MUNI
SCI

C8116

Antibodies as immunochemical tools

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Summary of interplay between T_H and B cells

Antigen

T cell epitope (binds to MHC, recognized by TCR)

B cell epitope (binds to BCR)

Antigen

Dendritic cell (DC)

1 DC acquires antigen and moves to lymph node

THelper

1 B cell acquires antigen

2 DC processes and presents antigen to CD4 T cell

THelper

2 B cell processes antigen and displays class II MHC-peptide complexes on surface

T cell activated

3a T cell interacts with B cell through TCR-MHC interaction

Activated THelper

4a B cell activated and secretes IgM

Plasma cell

IgM

3b T cell interacts with B cell through TCR-MHC interaction and by CD40L-CD40 interaction → IL-4

Activated THelper

AID ↑

CD40L CD40

4b Hypermutation Class switch recombination

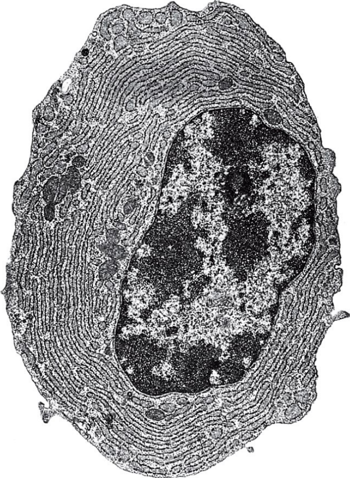
Other isotypes High affinity immunoglobulin

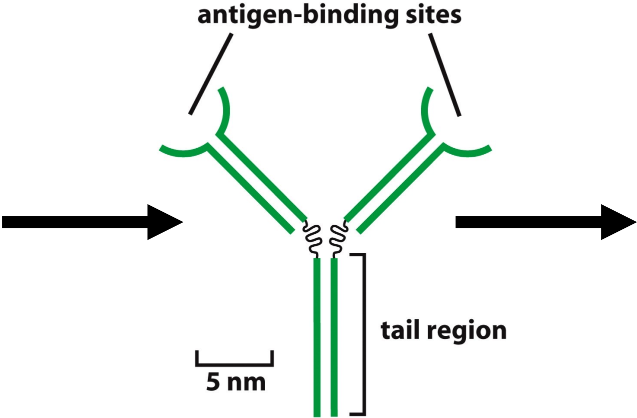
Antibodies as immunochemical tools

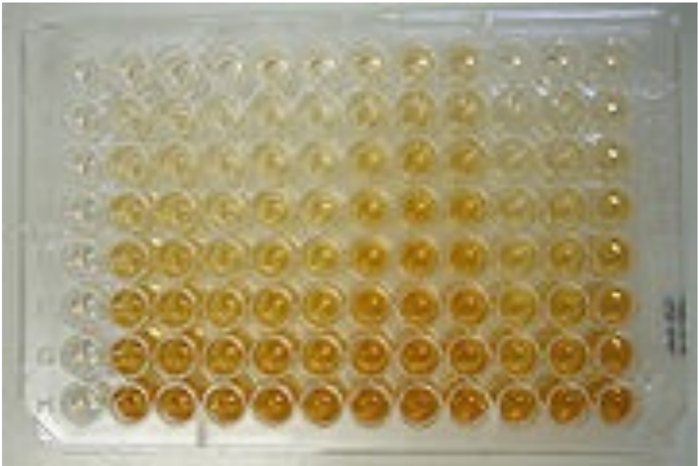
Immunology

The “tools“: antibodies

Immunoassay





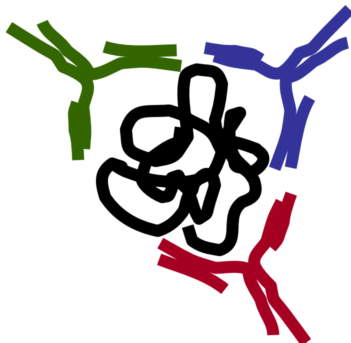


Polyclonal vs. monoclonal antibodies

polyclonal

Antibodies that are collected from sera of exposed animal

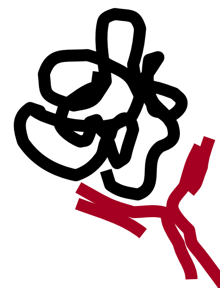
recognize
multiple antigenic sites
of injected substance



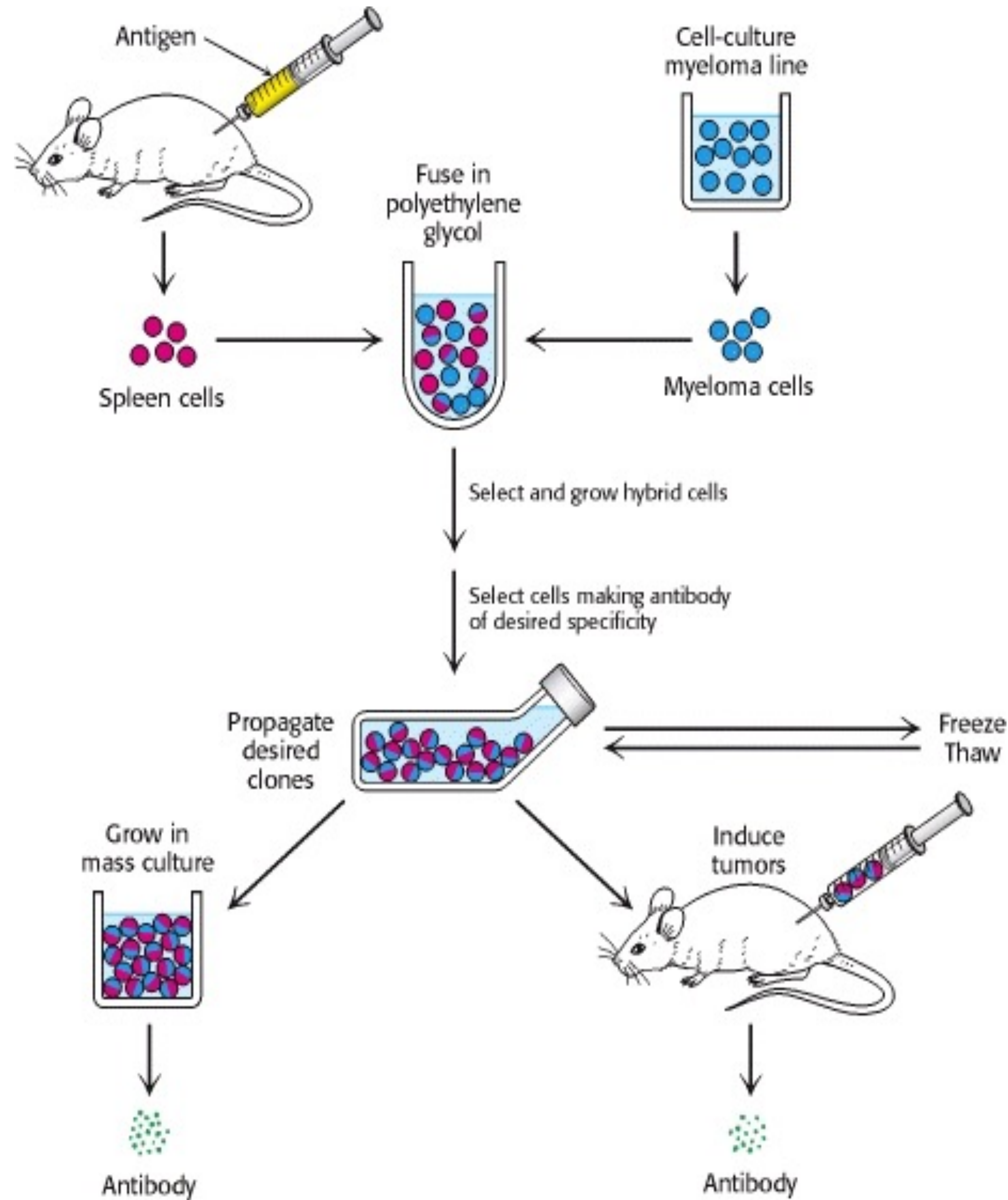
monoclonal

Individual B cell hybridoma is cloned and cultured.
Secreted antibodies are collected from culture media

recognize
ONE antigenic site
of injected substance



Generation of monoclonal antibodies

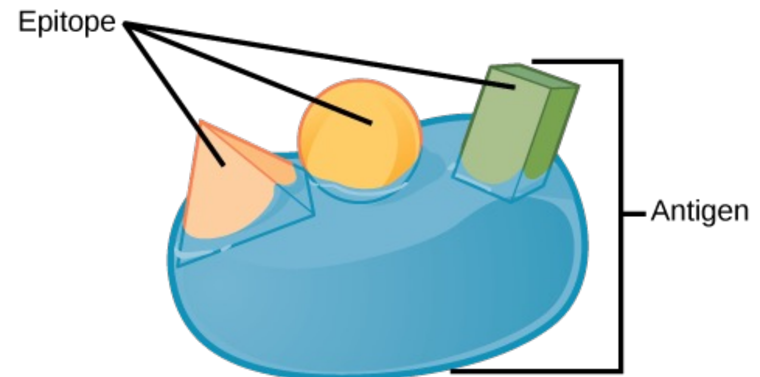


Antibodies: Definitions

Antibodies, or immunoglobulins (Igs), are γ-globulin proteins folded into well defined three-dimensional structures synthesized by living organisms, e.g. mice, rabbits or goats, or by living cells, in response to the presence of a foreign substance known as the antigen.

Immunogen: Molecule that is capable of eliciting an immune response by the immune system of an organism.

Antigen: Molecule that is able to bind to the product of that immune response: the antibody.

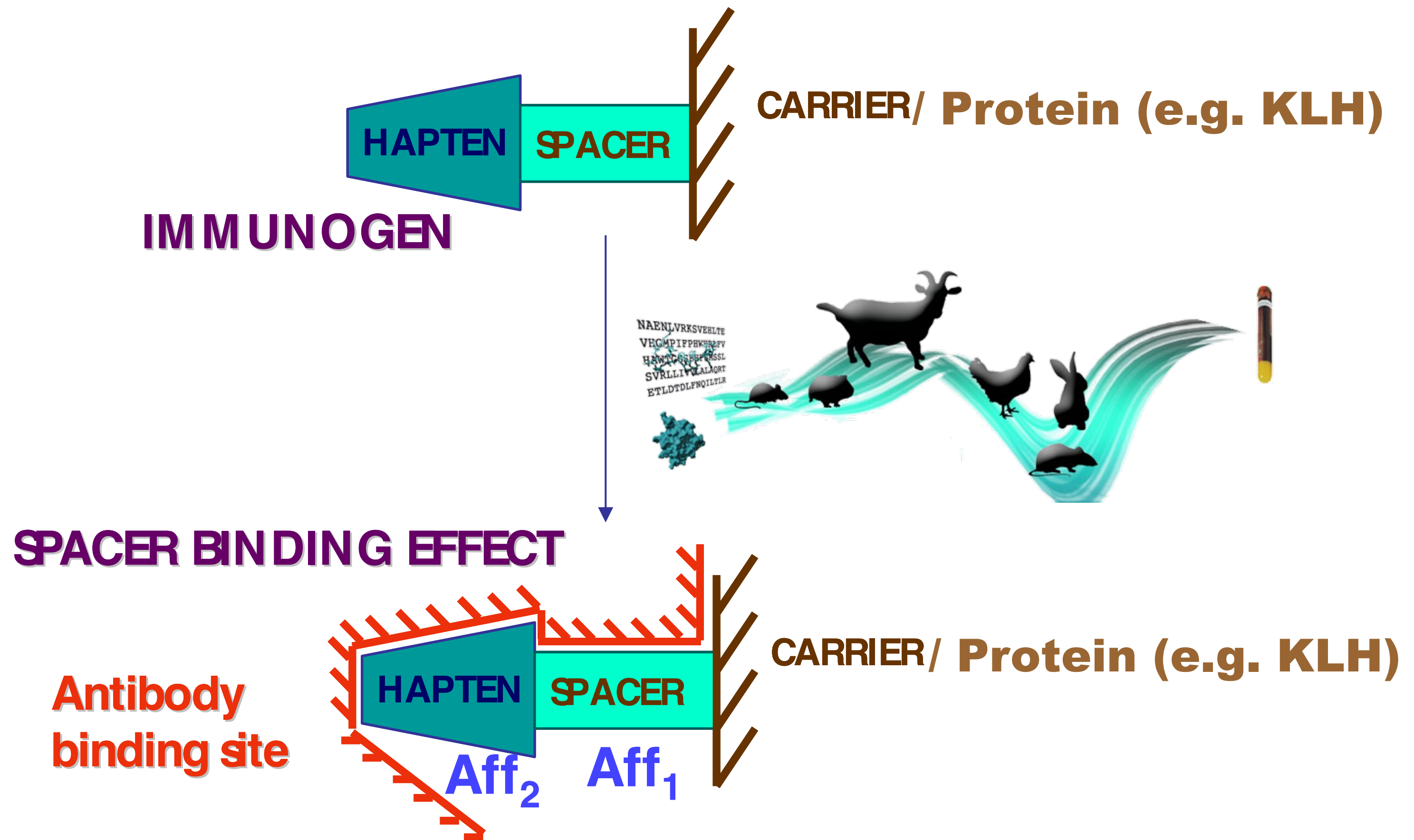


Epitope: An epitope is a specific location on the surface of an antigen that has a particular molecular structure and that is recognized by a particular antibody or a set of specific antibodies that the epitope elicits during the immune response.

Hapten: Small molecules (< 5000 Dalton) that need to be conjugated to a carrier protein (e.g. bovine serum albumin (BSA), keyhole limpet hemocyanin (KLH) or ovalbumin) to elicit the immune response.

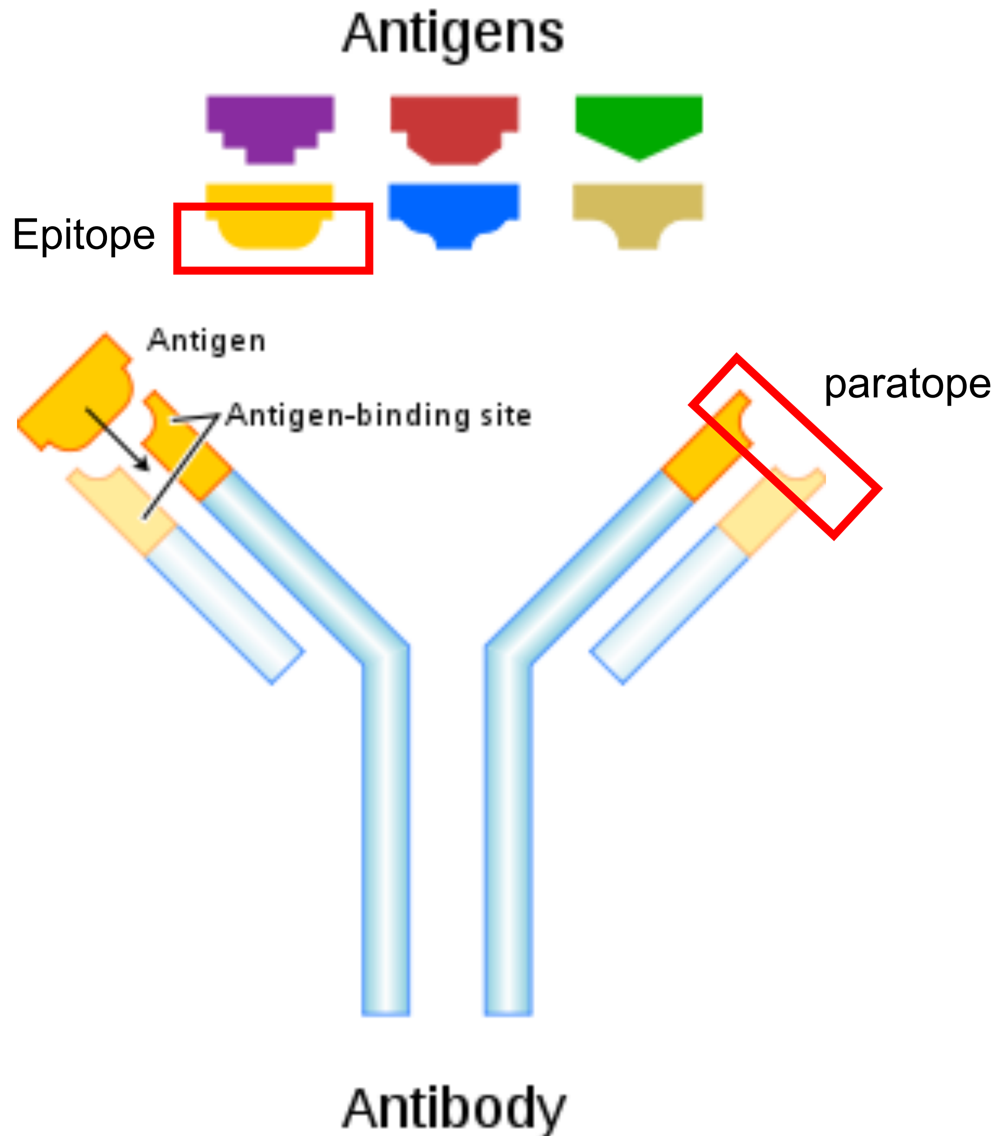
Antibodies: Definitions

Immunogens are always antigens but not all antigens are immunogens



Antibodies as immunochemical reagents

=> Antibodies are used as bioanalytical reagents to specifically detect and quantify other molecules



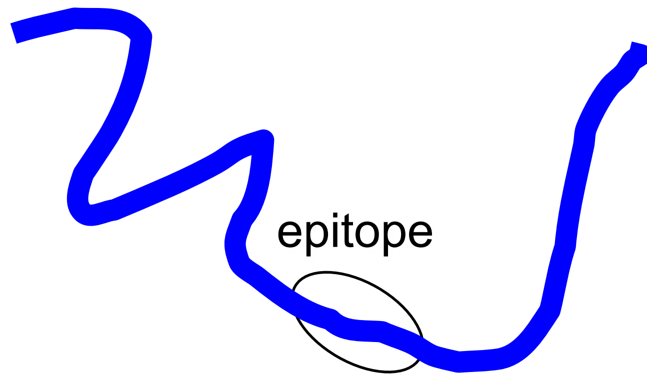
Continuous vs. discontinuous eptiopes

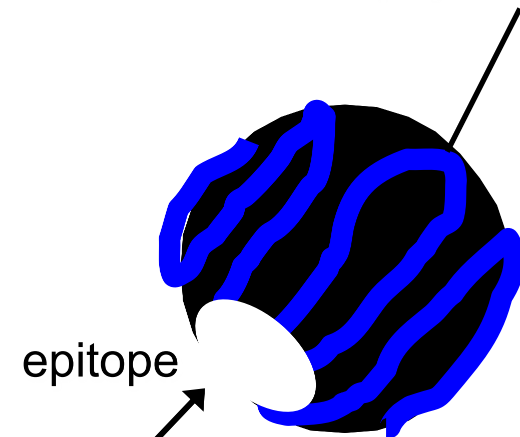
Continuous epitope:

short peptide or denatured protein structure, epitope consist of **sequential** amino acids

Discontinuous epitope:

present only in 3-dimensional protein structure, epitope comprises **non-sequential** amino acids

