

# OPTICS

## 1. Principles

- Source of Lights (focusing, lens)
- excitation and emission (dyes)
- Detection (filters, PMTs)

## 2. Dyes - staining

- Spectra – Excitation & Emission

## 3. Signal amplification

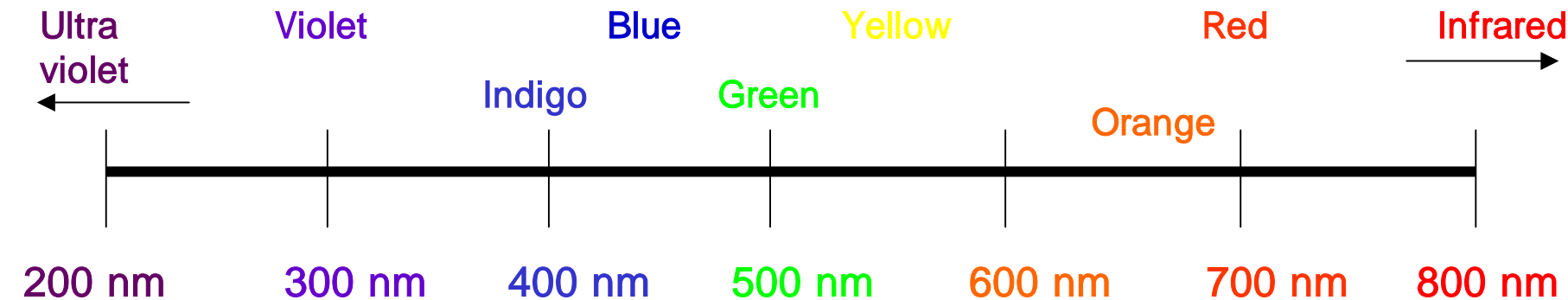
# The Characteristics of Light

- **Light** – a stream of photons with particle & wave characteristics and a spectrum of colors
- **Visible light** – a part of the spectrum of **electromagnetic radiation** [from cosmic rays, 0.001nm, to very low radio frequency waves, 1000nm] – “**polychromatic**”
- **Polychromatic vs. monochromatic (light of a single wavelength)**
- **wavelength, frequency, and intensity** – characteristics of light
  - **wavelength**: constructive or destructive interference
  - **frequency**: inversely proportional to the wavelength
  - **intensity**: energy per unit time
  - **high energy, a short wavelength, high frequency (x-rays) vs. low energy, a long wavelength, low frequency (radio waves)**

