Analysis of a 12-Color Panel using a Compensation-Free Flow Cytometer with 6 Fluorescence Detectors

CYTO 2022 Session 15: Cytometric Technologies #2 - Multiplexing+

Monday, June 6

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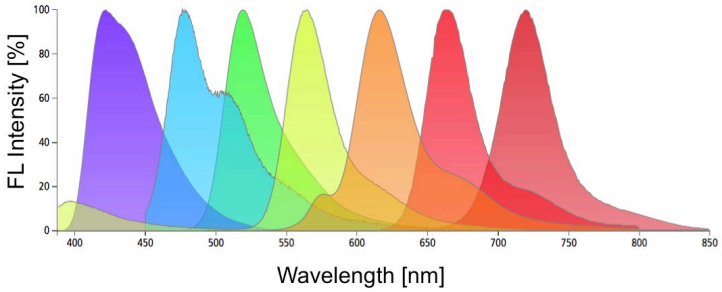
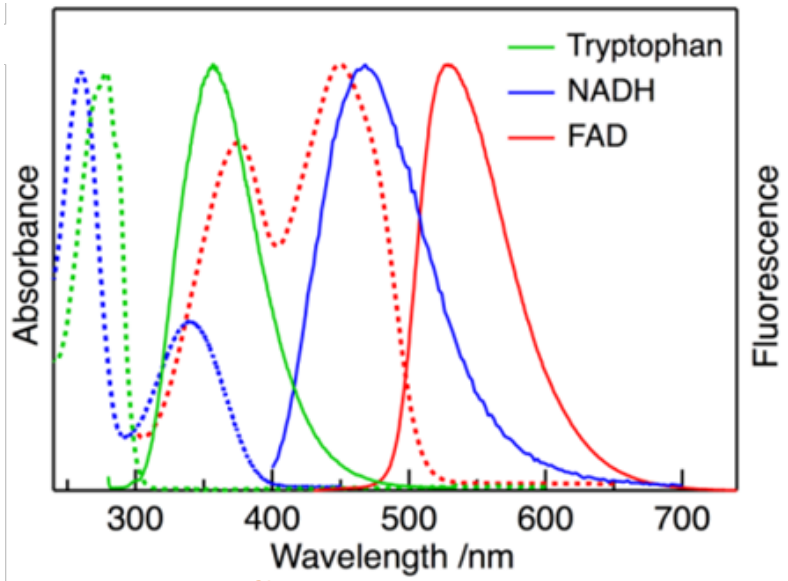
Time-resolved flow cytometry

addresses pains in fluorescence flow cytometry

- Spectral spillover → limited # channels
  - PAIN: long, complex runs
  - PAIN: incomplete information
- Spectral spillover → compensation
  - PAIN: population spreading
  - PAIN: wasted time
- Autofluorescence → high background
  - PAIN: reduced sensitivity
  - PAIN: reduced dynamic range





Utilize fluorescence lifetime in addition to fluorescence spectrum

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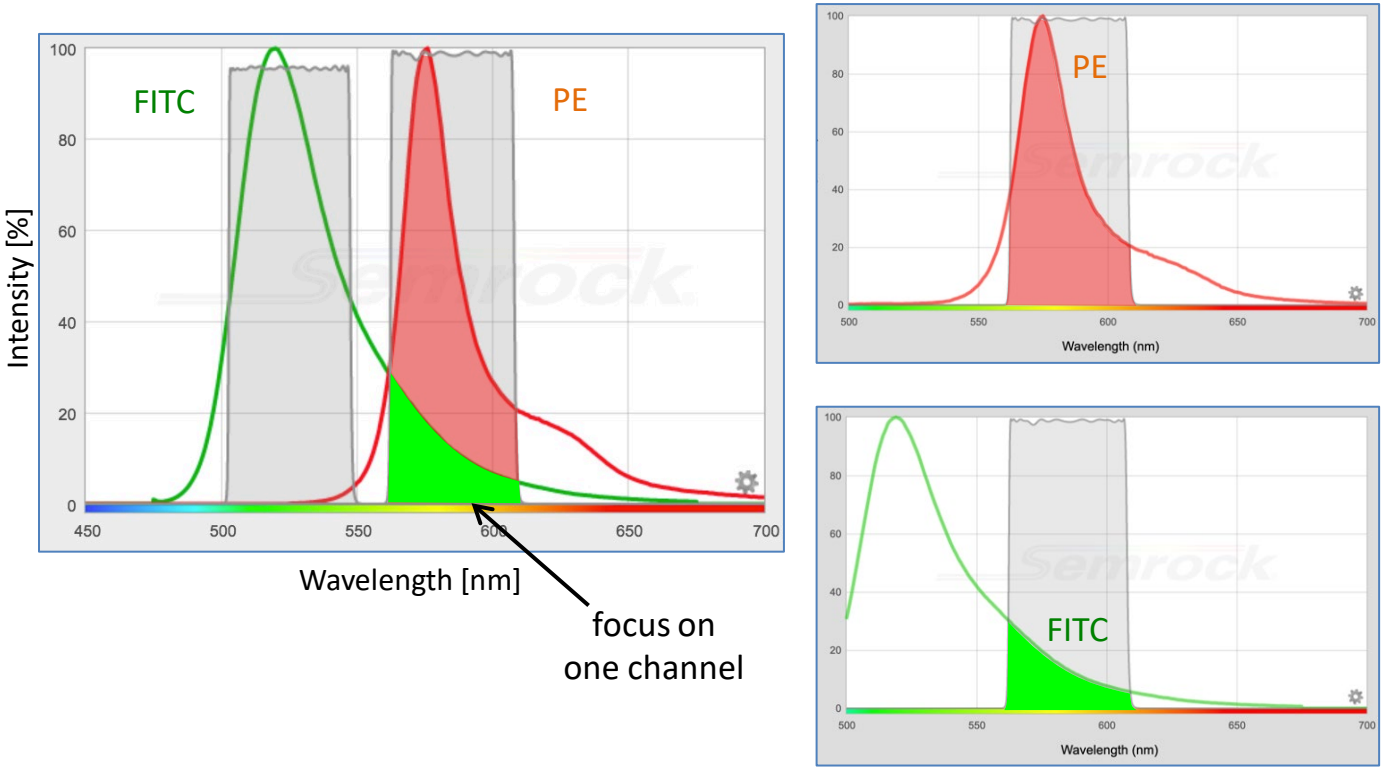
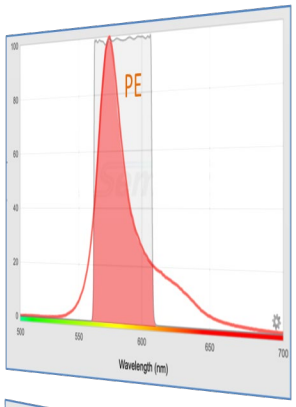
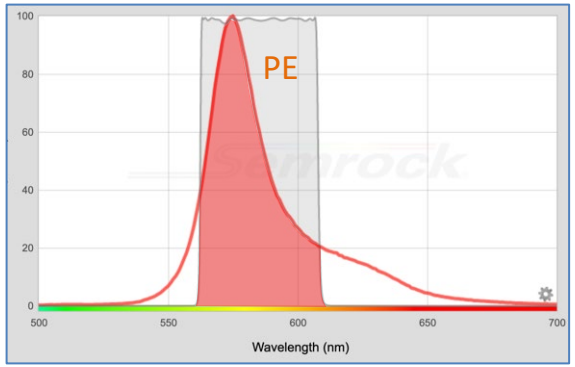
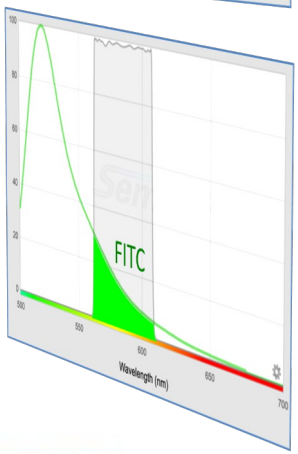
Problem: Overlapping Fluorophore Emissions

