Flow cytometry: Principles and Applications

CME in Hematology 2014
Pune

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My talk

Diagnosis of leukemia / lymphoma
FCM: principles and applications
FCM: Issues and troubleshoots
Diagnosis of leukemia / lymphoma
FCM: principles and applications
FCM: Issues and troubleshoots
Diagnosis of leukemia / lymphoma
Tumor cells may be mature looking or of blastic morphology.
Some blasts are classical....
like...
Others are semi classical....
And others could be complicated..
Leukemia lymphoma diagnosis
Leukemia lymphoma diagnosis

• Morphology
Leukemia lymphoma diagnosis

- Morphology
- Ancillary techniques
Leukemia lymphoma diagnosis

• Morphology
• Ancillary techniques
  - Immunophenotyping (IHC and FCI)
Immunophenotyping

Neutrophils CD13+, CD16+

BCL2
Leukemia lymphoma diagnosis

- Morphology
- Ancillary techniques
  - Immunophenotyping
  - Cytogenetics
Cytogenetics

Interphase FISH: BCR/ABL fusion

t(9;22)

der(15):
PML/RARA

der(17)
Leukemia lymphoma diagnosis

• Morphology
• Ancillary techniques
  - Immunophenotyping
  - Cytogenetics
  - Molecular diagnostics
Molecular Diagnostics

NED (Black peak): NPM gene
6 FAM (Blue peak): FLT 3 gene

NPM
169 bp

FLT3
Peak 1: 349 bp
Peak 2: 412 bp
Difference: 63 bp
What is the role of ancillary techniques??
Role of a Pathologist

- Diagnostic label
Role of a Pathologist

• Diagnostic label
• Prognostic marker
Role of a Pathologist

• Diagnostic label
• Prognostic marker
• Predictive markers
Role of a Pathologist

• Diagnostic label
• Prognostic marker
• Predictive marker
• Minimal residual disease (treatment effectiveness)
Thus all these ancillary techniques help in...

- Diagnosis
- Prognosis / Risk stratification
- Prediction
- Treatment effectiveness (MRD detection)
Most important...

Morphology + Immunophenotyping
Diagnosis of leukemia / lymphoma

Flow cytometry principles and applications

Issues and troubleshoots
IPT helps in subtyping the lineage of the tumors...
What is Immunophenotyping?

• Uses antibodies to identify, locate, and stain specific protein molecules in tissue or in fluids.

• Reaction visualized by a marker (fluorescent dye, enzyme, colloidal gold etc)

• Diagnosis, sub-typing, prognosticating and as a predicting marker of therapeutic response.
Methods for IPT...

Immunohistochemistry
Flow cytometry
Immunofluorescence
**Immunohistochemistry:**
Histopathology, paraffin embedded biopsy

**Flow cytometry:**
Peripheral blood, bone marrow aspirate, body fluids
Lymph node biopsy and aspirate
IHC by Routine Microscopy -
bio{common sens, text is too crowded to read}

architecture
cytology
mostly single color

FCM - fluids
multicolor immunophenotyping
IHC

- Popular in solid tissues
- Subtype tumors
- Architecture + cytology
- But is mostly single color
Some markers best on IHC like epithelial markers, RCT panels

Others on FCM like FMC7, HCL markers
C-erbB2 and Herceptin

Predictive markers

C-kit and imatinib in GIST
IHC is a must in lymphoma diagnosis
Example - DLBCL

• Commonest type of lymphoma

• 50% get cured, rest 50% not...

• Waste basket

• Can we differentiate good ones from bad ones
CD20 and Rituximab

Standard of diagnosis
Predictive markers
Hans Algorithm - DLBCL

CD10

- BCL6

- Non GCB

+ GCB

+ Mum1

- GCB

+ NonGCB
CD 20

CD 3

CD 10

bcl6

bcl2

Mum1

Diagnosis

Mib1+ >80%
Advantages of IHC - architectural relationships and ability to detect scanty tumor cells, as in HL or TCRBCL.

