# Full Spectrum Flow Cytometry for high-parameter analysis

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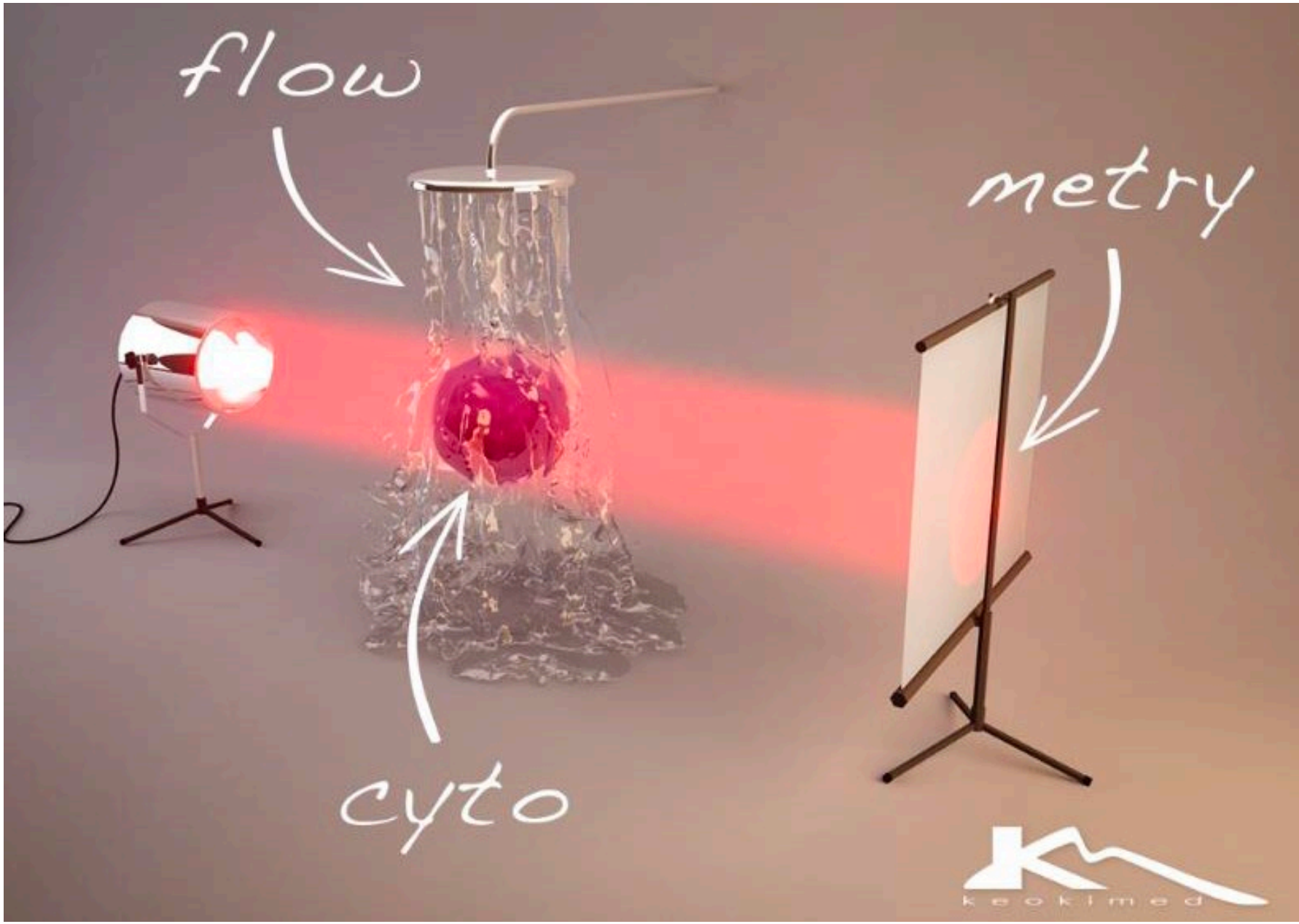
KU Leuven

# Flow Cytometry

A population of cells is hydrodynamically focused into a single cell stream and pulsed with lasers to record fluorescence at different wavelengths and to measure their characteristics.



# **Flow Cytometry** – powerful, high-parameter, single cell proteomics

What can be measured?

1) Physical/morphological cell characteristics (scatter)
   - Size, cellular complexity
2) Phenotypic profiling and subset definitions
   - Cell surface
   - Transcription factors
3) Functional profiling and cellular states
   - Cytokines/chemokines
   - Cytotoxic potential/degranulation
   - Activation/effector mechanisms
   - Migratory potential
   - Proliferation/viability/cell cycle/apoptosis
   - Signalling (phospho-proteome)

# Broad application

**Research and diagnostics**

- Pre-clinical disease models
- Clinical samples

**Immune-Monitoring** = deep immuno-phenotyping (e.g. longitudinal clinical trials)

**Immunology** and related fields (immuno-oncology, neuroimmunology, infectious disease monitoring, …), Haematology, **Oncology**, Neurology, Virology and more

- Dissect cellular composition of blood or tissue samples
- Biomarker analysis: Identify pattern/markers related to disease or disease stage or treatment
- Analyse cell viability, cell death, proliferation or cell cycle
- Functional profiling of cells (e.g. stimulating/inhibiting molecules, chemokines, cytokines)
- …

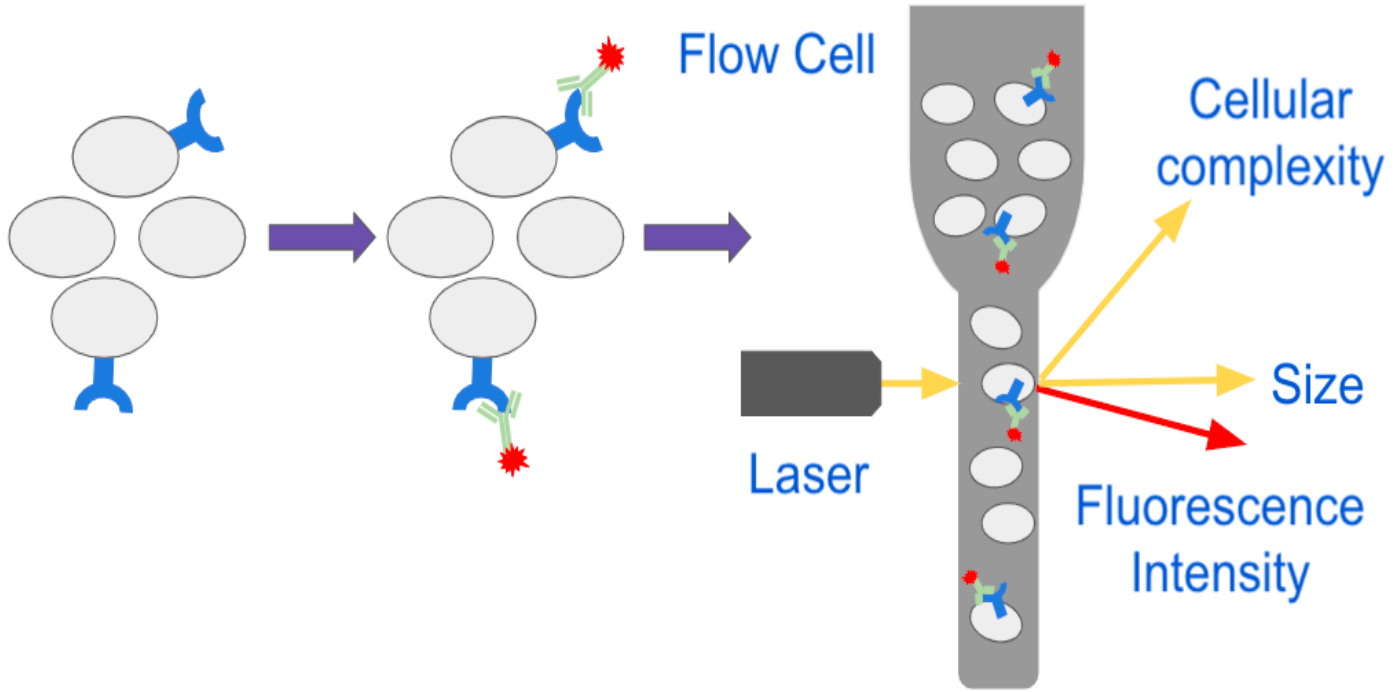
# What does flow cytometry NOT do?

