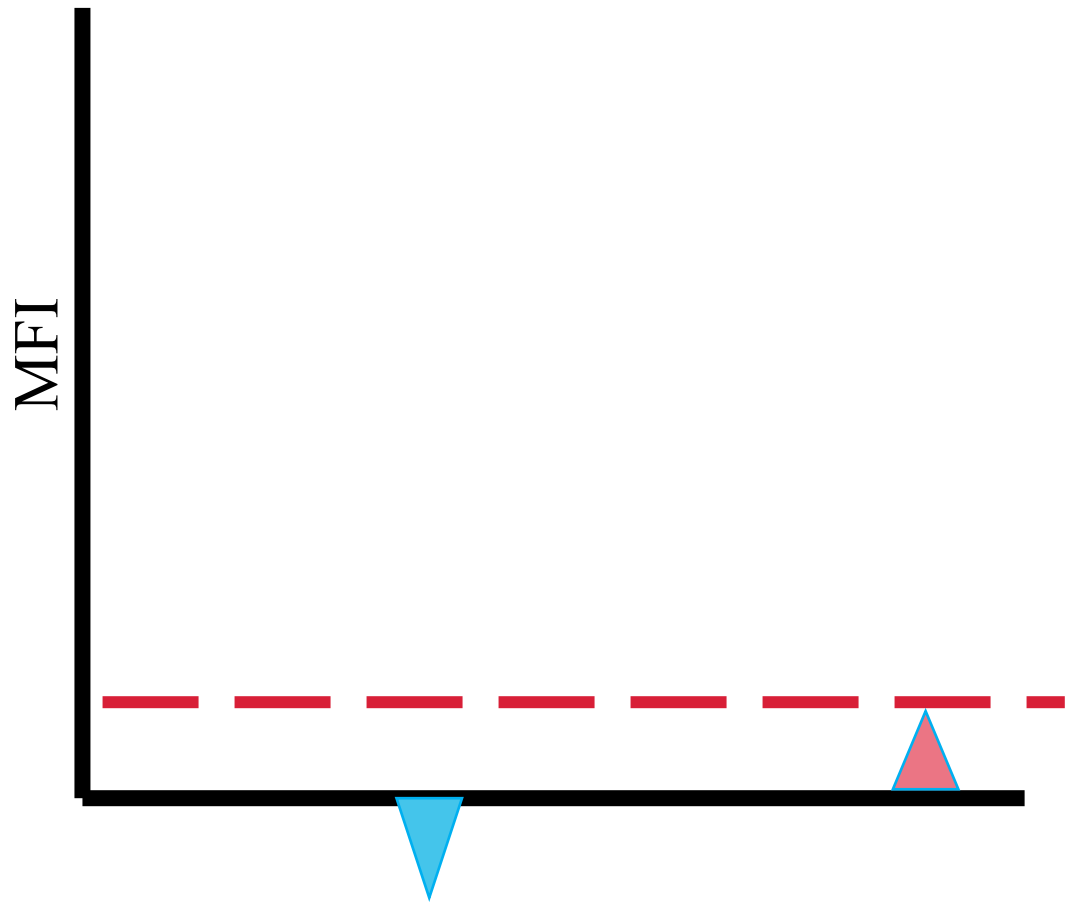
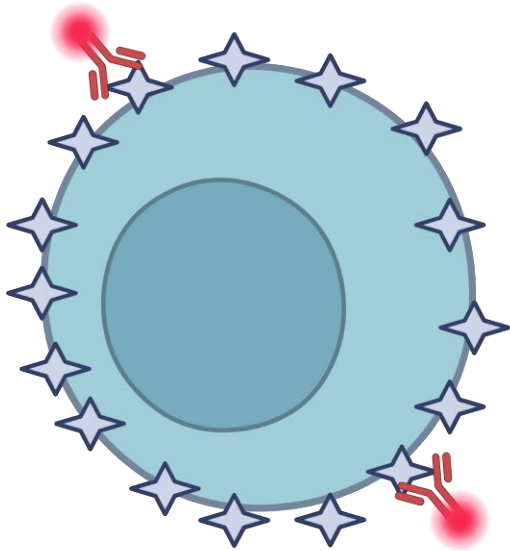
Conceptually



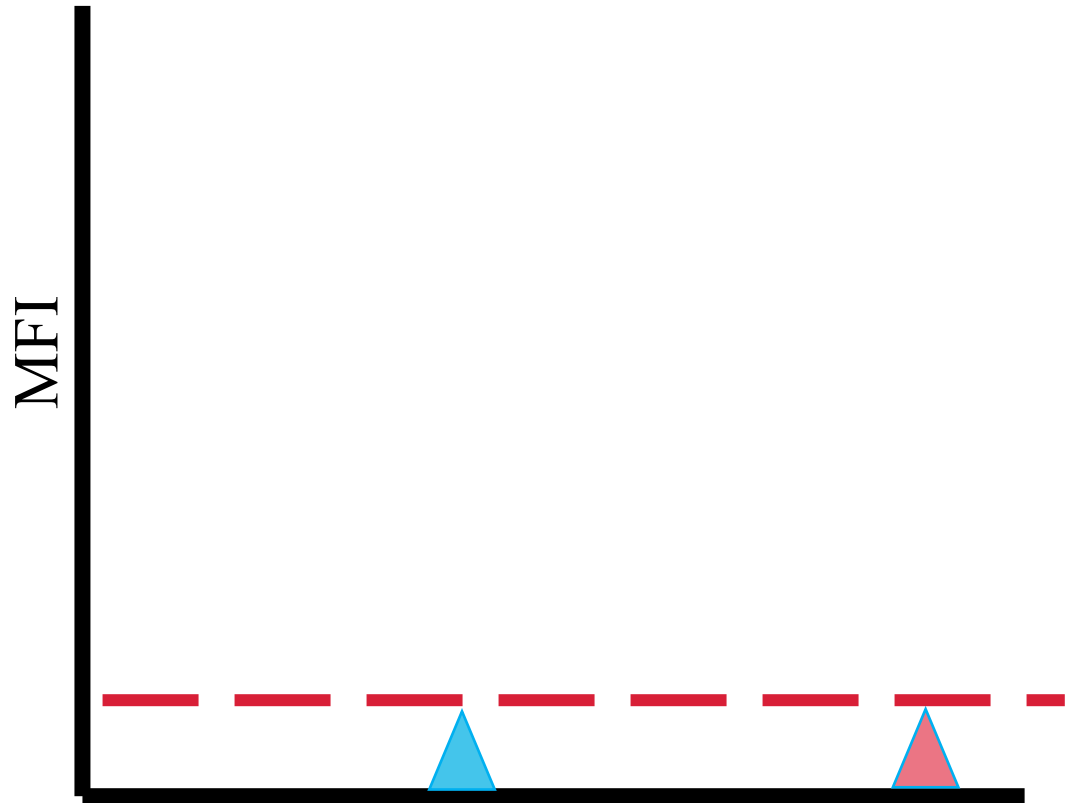
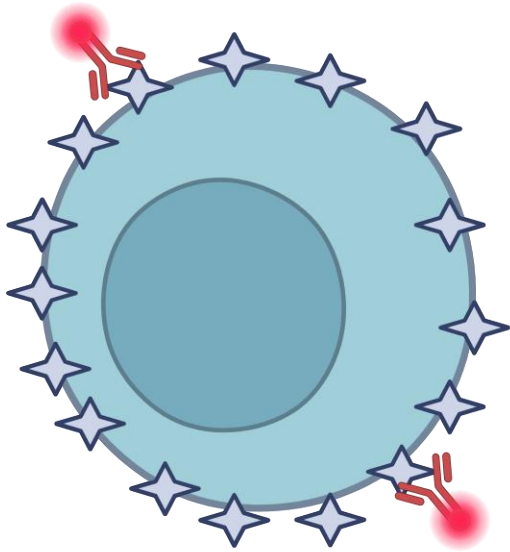
If the appropriate amount of autofluorescence is subtracted



