MUNI
SCI

C8116 Immunochemical techniques

Immunoassays

Spring term 2024

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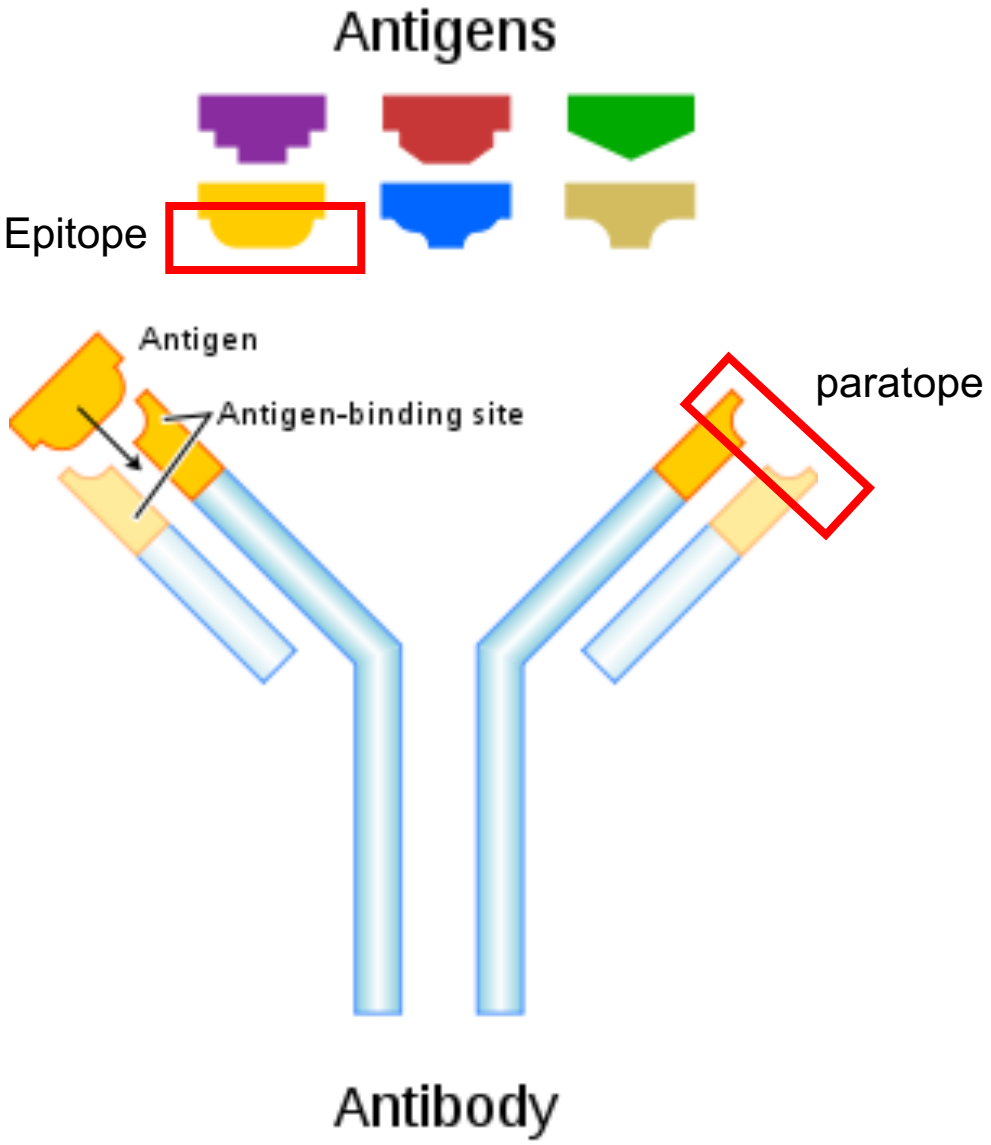
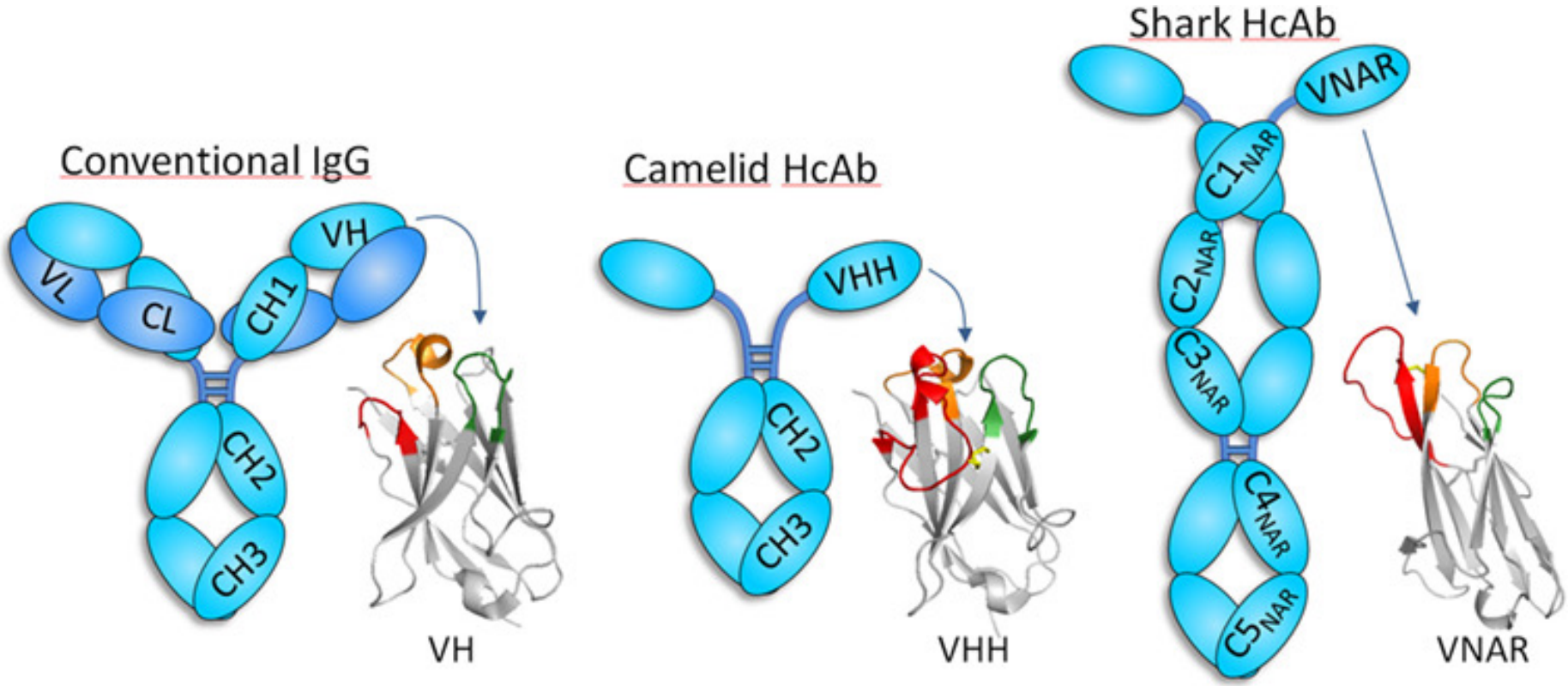
Department of Biochemistry
March 26th, 2024

Antibodies as immunochemical reagents

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



From heavy chain antibodies to nanobodies



our own most common antibody

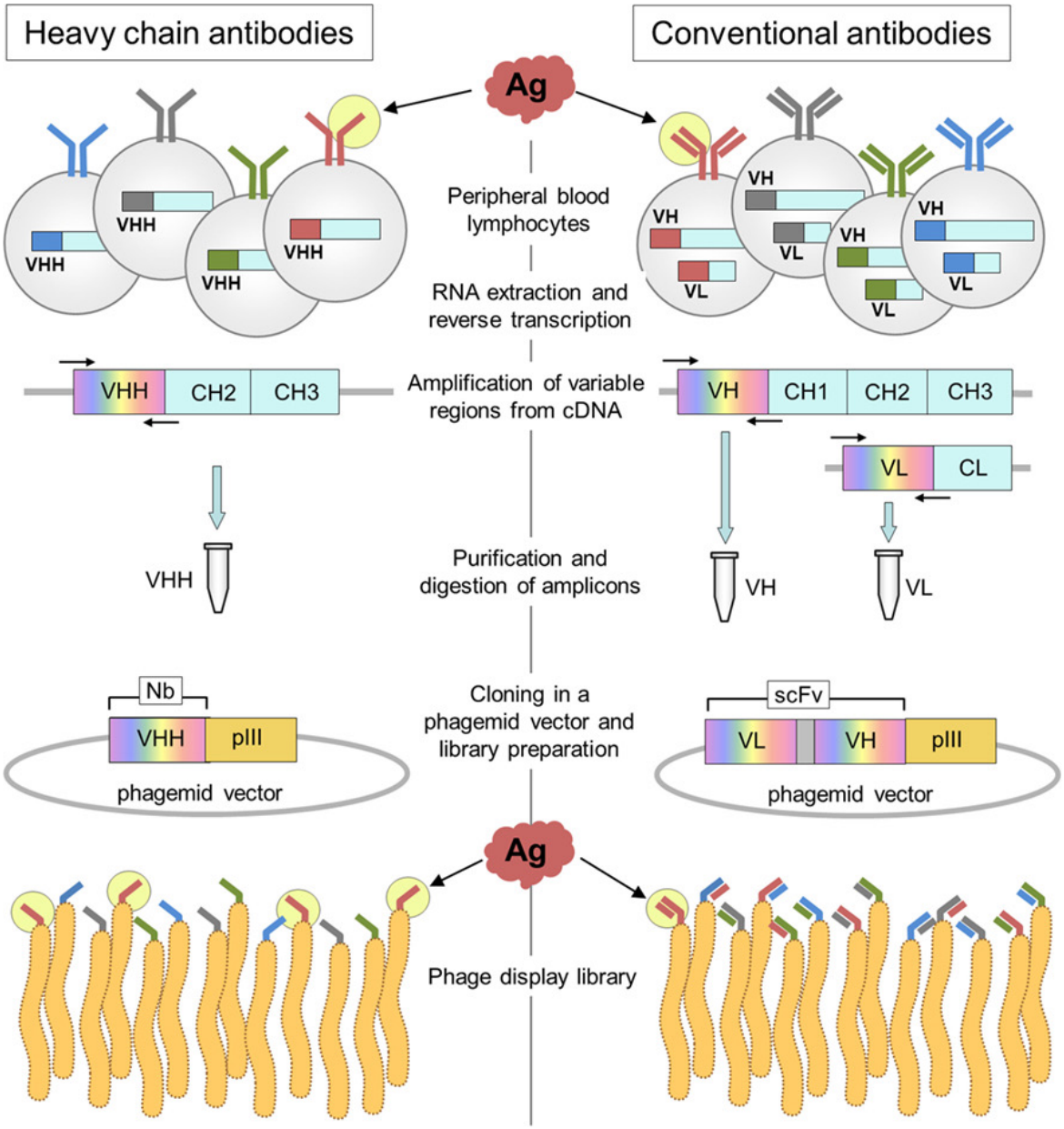
heavy chain antibodies

(velbloud, dromedár, lama)

(žralok)

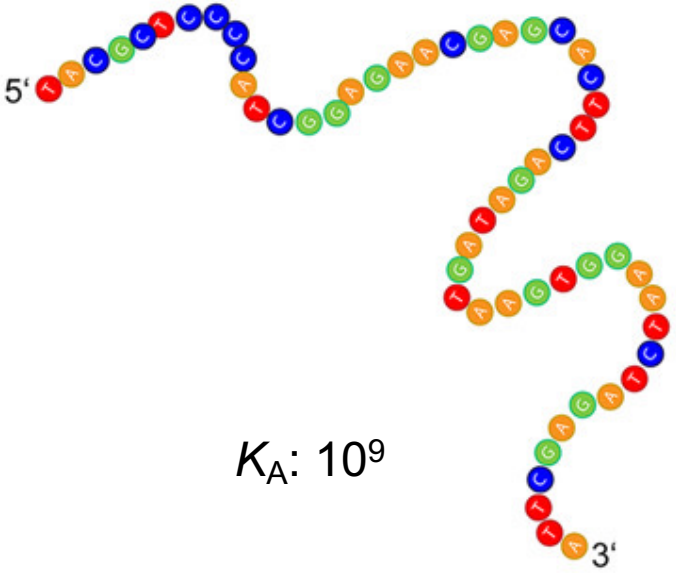
Front. Immunol., 2017 https://doi.org/10.3389/fimmu.2017.00977

Phage display



Aptamers

RNA or DNA aptamer

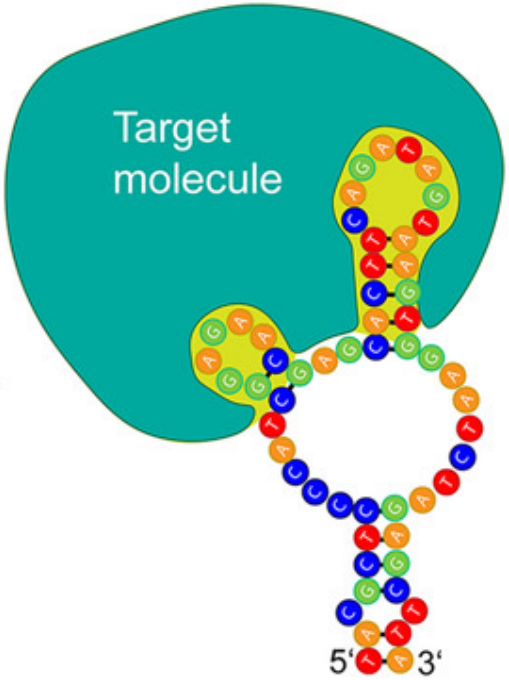


K_A: 10^9

Folding

Complementatary
base pairing

Target
binding

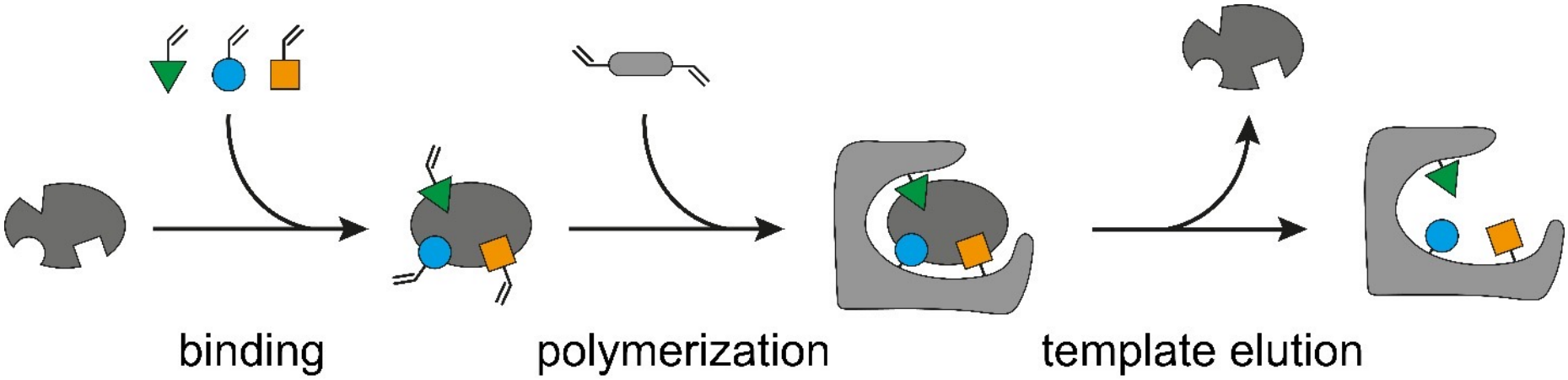


Binding through:
(1) 3-dimensional, shape-dependent interactions
(2) hydrophobic interactions, base-stacking, intercalation

Molecularly imprinted polymer (MIP)

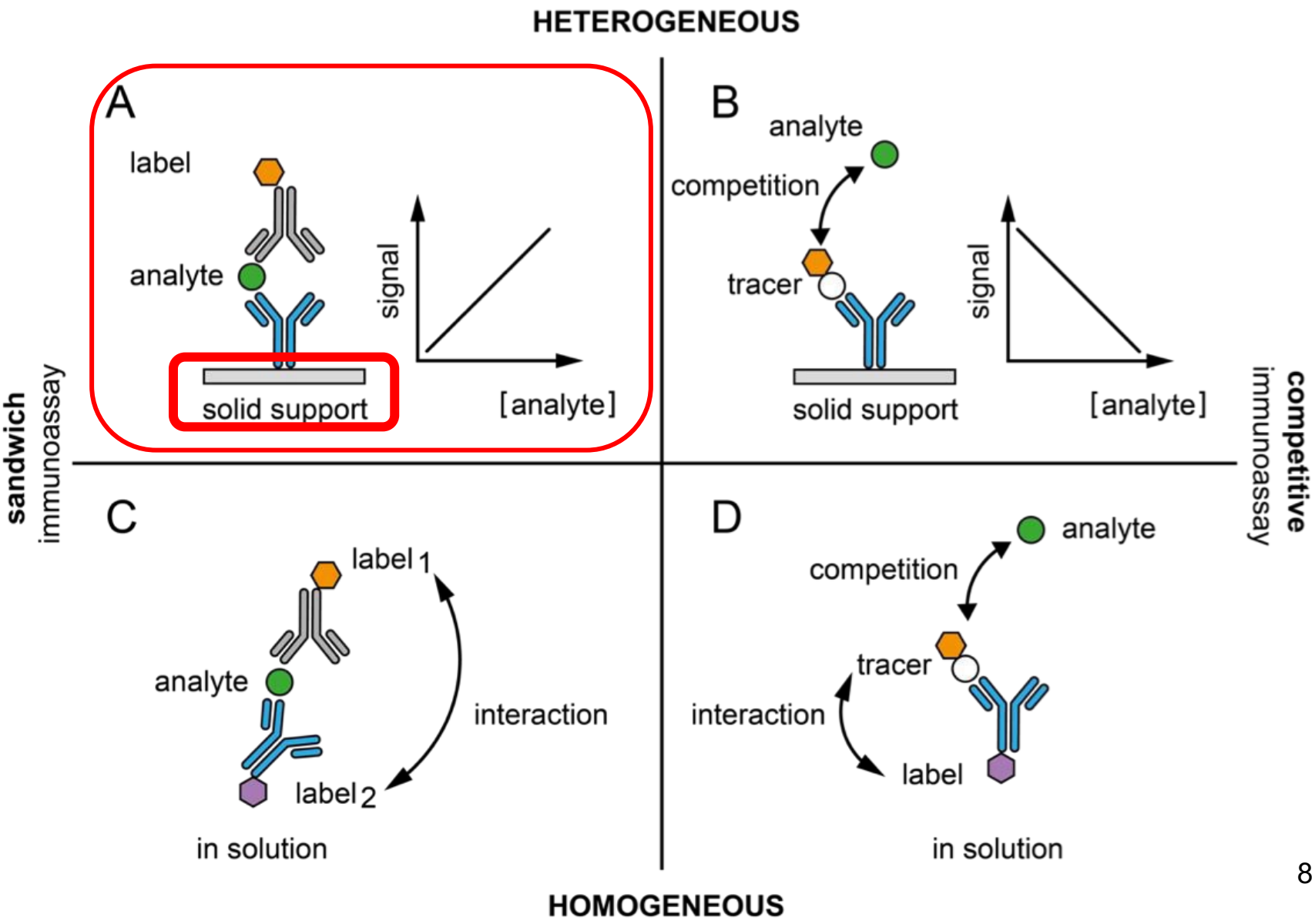
"Plastic antibodies"

MIPs are generated by:
polymerization of monomers in presence of the template analyte

