Lecture 3
Fluorecence Detection and Dyes for DNA Sequencing

<u>DNA sequencing on slab gel in 1993</u>

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

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

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

Analytical Chemistry to the rescue again!!!!

<u>Definitions for CE</u>

atto: 10^{-18}

zepto: 10^{-21}

yocto: 10^{-24}

<u>Limits of Detection for CE</u>

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

Typical Detection Limits for CE

With specialized
<u>LIF methods:</u>
MLOD = 1 molecule
CLOD = 10^{-16} M

Detector	MLOD (moles)	CLOD (M)
Direct absorbance	10^{-13}–10^{-16}	10^{-5}–10^{-7}
Indirect absorbance	10^{-12}–10^{-15}	10^{-4}–10^{-6}
Laser-induced fluorescence (LIF)	10^{-18}–10^{-21}	10^{-9}–10^{-12}
Indirect fluorescence	10^{-14}–10^{-16}	10^{-6}–10^{-8}
Chemilumin-escence (CL)	10^{-14}–10^{-16}	10^{-7}–10^{-9}
Refractive index (RI)	10^{-13}–10^{-15}	10^{-5}–10^{-7}
Thermooptical absorbance	10^{-15}–10^{-18}	10^{-5}–10^{-7}
Radioactivity	10^{-14}–10^{-18}	10^{-6}–10^{-10}
Raman	10^{-12}–10^{-15}	10^{-3}–10^{-5}

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. Most 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

Laser Power

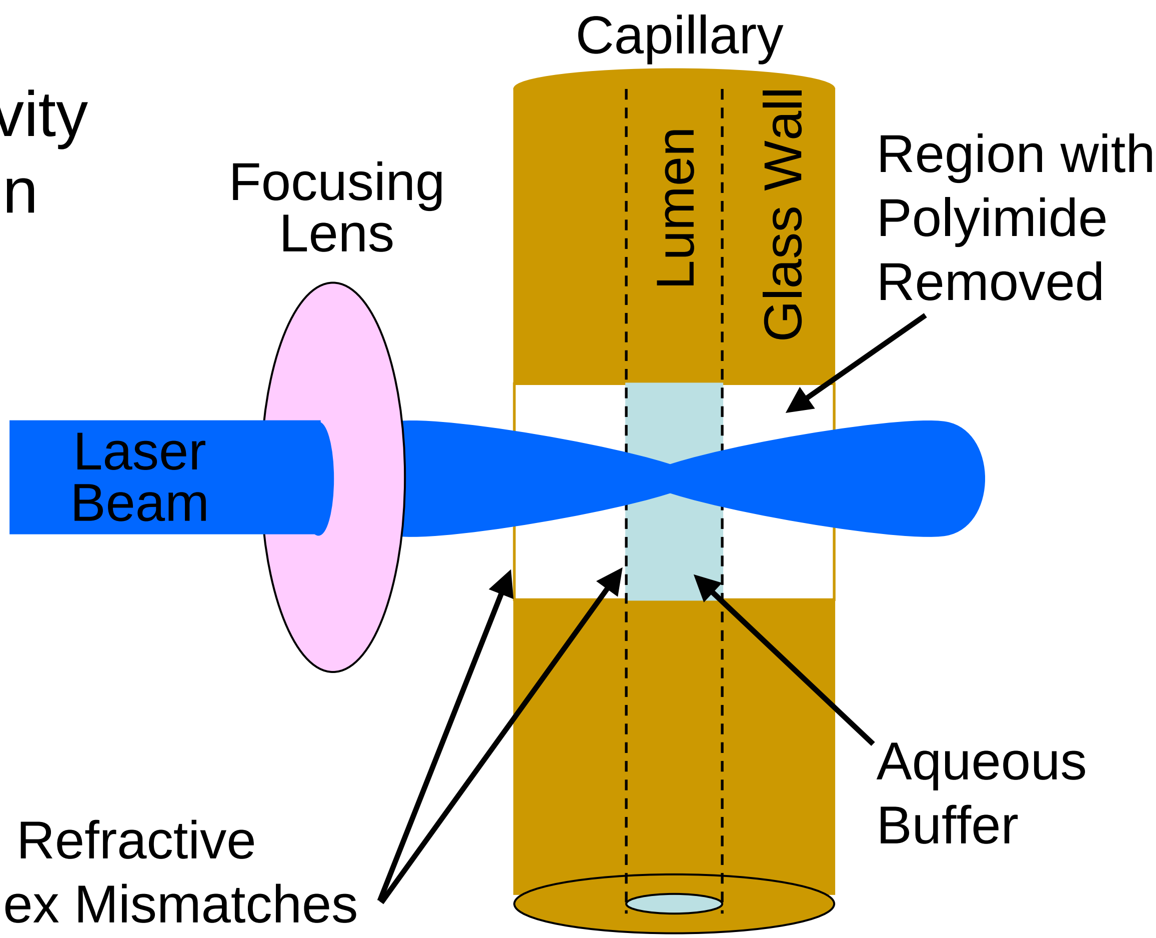
1. Fluorescence α laser power
   until saturate the fluorophore
2. Background/scattered light α laser power

Need to optimize the laser power!
More is not always better!

Detection Cell/Window

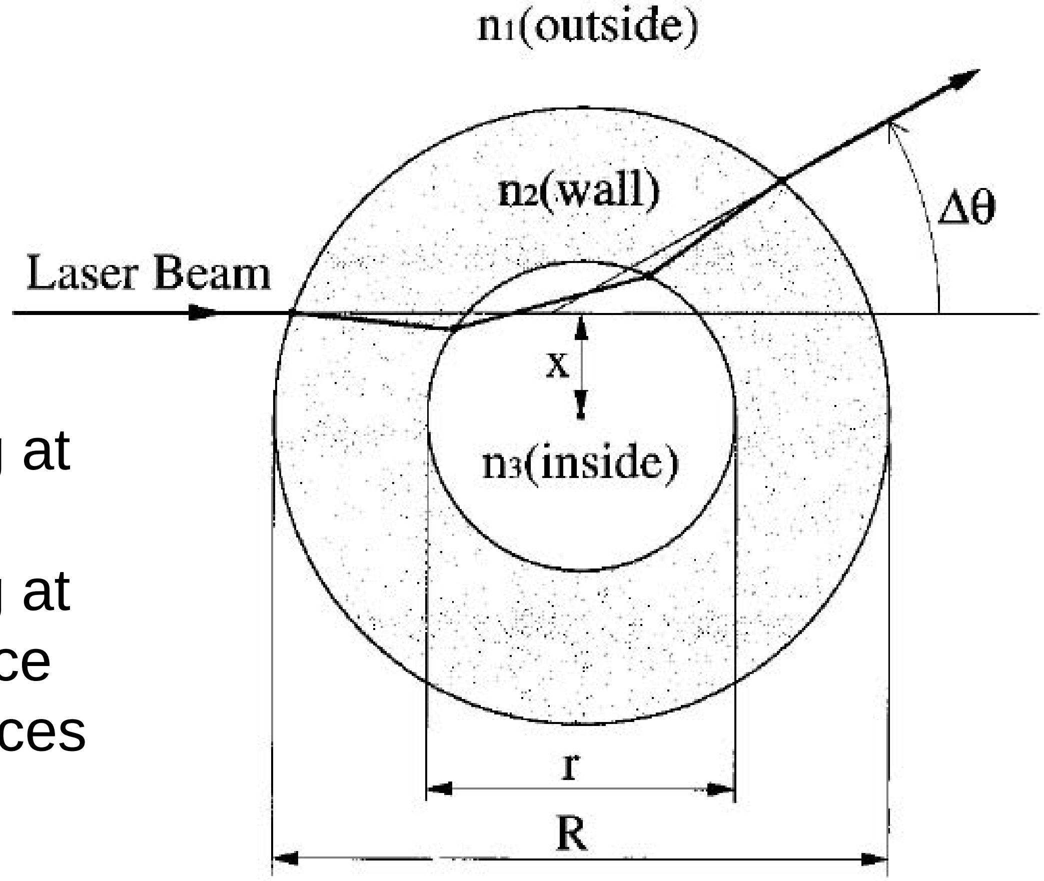
"On-Column"

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



Detection Cell/Window

"On-Column"



Issues:

1. Rayleigh scattering at air:glass interface
2. Rayleigh scattering at glass:water interface
3. Curved glass surfaces
4. Glass impurities

