Lecture 14

Antibody Methods

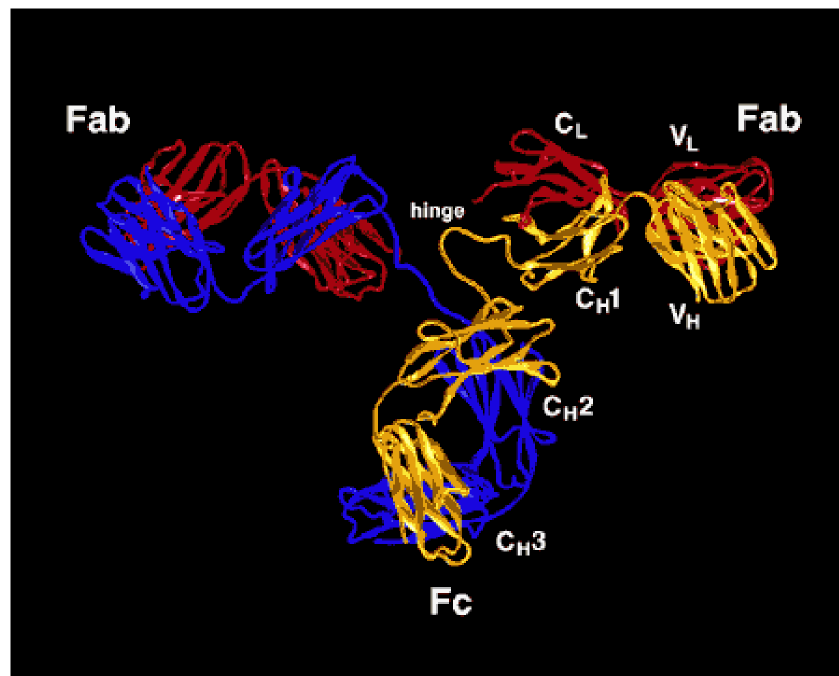
Putting the immune system to work.

Structure of an antibody molecule.

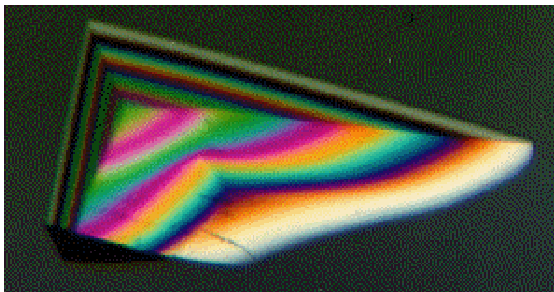
Antibody molecules are constructed from two light chains (kappa or lambda) and two heavy chains (alpha, delta, epsilon, gamma, or mu). The isotype of the antibody is defined by the heavy chain used (alpha -> IgA, delta -> IgD, etc) and each isotype has a defined function in the body.

In the figure below (a crystallographic structure), the heavy chains are blue and gold and the light chains are red. The light chains have two domains: C_L and V_L, representing constant and variable regions. The heavy chains have a variable domain (V_H) and three different constant domains. The stem of the antibody shown at bottom (the portion containing only heavy chain) is called the Fc domain, and the two arms (left and right) are called Fv domains, where the c and v refer to "constant" and "variable". The arms can be cleaved from the Fc domain separately (so that each arm is free) using the protease papain, and that product is called an Fab fragment. A different protease (pepsin) can cleave the antibody so that the Fc domain is removed but the two Fab arms are still connected by a disulfide linkage. Such a bifunctional product is called an $F(ab')_2$ fragment.

image source: http://www.antibodyresource.com/intactab.html



The model



The protein crystal used to generate the data for the model

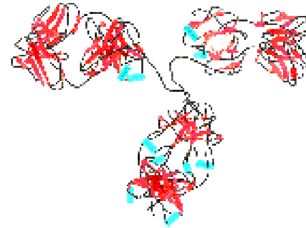
The top picture is a labeled ribbon drawing of the first intact antibody (IgG2A) every crystallized. The antibody is assymetric, reflecting its dynamic character. There are two local dyads in the molecule. One relates the heavy chains in the Fc, the other relates the constant domains of the Fabs. The bottom picture is the crystal from which the above structure was deduced. The longest edge of this crystal is roughly 1 mM across. For more information on this antibody, see:

1.Harris, L. J., Larson, S. B., Hasel, K. W., Day, J., Greenwood, A., McPherson, A. Nature 1992, 360, 369-372.
2.Harris, L. J., Skaletsky, E., McPherson, A. Proteins: Struct. Funct. Genet. 1995, 23, 285-289.

Source: http://www.antibodyresource.com/intactab.html

Turning to view it from another angle:

From another angle...



Source: http://www.antibodyresource.com/rot1.html by Stefan Hemmerich

For continuing your studies, you may be interested in a list of crystallographic antibody structures in the PDB database. Remember that you can use the PDB code with the program Protein Explorer.

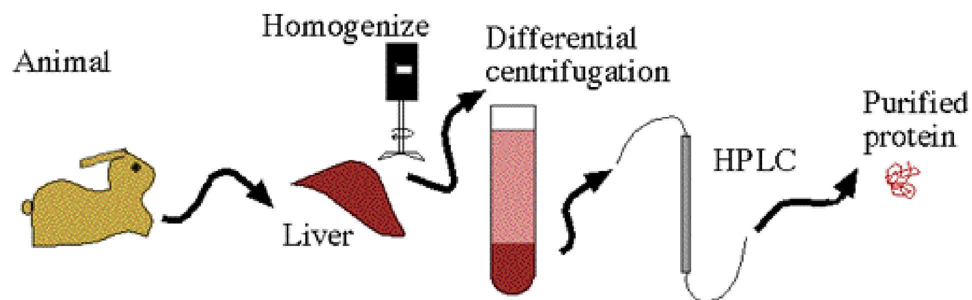
Polyclonal antisera

As a practical matter, how do we obtain an antibody directed against a purified antigen?

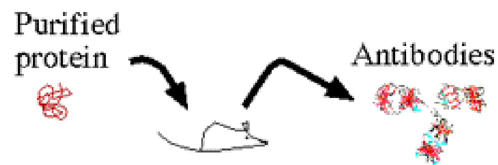
The first thing to consider is the source and purity of the antigen. A recombinant protein can be expressed to very high levels in E. coli, but as we have discussed previously there are legitimate concerns about:

- proper folding
- post-translational modifications

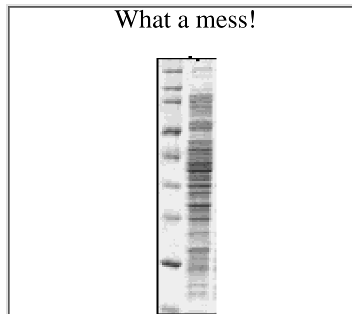
Suppose a researcher is using antibodies to study a eukaryotic protein. The researcher could purify an antigen from its native eukaryotic source, but unless the antigen is expressed at a high level it may be difficult to obtain an acceptable level of purity. The process might look something like this:



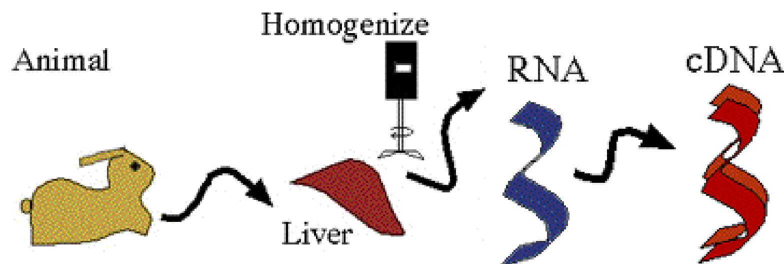
Now you have a purified protein, and you can use it as an immunogen in an animal. The animal makes antibodies which can be used in diagnostic work.