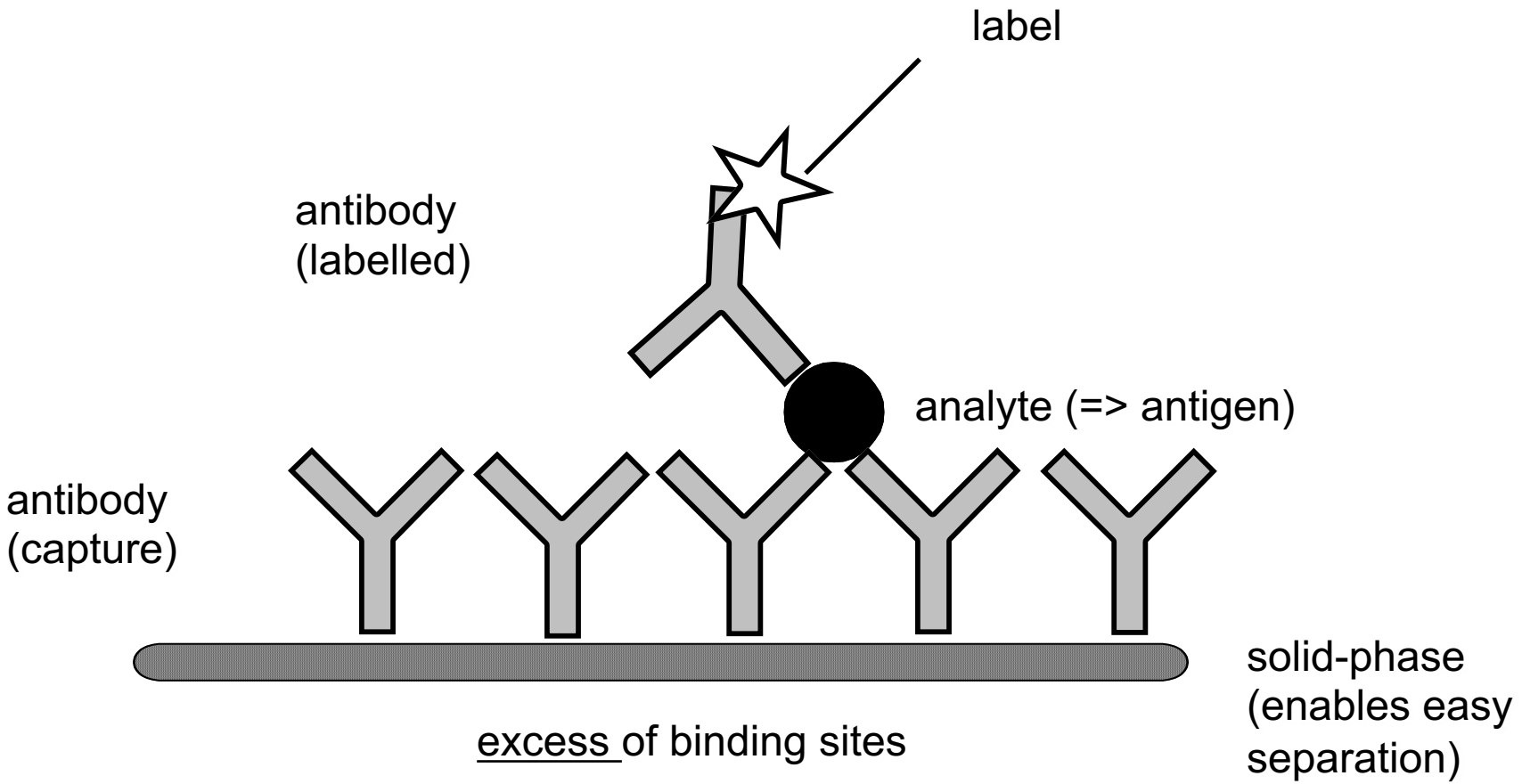


Immunoassays

A rough categorization of immunoassays



Sandwich immunoassay



Enzyme-linked immunosorbant assay (ELISA)

Enzyme	Properties
peroxidase galactosidase	rarely found in biosamples, high activity
phosphatase glucose oxidase	rarely found in biosamples, moderate activity
catalase	high activity but often present in samples
protease	low activity

very common

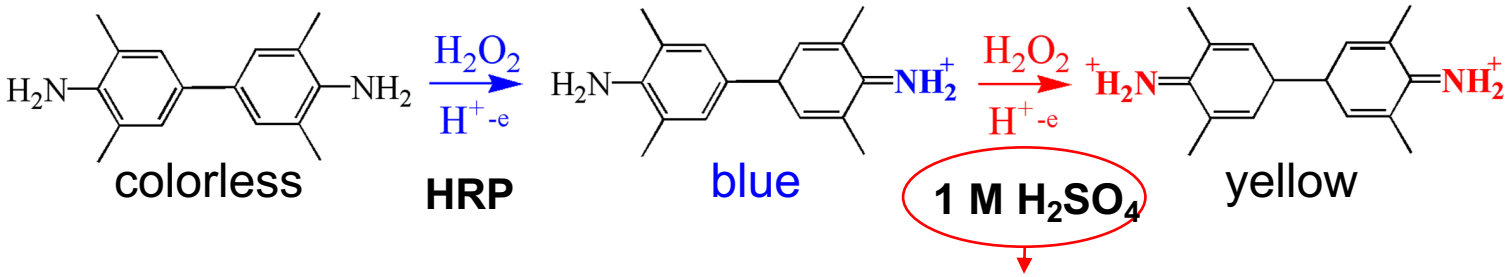
less suitable

=> Effect: strong signal amplification

(one enzyme label generates 100 - 1000 chromophores / fluorophores per second!)

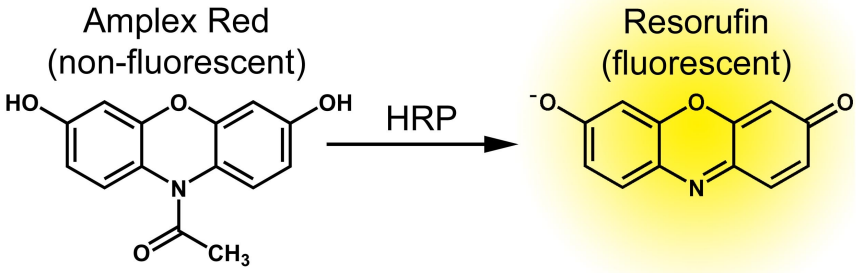
Enzyme-mediated signal generation

(a) a chromogenic substrate (3,3‘,5,5‘-Tetramethyl-benzidine (TMB)):



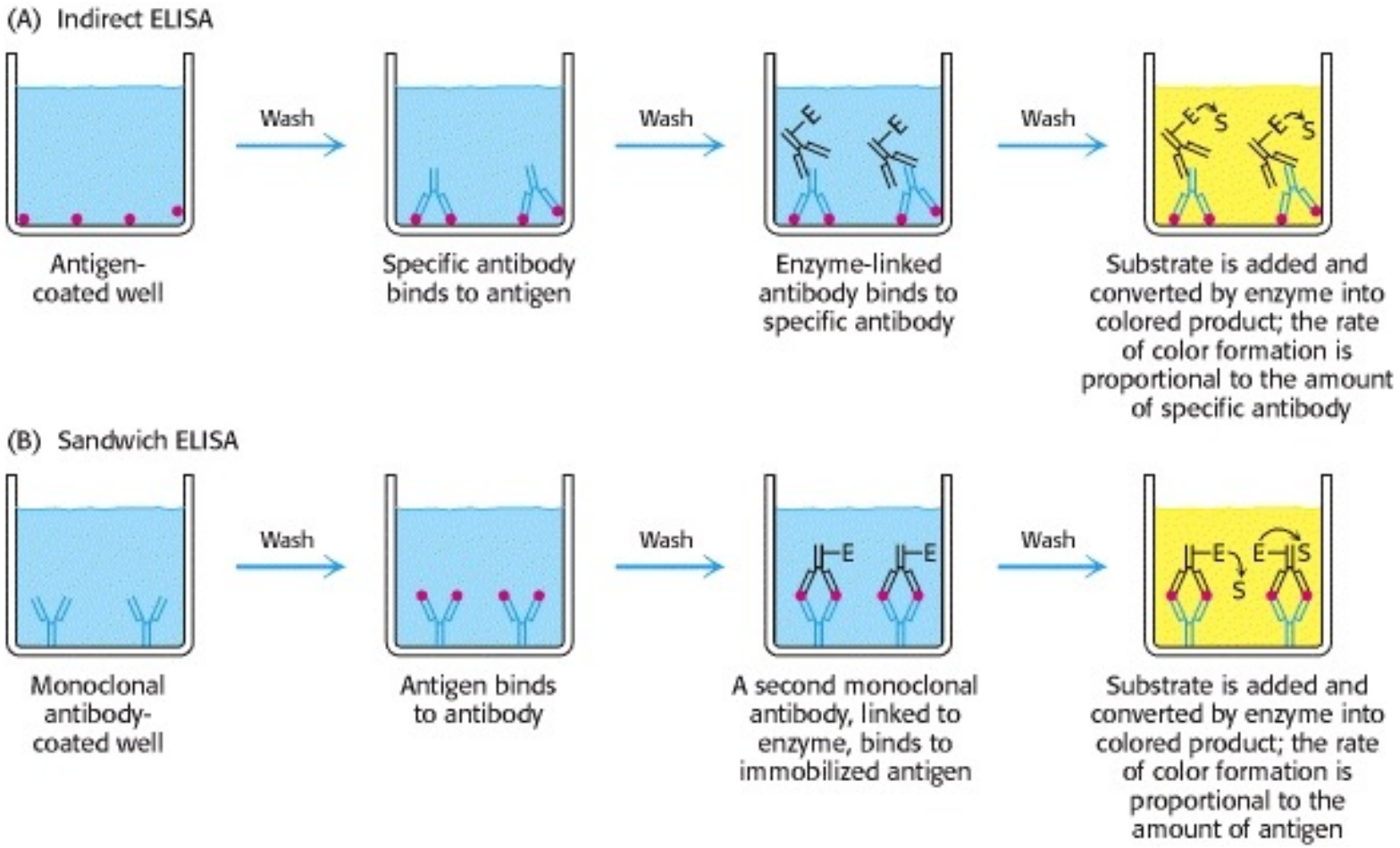
Stops the enzyme reaction:
Endpoint measurement

(b) a fluorogenic substrate:



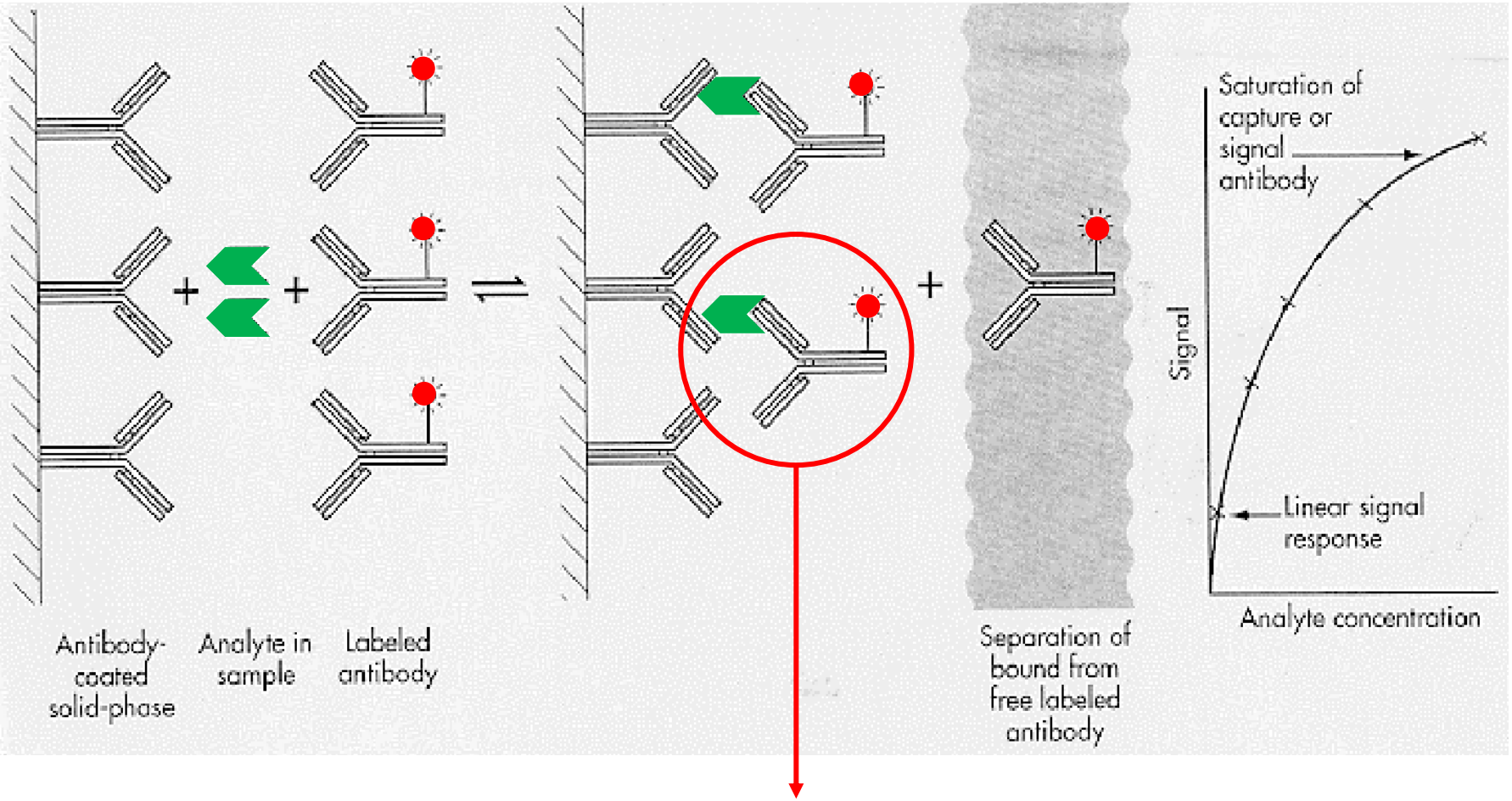
- Coloration depends on the amount of enzyme-labeled secondary Ab;
- microtiter plate reader; absorbance at 450 nm expressed as Optical Density (OD)

Alternative non-competitive ELISA formats



Immunometric assay

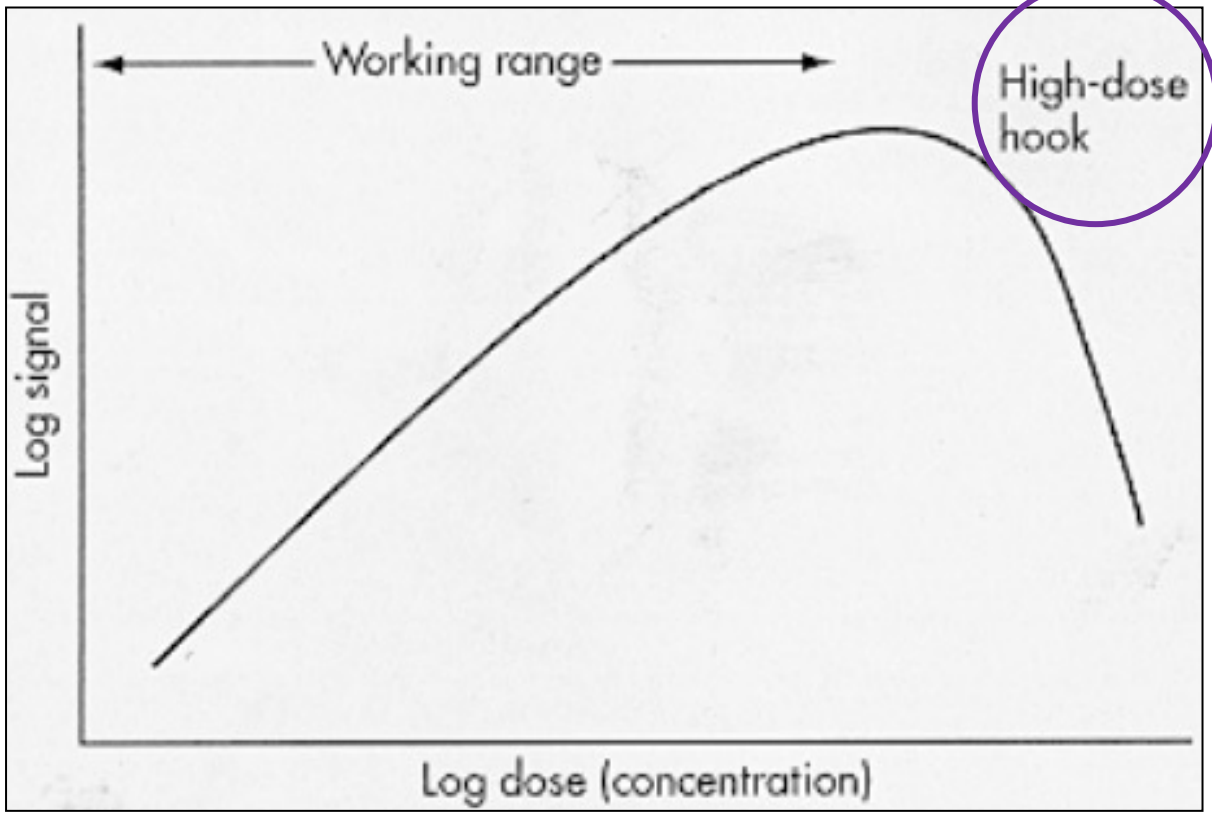
one-step assay



=> analyte is detected directly,
i.e. signal from immune complexes containing analyte

Immunometric assay

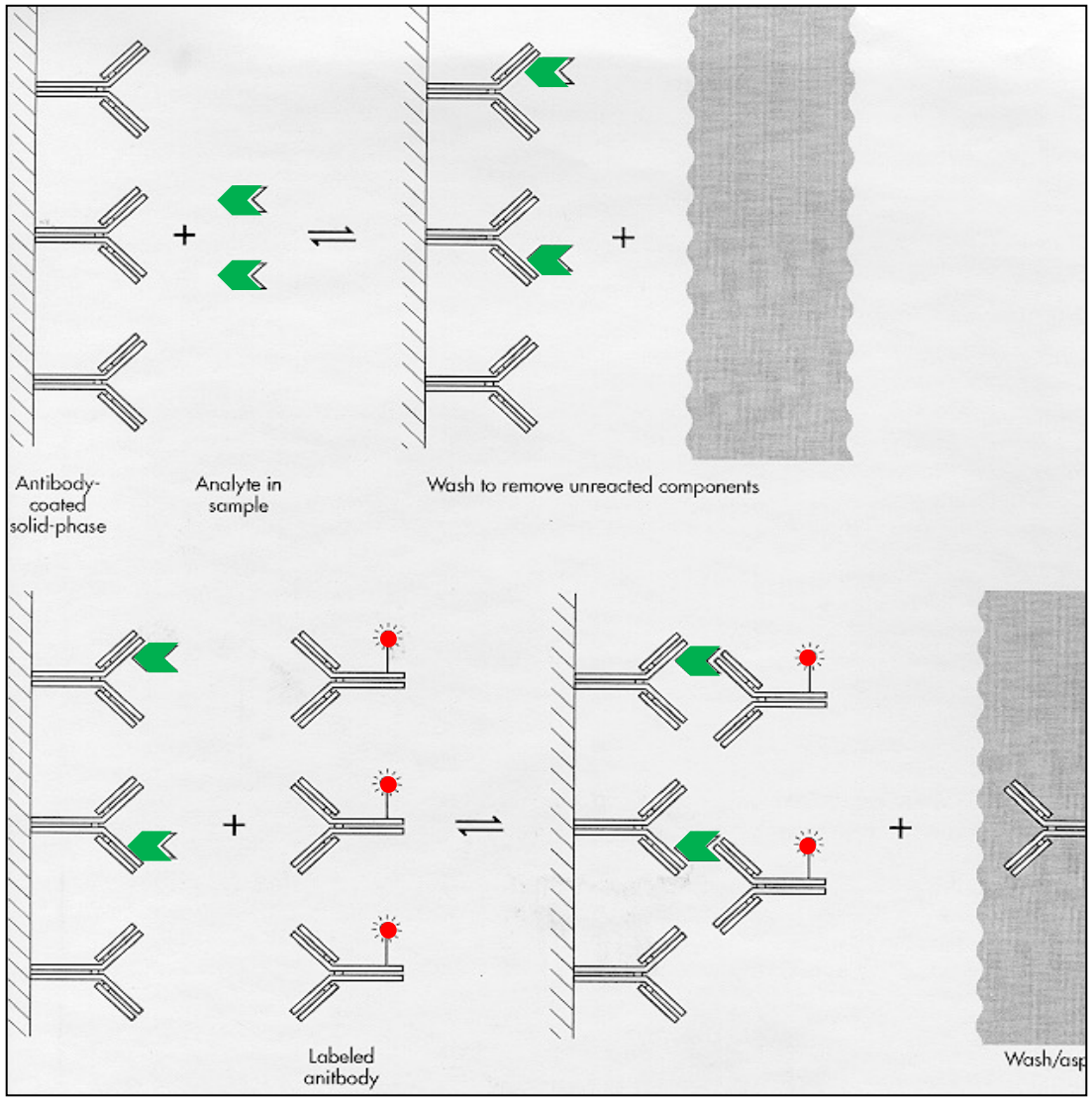
one-step assay



When capture antibody becomes saturated, free analyte in solution binds to the detection antibody and prevents it from binding to the antigen on the solid phase

Immunometric assay

two-step assay



=> avoids **high dose hook effect**

ELISA: data analysis

An-Ab binding:

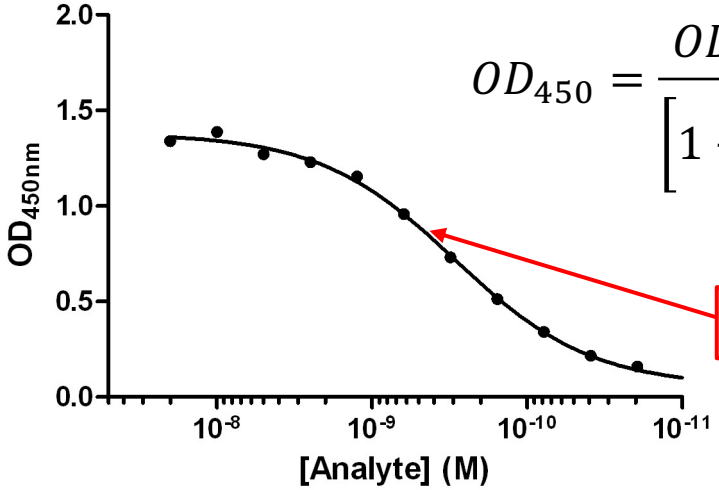
$$[An] + [Ab] \underset{k_2}{\overset{k_1}{\rightleftharpoons}} [AnAb]$$

$$K = \frac{[AnAb]}{[An][Ab]}$$

Surface-bound immune complex

4-parameter logistic function

$$OD_{450} = \frac{OD_{max} - bg}{\left[1 + \left(\frac{[An]}{C_{50}}\right)^{s}\right]}$$

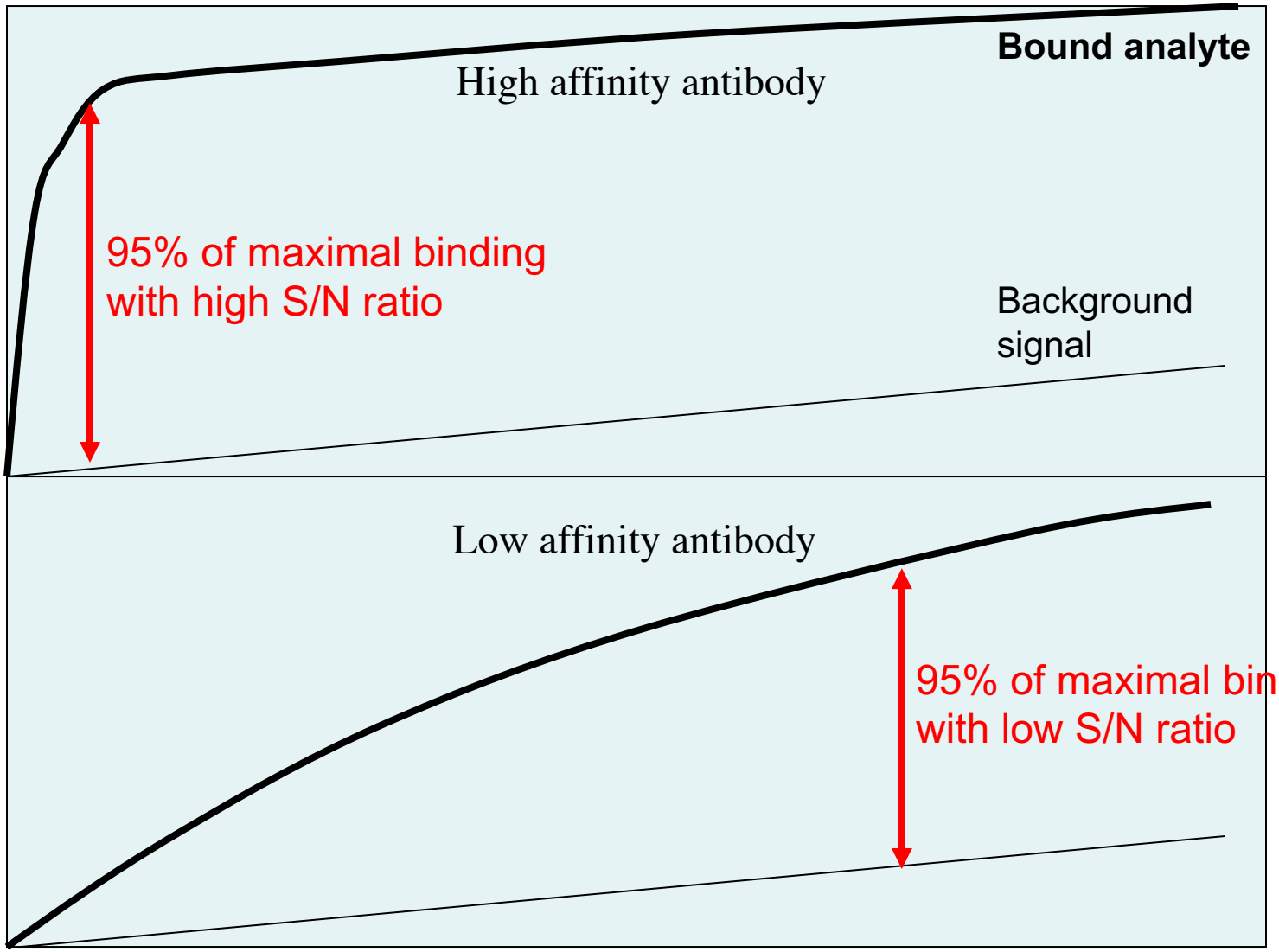


Variables: "optical density" (*OD = absorbance)* and [*An*]

Fitted parameters:

- OD_{max} (signal at saturation)
- *bg* (background signal)
- C_{50} (midrange concentration)
- *s* (slope)

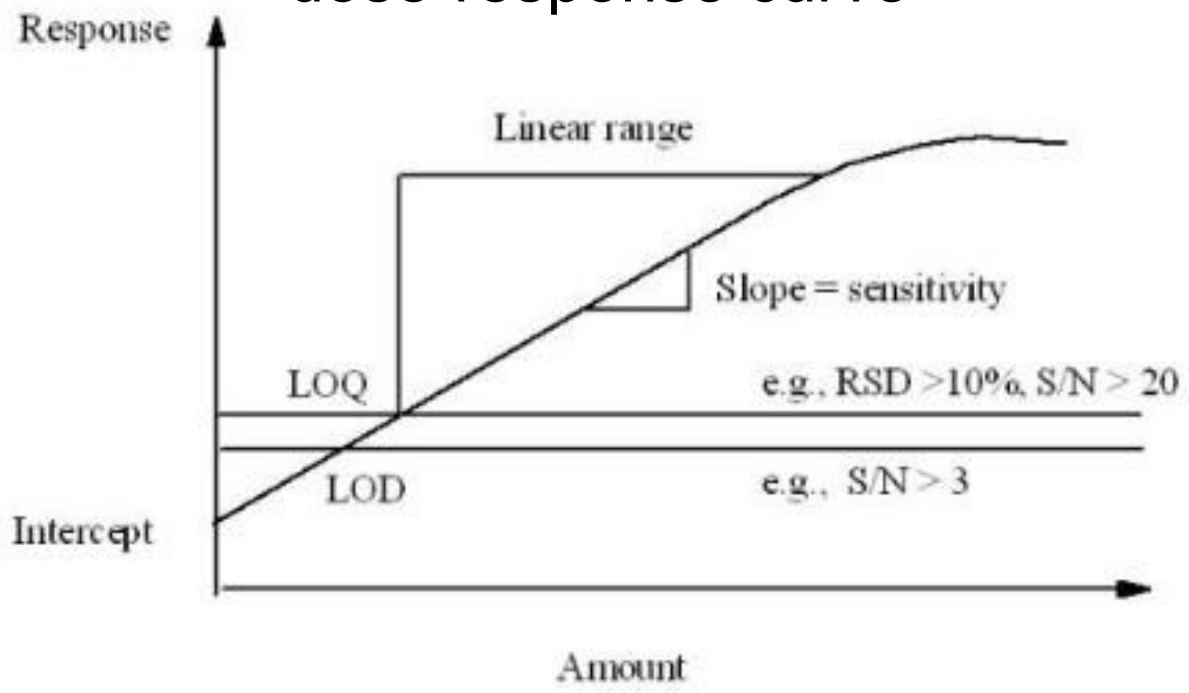
Detection limit of non-competitive assay



[labeled detection Ab]

Immunometric assay

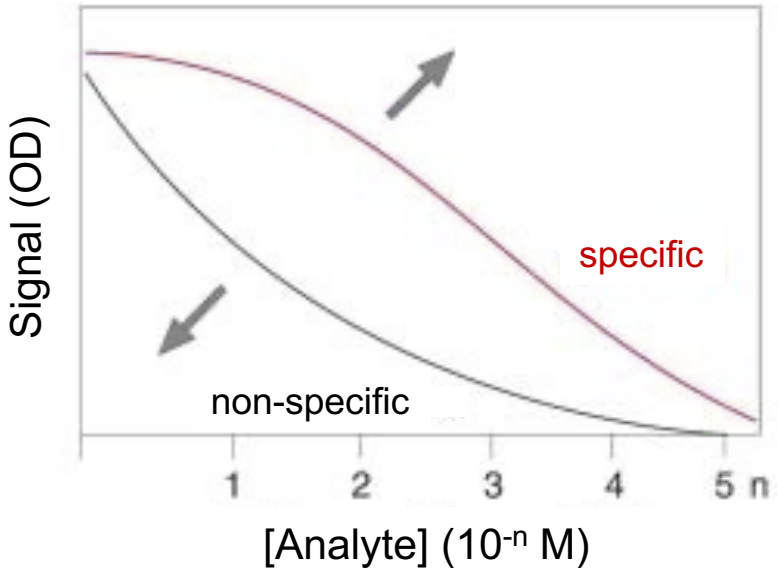
dose-response curve



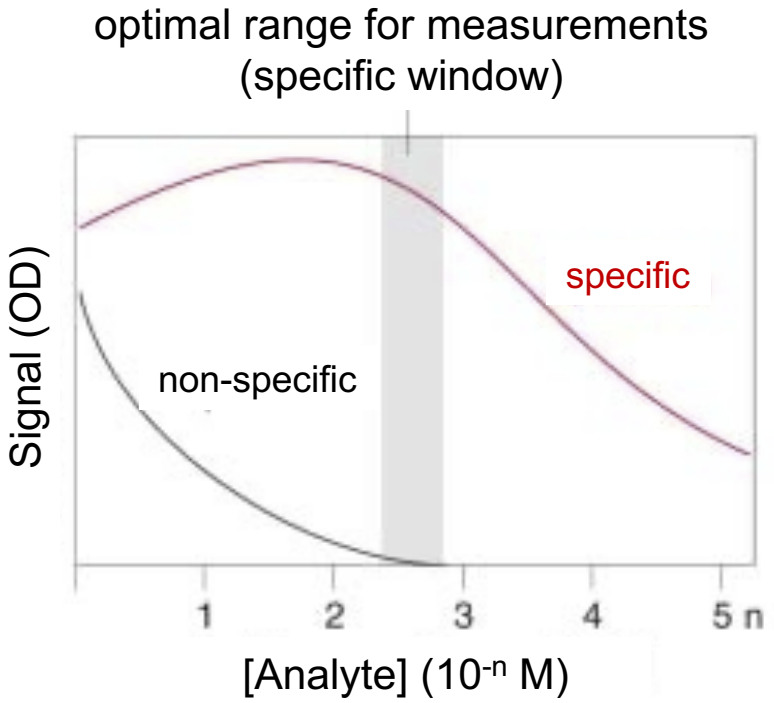
Limit of detection (LoD) vs. limit of quantification (LoQ)	
LoD	**LoQ**
The smallest concentration of an analyte in a test sample that we can easily distinguish from zero	The smallest concentration of an analyte in a test sample that we can determine with acceptable repeatability and accuracy

Optimization of immunoassays

Non-optimized assay



Optimized assay

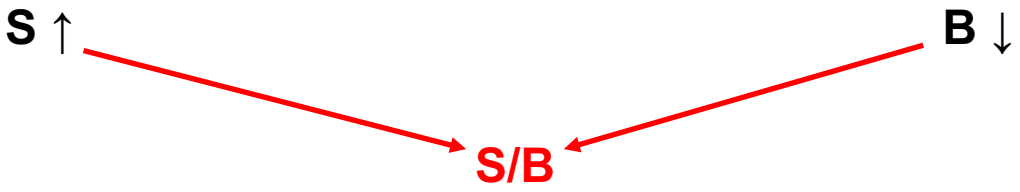


Labeling strategies

Fluorescence/luminescence
=> high-specific activity

Various detection modes can be combined with each other

Signal amplification	Background reduction
- brighter fluorescence improved quantum yield / quantum dots	**- electrochemistry** amperometry, voltametry, impedimetry
- **multiple labeling** attaching several reporter molecules / dye-doped nanoparticles / liposomes	**- (electro-)chemiluminescence** luminol / ruthenium-bipyridyl-complex
- **signal cascades** subsequent amplification steps	**- time-resolved fluorescence** lanthanide complexes
- **enzyme amplification** horseradish peroxidase	**- anti-Stokes photoluminescence** photon-upconversion, UCNPs

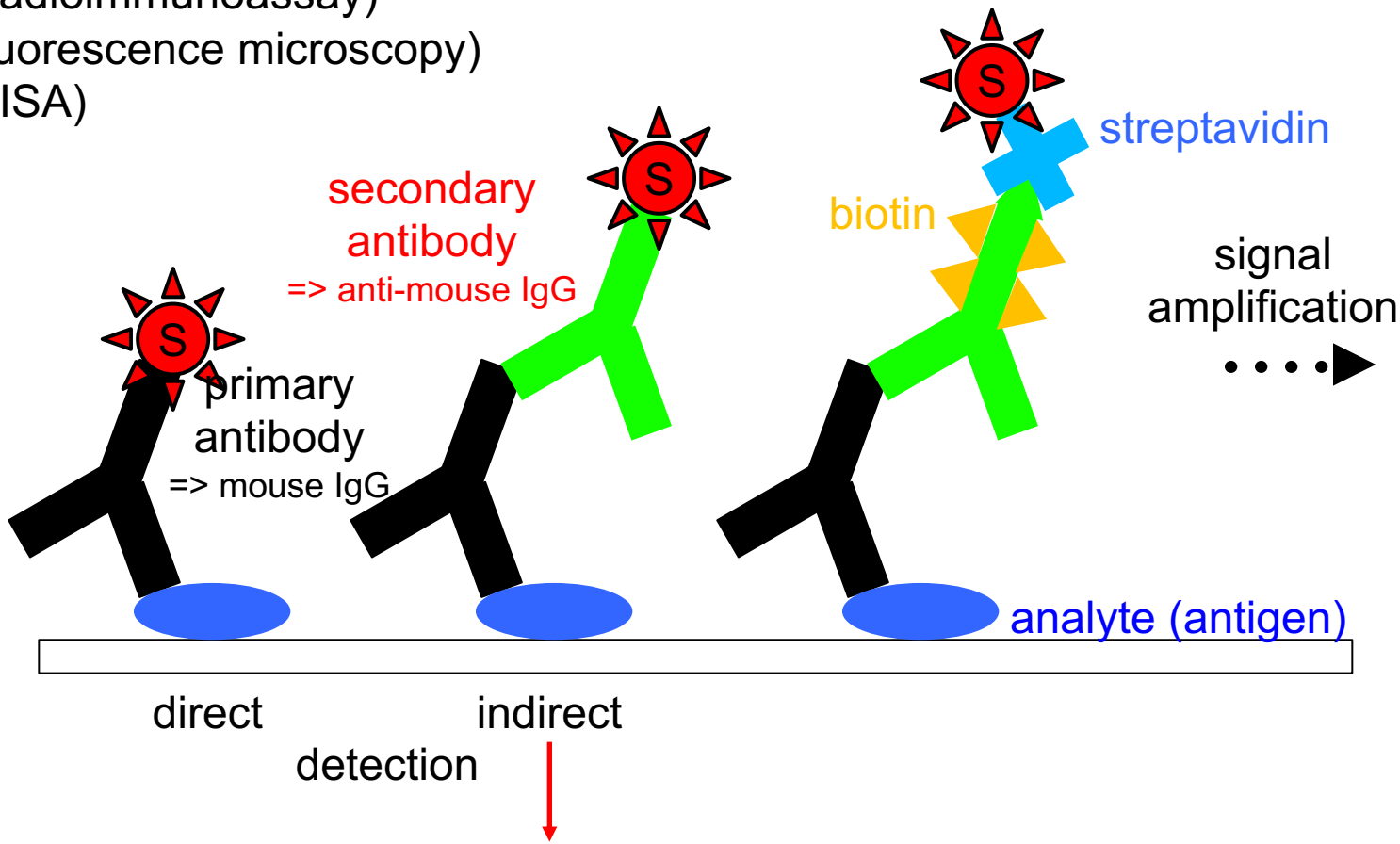


Here we only talk about the **optical signal and background**
=> even the best S/B is useless if the label binds to the surface
(non-specific binding)

Signal cascades

S (signal) examples:

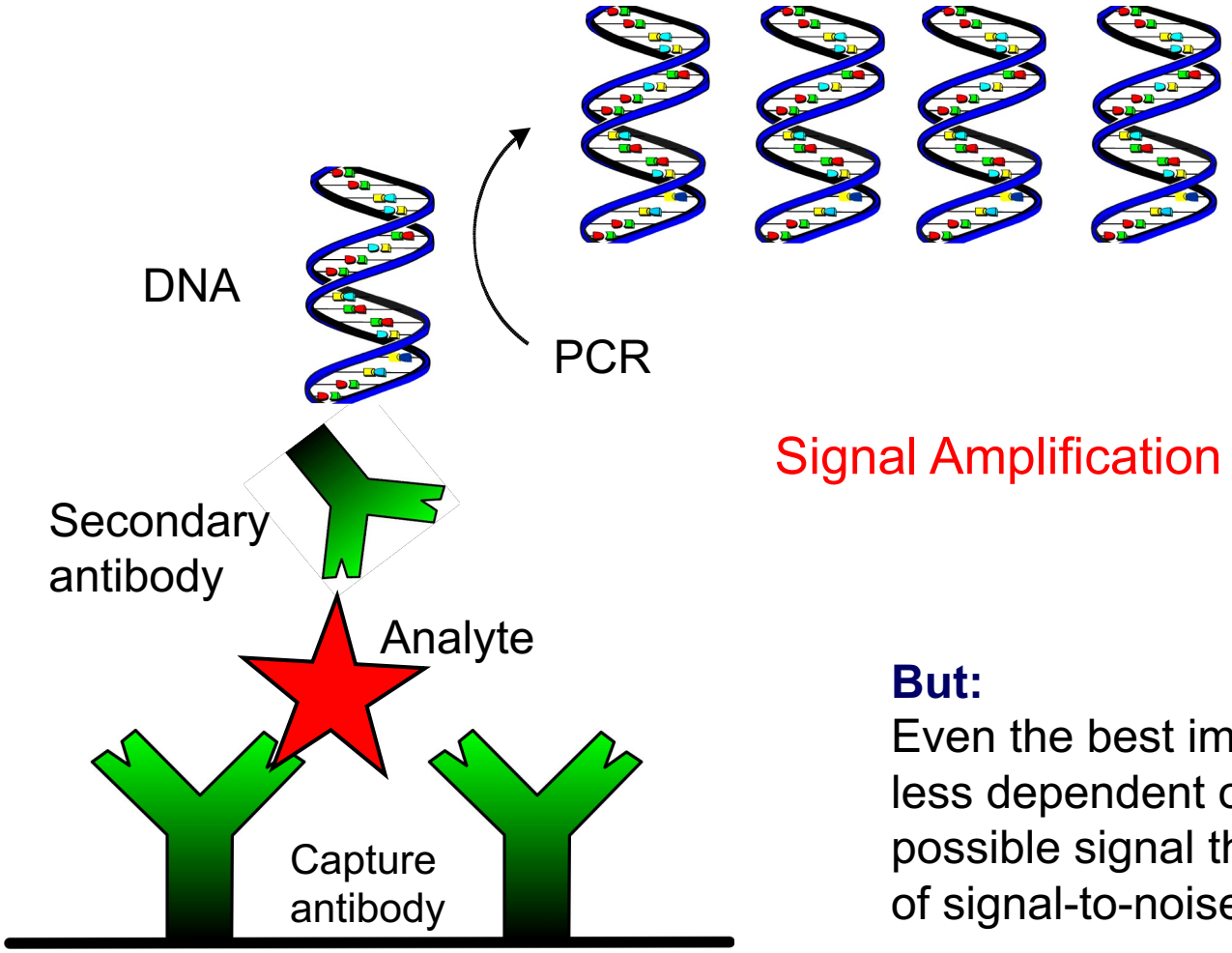
- radionuclide (radioimmunoassay)
- fluorophore (fluorescence microscopy)
- enzymatic (ELISA)



Advantages:

- only one type of secondary antibody is needed for many types of primary antibodies
- higher sensitivity => polyclonal secondary antibody can bind to different sites of the primary antibody

Immuno-PCR

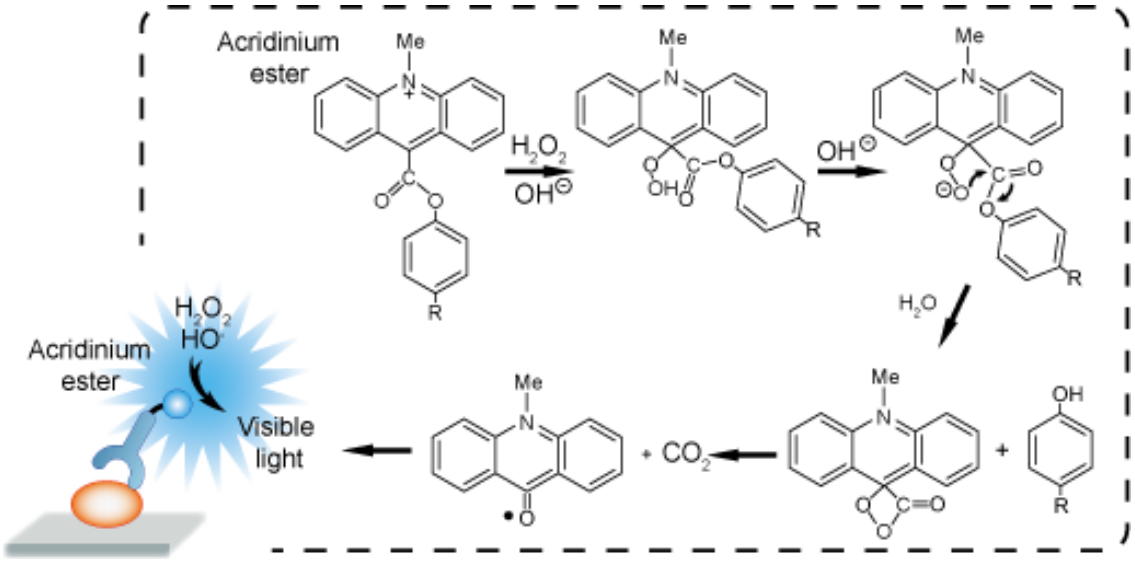


Signal Amplification by PCR

But:
Even the best immunosassay is less dependent on the highest possible signal than on the ratio of signal-to-noise (SNR).

