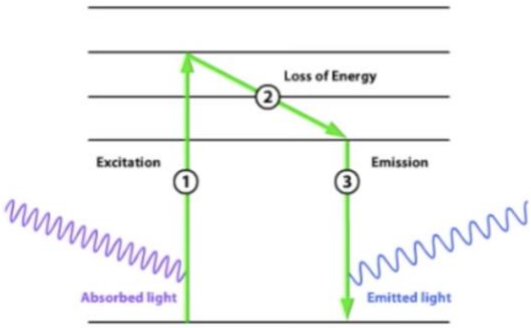
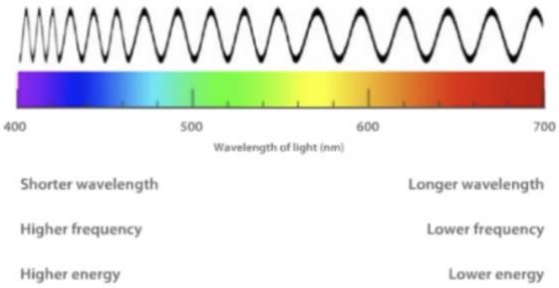
Fluorescence

- **Fluorescence** is the emission of light by a **fluorophore** (that absorbs light) at a particular wavelength which we detect as particular colours
- Fluorescence is used to mark particular regions or organelles within the cell
- In its ground state, a fluorophore is of low energy, is stable and does not fluoresce
- When a fluorescent molecule absorbs a photon from an external light source of the correct energy (wavelength) it excites an electron from a lower energy level (S0) to a higher energy level (S1)
- Molecules stay in a transient excited state for a very short time, then the electron falls back to the S0 level and the excess energy is released and emitted as light from a different photon (fluorescence)
- Some energy is lost through molecular vibrations (dissipates as heat) during the transient excited lifetime/state, so the emitted light (emission) is always of lower energy and longer wavelength than the absorbed light (excitation)
- Colour of light emitted is different from the colour of the light absorbed

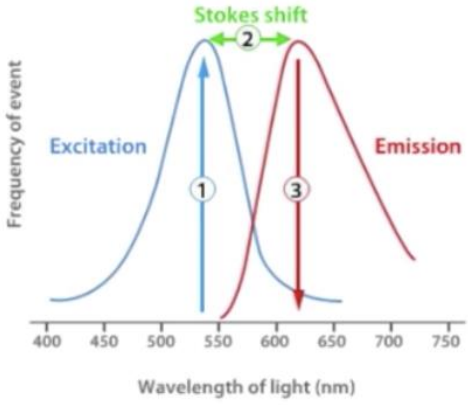




- The fluorophore's structural instability during the excited state makes it susceptible to degradation – high intensity illumination can result in a structural change in the fluorophore so that it can no longer fluoresce (process called **photo bleaching**)
- When a sample has been photo bleached, the fluorophores no longer jump to an excited state and fluorescence does not occur
- Fluorescent dyes/molecules have an excitation range, however some wavelengths within the range are more effective at exciting the fluorophores than others
- Light is emitted at a range of wavelengths and a molecule may emit at a different wavelength with each excitation event due to degradation/changes that occur in the excited state

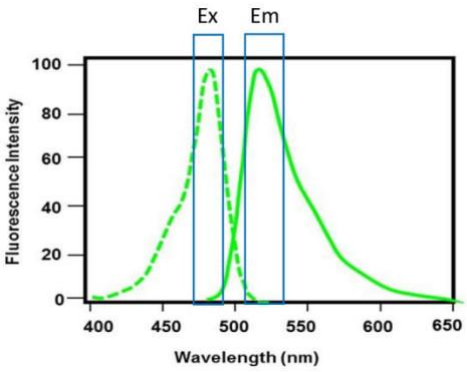
Measuring Fluorescence

- Fluorescence spectrum can be measured using a spectrofluorometer which can scan over a range of excitation wavelengths and detect emitted light over a range of wavelengths
- We don't need to determine the full spectra, light filters (band pass filters) that pass only defined parts of the excitation or emission spectrum of the molecule are used
- The wavelength at which the fluorophore is most efficiently excited at, is the **excitation maximum**
- The wavelength at which the fluorophore most efficiently emits light at is the **emission maximum**

