Applications of Flow Cytometry in Veterinary Medicine

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Overview

1. Instrumentation
   A. Essential components
   B. Basic principles of light scatter and fluorescence
2. Sample preparation/processing
3. Application of flow cytometry
   A. Detection of surface antibody
   B. Lineage determination of malignant neoplasms
   C. Define an antibody panel
   D. Identifying clonality (PARR test)
4. Case examples

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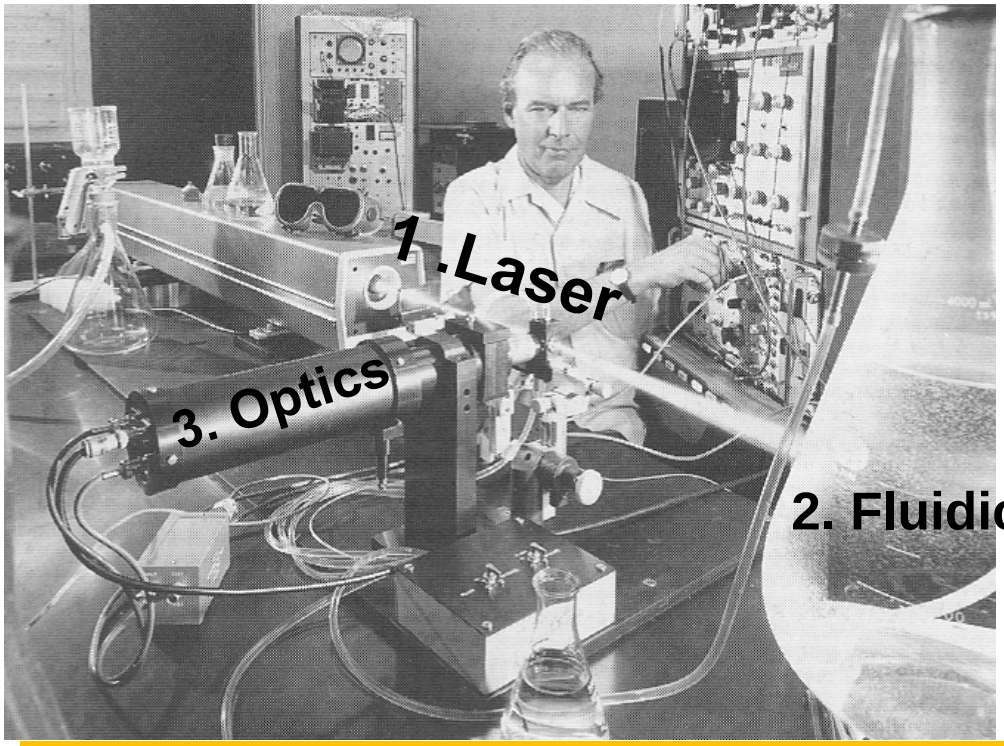
Flow cytometers

- *"flow cyto – meter" = measurement of cells in a fluid stream*
  - Measure multiple characteristics of cells by light scatter and fluorescence using lasers
  - Quantify leukocytes and differentiate cell types
  - Fluorescent dye labeled antibodies to cluster of differentiation antigens identify subsets of cells
    - i.e. CD4 or CD8 lymphocytes
      - immunodeficiency syndromes
      - lineage of lymphoma and leukemia
    - DNA content, apoptosis, viability, metabolism, proliferation

First Flow Cytometer, an “old tool”

4. Electronics



Basic Components

1. Laser –excitation optics
2. Fluidics
3. Collection Optics
4. Electronics

Marvin Van Dilla, Trujillo TT, Mullianey PF, Coulter JR (1969). Cell microfluorimetry: A method for rapid fluorescence measurement. Science 163:1213-1214.

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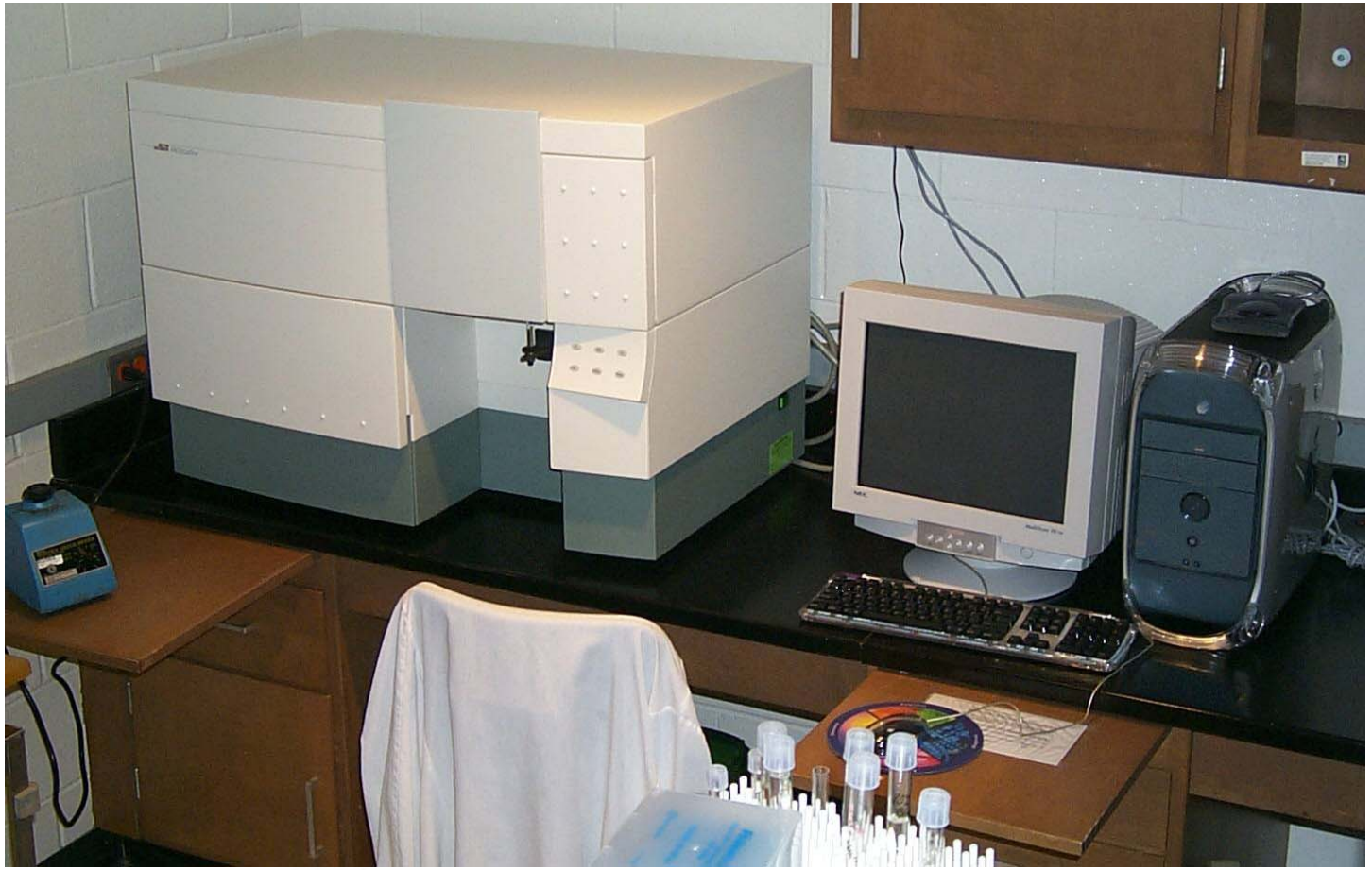
Analytical Flow Cytometer

FACSCalibur



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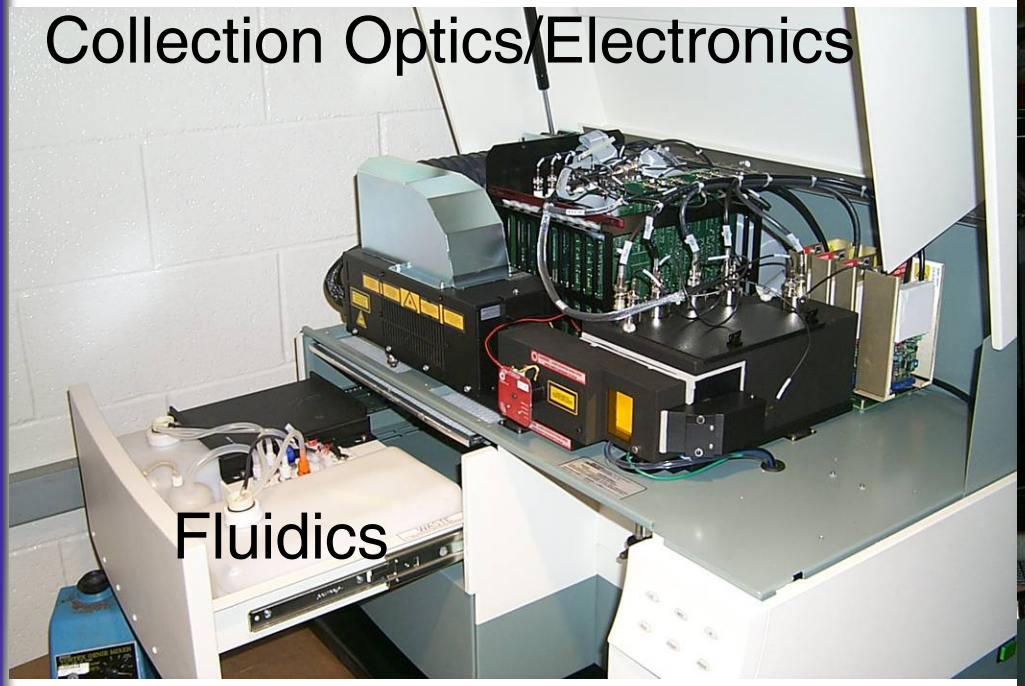
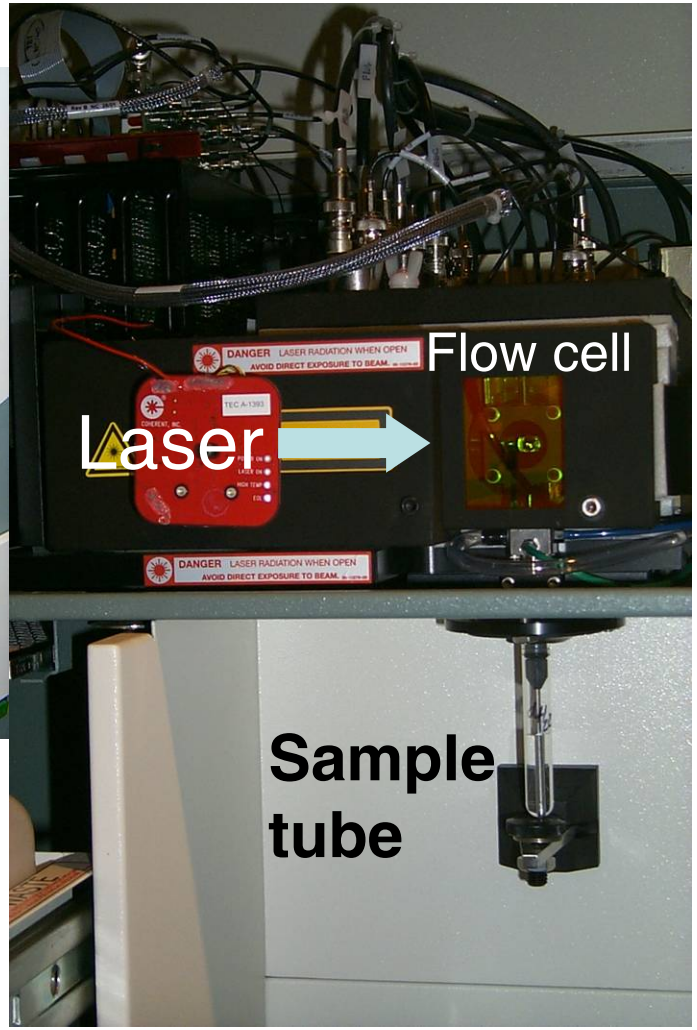
Components of a bench top analytical flow cytometer





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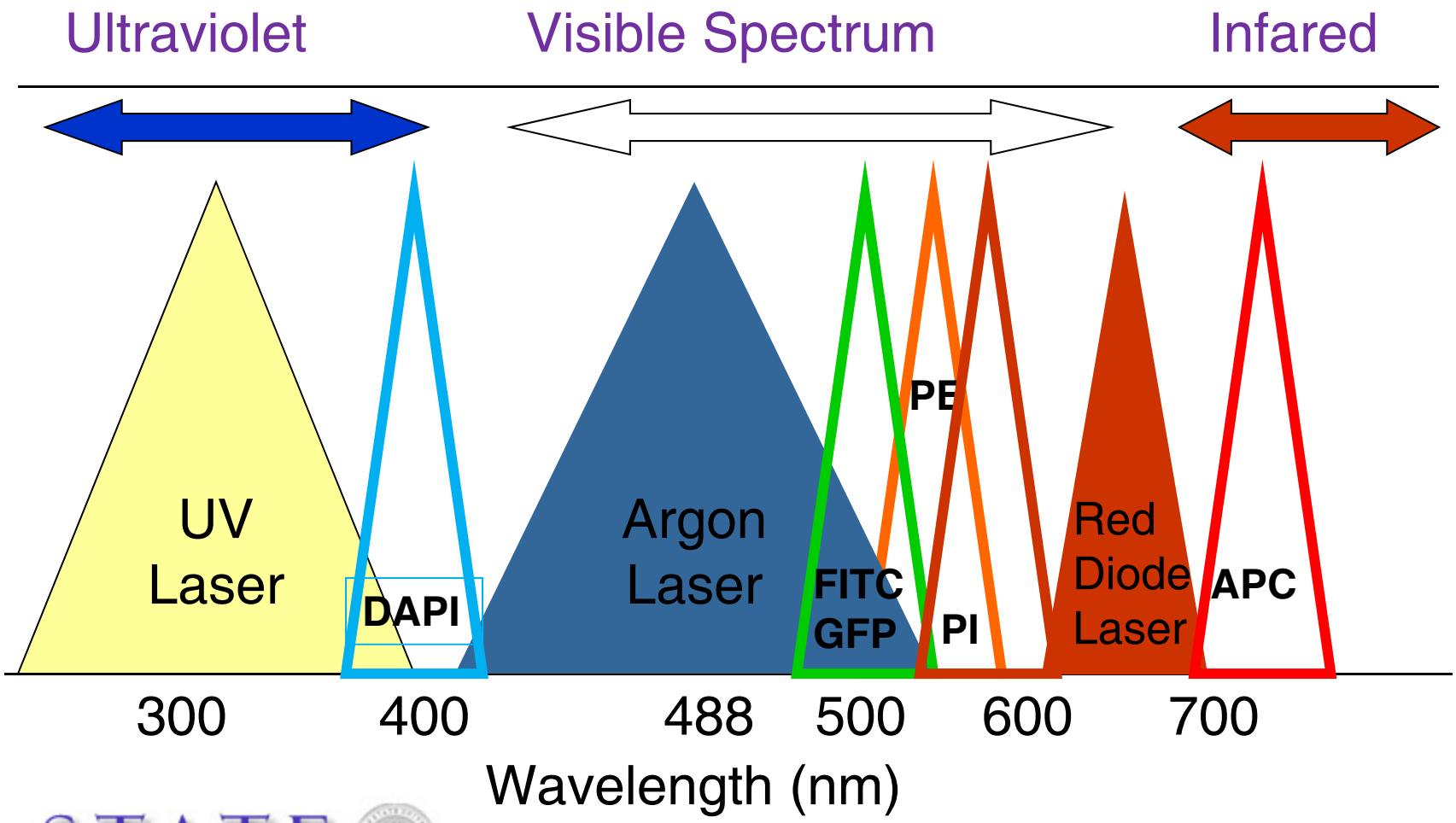
LASER

