

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

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THE UNIVERSITY OF
MELBOURNE

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



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

Cytometry
PART A

Journal of Quantitative
Cell Science



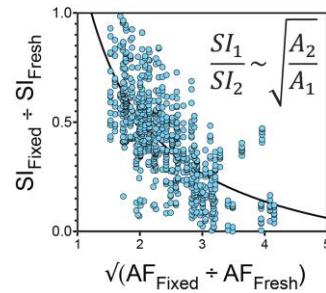
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Unlocking autofluorescence in the era of full spectrum analysis: Implications for immunophenotype discovery projects

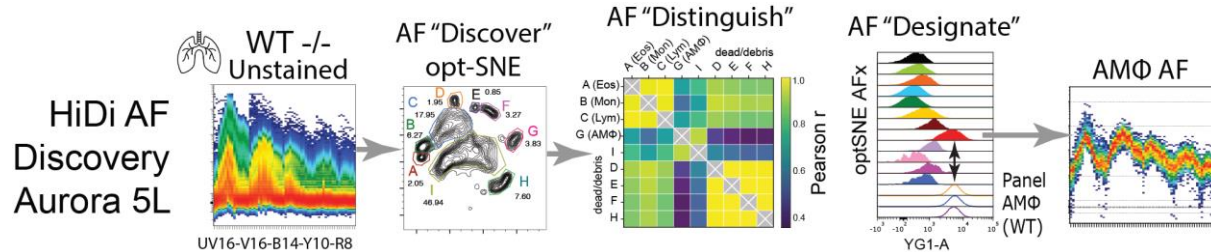
Vanta J. Jameson, Tina Luke, Yuting Yan, Angela Hind, Maximilien Evrard, Kevin Man, Laura K. Mackay, Axel Kallies, Jose A. Villadangos, Hamish E. G. McWilliam, Alexis Perez-Gonzalez

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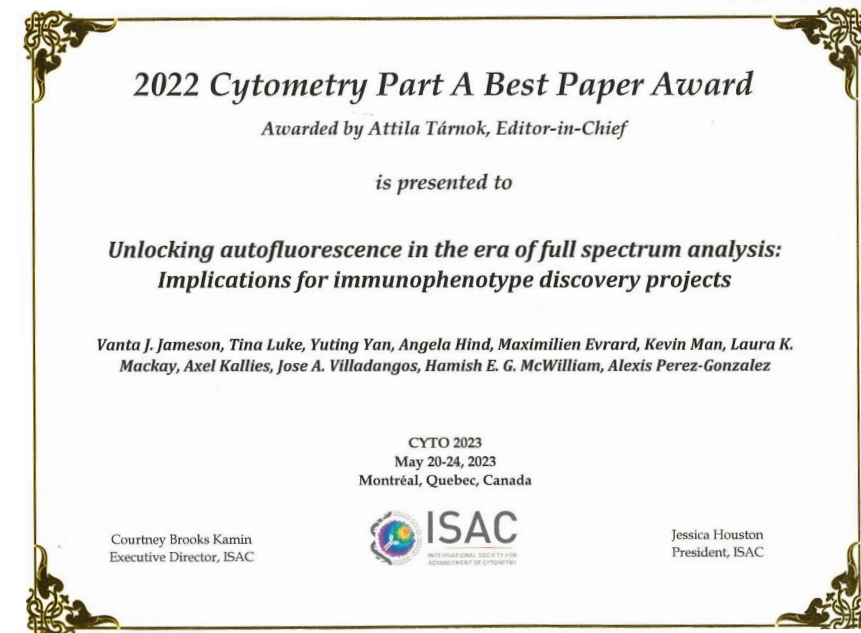
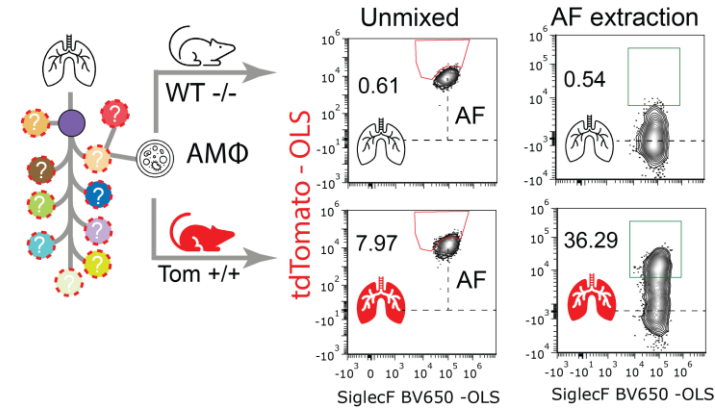
1. Autofluorescence in sensitivity theory



2. HiDi AF resolution—novel application for spectral cytometers



3. Practical application: biomarker discovery



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

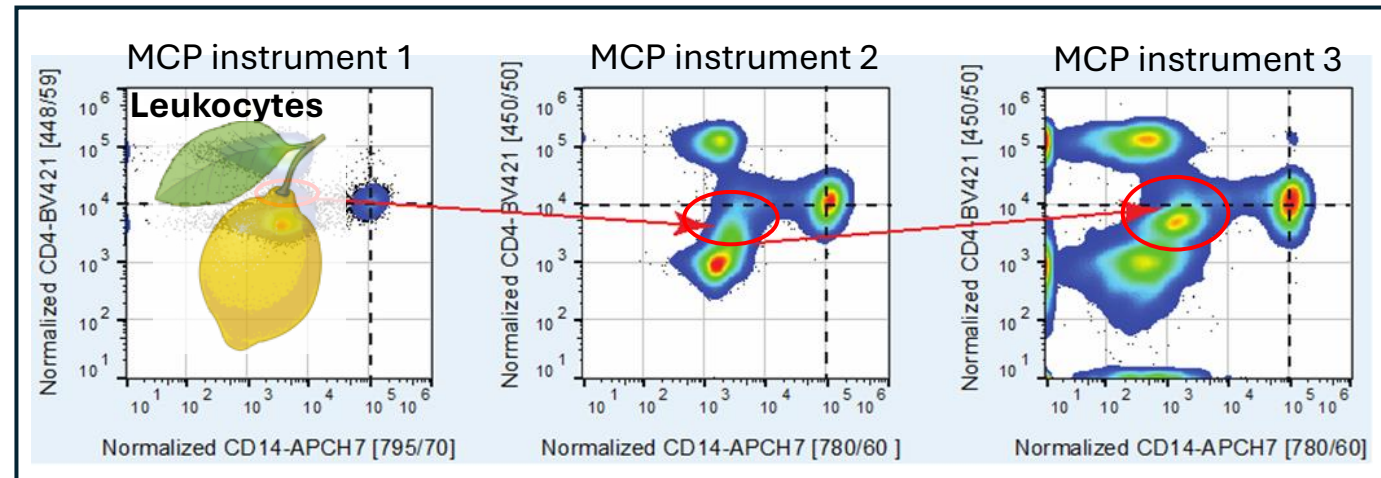
- **QC** and performance tracking – **beyond the basic**
 - Identify and intervene before data is compromised
- Testing and **understanding of instruments before opening service**
 - Abilities/ limitations, discovery of new applications
- **Benchmark** existing **cytometers** and new tech

Considerations for purchase

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

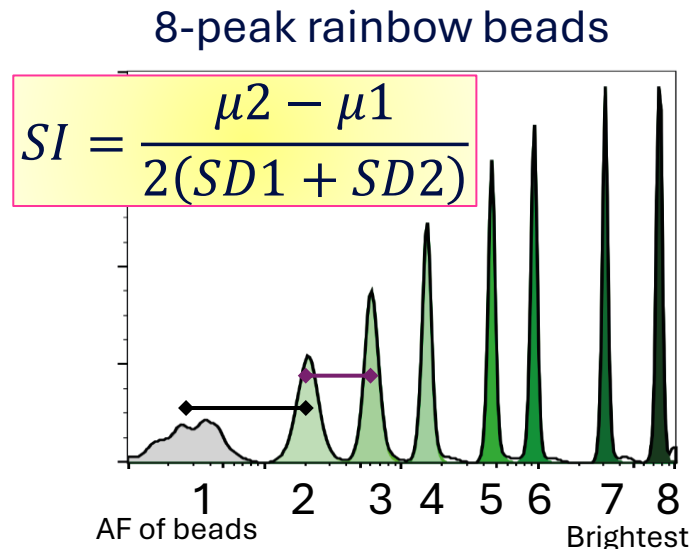
Why the effort?

