MUNI
SCI

C8116 Immunoaffinity techniques

Advanced microscopy

Spring term 2024

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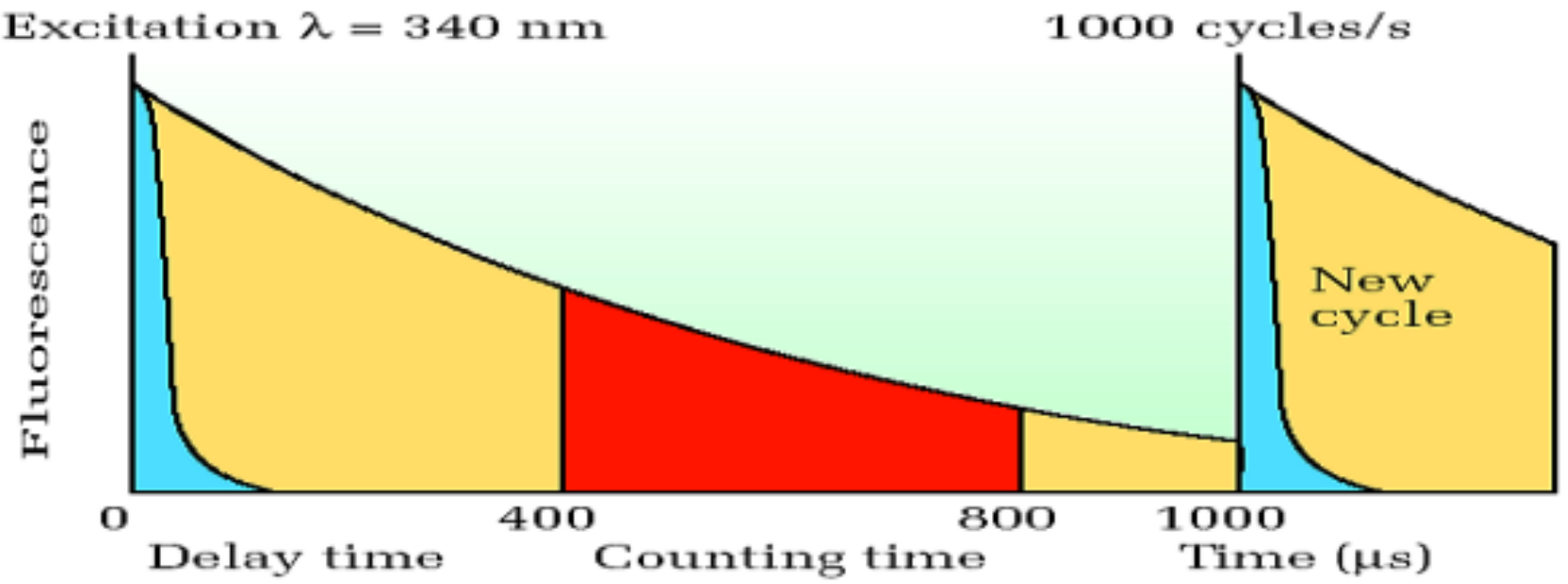
Department of Biochemistry
April 30th, 2024

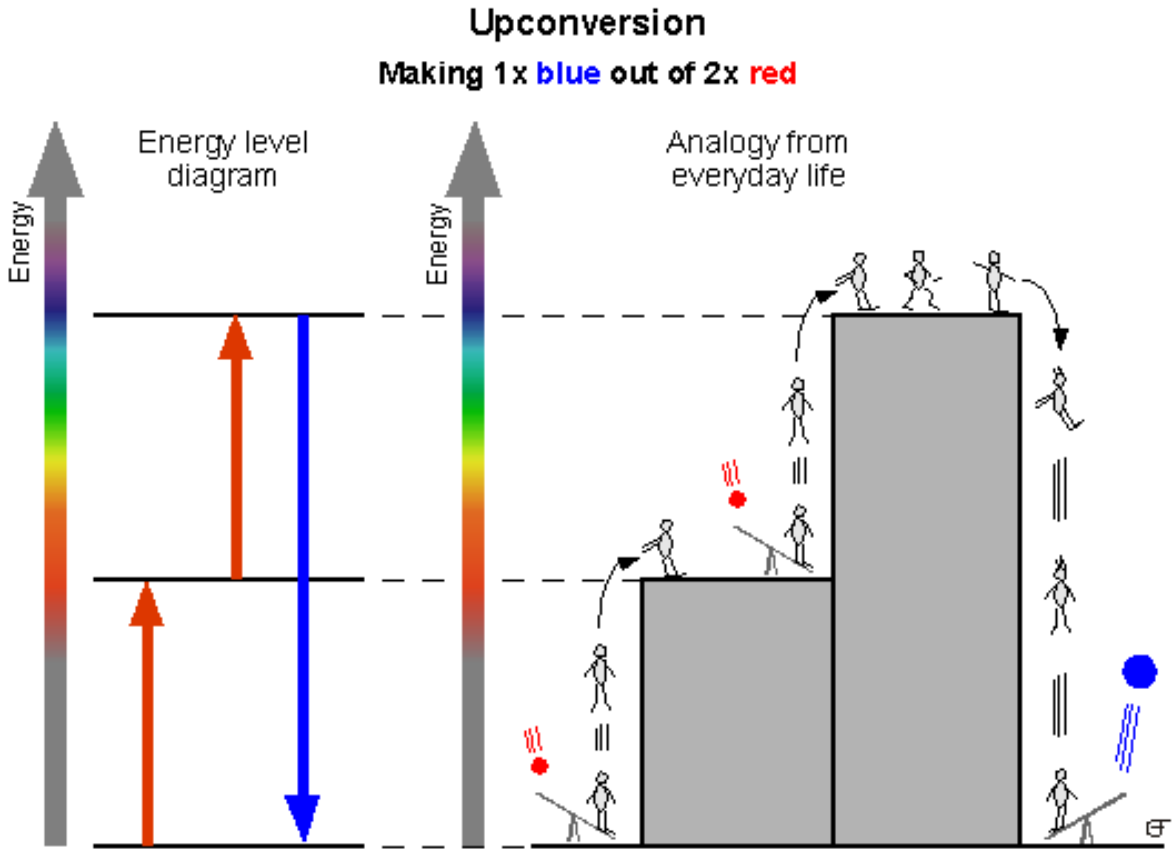
Time-resolved fluorescence

Europium-chelate; emission at 615 nm, decay time ~ 1 ms



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
$4f^{1}5d^{1}6s^{2}$	$4f^{3}6s^{2}$	$4f^{4}6s^{2}$	$4f^{5}6s^{2}$	$4f^{6}6s^{2}$	$4f^{7}6s^{2}$	$4f^{7}5d^{1}6s^{2}$	$4f^{9}6s^{2}$	$4f^{10}6s^{2}$	$4f^{11}6s^{2}$	$4f^{12}6s^{2}$	$4f^{13}6s^{2}$	$4f^{14}6s^{2}$	$5d^{1}6s^{2}$

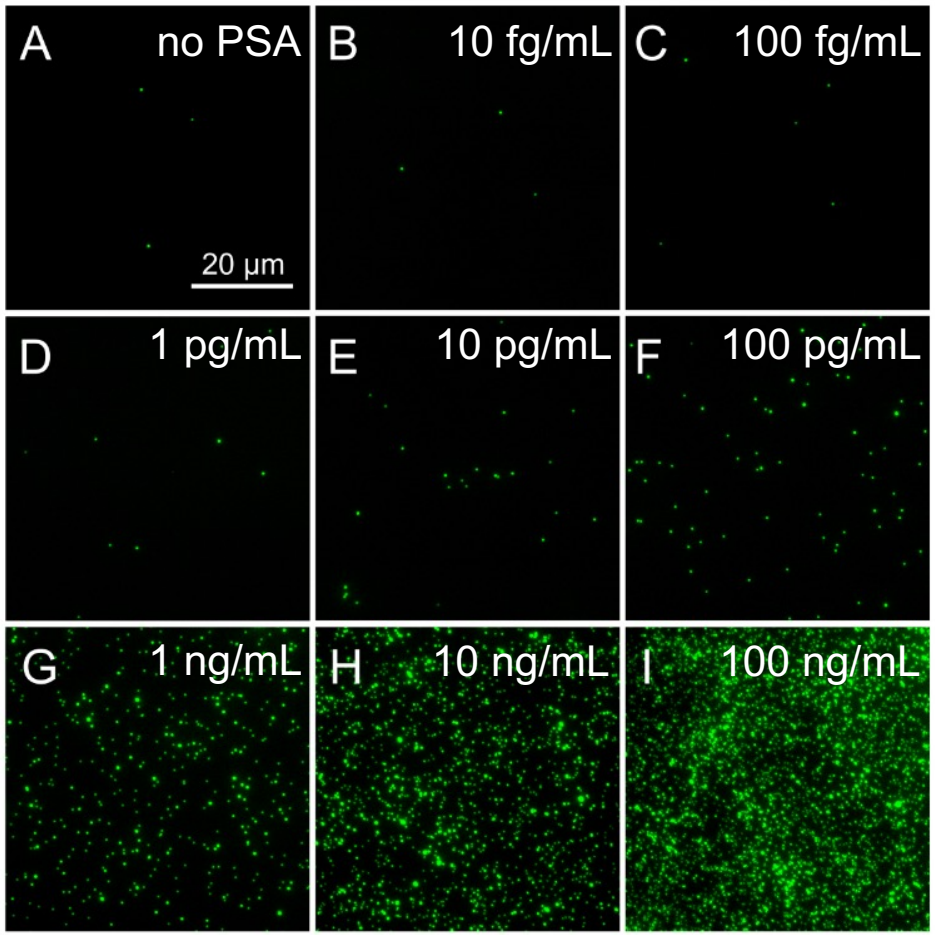
Photon-upconversion

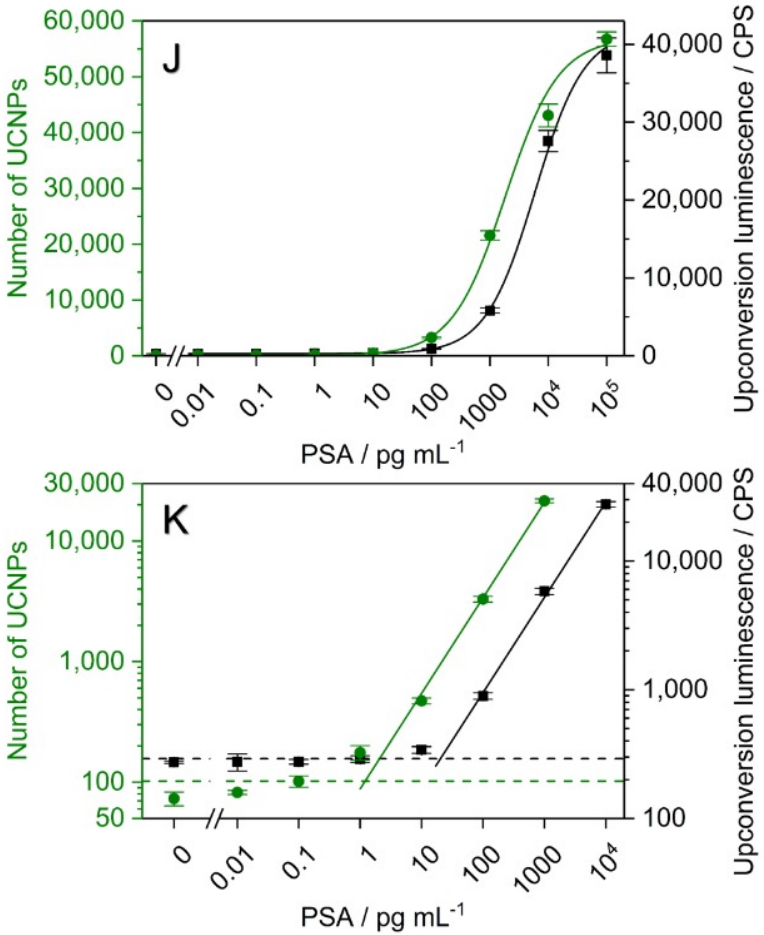


Sequential absorption of 2 or more photons via long-lived transition states
=> More time for absorbing a further photon

Counting single immune complexes

non-specific binding only





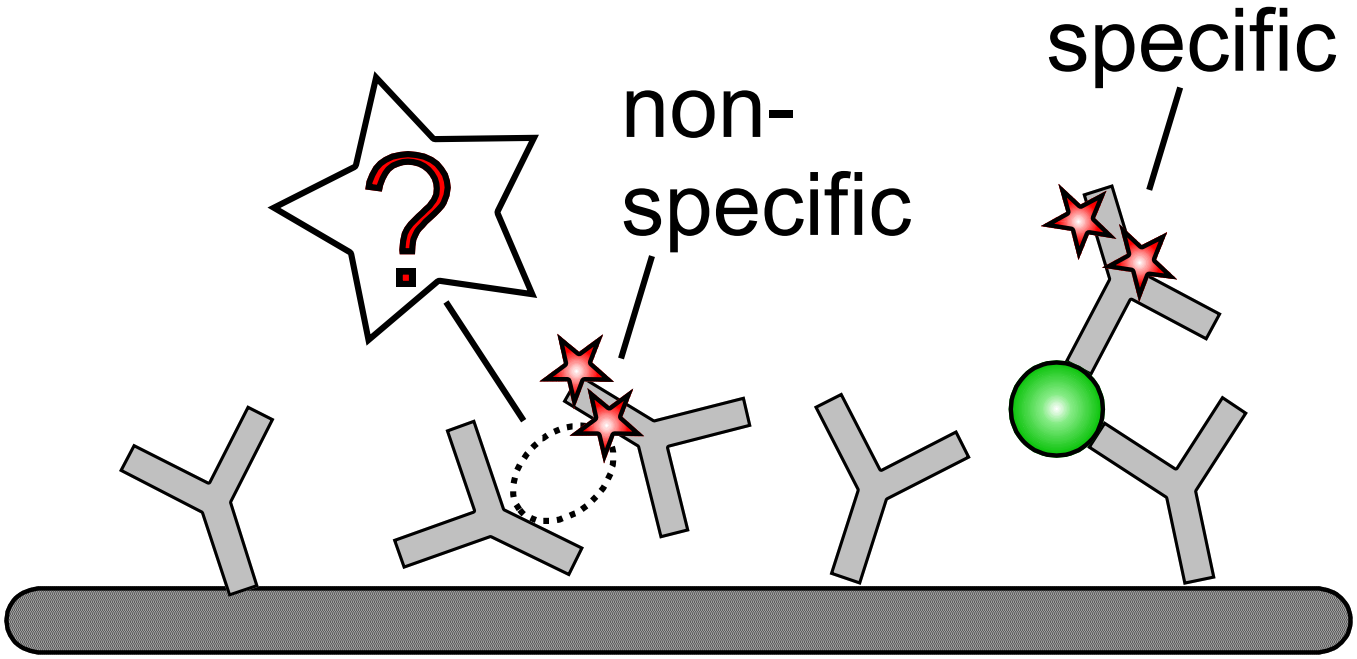
=> Detectable as diffraction limited spots

Anal. Chem. (2017) 89, 11825

Single molecule (digital) assays

Digital immunoassay allow for the detection of single analyte molecules, but this should not be confused with the **highest analytical sensitivity**

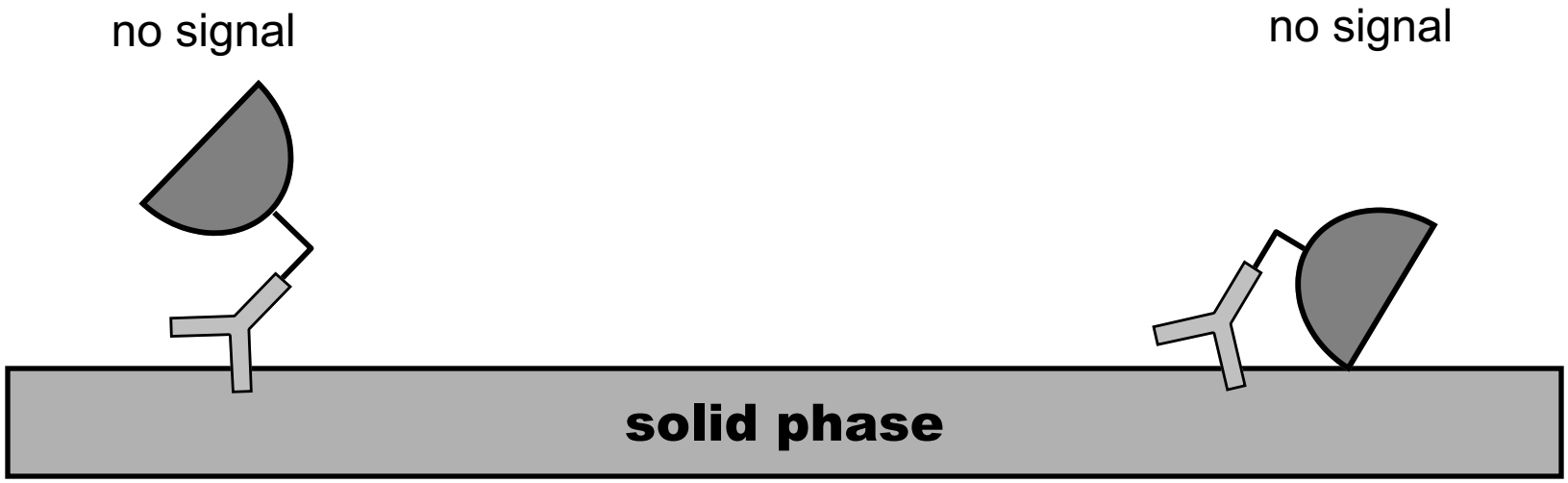
=> non-specific binding of labeled component (and its variation) defines the actual limit of detection



“Smart“ reporters for heterogeneous immunoassays

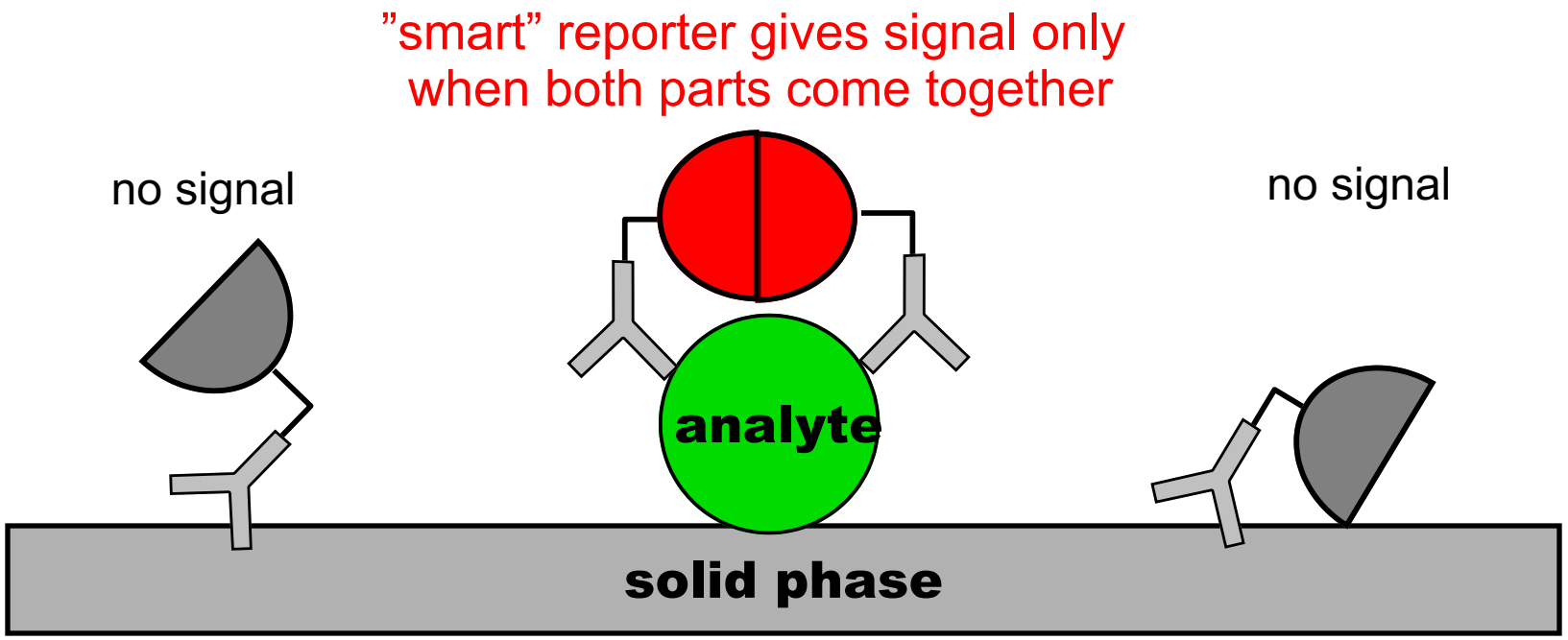
“Smart“ reporters

How to avoid signal from nonspecific binding?

