Activity 2: Fluorescence Microscopy

If you search for "Light microscopy images" on the internet, some of the images you will see will outline very specific features within cells, sometimes in beautiful bright colours (Figure 1), not really resembling the ones you saw in the previous Activity. Those micrographs are taken using an extremely powerful technique called **fluorescence microscopy**, which is perhaps the most extensively used type of microscopy in modern cell and molecular biology. In this Activity, we will figure out how it works, and will touch upon the myriads of ways it can be used in research and even in the clinic.

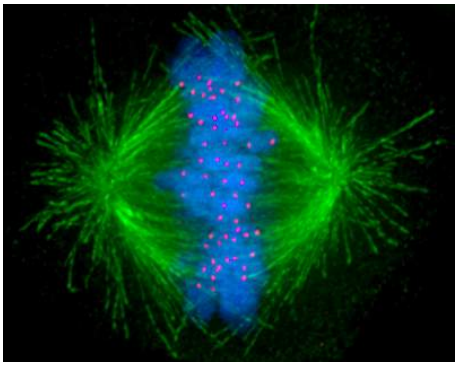


Figure 1: A fluorescence microscopy image of a mitotic spindle – compare this with the bright field and phase contrast images in Activity 1. Image by Afunguy (Wikimedia Commons).

The phenomenon of fluorescence

First we have to once again delve into physics and chemistry to understand – on a basic level – the phenomenon of fluorescence. Fluorescence is a subtype of photoluminescence. Luminescence in general means spontaneous light emission by a substance not resulting from heat. In photoluminescence, this emission is triggered by absorption of light, and fluorescence is a particular kind of such a process, where light is emitted very shortly after excitation and usually has a longer wavelength.

Behind fluorescence is the quantum mechanical concept of atomic energy levels, first proposed by Niels Bohr[1]. Watch this beautiful video about fluorescence, and answer the questions below.

Question 1 Why are high-visibility vests so bright? Would a picture of such a vest have this property?

[1]If you would like to know more about this, or need a refresher, have a look at the last section "Models of the Atom and Line Spectra" on the AK Lectures Quantum Theory series, in paticular the videos about the Bohr model.

Question 2 How do you think the intensity of excitation light affects the intensity of emission?

Fluorescence microscope

A fluorescence microscope allows one to image fluorescent molecules or structures within a sample. It is somewhat different from a conventional light microscope in that its optical elements now do not only include lenses. These optical elements have properties in the wavelength, or colour domain, and include monochromatic light sources, filters and dichroic mirrors.

- **Monochromatic light sources** (such as xenon arc or mercury-vapor lamps, lasers and LEDs) provide light in a very narrow spectral range. This enables one to specifically excite only the fluorophores whose absorption spectrum covers this range. They are also normally much brighter than the light sources used for general bright-field microscopy.
- **Optical filters** selectively transmit light in a particular range of wavelengths, while all other light is either absorbed or reflected.
- **Dichroic mirrors** are mirrors that transmit light in a particular range of wavelengths and are highly reflective for all other light. In practice, dichroic mirrors often reflect light above or below a certain threshold wavelength. They are used to separate the excitation and the emission light.

Have a look at this visualisation of the inner workings of a fluorescence microscope. Note that the second part of this video describes a special subtype of fluorescence microscopy called confocal microscopy, which we are not going to cover.

Task 1 Draw a scheme of a fluorescence microscope. Make sure to mark the optical devices specific to a fluorescence microscope.

Question 3 What "architectural" feature distinguishes fluorescence microscopes from the bright field microscopes?[2] Why would such a design be impossible with non-fluorescence microscopes?

Question 4 Is it possible to combine a conventional light microscope and a fluorescence microscope? If so, how?[3]

Task 2 Usually the detectors used for microscopy can only detect the intensity of the light, not its wavelength. Can you come up with a design for a fluorescence microscope that would allow one to image a sample where different structures are marked by different fluorophores, like in Figure 1?[4]

[2] If you cannot spot it, think about the condenser.