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



Powerful tool, routinely used at MCP for:

- Instrument design and configuration



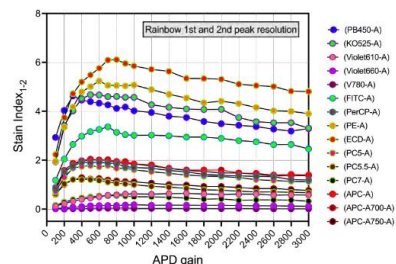
- Factory/field Consistency btw instruments



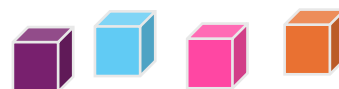
- Stability/baseline definition



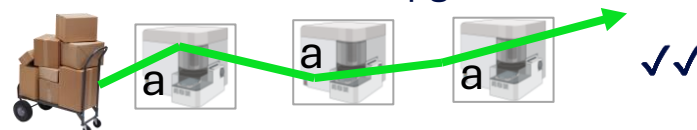
- Optimal detector settings (gain-trations)



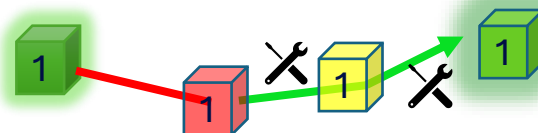
- Benchmark novel vs existing technologies



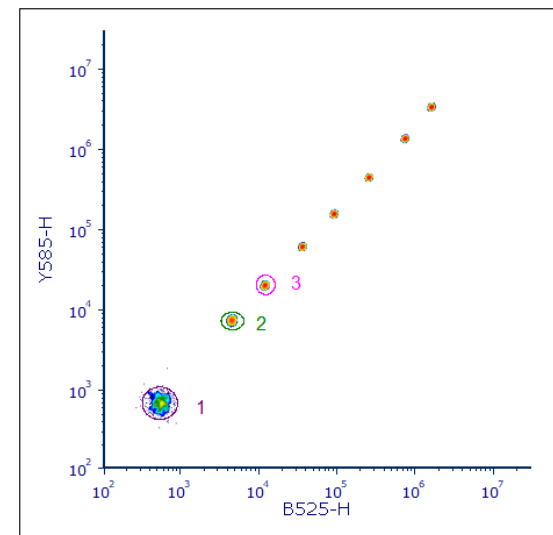
- Confirm installation and upgrade success



- Instrument troubleshooting and repair

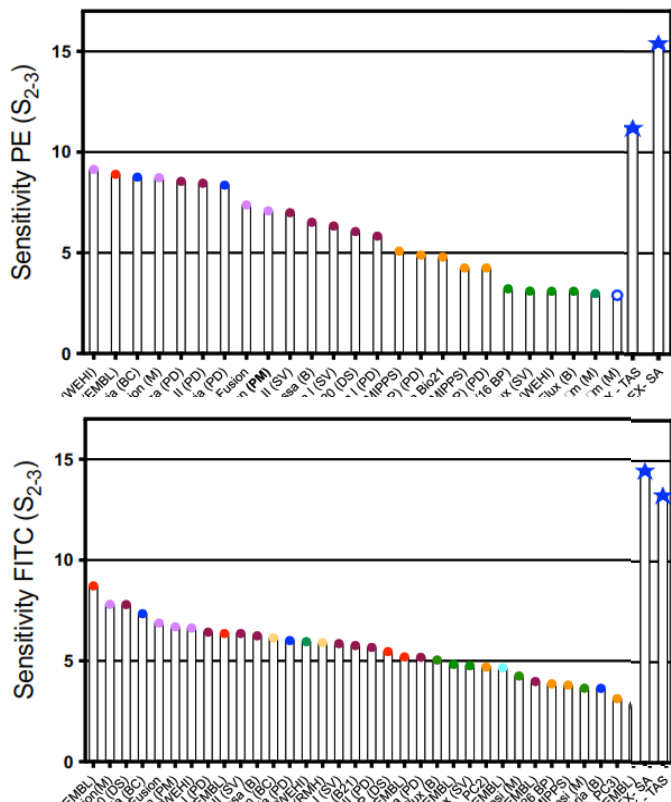


- Matching instruments across sites

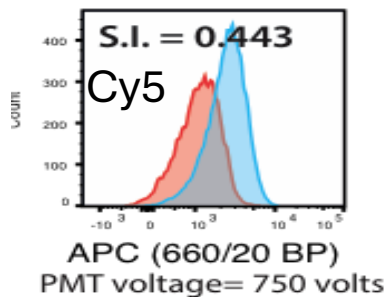


Sensitivity benchmarking – SI for CytoFLEX – freak of an instrument!

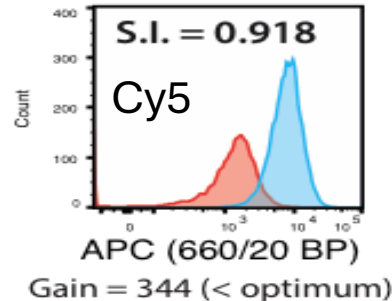
Parkville (2016) / EMBL cytometers – based on QC settings. SI rankings in FITC and PE detectors