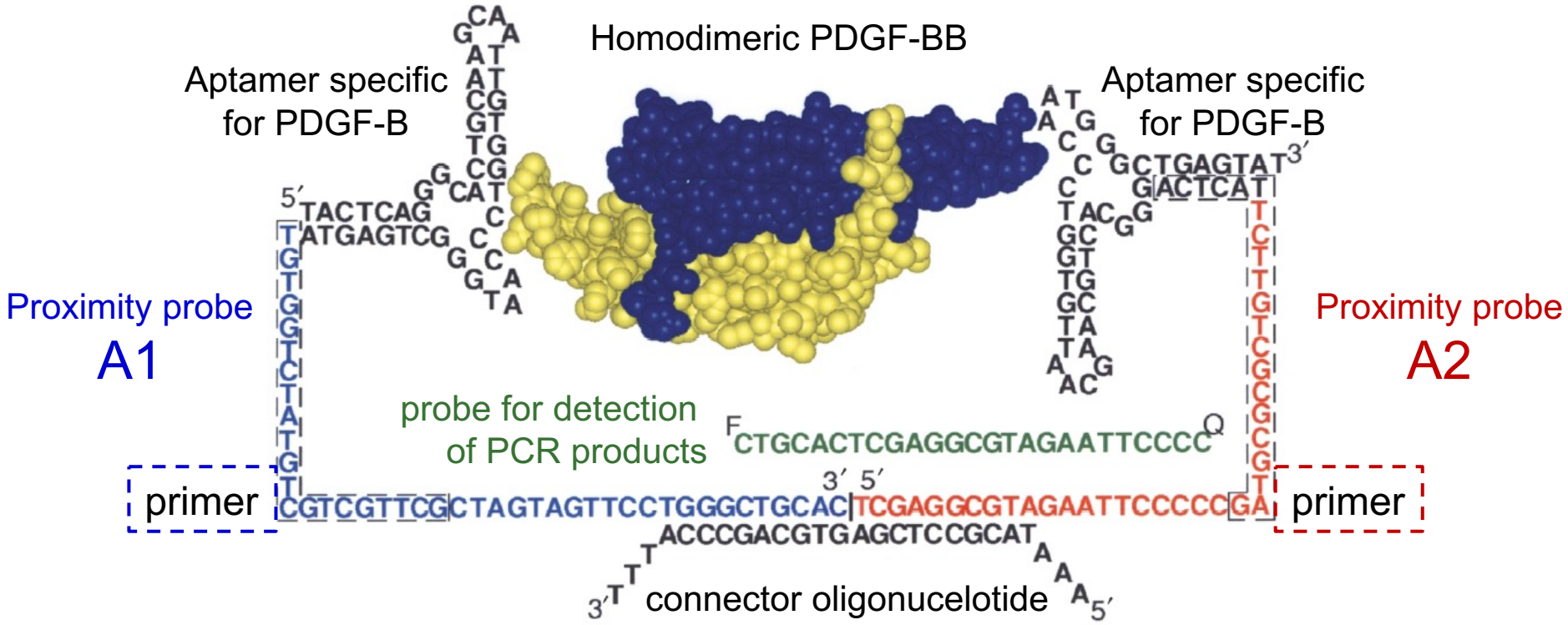


“Smart“ reporters

Modulation of high specific activity signal upon recognition of analyte



Proximity ligation

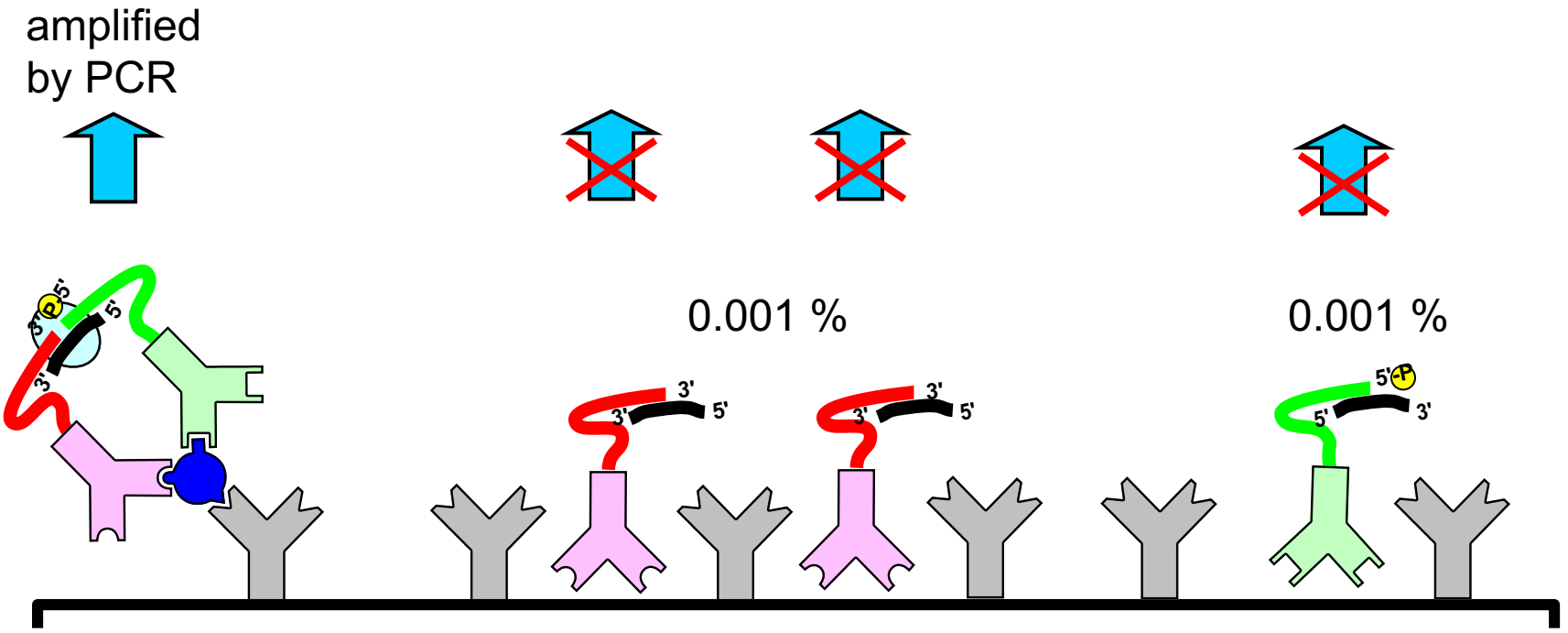


F: fluorescent dye fluorescein
Q: quencher TAMRA

Fredriksson (2002): Protein detection using proximity-dependent DNA ligation assays. *Nat. Biotechnol.* **20:** 473-477

Proximity ligation in immunoassays

sandwich immunoassay with two DNA-labeled detection antibodies and one solid-phase bound capture antibody



Proximity ligation in immunoassays

*only **specific binding** is detected*

- **to produce signal**, two labeled antibodies need to simultaneously bind to different epitopes of the same analyte

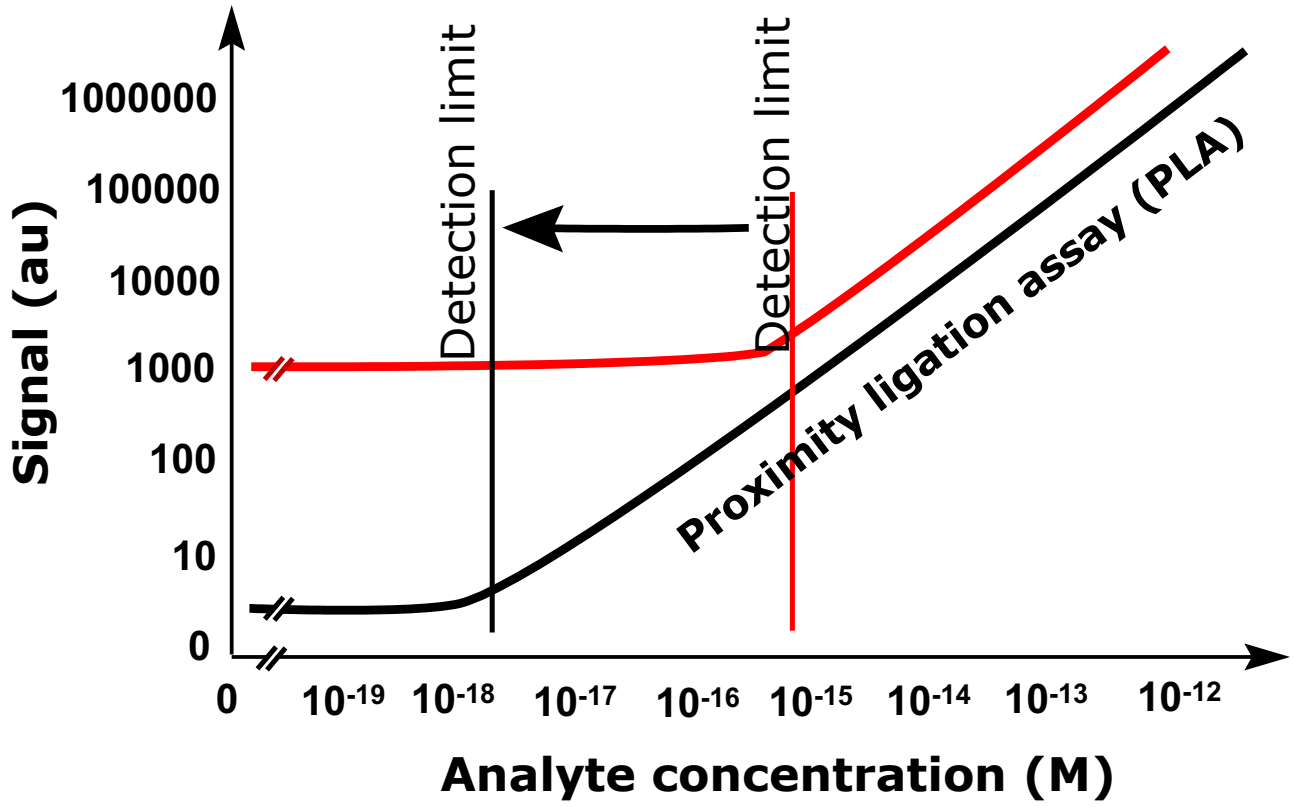
- **non-specifically bound** individual antibodies do not produce any signal

=> highly sensitive technology for protein detection

but: complex to perform, in total 3 antibodies against different epitopes are required

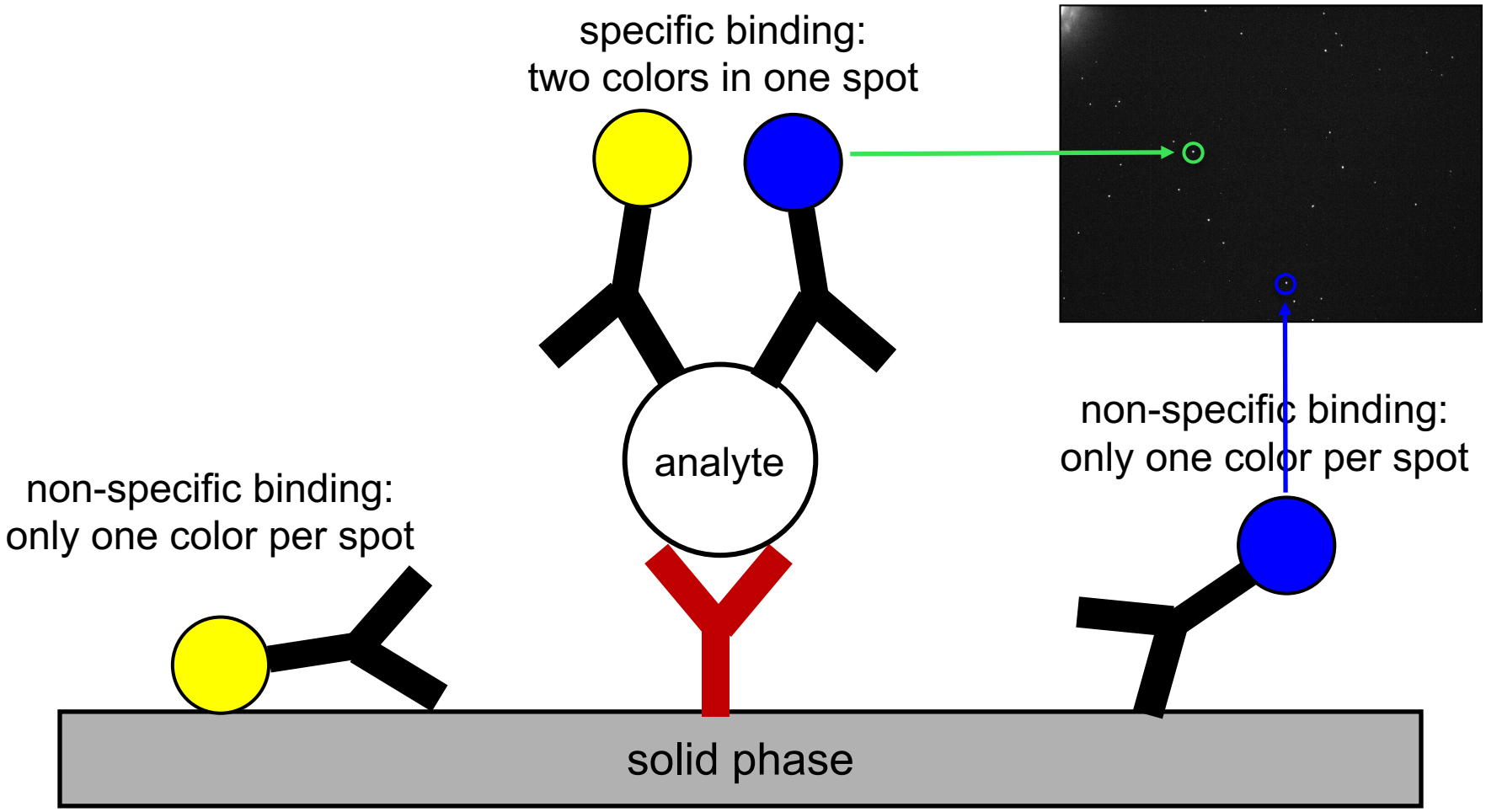
Proximity ligation in immunoassays

=> enables more sensitive detection than other assays due to high specificity in signal generation



Digital immunoassays: two-color colocalization

=> detection of 2 **non-interacting** reporters



=> one of the next projects in our lab

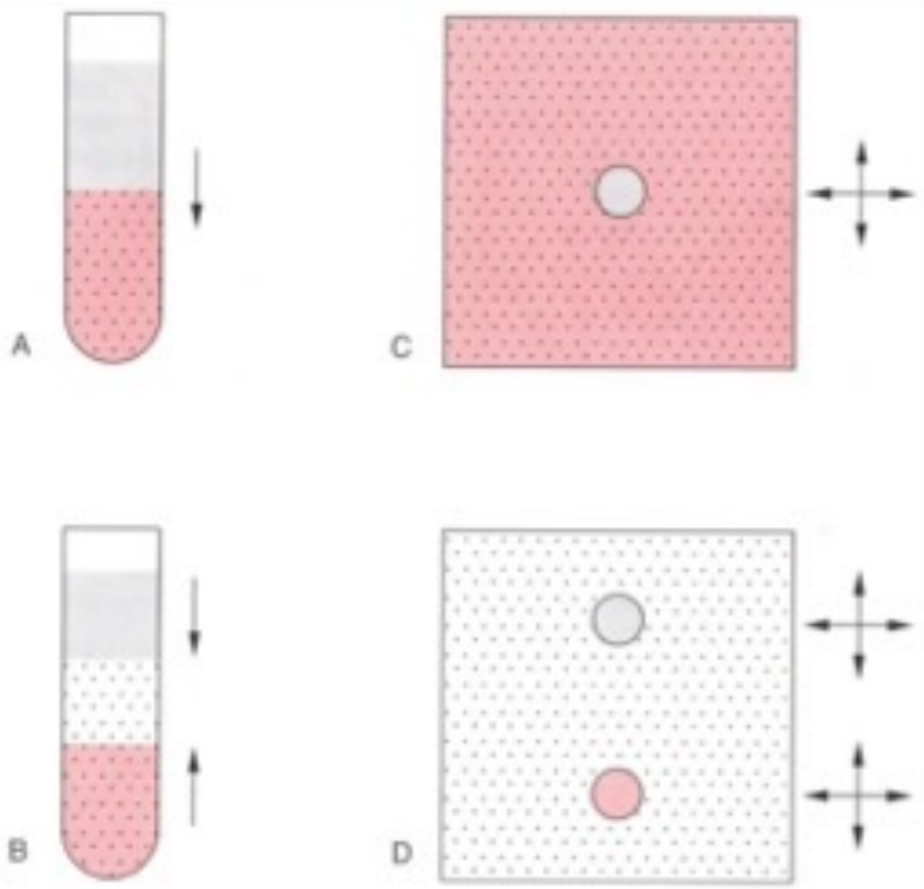
Immunoblotting

=> Antibodies for the detection of proteins immobilized on a membrane

Recapitulation: Immune precipitating systems

simple diffusion

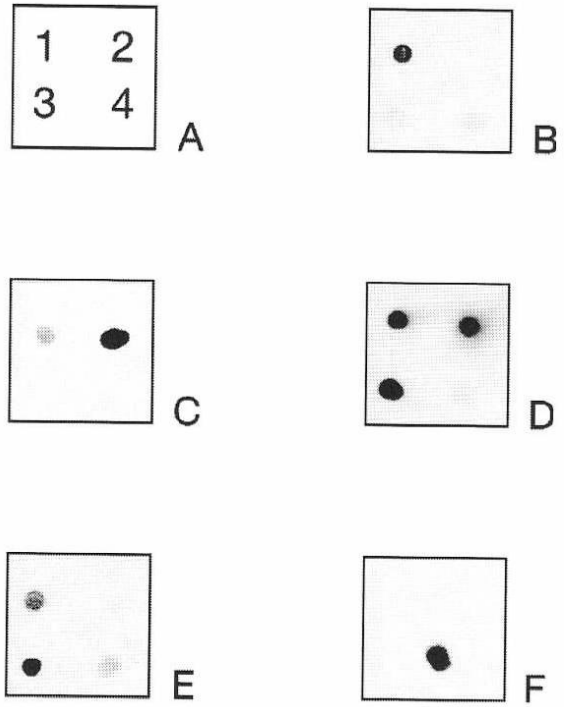
double diffusion



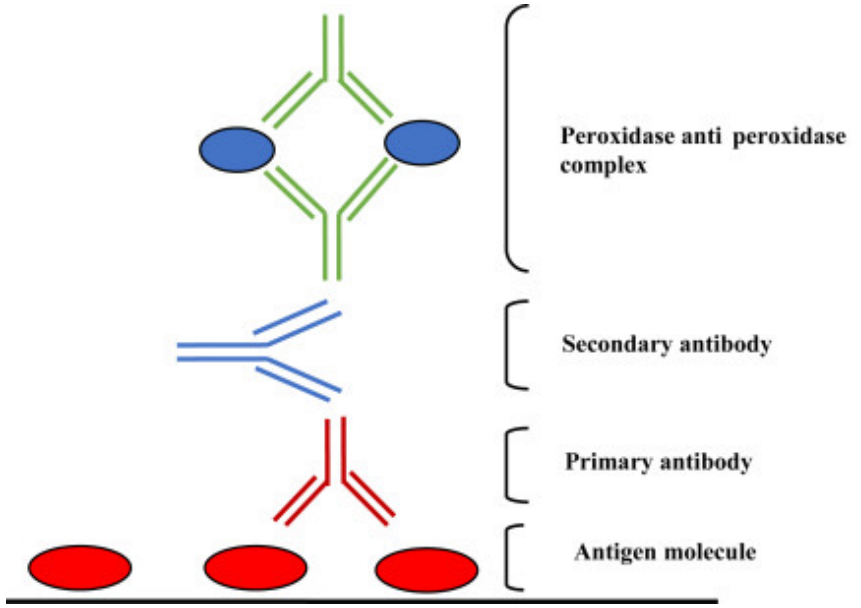
A) Oudin
B) Oakley / Fulthorpe
C) Mancini
D) Ouchterlony

Simple form: dot blot

Example of epitope mapping:



Detection scheme:

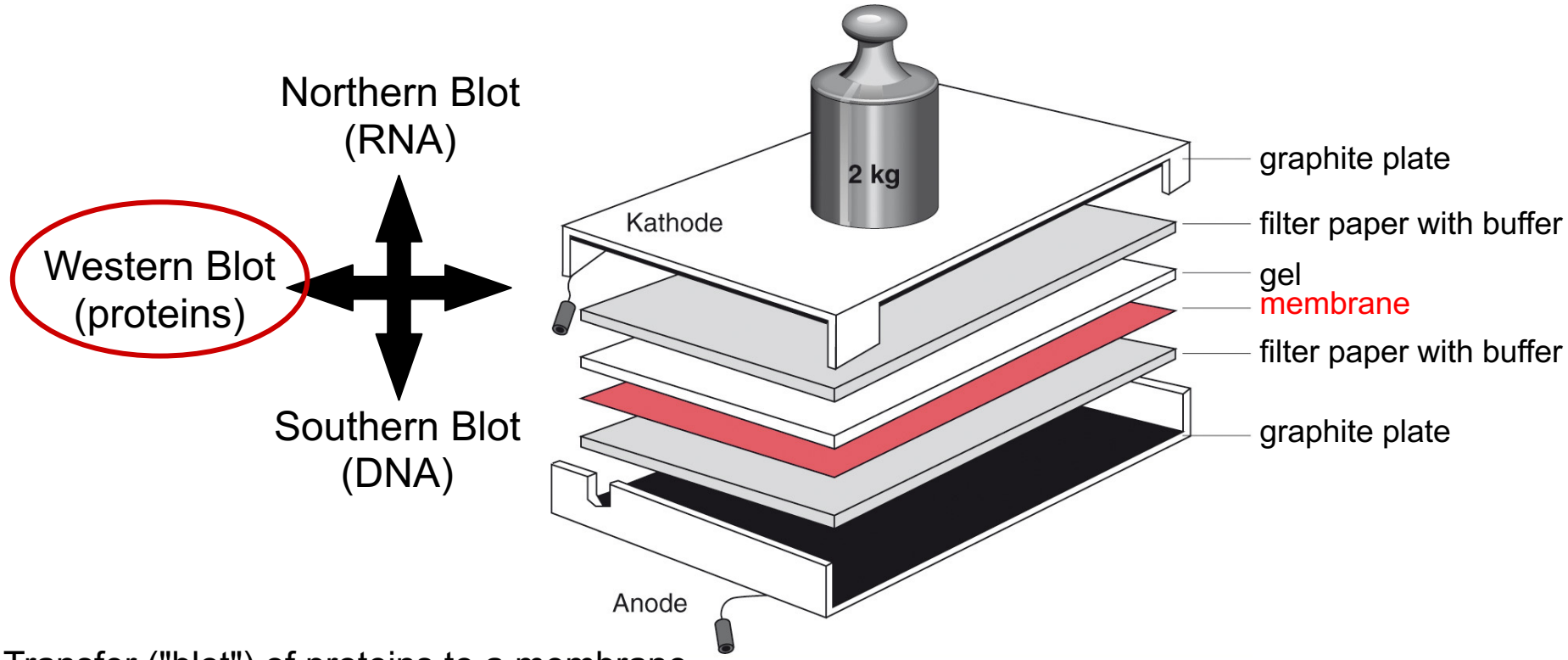


Peroxidase-antiperoxidase method (PAP)
=> Increases the amount of enzyme / signal strength

β_2-microglobulin (β_2-m, 0.05 mg/mL)
are spotted on a nitrocellulose membrane:
(1) amino acids 1-99 (intact peptide)
(2) amino acids 1-19 (fragment)
(3) amino acids 9-24 (fragment)
(4) amino acids 20-36 (fragment)