Chemiluminescent labeling

acridinium ester

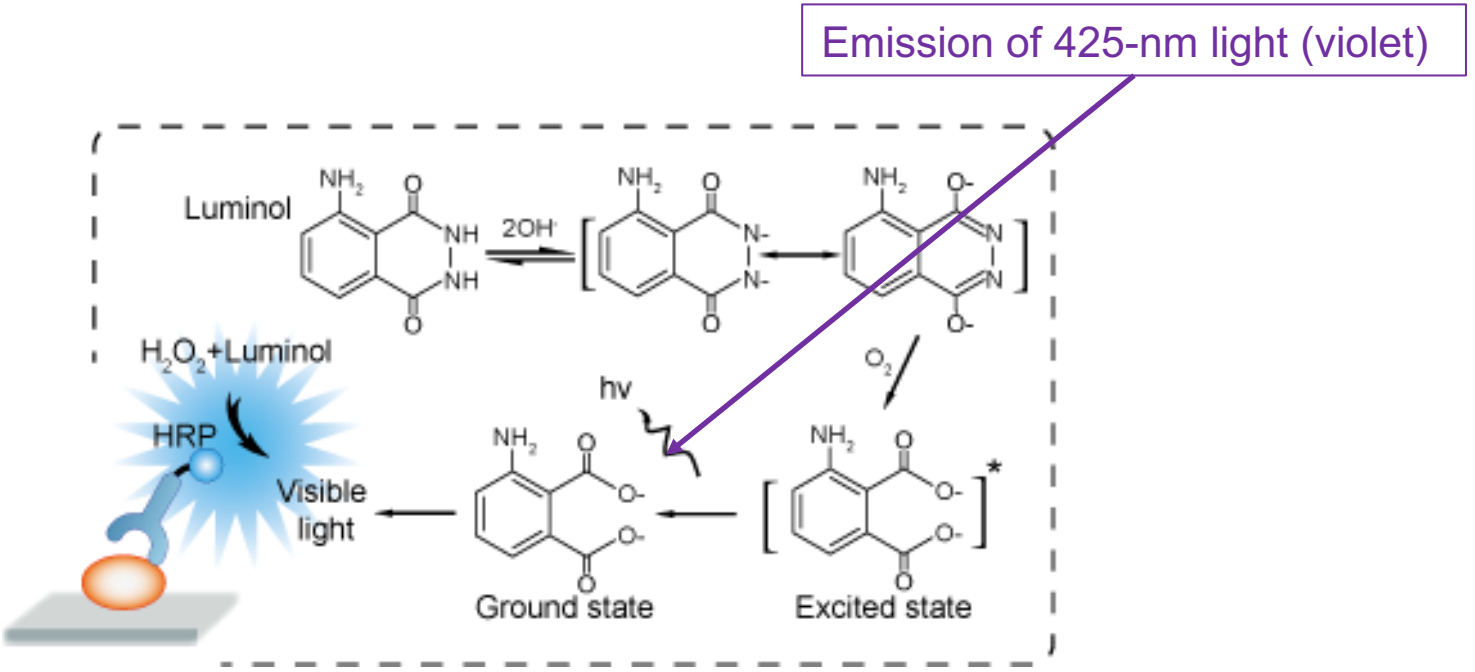


=> no enzyme required, but only **one photon per molecule**
(low specific-acitivity label)

Weeks, I. (1983) *Clin. Chem.* 29, 1474-1479

Chemiluminescent labeling

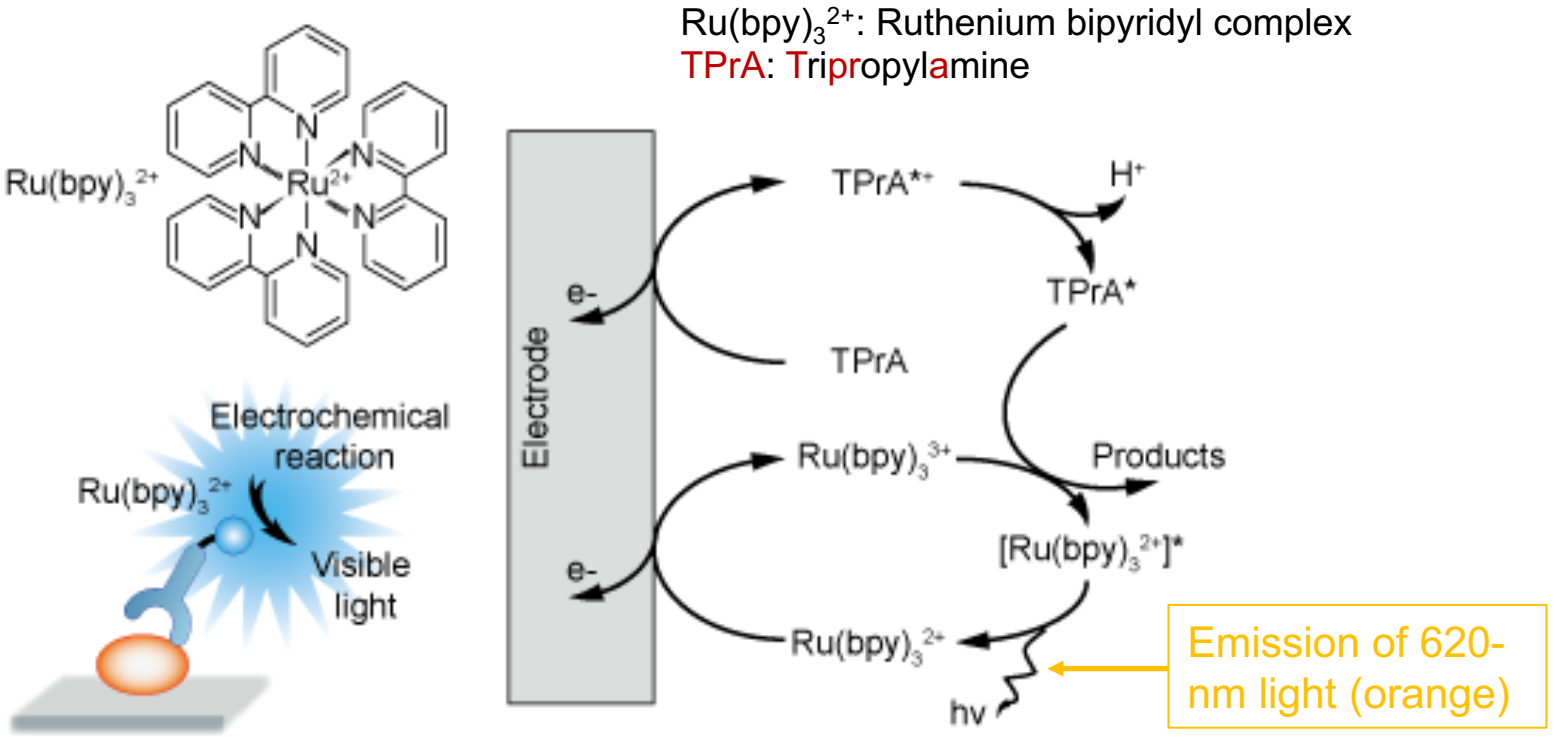
luminol



=> HRP oxidizes luminol:
one photon per catalytic turnover event

Electro-chemiluminescent labeling systems

=> Light emission mediated by a redox reaction



=> no enzyme required

=> the label can cycle between an oxidized and reduced state to generate many photons per molecule

(Electro-)chemoluminescent labels

Advantages: no need for excitation light
=> Simpler and more compact instrumentation
=> No autofluorescence or light scattering: background-free detection

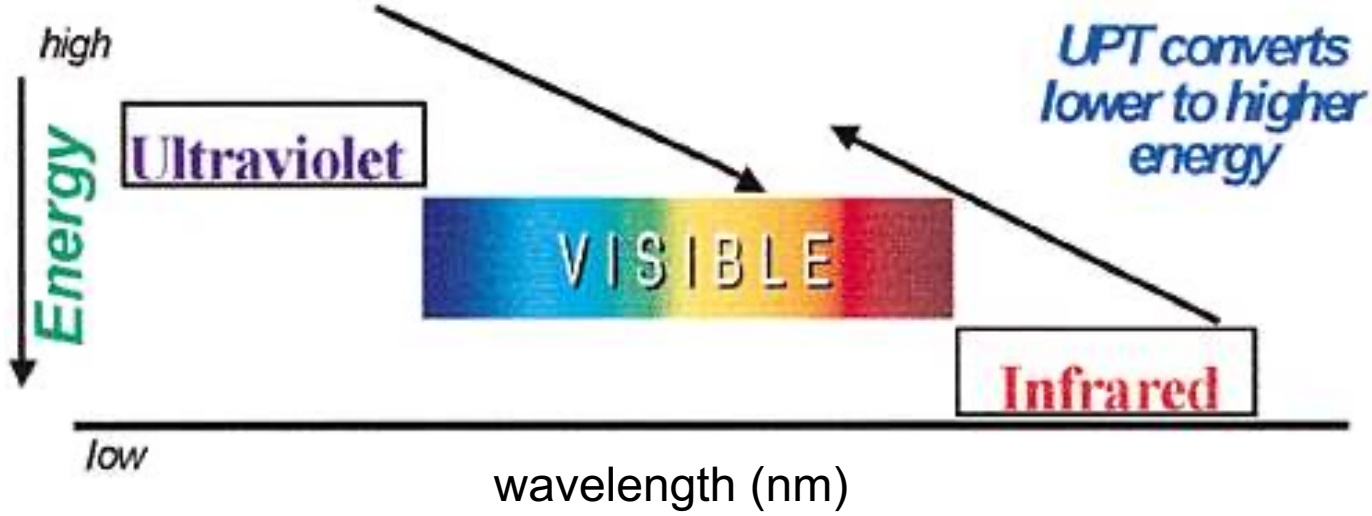
Disadvantages:
Each (electro-)chemical or enzymatic turnover event
only results in the emission of a single photon
=> weaker overall signal / not the highest activity
(as compared to fluorescence)

Time-resolved lanthanide fluorescence

=> Guest lecture on April 23rd

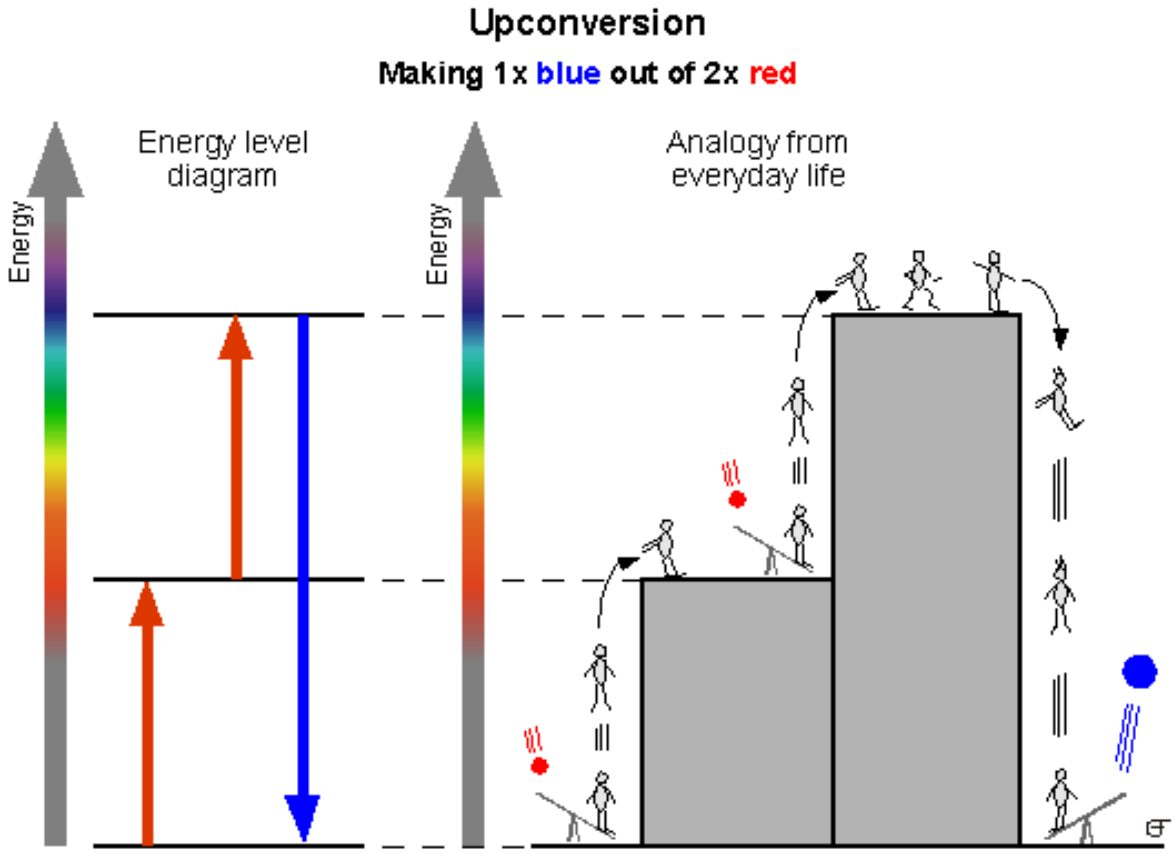
Photon-upconversion nanoparticles (UCNPs)

UCNPs: anti-Stokes emission



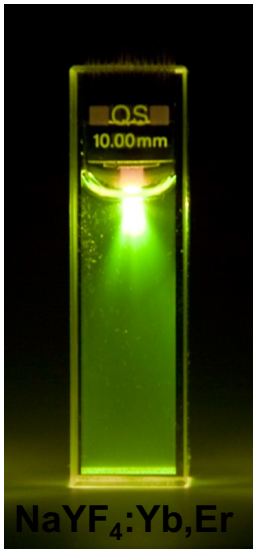
van de Rijke et al. (2001) *Nature Biotechnology* 19, 273–276

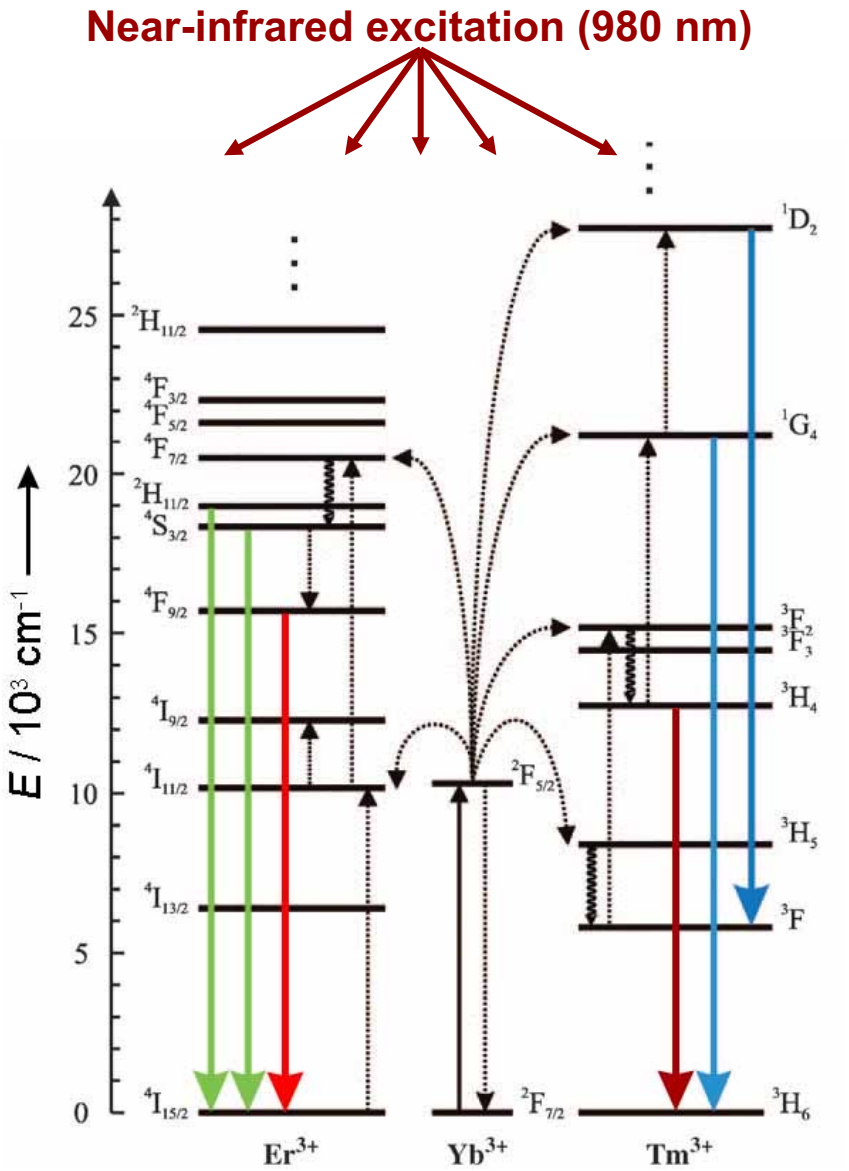
Sequential absorption of two or more photons



Sequential absorption of 2 or more photons via long-lived transition states
=> More time for absorbing a further photon