[3] Hint: look at Question 3 again!

[4] This is a hard task! Think about how you can separate the different colours either in space or in time.

Applications

After taking a dip into chemistry, physics and engineering, let us go back to the objects that fluorescence microscopes are used to study – namely, cells. You probably have already started wondering whether all their different components that are imaged using this technique are naturally fluorescent, and the answer is no. We will now take a look at a variety of ways in which life scientists are able to "light up" the features of interest and how this is applied to study... well, everything.

Fluorescent dyes

The simplest way to do that is to use **fluorescent dyes** (at least from the point of view of a biologist, not necessarily of the chemist who has to come up with those molecules!). Such dyes **bind specifically** to the components of cells with particular physical properties, e.g. hydrophobic membranes. A large variety of dyes exists, allowing researchers to highlight different organelles. Perhaps the most well-known example is 4',6-diamidino-2-phenylindole, or DAPI, which binds DNA and emits blue light. This is how the chromosomes were stained in Figure 1[5].

Visualising specific nucleic acid sequences and proteins

In many cases, however, researchers want to be able to see not simply a specific organelle or type of macromolecule, but the location of a **particular DNA/RNA sequence or protein**. The methods that allow one to achieve this both rely on **probes**, which are biomolecules that can recognise the target, conjugated to a fluorophore.

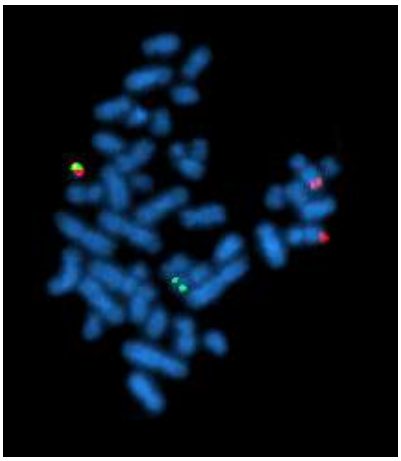
Fluorescence *in situ* hybridisation (FISH)

The technique used for labelling **particular nucleic acid sequences** is called **fluorescence *in situ* hybridisation**, or **FISH**. The probes in this case are themselves short nucleic acid sequences, or **oligonucleotides**, that are **complementary** to the sequence of interest. The probes with fluorophores attached to them will hybridise with the target sequence wherever this sequence is located in the cell (hence *in situ* – in place). By using either RNA or DNA oligonucleotide probes, FISH can be applied to visualise a particular RNA or DNA sequence, respectively. This Nature Education article describes the technique in detail – have a read through if you wish, or just look at the graphical explanation in Figure 1.

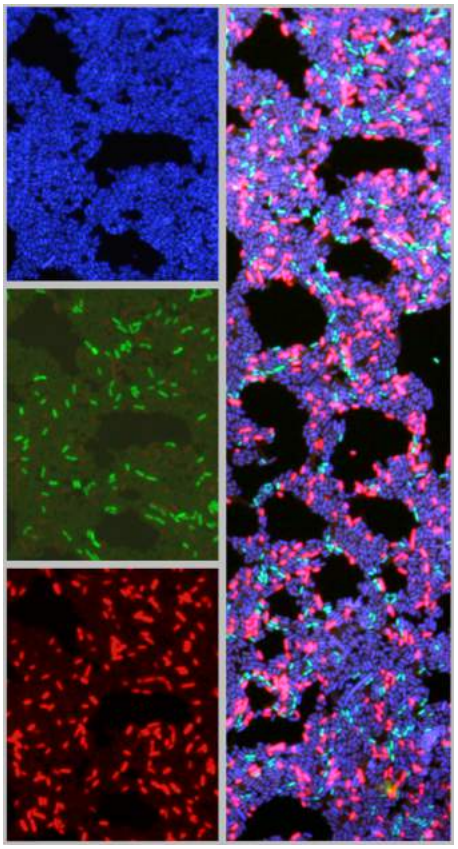
FISH is widely applied in scientific and clinical labs. DNA FISH, for example, can detect chromosomal abnormalities in hereditary diseases or in cancer. Figure 2a shows a FISH assay for chronic myeloid leukaemia, characterised by a translocation between chromosome 9 and 22, resulting in a tiny so-called Philadelphia chromosome. RNA FISH is used to study gene expression in organisms - check