Light Amplification by Stimulated Emission of Radiation



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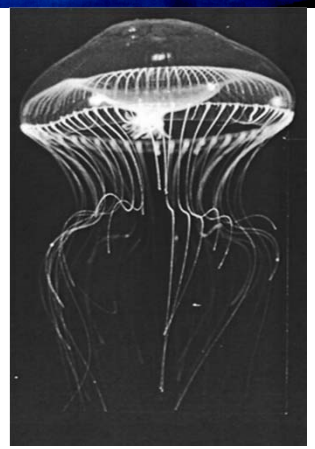
Fluorescence colors

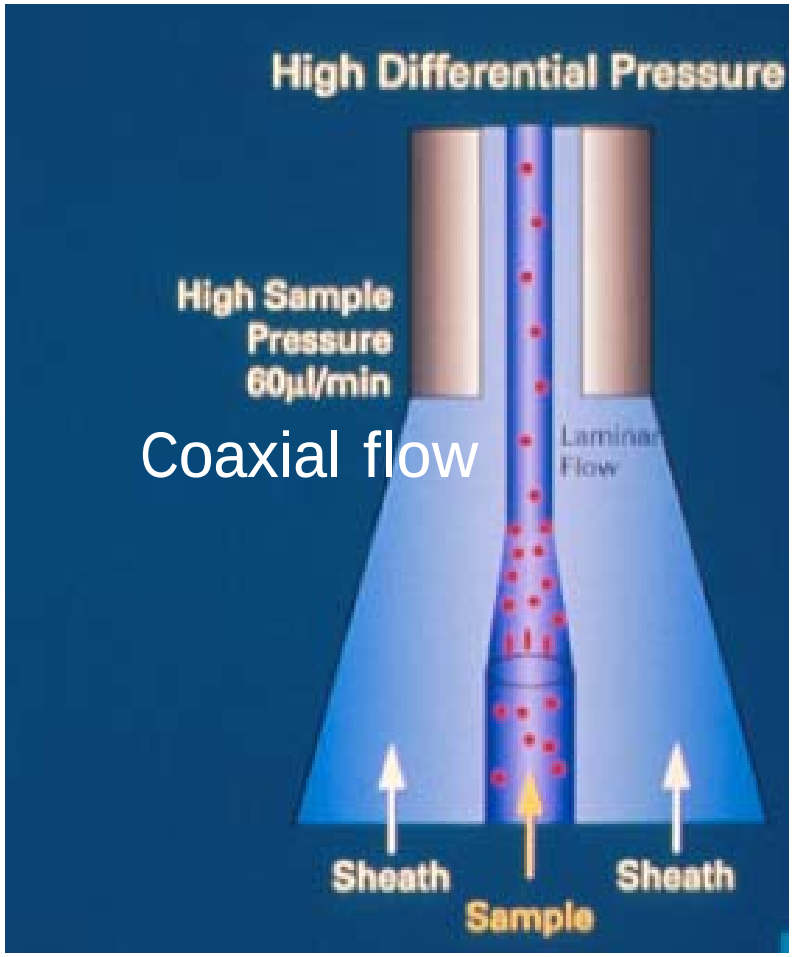
- First fluorochromes or fluorophores
  - Fluorescein (FITC)
  - Seaweeds/cyanobacteria ⟹
    - Phycobiliproteins (PE)
    - Allophycocyanin (APC)
  - Jelly fish - Green fluorescent protein (GFP) ⟹
  - Propidium iodide (PI)



Aequoria victoria

Fluidics

- Pressure lines and sheath fluid introduce sample stream into laser beam
- Sample pressure is higher than sheath fluid and introduces cells in single file



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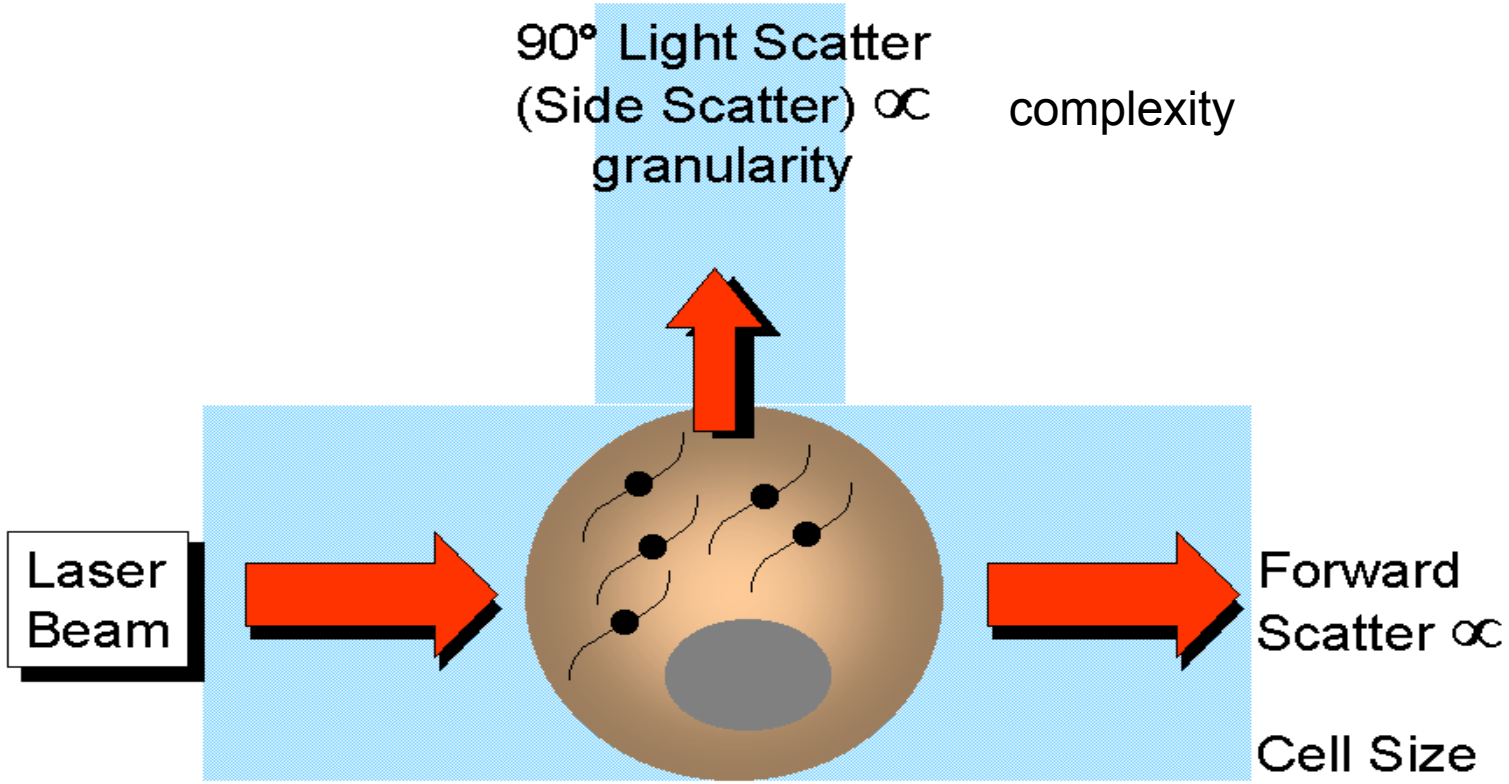
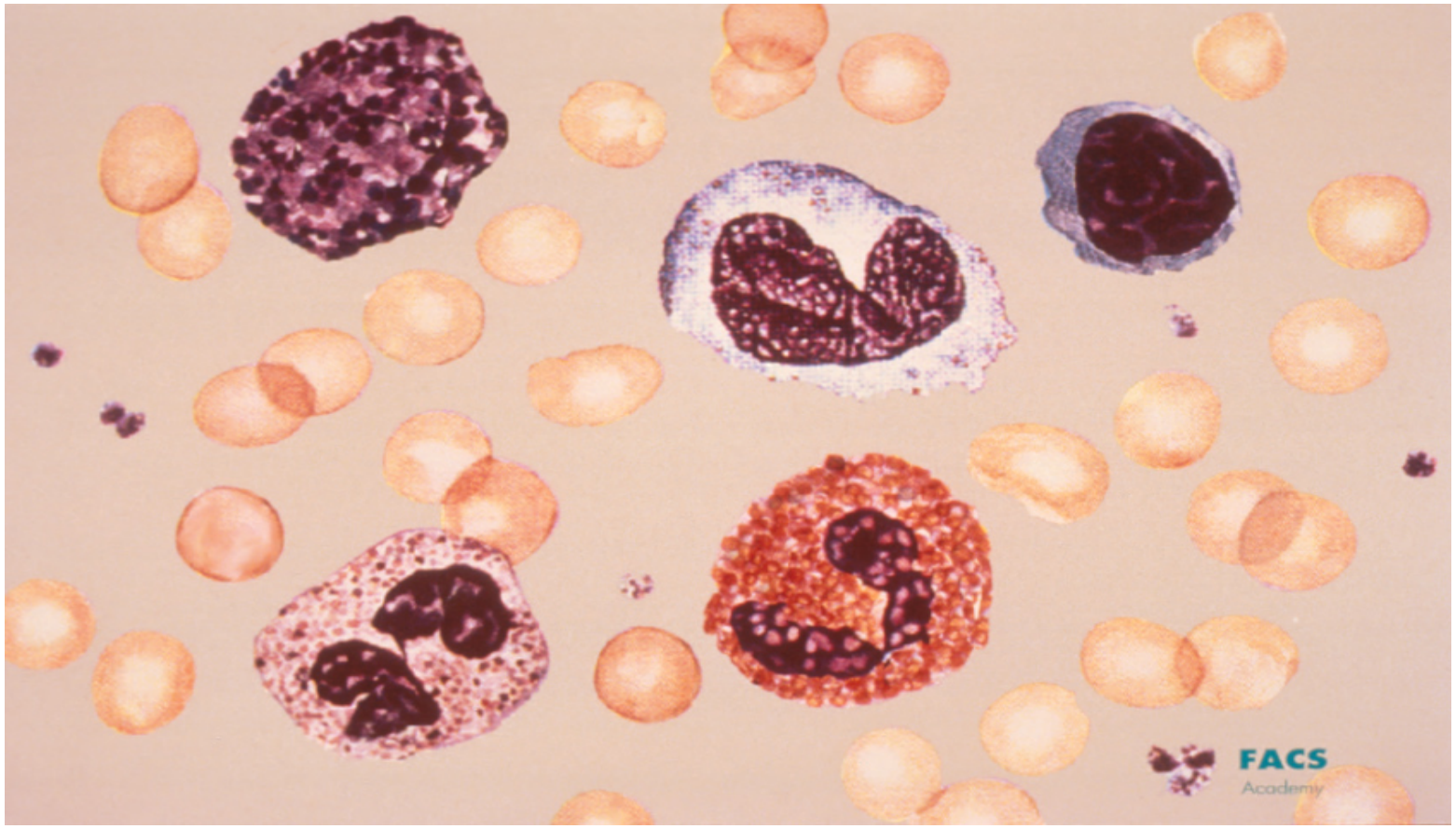
Basic light scatter properties of cells



Differential light scatter properties of cells define individual cells



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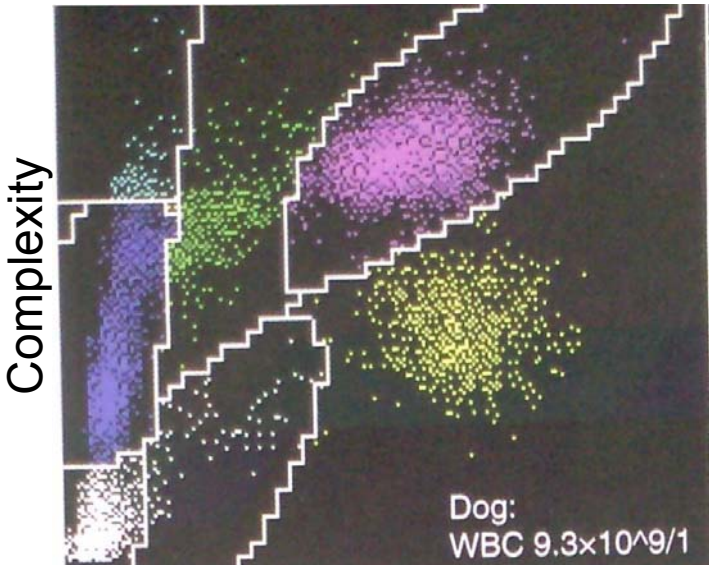
Hematology Analyzers
Advia 2120i use laser light scatter to differentiate cell populations



RBCS/platelets
Lymphocytes
Monocytes
Neutrophils
Eosinophils

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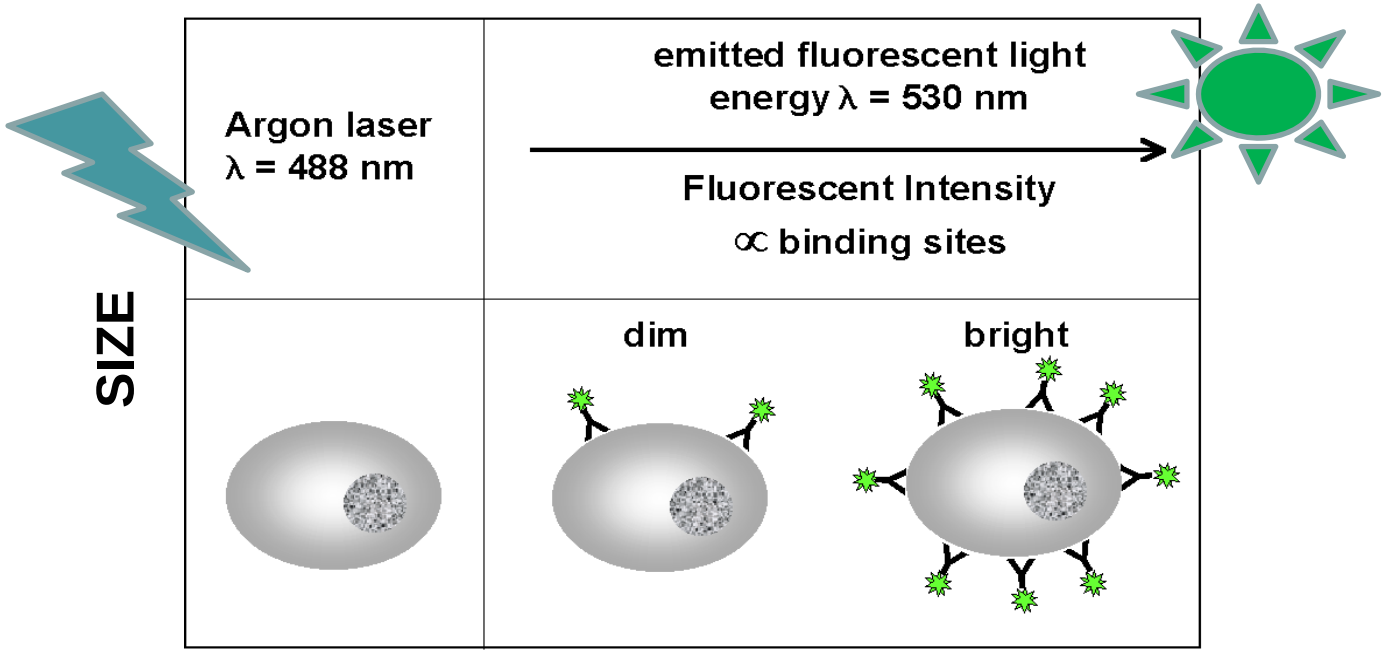
Collection Optics

