

Midterm

Extra Office Hours

Take

Regular Office Hours: Tuesdays 11-12  
Extra office hours: Wed, Feb 7 12-1pm  
Thurs, Feb 8 11am-12  
Fri, Feb 9 2-4pm

I WILL NOT BE HOLDING OFFICE HOURS ON TUESDAY Feb 13!!

Dina, Tim, and I encourage all confused students to come to our office hours and discussion sections so we can try to help un-confuse you.

No class on Tuesday Feb 13.

First midterm: Thurs Feb 15 at 6pm in 155 Dwinelle (not 2050 VLSB as listed in the original schedule).

Midterm will focus on material covered in lectures and will be designed to be taken in 90 min. (We have the room till 8pm.)

The CSIs will conduct a review session in our regular class period on Thursday Feb 15.

**TABLE 6-3 Sensitivity of various immunoassays**

Assay	Sensitivity* ( $\mu\text{g antibody/ml}$ )
Precipitation reaction in fluids	20-200
Precipitation reactions in gels	
Mancini radial immunodiffusion	10-50
Ouchterlony double immunodiffusion	20-200
Immunoelectrophoresis	20-200
Rocket electrophoresis	2
Agglutination reactions	
Direct	0.3
Passive agglutination	0.006-0.06
Agglutination inhibition	0.006-0.06
Radioimmunoassay (RIA)	0.0006-0.006
Enzyme-linked immunosorbent assay (ELISA)	$\sim 0.0001-0.01$
ELISA using chemiluminescence	$\sim 0.00001-0.01^\dagger$
Immunofluorescence	1.0
Flow cytometry	0.006-0.06

\*The sensitivity depends on the affinity of the antibody used for the assay as well as the epitope density and distribution on the antigen.  
†Note that the sensitivity of chemiluminescence-based ELISA assays can be made to match that of RIA.  
SOURCE: Updated and adapted from N. R. Rose et al., eds., 1997, *Manual of Clinical Laboratory Immunology*, 5th ed., American Society for Microbiology, Washington, DC.

The extraordinary specificity of antibodies for their antigens, and the ability to generate polyclonal and monoclonal antibodies to virtually anything, makes them fantastically useful reagents for detecting and quantitating substances.

A large number of different assays have been developed to detect antigens based on antibody binding that can be used in fluids, tissues, or cells.

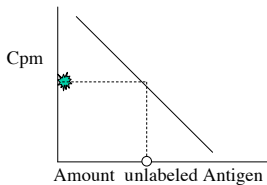
## Immunological Techniques

Monoclonal Antibodies

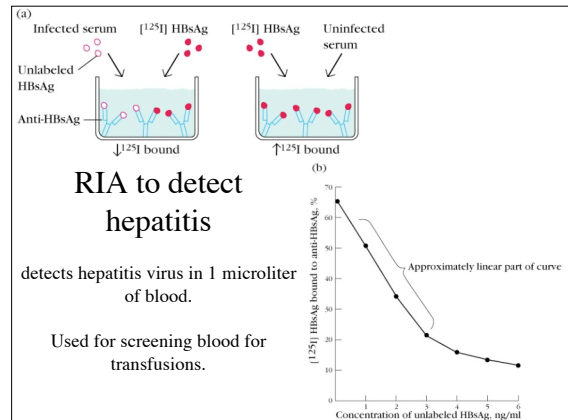
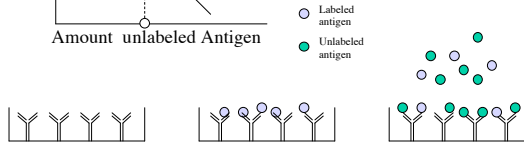
Radioimmune Assay (RIA)  
Enzyme Linked Immune Sorbant Assay (ELISA)  
Western blot  
Immunoprecipitation  
Immunofluorescence

