Multiparametric Flow Cytometry: Applications in Liquid Biopsy

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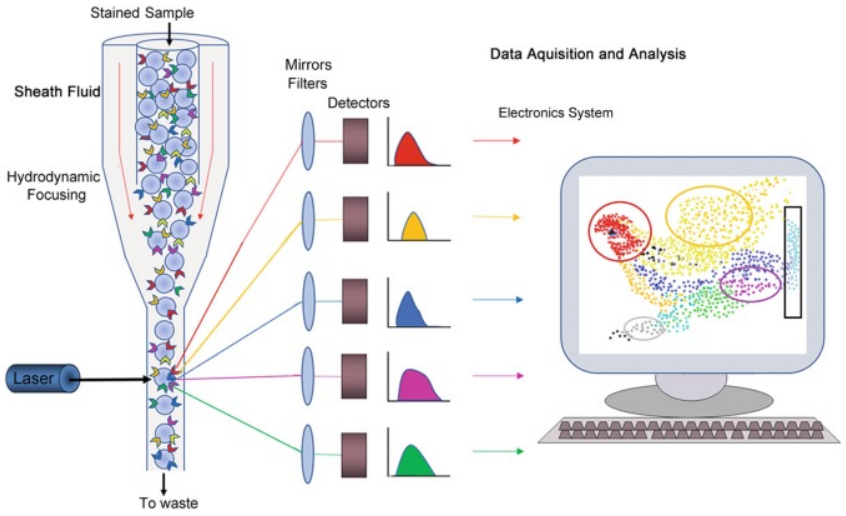
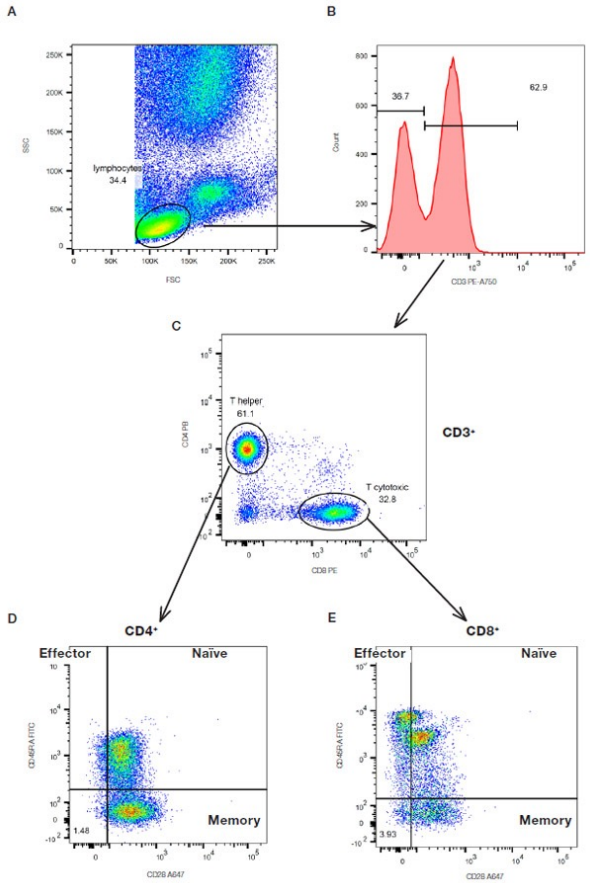
Disclosure

I have no conflicts of interest to disclose

Multiparameter Flow Cytometry (MFC)





Basic mechanism

Biological sample

⬇

Label it with a fluorescent marker

⬇

Cells move in a linear stream through a focused light source (laser beam)

⬇

Fluorescent molecule gets activated and emits light that is filtered and detected by sensitive light detectors (usually a photomultiplier tube)

⬇

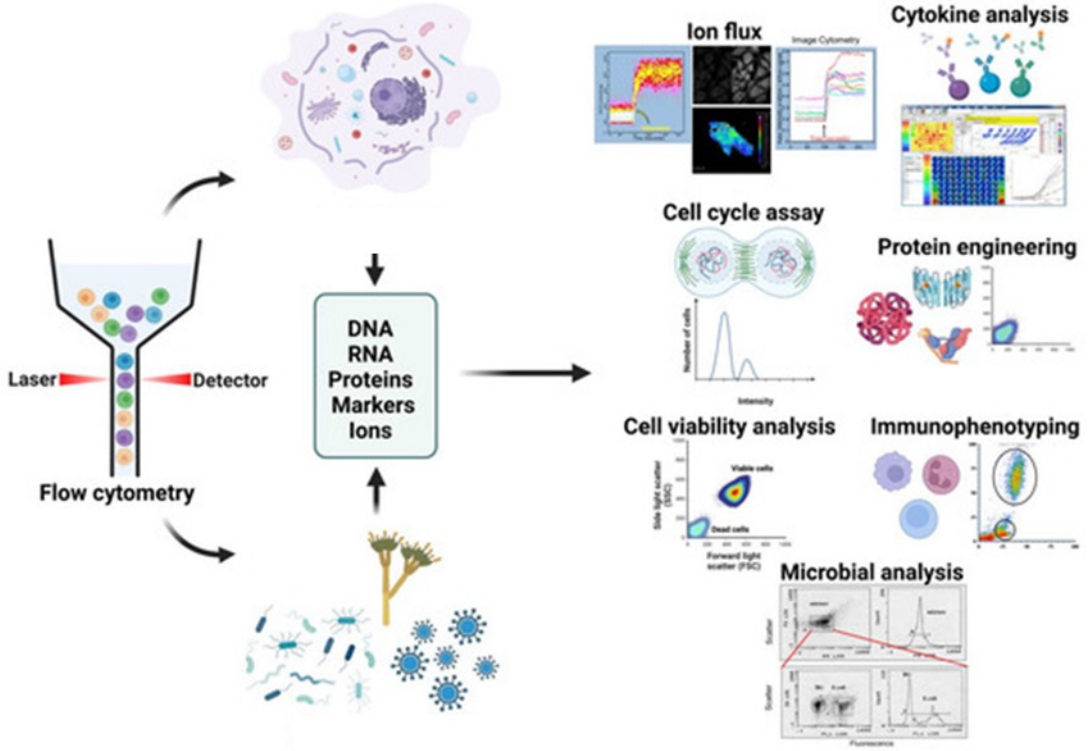
Conversion of analog fluorescent signals to digital signals

MFC Applications

MFC allows the quantitative and qualitative analysis of several properties of cell populations from any type of fresh unfixed tissue or body fluid.

The properties measured include a particle's related size, relative granularity or internal complexity, and relative fluorescence intensity.

