MUNI
SCI

C8116 Immunochemical techniques

Advanced microscopy IV

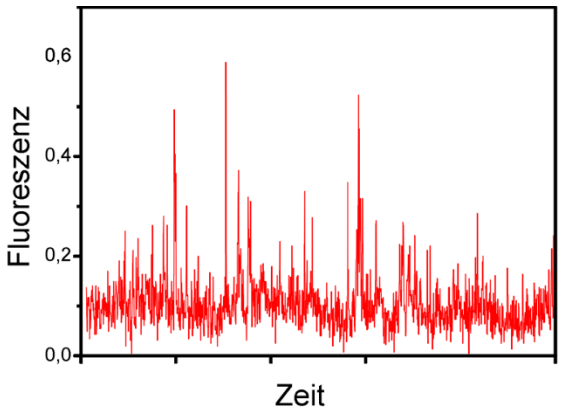
Spring term 2025

Hans Gorris
Department of Biochemistry
May 13th, 2025

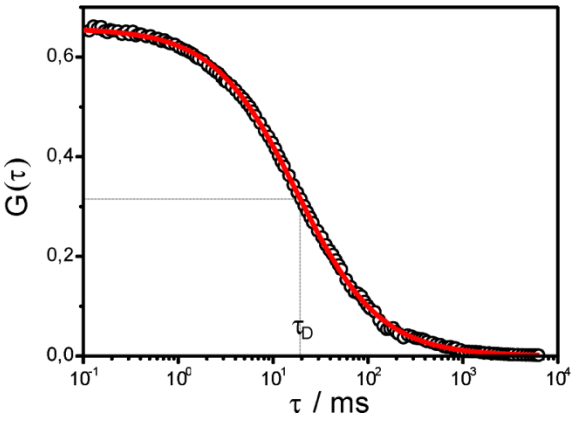
Fluorescence correlation spectroscopy

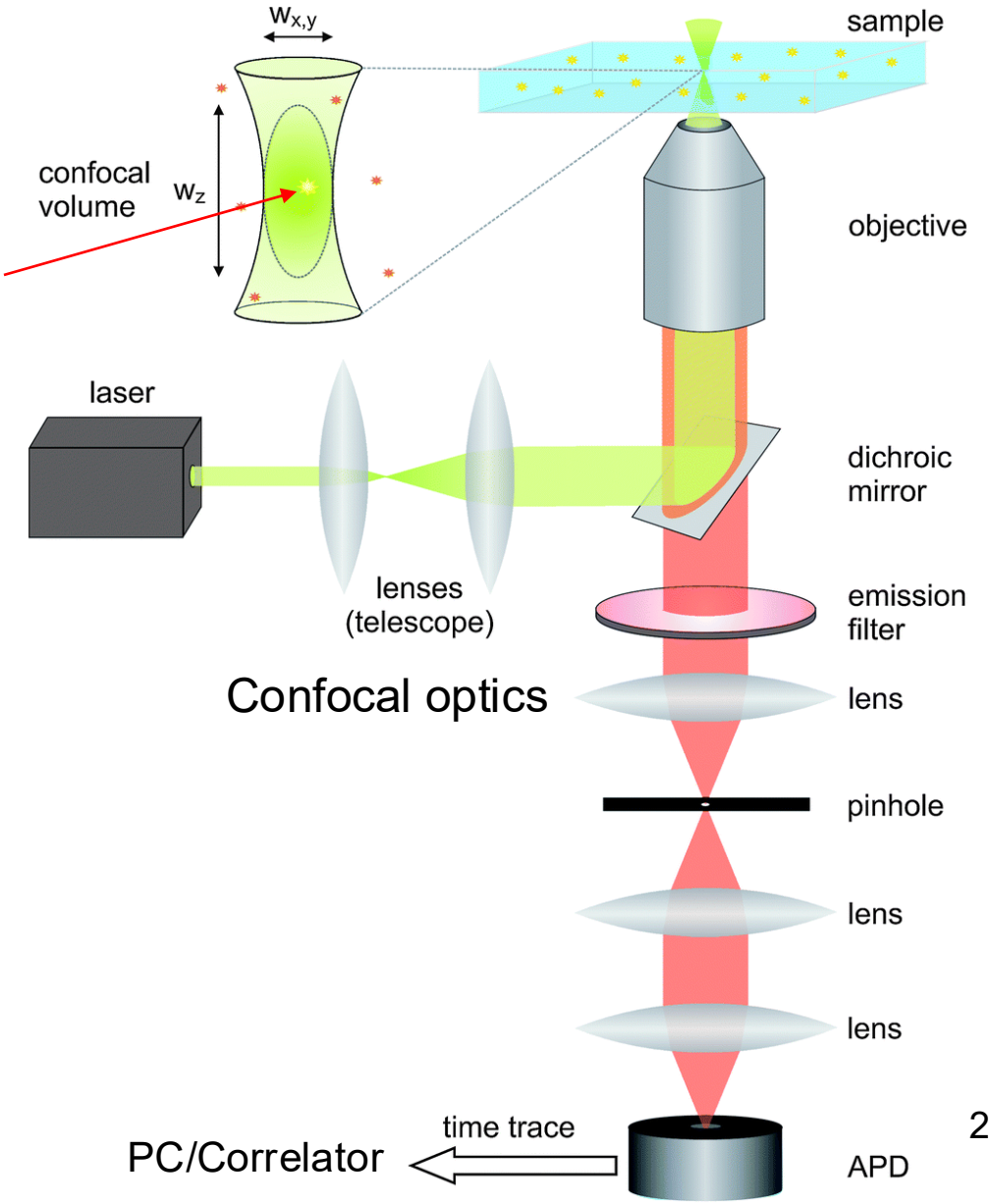
low fluorophore concentration
(~ 0.1 nM = 10^{-10} M)
+ very small focal volumen
(fL = 10^{-15} L)
=> single molecule in focal volume

Raw data



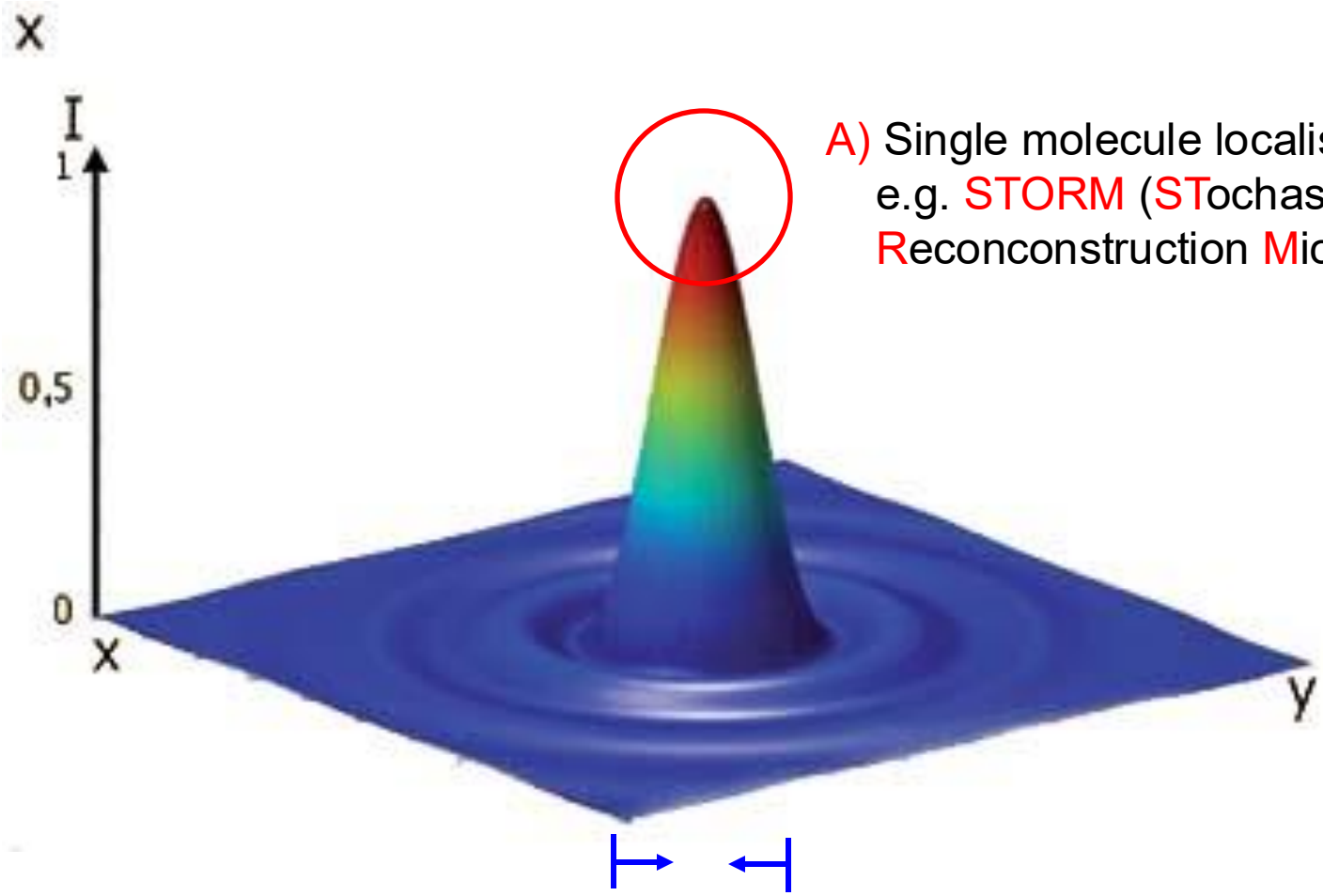
Autocor-
relation
function





Microscopy beyond the diffraction limit

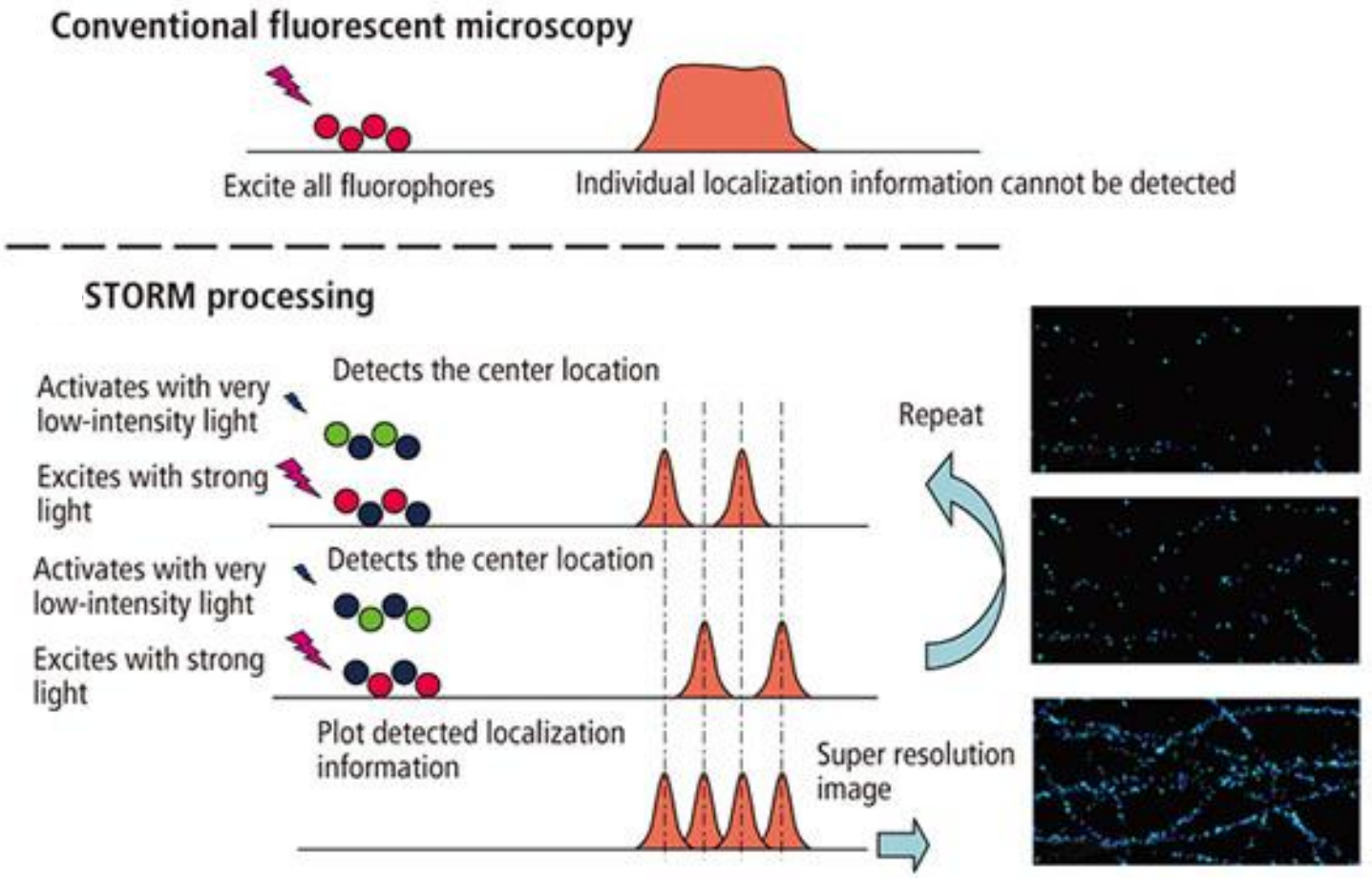
Using non-linear optical processes



A) Single molecule localisation (2006)
e.g. STORM (STochastic Optical Reconconstruction Microscopy)

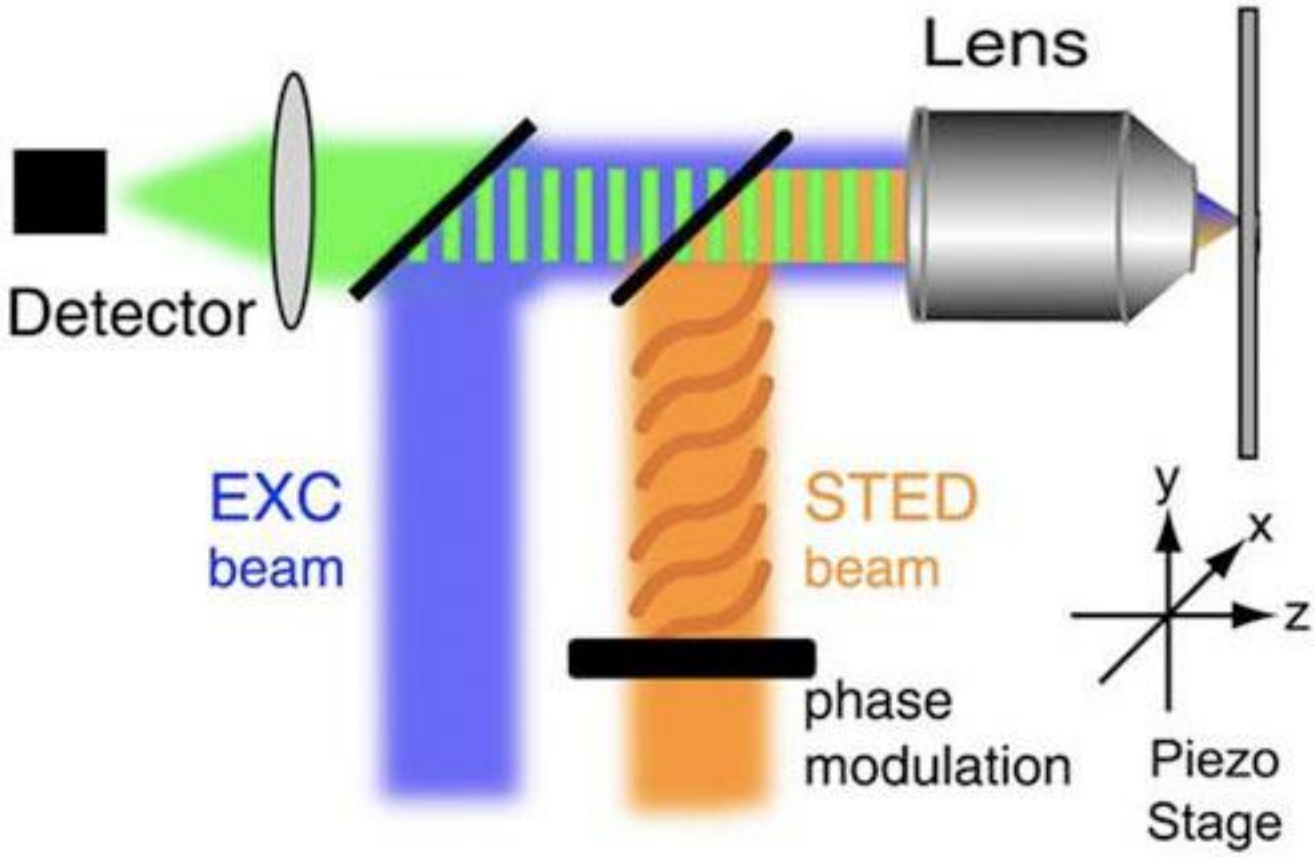
B) Structured illumination (1994/1999)
z.B. STED (STimulated Emission Depletion)

STORM microscopy



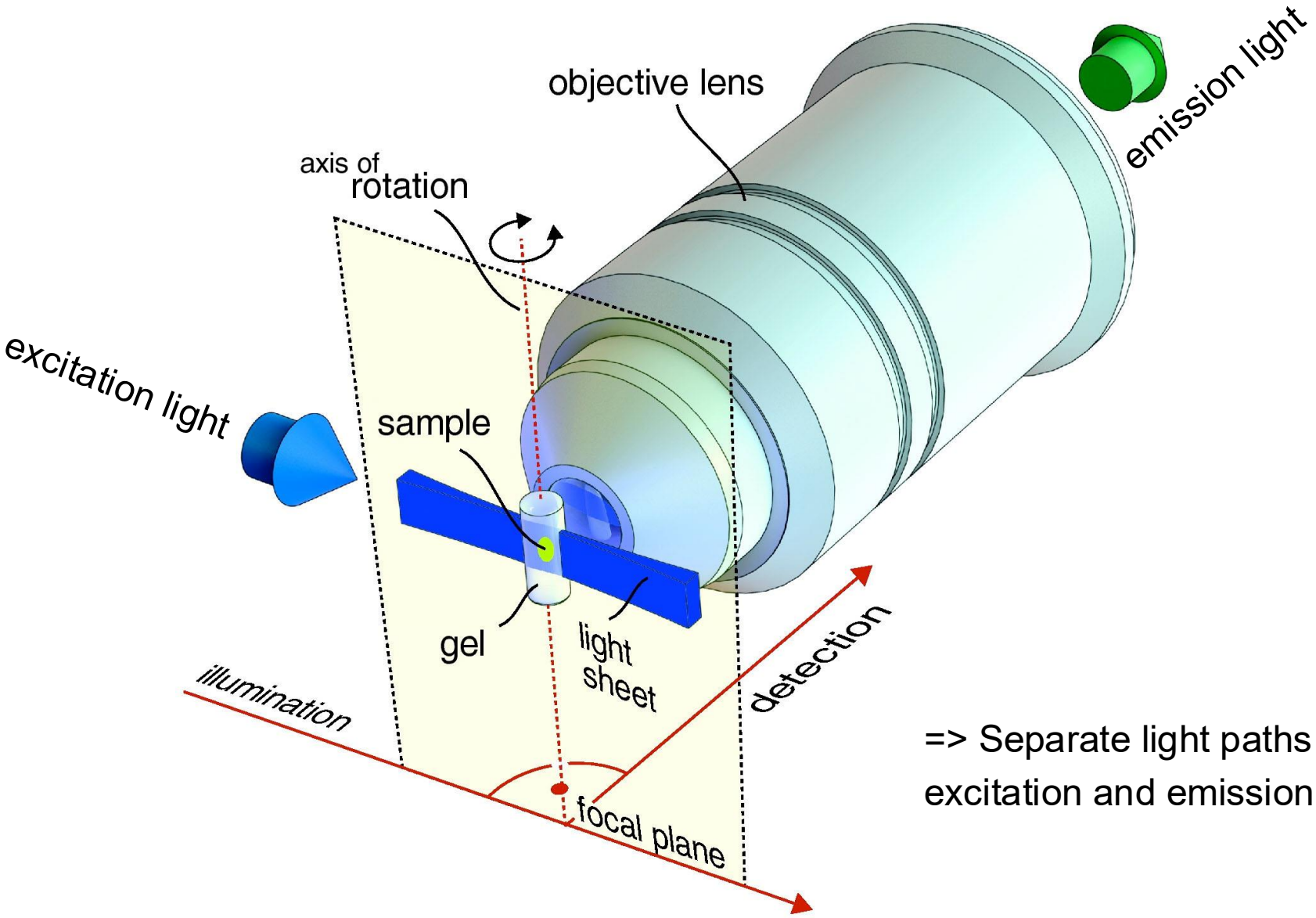
=> Maximum of the point spread function of a single fluor. molecule can be determined precisely
But: 1000-10.000 images required to put together a high-resolution image
=> Need for high computational power / appropriate „switchable“ fluorophores