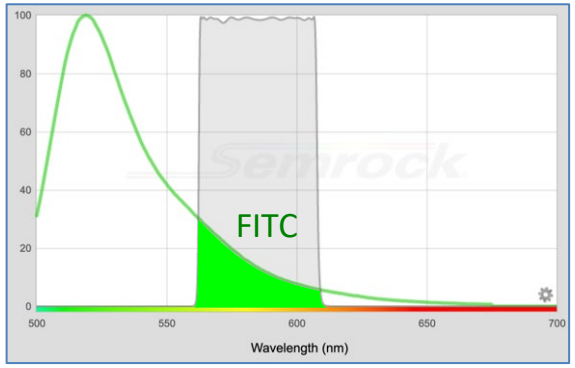


Problem: Overlapping Fluorophore Emissions









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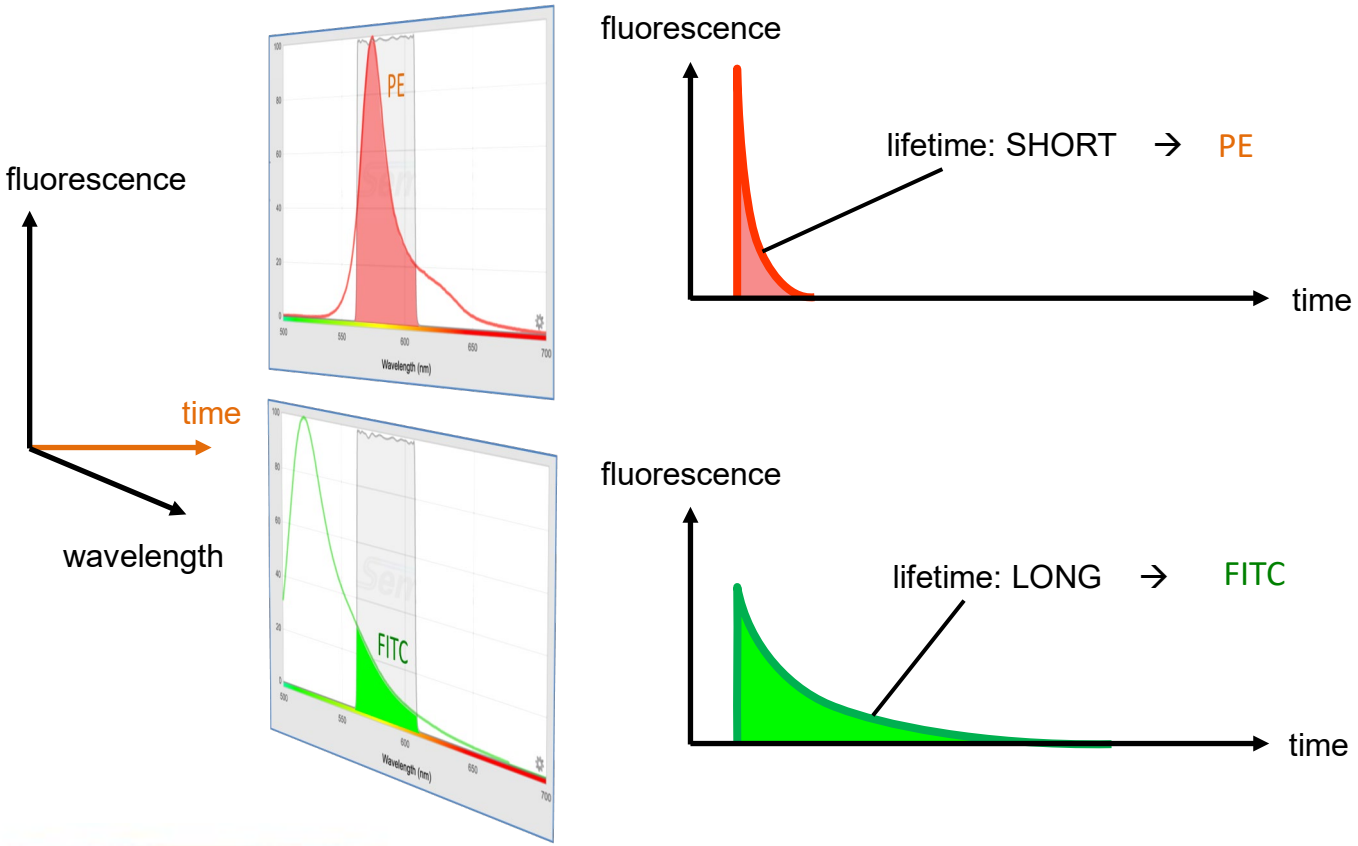
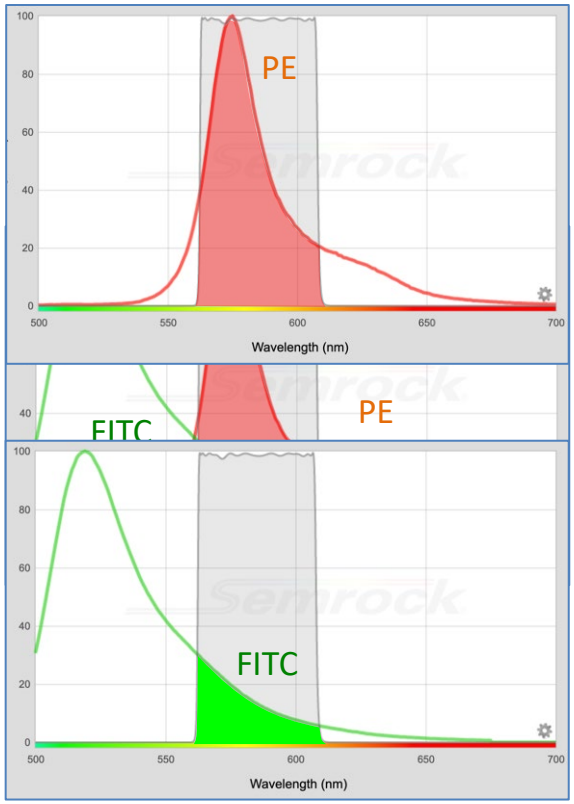
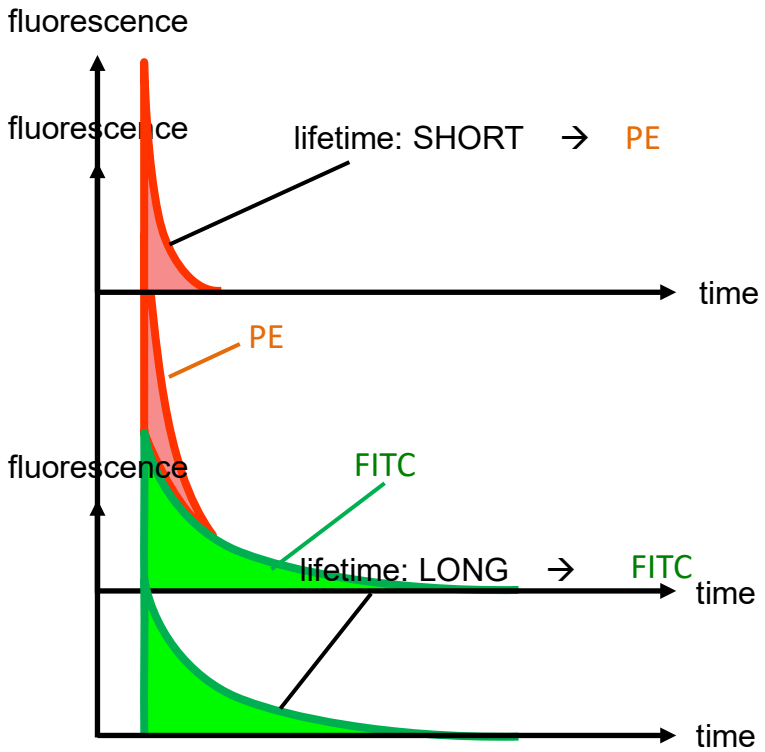
Time Enables Distinction of Overlapping Emissions