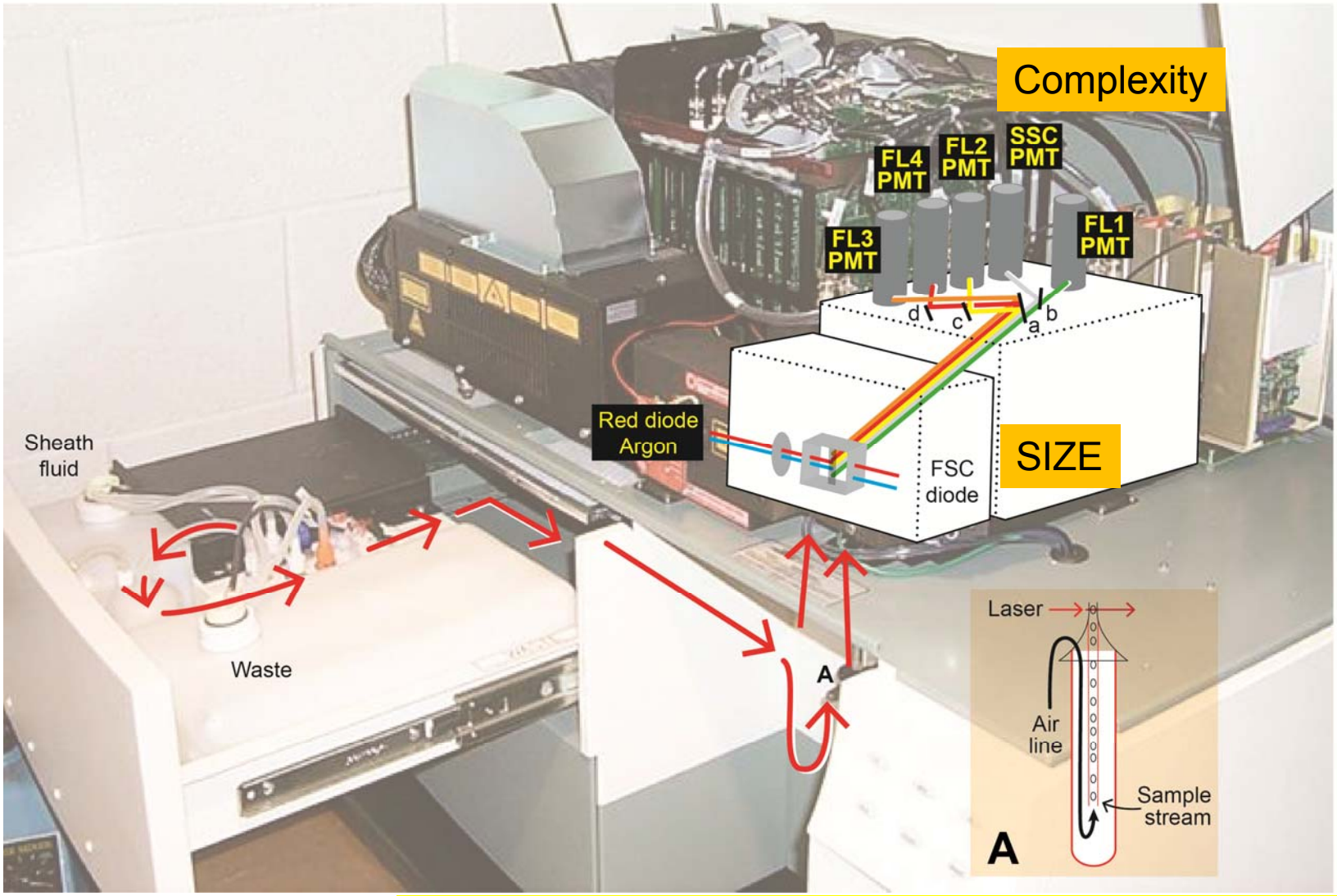
- Collection Optics
  - Mirrors, Filters, Detectors
  - Collect and filter wavelengths of light that come from the particle-laser beam interaction
    - Light scatter
    - Fluorescence

Basic principles of fluorescence



Laser excites fluorescently labeled antibodies to CD molecules

Collection Optics & Detectors



M. Wilkerson. Principles and Applications of Flow Cytometry and Cell Sorting in Companion Animal Medicine. Vet.Clincs of North America. Hematology.Vol. 42. 2012.

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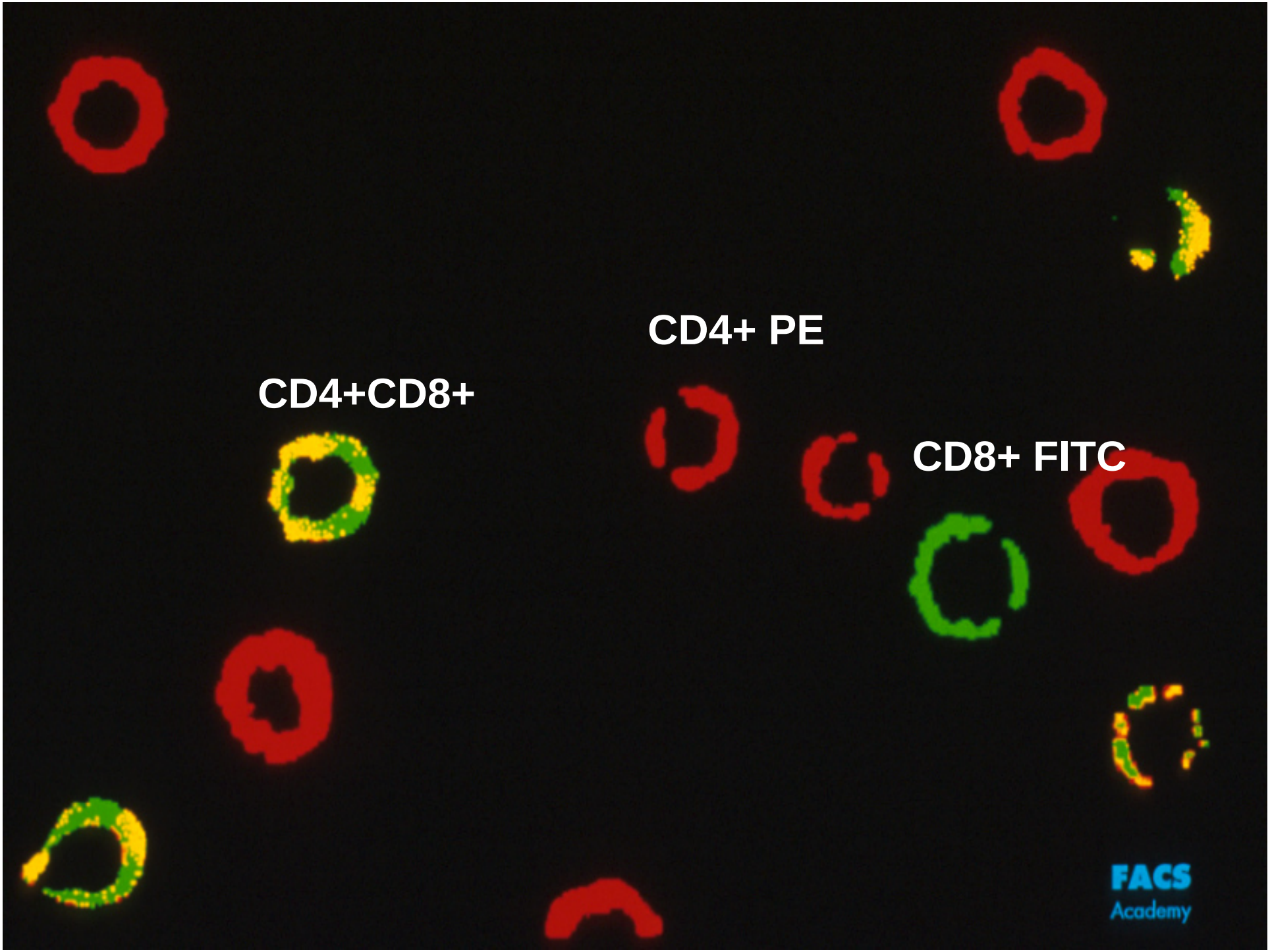
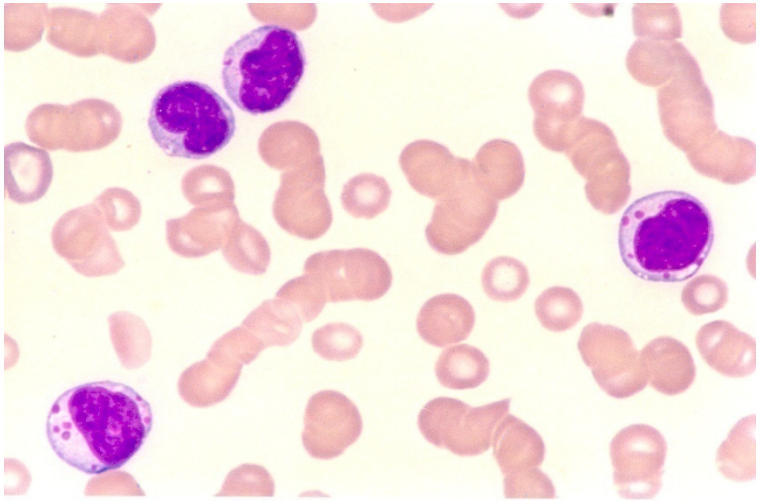
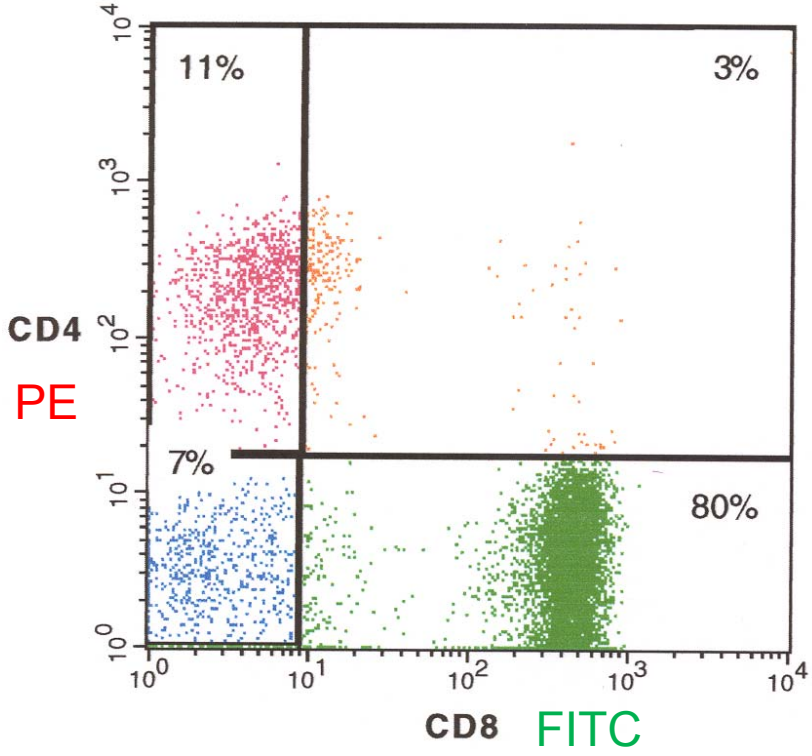

CD4+ PE
CD4+CD8+
CD8+ FITC
FACS
Academy

Large granular lymphocytosis



Lymphocytosis = 13,000/µL



Does the dog have Ehrlichiosis or lymphocytic leukemia?

Heeb, Wilkerson, Chun. JAAHA. 2003:39:379-384

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When should immunophenotyping be ordered?

- 2006 Consensus recommendations to use FC IP to screen for malignancy in people:
  - Bicytopenia and pancytopenia
  - Elevated leukocyte concentration
  - Presence of atypical cells or blasts in blood, bone marrow, or body fluids
  - Plasmacytosis or monoclonal gammopathy
  - Organomegaly and tissue masses

Key Role in Diagnosis and Classification

- Mature lymphoid neoplasms
- Plasma cell neoplasms
- Blastic malignancies
- Maturing myeloid and monocytic malignancies
- Monitoring treatment of a previously diagnosed hematolymphoid neoplasia
- Detection of minimal residual disease
  - 0.1 to 0.01% abnormal cells

Immunophenotyping in Veterinary Medicine

- Immunophenotyping using monoclonal antibodies to cluster differentiation (CD) molecules better defines the lineage of hemic neoplasia than morphologic assessment
  - B lymphomas respond more readily to therapy
  - Leukemias can be differentiated into
    - Myeloid vs. Lymphocytic
    - Acute leukemias identified by CD34 expression
    - Chronic lymphocytic leukemia in dogs is principally a CD8+ cell lineage (CD4+ in cats)
      - W. Vernau and P. Moore, 1999. Vet. Immun. Immunopathol.

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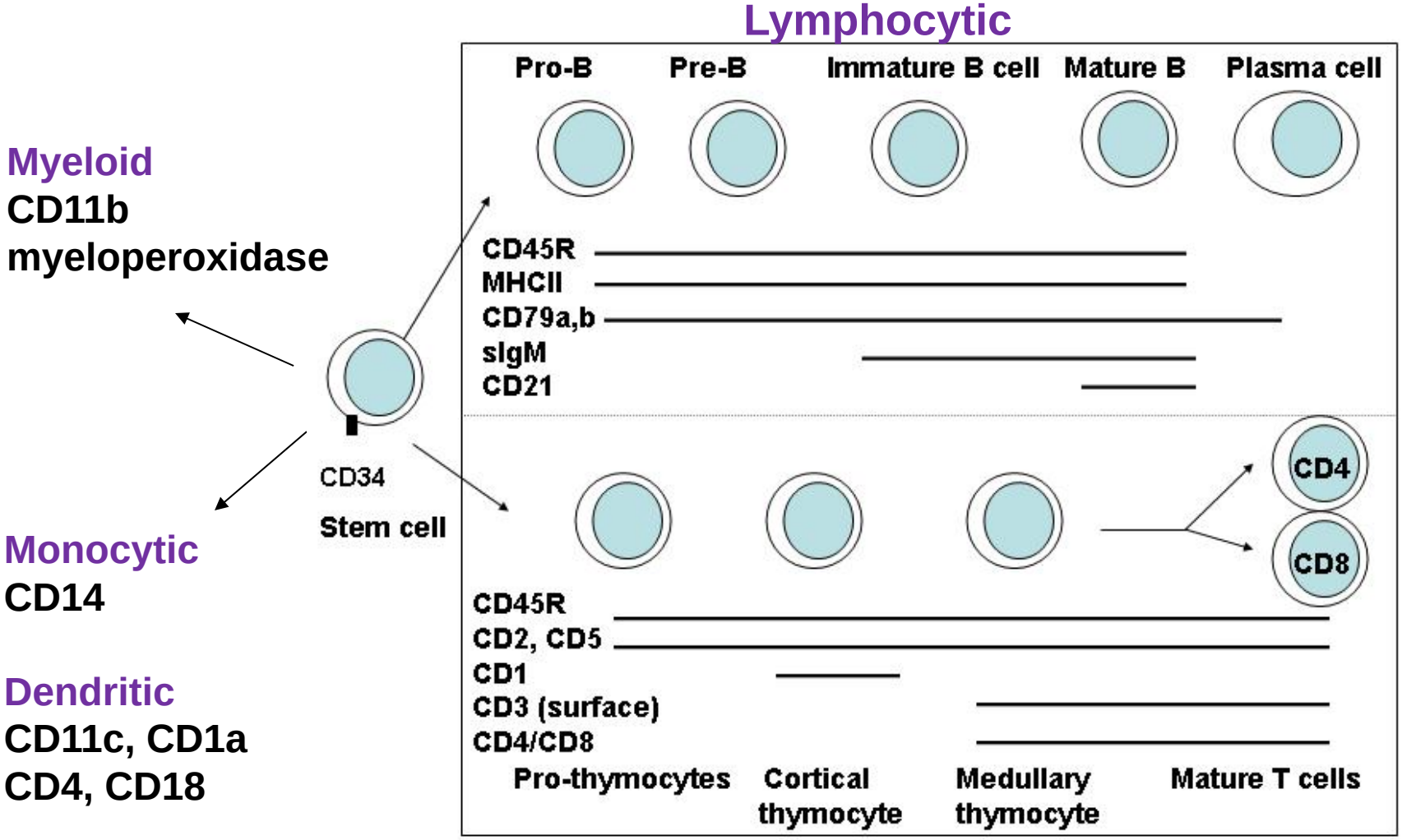
Cell lineage differentiation defined by Cluster of Differentiation molecules



