BIOC4004 - Industrial Biochemistry
Lecture 11 - Mon Feb 09, 04

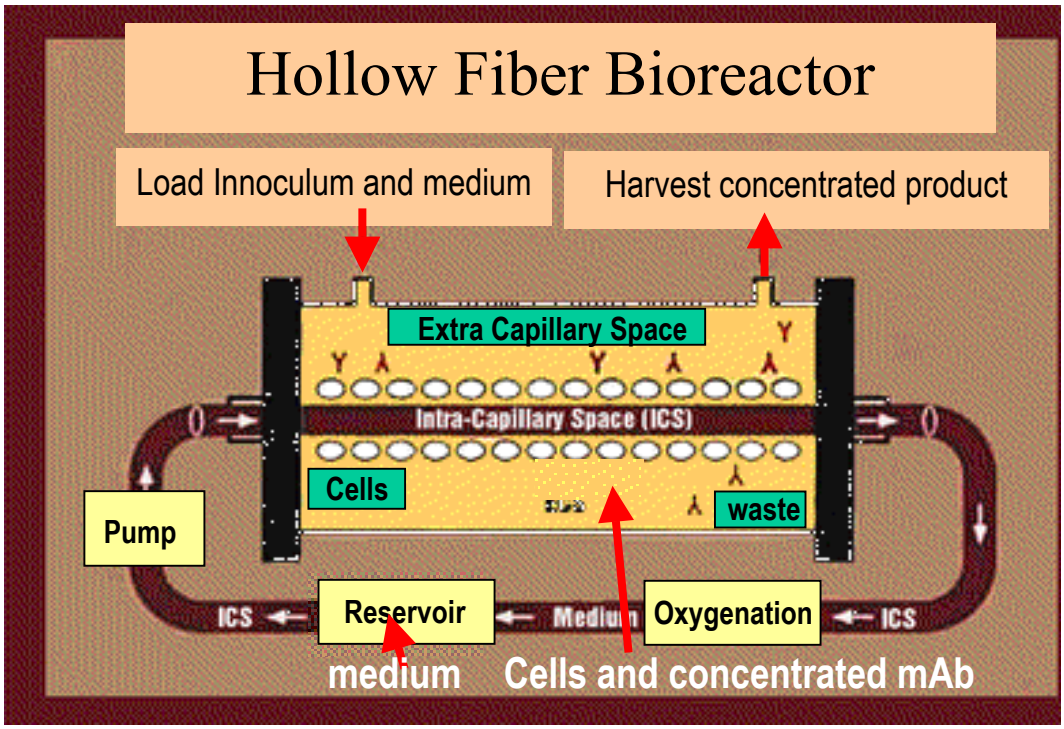
Topics for the Day:

- Antibody production
- Antibody fragments
- Phage-display
- Antibody engineering

Extension for Research proposal Topics :

due next Friday (Feb 13th, 2004)

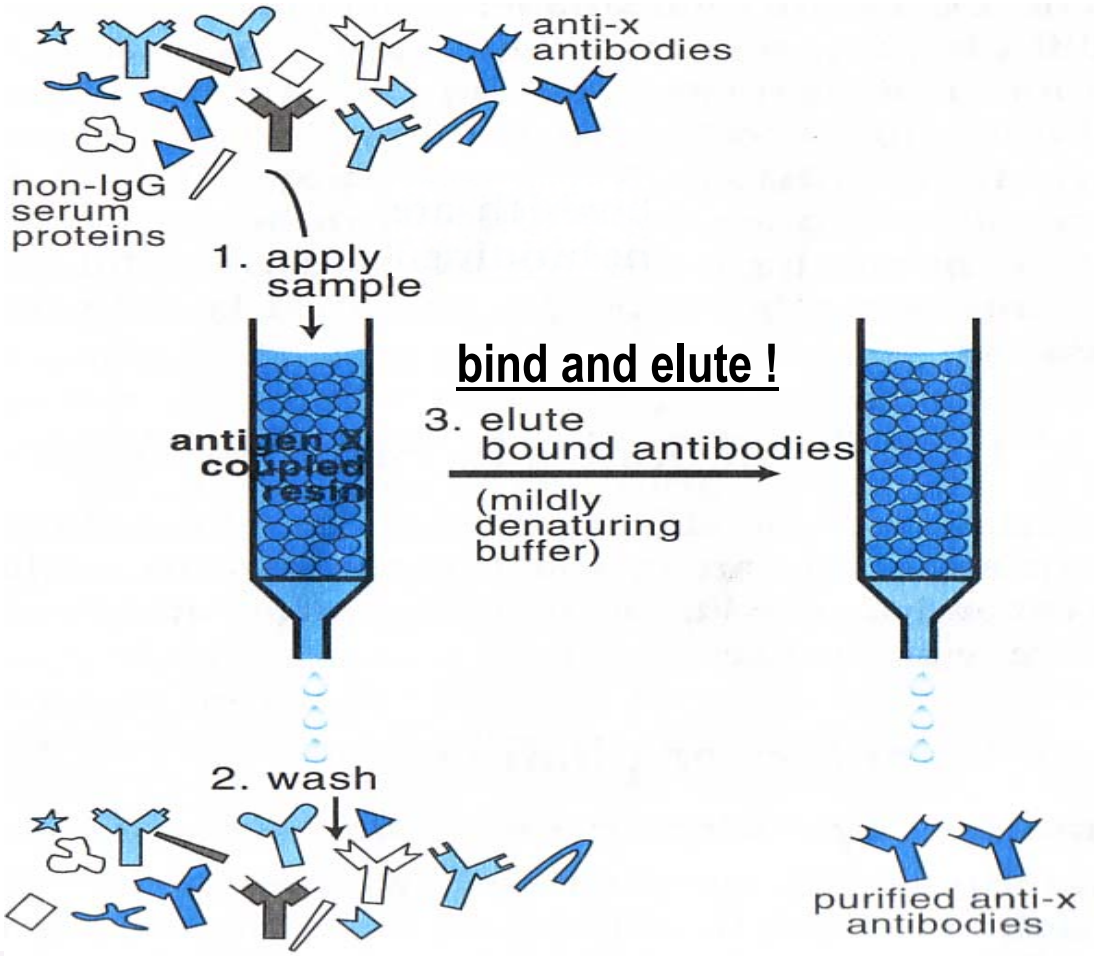
In Vitro production of Monoclonal Antibodies