- CELL/PARTICLES COUNTING
- CELL SORTING
- ANALYSIS
  - Immunophenotyping
  - Viability assay
  - Functional assays
  - Intracellular analysis (DNA, Proteins, Cytokines)



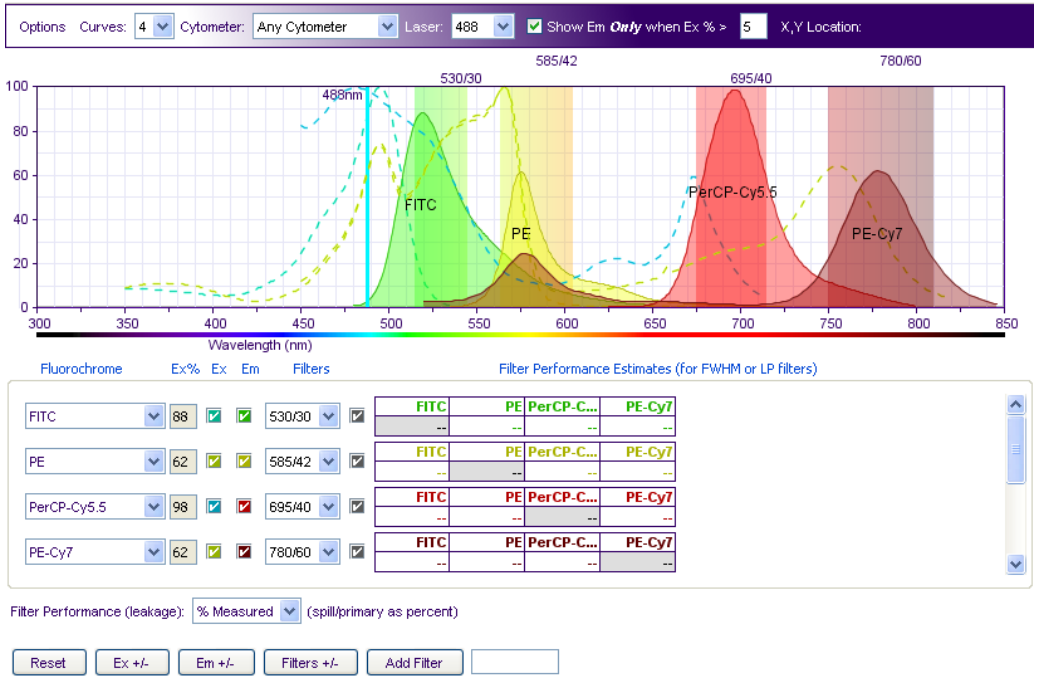
Multiparameter Flow Cytometry (MFC)

pre-analytical phase

- Cytometer setting
- Monoclonal antibody panel design (FluoroFinder, SpectraViewer)
- Monoclonal antibody titration
- Sample staining

- **Antigen expression/density**
- **Fluorochrome brightness**

Minimize the potential spectral overlap

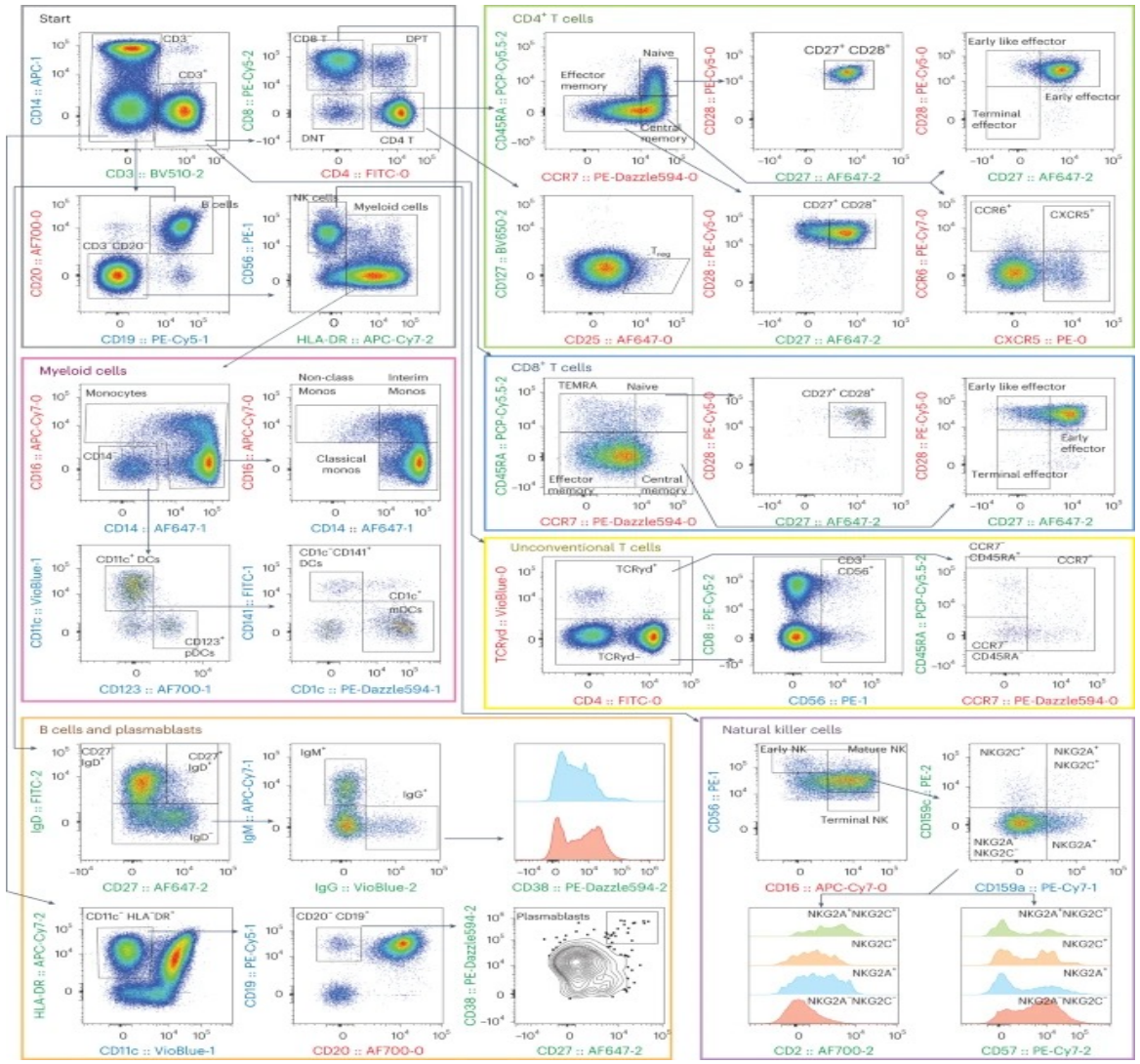


Panels	FITC	PE	PerCP-Cy5.5	PE-Cy7	APC	APC-H7	V450	V500
1	CD2	CD34	CD5	CD33	CD117	CD10	CD19	CD45
2	MPO-7	TdT	CD3	CD13	CD34	CD4	CD19/CD7	CD45
3	CD33	CD13	CD34	CD56	CD117	CD4	HLA-DR	CD45
4	CD15	CD123	CD34	CD25	CD117	CD7	HLA-DR	CD45
5	CD64	CD4	CD33	CD56	CD11B	CD14	HLA-DR	CD45