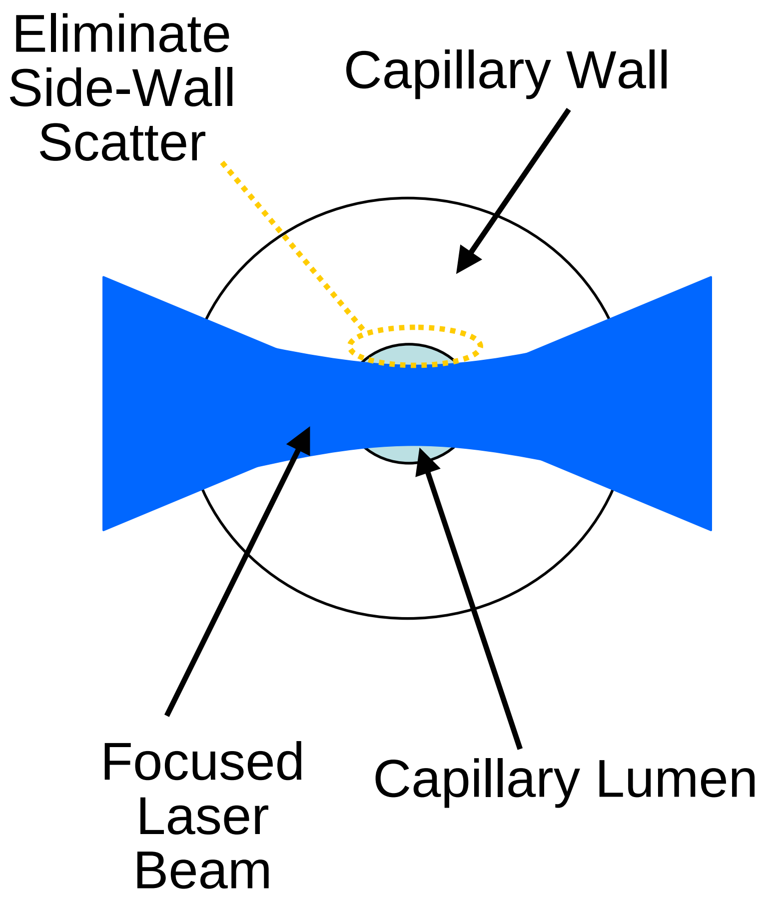
Anazawa et al, Anal. Chem. 1996, 68:2699.

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. Tilt capillary at Brewster's angle.
4. Use confocal excitation/emission (see subsequent section)

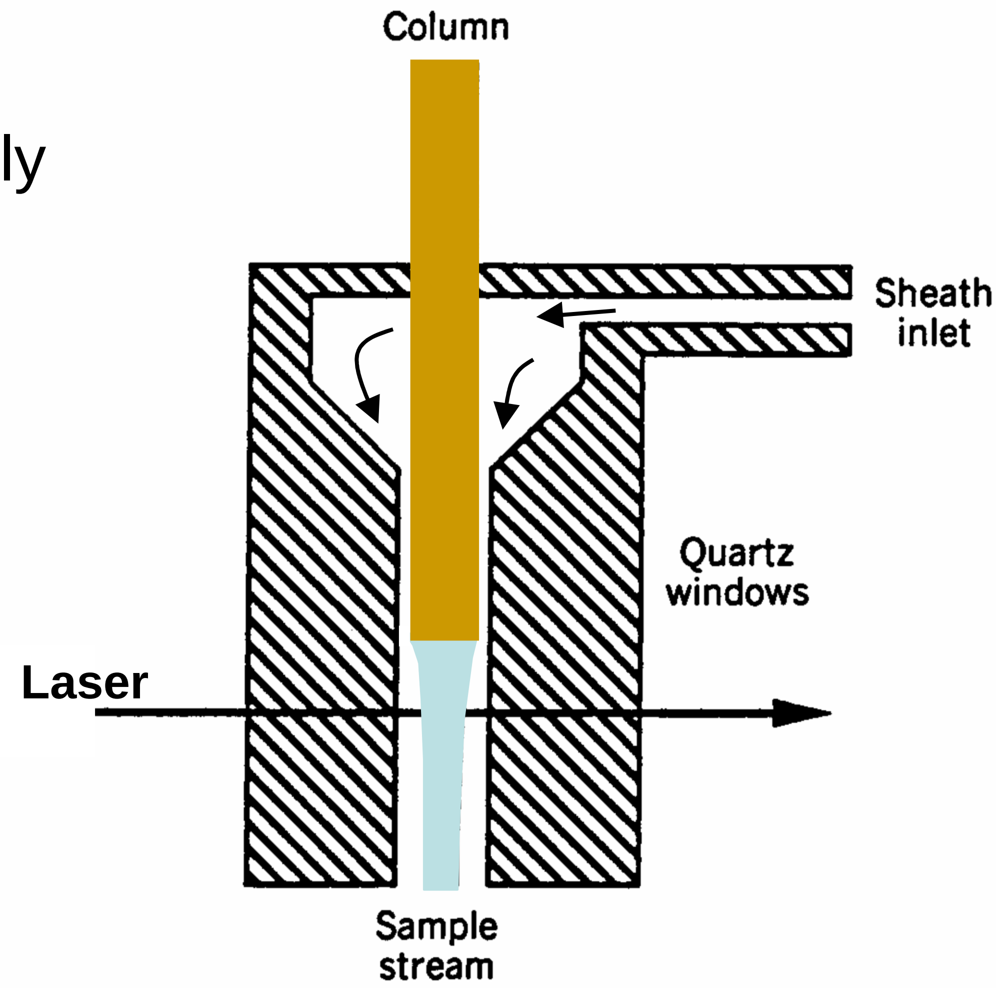


Detection Cell/Window

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

1. Eliminates index of refraction mismatch.
2. Flat, high quality quartz window
3. Ultra-high sensitivity

Issues:
Complexity

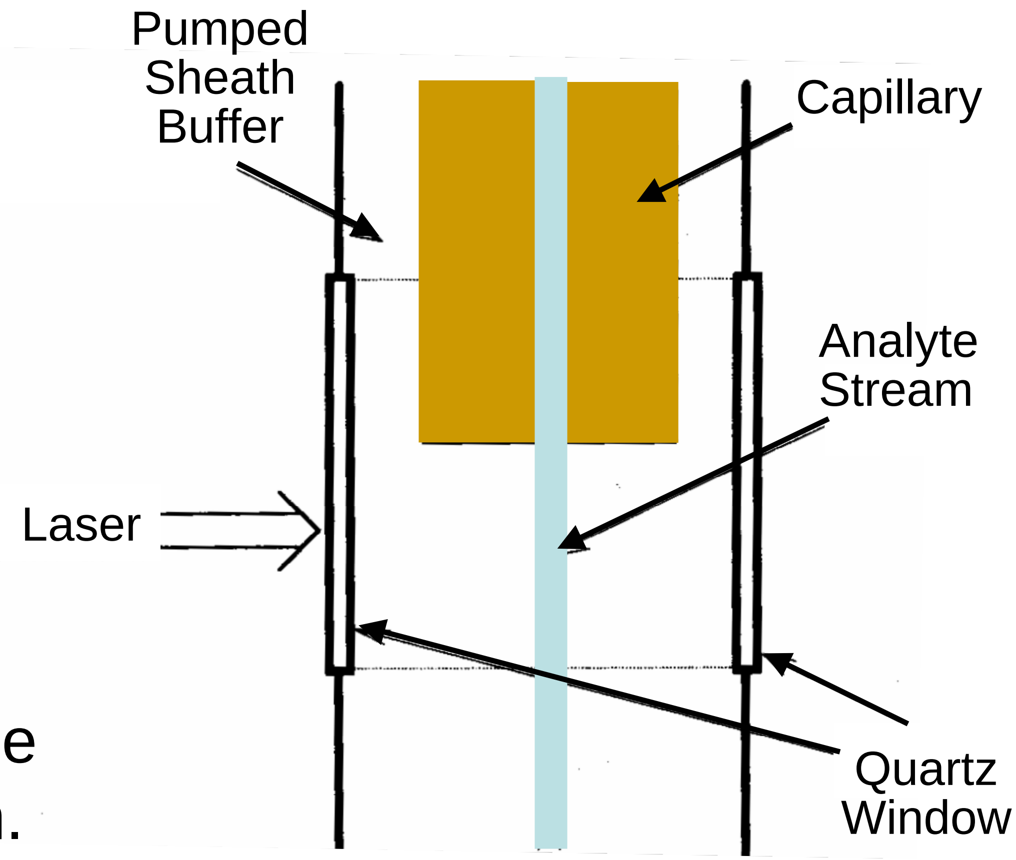


Cheng & Dovichi, 1988, Science, 242:562.

