FLOW CYTOMETRY CORE FACILITY KU LEUVEN

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KU Leuven Flow and Mass Cytometry Core Facility

Spectral Flow
Pitfalls and Possibilities

Vera Dermesrobian

11 March 2026

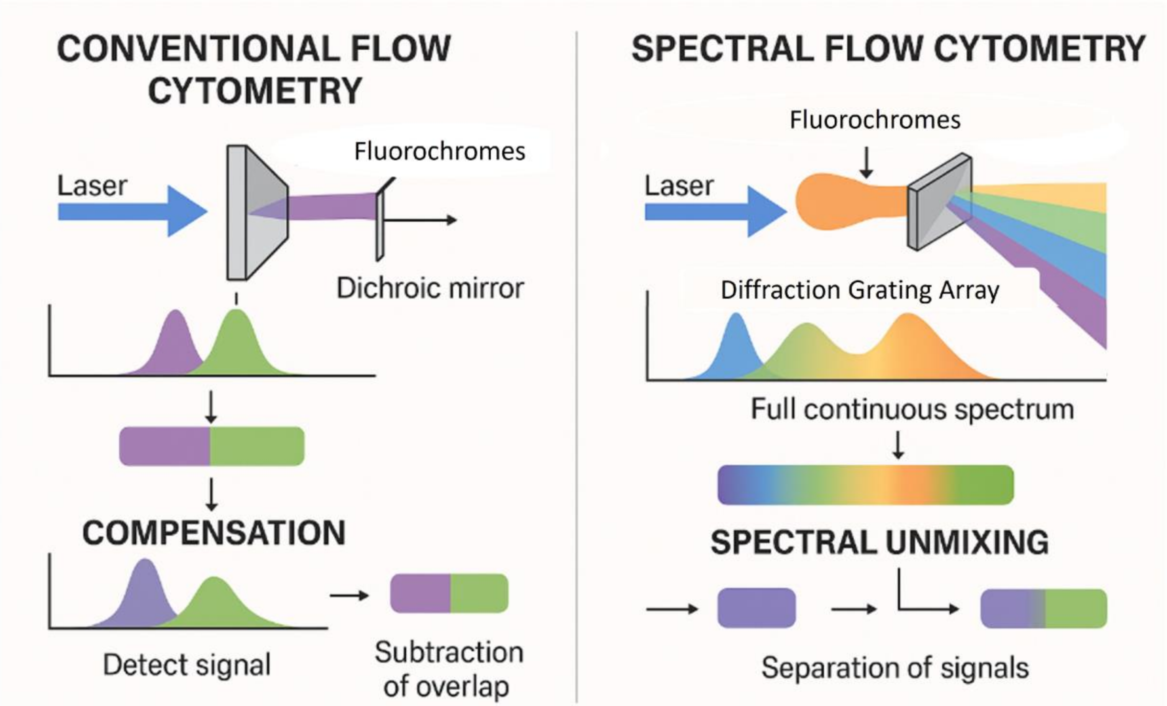
Why Was Spectral Flow Developed?

The field was pushing its limits.

- Increasing panel complexity.
- Limited filter-based detection in conventional flow.
- Overlapping fluorochromes.
- Restricted fluorochrome choices.

The technology evolved because the biology demanded it.

What Actually Changes?



Czechowska et al. 2025

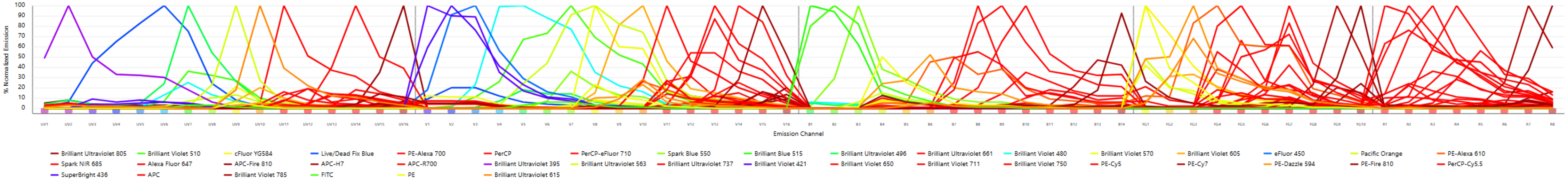
- Filter-based
- Peak emission
- Compensation

- Spectral signature
- Full spectrum
- Unmixing

Conventional cytometry samples fluorescence. Spectral captures it.

What Is Spectral Flow Measuring?

- Full emission profile across detectors.
- A spectral “fingerprint” for each fluorochrome.
- Signal contribution of each dye in every event.