STED microscopy: instrumental setup



EXC und STED are pulsed lasers with defined timing of pulses

Light sheet microscopy

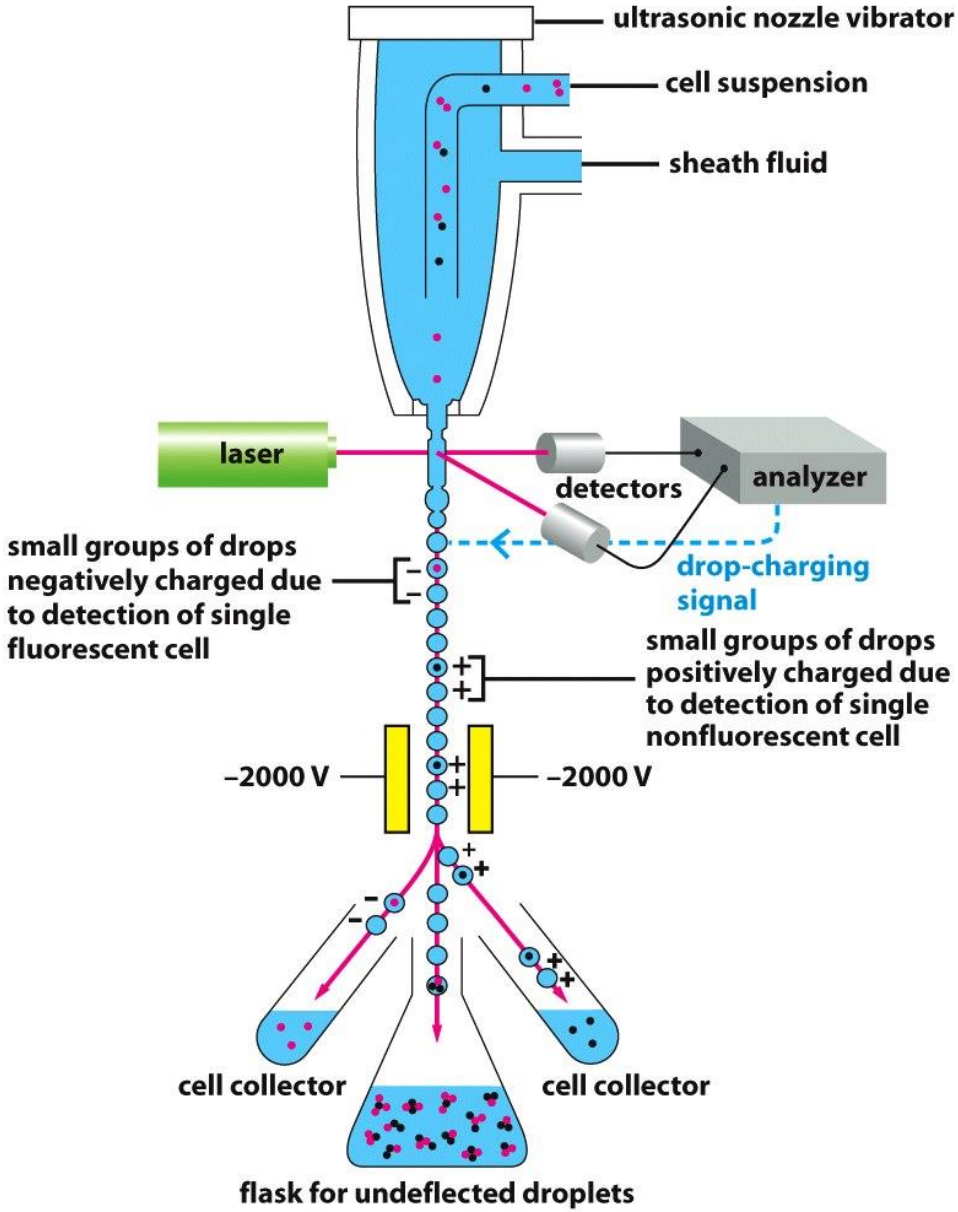


=> Separate light paths for excitation and emission light

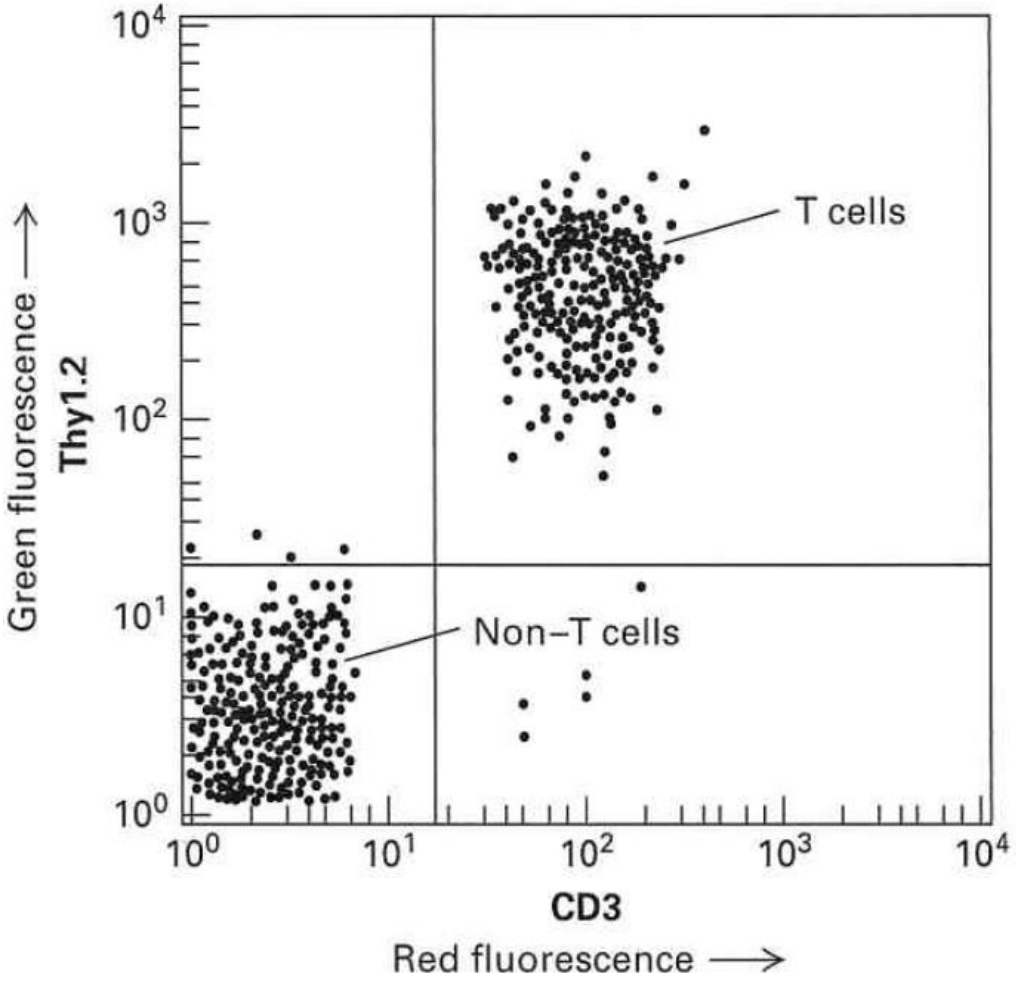
Fluorescence activated cell sorting

(FACS)

Fluorescence activated cell sorting (FACS)



Separation of T cells

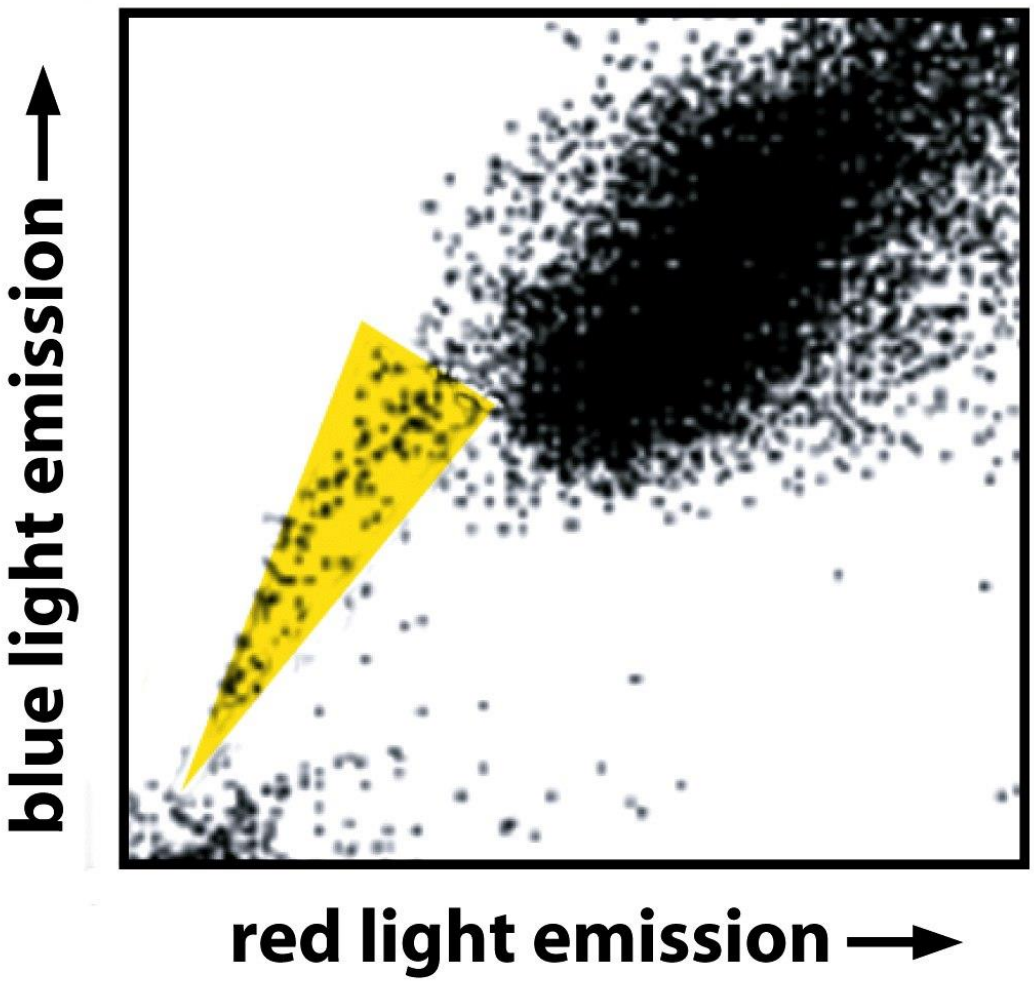


CD3 cell surface protein (T cell marker): Specific antibody labeled with red fluorophore

Thy1.2 cell surface protein (T cell marker): Specific antibody labeled with green fluorophore

=> Only T cells express the cell surface markers and can be isolated

Identification of cancer stem cells



Less fluorescent cancer stem cells (highlighted in yellow) are identified by cell sorting

Luminescent nanoparticles

=> Replacement for fluorescent dyes

Nanoparticles (size definition: 1-100 nm)

- Absorption (e.g. gold nanoparticles)
- Luminescence (similar use as organic dyes)
- Förster resonance energy transfer (FRET)
- Raman (plasmonic) enhancement: SERS
- Contrast enhancement (e.g. in TEM)
- Magnetism

Limitations of fluorescent dyes

1. Single fluorescent molecules are not visible with a conventional fluorescence microscope (high background signal because of autofluorescence etc.).
2. Fluorescence depends on the chemical environment (e.g. solvents, pH variation or protein binding).
3. Many fluorophores are hydrophobic and are deposited in certain cellular compartments => artefacts.
4. Cytotoxic effects of some fluorophores.
5. Labeling requires not only fluorescent signal generation but also targeting (e.g. Immuno labeling: targeting is mediated by an antibody).
6. Ratiometric measurements require two fluorescent signals.

Luminescent nanoparticles: an alternative

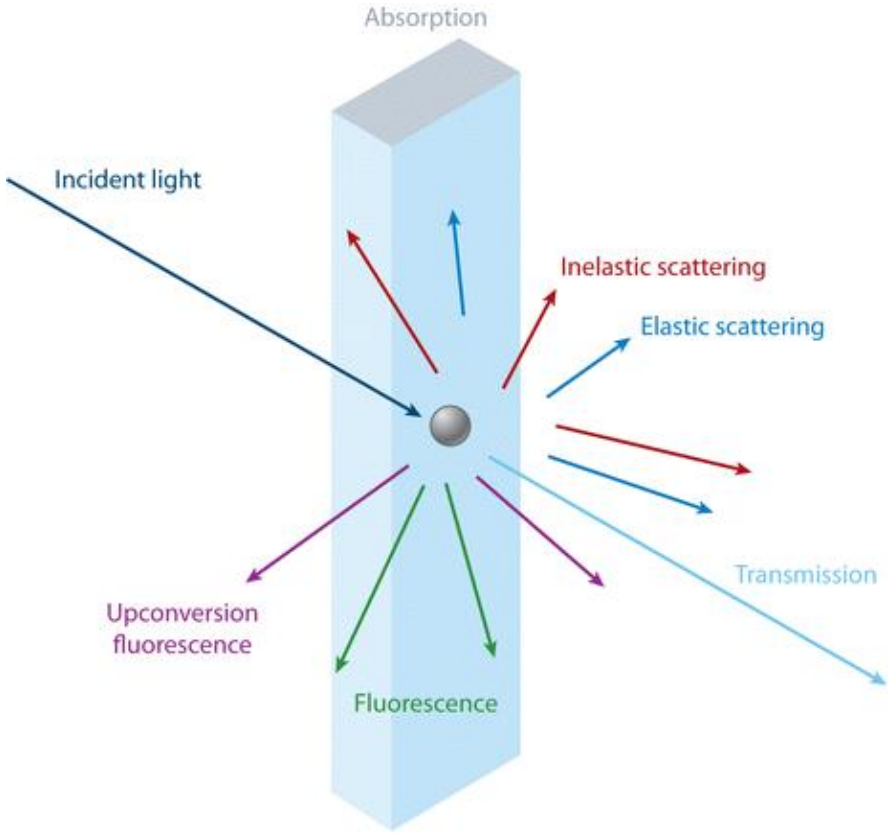
Advantages:

- Signal enhancement
- Chemical and photo-stability
- Tunable emission
- Carrier system (e.g. for targeting)
- Can combine various features => hybrid nanoparticles
  (e.g. luminescence + magnetism + NMR contrast, etc.)
- Measuring various parameters (pH, oxygen, ions)
- Parallel measurements of different analytes
  (e.g. different proteins: multiplexing)
  => Vision: “Lab-on-a-bead“

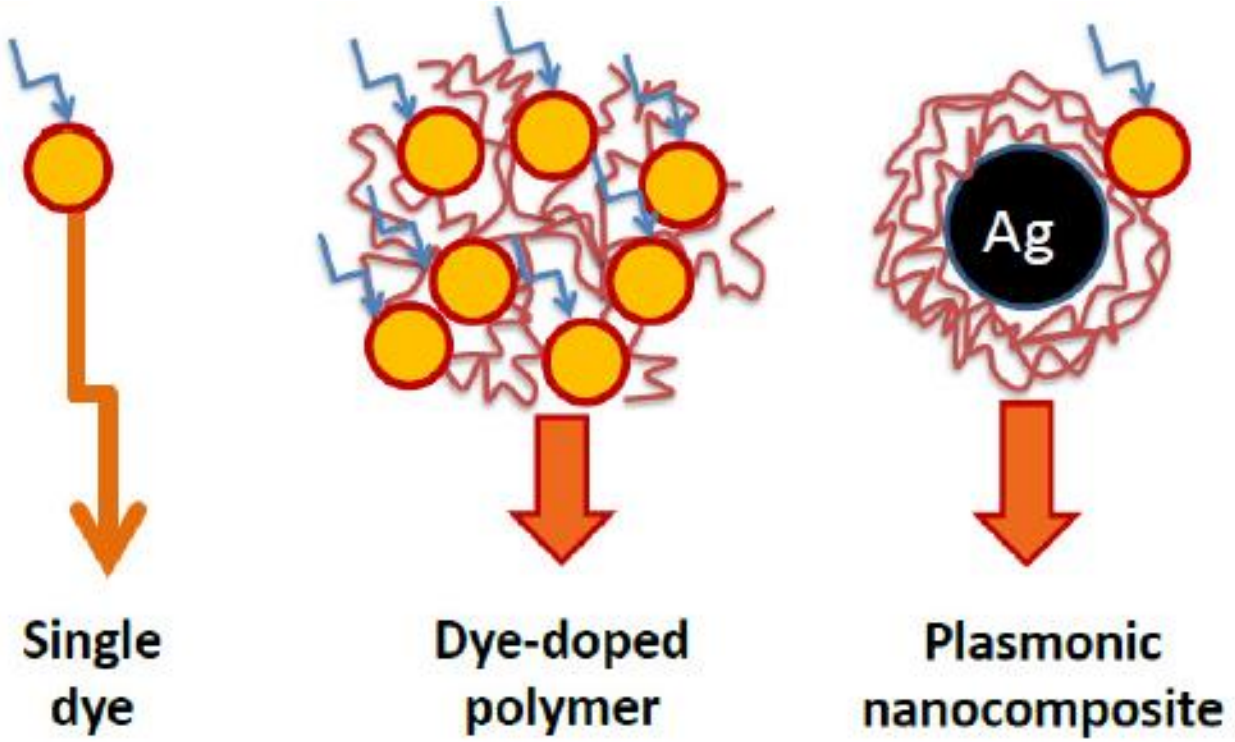
Disadvantages:

- quite large
- synthesis can vary from batch to batch

Nanoparticles: Optical information



Luminescent nanoparticles: signal enhancement



Different types of luminescent nanoparticles

- Polymer nanoparticles
- Silica nanoparticles

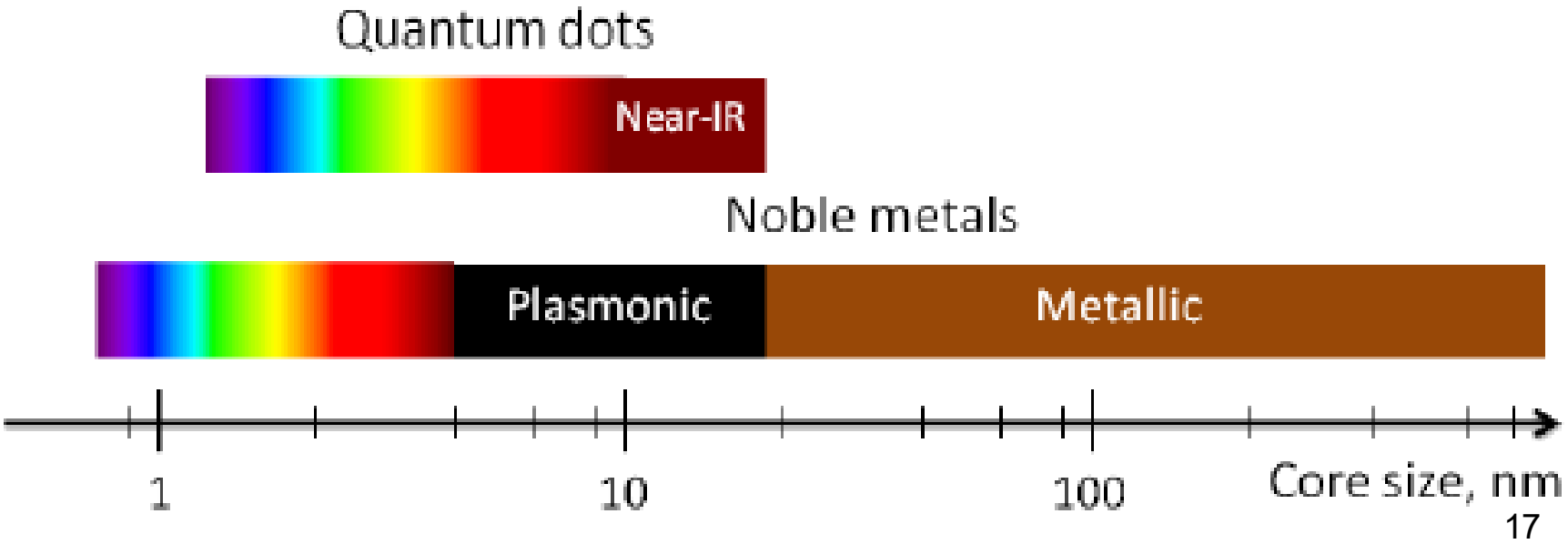
} based on organic fluorophores

- Quantum dots (QDs)
- Metallic nanoparticles

} size-dependent luminescence

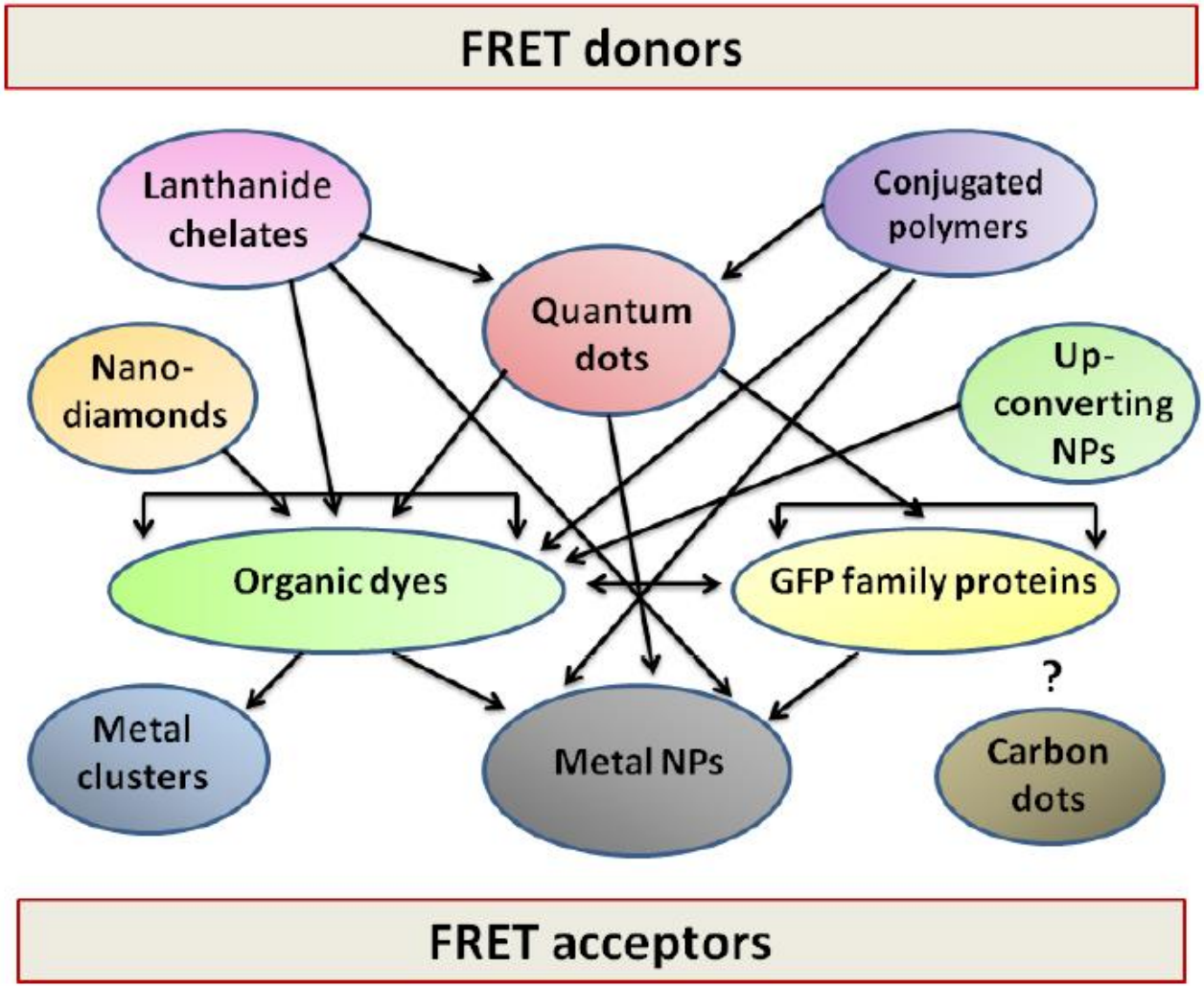
- Photon-upconversion nanoparticles (UCNPs)

} based on nanomaterial properties



A.P. Demchenko, Methods Appl. Fluoresc. (2013) 1: 022001

Luminescent nanoparticles for FRET



Polymer nanoparticles

=> Encapsulation of fluorescent dyes in a polymer matrix

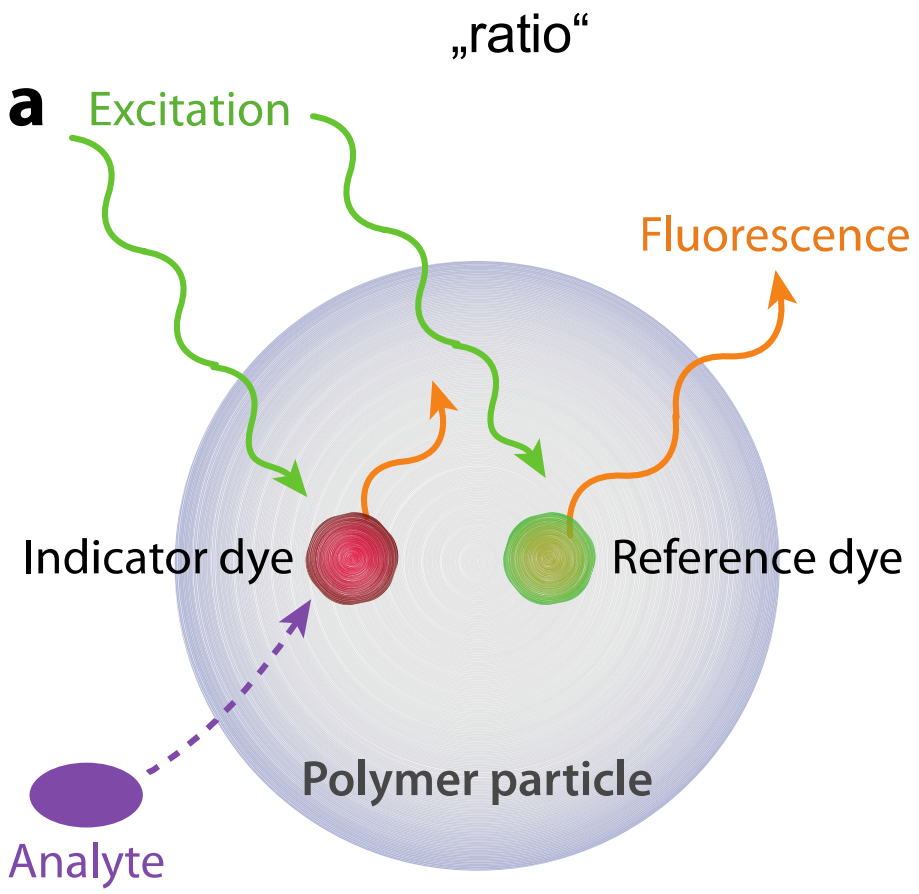
Polymer nanoparticles

1. Very bright

2. Variable polymer matrix (e.g. polyacrylonitrile, polystyrene):
   => enclosing one or several fluorophore(s)
   => can protect the fluorophore(s) from the environment
   => selection of pore sizes (sieve effect) to allow certain analytes access to the fluorophores
   => ratiometric measurements

3. Flexible design of the nanoparticle surface
   => hydrophilic groups (e.g. PEG)
   => ligands for targeting
   => fluorescent indicators

Polymer nanoparticles: fluorescence sensing



PEBBLE
(Probes Encapsulated By Biologically Localized Embedding)

Y. K. Lee, R. Smith, R. Kopelman, *Annu. Rev. Anal. Chem.* (2009) 2: 57-76

Polystyrene nanoparticles: hydrophobic

A) Preparation:

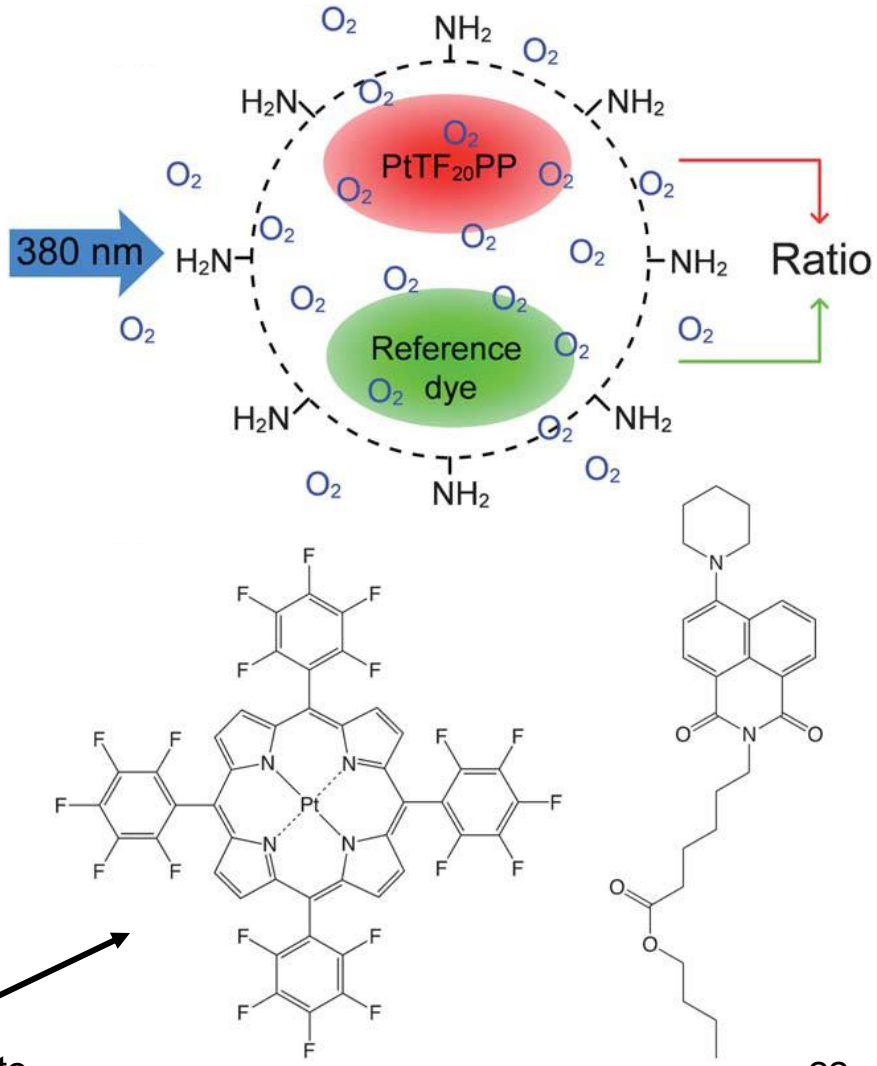
1. Polystyrene swells in organic solvents (THF); addition of fluorophore (hydrophobic)
=> Fluorophore is enclosed in nanoparticle matrix

2. Solvent exchange against buffer
=> Fluorophore is permanently entrapped in nanoparticle (in hydrophobic environment)

B) Inserting into cells

C) Fluorescence microscopy

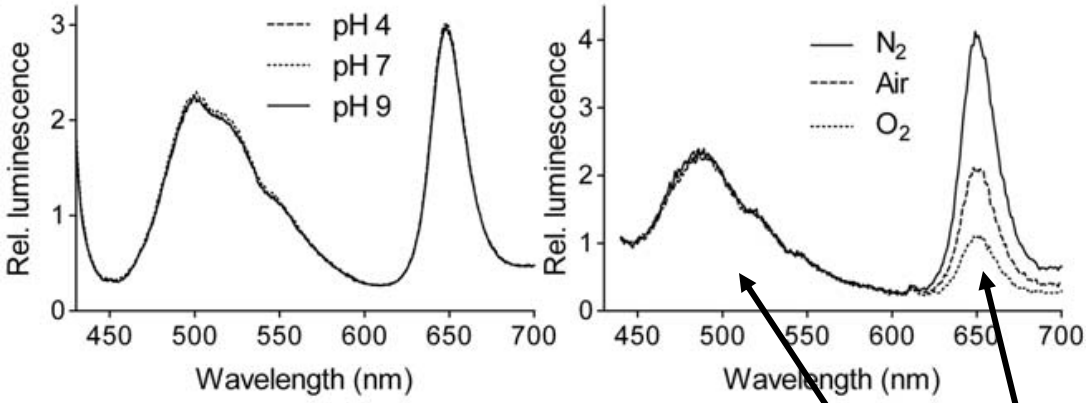
Here: Excitation with a single wavelength!

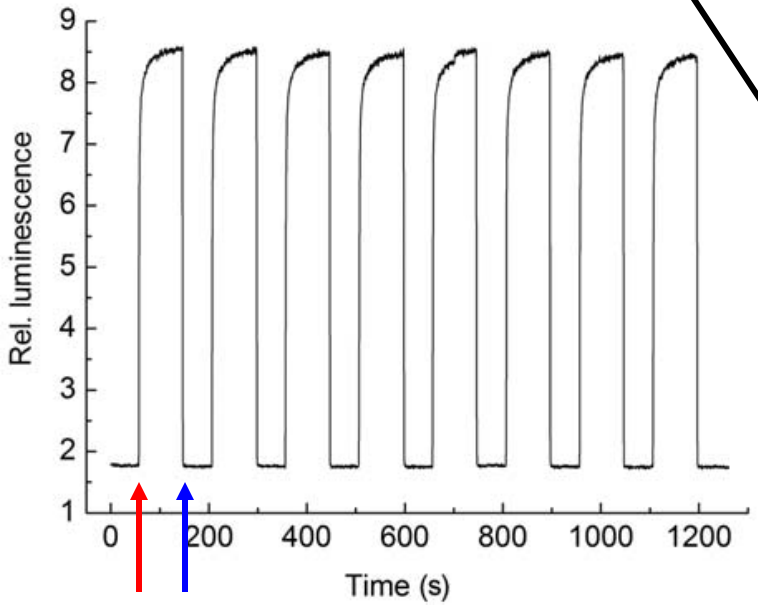


Oxygen-sensitive Platinum(II)meso-tetrakis-(pentafluorophenyl)-porphyrinato ($PtTF_{20}PP$) complex.

