Scattering & Fluorescence

Rayleigh Scattering- Elastic Scattering

"photon bouncing off a molecule/atom"

Scattered light has same λ as incident light.

Raman Scattering- Inelastic scattering

Incident light alters vibrational or rotational energy

Happens to ~1 in 10^7 photons

Scattered light has longer λ (lower energy)

than the incident light.

Fluorescence- Absorbed light electronically excites a molecule

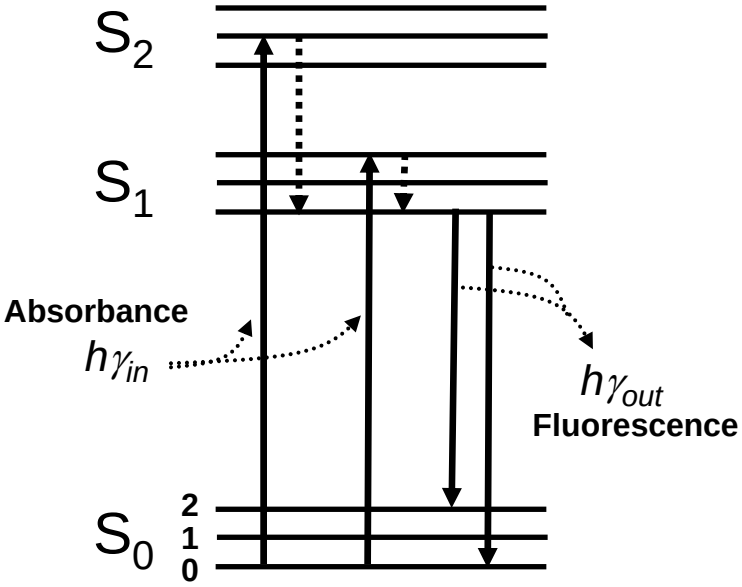
Decay after a resonance lifetime

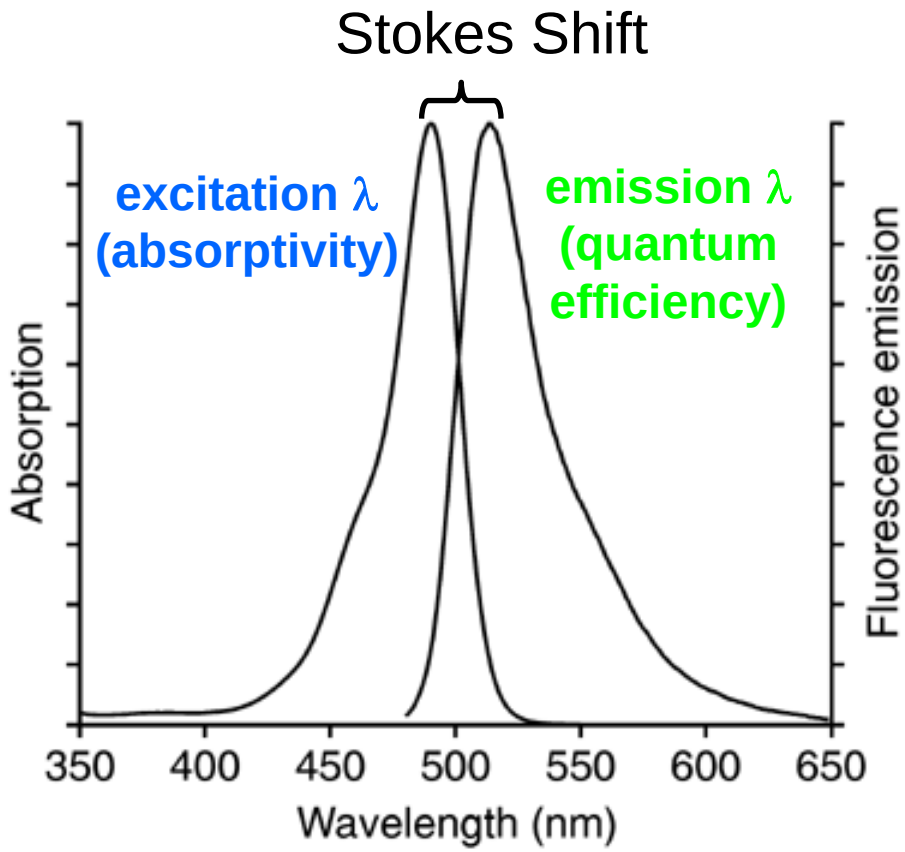
Emitted light typically has a longer λ than incident light

Can have a very high probability of occurring

Fluorescence

Jablonski Diagram





S_0, S_1, S_2 = Singlet ground, first, & second electronic states

0,1,2 = Vibrational energy levels

Lecture 3
Fluorecence Detection and Dyes for DNA Sequencing

DNA sequencing on slab gel in 1993

Sensitivity- 10^{-17}-10^{-18} moles of fluorophore/band

(~500 bands per lane- A lot!!!!!)

Can't sequence genome if need this much DNA!!!!

Analytical Chemistry to the rescue again!!!!

Definitions for CE

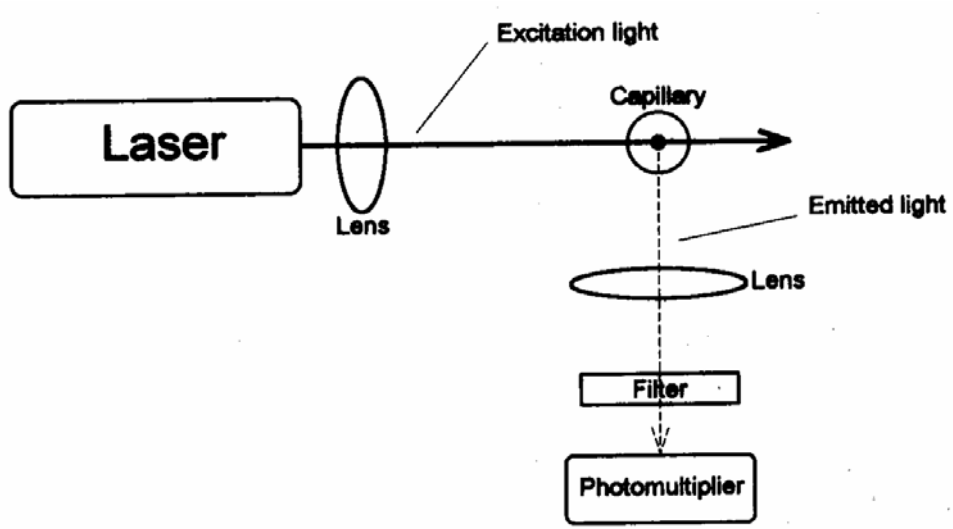
- atto: 10^{-18}
- zepto: 10^{-21}
- yocto: 10^{-24}

Limits of Detection for CE

1. Concentration (CLOD)- molar
2. Mass (MLOD)- moles

Fluorescence Detection By CE

Components:
Excitation Source
Detection Cell/Window
Light Collection Optics
Detector



Excitation Source- Laser
(for fluorescence measurements by CE)

Coherent, Low Divergence,
Monochromatic, High photon flux
High beam quality- focused to small spot size.

Excitation Source

Argon Ion Laser- Major lines at 488 & 514 nm

1. Very popular
2. Plethora of fluorophores exciting at 488 nm
   (fluorescein, its relatives, & others)
3. Small & relatively rugged
4. Low noise versions
5. Long lifetimes & relatively inexpensive
6. Used to sequence the human genome

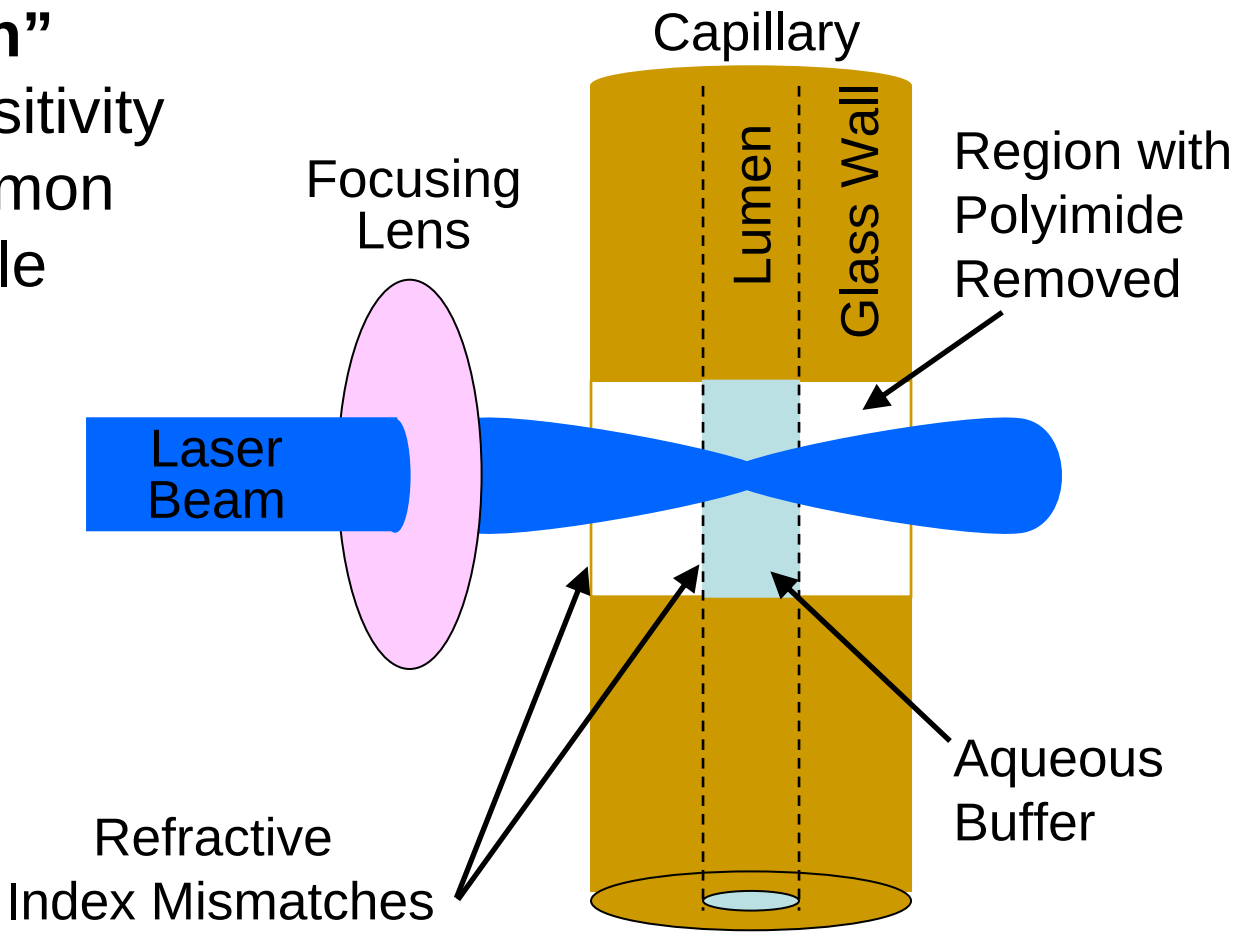
"Going the way of the typewriter!!!"

