Your pathway to new discoveries: from Spectral Flow Cytometry analysis to Single-Cell Multiomics

MB&C COURSE 2024

9th February 2024, Diepenbeek

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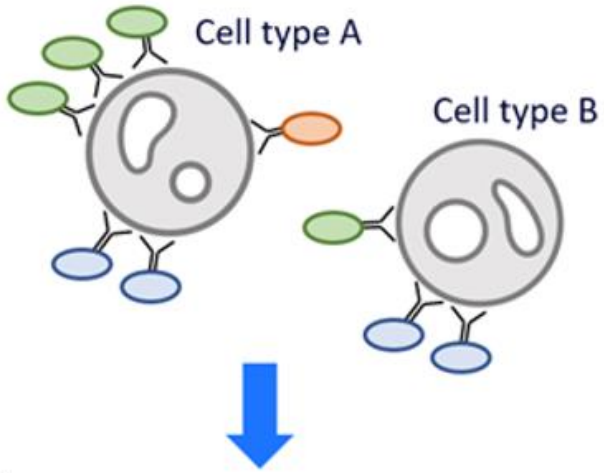
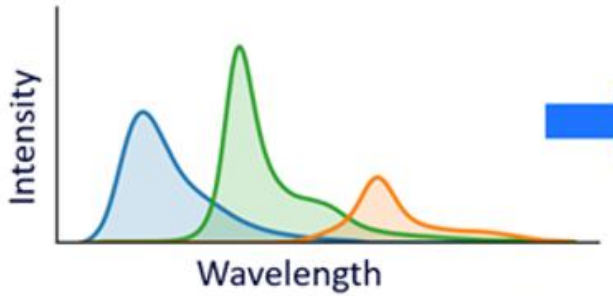
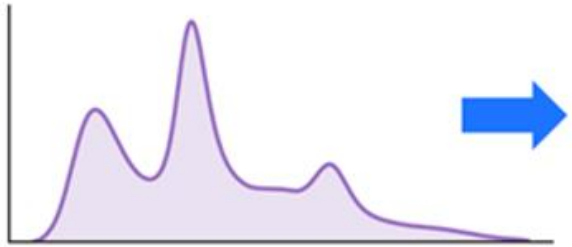
Flow cytometry is a process of signal transformation

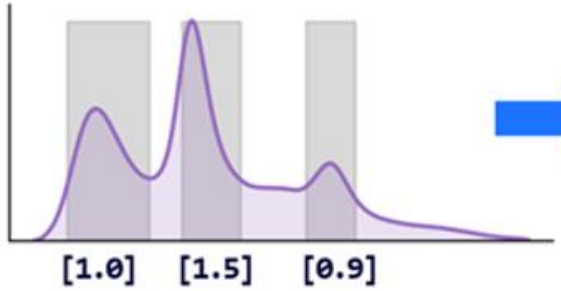




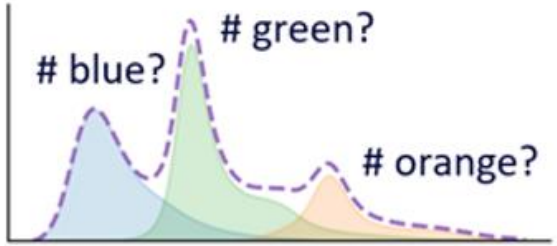
1. Fluorochromes on the cell emit colored light in proportion to their abundance.



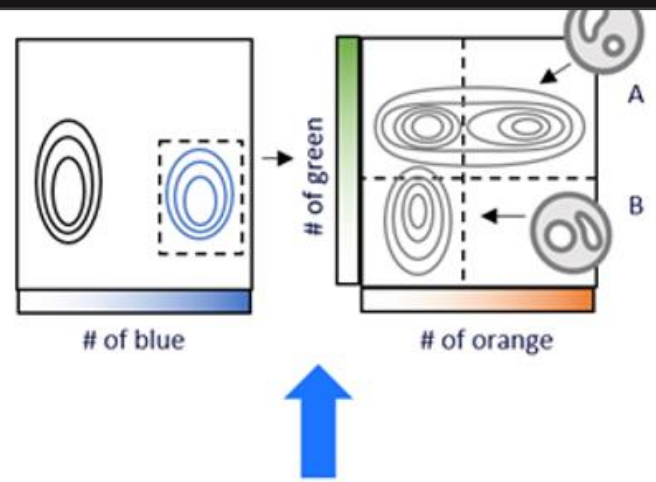
2. The total emitted light is the sum of each fluorochrome's contribution.



3. The cytometer samples some of the colored light into a digital signal.



4. Each fluorochrome's contribution is inferred from the measured digital signal.



Conventional and spectral flow cytometry

Goal:
Determine the contribution from each fluorochrome to the total measured signal

Two differences between spectral and conventional flow:

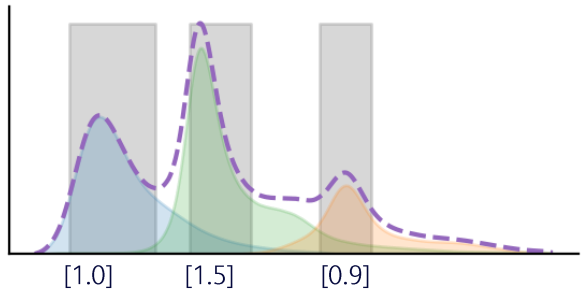
Difference 1 (hardware)

- *"How we convert colored light to digital signals"*
- Spectral uses more detectors than fluorochromes

Difference 2 (software)

- *"How we convert digital signals to biological data"*
- Spectral uses unmixing instead of compensation

Conventional

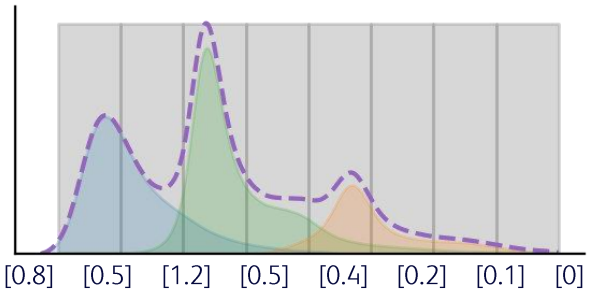


One detector per fluorochrome

"Compensation"

$$\begin{bmatrix}1.0\\1.5\\0.9\end{bmatrix} \xrightarrow{\text{math}} \begin{bmatrix}\text{2 blue}\\\text{3 green}\\\text{1 orange}\end{bmatrix}$$

Spectral



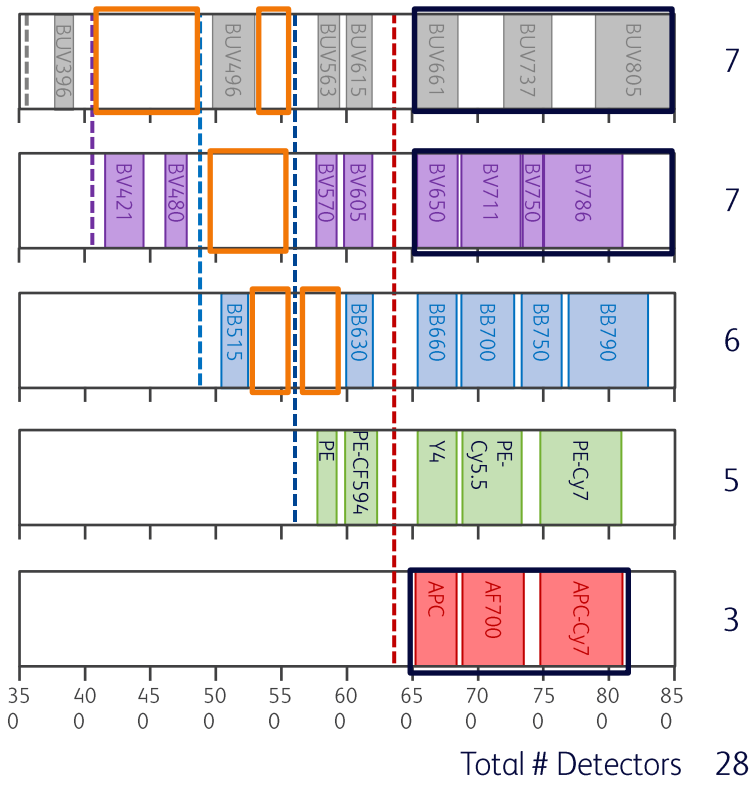
More detectors than fluorochromes

"Spectral unmixing"

$$\begin{bmatrix}0.8\\0.5\\1.2\\0.5\\0.4\\0.2\\0.1\\0\end{bmatrix} \xrightarrow{\text{math}} \begin{bmatrix}\text{2 blue}\\\text{3 green}\\\text{1 orange}\end{bmatrix}$$

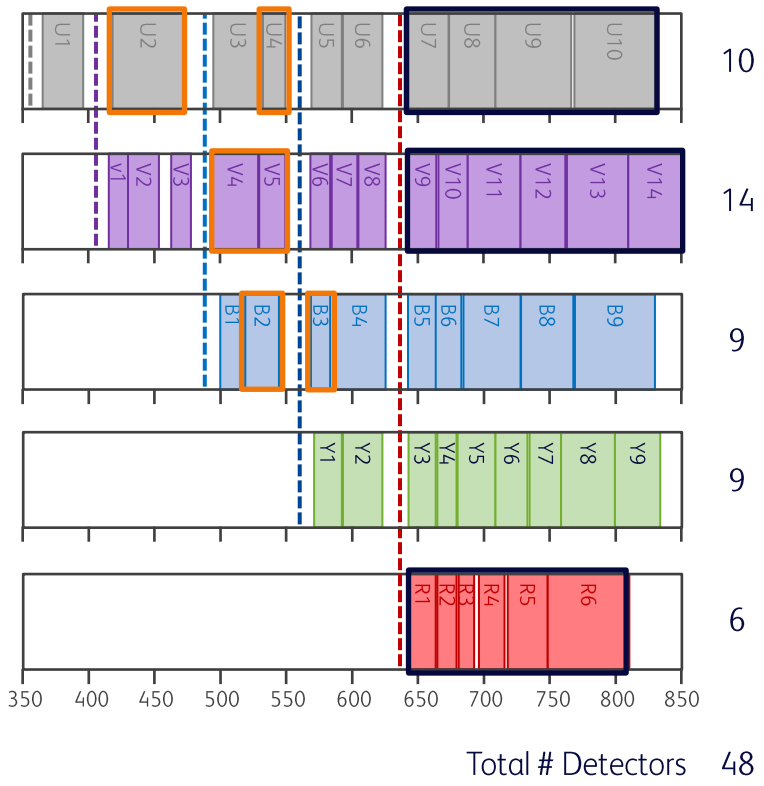
A spectral instrument uses the full visible spectrum

BD FACSymphony™ A5 Flow Cytometer



- Filters detect emission peaks
- Filters avoid laser lines

BD FACSymphony™ A5 SE Flow Cytometer



- Filters span the full spectrum
- Filters avoid laser lines

Additional detectors added to give more information across the spectrum

What remains the same?