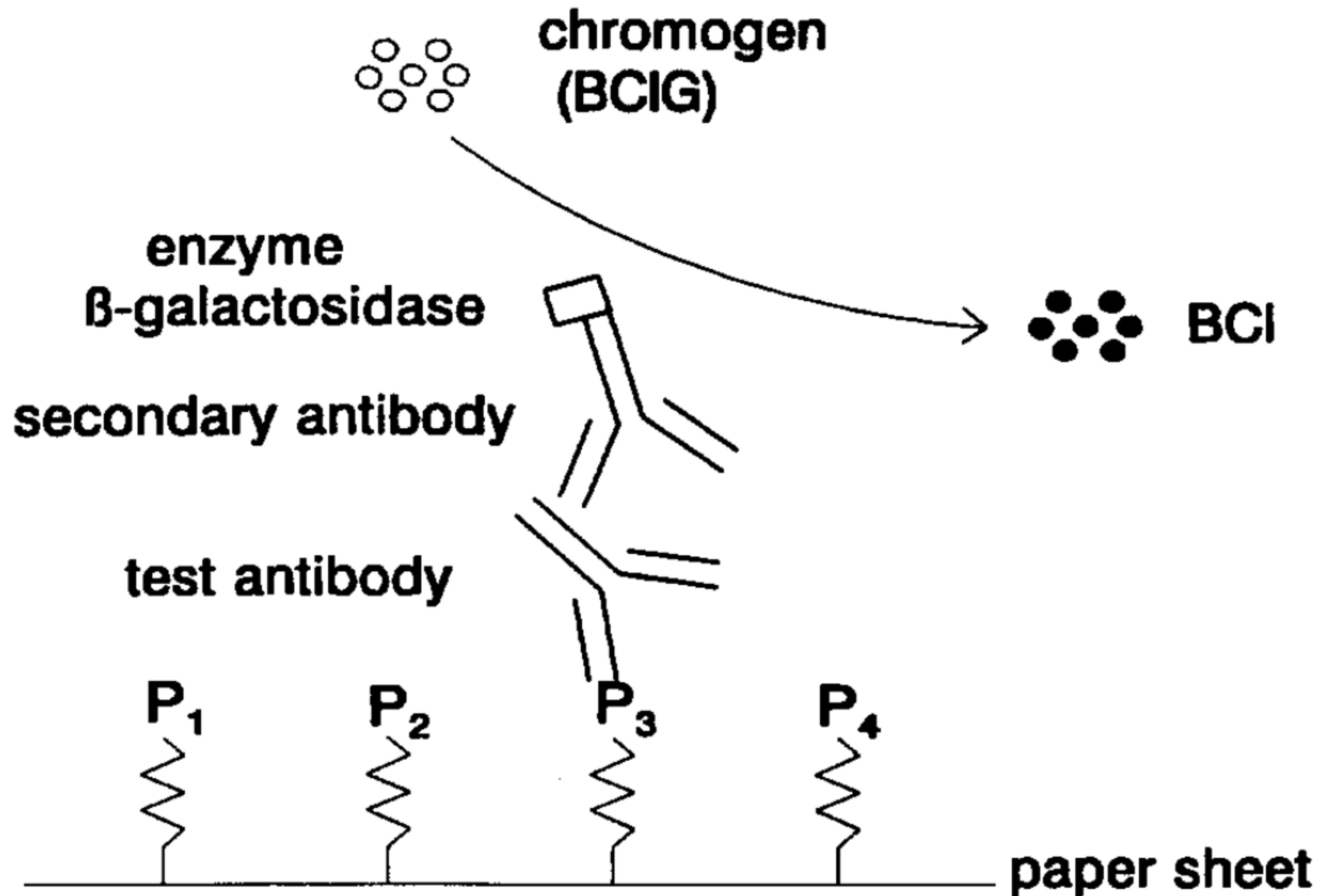




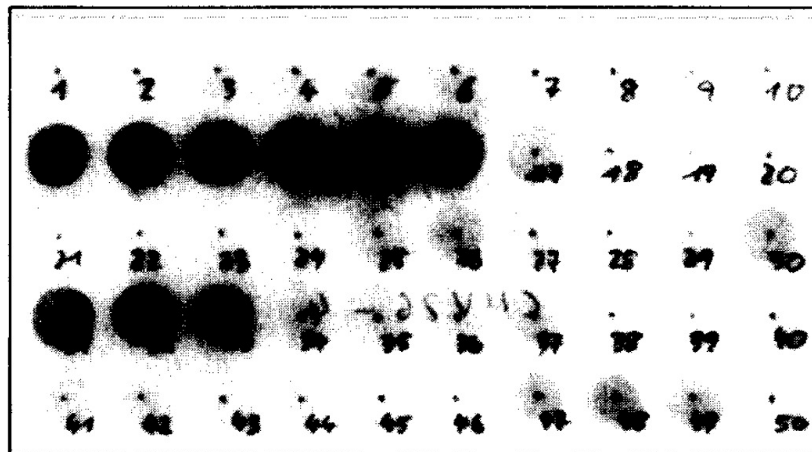
antibody binding/recognition site

Excursion: Epitope mapping

How do we know to what epitope an antibody binds?



Epitope mapping



CMV26-decapeptide scan

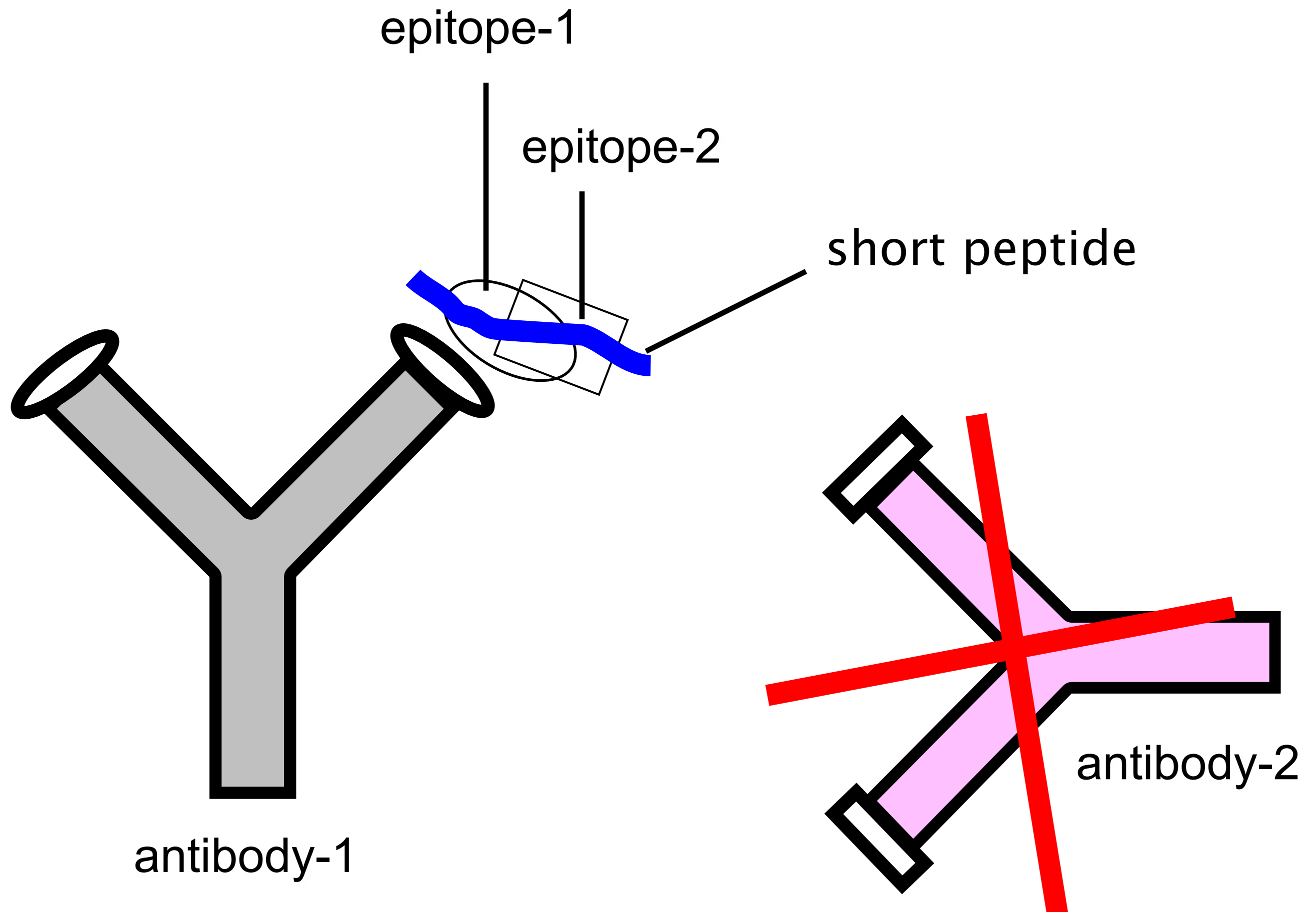
```
          10        20        30        40        50
IEGRGKSRGGGGGGGSLSSLANAGGLHDDGPGLDNDLMNEPMGLGGLGGGGGGGGKKH
```

1. IEGRGKSRGG
2. EGRGKSRGGG
3. GRGKSRGGGG
4. RGKSRGGGGG
5. GKSRGGGGGG
6. KSRGGGGGGG
7. SRGGGGGGGS
8. RGGGGGGGSL
9. GGGGGGGSLS
10. GGGGGGSLSS
11. **GGGGGSLSSL**
12. **GGGGSLSSLA**
13. **GGGSLSSLAN**
14. **GGSLSSLANA**
15. **GSLSSLANAG**
16. **SLSSLANAGG**
17. LSSLANAGGL
18. SSLANAGGLH
19. SLANAGGLHD
20. LANAGGLHDD
21. ANAGGLHDDG
22. NAGGLHDDGP
23. AGGLHDDGPG
24. GGLHDDGPGL
25. GLHDDGPGLD
26. LHDDGPGLDN
27. HDDGPGLDND
28. DDGPGLDNDL
29. DGPGLDNDLM
30. **GPGLDNDLMN**
31. **PGLDNDLMNE**
32. **GLDNDLMNEP**
33. **LDNDLMNEPM**
34. DNDLMNEPMG
35. NDLMNEPMGL
36. DLMNEPMGLG
37. LMNEPMGLGG
38. MNEPMGLGGL
39. NEPMGLGGLG
40. EPMGLGGLGG
41. PMGLGGLGGG
42. MGLGGLGGGG
43. GLGGLGGGGG
44. LGGLGGGGGG
45. GGLGGGGGGG
46. GLGGGGGGGG
47. LGGGGGGGGK
48. GGGGGGGGKK
49. GGGGGGGKKH

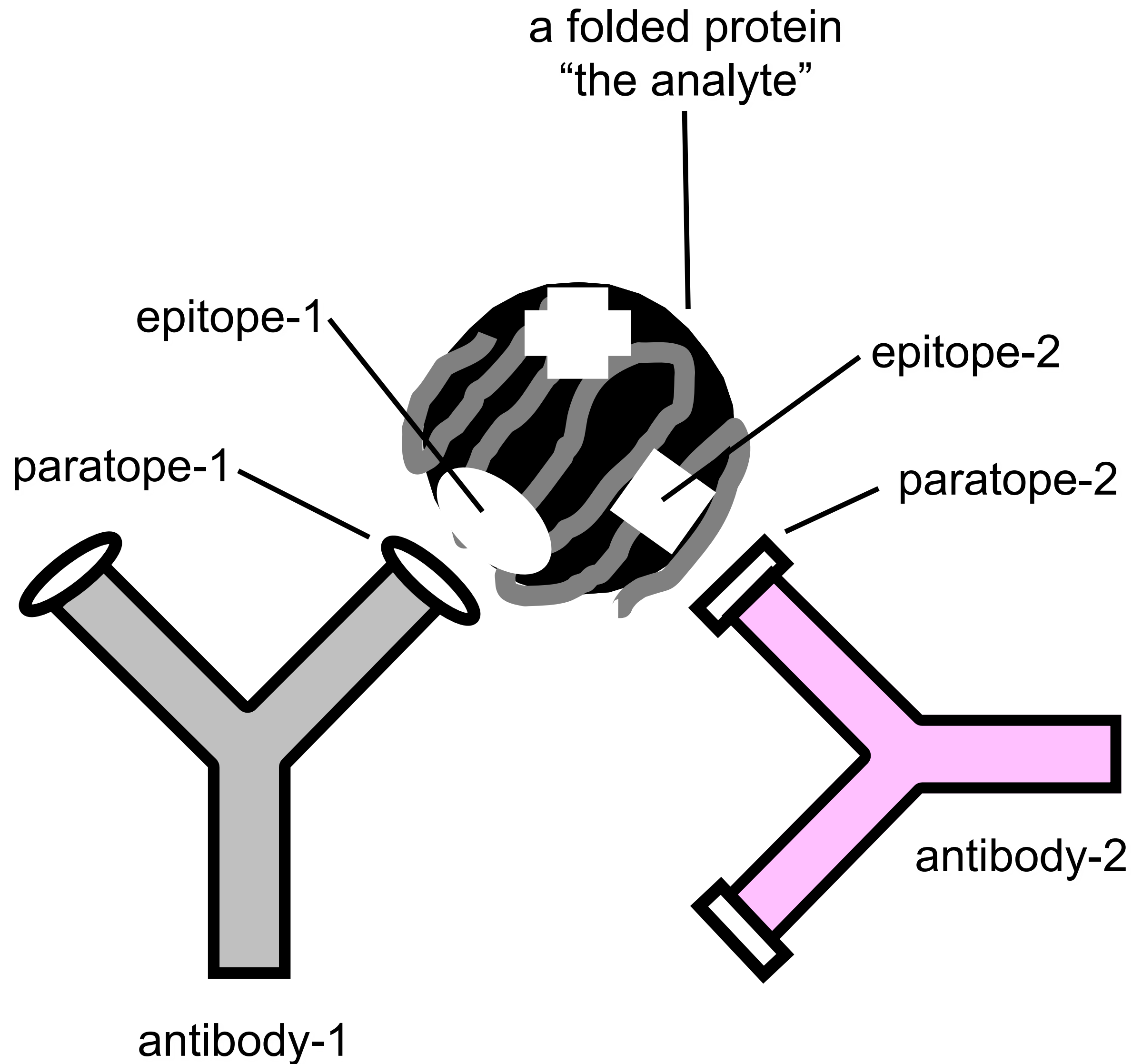
=> But only continuous epitopes

Overlapping eptiopes

Even small analytes can have multiple epitopes, but antibody binding to one epitope **blocks** another epitope, i.e. these epitopes are **overlapping**



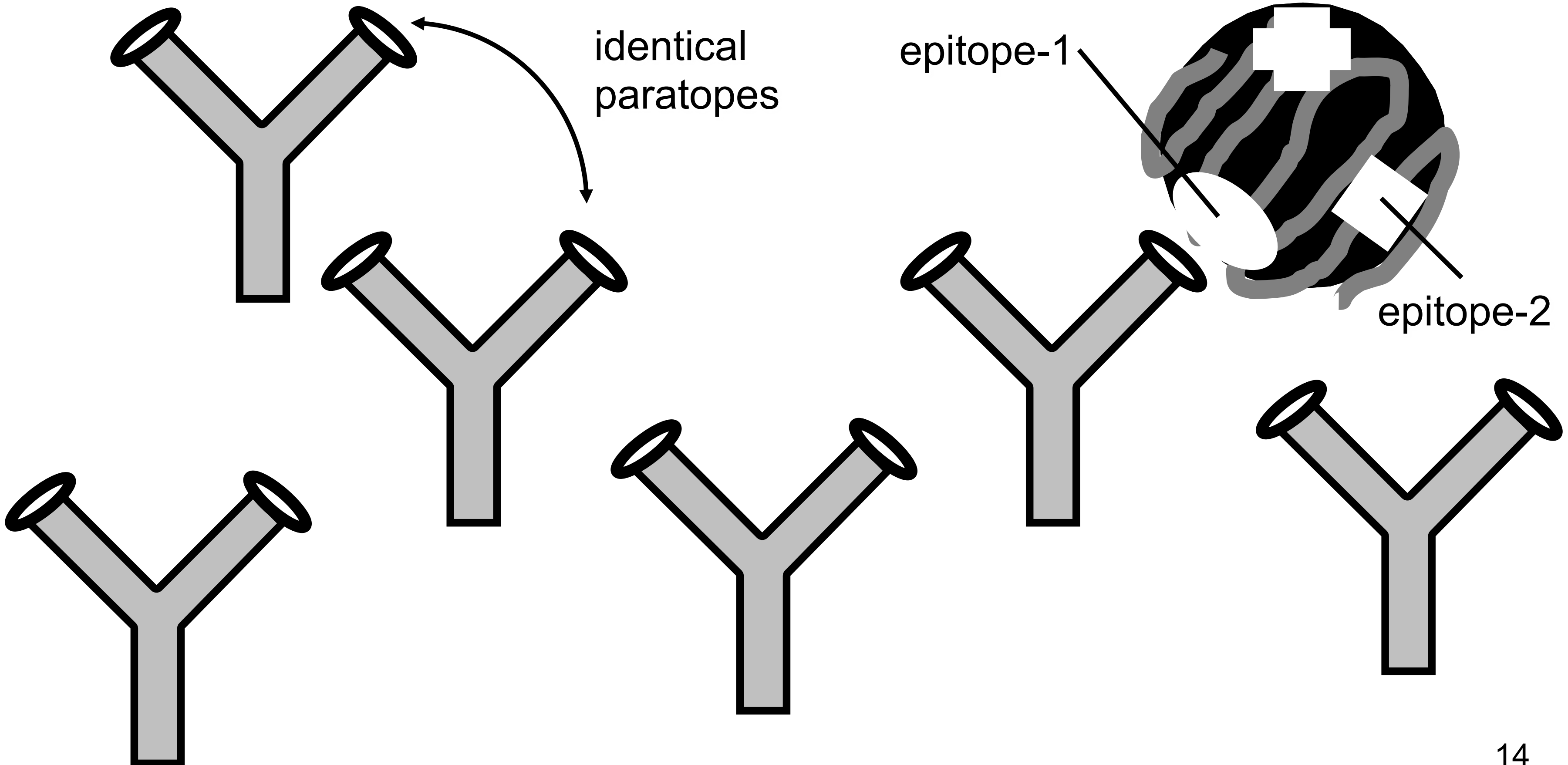
Non-overlapping eptiopes



Monoclonal antibody reagent

all antibodies are from the **same B cell clone** => reagent consist of identical antibodies, and all recognize and are specific for only one identical epitope

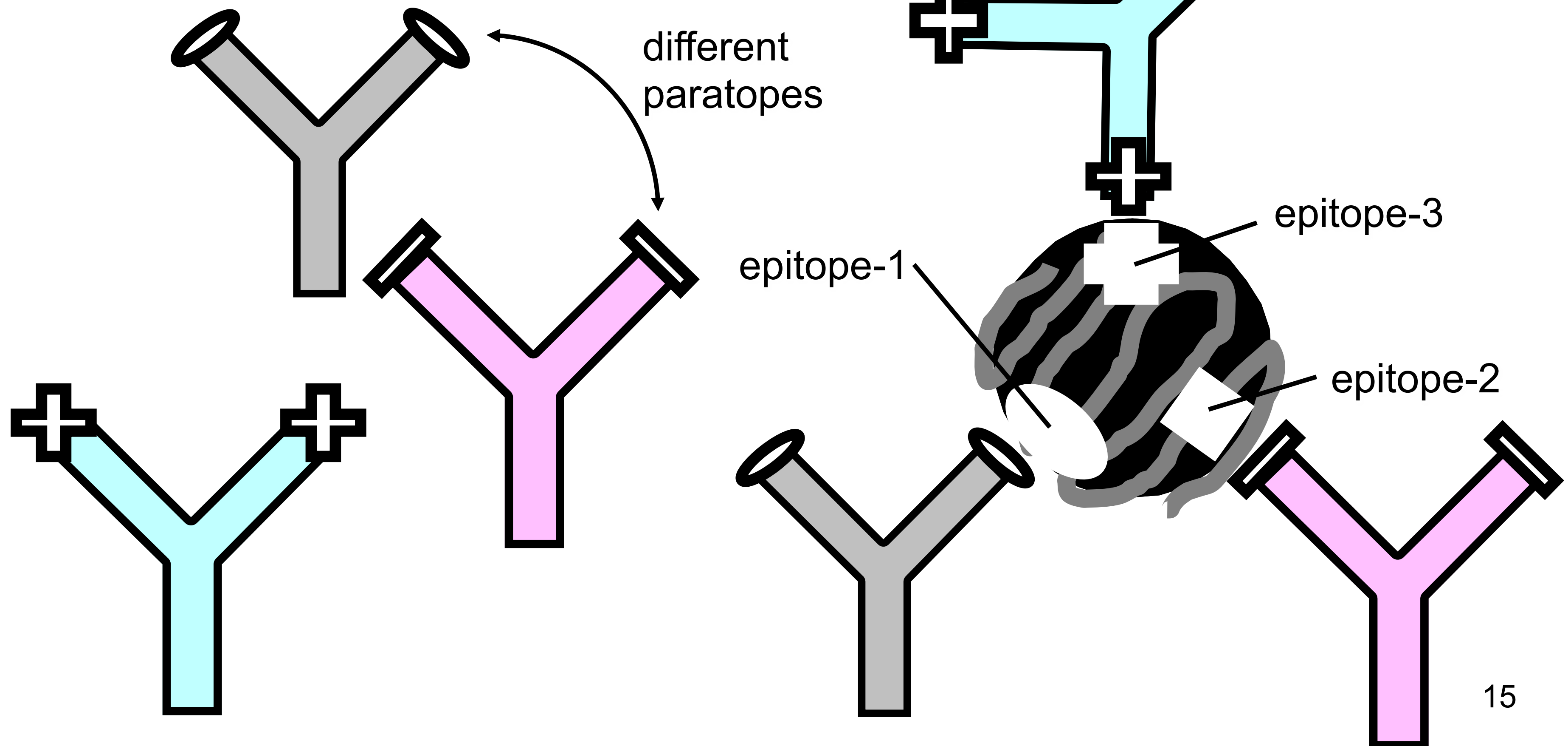
.. will bind only to one specific epitope in the analyte - unless there are multiple identical epitopes in the same analyte



Polyclonal antibody reagent

Mix of **different B cell clones**
=> reagent consist of antibodies that have different paratopes and recognize different epitopes, but exact composition is not known

… will bind simultaneously to one or several non-overlapping epitopes in the analyte



Antibody affinity

Affinity of an antibody

Tight fit / high affinity	Less interaction / lower affinity	Little interaction / very low affinity
Ab_1	Ab_2	Ab_3
A	B	C