Most popular now-
Solid-State, Blue Lasers- available at 473 or 488 nm.

Detection Cell/Window

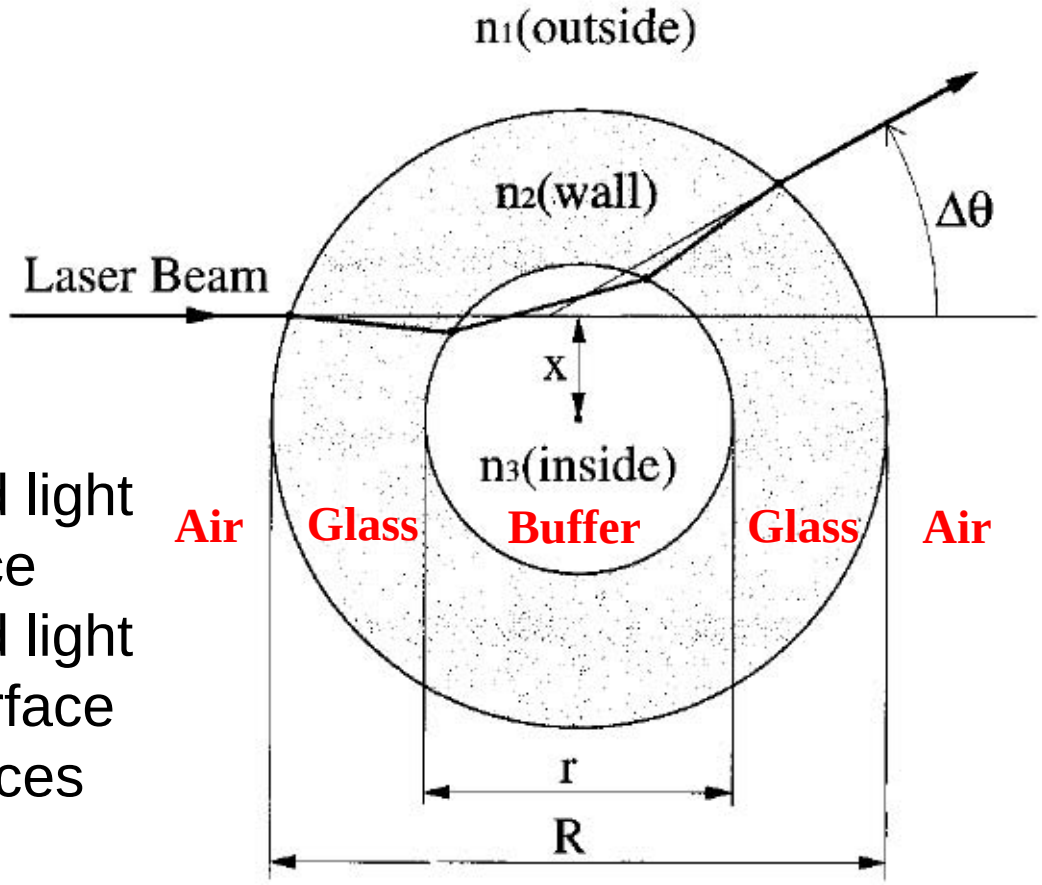
“On-Column”

1. Good sensitivity
2. Most common
3. Very simple
4. Low cost



Detection Cell/Window

“On-Column”



Issues:

1. Reflected/scattered light at air:glass interface
2. Reflected/scattered light at glass:water interface
3. Curved glass surfaces
4. Glass impurities

Anazawa et al, Anal. Chem. 1996, 68:2699.

Detection Cell/Window

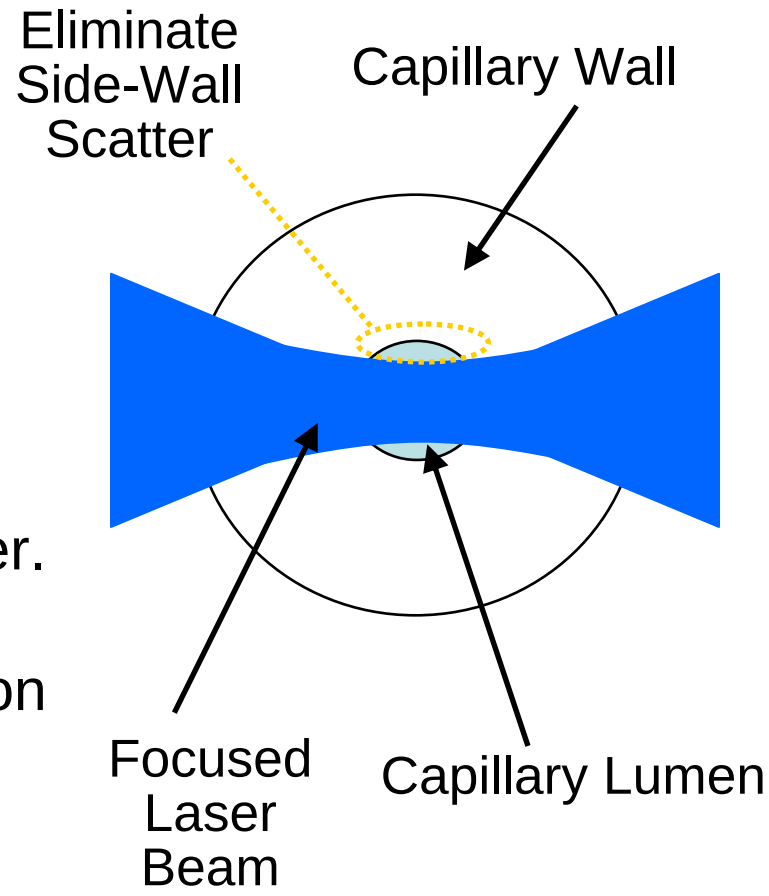
“On-Column”

Partial solutions:

1. Best sensitivity with visible-λ fluorophores.

2. Focus laser beam to a size smaller than the lumen diameter.

3. Use confocal excitation/emission (see subsequent section)

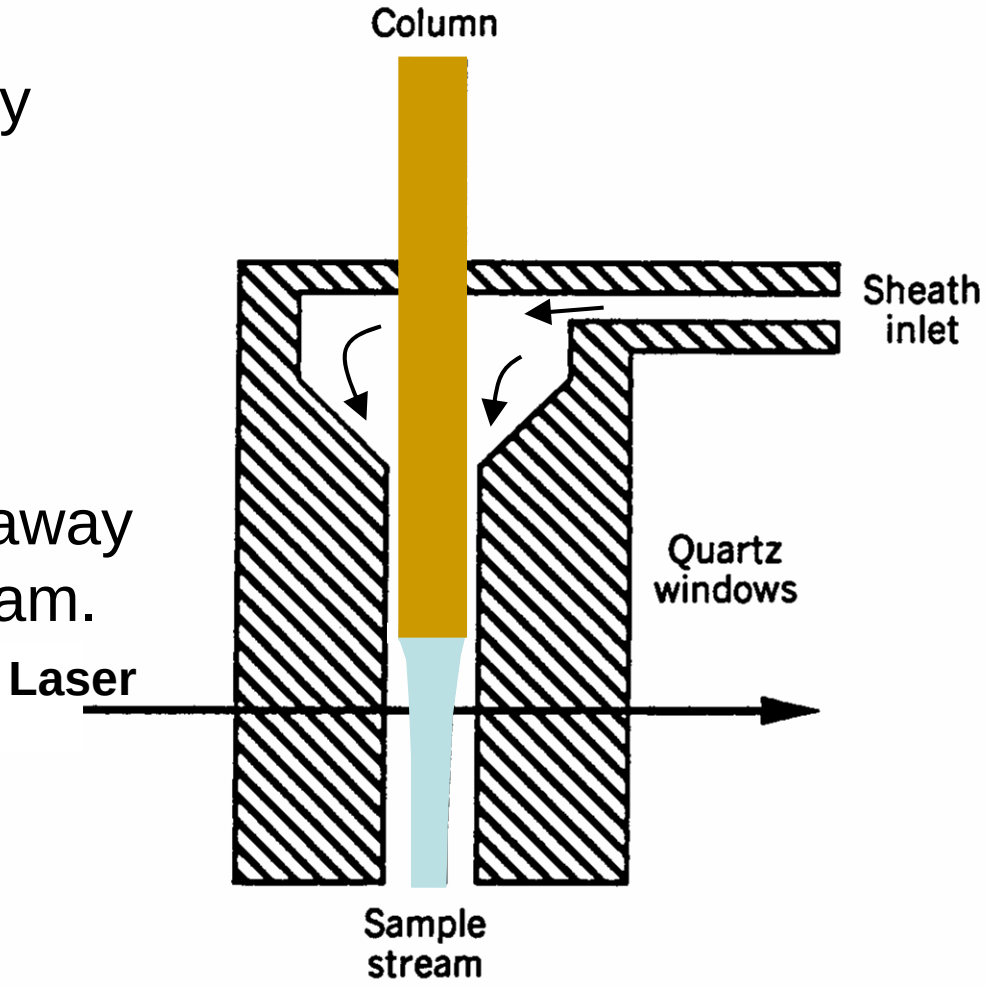


Detection Cell/Window

“Off-Column”- Spatially & Spectrally Separates Fluorescence from High Background

1. Moves index of refraction mismatch away from the analyte stream.
2. Flat, high quality quartz window
3. Ultra-high sensitivity