OMIP-069 on FluoroFinder

Spectral Unmixing – Powerful, But Conditional

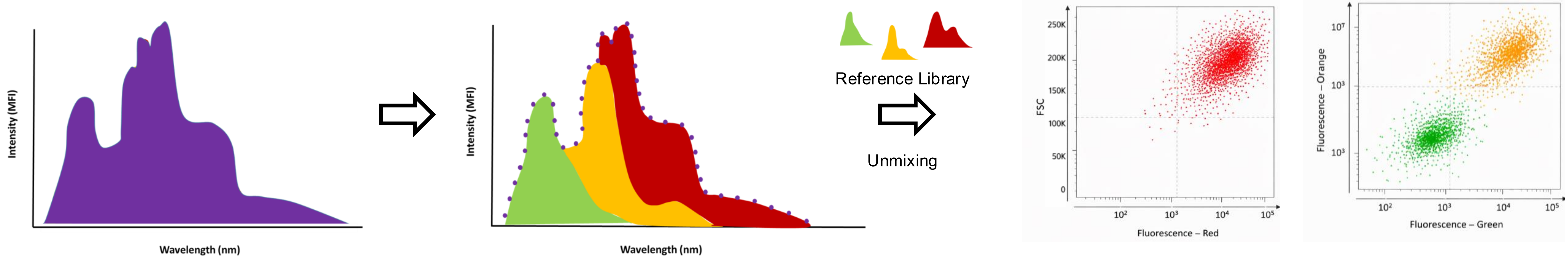
What Unmixing Does:

- Uses signal from all detectors to reconstruct one spectral profile per event.
- Identifies individual dye contributions in the total signal.
- Separates fluorophores into “pure” intensity values.

What it Depends On:

- Accurate single stain reference controls.
- Stable instrument performance.

Spectral is only as good as its reference library.



The Possibilities
High(er)-Parameter Panels

Full Spectrum Detection Enables:

- Separation by spectral signature, not peak emission.
- Resolution of highly overlapping fluorochromes.
- Expanded dye combinations feasibility.

→ 40 – 50 parameters panels reported.

Spectral increases flexibility, not infinite capacity.

ISAC CYTOMETRY

OMIP-069: Forty-Color Full Spectrum Flow Cytometry Panel for Deep Immunophenotyping of Major Cell Subsets in Human Peripheral Blood

Lily M. Park,[1] Joanne Lannigan,[2] Maria C. Jaimes[3]*

Received: 4 April 2024 | Revised: 11 September 2024 | Accepted: 14 September 2024
DOI: 10.1002/cyto.a.24900
OMIP
ISAC CYTOMETRY

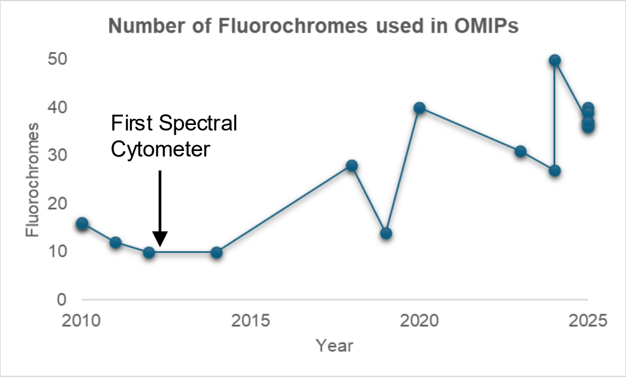
OMIP-109: 45-color full spectrum flow cytometry panel for deep immunophenotyping of the major lineages present in human peripheral blood mononuclear cells with emphasis on the T cell memory compartment

Lily M. Park[1] | Joanne Lannigan[2] | Quentin Low[1] | Maria C. Jaimes[1] | Diana L. Bonilla[1]

Received: 19 January 2024 | Revised: 27 March 2024 | Accepted: 2 April 2024
DOI: 10.1002/cyto.a.24841
OMIP
ISAC CYTOMETRY

OMIP-102: 50-color phenotyping of the human immune system with in-depth assessment of T cells and dendritic cells

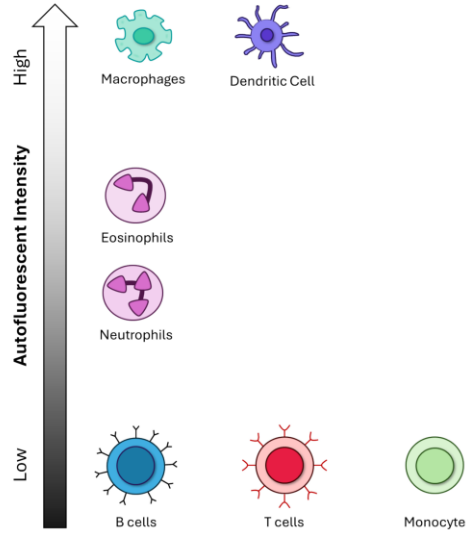
Andrew J. Konecny[1,2] | Peter L. Mage[3] | Aaron J. Tyznik[4] | Martin Prlic[1,2] | Florian Mair[1,5]