# The Visible Spectrum of Light

Short Wavelength  
High Frequency  
High Energy

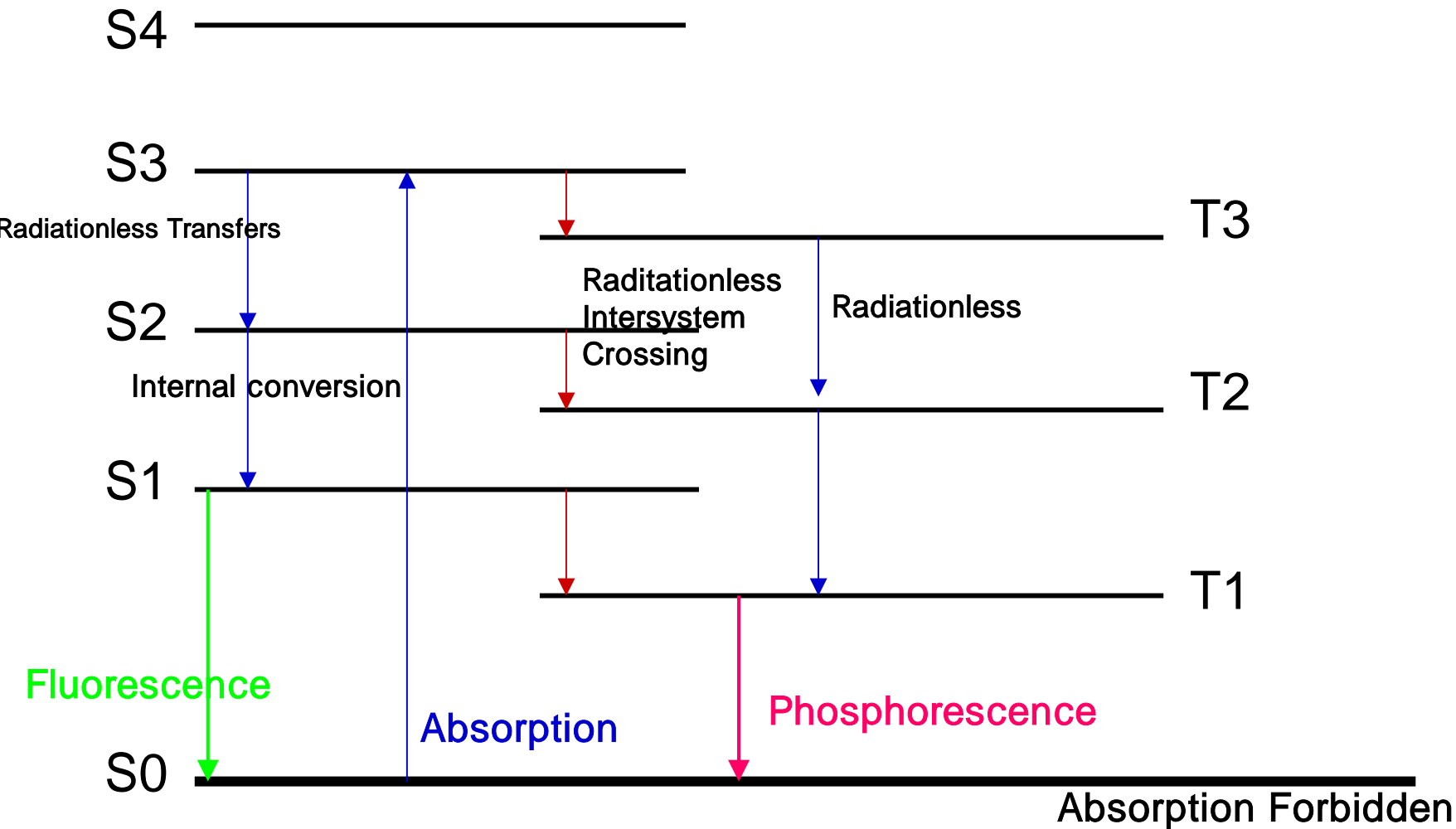
Long Wavelength  
Low Frequency  
Low Energy



# Types of Light Emission

1. **Incandescence** – polychromatic light emission due to the physical heating of an object (e.g. light bulb)
2. **Luminescence** – monochromatic or polychromatic light emission due to the excitation of electrons into higher energy states
  - electroluminescence (gas discharge lamps)
  - bioluminescence (light emitted from biological systems)
  - **chemiluminescence (light emitted from chemical system - most fluorescent molecules)**
3. **Chemiluminescence: Fluorescence vs. Phosphorescence**  
**[fluorescence]**
  - emission of light from excited atoms or molecules by the absorption of particulate or electromagnetic radiation.
  - the promotion of the electrons of the molecule into higher energy and spin states.
  - occurred when the electron relaxes back to the ground state.
  - last nano- to pico-seconds.

# Energy Level Diagram of Fluorescence and Phosphorescence



# Fluorescence

- due to emission of photons as an electron relaxes from the  $S_1$  excited state to the  $S_0$  ground state.
- fluorescence occurs exclusively from the singlet state.
- fluorescence occurs at shorter wavelengths (higher energy) than phosphorescence due to lower energy state of the lowest triplet state than the lowest singlet state.
- fluorescence has a higher energy emission

## [Phosphorescence]

- electron spin states changed from singlet to triplet due to the promotion of electrons to higher energy states.
- last milli- to nano-seconds.
- due to relaxation of the excited electron from the triplet state  $T_1$  to the ground state  $S_0$ .
- phosphorescence occurs only from the triplet state

# Fluorescence

Fluorescence is due to the **presence of free electrons in the molecule** and is often associated with **double bonds, aromatic rings, and heterocyclic rings**. When exciting radiation strikes the molecule, the electrons are excited into higher energy states. Fluorescence occurs when the excited **electrons relax from the S1 excited state to the S0 ground state**. The life time of fluorescence emission is from  $10^{-7}$  to  $10^{-10}$  seconds.