Issues:
Complexity

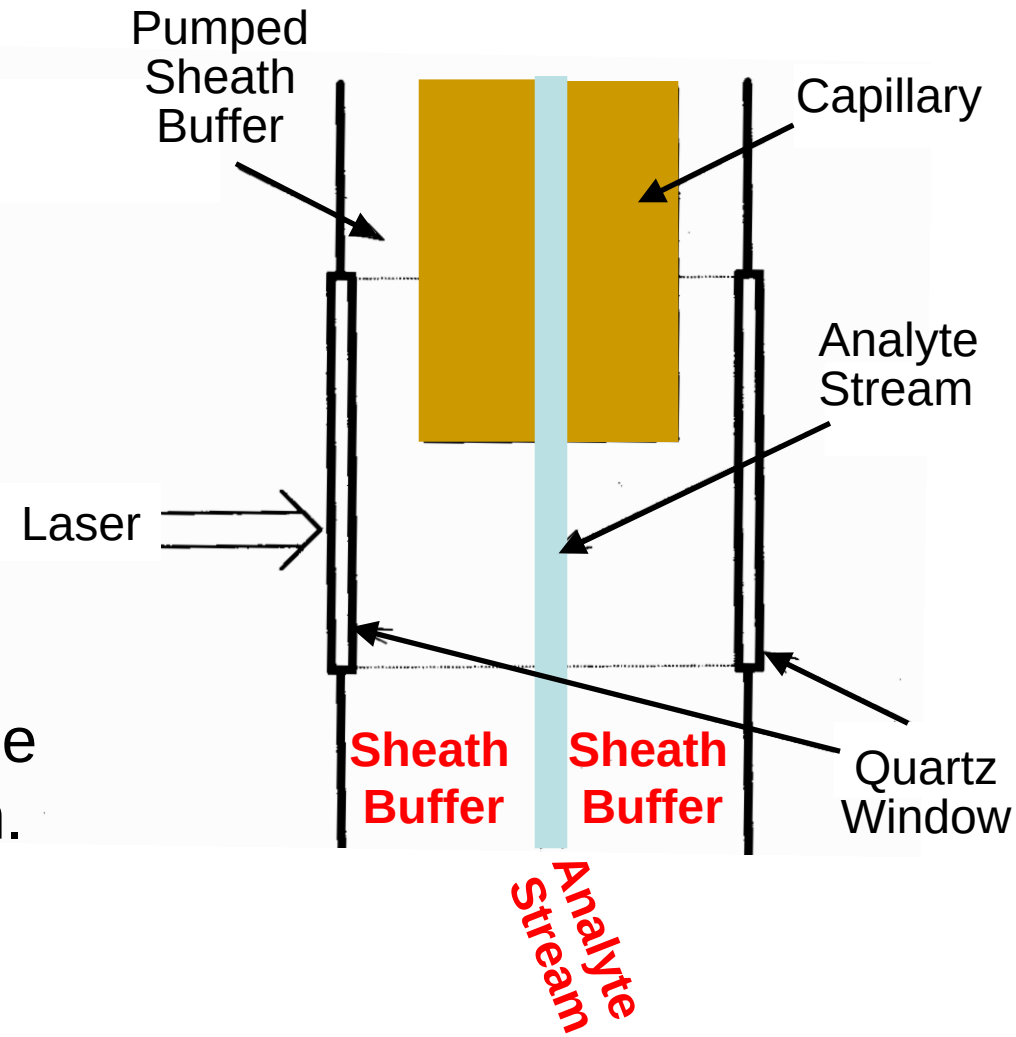


Cheng & Dovichi, 1988, Science, 242:562.

Detection Cell/Window

“Off-Column”

1. Rate of sheath flow controls diameter of analyte stream. (no band broadening)
2. Laminar flow at low rates- No mixing with sheath fluid.
3. Laser is focused to the size of analyte stream.



Light Collection & Detector

Detector: Photomultiplier Tube (PMT)
wide dynamic range
high sensitivity
low cost

Light Collection:

1. Maximize collection of fluorescent light
2. Minimize collection of background light
   a. Reflected/scattered light from interfaces
   b. Rayleigh scattering- particulates
   c. Raman scattering from water
   d. Background fluorescence
   (buffer solution or glass walls)

Maximizing Collection of Fluorescent Light

Fluorescence- Isotropic *i.e.* emitted in all directions

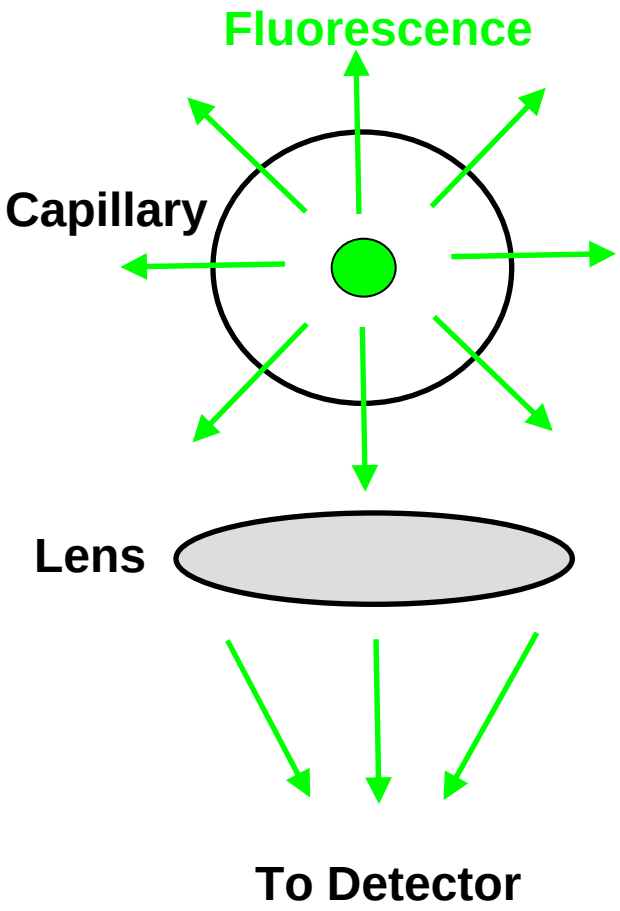
Need a Lens for Light Collection
(typically microscope objective)

Fraction of light collected =
Collection Efficiency =

$$\sin^2[0.5 \arcsin(NA/n)]$$

where
NA = numerical aperture
n = index of refraction of medium around lens = 1 (for air)

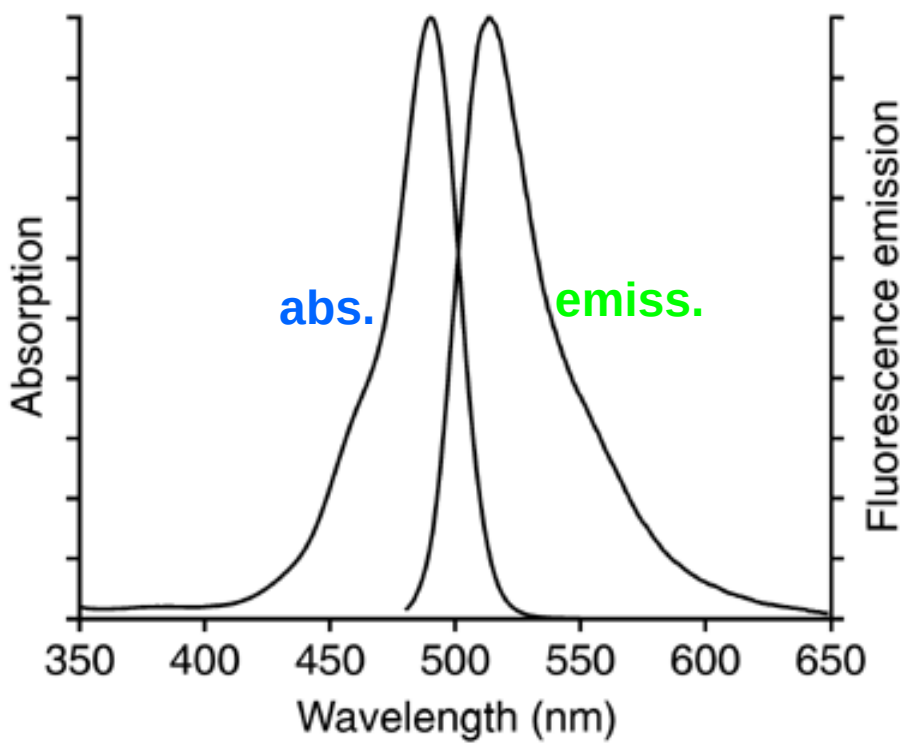
High NA microscope objectives give the best S/N.



Minimizing Background Light- Raman

Problematic Raman Band
(using 488 nm laser line)
is at 585 nm for water.

Fluorescein Spectra



For best S/N, must spectrally separate Raman from fluorescein emission.

Requires a bandpass filter:
~500 nm < λ < ~570 nm

Note: Filters- high transmission (>70%) in selected region but gratings (monochromators) have poor transmission (<1%).

Minimizing Background Light

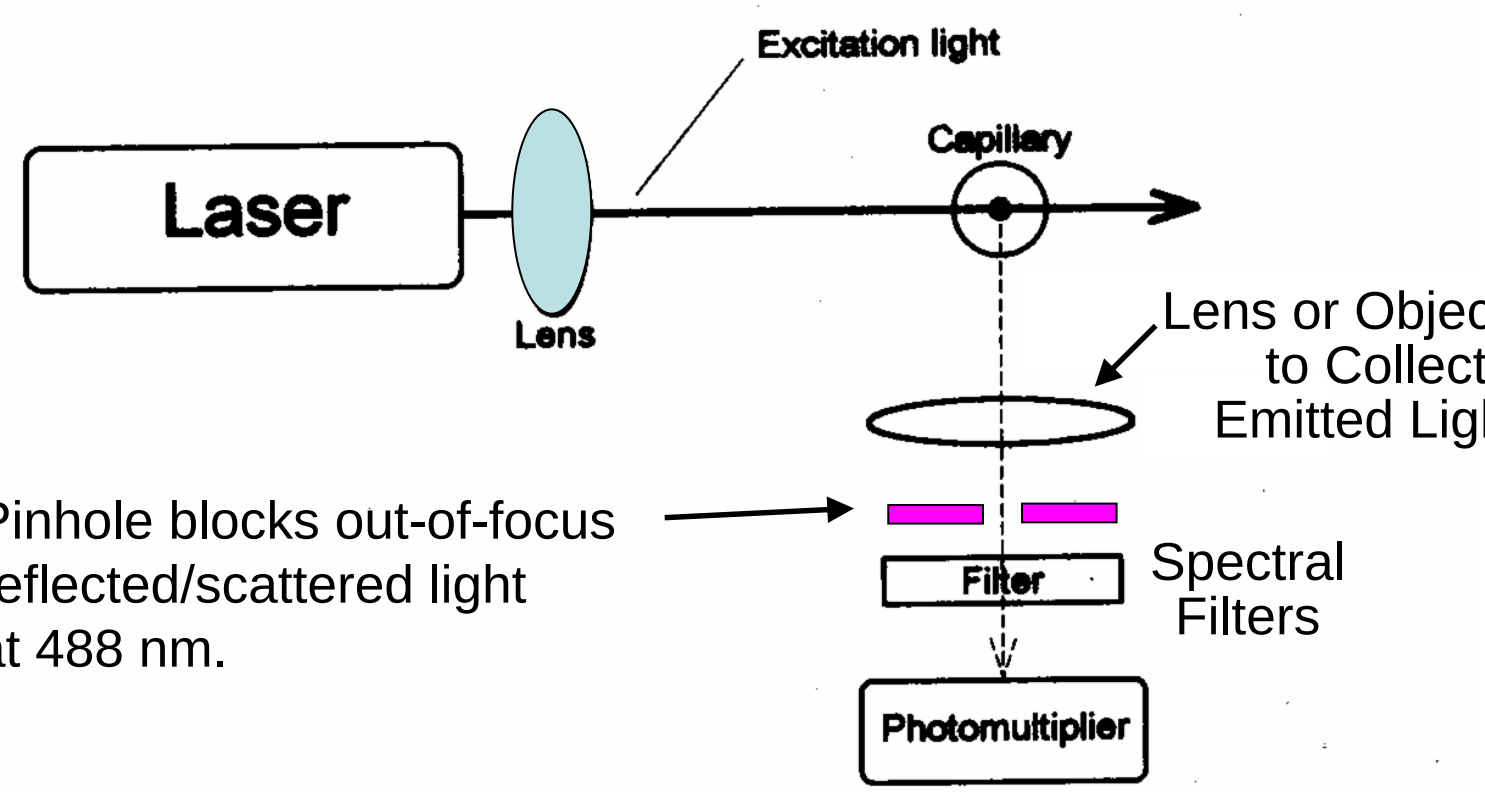
Reflected Light/Rayleigh Scattered Light-
at 488 nm (same λ as excitation).