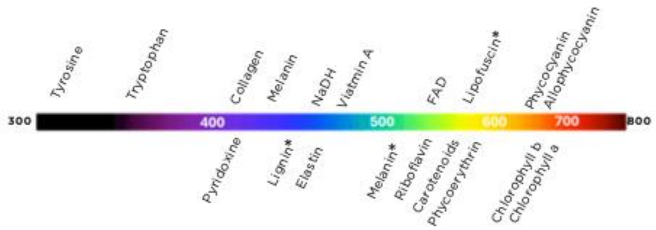
The Possibilities
Autofluorescence Extraction

Autofluorescence (AF) stops being a background and becomes a measurable informative parameter.

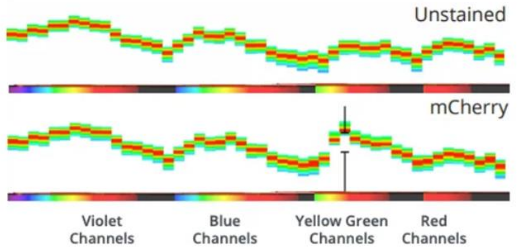
- Included in the unmixing matrix as an independent signature.
- Used to improve resolution in highly autofluorescent samples.
- Particularly useful for: tissue digests, myeloid populations, …

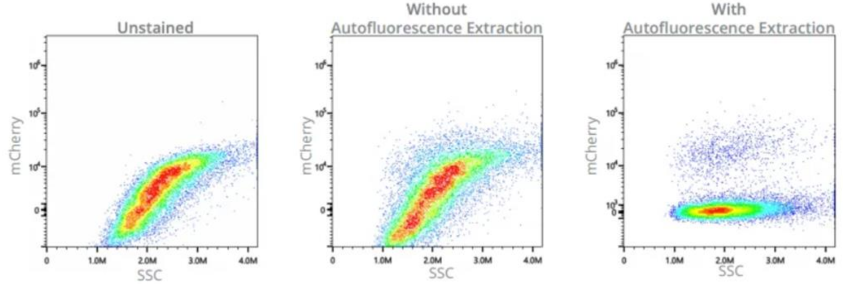


Knab et al. 2025



Beckman Coulter





Cytek Biosciences

The Possibilities
Autofluorescence Extraction

Autofluorescence (AF) stops being a background and becomes a measurable parameter.

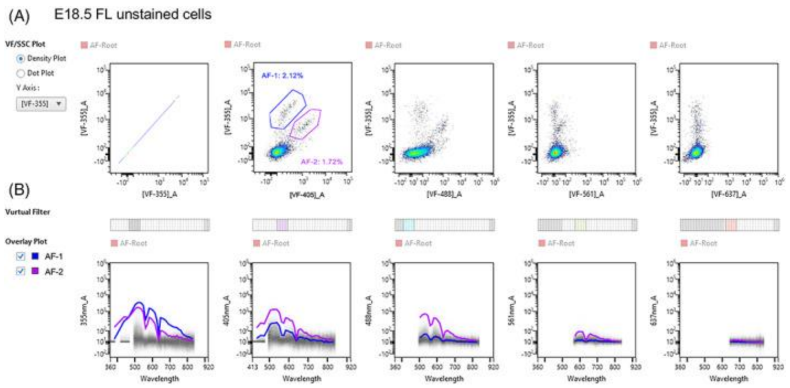
- Used to identify new cell subsets.

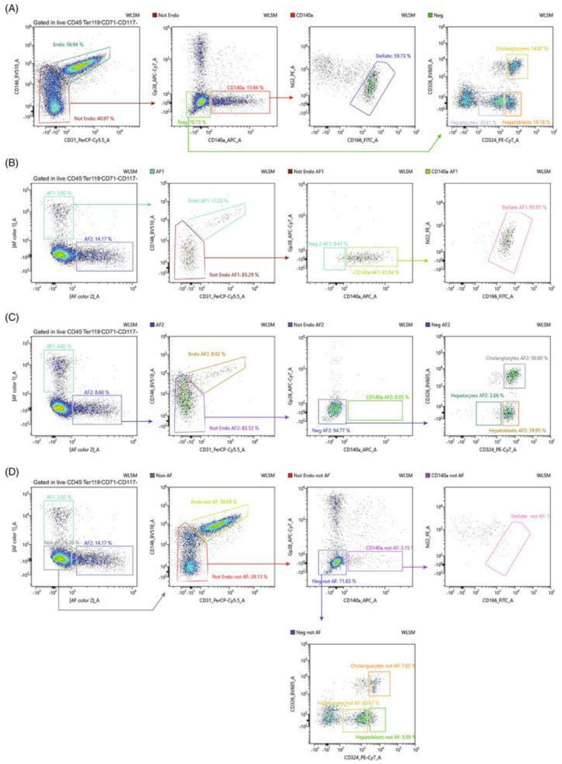
ORIGINAL ARTICLE

ISAC CYTOMETRY Journal of Quantitative Cell Science PART A

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]