# Light Absorption and Emission by Fluorescent Dyes

1. Absorption spectra: what wavelength the dyes absorb
2. Excitation spectra: what wavelength excite electrons into higher orbitals.
3. Emission spectra: what wavelength are emitted when the electron return to the ground state.
  - The absorption and excitation spectra are very similar or identical in most cases.
  - **The fluorescence emission wavelength for the conventional fluorescence used in microarray always longer than the wavelength of the excitation light.**
  - Fluorescence emission: orders of magnitude weaker than the excitation light
  - Fluorescence: linear – the power of emission light directly proportional to the power of the excitation light.
  - **“Stokes Shift”**: **The wavelength displacement of the emission spectrum with respect to the excitation spectrum.**

# Fluorescent Related Issues

- 1. Fluorescence Quantum Yield:** the amount of fluorescence a molecule emits in response to radiation absorption. (return of excited electrons to the ground state through the emission of fluorescence?)
- 2. Fluorescence Quenching:** mechanisms that reduce fluorescence quantum yield – **quenching mechanism, 4 different mechanisms exist.**
  - 1) Self quenching:** through interactions with other **unexcited fluorescent molecules** (through collision?)
  - 2) Energy transfer:** interaction between the singlet state of **the fluorescent molecules** and **the quenching molecules** (through collision)
    - **Charge transfer:** an electron is transferred between **the fluorescent molecule** and **the quenching molecule.**
    - **Intersystem crossing:** an electron of **the fluorescent molecule** enters the triplet state due to interaction with **quenching molecule.**

# Fluorescent Related Issues

- 3. Autofluorescence:** innate fluorescence of biological molecules (cells, DNA, RNA, Proteins, etc ..)  
- **it's an important source of interference specifically with weak fluorescence obtained from the real samples.**

**Ex) 1) flavin and pyridine nucleotides – fluoresce in the blue-excited green and UV excited blue regions.**

**2) Blue fluorescent lipofuschins and protoporphyrin**

**3) Amino acids tyrosine and tryptophan fluoresce @ 280 nm and phenylalanine fluoresces @ 260nm.**

- 4. Background Fluorescence:** generated from the extraneous source; specific chemicals, materials non-specifically bound to fluorescence materials – **most likely eliminated from the experimental procedure.**

# Fluorescent Dyes

## 1. Dyes excited near 488 nm wave length (Blue Argon Laser)

<u>Fluorescent Dye</u>	<u>Excitation, nm</u>	<u>Emission, nm</u>
5-FAM	494	518
Alexa™ 488	494	520
Calcein	494	517
Calcium Green™	506	533
Cy2™	489	506
FAM	488	508
FITC (5-FAM derivative)	494	518
FluorX™	494	519
GFP	488	558
GFP red shift (rsGFP)	488	507
Magnesium Green™	506	531
Oregon Green™ 488	496	524
Oregon Green™ 500	503	522
Rhodamine 110	496	520
Rhodamine 123	507	529
Rhodamine Green™	502	527
RiboGreen™	500	525
SYBR Green	497	520
Sypro Ruby	450	610
Yo-Pro™ - 1	491	509
YOYO™ - 1	491	509
Acridine orange	Blue	green

# Fluorescent Dyes

## 2. Dyes excited near 514 nm wave length (Blue - Green Argon laser)

<u>Fluorescent Dye</u>	<u>Excitation, nm</u>	<u>Emission, nm</u>
Alexa™ 532	531	554
BODIPY® 530/550	530	550
JOE	522	555
JOE - 514	514	549
TO-PRO™ - 1	514	533
TOTO® - 1	514	533
EtBr	518	605
Propidium Iodide	~515	~600

