MTiLS: Introduction to flow cytometry and cell sorting

IMB Flow Cytometry Core Facility

Source: StarcellBio

Dr. rer. nat. Jesús Gil-Pulido
Not only basic research, but also used in clinical settings

Diagnosis and staging of lymphomas and leucemias

Minimal residual disease

Solid organ transplantation (monitoring)

HIV infection

Immunodeficiencies
Fetomaternal hemorrhage...
Overview

• What is flow cytometry?
  • FSC – SSC (relative size, granularity / complexity)
  • Fluorescence
• Looking inside a flow cytometer
  • Fluidics
  • Optics
  • Electronics
• Data presentation and gating
• Introduction to electrostatic cell sorting
• Applications of flow cytometry & cell sorting
• Examples of current flow cytometers
• Advantages of flow cytometry over other techniques
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What is flow cytometry?

Flow - Fluid
Cyto - Cell
Metry - Measurement

Meaning that a feature from a “cell” in suspension will be measured (at single cell level).

How do flow cytometers do that?
Thanks to lasers and detectors
„…*in suspension*…“

No solid tissue

...only cell in suspension

- Mechanical disruption
- Enzymatic dissociation
What is flow cytometry?

**Flow** - Fluid

**Cyto** - Cell

**Metry** - Measurement

Meaning that a **feature** from a “cell” in **suspension** will be measured (at single cell level).

**But how does a flow cytometer do that?**

Thanks to lasers and detectors

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01-07-2019
Which features can be measured?

1. Characteristic of the „cell“ (relative size, complexity/granularity)

2. Fluorescence
1. Characteristic of the „cell“

How can a flow cytometer do that? Thank to the interaction of light with the cells.

Source: FineArtAmerica (all)
1. Characteristic of the „cell“: FSC and SSC

FSC = **Forward Scatter** (diffraction) → Relative size of a particle (signal collected at ~10°)

SSC = **Side Scatter** (reflection/refraction) → Granularity/complexity (signal collected at ~90°)

The bigger the cell, the more diffracted light will be collected.

The more complex a cell is, the more light will be reflected/refracted and collected by the detector!
1. Characteristic of the „cell“: FSC and SSC

- **FSC**: Forward Scatter (diffraction) → Relative size of a particle (signal collected at ~10°)

- **SSC**: Side Scatter (reflection/refraction) → Granularity/complexity (signal collected at ~90°)

The more complex a cell is, the more light will be reflected/refracted and collected by the detector!

*Source: Adapted from ThermoFisherScientific*

- The bigger the cell, the more diffracted light will be collected.

*Source: ThermoFisherScientific*
1. Characteristic of the „cell“: FSC and SSC

**Conventional flow cytometers**

Bacteria 0.5 µm  
Phytoplankton 2 µm  
Red Blood Cell 6 µm  
BD CBA Bead 7.5 µm  
Lymphocyte 8 µm  
Neutrophil 12 µm  
Monocyte 14 µm  

Smaller particles (<0.5µm): Extracellular vesicles (0.03µm – 1µm)  
Larger particles (>50µm): Whole organisms or cell clusters: *C. elegans*, *Drosophila* larvae, pancreatic islets (100µm – 1800 µm)
1. Characteristic of the „cell“: FSC and SSC

Lysed human blood

Source: Nordic Mubio

Neutrophils

Source: FineArtAmerica

Monocytes

Source: SciencePhoto Library

Lymphocytes

Source: SciencePhoto Library

HeLa cells in culture

Source: Bioprotocols

Exososomes

Source: Barts and The London
1. Characteristic of the „cell“: FSC and SSC

- **Apoptotic Cells**
- **Dead Cells**
- **Bigger Cells or Aggregates**
- **Live Cells**

**Forward Scatter FSC**

**Side Scatter SSC**

More Granular
Flow cytometry properties: FSC, SSC and fluorescence

1. Characteristic of the „cell“ (relative size, complexity/granularity)

2. Fluorescence
2. Fluorescence

- Molecules with the ability to fluorescence are fluorophores

**Excitation:** absorbance of photons

**Emission:** release of photons

*Source: ThermoFisherScientific*
2. Fluorescence in flow cytometry

How many fluorophores are?

