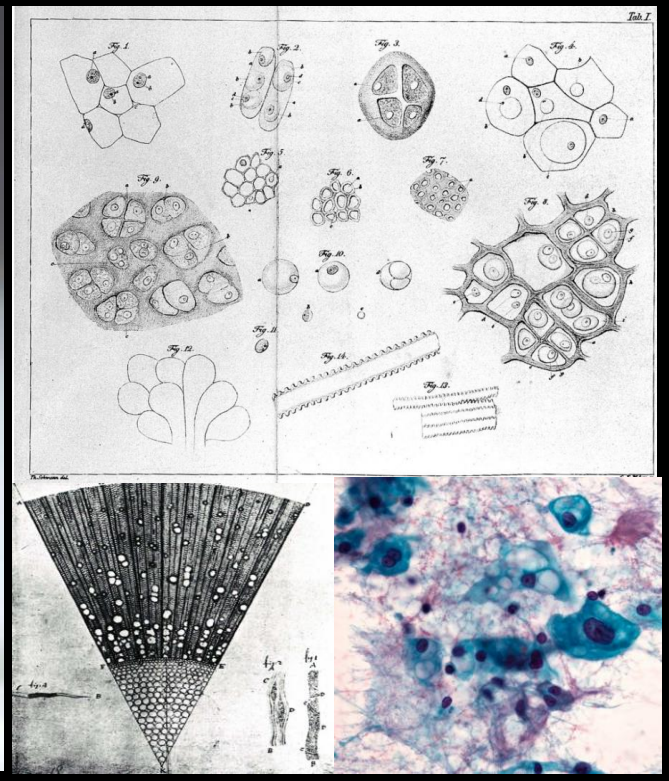
# Go with the Flow: Clinical Insights Through Flow Cytometry

Alexis S. Dadelahi PhD



# Seeing is Believing

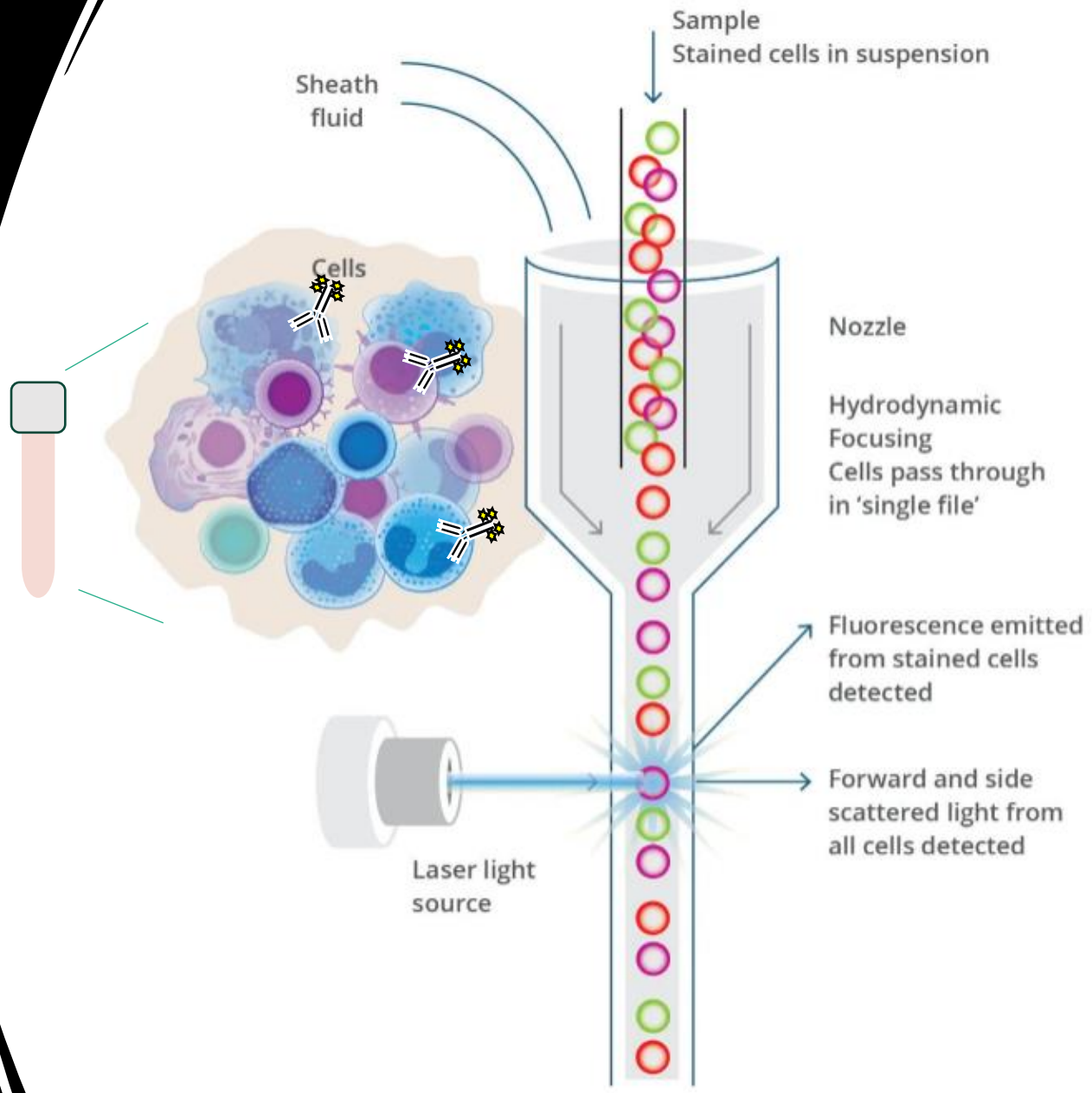
Cytometry- “cell measurement”



https://www.sciencemuseum.org.uk/objects-and-stories/medicine/microscope#:~:text=It's%20not%20clear%20who%20invented,30%20times%20its%20normal%20size; https://en.wikipedia.org/wiki/Antonie_van_Leeuwenhoek; https://daily.jstor.org/the-anatomists-of-ancient-alexandria/; https://wellcomecollection.org/works/vbh2vyy4

# What is **Flow** Cytometry?

- Technology that analyzes the physical and chemical characteristics of particles in **fluid** as it passes through at least one laser.
  - Single cell (and beyond) interrogation
  - HIGH Throughput (populations)
  - Utilizes both light scatter and fluorescence

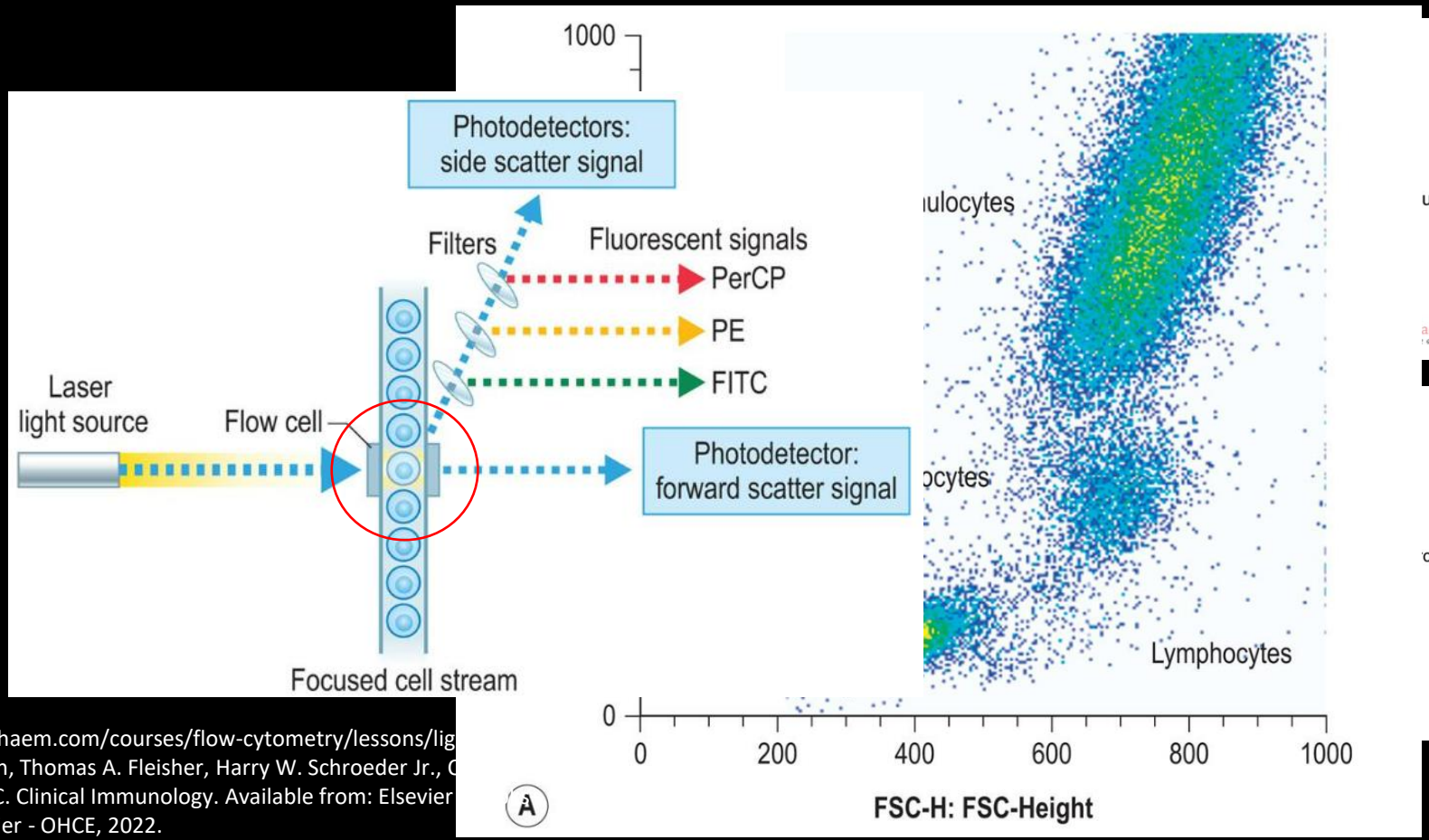


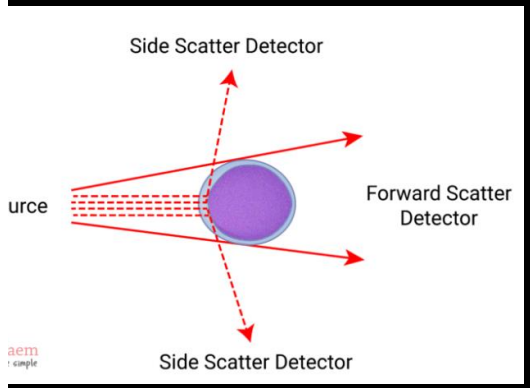
# Learning Objectives:

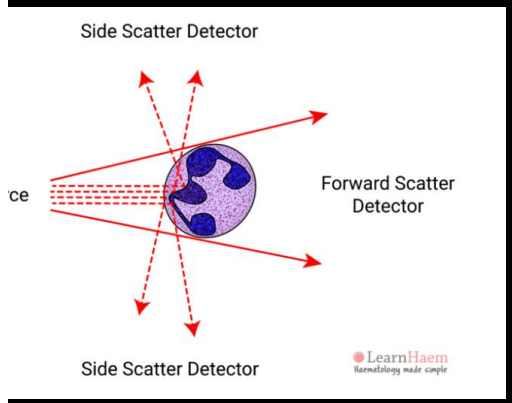
- Review the principles of flow cytometry and examine approaches for successful assay design.
- Discuss various applications of flow cytometry for clinical use.
- Evaluate and apply clinical cytometry techniques to address clinical questions.

# Flow Cytometry: The Interrogation Point

- Light scatter
  - Forward scatter → Rough estimate of cells size
  - Side scatter →Rough estimate of cell "complexity" (granules, organelles, density)
- Together, provide information about cells in our fluid suspension
  - Scatter characteristics differ by population



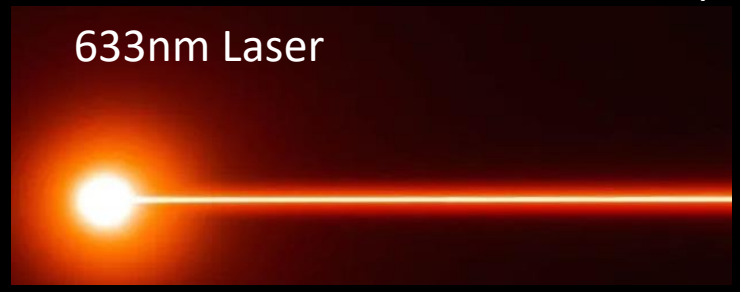



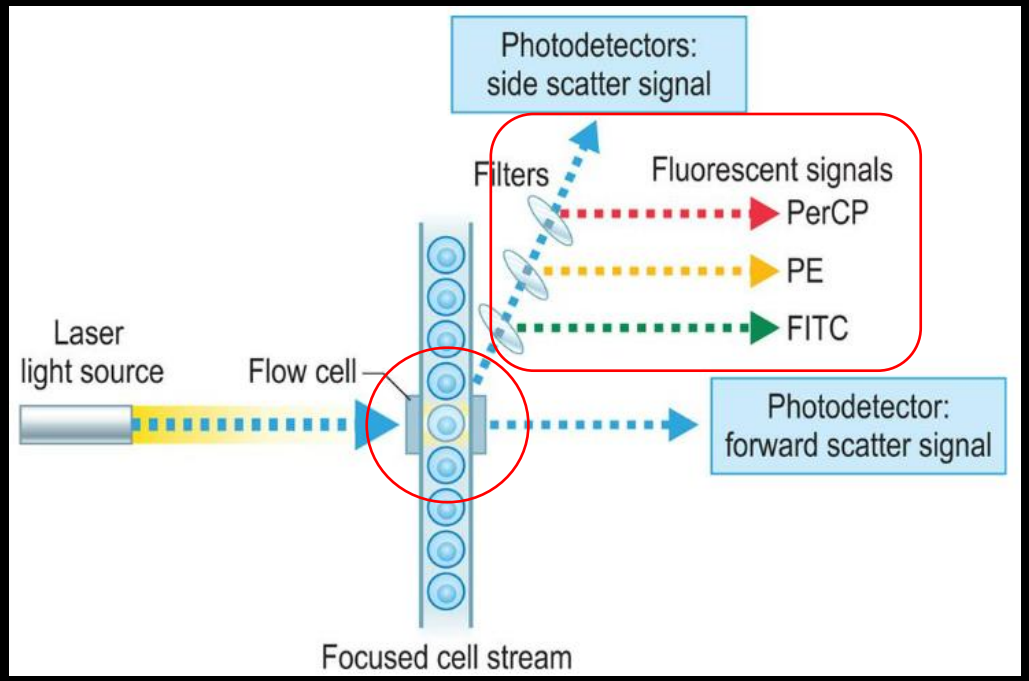

https://www.learnhaem.com/courses/flow-cytometry/lessons/lig
Jenn, Robert R. Rich, Thomas A. Fleisher, Harry W. Schroeder Jr., C
Weyand, David B. C. Clinical Immunology. Available from: Elsevier
(6th Edition). Elsevier - OHCE, 2022.

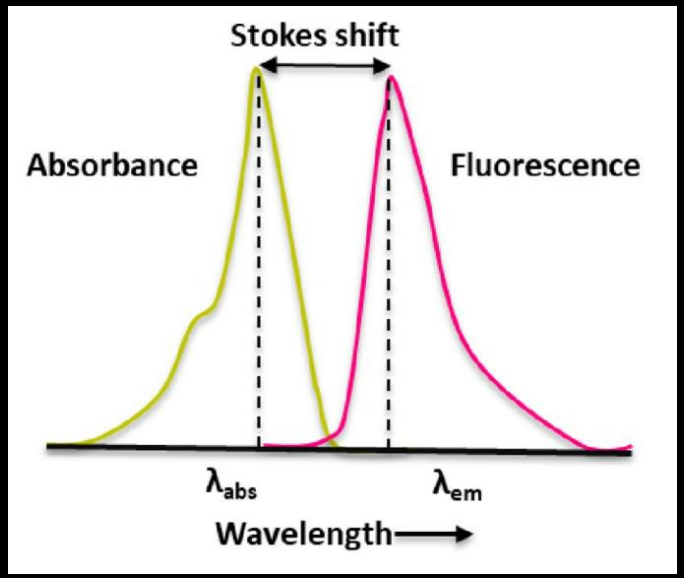
# Flow Cytometry: The Interrogation Point

- Fluorescence
  - Predictable emission wavelength following laser excitation

Fluorophore excited at 633nm



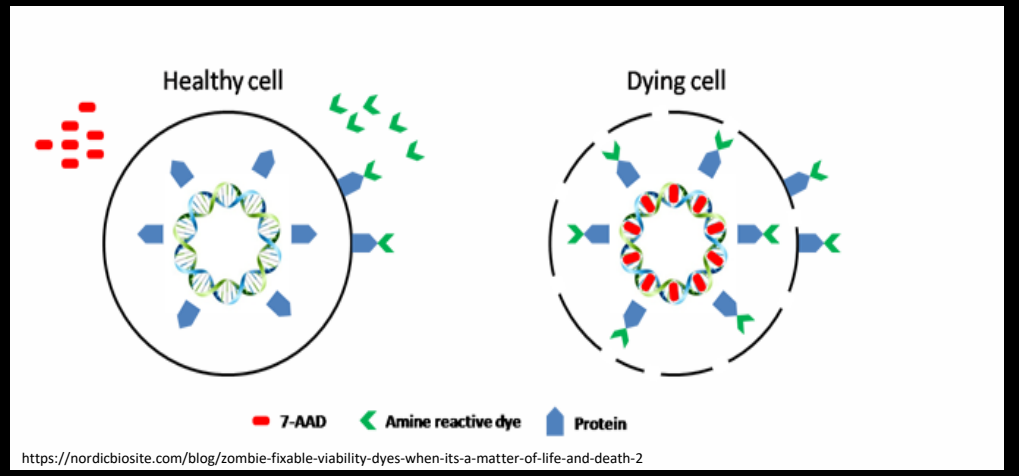




https://www.linkedin.com/posts/mark-hilliard-4b4b9830_science-scienceandtechnology-physics-activity-7229056594846584833-zgkZ/;

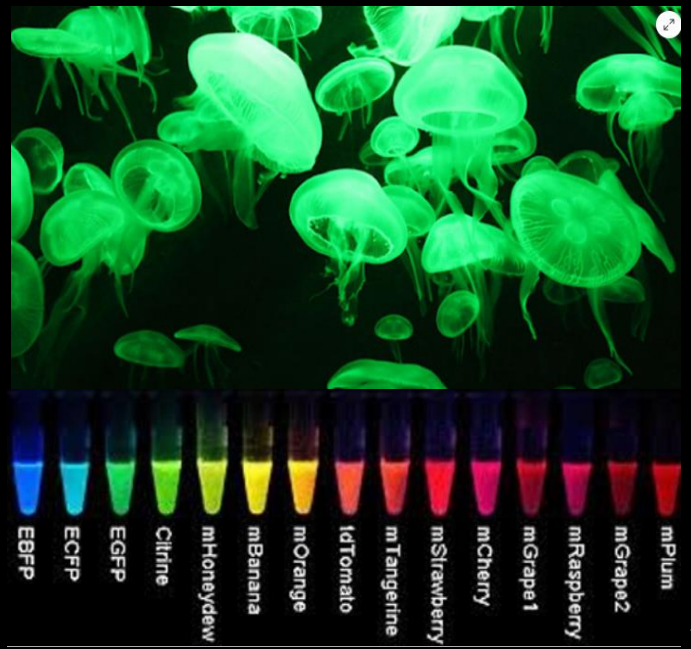
# Fluorescence in Flow Cytometry…Its to *Dye* For

- Types of fluorescent reagents
  - “dyes” or fluorescent chemicals → viability, proliferation, DNA content, function etc.
  - Fluorescent proteins → green fluorescent protein, red fluorescent protein etc.
  - Antibodies chemically bound (conjugated) to various fluorophores