Reference dye

Ratiometric measurement of oxygen





alternating: N_2 O_2

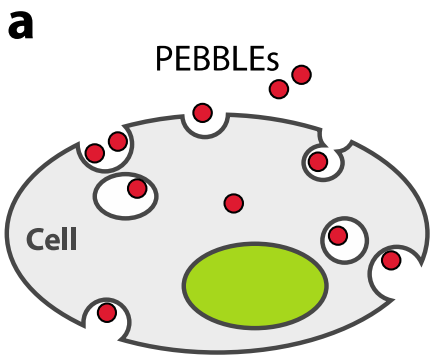
Polystyrene matrix (hydrophobic) has several functions:

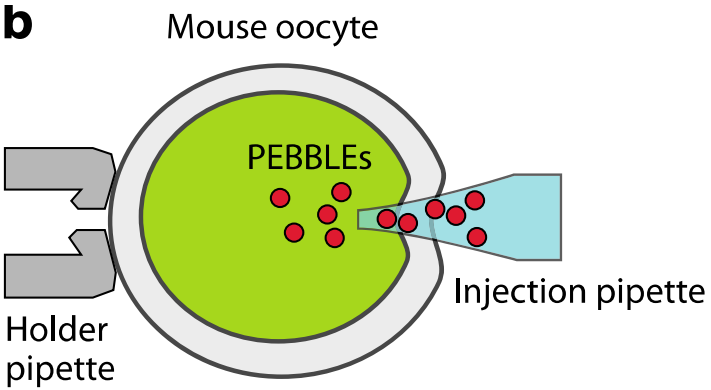
1. Permanent encapsulation of two fluorescent dyes
2. Provides access for oxygen but not for protons
3. Amino groups on surface result in a good dispersibility in water

PEBBLE

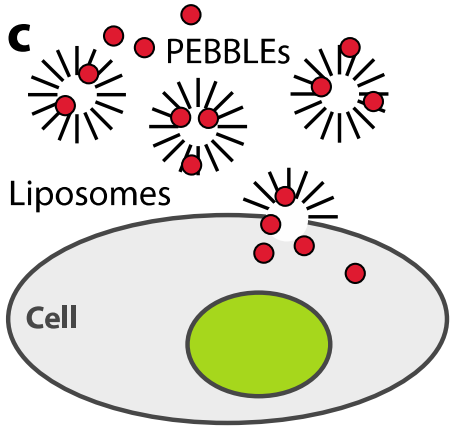
1. is specific for oxygen
2. enables „ratiometric“ measurements

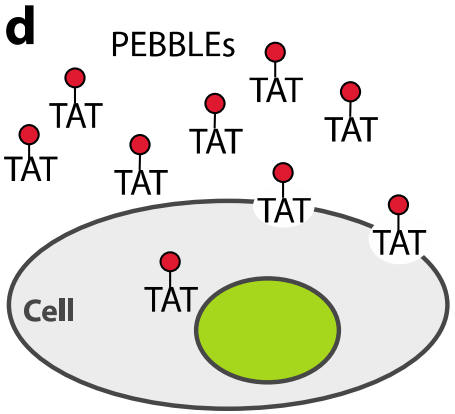
Insertion of nanoparticles into cells

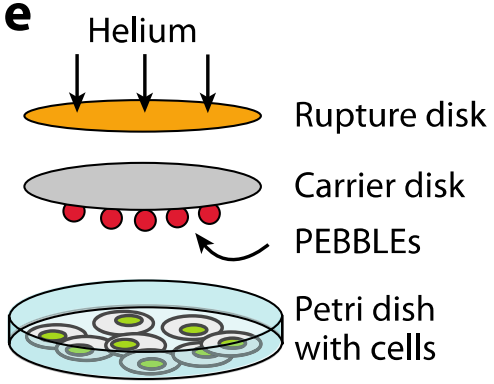




a) endocytosis
b) injection
c) liposomes
d) Membrane-penetrating peptide
e) Gene gun

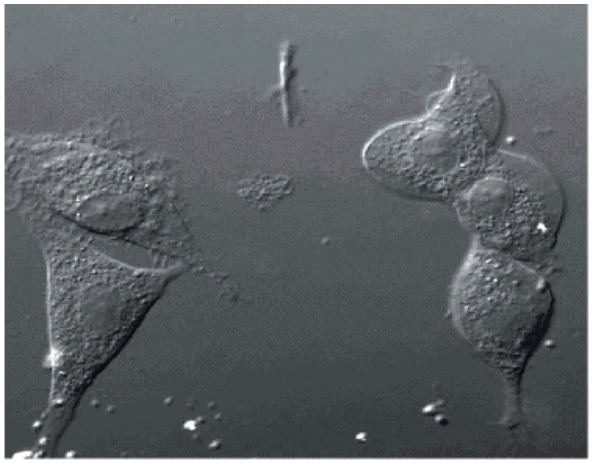






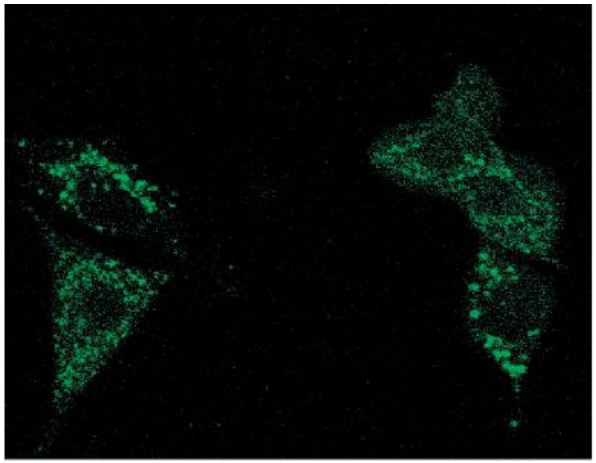
Ratiometric measurement using PEBBLEs

Interference contrast



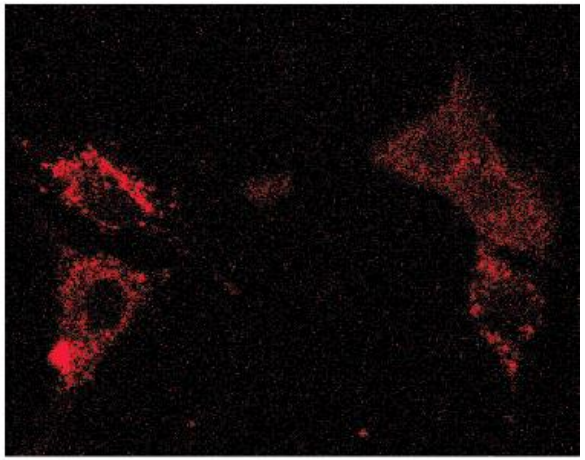
(a)

Reference dye

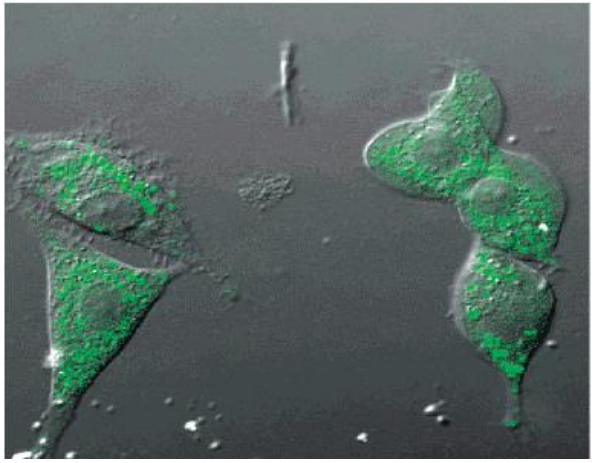


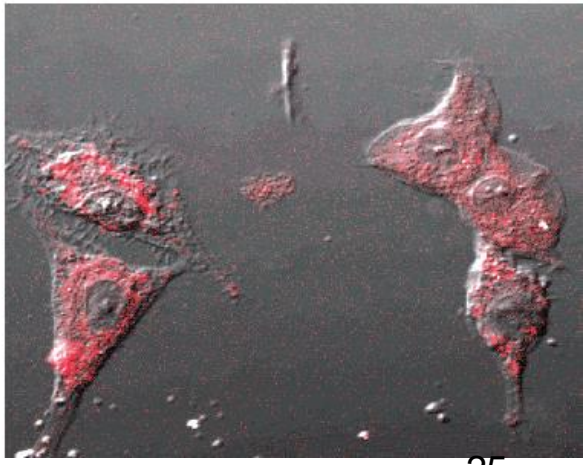
(b)

Oxygen probe



(c)





=> Inserted by “gene gun“

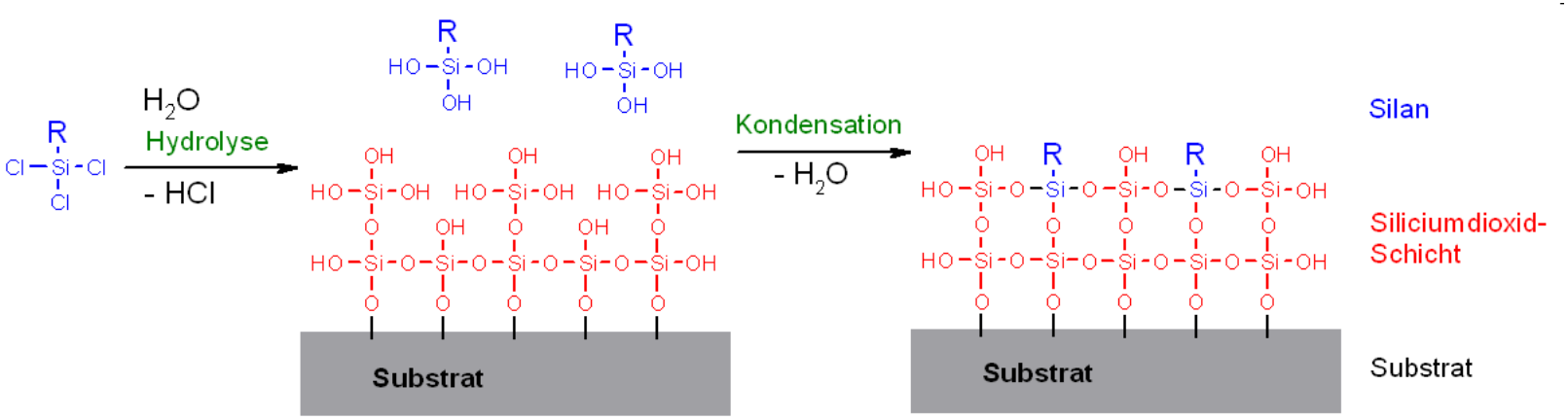
Silica nanoparticles

=> Encapsulation of fluorescent dyes in a silica matrix

Silica nanoparticles

Features:

- very stable (inorganic matrix)
- simple synthesis under mild conditions
- hydrophilic (=> dispersible in water)
- biocompatible
- simple surface modification (silanization)



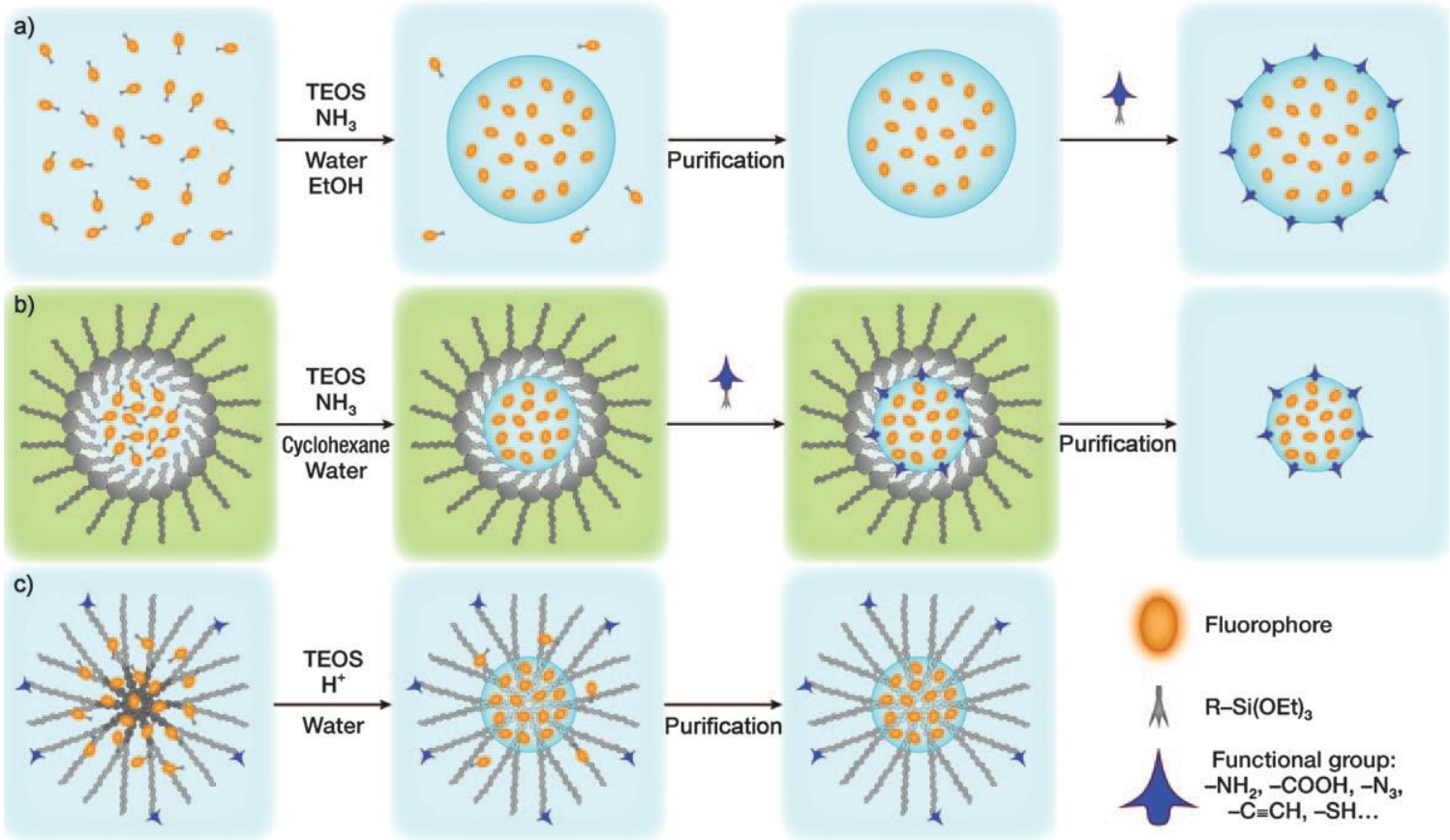
- can be combined with organic silanes (Ormosil)

Silica nanoparticles: synthesis

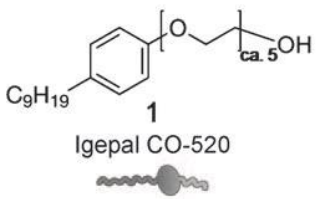
Stöber method

reverse microemulsion (water in oil)

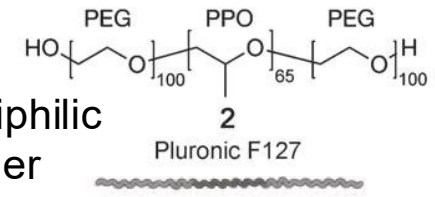
amphiphilic polymers



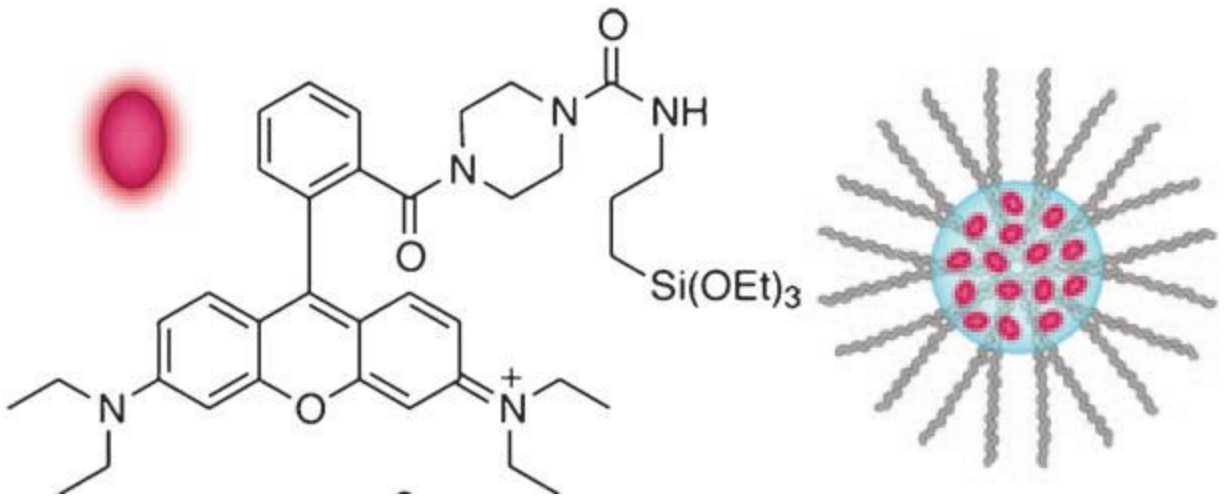
detergent

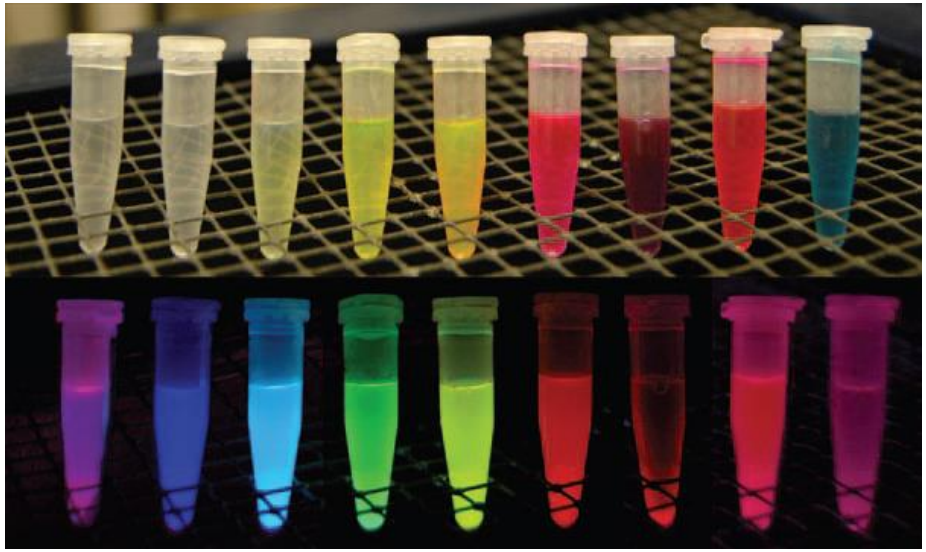


amphiphilic polymer



Silica nanoparticles: variable composition

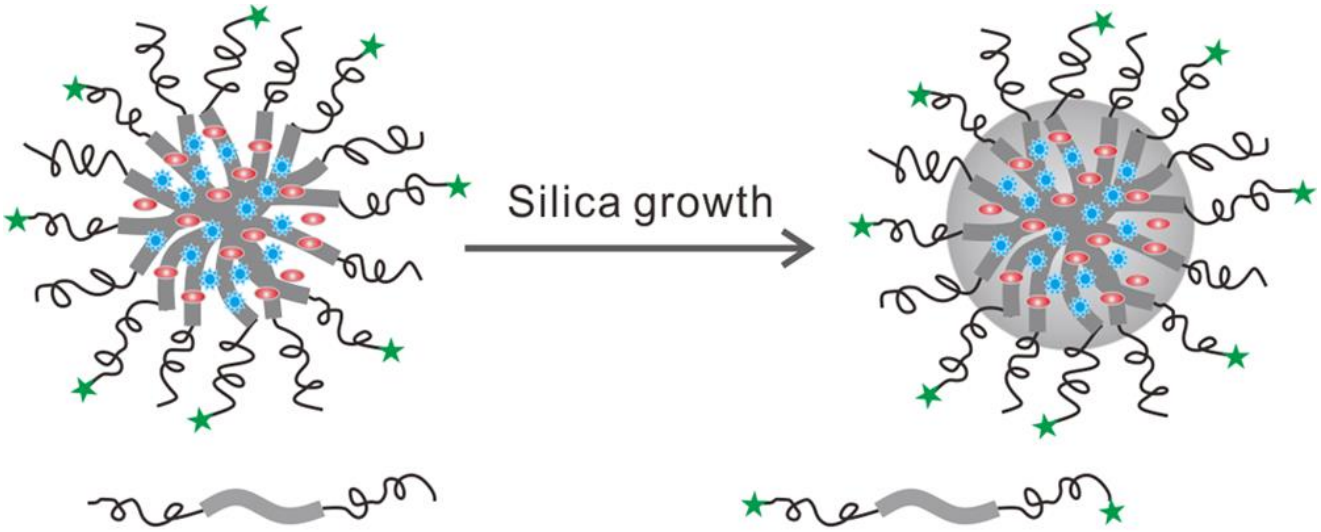




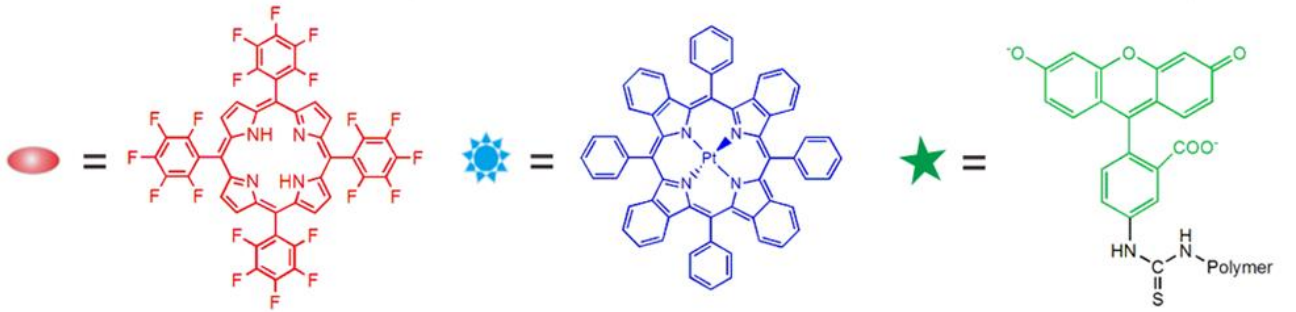
daylight

UV light

Silica nanoparticles for ratiometric measurements



12 nm
in diameter



Tetrakis(pentafluorophenyl) porphyrin (TFPP)

