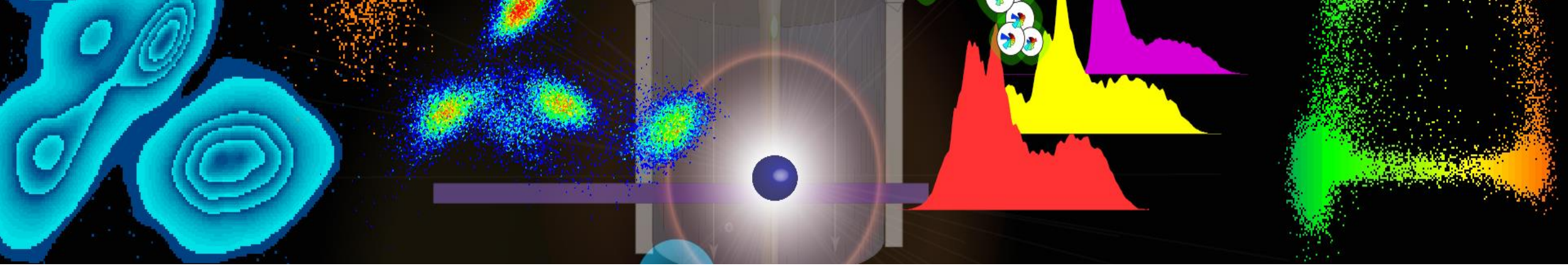


Contributions of the Melbourne Cytometry Platform to the enhancement of scientific discovery at UOM and beyond…

Vanta Jameson, PhD

Melbourne Cytometry Platform: Doherty node manager

Department of Microbiology and Immunology
Doherty Institute for Infection and Immunity
The University of Melbourne

https://biomedicalsciences.unimelb.edu.au/cytometry

Melbourne Cytometry Platform

- Shared resource laboratories across 4 locations (nodes) at Parkville campus of the UOM
- 6 dedicated staff – Platform manager, node managers and technical specialists
- 25 cytometers – analysers (20) and sorters (5)
- Annually support cytometry of ~400 researchers– UOM students and academics, external scientists
    - immunology, microbiology, cell & developmental biology, neuroscience, small particles, marine biology, food science, bioengineering, earth sciences
- Training & induction- theory, hands-on ~200 users p/a

Challenges: Funding and Time

- **$** to replace aging/ obsolete cytometers
- **Time**:
    - Staff development within saturation levels of service
    - Attend conferences
    - SRL accreditation
    - Prepare manuscripts

Doherty Institute

MELBOURNE BRAIN CENTRE

bio21 institute

MELBOURNE DENTAL SCHOOL

One paper, three stories, six years and a pandemic (not "the budget of a small country")

Cytometry PART A | Journal of Quantitative Cell Science | ISAC

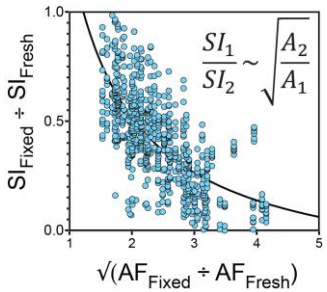
Original Article | Open Access

Unlocking autofluorescence in the era of full spectrum analysis: Implications for immunophenotype discovery projects

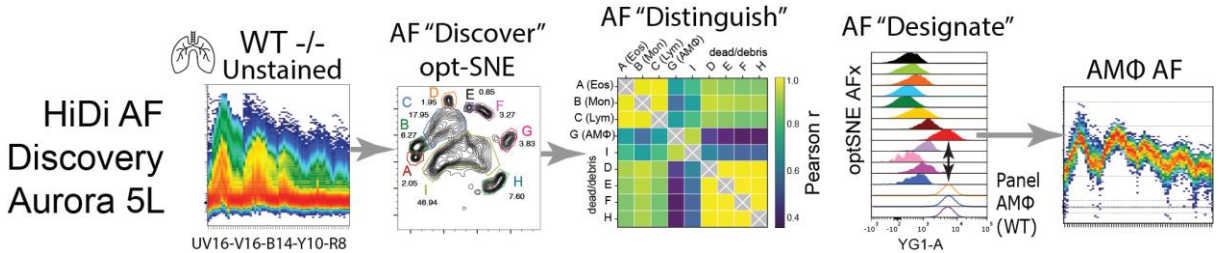
Vanta J. Jameson, Tina Luke, Yuting Yan, Angela Hind, Maximilien Evrard, Kevin Man, Laura K. Mackay, Axel Kallies, Jose A. Villadangos, Hamish E. G. McWilliam, Alexis Perez-Gonzalez

First published: 29 March 2022 | https://doi.org/10.1002/cyto.a.24555 | Citations: 8

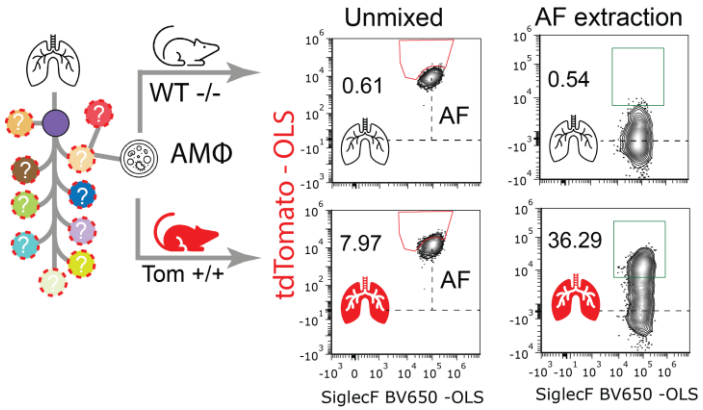
1. Autofluorescence in sensitivity theory

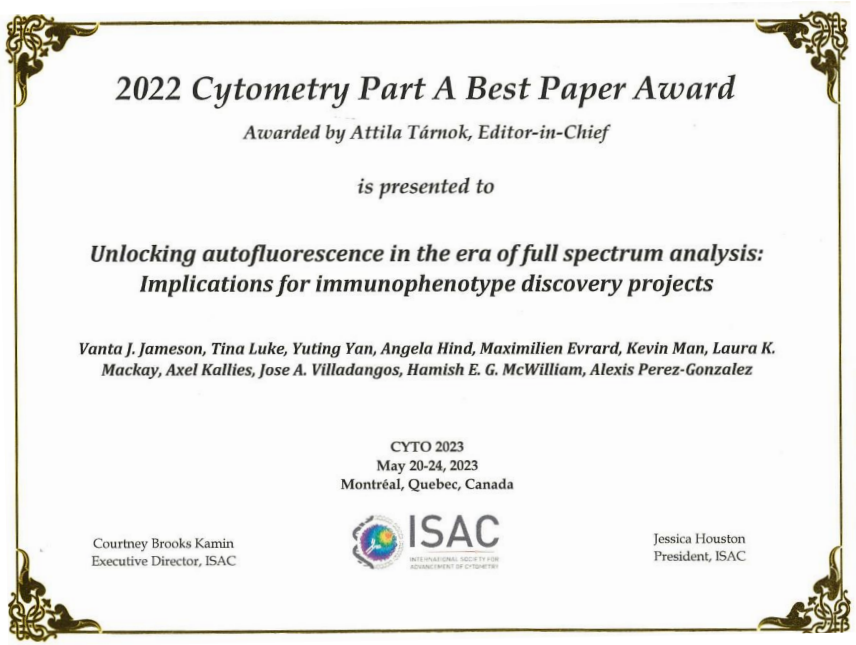


2. HiDi AF resolution– novel application for spectral cytometers



3. Practical application: biomarker discovery





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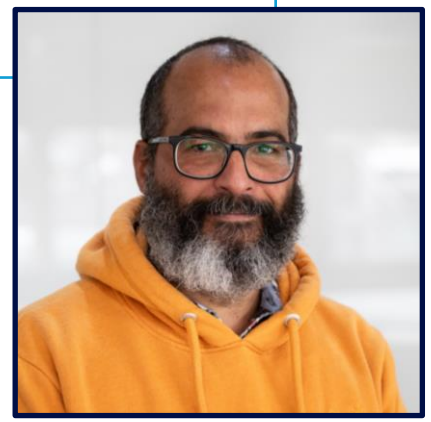
Evolution of the Melbourne Cytometry Platform – to deliver research excellence