Anti-CD4 FITC  Anti-CD3 APC  Anti-CD45 PE-Cy7



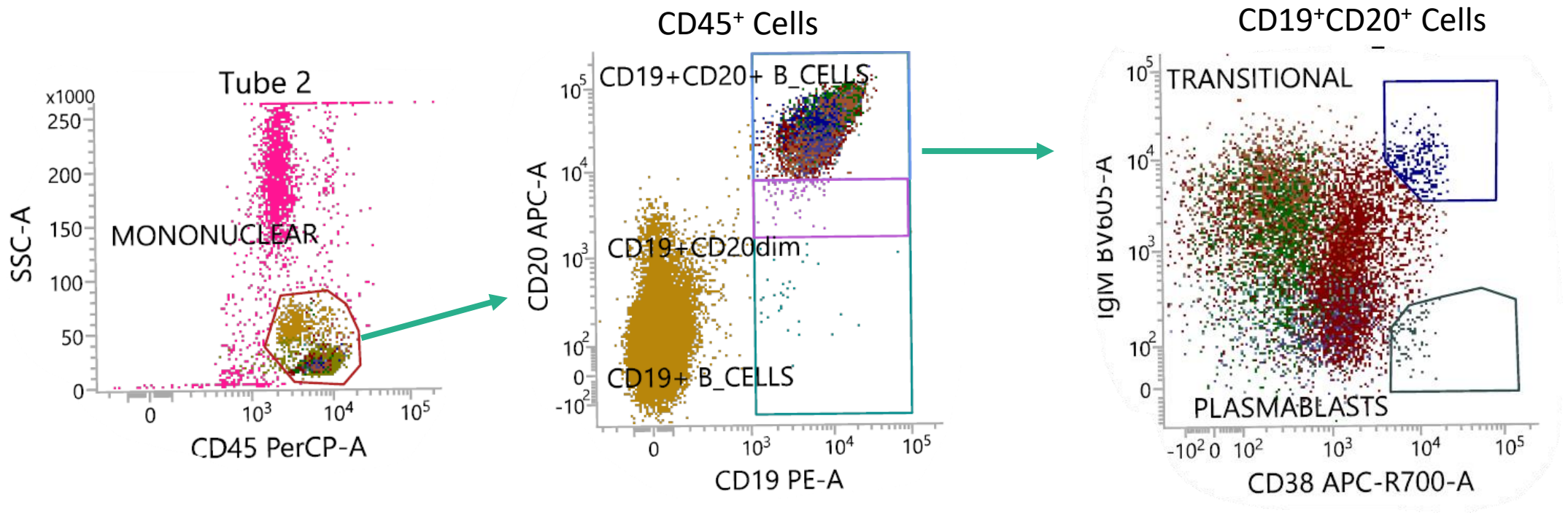

https://nordicbiosite.com/blog/zombie-fixable-viability-dyes-when-its-a-matter-of-life-and-death-2




https://www.thescientificteen.org/post/green-fluorescent-protein-what-is-this-mystical-substance-and-why-is-it-so-important;

# Gating

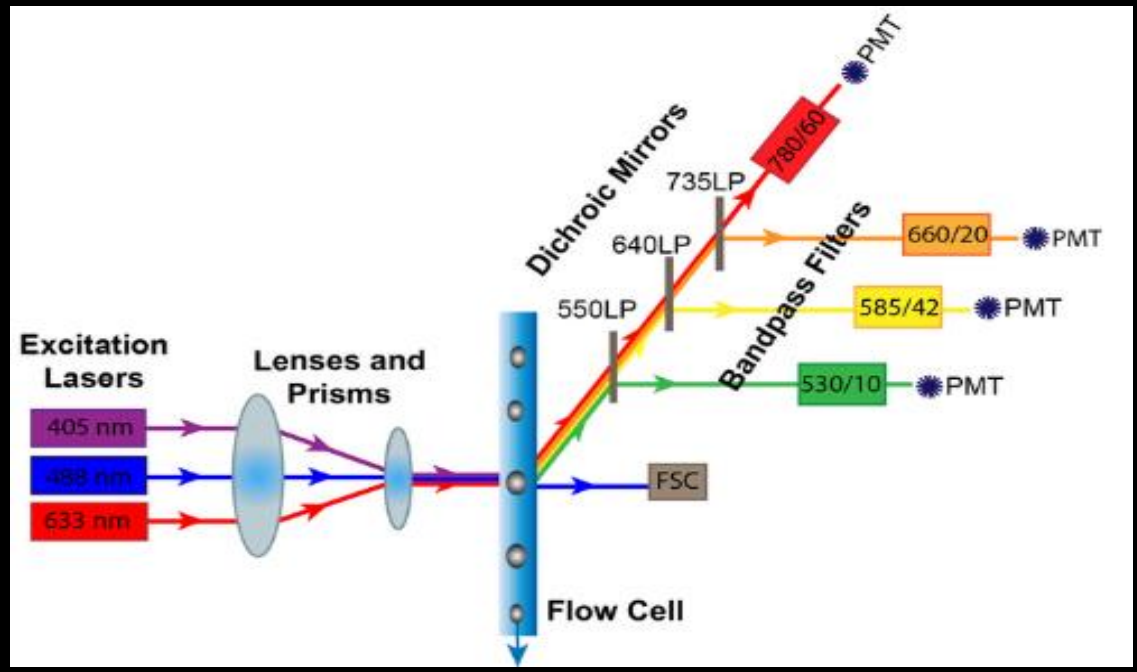
- Defining Populations of interest using what we know about light scatter and cellular characteristics




Jenn, Robert R. Rich, Thomas A. Fleisher, Harry W. Schroeder Jr., Cornelia M. Weyand, David B. C. Clinical Immunology. Available from: Elsevier eBooks+, (6th Edition). Elsevier - OHCE, 2022.

# More Lasers = More Colors

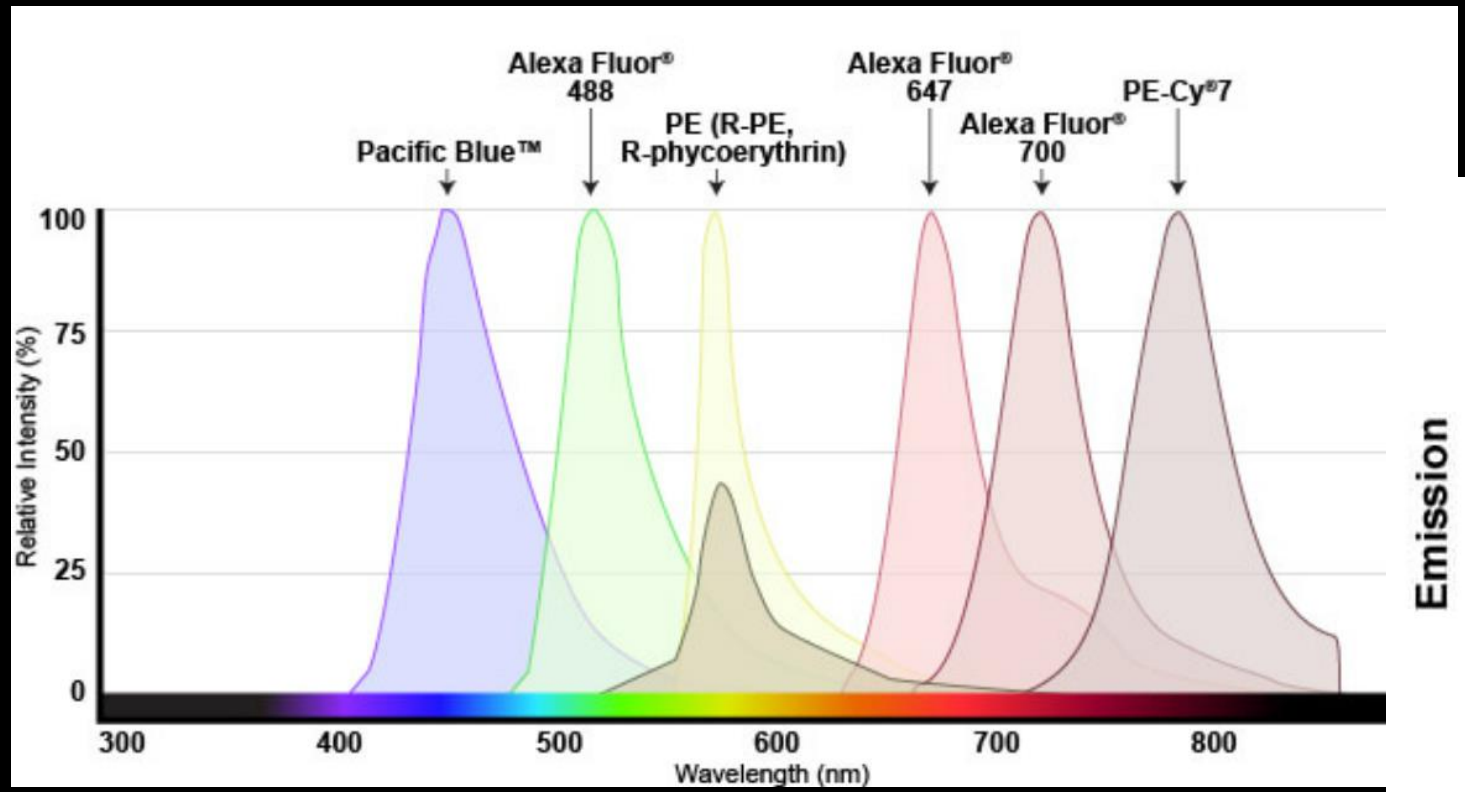
- Increasing number of lasers increases the number of fluorophores available for use
  - UV (350nm)
  - Violet (405nm)
  - Blue (488)
  - Green (532nm)
  - Yellow (560nm)
  - Orange (610nm)
  - Red (633nm)
- More colors improve our ability for multiparameter analysis (many things at once)

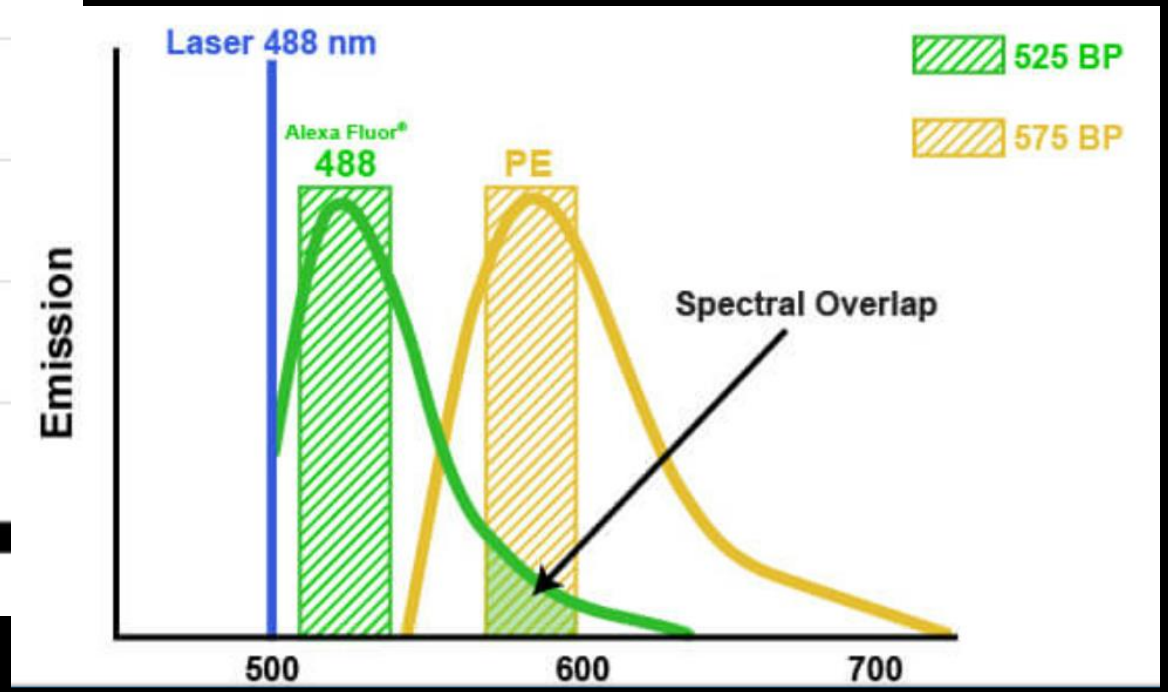



https://bitesizebio.com/31638/flow-cytometry-optics-system/;
https://stock.adobe.com/search?k=rainbow+wallpaper;

# More Colors, More Problems

- Increasing the number of fluorophores, increases panel complexity
  - Unique emission spectra
  - Overlap (spillover) increases as number of fluorophores increases



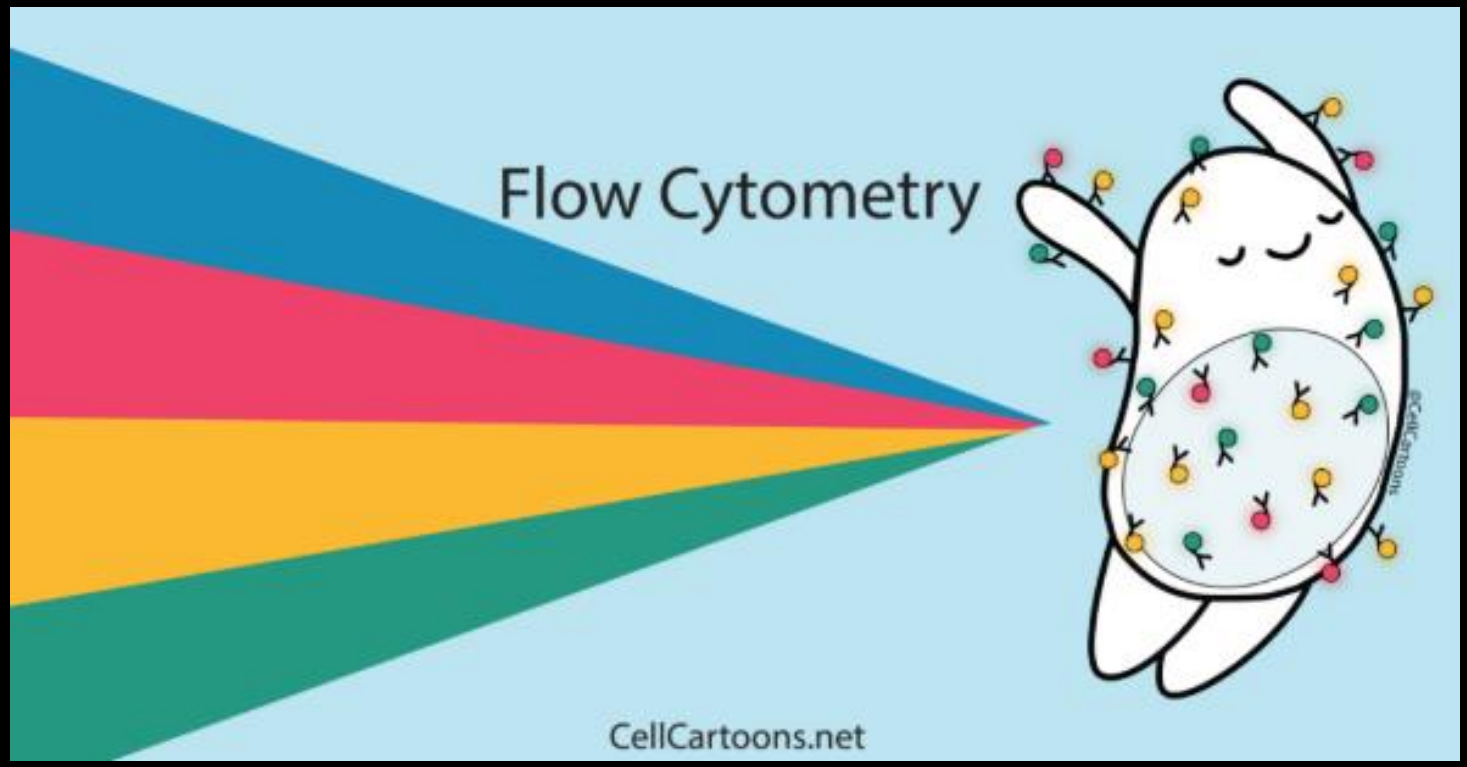






www.cellsignal.com/applications/flow-cytometry/flow-cytometry-overview

# Advantages of Flow Cytometric Analysis

- Multiparameter analysis→ observe many things at once
  - Populations, subpopulations and single cells
  - Plethora of staining reagents available
  - Extracellular and intracellular investigation possible
- Fixed or live cell analysis
- High throughput platform
- Highly sensitive and specific



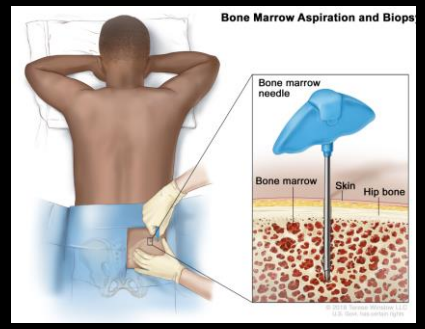
# Factors Affecting Panel Design

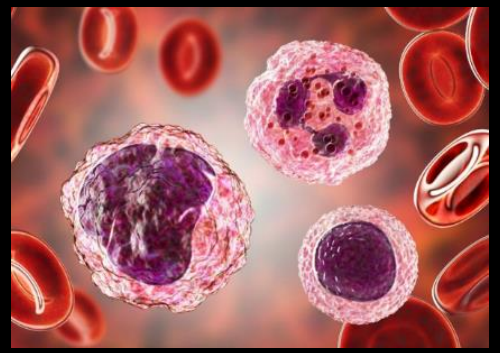
**WHAT**: What exactly is the goal→ informs what to target

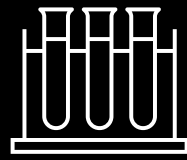
**WHERE**: Intracellular, extracellular

**HOW**: Fixation vs live? Permeabilization? Quantitative vs qualitative?

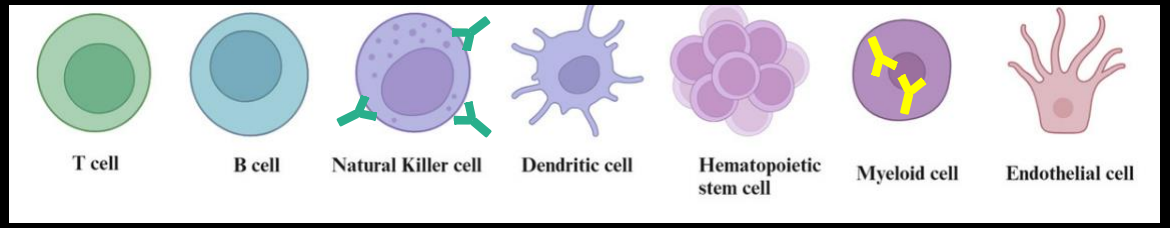
**READ OUT**: How will data be analyzed to interpret results? Quantitative vs Qualitative assays?