Multiparameter Flow Cytometry (MFC)

Analytical phase

- Compensation
- Data interpretation/analysis
- Gating strategy



Multiparameter Flow Cytometry (MFC)

Advantages

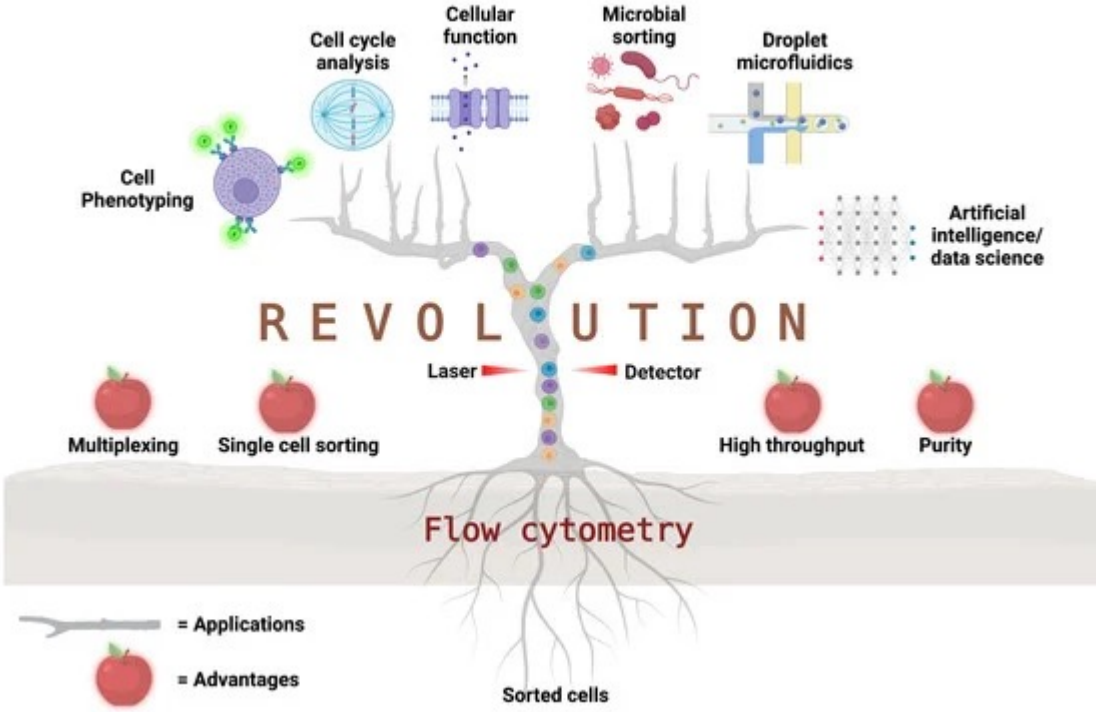
- ✓ **Rapid analysis:** millions of cells analyzed in a few minutes
- ✓ **High sensitivity:** Detects rare cell populations
- ✓ **Multiparametricity:** Analyze multiple characteristics of each cell
- ✓ **Information at a single cell level**
- ✓ **Rapid turnover of results**

Challenges and Limitations

- ✗ **Spectral overlap:** the number of fluorochromes is limited to avoid interference
- ✗ **Operator Variability** (e.g. manual gating) **and Limited Reproducibility**
- ✗ **Experience is critical:** inexperienced users may introduce significant errors
- ✗ **Difficulty in Interpreting Complex Data:** identifying different subpopulations requires experience and deep knowledge of cellular markers, particularly in high-parameter panels (>10-15 colors)

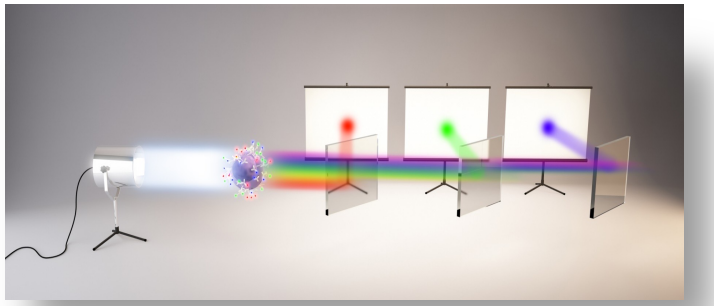
Innovative technologies

- **Spectral cytometry**
- **Mass cytometry**
- **Imaging MFC**
- **Cytometry coupled with sequencing**: Integration with omics techniques for in-depth understanding
- **Microfluidics and Automation**: Miniaturization for faster, more efficient analysis