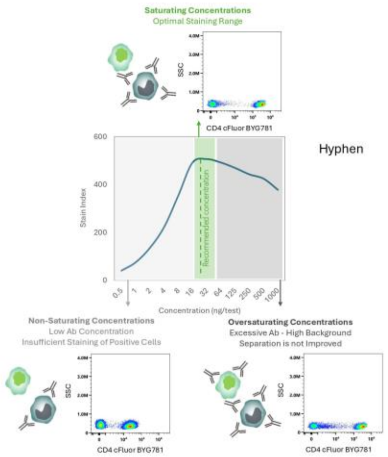


The Pitfalls

Panel Design is Harder, Not Easier

Panel Design Principles Remain.

- Fluorophore assignment: bright → dim markers.
- Antibody titration & stain index calculation.
- Backbone vs weak markers and co-expression rules.
- Fluorescence spread.



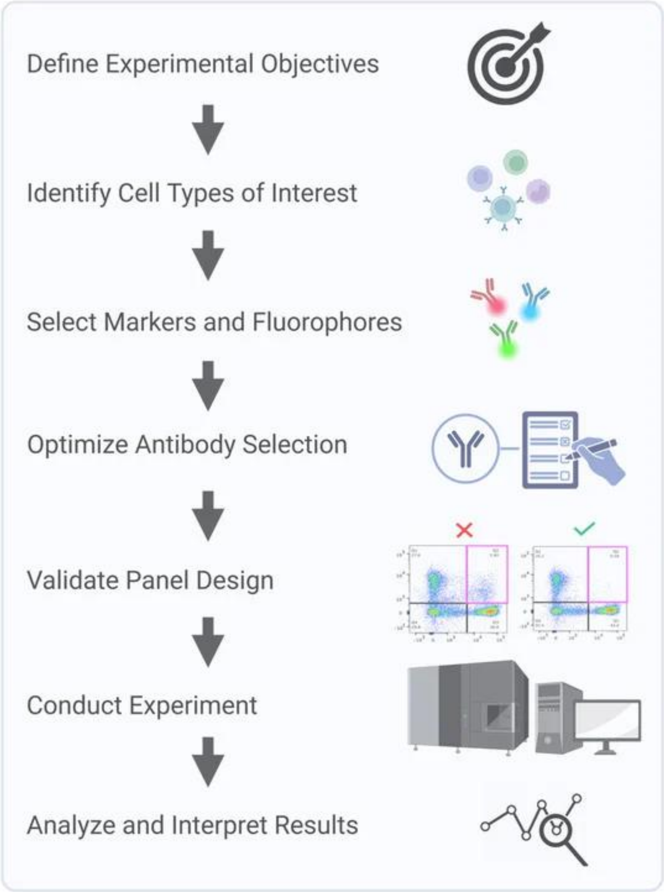
Jaimes et al. 2024

Fundamentals still apply, but spectral adds extra considerations.

The Pitfalls
Panel Design is Harder, Not Easier

New Spectral Considerations:

- Spectral similarity.
- Complexity index.
- Unmixing-dependent spread.
- AF handling.
- Tandem dye breakage effects.

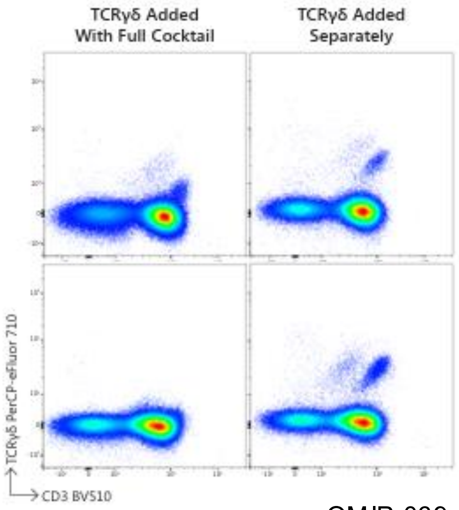


Czechowska et al. 2025

The Pitfalls
Sample Handling – Preparation and Staining

- Digestion, fixation, and permeabilization can alter signal, fluorochrome stability, and autofluorescence.
- Extended or sequential staining can improve resolution for dim or co-expressed markers.

Sample preparation and staining protocol can make or break your data.