- Cells and mAb are produced in small chamber separated from medium
  - volume of medium can be large
  - cell growth chamber is kept small (1-15 ml)
- Barrier is a semipermeable fiber
  - cells and mAbs can't diffuse out
  - nutrients and growth factors can diffuse in
  - cell waste products can diffuse out (dilution !!!)
- Medium can be replaced without losing cells or mAbs
- Cells and mAbs can be harvested independently of growth medium
- Compartmentalization makes it possible to achieve mAb concentrations comparable with those in mouse ascites

The hollow-fiber bioreactor is designed to provide total yields of 500 mg + of mAb

Purifying Antibodies



Quality control tests

Appearance of contents of thawed vial
Total protein concentration by absorbance at 280nm
Immunoglobulin content
SDS-polyacrylamide gel electrophoresis
Isoelectric focusing on polyacrylamide gel
Acid Native polyacrylamide gel electrophoresis
Endotoxins by chromogenic LAL
Pyrogen test
Sterility by aerobic and anaerobic culture
pH and Conductivity
Osmolarity by red cell lysis
Immunoglobulin species and isotype by immunodiffusion
Purity by HPLC on column specific for IgG
Residual DNA (by dot blot hybridization)
Residual protein A (by ELISA)
Residual bovine IgG (by ELISA)
Activity by immunofluorescence
Check on numbers of labels printed, used and destroyed

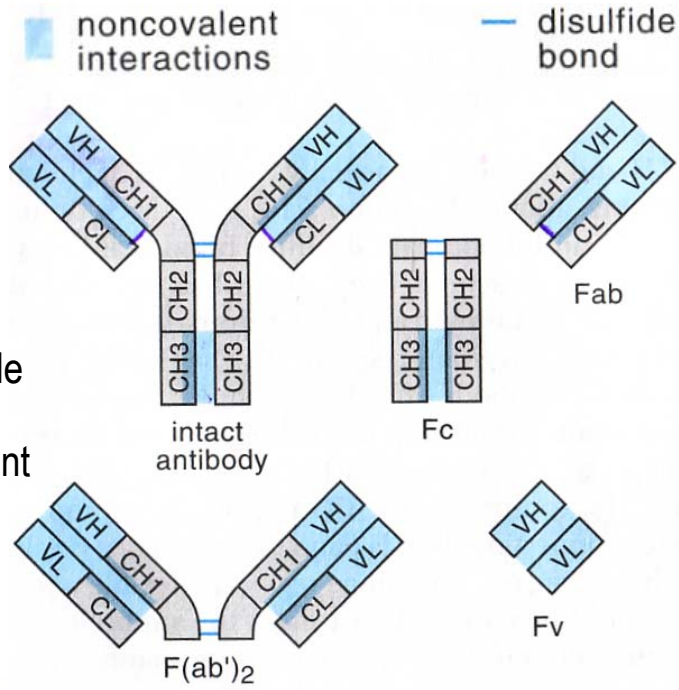
- Use affinity chromatography with
  - Protein A (*S. aureus*)
  - Protein G (*Streptococcus*)
  - **The actual antigen !!!!**
- Followed by Ion Exchange
  - S-sepharose
- Polished with Size exclusion
  - Superdex-200

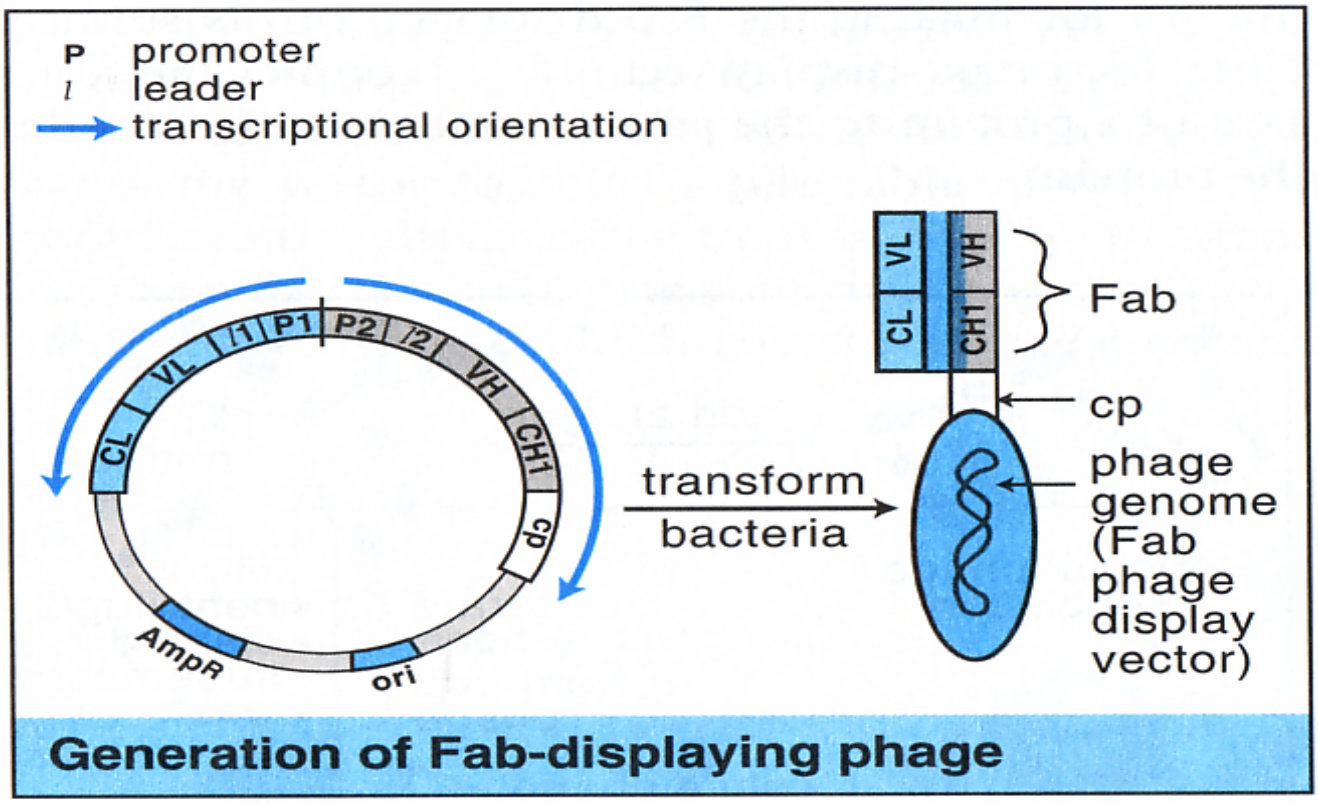
Those clever molecular biologists: Phage-Display