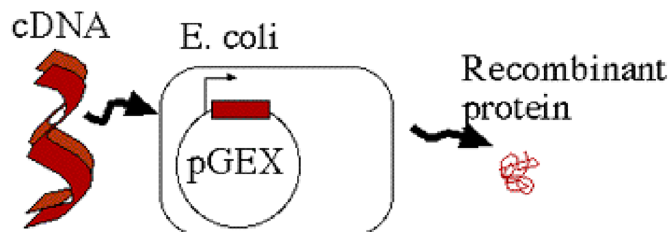
How do you get rid of all of the other cellular proteins that you don't want the laboratory animal to see? Is 99.9% purity high enough? This may be a significant problem because the laboratory animal will develop an immune response against any impurities in the preparation, just as it would to the protein of interest. If the antiserum is to be used for identifying the location or amount of an antigen, the presence of antibodies against other native proteins in the same organism would be a problem. If you performed a western blot using an antiserum that was nonspecific, you might get something like this:



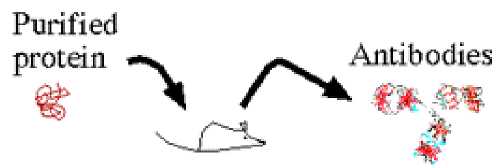
There is a considerable advantage to preparing an antigen in a non-native host such as E. coli, because even if the laboratory animal develops an immune response against a contaminant, that contaminant would be an E. coli protein. Antibodies against E. coli proteins are unlikely to interfere with the application of the antiserum against eukaryotic cells, because the proteins from eukaryotes and prokaryotes are dissimilar. Using a recombinant protein as an immunogen requires a process that might look something like this:



Why do you make a cDNA instead of working directly with genomic DNA? Well, there may be a bit of a problem with the fact that genomic DNAs contain both introns and exons. mRNA only contains exons, so you can isolate a contiguous coding sequence. So - we clone a cDNA into a bacterial expression vector, and let the bacteria make the recombinant protein.



Now that can be injected into an animal to make an antibody directed against the recombinant protein.



Still, the recombinant proteins extracted from E. coli may be improperly folded (particularly if the proteins must be solubilized from inclusion bodies) and may not resemble the native eukaryotic protein with respect to glycosylated or phosphorylated amino acids. That could be a problem, and the researcher may choose to express his protein from a eukaryotic expression vector system (such as in yeast, insect cells, etc.)

Immune response

An antigen can be injected into mice or rabbits (for example) along with an adjuvant to enhance the immune response. The irritation caused by the adjuvant is important in drawing attention from immune cells (such as macrophages and T lymphocytes) to the site of the wound. Do you remember when you were little that adults told you immunizations don't hurt? Well, they were kidding. The immunizations often include irritating agents that do hurt, and for a reason.

After the first injection of antigen, the animal will begin to mount an immune response, generally using IgM isotype antibodies. The response peaks and begins to decrease in magnitude about 3-4 weeks after injection. Then, it's time to "boost" the response with another injection. The immune response to the second immunization is much greater in magnitude and arrives after a shorter period of time. In addition, the IgM isotype reaction is lessened and more of the reaction is of the IgG type. A laboratory animal may be "boosted" several more times, with "bleeds" taken a week or so after each injection to check the antibody titer (reactivity). In general, the titer increases with each boost.

Note that serum taken from the laboratory animal is polyclonal at this point. That is, the antibodies directed against the injected antigen are sharing the bloodstream with antibodies unrelated to the experiment. That is, when the rabbit caught the "bunny sniffles" during the experiment, the antibodies against Bunny Sniffle Virus were also generated and are a component in the unfractionated serum.

In the case of antiserum directed against a GST fusion protein, some antibodies will be directed against the Schistosoma japonicum glutathione S transferase enzyme and others will be directed against the C-terminal fusion domain (i.e. the peptide of interest). The anti-GST antibodies are unlikely to interfere with your application of the antiserum (just as the anti-Bunny Sniffle Virus antibodies are unlikely to interfere, unless you are studying a related Human Sniffle Virus).

