FLOURESENCE

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OUTLINE

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- QUANTUM YIELD OF FLUORESCENCE
- FLUORESCENCE LIFETIME
- FLUORESCENCE IN NATURE
- APPLICATIONS OF FLUORESCENCE
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FLUORESENCE

- Fluorescence is the emission of light by a substance that has absorbed light or other electromagnetic radiation.
- The emitted light has a longer wavelength - therefore lower energy than the absorbed radiation.
- The most striking example of fluorescence occurs when:
  - The absorbed radiation is in ultraviolet region of spectrum - while the emitted light is in the visible region - which gives the fluorescent substance a distinct color.
  - Fluorescent materials cease to glow immediately when the radiation source stops.

Fluorophores: are molecules that absorb light or EM radiation and emit light
Different fluorophores absorb different wavelengths of of light –
Each fluorophore has specific excitation (absorption)spectrum
It also has a specific emission spectrum.

FLUORESENCE

Fluorescence occurs when an orbital electron of a molecule or atom - relaxes to its ground state by emitting a photon from an excited singlet state.

$$S_0 + h\nu_{ex} \rightarrow S_1$$ ← Excitation

$$S_1 \rightarrow S_0 + h\nu_{em} + heat$$ ← Fluorescence (emission)

With h - Plank's constant and ν - frequency of light

S_0 is the ground state of flourecent molecule

S_1 is the first excited state

The specific frequencies of excitation and emitted light are dependent on the particular system.

FLUORESENCE

- A molecule in S_1 can relax by various competing pathways:

  Non-Radiative : In this relaxation the excitation energy is dissipated as heat or vibrations to the solvent

  Phosphorescence: Excited organic molecules can also relax via conversion to a triplet state - which may subsequently relax via **phosphorescence** - or by a secondary non-radiative relaxation step.

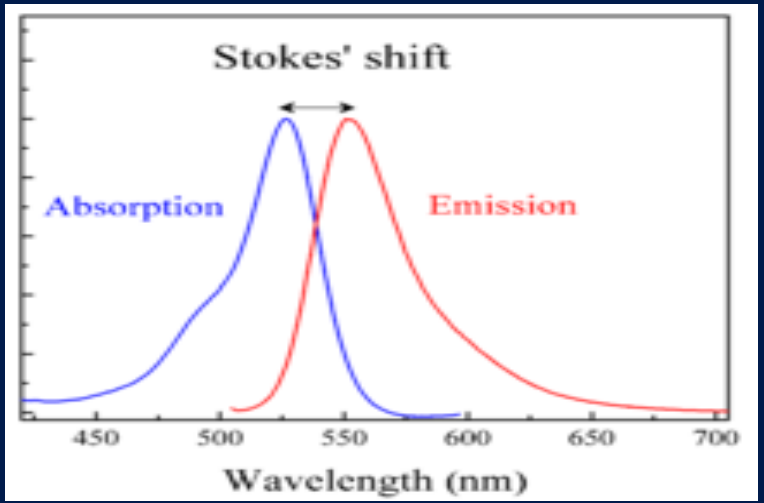
  Fluorescence quenching: Relaxation from S_1 can also occur through interaction with a second molecule through **fluorescence quenching**-

  Quenching refers to any process which decreases the **fluorescence** intensity of a given substance

A triplet is a quantum state of a system with a spin of 1.

