18th EBF Open Symposium

Tune in to Tomorrow
Science in High Definition

WS 11: Flow Cytometry

Barcelona, 18-20 November 2025

Agenda and aim

- Discussions on the challenges and solutions applicable to flow cytometry sample analysis
- Two focus areas:
  - Receptor Occupancy (RO) Flow Cytometry Assays
  - High-Parameter Flow Cytometry (HPFC)

- A short intro to the discussions: 10-15 minutes
- Regroup into a relevant table: 5 minutes
- Round table/panel ~ 60 minutes on a selected topic for each table

- Aim: Notes from the round tables will be used by EBF to form harmonizations and strategies for future recommendations

The round tables

- Note takers for round table discussions:

Traditional and Receptor Occupancy (RO) Flow Cytometry Assays	High-Parameter Flow Cytometry (HPFC)
Hanna Widmaier (Nuvisan)	Enrique Gomez Alcaide (Roche)
Dina Silke Malling Damlund (Lundbeck)	Thea Hogan (GSK)
James Munday (LabCorp)	? Will we need more (will adapt before WS)
Sandra Henkelman (QPS)	? Will we need more (will adapt before WS)

The topics for the round tables

- Each table discuss and take notes on minimum 3 topics below
- Your major challenges
- Suggestions on resolving the challenges

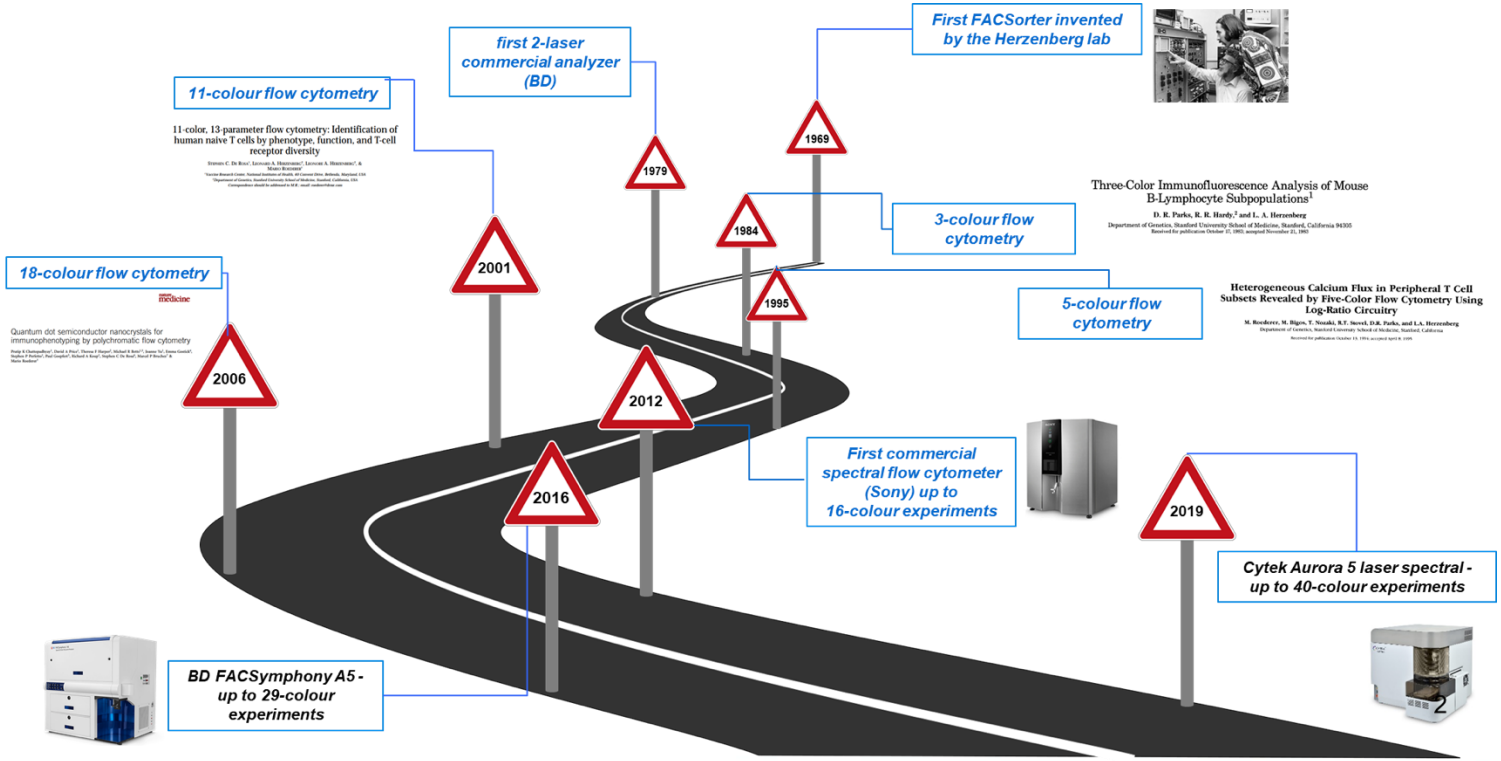
Traditional and Receptor Occupancy (RO) Flow Cytometry Assays	High-Parameter Flow Cytometry (HPFC)
Conjugation, stability testing or other technical challenges for custom-labelled reagents including bridging?	When are HPFC assays required?
Biological samples type to develop/validate RO assays on disease specific / activation-dependent targets?	Challenges in panel design?
Metrics to assess receptor occupancy?	Data analysis strategies
Normalization of receptor occupancy data?	Current needs and future requirements?
Issues with the different assay formats (Labeled Drug/Secondary detection antibodies/Competitive / non-competetive format/Labeled ligand/Other)	Other?

