Putting the Lumen into Luminescence: Fluorescence Microscopy

The cell: an amazingly complex object, so crucial to our existence that it has been the subject of intense research for centuries, yet one which is surprisingly difficult to scrutinise. James Bennett examines the recent contributions of physics to the field of fluorescence microscopy.

Hooke's `Microscopical Pores'

In modern times it is easy to take the existence of cells for granted, but it was not always so. The smallest entities capable of sustaining all the mechanisms of life were first observed in 1665 by Robert Hooke, who published the first great work on microscopy, *Micrographia*[1].

> *"I no ſooner diſcern'd theſe (which were indeed the firſt microſcopical poreſ I ever ſaw, and perhapſ, that were ever ſeen, for I had not met with any Writer or Perſon, that had made any mention of them before thiſ) but me thought I had with the diſcovery of them, preſently hinted to me the true and intelligible reaſon of all the phenomena of cork."*
> *-Robert Hooke, 1665*

We owe our current understanding of cells and their bewildering array of functions to the dedicated biologists who have studied them ceaselessly, but underpinning these great bodies of knowledge are the significant contributions of those who envisaged, designed and constructed the imaging technologies which are so critical to successful cell biology.

The key problem is this: the vast majority of cells are immensely small, being only a few tens of millionths of a metre across, and essentially transparent. To compound the problem, cells also contain a phenomenal number of sub-components, *and* these components are dynamically reshaped over timescales ranging from microseconds through to decades[2].

Successful imaging techniques need to circumvent these drawbacks in order to allow any hope of probing cellular functions.

One solution is fluorescence microscopy.

Troubles with Transparency

Before we consider the importance of fluorescence techniques we should take a detour to better understand the problems that `traditional' micrographers are faced with.

With simple light microscopes we can choose two modes of imaging. The first, transmission imaging, is similar in concept to a slide projector or stained glass window. In this case the amount of light which reaches your eyes, and the colour you see, is determined by *absorption*. Alternatively, we can use a reflection method: light is incident on the sample and a portion of the reflected light enters your eyes, carrying information about its size, shape and other characteristics. This image, like the print you see on this page, is essentially due to *scattering* properties.

Unfortunately, cells are generally transparent, meaning that absorption is weak, making cell imaging by transmission microscopy quite difficult. It is possible to boost the contrast of such an image by staining or using unconventional illumination, but these techniques come with a price.

Whilst immensely useful for improving contrast in optical transmission microscopy, stains are generally unable to be used in the study of dynamic cellular processes because of their cytotoxicity, not to mention carcinogenicity.

Contrast may also be increased by exploiting the natural absorption bands of nitrogen and carbon in the cell, but doing so requires the creation of specific soft X-ray wavelengths, the so-called 'water window', which are inconvenient to work with because of their propensity for causing cell damage in both sample and scientist[3].

Reflection microscopy fails to capture significant amounts of detail because it relies on the existence of sharp changes in refractive index. The refractive index of a material compares the speed of light in it to light speed in space, where the latter is a well-known constant[4].

Wherever two media meet light can be split into reflected and transmitted components, with the brightness of each determined by the difference in refractive indices across the boundary between the media. Since the refractive indices of cellular components generally do not vary far from that of water[5] reflections are dim, so reflected light images are of low contrast and intensity.

The only ways of getting around this suffer from the same limitations as encountered above; staining the sample for increased reflectivity is very often fatal to it, as is simply increasing the power of the illumination. The latter phenomenon of light-induced cell death is known as phototoxicity, or, more quirkily, as `optocution'[6].

What we *really* need is some method which doesn't hinge on the optical properties of the cell...

Light in the Darkness

A multitude of oceanic creatures, predator and prey alike, have been imbued with a fantastic power of luminescence which allows them to be located even in the darkest of environments. Fluorescence microscopy gives this same power to specific cellular components, right down to the single-molecule scale.

Attaching small fluorescent markers to objects is a simple but powerful experimental technique, because it effectively inverts the problems which plague transmission and reflection microscopy. Since the imaging light source is now *inside* the sample the cell's high transmissivity becomes a desirable property, rather than a hindrance.

Physics of Fluorescence

The physical principles behind fluorescence microscopy are largely independent of the particular light-emitting marker used, termed the fluorophore.

Fluorescent markers don't have on/off switches; they exist either in a low-energy ground state or a high-energy excited state. Fluorescence is not due to occupation of either of these states: rather, light is the cause of an upward transition (ground to excited) and the result of a downward transition, or decay. Once a marker is in an excited state the downward fluorescent transition becomes possible.

We can view these transitions as being accompanied by the absorption or emission of a 'packet' of light energy, known as a photon. Fluorophores have obviously been tutored by Master Yoda, because a fluorophore either absorbs a whole photon or nothing.

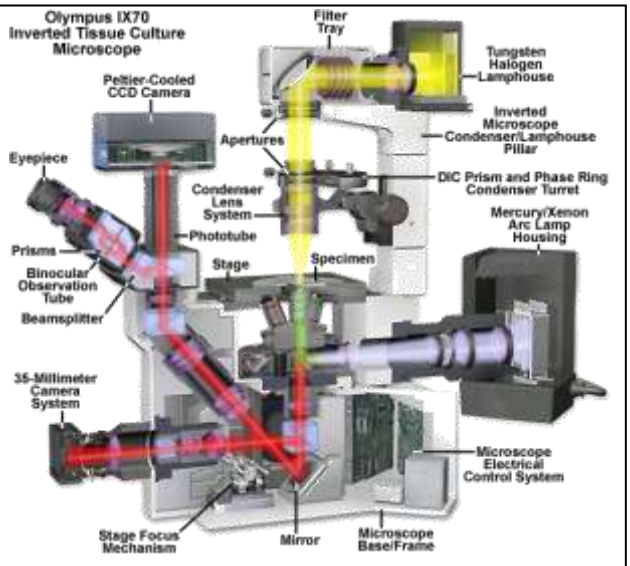
"Do or do not: there is no try." -
Jedi Master Yoda

The choice between these alternative outcomes is governed by quantum mechanics: only photons of some very specific energies are allowed to be absorbed, whilst all others are rejected. Which energies are palatable is determined by the internal structure of the fluorophores.

The photons required to excite fluorescent markers are supplied by the pump laser. Once this energy is absorbed, exciting the fluorophores, some is dissipated as heat. The majority remains to be re-emitted during the downward transition– fluorescence.

Near the turn of the last century Planck determined that the energy carried by a photon is inversely proportional to its wavelength, so a small amount of energy loss translates to an increase in the fluoresced wavelength relative to the pump[7]. This makes it possible to block out the latter with some cunningly-designed filters. What is left is a clean fluorescence signal emanating from within the cell, with a colour characteristic of the particular fluorophore used[8].

You would be forgiven for thinking that a fluorescence microscope might require no external light source. In practice it is usual to retain all of the components of a regular light microscope because this increases flexibility and simplifies the initial stages of cell location and identification. Somewhat counter-intuitively, a fluorescence microscope has an additional light source. This light, often a laser, is known as the *pump* (Image credit 2).



Direct illumination of a fluorophore is not always necessary: there are underhand and ingenious alternatives for delivering energy to them.

Attack of the Acronyms

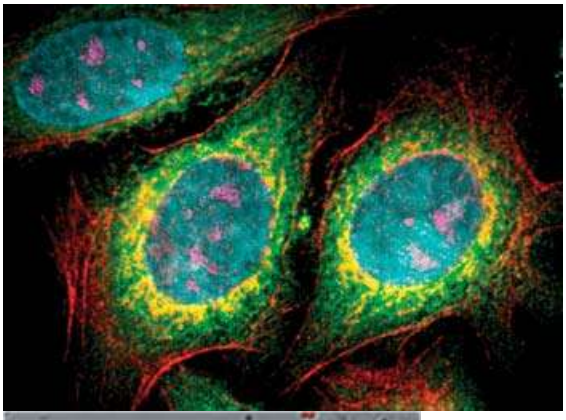
Biologists, like most scientists, are prone to overusing acronyms. When considering fluorescence microscopy there are two to bear in mind: TIRF and FRET.

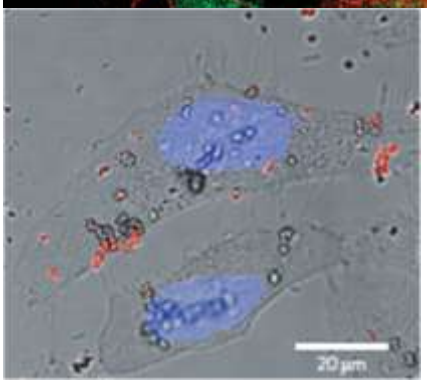
The first of these, total internal reflection fluorescence (TIRF), employs an obscure electromagnetic effect to allow sensitive measurements of the distance between fluorophore and microscope slide.

Total internal reflection occurs when the refractive index difference between two media is so large that their interface begins to act like a one-way mirror. You may have noted this effect whilst swimming, reading your watch at an angle (try it!) or enjoying a spectacular rainbow.

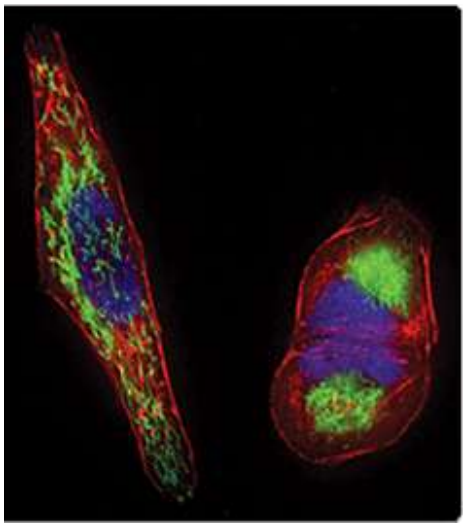
This simple picture doesn't tell the full story: electromagnetic theory requires that some light manages to penetrate through to the other side, but only in a very narrow region immediately beyond the interface[9]. This so-called evanescent (exponentially decaying) light wave can excite a fluorophore

Cells in Technicolour





Fluorescence microscopy is one of the workhorses of the modern cell biology world because of its fantastic flexibility and image quality.
Left: the internal structure of cells revealed by quantum dots. The cytoskeleton is labelled in red and green, the nucleus in blue, particular nuclear proteins fluoresce pink and mitochondria, the cellular powerhouses, show up as yellow (Image credit 3).
Right: fluorescent proteins and dyes label the workings of human cancer cells. In this image the green and red fluorescent proteins are localised to attachment points in the cytoskeleton and the blue fluorescence is due to a DNA-binding dye (Image credit 4).
Left: a transmission microscope image of similar human cancer cells overlaid with the red fluorescence signal due to single nanodiamonds which have been ingested by the cell (Image credit 5).



just like its regular (propagating) cousin[10].

TIRF uses this effect to create fluorescence by letting the pump light undergo total internal reflection from the boundary between the microscope slide and the cell. The small leakage of energy into the evanescent field powers the luminescence. Furthermore, because this field rapidly decays away from the slide it is possible to determine a fluorophore's distance from it by simply measuring the amount of luminescence[10].

A second method, FRET, is capable of showing exactly where molecular interactions are occurring[11].

Förster resonance energy transfer (FRET) requires two different types of fluorophores selected in a very specific manner: the photons which may be emitted by one, which we shall call A, must have energies which coincide with those allowed to be absorbed by the other, labelled B[12]. We say that the emission and absorption spectra overlap. If this condition is satisfied then an appropriately-selected pump laser will excite A but not B: remember, absorption of a photon is an all-or-nothing process, and the energy of a photon required to excite A is too large for B to incorporate[7].

The overlap between the emission and absorption spectra of A and B also allows energy transfer between them *without photons*, provided that they remain in close proximity: typically within ten billionths of a metre[12]. After A absorbs a photon it transfers a portion of this energy to B, which then emits light at a longer wavelength[11].

Now suppose we wish to observe when and where an interaction between two proteins occurs. Simply attach marker A to one protein and B to the other. With no interaction taking place only the fluorescence generated by A will be observed, because B is too far away for efficient energy transfer. The moment that longer wavelengths are detected we know that an interaction is taking place and can quickly identify the location.

TIRF, FRET and other related techniques are often used in conjunction with more conventional microscopy since they provide complementary data, giving us a clearer view of cellular processes.

Microscopist or Pyrotechnician?

Surprisingly, the same physics that determines the colour and behaviour of our fluorophores is also responsible for the brilliance and variety of colours seen in fireworks. A pyrotechnician carefully laces their fireworks with metals and other compounds to give the desired colours: carbon gives gold, aluminium white and so forth. This makes fluorescence microscopists, in a sense, pyrotechnicians, choosing which fluorophores to use to achieve the effect they desire.

The number of fluorescent markers available to the modern scientist is remarkable, but the majority may be

loosely categorised into three flavours: organic fluorophores, inorganic nanostructures and organic nanocrystals.

There is a fourth category, small organic dyes, but since these are generally cytotoxic we shan't consider them here[13].

The organic compound club does not have stringent membership criteria: any material composed primarily of carbon, perhaps with traces of hydrogen, oxygen, nitrogen and sulphur, is classified as organic[14]. This terminology has the counterintuitive feature that an interstellar diamond, being carbon-based, is `organic' even though it is light-years from the nearest lifeform!

Disco(soma and) Jellyfish

The mainstay of biological fluorescence microscopy is the organic fluorophore. Being of biological origin means that most cells are perfectly comfortable with their presence, although there are exceptions.

Most ubiquitous amongst fluorescent markers is green fluorescent protein, lovingly referred to as GFP, which was drawn from the genome of a jellyfish[15,16]. Red, yellow and orange fluorophores have been found in members of the *discosoma* genus of corals[8,15], and many others besides: this list is by no means exhaustive!

These markers are also quite bright, allowing for high signal-to-noise ratios when imaging. However, their real forte is flexibility of production, delivery and localisation. A full review of the possibilities in these areas would constitute a textbook, so we shall restrict ourselves to a few specific examples.

Cells industriously synthesise proteins, reading the relevant instructions from blueprints encoded in their DNA[17]. Researchers are able to selectively attach extra sequences which code for fluorescent proteins. The result is that every time the cell reads the modified gene, intending to create one protein, it also manufactures a fluorophore which is chemically bound to the desired product[16].

The 2004 experiments of Shaner and colleagues[8] used this to spectacular effect. Beginning with fluorophores derived from wild creatures, this team were able to produce red fluorescent proteins (RFPs) which were chemically simpler and less susceptible to changes in luminescence than their predecessors.

They artificially and rapidly evolved the original genes coding for RFPs in the laboratory, then inserted them into human cancer cells. These cells began to produce fluorescent α-tubulin, a protein involved in the formation of the cell's internal skeleton, the cytoskeleton. More importantly, the `chimeric' tubulin molecules were readily incorporated into the cytoskeleton, allowing it to be imaged by fluorescence microscopy[8].

This same piggy-backing mechanism is crucial to the success of FRET[11].

Organic fluorophores may be delivered to the cell in other ways: they might be packaged in vesicles which are then absorbed by the cell, bonded to antibodies which latch onto particular structures or attached to signalling molecules which allow fluorophore uptake by only specific cells[16].

The chief drawback of these fluorescent proteins and related compounds is their low photobleaching threshold. After a short observation time, typically less than five minutes, the fluorophores become degraded to the point where they no longer respond to the pump light. This is where the other categories of fluorescent markers come into their own[18,19].

Going Dotty

Inorganic nanocrystal fluorophores, hailing from quantum spectroscopy laboratories, are relative newcomers to the lands of biology. Minute semiconductor structures, known as quantum dots, come in a variety of shapes and sizes. They are strongly fluorescent objects with a wide absorption spectrum and narrow emission band, highly desirable properties for fluorescence microscopy[18,20].

Quantum dots take their name from the quantum confinement effect. In its excited state a quantum dot plays host to a single electron which is able to roam about its crystal container. Wave mechanics predicts that whenever an electron is confined like this its possible speeds in different directions are quantised. In the laboratory this effect manifests as longer fluorescence wavelengths for larger dots[7].

The most common quantum dots are self-assembled cadmium sulphide crystals precipitated from solution under carefully controlled conditions[18].

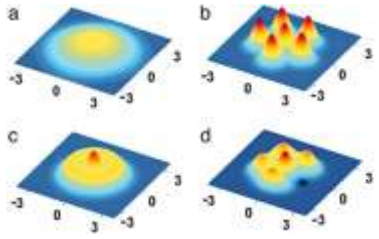
One negative is that in their raw state quantum dots are hydrophobic and cytotoxic[19]. Surface modifications have been used to overcome these issues, even creating opportunities to attach biological probes to the quantum dot[18]. This process, known as functionalisation, enables us to mimic some of the delivery techniques discussed in the context of organic fluorophores[21].

A second drawback is that quantum dots, although very bright, undergo random transitions to and from a `dark state' which fails to fluoresce. This blinking behaviour is believed to be due to the excited electron becoming trapped in surface imperfections[19]. Whilst this is not good news for single particle tracking, quantum dots remain an innovative alternative fluorophore with as-yet unexplored applications.

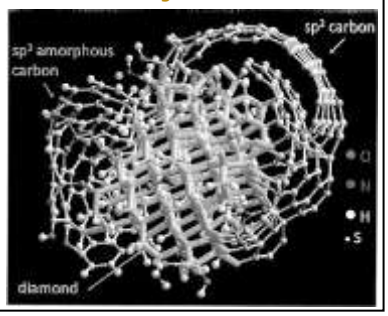
The Lord of the Bling

The lord of all nanoscopic organic crystals is the fluorescent nanodiamond. Remarkably bright, stable against blinking and nontoxic, the gemstones are desirable for long-term *in vivo* microscopy and single-particle tracking[22]. Experiments with nanodiamonds have achieved continuous observation times of ten hours[23]. Nanodiamonds also have a surface chemistry amenable to functionalisation, allowing them to enjoy the same bioconjugation properties as quantum dots[21].

Nanodiamonds ranging in size from 5 to 50 nanometres across are manufactured by high-pressure high-temperature synthesis, chemical vapour deposition, detonation of explosives or by burning away the outer layers of an existing diamond[24].



Left: the quantum confinement effect means that electrons in a quantum dot are more likely to be found in some places than others (Image credit 6).



Right: a schematic of a detonation nanodiamond showing the true diamond portion and the surface defects. The labels sp^3 and sp^2 refer to different shapes of chemical bond (Image credit 7).

Currently the palette is somewhat restricted, containing only three colours: red, green and blue.

In 2009 Mochalin and Gogotsi[25] discovered the blue fluorescent nanodiamond. These respond to UV light and go by the snappy name of ND-ODA: the nanodiamond (ND) is chemically coated with a compound named octadecylamine (ODA). ODA is hydrophobic, so tends to separate out of suspension in water but not in fats or oils like those that compose the cell's membrane[26]. As such, it is hoped that ND-ODA will allow specific targeting of lipid structures in biological specimens.

The origin of red fluorescence in nanodiamonds is not attributable to the diamond itself, but to an impurity. Any real diamond will not have an ideal crystal structure: common defects include atoms of the wrong type, known as substitutional impurities, and sites where atoms are missing altogether, termed vacancies.

Red fluorescence is due to nitrogen-vacancy (NV) centres, formed from a vacancy adjacent to a nitrogen impurity[27]. The latter are naturally present in most diamonds, and the number of the former can be artificially increased by bombardment with high-energy particles. Heating the crystal allows vacancies to diffuse, resulting in their capture by a nitrogen atom or destruction at the diamond surface[28].

NV centres are under consideration for a raft of interesting applications, ranging from drug delivery through to precision magnetometry and solid-state quantum computing[29]. The cross-over between these uses and fluorescence microscopy is surprisingly strong.

A prime example is the recent experiment of McGuiness and colleagues[23], who used fluorescence microscopy and quantum mechanics to three-dimensionally track the positions and orientations of NV nanodiamonds in living cancer cells. Nanodiamond orientation may be determined by analysing their magnetic properties whilst interacting with microwave light.

Green nanodiamonds are somewhat less bright than their NV cousins, but share their desirable stability and biocompatibility.

The first source of green emission is a large lattice impurity called an H3 centre[25]. These are composed of a vacancy sandwiched between two nitrogen atoms. H3 centres are produced by accelerating nitrogen ions into the diamond, making them more expensive to manufacture than NV nanodiamonds[30].

Amusingly, the second source of green fluorescence is simply the graphite-like coating found on ultra-small detonation nanodiamonds[21]. This layer of material allows the nanocrystal to emit green light when excited with ultraviolet rays. Crucially, fluorescence is maintained even when the surface of the crystal is functionalised.

A Flamboyant Future

What we have seen here – the physical basis of fluorescence microscopy, ingenious techniques such as TIRF and FRET, the flexibility of biological fluorescent molecules, tuneable emission characteristics of quantum dots and incredible stability and brightness of nanodiamonds – constitutes but a small glimpse into the current state of fluorescence microscopy in biology.

Since Hooke first viewed his `microscopical pores' we have progressed far beyond his expectations. Who knows where the next 336 years of microscopy will take us? The only safe bet, and the only one that does justice to the dedication and imagination of researchers, is that we will continue to find new and unexpected ways of putting the lumen into luminescence!

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Image Credits

1. Diagram of cork cells seen through early microscope, see ref. 1.
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Miscellany

1. `Jedi Master Yoda', copyright Star Wars Episode V: The Empire Strikes Back, LucasFilm (1980)
2. A lumen is simply the interior of any biological sack or vessel, such as the inside of the cell or organelles.