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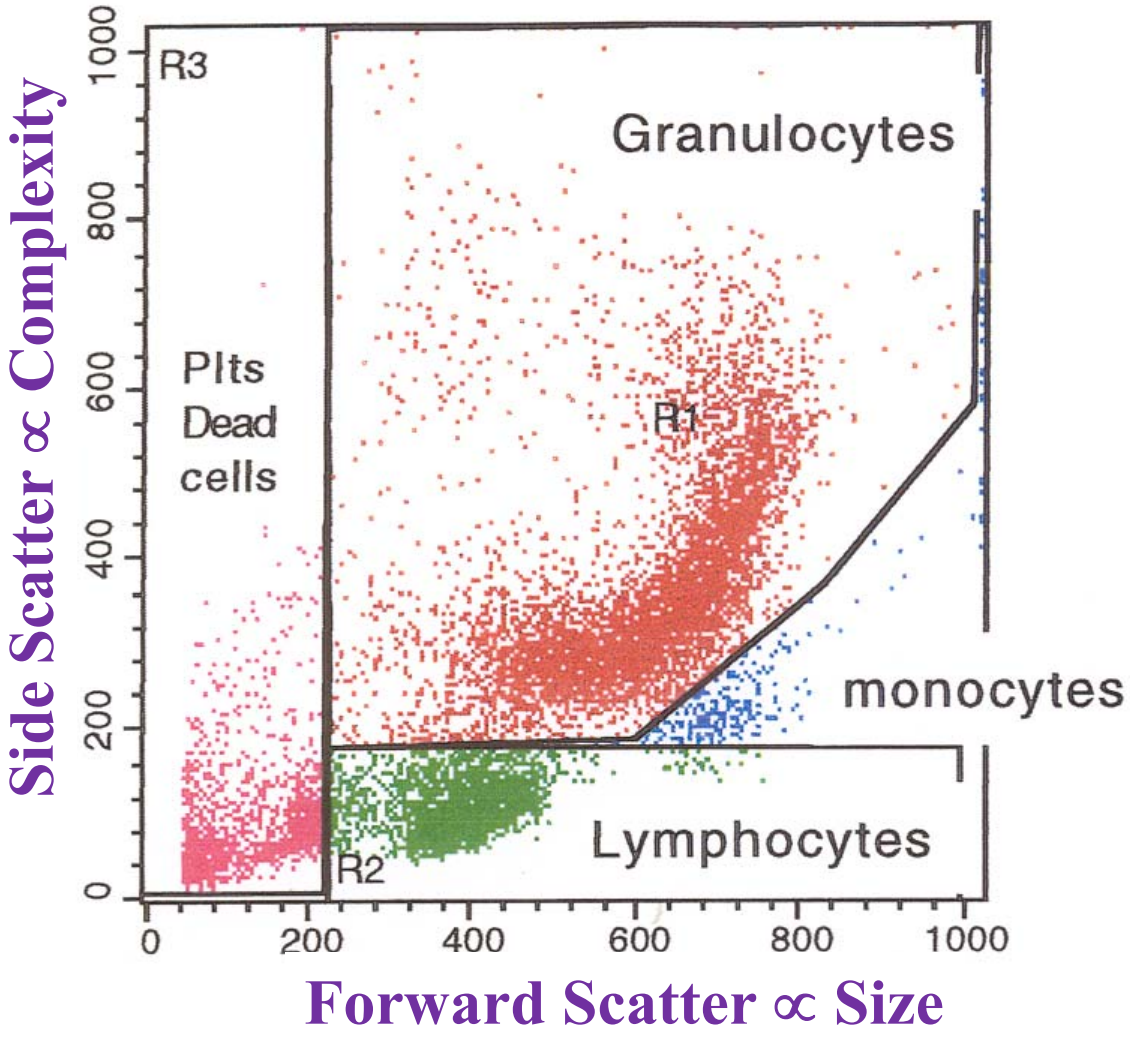
Selecting an Antibody Panel

CD molecule	T cell	B cell	Others
CD45			Pan leukocyte
CD34			Stem cell
CD3 or CD5	T cells		
CD4	T helper		
CD8	T cytotoxic		
CD79a (cytoplasmic)		Pro B cell to Plasma cell	
sIgM, CD22		Immat./Mat. B	
CD21		Mature B	
CD14			Monocytes
CD11b			Granulocytes
CD11c/CD1a			Dendritic cells

Sample preparation and processing

- Blood or bone marrow in EDTA anti-coagulated collection tubes
- Tissue samples (lymph nodes)
  - Needle biopsy of multiple areas
  - Best to have 500,000 to 1 million cells/mL
  - Add sample to a tube of 1 – 2 mL of saline
  - Send overnight carrier on cool packs

Scatter Plot of blood leukocytes



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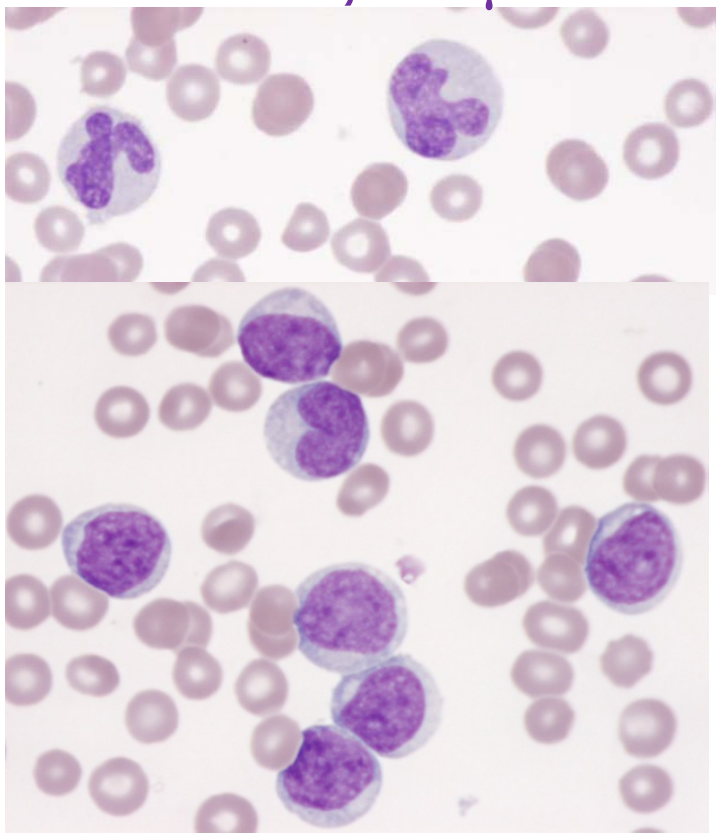
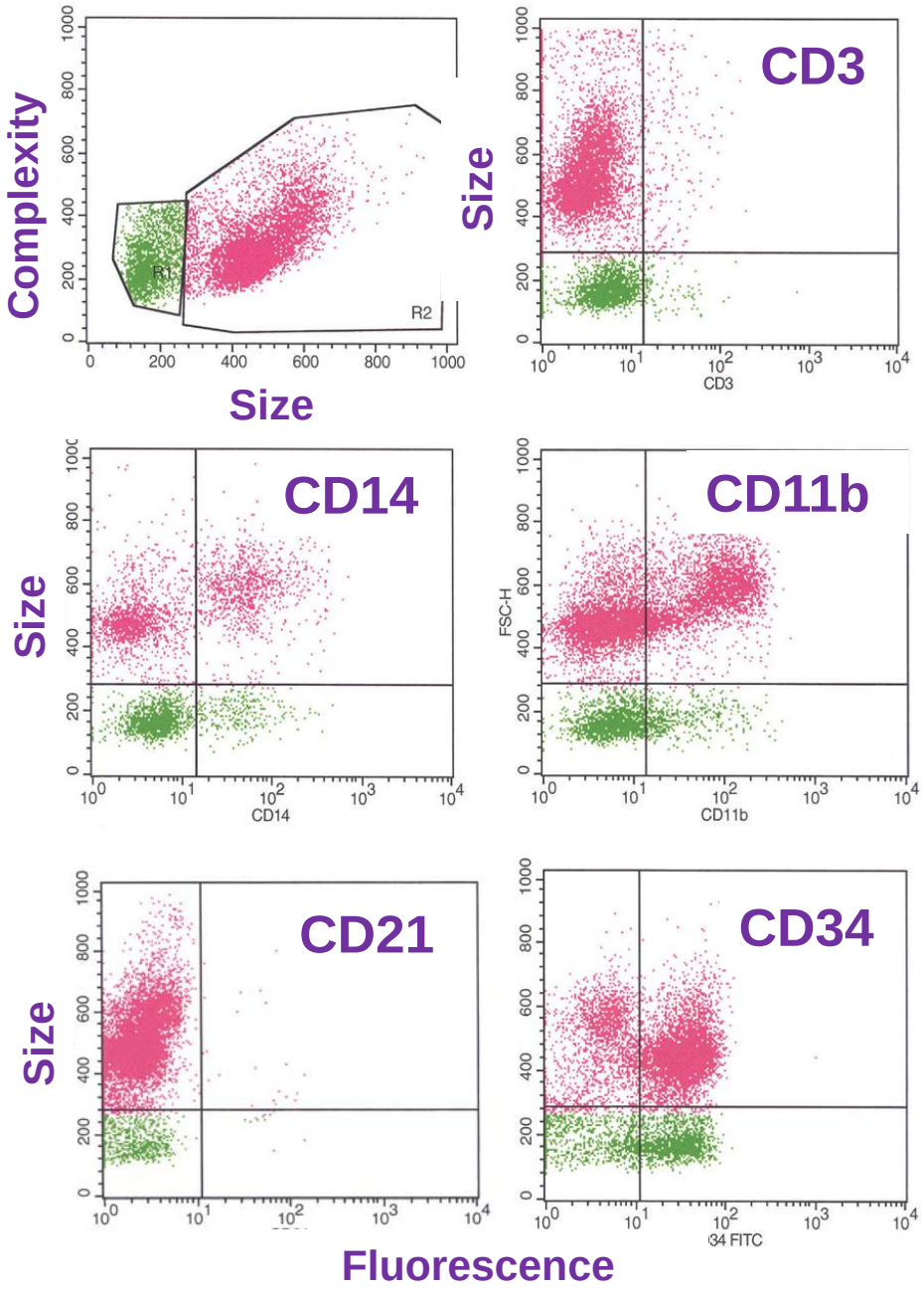
Leukemia

13 yr Spaniel
WBC = 120,000/μL



Acute Leukemia (CD34+)
Possible Myelomonocytic



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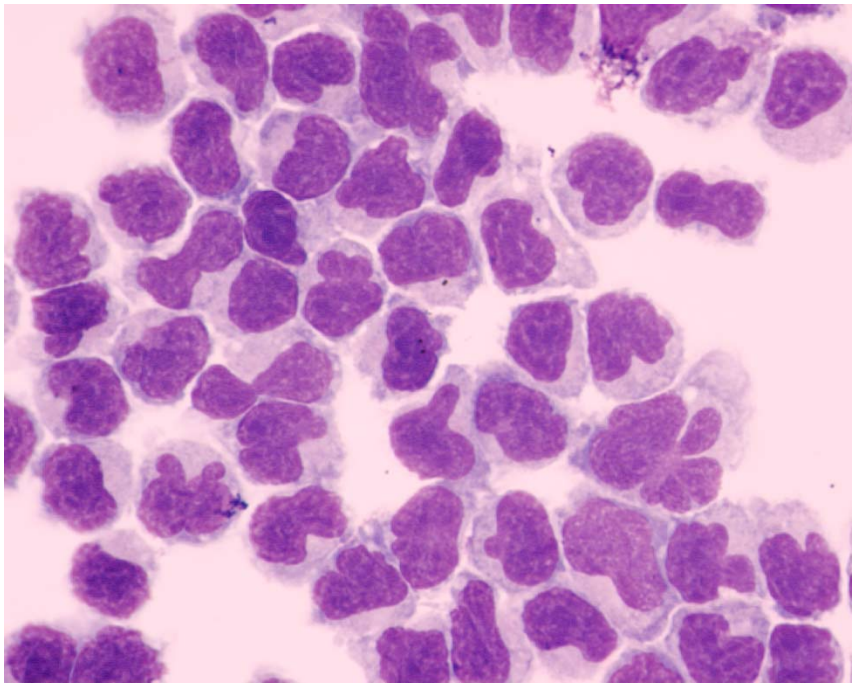
Guidelines for lymphoid malignancy

- In blood, lymphocyte concentration above reference range for lab and one of the following
  - 80% of lymphocytes with a single phenotype or
  - 60% of lymphocytes with a single phenotype and a positive clonality assay (PARR) or
  - Presence of lymphocytes with an aberrant phenotype for peripheral blood
- In tissue
  - Expansion of homogenous population of single phenotype
  - Presence of aberrant phenotype

Immunophenotyping of Fluids

- Cerebral spinal fluid
- Dog with ataxia
- Epidural lesion at T4
- Total nucleated cell count = 3,175/μL
- Protein = 145 mg/dL
- Cytology
  - Pleocytocysis
  - Monocytic or lymphocytic?



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Diagnosis of CSF case

- Imunophenotyping panel
  - CD45 (pan leukocyte) 99% cells
  - **CD3+ (T cell marker) 87% cells**
  - CD21 (mature B cell) 3% cells
  - CD14 (monocyte) 18% cells
- Necropsy
  - Multicentric lymphoma
  - Lymph nodes and Liver
  - Invasion of spinal nerves

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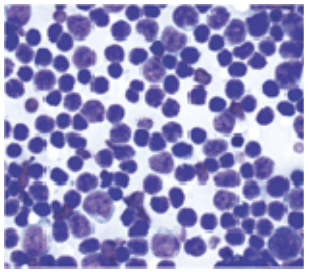
Adult Dog with mediastinal mass

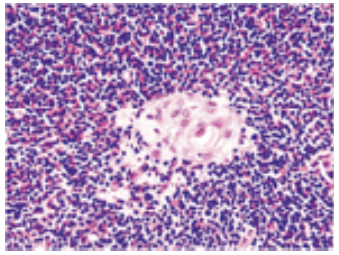
Cells from needle aspirate labeled with fluorescent antibodies to CD4 and CD8