Strategies:

1. Spectral filtering
    - Bandpass filter as with Raman
    - Notch/Razor filter-
        - Very high light rejection (OD > 6) at excitation λ
        - Very high transmission (>90%) at fluorophore emission

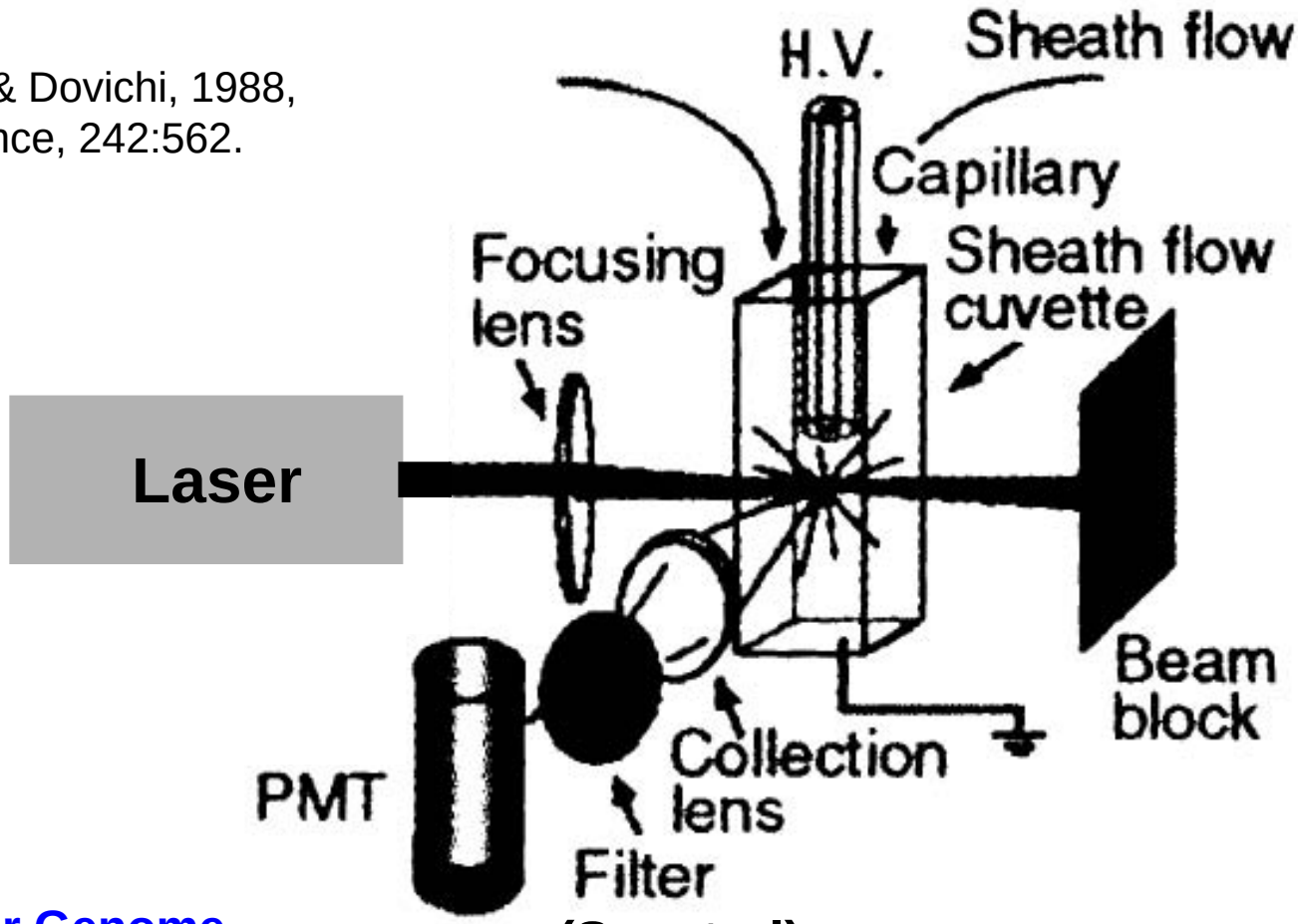
2. Spatial filtering
    - Optical Geometry-
        - a. Orthogonal
        - b. Epifluorescence (Confocal)
    - Apertures- Pinholes, Obscuration Bars

Orthogonal Optical Geometry
(On-Column Detection)



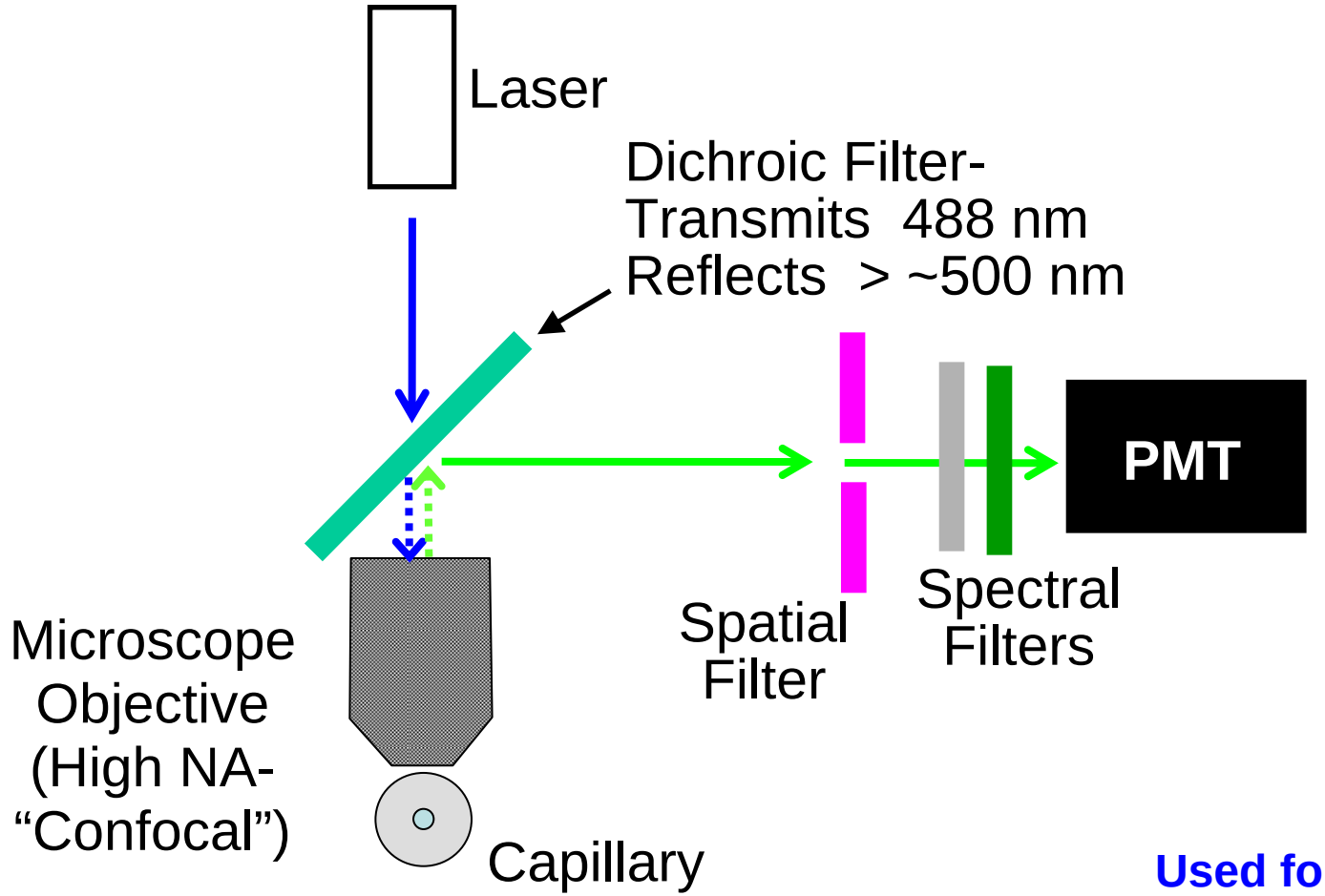
Orthogonal Optical Geometry
(Off-Column Detection)

Cheng & Dovichi, 1988,
Science, 242:562.



Used for Genome Sequencing

Epifluorescence Optical Geometry
(On-Column Detection)

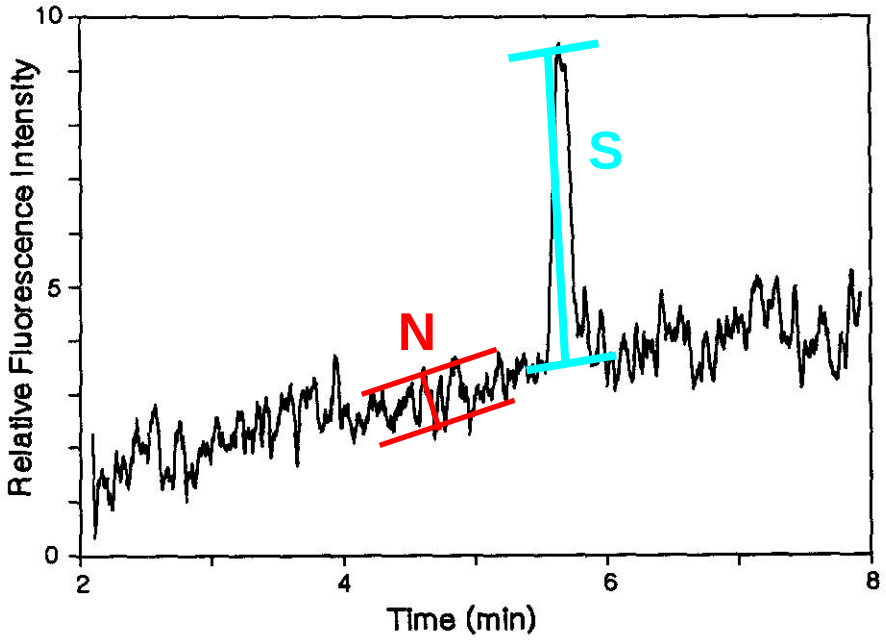


Used for Genome Sequencing

Optimizing Signal to Noise

1. Fluorescence increases linearly with laser power (until all fluorophores are excited).
2. Scattered light increases linearly with laser power
3. Noise in background increases as (laser power)$^{1/2}$.

S/N ~ (laser power)$^{1/2}$



Define detection limit as a S/N of 3:

On-column detection limits of ~10^{-20} moles/band.

Off-column (sheath flow) detection limits of ~10^{-21} moles/band.

Specialized Cases: Sheath flow- 1 molecule

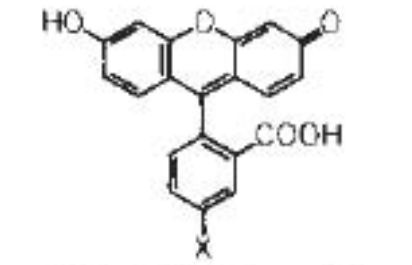
Fluorescent Dyes for DNA Sequencing

Need Four Dyes With These Attributes:

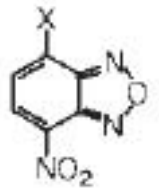
1. A common excitation λ.
2. High yet similar molar absorbances.
3. Four well-separated emission λ.
4. High yet similar quantum efficiencies.
5. Minimal and similar μ shifts when attached to DNA strands.
6. Common set of fluorescent reagents for all sequencing.

