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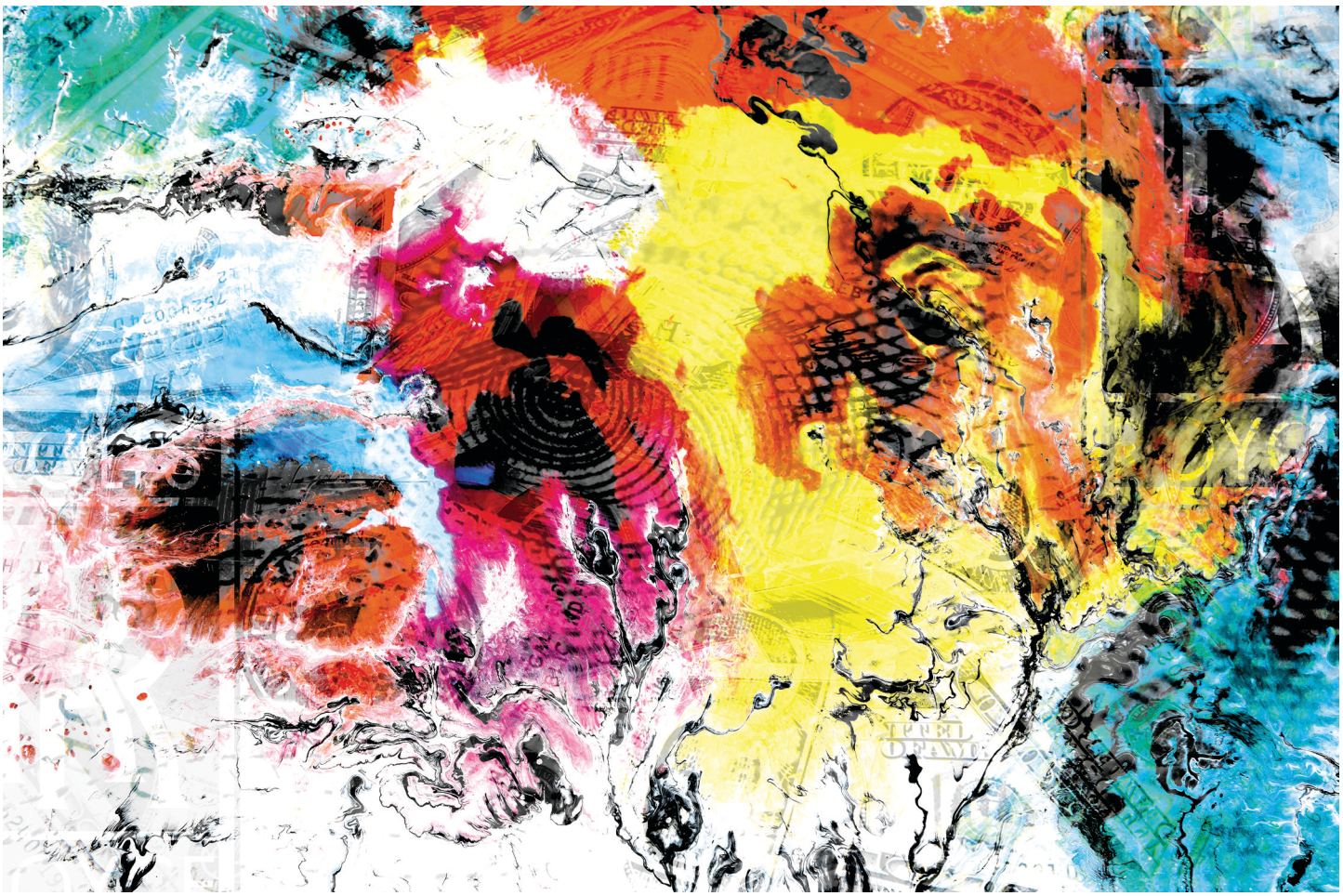
S296_1

Exploring cells with digital fluorescence microscopy



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Introduction

For centuries, humans have wondered what lies beyond what we can see. With the invention of the microscope, we unlocked an entirely new dimension, revealing the delicate architecture of cells, the vibrant complexity of bacteria, and even the atoms that build our world. Microscopy is not just a tool, it's a portal into the hidden stories of life, science, and discovery. This powerful technology has transformed our understanding of biology, medicine, and materials, and continues to push the boundaries of what is possible.

But what if we wanted to do more than just see? What if we wanted to highlight specific parts of this hidden world, make them glow, move, and tell their own stories? That's where fluorescence microscopy comes in.

Fluorescence microscopy doesn't just show us what's there, it lets us light up the inner workings of life in stunning colours. By tagging molecules with fluorescent markers, we can track proteins inside cells, follow the spread of disease, or even watch neurons communicate in real time. It's like turning on the lights in a dark room and discovering not just objects, but activity, interaction, and purpose.

This course will explore how this glowing frontier of microscopy is helping us unlock secrets at the cellular level and pushing science to new and exciting frontiers.

You will begin by building a solid foundation in the basic principles that underpin microscopy. From there, you will get a brief overview of the various types of microscopy and how they reveal different aspects of the microscopic world. Then, the course will dive deeper into the captivating realm of fluorescence microscopy, where glowing markers bring cellular processes to life in vivid detail. Along the way, you will revisit key cellular structures through an interactive 3D cell model, before applying your knowledge in the final section by exploring real biological samples using a digital fluorescence microscope.

Throughout the course you will encounter many micrographs, images taken with a microscope, and some videos that illustrate the power of microscopy. Questions and quizzes will help you to test your understanding throughout the course.

To gain maximum benefit from the course, you should have a basic understanding of the structures you can find inside cells. The free OpenLearn course 'A tour of the cell' can help you to acquire or deepen this knowledge. It also shows many images taken with electron microscopy, perfect if you are curious about this technique. If you are interested in exploring light microscopy in more detail including how to stain samples for histological examination, the free OpenLearn course 'Histology, microscopy, anatomy and disease' may also be of interest to you.

This OpenLearn course is an adapted extract from the Open University course S296 *Cell and molecular biology*.

Learning outcomes

After studying this course, you should be able to:

- outline some of the key principles of microscopy (with a focus on fluorescent microscopy) and the differences between light, fluorescence (light) and electron microscopy
- describe some labelling techniques used in fluorescence microscopy
- explore the structures and organelles found in a cell via fluorescence microscopy
- understand how to use a digital fluorescence microscope to collect data and images
- interpret images and data collected using fluorescence microscopy.

1 Introduction to microscopy

In this first section, you will be introduced to the units and sizes commonly used to measure structures within cells, some of which you may already be familiar with. You will then explore the factors that determine how much detail a microscope can reveal and be introduced to the concept of resolution. By applying this concept, you will understand why an electron microscope can reveal cellular structures in far greater detail than a light microscope.

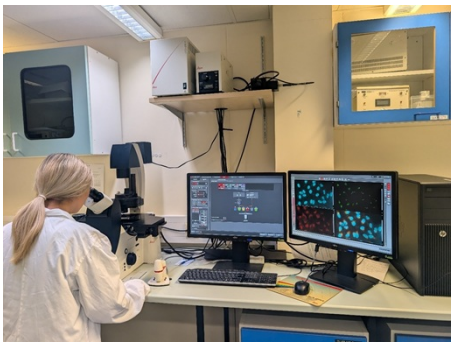


Figure 1 PhD student using a fluorescence microscope in The Open University's laboratories.

1.1 Why do we need microscopy?

Almost all cells are too small to be seen with the naked eye, so the study of cellular structure only began with the development of lenses and microscopes that could magnify cells many hundreds of times. A typical bacterium, for example, is about one micrometre in diameter and no more than a few micrometres in length (Box 1).

Box 1 Units used to measure the size of cells

To get down to the scale of cells, a unit of length is needed that is one-thousandth of a millimetre. This unit is the micrometre – abbreviated to μm (μ is the Greek letter mu) and sometimes referred to as a micron.

An even smaller unit, called the nanometre (abbreviated to nm), is needed when describing the size of subcellular components such as **organelles**, the membrane-bound structures inside eukaryotic cells that have a specific function. A nanometre is one-thousandth of a micrometre (Table 1).

Table 1 SI units of length.

