Luminescent lanthanides

Evolution of reporter systems for immunoassays

Part I – Luminescent lanthanides and time-resolved fluorescence

Tero Soukka 23.4.2024

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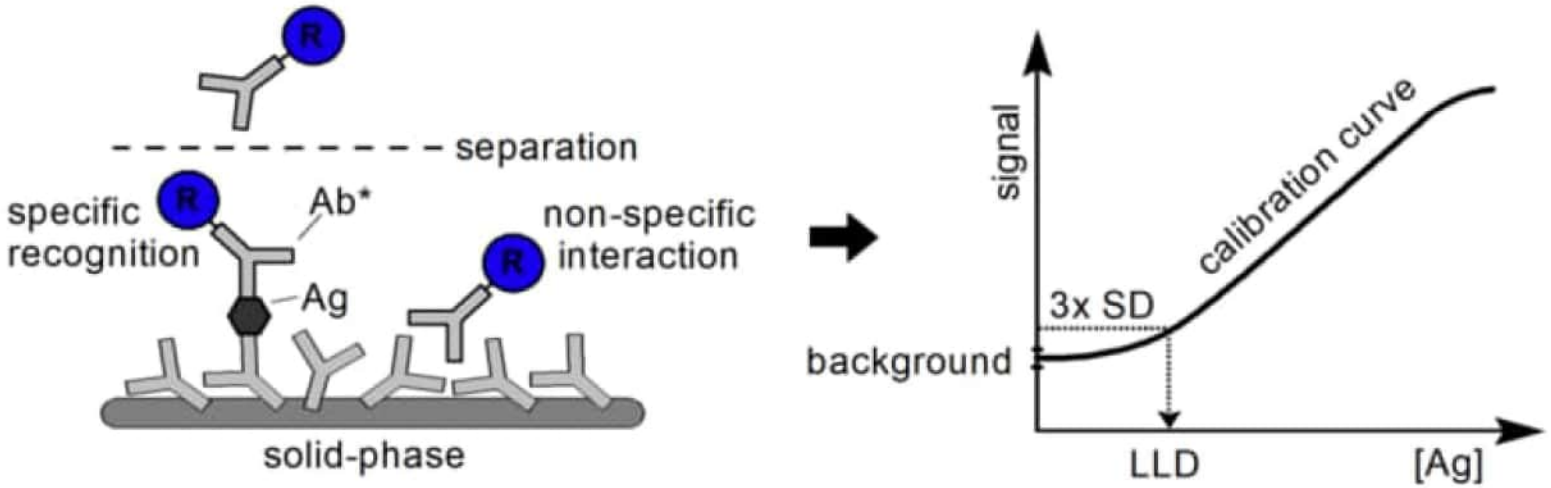
Immunoassays are used to quantify molecules
of interest based on specific recognition by antibodies

assay sensitivity, i.e. lower limit of detection, is defined by

- **binding affinity of the labeled antibody**

  * Soukka, T. *et al.* (2001) *Anal. Chem.* Anal Chem 73: 2254-2260

- **detectability of the label attached to the antibody**
- **non-specifically bound fraction of the labeled antibody**



Jackson, TM and Ekins, RP (1986) *J Immunol Methods* **87**: 13. https://doi.org/10.1016/0022-1759(86)90338-8

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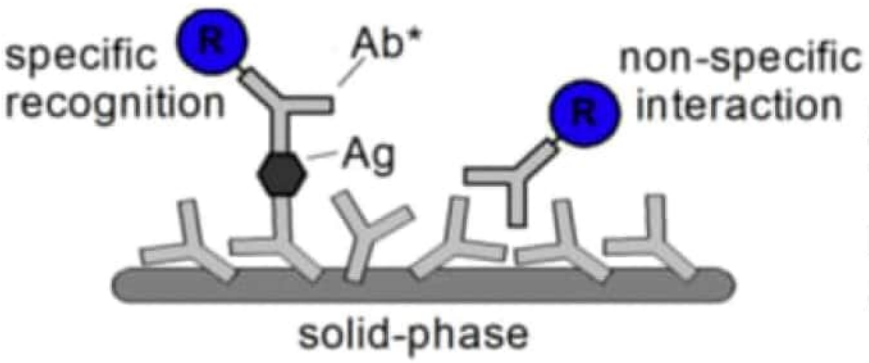
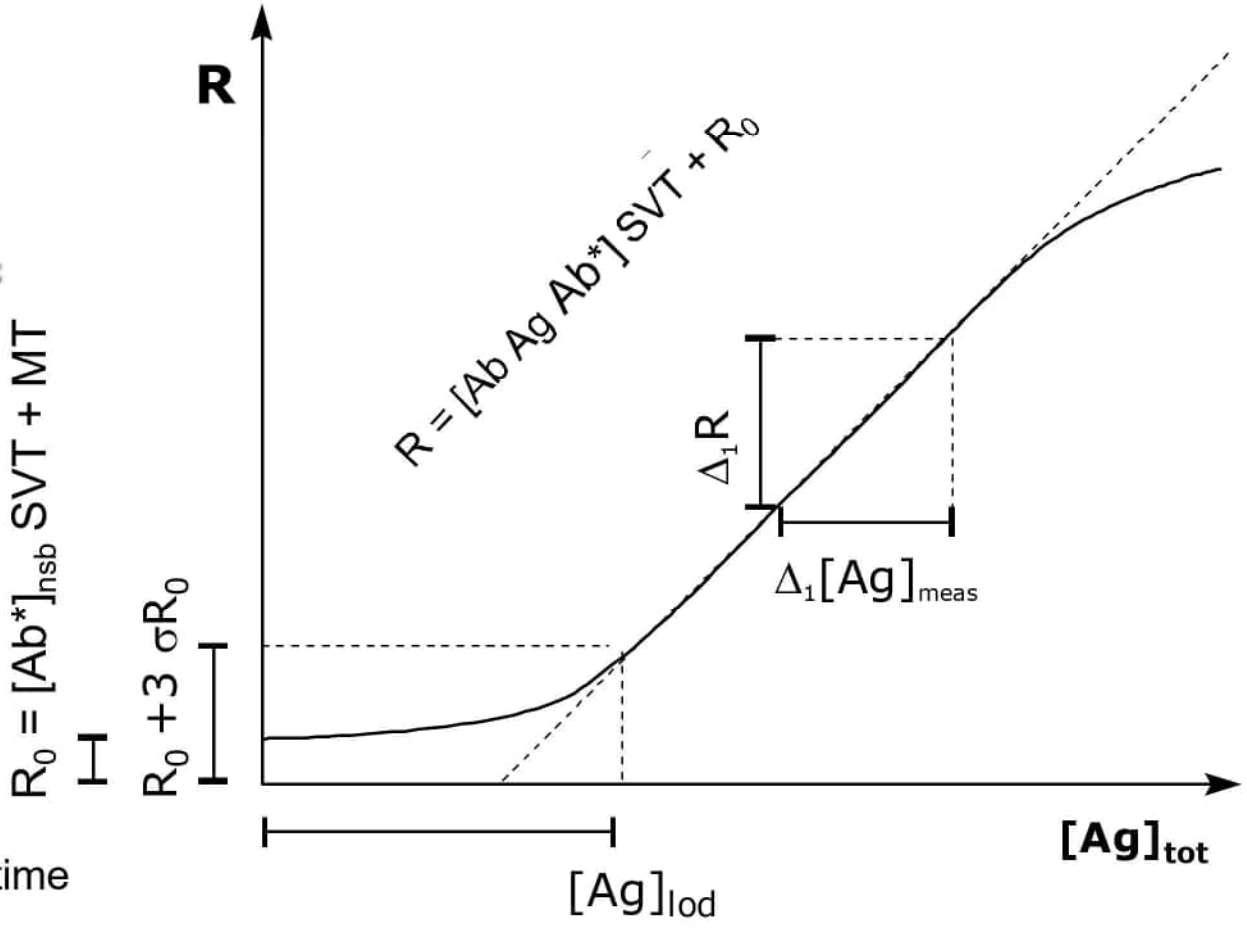
sandwich-type immunoassay



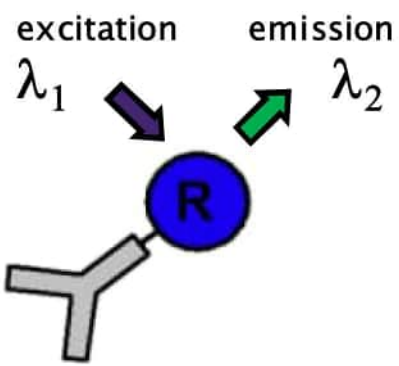
theoretical immunoassay limit of detection

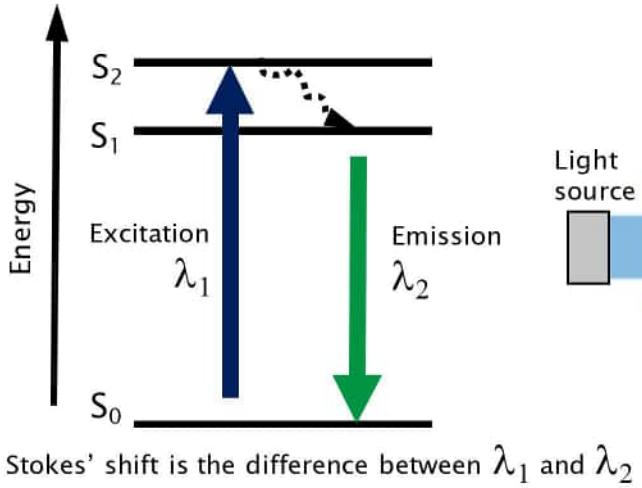


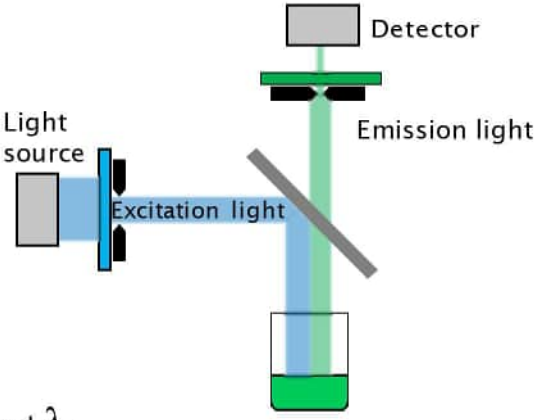
SVT = specific activity rate/vol/time x vol x time
MT = backround rate/time x time

Gorris, H.H. and Soukka, T. (2022). *Anal Chem* 94: 6073-6083. https://doi.org/10.1021/acs.analchem.1c05591

Fluorescent labels in immunoassays
enable rapid, accurate and quantitative detection





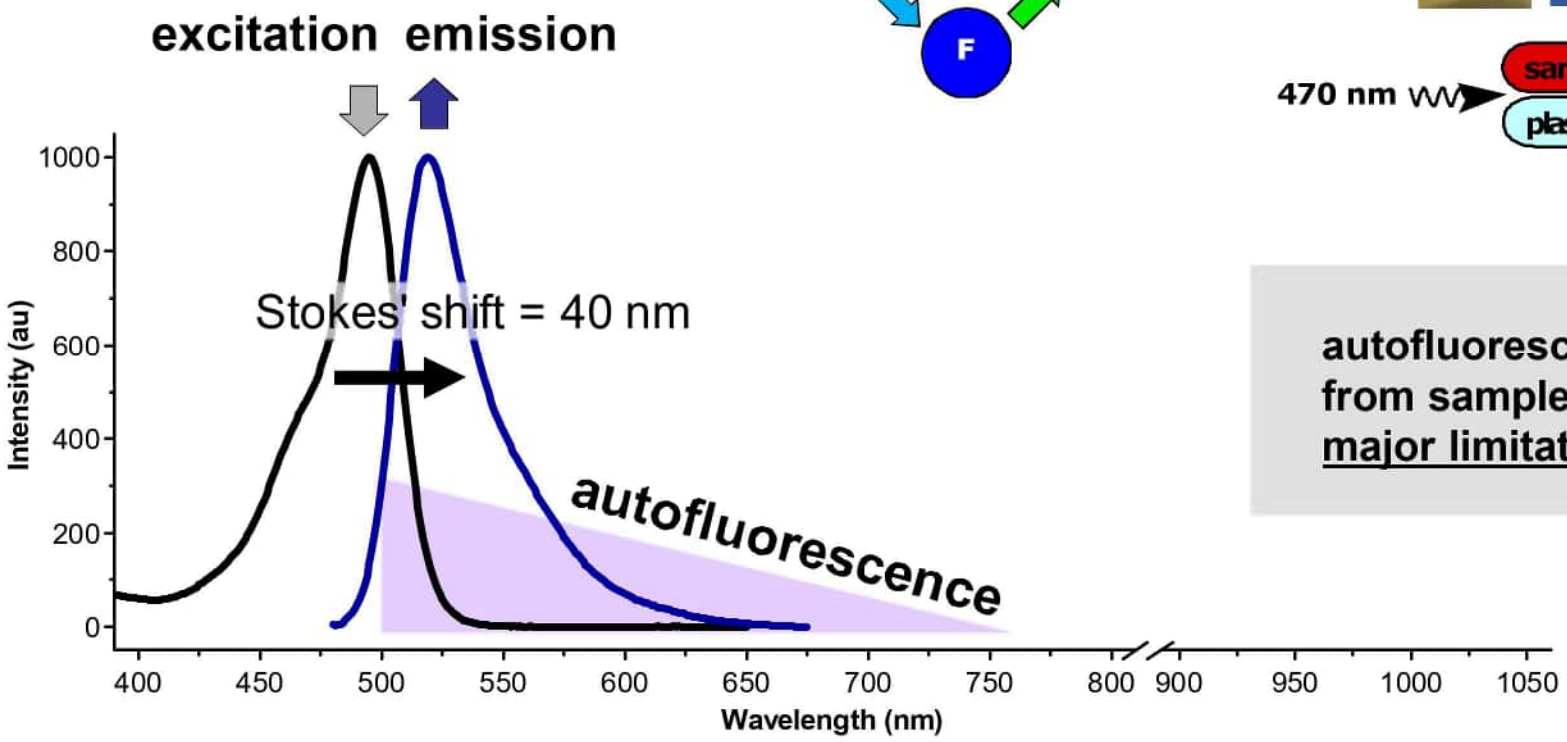


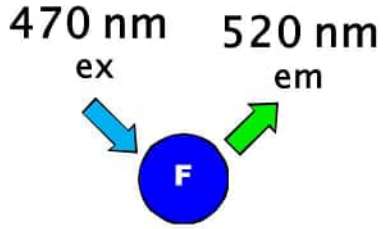
fluorescent labels in immunoassays provide
- **high specific activity**
  (number of detectable events per time unit per label)
- **multiplexing capability**
  (using fluorescent labels with different spectral properties or spatial information on arrays)

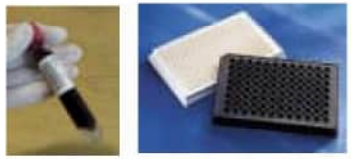
Kricka, LJ. and Park, JY. (2014). Pathobiology of Human Disease, 3207–3221. doi:10.1016/b978-0-12-386456-7.06302-4

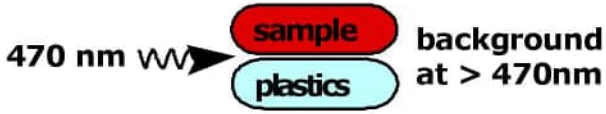
Autofluorescence

in immunoassays with conventional fluorophores









autofluorescence originating from sample and plastics is major limitation in detectability

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Recognition of time-resolved fluorescence

for reduction of the autofluorescence background in immunoassays

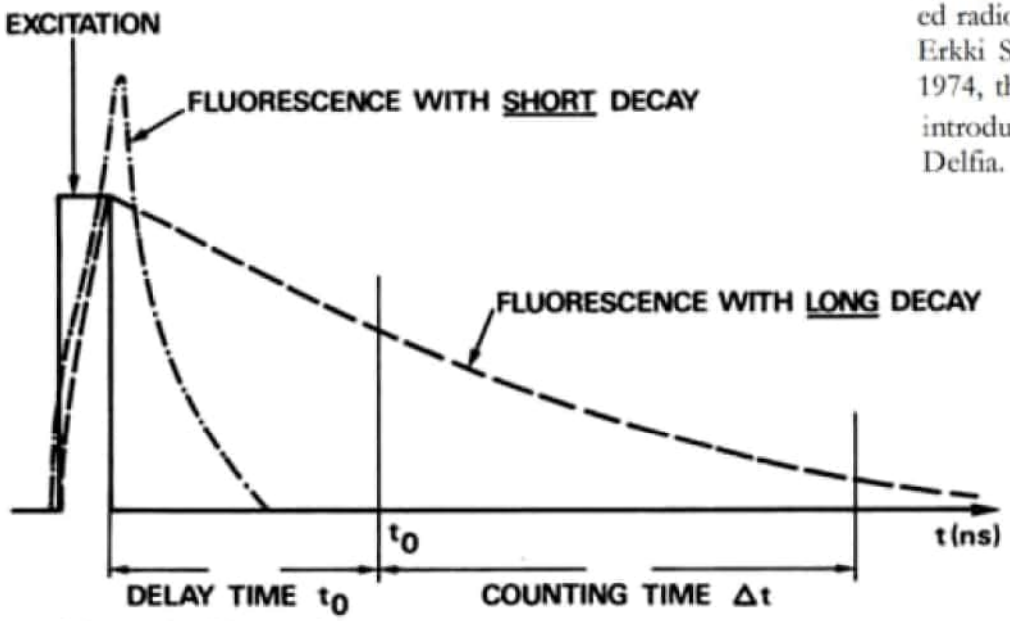


Fig. 4. Diagram of time-resolved fluorometric measurements

In time-resolved measurements the sample is excited with a short light-pulse (about 1 ns), measurement of fluorescence is started after a certain time (delay time t_0) has elapsed, during which time the short decay time background is reduced to almost zero. The fluorescence of a probe with a long lifetime is measured at certain intervals (t, counting time), starting from time t_0

Wallac. Wallac Ltd was founded by Jorma Wallasvaara in 1950 in Turku. The company specialized in the production of laboratory instruments. The early product lines included radiometers, which were the company's main product until the 1980s. In the 1970s, Erkki Soini started to study tracer compounds that could replace radioisotopes. In 1974, the company began to study time-resolved fluorescence. In 1984, the company introduced a new product based on this technology, the immunological assay method, Delfia. In the 1990s, this became the company's main product line.