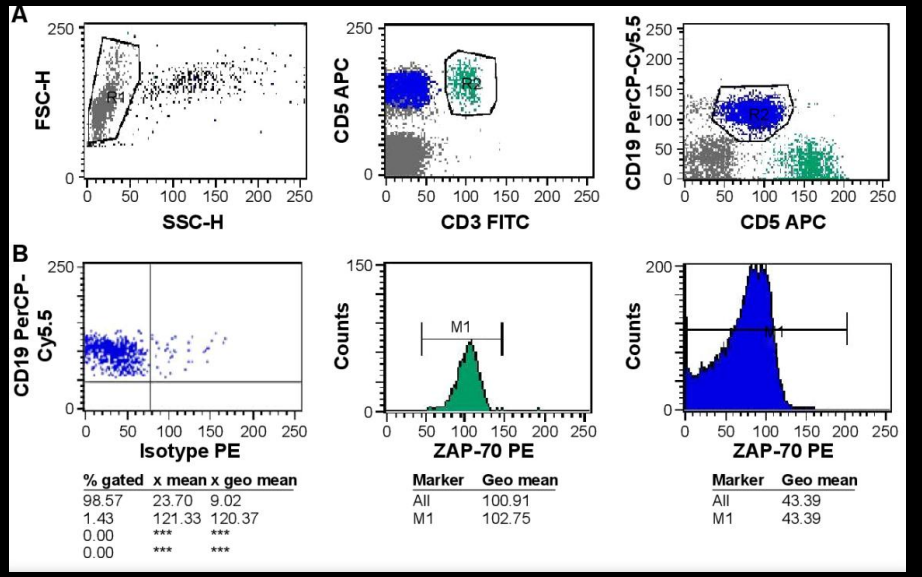




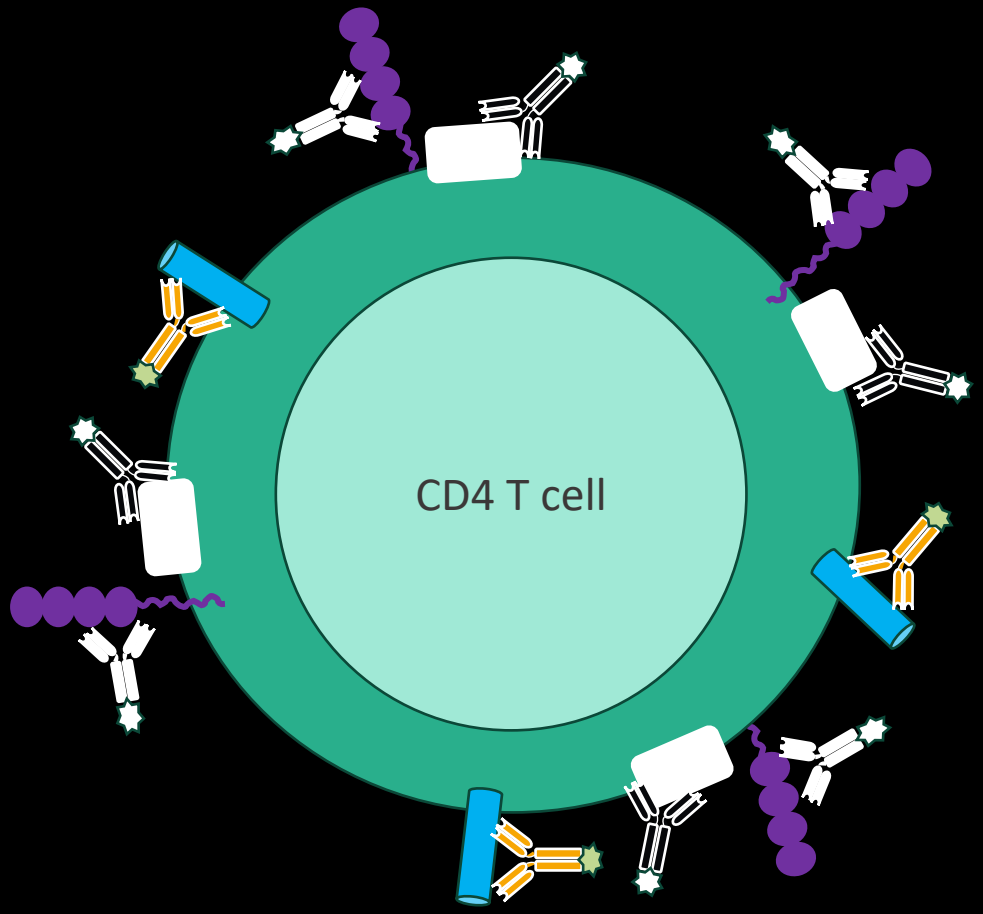
Sample Type



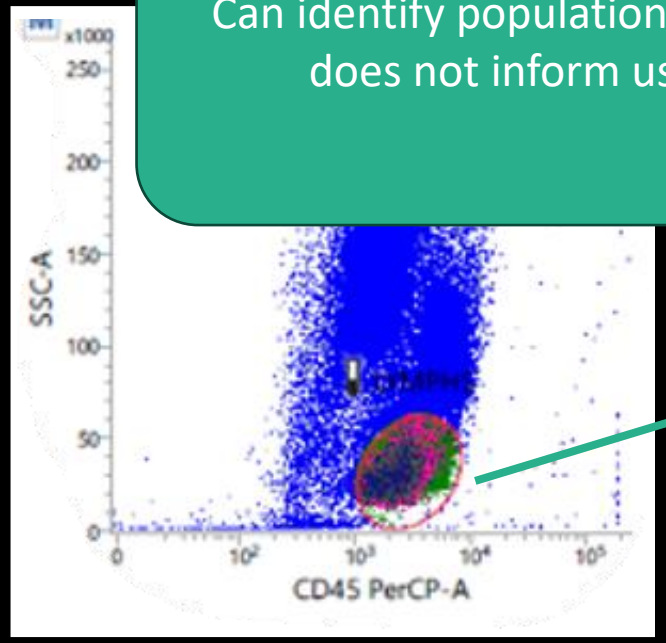



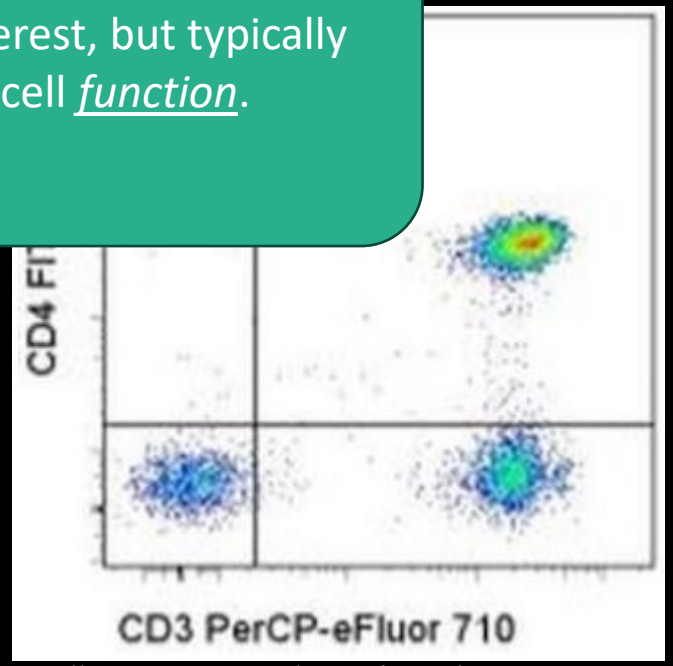

https://nci-media.cancer.gov/pdq/media/images/554337.jpg; https://www.the-scientist.com/accelerating-immune-research-with-cryopreserved-peripheral-blood-mononuclear-cells-70242; https://www.assaygenie.com/blog/immunophenotyping-by-flow-cytometry;
Wu, Yu-Jie et al. Using the geometric mean fluorescence intensity index method to measure ZAP-70 expression in patients with chronic lymphocytic leukemia. *OncoTargets and therapy* 9 (2016): 797 - 805.

# Extracellular Panel Approaches




Can identify populations of interest, but typically does not inform us about cell *function*.



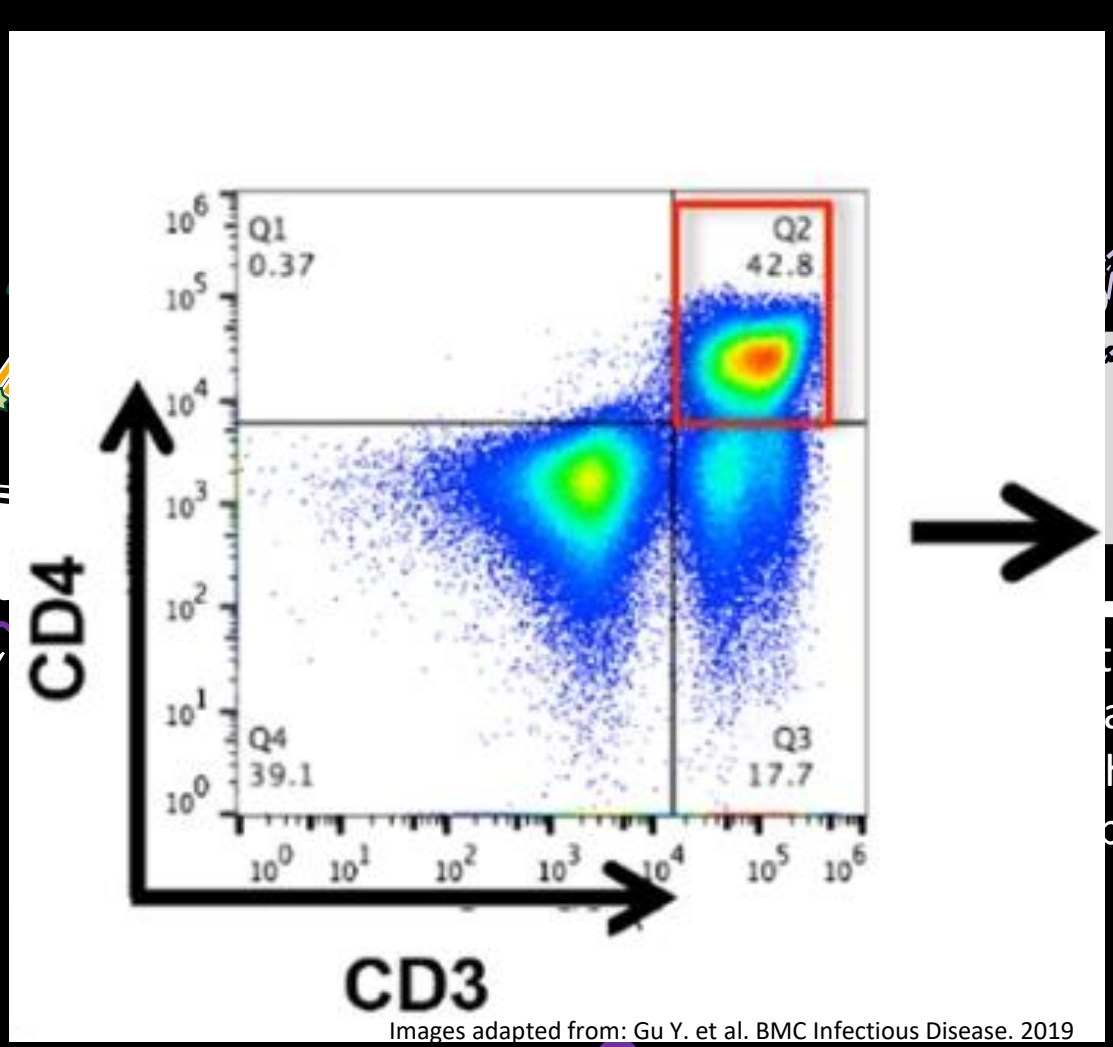

https://www.thermofisher.com/antibody/product/CD3-CD4-CD8a-Antibody-clone-SK3-SK7-SK1-Cocktail/22-0306-71

CD45-pan leukocyte marker

CD4-T subset-specific co-receptor

CD3-T cell signaling molecule (pan T cell marker)

# Panel Design: Intracellular Targets

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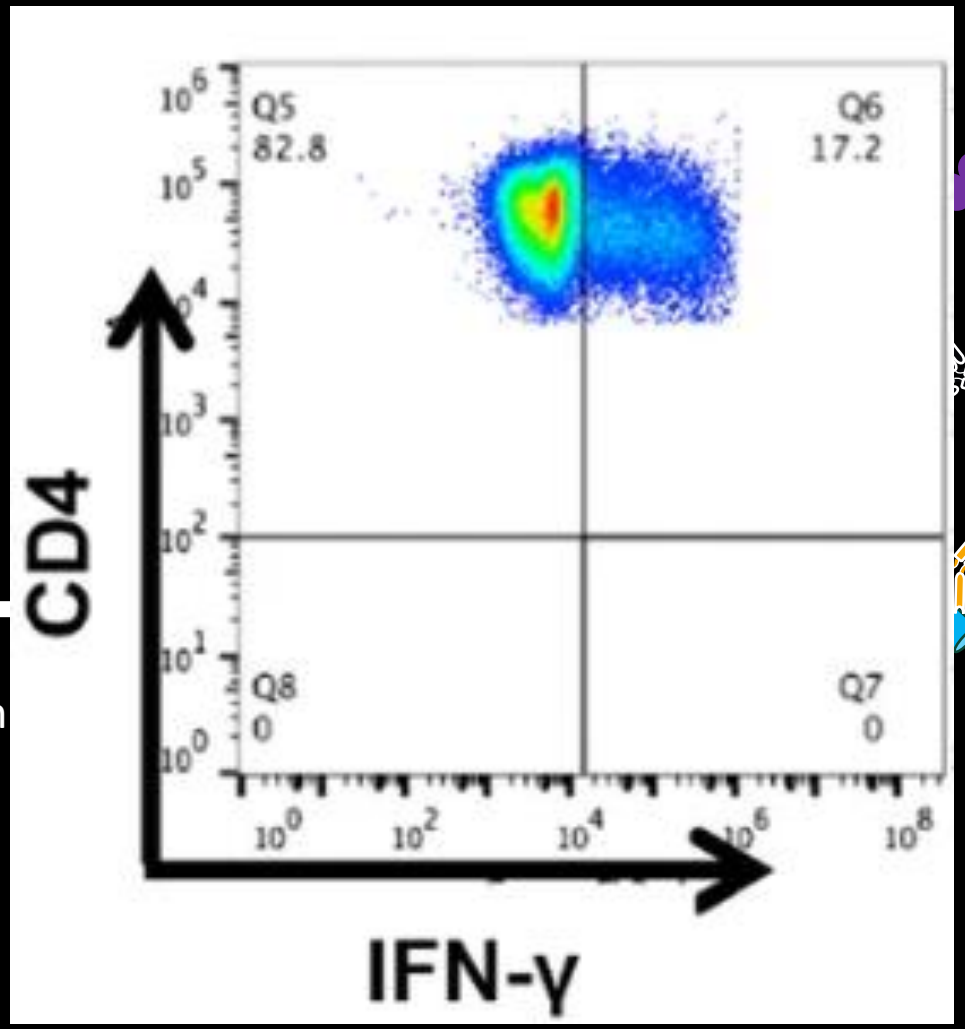



Images adapted from: Gu Y. et al. BMC Infectious Disease. 2019

CD45-pan leukocyte marker

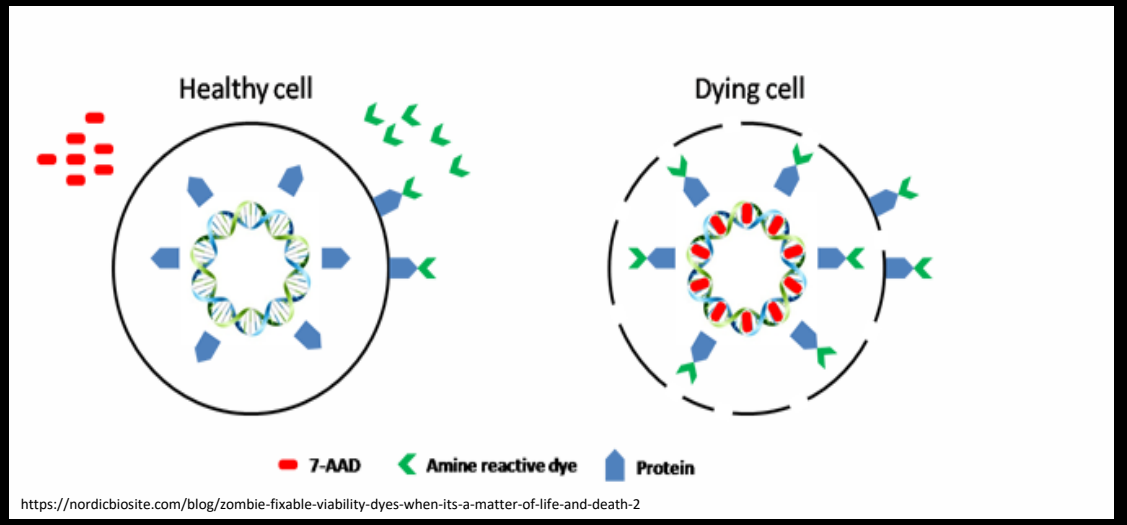
CD4-T subset-specific co-receptor

CD3-T cell signaling molecule (pan T cell marker)

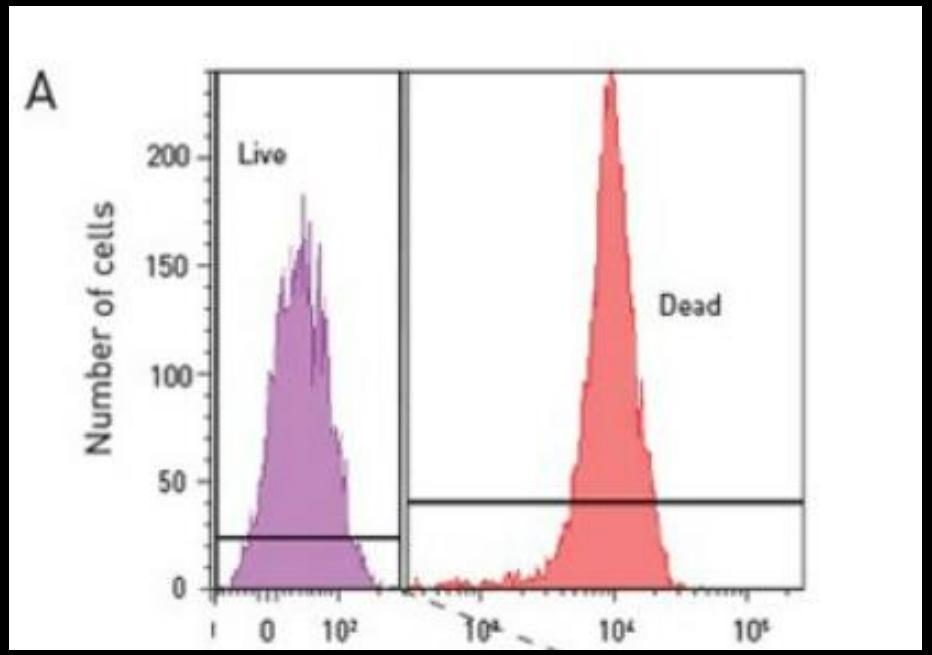
IFN-γ (cytokine)

# Panel Design: Intracellular Targets

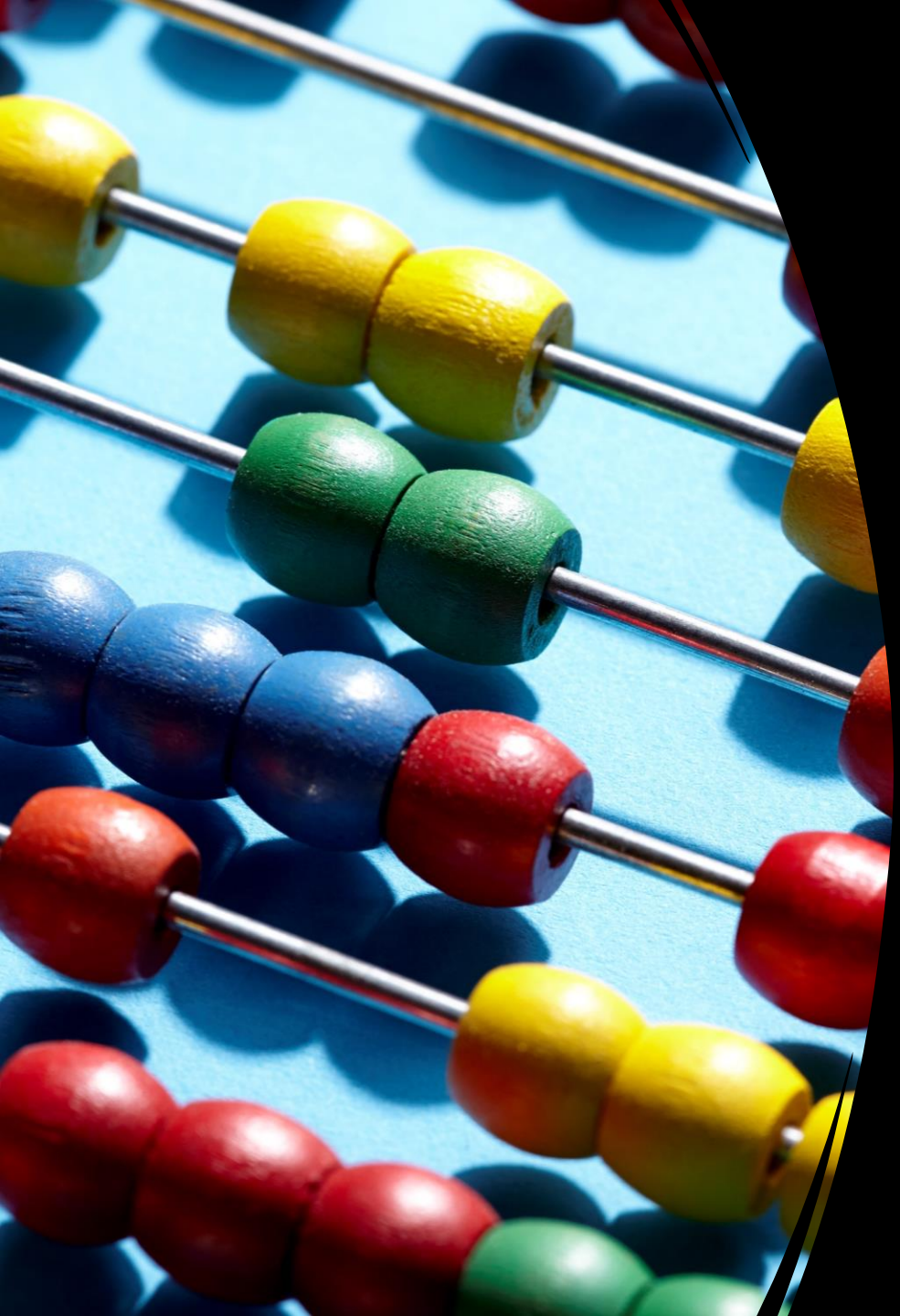
- Viability-DNA binding or amine reactive dyes
- Proliferation-DNA binding, pyrimidine analogs,
- Other-transcription factors, phosphorylated proteins, , chemical changes etc.



