AKADEMIE FÜR IMMUNOLOGIE

SPRING SCHOOL ON IMMUNOLOGY

Ettal, Bavaria, March 9-14, 2025

Single cell analysis and sorting.

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Notes

Materials from companies used in this presentation are for illustration of scientific and technical aspects, and not a recommendation to use their products.

Some of the texts in this presentation have been derived AI from chats like Google Gemini, and Microsoft Copilot .

Abundant instructions on using cytometry including cell separation are available in the publication authored by more than 200 experts in the field:
Cossarizza, Andrea, et al. "Guidelines for the use of flow cytometry and cell sorting in immunological studies."
European journal of immunology 51.12 (2021): 2708-3145.
In this presentation it is referred to as “Guidelines” with page numbers.

Single-cell analysis technologies for immunology research

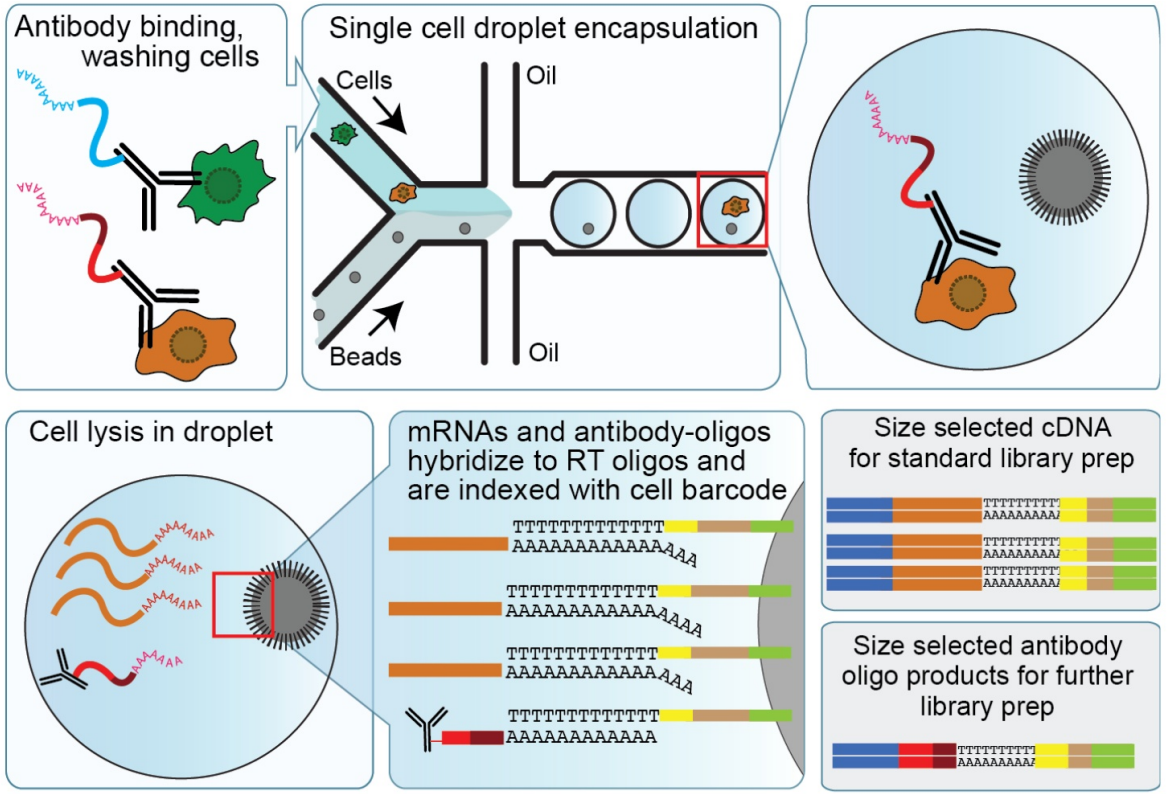
- **Single-Cell RNA Sequencing (scRNA-seq)**
- **Flow Cytometry,** fluorescent or mass labelling (CyTOF)
- **Microscopy-Based Techniques:**
    - **Imaging flow cytometry**
    - **Super-resolution microscopy**
    - **Spatial Transcriptomics**

Outcomes

- **Identify new cell types and subpopulations**
- **Understand immune responses**
- **Learn about spatial arrangement of tissues**
- **Develop new therapies:**

Derived from a Gemini response on technologies for immunology.

Single Cell Analysis by NGS (scRNA-seq)



