TELLING FLUORESCENCE APART

Cristina Teodosio

Belfast, September 22nd 2022

1

MULTICOLOR FLOW CYTOMETRY

1968
1969

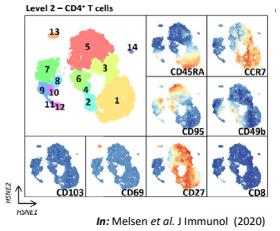
NICHE LABORATORY TECHNIQUE

ICP 11 FLOW CYTOMETER
Wolfgang Göhde, University of Münster, Germany

ROUTINE TOOL
IN RESEARCH & CLINICAL LAB

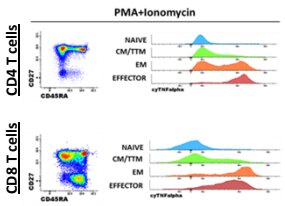
2022

Immunophenotype

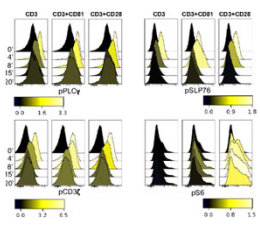


In: Melsen *et al.* J Immunol (2020)

Activation



Signaling



In: Sagi *et al.* PNAS (2012)

- *Gene expression*
- *Cell cycle*
- *Membrane potential*
- *Enzymatic activity*
- *Phagocytosis*
- *Calcium flux*
- *pH changes*
- *etc…*

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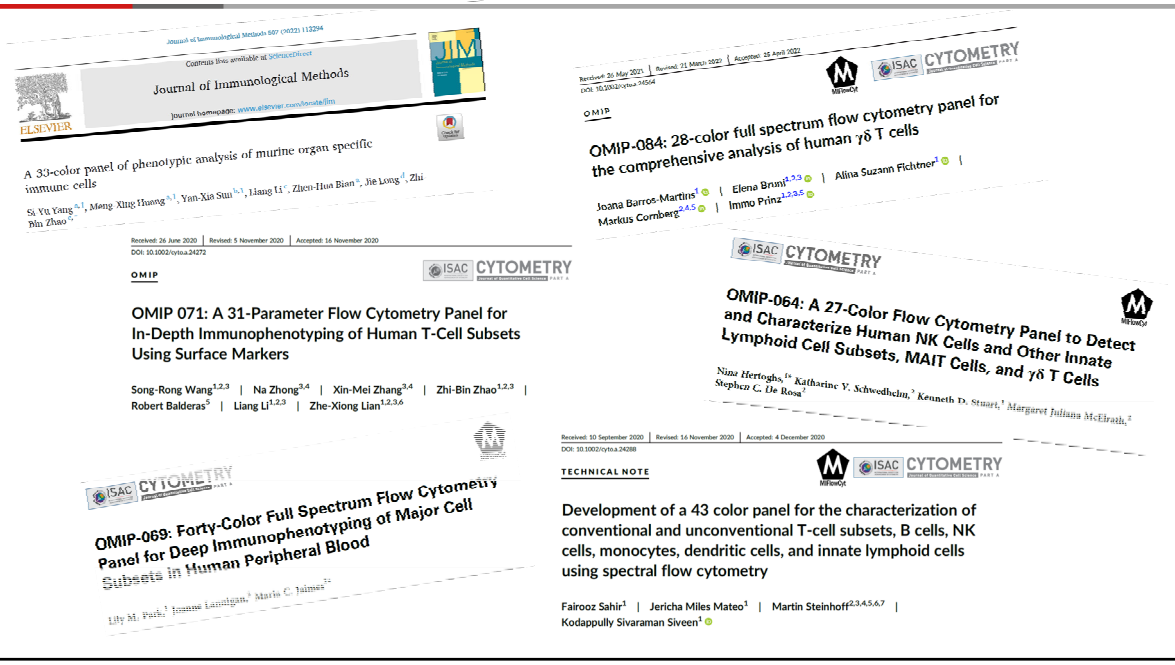
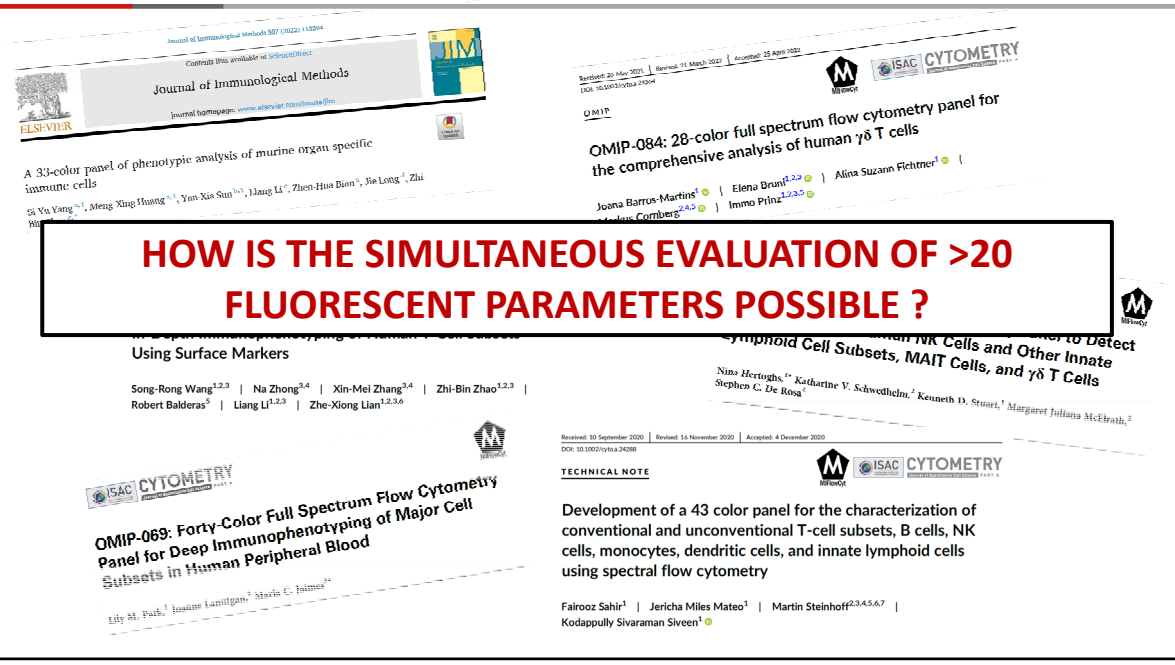
MULTICOLOR FLOW CYTOMETRY



3

MULTICOLOR FLOW CYTOMETRY



HOW IS THE SIMULTANEOUS EVALUATION OF >20 FLUORESCENT PARAMETERS POSSIBLE ?

