Lecture 10

Basics of Absorption and Fluorescent Spectroscopy

Fiber-optical biosensors.

Real time PCR. Pyrosequencing.

OPTICAL SENSING: BACKGROUND

Absorption spectroscopy

- **The Beer-Lambert law**

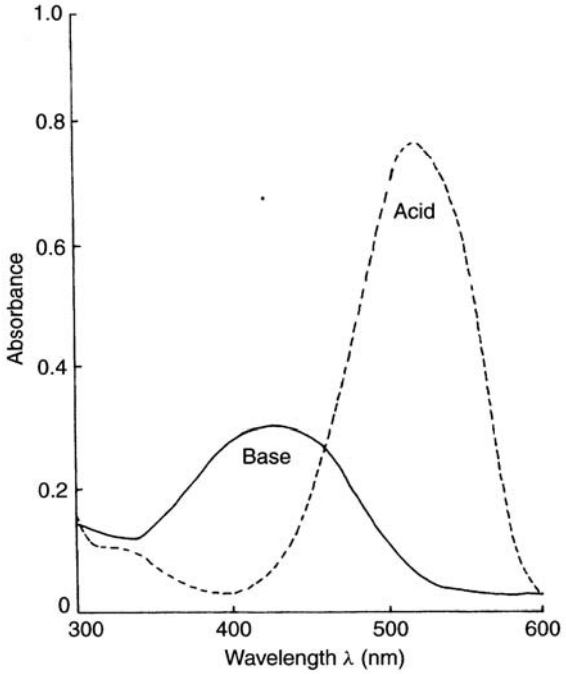
$$\log(I/I_0) = A = \varepsilon C l$$

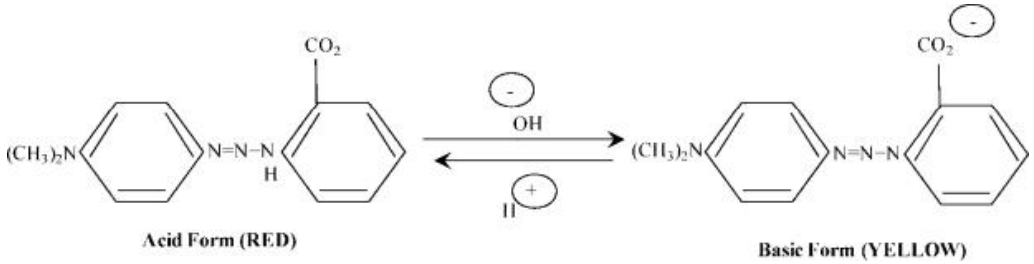
Adsorbance — Extinction coefficient — Concentration — Pathlength

✓ The sensitivity is proportional to the pathlength

What else can we measure with absorption?

- pH measurements:
  - Methyl Red dye has well separated absorption maxima for base and acidic state



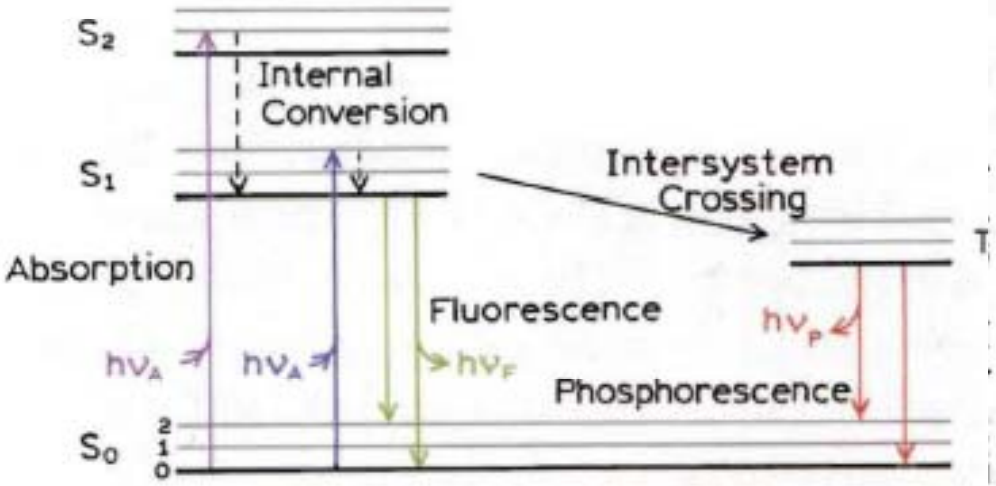


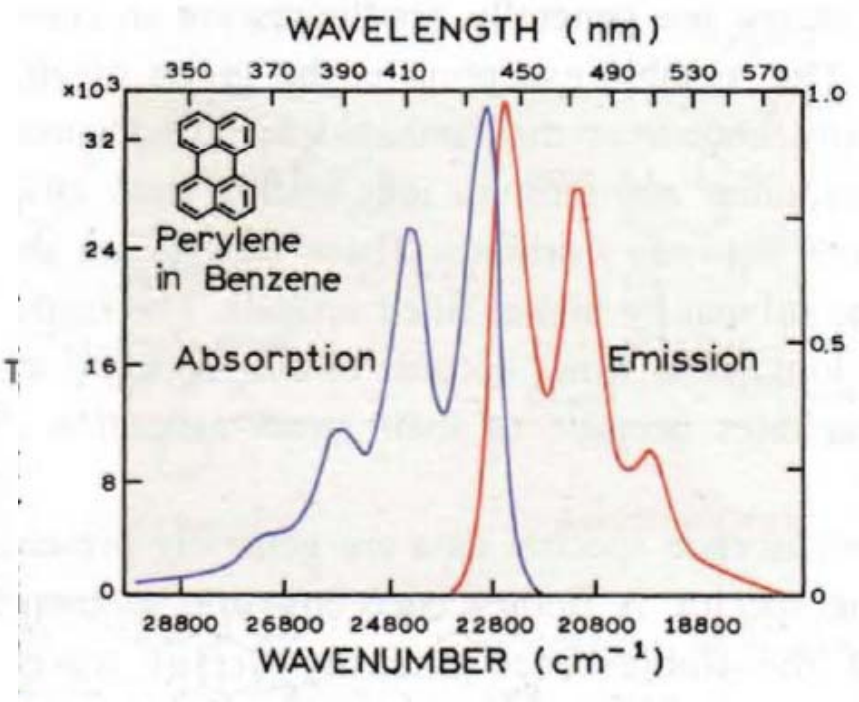
- Was used in the extrinsic geometry to measure blood pH in the range 7.0-7.5 (±0.01)

Photochemistry

- Typical times involved

Electronic transition caused by adsorption:	10^{-16} – 10^{-15} s
Fluorescence:	10^{-12} – 10^{-6} s
Intersystem crossing:	10^{-12} – 10^{-4} s
Phosphorescence	





Photochemistry

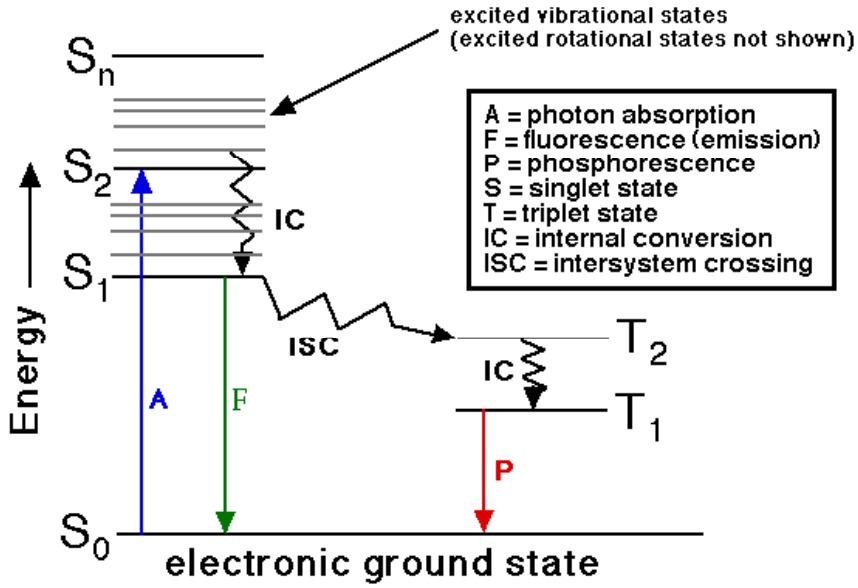
- Mechanism of decay of excited singled state

Absorption: $S + h\nu_i \longrightarrow S^*$ $\quad v_{abs} = I_{abs}$

Fluorescence: $S^* \longrightarrow S + h\nu_f$ $\quad v_f = k_f[S^*]$

Internal conversion: $S^* \longrightarrow S$ $\quad v_{IC} = k_{IC}[S^*]$

Intersystem crossing: $S^* \longrightarrow T^*$ $\quad v_{ISC} = k_{ISC}[S^*]$



If the absorbance of sample is not too high [S*] can be assumed small and constant:

$$\frac{d[S^*]}{dt} = I_{abs} - k_f[S^*] - k_{IC}[S^*] - k_{ICS}[S^*] = 0$$

$$I_{abs} = (k_f + k_{IC} + k_{ICS})[S^*]$$

$$\phi = \frac{v_f}{I_{abs}} = \frac{k_f[S^*]}{(k_f + k_{IC} + k_{ICS})[S^*]} = \frac{k_f}{k_f + k_{IC} + k_{ICS}}$$

Fluorescence life time

$$[S^*]_t = [S^*]_0 e^{-t/\tau_0}$$

$$\tau_0 = \frac{1}{k_f + k_{IC} + k_{ICS}}$$

Photochemistry

- **<u>Primary quantum yield:</u>**
  a number of photophysical or photochemical events that lead to a primary product per number of photons absorbed

$$\phi = \frac{\text{number of events}}{\text{number of photons absorbed}} = \frac{v}{I_{abs}}$$

$$\sum \phi_i = \sum_i \frac{v_i}{I_{abs}} = 1 \text{ and } \sum_i v_i = I_{abs}$$

$$\phi_i = \frac{v_i}{\sum_i v_i}$$

Photochemistry

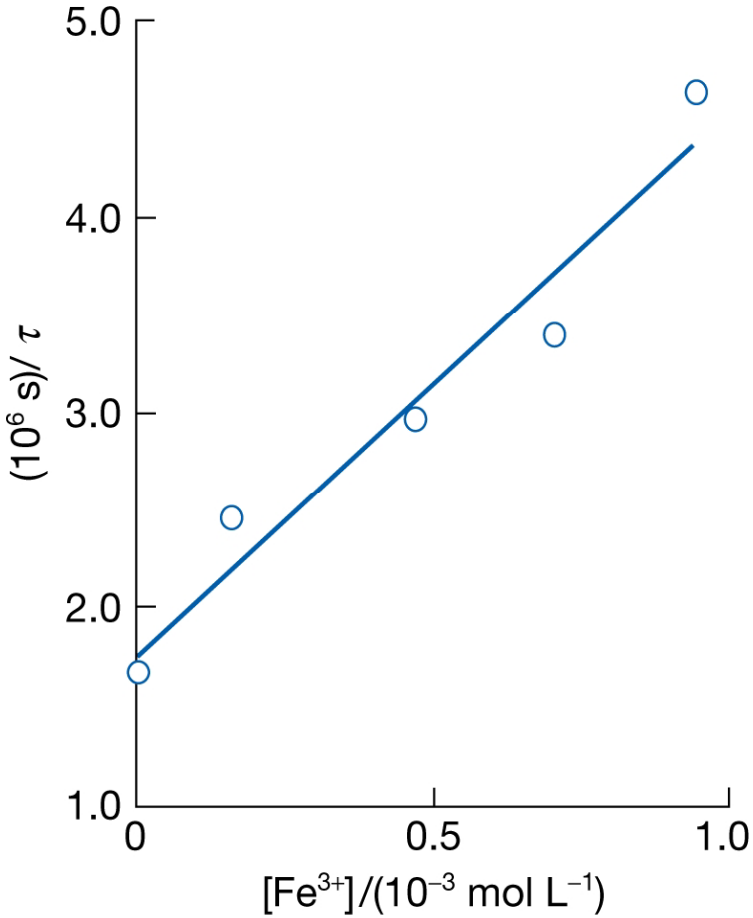
- Quenching – shortening of the lifetime of the excited state
- Can be described by opening an additional channel for deactivation of S*:

Quenching: $S^* + Q \longrightarrow S + Q \qquad v_Q = k_Q[S^*][Q]$

$$\frac{d[S^*]}{dt} = I_{abs} - k_f[S^*] - k_{IC}[S^*] - k_{ICS}[S^*] - k_Q[S^*][Q] = 0$$

$$\phi = \frac{k_f}{k_f + k_{IC} + k_{ICS} + k_Q[Q]}$$

Stern-Volmer plot: $\dfrac{\phi_f}{\phi} = 1 + \tau_0 k_Q[Q]$



Photochemistry

- Quenching mechanisms

Collision deactivation $S^* + Q \rightarrow S + Q$

Resonance energy transfer $S^* + Q \rightarrow S + Q^*$

Electron transfer $S^* + Q \rightarrow S^- + Q^+ \ or \ S^+ + Q^-$

Photochemistry

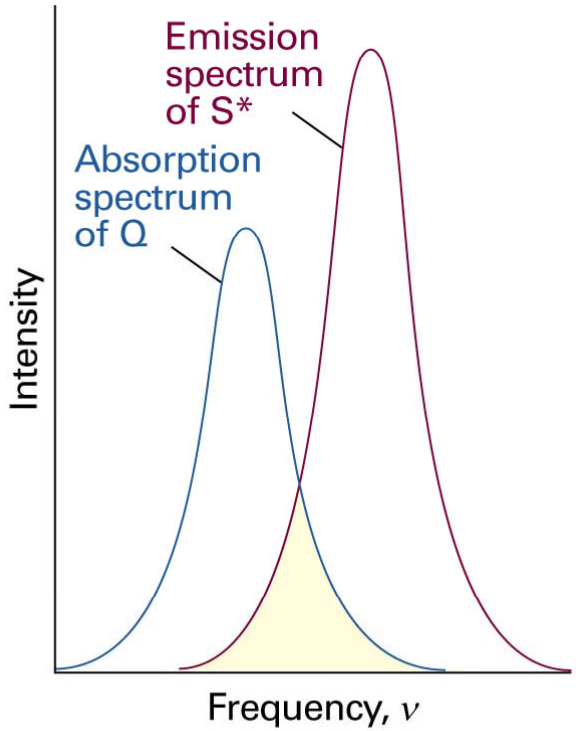
Resonance energy transfer $S^* + Q \rightarrow S + Q^*$

Transfer efficiency $E_T = 1 - \dfrac{\phi_f}{\phi_{f,0}}$

- **Förster theory energy transfer** (proposed by T.Förster in 1959)
  - Energy donor and acceptor are separated by a short distance
  - Photons emitted by an excited state of the donor can be absorbed by the acceptor

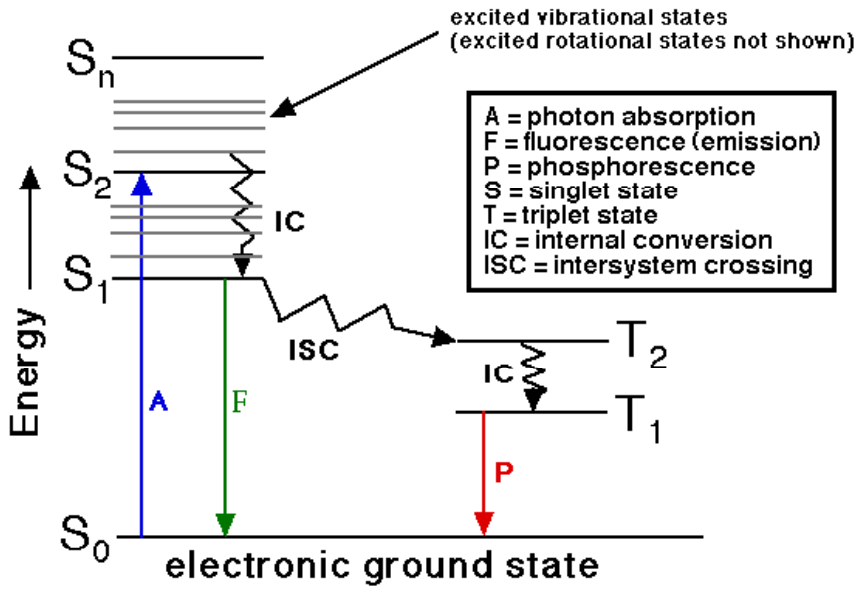
Transfer efficiency $E_T = \dfrac{R_0^{\,6}}{R_0^{\,6} + R^6}$

Donor†	Acceptor	R_0/nm
Naphthalene	Dansyl	2.2
Dansyl	ODR	4.3
Pyrene	Coumarin	3.9
IEDANS	FITC	4.9
Tryptophan	IEDANS	2.2
Tryptophan	Haem (heme)	2.9



Fluorescent measurements

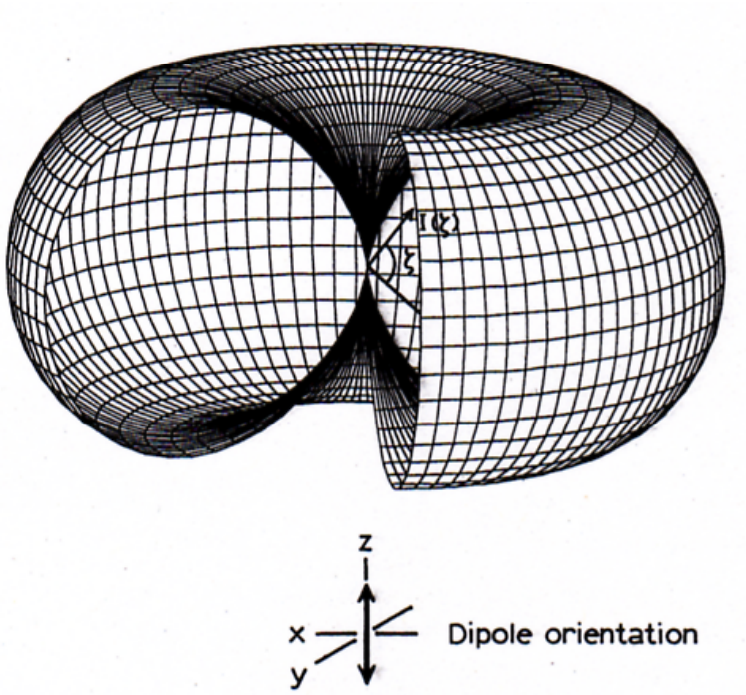
- Common sensing approaches:
  - Intensity measurements:
    - Binding of a fluorescently labelled probe
    - FRET
    - Quenching
  - Depolarization



Fluorescent anisotropy

- Emitting fluorophore behaves like a radiating dipole
- Radiation field of a single fluorophore:

$$E(\theta, r) = k\frac{\sin(\theta)}{r}$$

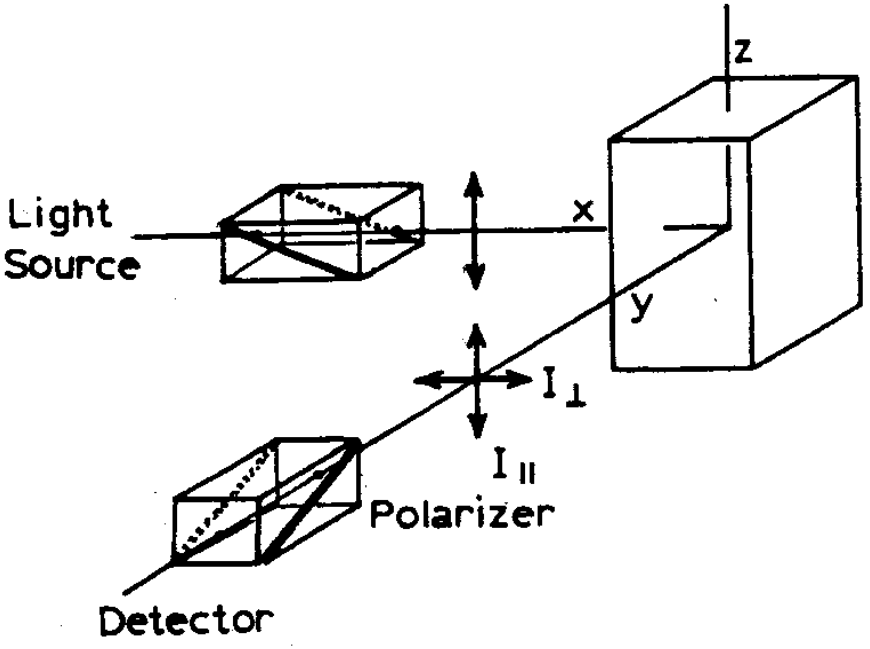


- Excitation with polarized light will preferentially excite molecule oriented in the direction of polarization

Fluorescent anisotropy

- Anisotropy ***r***:

$$r = \frac{I_{\parallel} - I_{\perp}}{I_T} = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}}$$



- Anisotropy is mainly decreased due to rotational diffusion during the lifetime of the excited state (typ. 1-10 ns)

Perrin equation

$$r = \frac{r_0}{1 + \tau/\theta}$$

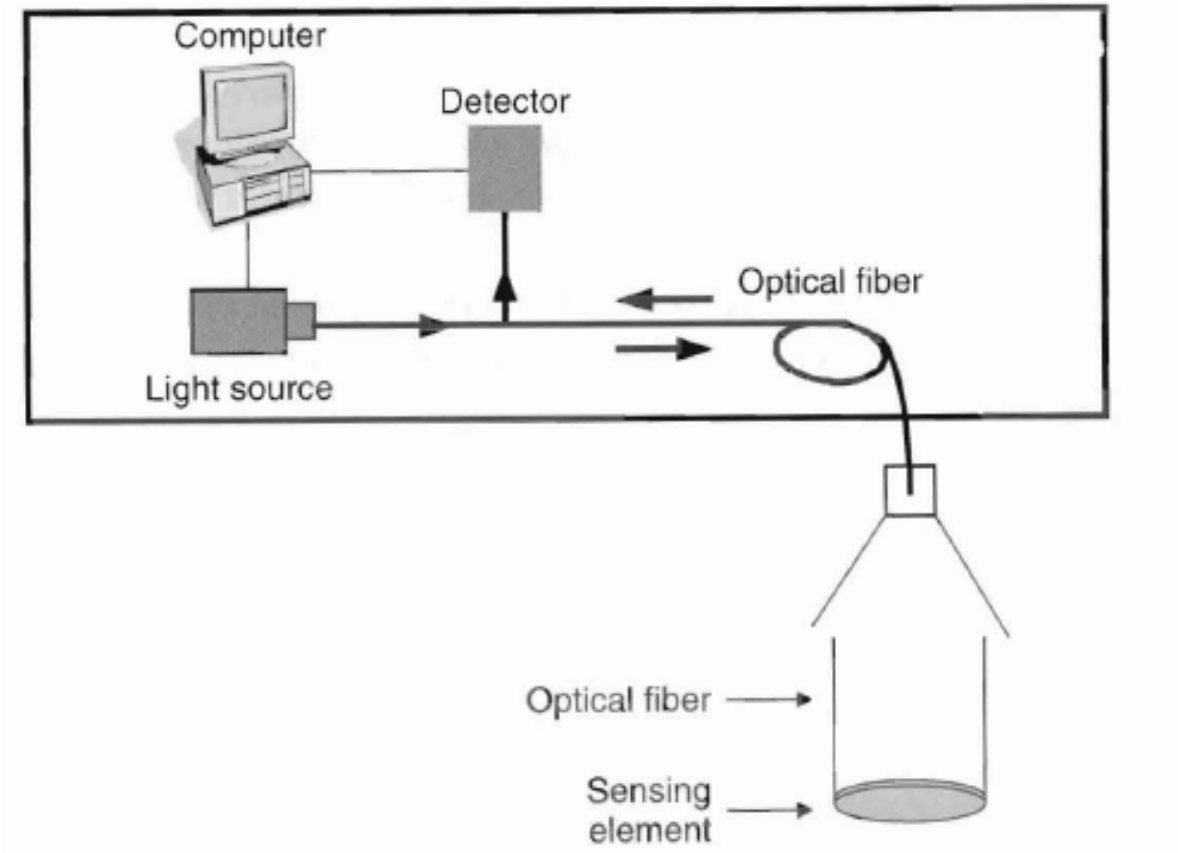
rotational correlation time for diffusion

fluor.lifetime

OPTICAL SENSING: APPLICATIONS

Bio-Optrode sensors

- Bio-Optrode: stands for “optical electrode” biosensor – fiber-optic based analytical device