Initial Four Dyes for DNA Sequencing

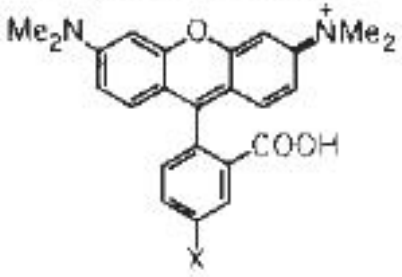
Each base-specific reaction (Sanger rxn) had a different dye-labeled primer:



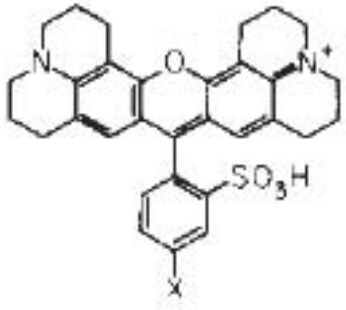
Fluorescein



NBD



Tetramethylrhodamine



Texas red

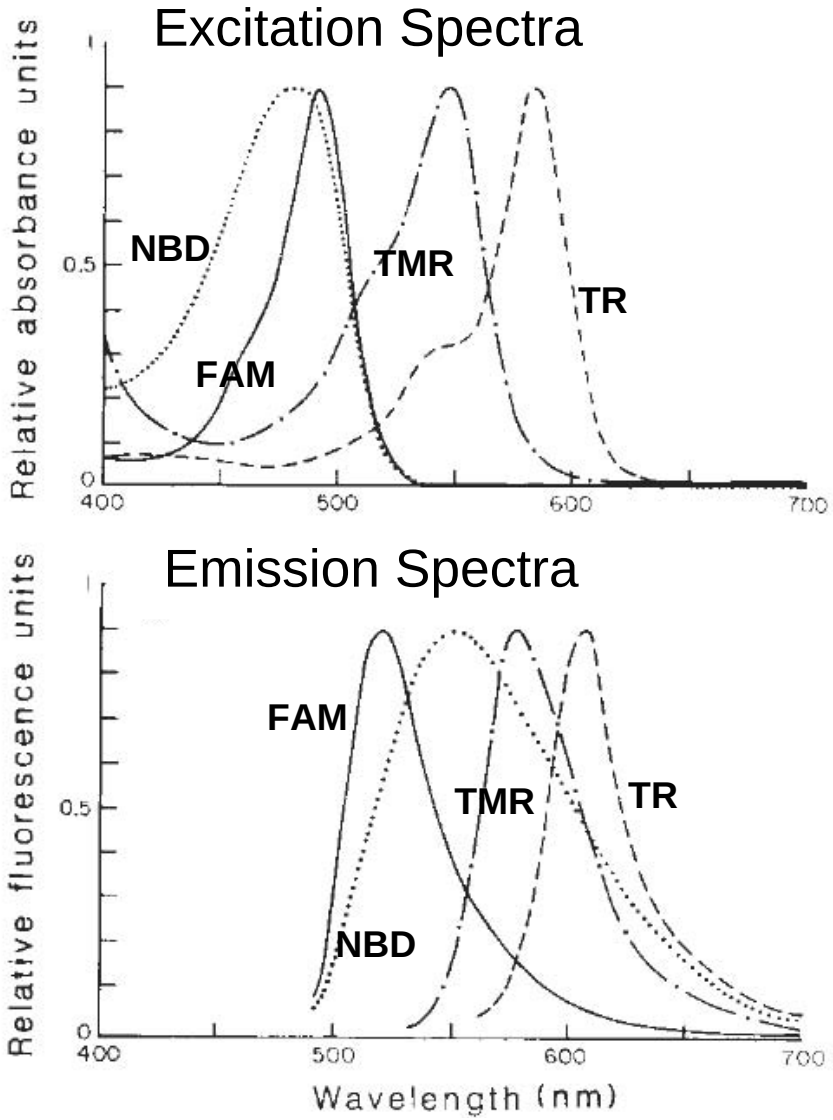
	Absorption max (nm)	Emission max (nm)
Fluorescein (FAM)	493	516
4-Chloro-7-nitrobenzo-2-1-diazole (NBD)	475	540
Tetramethyl-rhodamine (TMR)	556	582
Texas Red (TR)	599	612

Smith, L.M. et al 1986, *Nature* 321:674-9.

Issues With Initial Four Dyes

Issues:

1. Required 2 excitation wavelengths.
2. Dyes were not equally bright.
3. Emission λ overlap.
4. μ shifts for the different dyes are not similar.
5. Need 4 different primers for each sequencing rxn.



Smith, L.M. et al 1986, *Nature* 321:674-9.

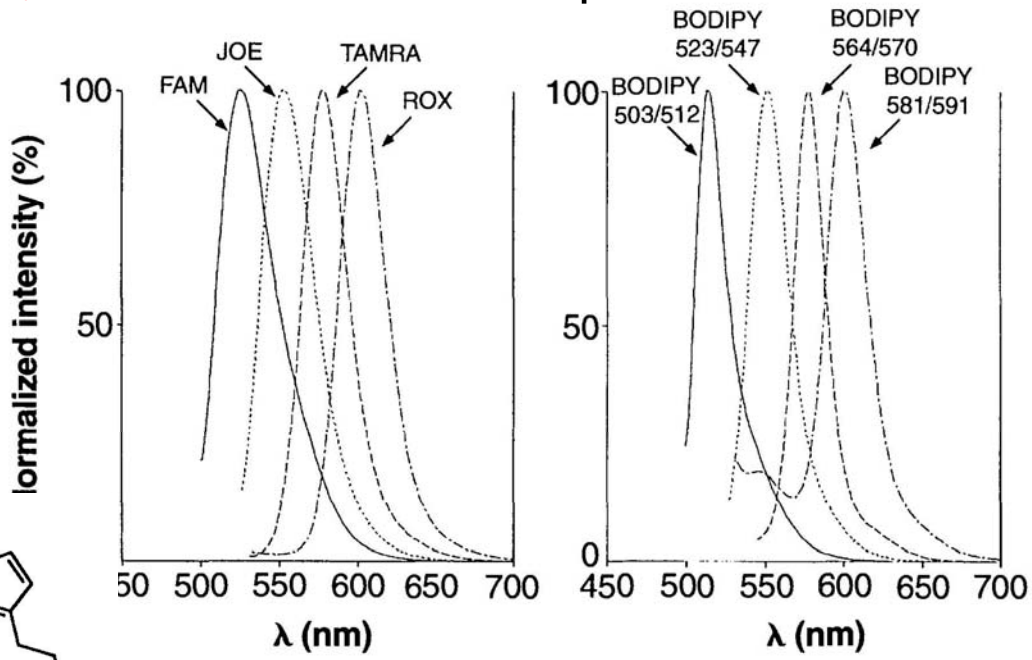
Improvements to Initial Four Dyes

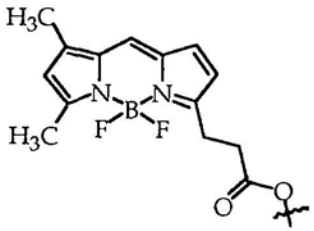
New Fluorophores:

1. Improved fluorescein and rhodamine derivatives-
   Fluorescein-derived: JOE; Rhodamine-derived: TAMRA & ROX
   Better spectral properties.
   Still require 2 excitation λ.
   Nonuniform shifts in μ.

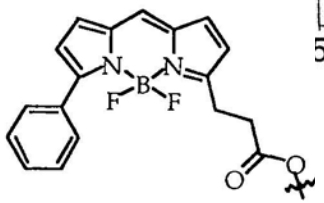
2. BODIPY dyes-
   Good spectral prop.
   Uniform shifts in μ.
   Still require 2 excitation λ.

Emission Spectra





BODIPY 503/512



BODIPY 523/547

Swerdlow et al 1990, Nucl. Acids Res. 18:1415;
Karger et al 1991, Nucl. Acids Res. 19:4955;
Metzker et al 1996, Science 271:1420.

Fluorescent Dyes for DNA Sequencing

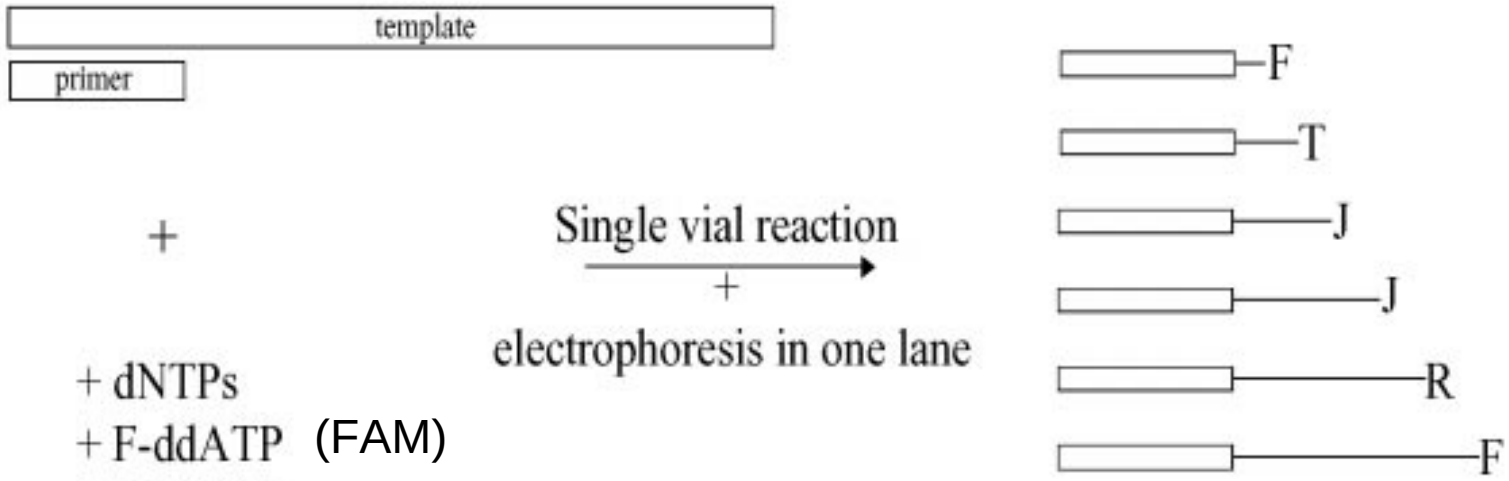
Need Four Dyes With These Attributes:

1. A common excitation λ.
2. High yet similar molar absorbances.
3. Four well-separated emission λ. Done
4. High yet similar quantum efficiencies. Done
5. Minimal and similar μ shifts when attached to DNA strands. Done
6. Common set of fluorescent reagents for all sequencing.

Improvements in Dye Labeling Technology

Dye-Labeled Terminators- Fluorophore is linked to the ddNTP terminator. Use the same 4 terminators for all sequencing reactions.

Note: These also required improvements in the polymerase so it could utilize the labeled terminators.