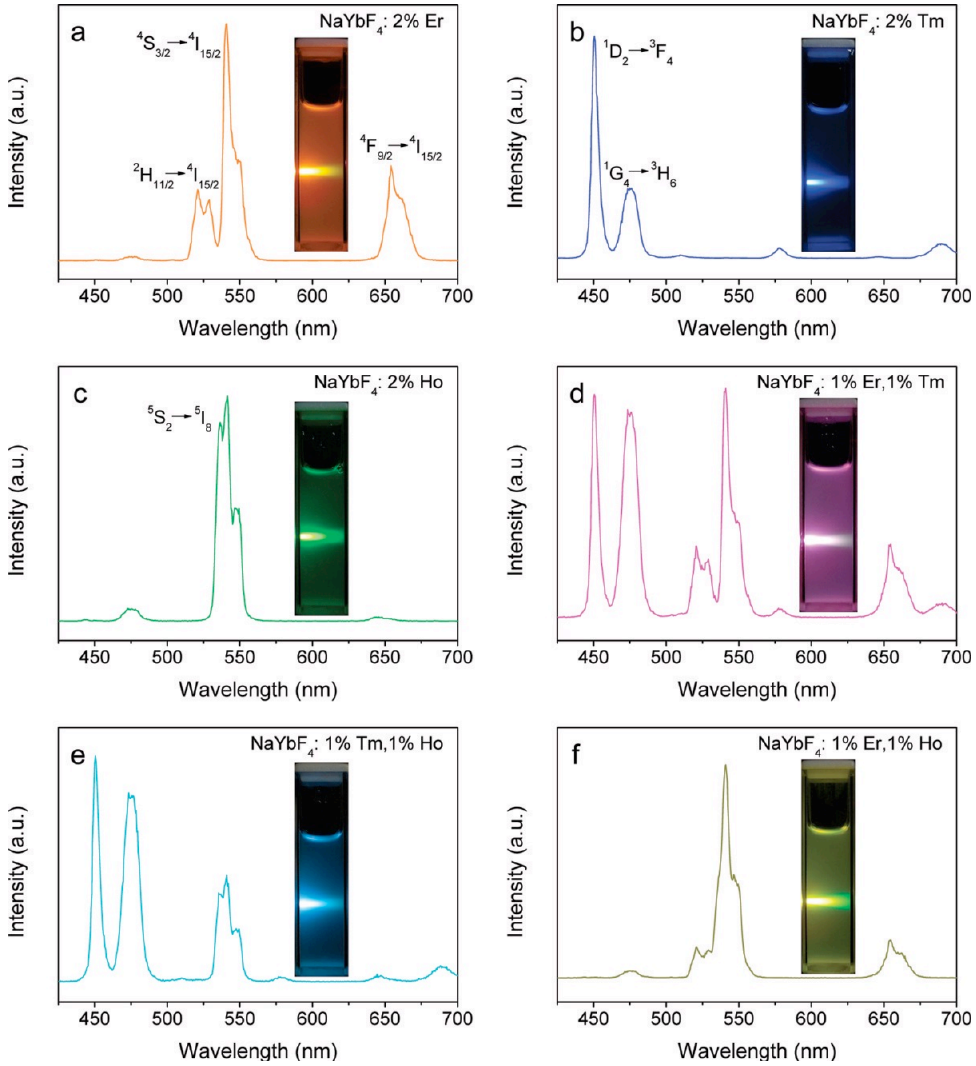
Upconversion luminescence of UCNPs





Luminescence of UCNPs depends on lanthanide dopant composition

Emission depends on lanthanide composition

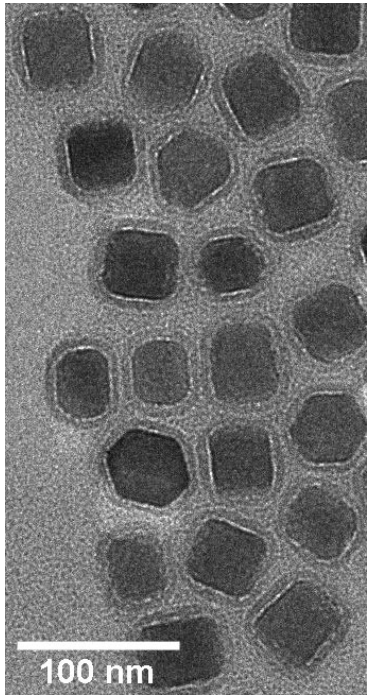


In time-resolved measurements:
different lifetimes

Here:
different emission signatures

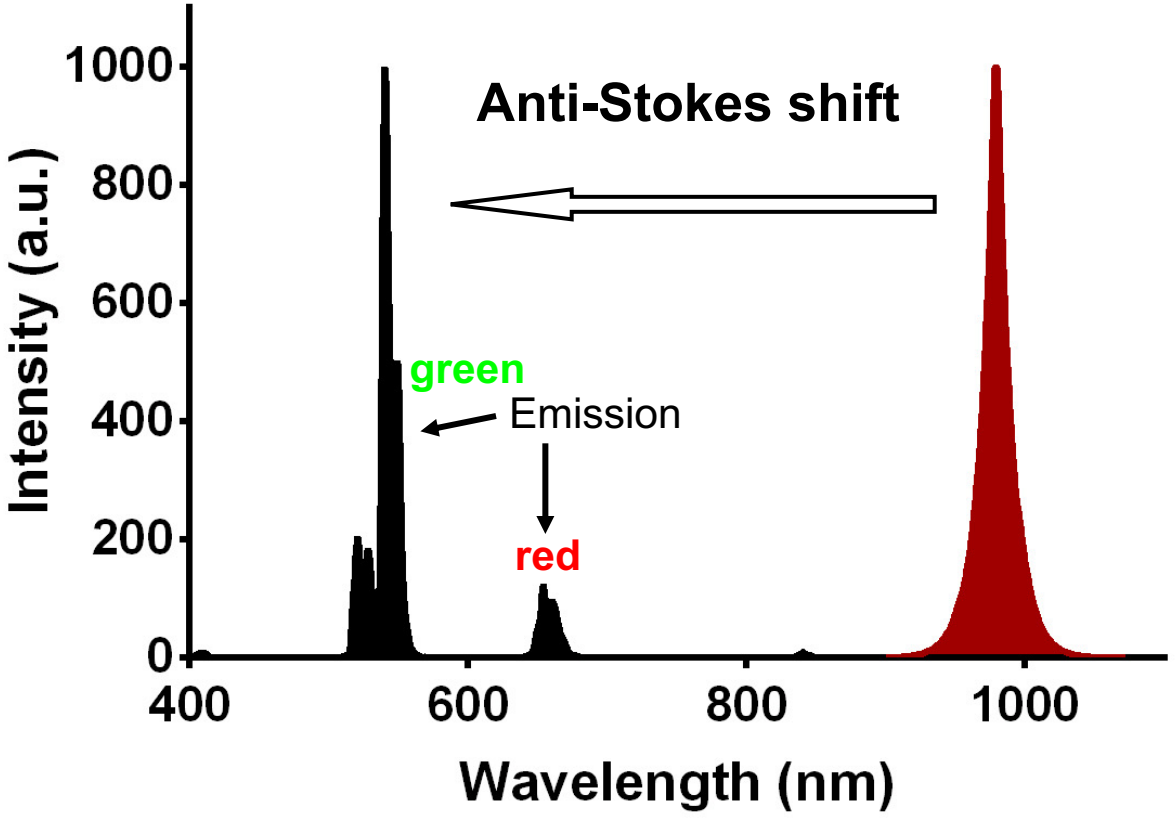
58	59	60	61	62	63	64	65	66	67	68	69	70	71
140	141	144	145	150	152	157	159	163	157	167	169	173	175
Ce	**Pr**	**Nd**	**Pm**	**Sm**	**Eu**	**Gd**	**Tb**	**Dy**	**Ho**	**Er**	**Tm**	**Yb**	**Lu**
Cerium	Praseodymium	Neodymium	Promethium	Samarium	Europium	Gadolinium	Terbium	Dysprosium	Holmium	Erbium	Thulium	Ytterbium	Lutetium
$4f^15d^16s^2$	$4f^36s^2$	$4f^46s^2$	$4f^56s^2$	$4f^66s^2$	$4f^76s^2$	$4f^75d^16s^2$	$4f^96s^2$	$4f^{10}6s^2$	$4f^{11}6s^2$	$4f^{12}6s^2$	$4f^{13}6s^2$	$4f^{14}6s^2$	$5d^16s^2$

UCNPs as background-free optical labels



TEM of UCNPs
$NaYF_4$:Yb,Er

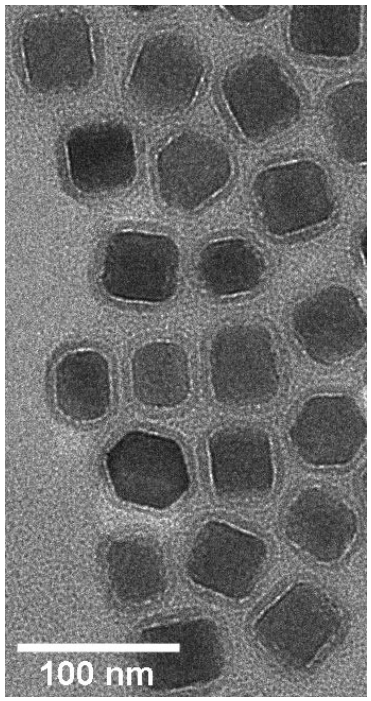
Hexagonal
crystal structure



Photon-upconversion is ca. 1,000,000 x
more efficient than 2-photon excitation

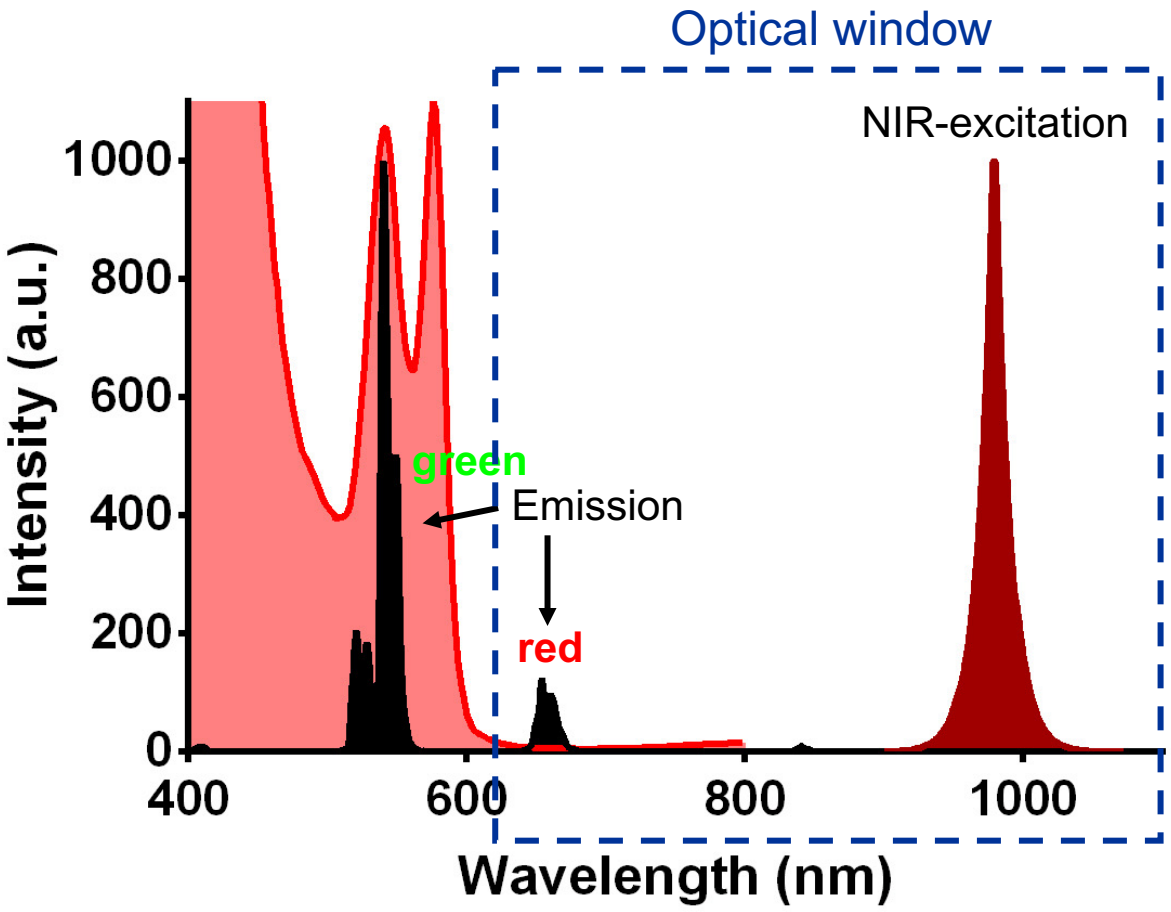
=> excitation by using a continuous 980-nm laser source

UCNPs as background-free optical labels



TEM of UCNPs
$NaYF_4$:Yb,Er

Hexagonal
crystal structure



No autofluorescence
Very low light scattering
} Background-free imaging

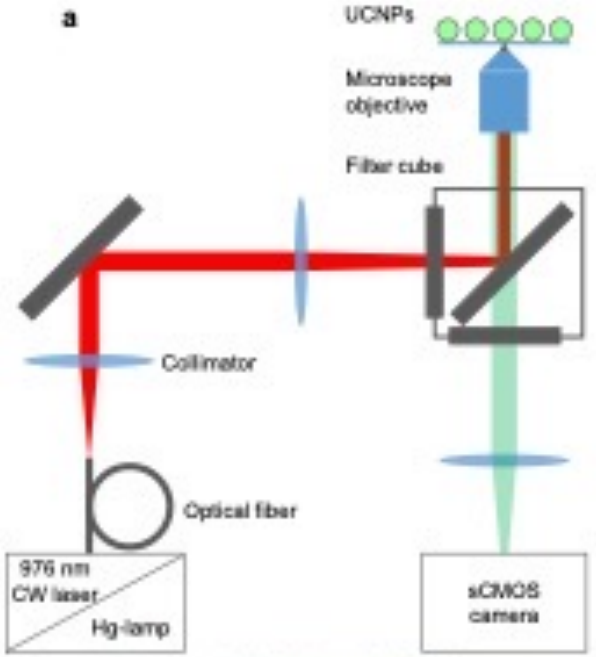
... and completely photostable

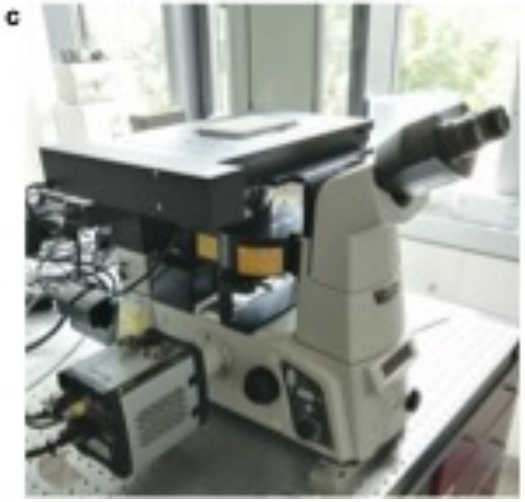
Advantages of UCNPs

Features	Unlike	Enables
Excitation by NIR light (980 nm)	Organic fluorophores / QD	1) Background-free diagnostic assays 2) Deep tissue / small animal imaging
Large anti-Stokes shifts	Org. fluorophores	Excellent separation of detection channels
Narrow and multiple emission bands of UV, visible or NIR light	Org. fluorophores	Multiplexing / ratiometric measurements
No photobleaching	Org. fluorophores	Long-time imaging
Paramagnetic (co-dopant: Gd^{3+})	Org. fluorophores	Hybrid nanoparticles: Magnetic resonance imaging (MRI)
Low toxicity	QD / radionuclides	Cellular imaging / easier handling

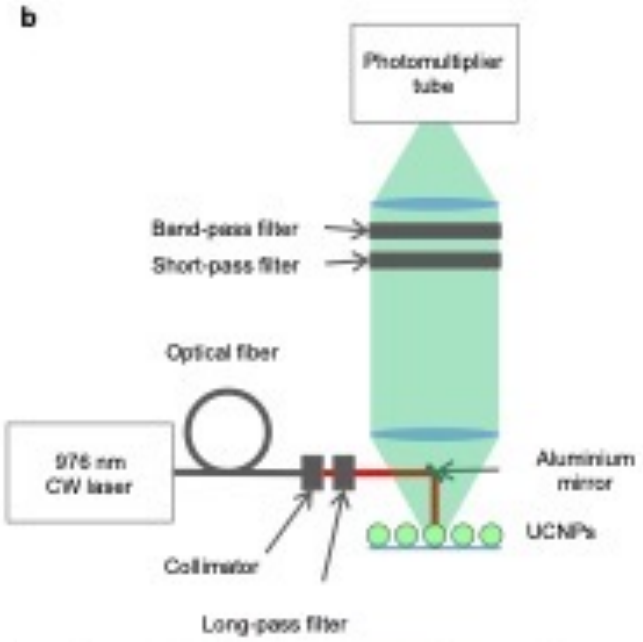
Instruments for the detection of UCNPs

Microsocope

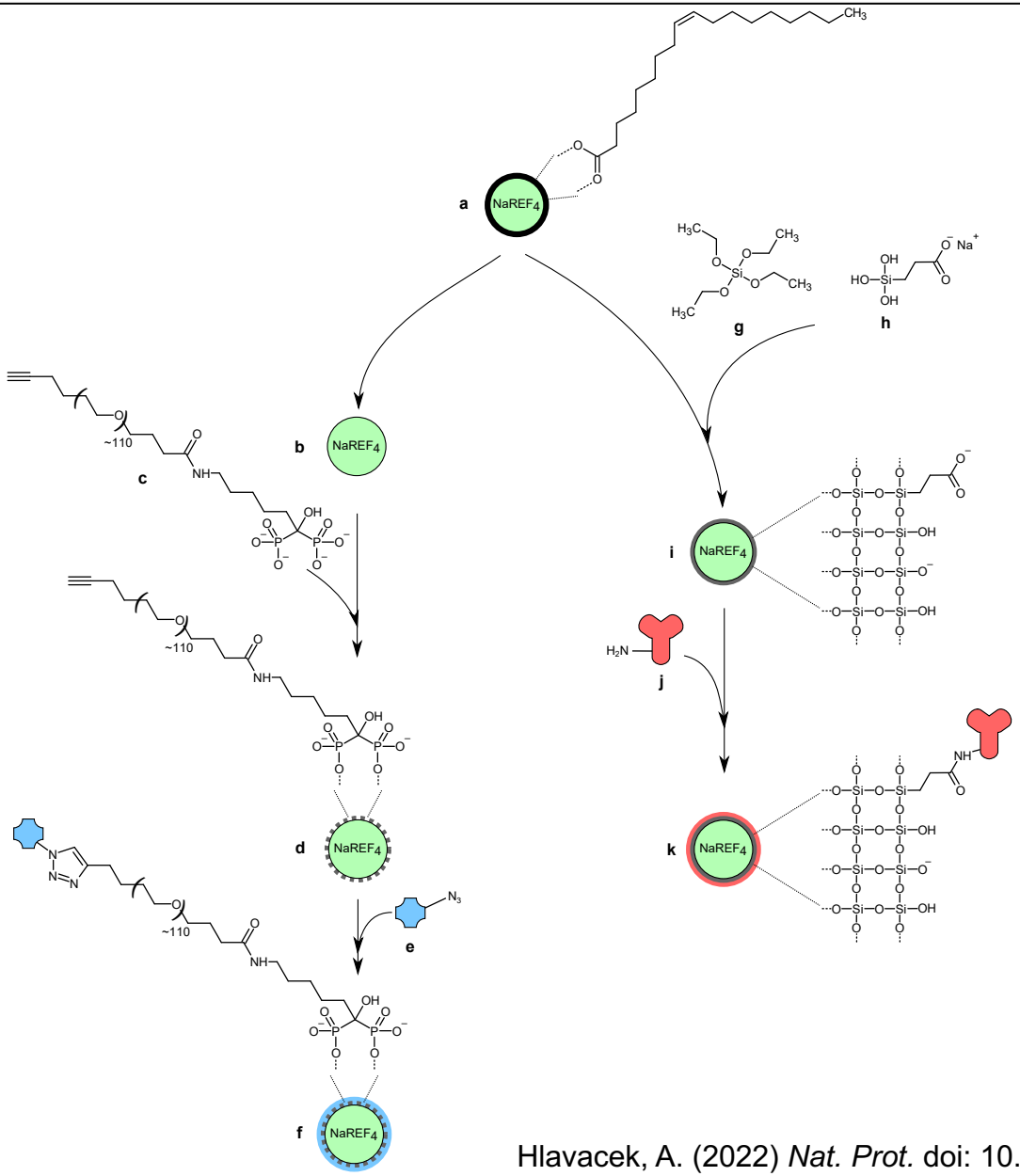




Microtiter plate reader

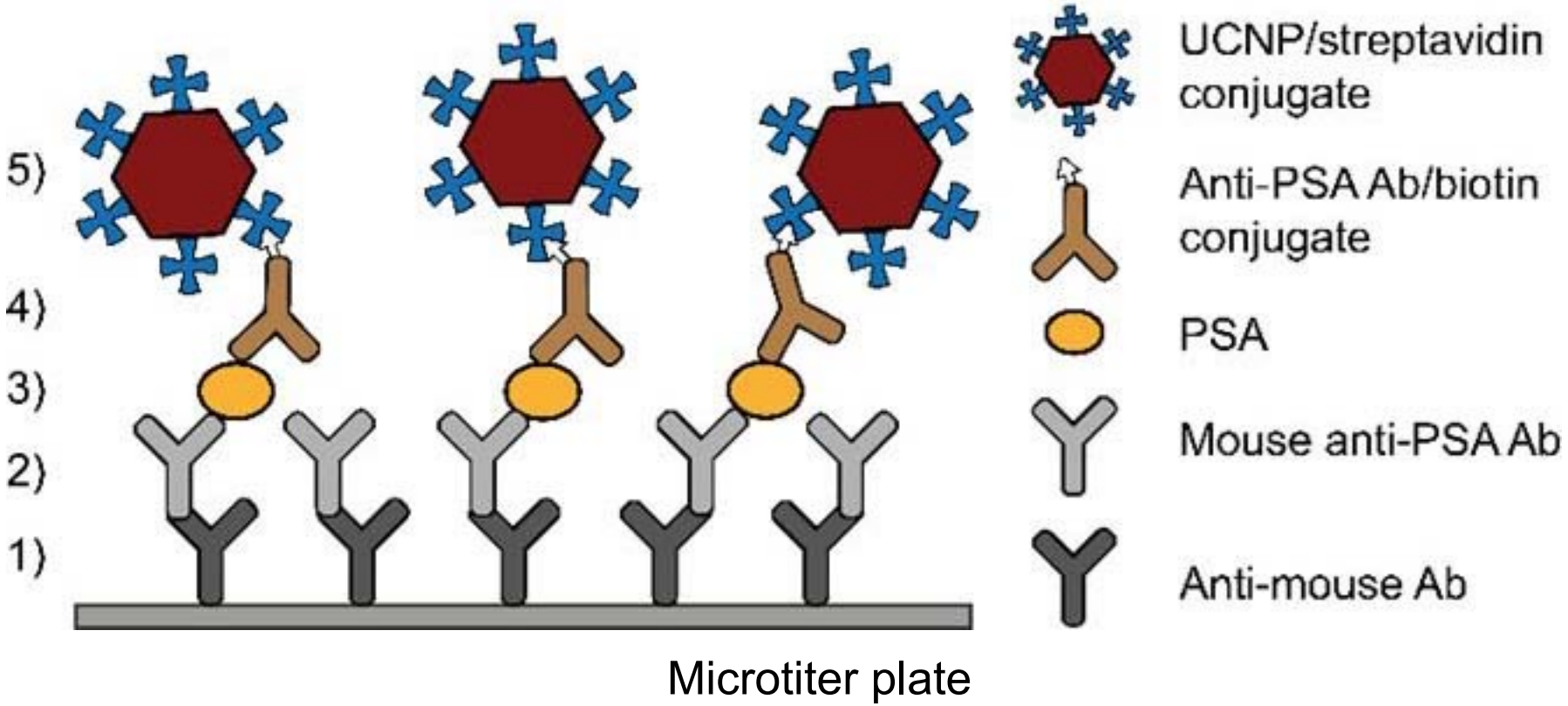


Surface functionalization of UCNPs



Hlavacek, A. (2022) *Nat. Prot.* doi: 10.1038/s41596-021-00670-7

Upconversion-linked immunosorbent assay (ULISA)