- Spatial information / tissue architecture is lost
  - high-parameter IHC = protein level
  - spatial transcriptomics = RNA level
  - CyTOF Hyperion

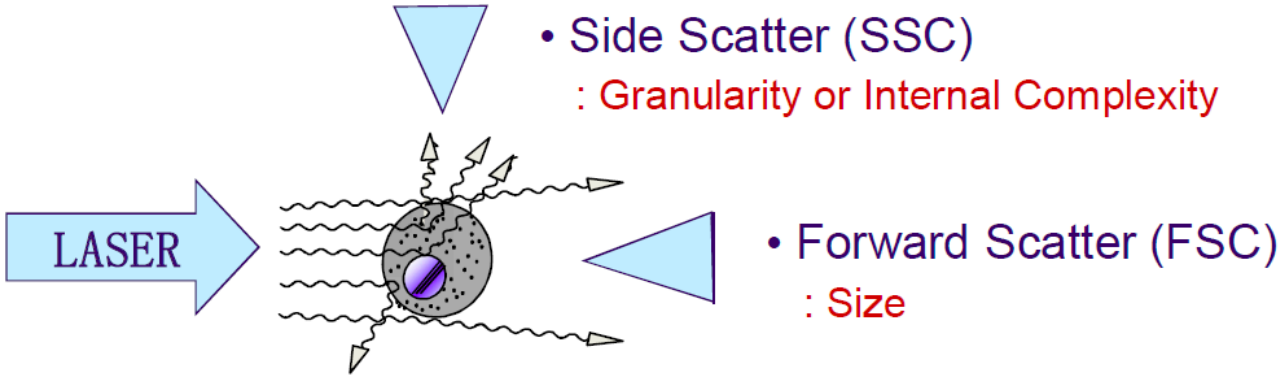
- Secreted proteins: what is produced is not necessarily secreted
  - IsoPlexis technology
  - (cytokine capture assays – not multiplexed)

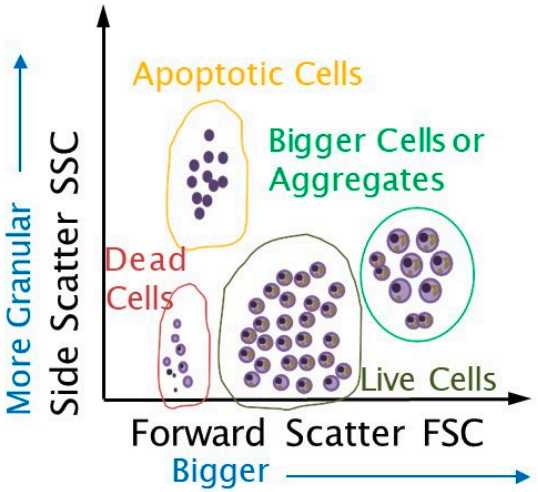
- No information on intracellular sublocation of proteins
  - Image Cytometry