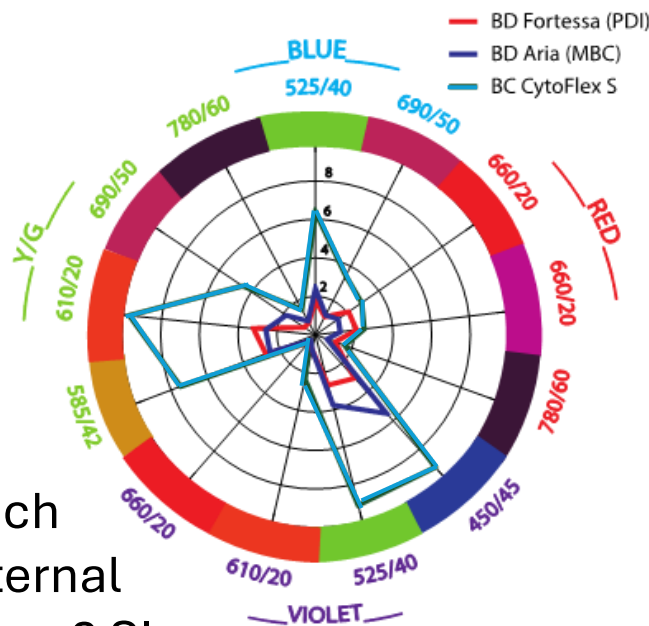
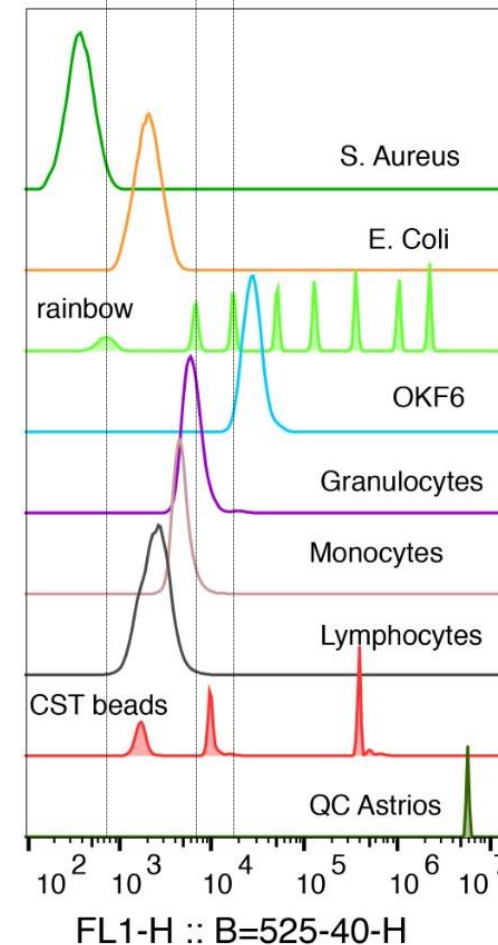
Cytometer X



CytoFLEX S



Why we care about low-end resolution

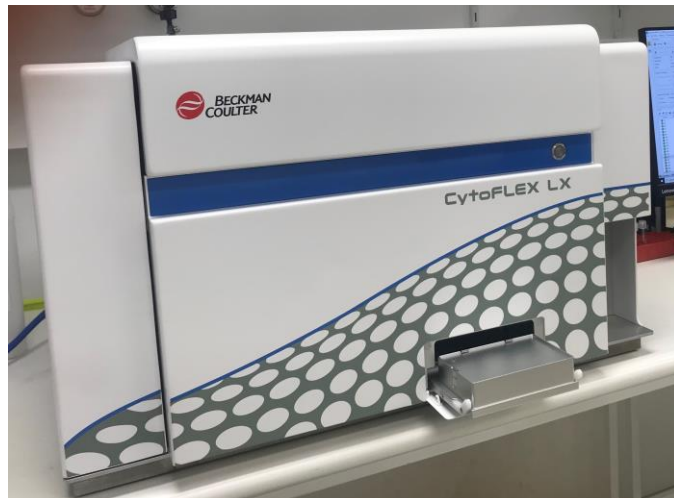


Each internal ring +2 SI

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



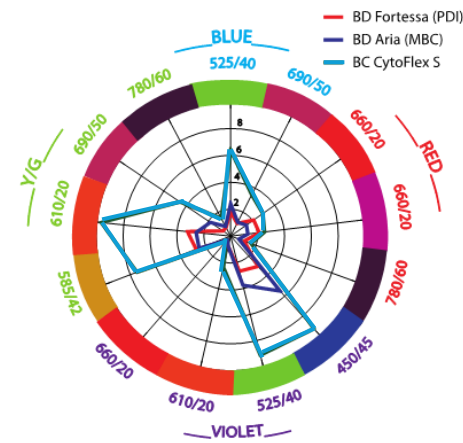
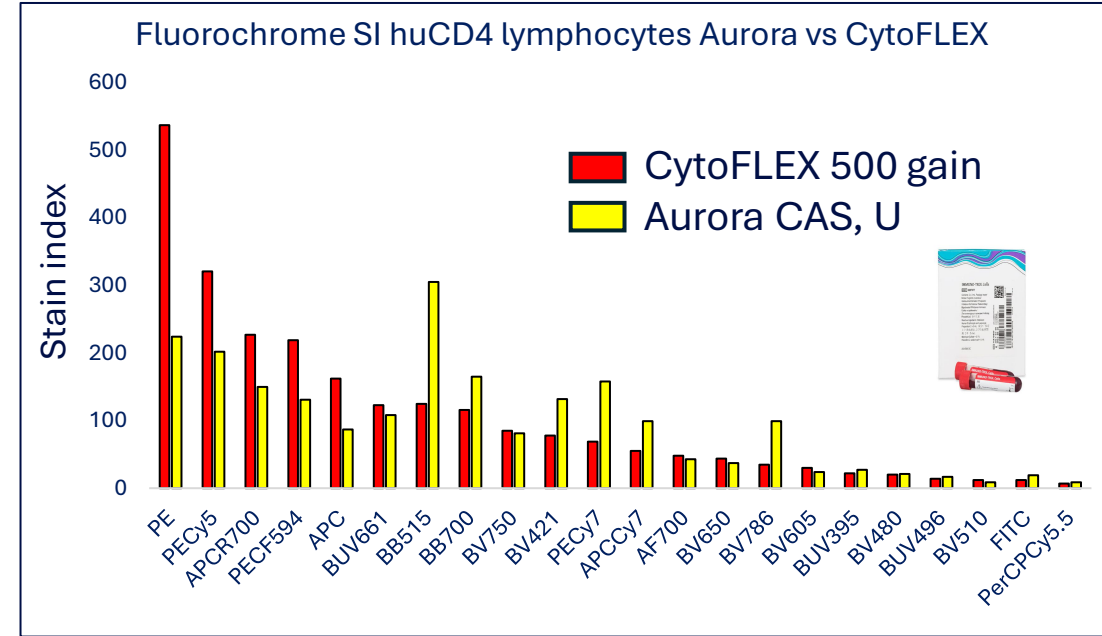
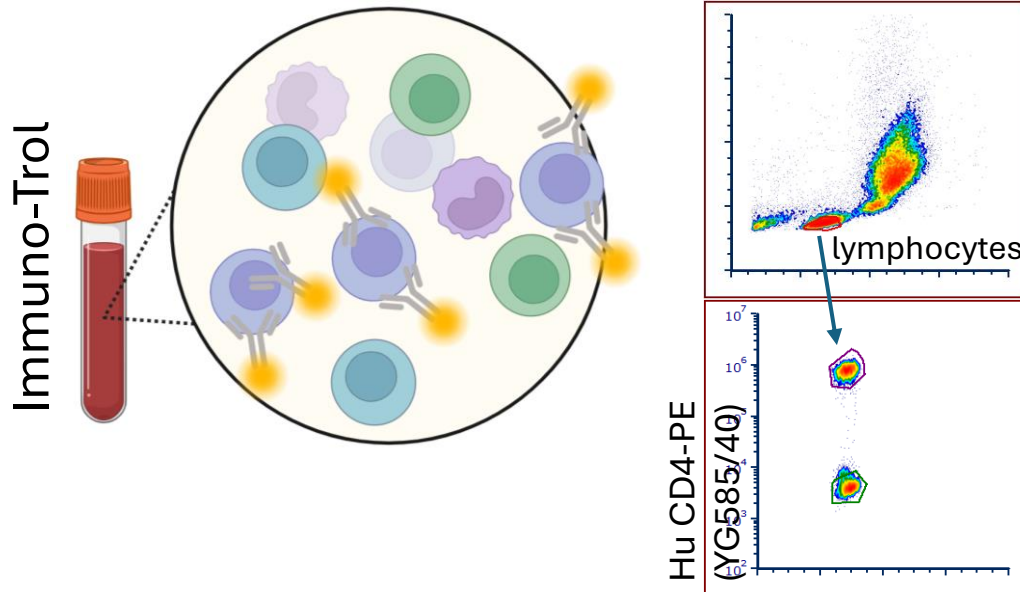
Immuno-Trol stabilised (fixed) human blood