The different "portions" of an antibody have different roles:

- Fc responsible for mediating
  - transport
  - cell recruitment

- VH and VL variable regions responsible for antigen binding
  - as long as VH and VL are present there can be antigen binding
    - F(ab')2
    - Fab
    - Fv





Generation of Fab-displaying phage

- Engineer a phage to code for the domains of in a Fab (or **Fv**) fragment
- The Fab/Fv fragment CDSs are fused to the phage coat protein CDS
- Phage "displays" Fab/Fv fragment on its surface (it's an expression vector, after all)
  - a Fab/Fv fragment-coated phage !!!!

Why Phage-Display is cool (part I)

- B-cells generate a **HUGE** diversity of antibodies (> 10^{11})
  - several genes involved
  - **MANY** systems to generate diversity:
    - shuffling of segments encoding the antigen binding sites
    - insertions and deletions
    - etc, etc, etc

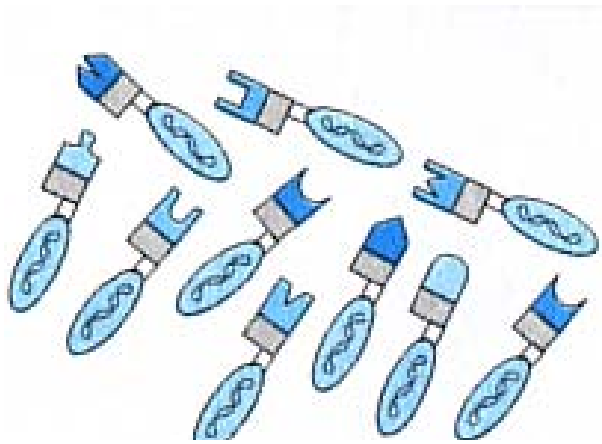
  However, everything is in the genes !!! The system is built to generate an antibody for **ANY** given antigen

  What if ?
  - we isolated a whole ton of B-cells (~ 10^8)
  - obtained their mRNA
  - synthesized cDNA from the Fab-encoding mRNA molecules
  - cloned all of these into a phage display vector

  you'd have **A PHAGE DISPLAY LIBRARY**

Phage-Display Libraries

- A collection of phage-display clones
- each clone displays a different Fab region
  - ie. a different antigen binding specificity



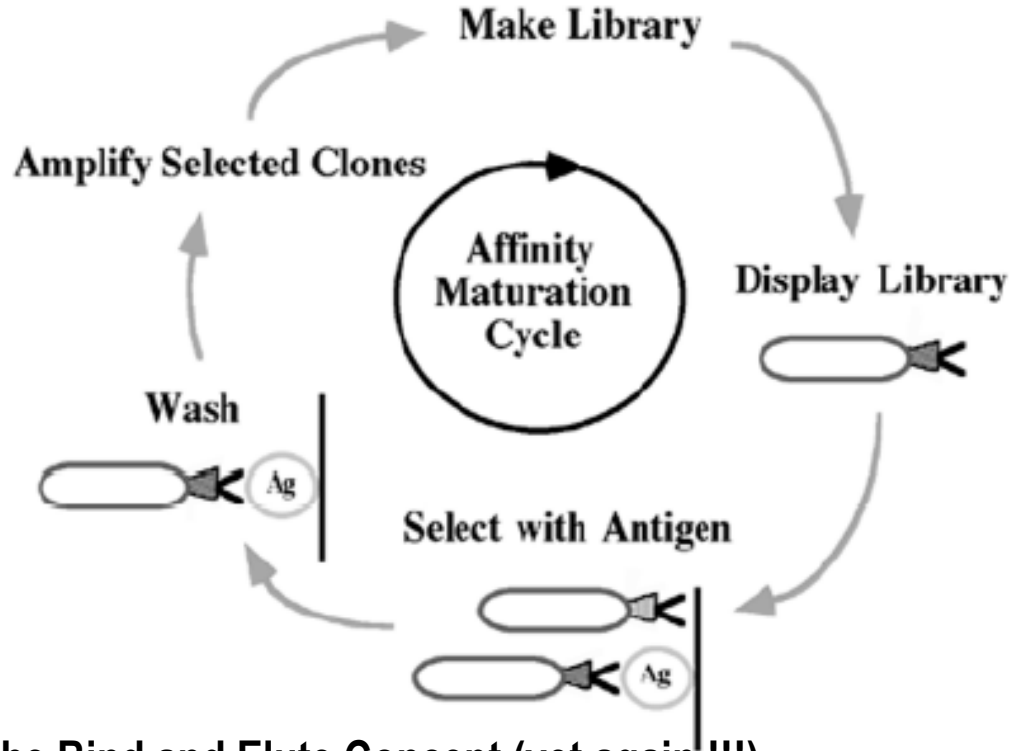
A phage-display library is the equivalent of having the initial population of B-cells:

- instead of a B-cell and its antibody ➔ a phage and its coat of Fab fusion-protein
- Plus these HUGE advantages
  - It's in *E. coli,* which is much easier to deal with !!!!
  - A phage carries the Ab fragment AND its corresponding DNA sequence !!!!
    - It's an expression vector
- The library can be screened for clones that are able to bind to a given antigen
  - this is called "**panning**"

Why Phage-Display is cool (part II)

Panning a Phage-display Library: Affinity Maturation

It's like doing the reverse of screening an expression library (or like affinity chromatography !!!)