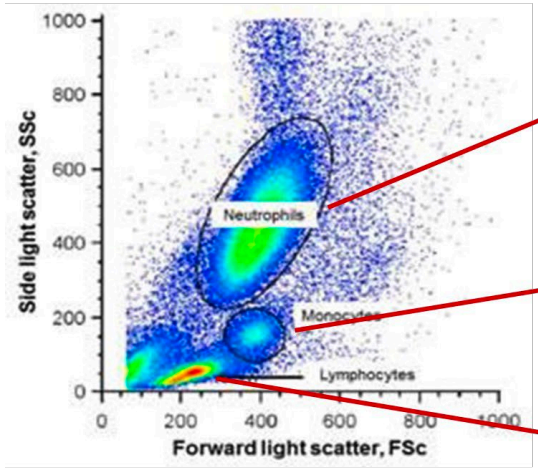
# The Principle

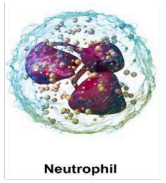


# Morphological characteristics

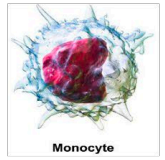


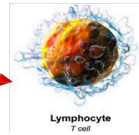




12 µm



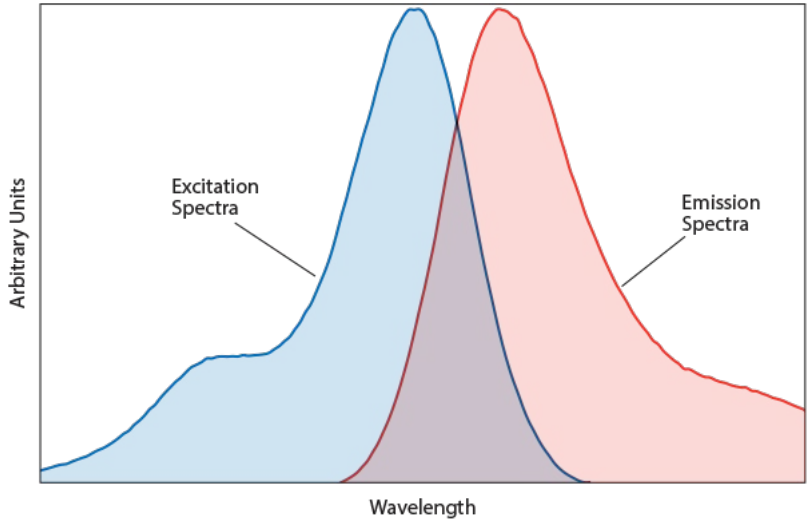

14 µm




8 µm

# Proteome profiling

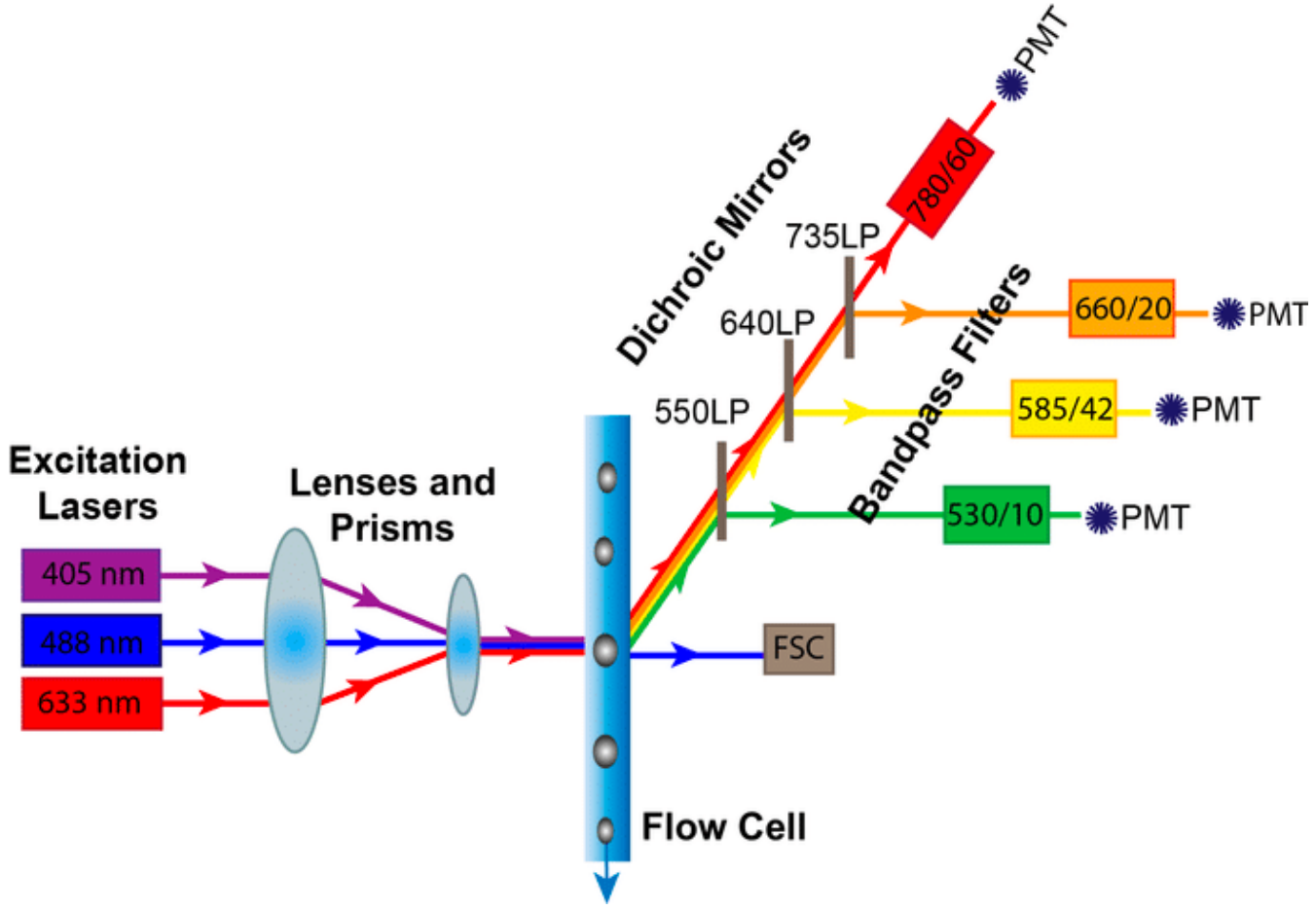
- Staining cells with fluorochrome-labelled antibodies





1) The flow cytometer must be able to excite the fluorochrome (correct laser wavelength).
2) The flow cytometer must be able to measure the emitted fluorescence (correct detector).

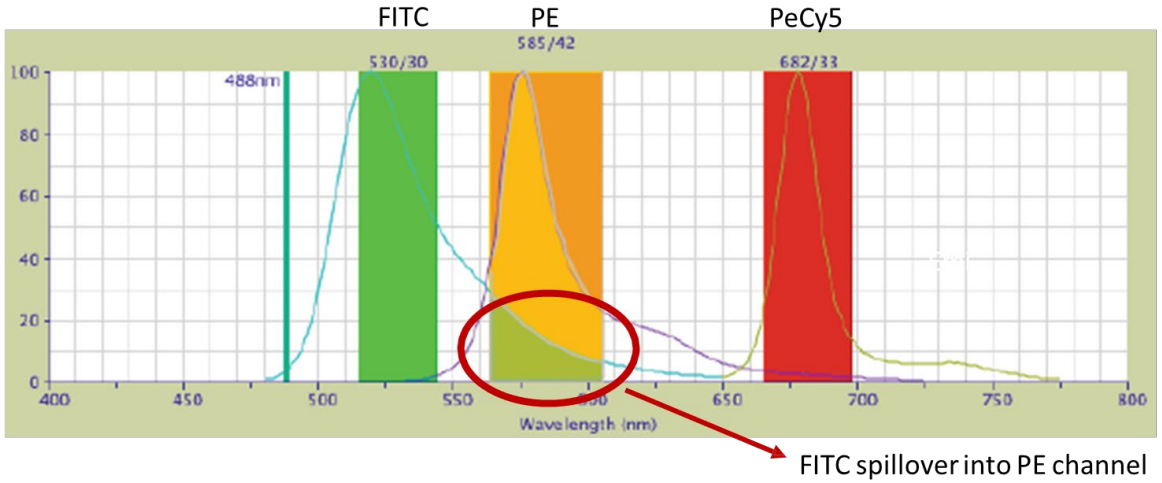
# Conventional Flow Cytometer



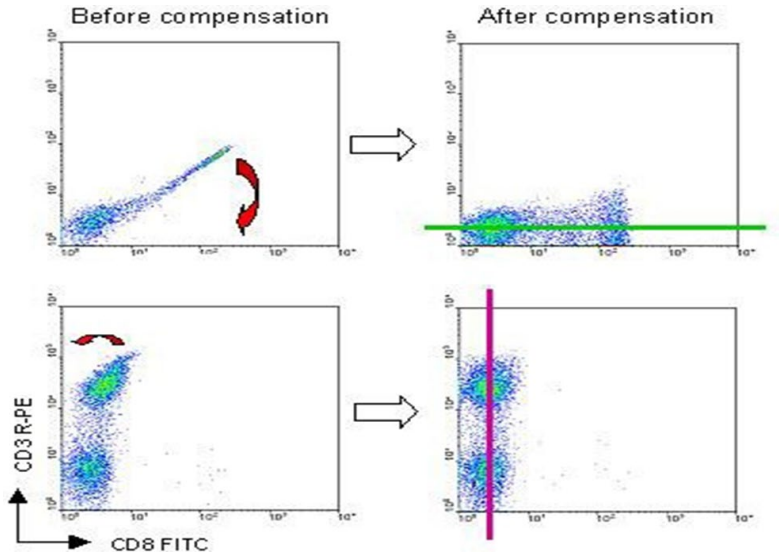

The number of detectors represents the maximal number of fluorochromes that can be detected

# Compensation

Process of correcting for spectral overlap in each detector via subtraction.




True PE signal = Primary PE signal - % of FITC spillover

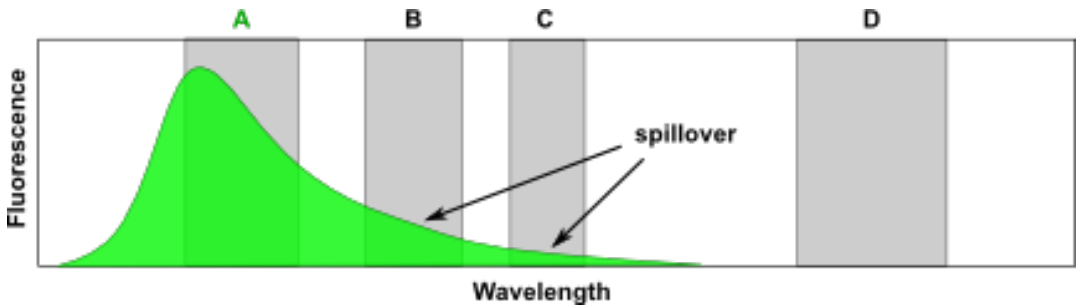


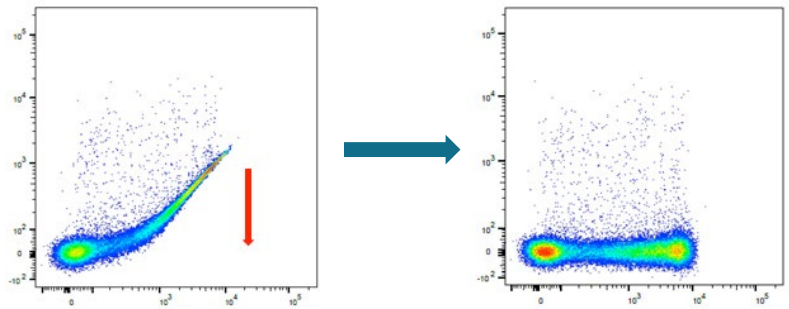

The need for compensation can be overcome in other flow cytometric technologies

- Mass cytometry
- Full spectrum flow cytometry

# Flavours of Cytometers

- **Conventional Flow Cytometry**
- Mass Cytometry
- Spectral Flow Cytometry
- Imaging Flow Cytometry