Stoeckius M et al(2018),dx.doi.org/10.17504/protocols.io.ngzdbx6

Cite-SEQ

Single Cell Analysis by NGS

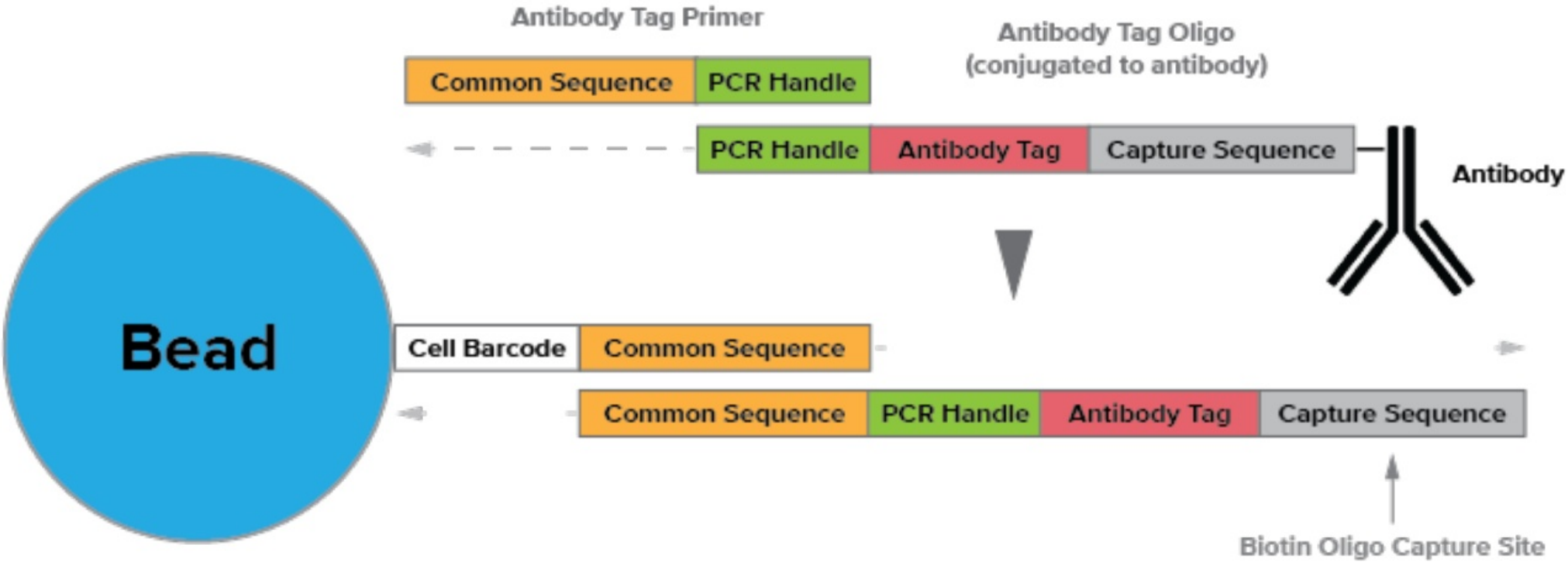


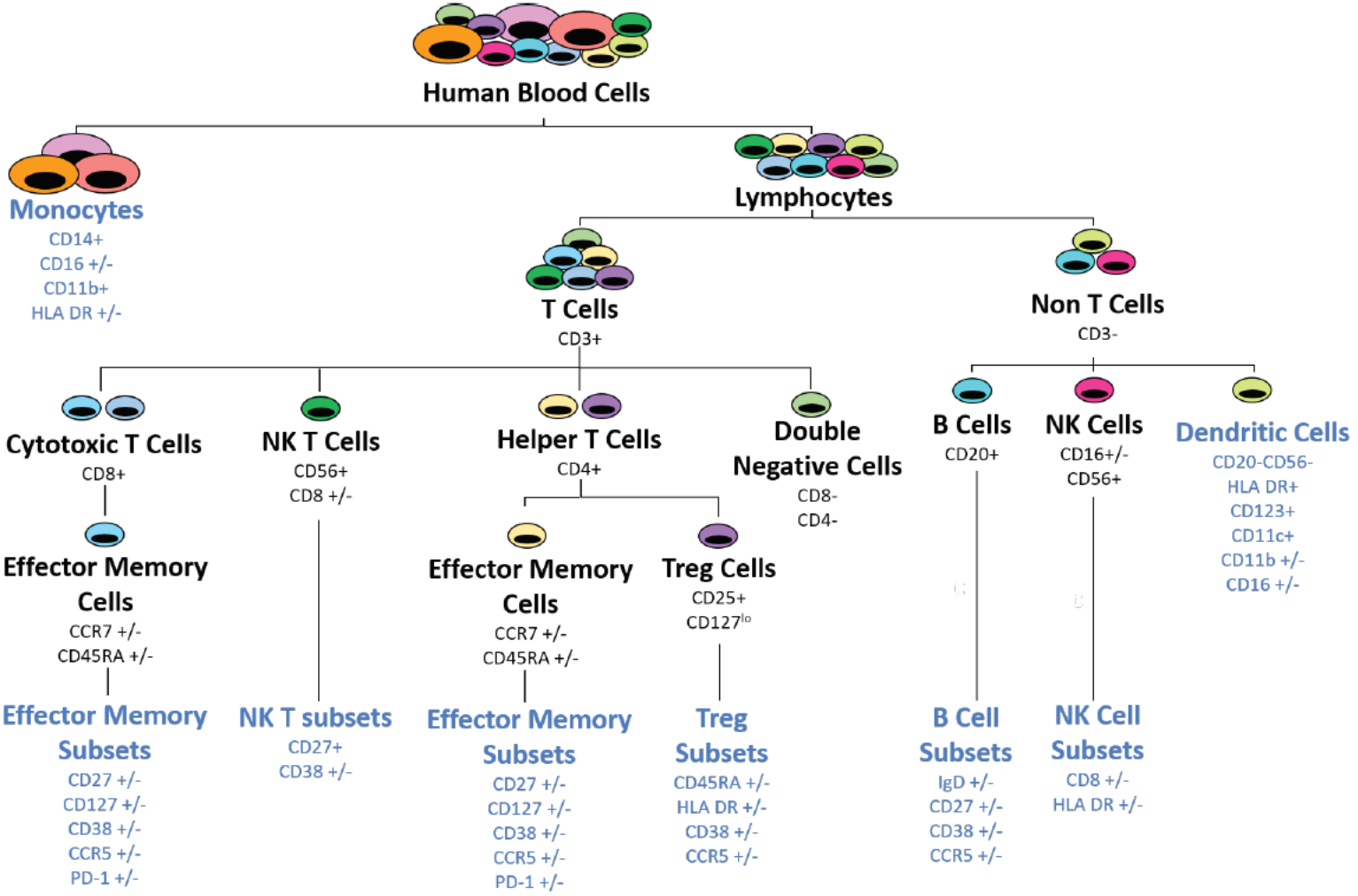
Figure 2. Antibody Tag Oligo construct.

Tapestri MissionBio

Single Cell Analysis by NGS

NGS based phenotyping		
	1% subset	10% subset
Markers	50	20
Fraction of interest	0.010	0.100
hi/low expression	100	10
reads/marker	10	10
total reads	1.00E+09	1.00E+07
time/read [s]	0.02	0.02
#rare cells analyzed	200	500
analysis time [d]	231	2
total cells analyzed	20000	5000
[s]/total cell	1000	40.00