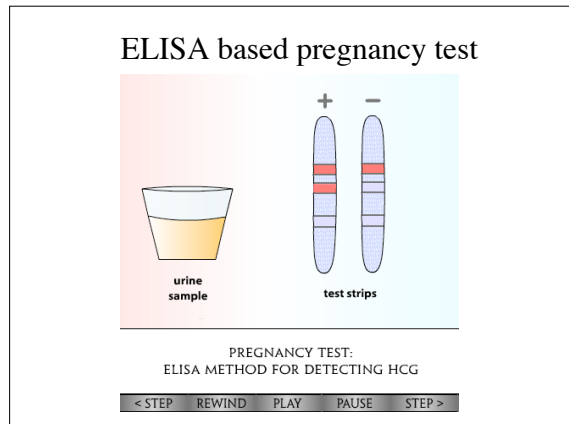
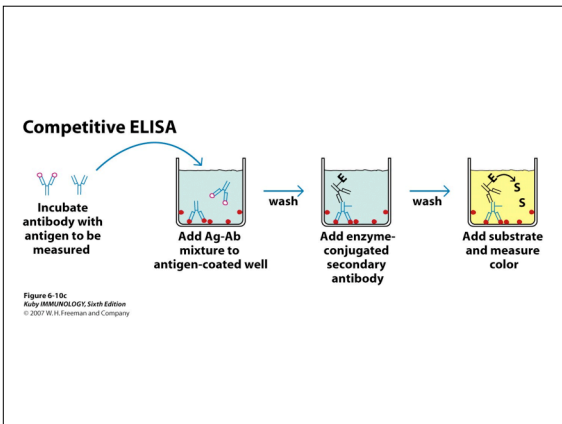
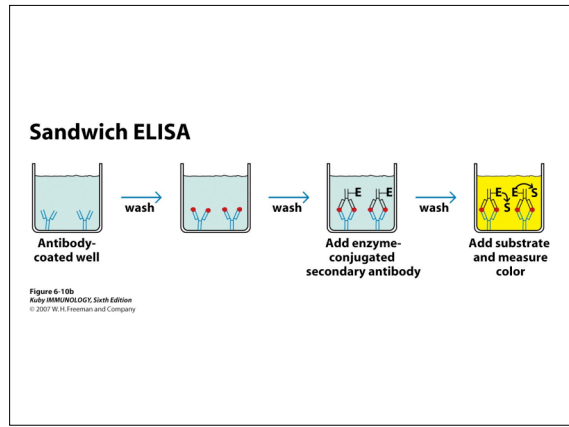
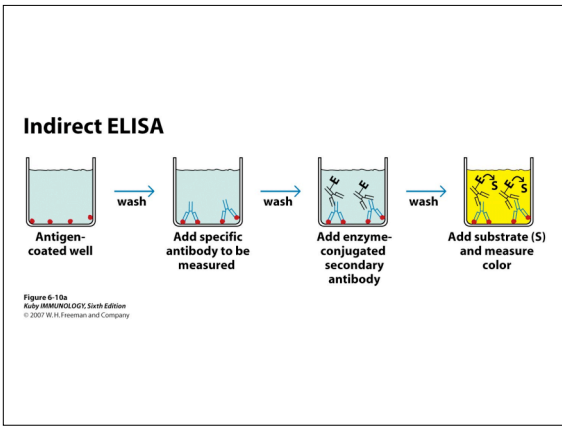
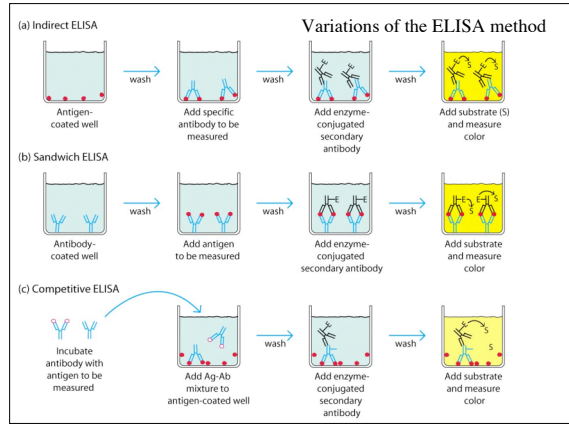
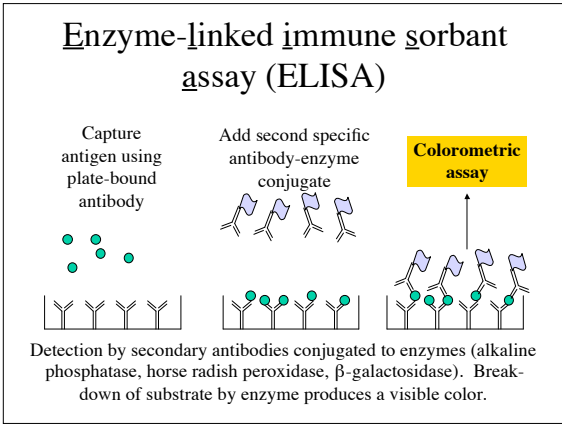
Flow cytometry  
Expression cloning

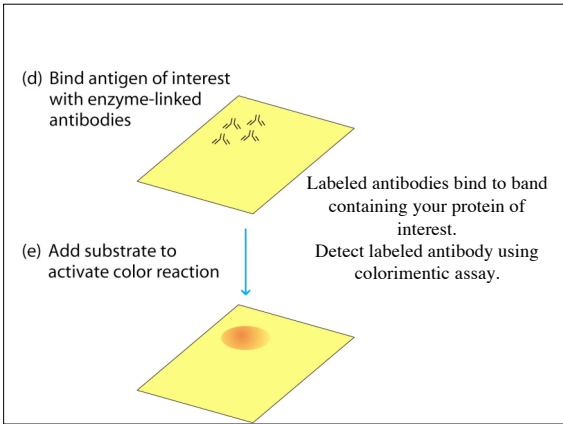
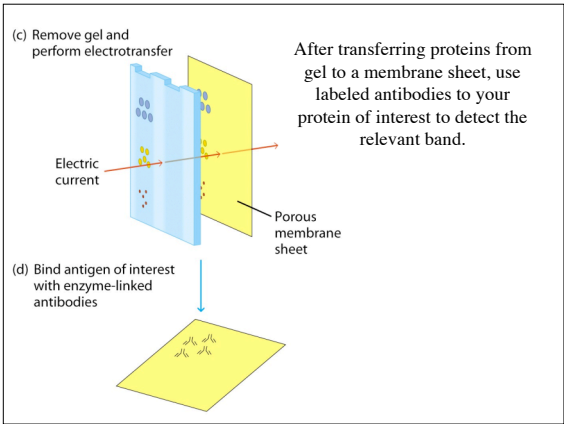
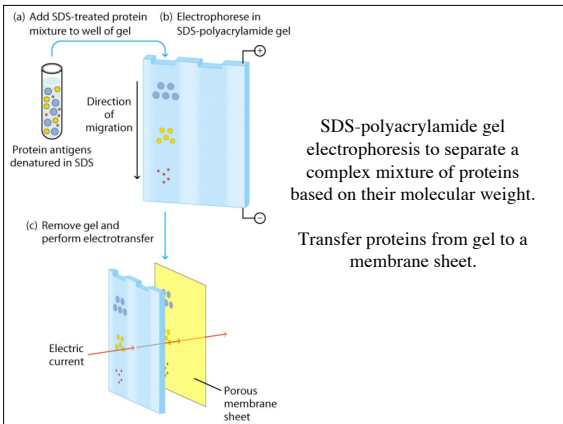
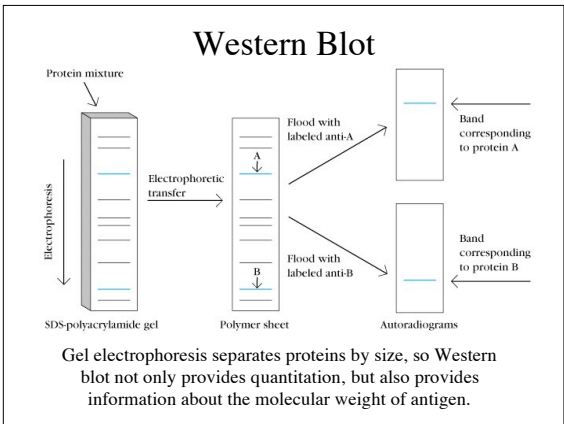
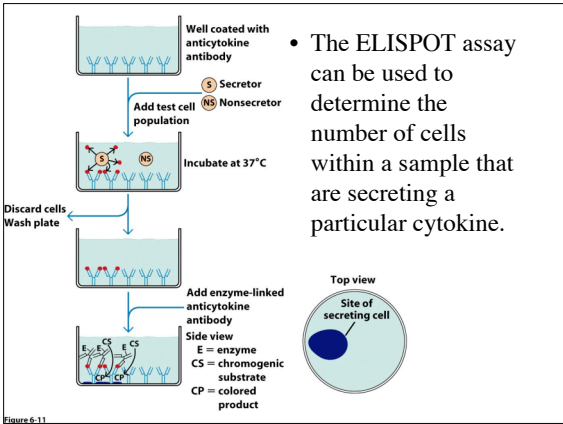
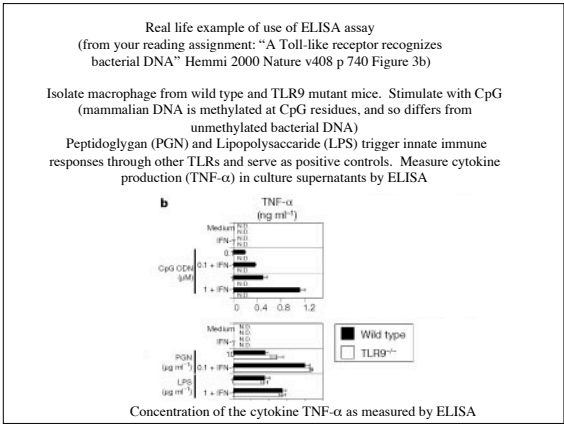
## Radioimmunoassay (RIA)