Fluorescent proteins („natural“)

Source: Microbiological Blogs

Source: NightSea.com
2. Fluorescence in flow cytometry

> How many fluorophores are?

**Synthetic compounds**

- Cyclic ring compounds: FITC, Texas Red, Alexa 488
- Tandem dyes: BV575, PE-Cy5, APC-Cy7
- Polymer and nanocrystal dyes: BV421, Quantum Dots

**Quantum Dots**

Source: Medium.com
2. Fluorescence: antibodies conjugated to fluorophores

- They specifically bind your antigen (i.e. FITC-antibody recognizing CD3 in T cells) → plenty of options (multiparametric flow cytometry)

- Conjugated directly with fluorophores or biotin (=secondary stainings)
2. Fluorescence

- Every fluorophore has an excitation and an emission range, which are **unique** (important for *spectral cytometry*).

- In flow cytometry, knowing excitation and emission ranges are **critical** (panel design).

![Fluorescence Diagram](https://www.thermofisher.com/)

**Emitting light will always have a longer wavelength = less energy** („Stokes shift“)

*Source: ThermoFisherScientific Spectral Viewer*
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Digital flow cytometer

Flow Cytometer

Fluidics

Electronics

Optics

LSRFortessa
Fluidics

- Responsible for moving your precious samples to the interrogation point (and to the waste)

Interrogation point (where „cells“ will be interrogated by the lasers)

Source: ThermoFisherScientific (Modified)
Fluidics: the sheath fluidic

**Flow - Fluid**

- Flow cytometers are filled with a fluid, the **sheath fluid**, which runs in a laminar way.

- The sample (in **suspension**) is introduced into the flow cytometer

  - Sample Injection Port (SIP)
  - High Throughput Sampler (HTS)
Fluidics: the flow cell

• Cells are going to be interrogated in the flow cell. This part of a flow cytometer is also where **hydrodynamic focusing** takes place.

Source: Innovation.ca
Fluidics: hydrodynamic focusing

Core diameter and cells at time of sample injection

Core diameter and cells at time of measurement

Hydrodynamic focusing
Fluidics: hydrodynamic focusing

- If two fluids differ enough in density and/or velocity, they behave as two independent fluids, without mixing. They form a two-layer stable flow (laminar)

Gulf of Alaska

Rio Negro Vs Rio Solimoes

Source: Hipertextual.com

Source: Nuestroclima.com
Fluidics: hydrodynamic focusing

Sample stream \( (p_{\text{sample}}) \) > Sheath fluid \( (p_{\text{sheath}}) \)

(different velocities)

Hydrodynamic focusing

Laminar flow

Source: BD Biosciences (modified)
Fluidics: the importance of the flow rate

Low: ≈ 12 µl/min
Medium: ≈ 35 µl/min
High: ≈ 60 µl/min

Source: BD Biosciences (modified)
Flow Cytometry Core Facility
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cytometry@imb-mainz.de

Low: ≈ 12 µl/ min

LOW
Decreased coincident events
Better signal resolution

HIGH
Increased coincident events
Lower signal resolution

High: ≈ 60 µl/ min

Source: BD Biosciences (modified)
Fluidics: the importance of the flow rate. Example

- **LOW** *(Decreased coincident events, Better signal resolution)*

- **HIGH** *(Increased coincident events, Lower signal resolution)*

*Source: BD Biosciences (modified)*
Fluidics: summary

1. It allows your sample to travel from the injection point to the interrogation point to the waste.

2. Hydrodynamic focusing makes your cells to pass in a single line through the interrogation point.

3. Flow rate impacts hydrodynamic focusing and therefore also your results. Higher flow rates > higher coincident events, less signal resolution; lower flow rates > lower coincident events, better signal resolution.
Digital flow cytometer

Flow Cytometer

 Fluidics

 Optics

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LSRFortessa
What is flow cytometry?

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Meaning that a feature from a “cell” in suspension will be measured (at single cell level).

How do flow cytometers do that?

Thanks to lasers and detectors
Optics: lasers, filters and detectors

Source: ThermoFisherScientific (Modified)
LASERS are the core of your flow cytometer!
The number of laser a flow cytometer is equipped with will vary among different machines. The more lasers, the more parameters to analyse!