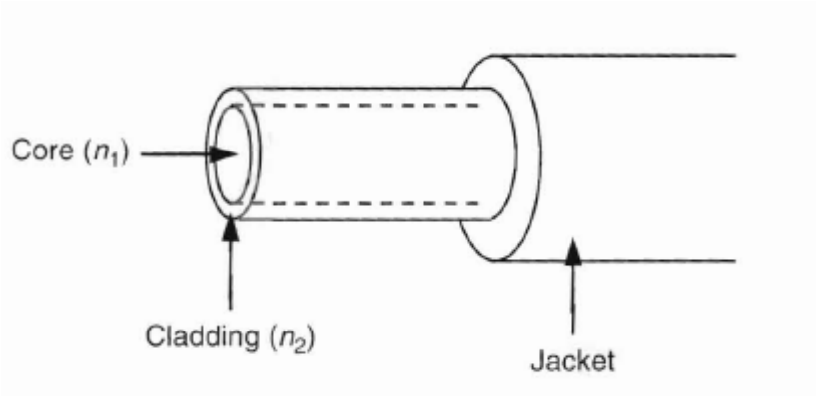


Bio-Optrode sensors

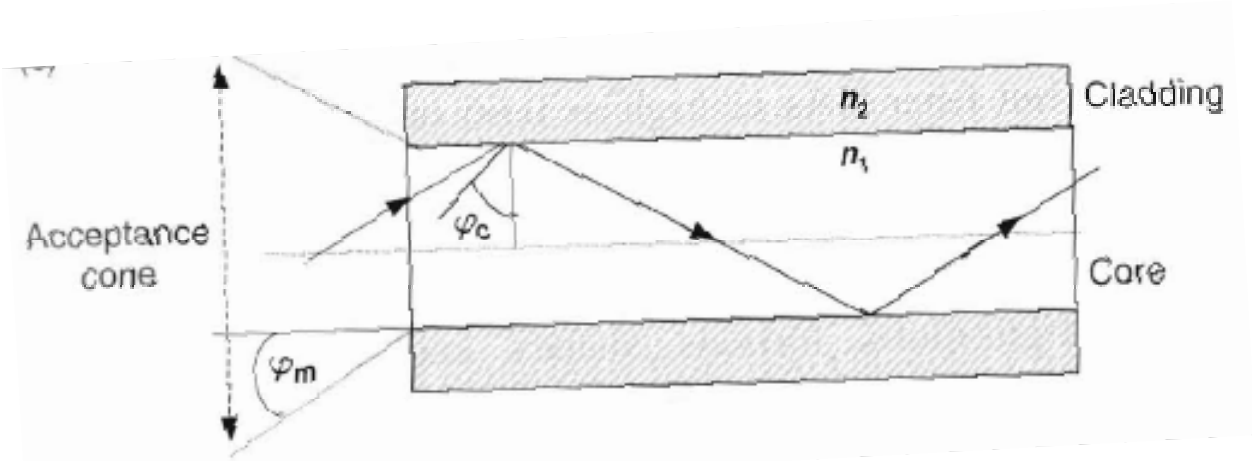
- Fiber-optic principles

$$n_2 > n_1$$

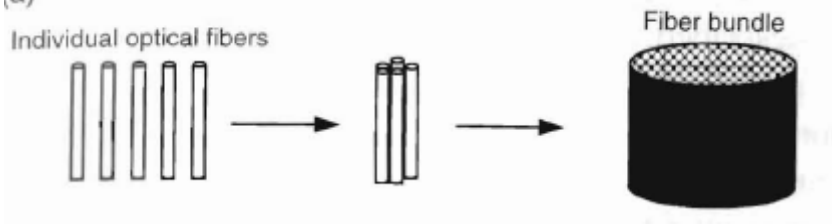
If the light strikes cladding at the angle larger than critical it propagates along the fiber



$$\sin \varphi_c = \frac{n_2}{n_1}$$



- Optical fibers can be combined into bundles, either coherent (ordered, can be used for imaging) or not (randomized)

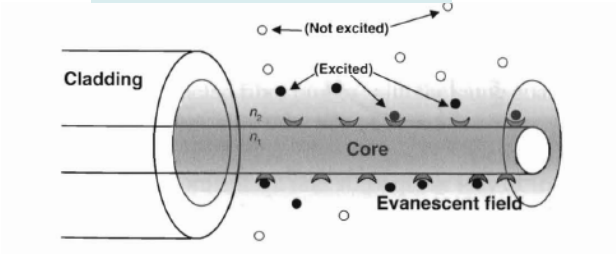


Bio-Optrode sensors

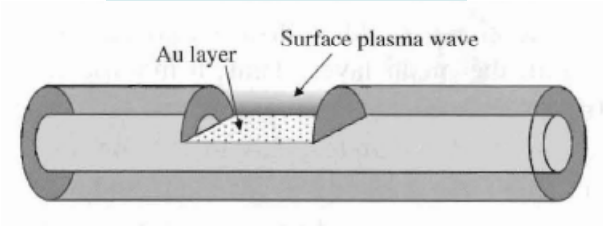
- **Optical phenomena employed**:
  - Fluorescence (intensity of fluorescence, lifetime, quenching, FRET, bio- and chemi-luminescence)
  - Adsorption
  - SPR

Intrinsic mode:
measurand acts directly on the waveguide.

evanescent field excitation



SPR



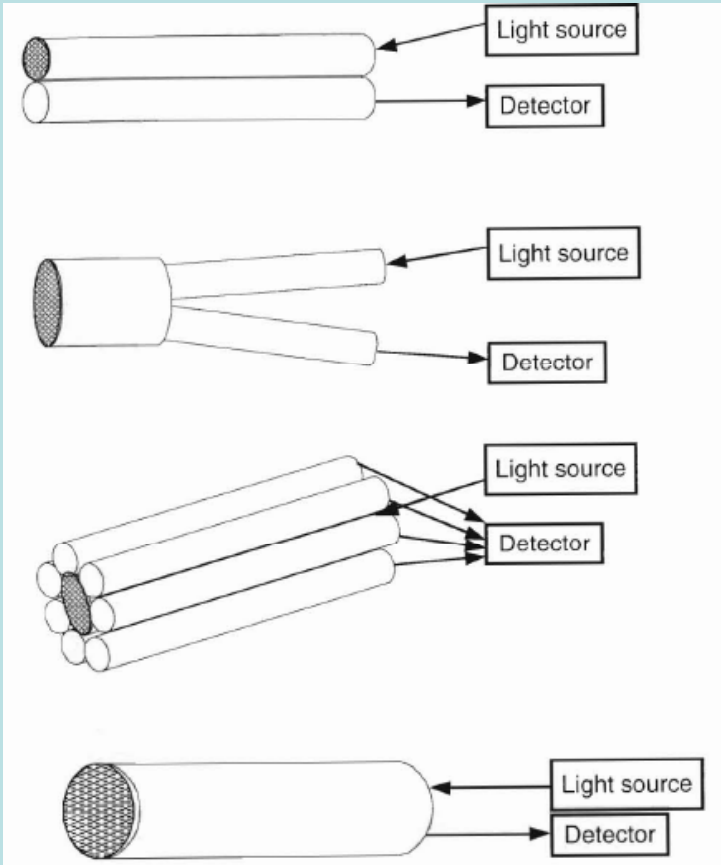
Extrinsic mode:

two fibers

bifurcated fiber

central illumination/ sensing fiber, surrounding detection fibers

single fiber

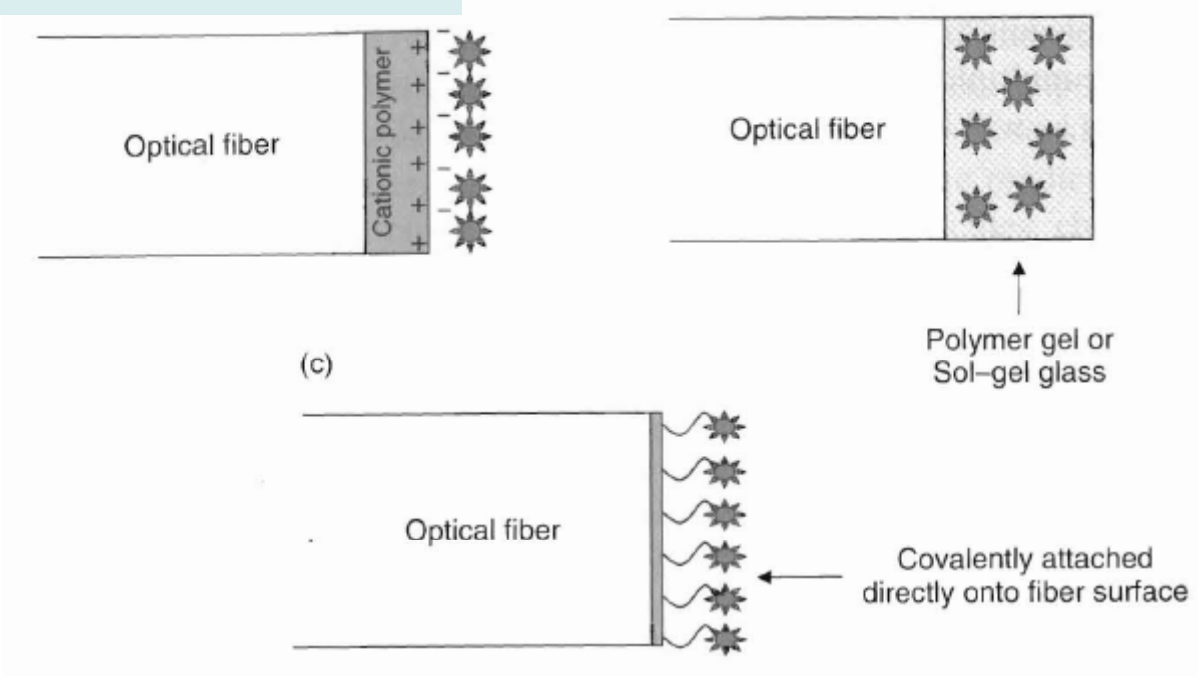


Bio-Optrodes: Immobilization

- **Aim**: immobilize bio-molecules **retaining their activity**, often immobilization is done together with the indicator dye
- Can be immobilized directly on a fiber or on an attached membrane

adsorption immobilization:
using electrostatic or hydrophobic interaction

entrapment in a polymerized layer (usually polyacrylamide gel, but also agarose and alginate gels, PVA, silicate)