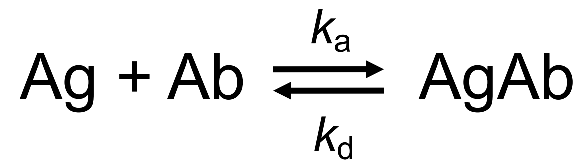
good epitope specificity decreasing epitope specificity

Antibody-antigen binding reaction

Ab (antibody)

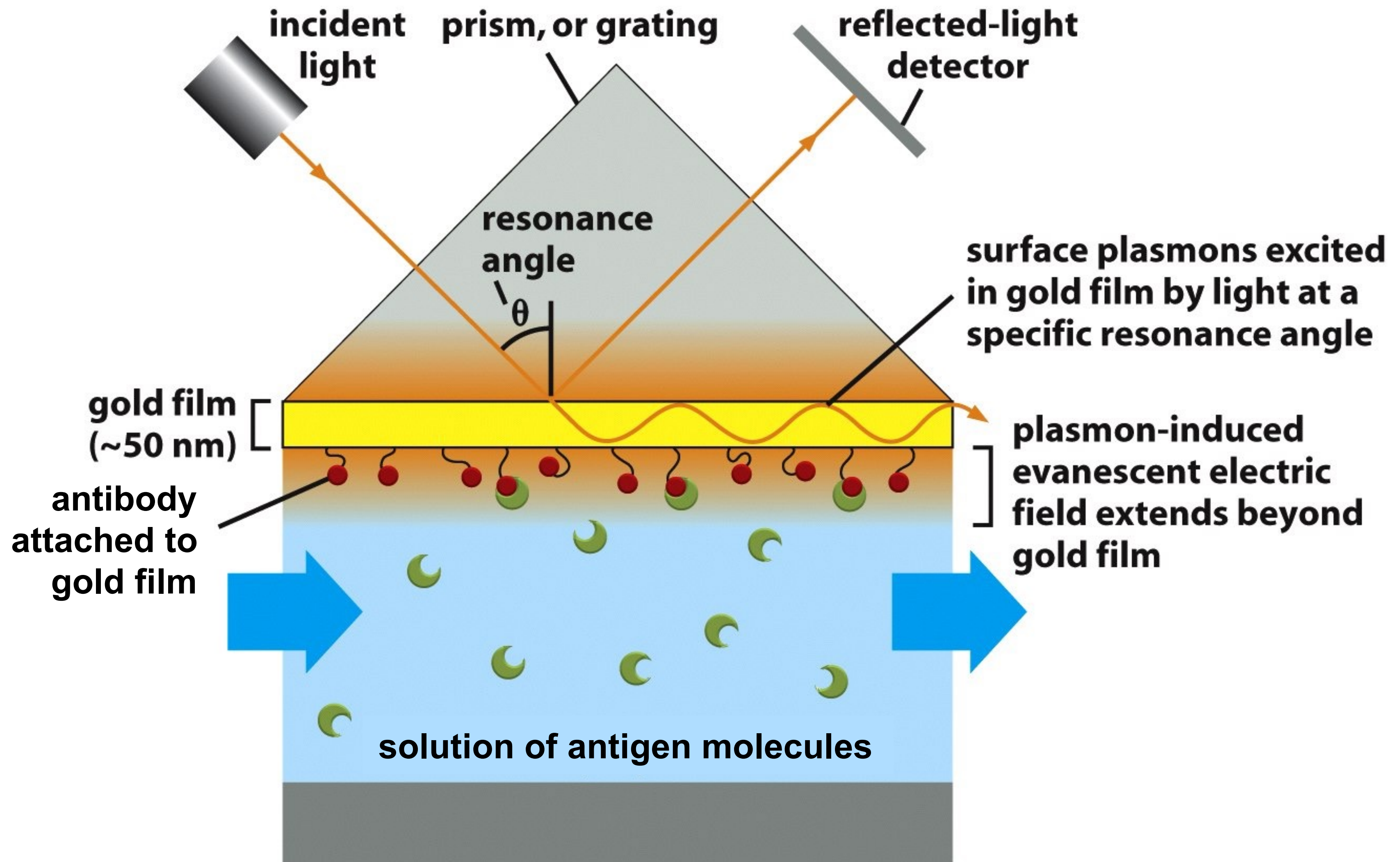
Ag (antigen/analyte)

AgAb



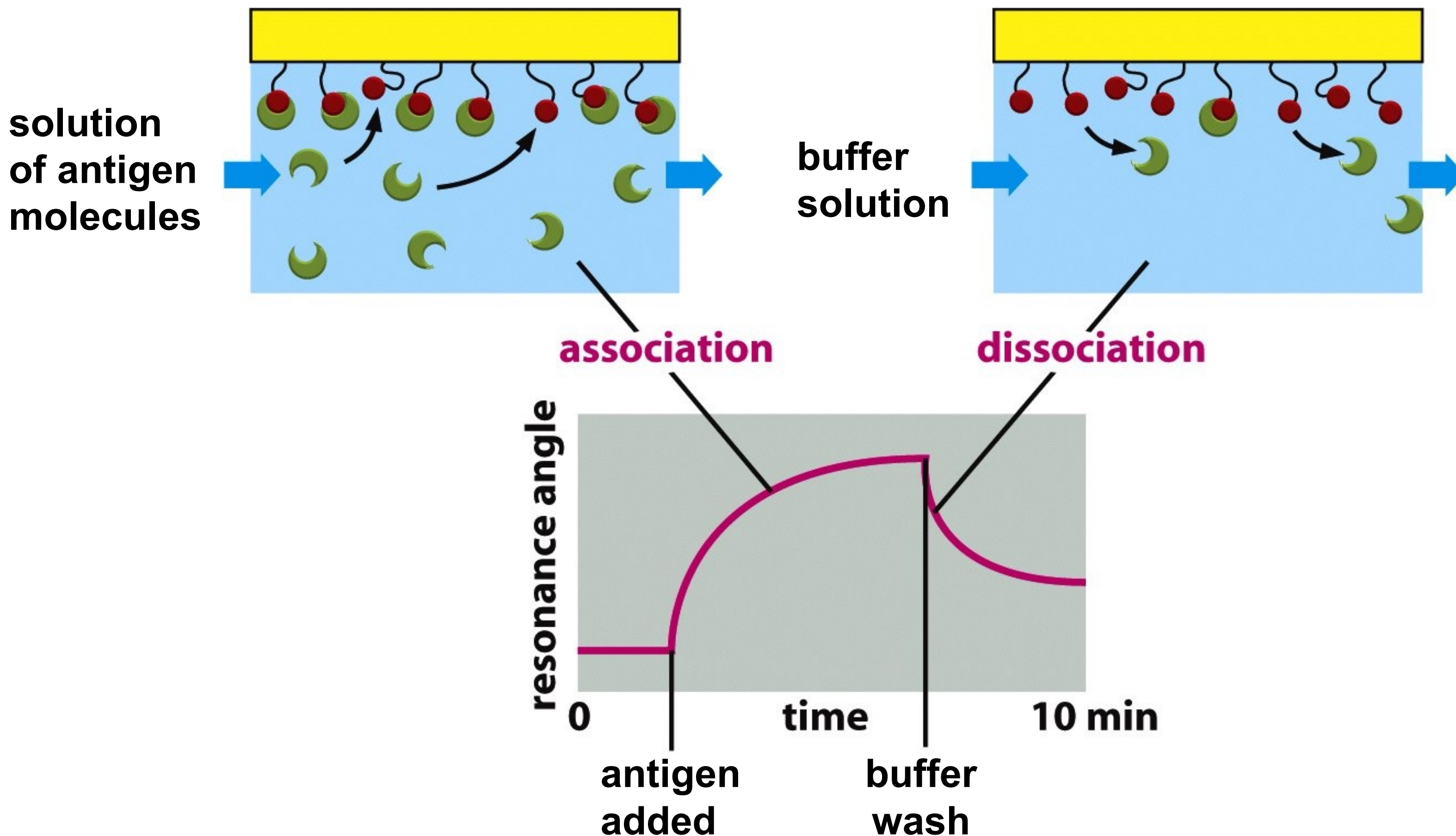
$$\mathrm{Ag} + \mathrm{Ab} \underset{k_\mathrm{d}}{\overset{k_\mathrm{a}}{\rightleftarrows}} \mathrm{AgAb}$$

Surface plasmon resonance (SPR)

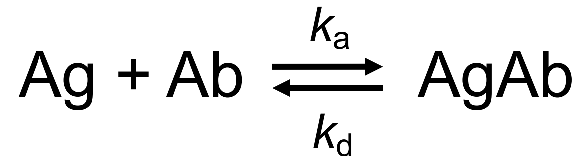


Determining the affinity of antibodies by SPR

(1) Binding of antigen to surface immobilized antibodies increases the refractive index of the surface layer.
(2) The resulting change of the resonance angel for plamson induction can be measured by a photodetector.



Affinity of an antibody



$$k_a[Ag][Ab] = k_d[AgAb]$$

reaction velocities at equilibrium:

$$K = \frac{k_a}{k_d} = \frac{[AgAb]}{[Ag][Ab]}$$

Bound antibody and antigen

free antigen

free antibody

k_a: association rate constant (on rate)

k_d: dissociation rate constant (off rate)

K: affinity constant

Affinity of an antibody

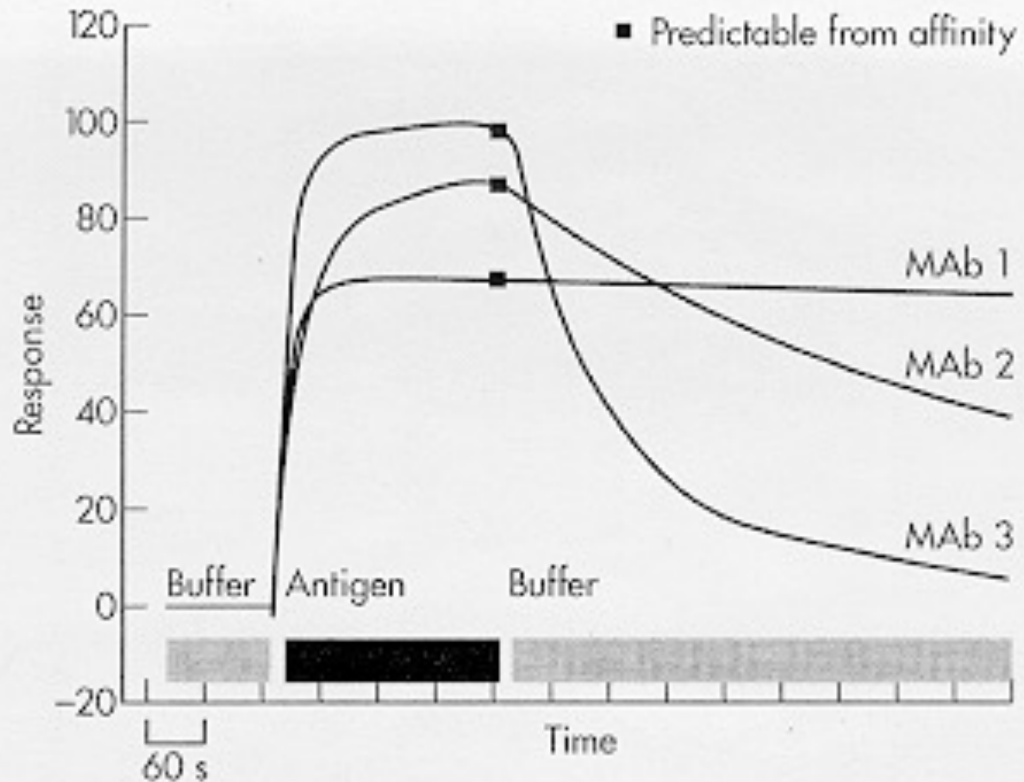


Fig. 1.6 Response curves illustrating the interaction of P24 antigen (125 nM) with three different monoclonal antibodies (MAbs).

MAb 1: extremely stable binding: off-rate is very slow

MAb 3: poorly suited for immuno-assay due to dissociation problems, e.g. during wash or during 2nd step if used as capture antibody

Sensorgrams for 3 moncolonal antibodies against HIV p24 surface Ag

Affinity of an antibody

$$K = \frac{k_a}{k_d} = \frac{[AgAb]}{[Ag][Ab]}$$

Bound antibody and antigen

free antigen

free antibody

approximate calculation of concentrations in equilibrium:

if $[Ag]_{tot} << [Ab]_{tot}$, only a very small antibody fraction is present in the complex $[AgAb]$

=> $[Ab] \approx [Ab]_{tot}$

$[Ag]_{tot} = [Ag] + [AgAb]$

$[Ab]_{tot} = [Ab] + [AgAb]$

$$[AgAb] = \frac{[Ab]_{tot}[Ag]_{tot}\,K}{([Ab]_{tot}\,K) + 1}$$

free (unbound) concentrations

Affinity of an antibody

Calculating the equilibrium concentration

$$[AgAb] = \frac{[Ab]_{tot}[Ag]_{tot}\,K}{([Ab]_{tot}\,K) + 1}$$

$[Ab]_{tot} = 1 * 10^{-9}$ M
$[Ag]_{tot} = 1 * 10^{-12}$ M (i.e. much smaller)

$K = 1 * 10^{9}\ M^{-1}$

by calculating we get $[AgAb] = 0.5 * 10^{-12}$ M (i.e. 50%)

"rule of thumb": when $[Ab]_{tot} = 1/K$ then $[AgAb] = 50\%\ [Ag]_{tot}$
$[Ab]_{tot} = 10/K$ then $[AgAb] = 90\%\ [Ag]_{tot}$
$[Ab]_{tot} = 0.1/K$ then $[AgAb] = 10\%\ [Ag]_{tot}$

Affinity of an antibody

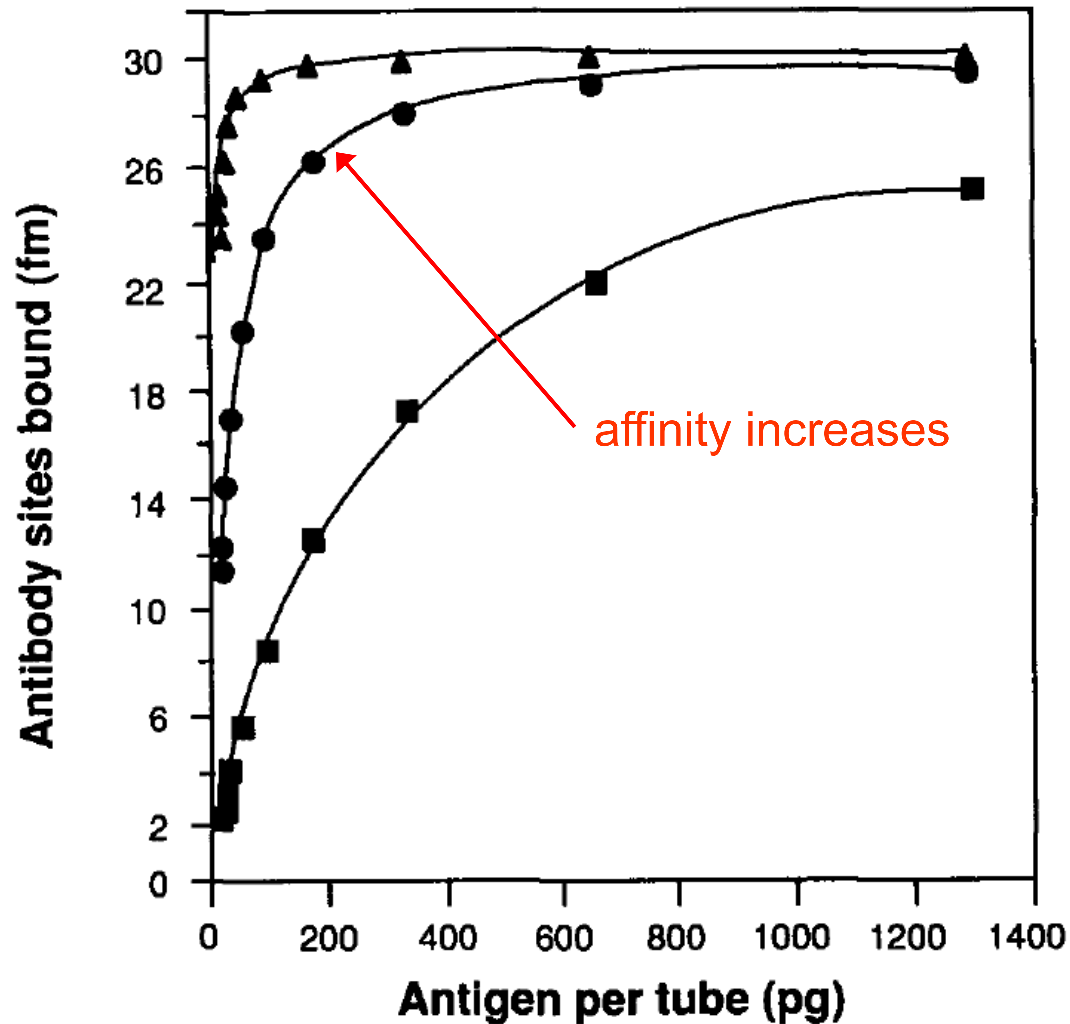
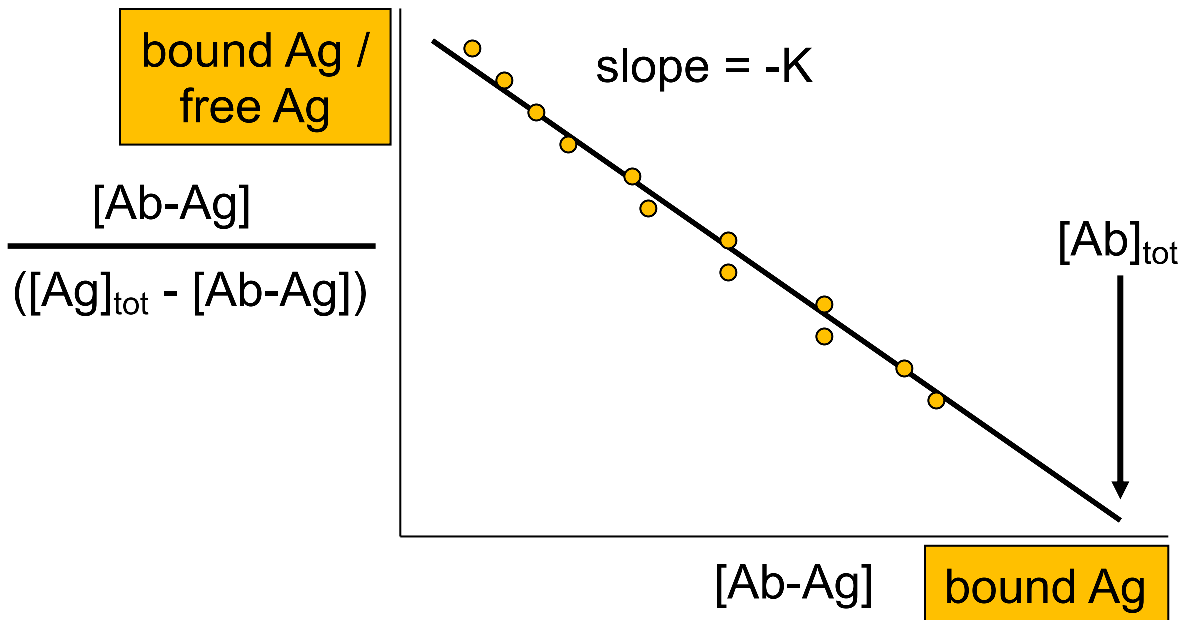


Figure 8.4 Estimation of filled antibody sites, at different concentrations of antigen, for three antibodies with different affinity constant (l/mol). ■ = 1×10^9, ● = 1×10^{10}, ▲ = 1×10^{11}.