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 costs

Light Collection:

1. Maximize collection of fluoresced light
2. Minimize collection of background light
   a. Rayleigh scattering
   b. Raman scattering
   c. Background fluorescence

Maxizing Collection of Fluorescent Light

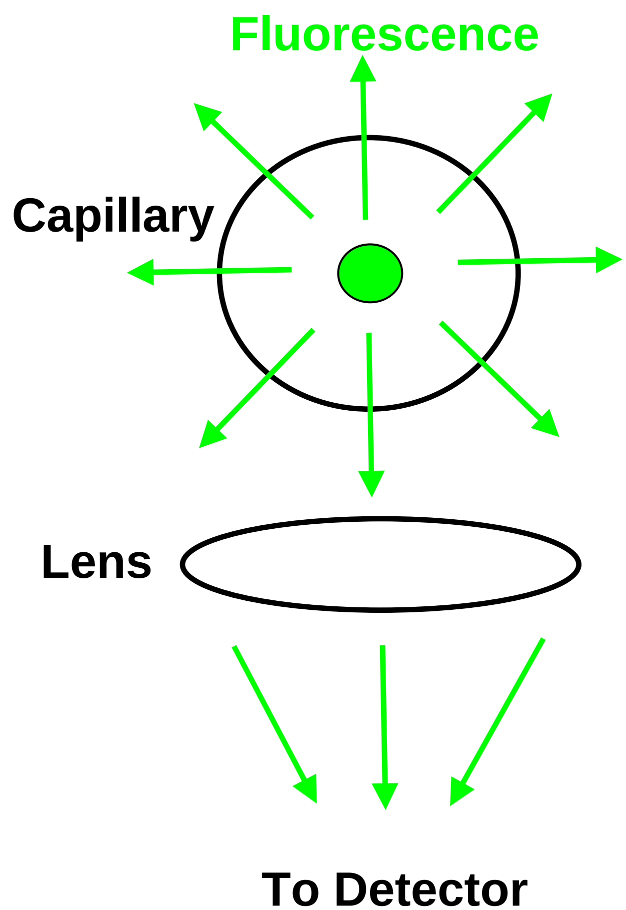
Fluorescence- Anisotropic *i.e.* emitted in all directions

Need a Lens for Light Collection

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)

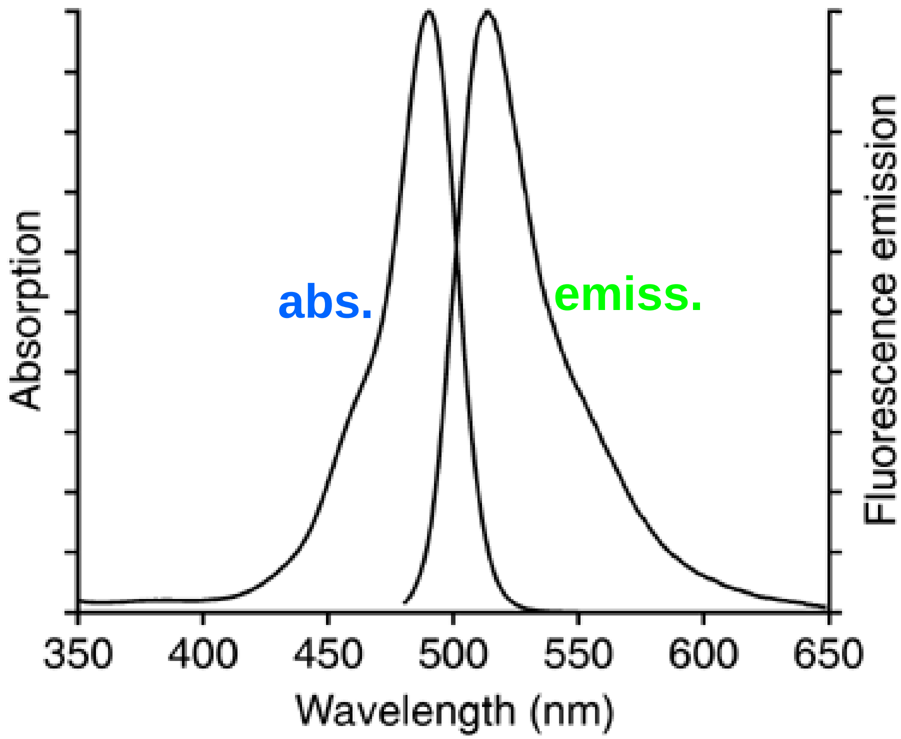
High NA microscope objectives
give the best S/N.



Minimizing Background Light- Raman

Main 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- Rayleigh

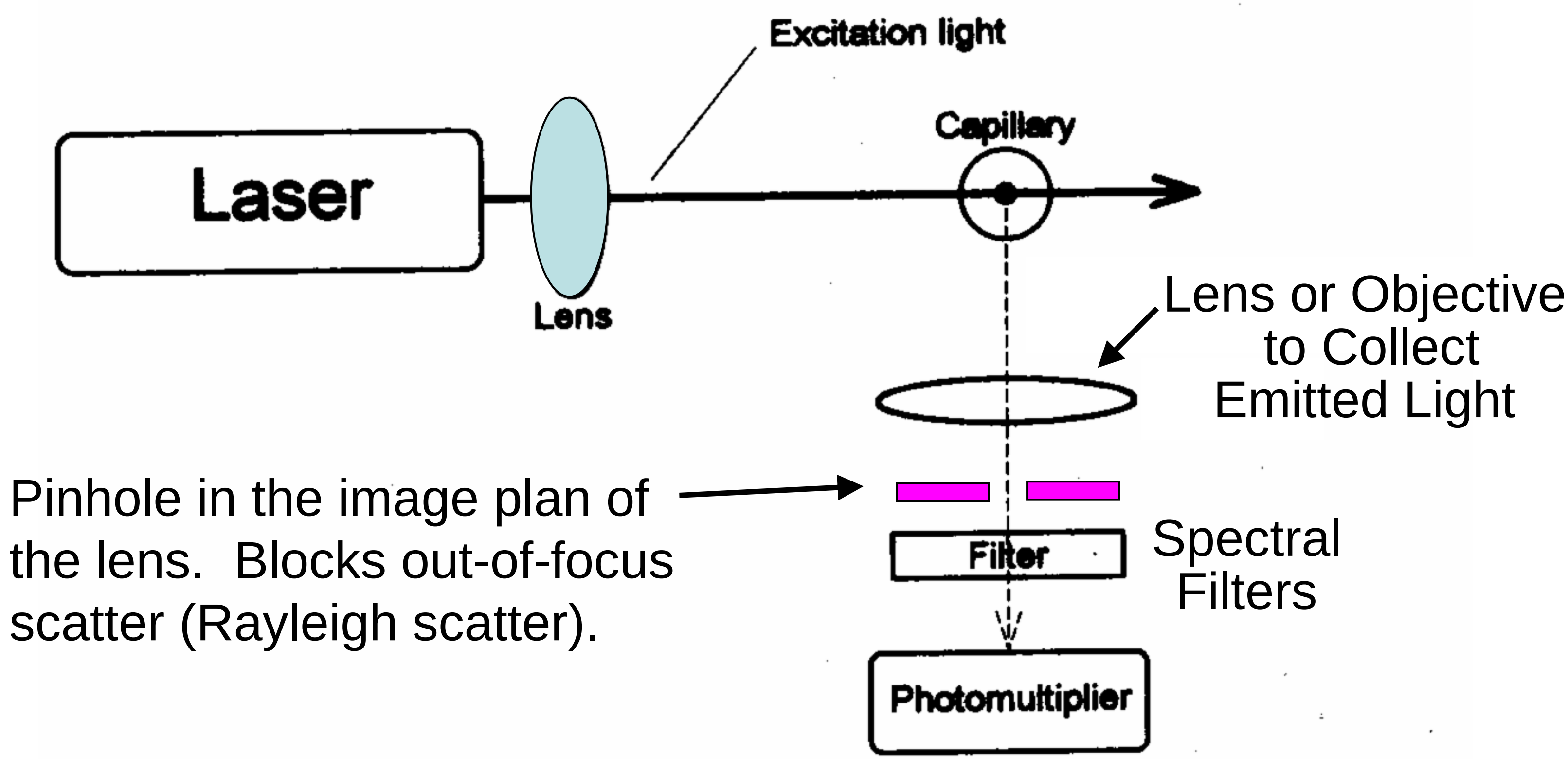
Rayleigh Scatter- at 488 nm (same as excitation).
Has an angular dependence.