Bio-Optrodes: Implementations

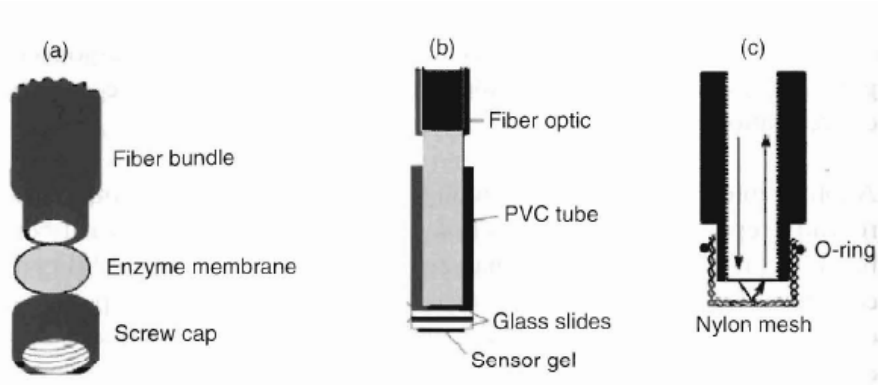
- **Enzymatic sensors**: some additional chemical or enzymatic (co-immobilized enzymes) reaction create optically detected analyte.

$$\text{Glucose} + O_2 \rightarrow \text{Gluconic acid} + H_2O_2$$

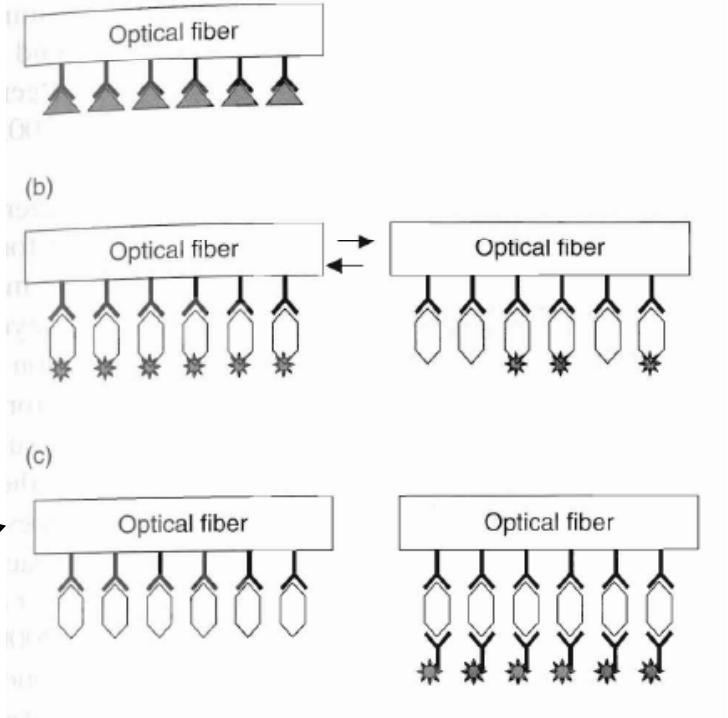
  - monitoring oxygen consumption using ruthenium complex
  - measuring H_2O_2 using luminol chemiluminescence
  - measuring H_2O_2 using europium tetracycline (EuTc)

Bio-Optrodes: Implementations

- **Affinity-based sensors**: uses antibody, receptors or nucleic acids. Often designed with disposable sensing layer cap

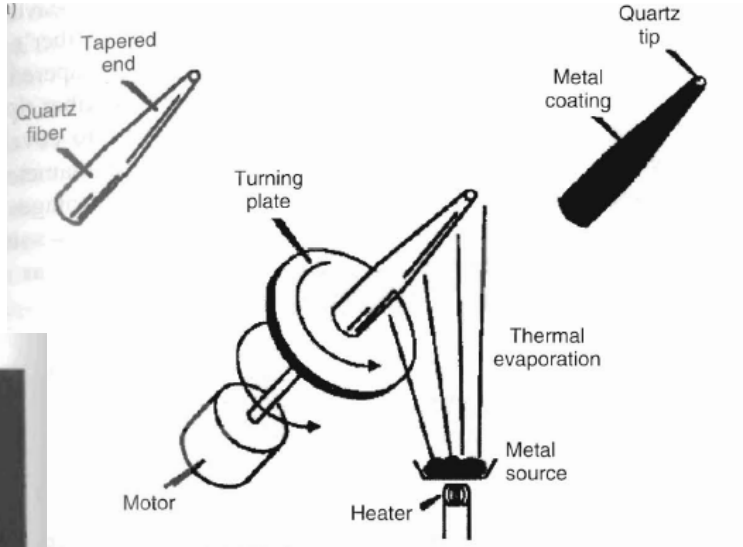


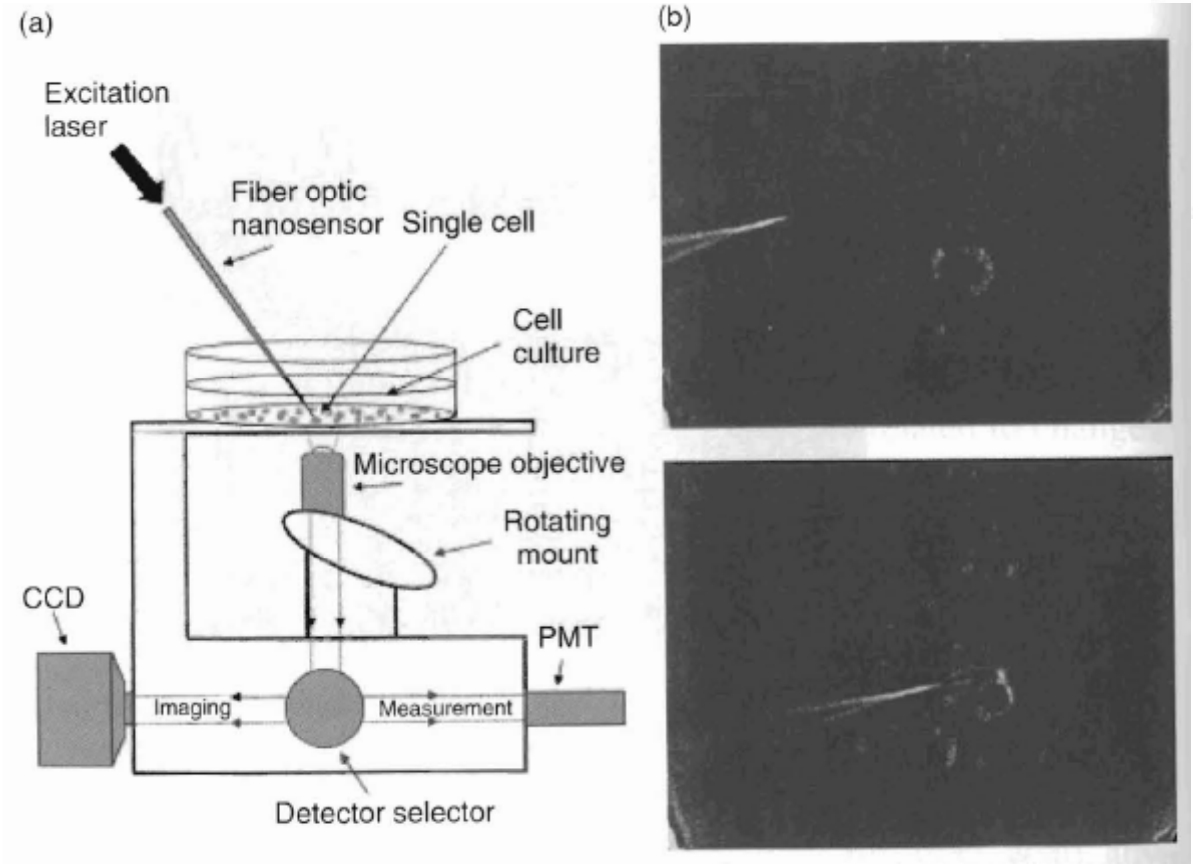
- **Typical assay schematics**
  - detection of self-fluorescent antigen
  - competition assay with fluorescently-labelled antigen
  - sandwich assay with fluorescently-labelled antibody



Bio-Optrodes: Implementations

- **Nano-optrode**: measuring within a single cell with picomole sensitivity

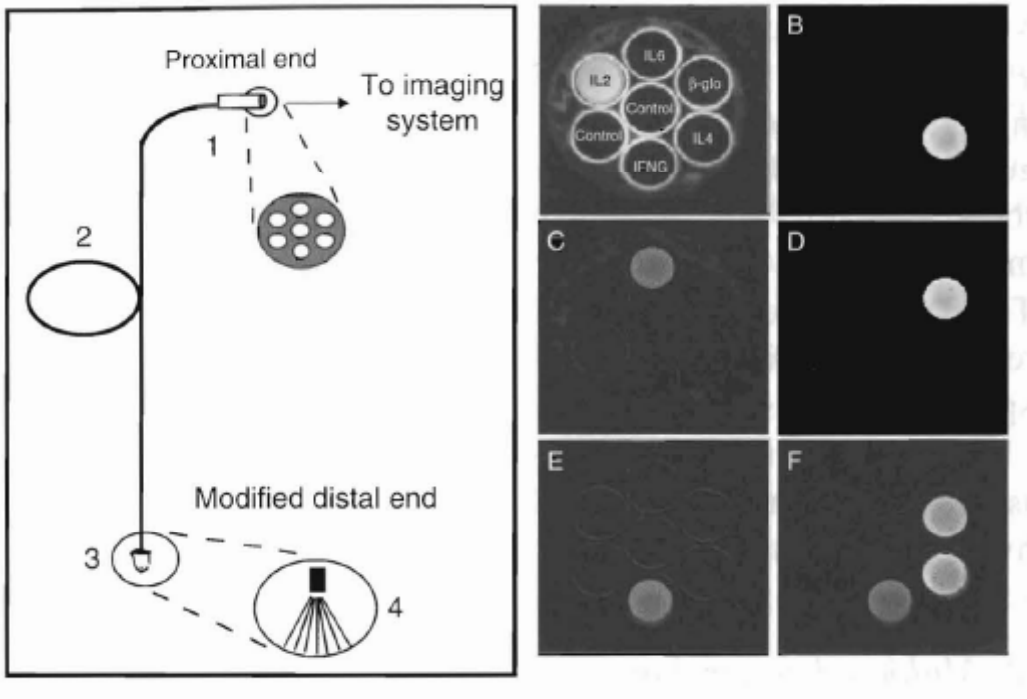




Bio-Optrodes: Implementations

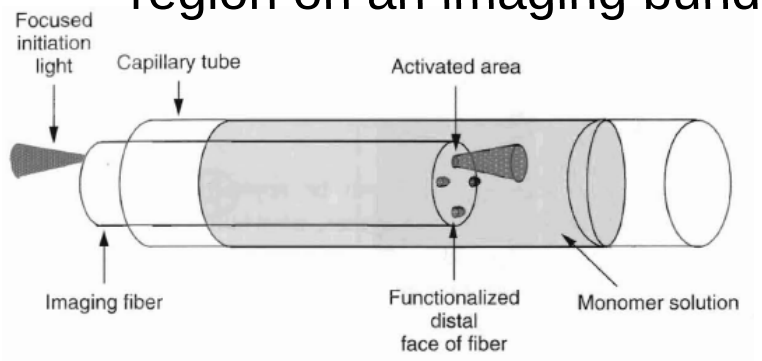
- **Multi-analyte sensors**: detection of several analytes simultaneously is essential in particular for clinical applications.