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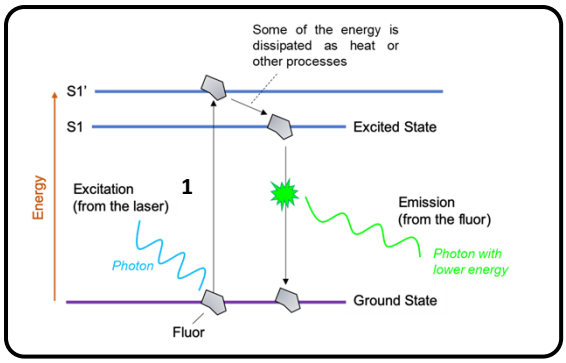
FLUORESCENCE & FLUOROCHROMES

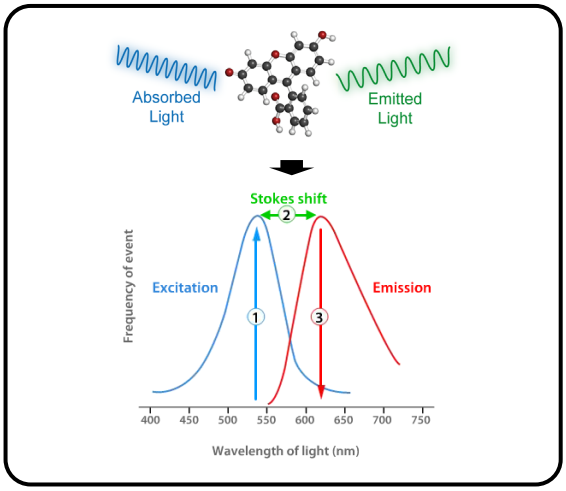
✓ *How fluorescence works?*

JABLONSKI DIAGRAM OF FLUORESCENCE



Modified from: https://marissafahlberg.com

STOKES SHIFT



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FLUORESCENCE, DYES & FLUOROCHROMES

LABELLING FLUOROCHROMES

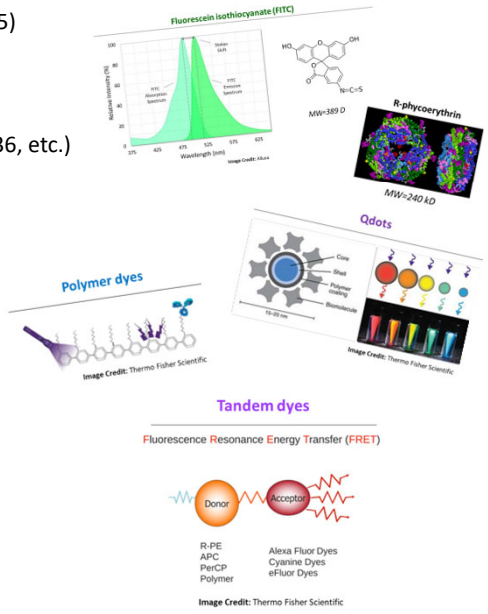
- **Small organic molecules** (e.g. FITC, Alexa Fluor, Pacific Blue, Cy5)
- **Large protein molecules** (e.g. PE, APC, PerCP)
- **Nanocrystals** (e.g. Quantum Dots – Qdots)
- **Organic polymers** (e.g. BV521, BUV395, BB515, Super Bright 436, etc.)
- **Tandem dyes** (e.g. PE-Cy7, PerCP Cy5.5, APC-H7, BV605, etc.)
- **Other/trademark** (e.g. NovaFluor™, Kiravia™, cFluor®, etc.)

FLUORESCENT PROTEINS

- Green Fluorescent Protein (GFP)
- Yellow Fluorescent Protein (YFP)
- Red Fluorescent Protein (dsRed)
- mCherry
- mBanana, etc.

FUNCTIONAL DYES

- **Nucleic acid-binding dyes** (e.g. DAPI, Propidium iodide, etc.)
- **Mitochondrial dyes** (e.g. Mitotracker, JC-1, etc.)
- **Calcium flux** (e.g. Indo-1, etc.)
- **Cell proliferation** (e.g. CFSE, Ki-67, etc.)
- **Etc…**



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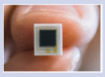
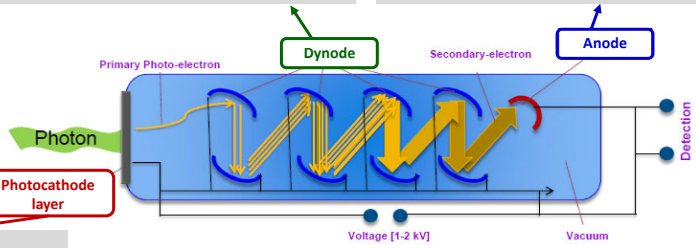
FLUORESCENCE DETECTION IN FLOW CYTOMETRY

TYPES OF DETECTORS	Photomultipliers (PMT)	Avalanche photodiodes (APD)	Silicon photomultipliers (SiPM)
			

- Electrons flow from dynode to dynode
- Each dynode generates secondary emission of more electrons
- Each dynode has a potential voltage more positive than the preceding dynode