https://nordicbiosite.com/blog/zombie-fixable-viability-dyes-when-its-a-matter-of-life-and-death-2



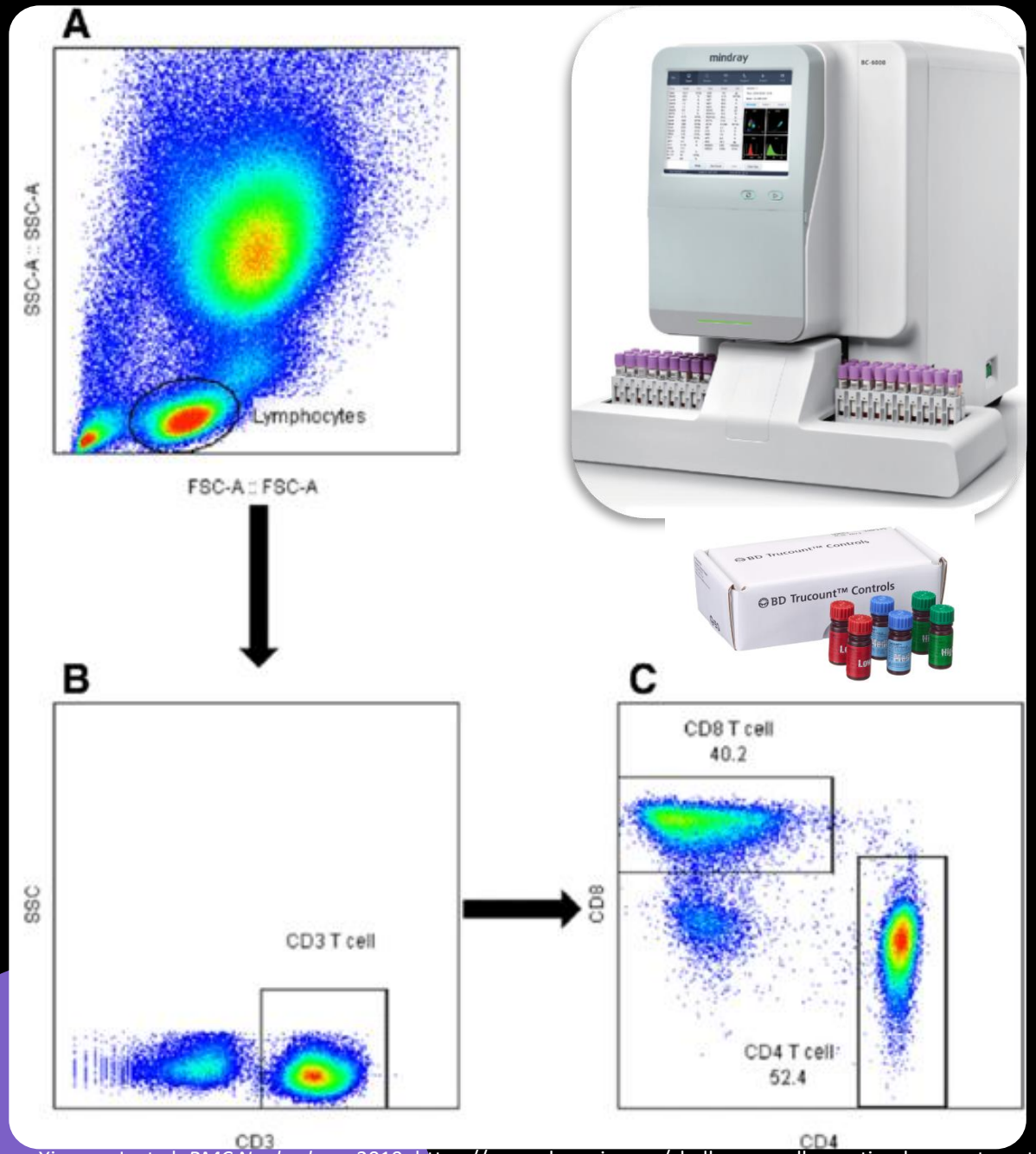

https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cell-viability-and-regulation/cell-proliferation/click-it-edu-assays.html; https://www.thermofisher.com/us/en/home/life-science/cell-analysis/cell-viability-and-regulation/cell-viability.html



# Who's There and How Much? Quantitative and Qualitative Flow Cytometry

- How is the assay reported?
  - Quantitative flow assays: reports out a quantitative result value (i.e. cells/µL)
  - Qualitative flow assays: detect the presence or absence of cell population where reported value is based on a relative frequency (i.e. % of $CD45^+$ lymphocytes)

# Quantitative Flow Cytometry







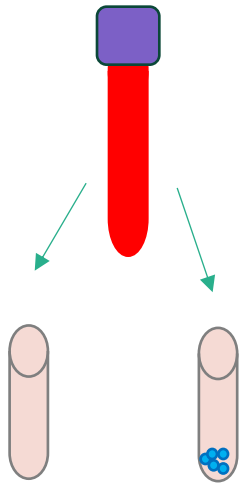



- Flow cytometric analysis can measure the frequency of cell subsets within the sample.
- Using this relative percentage, absolute cell counts for subpopulations can be back calculated from absolute cell counts or reference material (known quantity of fluorescent beads)



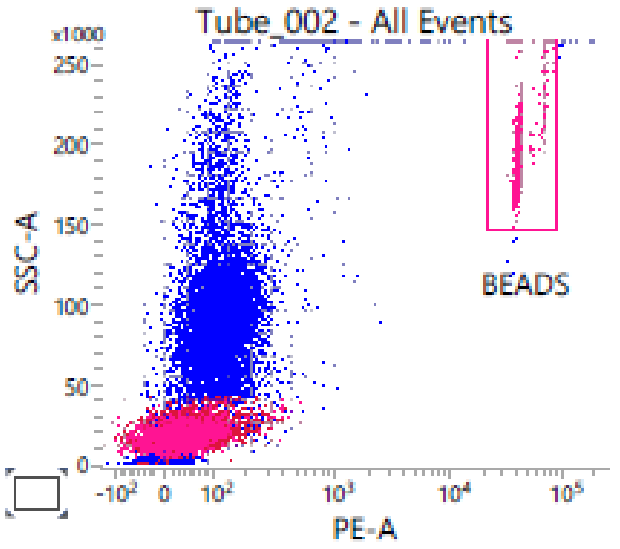
Xiaoyan J, et al. *BMC Nephrology.* 2019; https://www.denovix.com/challenges-cell-counting-hemocytometer/ ; https://www.mindray.com/en/products/laboratory-diagnostics/hematology/5-part-differential-analyzers/bc-6000; https://www.bdbiosciences.com/en-ie/products/reagents/flow-cytometry-reagents/clinical-diagnostics/multicolor-cocktails-and-kits-ivd-ce-ivds/controls.340335?tab=product_details

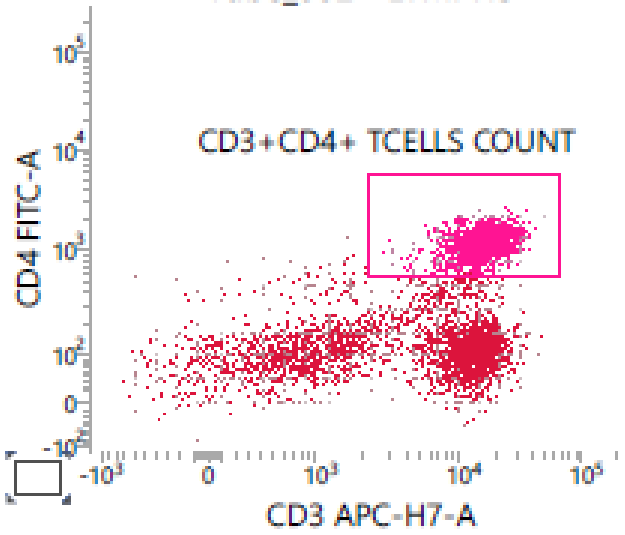
Patient sample



Tube 1: aliquot of patient sample stained with all markers of interest

Tube 2: aliquot of patient sample stained with lineage markers, and combined with known quantity of fluorescent beads



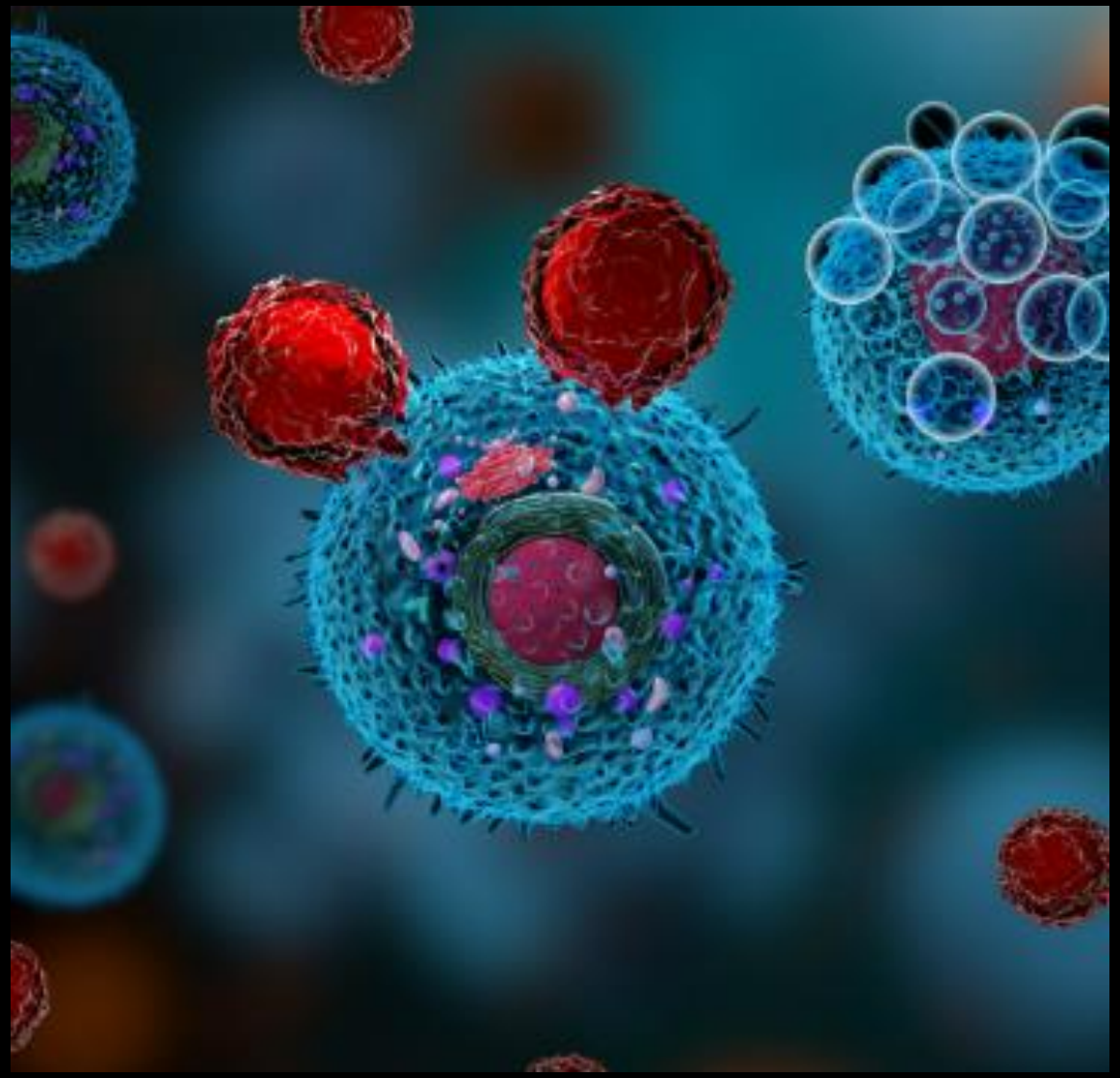

# Quantitative Flow Cytometry-HIV Monitoring

- Percentage and total number of lymphocytes can often aid in informing next diagnostic steps
- Enumeration of CD4 T cells and HIV
  - CD4 T cell counts ≤200 cells/µL is AIDS-defining event
  - Useful for monitoring disease progression, and prevention of opportunistic infection

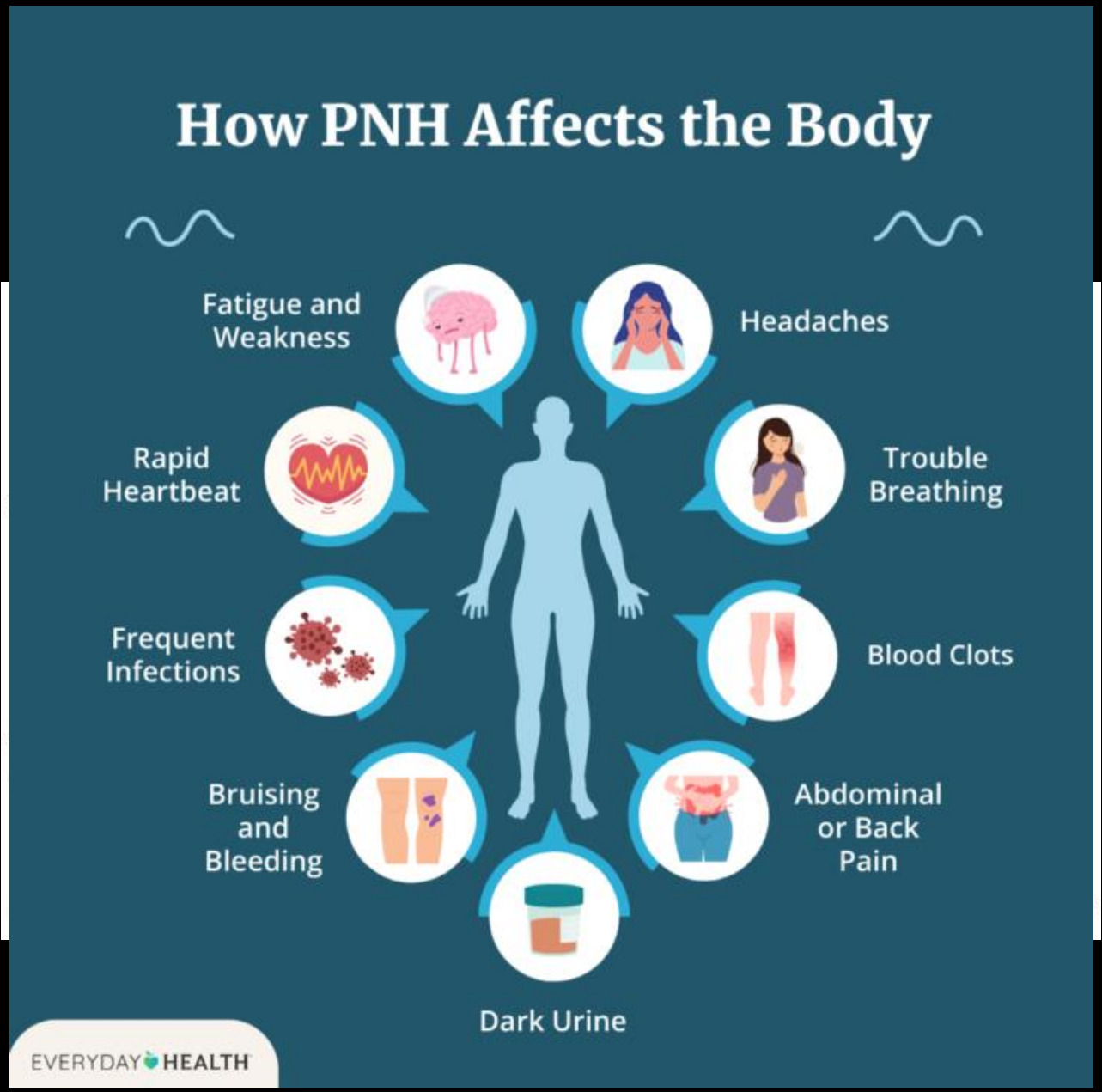
https://interactivebiology.com/3574/aids-and-mechanism-of-hiv-infection/; Schnizlein-Bick CT et al. Evaluation of TruCount Absolute-Count Tubes for Determining CD4 and CD8 Cell Numbers in Human Immunodeficiency Virus-Positive Adults. *Clinical and Diagnostic Laboratory Immunology*. 2000. 7(3):336-343; https://hivinfo.nih.gov/understanding-hiv/fact-sheets/stages-hiv-infection

# Qualitative Clinical Flow Cytometry

- Assessing the presence or *absence* of…
  - Cell population(s)
  - Target(s) of interest on a cell population
  - Expression level of targets of interest (how much?)
- Often useful for diagnosis/support of diagnosis



https://bioxcell.com/trem2-modulation-remodels-the-tumor-myeloid-landscape-enhancing-anti-pd-1-immunotherapy/




# Paroxysmal Nocturnal Hemoglobinuria (PNH)

- Rare blood disorder characterized by a *reduction* or *absence* of glycosyl phosphatidylinositol (GPI)-anchored membrane proteins
  - Lack of GPI expression on red blood cells (RBCs) and white blood cells (WBCs) leads to increased susceptibility to complement activity → increased cell lysis