To set the scene

- A bit of history and background High-Parameter Flow Cytometry (HPFC) - Enrique Gomez Alcaide on behalf of the EBF Flow Cytometry team
- If time allows: Refresher on the survey presented this morning on RO assays –

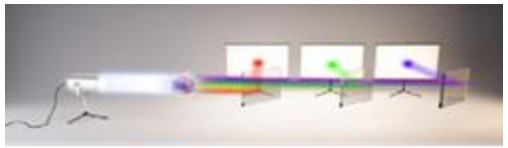
- Round tables:
  - Remember not only discuss your issues
  - Focus on ideas to solve them!

Evolution of Flow Cytometry over Time

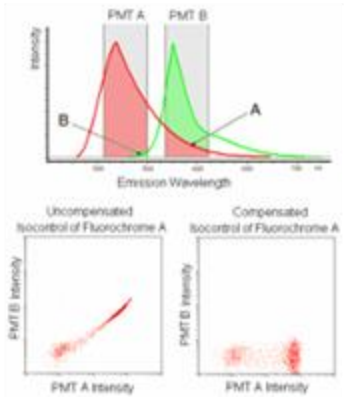


HPFC assays in clinical trials - Spectral flow cytometry

Revolution in the field



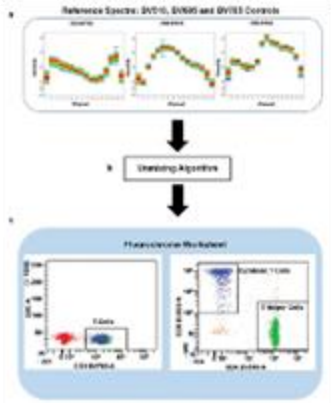
Conventional



10-plex

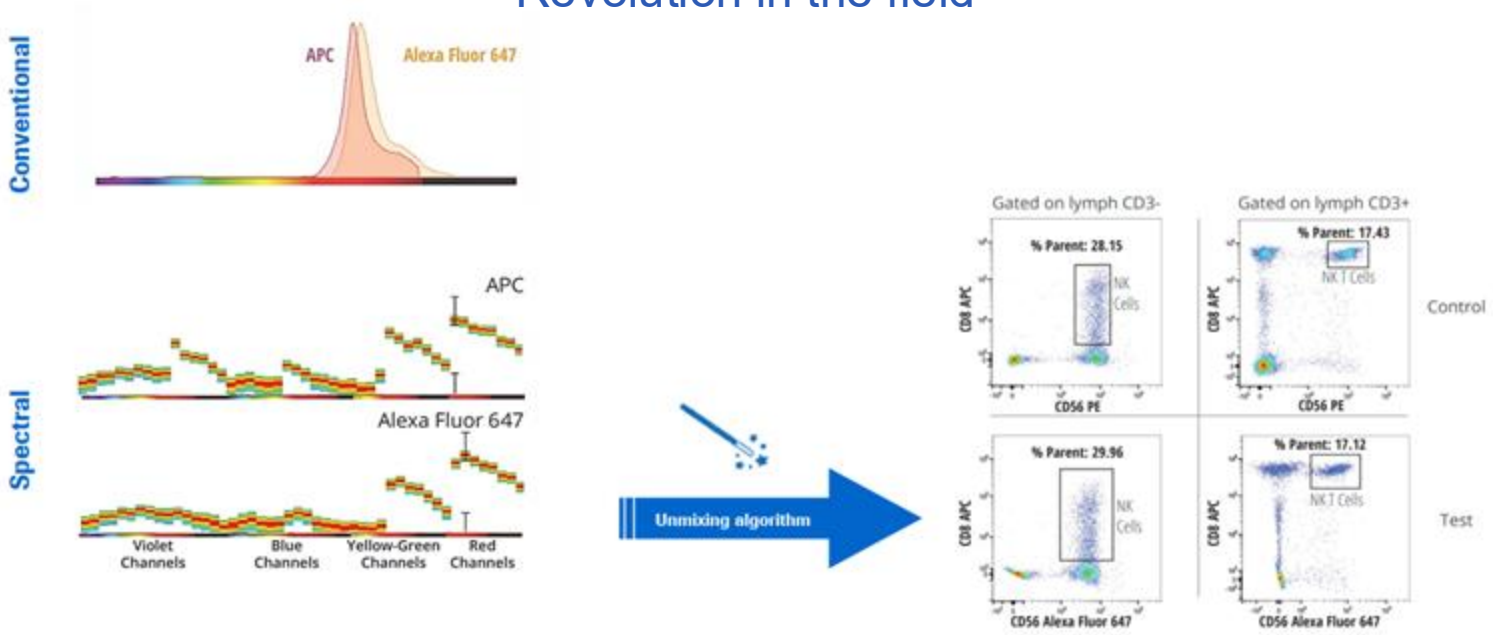
>40-plex

Spectral



HPFC assays in clinical trials - Spectral flow cytometry

Revolution in the field

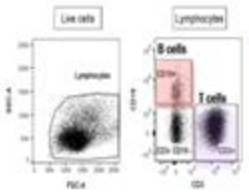


Spectral Flow cytometry allows combinations of fluorochromes that traditionally were not possible to be used due to overlapping emission patterns

High-parameter flow cytometry assay data analysis in clinical trial

Paradigm-shift : Manual gating vs. Computational tools for data visualization and data analysis

Manual gating



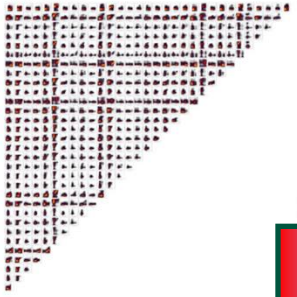
Examples:
FlowJo
FCS Express
Kaluza

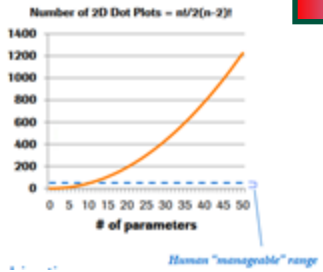
Advantages
Applicable to any sample
Historically the gold standard
Hypothesis driven