✓ all flow cytometric applications

✗ limited number of markers (~20-25 parameters)

✗ not all dyes can be combined

✗ suffers from spill-over of fluorescence emission between markers

✗ requires pre-analysis data processing

✗ may not reflect antibody-conjugate quality

✗ autofluorescence of cells impacts sensitivity / resolution

# Full Spectrum Flow Cytometry – a transformative technology –

**Conventional / Polychromatic Flow Cytometry**

- Employs mirrors & optical filters to reflect, block, or transmit photons based on wavelength
- Uses ONE detector – ONE fluorophore setup
- Only a fraction of the emission spectra are recorded
- Fluorophores are defined through specific wavelength
- Similar fluorphores are difficult to distinguish
- Requirement for several staining panels
- Autofluorescence can hamper analysis

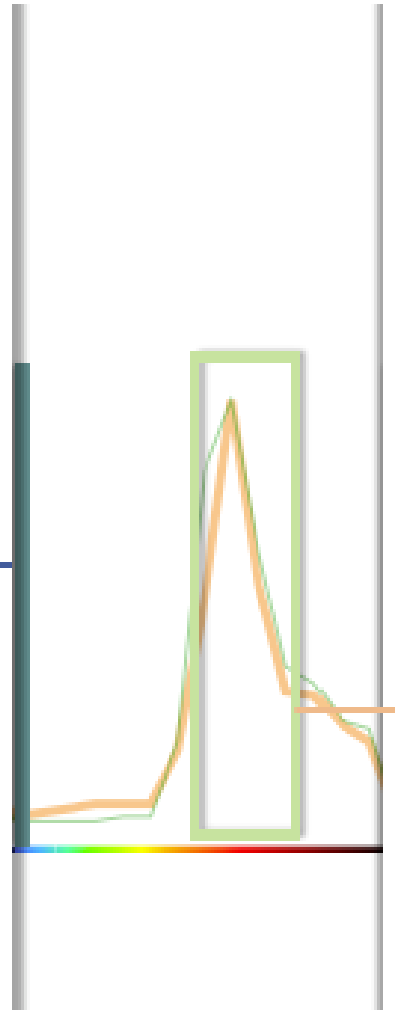
**Spectral Flow Cytometry**

- Employs dispersive optics such as prisms to disperse photons according to wavelength across an array of detectors
- All detectors capture a signature of the full emission spectra of fluorophores, ie. collect entire spectral profile (depending on available lasers)
- Fluorophores with similar emission spectra can be distinguished
- Merging of panels, design of complex panels – saves sample and reagents
- Autofluorescence is treated as another ‘color’ and can be removed allowing for clearer identification of dim fluorescent signals

# Conventional Spectrum

BB700 and PerCP-Cy5.5

only Blue laser excitation
taken into account



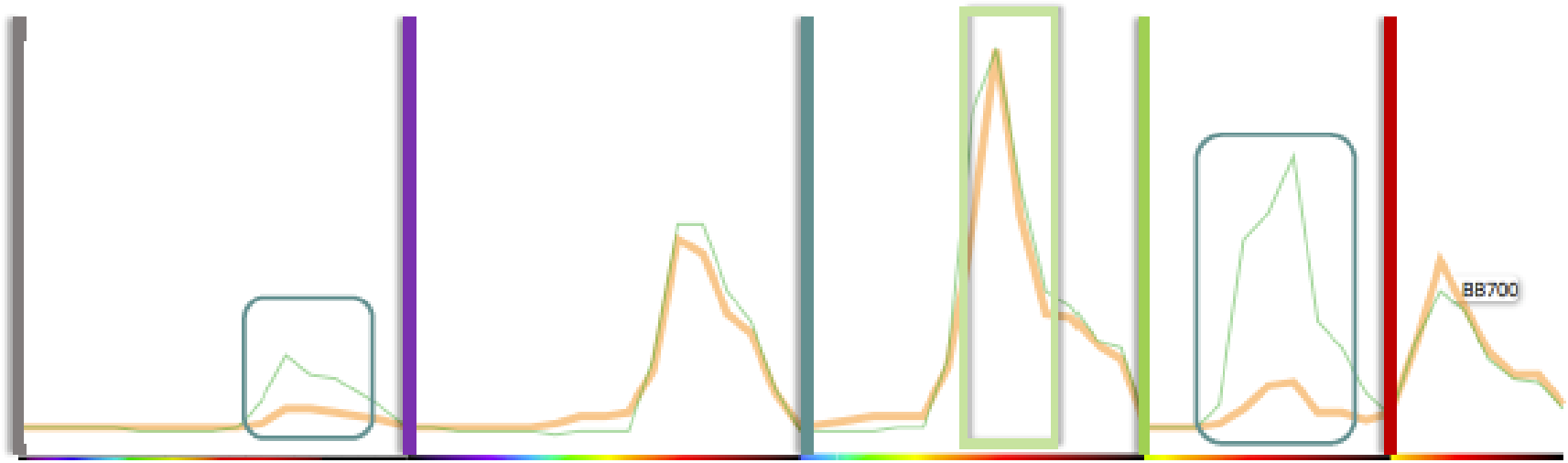
Both Fluorochromes peak
into same bandpass filter

Can't be combined

# Full Spectrum Signature

BB700 and PerCP-Cy5.5

Uses Signal generated by every laser




Fluorochrome is measured in all available detectors

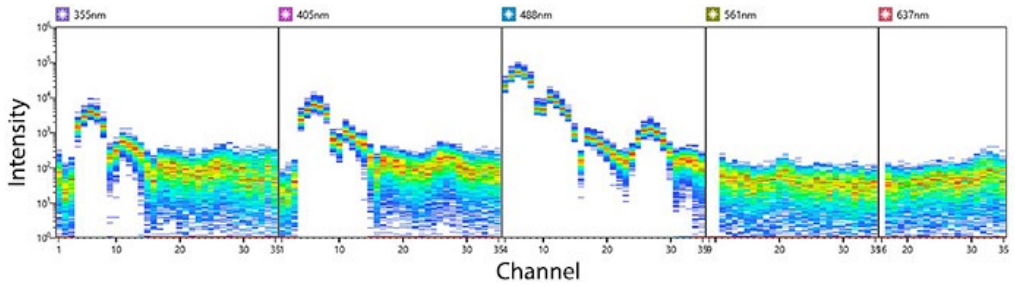
↓

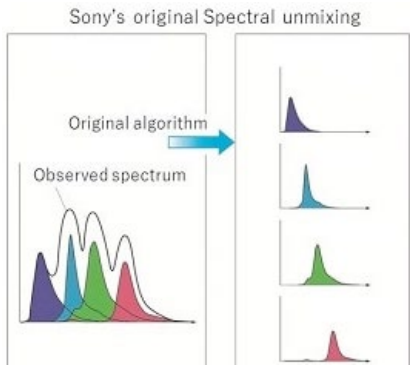
Distinguish fluors with 2% spectral difference
(Similarity Index™ ≤0.98

CYTEK
TRANSCEND THE CONVENTIONAL

# Flavours of Cytometers

- Conventional Flow Cytometry
- Mass Cytometry
- **Spectral Flow Cytometry**
- Imaging Flow Cytometry



