MUNI
SCI

C8116 Immunochemical techniques

Immunoassays II

Spring term 2024

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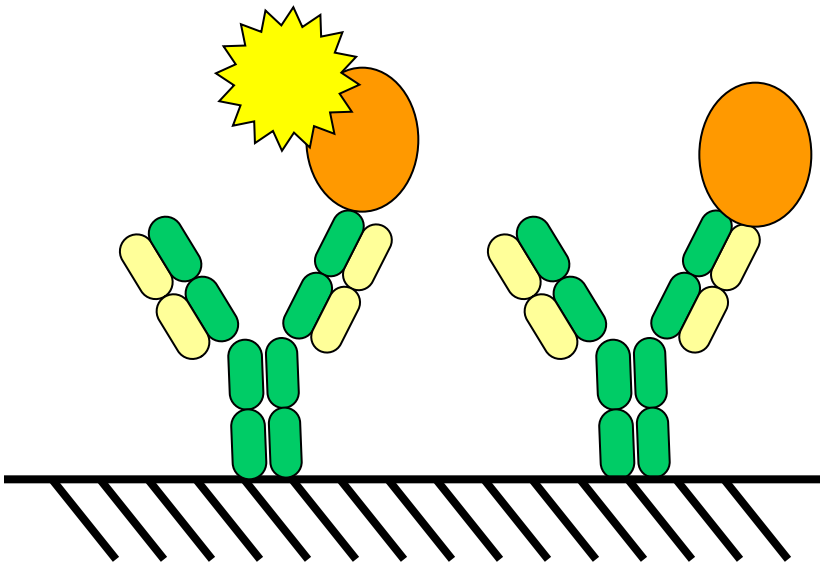
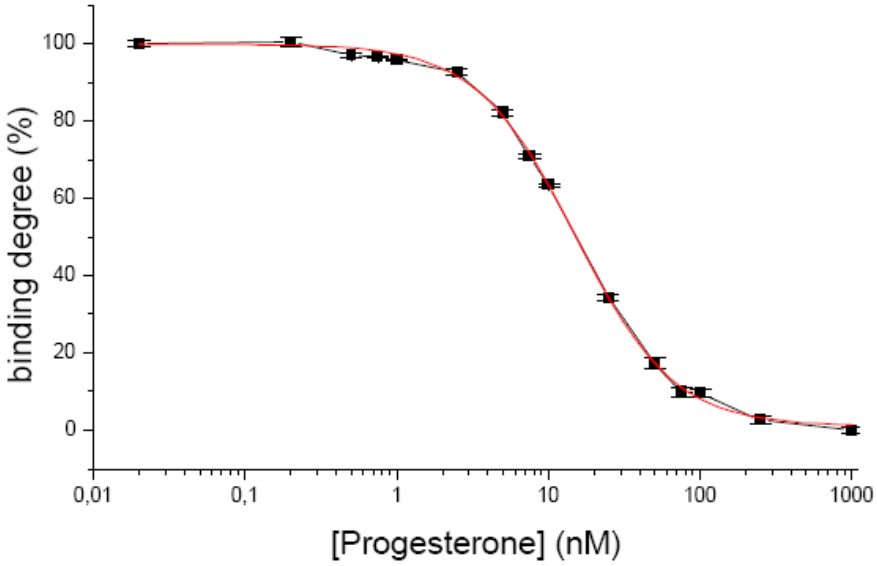
April 16th, 2024

Competitive immunoassay

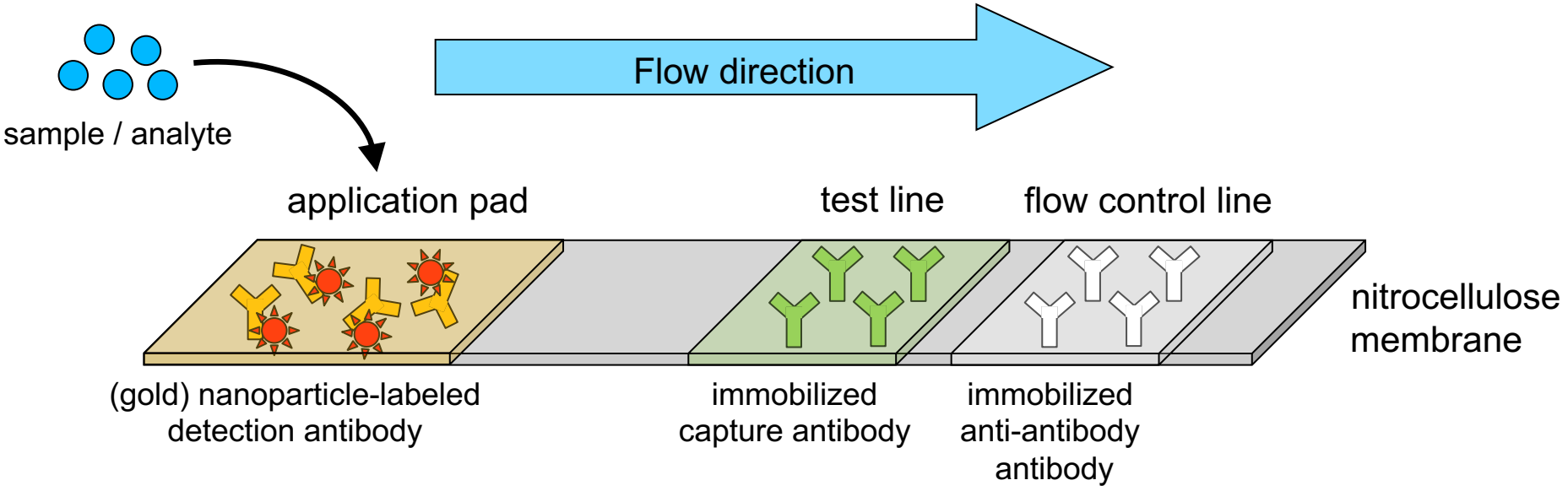




Note: The sandwich ELISA is not applicable to small molecules such as steroid hormones (e.g. progesterone), because they do not possess <u>two</u> epitopes for binding both the capture Ab and the detection Ab.

Lateral flow assay

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



Digital (single-molecule) assays

based on:

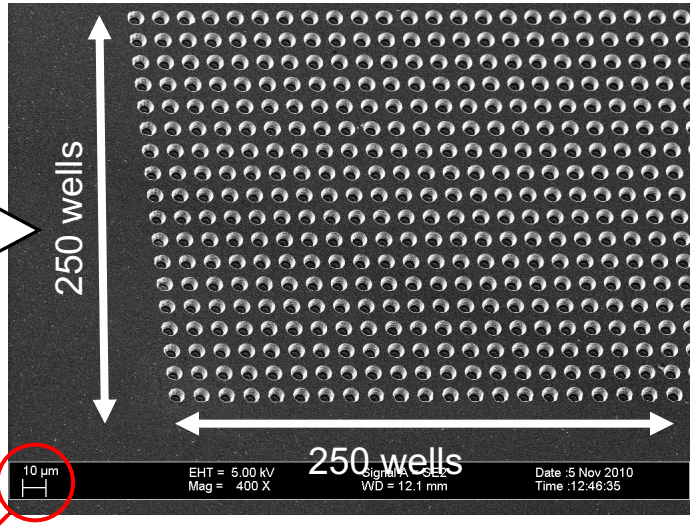
- life-time luminescence
- upconversion nanoparticles
- enzyme labels
- fluorescent labels

Minimizing the size of wells

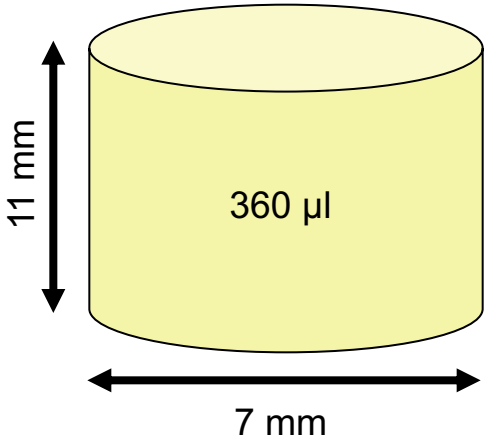
Microtiter plate (96 wells)

Increasing the number of wells by a factor of 10^3

Femtoliter array (62.500 wells)

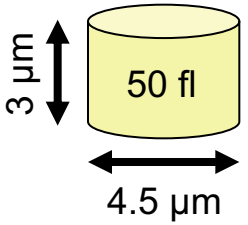


SEM: scale bar (10 µm)



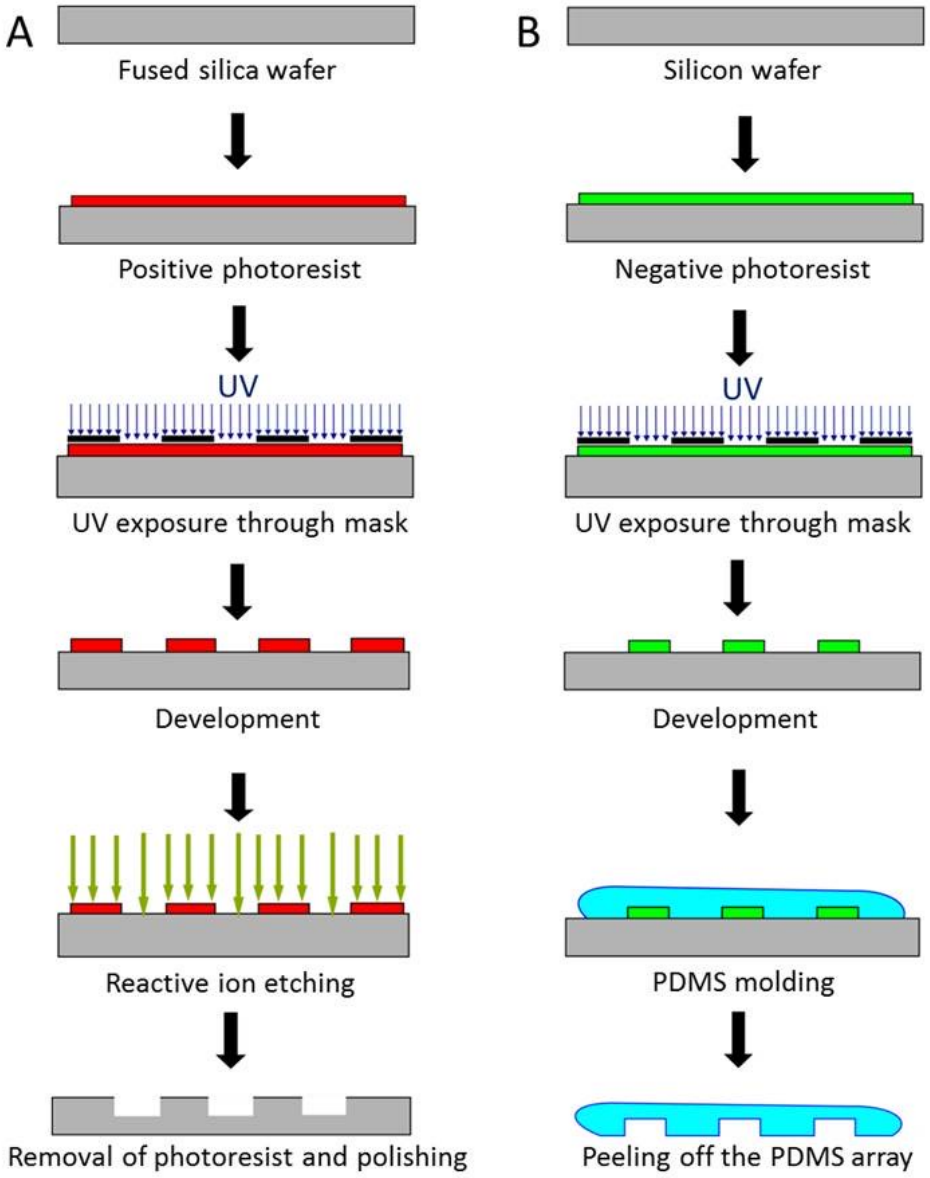
Microtiter plate well

Reduction of the volume by a factor of 10^{10}

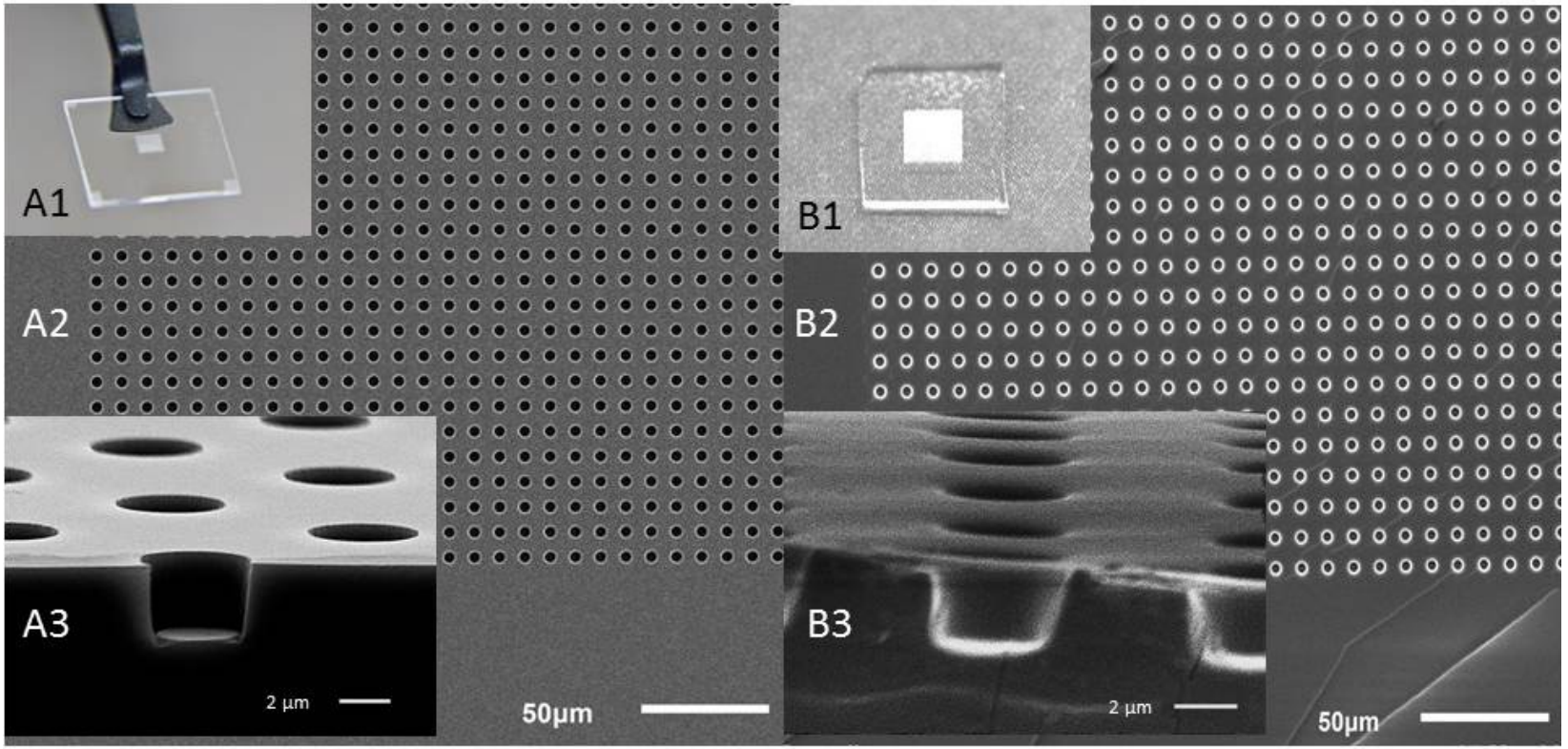


Femtoliter well

Femtoliter arrays generated by photolithography



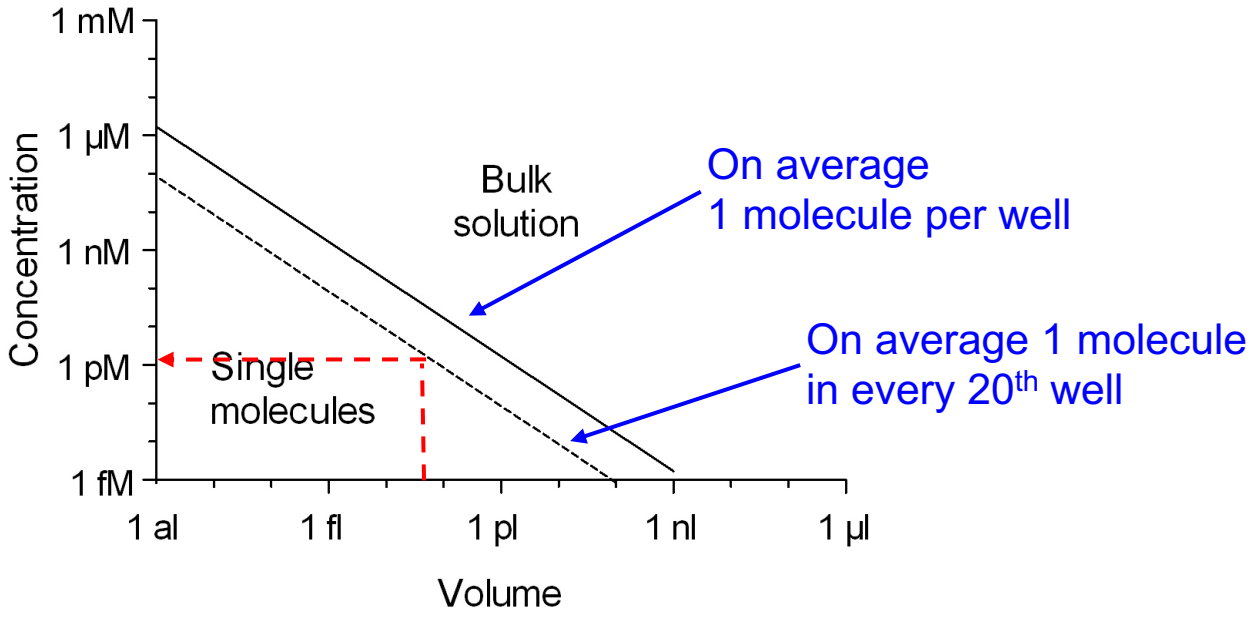
SEM images of femtoliter arrays



Isolation of single molecules in femtoliter arrays

Expected number of molecules in a given volume:

Volume			1 µM	1 nM	1 pM
$(1\ mm)^3$	1 µL	10^{-6} L	6×10^{11}	6×10^{8}	6×10^{5}
$(100\ \mu m)^3$	1 nL	10^{-9} L	6×10^{8}	6×10^{5}	6×10^{2}
$(10\ \mu m)^3$	1 pL	10^{-12} L	6×10^{5}	6×10^{2}	< 1
$(1\ \mu m)^3$	1 fL	10^{-15} L	6×10^{2}	< 1	
$(100\ nm)^3$	1 aL	10^{-18} L	< 1		



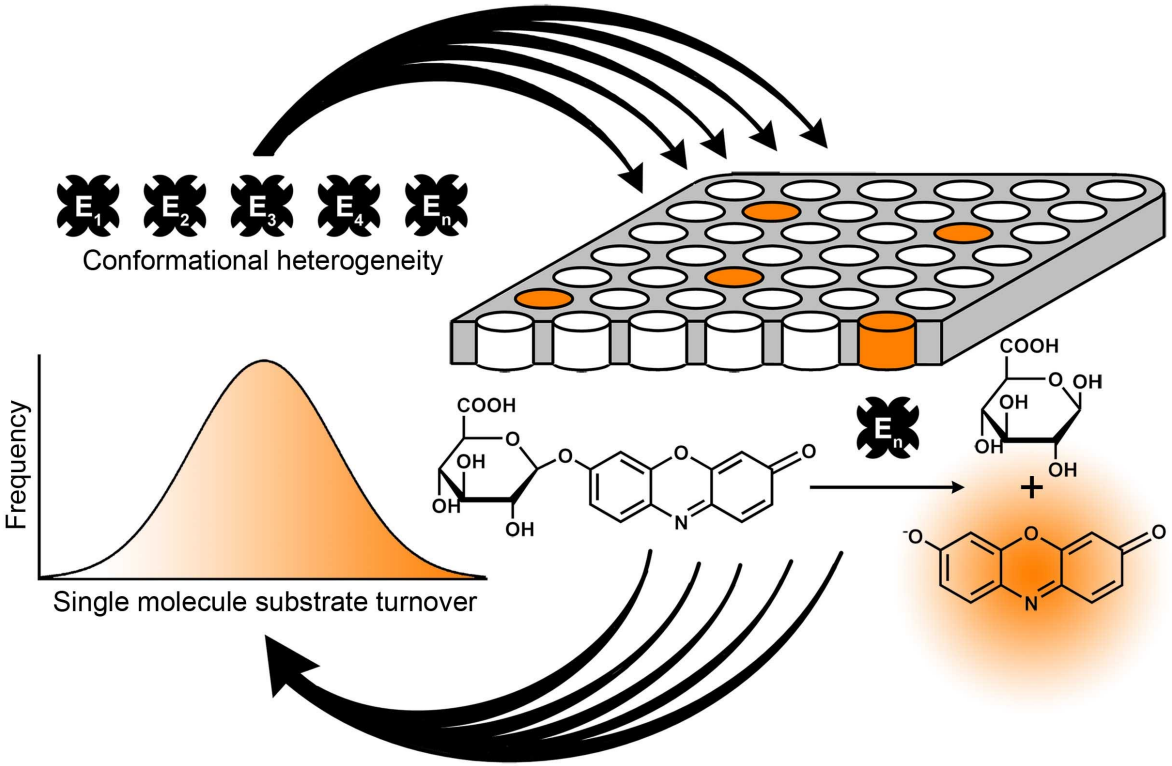
Here:
Volume of well: 40 fL
Enzyme conc.: 1.8 pM
~5 % of the wells contain a single enzyme molecule