Prober et al, Science, 1987, 238:336;
Rosenblum et al, Nucl. Acids Res. 1997, 25:4500

Fluorescent Dyes for DNA Sequencing

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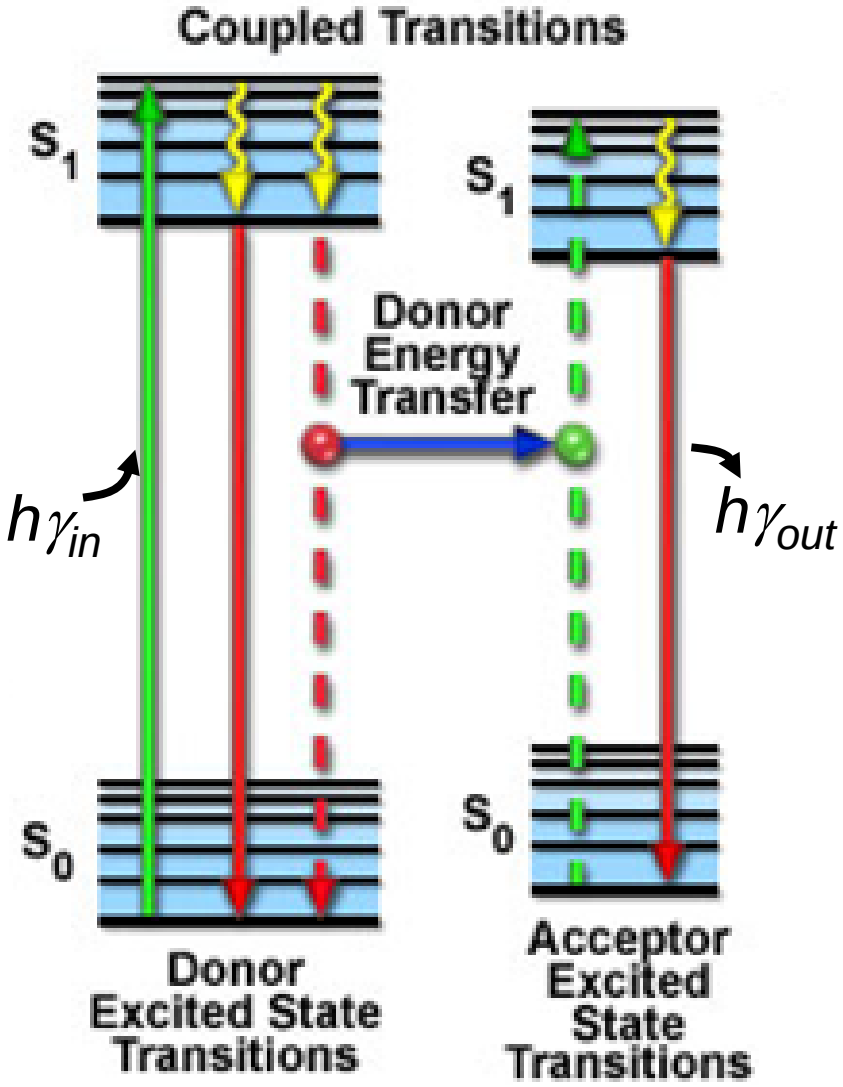
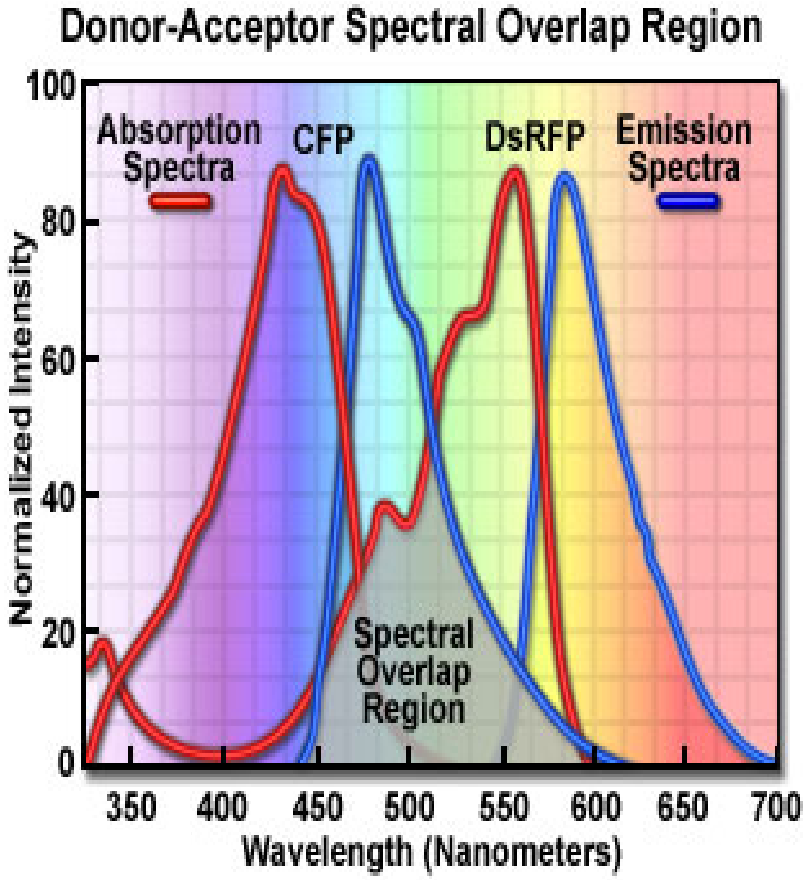
4. High yet similar quantum efficiencies. Done
5. Minimal and similar μ shifts when attached to DNA strands. Done
6. Common set of fluorescent reagents for all sequencing. Done

Fluorescence Resonance Energy Transfer



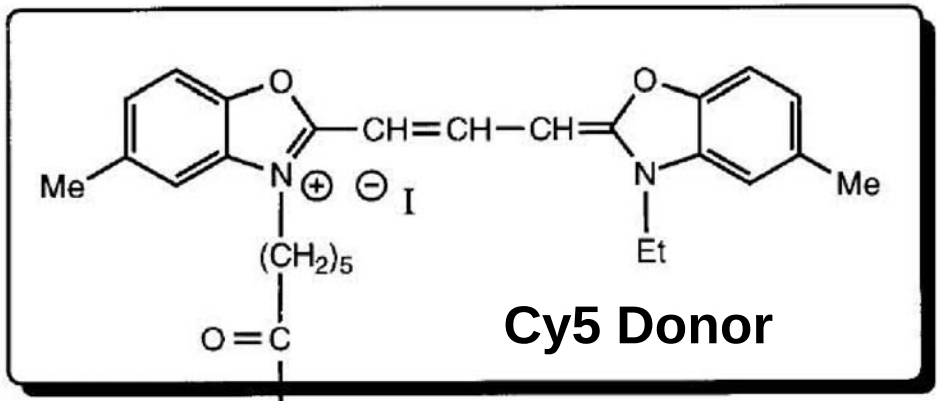


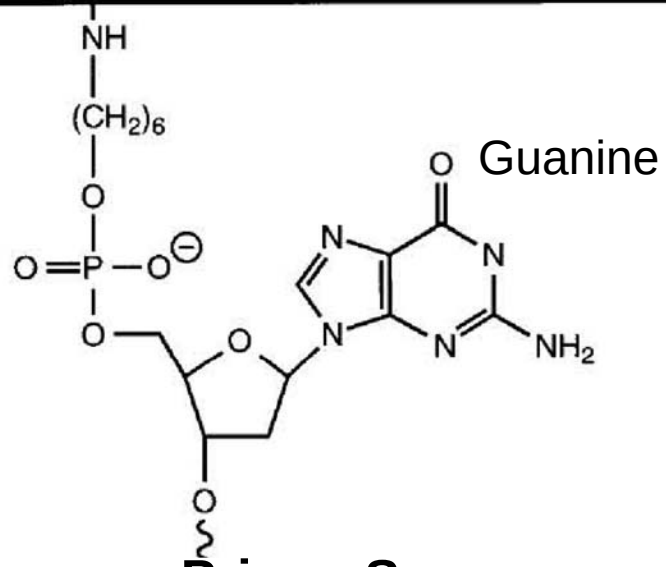
Energy Transfer Dyes on Primers Permit Single Wavelength Excitation!

Two dyes per primer:

1. Common donor
   FAM or Cy5
   (common exc.- 488 nm)
2. DNA spacer between dyes
3. Different acceptors
   FAM, JOE, TAMRA, ROX

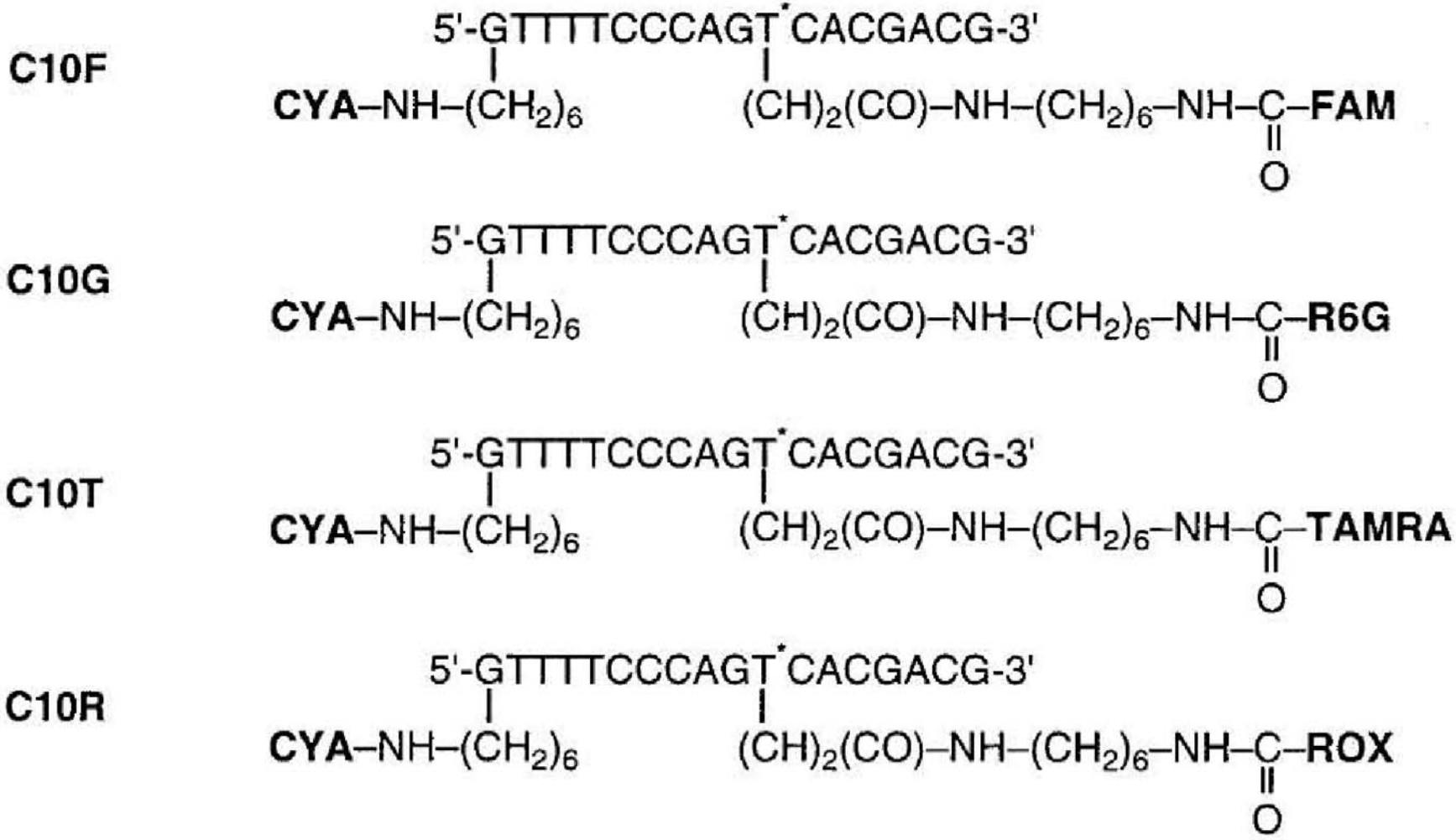
Developed by Mathies' and Glazer's labs. Their paper using a FAM donor (PNAS 1995, 92:4347) is on the web site.