Strategies:

1. Spectral filtering
   - Bandpass filter as with Raman
   - Notch filter- Very high light rejection (OD > 6) in a very narrow λ range (~10 nm)

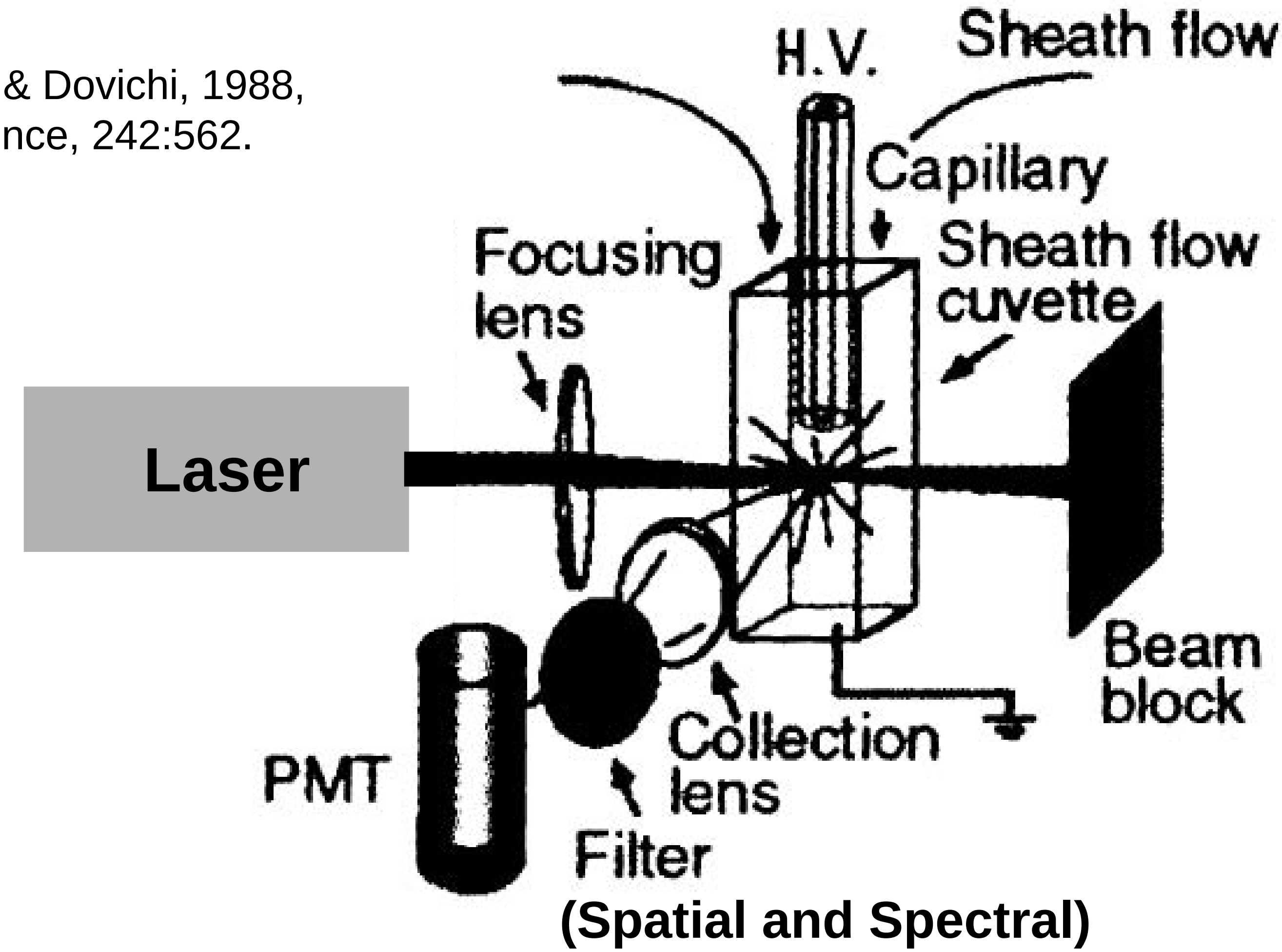
2. Spatial filtering
   - Optical Geometry-
     - a. Orthogonal
     - b. Epifluorescence (Confocal)
   - Apertures- Pinholes, Obscuration Bars

Orthogonal Optical Geometry
(On-Column Detection)

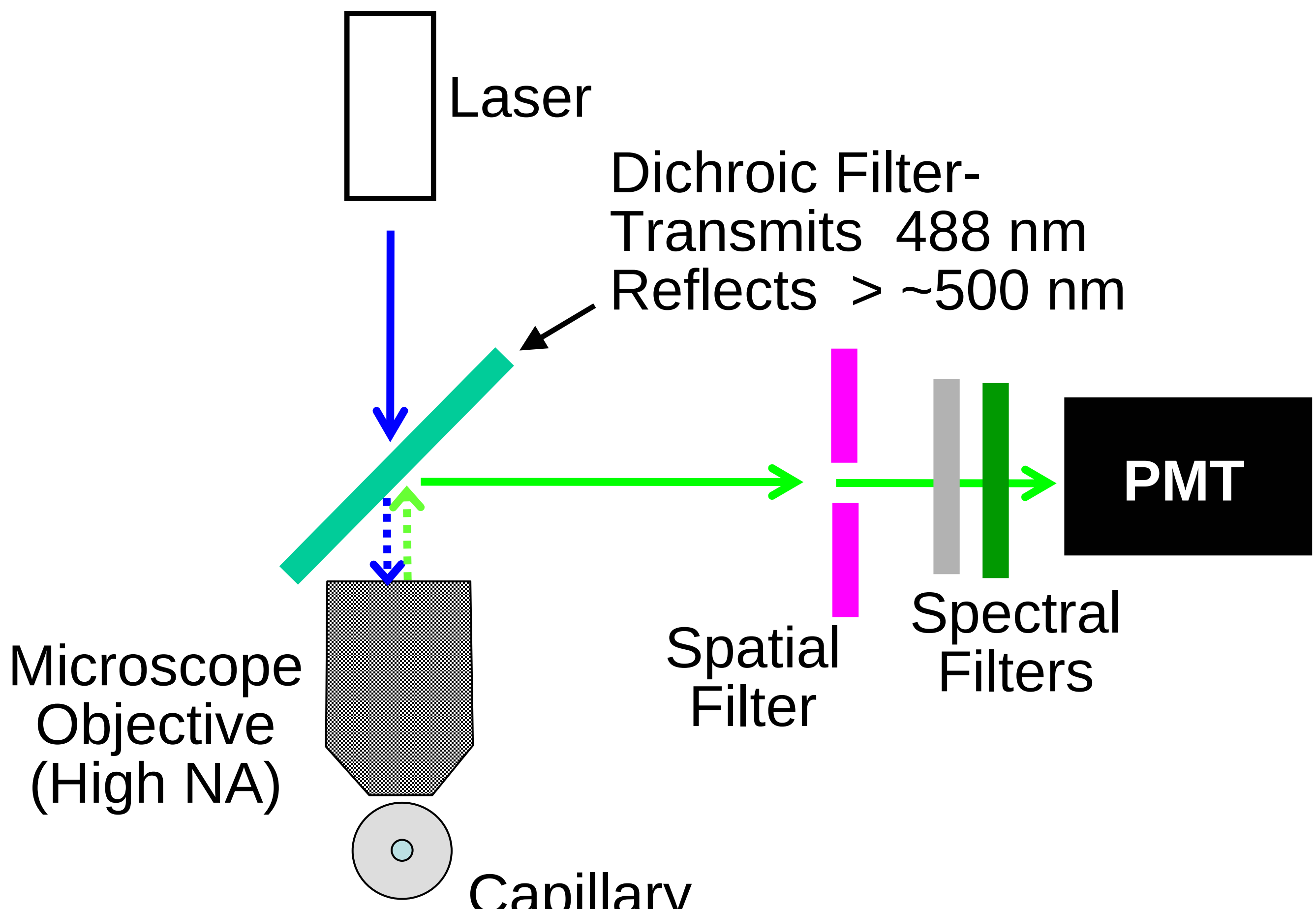


Orthogonal Optical Geometry
(Off-Column Detection)

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



Epifluorescence Optical Geometry
(On-Column Detection)

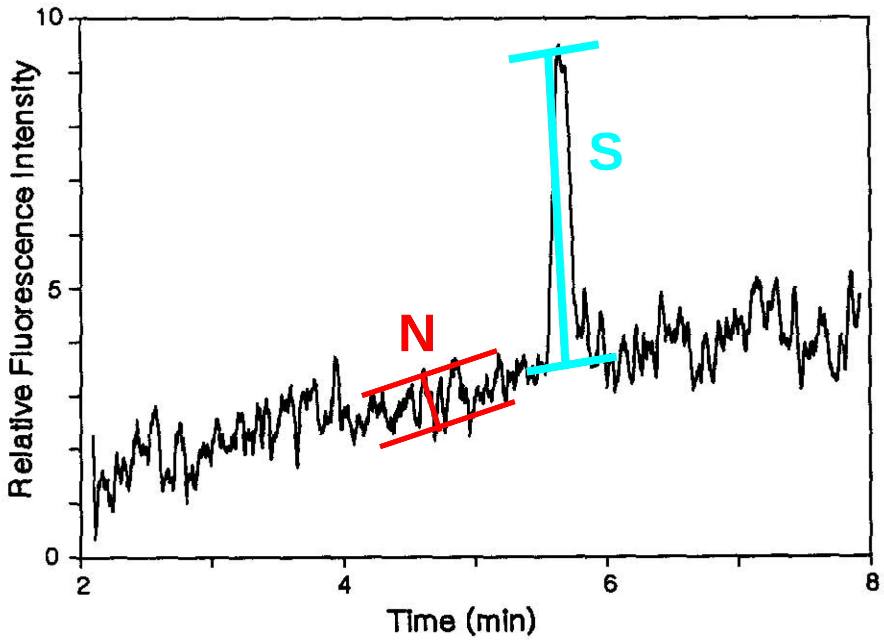


Optimizing Signal to Noise

Detection Limits:
[Analyte] with S/N~3

1. Fluorescence increases linearly with laser power.
2. Noise in background increases as (laser power)$^{1/2}$.

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



Typically with an S/N of 3,
On-column detection limits of ~10^{-20}/band.
Off-column (sheath flow) detection limits of ~10^{-21}/band.
Specialized Cases: Sheath flow- 1 molecule