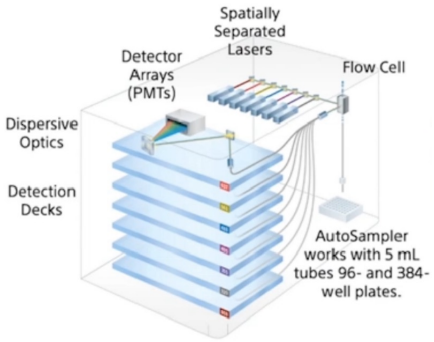


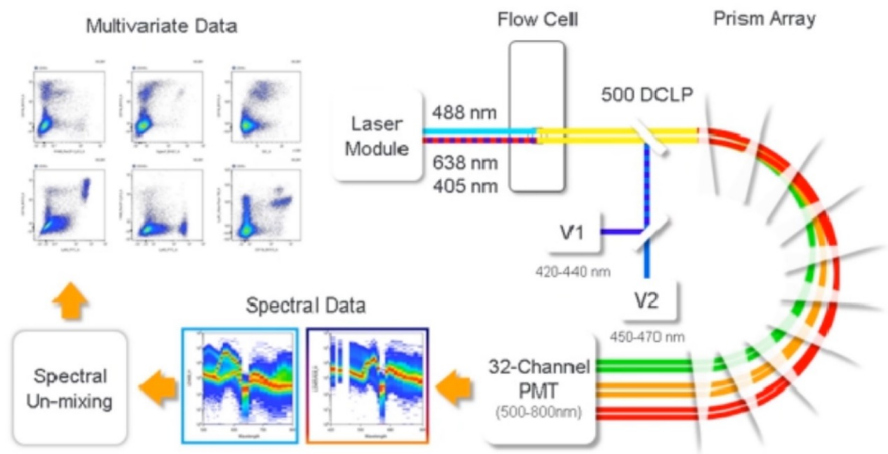
MFC vs Spectral Cytometry

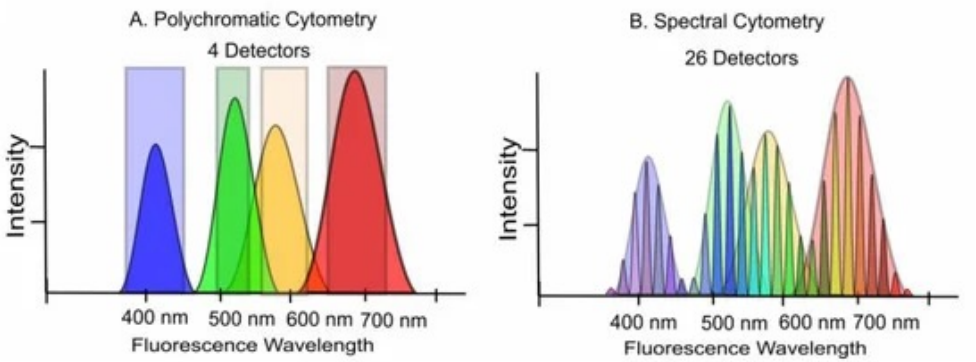
Conventional Flow Cytometers



Spectral Cytometers

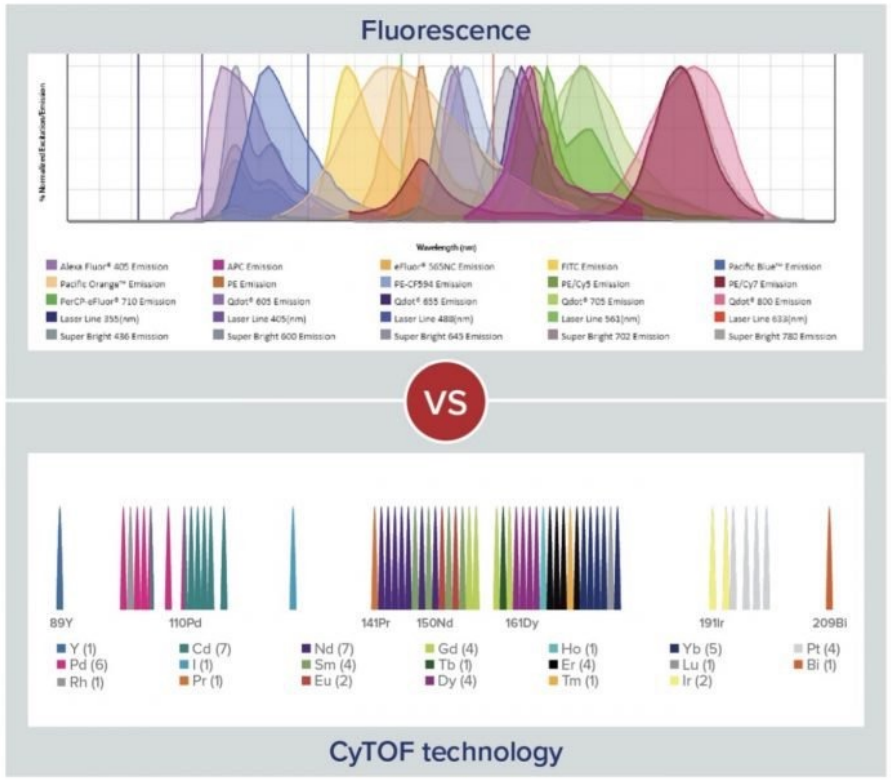


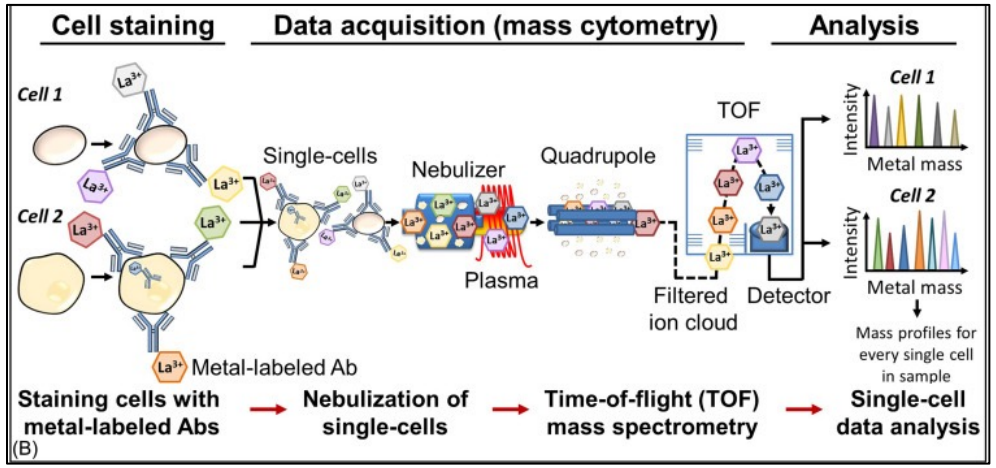




- **Antigen density and expression levels**
- **Fluorochrome choice/ conjugation availability**
- **Spectral unmixing**
- **Ab TITRATION/ STAIN INDEX**
- **Control: Unstained, positive control, FMO**
- **Complex gating strategies**