Affinity of an antibody: Scatchard plot

linearization:

$$K\,([Ab]_{tot} - [Ab\text{-}Ag]) = \frac{[Ab\text{-}Ag]}{([Ag]_{tot} - [Ab\text{-}Ag])} = \frac{\text{bound Ag}}{\text{free Ag}}$$

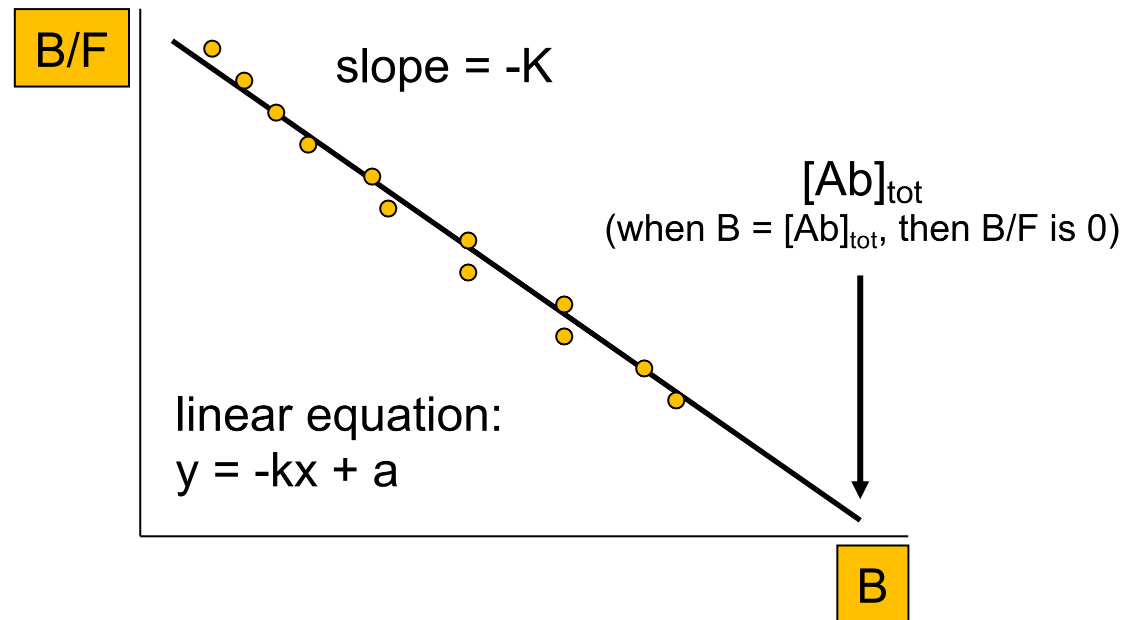


Affinity of an antibody: Scatchard plot

B = [Ag-Ab]   F = [Ag] = $[Ag]_{tot}$ – [Ag-Ab]

K ($[Ab]_{tot}$ – B) = B/F => B/F = – K B + K $[Ab]_{tot}$

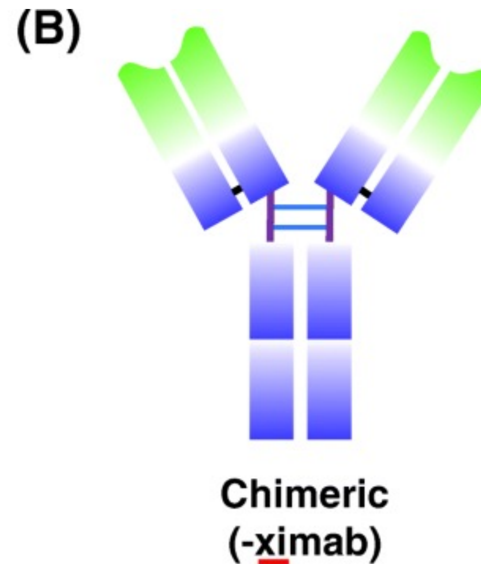
constant



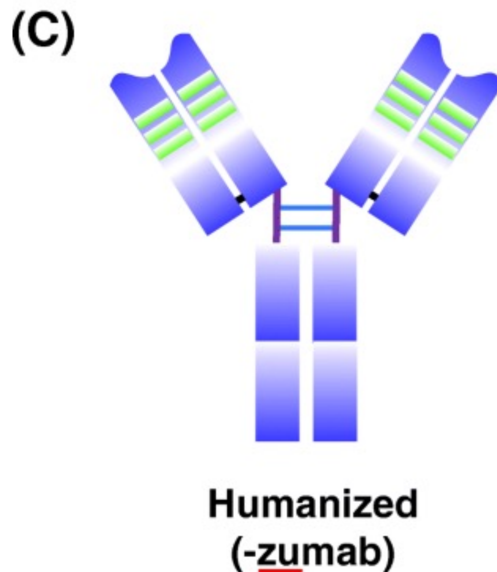
=> Typcially replaced by non-linear fitting using computer programs

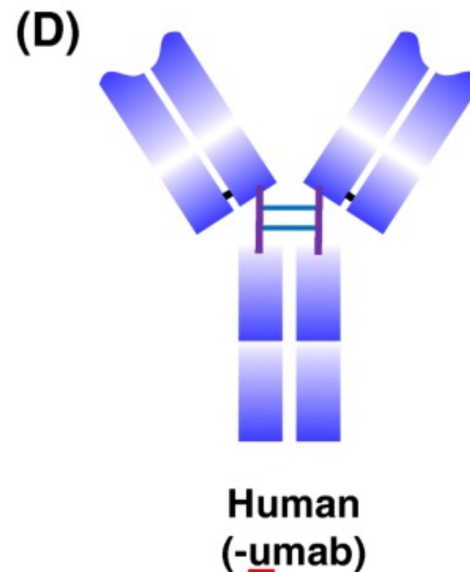
Antibody engineering

Excursion: Antibody engineering for therapy

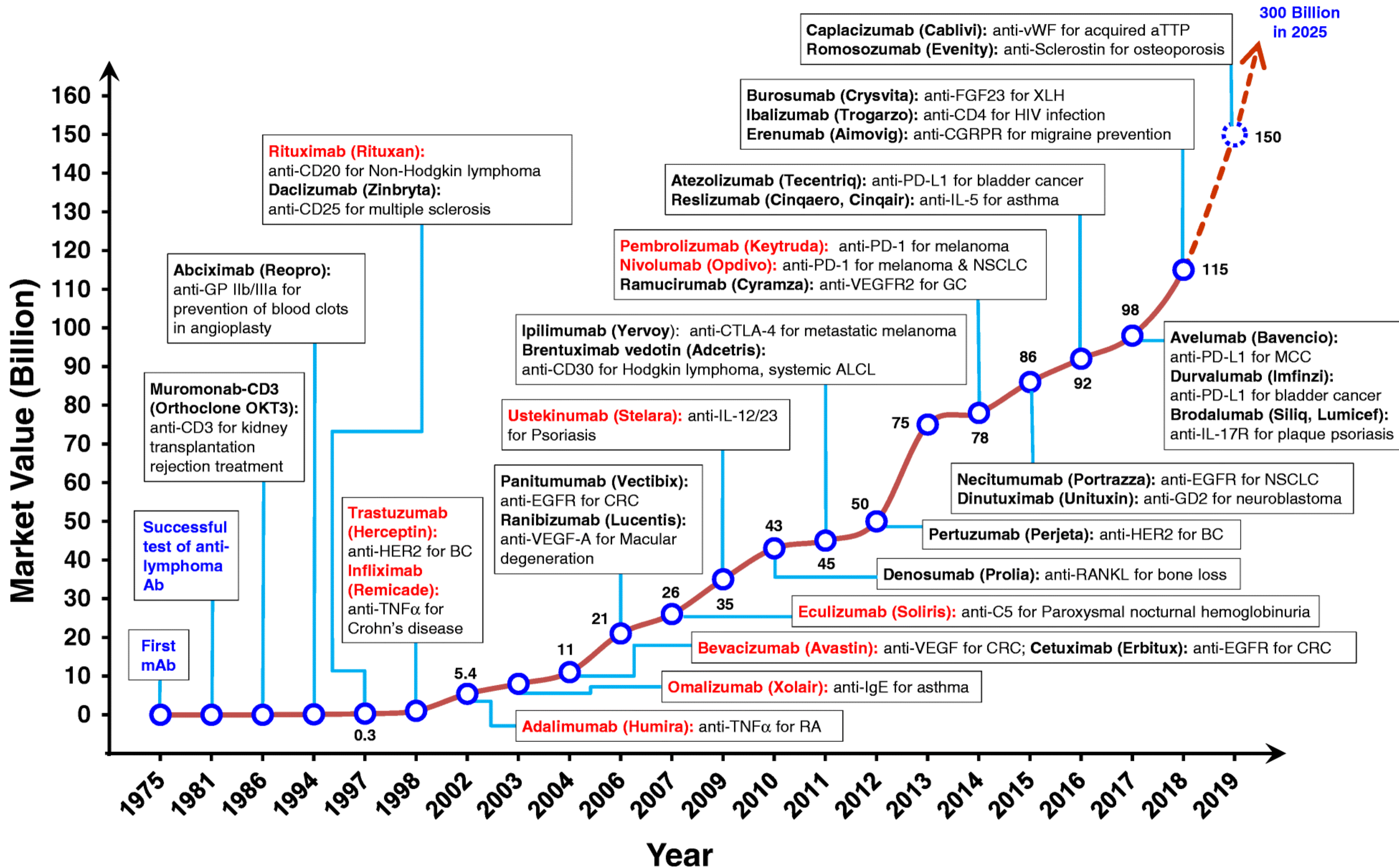


Natural antibodies (raised in mice) are potentially immunogenic
=> Potential side effects





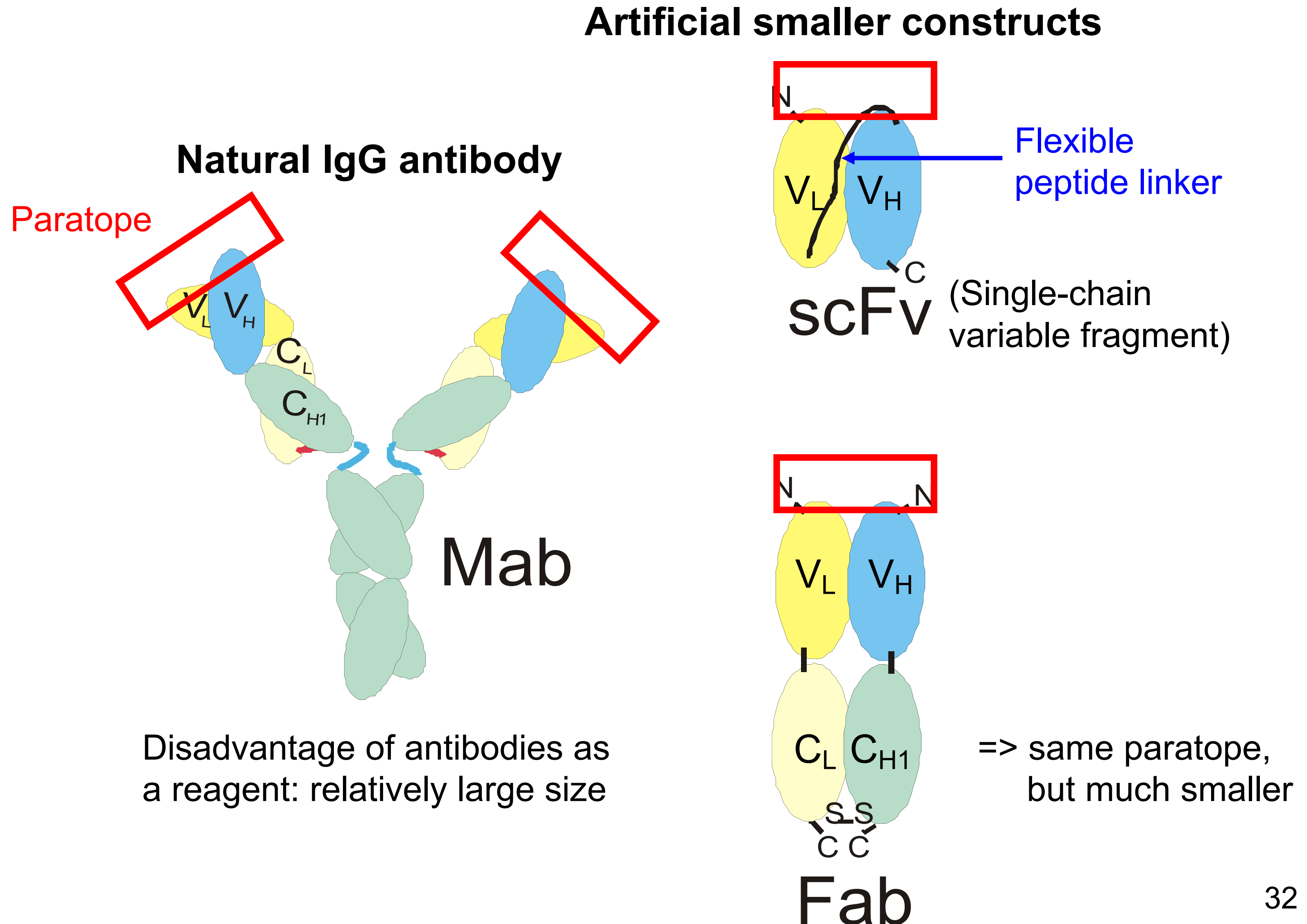
Therapeutic antibodies (market value)



Humanized antibodies

No.	Drug	Indication (1st US FDA Approval Year)	Company	2018 Revenue (USD, billion)
1	Adalimumab	Rheumatoid arthritis (2002)	AbbVie	$19.9 bn
	(Humira)	Psoriatic arthritis (2005)		
		Ankylosing spondylitis (2006)		
		Juvenile Idiopathic Arthitis (2008)		
		Psoriasis (2008)		
		Crohn's disease (2010)		
		Ulcerative colitis (2012)		
		Hidradenitis suppurativa (2015)		
		Uveitis (2018)		
2	Nivolumab	Melanoma (2015)	Bristol-Myers Squibb	$7.6 bn
	(Opdivo)	Non-small cell lung cancer (2015)		
		Renal cell carcinoma (2015)		
		Head and neck squamous cell (2016)		
3	Pembrolizumab	Melanoma (2014)	Merck & Co	$7.2 bn
	(Keytruda)	Head and neck cancer (2016)		
		Non-small cell lung caccer (2015)		
		Lymphoma (2018)		
		Cervical cancer (2018)		
		Microsatellite instability-high cancer (2018)		
4	Trastuzumab	Breast cancer (1998)	Roche	$7.0 bn
	(Herceptin)	Gastric cancer (2010)		
5	Bevacizumab	Colorectal cancer (2004)	Roche	$6.8 bn
	(Avastin)	Non-small cell lung caccer (2006)		
		Breast ERB2 negative cancer (2008)		
		Renal cell carcinoma (2009)		
		Glioblastoma (2011)		
6	Rituximab,	Non-Hodgkin's lymphoma (1997)	Roche	$6.8 bn

Recombinant antibody fragments



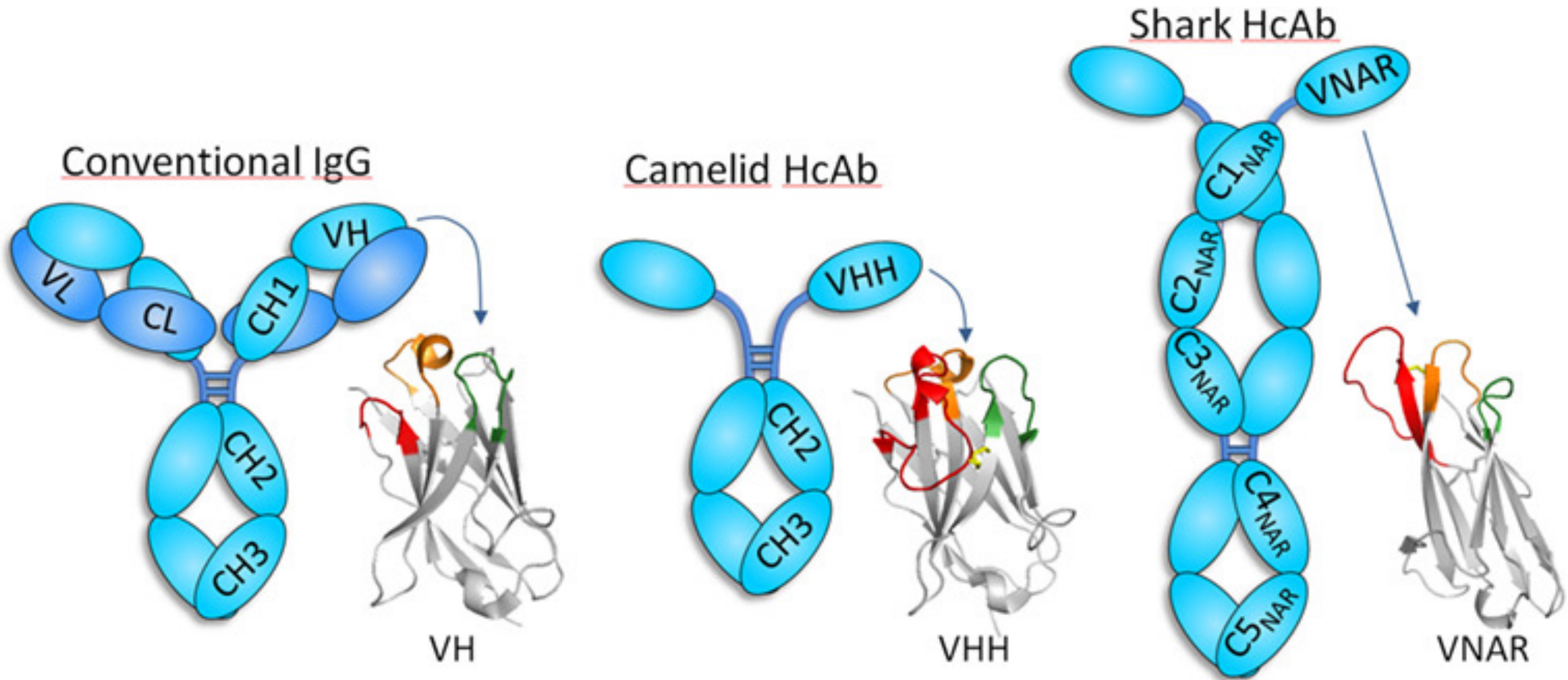
Recombinant antibody fragments

Immortalization of hybridomas through cloning
or
generation of new **antibodies without immunization**

- Greater speed of **production** *(E. coli batch fermentation)*
- New specificities especially for **poor immunogens**
- Possibility to **fine-tune** antibody specificity and affinity
- Possibility to **tailor make** the antibody to perform special tasks
    - tags, handles (for conjugation, immobilization)
    - fusing to other protein (e.g. enzymes)

Likely to be increasingly used in **miniaturised systems** to enable full control of antibody performance.

Heavy chain antibodies



our own most common antibody

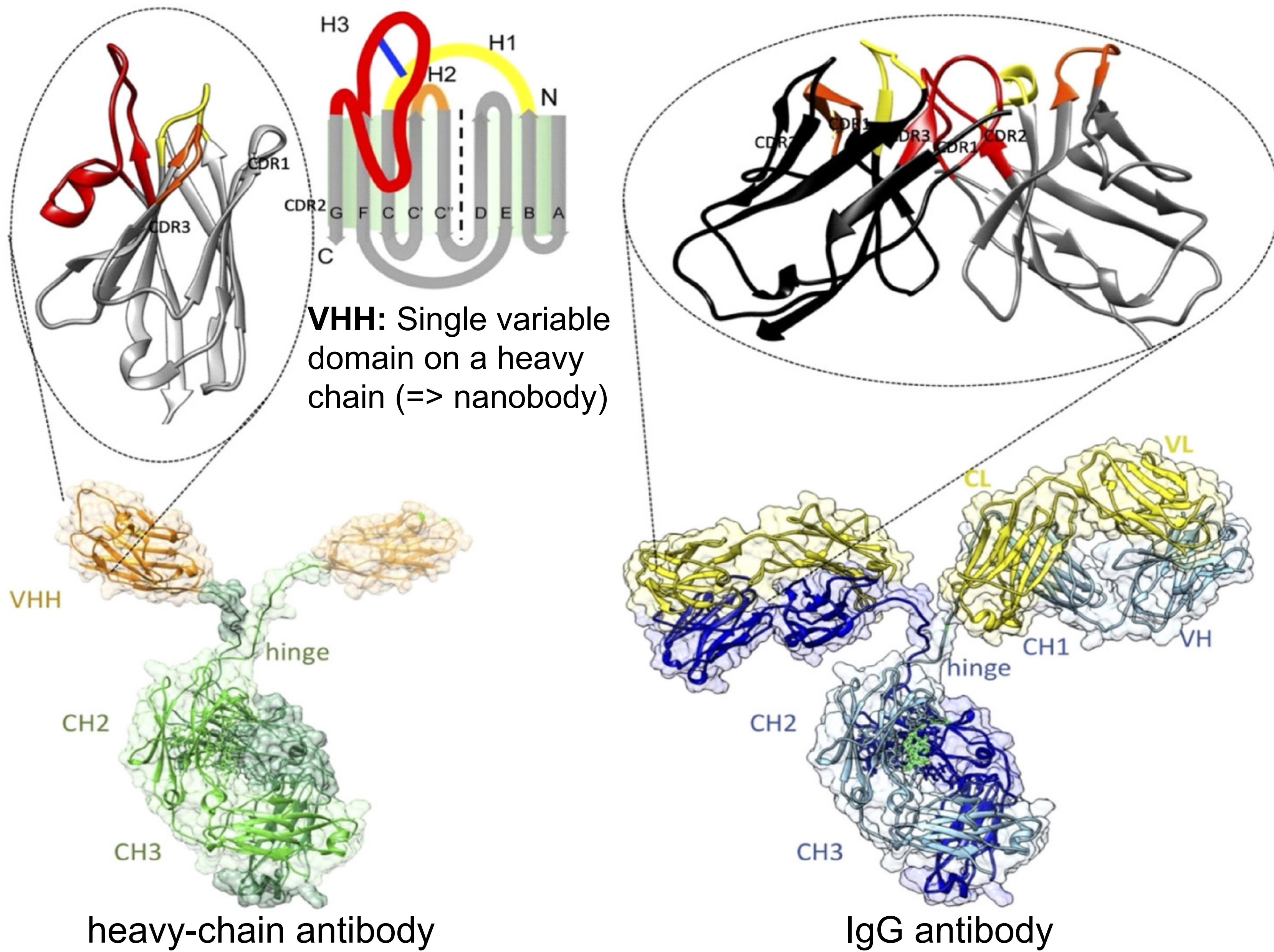
heavy chain antibodies

(velbloud, dromedár, lama)