=> Various antibodies (B-F) bind to different parts of β_2-m

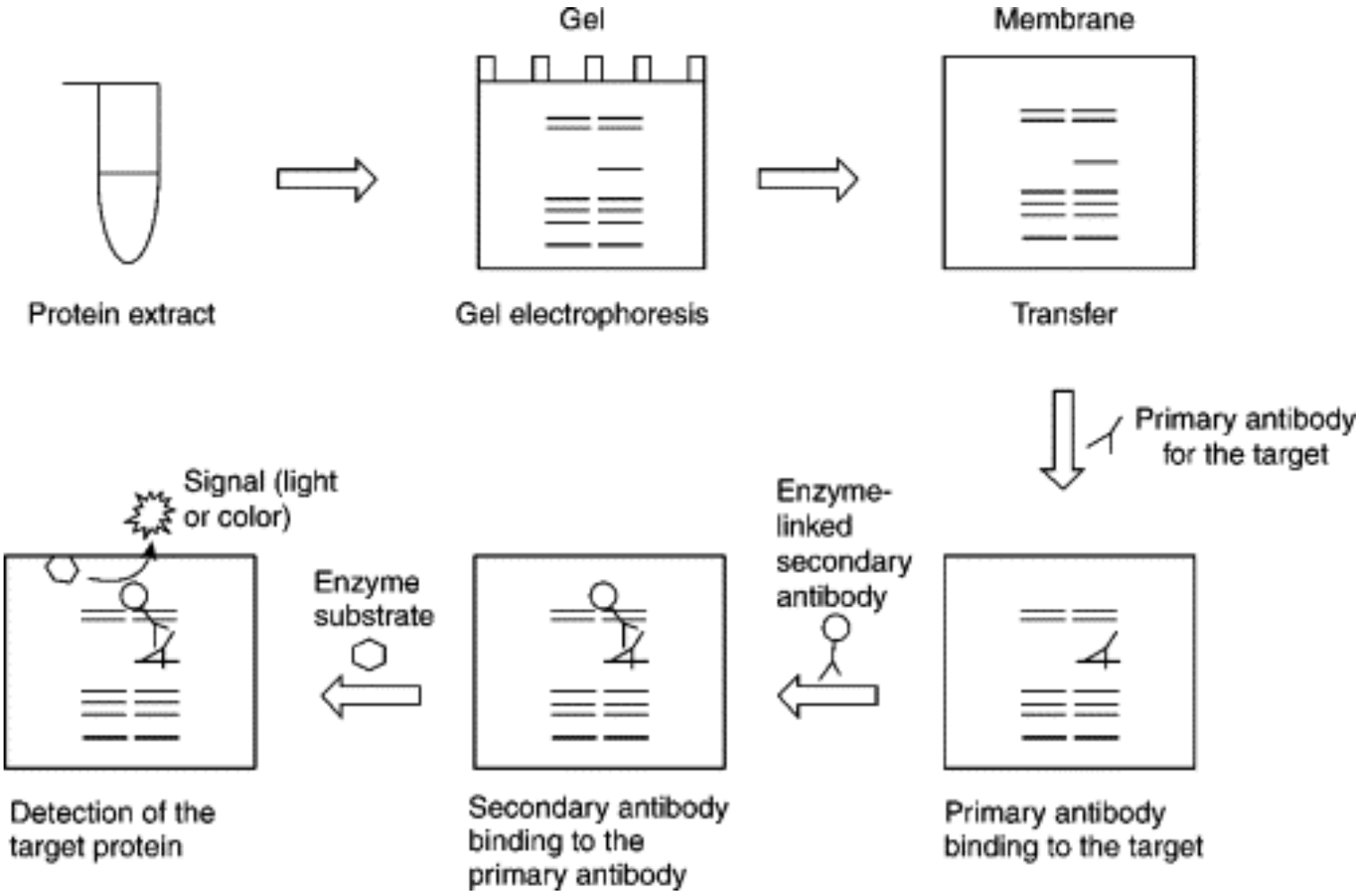
Western blotting



Transfer ("blot") of proteins to a membrane
=> typically nitrocellulose or PVDF
(high non-specific binding of proteins;
blocking required before detection reagents can be applied)

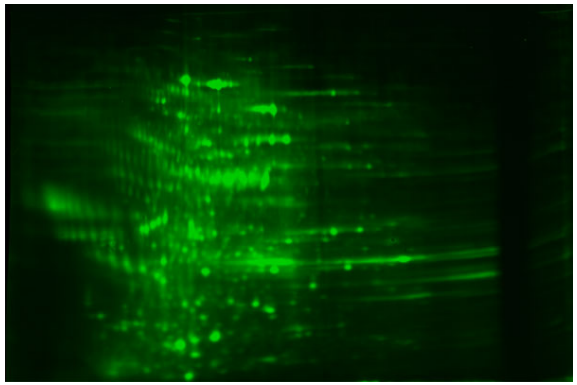
Then protein detection
- via (labeled) antibodies specific for the target protein
- by mass spectrometry
- proteolytic degradation for sequencing

Western blotting

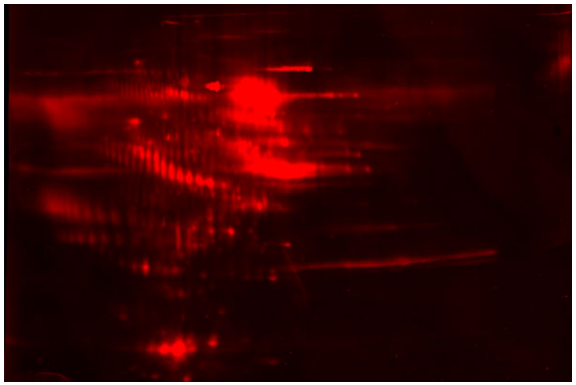


Western blot detection

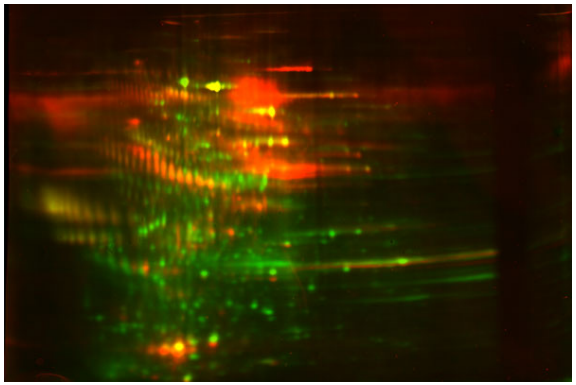
Fluorescent detection: Host cell protein (HCP) analysis



Fluorescent total protein pattern



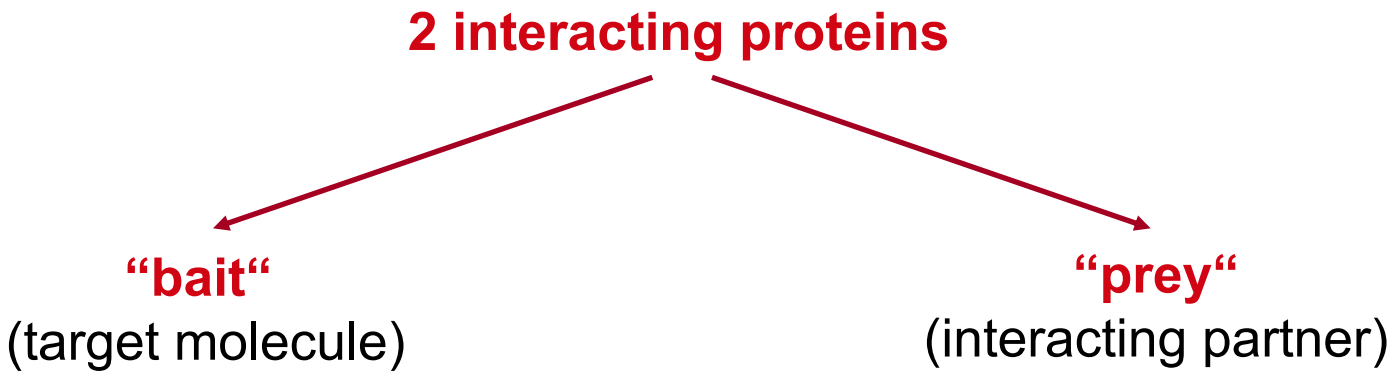
HCP-specific immunostaining, detection by Cy3-secondary antibody conjugate



Overlay

Analysis of protein-protein interactions

Analysis of protein-protein interactions



A) ***In vitro***

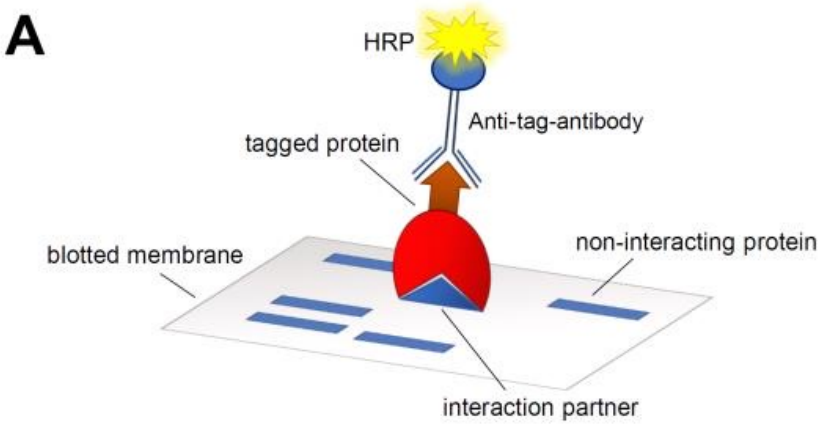
=> protein interactions investigated in a test tube

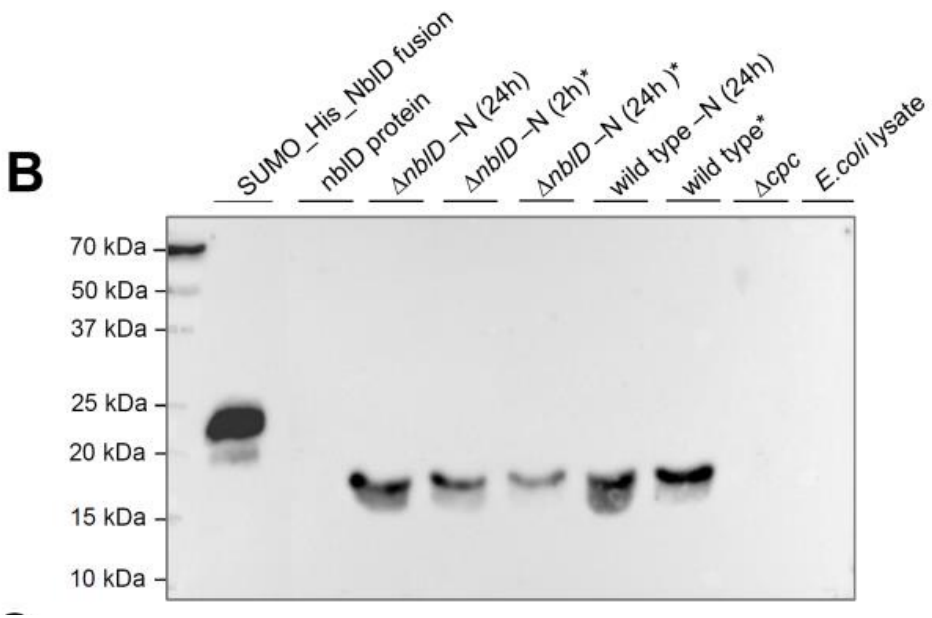
B) ***In vivo***

=> protein interactions investigated in living organisms

Far Western Blotting

Proteins blotted on a membrane (bait) are incubated with interacting proteins (prey)

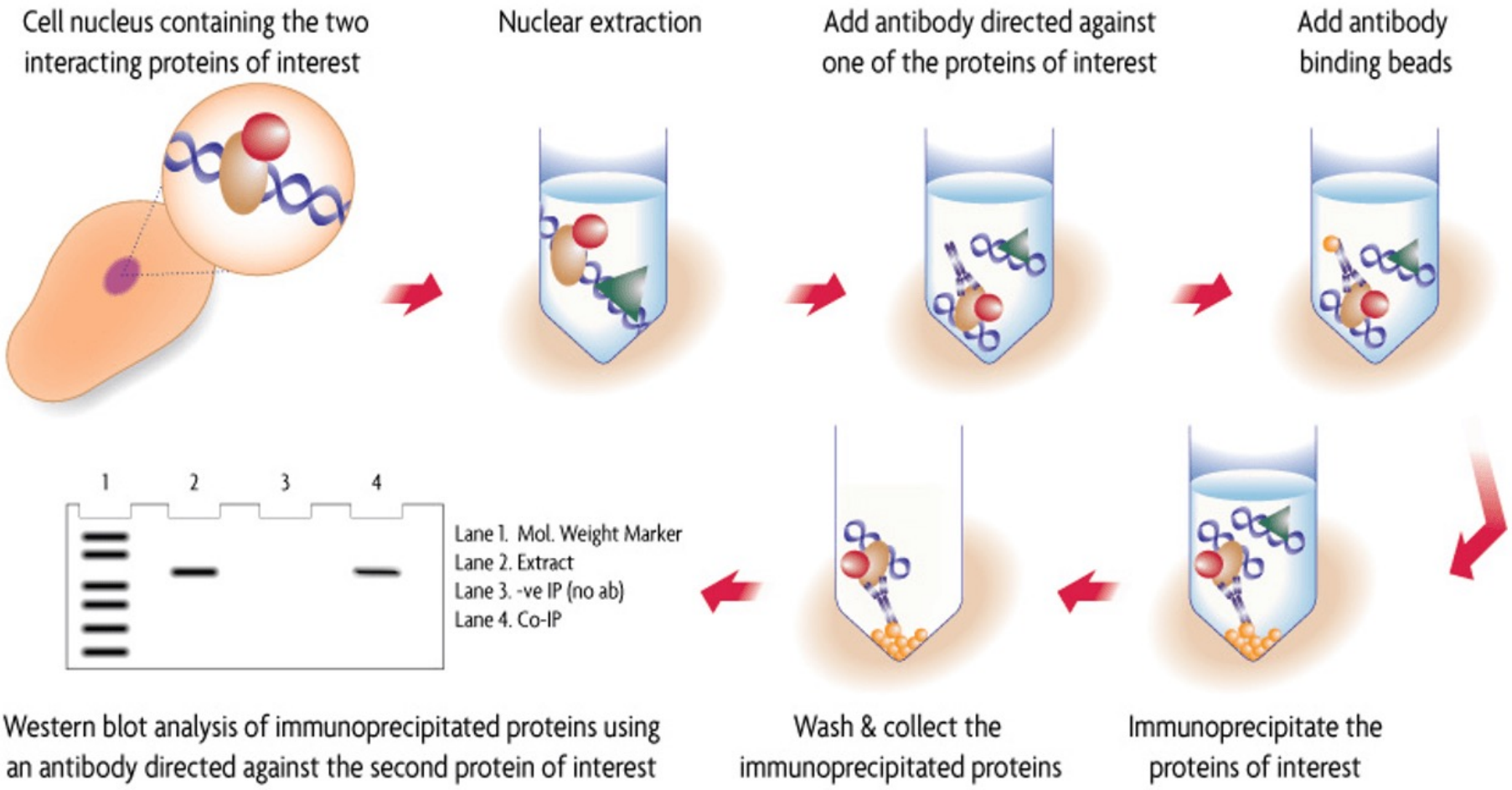




(1) potential interaction partners are transferred from the gel to a membrane
(2) tagged protein is added
(3) HRP-coupled secondary antibody binds to protein tag

Chemiluminescent signal

Co-immunoprecipitation



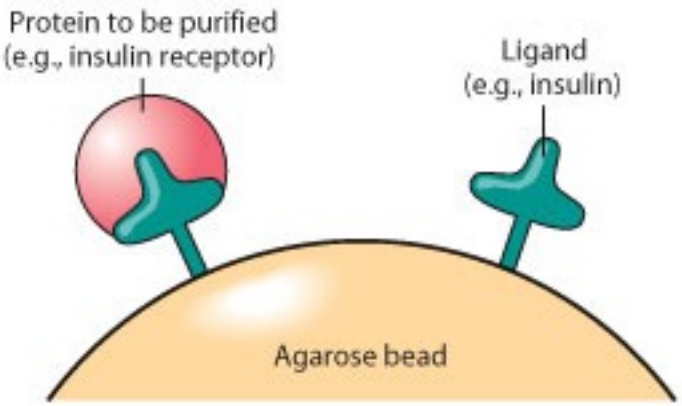
=> e.g. use of protein A coated beads for immobilizing antibodies

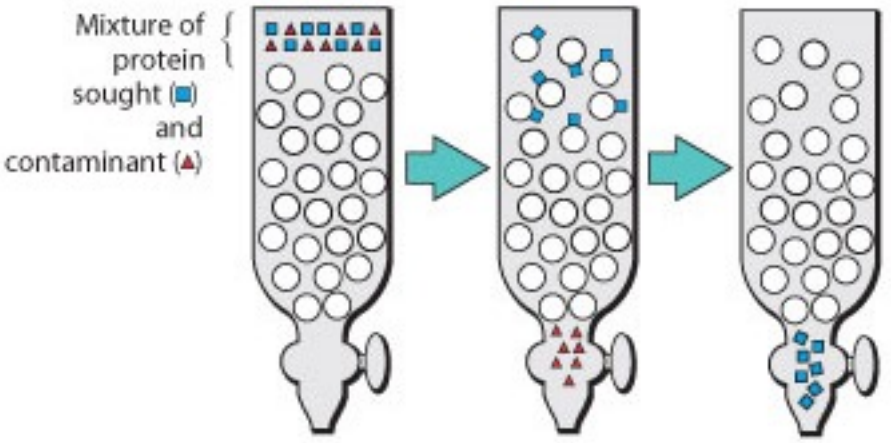
Affinity chromatography

One binding partner (here: insulin) is immobilized on solid support (bead), the other (the *"analyte"*; here the insulin receptor) is contained in the (usually complex) sample.

1. Receptor (red) specifically binds to ligand (green) when passing the column

2. Bound receptor is then washed off with a chaotropic reagent or with acid





Variations of affinity chromatography

1. Biospecific / biomimetic binding pairs:

- ligand / receptor
- antibody / hapten
- substrate / enzyme
- single stranded DNA
- lectin / carbohydrate

→ The column is usually covalently modified with the first binding partner.

2. Metal chelate

- His-tag

→ Requires recombinant protein

Binding constant (K_D) should be 10^{-5} - 10^{-7} M

e.g. biotin-streptavidin (K_D = 10^{-14} M) less suitable => almost irreversible binding

=> highest selectivity compared to other types of chromatography

=> high capacity for target protein

But: - more knowledge about target protein required

- longer preparation time

Preparation of recombinant proteins

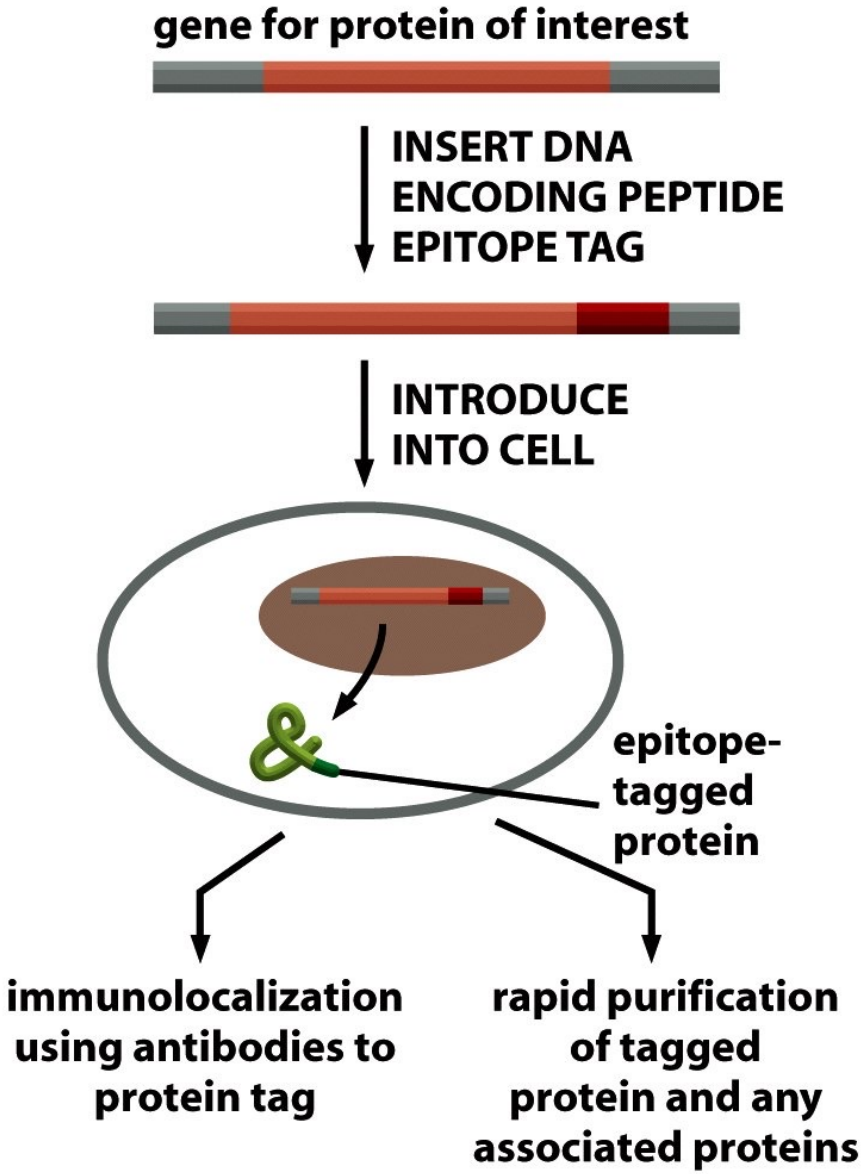
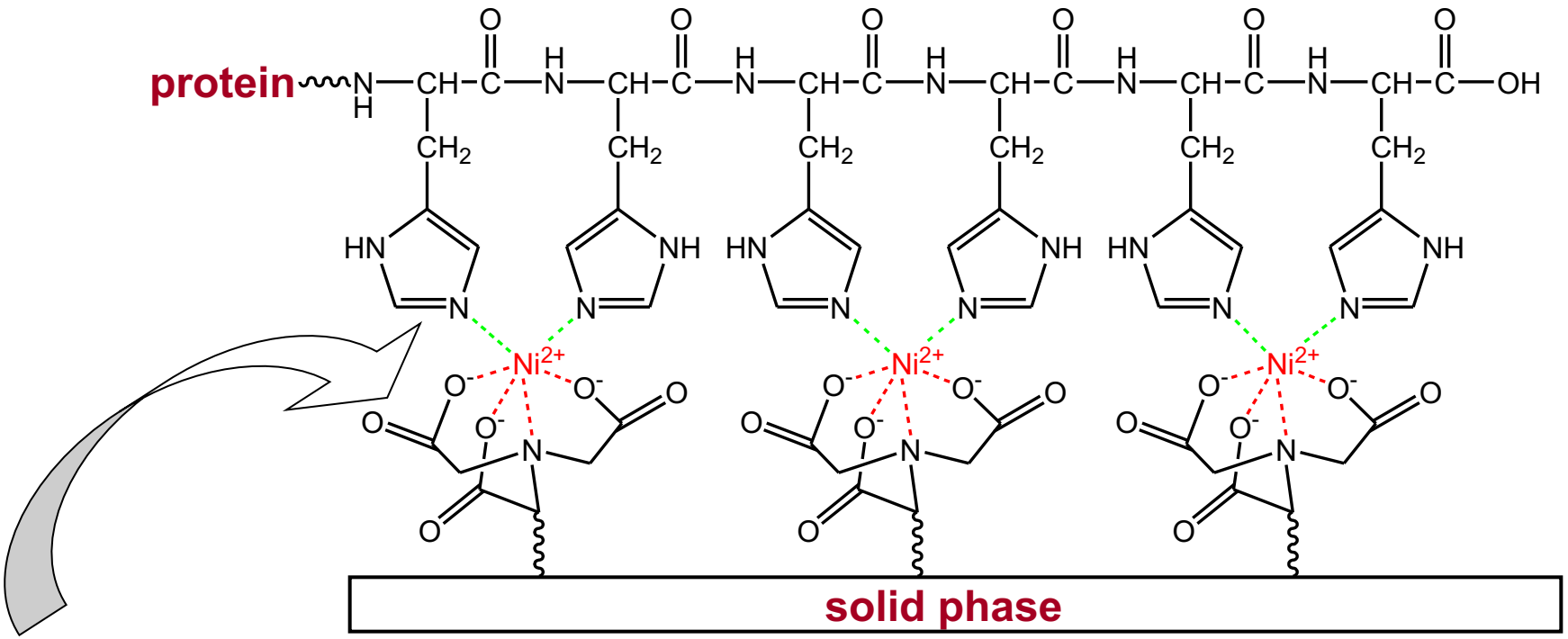
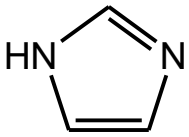


