

Immunological Techniques in Research and Clinical Medicine

Philip L. Cohen, M.D.

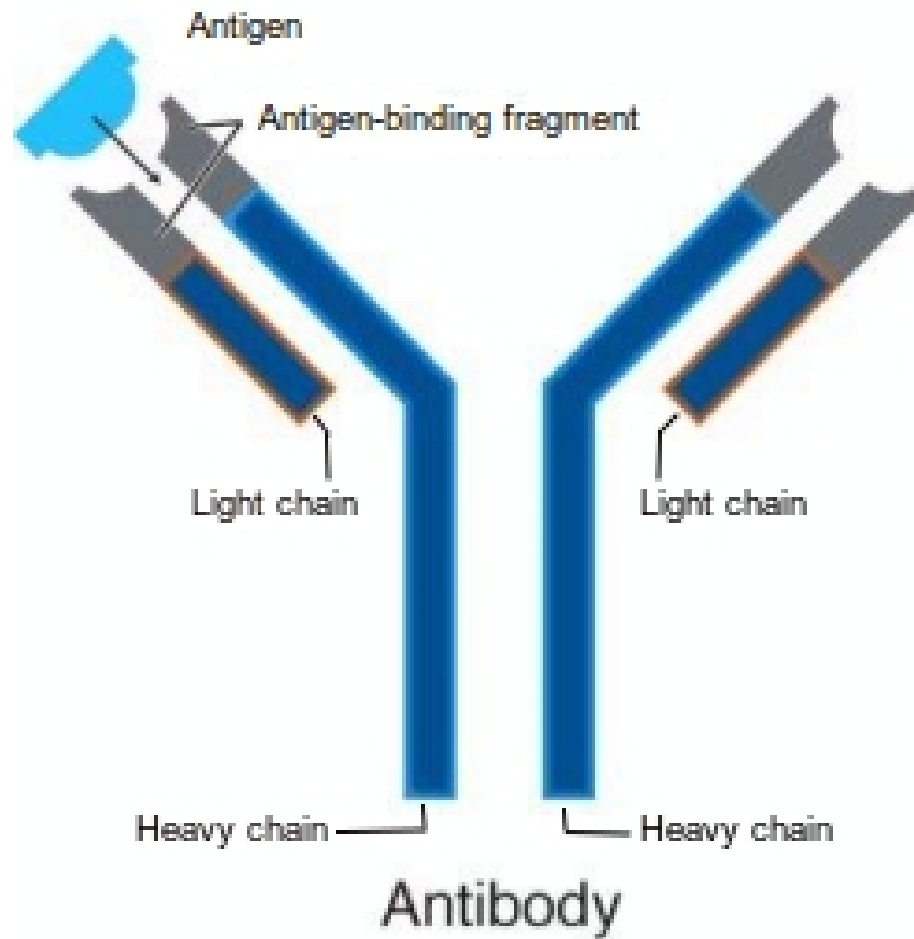
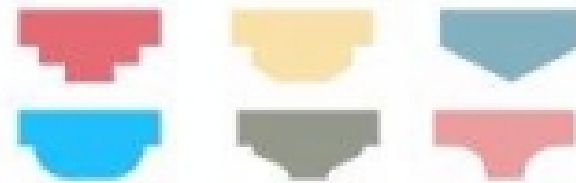
Chief of Rheumatology, LKSOM

10 March 2016

Antibodies – Remarkable Tools for Research and Diagnosis

- You can make an antibody to practically anything
- Monoclonal antibodies have a single specificity, so the immunogen need not be pure (e.g., whole cells or lysates)
- Antibodies are stable (decades at -20C!)
- They can be covalently coupled to enzymes, chromophores, biotin, and many other things..

Antigens



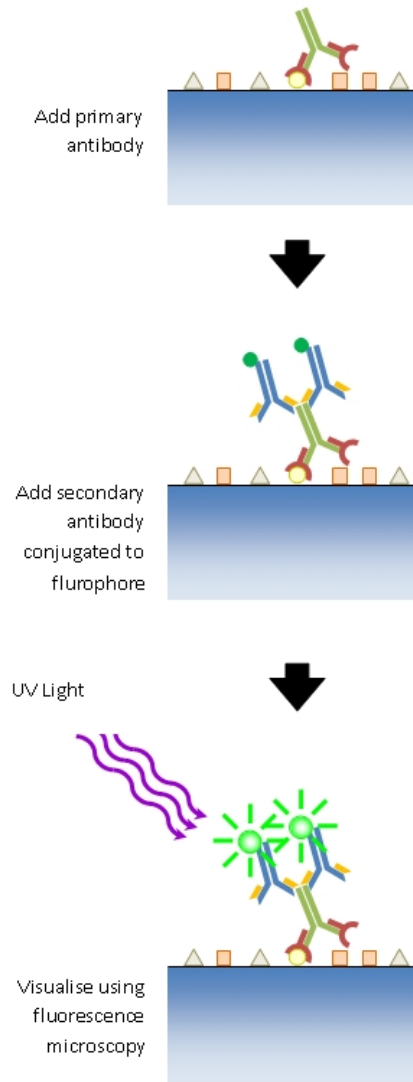
Albert Coons, M.D.
1912-1978



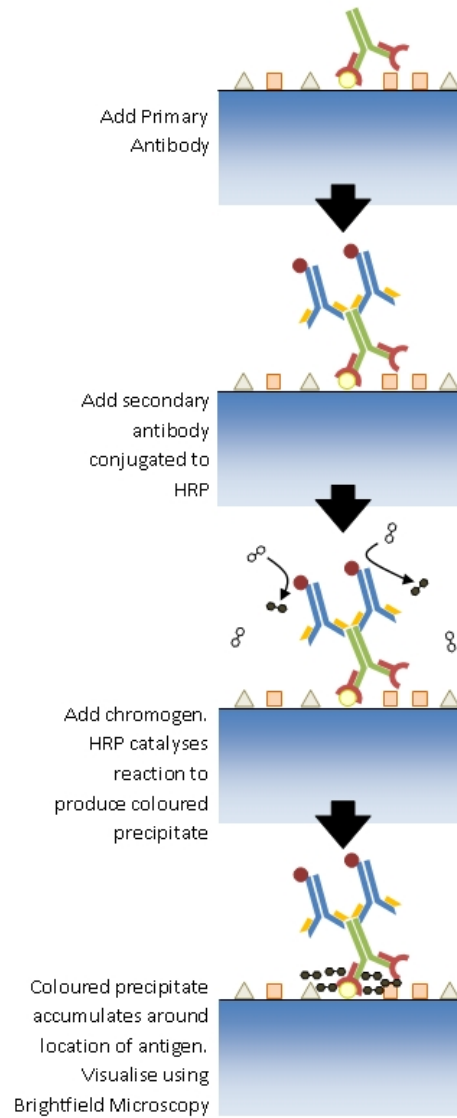
Coons' Insight

- Antibodies can be “tagged” with small fluorescent molecules and still retain their binding specificity
- These “tagged” antibodies can be used as probes to visualize specific molecules in tissues, cells, or anywhere