Poisson distribution:

$$P_\mu(v) = e^{-\mu}\frac{\mu^v}{v!}$$

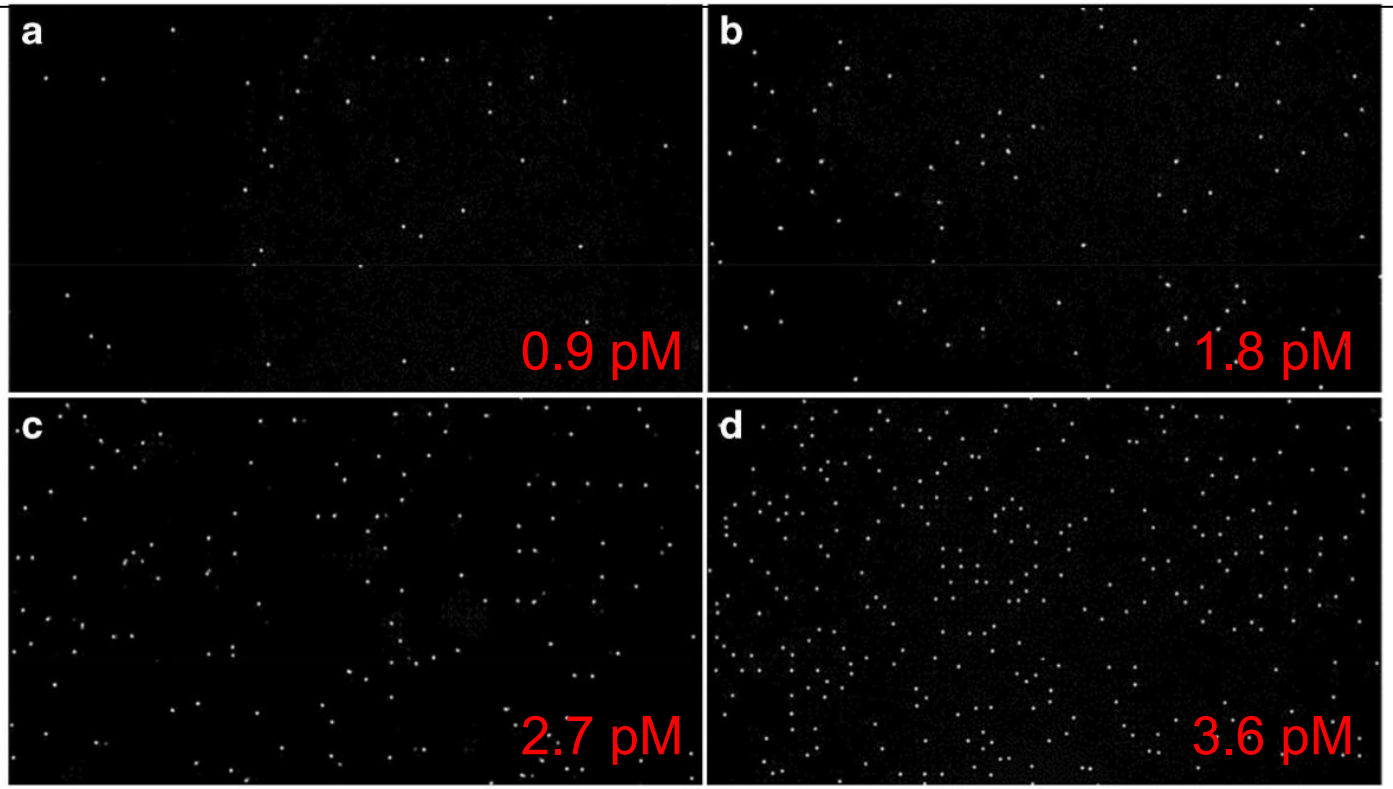
with:
μ = average occupancy (0.05)
$P_\mu(v)$ = probability of finding exactly v (i.e. 0,1,2,3 …) molecules in any given well

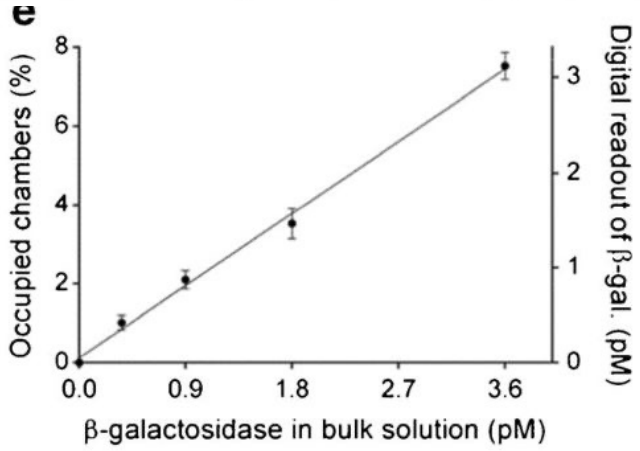
Single enzyme molecule reaction



Observing single enzyme molecules

Counting individual enzyme molecules



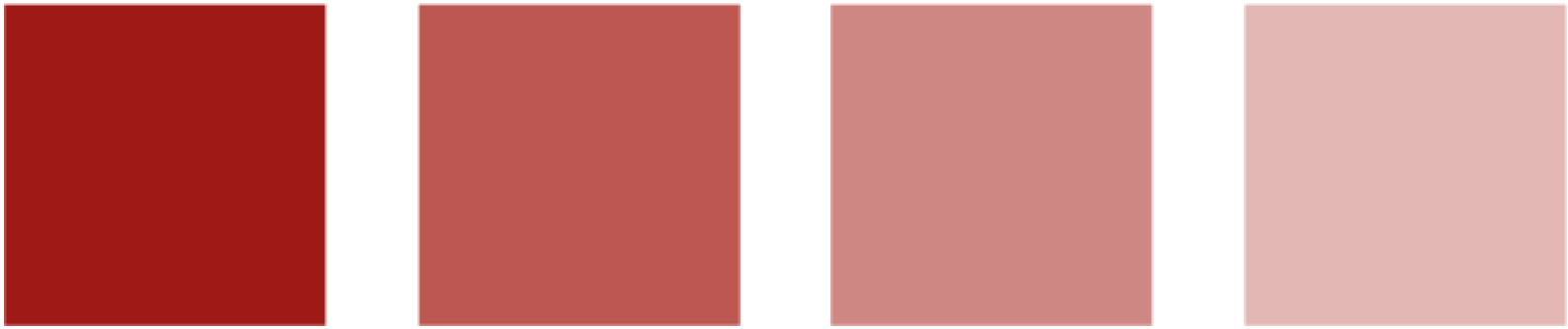


Linear relationship between number of fluorescent chambers and enzyme concentration

Anal. Bioanal. Chem. (2015) **407**, 7443

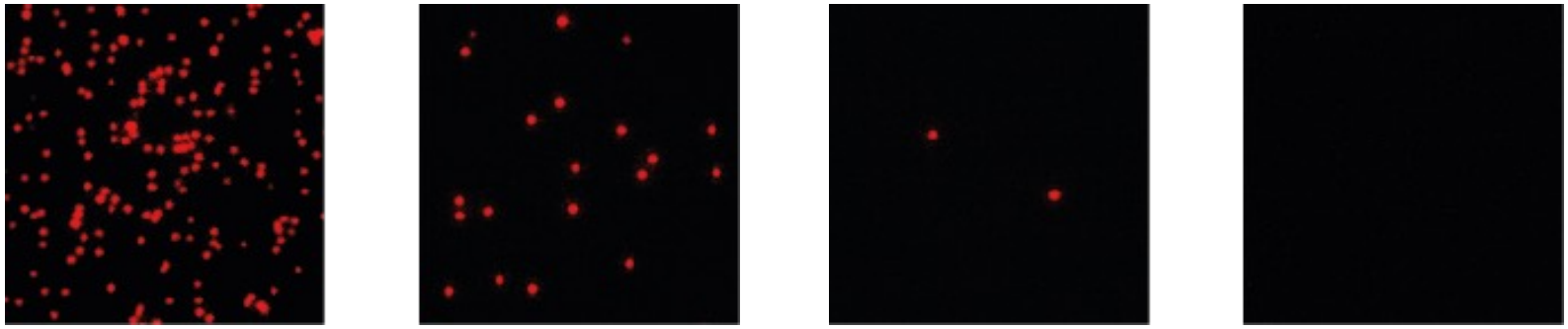
Surpassing the traditional detection limit

Conventional immunoassay (analog readout)



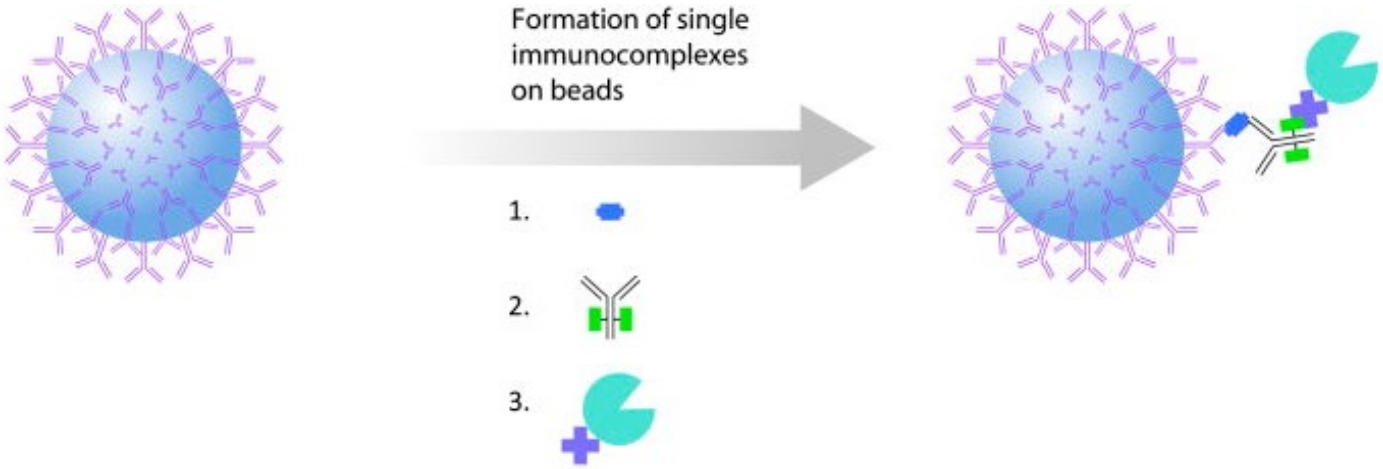
=> Millions of molecules needed to reach detection limit

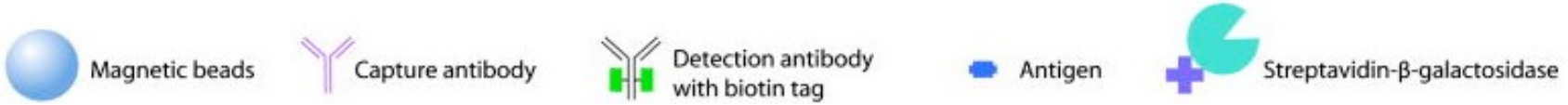
Single-molecule immunoassay (digital readout)



=> One molecule needed to reach detection limit

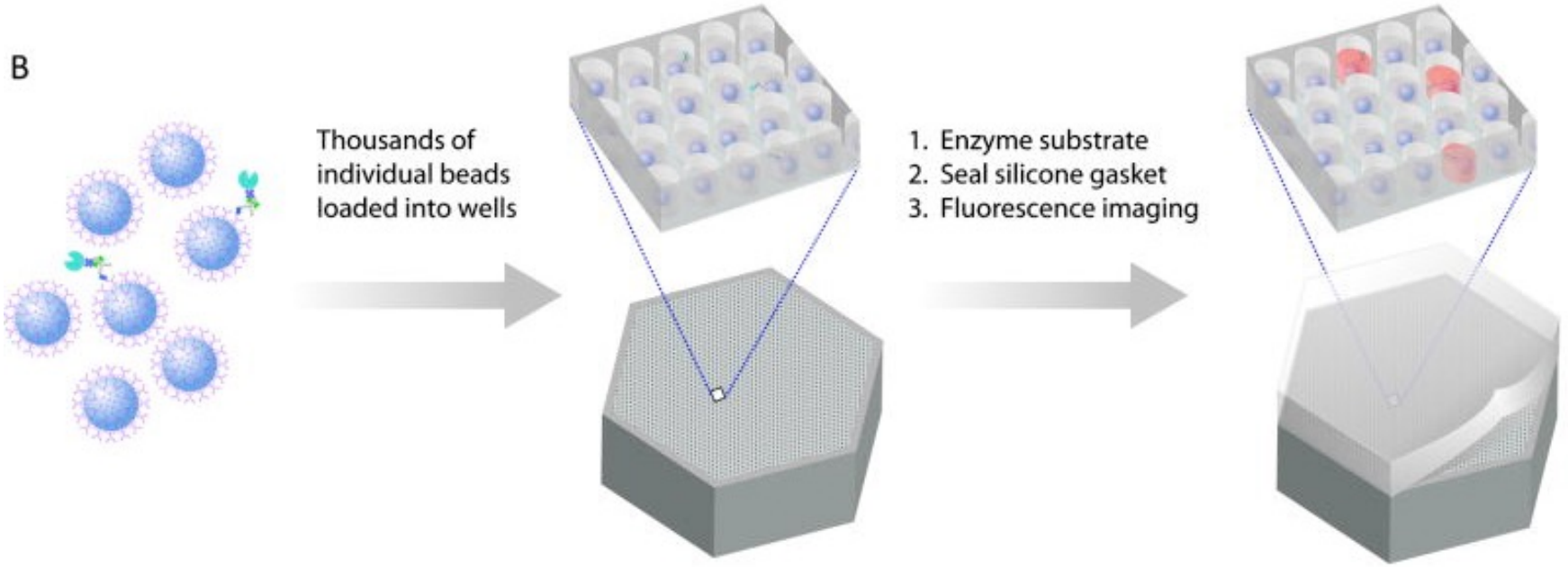
Single-molecule ELISA on beads (Quanterix)

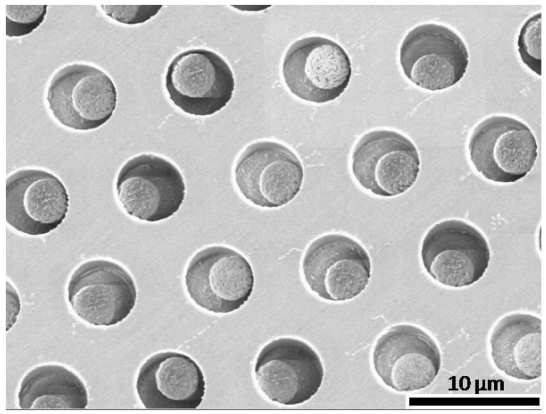


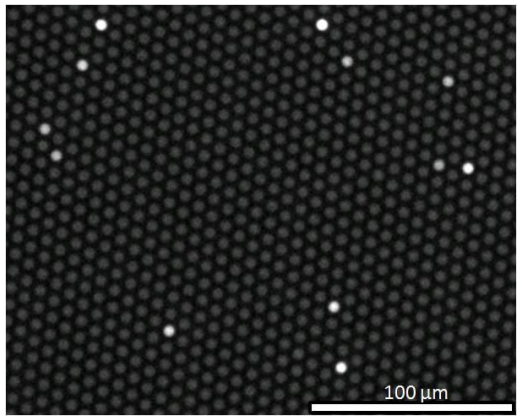


Nature Biotechnology (2010) **28**, 595

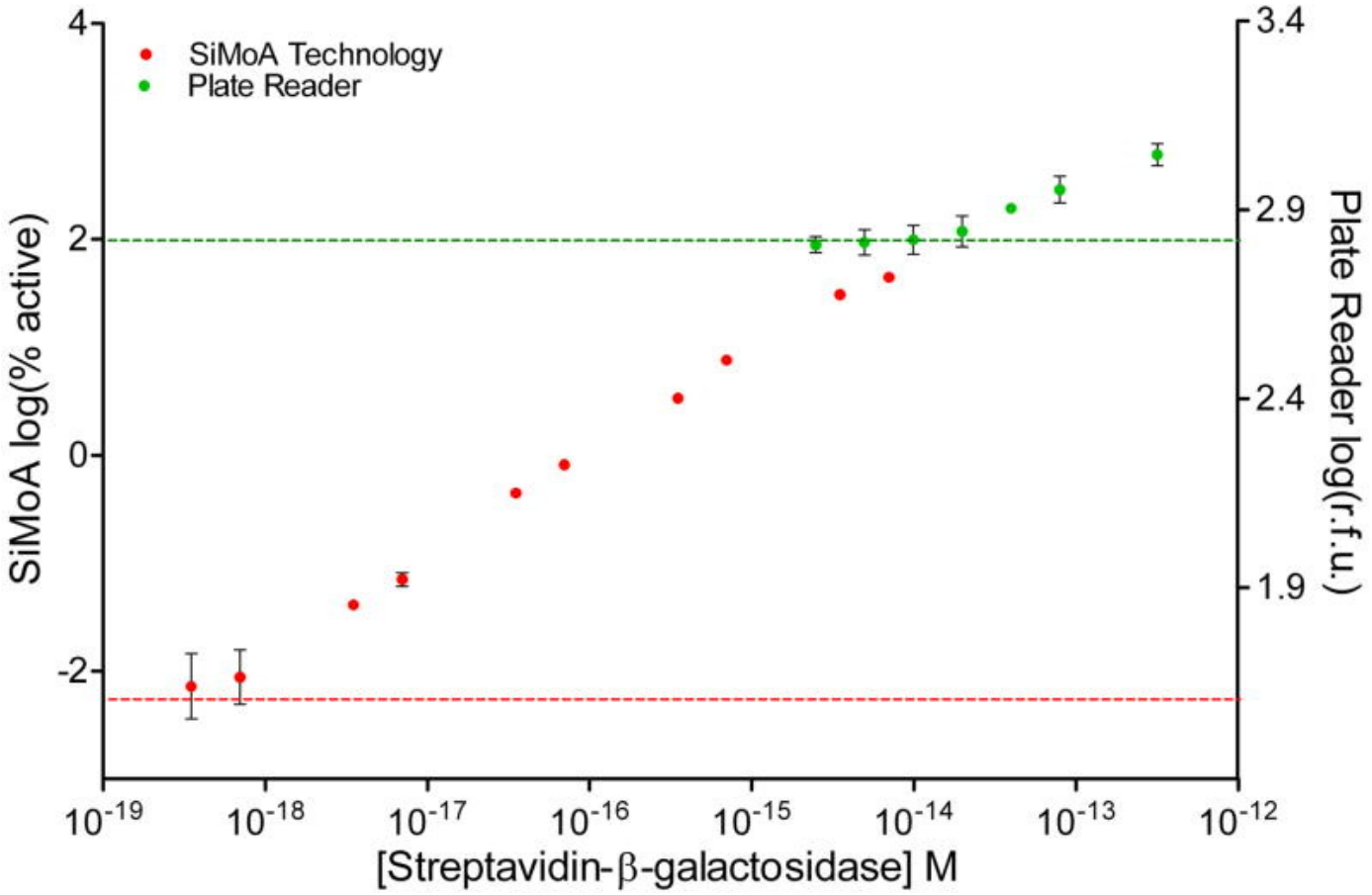
Single-molecule ELISA on beads (Quanterix)





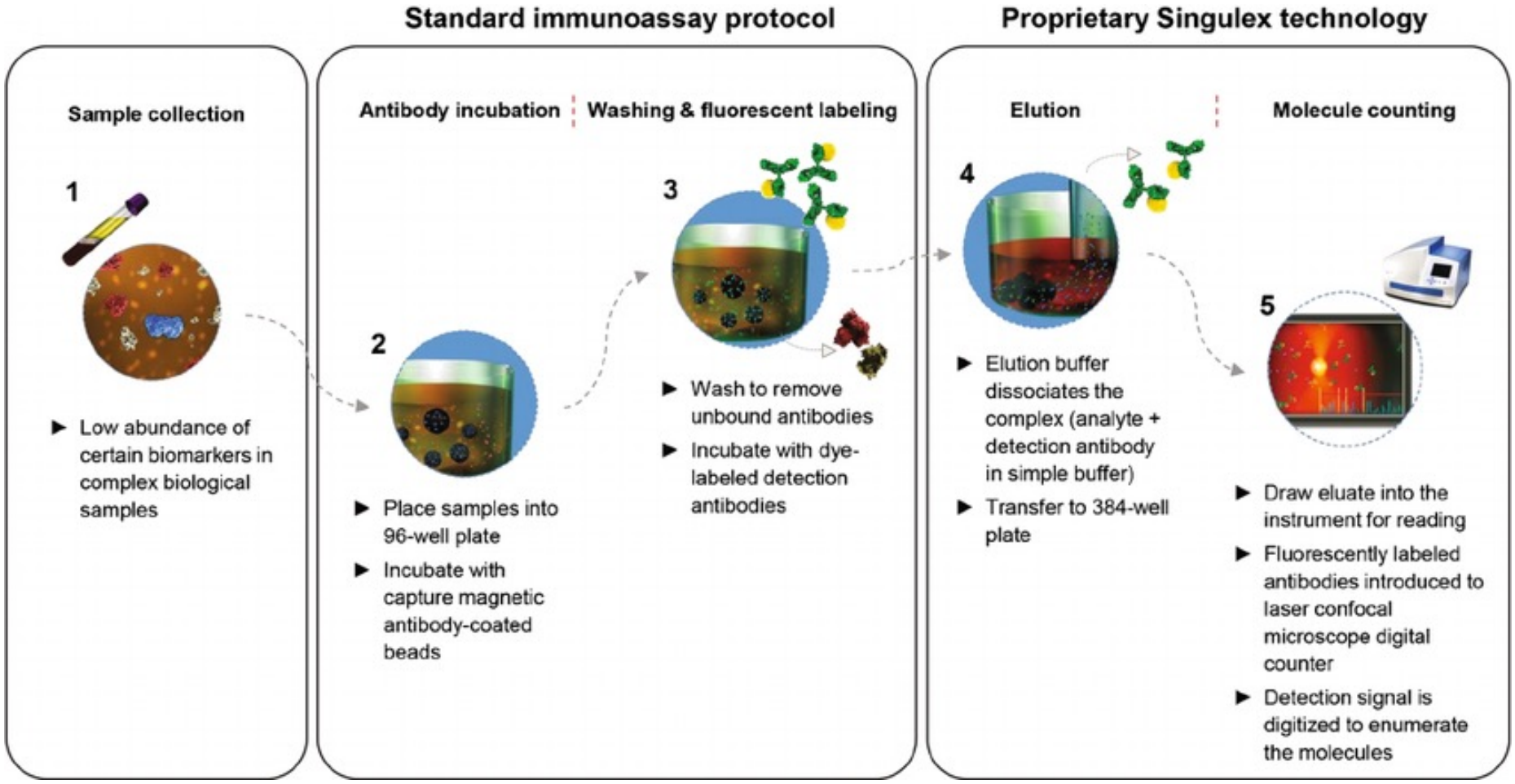


Single-molecule ELISA on beads (Quanterix)



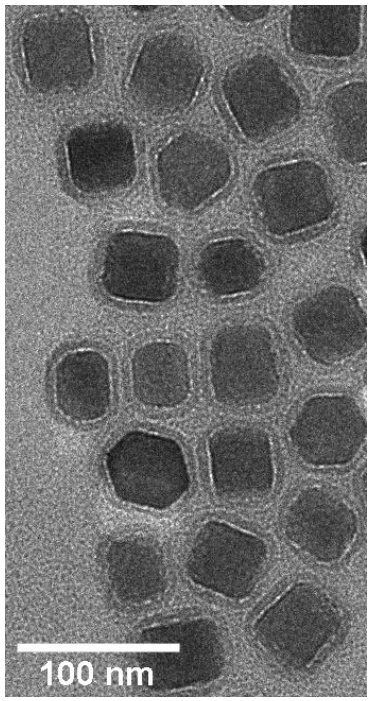
=> Digitization of enzyme-linked complexes greatly increases sensitivity compared with bulk, ensemble measurements.

