

**Flow Cytometry in the Clinical Laboratory:
General Immunophenotyping Considerations**





Raymond E Felgar, MD, PhD

Director, Hematopathology and Flow Cytometry Lab

University of Rochester Medical Center

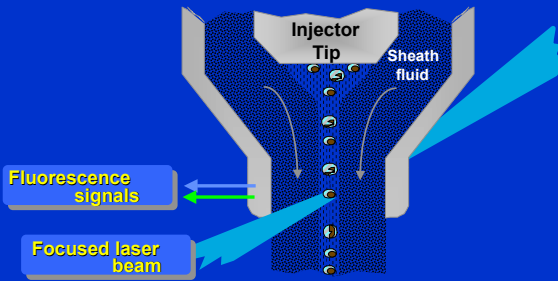
Topics to Cover

- Review basics of flow immunophenotyping
- Review some of the basic markers and principles of hemepath-related diagnostic markers
- Discuss basics of panel design, with examples from our lab
- Review some sample flow analyses (recent cases our lab)
- Review basics of data storage

Basic Principles of Lymphoma/Leukemia Typing

- Cells are labeled with fluorochrome signals via tagged antibodies
- Cells are run through a fluidic stream so that single cells can be analyzed one at a time
- Multiple parameters are collected on each cell:
 - Forward angle light scatter (cell size)
 - 90 degree light scatter (Side-Scatter, Cell Granularity)
 - Fluorescent signal information (FL1, FL2, FL3, FL4, etc.)
 - Most clinical labs use up to 4 color analysis (5 or 6 soon?)
 - Research applications can now allow up to 14 color analysis

Flow Cell



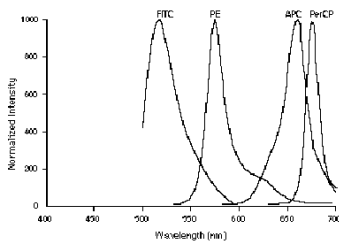
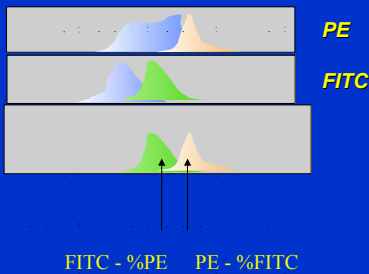


Figure 3-4 Emission spectra of the fluorochromes shown in Figure 3-3

Basic Principles of Lymphoma/Leukemia Typing: Compensation

- Unfortunately, in reality, emission signals overlap and therefore may not be detected as single, distinct signals or be counted as two different signals
- To correct for this, a portion of each overlapping signal is subtracted from the other overlapping signal
- This concept is known as compensation and can be done by the computer software (most Coulter instruments) or by the flow cytometer prior to signal storage in listmode (most BD instruments)

Compensation Illustrated



Data Display and Analysis

- Data can be analyzed and displayed in a variety of formats:
- Single parameter histograms (signal intensity, relative quantitation of antigen expression)
- Dot Plots (Display distribution of 2 antigens on an x-y plot)
- 3D plots (2 D dot plot with number of events on z axis)
- 3D plots of 3 antigens (cloud plot, attractor plot)

Data Display and Analysis

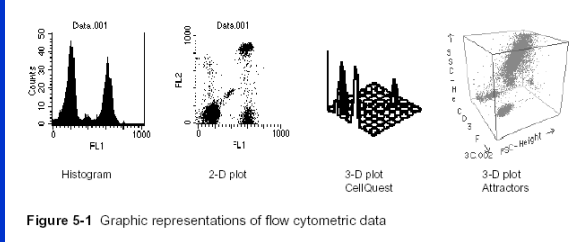


Figure 5-1 Graphic representations of flow cytometric data

Lymphoma and Leukemia Immunophenotyping: Basic Principles

- First and foremost, assign probable cell lineage (B, T, or myeloid)
- Within B or T cell lineage, assign to most likely corresponding normal cell type (e.g. follicular, marginal cell, mantle cell) or stage of differentiation (precursor B, precursor T)

Lymphoma and Leukemia: Lineage Markers

- B-Cell:
- CD19, CD20, CD22
 - Clonality: All kappa or all lambda
- T-Cell:
- CD2, CD3, CD5, CD7
- Myeloid:
- CD13, CD33, CD117 (c-kit)

Markers of Immaturity

B-Precursor:

- TdT, cCD22, lack of surface immunoglobulin, CD10 (in right context), CD34

T-Cell:

- CD1a, lack of surface CD3 with cytoplasmic CD3, CD4-/CD8- dual neg, CD4+/CD8+ dual pos, CD34

Myeloid:

- CD117 (c-kit), CD34, presence of HLA-DR

Data Display and Analysis: Gating

- Choose subpopulations of cells to study based on common parameters measured on all cells
- Can be based on light scatter (FSC, SSC), fluorochrome signal (CD45 for leukocytes, CD19 for B cells, etc.) or combination of above
- Two most common methods:
 - FSC vs SSC (Good for uniform populations such as tissue suspensions, cell lines)
 - CD45 vs SSC (Good for blood and bone marrow)