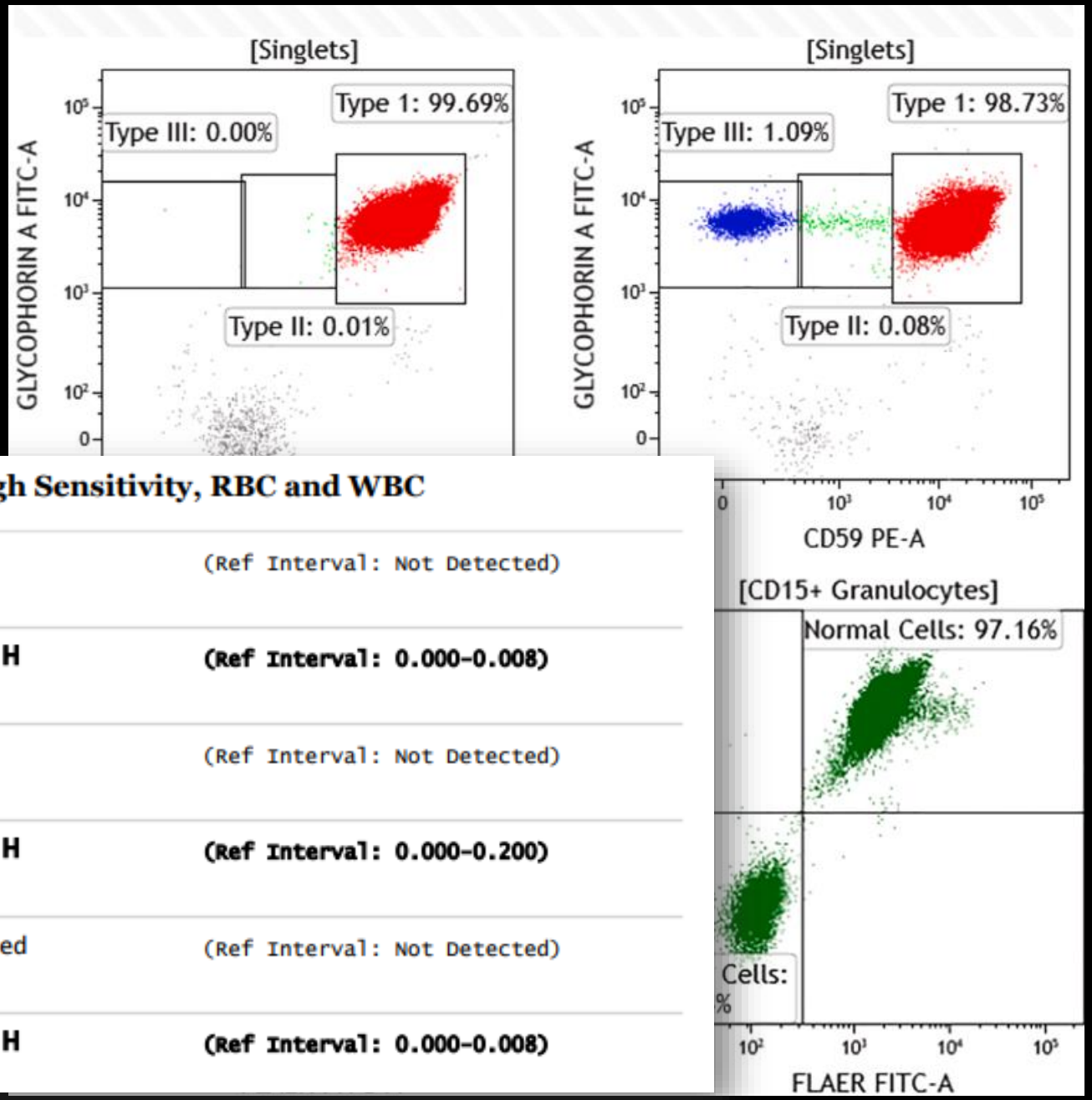
Brodsky RA. *J Clin Invest*. 2019; https://www.everydayhealth.com/blood-disorders/paroxysmal-nocturnal-hemoglobinuria/guide/;


# PNH: Detecting Deficiency

- Flow cytometric analysis of GPI-anchored proteins on RBCs and WBCs is the gold standard for diagnosis and monitoring of disease
- Purpose of the assay demands special considerations
  - Tasked with measuring what *isn't* there
  - Limited number of events detected within a given population → Rare event analysis
    - Minor PNH clones <1%-4% of total cells
    - PNH clones are sensitive to destruction

# Detecting PNH Clones with Flow Cytometry

- Sufficient sensitivity → Increase events (≥100,000)
- Assess both RBC and WBC for presence of GPI-anchore
  - RBC
    -
    -
  - WB
    deri
    GPI-
  - Line
    -
    -
    -




**Paroxysmal Nocturnal Hemoglobinuria (PNH), High Sensitivity, RBC and WBC**
ARUP test code 2005006

| | | | |
|---|---|---|---|
| Neutrophil PNH Phenotype | Detected | | (Ref Interval: Not Detected) |
| FLAER and CD157-deficient neutrophils | **1.378 %** | **H** | **(Ref Interval: 0.000-0.008)** |
| Monocyte PNH Phenotype | Detected | | (Ref Interval: Not Detected) |
| FLAER and CD157-deficient monocytes | **3.142 %** | **H** | **(Ref Interval: 0.000-0.200)** |
| RBC PNH Phenotype | Minor Detected | | (Ref Interval: Not Detected) |
| Total (II and III) CD59-deficient RBC | **0.557 %** | **H** | **(Ref Interval: 0.000-0.008)** |

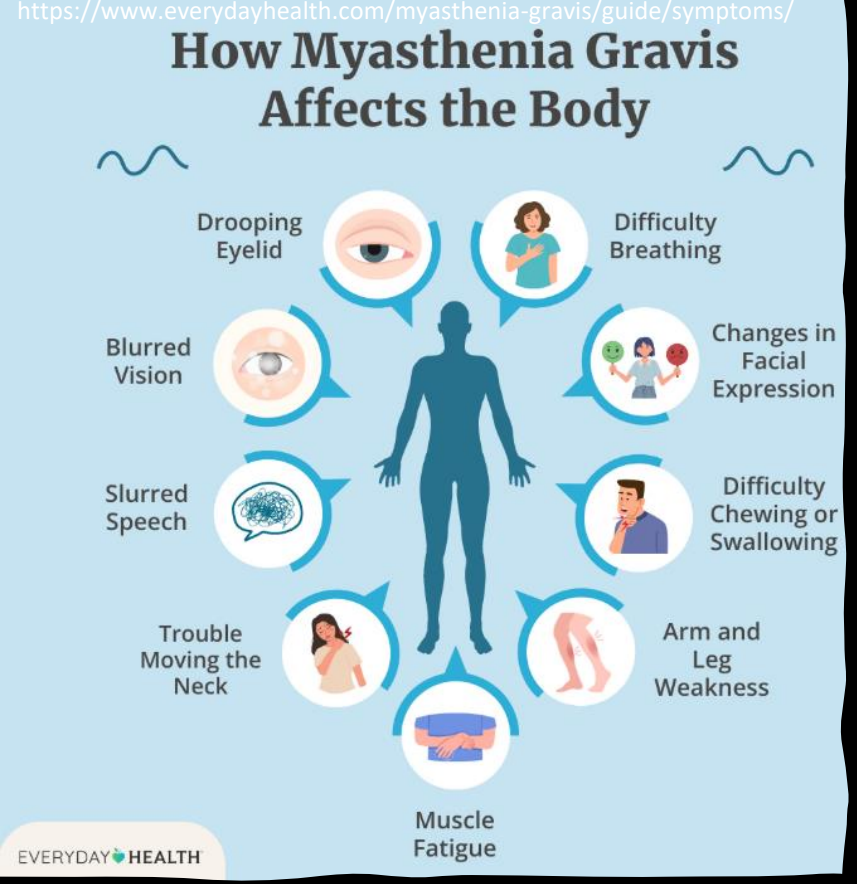
https://www.semanticscholar.org/paper/Case-1-%3A-Rapid-Expansion-of-the-PNH-Clone-in-a-With-Shrit-Scott/f3f39feb35521361c868f55df67aaa19300e763f/figure/0; Dadelahi et al. *Clinics in Laboratory Medicine*. 2024

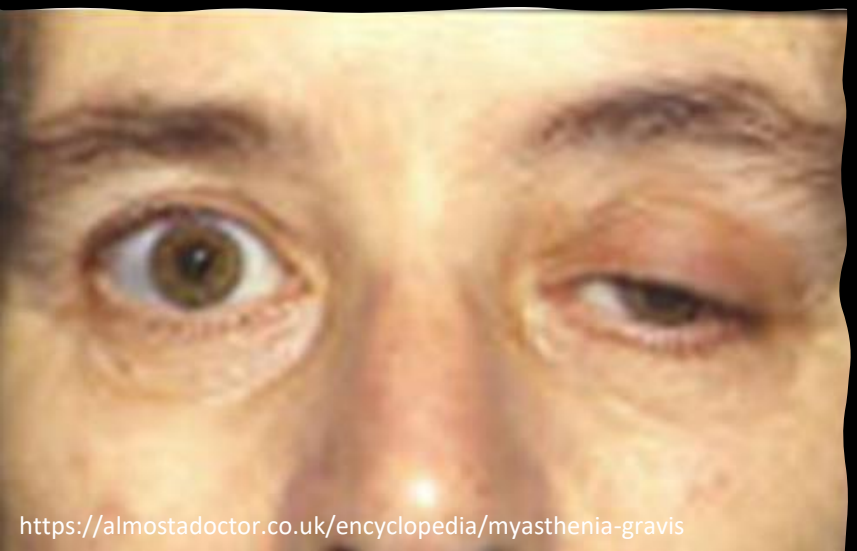
https://www.ere.net/articles/the-three-hallmarks-of-a-dysfunctional-workplace


# Form ≠ Function: Investigating Cell Function Using Flow Cytometry

- Presence of a particular marker can indicate cell phenotype, lineage, and suggest function, but the presence of a marker alone does not necessarily indicate *functionality*
- Flow cytometric techniques can still be leveraged to investigate cell function, with clever assay design
  - Read out for function: phosphorylation, proliferation, cytokine production, specific protein regulation etc.
- Powerful strategy for investigating functional immune responses

https://www.everydayhealth.com/myasthenia-gravis/guide/symptoms/



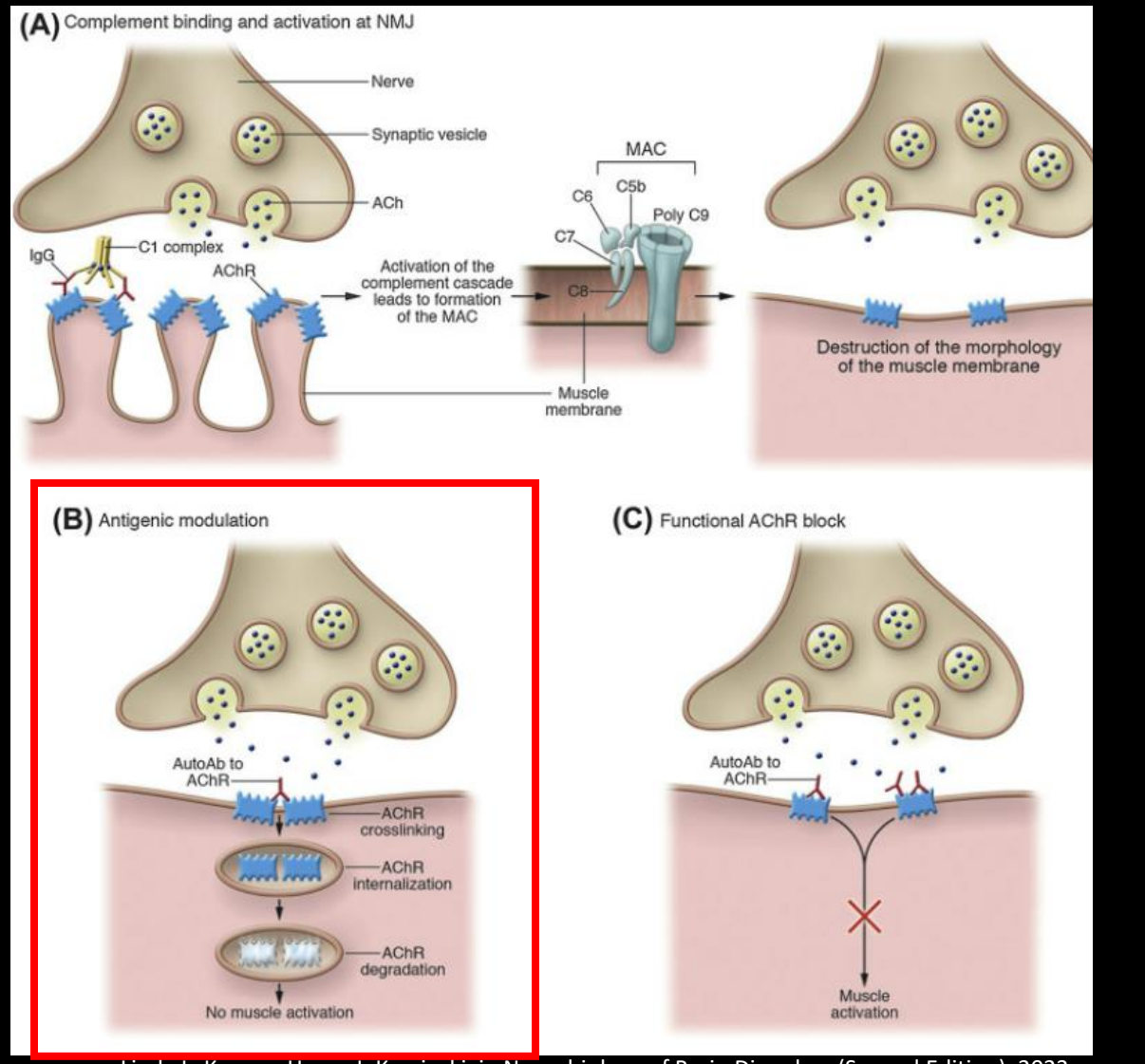
https://almostadoctor.co.uk/encyclopedia/myasthenia-gravis

# Functional Assays: Myasthenia Gravis Modulating Autoantibody Detection

- Myasthenia Gravis- autoimmune disorder in which autoantibodies are generated against the neuromuscular acetylcholine receptors (AChR)
  - Disruption of neuromuscular transmissions → voluntary muscle weakness and fatigue
  - Fluctuating weakness that worsens with activity, improves with rest

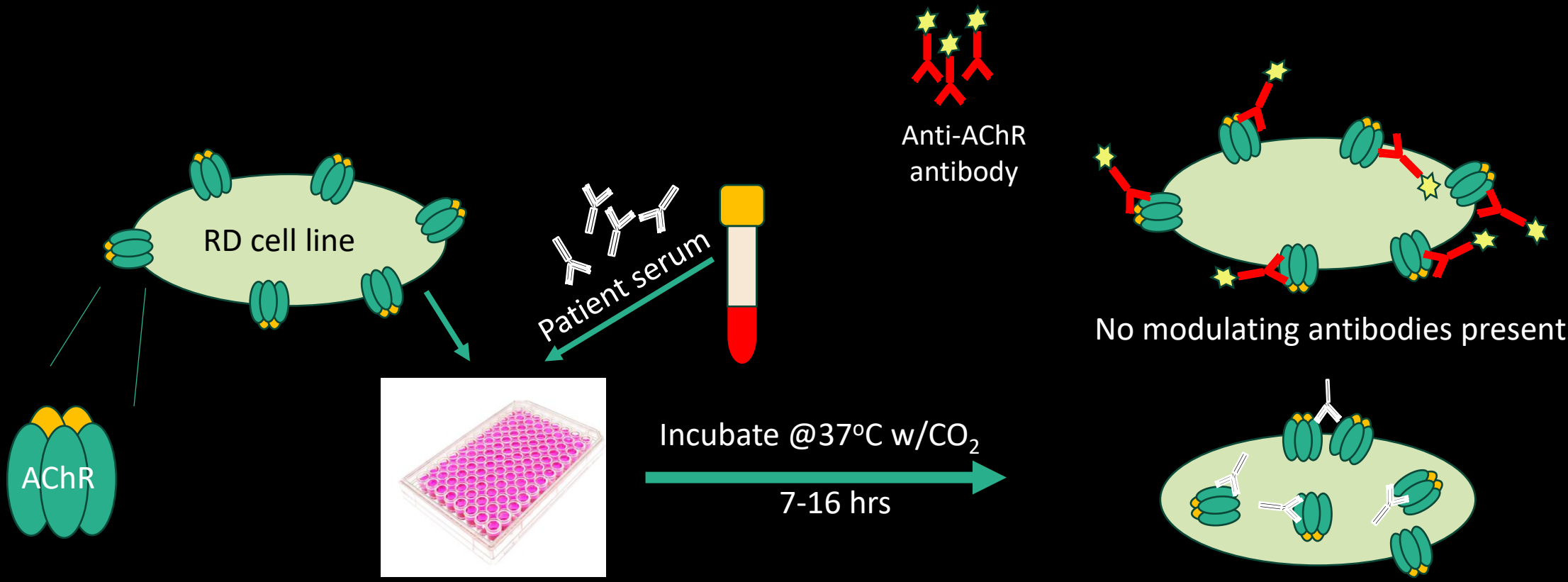
# Functional Assays: Myasthenia Gravis Modulating Autoantibody Detection

- Myasthenia gravis diagnosis is often based on correlating clinical presentation with laboratory confirmation of autoantibody against AChR
- Additional characterization of autoantibody may aid in characterization and prognosis of disease
  - Modulating antibodies are correlated with enhanced disease severity




Linda L. Kusner, Henry J. Kaminski, in Neurobiology of Brain Disorders (Second Edition), 2023

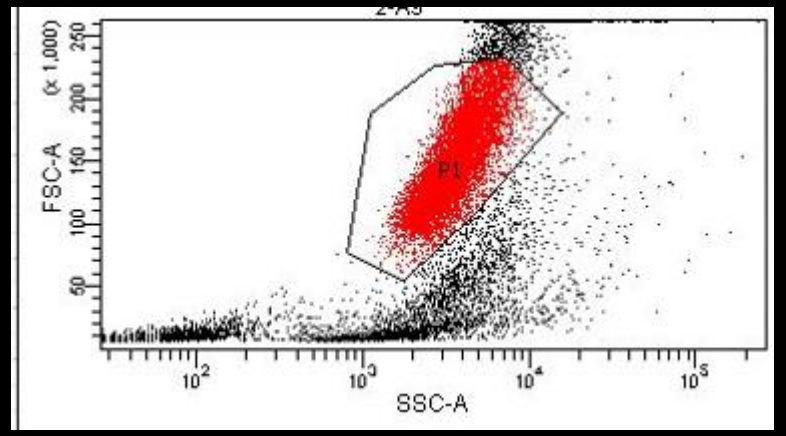
Mané-Damas M, et al. Novel treatment strategies for acetylcholine receptor antibody-positive myasthenia gravis and related disorders. *Autoimmunity Reviews*. 2022. 21(7).