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

$$\text{stain index (SI)} = \frac{MFI_{pos} - MFI_{neg}}{2(SD_{neg})}$$



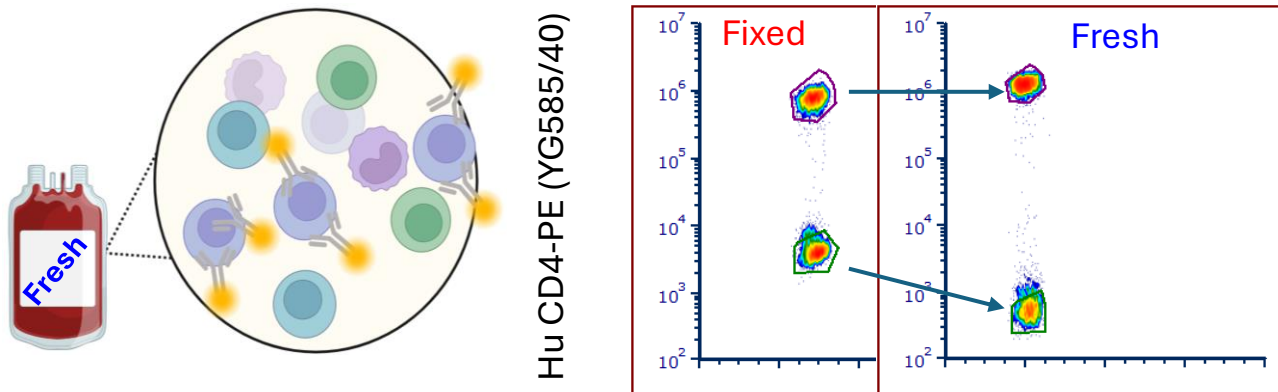
Stain Index results:

CytoFLEX should >> Aurora ...

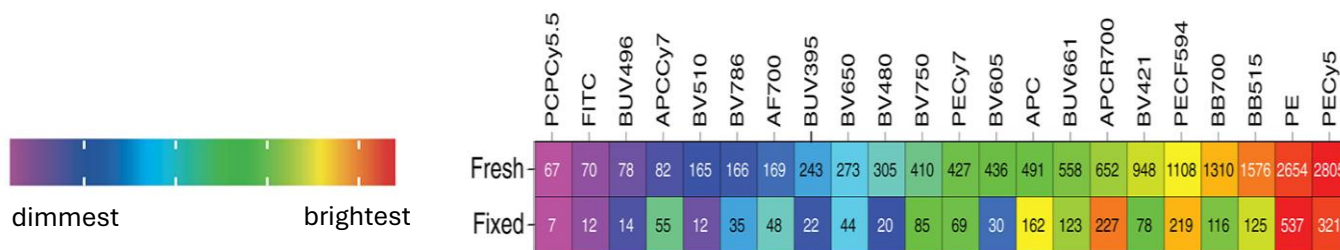
data not as impressive as expected based on 8-peak rainbow Separation Index values

Operational sensitivity of CytoFLEX LX: high and low AF

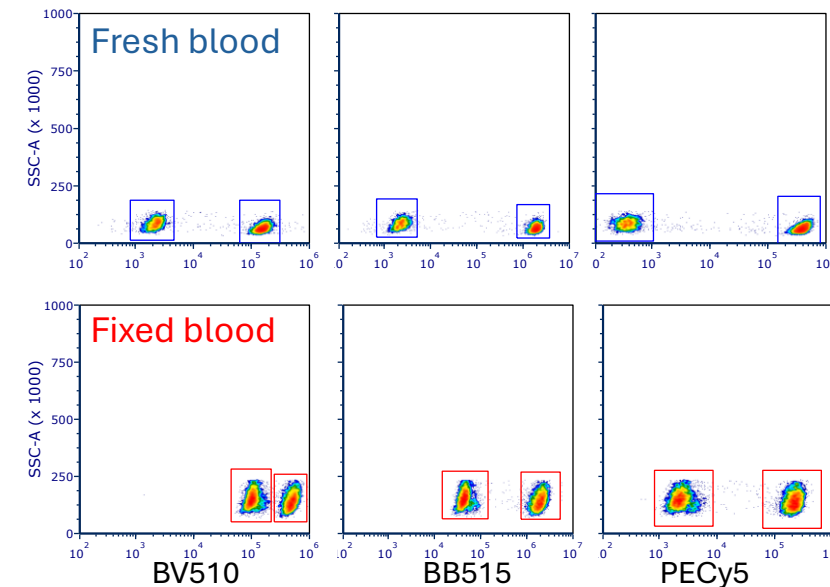
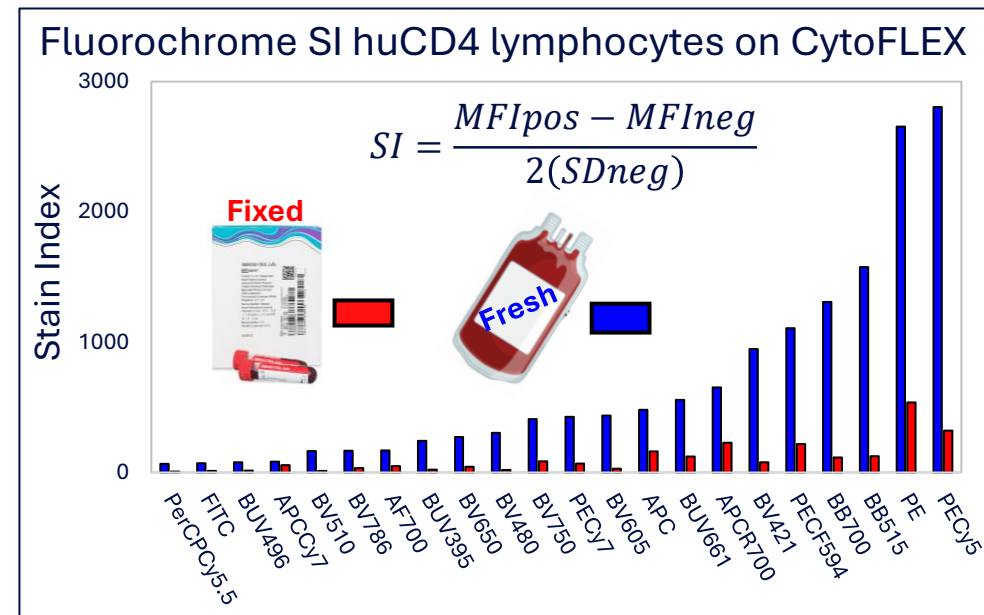
- Fresh blood – Stain index up to 8.7x higher than Immuno-Trol