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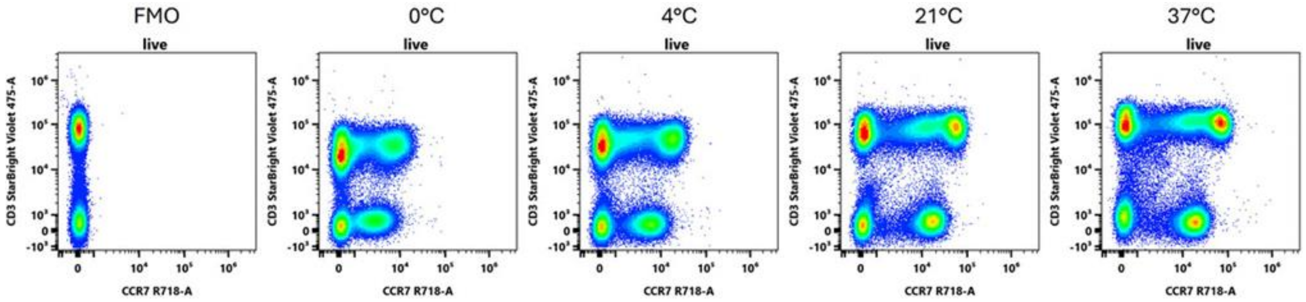


Effect of staining temperature on CCR7 and CD3 resolution in human PBMCs.
Burton et al. 2025

The Pitfalls

Single-Stain Controls Are Critical

Key rules for spectral reference controls:

- Positive population ≥ brightness of experimental sample.
- Positive and negative populations must have similar autofluorescence.
- Use the exact same antibody conjugate (same fluorochrome and lot).
- Reference controls must be processed identically to the full sample.

Unmixing quality depends entirely on reference controls.

The Pitfalls

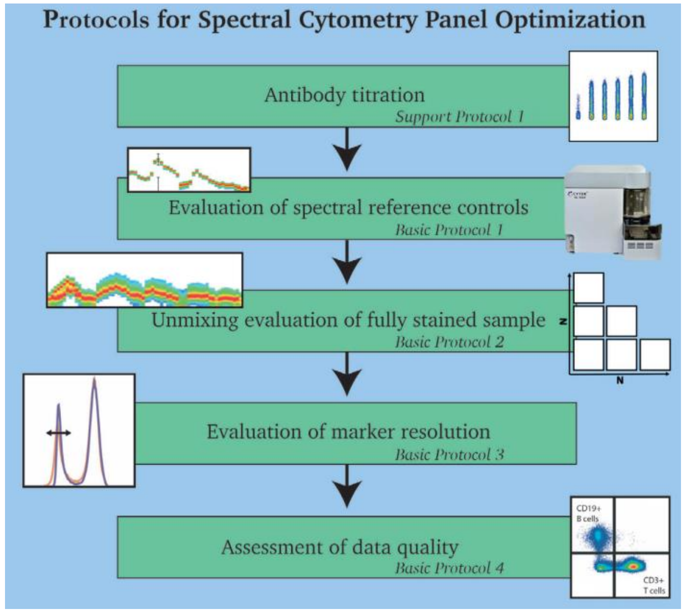
Single-Stain Controls Are Critical

Always evaluate the spectral signature.

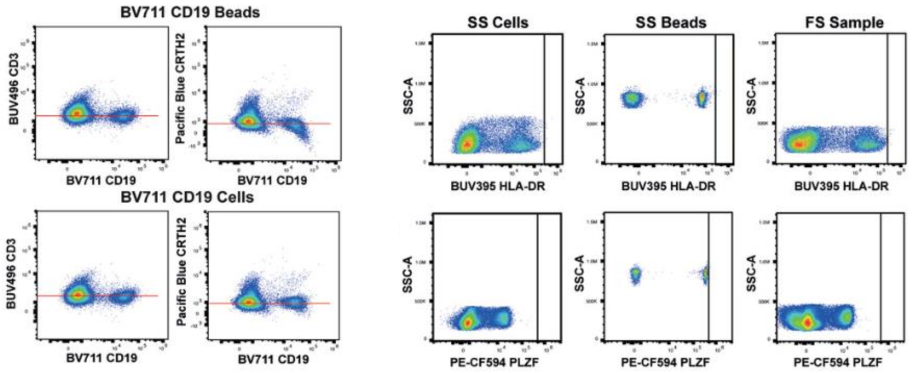
- Verify the spectral profile of reference controls.
- Check tandem dye stability.

Cells vs beads.

- Test both beads and cells.
- Use the control that best unmix the full sample.



Ferrer-Font et al. 2021



Ferrer-Font et al. 2021

The Pitfalls

Controls Are Critical

- Unstained control to assess AF.
- FMO controls for objective gating when populations are not clearly separated.
- FMM controls can help in large panels (20+ markers).
- Match controls to the same tissue or sample type whenever possible.