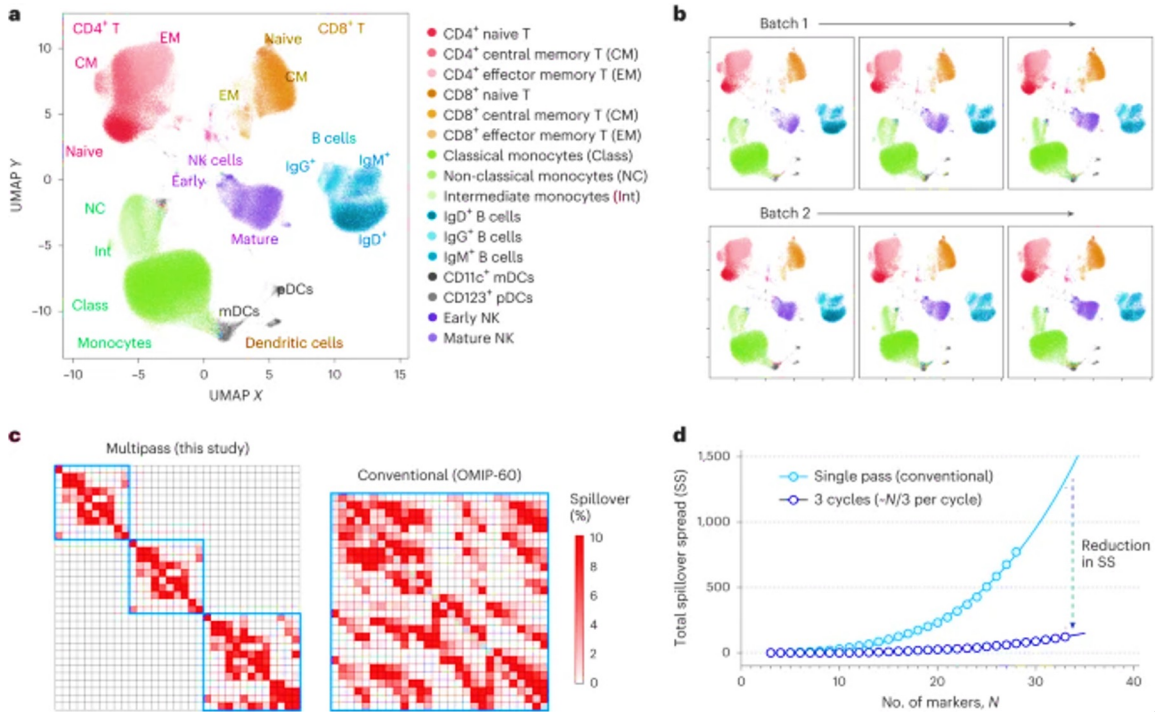
MFC vs Mass Cytometry

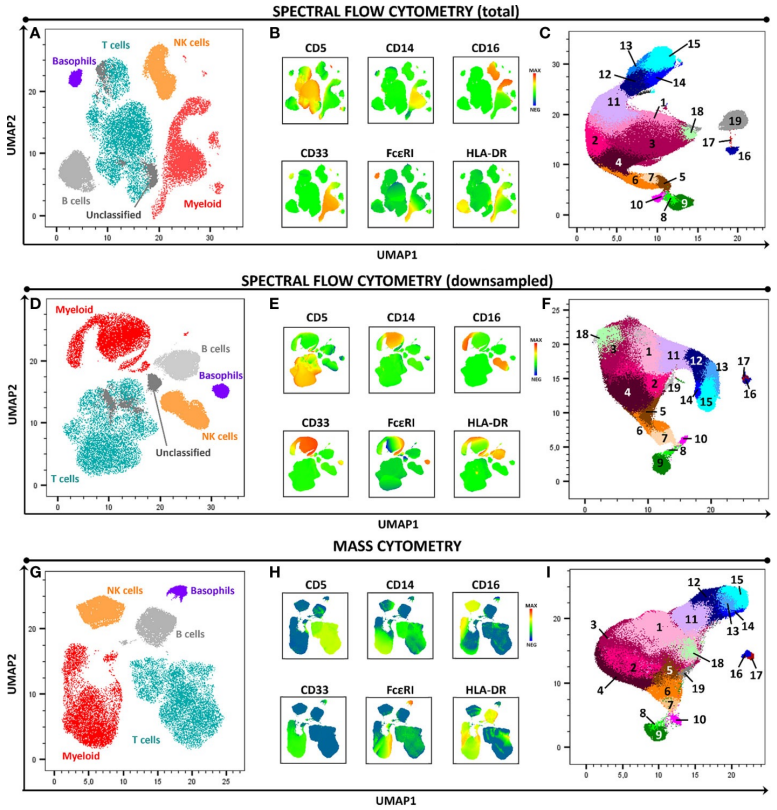




- ✓ **Analyze up to 40-50 markers per cell**
- ✓ **Eliminates interference due to fluorescence**
- ✓ **It provides an ultra-detailed profile of the cells analyzed**
- ✗ **Slower analysis than classic MFC**
- ✗ **Expensive/less common instruments in clinical laboratories**
- ✗ **Destructive process: the analyzed cells cannot be recovered for further study**