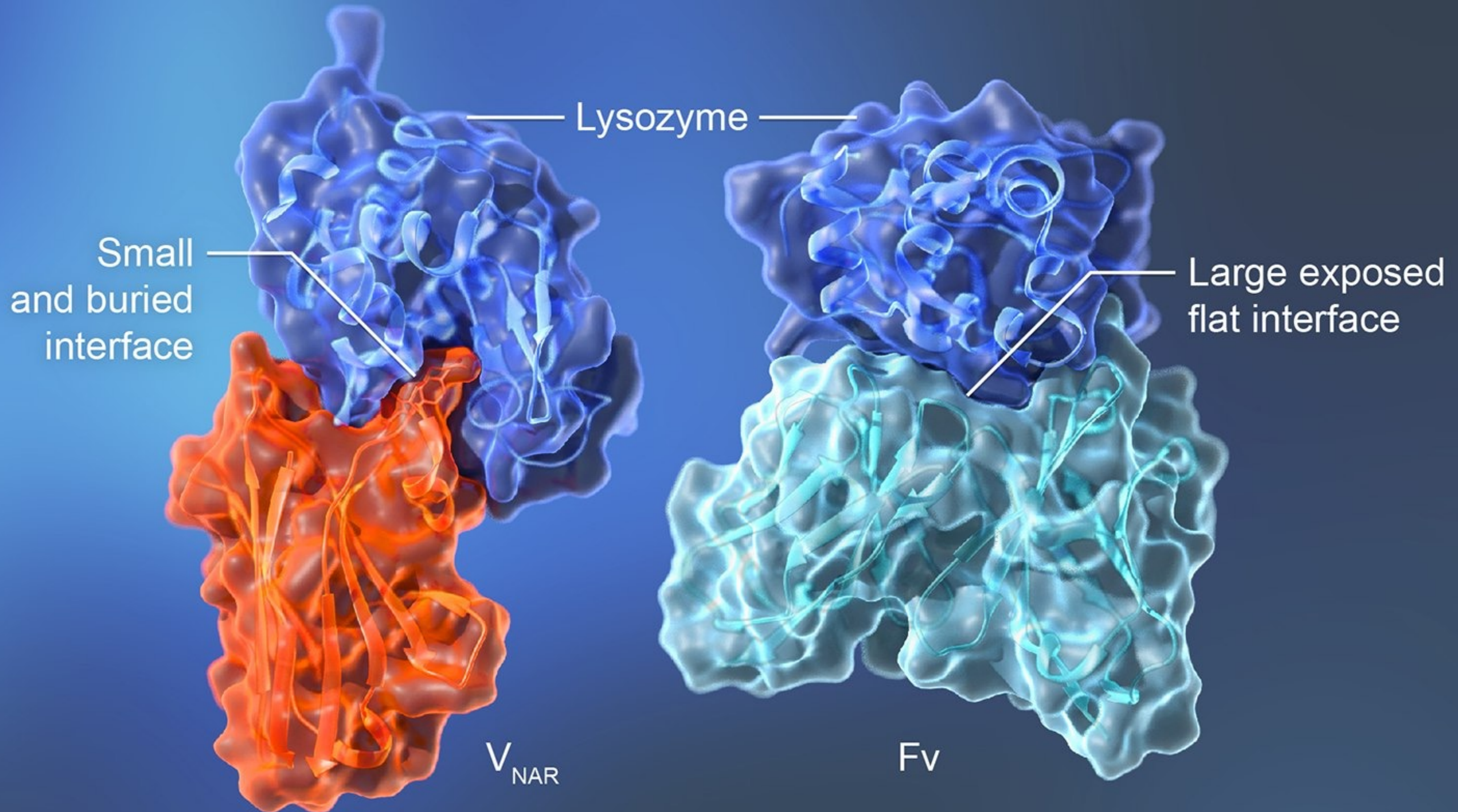
(žralok)

Front. Immunol., 2017 https://doi.org/10.3389/fimmu.2017.00977

From heavy chain antibodies to nanobodies



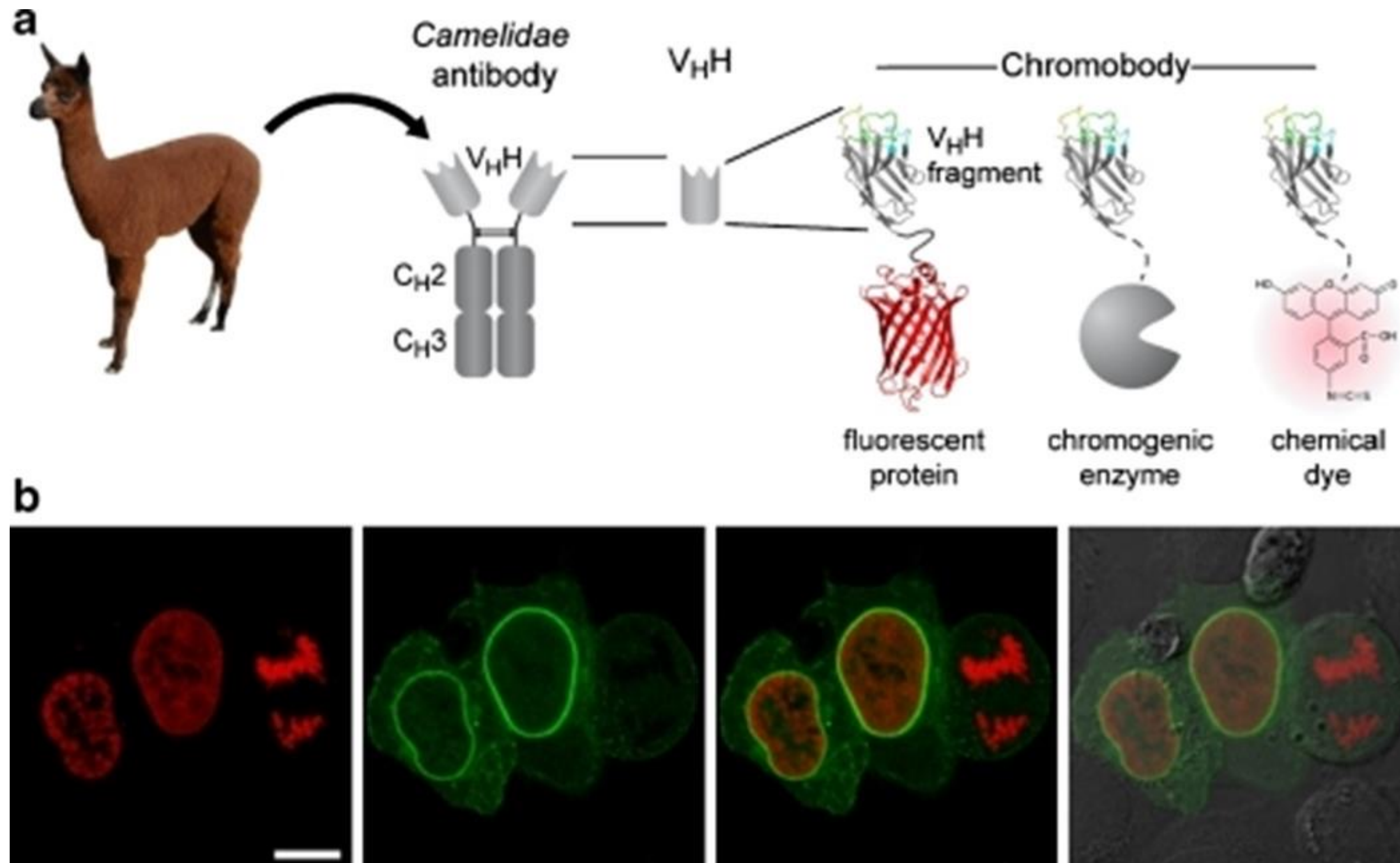
Nanobodies: Detection of hidden epitopes



Advantages of nanobodies

- Mass: ca. 15 kDa (IgG: 150 kDa), 2.5 nm diameter (IgG 15 nm)
- High solubility
- Rapid targeting and fast blood clearance
- Detection of “hidden“ epitopes
- Easy cloning: Recombinant engineering and protein expression *in vitro* in bacterial production systems are much simpler
- Very stable and heat resistant (no cold storage required)
- Simple genetic structure allows easy re-engineering of nanobodies to introduce new antigen-binding characteristics or attach labels

Recombinant nanobodies

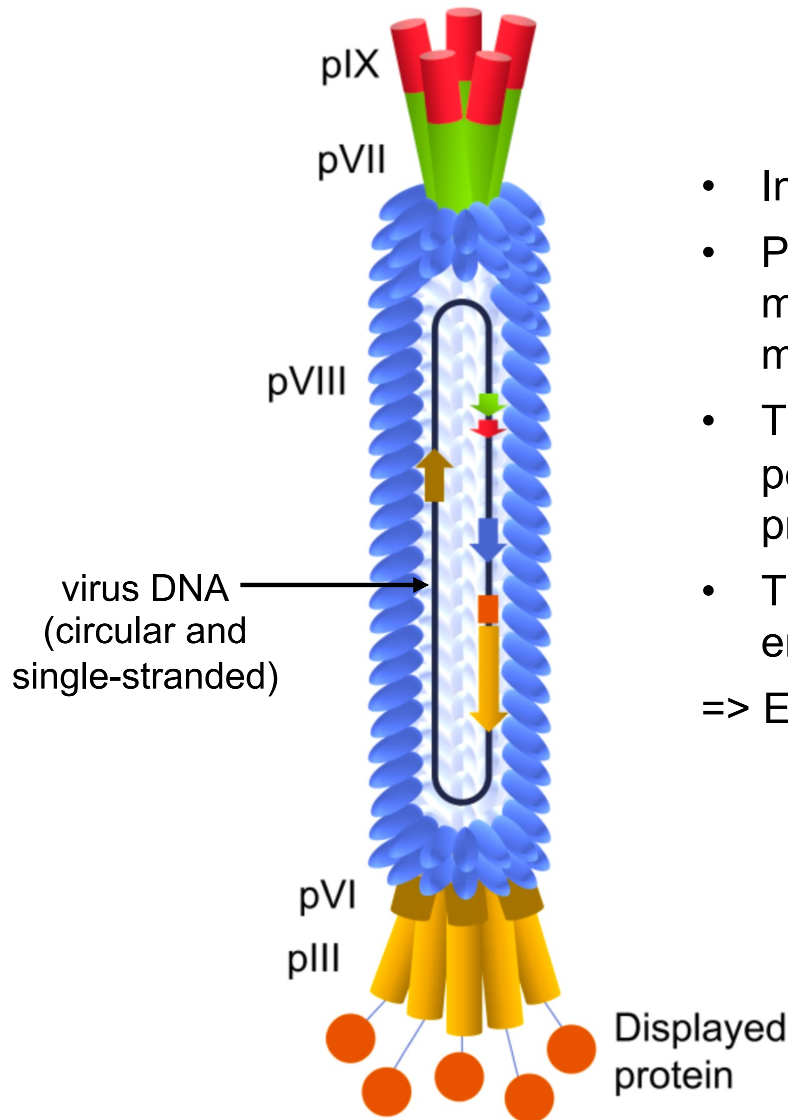


a Chromobodies.

b Detection of the nuclear lamina with lamin chromobody in living cells. Confocal images of HeLa cells coexpressing lamin chromobody (*green*) and red fluorescent histone H2B as a mitosis marker. Scale bar: 10 μm

Anal. Bioanal. Chem. (2010) 397: 3203–3208

Phage display using filamentous phage M13

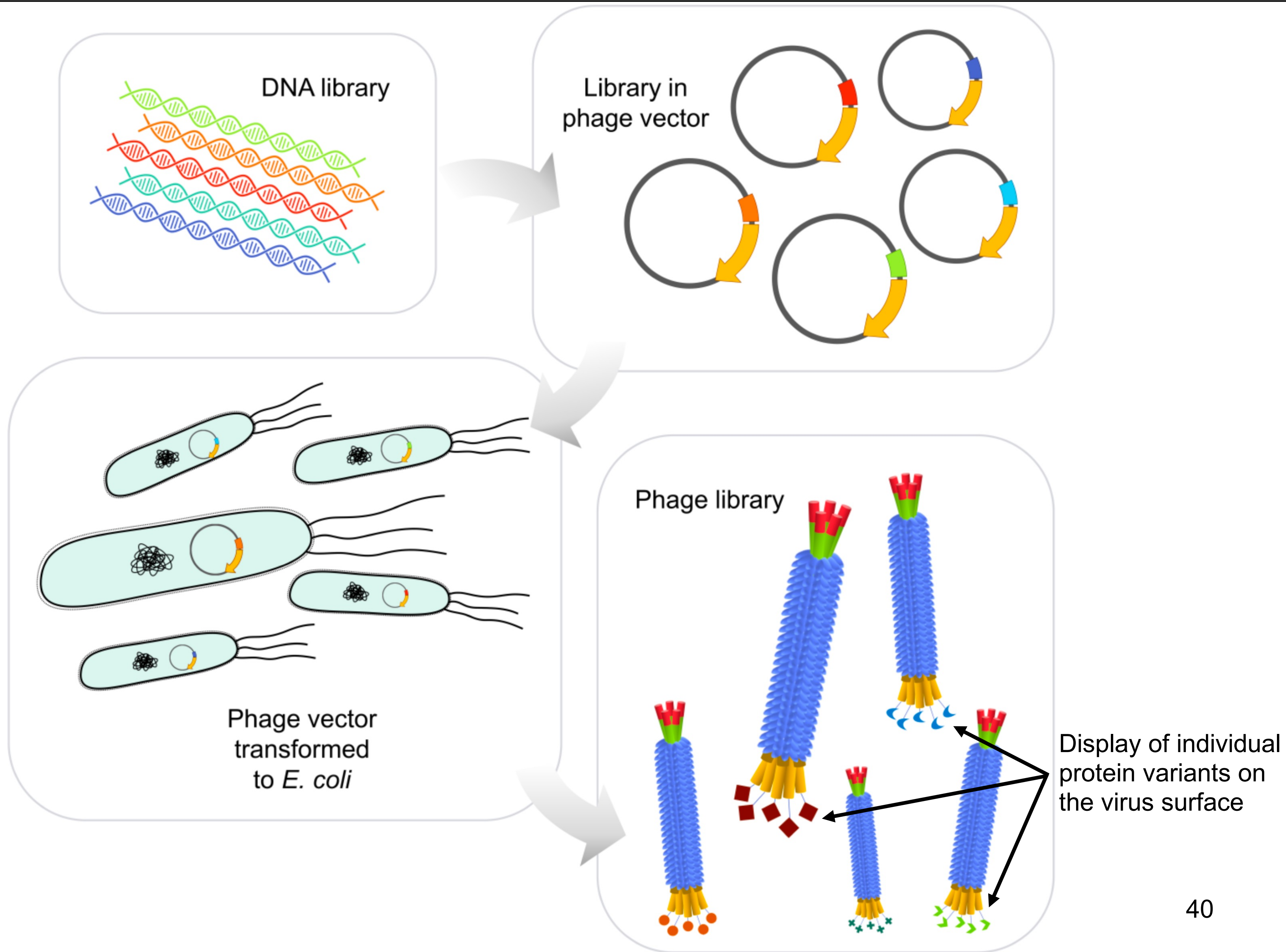


- Infects / replicates in *E. coli*
- Protein coat:
  major coat protein: pVIII
  minor coat proteins: pIII, pVI, pVII, pIX
- The phage can be engineered to display foreign peptides or proteins as a fusion with one of the coat proteins, most commonly pIII.
- The genomic DNA encoding for the coat proteins is enclosed within the protein coat.

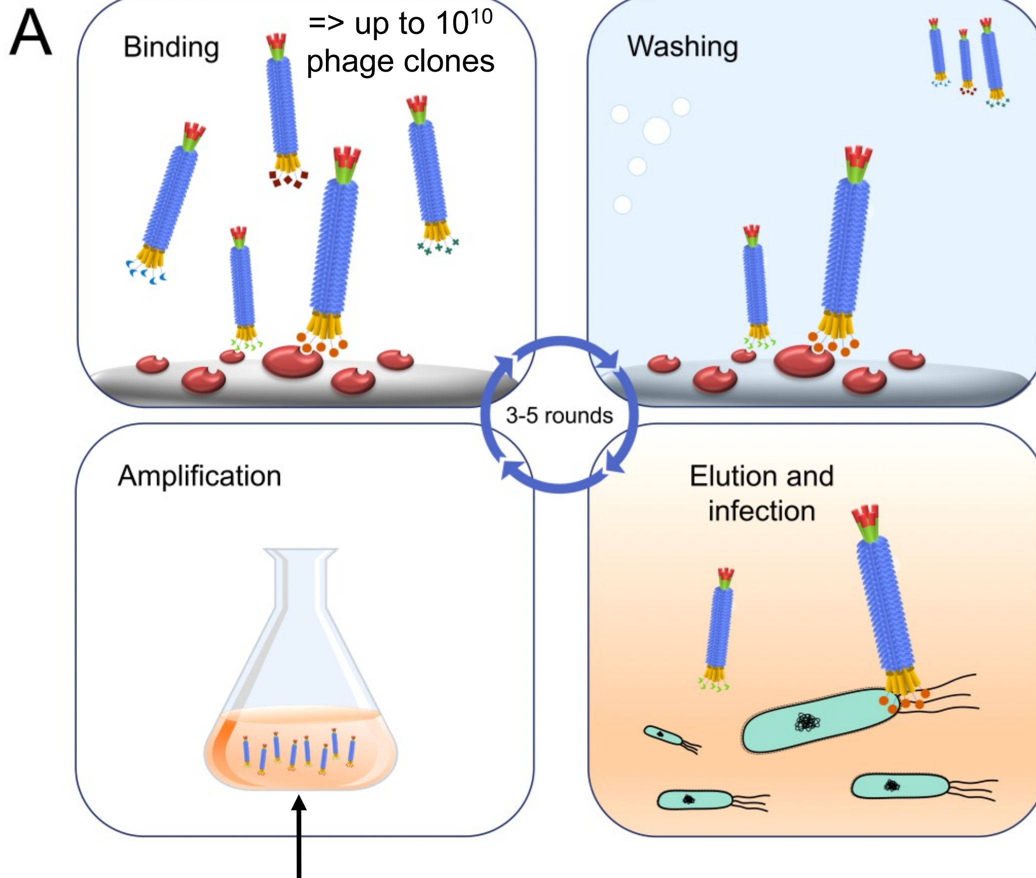
=> Each protein remains connected to its encoding DNA

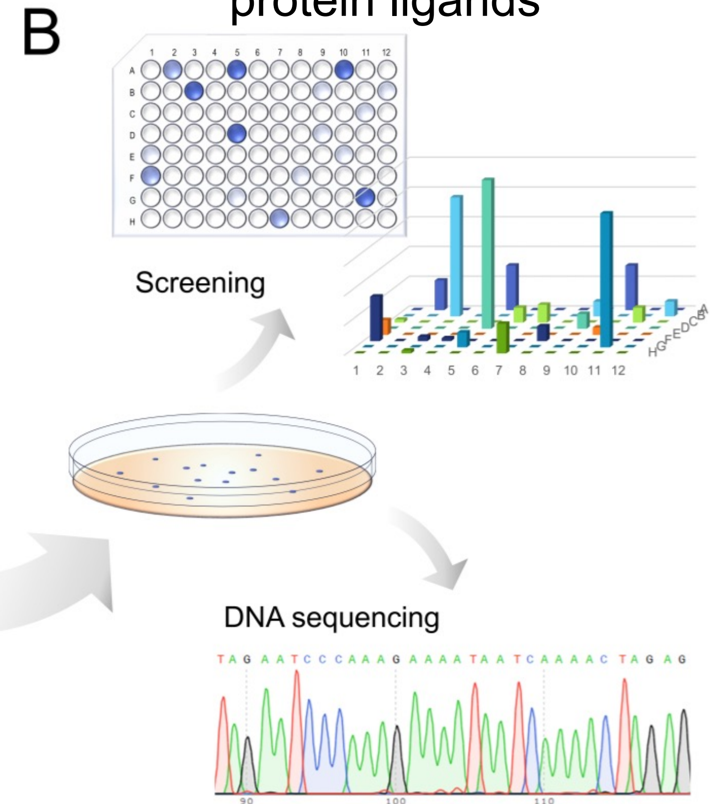
George Smith / Greg Winter:
Nobel prize in chemistry 2018