- Thymic lymphoma?
- B cell lymphoma?
- Thymoma? 🙂



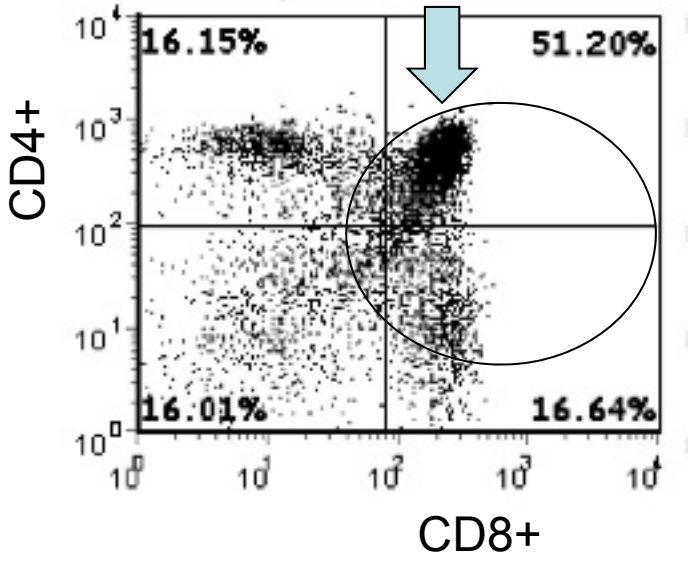
cytology

Small lymphocytes



histopath

CD4+CD8+

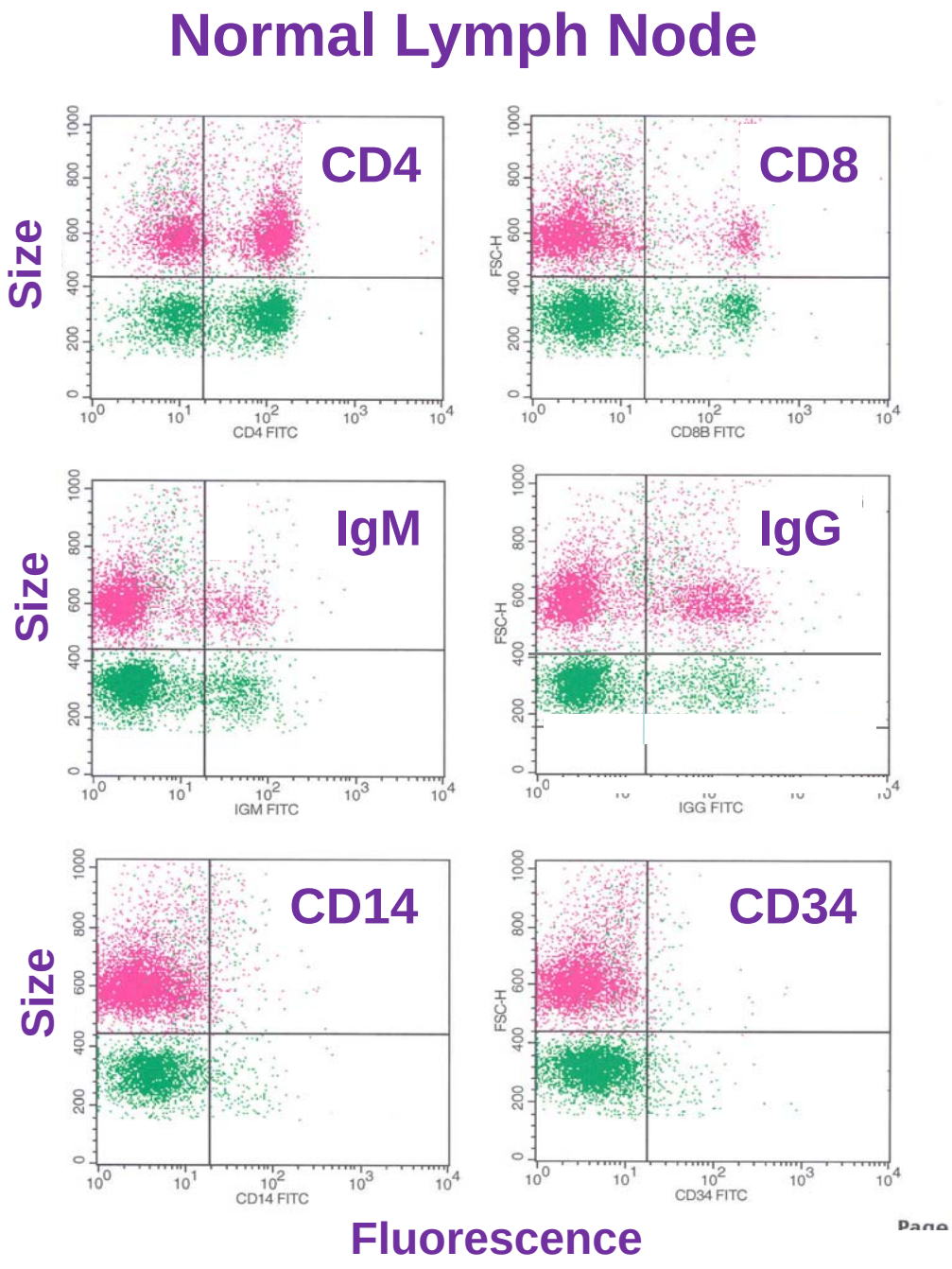


Is this lesion polyclonal or monoclonal? What would You expect with PARR?

See Lana et al., 2006 JVIM and Lara-Garcia et al., 2008 VCP.

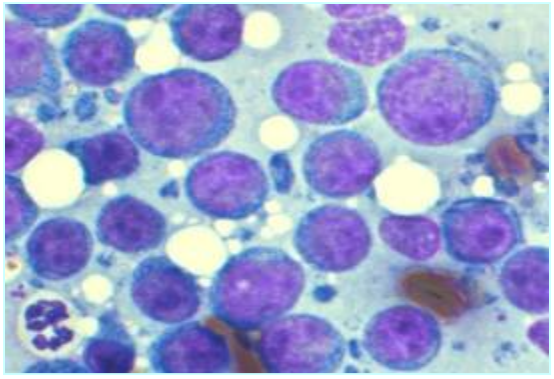
Antibody specificity

- **Dot plot analysis**
  - **Complexity vs. size**
    - Place gates on cells based on size
    - Green = Small cells
    - Pink = Large cells
  - **Size vs. Fluorescence**
    - Cells stained for CD markers are detected to the right of the vertical line

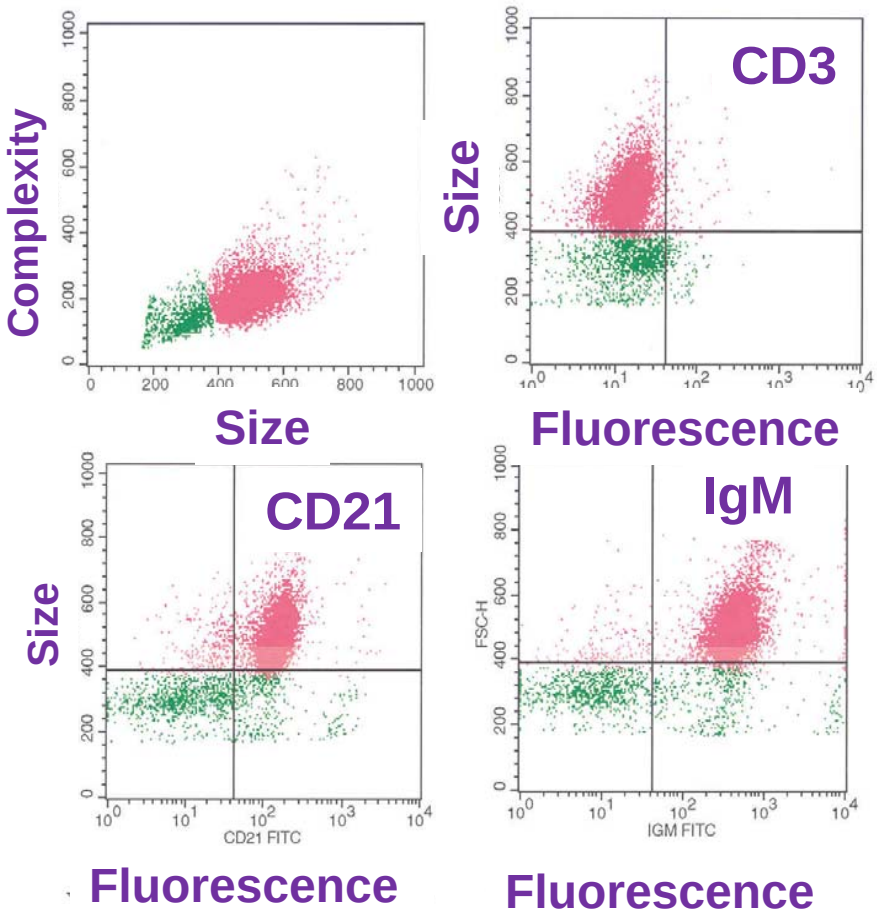


Lymphoma cases

- **5 yr old Labrador**
- **Large lymphoblastic cells**
  - ❑ **CD3 negative**
  - ❑ **CD21+ IgM+**



B cell lineage



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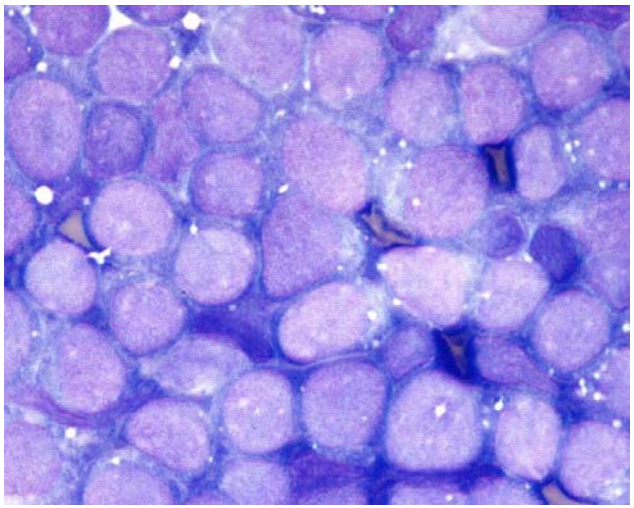
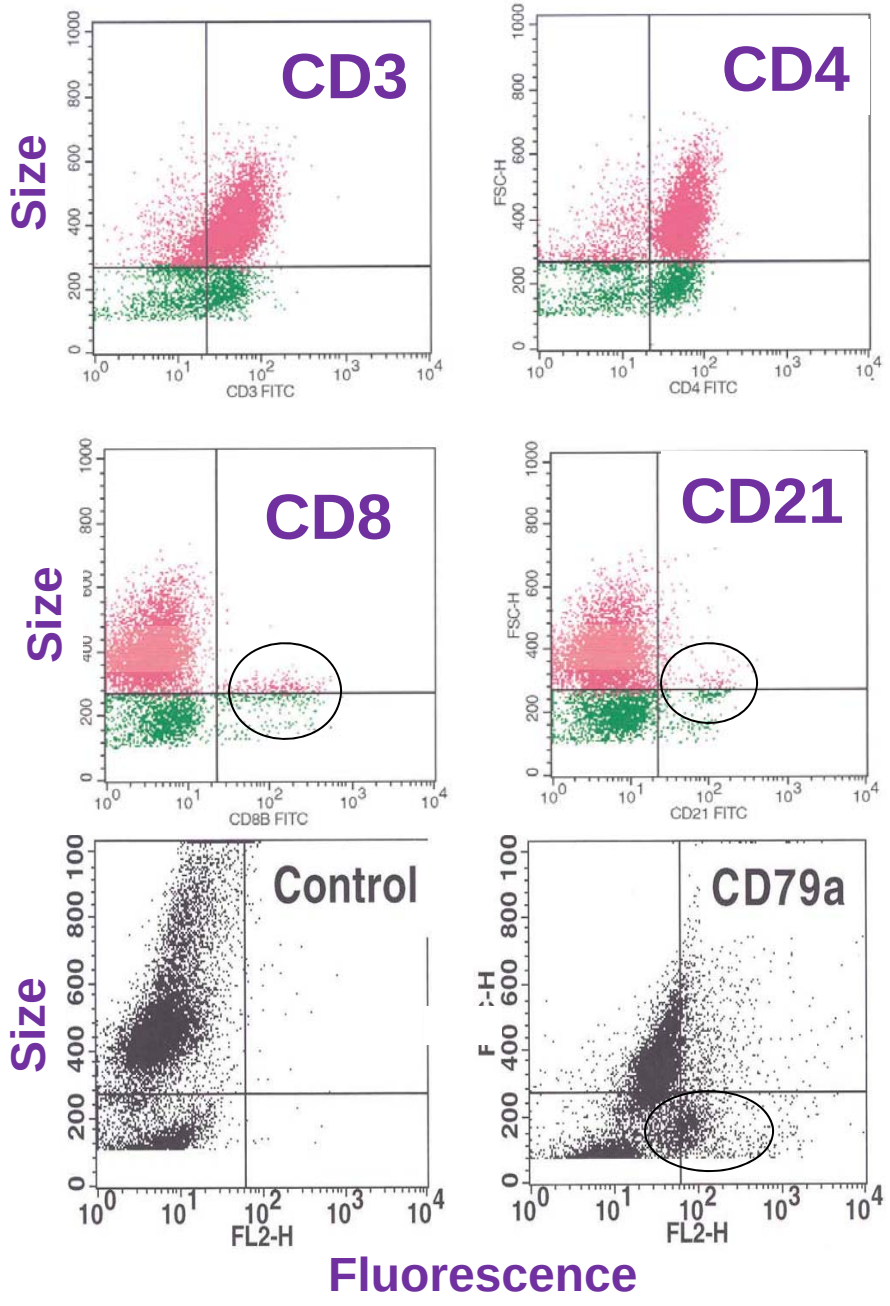
Case 2

9 year old mix breed dog

- **Large & small cells**
  - **CD3+ CD4+**
- Few small cells (resident)
  - CD8+
  - CD21+CD79a+



T cell lineage



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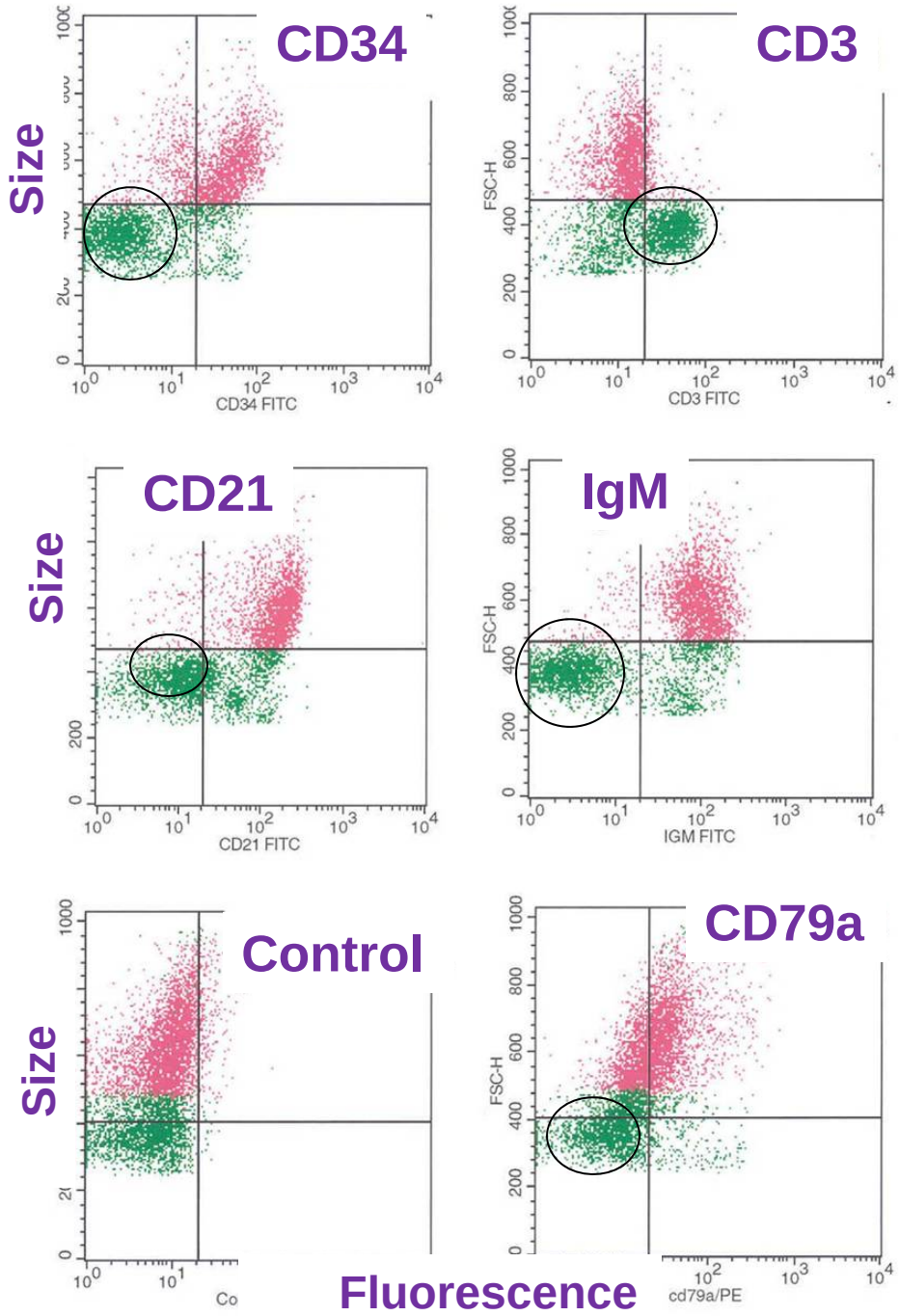
Case 3
7 year old Pointer

- Large cells
  - CD34+ CD21+ IgM+ CD79a wk+
- Small cells
  - CD3+
- Significance of CD34?
- Which cells are neoplastic?