Regardless of the number of laser your machine is equipped with, laser needs to be directed to the flow cell...
**Optics: lasers**

**BD LSRII**

*Source: Washington University*
Optics: lasers

Collecting light from specific laser (i.e.: blue laser)

Note: emitted light from different lasers will be collected in different fiber optic cables
2. Fluorescence

- Every fluorophore has an excitation and an emission range, which are unique (important for spectral cytometry)

- In conventional flow cytometry, knowing excitation and emission ranges are critical

A conventional flow cytometer is not going to measure the whole emission spectrum > we need filters!

Emitted light will always have a longer wavelength = less energy („Stokes shift“)

Source: ThermoFisherScientific Spectral Viewer
With wavelength discrimination: dichroic mirrors

Source: BD Biosciences
Optics: detectors

- Detectors are devices that sense the light, then convert it to an electronic signal.
Optics: detectors. The PMT

- Amplification: up to $10^8$ (voltage dependent)
- Supply voltage: up to 1000 V >> You can change using Flow’s Software
- PMT provides current output proportional to light intensity

Source: Florida University
Optics: detectors. The PMT. Voltages

PMT voltage too high: positive signal off scale

Reduced PMT voltage: positive signal fully resolved

Source: Thermo Fisher Scientific
Optics: detectors. The PMT. Trigons and octagons

Source: BD Biosciences
Optics: detectors. The Trigon

Fluorescence emission

Source: BD Biosciences (modified)
Optics: detectors. The Octagon

Fluorescence emission

Source: BD Biosciences (modified)
Optics: spectral flow cytometry, a new era in flow

- In conventional flow cytometry: a specific wavelength of the emission of a fluorophore is collected. Thus, if two fluorophores excited by the same laser are detected by the same detector they cannot be used in flow cytometry (e.g. APC – AF647).

- Spectral flow cytometry changes this paradigm and it analyzes the emission “fingerprint” of each fluorophore! They can be used at the same time!

Source: ThermoFisher Scientific
Optics: summary

1. Lasers with specific emission wavelengths excite different fluorophores.

2. Emitted light (fluorescence and scatter) is organized into wavelength ranges using filters.

3. The specific wavelength ranges are sent into the detectors (PMTs).

4. Spectral flow cytometers do not only detect a small portion of the emission range of a fluorophore, but it records the whole „emission fingerprint“, allowing the detection of fluorophores that conventional flow cytometry cannot resolve.
Digital flow cytometer

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01-07-2019
Electronics: voltage pulse by single cells

- From photoelectric current to dots on a plot (via Analog to Digital Converter)
Electronics: voltage pulse by single cells

- From photoelectric current to dots on a plot (via Analog to Digital Converter)
Electronics: voltage pulse by single cells

- From photoelectric current to dots on a plot (via Analog to Digital Converter)
Electronics: voltage pulse by aggregates

- Aggregates negatively impact your precious data: identify them!
Electronics – Doublet discrimination

- Remove your doublets before start analyzing/sorting any parameter

Doublets/ Aggregates/cell clusters

- Doublet exclusion possibilities: FSC or SSC.

Single cells
Electronics – How do aggregates impact your results?

My sample has the two proteins, great!

• Blue and red signal are coming from two different cells, not from only one!

Blue dye is associated with your protein A

tdTomato is associated with your protein B
1. The current is converted into a pulse, characterized by an area, width and height.

2. Characteristics of the pulse can be used for removing doublets, which can impact your results.

3. Each pulse is converted and represented as a dot in a plot.
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Data presentation

• Each pulse will be processed and presented as a graphic in the software.

• You can choose between different data presentation styles according to your experiment (or you PI, reviewer...etc.).
Data presentation

Plot types

• **Histograms**
• Dot plots
• Contour plots
• Density plots

Data display

• Linear scale
• Logarithmic scale
• Biexponential scale
Data presentation

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Data presentation: gating

- At the end of your experiment, you will generate a FCS (Flow Cytometry Standard) file.
- FCS files can be analyzed by different softwares (FlowJo, DIVA Software... etc.)

- Gating is the process of narrowing your population of interest.
- The selected gating strategy impacts your results

What is important to know when gating your cells of interest?

- Doublet Discrimination
- Dead Cells Exclusion
Data presentation: gating

Example: you want to see how many of your cells are RFP⁺
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Cell sorting – a brief overview

• There are different ways to sort (separate) cells (magnetic, mechanical, microfluidic, electrostatic).