Time Enables Distinction of Overlapping Emissions





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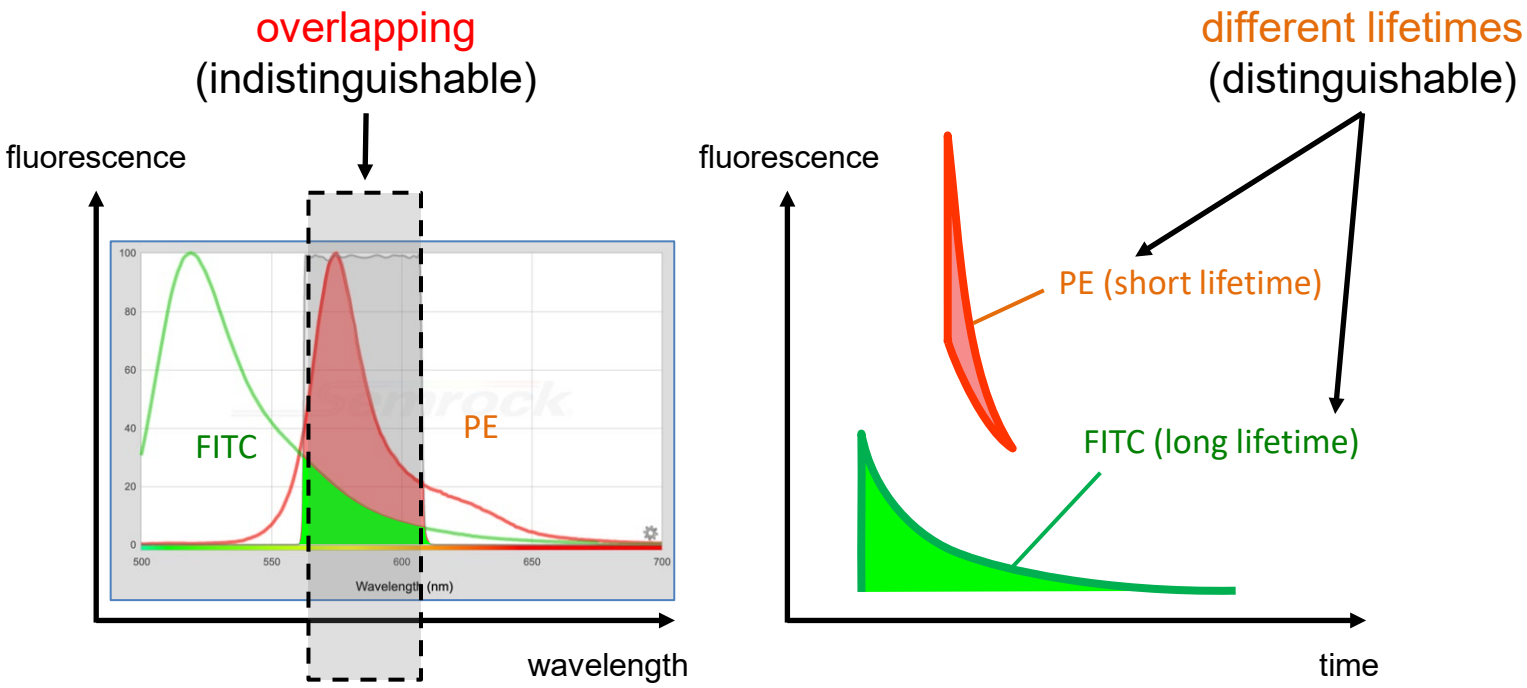
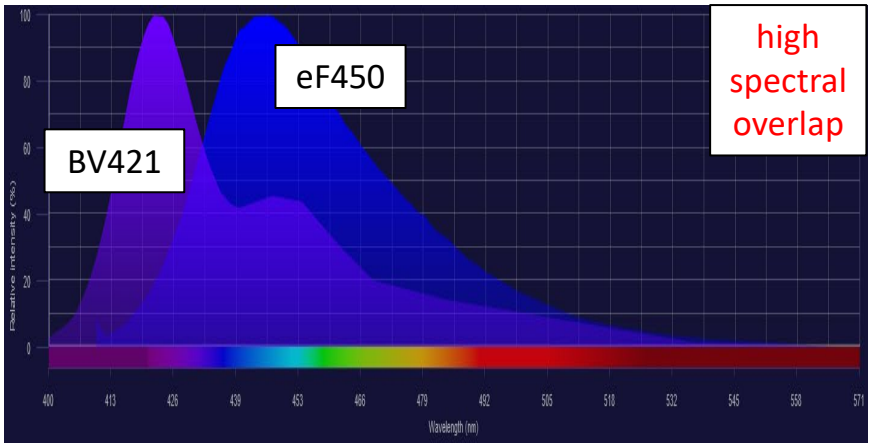
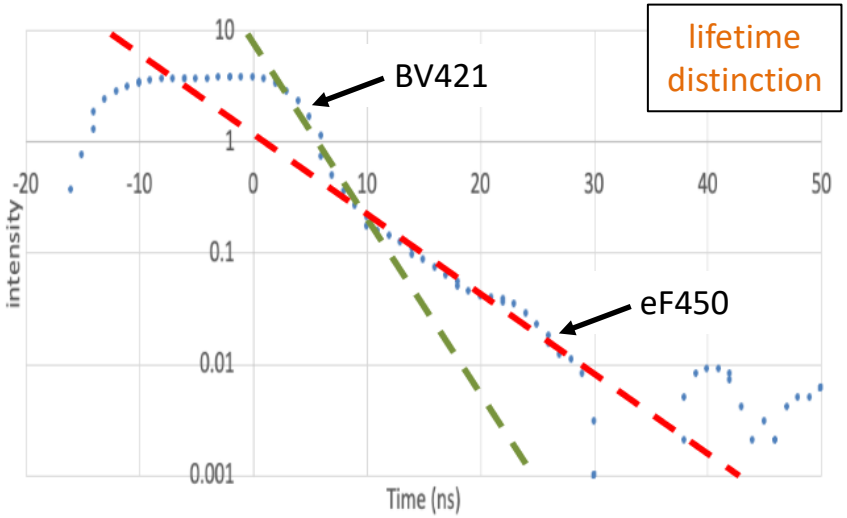