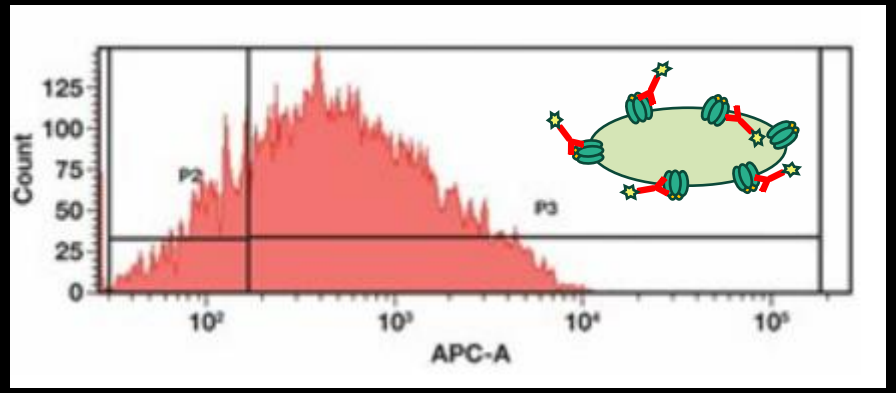

Anti-AChR antibody
RD cell line
Patient serum
AChR
Incubate @37°C w/CO2
7-16 hrs
No modulating antibodies present
Modulating antibodies present

# Functional Assays: Myasthenia Gravis Modulating Autoantibody Detection

- Fluorescent signal intensity is used to calculate %Modulation
- Compare intensity of signal to maximum calibrator (no autoantibody) and negative controls

| Reference Interval | | |
|---|---|---|
| 0-45% modulation | = | Negative |
| ≥46% modulation | = | Positive |



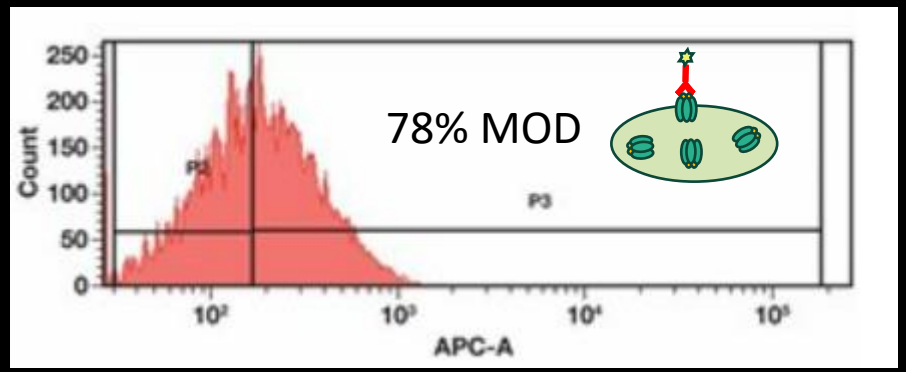
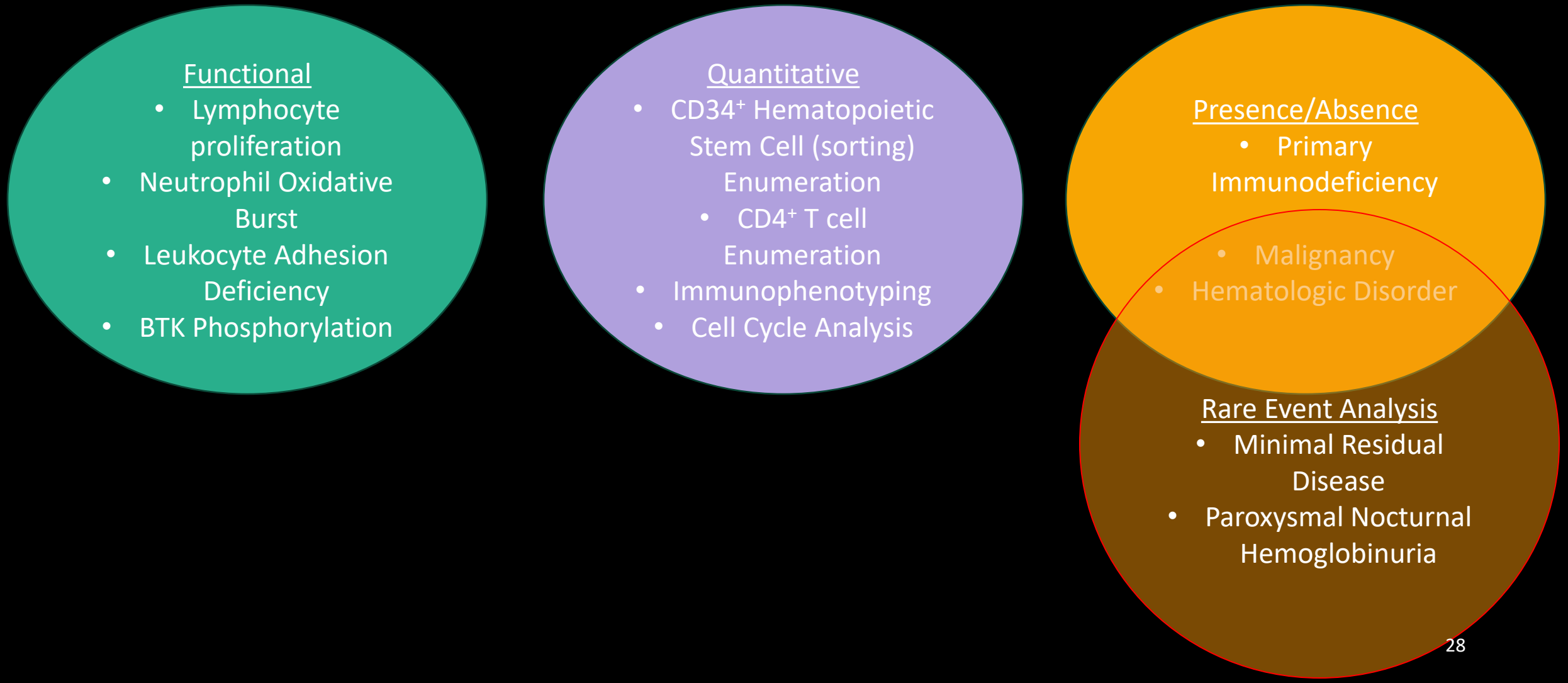




Figure adapted from: Bucky K. Lozier, Thomas R. Haven, Mark E. Astill, Harry R. Hill, Detection of Acetylcholine Receptor Modulating Antibodies by Flow Cytometry, *American Journal of Clinical Pathology*, Volume 143, Issue 2, February 2015, Pages 186–192, https://doi.org/10.1309/AJCPYEOR6SGE8ZLU

# Clinical Applications of Flow Cytometry

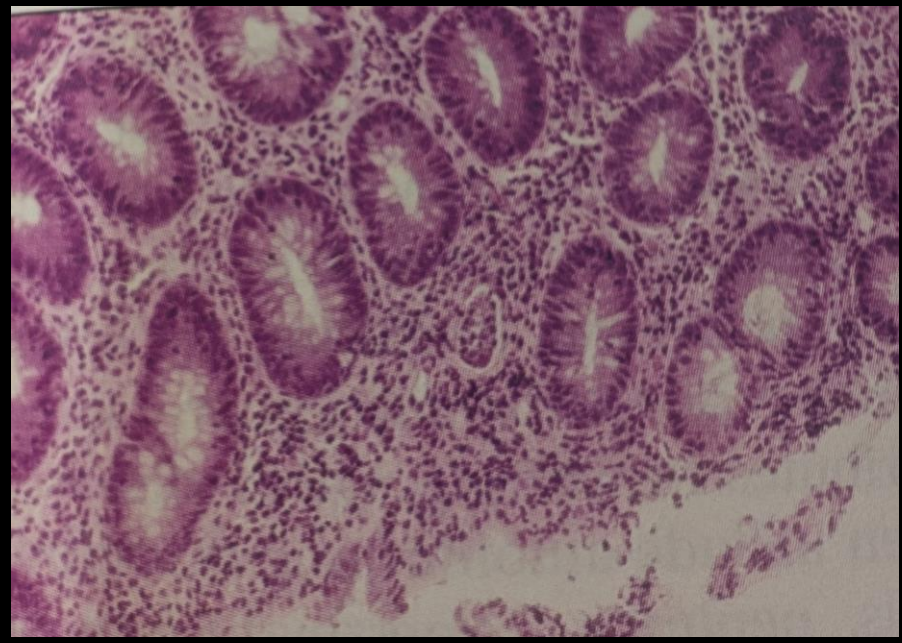


# Case 1: Infantile Onset Type 1 Diabetes

- 4 mo old male presents to the clinic with an intractable diarrhea and failure to thrive. Antibiotic treatment was administered, but watery stools persisted.
- At 6 mo old, he developed high glucose levels and glucose in the urine.
  - Diagnosed with Type 1 Diabetes and referred to endocrinology.
- Further work-up revealed enlarged lymph nodes and spleen, but his white blood cell count (7300 /µl), hemoglobin (11.3 g/dl), and platelet count (435,000/µl) were normal.
- Autoantibodies to both glutamic acid decarboxylase (GAD65 antigen) and pancreatic islet cells were detected.

# Case 1: Cont'd.

- Despite insulin treatment, diarrhea persisted, as well as continued failure to thrive, prompting a duodenal biopsy.
  - Biopsy revealed almost total villous atrophy, with dense infiltration of T cells and plasma cells.
- Follow up discussion with the family revealed no major health complications excluding a case of atopic dermatitis shortly after birth of the patient.
- Mother revealed that another son had perished in infancy with severe diarrhea and a low platelet count.





Geha, Raif, and F. R. E. D. Rosen. *Case Studies in Immunology: Immune Dysregulation, Polyendocrinopathy, Enteropathy X-linked Disease: A Clinical Companion*. WW Norton & Company, 2010.

# Immune Dysregulation, Polyendocrinopathy, Enteropathy, X-Linked (IPEX) Disorder

- Familial autoimmune syndrome with an X-linked recessive pattern of inheritance:
  - Early onset enteropathy
  - Dermatitis
  - Endocrinopathy → Type I Diabetes, thyroid disease
    - Often with autoantibodies

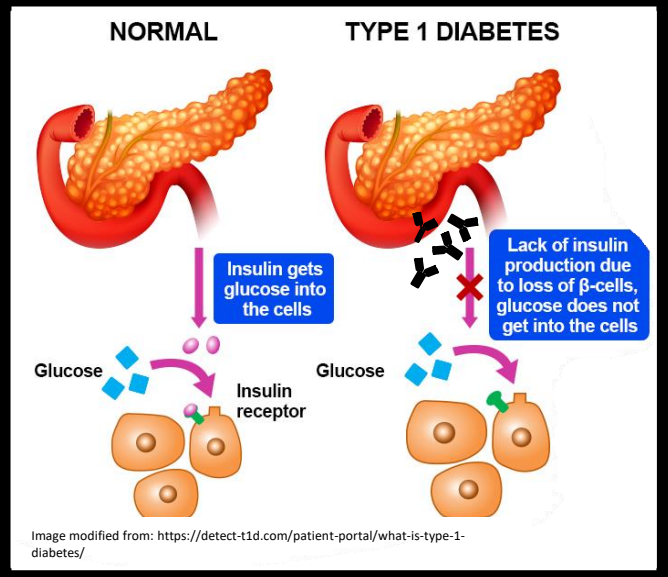
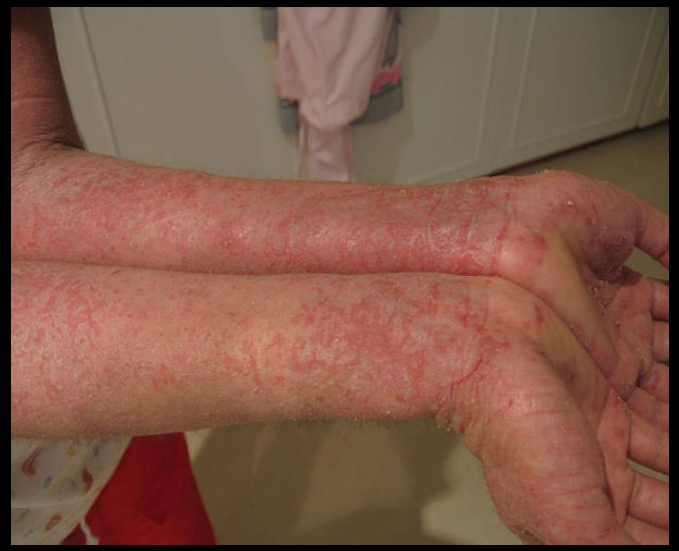
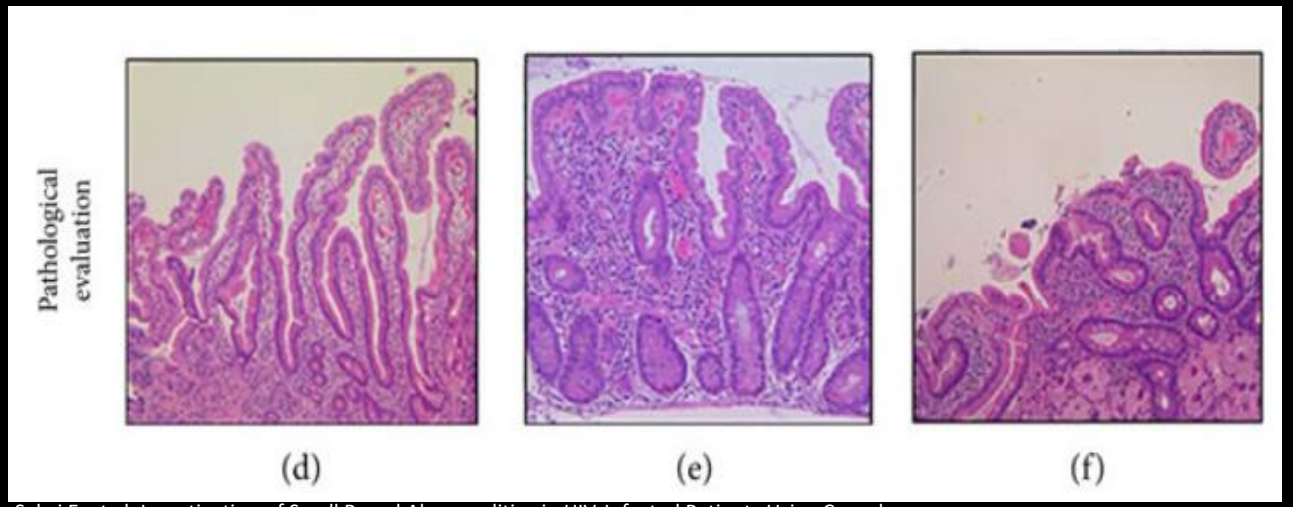


Image modified from: https://detect-t1d.com/patient-portal/what-is-type-1-diabetes/


https://www.lecturio.com/concepts/ipex-syndrome/



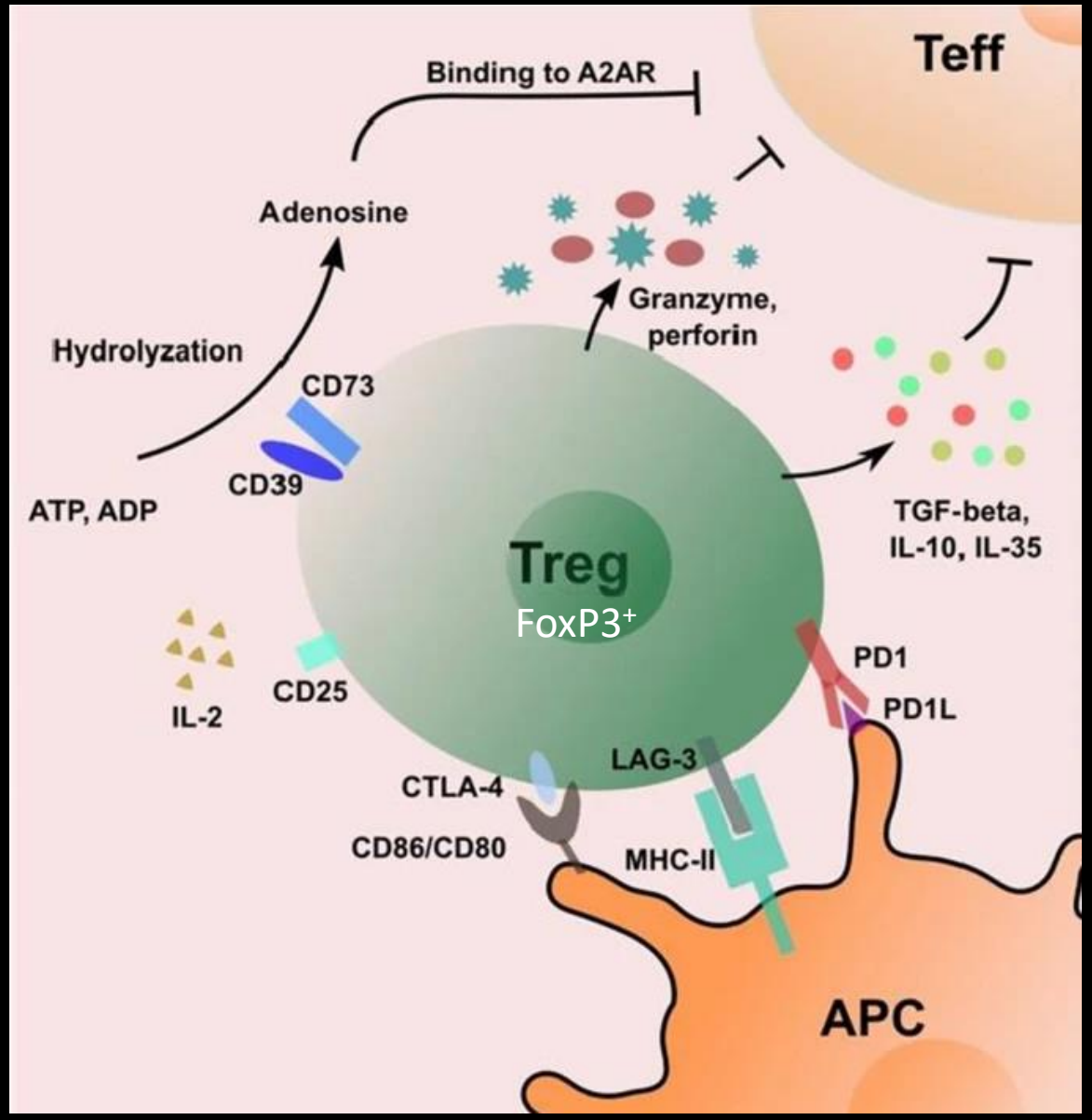
Sakai E, et al. Investigation of Small Bowel Abnormalities in HIV-Infected Patients Using Capsule Endoscopy. *Gastro Research and Practice*. 2017 (5):1-7

Torgerson TR. (2020). IPEX and IPEX-like disorders. In: Sullivan KE, Stiehm ER (eds) Stiehm's Immune Deficiencies. Inborn Errors of Immunity. Elsevier, Academic Press.

# Etiology of IPEX→ Regulation Breakdown

Dysfunctional or absence of $T_{reg}$ cells leads to IPEX

- $T_{reg}$ cells: subset of CD4 T cell involved inhibiting immune system
- Expression of the transcription factor forkhead protein 3 (FoxP3) commits to $T_{reg}$ phenotype
- Variants or loss of FoxP3 expressing cells leads to autoimmunity






Yan, S. et al. Regulatory T cells in rheumatoid arthritis: functions, development, regulation, and therapeutic potential. *Cell. Mol. Life Sci.* **79**, 533 (2022

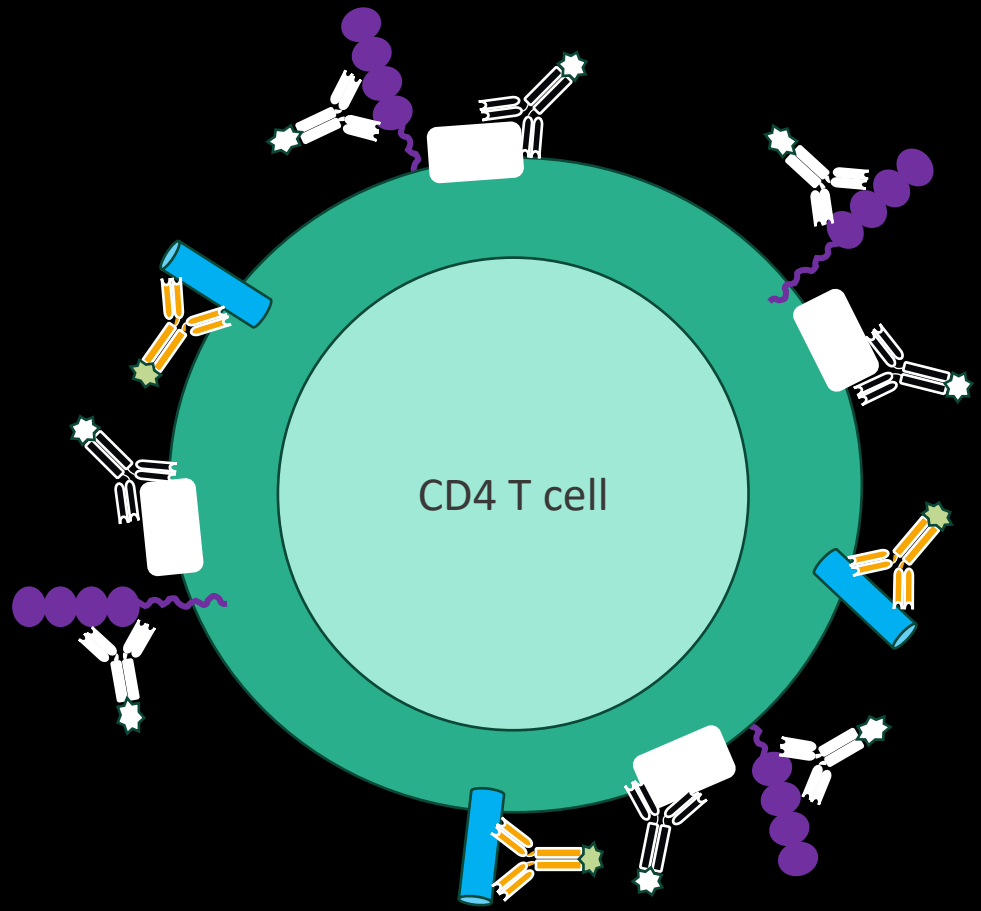
# Diagnosis of IPEX

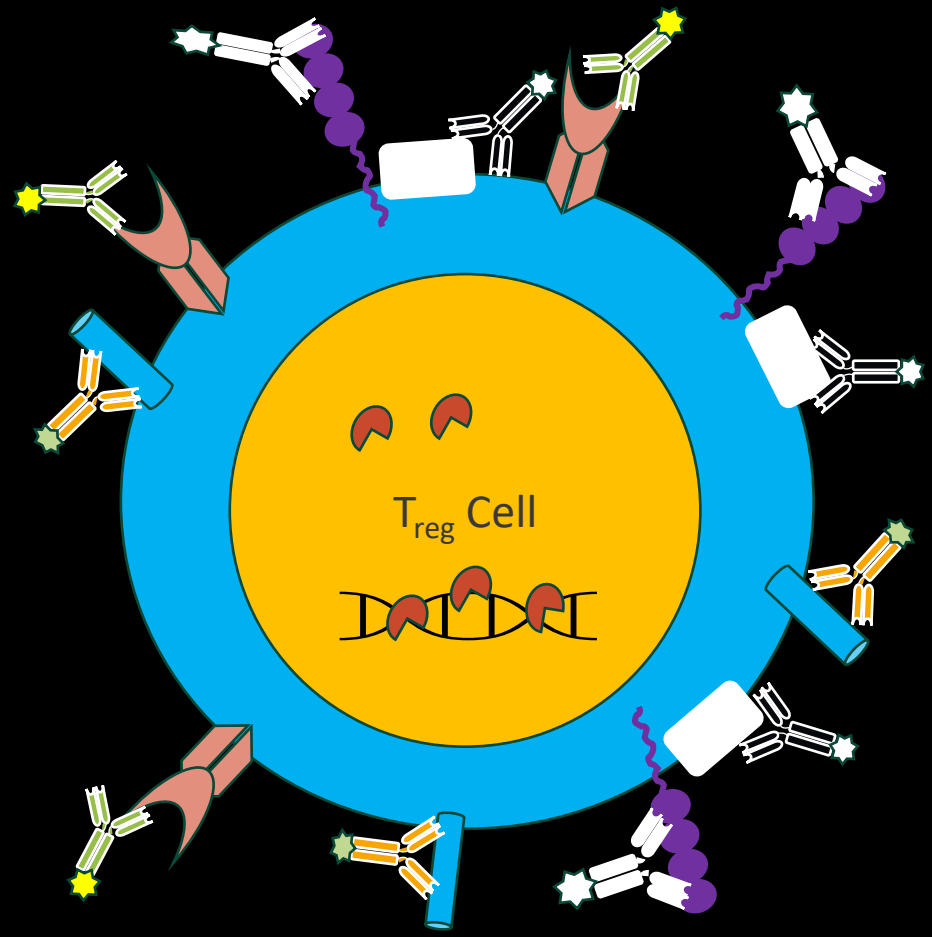
- Currently, identification of a pathogenic variant in *FOXP3* is considered the gold standard for establishing a diagnosis of IPEX
- Identification of variants can be difficult→ non exon variants, deletion/duplication, variants of unknown significance etc.
- Adjunct assays targeting detection of $T_{reg}$ cells and/or FoxP3 expression can supplement, confirm pathogenicity of genetic testing.