Unit (symbol)	Multiple in metres	Multiple in micrometres
metre (m)	$1\ \mathrm{m}$	$10^{6}\ \mu\mathrm{m}\ \left(1\,000\,000\ \mu\mathrm{m}\right)$
centimetre (cm)	$10^{-2}\ \mathrm{m}\ \left(1/100\,\mathrm{m}\right)$	$10^{4}\ \mu\mathrm{m}\ \left(10\,000\ \mu\mathrm{m}\right)$
millimetre (mm)	$10^{-3}\ \mathrm{m}\left(1/1000\,\mathrm{m}\right)$	$10^{3}\ \mu\mathrm{m}\ \left(1000\ \mu\mathrm{m}\right)$

micrometre (μm)	$10^{-6}\,\mathrm{m}\,(1/1000\,000\,\mathrm{m})$	$1\,\mu\mathrm{m}$
nanometre (nm)	$10^{-9}\,\mathrm{m}\,(1/1000\,000\,000\,\mathrm{m})$	$10^{-3}\,\mu\mathrm{m}\ (1/1000\ \mu\mathrm{m})$

If you are not familiar with the very small units of measurement mentioned in Box 1, you should study Figure 2 and work through the questions that follow. Familiarity with the relative sizes of various molecules and organisms will be helpful for any future study in this area.

Figure 2 The relative sizes of cells, organelles and molecules arranged on a logarithmic scale: (a) shows structures larger than 100 nm which can be visualised using light and electron microscopy; (b) shows those smaller than 100 nm which can only be visualised in an electron microscope.

How many nanometres are there in one millimetre?

Answer

There are 1000 (10^3) nm in 1 μm and there are 1000 (10^3) micrometres in 1 mm. So, there are 1000 × 1000 = 1 000 000 (or 10^6) nm in 1 mm.

What type of scale is used in Figure 2 and why is it used?

Answer

It is called a logarithmic scale. Each unit is ten times greater than the previous unit, which is helpful when you want to show a very wide range of values using the same scale.

In Figure 2, approximately what size is the structure labelled as X?

Answer

Just under 0.3 nm, which is the size of a water molecule.

What is the size range (from smallest to largest) of bacteria in micrometres and in nanometres?

Answer

0.8–10 μm, which is 800 nm to 10,000 nm (or $1 \times 10^3\mathrm{nm}$ to $5 \times 10^3\mathrm{nm}$).

Next, you'll be introduced to the important difference between magnification and resolution, which helps you to understand why some microscopy techniques allow you to see more detail.

1.2 Magnification and resolution are important when studying details

You should now be familiar with the sizes of cells and structures that you will see when using the digital fluorescence microscope. Before you get to that, it's important to understand the difference between two different terms: magnification and resolution. **Magnification** describes by how much a microscope or a digital camera can enlarge, or magnify, an object. This increases the size in which you can see a structure – think about the zoom a camera can offer you. However, whilst the structure is enlarged, it might become blurry and you can't see the details. The ability of being able to separate two neighbouring objects in an image is described with the term **resolution** (i.e. the two objects can be resolved; they do not appear as a single object). You might be familiar with the concept of resolution from using digital cameras. A camera with more Megapixels has a higher resolution, meaning that it will show more details when enlarging the image compared to an image taken with a camera that has fewer Megapixels. A typical light microscope has a maximum resolution of around 200 nm, whilst electron microscopy can achieve a resolution of around 0.1 nm.

Inspect Figure 3 and state which of the panels has been taken on a microscope with a higher resolution.

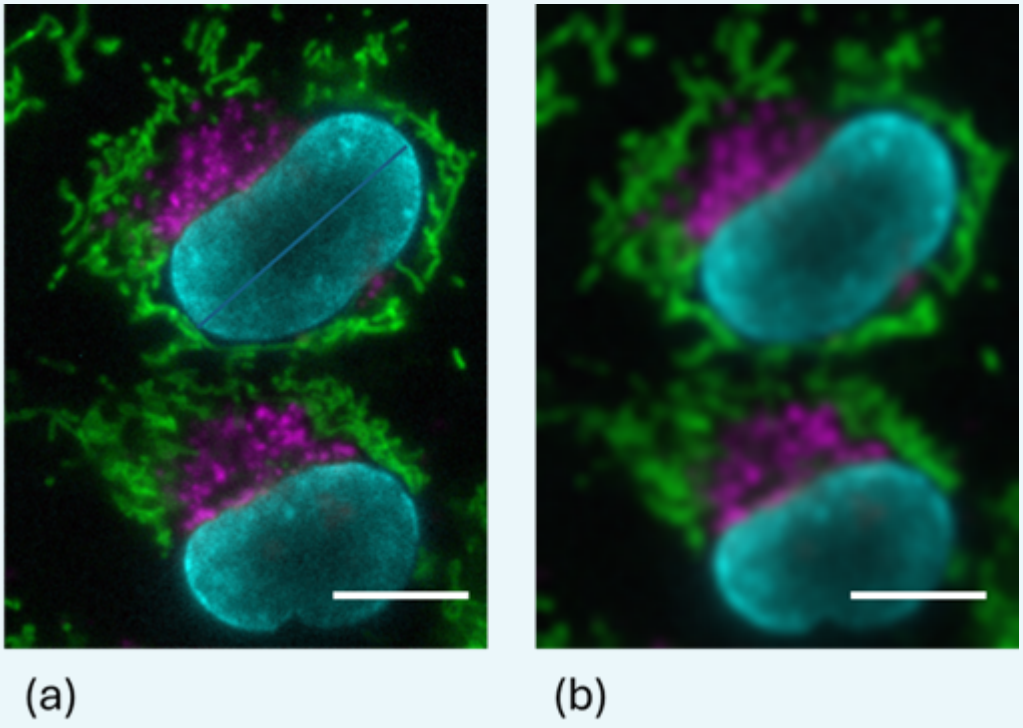


Figure 3 Two fluorescence micrographs of the same structure inside a cell, taken at two different resolutions. The scale bar equals 10 µm.

○ Panel (a)

Correct. You can identify this because both micrographs are shown with the same magnification and panel (a) shows more details.

○ Panel (b)

Incorrect. Remember that an image taken with a higher resolution will show more details when presented with the same magnification.

So, it’s not just about zooming in (magnification), it’s about how clearly you can see once you’ve zoomed in – and that’s resolution.

2 Types of microscopy

The three different microscopy techniques most commonly used to study cells are light microscopy, fluorescence (light) microscopy and electron microscopy. The first two use light to visualise the sample, while electron microscopy uses a beam of electrons. Because cells have a very limited contrast, they are difficult to see clearly under any microscope without **staining**. Various staining techniques can be used to visualise structures, or even specific proteins, inside cells. The choice of staining method depends on the type of microscopy, and the particular research question being addressed.

Each type of microscopy has its own advantages and limitations, which you will explore as you learn more about the three types. Examples of cells visualised with each technique are shown in Figure 4.