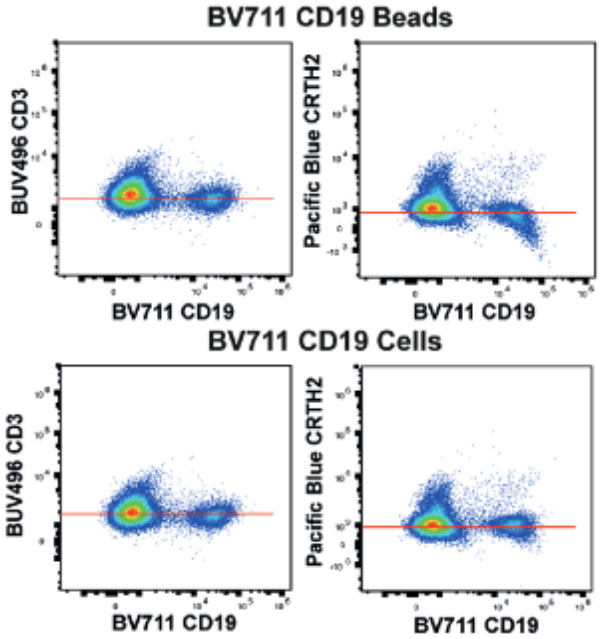

✓ all mentioned applications
✓ simplified panel design
✓ reads entire spectrum (spectral fingerprint)
✓ can combine more (similar) fluorochromes → very high parameter panels (35+)
✓ no compensation -> uses spectral unmixing
✓ autofluorescence subtraction
✓ determine antibody-conjugate quality
✓ efforts of fluorochrome development focused on spectral applications
✓ very high throughput

# General panel design rules still apply

- antibody titrations
  - pre-gate on expressing cells when titrating a rare marker
  - if necessary, titrate on surrogate cells and validate on tissue of interest
  - calculate stain index and chose ab concentration where SI peaks (without positive shift of negative population)
- fluorophore assignment
  - bright fluorophores for low abundance antigens and vice versa
  - avoid very similar fluorophores for co-expressed antigens
  - know your markers, their expression levels and co-expression pattern
- test different ab clones per marker if optimal one not previously determined
- fluorochromes that create most spillover best assigned to dump or viability channel
  - depends on instrument configuration
- usually AF is high in the UV, V and B regions, therefore preferably choose dyes excited by Y/G or R lasers for highly autofluorescent cells

# complex panels require detailed optimization

- carefully examine AF peaks in unstained sample and avoid allocating fluorophores that emit where AF dominates
- try beads vs cells as single stained -> determine per fluorochrome which unmixes fully stained sample best (NxN matrix)

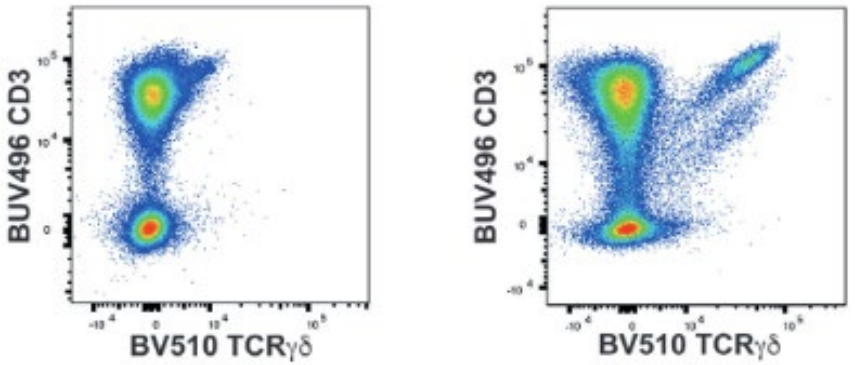



E.g. CD19 on cells outperforms CD19 on beads for unmixing

# complex panels require detailed optimization

- carefully examine AF peaks in unstained sample and avoid allocating fluorophores that emit where AF dominates
- try beads vs cells as single stained -> determine per fluorochrome which unmixes fully stained sample best (NxN matrix)
- evaluate marker resolution in the fully stained sample
  - loss of resolution? sequential staining or different choice of fluorochromes may help, spillover spreading matrix (SSM) can indicate which fluorochromes are problematic

**Before** and **After**
sequential staining



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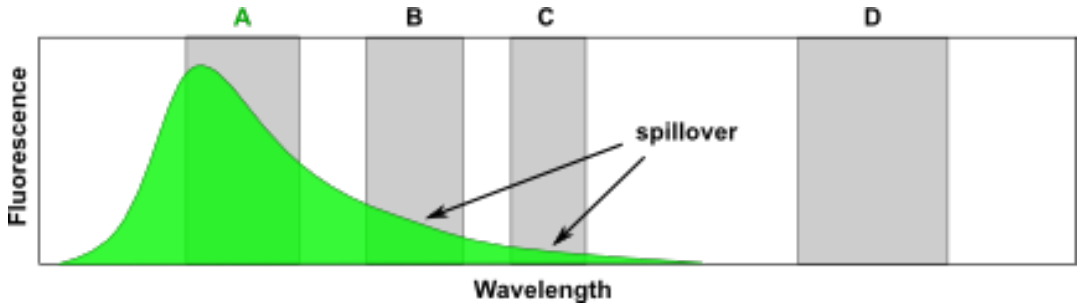
  - increased spread of neg population? check that separation is not impacted, co-expression of markers stained with very similar fluorochromes not considered?

# controls for accurate unmixing and analysis

- spectral reference controls and unstained cells
  - beads or cells
  - must be treated IDENTICAL to fully stained samples
  - pos population should be brighter than fully stained sample
  - pos & neg population should have same AF
  - use exact tandem fluorochrome-labelled ab as in fully stained sample
  - the fluorochrome signature of reference controls needs to be assessed to match the expected one (especially with beads)
- unstained control
  - match tissue/sample type(s)
  - assess AF
- fluorescence-minus-one (FMO) for objective cut-offs (gating controls)
  - when pos cells are not clearly separated
  - useful to assess spread of neg cells
- fluorescence-minus-multiple (FMM) can be useful for larger (20+) panels

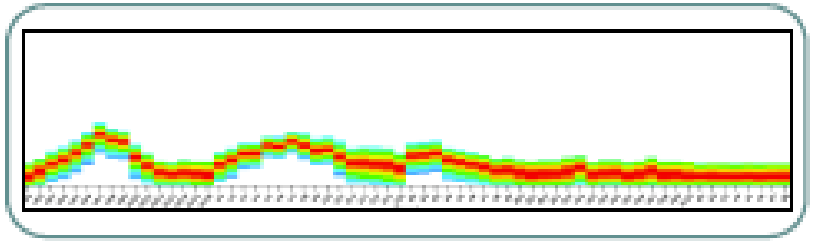
# Spectral Unmixing (instead of compensation)