- Voltage output or signal
- The higher the voltage applied to each PMT, the more electrons are generated → The greater the amplification



- Sensitive to light
- Generates photoelectrons when hit with photons

Modified from: https://www.thermofisher.com

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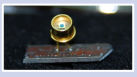
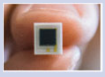
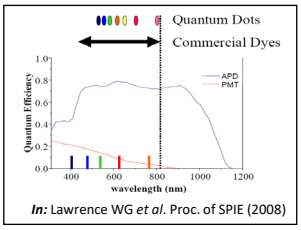
FLUORESCENCE DETECTION IN FLOW CYTOMETRY

TYPES OF DETECTORS	Photomultipliers (PMT)	Avalanche photodiodes (APD)	Silicon photomultipliers (SiPM)
			
Peak quantum efficiency (η) %	<40	<90	<40 (PDE)
Gain (μ)	10^5-10^6	<100 (can increase up to 10^5/10^6 with Geiger mode)	10^5-10^6
Spectral coverage (nm)	115-900	190-1200	320-900



In: Lawrence WG *et al.* Proc. of SPIE (2008)

Quantum efficiency, ratio of the number of photoelectrons released in a photoelectric process to the number of radiation quanta absorbed

Photon detection efficiency (PDE), expresses a fraction of the incoming photons contributing to the output signal.

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FLUORESCENCE DETECTION IN FLOW CYTOMETRY: VOLTAGE PULSE

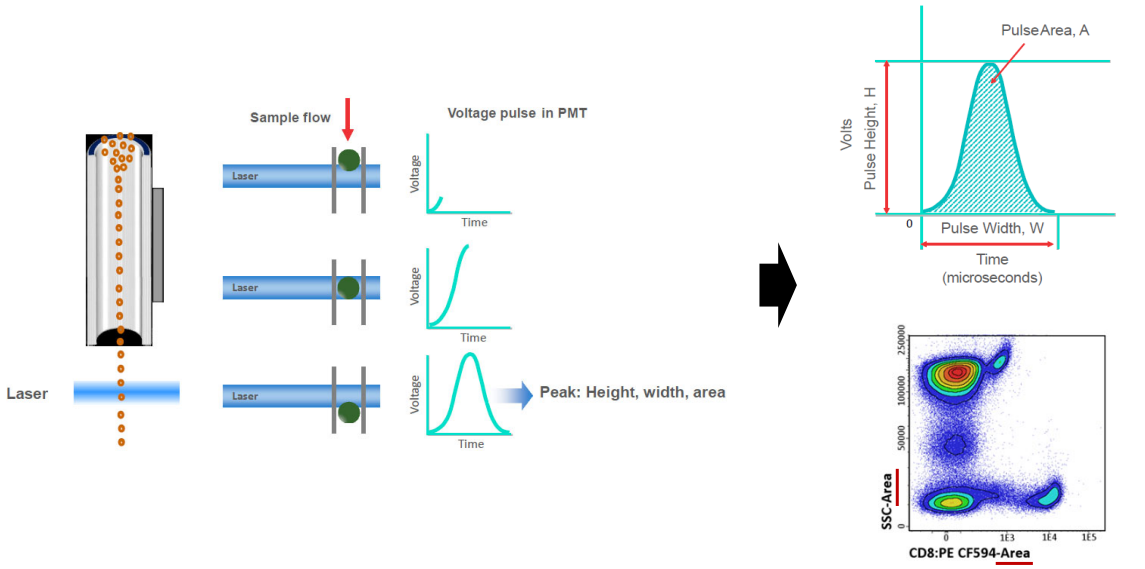

Image Credit: Thermo Fisher Scientific

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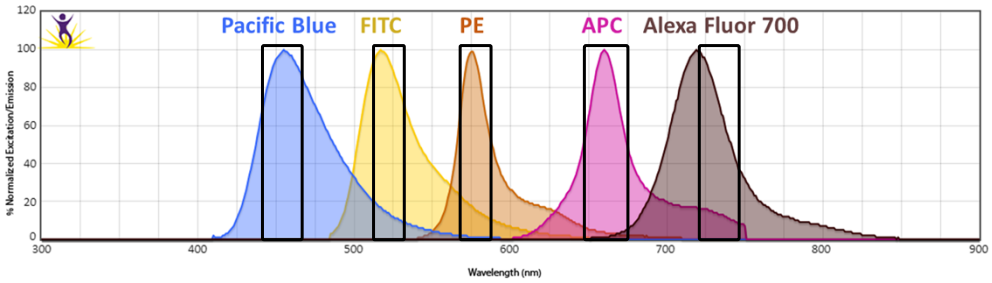
SPECTRAL OVERLAP



How to tell the fluorescence apart?

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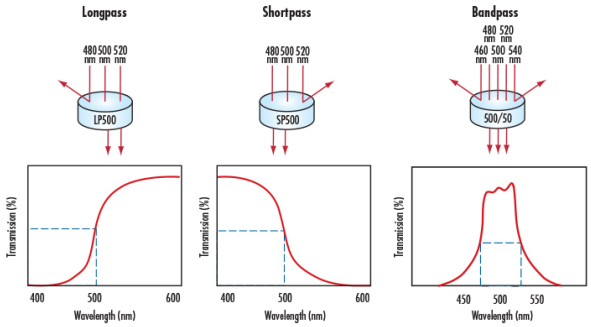
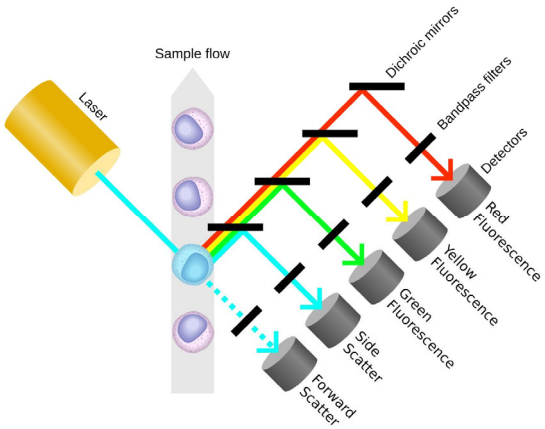
SPECTRAL OVERLAP: CONVENTIONAL FLOW CYTOMETRY