The Bind and Elute Concept (yet again !!!)

incubate phage with antigen

- wash unbound phage
- Elute bound phage from antigen
    - use low pH
- You now have antigen-binding phage-display clones
- You can pop the phage back into *E coli* to amplify the clone

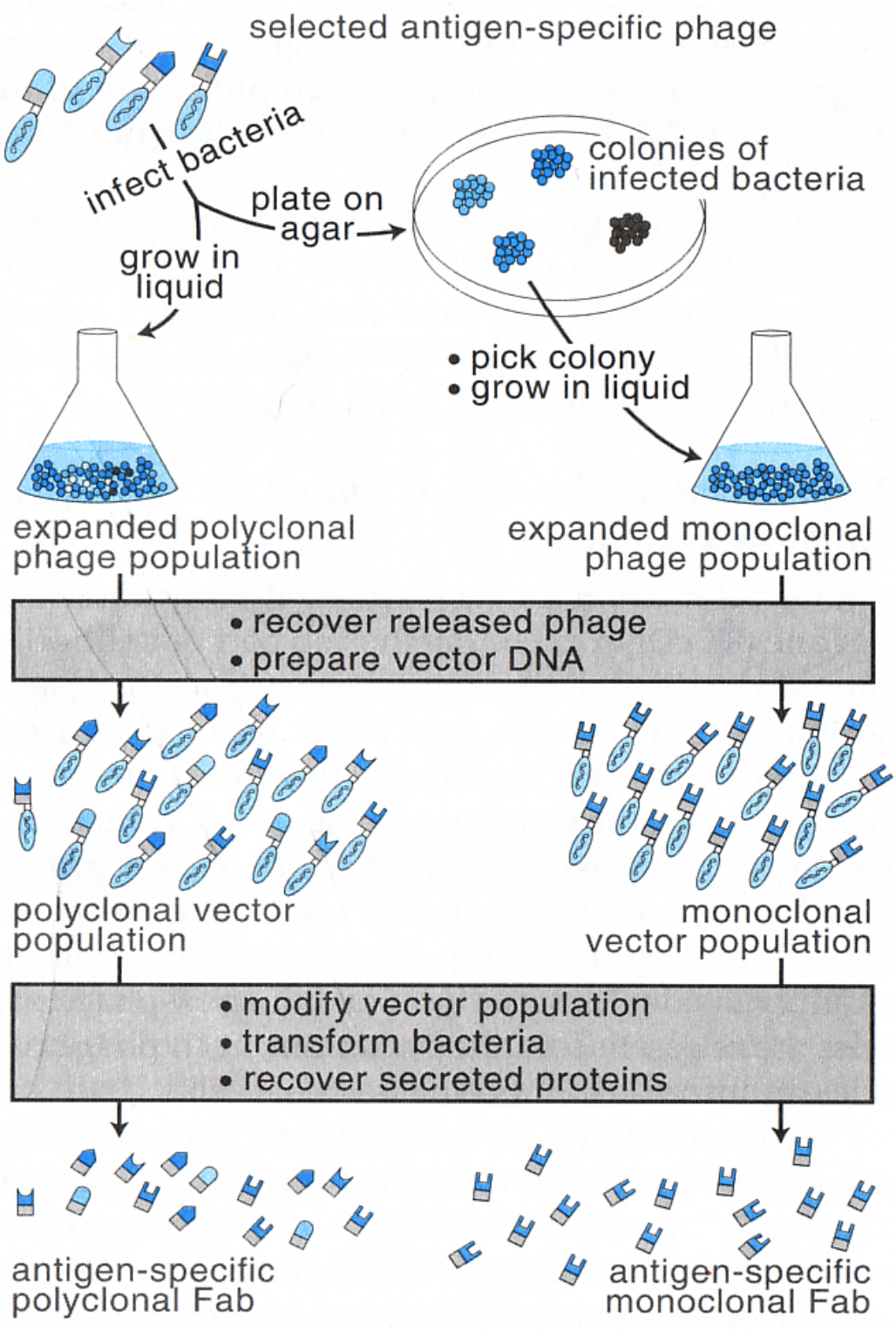
The Result: you can find a clone that expresses a Fab fragment that binds to your antigen of interest:

- a marker on the surface of a cancer cell ?
- Anything your heart desires !!!

Coupling of function to genetic information by the phage display technology

Why Phage-Display is cool (part III)

Panning a Phage-display Library (cont)