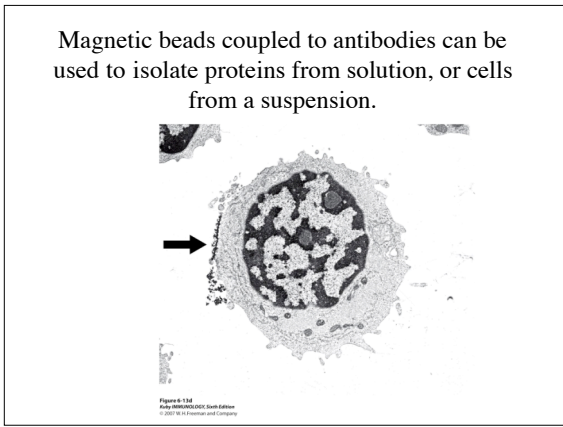
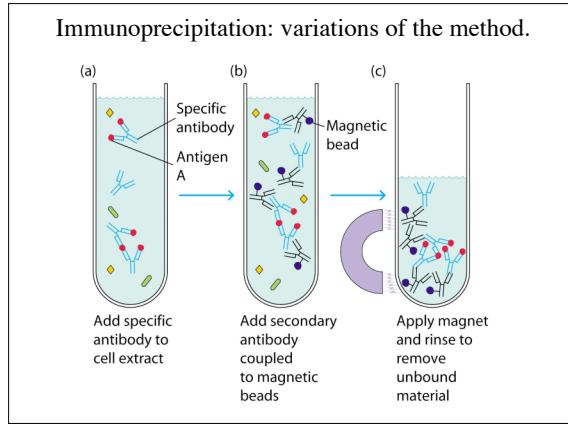
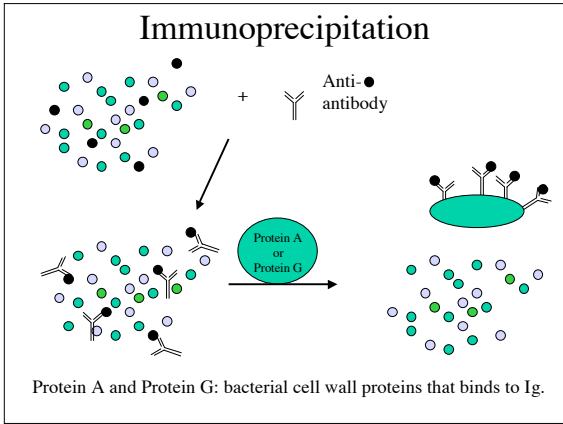


- 1960 Yalow and Berson (Nobel Prize)
- Very sensitive: can detect material present at concentrations of  $<0.001$  micrograms/ml.
- Takes advantage of protein binding to plastic of tissue culture dish.
- Generate standard curve with known amounts of unlabeled antigen
- Measure unknown using standard curve.



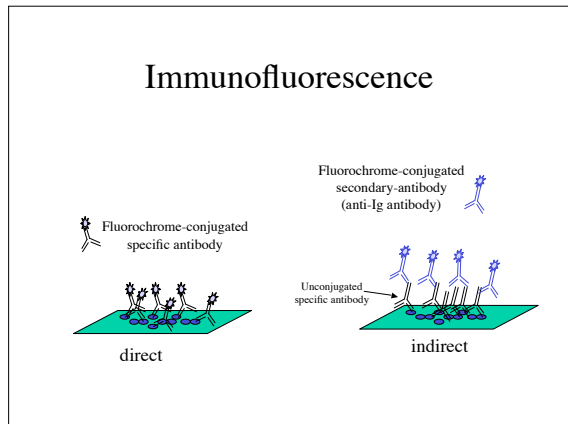
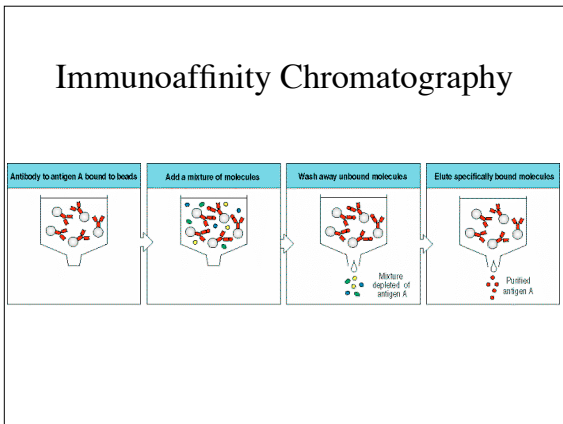