Figure 8-15 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Protein purification: His_6 tag

=> Insert six times the codon CAT or CAC after the DNA sequence for the protein



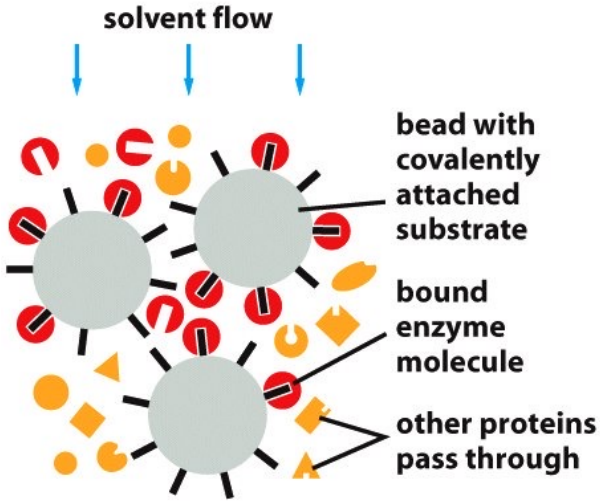


imidazole

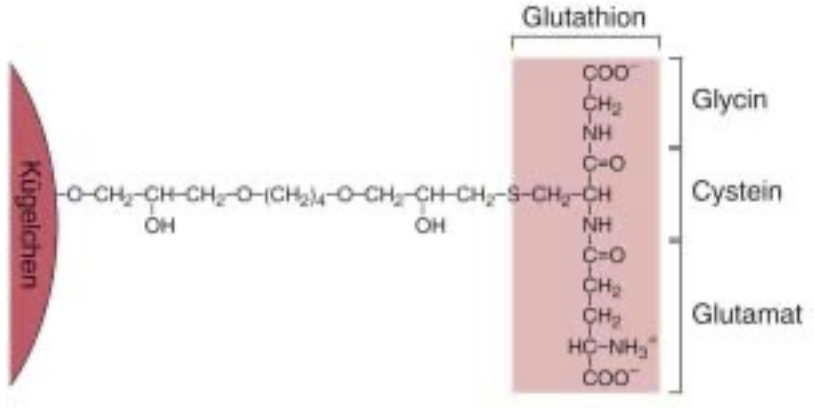
Washed off from the column
by a small molecule competitor

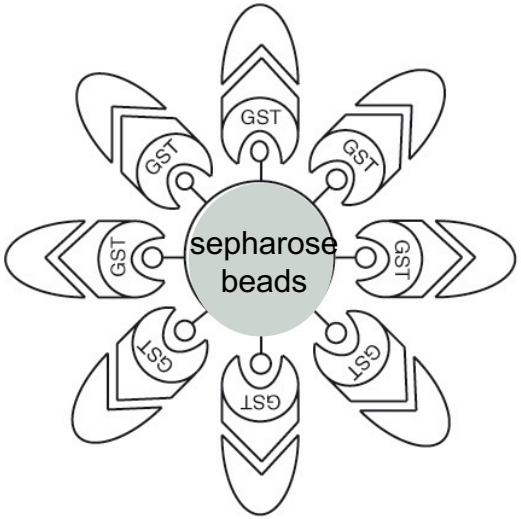
Protein-protein interactions: GST pulldown assay

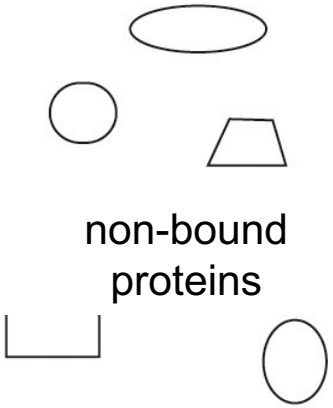
Affinity chromatography



Surface structure of glutathione beads







prey protein (carries a radioactive label, e.g. ^{35}S)

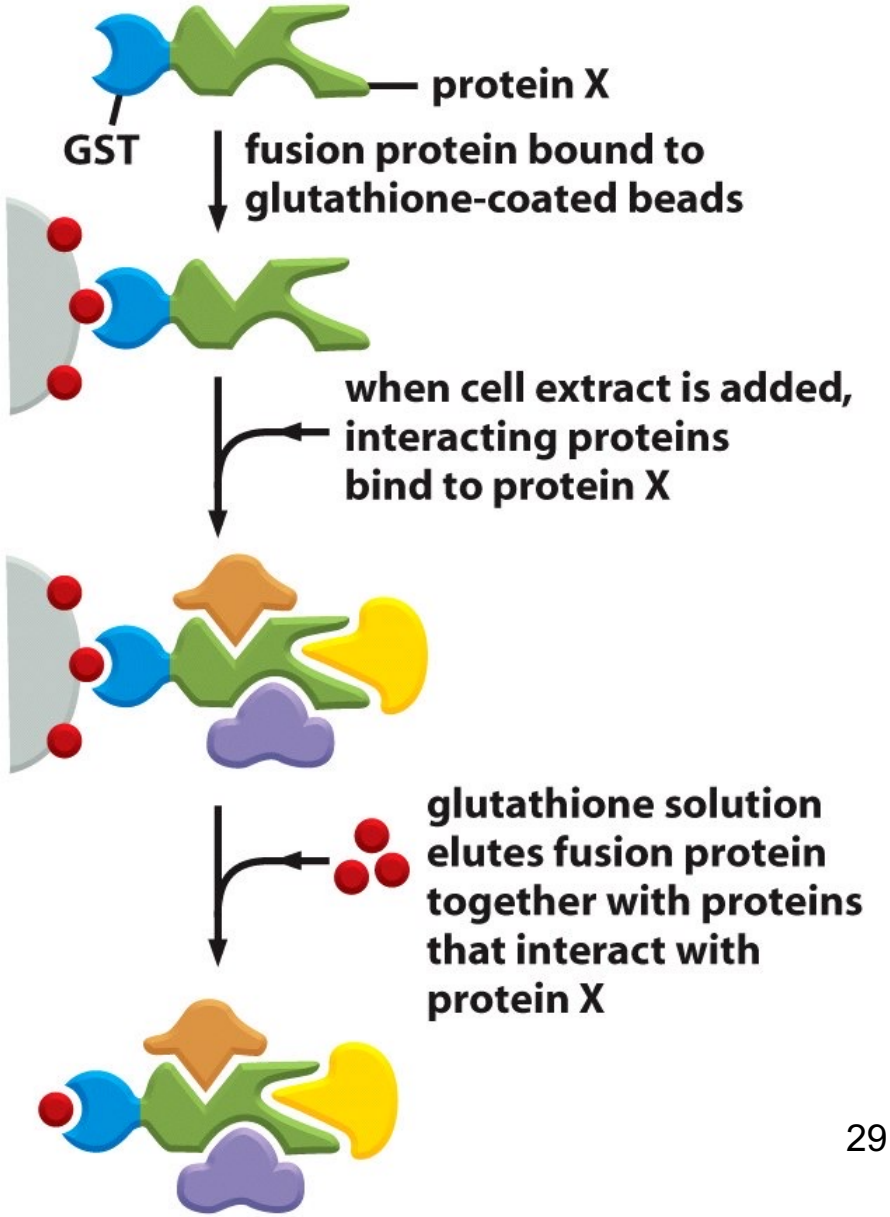
bait protein

glutathion S-transferase

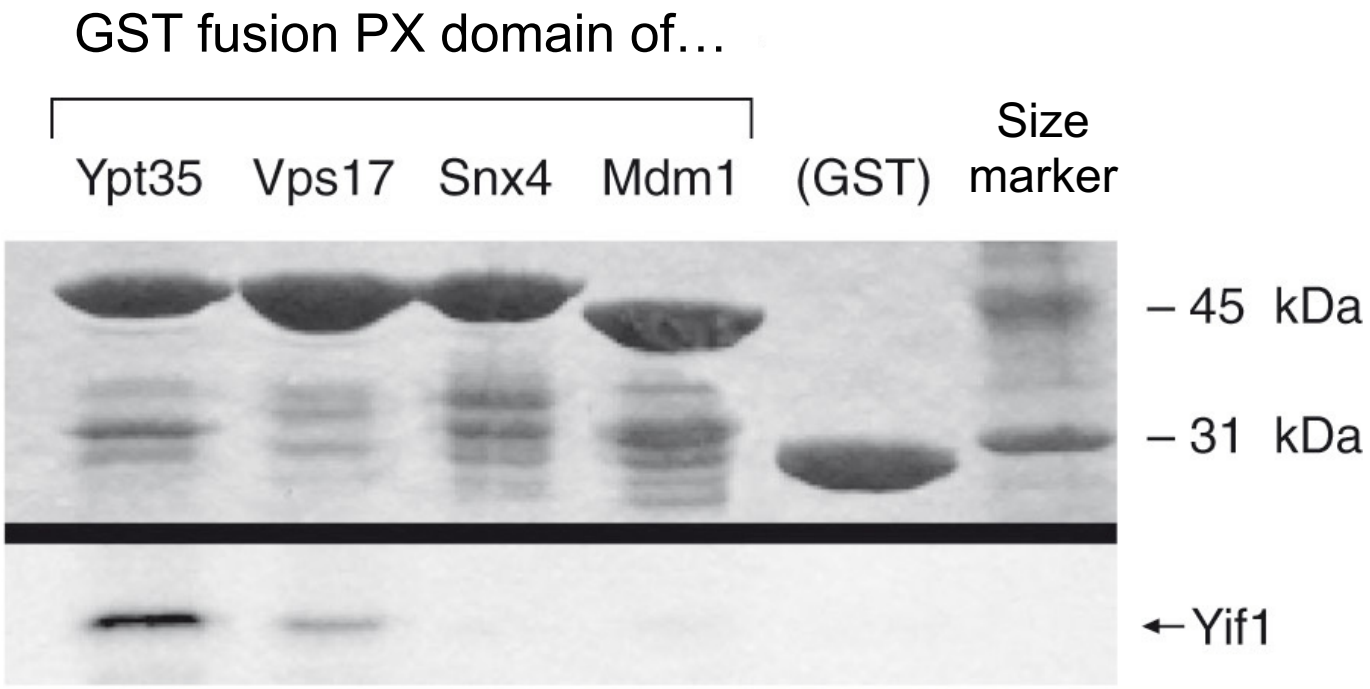
glutathion

GST pulldown assay: co-binding

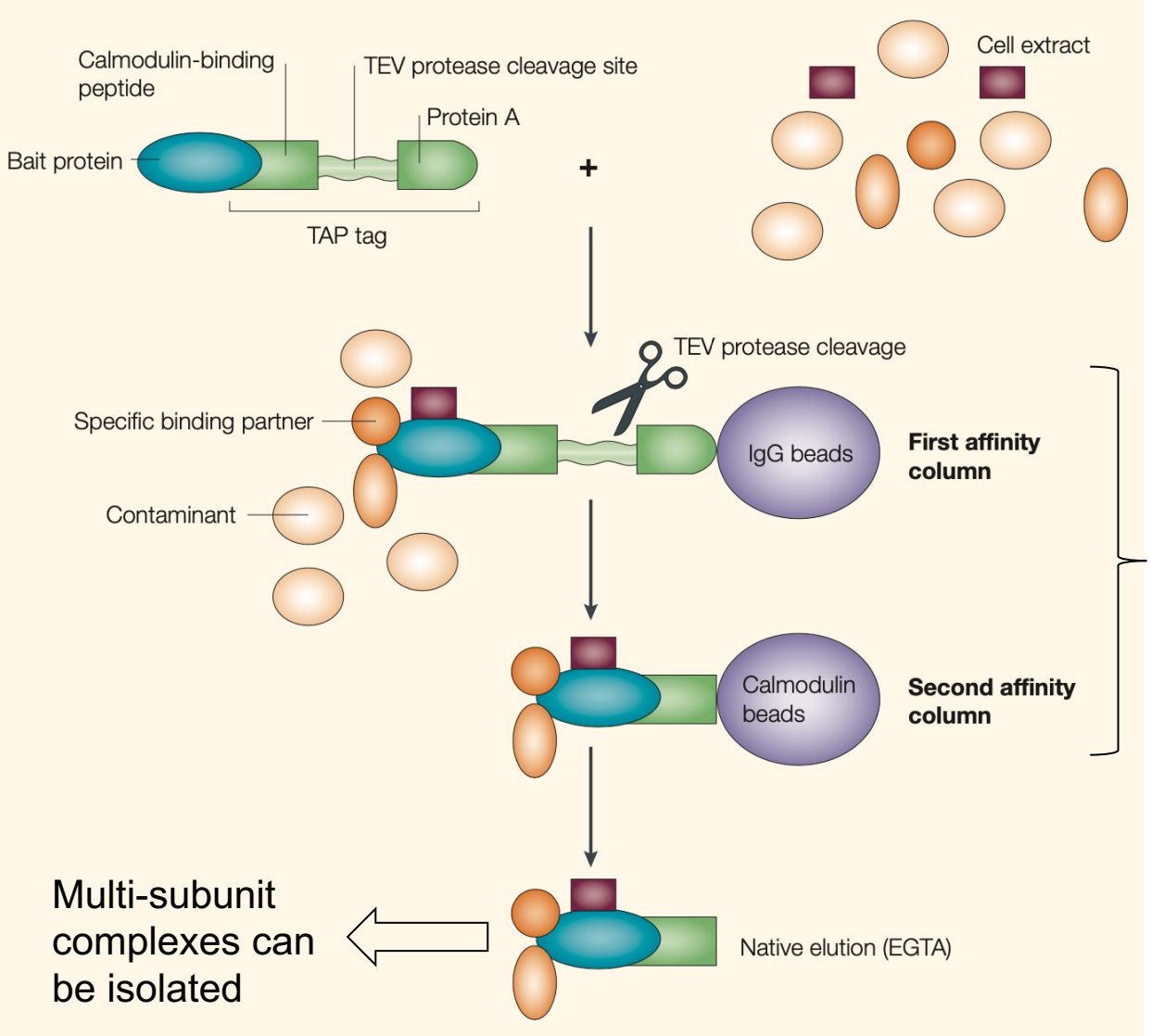
Recombinant DNA techniques are used to make fusion between protein X and glutathione S-transferase



GST pulldown assay: gel and autoradiogram

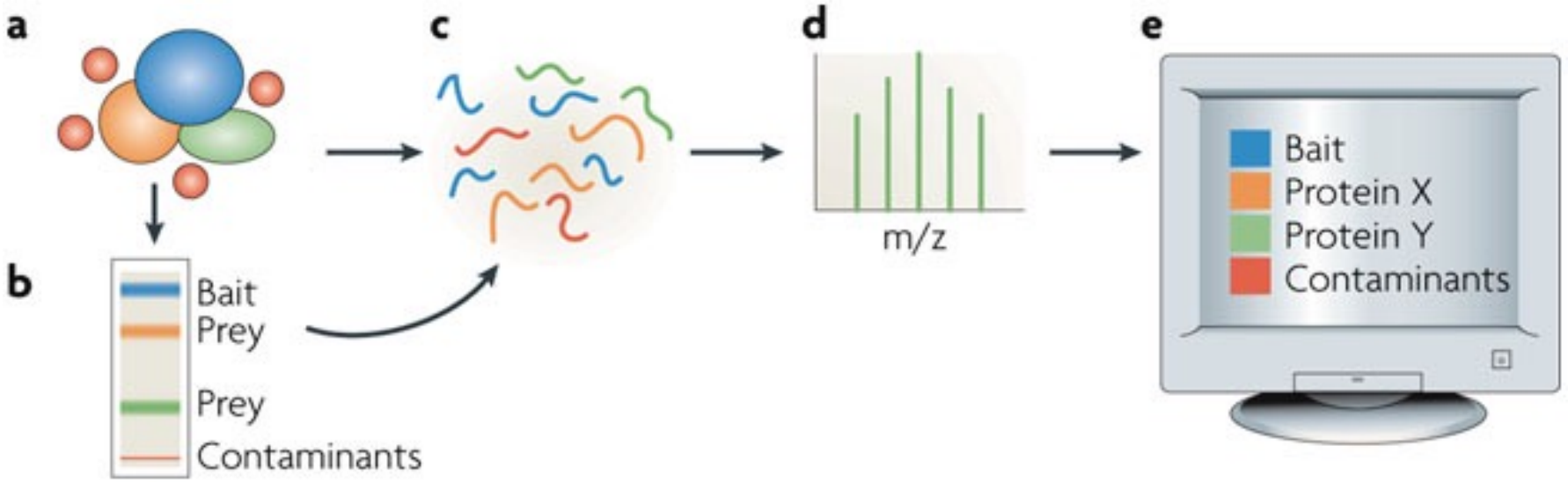


Tandem Affinity Purification: TAP tagging



=> 2 washing steps, less non-specific binding, milder conditions

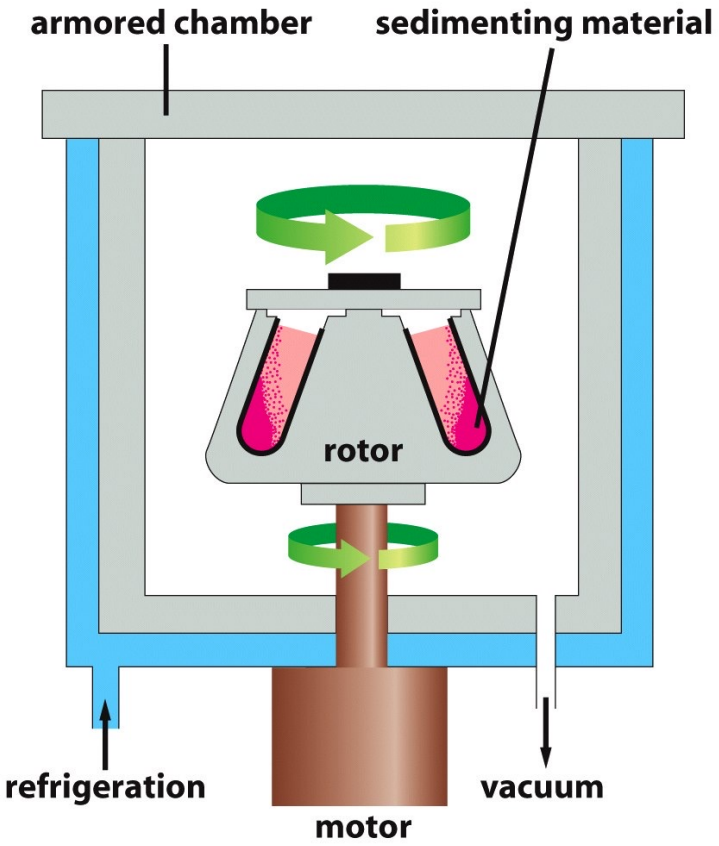
Protein complexes analyzed by MS



Nature Reviews | Molecular Cell Biology

Analytical ultracentrifugation

Separation and detection of protein-protein complexes



Svedberg equation:

$v = [d^2 (\rho_p - \rho_m) g]/18\eta$

v = rate of sedimentation

d = diameter of particle

$(\rho_p - \rho_m)$ = difference in the density of particle and medium (water/sucrose)

g = gravity applied (routinely at 10,000; ultracentrifugation: 150,000)

η = viscosity of medium

g depends on the rotational speed **and** the rotor diameter!