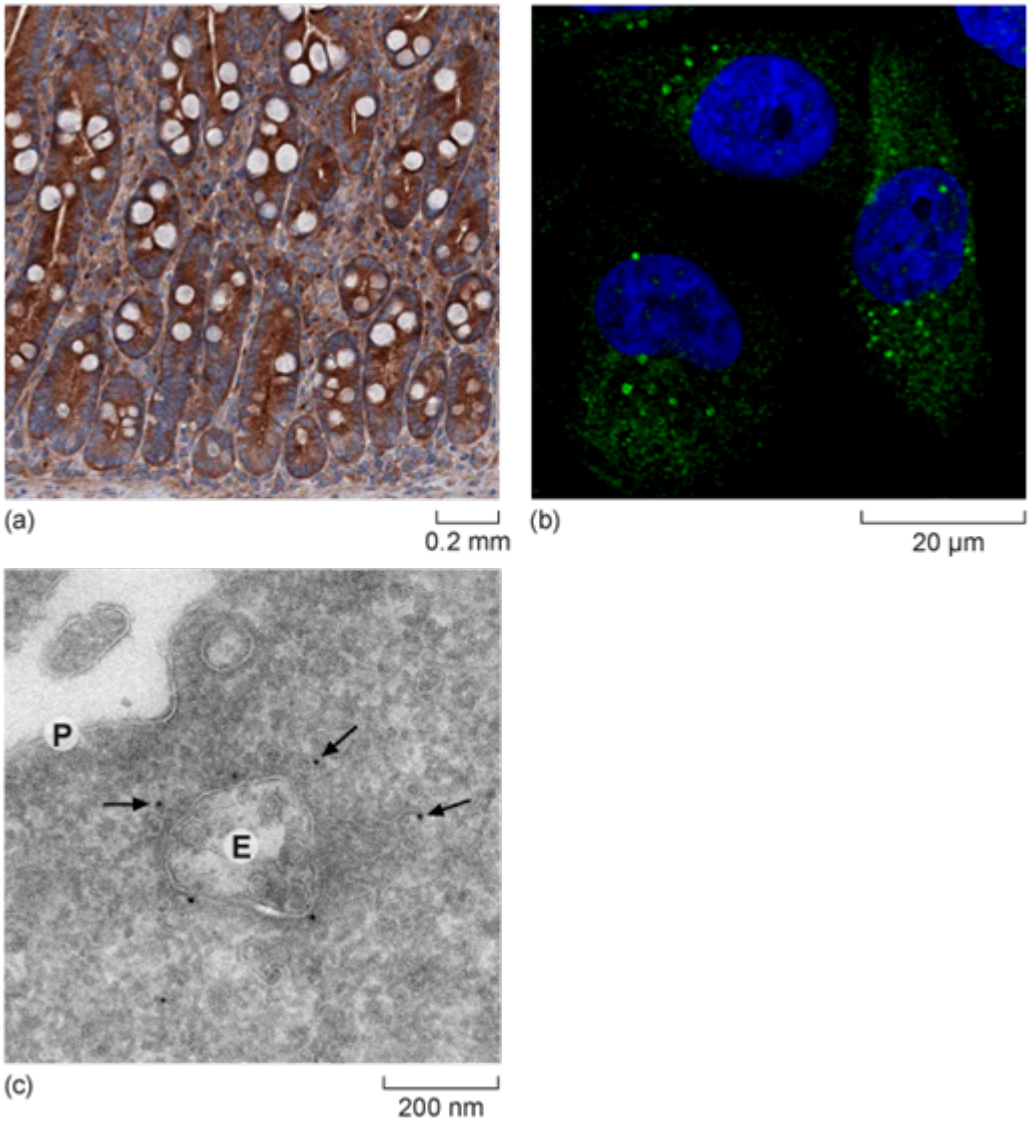


Figure 4 (a) Light microscopy image showing the protein of interest stained brown. (b) Fluorescence microscopy image with the protein of interest labelled in green and cell nuclei in blue. (c) Transmission electron microscopy (TEM) image. The dark dots indicated by arrows represent the protein of interest. ‘E’ marks an endosome, and ‘P’ indicates the plasma membrane.

What difference can you observe in the scale bars shown in Figure 3?

Answer

The scale bars range from 0.2 mm to 200 nm, indicating that the images taken with a light microscope reveal fewer details than those taken with fluorescence (light) microscopy. However, they allow us to see the whole cells.

Which of the panels in Figure 3 was taken with the highest resolution?

Answer

The image taken with a transmission electron microscope has the highest resolution and reveals most details.

Box 2 Use of scale bars in microscopy

The magnification of a structure in a **micrograph**, an image taken using a microscope, results as a combination of several steps in which the original structure is magnified. First, the microscope objective applies a magnification. Then the camera used for taking the image can apply an additional magnification. Finally, the computer software displaying the image can also apply magnification or display the image in various sizes, depending on your screen. That's why stating the magnification is not the most useful way to show the size of a structure, and where the use of scale bars is critical.

A **scale bar** is added to a micrograph to illustrate the size of the cells or structure independent of the equipment used to display it, and independent of the size of the image that is displayed. It can be shown within or underneath the micrograph. In multi-panel figures, one scale bar might apply to several images.

You will now be introduced to the components of light microscopes.

2.1 Components of a light microscope

All microscopes use a series of magnifying lenses to enable very small objects such as cells to be seen by the human eye. A **light microscope** (or optical microscope) uses visible light to illuminate the sample and glass lenses to focus and magnify the image. **Light microscopy** can magnify an object up to about 1000 times the original size, obtain a resolution of around 200 nm and it can be used with either living or **fixed cells**. The box below briefly explains how a light microscope works.

Box 3 How a light microscope works

In a light microscope, three lenses are important for forming the magnified image: the condenser, the objective and the camera (Figure 5).

Figure 5 A compound (multiple lens) light microscope with a diagram illustrating how the condenser focuses light on the sample and transmits the light through the objective and eyepiece lenses to the observer.

The condenser focuses a beam of light onto the sample placed on the stage. In an upright microscope, like the one shown in Figure 5, the light source and condenser are both located beneath the stage. The focused beam of light is transmitted through the sample and then passes through the objective, which magnifies the image and passes it to the eyepiece(s), or to a camera that directs the captured image to a computer screen. The image is brought into sharp focus by moving the sample closer to, or further from, the objective. Most microscopes have several objectives ranging from ×4 to ×100 magnification (note that '×' means times and indicates the times-fold magnification). The eyepiece adds further magnification, often ×10. For example, the total magnification achieved by a ×4 objective combined with a ×10 eyepiece will be 4 × 10 = ×40. Often, additional magnification is added by the camera taking the images, or within computer software.

To increase the contrast and visualise specific structures in cells or tissues, different staining techniques can be used. Light microscopy is very important as a technique to detect changes in cell structure during the development of diseases and are routinely used in pathology labs around the world. If you are interested to learn more about histology, the study of the microscopic structure of complex plant and animal tissues, the free OpenLearn course 'Histology, microscopy, anatomy and disease' provides a lot more information. In this course, you will now take a look at fluorescence microscopy.

2.2 Fluorescence (light) microscopy

A particularly insightful technique that scientists use to obtain information about the structure and function of the inner workings of cells is by using fluorescent light for visualiation. Here, we don't include the term 'light' in the name and just call it **fluorescence microscopy** (also called fluorescence imaging).

Fluorescence is very much part of the natural world. You may be aware of fluorescent lighting, perhaps in your home or workplace, but fluorescence is also abundant in minerals, plants and animals. The jellyfish *Aequorea victoria* is an example of an animal that uses fluorescence as part of its defence against predators (Figure 6).

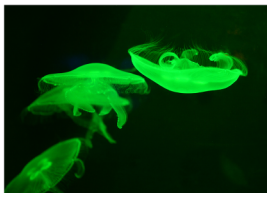


Figure 6 Green fluorescent protein fluorescence in the jellyfish *Aequorea victoria*.

This jellyfish became famous when the gene that encodes the protein that makes it appear fluorescent, green fluorescent protein (GFP), was isolated and cloned. GFP has since been used extensively in cell biology research, along with many other fluorescent proteins that have different colours.

The awarding of the 2008 Nobel Prize in Chemistry to Roger Tsien, Osamu Shimomura, and Martin Chalfie for their work in characterising green fluorescent protein (GFP) highlights the profound impact of fluorescent proteins on biological research. Their groundbreaking contributions have revolutionised how scientists visualise and study cellular processes in real time. (For more information on GFP and its applications, see the 'Further reading' section at the end of this course.)

2.3 Components of a fluorescence microscope

Fluorescence microscopes are similar to conventional light microscopes but include special filters that allow specific wavelengths of light to illuminate the sample and detect the emitted fluorescence (Figure 7). While understanding the technical details of these filters is not necessary for this course, it is helpful to know that they enable the detection of fluorescent signals from labelled cell and tissue components. When working with living cells growing in a dish or flask, researchers often use a so-called inverted microscope. In an inverted microscope, the objectives are located below the sample and the light is directed from above, making it easier for the researcher to access and manipulate the sample.

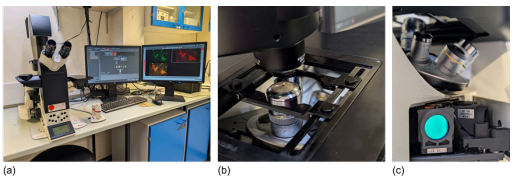


Figure 7 Inverted fluorescence (light) microscope at The Open University. (a) shows the whole microscope including the computer used to control the microscope and acquire the images. (b) Detailed view of the objectives, placed underneath the sample. (c) Filters that allow for selecting light of a specific wavelength to illuminate the sample.

Like conventional light microscopy, fluorescence microscopy can be used in living or fixed cells. There are several methods for staining specific structures and proteins within cells for visualisation using fluorescence microscopy. Because the structures are viewed against a dark background, fluorescence microscopy provides much greater contrast, making it easier to clearly identify and distinguish cellular structures.

Why might you want to use an inverted microscope and have access to the sample when working with living cells?

Answer

You might want to exchange the medium that is covering the cells to ensure they are kept alive, or in order to add compounds to stimulate the cells so you can observe their response in real time.

2.4 Electron microscopy

Although it's not the focus of this course, **electron microscopy** was mentioned because of its higher resolution and ability to study ultrastructure in detail. You will now be briefly introduced to two important types of **electron microscope** (EM).

1. *Transmission electron microscopy*: a beam of electrons is accelerated in a **transmission electron microscope** (TEM) at high velocity through a sample, which is a very thin section (less than 10 nm). Samples can range from tissue sections to purified protein complexes. Electrons cannot pass through glass; instead, magnets are used as the 'lenses' that control and focus the path of the electron beam. The interior of the microscope is under vacuum to prevent scattering of the electrons by air molecules. Electrons that have passed through the sample reach the detector, where they activate a fluorescent screen, or are captured with a digital camera, forming the image (Figure 8a and b). Electron microscopes first became available in 1939 and assisted in the discovery of organelles like the Golgi apparatus (Figure 8c).
2. *Scanning electron microscopy*: a technique for studying the surface of intact cells using a **scanning electron microscope** (SEM). The sample is first coated with a thin metallic layer that deflects an electron beam onto the detector, giving a very fine detail of the surface features (Figure 8d).