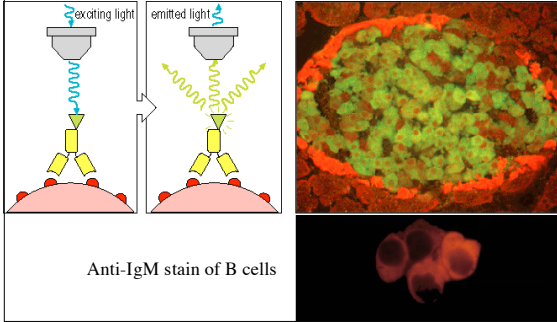


And check out “A Toll-like receptor recognizes bacterial DNA” Hemmi 2000 Nature v408 p 740  
Figure 3 f and g

For real-life examples of Immunoprecipitations and Western blots to analyze TLR signaling intermediates in wild type and TLR9- macrophage in response to different TLR agonists.



Immunofluorescence can provide spatial information about cells or molecules that react with antibodies.



Excitation and emission wavelengths of some commonly used fluorochromes		
Probe	Excitation (nm)	Emission (nm)
R-phycoerythrin (PE)	480; 565	578
Fluorescein	495	519
PerCP	490	675
Texas Red	589	615
Rhodamine	550	573

Figure A-16 Immunobiology, 6/e. © Garland Science 2005

## Immunological Techniques

Monoclonal Antibodies

Radioimmune Assay (RIA)

Enzyme Linked Immune Sorbant Assay (ELISA)

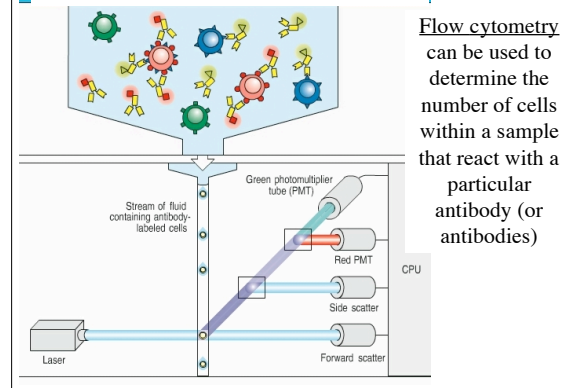
Western blot

Immunoprecipitation

**Flow cytometry**

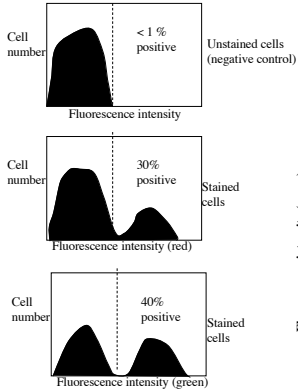
Expression cloning

Mixture of cells labeled with fluorescent antibodies



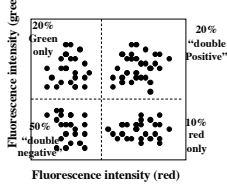
Flow cytometry can be used to determine the number of cells within a sample that react with a particular antibody (or antibodies)

1 parameter histograms:

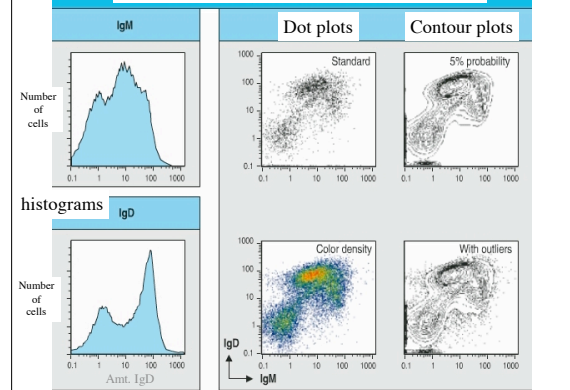


**Flow cytometric analysis of cells stained with 2 different labeled antibodies**

2 parameter dot plot:



Analysis of cells stained with labeled antibodies



## The Power of Flow Cytometry

### Quantitative:

Accurately determine relative fluorescent levels (protein levels) on individual cells.  
Accurately determine the number of fluorescent cells within a population.

### Sensitive:

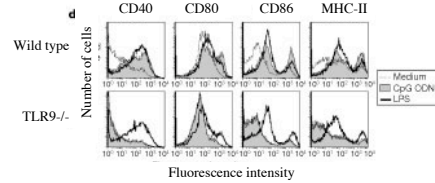
Analysis can be performed with  $<10^4$  cells.

### Flexible:

Fluorescent labeled antibodies specific for many cell surface proteins are readily available. Can simultaneously stain for  $>4$  markers.

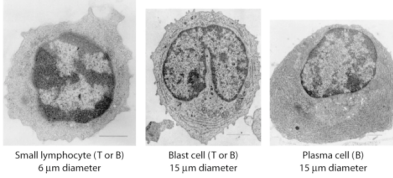
Real life example of use of flow cytometry  
(from your reading assignment: "A Toll-like receptor recognizes bacterial DNA" Hemmi 2000 Nature v408 p 740 Figure 3d)

Isolate macrophage from wild type and TLR9 mutant mice. Stimulate with CpG (or LPS as positive control). Examine expression of activation markers by flow cytometry.