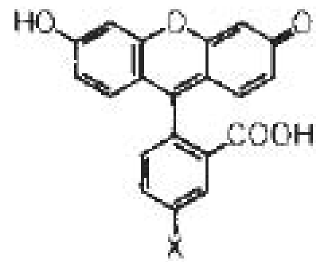
Fluorescent Dyes for DNA Sequencing

Need Four Dyes With These Attributes:

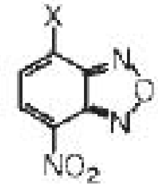
1. A common excitation λ.
2. High yet similar molar absorbances.
3. Four well-separated emission λ.
4. High yet similar quantum efficiencies.
5. Minimal and similar μ shifts when attached to DNA strands.
6. Common set of fluorescent reagents for all sequencing.

Initial Four Dyes for DNA Sequencing

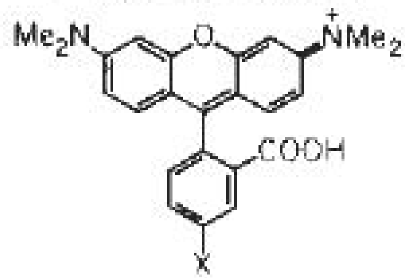
Each base-specific reaction (Sanger rxn) had a different dye-labelled primer:



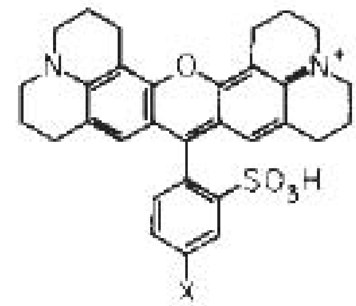
Fluorescein



NBD



Tetramethylrhodamine



Texas red

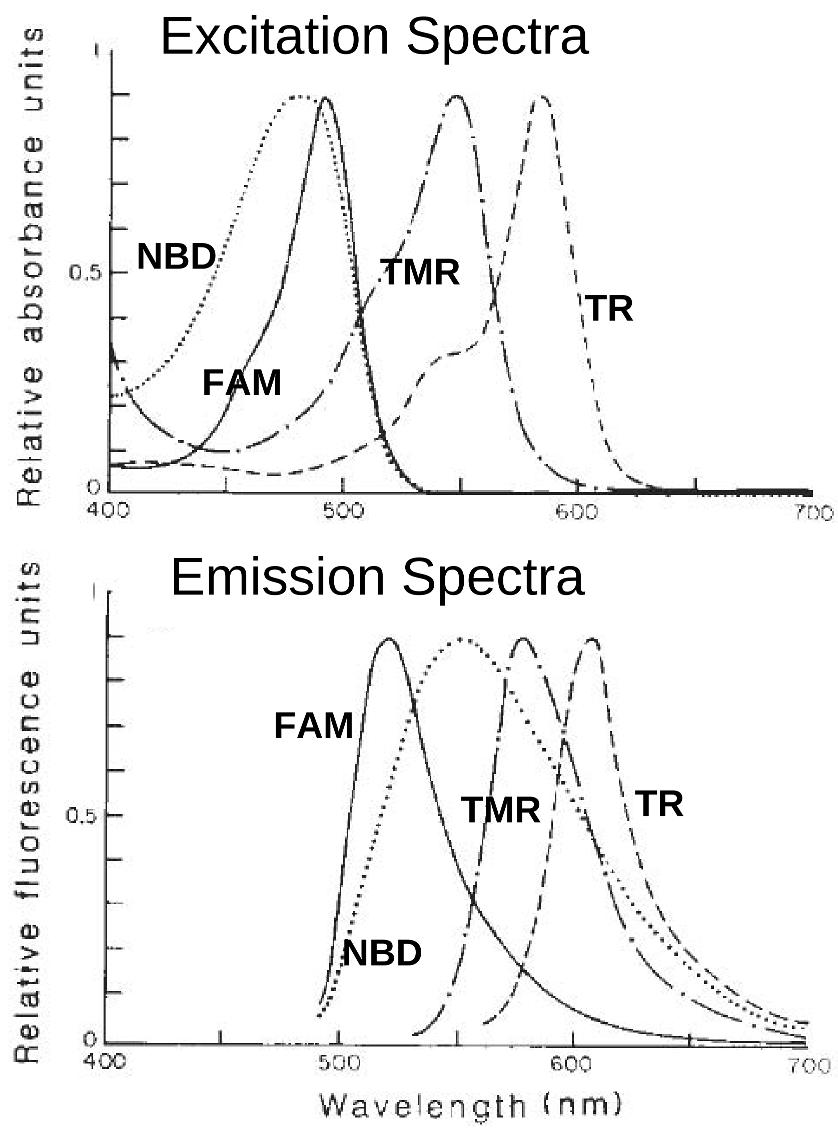
	Absorption max (nm)	Emission max (nm)
Fluorescein (FAM)	493	516
4-Chloro-7-nitrobenzo-2-1-diazole (NBD)	475	540
Tetramethyl-rhodamine (TMR)	556	582
Texas Red (TR)	599	612

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.

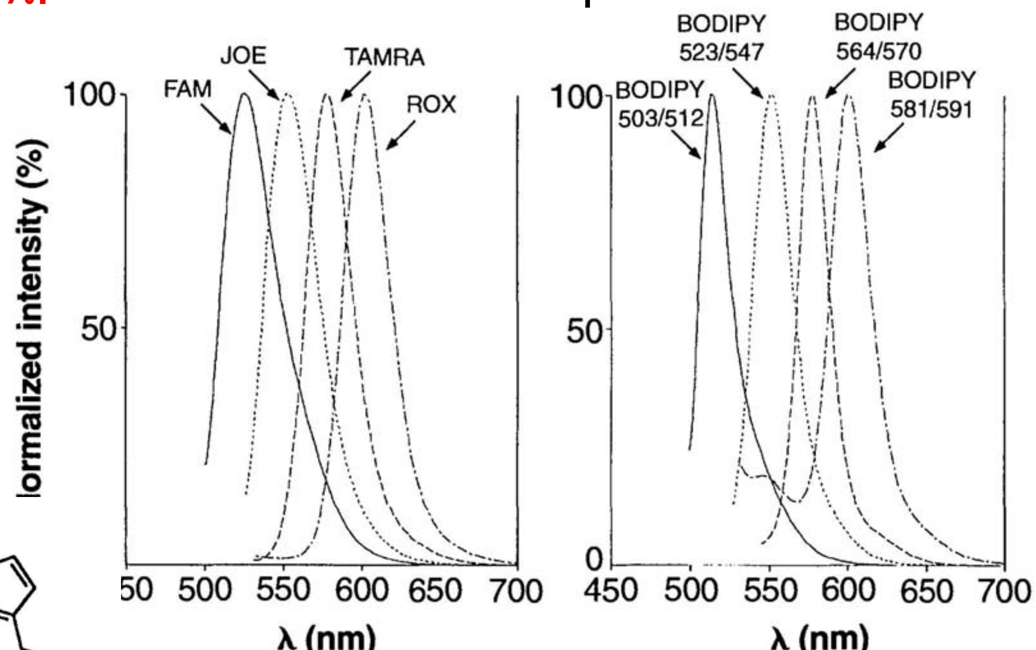
Improvements to Initial Four Dyes

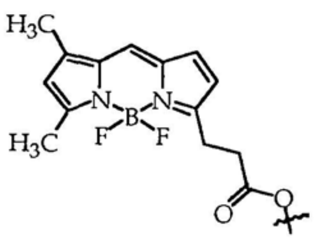
New Fluorophores:

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.

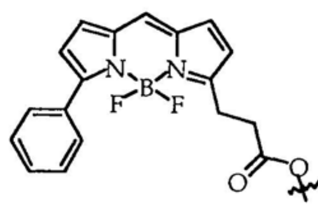
2. BODIPY dyes-
   Good spectral prop.
   Uniform shifts in μ.
   Still require 2 excitation λ.