Construction of phage displayed protein libraries

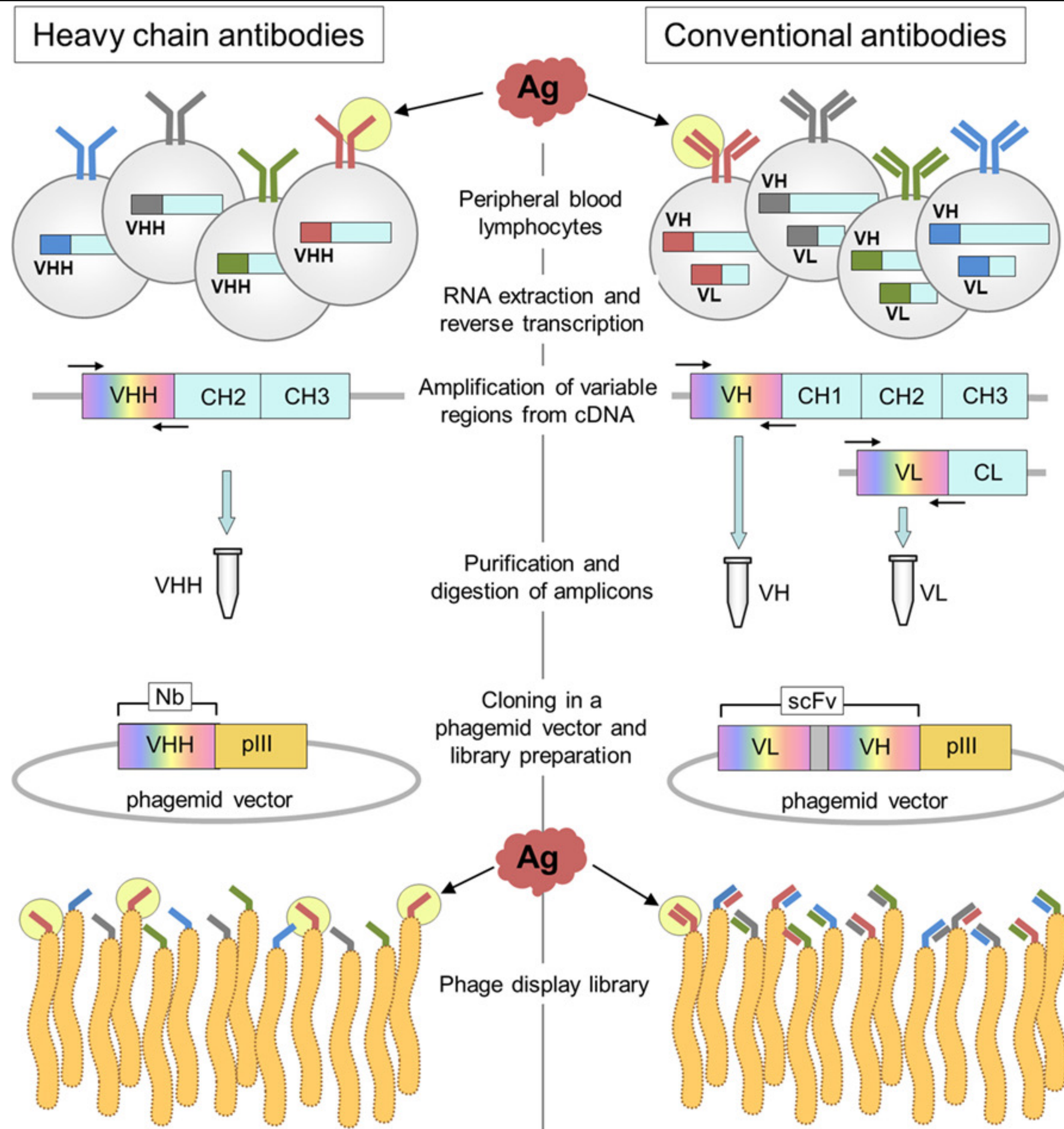


Protein engineering by *in vitro* evolution



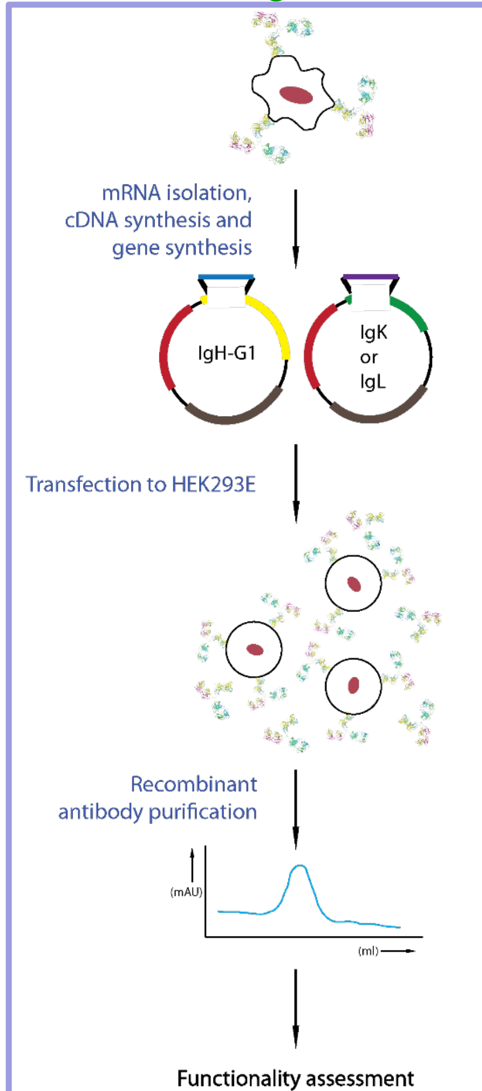


Single-domain antibody (nanobody)



Production of recombinant antibodies

Expression system
(Gene of Ab fragmentknown)



Phage display
(Gene of Ab fragment unknown)

Human antibody library

Phage library displaying human antibody fragments

Antibody Fragment (scFv)

Panning

Next panning cycle

Library selection

Antigen column

Washing

Elution

Amplification in bacteria

Screening for specificity

Cloning

IgG expression vector

Transfection

Mammalian Cell

Nucleus

Translation

Secretion

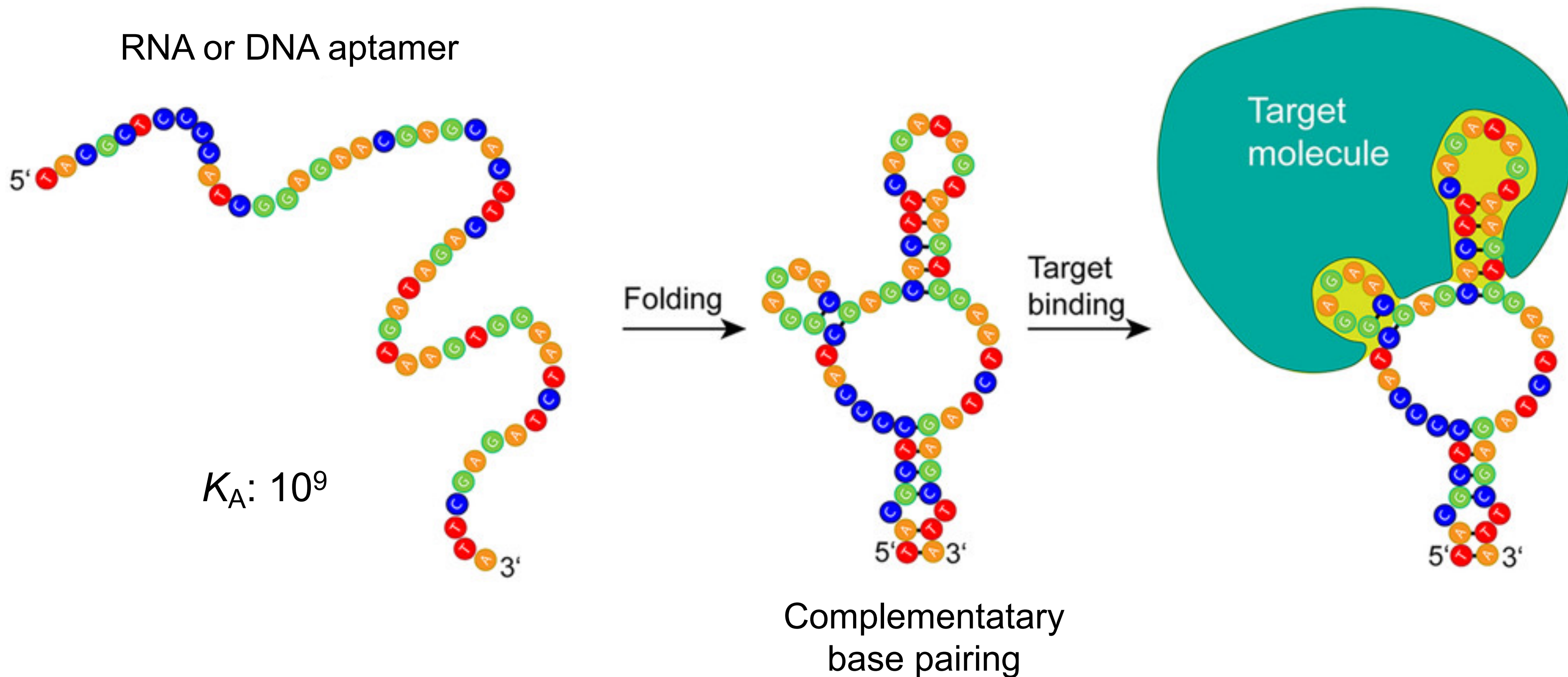
Purification

scFv

Fc

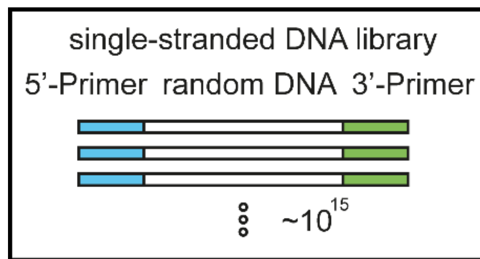
Alternatives for antibodies

Aptamers



Binding through:
(1) 3-dimensional, shape-dependent interactions
(2) hydrophobic interactions, base-stacking, intercalation

SELEX*



PCR

dsDNA pool

transcription

RNA pool

target immobilized on microsphere

selection

separation

unbound RNA

elution

selected RNA

reverse transcription

cDNA

PCR

dsDNA

transcription

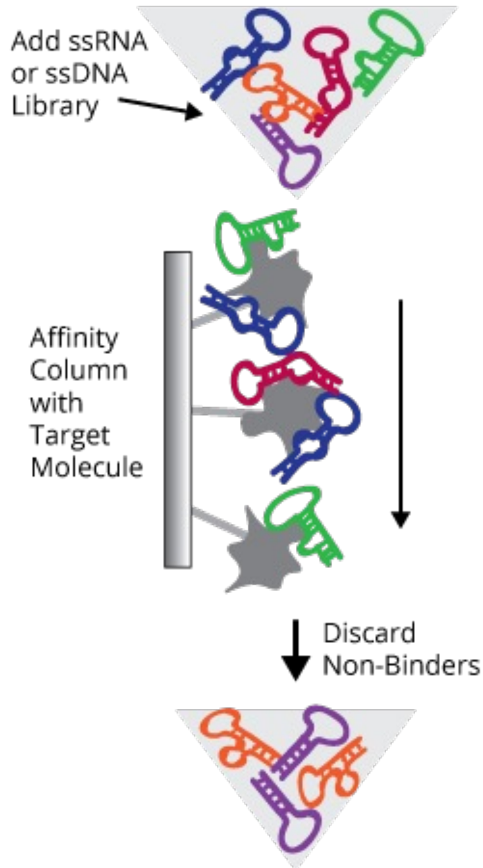
cloning & sequencing

Aptamer

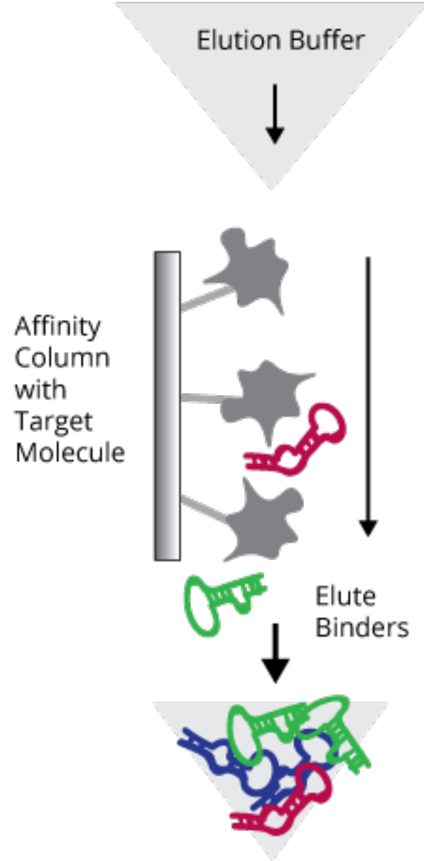
*systematic evolution of ligands by exponential enrichment
=> *in vitro* selection

SELEX*

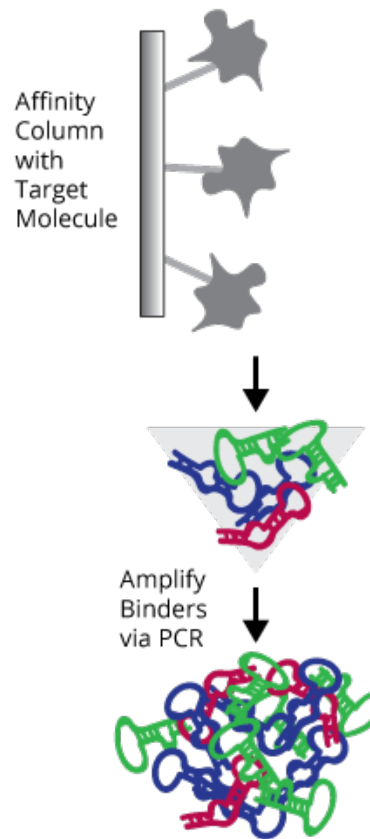
Step 1: Bind oligonucleotide library and discard non-binder



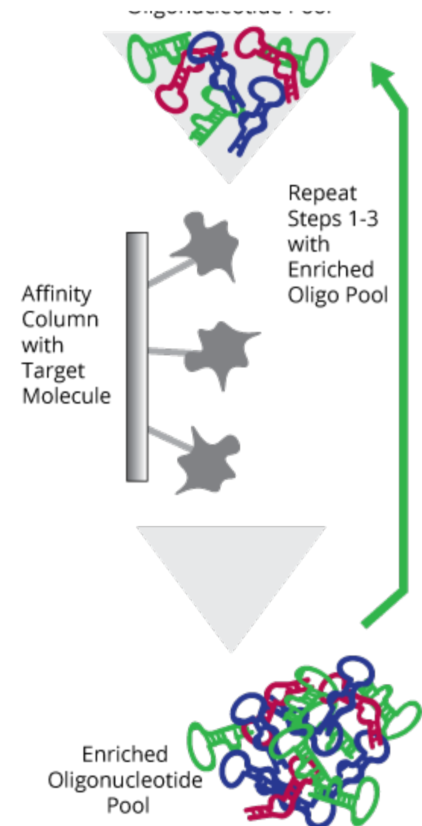
Step 2: Elute oligonucleotides that bind desired targets



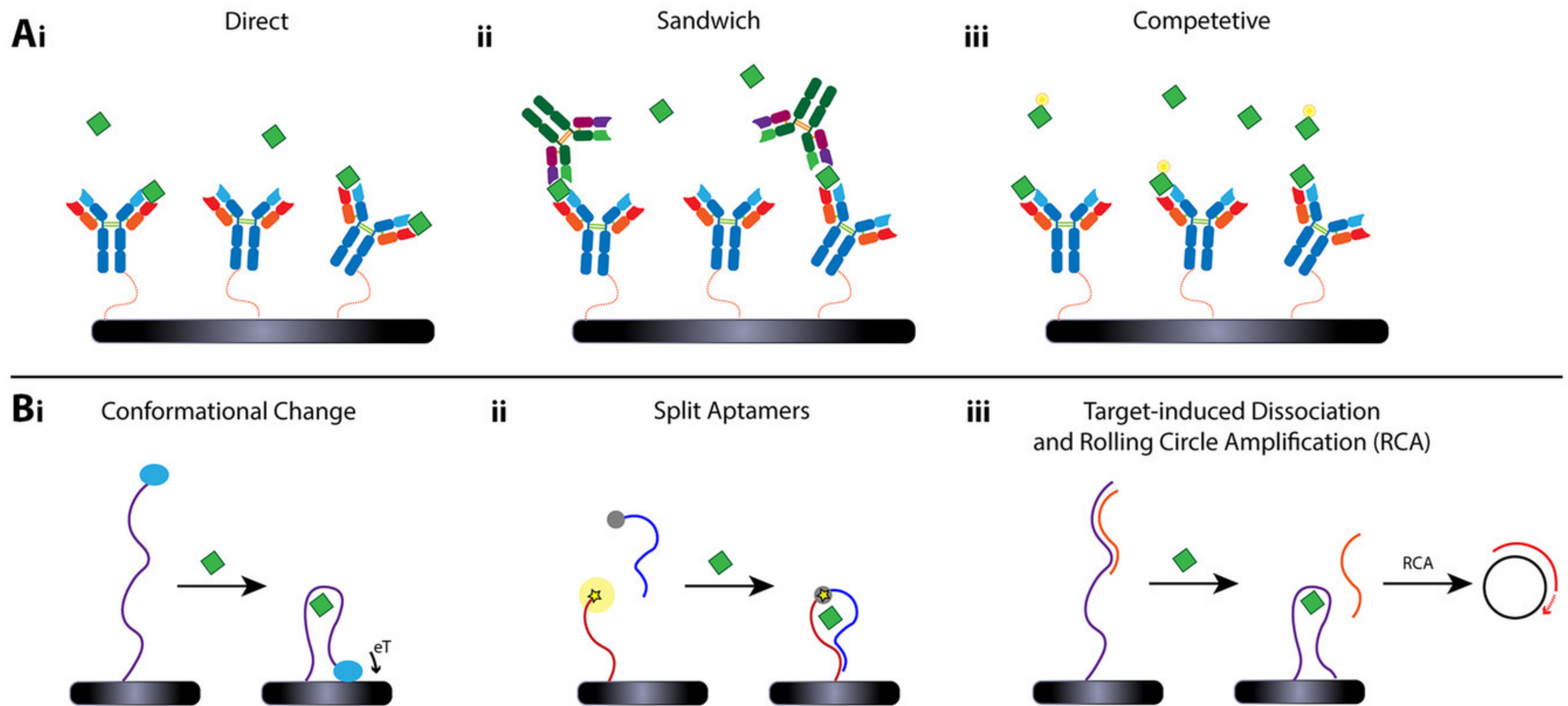
Step 3: Perform PCR to amplify eluted binders



