10-Color Flow Cytometry for the Efficient Diagnosis of Lymphoid Neoplasms

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Greetings from Seattle!
Overview

• Rationale for 10-color flow cytometry (FC)

• Keys to setting up a 10-color FC laboratory

• 9- and 10-color immunophenotyping, with an emphasis on challenging cases
  • Immature lymphoid neoplasms
  • Mature lymphoid neoplasms
  • Plasma cell neoplasms

Why 10-Color Flow?

• Gets much more information from small samples (FNA, CSF)
  - 10-color yields 7.5x more info. than 4-color flow (45 vs. 6 dot-plots)
  - 10-color yields 3x more info. than 6-color flow (45 vs. 15 dot-plots)
  - 10-color yields 1.6x more info. than 8-color flow (45 vs. 28 dot-plots)

• Routinely collect > 100,000 viable cells (> 300,000 viable cells for MRD work), so identify small abnormal populations missed by lesser flow

• In even small biopsies, can almost always make definitive 2008 WHO diagnoses when combine flow with histology and limited IHC/FISH

• Streamlined specimen setup / reporting = FASTER turnaround

9/10-Color FC Implementation-1: Choice of Flow Cytometer

- Signed out 9-/10- color clinical flow data from BD LSRIIs at the U. of WA from 2004-5, so wanted at least 6 colors, with definite ability to expand to 8 colors, for lab efficiency and better handling of small specimens and MRD

- Not possible with the 6-color FACSCanto in May 2005


- Beckman-Coulter 3-laser/10-color Gallios available now
Implementation-2: Choice of Antigens (27) to Evaluate Routinely

B-cell Ags:  Kappa, Lambda, CD19, CD20, CD10, CD38, CD45
T & NK Ags:  CD2, CD3, CD4, CD5, CD7, CD8, CD56, CD16
Pan-myeloid Ags:  CD13, CD33, CD45
Myeloid blast-associated Ags:  CD34, CD117, HLA-DR, CD38
Granulocyte-associated Ags:  CD10, CD14, CD15, CD16, CD117, CD11b
Monocyte/dendritic cell-associated Ags:  HLA-DR, CD4, CD14, CD15, CD38, CD64, CD123, CD36, CD11b
Erythroid-associated Ags:  CD38, CD71, CD117
Implementation-3: Principles of Antibody Panel Design

- Goal: Maximize information obtained per unit of specimen, unit of cytometry staff effort, and unit of cytometer time
- Maintain across-tube comparability (e.g., CD45/SS gating in all) while minimizing antibody redundancy
- Pair strongly expressed antigens (e.g., neutrophil CD16) with dimmer fluorochromes (e.g., FITC, Alexa700), and vice versa
- Combine antibodies to maximize ability to identify abnormal cell populations (e.g., pairing CD34 and CD7 antibodies to look at blasts)
- All things being equal, choose IgG1 over IgG2 antibodies (see Wood BL & Levin GR. *Cytometry B Clin Cytom*. 2006;70:321-8)

Implementation-4: Fluorochrome Choice

- Availability: Single vs. tandem dyes, stock vs. custom reagents
- Excitation of dye: function of excitation spectra (related to size of dye) and laser type
- Measuring fluorescence of dye: function of emission spectra and optics of instrument

Implementation-5: Ab Panels in Practice

- Sought to screen B and T cells with 1 tube each, and myeloid cells with 2 tubes
- Sought a single main add-on tube for each of the following: CLL/MCL, HCL/MZL, plasma cells, B-ALL, T-ALL, AML
- Sought to maximize use of ASRs and off-the-shelf reagents, and minimize custom antibodies, hence original choice of Alexafluor 700 as “ninth” color in 2005
- Compromises often required for antibodies & fluorochromes

Lymphoid Panel in 2000:
4-Color Flow, Single Laser (13 Ags)

<table>
<thead>
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<td>CD5</td>
<td>CD56 /</td>
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Lymphoid Panel in 2010: 10-Color Flow (15 Ags + Viability Dye)

Implementation-6: Creating Antibody Cocktails

- Encourages use of standardized reagents within laboratory
- Time savings for technical staff
- Enhances assay-to-assay reliability by:
  - Reducing chances of pipetting incorrect antibody
  - Reducing chances of pipetting incorrect volume of antibody, as relatively large volumes used in cocktails
- Recent FDA limitations limit available ASR cocktails
**Ab Optimization / Cocktail Validation**

- Titrate antibodies individually, on appropriate cell population(s), to optimize signal:noise ratios