Lifetime distinction for high spectral overlap case

High spectral overlap

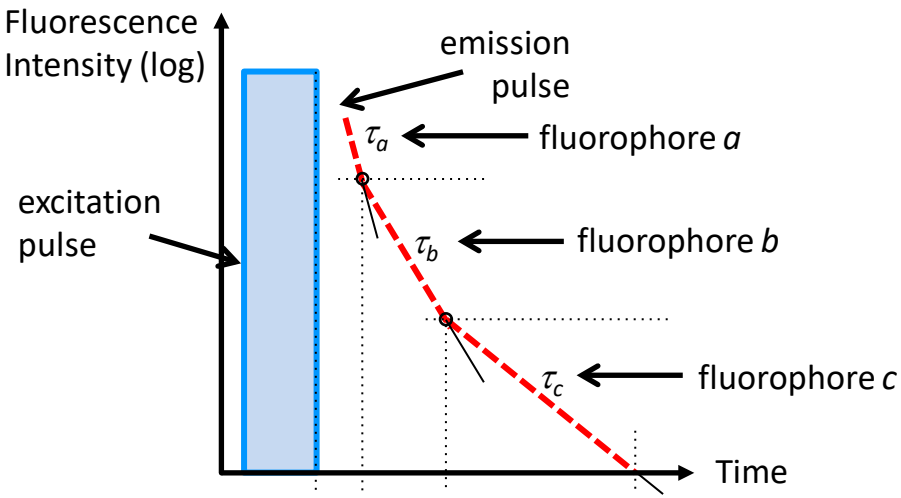


Lifetime distinction

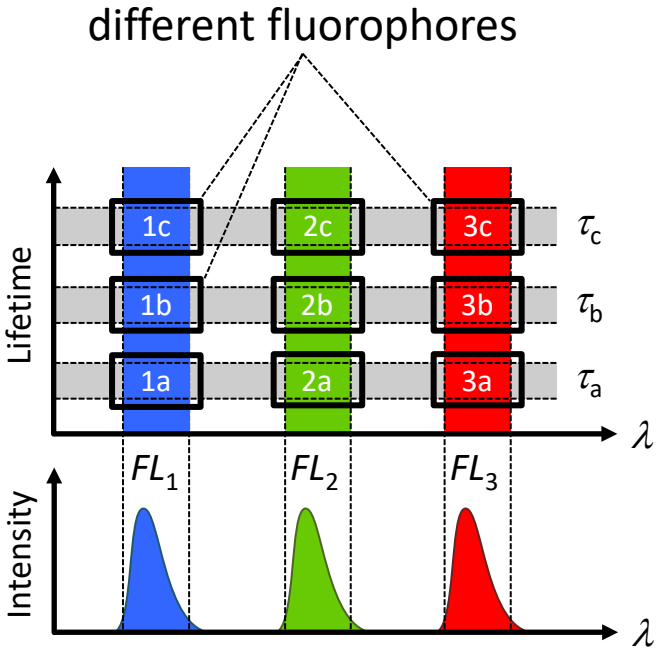


High multiplexing with lifetime distinction

- Distinguish fluorophores based on color AND lifetime
  - → reduce spectral spillover
  - → **compensation-free analysis**



different lifetimes
in each spectral band



Arno compensation-free flow cytometer

- Six photomultiplier tube modules detect 12 fluorophores
  - Time-resolved flow cytometry detects 2 fluorescence lifetimes
- Two fiber-coupled high-bandwidth digitally modulated lasers
  - 405 nm, 300 mW if run CW
  - 488 nm, 200 mW if run CW
- Spatially separated laser focus regions in the flow cell
- *Shasta* fluidics module
  - quadruple-regulated pressure control module
  - high stability of the sample core stream
  - expanded range of flow rates (transit times from 2 to 100 µs)
    - transit time of 100 µs used in the experiments

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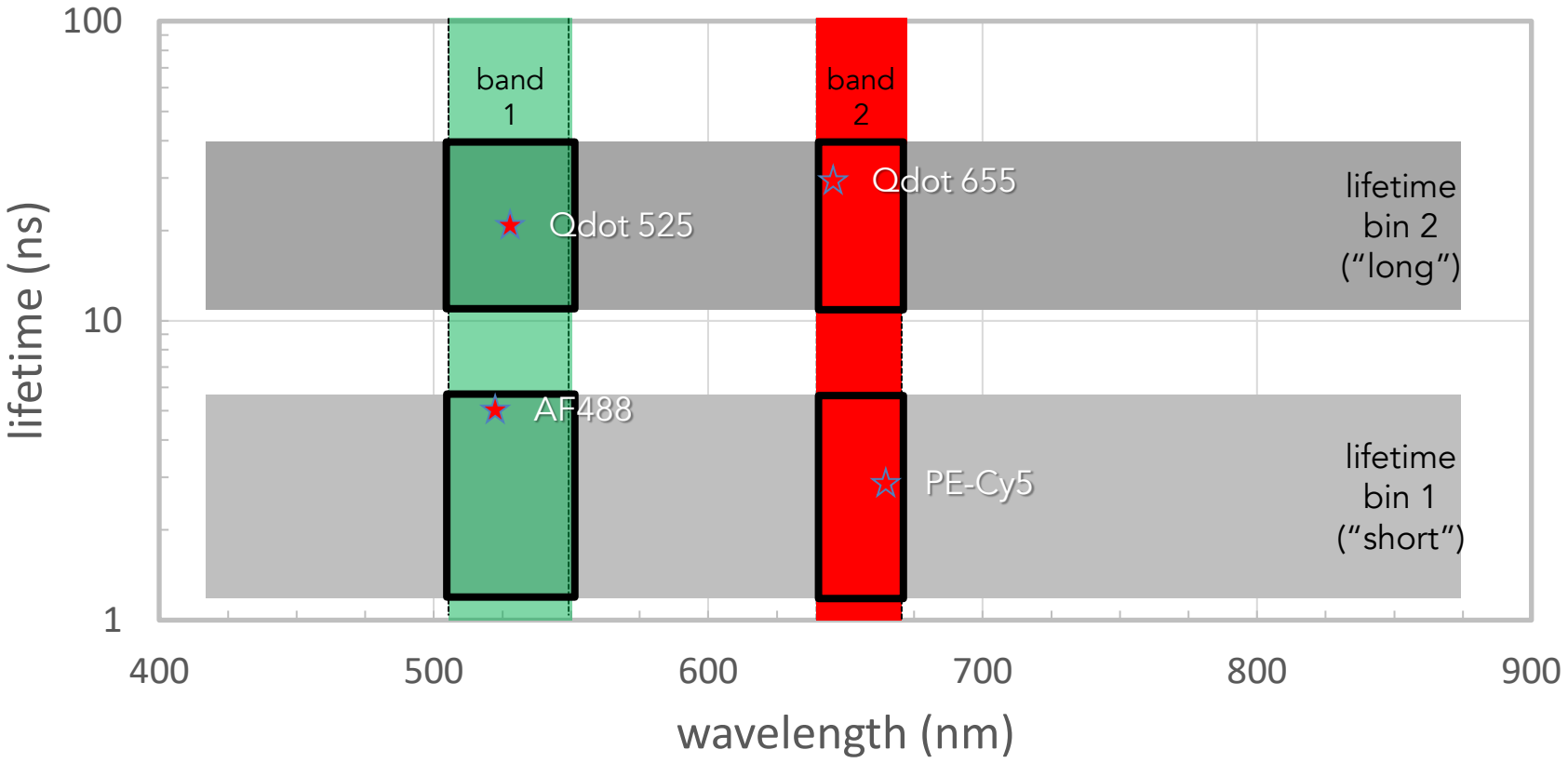
4 Fluorophores: 2 Spectral Bands & 2 Lifetimes