All controls must undergo the same handling as the fully stained sample.

The Pitfalls
Unmixing Models Differ

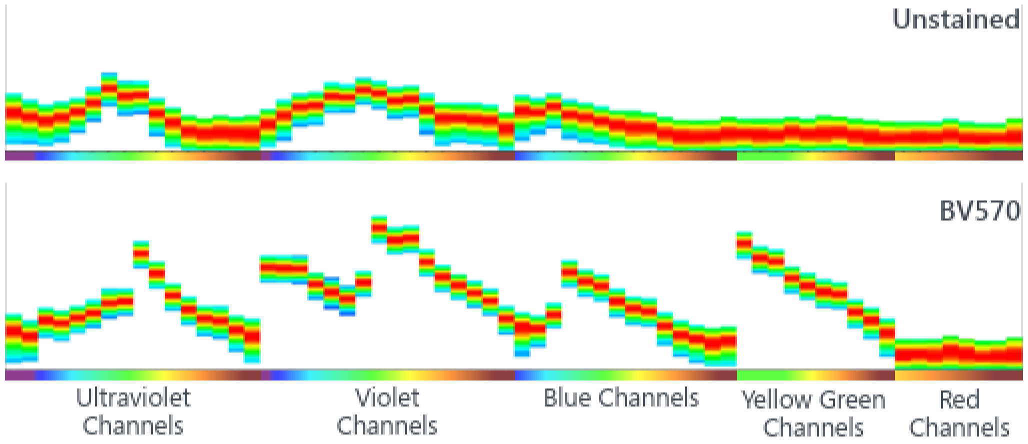
Unmixing Algorithm	Overview	Advantages	Limitations
Ordinary Least Squares (OLS)	Assumes linear combinations, constant noise across detectors.	Fast computation; good for high-intensity signals.	Sensitive to variable noise; may produce negative values; poor for dim markers.
Non-Negative Least Squares (NNLS)	Constrains OLS solutions to non-negative values.	Prevents negative intensities; robust for moderate signals.	May zero out weak true signals, reducing sensitivity.
Weighted Least Squares (WLS)	Applies weights to detectors based on noise level.	Improved dim marker resolution; accounts for heteroscedasticity.	Requires accurate noise estimation; more complex.
Poisson Regression-Based Unmixing	Models photon counting noise (variance proportional to mean).	Superior preservation of low-intensity signals; best biological match.	Computationally intensive; slower on large datasets.
Variance-Stabilizing Transformation (VST)	Stabilizes variance before linear unmixing.	Simplifies unmixing; efficient for moderate noise correction.	Approximate; depends on transformation quality.

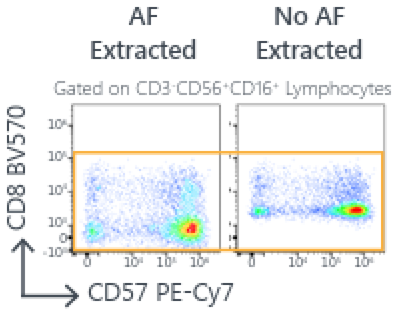
Czechowska et al. 2025

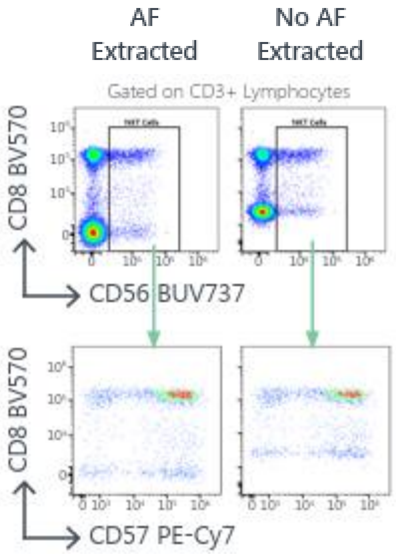
The Pitfalls
Autofluorescence Extraction Can Mislead

AF signatures are biologically context dependent: Cell type, metabolic state, and sample handling influence autofluorescence.

- AF can interfere with dim marker detection.
- AF spectra can overlap with fluorochromes, reducing marker separation *(Panel Design)*.
- Heterogeneous AF within samples complicates the extraction.