Figure 8 (a) A transmission electron microscope in the imaging facility at The Open University. (b) Diagram illustrating the components of an electron microscope. (c) Transmission electron micrograph of a frog leukocyte (white blood cell). (d) Scanning electron micrograph of a HeLa cell, illustrating that cells are not flat and that their surface has many extensions.

Electron microscopy can only be used in fixed cells. During the sample preparation, heavy metals are used to increase the contrast in the samples, for example to clearly see cellular membranes.

3 The science behind fluorescence and its applications in microscopy

The following section will introduce you to the theory behind fluorescence. Understanding this concept will help you to understand how it is possible to see several different colours in one image taken on a fluorescence microscope.

3.1 Fluorescence is activated by light

What makes fluorescent molecules like GFP ‘glow’ the way they do? To understand this, it is necessary to look at the molecular level and specifically at the arrangements of atoms. Atoms contain a central nucleus around which electrons move in electron shells (or orbitals). The energy state of electrons increases from the innermost to the outer shells.

Fluorescent molecules, also called **fluorophores**, generally contain rings of carbon atoms (called aromatic rings). These molecules can absorb incoming energy (usually in the form of light). As a result of the absorption of energy, electrons within the molecules change from a resting condition called the ‘ground state’ to an ‘excited state’ on a shell further away from the nucleus for a very brief period of time. Eventually, the excited electrons return to their ground state, and as they do this, they emit some of the energy they had originally absorbed as photons of light (Figure 9).

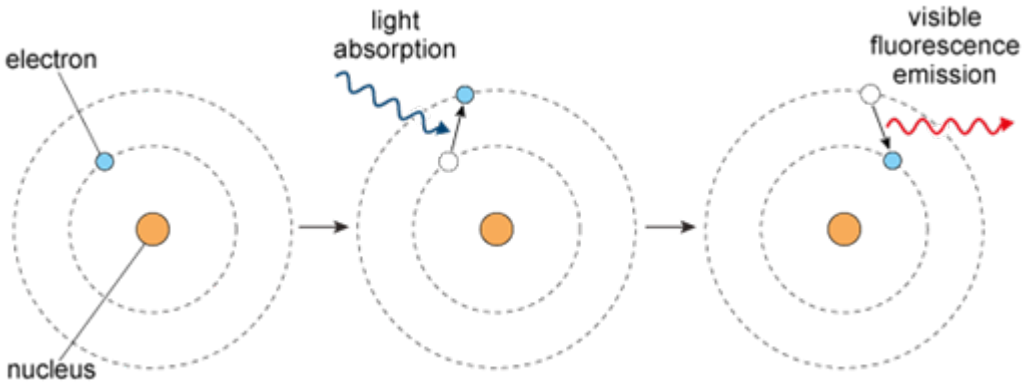


Figure 9 A single atom within a molecule is shown absorbing energy, which excites an electron from its ground state to an excited state. When the electron returns to its ground state, light is emitted.

3.2 The colour of the emitted light depends on the fluorescent molecule

The light emitted by a fluorescent molecule is a different colour to the light that it absorbs. This is because electrons in their excited state lose a tiny amount of energy before they return to the ground state. Fluorescent molecules absorb light at specific wavelengths in the visible spectrum, which is depicted in Figure 10. If you examine the image, you will notice that the wavelength of light increases as the spectrum progresses from blue to red, while the energy of the light decreases. Therefore, the light used to excite a fluorescent molecule (e.g. blue or violet light) has more energy and a shorter wavelength than the light the molecule emits (often green, yellow, or red).

This shift in colour (known as the Stokes shift) is crucial in fluorescence microscopy. It allows researchers to clearly distinguish between the excitation light and the emitted fluorescence, enabling accurate visualisation of specific targets within cells and tissues.

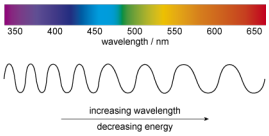


Figure 10 The visible spectrum, showing the colours with their corresponding wavelength in nanometres (10^{-9} m).

If a fluorescent probe absorbed light in the green part of the visible spectrum, what colour range might its emitted light have?

Answer

The emitted light would have a longer wavelength (less energy) than green light, and so would be in the yellow-to-red part of the visible spectrum.

The wavelength of light absorbed by a fluorescent molecule is characteristic of that particular molecule, as is the wavelength of light it emits. The absorption and emission of specific colours by different fluorescent molecules allows researchers to investigate many different aspects of cell biology at the same time within a single biological sample.

In the next section, you will learn how different structures inside cells can be stained differently to obtain the images you will encounter throughout the rest of the course. But before that you will embark on a ‘Journey into a cell’, an immersive exploration that showcases the remarkable capabilities of both fluorescence and electron microscopy.

4 Applying fluorescence microscopy to make cells colourful

You will now see examples of cells being visualised with fluorescence microscopy, and learn about different methods used by researchers to label specific structures inside cells, in order to see them using fluorescence microscopy.

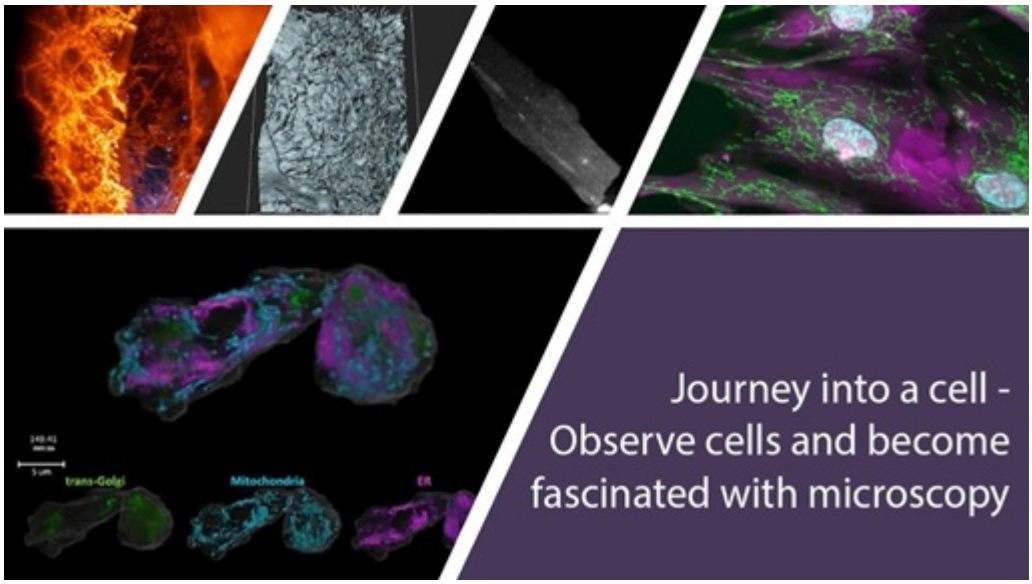
4.1 Journey into a cell

Video 1 provides you with a ‘Journey into a cell’, which uses images and videos to illustrate how cell structures and organelles can be viewed within living cells, and how multiple fluorescent probes can be used within the same cell or tissue to build high-resolution two- and three-dimensional representations. The following sections will explain some of the techniques mentioned in the video in more detail.

Watch the video and then answer the following questions.

Video content is not available in this format.

Video 1 ‘Journey into a cell’



Which technique allows scientists to mark and visualise structures inside living cells?

Answer

Fluorescence microscopy (not electron microscopy).

How can you identify the nucleus in a cell?

Answer

By applying a DNA-binding probe to visualise it by its fluorescence, or by the space it occupies being left ‘dark’ when probes are used that detect other cellular organelles that are present in the cytoplasm.

Can you think of a reason why using more than three colours in fluorescence microscopy might prove difficult?

Answer

It can be difficult to distinguish the colours/organelles stained.

What conclusions can you draw from experiments done with the fluorescent staining of living cells and tissues that you would not be able to draw using fixed material (where the cells/organs were killed before staining)?

Answer

The ability to use living cells and tissues means that you can study cells in their native environment and also examine interactions between cells and how they change over time. You can also observe the movement of organelles and/or molecules inside cells in real time.

What example was given in Video 1 of fluorescence being used to measure the change in concentration of an ion over time?

Answer

Calcium ion changes in the cardiac myocyte.

From your ‘Journey into a cell’, you should now have a good understanding of how fluorescence can be used to study cells and tissues. Some of the techniques mentioned in the video will be explained in more detail in the following sections.

You will now learn about some techniques that are used to stain structures and proteins inside cells before they can be inspected using fluorescence microscopy. You will see examples of these staining techniques later when you inspect cells in the digital fluorescence microscope.

4.2 Using dyes (or probes) to stain structures

You learned about fluorescent molecules (also known as fluorophores) earlier in this course, and that it is possible to visualise several structures inside cells at the same time using fluorophores that emit different colours of light (Figure 11).