Spectral and Mass Cytometry data



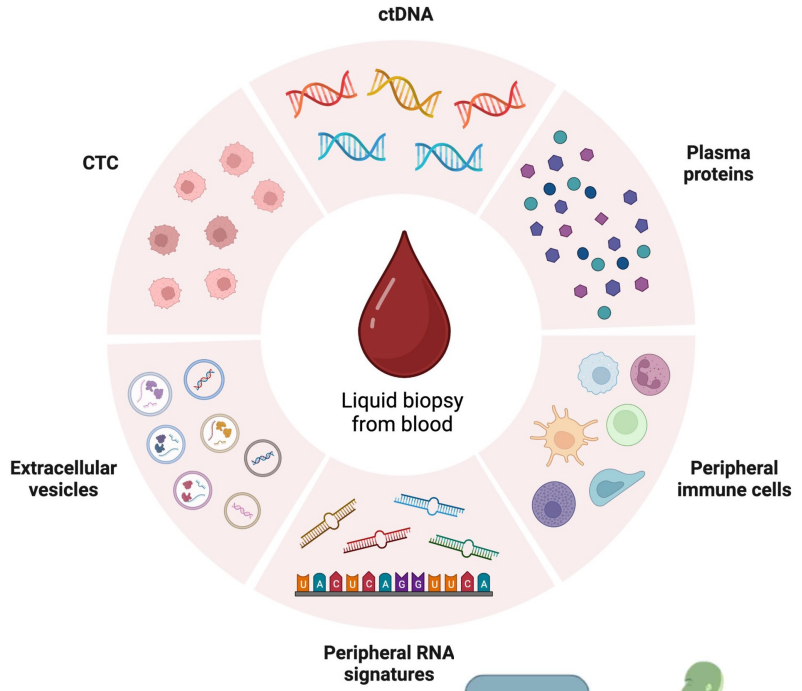


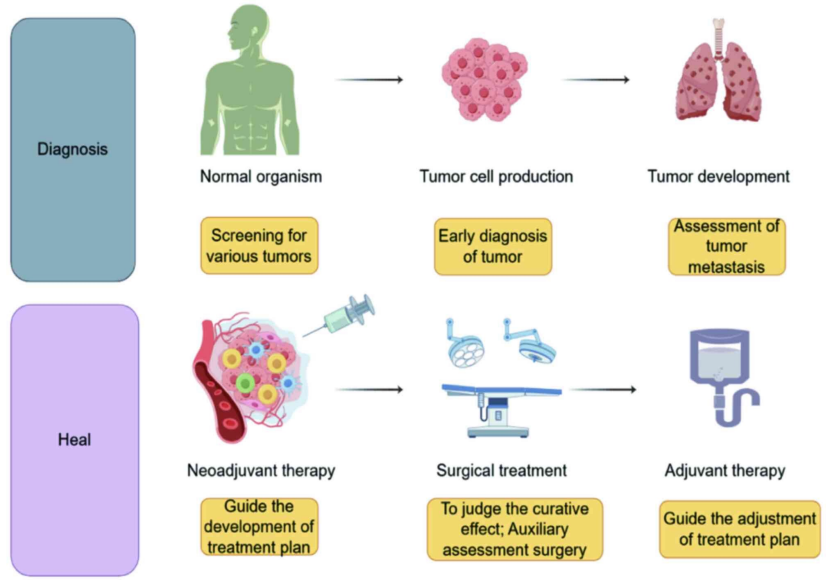
- Spectral/mass cytometry data must be processed using advanced software (**FlowJo, Cytobank, FCS Express**) and automated analysis methods/algorithms (t-SNE, h-SNE, UMAP and FlowSOM)
- Algorithms reduce the dimensionality of the data and allow easy visualization of high-dimensional data

Spectral/mass cytometry data allow the analysis of increasingly complex cellular expression profiles and the identification of previously unknown cell subsets

MFC Applications in Liquid Biopsy

- Circulating tumor cells (CTCs)
- Minimal residual disease (MRD)
- Immune system subpopulations
- Extracellular vesicles/Exosomes





Circulating tumor cells (CTCs)

Solid Tumor	CTC Surface Markers	Function
Breast cancers	EpCAM, HER2, CK8/18/19	HER2 for targeted therapy, EpCAM for isolation
Lung cancer(NSCLC/SCLC)	EpCAM, N-caderina, CK, TTF-1	TTF-1 for Adenocarcinomas, N-Cadherin for EMT
Colorectal cancer (CRC)	EpCAM, CK, CD133, EGFR	CD133 for Cancer Stem Cells
Gastric cancers	EpCAM, CK, HER2	HER2 per terapia anti-HER2
Pancreatic Cancer	EpCAM, CK, vimentina, CXCR4	CXCR4 associated with metastasis and drug resistance
Prostate cancer	EpCAM, PSMA, AR-V7	AR-V7 for resistance to anti-androgen therapy
Ovarian cancer	EpCAM, CK, CA125	CA125 for therapeutic monitoring
Melanoma	MCAM, ABCB5, MART-1, S100	ABCB5 for Melanoma Cancer Stem Cells

Haematological disorders	Surface Markers	Function
Chronic Lymphocytic Leukemia (CLL)	$CD5^+$, $CD19^+$, $CD20^+$, $CD23^+$	B cell identification
Chronic Myeloid Leukemia (CML)	$CD33^+$, $CD13^+$, $CD117^+$, $CD34^+$	Leukemia stem cell markers
Acute Myeloid Leukemia (AML)	$CD34^+$, $CD13^+$, $CD33^+$, HLA-DR, $CD117^+$	Leukemia stem cell & therapy targets
Acute lymphoblastic leukemia (ALL)	$CD10^+$, $CD19^+$ (B-ALL) or $CD3^+$ (T-ALL), TdT^+	Differentiation of B/T-ALL
Diffuse large B-cell lymphoma (DLBCL)	$CD19^+$, $CD20^+$, $CD22^+$, $CD79a^+$	CD20 as rituximab target
Follicular lymphoma (FL)	$CD10^+$, $CD19^+$, $CD20^+$, $BCL2^+$	BCL2 linked to treatment resistance
Linfomas in Burkitt	$CD10^+$, $CD19^+$, $CD20^+$, MYC^+	MYC indicates aggressiveness
Linfoma di Hodgkin	$CD30^+$, $CD15^+$, $CD45^-$, $PAX5^+$	CD30 as brentuximab target
Peripheral T Lymphomas (PTCL)	$CD3^+$, $CD4^+$, $CD5^+$, $CD7^+$, $CD30^+$	CD30 as a therapy target
Myeloma	$CD45^-$, $CD138^+$, CD38+, CD19-	CD138 plasma cell marker

Rarity → 1–10 CTCs per billion blood cells

False Positives → Normal cells may express CTC-like markers

Heterogeneity → CTCs become undetectable

Solid Tumor

- **Epithelial-Mesenchymal Transition (EMT) Process** → Loss of **EpCAM, CK**
- **Intra-patient heterogeneity**

Haematological disorders

- **Selective pressure of therapy**→ Resistant subclones may lose target antigens (e.g., CD19 in B leukemias after anti-CD19 therapy)
- **Immunoediting** → Modulation of antigenic expression to escape the immune system
- **Clonal heterogeneity** → Cells with the same genetic profile can express different markers depending on the stage of the disease or their origin

Circulating tumor cells (CTCs)

Heterogeneity and loss/modulation of markers in CTCs is a critical limitation of MFC-based techniques, requiring more advanced strategies:

- Developing larger panels to identify more complex and specific immunophenotypic profiles
- Identification of disease-specific biomarkers stable over time
- More flexible gating strategies to avoid exclusive dependence on a single marker

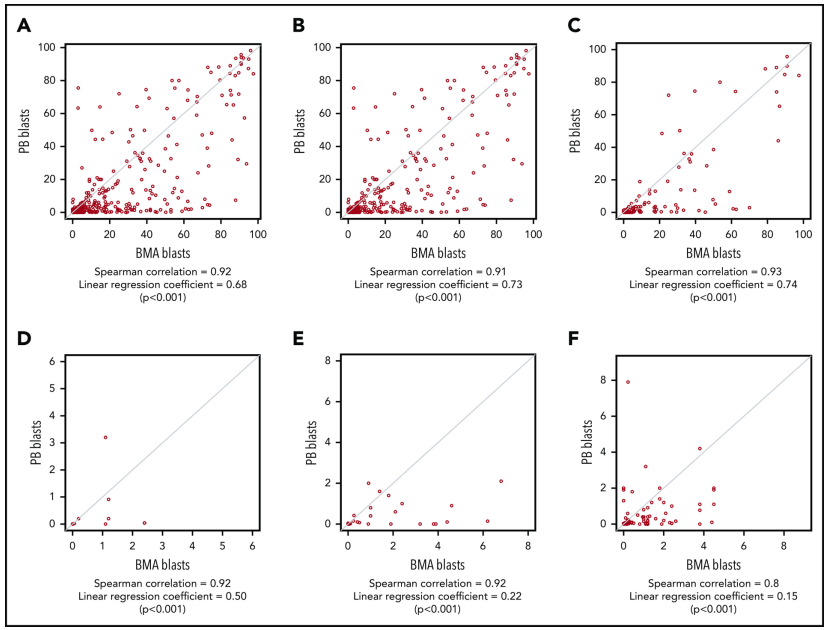
Minimal residual disease (MRD)