Flow Cytometry



CYTEK Biosciences 2018

Flow Cytometry

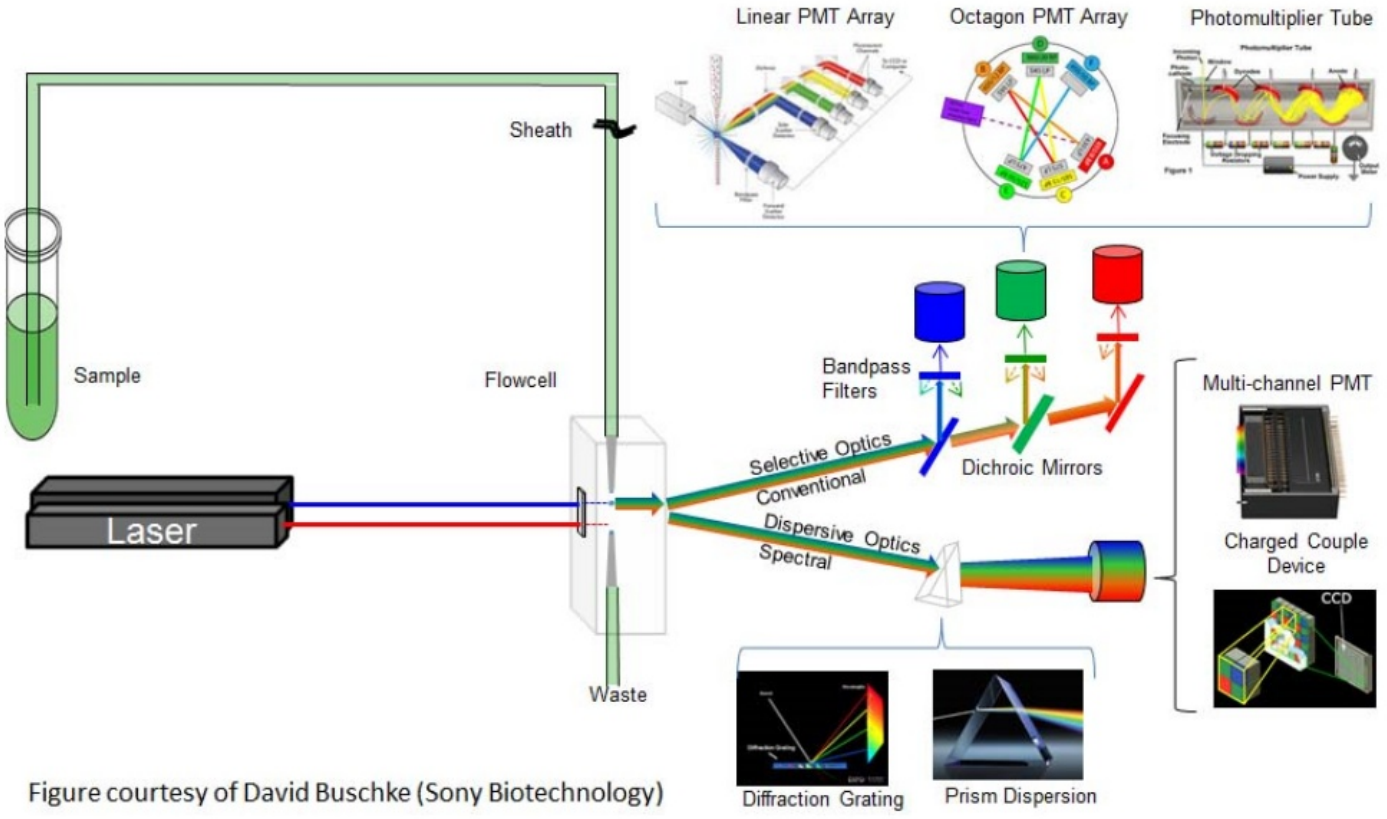
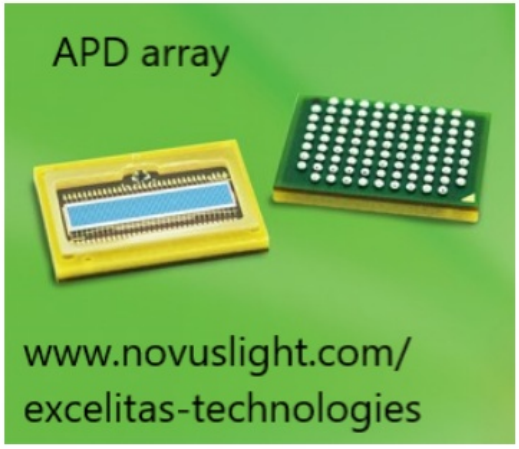
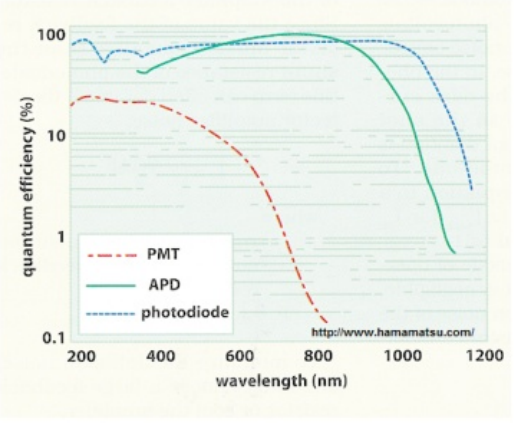
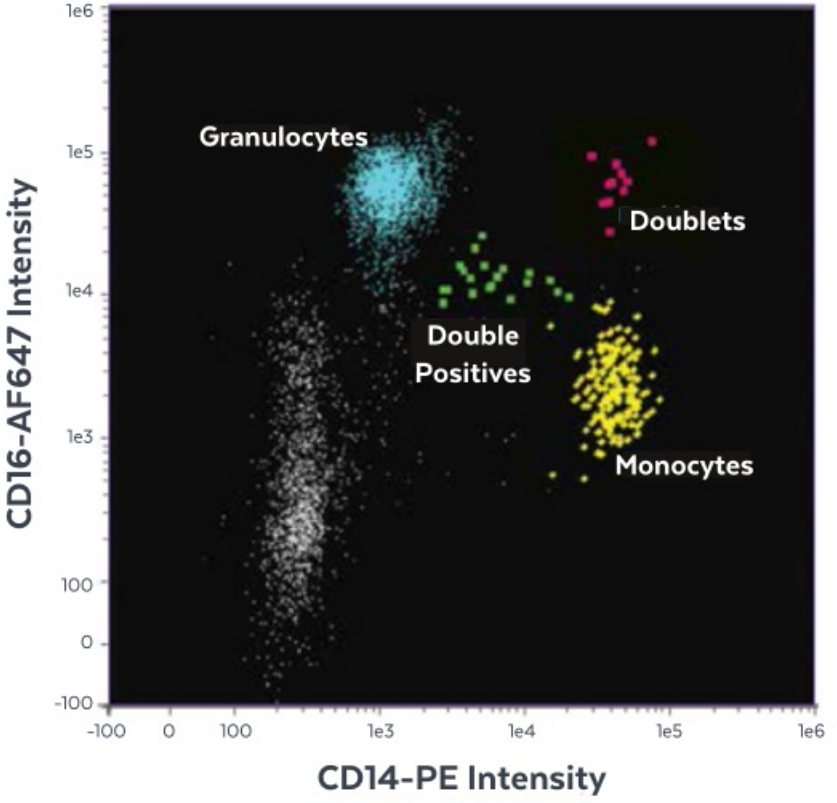