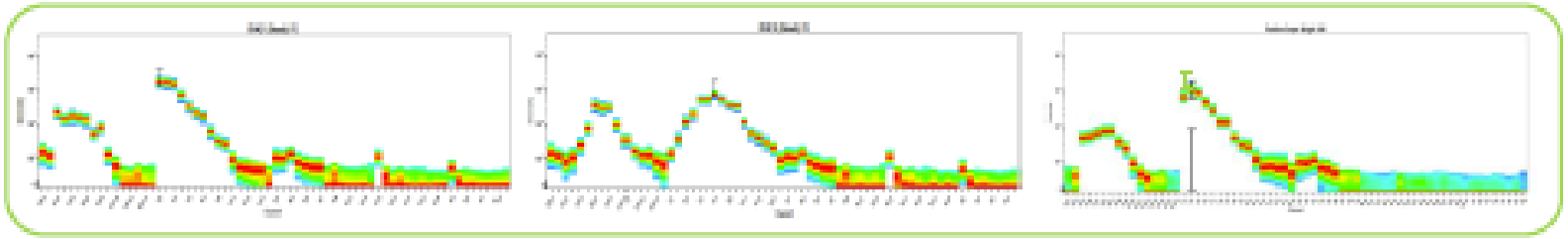


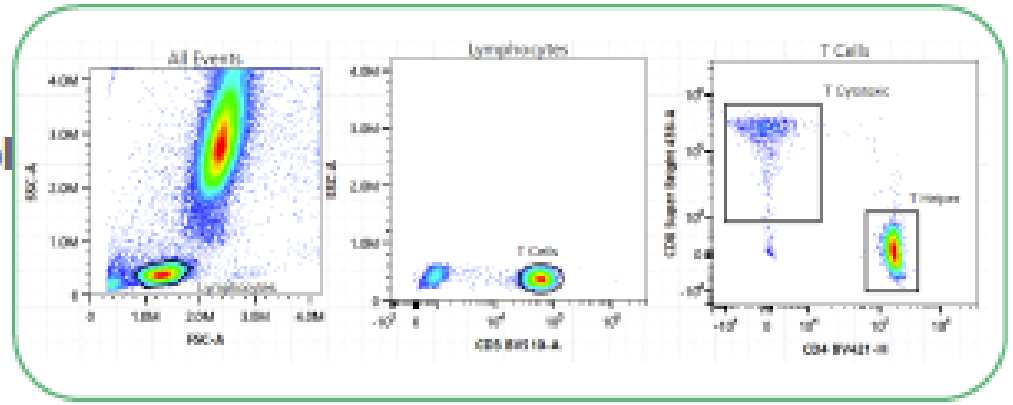

Instead of removing spectrum information in other channels through compensation …

# Spectral Unmixing (instead of compensation)

1. Run **UNSTAINED control**
2. Run **REFERENCE controls**
3. **Unmix**
4. Run **multicolor sampl**







# Instruments and vendors

***Analysers***

- Sony – first commercial spectral analyser, now ID7000 with up to 7 lasers
- Cytek – Aurora (up to 5 lasers) and Northern Lights (up to 3 lasers)
- BD Symphony A5 SE (spectral-enabled)

***Cell sorters*** (unmixing in real-time to make sorting decisions)

- Cytek Aurora CS
- Thermo Big Foot (cell sorter)
- Sony to release its spectral sorter
- BD FACSDiscover S8 (spectral imaging sorter)

# Applications – **Deep Immune-Monitoring**

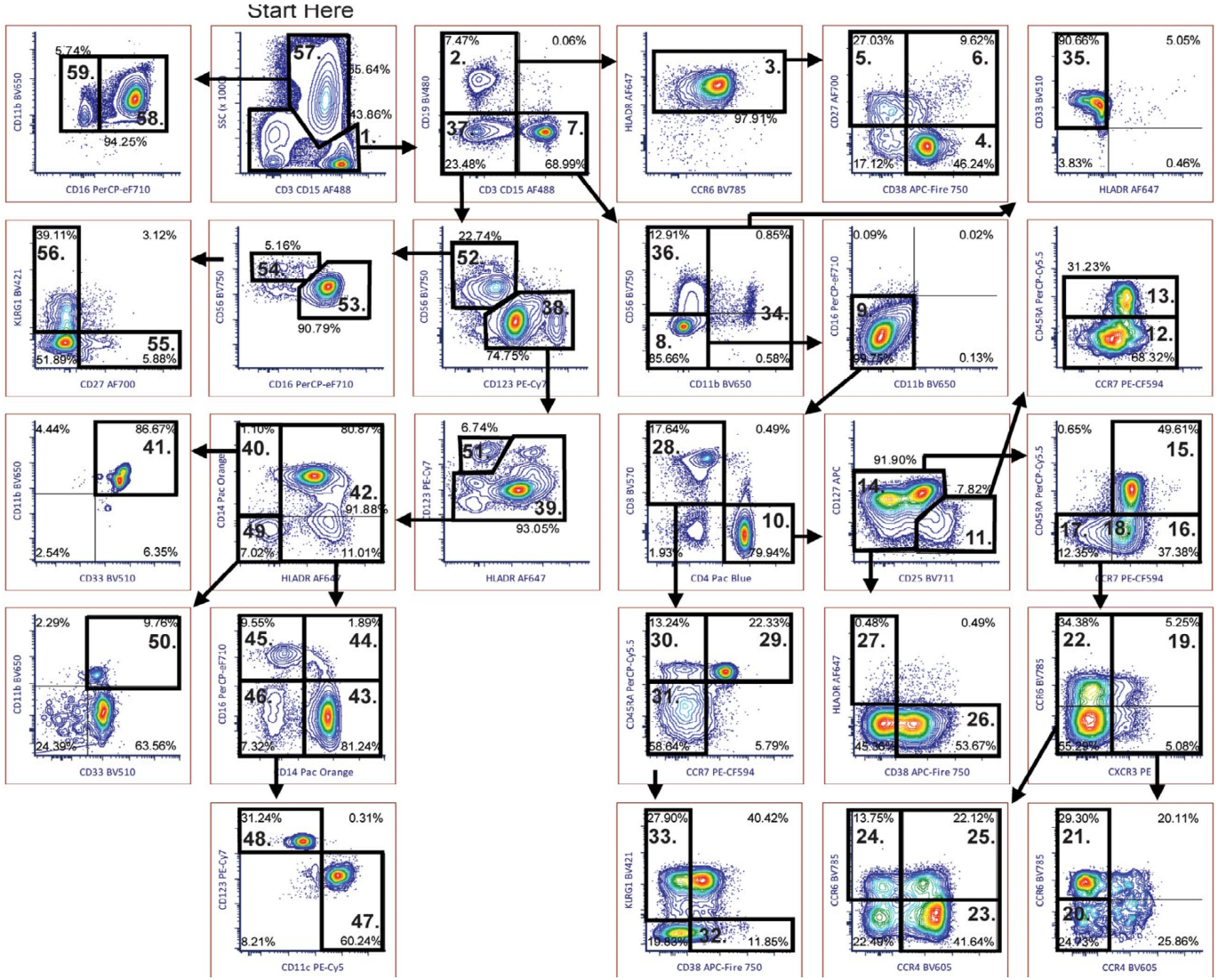
- Off-the-shelf staining kits
- Optimised Multicolor Immunofluorescence Panels (OMIPs)
- Published antibody panels
- Own antibody panel design
  - Use panel design tools such as FluoroFinder or Cytek
  - Use panel design services by commercial vendors

- pitfalls / considerations to careful control for
  - decomposing tandem dyes
  - probe-probe interactions between molecules bound in/on a cell



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

# Applications – **Deep Immune-Monitoring**




Example 1

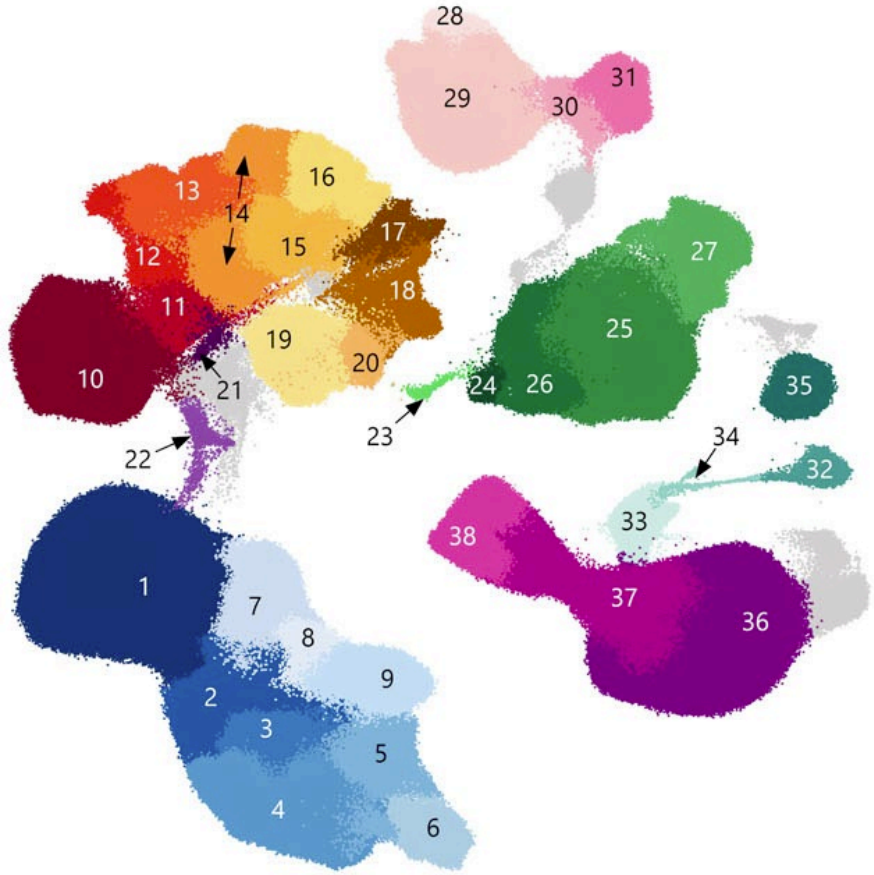
- 25-color panel on spectral flow cytometer
- Gating 50 populations -> but >400 marker combinations possible