2016-current
MCP managed instruments
Collaborative: nodes under a single management framework
Global education: training materials, workshops, seminars, lecturing, student supervision
Purchasing: competitive **grants** and collaborative researcher contributions: **merit, value add**
Staffing: full-time, **continuing**; career progression/ **development prioritised**
Proactive cytometrists dedicated to technology expertise and research outcome
Advanced QC, sensitivity, benchmarking
Regular acknowledgement, **collaborations**, **authorship**



2 priority areas (among others):

1. **Service** excellence– strong **rapport** between researchers and Platform
2. **Advanced** instrumentation **testing** and **investment** based on **merit**

1. Dedication to service for excellent scientific outcome

- **Proactivity**
  - Fair management of resources
  - Instrument care
  - ‘Can do’ mindset, troubleshooting, finding solutions
- **Expertise** (technology)
  - Capabilities/ limitations, direct to most appropriate resource
  - Fine-tuning, optimising assays
- **Education** (practical and theory) – knowledge is power
  - Hands-on training, troubleshooting, identifying problems
  - Workshops, seminars, lectures, tutorials
- **Engagement**
  - Communication – townhall meetings, user surveys
  - Consultation – experimental and panel design
  - **Collaboration**: **mutually beneficial** for researcher and MCP- expertise gained will benefit others

2. Cytometer testing: the SRL's duty of care

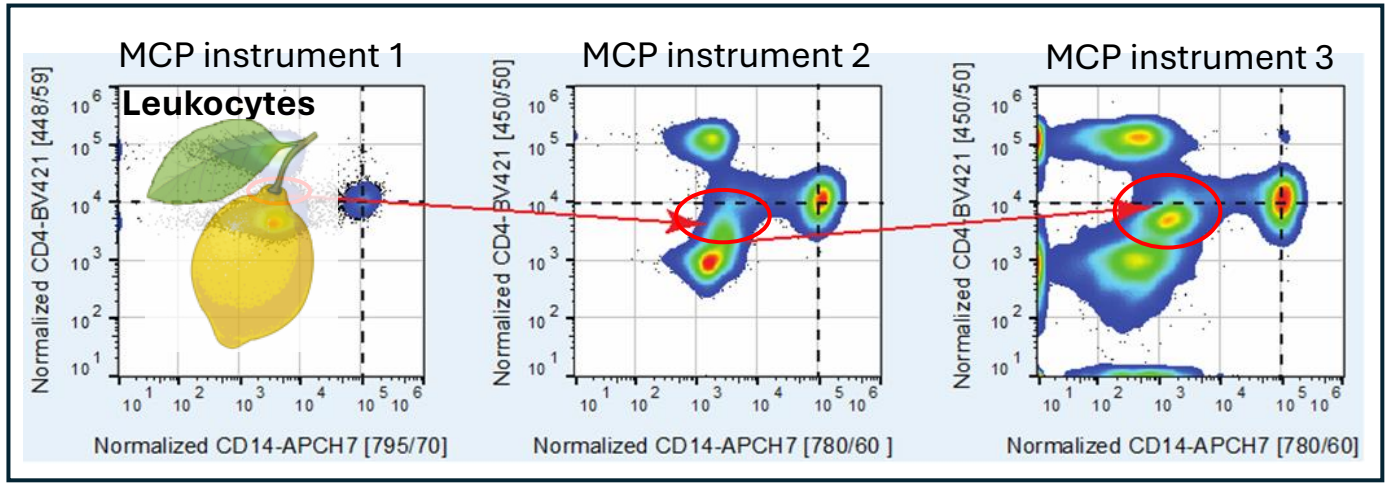
- **QC** and performance tracking – **beyond the basic**
  - Identify and intervene before data is compromised
- Testing and **understanding of instruments before** opening **service**
  - Abilities/ limitations, discovery of new applications
- **Benchmark** existing **cytometers** and new tech

Considerations for purchase

- Project compliant, demand
- Robust, consistent performance
- Added capabilities, future-proof
- Value for money
- **Sensitivity/ resolution**

Why the effort?

- Researchers cautious to adopt new technologies
- Poor signal resolution 'red-flags' tech
- Bad experiences go 'viral' condemning instruments to lemon status
- An instrument not used = resources wasted, impact on budget



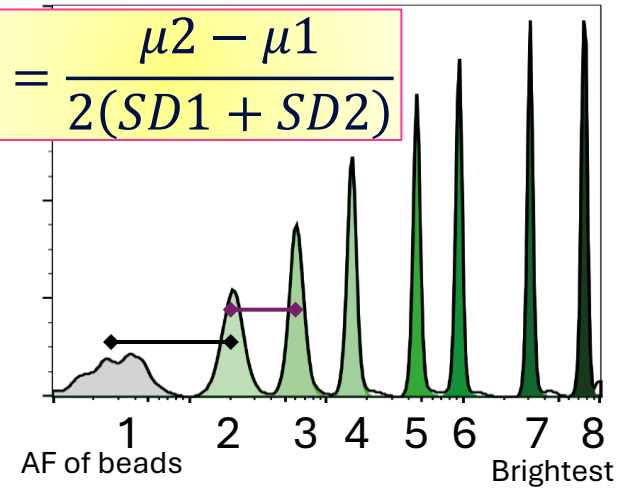
Instrument sensitivity assessment

- **Separation Index** (SI): robust metric to measure resolution of signals

$$SI = \frac{\mu 2 - \mu 1}{2(SD1 + SD2)}$$

- Higher SI = better resolution between 2 peaks (usually 1-2 or 2-3)

8-peak rainbow beads



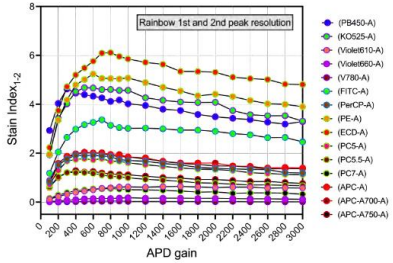
Powerful tool, routinely used at MCP for:

- Instrument design and configuration

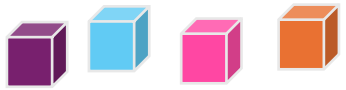
- Factory/field Consistency btw instruments

- Stability/baseline definition

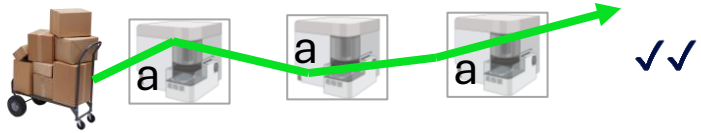
- Optimal detector settings (gain-trations)



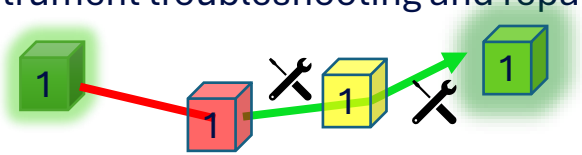
- Benchmark novel vs existing technologies



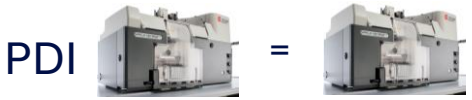
- Confirm installation and upgrade success