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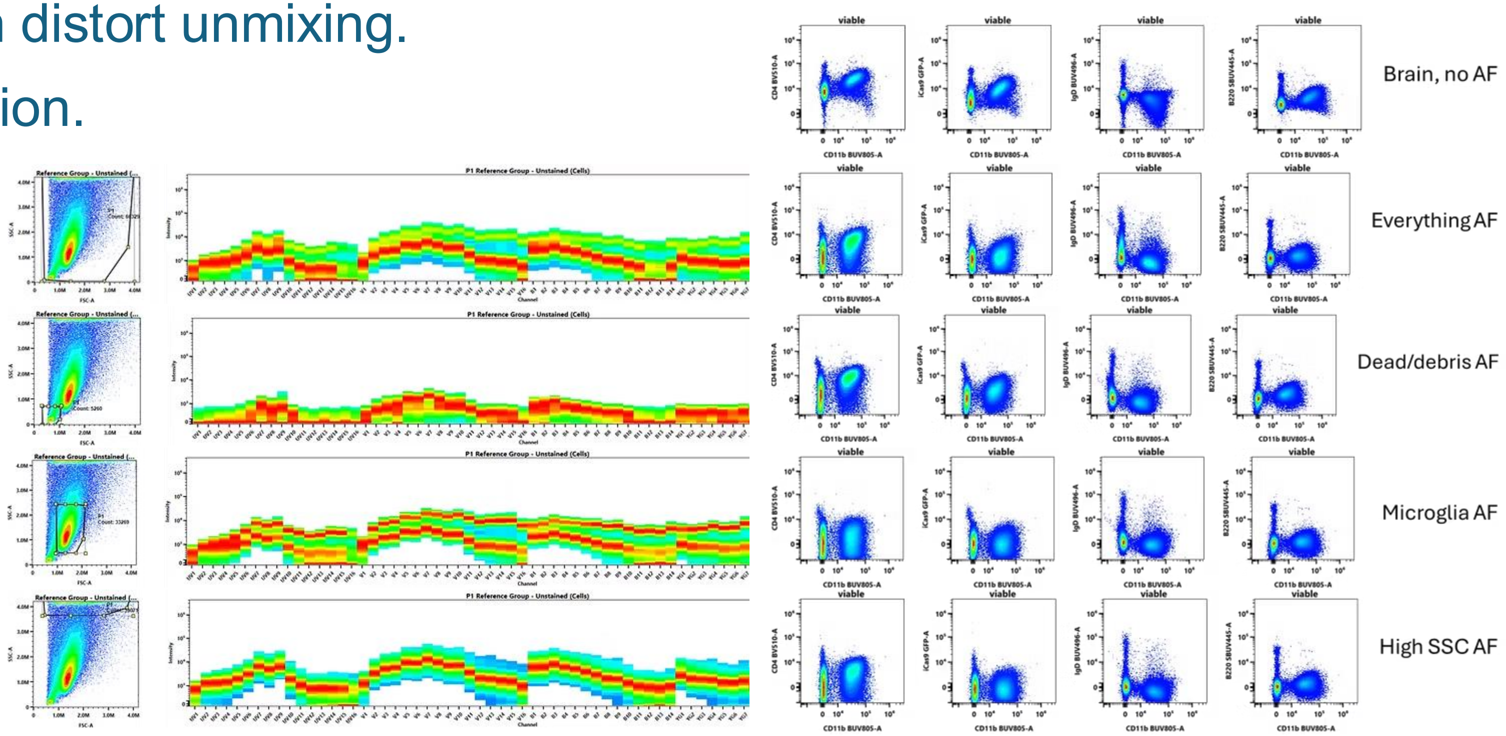
The Pitfalls
Autofluorescence Extraction Can Mislead

Gating for AF matters.

- AF spectrum depends on the cells included in the gate.
- Exclude debris, dead cells, and doublets.
- Incorrect gates can distort unmixing.
- Validate AF correction.



Colibri Cytometry

The Pitfalls

Spread Still Exists

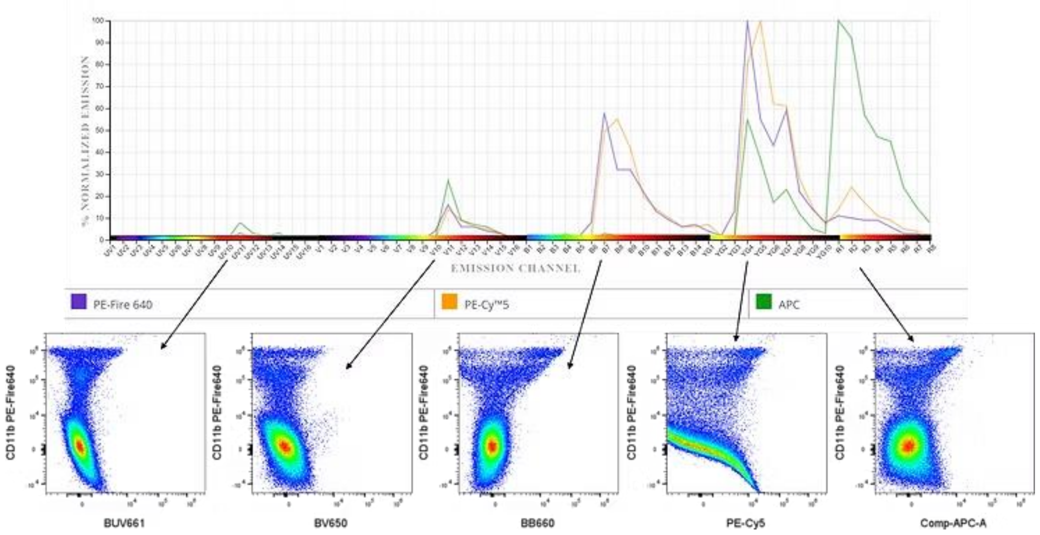
Spectral flow does not eliminate spread.