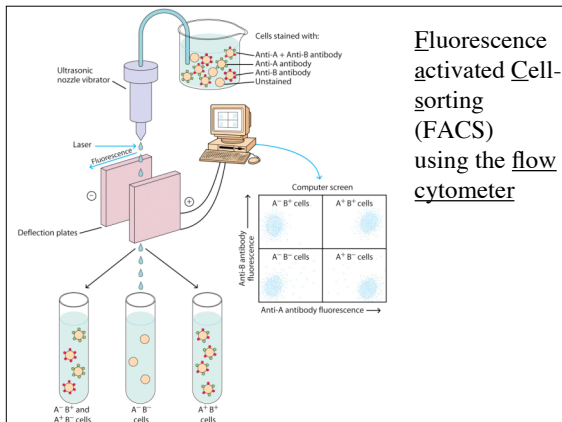
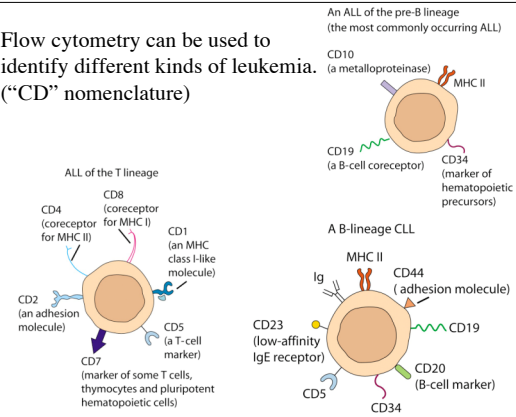


Note: CD40, CD90, CD86, and MHC-II are all important for T cell activation by macrophage (innate immunity stimulating adaptive immunity).

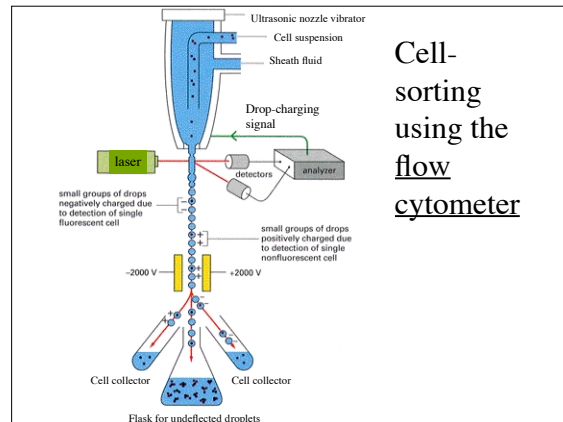
The generation of monoclonal antibodies specific for cell surface proteins, coupled with flow cytometry, provides a powerful tool for identifying different lymphocyte populations.



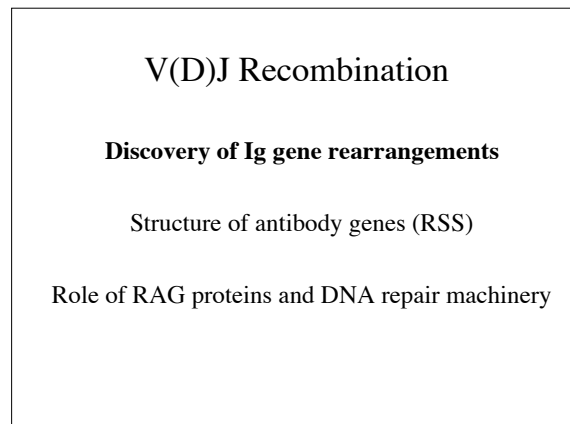
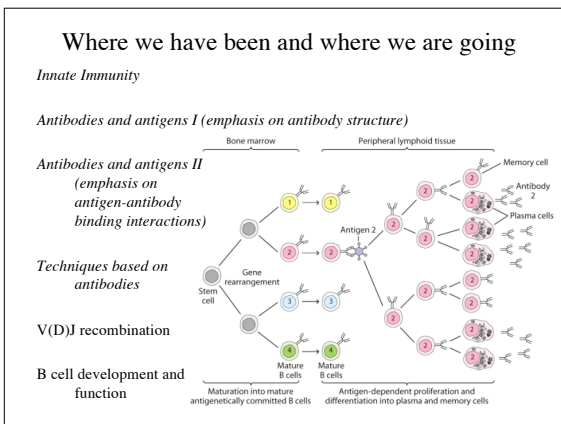
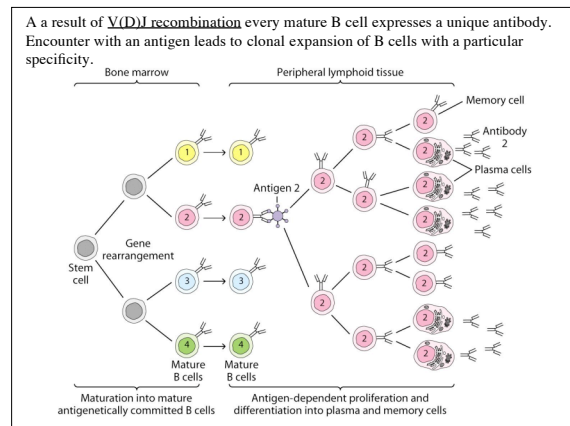
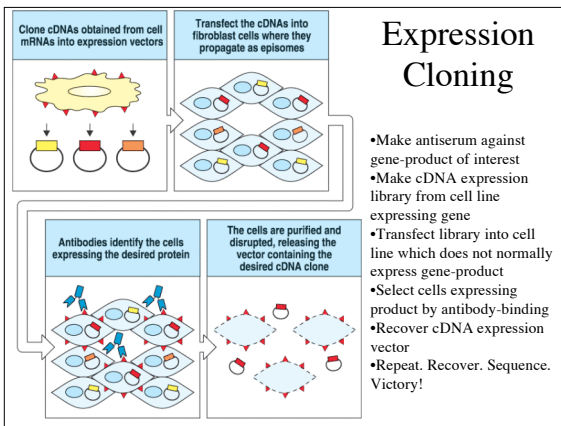
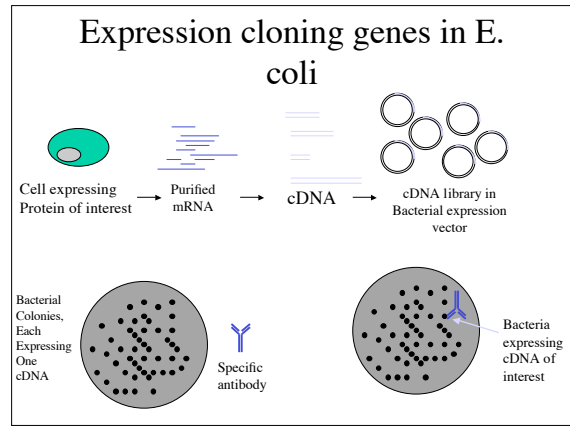
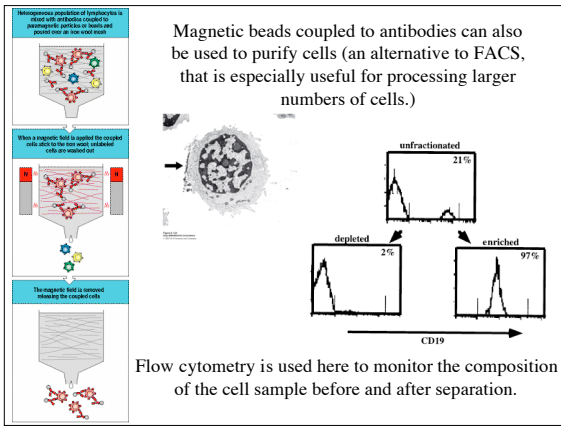
Flow cytometry can be used to identify different kinds of leukemia. ("CD" nomenclature)



