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

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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.

**Radioimmunoassay (RIA)**

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

### Table 4.1: Sensitivity of various immunossays

<table>
<thead>
<tr>
<th>Assay</th>
<th>Sensitivity (lg antibody/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Precipitation reaction in fluids</td>
<td>20-200</td>
</tr>
<tr>
<td>Precipitation reaction in gels</td>
<td>10-100</td>
</tr>
<tr>
<td>Marine radial immunodiffusion</td>
<td>10-200</td>
</tr>
<tr>
<td>Enzyme-linked immune sorbent assay (ELISA)</td>
<td>1&lt;sup&gt;-10&lt;/sup&gt; - 1&lt;sup&gt;-9&lt;/sup&gt;</td>
</tr>
<tr>
<td>Enzyme-linked immunosorbent assay (ELISA)</td>
<td>1&lt;sup&gt;-9&lt;/sup&gt; - 1&lt;sup&gt;-8&lt;/sup&gt;</td>
</tr>
<tr>
<td>Immunofluorescence</td>
<td>1.0</td>
</tr>
<tr>
<td>Enzyme immunoassay</td>
<td>0.001 - 0.005</td>
</tr>
<tr>
<td>Enzyme linked immunosorbent assay (ELISA)</td>
<td>0.0001 - 0.005</td>
</tr>
</tbody>
</table>

*Note: sensitivity depends on the affinity of the antibody used for the assay as well as the antibody density and distribution on the substrate.

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**Immunological Techniques**

- **Monoclonal Antibodies**
- **Radioimmune Assay (RIA)**
- **Enzyme Linked Immune Sorbant Assay (ELISA)**
- **Western blot**
- **Immunoprecipitation**
- **Immunofluorescence**
- **Flow cytometry**
- **Expression cloning**

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**Radioimmunoassay (RIA)**

- Detects hepatitis virus in 1 microliter of blood.
- Used for screening blood for transfusions.
Enzyme-linked immune sorbant assay (ELISA)

Capture antigen using plate-bound antibody
Add second specific antibody-enzyme conjugate

Colorometric assay

Detection by secondary antibodies conjugated to enzymes (alkaline phosphatase, horse radish peroxidase, β-galactosidase). Breakdown of substrate by enzyme produces a visible color.

Indirect ELISA

Antigen-coated well
Add specific antibody to be measured
Add enzyme-conjugated secondary antibody
Add substrate and measure color

Sandwich ELISA

Antigen-coated well
Add specific antibody to be measured
Add enzyme-conjugated secondary antibody
Add substrate and measure color

Competitive ELISA

Incubate antibody with antigen to be measured
Add Ag-Ab mixture to antigen-coated well
Add enzyme-conjugated secondary antibody
Add substrate and measure color

ELISA based pregnancy test

PREGNANCY TEST
ELISA METHOD FOR DETECTING HCG
Real life example of use of ELISA assay
(from your reading assignment: “A Toll-like receptor recognizes bacterial DNA” Hemmi 2000 Nature v408 p 740 Figure 3b)

Isolate macrophage from wild type and TLR9 mutant mice. Stimulate with CpG (mammalian DNA is methylated at CpG residues, and so differs from unmethylated bacterial DNA). Peptidoglycan (PGN) and Lipopolysaccaride (LPS) trigger innate immune responses through other TLRs and serve as positive controls. Measure cytokine production (TNF-α) in culture supernatants by ELISA.

![ELISA Assay Diagram]

Concentration of the cytokine TNF-α as measured by ELISA

- The ELISPOT assay can be used to determine the number of cells within a sample that are secreting a particular cytokine.

![ELISPOT Assay Diagram]

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· Western Blot

Gel electrophoresis separates proteins by size, so Western blot not only provides quantitation, but also provides information about the molecular weight of antigen.

![Western Blot Diagram]

SDS-polyacrylamide gel electrophoresis to separate a complex mixture of proteins based on their molecular weight.

 Transfer proteins from gel to a membrane sheet.

![SDS-PAGE Diagram]

Labeled antibodies bind to band containing your protein of interest. Detect labeled antibody using colorimetric assay.
Immunoprecipitation

Protein A and Protein G: bacterial cell wall proteins that binds to Ig.

Immunoprecipitation: variations of the method.

Magnetic beads coupled to antibodies can be used to isolate proteins from solution, or cells from a suspension.

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.