Platinum(II) tetraphenyl tetrabenzoporphyrin (PtTPTBP)

Fluorescein isothiocyanate (FITC)

Reference dye

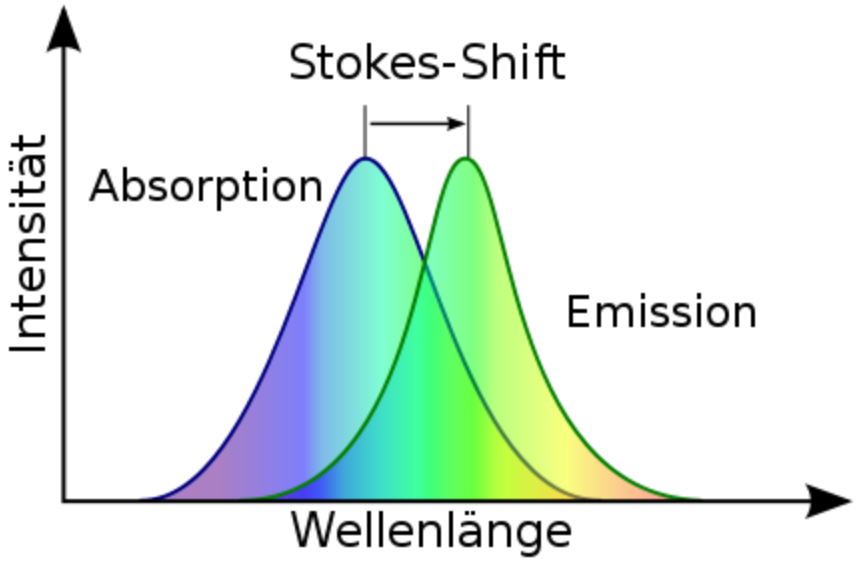
Oxygen probe

pH probe

Limitations of polymer and silica nanoparticles

Fluorophores encapsulated in nanoparticles (silica/polymer) provide many advantages,
But still suffer from some disadvantages of conventional dyes:

1. Photobleaching
2. broad / overlapping emission bands (disdavantage in multiplexing)
3. limited Stoke‘s shifts (absorption / emission overlaps: background)
4. excitation with short-wavelength light (autofluorescence / light scattering)



=> Filter sets to separate excitation and emission light

Quantum dots

Quantum dots (QD): Introduction

Used for 20 years in bioanalysis

=> Semiconductor nanoparticles (Ø ca. 2 - 10 nm)
consisting of heavy metal salts
e.g. CdSe, CdTe, InP, InAs (toxic)

- The emission wavelength depends on diameter and material

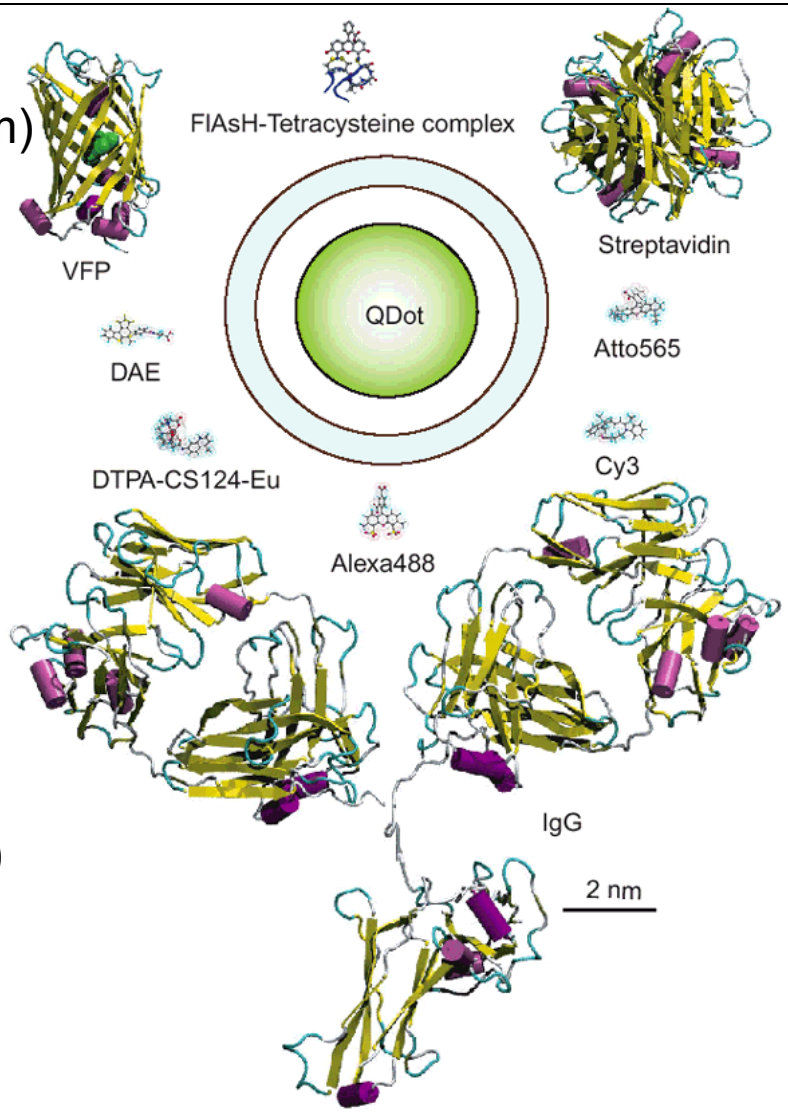
- can be excited together (absorb light of wavelength, that has enough energy to cross the energy gap)

- high excitation coefficients

- narrow emission bands (provided the size distribution is homogeneous)

- photostable

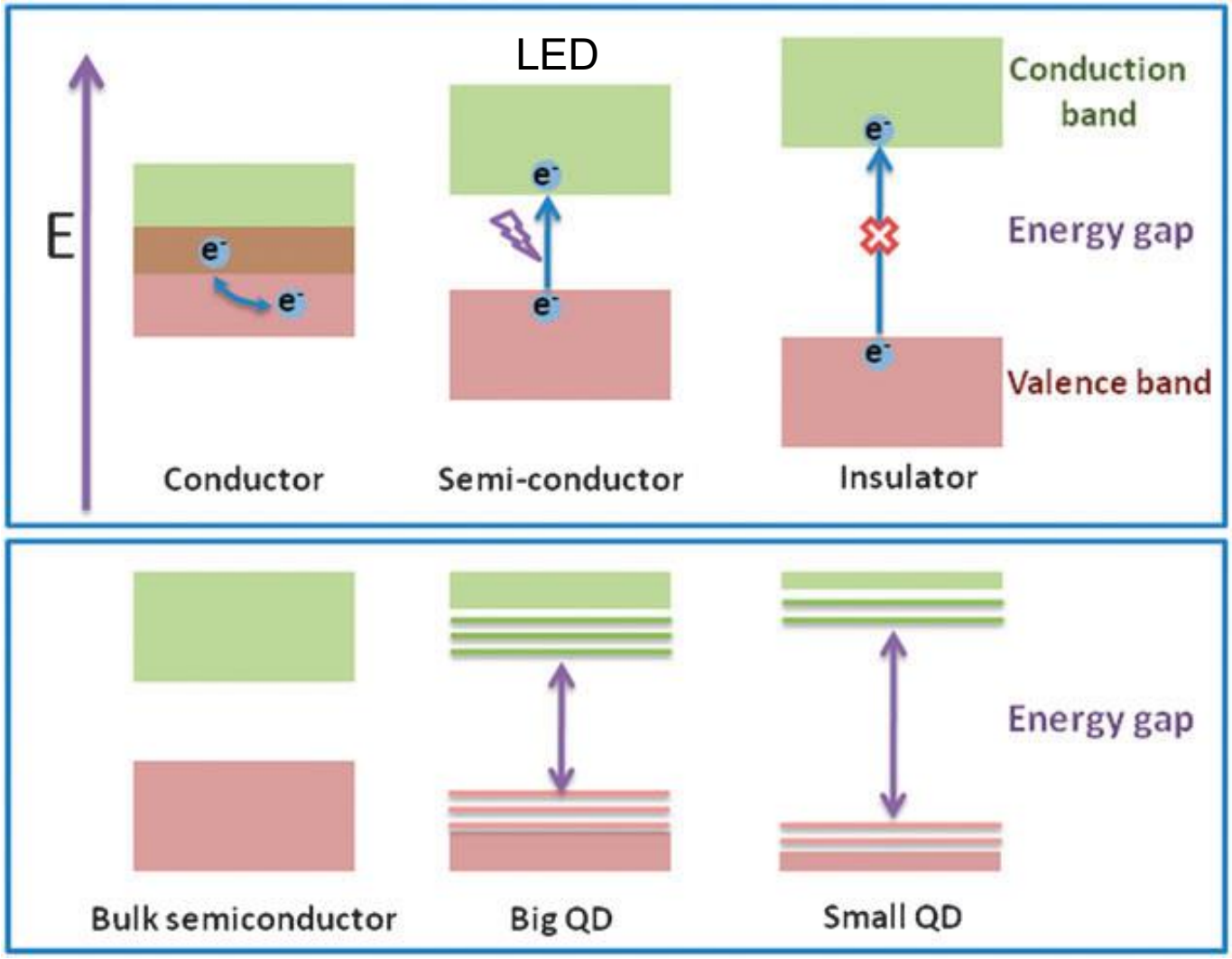
- QDs can be encapsulated/surface silanized
=> avoiding toxic effects
=> ligands etc. (see silica-NP)



Comparison of sizes

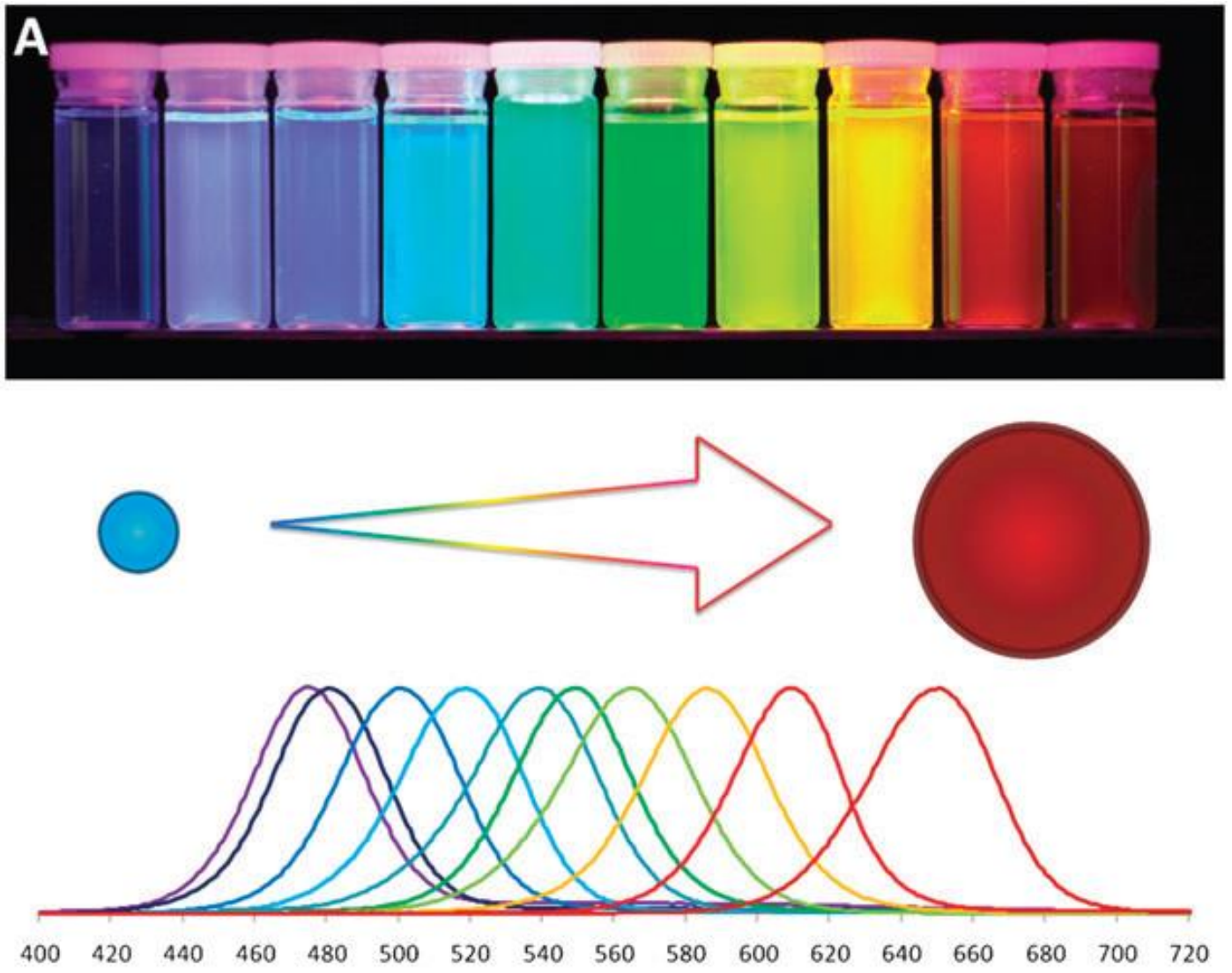
Nobel prize in Chemistry 2023: Anal. Bioanal. Chem. (2024) vol 416, pp 3283–3293