- Confirm similar antibody-associated fluorescence on singly-stained cells vs. cells stained with 9/10-color cocktails

- If already using fewer-color cocktails, confirm similar performance vs. 9/10-color cocktails

**Implementation-7: Assay Validation During Lab Set-up**

- Used ~50 normal peripheral bloods and ~20 well-defined hematolymphoid cell lines (from ATCC and DMSZ) to confirm expected immunoreactivity

- Cross validation on ~30 clinical specimens kindly provided by several outside laboratories, with uniform concordance with available outside flow interpretations

- Initial validation period: ~4 months from LSRII set-up
Data Analysis-1: General Gating

Doublet Elimination  FS/SS Gating  WBC Gating

Data Analysis-2: Lineage Gating

Viable Cells  Blasts:  B cells:  T cells:

CD45/SS  CD34/SS  CD19/SS  CD3/SS
Data Analysis-3: Compensation and Data Scaling

Case Studies: Thoroughly Understand the Normal in Order to Recognize the Abnormal

B-lymphoid Neoplasms

Normal Bone Marrow: B Cells and Plasma Cells
Case Study 1: 73 y.o. male with lymphocytosis
Final Dx 1: CLL/SLL-like Monoclonal B Cell Lymphocytosis (~2400/uL) with Trisomy 12 by FISH; FLOW KEY: CD200+


Case Study 2: 48 y.o female with pancytopenia, inaspirable marrow, and scanty BM biopsy
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Final Dx 2: Hairy cell leukemia;
FLOW KEY:
CD103+, CD25+

H&E
CD25
TRAP
Case Study 3: 72 y.o. female with right axillary lymphadenopathy, small biopsy
Final Dx 3: Follicular lymphoma, Grade 1-2; FLOW KEY: bcl-2+, low CD10+

Case Study 4: 21 y.o female with omental mass and scanty specimen
Case Study 4: 21 y.o female with omental mass and scanty specimen

Final Dx 4: Burkitt lymphoma, MYC+ by FISH; FLOW KEY: CD10+, CD38+, Bcl-2-negative
Case Study 5: 58 y.o. male with hx of myeloma
Final Dx 5:  t(11;14)+ plasma cell myeloma, positive for CD20 & surface light chain; FLOW KEY: CD138+, bright CD38+, low CD45+, low CD19+, CD22-negative


Case Study 6: 25 y.o. female with C7-T4 paraspinal mass

Initial IHC in SBRCT Workup

CD34  CD43
Case Study 6: 25 y.o. female with C7-T4 paraspinal mass

Second round of IHC to Establish Lineage (but CD45-)

CD79a

CD33
Final Dx 6: B-Lymphoblastic Lymphoma with Positive CSF and Minimal Marrow Involvement; FLOW KEY: CD45-neg., CD19+

Case Study 7: 35 y.o. female, s/p SCT for CHL
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Final Dx 7:
Relapsed CHL, CD4+;
FLOW KEY: CD45-neg

Reference: Asano N, et al.
J Clin Oncol. 2006;24:4626-33
T-lymphoid Neoplasms

Normal Thymus: Immature T Cells
Normal Bone Marrow: Mature T Cells

Case Study 8: 54 y.o. male with marked peripheral lymphocytosis
Case Study 8: 54 y.o. male with marked peripheral lymphocytosis

Final Dx 8: T-Prolymphocytic Leukemia, (inv[14]+);
FLOW KEY: Dual CD4+/CD8+ with MATURE CD34-/TdT-/CD1a- phenotype
Case Study 9: 62 y.o. female, R/O AML
Final Dx 9: Anaplastic large cell lymphoma, ALK+;
FLOW KEY:
CD30+, bright
CD45+, CD7+, cyto CD3+, aberrant CD13+


Case Study 10: 80 y.o. female with adenopathy
Case Study 10: 80 y.o. female with adenopathy

Final Dx 10: Angio-immunoblastic T Cell Lymphoma (missed by 6-color FC elsewhere);
FLOW KEY: CD4+, CD5+, surface CD3- & TCR-α/β-negative

CXCL-13
Case Study 11: 38 y.o. male, pleural fluid
Final Dx 11: T-Lymphoblastic Lymphoma (cytokeratin-negative mediastinal mass); FLOW KEY: Abnormally low CD1a and high CD7 for benign thymocytes

Non-Hematopoietic Neoplasms That Can Mimic Lymphoma
Case Study 12: Lung biopsy in 68 y.o. female
Final Dx 12: Small Cell Neuroendocrine Carcinoma; FLOW KEY: CD45-negative, strongly CD56+ population


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The End