Detection:

UV absorption and/or interference optical refractive index through two windows of quartz glass in the rotor

Analysis of protein-protein interactions *in vitro*

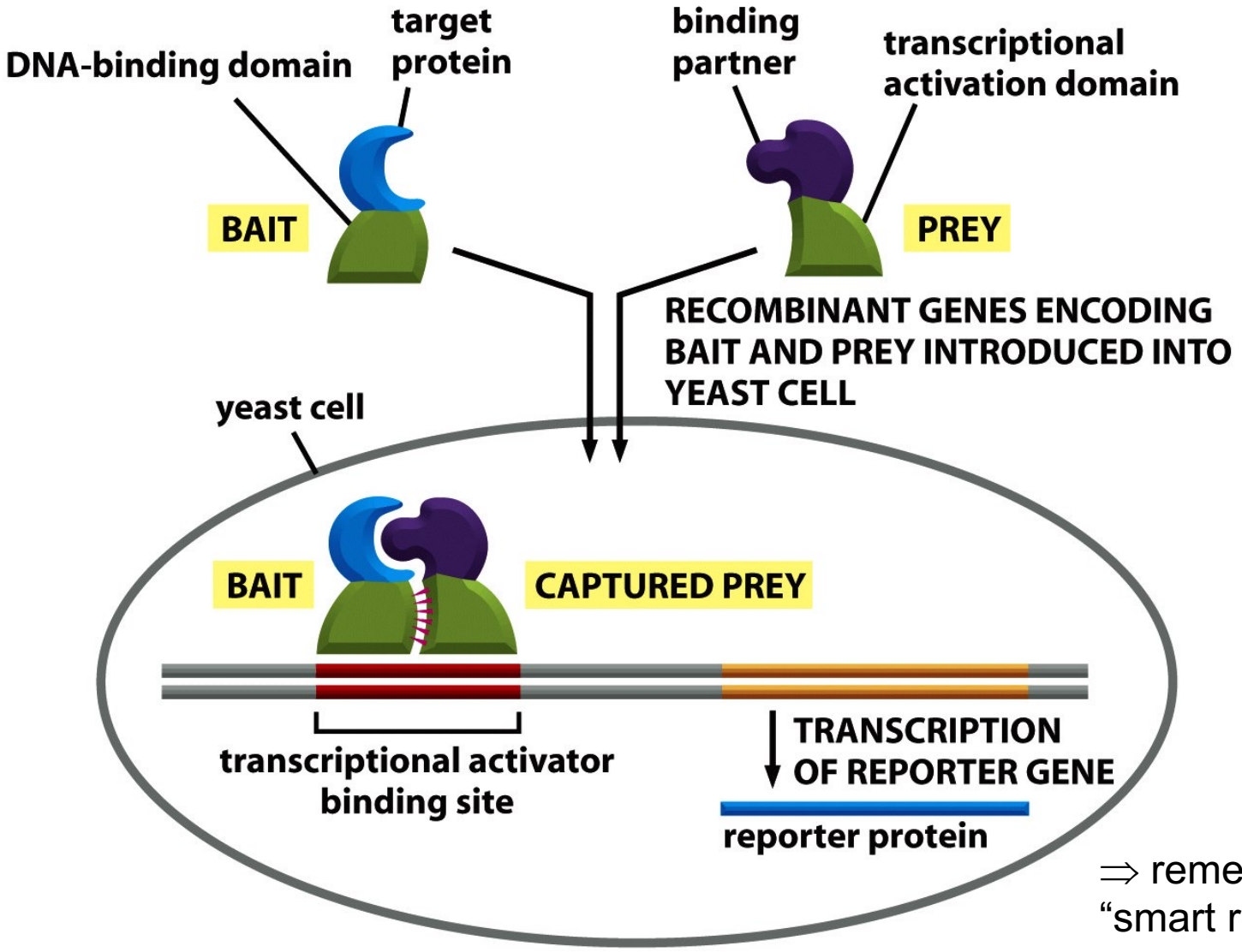
Method	Advantages	Disadvantages
Far Western blotting	• Both cloning and heterologous expression and detection by specific antibodies is possibly if antibody is available	
Co-immuno-precipitation	• Does not require cloning and heterologous expression • Rapid if antibody is available	• Not generic: requires access to specific antibodies
Affinity pulldown	• Generic ability to purify low-abundance protein complexes	• The presence of a protein tag may influence results • Competition with the endogenous complex
Tandem affinity purification (TAP)	• Generic ability to purify low-abundance protein complexes • Mild conditions used throughout	• The presence of a protein tag may influence results • Competition with the endogenous complex
Analytical ultracentrifugation	• Does not require cloning and heterologous expression • Rapid if antibody is available	• Expensive equipment required

Yeast 2-hybrid system (Y2H)

In vivo

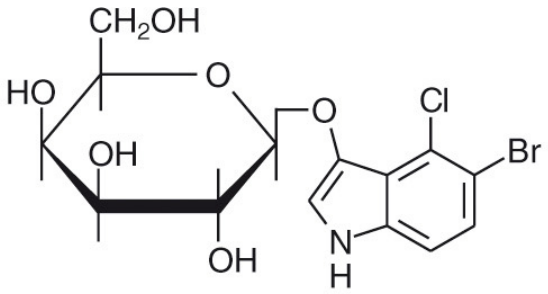
$\Rightarrow$ Protein-protein interactions are investigated in their natural environment

Y2H: Protein fragment complementation assay

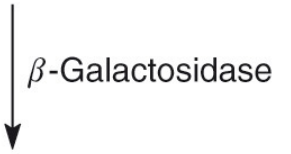


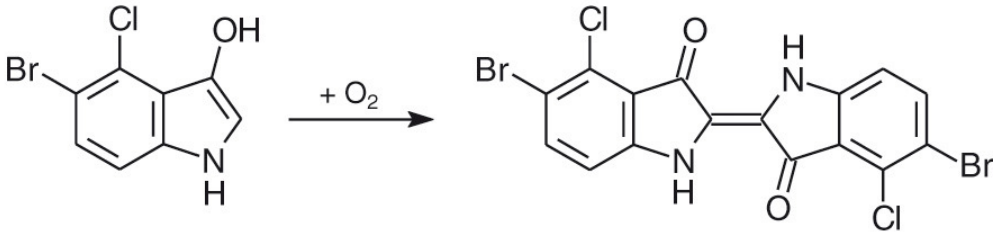
⇒ remember:
“smart reporters”

Detection of reporter gene expression



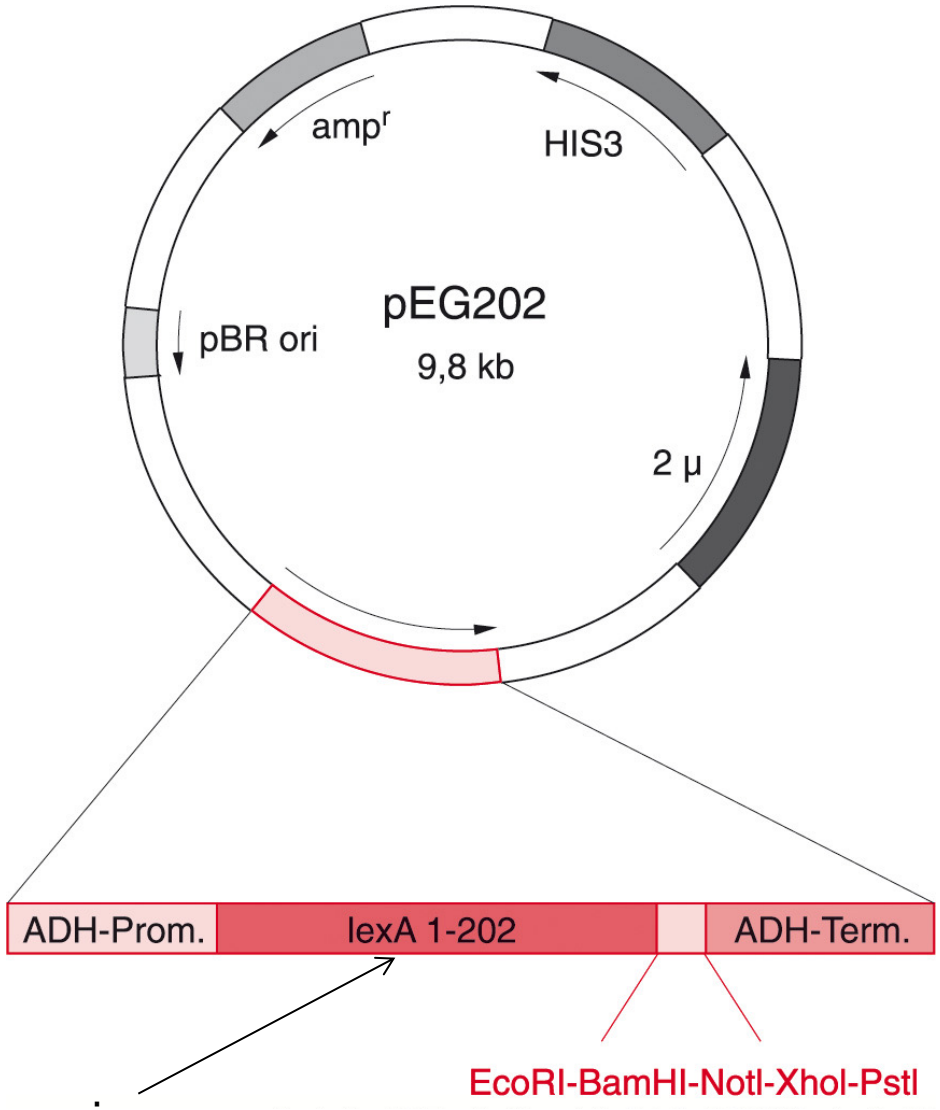
5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)
(no color)



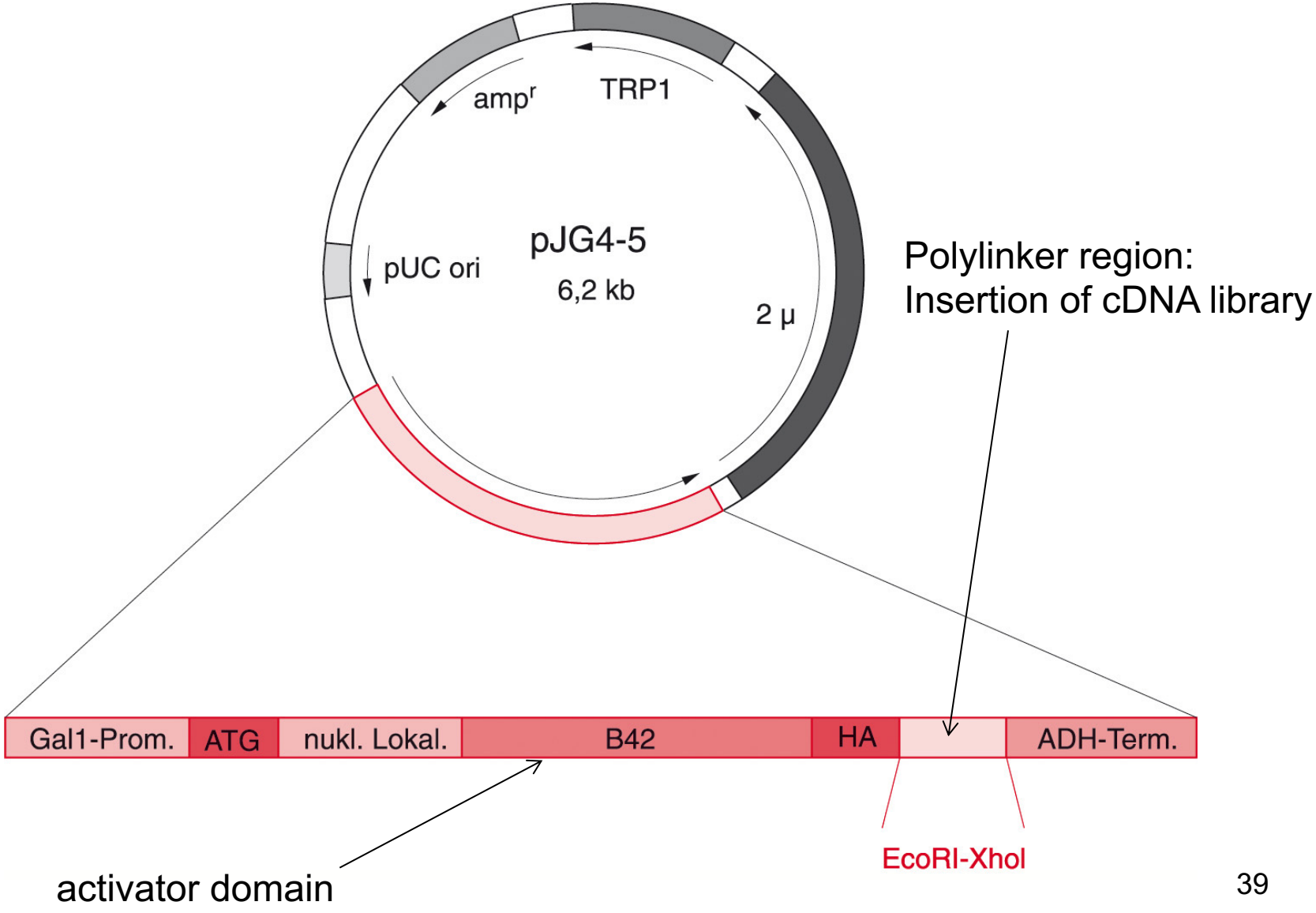


5,5‘-dibromo-4,4‘-dichloro-3-indigo
(blue)

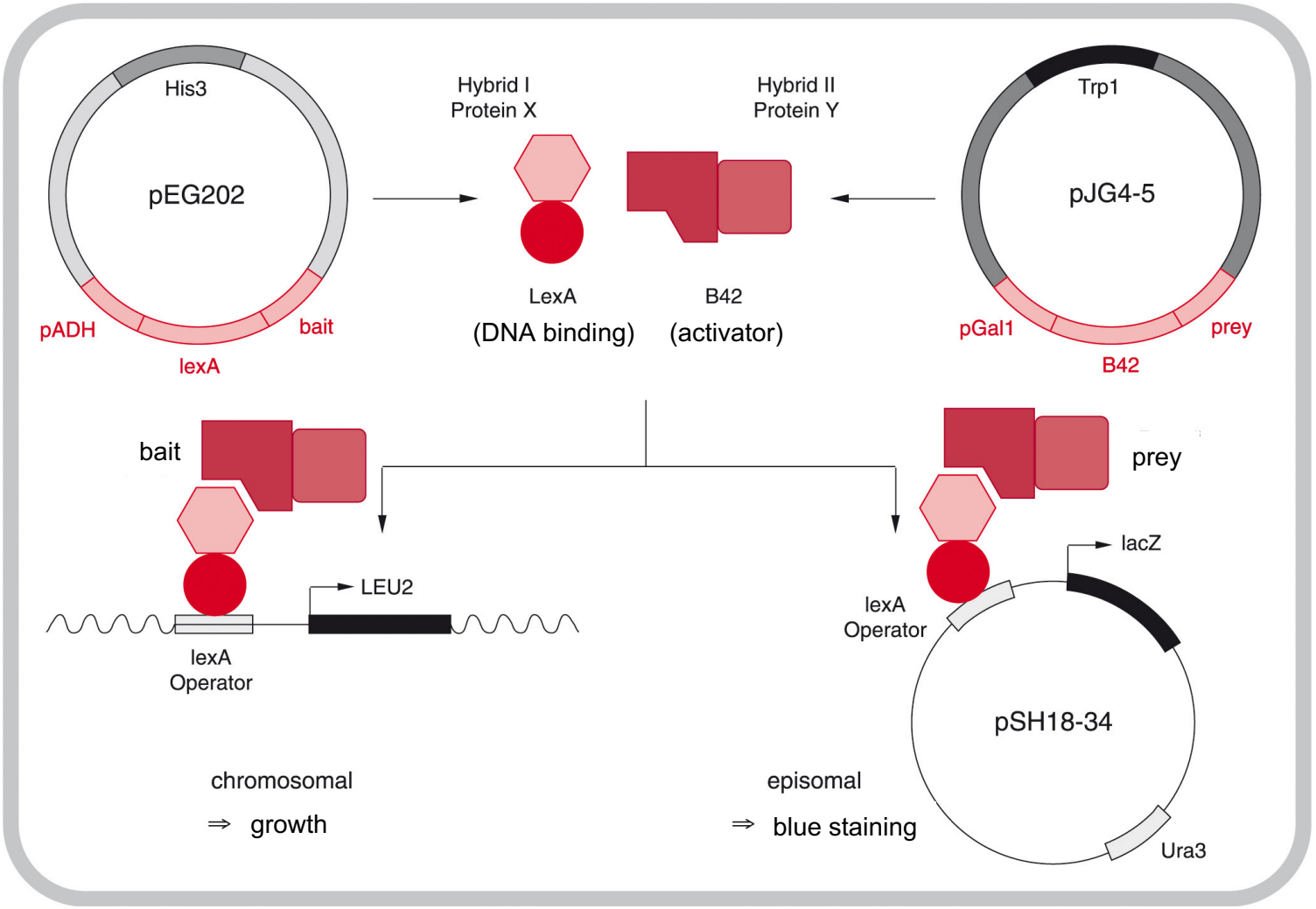
Vector for coding bait fusion protein



Vector for coding prey fusion protein



Yeast two-hybrid system



Yeast two-hybrid system

Large libraries of cDNA can be screened for protein interactions in their natural environment

But many false negative and false positive results (up to 70 %)
- Fusion proteins are overexpressed
- Proteins that are loacted in different cellular compartments interact
- Fusion proteins may inhibit interactions
- Posttranslational modifications are missing
- The transcription can only occur in the nucleus: fusion proteins must be transported into the nucleus

=> Further analyses are required to confirm a newly discovered protein-protein interaction

Can be extended to detect:
- protein-DNA interactions (yeast one-hybrid system)
- DNA-DNA interactions

Can be performed in other organisms: *E. coli*

Predicting protein-protein interactions from databases

in silico

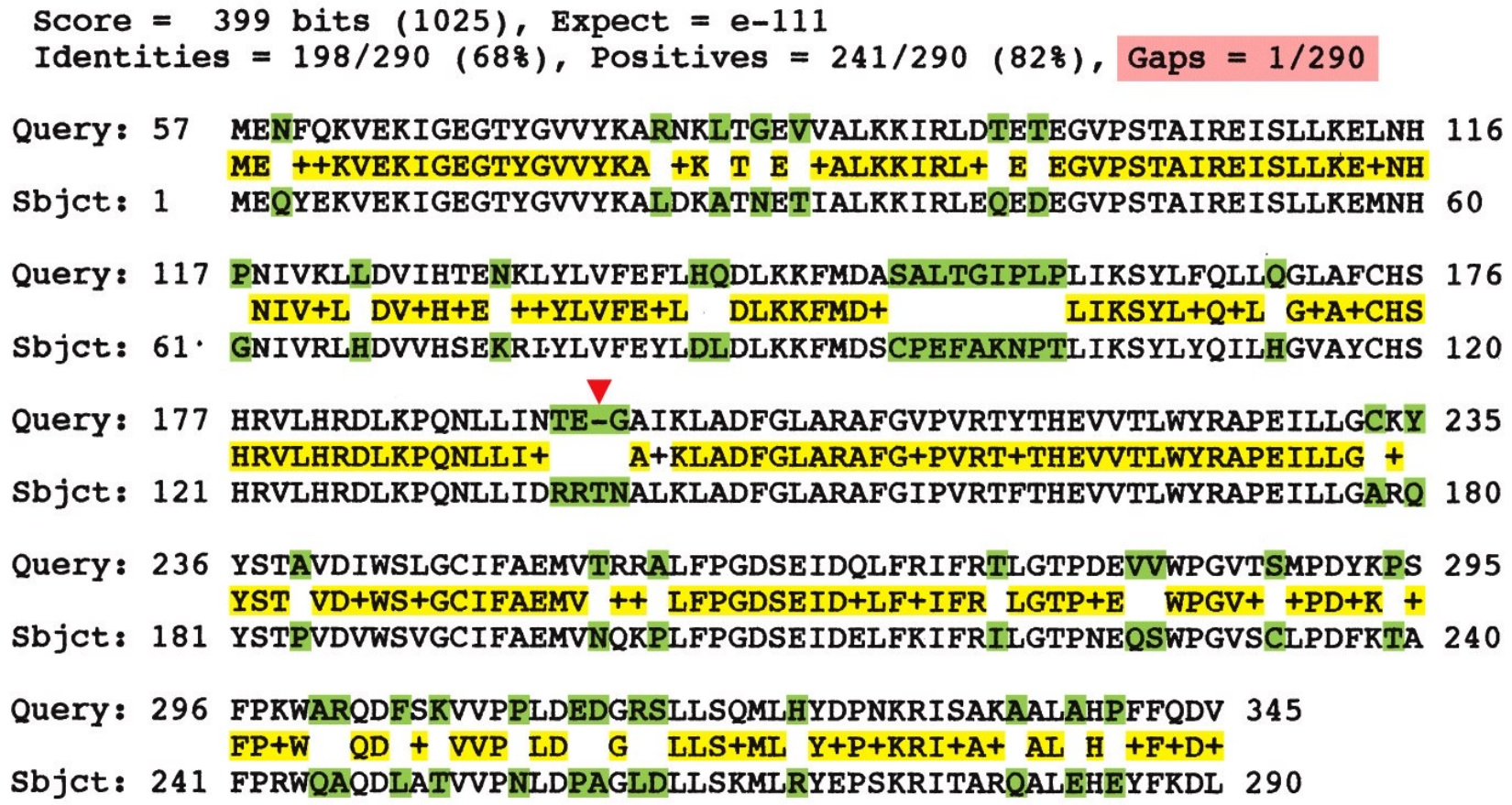


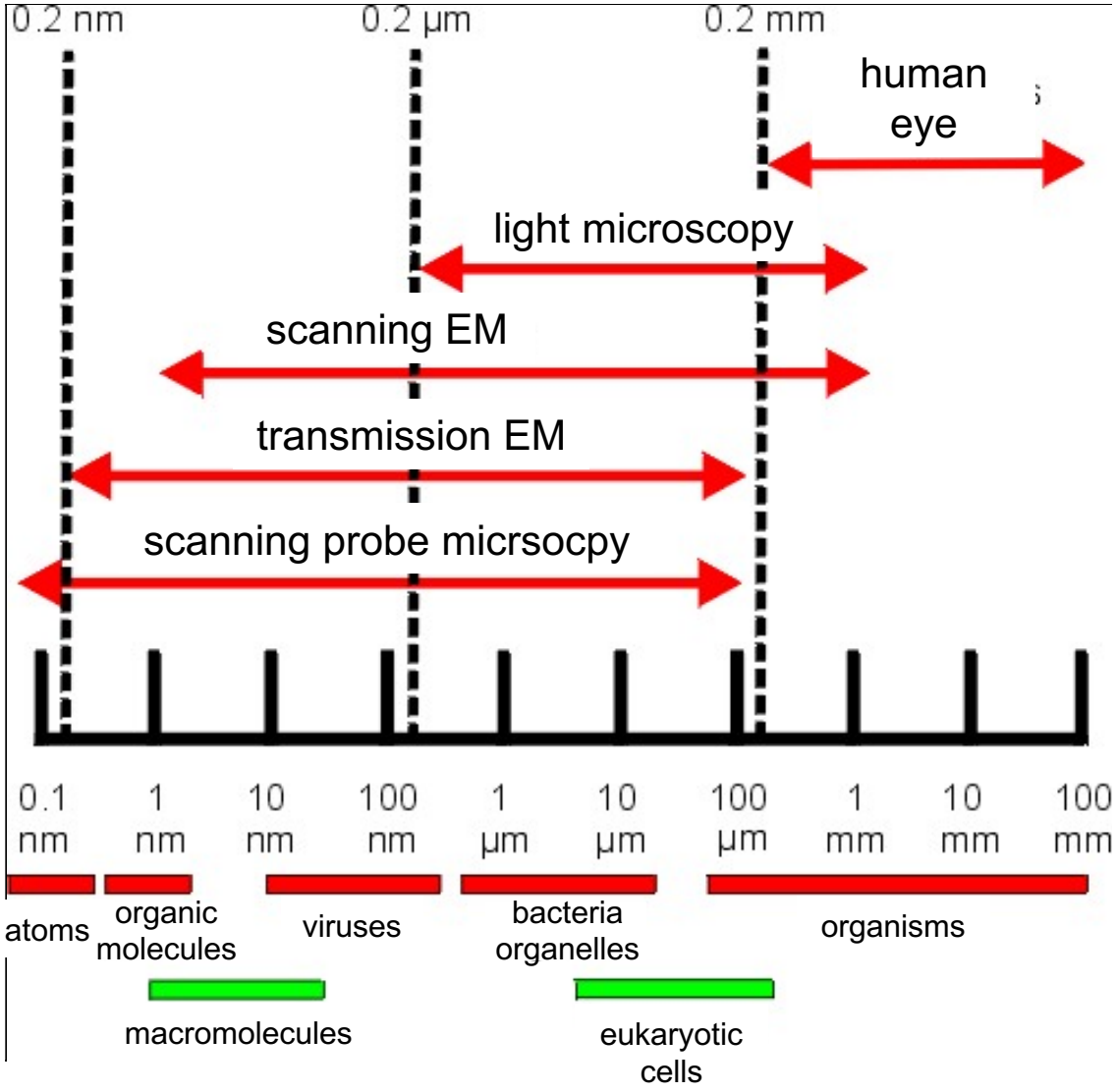
Figure 8-30 Molecular Biology of the Cell 5/e (© Garland Science 2008)

Microscopy
fundamentals -> advanced

Light microscopy

- Foundations of light microscopy / optical resolution
- Dark-field / phase contrast microscopy
- Fluorescence microscopy: advantages and limitations
- Confocal / multiphoton microscopy
- Total internal reflection microscopy
- Single molecule fluorescence microscopy
- Microscopy beyond the diffraction limit (STED / STORM)
- Fluorescence correlation spectroscopy
- Light sheet microscopy