Experimental workflow

- Sample preparation
- Sample staining
- Sample acquisition

Controls

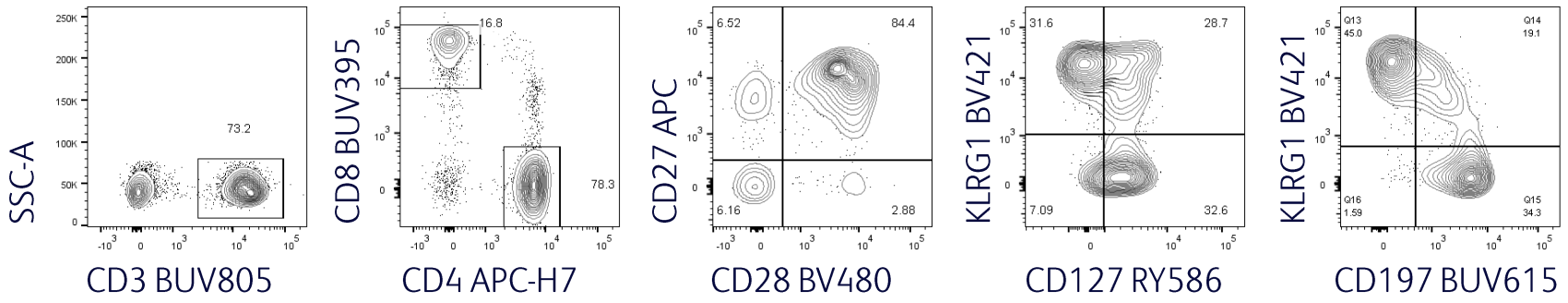
- Appropriate single stain controls for accurate unmixing
- Biological and FMO controls

Panel design approach

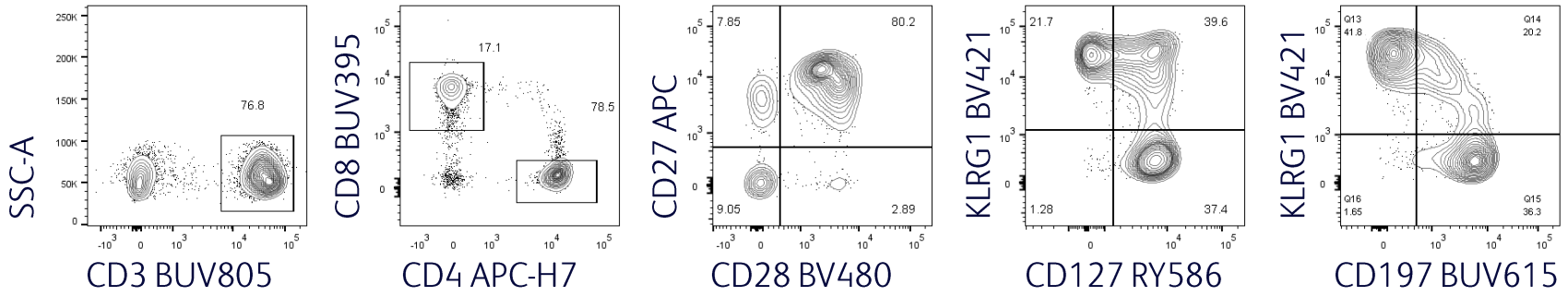
- Know your biology, fluorochromes and instruments
- Managing spread

Similar design and resolution of a 12-color biological panel

BD FACSymphony™ A5 Cell Analyzer – Conventional compensation



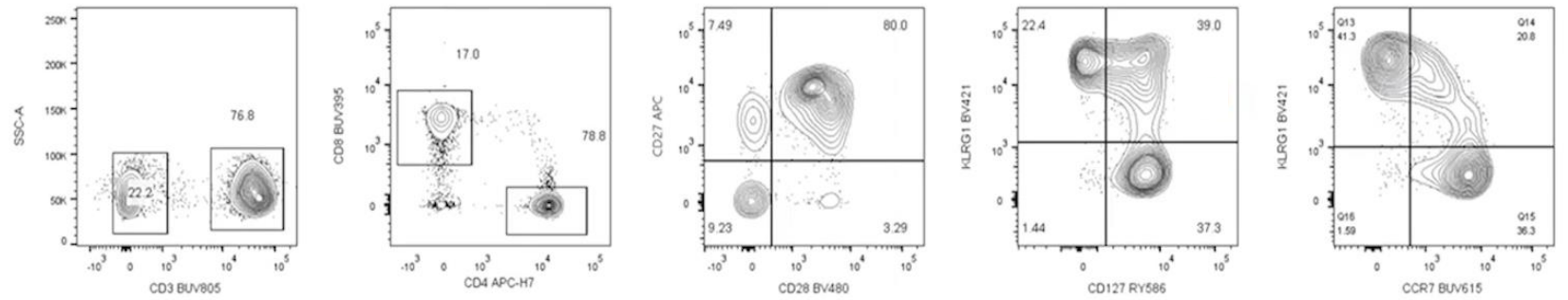
BD FACSymphony™ A5 SE Cell Analyzer – Spectral unmixing



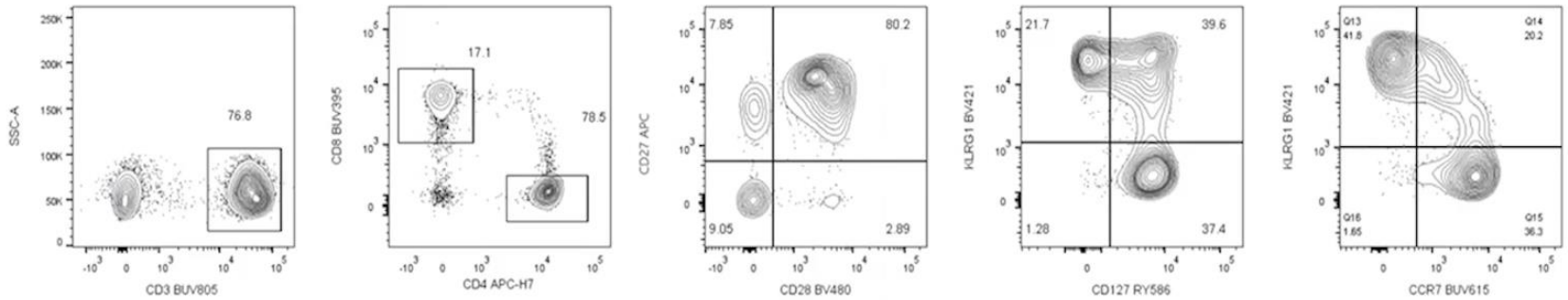
Same sample run on two different instruments with different settings and configuration

Similar design and resolution of a 12-color biological panel

BD FACSymphony™ A5 SE Cell Analyzer – Conventional compensation



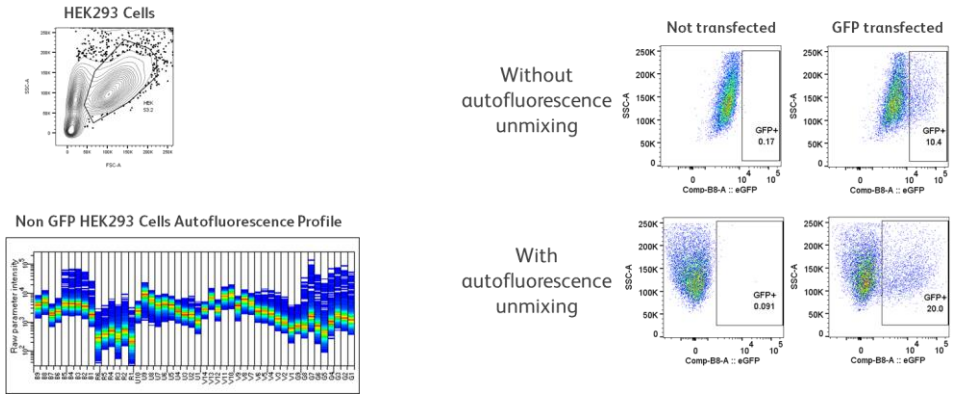
BD FACSymphony™ A5 SE Cell Analyzer – Spectral unmixing



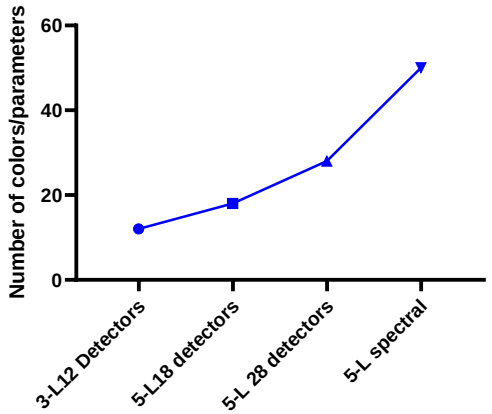
Same sample run on the same instrument with the same settings and configuration