DICHROIC MIRRORS & FILTERS



In: https://www.edmundoptics.co.uk



In: Teague *et al*, bioRxiv (2022)

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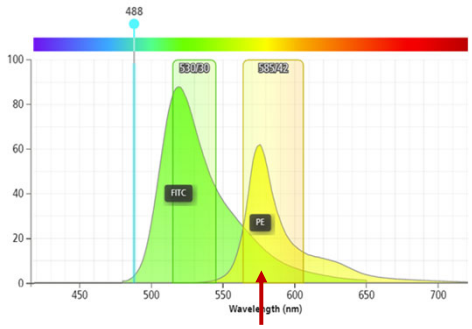
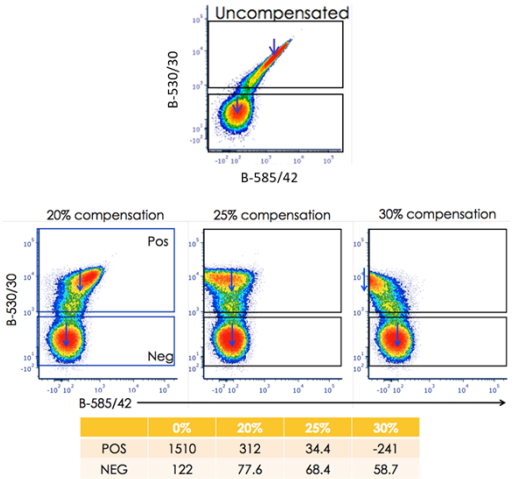
SPECTRAL OVERLAP: CONVENTIONAL FLOW CYTOMETRY



Spillover of FITC in PE detector

COMPENSATION



	0%	20%	25%	30%
POS	1510	312	34.4	-241
NEG	122	77.6	68.4	58.7

In: https://expert.cheekyscientist.com

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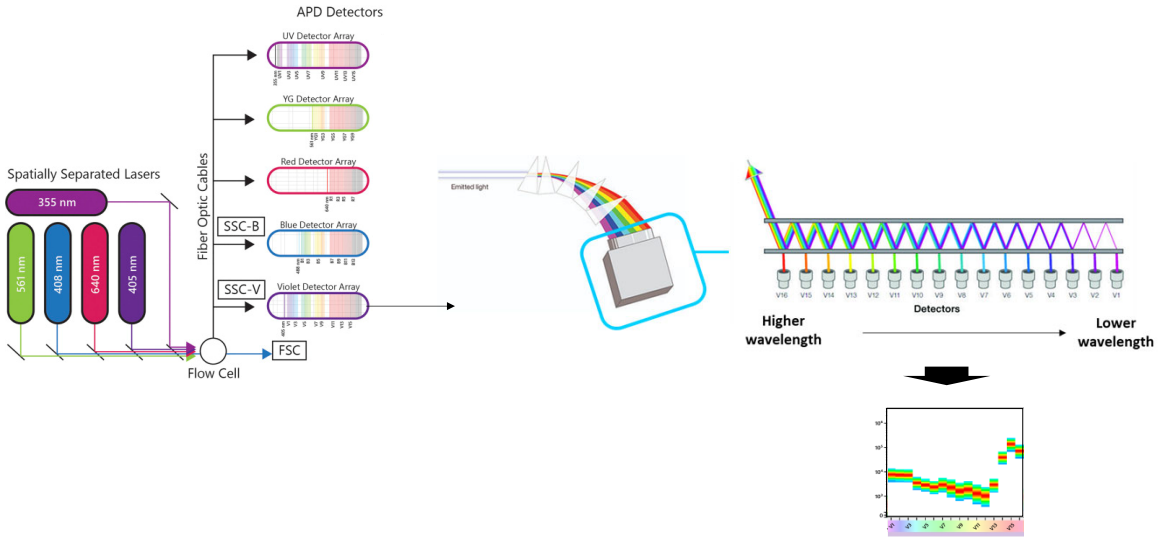
SPECTRAL OVERLAP: SPECTRAL FLOW CYTOMETRY



APD, Avalanche photodiodes;

Modified from: *Bonilla et al.* Front Mol Bio (2021) and https://www.thermofisher.com

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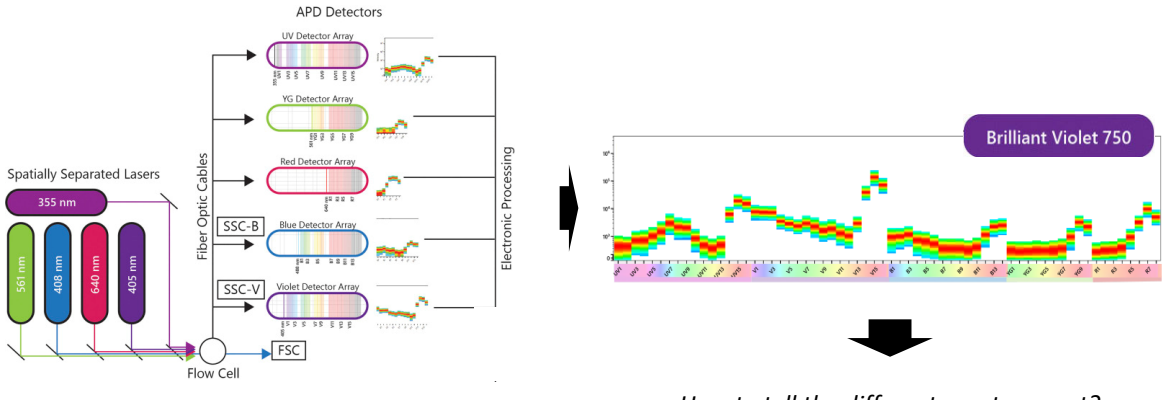
SPECTRAL OVERLAP: SPECTRAL FLOW CYTOMETRY



How to tell the different spectra apart?