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Significance of CD34 expression in human lymphoma

- Schmidt et al., **Aberrant Antigen Expression Detected by Multiparameter Three Color Flow Cytometry in Intermediate and High Grade B-Cell Lymphomas.** 1999. Leukemia and Lymphoma

15% of the B cell lymphomas were CD34+

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Immunophenotypic markers of prognosis in canine lymphoproliferative disorders

- Study at CSU of 96 dogs with lymphocytosis including stage V lymphoma. Williams et. al. 2008. JVIM
  - CD34+ phenotype in the blood had shorter survival time (average of 16 days) compared to lymphocytosis that expressed mature B and T cell antigens (300 – 500 days)
  - CD21+ lymphocytosis composed of large cells had shorter survival times compared to small cell CD21+ lymphocytosis
- Rao, et. al., JVIM. 2011
  - Lack of MHC II expression in B cell lymphomas had a poor outcome (Rao, et al., JVIM. 2011.)
- Avery et. Al. Abstract, ACVP Proceedings 2012
  - T cell lymphomas not expressing CD5 had a better prognosis

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Case 4: 2-year old male Golden retriever with peripheral lymphadenopathy

Laboratory Data Abnormalities

Hypercalcemia	[19.4 mg/dL]	Ref. Range [9.7-12.1 mg/dL]
Thrombocytopenia	36,000/μL	Ref. Range [164,000-10,000/μL]
Lymphopenia	800/ μL	Ref. Range [1,500-5,000/μL]

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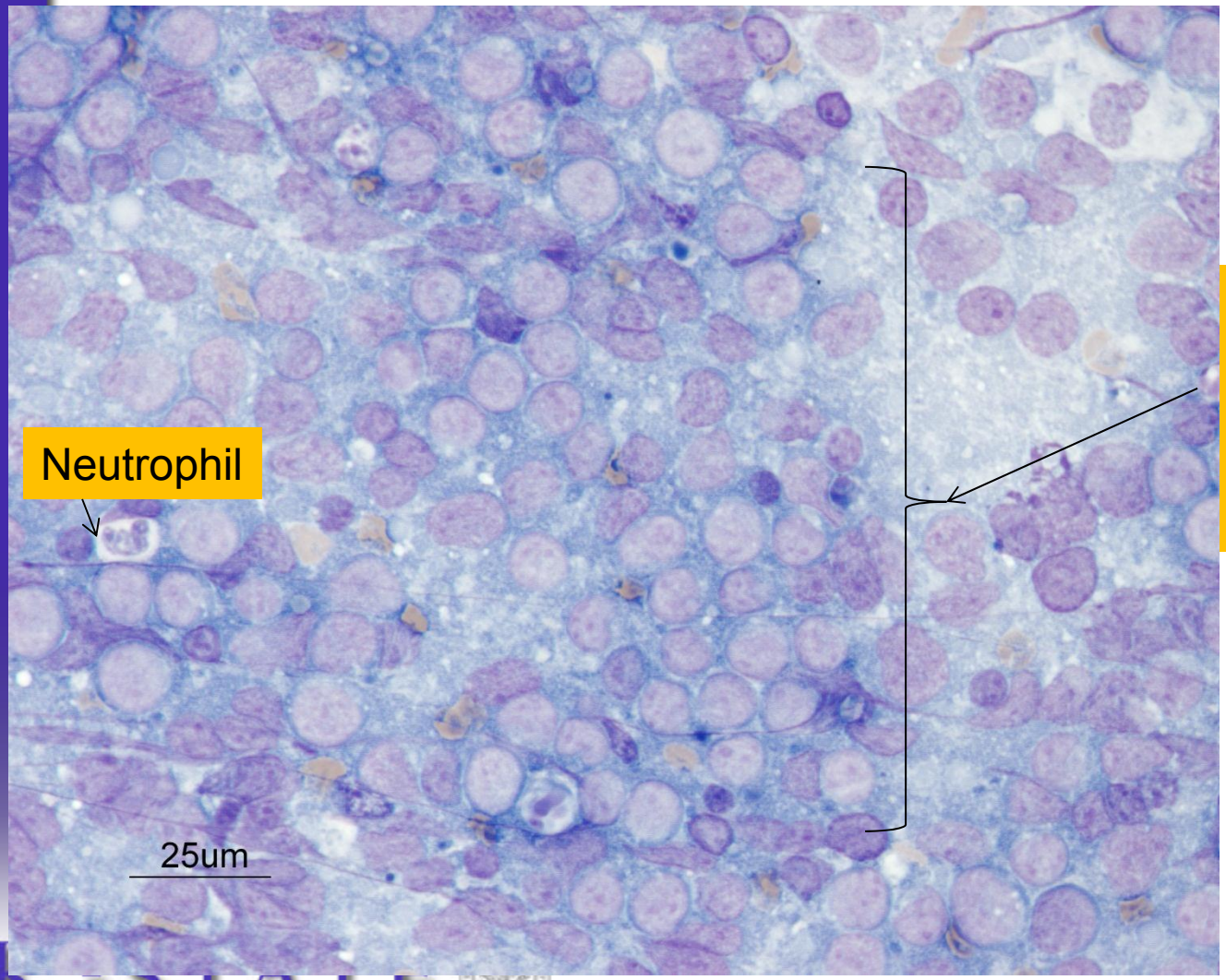
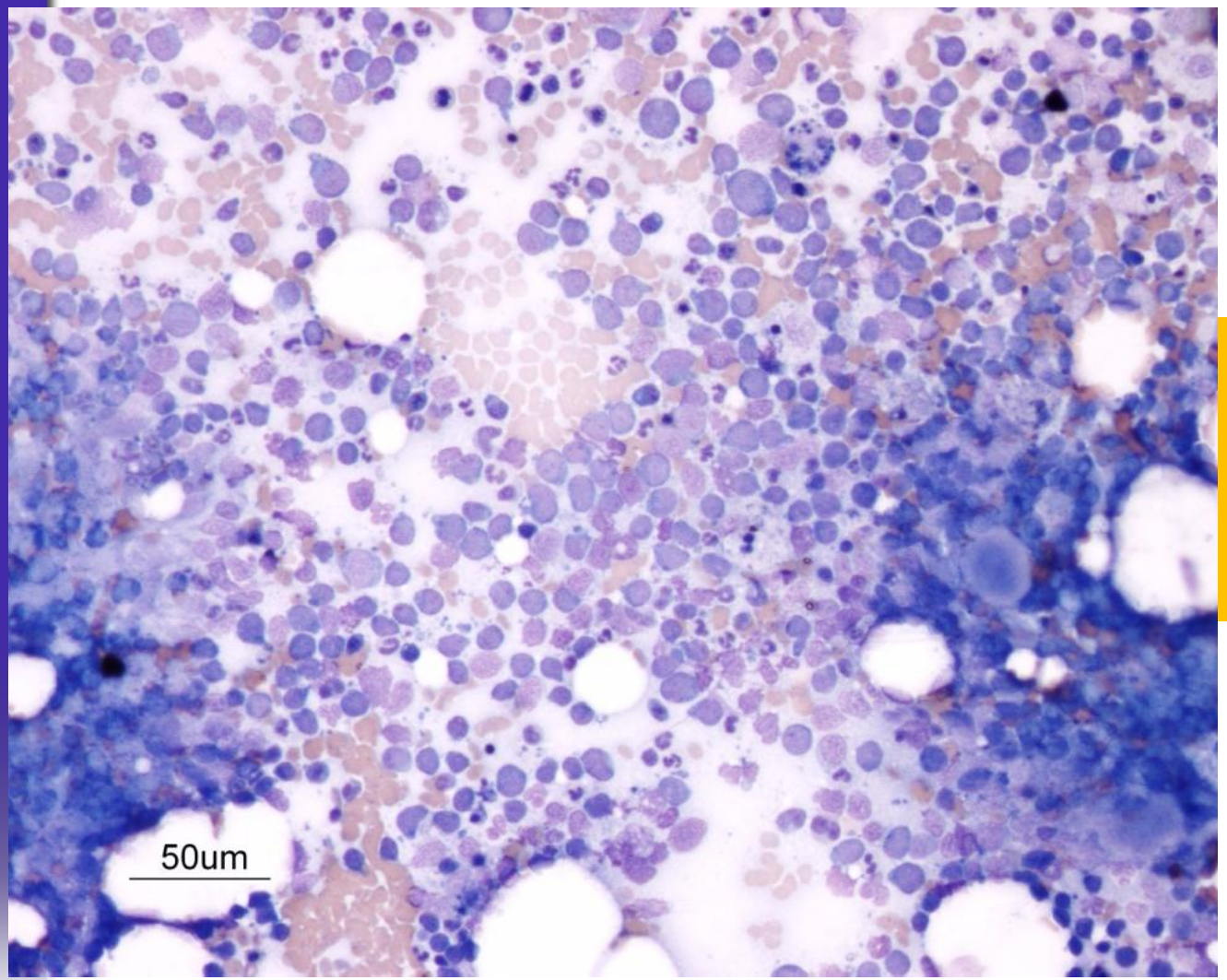
Aspirate of prescapular lymph node



90% of cells are immature lymphocytes

Bone Marrow Aspirate



Immature lymphocytes replace normal bone marrow

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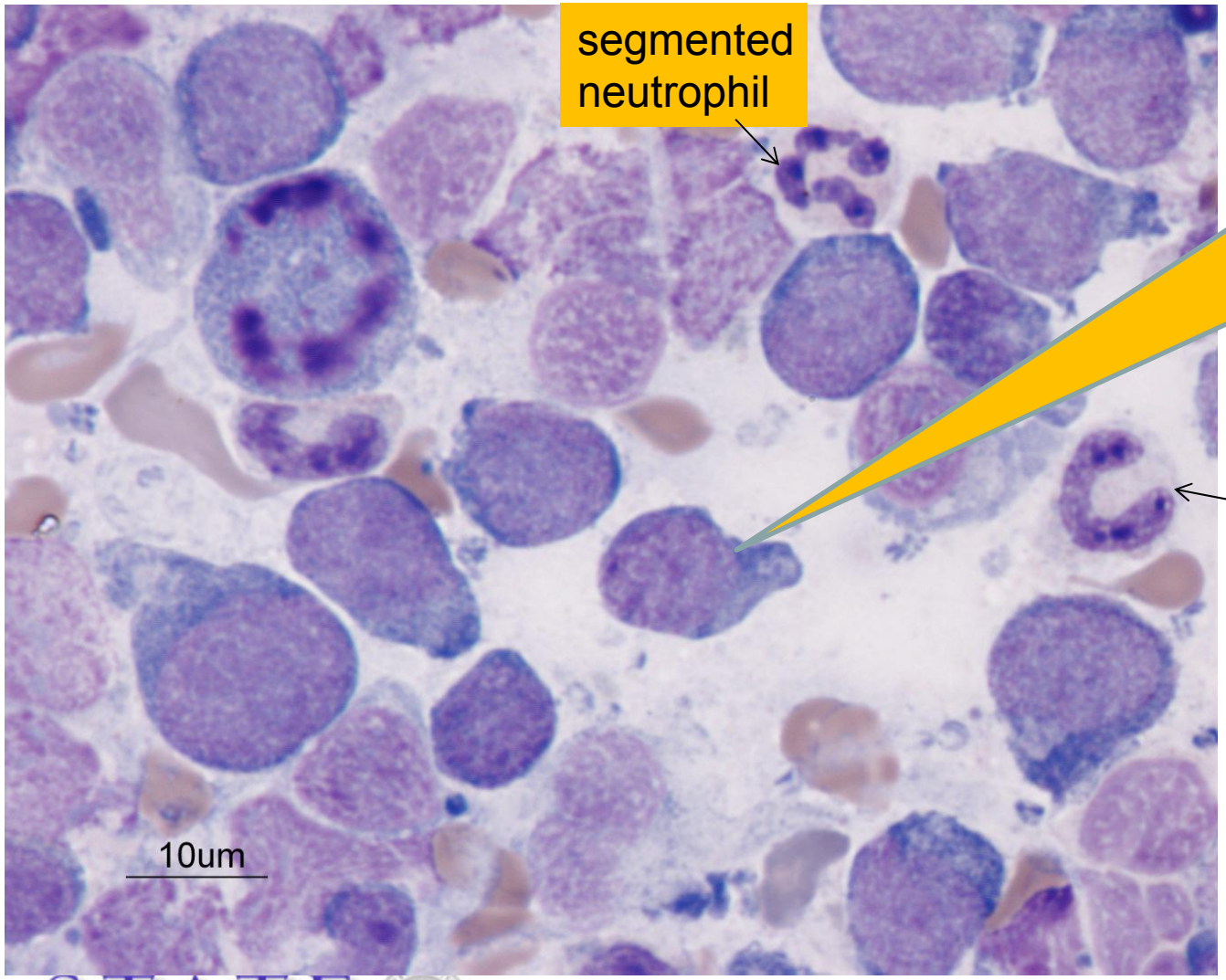
High power image of Bone Marrow



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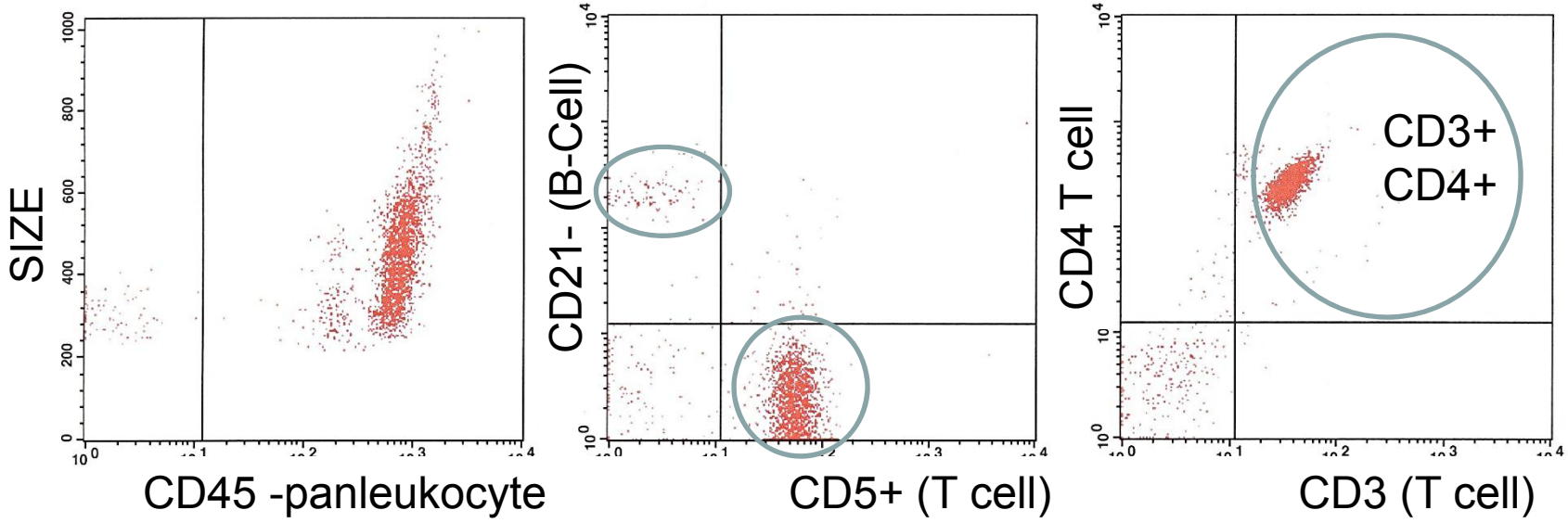
Diagnosis = Stage V lymphoma
Additional Tests: Immunophenotyping using flow cytometry to determine cell lineage

Majority of lymphocytes in lymph node aspirate express T cell antigens (CD5, CD3, CD4)

Cell lineage = ?