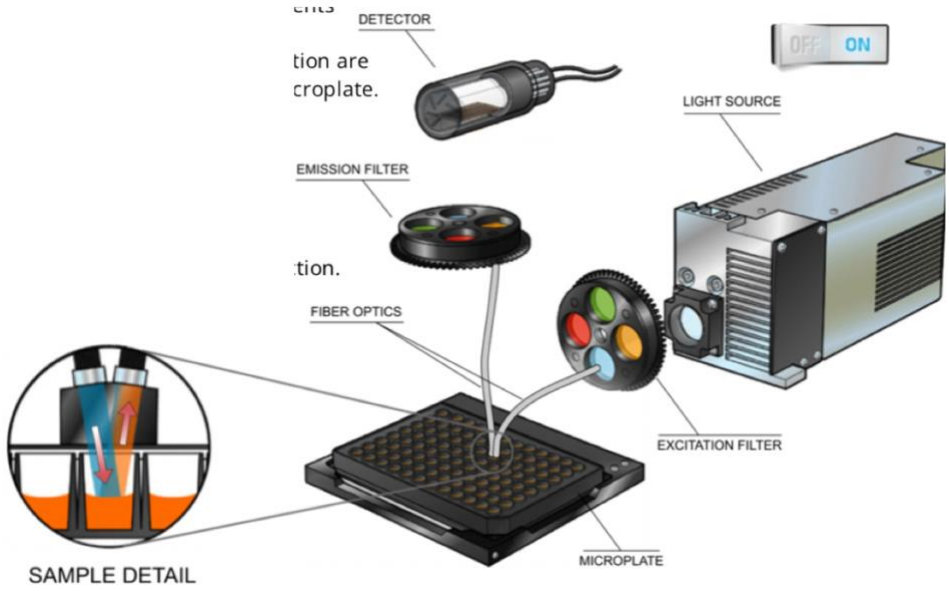


- Emission at lower or higher wavelengths affects only the intensity of the light emitted
- The difference between the excitation and emission maxima is called the **stokes shift**

eg. To excite fluorescein, a filter that passes 475 – 590 nm of light would be used, and to detect fluorescence emission of fluorescein, a filter that passes 510 - 530 nm light is used



- Fluorescence is detected by the EVOS fluorescence microscope and quantitatively by a plate reader
- Fluorescence can be measured quantitatively by a plate reader. A standard curve must first be created for each of the fluorescent molecules used. This is done by using the plate reader to measure the absorbance of the fluorescent molecule over a range of known concentrations. Then, the sample containing fluorophores of unknown concentration can be placed in the plate reader and their absorbances can be obtained. From this, the standard curve can be plotted on a graph and the equation of its trendline is then used to calculate the unknown concentrations of the fluorophores.
- Plate readers measure the absorbance of the sample and the concentration is found using the equation of the standard curve
- Each fluorescent molecule produces an image that can be overlayed with others, to highlight multiple regions of a cell



Excitation Light Sources

- **Broadband sources** (mercury-arc, tungsten halogen lamps) produce white light over a wide range of wavelengths
    - Wavelengths needed for excitation from broadband sources must be filtered using optical filters
- **Laser sources** (argon laser) produce blue light over 1 or a few wavelengths
- **LEDs** produce green light (low cost, low energy consumption, long lifetime)

Optical Filters

- Can be coloured glass or interference filters
- Optical filters allow light of a certain wavelength to pass through, whilst blocking others

- A filter that transmits a narrow range of wavelengths is called a **bandpass excitation filter**
- Filters also eliminate stray light from other sources such as from the excitation source, so the only light we see being emitted is the fluorescent light
- **Longpass emission filters** block out excitation light, only allowing the emitted light to pass through (for a sample with a single dye)
- **Bandpass emission filters** isolate the emissions from each dye (for a sample with multiple dyes)
- **Excitation filters** isolate a specific wavelength to pass through the sample
- **Dichroic mirrors** are a beam-splitting mirror between the excitation and emission filters
- **Emission filters** block out any unwanted fluorescence signals
- **Cross-talk** is when emission of 2 or more dyes overlaps and is detected simultaneously

Fluorescent Dyes

- **Fluorescent dyes** are small chemical fluorophores
- Fluorescein (green), V450 (blue) and rhodamine (red)
- Fluorescent dyes often require the cells to be killed
- Have an aromatic fluorophore in their structure responsible for absorbing and emitting light
- Dyes are made functional with other chemical modifications, allowing them to attach to specific targets when incubated with living cells
  eg. dyes such as MitoTracker are reactive and covalently label proteins on entering the mitochondrion. DAPI (4',6diamidino-2-phenylindole) is used for labelling the nucleus as it fluoresces blue only when bound (non-covalently) to double stranded DNA
- Fluorescent dyes must be used at correct concentrations for efficient labelling of cells