• We will focus on electrostatic sorting (most extended)

• In any case, cell sorting allows you to enrich a population of interest.
Electrostatic cell sorting – the principle

- A vibrating mechanisms („transducer“) causes a liquid stream to break into single droplets.
- One drop contains one cell (in theory).
- Cells are interrogated before the droplet breaks off.
- At the stream break-off point, the droplet is charged, if it contains cell of interest.
- Charged droplets are deflected in an electric field and collected in the respective sorting device.
Possible collection devices for cell sorting

- **Tubes**
  - Source: Eppendorf
  - Source: Amazon
  - Source: StemCell Technologies

- **Microscopy slides**
  - Source: Parco Scientific

- **Microtiter plates (it allows you single cell sorting)**
  - 96-well plates
    - Source: Cole Parmer
  - 384-well plates
    - Source: Axon Lab
  - Source: Eppendorf
  - Source: StemCell Technologies
  - Source: Amazon

- **Ibidi chamber**
  - Source: Amersham Biosciences
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Applications: phenotyping (Multiparametric FC)

- If you know **surface** or **intracellular** antigens being expressed in a specific cell population, you can use multiparametric FC for the identification of populations (very popular application).

- For example, peripheral blood can be analyzed for the detection of B cells, T cells, monocytes...and even its activation status. **Up to 50 parameters at the same time.**

- Immunophenotyping, for example, is a really important tool nowadays to monitor patient responses after immunotherapy drug administration.

- In theory, your imagination is the limit!*
  *and, of course, the availability of reagents...
Applications: phenotyping

Characterization of dendritic cells (a complex subset to look at...) in different mice organs
Applications: transfection/transduction/transformation efficiency

- You could look under the microscope, but quantification might be tedious >> Flow cytometry is really fast and gives you a fast % of + cells.

- AND if you only want + cells...→ **cell sorting!**
Applications: phospho-protein profiling

→ Typically: phospho-protein detection is done by Western Blot
→ Currently there are several antibodies recognizing non-phosphorilated and phosphorilated version.

Western Blot
- No opportunity to view variability
- Requires sorting of subsets to gain access to intracellular antigens
- Requires larger numbers of cells $10^6$

Flow Cytometry
- Differentiation possible
- Subset Typing via surface markers possible
- Possibility to observe heterogeneity in the population
- Requires fewer cells $10^3$-$10^4$

http://wwwbdbiosciencescom/research/phosflow/
Applications: phospho-protein profiling

→ You can even easily analyze the status of phosphorilation over time

- Kinetics of MAPK p38 on CD4+ T cells out of a mixture of cells
Applications: calcium flux

• Why any interest in study Ca2+ flux?
  • Ca2+ is related with cell activation. You may want to check Ca2+ levels after several treatments, to see whether Ca2+ is affected and thus, also activation status of a cell (by multiparametric flow cytometry together with surface markers)
  • Remember: Ca2+ is one of most important second-messengers in a cell.

• How do you measure Ca2+ flux? With dyes whose emission spectra changes after binding Ca2+:
  • Indo-1: 475nm to 400nm after Ca2+ binding (excitation: UV) > We measure the ratio
  • Fluo-4: 100x more emission at 506nm after Ca2+ binding (excitation: blue laser)
Applications: calcium flux with Indo-1

- Stimulation of Jurkat T cells, monitoring of cell activation over time

\[
\text{Indo-Ca}^2+\text{bound (400nm)} \quad \text{If Ca}^2+\text{bound} > \text{Ca}^2+\text{free then higher Ratio = Ca}^2+\text{flux inside the cell}
\]

\[
\text{Indo-Ca}^2+\text{free (475nm)}
\]

Source: Link
Applications: Apoptosis

Apoptosis = programmed cell death. During the process several molecules can be analyzed by flow cytometry to follow and characterize apoptosis.

Example 1: phosphatidylserine translocation

Source: Novus Biological
Applications: apoptosis (PS by Annexin V)

Source: Applications in flow cytometry: Tim Schenkel BD Biosciences Training Center Heidelberg
Applications: Apoptosis

Apoptosis = programmed cell death. During the process several molecules can be analyzed by flow cytometry to follow and characterize it.