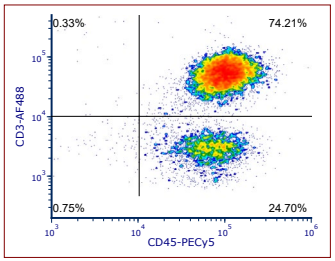
4-fluorophore compensation-free panel high lifetime separation

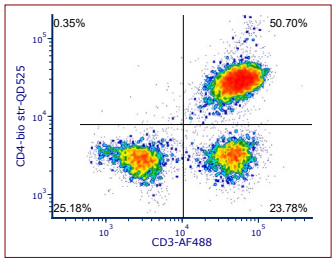
marker	fluorophore	bin	lifetime (ns)	filter range (nm)	laser (nm)
CD3	Alexa Fluor 488	FL1 (CH1) short	4.9	FL1: 503-548	488
CD4 - biotin	streptavidin - Qdot 525	FL1 (CH1) long	22		
CD45	PE-Cy5	FL2 (CH2) short	3	FL2: 645-675	
CD8	Qdot 655	FL2 (CH2) long	28		

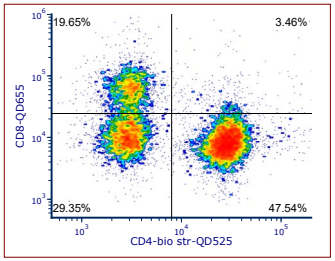
Cyto-Trol lyophilized lymphocytes

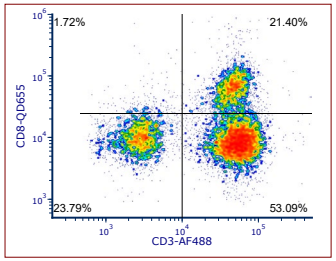
Marker	Expected positive population	Measured positive population
CD3	80±8%	74%
CD4	54±7%	51%
CD8	28±7%	21%