It is an important experimental control to collect a "pre-immune bleed" from the lab animal before beginning a course of injections, just to be sure that the immune reaction was newly created by the injection.

Antibodies can be purified from crude serum using methods such as ammonium sulfate precipitation, gel filtration chromatography, or affinity chromatography. For example, immunoglobulins tend to have the ability to bind to Protein A (from Staphylococcus A), and protein A-sepharose is a common affinity matrix.

Monoclonals

The types of antisera that have been described so far are collections of antibodies. Antibodies raised against a single antigen will tend to have many different specificities, which is to say that some antibodies will recognize one side of the molecule and other antibodies will recognize another side.

You can obtain monoclonal antibodies (a single specificity) by the following method.

1. Immunize rats (or more usually mice) with an antigen.

2. A week or two after the first boost, kill the animal and remove its spleen (containing activated B lymphocytes that are making antibodies). Some of the spleen cells will be responding to the injected antigen.

3. Fuse individual splenic B lymphocytes with a plasmacytoma (a B lymphocytic tumor that secretes large amounts of immunoglobulin). It is common to use a plasmacytoma that does not make an endogenous antibody, but has the capability of expressing antibodies.

4. Grow these hybrid cells (called hybridomas) and sequester groups of them in the wells of microtiter dishes. The hybridomas will secrete antibodies into the culture medium, and these "supernatant" antibodies can be collected and tested.

5. Once a "well" in the plate has been identified as being positive, that is that it has a hybridoma secreting antibody against an antigen of interest, it is important to isolate the hybridoma in pure form. The cell line is diluted to the point where each well contains a single cell, and the culture is "cloned" because it is then single-cell in origin. This means that the antibody being generated by the cells is from a single rearrangement of the immunoglobulin locus and is monoclonal.

6. The hybridoma can be grown in vitro, and the culture supernatant collected and purified. This will yield about 10-100 micrograms of antibody per ml of culture. Alternatively, the antibody can be injected into the peritoneal cavity of a pristane-primed (or mineral oil primed) mouse (or rat), and an ascites tumor will develop. The ascites fluid can be collected and will contain approximately 1 to 10 mg protein per ml. The in vivo methods are frowned upon because they cause pain to animals (not that collecting fetal calf serum for culture is painless), and there is some risk that the ascites will contain endogenous polyclonal antibodies against other specificities, viral pathogens, etc.

7. Hybridoma cells can be cryopreserved

As an example of an application, see OneLambda's list of monoclonal anti-HLA antibodies

Humanization

One problem with using mouse monoclonals (or rat monoclonals) for immunotherapy is that the Fc regions are recognized as foreign by the patient.

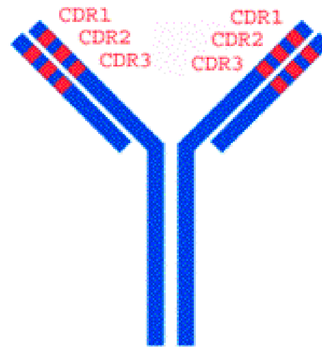
Surely you remember the story of Balto, the brave sled dog, who delivered an antiserum against diptheria to an isolated town in Alaska? Diptheria used to be treated with horse sera that contained antibodies against the bacterial pathogen. If you injected a person with the specific anti-diptheria horse serum, the horse antibodies would bind to the bacterial antigens and inactivate them. Why a horse? Because they're really big, and you can get a lot of serum from them. We call this treatment with donated antibodies a form of "passive immunity" because the recipient did not need to develop an immune response. It's a similar story with snakebite antivenom - you treat an

individual with purified antisera from livestock that have been immunized with the venom.

So - now I hear you asking "Since the horse antibodies are foreign proteins, why doesn't the recipient develop antibodies that bind to the horse antibodies?" That's a good question!

