Flow Cytometry (FCM) Basics and Principle

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What is flow cytometry?

- Flow Cytometry is the **measurement** of **cellular** properties (cytometry) as the cells are moving in a **fluid stream (flow)**, past a stationary set of detectors.
The evolution of flow cytometers has occurred in four phases:

- Microscopy
- Dye chemistry
- Electronics
- Computers

All above with global biomedical needs, produced an instrument called as ‘flow cytometer’.
Flow cytometry - Applications

**Diagnostics**

1. Immunophenotyping of Leukemias and Lymphomas
2. CD34 counts
3. DNA Analysis using PI
4. CD4 counts

**Research**

1. Intracellular ions and enzymes
2. Cell viability using DNA binding dyes (PI)
3. In situ hybridization
4. Phagocytosis (PI)
5. Suspension array analysis
Cytomtics™ FC500
Principle

Flow cytometer analyses the physical (size and complexity) and the chemical properties (immunofluorescence) of the cells, as they pass through a coaxial stream of fluid.

It is a rapid and quantitative way of examining multiple features of individual cells simultaneously.
Principles of flow cytometry:

Fig. 1 - Schematic view of the main components of the Cytometer. 1 – Fluidic system; 2 – Lasers; 3 – Optics; 4 – Detectors; 5 – Electronics and computer system; 6 – interrogation point.

Fig. 2 - Schematic representation of the hydrodynamic focusing in flow cytometer.

Thousands of cells per second through a laser beam.
Components of FCM

1. FLUIDICS
2. OPTICS
3. ELECTRONICS
Fluidics

- Provides a highly controlled fluid stream.
- Provides exact location of a cell in three dimensions.
- Maintains sample handling compartment (Flow Cell).
Fluidics......

- The sample with its own isotonic fluid, is introduced at a higher differential pressure than the sheath stream into the nozzle through the **Sample injection Port (SIP)**.

- The laminar flow induced by the nozzle imparts a **hydrodynamic focusing effect** to locate the sample stream in the center of the sheath stream.

- **Coaxial stream** within a stream is created.
Hydrodynamic focusing
Optics

1. Light source
2. Forward Scatter
3. Side Scatter
4. Light detectors – PMT and photodiodes
5. Filters and Mirrors
Light source

• Need to have a light source focused on the same point where cells have been focused (the illumination volume)

• Two types of light sources
  – Lasers
  – Arc-lamps
Light source....

- **Lasers**
  - can provide a single wavelength of light (a **laser line**) or (more rarely) a mixture of wavelengths
  - can provide from milliwatts to watts of light.

- provide **coherent** light.

- **Argon gas laser**: Strong emission at 480nm.
Light scatter

- **Forward:**
  - Detected in the same axis as laser.
  - Size and shape of cells.

- **Side:**
  - Detected at 90 degrees.
  - Granularity and complexity of cells.
Fluorochromes

- **Fluorescence** is absorption of light of lesser wavelength and then emission of light of longer wavelength.
- Fluorochromes are tagged to monoclonal antibodies.
- These absorb the 488nm light from the laser source and emit lights of longer wavelengths.
- Commonly used ones are FITC, PE, PerCP, PE-Cy5 etc.
<table>
<thead>
<tr>
<th>Fluorochrome</th>
<th>Excitation Wavelength</th>
<th>Emission Wavelength</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC</td>
<td>488</td>
<td>530</td>
</tr>
<tr>
<td>Phycoerythrin (PE)</td>
<td>488</td>
<td>580</td>
</tr>
<tr>
<td>PE-CY5</td>
<td>488</td>
<td>620/670</td>
</tr>
<tr>
<td>Propidium iodide (PI)</td>
<td>488</td>
<td>620</td>
</tr>
<tr>
<td>Peridinin chlorophyll (PerCP)</td>
<td>488</td>
<td>670</td>
</tr>
<tr>
<td>Allophycocyanin</td>
<td>633(HeNe)</td>
<td>670</td>
</tr>
<tr>
<td>CY5</td>
<td>633(HeNe)</td>
<td>670</td>
</tr>
</tbody>
</table>
Fluorescence Emission Spectrum
Fluorochromes do not emit at one wavelength, but rather in a spectrum.

This results in some overlap (or bleeding) of the emissions of one fluorochromes into the other, which cannot be eliminated by optical filters.

The fluorescence from FITC gives a weak signal in the optical path used to detect PE. This “bleeding over” is corrected by compensation.

Compensation is an electronic method that subtracts a percentage of the fluorescence signal determined to be due to spectral overlaps.
Compensation
Light detectors and separators

- **Photodiodes** detect intense light like forward scatter and convert them into electronic signals.