Photophysics of quantum dots

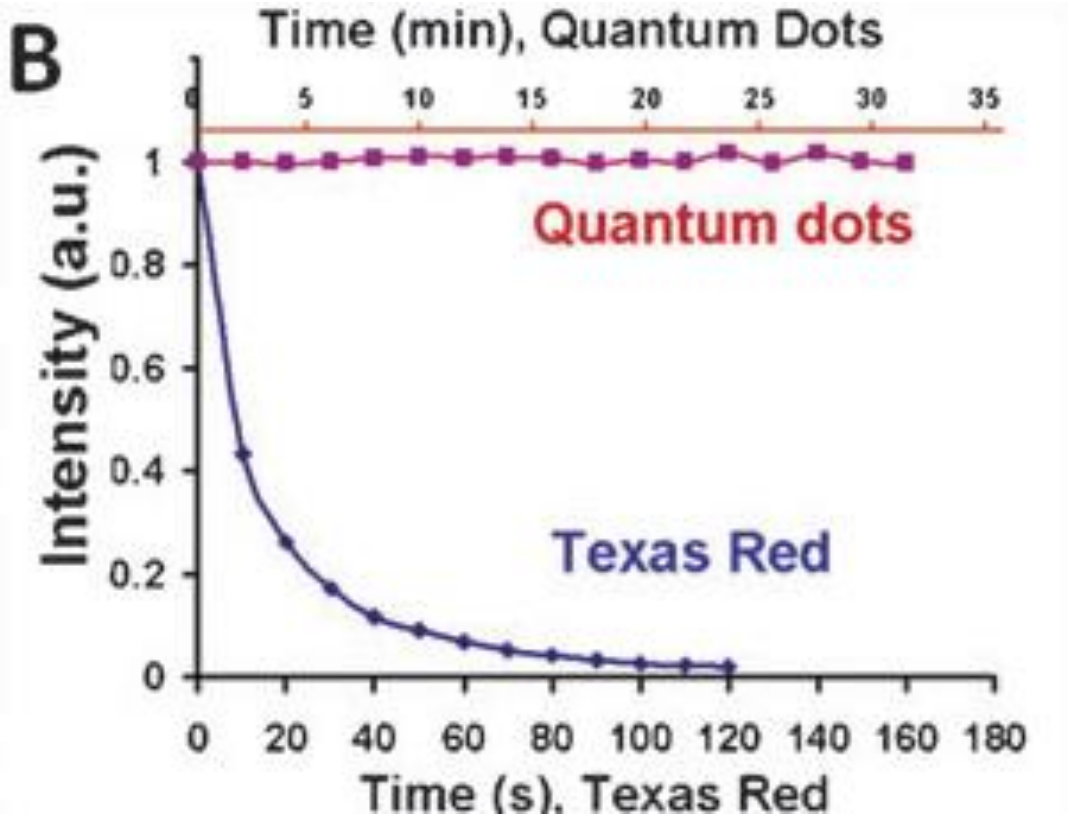


Energy gap gets larger in smaller QDs => emission of short wavelength light

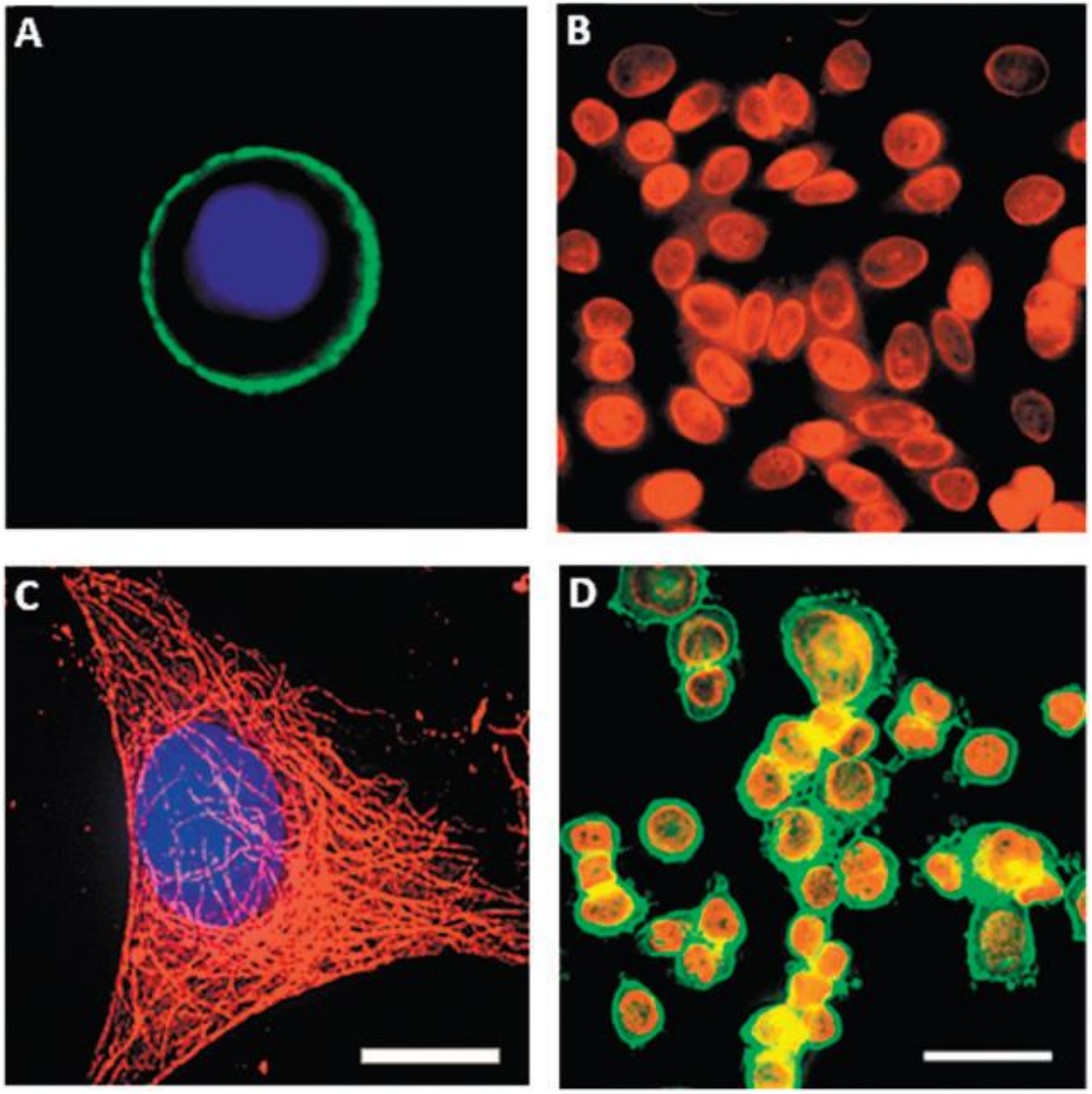
Luminescent properties of quantum dots



High photostability of quantum dots



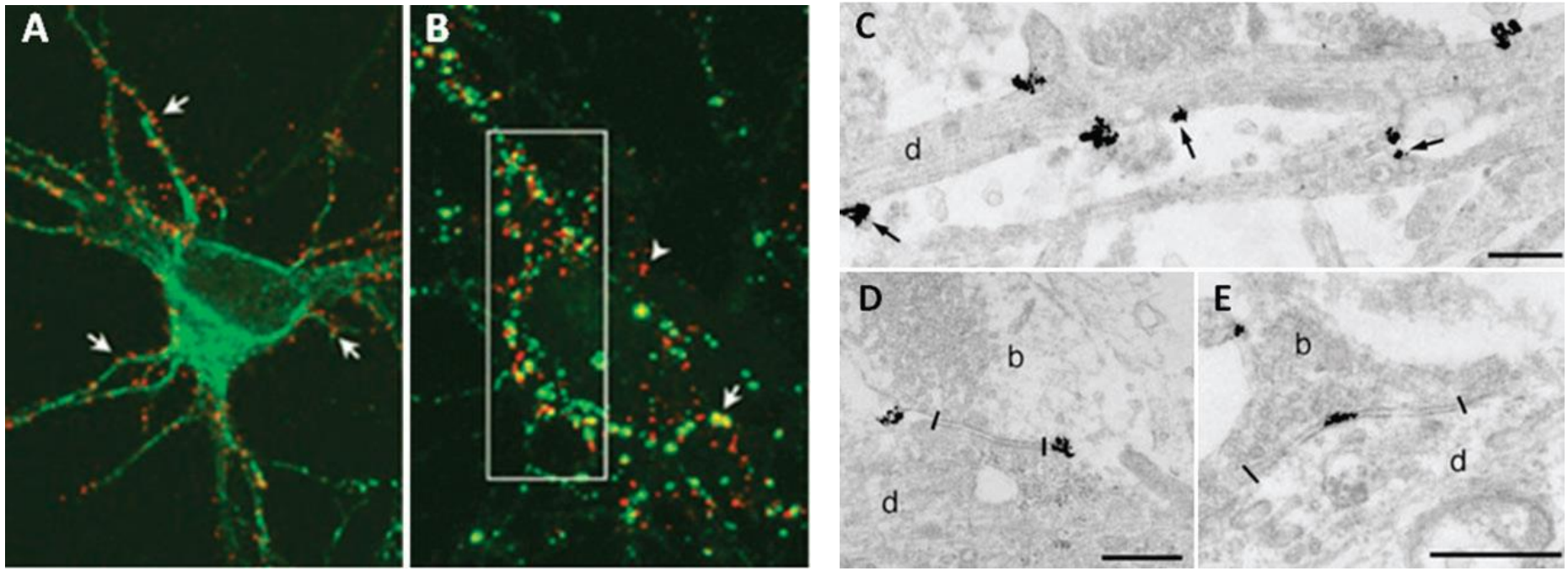
Quantum dots in fluorescence microscopy



Specific antibody labeling
A) of a membrane protein
B) of a nuclear protein
C) of microtubules
D) of both B and C
=> using the same excitation wavelength (UV)

Counter staining of the nucleus by using a fluorescent dye (DAPI)

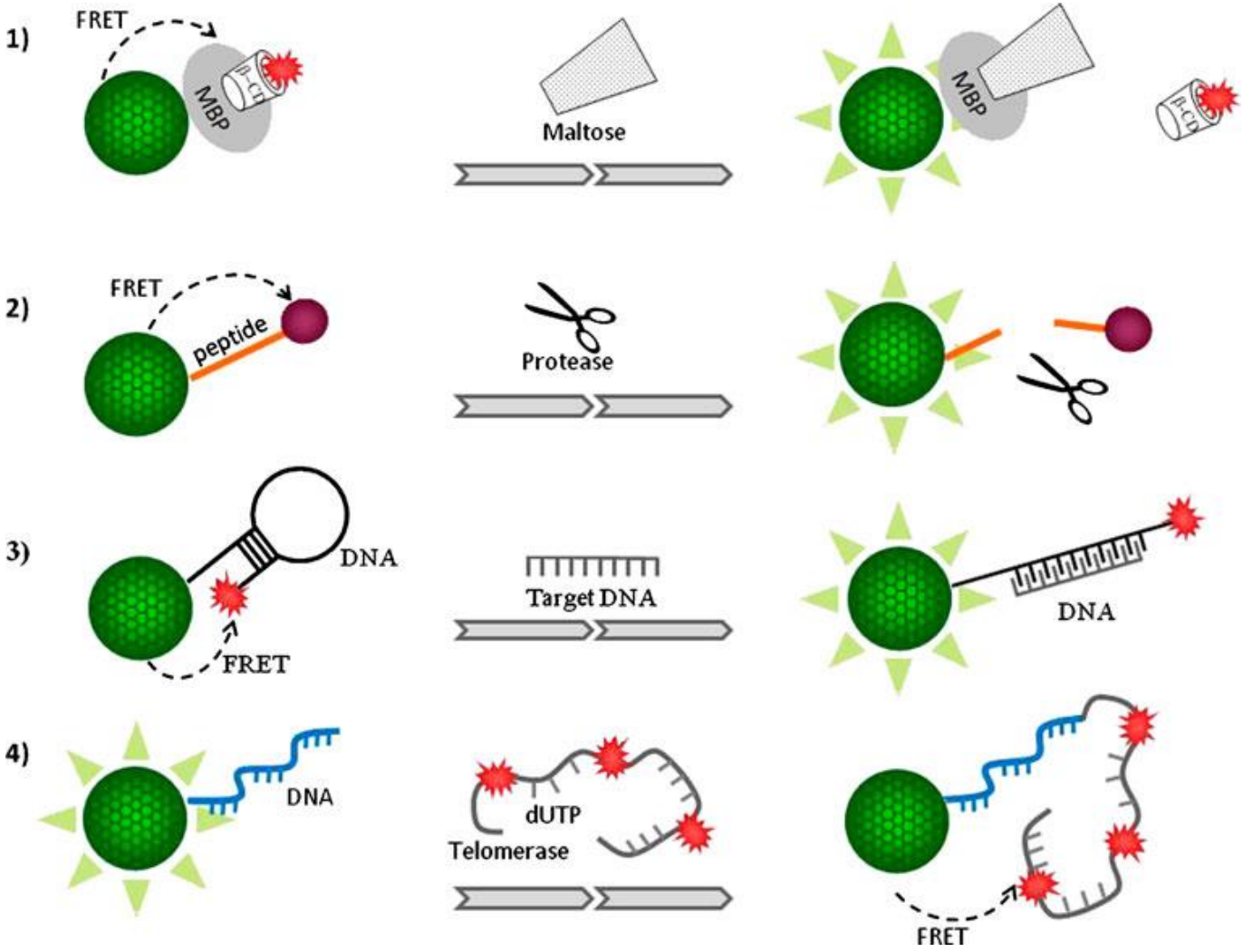
Quantum dots in fluorescence microscopy



QD-labeled antibody against glycin receptor on nerve cells (neurons)

Visible by luminescence and TEM (heavy metals)!

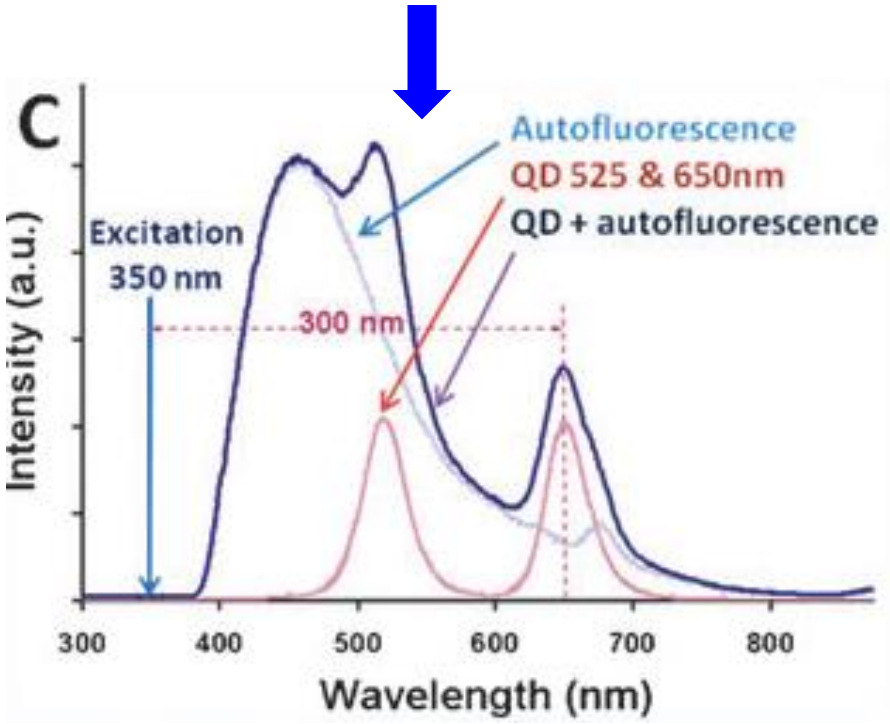
Quantum dots: sensor applications



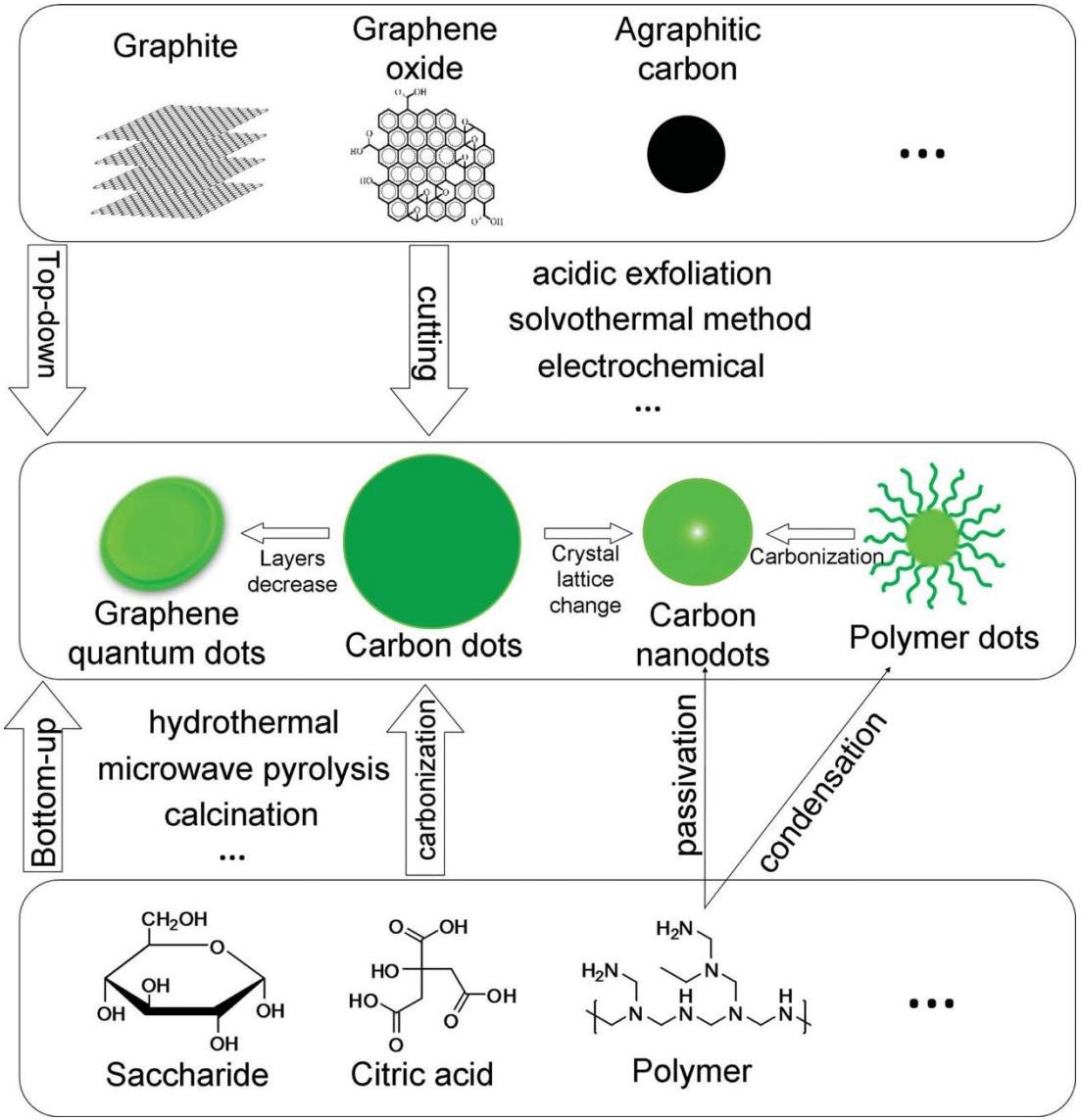
Limitations of quantum dots

1. Toxicity (heavy metal ions)
2. Blinking (invisible for some time)
3. Excitation with short wavelength light

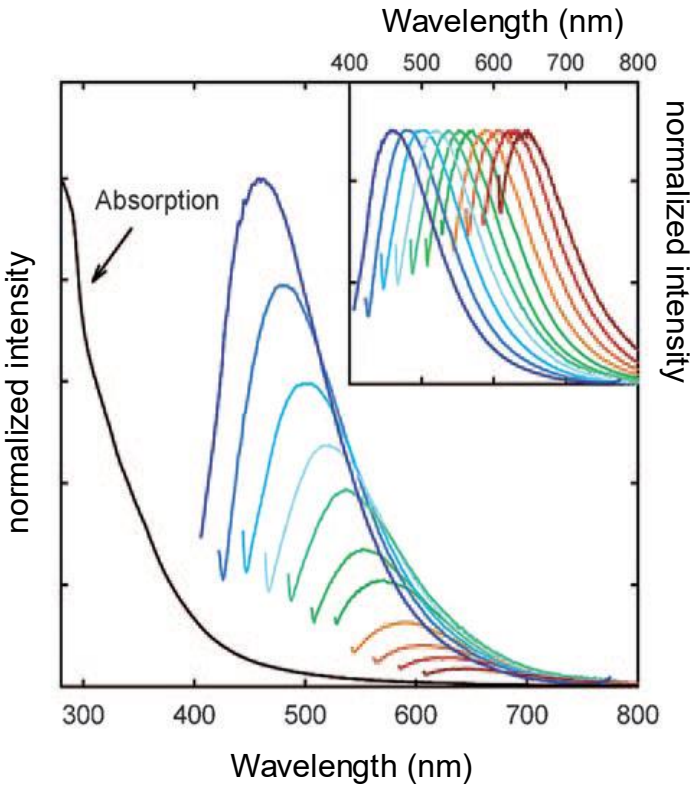
New development:
carbon nanodots (C dots)

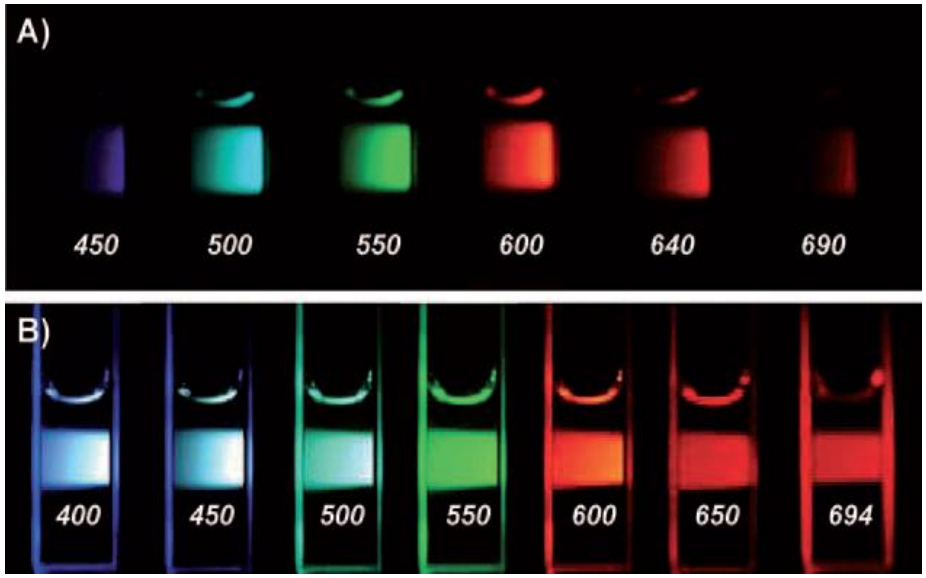


Synthesis of carbon dots



Luminescent properties of carbon dots

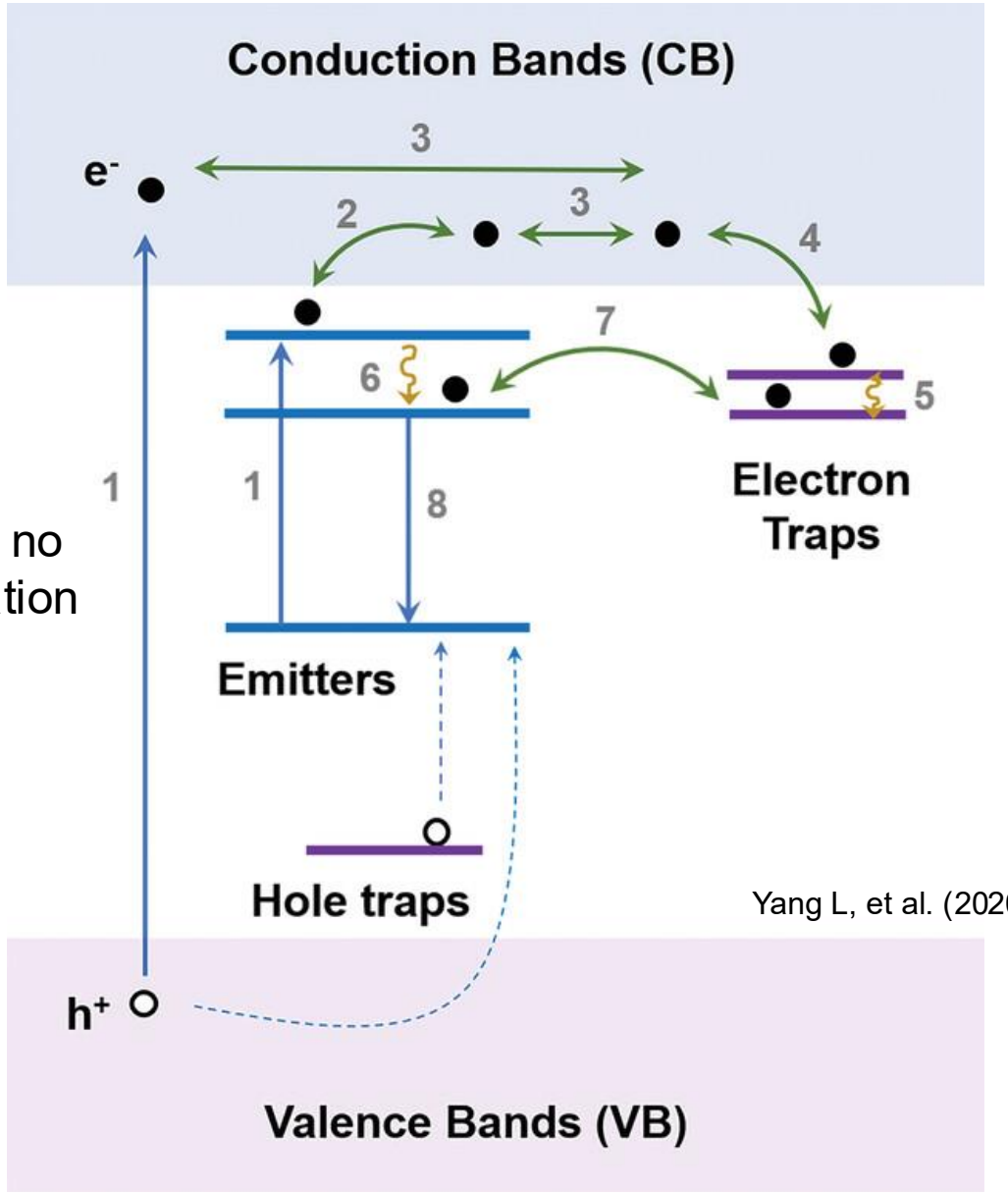




Persistent luminescent nanoparticles (PLNP)

