Flow cytometry in clinical practice

Pitfalls, Advances, and Opportunities

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Outline

- Why?
- What?
- When?
- How?
- Who?

Why should I care about flow cytometry?

- Standard of care in diagnosis of hematolymphoid malignancy

What can flow cytometry do for me?

- Rapid whittling of the differential diagnosis in less than 4 hours.
- Example: Cervical Lymphadenopathy- ddx reactive, carcinoma, lymphoma, sarcoma
- Flow can tell you:
  - lymphoma, reactive, small cell carcinoma
  - B cell vs T cell lymphoma

When should I order flow?

It depends….

1. Lymphadenopathy without a clear primary
2. Leukocytosis of unclear cause (particularly lymphocytosis)
3. Leukopenia (with a relative lymphocytosis)
4. **Almost* any bone marrow biopsy

When should I not order flow?

(You may not know *a priori)*

1. Leukocytosis with a clear cause
2. Infectious etiologies
3. Non-heme malignancies
4. MDS/MPN in the peripheral blood
5. CSF fishing expedition – very low yield*

Evidence for CSF flow

- Screening in neurologic symptoms
  – sensitivity 13%
- Monitoring for CSF involvement post therapy
  – sensitivity 78-100%

ORIGINAL ARTICLE

Cerebrospinal Fluid Flow Cytometry: Utility in Central Nervous System Lymphoma Diagnosis

Ka Loong Kelvin Au, Sarah Latonas, Afshin Shameli, Iwona Auer, Christopher Hahn

ABSTRACT: ***Background:*** Flow cytometry of the cerebrospinal fluid (CSF) is used in isolation or as an adjunct to cytology to increase the sensitivity of detecting central nervous system (CNS) lymphoma. We aimed to evaluate the sensitivity of CSF flow cytometry as a diagnostic screening tool for primary CNS lymphoma in patients presenting with undifferentiated neurologic symptoms. ***Methods:*** We retrospectively reviewed all CSF samples received by the Calgary Laboratory Services Flow Cytometry Laboratory from 2012 to 2015. Clinical data, laboratory investigations, radiologic imaging studies, and pathological data were analyzed. Clinical review extended to 2 years post-CSF flow cytometric testing. ***Results:*** Only 43/763 (5.6%) samples of CSF flow cytometry in 28/573 (4.9%) patients were found to be positive for a hematological malignancy in patients with undifferentiated neurologic symptoms. The overall sensitivity of the test was 13.8% with 25 patients with negative CSF flow cytometry later having a positive biopsy for CNS lymphoma. CSF flow cytometry was negative in all cases when at the time of CSF examination the patient did not have a previous hematological malignancy or findings of abnormal enhancement on MRI ($n = 249$). ***Conclusion:*** CSF flow cytometry has low utility in screening for primary CNS lymphoma in the absence of a previous history of hematologic malignancy or findings of abnormal enhancement on MRI.

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Review Article

Flow Cytometric Characterization of Cerebrospinal Fluid Cells

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Flow cytometry facilitates the detection of a large spectrum of cellular characteristics on a per cell basis, determination of absolute cell numbers and detection of rare events with high sensitivity and specificity. White blood cell (WBC) counts in cerebrospinal fluid (CSF) are important for the diagnosis of many neurological disorders. WBC counting and differential can be performed by microscopy, hematology analyzers, or flow cytometry. Flow cytometry of CSF is increasingly being considered as the method of choice in patients suspected of leptomeningeal localization of hematological malignancies. Additionally, in several neuroinflammatory diseases such as multiple sclerosis and paraneoplastic neurological syndromes, flow cytometry is commonly performed to obtain insight into the immunopathogenesis of these diseases. Technically, the low cellularity of CSF samples, combined with the rapidly declining WBC viability, makes CSF flow cytometry challenging. Comparison of flow cytometry with microscopic and molecular techniques shows that each technique has its own advantages and is ideally combined. We expect that increasing the number of flow cytometric parameters that can be simultaneously studied within one sample, will further refine the information on CSF cell subsets in low-cellular CSF samples and enable to define cell populations more accurately.

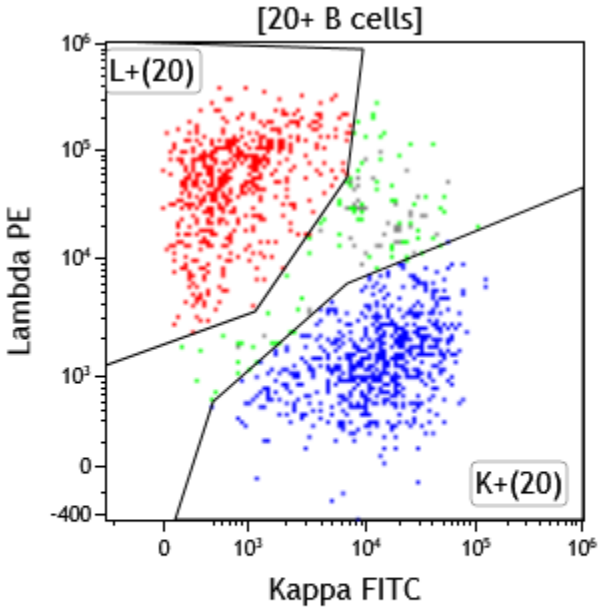
How do I use recent(ish) advances in flow diagnosis?

- T cell clonality
- Myelodysplastic syndrome evaluation
- CMML
- Minimal residual disease testing

T cell clonality

- Clonality (Monotypia) Determination
  - “Killer application” of flow cytometry
  - Worked well in B cell process
  - Light chain selection takes place early in B cell development and neoplastic processes

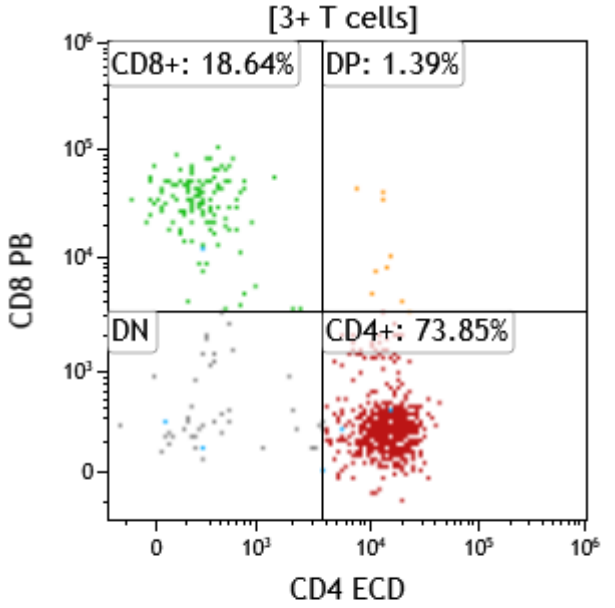
n.b. Not all monotypic things are clonal



Gate	Number	%Gated
All	1,393	100.00
K+(20)	699	50.18
L+(20)	565	40.56

CD4:CD8 Ratio

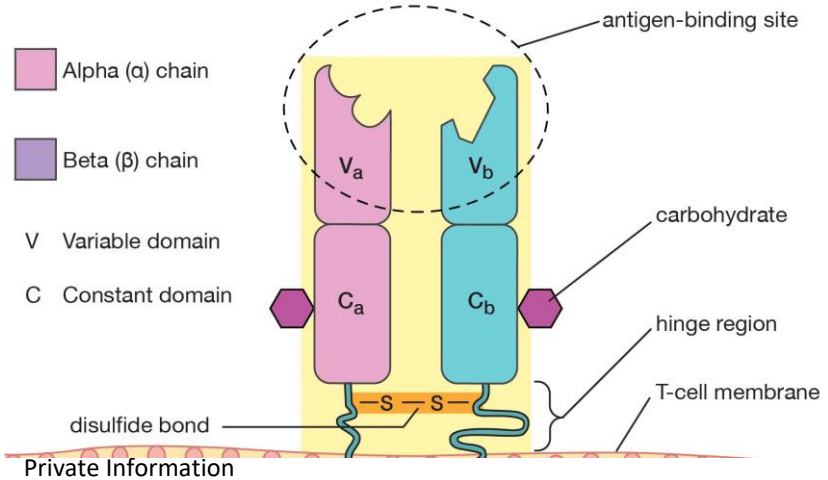
- Surrogate for clonality
  - Used in conjunction with phenotypic aberrancy
  - Lots of pitfalls
    - Reactive conditions
    - Expansion of reactive subsets
  - Relatively wide range of normal ratios 4-6:1 to 1-0.5:1 depending on who you ask