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

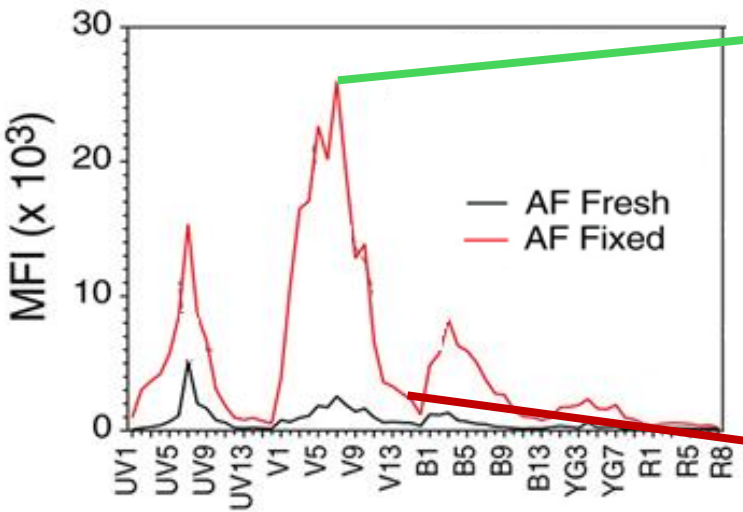


- **Operational brightness depends on: Fluorochrome brightness AND instrument sensitivity AND autofluorescence!!!**



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

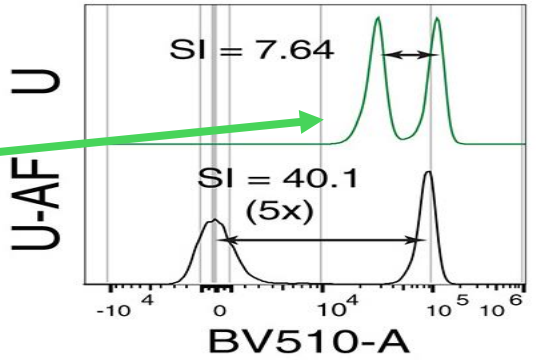
Aurora 5L raw detectors displaying AF of unlabelled fresh and fixed lymphocytes



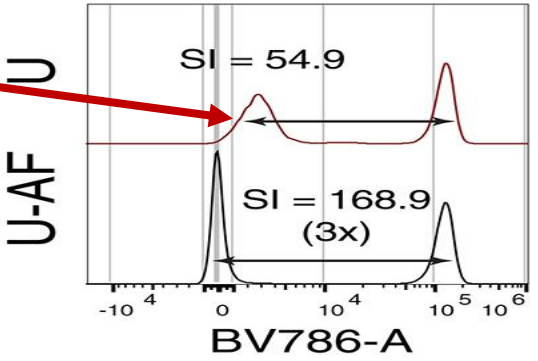
Aurora 5L detectors

U – unmixed
U-AF – unmixed with AF extraction

SI improvement after AF extraction on unmixed fixed lymphocytes

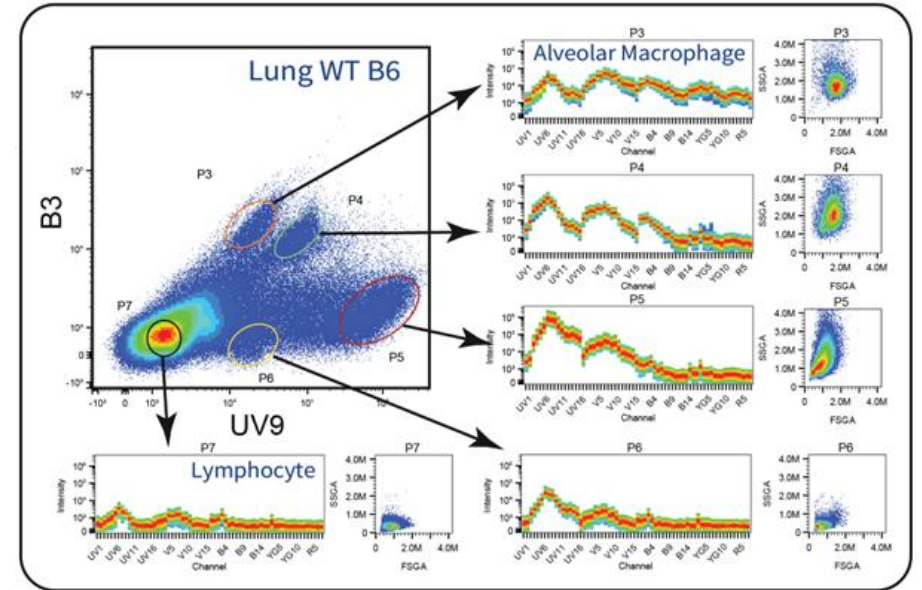


BV510 emission in area of high AF (raw V6)



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 $\sim 10^4$**
- **AF in resolution theory**

Discovery project – expression of MR1 in immune subsets

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

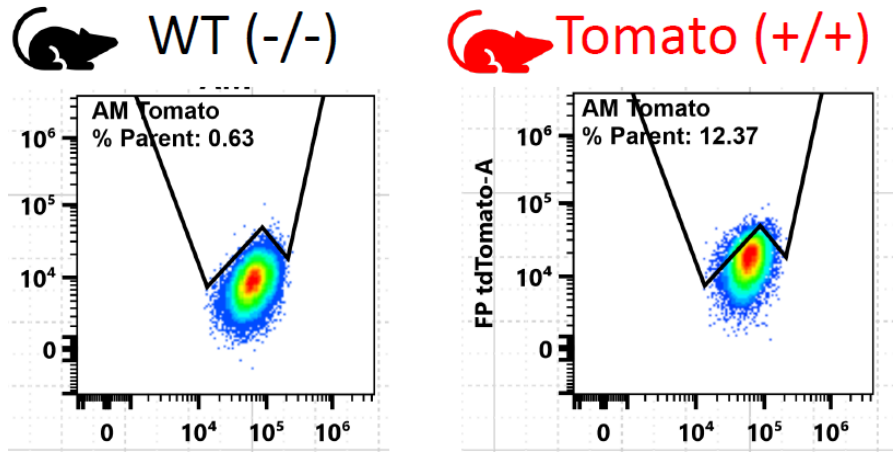
- Tissue and cellular subset **expression of MR1** (involved in antigen presentation) **unknown**
- Transgenic mouse model with **tdTomato-MR1 gene reporter**. Expectation **expression very low**
- Tissues of interest: **spleen, skin** and **lung** (AF populations)
- Lab’s **initial** independent **phenotyping**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