Some antibodies may be better evaluated in paraffin tissue (eg, **CyclinD1**, CD15, and the presence of Bcl-2, Bcl-6, cyclin D1, ALK-1, and cytoplasmic kappa and lambda).

Likewise, some markers work better on FCI (CD13, CD14, CD19, CD33, etc). Rare markers for BPDCA etc.

**FCM** is important for fluids.............
2. FCM
1953 - The first **impedance-based** flow cytometry device, using the coulter principle (Wallace A Coulter).

1968 - The first **fluorescence-based** flow cytometry device (ICP 11) by Wolfgang Göhde, University of Munster.
Measurement of cellular properties as the cells (or nuclei, microorganisms, chromosomes, and latex beads) move in a fluid stream, *past a stationary set of detectors (thousand events per second)*

It analyses
- physical, as well as
- chemical properties (immunofluorescence)

Quantitative single cell analysis
Fluorescent dyes may bind or intercalate with different cellular components such as DNA or RNA.

Additionally, antibodies conjugated to fluorescent dyes can bind specific proteins on cell membranes or inside cells.

Commonly used dyes include PI, PE, FITC, although many other dyes are available. Tandem dyes with internal fluorescence resonance energy transfer can create even longer wavelengths and more colors.
Components of a Flow Cytometer

- Fluidics: a flow cell with sheath fluid (hydrodynamic focussing)
Components of a Flow Cytometer

- **Fluidics**: a flow cell with sheath fluid (hydrodynamic focusing)

- **Optics**: LASERS, single wavelength, coherent light (however incoherent light is of random phase varying with time and position)
Components of a Flow Cytometer

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• a detector and Analogue-to-Digital Conversion (ADC) system - which generates FSC and SSC as well as fluorescence signals from light into electrical signals that can be processed by a computer
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• an amplification system – linear or logarithmic
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- an amplification system – linear or logarithmic

- a computer for analysis of the signals
Sample preparation

Draw blood from subject

Separate mononuclear cells using a Ficoll gradient

Cryopreserve

Stain with fluorescent antibody conjugates
Sample preparation

- Draw blood from subject
- Separate mononuclear cells using a Ficoll gradient
- Cryopreserve

Instrument setup

- Baseline PMT voltage determination
- Optimize fluorescence detector sensitivity
- Stain with fluorescent antibody conjugates
1. Physical properties

Scatter pattern
Forward scatter

Size

Side scatter

Granularity
LASER Sample in a hydrodynamically focused stream

Detectors

Amplification and computer

Scatter only
2. Chemical properties

FCI
Laser

Sample in a hydrodynamically focused stream

Scatter plus FCI

Amplification and computer

Detectors
Sample in a hydrodynamically focused stream

Laser

Focal Optics

Cell Sorter

Detectors

Amplification and computer

PMT

Detector

Dichroic Filter

Lens and Filters

Spectral Cells

Solid Cells

LASER
Data shown either as a

- single parameter histograms, or
- two parameter correlated plots

Data may be shown as

• Linear scale  The scale on which the output is directly proportional to the input.

• Logarithmic scale  The scale on which the values increase logarithmically.
DNA ploidy

Count

256
128

384
256
128

S phase

DNA content, linear scale

G₀G₁

G₂M

Overlay Plot

Overlay Plot

[CD45 Lymph]
[ALL Blasts]

DNA ploidy
Two parameter (dot plot), no dye, linear scale
Two parameter (dot plot), dye, linear versus log scale
Multiple parameter (dot plots), multiple dyes, FCI, linear/log scale.
Types of FCM

- Single Laser or Multiple Lasers
  (1 laser three color, 4 lasers 18 fluorescence detectors)

- Sorter (so as to purify populations of interest)

- Laser scanning cytometers
Advantages of a FCM

• Study of cells, chromosomes and particles (analysis, counting and sorting)

