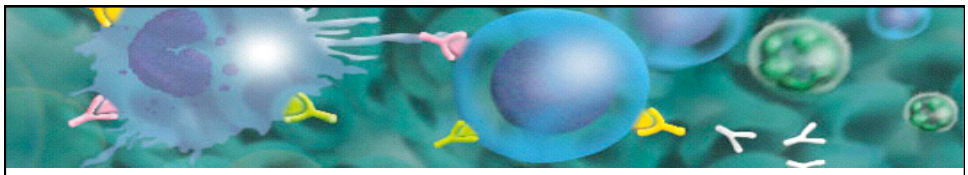
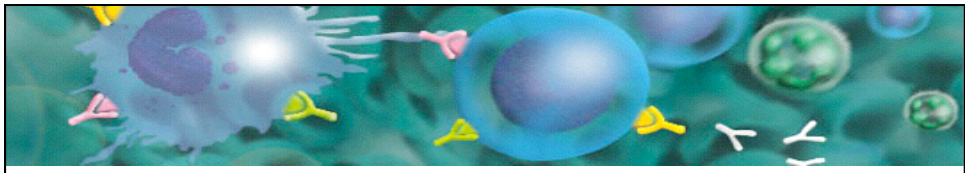


Flow Cytometry

MRC | Laboratory of Molecular Biology

Maria Daly
Flow Cytometry Facility Manager
MRC-LMB



Talk Overview

- Our Facility
- What is Flow Cytometry?
- Components of a Flow Cytometer
- Applications in Biology

Our Facility: the People

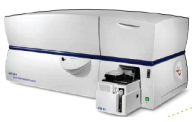
Maria Daly Fan Zhang Martyn Balmont

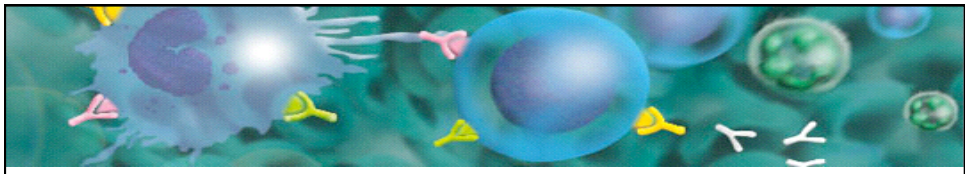
Our Facility at the LMB

MoFlo High Speed Sorter:
- 4 lasers, 8 colour detection

Synergy dual channel High Speed Sorter :
1) 5 lasers, 15 colour detection
2) 3 lasers, 10 colour detection

LSRII Analyser:
4 lasers, 14 colour detection





2 x Fortessa Analysers:

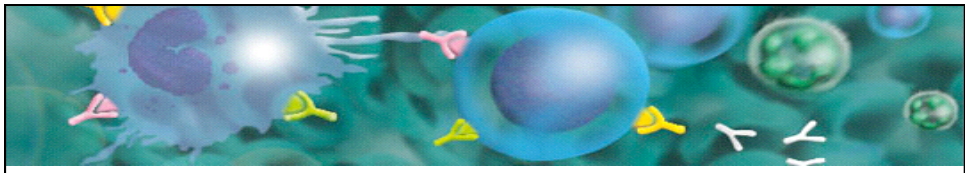
1. 4 lasers, 16 colour detection
2. 5 lasers, 18 colour detection

1 x Sony SP6800 Spectral Analyser:

This is a 3 laser, 34 PMT detection system which captures the spectral fingerprint of each fluorochrome

- 405nm, 50mW laser, with 2 PMT detectors (420-440, 460-480nm)
- 488nm, 50mW laser
- 640nm, 40mW laser

Emission spectra from above 3 excitation lasers is detected across a band of 32 fluorescence detectors detecting emission wavelengths from 500 – 800nm

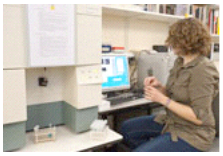


Eclipse Analyser:

- 3 lasers, 5 colour detection

FACSCalibur Analysers:

- 2 lasers, 4 colour detection



Publications supported by the Facility from October 2016 – September 2017

Gammons M. V., Rutherford T. J., Steinhart Z., Angers S., Bienz M.
Essential role of the Dishevelled DEP domain in a Wnt-dependent human-cell-based complementation assay
Journal of Cell Science 129(20):3892-3902. 15 October 2016
Division: Protein & Nucleic Acid Chemistry

Bottermann M., Lode H. E., Watkinson R. E., Foss S., Sandlie I., Andersen J. T., James L. C.
Antibody-antigen kinetics constrain intracellular humoral immunity
Scientific Reports 6:37457. 24 November 2016
Division: Protein & Nucleic Acid Chemistry

Wu Y. L., Stubbington M. J., Daly M., Teichmann S. A., Rada C.
Intrinsic transcriptional heterogeneity in B cells controls early class switching to IgE
Journal of Experimental Medicine 214(1):183-196. 1 January 2017
Division: Protein & Nucleic Acid Chemistry

McEwan W. A., Falcon B., Vaysburd M., Clift D., Oblak A. L., Ghetti B., Goedert M., James L. C.
Cytosolic Fc receptor TRIM21 inhibits seeded tau aggregation
Proceedings of the National Academy of Sciences of the United States of America 114(3):574-579. 17 January 2017
Division: Protein & Nucleic Acid Chemistry, Neurobiology

Saluzzo S., Gorki A. D., Rana B. M., Martins R., Scanlon S., Starkl P., Lakovits K., Hladik A., Korosec A., Sharif O., Warszawska J. M., Jolin H., Mesteri I., McKenzie A. N., Knapp S.
First-Breath-Induced Type 2 Pathways Shape the Lung Immune Environment
Cell Reports 18(8):1893-1905. 21 February 2017
Division: Protein & Nucleic Acid Chemistry

van Tienen L. M., Mieszczanek J., Fiedler M., Rutherford T. J., Bienz M.
Constitutive scaffolding of multiple Wnt enhanceosome components by Legless/BCL9
ELife 6: e20882. 15 March 2017
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Houlihan G., Arangundy-Franklin S., Holliger P.
Exploring the Chemistry of Genetic Information Storage and Propagation through Polymerase Engineering
Accounts of Chemical Research 50(4):1079-1087. 18 April 2017
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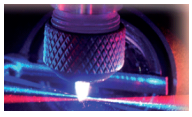
Šviković S., Sale J. E.
The Effects of Replication Stress on S Phase Histone Management and Epigenetic Memory
Journal of Molecular Biology 429(13):2011-2029. 20 June 2017
Division: Protein & Nucleic Acid Chemistry

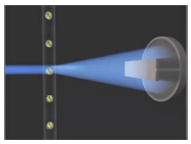
Burgos-Barragan G., Wit N., Meiser J., Dingler F. A., Pietzke M., Mulderrig L., Pontel L. B., Rosado I. V., Brewer T. F., Cordell R. L., Monks P. S., Chang C. J., Vazquez A., Patel K. J.
Mammals divert endogenous genotoxic formaldehyde into one-carbon metabolism
Nature 548(7669):549-554. 31 August 2017
Division: Protein & Nucleic Acid Chemistry

Flow Cytometry



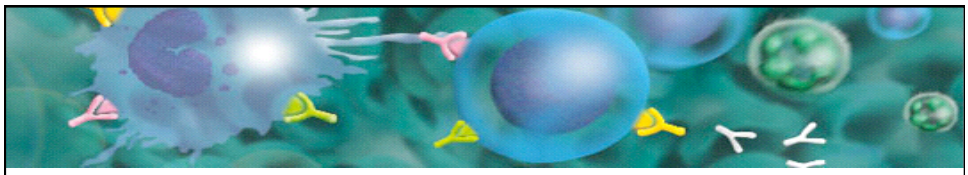
The value of the technique:

- measurements of large numbers of single cells in suspension within a short period of time



The major disadvantage:

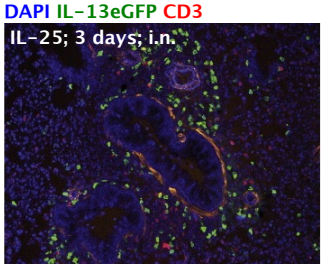
- It requires a suspension of single cells or other particles with minimum clumps and debris
- The tissue architecture and any information about the spatial relationship between different cells are lost when single cells are prepared



Cytometry

- Localization of antigen is possible
- Poor enumeration of cell subtypes
- Tissue architecture