Lot 729101

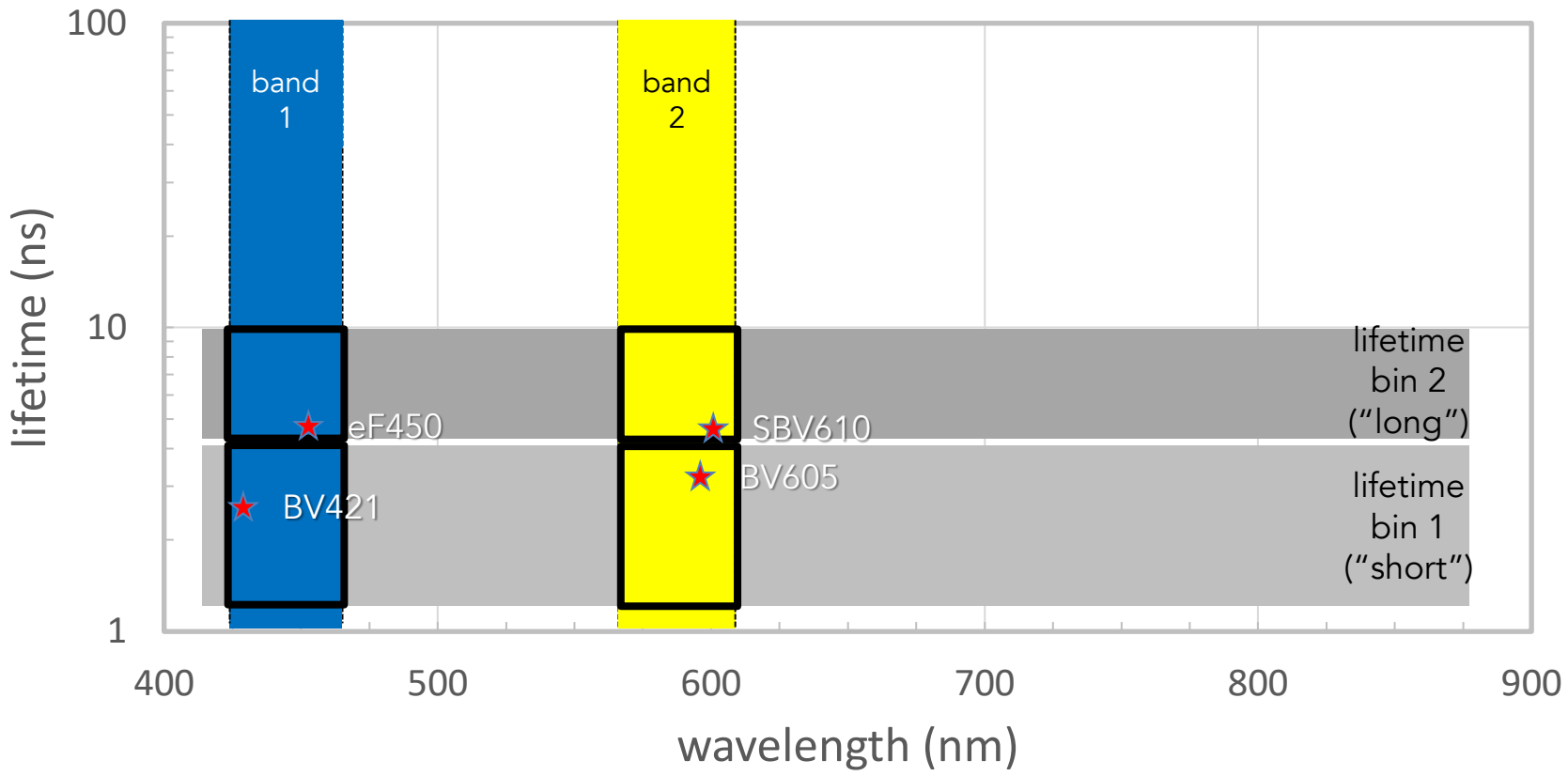








4 Fluorophores: 2 Spectral Bands & 2 Lifetimes



4-fluorophore compensation-free panel

low lifetime separation

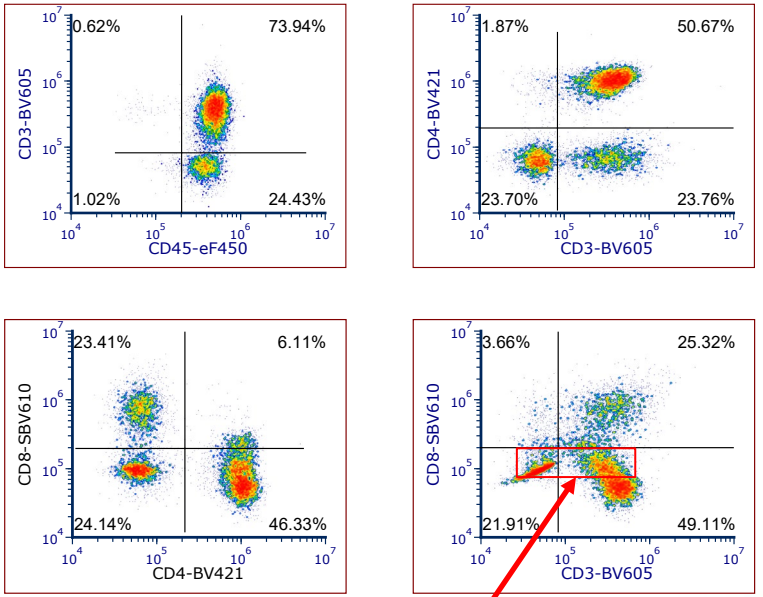
Lifetime distinction achieved with < 20% difference in lifetime (3.6 ns vs. 4.2 ns)

marker	fluorophore	bin	lifetime (ns)	filter range (nm)	laser (nm)
CD4	BV421	FL1 (CH1) short	2.5	FL1: 422-460	405
CD45	eFluor 450	FL1 (CH1) long	4.7		
CD3	BV605	FL2 (CH2) short	3.6	FL2: 570-616	
CD8	StarBright Violet 610	FL2 (CH2) long	4.2		

Cyto-Trol lyophilized lymphocytes

Marker	Expected positive population	Measured positive population
CD3	79±8%	74%
CD4	53±7%	51%
CD8	29±7%	25%

Lot 729109



Fitting artifacts
(very similar lifetimes)

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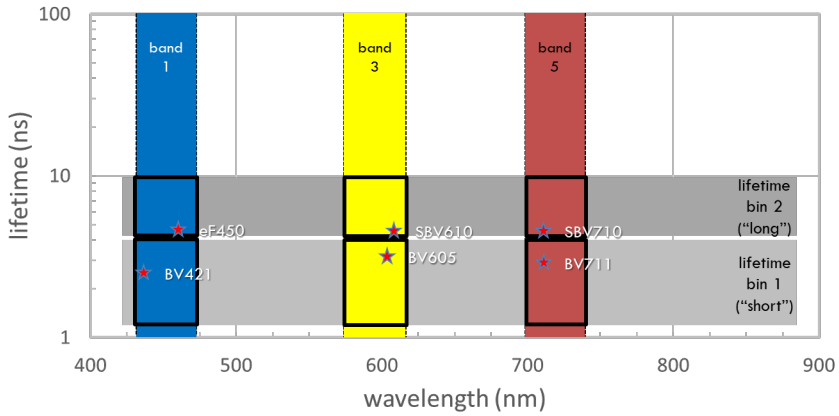
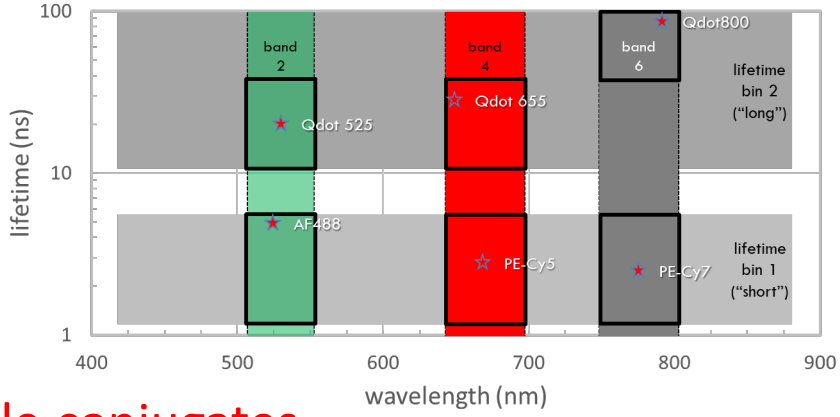
12 Fluorophores: 6 Spectral Bands & 2 Lifetimes

- 405-nm laser excited fluorophores
  - Brilliant Violet 421
  - eFluor 450
  - Brilliant Violet 605
  - StarBright Violet 610
  - Brilliant Violet 711
  - StarBright Violet 710
- 488-nm laser excited fluorophores
  - Alexa Fluor 488
  - Qdot 525
  - PE-Cy5
  - Qdot 655
  - PE-Cy7
  - Qdot 800





Commercially available conjugates

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12-fluorophore panel
peripheral blood mononuclear cell (PBMC) sample

marker	fluorophore	purpose	lifetime (ns)	filter range (nm)	laser (nm)
CD56	Brilliant Violet 421	NK cells	2.5	FL1: 422-460	405
CD2	eFluor 450	T cells	4.5		
CD4	Alexa Fluor 488	CD4 T cells	4.9	FL2: 503-548	488
CD197 (CCR7) - biotin	streptavidin - Qdot 525	differentiation	22		
CD45	Brilliant Violet 605	WBCs	3.6	FL3: 585-604	405
CD3	StarBright Violet 610	T cells	4.2		
CD19	PE-Cy5	B cells	3	FL4: 645-675	488
CD74 (HLA-DR)	Qdot 655	APCs	28		
CD8	Brilliant Violet 711	CD8 T cells	3.7	FL5: 708-732	405
CD45RA	StarBright Violet 710	differentiation	4.4		
CD20	PE-Cy7	B cells	2.5	FL6: 770 LP	488
CD14	Qdot 800	monocytes	92		