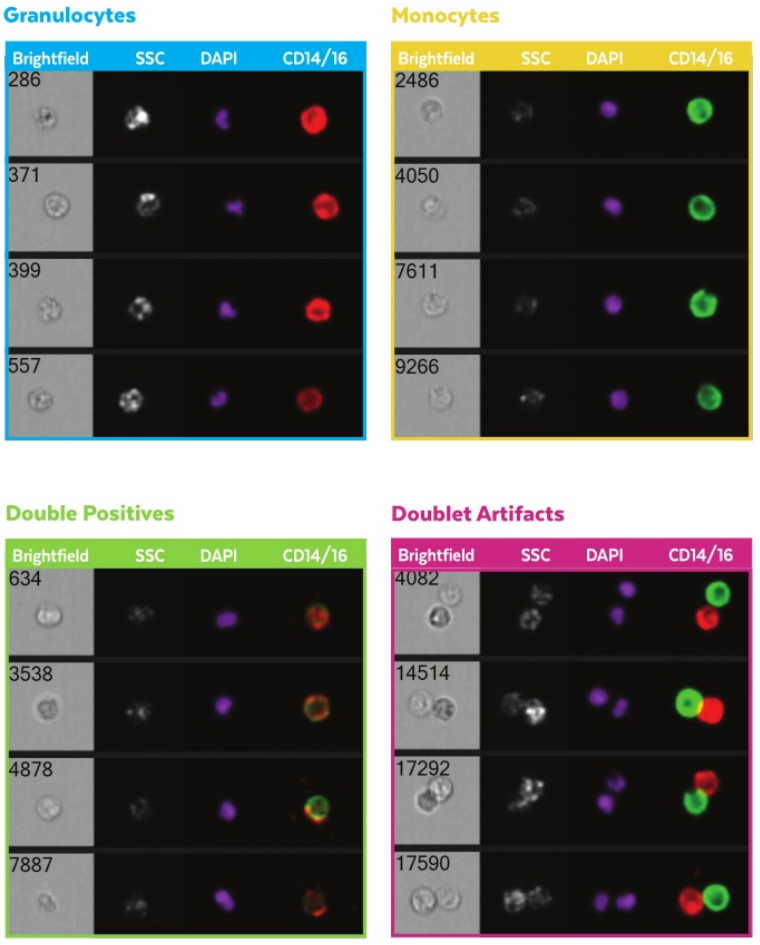
Figure courtesy of David Buschke (Sony Biotechnology)





Imaging Flow Cytometry





Amnis Imagestream (early imaging flow cytometer, presently additional commercial systems)

Cycling single cell multi-parameter analysis

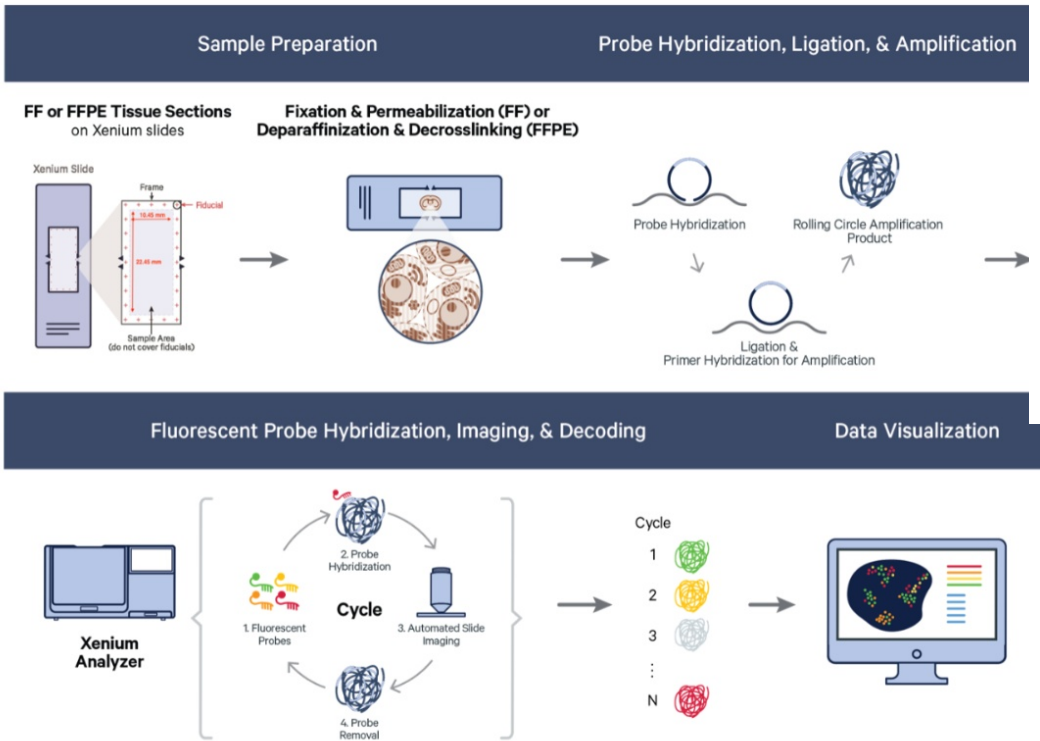
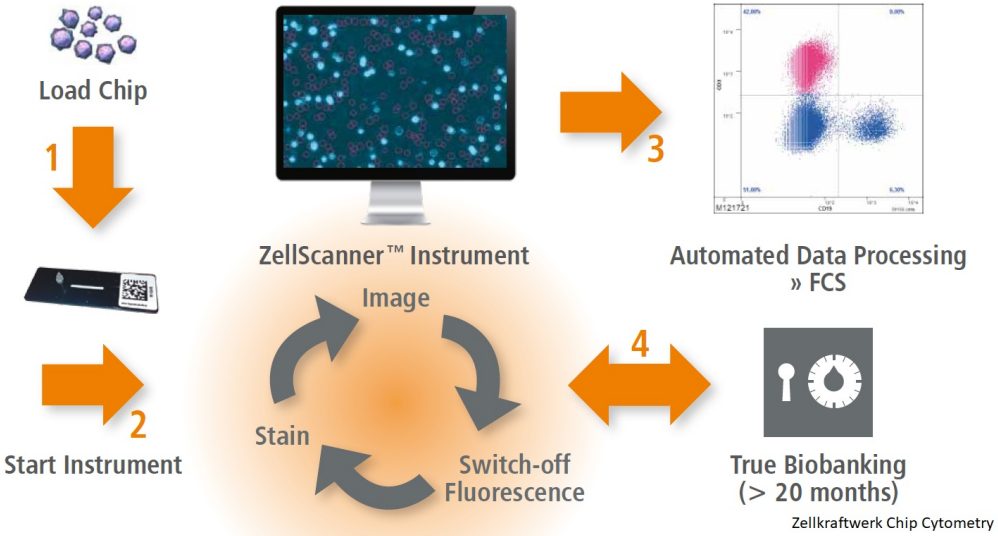
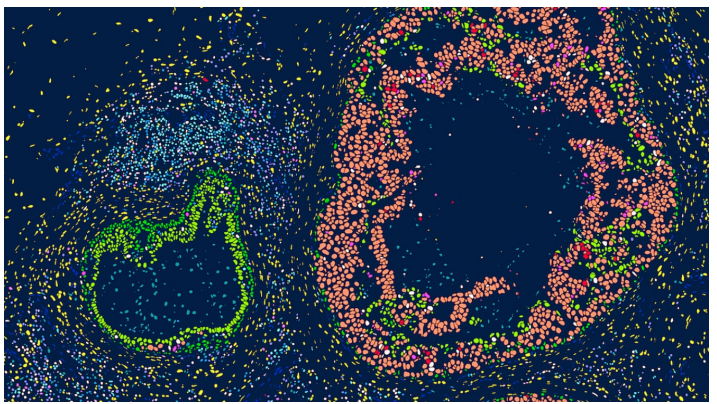


Figure 1 – Xenium General Workflow (Diagram from 10x Genomics)