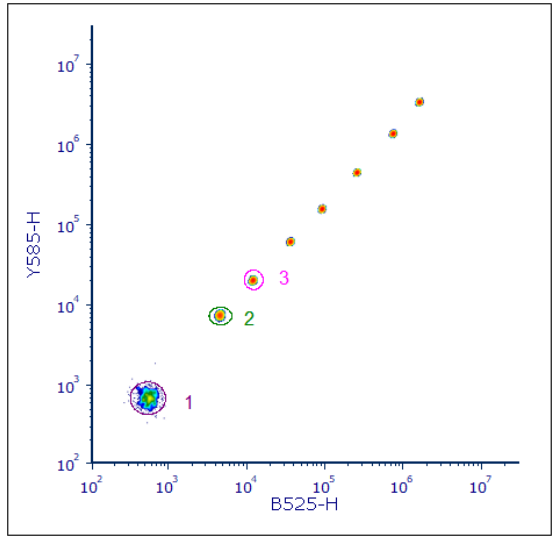


- Instrument troubleshooting and repair



- Matching instruments across sites





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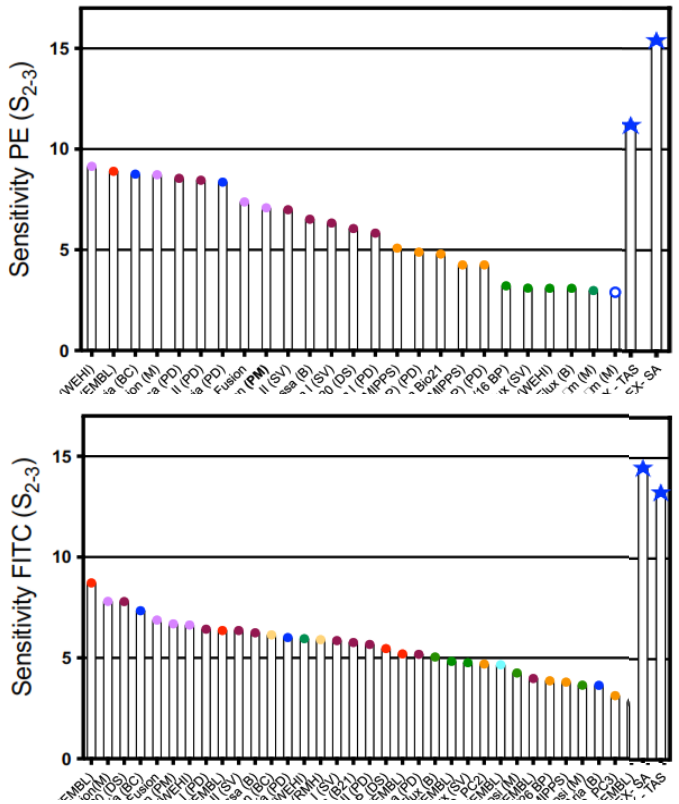
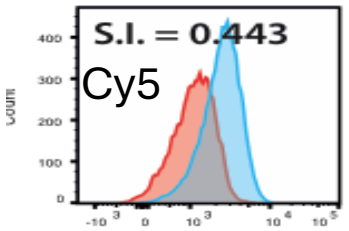
Sensitivity benchmarking – SI for CytoFLEX – freak of an instrument!

Parkville (2016) / EMBL cytometers – based on QC settings. SI rankings in FITC and PE detectors

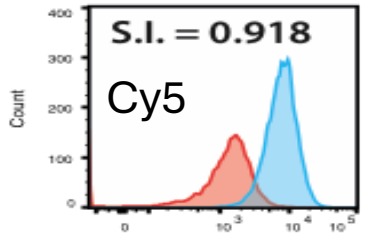


Cytometer X

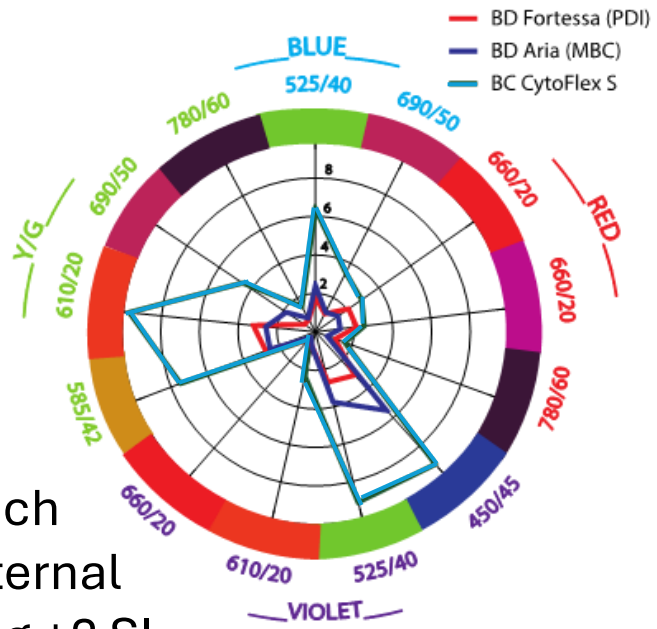


PMT voltage= 750 volts

CytoFLEX S

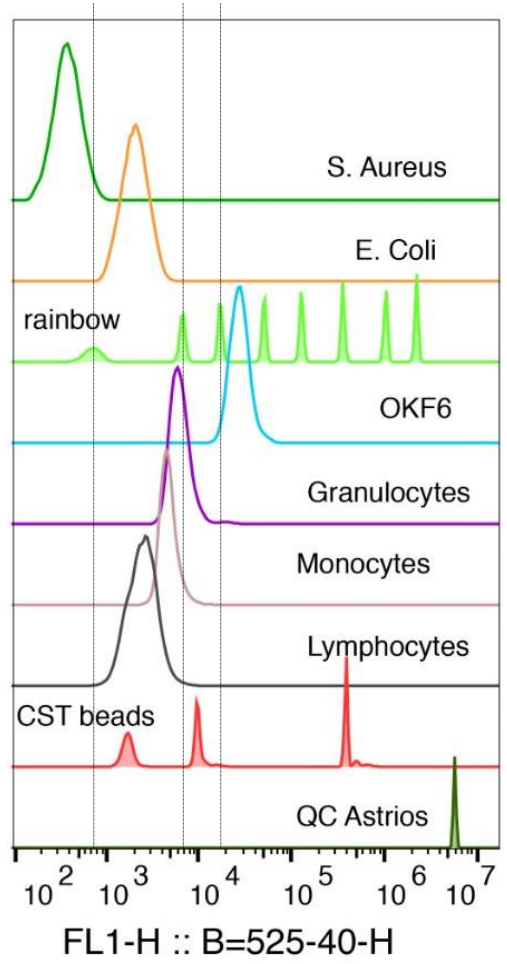


Gain = 344 (< optimum)



Each internal ring +2 SI

Why we care about low-end resolution



2016-2018: purchases for node-specific needs

Instruments purchased placed at nodes to best suit performed applications

CytoFLEX – dim signal resolution, microbiology applications - installed at **Melb Brain Centre** and **Dental School**

Aurora – sensitivity comparable to Fortessa, fluorescent probe 'agnostic', large panels - installed at **Doherty Institute**

Operational testing of our new purchases and existing cytometers

- **Detector sensitivity/ Gain-tration** (8-peak)
- Compare **sensitivity between like** cytometers (MBC CytoFLEX S, LX and DS CytoFLEX LX) (8-peak)
- Comparison between **spectral and conventional –** spread/spillover (panel transference)
- **Operational brightness of fluorochromes:** performance in Aurora and CytoFLEX