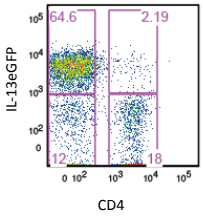
DAPI IL-13eGFP CD3



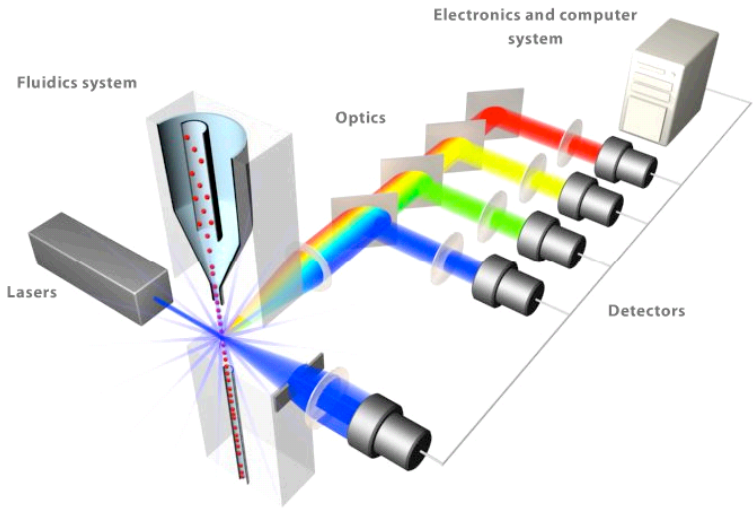
Jillian Barlow and Veera Panova, MRC-LMB

Flow Cytometry

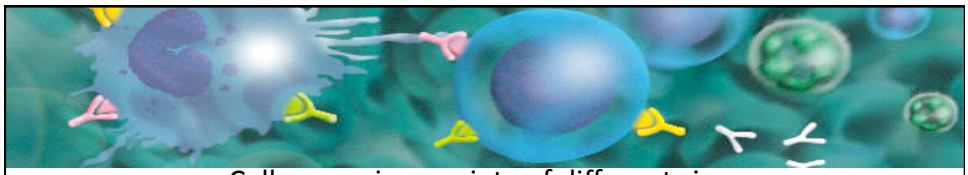
- Cannot tell you where antigen is
- Can analyze many cells in a short time frame
- Can look at numerous parameters at once



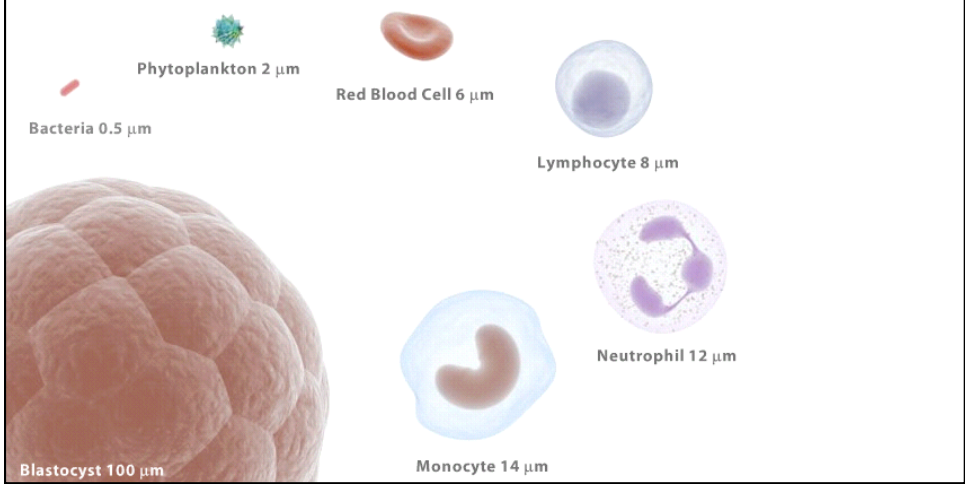
Components of a Flow Cytometer



http://www.thermofisher.com/uk/en/home/support/tutorials.html



Cells come in a variety of different sizes
Make sure they will fit the flowcell or nozzle you use



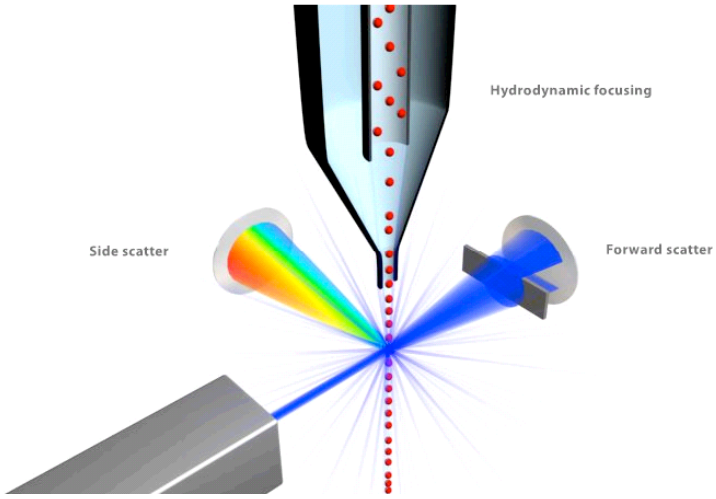
Sample Preparation

- Sample preparation is key to getting good data
- A single cell suspension is necessary
- Dissociate cells with appropriate reagents
- Titrate your antibodies to find optimal conc.
- Filter cell samples which are aggregated through a nylon mesh to remove clumps
- 70µm, 100µm, or 150µm as appropriate
- Rubbish in = Rubbish out

Obtaining a single cell suspension

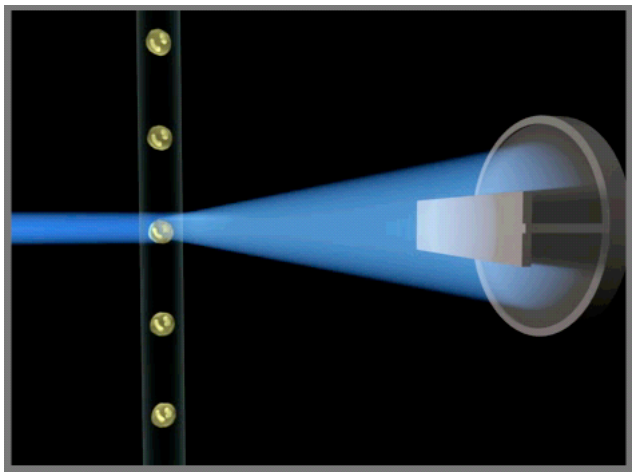
- Some cells come naturally in suspension
  - splenocytes
  - cell lines e.g. Jurkat,
- Some cell lines grow adherent on plastic
  - remove from plastic with e.g. EDTA, Trypsin, Accutase
- Tissues are more difficult and need mechanical or enzymatic dissociation
  - Collagenase
  - Press through a fine mesh
- The sample should contain as little debris and as few clumps as possible