MRD is defined as the persistence of a small number of malignant cells after the initial treatment, undetectable by morphologic or conventional screening methods

Liquid biopsies offer several advantages over bone marrow (BM) for **MRD** detection:

- Less invasive
- Provide a more comprehensive overview of tumor heterogeneity
- Enable repeated sampling
- Allow better monitoring of response dynamics to specific treatments

Relationship between bone marrow and PB blast percentage measured by MFC in patients with AML

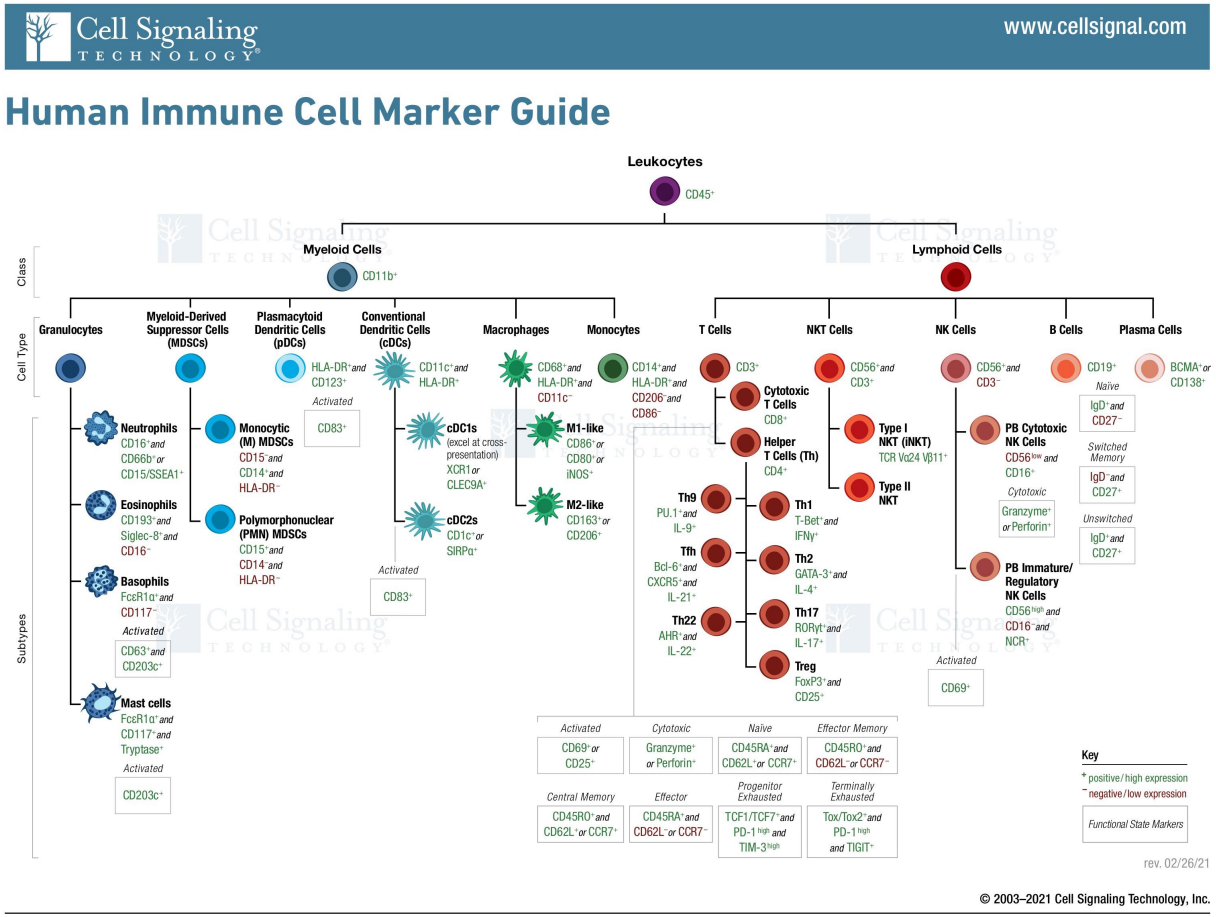


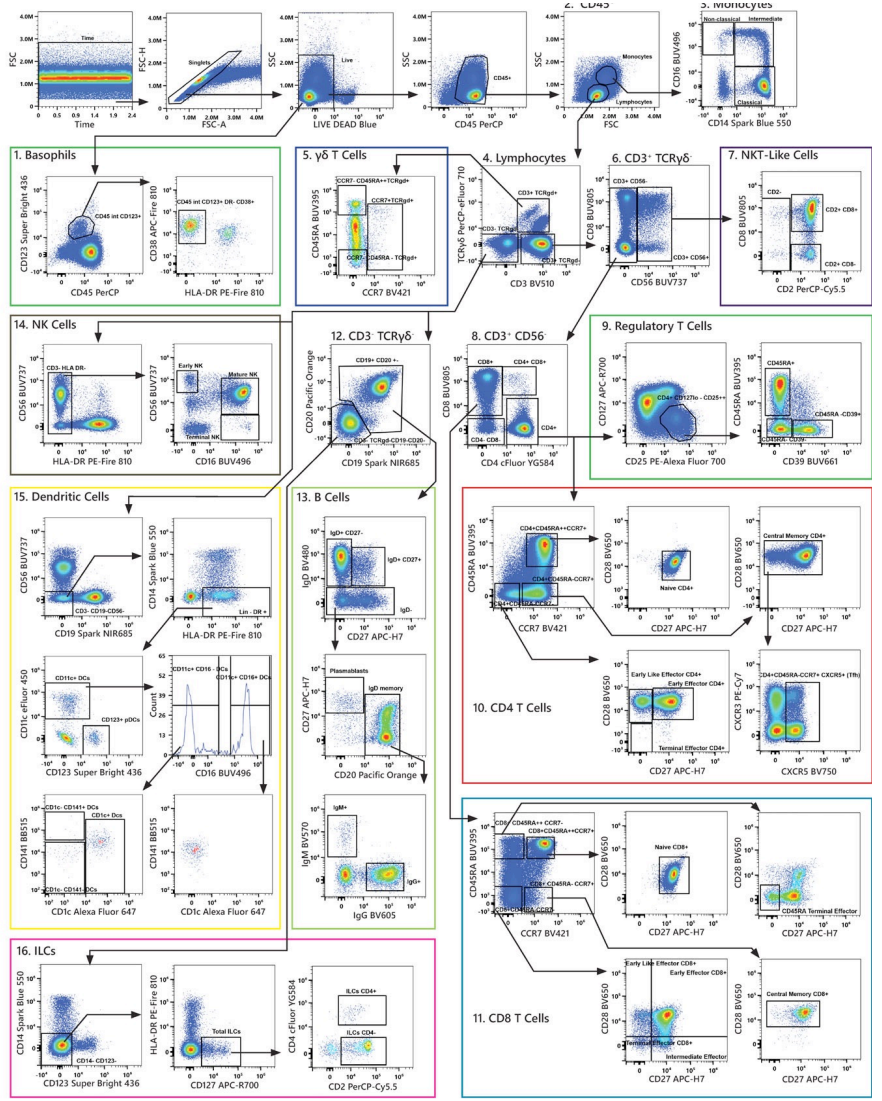
A. all sample pairs
B. first sample pair for each patient
C. PB and BM samples obtained on the same day
D. pairs obtained within 30 days before HCT
E. pairs obtained within 90 days after HCT
F. pairs for which BM blasts were <5% by both flow cytometry and morphology