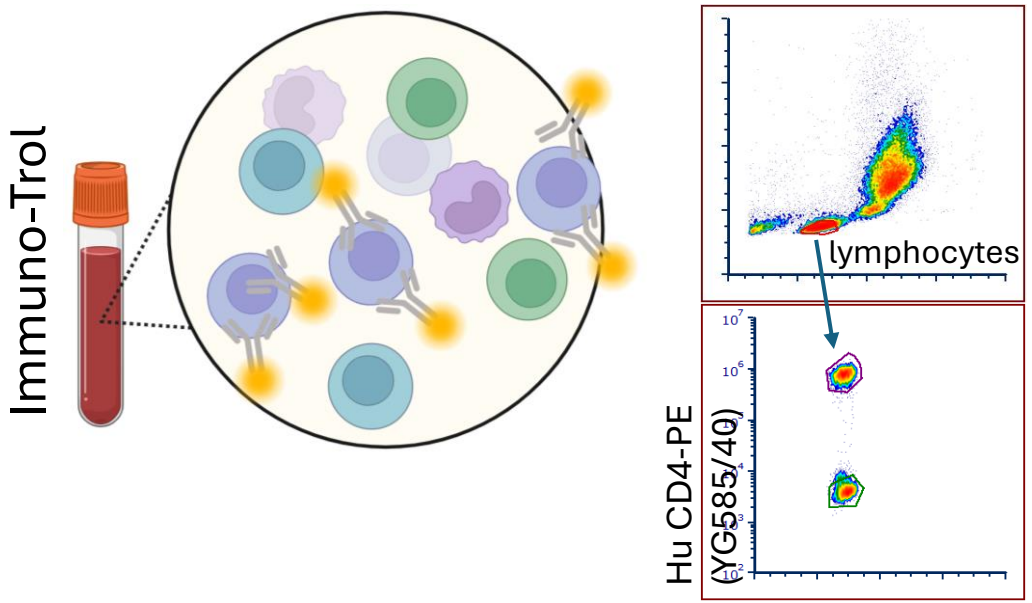
26-fluorochrome huCD4 test kit

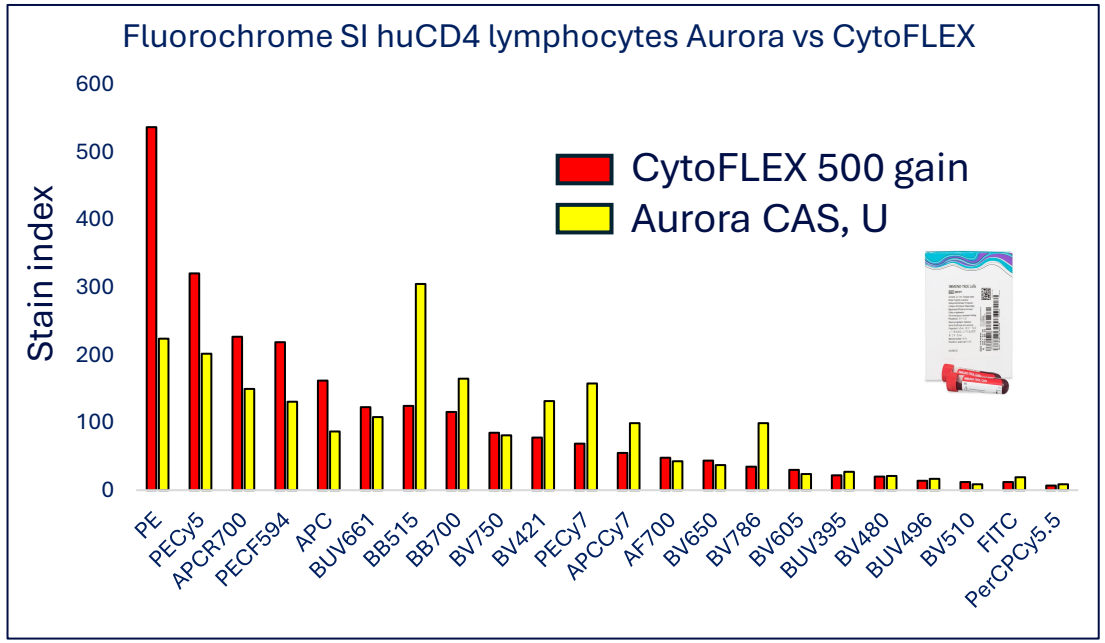
Immuno-Trol stabilised (fixed) human blood

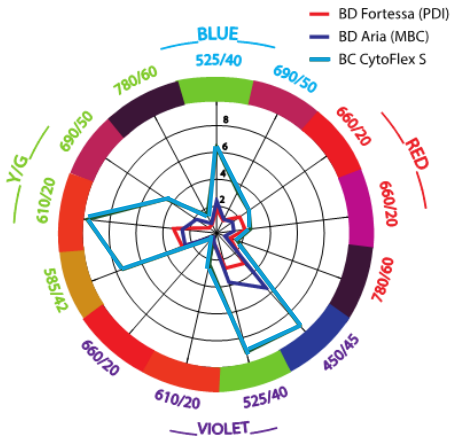
Fluorochrome operational brightness: CytoFLEX vs Aurora

Operational brightness (Stain Index SI)– related to separation index, but combination of: **Fluorochrome brightness AND instrument sensitivity**. The higher the number, the better the resolution

$$stain\ index\ (SI) = \frac{MFIpos - MFIneg}{2(SDneg)}$$







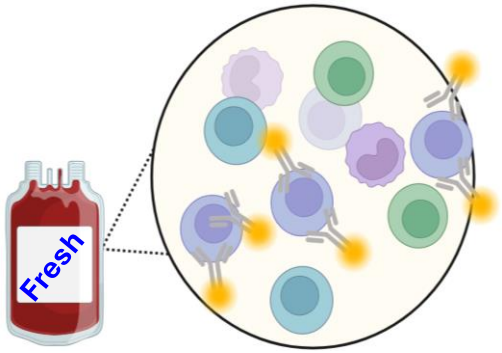
Stain Index results:

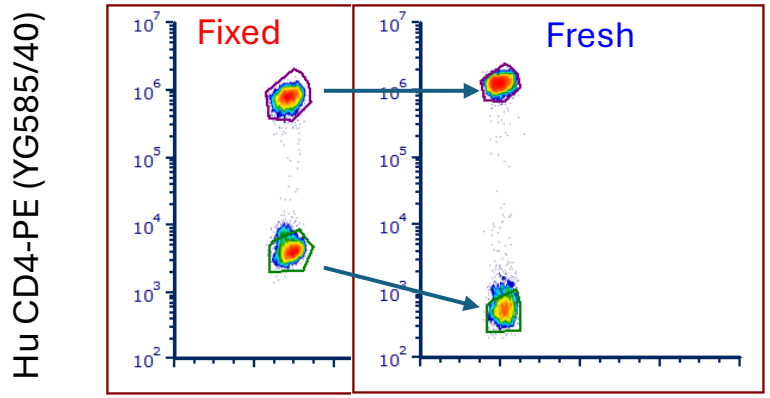
CytoFLEX *should* >> Aurora …

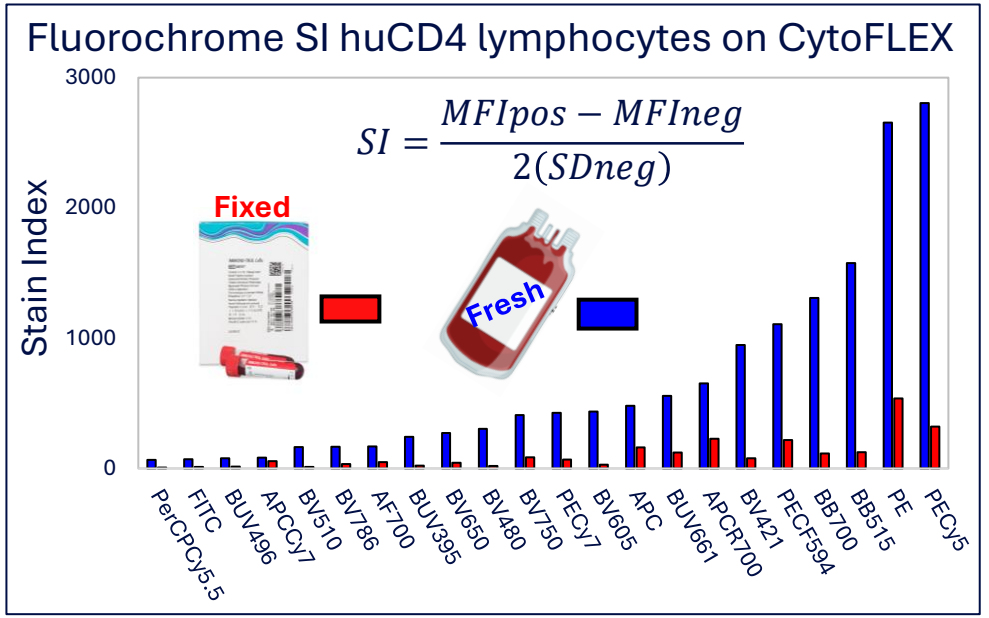
data not as impressive as expected based on 8-peak rainbow Separation Index values

Operational sensitivity of CytoFLEX LX: high and low AF

- Fresh blood – Stain index up to 8.7x higher than Immuno-Trol

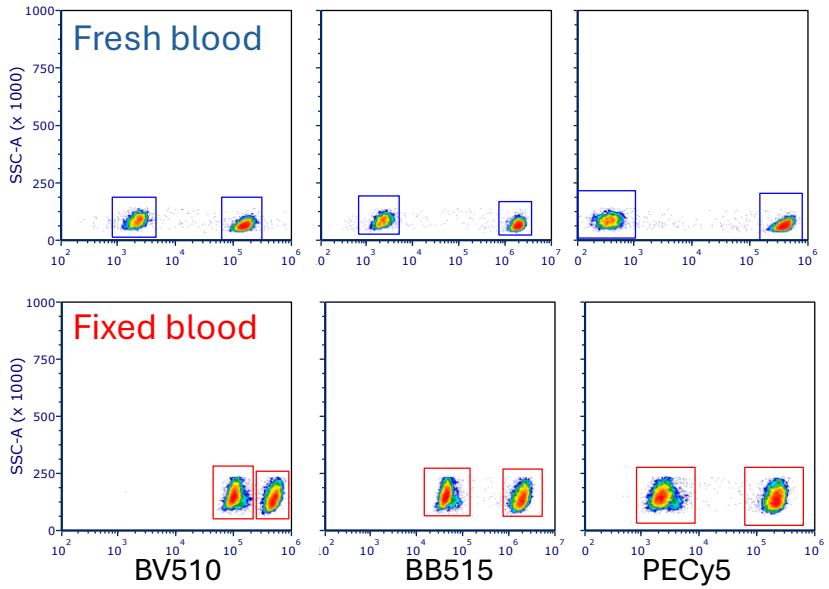






- High autofluorescence (AF) of unlabelled cells impacts on resolution
- Brightness of fluorochromes re-ordered depending on AF of carriers