Advantages of spectral flow cytometry

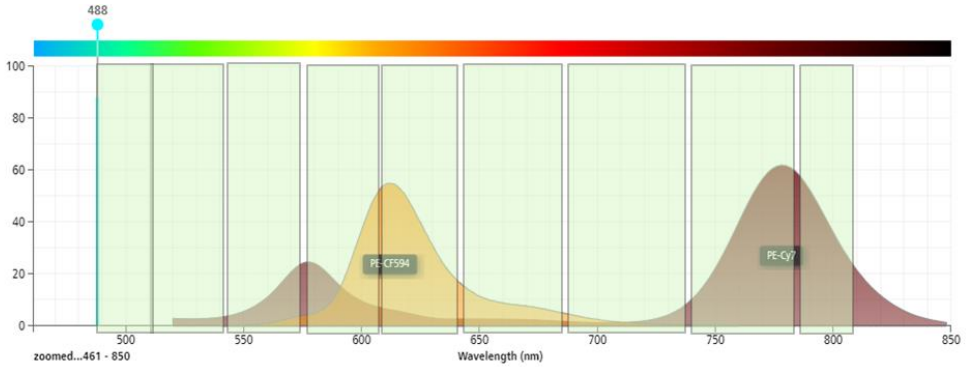
Autofluorescence extraction



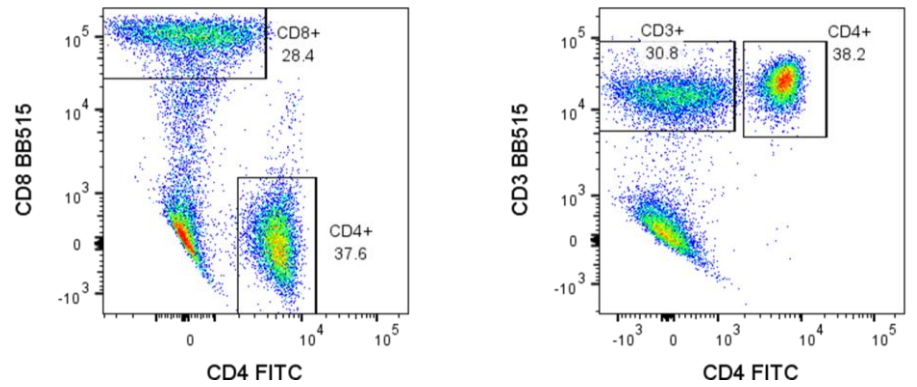
Increased panel size



Increased fluorochrome choice flexibility

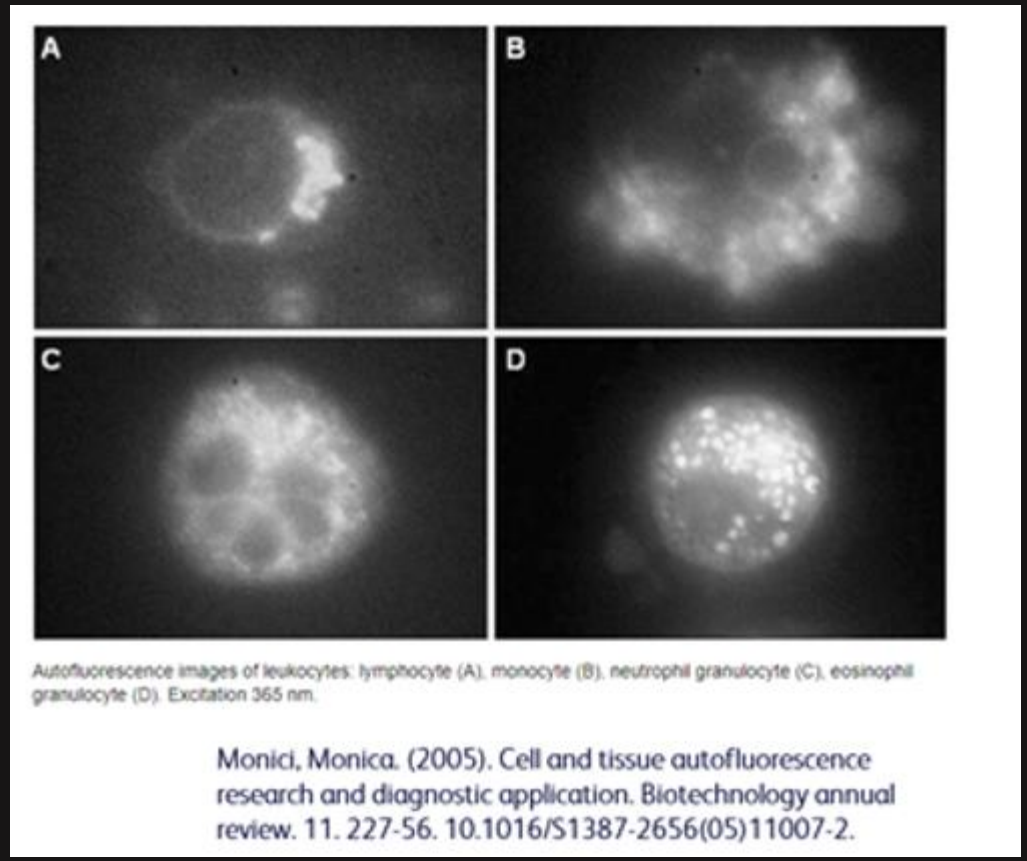


Use of highly overlapping dyes



Autofluorescence extraction

- The natural fluorescence of cells
- Cells contain compounds and organelles that are naturally fluorescent
- More pronounced in larger cells or cells with higher granularity (side scatter). For example, lymphocytes have lower autofluorescence than granulocytes
- Metabolic activity and experimental conditions also influence autofluorescence (proliferation, activation…).

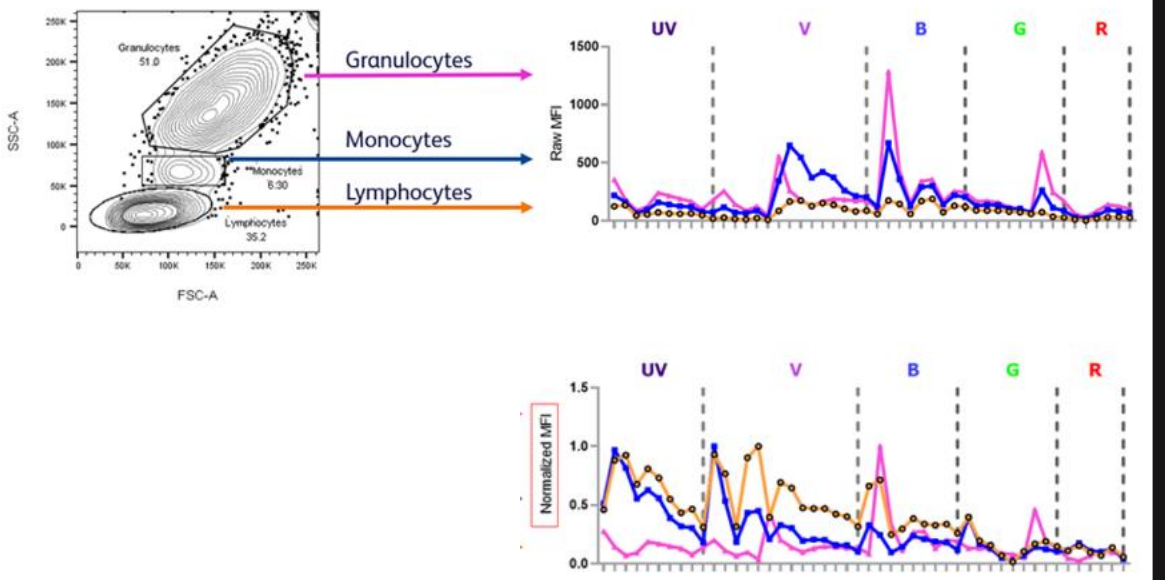


Autofluorescence images of leukocytes: lymphocyte (A), monocyte (B), neutrophil granulocyte (C), eosinophil granulocyte (D). Excitation 365 nm.

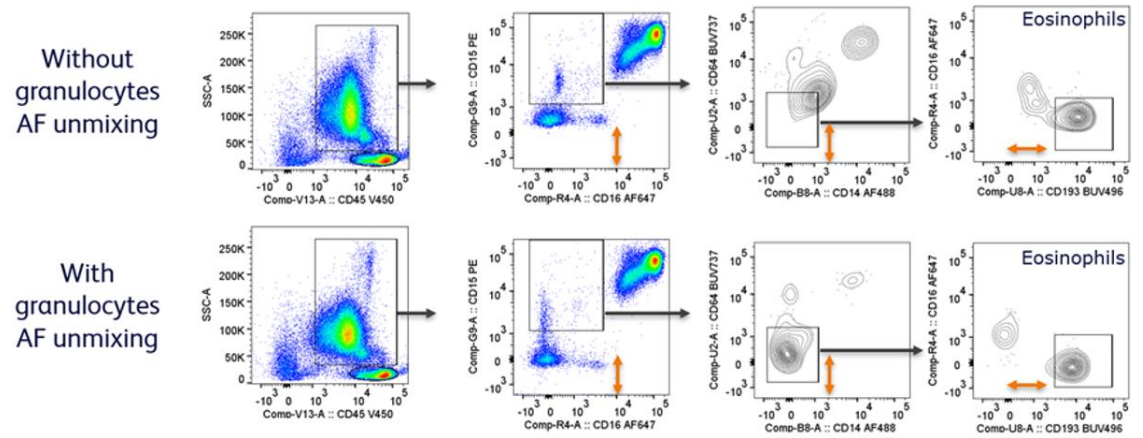
Monici, Monica. (2005). Cell and tissue autofluorescence research and diagnostic application. Biotechnology annual review. 11. 227-56. 10.1016/S1387-2656(05)11007-2.