Modified from: *Bonilla et al.* Front Mol Bio (2021) and https://www.thermofisher.com

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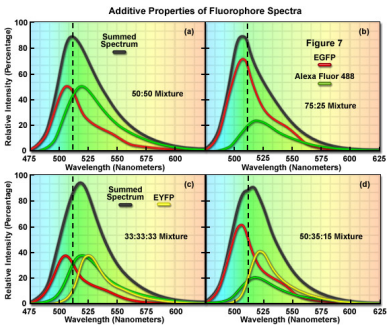
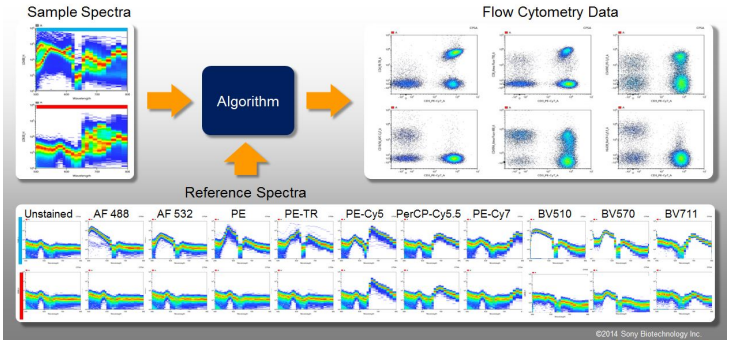
SPECTRAL OVERLAP: SPECTRAL FLOW CYTOMETRY

✓ **SPECTRAL UNMIXING**



In: https://zeiss-campus.magnet.fsu.edu



✓ Possibility for **new fluorochrome combinations**

✓ **Selection of fluorochromes is not limited by instrument compatibility** (i.e., available detectors and filters).

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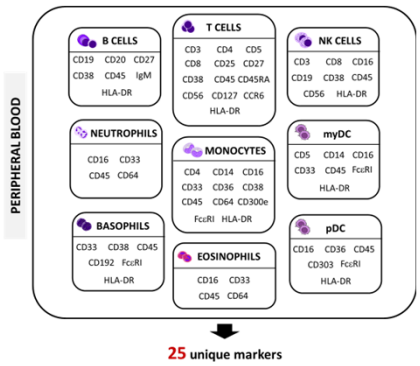
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

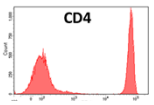
1. FLUOROCHROMES & BIOLOGY



25 unique markers

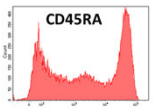
ANTIGEN CLASSIFICATION

Primary:
- Well characterized
- Easily classified as positive or negative
- Typically define broad subsets or lineages
- *Examples:* CD3, CD4, CD19



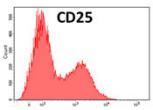
Dimmer fluorochromes can be used

Secondary:
- Well characterized
- Typically expressed at a higher density
- Often expressed over a continuum
- *Examples:* CD27, CD28, CD45RA, CD45RO



Selection depends on the cell population of interest

Tertiary:
- Expressed at low levels
- Variable upon activation
- Unknown
- Critical
- *Examples:* CD25, STAT5, FoxP3



Use bright fluorochromes

Adapted from: Mahnke YD *et al.* Clin Lab Med (2007)

myDC, myeloid dendritic cell; **pDC**, plasmacytoid dendritic cell

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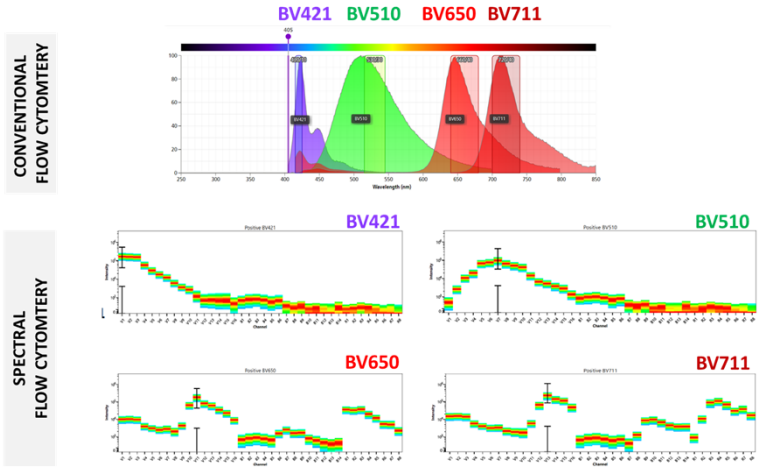
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

2. FLUOROCHROME SELECTION

✓ **Uniqueness**



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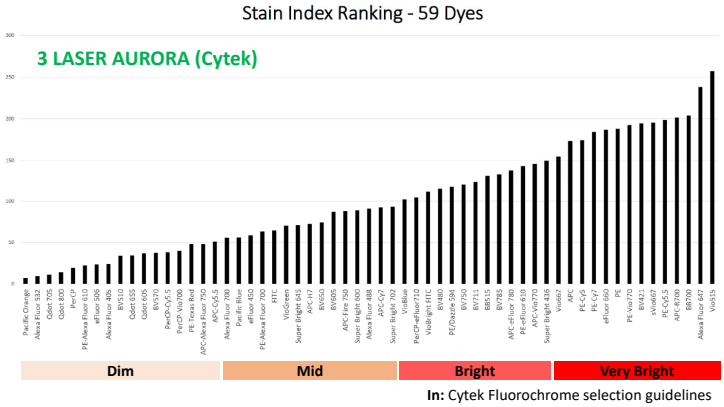
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