Figure 11 HeLa cells labelled with two fluorescent dyes: one that binds to DNA and one that accumulates inside mitochondria. (a) Micrograph taken after illuminating the cells with ultraviolet (UV) light to excite the dye bound to DNA, which then emits blue light. (b) A second micrograph taken after illuminating the cells with green light to excite the dye localised in mitochondria, which then emits red light. (c) The images are merged using computer software to show the fluorescence of both dyes at the same time.

If you want to visualise red fluorescence, like the one in mitochondria shown in Figure 11, why would you use green light to excite the fluorophore?

Answer

Referring back to the spectrum of visible light shown in Figure 10, and the principle of fluorescence, the excitation wavelength must be shorter than the emission wavelength. Green light has a shorter wavelength and more energy, so it can be used to cause emission of red light.

Staining of structures can be done with, for example, **fluorescent dyes**, stains, probes, or labels. These terms are often used interchangeably in science literature. **Fluorescent indicators** change their properties, for example their brightness, depending on their environment. For example, they get brighter when the concentration of a certain ion changes.

The concentration of which ion can be measured by a fluorescent indicator that can show the change of the intracellular pH value?

Answer

The concentration of H^+ ions (protons). An increase in the proton concentration causes a drop in the pH (it becomes more acidic). A decrease in the proton concentration causes an increase of the pH (it becomes more alkaline).

Many fluorescent dyes and fluorescent indicators are designed to be membrane-permeable and can be taken up by living cells by simply immersing a tissue section, or cells grown on a glass coverslip, in a solution containing the dye or indicator, and then rinsing off the excess.

4.3 Immunolabelling

Immunolabelling is a technique used to detect specific proteins or structures in cells or tissues by using **antibodies** — molecules that naturally recognise and bind to particular targets. A so-called primary antibody (yellow Y-shapes in Figure 12a) binds to a target, for example the protein actin. Primary antibodies are raised in different animals. The so-called primary antibody is either directly attached to a fluorescent dye or detected by a second fluorescently labelled antibody (green and red Y-shapes in Figure 12a). When

viewed under a fluorescence microscope, the dye lights up, allowing researchers to see where the protein is located in the sample. A common variation of this technique is **double immunolabelling**, where two different proteins are labelled at the same time using antibodies tagged with two distinct fluorescent dyes, or by using two primary antibodies that were raised in different animal species. These primary antibodies are recognised specifically by different secondary antibodies, labelled with different fluorophores.

Double immunolabelling allows scientists to observe the spatial relationship or co-localisation of different proteins within the same cell or tissue (Figure 12). Immunolabelling can only be performed in fixed and permeabilised cells, because the large antibody molecules would otherwise not be able to enter the cells. By using labels other than fluorophores, immunolabelling can also be visualised with light and electron microscopy.

Figure 12 Double immunolabelling of the cytoskeleton. (a) Illustration of the principle of double immunolabelling using two primary antibodies and two differently labelled 'secondary' antibodies. Note that the epitopes on the primary antibodies, to which the secondary antibodies bind, differ in shape. (b) Double immunofluorescence staining in cells using a primary antibody against actin (the secondary antibody was labelled with a green fluorophore) and tubulin (the secondary antibody was labelled with a red fluorophore). The nucleus is shown in blue.

Double immunolabelling to detect two proteins, A and B, would not work if the primary antibodies for A and B were both mouse antibodies. Can you suggest why?

Answer

Each protein would bind a mouse primary antibody. The anti-mouse secondary antibody subsequently applied to detect the primary antibodies would then bind to both primaries, and so they would both be labelled with the same detection molecule (e.g. the same colour of fluorescence) and it would not be possible to see the separate locations of proteins A and B.

4.4 Expression of fluorescent proteins

Some dyes can be used in living cells, but one of the most powerful and widely used labelling techniques in live cultured cells, and even whole organisms, is to genetically modify them to express a fluorescent protein. Interestingly, certain animals naturally produce fluorescent proteins as part of their defence against predators. One well-known example is the jellyfish *Aequorea victoria*, which produces GFP, a molecule that you have encountered earlier in the course (Figure 13a).

Scientists can take the gene that encodes GFP and fuse it to a gene of interest and then introduce this modified gene into cells (Figure 13b) or even whole animals (Figure 13c and d). The resulting GFP-fusion protein can be visualised in real time under a fluorescence microscope, allowing researchers to track where the protein is located inside the living cell. In most cases, the fusion protein behaves like the original protein, maintaining its normal function and proper localisation within the cell.

Figure 13 GFP expression. (a) The jellyfish *Aequorea victoria* naturally expresses GFP

in the light-emitting organs at the bottom of its bell. (b) HeLa cells expressing GFP on their ER. (c) GFP expressed in neurons of the worm *Caenorhabditis elegans*. (d) A mouse expressing GFP.

You have now learned about the different types of microscopy, the principles of fluorescence and how cellular structures using fluorescence. In the next section you will briefly explores why fluorescence microscopy is considered such a versatile technique in cell biology.

4.5 Similarities and differences between the three types of microscopy

After learning about three types of microscopy that are often used to study cells, and about commonly used different ways to make structures in cells fluorescent, you are now encouraged to test your learning and understanding by filling in the following table and answering the quiz questions.

Using your knowledge from this course, fill in Table 2. To give you an idea how much detail to include, the first column has been filled in. You will find a filled-in version in the answer underneath.

Table 2 Comparison of the three types of microscopy introduced in this course and their applications.

Feature	Light microscopy	Fluorescence microscopy	Electron microscopy
Source of illumination	Visible white light	*Provide your answer...*	*Provide your answer...*
Resolution	~ 200 nm	*Provide your answer...*	*Provide your answer...*
Magnification (maximum)	Up to ~1,000×	*Provide your answer...*	*Provide your answer...*
Staining required?	Often needed (e.g. dyes)	*Provide your answer...*	*Provide your answer...*
Live imaging?	Yes	*Provide your answer...*	*Provide your answer...*
Contrast?	Low (without stains)	*Provide your answer...*	*Provide your answer...*
Is specific labelling possible?	Limited (general stains)	*Provide your answer...*	*Provide your answer...*

Type of image produced	Brightfield image (natural contrast or stained)	*Provide your answer...*	*Provide your answer...*
Sample preparation	Simple and fast	*Provide your answer...*	*Provide your answer...*
Main applications	Basic cell structure, tissues	*Provide your answer...*	*Provide your answer...*

Answer

Here is a filled-in version of the table with information from this course.

Table 2 (completed) Comparison of the three types of microscopy introduced in this course and their applications.

Feature	Light microscopy	Fluorescence microscopy	Electron microscopy
Source of illumination	Visible white light	Specific wavelengths of light (often UV or laser)	Beam of electrons
Resolution	~ 200 nm	~ 200 nm	~0.1–1 nm (much higher due to electron wavelength)
Magnification	Up to ~1,000×	Up to ~1,000×	Up to ~1,000,000 for TEM Up to ~2,000,000 for SEM
Staining required?	Often needed (e.g. dyes)	Yes, fluorescent dyes or proteins	Yes, heavy metals
Live imaging?	Yes	Yes	No (requires vacuum and fixed/dehydrated samples)
Contrast?	Low (without stains)	High (fluorescent signal on dark background)	Very high due to electron scattering
Is specific labelling possible?	Limited (general stains)	Yes, highly specific using antibodies or tagged proteins	Limited; specific structures identified by morphology
Type of image produced	Brightfield image (natural contrast or stained)	Fluorescent image on dark background	Black and white

Sample preparation	Simple and fast	Moderate (labelling, fixing if needed)	Complex, time-consuming
Main applications	Basic cell structure, tissues	Protein localisation, live-cell imaging, molecular tracking	Ultrastructure, organelles, viruses, nanostructures

You are part of a research team investigating the localisation and function of a specific protein in nerve cells. In your studies, you want to study different aspects of this protein. Unless specifically mentioned, the protein has not been genetically modified to carry a fluorescent marker. You have access to three types of microscopes: a standard light microscope, a fluorescence microscope, and an electron microscope. Questions 1-4 explain experiments your research team wants to perform. Based on the features of each microscopy technique (illumination, resolution, sample preparation, and ability to image live cells), which microscope would you choose for each of the experiments and why?

Question 1

Your goal is to identify if this protein is found in ribosomes, which have a diameter of around 25 nm.

Answer

To identify if the protein is localised in ribosomes, using the electron microscope is the most suitable option. Here's why:

- Resolution:
  The electron microscope is the only one of the three types that has a high enough resolution to visualise ribosomes. It has a resolution of 0.1 – 1 nm.
- Specific labelling:
  You would use immunolabelling to visualise the protein in the cell, and you would identify ribosomes by their characteristic appearance.
- Live-cell imaging:
  To identify the localisation, you don't need to work with living cells. Fixing the samples during the preparation for electron microscopy ensures that structures and proteins are found in the place where they were present in the living cell.