The simple answer is that they do, and so you may not want to get a lot of snakebites. After you've had a few shots of antivenom from the same livestock species, you run the risk of an allergic reaction to the foreign antibodies. It is the constant regions of the antibodies that make them "horse" or "human".

What parts of the antibody are variable (specifying antigen binding) ane what parts are constant for an animal? The figure below shows (in red) the position of CDRs (complementarity determining regions). The peptide sequence between CDRs is called the framework region (FR).

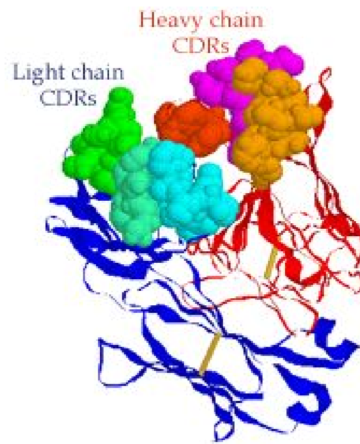


Looking at the sequences of antibodies (such as the kappa light chains below), the complementarity determining regions (CDR, in red) are separated by framework regions (FR, in blue) that are less variable.

L1-L2-L3	Locus	FR1 12345678901234567890123	CDR1 45678901abcdef234	FR2 567890123456789	CDR2 0123456	FR3 78901234567890123456789012345678	CDR3 9012345
2-1-(1)	O12	DIQMTQSPSSLSASVGDRVTITC	RASQSISS------YLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP
2-1-(1)	O2	DIQMTQSPSSLSASVGDRVTITC	RASQSISS------YLN	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQSYSTP
2-1-(1)	O18	DIQMTQSPSSLSASVGDRVTITC	QASQDISN------YLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP
2-1-(1)	O8	DIQMTQSPSSLSASVGDRVTITC	QASQDISN------YLN	WYQQKPGKAPKLLIY	DASNLET	GVPSRFSGSGSGTDFTFTISSLQPEDIATYYC	QQYDNLP
2-1-(U)	A20	DIQMTQSPSSLSASVGDRVTITC	RASQGISN------YLA	WYQQKPGKVPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISSLQPEDVATYYC	QKYNSAP
2-1-(1)	A30	DIQMTQSPSSLSASVGDRVTITC	RASQGIRN------DLG	WYQQKPGKAPKRLIY	AASSLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	LQHNSYP
2-1-(1)	L14	NIQMTQSPSAMSASVGDRVTITC	RARQGISN------YLA	WFQQKPGKVPKHLIY	AASSLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	LQHNSYP
2-1-(1)	L1	DIQMTQSPSSLSASVGDRVTITC	RASQGISN------YLA	WFQQKPGKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP
2-1-(1)	L15	DIQMTQSPSSLSASVGDRVTITC	RASQGISS------WLA	WYQQKPEKAPKSLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQYNSYP
2-1-(1)	L4	AIQLTQSPSSLSASVGDRVTITC	RASQGISS------ALA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQFNSYP
2-1-(1)	L18	AIQLTQSPSSLSASVGDRVTITC	RASQGISS------ALA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQFNSYP
2-1-(1)	L5	DIQMTQSPSSVSASVGDRVTITC	RASQGISS------WLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP
2-1-(1)	L19	DIQMTQSPSSVSASVGDRVTITC	RASQGISS------WLA	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	QQANSFP
2-1-(1)	L8	DIQLTQSPSFLSASVGDRVTITC	RASQGISS------YLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTEFTLTISSLQPEDFATYYC	QQLNSYP
2-1-(1)	L23	AIRMTQSPFSLSASVGDRVTITC	WASQGISS------YLA	WYQQKPAKAPKLFIY	YASSLQS	GVPSRFSGSGSGTDYTLTISSLQPEDFATYYC	QQYYSTP
2-1-(1)	L9	AIRMTQSPSSFSASTGDRVTITC	RASQGISS------YLA	WYQQKPGKAPKLLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISCLQSEDFATYYC	QQYYSYP
U-1-(1)	L24	VIWMTQSPSLLSASTGDRVTISC	RMSQGISS------YLA	WYQQKPGKAPELLIY	AASTLQS	GVPSRFSGSGSGTDFTLTISCLQSEDFATYYC	QQYYSFP
2-1-(1)	L11	AIQMTQSPSSLSASVGDRVTITC	RASQGIRN------DLG	WYQQKPGKAPKLLIY	AASSLQS	GVPSRFSGSGSGTDFTLTISSLQPEDFATYYC	LQDYNYP
2-1-(U)	L12	DIQMTQSPSTLSASVGDRVTITC	RASQSISS------WLA	WYQQKPGKAPKLLIY	DASSLES	GVPSRFSGSGSGTEFTLTISSLQPDDFATYYC	QQYNSYS

