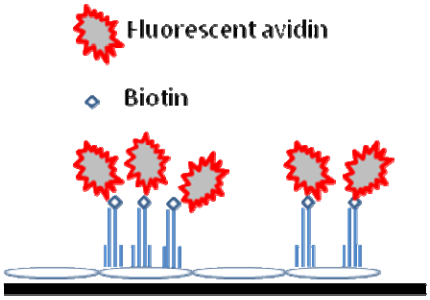
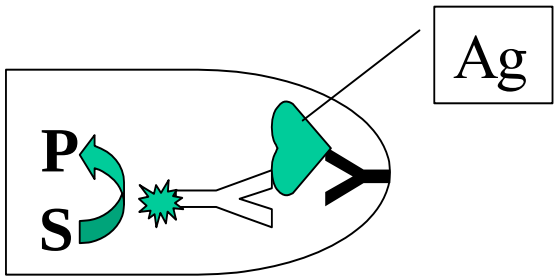
IMMUNOLOGICAL TECHNIQUES 2

Here we consider briefly a few common and highly useful techniques, widely taken advantage of by workers in all sorts of fields (for example, psychologists measuring cortisol in blood or saliva, epidemiologists looking at HIV seropositivity, ecologists looking for toxic substances in water, forensic pathologists, and on and on.)

BIOTIN & AVIDIN. Biotin is one of the B vitamins. It happens to bind with high affinity to a protein from hens' eggs called avidin, and also to a bacterial protein, streptavidin. This allows another nice technique: to detect whether an antibody has bound its antigen, the antibody can be biotinylated—have biotin chemically bound to it—a relatively simple procedure. Once bound, its presence can be revealed by adding fluorescent or enzyme-tagged avidin. The advantage is amplification: each avidin can have several fluorescent molecules coupled to it, and each antibody several biotins, so the light emitted is much brighter than if you had fluorescent-labeled your antibody directly.



ELISA: ENZYME-LINKED IMMUNOSORBENT ASSAY. If an antigen is at least *divalent*, an excellent technique is a **sandwich or capture ELISA** to measure its concentration in a biological fluid or other solution. This is repeated here just for your convenience. Let's say it's a patient's serum and we're measuring myocardial creatine kinase isoform MB in the serum, which might be elevated shortly after a heart attack. You start by making or buying two monoclonal antibodies to human CK-MB, each to a different epitope. Put one mAb on a plate so that it's stuck there. We assume that at least some of the antigen-binding sites are available. Add the patient's serum. Wash off anything that isn't bound. Then add the second antibody, which will stick to the other epitope on the antigen in proportion to how much antigen's there. The second antibody has an enzyme coupled to it—usually peroxidase—and it completes the sandwich. Now add a colorless peroxidase substrate (**S**) that produces a colored product (**P**). Finally, measure the intensity of the product color in a plate spectrophotometer, also called an ELISA reader.

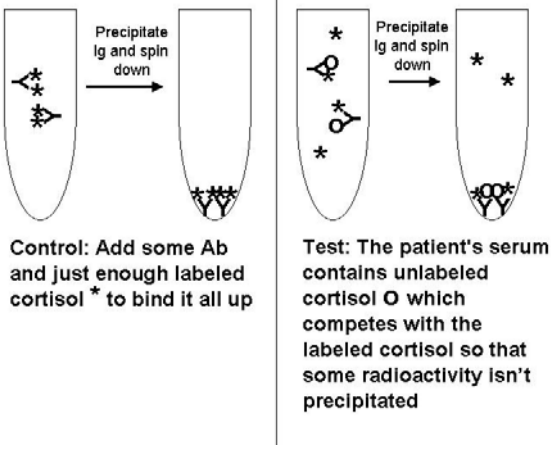


ELISPOT. Do you want to know if your clever new treatment changed the patient's balance of Th1, Th2, and Treg cells? Take some blood, stimulate the T cells with a mitogen, and then pour them onto 3 wells, to which an antibody to IFNγ, IL4, or TGFβ has first been bound. The cells will settle; give them some time to release their favorite cytokine, which will bind to the surrounding antibodies if it's the right well. Then wash the wells to remove cells and medium, and pour in biotinylated anti-IFNγ or anti-IL4 or anti-TGFβ; wash and then add avidin to which an enzyme (usually peroxidase) is bound. Add the substrate and look through the microscope for small blue (product) dots. Compare the ratios of dot numbers in the 3 plates. This is a kind of ELISA done on individual cells, hence the name. There are robots that can do this, too.

COMPETITION IMMUNOASSAYS. The most widely used immunoassays are variants of the ELISA. Remember, though, that ELISAs which measure antigen use one antibody to grab the antigen, and another to reveal that it has been grabbed. This means that the antigen has to have at least 2 different epitopes. But there are many molecules we would like to measure, that are so small they are in the size range of single epitopes. We can make antibodies against them, by coupling them first to an immunogenic carrier protein, but they are still single epitopes (univalent) in the form that we want to measure them, for example as a drug or hormone in blood. So we need a different concept for these important molecules.