• Thousand of particles per second

• Multiparametric analysis at a single cell level

• Pattern studies

• Sorting
Research Applications

- Autofluorescent Proteins
- Antigen or Ligand Density
- Apoptosis
- Enzyme activity
- DNA, RNA content and changes in the cell cycle
- Membrane Potential
- Cytokine receptors and it's synthesis
- Drug uptake and efflux
- Phagocytosis
- Viability
- Changes in Intracellular pH
- Changes in Intracellular calcium
- Changes in Intracellular glutathione
- Changes in Oxidative Burst
- Drug discovery and vaccine development
Diagnostic Applications

1. Monitoring AIDS patients
2. Immunophenotyping
3. Monitoring MRD
4. CD34 counts
5. Reticulocyte Counts
6. PNH
7. DNA analysis of S-phase fraction
8. Platelet counts
Start a clinical cytometry facility
Do we really need one? Centralized labs

Hospital / institute based or a stand alone lab

Reagents/maintenance expensive

Stake holders:
Management support
Cytometrist/Pathologist/Scientist
Oncologist/Hematologist support
Vendor support
Clinical cytometry
RBCs
WBCs
Platelets
Others

Cytoplasmic/nuclear characters
WBCs

Lysis of red cells
Acquire WBCs without any antibodies
FCM - Based on scatter pattern

- Lymphocytes
- Monocytes
- Neutrophils
Scatter pattern, FSC vs SSC
Cells of interest
Special gating procedures can help separate tumor cells from normal lymphocytes.

CD45 gating for blasts

CD19 gating for B cell lymphomas
Cells of interest and different types of Gating

1. FSC and SSC
2. CD45 and SSC
3. CD19 and SSC
4. CD3 and SSC
5. others
Normal Peripheral blood
Forward vs side scatter

Normal Bone marrow
Forward vs side scatter
Gating

Scatter pattern, FSC vs SSC
Gating

Scatter pattern, FSC vs SSC
Gating
Scatter pattern, FSC vs SSC
What is an abnormal pattern??
What is an abnormal in this case?
Where do the blasts of acute leukemia appear in scatter pattern plot??
Where do the blasts of acute leukemia go??
Lots of blasts??

Scanty blasts??

Side

Forward
Where do the tumor cells of Hairy cell leukemia go?
Where do the hairy cell leukemia go in flow plots?
How to separate cells of interest..

Special gating procedures can help separate tumor cells from normal lymphocytes
Lysis of red cells

CD 45 gating for blasts

Similarly CD19 gating for HCL tumor cells

Add Antibodies e.g., CD45
Special gating based on CD45..
WBCs

- Neutrophils
- Lymphocytes
- Monocytes
- Eosinophils
- Basophils

Very few stem cells (more in BM)
Normal BM – CD45 gating
CD45 Gating in BM

So many clusters
CD45 Gating
CD45 Gating
CD45 Gating
CD45 Gating
CD45 Gating
X axis vs Y axis
CD45 Gating
CD19 Gating, new case of CLL

CD19 Gating, CLL, post treatment
T cell lymphocytosis

60-75% of lymphoid cells in peripheral blood are T-cells
Cells of interest may be scanty
When to call it positive?
What is positive?
What is positive?
Can we differentiate these dots....
gamma delta T cells

alpha beta T cells and monocytes

B-cells

Other cells

CD19+ : 9.102%

Non B Cells : 84,287
## Fluorochromes

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<th>Dye</th>
<th>Excitation</th>
<th>Emission</th>
<th>Molecular Weight</th>
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<td>520 nm</td>
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<tr>
<td>PE</td>
<td>488 nm</td>
<td>578 nm</td>
<td>240 000 Da</td>
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<tr>
<td>ECD</td>
<td>488 nm</td>
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<tr>
<td>PC</td>
<td>488 nm</td>
<td>668 nm</td>
<td>105 000 Da</td>
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<td>PerCP</td>
<td>488 nm</td>
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<tr>
<td>APC</td>
<td>613 nm</td>
<td>665 nm</td>
<td>105 000 Da</td>
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</table>

Each antibody is tagged with a different fluorochrome.