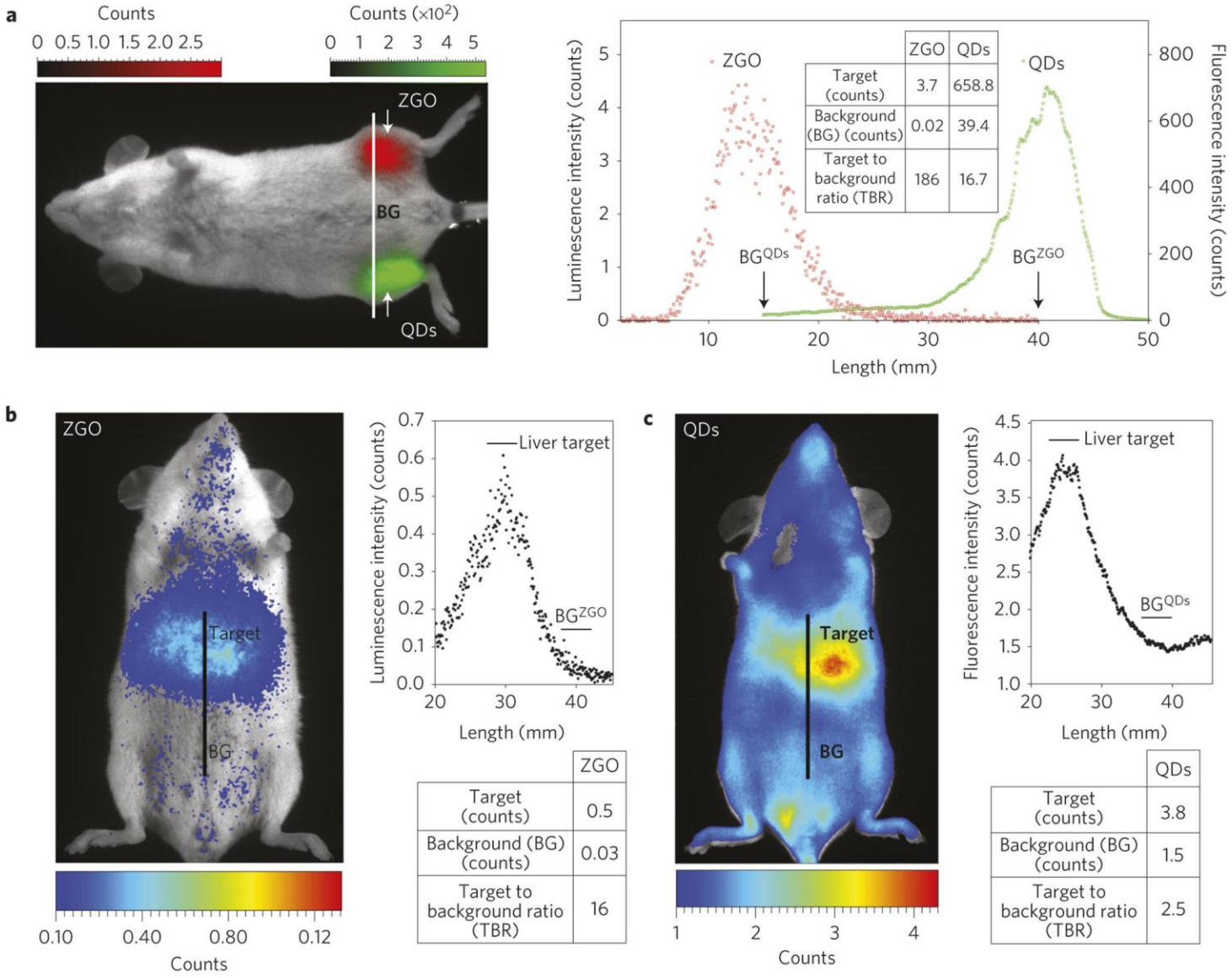
Persistent luminescent nanoparticles

=> During imaging, no fluorescence excitation is needed.



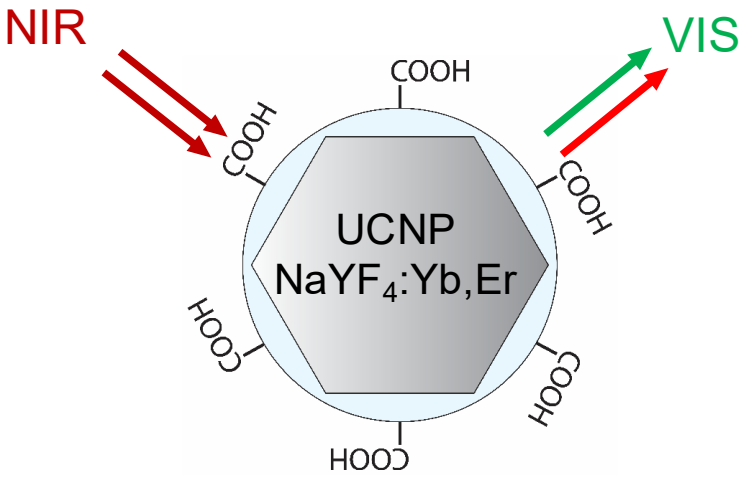
Yang L, et al. (2020) *Adv. Opt. Mater.* 2202382

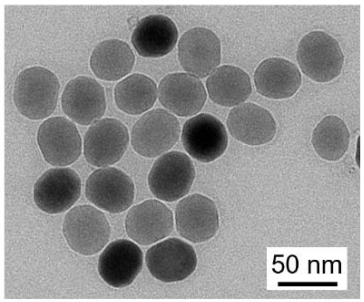
Persistent luminescent nanoparticles



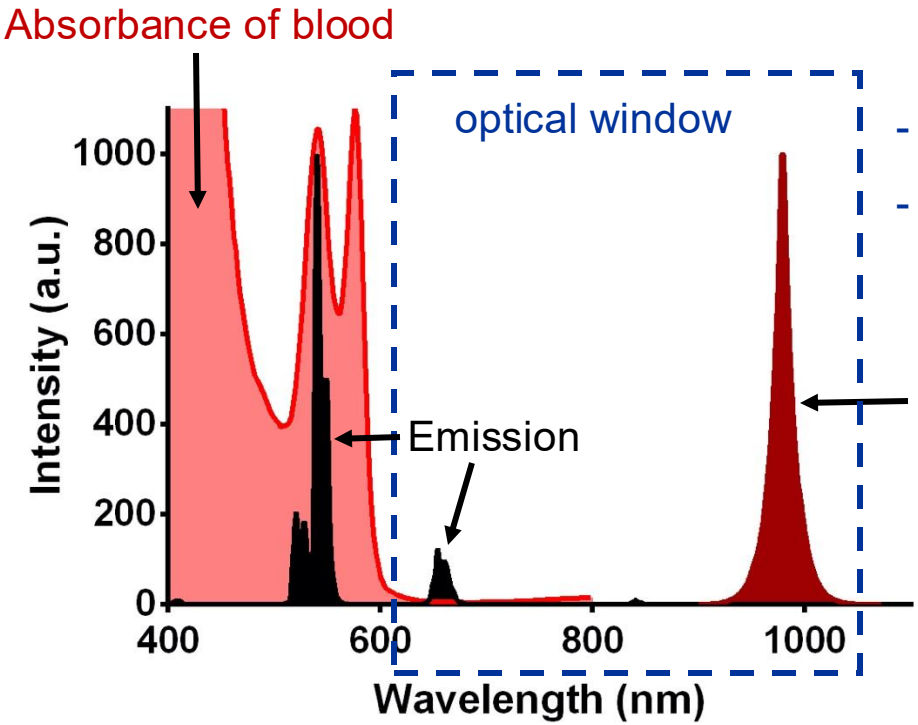
Photon-upconversion nanoparticles (UCNP)

Repetition: UCNPs





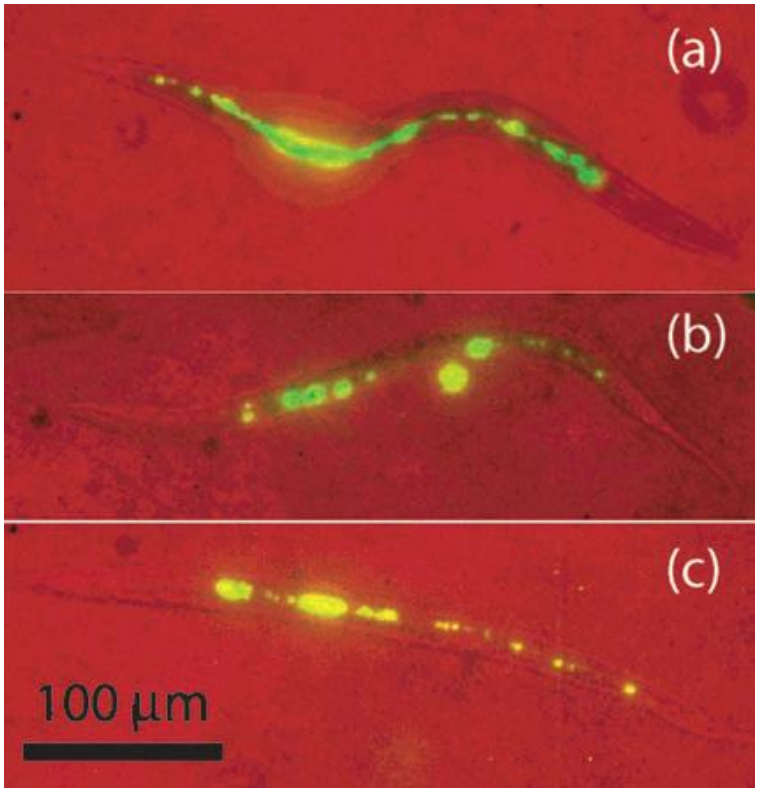
- completely photostable
- ca. 1 million-fold more efficient than 2-photon microscopy



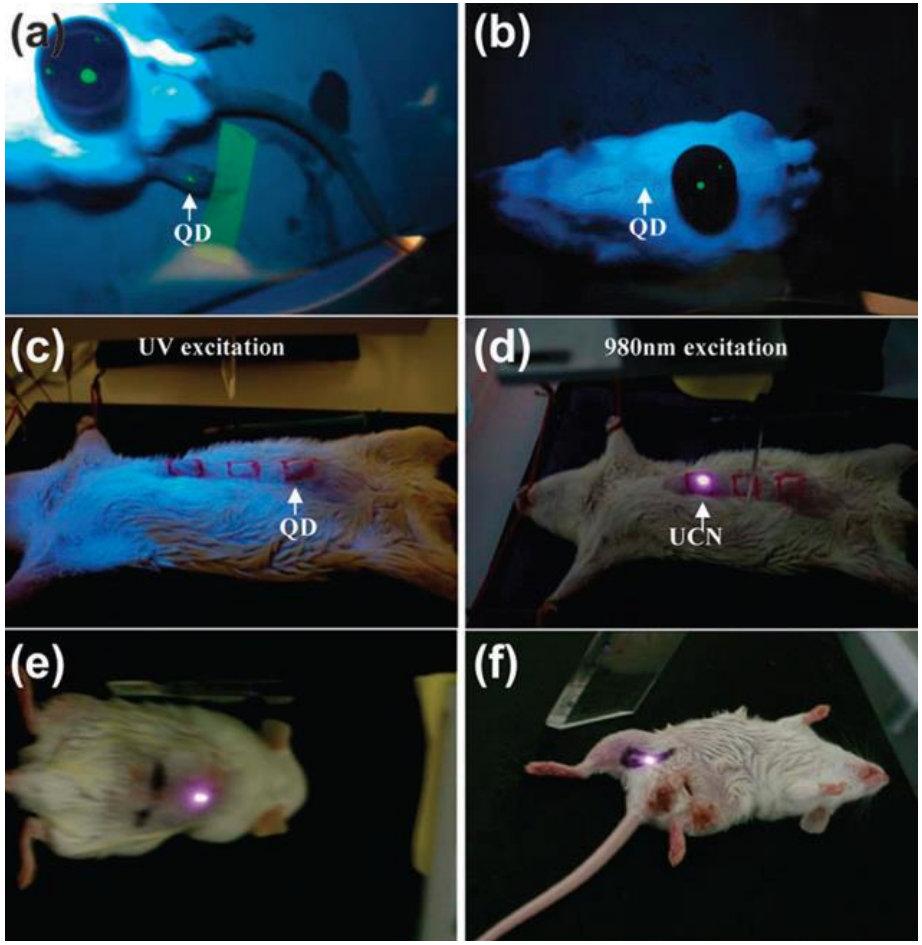
- no autofluorescence
- very low stray light

} background-free detection

UCNPs for small animal (*in vivo*) imaging



UCNPs in a Nematode worm under 980 nm-excitation

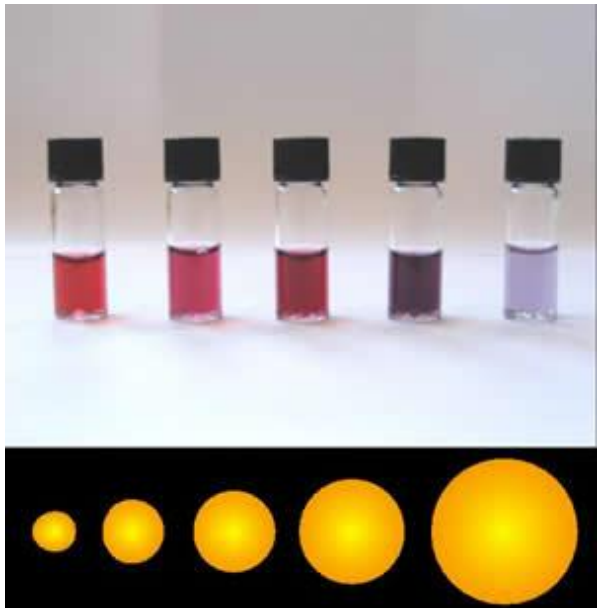


Quantum dots are only visible through the naked skin but UCNPs also in deeper layers of tissue

Gold nanoparticles

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 => contrast agent for TEM (as explained earlier)
  3. collective oscillations of valence electrons in metal grid
     in resonance with frequency of visible light (comparable to SPR)



absorption by localised
surface plasmon resonance

↓

size-dependent

Lycurgus cup (4th century AD)