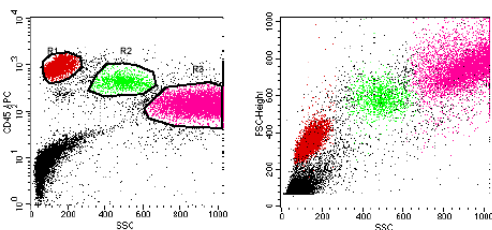
Data Display and Analysis: Gating

BLOOD

R1 = Lymphocytes

R2 = Monocytes

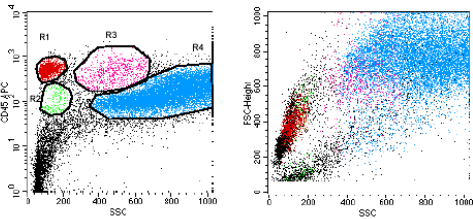
R3 = Granulocytes



Data Display and Analysis: Gating

BONE MARROW

R1 = Lymphocytes R2 = Blasts R3 = Monocytes R4 = Granulocytes and Myeloid Precursors



Data Display and Analysis: Choice of Panels

- Factors to consider when deciding what panels to set up:
 - Informative antigen pairs
 - CD19 or CD20 with kappa and lambda to look for B-cell clone
 - Pan-T cell markers to look for abnormal T cell population (CD2, CD3, CD5, CD7)
 - Relative signal intensities of fluorochromes (APC = PE > FITC > PerCP (or PerCP-Cy5.5))
 - Cost (Low sample volumes may preclude large stock of 4 color reagents)

Data Display and Analysis: Choice of Panels

- Other factors to consider:
 - Nature of samples to be run
 - Lymph nodes and tissues may not generally need CD45 based gating-light scatter sufficient
 - BMs and Bloods – CD45 gating more useful
 - Types of cases (i.e. diagnoses encountered in local practice)
 - Case volume (Some labs with large sample numbers run one large panel on everything)
 - Expertise of technical staff
 - Do you want to use automated tube loader and/or stainer technologies? (Up front standardization saves on run time.)

General Principles for Clinical Flow Labs

- Other general principles of clinical flow:
 - In general, the more standardized possible, the better
 - Avoid making home-brew solutions
 - Most expensive cost is usually technical labor

Panels

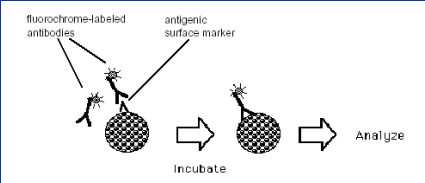
- BASIC PANEL (FITC/PE/PerCP/APC):**
- ___ IgG1 F/IgG1 PE/IgG1 PerCP/IgG1 APC
 - ___ Fab Fite/IgG1 PE/---/IgG1 APC
 - ___ CD3/CD19/CD14/CD45
 - ___ CD3/CD4/CD8/CD45
 - ___ CD7/CD5/CD3/CD45
 - ___ CD33/CD13/CD34/CD45
 - ___ CD57/CD16/CD3/CD45
 - ___ Kappa/lambda/CD19/CD45 _____ Wash and incubated
 - ___ Kappa/CD19/CD14/CD45
 - ___ Lambda/CD19/CD14/CD45

Panels

- Lymphoma Panel Add-ons:**
- B CELL LYMPHOMAS AND CHRONIC LEUKEMIAS:**
 - CD20/CD5/CD19/CD45
 - CD20/CD10/CD19/CD45
 - CD20/CD23/CD19/CD45
 - FMC-7/CD20/CD19/CD45
 - CD20/CD22/CD19/CD45
 - CD5/CD38/CD19/CD45
 - Hairy Cell Leukemia Screen:**
 - CD103/CD11c/CD19/CD45
 - CD20/CD25/CD19/CD45
 - T Cell Lymphoma/Leukemia:**
 - TCR $\alpha\beta$ /TCR $\gamma\delta$ /CD3/CD45
 - CD3/CD5/CD8/CD45
 - CD3/CD2/CD45
 - CD7/CD1a/CD3/CD45
 - CD5/CD25/CD3/CD45
 - CD5/CD30/CD3/CD45

Staining Methods

-Generally use direct stain, lyse, wash methods for surface marker analysis using directly labeled antibodies



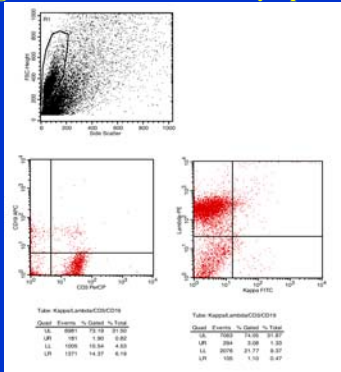
Staining Methods

- Analysis can then be done on fresh samples immediately after staining
- Or (preferably) samples can be lightly fixed (1% formalin or paraformaldehyde) and analyzed immediately or up to several days after staining
- Advantage of fixation is that it stabilizes light scatter properties and prevents patching and capping