Weak FP expression

Subset expression unknown

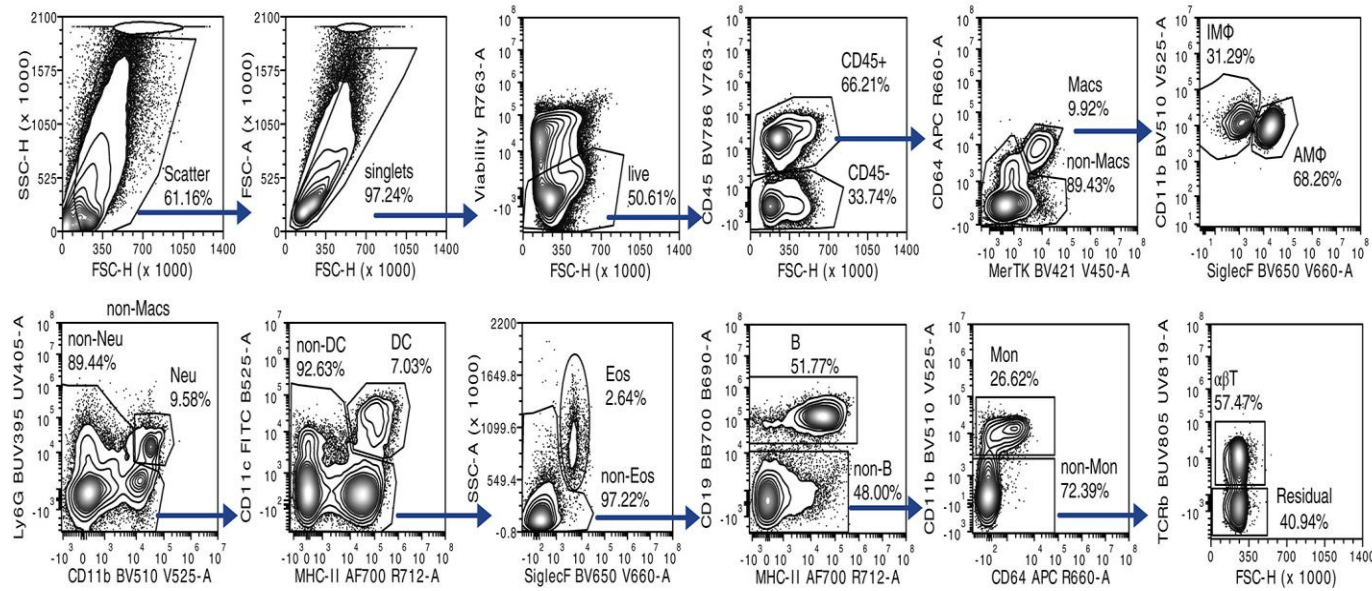
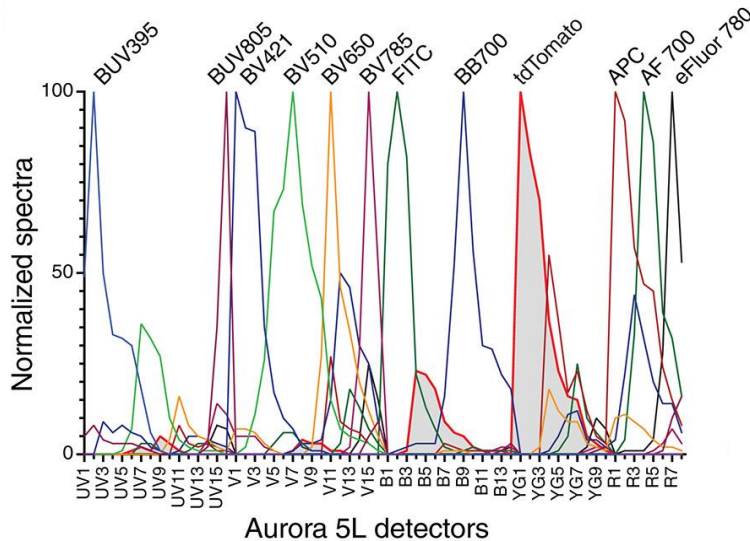
The **PERFECT** biological model to apply cytometer testing, autofluorescence findings

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	8.20	18.57		

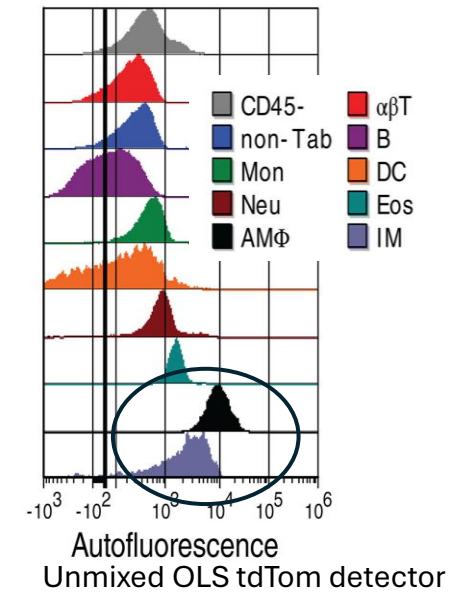
Broad panel design to fit both CytoFLEX and Aurora 5L

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

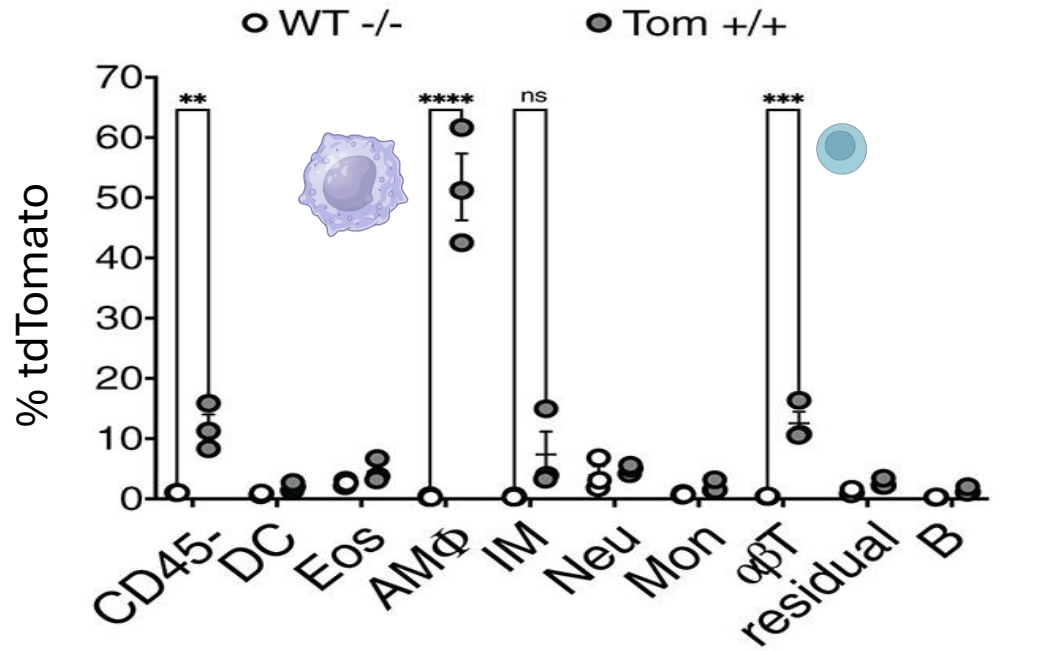


Sensitivity versus AF extraction??