Autofluorescence removal can improve resolution: granulocytes

Differences in autofluorescence can be measured within the same sample



Spectral

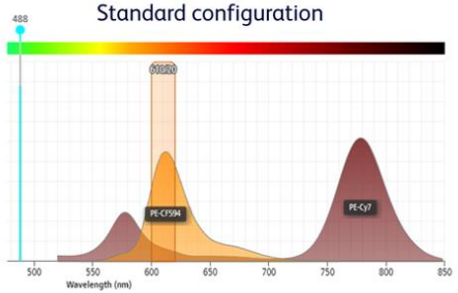


Fluorochrome choice flexibility

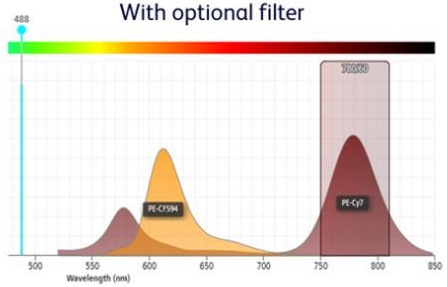
Conventional

Fluorochrome choice depends on laser availability and filter configuration

Standard configuration



With optional filter

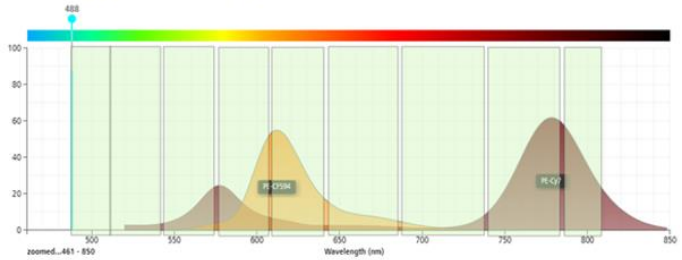


Either PE-CF594 **or** PE-Cy7 can be detected in this instrument

Spectral

Fluorochrome choice only depends on laser availability

Any spectral flow cytometer with a blue laser

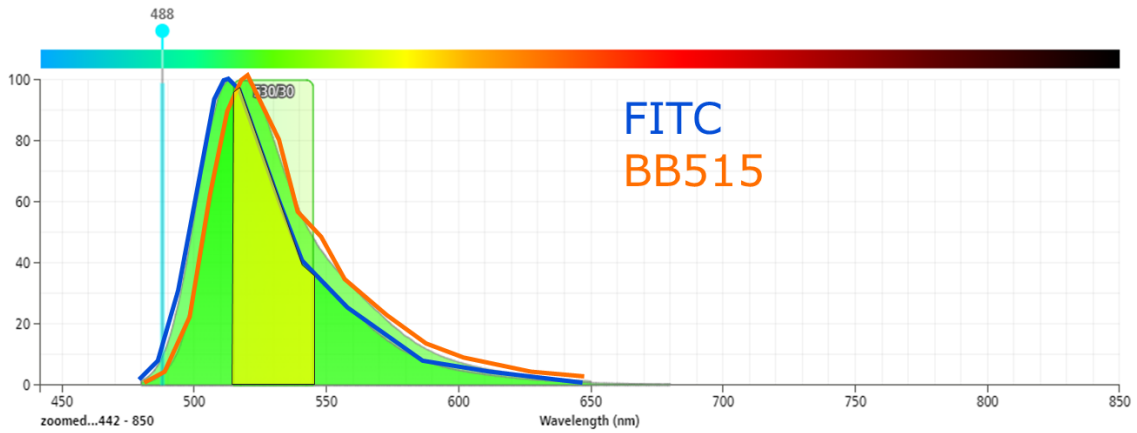


- By collecting the full spectrum, fluorochrome choice is not dictated by filter configuration anymore
- Any fluorochrome excited by a suitable laser can be detected
- This also results in higher flexibility and multiplexing capability, as **no fluorochrome needs to be sacrificed** to detect a new fluorochrome of interest

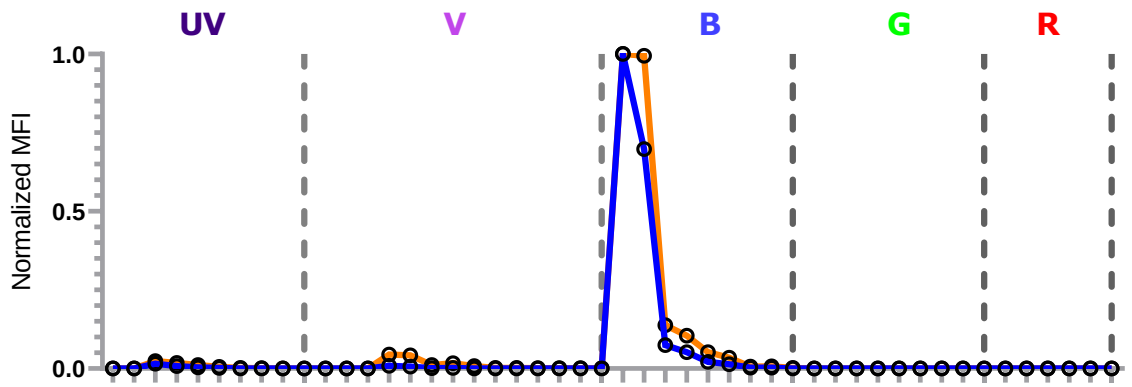
Use of highly overlapping dyes

Simultaneous use of highly similar fluorochromes undistinguishable in conventional flow cytometry enabled the design of large panels

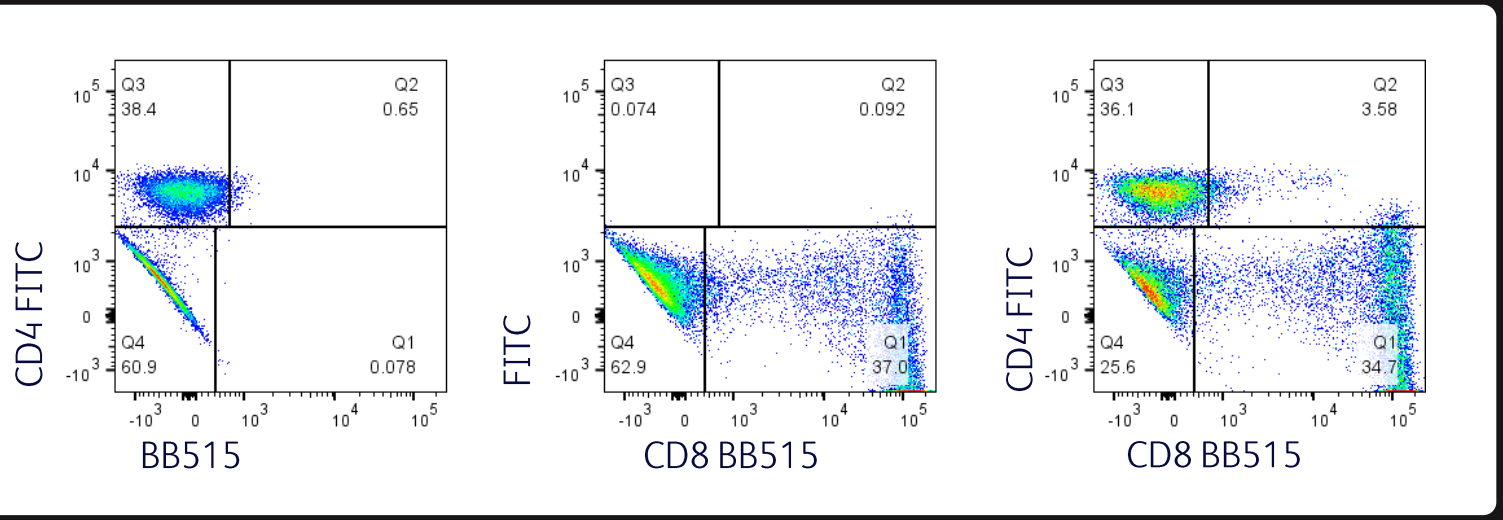
Conventional



Spectral



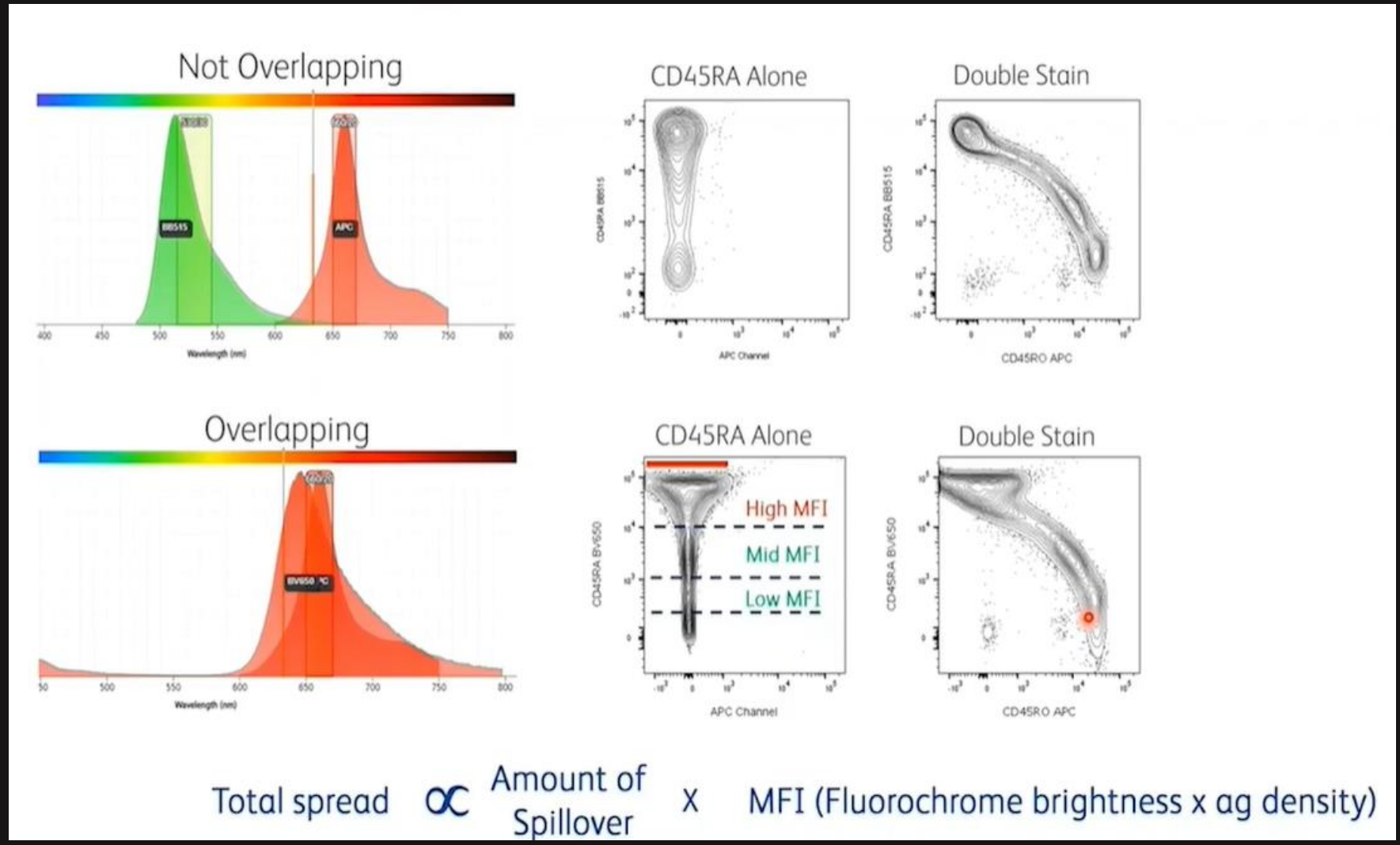
Use highly overlapping fluorochromes together