The lab uses a variety of immunoassays that depend on **competition** for binding to an antibody between molecules in a patient sample, and a standard amount of the same molecules that have been labeled in some detectable way. A fixed amount of antibody is used. The more of the molecule there is in the patient sample, the fewer labeled molecules get to bind the antibody; they are outcompeted. We then need a method to separate the antibody with its bound antigen from the rest of the mixture, and observe how much label is attached. Since all that's required is for the molecule to bind the antibody, these work well with *univalent antigens* like most drugs and non-protein hormones.

As an example, let's talk about a generalized **radioimmunoassay (RIA),** one of the earliest competition assays still used, mainly in research labs. We want to measure the steroid hormone cortisol in human serum. What we do is prepare a rabbit antiserum to cortisol (not so easy, as cortisol of course isn't an immunogen; we turn it into one by coupling it to an immunogenic protein, a lot like we do when we make a conjugated vaccine). Then we buy some radioactive cortisol, cort*. When we mix a standard amount of cort* with the antibody, it will bind, but there will be no precipitation (Q: do you understand why? A: cortisol is univalent.) Nevertheless, if we add something that precipitates immunoglobulins (such as ammonium sulfate, or even the right amount of goat anti-human Ig), the precipitated antibodies will carry the antigen, cort*, down with them; if it was not bound by antibody, the cort* would remain in the solution. We count the radioactivity in the spun-down pellet. If we repeat this procedure in the presence of a measured amount of a patient's serum, the cortisol in the serum, which isn't radioactive, will *compete* with the cort* for binding to the rabbit anti-cortisol; therefore less cort* will be bound, and when we precipitate the horse antibody with our ammonium sulfate we'll find fewer counts in the precipitate, and more in the supernatant. You can see that the number of counts precipitated would be inversely proportional to the amount of cortisol in the patient's serum, and that some kind of standard curve would allow you to quantify it precisely. This is rather a long-winded explanation, but this is a very important technique[1] by which vital substances are measured in the research lab. It only requires some kind of radiation counter, which most have access to.



[1] It won Rosalyn Yalow the 1977 Nobel Prize in medicine.

Fluorescence polarization immunoassays (FPIA) have replaced radioimmunoassays in big clinical labs, as they also work with univalent antigens but don't require radioactivity. Like the RIA they are competition assays. Let's say you want to measure digoxin in a patient's blood (to see whether he's adequately dosed, or overdosed, with this powerful drug). You buy some specially prepared fluorescent digoxin. Small molecules spin rapidly in solution, so when they are illuminated with a UV laser beam, fluorescent light is emitted randomly in all directions. But if you add an antibody to the digoxin, the complex is relatively large and spins much more slowly; when light hits it, fluorescence tends to be emitted at a fixed angle to the incident light; that is, it is *polarized*. Photomultiplier tubes with polarizer filters can detect this. Now the test: as in the RIA, we add some of the patient's serum to the antibody and labeled digoxin, and if there's digoxin in the serum, it will compete with the labeled drug for the antibody, and that means some fluorescent digoxins *won't* be bound by antibody, and there will be less polarized fluorescence emitted. Long to say but quick to do: in a big lab, these are done by large mysterious machines fueled by reagents supplied by the machine manufacturers, and fed robotically with test samples.

The lab also uses chemoluminescent and fluorescent microparticle immunoassays, which are similar in principle, but use different methods to separate free from bound antigen or to detect it.