AF of WT mouse leukocyte subsets



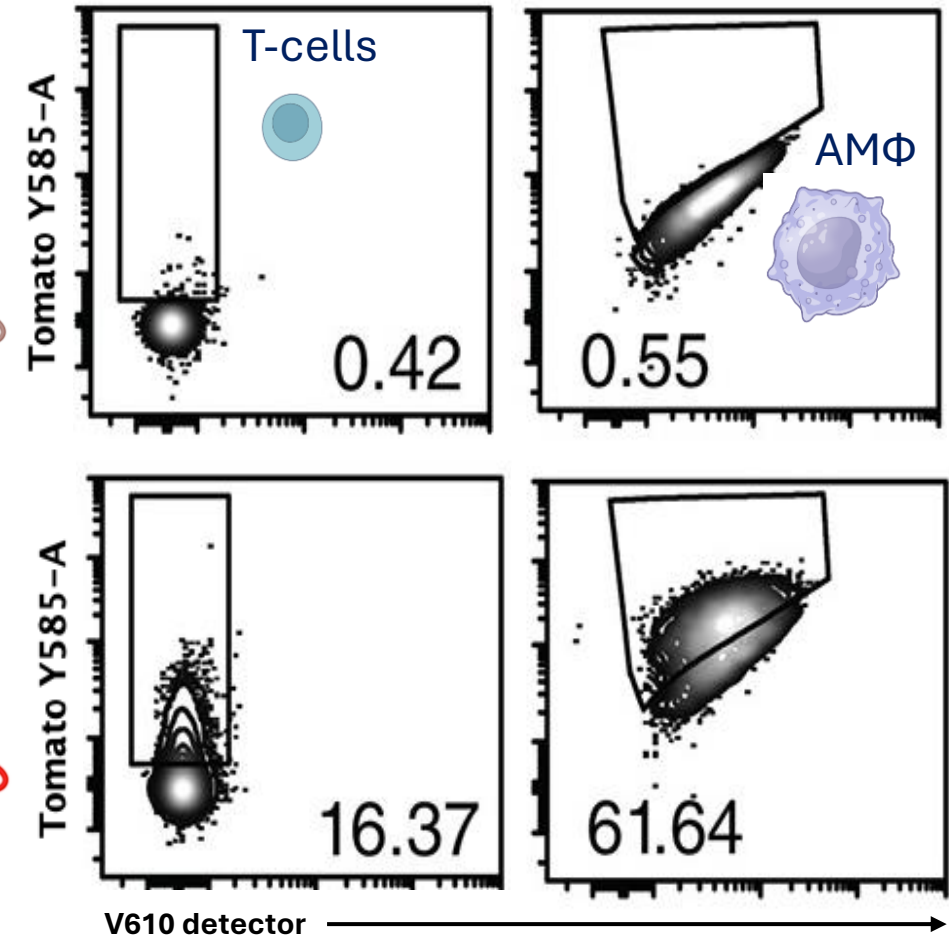
CytoFLEX LX reveals tdTomato-MR1 expression in low and high AF cells in lung



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

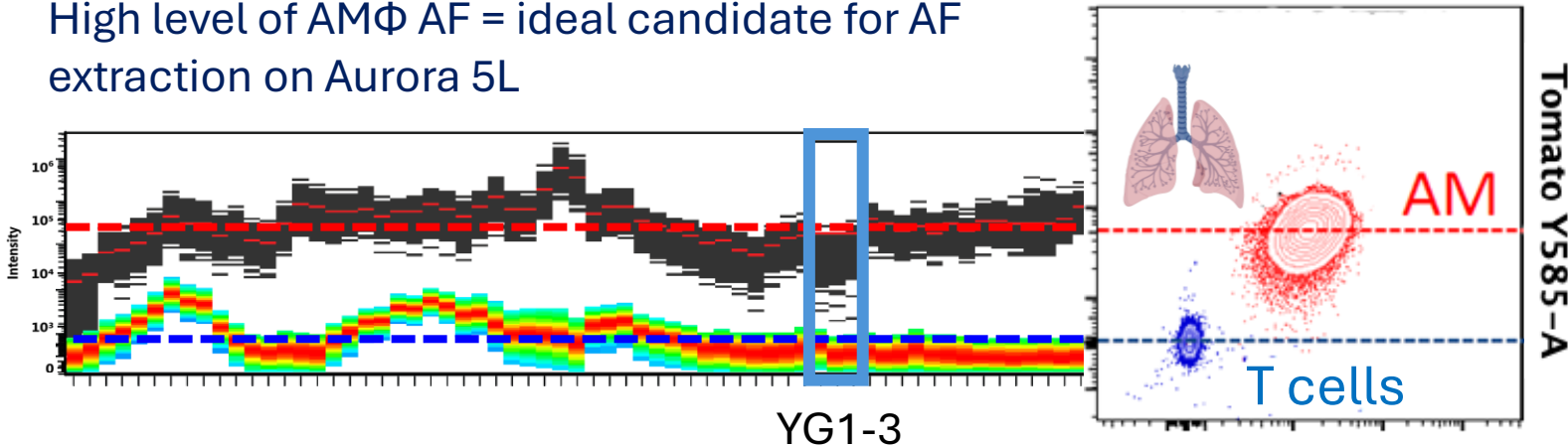


CytoFLEX data

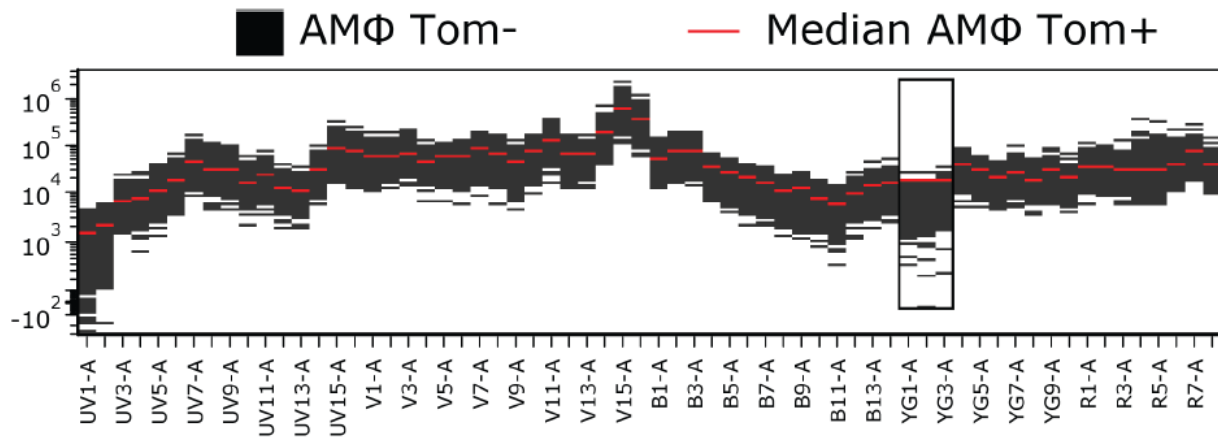


AM Φ -specific AF extraction resolves tdTom+ cells in lung

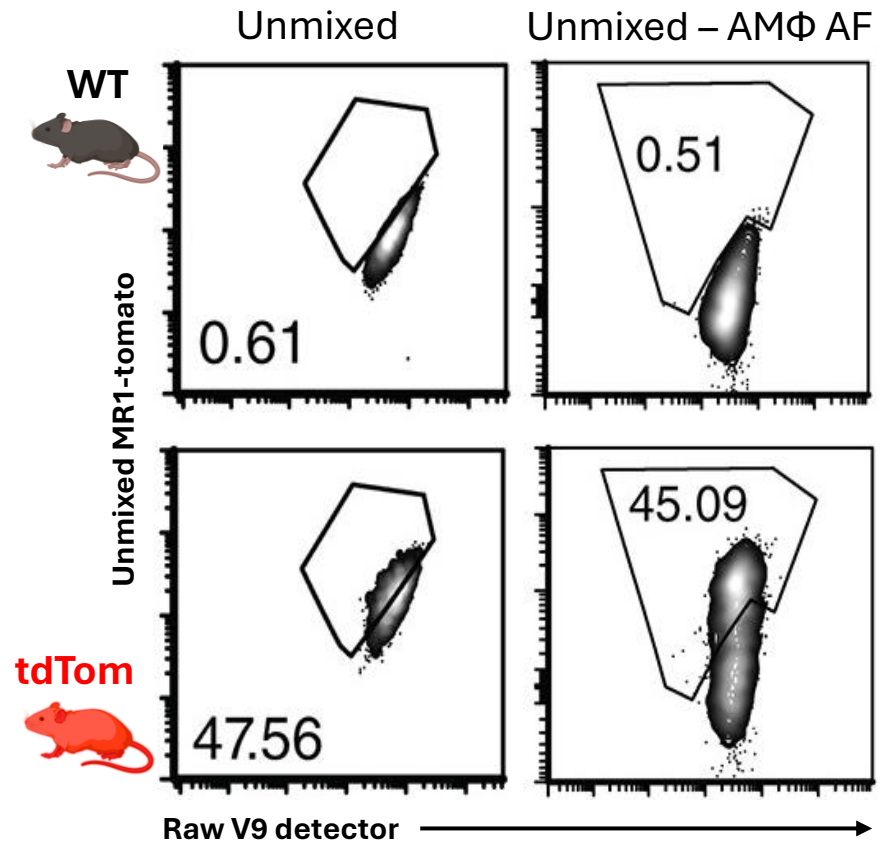
High level of AM Φ AF = ideal candidate for AF extraction on Aurora 5L