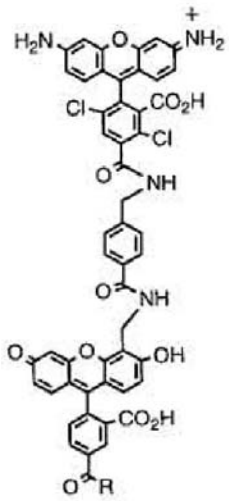
Primer Sequence with Attached Acceptor

Primers with Cy5 Donor and An Acceptor

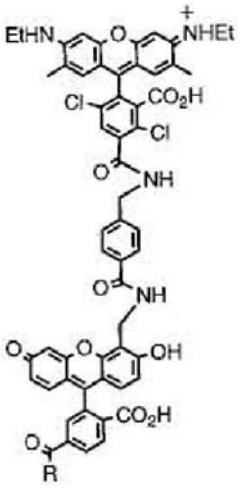


Hung et al, 1996, Anal. Chem. 243:15.

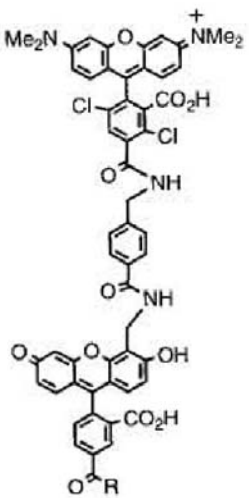
Energy Transfer Pairs for Terminators



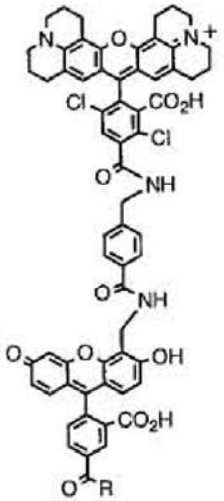
5CFB-dR110



6CFB-dR6G



5CFB-dTMR

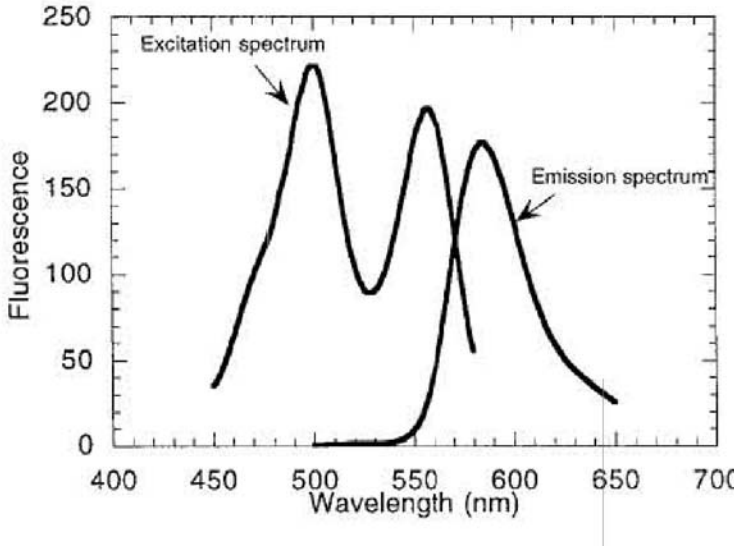


5CFB-dROX

Rhodamine Derivatives (Acceptor)

Linker

Carboxy-Fluorescein (Donor)

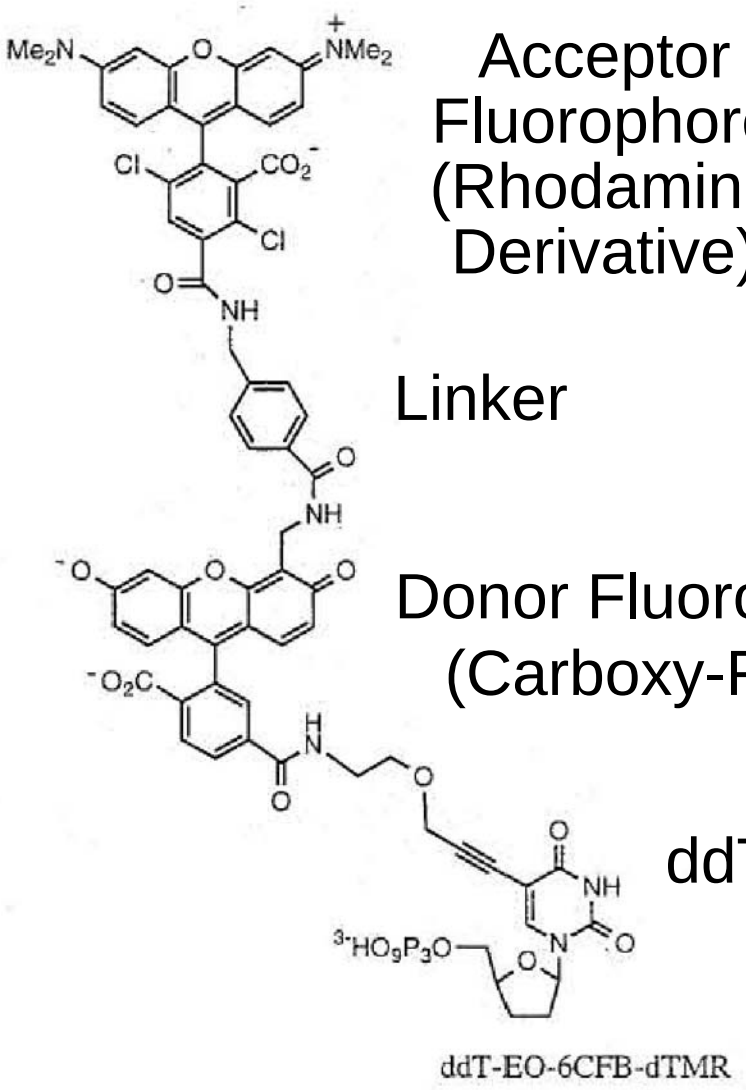


Excitation Spectra-
Peaks from FAM & Rhodamine

Emission Spectra-
Single Rhodamine Dye Peak

Lee et al, 1997, Nucl. Acids Res. 25:2816.

Energy Transfer Terminators- Single λ Excitation



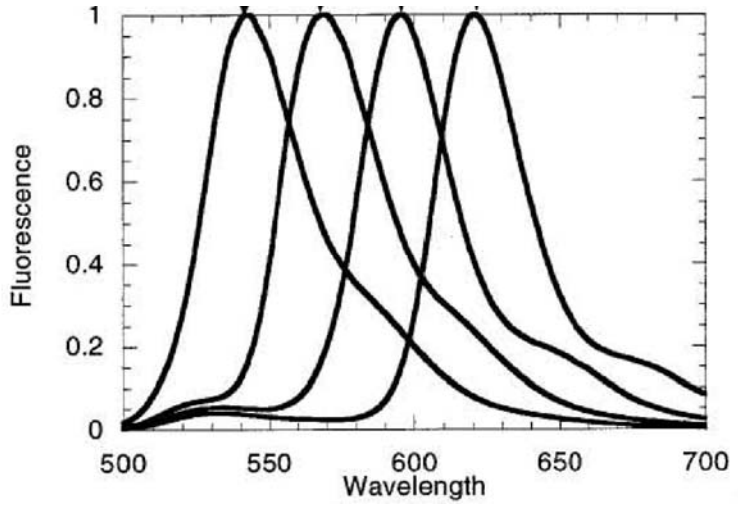
ddT-EO-6CFB-dTMR

Acceptor Fluorophore (Rhodamine Derivative)

Linker

Donor Fluorophore (Carboxy-FAM)

ddTTP



Emission Spectra of the Four E.T. Terminators *("Big-Dye" Terminators™ of PE Applied Biosystems)*

Rosenblum et al, 1997, Nucl. Acids Res. 25:4500.

Fluorescent Dyes for DNA Sequencing

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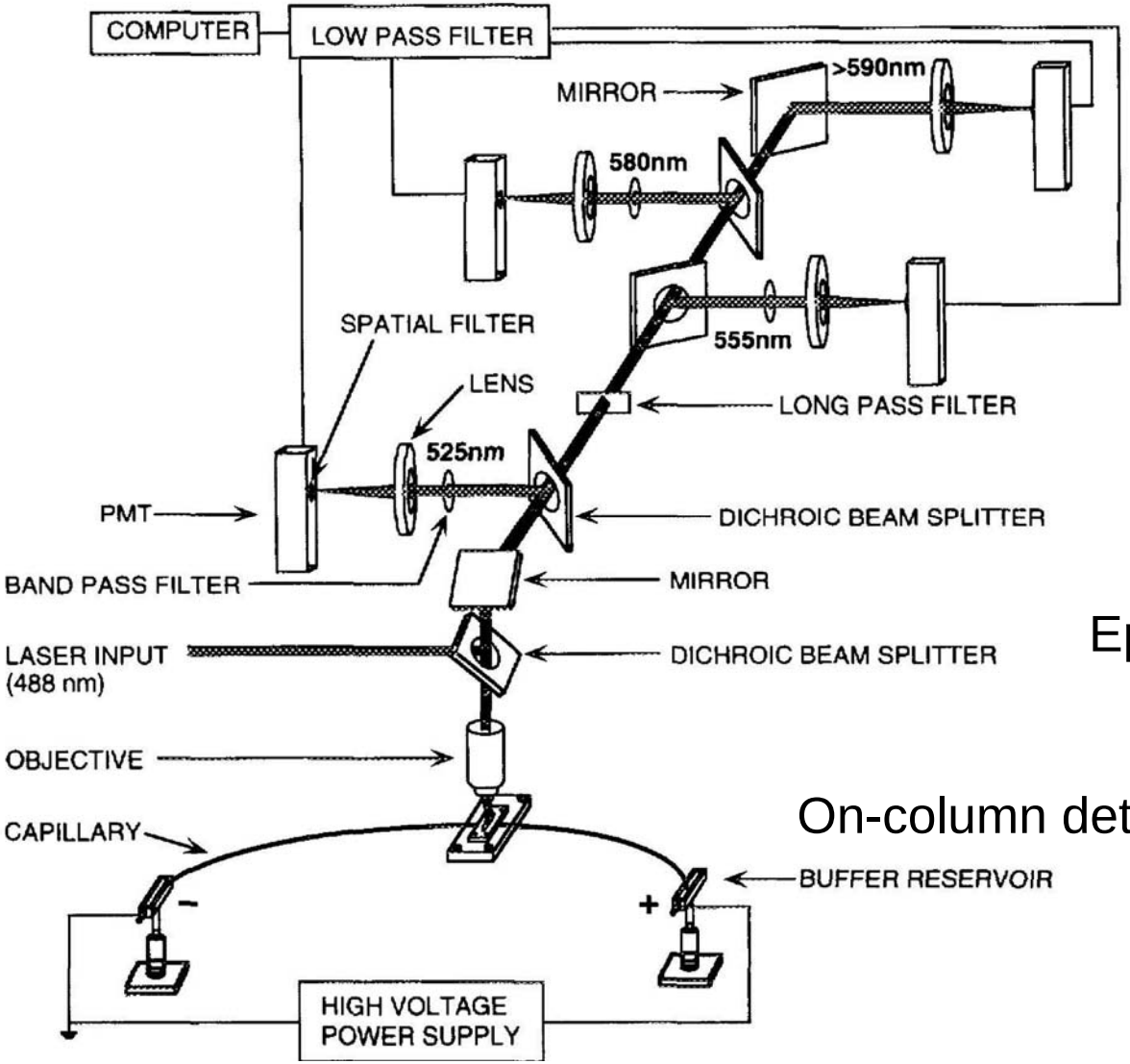
4. High yet similar quantum efficiencies. Done
5. Minimal and similar μ shifts when attached to DNA strands. Done
6. Common set of fluorescent reagents for all sequencing. Done