Hypercalcemia common with T Cell Lymphomas

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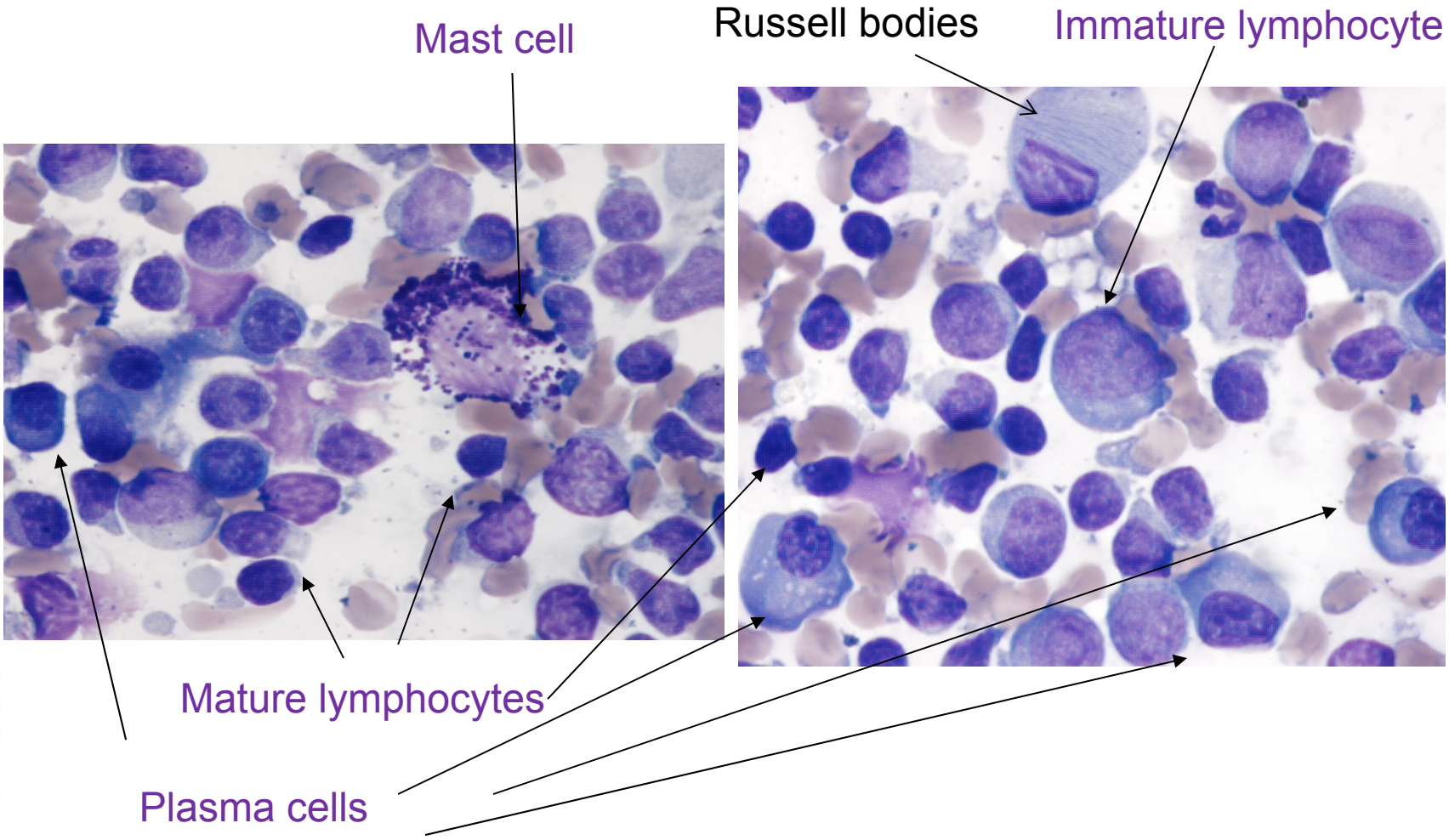
Lymphoma vs reactive lymph node?

- Lymphoma = neoplasia of lymphocytes
  - Clonal expansion of T or B cells
    - Single receptor specificity
    - Immature morphology
- Reactive lymph node
  - Antigenic stimulation causes expansion of multiple clones of T and B cells (polyclonal)
    - Multiple receptor specificities
    - Mature morphology and presence of plasma cells

Reactive lymph node



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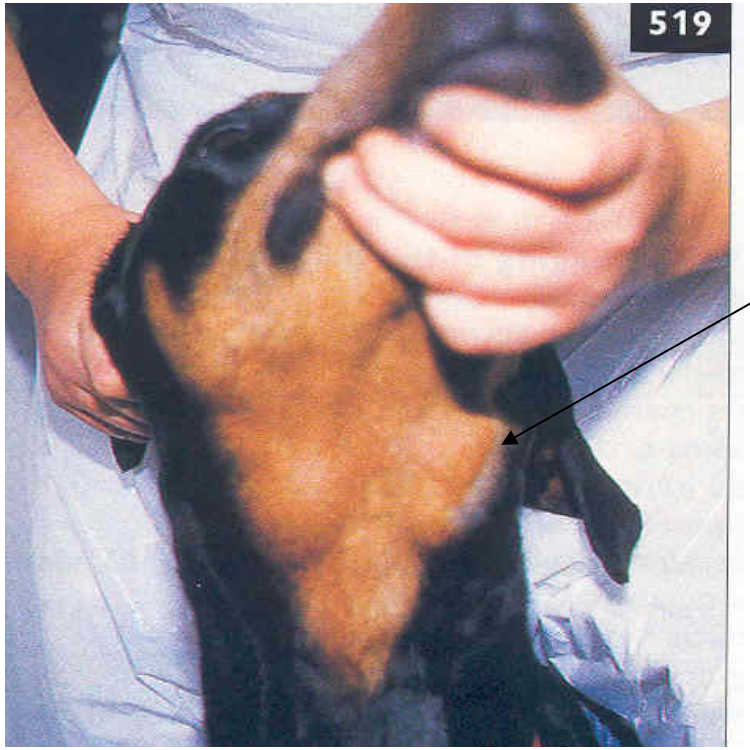
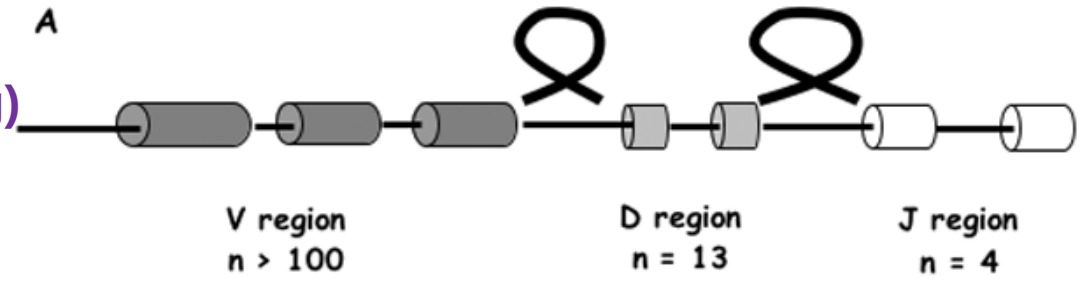
How do you distinguish reactive lymph node enlargement from lymphoma?



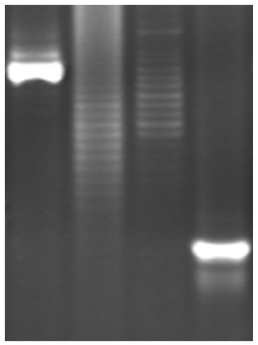
- Grossly bilateral lymphadenopathy suggests neoplasia

PCR for antigen receptor (PARR) rearrangements to identify clonality

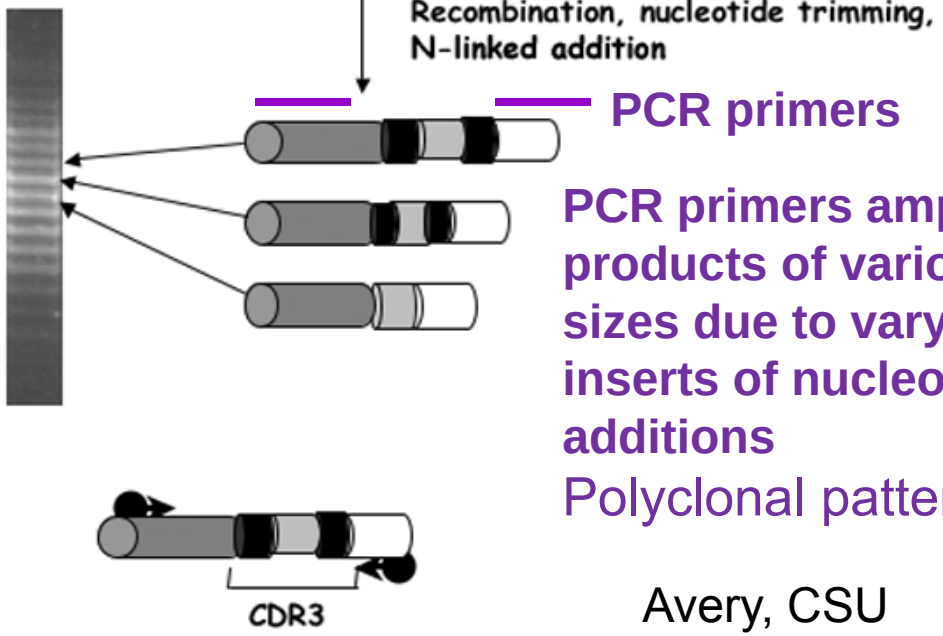
TCR
BCR (Ig) genes



control
Cμ
+ _Ig_TCR



Single band indicates monoclonal population of T cells



PCR primers

PCR primers amplify products of various sizes due to varying inserts of nucleotide additions

Polyclonal pattern

Avery, CSU

Primers amplify antigen binding site

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Recombination

Light chain or α

Heavy chain or β

Germline DNA

exons

introns

V_{1-40} J_{1-6} C

V_{1-51} D_{1-27} J_{1-5} C_{H1} C_{H2} C_{H3}

DJ rearranged

VJ or V-DJ

mRNA

AAA

AAA

CDR3 encodes for Ag binding site

CDR3

V_L C_L

V_H C_H

BCR or Antibody protein

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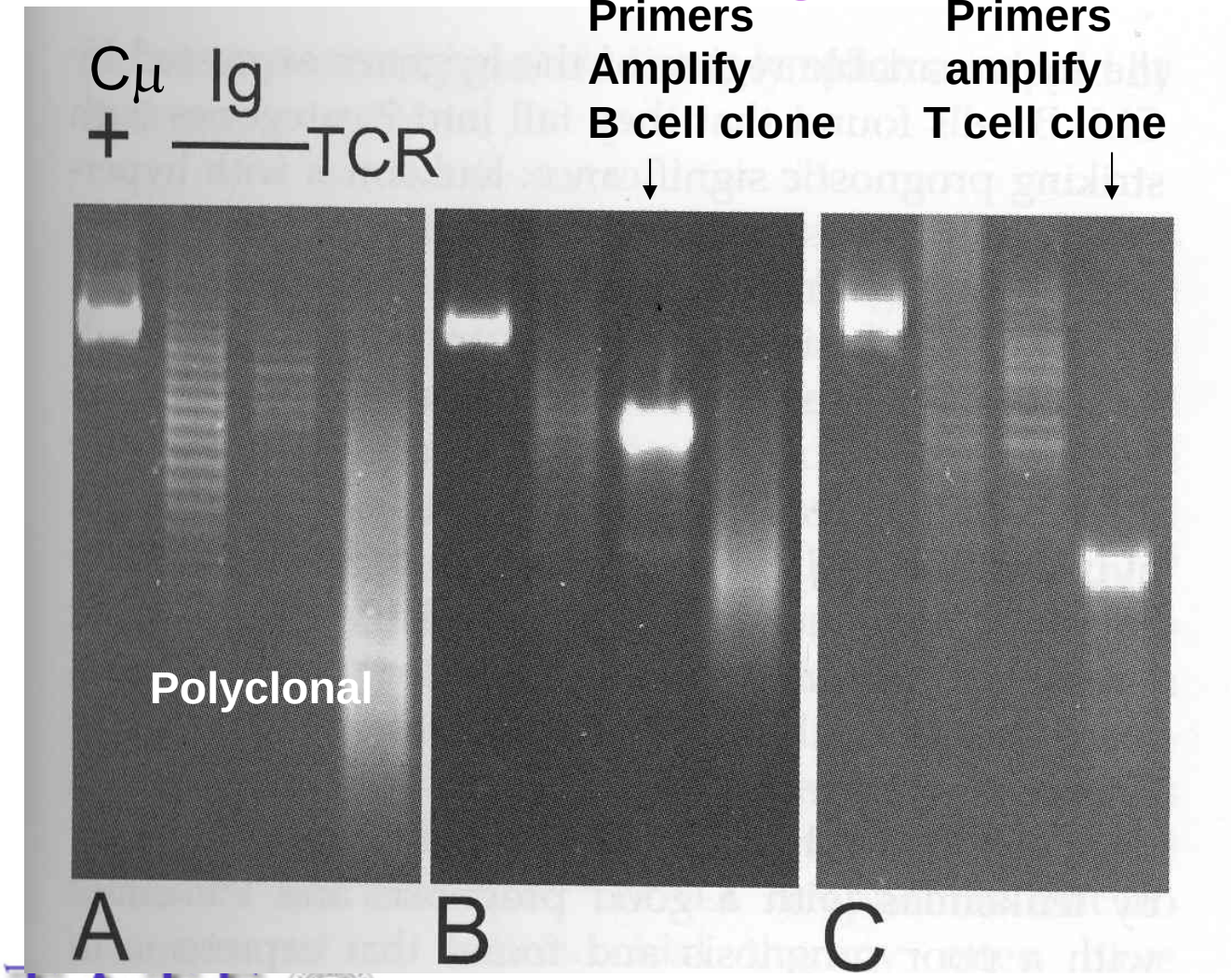
PCR for antigen rearrangement



Dr. Avery
CSU

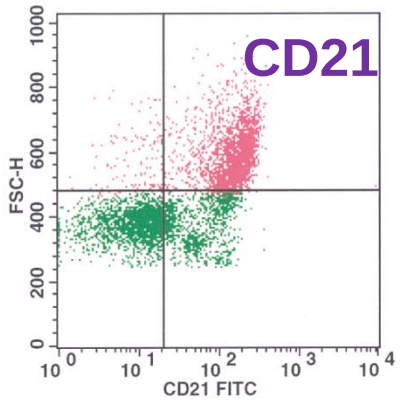
Important information on the PARR test

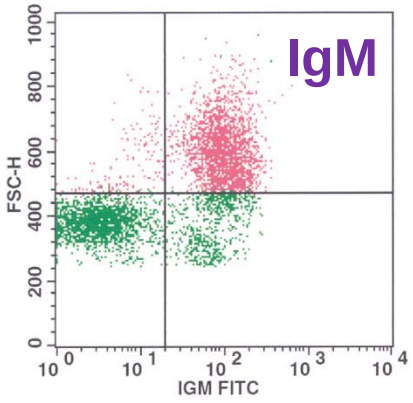
1. Sensitivity & Specificity: 75% & 92%
2. Detects 1 neoplastic lymphocytes in 100 heterogeneous nonneoplastic cells
3. Detects neoplastic lymphocytes in PB 2.5 times more than microscopic evaluation
4. Not prognostic for disease-free interval or duration of survival
5. Confirmed B-cell lymphosarcoma in aqueous humor sample
6. **False negatives** – primers not specific, somatic recombination, NK cells
7. **False positive** - -pseudoclonality (amount and quality of DNA)

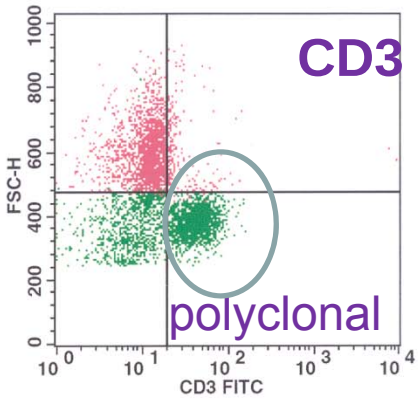
- Burnett et al., 2003 Vet. Path.
- Keller et al., 2004. Vet Clin. Path.
- Lana et al., 2006. JVIM
- Pate et al., 2011. JAVMA
- Werner et al., 2005 Vet. Path.