Torgerson TR. (2020). IPEX and IPEX-like disorders. In: Sullivan KE, Stiehm ER (eds) Stiehm's Immune Deficiencies. Inborn Errors of Immunity. Elsevier, Academic Press; https://primaryimmune.org/understanding-primary-immunodeficiency/types-of-pi/ipex-syndrome

# Regulatory T Cell Panel, FoxP3-Quantitative Assay




CD4-T subset-specific co-receptor

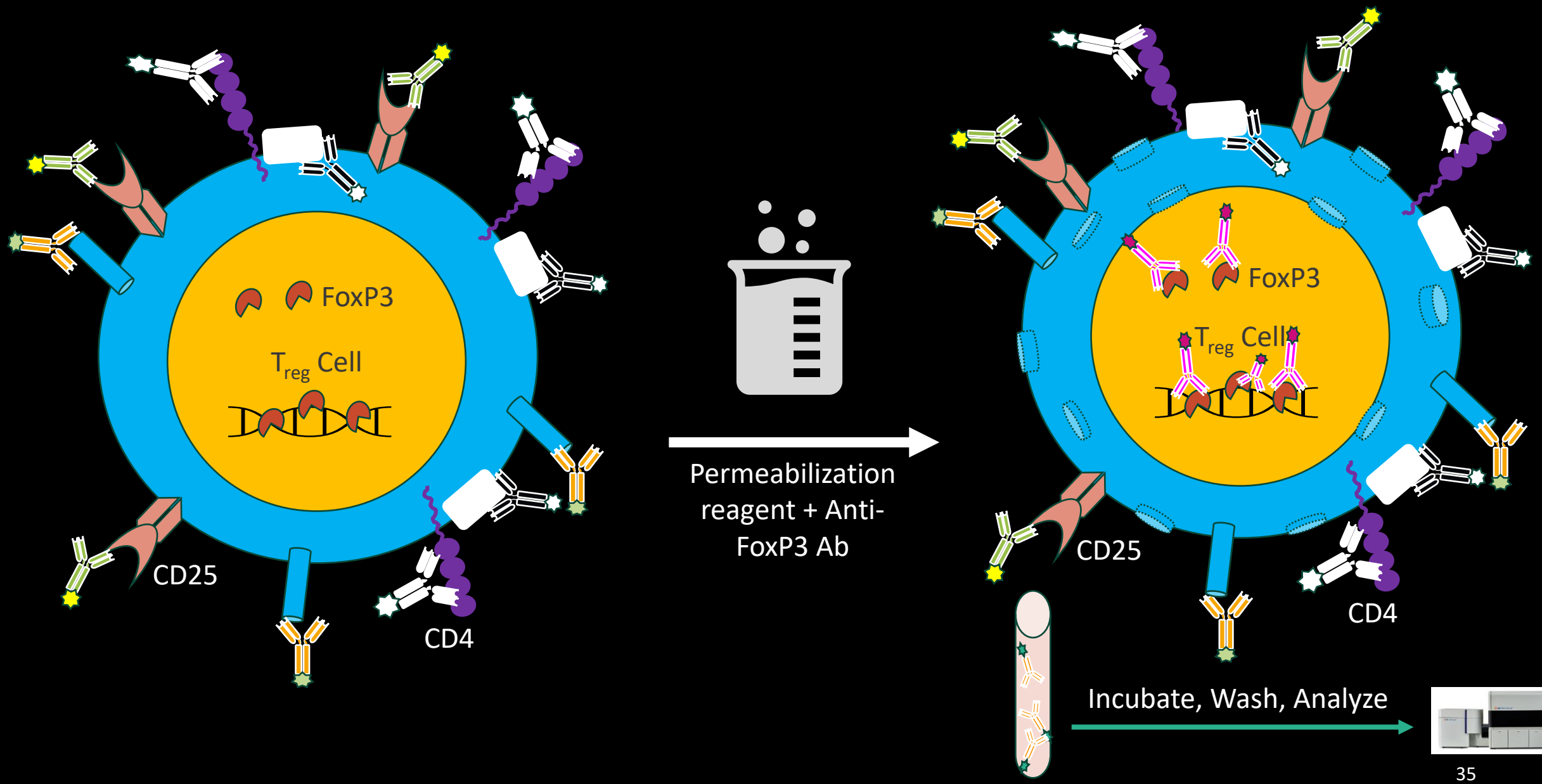
CD45-pan leukocyte marker

CD3-pan T cell marker

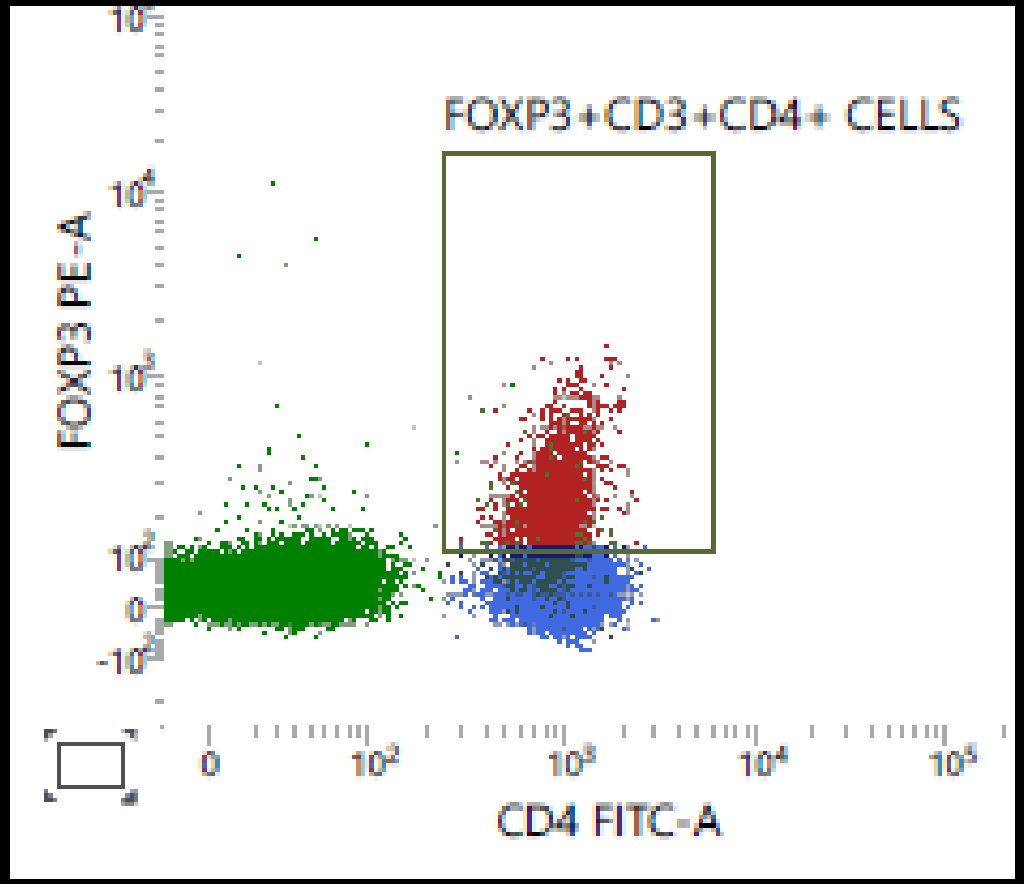
CD25-High expression on $T_{reg}$

FoxP3-$T_{reg}$ specific

# Regulatory T Cell Panel FoxP3 Staining



## Normal Control

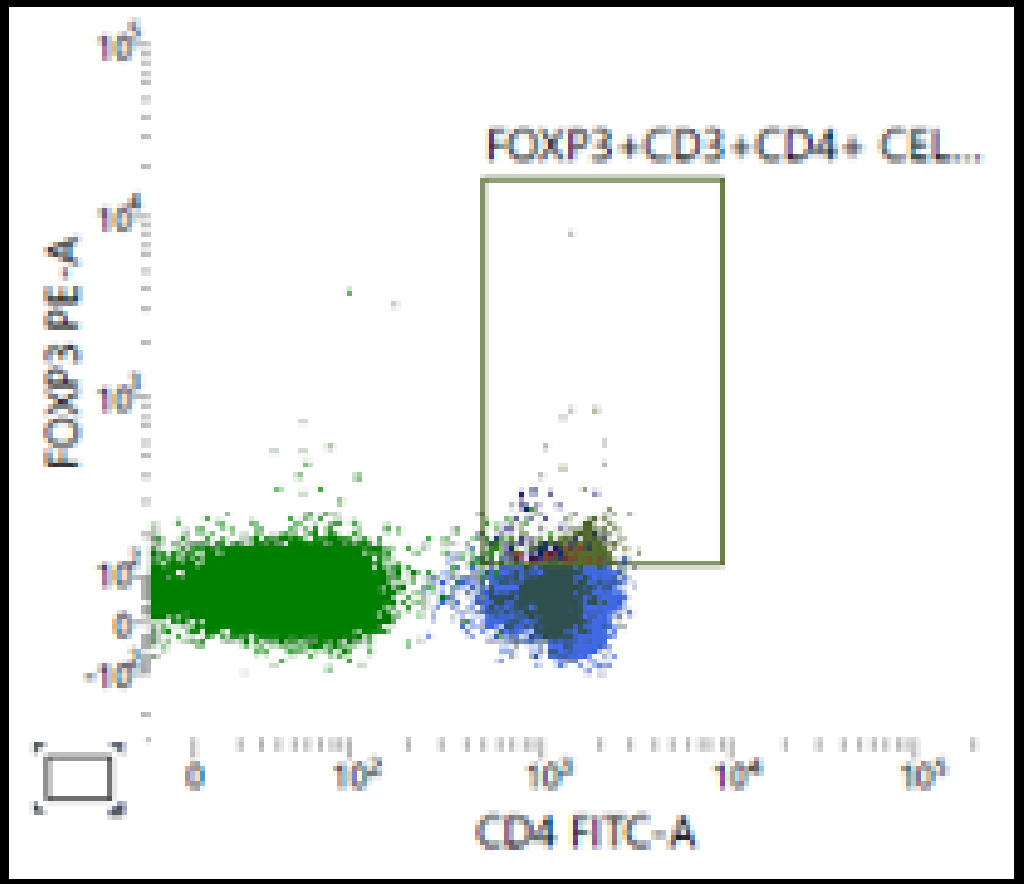



**RESULTS**

**FOXP3TREGS %: 11.9**

**FOXP3TREGS Abs: 154**

## Suspect IPEX patient



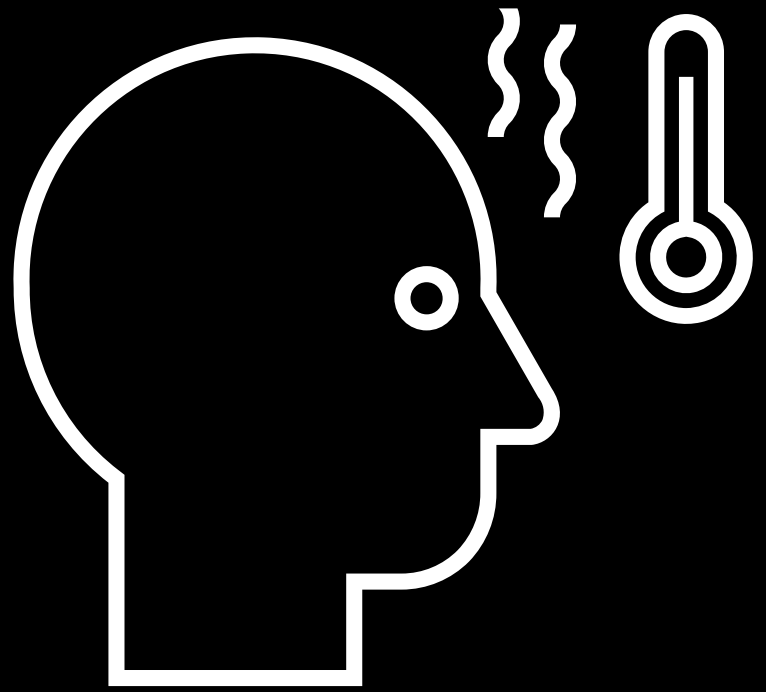

**RESULTS**

**FOXP3TREGS %: 0.6**

**FOXP3TREGS Abs: 6**

# Case 2: A Curious Case of Neutropenia

- A 67-yr old male with hyperlipidemia (high cholesterol) presents to the clinic with a fever lasting > 3 days.
- Aside from fever, the patient's physical condition was unremarkable.
- Initial blood test revealed leukocytopenia (low white blood cell count).



# Case 2: Cont'd

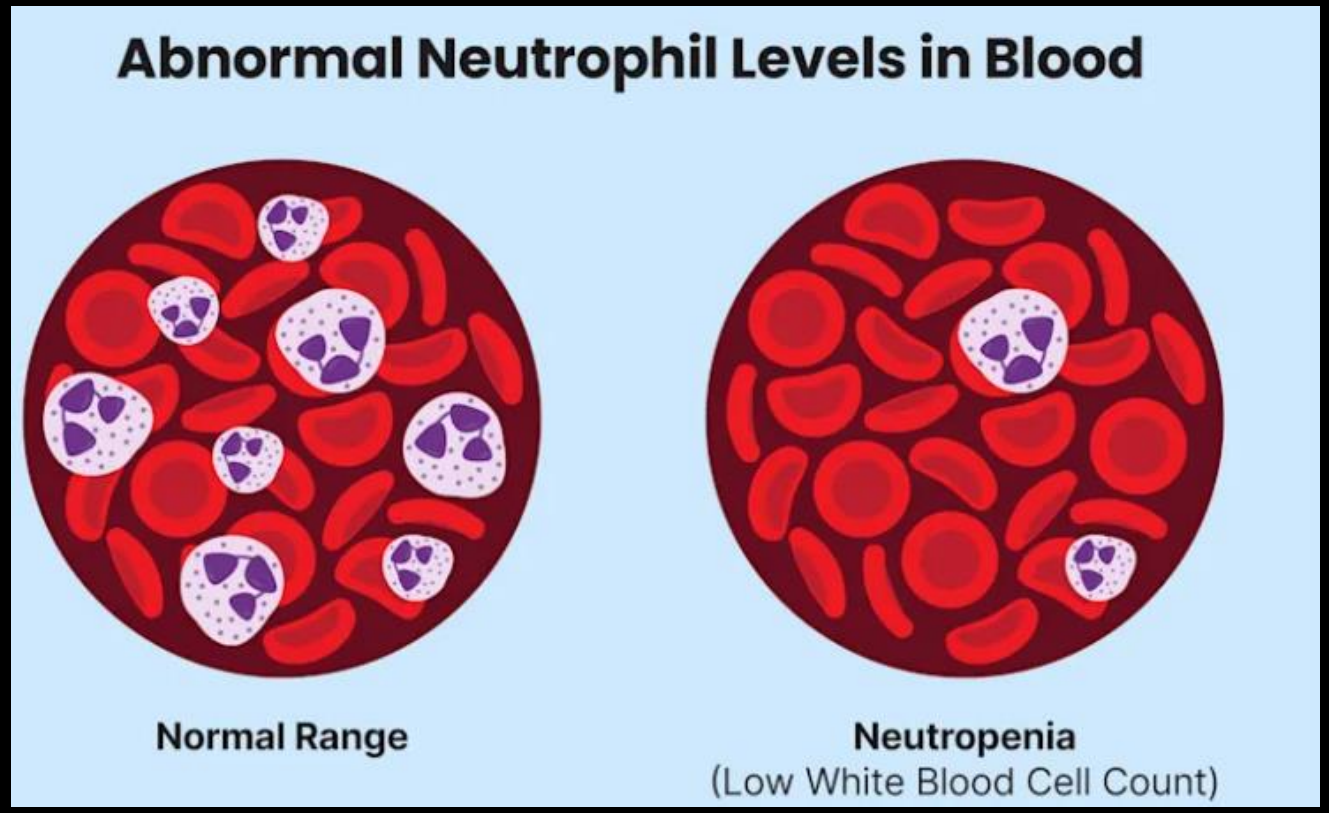
- Total WBC count was 1100/µl, with a neutrophil count of 33/µl (**LOW**)
- Bone marrow aspiration revealed no morphological dysplasia in any of the hematological cell lineages, nor abnormal cell populations
- A CT scan displayed no signs of infection, swollen lymph nodes, or hepatosplenomegaly

What could be mediating neutropenia in this patient?!