Step 4: Repeat steps 1 through 3 using enriched oligonucleotide pool

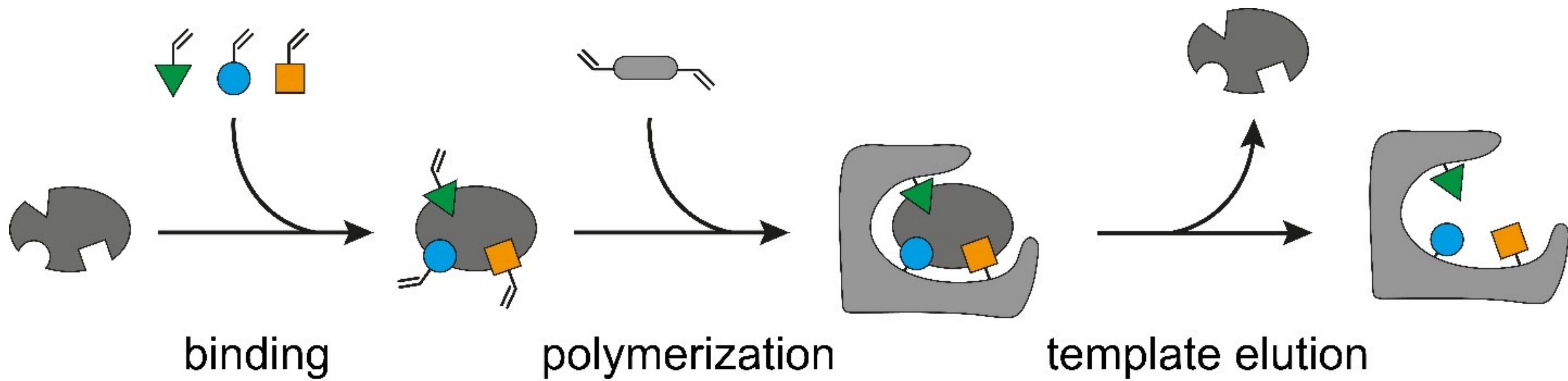


Aptamers: Assay designs



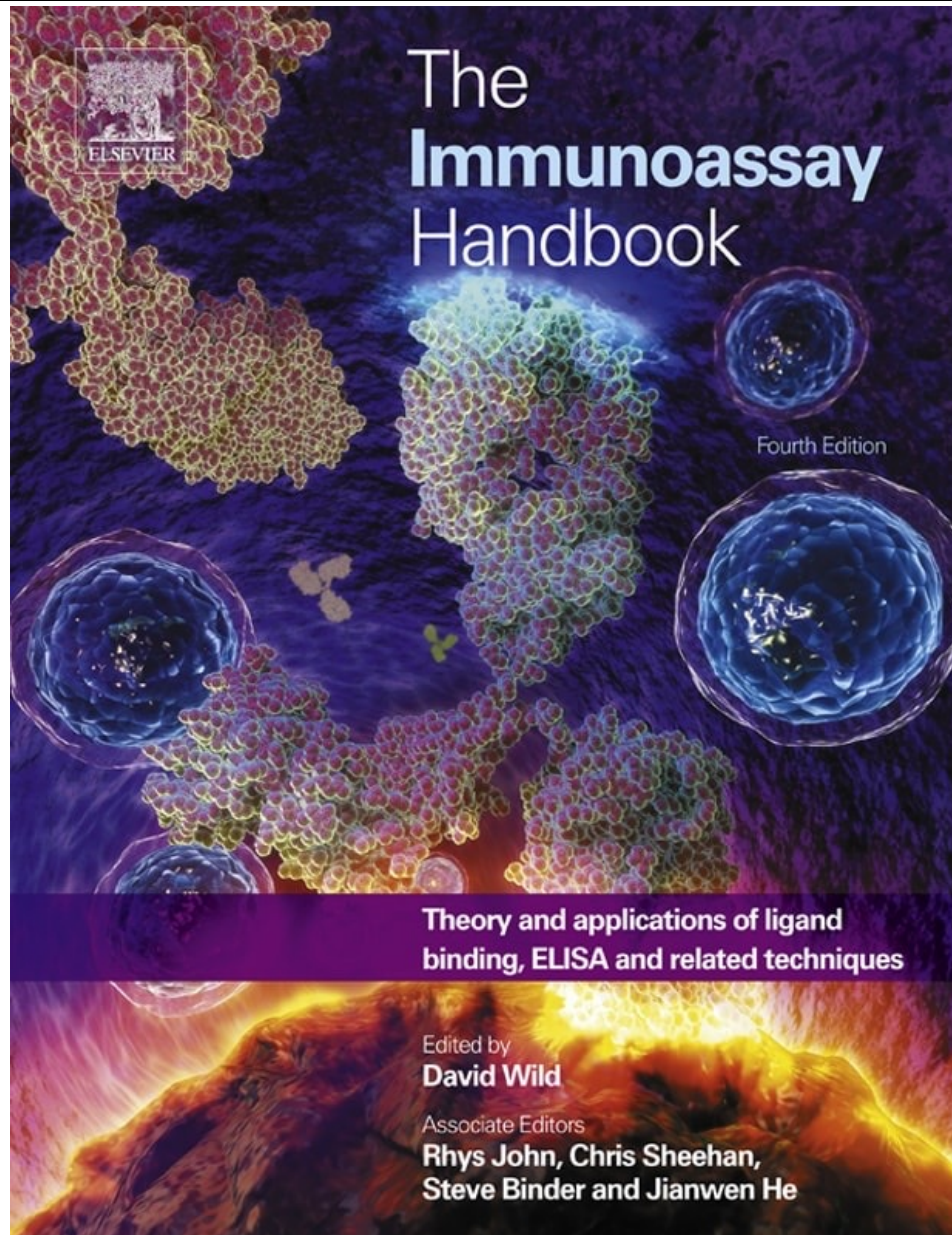
Molecularly imprinted polymer (MIP)

“Plastic antibodies“

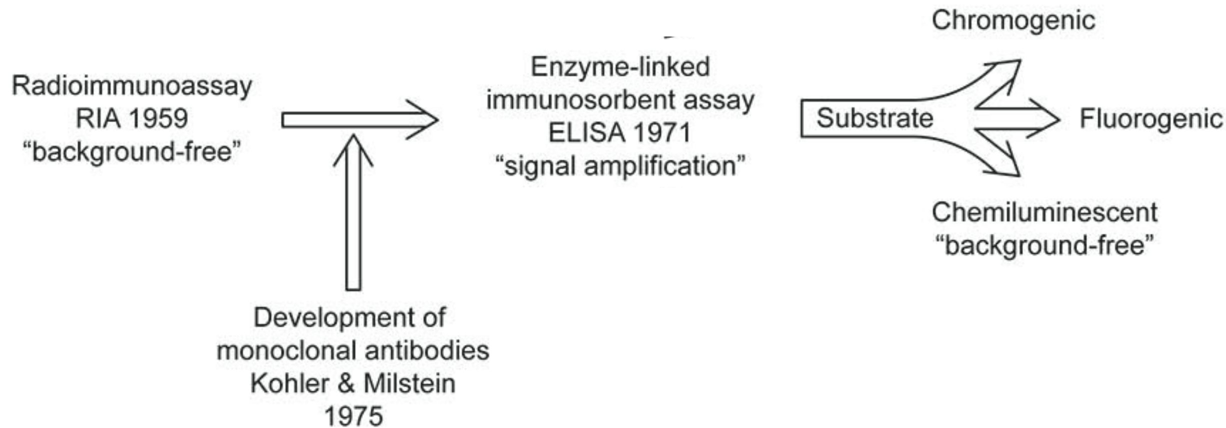


Immunoassays

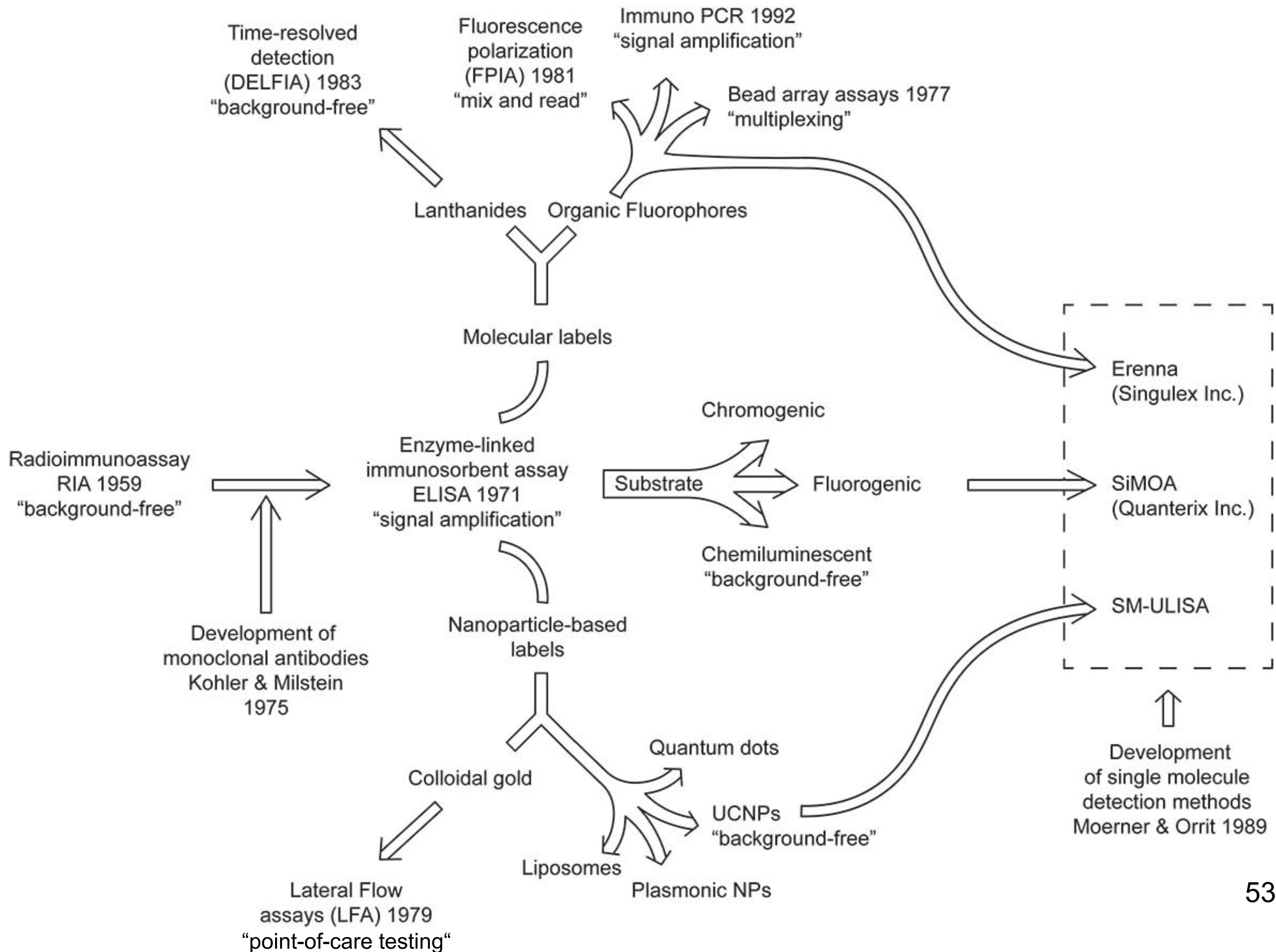
Literature for in-depth reading



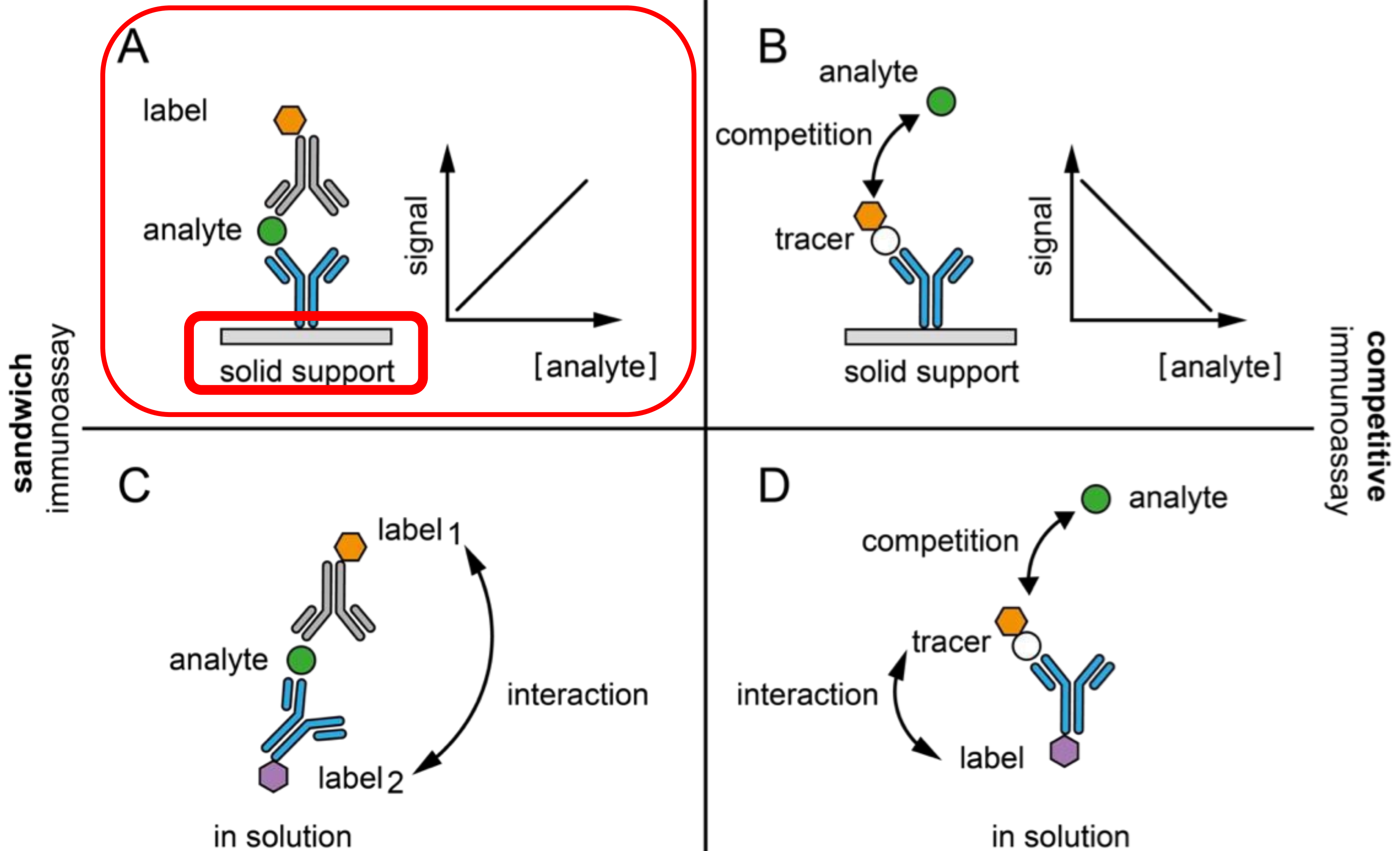
History of immunoassays



History of immunoassays



A rough categorization of immunoassays



Solid phase matrix

in heterogeneous non-competitive sandwich immunoassays

Performance-related issues:

1) low background in detection system
2) immobilization qualities:
   - high capacity
   - suitable and easy coupling chemistries
   - large surface
   - maintained reactivity of capture protein
   - no leakage
3) easy handling
4) inert in binding the labelled antibody/analyte => low background
5) effectively washed => low background
6) antibody excess through **high density** - surface measurement
7) antibody excess through **large surface** - integrating measurement

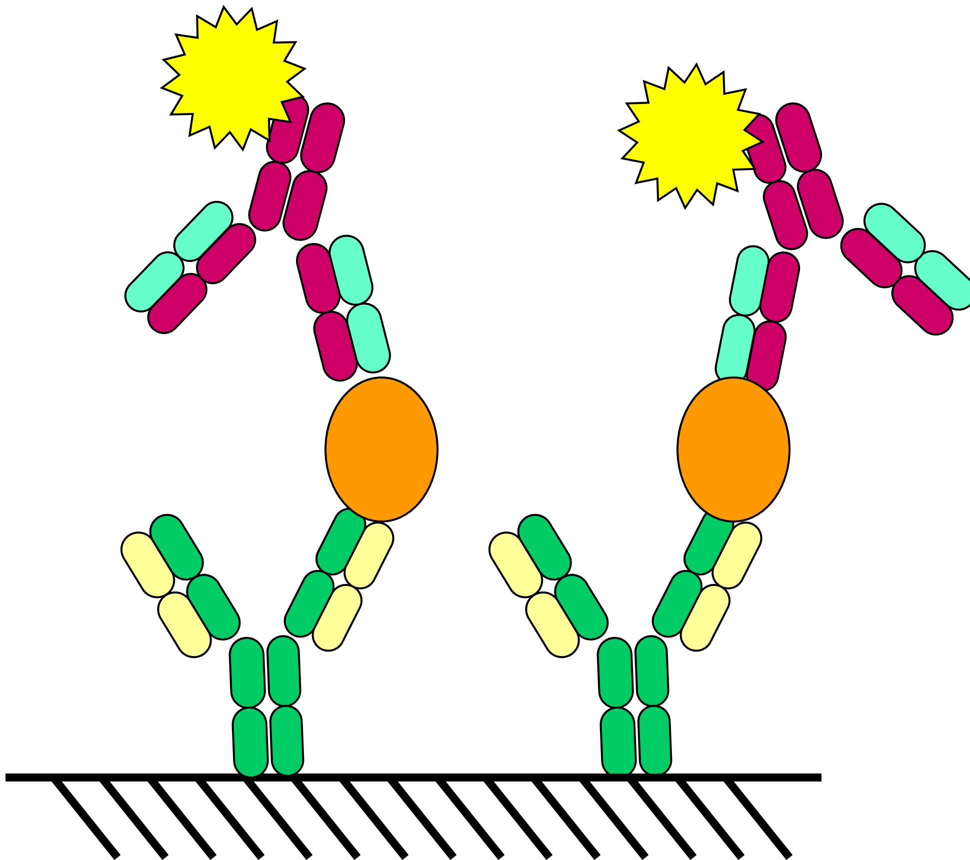
Solid phase matrices

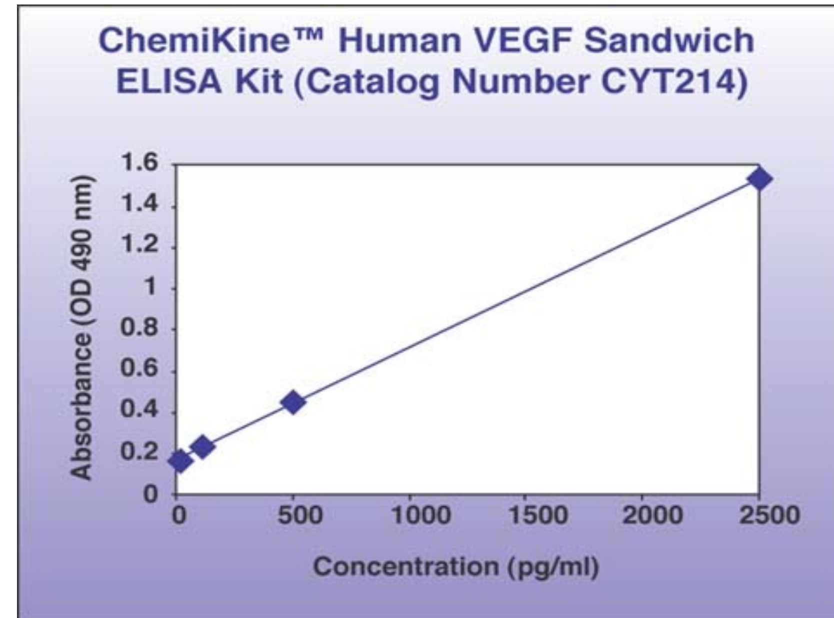
Size	Examples	Advantages	Disadvantages
Small particle / "beads" (< 20 µm)	Latex Microcrystalline cellulose Fine porous glass Magnetic beads Liposomes Starburst™ dendrimers	Dispensing as for liquids Agitation not required High antibody binding capacity	Centrifugation required (unless used with a membrane capture) Long magnetic precipitation
Medium particle (< 1 mm)	Sepharose beads Sephacryl beads Sephadex beads	Centrifugation not required Short magnetic separation	Agitation required Slower binding kinetics than above Moderate antibody binding capacity
Single particle	Polystyrene	Centrifugation not required Agitation not required	Some variability in antibody coupling Lower antibody binding capacity Difficulty in dispensing Poor binding kinetics
Fibers	Membranes Glass fibers Nylon Silicon rubber	Centrifugation not required Agitation not required No dispensing of reagent Simple to use	Medium antibody binding capacity Can be fast binding kinetics
Solid surface	Coated tubes Dipsticks Microtiter plates (MTP)	Centrifugation not required Agitation rare No dispensing of reagent Simple to use	Variability in antibody coupling Lowest antibody binding capacity Slowest binding kinetics

Most frequently used solid phase matrices

Non-competitive immunoassay

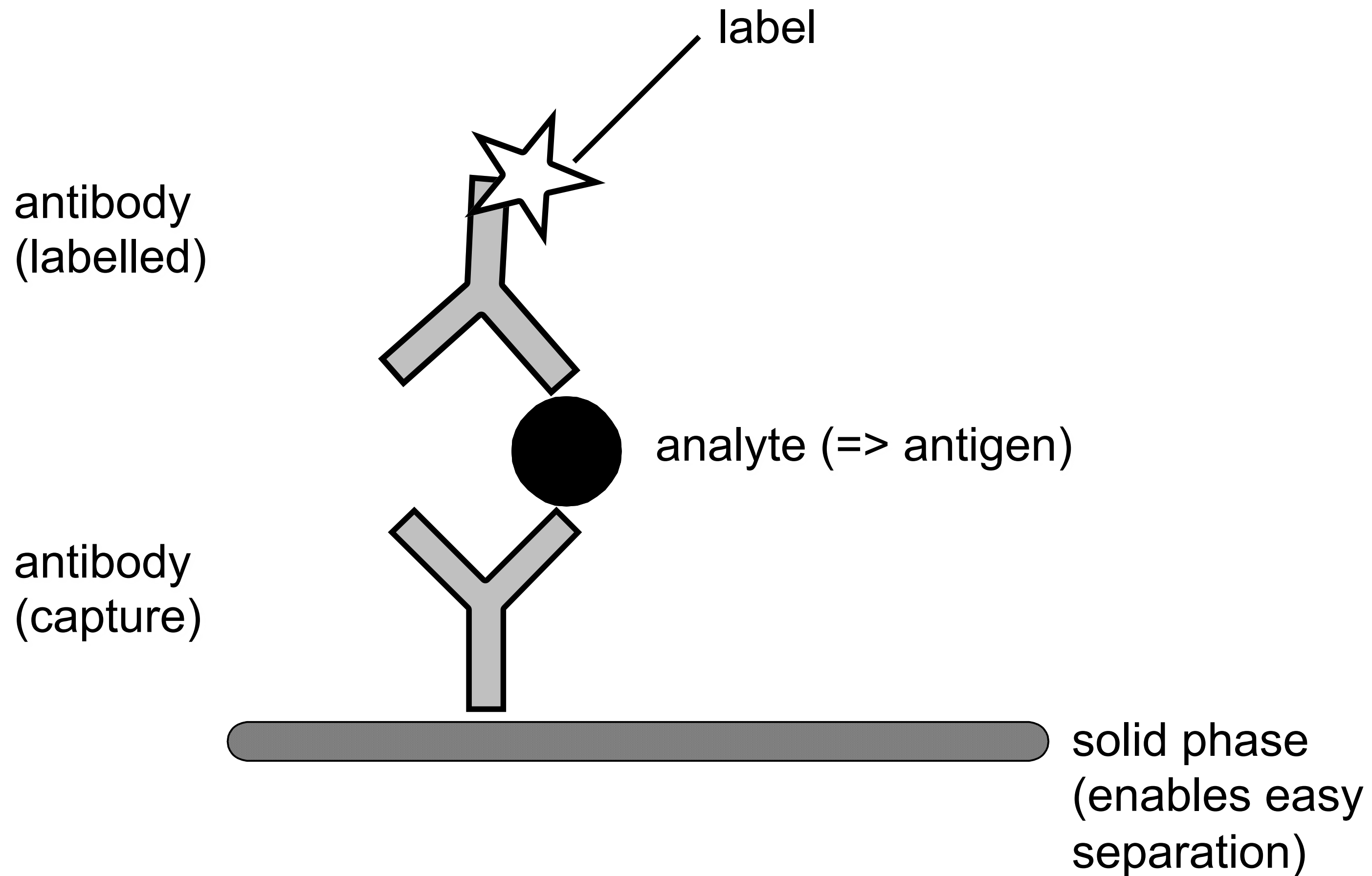
("sandwich" immunoassay)