Immunofluorescence

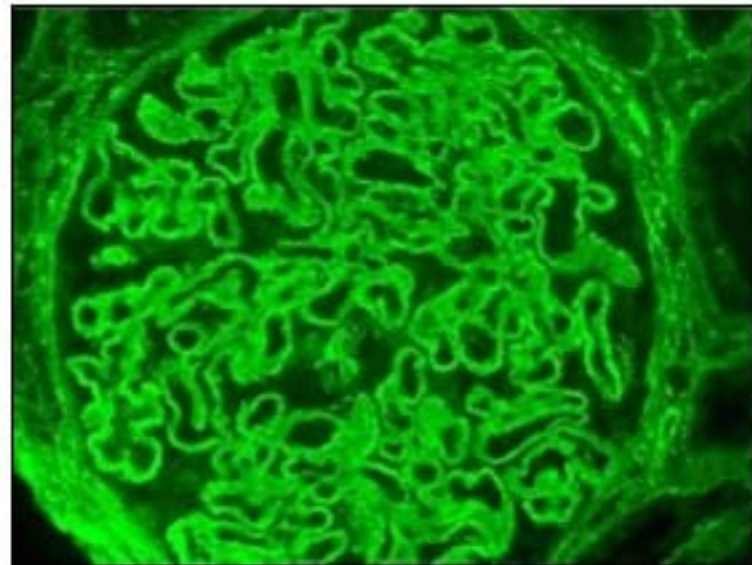


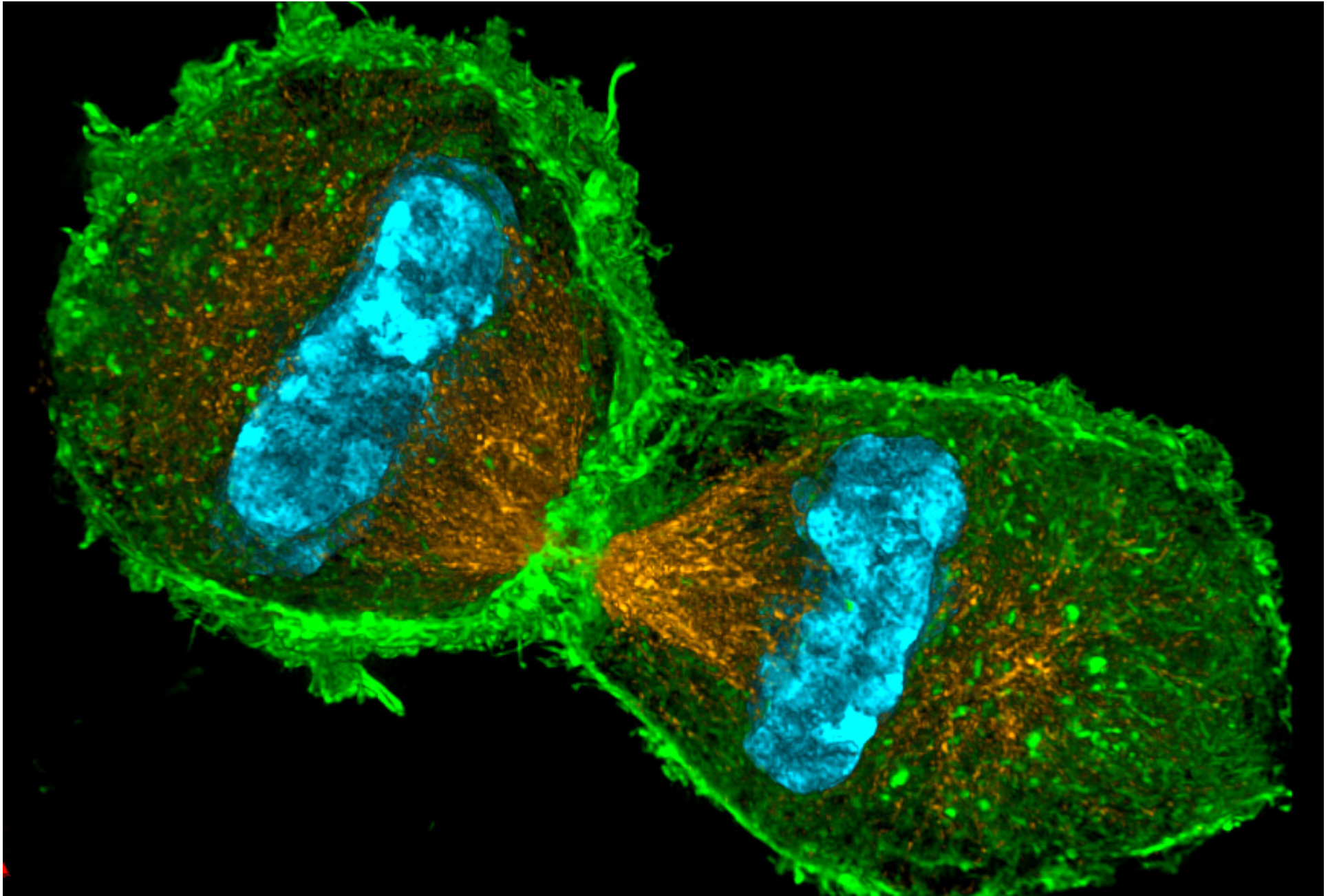
Immunohistochemistry



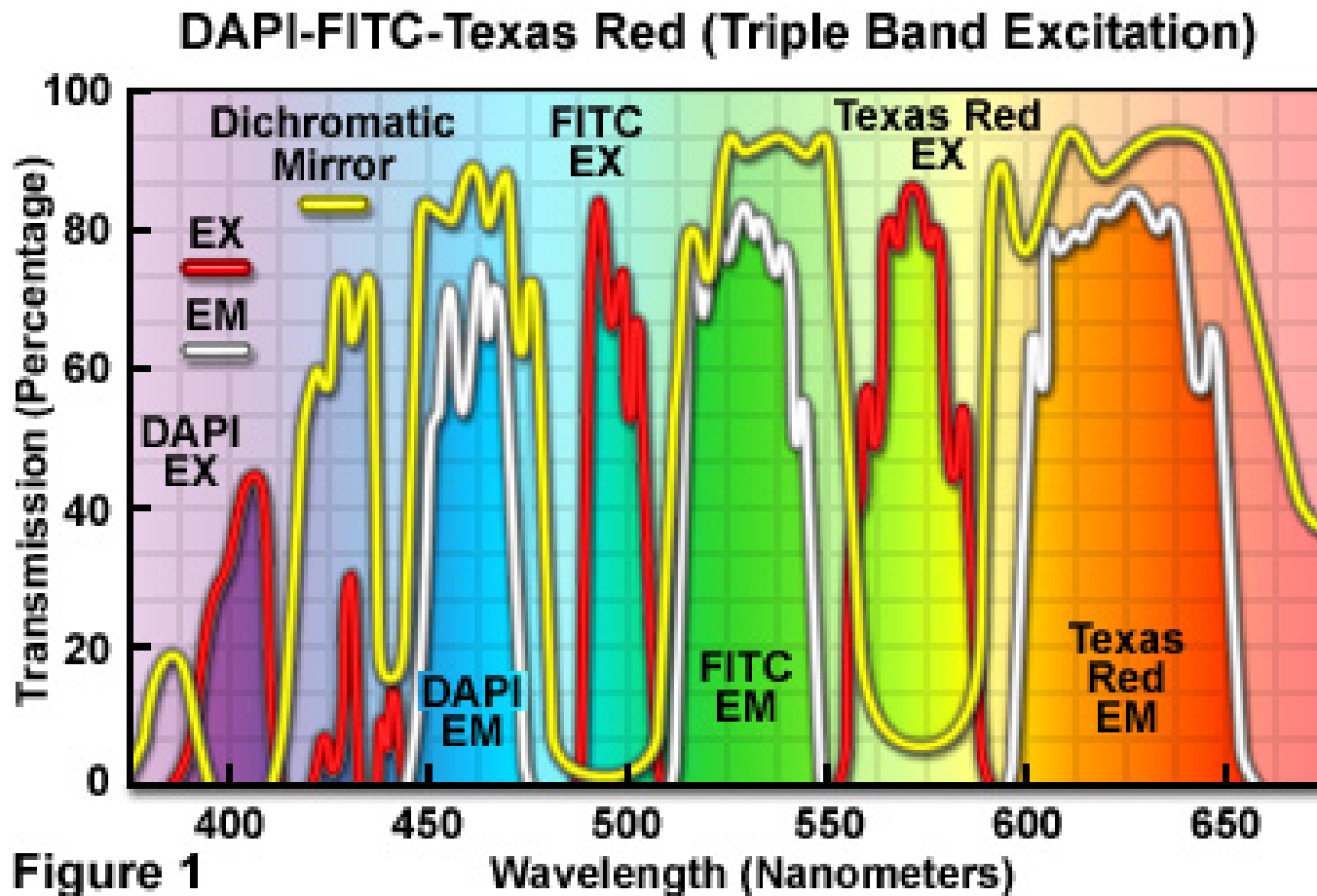
Membranous nephropathy

- **IF:**
 - Strong fine granular staining for IgG and less intense granular staining for C3 along capillary loops
 - IgM, IgA
 - The presence of IgA and C1q in the deposits has been proposed as a marker for SLE MGN



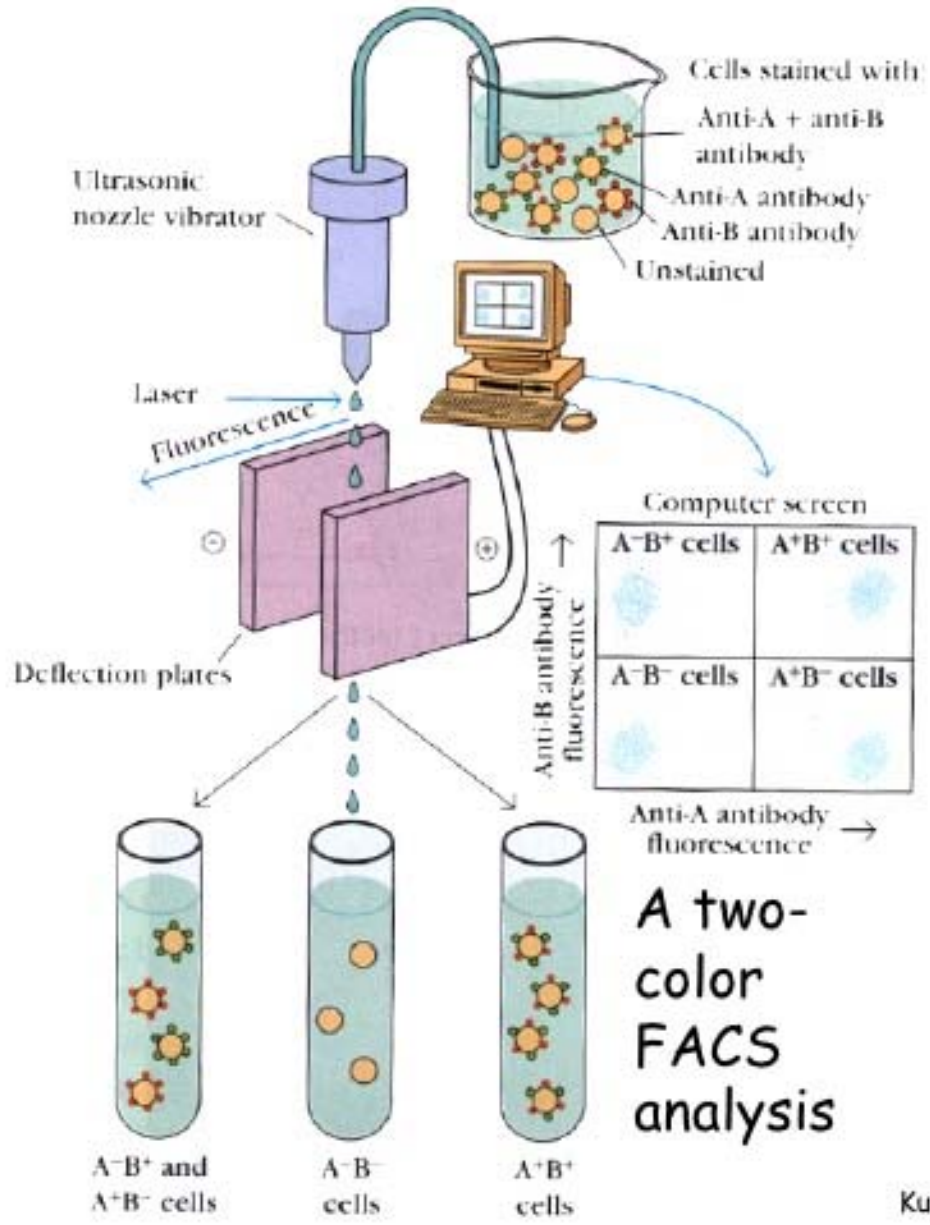


Multiple Color Immunofluorescence

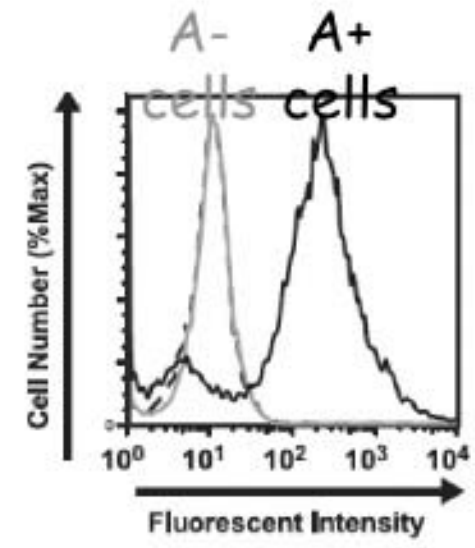


Some Considerations for Fluorescence Staining

- Usually requires freshly snap-frozen tissue
- Conjugated antibodies are less stable than native molecules, are light-sensitive
- For fixed tissues, immunohistochemistry is preferred
- Can be quantitated using laser capture fluorescence microscopy
- Confocal and dual photon microscopy