Sensitivity Reducing Factors

https://pbs.twimg.com/media/EWHoc2gXkAAUGlh.jpg

https://www.pikrepo.com/fsizb/green-pine-trees-under-blue-sky-during-daytime

Light background

Spectral overlap

Electronic noise

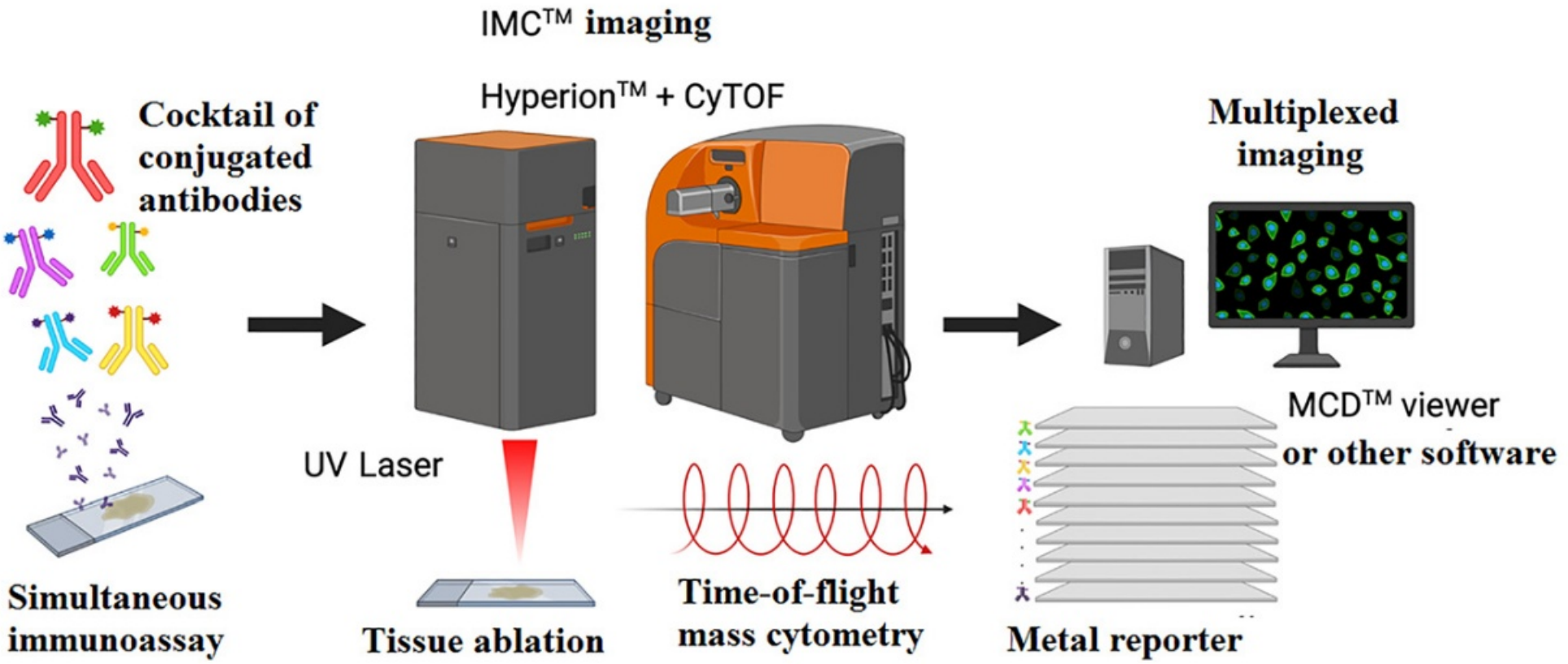
Photon shot noise

…

Unbound dye

Non-specific binding

Mass Cytometry



Cereceda K et al: doi.org/10.1515/almed-2021-0075

Single Cell Proteomics

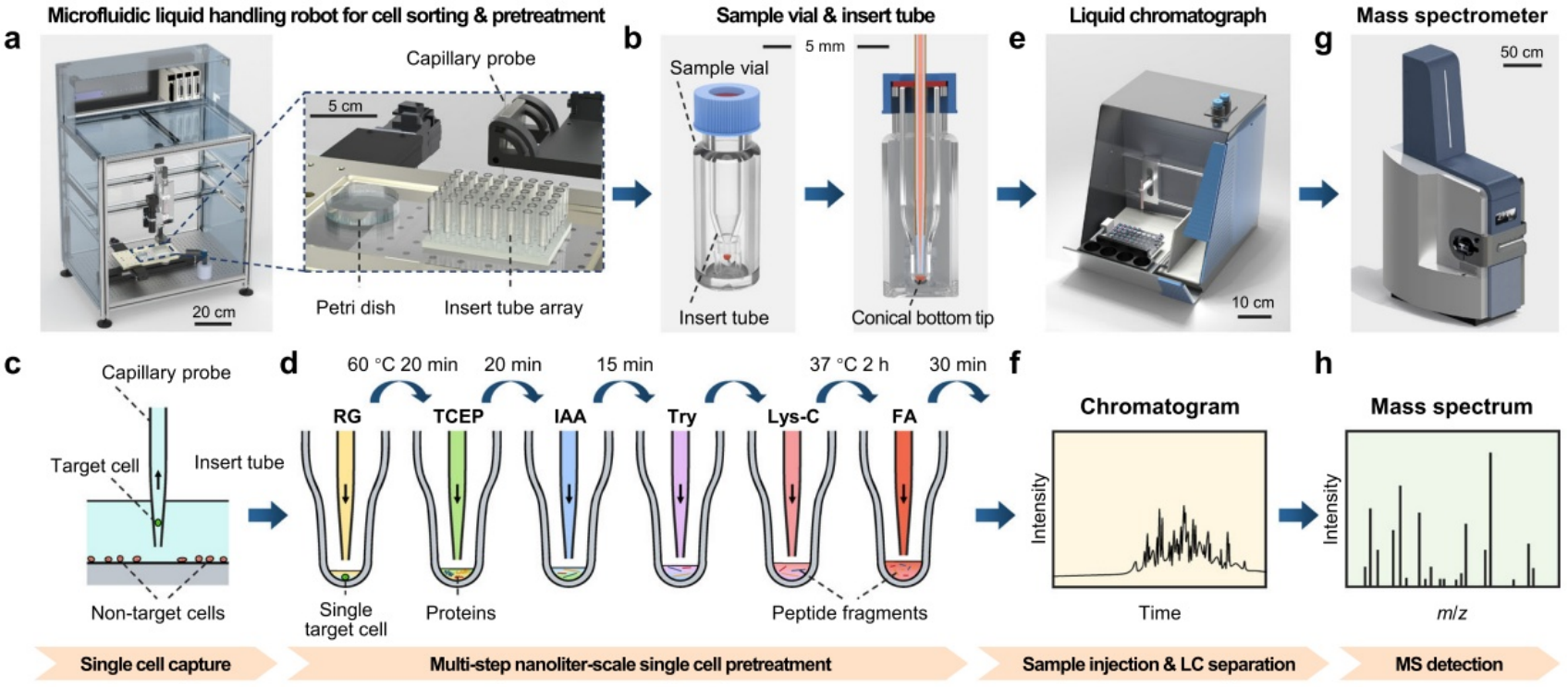
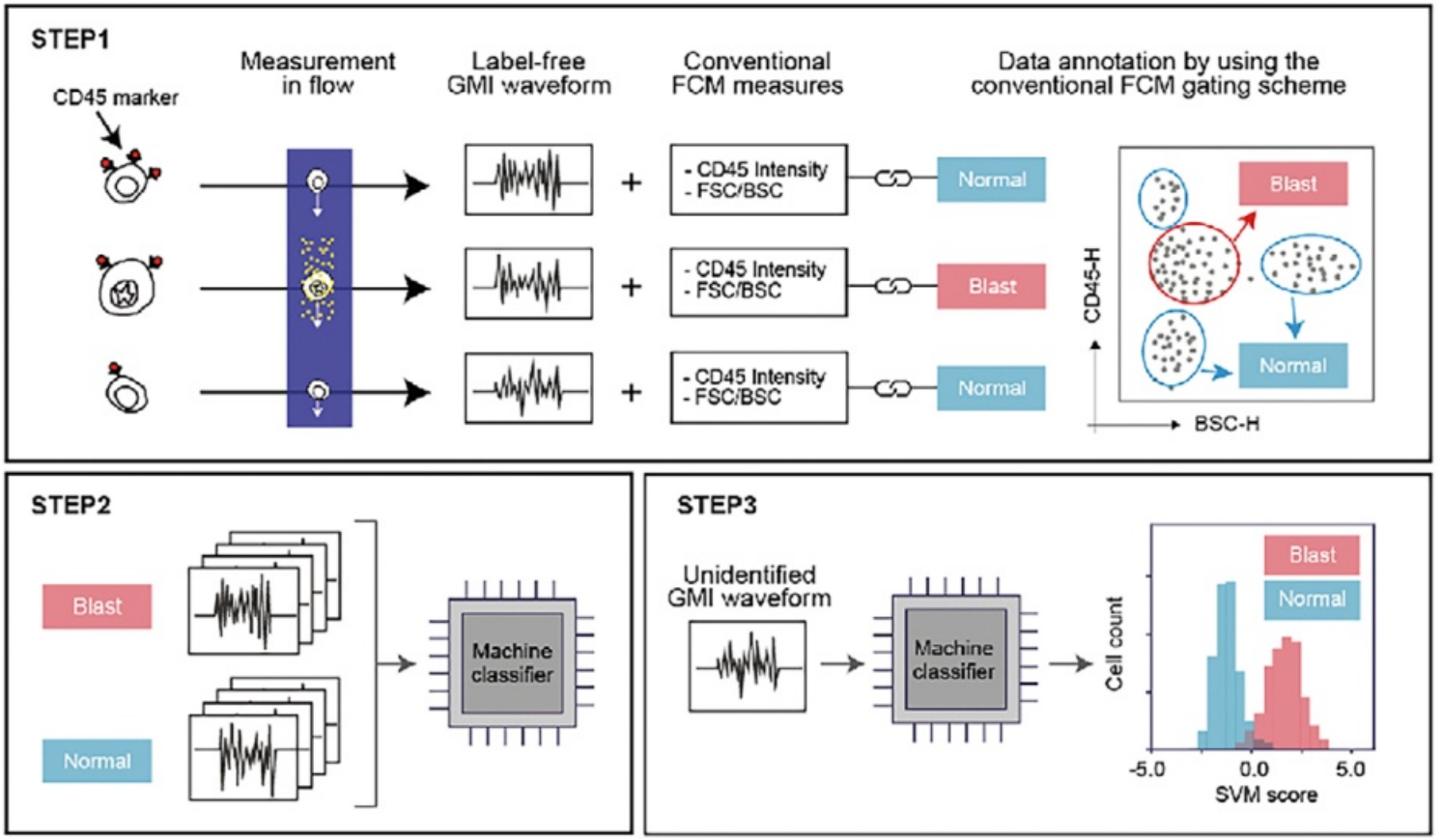
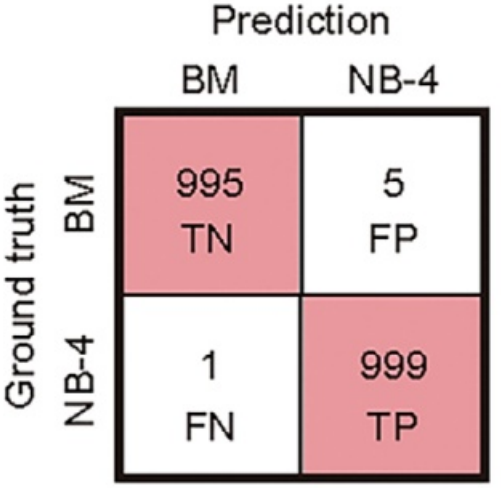


Fig. 1 | Schematic diagram of the PiSPA workflow for single cell proteomic analysis. The PiSPA workflow was conducted using a probe-based microfluidic liquid handling robot for cell sorting and pretreatment, a commercial LC system with an autosampler, and a tims-QTOF mass spectrometer. The microfluidic liquid handling robot (**a**) with an insert tube array (**b**) completed the sorting of single cells and the multi-step pretreatment of the single cell samples with the automated pick-up operation mode, including sorting of single target cells (**c**), nanoliter-scale cell lysis (RG, RapiGest SF), protein reduction (TCEP, tris (2-carboxyethyl) phosphine), alkylation (IAA, iodoacetamide), enzymatic digestion (Try, trypsin; Lys-C, Endo-proteinase Lys-C) and termination of the digestion (FA, formic acid) (**d**). Insert tubes coupled with sample vials were used as the nanoliter microreactors for sample pretreatment of single cells (**b**). After sample pretreatment, the insert tubes & sample vials were used as sample tubes for the autosampler of the LC system to perform the sample injection (**e**), LC separation (**f**) and subsequent MS detection of the digested peptide components from single cells (**g**, **h**).