Detection of Multiple Emission Wavelengths



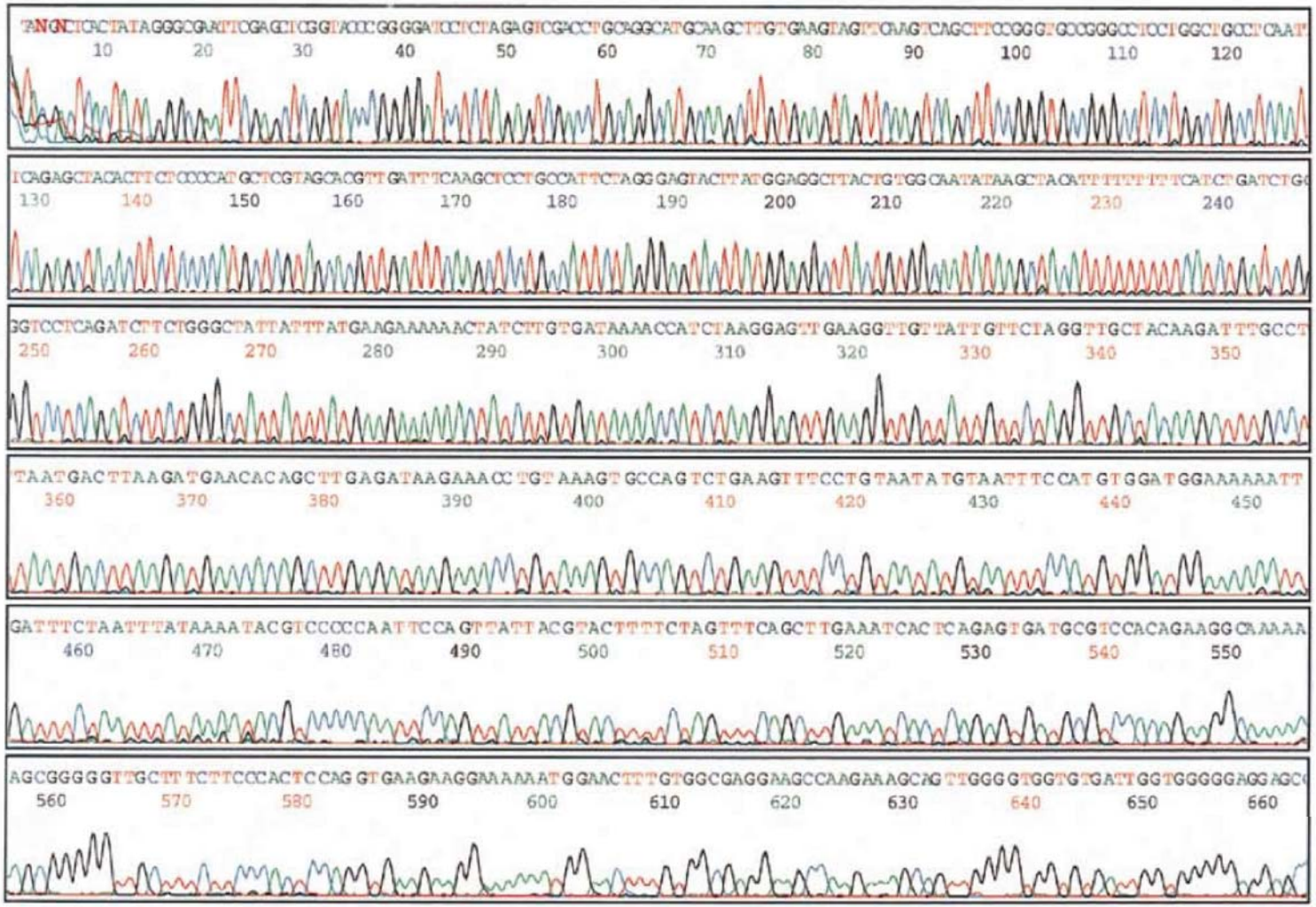
A succession of dichroic beam splitters direct different λ to the appropropriate PMT.

Epifluorescence geometry

On-column detection

Ju et al, 1995, Anal. Biochem. 231:131.

Sequencing Run- Energy Transfer Terminators



Lee et al, 1997, Nucl. Acids Res. 25:2816.

References- Detection

1. Wu S, Dovichi NJ. (1989) High-sensitivity fluorescence detector for fluorescein isothiocyanate derivatives of amino acids separated by capillary zone electrophoresis. **Journal of Chromatography**, 480: 141-155.

2. Li L, McGowan LB. (2000) Improving signal to background ration for on-the-fly fluorescence lifetime detection in capillary electrophoresis. **Electrophoresis**, 21: 1300-1304.

3. Yeung ES, Wang P, Li W, Giese RW. (1992) Laser fluorescence detector for capillary electrophoresis. **Journal of Chromatography**, 608: 73-77.

4. Swerdlow H, Wu S, Harke H, Dovichi NJ. (1990) Capillary gel electrophoresis for DNA sequencing: Laser-induced fluorescence detection with the sheath flow cuvette. **Journal of Chromatography**, 516: 61-67.

4. Roach MC, Gozel P, Zare RN. (1988) Determination of methotrexate and its major metabolite, 7-hydroxymethotrexate, using capillary zone electrophoresis and laser-induced fluorescence detection. **Journal of Chromatography**, 426:129-140.

5. Lee TT, Yeung ES. (1992) High-sensitivity laser-induced fluorescence detection of native proteins in capillary electrophoresis. **Journal of Chromatography**, 595: 319-325.

6. Chen DY, Swerdlow HP, Harke HR, Zhang JZ, Dovichi NJ. (1991) Low-cost, high-sensitivity laser-induced fluorescence detection for DNA sequencing by capillary gel electrophoresis. **Journal of Chromatography**, 559: 237-246.

7. Clark SM, Mathies RA. (1993) High-speed parallel separation of DNA restriction fragments using capillary array electrophoresis. **Analytical Biochemistry**, 215: 163-170.

8. Drossman H, Luckey JA, Kostichka AJ, D'Cunha J, Smith LM. (1990) High-speed separations of DNA sequencing reactions by capillary electrophoresis. **Analytical Chemistry**, 62: 900-903.

9. Cheng YF, Dovichi NJ. (1988) Subattomole amino acid analysis by capillary zone electrophoresis and laser-induced fluorescence. **Science**, 242: 562-564.

10. Lee TT, Yeung ES. (1996) Capillary electrophoresis detectors: Lasers. **Methods in Enzymology**, 270: 419-449.

11. Swinney K, Bornhop DJ. (2000) Detection in capillary electrophoresis. **Electrophoresis**, 21: 1239-1250.

12. Johnson, M.E., Landers,J.P. (2004) Fundamentals and practice for ultrasensitive laser-induced fluorescence detection in microanalytical systems. **Electrophoresis** 25: 35133527.

References- Fluorescent Dyes

1. Zakeri H, Amparo G, Chen SM, Spurgeon S, Kwok PY. (1998) Peak height pattern in dichloro-rhodamine and energy transfer dye terminator sequencing. **Biotechniques**, 25: 406-414.

2. Franca LTC, Carrilho E, Kist TBL. (2002) A review of DNA sequencing techniques. **Quarterly Reviews of Biophysics**, 35: 169-200.

3. Hung SC, Ju J, Mathies RA, Glazer AN. (1996) Cyanine dyes with high absorption cross section as donor chromophores in energy transfer primers. **Analytical Biochemistry**, 243: 15-27.

4. Hung SC, Ju J, Mathies RA, Glazer AN. (1996) Energy transfer primers with 5- or 6-carboxyrhodamine-6G as acceptor chromophores. **Analytical Biochemistry**, 238: 165-170.

5. Ju J, Kheterpal I, Scherer JR, Ruan C, Fuller CW, Glazer AN, Mathies RA. (1995) Design and synthesis of fluorescence energy transfer dye-labeled primers and their application for DNA sequencing and analysis. **Analytical Biochemistry**, 231: 131-140.

6. Ju J, Glazer AN, Mathies RA. (1996) Energy transfer primers: A new fluorescence labeling paradigm for DNA sequencing and analysis. **Nature Medicine**, 2: 246-249.

7. Ju J, Ruan C, Fuller CW, Glazer AN, Mathies RA. (1995) Fluorescence energy transfer dye-labeled primers for DNA sequencing and analysis. **Proceedings of the National Academy of Science, USA**, 92: 4347-4351.

8. Kricka LJ. (2002) Stains, labels and detection strategies for nucleic acids assays. **Annals of Clinical Biochemistry**, 39: 114-129.

9. Lee LG, Spurgeon SL, Heiner CR, Benson SC, Rosenblum BB, Menchen SM, Graham RJ, Constantinescu A, Upadhya KG, Cassel JM. (1997) New energy transfer dyes for DNA sequencing. **Nucleic Acids Research**, 25: 2816-2822.

10. Metzker ML, Lu J, Gibbs RA. (1996) Electrophoretically uniform fluorescent dyes for automated DNA sequencing. **Science**, 271: 1420-1422.

11. Rosenblum BB, Lee LG, Spurgeon SL, Khan SH, Menchen SM, Heiner CR, Chen SM. (1997) New dye-labeled terminators for improved DNA sequencing patterns. **Nucleic Acids Research**, 25: 4500-4504.