What we can “see“

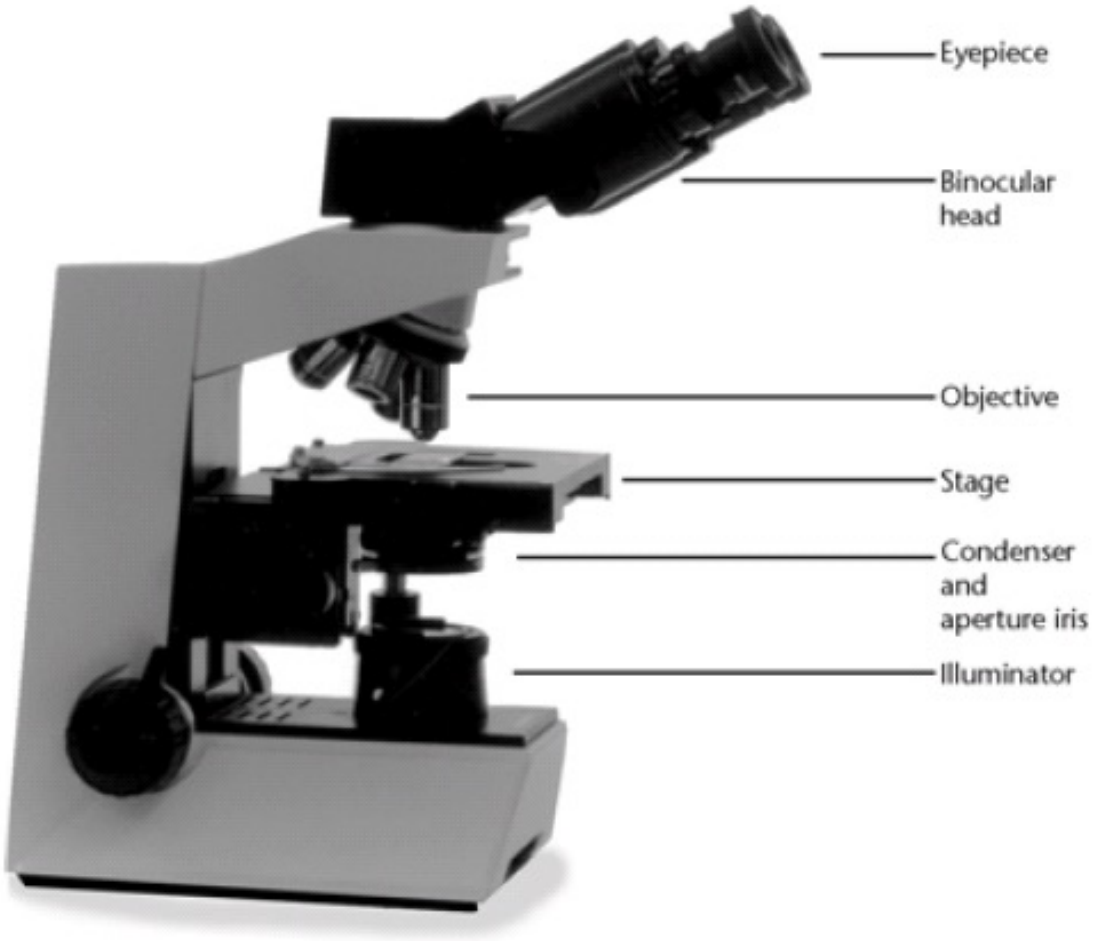


Foundations of light microscopy

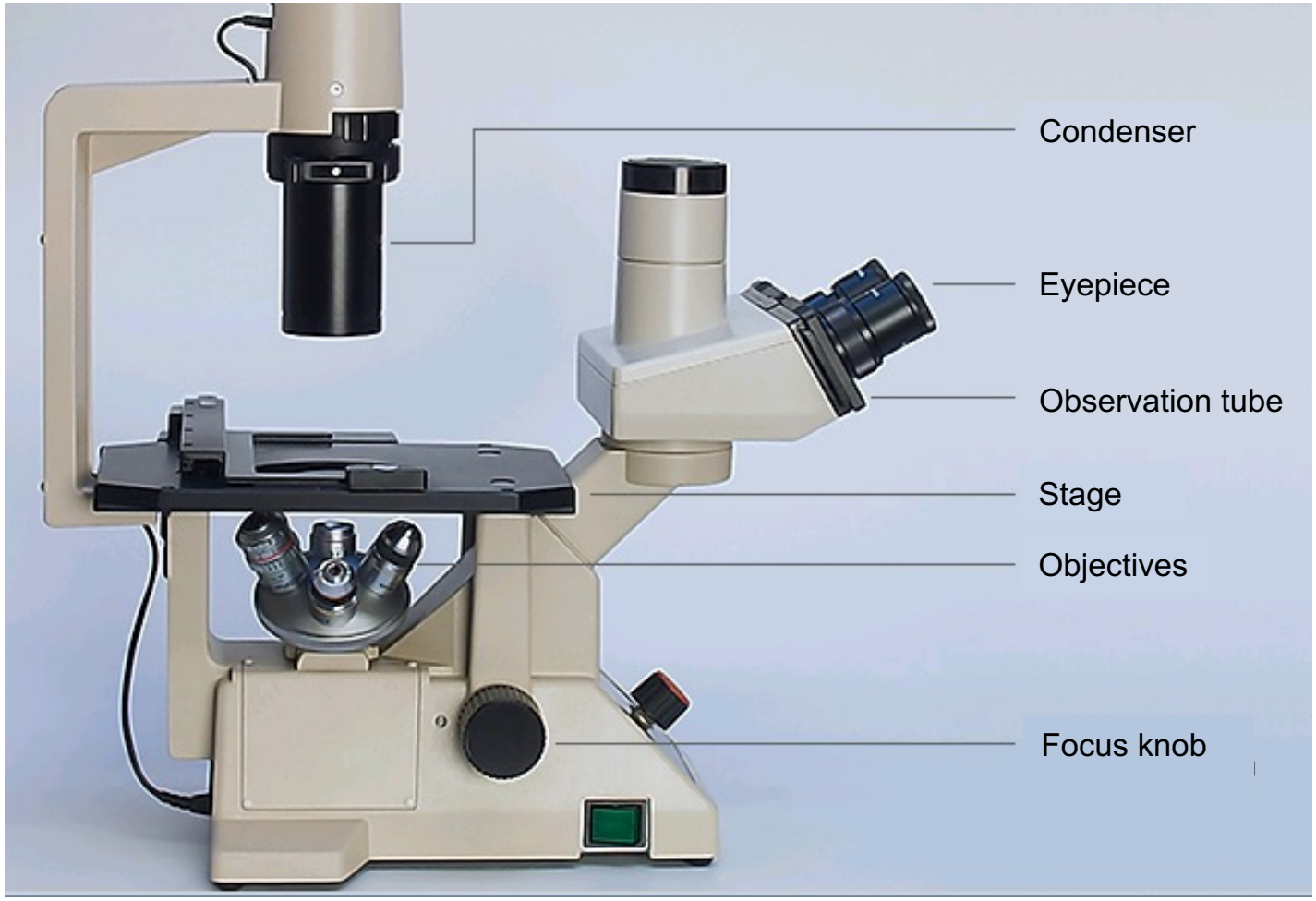
A very useful online guide to microsocpy:

https://www.microscopyu.com/microscopy-basics

Light microscopy: Upright microscope

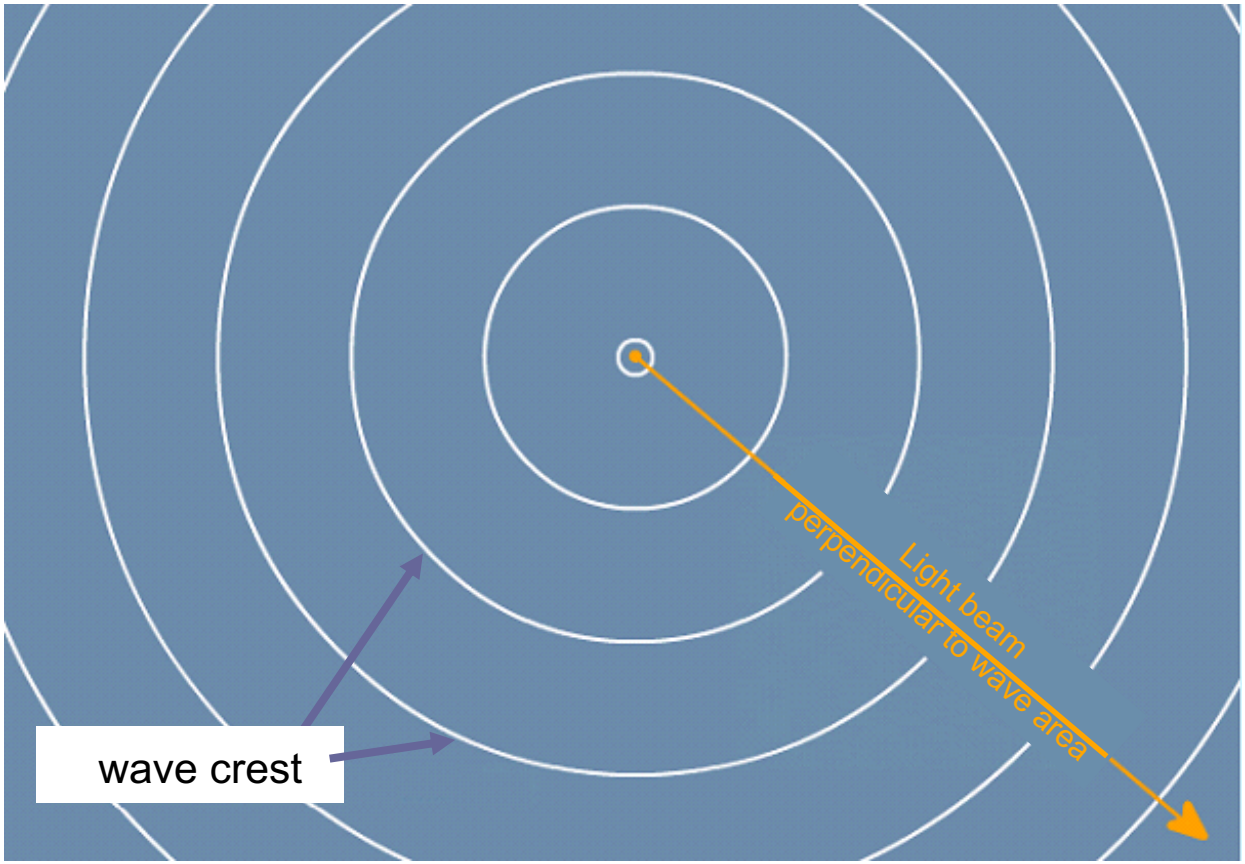


Light microscopy: Inverted microscope



Huygens principle

Wave propagation:
Each point on a wavefront is the source of a new spherical wavelet.
The sum of these spherical wavelets forms the wavefront.
Valid for any type of wave: water waves, sound waves, electromagnetic waves (light).



Light refraction

Light is transmitted in various transparent materials with different speeds (c):

Refractive index $n = c_0 / c_1$

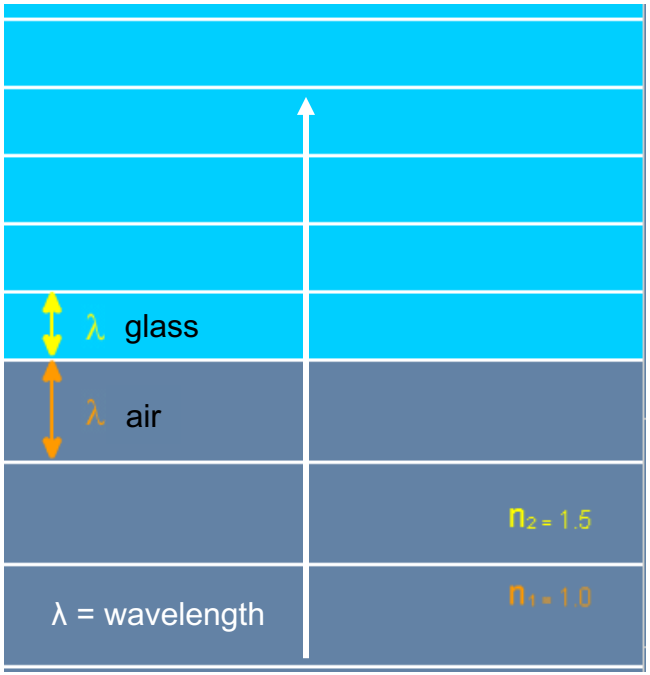
Snellius law of refraction:

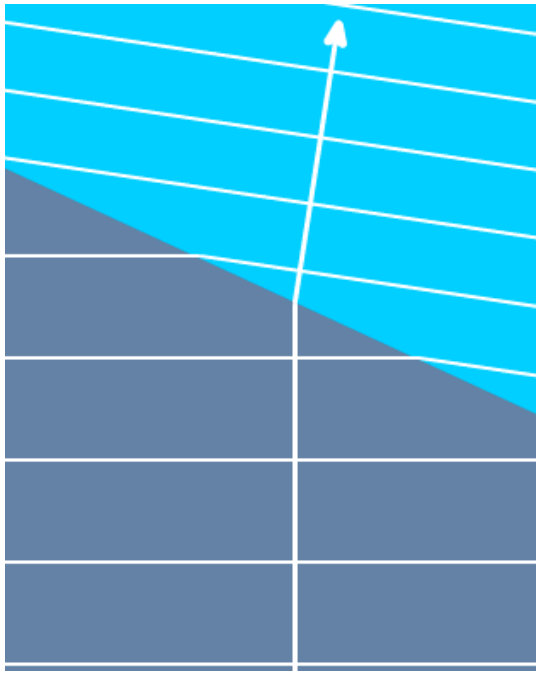
$n_1 \sin\alpha = n_2 \sin\alpha'$

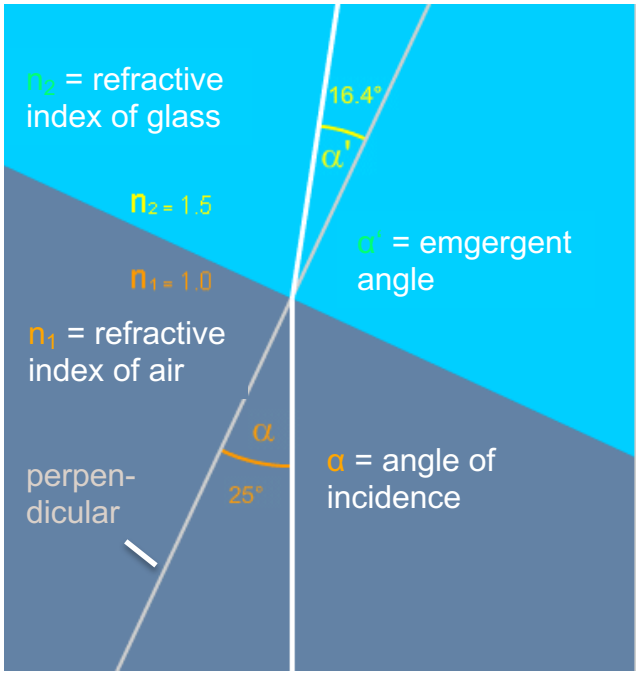
α: angle of incidence
α‘: emergent angle
=> relative to perpendicular

Light refraction

Light refraction at air-glass interface $\frac{\sin\alpha}{\sin\alpha'} = \frac{n_2}{n_1}$





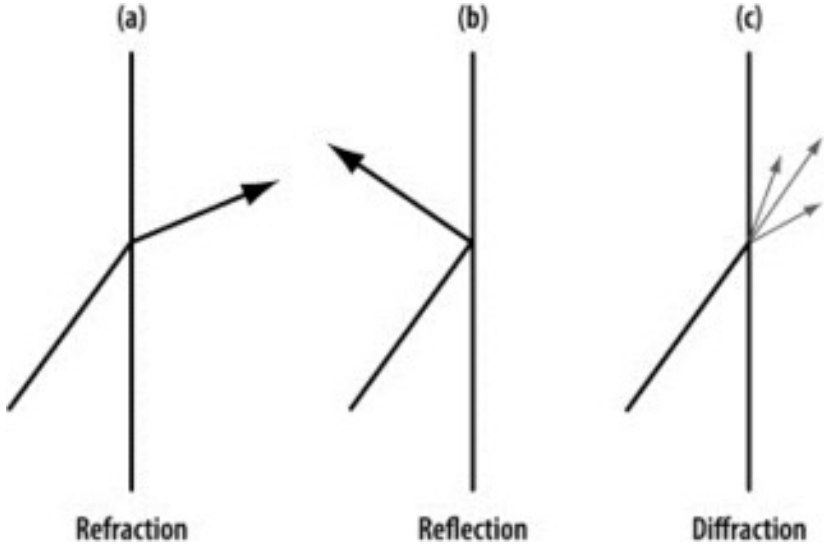


Light entering an optically DENSER medium is refracted TOWARDS perpendicular.

Light entering an optically THINNER medium is refracted AWAY from perpendicular.

Summary of wave propagation

When a light wave encounters an object, it may be reflected, absorbed, refracted, diffracted, or scattered depending on the composition of the object and the wavelength of light.

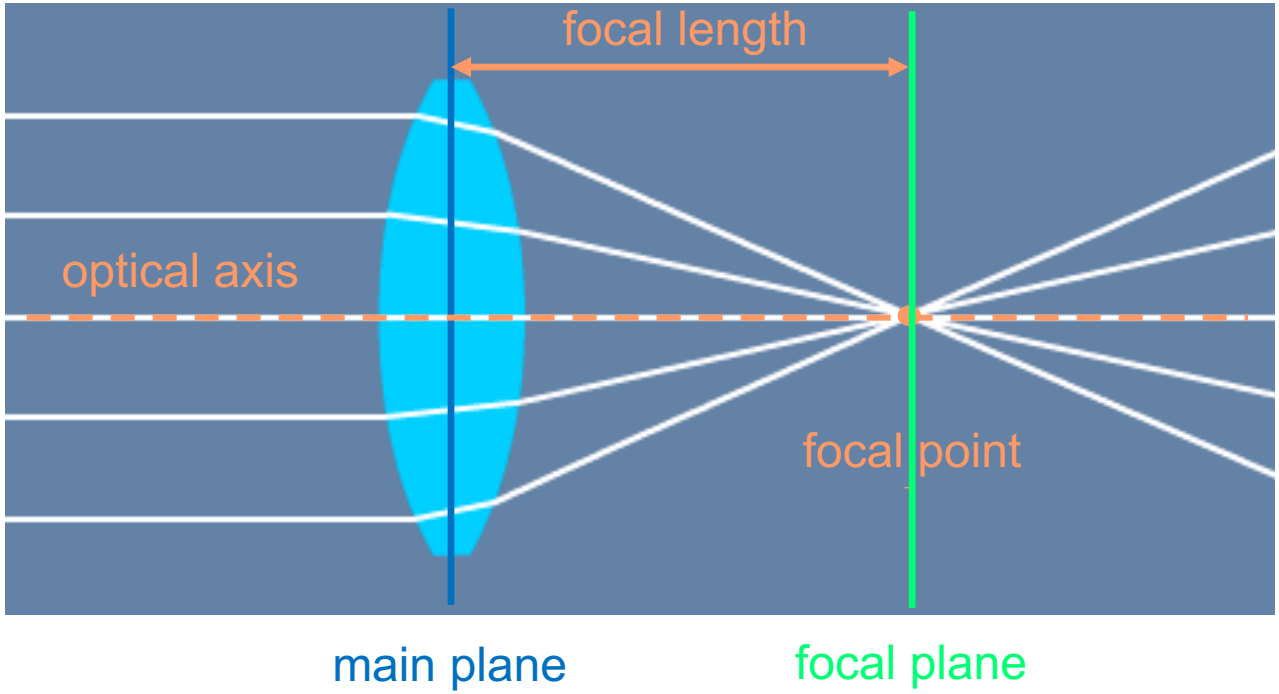


- **Refraction:** Light wave changes direction as it passes from one medium (n_1) to another medium (n_2) as a result of differences in speed of light: in vacuum > air > water > glass.
- **Reflection:** Light wave hits an object and bounces off. Very smooth surfaces such as mirrors reflect almost all incoming light.
- **Diffraction:** Interference or bending of waves around the corners of an obstacle or through an aperture into the region of geometrical shadow of the obstacle/aperture.