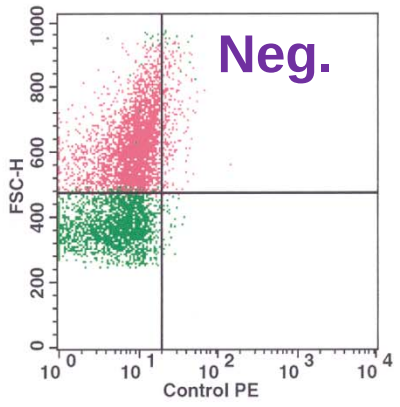
Case 5 Mixture of B and T cells
Large B lymphocytes are clonal

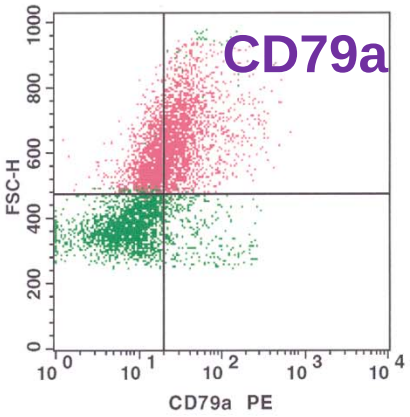
SIZE

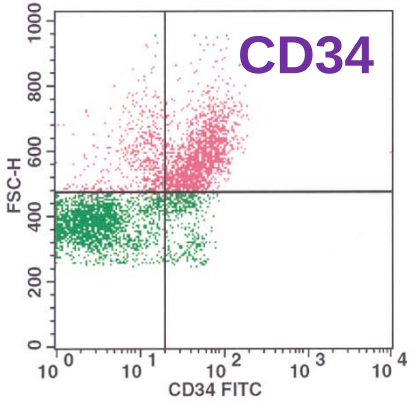


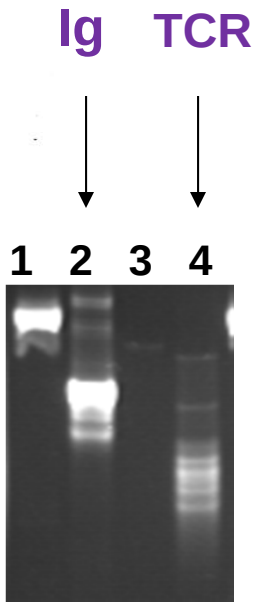












T cell Rich
B cell Lymphoma

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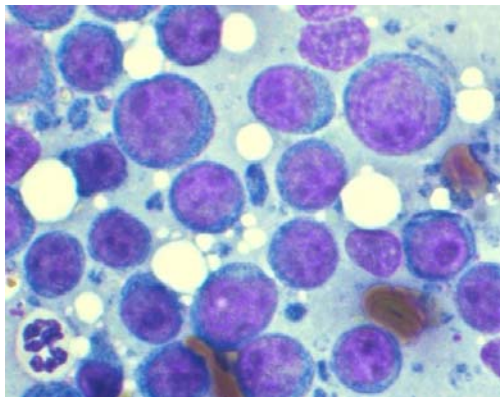
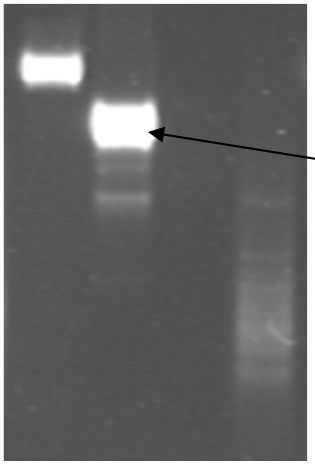
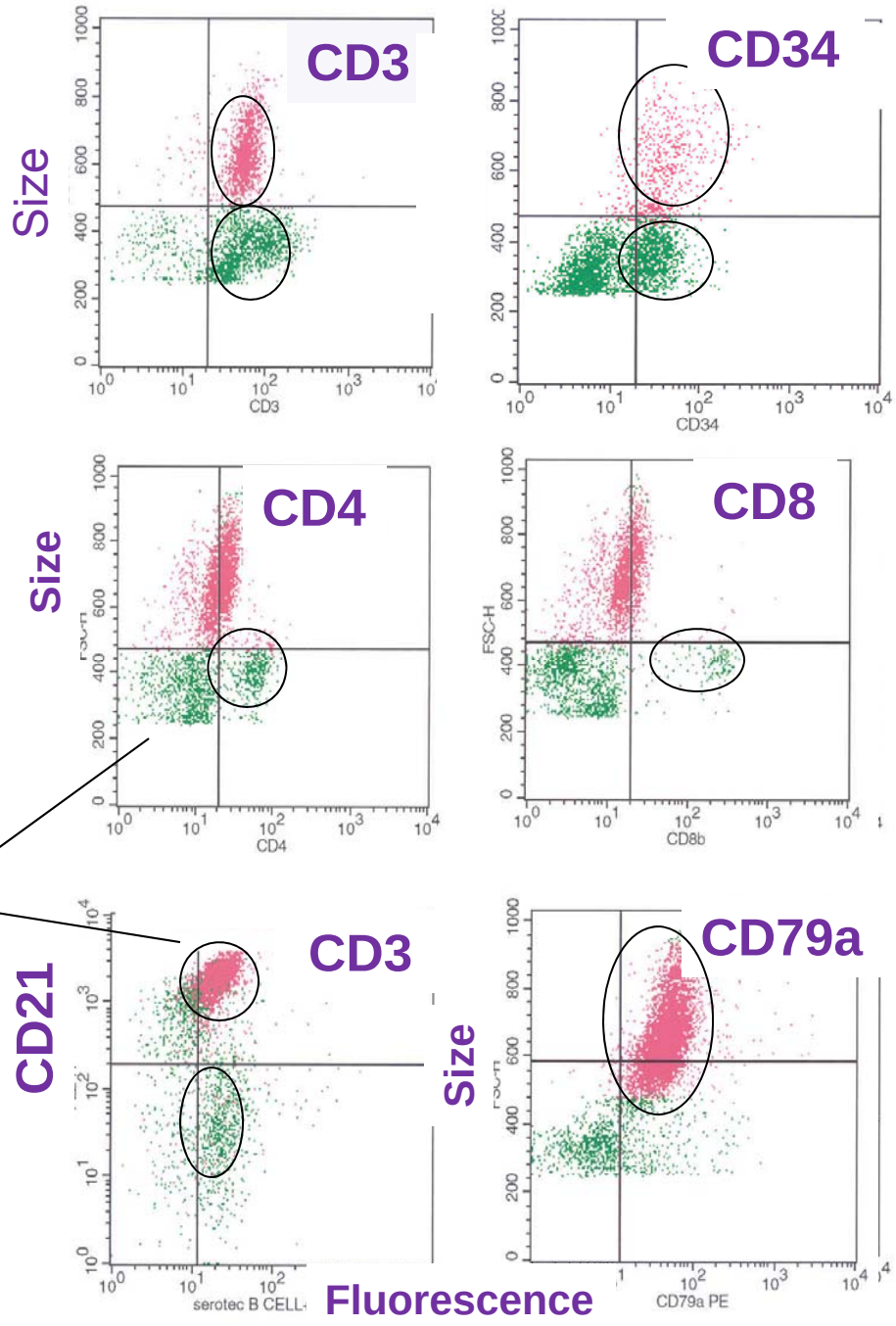
Case 6
7 yr Labrador cross

- **Large & small cells**
  - CD3+, CD34+
- **Small cells**
  - CD4+, CD8+ bright
- **Large cells**
  - CD21+/CD3+, CD79a+





B cell lineage



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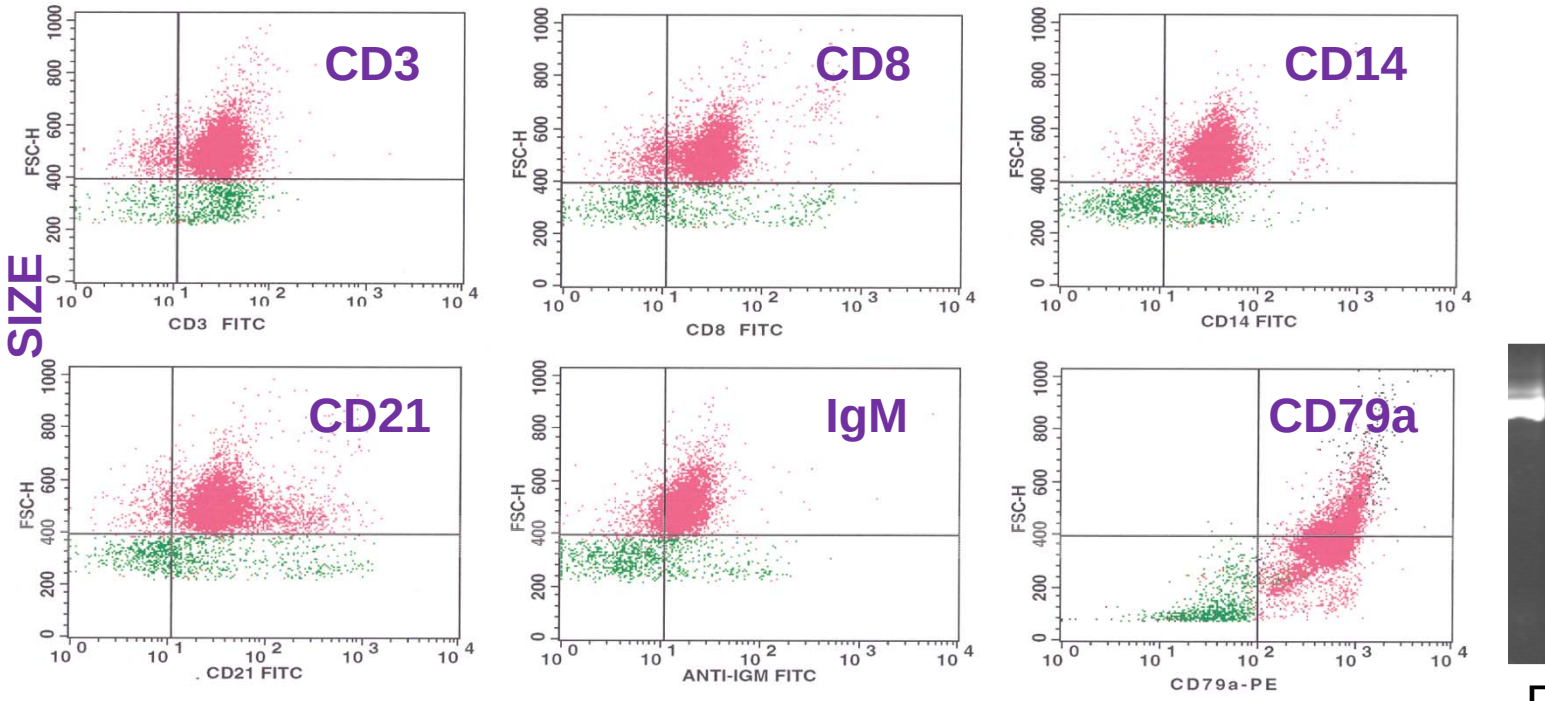
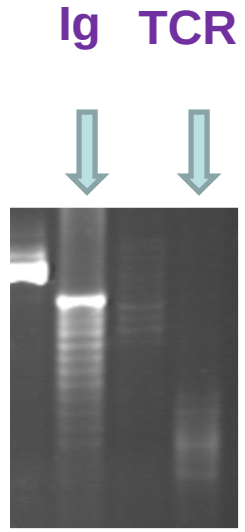
Case 7: Aberrant T cell and monocyte antigen expression in a B cell clone





PARR indicates B cell clonality

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Reports of aberrant CD expression and gene rearrangements

- Gelain et al., 2008. Aberrant phenotypes and quantitative antigen expression in different subtypes of **canine** lymphoma by flow cytometry.
  - B – decreased CD79 and expression of CD34
  - T – decreased CD45 expression
  - CD3 or CD5 expression without CD4 or CD8
  - CD79 and not CD21 for B cells
- Wilkerson et al., 2005. Lineage differentiation of canine lymphoma/leukemias and aberrant expression of CD molecules.
  - CD8, CD14, and CD21
- Kyoda et . al. 1997. Prognostic significance of immunoglobulin heavy chain gene rearrangement in patients with acute myelogenous leukemia.

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Conclusions

1. Flow cytometry Immunophenotyping useful tool in diagnosis/prognosis of canine lymphoproliferative and hematopoietic neoplasias
   - Providing the sample can be dispersed in suspension
   - Correlates with cytomorphologic features
2. Broad immunophenotyping panels and multi-color analysis improves diagnostic capabilities.
3. Lineage infidelity is common at early stages of hematopoietic differentiation. Aberrant expression of CD molecules occurs in canine lymphomas/leukemias and could be used to screen for neoplasic disorders, minimal residual disease or monitor relapse.
4. PARR done when cytology, histopathology, and immunophenotyping is ambiguous.

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- **Avery, A. 2009.** Molecular diagnostics of hematologic malignancies. Top Companion Anim Med 24: 144-150.
- **Burnett, R. C., W. Vernau, J. F. Modiano, C. S. Olver, P. F. Moore, and A. C. Avery. 2003.** Diagnosis of canine lymphoid neoplasia using clonal rearrangements of antigen receptor genes. Vet Pathol 40: 32-41.
- **Gelain, M. E., M. Mazzilli, F. Riondato, L. Marconato, and S. Comazzi. 2008.** Aberrant phenotypes and quantitative antigen expression in different subtypes of canine lymphoma by flow cytometry. Vet Immunol Immunopathol 121: 179-188.
- **Keller, R. L., A. C. Avery, R. C. Burnett, J. A. Walton, and C. S. Olver. 2004.** Detection of neoplastic lymphocytes in peripheral blood of dogs with lymphoma by polymerase chain reaction for antigen receptor gene rearrangement. Vet Clin Pathol 33: 145-149.
- **Lana, S., S. Plaza, K. Hampe, R. Burnett, and A. C. Avery. 2006.** Diagnosis of mediastinal masses in dogs by flow cytometry. J Vet Intern Med 20: 1161-1165.
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- **Pate, D. O., B. C. Gilger, S. E. Suter, and A. B. Clode. 2011.** Diagnosis of intraocular lymphosarcoma in a dog by use of a polymerase chain reaction assay for antigen receptor rearrangement. J Am Vet Med Assoc 238: 625-630.
- **Kyoda, K., S. Nakamura, S. Matano, S. Ohtake, and T. Matsuda. 1997.** Prognostic significance of immunoglobulin heavy chain gene rearrangement in patients with acute myelogenous leukemia. Leukemia : official journal of the Leukemia Society of America, Leukemia Research Fund, U.K 11: 803-806.
- **Werner, J. A., J. C. Woo, W. Vernau, P. S. Graham, R. A. Grahn, L. A. Lyons, and P. F. Moore. 2005.** Characterization of feline immunoglobulin heavy chain variable region genes for the molecular diagnosis of B-cell neoplasia. Vet Pathol 42: 596-607.
- **Wilkerson, M. J., K. Dolce, T. Koopman, W. Shuman, R. Chun, L. Garrett, L. Barber, and A. Avery. 2005.** Lineage differentiation of canine lymphoma/leukemias and aberrant expression of CD molecules. Vet Immunol Immunopathol 106: 179-196.
- **Williams, M. J., A. C. Avery, S. E. Lana, K. R. Hillers, A. M. Bachand, and P. R. Avery. 2008.** Canine lymphoproliferative disease characterized by lymphocytosis: immunophenotypic markers of prognosis. J Vet Intern Med 22: 596-601.

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