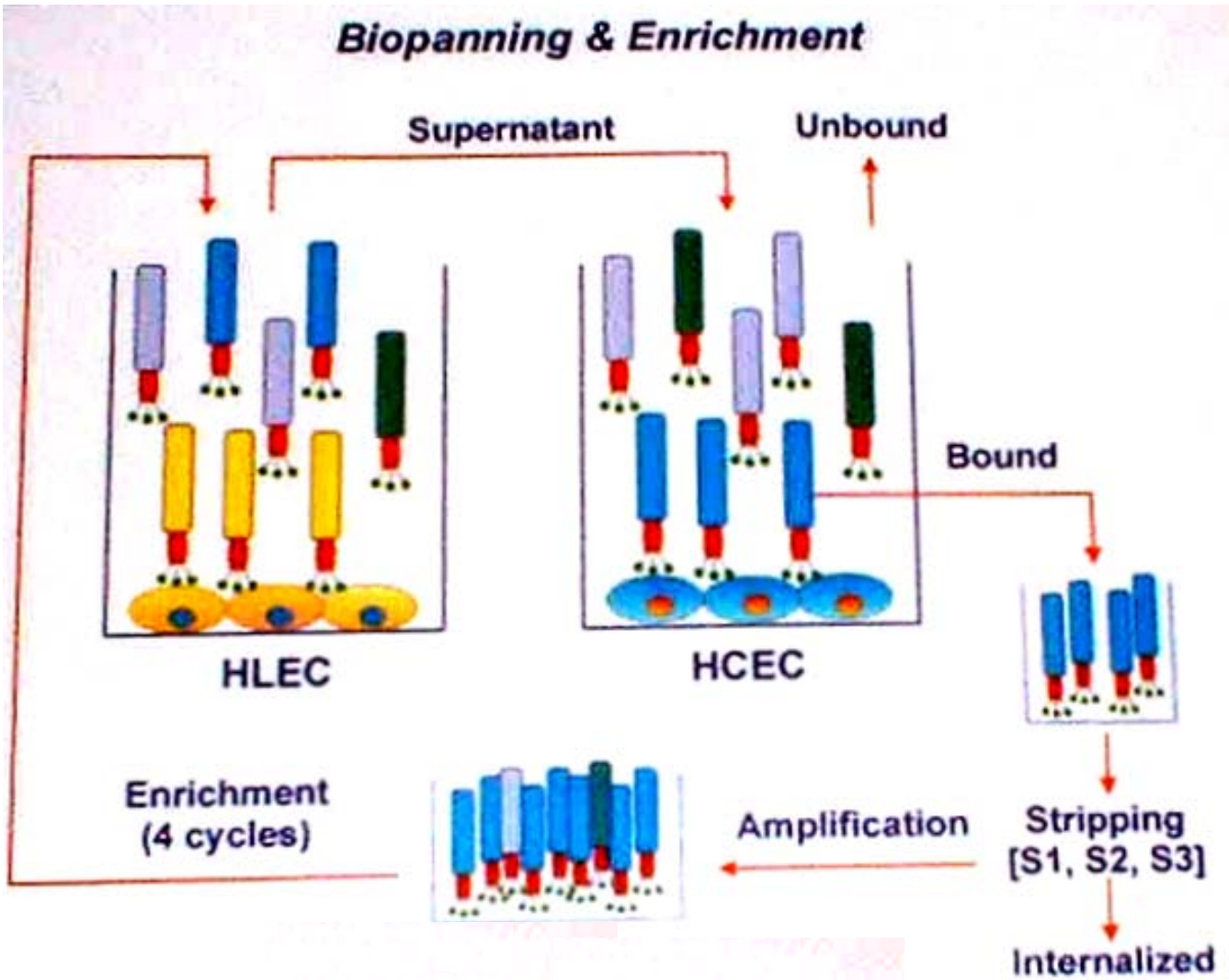


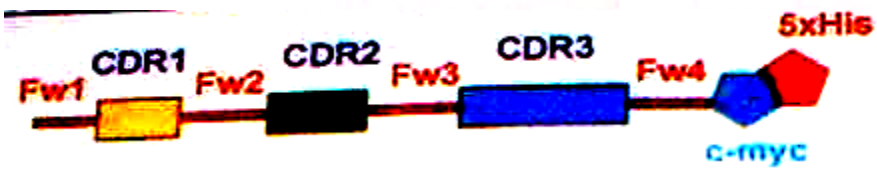
- an alternative to making antibodies in mammalian cells

Incorporating biological activity into Panning: Biopanning

Problem: Antibodies can't cross Blood-Brain Barrier, may need to develop Ab that can do it

Solution: use in vitro BBB model to select clones that can go across BBB and can be internalized

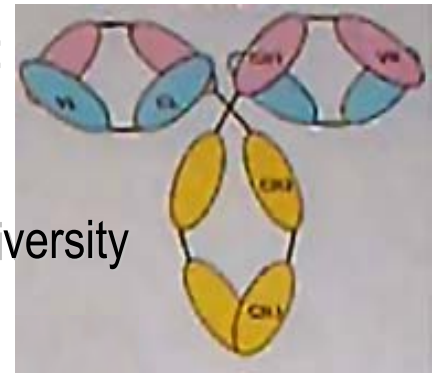




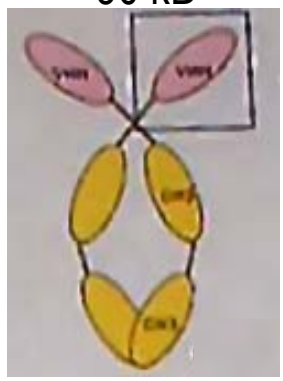
- c-myc tag allows for immunohistochemistry to monitor internalization

Single-chain antibodies

Conventional IgG
150 kD



Camelid IgG
90 kD



Camelid species have weird IgG:

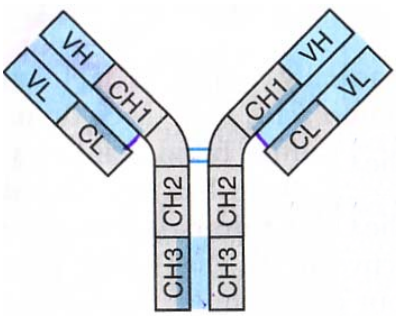
- lack light chain
  - light chain contributes to diversity
  - camels don't suffer for it

- no light-chain means less bulk !
- more stable than recombinant antibody fragments
- can get into nooks that conventional Abs can't
- can access antigenic epitopes that conventional Abs can't

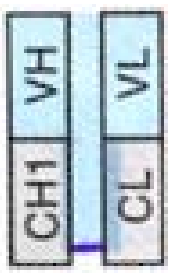
- good for immunotherapeutics and immunodiagnostics

Thank you Mother Nature !!!!

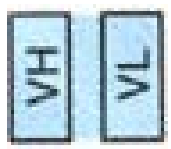
What's the smallest antibody fragment able to bind antigen ?