PBMCs stained then fixed

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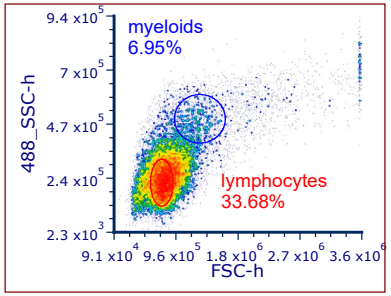
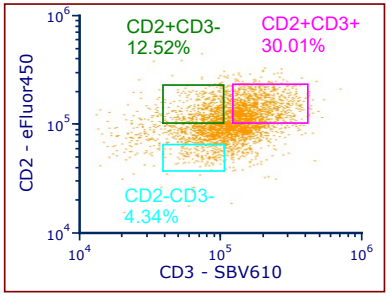
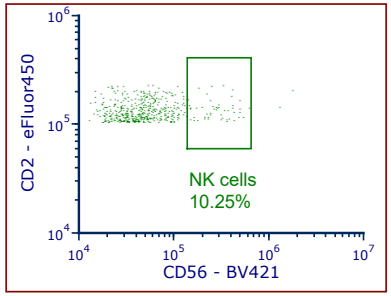
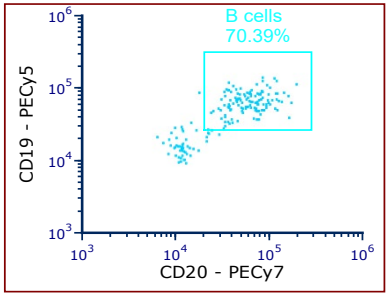
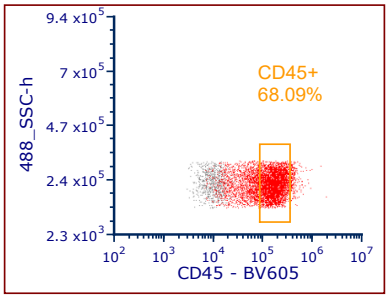
12-fluorophore panel compensation-free results