Fluorescent Proteins

- **Fluorescent proteins** are proteins with genetically encoded fluorophores that tag/fuse to proteins of interest
- The fluorophore is formed from side chains of a small number of amino acids and is covalently linked to the inside of the protein
- Side chains are brought in close proximity during protein folding and an autocatalytic process causes rearrangement of bonds to form the fluorophore
- The DNA sequence of the fluorescent protein (and therefore amino acid sequence) determines the emission colour
- Different colour fluorescent proteins can be expressed and detected together in the same cell
- Unlike dyes, fluorescent proteins do not enter cells by incubation
- Fluorescent proteins must be expressed inside the cell from a gene integrated into the genome or introduced on a plasmid
- DNA sequences can be modified to encode for fluorescent proteins which localise to different compartments of the cell after translation (eg. mitochondrion)
- The sequence may encode a specific protein of interest which will therefore be expressed and joined (tagged) with the fluorescent protein (forming a translational fusion protein)

- Levels of expression and the location of the protein of interest may then be monitored in the cell using fluorescence microscopy
- GFP (green fluorescent protein) is a common protein used to tag regions within a cell because it is small and can be attached to the end of proteins
- GFP chromophore is made up of 3 amino acids
- If an amino acid is mutated, the colour that is emitted changes

Benefits of Fluorescent Proteins

- Uniquely genetically encoded
- Non-invasive
- Non-toxic
- No cofactors required (except O_2)
- Highly specific labelling of compartments and proteins (eg. targeting the mitochondrion)
- Usually do not interfere with function of proteins to which they are fused.
- Easily visualised
- Diverse range of properties (eg. useful as biosensors)

Biosensors

- Fluorescent proteins can be used as **biosensors** which act to monitor and transmit information about cellular processes
- If fluorescence is insensitive to changes in the environment of the cell, it is a passive reporter (used to mark the cell, fuse to proteins or measure gene promotor activity)
- If fluorescence emission is sensitive to changes in the environment of the cell, it is an active reporter and acts as a biosensor
    - Sensitive to pH, protein phosphorylation, protease action, protein-protein interactions, alteration in calcium and metabolite concentrations

Model Organisms

- Everything is related
- What we learn from one species can be applied to another species
- Model organisms are used to better understand cellular processes (apoptosis, cell division, signalling pathways, gene expression, Hox genes and miRNA etc)

Escherichia coli (bacteria)

- Single cell prokaryote (simplest prokaryote model organism)

- Circular chromosome, 4.6 million nucleotides, 4300 genes
- Ideal model for studying DNA replication, genetic code, gene expression and protein synthesis
- Easy to grow and study, forms colonies, rapid growth
- Small genome compared to mammalian cells – genetic analysis
- Synthesise all their own amino acids, nucleotides – mutants used to define pathways

TABLE 1–2 Some Model Organisms and Their Genomes

Organism	Genome size* (nucleotide pairs)	Approximate number of genes
Escherichia coli (bacterium)	4.6×10^6	4300
Saccharomyces cerevisiae (yeast)	13×10^6	6600
Caenorhabditis elegans (roundworm)	130×10^6	21,000
Arabidopsis thaliana (plant)	220×10^6	29,000
Drosophila melanogaster (fruit fly)	200×10^6	15,000
Danio rerio (zebrafish)	1400×10^6	32,000
Mus musculus (mouse)	2800×10^6	30,000
Homo sapiens (human)	3200×10^6	30,000

*Genome size includes an estimate for the amount of highly repeated DNA sequence not in genome databases.

Saccharomyces cerevisiae (yeast)

- Single cell eukaryote (simplest eukaryote model organism)
- 13 million nucleotides (3 x that of E.coli), 6600 genes
- Nucleus, subcellular organelles
- Can be manipulated using techniques applicable to E. coli (eg. making mutants)
- Yeast mutants important for understanding autophagy
- Small, easy to grow rapidly by budding
- 16 linear chromosomes (haploid and diploid forms)

Caenorhabditis elegans (roundworm)

- Multicellular hermaphrodites
- Genome smaller than that of higher eukaryotes
- 20,000 genes (3 x yeast but similar to humans)
- Simple, 959 cells in adult worms (plus 1000-2000 germ cells)
- Embryonic origin and development of each cell is known in the adult
- Easily grown and genetically manipulated in the lab, transparent
- Used for identifying critical genes that control development and cell division
- Similar genes function in higher eukaryotes
- Short life cycle, large brood size

Arabidopsis thaliana (plant)

- Simple, 15,000 unique genes
- Easy to grow in the lab, short generation time
- Used for analysing the process of flowering

Vertebrates

- Human genome 3 x 10^9 base pairs and 20,200 genes
- 200 different cell types
- Isolated cells in culture
- Important for understanding DNA replication, DNA synthesis, protein synthesis and cell division
- Manipulation of growth media allows signalling events that control growth and differentiation in intact organism
- Study of highly specialised cells

- Mice have similar genome to humans and homologous genes
- Mice are used as model organisms to analyse mutations in humans
- Mice can easily be genetically altered (transgenic mice) and gene editing (CRISPR/Cas9)