2. FLUOROCHROME SELECTION

✓ Uniqueness

✓ **Fluorochrome brightness**



In: Cytek Fluorochrome selection guidelines

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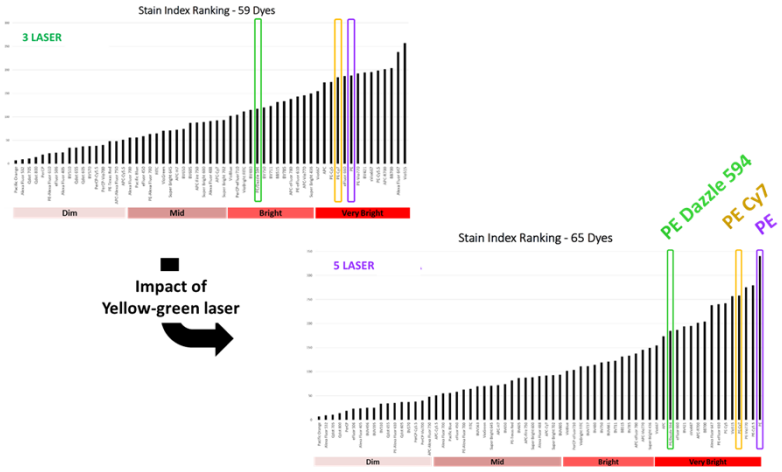
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

2. FLUOROCHROME SELECTION

- ✓ Uniqueness
- ✓ **Fluorochrome brightness**



In: Cytek Fluorochrome selection guidelines

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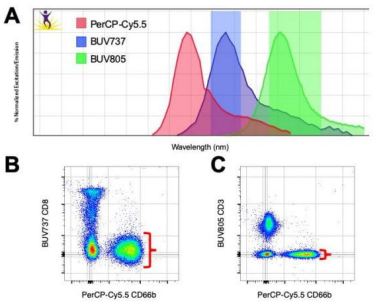
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

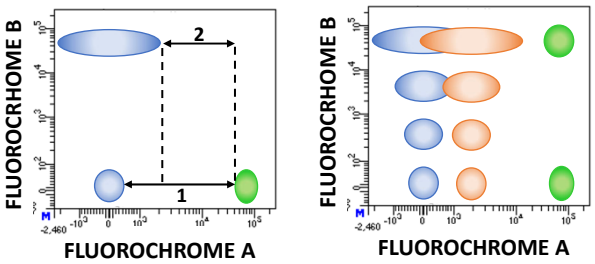
2. FLUOROCHROME SELECTION

- ✓ Uniqueness
- ✓ Fluorochrome brightness
- ✓ **Spread**



In: https://voices.uchicago.edu

✓ ***Spread (spillover-spreading error) impact in resolution***



Resolution of a marker conjugated with Fluor A DECREASES in presence of Fluor B

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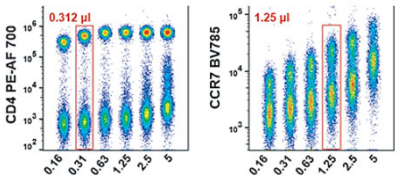
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

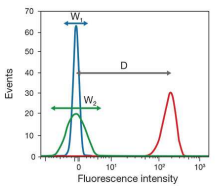
✓ **Optimization of the reagents**

✓ ***Reagent titration***

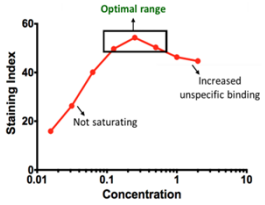


In: Jachimowicz *et al*, Agilent technical note

STAINING INDEX



$$\frac{median_{pos} - median_{neg}}{2 * rSD_{neg}}$$



In: https://www.thermofisher.com

Modified from: https://expert.cheekyscientist.com

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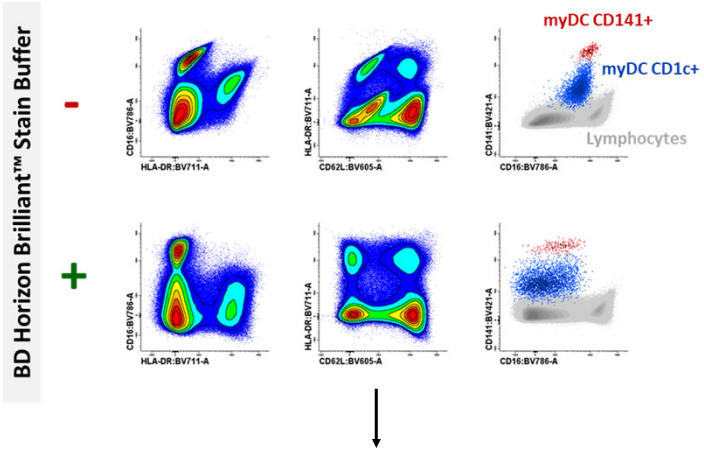
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

✓ **Optimization of the reagents**

✓ ***Non-specific polymer interactions***



• Usage of **polymer stain buffer** in stainings with >2 polymer dyes

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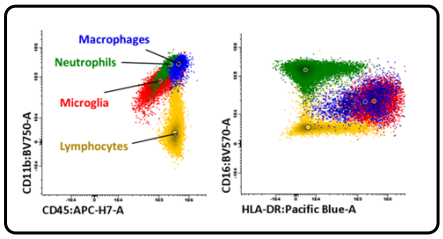
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

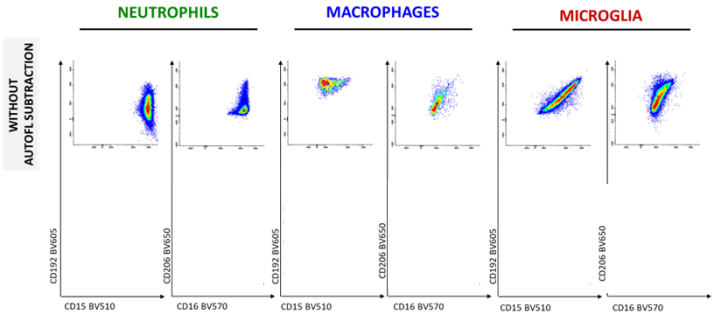
- ✓ Optimization of the reagents
- ✓ **Autofluorescence** (350-550nm)