Flow cytometry -- Fluorescence Activated Cell Sorting (FACS)



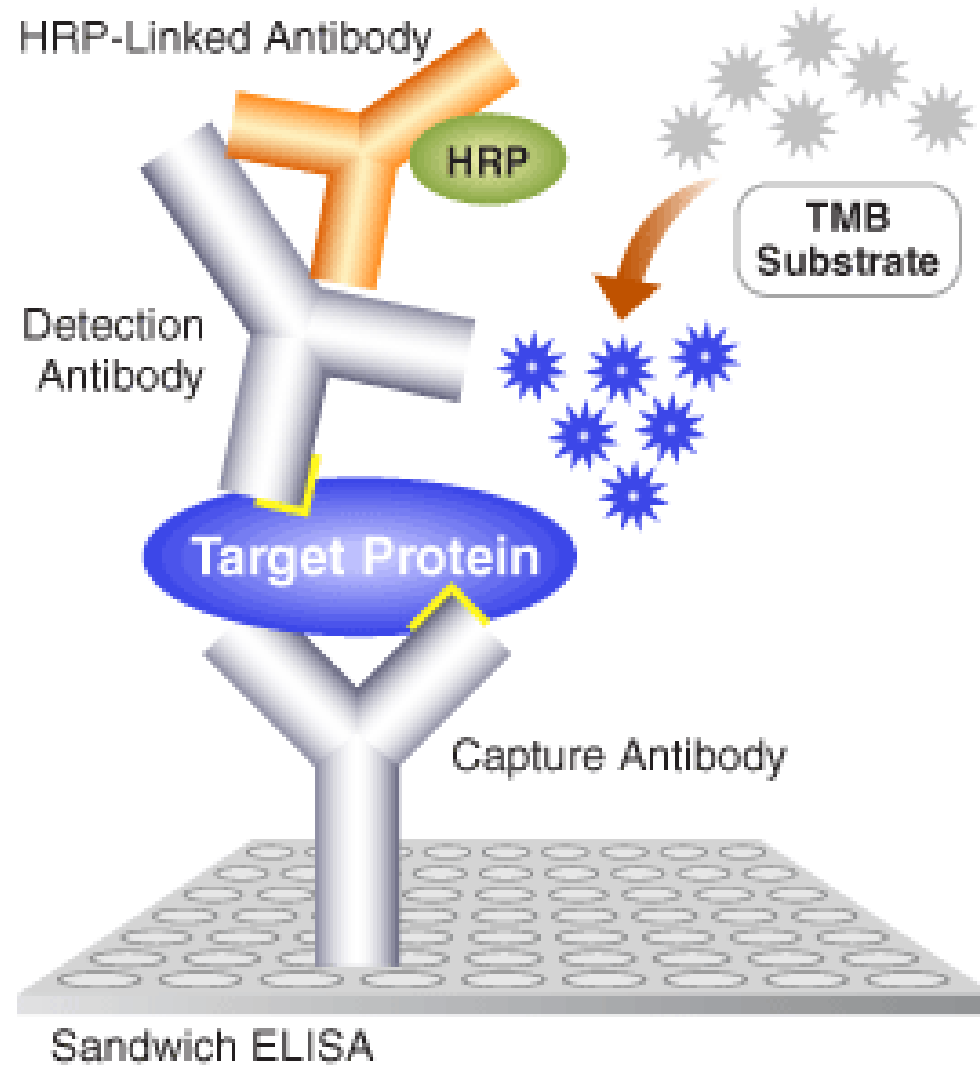
Single color
 FACS
 analysis
 (e.g., using
 anti-A
 antibody) --

Kuby, Kindt, Goldstein, Osstarr Immunology Textbook

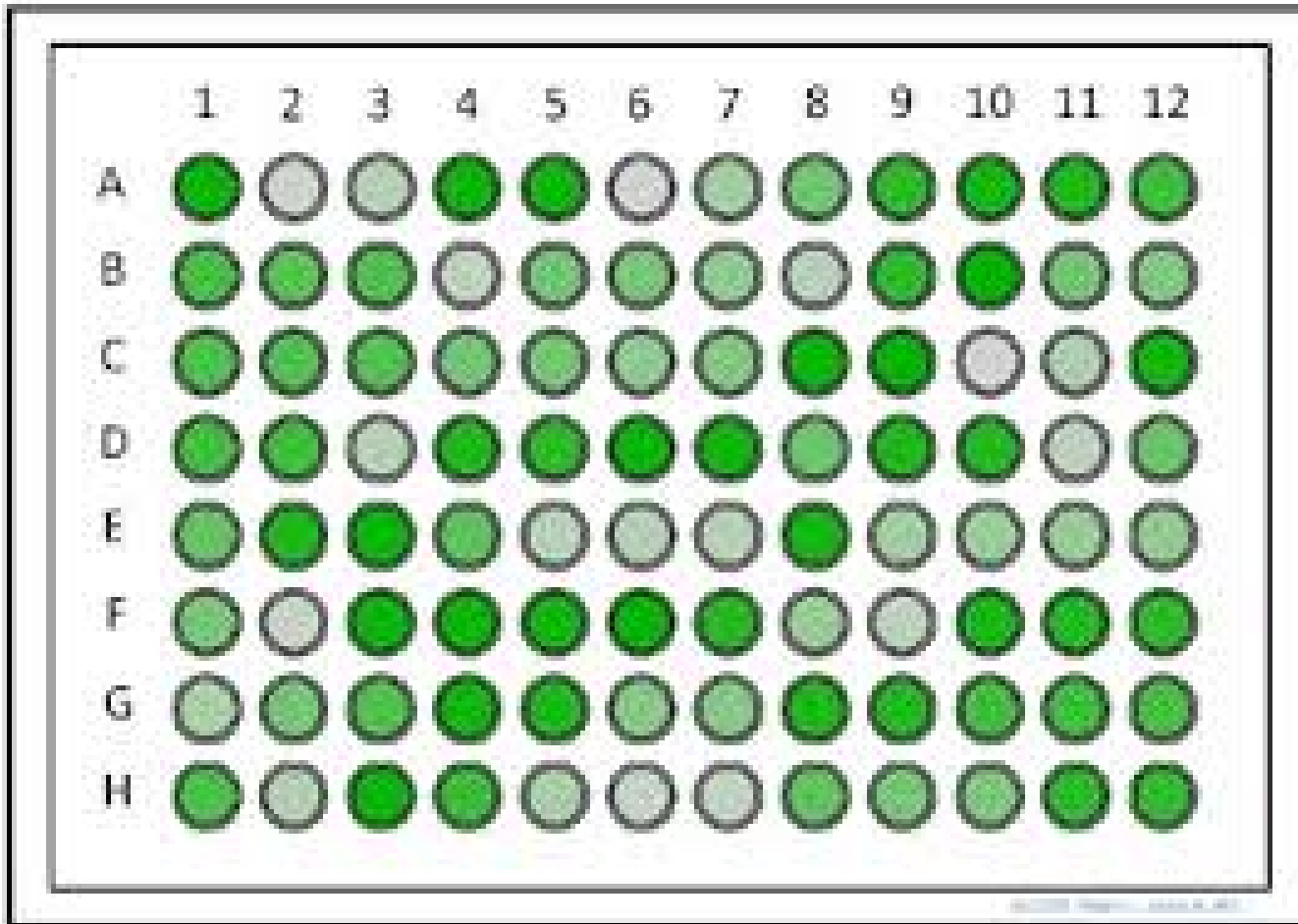
Antibodies as Tools for Quantitating Proteins and Almost Everything Else

- Monoclonal abs are exquisitely specific
- Essentially any molecule can be quantitated
- It helps to have two monoclonals with different specificity (sandwich assays)
- Examples of what's measured: hormones, drugs, cytokines, tumor-derived proteins (e.g. PSA)
- Sensitivity to ng levels at least