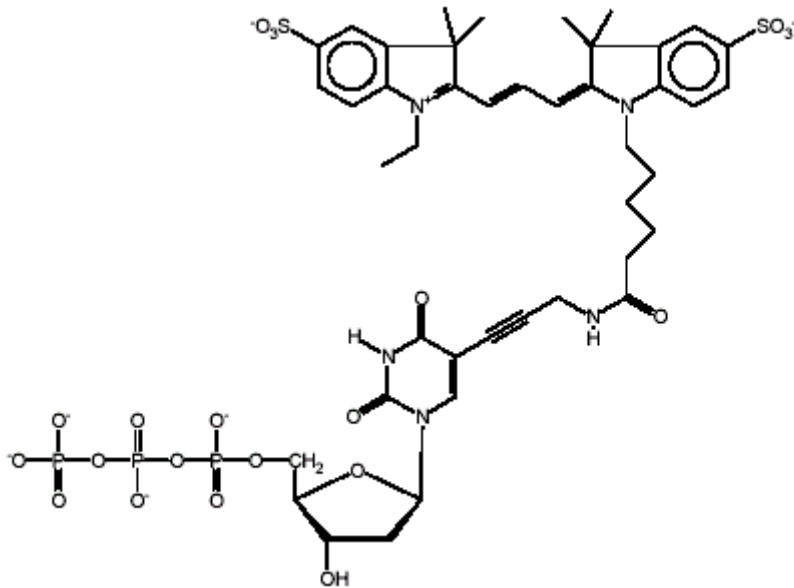
# Fluorescent Dyes

## 3. Dyes excited near 543.5 nm wave length (He-Ne laser)

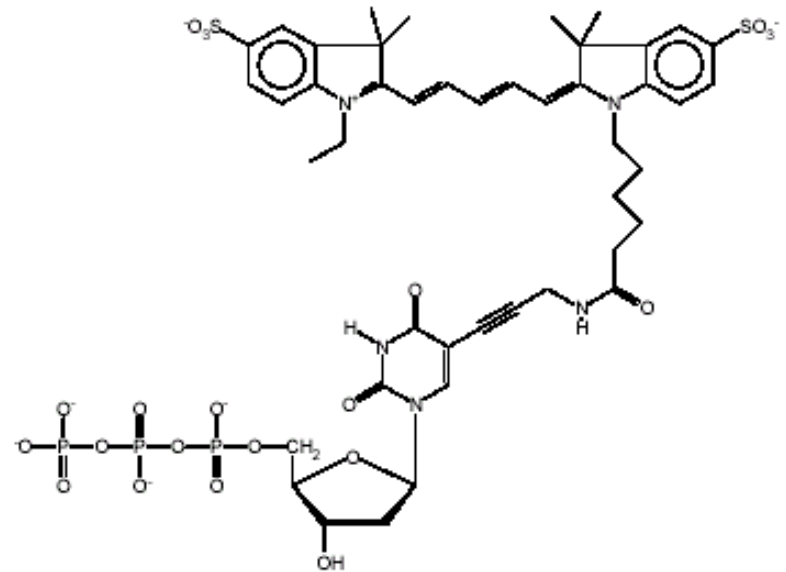
<u>Fluorescent Dye</u>	<u>Excitation, nm</u>	<u>Emission, nm</u>
Alexa™ 546	565	570
Alexa™ 555	555	565
Alexa™ 568	579	604
BODIPY® 558/568	558	570
BODIPY® 564/570	564	568
BODIPY® TMR	542	574
Calcium Orange™	549	576
Cy3.5™	561(?)	596
Cy3™	550	570
Dil	549	565
dsRed	558	583
Magnesium Orange™	550	575
Nile Red	549	559
PBXL - 1	545	666
Phycoerythrin, R&B	565	575
Pyronin Y	555	580
R-Phycoerythrin (R-PE)	565	575
Rhodamine B	555	580
Rhodamine Phalloidin	542	565
Rhodamine Red™	570	590
TAMRA	555	580
TRITC	566	580