Godwin CD, et al. Blood. 2021

Cut-off???

Immune system subpopulations





Evaluation of inhibitory Immune checkpoint expression

Extracellular vesicles/Exosomes

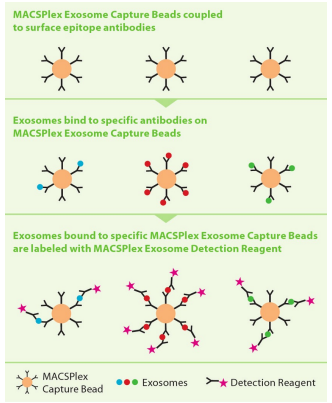
EV Subtype	Origin	Size (nm)	Markers	Release Mechanism
Exosomes	Multivesicular bodies	50–150	CD9, CD63, CD81, TSG101, ALIX, HSP70	Fusion with the plasma membrane (ESCRT-dependent or independent)
Microvesicles	Plasma membrane	100–1000	Integrins, Selectins, CD40, Tissue Factor	Cytoskeletal reorganization and membrane budding
Apoptotic Bodies	Plasma membrane	100–5000	Annexin V, C3b, Thrombospondin, Annexin A1	Cellular fragmentation during apoptosis

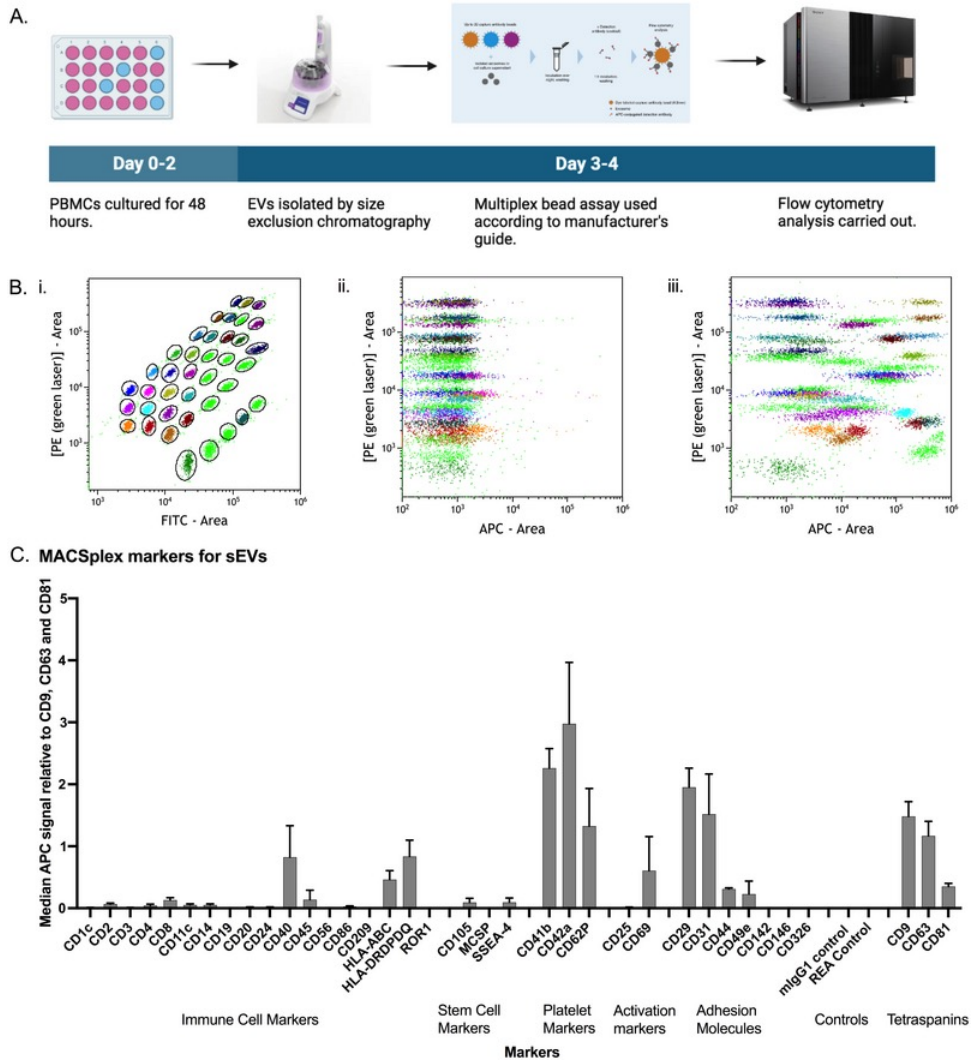
Challenges of Direct EV Analysis

- **Small size** → Difficult detection with standard cytometers
- **Low signal intensity** → Limited fluorescence per EV
- **Debris contamination** → Requires accurate discrimination
- **Lack of standardization** → No clear calibration references

Bead-Based Approaches

- **Increased target size** → Improved detection in flow cytometry.
- **Enhanced signal intensity** → Higher fluorescence per event
- **Reduced debris interference** → More specific EV selection
- **Multiplexing capability** → Enables analysis of multiple EV subpopulations





Multiparametric Flow Cytometry: Applications in Liquid Biopsy

Conclusions

- Multiparametric Flow Cytometry is a powerful tool for analyzing cells in liquid biopsy
- It offers significant opportunities for early detection, monitoring, and understanding of diseases
- Continuous technological developments promise to further expand its applicability

Future innovations

- Development of more sensitive and automated cytometers
- New biomarkers for more accurate characterization
- Integration with other technologies (e.g., DNA sequencing)
- Combined approaches to improve disease monitoring (e.g., cytometry + imaging)

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