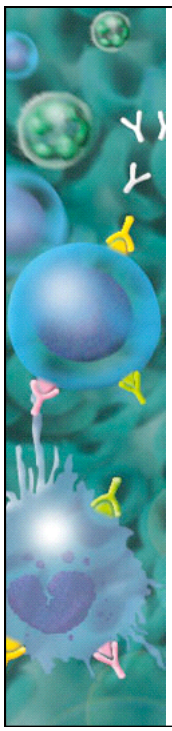
Hydrodynamic focusing



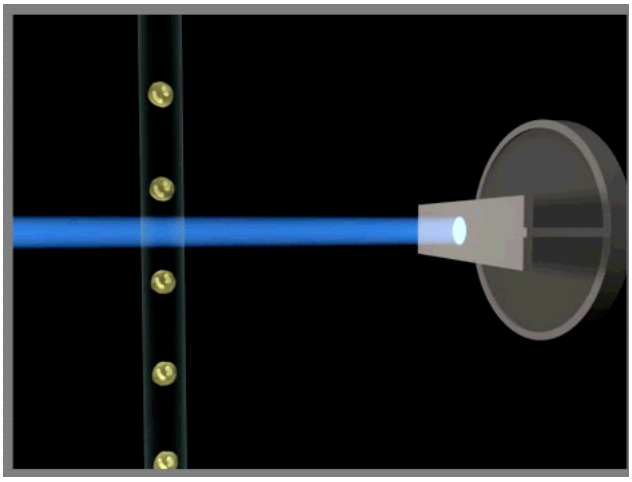
Single Particles in a stream

Forward Scatter

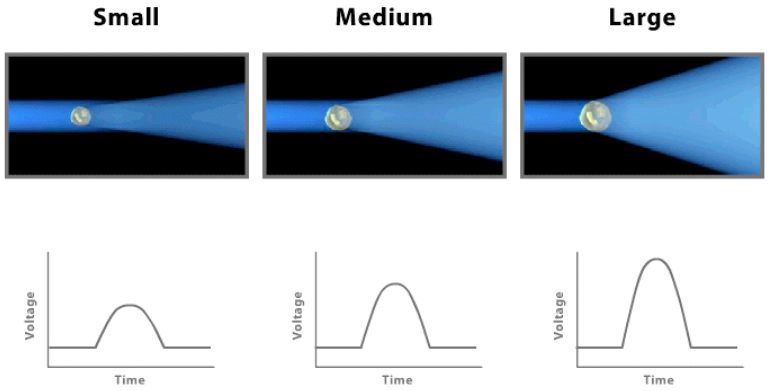




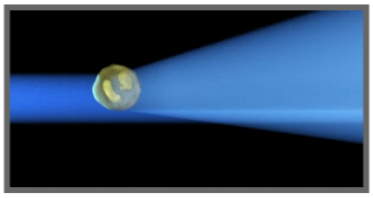
Forward Scatter

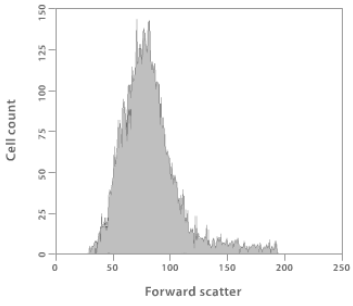


Forward Scatter Pulses

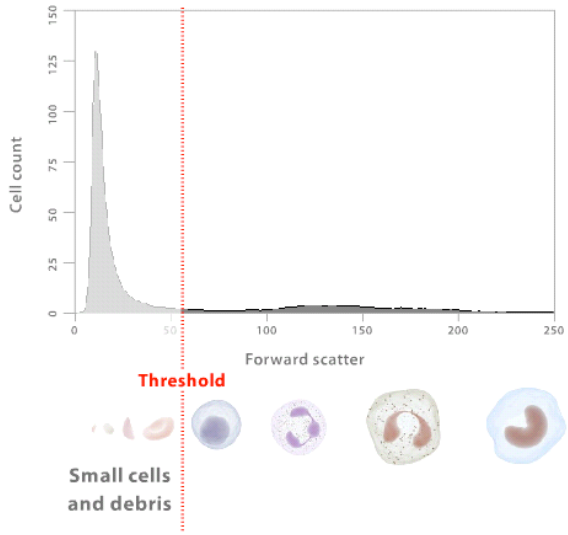


Forward Scatter Histogram

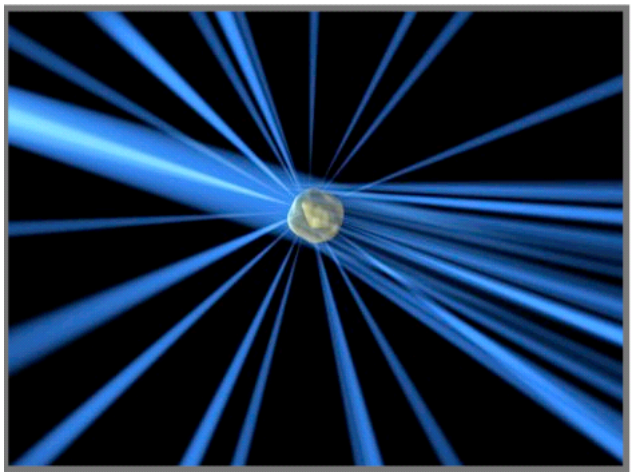




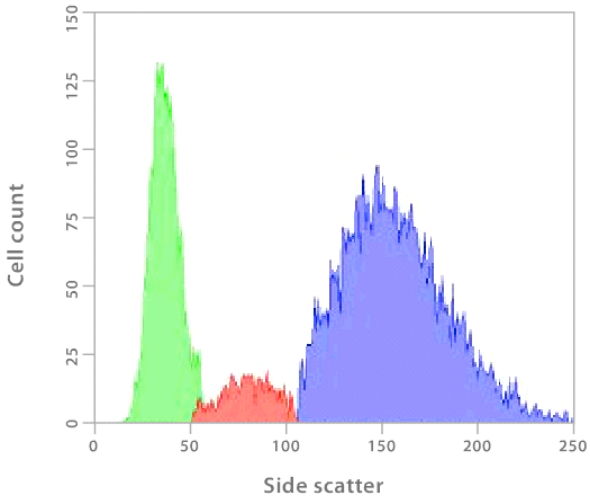
Threshold



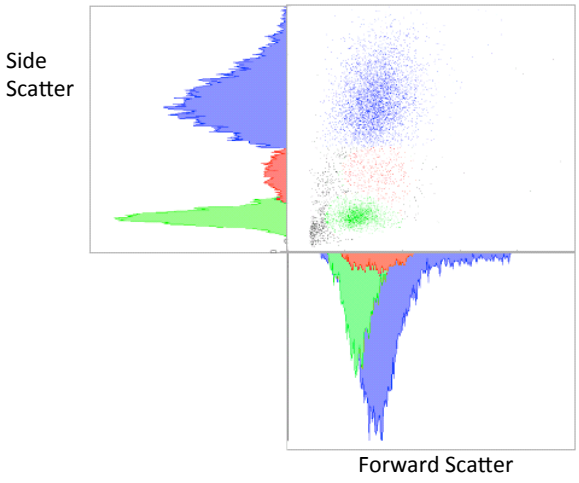
Side Scatter



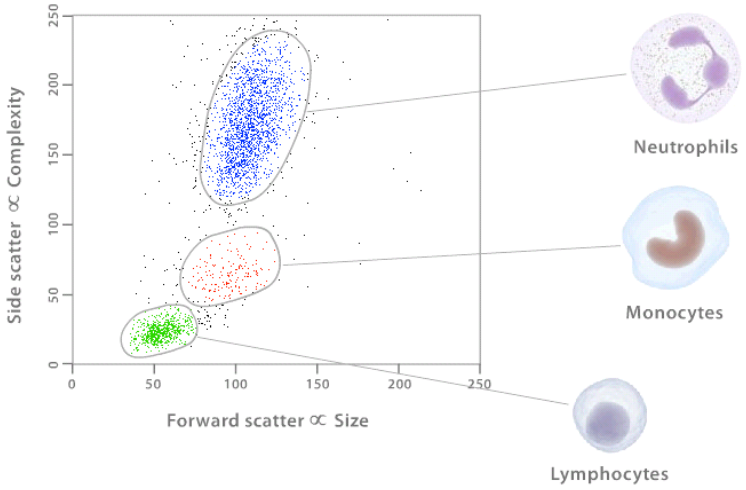
Side Scatter



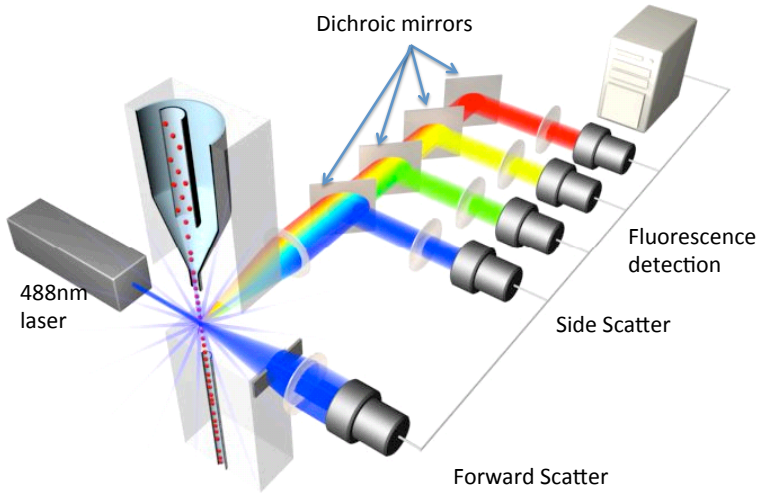
2 Parameter Dot Plot



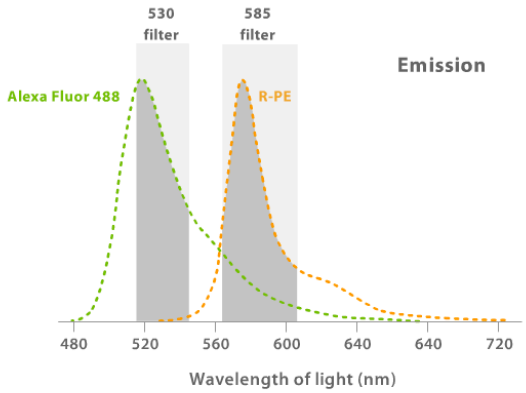
Whole Blood Dot Plot



Optical Layout

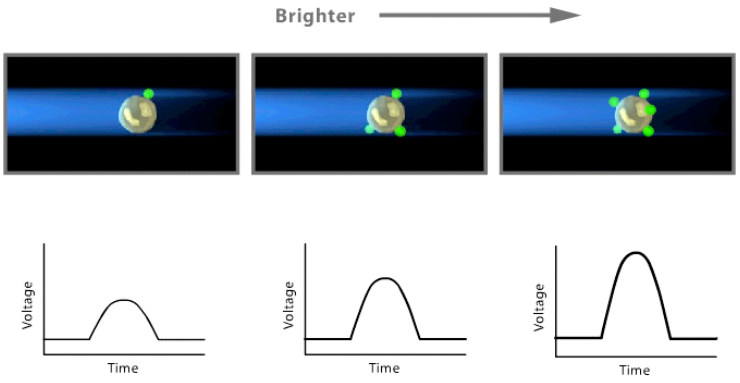


Filters in front of detectors to separate Fluorescence λ

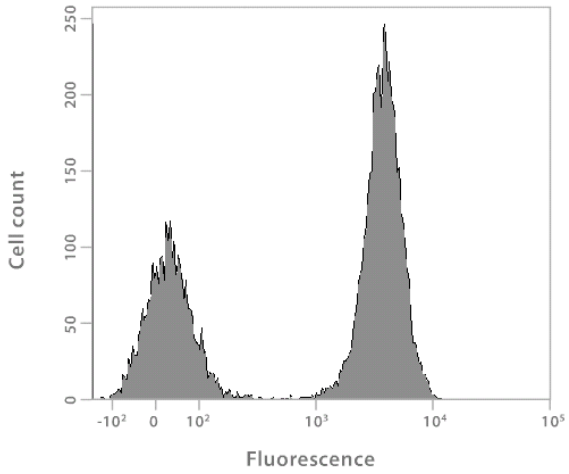


So, run single colour controls to assess the spectral overlap

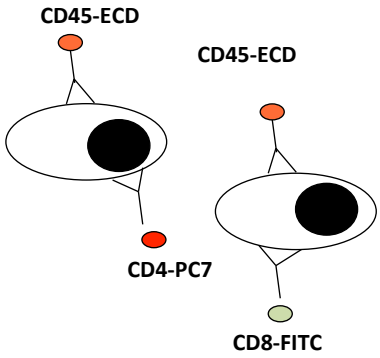
Fluorescence

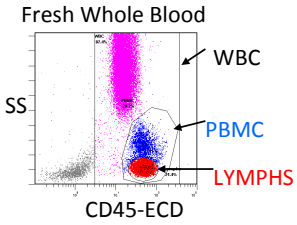


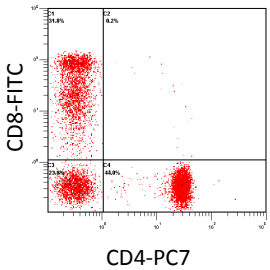
Fluorescence Histogram



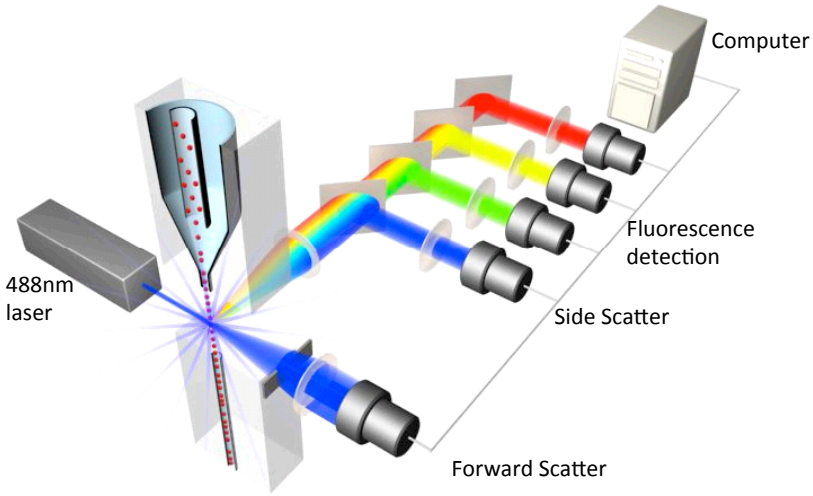
Fluorescence Dot Plot





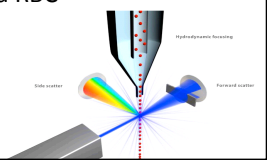


Optical Layout



Other considerations

- Avoid co-incidence
  - Dilute the sample to a concentration the instrument can handle
- Get rid of unwanted cells
  - Lyse RBC
    - Ammonium Chloride
    - RBC lysing reagents e.g acid lysis
    - Carbonic anhydrase
  - Ficoll or Lympholyte M
    - Separates mononuclear cells from Granulocytes and RBC

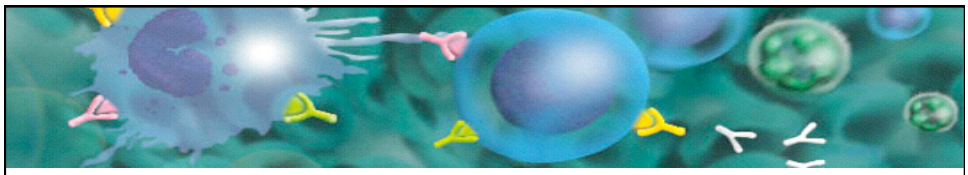


Always include controls

- The instrument settings for collecting cell measurements are set by you
- Use controls to set your background values