Digital assays: Single fluorophore counting (Singulex)



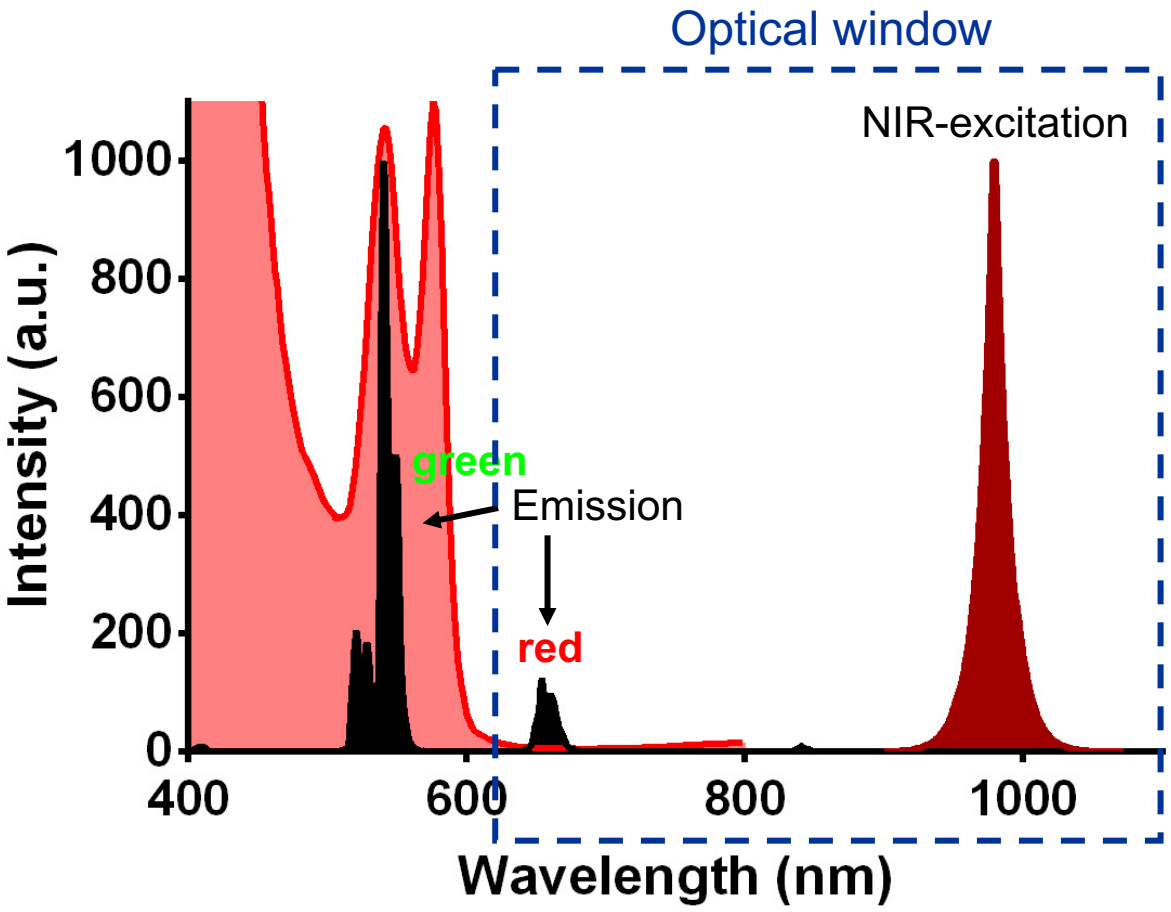
=> A separtion between antigen capture and detection is required to avoid optical background interference.

UCNPs as background-free optical labels



TEM of UCNPs
$NaYF_4$:Yb,Er

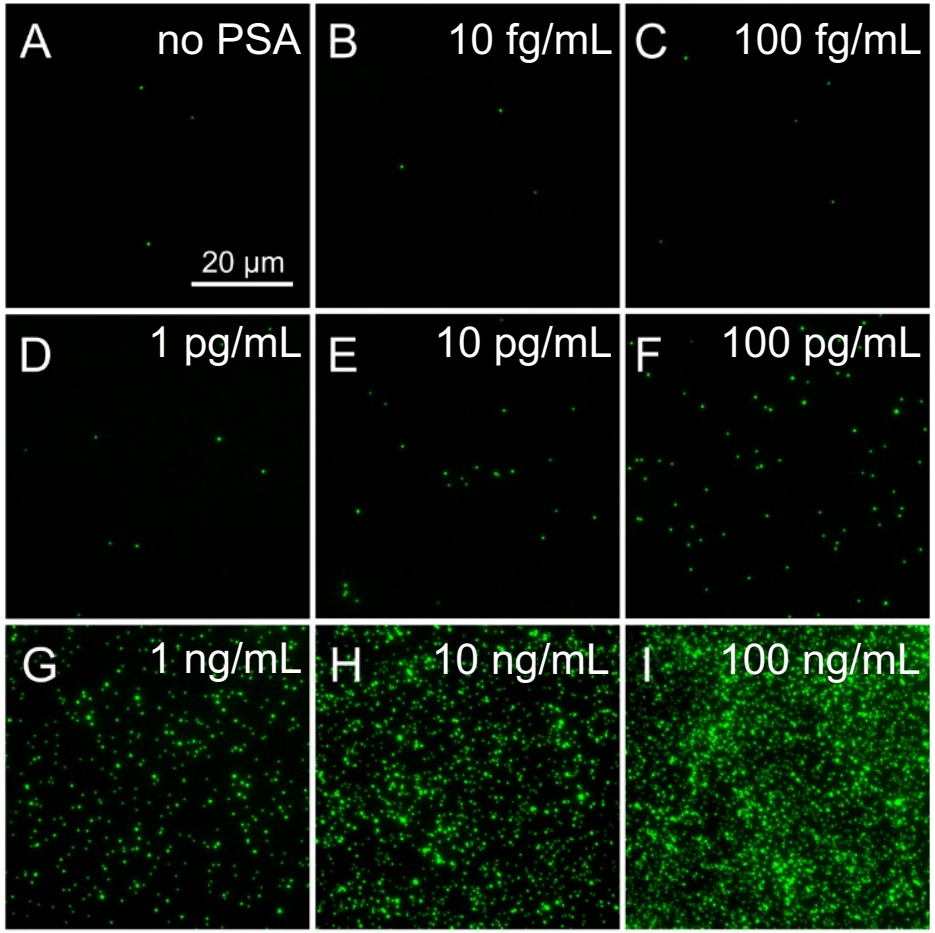
Hexagonal
crystal structure

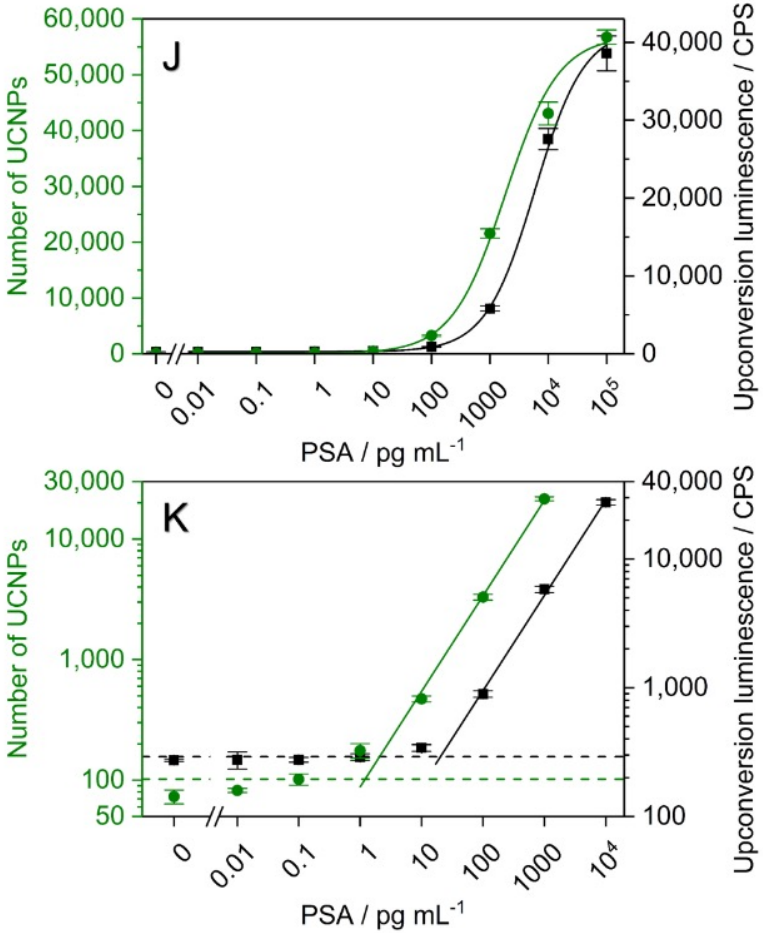


No autofluorescence
Very low light scattering
} Background-free imaging

... and completely photostable

UCNPs for digital assays





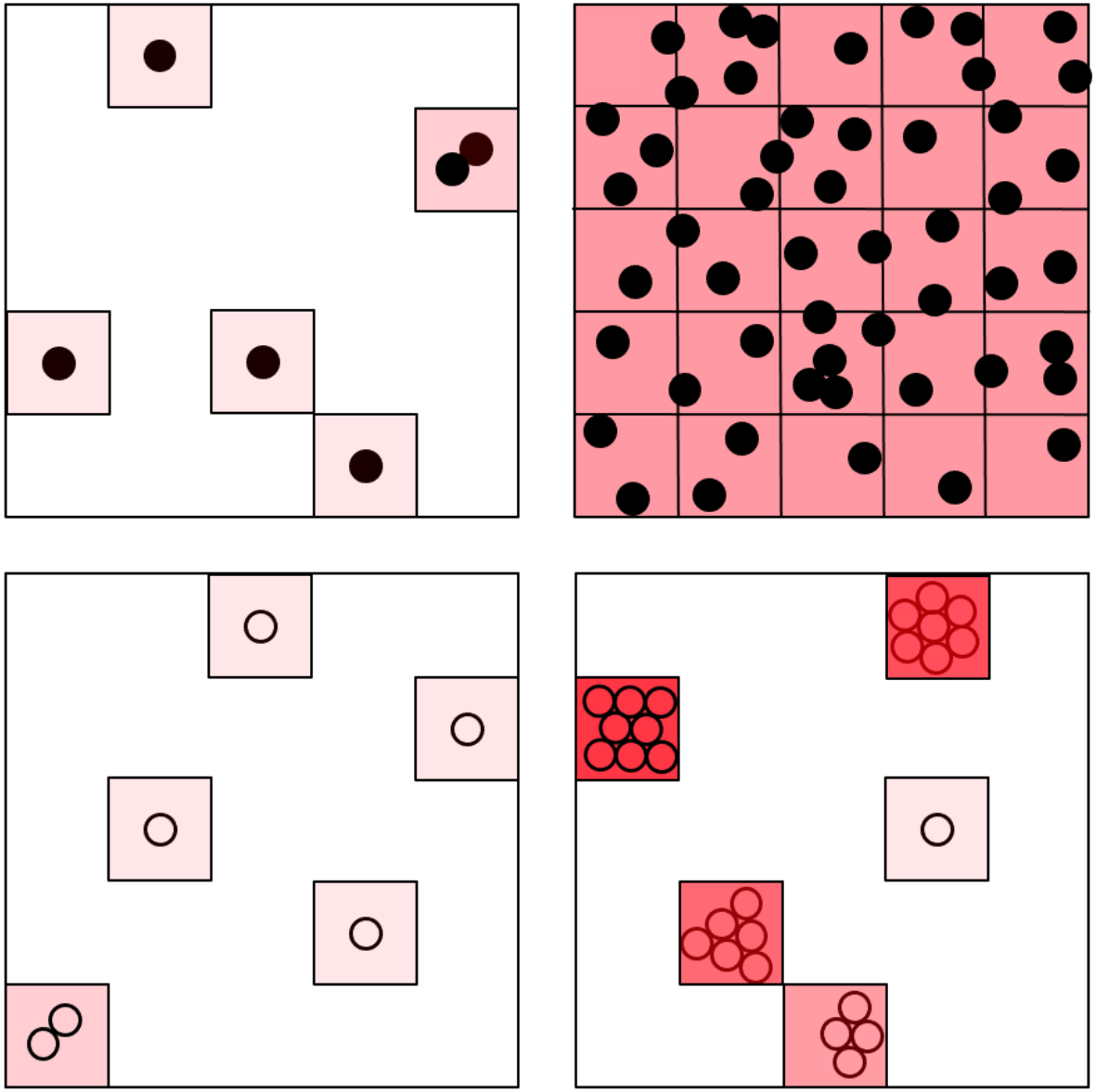
Single UCNPs are detectable as diffraction-limited spots
Excitation power: ~640 W/cm^2

Anal. Chem. (2017) 89, 11825

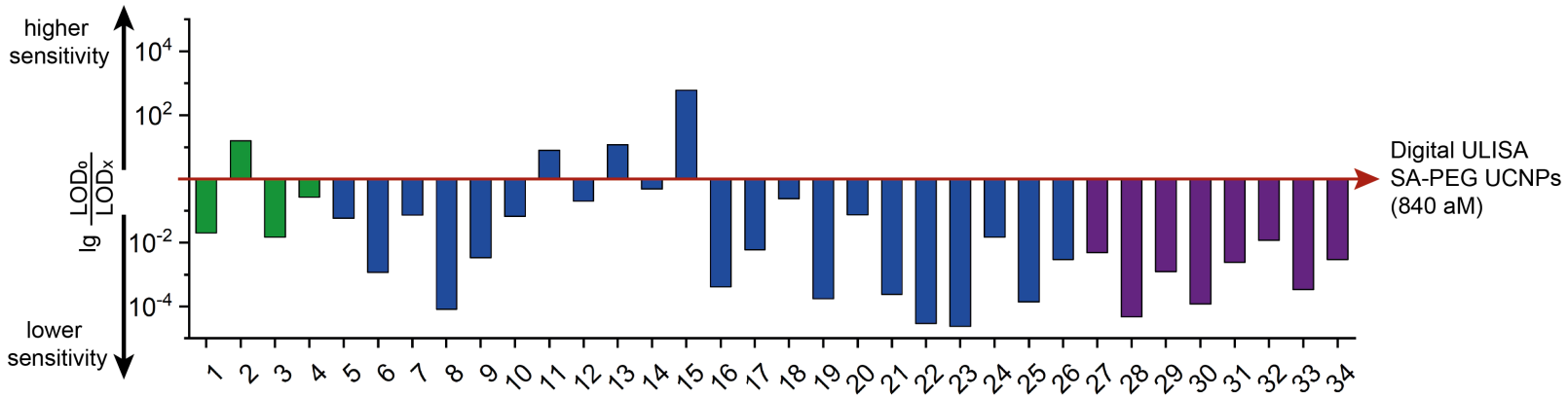
Analog vs. digital readout

specific binding

non-specific binding



Detection limits of various immunoassays



Digital Assays

1 Digital ULISA Ab-silica UCNPs (42 fM)

2 Digital ELISA in femtoliter arrays (52 aM)

3 Single-particle time-resolved fluorescence (50 fM)

4 Singulex Erenna (single molecule counting in capillaries) (3.9 fM)*
*LOD for cardiac troponin 1

Analog Assays

5 Analog ULISA SA-PEG UCNPs (14 fM)

6 Analog ULISA Ab-silica UCNPs (0.7 pM)

7 AuNP-based bio-barcode assay (11 fM)

8 AuNP-enhanced surface plasmon resonance (10 pM)

9 Chemiluminescence imaging immunoassay (0.24 pM)

10 Colorimetric assay with Mesoporous silica NPs (12.5 fM)

11 Electrochemical sensor with Au-Ag-Cu_2O NPs (105 aM)

12 Electrochemical sensor with AuNP hybrid nanomaterial (4.2 fM)

13 Electrochemical sensor with peptide-DNAzyme conjugates (70 aM)

14 Electrochemiluminescence immunoarray (1.7 fM)

15 Electrochemiluminescence with conductive nanospheres (1.4 aM)

16 Electrochemiluminescence with MOF/Au/G-Quadruplexes (2 pM)

17 Immuno-PCR (0.14 pM)

18 Localized SPR (3.5 fM)

19 Microbead-based immunoassay (4.7 pM)

20 Photoelectrochemistry with rolling circle amplification (11 fM)

21 Plasmon excited quantum dots (3.5 pM)

22 Quantum dot-based FRET immunoassay (28 pM)

23 Quantum dot-encoded microbeads (35 pM)

24 Time-resolved fluorescence (56 fM)

25 Multianalyte microarray (5.9 pM)

Commercial Assays

26 Abcam ab113327 (0.28 pM)

27 Abcam ab188389 (0.17 pM)

28 Biorbyt orb339660 (17 pM)

29 LifeSpan BioSciences LS-F25971 (0.67 pM)

30 LifeSpan BioSciences LS-F5207 (7.0 pM)

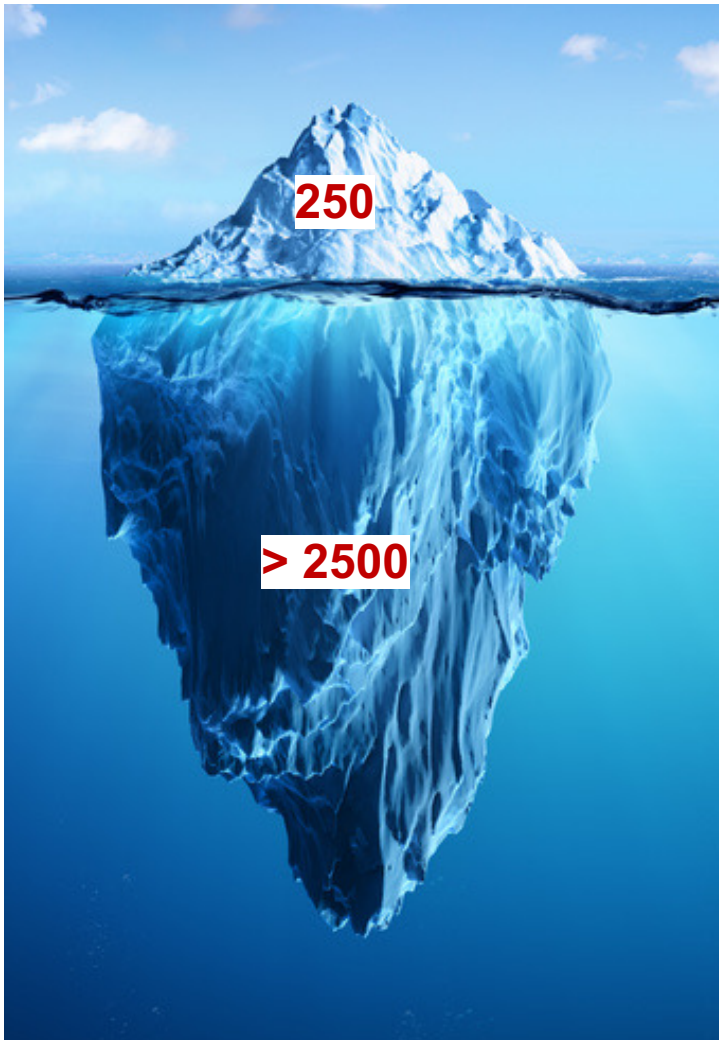
31 OriGene EA100514 (0.35 pM)

32 Roche Elecsys total PSA (0.07 pM)

33 R&D Systems DKK300 (2.4 pM)

34 Thermo Fisher Scientific EHKLK3T (0.28 pM)

We only see the tip of the iceberg



10^{-3} M

Current protein blood tests (ELISA)

10^{-12} M

Large unused potential of diagnostic biomarkers
(human genome: 25000 genes)

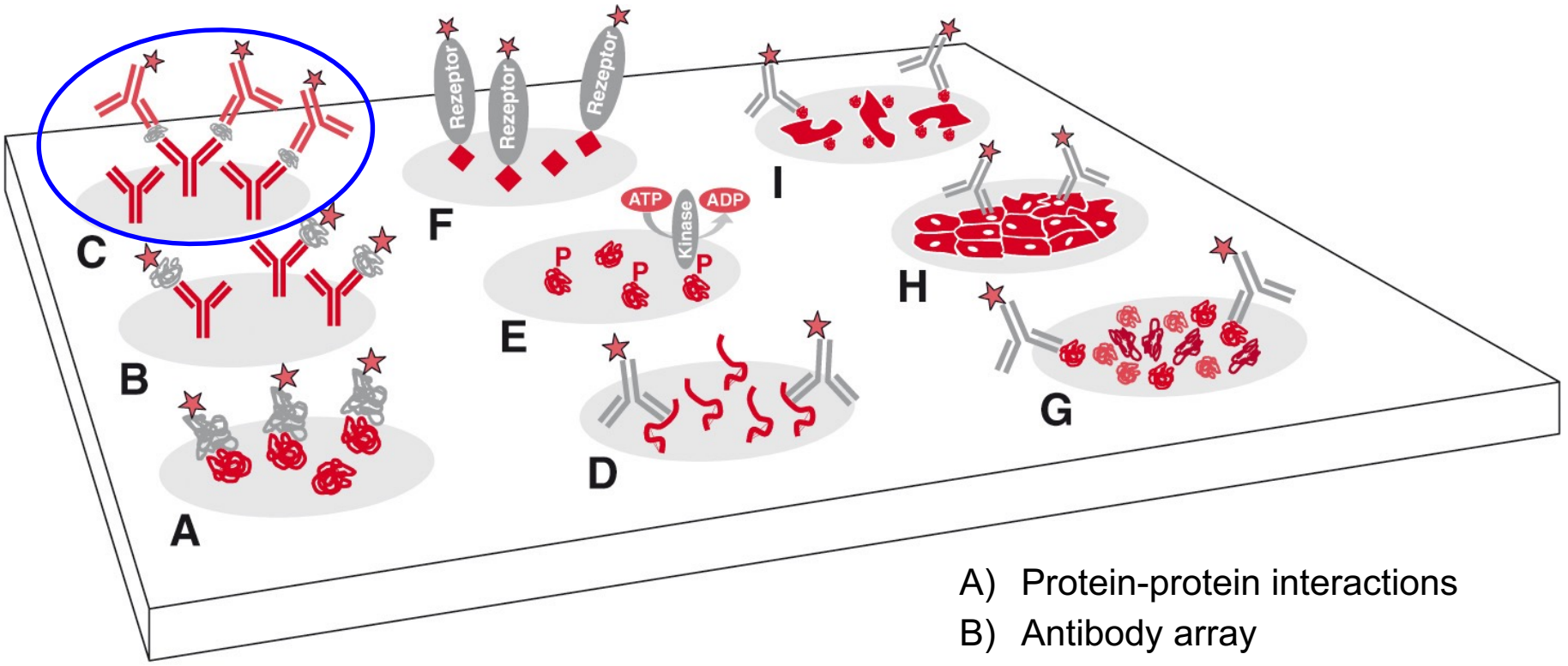
Challenges:

- Limited sensitivity
- Limited dynamic range
- Imprecision of results
- Large sample size needed