**Tandem dyes**
FCM - Applications
1. Multicolor Immunophenotyping
What is Multicolor IPT? 3 or more colors

Single laser: 3-4 colors
Two lasers: 6 colors
Three lasers: 8 colors plus

More lasers - more colors, more antibodies, more fluorochromes, tandem dyes, better data, more information, flexibility, however, more issues
Single color means one antibody

Two color means two antibodies
4 color with CD45 gating

*CD45 as a tracking marker*

- Myeloid tube
- T cell tube
- B cell tube
- MDS tube
- Other tube

Myeloid tube: CD45
T cell tube: CD45
B cell tube: CD45
MDS tube: CD45
Other tube: CD45

Four colors
Immunophenotyping helps in detecting:
Immunophenotyping helps in detecting:

- presence or absence of an antigen
- intensity of expression
- presence of blasts
- clonality: LCR, V-beta repertoire
- scanty cells
- maturation patterns

CD3 antibody by FCM detects the fully assembled TCR-CD3 complex, which is present on the surface of T cells only.

In contrast, the CD3 IHC stain usually detects only the epsilon component of CD3, therefore cannot distinguish between T & NK cells.
B-cell clonality
Clonality of B cells
Polyclonal B cells
Monoclonal B cells
T cell normal and abnormal
Normal T cell subsets

70% of lymphoid cells in peripheral blood are T cells
Antibodies used for Leuk/Lym diagnosis

- Acute leukemia
- Lymphoma/CLPDs
Reagents required in acute leukemia

T-cell tube – CD45, CD2, CD3, CD4, CD5, CD7, CD8
B-cell tube – CD45, CD19, CD20, CD10,
Myeloid cells – CD45, CD13, CD33, CD117
Myeloid tube – CD45, CD14, CD16, CD64
Cytopl. tube – CD45, Tdt, cCD3, cCD79a, cCD22, AntiMPO
Others – CD45, CD34, HLADR and so on
Reagents required in lymphomas

Screening tube -
B-cell tube – CD45, CD19, CD20, CD10, kappa, lambda
CLL tube – CD45, CD19, CD20, CD5, CD23
T-cell tube – CD45, CD2, CD3, CD4, CD5,CD7, CD8
HCL tube: CD45, CD19, CD11c, CD25, CD103, CD123
NK cell tube: CD45, CD3, CD16, CD56, CD8
PC tube: CD45, CD38, CD138, cytoK, cytoL, CD56, CD19
Others....
Indian Guidelines
Approach to Acute Leukemia and CLPDs

IJPM, 2008
How to make panels?

- literature based,
- training based,
- trial or hunch based
• Panels are decided based on morphology and clinical indications

• 4-8 color FCI
Let us construct a 4 color AL panel
Four color AL panel

a cocktail of antibodies of similar lineage...

CD3 - FITC
CD4 - PE
CD8 – PerCP
CD45 – TR

a cocktail of antibodies of different lineages...

CD3 - FITC
CD19 - PE
CD13 – PerCP
CD45 – TR
Four color panel

- CD3 - FITC
- CD19 - PE
- CD13 – PerCP
- CD45 – TR

T-cell tube
- CD3 - FITC
- CD4 - PE
- CD8 – PerCP
- CD45 – TR

a cocktail of antibodies of different lineages

- CD3 - FITC
- CD19 - PE
- CD13 – PerCP
- CD45 – TR
# Four color panel - 4/5 tubes

<table>
<thead>
<tr>
<th>T-cell tube</th>
<th>B-cell tube</th>
<th>Myeloid- tube</th>
<th>Miscellaneous</th>
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<td>CD8 – PerCP</td>
<td>CD10 - PerCP</td>
<td>CD117 – PerCP</td>
<td>CD7 – PerCP</td>
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# 8 Color Acute Leukemia Panel