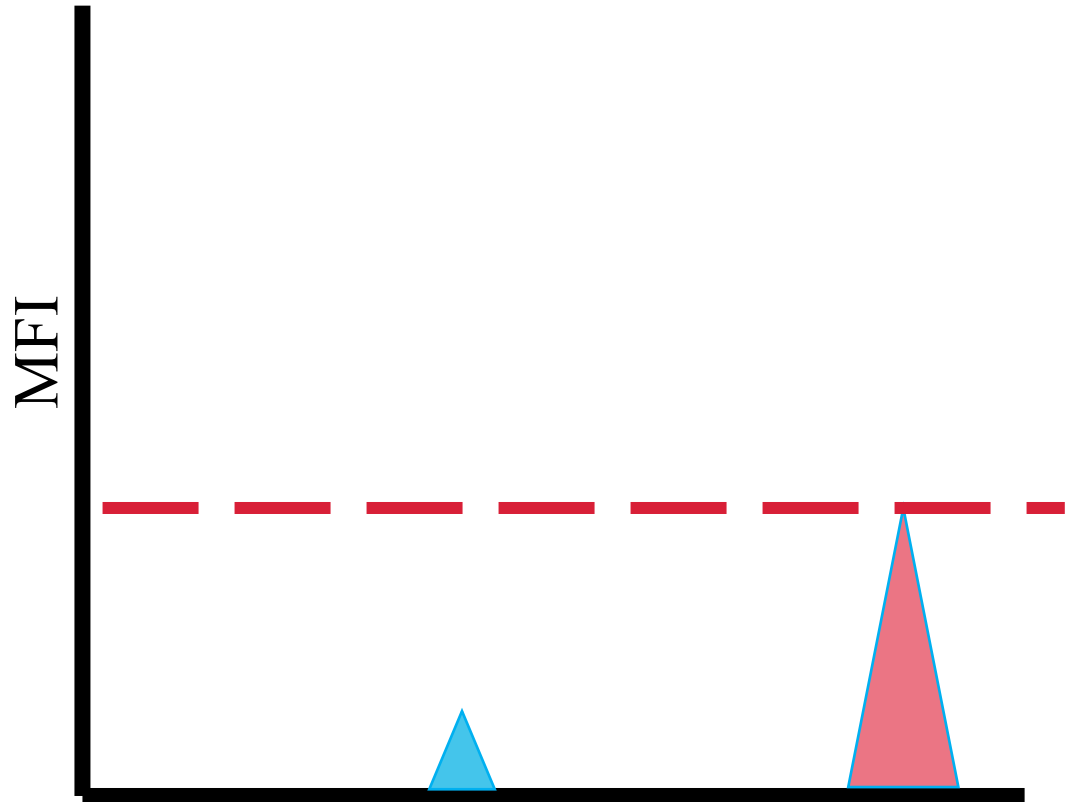
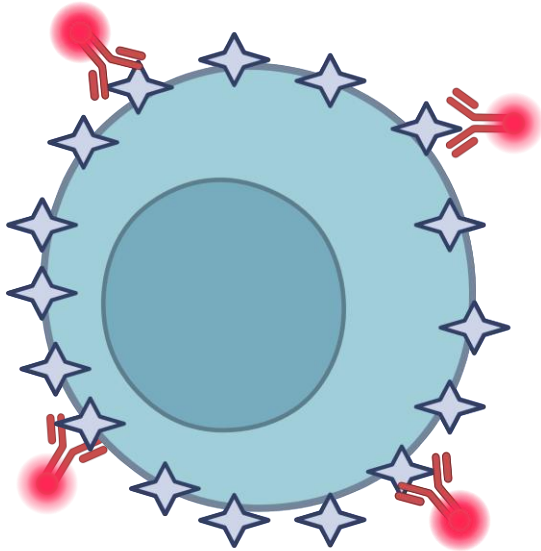
If too much autofluorescence is subtracted