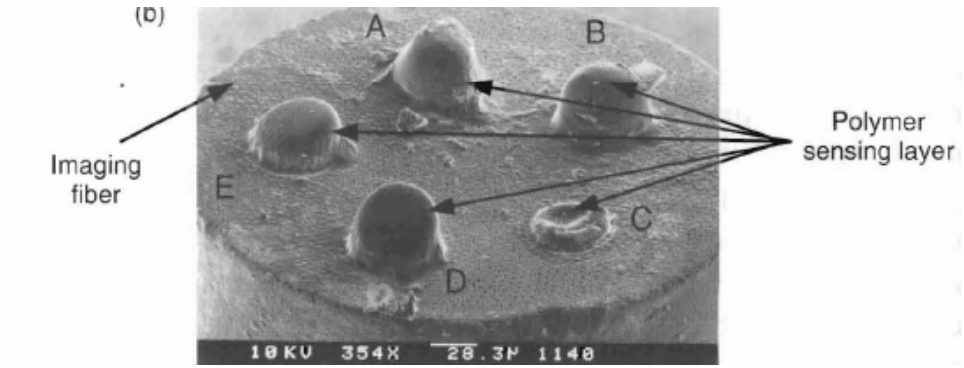
- **common approaches:**
  - bundle of individual functionalized fibers



Ferguson et al, Nature Biotech.14, 1681 (1996)

- forming discrete sensing region on an imaging bundle





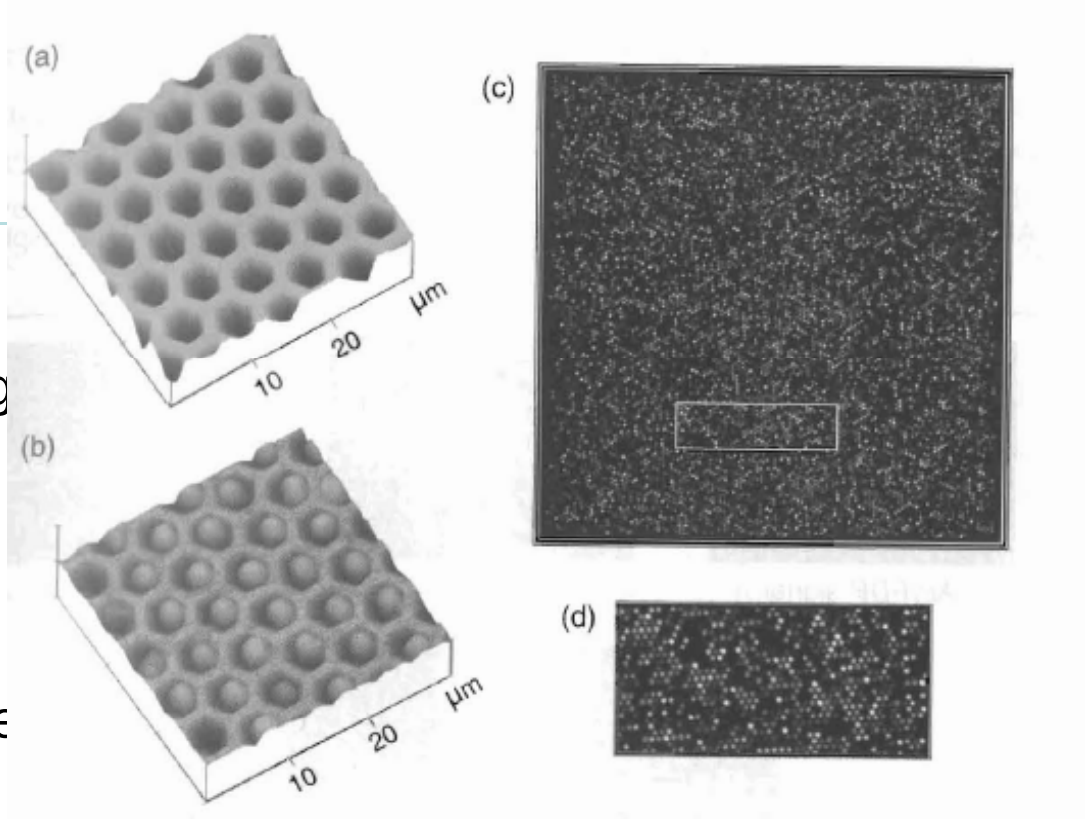
Ferguson et al, Anal.Chem.Acta.340, 123 (1997)

Bio-Optrodes: Implementations

- **Microwell** approach to multianalyte detection**:**

Strategy:

- microwells fabricated by etching the cores of individual fibers in imaging bundle
- sensing microspheres are randomly distributed, each type of spheres carries unique marke (e.g. ratio of various dyes)
- image of a microwell array is taken with a CCD camera



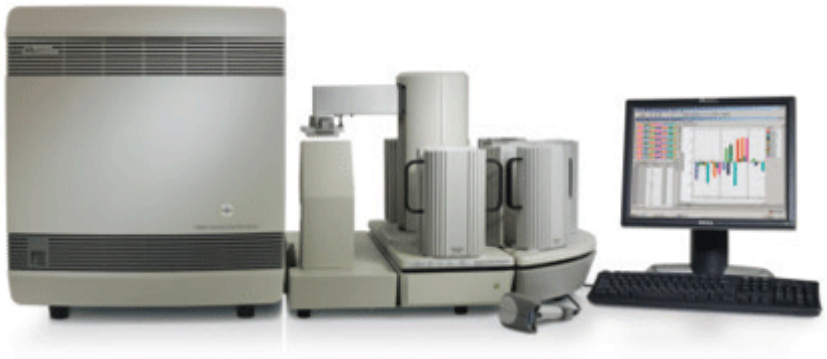
Walt et al, Science 287, 451 (2000)

Advantages:

- presence of multiple replicates reduces false positive and negatives
- higher signal-to-noise ratio due to averaging from multiple locations

Real Time PCR

- Real Time PCR
  - Ability to monitor the process as it occurs
  - **Quantification of the DNA** amount is possible as measurements are taken also in the linear phase (compare, usual PCR – end point assay)
  - **No post processing** necessary (gel electrophoresis etc.)
  - Increased **dynamic range** of detection



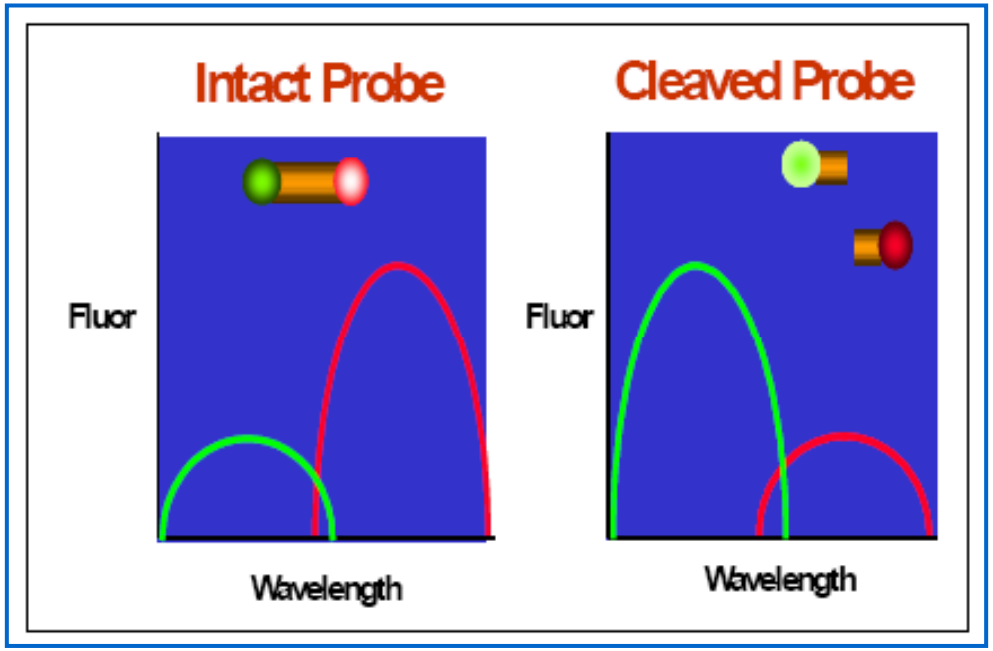
Applied Biosystems 7900HT Real Time PCR, using TaqMan® 384-well array and robotic loading

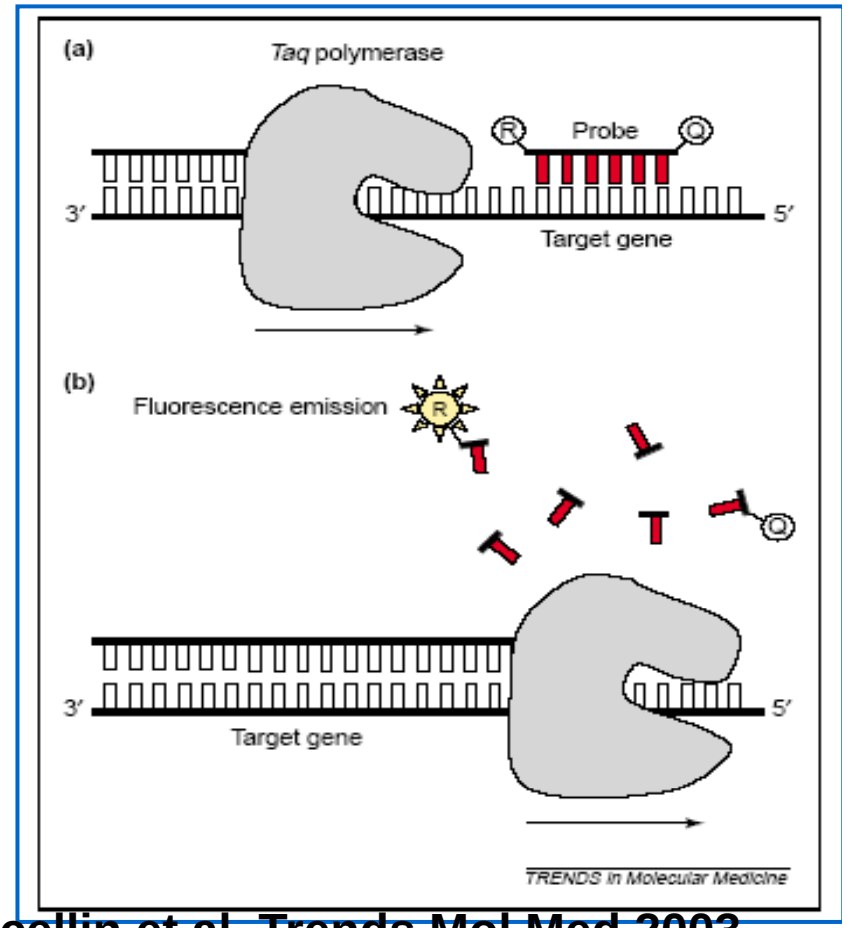
Real Time PCR

- **SYBR Green I technique:** SYBR Green I fluorescence is enormously increased upon binding to double-stranded DNA. During the extension phase, more and more SYBR Green I will bind to the PCR product, resulting in an increased fluorescence. Consequently, during each subsequent PCR cycle more fluorescence signal will be detected.
- **Hydrolysis probe technique:** The hydrolysis probe is conjugated with a quencher fluorochrome, which absorbs the fluorescence of the reporter fluorochrome as long as the probe is intact. However, upon amplification of the target sequence, the hydrolysis probe is displaced and subsequently hydrolyzed by the Taq polymerase. This results in the separation of the reporter and quencher fluorochrome and consequently the fluorescence of the reporter fluorochrome becomes detectable. During each consecutive PCR cycle this fluorescence will further increase because of the progressive and exponential accumulation of free reporter fluorochromes.
- **Hybridization probes technique:** In this technique one probe is labelled with a donor fluorochrome at the 3' end and a second –adjacent- probe is labelled with an acceptor fluorochrome. When the two fluorochromes are in close vicinity (1–5 nucleotides apart), the emitted light of the donor fluorochrome will excite the acceptor fluorochrome (FRET). This results in the emission of fluorescence, which subsequently can be detected during the annealing phase and first part of the extension phase of the PCR reaction. After each subsequent PCR cycle more hybridization probes can anneal, resulting in higher fluorescence signals.

