INTRODUCTION TO FLOW CYTOMETRY

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Overview or The Many Parts of Flow

- What is Flow Cytometry?
- Fluidics
- Light Scattering
- Fluorescence
- Data presentation
- Interpreting the data
- Applications
- Sorting

Flow Basics

Data Analysis

Motivation and Experiments
Cellular Parameters

- Granulocytes
- Basophils
- Neutrophils
- Eosinophils
- Monocytes
- Lymphocytes
Cellular Parameters
What Is Flow Cytometry?

- Flow ~ cells in motion
- Cyto ~ cell
- Metry ~ measure
- Measuring properties of cells while in a fluid stream
History and uses of Flow Cytometry

It can be used for...

- Immunophenotyping
- DNA cell cycle/tumor ploidy
- Membrane potential
- Ion flux
- Cell viability
- Intracellular protein staining
- pH changes
- Cell tracking and proliferation
- Sorting
- Redox state
- Chromatin structure
- Total protein
- Lipids
- Surface charge
- Membrane fusion/runover
- Enzyme activity
- Oxidative metabolism
- Sulfhydryl groups/glutathione
- DNA synthesis
- DNA degradation
- Gene expression

Publications citing 'flow cytometry'

The use of flow in research has boomed since the mid-1980s.
Flow Cytometer
What Happens in a Flow Cytometer?

1. Fluidics
   Cells in suspension flow in single-file through an illuminated volume where they scatter light and emit fluorescence.

2. Optics
   that is collected, filtered and converted to digital values that are stored on a computer.

3. Electronics
   
http://flow.csc.mrc.ac.uk/?page_id=5
Fluidics – Hydrodynamic Focusing

- When conditions are right, the sample fluid flows in a central core that does not mix with the sheath fluid.
- The introduction of a large volume into a small volume in such a way that it becomes “focused” along an axis is called **Hydrodynamic Focusing**.

Adapted from Purdue University Cytometry Laboratories
Light Scattering

coherent light source (488nm, blue)

Forward Scatter FSC
small angle scattering (0.5-5°) (roughly) size and shape (blue)

FSC Intensity
Light Scattering

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Side Scatter SSC
large angle scattering (15-150°), darkfield, complexity and granularity (blue)
Light Scattering

coherent light source (488nm, blue)

Side Scatter SSC
large angle scattering (15-150°), darkfield, complexity and granularity (blue)
Histogram

Forward Scatter FSC

Count

0 10 100 1000 10000

smaller  bigger
Dot Plot

- **Side Scatter SSC**: More Granular
- **Forward Scatter FSC**: Bigger

Regions:
- **Live Cells**
- **Dead Cells**
- **Apoptotic Cells**
- **Bigger Cells or Aggregates**
Light Scattering in Whole Blood
Colours

- Antibodies
- Fluorescent dyes
- Fluorescent proteins
Fluorescence

- Absorbed excitation light
- Exited state
- Emitted fluorescence light
- Green light (Lower energy)
- Blue light (High energy)
- Ground state
Fluorescence

Fluorescence emission
lower energy than source light
(green)

cohereent light source
(488nm)

Fluorescence detector

green and blue fluorescence

Fluorescence
Filter Properties

**Long-pass filters** transmit wavelengths above a **cut-on** wavelength

- 550LP
  - Emission light
  - > 550nm

**Band-pass filters** transmit wavelengths in a narrow range around a specified wavelength

- 510/20BP
  - Emission light
  - 500-520nm

Stokes shift

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Wavelength (nm)

400  450  500  550  600
Fluorescence

Fluorescence detection involves a coherent light source (488nm) that emits light. When this light interacts with a fluorescent molecule, it excites the molecule to an excited state. Upon relaxation, the molecule emits light with lower energy than the source light, resulting in green fluorescence. The emitted light passes through a bandpass filter to block any light except for the green fluorescence. The fluorescence detector then measures the intensity of the green fluorescence.
More Fluorescent Parameters…
Dichroic Mirror