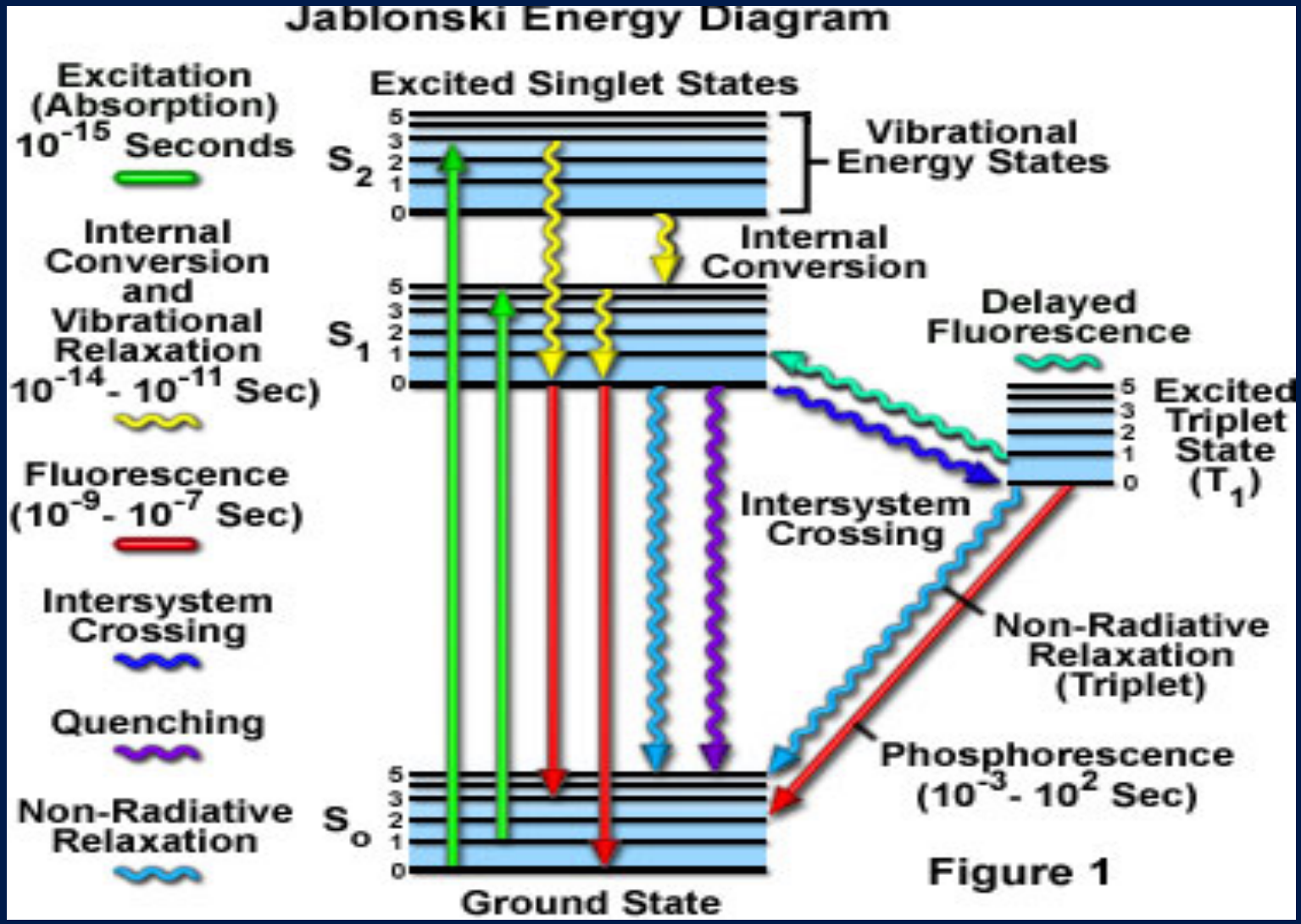
FLUORESCENCE

- **Stokes shift:** is the difference between positions of the band maxima of the absorption and emission spectra of the same electronic transition.



- **RESONANCE FLUORESCENCE*:*** If the emitted radiation have the same wavelength as the absorbed radiation.

FLUORESCENCE



QUANTUM YEILD OF FLUORESCENCE

- Quantum yield gives the efficiency of the fluorescence process.
- It is defined as the ratio of the number of photons emitted to the number of photons absorbed.

$$\Phi = \frac{\text{Number of photons emitted}}{\text{Number of photons absorbed}}$$

- The maximum fluorescence quantum yield is 1.0 (100%) - each photon absorbed results in a photon emitted.
- Compounds with quantum yields of 0.10 are still considered quite fluorescent.

QUANTUM YEILD OF FLUORESCENCE

- There many processes that effects the quantum yield
  - Dynamic collisional quenching
  - Resonance energy transfer: a mechanism describing energy transfer between two light-sensitive molecules
  - Internal conversion- is non-radiative and occurs between rotational and vibrational levels of molecule
  - Intersystem crossing- between singlet to triplet states and vice versa

FLUORESCENCE LIFETIME

- The fluorescence lifetime refers to the average time the molecule stays in its excited state before emitting a photon
- Fluorescence typically follows first-order kinetics:

$$[S1] = [S1]_0 e^{-\Gamma t}$$

[S1] is the concentration of excited state molecules at time t

$[S1]_0$ is the initial concentration

Γ is the decay rate or the inverse of the fluorescence lifetime

FLUORESCENCE LIFETIME

- Various radiative and non-radiative processes can de-populate the excited state.
- In such case the total decay rate is the sum over all rates:

$$\Gamma_{tot} = \Gamma_{rad} + \Gamma_{nrad}$$

Γ_{tot} is the total decay rate

Γ_{rad} the radiative decay rate

Γ_{nrad} the non-radiative decay rate

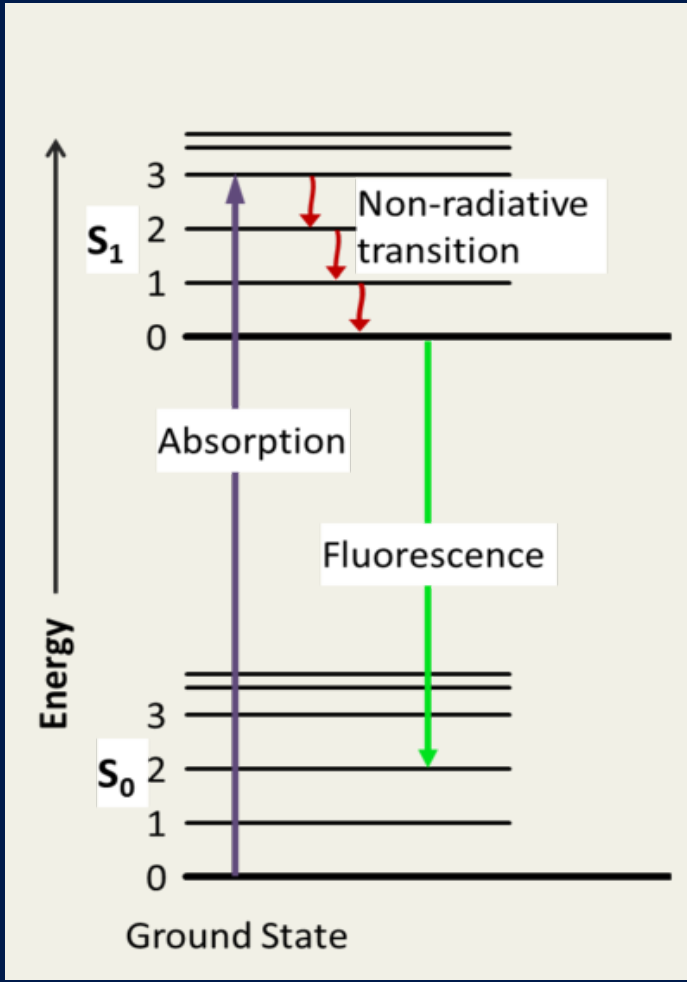
If the rate of spontaneous emission - or any of the other rates are fast - the lifetime is short.

FLUORESCENCE LIFETIME

- For commonly used fluorescent compounds - typical excited state decay times for photon emissions with energies from the UV to near infrared are within the range of 0.5 to 20 nanoseconds
- The fluorescence lifetime is an important parameter for practical applications of fluorescence such as **fluorescence resonance energy transfer** and **Fluorescence-lifetime imaging microscopy**.