# Cy3 & Cy5-dUTP

Cyanine 3



Cyanine 5



# Fluorescent Dyes

## 4. Dyes excited near 594 nm wave length (Yellow He-Ne Laser)

<u>Fluorescent Dye</u>	<u>Excitation, nm</u>	<u>Emission, nm</u>
Alexa™ 594	590	615
Calcium Crimson™	590	615
PBXL-3	614	662
ROX	580	605
Texas Red®	595	615

# Fluorescent Dyes

## 5. Dyes excited near 612 nm wave length (Orange HeNe laser)

<u>Fluorescent Dye</u>	<u>Excitation, nm</u>	<u>Emission, nm</u>
C-Phycocyanin	620	648
R-Phycocyanin	618	642
Yo-Pro™ - 3	612	631
YOYO™ - 3	612	631

# Fluorescent Dyes

## 6. Dyes excited near 632.8 nm wave length (Red HeNe laser)

<u>Fluorescent Dye</u>	<u>Excitation, nm</u>	<u>Emission, nm</u>
Alexa™ 647	649	666
Alexa™ 660	663	690
Allophycocyanin (APC)	650	660
BODIPY® 630 - 650	630	650
Cy5.5™	675	694
Cy5™	649	670
Did Dilc(5)	644	665
Red Reflect	644	633
Thiadicarbocyanine	651	671
TO-PRO™ - 3	642	660
TOTO® - 3	642	660

# Fundamentals in Fluorescence Technology

## 1. Source of light for excitation

- control (monochrome) & focusing (alignment of lenses)

## 2. Discrimination Part for various fluorescence

- excitation, emission, autofluorescence
- filters and alignment

## 3. Detection System for a selected fluorescence

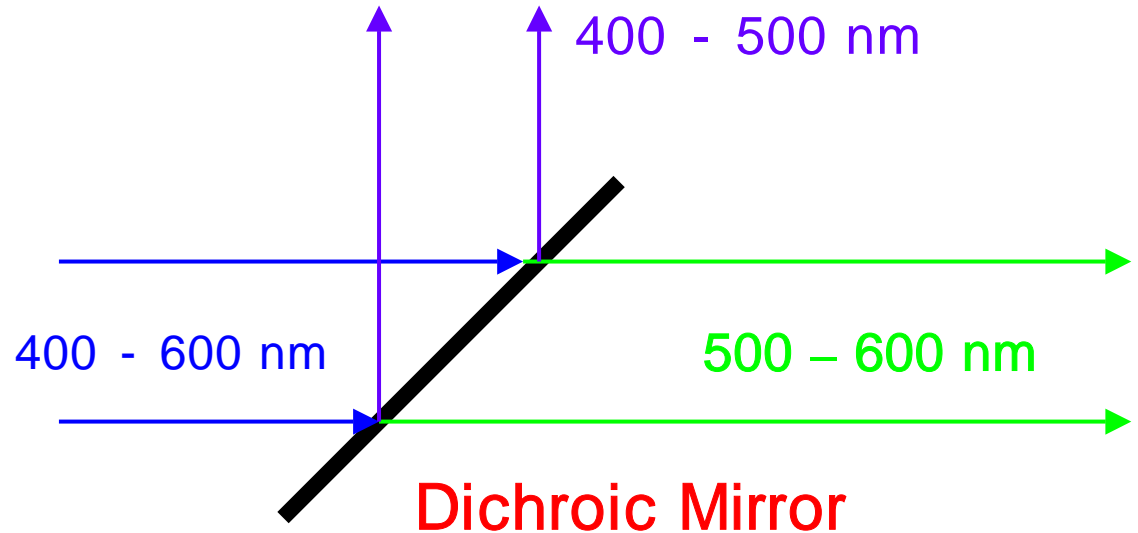
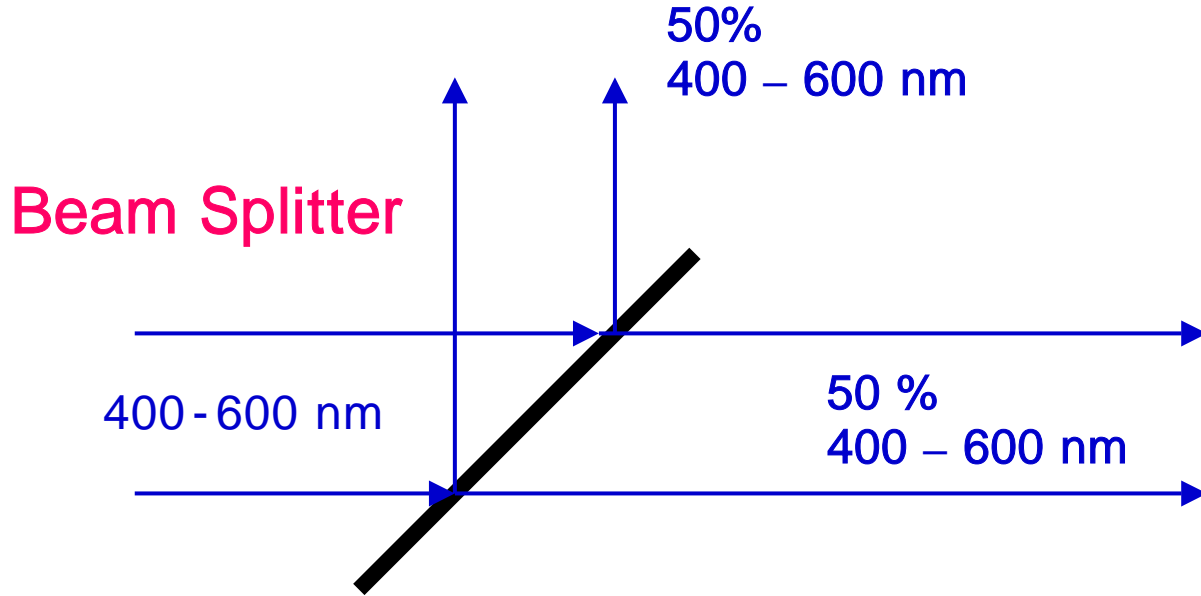
- PMTs for amplification
- signal conversion: electrons – current – volt