Emission Spectra





BODIPY 503/512



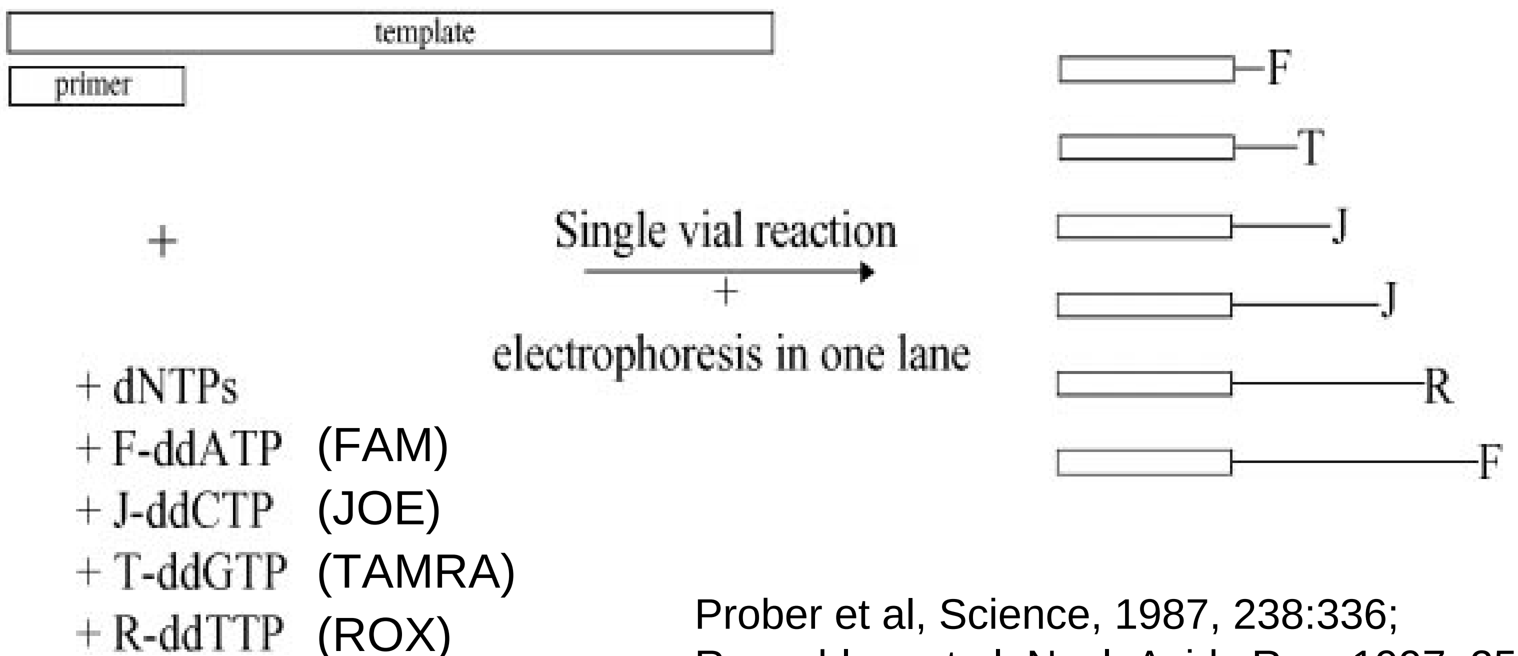
BODIPY 523/547

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.

Improvements in Dye Labelling Technology

Dye-Labelled 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 labelled terminators.



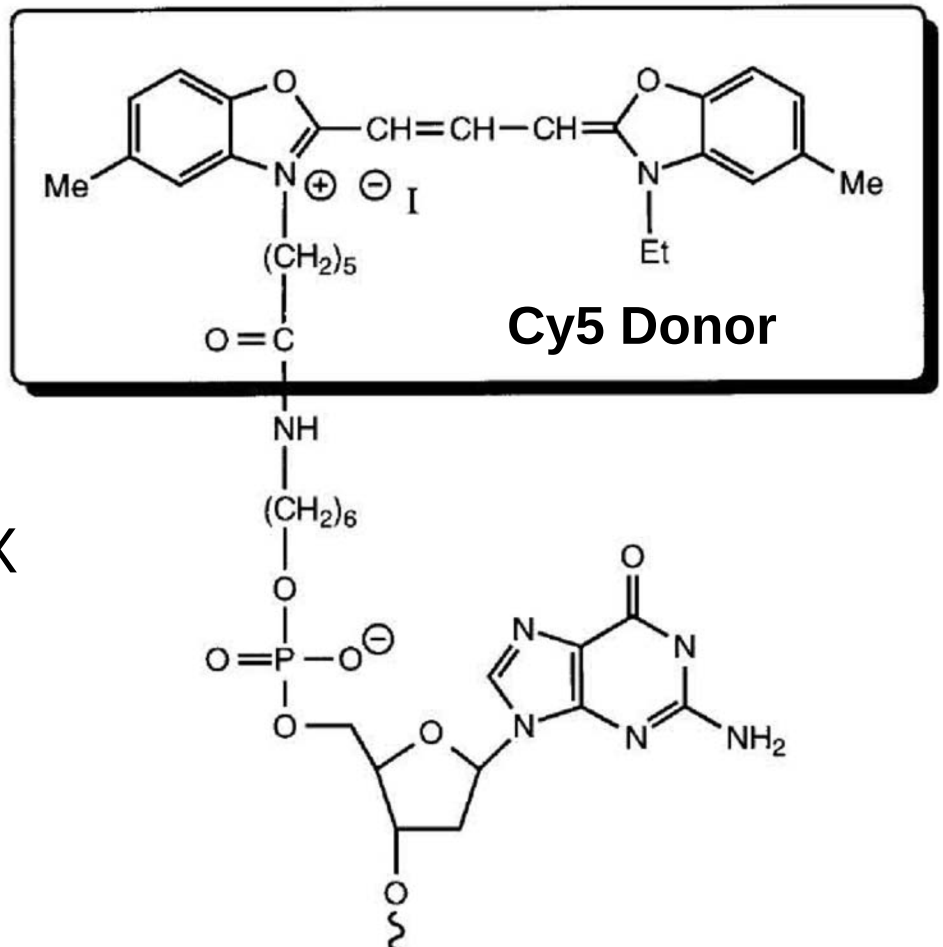
Prober et al, Science, 1987, 238:336;
Rosenblum et al, Nucl. Acids Res. 1997, 25:4500

Energy Transfer Dyes on Primers Permit Single Wavelength Excitation!

Two dyes per primer:

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

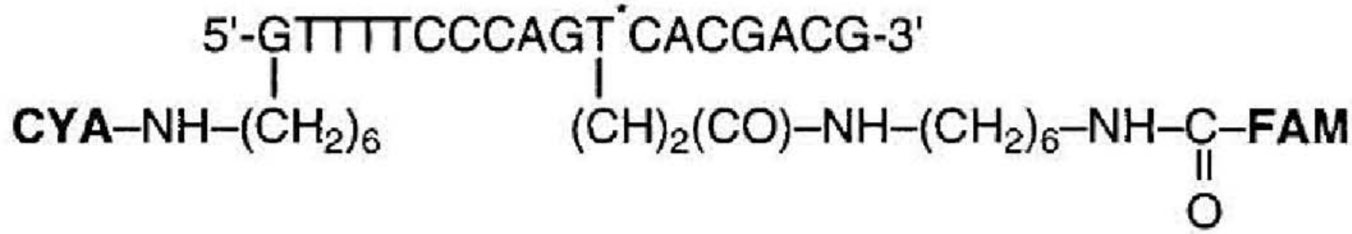
Developed by Mathies' and Glazer's labs. We'll discuss their paper using a FAM donor (PNAS 1995, 92:4347) on Thursday.