CLIN. CHEM. 25/3, 353–361 (1979)

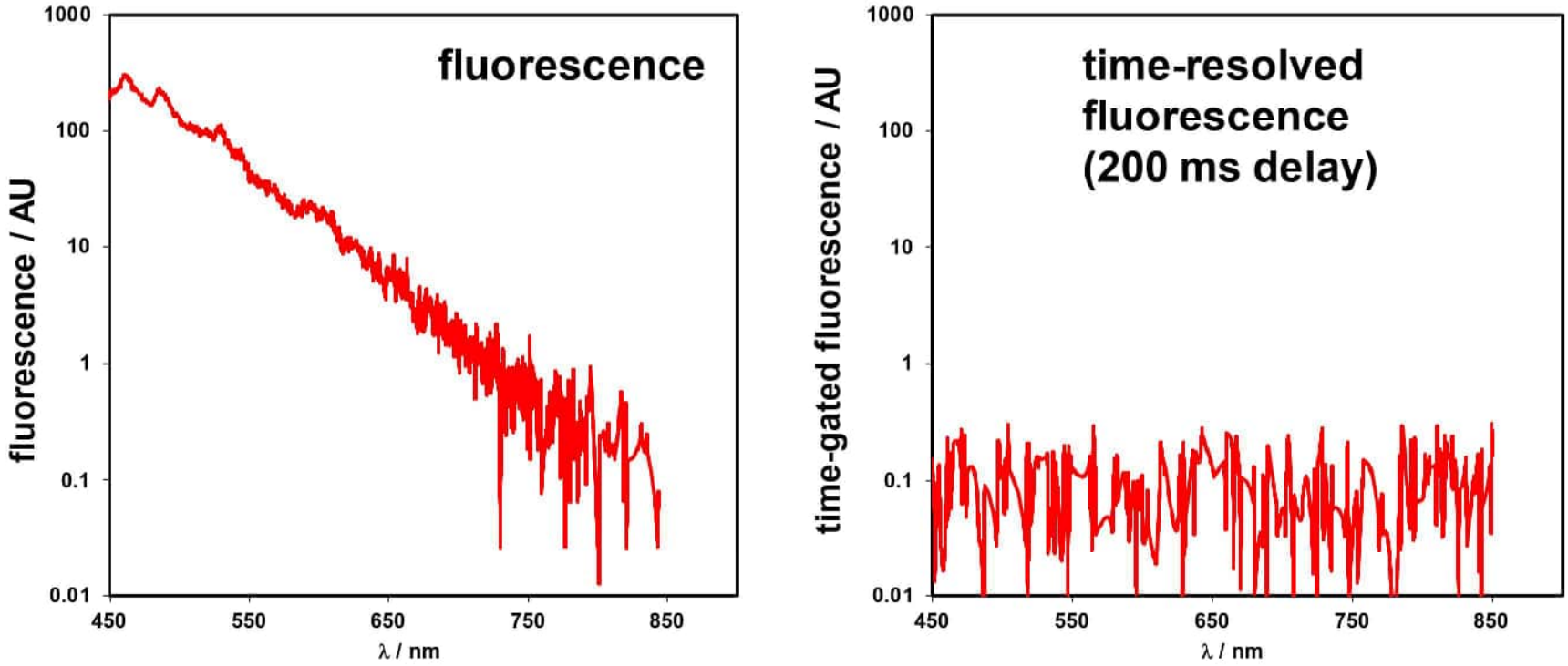
Fluoroimmunoassay: Present Status and Key Problems

Erkki Soini[1] and Ilkka Hemmilä[2]

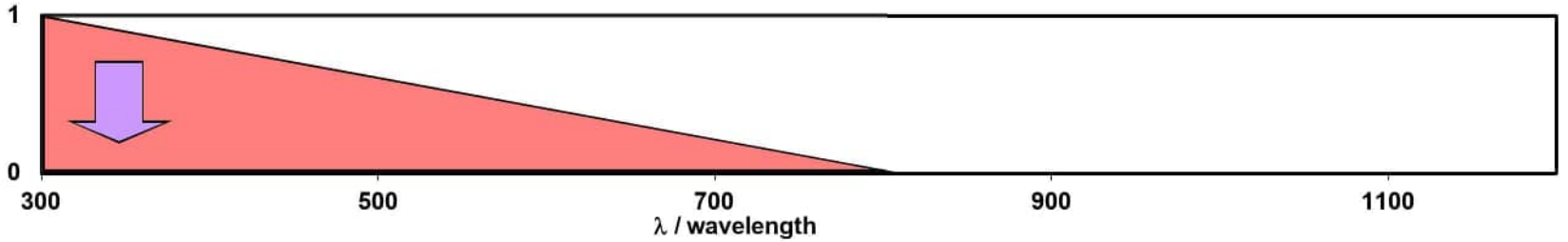
Fluorescence immunoassay of biological fluids (for example, blood samples) is discussed. We attempt to chart present methods of assay as well as new possibilities. Different fluorescent probes, their detection limit, and methods for reduction of background are discussed; methods for separating the free and bound fraction are also reviewed. Special consideration is given to the possibilities of enhancing sensitivity by developing both instruments and chemical methods, and in particular to the possibilities inherent in time-resolved fluorometric applications and to the use of metal chelates in this application.

Soini, E. and Hemmilä, I. (1979) *Clin Chem.* 25: 353-361

Background reduction in time-resolved fluorometry



- 1:50 diluted whole blood, uv-excitation at 340 nm

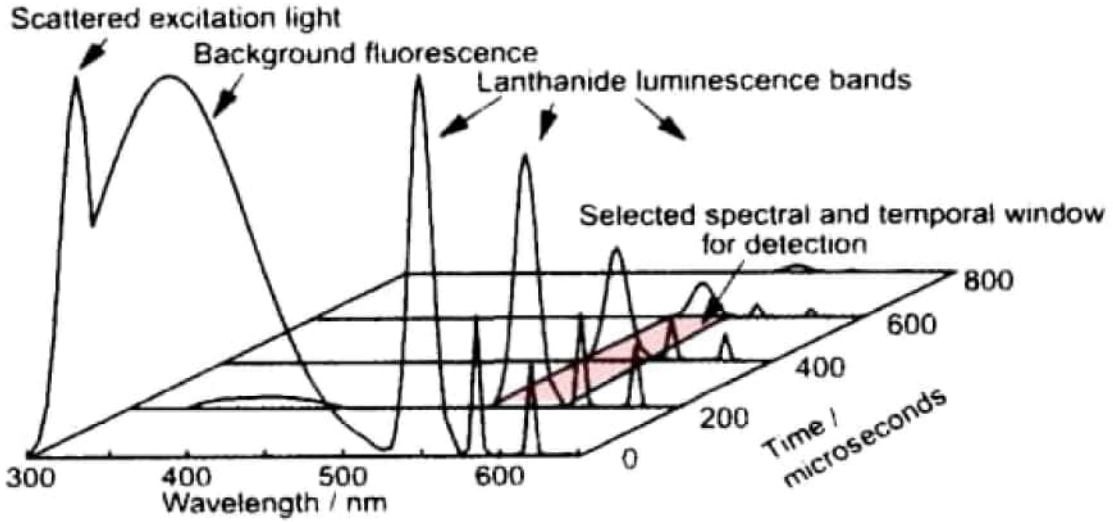


Elimination of autofluorescence

using time-resolved fluorometer for lanthanide chelates

Lanthanide chelates and time-resolved fluorescence detection provide potentially very high detection sensitivity for the following reasons:

- Signal/photon emission ("specific activity") can be increased by a stronger excitation
- Background fluorescence from sample "blank" can be discriminated by using time-resolved detection
- Lanthanide concentrations in biological samples are normally negligible
- Lanthanide labels are biochemically inert (no interaction with the sample)



Principle of time-resolved detection of lanthanide chelate luminescence. Luminescence is counted at lanthanide specific wavelength band is collected at delayed time-window.

Soini, E. and Kojola, H. (1983) *Clin Chem.* 29: 65-68

Review:
Gudgin Dickson, E.F., et al. (1995) *J. Photochem Photobiol B: Biol*. 27: 3-19

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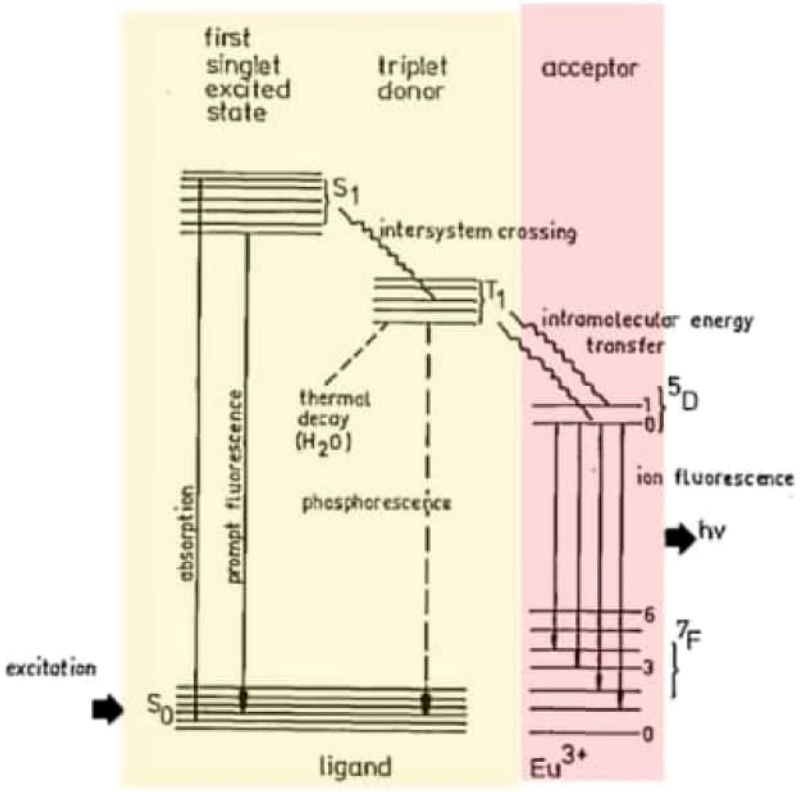
Long lifetime luminescence emission

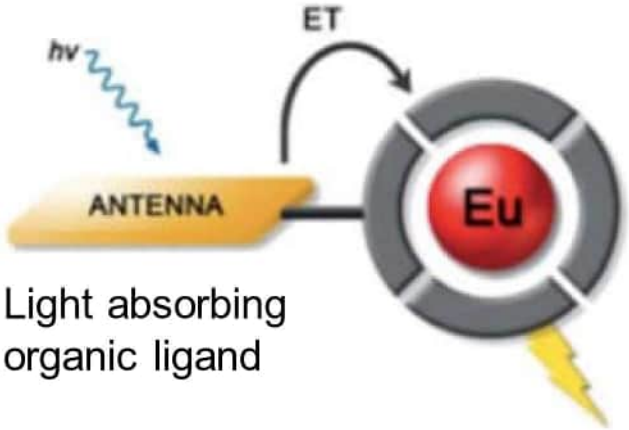
of rare earth metal complexes of organic light-harvesting ligand



Schematic diagram of the radiative processes of the chelate leading to Eu metal ion fluorescence.

Soini, E. and Hemmilä, I. (1979) *Clin Chem.* 25: 353-361

Antenna ligand needed for excitation: direct ion absorption is low due to forbidden electric dipole f-f transitions.



Light absorbing organic ligand

Coordinated water (H_2O) is strong quencher of europium(III).

Prediction of number of water molecules in the first coordination sphere of a europium(III).

$$q = 1.11\,[\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - 0.31]$$

(Horrocks equation)

Supkowski, R.M. and Horrocks, Jr. W.D. (2002) *Inorg Chim Acta* 340:44-48

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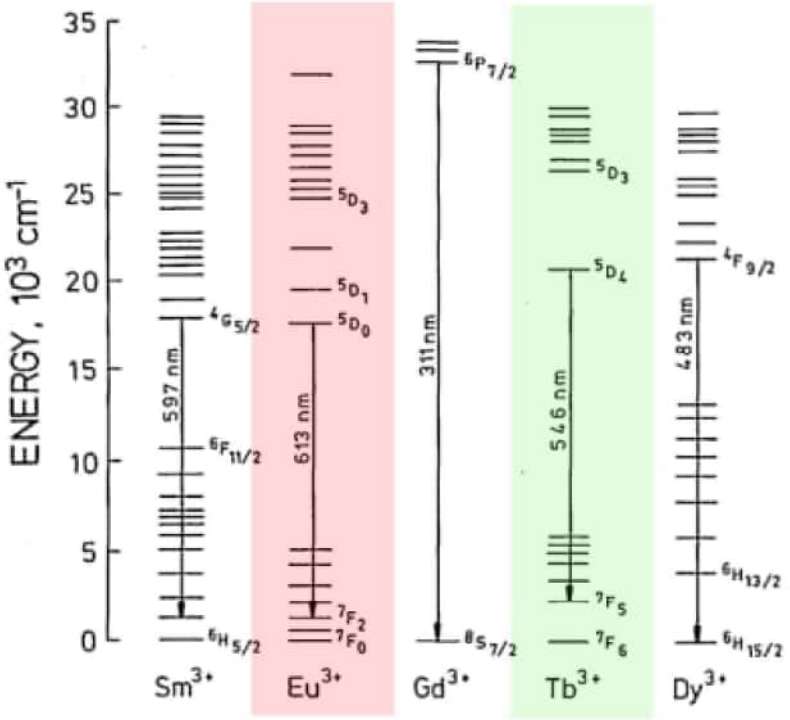
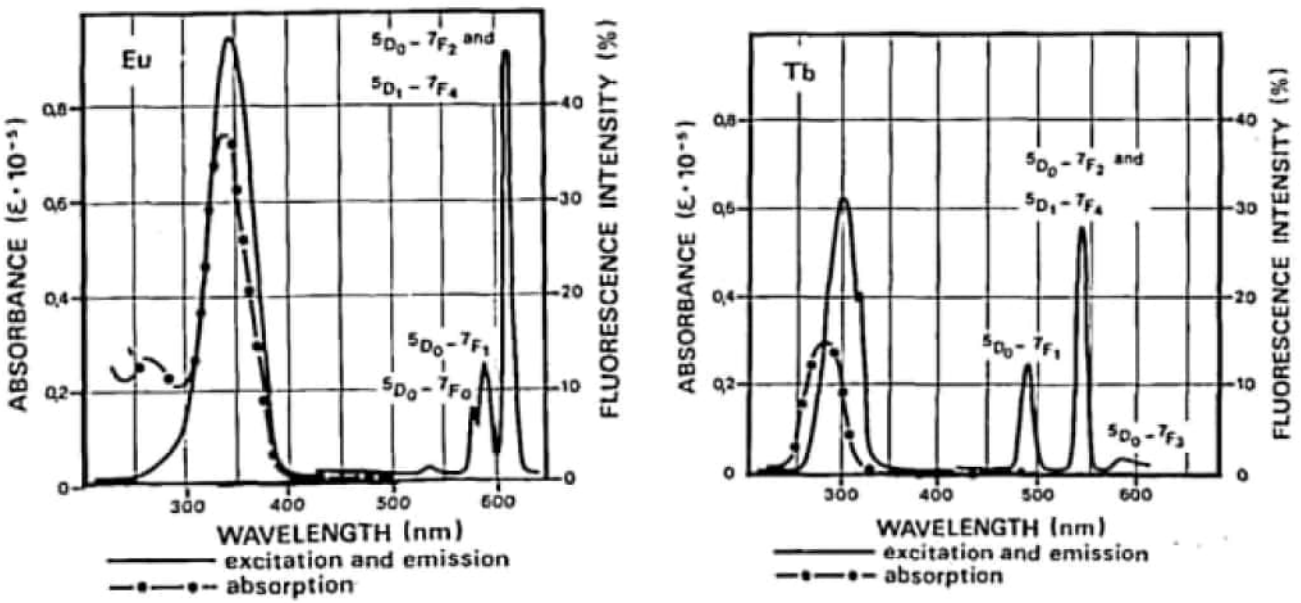
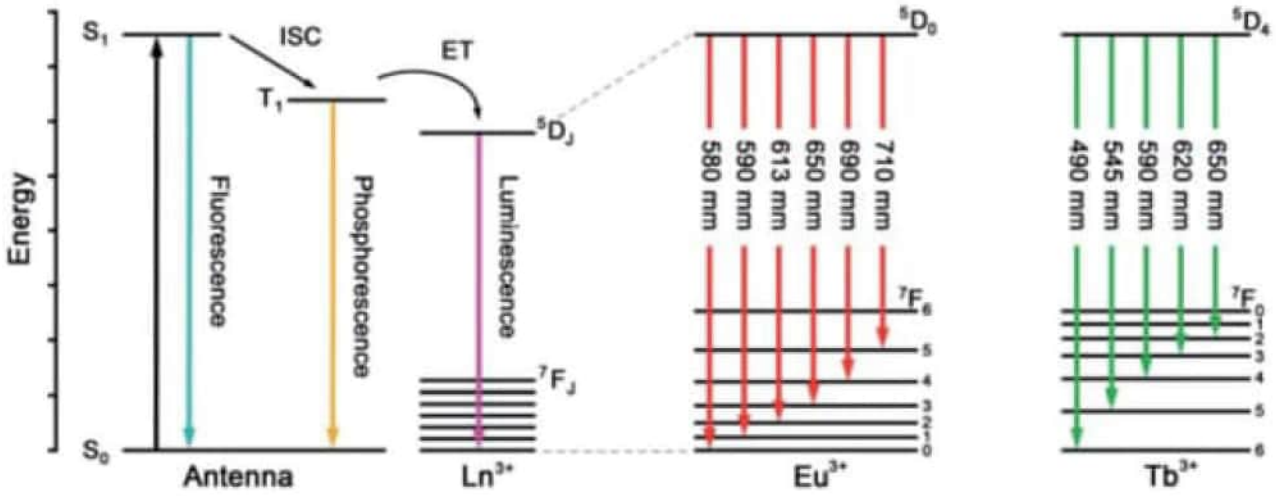


Principal emission lines of certain lanthanide ions.