- **FISH, Confocal Microscopy, Flow Cytometry**
- **Fluorometer, DNA chip scanner, Real-time PCR**
- **Luminometers – no excitation required**

# Fluorescence Detection

- Fluorescence detected in the orthogonal plane. (figure 5.4)
- Crossed cylindrical lenses to accurately focus the light radiating from the interrogation point into the detector.
- multiple PMTs to allow multiple simultaneous fluorescence measurements on a single sample.
- three aspherical lenses and a pinhole to eliminate extraneous light – It has to be make sure that only light originally emitted or scattered from the interrogation point enters the PMT for analysis. → extraneous light discrimination!
- Objective Lens: updated?
- Filters and dichroic mirrors between the interrogation point and the PMTs: to pass only selected single wavelength of interest into a single PMT. → wavelength discrimination!

# Beam Splitter and Dichroic Mirror



# Photodetection

**“Light scatter and fluorescence are emitted in all directions and are detected with electro-optical sensors”**

## **Photodiodes and Photomultiplier tubes (PMT):**

- **The photoelectric effect: “Einstein’s explanation- Nobel Prize**  
**“When photons strike the charged surface of a cathode in a vacuum, they deliver a discrete amount of energy to the surface of the cathode. If this energy (frequency) is high enough to overcome the force that binds the electrons to the atoms of the cathode, one electron will be emitted for each photon that strikes the cathode.”**
- 1) **The photoelectric effect = f(wavelength)**
- 2) **Both photodiode and PMT use the effect to generate the electric signals.**
  - photodiode – direct use**
  - PMTs – use to generate electrons amplified by dynodes.**

# The Photodiode

- **Function: similar to semiconducting silicon layers**

1) no need the input of external power: use of photoelectric effect to generate an electric potential

2) input to the photodiode: light

output of the photodiode: electricity which must be amplified.

## The Photomultiplier Tube (PMT)

- **Used when very dim light must be sensed.**

1) requires external power to make low level light measurements.