Immuoaffinity Chromatography

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

Anti-IgM stain of B cells

Immunological Techniques

Monoclonal Antibodies

Radioimmune Assay (RIA)

Enzyme Linked Immune Sorbant Assay (ELISA)

Western blot

Immunoprecipitation

Flow cytometry

Expression cloning

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

Mixture of cells labeled with fluorescent antibodies

Flow cytometric analysis of cells stained with 2 different labeled antibodies

1 parameter histograms:

<table>
<thead>
<tr>
<th>Cell number</th>
<th>Fluorescence intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>&lt; 1% positive</td>
<td>Unstained cells (negative control)</td>
</tr>
<tr>
<td>30% positive</td>
<td>Stained cells</td>
</tr>
<tr>
<td>40% positive</td>
<td>Stained cells</td>
</tr>
</tbody>
</table>

2 parameter dot plot:

<table>
<thead>
<tr>
<th>Fluorescence intensity (green)</th>
<th>Fluorescence intensity (red)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% &quot;double positive&quot;</td>
<td>20% &quot;double negative&quot;</td>
</tr>
<tr>
<td>10% &quot;red only&quot;</td>
<td>50% &quot;green only&quot;</td>
</tr>
</tbody>
</table>

Analysis of cells stained with labeled antibodies
The Power of Flow Cytometry

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

Sensitive:
Analysis can be performed with <10⁴ cells.

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

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
Magnetic beads coupled to antibodies can also be used to purify cells (an alternative to FACS, that is especially useful for processing larger numbers of cells.)

Flow cytometry is used here to monitor the composition of the cell sample before and after separation.

Expression cloning genes in E. coli

A result of V(D)J recombination every mature B cell expresses a unique antibody. Encounter with an antigen leads to clonal expansion of B cells with a particular specificity.

Where we have been and where we are going

V(D)J Recombination

Discovery of Ig gene rearrangements

Structure of antibody genes (RSS)

Role of RAG proteins and DNA repair machinery
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 γ and µ chains)
- Germ-line vs somatic variation models
- Dryer 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...

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
**Detecting Ig gene rearrangement using 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

**Multigene organization of Ig genes: light chain genes**

- $V_K$ gene segments
- $J_K$ gene segments
- $C_K$ exons

Light chains encoded by 2 gene loci: kappa and lambda

Each light chain encoded by 3 kinds of gene segments:
V (variable), J (joining), C (constant)

A V and J segment are brought together by somatic DNA rearrangement process: “V(D)J recombination”

**Multigene organization of Ig genes: heavy chain genes**

- $V_H$ gene segments
- $D_H$ gene segments
- $I_H$ gene segments
- $C_H$ exons

Heavy chains encoded by a single gene locus.

Each heavy chain encoded by 4 kinds of gene segments:
V (variable), D (diversity), J (joining), C (constant)

V, D, and J segments are brought together by somatic DNA rearrangement process: “V(D)J recombination”

**Multigene organization of Ig genes**

V and J gene segments brought together in DNA before transcription. (RNA slicing removes introns.)
**V(D)J Rearrangement: heavy chain**

V<sub>H</sub> gene segments
First 96 aa's of Ig HC

D<sub>H</sub> gene segments
3-6 aa's HC

J<sub>H</sub> gene segments
10-12 aa's HC

C<sub>H</sub> exons

Gene rearrangement

Transcription

RNA splicing

mRNA

V, D, and J gene segments brought together in DNA before transcription. (RNA slicing removes introns.)

**Gene rearrangement juxtaposes promoter and enhancers**

Promoters: relatively short nucleotide sequences within ~200 bp of transcriptional start site that initiate transcription in a certain direction.

Enhancers: nucleotide sequences located up-stream or down-stream of a gene that activated the promoter in an orientation independent manner.

**Combinatorial Diversity in humans**

<table>
<thead>
<tr>
<th>Gene segments</th>
<th>Heavy chain</th>
</tr>
</thead>
<tbody>
<tr>
<td>V&lt;sub&gt;H&lt;/sub&gt;</td>
<td>V&lt;sub&gt;H&lt;/sub&gt;</td>
</tr>
<tr>
<td>D&lt;sub&gt;H&lt;/sub&gt;</td>
<td>D&lt;sub&gt;H&lt;/sub&gt;</td>
</tr>
<tr>
<td>J&lt;sub&gt;H&lt;/sub&gt;</td>
<td>J&lt;sub&gt;H&lt;/sub&gt;</td>
</tr>
</tbody>
</table>

**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.

Allelic exclusion of Ig genes: ensures that most B cell will express a single antibody specificity (allele: two or more alternative forms of a gene.)

Rearranging gene segments are flanked by a conserved “rearrangement signal sequence”

Rearranging gene segments are flanked by a conserved “rearrangement signal sequence”

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 $V_H$ don’t join with $J_H$, etc.
Flexibility in joining of gene segments contributes to junctional diversity.

Note most rearrangements are non-productive!
(Only 1/3 rearrangements preserves the correct reading frame of the J segment.)

P and N nucleotide addition also contribute to junctional diversity.

(a) P nucleotide addition

(b) N nucleotide addition

P nucleotides are generated by resolution of hairpin structures. N nucleotides are added by an enzyme called terminal deoxynucleotidyl transferase (TdT).