Source: http://www.mrc-cpe.cam.ac.uk/imt-doc/public/INTRO.html

The heavy chain variable regions would also have three CDRs, and the six CDRs (3 from V_L and 3 from V_H) would form the binding site of the antibody. In the three dimensional structure, these appear at the tips of the Fab fragment (as shown by the model of anti Taq polymerase Fab below):



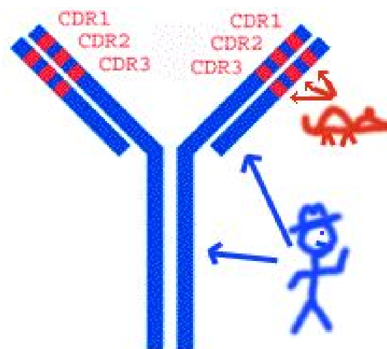
Here is the Fab fragment from an antibody directed against Taq polymerase.

I have adjusted the image so that the CDR regions of the light chain are colored in shades of blue-green (spacefilling), and CDR regions of heavy chain colored in shades of red-orange (spacefilling). Note that these CDR regions form the tip of the complex, and form the surface that defines the specificity of the antibody. Do you remember that we had a word "isotype" that we used to describe the class of the antibody? There's also a word that we use to describe the binding specificity of an antibody - the portion of the antibody molecule that is responsible for binding to an antigen. We call it the idiotype.

Another view of the CDR regions

http://www.antibodyresource.com/rot2.html by Jose Saldanha

So, what we would really like is to be able to make a mouse monoclonal antibody against a target, then use recombinant DNA techniques to make the antibody look more human than mouse in origin:



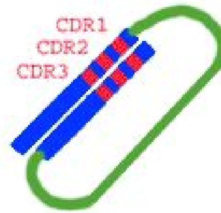
How do we do that? We take the constant and framework regions from human DNA, and combine them with the CDR domains from the mouse monoclonal antibody, as isolated from the DNA in the mouse hybridoma.

An application of humanization: Herceptin® (anti-HER-2), directed against a transmembrane receptor protein (HER-2/neu) that is over-expressed on some human mammary carcinomas. This humanized antibody has been approved as a treatment for human metastatic breast carcinoma (as of September 25 1998 the FDA approved Genentech's Herceptin® HER-2 monoclonal antibody for use)

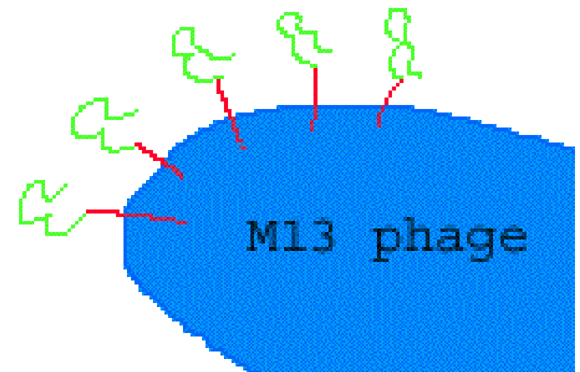
Recombinant antibodies

With the ability to manipulate DNA sequences easily, it is now possible to work with antibody-encoding genes directly, as we just described for the humanization of a mouse monoclonal. The heavy and light chains of an antibody can be expressed in E. coli or eukaryotic organisms (such as plants), and some functional antibodies can be generated.