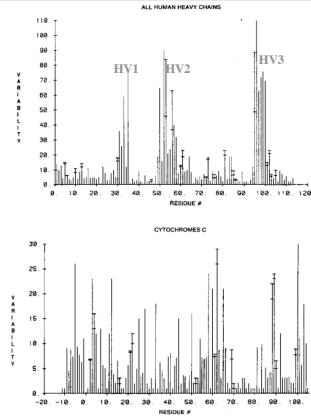
Fluorescence activated Cell-sorting (FACS) using the flow cytometer



Cell-sorting using the flow cytometer



## Variability of Ig Sequences



## The puzzle of antibody diversity

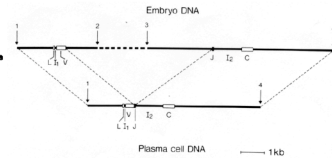
- Limitless array of Ig sequences (too large to be encoded in genome)
- Variation limited to V domain.
- Identical V segment could be associated with two different C regions (myeloma protein with  $\gamma$  and  $\mu$  chains)
- Germ-line vs somatic variation models
- Dreyer and Bennett (1965) the 2 gene model; a violation of the "one gene, one polypeptide" rule
- 1976: the emerging tools of molecular biology open the way for the breakthrough...

## The breakthrough paper

Cell, Vol. 15, 1-14, September 1978, Copyright © 1978 by MIT

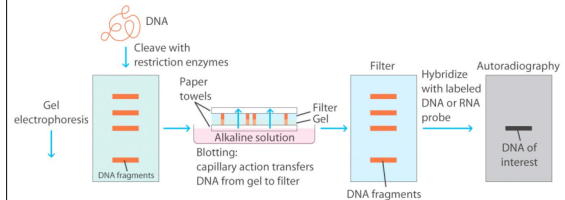
### A Complete Immunoglobulin Gene Is Created by Somatic Recombination

Christine Brack, Minoru Hiraoka,  
Rita Lenhard-Schuller and Susumu Tonegawa  
Basel Institute for Immunology  
Grenzacherstrasse 487  
Postfach 4000 Basel 5  
Switzerland

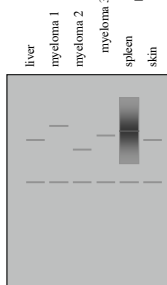


## Review of Southern blot method

Restriction endonucleases cleave at specific sequences in DNA and can be used to generate a physical map of DNA.  
(e.g. EcoRI cleaves at the sequence: 5'-GAATTC-3')



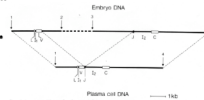
## Surprising Southern blot



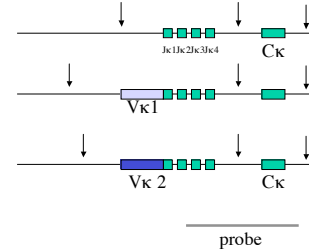
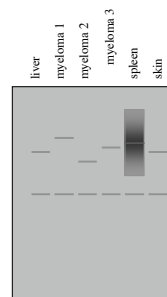
Cell, Vol. 15, 1-14, September 1978, Copyright © 1978 by MIT

### A Complete Immunoglobulin Gene Is Created by Somatic Recombination

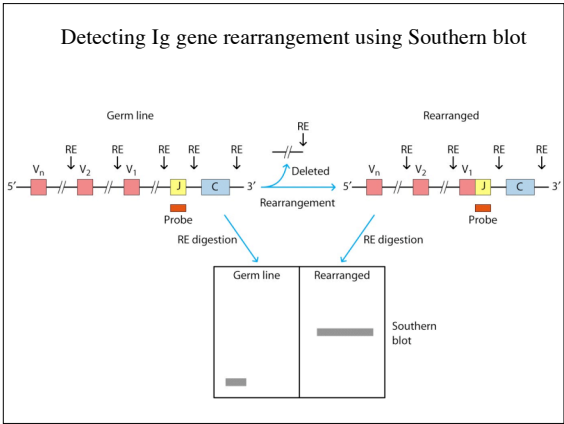
Christine Brack, Minoru Hiraoka,  
Rita Lenhard-Schuller and Susumu Tonegawa  
Basel Institute for Immunology  
Grenzacherstrasse 487  
Postfach 4000 Basel 5  
Switzerland