Although feasible, the simultaneous use of FITC and BB515 is challenging and limited due to high overlap resulting in high spread

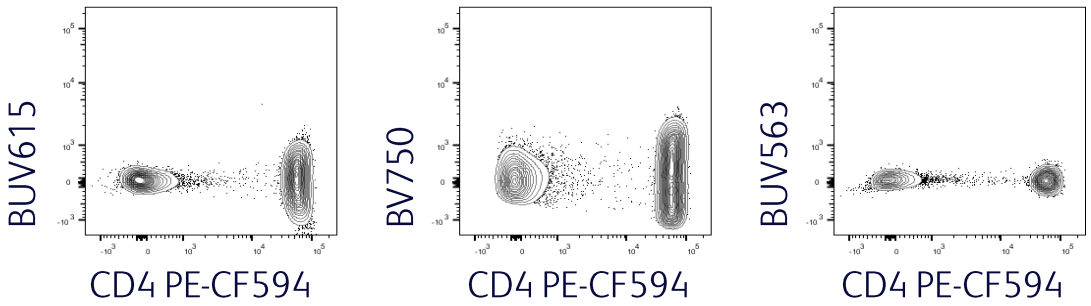
Fundamental panel design principles common to conventional and spectral flow cytometry

Spread can impact the biological resolution

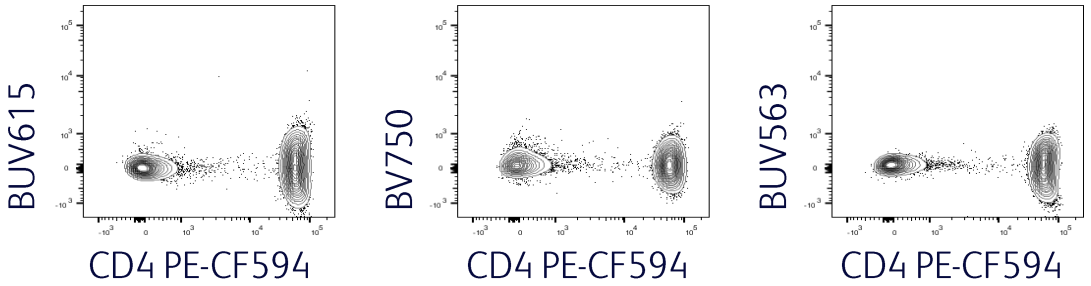


Spectral flow cytometry does NOT eliminate spread

BD FACSymphony™ A5 Cell Analyzer – Conventional compensation



BD FACSymphony™ A5 SE Cell Analyzer – Spectral unmixing



We still need to deal with spread in spectral flow cytometry in the same way as in conventional flow cytometry

The same panel design principles still apply for spectral flow cytometry

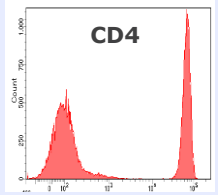
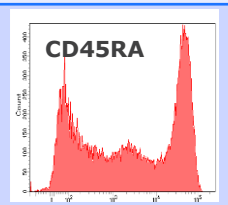
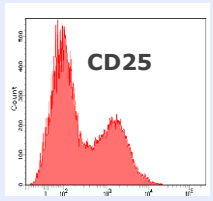
1. Understanding the biology:
- Level of co-expression
- Antigen density

2. Know your fluorochromes:
- Brightness (to be matched with antigen density)
- Spectral overlap

3. Know your instrument:
- Configuration
- Spread

Know the biology: co-expression and antigen density

Antigen categorization based upon expression pattern

Primary	typically **high** expressed and identify broad subsets or lineages; expression is usually **"on" or "of"** Examples: lineage markers like CD3, CD4, CD8, CD45	
Secondary	expressed at **intermediate** levels or over a **continuum** Examples: CD27, CD28, CD45RA, CD45RO	
Tertiary	expressed at **low** levels, **uncharacterized** antigens Examples: chemokine receptors	

Useful information in literature

OMIPs Collection

Optimized Multicolor Immunofluorescence Panel (OMIP) is a special peer-reviewed *Cytometry Part A* publication type that reports on newly designed and optimized multicolor panels for flow cytometry, fluorescence microscopy, image cytometry, and other polychromatic fluorescence-based methods. The first two OMIPs were published in the September 2010 issue of the Journal.

OMIPs are aimed: (1) to alleviate the development time for researchers in need of the same or highly similar panels, (2) to provide a starting point for the creation of novel OMIPs, and (3) to give the developers of the panels credit via citation or the publication.

CD Maps—Dynamic Profiling of CD1–CD100 Surface Expression on Human Leukocyte and Lymphocyte Subsets

Tomas Kalina[1,2†], Karel Fišer[1†], Martin Pérez-Andrés[3,4], Daniela Kuzilková[1], Marta Cuenca[5], Sophinus J. W. Bartol[6], Elena Blanco[3,4], Pablo Engel[5], and Menno C. van Zelm[6,7*], on behalf of the Human Cell Differentiation Molecules (HCDM) organization

https://doi.org/10.3389/fimmu.2019.02434

https://public.tableau.com/app/profile/fanny2212/viz/OMIP_ISAC/Menu

Know the fluorochrome characteristics: brightness and spectral overlap

Tools and resources

BD Life Sciences Relative Fluorochrome Brightness

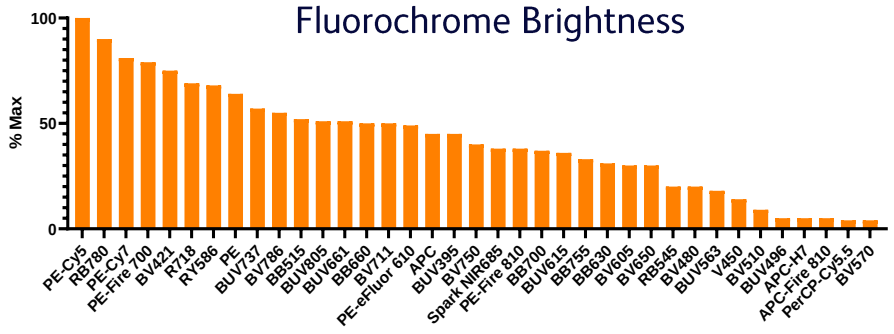
bdbiosciences.com/colors

This table provides general guidance with respect to the relative capability of different fluorochromes to resolve dimly stained populations; it is not a representation of absolute fluorescence. Rankings were determined by comparing the stain index (resolution) of cells stained with multiple formats on several clones run on a variety of flow cytometers. Many factors can influence the relative fluorochrome/reagent performance on a given instrument, including laser power, PMT voltage, optical filters, antibody clone and biological sample.

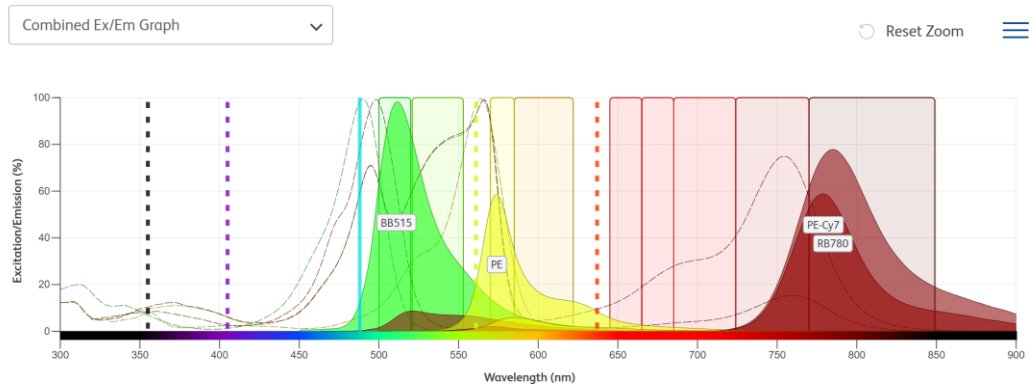
Laser	Fluorochrome: Very Bright	Bright	Moderate	Dim
Ultraviolet (355 nm)		BD Horizon™ BUV563 BD Horizon™ BUV661 BD Horizon™ BUV737	BD Horizon™ BUV395 BD Horizon™ BUV496	BD Horizon™ BUV805
Violet (405 nm)	BD Horizon™ BV421 BD Horizon™ BV650 BD Horizon™ BV711	BD Horizon™ BV480 BD Horizon™ BV605 BD Horizon™ BV786	BD Horizon™ BV510	BD Horizon™ V450 BD Horizon™ V500
Blue (488 nm)	BD Horizon™ BB515 BD Horizon™ BB700 BD Horizon™ PE-CF594 PE-Cy™5	PE PE-Cy™7	FITC Alexa Fluor® 488 PerCP-Cy™5.5	PerCP
Yellow/Green (561 nm)	PE BD Horizon PE-CF594 PE-Cy5 PE-Cy7			
Red (640 nm)		APC Alexa Fluor® 647 BD Horizon™ APC-R700		Alexa Fluor® 700 APC-H7 APC-Cy7