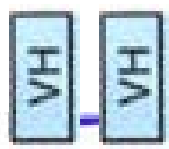
intact Ab



Fab



Fv



sdAb

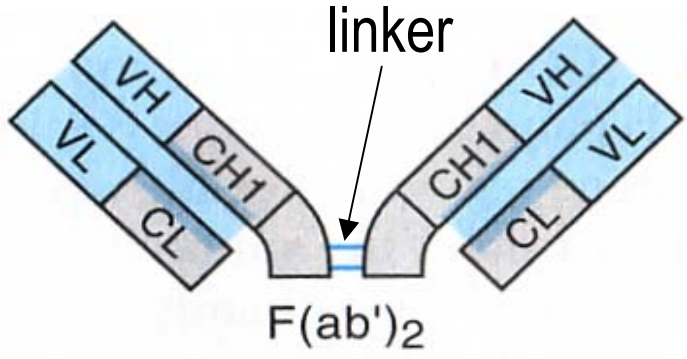
"Affinity", "Avidity", "Valence"

- An antibody's binding strength towards its epitope on an antigen is determined by a combination of affinity and avidity.

- Avidity is affected by **valence** (number of binding sites on the Ab)

- phage-display clones may have ok affinity, but because of monovalancy, have limited avidity

How to solve the problem ? Multimerization

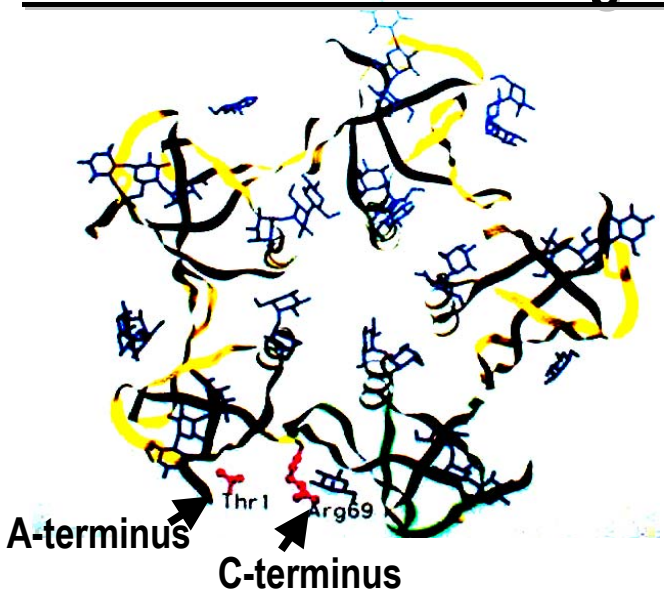


What about using some other type of "scaffold" ?
Zhang *et al. J Mol Biol. 2004 Jan 2;335(1):49-56.*

-Shiga-like 1 verotoxin from *E. coli:*

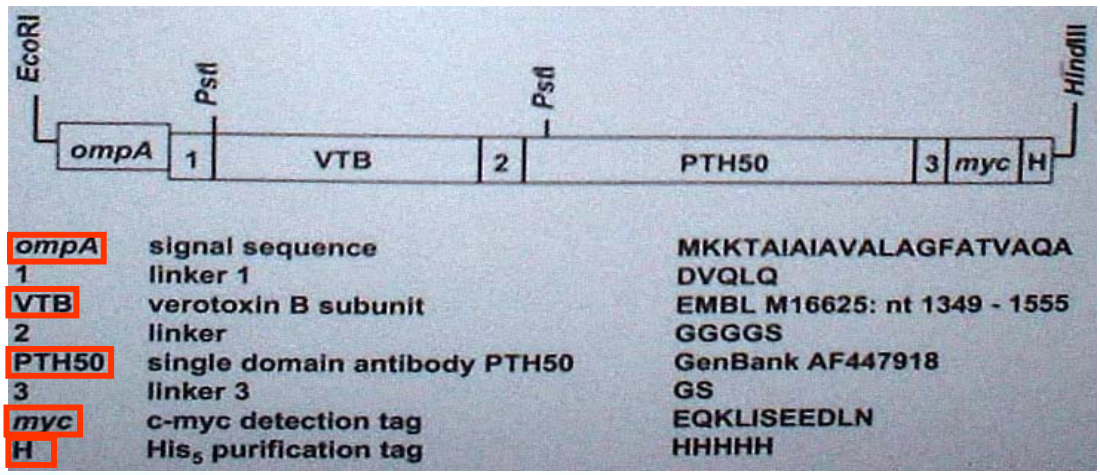
- **AB_5** pentamer (1 catalytic subunit A + pentamer of Subunit B)
- Subunit B :
  - small: 69 AA
  - self-pentamerizes (ring-like structure)
  - fusion protein with antibody
    - formation of a pentavalent antibody