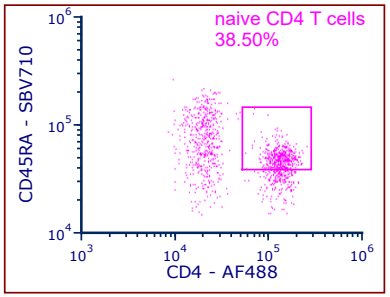


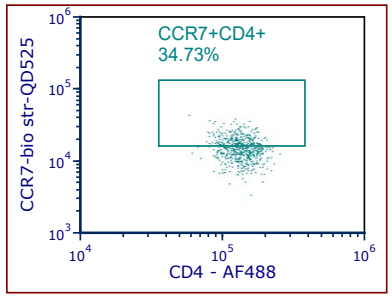


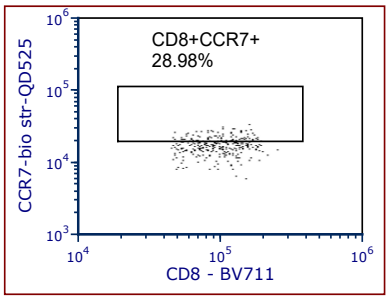


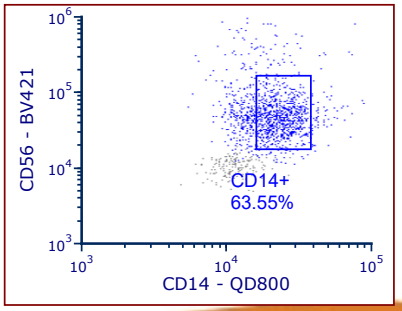


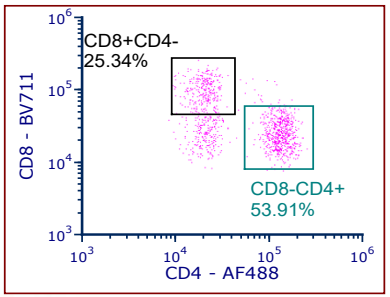


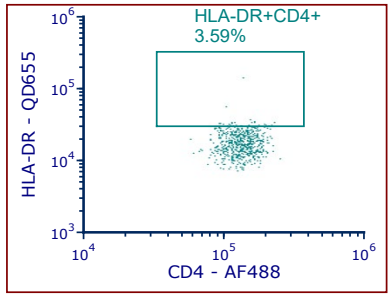


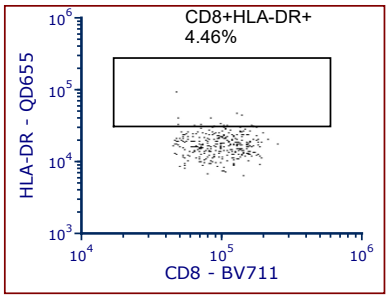












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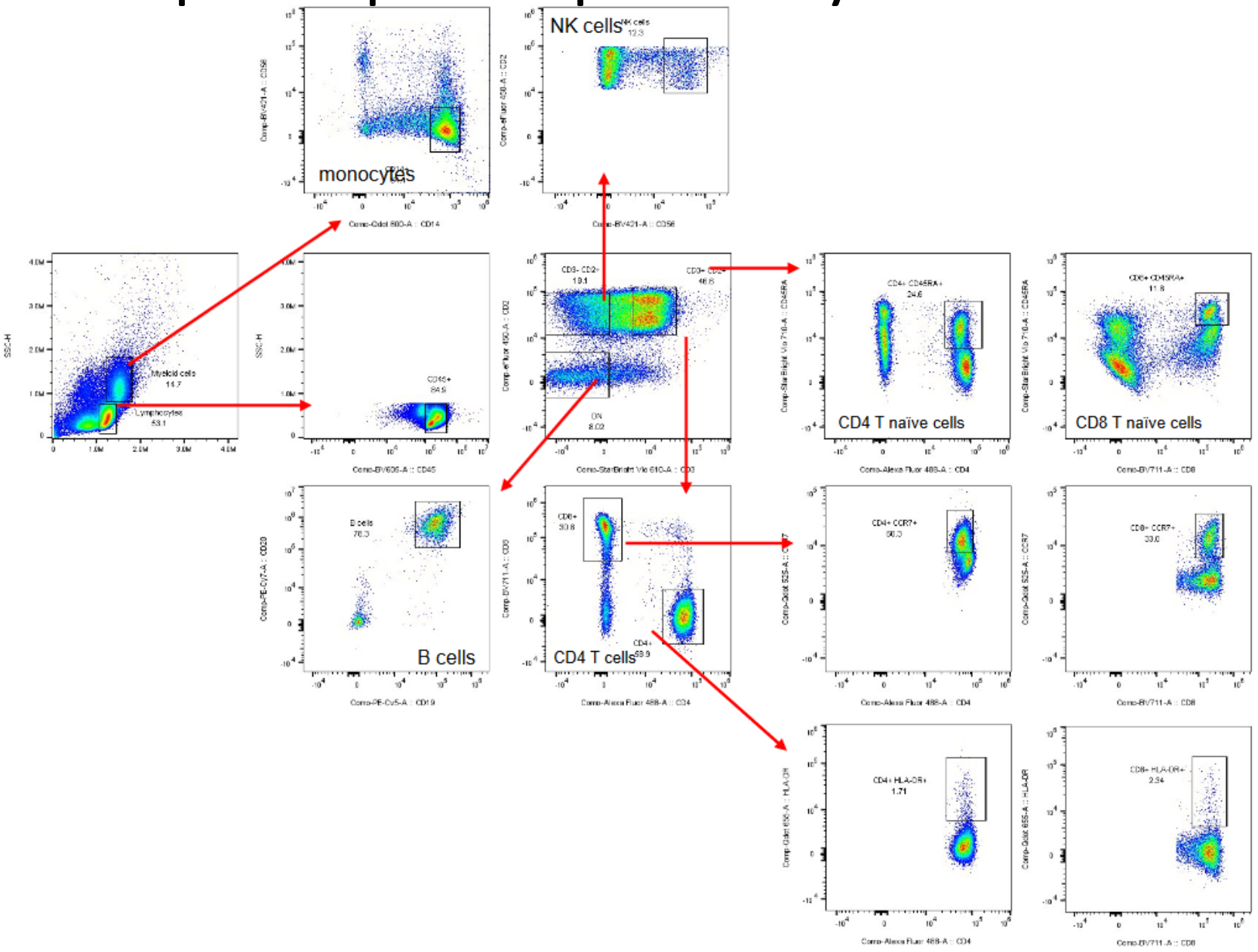
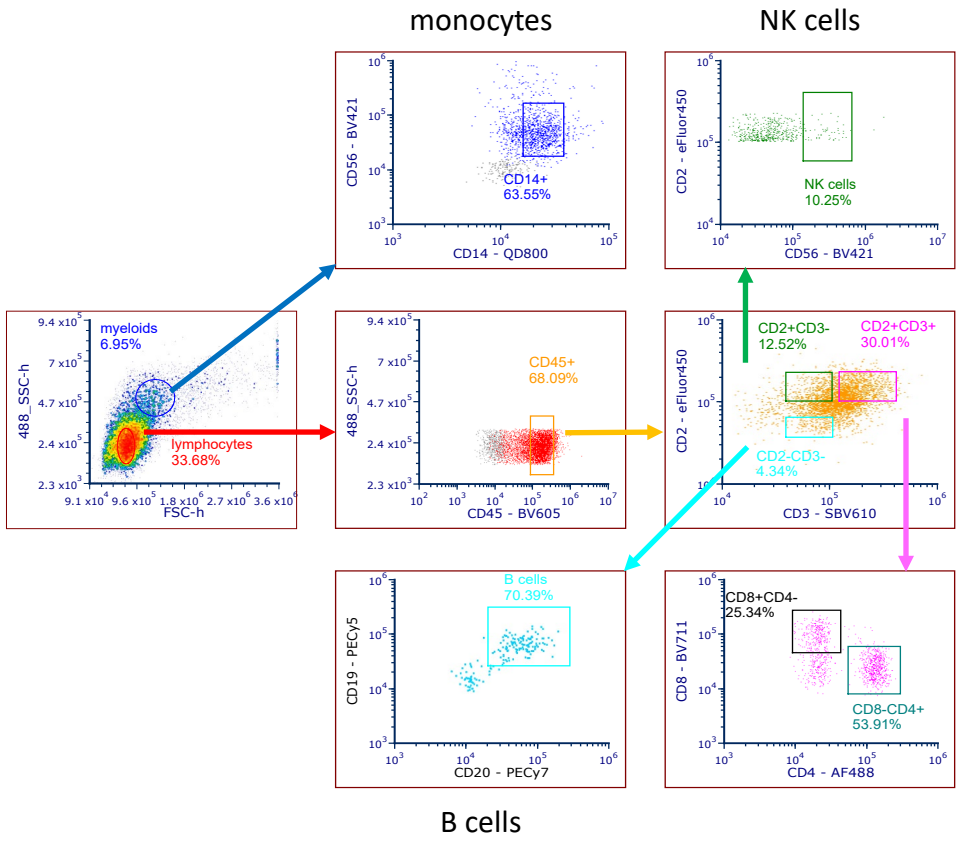
12-fluorophore panel spectral cytometer results

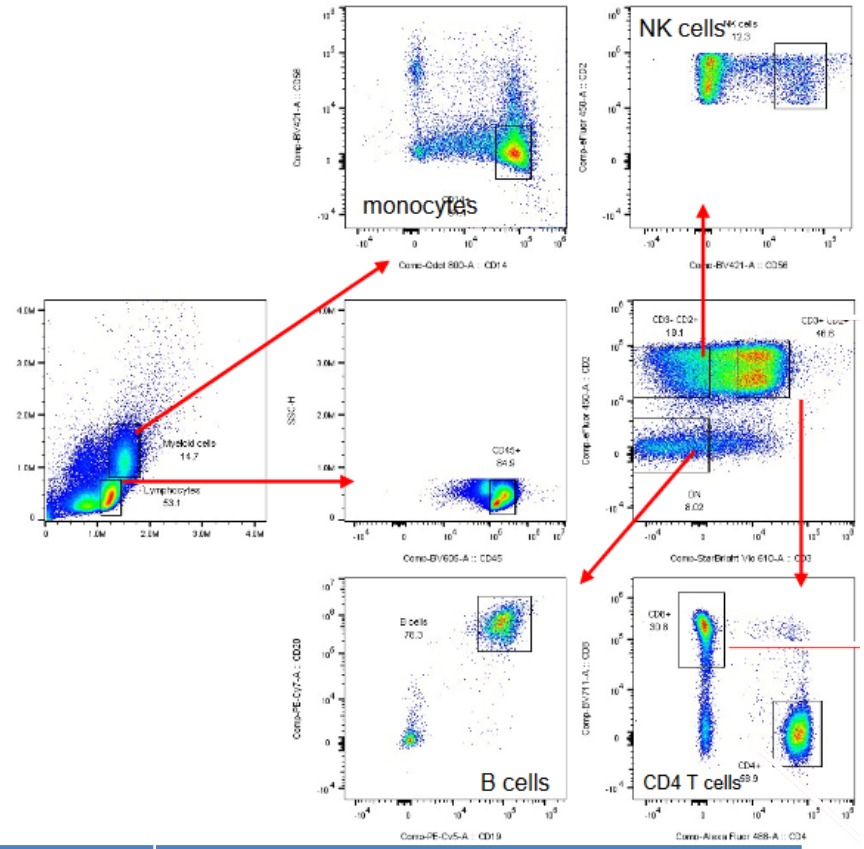


12-fluorophore panel comparison of results

Compensation-free cytometer



Spectral cytometer



Species	Compensation-free population	Spectral cytometer population
CD8 T cells	25% of CD3+CD2+	31% of CD3+CD2+
CD4 T cells	54% of CD3+CD2+	59% of CD3+CD2+
NK cells	10% of CD3-CD2+	12% of CD3-CD2+
monocytes	64% of myeloids	65% of myeloids
B cells	70% of CD3-CD2-	78% of CD3-CD2-

Summary

- Time-resolved flow cytometry → compensation-free analysis

- Demonstrated compensation-free panel analyses
  - 4-fluorophore lymphocyte panels on Cyto-Trol cells
    - one with 488-nm pulsed laser and another with 405-nm pulsed laser
    - 2 fluorescence detectors & 2 lifetime bins
    - verified with Cyto-Trol cell lot information
  - 12-fluorophore panel on PBMCs
    - simultaneous 405-nm & 488-nm pulsed lasers
    - 6 fluorescence detectors & 2 lifetime bins
    - verified with spectral cytometer analysis

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