Adachi Y et al. Two Cases of Autoimmune Neutropenia Complicated with Other Lineages of Autoimmune Cytopenia, Successfully Treated with Prednisolone. *Intern Med*. 202. 60(8):1271-1277

# Secondary Neutropenia

- Neutrophil: primary mediator of rapid innate immune response
- Neutrophil count below 500 cells/µL greatly increases risk of infection
  - Mild: 1000-1500 cells/µL
  - Moderate: 500-1000 cells/µL
  - Severe: <500 cells/µL
- Common manifestation of severe disease → unspecific (broad differential diagnosis)

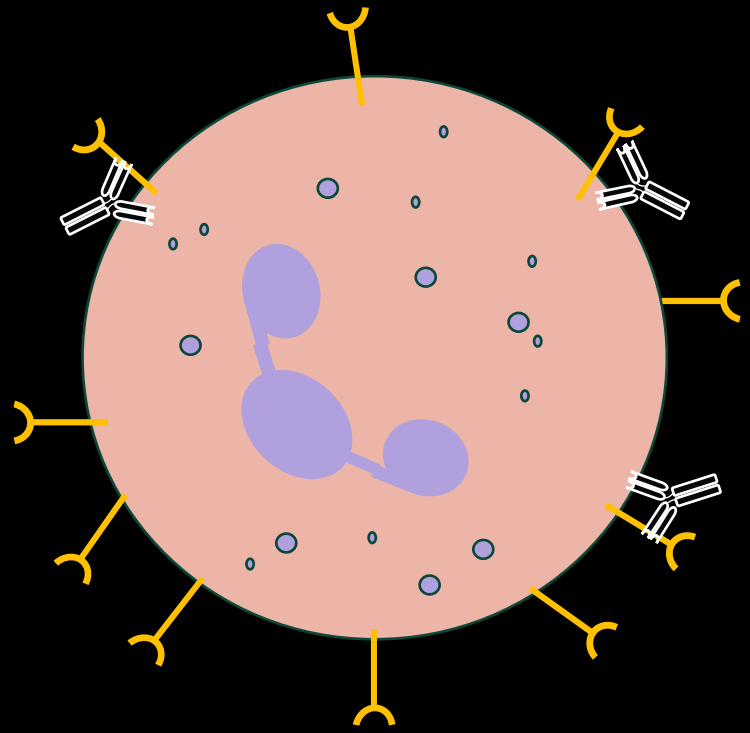



- Autoimmune disease
- Lymphoproliferative syndromes
- Inborn errors of immunity
- Drug-induced
- Chronic infection
- Endocrinopathies

Rezaei N, de Vries E, Gambinerie E, Meyts I, Haddad E. (2020). Common Presentations and Diagnostic Approaches. In: Sullivan KE, Stiehm ER (eds) Stiehm's Immune Deficiencies. Inborn Errors of Immunity. Elsevier, Academic Press; Malech HL et al. The Role off Neutrophils in the Immune System: An Overview. *Methods Mol Biol*. 2014:1124:3-10.

# Anti-Neutrophil Antibodies and Neutropenia

- Autoimmune neutropenia (AIN): hematological diseases caused by autoantibody induced  destruction of neutrophils
  - Detection of neutrophil autoantibody can help exclude other causes of neutropenia, and inform treatment strategies
    - Granulocyte-colony stimulating factor (G-CSF)
    - Immunosuppressive drugs (prednisolone)
    - IVIG, rituximab, alemtuzumab



**-opsonization = increased clearance**
**-agglutination and phagocytosis**
**-complement-mediated destruction**

Fujita M, et al. A rare case of adult autoimmune neutropenia successfully treated with prednisolone. *Intern Med.* 2018 56(11):1415-19; Adachi Y et al. Two Cases of Autoimmune Neutropenia Complicated with Other Lineages of Autoimmune Cytopenia, Successfully Treated with Prednisolone. *Intern Med*. 202. 60(8):1271-1277; Autrel-Moignet A, Lamy T. Autoimmune neutropenia; Autoimmune Cytopenias Quarterly medical Review. 2014. 43: e105-e118.

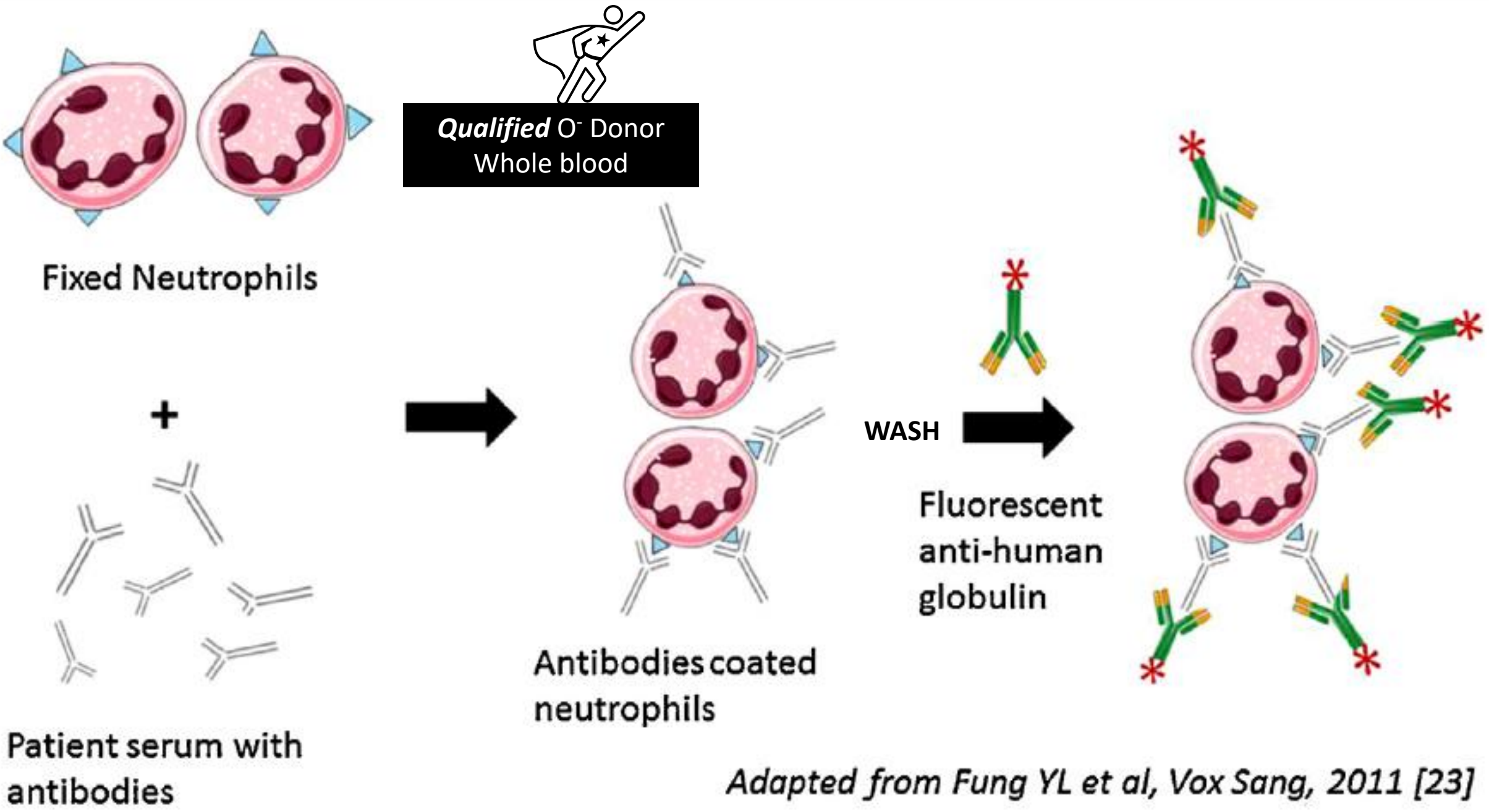
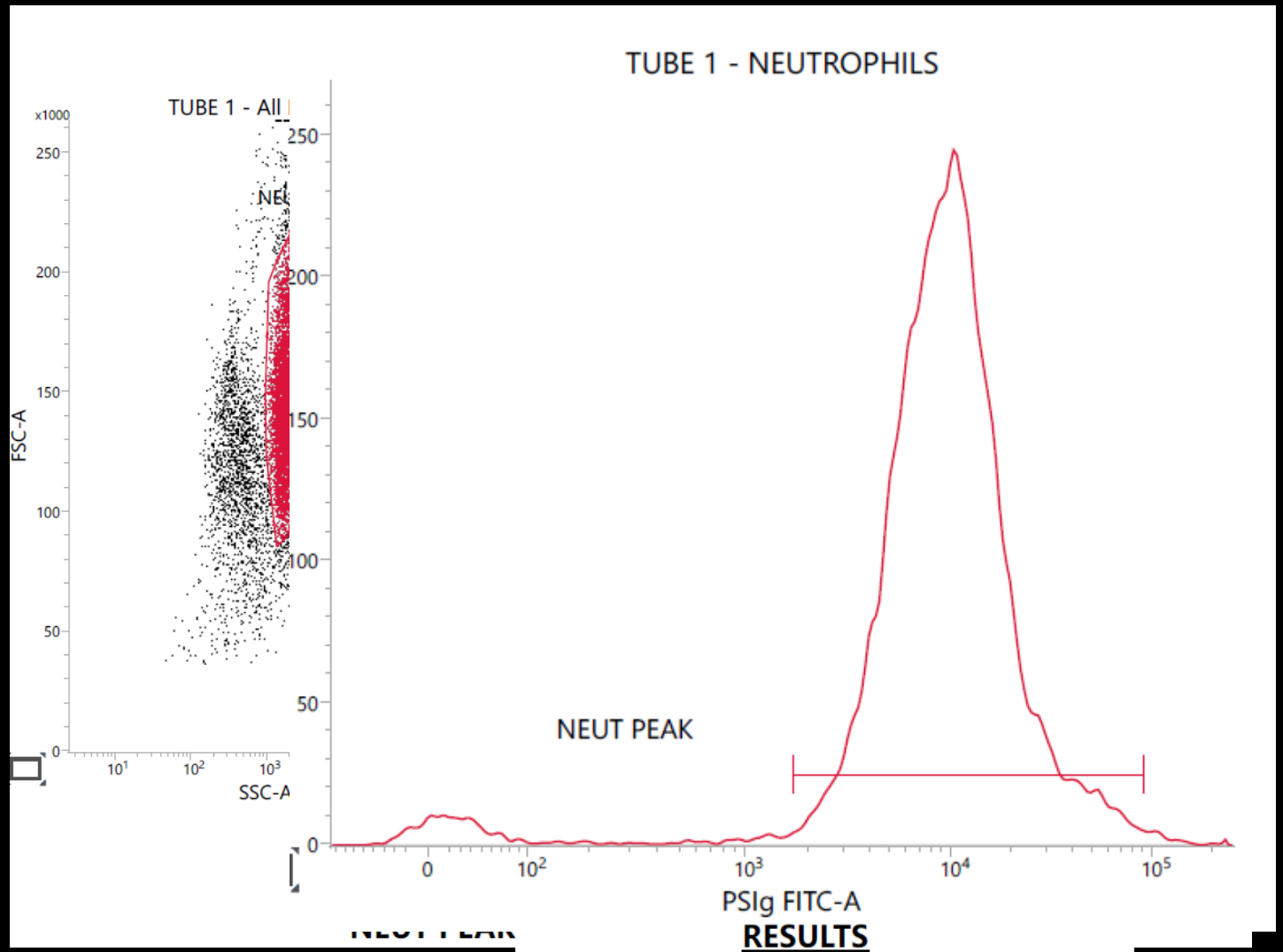
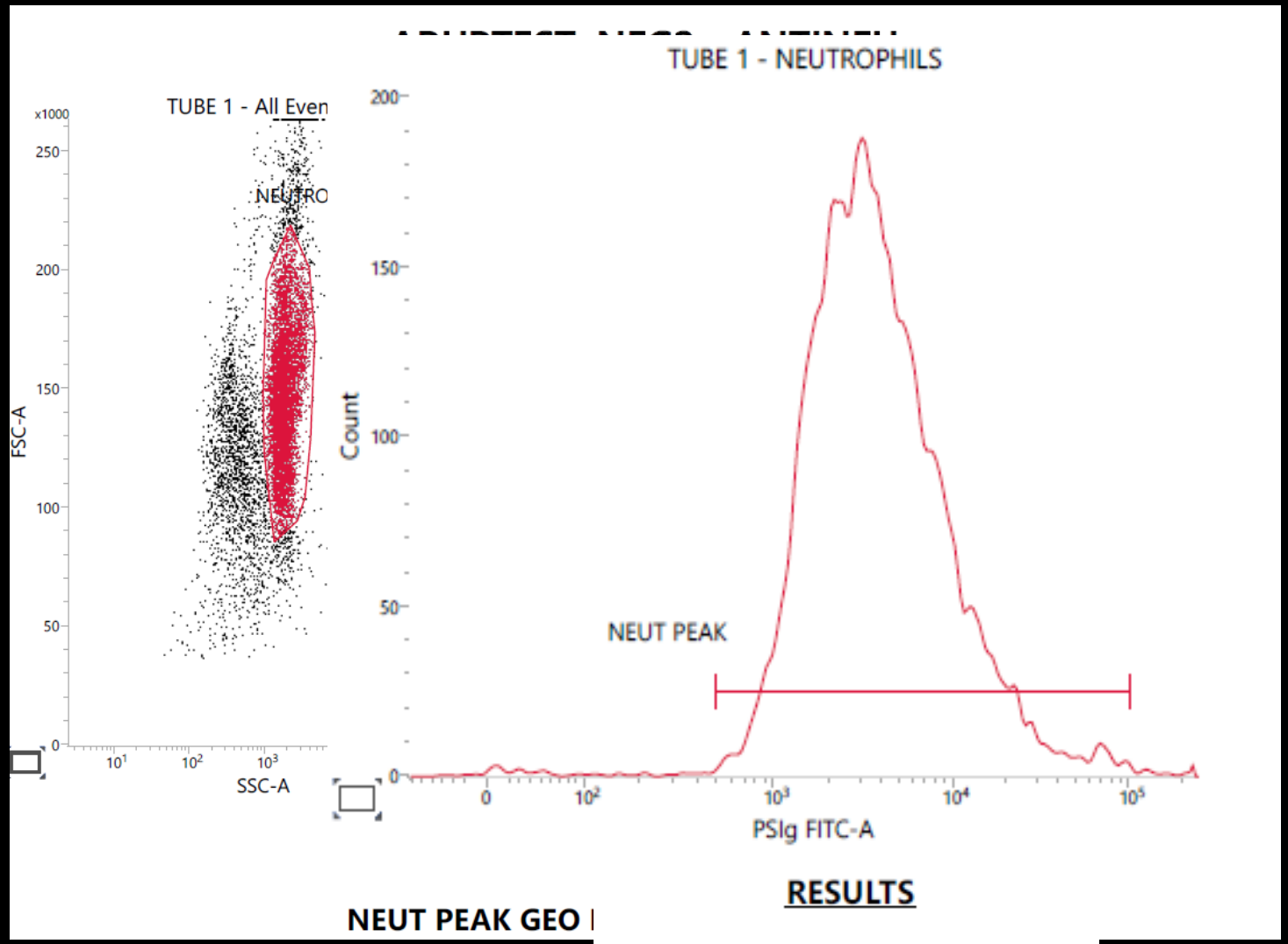



Image adapted from: Autrel-Moignet et al. *Autoimmune Cytopenias Quarterly Medical Review*. 2014.

# Anti-Neutrophil Antibody Assay Result Interpretation:

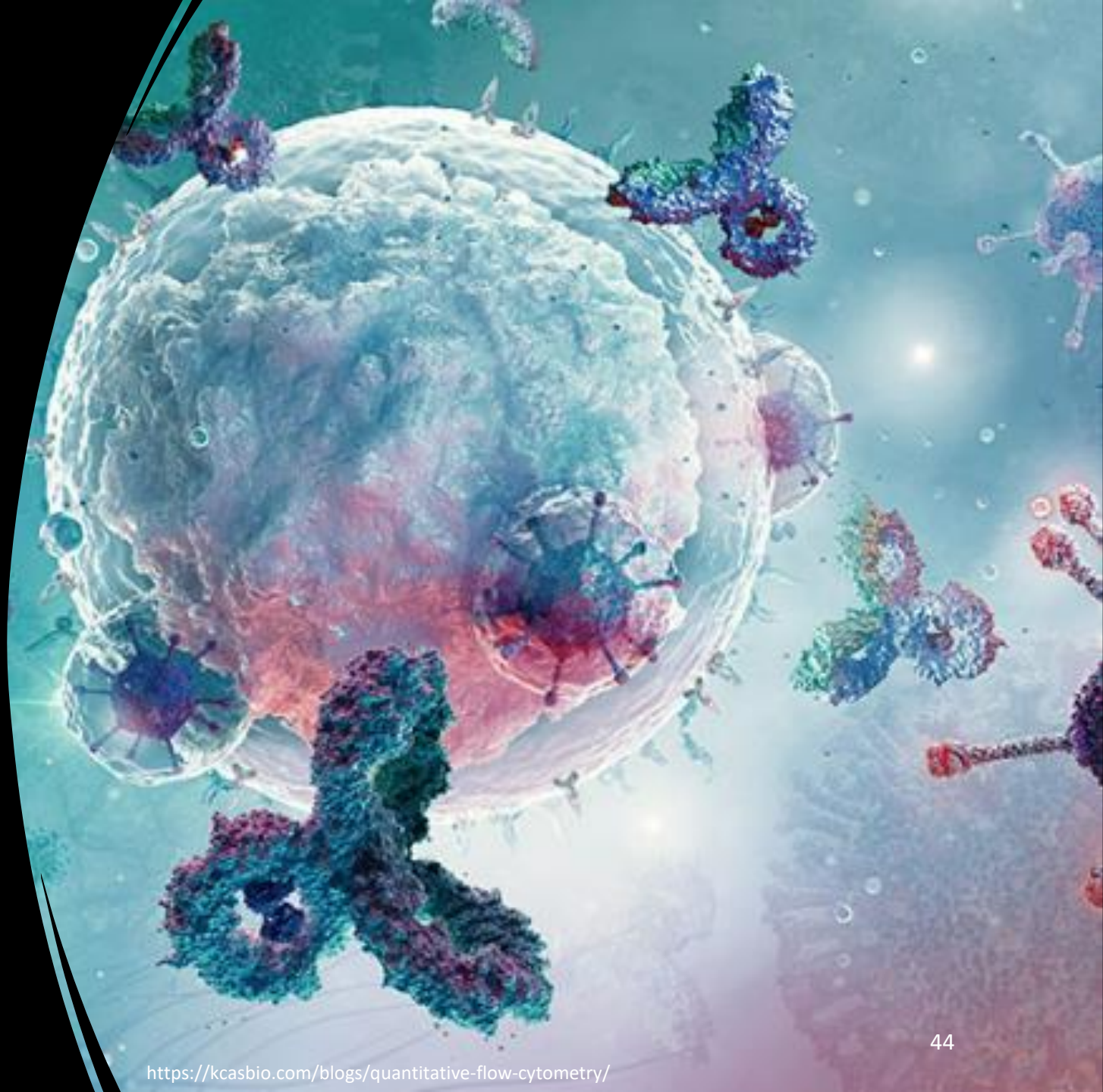


# Anti-Neutrophil Antibody Assay Result Interpretation: Weak Positives



# Flow Cytometry: In*cyte* for the Clinic

- Flow cytometry is a powerful tool for analyzing clinical samples.
  - Multiparametric analysis
  - High sensitivity and through put
  - Versatile: Live, fixed, quantitative, functional assays, rare event analysis, sorting
- Versatility of flow cytometry allows its application to diverse clinical scenarios where it can be a key factor in informing practice, diagnosis, and monitoring of disease.



https://kcasbio.com/blogs/quantitative-flow-cytometry/

# Thank You!

# Knowledge Check

Further work up with a *FoxP3* genetic panel did not detect a variant in the patient. Which of the following flow cytometric approaches would you consider most useful to further investigate the possibility of IPEX?

A. Total CD4 and CD8 T cell enumeration via flow cytometry.
B. T cell proliferation assay via flow cytometry.
C. Quantify total FoxP3 expressing cells.
D. Intracellular cytokine measurement.

# Knowledge Check:

For a quantitative, intracellular flow assay, which step(s) are crucial to include?

A. Fixation

B. Permeabilization

C. Collection of enough target cell events

D. Use of a normal reference control

# Knowledge check

Which of the following factors should be considered when designing a panel to detect anti-neutrophil antibodies using flow cytometry?

A. An assessment of neutrophil viability.

B. Permeabilization to detect *intracellular* anti-neutrophil antibodies.

C. Criteria for a minimal number of neutrophils analyzed per sample to meet sensitivity requirements.

D. All of the above.

# Knowledge Check

How can we use florescence intensity to resolve borderline results in this flow cytometric assay?

A. Use a reference material, such as BD TruCount™ beads
B. “Eye-ball it”
C. Use negative and normal controls to establish positive cut-off
D. Borderline results suggest pre-analytical issue, retest