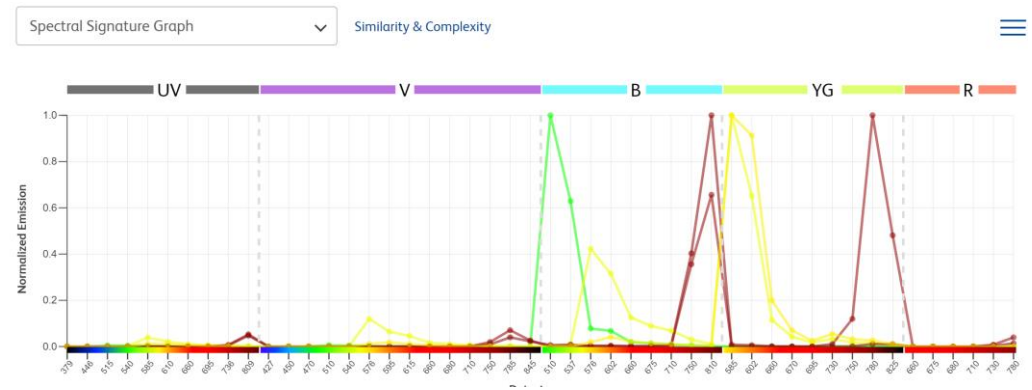
BD

Stain Index

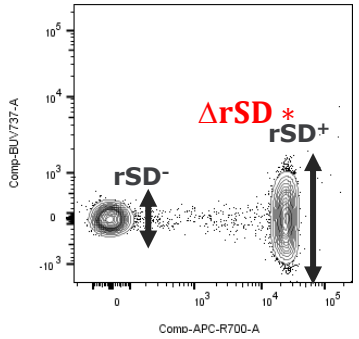


BD SpectrumViewer





Tools to predict spread: CD4 Total Spread Matrix

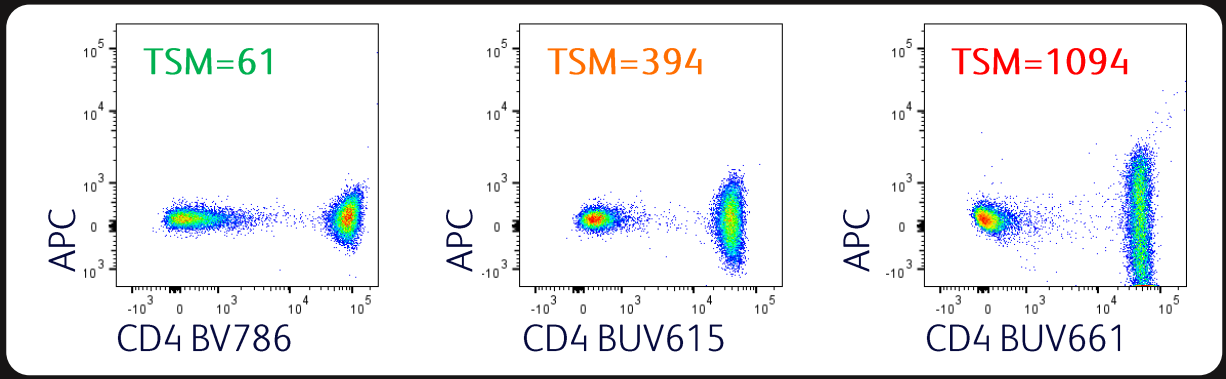


*Mathematically equivalent to

$$\Delta\sigma_C = \sqrt{{}^{S}\sigma_C{}^2 - {}^{R}\sigma_C{}^2}$$

	FITC	PerCP-Cy5.5	RB780	APC	AF700	APC-Cy7	BUV395	BUV496	BUV563	BUV615	BUV661	BUV737	BUV7805	BV421
FITC	0	0	28	0	0	0	0	0	14	35	0	0	0	0
PerCP-Cy5.5	0	0	179	223	252	70	0	0	28	45	120	269	110	0
RB780	89	132	0	100	143	92	0	0	0	29	108	266	377	89
APC	0	336	69	0	609	113	0	0	71	130	238	335	87	25
AF700	0	191	72	273	0	108	0	89	123	488	178	280	83	9
APC-Cy7	0	44	46	140	188	0	0	0	0	23	59	151	198	0
BUV395	0	0	0	19	0	0	0	35	0	26	19	0	0	0
BUV496	0	0	19	73	0	0	84	0	116	146	81	94	33	2
BUV563	54	78	42	181	47	19	84	85	0	321	187	205	62	22
BUV615	0	244	98	394	138	61	75	4	173	0	411	508	177	0
BUV661	0	392	156	1094	740	137	78	0	75	167	0	986	303	24
BUV737	0	146	453	181	601	116	77	0	0	22	193	0	549	4
BUV7805	0	0	26	8	39	67	103	0	0	0	20	140	0	5
BV421	0	0	13	0	0	0	63	65	25	37	1	2	0	0
BV480	88	102	22	53	21	0	23	148	73	118	58	67	30	92
BV570	0	205	46	100	64	25	13	56	152	188	123	137	46	262
BV605	0	538	130	292	176	71	19	37	166	806	343	418	152	266
BV650	0	619	116	451	349	81	0	0	65	180	498	472	164	247

- Accurately measures the total spread: difference in rSD between the positive and negative population
- Color coding can be used to identify different levels of spread
- The values are directly proportional to the amount of spread
- The same value across different rows means the same amount of spread

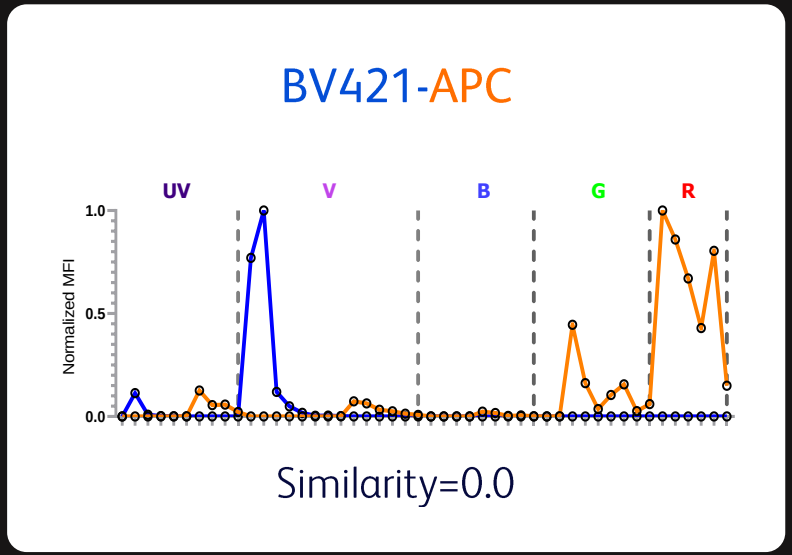


Additional challenges and tools when designing 35+ color panels in spectral flow cytometry

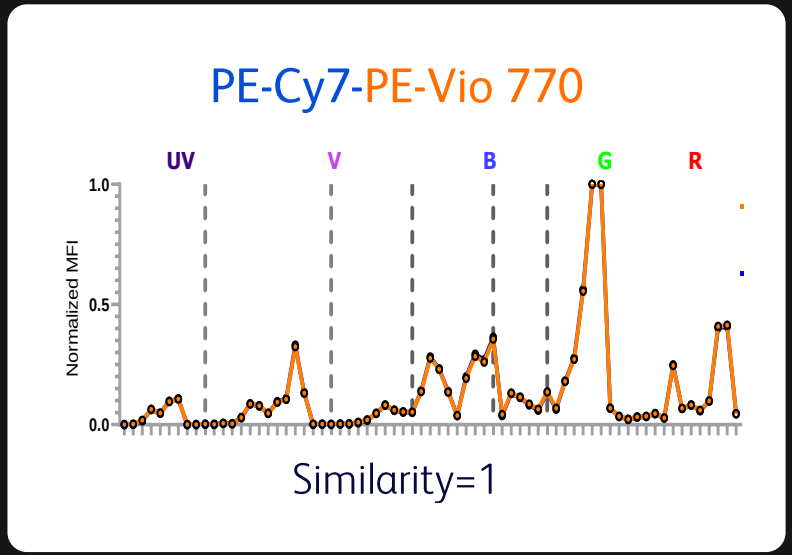
The use of highly overlapping dyes in high parameter panel design

Similarity index is a quantitative tool to determine the level of overlap between the emission profiles of two fluorochromes

A similarity index of 0 means the two fluorochromes have completely distinct emission profiles