Glioblastoma immune infiltrates



Responsible scientists: *Effie Stavrakaki, Cristina Teodosio*

✓ ***Populations with different autofluorescence spectrum***



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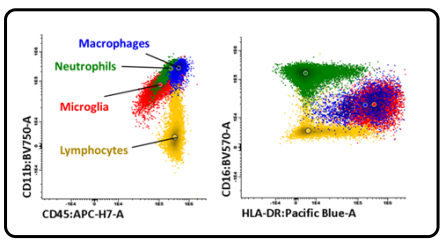
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

- ✓ Optimization of the reagents
- ✓ **Autofluorescence** (350-550nm)

Glioblastoma immune infiltrates



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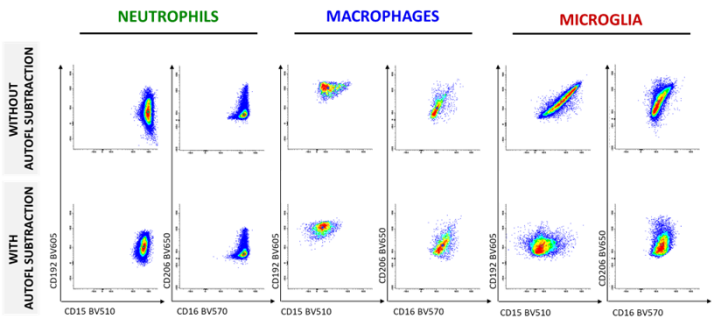

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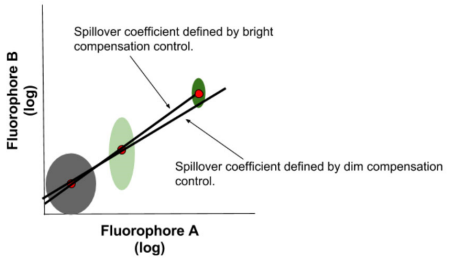
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

- ✓ Optimization of the reagents
- ✓ Autofluorescence
- ✓ **Compensation/reference controls**

✓ ***Criteria for good compensation/reference controls***

1. Compensation control must be **as bright as, or brighter, than the experimental stain.**



In: https://expert.cheekyscientist.com

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FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

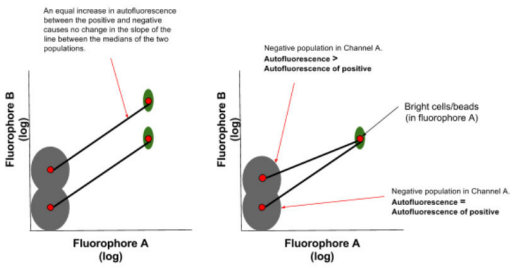
3. UNSPECIFIC FLUORESCENCE

- ✓ Optimization of the reagents
- ✓ Autofluorescence
- ✓ **Compensation/reference controls**

✓ ***Criteria for good compensation/reference controls***

1. Compensation control must be **as bright as, or brighter, than the experimental stain.**

2. **Autofluorescence should be the same** for the **positive and negative** populations used for the compensation calculation in each channel.



In: https://expert.cheekyscientist.com

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FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

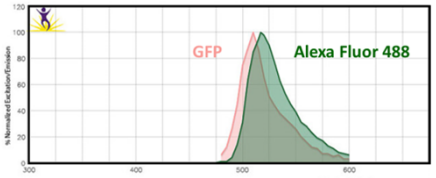
- ✓ Optimization of the reagents
- ✓ Autofluorescence
- ✓ **Compensation/reference controls**

✓ ***Criteria for good compensation/reference controls***

1. Compensation control must be **as bright as, or brighter, than the experimental stain.**

2. **Autofluorescence should be the same** for the **positive and negative** populations used for the compensation calculation in each channel.

3. The **fluorophore used** must be the **exact fluorophore** used in the **experimental sample** (same flurochrome, same lot if tandem, same protocol)



In: https://expert.cheekyscientist.com

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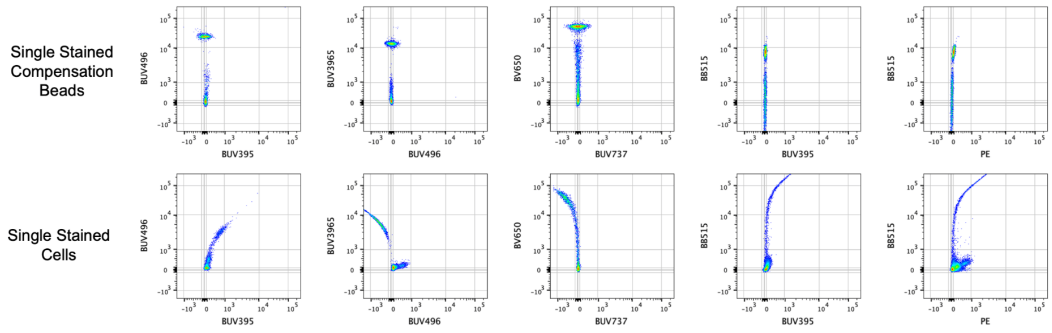
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

- ✓ Optimization of the reagents
- ✓ Autofluorescence
- ✓ **Compensation/reference controls**

✓ ***Beads vs. cells***



In: https://voices.uchicago.edu

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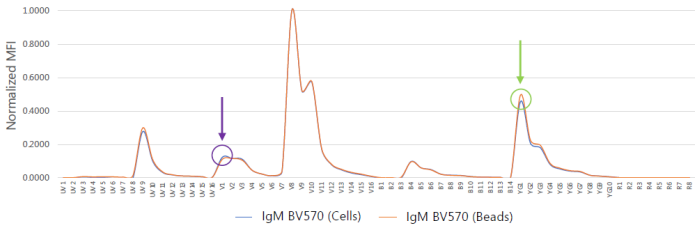
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