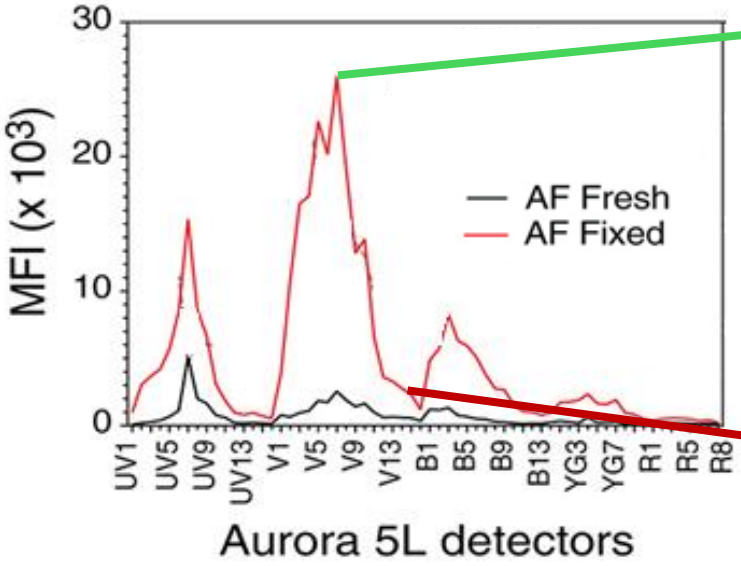
	PCPCy5.5	FITC	BUV496	APCCy7	BV510	BV786	AF700	BUV395	BV650	BV480	BV750	PECy7	BV605	APC	BUV661	APCR700	BV421	PECF594	BB700	BB515	PE	PECy5
Fresh	67	70	78	82	165	166	169	243	273	305	410	427	436	491	558	652	948	1108	1310	1576	2654	2805
Fixed	7	12	14	55	12	35	48	22	44	20	85	69	30	162	123	227	78	219	116	125	537	321



- **Operational brightness depends on: Fluorochrome brightness AND instrument sensitivity AND autofluorescence!!!**

Aurora 5L: Fluors that peak in regions of high AF benefit from AF extraction

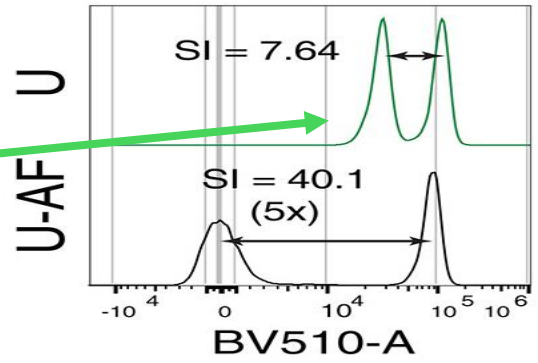
Aurora 5L raw detectors displaying AF of unlabelled fresh and fixed lymphocytes



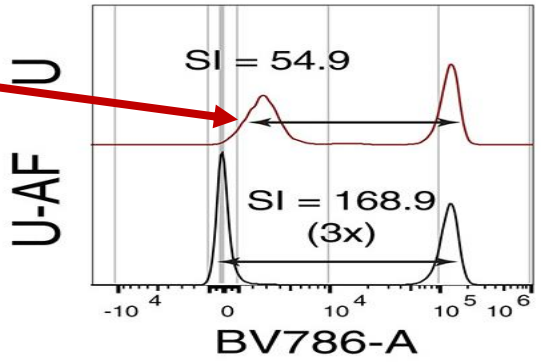
U – unmixed

U-AF – unmixed with AF extraction

SI improvement after AF extraction on unmixed fixed lymphocytes

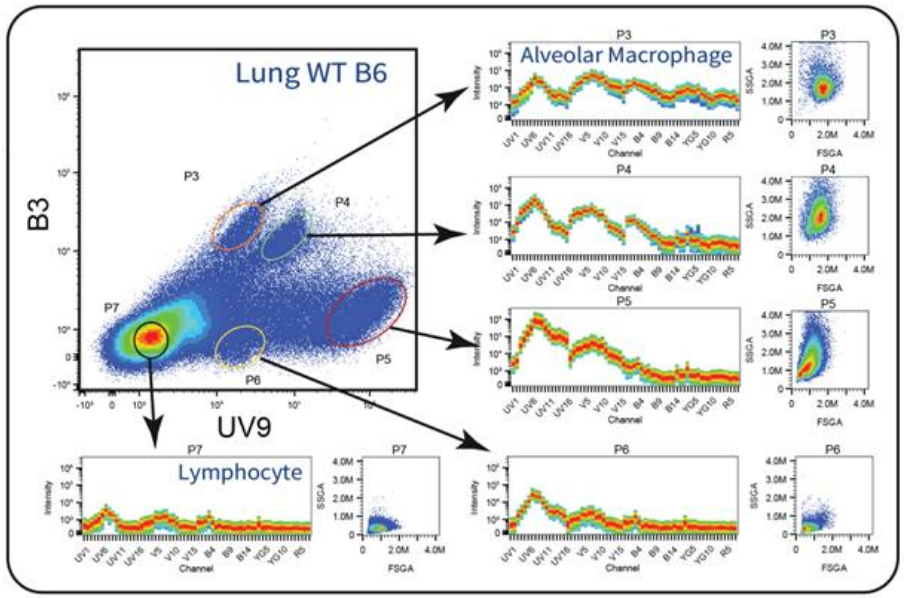


BV510 emission in area of high AF (raw V6)



BV786 emission in area of low AF (raw V14)

High AF cells likely to benefit from cell-specific AF extraction



Benefit of AF extraction in low AF setting marginal, maths to predict level of AF at which AF extraction should be applied

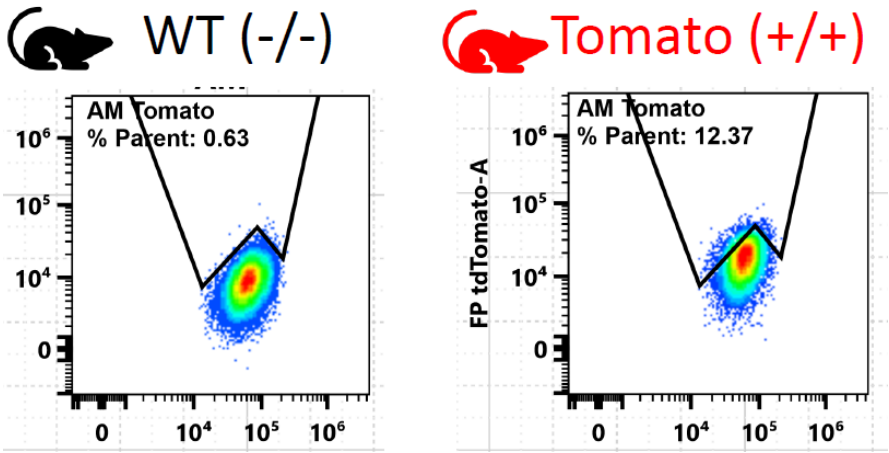
Cytometer testing – sensitivity and autofluorescence

- **CytoFLEX – exquisite sensitivity** in YG detectors (PE & PE tandems, tdTomato, RFP, mCherry) regardless of carrier AF
- '**Brightness**' of fluorochromes **dependent on AF** of carrier particle
- **High AF reduces** operational **brightness** of all fluorochromes
- **AF extraction** greatest **benefit** when carrier particle **AF MFI ~10^4**
- **AF** in **resolution theory**