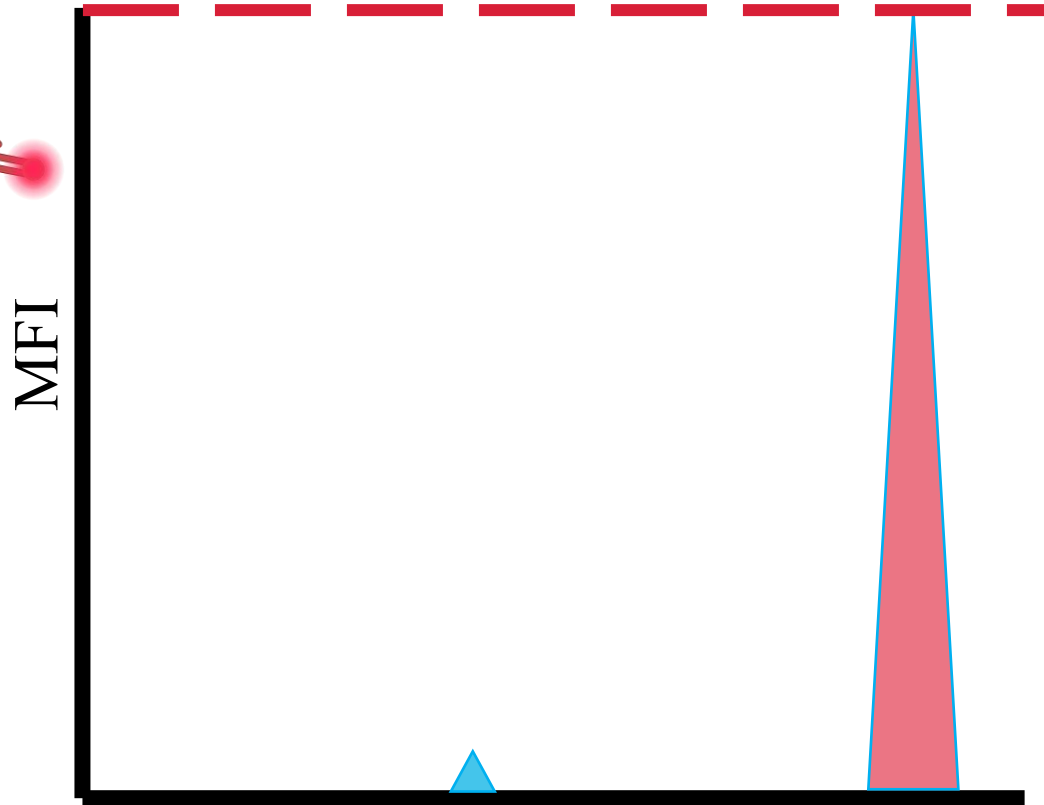
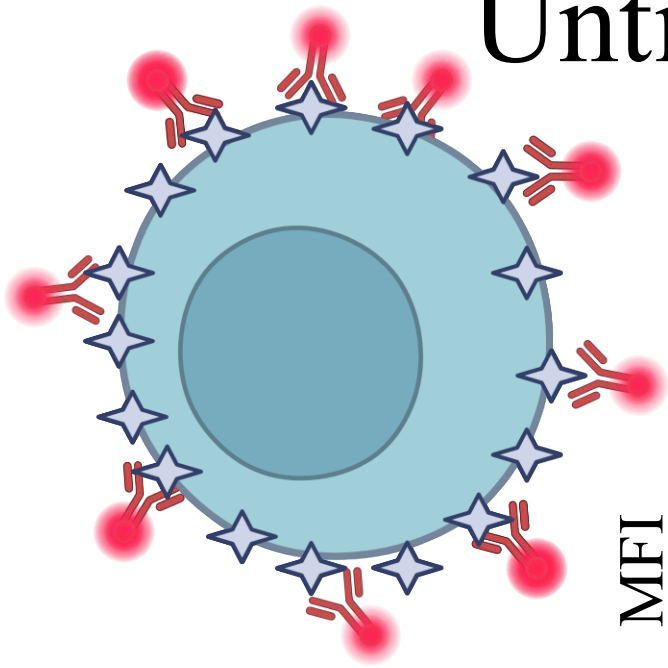
If too little autofluorescence is subtracted



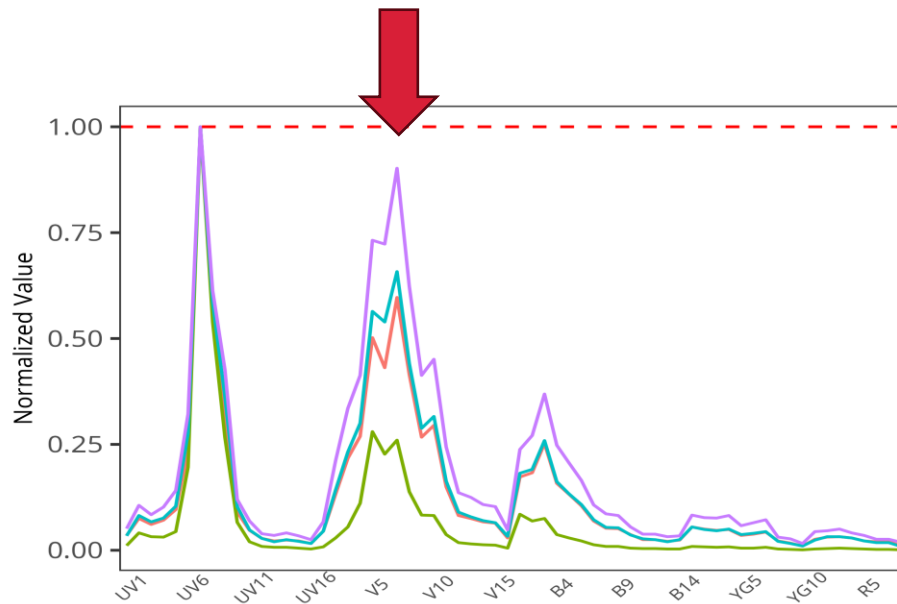
The effect of residual autofluorescence on the signature decreases as the fluorophore brightness increases