- **Photomultiplier tubes**: Very sensitive and detect weak signals and amplify them into electronic signals.
Filters

- **Long pass filters** transmit wavelengths above a cut-off wavelength
- **Short pass filters** transmit wavelengths below a cut-off wavelength
- **Band pass filters** transmit wavelengths in a narrow range around a specified wavelength
Example Channel Layout for Laser-based Flow Cytometry
Electronics

- **Digital**: Analogue to Digital Converter (ADC). This can be read directly to the display on the oscilloscope or computer screen as graphics and numerical.

- **Software**: Different programs are available as Cell Quest Pro, Modfit, Simulset, CD4/8, Retic Count, HLA-B27.

- **Hardware**: Stores acquired data.
Diagrammatic representation of working of a flow cytometer

- Fluidics
- Optics
- Electronics
Procedure

- Specimens - peripheral blood, bone marrow and body fluids
- Processed within 4-6 hrs of sample collection
- Make a single cell suspension of mononuclear cells
- Cell lysis method: NH4cl based solution
- Add fluorochrome tagged antibodies
- Incubate
- Preparation of cytospin smears for morphological correlation
- Sample is brought to FCM
- Acquisition
- Analysis
- Data interpretation along with morphological correlation
- Dispatch of report

Turn around time: 1 day
Factors before FCM analysis

• **Manual Differential cell counts** before processing.

• **Cytospin spots.**

• **Blast percentage** as a guide in *gating* during the FCM analysis.

• **Viability** count also should be made prior to staining, as dead cells are more likely to stain nonspecifically than viable cells.
Gating

- **FSC v SSC**
  - based on size & granularity of the cells.

- **CD45 v SSC**
  - based on dim positive blasts
Gating

• A major asset of this technology is the ability to separately analyze discrete subpopulations within a single acquired specimen.

• Thus, samples with mixed populations of cells (such as leukocytes from peripheral blood) may be stained and interrogated by flow cytometry with each subpopulation of cells (e.g., lymphocytes, monocytes, and granulocytes) being analyzed separately.

• Gating can be done either during acquisition or during analysis of listmode files.
Primary panel for Acute Leukemia

- **Lymphoid:**
  - B-cells: CD10, CD19.
  - T-cells: CD3, CD4, CD7, CD8

- **Myeloid:** CD13, CD33, CD117, CD14.

- **Non-lineage:** HLA-DR, CD34, TdT

- **Positive Control:** CD45 (LCA)

- **Negative Control:** Isotype IgG1
Secondary panel for Acute Leukemia

- B-lineage specific: cytoCD22 / cytoCD79a
- T-lineage specific: cytoCD3, TCR
- Myeloid lineage specific: anti-MPO
- Other Markers: CD41, CD61 and Glycophorin A, CD56.
- Plasma cell markers: CD 38, CD 138
Two Colour Analysis
2 parameter dot plot

[Graph showing dot plot analysis of CD45, CD19, CD45, CD3, CD19 PE, CD3 FITC, Lym, Mon, Gran]
2 Parameter Scatter Plots

- Single Positive PI Population
- Double Positive Population
- PE FL
- Negative Population
- FITC FL
- Single Positive FITC Population
Co-expression of two markers on single cells
CD19 - R-PE.CY.5
Lambda - PE

CD79b - PE
FMC7 - FITC

File: 006
Quad  Events  % Gated
UL    9      0.18
UR    4716   93.87
LL    264    5.25
LR    35     0.70

File: 007
Quad  Events  % Gated
UL    553    10.99
UR    55     1.09
LL    4184   83.13
LR    241    4.79

CD5 +  93%
CD19 + 92%
CD20 + 95%
CD23 + 99%
SmIg + 99%
Lambda+ 94%

Diagnosis: CLL
Quality Control

• Alignment & calibration of FCM
  Fluorescent beads
  Adjustment of PMT settings
• Maintenance of a historical database
  Distribution of a marker on normal cell population

• Negative control
• Positive control

• Sample handling & preparation
  Collection & transportation of specimen
  Manual differential cell counts
  Cytospin smears
Advantages of FCM in Immunophenotyping

- High degree of efficiency, sensitivity and reproducibility.

- Can detect scanty neoplastic cells in a heterogeneous population, which can further be increased by various types of gating.

- Large number of cells can be assessed very rapidly.

- Multiple parameters can be simultaneously studied especially useful in detection of MRD.

- Retrieval of stored data is possible.
Why to do FCM in all cases of leukemia?

• In spite of detailed morphological and cytochemical studies, it is not possible to diagnose and subtype all the cases of leukemias.

• The distinction is important because prognosis and therapy of various hematological malignancies are significantly different.
Acknowledgments

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