The excitation and emission spectra of Eu^{3+} and Tb^{3+}. The metal ion emits energy as narrow-band emission. The excitation band is typically broad and the Stokes shift more than 200 nm.

Soini, E. and Lövgren, T.. (1987). *CRC Crit Rev Anal Chem* 18: 105–154. doi:10.1080/10408348708542802



Emission characteristics of lanthanide chelates containing various lanthanide ions

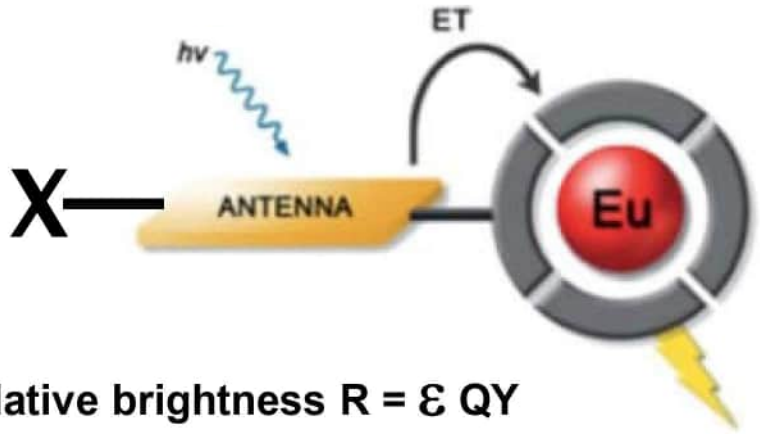
Lanthanide ion	Approx. peak wavelength of principal emission band (nm)	Color of principal emission	Approx. luminescence lifetime (μs)
Sm (III)	645	red	30–100
Eu (III)	615	orange	100–1000
Tb (III)	545	green	400–2000
Dy (III)	573	yellow	1–10

Typical properties of most organic fluorophores, and nonspecific background signals, compared with lanthanide chelates

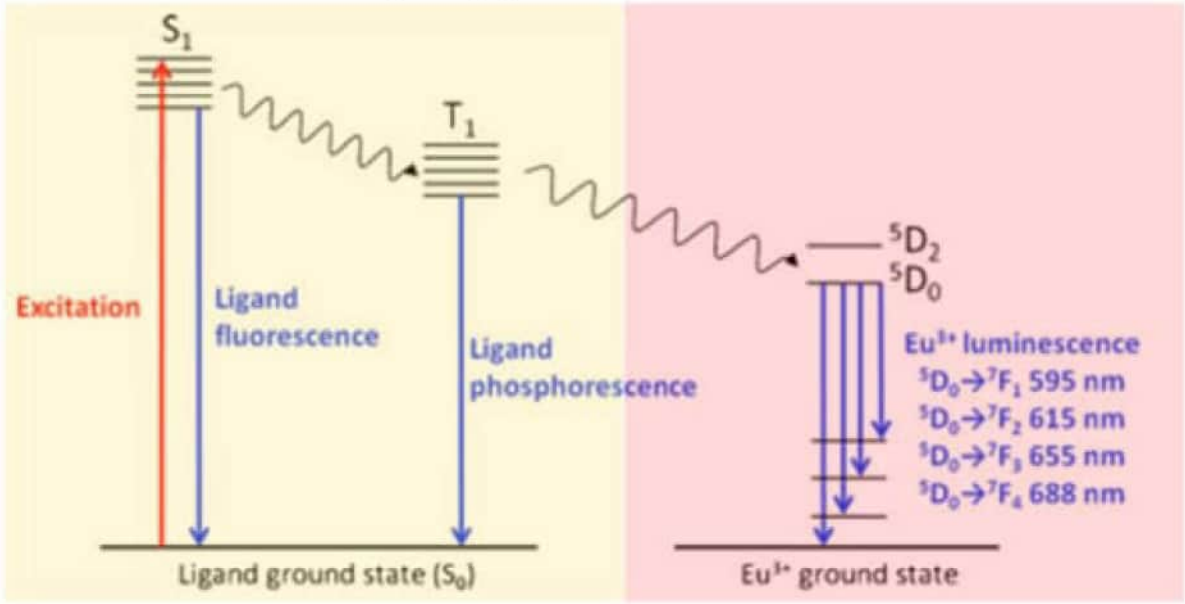
Property	Organic fluorophores and nonspecific background signals	Luminescent lanthanide chelates
Wavelength of absorption and emission bands	Anywhere in ultraviolet/visible region	Absorption in ultraviolet, emission in visible
Width of absorption and emission bands	Both broad	Absorption broad, emission several narrow bands of 1–20 nm each, separated by tens of nm
Stokes' shift between absorption and emission bands	Small (0–50 nm)	Large (150–300 nm)
Luminescence yield	Up to 100%	Usually lower, particularly in water
Luminescence lifetime	Fluorescence lifetimes on order of 1 to 100 ns	10 μs to 10 ms
Sensitivity of luminescence to variations in environment and temperature	Usually significant	Much less, within range of stable chelate formation
Chemical and photochemical stability	Usually chemically stable; may be photochemically unstable	Ligand and chelator binding to lanthanide ion often not strong; may be photochemically unstable

Requirements of luminescent Ln(III) chelates

as labels for protein conjugation and immunoassay application



Relative brightness R = ε QY



- reactive group for bioconjugation to proteins
- **antenna with efficient light absorption (ε) and triplet level suitable for energy-transfer to Ln^{3+}**
- **kinetically stable coordination of the antenna ligand to Ln^{3+}**
- water molecules replaced from the coordinating sphere to improve QY (coordination number 9)

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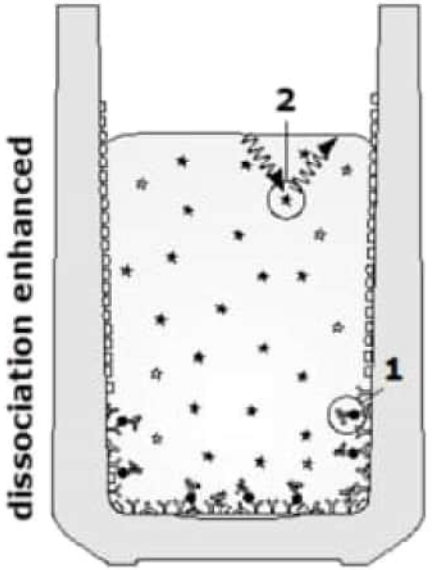
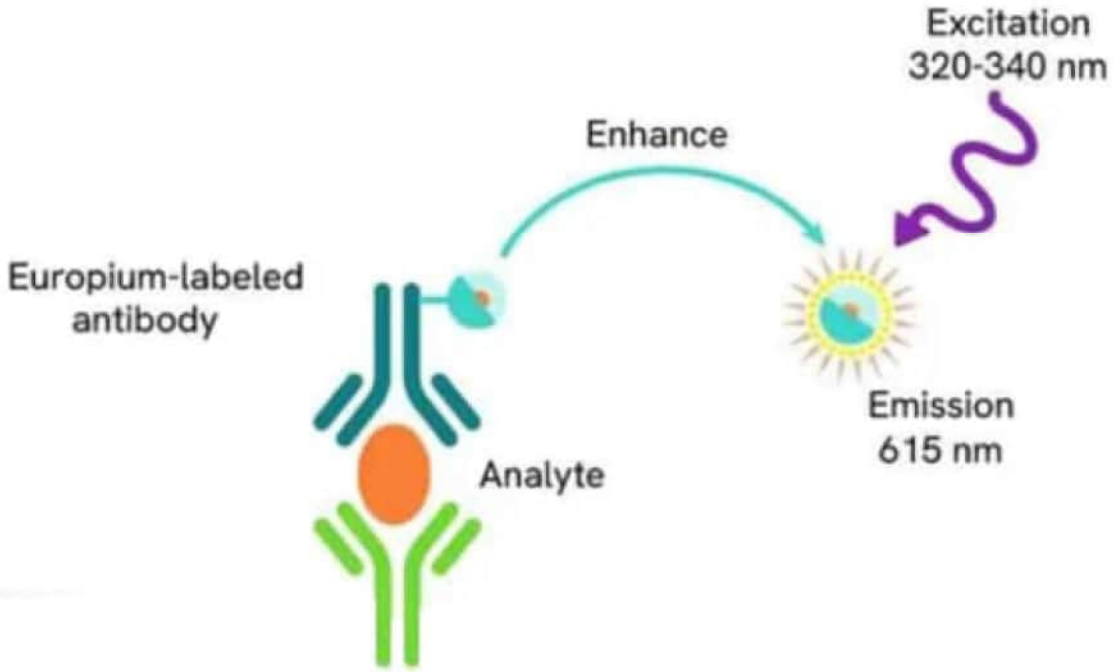
Initially it was <u>difficult to combine</u> all the requirements in a single molecule

DELFIA label technology

dissociation enhanced lanthanide fluoroimmunoassay

was developed to circumvent the challenges





Two ligands

- (1) first to conjugate Eu^{3+} to antibodies

stable complex during assay – no antenna

- (2) second to form luminescent complex with Eu^{3+} in solution

highly efficient antenna

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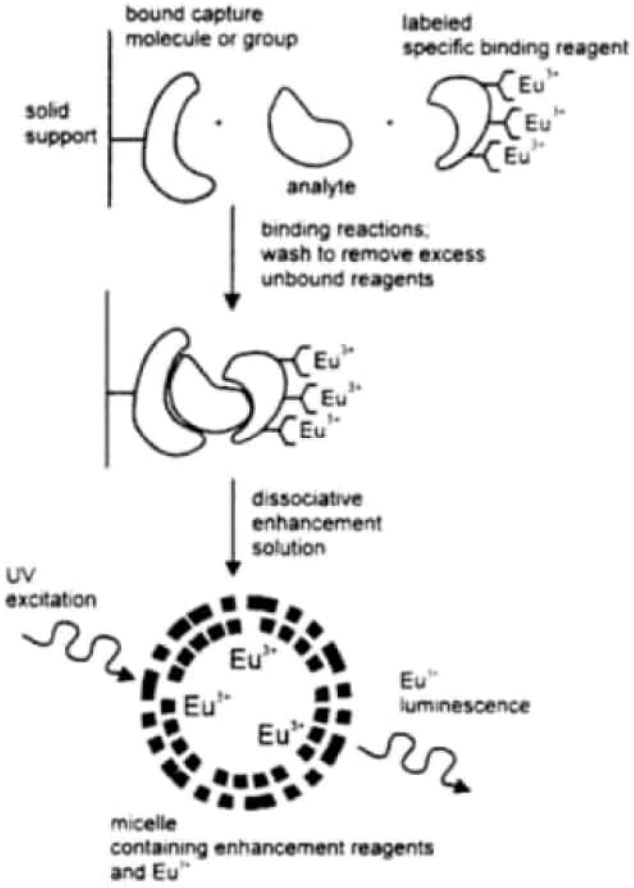
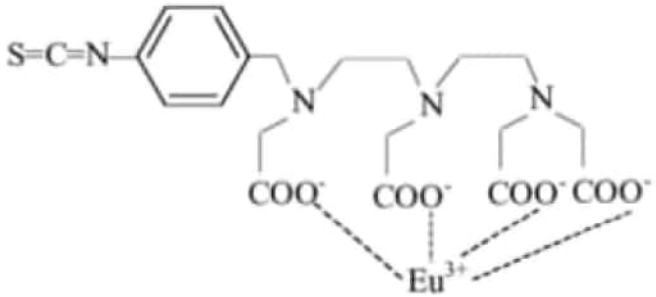
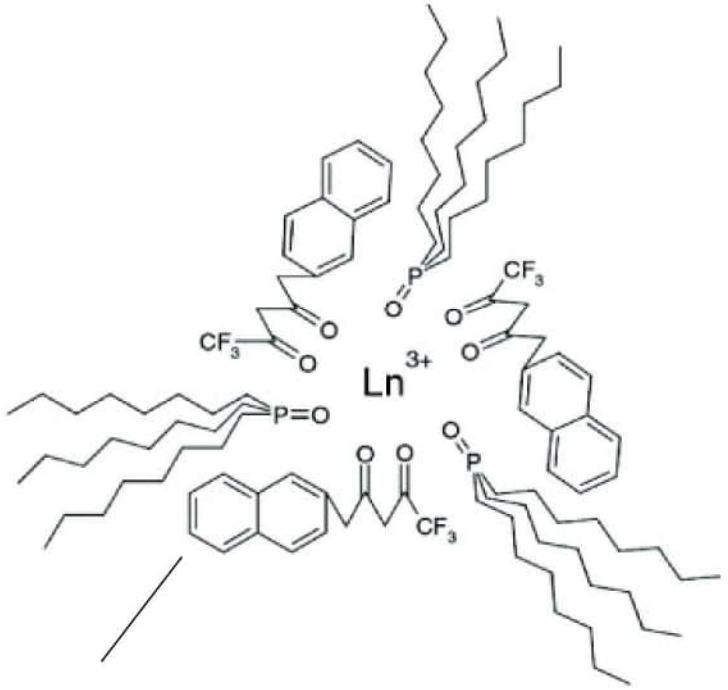
DELFIA enhancement solution

- **“non-luminescent” small EDTA-like N1-Eu-chelate** is coupled to protein and Eu^{3+} is dissociated to enhancement solution before forming new ”ideal” highly luminescent complex with 2-NTA in micellar structure with minimal interaction of water



ITC-N1-Eu-chelate

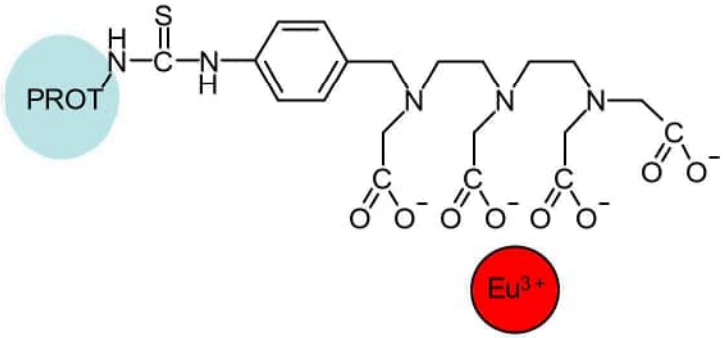




2-NTA

1-(2-naphtoyl)-3,3,3-trifluoroacetone

Non-fluorescent lanthanide chelate and enhancement



Addition of enhancement solution

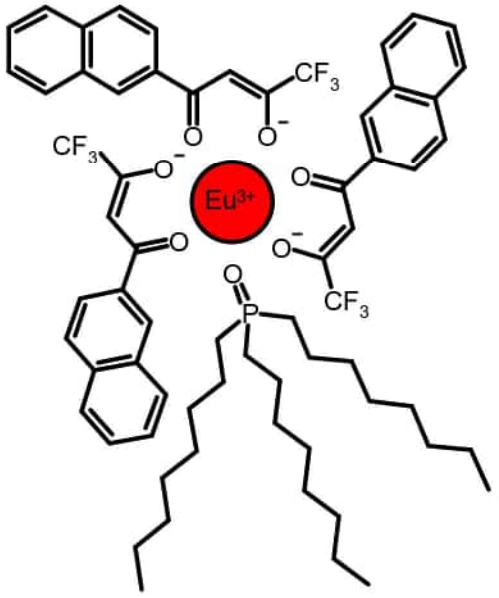
- low pH
-> ion dissociates
- excess of new ligand
-> fluorescent complex