## Surprising Southern blot

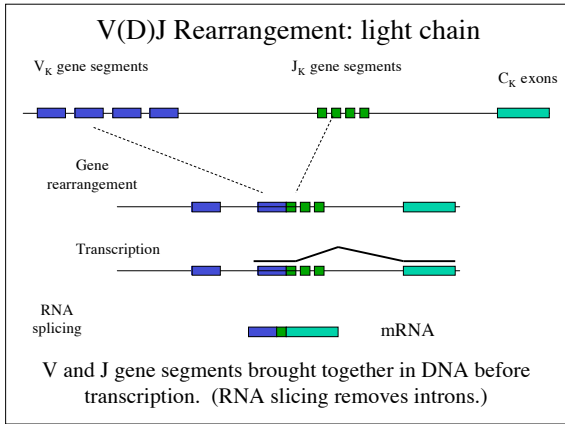
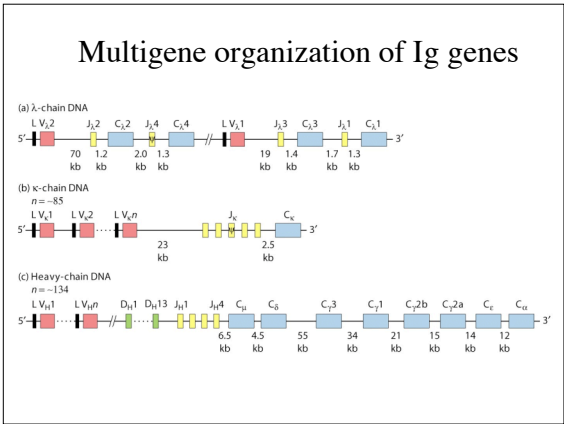
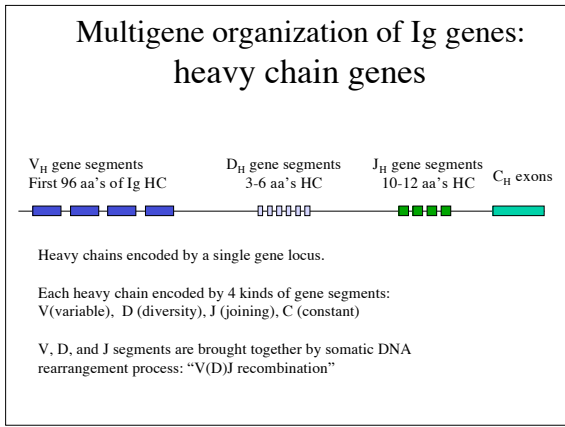
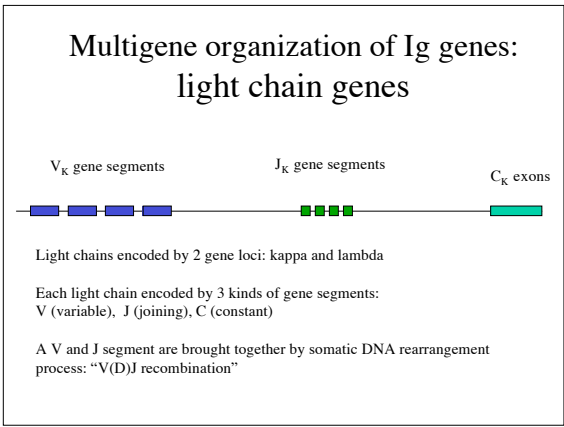


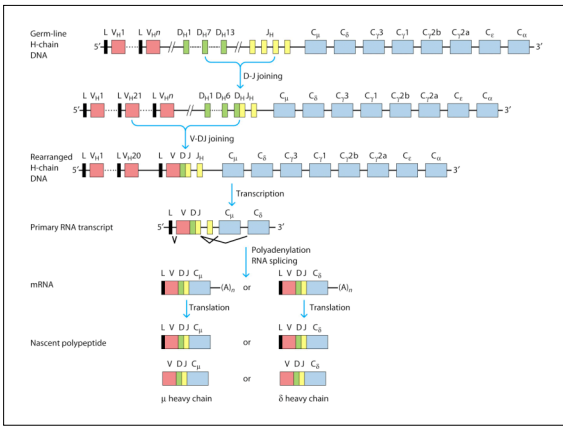
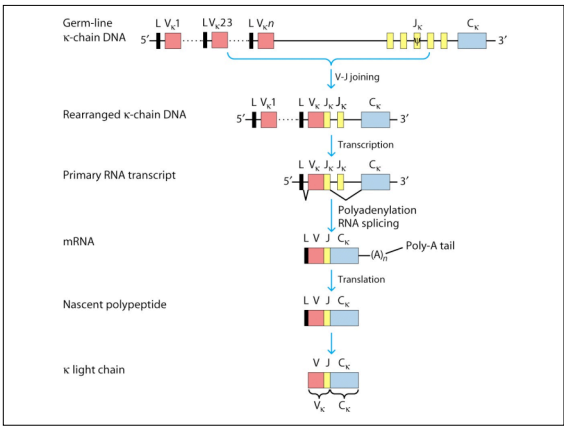
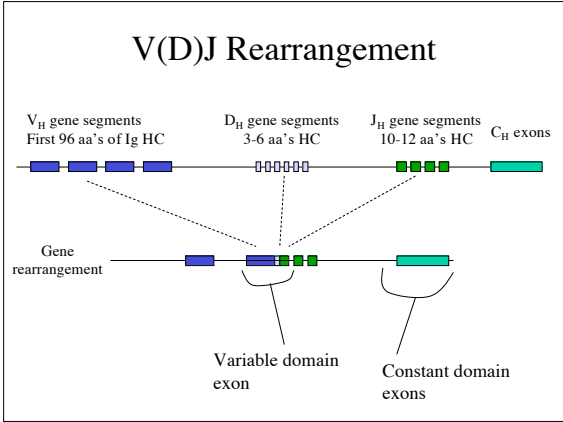
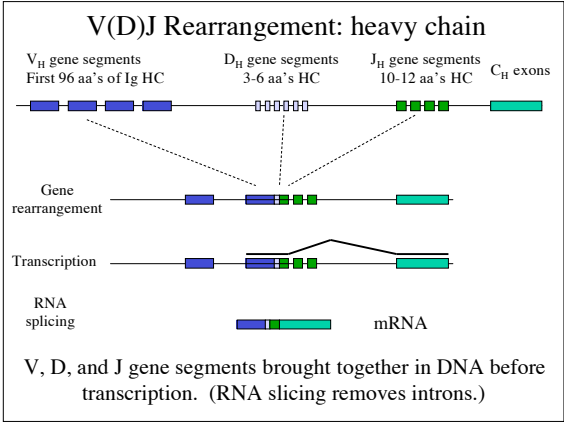