Question 2

Your goal is to identify if this protein is present in lysosomes, which have a diameter of 0.5 – 1 µM. Your research team commonly uses a fluorescent dye that you know specifically labels lysosomes.

Answer

To identify if a specific protein is present in lysosomes, which can be identified with a fluorescent dye, using the fluorescence microscope combined with immunolabelling is the most suitable option. Here's why:

- Specific labelling:

  The protein is not tagged with a fluorescent marker, which means you need to visualise it using immunolabelling. Lysosomes can be specifically labelled with the fluorescent dye, so you want to visualise your immunolabelling with fluorescence microscopy. Light microscopy cannot visualise your protein with such high specificity.
- Live-cell imaging:

  For the purpose of this experiment, you cannot use live cell imaging because the process of immunolabelling needs the cells to be fixed and permeabilised.
- Resolution:

  The resolution of fluorescence microscopy is ~200 nm, which is sufficient for visualising lysosomes.

Question 3

Your goal is to track this protein in real time to understand how its behaviour changes in response to different stimuli. For this experiment, the protein has been genetically tagged with a fluorescent marker.

Answer

To track a specific protein in real time within living nerve cells, using the fluorescence microscope is the most suitable option. Here's why:

- Specific labelling:

  The protein has been tagged with a fluorescent marker, which means fluorescence microscopy is required to excite and detect that specific signal. Neither light microscopy nor electron microscopy can visualise tagged proteins with such specificity.
- Live-cell imaging:

  Fluorescence microscopy allows imaging of living cells. Electron microscopy cannot be used for live samples, as it requires the sample to be fixed, dehydrated, and placed in a vacuum. Light microscopy can image live cells but cannot track specific proteins unless general staining is sufficient, which isn't in this case.
- Real-time observation:

  Fluorescence microscopy can capture dynamic processes over time, making it ideal for tracking the movement of proteins or organelles within cells.
- Resolution:

  While not as high as electron microscopy, the resolution (~200 nm) is sufficient for visualising protein localisation and movement within the broader cellular context, especially with the help of time-lapse imaging or even super-resolution techniques if needed.

Question 4

You have already confirmed that cells from individuals with a certain disease express a higher level of the protein you are studying. Now you want to know if the overall cell structure is changed in cells from individuals with the disease. You don't need to visualise the protein itself.

Answer

Changes in cellular structure, without the need to identify specific proteins, is best done with a light microscope. Here's why:

- Specific labelling:

  Light microscopes are not good to visualise specific labelling due to the lower contrast of the samples. However, specific labelling is not necessary in this study, and the sample preparation for light microscopy is often quicker and cheaper than that for the other techniques. That's why light microscopy is often used in pathology labs to study changes in cellular structure in disease development.
- Live-cell imaging:

  There is no need for live-cell imaging in this study.
- Resolution:

  Studying changes in the overall cellular structure does not need a high resolution, so light microscopy is suitable.

5 Why is fluorescence microscopy so versatile?

The remainder of this course will focus on fluorescence microscopy, widely regarded as one of the most versatile techniques for visualising cells and proteins. Here are the key reasons for its versatility:

- **Specificity**: fluorescent dyes or proteins can label specific structures, organelles, or molecules, such as DNA, mitochondria, or individual proteins. Using antibodies (immunolabelling), researchers can target virtually any protein of interest.
- **Multiplexing**: different fluorescent dyes emit light at different wavelengths, thus allowing multiple targets to be visualised simultaneously in different colours within the same sample.
- **Compatibility with other techniques**: fluorescent microscopy could be combined with molecular and genetic techniques to visualise genetically engineered proteins inside cells.
- **Live-cell imaging**: the main benefit of the above compatibility is that researchers can track dynamic processes such as the expression or the movement of a protein, the behaviour of an organelle in living conditions.
- **Enhanced capabilities**: different types of fluorescent microscopy (such as confocal microscopy) offer some important enhancements.
- **Quantification and analysis**: fluorescence intensity can be measured, allowing for quantitative data collection on things like protein concentration, gene expression levels, or ion concentrations.

The following sections provide a bit more insight on the points mentioned above.

5.1 Fluorescence microscopy allows observing specific structures individually and combined

Fluorescence microscopy makes it possible to observe specific cellular structures both individually and in combination. After staining different structures within a cell using fluorophores of different colours, the microscope's separate channels can be used to select specific wavelengths of light to excite each dye one at a time. This allows for the capture of individual images, each showing a single labelled structure (Figure 14a and b, and Video 1). These individual fluorescence images are then digitally merged or overlaid using computer software, creating a combined image that reveals the spatial relationship between all stained structures within the cell or tissue (Figure 14c).

Figure 14 (repeat of Figure 11) HeLa cells labelled with two fluorescent dyes: one that binds to DNA and one that accumulates inside mitochondria. (a) Micrograph taken after illuminating the cells with ultraviolet (UV) light to excite the dye bound to DNA, which then emits blue light. (b) A second micrograph taken after illuminating the cells with green light to excite the dye localised in mitochondria, which then emits red light. (c) The images are merged using computer software to show the fluorescence of both dyes at the same time.

Capturing separate images and merging them allows detailed studies of the localisation of structures and proteins. You will see more examples when using the digital fluorescence microscope yourself.

5.2 Fluorescence microscopy allows visualising

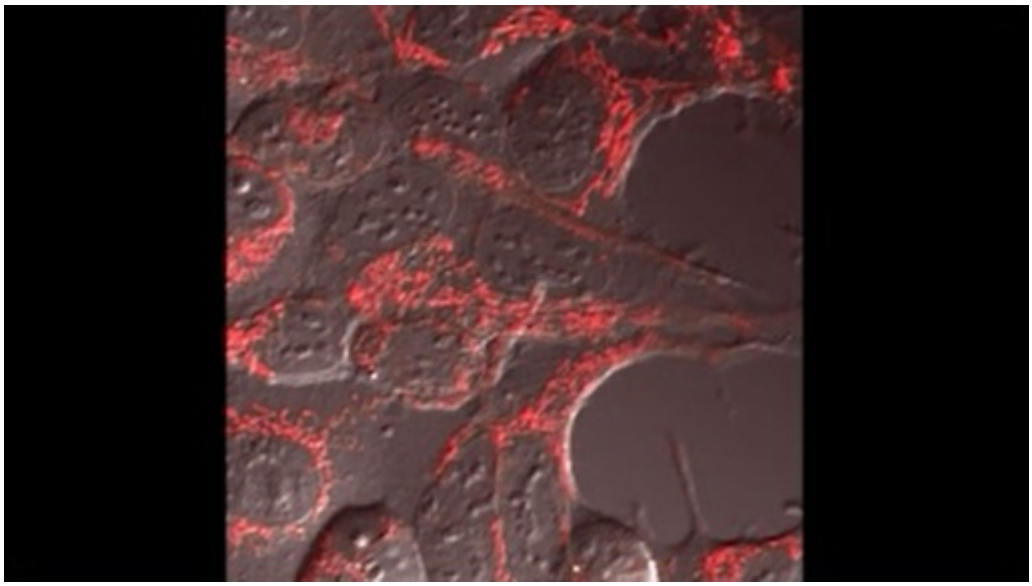
dynamic processes

Fluorescence microscopy is a powerful tool for observing dynamic processes like the movement of mitochondria inside cells (Video 2).

While you might picture mitochondria as many small, oval-shaped structures scattered throughout the cell, fluorescence imaging reveals that in many cases, they actually form an interconnected, network-like structure. This network is often in motion, constantly changing shape and position. As a result, the simplified appearance of mitochondria in static micrographs doesn't fully reflect their complex and dynamic nature.

Video content is not available in this format.

Video 2 Mitochondrial dynamics. Mitochondria in HeLa cells expressing a red fluorescent protein were visualised using fluorescence microscopy. Constant movement of mitochondria can be seen in all cells. The cell towards the bottom left shows changes in the shape of its elongated mitochondria.



Why is it not possible to obtain a video like Video 2, showing mitochondrial movement, when using electron microscopy?

Answer

To be used in electron microscopy, the cells need to be fixed. They are no longer alive and no movement will take place inside the cells.

Visualising these dynamic processes greatly helps with understanding how a cell functions. Comparing how such processes are affected, for example in the context of a disease, can help to understand how diseases develop and affect cellular behaviour.

5.3 Specialised applications: Confocal microscopy

Specialised applications of fluorescence microscopy are continuously being developed, and the examples presented here present only a snapshot – this list will undoubtedly expand in the future. While this course does not cover these advanced techniques in detail, they are introduced to give you an idea of the wide-ranging possibilities fluorescence microscopy offers.

