Activity 3: Frontiers in light microscopy

In the final Activity, we will scratch the surface of the fascinating new developments in light microscopy, which nowadays allow scientists to see things that nobody has ever seen before.

Breaking the barrier: how we outsmarted physics

Remember Abbe's fundamental resolution limit of light microscopy, due to which any objects closer than ∼0.2 µm in space cannot be distinguished? Well, the 2014 Nobel Prize in Chemistry was awarded to the three pioneers who were not cast down by laws of physics. Of course, Abbe's limit still holds, but those extraordinary scientists (along with many others) found a way around it and developed methods now collectively known as **super-resolution fluorescence microscopy**. The Nobel Prize website gives an excellent summary of these techniques and the history of their development. Have a read through it, and we will then have a closer look at one of these techniques known as single-molecule localisation microscopy.

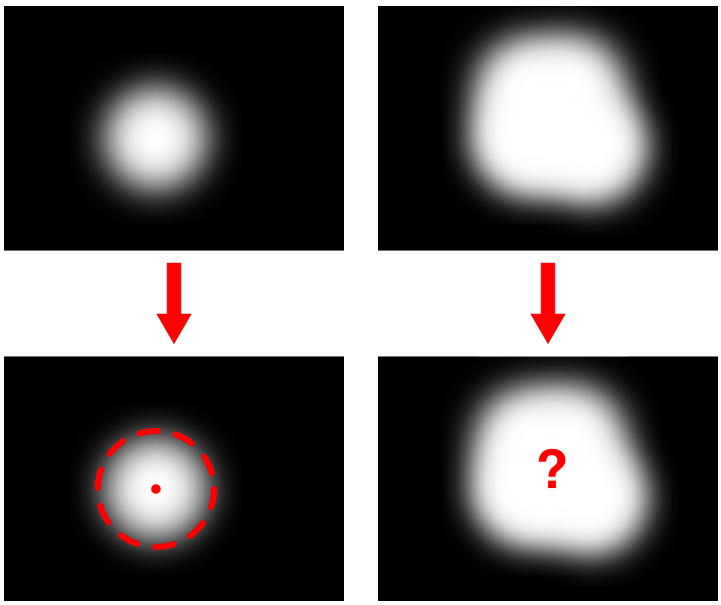
Single-molecule localisation microscopy

The problem that Abbe's limit imposes is not absolute – it is relative, as is the concept of resolution. When we have a single light emitter, we know enough about the properties of Airy disks to be able to determine the position of the source quite accurately (Figure 1a). However, when many emitters are close (closer than Abbe's limit) to each other, their images overlap and blend, making it impossible to figure out what is going on (Figure 1b).

The trick that **single-molecule localisation microscopy (SMLM)** uses to bypass this is to **separate** these **emitters in time** rather than in space. In other words, only a few emitters that are far enough apart are visible at a time. After their image has been recorded, they are turned off, and a new set is turned on. This operation is repeated over and over, generating a movie of flashing dots. If all the frames in this movie were just superimposed, we would get our conventional diffraction-limited micrograph. However, if each frame is analysed individually, the light peaks in it can be used to determine the localisation of each fluorophore with greater accuracy. The resulting reconstructed image has a much higher resolution, up to several nanometers. This is a great animation of the principle of SMLM.

Blinking fluorophores

As described above, SMLM is based on the ability of fluorophores to be switched on and off, often referred to as **"blinking"**. There are many ways in which this is



(a) For a single emitter, its position can be determined rather accurately from its diffraction-limited image.

(b) Several emitters that are closer than Abbe's resolution limit are impossible to resolve with conventional microscopy.

Figure 1: Consequences of Abbe's resolution limit.

achieved (hence, there are many different types of SMLM), using both organic fluorophores and fluorescent proteins. Broadly, the most widely used SMLM techniques nowadays utilise two principles: activation by light or by binding events.

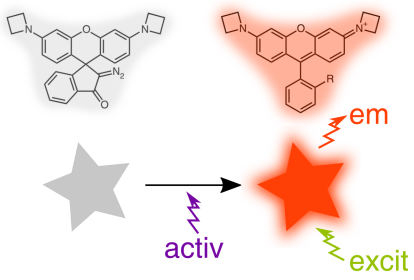
Activation by light. Activation by light includes

- **photoactivation**, where a non-fluorescent molecule is irreversibly activated (Figure 2a);
- **photoconversion**, where a fluorescent molecule irreversibly changes its emission spectrum (Figure 2b); and
- **photoswitching**, where a molecule can be reversibly switched between the fluorescent and non-fluorescent states (Figure 2c).

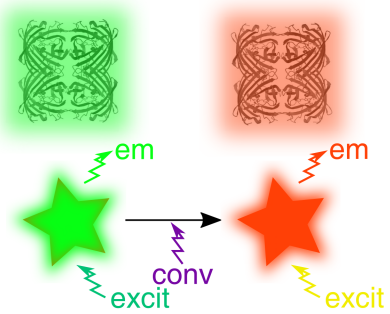
In the case of photoactivation and photoconversion, the molecules are then switched off irreversibly through **bleaching** – a process where strong excitation leads to changes in chemical structure of a fluorophore, rendering it non-fluorescent.

The change in fluorophore behaviour occurs when it encounters a photon of a special activating/converting/switching wavelength. Therefore, for SMLM, the sample is illuminated by both the excitation light and the activating/converting/switching light.

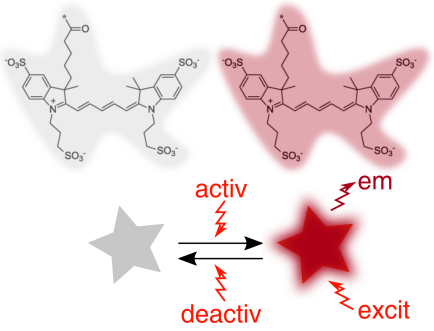
Question 1 How do you think the blinking behaviour can be controlled to yield a low enough number of active fluorophores in each frame?



(a) Photoactivatable dyes and proteins, e.g. photoactivatable Janelia Fluor 549.



(b) Photoconvertible dyes and proteins, e.g. Eos protein (structure from Nienhaus et al. (2005)).



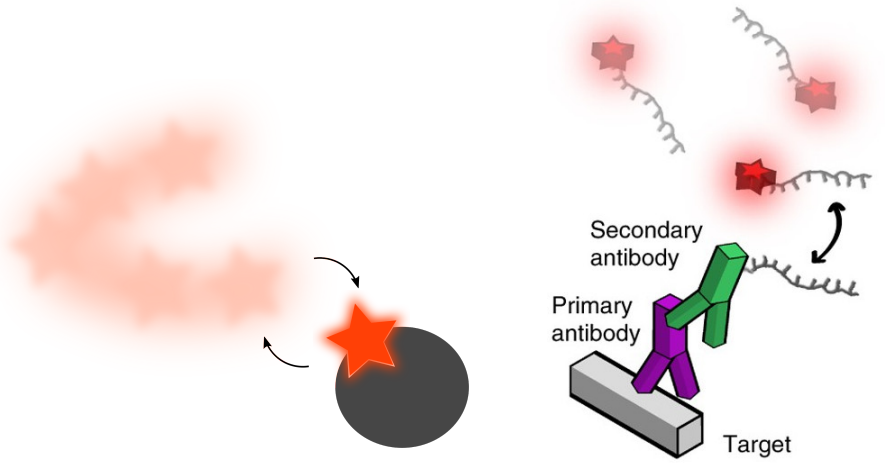
(c) Photoswitchable dyes and proteins, e.g. Alexa Fluor 647.

Figure 2: Types of fluorophores used for light-activated SMLM. Shown are structures of example fluorophores. activ, activation, excit, excitation, em = emission.

Activation by binding. When fluorophores freely diffusing in solution are imaged with suitably long exposure, they do not generate a sharp image – instead, just like in long-exposure photography, all we see is haze. However, once a fluorophore is immobilised, it will produce a signal (Figure 3a). In addition, some fluorophores increase in fluorescence intensity upon binding to a substrate, such as DNA or membranes.

These two phenomena are used in a range of SMLM techniques, collectively known as **PAINT (Point Accumulation for Imaging by Nanoscale Topography)**. This method relies on fluorophores, sometimes conjugated to suitable ligands, **reversibly binding** to the structures of interest (Figure 3a). For example, the fluorescent dye Nile Red can be used as a PAINT probe for membranes. Another ingenious variation is **DNA-PAINT**, where the protein of interest is labeled with an antibody that has a single-stranded oligonucleotide attached to it. The complementary imager strand, conjugated to a fluorophore, is used as a reversibly binding probe (Figure 3b).

Question 2 How do you think the blinking behaviour can be controlled to yield a low enough number of active fluorophores in each frame in simple PAINT imaging?



(a) PAINT relies on the increase in signal intensity that occurs when a fluorescent molecule binds its substrate.

(b) The principle of DNA-PAINT (Schnitzbauer et al. 2017).

Figure 3: Blinking in PAINT arises through reversible binding of fluorescent probes.

What additional parameter can be tuned in DNA-PAINT?

Single-particle tracking

The goal of SMLM is to produce a high-resolution image by recording the position of as many fluorophores bound to the structure of interest as possible. This is normally done in fixed samples, since that allows the imaging to proceed for a long time without the cells or the contents within them moving or stirring. In contrast, **single-particle tracking** utilises the same principle of precise localisation of dispersed molecules, but in **live cells** and with a different purpose. Instead of turning the fluorophores off as soon as we know their position, they are kept on for as long as possible, and their **movement** within living cells is **followed and analysed**. Physical models of diffusion can help to extract useful information about the properties of the tracked molecules, such as whether they are diffusing on their own or interacting with a partner, if their movement is directional (e.g. along a strand of DNA), or whether it is affected by the local environment in the cell (e.g. molecular crowding).

What can we see now?

Although methods for determining the structure of proteins and other macromolecules have existed for many decades, their precise arrangement in cells and the ways in which they work together in an endogenous environment have been obscure. Super-resolution microscopy and single-particle tracking have enabled scientists to bridge that gap. These techniques have revolutionised microbiology and virology, molecular neuroscience, chromatin biology and epigenetics, membrane biology and many other fields. You can learn more about that on this amazing resource by

ONI[1].

How to see what happens inside a mouse's head

In the last two decades, extremely **compact** and **lightweight** fluorescence microscopes, or **miniscopes**, have been developed. Their size has for the first time enabled researchers to mount them **directly onto small animals**, such as birds and mice. The lens of such a microscope can be surgically inserted right into the animal's brain, allowing the observation and recording of spatiotemporal activity of many neurons. In this way, the animal's behaviour is not restrained by the method of investigation, opening up a wealth of possibilities for correlating behaviour with neural activity.

Gradient Refractive Index (GRIN) lenses

So far we have talked about imaging single cells or thin tissue slices. Most of the light passes through these samples, and only a very small fraction is absorbed or scattered. We learned in the first Activity that this interaction between light and matter produces contrast in microscopic images, and that one of the problems of simple bright-field microscopy is that there is not enough absorption and scattering. However, in a physiological scenario, the many layers of cells located on top of each other lead to an opposite effect. Therefore, the issue with **deep-tissue imaging** in general, and with imaging the brain in particular, is the **depth of light penetration**. Even with special techniques, the maximum penetration into uncleared brain tissue is about 1 mm.

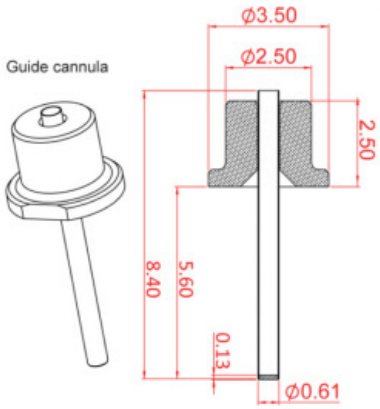
The solution to this problem is to insert the objective of the microscope inside the area of interest in the brain. If you have seen a traditional microscope, you are probably now imagining a huge hole in the skull and flesh of a poor mouse, which has to accommodate an objective about a centimeter in diameter. Indeed, the objective lens is required to collect the light, alongside additional optics within the barrel that will transmit the image further. Fortunately for mice, this is not how it is done.

The technology that allowed deep-tissue *in vivo* imaging to become reality is **Gradient Refractive Index**, or **GRIN**, **optics**. As the name suggests, such devices have a variable refractive index. A GRIN lens used for miniaturised microscopy typically is a rod that has a high refractive index near the central axis and a lower index around the edges (Figure 4a). Such a lens is able to bend light owing to this **gradient** rather than the surface curvature (Figure 4b). As a result, the GRIN lens has several advantages.

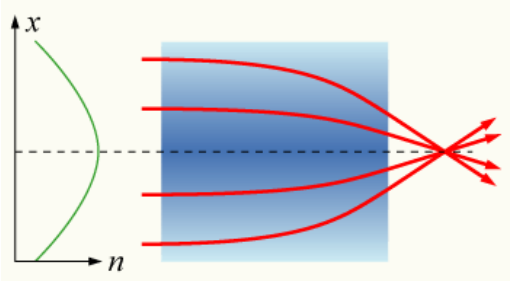
- The **rod-shaped** lens is able to **relay** the light it gathers throughout the length of its body, as opposed to a small conventional lens that needs space and/or additional optics behind it to do that.

[1]Oxford NanoImaging – the company that makes the smallest super-resolution microscope in the world!

- The **optical power** of the GRIN lens can be **tuned** by the **gradient** of the optical density, as well as its **thickness** and **length**. For superficial imaging, lenses with a diameter of about 1 mm are generally used, while for deep tissue penetration, lenses 0.3-0.5 mm in diameter are common.
- The high optical power combined with the flat surface of the lens enable it to have a very **short working distance (0.1-0.2 mm)**. This means that light from this distance can efficiently penetrate the tissue and reach the objective.



(a) Schematic and dimensions of a rod-shaped GRIN lens. (Bocarsly et al. 2015).



(b) Ray diagram of light, focused by a GRIN lens. A schematic plot of the optical density n versus the radial coordinate x is shown. Image courtesy: Piachoo v2, Wikimedia Commons.

Figure 4: Properties of GRIN lenses.

Miniscope design

Figure 5 illustrates a typical setup of a fluorescence miniscope. It is very reminiscent of a conventional fluorescence microscope, with the excitation source and filter, a dichroic mirror, an emission filter and a detector, but is much lighter – the model shown weighs about 3 grams. Another key difference is, of course, the GRIN objective and relay lenses.

What can we see?

What possibilities does such an approach offer? Miniscopes can be used in a variety of ways, but we will focus here on the advantages it has when applied in neuroscience.

1. Observing events happening on a **cellular level** within **living subjects**.
2. The subjects can **move freely**, exhibiting **natural behaviour**.

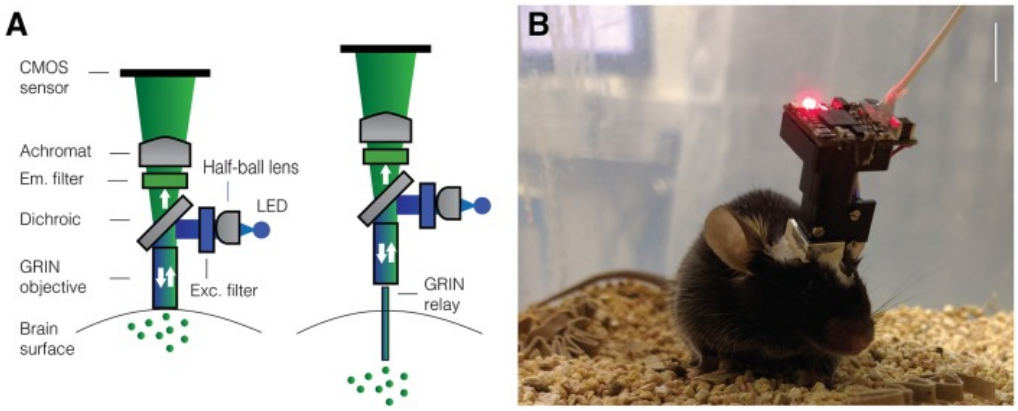


Figure 5: A. A typical design of a miniscope. Notice the GRIN lenses that allow both to deliver the excitation light to the brain and to collect the emitted light. The miniscope on the left is used for imaging the surface of the brain, while the thinner GRIN relay lens of the miniscope on the right can be inserted deep into brain tissue. B. A mouse carrying a 3-g UCLA miniscope. Scale bar, 10 mm. Image from Aharoni and Hoogland (2019).

3. Regular time-lapse imaging of the same cells in the same subject for **months** and even more than a year.

The specific applications of this technique depend, of course, on where the light (the fluorescence emission) is coming from, i.e. from the labeling strategy.

Monitoring vascularisation

Some fluorescent dyes (e.g. fluorescein) can be added directly into the bloodstream of animals, making their vessels fluorescent. Furthermore, cells (mostly red blood cells, which are most abundant) can be seen as shadows on this fluorescent background. As a result, on short timescales, the speed of blood circulation can be measured in different conditions, while formation and stability of vessels can be monitored in the long term. For example, in this video (this link will download it onto your computer), the bloodstream in a particular area of the cerebellum, associated with locomotion, is shown as the subject mouse walks or runs (Ghosh et al. 2011).

Question 3 Can you think of any other applications where monitoring blood vessels and blood flow could be interesting and/or useful?

Tracking neuron activity with calcium sensors

Calcium is a very important player in **signal transduction** in neurons. When the electric signal (action potential) travels along the neuron and reaches its tip, it needs to trigger the release of special chemicals, neurotransmitters, that will pass the signal on to the next neuron. The first step in this process is the influx of calcium, which subsequently leads to release of neurotransmitters. Therefore, calcium can be used as a marker of neuronal activity.

In Activity 2, we learned how a fluorescent calcium sensor called **GCaMP** works. The GCaMP sensor is a protein, and therefore the gene encoding for it can be inserted into live animals, using generic engineering tools. This video (again, this link will download it onto your computer) shows neuronal activity in the CA1 area of the hippocampus as the mouse explores a circular arena (Ziv et al. 2013). Hippocampus plays a major role in learning and memory, as well as spatial memory and navigation. Therefore, correlating firing patterns of individual neurons with behaviour of animals in new environments has an exciting potential to elucidate the mechanisms of memory formation.

References

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