FLOURESENCE

- After an electron absorbs a high energy photon the system is excited electronically and vibrationally.
- The system relaxes vibrationally, and eventually fluoresces at a longer wavelength.



RULES

- There are several general rules that deal with fluorescence

Kasha–Vavilov rule:

- The Kasha–Vavilov rule dictates that the quantum yield of fluorescence is independent of the wavelength of exciting radiation.
- The Kasha–Vavilov rule does not always apply and is violated in many simple molecules
- The fluorescence spectrum shows very little dependence on the wavelength of exciting radiation.

Mirror image rule:

- For many fluorophores the absorption spectrum is a mirror image of the emission spectrum

FLUORESCENCE IN NATURE

- There are many natural compounds that exhibit fluorescence - and they have a number of applications
  - Biofluorescence
  - Aquatic biofluorescence
  - Abiotic fluorescence
- **Biofluorescence** - is the absorption of electromagnetic wavelengths from the visible light spectrum by fluorescent proteins in a living organism - and the re-emission of that light at a lower energy level.
- This causes the light that is re-emitted to be a different color than the light that is absorbed

Biofluorescence

- Stimulating light excites an electron, raising energy to an unstable level.
- This instability is unfavorable - so the energized electron is returned to a stable state almost as immediately as it becomes unstable
- This return to stability corresponds with the release of excess energy in the form of fluorescent light.
- This emission of light is only observable when the stimulant light is still providing light to the organism/object.

Biofluorescence

- **Chromatophores:** are pigment-containing and light-reflecting cells- or groups of cells

- Fluorescent chromatophore*:* Pigment cells that exhibit fluorescence and function somatically are similar to regular chromatophores

- **Fluorescent chromatophores:** can be found in the skin -e.g. in fish - just below the epidermis - amongst other chromatophores.

AQUATIC BIOFLOURESCENCE

- Water absorbs light of long wavelengths – so less light from these wavelengths reflects back to reach the eye
- Therefore- warm colors from the visual light spectrum appear less vibrant at increasing depths.
- Water scatters light of shorter wavelengths- meaning cooler colors dominate the visual field in the photic zone.
- Light intensity decreases 10 fold with every 75 m of depth _ so at depths of 75 m- light is 10% as intense as it is on the surface - and is only 1% as intense at 150 m as it is on the surface.

The photic zone is the depth of water where almost all of the photosynthesis occurs and about 90% of all marine life lives in this zone

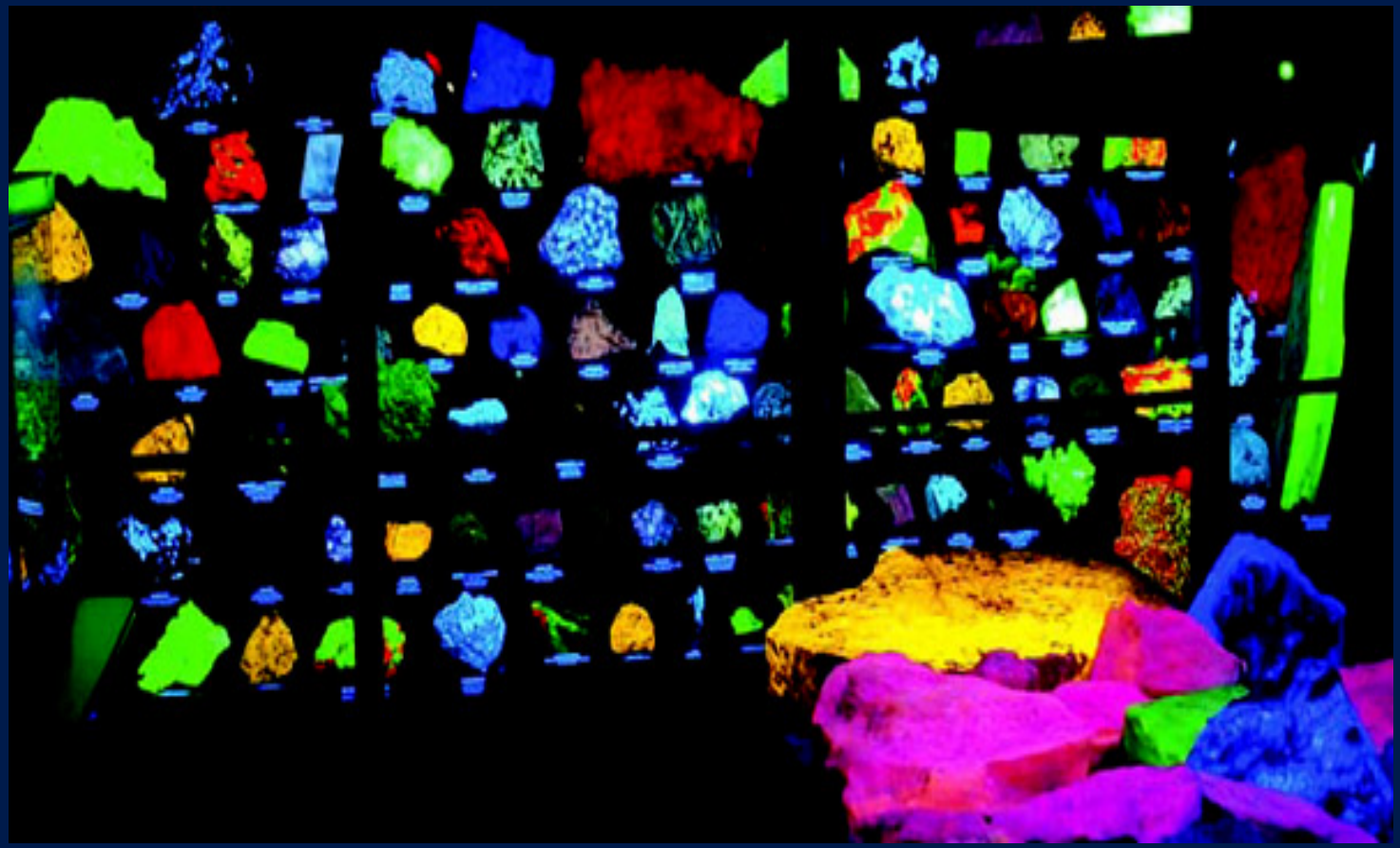
AQUATIC BIOFLOURESCENCE

- As any type of fluorescence depends on the presence of external sources of light -biologically functional fluorescence is found in the photic zone - where there is not only enough light to cause biofluorescence - but enough light for other organisms to detect it.