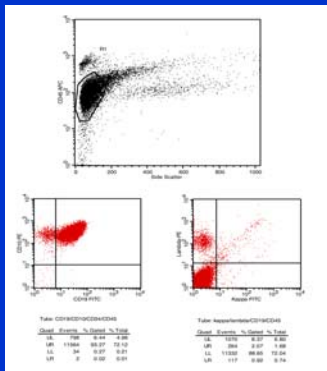
Staining Methods: Cytoplasmic

- Cytoplasmic or nuclear antigens (myeloperoxidase, TdT) can be assessed
- First, stain for desired surface markers
- Fix cells (10% formalin) for 15 mins.
- Wash
- Add cytoplasmic antibody with a permeabilization reagent (weak detergent)
- Wash again, store in light fixative, analyze

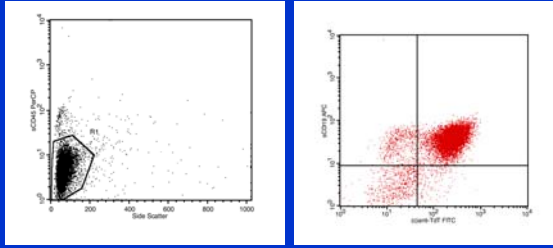
Sample Cases: Clonal B-cell (Single tube- Gastric MALT lymphoma)



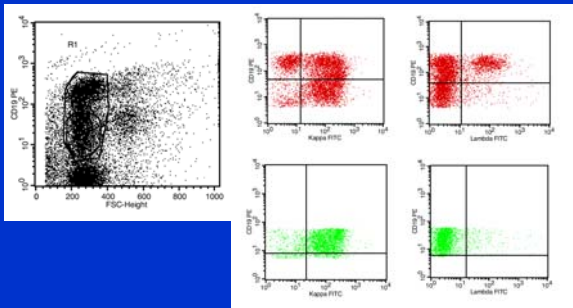
Sample Cases: B-precursor ALL (BM)



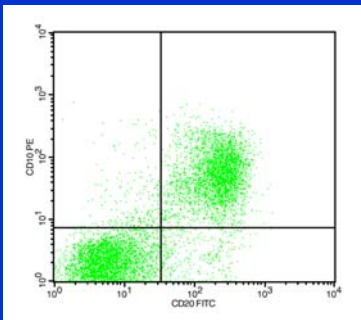
Sample Cases: B-precursor ALL (Extramedullary – Testicular Mass)



Follicular Lymphoma

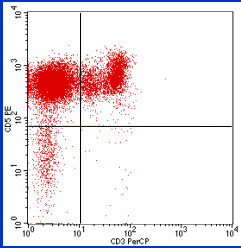


Follicular Lymphoma (Gate = CD19 dim cells)

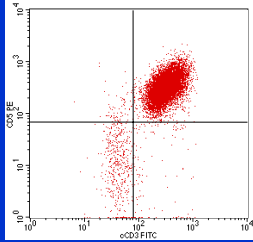


Sample Case: T-Lymphoblastic

Partial Surface CD3+



Strongly Cytoplasmic CD3+



Data Storage

- In general, all data points for each tube analyzed are saved as a single data file (listmode file)
- All listmode files are grouped together with analysis file and saved on hard drive
- For long-term data storage and archiving, data should be transferred to magneto-optical, optical or tape media

Data Storage

- Storage Media
 - Magneto-Optical Drives:
 - Heats media with laser, saves data electromagnetically
 - When media cools, data “locked” into medium
 - Advantages are large capacity (5 GB per disk), can be written over or erased (if needed)
 - Disadvantages: Cost (\$1600 or more for one drive), not easy to replace or to have alternate reader
 - CD-R disks
 - High quality CDs theoretically can store data for Decades (or longer), easy to access data
 - Capacity (700 MB per disk, about 3 weeks data for our lab)

Data Storage

- Storage Media
 - DVDs
 - Similar to CDs, Higher capacity (4.5 GB, 4 months of data)
 - Tape Drives
 - Useful for long term storage
 - High capacity, relatively low cost
 - Less flexible in retrieving data, not random access device

Data Storage and Retrieval

- If using different platforms (eg. Both BD and Coulter), may need software to convert data files to standardized format
- Alternatively, some non-vendor software programs can handle either BD or Coulter generated data (WinList software, Verity House, available in both PC and Mac formats)
- Some software packages (WinList) also allow for compensation correction (if data are undercompensated)

Summary

- Flow cytometry can provide useful information about tumor cell lineage, stage of differentiation, probable normal cell counter part
- Choice of panels and methods of gating critical to data interpretation
- Data can be stored long-term for later reanalysis
- Operation of a successful clinical flow cytometry lab requires a good working knowledge of software capabilities, data storage devices, and general types of cases

Ciao!