Principle of the Sandwich ELISA

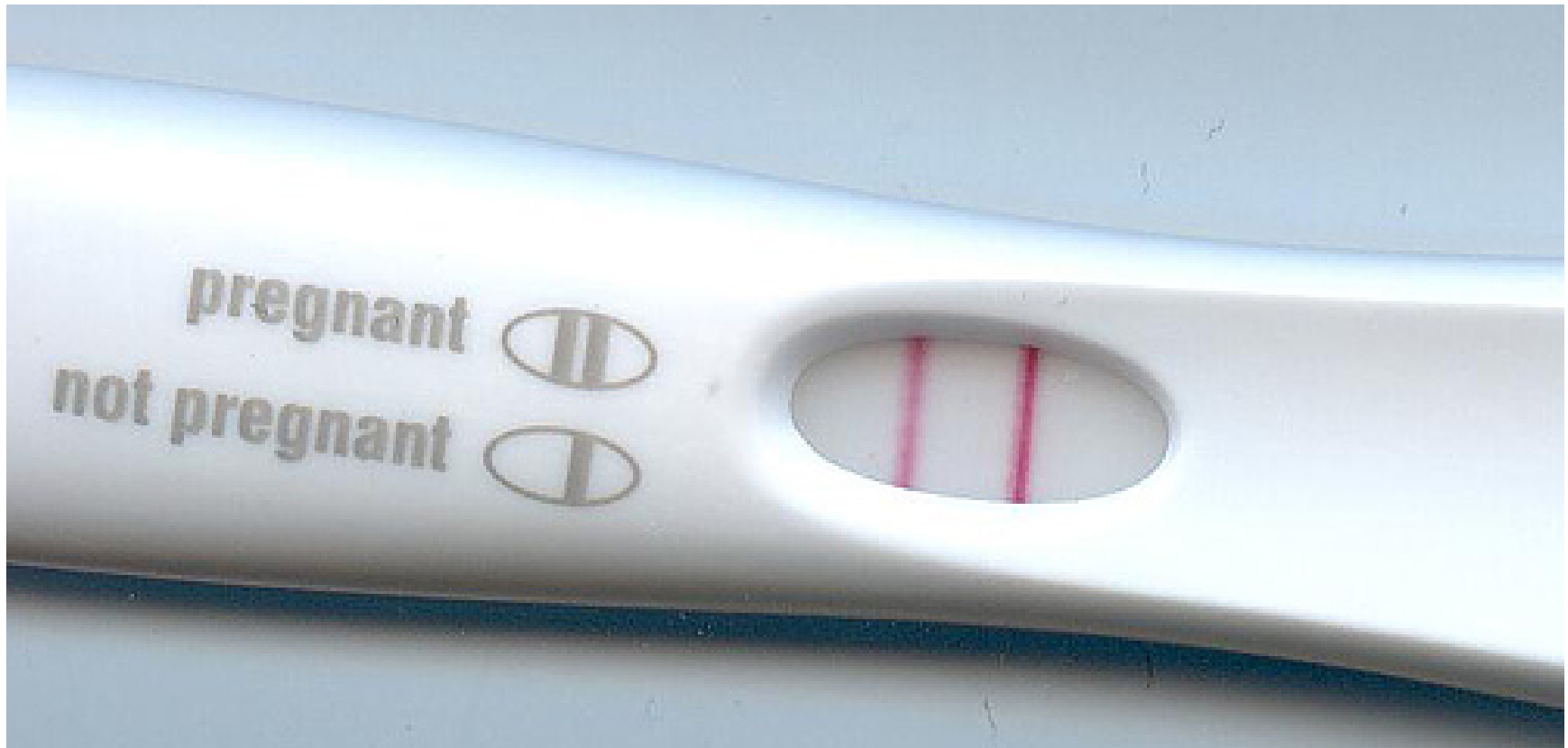


ELISA Plate

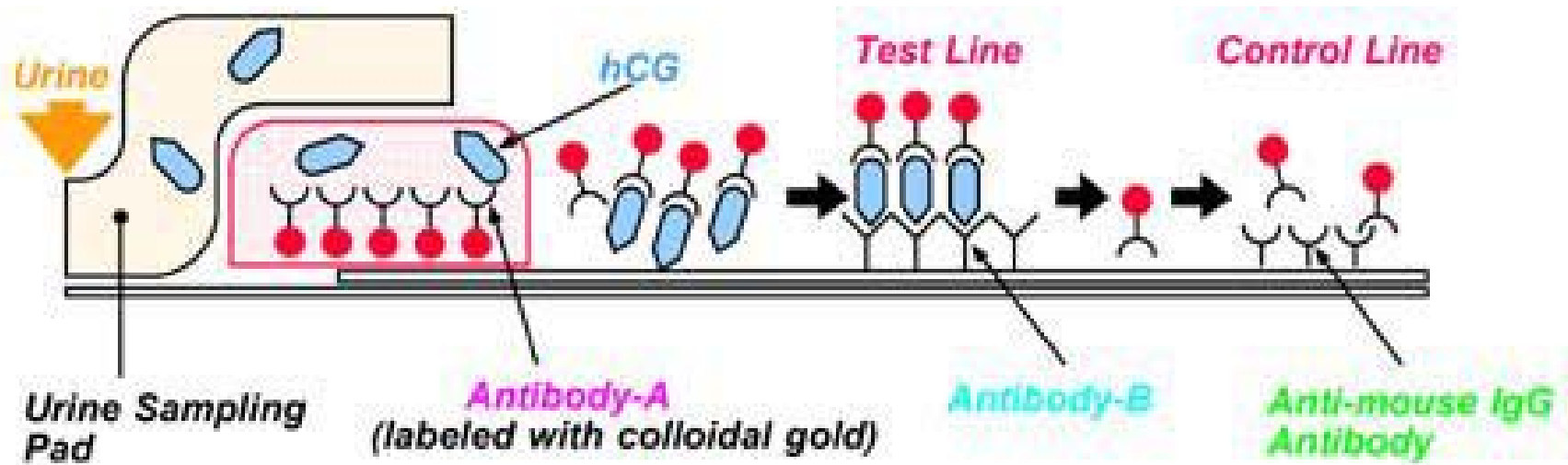


ELISA Plate Reader





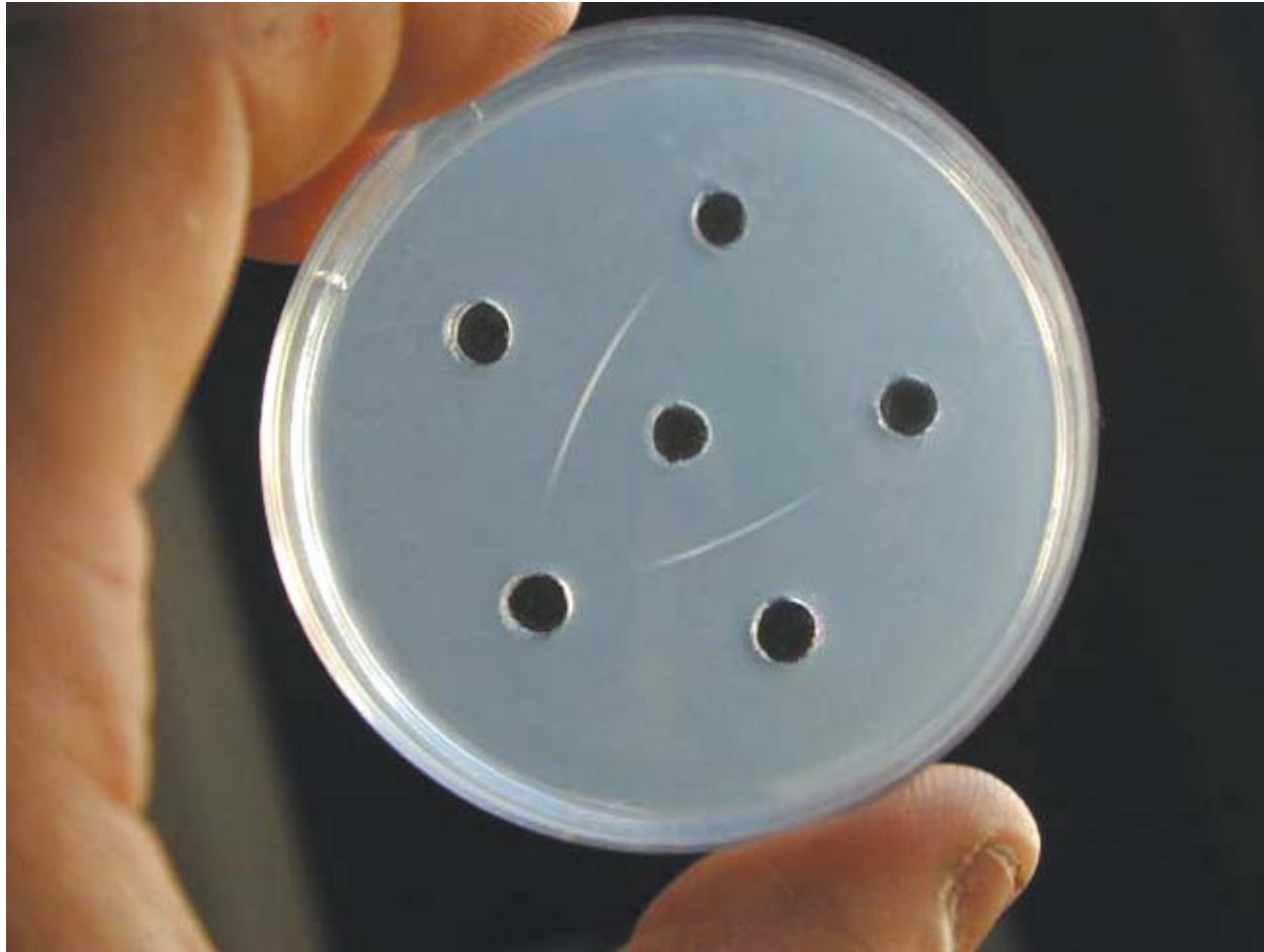
An Important ELISA Application



































































































Methods for Diagnosis

- Immunodiffusion (Ouchterlony)
- Hemagglutination and latex agglutination
- Complement fixation
- ELISA
- Western Blot and other electrophoretic methods

Immunodiffusion Detection of Antibodies

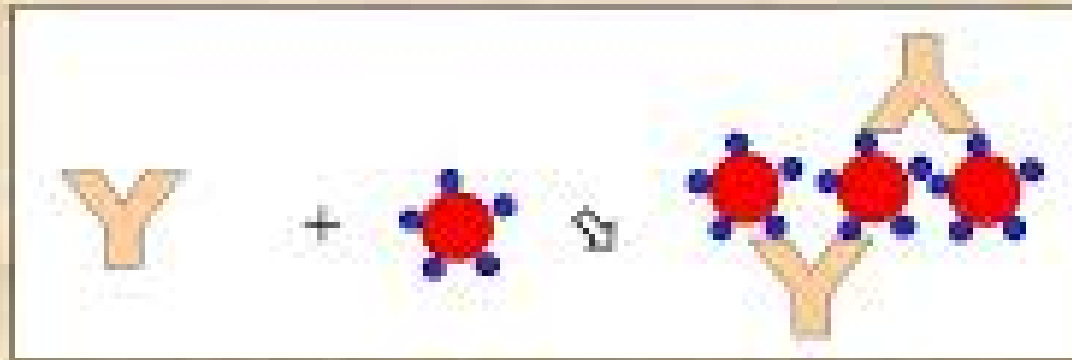


Hemagglutination on a Microtiter Plate

Patient	1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512	1/1024	Pos.	Neg.	Titer
1													64
2													8
3													512
4													<2
5													32
6													128
7													32
8													4