=> All these phenomena can be explained by Huygens principle of wave propagation

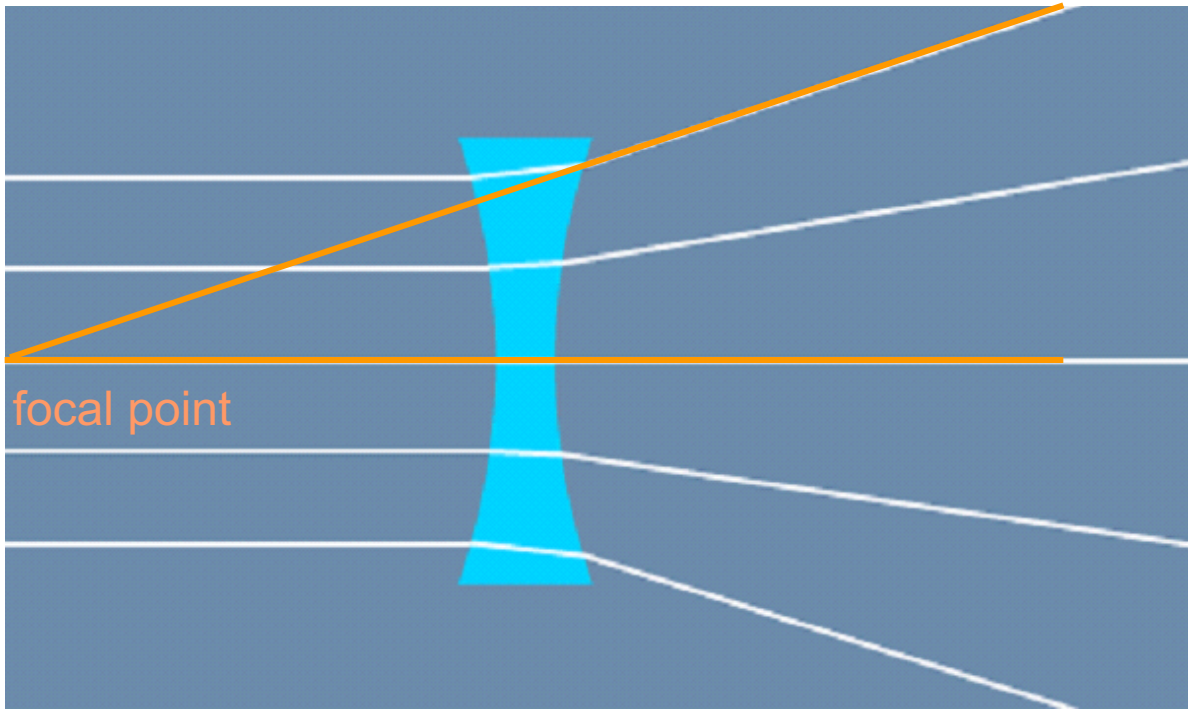
Collective lens

Convex glass (spherical)

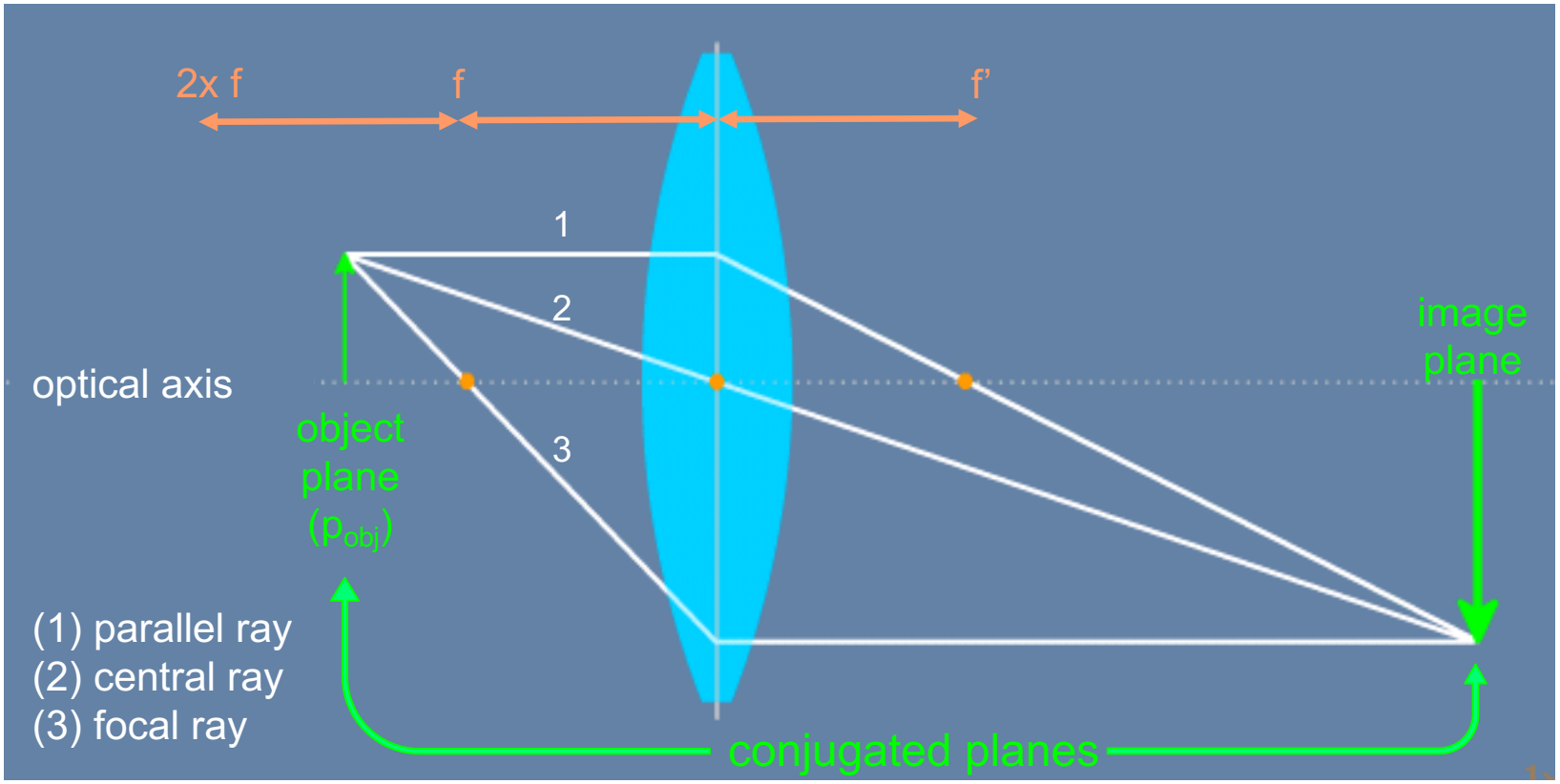


Diverging lens

Concave glass (spherical)



Collective lens



We obtain a real image (upside down) if object is placed:

(A) in focal plane ($p_{obj} = f$): parallel rays emerge after lens; i.e. image is not focused

(B) between simple and double focal length ($f < p_{obj} < 2f$): magnified image

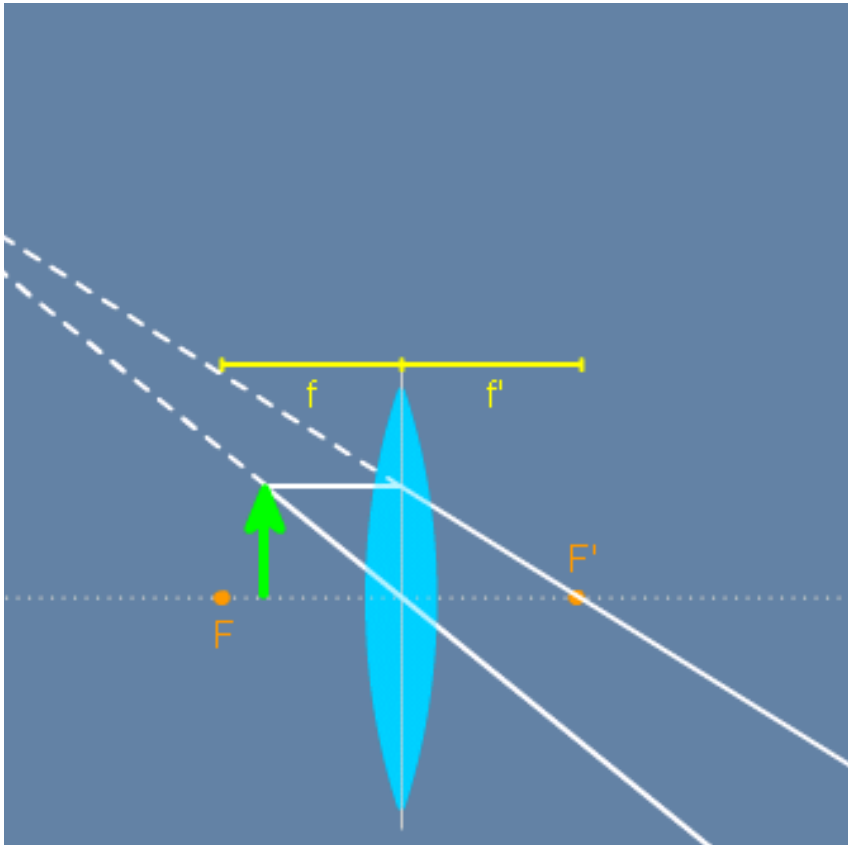
(C) in double focal length ($p_{obj} = 2f$): image has the same size as object

(D) beyond double focal length ($p_{obj} > 2f$): demagnified image

Collective lens

Object placed between focal point and lens ($p_{obj} < f$)

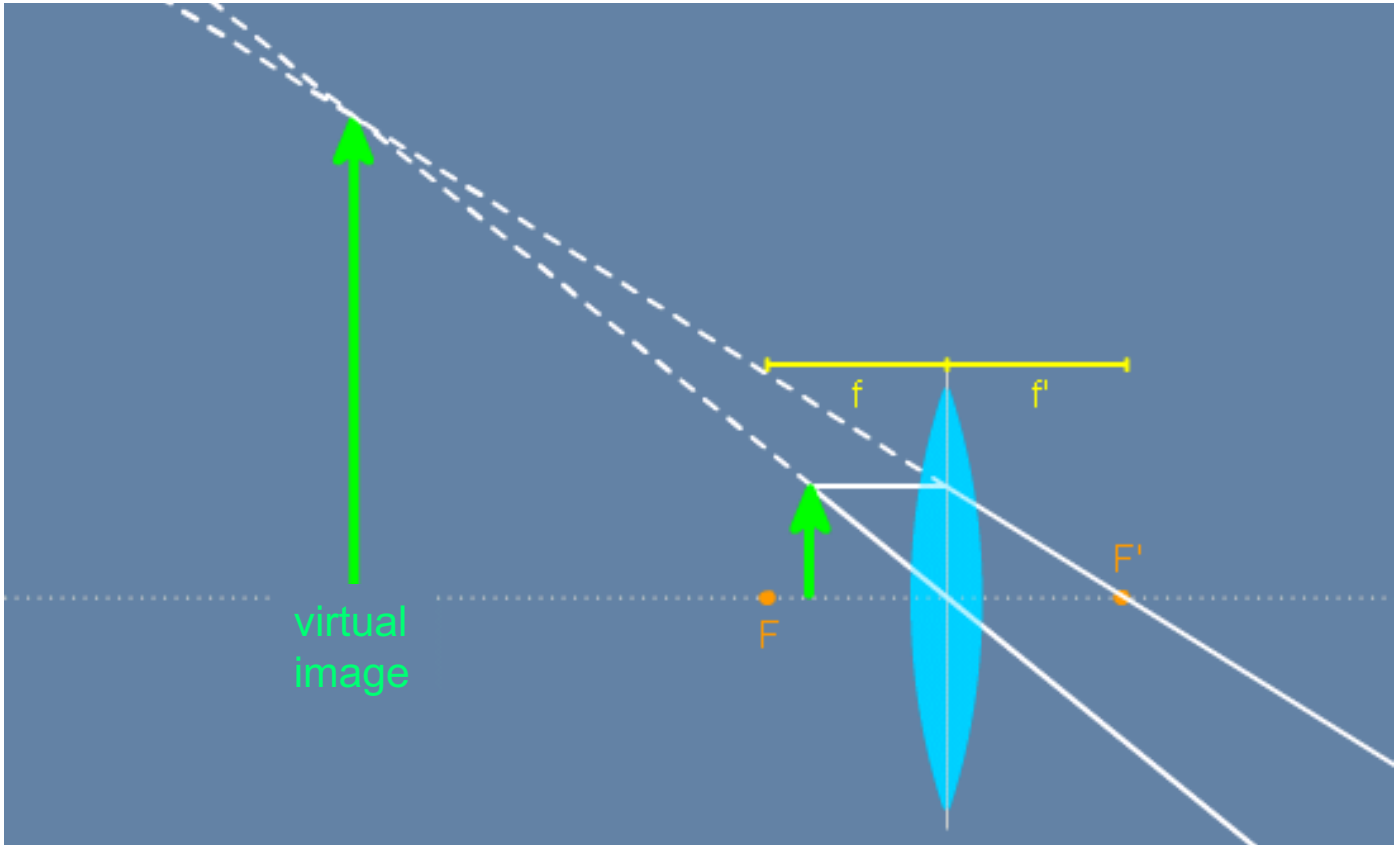
=> Diverging rays after lens, i.e. image cannot be focused



Collective lens

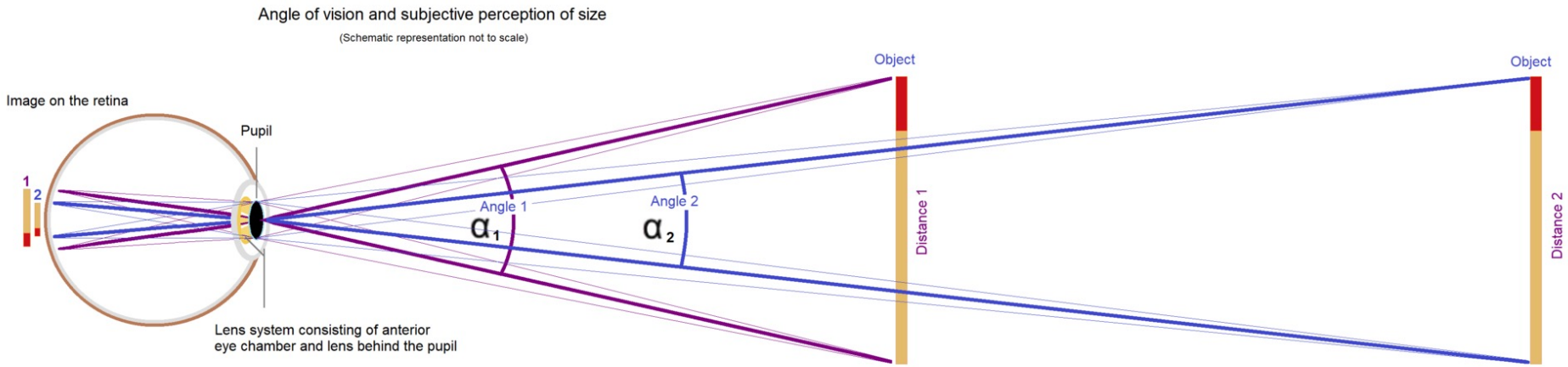
Object placed between focal point and lens ($p_{obj} < f$)

=> Diverging rays after lens, i.e. image cannot be focused

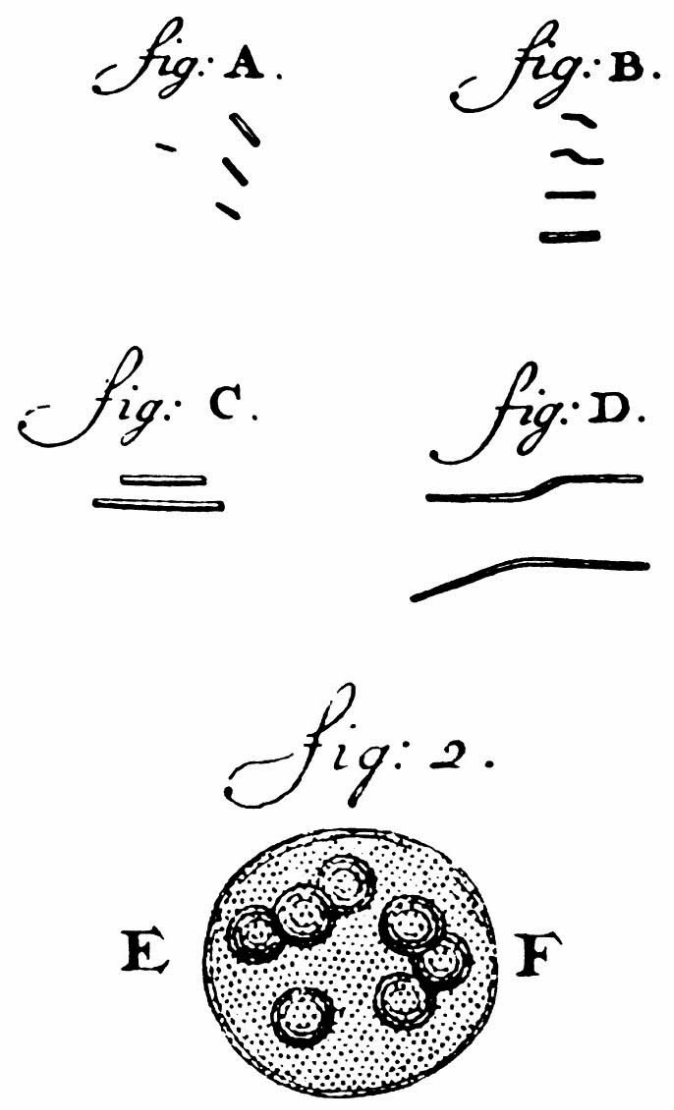


Magnified virtual image behind object (loupe).

Visual angel



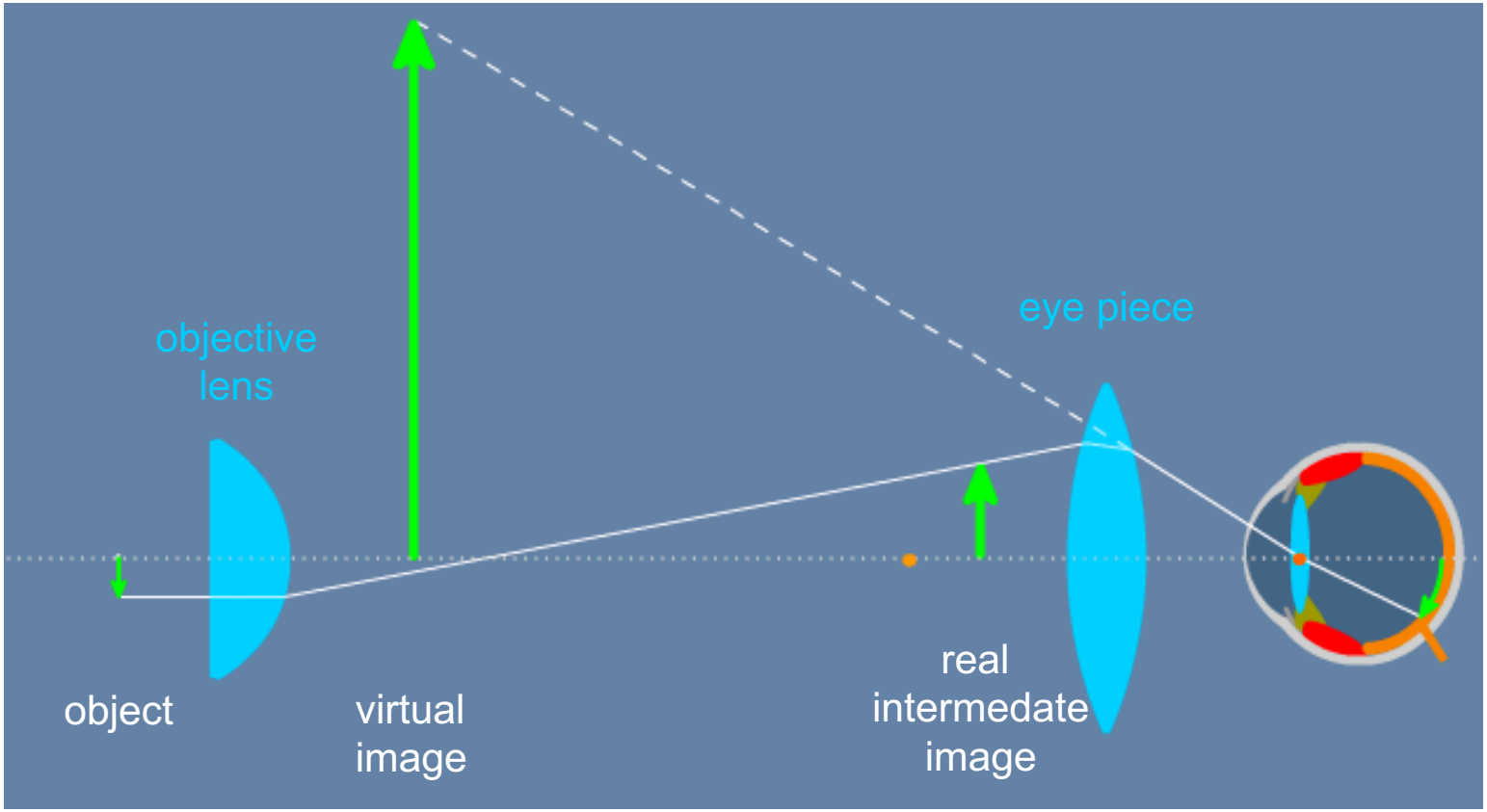
Anton van Leeuwenhook (1632-1723)