- The visual field in the photic zone is naturally blue, so colors of fluorescence can be detected as bright reds, oranges, yellows, and greens.

ABIOTIC FLUORESCENCE

- Gemstones, minerals, may have a distinctive fluorescence or may fluoresce differently under short-wave ultraviolet, long-wave ultraviolet, visible light, or X-rays.
- Crude oil (petroleum) fluoresces in a range of colors, from dull-brown for heavy oils and tars through to bright-yellowish and bluish-white for very light oils and condensates.
- Organic solutions such anthracene or stilbene, dissolved in benzene or toluene, fluoresce with ultraviolet or gamma ray irradiation.
- Fluorescence is observed in the atmosphere when the air is under energetic electron bombardment.



fluorescent minerals

ABIOTIC FLUORESCENCE

- In cases such as the natural aurora, high-altitude nuclear explosions, and rocket-borne electron gun experiments, the molecules and ions formed have a fluorescent response to light.
- Vitamin B2 fluoresces yellow
- Tonic water fluoresces blue due to the presence of quinine.
- Highlighter ink is often fluorescent due to the presence of pyranine
- Banknotes, postage stamps and credit cards often have fluorescent security features.

APPLICATIONS OF FLUORESCENCE

- Lighting
  - The common fluorescent lamp relies on fluorescence _ contains a coating of a fluorescent material - called the phosphor - which absorbs the ultraviolet and re-emits visible light.
  - Fluorescent lighting is more energy-efficient than incandescent lighting elements.

APPLICATIONS OF FLUORESCENCE

SPECTROSCOPY

- Usually the setup of a fluorescence assay involves a light source – which emit many different wavelengths of light.
- A single wavelength is required for proper analysis – light is passed through an excitation mono-chromator -and then that chosen wavelength is passed through the sample cell
- After absorption and re-emission of the energy -many wavelengths may emerge due to Stokes shift.
- To separate and analyze them-the fluorescent radiation is passed through an emission monochromator- and observed selectively by a detector

APPLICATIONS OF FLUORESCENCE

- Biochemistry and medicine
  - Fluorescence in the life sciences is used generally as a non-destructive way of tracking or analysis of biological molecules by means of the fluorescent emission at a specific frequency where there is no background from the excitation light - as relatively few cellular components are naturally fluorescent
  - A protein or other component can be – labelled with an extrinsic fluorophore - a fluorescent dye that can be a small molecule, protein, or quantum dot

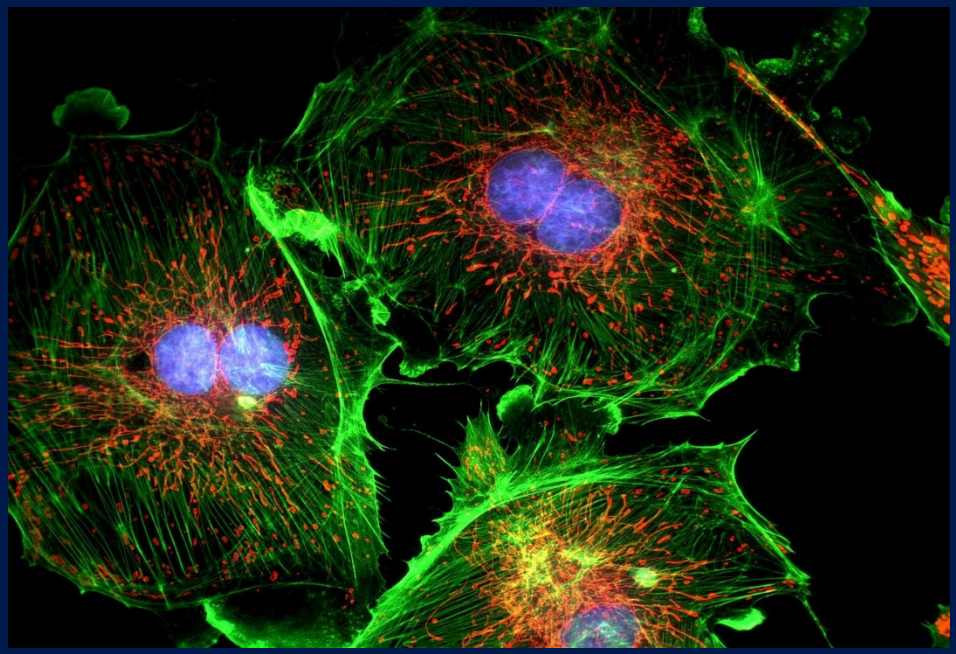
APPLICATIONS OF FLUORESCENCE

- Microscopy
  - When scanning the fluorescent intensity across a plane one has fluorescence microscopy of tissues, cells, or subcellular structures _
  - That can be done by labeling an antibody with a fluorophore and allowing the antibody to find its target antigen within the sample.
  - Labelling multiple antibodies with different fluorophores allows visualization of multiple targets within a single image (multiple channels).

APPLICATIONS OF FLUORESCENCE

FLIM: Fluorescence Lifetime Imaging Microscopy _ can be used to detect certain bio-molecular interactions that manifest themselves by influencing fluorescence lifetimes.