- Use single colour - samples to set your colour compensation and position of positive cells
- Choose dyes used in your experiments wisely
  - ✓ brightest dye on least expressed antigen
  - ✓ choose dyes with least spectral overlap or which use different lasers for excitation

Labelling Cells with Fluorescent Dye/Marker

- Otpimise the staining conditions for YOUR cells in your model system
  - Immunophenotyping
  - DNA analysis
  - Intracellular Cytokine Staining
  - CFSE proliferation assay



Immunophenotyping

Protocol:
- 100µl sample
- 10µl antibody
- 10 mins RT
-Wash with PBS + 2.5% FCS
- Centrifuge to pellet, remove supernatant
- Re-suspend in PBS + 2.5% FCS
- Analyse

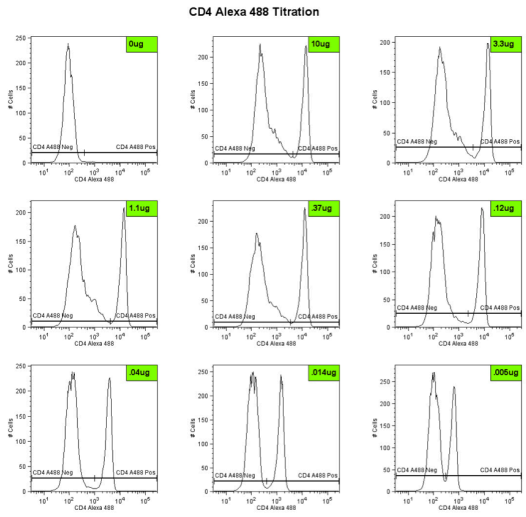
Immunophenotyping

- Read the product insert sheet
  - Find the concentration of the reagent e.g. mg antibody/ml
  - Read off range of antibody concentration manufacturer recommends for use
- Titrate your antibodies to find the optimal concentration for use
  - Keep final volume constant e.g. 100µl
  - Antibody concentration is vital, cell numbers may vary
  - Add range of antibody concentration at constant volume
  - Find saturating concentration for use
- Be consistent
  - Use same staining conditions each time you do the experiment

Antibody Titration

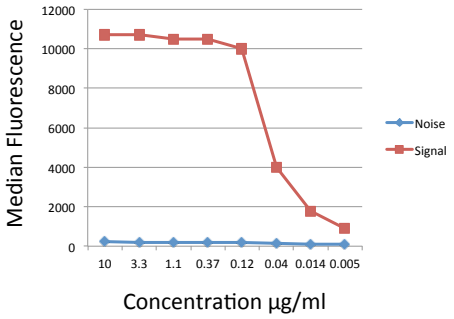
- For most purposes, the main objective is to maximize signal : noise (pos/neg separation)
  - This may occur at less than saturated staining
  - This may or may not be the manufacturer's recommended titer
- Titer is affected by:
  - Staining volume (e.g., 100 μL)
  - Number of cells (not critical up to ~$5x10^6$)
  - Staining time and temperature (e.g., 10 min RT)
  - Type of sample (whole blood, PBMC, splenocytes.)