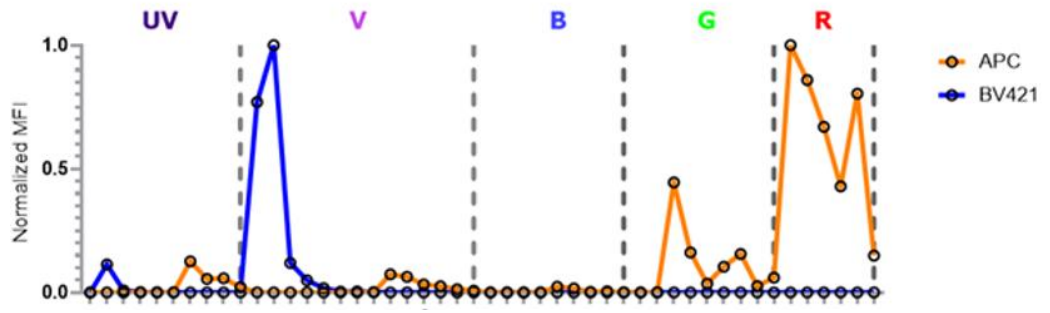


A similarity index of 1 means the two fluorochromes are identical

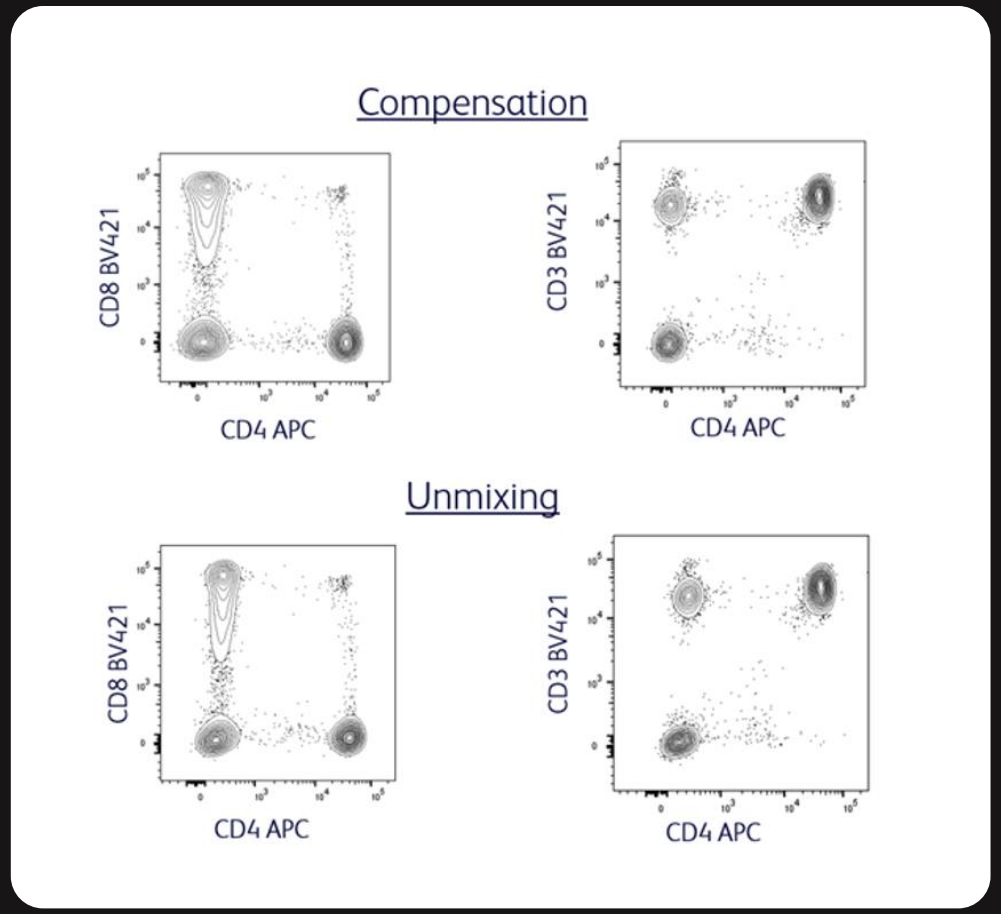


Completely separate fluorochromes

Similarity = 0.0

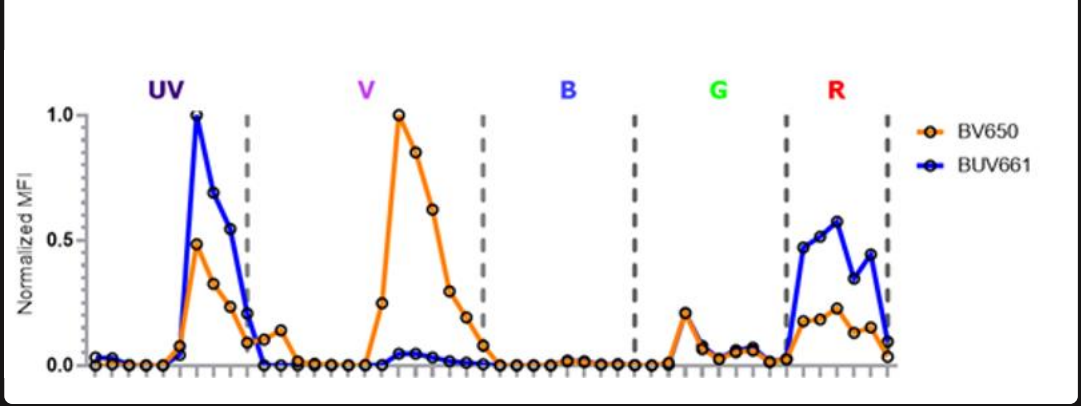


Ideal fluorochrome combinations
No spectral overlap = no spread



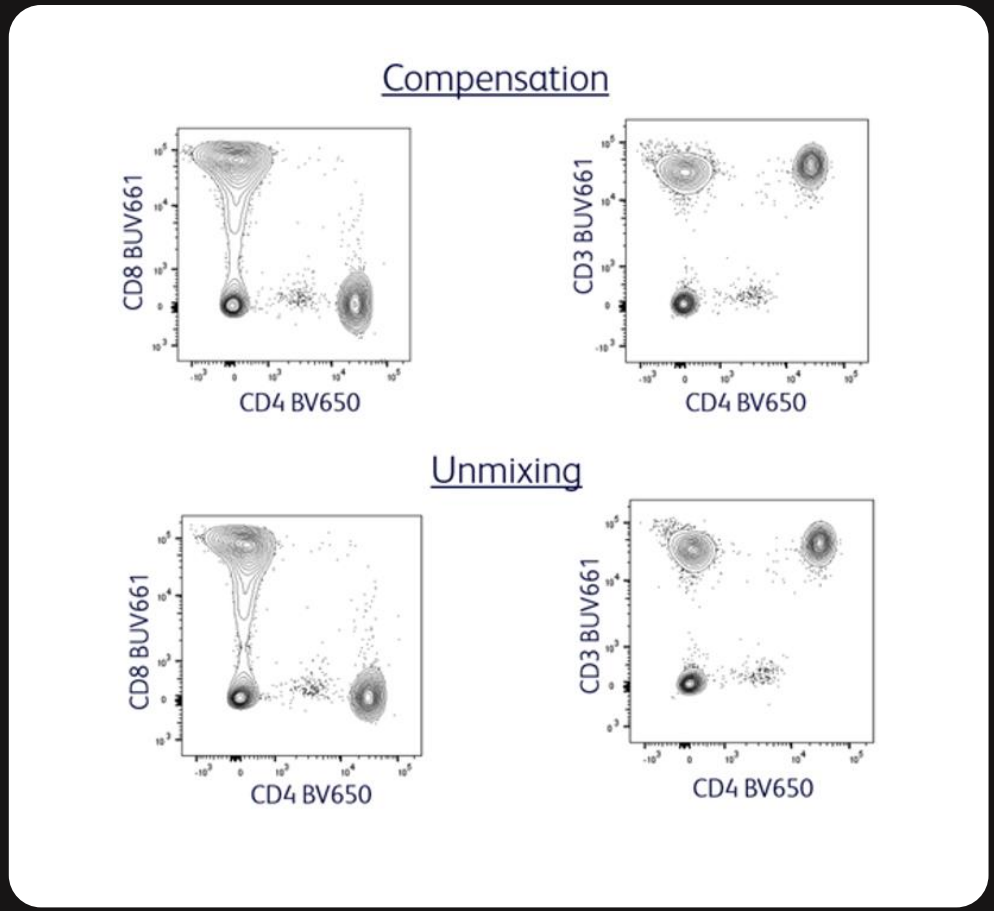
Partially overlapping fluorochromes

Similarity = 0.5



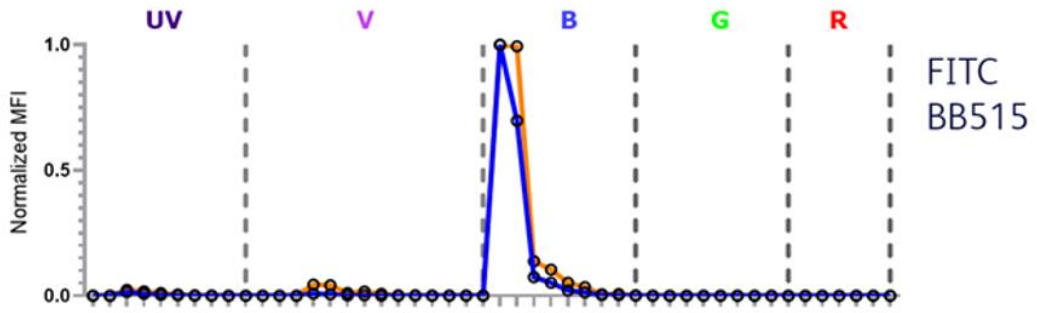
Can be separated by both unmixing and compensation

Panel design rules need to be followed to manage spread



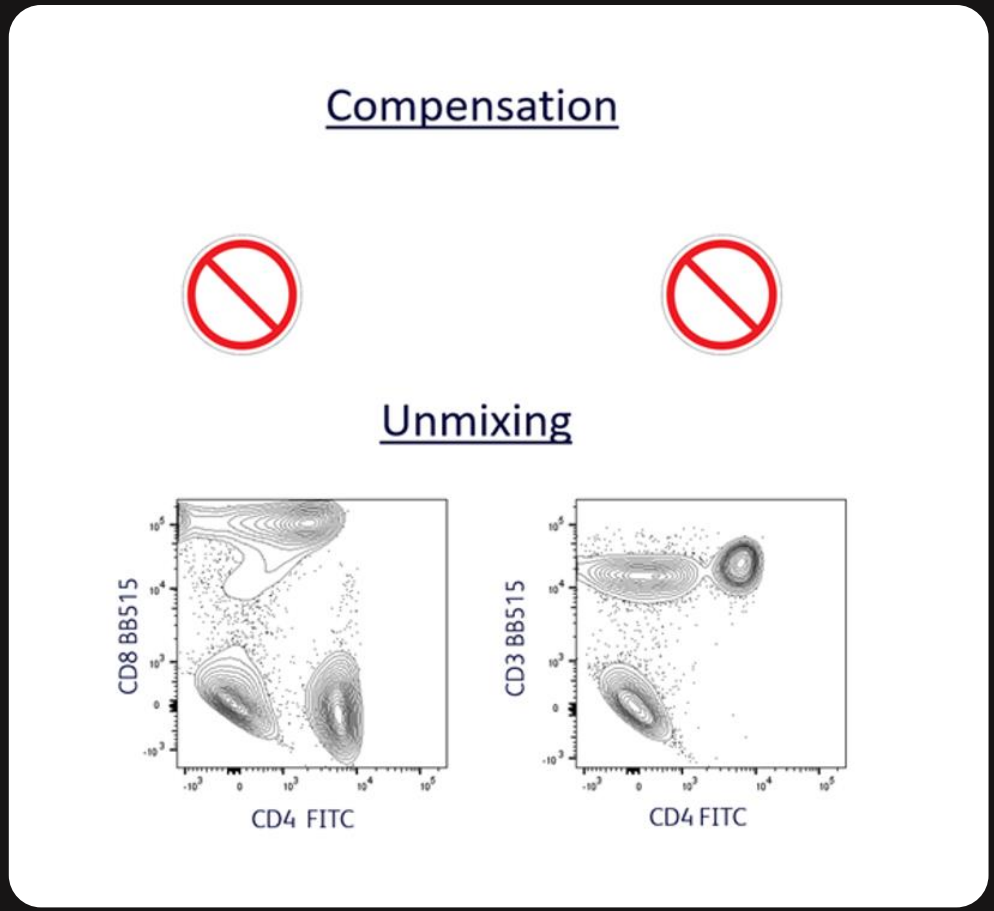
Highly overlapping fluorochromes

Similarity = 0.98



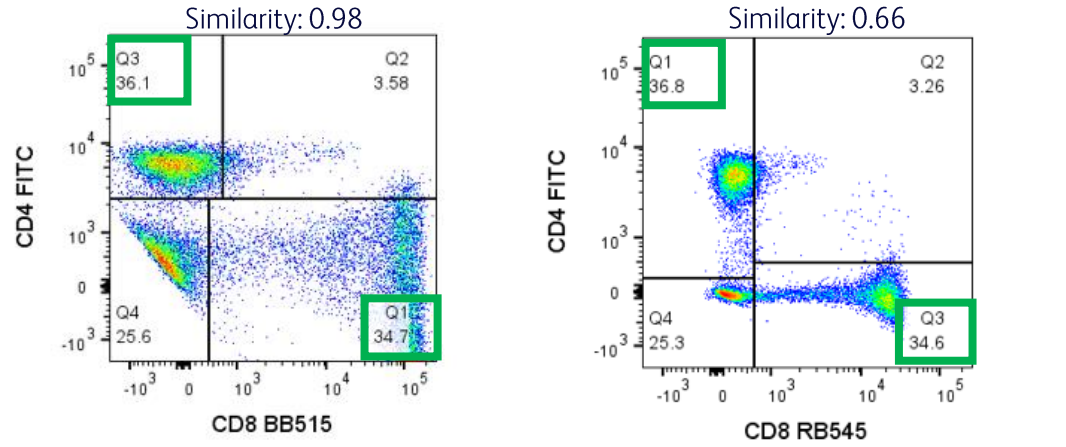
Cannot be resolved in conventional flow cytometry