Disadvantages
Subjective and biased
Reproducibility challenging
Time and labour intensive
Not scalable

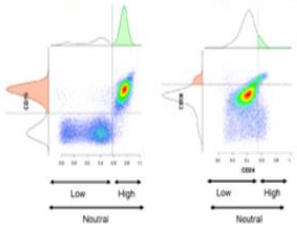
Current gold standard

32 parameters
496 plots





Algorithm-assisted gating (supervised)



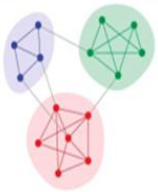
Examples:
FlowDensity
FlowType
FlowLearn

Advantages
Reproducible
Scalable
Automated
Hypothesis driven

Disadvantages
Requires computational skills
Initial setup effort

Ideal for high-level routine biomarker analysis

Algorithm-based gating (unsupervised)



Examples:
FlowSOM
Phenograph
SPADE

Advantages
Unbiased
Scalable
Automated
Exploratory analysis

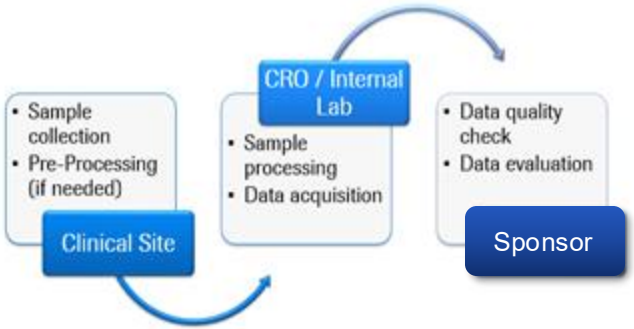
Disadvantages
Sometimes hard to interpret
Raw data obscured

Ideal for ad hoc exploratory biomarker analysis

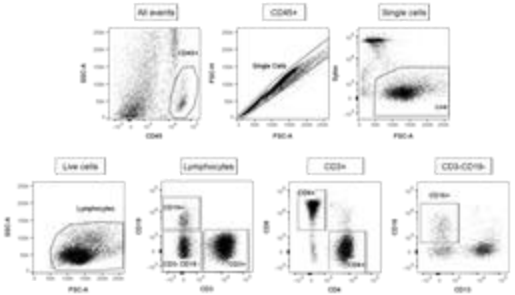
Figures From: Sean C. Bendall et al. Trends Immunol. 2012.

Challenges of implementing HPFC data analysis

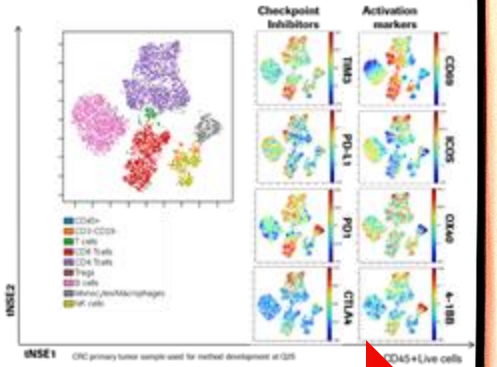
Data management, data analysis & data QC



Low-parameter flow cytometry (max 10 markers)



High-Parameter flow cytometry (> 50 markers)

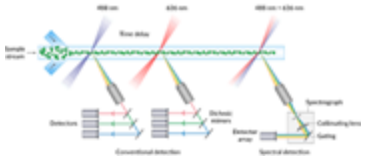


Increased Complexity

Increase in assay complexity challenges the central labs assay validations and the **data management of results**

High parameter flow cytometry in clinic: pros & cons

Conventional flow



(up to 10-16 parameters)
Low-parameter FC

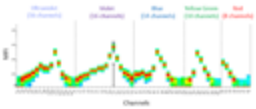
Advantages

- Straight forward protocols/analysis
- Short TAT for data access
- Reduced list of reportables (key ones)
- Cost per sample reduced

Challenges

- Reiteration of moAb used per tube (i.e. CD45, CD3, CD4, CD8...)
- Volume of blood (several tubes)
- Limited exploratory capacity
- Limited reverse translational research applications
- Total costs – High

Spectral flow



(up to 40 parameters)
High parameter FC

- Reduced patient material
- Standardization across studies
- (Reverse translational research)
- Flexibility in panel designs
- Higher exploratory capacity
- Unbiased analysis
- Reduced costs?