DELFIA label technology

dissociation enhanced lanthanide fluoroimmunoassay

-> spatial information is lost

-> <u>unsuitable</u> for direct measurement from solid surface



Hemmila I, et al. (1984) *Anal Biochem* 137: 335-343

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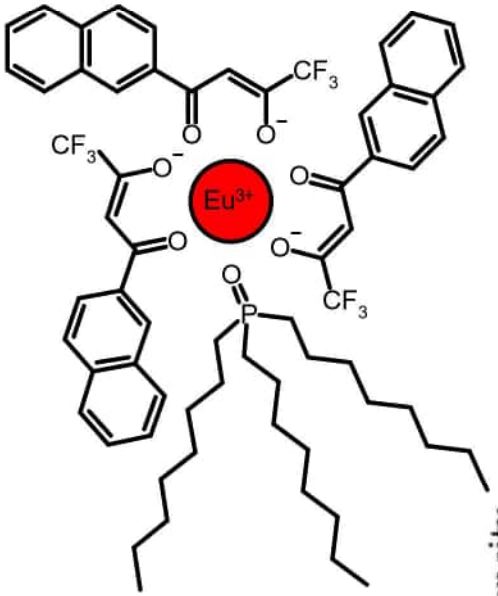
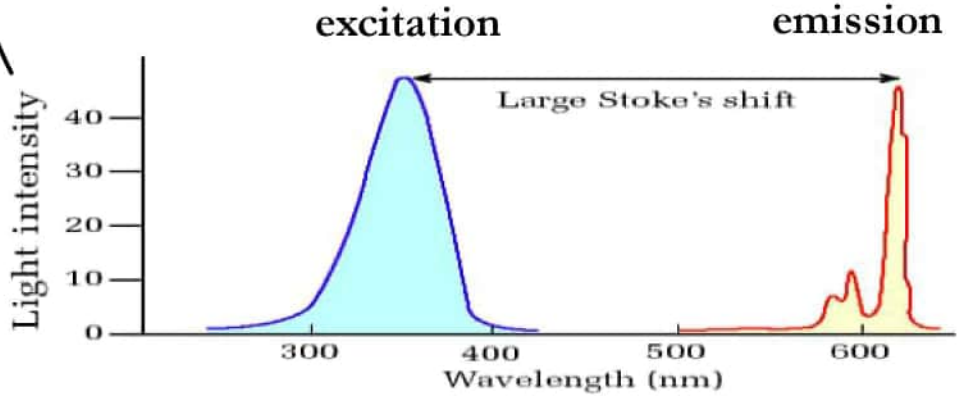
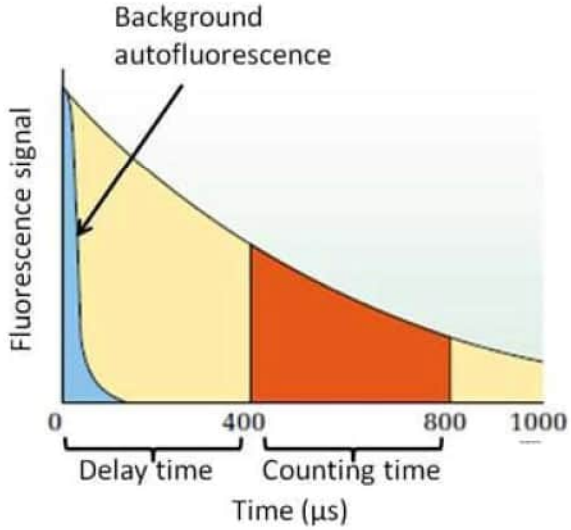
Highly sensitive detection of Eu^{3+} with time-resolved fluorometry



- **large Stoke's shift**
- **narrow emission peaks**
- **long fluorescence lifetime (~ 1 ms)**





Soini E, Hemmilä I (1979) *Clin Chem* 25: 353-361

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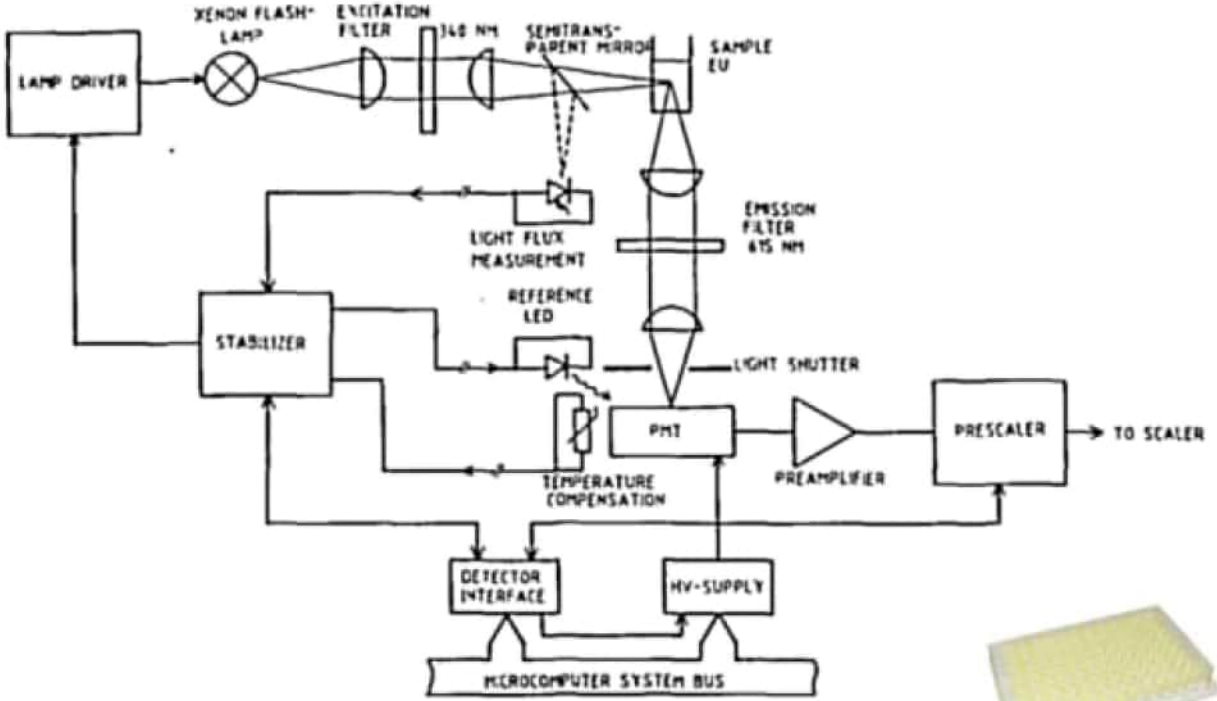
Principle of pulsed time-resolved fluorometer

Europium-chelate; emission at 615 nm, decay time ~ 0.7 ms – typically collection of integrated signal after 1000 Xe-flash pulses using 400 us delay and 400 us window.



fused silica/quartz optics

high power Xe-flash excitation
(with afterglow up to 50 us)

UV-excitation filter
with efficient VIS/NIR blocking

narrow high-transmission band-pass filter for emission band

low dark count PMT and photon counting

special "yellow" microplates
with UV-quencher for minimal background

Soini E, Kojola H (1983) *Clin Chem* 29: 65-68

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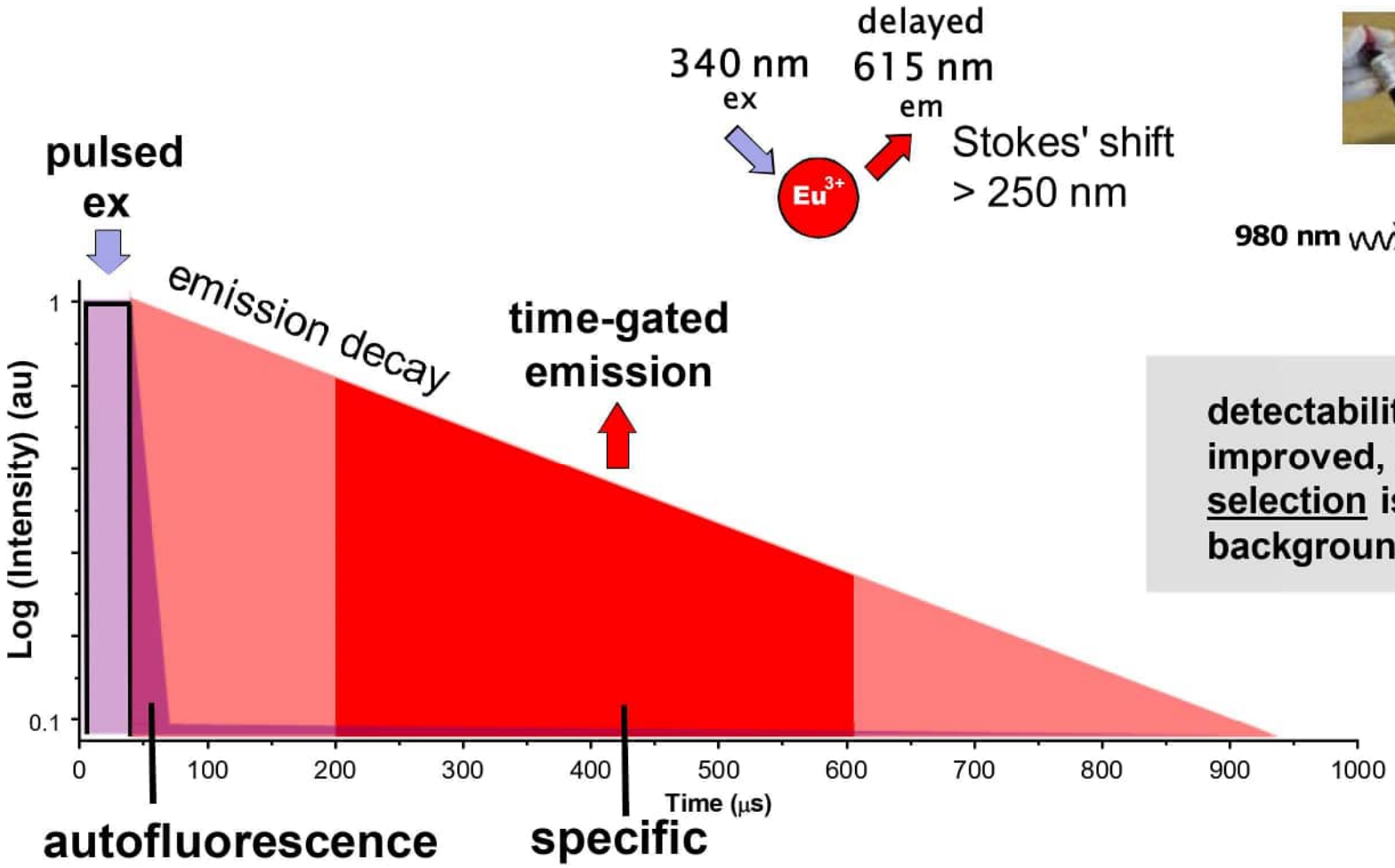
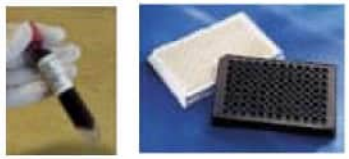
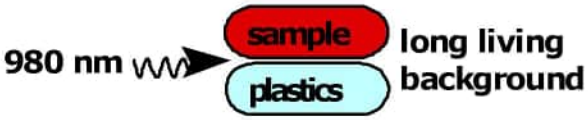
Time-resolved fluorometry

of lanthanide luminescence to suppress autofluorescence







detectability is significantly improved, but careful material selection is required to avoid background

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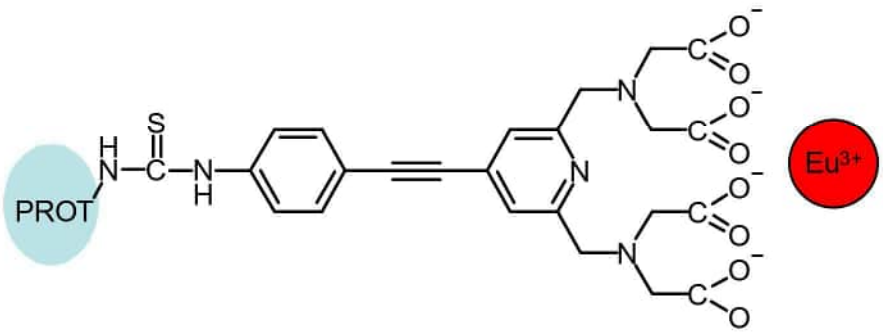
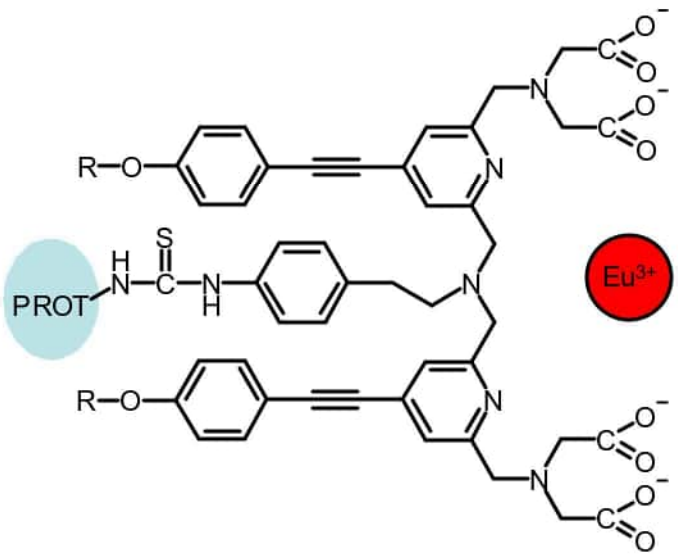
Development of intrinsically fluorescent lanthanide chelates

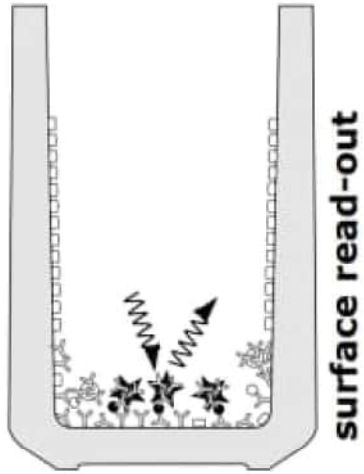


→ spatial information is preserved

Takalo, *et al.* (1994) *Bioconjug Chem* **5**: 278-282.