Hydrolysis probe techniques

- **TaqMan probes**
- The techniques is based on the following two phenomena:
  - FRET (Förster Resonant Energy Transfer)
  - Polymerases 5’ exonuclease activity

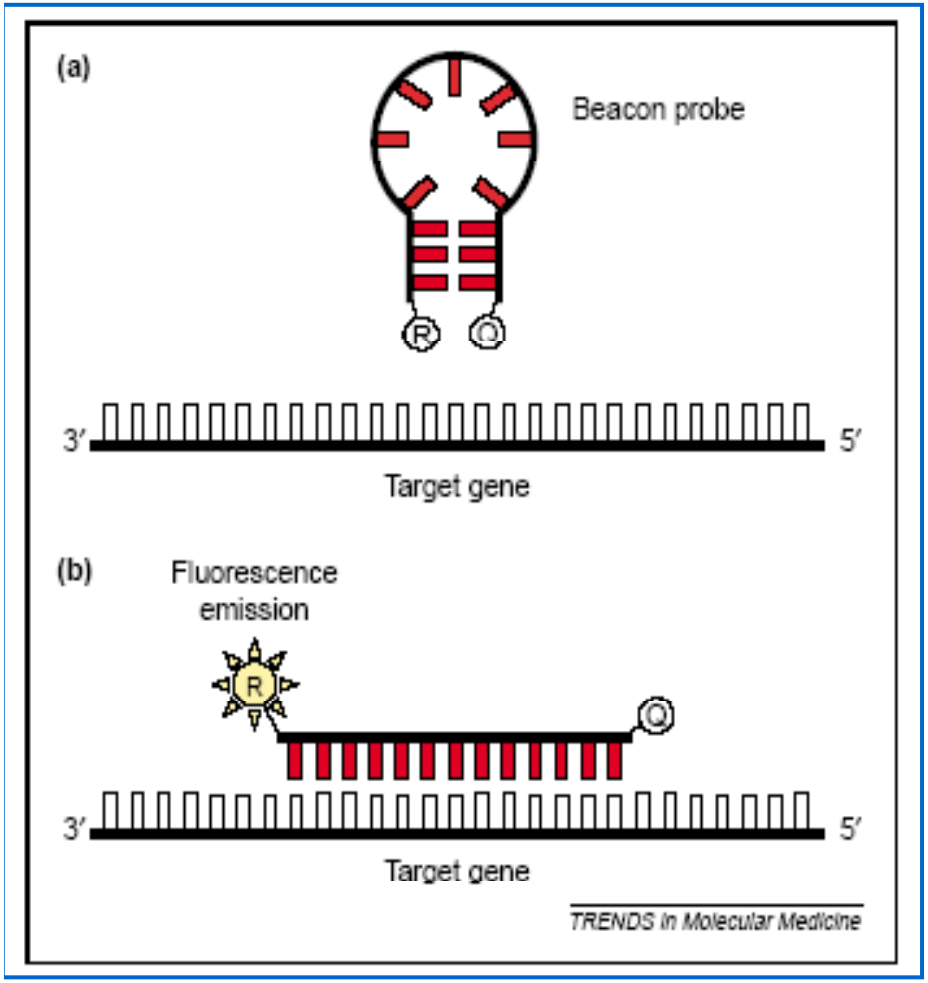




Mocellin et al. Trends Mol Med 2003

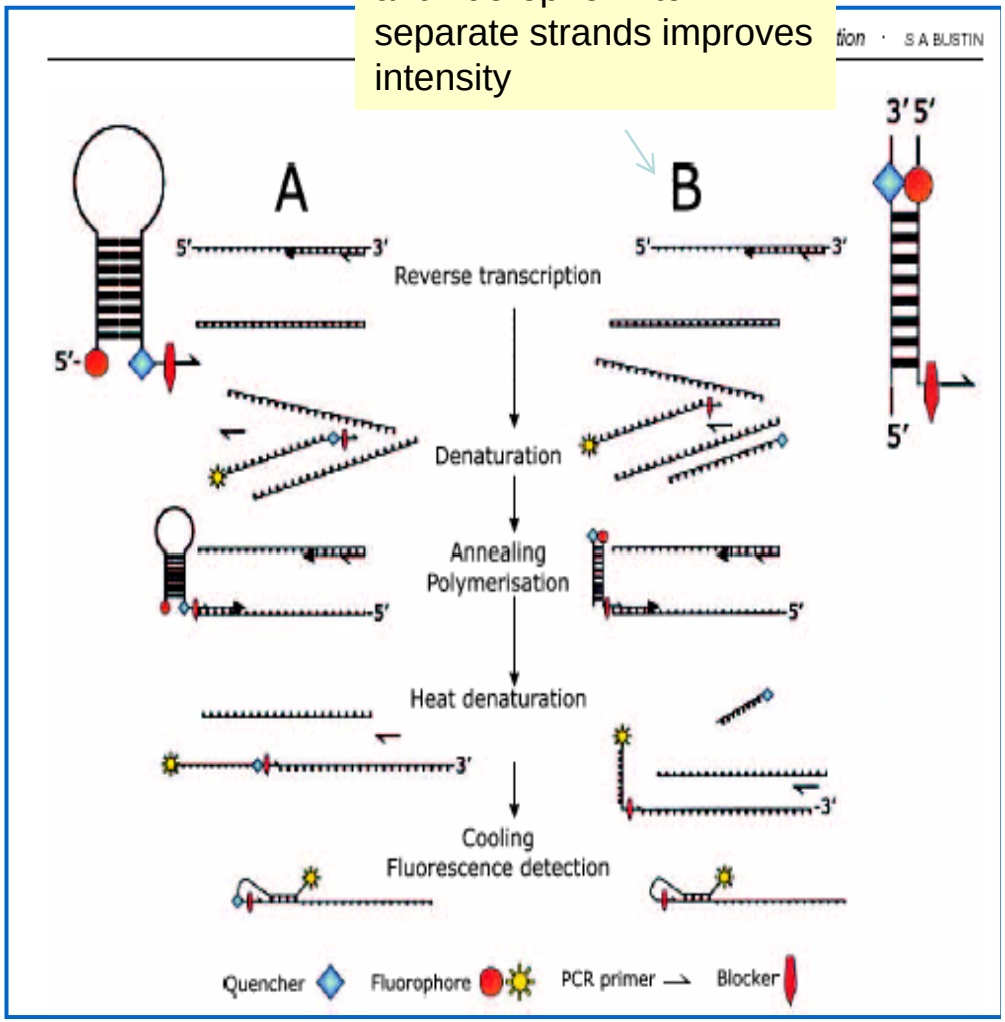
Hybridization techniques

- Molecular Beacons and Scorpions



Mocellin et al. Trends Mol Med 2003

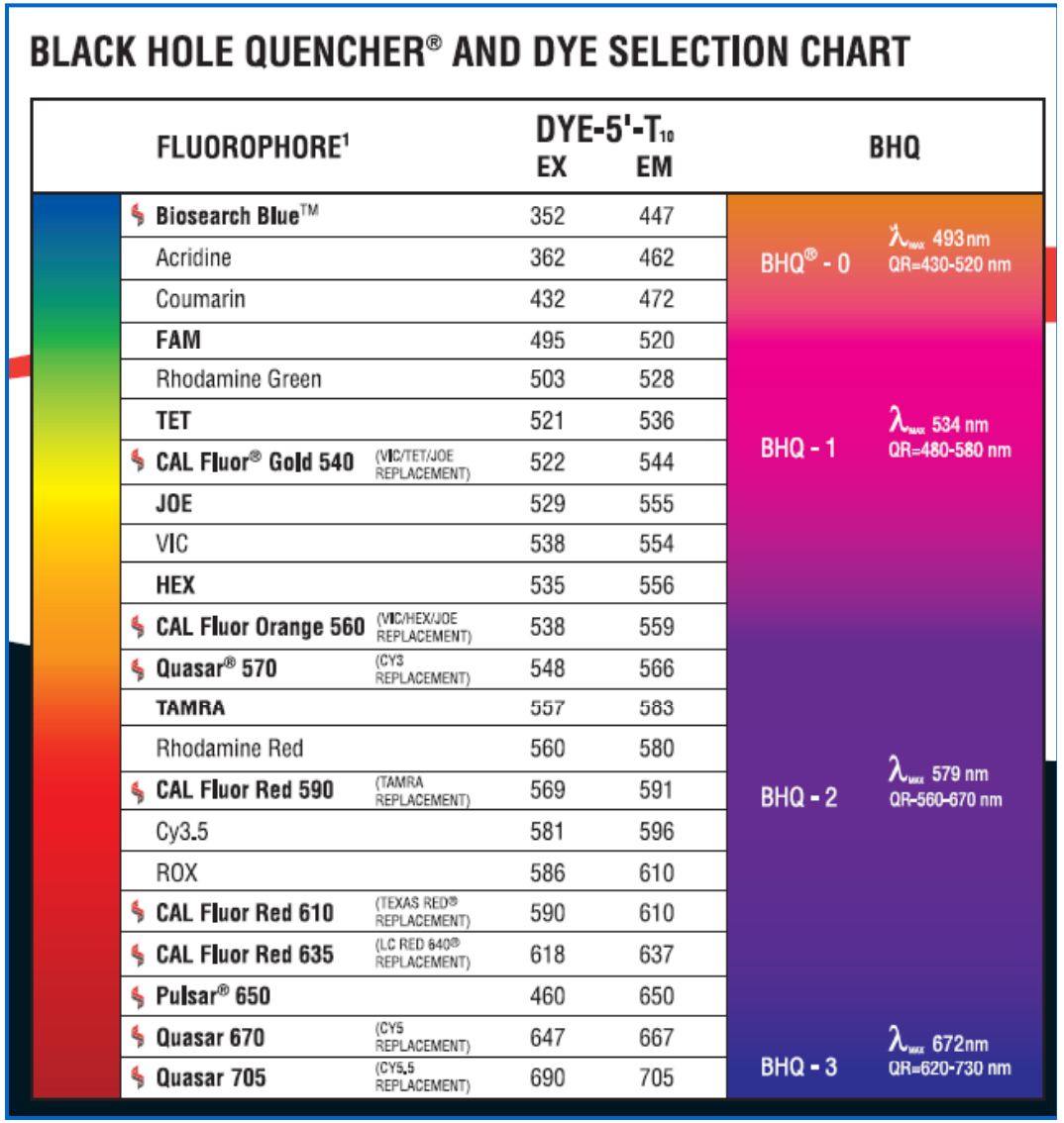
Separation of quencher and fluorophor into separate strands improves intensity