[5]Note that since detectors usually do not distinguish the wavelength of light, the micrographs are really black-and-white, and an arbitrary pseudo colour is applied to them during post-processing. However, DAPI does emit blue light in reality.

this lovely video about using *in situ* hybridisation[6] to look at cell division in a columbine flower bud. Another example of RNA FISH application in identification of microbial species in a biofilm is shown on Figure 2b, which can be useful to study interactions between those species.



(a) DNA FISH, showing the *ABL1* gene on chromosome 9 (green) and the *BCR* gene on chromosome 22 (red), as well as their fusion on the Philadelphia chromosome (green and red). Courtesy: Wikimedia Commons.



(b) RNA FISH against the ribosomal RNA, used to identify *Listeria monocytogenes* (green), *Salmonella enterica* (red) and *Escherichia coli* in a biofilm (Almeida et al. 2011).

Figure 2: Examples of DNA and RNA FISH applications.

Question 5 The blue colour in both panels of Figure 2 is not coming from a FISH probe – what do you think it denotes? How is it possible to identify *Escherichia coli* in Figure 2b?

Question 6 Why is RNA, rather than DNA FISH used in the study in Figure 2b?

[6]In this particular case, fluorescence is not used to visualise hybridisation – it is done by a different technique called immunohistochemistry.

Immunofluorescence

Immunofluorescence, the method that allows visualising a **particular protein** in a sample, relies on the specific interaction between an antigen and an **antibody**. Antibodies in this case are raised against the protein of interest and are either directly conjugated to a fluorophore (direct method), or are recognised by a fluorescently labeled secondary antibody (indirect method). Watch this video to find out the details of how it works. Immunofluorescence, just as FISH, is used virtually in every field of biological research, as well as in the clinic, for example in the diagnosis of infectious or autoimmune diseases.

Question 7 What do you think could be the advantages of direct or indirect immunofluorescence?

Question 8 Why does an antibody from a different species need to be used as a secondary antibody in indirect immunofluorescence?

Question 9 In Figure 1, immunofluorescence against a particular protein is shown in green. Can you guess what protein that is?

Fluorescent tags

Immunofluorescence is an amazing tool, but it has a very serious limitation: to allow the antibodies to enter, cells have to be fixed and permeabilised – basically, killed. Therefore, it cannot be used to study the **dynamics of protein expression and localisation**. In addition, for some proteins, a specific enough antibody cannot be generated. The method that allowed scientists to overcome these issues and look at proteins in living cells combines two breakthroughs: **genetic engineering** and identification of the **green fluorescent protein (GFP)**.

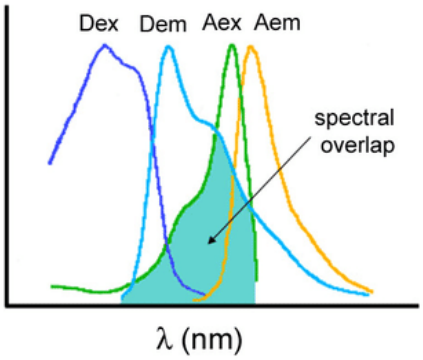
GFP is a protein found naturally in jellyfish, discovered by Osamu Shimomura in the sixties. It is a rather special protein, so no wonder it has featured as the Protein Data Bank Molecule of the Month back in 2003, where you can read more about it and its derivatives. In the simplest case, the gene of interest can be expressed from a plasmid – an autonomous circular piece of DNA that can be introduced into cells – together with GFP or another fluorescent protein as a "tag". Check out how it is done here. As a result, the location of a protein can be visualised in intact cells or even organisms.