→ direct measurement from (dry) solid surface (no need for enhancement)



→ donors in fluorescence resonance energy-transfer assays (FRET)

von Lode, et al. (2003) *Anal Chem* **75**: 3193-3201. https://doi.org/10.1021/ac0340051

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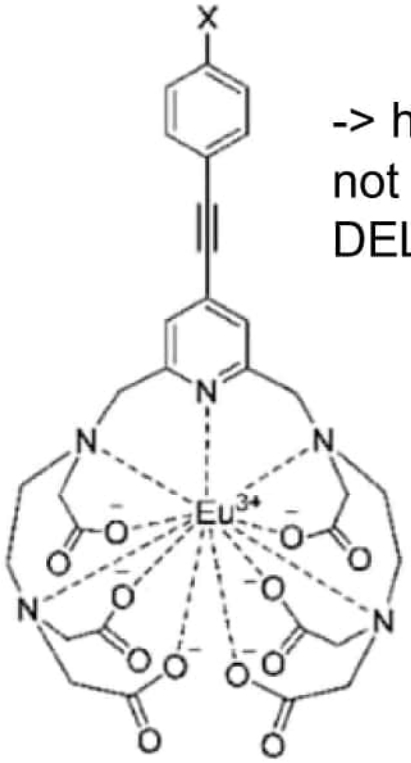
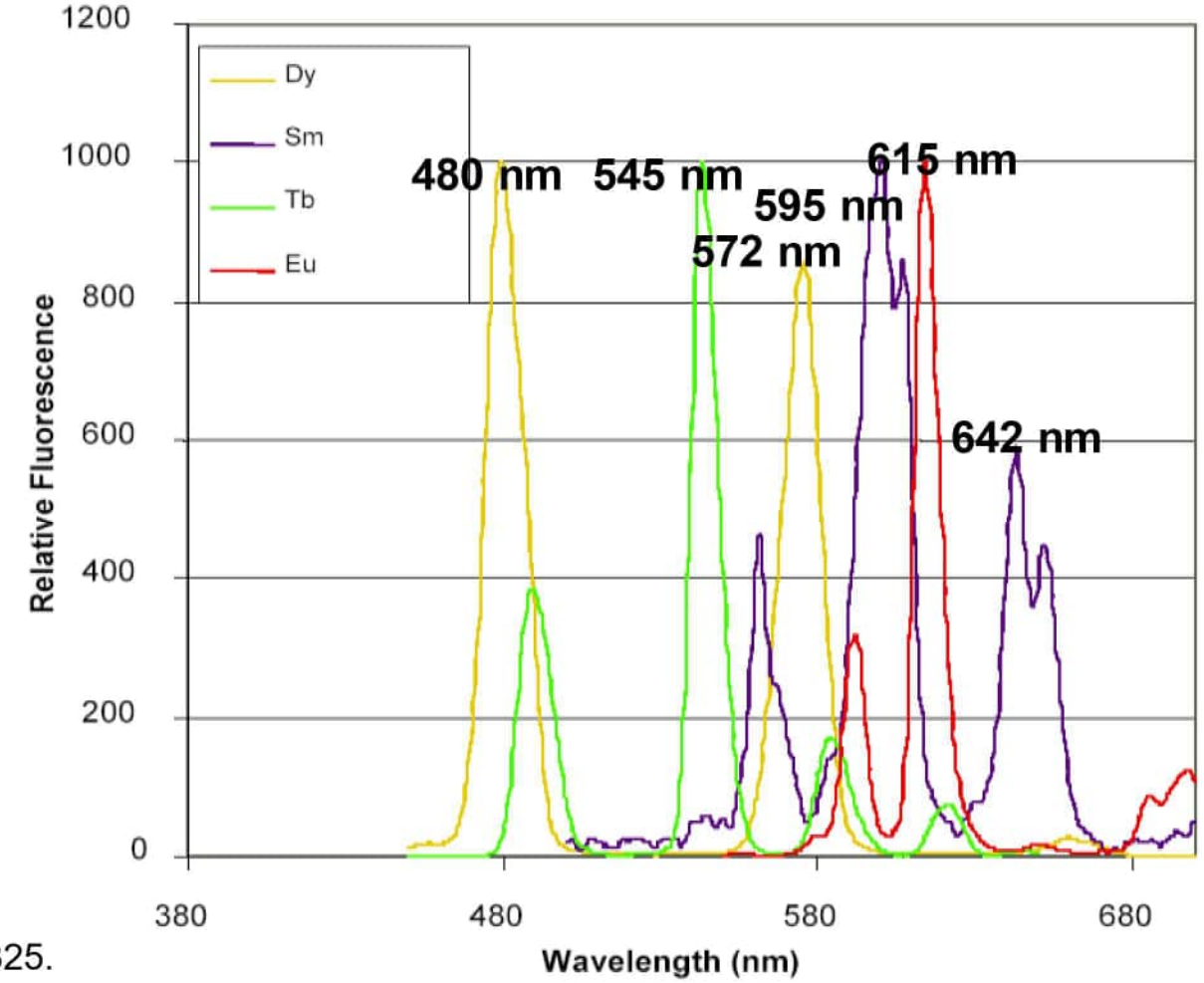
Intrinsically fluorescent Eu, Tb, Sm and Dy chelates



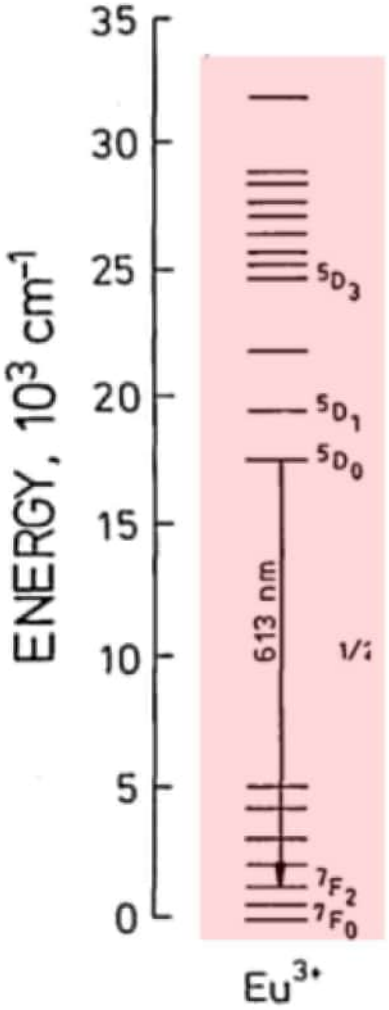
-> highly stable, but not as bright as DELFIA complexes

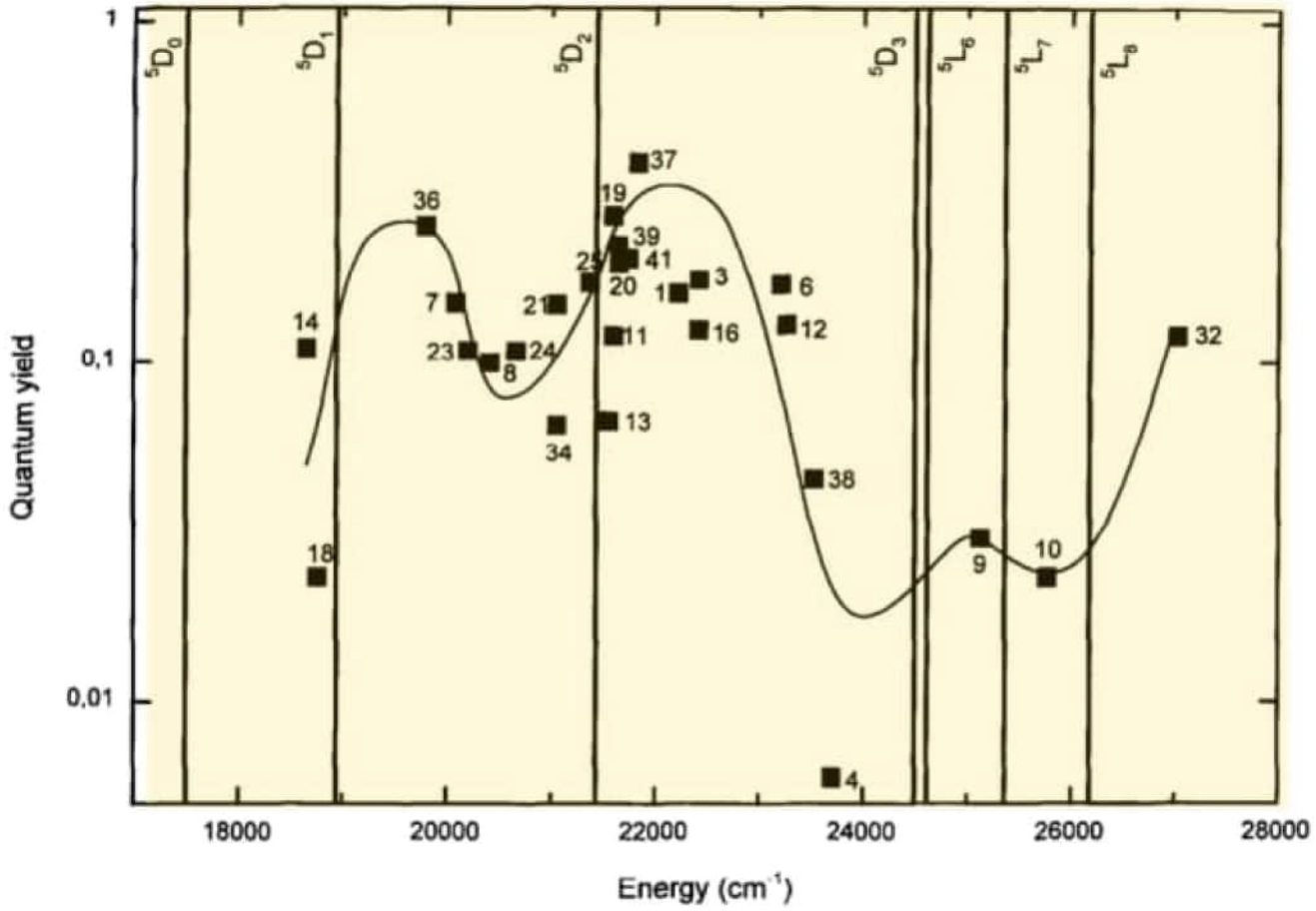
Pulsed excitation at 320-340 nm; time-resolved fluorometry.



Karvinen J, et al. (2004*) Anal Biochem*. 325: 317-325.

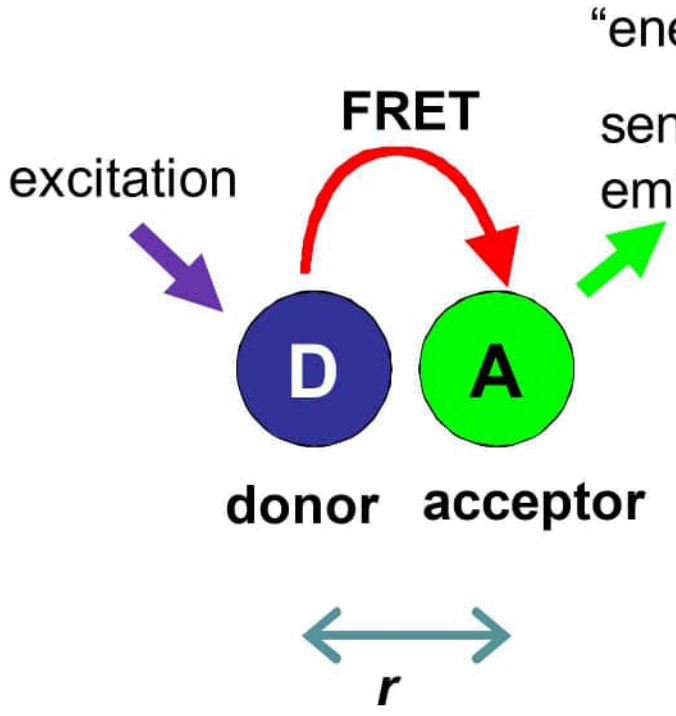
Correlation between the lowest triplet state energy level of the ligand and lanthanide(III) luminescence quantum yield





Latva, M. et al. (1997) J Lumin 75: 149-169.

FRET
Fluorescence resonance energy transfer



$$E = \frac{1}{1 + (r/R_0)^6}$$

R_0 = 50 % efficiency distance (~3 – 8 nm)

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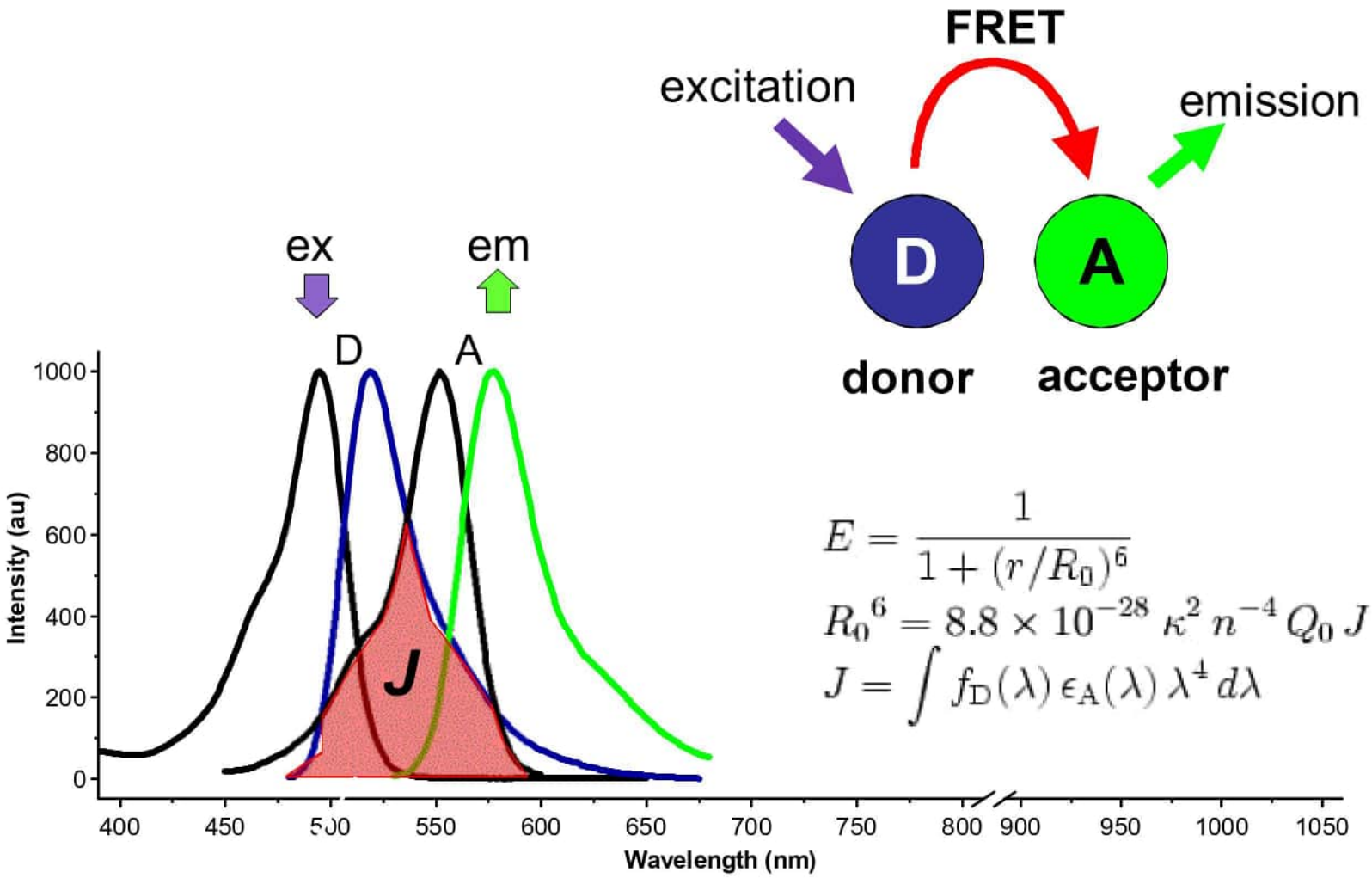

$$E = \frac{1}{1 + (r/R_0)^6}$$

$$R_0^{\,6} = 8.8 \times 10^{-28}\, \kappa^2\, n^{-4}\, Q_0\, J$$

$$J = \int f_{\mathrm{D}}(\lambda)\, \epsilon_{\mathrm{A}}(\lambda)\, \lambda^4\, d\lambda$$

spectral overlapping

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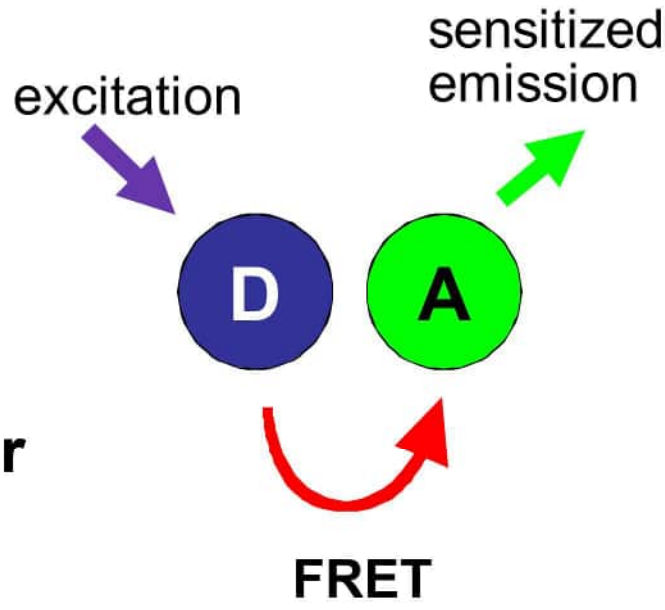
Conventional FRET