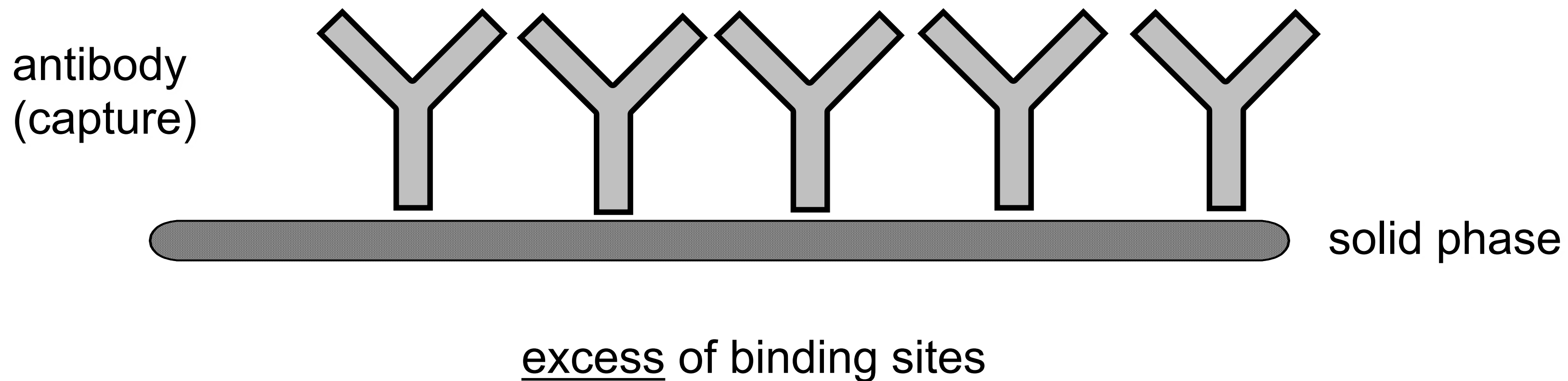
Non-competitive immunoassay

("sandwich" immunoassay)



Non-competitive immunoassay

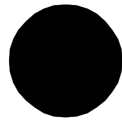
a capture antibody specific for a single epitope of the analyte is coated on a solid phase
(e.g. on a microtiter plate)
(=> monoclonal antibody preferred)

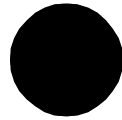


Non-competitive immunoassay

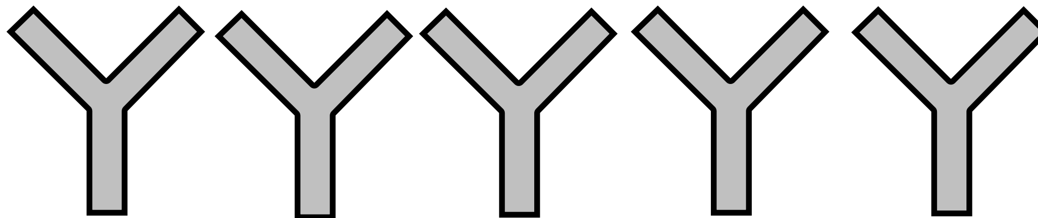
sample containing the analyte (at least <u>two non-overlapping epitopes</u>) is added; incubation for binding

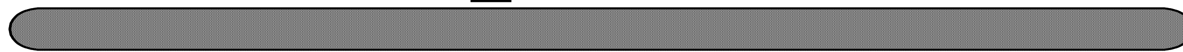
analyte





antibody (capture)



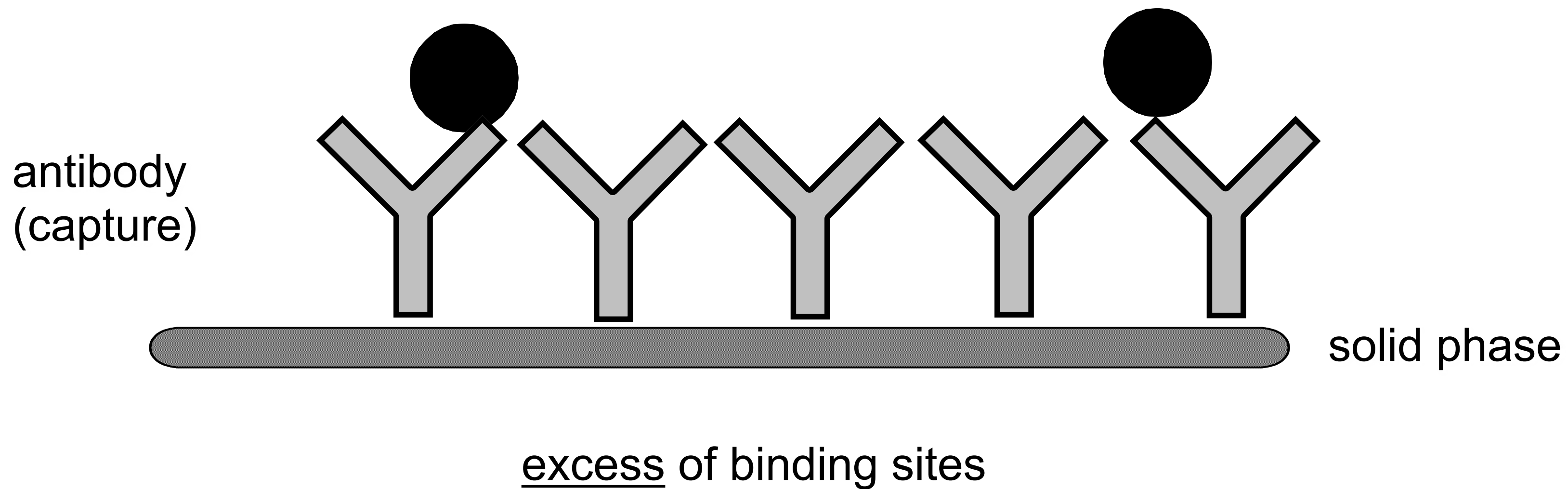


solid phase

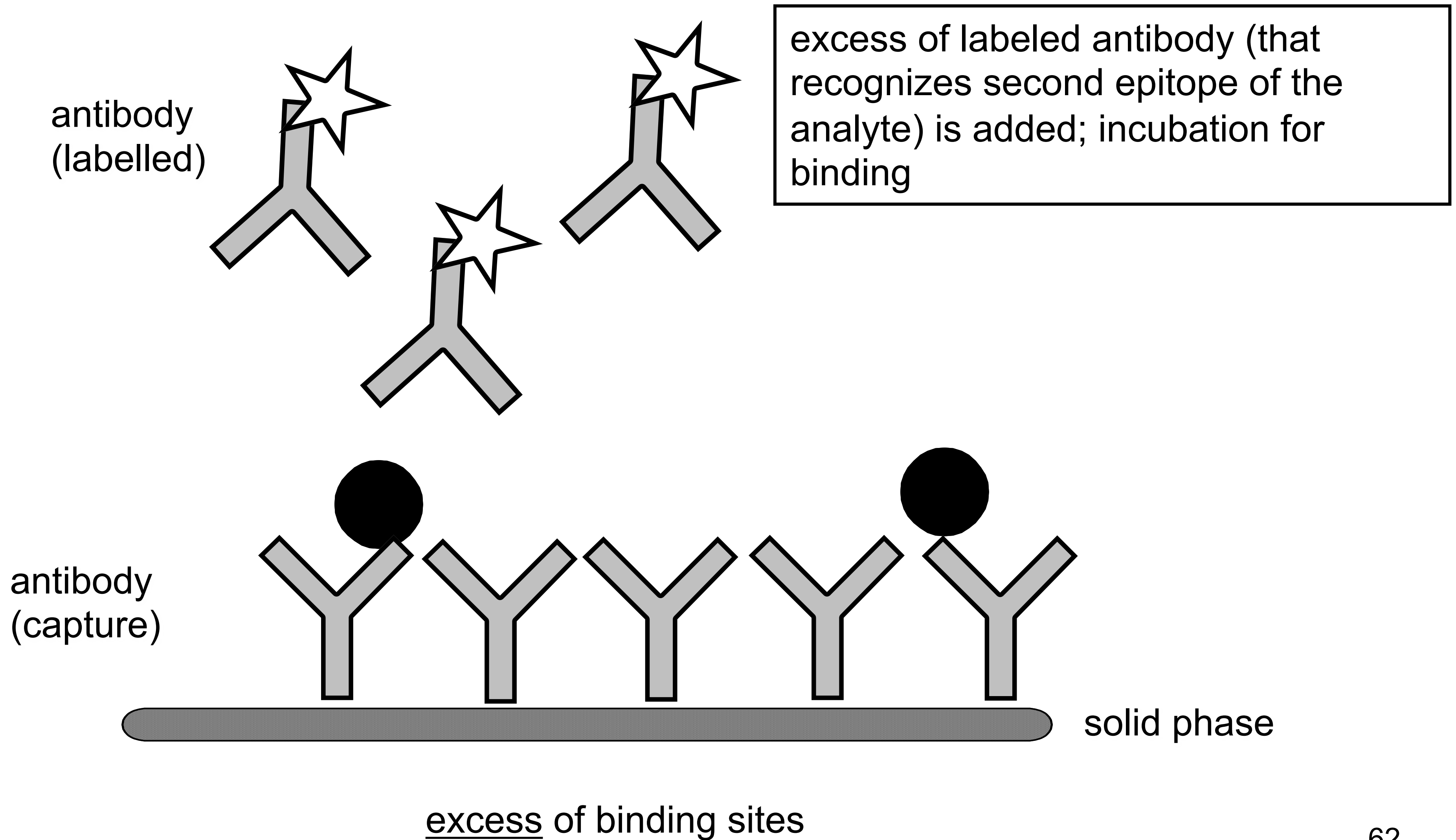
<u>excess</u> of binding sites

Non-competitive immunoassay

analyte is bound; in two-step assay: sample is washed away with excess of analyte

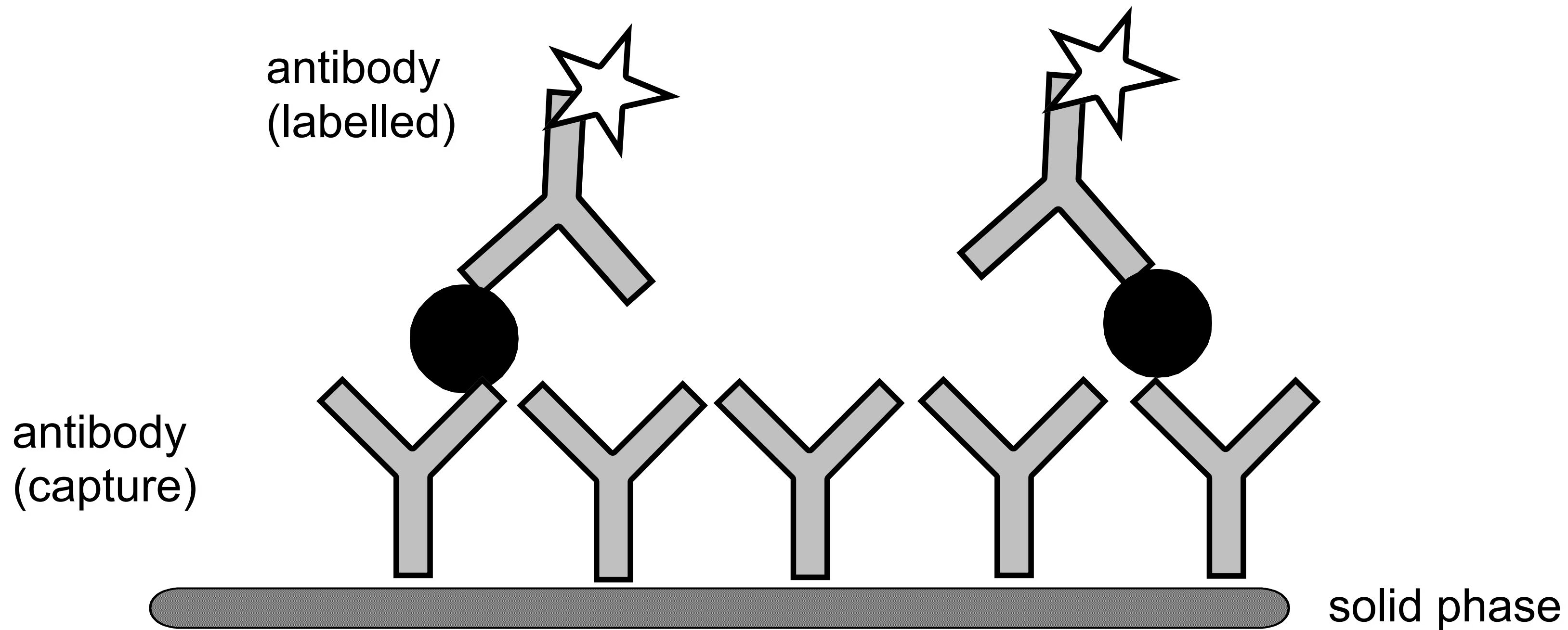


Non-competitive immunoassay

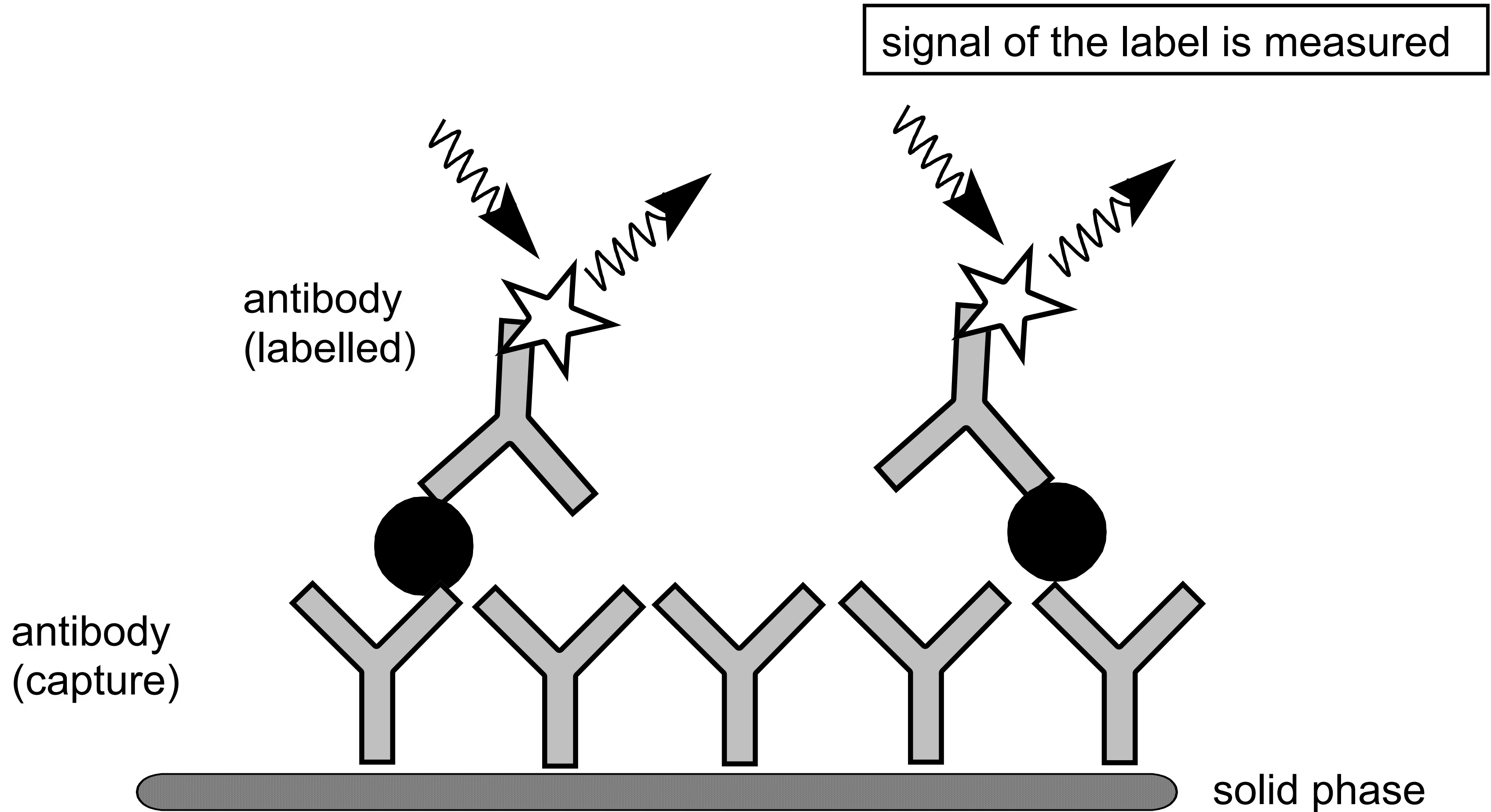


Non-competitive immunoassay

labeled antibody is bound;
excess is washed away

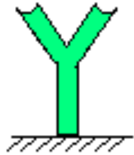


Non-competitive immunoassay

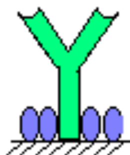


Enzyme-linked immunosorbant assay (ELISA)

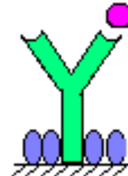
Thorough washing steps required



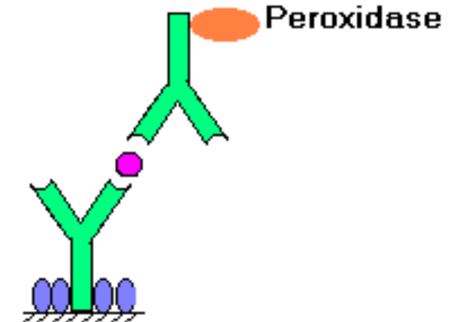
A. Coating anti-IgG



B. Blocking



C. React sample antigen



D. React POx labelled secondary antibody

Non-covalent absorption of capture antibody to polystyrene surface (microtiter plate)

Block surface with BSA or detergents to prevent non-specific binding of other proteins

Add sample that contains the antigen (the analyte), e.g. tumor markers, viruses, or antibodies in serum.

1. Add enzyme-labeled detection antibody (e.g. horseradish peroxidase); wash
2. Add chromogenic reagent (e.g. TMB)
3. Add "stop solution" (e.g. H_2SO_4)

Blocking is essential to avoid non-spec. binding

Normal serum
Normal serum (1-5% w/v) carries antibodies that bind to reactive sites and prevent non-specific binding of the secondary antibody. Serum is rich in albumin and other proteins that readily bind to non-specific protein binding sites of the sample.

Protein solutions
Blocking buffers often contain proteins such as bovine serum albumin (BSA), gelatin or nonfat dry milk (1-5% w/v). These inexpensive and readily available proteins are present in large excess compared to the antibody, so they compete with the latter for binding to nonspecific sites in the sample. Many labs developed homemade blocking buffers. It is important that blocking buffers are free of precipitates and other contaminants that can interfere with the detection.

Commercial buffers
Ready-made blocking buffers can contain highly purified single proteins or proprietary protein-free compounds. Many options are available that perform better than gelatin, casein or other proteins used alone, and they have improved shelf lives compared to homemade preparations.

Blocking tips

- Monitor both background (negative control) and signal strength (positive control) with various blocking reagents.
- Choose the blocking buffer that yields the highest signal-to-noise ratio.
- Ensure that there are no substances in the blocking buffer that interfere with a particular assay. Non-fat dry milk, for example, contains biotin and is inappropriate for use with any detection system that includes a biotin-binding protein.
- For optimal assay conditions, use the same blocking buffer for diluting the antibody that is used for the blocking step.