- Increase complexity and validation timelines
- Technical issues with unmixing process
- Increased data generation – New analysis tools required
- Difficult CRO management (increase of queries & TAT data delivery, …)

A plentiful automated analysis tools are available but lacking robustness and reproducibility, delaying its implementation in routine clinical data analysis

If time allows RO summary of surveys

RO summary on experience on issues with custom labels for RO assays

- Issues most often relate to conjugation and stability
  - Unknown stability
  - Lot-to-lot variability and Conjugation Inconsistency
  - Fluor intensity normalization
  - Stearic hinderance based on conjugation
  - Release of biotin or surplus amount of free biotin.
  - Decrease in DOL (degree of labeling) occurring during extended storage
  - Photobleaching, light exposure, or freeze-thaw cycles, lead to signal loss over weeks or months can result in signal loss in long-term studies
  - Lack of CoA of the custom-labelled material
- Other issues relate to samples
  - Samples not fresh enough / Correct collection tubes but forgot to centrifuge/prepare cells correct at clinical site

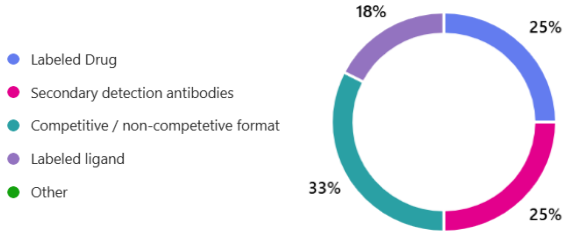
Best practice to Bridge between lots?

RO summary on experience on samples, metrics and normalization

- Samples for method development/validation?
  - Top 5 ranking order: Healthy volunteer whole blood; Healthy volunteer WBCs/PBMCs; Patients whole blood; Patients WBC; Cell lines
  - Others involve: Stimulated whole blood or PBMC from healthy subjects; Animal PBMCs / whole blood; Commercial tissues (BMMA samples); Lyophilised cells (eg vericells); Patients whole blood may need to be used to confirm assay condition in pre-validation step
- Metrics to access RO?
  - Top 3 ranking order: %RO (based on dual labelling, competitive and noncompetitive); MFI of bound/unbound populations; Ratio of bound total receptor
  - Others included: Integrate %RO and integrated MFI; MESF (normalized MdFI and MFI): Normalized MFI values, e.g. MESF values; % Bound (Competing Format)
- How to normalize?
  - Top 5 ranking order %RO of baseline; Subtraction of control/isotype MFI; Fold change MdFI; Fold change MFI; Internal cell population standard
  - Others included: Not a clear preference, study/assay dependent; Saturated control, baseline, and total receptor; Control samples, saturated (exogenous drug) samples, integrated MFI; % RO relative to saturated control/ sample timepoint

RO summary on experience on issues when developing various assay formats

- Which type of format used is case by case depending on CoU



- Issues include:
  - Identification and availability of both competitive/non-competitive antibodies
  - Difficulties to find commercial non-competitive clones in total vs free version of RO
  - Binding affinities
  - Finding a total receptor antibody that do not cause steric hinderance.
  - No saturation with the secondary antibodies
  - Labelled drug approach without secondary not always sensitive enough
  - High background/nonspecific binding when using secondary reagents,
  - Sample processing (snapshot) may prevent the RO detection
  - Internalization/receptor recycling after binding

To the round tables

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Contact Information

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