Cell and molecular biology: detection of colocalization using fluorescence-labelled antibodies for selective detection of the antigens of interest using specialized software, such as CoLocalizer Pro.



Fluorescent paint lit by UV tubes

GREEN FLUORESCENT PROTEIN

- The green fluorescent protein (GFP) is a protein composed of 238 amino acid residues that exhibits bright green fluorescence when exposed to light in the blue to ultraviolet range
- Although many other marine organisms have similar green fluorescent proteins, GFP traditionally refers to the protein first isolated from the jellyfish Aequorea victoria
- The GFP from A. victoria has a major excitation peak at a wavelength of 395 nm and a minor one at 475 nm.
- Its emission peak is at 509 nm, which is in the lower green portion of the visible spectrum
- The fluorescence quantum yield (QY) of GFP is 0.79.

GREEN FLUORESCENT PROTEIN

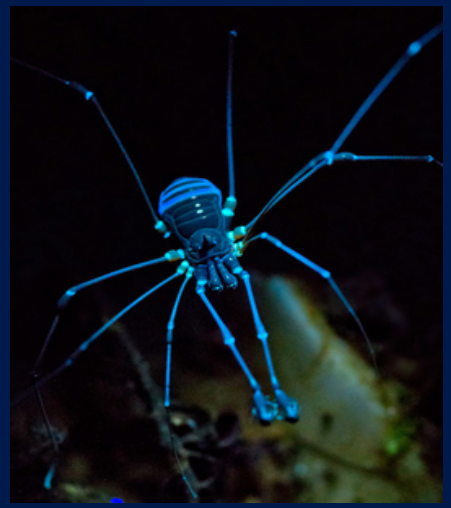
Aequorea victoria

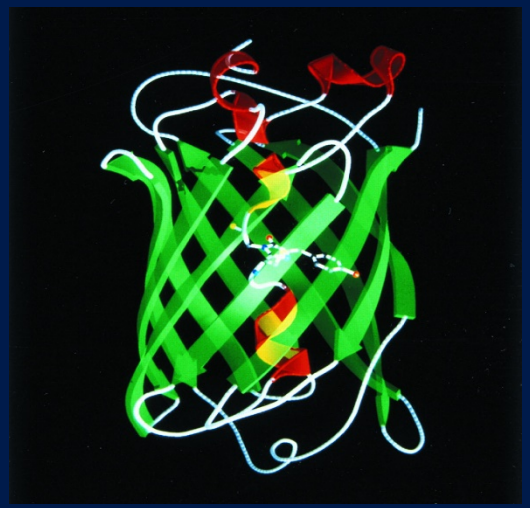
GREEN FLUORESCENT PROTEIN

- GFP makes an excellent tool in many forms of biology due to its ability to form internal chromophore
- In cell and molecular biology - the GFP gene is frequently used as a reporter of expression
- It has been used in modified forms to make biosensors
- Many animals have been created that express GFP

GREEN FLUORESCENT PROTEIN

- GFP can be introduced into animals or other species through transgenic techniques and maintained in their genome and that of their offspring.
- Scientists Roger Y. Tsien, Osamu Shimomura, and Martin Chalfie were awarded the 2008 Nobel Prize in Chemistry on 10 October 2008 for their discovery and development of the green fluorescent protein.

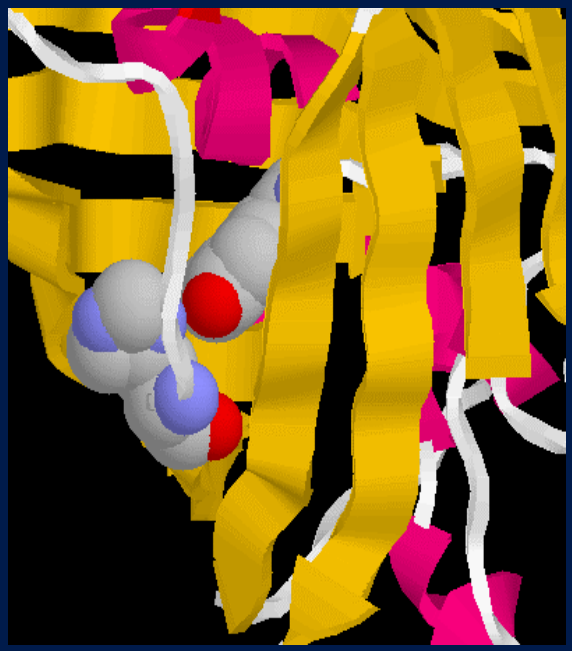




GREEN FLUORESCENT PROTEIN

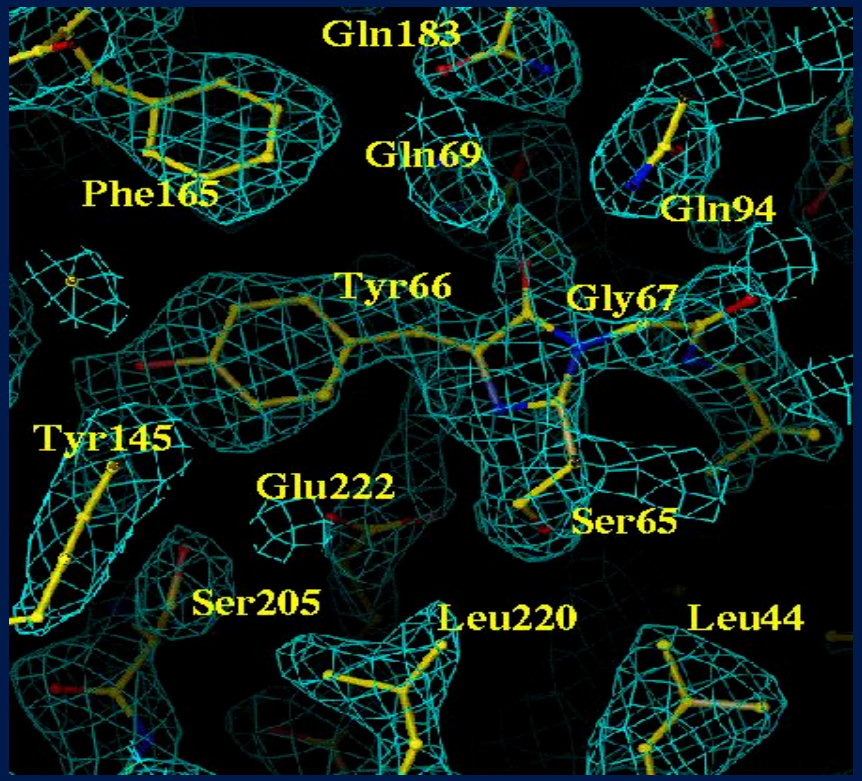
- GFP is composed of 238 amino acids
- Each monomer composed of a central α-helix surrounded by an eleven stranded cylinder of anti-parallel β-sheets
- Cylinder has a diameter of about 30A and is about 40A long
- Fluorophore located on central helix