Yu W et al. 2024 https://doi.org/10.1038/s41467-024-45659-4

Label-free machine learning supported single cell analysis





Kawamura Y el al. 2024 DOI: 10.1002/cyto.a.24821

Cell sorting

- **Fluorescence-Activated Droplet Cell Sorting** (FACS)
- **Bulk Cell Selection** eg MACS

Applications

- **Studying Immune Cell Function**
    - **Cell Proliferation**
    - **Cytokine production**
    - **Cytotoxicity**
- **Developing clonal cell lines for protein production**
- **Immunotherapy Development**
    - **Cell therapy**
    - **Vaccine development**
- **Disease Research**
    - **Studying immune responses**
    - **Identifying biomarkers**
- **Cell Subset Proteomics**

Cell Selection Applications and Requirements

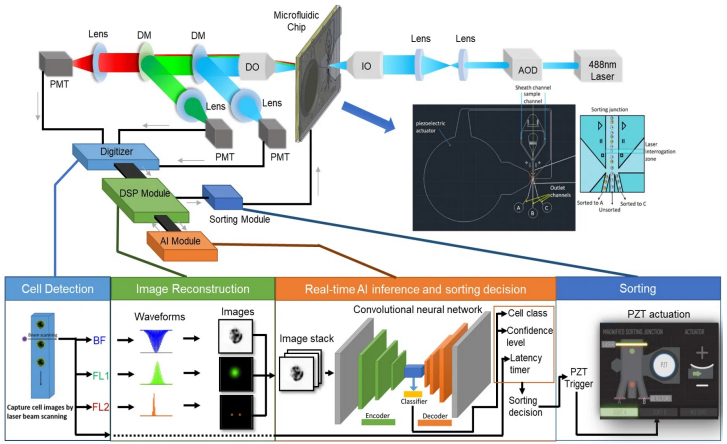
Single Cells
Creation of single clone hybridomas for antibody production
Single Cell Genomics
Single Cell Proteomics

Few cells
Studying immunoglobulin class switch
Weissman identifying root hematopoietic stem cell
Immune response profiles

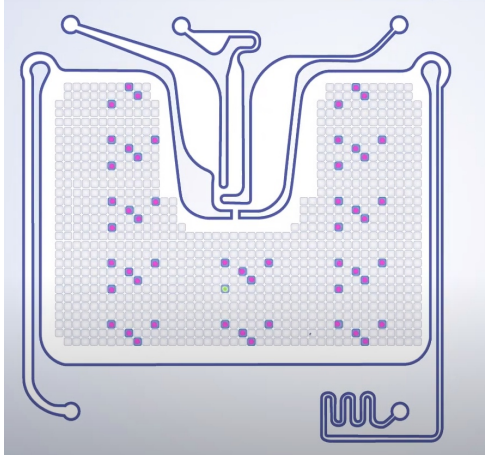
Many Cells
Metabolomics
Cell Therapy e.g. 2E8 need sorting to obtain 2E6 CD34+ cells (5.5hr at 1E4/s)

Sequential Single Cell Selection Technologies

- Limiting Dilution
- Droplet Sorting (spectral analysis, imaging, morphology fingerprint and AI,...)
- Fluid Channel MEMS walve
- Fluid Channel stream switching
- Dielectric movement with microscopic observation
- Optical tweezers



https://nanocellect.com/image-guided-cell-sorting/



siliconbiosystems.com/en-us/DEPArray-PLUS

Benefit of parallel pre-enrichment before sequential sorting

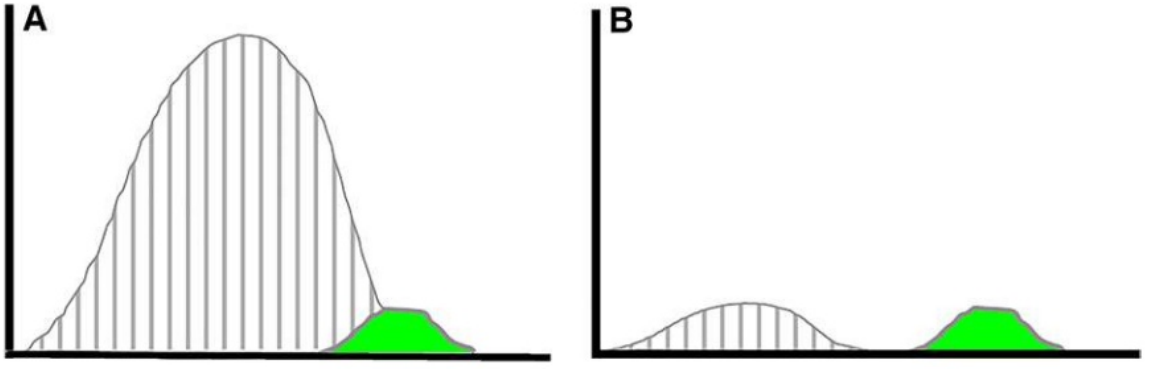


Figure 11. Improvement of population discrimination after pre-enrichment. Cytometer histograms of unwanted (gray lines) and wanted (solid green) populations. (A) A large excess of an unwanted population may create substantial overlap with the target population, making it impossible to achieve a good single-cell sort. (B) After a pre-enrichment bulk sort, which removes most of the unwanted population a good discrimination between the two populations can be achieved.

Guidelines pg1610

Modern parallel cell separation technologies

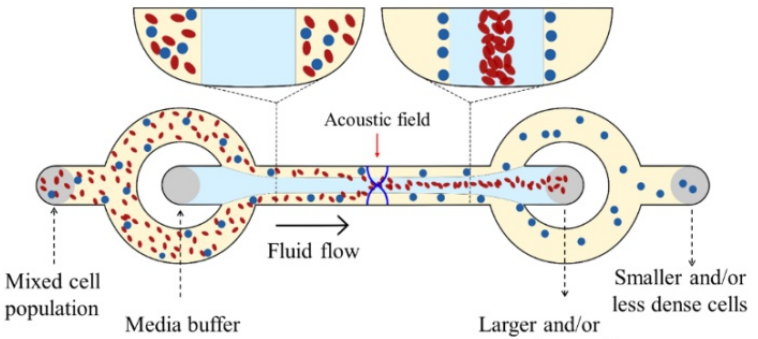
Older methods (next slide)

Magnetic (magnetic force with magnetic binding reagents)

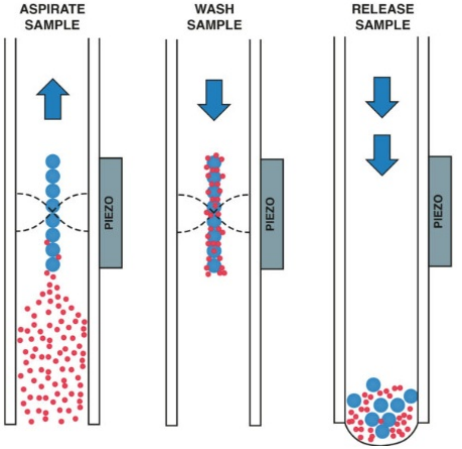
Acoustic (deflection, trapping)