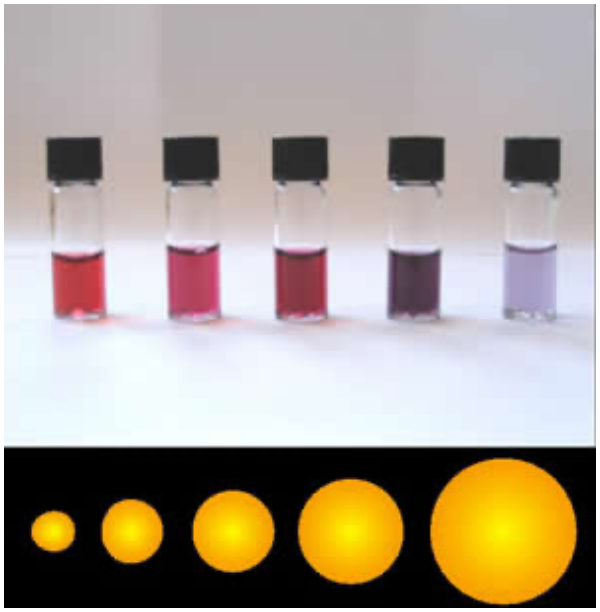


Mickert MJ (2019) *Anal. Chem. 91,* 9435

Gold nanoparticles

Introduction to gold nanoparticles (colloidal gold)

- known since ancient times (glass staining)
- modern synthetic approaches: size control in the range of 2 to 100 nm
- synthesis: reduction of $HAuCl_4$ in aqueous solution e.g. by citrate
- simple surface modification e.g. via self-assembled monolayer (SAM): thiols
- properties:
  1. chemically stable
  2. high electron density
  3. collective oscillations of valence electrons in metal grid
     in resonance with frequency of visible light



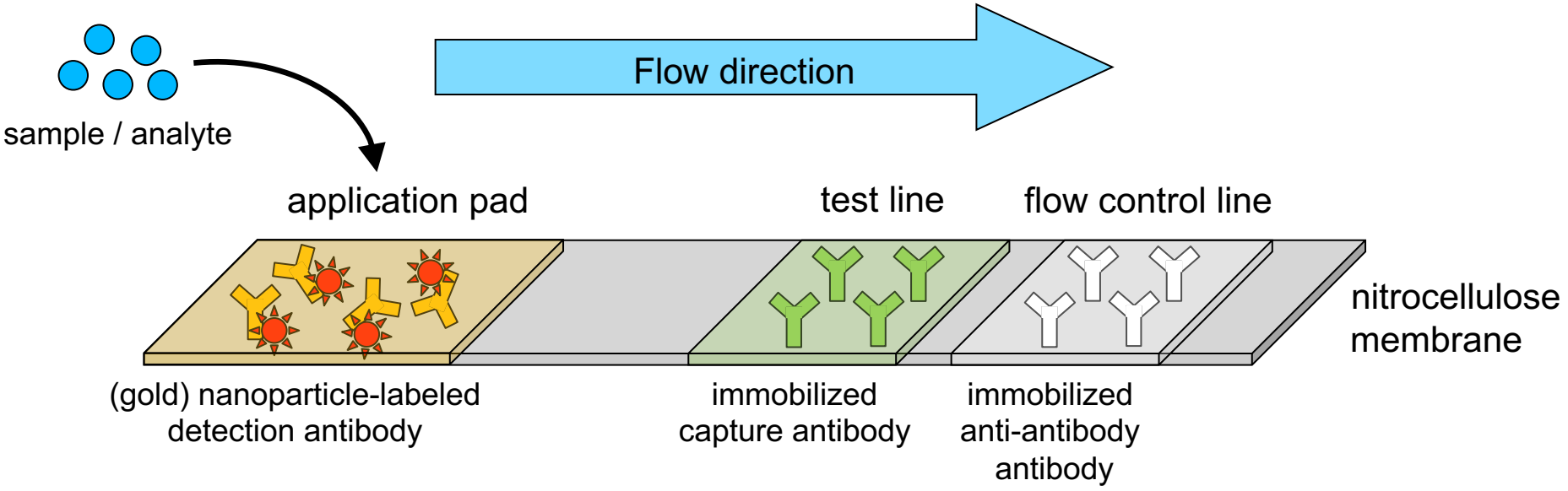
absorption by localised
surface plasmon resonance

↓

size-dependent

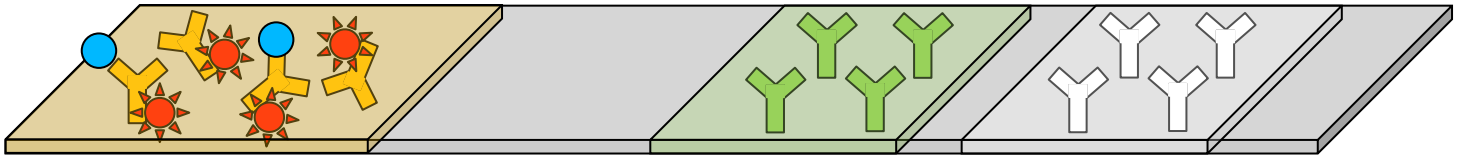
Lateral flow assay

- Separation-based assay using capillary flow in nitrocellulose membrane
- qualitative result: yes/no answer
- pregnancy test measures hCG (human chorionic gonadotropin)

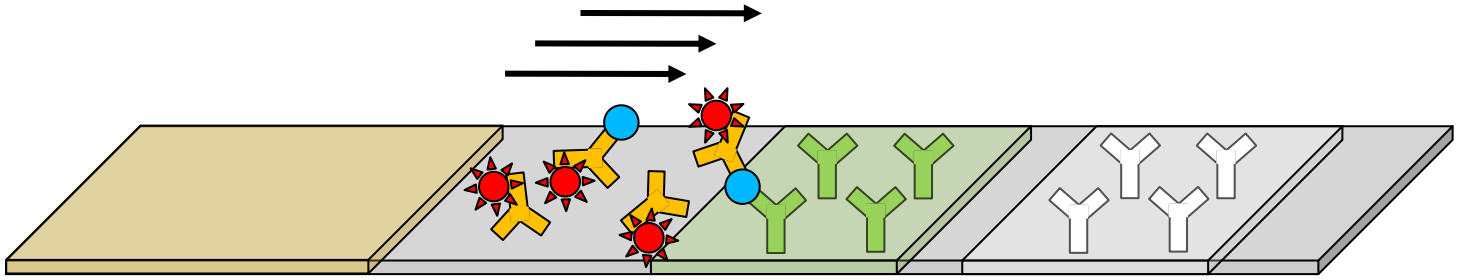


Lateral flow assay

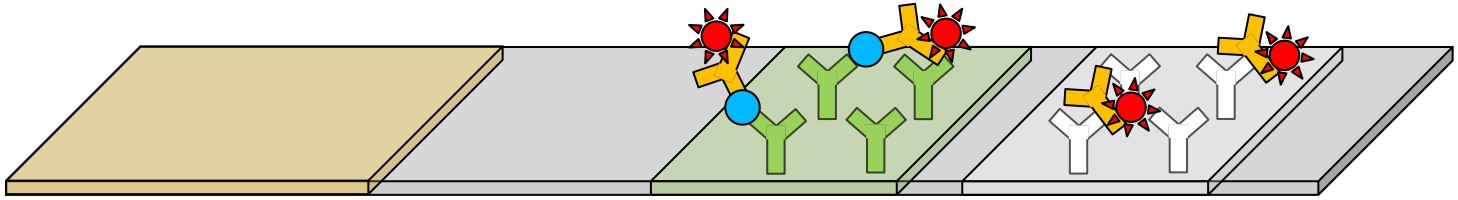
(1) Analyte is bound to labeled antibody



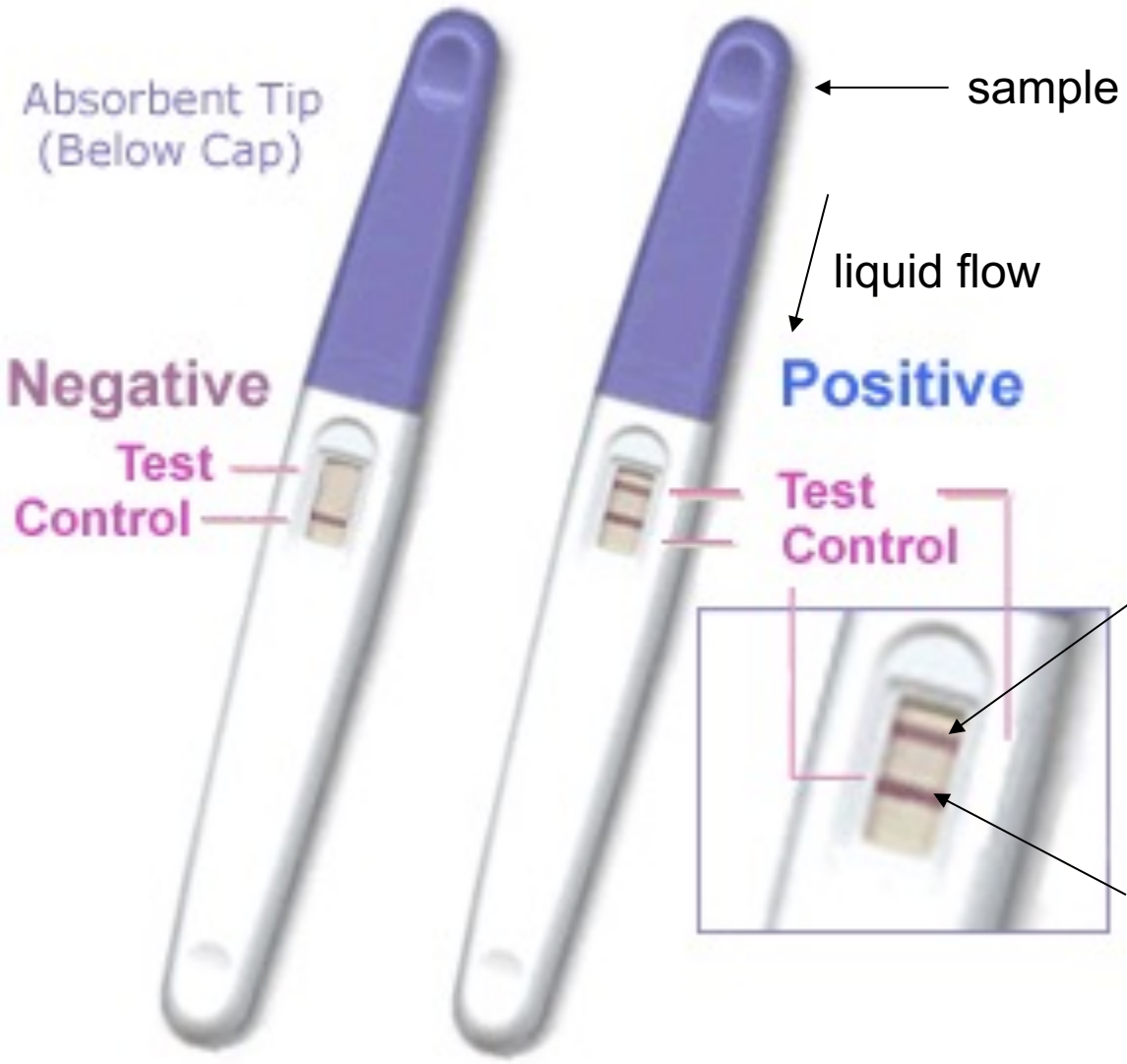
(2) Analyte-labeled antibody complex and non-bound labeled antibody move with flow



(3) Analyte-labeled antibody complex is bound to immobilized capture antibody; labeled antibody is bound to immobilized anti-antibody antibody



Lateral flow assay



1st line (test result)

(analyte + labeled antibody)

-> if not visible, HCG not high
-> not pregnant

2nd line (control)

-> proof that flow and labeled antibodies have worked

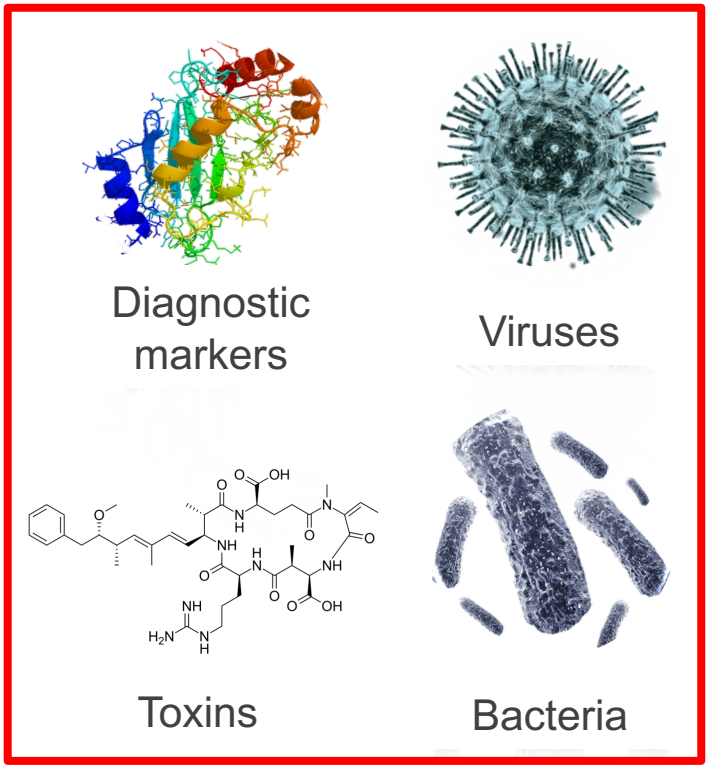
Excursion: Biosensors

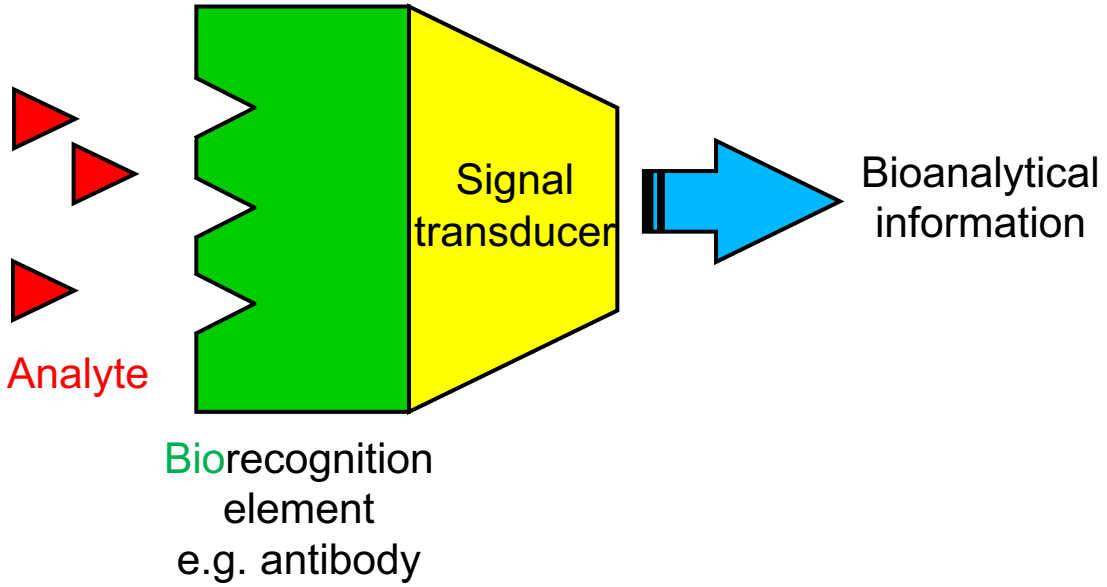
Injection of sample urine (containing hCG) to the dorsal lymph sac of femal frog