Althose these can be resolved in spectral flow, their use is limited and challenging due to the very high spread

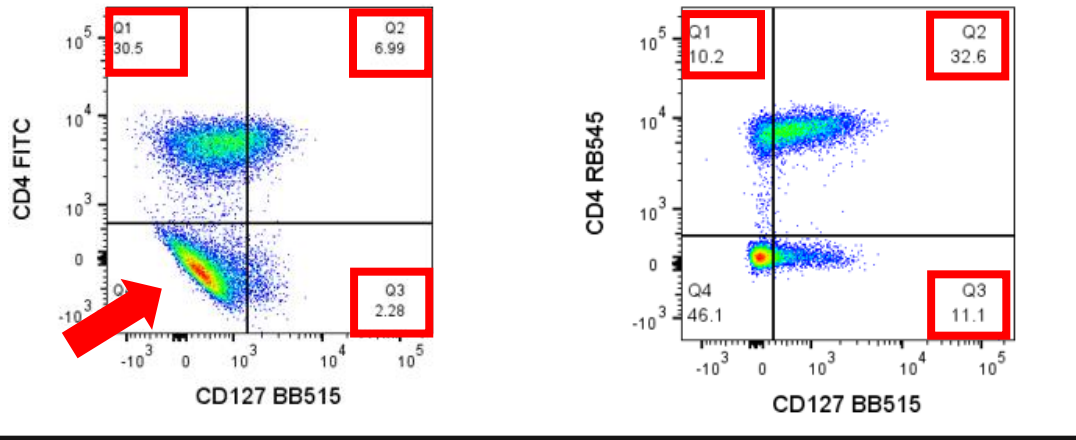


How to use highly overlapping fluorochromes together

High expressed, not-coexpressed markers



Low expressed markers



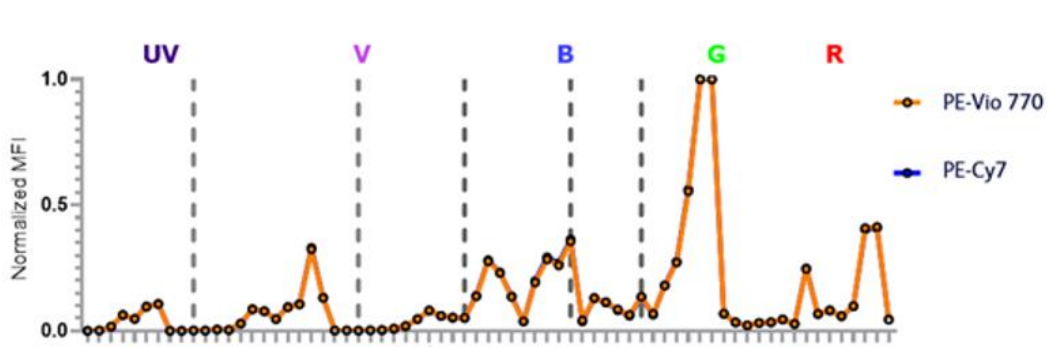
High spread and background in the negative population can significantly reduce marker resolution

Biological resolution can still be maintained for **highly expressed and clearly separated, orthogonal markers**

Biological resolution of markers expressed at low levels will be lost irrespective of co-expression patterns

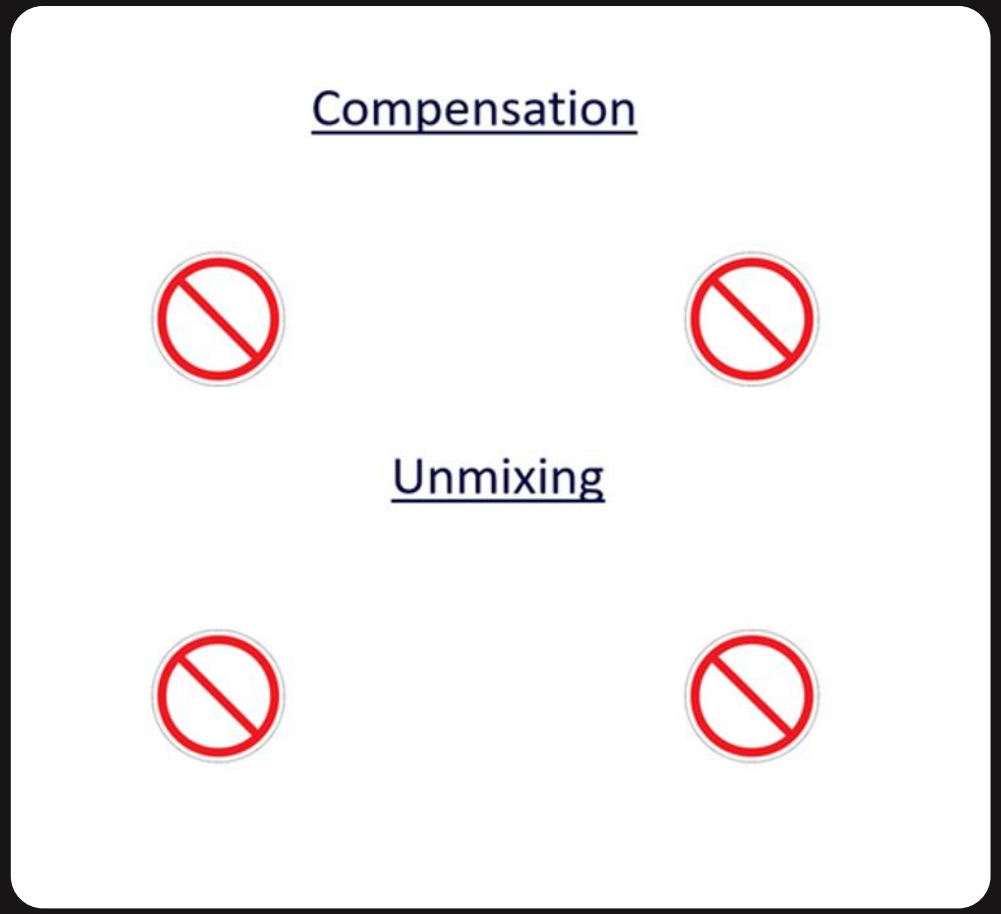
Identical fluorochromes

Similarity = 1



Fluorochromes with identical emission profile, despite different name

These fluorochrome combinations can't be used in conventional nor spectral flow cytometry



How to use the Similarity Index?

To exclude identical fluorochromes

To identify and avoid highly overlapping fluorochromes when possible

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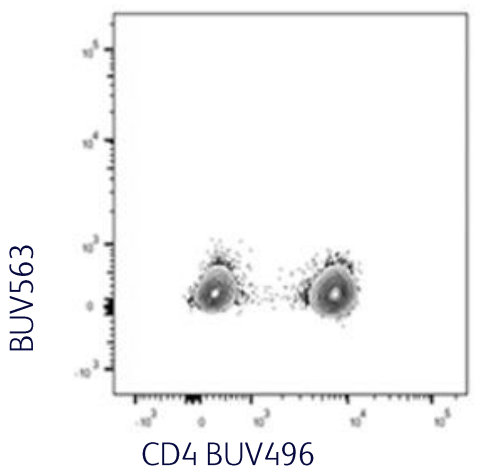
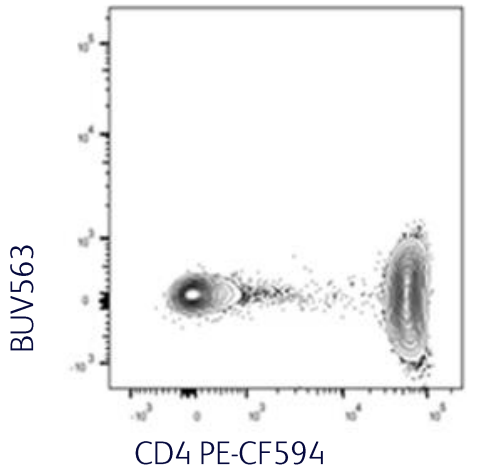
Not to select reagents for partially overlapping dyes: not the best tool to predict spread because it doesn't take into account brightness





Fluorochrome	Stain Index	Similarity Index (BUV563)	TSM
BUV496	25	0.5	116
PE-CF594	124	0.34	388

Summary

Conventional and spectral flow cytometry use a different approach to measure the individual contribution of each fluorochrome to the total measured signal

- Conventional: collection of discrete emission profiles through a 1:1 fluorochrome and detector ratio
- Spectral: collection of emission across the entire spectrum

Collection of the entire emission spectrum offers some advantages:

- The possibility to extract autofluorescence and to obtain better resolution for highly autofluorescent samples
- Higher fluorochrome choice flexibility
- Development of larger multicolor panels

Spectral flow cytometry does not eliminate spread

- The same panel design principles should be followed to avoid loss of resolution due to spread

The same tools can be used to guide panel design

- Total spread matrix, fluorochrome brightness reference

The Similarity index can be used to exclude identical dyes, and to identify and exclude highly similar dyes whenever possible

Thank you!

Wednesday, February 14, 2024 at 17h

NEW BD Webinar: Revealing cellular diversity with the power of spectral cytometry

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