## V(D)J Recombination

Discovery of Ig gene rearrangements  
**Structure of antibody genes (RSS)**  
 Evidence for role of RAG proteins and DNA repair machinery



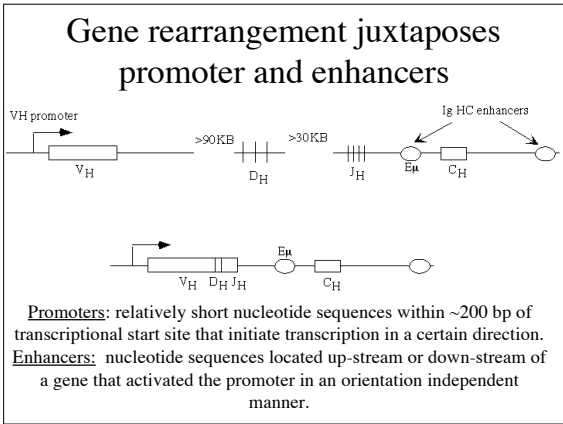


### Combinatorial Diversity in humans

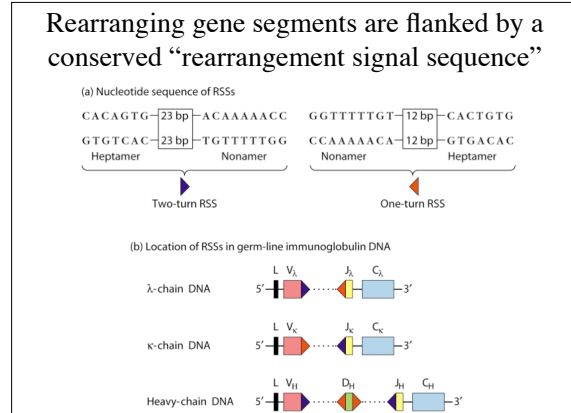
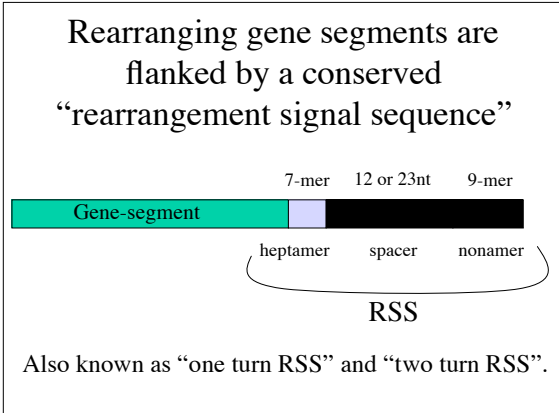
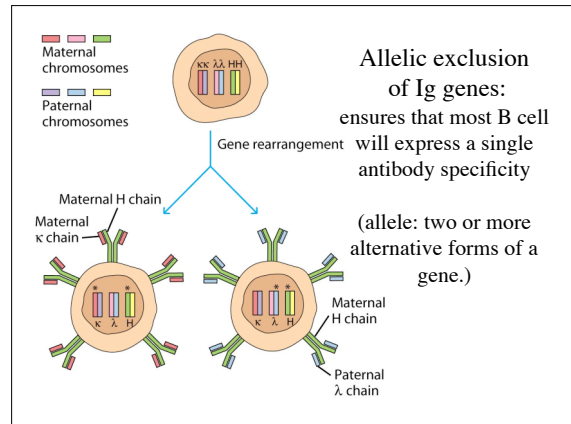
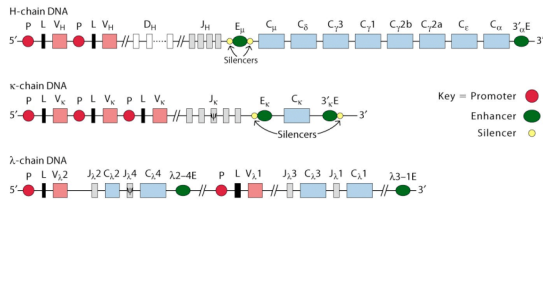
TABLE 5-2 Combinatorial antibody diversity in humans and mice

Multiple germ-line segments	Heavy chain	LIGHT CHAINS	
		κ	λ
ESTIMATED NUMBER OF SEGMENTS IN HUMANS*			
V	48	41	34
D	23	0	0
J	6	5	5
Combinatorial V-D-J and V-J joining (possible number of combinations)	$48 \times 23 \times 6 = 6624$	$41 \times 5 = 205$	$34 \times 5 = 170$
Possible combinatorial associations of heavy and light chains†	$6624 \times (205 + 170) = 2.48 \times 10^6$		+

Junctional diversity (flexible joining of segments, P and N region additions at junctions) also contributes substantially to the total diversity of antibodies.



Ig promoters are actively transcribed when they are brought close to enhancers due to gene rearrangement.



The 12/23 Rule

Only gene segments flanked by RSSs with dissimilar spacers can undergo V(D)J recombination with one another.

Ensures that V segments don't join with other Vs, that V<sub>H</sub> don't join with J<sub>H</sub>, etc.