=> Frog starts ovulation within 12 hours

=> an example of a “biosensor“

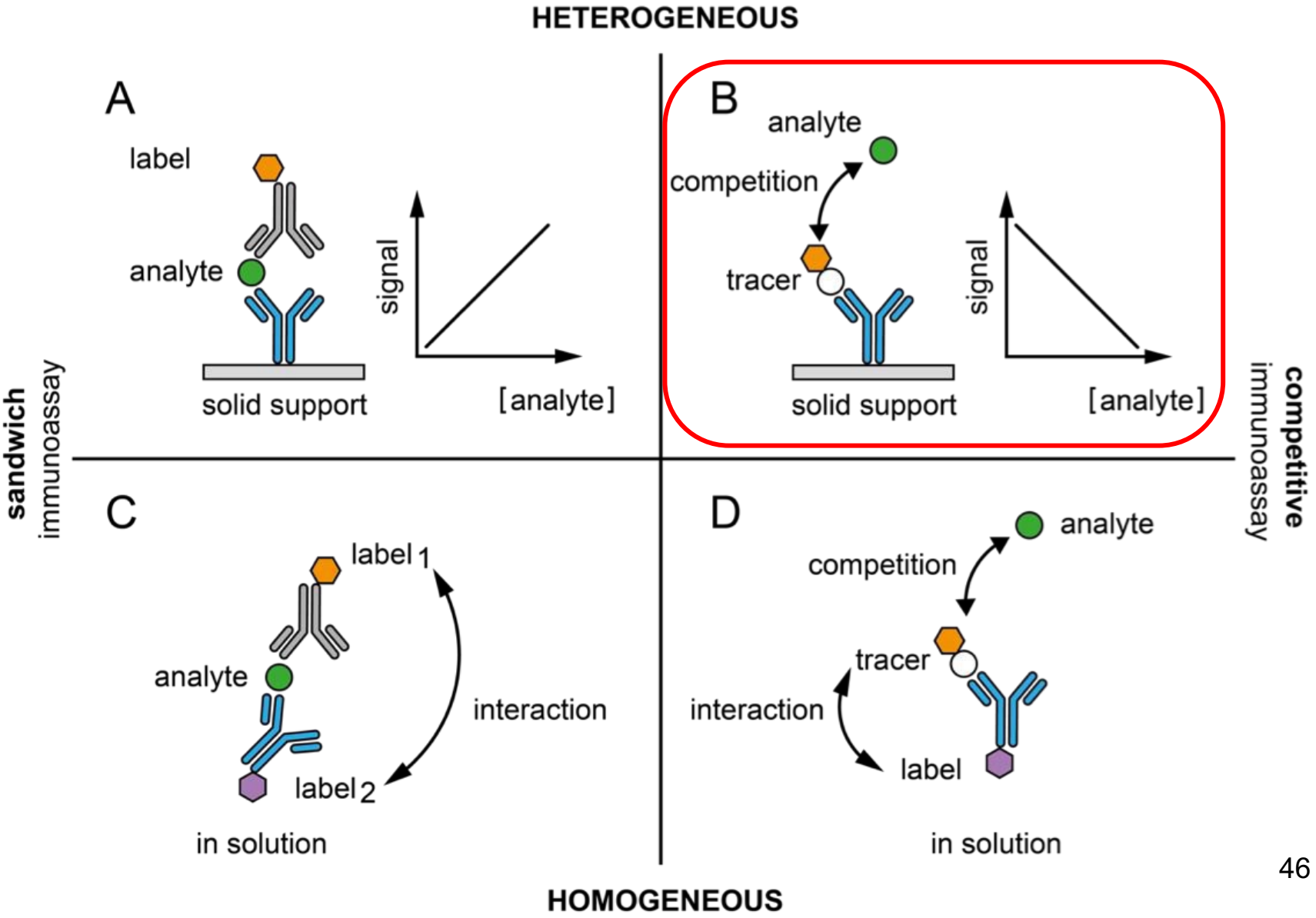
Scheme of a biosensor



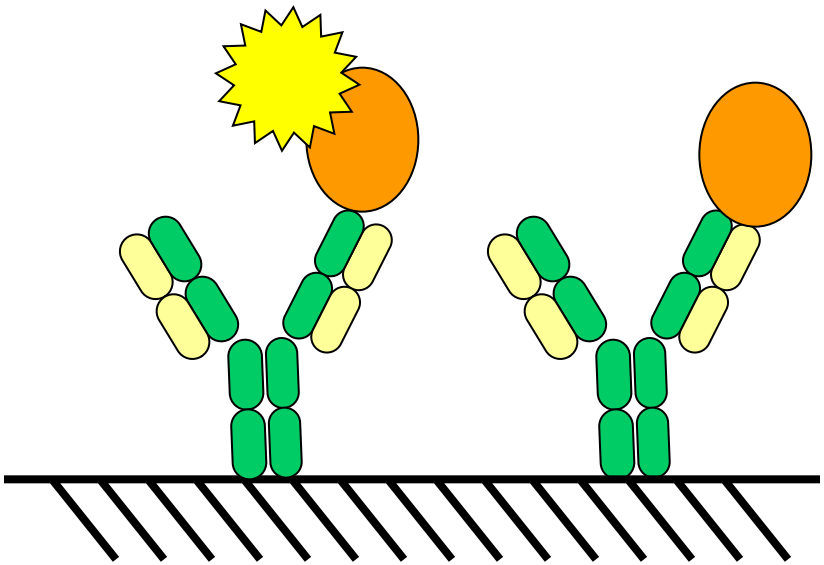


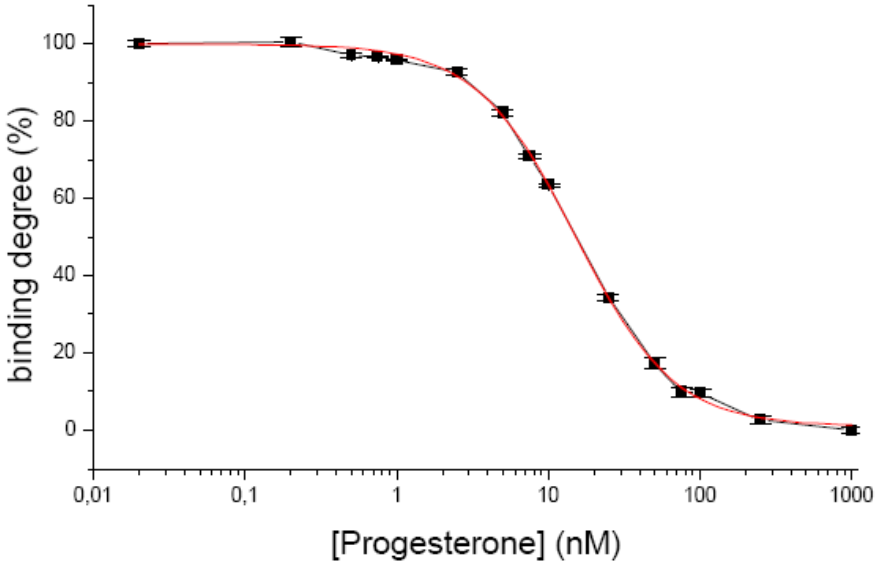
=> What is the difference between an immunoassay and a biosensor?

A rough categorization of immunoassays



Competitive immunoassay



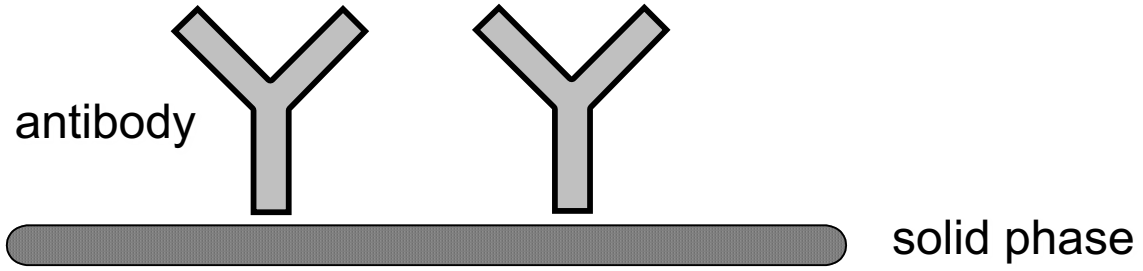


Note: The sandwich ELISA is not applicable to small molecules such as steroid hormones (e.g. progesterone), because they do not possess two epitopes for binding both the capture Ab and the detection Ab.

Competitive immunoassay

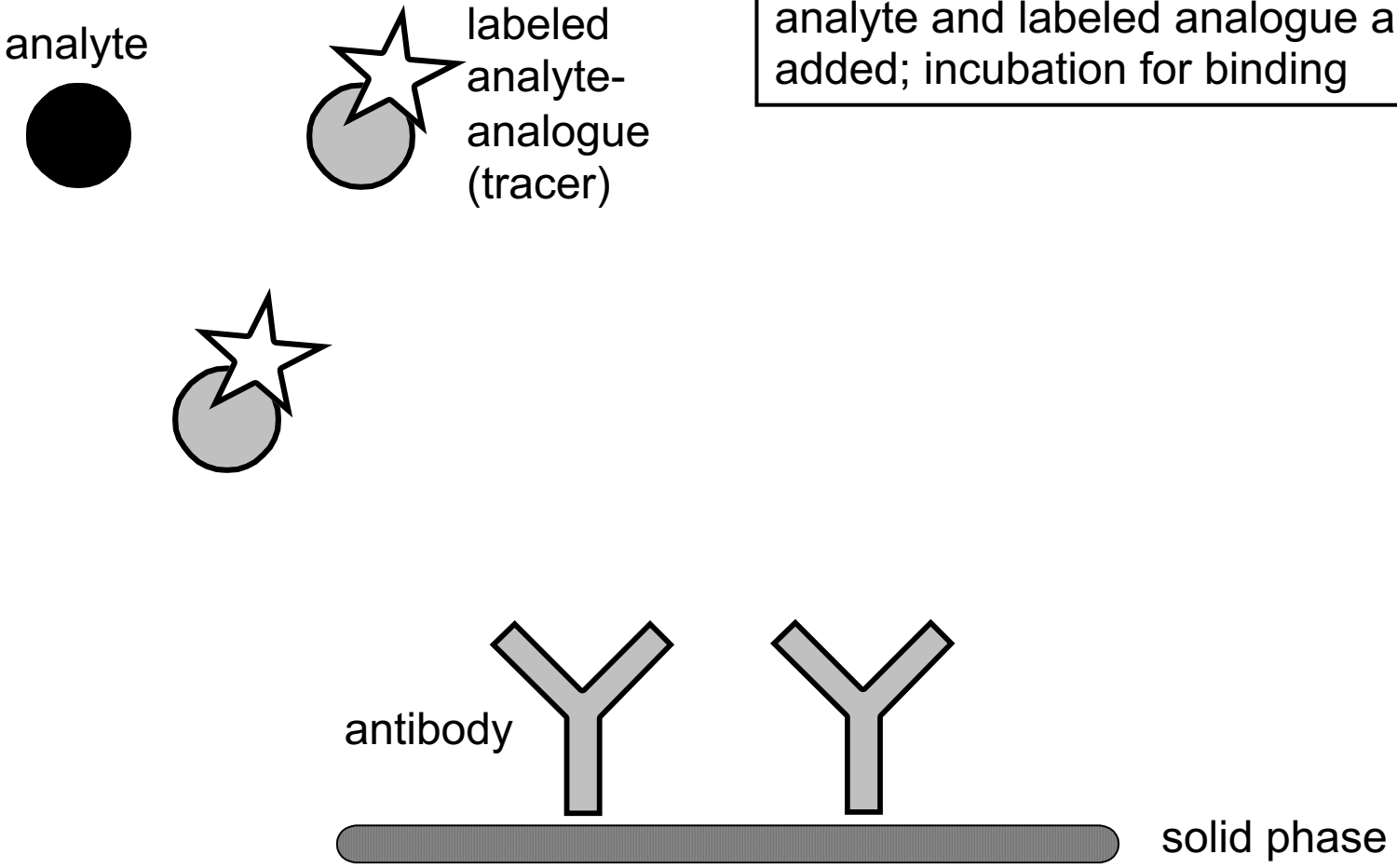
antibody against analyte either recognizing

- single epitope = monoclonal ab, or
- multiple epitopes = polyclonal ab



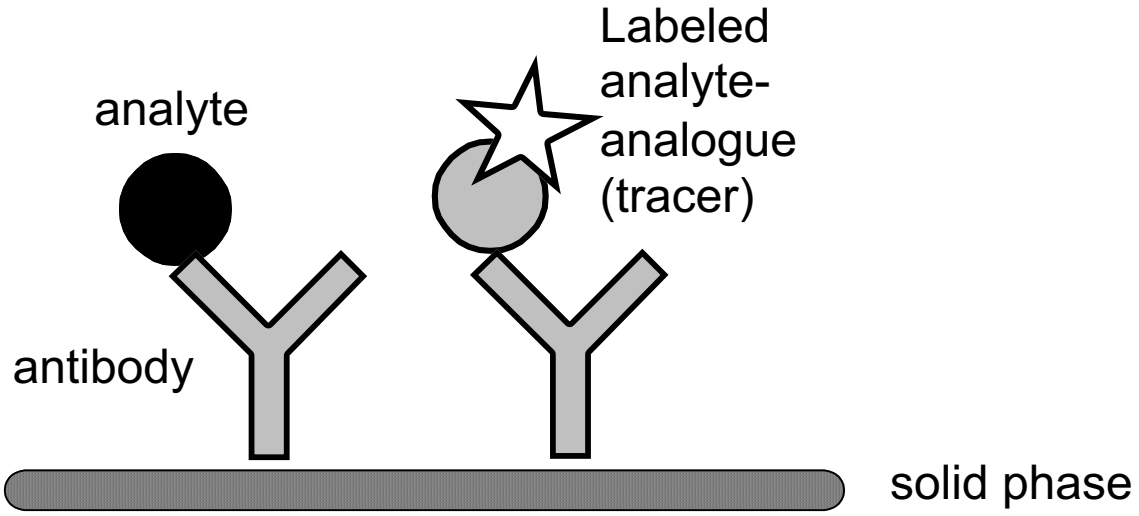
limited amount of antibodies

Competitive immunoassay



Competitive immunoassay

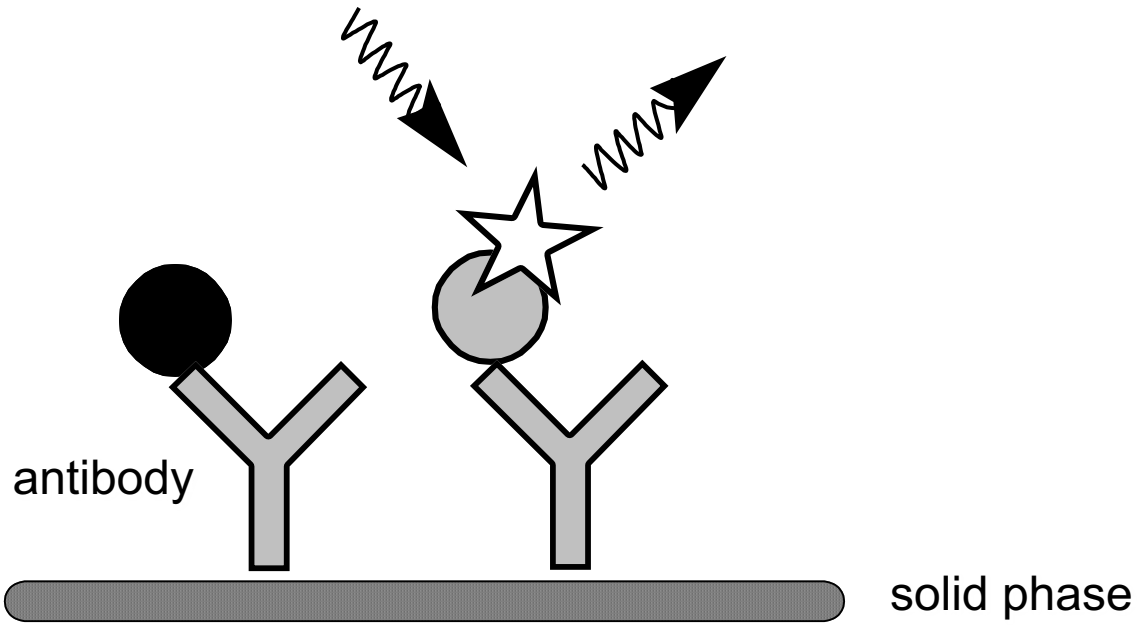
non-bound analyte and analogue are washed away



competition in binding to a limited number of antibodies

Competitive immunoassay

signal of the label is measured

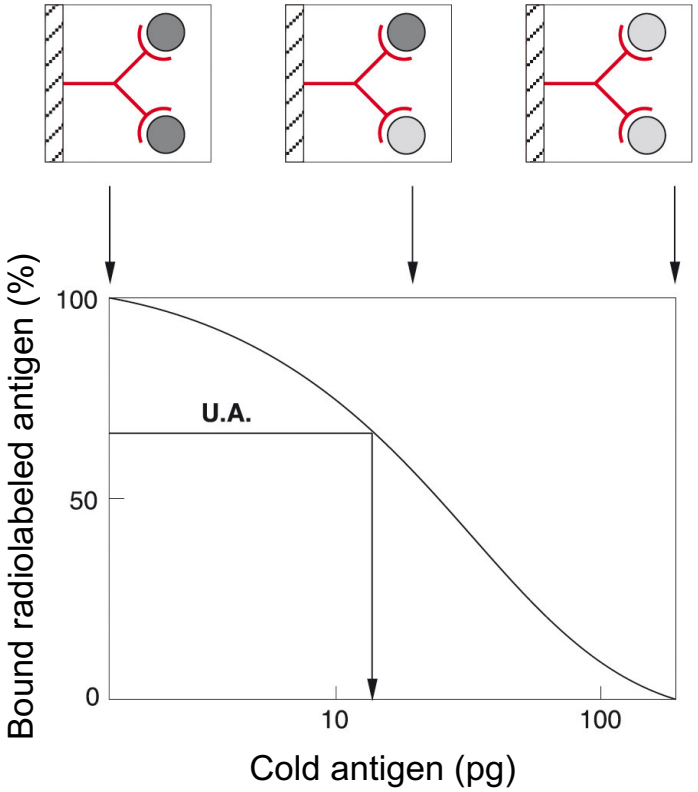


limited amount of antibodies

Radioimmunoassay (RIA)

- First kind of immunoassay: 1950s (Rosalyn Yalow, Nobel price in 1977) and still in use (very sensitive and background-free)
- In addition to the analyte, a second antigen that carries a radioactive (“hot”) label is needed (concentration must be known) => tracer
- Radionuclides: typically ^{125}I, ^{3}H => safety precautions are needed
- Mainly used for small molecule analytes such as hormones in a competitive immunoassay (originally developed for insulin), other example: renin, a marker for hypertension (concentration in serum: 10^{-12} M)

Radioimmunoassay (RIA)



Labeled ("hot") antigen

Unlabeled ("cold") antigen

Antibody specific for and

U.A. Unknown antigen concentration can be determined from standard curve

Solid phase

Radioimmunoassays (RIA)

- On the first sight:
  radionuclides are perfect labels for immunoassays => background-free
  (there is no intrinsic radioactivity in sample, test tube of instrument)

- It is obvious: radioactive labels (e.g. ^{125}I, ^{3}H) require special safety precautions

- **But also:**
  Each decay event of a radionuclide is **detectable only once**

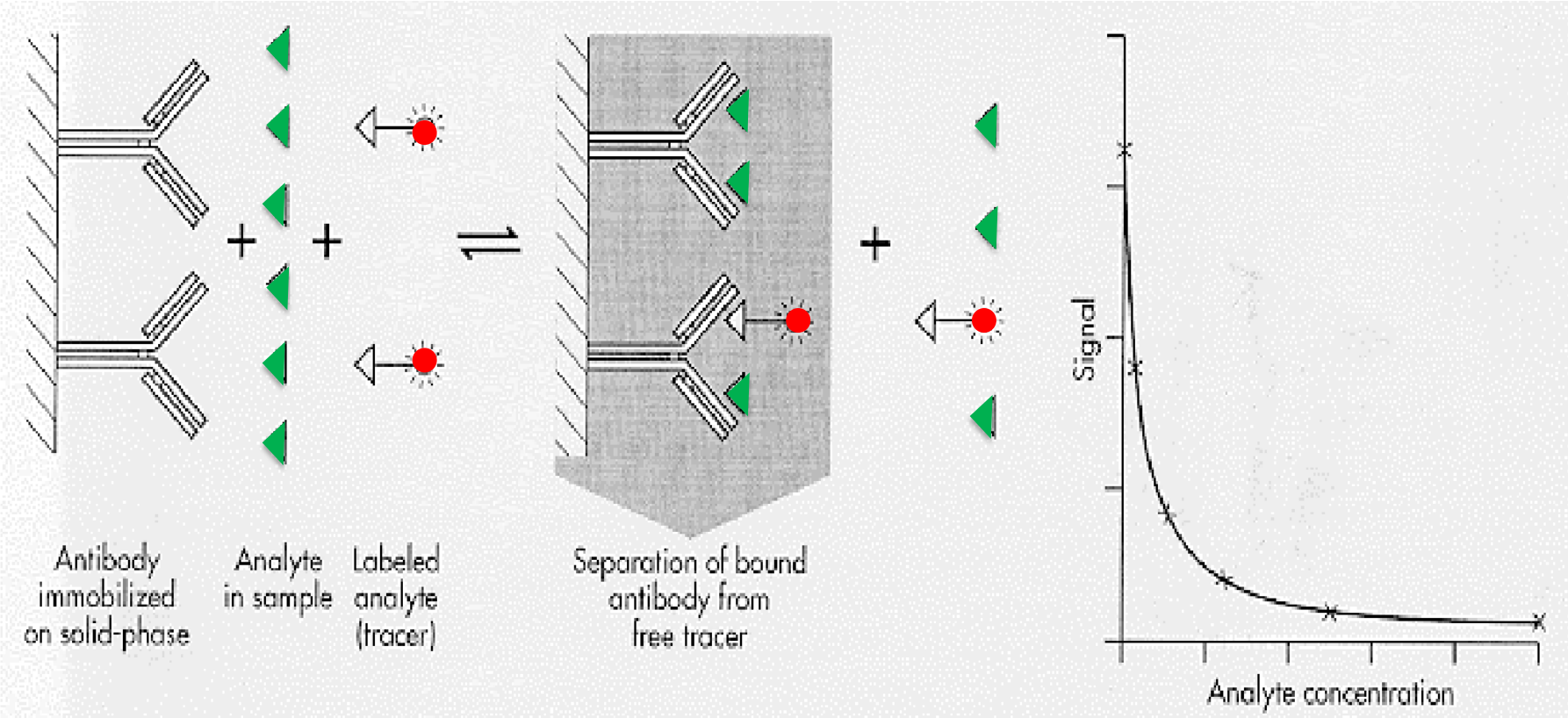
  ^{125}I (gamma rays): $t_{1/2}$ = 60 days / 20 - 48% of radiation is detected
  ➔ if there is one radiolabel per detection antibody molecule, more than 2500 labeled Ab molecules are needed to detect **one decay event / hour**
  ➔ "low-activity" labels need long signal acquisition times

- Using nuclides of shorter half-lives (providing more decay events / second)
  is not an option because
  => their shelf life is reduced accordingly
  => a higher activity leads to radiodamage of biomolecules