The Active Site



GREEN FLUORESCENT PROTEIN

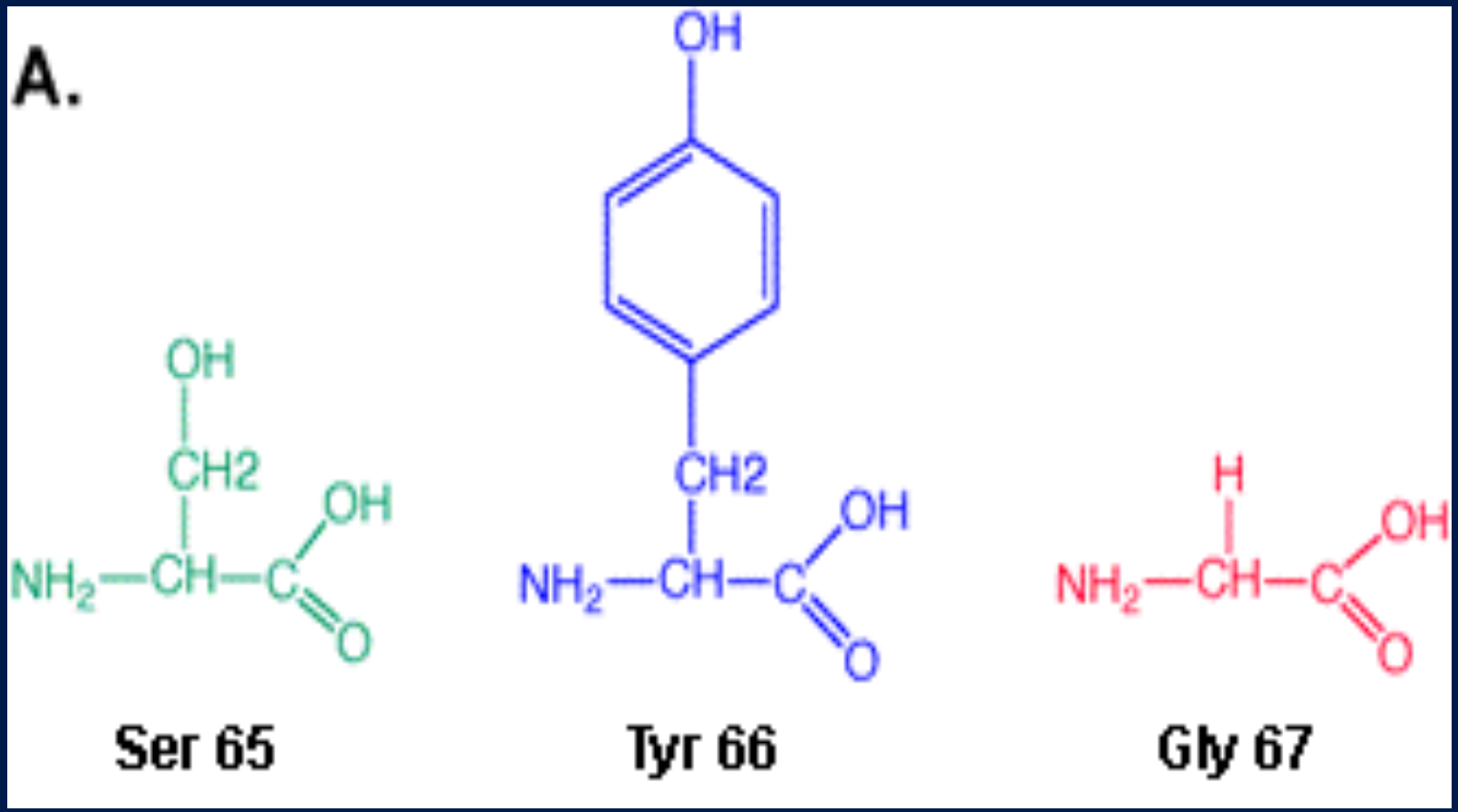
- The Fluoropore Active Site
  - Ser65-Tyr66-Gly67
  - Deprotonated phenolate of Tyr66 is cause of fluorescence
  - Forster Cycle (1949- Theodor Forster)
  - Proton transfer to His148



GREEN FLUORESCENT PROTEIN

- Fluorophore formation
  - One limitation of GFP is its slow rate of fluorescence acquisition *in vivo*
  - Renaturation most likely by a parallel pathway
  - Oxidation of Fluoropore (2-4 hours)
  - Two step process