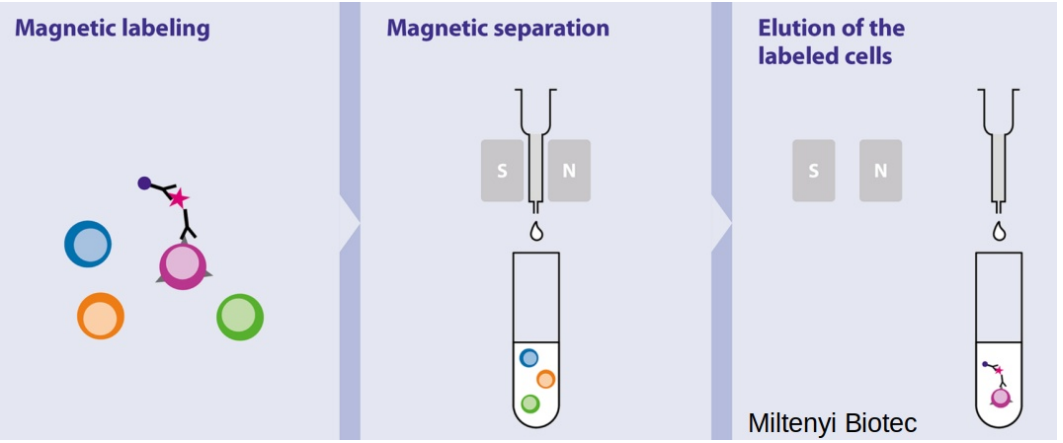
Hydrodynamic (fluid steaming forces)

Optical (laser tweezers)



https://acousort.com/technology/





Historical Parallel Selection Methods

I. Separation by physical parameters

- Density e.g. Ficoll, Percoll
- Lysis e.g. erythrocyte removal
- Adhesion e.g. nylon wool

II. Cell separation by immunological parameters

- Complement mediated specific lysis of Ab-coated cells
- Specific adherence of cells to Ab- coated plastics
- Rosetting
- Avidin columns
- Change of buoyancy by cell-cell contact across surface molecules

III. Separation using biological characteristics

- Fe-Phagocytosis

IV. Separation by biochemical characteristics

- L-leucine methyl ester (microglia, macrophages)
- Antibiotic resistance
- Selection of gene-targeted cells

From: "Historical and Useful Methods of Preselection and Preparative Scale Sorting: Charlotte Esser
I. BACKGROUND." Cell Separation Methods and Applications. CRC Press, 1997. 21-34; Table 9

Acknowledgements

BD Biosciences

Miltenyi Biotec

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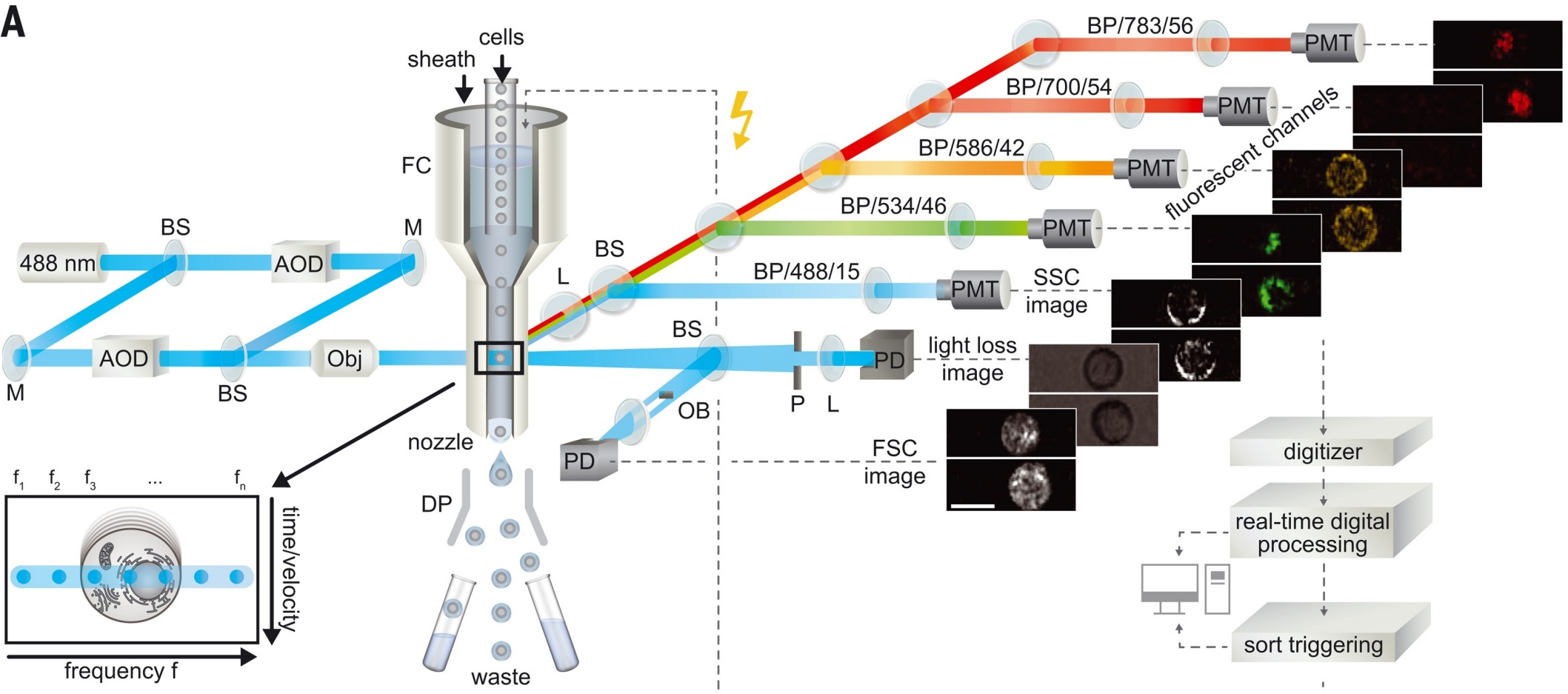
Ming Yan

Stefan Miltenyi

Imaging Flow Cytometry



Schraivogel D et al. Science 375.6578 (2022): 315ff

Parameter combinations for optimal sensitivity

Rare Cell Analysis by Flow Cytometry

Optimization of the Limit of Detection by Multi-Parameter Analysis

Fluorescence Parameter Combination	Limit of Detection (rare cells/million other cells)
++	50
+-	0.5
+-+	0.01

For the AmCell CD34 count the +- approach is approximated. The exclusion reagents are CD45 and CD15 conjugated to fluorescein, the positive marker for progenitor cells is CD34, conjugated to R-phycoerythrin. The sensitivity listed above from the references is not reached completely, because some instrument and software components were not used.

Gross H.J., Verwer B., Houck D., Recktenwald D.: Detection of Rare Cells at a Frequency of One per Million by Flow Cytometry. **Cytometry 1993**

Gross H.J., Verwer B., Houck D., Hoffman, R., Recktenwald D.: Model Study Detecting Breast Cancer Cells in Peripheral Blood Mononuclear Cells at Frequencies as low as 10-7. **Proc. natl. Acad. Sci. USA 1995**

- more sensitivity with more parameters
- exclusion parameter is essential