Discovery project – expression of MR1 in immune subsets

MCP collaboration: Yuting Yan, Hamish McWilliam (Jose Villadangos group)

- Tissue and cellular subset **expression of MR1** (involved in antigen presentation) **unknown**
- Transgenic mouse model with **tdTomato-MR1 gene reporter.** Expectation **expression very low**
- Tissues of interest: **spleen, skin** and **lung** (AF populations)
- Lab's **initial** independent **phenotyping** on Aurora and Fortessa **"didn't work"** → MCP for best approach



Weak FP expression

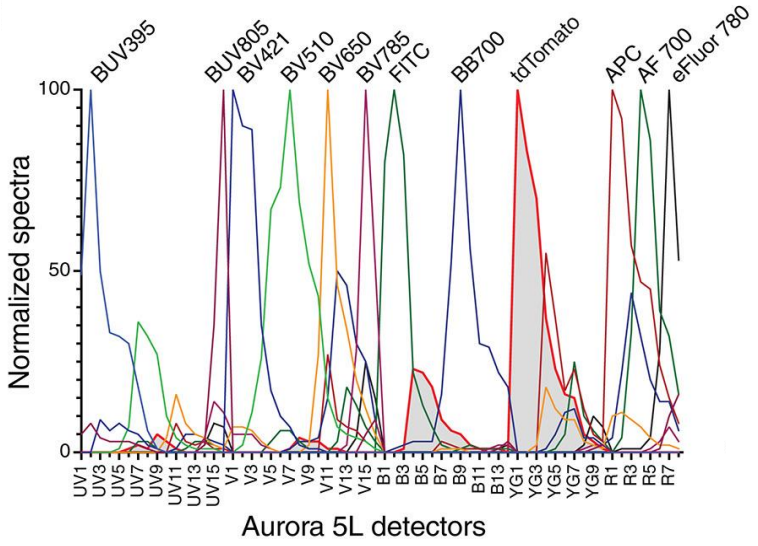
Subset expression unknown

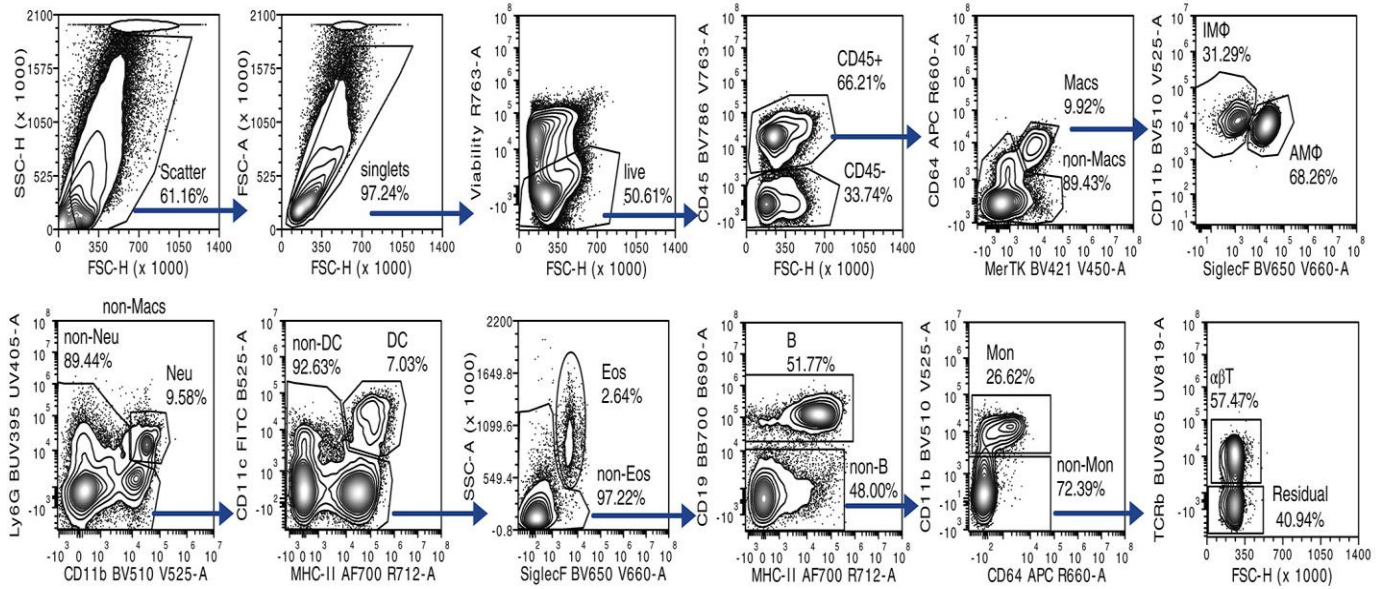
The **PERFECT** biological model to apply cytometer testing, autofluorescence findings

Cells with low and high AF

COLLABORATION!

Panel/ experimental design to identify low tdTomato expression in mixed lineage samples



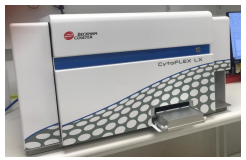


FlowJo SSM

	BUV395	BUV805	BV421	BV510	BV650	BV785	FITC	BB700	tdTomato	APC	AF 700	eFluor 78	spreading inflicted
BUV395	0.00	1.43	0.00	0.00	0.37	0.82	0.00	0.00	0.00	0.04	0.68	0.00	3.33
BUV805	3.08	0.00	0.00	1.87	0.00	3.58	1.19	0.57	0.44	0.85	0.00	0.35	11.93
BV421	17.73	0.00	0.00	0.00	0.00	1.03	0.00	0.00	0.00	0.00	1.22	0.00	19.98
BV510	0.00	0.00	0.30	0.00	1.17	0.00	0.00	0.00	0.00	0.43	1.03	0.00	2.93
BV650	0.00	3.31	0.59	0.00	0.00	3.53	0.00	0.39	0.00	2.51	8.96	2.05	21.34
BV785	1.12	1.98	0.27	0.60	0.36	0.00	0.00	0.00	0.31	0.24	0.38	0.40	5.66
FITC	4.86	0.54	0.00	0.51	0.31	0.00	0.00	0.32	0.19	0.00	0.00	0.00	6.73
BB700	0.00	9.44	1.65	0.73	2.92	8.65	1.53	0.00	0.51	1.67	10.40	7.45	44.95
tdTomato	15.95	0.00	1.82	0.00	1.69	0.00	0.00	1.01	0.00	0.47	0.00	0.47	21.42
APC	1.04	5.87	1.09	0.15	5.37	5.72	1.12	0.37	0.18	0.00	3.66	3.78	28.35
AF 700	0.00	6.64	1.17	0.06	0.24	6.17	1.11	0.36	0.12	0.34	0.00	4.08	20.27
eFluor 780	173.3	86.52	14.95	3.02	3.20	35.35	5.73	1.26	2.36	1.88	12.87	0.00	340.47
spreading received	217.11	115.72	21.84	6.94	15.63	64.84	10.68	4.29	4.12	9.20	18.57		

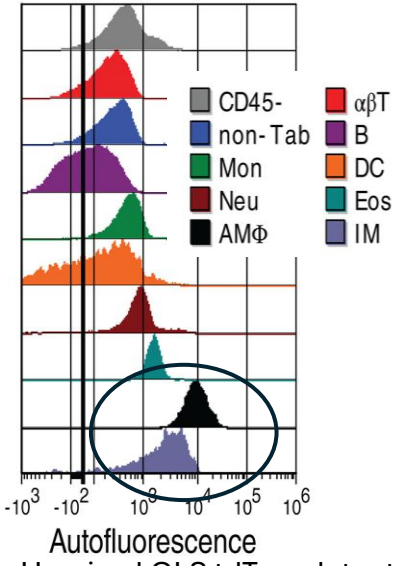
Broad panel design to fit both CytoFLEX and Aurora 5L

- Sensitivity of CytoFLEX versus Aurora AF extraction
- Avoid spillover into tdTomato