# OMIP-069 – Human PBMC immune-phenotyping

**40-color panel for spectral flow cytometer**

Describes activation and functional subsets of

- Basophils
- Monocytes
- gd T cells
- NKT-like cells
- NK cells
- Regulatory T cells
- B cells
- Conventional CD4 T cells
- CD8 T cells
- Dendritic cells
- Innate lymphoid cells




1 Naive CD4⁺
2 Central Memory CD4⁺
3 Early Effector CD4⁺
4 Early-Like Effector CD4⁺
5 Early Effector CD4⁺ PD-1⁺
6 CD4⁺ CD57⁺
7 CD4⁺ CD127$^{lo}$ CD25$^{++}$ CD45RA⁺
8 CD4⁺ CD127$^{lo}$ CD25$^{++}$ CD45RA⁻ CD39⁺
9 CD4⁺ CD127$^{lo}$ CD25$^{++}$ CD45RA⁻ CD39⁻

10 Naive CD8⁺
11 Central Memory CD8⁺
12 Early Effector CD8⁺
13 Intermediate Effector CD8⁺
14 CD8⁺ CD45RA⁺ CCR7⁻
15 CD8⁺ CD45RA⁺⁺ CCR7⁻
16 CD45RA Terminal Effector CD8⁺
17 CCR7⁻ CD45RA$^{++}$ TCRγδ⁺
18 CCR7⁻ CD45RA$^{lo}$ TCRγδ⁺

19 CD2⁺ CD8⁺ NKT-like
20 CD2⁺ CD8⁻ NKT-like
21 CD4⁺ CD8⁺
22 CD4⁻ CD8⁻
23 ILCs
24 Early NK
25 Mature NK
26 CD159a⁺ NK
27 Mature NK CD159c⁺

28 IgD⁺ CD27⁺ B cells
29 IgD⁺ CD27⁻ B cells
30 IgD Memory
31 IgG⁺ B cells
32 pDCs
33 CD1c⁺ DCs
34 CD1c- CD141⁺ DCs
35 CD45$^{int}$ CD123⁺ HLA-DR⁻ Basophils
36 Classical Monocytes

37 Intermediate Monocytes
38 Non-classical Monocytes

# Applications – high-dimensional immune-monitoring

- Identification and quantification of cell populations
- longitudinal immune-monitoring
- Description of cell population and gradually zooming into cell populations through various protein markers
- insight on maturation, activation, function & migratory potential
- clinical samples and pre-clinical models
- 100s of cell subsets in a single panel
- unsupervised analysis
- identification and follow-up of
  - biomarker-to-disease correlation
  - biomarker-to-treatment correlation
- genotype-to-phenotype correlation

# Applications – **Autofluorescence as parameter**

- source of background that interferes with the signal from dim fluorophores and/or low abundance markers
  - resolution of dim signals from various sources of background

- can arise from several endogenous metabolites, amino acids and other molecules

- rich source of information about cell state / cell content

# Applications – **Autofluorescence as parameter**

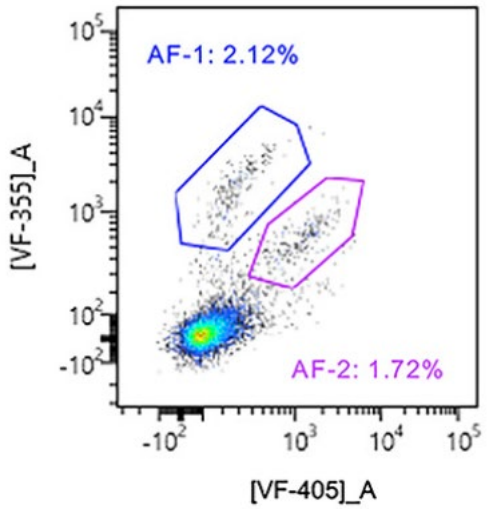
Cytometry PART A
Journal of Quantitative Cell Science
ISAC

Original Article | Open Access

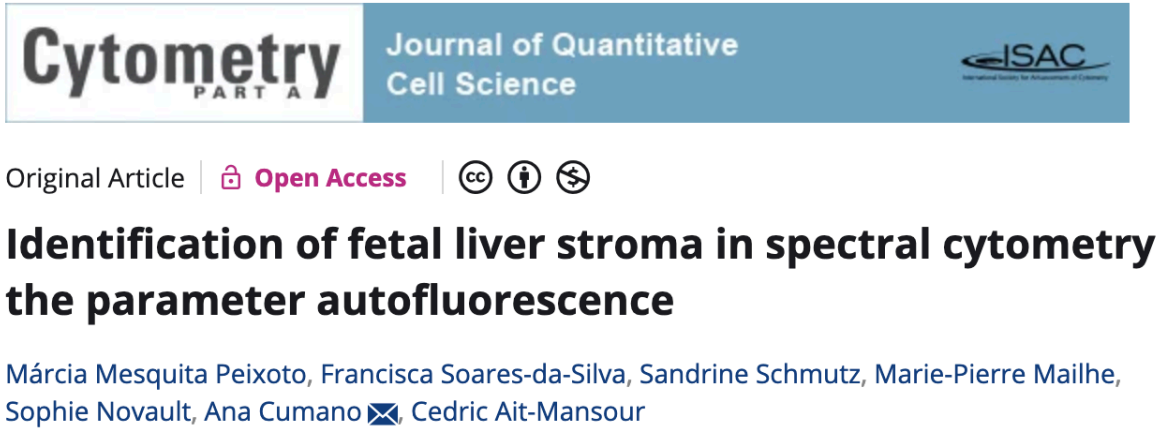
**Identification of fetal liver stroma in spectral cytometry using the parameter autofluorescence**

Márcia Mesquita Peixoto, Francisca Soares-da-Silva, Sandrine Schmutz, Marie-Pierre Mailhe, Sophie Novault, Ana Cumano, Cedric Ait-Mansour

- fetal liver stroma stained for ID7000 plus use ‘autofluorescence finder’ option on unstained sample
- identified two distinct autofluorescence emission spectra