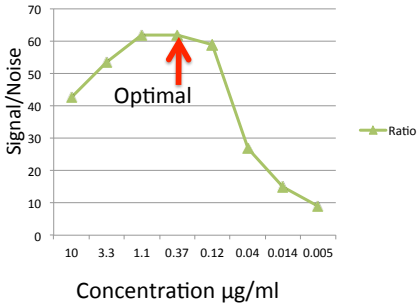
Antibody Titration



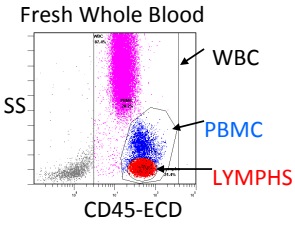
The University of Chicago Flow Cytometry Facility

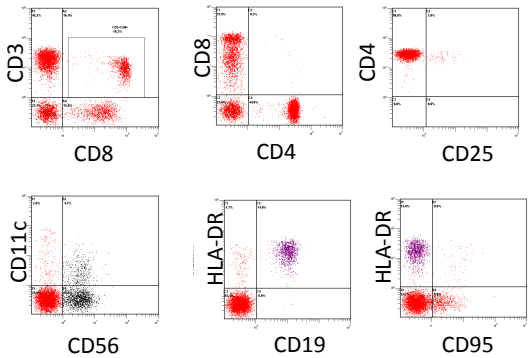
Antibody Titration





Immunophenotyping



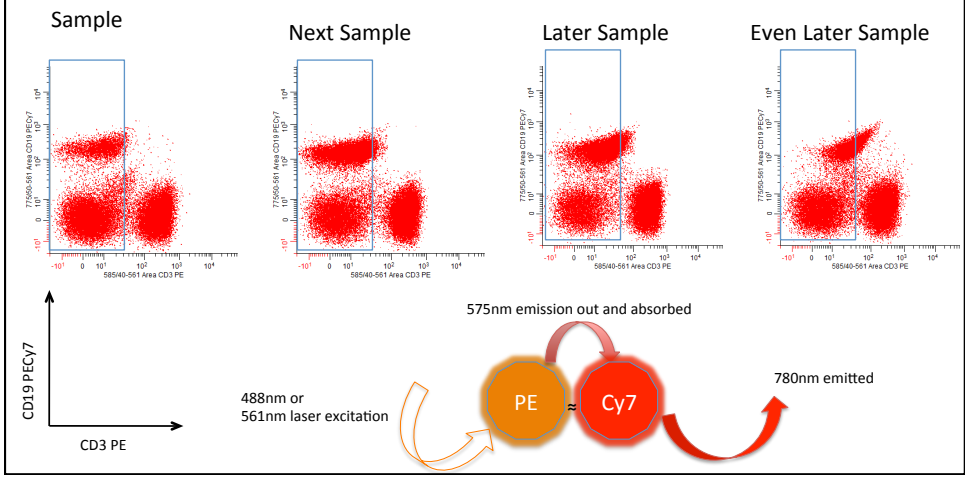


Protocol:
- 100µl sample
- 10µl antibody
-10 mins RT
- RBC lyse
- Wash
- Analyse

Titrate antibodies to find optimal concentration for use

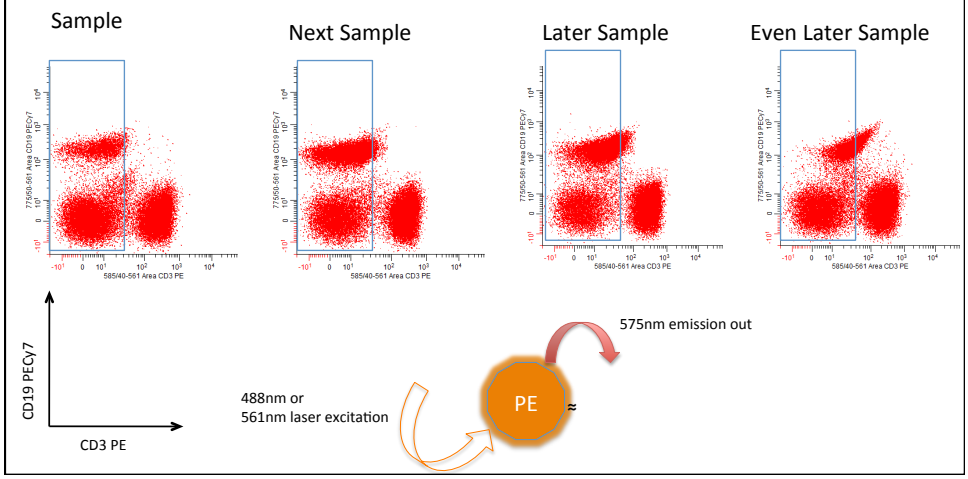
Be Careful of Reagent Degradation!

Light will photobleach your tandem – protect your antibody-labelled samples from light at all times



Be Careful of Reagent Degradation!

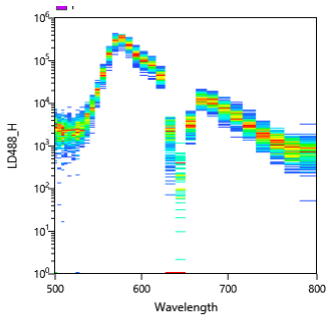
Light will photobleach your tandem – protect your antibody-labelled samples from light at all times



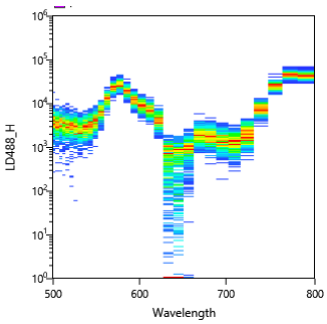
Spectral Analyser

We can analyse the spectrum of Fluorochromes and reveal the degradation

PE spectrum



PECy7 (degraded) spectrum



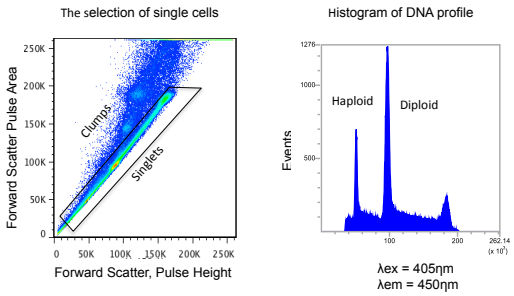
Emission wavelength in ηm

Cell Cycle Analysis

A number of dyes can be used e.g.