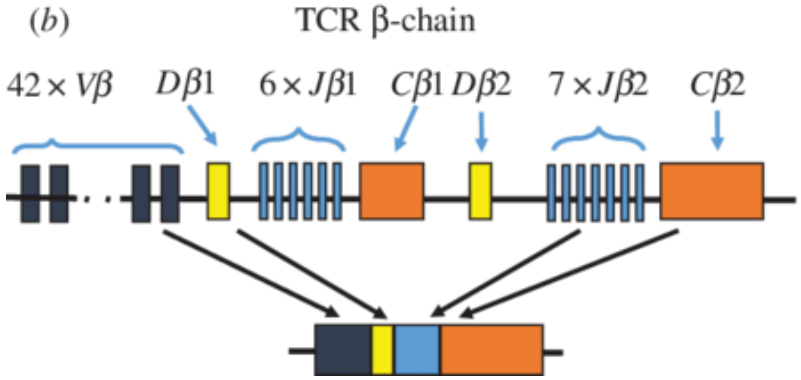


Gate	Number	%Gated
All	719	100.00
CD4+	531	73.85
CD8+	134	18.64
DN	44	6.12
DP	10	1.39

T cell receptor constant region

- New antibodies raised against constant beta region 1 (JOVI.1)
- TCR expression requires expression of the constant regions
- There are two constant genes that can be selected

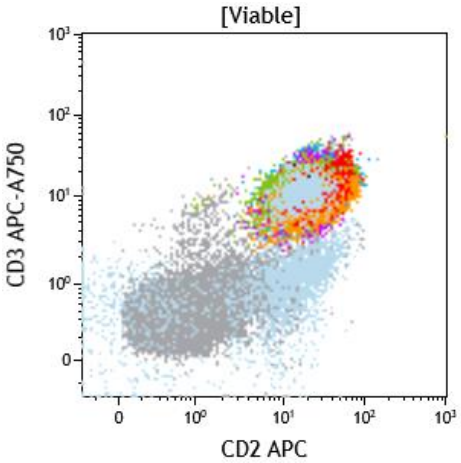


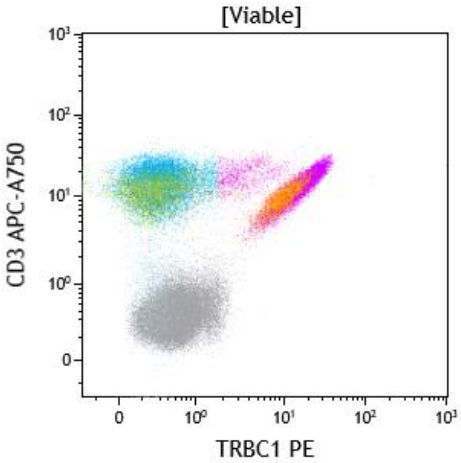


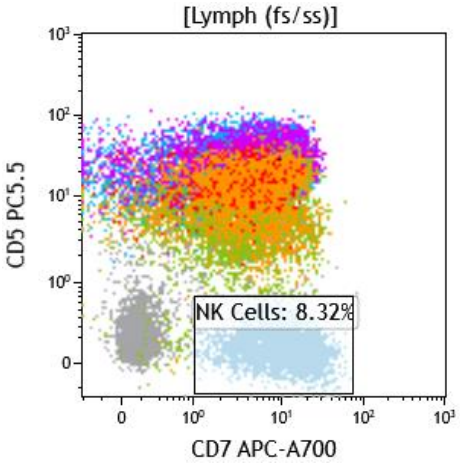
Running on one cylinder…

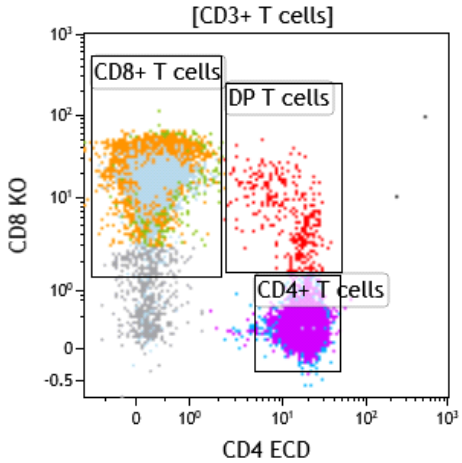
- We have one antibody against TRBC1
- No antibodies exist for TRBC2 (yet)
- Therefore, a positive result for TRBC1 binding means TRBC1 expression
- But, a negative result does not necessarily mean TRBC2 expression (could be TCR-γδ)
- CD3 required for TCR expression, therefore CD3- cases will be negative for TCR-{anything}

Normal/Reactive Case

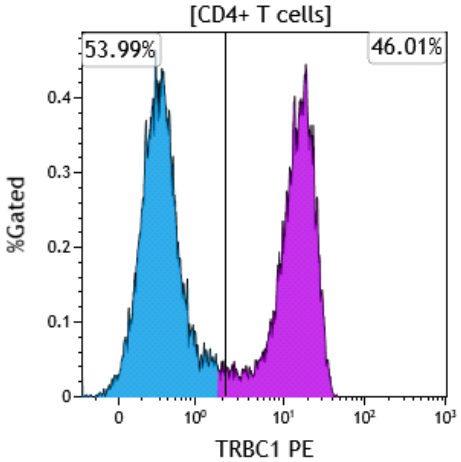


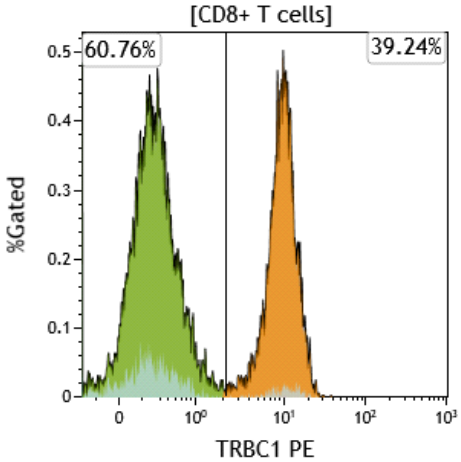


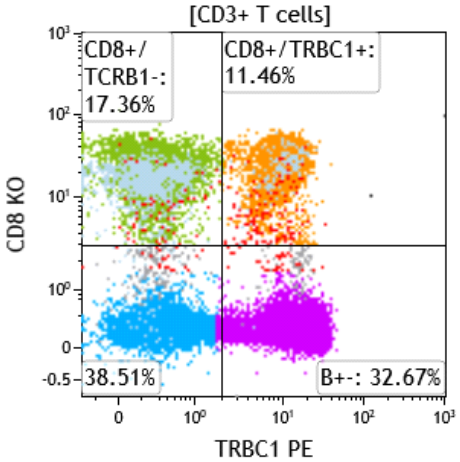


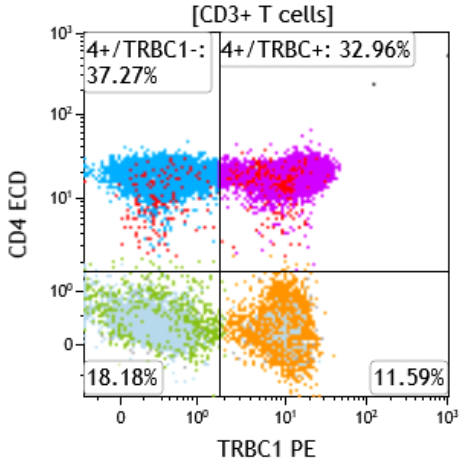


Gate	%Gated
All	100.00
CD4+ T cells	69.13
CD8+ T cells	28.60
DP T cells	0.87





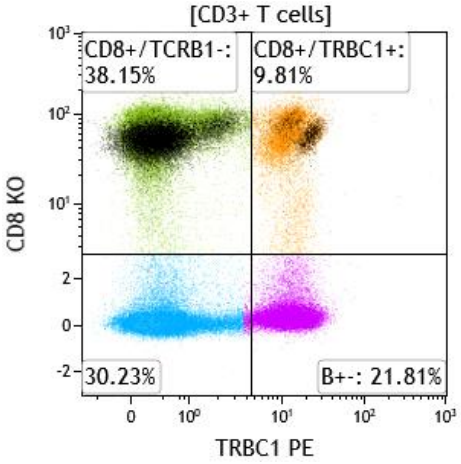


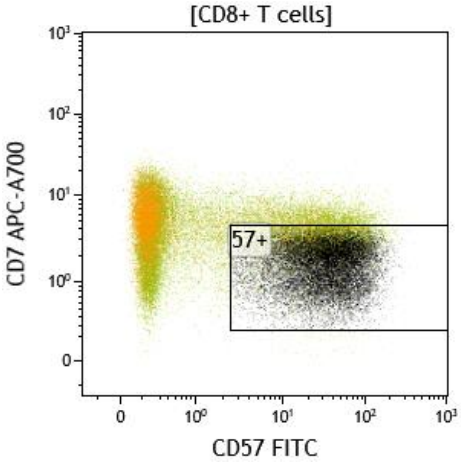


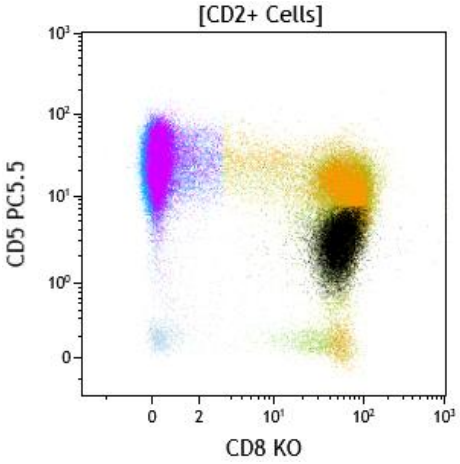
Gate	Number	%Gated
All	24,945	100.00
D-	13,469	53.99
D+	11,476	46.01

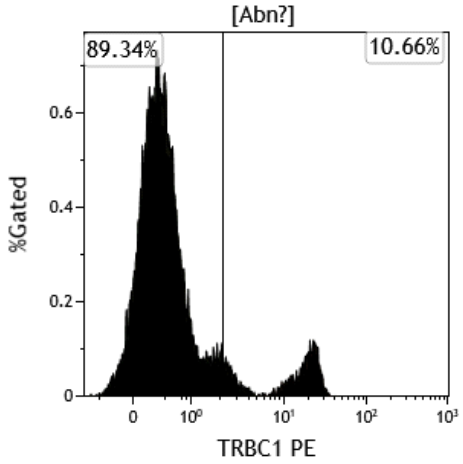
Gate	Number	%Gated
All	10,318	100.00
E-	6,269	60.76
E+	4,049	39.24

LGL example



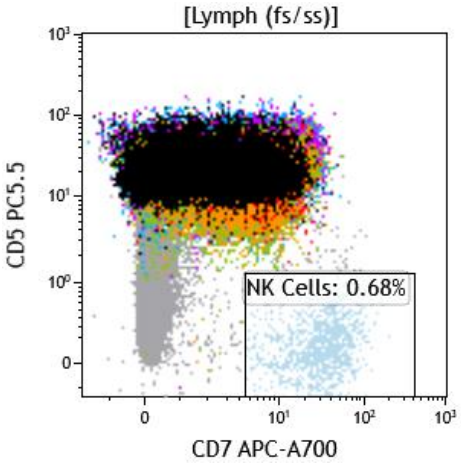


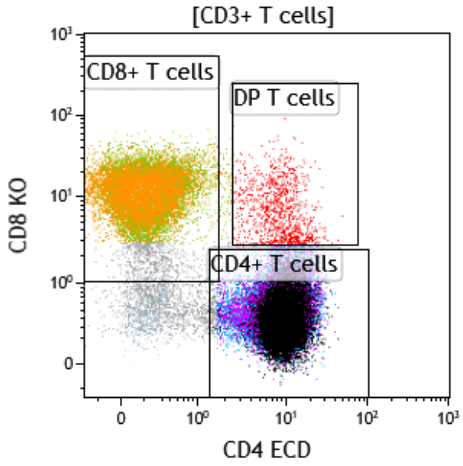




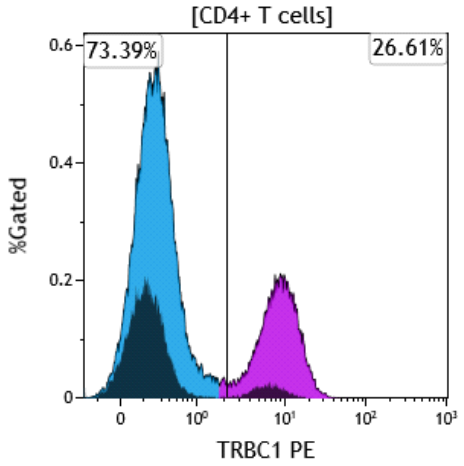
Gate	Number	%Gated
All	16,081	100.00
C-	14,367	89.34
C+	1,714	10.66

TFH example

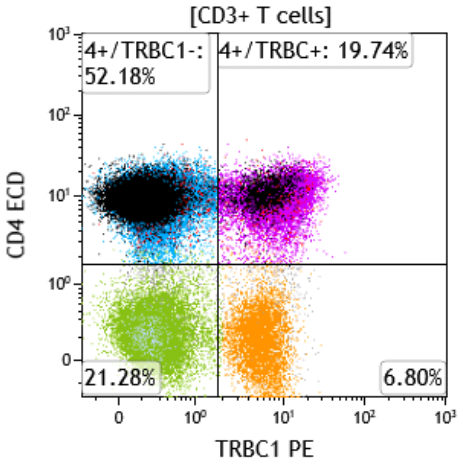


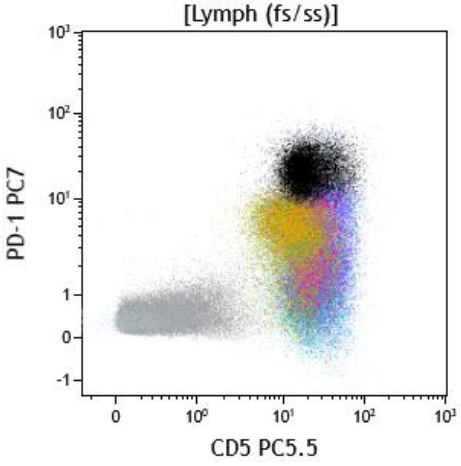


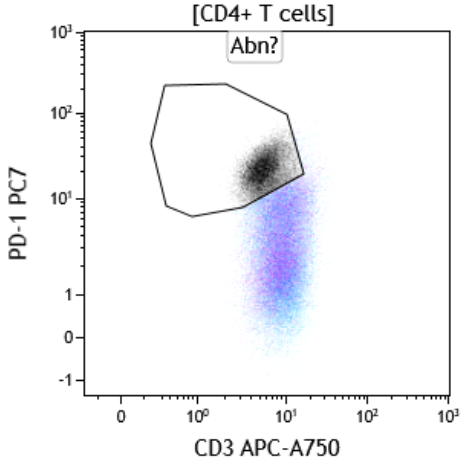
Gate	%Gated
All	100.00
CD4+ T cells	70.66
CD8+ T cells	26.03
DP T cells	1.21

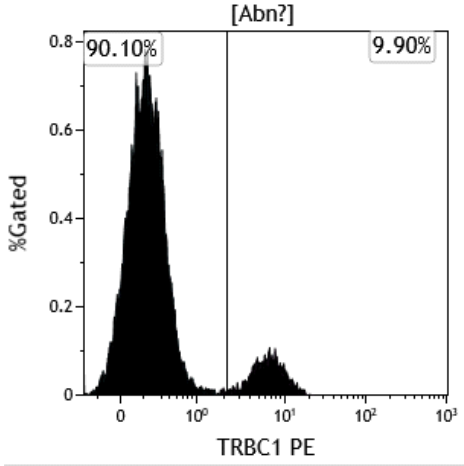


Gate	Number	%Gated
All	61,294	100.00
D-	44,982	73.39
D+	16,312	26.61









Gate	Number	%Gated
All	16,369	100.00
C-	14,748	90.10
C+	1,621	9.90

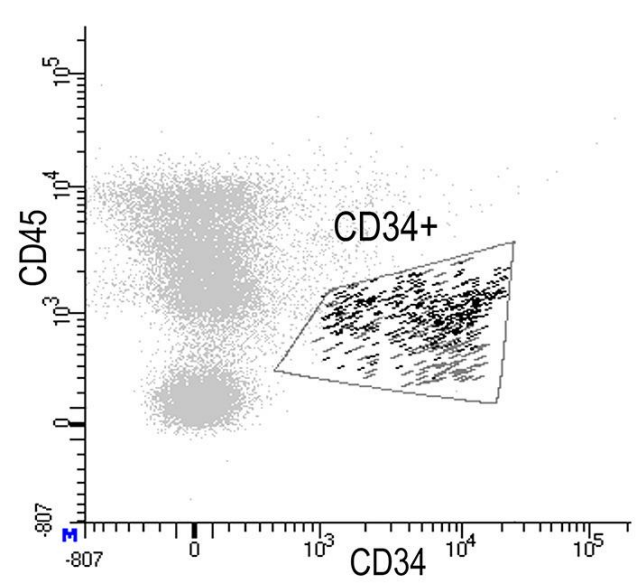
The future of T cell clonality

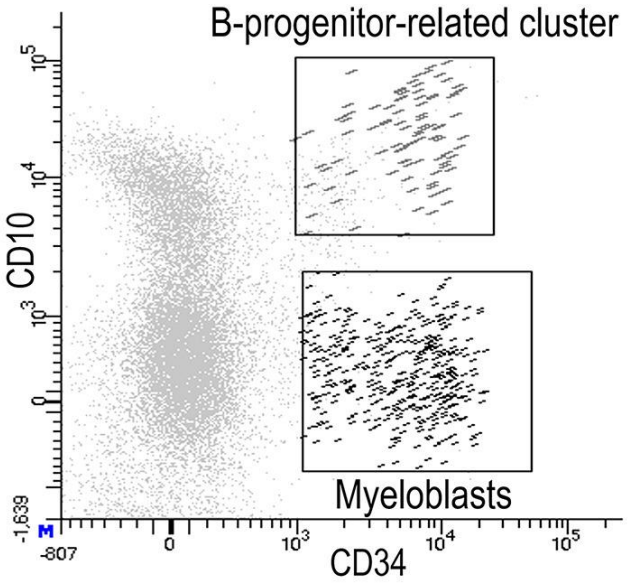
- Will likely be incorporated into screening panels
  - Akin to Kappa/Lambda for B cell lymphomas

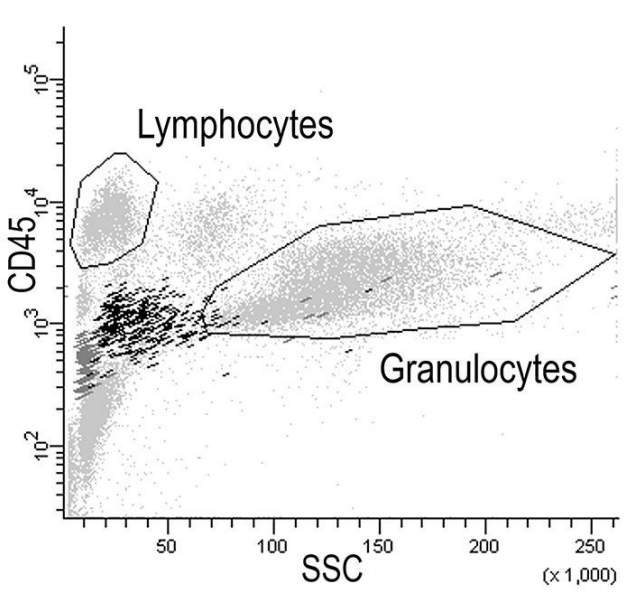
MDS evaluation by flow*

- Not a diagnostic criteria by WHO5e or ICC
- Ogata Scoring

Ogata Score







Parameter	Cut-off values	Score*
Myeloblast (% of CD45+ cells)	> 2 %	1
B-progenitor-related cluster size (% of CD34+)	< 5 %	1
Lymphocyte to myeloblast CD45 ratio	≤ 4 or ≥ 7.5	1
Granulocyte to lymphocyte SSC ratio	≤ 6	1

*MDS is indicated for samples obtaining ≥ 2 points

Ogata Score

- Easy to implement: Just about any flow panel can pull it off
- Objective criteria
- Recommend 1000 CD34+ cells for statistical rigor

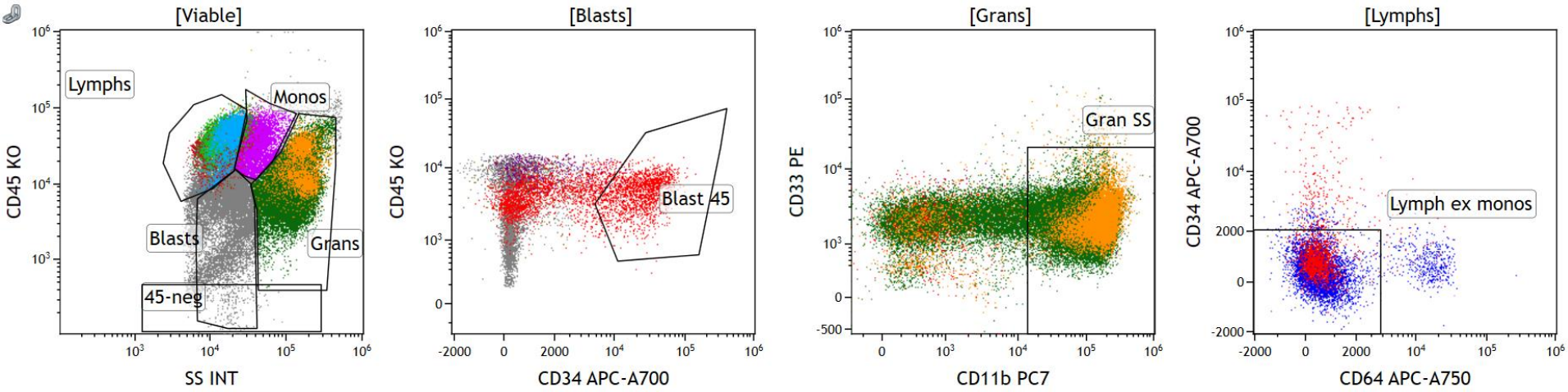
- Specific but not sensitive

Ogata K, Della Porta MG, Malcovati L, Picone C, Yokose N, Matsuda A, Yamashita T, Tamura H, Tsukada J, Dan K. Diagnostic utility of flow cytometry in low-grade myelodysplastic syndromes: a prospective validation study. Haematologica. 2009 Aug;94(8):1066-74. doi: 10.3324/haematol.2009.008532. Epub 2009 Jun 22. PMID: 19546439; PMCID: PMC2719029.

	Flow score using 4 parameters									Fl					
	0	1	2	3	4	Cases positive / cases examined	Sensitivity (%)	Specificity (%)	Likelihood ratio	0	1	2	3	4	5
Japanese cohort															
Non-clonal cytopenia	35	7	1	0	0	1/43				35	7	1	0	0	0
All low-grade MDS patients	12	14	7	11	2	20/46	44 (29-59)	98 (88-100)	18.7 (3.6-108.8)	2	11	15	12	5	1
Patients with conventional markers	4	4	5	6	1	12/20	60 (36-81)	98 (88-100)	25.8 (5.2-151.5)	0	4	7	7	1	1
Patients without conventional markers	8	10	2	5	1	8/26	31 (14-52)	98 (88-100)	13.2 (2.4-80.7)	2	7	8	5	4	0
Italian cohort															
Non-clonal cytopenia	38	20	5	0	0		5/63			22	12	4	0	0	0
All low-grade MDS patients	8	18	37	20	5	62/88	71 (60-80)	92 (82-97)	8.9 (4.2-20.4)	3	6	26	25	6	0
Patients with conventional markers	1	7	13	9	3	25/33	76 (58-89)	92 (82-97)	9.5 (4.6-20.7)	1	3	5	5	5	0
Patients without conventional markers	7	11	24	11	2	37/55	67 (53-79)	92 (82-97)	8.5 (4.0-19.5)	2	3	21	20	1	0

[1]*Data are the diagnostic power of the "flow score 2 or more." Data in parentheses are 95% CI.*

Implementation



Ogata Score Calculation

Myeloblasts out of 45+ cells (>2%)	2.51
H-Gones out of 34+ (<5%)	17.53
L v M 45 ratio (<4 or >=7.5)	6.94
G v L SS ratio (<=6)	8.35

Let a hundred flowers bloom; let a hundred schools of thought contend.

TABLE 1 Diagnostic MDS FCM-scores

	Progenitors	Granulopoiesis monopoiesis	Nucleated red cells	Output	
FCSS[a] (40 parameter)	> % abn myPC	abn pattern		0–1	Normal
				2–3	Moderate
				≥4	Severe
Ogata-score (4 parameter)	> % myPC	abn SSC		0–1	Low
	abn CD45 MFI			≥2	High
	< % lyPC				
RED-score[b] (3 parameter)			abn CD36	≥3	Suggestive of MDS
			abn CD71		
			abn Hb level		
ELN-NEC (3 parameter)			abn CD36 abn CD71 abn CD117	≥5	Erythroid dysplasia
iFS[c] (44 parameter)	> % myPC abn CD45 MFI < % lyPC	abn pattern	abn CD36 abn CD71 abn CD117	A B C	No MDS related features Limited number of MDS associated changes Features consistent with MDS

Abbreviations: abn, abnormal; ELN-NEC, European leukemia net-nucleated erythroid cells; FCSS, flow cytometry scoring system; iFS, integrated flow score; lyPC lymphatic progenitors; myPC myeloid progenitors.

[a]For FCSS, in granulopoiesis as well as monopoiesis, the following parameters were analyzed: SSC, CD34, HLA-DR, CD11b, CD33, CD13, CD16, and aberrant expression of lymphatic antigens; additionally in granulopoiesis: CD45, asynchronous shift to the left, abn lymphoid-to myeloid ratio and in monopoiesis: CD14.

[b]Cell preparation consisted of a wash—stain—no lyse procedure, as reported by Mathis et al., 2013.

[c]iFS is a combination of the parameters of Ogata-score, most of the parameters of FCSS (plus CD15 in granulopoiesis and monocytic-to-lymphoid ratio in monopoiesis, and ELN-NEC. Further information about the scoring details are reported by Cremers et al., 2017 (Table 2C).

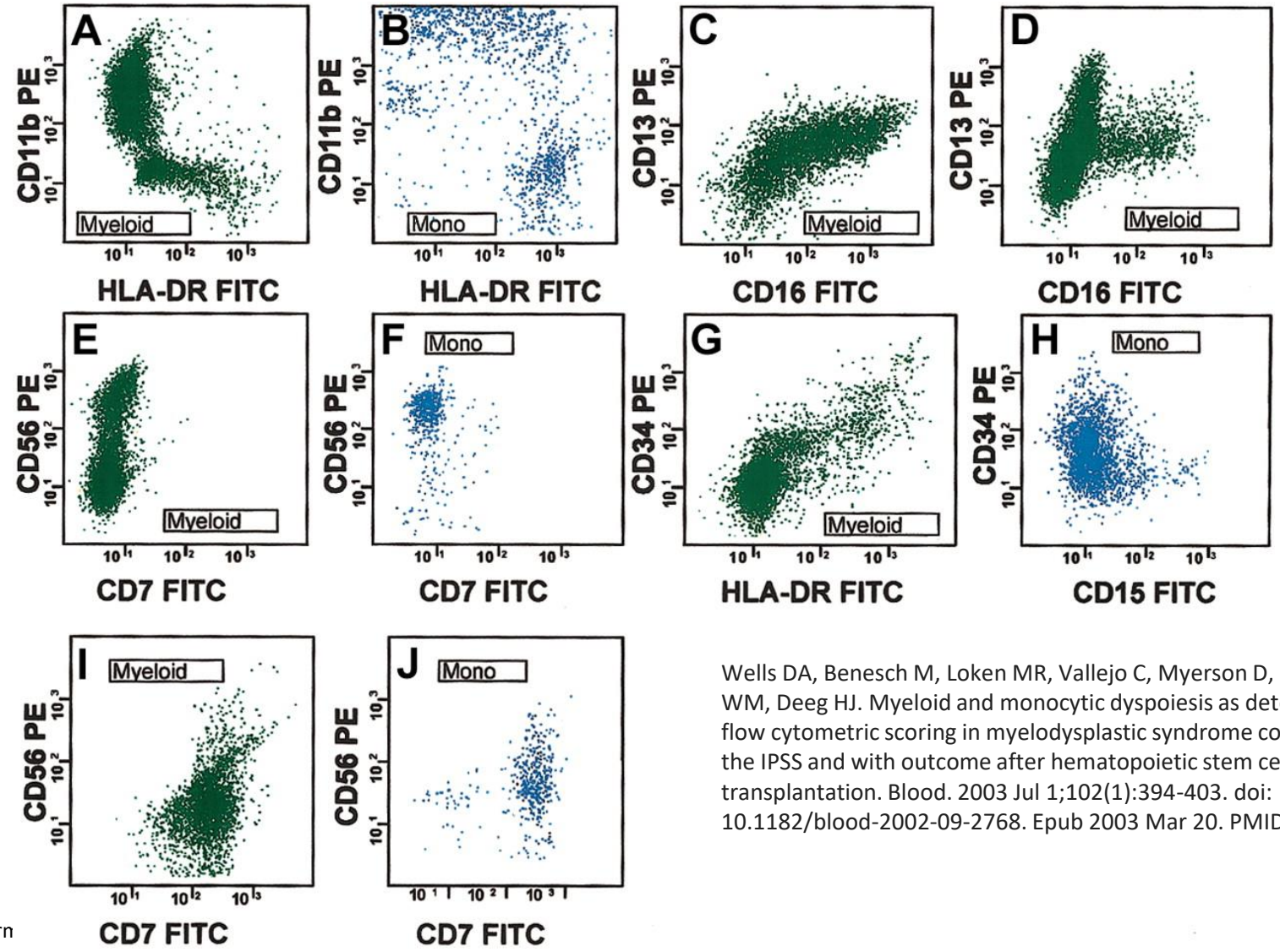
iFS

Table 2A. The parameters that describe the original integrated MDS-FC score, the erythroid score and the diagnostic score.

Diagnostic Score	Myeloid progenitors	Granulocytes**	Monocytes**	Erythrocytes
Two of the following: Increased percentage of myeloid progenitor cells Abnormal expression of CD45 on myeloid progenitor cells Decreased SSC on granulocytes Decreased percentage of B-cell progenitor cells	>5% myeloid progenitors **OR:** <5% myeloid progenitors with one of the following: Lymphoid markers present (CD2, CD5, CD19, CD25, CD56) **OR:** <5% myeloid progenitors with two of the following: Decrease in CD45 expression Abnormal expression of CD34 Abnormal expression of CD117 Abnormal expression of CD13 Abnormal expression of CD33 Abnormal expression of HLA-DR Expression of CD11b Expression of CD15*	Two of the following: Decreased SSC Abnormal CD11b/CD13 Abnormal CD16/CD13 Expression of HLA-DR Lack of CD33 expression Asynchronous shift to the left Abnormal expression of CD15 **OR:** Presence of lymphoid markers **OR:** Presence of CD34 on mature myeloid cells **OR:** Myeloid/Lymphoid ratio < 1	Two of the following: Abnormal CD45/SSC Decreased/increased number as compared to lymphocytes Abnormal CD11b Abnormal HLA-DR Abnormal CD11b/HLA-DR Abnormal expression of CD14 Abnormal expression of CD13 Loss of CD16 Abnormal expression of CD33 **OR:** Presence of lymphoid markers **OR:** Presence of CD34 on mature monocytic cells	Two of the following***: Increased CD36 coefficient of variation Increased CD71 coefficient of variation Decreased expression of CD71 Decreased / increased percentage of CD117 positive within nucleated erythroid cells

If a cell compartment is considered abnormal, a '+' is assigned in Tables 2B-2C.*Note that normal myeloid progenitors might also express CD15. **The granulocytic and monocytic cell compartments were integrated into one compartment in Table 2C (the iFS). ***in case of aberrant CD71 percentage and CD117 percentage one extra abnormality is mandatory. This figure is adapted from Wells *et al.*, scores adjusted as by Cutler *et al.*, and Cremers *et al.*[7,17,25]

Abnormalities



Wells DA, Benesch M, Loken MR, Vallejo C, Myerson D, Leisenring WM, Deeg HJ. Myeloid and monocytic dyspoiesis as determined by flow cytometric scoring in myelodysplastic syndrome correlates with the IPSS and with outcome after hematopoietic stem cell transplantation. Blood. 2003 Jul 1;102(1):394-403. doi: 10.1182/blood-2002-09-2768. Epub 2003 Mar 20. PMID: 12649150.

But that's not all….

Table 2B. The addition of the erythroid evaluation to the diagnostic score.[13,14]

Diagnostic score	0	0	1	1	≥2	≥2
Aberrant erythroid	-	+	-	+	-	+
MDS according to FC	No	No	No	Yes	Yes	Yes

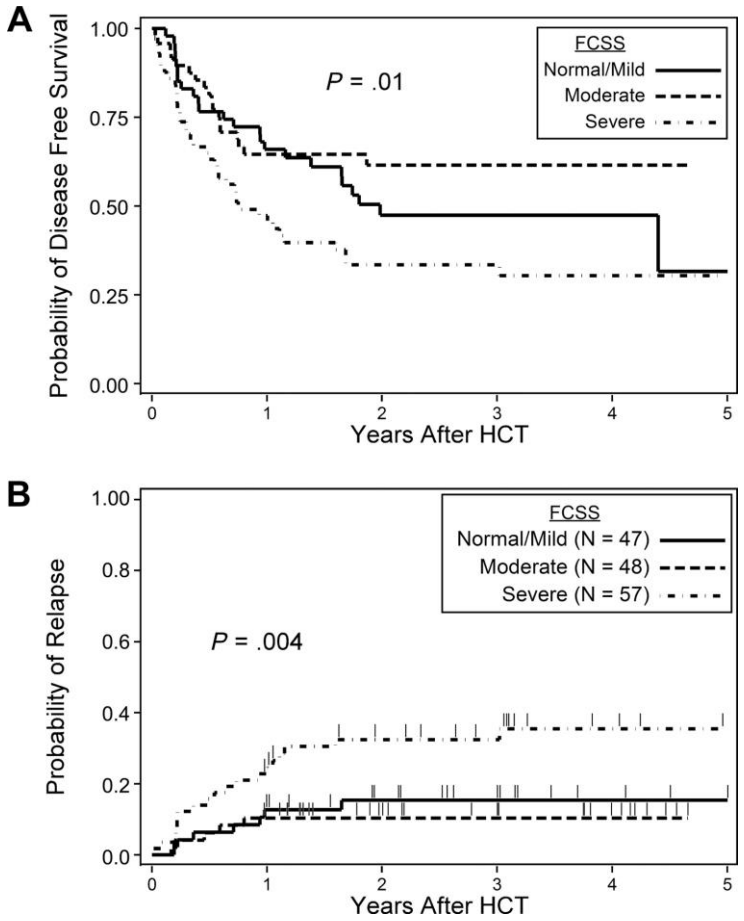
Table 2C. The addition of the erythroid evaluation to the integrated MDS-FC score (iFS).[16]

Diagnostic score	<2 abnormalities								≥2 abnormalities							
Aberrant myeloid progenitors	-	-	-	-	+	+	+	+	-	-	-	-	+	+	+	+
Aberrant neutrophils (≥2 other aberrancies)																
Aberrant monocytes (CD56 / ≥2 aberrancies)	-	-	+	+	-	-	+	+	-	-	+	+	-	-	+	+
Original iFS*	A	A	A/B	A/B	A/B	A/B	C	C	A/B	A/B	B/C	B/C	B/C	B/C	C	C
Aberrant erythroid (≥2 aberrancies)	-	+	-	+	-	+	-	+	-	+	-	+	-	+	-	+
New iFS*	A	B	B	C	B	C	C	C	A/B	C	C	C	C	C	C	C
Labeled MDS	No	No	No	Yes	No	Yes	Yes	Yes	No	Yes	Yes	Yes	Yes	Yes	Yes	Yes

The four-parameter diagnostic score as described by Della Porta *et al.*,[14] Aberrant myeloid markers, neutrophils and monocytes based on the modified FCSS score. Aberrant myeloid markers as describes in table 2A; more than 2 points per lineage. Aberrant erythroid markers as recommended by the ELNet iMDS-flow, described in Table 2A and the tandem-paper. *Category A 'no MDS-related features', B 'limited number of changes associated with MDS', or C 'features consistent with MDS'. Choice for A or B and B or C depends on the kind and number of aberrancies that are encountered. Note that patients with ≥2 points in the diagnostic score can still be labeled as no MDS by the iFS when there are no other abnormalities.

So what?

- Strict application of the FCSS or iFS system is unusual in US labs
- But it seems to work
  - Several validations demonstrated over 15 years



Scott BL, Wells DA, Loken MR, Myerson D, Leisenring WM, Deeg HJ. Validation of a flow cytometric scoring system as a prognostic indicator for posttransplantation outcome in patients with myelodysplastic syndrome. Blood. 2008 Oct 1;112(7):2681-6. doi: 10.1182/blood-2008-05-153700. Epub 2008 Jul 7. PMID: 18606877; PMCID: PMC2556605.

Features of CMML by flow

- Homogenization of the mature monocytic subsets
- Classical monocytes comprising >94% of all monocytes
  - Sensitivity and specificity values of 90.6% and 95.1% for CMML

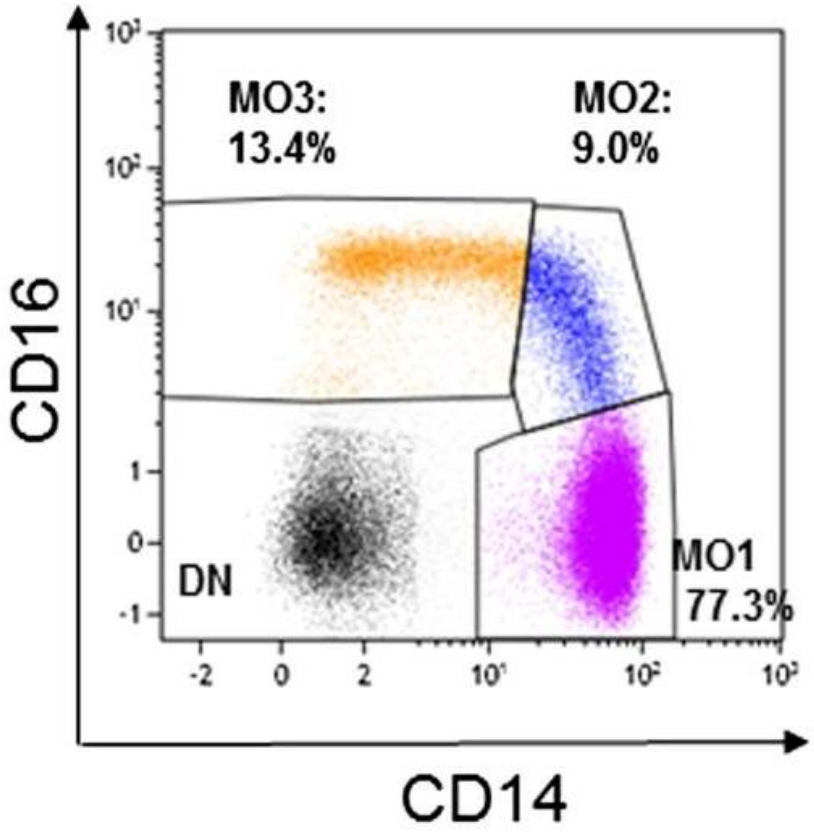
Selimoglu-Buet, Dorothée et al. “Characteristic repartition of monocyte subsets as a diagnostic signature of chronic myelomonocytic leukemia.” *Blood* vol. 125,23 (2015): 3618-26. doi:10.1182/blood-2015-01-620781

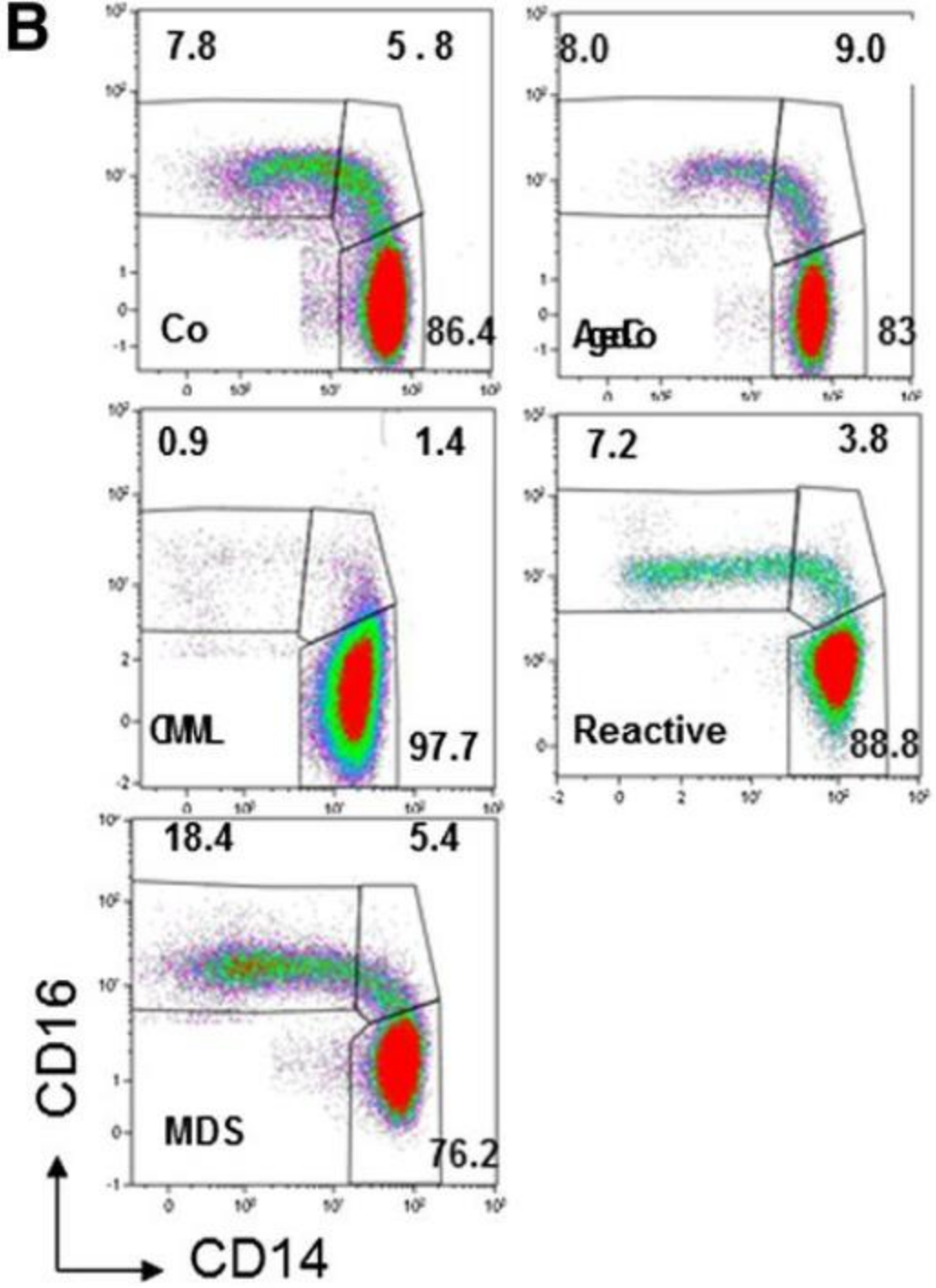
Table 6 Diagnostic criteria of chronic myelomonocytic leukaemia.

From: The 5th edition of the World Health Organization Classification of Haematolymphoid Tumours: Myeloid and Histiocytic/Dendritic Neoplasms

Prerequisite criteria
1. Persistent absolute (≥0.5 × 10^9/L) and relative (≥10%) peripheral blood monocytosis.
2. Blasts constitute <20% of the cells in the peripheral blood and bone marrow.[a]
3. Not meeting diagnostic criteria of chronic myeloid leukaemia or other myeloproliferative neoplasms.[b]
4. Not meeting diagnostic criteria of myeloid/lymphoid neoplasms with tyrosine kinase fusions.[c]
Supporting criteria
1. Dysplasia involving ≥1 myeloid lineages.[d]
2. Acquired clonal cytogenetic or molecular abnormality.
3. Abnormal partitioning of peripheral blood monocyte subsets.[e]
Requirements for diagnosis
- Pre-requisite criteria must be present in all cases.
- If monocytosis is ≥ 1 × 10^9/L: one or more supporting criteria must be met.
- If monocytosis is ≥0.5 and <1 × 10^9/L: supporting criteria 1 and 2 must be met.
Subtyping criteria
- Myelodysplastic CMML (MD-CMML): WBC < 13 × 10^9/L
- Myeloproliferative CMML (MP-CMML): WBC ≥ 13 × 10^9/L
Subgrouping criteria (based on percentage of blasts and promonocytes)
CMML-1: <5% in peripheral blood and <10% in bone marrow
CMML-2: 5–19% in peripheral blood and 10-19% in bone marrow

[e]Based on detection of increased classical monocytes (>94%) in the absence of known active autoimmune diseases and/or systemic inflammatory syndromes.





MRD Testing

- Low levels of disease after therapy is predictive of relapse
  - *dose response
- Multiple modalities available
  - PCR, NGS, flow cytometry

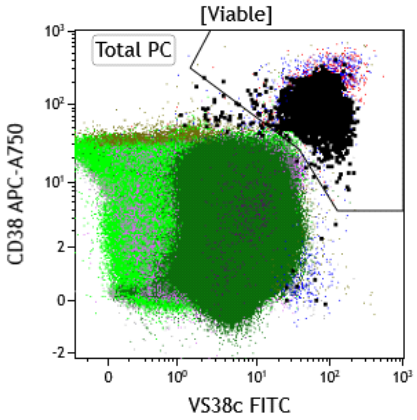
Flow Based MRD testing

- Standard of care in B-LBL
- Widely used in AML and Myeloma
- Some use in B-NHL (CLL/SLL)

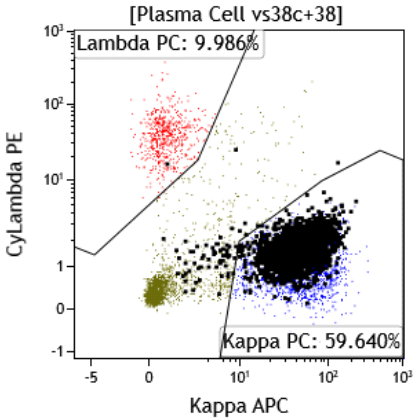
Basic Principle

- Evaluate lots of cells and find a few dozen abnormal ones
  - Phenotypically aberrant: gain or loss of antigen expression, homogeneous expression where you expect a stereotypic maturational path

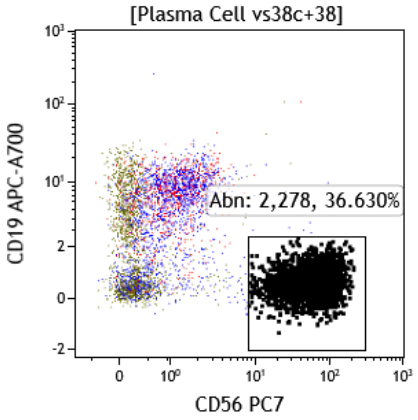
Myeloma MRD



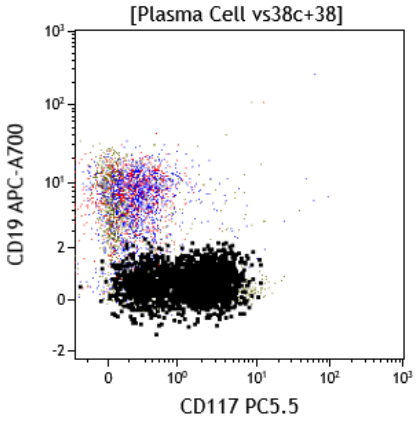
Gate	Number	%Gated
All	4,554,909	100.000
Total PC	4,508	0.099

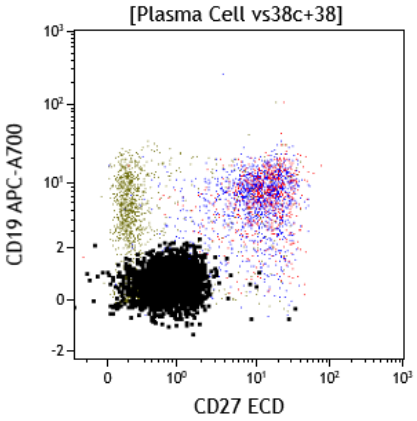


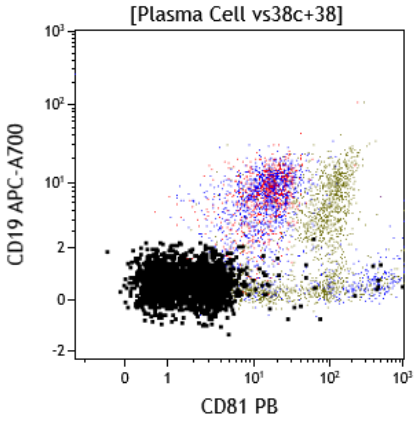
Gate	Number	%Gated
All	6,219	100.000
Kappa PC	3,709	59.640
Lambda PC	621	9.986



0.05% of leukocytes



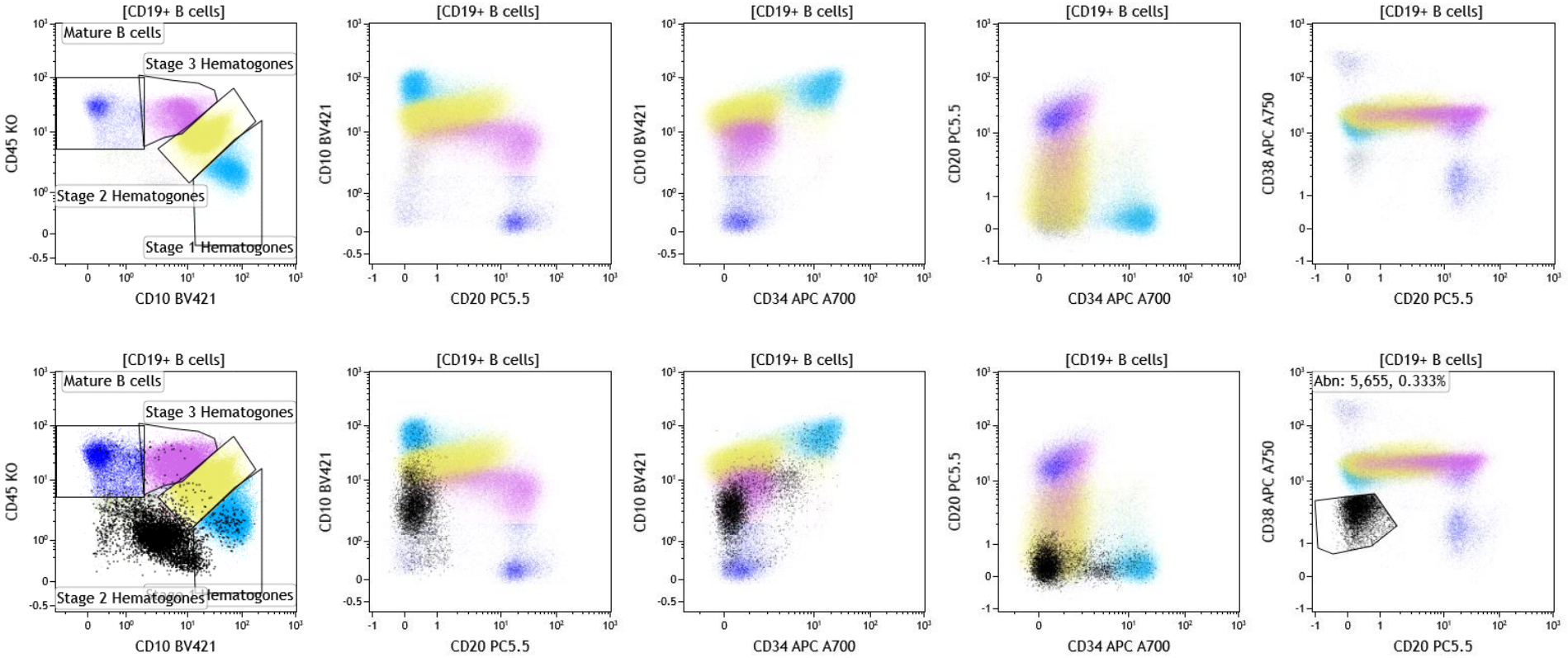




Additional Markers in MM MRD

- Vs38c
  - Relatively specific marker for plasma cells, cytoplasmic localization, likely immune to targeted therapies
- CD27
  - Often decreased in myeloma (50% of cases)
- CD81
  - Can be decreased or increase in myeloma (usually decreased, 50% of cases)
  - Bright CD81 is seen in hematogones (19+/38+ cells)

B-LBL MRD



Maturation based MRD

- Know the normal patterns of maturation
- Know typical derangements seen in post therapy/stressed marrows

Flow MRD in the era of targeted therapies

- Myeloma
  - Anti-CD38 therapy (daratumumab)
- B cell lymphomas/Leukemias
  - Anti-CD20 and Anti-CD19 therapies
- Necessitates novel gating markers to circumvent key marker loss
  - VS38c, IRF4(MUM1)
  - CD24+CD66b

Opportunities in flow cytometry

- Tech only or TC/PC split flow cytometry

  n.b. Not performed at ARUP

- TC has high fixed cost/ low marginal cost

- Maybe its worthwhile? Maintain skills, professional satisfaction, revenue* or RVU generation
- PC codes
  - 88187/8/9=16+ color eval

Growing in popularity

- Offered by many commercial reference laboratories
- Different models
  - Analysis can done by the referring pathology lab
  - Digital PDF vs raw data

Nota bene

For laboratories performing only the interpretation component of flow immunophenotyping data *(the flow technical component is performed at an outside flow laboratory), the following Flow Cytometry Checklist requirements apply: FLO.18385, FLO.23706, FLO.30640, FLO.30730, and FLO.30790. Additionally, requirements located in the All Common Checklist addressing proficiency testing, quality management, procedure manual, specimen rejection, and results reporting are applicable.*

- CAP proficiency exists for referring lab

Flow Cytometry, Interpretation Only FL5		
Procedure	Program Code	Challenges per Shipment
	FL5	
Flow cytometry, interpretation only of leukemia/lymphoma	▮	3

Program FL5 is for laboratories that receive flow cytometry analyses from referring laboratories to perform the interpretation of patient results.

*Disclosure: I am a member without pay on the CAP's Diagnostic Immunology and Flow Cytometry Committee

Setting up a flow lab?

- Highly unlikely in this financial environment

- Lack of skilled med techs
- High startup and fixed costs
- Upgrading with each generation of technology is out of reach

Conclusion

- Flow is the standard of care in hematolymphoid diagnosis
- Flow is fast and efficient
- Flow is not a panacea for good clinical judgement and has a limited set of indications
- Advances in flow have increased its utility
- TC/PC splits are an opportunity to improve your practice