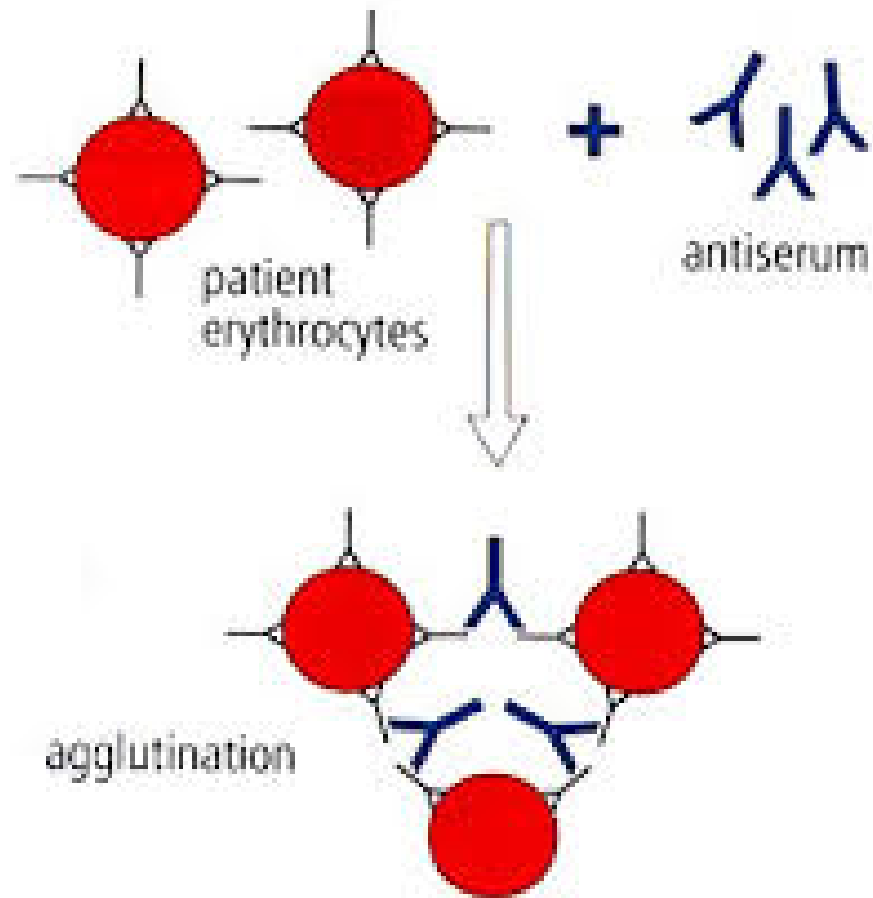
Passive Agglutination/Hemagglutination

- Definition - agglutination test done with a soluble antigen coated onto a particle

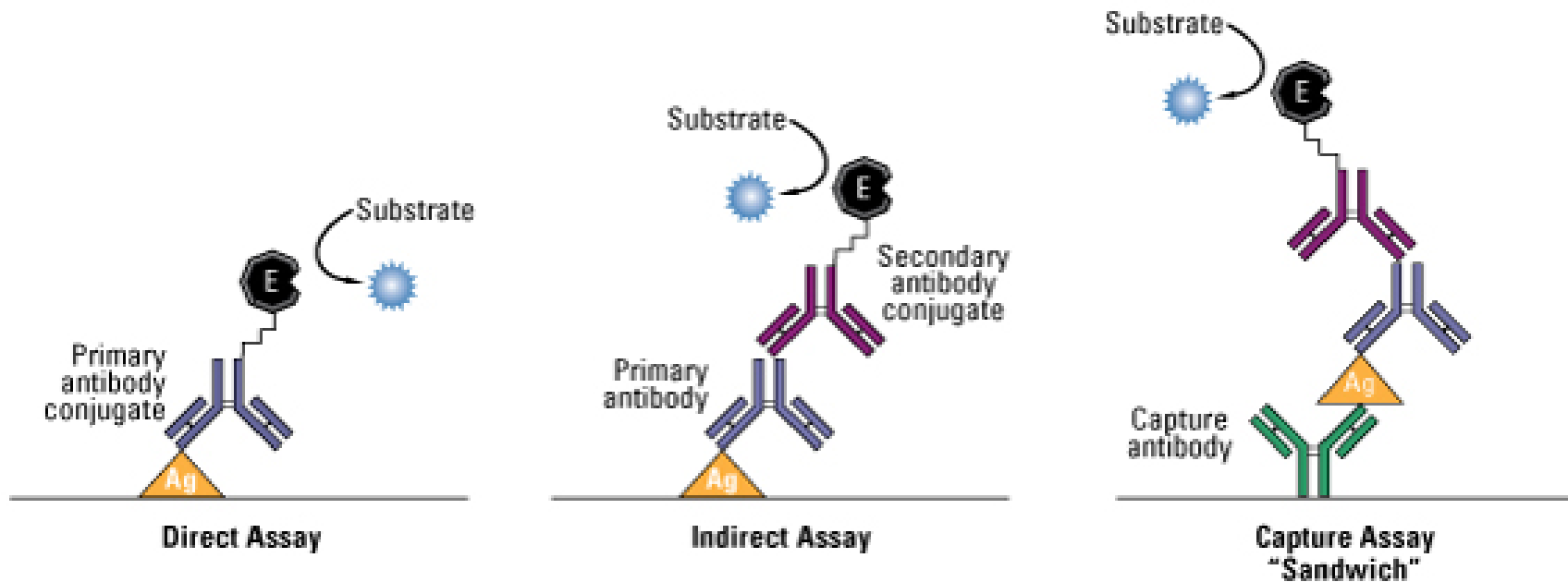


- Applications
 - Measurement of antibodies to soluble antigens

Direct Coombs Test



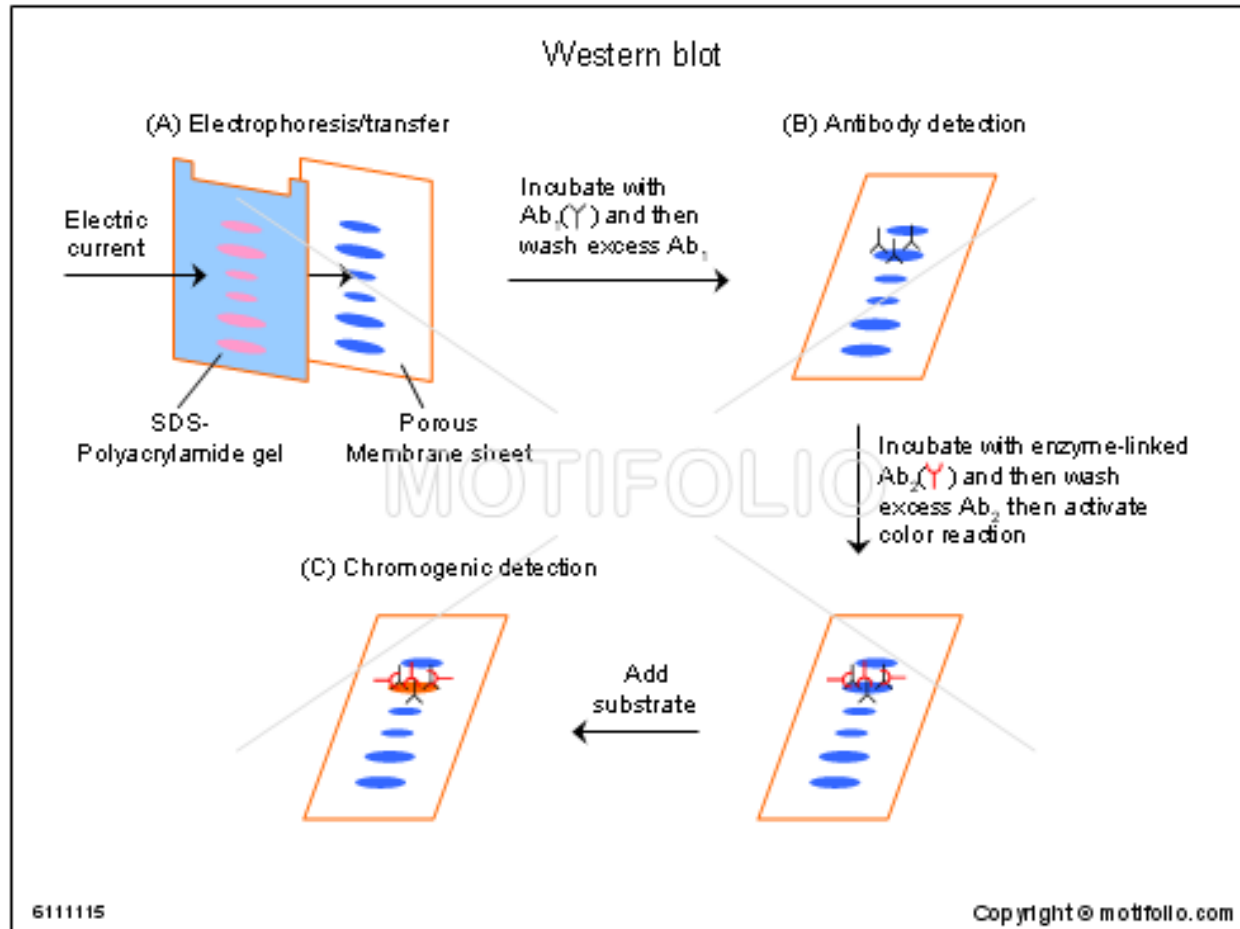
ELISA Principles



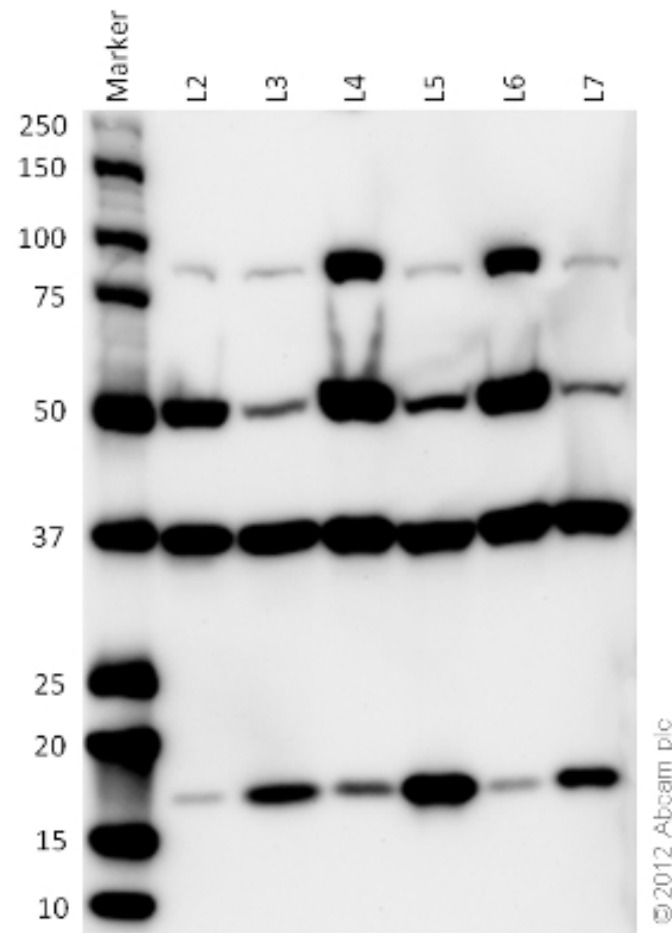
Some Points About ELISAs

- Understand O.D.s (optical densities)
- How to make ELISAs quantitative
- Issues of Specificity and Stickiness