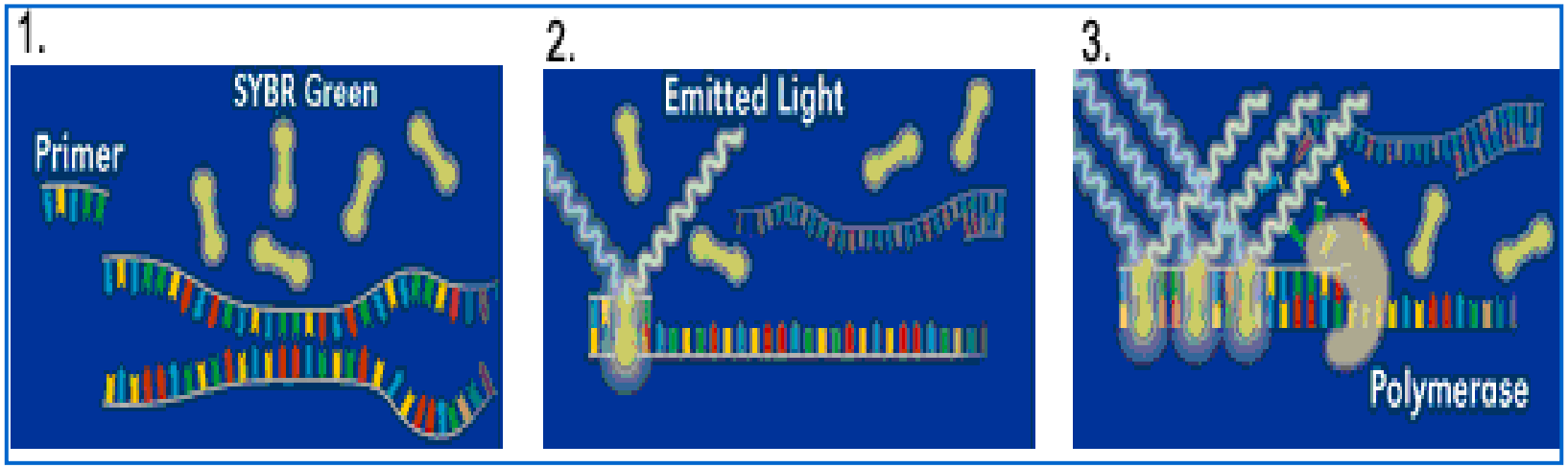
Bustin SA. J Mol Endocrinol 2002

Black Hope Quenchers™

- Advantages of Black Hole Quenchers ™:
  - No native fluorescence
  - Covers VIS and NIR
  - Maximizes spectral overlap



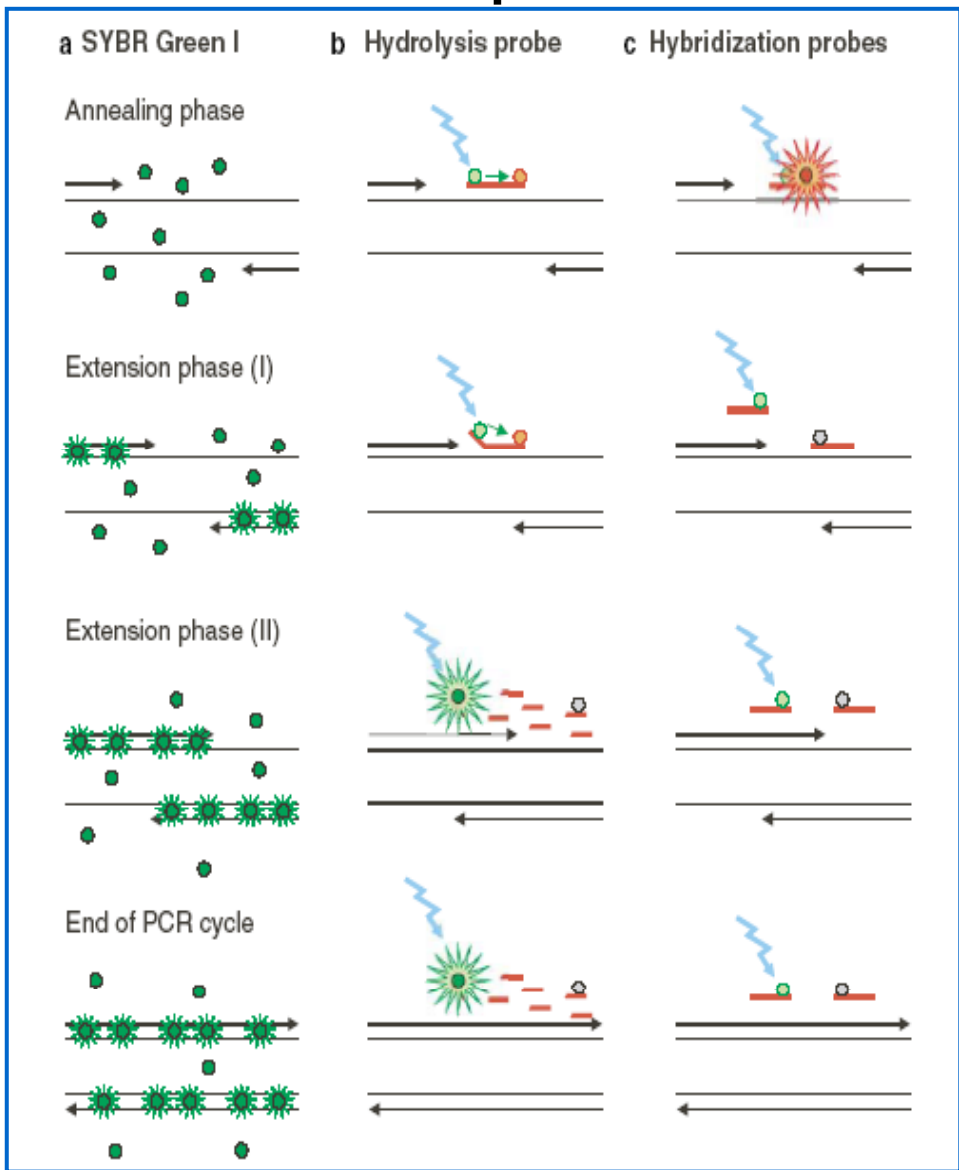
SYBR Green



- **SYBR Green greatly increases fluorescence intensity upon intercalation into dsDNA (Still, background fluorescence should be checked before amplification and substracted)**
- **During elongation the signal is proportional to the length of dsDNA present**
- **Upon denaturation of the DNA for the next heating cycle, the dye molecules are released and the fluorescence signal falls.**

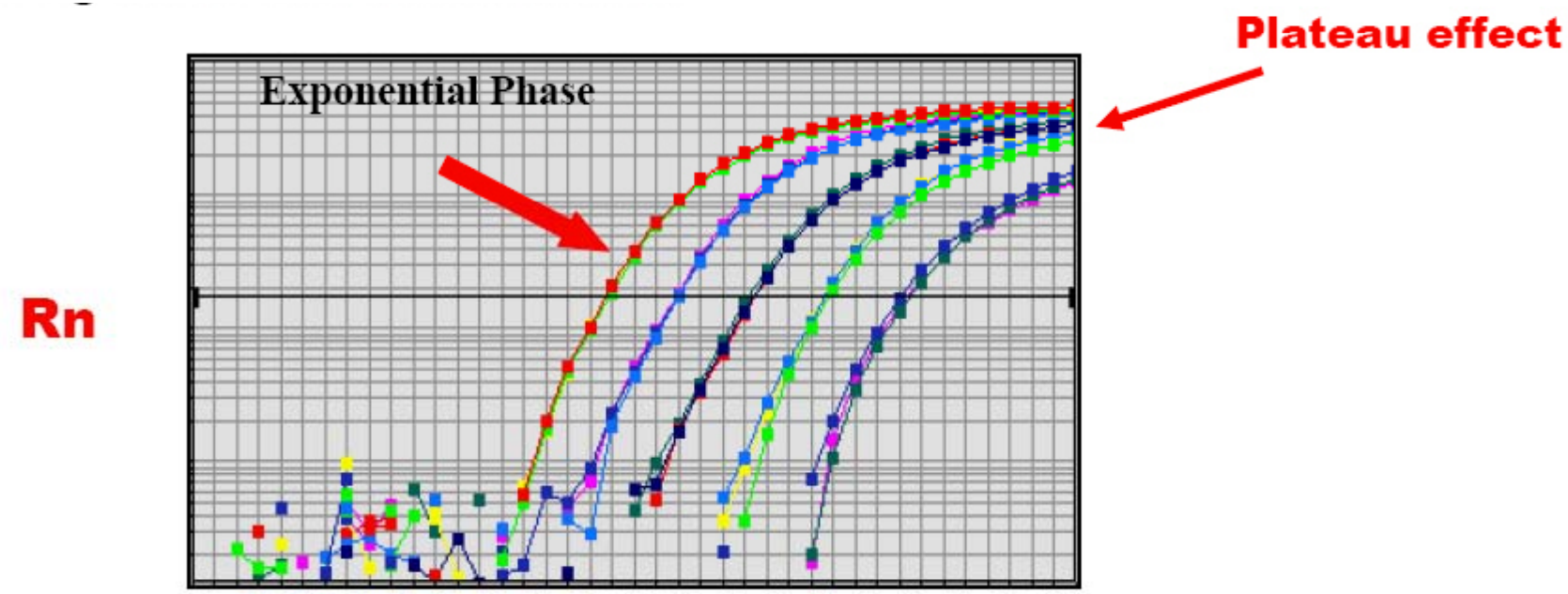
Real-time PCR techniques

- Comparison of the RT-PCR techniques ”phase-by-phase”



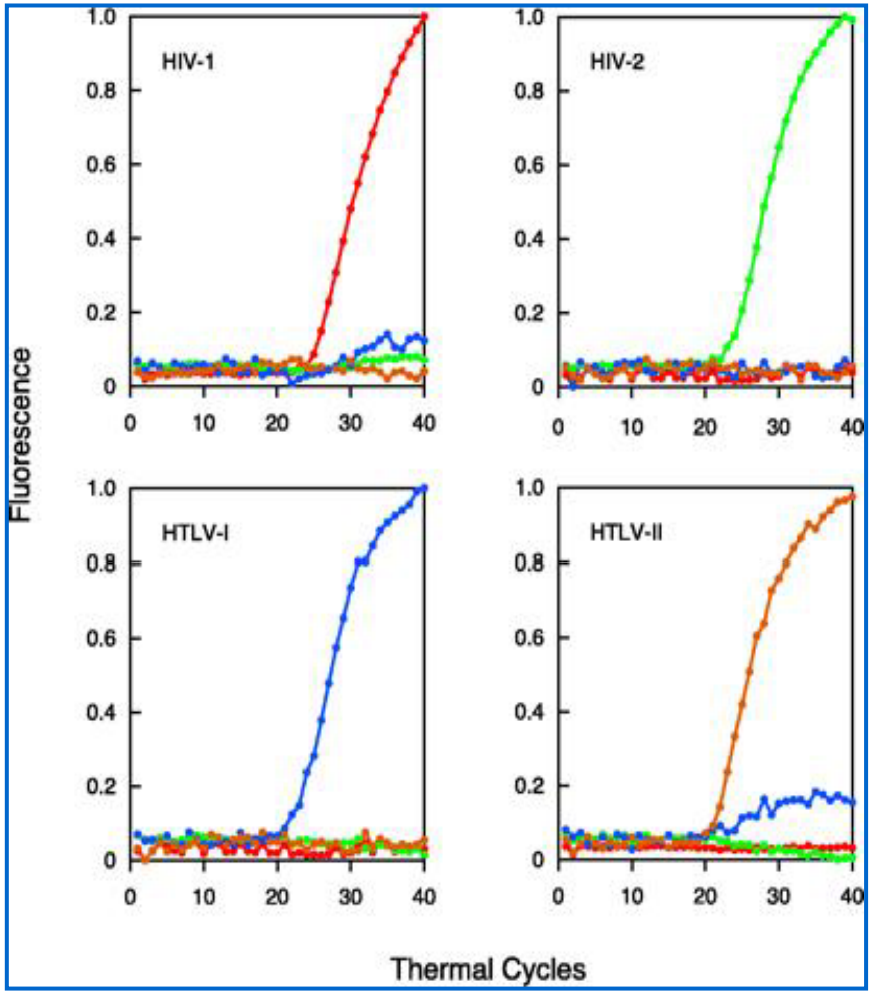
Real-time PCR

- RT-PCR measurement on 5-fold dilution series



Multiplex RT-PCR

- **Real-time detection of four different retroviral DNAs in a multiplex format**