Spread is the increase in the variability of the signal in one detector caused by overlapping emission from other fluorochromes.

- Highly overlapping fluorochromes increase spread.
- Photon statistics and detector noise still govern spreading.

Tools to assess spread:

- Spillover Spreading Matrix (SSM).
- Unmixing-dependent spreading (HotSpot).



Colibri Cytometry

The Pitfalls
Data Analysis and Interpretation Complexity

High-dimensional panels increase analytical complexity.

Risks:

- False discoveries.
- Overclustering.
- Artifacts misinterpreted as biology.
- Batch effects between runs can confound results.

High dimensionality ≠ high confidence.

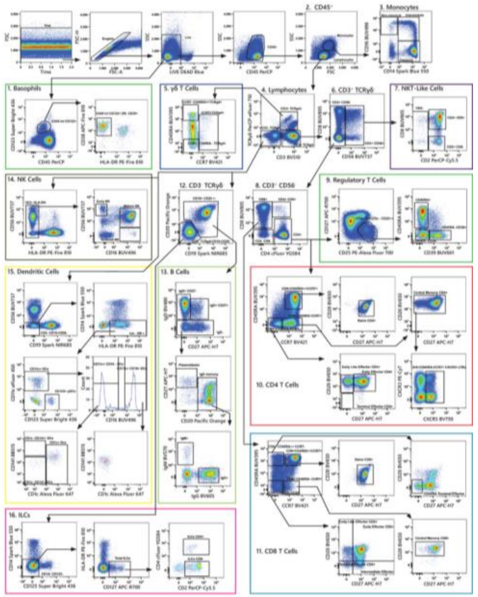
The Pitfalls

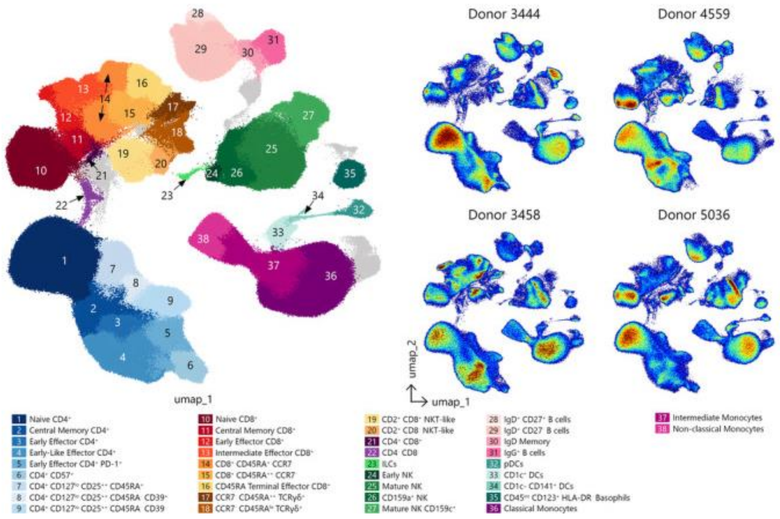
Data Analysis and Interpretation Complexity

Advanced computational tools (UMAP, FlowSOM, CITRUS, t-SNE, etc.) can aid analysis and visualization.

Results must be cross-checked:

- Validate against conventional gating.
- Replicate with independent methods.





OMIP-069

Spectral Flow in a Core Facility

Expectation	Reality
Free fluorochrome choice	Panel design still matters
Unmixing fixes panel issues	Unmixing cannot fix poor design
Controls similar to conventional flow	Controls are even more critical
Limited advantage over conventional flow	Strong advantages once adopted
A replacement for conventional flow	Ideal for large panels and autofluorescent samples

Spectral flow expands possibilities, but good cytometry practices remain essential.

Key Takeaways

- Spectral flow is a powerful extension of flow cytometry, not a replacement of its fundamentals.
- Good controls are more important, not less.
- Thoughtful panel design and experimental planning remain essential.
- Autofluorescence extraction can be a major advantage when used properly.

Spectral flow can do everything conventional flow does and much more, but it should be used when the biological question truly benefits from it.

THANK YOU