Biosensors

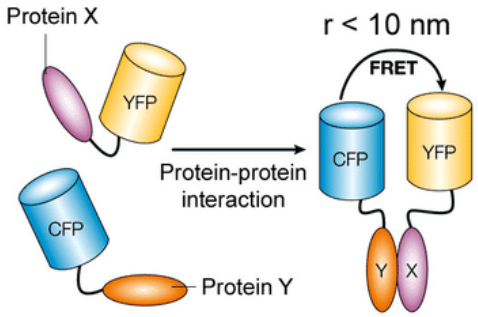
Finally, fluorescence can be applied not only to find out whether a protein is expressed and where it is located, but also to obtain more specific information about the cellular processes. Such techniques are called **biosensors**, and they can report on a vast variety of events and conditions, such as protein-protein interactions, concentrations of particular ions and pH. We will only touch upon a couple of examples from this blooming field.

FRET – a molecular ruler

FRET, which stands for **Förster resonance energy transfer**, is a mechanism of radiationless energy transfer between two fluorophores, a **donor** and an **acceptor**. FRET occurs when the emission spectrum of the donor overlaps with the absorption spectrum of the acceptor (Figure 3a) and when they are **sufficiently close (<10 nm)** to each other (Figure 3b). Therefore, FRET can be used to detect when the two fluorophores come into contact. By placing the two fluorophores onto the same protein, its conformational changes can be studied; when the fluorophores are on different proteins, interactions between them can be detected (Figure 3b).



(a) The spectral overlap between the emission spectrum of the donor and the excitation spectrum of the acceptor is required for FRET. Spectra for CFP (donor) and YFP (acceptor) are shown as an example. D, donor, A, acceptor, ex, excitation, em, emission.



(b) FRET can be used to detect interactions between proteins of interest, fused to fluorescent proteins that make up a FRET pair.

Figure 3: The two conditions for FRET: spectral overlap and proximity. Image credit: Scolari (2009).

Question 10 The cyan and yellow fluorescent proteins (CFP and YFP) are a classic FRET pair. Looking at the diagrams in Figure 3, explain what happens upon illuminating the sample with violet light, if the proteins are interacting and if they are not.

Sensing calcium

Another very widely used exampe of a biosensor is **GCaMP** – a **calcium ion sensor**, consisting of **GFP**, **calmodulin** and **M13 peptide** (Figure 4).

Structurally speaking, GFP has a shape of a barrel with the fluorescent core hidden inside it. The function of the barrel is to keep water away from the centre – in an aqueous environment, the core fluorescence is greatly diminished. A specially engineered version of GFP, cpGFP, is used in GCaMP. The polypeptide chain of cpGFP has free ends in the middle of the barrel (rather than at the tip), and these

ends are fused to calmodulin and M13. This creates a channel, which allows water to enter the barrel, reducing fluorescence (Figure 4, left).

Calmodulin is a calcium-binding protein. In the presence of calcium ions, calmodulin changes conformation. This change enables it to interact with the M13 peptide. As a result, in the context of GCaMP, these proteins come together and block the channel in the cpGFP barrel (Figure 4, right). Overall, GCaMP becomes fluorescent when bound to calcium, making it a great tool to measure calcium concentrations in different conditions.

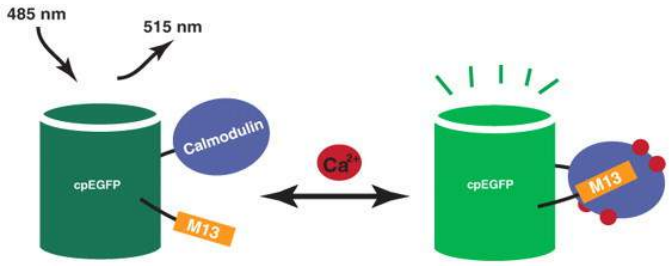


Figure 4: The GCaMP calcium sensor becomes fluorescent in the presence of calcium due to a conformational change. Image from Tian et al. (2012).

References

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