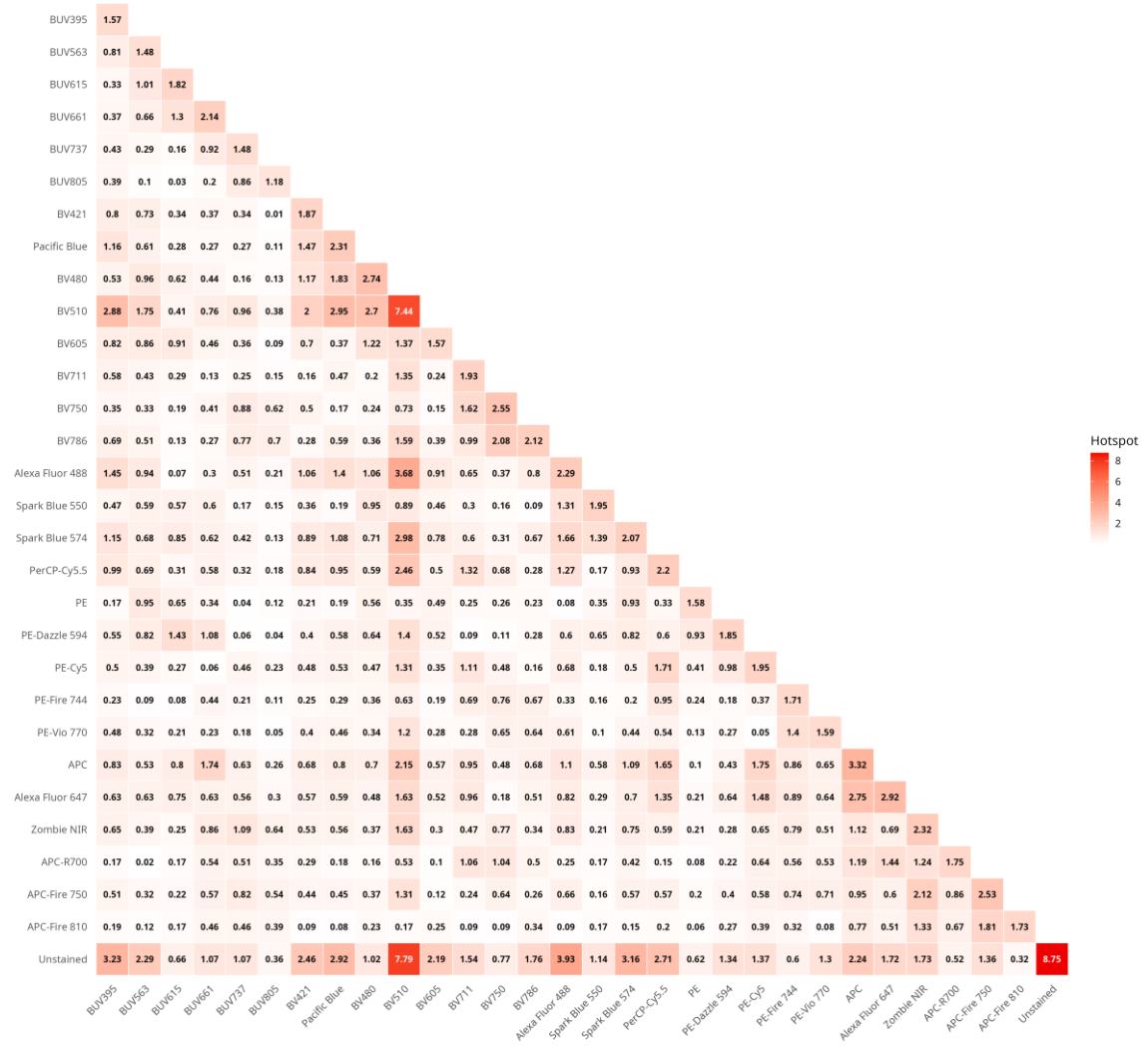
Until contribution minimal



Residual autofluorescence after background subtraction



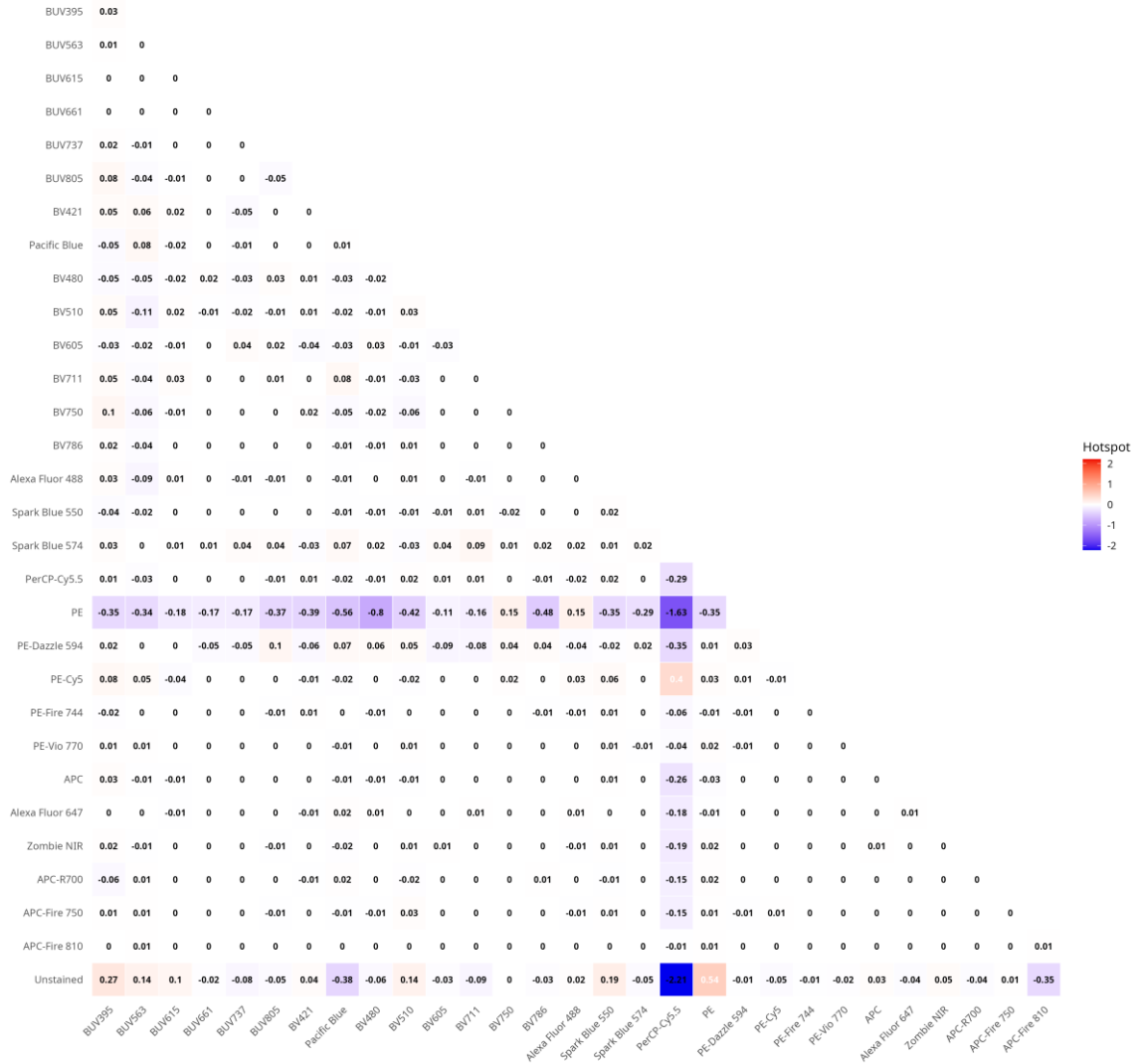
Increased Collinearity



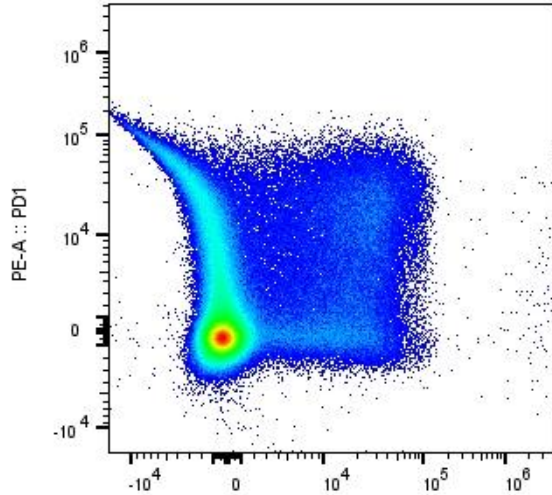
Increased Collinearity

PE →