<table>
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<tr>
<th>Tube No.</th>
<th>V500</th>
<th>Brilliant Violet 421</th>
<th>FITC</th>
<th>PE</th>
<th>PerCP-Cy5.5</th>
<th>PE-Cy7</th>
<th>APC</th>
<th>APC-H7</th>
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<td>CD10</td>
<td>CD19</td>
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<td>CD10 341092 HI10a 100 tests Mouse IgG1</td>
<td>CD133 130-090-826 AC133 100 tests Mouse IgG1</td>
<td>CD20 641396 L27 100 tests Mouse IgG1</td>
</tr>
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</table>
Comprehensive panel of antibodies used for L/L diagnosis
Case 1
5 year old boy with fever – 1 month

Classical 3-4 color
Diagnosis
MPO negative

NSE negative

Diagnosis
FSC vs SSC

3 color IPT – Common practice
File: 6

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<th>Quad</th>
<th>Events</th>
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HLA-DR + 84%

CD10 + 98%

CD19 + 98%
CD10+, CD19+, HLADR+

B cell ALL, Burkitt’s Lymphoma

FL, DLBCL
CD10+, CD19+, HLADR+

- B cell ALL, Burkitt’s Lymphoma
- FL, DLBCL
- Hematogones (Tdt+/-)

Thus morphology, adequate panels with patterns analysis, cytogenetics and clinical history
Case 2

12 year old girl, presented with fever for 2 weeks.

*Peripheral blood smear reveals high counts with blast like cells*
Acute leukemia
cytochemical MPO negative

What next?
FCI done

B cell tube – Negative
Myeloid tube – Negative
All T cell markers including CD3 – Negative

Blasts expressed only CD7
T-cell markers
- CD3
- CD7
- CD2
- CD5
- CD4/CD8
What do you next?
Do cytoplasmic markers
Diagnosis

T-cell ALL

Note: Blasts may be surface CD3 negative
Tissue flow
45 year old male,
left cervical LN since 1 month

Lymph node FNAC done
45 year old male, LN - fnac
Issues of 3 color IPT

Diagnosis: Mantle Cell Lymphoma
Subtyping of Small B cell lymphomas
Nodular pattern
Common B cell NHLs expressing CD5 (T-cell marker)

1. CLL – express CD23
2. MCL – express cyclin D1 (*not by flow*)
Lymphoma Diagnosis

Gold standard is biopsy plus IHC
FCM and lymphoma diagnosis

- Best for CLPDs
- Mostly B cell type and panels are well defined
- Also used for lymph node biopsy, aspirate, fluids and other tissues
T cell lymphomas are rare

Need elaborate panel

• Altered expression of pan T cell markers like CD2, CD3, CD5 and CD7 (also seen in IM, CMV)

• Subset restriction of CD4 or CD8 (also seen in IM, HIV, AID)

• Increased expression of markers like CD25 with/without CD4

• Aberrant expression of CD10, CD30, CD103, Tdt, Alk1 etc.

• Aberrant expression of CD16, CD56

• V beta repertoire

• HTLV-1 serology, PCR for TCR gene rearrangements
Lymphoma Screening Tube

- 15 antibodies in one tube
- Mutually exclusive Abs
Lymphoma screening tube
15 color panel

CD45 Alexa 700
CD38
CD19+TCR GD V421
CD3+CD14 V500
CD5 PC7
CD4+ CD20
CD7+Cyto lambda PE
CD8+ cyto Kappa FITC
CD10 APC
CD34 ECD
CD38 Alexa750
Plasma cell Neoplasms
8 color Myeloma panel

Gating strategies
First tube for Myeloma, TMH

- CD38 FITC
- CD19 V450
- CD20 APCH7
- CD27 PE
- CD28 APC
- CD45 V500
- CD117 PEcy7
- CD56 PerCP cy5.5
Second myeloma tube, TMH

- Cyto Lambda FITC
- Cyto Kappa PE
- CD45 APC H7
- CD38 PerCP-Cy5.5
- CD19 PE-Cy7
- CD56 APC
- Blank
- Blank

PG Subramanian
Case of plasma cell dyscrasia (eight color)