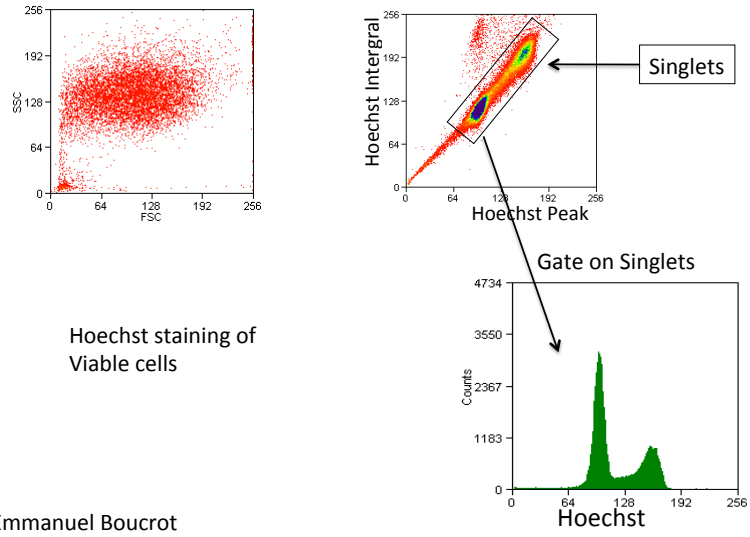
- Propidium Iodide
- Hoechst
- DAPI
- DRAQ5

Cell Cycle Analysis



Data from Ben Taylor (PNAC), MRC-LMB

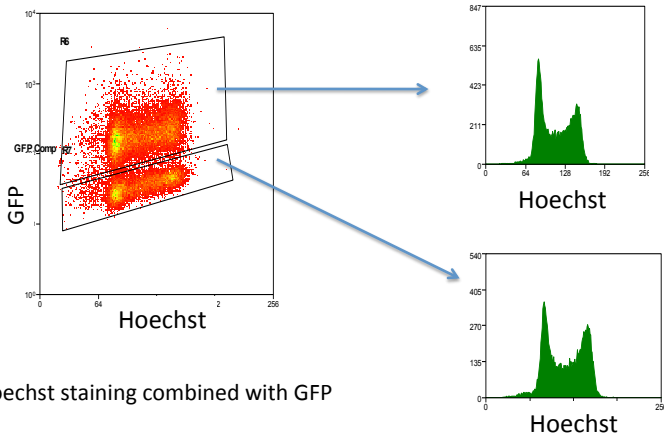
Cell Cycle Analysis



Hoechst staining of
Viable cells

Emmanuel Boucrot

Cell Cycle with GFP



Hoechst staining combined with GFP

No fixation required thus leaving GFP expression unaffected

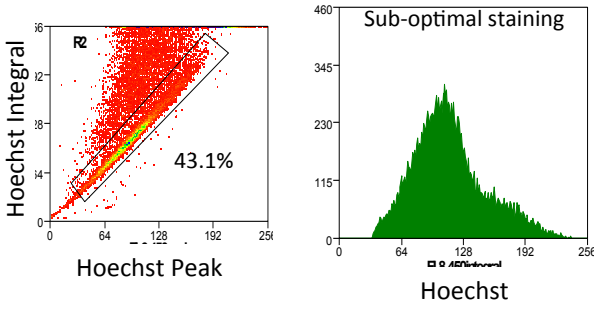
Ivan Rosado
PNAC, MRC-LMB

Cell Cycle

Find the optimal staining concentration for your cells

Keep cell concentration and TIME of staining at required temperature consistent between experiments!!

Disaggregate population
Minimize clumps

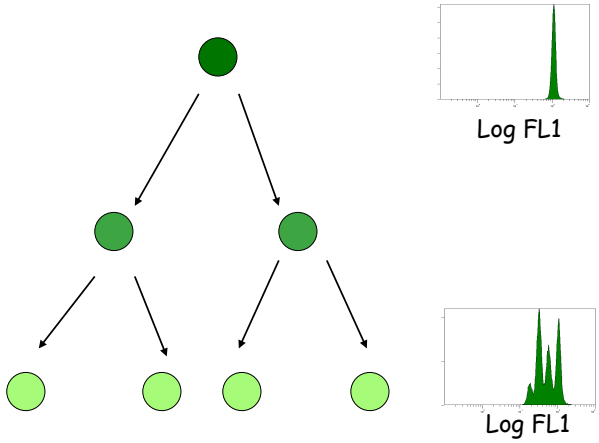


Proliferation using CFSE

Protocol:
- Wash sample with PBS, no protein
- Re-suspend cells in 5uM CFSE
- 10 mins at 37°C
- Wash
- Culture
- Analyse

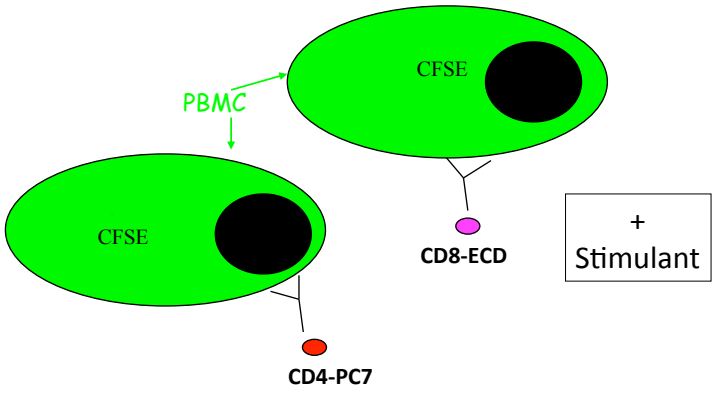
Proliferation using CFSE

As the CFSE loaded cell divides, the fluorescence is halved



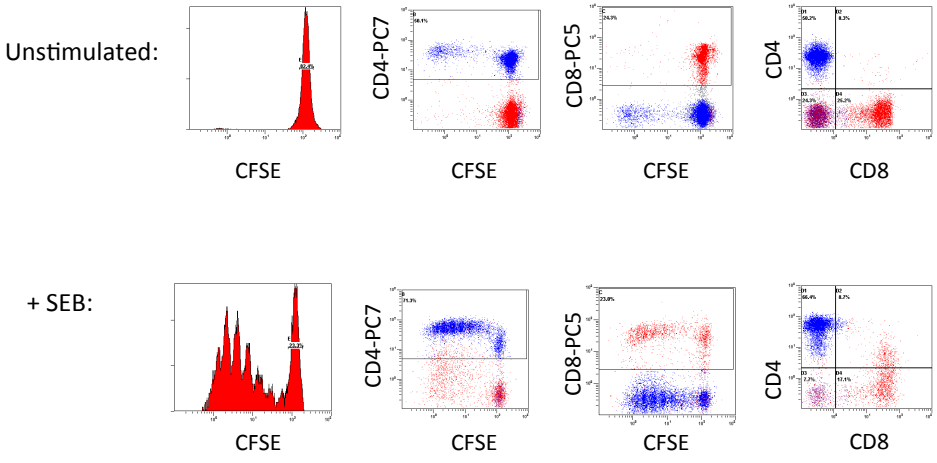
Alternative colours:
- CellTrace Violet Cell Proliferation Kit (Invitrogen)
- Cell Proliferation Dye eFluor 670 (eBioscience)

Measurement of Cell Division and Phenotype



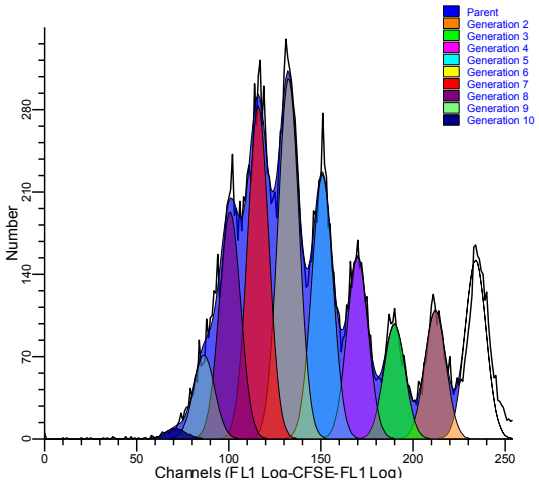
Set up in culture and monitor over 7 days to look at response

Human PBMC, Day 7



Example calculation of Precursor Frequency (Modfit)

Culture 4: (55928-CFSE + PMA+Ionomycin),



Proliferation Wizard Basic Model

File: 10_m4a Specimen R_1 00000382 010.LMD
Date acquired: 16-Feb-06
Date analyzed: 22-Feb-2006

Parent: 9.49 % at 234.00

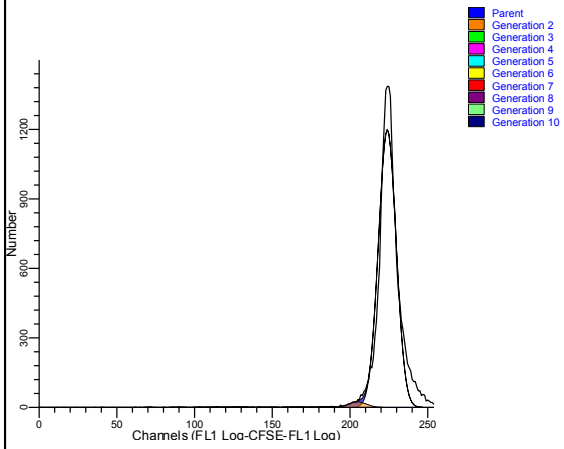
Generation 2: 6.83 % at 212.13
Generation 3: 6.10 % at 189.93
Generation 4: 9.66 % at 169.90
Generation 5: 14.06 % at 150.82
Generation 6: 19.16 % at 132.43
Generation 7: 17.65 % at 115.96
Generation 8: 12.05 % at 100.68
Generation 9: 4.46 % at 86.61
Generation 10: 0.55 % at 71.26