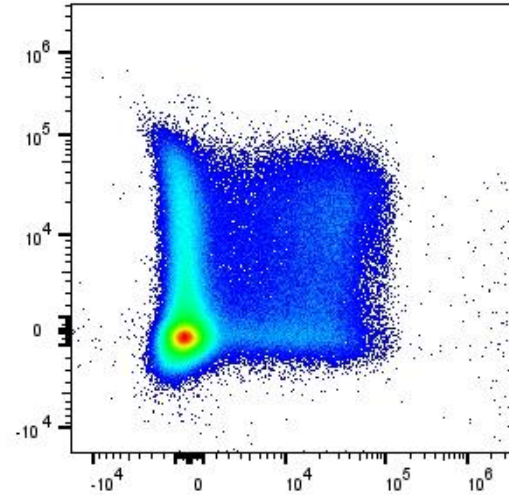
Unstained →



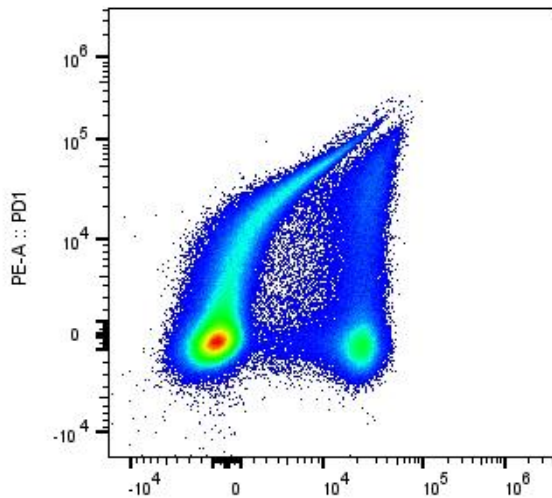
PE + residual AF PE – residual AF



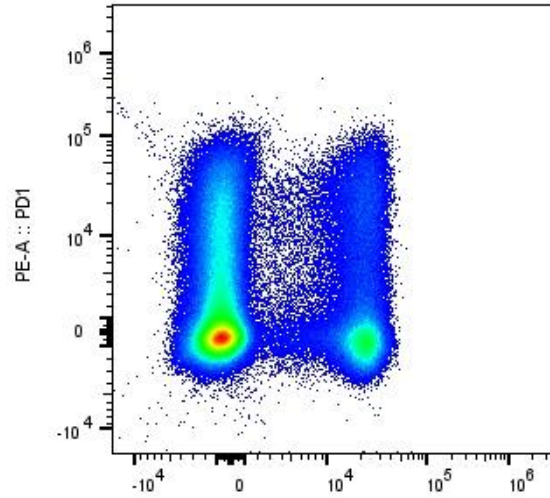
BV480-A :: CD161



BV480-A :: CD161

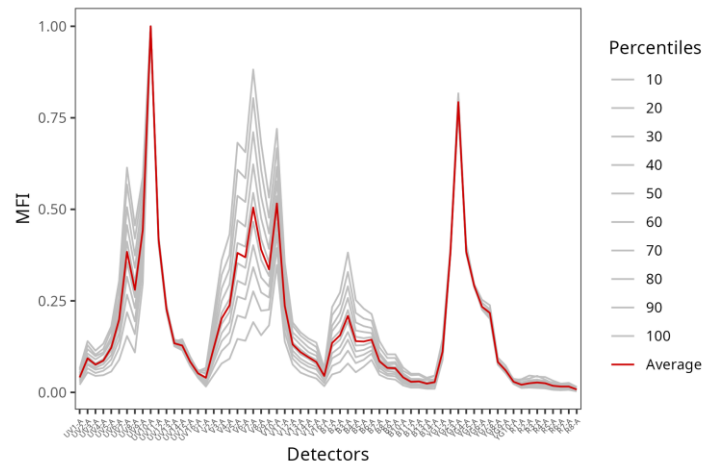
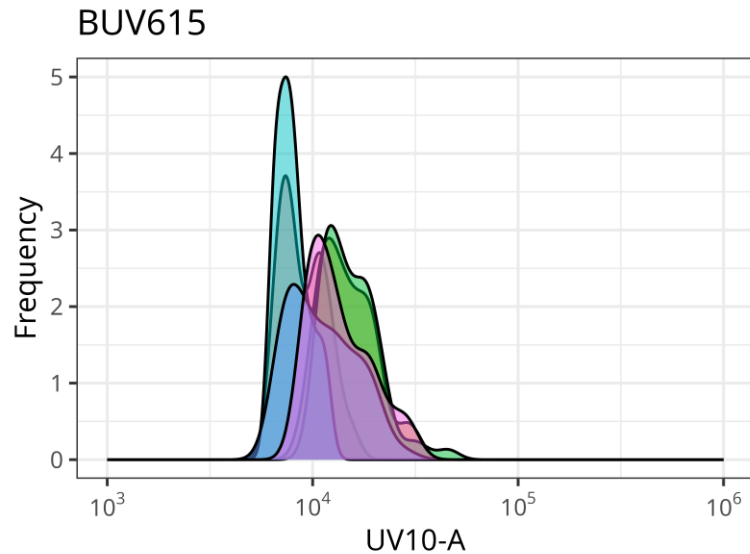