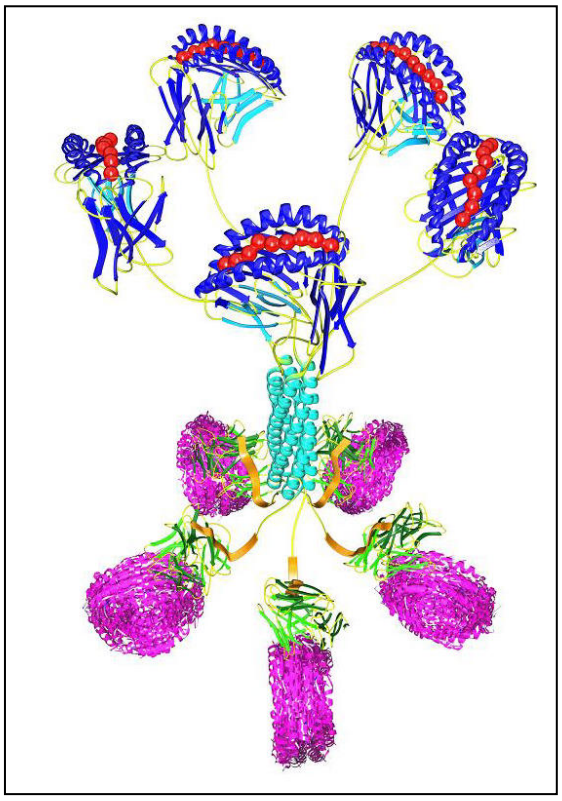
MULTIPLEX CYTOKINE MEASUREMENTS. More and more, as we gain knowledge of the complexity of cytokine and chemokine signaling, we want to measure not one, but a whole cascade of cytokines in blood, tissue fluids, or cell culture supernatants. There are plate-based arrays for this, proteomic versions of gene chips, but **Luminex** has come up with some very appealing technology. It is, in brief, a lot of sandwich ELISA tests on single tiny beads. They make 6-micron polystyrene microbeads that incorporate varying ratios of 2 fluorescent dyes that emit at different wavelengths in the red/infrared (R/IR) region. At present they make 100 different sorts of beads, each with a slightly different ratio of the two dyes; they are accurately distinguishable in a specialized flow cytometer. To set up a typical assay, a set of beads of one particular R/IR ratio has coupled to it, let us say, a monoclonal antibody to TNF; another set of beads gets an antibody to IL-1; and so on and on. In theory 100 different cytokines (or any other set of antigens of interest) could be detected on a single biological sample at one time. The beads are mixed and then exposed to the biological sample, and any cytokine present will bind to the appropriate beads in proportion to its concentration in the sample. The beads are washed and a set of second monoclonal antibodies is added, to each of the antigens that can be detected by the test set; these have biotin coupled to them. Now you have a sandwich. After incubation, fluorescent phycoerythrin-coupled streptavidin is added, which binds to the biotin-labeled monoclonal. The mixture of beads is then run through the flow cytometer. It identifies each bead by its characteristic R/IR fluorescence ratio, and simultaneously quantifies the amount of PE fluorescence on the bead. The readout, then, is: Bead set #56, the TNF capture bead, shows XX units of fluorescence of PE in patient sample 1, equal to a value of 1.7 ng/mL as determined with standards. And so on. Rumor is you can't get an NIH grant to study cytokine regulation any more if you don't do multiplex! The Cancer Center's Flow Cytometry core makes Luminex available to researchers.

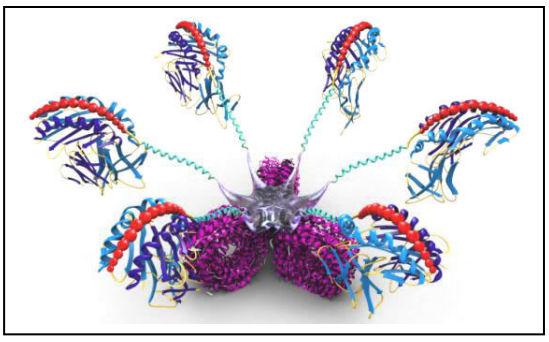
Note, you could couple antibodies to hormones to the beads, or hormone receptors to capture active hormones. You can type people for HLA alleles: one company offers a service in which patient DNA is amplified with generic HLA PCR primers, and then added to a set of 100 Luminex beads coupled with allele-specific DNA probes.[2]

A plate-based multiplex assay system for cytokines, made by Meso Scale Discovery (MSD), is currently very popular at CU, with instruments on the AMC (Barbara Davis) and NJH campuses.

ANTIGEN-SPECIFIC T CELLS TECHNIQUE: MHC MULTIMERS. How many CTL against HIV, or their own islet cells, do patients have during the course of their illness and treatment? This is an important question for research, and eventually for guiding rational therapy. Traditionally it was done by a very tedious limiting dilution assay, which was variable and could never be scaled up. The question can be boiled down to: How many cells in this patient's blood have TCRs that bind with high affinity to such-and-such an immunodominant peptide presented on HLA-A2 or HLA-B7 (or whatever the patient's Class I alleles are)? We could make some HLA-A2 fluorescent, mix it with the peptide to load up its antigen-presenting groove, and then see how many CD8 cells in the patient's blood it bound to. But it doesn't work well, as the affinity of TCR-peptide-MHC is low, and the loaded MHC tends to fall off the cell during the analysis. So now it is possible to buy from several manufacturers many alleles of MHC tetramers or pentamers and beyond, which you can load with the peptide of your choice, and expose to T cells: and because the *avidity* of multiple binding sites is much higher than the *affinity* of a single binding, the multimers stay on the cell and you can do flow cytometry to see them. Exactly the same thing can be done for quantifying specific Th cells.

To the right is the ProImmune Pro5® MHC Class I Pentamer. There are 5 class I antigen-binding domains at top that have been loaded with a peptide. They are joined together by a coiled-coil domain, to which is attached 5 protein domains that can be heavily modified with fluorescent reporters (at the bottom of the picture).





And above is the ProImmune MHC Class II Ultimer™ which uses 6 peptide-loaded Class II MHC antigen-binding domains to detect antigen-specific CD4 T cells. Slick.

[2] http://www.proimmune.com/ecommerce/page.php?page=typing

Learning Objectives for 27: Immunological Techniques 2

1. Explain the principle of a competition immunoassay.
2. Discuss the way biotin-avidin may be used to detect antibody presence.
3. Compare and contrast the sorts of antigens that can be measured in an ELISA-based assay, and those that require a competition immunoassay.
4. Explain in principle the use of MHC/peptide multimers to detect antigen-specific T cells.