Confocal microscopy (Figure 15a) enhances image clarity by eliminating out-of-focus light, using a pinhole and laser scanning system. This allows for the capture of a series of optical sections – thin, focused slices – at different depths within a specimen. These images form a z-stack (Figure 15b), which computer software can reconstruct into a highly detailed three-dimensional (3D) image of the cell or tissue (Figure 15c). This is particularly useful for studying complex structures in thick specimens, such as tissues or organoids, and provides insights into spatial relationships within cells.

Figure 15 (a) A confocal microscope at The Open University, used to take some of the fluorescence micrographs in this course. (b) Illustration of the process involved in taking optical sections. (c) 3D reconstruction of blood vessels (green), surrounded by astrocytes (shown in blue and red) within brain tissue.

5.4 Specialised applications: Studying molecular dynamics

Fluorescence microscopy can also be used to investigate real-time molecular interactions and dynamics within live cells. Two commonly used techniques are:

- **Fluorescence Resonance Energy Transfer** (FRET): measures the energy transfer between two fluorescent molecules in close proximity, allowing researchers to study protein–protein interactions, conformational changes, and signal transduction pathways at the nanoscale.
- **Fluorescence Recovery After Photobleaching** (FRAP): involves irreversibly bleaching a region of fluorescence and monitoring the movement and replacement of fluorescent molecules into that region over time. This helps in understanding protein mobility, membrane fluidity, and molecular binding dynamics.

5.5 Specialised applications: Super-resolution microscopy

Traditional light microscopy is limited by the diffraction of light, which restricts resolution to around 200 nanometres. **Super-resolution microscopy** breaks this barrier by using advanced optics, fluorescent molecule behaviour, and computational techniques to achieve resolutions as fine as ~25 nanometres. This allows researchers to visualise structures previously invisible with standard fluorescence methods. Key techniques include:

- **Structured Illumination Microscopy (SIM)**: projects a patterned light grid onto the sample and reconstructs images computationally. SIM doubles resolution (to ~100 nm) and is ideal for live-cell imaging due to its low light exposure.

- **STORM (Stochastic Optical Reconstruction Microscopy)**: uses randomly blinking dyes to localise individual molecules and reconstruct ultra-high-resolution images of fixed samples.
- **PALM (Photoactivated Localization Microscopy)**: similar to STORM, but uses photoactivatable fluorescent proteins, achieving comparable resolution (~20–30 nm).

Each technique offers unique advantages, expanding what can be visualised at the molecular level and enabling deeper insight into cell structure and function.

Super-resolution microscopy was able to achieve a resolution of ~25 nm when this course was written, which is far beyond the limit of light microscopy.

> Which cellular structure with a diameter of ~25 nm can you think of, that you could visualise with super-resolution microscopy?
>
> **Answer**
>
> Ribosomes have a diameter of ~25 nm, and you learned earlier that you need the resolution of an electron microscope to visualise these.

When visiting the digital fluorescence microscope, you will see slides of various cellular structures. Prior to that you now have a chance to inspect an interactive 3D cell to get a reminder about the function and localisation of different structures. After that, you'll be introduced to the digital fluorescence microscope.

6 Inspecting structures in a 3D cell

The interactive 3D animal cell below helps you to see the arrangement of animal cell components.

- Select each cell component from the drop-down list to read explanations.
- Click on the micrographs to see the structures in more detail.

Interactive content is not available in this format.

Interactive 3D animal cell

When you have explored the whole cell, attempt the following questions.

Question 1

Based on your observations in the 3D interactive cell, put the following structures into the correct order from the largest to the smallest.

nucleus

mitochondria

nucleolus

secretory vesicle

ribosomes

Match each of the items above to an item below.

(largest)

(smallest)

Question 2

What is the name of the membrane system that begins at the nuclear envelope and spreads throughout the cell?

Provide your answer...

Answer

The endoplasmic reticulum (ER). The nuclear envelope is continuous with the rough ER (RER), which is studded with ribosomes on its outside. Further away from the

nucleus, the RER continues into the smooth ER (SER), which no longer has ribosomes on its membrane. You will see different shapes of the ER when inspecting slides in the digital fluorescence microscope.

Question 3

What are the three main components of the cell membrane?

Provide your answer...

Answer

Lipids, mostly phospholipids (forming a lipid bilayer), membrane proteins and sugars. The sugars are attached to proteins (forming glycoproteins) or lipids (glycolipids). When inspecting cells in the digital fluorescence microscope, you will see that the cell membrane is not smooth, but has many extensions.

Question 4

What is the membrane structure of mitochondria, and which type of microscopy allows studying it?

Provide your answer...

Answer

Mitochondria are surrounded by a double membrane. The inner membrane is folded into structures called cristae. The space between the inner and outer membrane is called the intermembrane space. The membrane structure can be studied using electron microscopy, which has a high enough resolution to clearly show both membranes.

Question 5

Which specialised organelle in animal cells fuses with vesicles to break down worn-out cell components?

Provide your answer...

Answer

Lysosomes fuse with vesicles containing old organelles and ingested material and break down the contents.

7 Using the digital fluorescence microscope

Finally, it's time for you to leave the theory behind and explore real samples using the digital fluorescence microscope (DFM). Start by watching Video 3 which introduces the DFM and how to use it. Please note that the microscope shown in the video contains a different set of slides than the one you will be working with. For example the 'Tutorial slide' featured in the video is labelled 'Training slide A' in your version of the DFM. Despite the difference in slide sets, both DFMs operate in the same way, and the instructions provided in the video are fully applicable to your microscope experience. Each slide includes some text explaining the type of staining used and highlighting the key features you should focus on when exploring the slides. For convenience, you might want to open the DFM in a separate tab so you can refer to the course questions while examining the slides.

Video content is not available in this format.

Video 3 An introduction to the digital fluorescence microscope. (This video mentions other resources from the full OU course – these can be ignored.)

Now go to the DFM (open the link in a separate tab/window so you can easily return here).

Examine **Slide 01: Training slide A – fluorescent dyes**. Inspect the staining for nuclei, mitochondria and cytoplasm using the respective channels (Channels 01–03), then look at the merged images (Channels 04–07) to familiarise yourself with the microscope. Note that if the view doesn't change when clicking on a specific channel, choose a different channel and then try again.

You will be asked to observe colour changes that can indicate a co-localisation of structures (i.e. them being in the same place). People can see colours differently, so you might not see a change to the colour exactly as the course describes. That's fine – you might have a different perception of colours. As long as you observe a clear colour change, this indicates a co-localisation of the respective structures.

After inspecting all channels, answer the following questions in the text boxes provided.

Question 1

How many nuclei are there on **Slide 01: Training slide A – fluorescent dyes**? Which channel did you use to count them and why?

Provide your answer...

Answer

There are seven nuclei (Figure 16). Each cell has a single nucleus, so the nuclear staining in Channel 01 (or in Channels 04, 05 or 06) is a good way to identify the nuclei on the slide.