Western Blot



Typical Western Blot



Uses of Western Blots

- For determining antibody specificity when the antigen is complex
- WBs are at best semi-quantitative
- Some antibodies “don’t blot”
- Need for probity in data presentation

Assays of Cellular Immunology

- DTH – “red bump in the skin”
- Most cellular assays depend on density separation of “PBM” – peripheral blood mononuclear cells

This Week's Citation Classic

CC NUMBER 45
NOVEMBER 8, 1982

Bøyum A. Isolation of mononuclear cells and granulocytes from human blood. *Scand. J. Clin. Lab. Invest.* 21(Suppl. 97):77-89, 1968.
[Norwegian Defence Research Establishment, Division for Toxicology, Kjeller, Norway]

A technique for isolation of lymphocytes is described. Blood is layered over a fluid with density of 1.077 g/ml. After centrifugation, red cells and granulocytes have formed a sediment at the bottom, and lymphocytes and monocytes are easily collected from the interface between plasma and the separation fluid. [The SCI® indicates that this paper has been cited in over 5,850 publications since 1968.]

Arne Bøyum
Division for Toxicology
Norwegian Defence
Research Establishment
N-2007 Kjeller
Norway

October 6, 1982

"When the work started in 1961, the goal was to isolate bone marrow lymphocytes for studies of immune reactions following bone marrow transplantation. Fortunately, I was happily unaware of the obstacles ahead, and unfortunately, ignorant of Newton's law of motion. I figured that, with the appropriate gradient design, the slowly sedimenting lymphocytes could easily be picked up after centrifugation. Today, it is with understanding rather than self-irony that I reread in my proposed research protocol that the work would last one to two years. It took six years and I sure remember those hours at the microscope.

"The first two-year period was one of striking discrepancy between effort and progress. No matter how fancy the gradient design, the lymphocytes never behaved as predicted. I even construct-

ed my own monstrous centrifuge. It never worked, and it is still there in the attic to remind me of my scientific infancy. With high polymer compounds as gradient material, the problem was that density and viscosity could not be varied independently. It occurred to me that this difficulty could be overcome using a mixture of two compounds. This turned out to be the first breakthrough. The choice of an X-ray contrast medium to adjust the density was a lucky one. Next, I ended up with different sugar polymers as partners for viscosity control. Moreover, for simplification, I switched from bone marrow to blood.

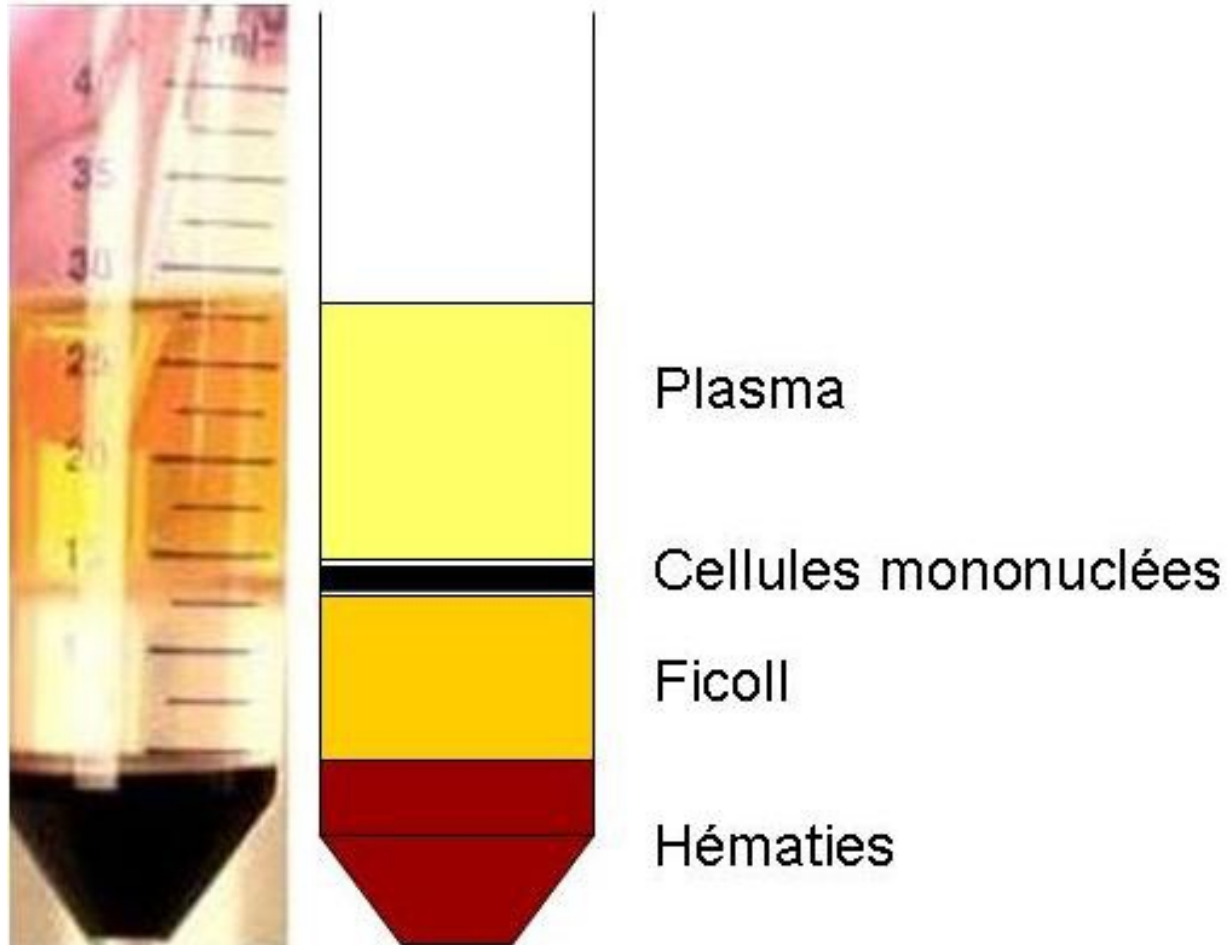
"A small episode in the lab radically changed the further work. On one occasion, when the gradient was already loaded and the centrifuge being used, while having to wait for a few minutes, I noted that the red cells started to aggregate at the interface, and fell rapidly to the bottom. So, I left the tube on the desk to see what finally happened. This is where a long study of sedimentation in a 1 g gravity field started. (My colleague who used the centrifuge still complains he never got the credit he deserved for his contribution.) I tested out every possible variable, and gradually learned something about the physicochemical mechanisms in a 1 g sedimentation process. This knowledge was then applied to centrifugal techniques. After a total of 3½ years I was able to obtain a pure suspension of mononuclear blood cells, but it took another year to perfect the technique. Altogether, it was a matter of finding the right density and composition of the separation fluid, and a suitable cell concentration. The technique is generally applicable to blood lymphocyte isolation.^{1,2} This paper has been highly cited because it has the advantage of being a simple one-step procedure."

Cited ~14,000 times!

1. Bøyum A. Separation of blood leucocytes, granulocytes and lymphocytes. *Tissue Antigens*. 4:269-74, 1974.

2. ———, Isolation of lymphocytes, granulocytes and macrophages. *Scand. J. Immunol. S(Suppl. 5):9-15, 1976.*

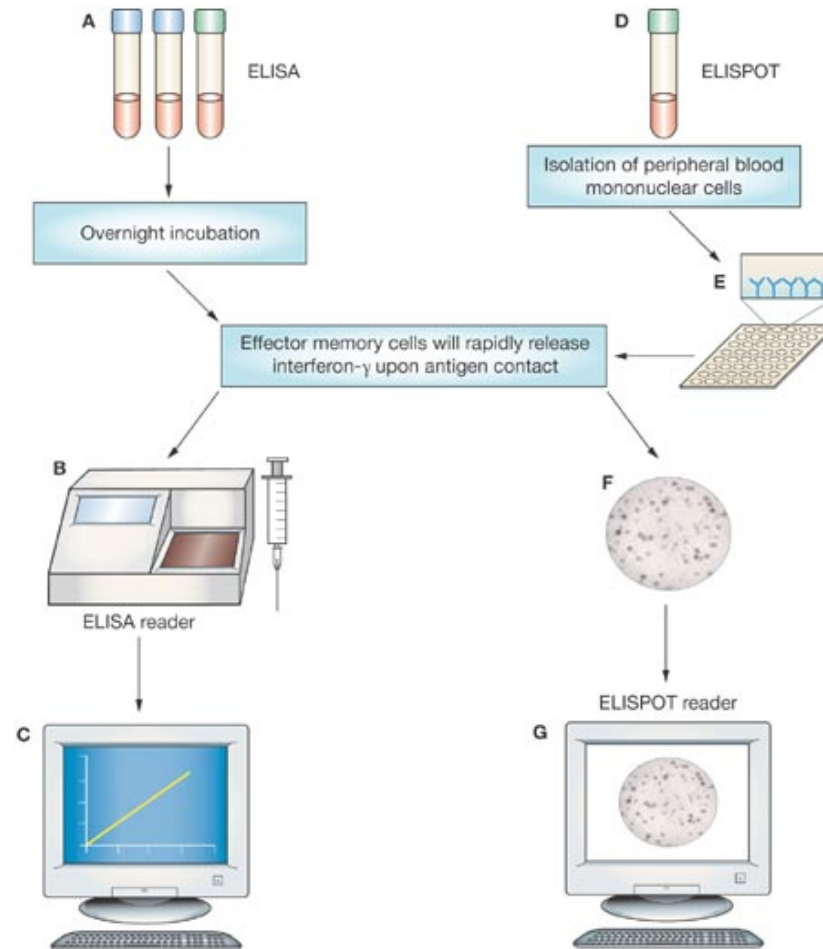
Ficoll Hypaque Separation of PBM



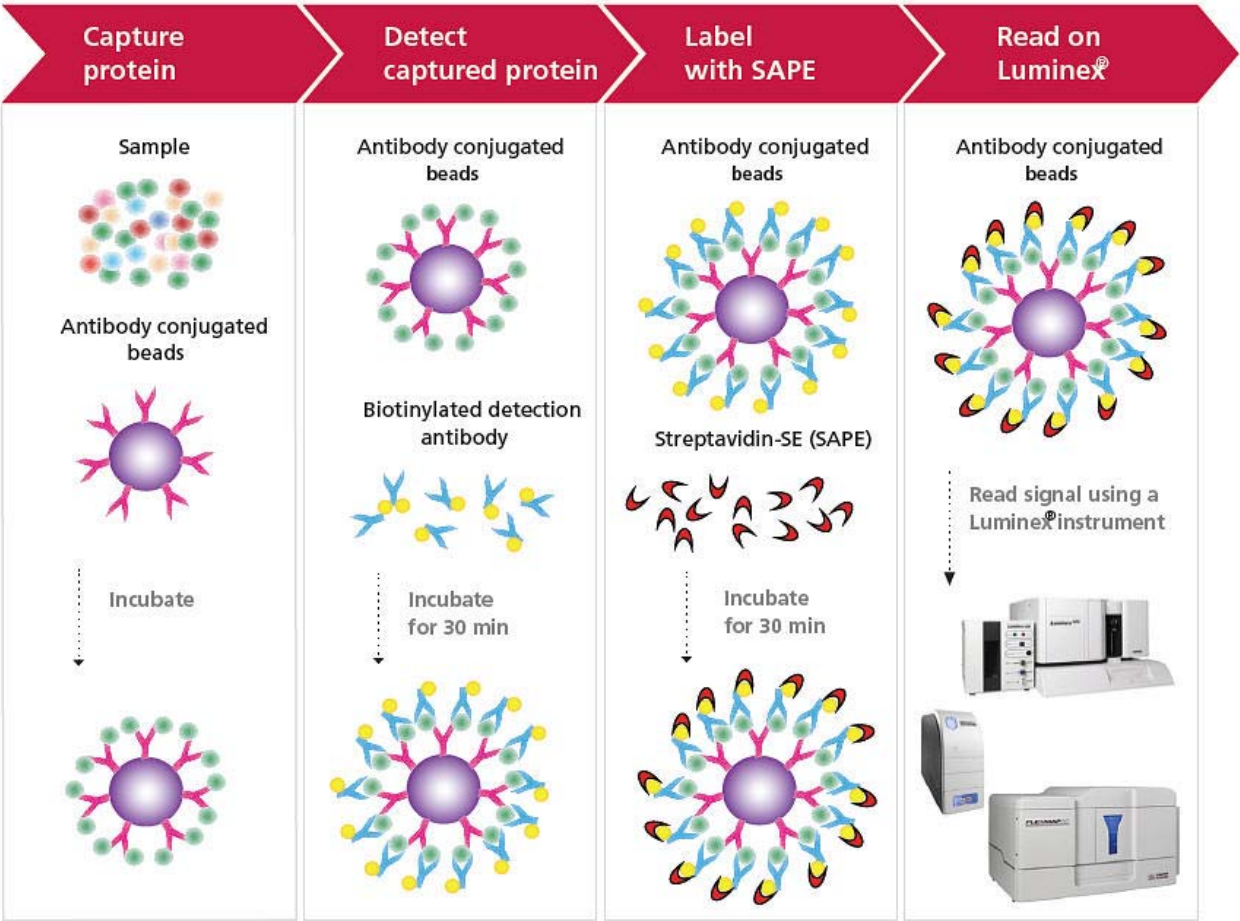
Using Isolated PBM to Study Immunity

- Counting cells via flow cytometry
- T-cell functional assays: antigen, aCD3, or mitogen-induced proliferation (CFSE preferred these days, formerly 3H thymidine incorporation into DNA)
- Cytotoxicity
- Cytokine production
- Cell mixing and culture expts.

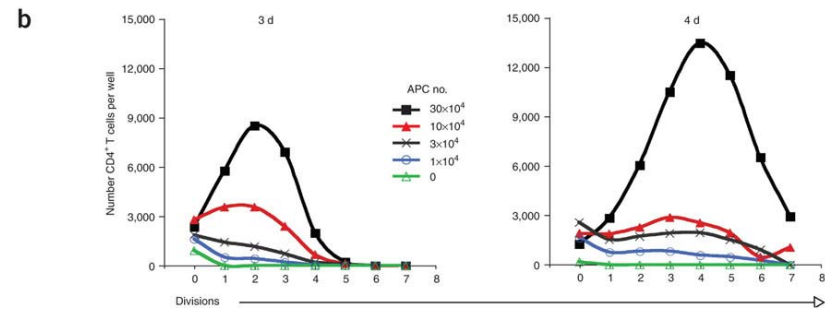
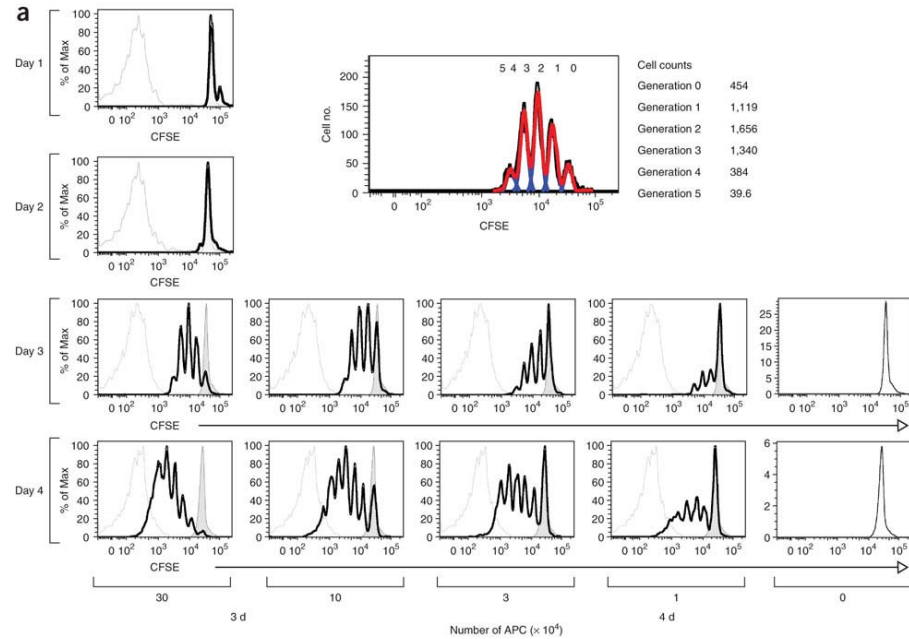
Quantiferon Gold – a Cytokine Assay



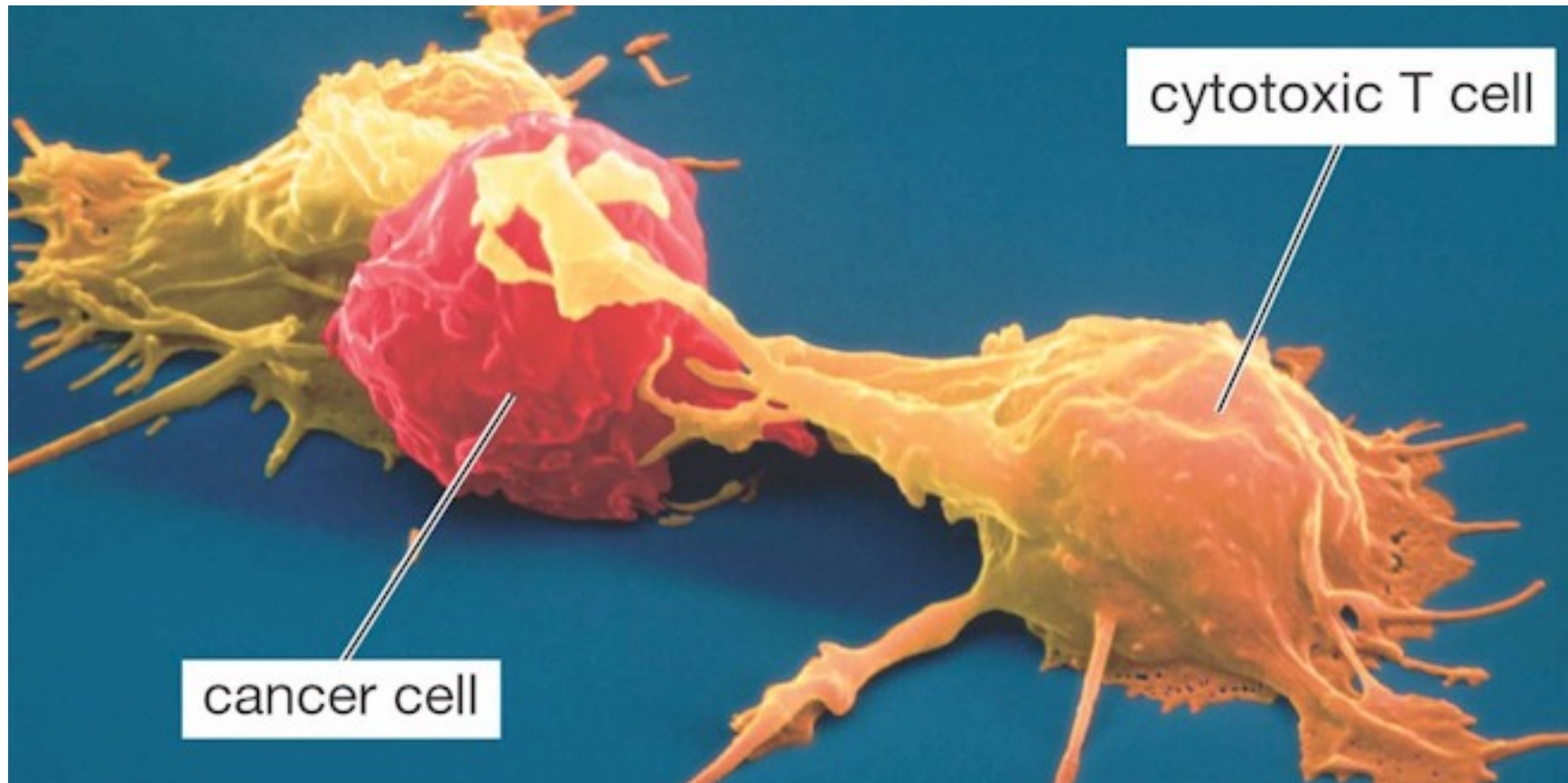
A Multiplex Cytokine Assay



Proliferation Measured by CFSE Dilution



Cytotoxic T Cells Destroying a Cancer Cell



Transcription Factors and T-cell Subsets

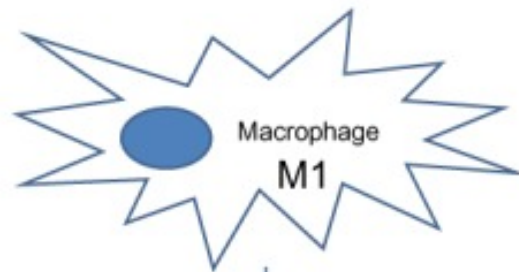
- FoxP3 for T regs, CD25+
- TH1 (T bet)
- TH2 (GATA 3)
- TH17 (RoR gamma T)