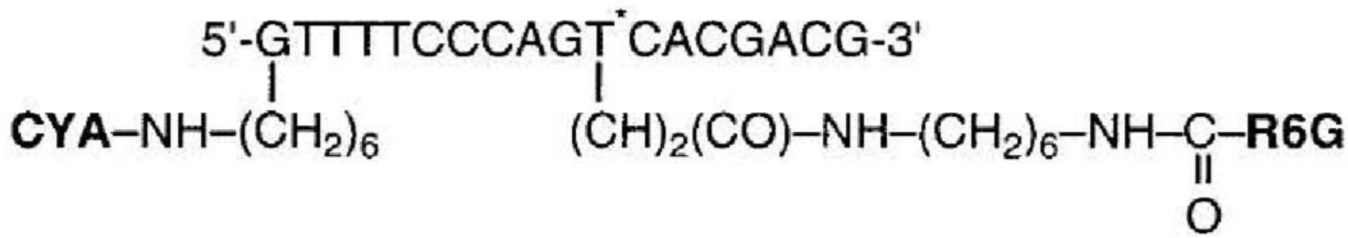
Primer Sequence with Attached Acceptor

Primers with Cy5 Donor and An Acceptor

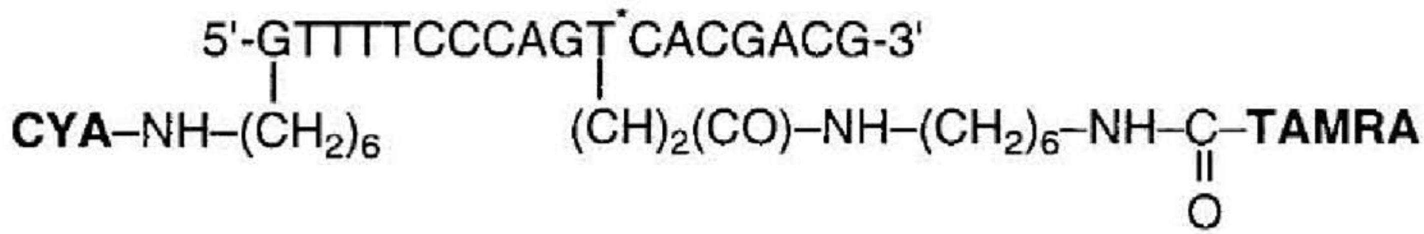
C10F



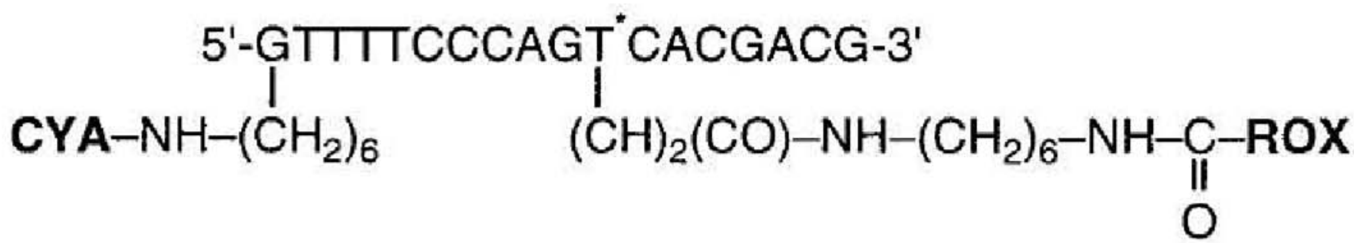
C10G



C10T

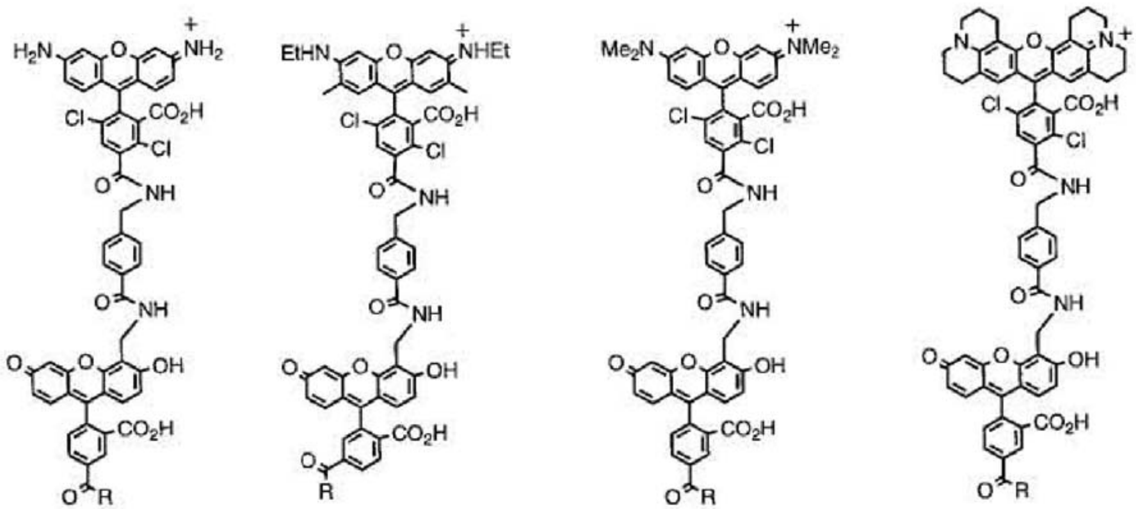


C10R



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

Energy Transfer Pairs for Terminators

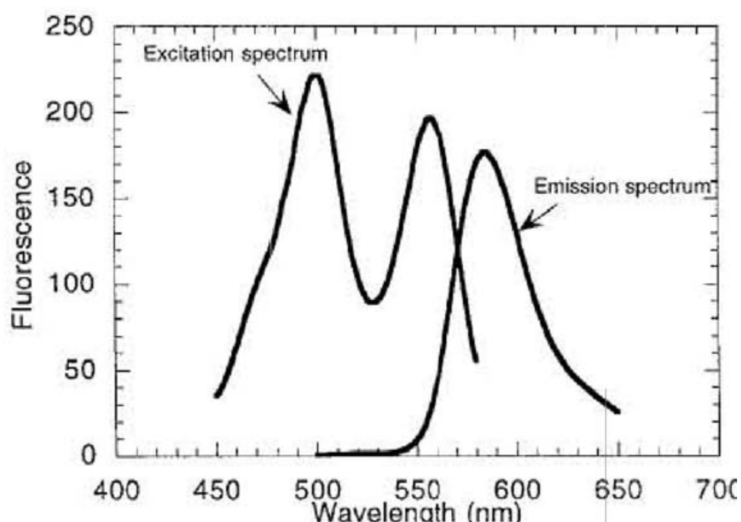


5CFB-dR110 6CFB-dR6G 5CFB-dTMR 5CFB-dROX

Rhodamine Derivatives

Linker

Carboxy-Fluorescein

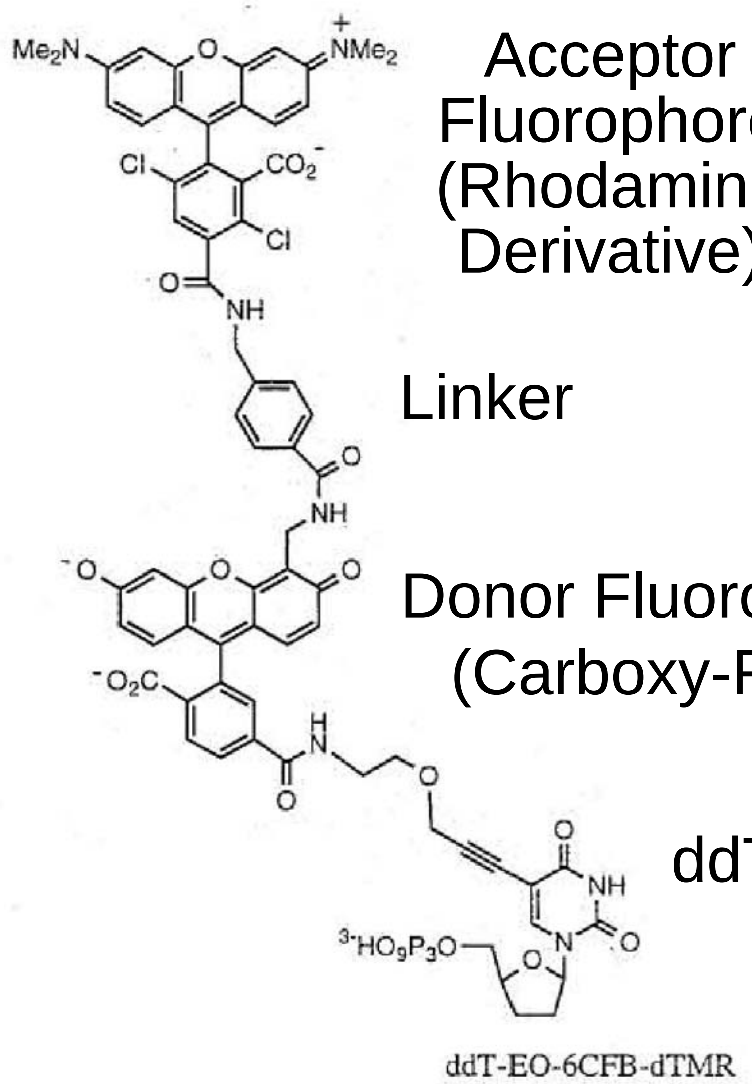


Excitation Spectra-
Peaks from FAM & Rhodamine

Emission Spectra-
Single Rhodamine Dye Peak

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

Energy Transfer Terminators- Single λ Excitation

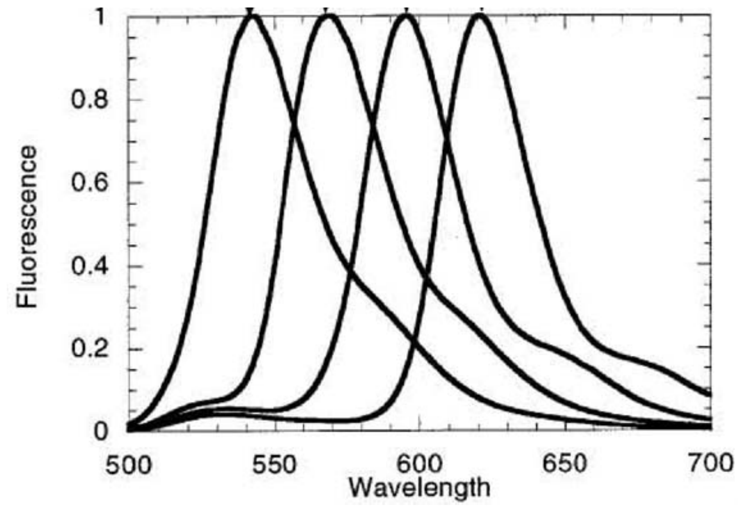


Acceptor Fluorophore (Rhodamine Derivative)