Example 2: phosphatidylserine translocation

Source: Novus Biological
Applications: Apoptosis

- You need dyes binding DNA: PI, Sytox, DAPI, 7-AAD...

Source: RayBiotech
## Applications: apoptosis

<table>
<thead>
<tr>
<th>Change</th>
<th>Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Translocation of phosphatidylserine from the inner to the outer leaflet of the plasma membrane</td>
<td>Annexin V</td>
</tr>
<tr>
<td>Changes of mitochondria membrane potential</td>
<td>BD MitoScreen test</td>
</tr>
<tr>
<td>Activation of caspases</td>
<td>e.g. activation of caspase-3</td>
</tr>
<tr>
<td>DNA fragmentation</td>
<td>APO BrdU Kit (TUNEL)</td>
</tr>
</tbody>
</table>

*Source: Applications in flow cytometry: Tim Schenkel BD Biosciences Training Center Heidelberg*
Applications: cell proliferation

The principle:

In each generation, the amount of dye is $\frac{1}{2}$ (theoretically)

Most common dyes: CFSE, CellTrace Violet...

Source: Luzyanina et al. Theoretical Biology and Medical Modelling 2007 4:26
Applications: cell cycle 1D and 2D

Cell cycle 1D = one parameter using DNA-binding dyes (as in apoptosis): PI, 7-AAD, DAPI...etc.

Cell cycle 2D = 1D + another parameter (normally an antibody), to fully reveal S-phase

Source: Beckman Coulter

Source: BioRad
Applications of cell sorting

• Enrichment of transfected cells (i.e. GFP+ only, mCherry+ only, double positive...etc.) or any cell of interest (you can sort up to 6 populations at the same time, at the IMB only 4)

• Single-cell sorting for single cell clone expansion (available only for some cells, as others might not like to be alone!)

• Single-cell sorting for single-cell sequencing (collaboration with Genomics)

Source: Cytosplore
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Examples of conventional flow cytometers

**BD Flow cytometers**

- FACSFortessa (18p.)
- BDFACSSymphony (50p.)

**Beckman coulter cytometer**

- Gallios (12p.)

**Miltenyi Biotech cytometer**

- MACSQuant (10p.)

**ThermoFisher Cytometer**

- Attune Nxt (16p.)
Examples of spectral flow cytometers

Cytek

Sony

Aurora (?)

SP6800
Imaging Flow Cytometry: ImageStream

• Combination of flow cytometry + imaging

• For each cell, one picture
Imaging Flow Cytometry: ImageStream

**Advantage**

- Information about signal distribution within the cell (spatial distribution) and about cell morphology of a high number of cells

**Disadvantages**

- Low data acquisition rate (5000 evt/sec)
- Only up to 10 colours
- Huge data amount per measurement
- Image quality
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Advantages of flow cytometry

- Quick sample processing (up to 10,000 events/s). Generation of high amounts of data.
- Possibility of automatization (e.g. using 96/384-well plates)
- „Easy“. Sample preparation can be easily done.
- Analysis of multiple cell types in one simple tube.
- Detection of rare population, e.g.: circulating tumour cells.
- Possibility of cell sorting > cell purification for downstream analysis (PCR, qPCR, culture, single cell sequencing...etc.)
BONUS I: Do not forget!

No solid tissue

...only cell in suspension

- Mechanical disruption
- Enzymatic dissociation

- X It cannot locate a component within the cell
- X It cannot see how the fluorescent compound is distributed in the cell
- X No detailed intracellular morphology
BONUS II: Is Flow Cytometry and FACS the same?

Flow cytometry  \(\xrightarrow{\text{not}}\)  FACS

**Fluorescent Activated Cell Sorting**  
(BD Trademark)

If you are only analyzing samples > Flow Cytometry

If you are going to sort your cells of interest > Cell Sorting

Everything will be clearer!
TAKE HOME „PHRASES“

Cells/Cells-like-particles in suspension

FSC – Relative Size
SSC – Cell complexity
Fluorescence

Hydrodynamic focusing

Sheath Fluidic

Lasers - Detectors

Many applications available! Enjoy!
Who are we?

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Where can you find us?

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THANKS FOR YOUR ATTENTION!

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Or come by to our Facility 😊

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