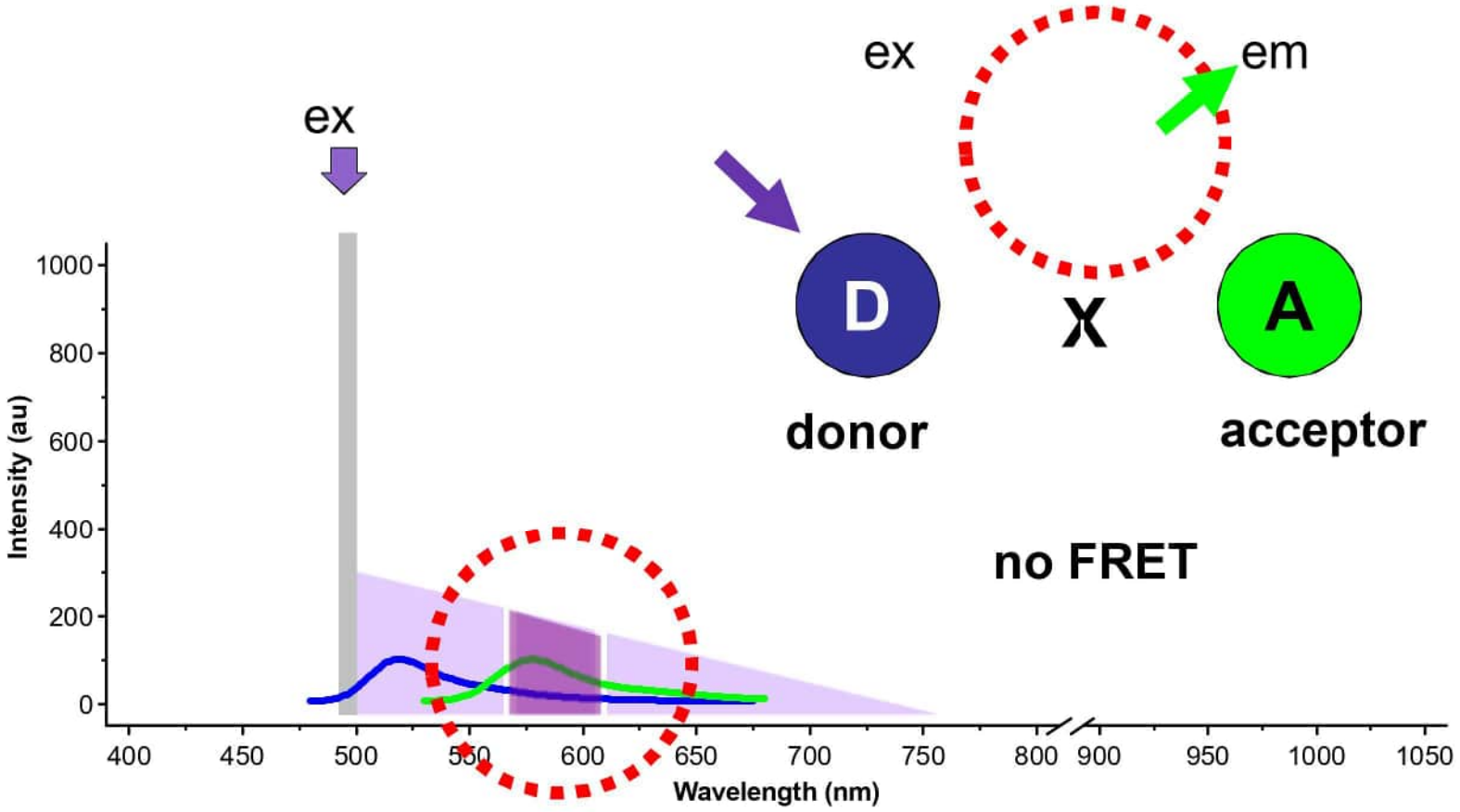
is excellent research tool, but has severe limitations

- **autofluorescence (background fluorescence)**
- **direct excitation of acceptor**
- **crosstalk of donor**



Autofluorescence background

in conventional FRET



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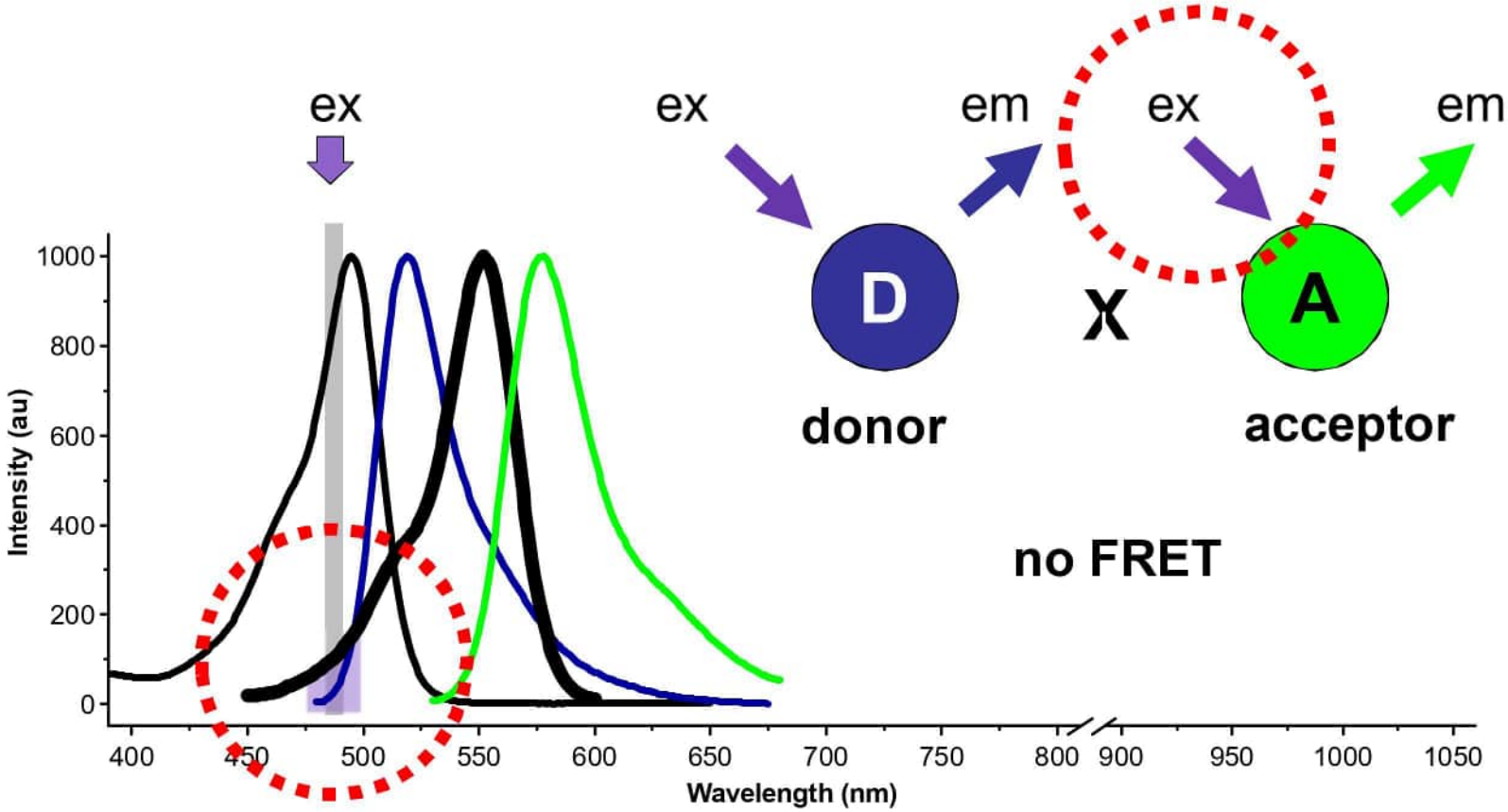
Acceptor is excited directly
in conventional FRET



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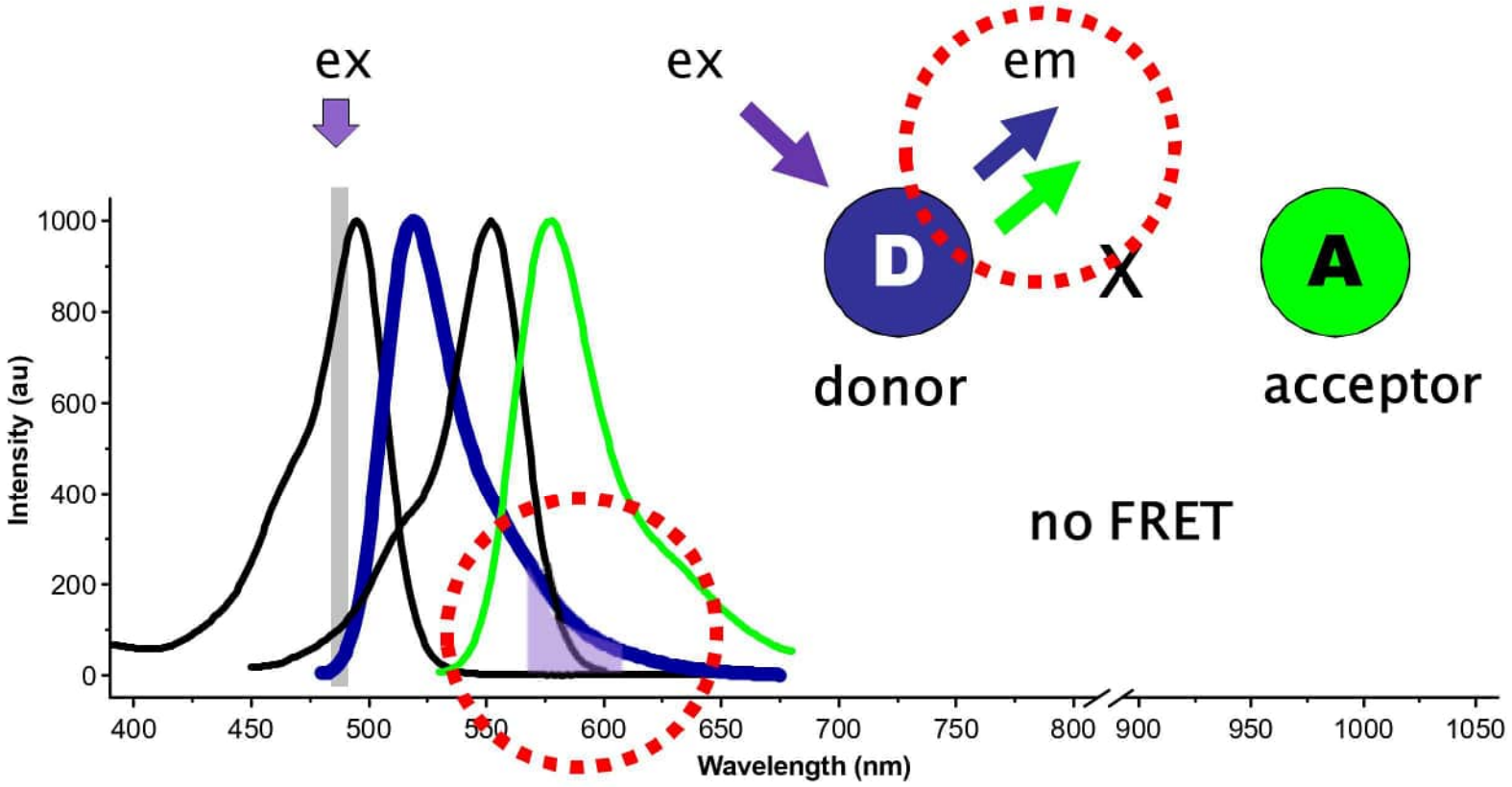
Crosstalk of donor emission
in conventional FRET



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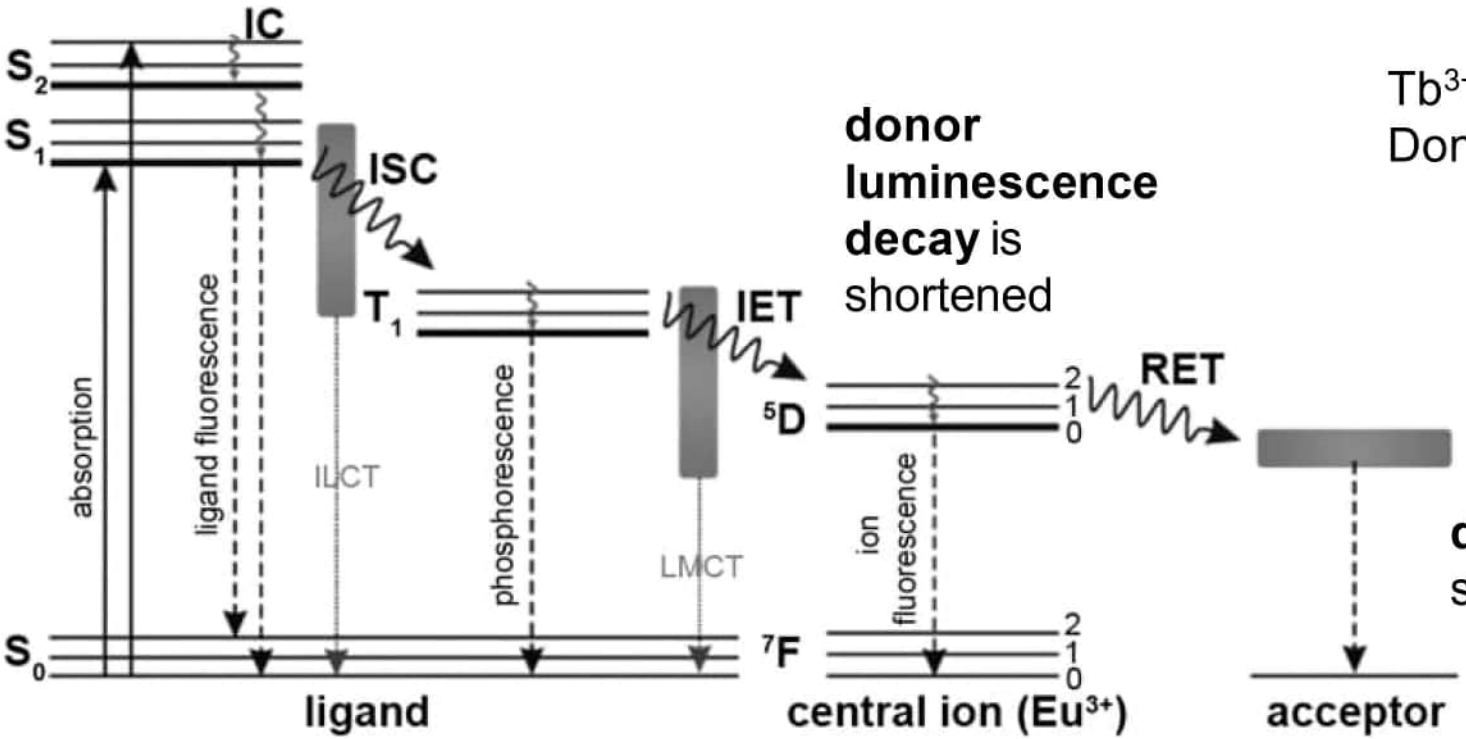
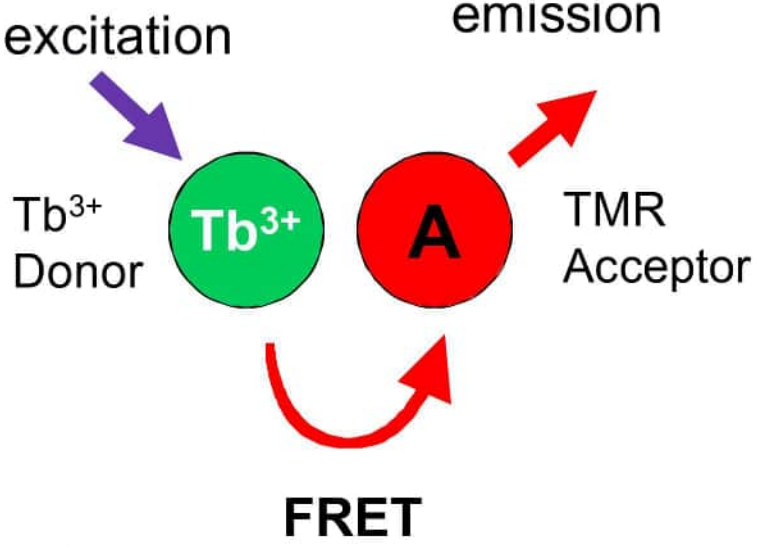
Time-resolved FRET (TR-FRET)

using Ln^{3+} donor with conventional acceptor fluorophore provides significant advantages



donor luminescence decay is shortened

delayed sensitized emission



delayed sensitized emission

Blomberg, K. et al. (1999) *Clin Chem* 45:6.