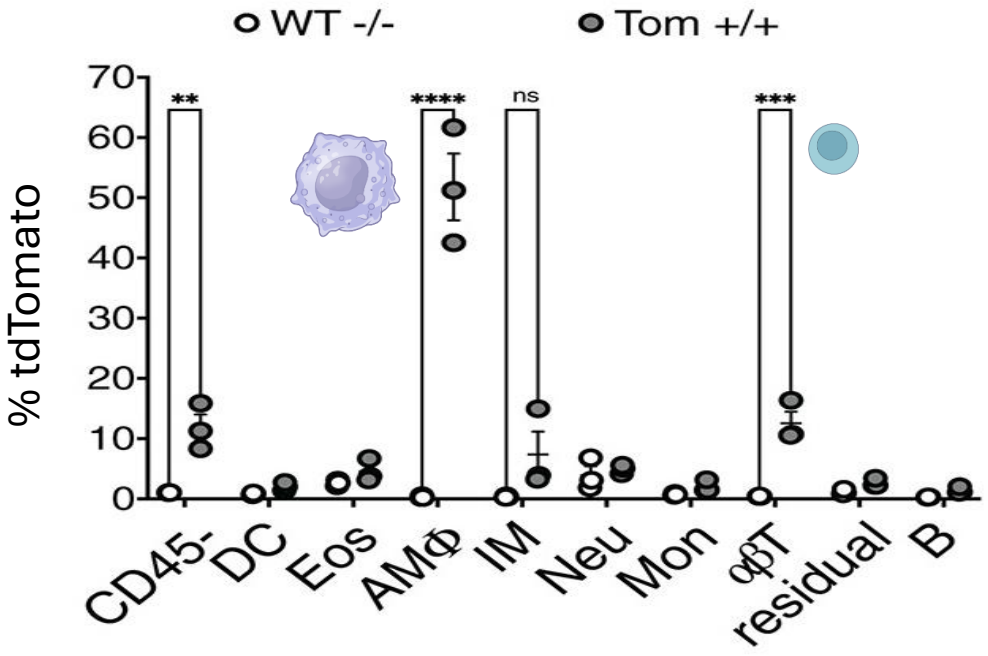


Sensitivity versus AF extraction??

AF of WT mouse leukocyte subsets

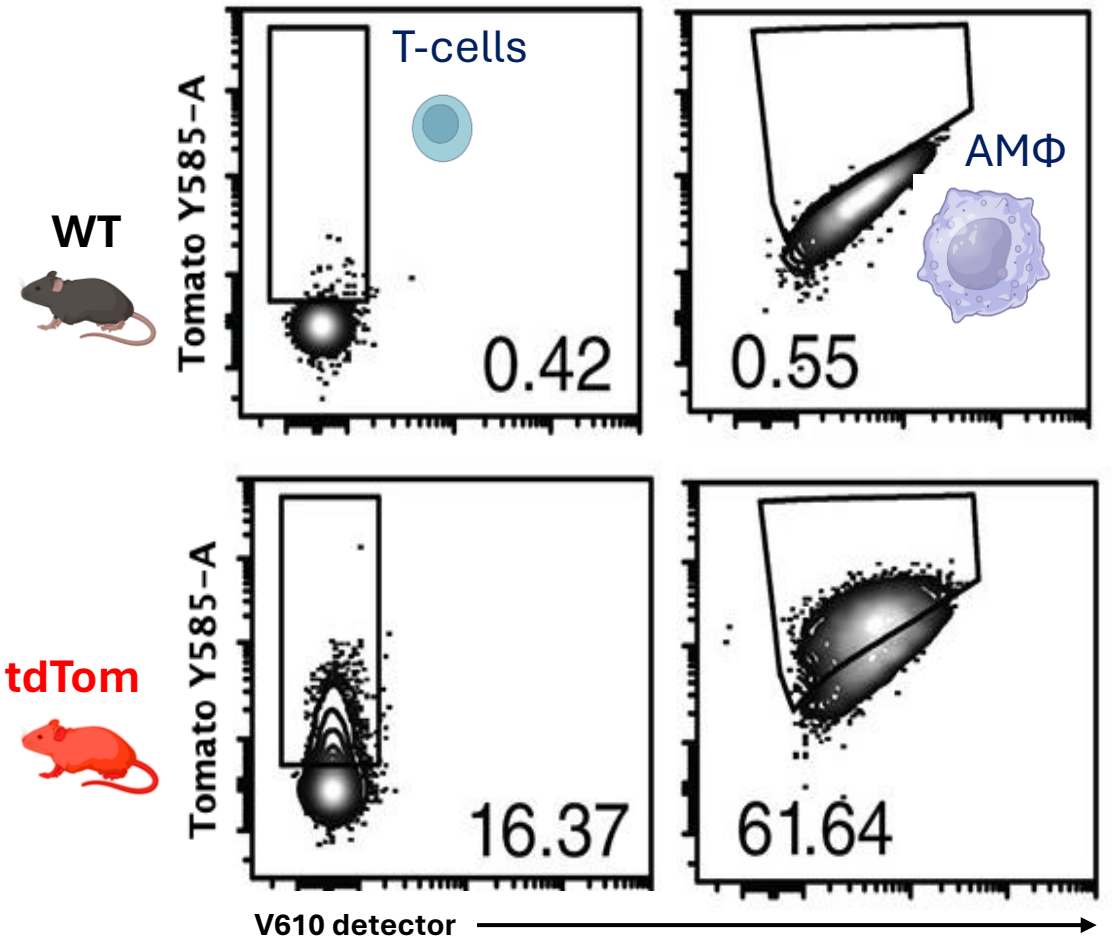


CytoFLEX LX reveals tdTomato-MR1 expression in low and high AF cells in lung



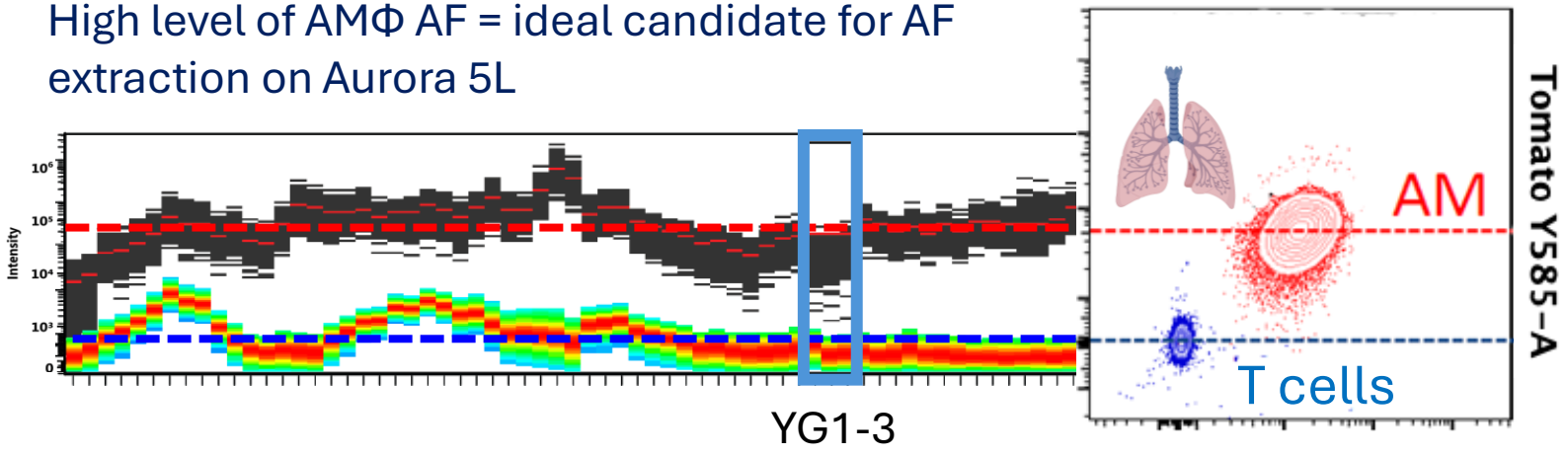
- Highest tdTom expression in lung alveolar macrophages (AMΦ) and αβT-cells
- AMΦ autofluorescence exceptionally high
- CytoFLEX sensitivity overcomes high AF