Vet JA et al. PNAS 1999

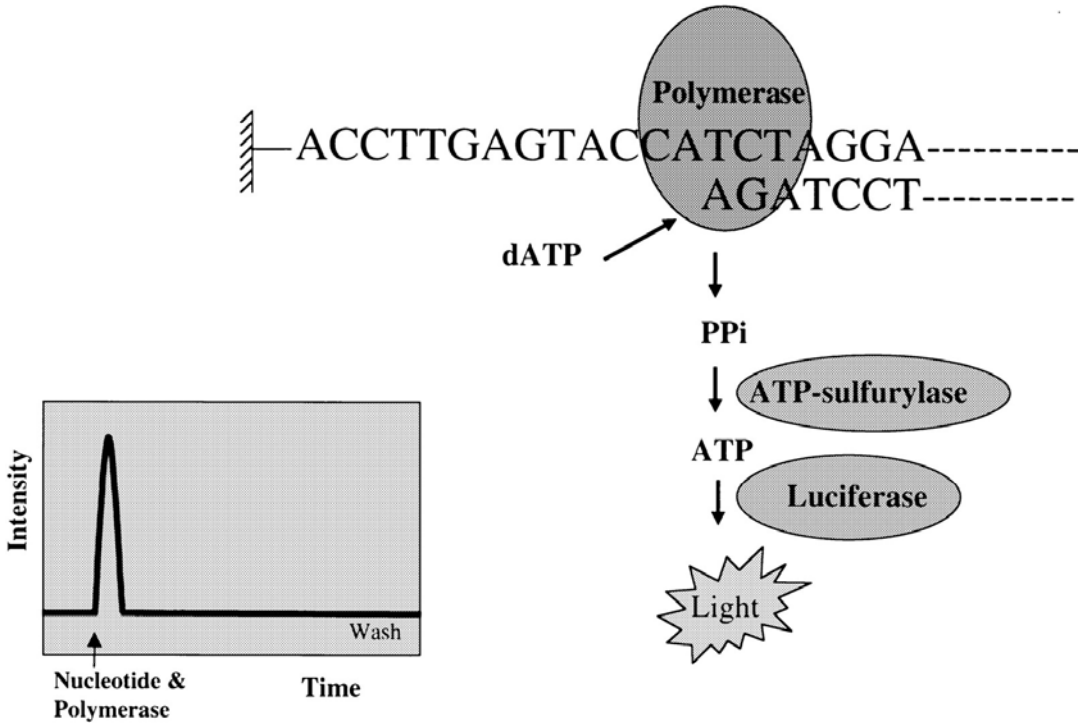
- **Molecular beacons with different fluorophores**
  - **HIV-1: fluorescein (red line)**
  - **HIV-2: tetrachlorofluorescein (green line)**
  - **HTLV-1: tetramethylrhodamine (blue line)**
  - **HTLV-2: rhodamine (red line). “False positive” in the last case due to choice of detection wavelength**

Pyrosequencing

- **PYROSEQUENCING is a unique method for DNA sequencing based on the "sequencing by synthesis" principle (Mostafa Ronaghi and Pål Nyrén, Analytical Biochemistry 1996 and Science 1998)).**
- **Commercialized by Pyrosequencing AB, licensed to 454 Life Science and Quiagen.**
- **Array format platform from 454 Life Sciences can currently sequence 100mln basepairs in 7h run**

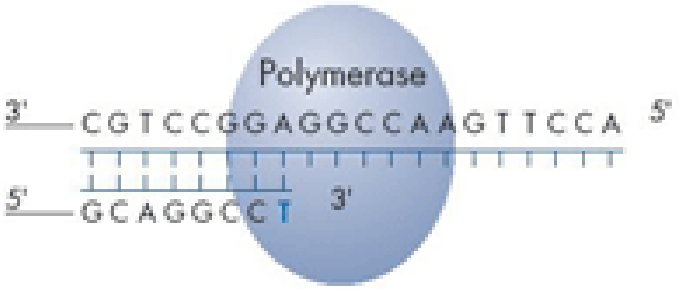
Pyrosequencing

- **The idea:**

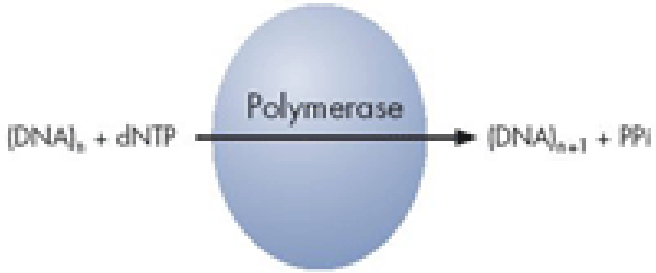


Pyrosequencing

- **Step 1**
  A sequencing primer is hybridized to a single-stranded PCR amplicon that serves as a template, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase, and apyrase as well as the substrates, adenosine 5' phosphosulfate (APS), and luciferin



- **Step 2**
  The first deoxribonucleotide triphosphate (dNTP) is added to the reaction. DNA polymerase catalyzes the incorporation of the deoxyribo-nucleotide triphosphate into the DNA strand, if it is complementary to the base in the template strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide.



http://www.pyrosequencing.com

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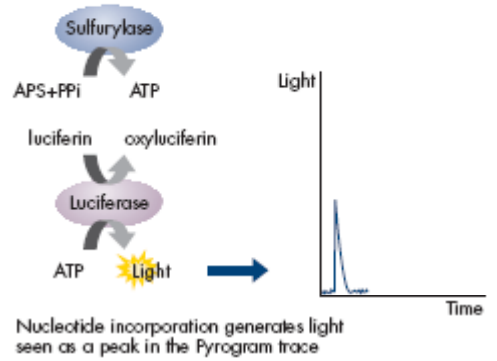
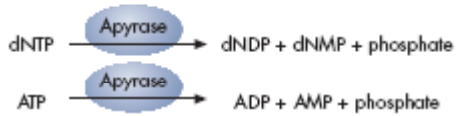
- **Step 3**
  ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate (APS). This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) chip and seen as a peak in the raw data output (Pyrogram). The height of each peak (light signal) is proportional to the number of nucleotides incorporated.



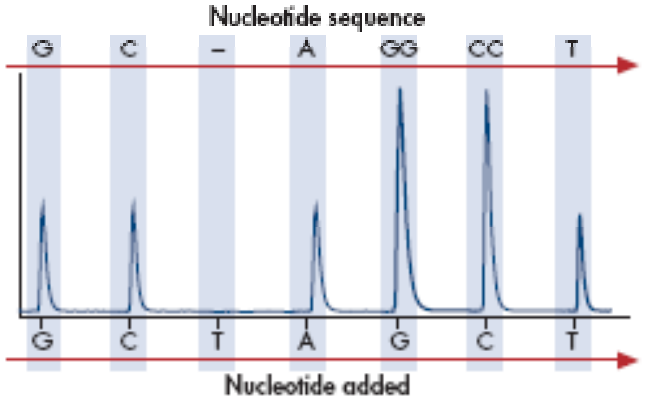
Nucleotide incorporation generates light seen as a peak in the Pyrogram trace

- **Step 4**
  Apyrase, a nucleotide-degrading enzyme, continuously degrades unincorporated nucleotides and ATP. When degradation is complete, another nucleotide is added.



Step 5

Addition of dNTPs is performed sequentially. It should be noted that deoxyadenosine alfa-thio triphosphate (dATP·S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it is efficiently used by the DNA polymerase, but not recognized by the luciferase. As the process continues, the complementary DNA strand is built up and the nucleotide sequence is determined from the signal peaks in the Pyrogram trace.



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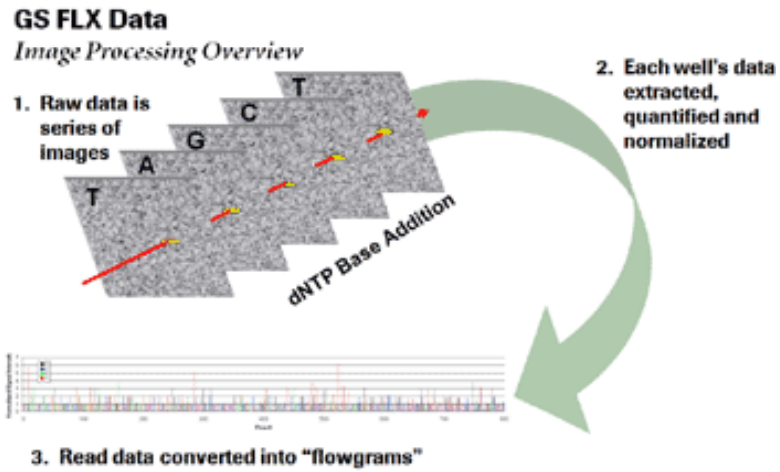
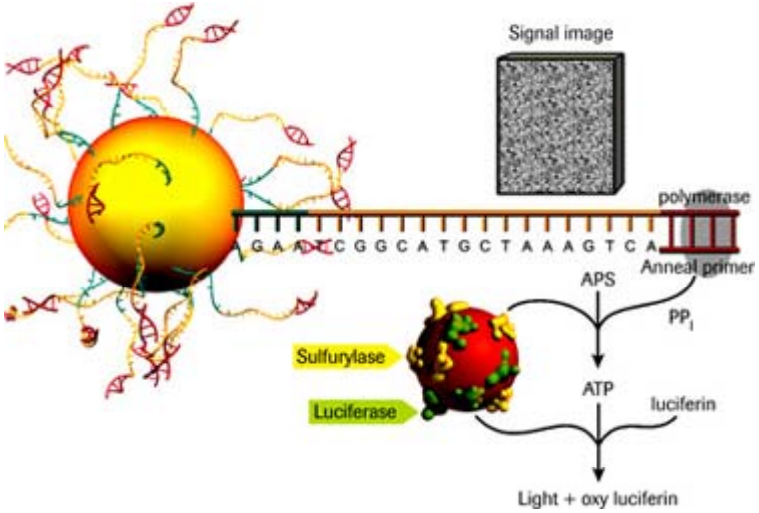
Pyrosequencing: 454 Genome Sequences

- Pyrosequencing in array format: High-Throughput sequencing system.

Throughput: 400-600 million high-quality, filter-passed bases per run*
1 billion bases per day
Run Time: 10 hours
Read Length Modal length = 500 bases, Average length = 400 bases
Accuracy Q20 read length of 400 bases (99% at 400 bases and higher for prior bases)
Reads per run: >1 million high-quality reads
Robustness: No complex optics or lasers; reagents have long shelf life





- Flash presentation: http://www.roche-applied-science.com/publications/multimedia/genome_sequencer/flx_presentation/wbt.htm