Spark Blue 574-A :: CD8

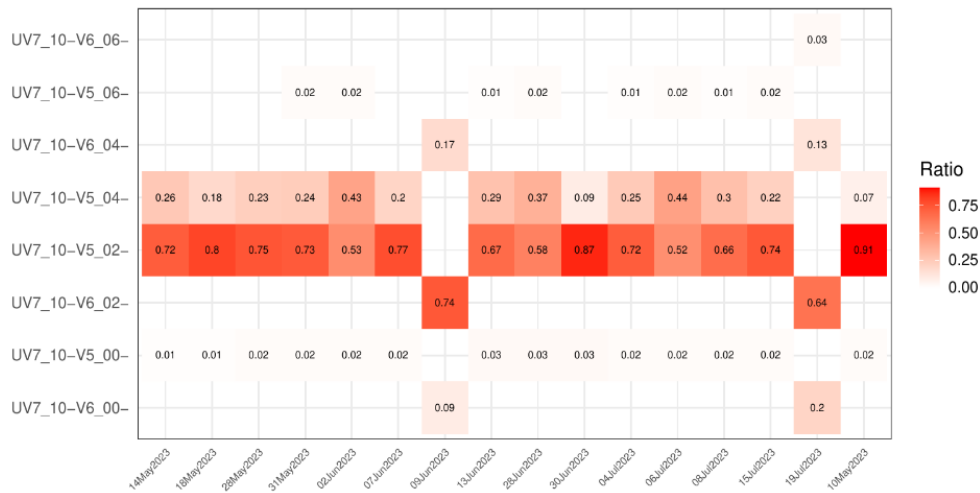


Spark Blue 574-A :: CD8

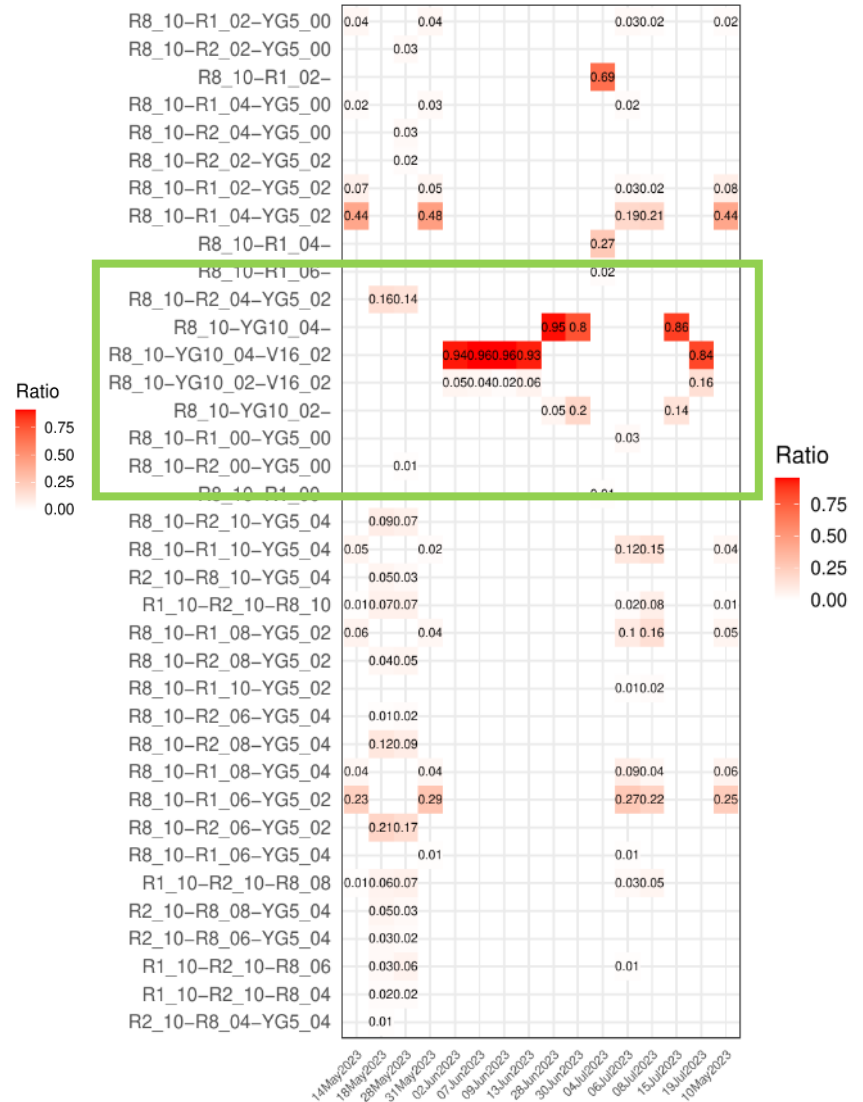
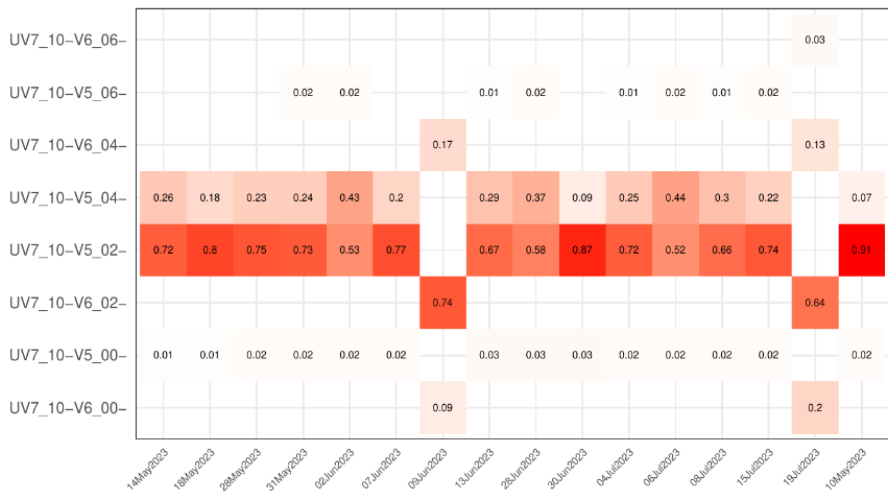
Some fluorophore-antigen combinations may just be too dim for cell controls