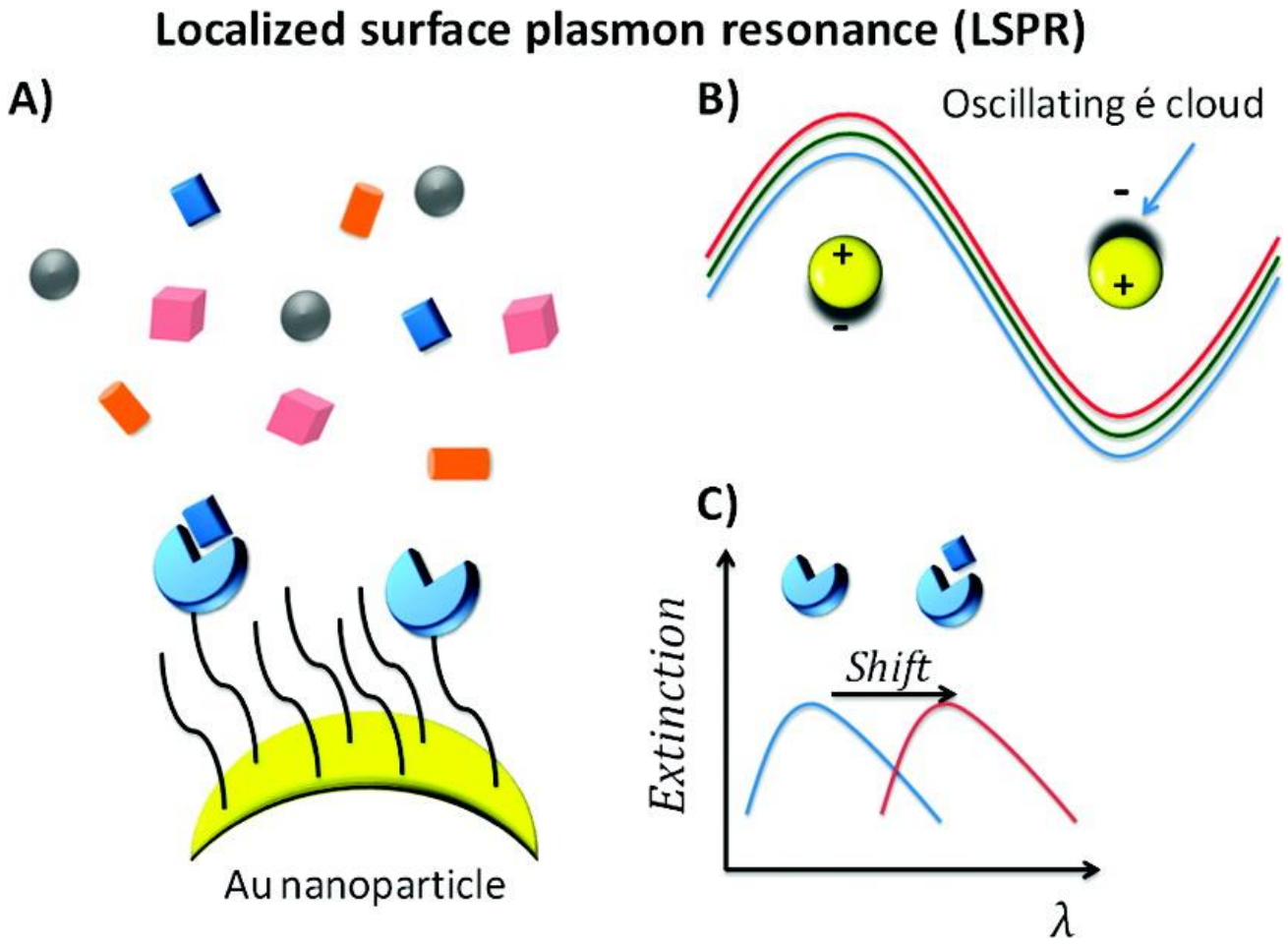
Front illumination

Back illumination

Applications of gold nanoparticles

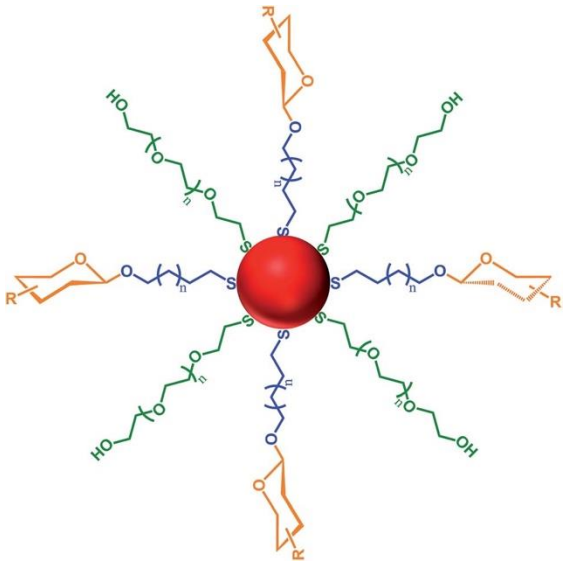
- Immuno labeling (bound to antibody)
  - → TEM => contrast agent
  - → Immunoassays => e.g. staining of lateral flow assays
- Detection of environmental contaminants (polycyclic aromates, heavy metal ions, pesticides, explosives)
  - → Signal amplification for Raman Spectroscopy by localised surface plasmon resonance => Surface enhanced Raman Spectroscopy
- Detection of proteins / DNA
  - → Change in localized surface plasmon resonance

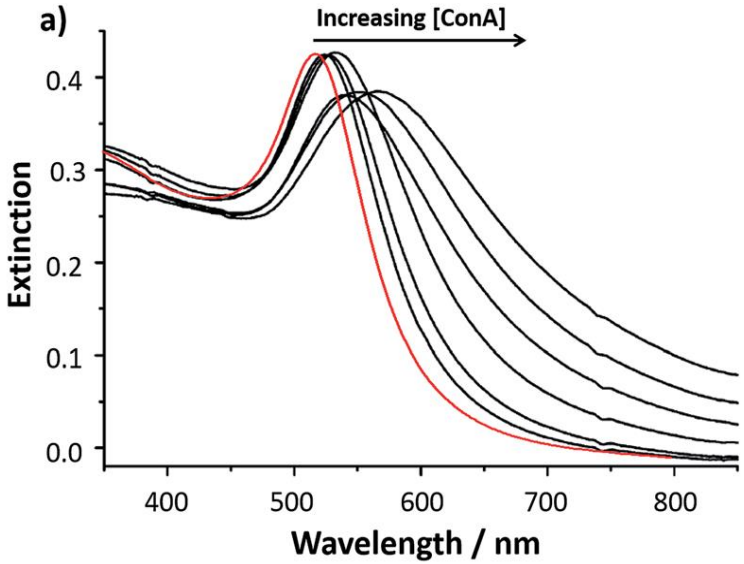
Gold nanoparticles: Plasmonic interactions

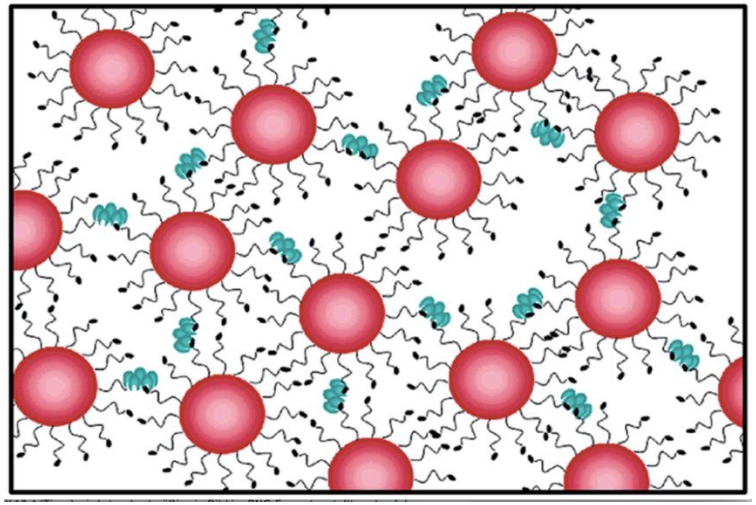


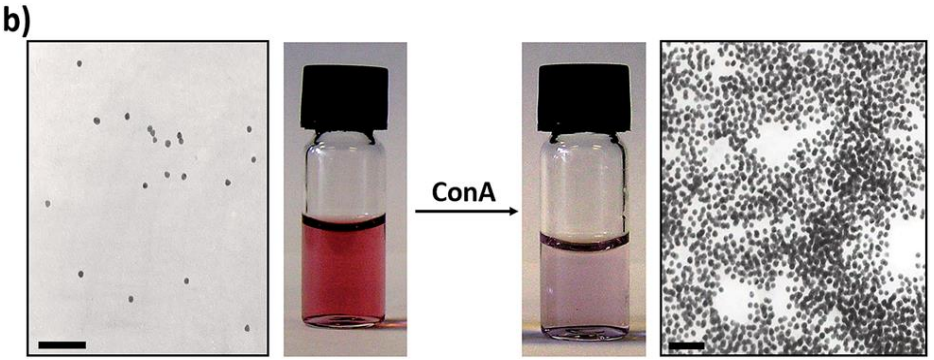
=> Colorimetric bioassay

Plasmonic interactions by gold NP aggregation









Readout:
- by eye
- UV/vis spectrometer

Characterization of nanoparticles

- more difficult compared to small molecules (fewer methods available)

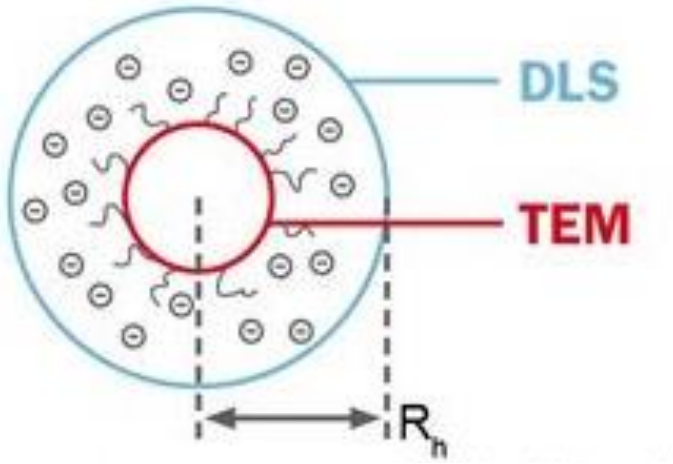
- **Transmission electron microscopy:**
  information about shape and size
  but: nanoparticles are not present as a suspension (as a colloid),
  but rather dried on surface under vaccuum

- **Dynamic light scattering** (also called: photon correlation spectroscopy):
  Information about hydrodynamic diameter in suspension
  requires:
  1. highly diluted samples
  2. very clean preparation
  3. exact temperature control

Stokes-Einstein equation:

$$D_h = \frac{k_B T}{3\pi\eta D_t}$$

Characterization of nanoparticles: TEM ⇔ DLS



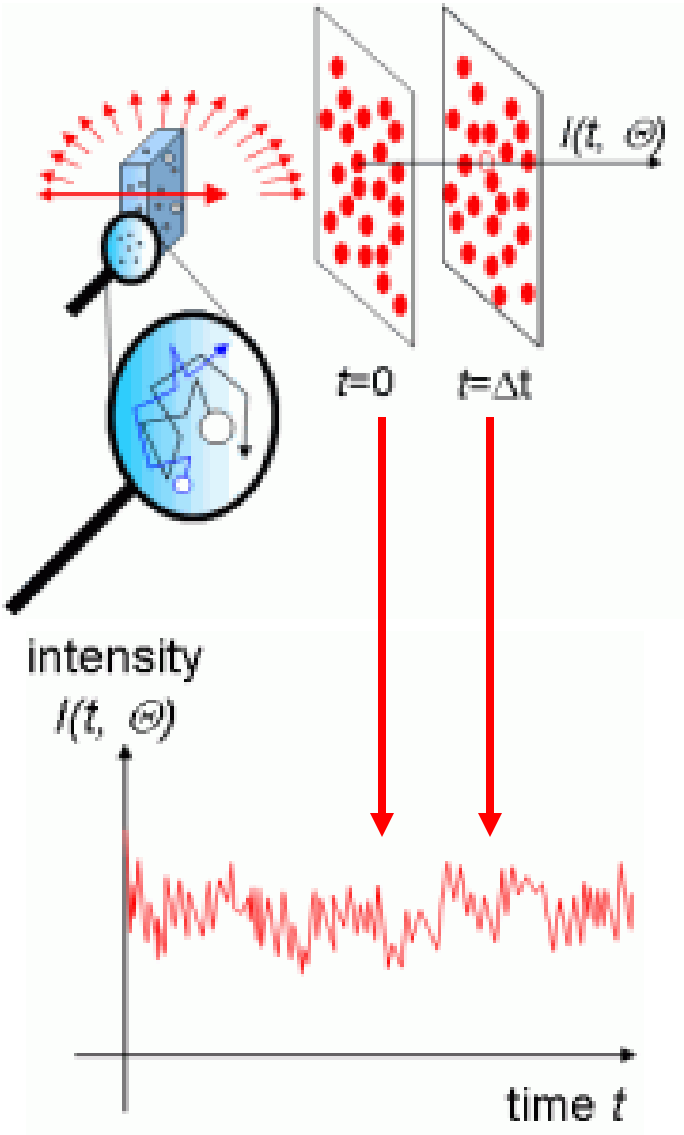
R_h: hydrodynamic radius

= Stokes radius

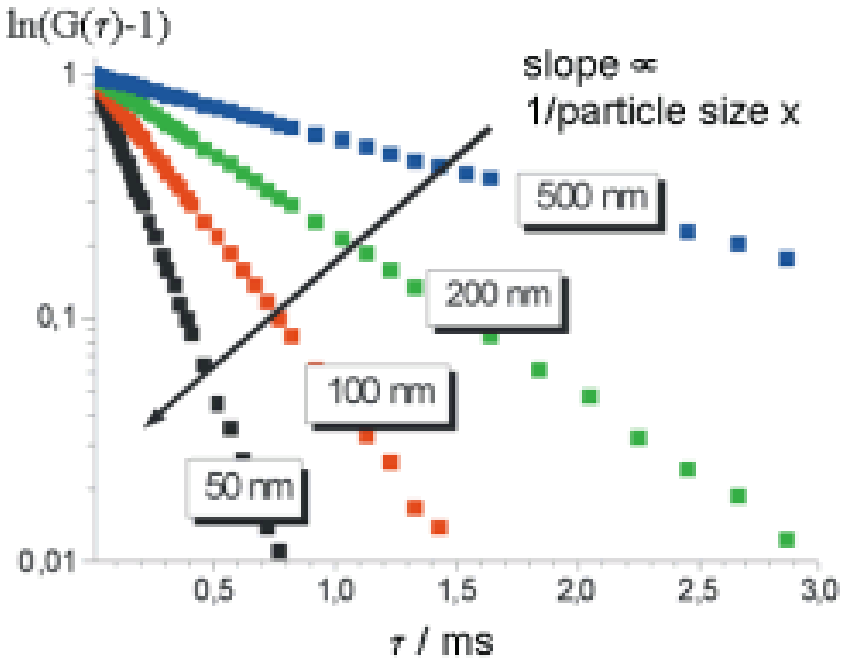
as a result of e.g.:

- ions that form solvation shell
- PEG

Dynamic light scattering (DLS)



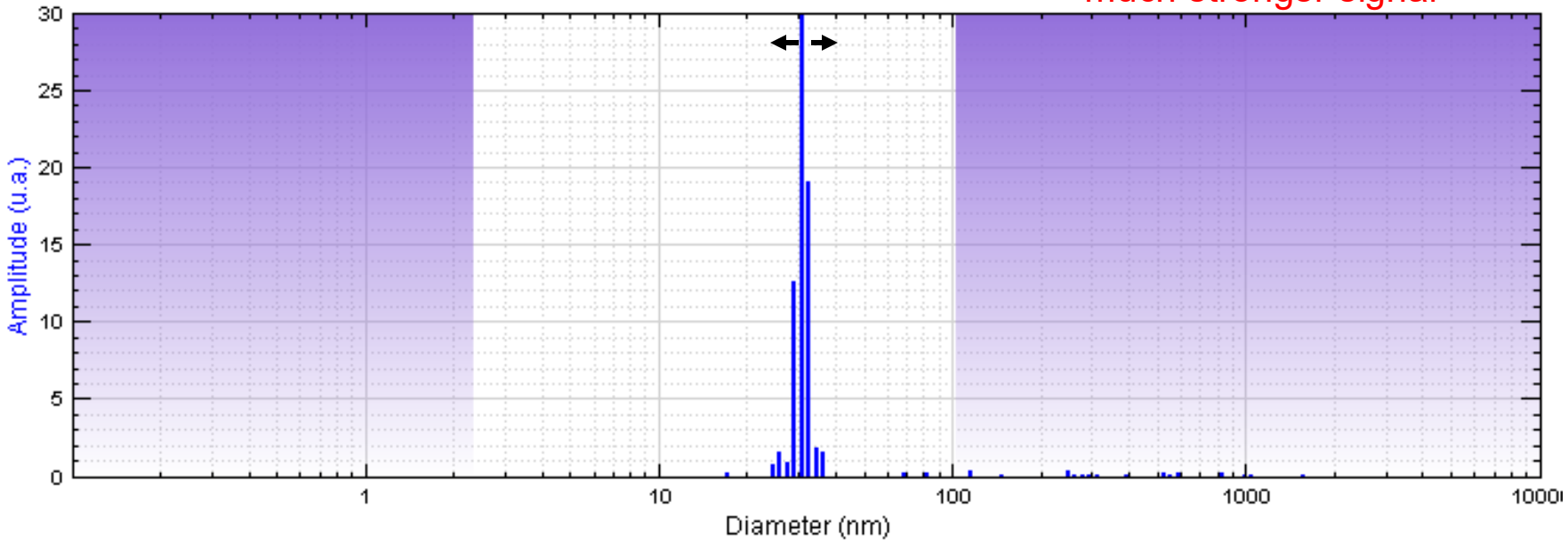
autocorrelation analysis:



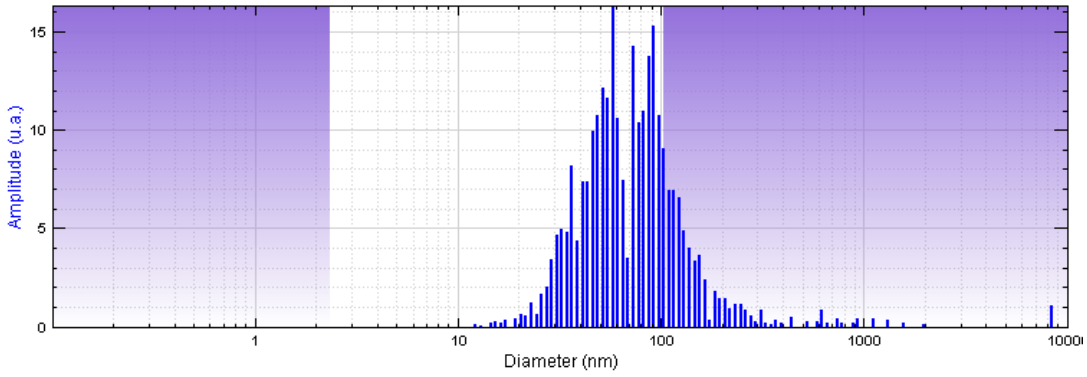
Hydrodynamic diameter

Determination of size distribution

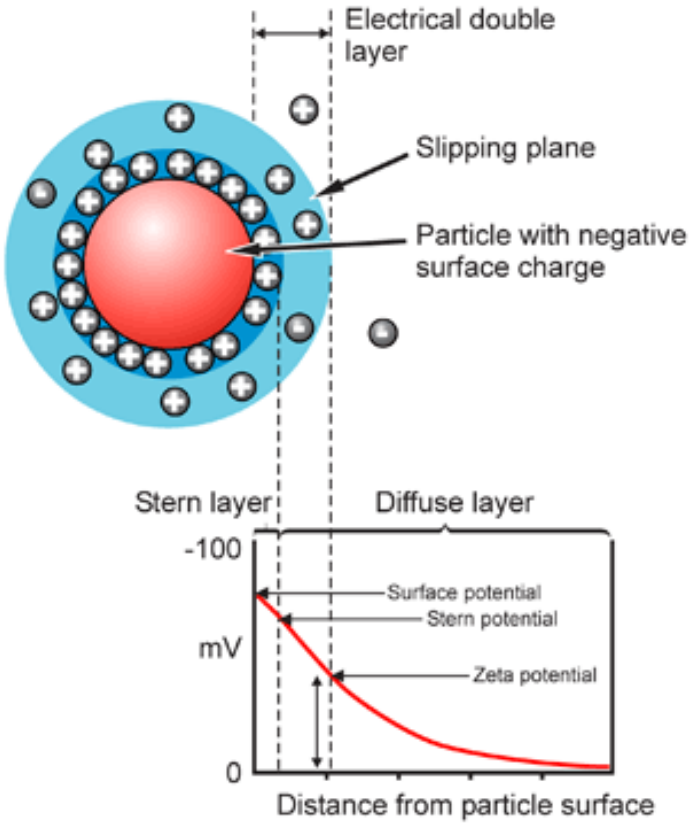
Larger particles result in a much stronger signal



Example of a heterogeneous nanoparticle distribution:



Zeta potential



For measuring the zeta potential, the nanoparticles are moved in an electric field
=> Part of the compensatory charge is stripped off by this movement
Velocity of the movement is indicates the zeta potential

Surface charge via:

- Ionisation of surface groups
  e.g. silica nanoparticles:
  negativ zeta potential via dissociation of silanol groups
  => well dispersible in water
- Adsorption of ions