At an other angle the filter can be used as a dichroic filter or dichroic mirror.
Channel Layout of a laser-based Flow Cytometer

- Photon-distribution to detectors according to energy-levels (wavelengths)
- Optical elements provide separation of channels and wavelength selection
Compensation

- Fluorochromes typically fluoresce over a large part of the spectrum
- Overlap between the colors emitted by different fluorochromes
- Mathematical compensation is used to reduce overlapping results
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Microscopy vs Flow Cytometry

**Microscopy**
- Localization of antigen is possible
- Poor enumeration of cell subtypes
- Limiting number of simultaneous measurements

**Flow Cytometry**
- Can not tell you where antigen is
- Fast
- Subpopulation analysis
- Multiparameter analysis
N-Dimensional Plots
Gating

• Is used to isolate a subset of cells on a plot

• Allows the ability to look at parameters specific to only that subset
Important Points on Analysis

• Visualization

• What kind of data are you looking for?
  • How much fluorescence?
  • What percent are positive?
  • How much more positive is \( x \) than \( y \)?
  • What is the ratio between green and red fluorescence?

• What kind of statistics are available
  • MFI (geometric or arithmetic)
  • \%-ages
  • CV
  • …
Applications of Flow Cytometry

- Cell function
- Immunophenotyping
- DNA and RNA analysis
- Sorting and cell isolation
- Cell death

Main applications:

Apoptosis (cell shrinks, chromatin condenses) → "Budding" → Apoptotic Bodies are phagocytosed; no inflammation

Necrosis (cell swells) → Cell becomes leaky, blebbing → Cellular and nuclear lysis causes inflammation
Application: Immunophenotyping

- Detection of cell surface molecules as example cluster of differentiation

www.med4you.at
Application: Clinical phenotyping

Normal blood sample

Blood from Patient
Application: Clinical phenotyping

www.med4you.at
Application: Cell function

- Intracellular staining of cytokines, cytoskeleton, enzymes, transcription factors, signaling molecules

![Diagram showing cell function and signaling pathways](image)
Application: DNA Analysis

- DNA content of individual cells gives information about their ploidy
- Suitable dyes: PI, 7-AAD, DAPI
- Combination with light scatter or immunofluorescence
Application: Cell Death

Measurements of cell death:
- Expression of proteins involved in apoptosis
- Activation of caspases
- Changes in the mitochondrial membrane potential
- Changes in the plasma membrane
- Cell shrinkage
- Chromatin changes
- DNA degradation
Application: Cell Expression
Principle of Sorting

- Sorting (separating) cells based on properties measured in flow is also called **Fluorescence-Activated Cell Sorting (FACS)**.
Application: Sorting

- **green fluorescence** vs. **red fluorescence**
  - dead cells vs. viable cells

- **yellow fluorescence** vs. **green fluorescence**
  - GFP positive cells 5.76%

- **sort** → analysis
- **sort** → culture
Advantages and Disadvantages

- Distinct cell populations defined by size and granularity
- Dead cells may be gated out for analysis
- Detection of weakly expressed surface antigens
- Multicolor analysis may be performed

- Sterility issues: antibiotics sufficient to control microbial contamination
- Viability of fragile cells: larger nozzle, 4°C, appropriate buffer
What are the services in our facility

**Analysis**
- BD Canto II
- Introduction 1h
- Booking / usage by yourself
- Advice if requested

**Sorting**
- BD AriaIII
- Appointment for experiment discussion
- Check cells for fluorescence
- Prepare the cells and let them sort
Summary

Links:

isac-net.org
www.cyto.purdue.edu
flowbook-wiki.denovosoftware.com
wwwbdbiosciences.com/research/multicolor/spectrum_viewer/index.jsp

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Electronics – Voltage Pulse

Laser Beam

Transmitted Light

Detector

Voltage

Time
Measurements of the Electronic Signal

- Voltage
- Time
- Area A
- High H
- Width W