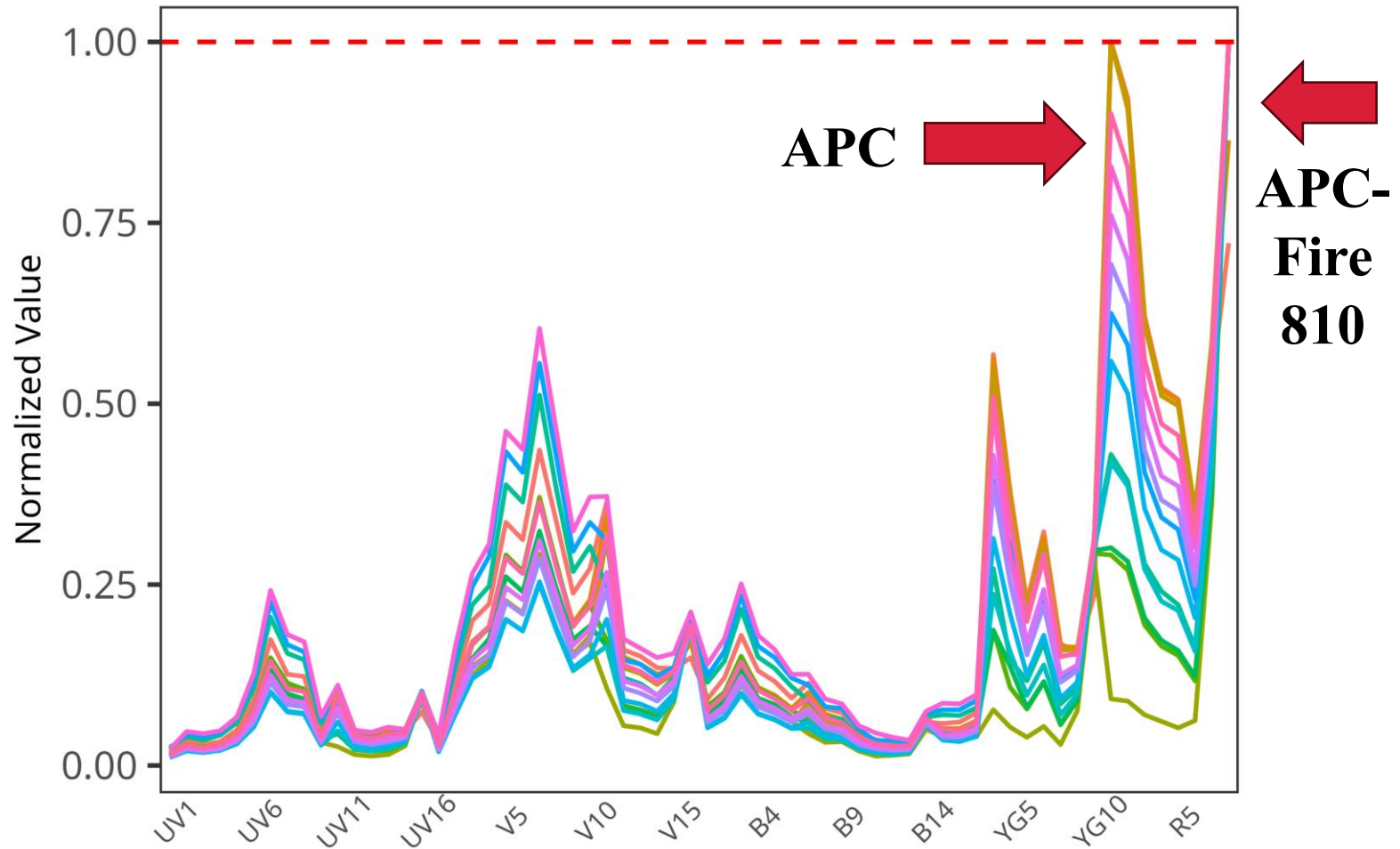
Fluorophore stability over time



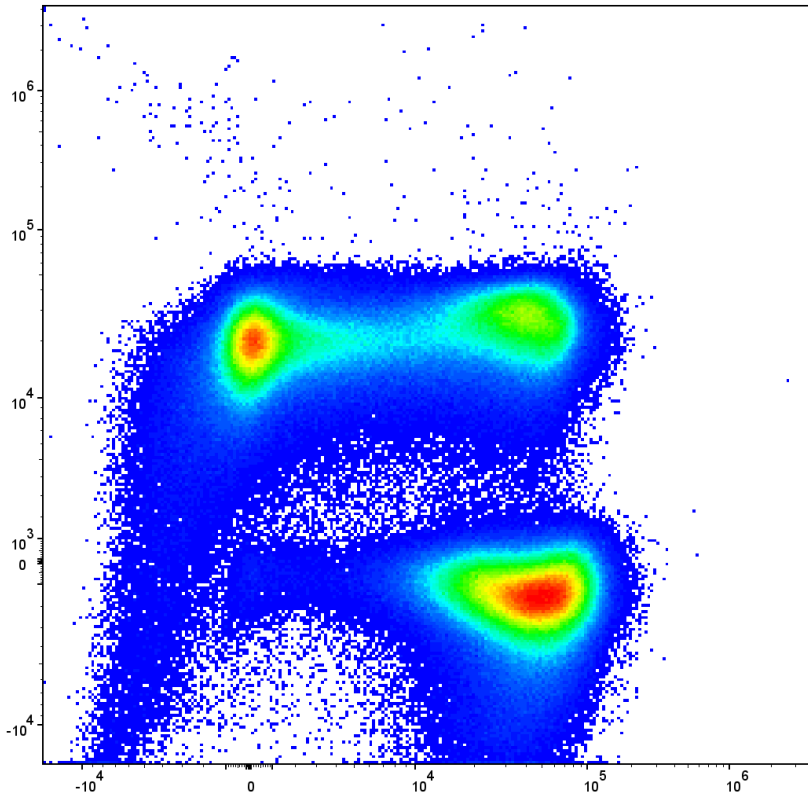
Detection of tandem degradation



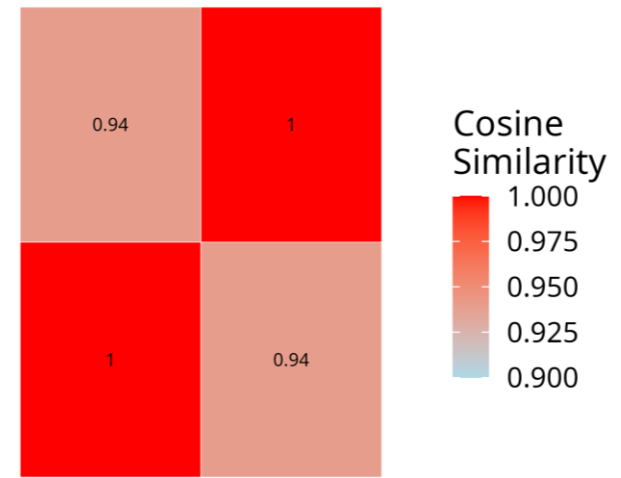
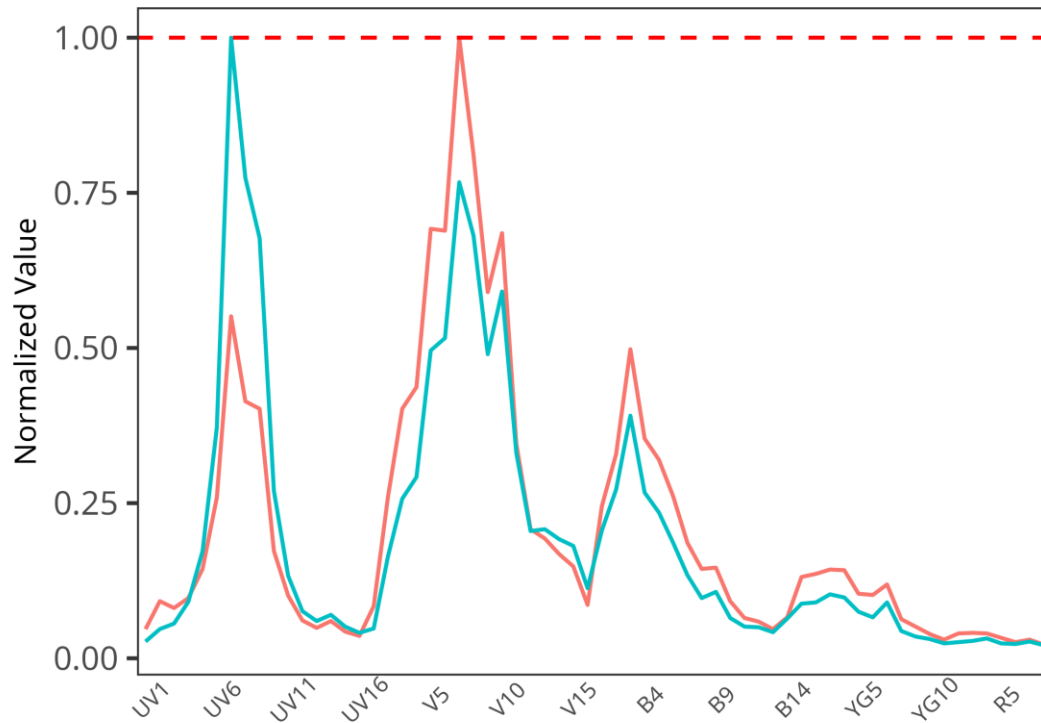
APC-Fire 810 -> APC



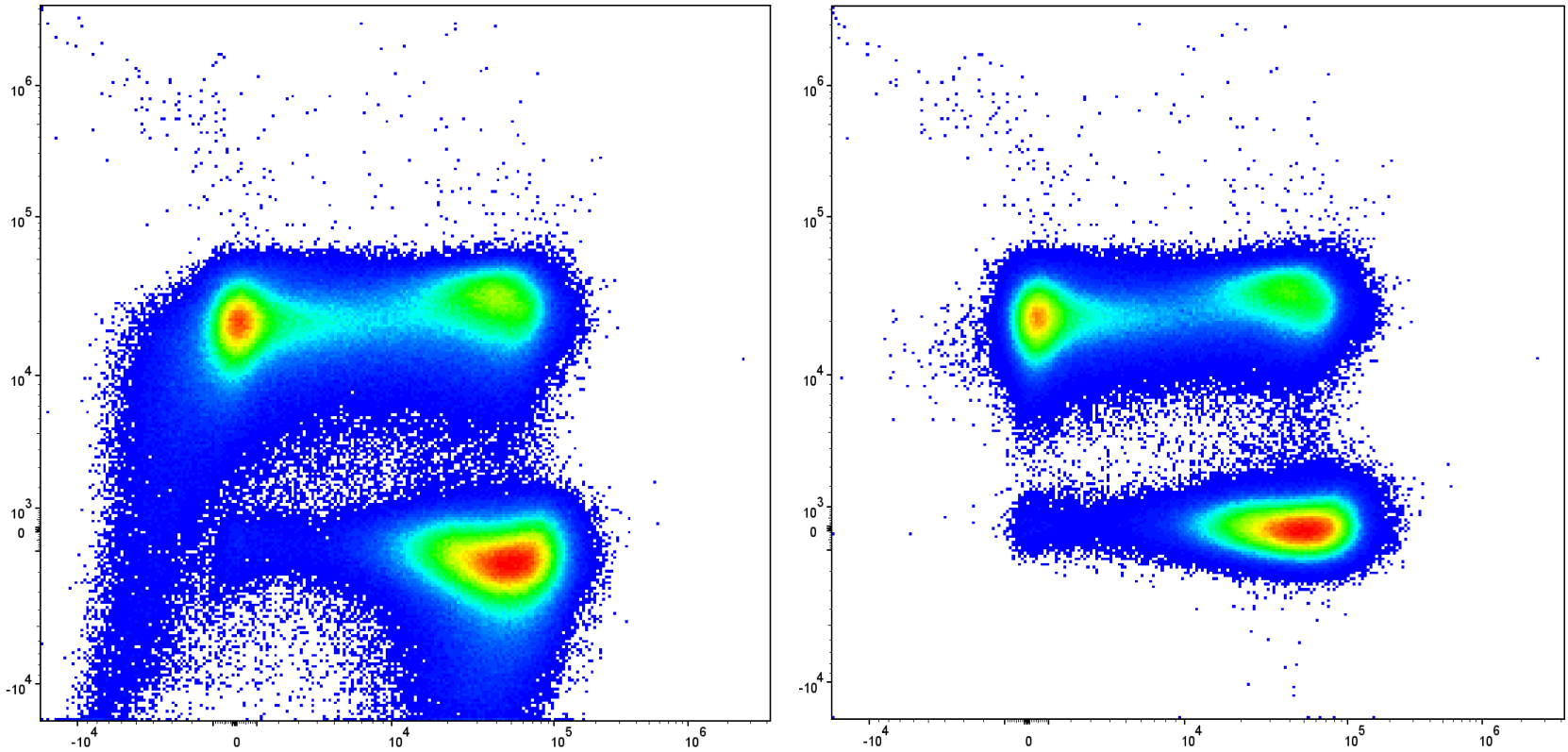
Autofluorescence Issues



Isolating autofluorescence



Isolating autofluorescence



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Dr. Eileen Barry
Dr. Brett Hassel

Flow Cytometry Discord
Flow Cytometry Reddit



Replicability & Reproducibility



My code to reproduce the analysis and figures for my CYTO posters can be found at <https://github.com/DavidRach/>

Our large dataset of single color and unstained raw .fcs files will be available on ImmPort soon (SDY3080)

My R packages are now on GitHub:
<https://github.com/DavidRach/Luciernaga>

Finishing documentation and catching occasional bug, will be submitting all to Bioconductor in the Fall

Questions?



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