up to 200-fold magnification

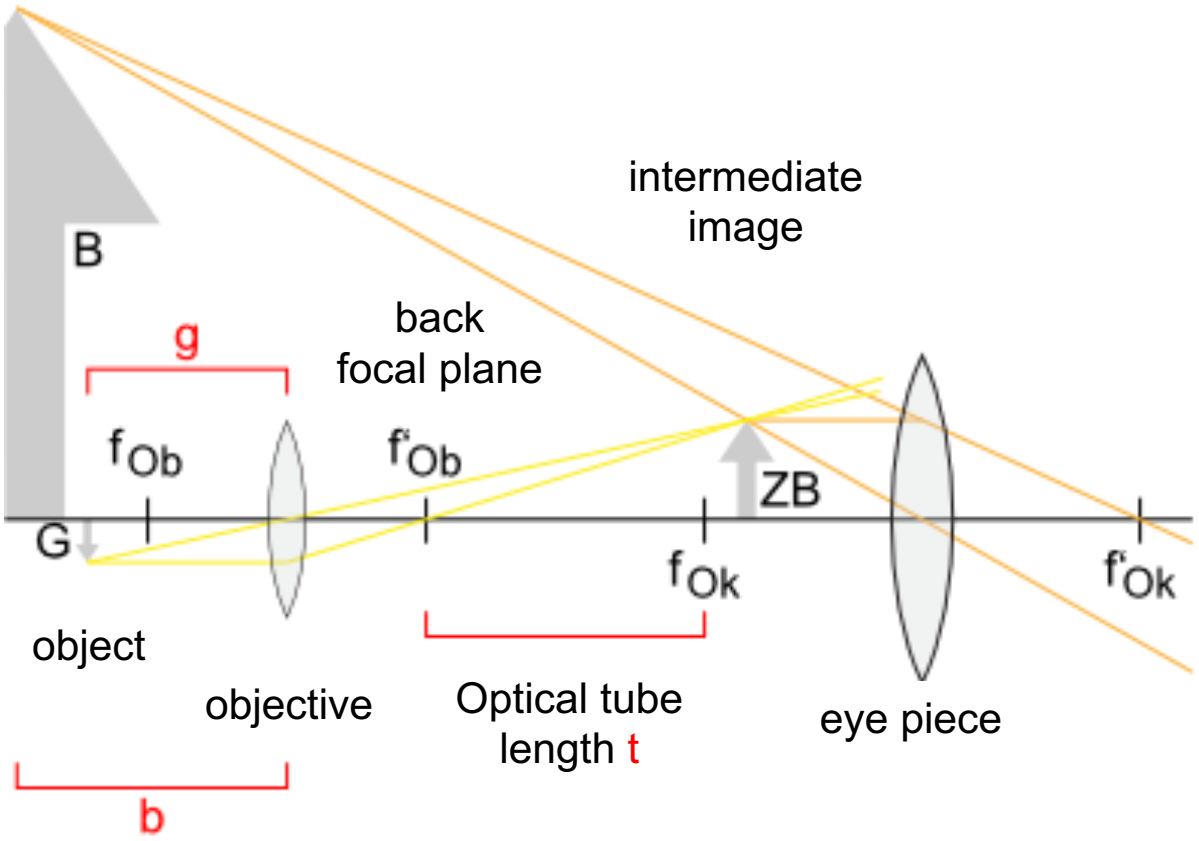
Light path of combined microscope

Combination of two collective lenses

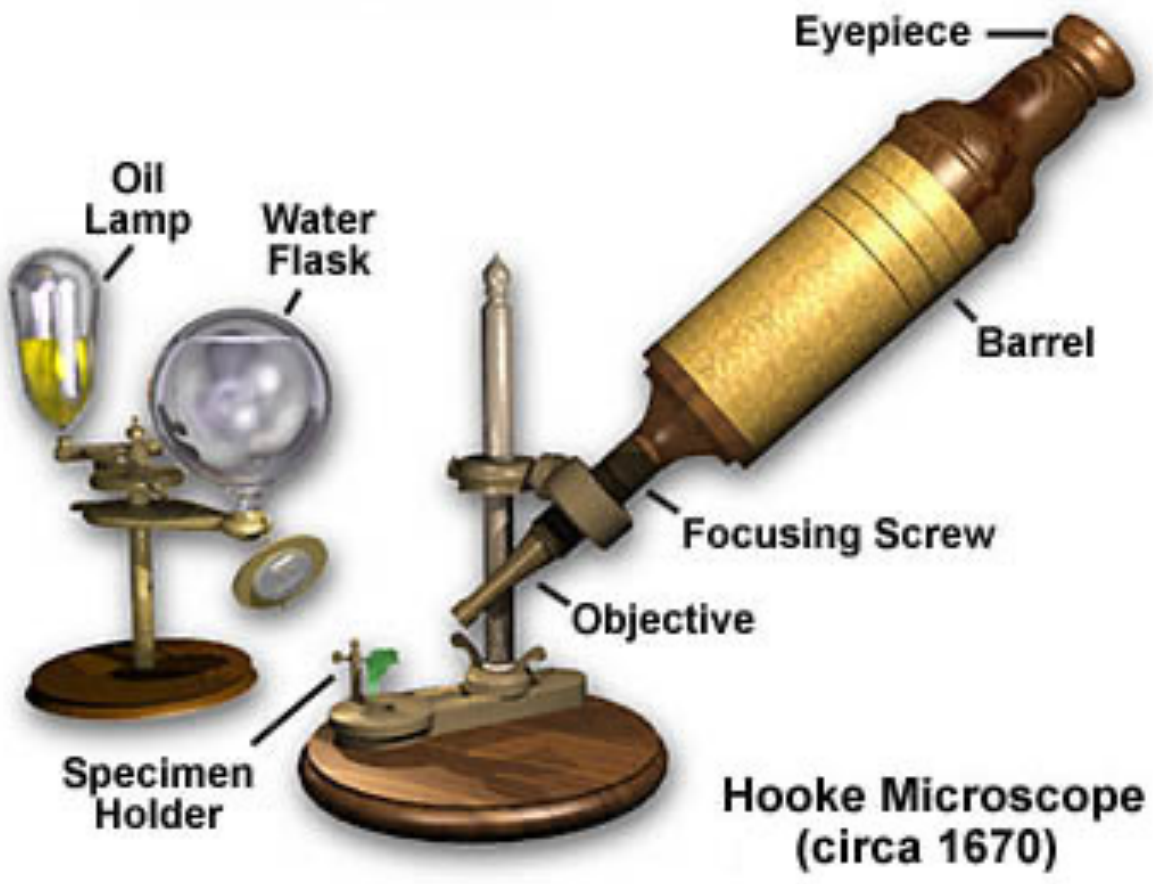


$$M_{total} = M_{objective} \times M_{eye\ piece}$$

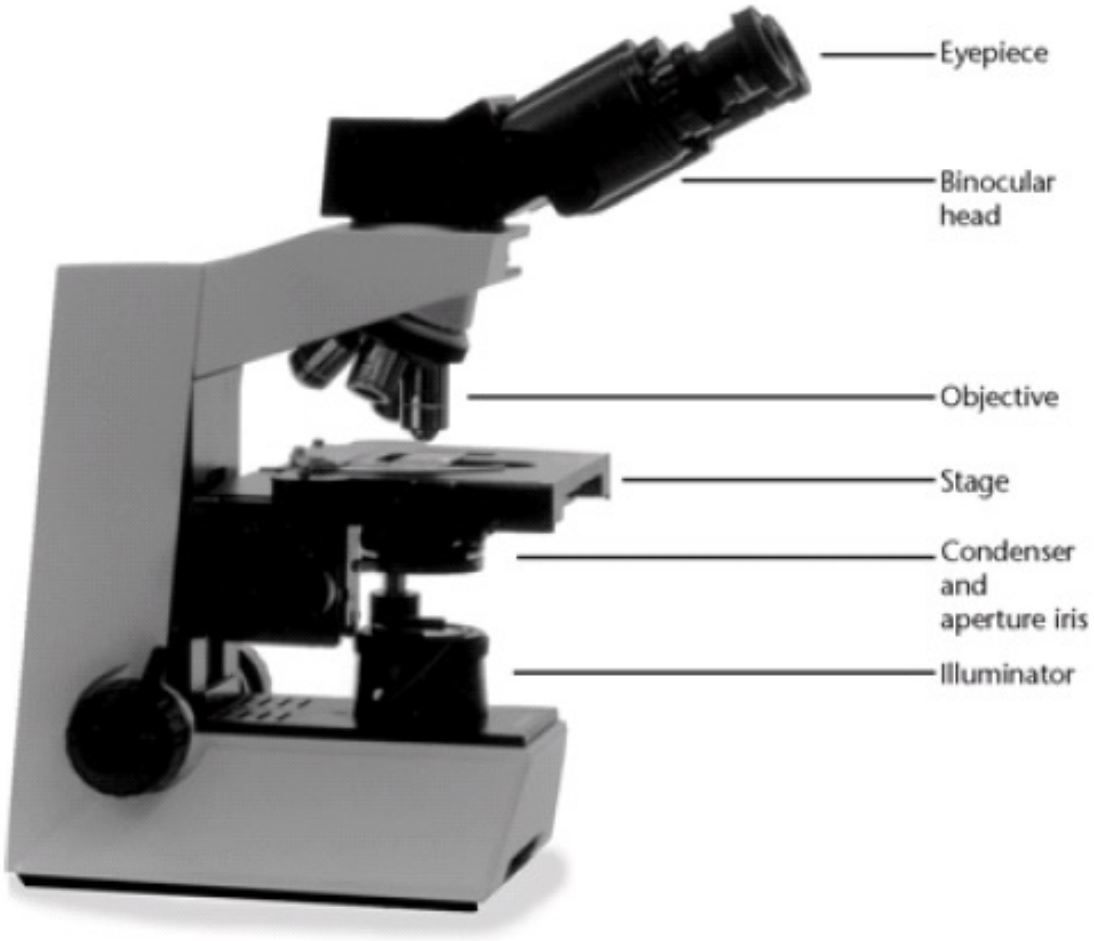
Imaging light path of an optical microscope



Setup of (historical) combined microscope

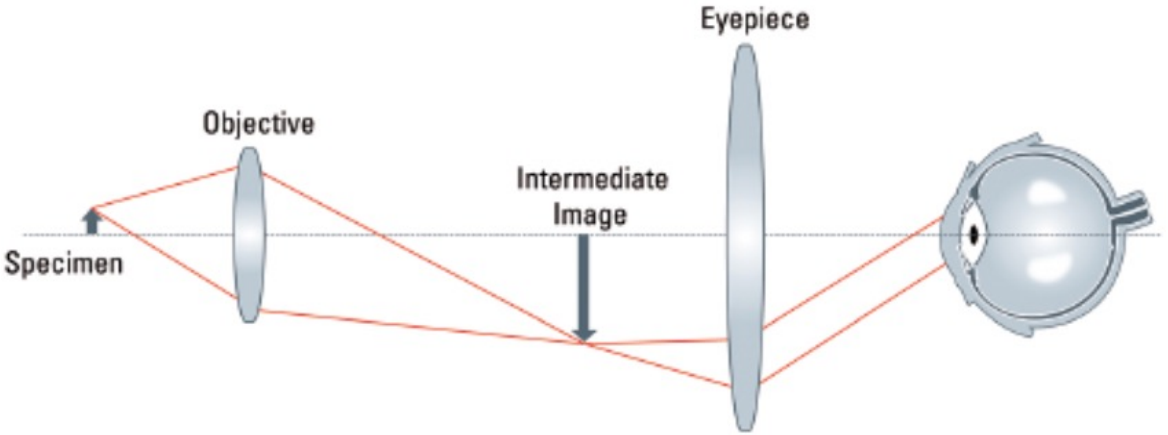


Setup of (modern) combined microscope

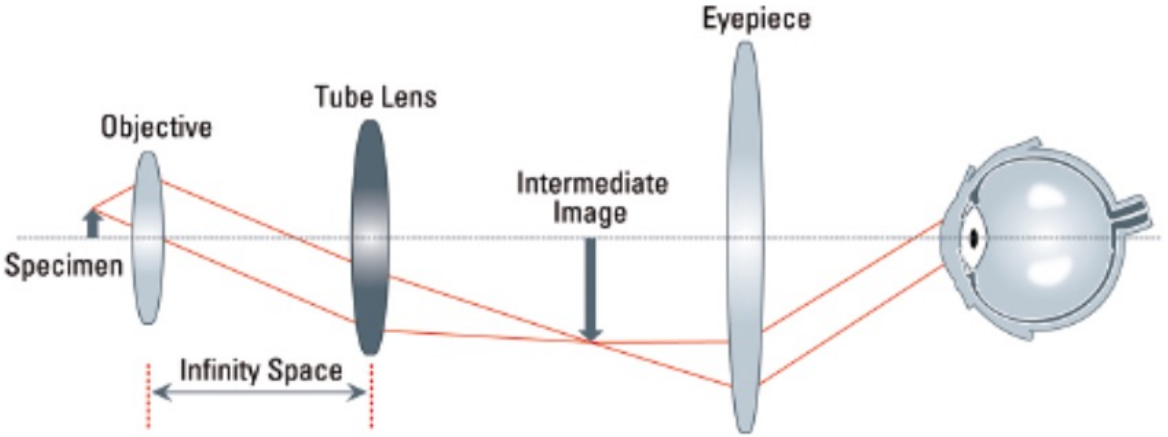


Modern microscopes are infinity corrected

finite optical system

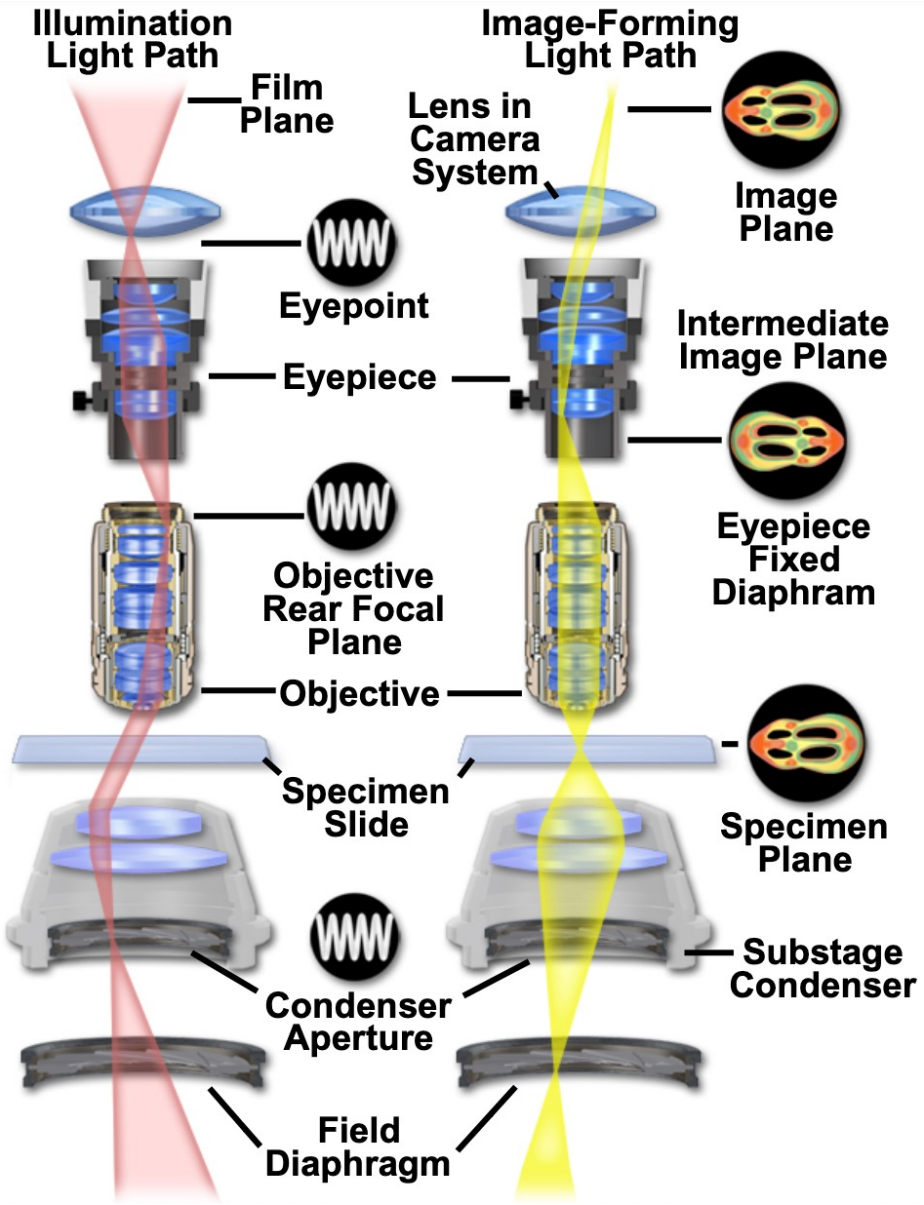


infinity-corrected optical system



Conjugate planes in an optical microscope

=> Köhler illumination



Bright-field microscopy

Light from the condenser passes through sample (transmission mode), is attenuated by absorbing materials and collected by the objective

Total magnification (M_{tot}) = $M_{objective}$ x $M_{eyepiece}$

- but there is a fundamental limit of resolution depending only on the objective:
  $\lambda/(2n*\sin\alpha)$ – note: M does *not* appear in this equation!

  with λ: wavelength of light
  n: refractive index
  α: half of acceptance cone

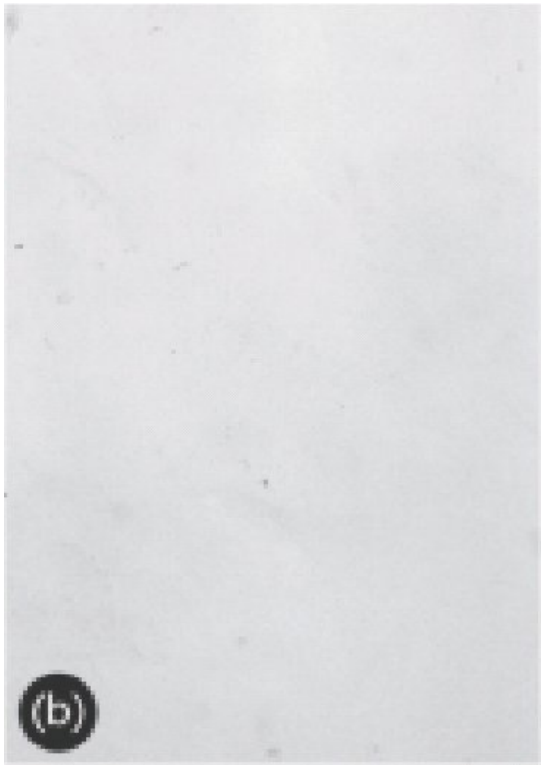
- higher magnifications are called empty magnification

- The objective forms an image in the the intermediate image plane that contains all information on the specimen accessible by the microscope! Any further image magnification by eyepiece or camera lenses only changes the size for easier observation or to fit the camera chip, but does not add any information.

=> The resolution and brightness/contrast of an objective are essential

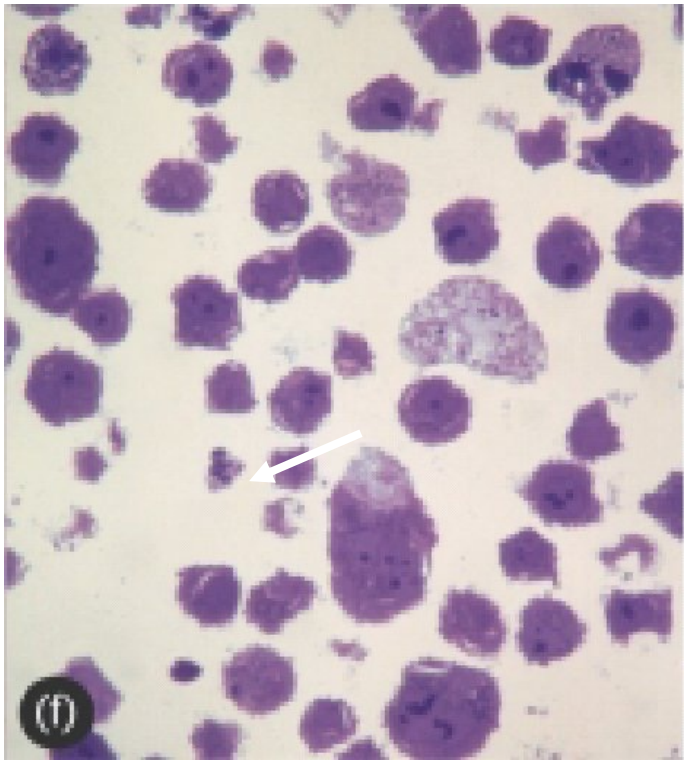
Standard (bright field) microscopy

cell sample



=> poor contrast because cells are
70% water
15% proteins
6% RNA
+ smaller amounts of others

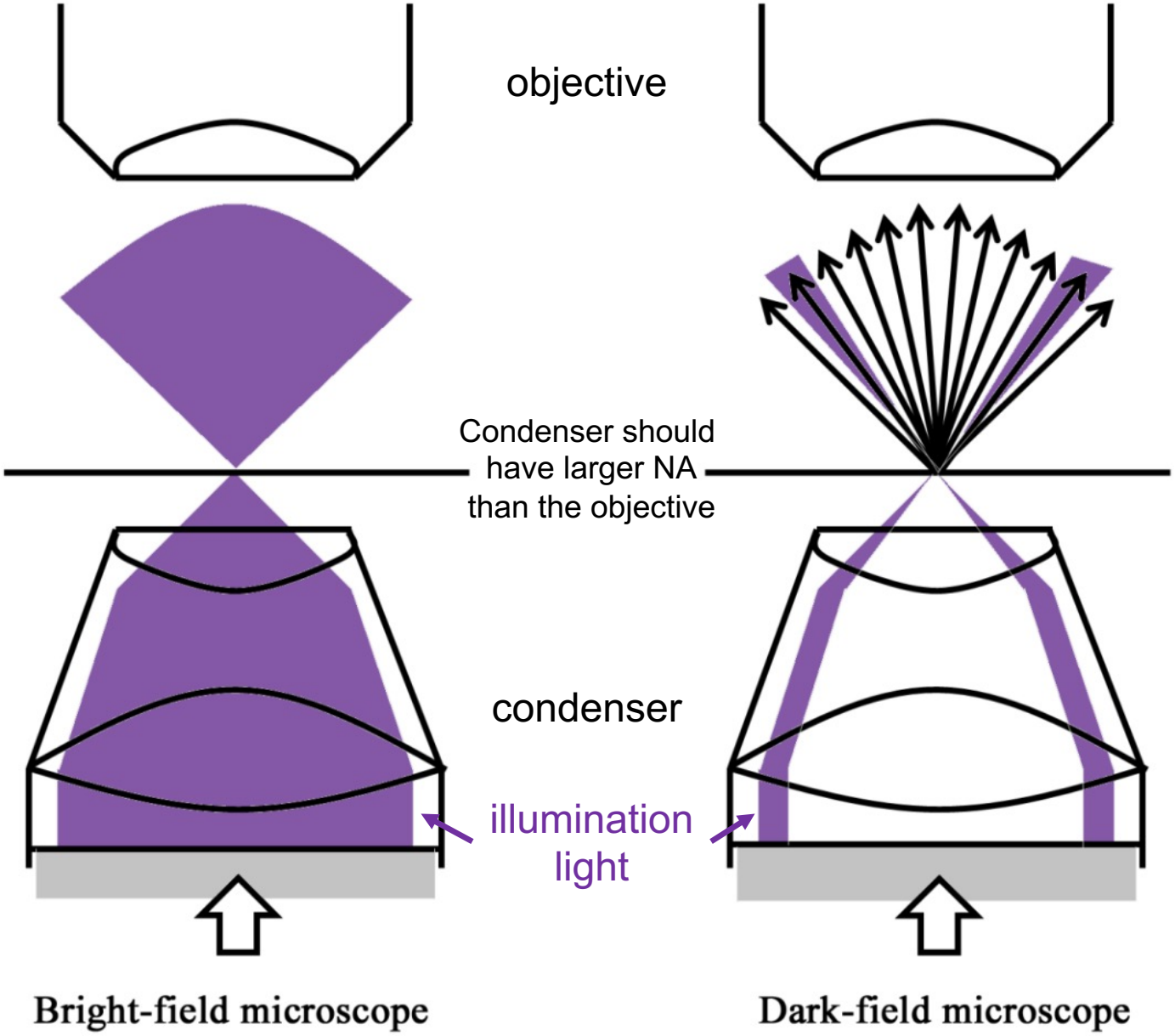
stained cells => higher contrast



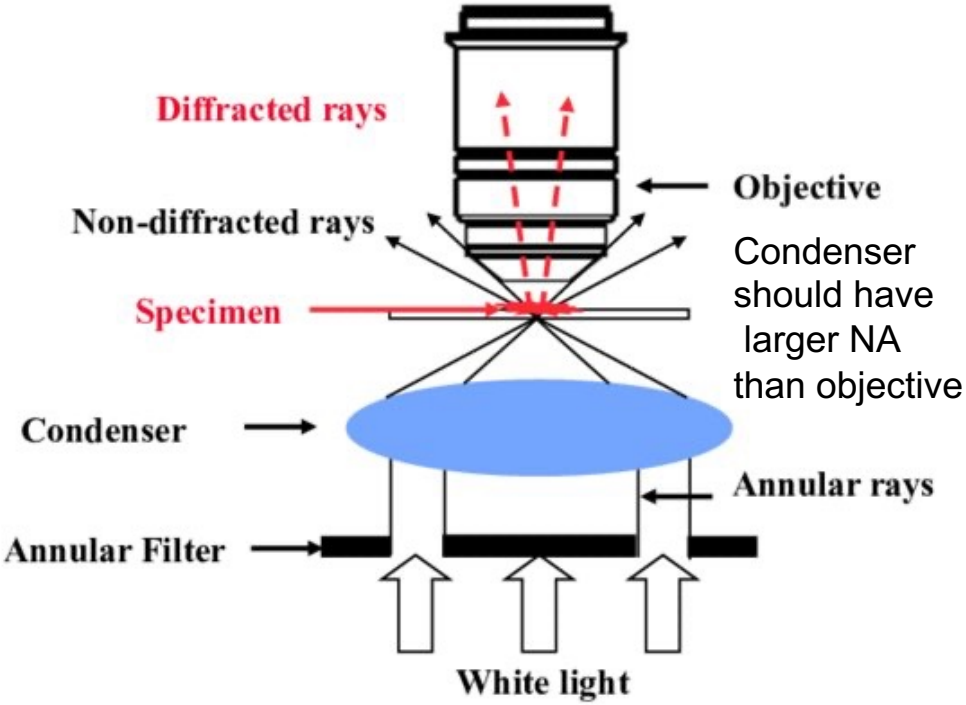
examples:
- Gram-staining (bacteria)
- Stained tissues (histology)

but: fixing/staining kills cells

Bright-field vs. Dark-field microscopy



Dark-field microscopy



Amphipod crustacean (25x magnification)

Dark-field microscopy prevents non-diffracted light from entering the objective. Only light rays diffracted by the specimen are collected by the objective. Thus, a bright image appears against a **dark background**, resulting in a much better image contrast compared to bright-field microscopy.
=> Enables observation of living cells/organisms.

In biology, dark-field microscopy has been replaced by improved techniques, but it has recently reemerged for the analysis of strongly light scattering (plasmonic) nanomaterials.