Gorris & Soukka, *Anal. Chem.* 2022, 94, 6073

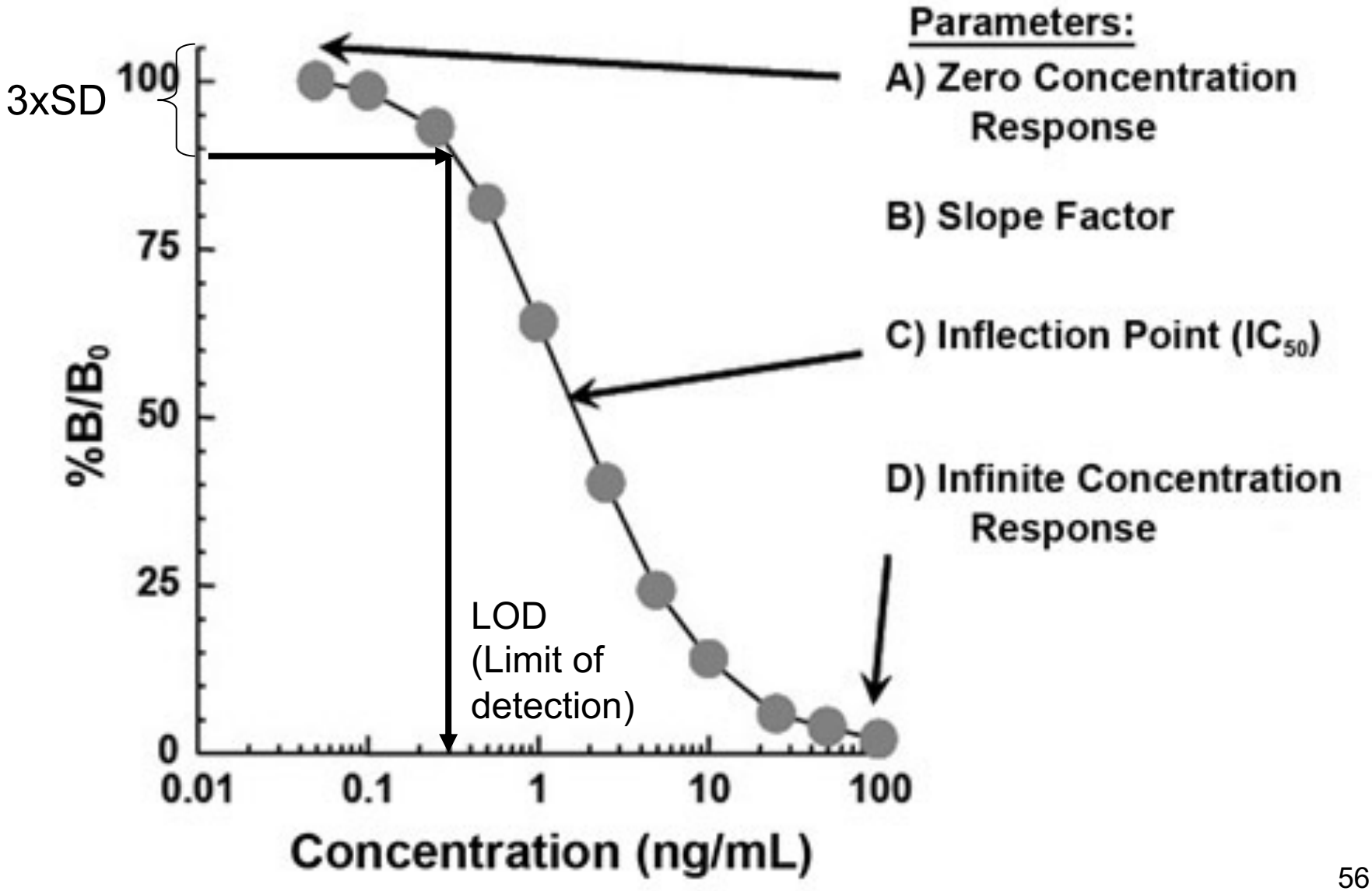
Competitive immunoassay

solid phase separation



=> analyte is measured indirectly
i.e. signal from those binding sites where the analyte is absent

Competitive immunoassay

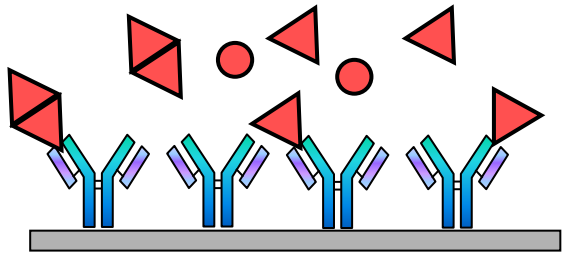


=> typically not as sensitive (i.e. higher LOD) than immunometric immunoassay

Cross reactivities

Cross reactivities (%) for structurally related compounds are determined in comparison to the main analyte at test mid points (IC_{50}) and are expressed in %:

$$CR(\%) = \frac{IC_{50}\ (\textbf{main analyte})}{IC_{50}\ (\textbf{cross reactant})}\ x100$$



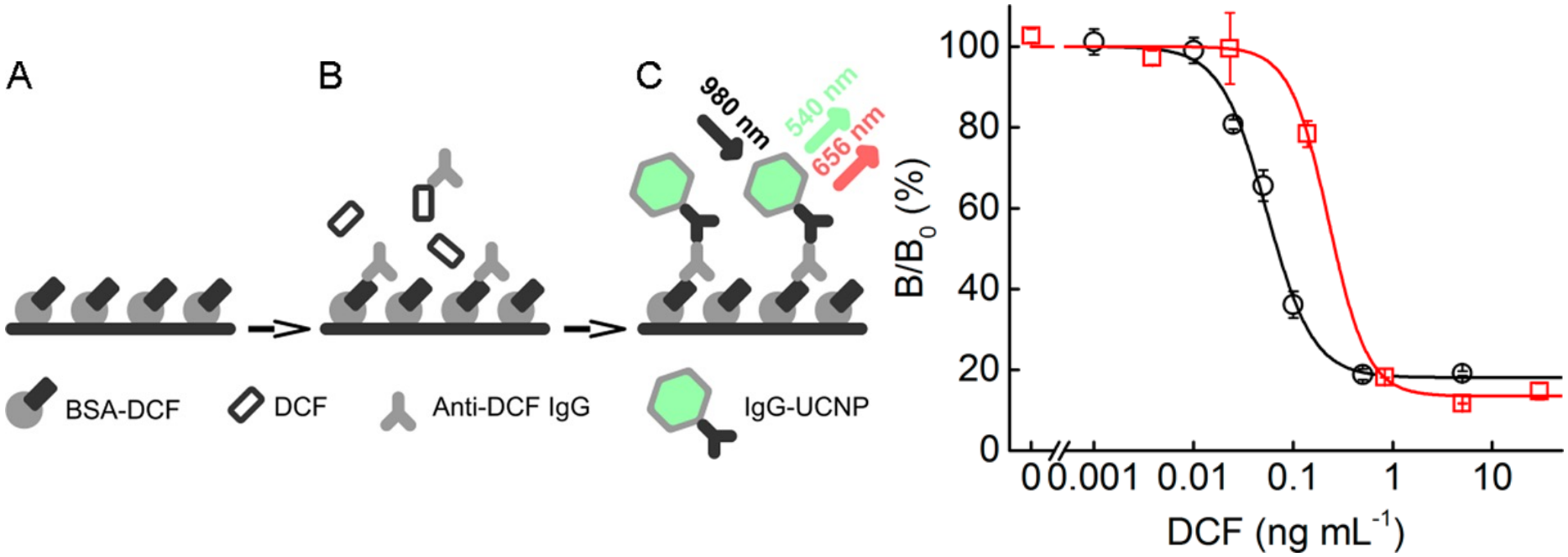
High cross reactivity: CR (%) > 10

Low cross reactivity: 1 < CR (%) < 10

No cross reactivity: CR (%) < 1

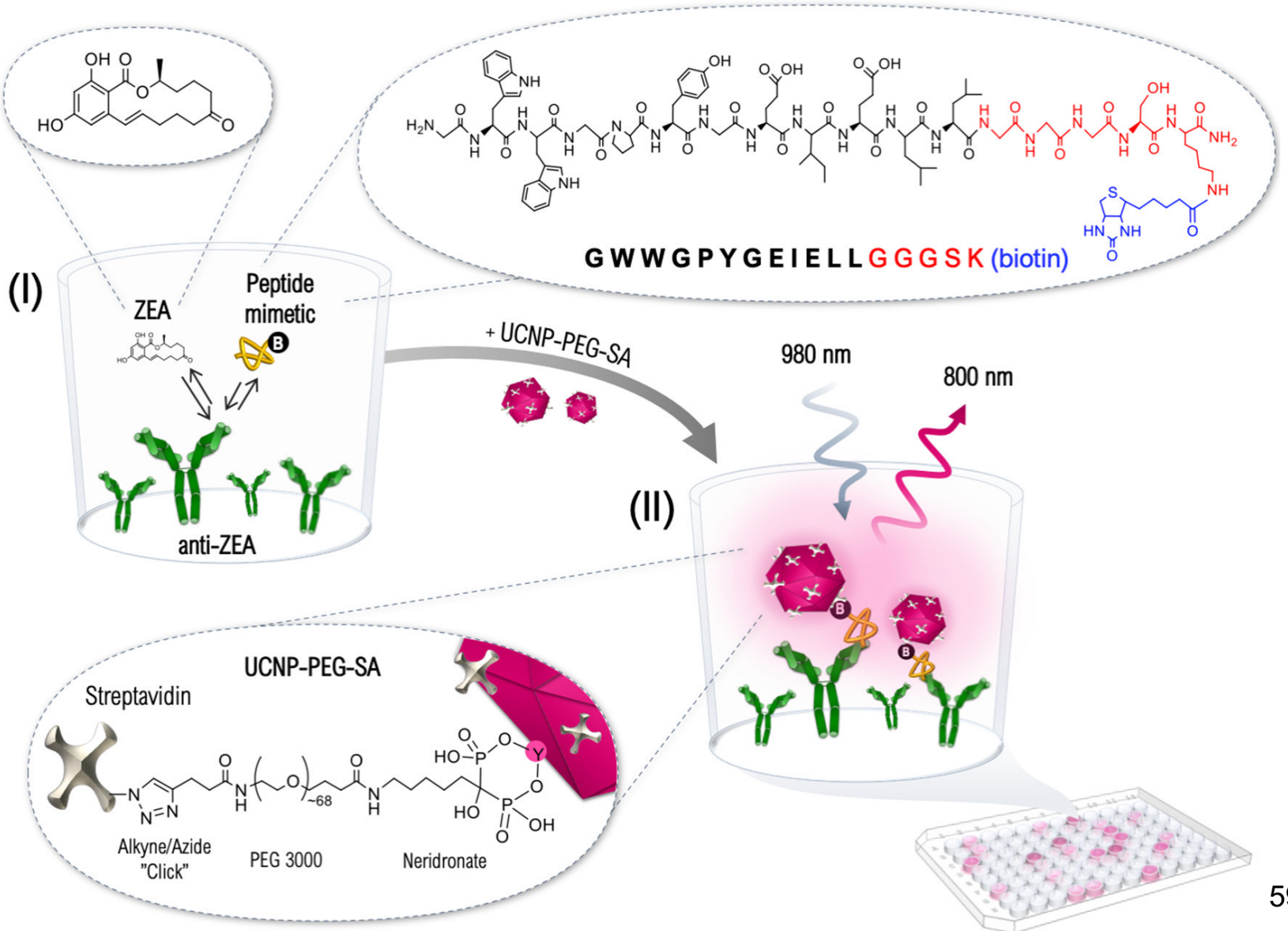
Trends Anal. Chem. 1995, 14:415–425

Competitive ULISA I



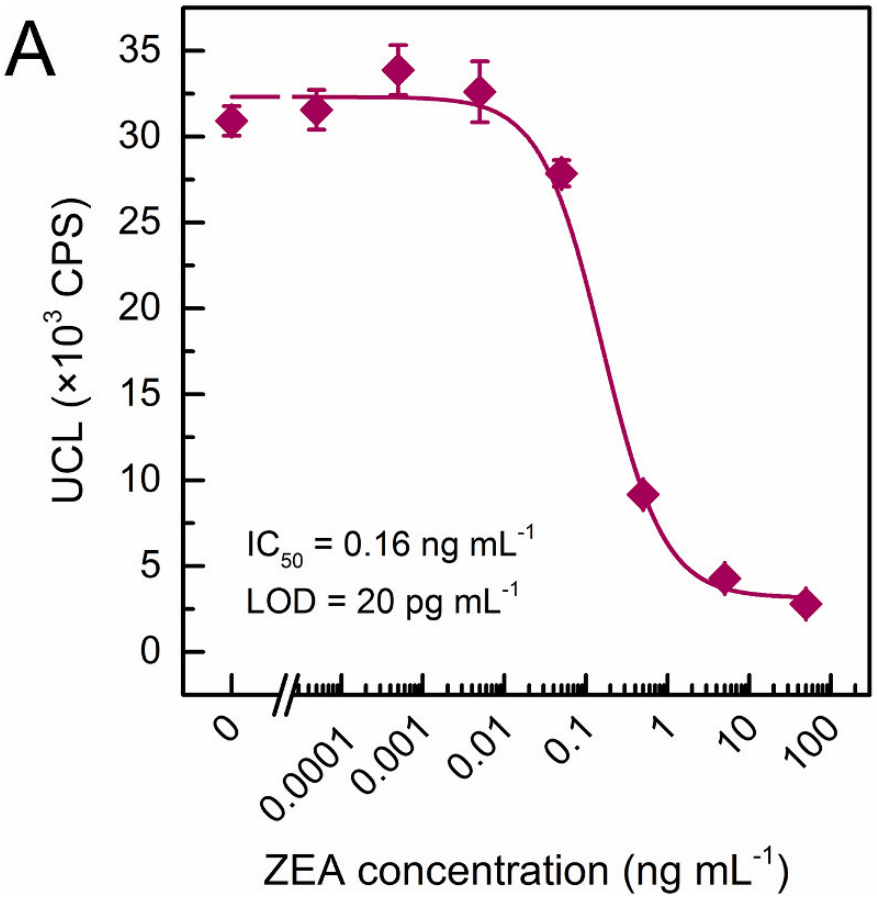
Hlavaček (2016) *Anal. Chem. 88,* 6011-6017

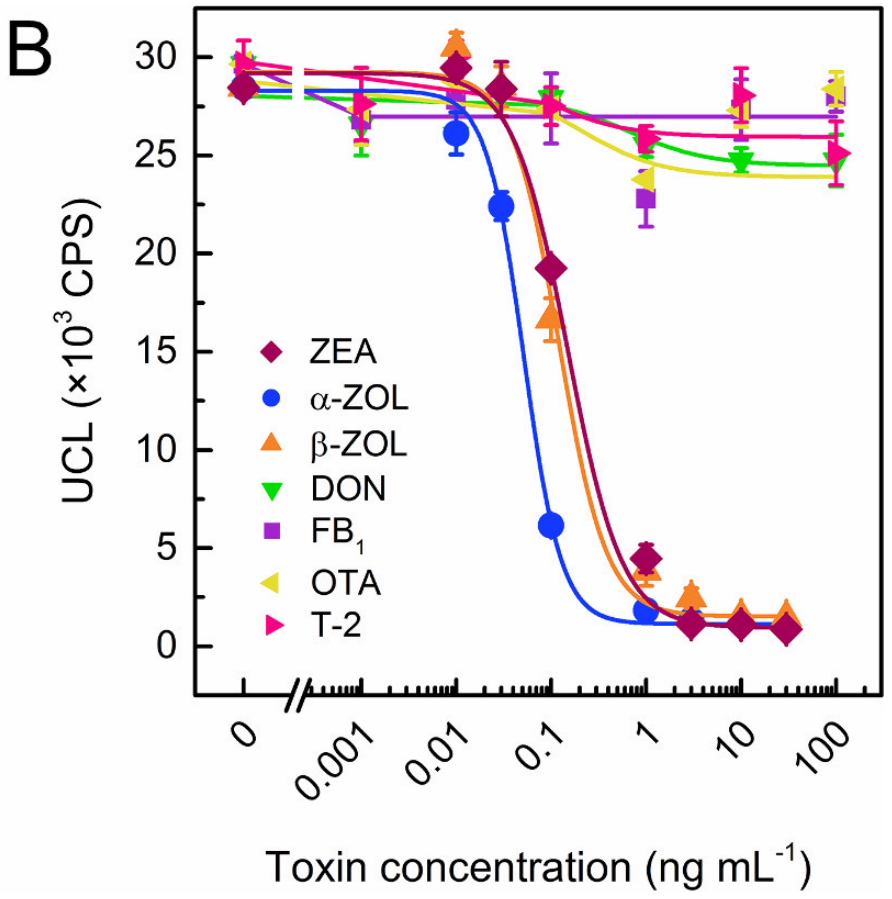
Competitive ULISA II



Peltomaa, R. (2020) *Biosens. Bioelectron. 170, 112683*

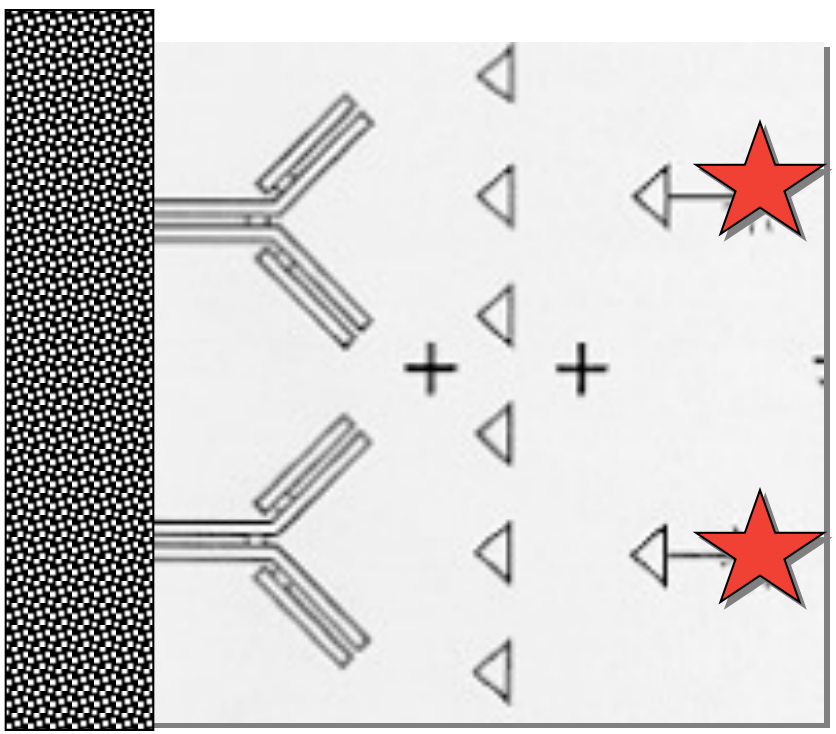
Competitive ULISA II



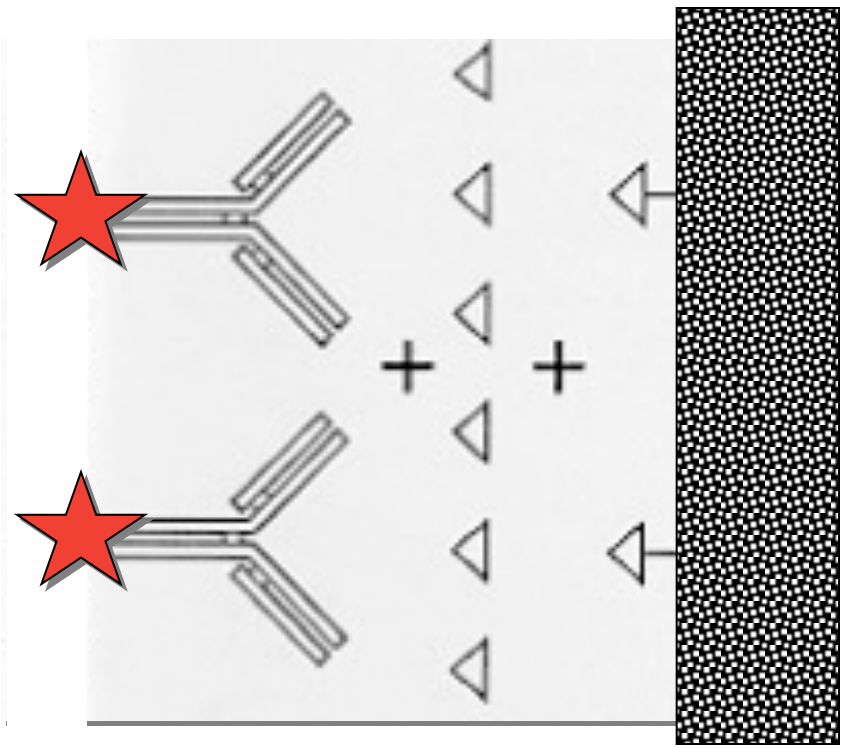


Peltomaa, R. (2020) *Biosens. Bioelectron. 170, 112683*

Competitive immunoassay: alternative formats



vs.



Labeled analyte serves as tracer

Labeled antibody serves as tracer

Indirect competitive immunoassay

Determination of saliva immunoglobulin IgA
(this test is commercially available)

Steps:

1) prepare an antibody-enzyme conjugate
2) add to analyte solution in excess
3) make a microplate with immobilized IgA
4) add solution
5) let react; wash; add substrate
6) measure color