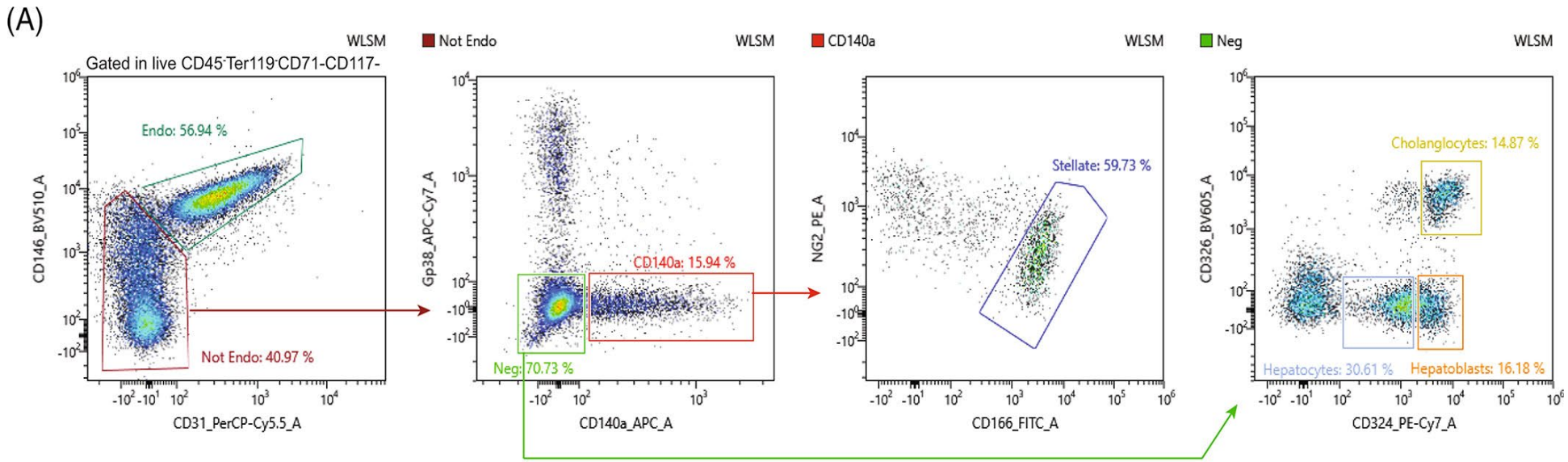
# Applications – **Autofluorescence as parameter**




- FL stroma stained for ID7000 plus use ‘autofluorescence finder’ option on unstained sample
- identified two distinct autofluorescence emission spectra
- Using autofluorescence as a fluorescence parameter we could assign the two autofluorescent signals to three distinct cell types and identified surface markers that characterize these populations
- autofluorescence used as a parameter in spectral FCM is a useful tool to identify new cell subsets that are difficult to analyze in conventional FCM

antibody staining identifies

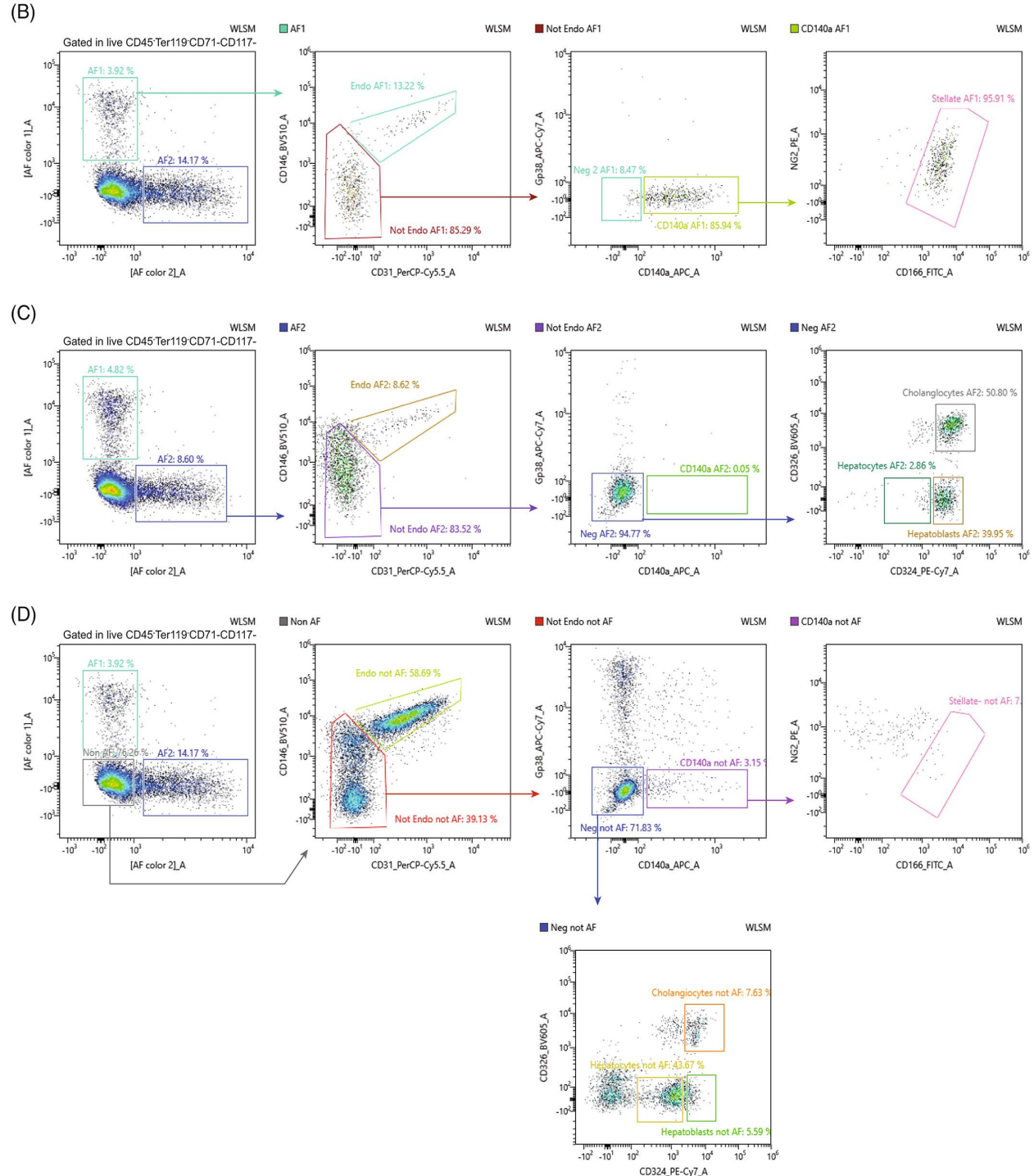
- stellate cells
- cholangiocytes
- hepatocytes
- hepatoblasts



Autofluorescence finder function identifies two distinct autofluorescence emission spectra

set these as parameters = 3 populations

- AF1 = stellate cells
- AF2 = cholangiocytes and hepatocytes
- ‘DN cells’ = enriched in hepatoblasts



# Spectral FC in clinical environments

- requires CE-marked instruments complient with the European In-Vitro Diagnostic Devices Directive (IVDD 98/79/EC) = CE-IVD instruments
  - Cytek Northern Lights-CLC
  - Cytek cFluor 6-color TBNK-SL assay
- limited application yet but will slowly outcompete polychromatic FC also in clinical/diagnostic settings
  - fusion / expansion of panels e.g.
  - ease of analysis
- requires adjustments of FC-related inspection lists

# promising clinical use cases call for efforts to ‘spectrally update’

- 27-color single-tube assay[1]
  - using a 3-laser Cytek Aurora for detection of measurable residual disease in patients diagnosed with acute myeloid leukemia
  - the lower limit of detection for identifying abnormal myeloblasts was 0.0013% in limiting dilution studies, indicating excellent sensitivity
- 24-color single-tube[2]
  - t-SNE successfully identified abnormal cell types, suggesting that machine learning algorithms may have diagnostic utility for high-dimensional flow cytometry datasets (obviating the need for standard gating strategies)

[1]Soh et al, Cytometry A, 2022

[2]published as part of the 2020 American Society for Hematology conference

# promising clinical use cases call for efforts to ‘spectrally update’

- 23-color single-tube assay[3]
  - on Cytek Northern Lights was compared against 4-tube assay on a BD FACSCanto II
  - comparable results with bone marrow aspirates from multiple myeloma (MM) and non-MM patients
  - very high concordance with quantification of plasma cell abundance in MM patients

[3]poster presented at the International Clinical Cytometry Society (ICCS) 2021 meeting

# Thank you.

Conventional

Vs.

Spectral