GREEN FLUORESCENT PROTEIN



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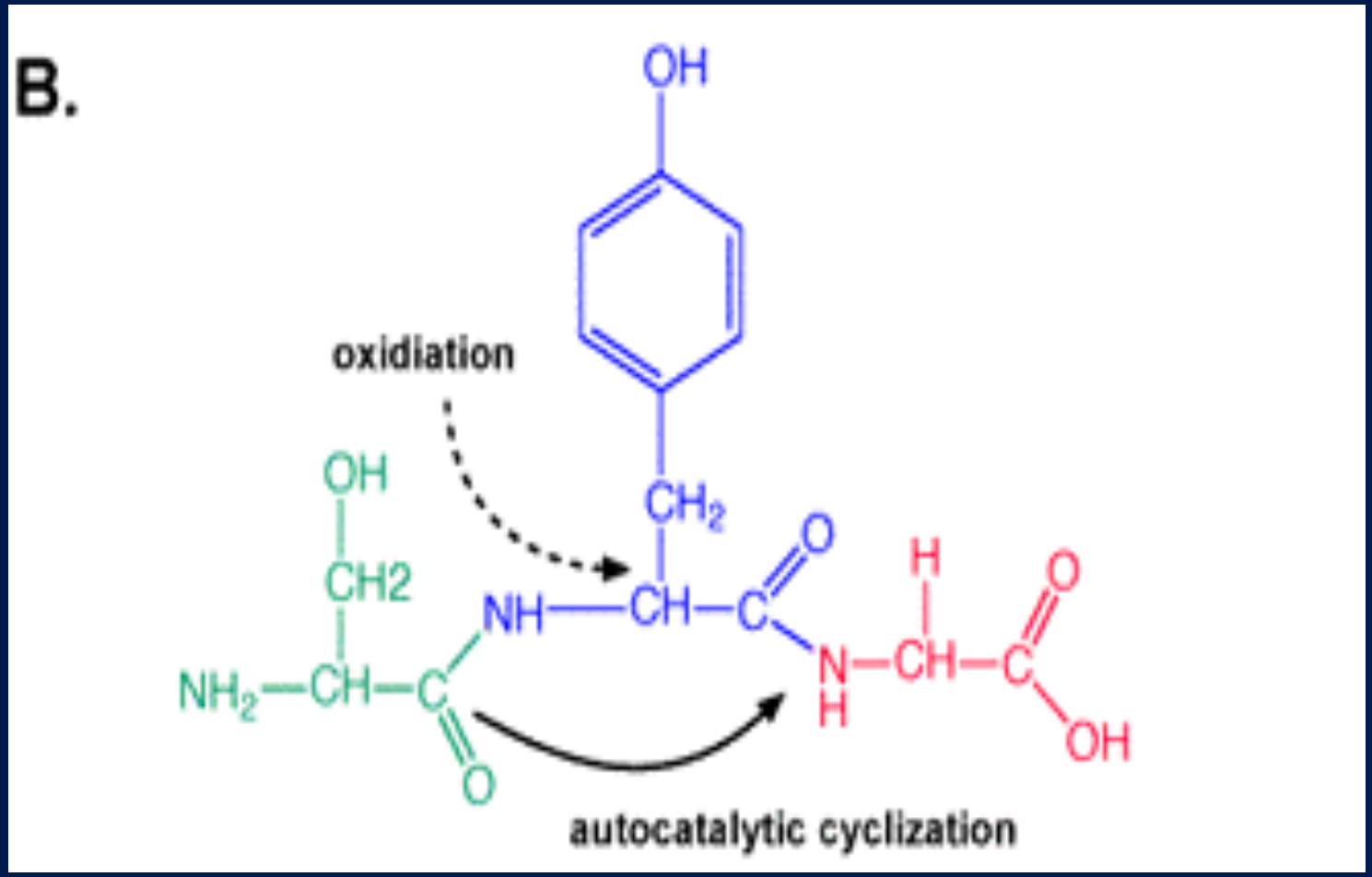

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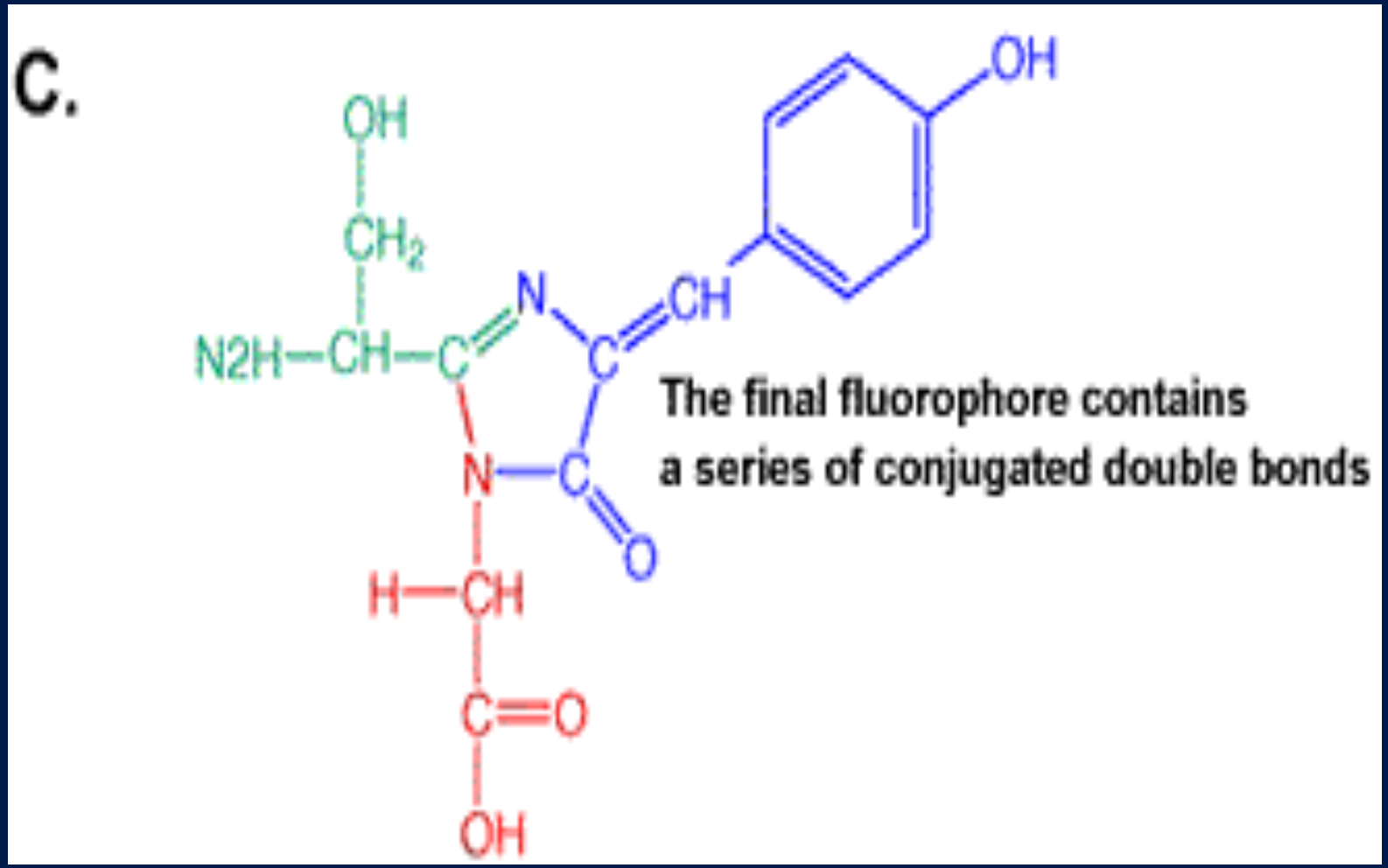