Pentamerization of Single-chain antibodies

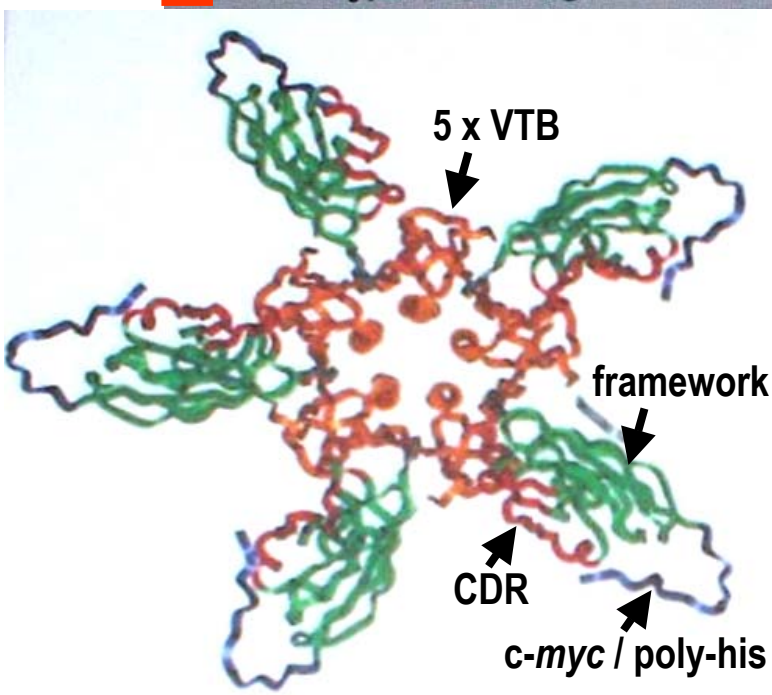


- A and C termini end-up at the periphery of pentamer

- a fusion partner would not affect pentamerization



ompA	signal sequence	MKKTAIAIAVALAGFATVAQA
1	linker 1	DVQLQ
VTB	verotoxin B subunit	EMBL M16625: nt 1349 - 1555
2	linker	GGGGS
PTH50	single domain antibody PTH50	GenBank AF447918
3	linker 3	GS
myc	c-myc detection tag	EQKLISEEDLN
H	His_5 purification tag	HHHHH



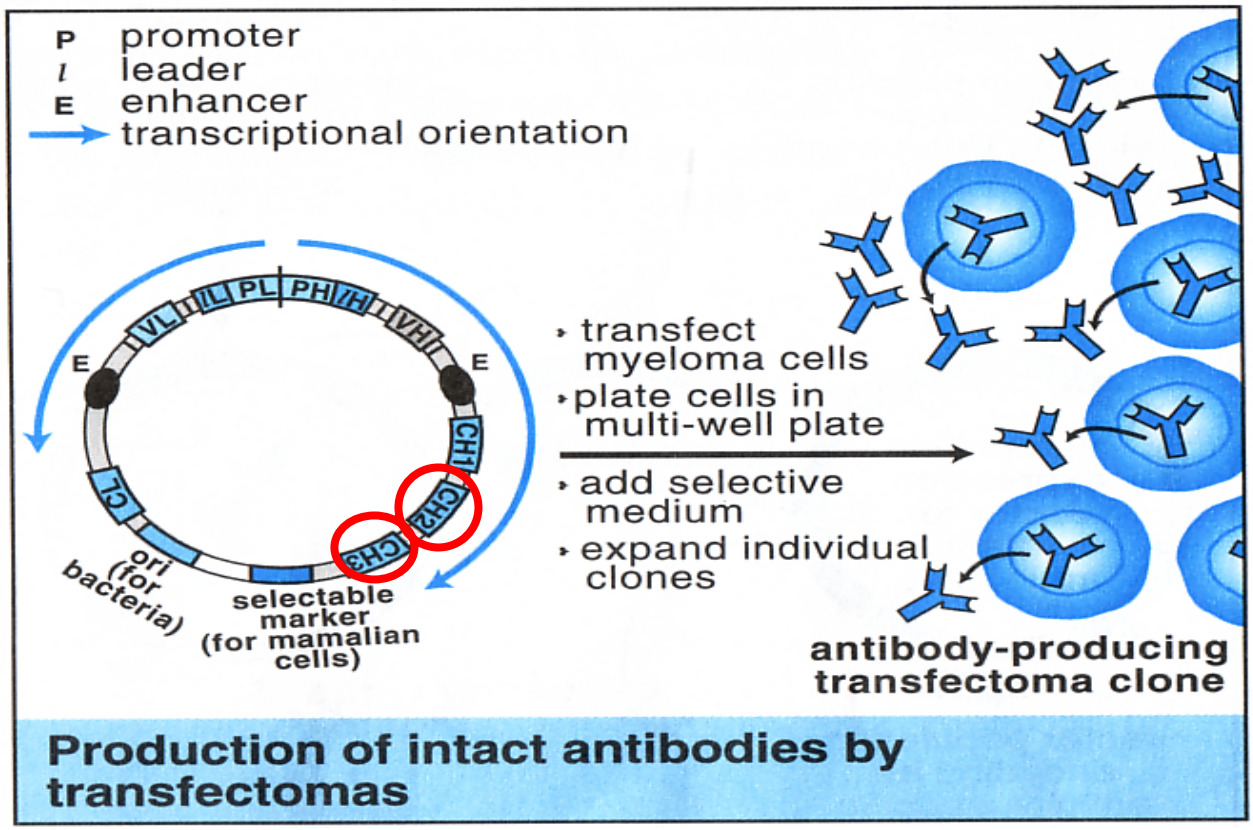
- Ab presented in planar fashion
  - multivalent binding

- Pentamerization led to a 2500-fold increase in binding affinity

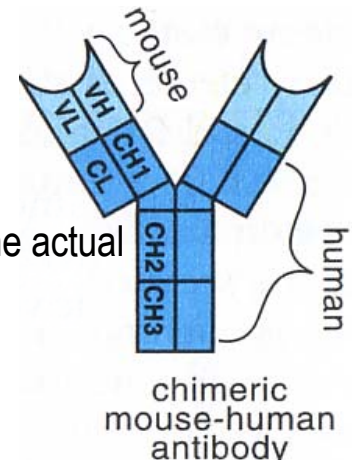
- Could exploit both A and C termini
  - decavalent ?
  - bi-specific ?