Linker

Donor Fluorophore (Carboxy-FAM)

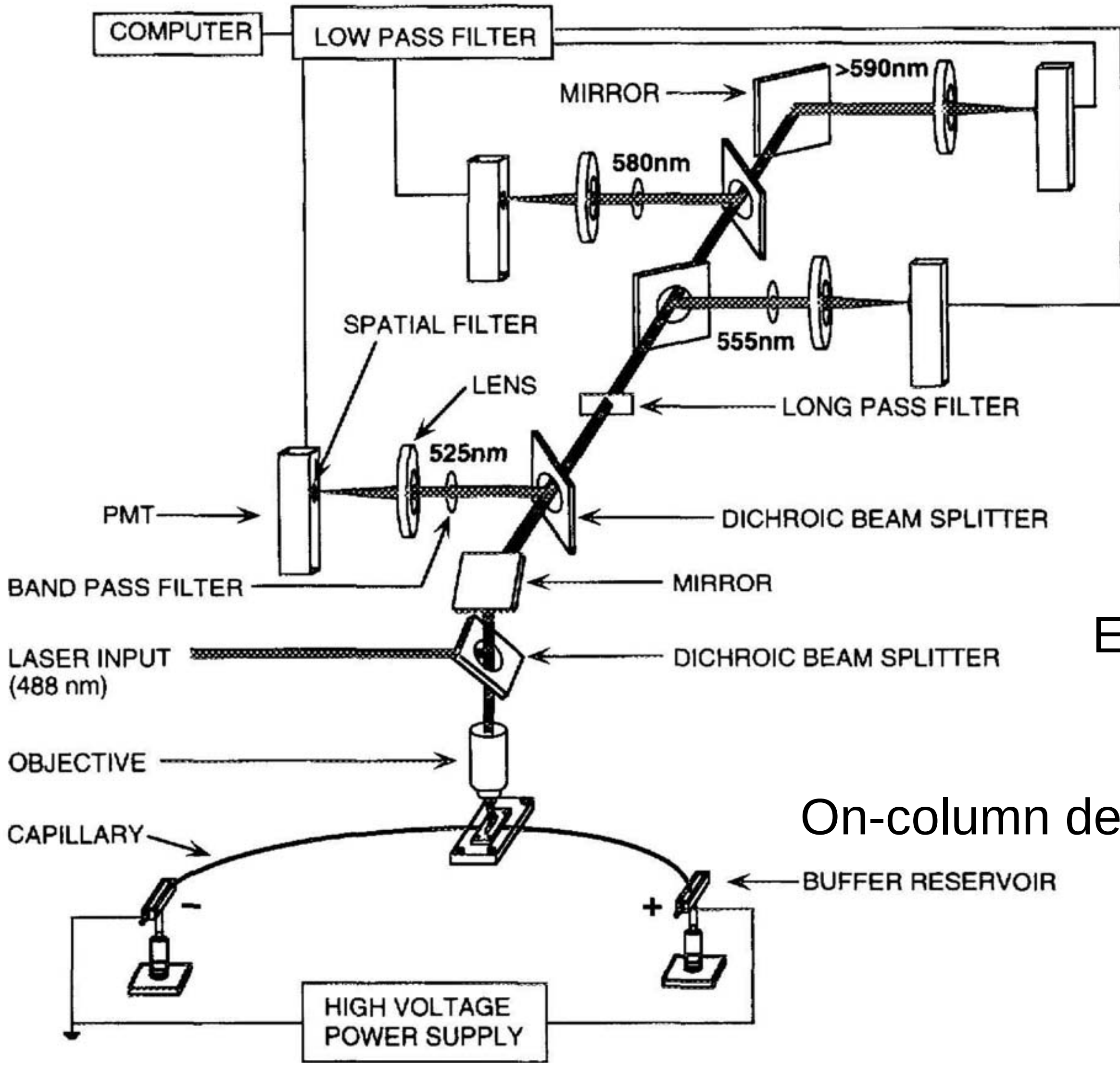
ddTTP



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

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

Detection of Multiple Emission Wavelengths



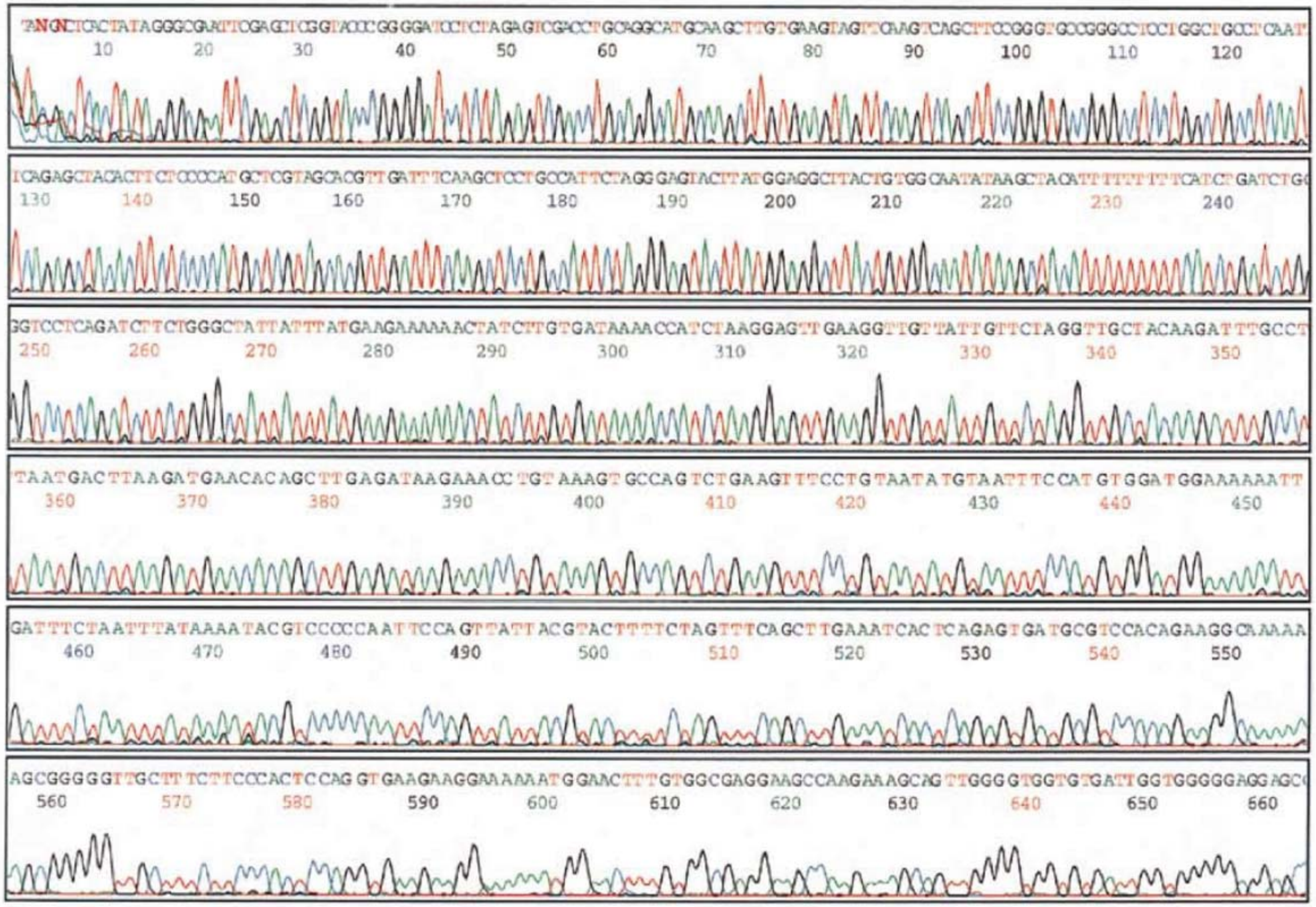
A succession of dichroic beam splitters direct different λ to the appropropriate PMT.

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.

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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.

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

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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.

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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.

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