Proliferation Index: 5.71
Nonproliferative Fraction: 0.54
Division Error Index: 1.00
Spacing of generations: 21.87

For cells at generation >= 3:
Upper Generation P.I.: 18.20
Precursor Frequency: 0.262734

Number of Cells Analyzed: 23443
Reduced Chi-Square: 1.837

Culture 1: (55928-CFSE), no restimulation



Proliferation Wizard Basic Model

File: 01_m1a Specimen R_1 00000373 001.LMD
Date acquired: 16-Feb-06
Date analyzed: 22-Feb-2006

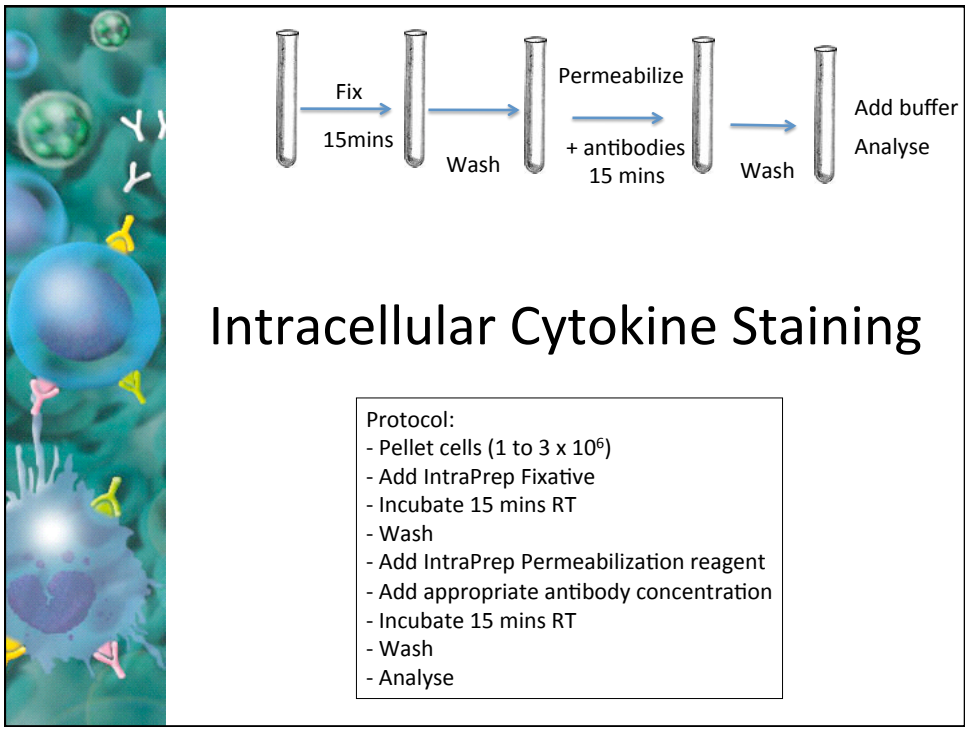
Parent: 97.22 % at 224.00

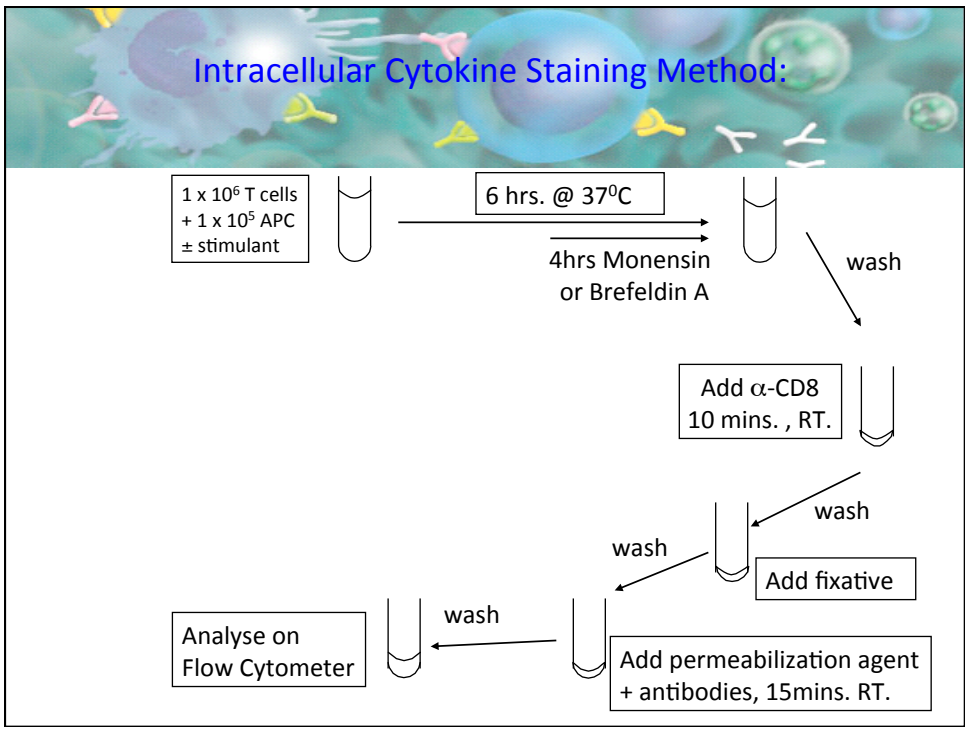
Generation 2: 1.95 % at 204.81
Generation 3: 0.23 % at 185.62
Generation 4: 0.14 % at 166.43
Generation 5: 0.08 % at 147.24
Generation 6: 0.16 % at 128.05
Generation 7: 0.11 % at 108.86
Generation 8: 0.06 % at 89.67
Generation 9: 0.04 % at 70.48
Generation 10: 0.01 % at 51.29

Proliferation Index: 1.02
Nonproliferative Fraction: 0.99
Division Error Index: 1.00
Spacing of generations: 19.19

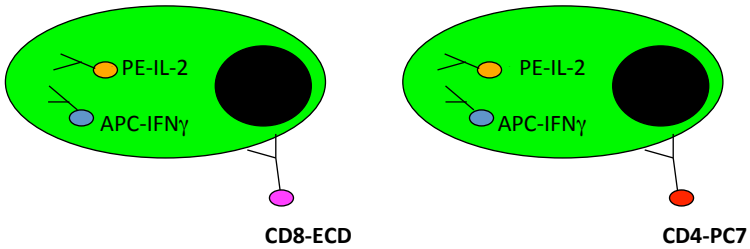
For cells at generation >= 3:
Upper Generation P.I.: 9.43
Precursor Frequency: 0.000894

Number of Cells Analyzed: 19175
Reduced Chi-Square: 7.583


Fix
15mins
Wash
Permeabilize
+ antibodies
15 mins
Wash
Add buffer
Analyse
Intracellular Cytokine Staining
Protocol:
- Pellet cells (1 to 3 x 10⁶)
- Add IntraPrep Fixative
- Incubate 15 mins RT
- Wash
- Add IntraPrep Permeabilization reagent
- Add appropriate antibody concentration
- Incubate 15 mins RT
- Wash
- Analyse

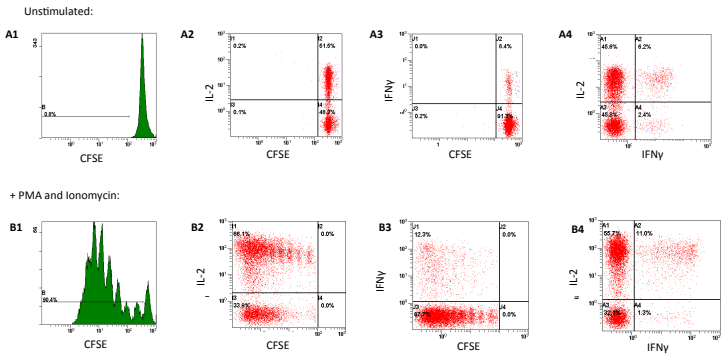

Intracellular Cytokine Staining Method:
1 x 10⁶ T cells
+ 1 x 10⁵ APC
± stimulant
6 hrs. @ 37⁰C
4hrs Monensin
or Brefeldin A
wash
Add α-CD8
10 mins. , RT.
wash
wash
Add fixative
Analyse on
Flow Cytometer
wash
Add permeabilization agent
+ antibodies, 15mins. RT.