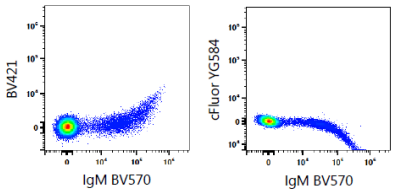
- ✓ Optimization of the reagents
- ✓ Autofluorescence
- ✓ **Compensation/reference controls**

✓ ***Beads vs. cells***



	Normalized MFI	
	V1	YG1
IgM BV570 (Cells)	0.1215	0.2051
IgM BV570 (Beads)	0.1092	0.2229

Single stained cells



In: Park *et al*, Cytometry Part A (2020)

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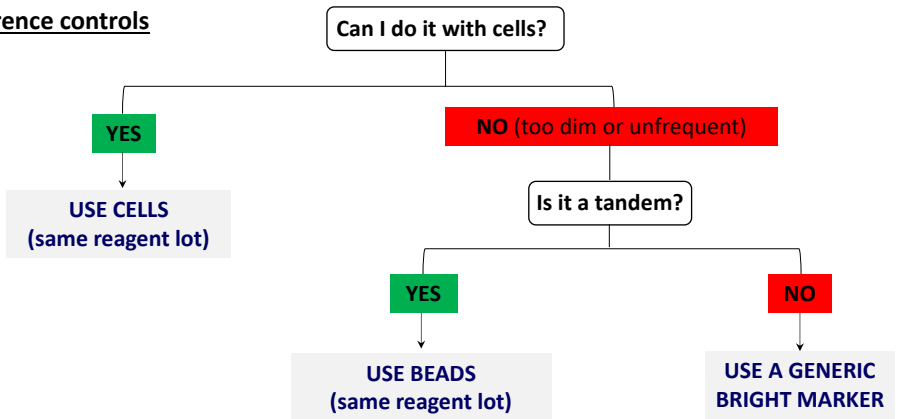
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

3. UNSPECIFIC FLUORESCENCE

- ✓ Optimization of the reagents
- ✓ Autofluorescence
- ✓ **Compensation/reference controls**



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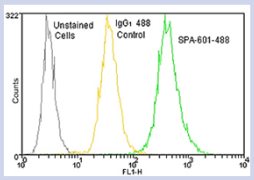
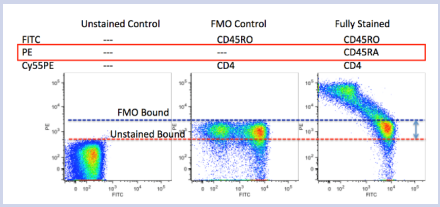
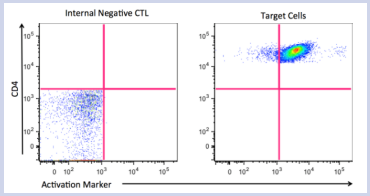
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

4. FLUORESCENCE & DATA ANALYSIS

✓ **Gating controls**

ISOTYPE CONTROL	FLUORESCENCE-MINUS-ONE (FMO)	INTERNAL NEGATIVE CONTROL (INC)
		
Background binding of a specific antibody isotype	Effects of spectral overlap in the channel of interest	Non-specific binding of the antibody

Images: https://expert.cheekyscientist.com & https://www.enzolifesciences.com

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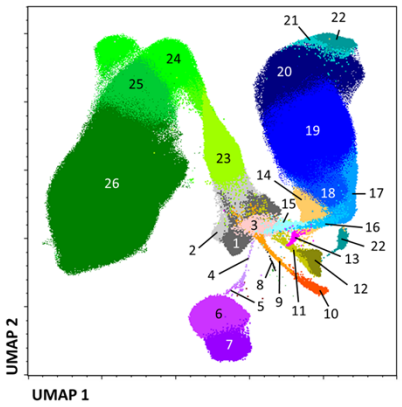
FLUORESCENCE: PANEL DESIGN & DATA ANALYSIS

28 COLOR PANEL FOR CHARACTERIZATION OF HUMAN BONE MARROW
[Bone marrow mononuclear cells]



HPC
1 Hematopoietic stem cells (HSC)
2 Other hematopoietic precursor cells (HPC)

Neutrophil lineage
3 Myeloblasts
4 Promyelocytes
5 Myelocytes
6 Metamyelocytes
7 Band + segmented neutrophils

8 Mast cells

Basophils
9 Basophil precursor cells
10 Mature basophils

Dendritic cells
11 pDC precursor cells
12 Mature pDC
13 CD141⁺ myDC
14 CD1c⁺ myDC

Monocytic lineage
15 Monoblasts CD34⁺/CD117⁺
16 Monoblasts CD34⁻/CD117⁺
17 Promonocytes CD14⁻
18 Promonocytes CD14^{lo}
19 Promonocytes CD14^{hi}
20 cMo
21 iMo
22 ncMo

Etythroid lineage
23 Pro-erythroblast CD34⁺ CD117⁺ HLA-DR⁺ CD105^{lo}
24 Pro-erythroblast CD34⁻ CD117⁺ HLA-DR^{lo} CD105⁺
25 Erythroblast CD34⁻ CD117⁻ HLA-DR⁻ CD105⁺
26 Erythroblast CD34⁻ CD117⁻ HLA-DR⁻ CD105⁻

Responsible scientists: *Cristina Teodosio, Kirsten Canté, Frank Staal*

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CONCLUSION

EXPONENTIAL GROWTH OF THE FLOW CYTOMETRY FIELD

- ✓ **Improvement of the way flow cytometers detect fluorescence**
- ✓ **Development of new fluorochromes**
- ✓ **Careful panel design and analysis strategies**
- ✓ **GOOD QUALITY DATA**

Shapiro's 7th Law:

No Data Analysis Technique Can Make Good Data out of Bad Data

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