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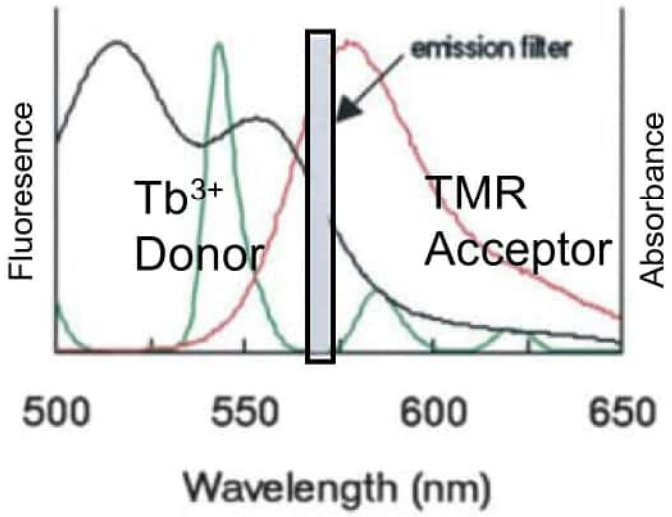
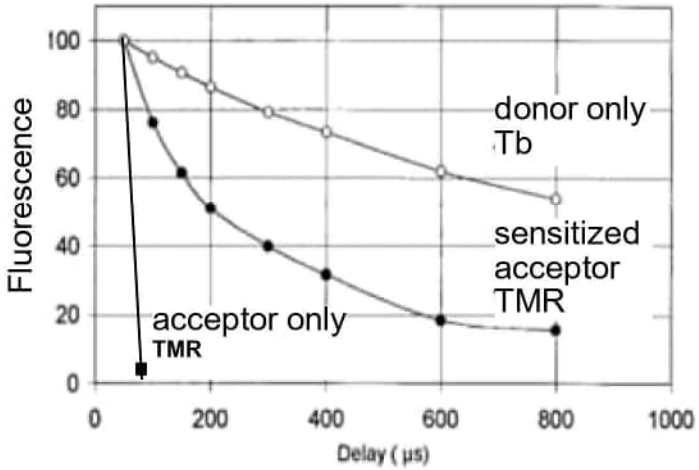
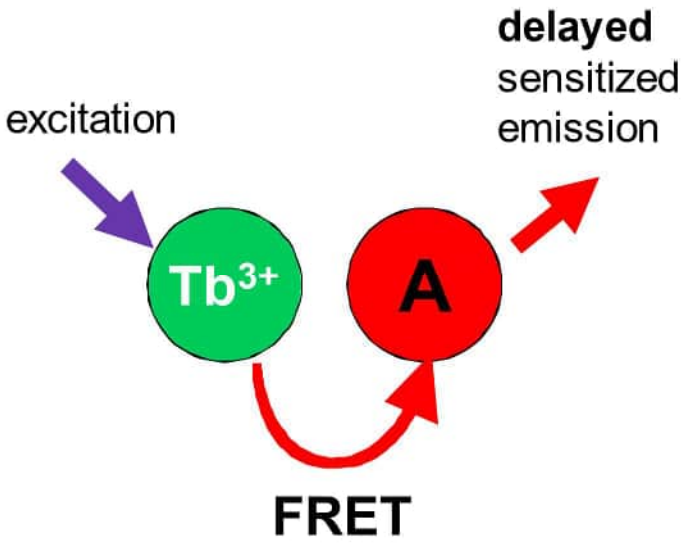


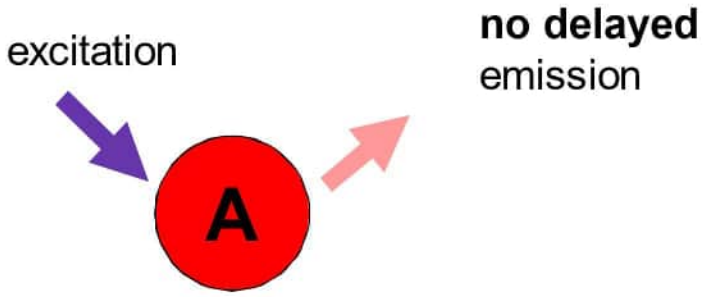




- **time-gating** resolves
  autofluorescence and

  short-living emission of
  direct excitation of acceptor

- **no crosstalk of donor** as
  donor emission is narrow banded

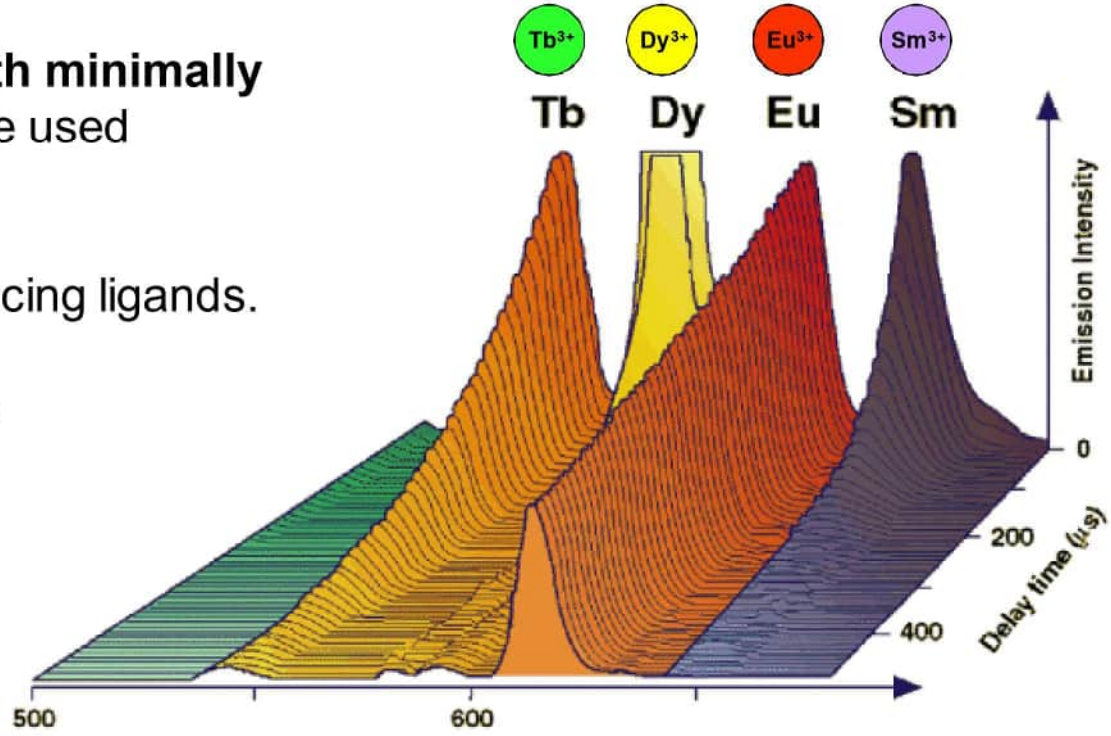


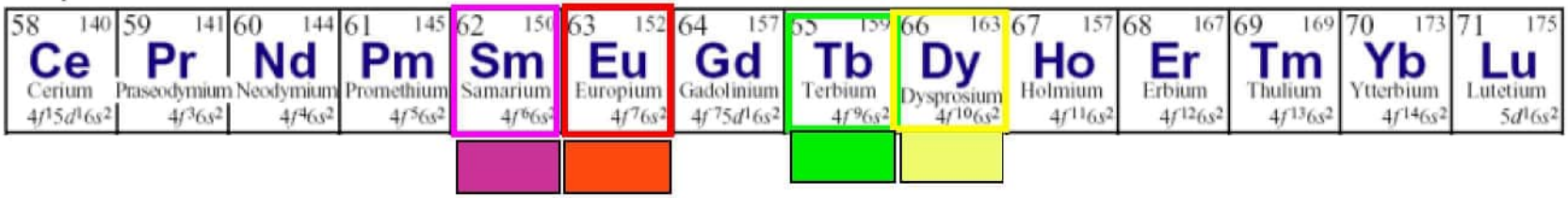
Multiparametric DELFIA label technology

Four luminescent lanthanides with minimally overlapping emission lines can be used simultaneous as labels

Eu/Sm + Tb/Dy with different enhancing ligands.

Tb 545 nm (100-1500 us, QY 40%)
Eu 615 nm (500-730 us, QY 70%)
Sm 642 nm (50 us, QY 2%)
Dy 572 nm (1-20 us, QY 2%)





Hemmiä, I. and Mukkala, V-M. (2001) *Crit Rev Clin Lab Sci* 38:441–519.

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Multiparametric/multiplexed assay

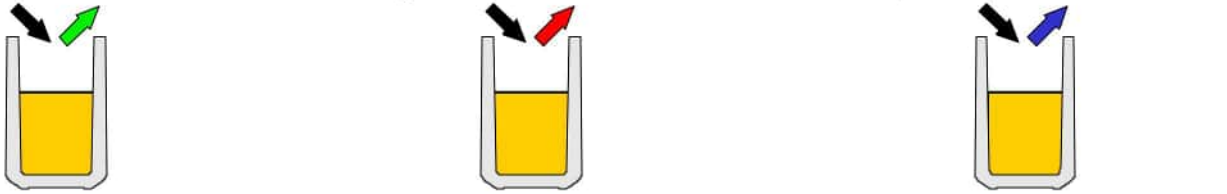
= assay that measures **more than one analyte** simultaneously from **the same aliquot of sample** in a single run/cycle of the assay

Multiplexed assay

to measure multiple analytes from single aliquot of sample

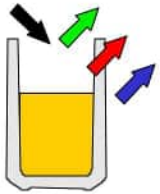
Separate assays

sample A result 1 sample A result 2 sample A result 3



Multiplexed assay

sample A result 1, result 2, result 3



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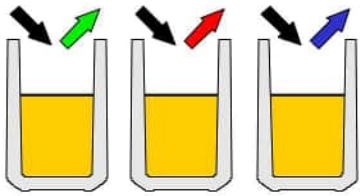
Multiplexed assay

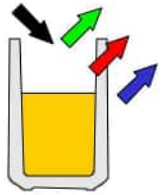
is more cost efficient to measure multiple analytes

Separate assays



3x consumables (3x reagents)
3x sample
3x work

Multiplexed assay



1x consumables (~1-3x reagents)
1x sample
1x work

-> more economical

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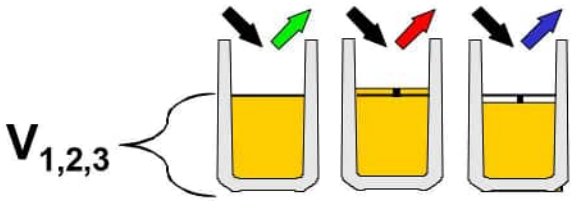
Multiplexed assay

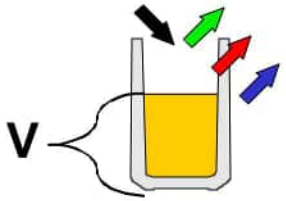
is more accurate in ratiometric measurements

Separate assays



$$ratio_s = \frac{result_1 \times v_1}{result_2 \times v_2}$$

Multiplexed assay



$$ratio_{mp} = \frac{result_1 \times \cancel{v}}{result_2 \times \cancel{v}}$$

-> effects of common errors in analysis are eliminated

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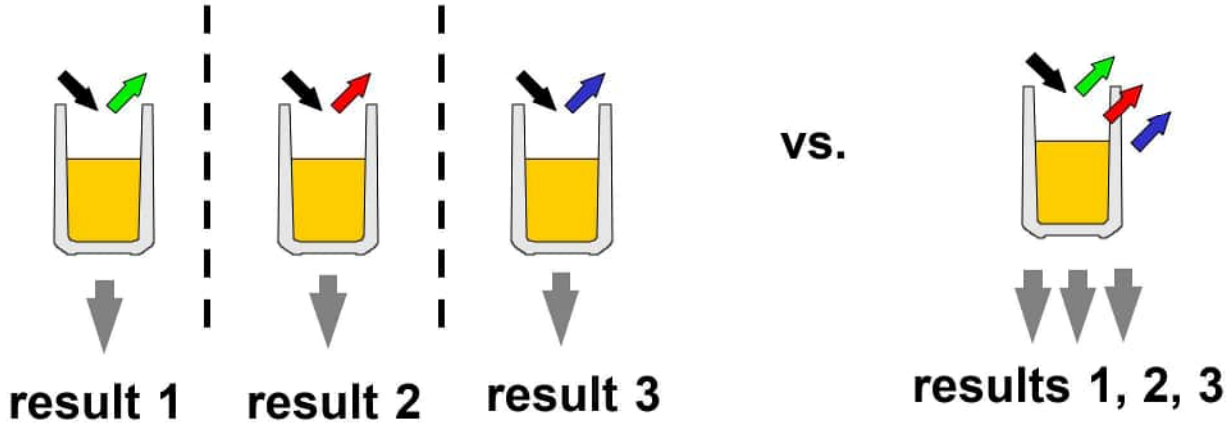
need in clinical diagnostics to measure multiple analytes

e.g. several infectious diseases share common basic symptoms, but the identification of the cause may be needed for selecting the proper treatment



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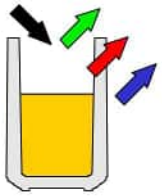
Mode of multiplexing

to enable separate measurement of multiple analytes

Emission (spectral multiplexing)



wavelength separation

result

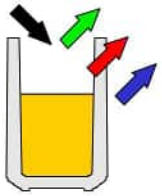
1 **2** **3**

band-pass filter

Mode of multiplexing

to enable separate measurement of multiple analytes

Emission (spectral multiplexing)



wavelength separation

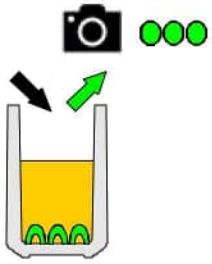
result

1 **2** **3**

band-pass filter

Spot position

(spatial array)



imaging/scanning

result

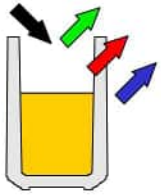
1 **2** **3**

solid-phase area

Mode of multiplexing

to enable separate measurement of multiple analytes

Emission (spectral multiplexing)



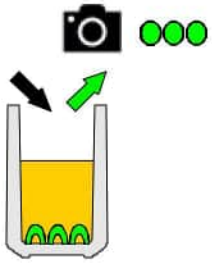
wavelength separation

result

1 **2** **3**

band-pass filter

Spot position
(spatial array)



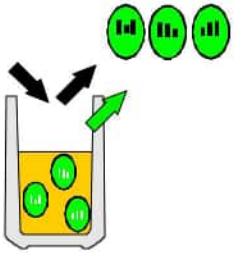
imaging/scanning

result

1 **2** **3**

solid-phase area

Optical barcode
(suspension array)



flow cytometry

result

1 **2** **3**

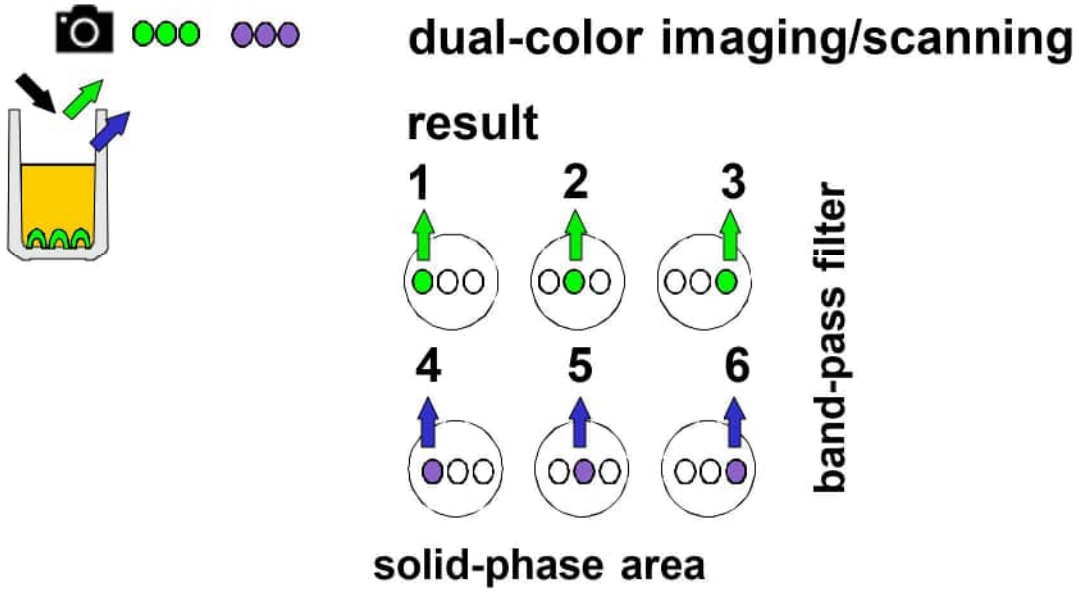
optical tag readout

Dual-mode of multiplexing

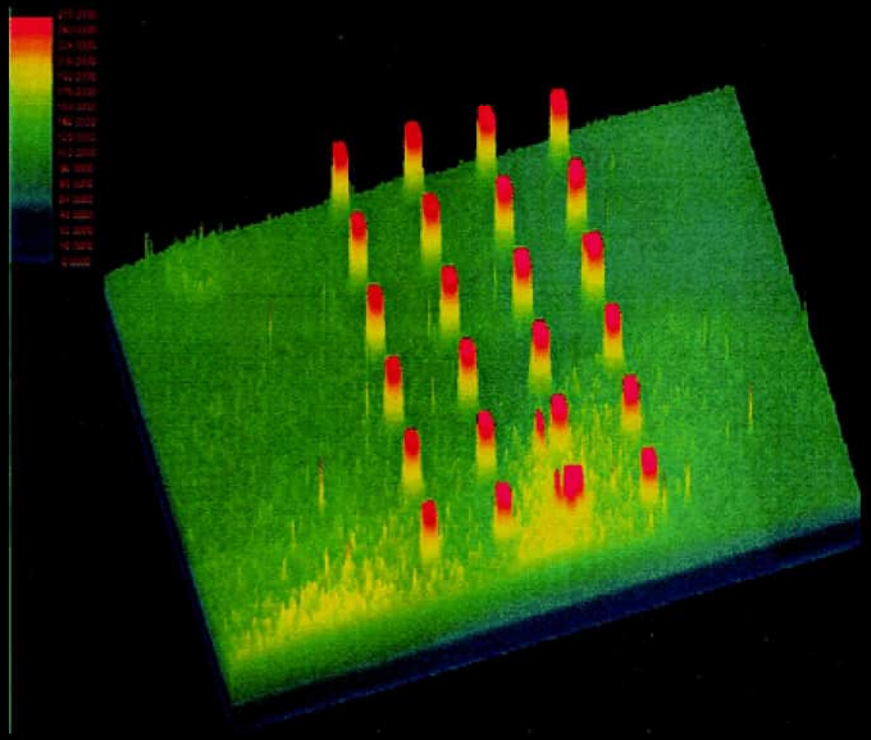
combining two modes to measure multiple analytes