CFSE and Immunophenotype and Intracellular Cytokines



5-Colour Combination with dual Cytokine

Day 7 +/- stimulation



IL-2 produced from first cell division onwards
IFNγ produced after a number of cell divisions

Sorting

MoFlo Cell Sorter

Upper Chamber

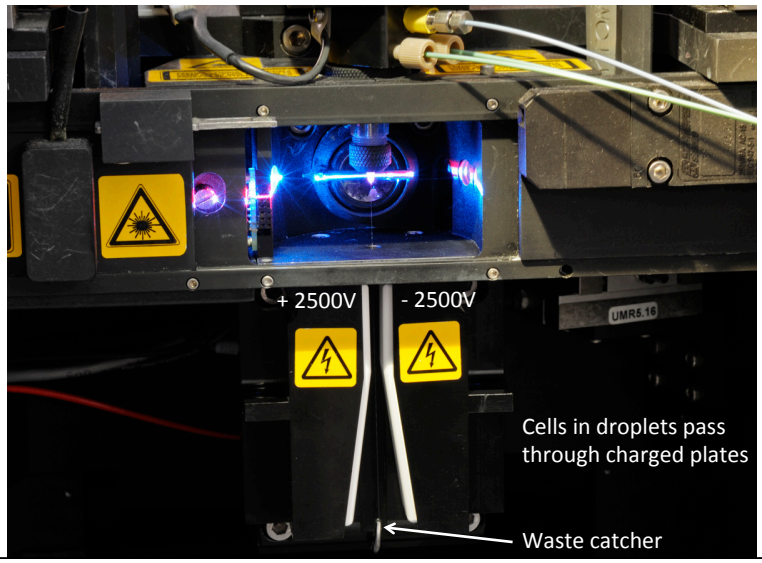
Lasers intercept sample stream

Stream view

The stream is vibrated at high frequency so that droplets are formed

Droplets can be charged either
- Positive (+)
- Negative (-)

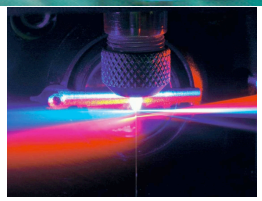
Our MoFlo Cell Sorter



MoFlo Cell Sorter

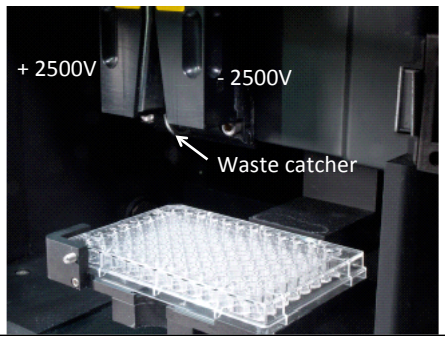
Upper Chamber

Lasers intercept sample stream

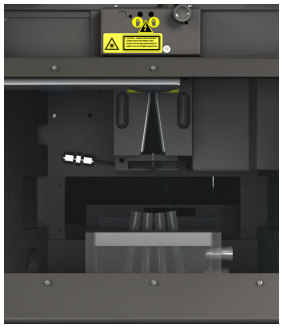


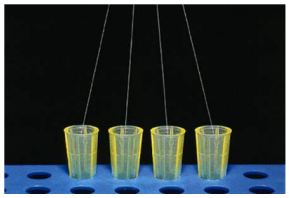
Lower Chamber

Stream droplets pass down through charged plates and deflected into collection tubes or plate

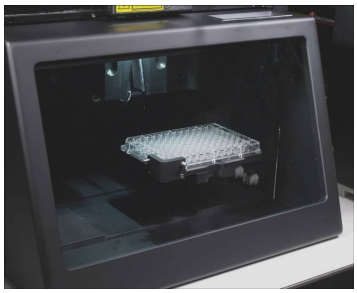


Sort into tubes, plates, whatever





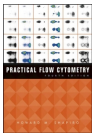
Purify the population you want based on fluorescence and light scatter

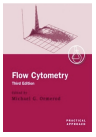


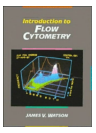
References

Reference Books:

- Practical Flow Cytometry. Howard M Sharpiro (free pdf)
- Flow Cytometry A Practical Approach. MG Ormerod
- Cytometric Analysis of Cell Phenotype and Function. McCarthy & Macey
- Introduction to Flow Cytometry. JV Watson.

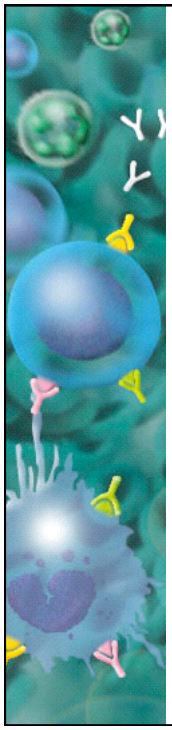






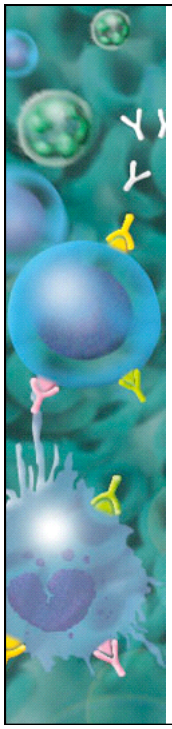
Web References:

- FLOW CYTOMETRY A basic introduction. Michael G. Ormerod
  http://flowbook.denovosoftware.com/



History

1965: L. Kamentsky (IBM Labs) and M. Fulwyler (Los Alamos Nat Lab) experimented with fluidic switching and electrostatic cell sorters.
1967: Mack Fulwyler purified (>95%) blood granulocytes and lymphocytes by passing cells through a Coulter orifice, then breaking the stream into droplets which could be charged, and then deflected into a collection vessel as they passed between voltage plates
1967: Kamentsky built the Rapid Cell Spectrophotometer (RCS) a syringe pump based sorter which measured nucleic acid content of cervical cells and cells size by light scatter
1968: First fluorescence-based flow cytometry device (ICP11) was developed by Wolfgang Göhde. Commercialized by German developer and manufacturer Partec through Phywe AG in Göttingen. The technology was termed Pulse Cytophotometry. Partec was acquired by Sysmex in 2013
1969: Ethidium bromide first used by Dittrich and Göhde
1971: The Cytofluorograph from Bio/Physice Systems Inc (later Ortho Diagnostics)
1972: L. Herzenberg (Stanford) developed a cell sorter which separated cells stained with fluorescent antibodies.
1973: Crissman and Steinkamp introduced Propidium Iodide



History

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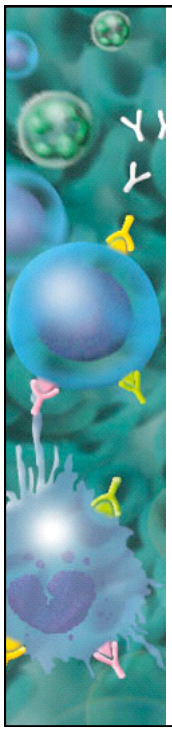
1973: Crissman and Steinkamp introduced Propidium Iodide
1973: PAS 8000 from Partec
1974: first FACS instrument from Becton Dickinson
1974: first commercial flow cytometric differential blood counter (Hemalog D)
1975: the ICP22 from Partec/Phywe
1976 Hoechst dyes introduced by Latt and Stetten
1977/78: The Epics from Coulter
1977: Stohr introduced DAPI
1978: The term Flow Cytometry was coined at the Conference of the American Engineering Foundation in Pensacola, Florida



Growth in Flow Cytometric Studies

March 5th 2010: PubMed search on

"flow cytometry"	: 107,139 citations (March 5th 2010)
	: 179,485 citations (March 8th 2016)
"cytometry"	: 110,489 citations (March 5th 2010)
	: 184,309 citations (March 8th 2016)

First citation on both lists: 1974 paper by Mack Fulwyler

The Society for Analytical Cytology, founded in 1978, established Cytometry as its journal title from the beginning, but only recently changed its name to the International Society for the Advancement of Cytometry.