AF obscures tdTom expression in Tom+ AM Φ



Extraction of AM Φ -specific AF resolves tdTomato+ AM Φ

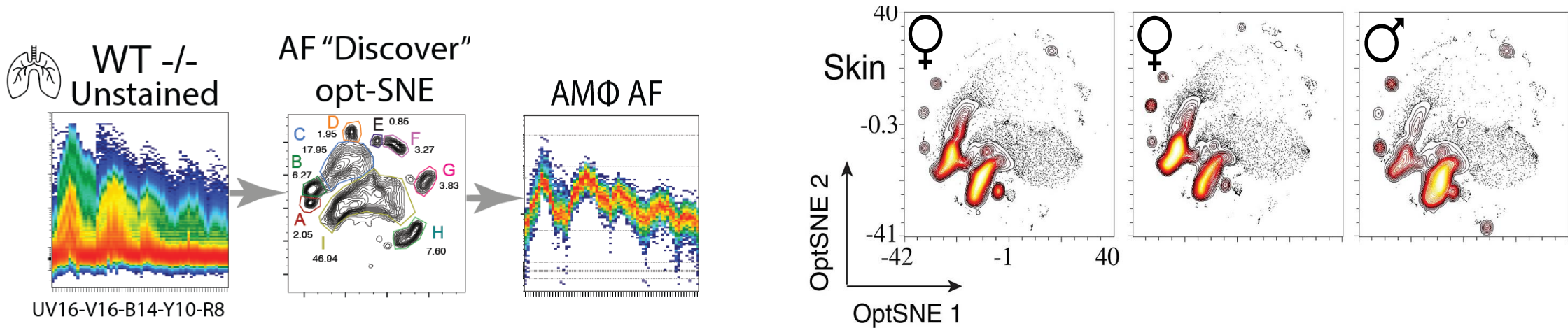


 preserves raw detectors after unmixing

Manuscript in review in **Science**

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*

Sorter PDR: Quantitatively evaluating a new sorter for the SRL
 Oliver Eltherington¹, Alison Morey¹, Vanta Jameson¹, Alexis Perez-Gonzalez¹
 Melbourne Cytometry Platform, Peter Doherty Institute, Department of Microbiology and Immunology, The University of Melbourne, Parkville, Australia

Introduction. Recently the Melbourne Cytometry Platform purchased an 5 laser Cytek Aurora Cell Sorter (CS). Since purchasing it we have performed several tests to quantify its performance. The platform already has four 5L Cytek Aurora analysers therefore it is essential we demonstrate to our users that the sensitivity of the CS is equivalent to its analyser counterparts. We also have four other sorters in the platform and it is essential the sorter performs as well or better than these as a sorter. As well as determining whether it is a good fit we want to see how far we can push the limits of its capabilities. This project addresses how well we can see the populations of interest and the sorters performance in terms of recovery. This informs how to set up the Aurora CS when sorting a particle that will push the stability of the instrument to the limit such as a 32 µm hydrogel Nanobead.

Keywords: Efficiency, Recovery, Sensitivity, Resolution, Separation Index, Signal-to-Noise Ratio, Sorting Accuracy, Sorting Error, Sorting Rate, Sorting Throughput, Sorting Volume, Sorting Yield, Sorting Precision, Sorting Accuracy, Sorting Error, Sorting Rate, Sorting Throughput, Sorting Volume, Sorting Yield, Sorting Precision, Sorting Accuracy, Sorting Error, Sorting Rate, Sorting Throughput, Sorting Volume, Sorting Yield, Sorting Precision.

1. The Aurora CS sensitivity matches the Aurora analyser
 1. Sphero™ Rainbow calibration particles (R bead) (Spherotech, RCP-30-5A) were acquired for 5 minutes (lowest flow rate, CS 8 µl/min). Aurora analyser: 35 µl/min on the Aurora 5L CS (100 µm & 70 µm nozzles) and four 5L Aurora analysers. A minimum of 2000 events for each peak was captured. The separation index was used to determine resolution between the "negative" and the dimmer rainbow peak for each detector (A). A higher separation index means an increased resolution between the peaks. As shown, sensitivity is very similar across the instruments.

2. Increasing the DFC does not compromise sensitivity or recovery
 A cell sorter should be high speed. Factory nozzle settings were resulting in slower efficient sort rates than our other sorters. Collection was done by altering PSI, frequency & amplitude while maintaining an efficient DFC ensuring the satellites merge and are fast moving. To quantify a sorter's recovery capabilities we used Rmax². An overview of the technique is shown in A. The factory and optimized settings for a 100 µm and 70 µm are shown in B. These will be different between instruments and nozzle. QC and automatic DCD were run before acquiring rainbow R bead beads and generating the separation index as in section 1. Rmax (C) was performed using a 1-drop purity sort mask (midway) before altering the nozzle settings to the optimized settings and repeating. Note: Calibrite™ beads (BD, 340486) were used in this Rmax and beads were assessed for 2 minute prior to 1 minute of sorting. Sort rate was maintained around 1200 events/sec and sort rate was maintained below 400 events/sec. Efficiency was maintained >95% and flow rate never exceeded 2 for C. Increasing the frequency increases the max efficient threshold event rate (1/PSI DDF) by 1.5x. It may also improve recovery according to Rmax without compromising sensitivity or recovery. Note: The larger error in the 100 µm Factory Rmax result could be due to lower CS events captured decreasing precision. Once optimized we could then compare it to the rest of our sorters. Note the efficiency is added in this graph and is similar across the machines except the BC Cytoflex 801 which had a higher efficiency. From this it is clear that the CS can run as fast or faster than the other sorters in the platform without compromising recovery.

3. Particle size influences recovery
 A. Schematic of the recovery test setup. B. Recovery test results showing recovery percentage vs particle size for different nozzle sizes. C. Recovery test results showing recovery percentage vs particle size for different nozzle sizes. D. Recovery test results showing recovery percentage vs particle size for different nozzle sizes.

4. Setting DCD using Sphero™ Rainbow beads
 A. Schematic of the DCD setting process. B. Recovery test results showing recovery percentage vs particle size for different nozzle sizes. C. Recovery test results showing recovery percentage vs particle size for different nozzle sizes.

Thank you

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Dept Anatomy and Physiology: Matthew Watt, Christine Wells, researchers and students

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Brian Baynes
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Melbourne Cytometry Community (2016)

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