The H and L chains do not even need to be expressed as separate polypeptides. The two can be fused with an intervening spacer, and the Fab unit expressed as a single polypeptide. We call that a single chain Fv antibody (ScFv). It might look something like this, with the spacer shown in green:



One application of this is to prepare a phage display library with coat proteins fused to randomized ScFv domains. The antibody domains can fold into a functional structure.



Now starting with a random collection of phage, it is no longer necessary to immunize animals! You can select physically for antibodies that bind to a target, from among all of the specificities represented by the library. Not only is this better for the mice, but it's better for the researcher. You don't need to wait for the animal to develop an immune response if you can isolate binding domains in vitro.

Once a successful phage with high affinity constant is identified, you can transplant the CDR domains as DNA, into the regular framework of antibody genes, and this can be transfected into a hybridoma cell line for antibody production. Or, you can use the ScFv as a binding specificity "as is".

In either case, you have the capability of controlling binding specificity at the level of recombinant DNA techniques.

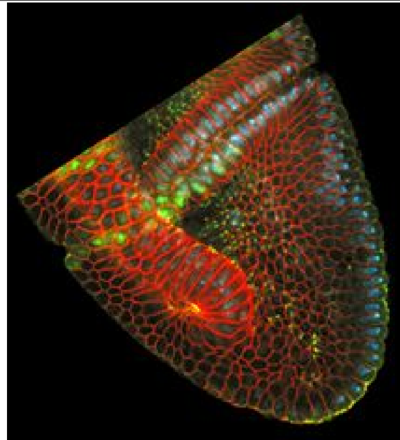
Using an antibody in research When you're planning an experiment that uses antibodies, you may find it necessary to prepare your own antisera for experimental research, or there are a number of commercial outfits that sell antibodies.

For example, try out the search engine at AbCam Inc.

Your next concern may be to detect where your antibody is binding, whether on a Western blot, ELISA, or slide of fixed cells. The general approach is to use a secondary antibody that is conjugated with a chemical or enzymatic label, and to detect the secondary antibody binding by a colorimetric (or luminescent) enzyme substrate, fluorescent tag, radioactive tag, gold particle, etc.

Zymed has a list of secondary antibodies that you may wish to peruse. For example, in the list of secondary antibodies to mouse immunoglobulin, you can see that you have a choice of mouse Ig isotype (IgA, IgE, etc.) host of origin (goat, rabbit, etc), and conjugate (biotin, alkaline phosphatase, gold, etc.)

Using fluorescent dyes, it is possible to detect multiple antibody binding events. For example, this is an image from Molecular Probes Inc. with the following notes:



The formation of the cephalic furrow in the anterior end of a developing Drosophila melanogaster embryo visualized with the help of several fluorescent stains. A **primary antibody to neurotactin** was visualized using a red-fluorescent Cy3 dye secondary antibody (Amersham Pharmacia Biotech Limited). **Primary antibodies to plasma membraneóbound myosin and to nuclear-localized even-skipped (Eve) protein** were visualized with green-fluorescent Alexa Fluor 488 goat antiómouse (A-11001) and goat anti-rabbit (A-11008) IgG (H+L) conjugates, respectively. The **nuclei were stained with blue-fluorescent Hoechst 33342** (H-1399). The sample was prepared by Eric Wieschaus, and the imaging was performed by Joe Goodhouse, Princeton University.

http://www.probes.com/servlets/photo?fileid=g001085&id=18

Antibodies are important tools in diagnostic tests, and are emerging as "smart drugs" in the pharmaceutical industry.

Stan Metzenberg
Department of Biology

California State University Northridge
Northridge CA 91330-8303
stan.metzenberg@csun.edu

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