Anderson & Anderson (2002) *Molecular & Cellular Proteomics 1*, 845

From microarrays to bead assays

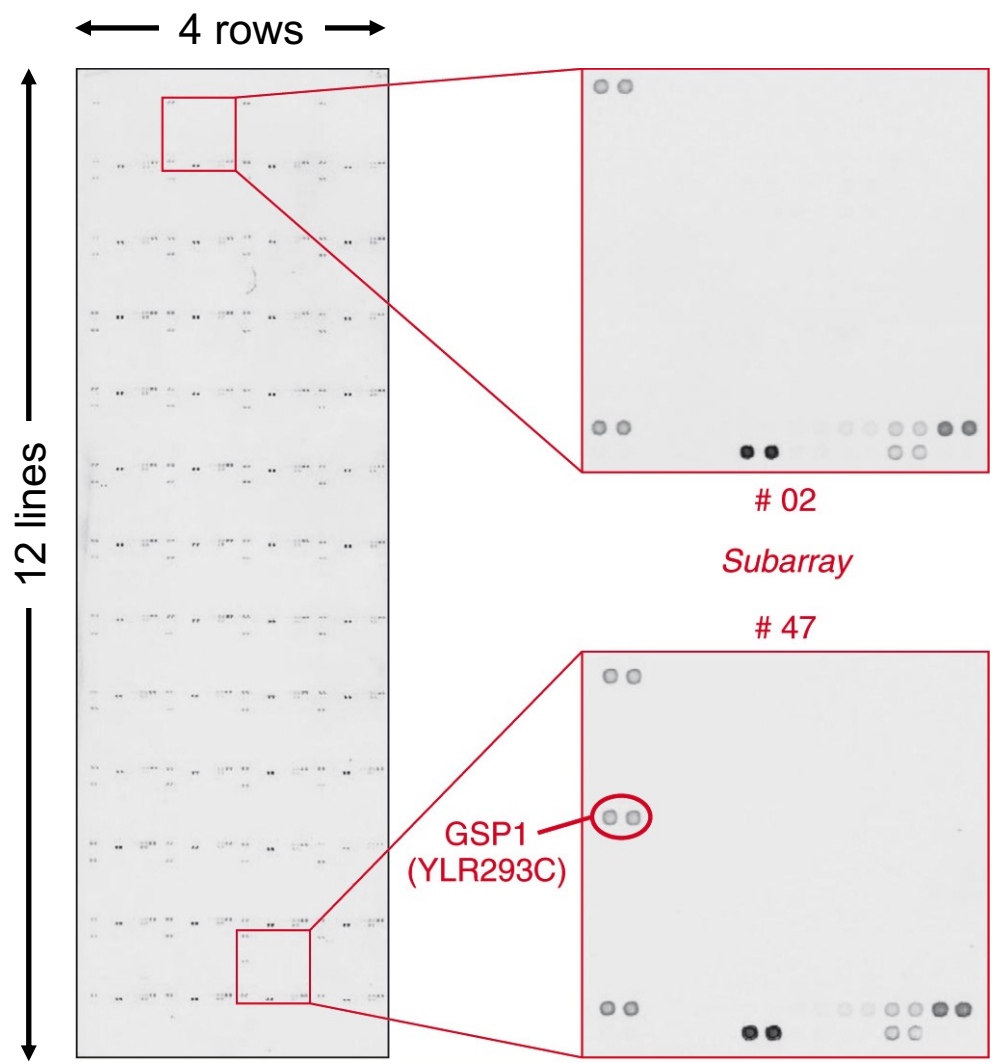
Various applications of microarrays



A) Protein-protein interactions
B) Antibody array
C) Sandwich immunoassay
D) Peptide array
E) Enzyme-substrate interactions
F) Ligand-receptor interactions
G) - I) Reverse microarrays

Protein micorarrays (Invitrogen)

48 subarrays with 4000 different yeast proteins



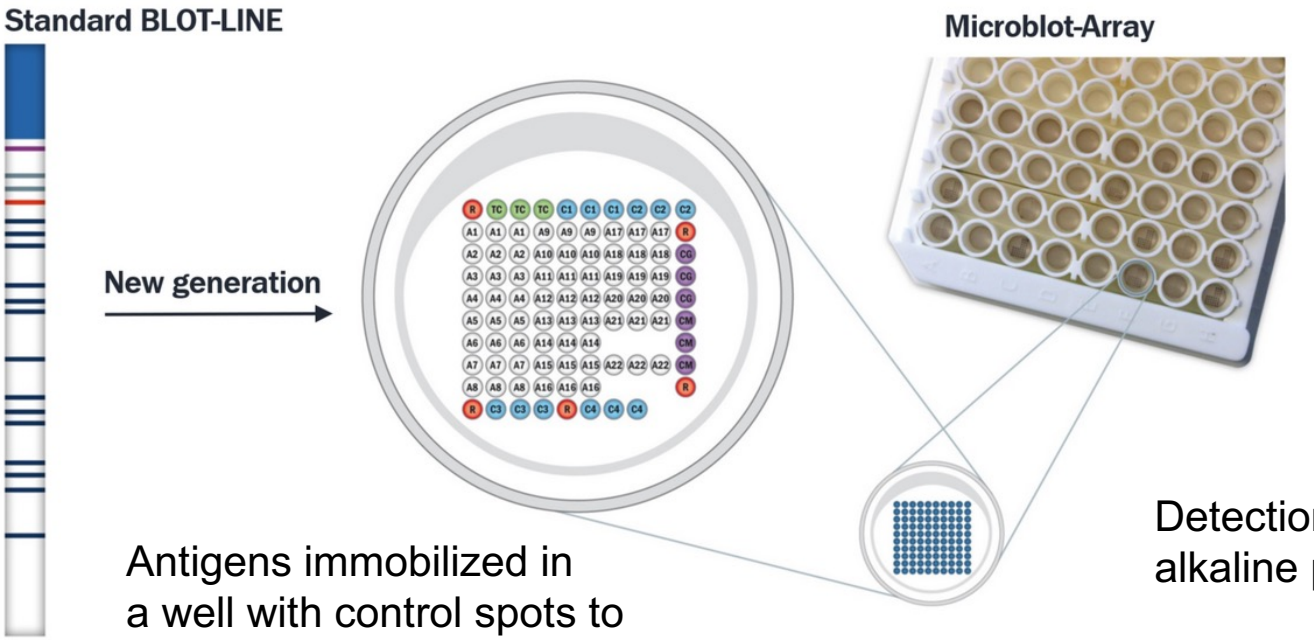
Protocol:

(1) Add the biotinylated protein MOG1 (involved in nuclear import)

(2) Add fluorescence-labeled streptavidin

=> Binding to interaction partner GSP1

TestLine



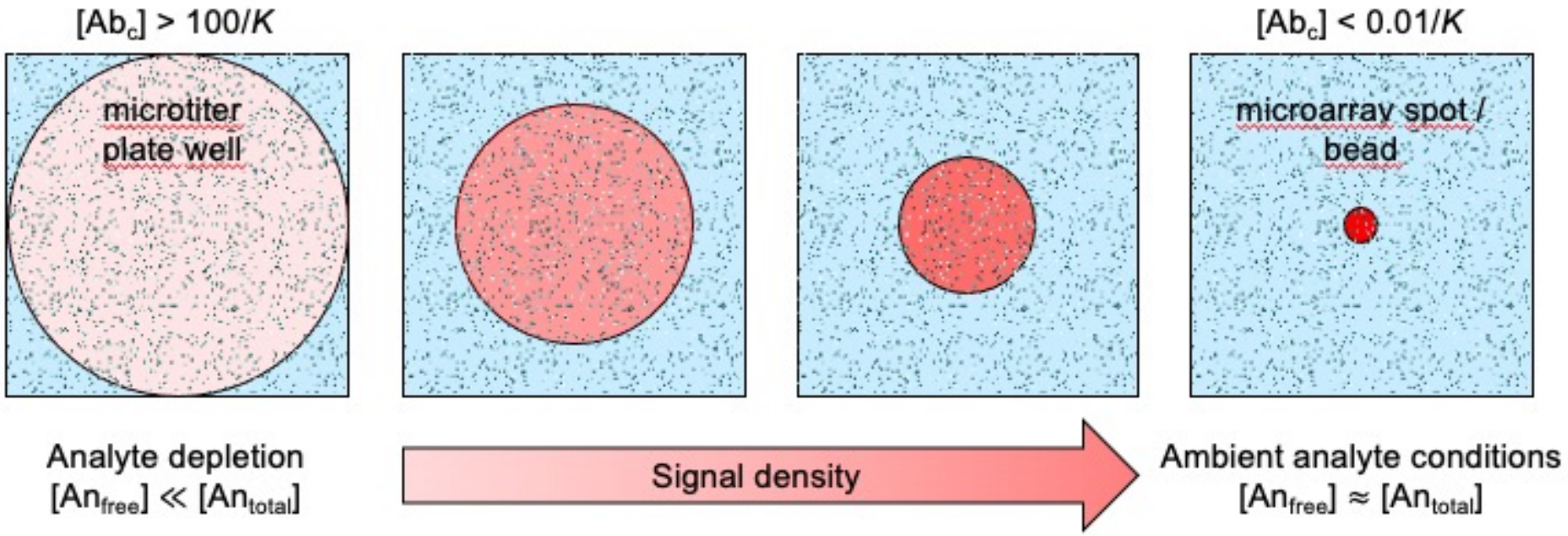
Antigens immobilized in
a well with control spots to

1) check the presence of conjugate
2) check functionality and sensitivity

Detection with conjugate:
alkaline phosphatase antibody

PROPERTIES	BLOT-LINE	MICROBLOT-ARRAY
Maximum antigens per strip/well	19	44
Tests per kit	20	up to 96
Maximum capacity per strip/well	21 bands	200 spots
Sample consumption per test	30 µl	10 µl

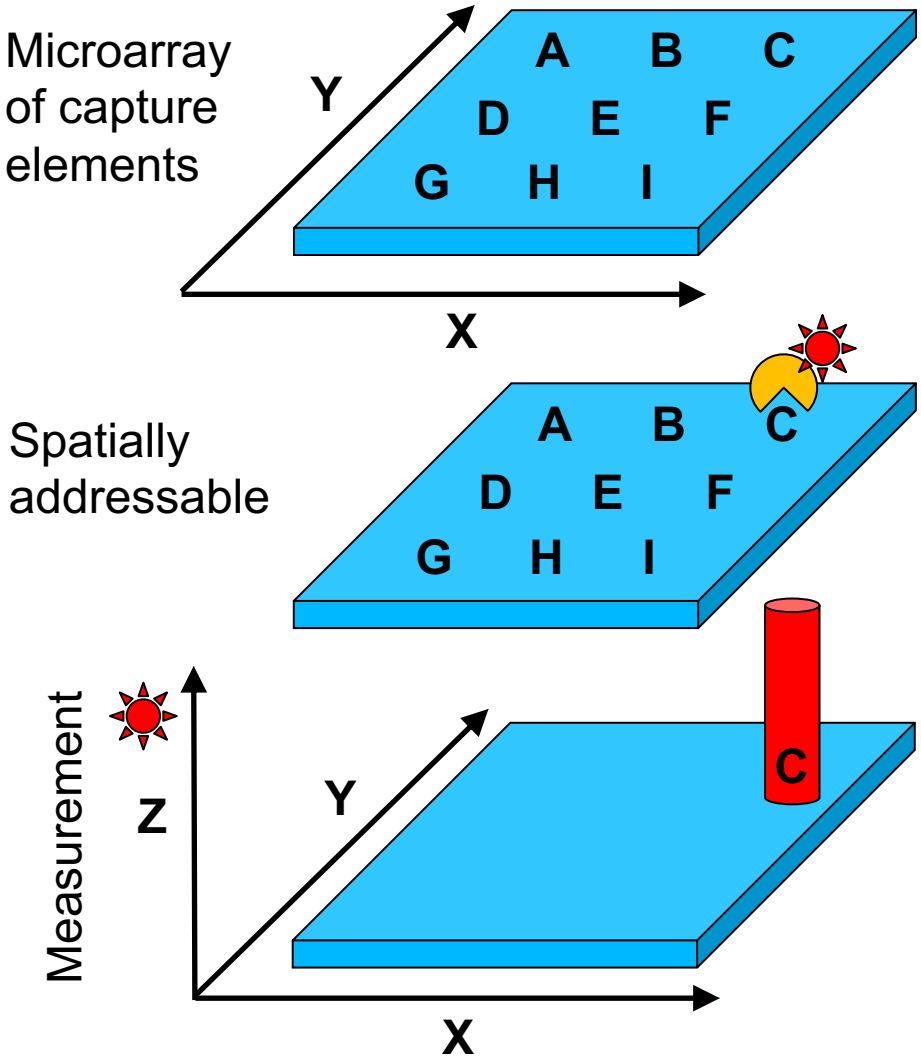
Microarrays: Ambient analyte assays

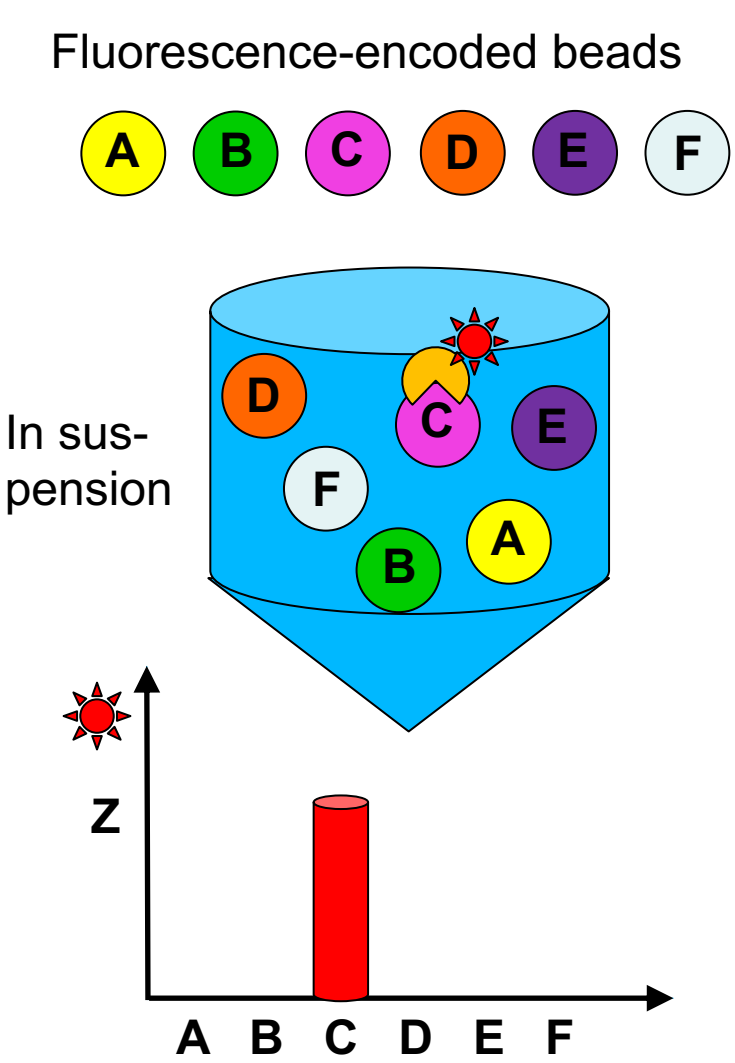


- Very small amounts of capture antibody do not reduce the analyte concentration of the sample: „Ambient analyte assay“
- On a small detetction area, there is less space for non-specific binding

=> High signal density correlates with high sensitivity

Planar array ⇔ Bead array



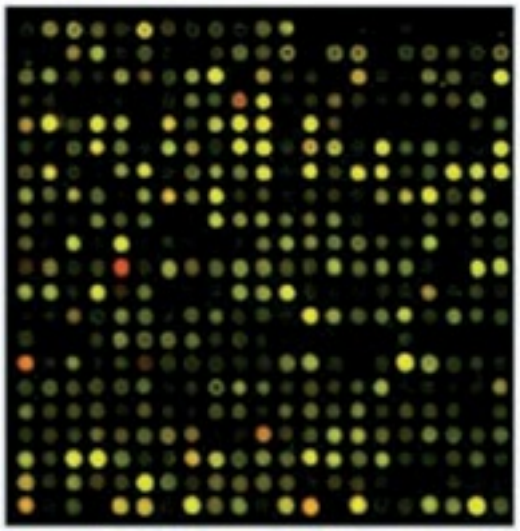


Beads: magnetic separation

Beads can be separated by applying
an external magnetic field

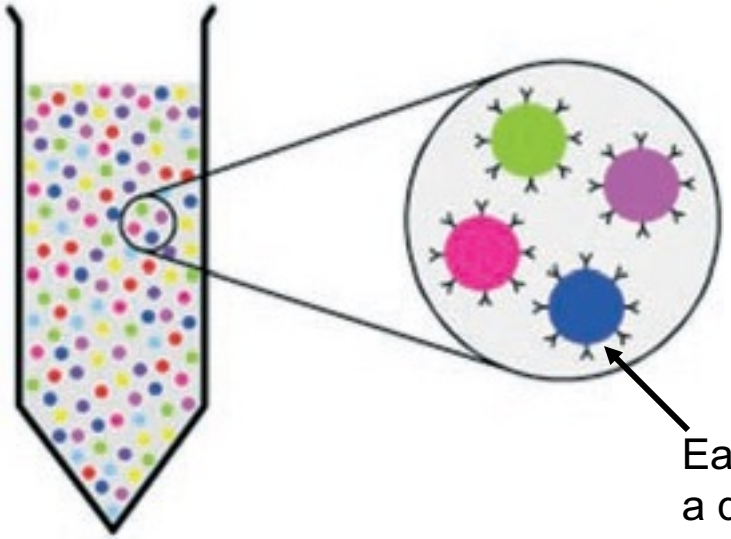
=> Allows for washing steps to
remove excess reagents

Microarrays: Multiplexing



Planar array

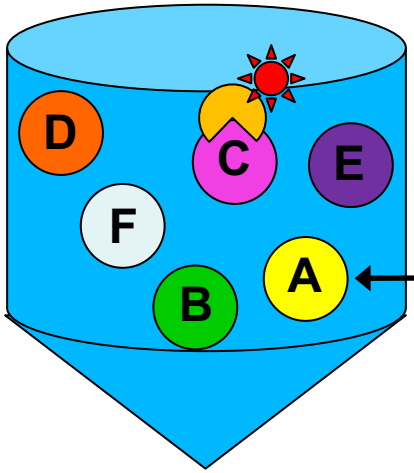
- Antibodies are immobilized on fixed positions on a solid support
- Each type of analyte can be directly addressed by its spatial location



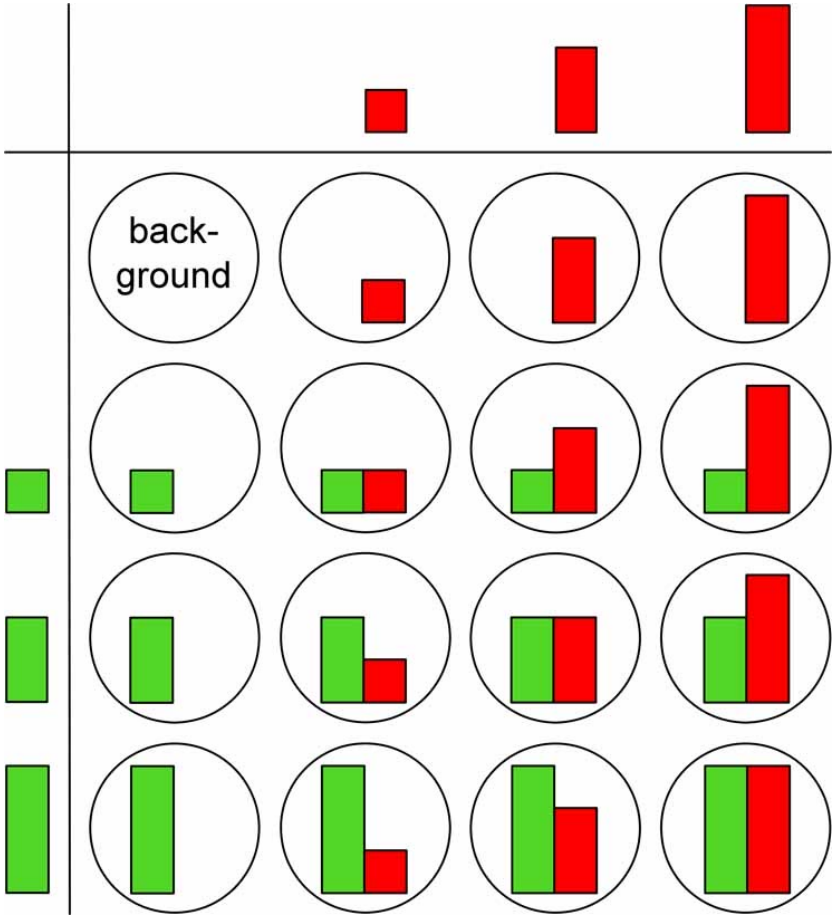
Bead array

- Antibodies are immobilized on the surface of beads
- An encoding strategy is required (e.g. code of different fluorophore combinations)

Beads: Fluorescent codes



Each encoded bead carries a distinct capture element



A

Bead encoding by using fluorophore combinations:

2 colors

4 intensities

Number of Codes = $i^f - 1$

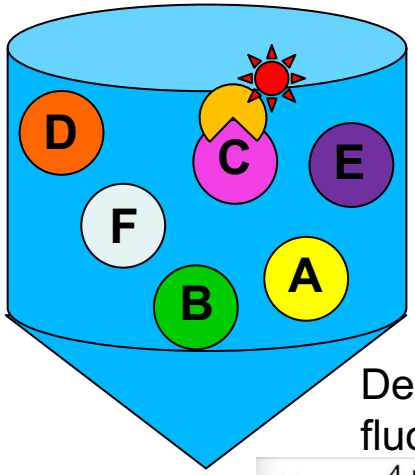
$4^2 - 1 = 15$ Codes

B

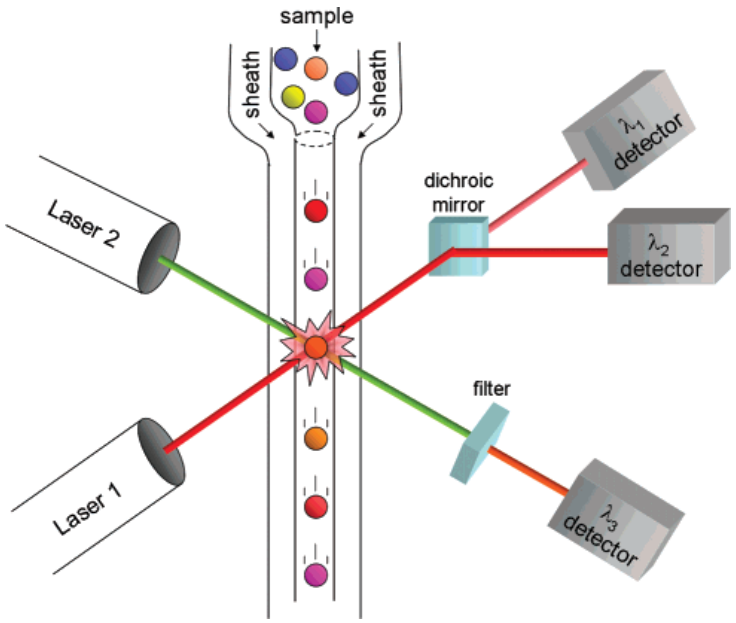
Another fluorophore (color) is required for the detection of analyte binding.

Readout of fluorescent codes

Beads in suspension

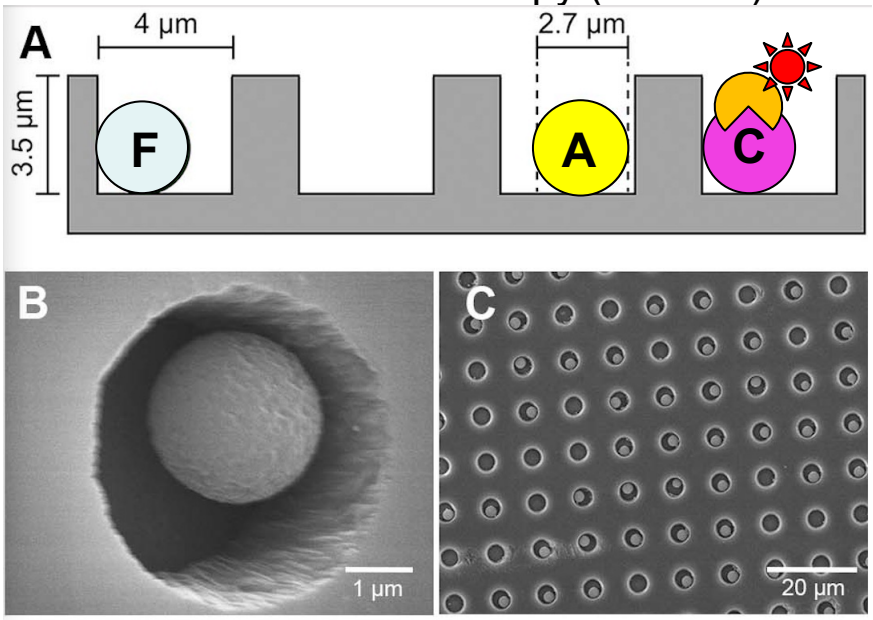


Flow cytometry (Luminex)



=> “on the fly”

Deposition on femtoliter arrays + fluorescence microscopy (Illumina)



=> randomly ordered

Three types of array formats

Planar (directed) arrays

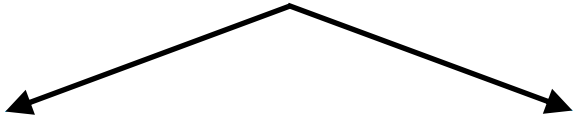
Positional encoding of probe elements on array

Advantages:
Simple readout
Very common

Disadvantages:
Probe molecules must be attached to each spot individually
⇒ Batch-to-batch variation
⇒ limited throughput

Suspension arrays **Randomly ordered arrays**

Fluorescence-encoded beads



Sorting by flow cytometry — Loading into femtoliter arrays

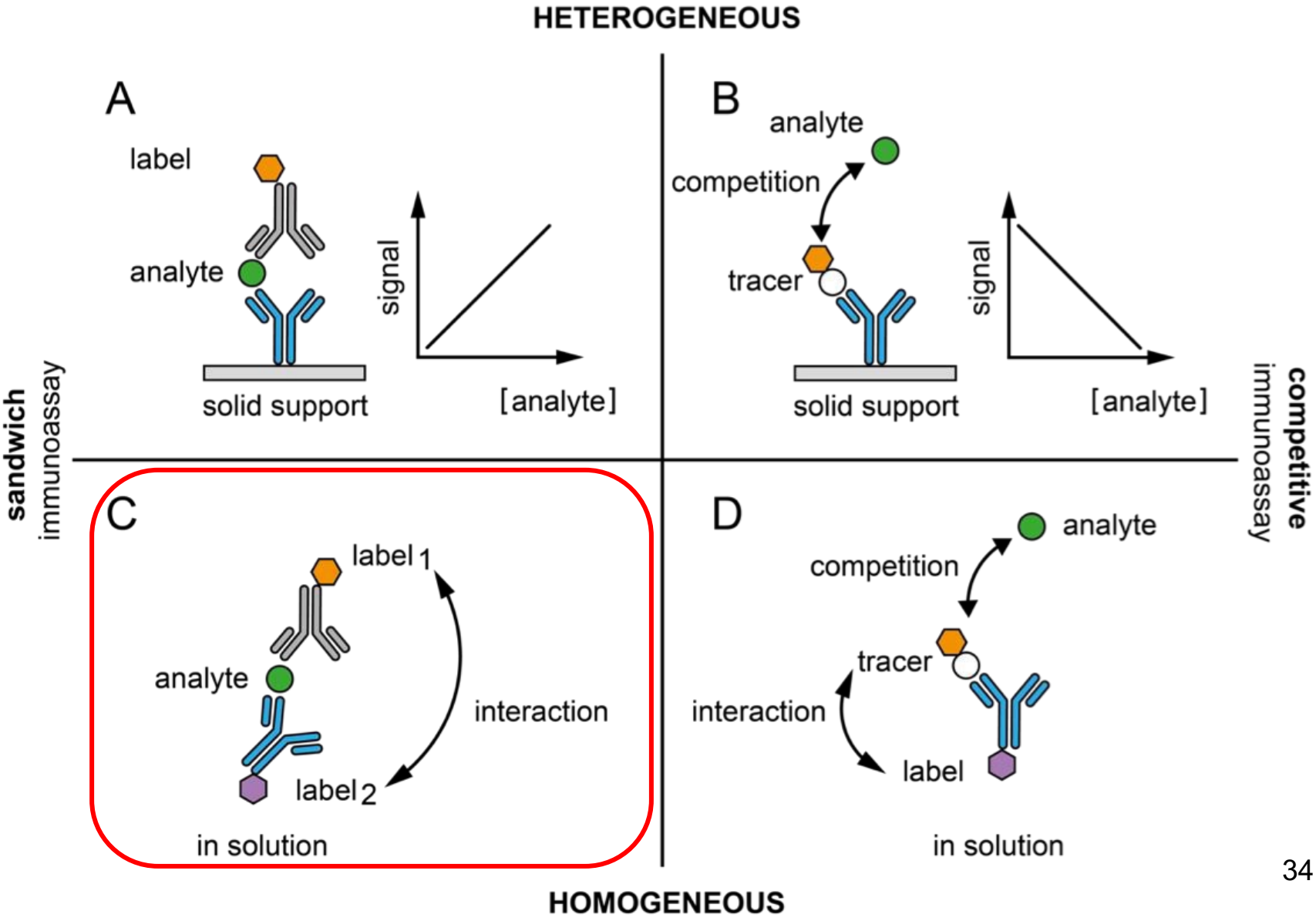
Advantages:
Homogeneous beads generated in a single batch
inexpensive
Only small sample volumes required

Disadvantages:
Each probe requires a specific code

=> Enables thousands of measurements in a small volume

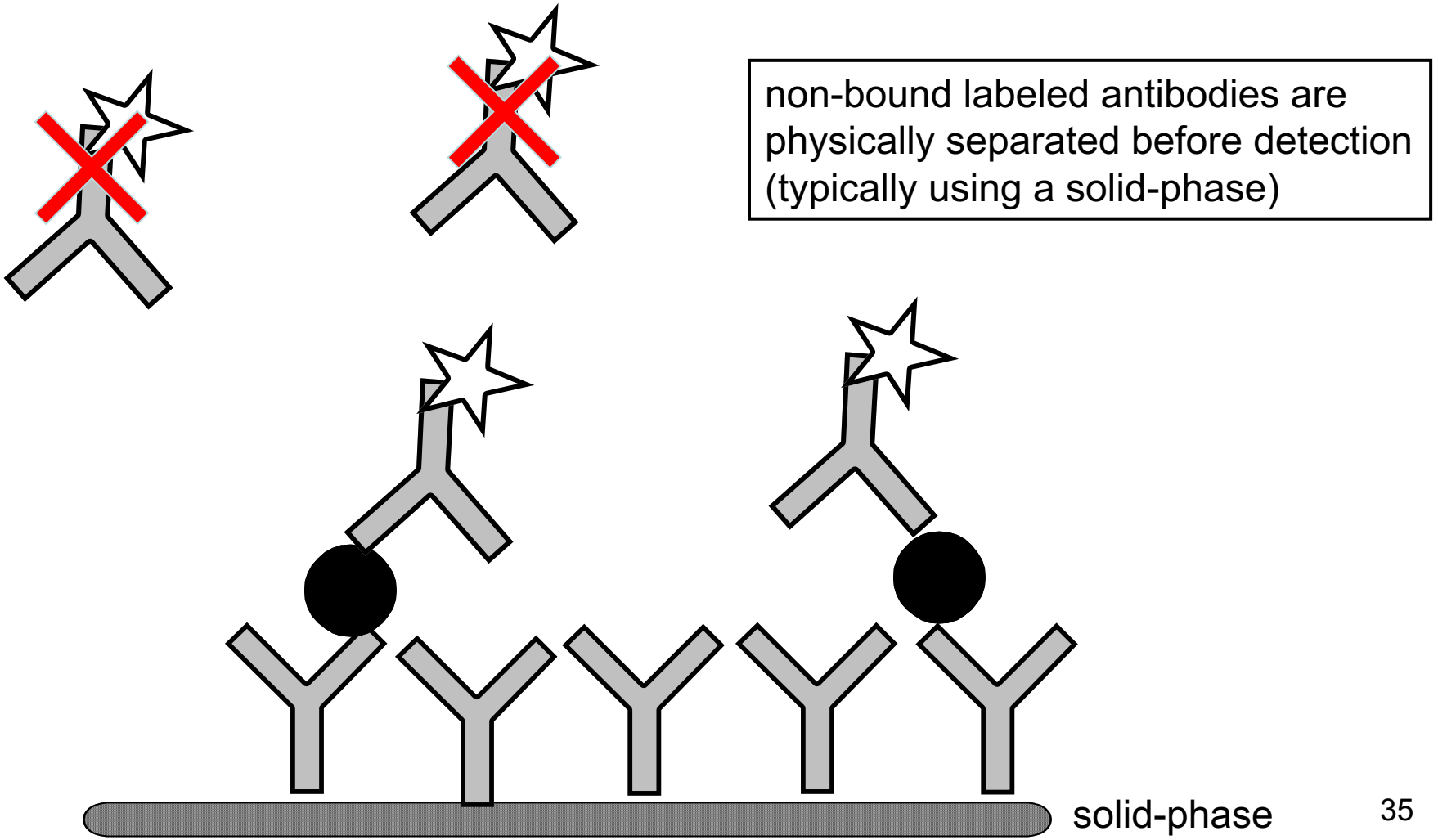
Homogeneous immunoassays

Heterogeneous vs. homogeneous immunoassay



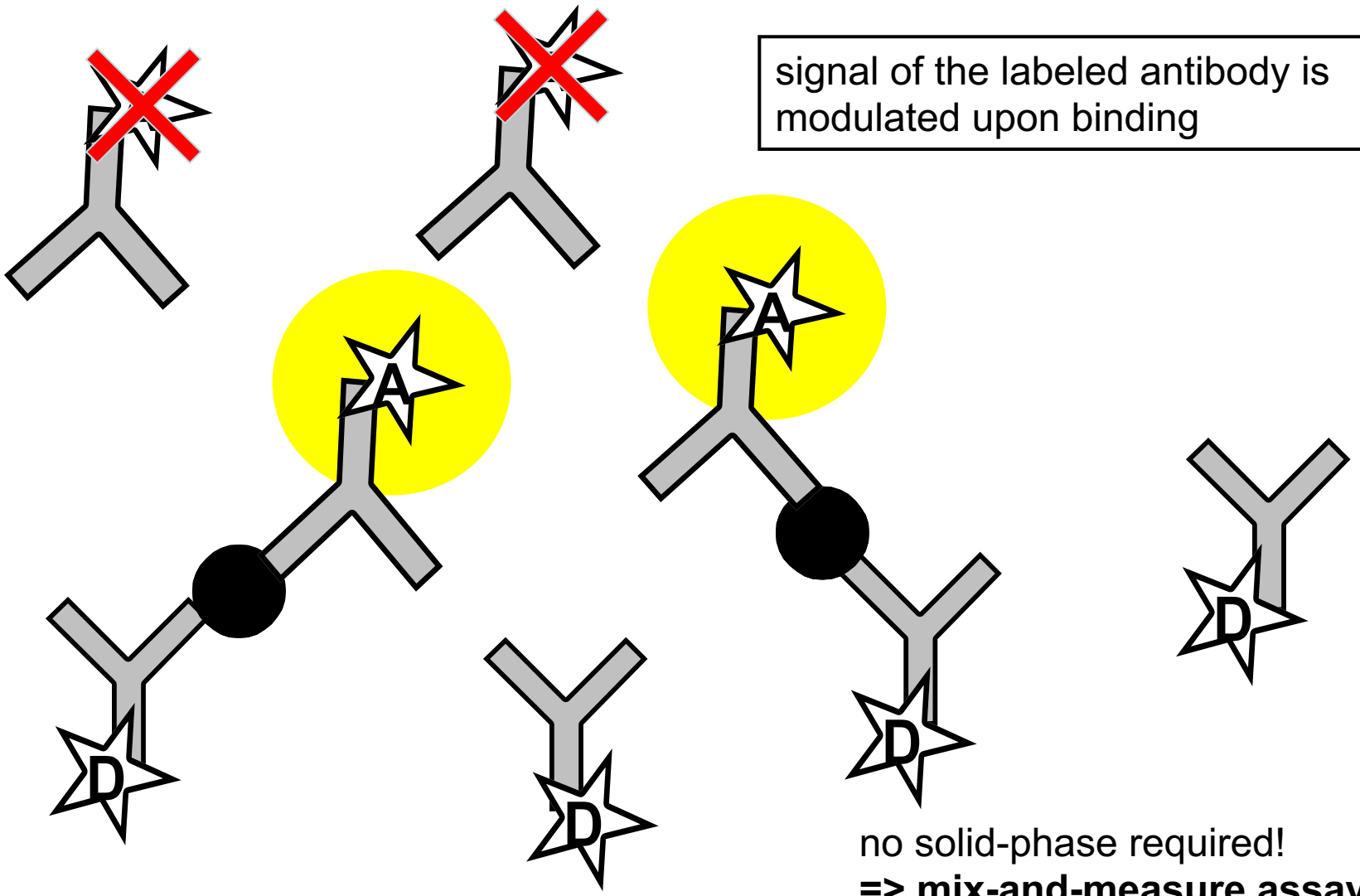
Heterogeneous assay

non-competitive "sandwich" immunoassay



Homogeneous assay

non-competitive "sandwich" immunoassay



signal of the labeled antibody is modulated upon binding

no solid-phase required!

=> mix-and-measure assay

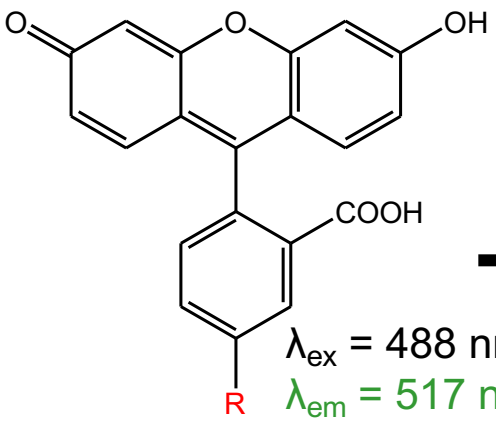
Examples how to modulate the detection signal

=> “smart” reporters for homogeneous assays

- Fluorescence resonance energy transfer (FRET)
- Luminescent oxygen channeling
- Fluorescence polarization
- Lanthanide complementation

Fluorescence Resonance Energy Transfer (FRET)

fluorescein

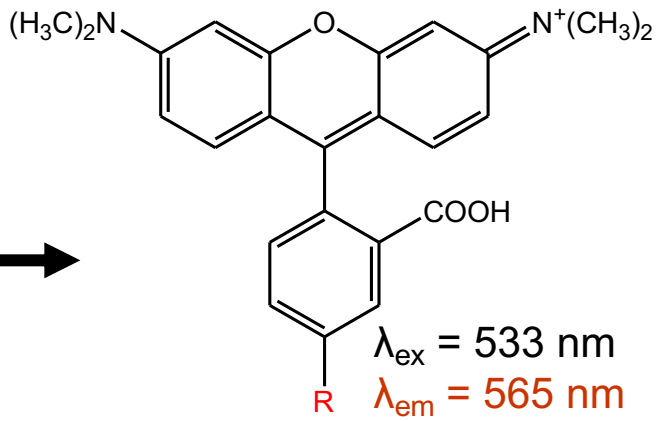


λ_{ex} = 488 nm

λ_{em} = 517 nm

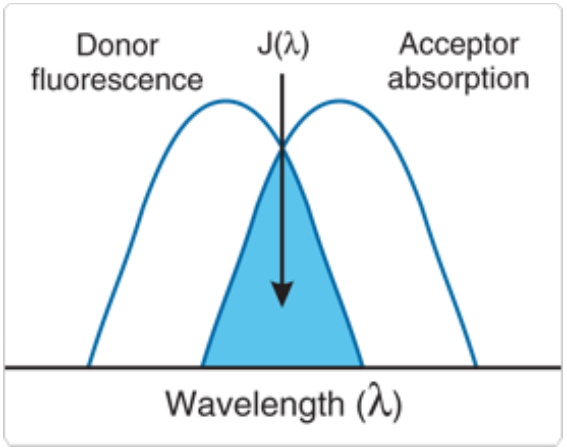
FRET

tetramethylrhodamine



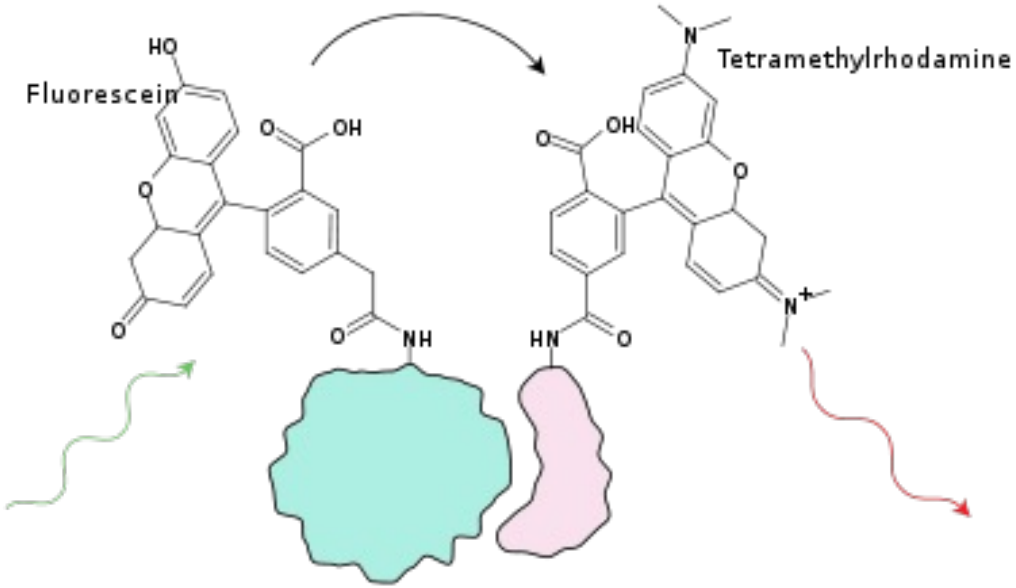
λ_{ex} = 533 nm

λ_{em} = 565 nm



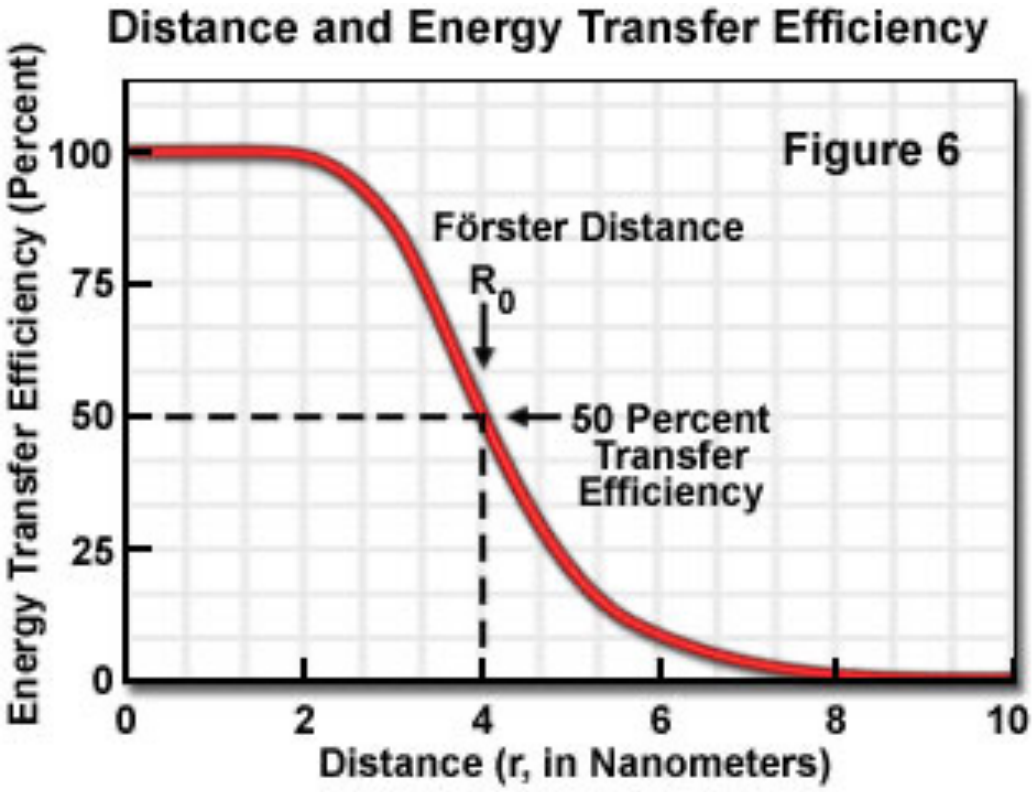
Donor

Acceptor



FRET: A nanoscale ruler

$$E_T = \frac{1}{1 + \left(\frac{R_{DA}}{R_0} \right)^6}$$



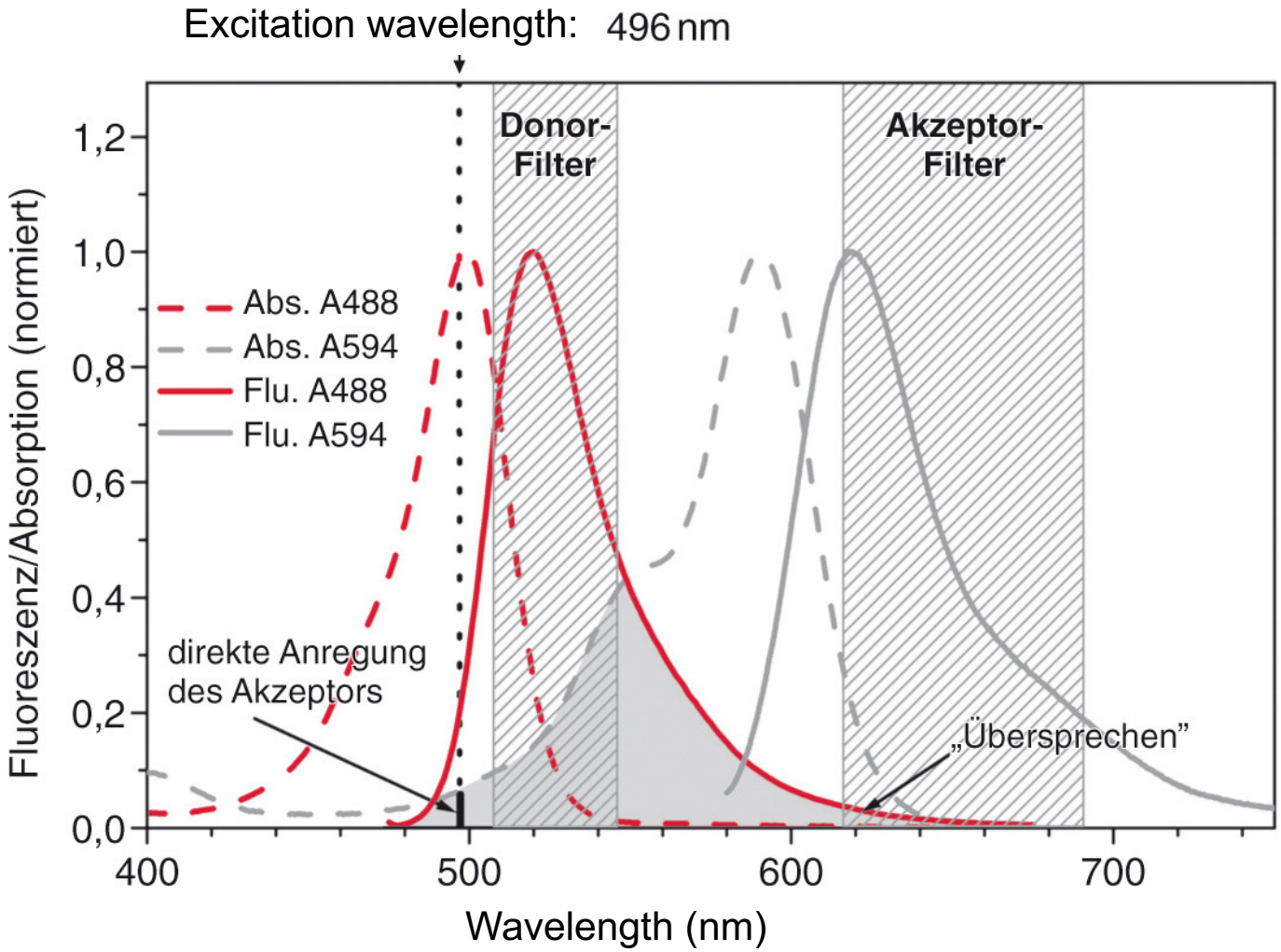
FRET: A nanoscale ruler

Förster Critical Distance for Common RET Donor-Acceptor Pairs

Donor	Acceptor	Förster Distance (Nanometers)
Tryptophan	Dansyl	2.1
IAEDANS (1)	DDPM (2)	2.5 - 2.9
BFP	DsRFP	3.1 - 3.3
Dansyl	FITC	3.3 - 4.1
Dansyl	Octadecylrhodamine	4.3
CFP	GFP	4.7 - 4.9
CF (3)	Texas Red	5.1
Fluorescein	Tetramethylrhodamine	4.9 - 5.5
Cy3	Cy5	>5.0
GFP	YFP	5.5 - 5.7
BODIPY FL (4)	BODIPY FL (4)	5.7
Rhodamine 6G	Malachite Green	6.1
FITC	Eosin Thiosemicarbazide	6.1 - 6.4
B-Phycoerythrin	Cy5	7.2
Cy5	Cy5.5	>8.0

(1) 5-(2-iodoacetylaminoethyl)aminonaphthalene-1-sulfonic acid
(2) N-(4-dimethylamino-3,5-dinitrophenyl)maleimide
(3) carboxyfluorescein succinimidyl ester
(4) 4,4-difluoro-4-bora-3a,4a-diaza-s-indacene

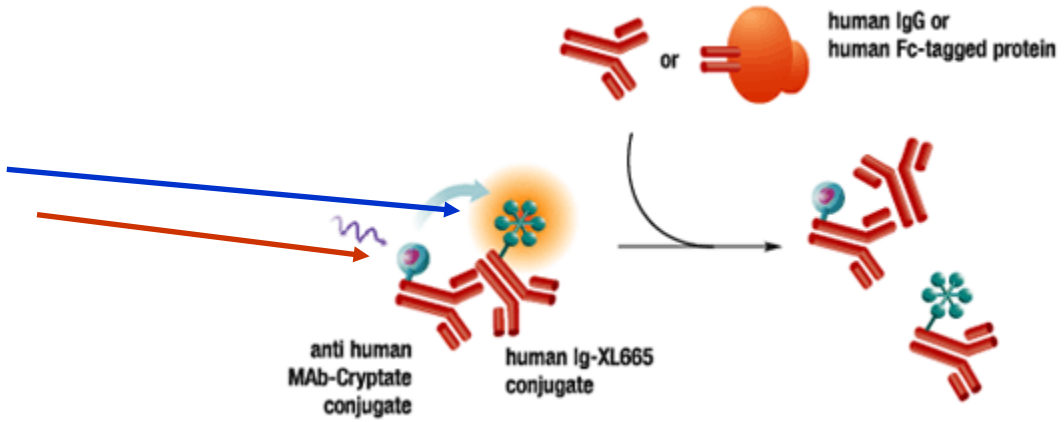
Detection of FRET



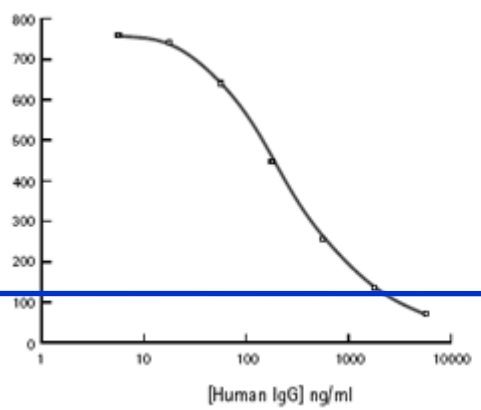
Competitive immunoassay based on FRET

In solution (cuvette, microtiterplate) => Immobilization not required

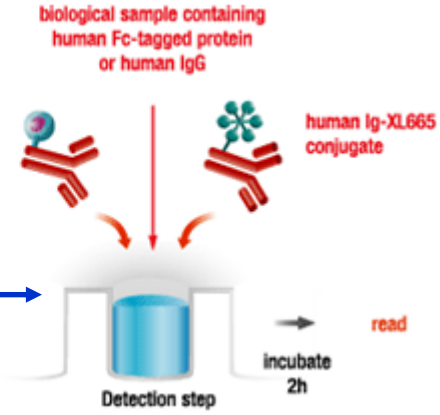
Example: Assay for IgG
2 reagents:
- IgG labeled with FRET acceptor
- *anti*-IgG labeled with FRET donor



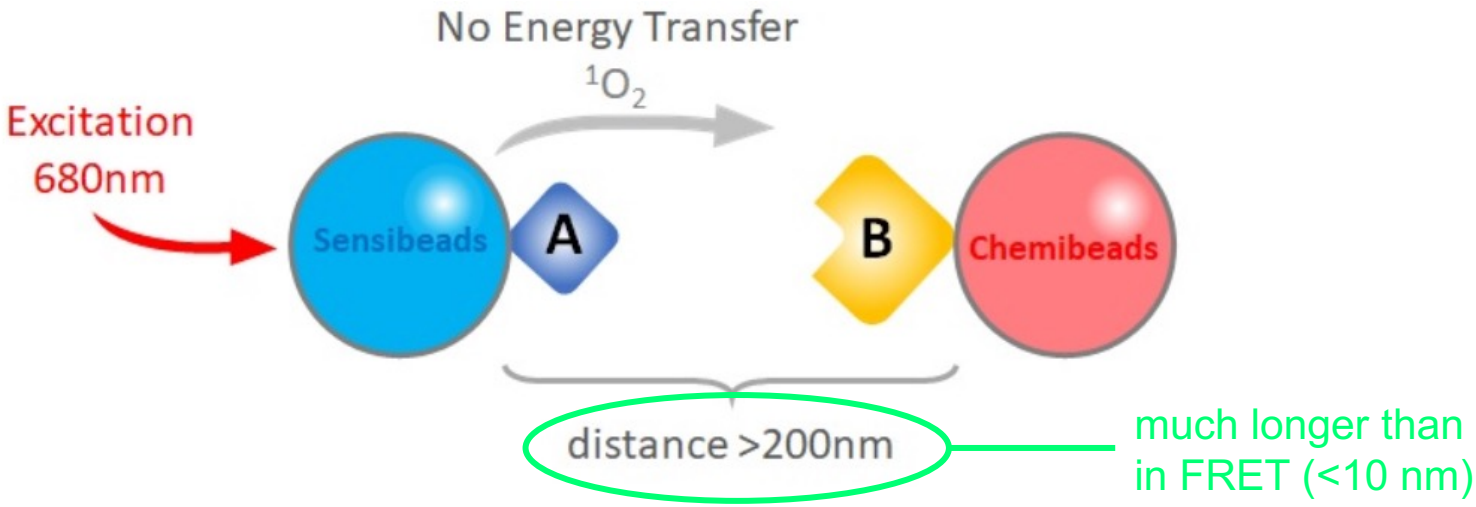
Principle:
IgG (analyte) and labeled IgG compete for binding to anti-IgG; if labeled IgG is present in large excess (= little analyte), FRET is efficient. Otherwise, there is little FRET.



Steps:
Add labeled IgG and labeled anti-IgG to the sample. That's it!

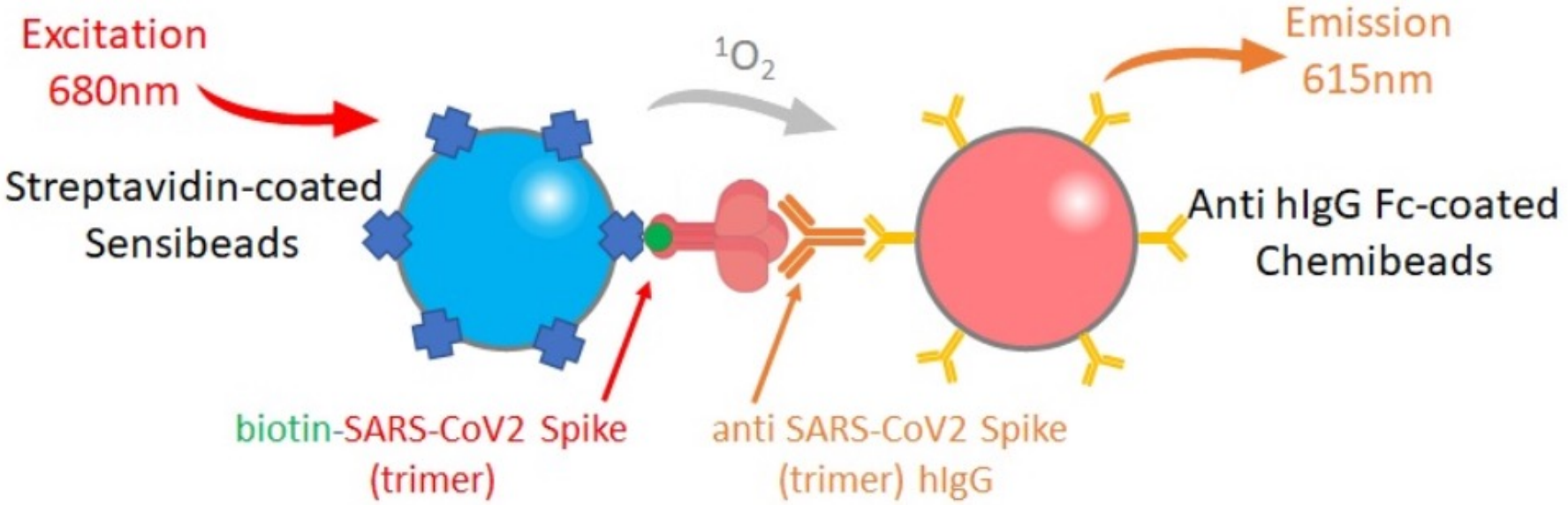


Luminescence oxygen channeling

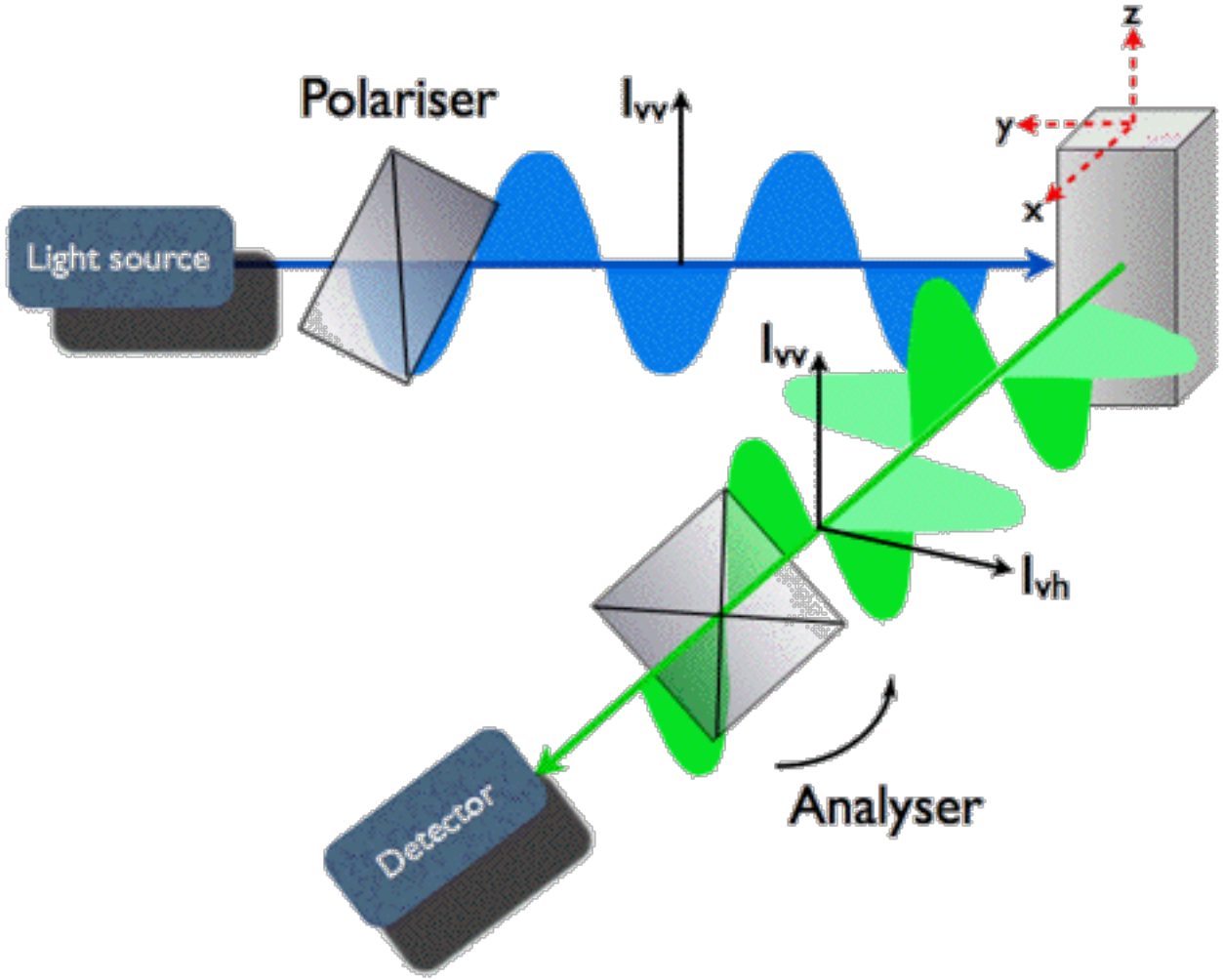


E. Ullmann et al. 1994, *PNAS 91*: 5427

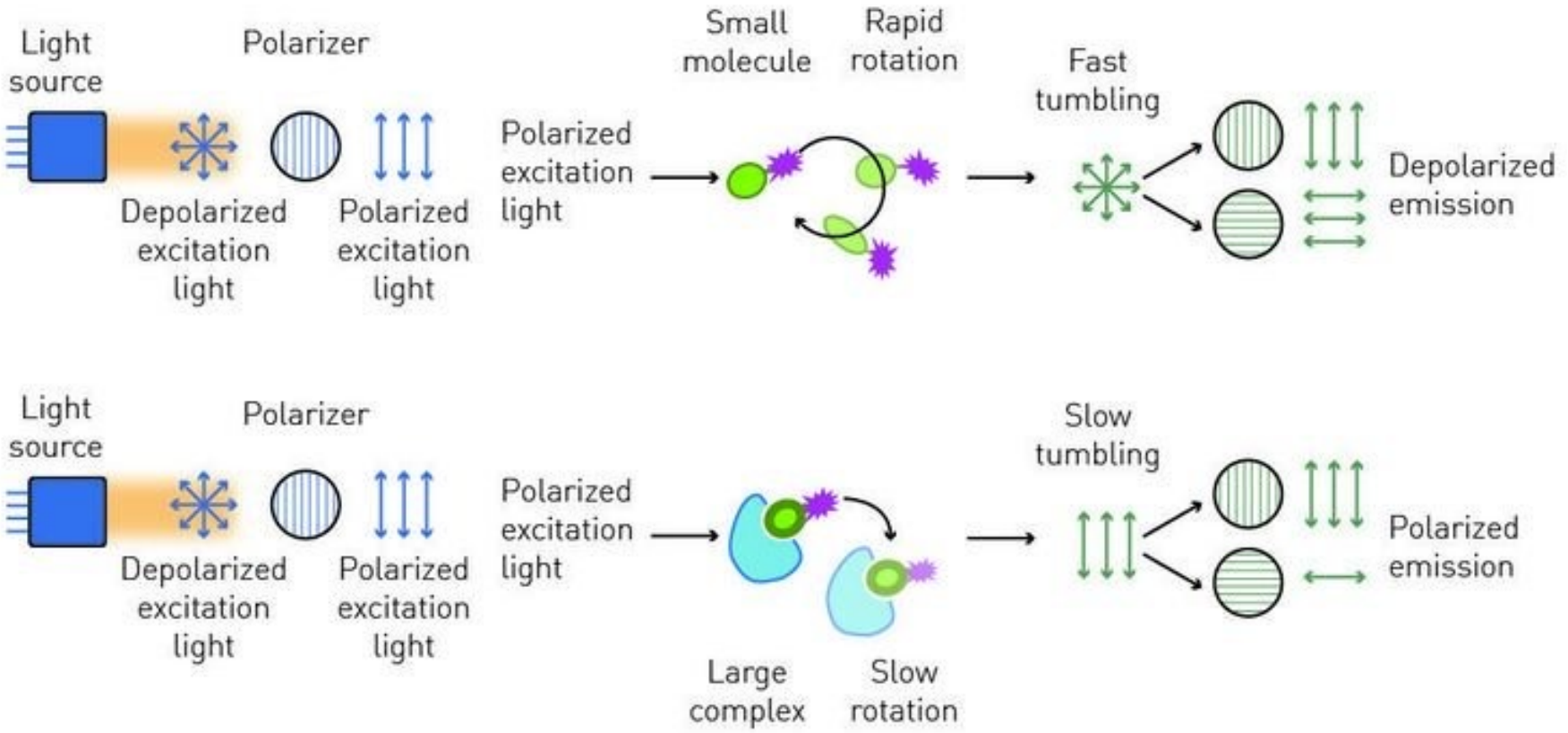
Luminescence oxygen channeling immunoassay



Fluorescence Polarization Immunoassay (FPIA)



Fluorescence Polarization Immunoassay (FPIA)



Fluorescence Polarization Immunoassay (FPIA)

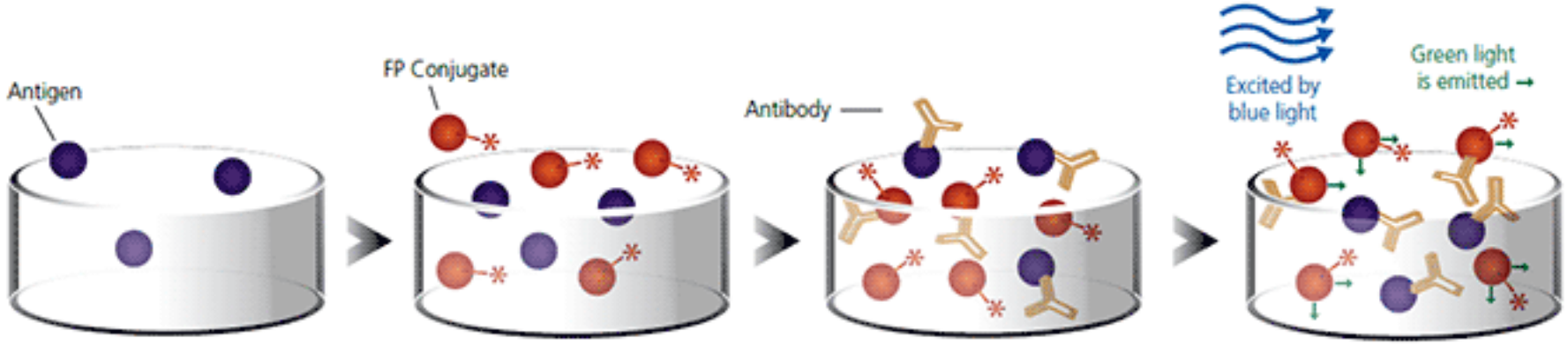
In solution (e.g in microtiter plate)

Reagents needed:
(a) labeled antigen; (b) antibody (a secondary antibody is not needed)

Reaction: competitive binding of free antigen and labeled antigen to labeled antibody.

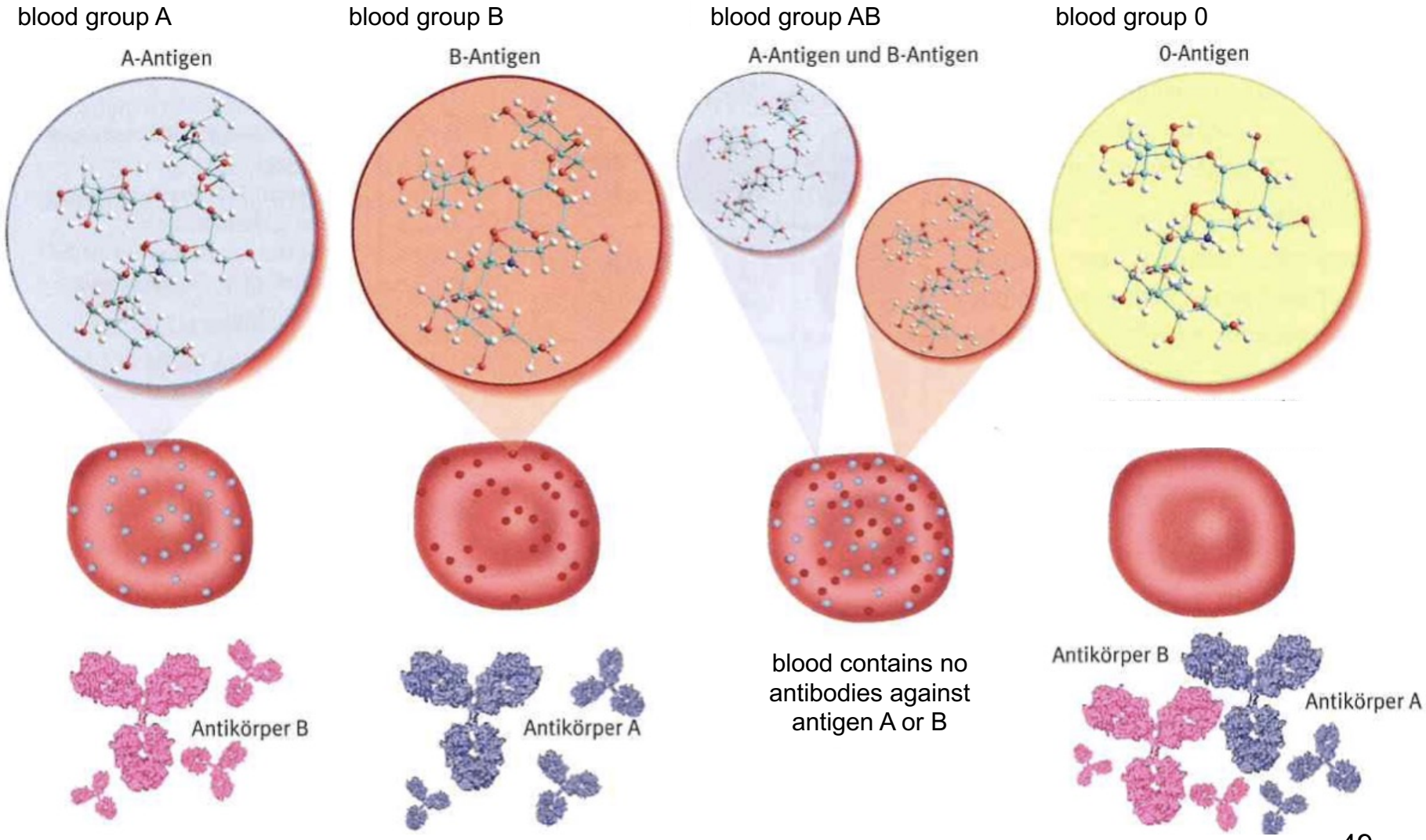
Labeled antigen ("FP conjugate") in solution tumbles and depolarizes light.

Labeled antigen bound to antibody tumbles more slowly => less depolarized light.



Immune agglutionation / precipitation

Blood type: different antigens on red blood cells



Immune agglutination

antigen-covered microscopic particles in suspension
(e.g. bacteria, blood cells, or latex particles)

+

Specific immune serum / antibodies

II

V

cross-linking forms large aggregates
that are not stable in suspension
(agglutination)

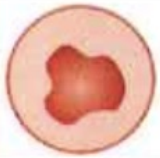
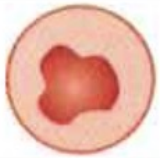
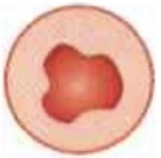
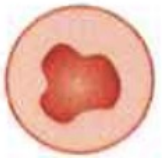
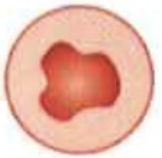
II

V

visible sedimentation

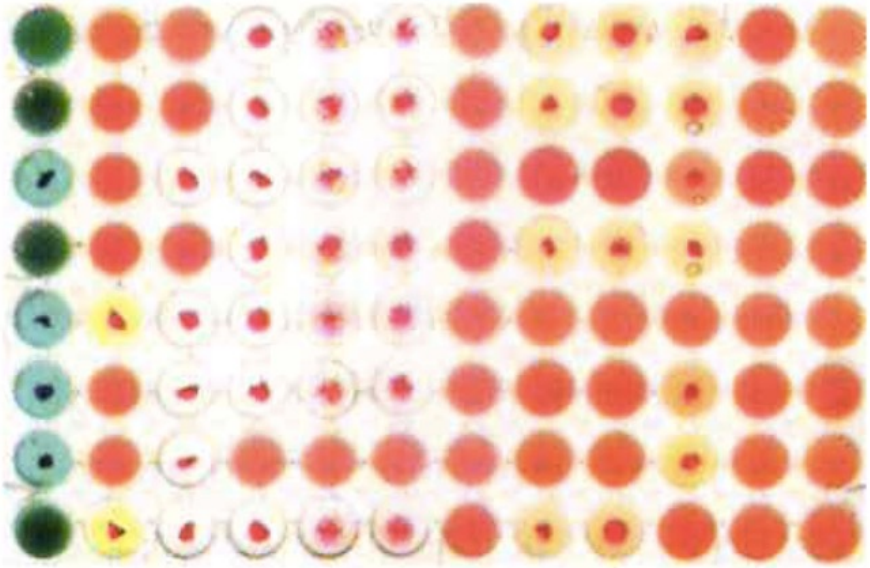
Advantages:
Cheap, easy, very sensitive,
but semi-quantitative

Immune agglutination: blood type

Test series			blood group
anti-A	anti-B	anti-AB	
			A
			B
			AB
			0

 agglutination  no agglutination

Determination of blood type in microtiter plate



Evaluation:
positive: agglutination, bead formation
negative: erythrocytes remain in suspension (homogeneous red fluid)

Immunoprecipitation

soluble antigen

+

specific antibodies

II

V

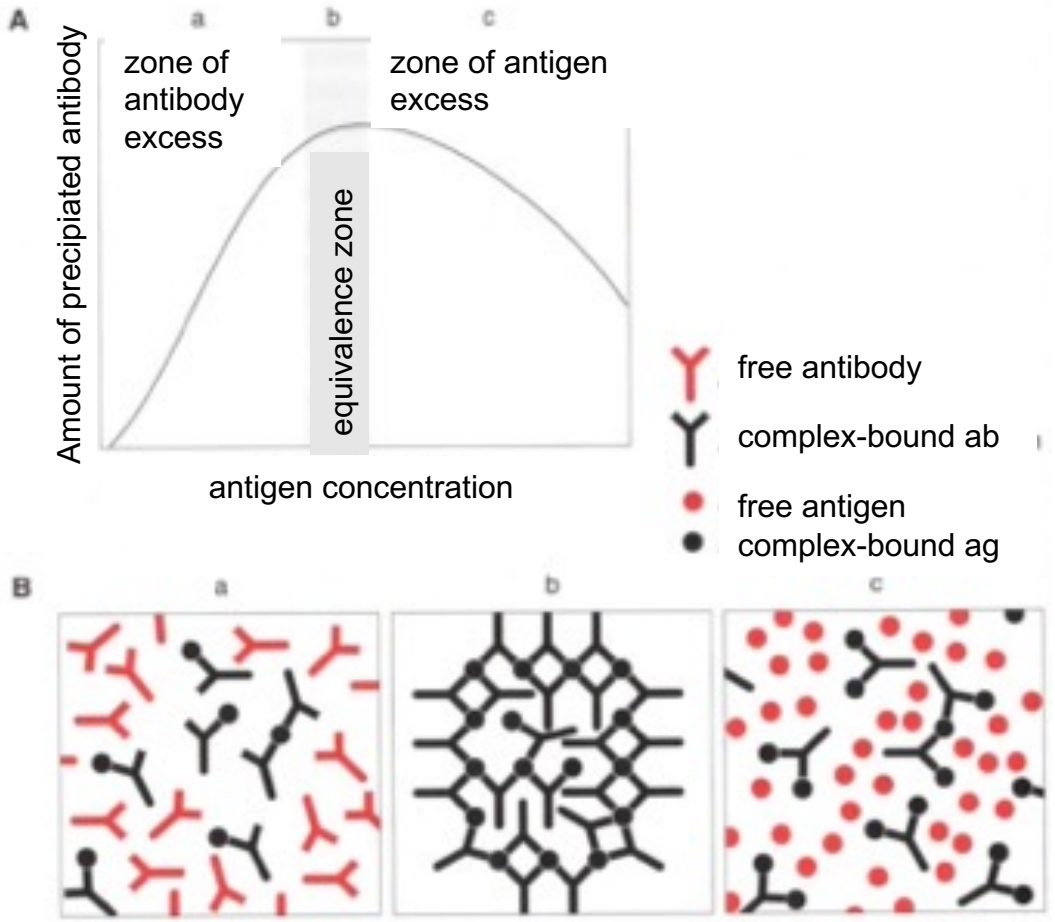
cross-linking forms insoluble antibody-antigen complexes

(=> remember: at least 2 or more epitopes required)

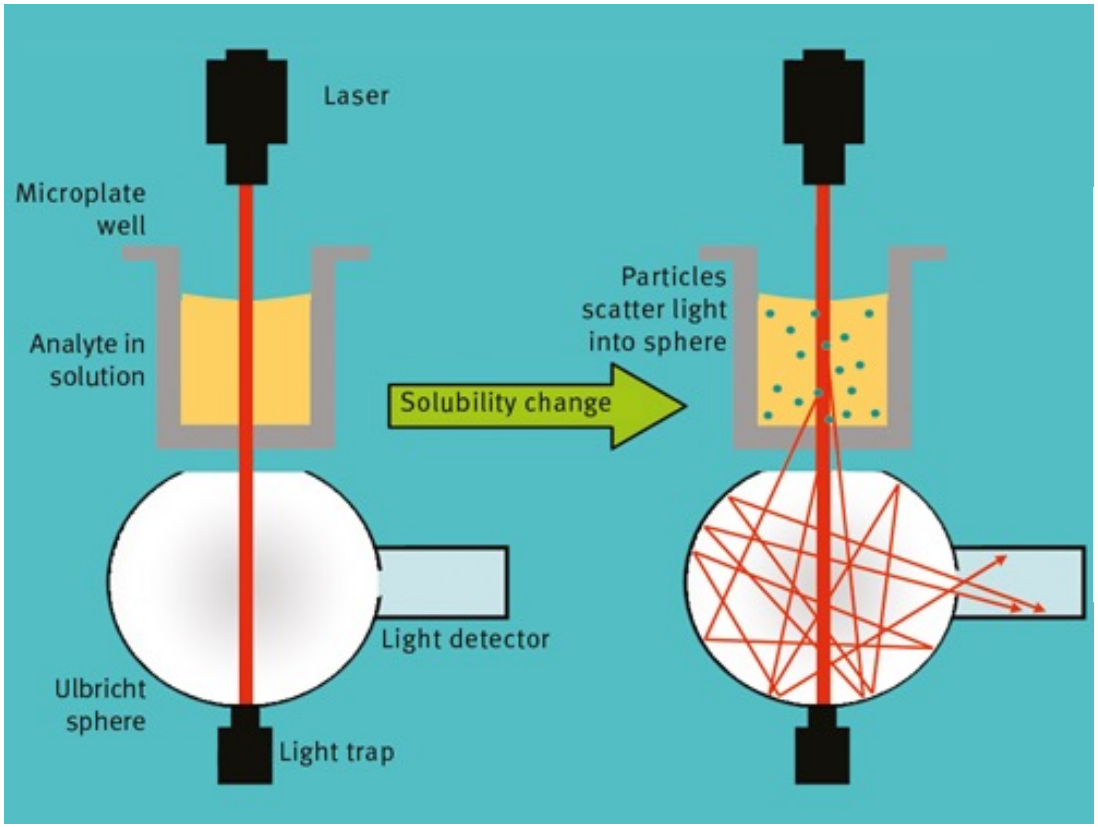
II

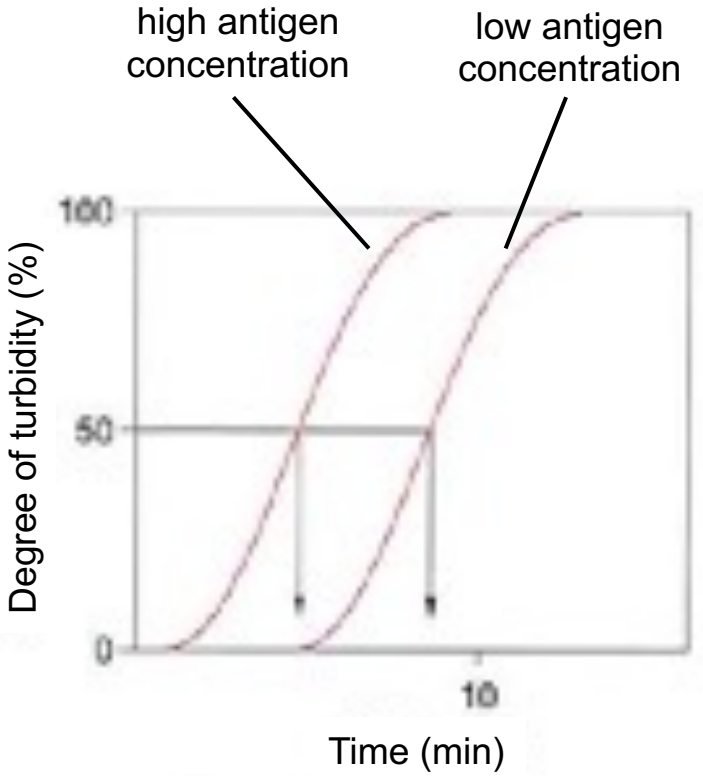
V

visible sedimentation



Nephelometry

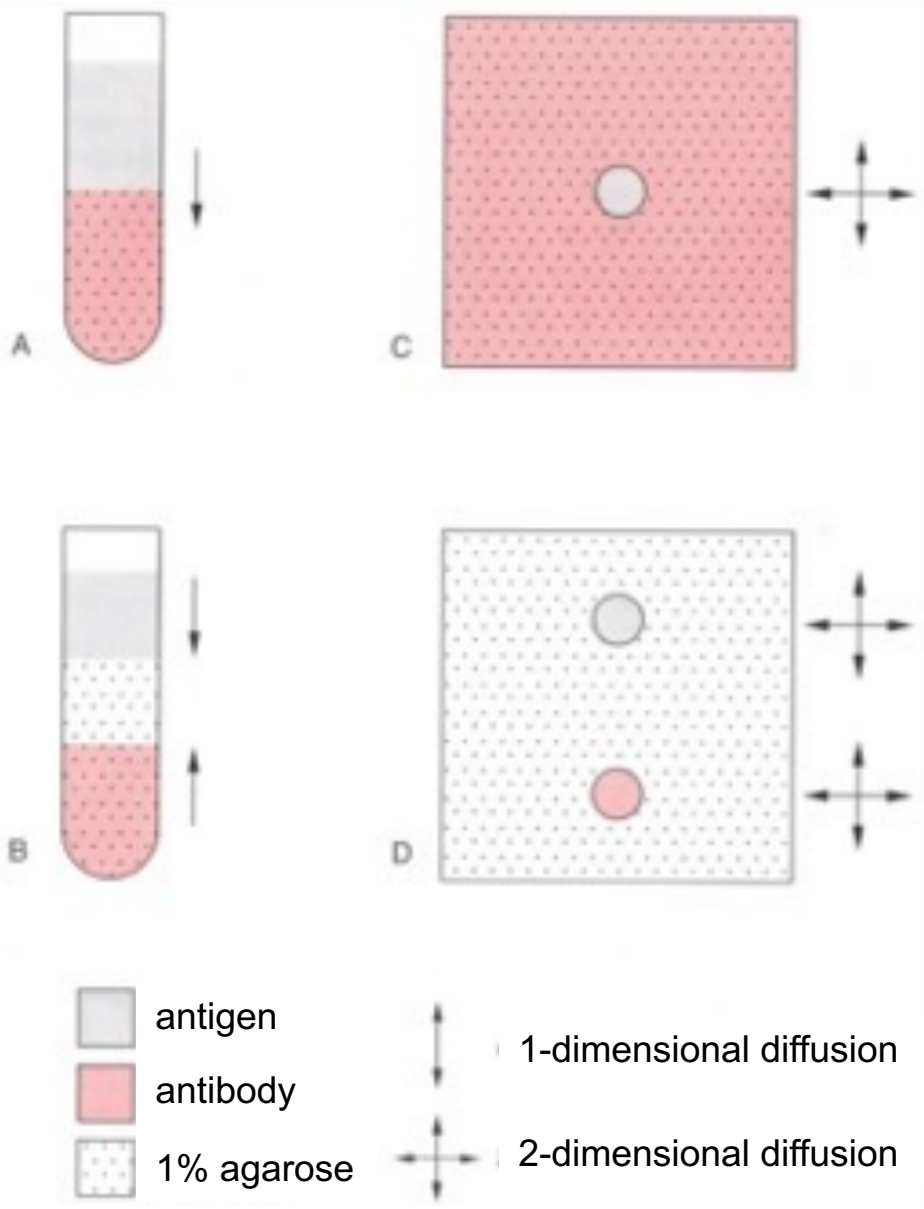




Classification of immunoprecipitation systems

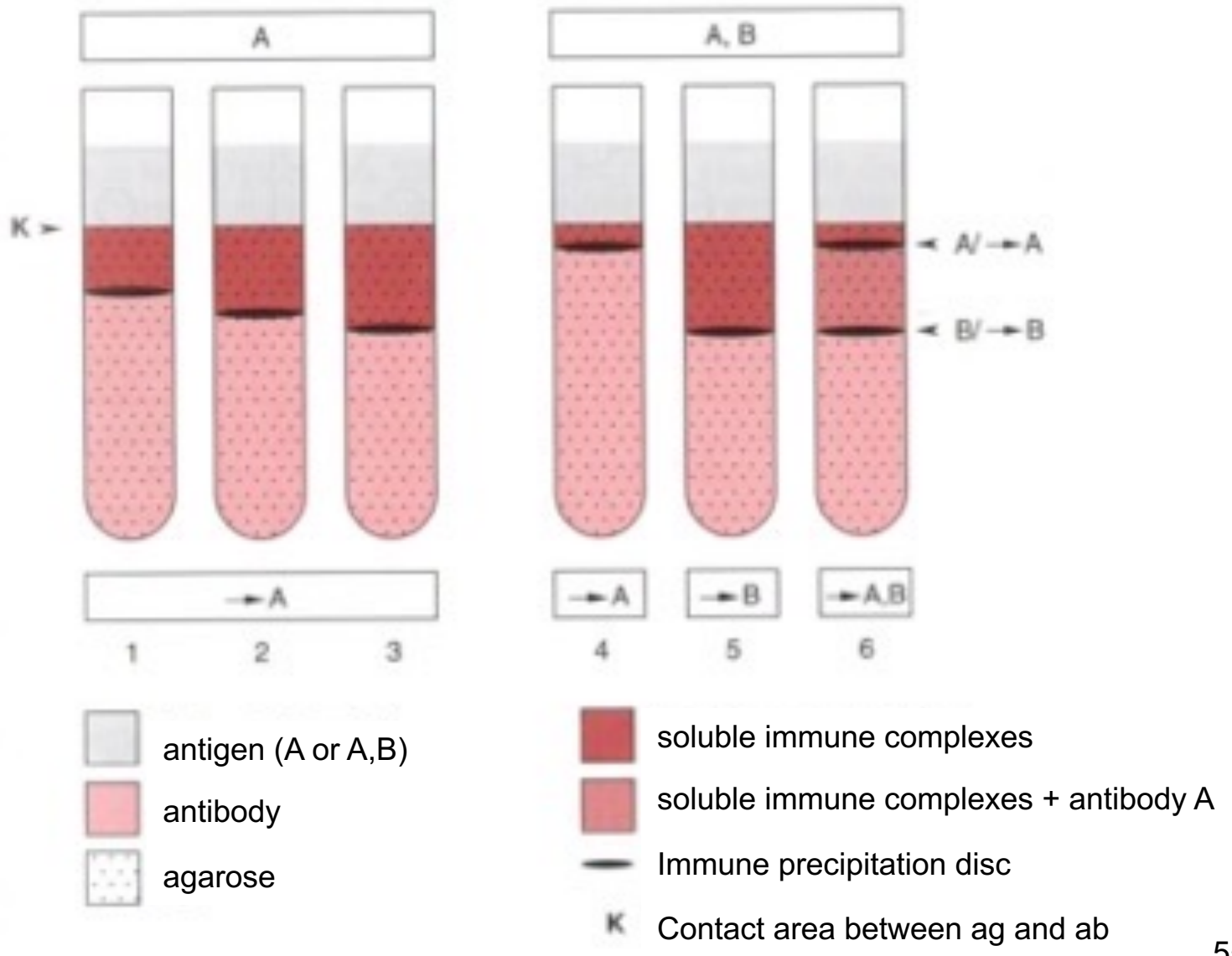
simple diffusion

double diffusion

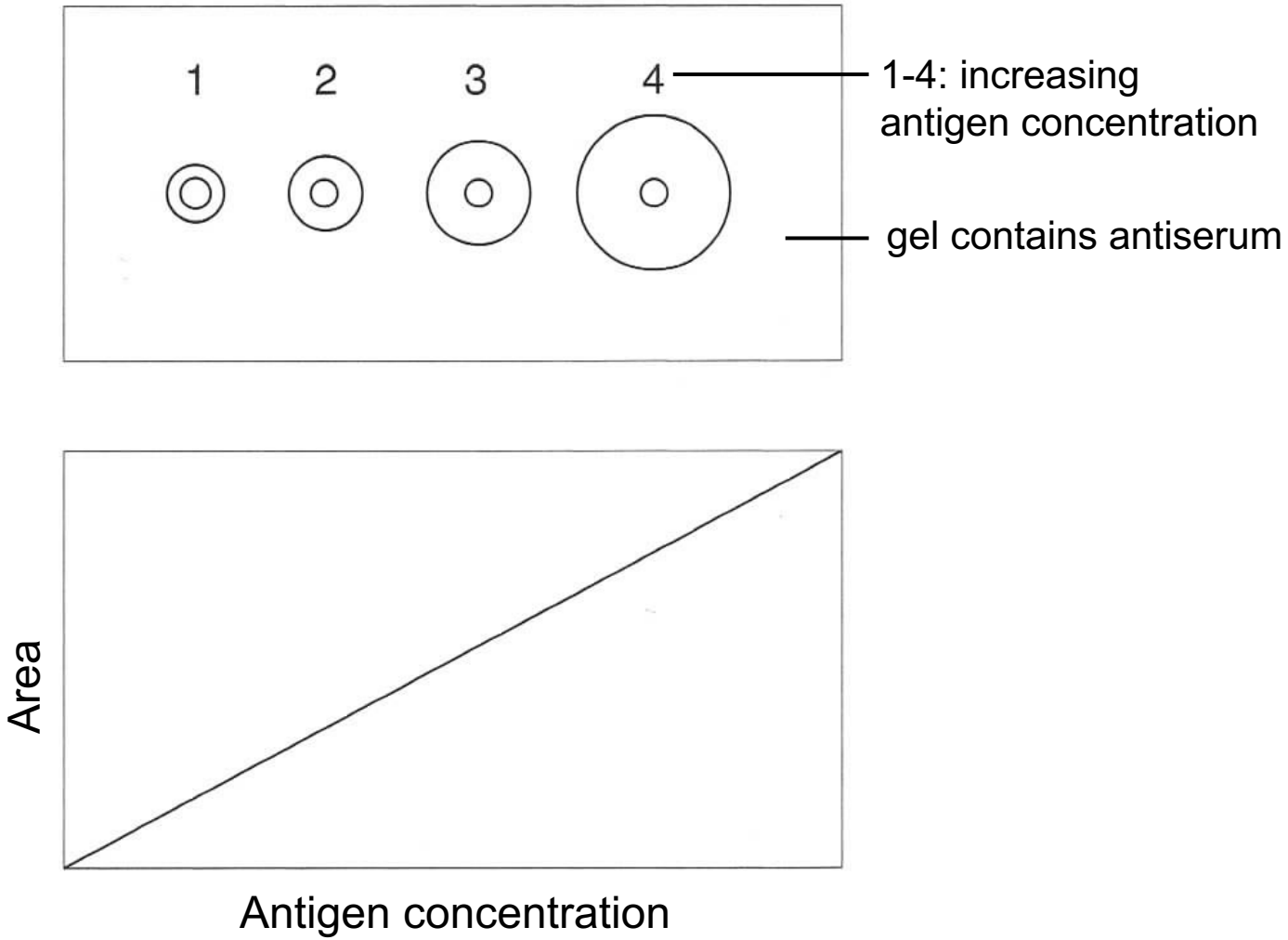


A) Oudin
B) Oakley / Fulthorpe
C) Mancini
D) Ouchterlony

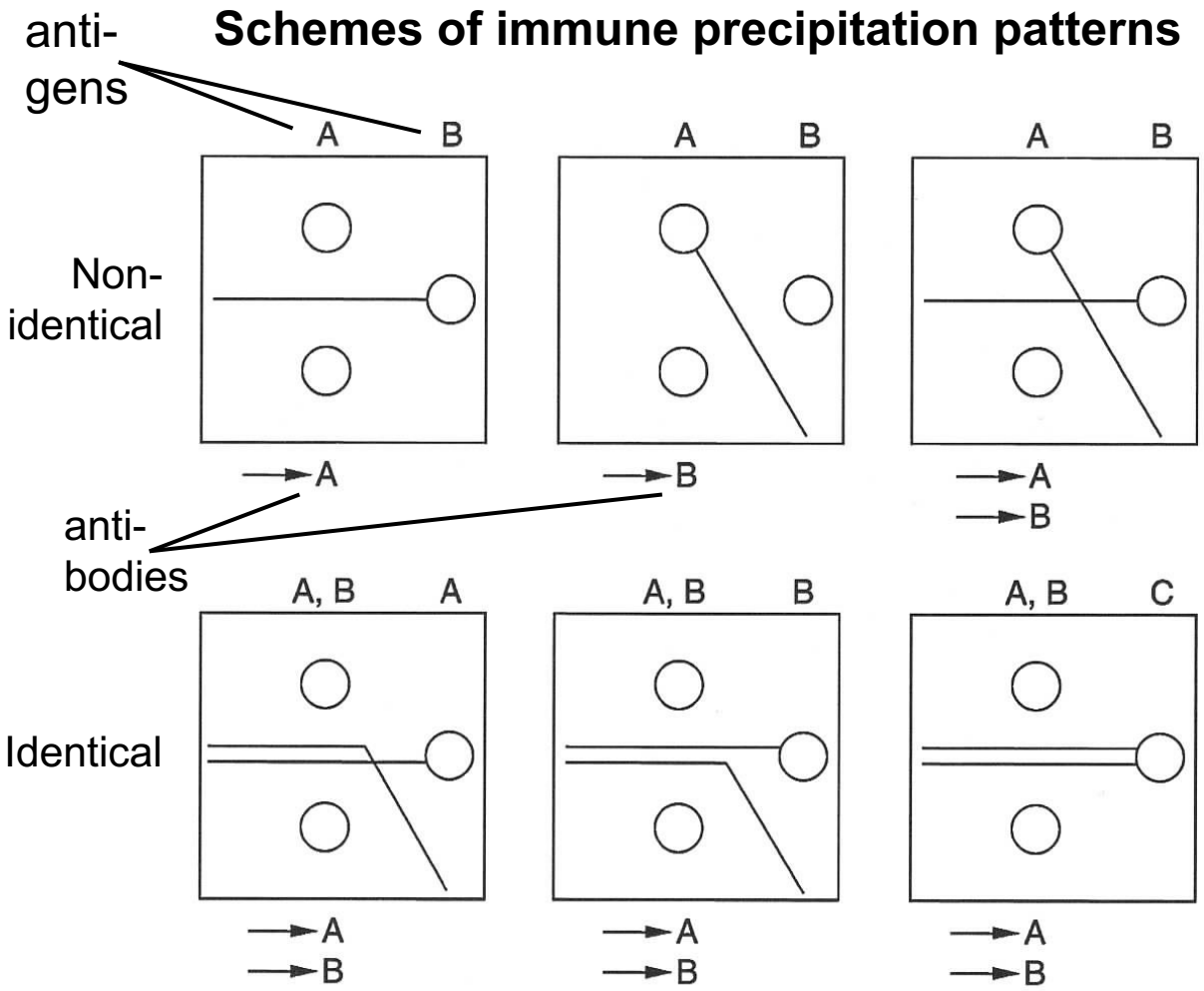
1-dimensional immune diffusion (Oudin)

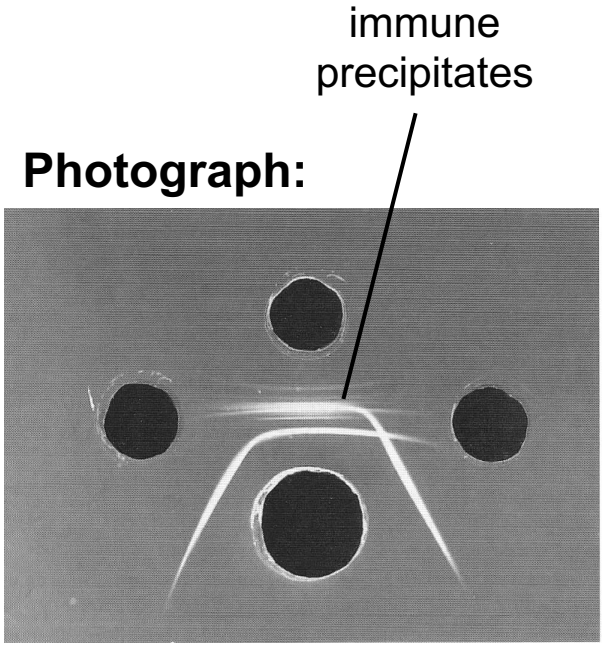


2-dimensional immune diffusion (Mancini)



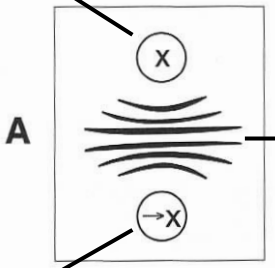
2-dimensional immune diffusion (Ouchterlony)





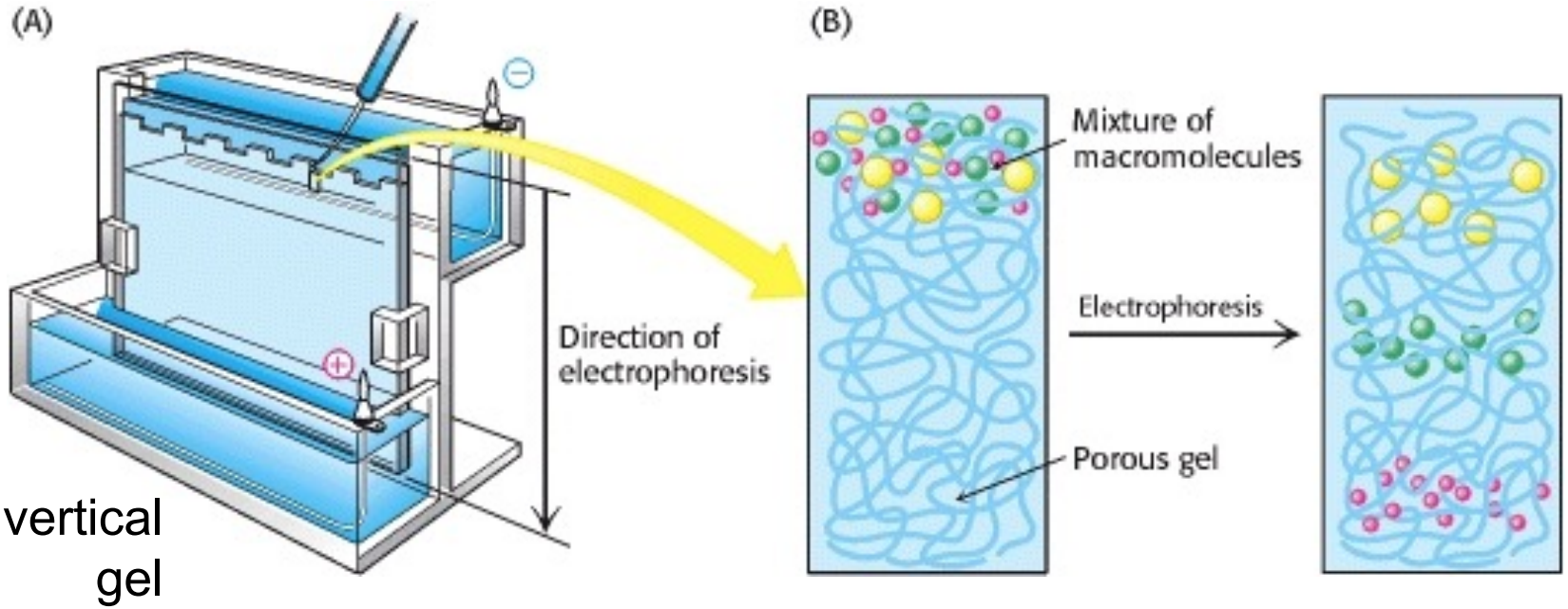
4 holes punched out
in agarose gel

Immune diffusion



Problem:
patterns cannot be resolved

Protein electrophoresis



Migration of proteins/antigens:

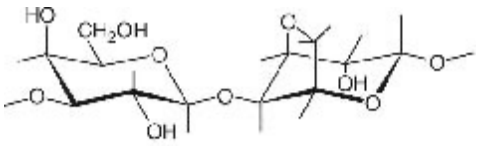
$$v = q * E / f_c$$

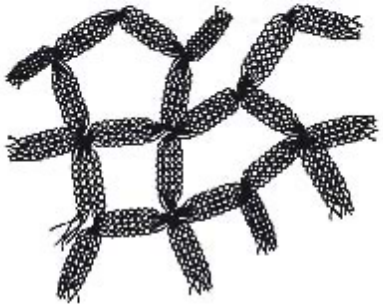
Matrices for protein electrophoresis

slab gel

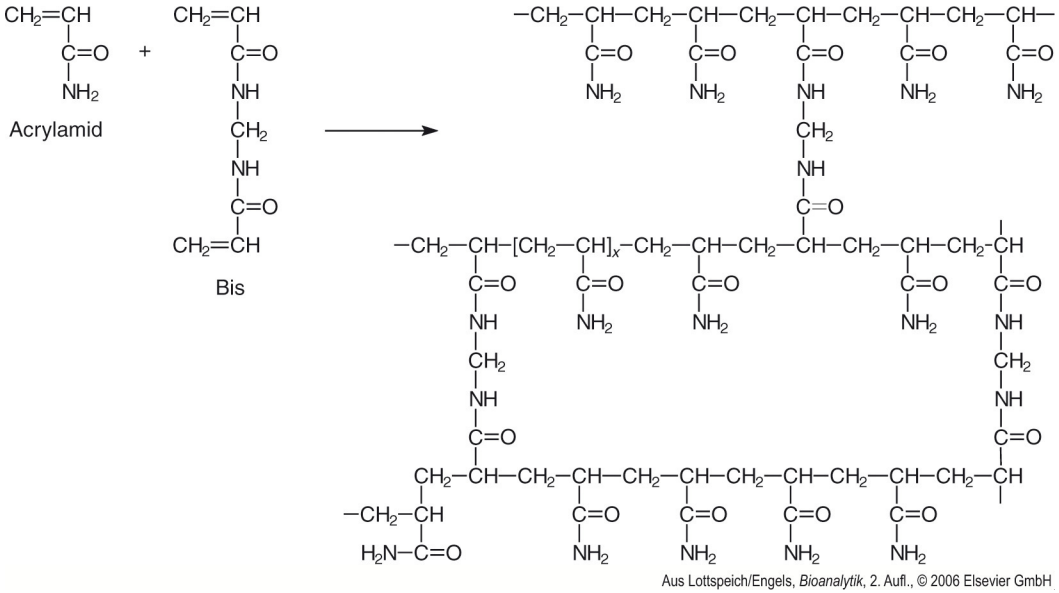
(prevents convective mixing and serves as molecular sieve)

agarose
(more common for DNA)





polyacrylamide (PAGE)



Aus Lottspeich/Engels, *Bioanalytik*, 2. Aufl., © 2006 Elsevier GmbH

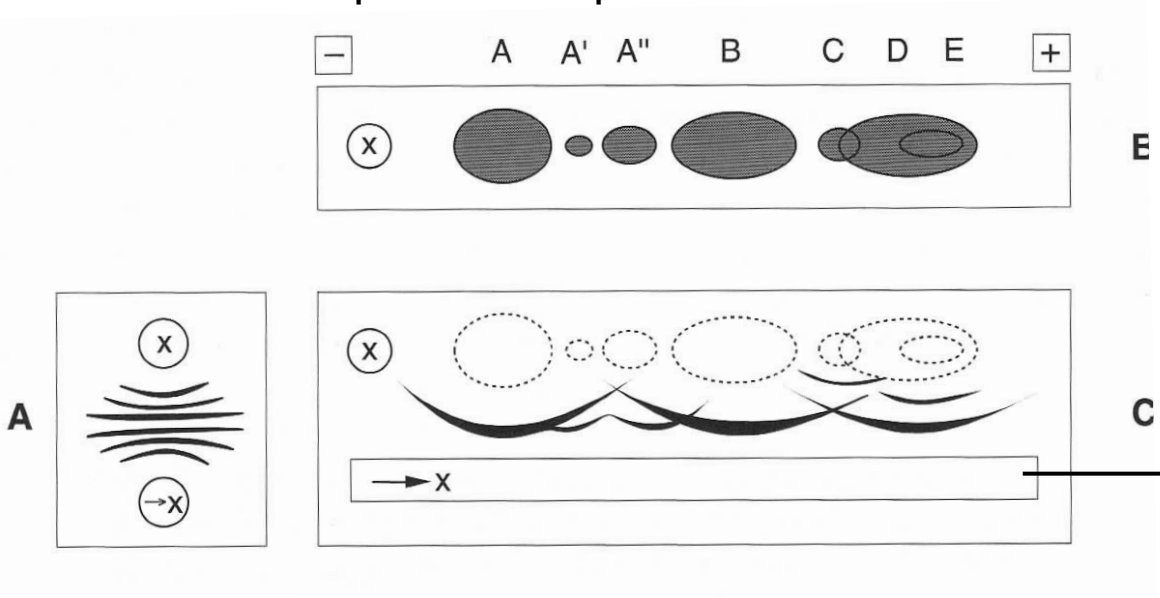
larger ← **pore size** → smaller

(1%: 150 nm) (5 nm)

=> both types of matrices are electrically non-conductive

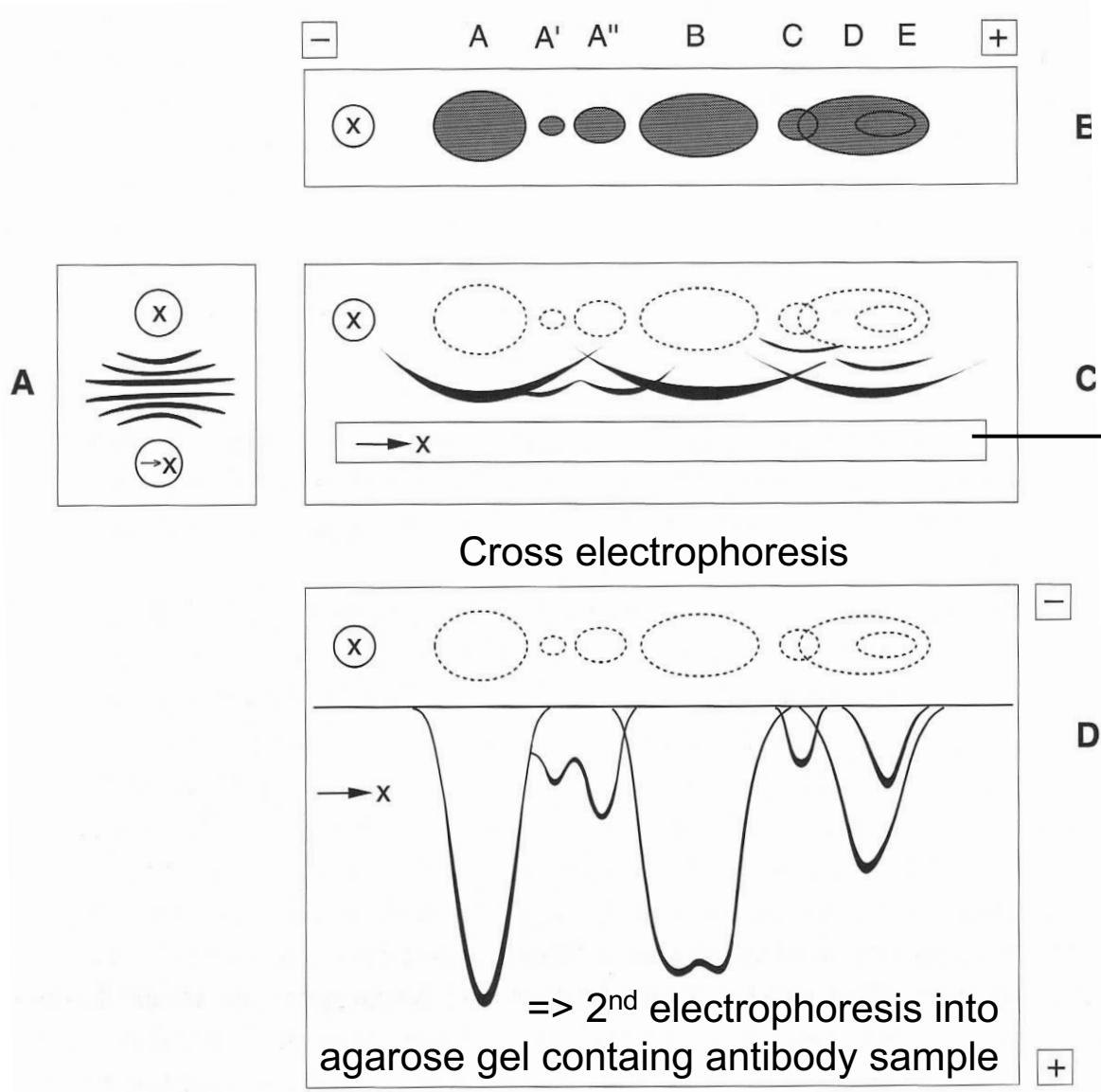
Immune electrophoresis

Separation of proteins in electric field



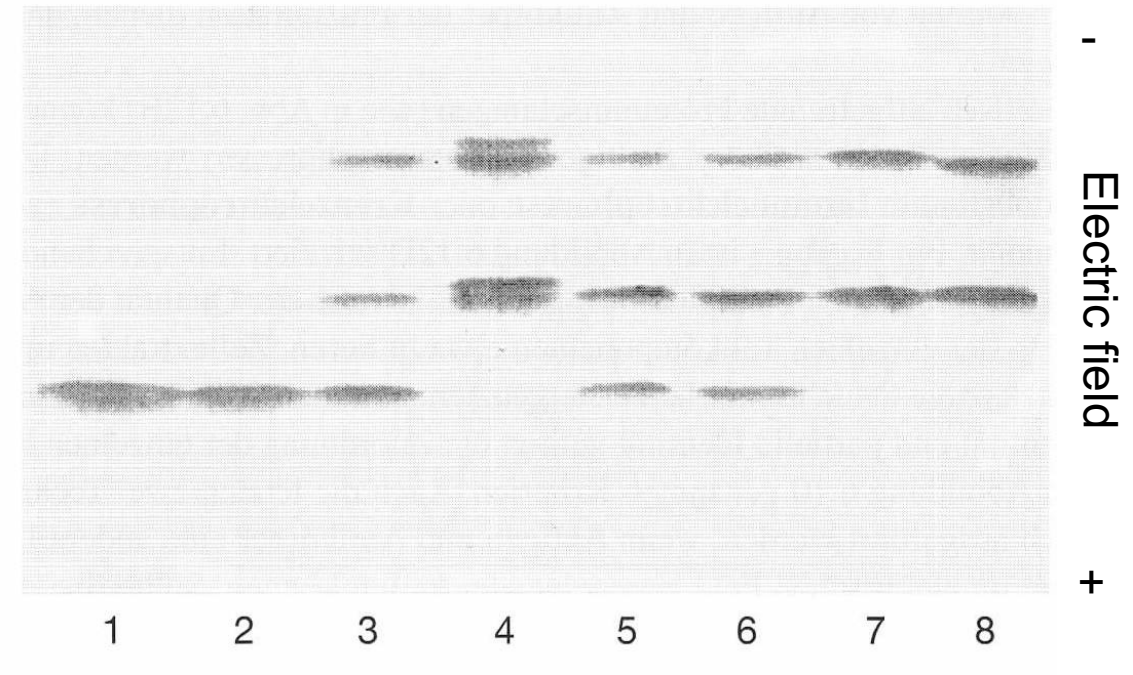
Cross electrophoresis

Separation of proteins in electric field



Immunofixation

1) electrophoresis in e.g. agarose gel
2) diffusion from the agarose gel onto
   a cellulose acetate membrane (**does not** bind proteins)
3) immune complexes precipitate on membrane and are not washed out



Guest lecture: next week

Prof. Tero Soukka
University of Turku, Finland
Department of Life Technologies/Biotechnology

1 pm: Evolution of lanthanide-based labels for immunoassays

2 pm: Research talk open for all

Thank you for your attention