Tailoring phage-display clones for human therapy

- Fab can bind antigen but can not elicit immune response
  - good as a detection reagent
  - no good as a therepeutant



- Take the Fab genes from a phage-display clone(CL, CH1, VL, VH)
- Put into shuttle-vector that has:
  - human CH2 and CH3 regions (the CH fragment)
  - mammalian promoters and enhancers
- Transfect: you get mammalian cell producing an intact Ab



The CH2 and CH3 regions (collectively, the CH region) mediate the actual immune response:

- Fab + CH (intact Ab) can elicit immune response
- Could be used as a therapeutic mAb

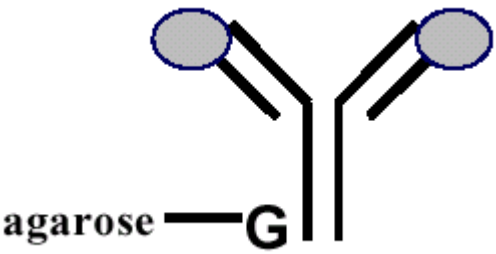
- B-cells for generating phage-display libraries are usually from mouse
  - if only mouse VL and VH are used, and the rest is derived from human sequences, the chimeric mAb can be used in human therapy

Immunoassays for Research and Diagnostics

The general procedure used in all immunoassays
1) allow antibody-antigen complex formation
2) Detection:
- directly label 1° antibody
- anti-antibody (2° Ab)
- antibody-binding protein
    - protein A (S. aureus)
    - protein G (Streptococcus)

- Radiolabeling
  - iodination
  - metabolically (Mabs)
- Fluorochromes
  - fluorescein
  - rhodamine
- Biotinylation
- Enzyme Crosslinking
  - alkaline phosphatase
  - horseradish peroxidase

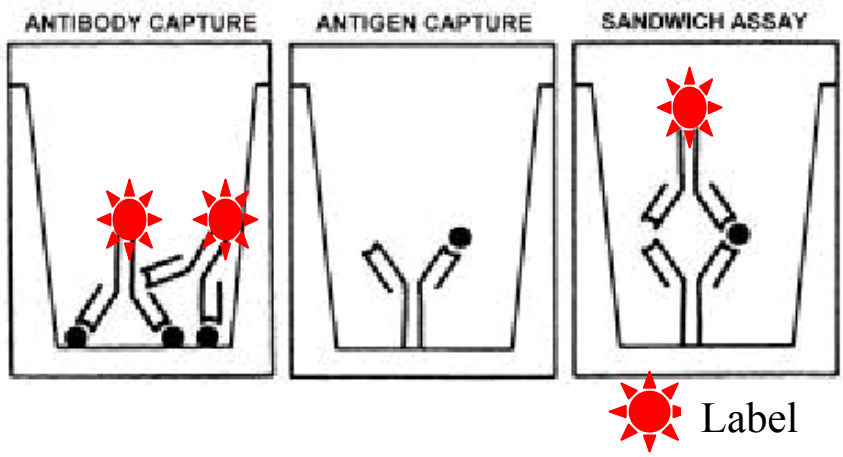
Labeling Abs



Affinity chromatography

1. Antigen electrophoresis
2. Transfer to membrane
3. Blocking
4. Incubate with 1° antibody
5. Wash
6. Incubate 2° antibody
7. Wash
8. Develop with substrate

Western blotting



1. Antigen binding
2. Blocking
3. Incubate 1° Ab
4. Wash
5. Incubate 2° Ab
6. Wash
7. Develop with substrate

ELISA

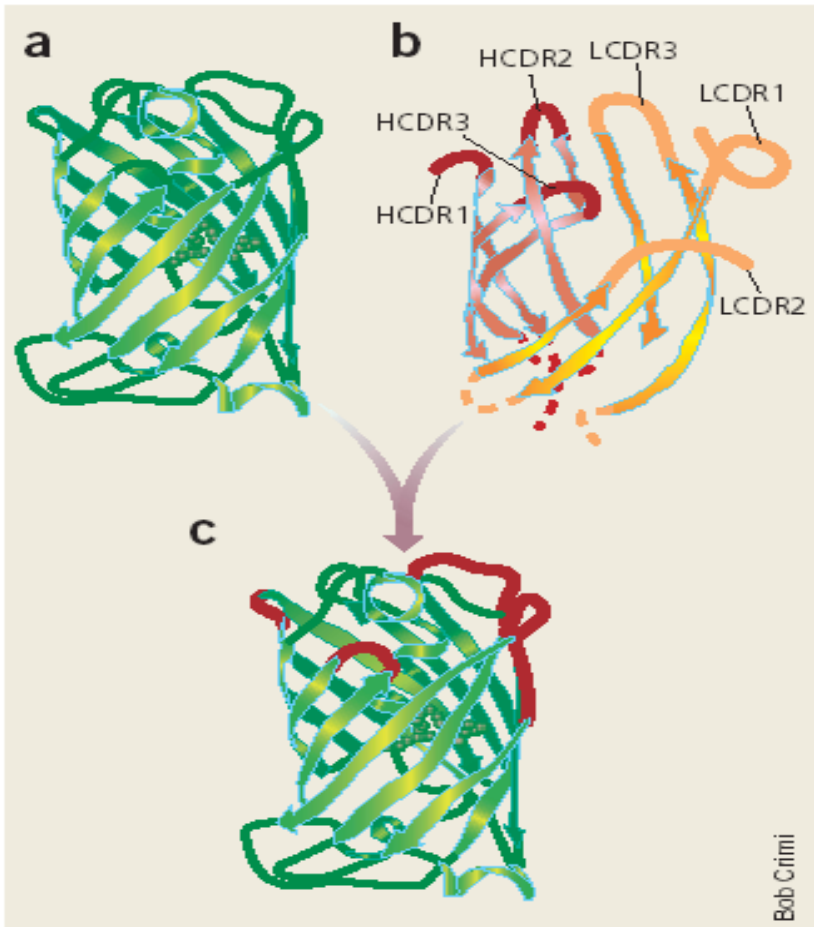
1. Prepare cells or tissue
2. Incubate 1° antibody
3. Wash
4. Incubate 2° antibody
5. Wash
6. View under UV illumination

Immuno Fluorescence

Fluorobodies

Zeytun *et al*., Nat Biotechnol. 2003 Dec;21(12):1473-9

GFP and the complementarity region of an Ab have similar structure
-"barrel" made-up of beta-strands linked by loops on top
- replace these loops with the CDR loops !
- Native GFP tends to lose activity with loop replacement
    - used "superfolder" mutant: more stable and more tolerant to additions



-fluorobodies have both binding **and** intrinsic fluorescence
    -No need to add tags for detection
    -Detection in real-time
    -Could be used in immunohistochemistry, FACS, ELISA

A review of Ab and Ab-derived fragments