ADVANTAGES OF GFP

- The biggest advantage of GFP is that it can be heritable _ depending on how it was introduced - allowing for continued study of cells and tissues.

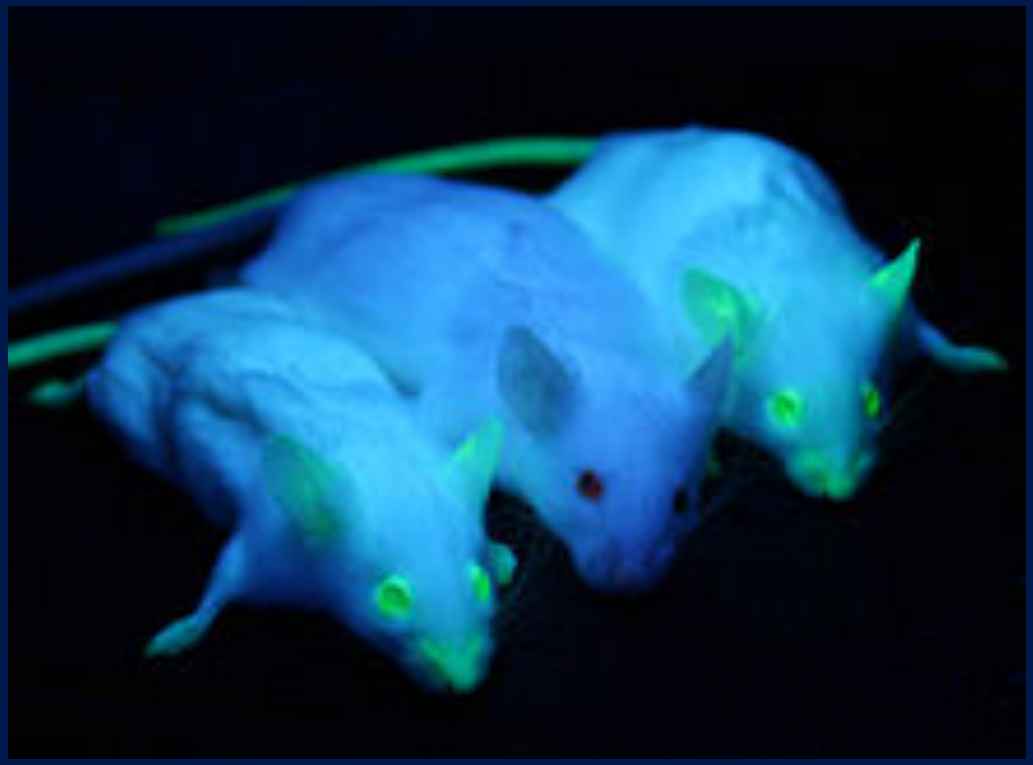
- Visualizing GFP is noninvasive- requiring only illumination with blue light.

- GFP alone does not interfere with biological processes, but when fused to proteins of interest - careful design of linkers is required to maintain the function of the protein of interest.

APPLICATIONS OF GFP

- Fluorescence microscopy
  - The availability of GFP and its derivatives has thoroughly redefined fluorescence microscopy and the way it is used in cell biology and other biological disciplines
  - While most small fluorescent molecules such as FITC (fluorescein isothiocyanate) are strongly phototoxic when used in live cells, fluorescent proteins such as GFP are usually much less harmful when illuminated in living cells.
  - This has triggered the development of highly automated live-cell fluorescence microscopy systems _ which can be used to observe cells over time expressing one or more proteins tagged with fluorescent proteins.

APPLICATIONS OF GFP



Mice expressing GFP under UV light (left & right), compared to normal mouse (center)

APPLICATIONS OF GFP

- Transgenic pets
  - Alba _ a green-fluorescent rabbit _ was created by a French laboratory using GFP for purposes of art and social commentary
  - The US company Yorktown Technologies markets to aquarium shops green fluorescent zebrafish (GloFish) that were initially developed to detect pollution in waterways.
  - NeonPets- a US-based company has marketed green fluorescent mice to the pet industry as NeonMice.


THANK YOU