2) **Photocathode: a negatively charged and emits electrons in response to light.**

**A series of dynodes: release more than one electron for each electron that strikes them.**

**An anode: accepts electrons from the dynodes and provide the output current signal through an electrode.**

3) **three types PMTs:**

**bialkali: a peak output of 40 mA/W @ 400 nm**

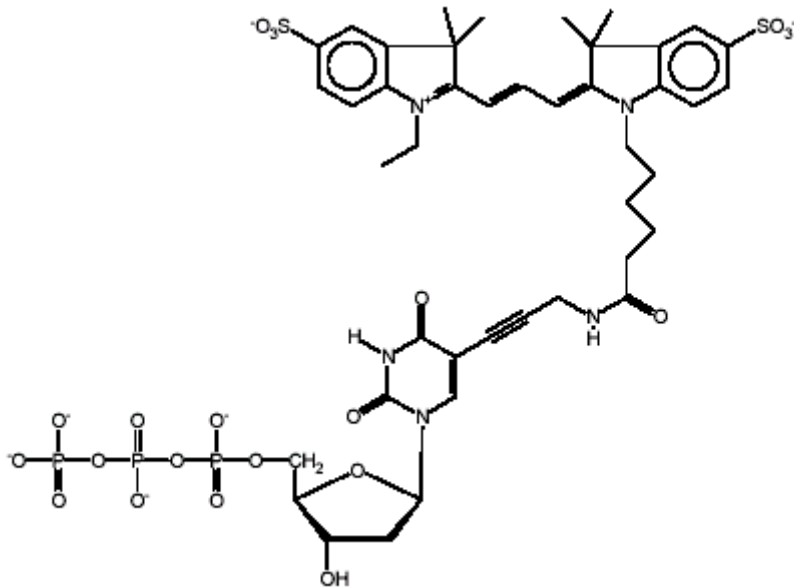
**gallium arsenide: an output of 50 mA/W from 300-850 nm.**

**multialkali: @ 750 nm**

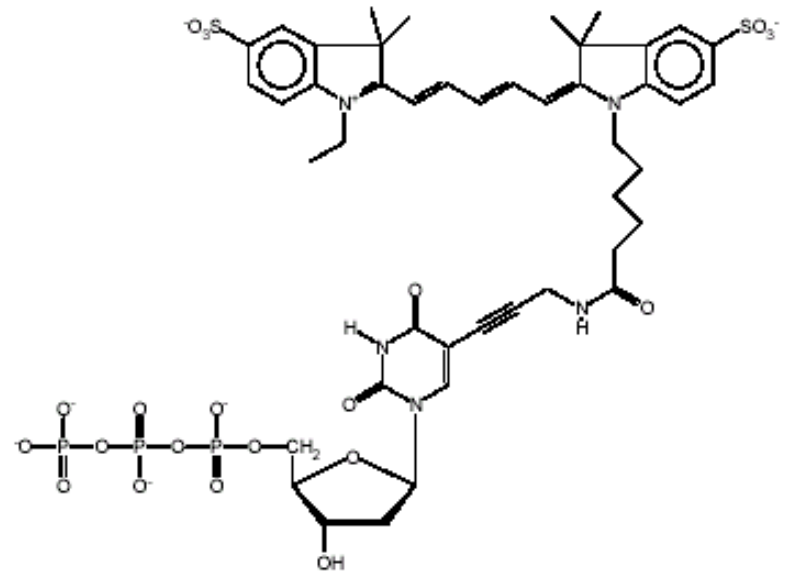
# **DNA Microarray Optics**

# Cy3 & Cy5-dUTP

## Cyanine 3



## Cyanine 5



# The Excitation Light (on **Microarray**)

## . **Flood illumination:**

- a large area of the sample is excited at one time.
- used with CCD camera type instruments and confocal scanner.

## . **Focused spot illumination:**

- tied in with light gathering and spatial addressing.
- very intense excitation for a very short time.

## . **Excitation wavelength:**

- based on the intended dyes
- placed on the left side of the dye's excitation peak.
- excessive excitation light – causing damage through **photobleaching** or **pollute** the fluorescence emission signal.

# The Confocal Scanning Arrangement in a microarray scanner