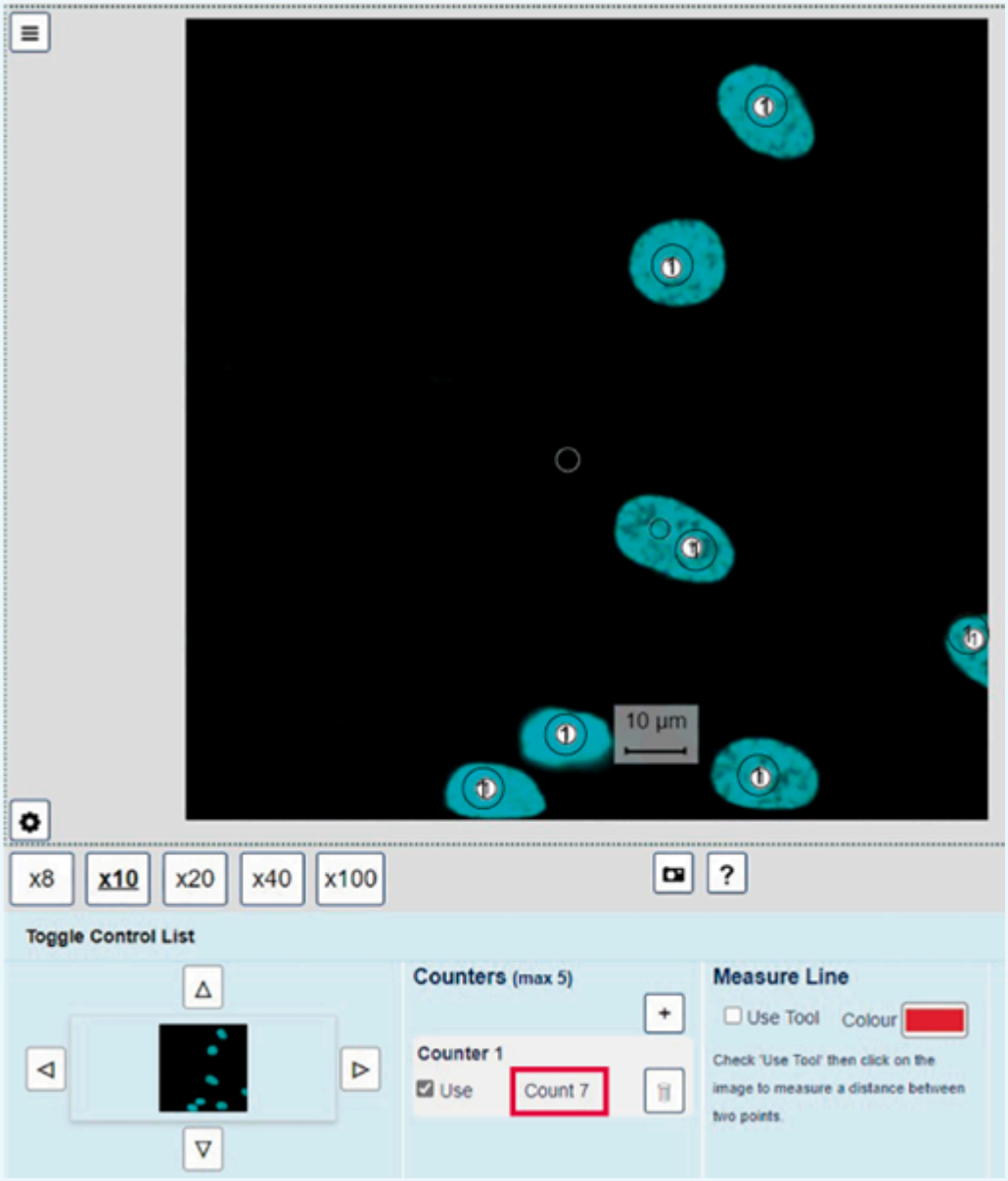


Figure 16 Screenshot illustrating the seven nuclei counted with the counting tool using the ×10 objective and Channel 01.

Question 2

What is the length and width of the nucleus at position X: 3016, Y: 575? For your measurements, assume that the nucleus has an elliptical shape. The length is the longest diameter (distance). The width is measured at approximately a right angle to the length, at the place where the ellipse is the widest. You can see examples of these measurements in the answer. Give your answer to one decimal place. Which channel and objective did you use to make your measurement? You can record your measurement(s) in Table 3 below.

Table 3 Measurements of cell nuclei

Lengths (longest lengths) to 1 decimal place (µm)			Widths (smallest lengths) to 1 deci	
Provide your answer...	*Provide your answer...*	*Provide your answer...*	*Provide your answer...*	*Provid*
Mean =	*Provide your answer...*		Mean =	*Provid*

Answer

Here's a completed version of the table.

Table 3 (completed) Measurements of cell nuclei

Lengths (longest lengths) to 1 decimal place (µm)			Widths (smallest lengths) to 1 decimal place (µm)		
18.7	18.3	18.9	12.4	12.5	12.8
Mean =	18.6		Mean =	12.6	

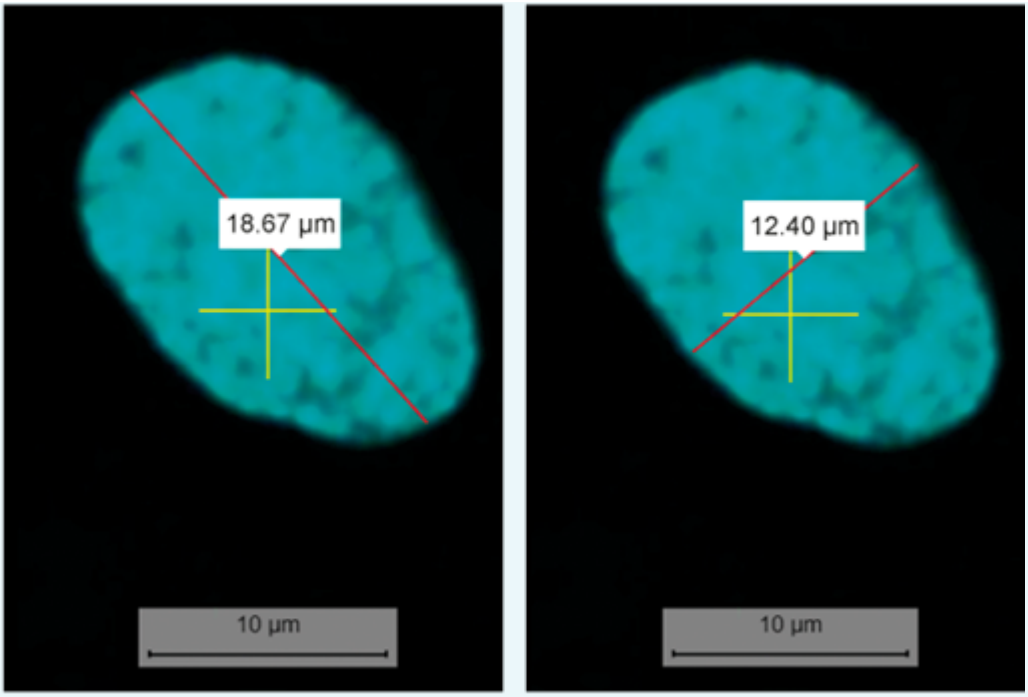


Figure 17 Measurements of (a) the length and (b) the width of the nucleus at X: 3016, Y: 575 using Channel 01 and the ×40 objective.

The measurement should be done in Channel 01 because it only shows the nuclei, thus clearly identifying the borders of the nucleus.

The largest objective that shows the whole nucleus should be used to make the most accurate measurement. From our experience, this was the ×40 objective. This may differ depending on your screen size.

Question 3

To develop your skills in making observations in micrographs, inspect **Slide 01: Training slide A – fluorescent dyes** and take notes about the appearance of the mitochondria and their localisation. Include information about which channels you use to make these observations.

Provide your answer...

Answer

Mitochondria form a tubular network (Channel 02) that spreads through the cytoplasm (Channels 06 and 07). They are absent from the nucleus (Channel 06 and 07; or flicking between Channels 01 and 02). In a few places, mitochondria can be seen in an area with only weak staining for the cytoplasm (e.g. X: 3731, Y: 2060; Channel 06).

Question 4

Now inspect **Slide 02: Training slide B – EGF receptor (cell membrane)** and practise determining if two structures co-localise, meaning they are present in the same place in a cell. Co-localisation can be identified by a colour change in the fluorescence micrographs (e.g. co-localisation of a green and a red structure results in a yellow colour; co-localisation of green and purple structures results in white – keep in mind though, that you might perceive any change to be a different colour).

To practise identifying co-localisation, go to the positions listed in the first column of Table 4 and observe the colour at this position in Channel 08. Use this information to state which structures might overlap at this position. Record your observations in Table 4 below. Note that one example answer is provided.

Table 4 Observations to identify the co-localisation of cellular structures

Position	Colour	Co-localisation	Explanation
X: 1963 Y: 714	yellow	microtubules and ER	The yellow colour indicates overlap of Channel 03 (microtubules) and Channel 04 (ER)
X: 2124 Y: 2586	*Provide your answer...*	*Provide your answer...*	*Provide your answer...*
X: 2095 Y: 2082	*Provide your answer...*	*Provide your answer...*	*Provide your answer...*

Answer

Here's a completed version of the table.

Table 4 (completed) Observations to identify the co-localisation of cellular structures

Position	Colour	Co-localisation	Explanation
X: 1963 Y: 714	yellow	microtubules and ER	The yellow colour indicates overlap of Channel 03 (microtubules) and Channel 04 (ER).
X: 2124 Y: 2586	red	none	The ER does not co-localise with structures nearby, e.g. the cell membrane (purple) or microtubules (green).
X: 2095 Y: 2082	white	cell membrane and microtubules	The white colour indicates co-localisation of Channel 01 (cell membrane) and Channel 03 (microtubules).

Question 5

In **Slide 06: Endoplasmic reticulum (ER)**, compare the distribution of the ER in the cells at positions X: 1400, Y: 2700 and at X: 3800, Y: 1600. Considering what you've learned so far and comparing to what you've seen in the other slides in the DFM, which appearance is typical for the ER?

Hint: use Channels 01 and 05 to answer the question.

Provide your answer...

Answer

The ER forms a network distributed throughout large parts of the cytoplasm in the cell at X: 1400, Y: 2700. In contrast, the ER is only present around the nucleus in the cell at X: 3800, Y: 1600. A distribution throughout the cytoplasm is more typical for the ER.

Channel 01 clearly shows the ER surrounding the nucleus in the cell at X: 3800, Y: 1600, better than the merged view in Channel 05. The ER network can be seen in Channel 05.

Question 6

This is more of a fun, light-hearted question. In **Slide 07: Golgi apparatus**, which nucleus resembles an emoji of a face with an open mouth: