Flow Cytometry

Talk Overview

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

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

**Synergy High Speed Sorters:**
1. 5 lasers, 15 colour detection
2. 3 lasers, 10 colour detection
3. 5 lasers, 17 colour detection

High-end Analysers

**2 x Fortessa Analysers:**
1. 4 lasers, 16 colour detection
2. 5 lasers, 18 colour detection

**LSRII Analyser:**
- 4 lasers, 14 colour detection

**CytoFLEX LX:**
- 6 lasers, 21 colour detection
Our Facility at the LMB

Eclipse
• 3 lasers, 5 colour detection

FACSCalibur
• 2 lasers, 4 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 detected across a band of 32 fluorescence detectors detecting emission wavelengths from 500 – 800nm

Selected publications supported by the Facility in 2018


1. Prion-like protein aggregates exploit the RHO GTPase to cofilin-1 signaling pathway to enter cells. Zhong Z., Grasso L., Sibilla C., Stevens T. J., Barry H., Bertolotti A. EMBO Journal 37(6). 1 March 2018


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

Flow Cytometry
Cytometry
• Localization of antigen is possible
• Poor enumeration of cell subtypes
• Tissue architecture

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

Jillian Barlow, MRC-LMB
Components of a Flow Cytometer


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

Forward Scatter
Forward Scatter

Forward Scatter Pulses

Small  Medium  Large

Voltage vs. Time
Forward Scatter Histogram

Threshold

Threshold

Small cells and debris
Side Scatter

Cell count

Side scatter
2 Parameter Dot Plot

Whole Blood Dot Plot
Fluorescence detection

Forward Scatter

Side Scatter

Dichroic mirrors

Filters in front of detectors to separate Fluorescence λ

So, run single colour controls to assess the spectral overlap
Fluorescence

Fluorescence Histogram

Histogram of cell count against fluorescence intensity.
Fluorescence Dot Plot

CD45-ECD
CD4-PC7
CD8-FITC

Fresh Whole Blood
WBC
PBMC
LYMPH5

Optical Layout

488nm laser
Forward Scatter
Side Scatter
Fluorescence detection
Computer
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

- Optimise 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
**Immunophenotyping**

**Fresh Whole Blood**
- WBC
- PBMC
- LYMPHS

**CD45-ECD**
- **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

**Sample**
- Next Sample
- Later Sample
- Even Later Sample

- 575nm emission out and absorbed
- 488nm or 561nm laser excitation
- PE
- Cy7
- 780nm emitted
Be Careful of Reagent Degradation!

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

Sample  Next Sample  Later Sample  Even Later Sample

Spectral Analyser

We can analyse the spectrum of Fluorochromes and reveal the degradation

Emission wavelength in nm
Cell Cycle Analysis

A number of dyes can be used e.g.
- Propidium Iodide
- Hoechst
- DAPI
- DRAQ5

Data from Ben Taylor (PNAC), MRC-LMB
Cell Cycle Analysis

Hoechst staining of Viable cells
Harvey McMahon’s Lab

Cell Cycle with GFP

Hoechst staining combined with GFP
No fixation required thus leaving GFP expression unaffected

KJ Patel’s Lab
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 5μM CFSE
- 10 mins at 37°C
- Wash
- Culture
- Analyse
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

Unstimulated:

+ SEB:

Example calculation of Precursor Frequency (Modfit)
Culture 1: (55928-CFSE), no restimulation

- Parent Generation 2: 224.00
generation 3: 1.95
Generation 4: 0.14
Generation 5: 0.08
Generation 6: 0.16
Generation 7: 0.11
Generation 8: 0.06
Generation 9: 0.04
Generation 10: 0.01

Proliferation Index: 1.02
Division Error Index: 1.00
Spacing of generations: 19.19

For cells at generation >= 3:
- Upper Generation P.I.: 9.43
- Precursor Frequency: 0.000004
- Number of Cells Analyzed: 39175
- Reduced Chi-Square: 7.583

Intracellular Cytokine Staining Protocol:
- Pellet cells (1 to 3 x 10^6)
- 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. $1 \times 10^6$ T cells + $1 \times 10^5$ APC ± stimulant
2. 6 hrs. @ 37°C
3. 4 hrs Monensin or Brefeldin A
4. Add $\alpha$-CD8 10 mins., RT.
5. Wash
6. Add permeabilization agent + antibodies, 15 mins. RT.
7. Analyse on Flow Cytometer

**CFSE and Immunophenotype and Intracellular Cytokines**

- PE-IL-2
- APC-IFNγ
- CD8-ECD
- PE-IL-2
- APC-IFNγ
- CD4-PC7

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
The stream is vibrated at high frequency so that droplets are formed. Droplets can be charged either:
- Positive
- Negative

Cells in droplets pass through charged plates.
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 Sharpio (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.
1972: L. Herzenberg (Stanford) developed a cell sorter which separated cells stained with fluorescent antibodies.
1973: Crissman and Steinkamp introduced Propidium Iodide.
History

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.