CytoFLEX data

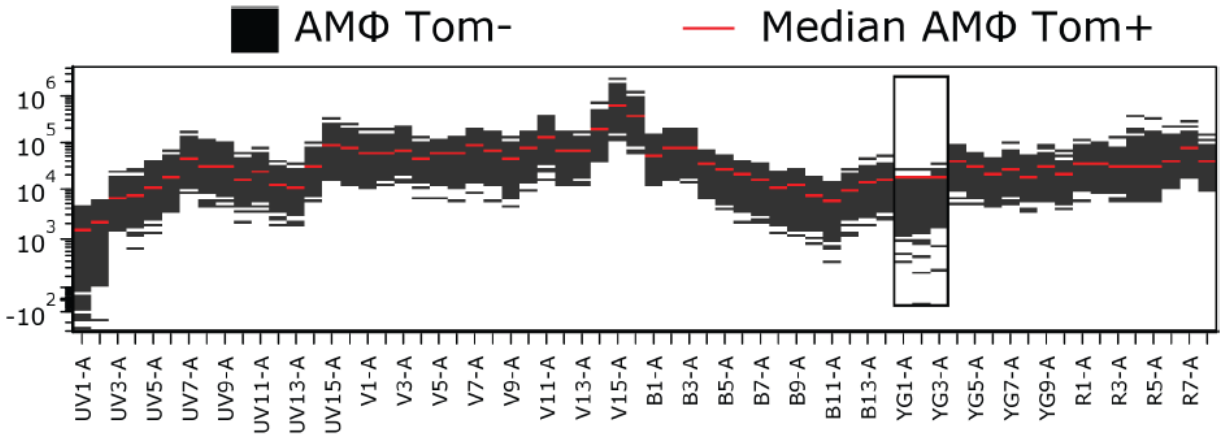


AMΦ-specific AF extraction resolves tdTom+ cells in lung

High level of AMΦ AF = ideal candidate for AF extraction on Aurora 5L

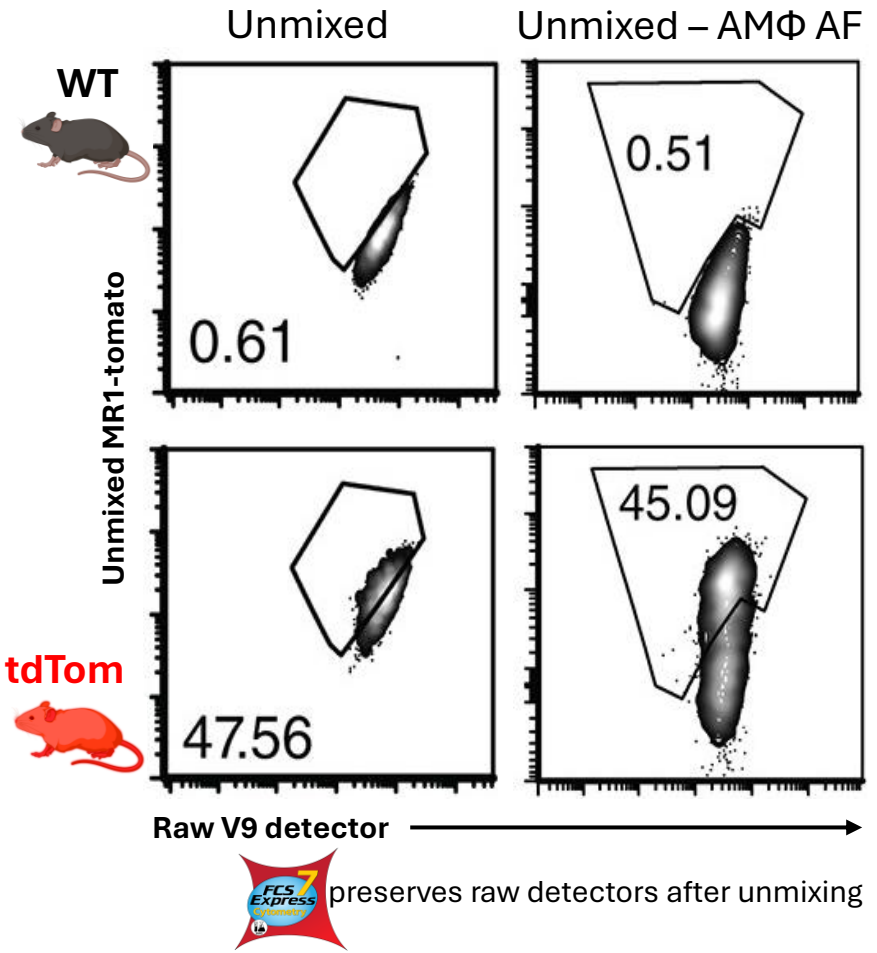


AF obscures tdTom expression in Tom+ AMΦ



Manuscript in review in **Science**

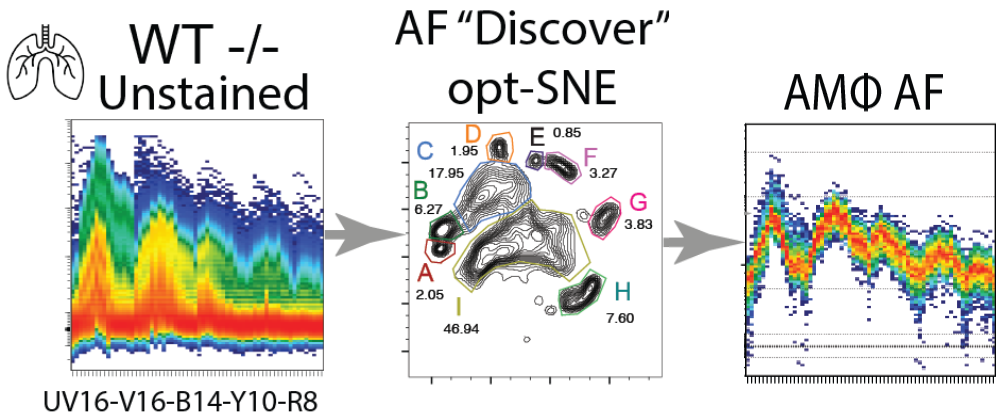
Extraction of AMΦ -specific AF resolves tdTomato+ AMΦ

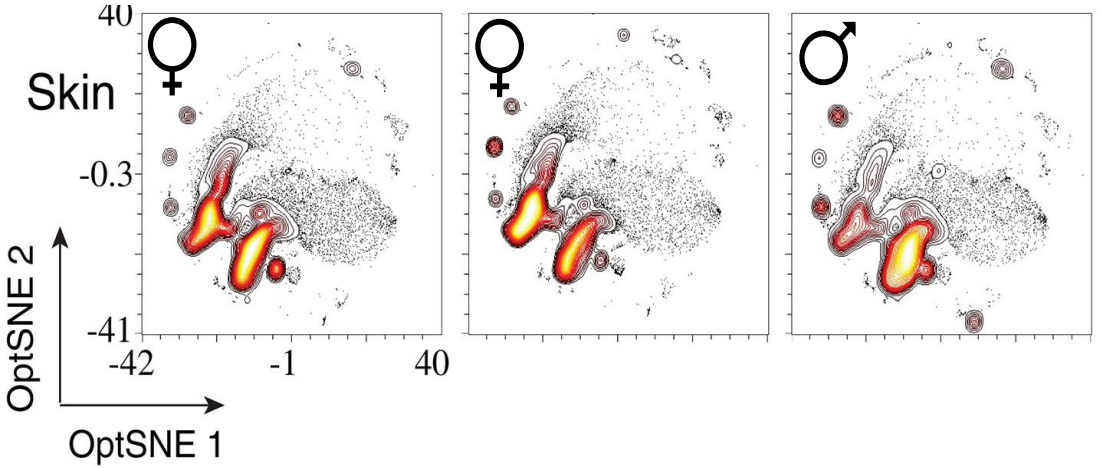


FCS Express preserves raw detectors after unmixing

Novel application for spectral cytometers: HiDi AF resolution

- Through instrument sensitivity testing, collaboration, can discover and develop new methodologies
  - Novel methodology: **full-spectrum AF** of unlabelled samples acquired on Aurora 5L sufficient to define/ **identify cellular** subsets using **opt-SNE**
  - Impactful for **discovery projects** - such as tdTomato cellular/ tissue distribution vs WT
  - Identification of **biologically relevant phenotypes**: male vs female skin, chronic vs acute inflammation
  - **UV and violet excitation** most important for resolving differences in AF in mammalian tissues





In conclusion…

- SRL staff are as critical a part of the scientific discovery process as researchers
- SRLs' strong interest in instrument performance good for researchers AND encourages production of better/ more sensitive cytometers
- Through duty of care, collection of day-to-day data and curiosity, discoveries that can be impactful for the broader scientific community are made
- Proactivity in service is *intellectually stimulating, rewarding* and *fun*

Thank you

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