Macrophage Subsets

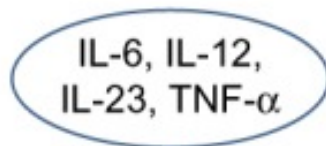
- Macrophages differentiate into functional subsets that can be recognized by surface markers and by cytokine profiles
- Originally M1 and M2, getting more complex

Macrophages Differentiate into Distinct Phenotypes

Classical activated macrophage



↑ production of inflammatory cytokines

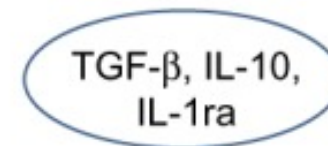


RA, Behcet's disease,
early stage of MS
gout, OA

Alternatively activated macrophage



↓ production of inflammatory cytokines
↑ tissue remodeling, angiogenesis, and wound repair



SpA,
late stage of MS
systemic sclerosis

Techniques to Study the Immune System Itself

- Is the patient immunodeficient?
- Does the patient have a malignancy of the immune system?
- Is the immune system causing injury?

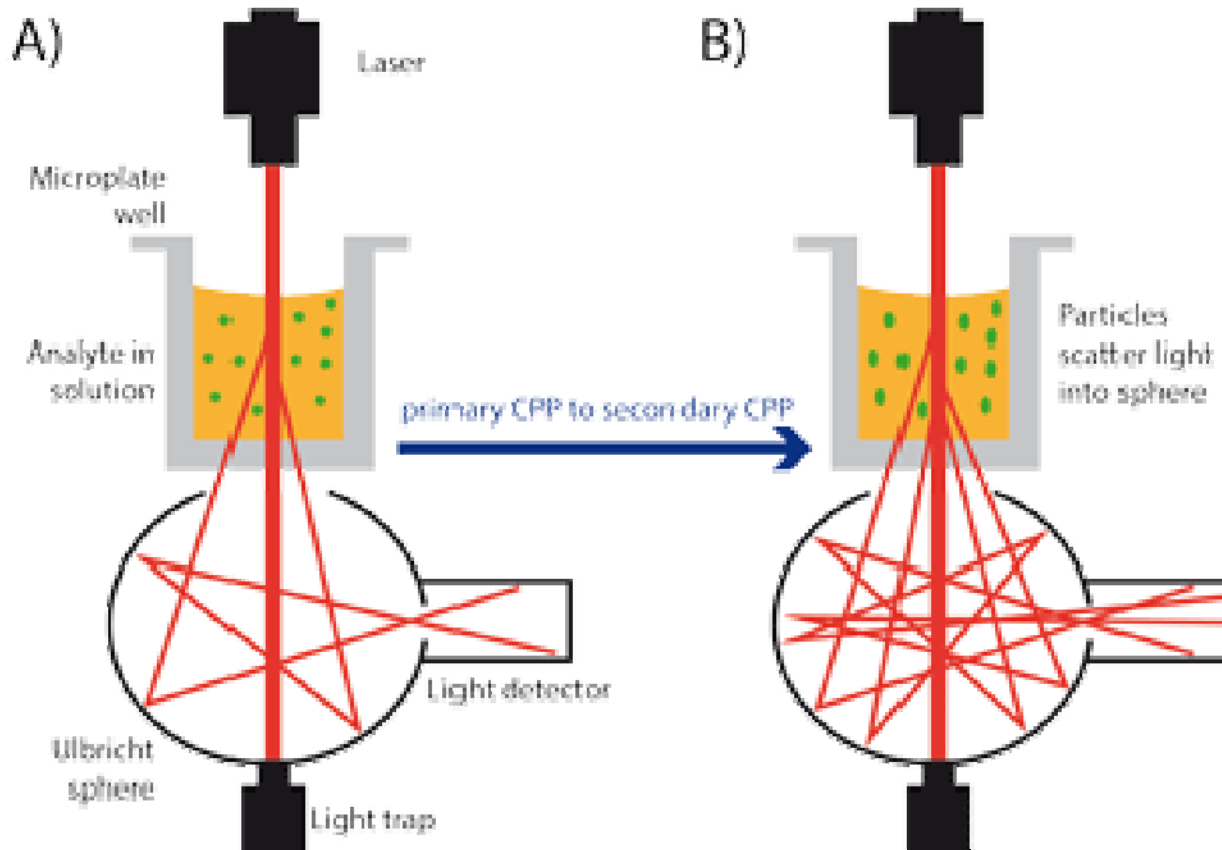
Assessing Humoral Immunity

- GLOBULIN levels (total protein minus albumin, or reported as globulin). Poor man's test
- IgG and subclasses 1-4
- IgA
- IgM
- Isoagglutinins
- Response to vaccination – pneumococcal, meningococcus

Complement Assessment

- C3, C4
- When to look at CH50?

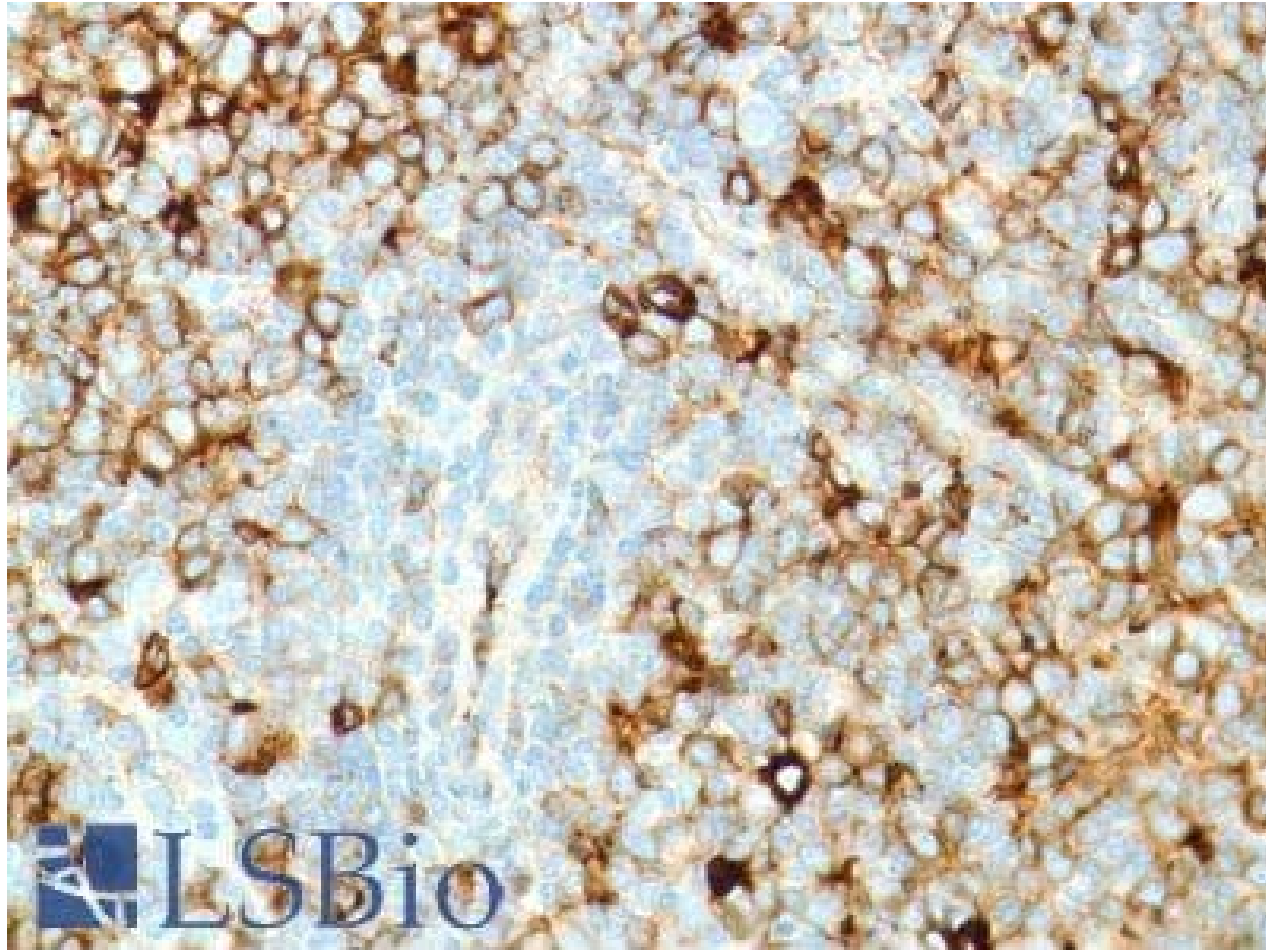
Principle of Nephelometry



Is it Cancer?

- Individual immunoglobulin molecules have either kappa or lambda light chains (about 60:40)
- In lymphoid tissues, cellular expression of kappa and lambda is likewise mixed
- So if lymphoid tissue has infiltrates of cells bearing (by IF or IHC) kappa or lambda only, the cells must be monoclonal and probably lymphoma
- Similar situation for T-cell receptor V genes

Lymphoma Stained with anti Kappa



The End