Emission color and spot position

(spectral and spatial multiplexing)



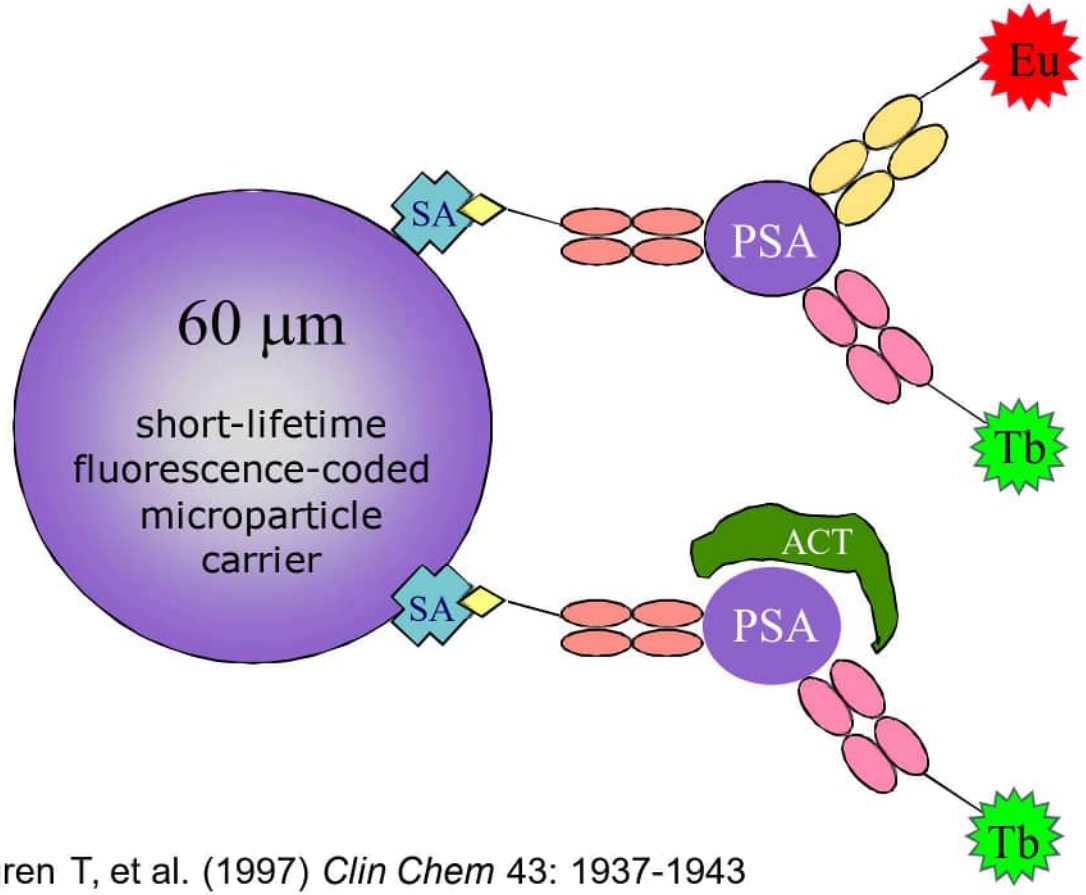
Protein array - biotinylated antibody spots



Detection of intrinsically fluorescent europium chelate-labeled streptavidin with time-resolved microimager.

Scorilas A, Bjartell A, Lilja H, Moller C, Diamandis EP (2000) Clin Chem 46: 1450-1455

Multiparametric liquid-array on categorized microparticles



Assays with time-resolved microfluorometer.

Lövgren T, et al. (1997) *Clin Chem* 43: 1937-1943

Hakala H, et al. (1998) *Nucleic Acids Res* 26: 5581-5588

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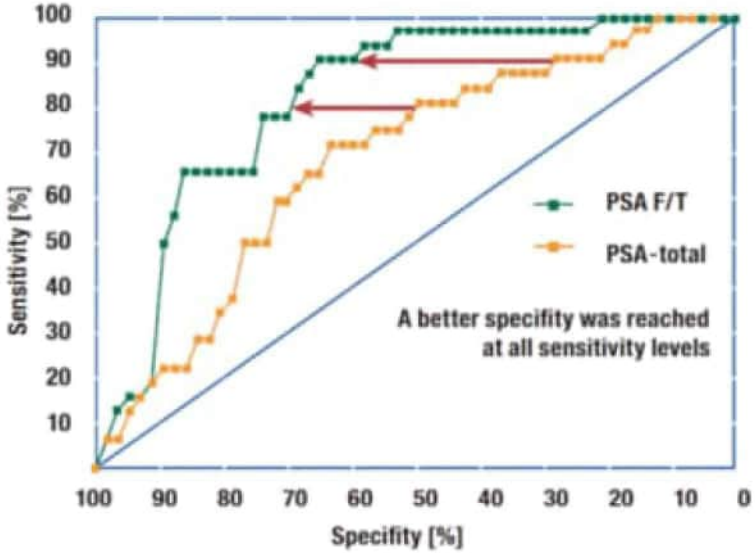
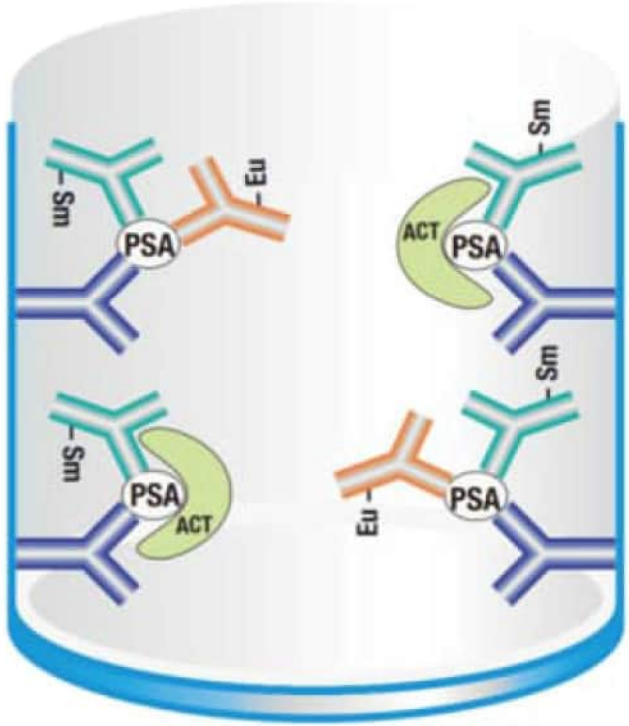
Prostatus free/total PSA immunoassay

based on Sm^{3+} and Eu^{3+} dual-label DELFIA technology



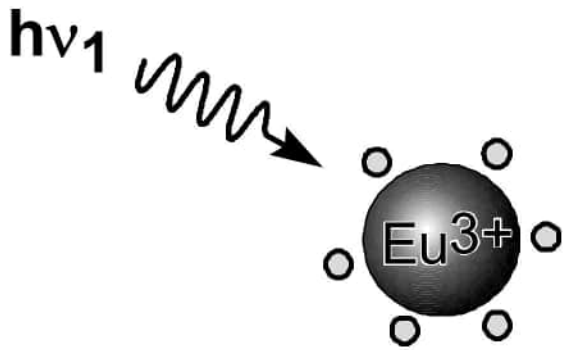
Free/total PSA ratio in serum provides improved discrimination of cancer compared to total PSA.



Sm-labeled antibody for total PSA
and Eu-labeled antibody for free-PSA

Two-plex assay is more accurate than ratio of two separate assays.

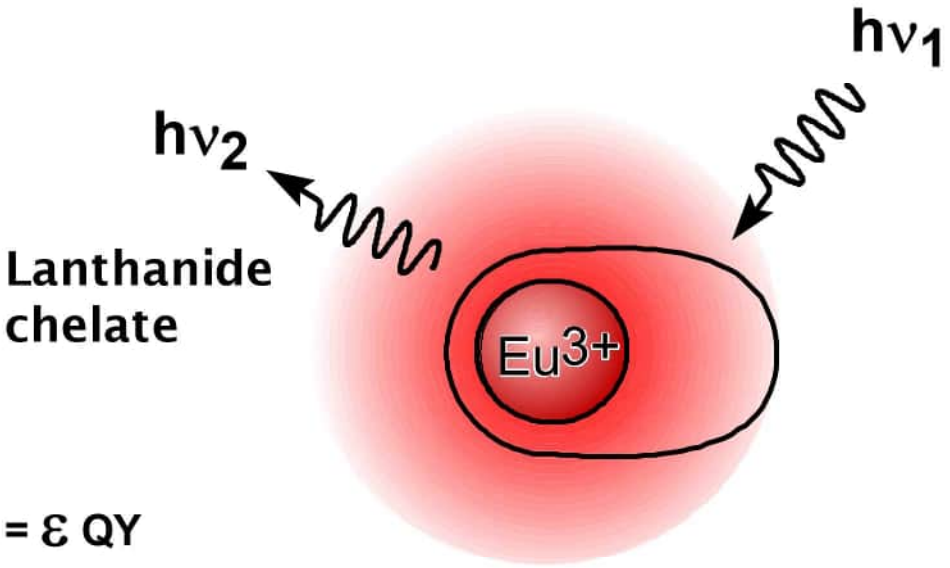
Bare lanthanide ions are
"non-nonluminescent"



- molar absorptivitity
  $< 1\ M^{-1}\ cm^{-1}$
- quenched by
  coordinated water molecules

⇒ practically no luminescence

Excitation through
light-harvesting organic ligand

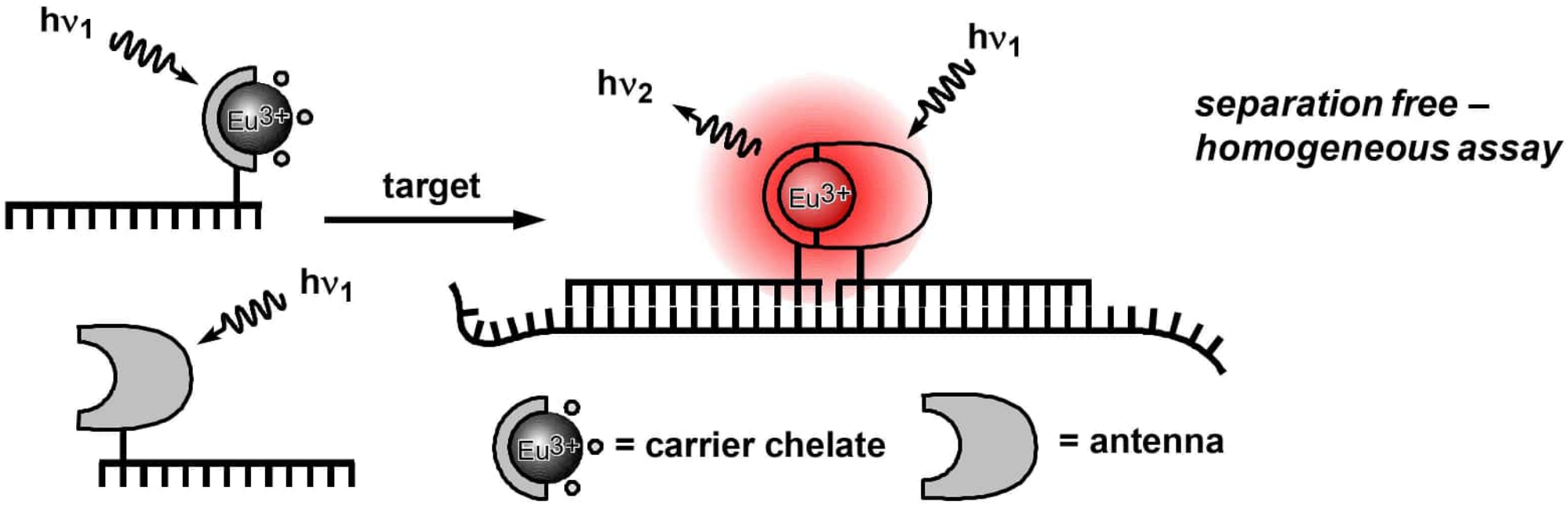


- molar absorptivitity
  $> 10000\ M^{-1}\ cm^{-1}$
- high quantum yield

⇒ highly luminescent (time-resolved detection enables low limit-of-detection)

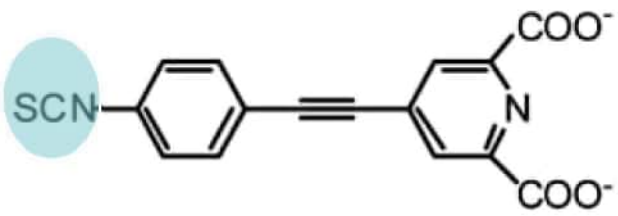
Relative brightness R = ε QY

Mixed-chelate complex formation through biomolecular interactions

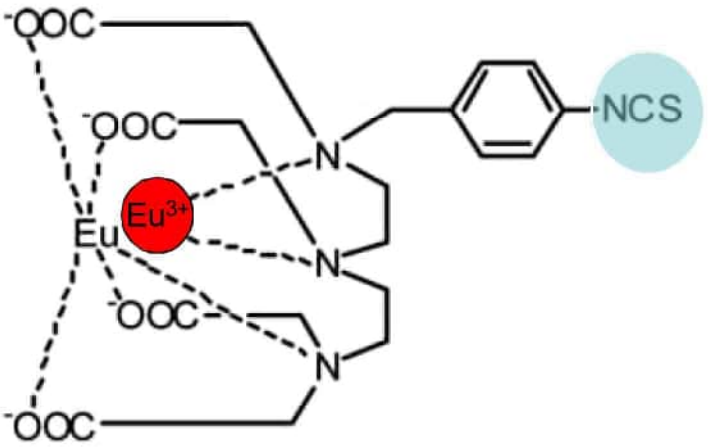


Karhunen U *et al.* (2010) *Anal Chem* 82: 751-574

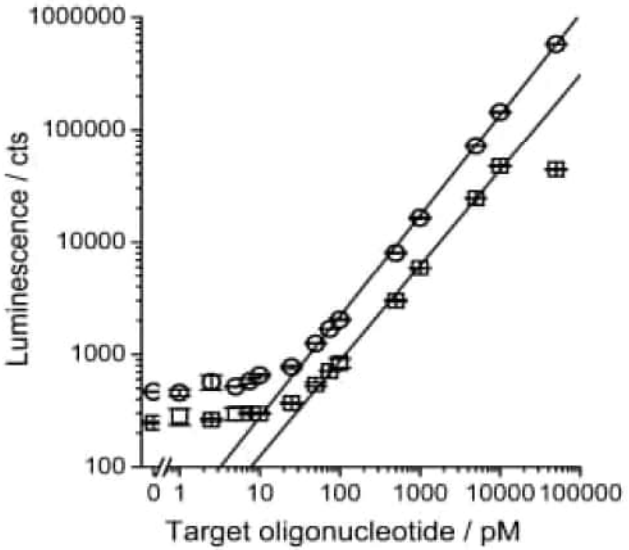
Switchable lanthanide luminescence



antenna ligand



ion carrier chelate



Chelate complementation

- fluorescent europium chelate divided to two label moieties

→ novel **homogeneous** reporter technology
(very high degree of modulation)

Karhunen et al. (2001) *Anal Chem* **82**: 751-754

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Summary

Luminescent lanthanides and time-resolved fluorescence

- millisecond time-gated luminescence detection efficiently **eliminates autofluorescence**
- low background **optical material selection is needed** for detection and consumables
- organic **light-harvesting antenna** ligand is required for efficient excitation of lanthanides
- lanthanide **chelate-dyed nanoparticles provide extreme detectability**
- **DELFIA technique** resembles enzyme assays as it requires enhancement step
- immunoassay **sensitivity** with lanthanide-chelate dyed nanoparticles is **limited by non-specific interactions**
- nanoparticle based solid-phase assays are prone to **steric limitations**

- most efficient luminescent lanthanides are Eu^{3+} and Tb^{3+} followed by Sm^{3+} and Dy^{3+}
- high-intensity UV-excitation and low emission intensity are challenges for detection
- time-gated luminescence imaging requires special instrumentation

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