Fluorescence microscopy

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Although fluorescence microscopy permeates all of cell and molecular biology, most biologists have little experience with the underlying photophysical phenomena. Understanding the principles underlying fluorescence microscopy is useful when attempting to solve imaging problems. Additionally, fluorescence microscopy is in a state of rapid evolution, with new techniques, probes and equipment appearing almost daily. Familiarity with fluorescence is a prerequisite for taking advantage of many of these developments. This review attempts to provide a framework for understanding excitation of and emission by fluorophores, the way fluorescence microscopes work, and some of the ways fluorescence can be optimized.

Many of the technical improvements in microscopes over the years have centered on increasing the contrast between what is interesting (signal) and what is not (background). Fluorescence microscopy is the quintessential example, as it aims to reveal only the objects of interest in an otherwise black background. Because of its intrinsic selectivity, fluorescence imaging has become the mainstay of microscopy in the service of biology. Over the past several decades, organic chemists have devised many thousands of fluorescent probes that provide a means of labeling virtually any imaginable aspect of biological systems. For example, the Molecular Probes Handbook, tenth edition online (http://probes.invitrogen.com/handbook/), which is perhaps the largest compendia of fluorescence applications for biologists, has 23 chapters, 14 of which describe the 3,000 or so fluorescent probes for a wide range of cell biological questions. The large spectral range of available fluorophores allows simultaneous imaging of different cellular, subcellular or molecular components. In addition, the co-opting of intrinsically fluorescent gene products, most notably green fluorescent protein (GFP) and its variants, has allowed molecular biologists to genetically tag protein components of living systems and usher in a new era for fluorescence. Lastly, the rapidly advancing innovations of laser scanning confocal and two-photon microscopes mean that fluorescence approaches now provide a powerful approach to seeing microscopic structures in three dimensions, even deep within tissues. For these reasons it is difficult to do cell or molecular biology without understanding the basics of fluorescence, and this trend is accelerating. According to PubMed, 127,804 articles used the word 'fluorescence' in their title or abstract in 2004—that is more than the total number of fluorescence papers in the previous five years (1999–2003) combined. Our aim is to describe concepts that underpin fluorescence microscopy and highlight some of the pitfalls frequently encountered. There are several more detailed accounts that focus on specific aspects of this large subject, which we will refer to frequently to guide more in-depth reading.

PRINCIPLES OF FLUORESCENCE

Excitation and emission

Fluorescence microscopy requires that the objects of interest fluoresce. Fluorescence is the emission of light that occurs within nanoseconds after the absorption of light that is typically of shorter wavelength. The difference between the exciting and emitted wavelengths, known as the Stokes shift, is the critical property that makes fluorescence so powerful. By completely filtering out the exciting light without blocking the emitted fluorescence, it is possible to see only the objects that are fluorescent. This

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approach to contrast is superior to absorption techniques in which objects are stained with agents that absorb light. With absorbance dyes, the amount of light absorbed becomes only infinitesimally different from the background for small objects. In fluorescence, however, even single fluorescent molecules are visible if the background has no autofluorescence.

Fluorophores

Molecules that are used by virtue of their fluorescent properties are called fluorophores. The outermost electron orbitals in the fluorophore molecule determine both its efficiency as a fluorescent compound and the wavelengths of absorption and emission. When fluorescent compounds in their so-called 'ground state' absorb light energy (photons), alterations in the electronic, vibrational and rotational states of the molecule can occur. The absorbed energy sometimes moves an electron into a different orbital that is on average farther away from the nucleus. This transition to an 'excited state' occurs very rapidly (in femtoseconds). Usually the excitation process also sets in motion molecular vibrations in which the internuclear distances vary over time. All this absorbed energy is eventually shed. Vibrational relaxation and fluorescence emission are the chief ways a fluorophore returns to its low-energy ground state.

Whereas many organic substances have intrinsic fluorescence (autofluorescence), and a few are useful for specific labeling of components in biological systems, the typical approach to fluorescence microscopy is to take advantage of synthesized compounds that have some degree of conjugated double bonds. Such compounds often have ring structures (aromatic molecules) with pi bonds that easily distribute outer orbital electrons over a wide area. These compounds are optimal for fluorescence microscopy because the energy differences between excited state and ground state orbitals are small enough that relatively low-energy photons in the visible part of the electromagnetic spectrum can be used to excite electrons into excited states. In general, the more conjugated bonds in the molecule, the lower the excited energy requirement and the longer the wavelength (redder) the exciting light can be. The emitted light is shifted in the same direction. In addition, the efficiency, as measured by fluorescent quantum yield, increases with the number of pi bonds.

In GFP, a post-translational alteration of three amino acids, (Ser-Tyr-Gly) located in an α helix running down the center of the barrel-shaped protein forms an imidazolidone ring with conjugated double bonds and planar structure[1]. This ring structure is the chromophore. Mutations in the ring structure that add more conjugated bonds shift the fluorescence excitation and emission to longer wavelengths—as in yellow fluorescent protein (YFP).

Jablonski diagrams

A useful approach to understand the details of the excitation and emission process is to render the process in the form of a diagram first conceived by Alexander Jablonski in the 1930s (Fig. 1a). Those interested in a more rigorous treatment of this subject might read the first few chapters in several excellent texts[2–4]. On the left side of the diagram are the singlet states. These states maintain the paired + ½ and – ½ spin states of the electrons as they are normally, with each electron in a pair having opposite spins (see **Box 1** for useful

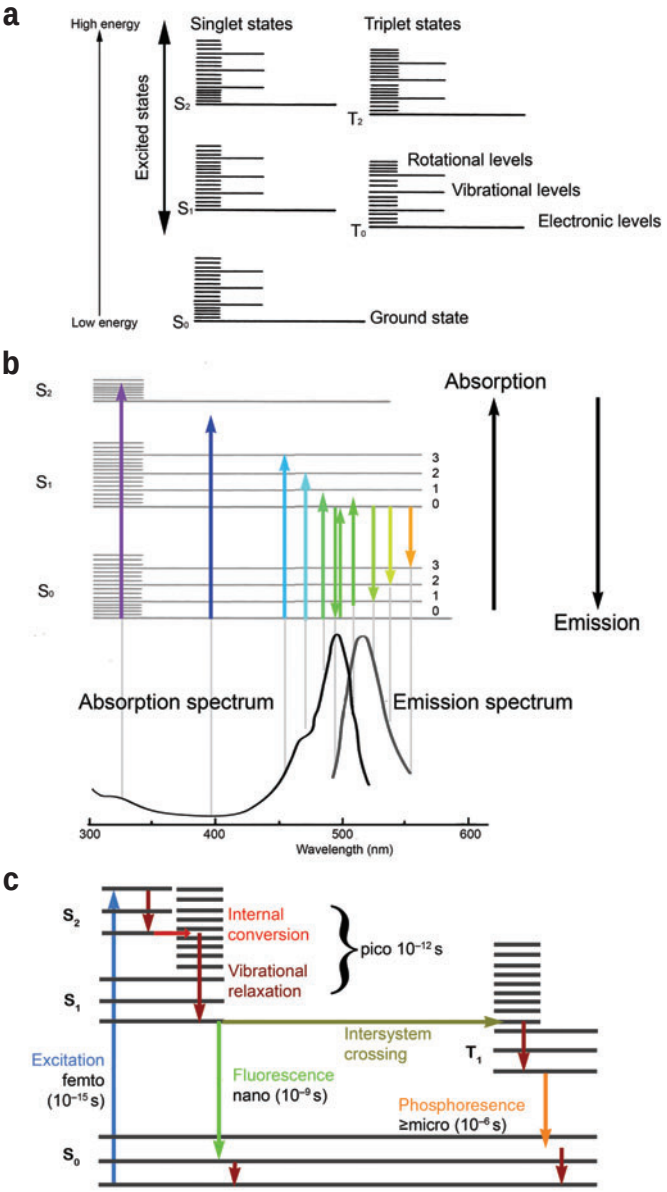

Figure 1 | Fluorescence fundamentals. (**a**) Jablonski diagram displaying the energy states of a molecule. Molecules not absorbing energy are mainly confined to the lowest vibrational states of the ground state S_0. (**b**) The spectral characteristics related to absorption and emission of energy by a molecule can be related to the size of the energy steps needed to bring a molecule from one energy level to another. The absorption and emission spectra of the common fluorophore FITC are shown below a Jablonski diagram. Each vertical gray line aligns the spectra with the energy of the absorbed (arrows pointing up) or emitted (arrows pointing down) photons. The arrows are colored to represent the wavelength of the photons. For example, the purple arrow to the left represents the energy of an ultraviolet photon that can cause the molecule to transition from the ground state to the second singlet excited state. The orange arrow on the right represents the lowest energy photon that can be emitted by this molecule as it drops back from the lowest energy state of S_1 to a high vibrational state of S_0. Note in the region of overlap of the absorption and emission spectra, some photons (for example, the green, up-pointing arrow) are absorbed when the vibrational level is not at its lowest level. Also note the symmetry between the absorption and emission curves owing to the similarity of transitions to vibrational states in S_0 and S_1. (**c**) The times that the various steps in fluorescence excitation and emission and phosphorescence take.

definitions). S_0 is the ground state and represents the energy of a molecule that is not being excited by light. S_1 and S_2 are excited singlet states in which an outer electron is boosted into a different orbital. S_2 contains more energy than S_1 and S_1 more energy than the ground state, S_0. On the right of the diagram are the triplet states in which an outer electron, boosted to a new orbital, has also undergone a subsequent reversal in spin so that a former pair of electrons are now parallel. From quantum theory, it is forbidden that an electron be in any spin state other than the two that exist in the paired electrons (+½ or –½); thus, for the electron to reverse spin it must go through a 'forbidden' transition, which is relatively unlikely. Nonetheless electrons can undergo 'intersystem crossing' between singlet and triplet states. This has important consequences, which will be discussed later.

Excitation spectra

When a fluorophore absorbs light, all the energy possessed by a photon is transferred to the fluorophore. This energy is inversely related to the photon's wavelength ($E = h \times c / \lambda$, where h is Plank's constant and c and λ are the speed and wavelength of light in vacuum, respectively). If the absorbed photon's energy is greater than that needed to exactly transition from the ground state to the lowest energy level of S_1, the molecule will also undergo a change in vibration, rotation and/or move into an even higher electronic orbital (S_2). Thus, there is a range of wavelengths that can excite a molecule. The minimum energy required for fluorescence, however, is from a photon that can cause the transition of an electron into a higher electronic excited state (that is, S_0 to S_1).

The time it takes a molecule to transition from the ground state to an excited state is extremely brief, on the order of femtoseconds (roughly equal to the time over which a photon of a particular wavelength traveling at the speed of light takes to cross paths with a molecule). Whereas one photon of the appropriate energy typically causes this transition, it is also possible for multiple photons to add their energy to bring a molecule to the excited state. For example, if two photons with half the energy (that is, twice the wavelength) of that needed to reach the excited state impinge on a molecule at the same time, their energies can sum and provide for two-photon excitation. Given the brevity of the absorption process, only enormously high light intensities provide sufficient probability for multiple low-energy (infrared) photons to be in the same place at nearly the same time.

The excitation spectrum of a fluorophore can be determined empirically by measuring the fluorescence yield by shining light of various wavelengths on a cuvette containing the fluorophore and recording the amount of fluorescent Stokes-shifted light that is produced at the peak wavelength of the fluorescence spectrum. The peaks and valleys in the excitation spectrum reflect the energy levels in the molecule's Jablonski diagram (Fig. 1b). For example, for many molecules there are only few vibrational or rotational states between the highest energy level of S_1 and the lowest level of S_2, hence for many fluorophores the absorption spectrum shows a dip between the longer wavelength peak related to exciting to an S_1 level and the shorter wavelength absorption to an S_2 level. Not surprisingly, given the threshold energy needed to get excitation, the energy for excitation has a sharper cutoff on the low-energy–long-wavelength side of the spectrum than the short-wavelength–high-energy side where there is no sharp cutoff; although practically speaking, the optical opacity of glass to ultraviolet light limits excitation in a fluorescent microscope beyond the near UV.

The probability that a fluorophore will absorb a photon is called its molar extinction coefficient, ε, in units of $M^{-1}cm^{-1}$. This property, which measures the probability of absorption of light as it passes through a solution containing the fluorophore, is similar (although the units are different) to the dye's 'cross-section', implying that the molecule can be imagined as a target and the bigger the cross-section, the more likely it is that it will catch a photon. The value of ε is specified for the wavelength that is the absorption maximum, which is where its cross-section is largest. Useful small organic fluorophores have ε values between ~25,000 and ~200,000 (the higher, the better the absorber). So-called 'enhanced' GFP (EGFP) has an excitation maximum shifted to 488 nm and an ε of approximately 60,000, which is five times higher than wild-type GFP with an excitation maximum of 470 nm. Dyes with high extinction coefficients tend to be useful when light intensity needs to be kept to a minimum, such as when imaging living tissues or when there are very few fluorophore molecules[5]. All

BOX 1 ELECTRONIC STATES

Spin. Spin is the amount of angular momentum associated with an elementary particle or a nucleus. Electrons have an intrinsic and invariable internal motion that corresponds to a spin of ½ and provides the electron with directional orientation. The electron also has an intrinsic magnetic moment directed along its spin axis. In the case of an atomic electron, the magnetic moment will be oriented parallel or antiparallel, with respect to the magnetic field, to the rest of the atom, which is indicated by the associated spin quantum number with values of +½ or –½.

Singlet state. Electrons normally reside as pairs with opposite spins within a single orbital; this results in a cancellation of their magnetic moments to produce a so-called singlet state.

Triplet state. A pair of nonbonding electrons may exist in two separate orbitals with the electron spins parallel to each other. Atoms or molecules with such a configuration will have an overall magnetic moment that may be parallel, perpendicular or antiparallel to the direction of a magnetic field. These three possible alignments correspond to three forms of slightly different energy, so the atom or molecule can exist in all three forms and is said to be in a triplet state. Triplet states tend to be relatively long-lived.

Ground state. This is the normal, nonexcited state of a molecule. When talking about the ground electronic state, a molecule may still have excess vibrational and rotational energy.

Excited state. When an electron absorbs sufficient energy, it can transition to a higher energy level (higher orbital) and thus create an atom or molecule in an excited state. Excited states tend to be relatively short-lived and return to electronic states of lower energy, ultimately to the ground state, by losing their excess energy. This energy loss may involve radiationless transitions—such as internal conversion, intersystem crossing or vibrational relaxation—or radiative transitions—such as fluorescence and phosphoresence.

other things being equal, a dye with a higher extinction coefficient will also give a greater signal for the same amount of light-induced background than a dye with a lower extinction coefficient.

Emission spectra

Once excited, the molecule uses several different pathways to ultimately lose the absorbed energy and return to the ground state (Fig. 1c). 'Internal conversion' is a transition between electron orbital states (such as S_2 to S_1). Strictly speaking, internal conversion allows isoenergetic transitions from low vibrational energies of one electronic state to high vibrational modes of a lower electronic state, so no energy is lost during this transition but the extra energy is eventually shed through vibrational relaxation. During vibrational relaxation the vibrational energy in the fluorophore is transferred to nearby molecules via direct interactions. In aqueous medium, water is the likely energy recipient. Notably, vibrational relaxation does not lead to any emitted photons. Internal conversion and vibrational relaxation takes picoseconds and typically bring the molecule back to the lowest energy level of S_1. Internal conversion sometimes transitions excited molecules all the way to the ground state S_0 but for most fluorophores the energy difference between the ground state vibrational modes and first singlet excited state is large enough that this path is not preferred. The excited molecule now has a similar vibrational state as it did in the ground state but the outer electron orbital still contains extra energy.

In good fluorophores, the preferred final energy path back to the ground state is the expulsion of a photon whose energy covers the gap between the lowest vibrational state of S_1 and any one of the vibrational or rotational states of S_0. The emission spectrum of a fluorophore is just the range of wavelengths this emitted photon can have (Fig. 1b). This spectrum can be empirically determined by exciting the fluorophore (typically at its peak absorbance wavelength) and using a device to measure the fluorescence spectrum. Given the nonradiative drop back to the lowest level of S_1, the wavelength of the emitted light is not related to the particular wavelength of the exciting light; thus one cannot shift the emission spectrum by changing the color of the exciting light. In most cases the outgoing emitted light is also not related to the incoming direction of the exciting photons so photons can be emitted in the opposite direction of the incoming photons, as occurs in standard epifluorescence microscopes described below.

Because the emission starts from the lowest level of S_1, the energy of the emitted photon is typically less than the absorbed photon as vibrational relaxation and internal conversion remove the excess energy—this is the origin of the Stokes shift. The magnitude of the Stokes shift varies considerably from one fluorophore to another. Generally large shifts have the advantage of easier separation of the exciting and emitted light. But not all fluorescent emission occurs at longer wavelengths than the exciting light. Often a minority the ground state fluorophores is in one of the higher vibrational states of S_0 when they are excited. In these cases a photon can drop back in a larger energy jump than was needed to reach S_1, accounting for the overlap of the emission and excitation spectra (Fig. 1b).

A more serious overlap concern relates to imaging multiple fluorophores in the same sample. Given the broad excitation and emission spectra of each fluorophore, even spectrally shifted fluorophores can be excited by the same wavelength and exhibit overlapping emission. These overlaps can cause confusing crosstalk or bleed-through between signals associated with different fluorophores in the same sample. This difficulty has been ameliorated by organic synthesis of ever wider spectral ranges of fluorophores (by adding more conjugated bonds) that now permit a user to choose fluorophores with little overlap in excitation and/or emission spectra (Fig. 2a,b). Crosstalk, however, remains a serious problem with fluorescent proteins that have very broad excitation and emission spectra relative to small organic chemical fluorophores (Fig. 2c). Here too, new wavelength-shifted variants may come to the rescue[6].

Although shifted to longer wavelengths, the spectrum of the emitted fluorescence has mirror symmetry with the main part of the spectrum of the absorbed light (Fig. 1b). This symmetry is related to final drop back to the ground state from the lowest vibrational state of S_1. The vibrational transitions going back to the ground state are the same as those that occurred during absorbance. In each case the molecule is beginning typically in the '0' vibrational state and moving to either

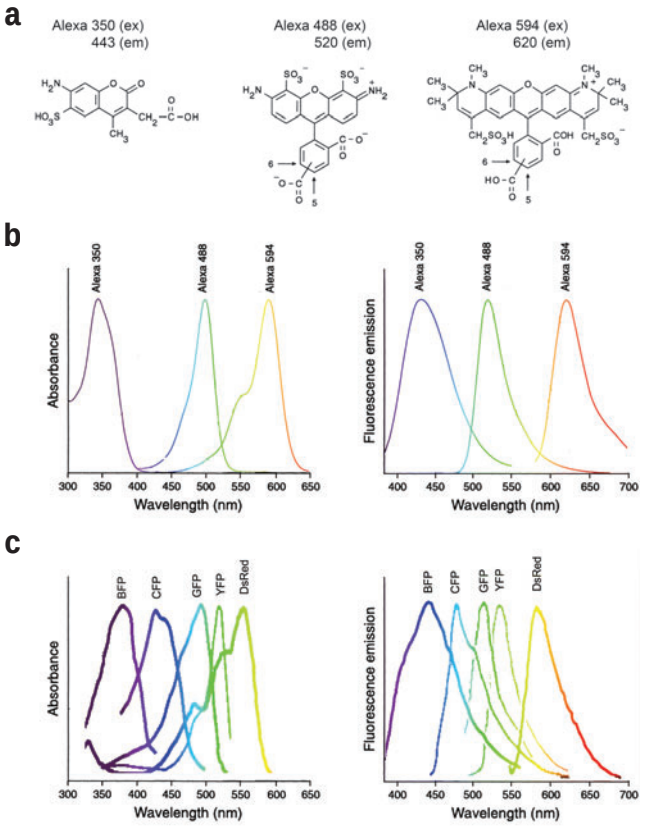

Figure 2 | The spectral and structural properties of fluorophores. (**a**) Three Alexa fluorescent molecules have conjugated double bonds but the excitation and fluorescence emission characteristics shift to longer wavelengths in proportion the degree of conjugated bonds. The 17 Alexa Fluor dyes, sold by Invitrogen's Molecular Probes span the near-UV, visible and near infrared. Along with the wavelength shift, the cross-section of the dyes increases. Alexa 350 has an extinction coefficient of 19,000 $cm^{-1}M^{-1}$, whereas for Alexa Fluor 750 it is 240,000 $cm^{-1}M^{-1}$. (**b**) The absorption spectra and emission spectra are largely nonoverlapping for these particular Alexa fluorophores. (**c**) In contrast, the mutated spectral variants of fluorescent protein from the *Aequorea* species of jelly fish and *Discosoma* species of reef coral have substantial overlap in both excitation and emission spectra. Recent attempts to generate more red-shifted fluorescent proteins have raised the possibility that eventually cross-talk will not be a major problem for fluorescent proteins either[6]. Data in **a** from[32]; **b**, from the Invitrogen pamphlet on Alexa Fluor Dyes; **c**, from the Clontech website.

the same or a higher vibrational state, for example, 0, 1, 2 or 3. These vibrational transitions have the same likelihood in either direction. For example, S_0 level vibrational level 0 to S_1 level 3 is comparable in likelihood to S_1 vibrational level 0 to S_0 level 3. Hence the absorption peaks and the emission peaks are the same but shifted to longer wavelengths for the emission. Knowing the absorbance and the emission spectrum for a fluorophore helps decide which filters are optimal, which lasers in scanning microscopes to excite dyes with, and how best to get excitation of one dye without crosstalk from another.

Intersystem crossing

Unfortunately, fluorescence emission is not the only way fluorophores in the excited state can lose energy. Another pathway of energy loss occurs after intersystem crossing by means of a forbidden transition to the triplet state (Fig. 1a,c). In many fluorophores, triplet state vibrational energy levels overlap with the lowest energy level in S_1. This overlap favors intersystem crossing followed by internal conversion to the lowest energy of T_1. The triplet state molecule now has no easy path back to the lower-energy singlet ground state, as this transition requires the triplet outer electron to again undergo a forbidden transition. Although some triplet-state molecules reach the ground state without light emission, in many cases light emission termed phosphorescence does occur, but it can take microseconds as it depends on the unlikely forbidden transition. In the meantime triplet-triplet transitions can move the molecule into higher triplet states if another photon is absorbed, further delaying any light emission. In laser scanning microscopes where the fluorescence is measured serially in rapid succession from one point to the next, the delay of the phosphorescent photon is often too long to allow it to be counted for the correct pixel, thus weakening the fluorescent signal. Furthermore, as molecules in the triplet state are unable to cycle through absorbance and emission rapidly, the state temporarily removes potentially fluorescent molecules from the total pool. Last, and perhaps the most problematic issue, is that triplet-state molecules can undergo photochemical reactions that cause irreversible bleaching and phototoxicity.

Quantum yield and fluorescence intensity

The quantum yield of a fluorophore is a measure of the total light emission over the entire fluorescence spectral range. It is measured as the ratio of fluorescence emission to nonradiative energy losses. With very intense light sources, such as lasers used in laser scanning microscopes, the quantum yield provides an accurate measure of the maximum intensity that can be obtained with a fluorophore. With dimmer light sources, the intensity depends critically on the product of the extinction coefficient and the quantum yield.

A fluorescent dye such as fluorescein has a high quantum yield of about ~0.9 and GFP has a quantum yield of ~0.8. Higher quantum yield not only increases fluorescence intensity but also means alternative competing photochemical processes associated with intersystem crossing, such as bleaching and free radical formation, are less likely. Except in situations where one is trying to do some photochemistry with a dye, the closer the quantum yield is to 1 the better. For example, eosin, a brominated derivative of fluorescein, has a quantum yield that is one-fifth that of fluorescein, but a 20-fold greater yield of a reactive intermediate (singlet oxygen) from interactions between its triplet state and oxygen. Eosin excitation can be used to photoconvert diamino benzidine into an electron-dense product to mark sites of fluorescence for electron microscopic work[7].

Excited state lifetime, energy transfer and photochemistry

The time the molecule remains excited before yielding a photon is called the excited state lifetime. This time is dominated by the duration the molecule spends in the lowest level of S_1, which is typically several nanoseconds before spontaneous decay to the ground state. The lifetime can be made briefer if another molecule nearby can absorb the energy. Such intermolecular interactions both reduce the fluorescent lifetime and the quantum yield. Fluorescence lifetime imaging microscopy (FLIM) probes alterations in the milieu in the immediate vicinity of a fluorescent molecule and is thus an important tool for studying molecular interactions between fluorescently labeled molecules and their interacting partners[8,9]. If the interacting partner is itself a fluorophore, the energy in an excited state molecule can excite the nearby fluorophore. This phenomenon, known as fluorescence resonance energy transfer (FRET) occurs when a very nearby fluorophore's absorption spectrum overlaps with the emission spectrum of the excited fluorophore. The donor's energy is absorbed by the acceptor, which then emits a photon shifted to a longer wavelength[10]. The strong distance dependence of this energy transfer allows FRET to assay changes in intramolecular distances that are far below the resolution of the light microscope.

Excited state molecules can also participate in a wide range of photochemical reactions[4]. Photouncaging, for example, is widely used in biology to release biologically active compounds after light induced release of a blocking group. Some fluorescent proteins can also undergo a change in structure and spectral properties with light. Notably, the red chromophore in Kaede, a fluorescent protein from a coral is generated from a green form of this protein that is converted by exposure to ultraviolet light[11,12].

PHYSIOLOGICAL FLUOROPHORES

At present, there are many probes that assay some aspect of physiological processes by accumulating in organelles such as mitochondria, endoplasmic reticulum, nucleus or synaptic vesicles[13,14]. Fluorophores have also been developed to take advantage of the fact that a fluorophore's absorption and emission properties can be highly sensitive to a changing milieu. Fluorescent sensors change their absorbance and/or emission spectra when bound to calcium ions[15,16] hydrogen ions[17] or other molecules of interest. Although many sensors allow only an estimation of the change in concentration of their target by undergoing a simple increase or decrease in fluorescence, some of these fluorophores can be used in 'ratiometric' assays that provide a quantitative way to measure concentration. By taking the ratio of the dye's behavior at two emission or absorption wavelengths—one being the wavelength sensitive to the target molecule and the other being one that is insensitive to the target—it is possible to normalize for changes unrelated to a change in target concentration, such as the distance through a cell, an inhomogeneous milieu or local dye concentrations that might make local absorbance or emission intensity vary for reasons other than changes in the local concentration of the molecule being studied. Doing ratiometric imaging correctly requires a full understanding of the sources of measurement errors and how the calculations are made. Fortunately there are a number of excellent reviews of this powerful quantitative technique (see[18]).

Genetically encoded fluorescent probes that monitor calcium and a variety of other intracellular signals are rapidly evolving, and are beginning to provide windows into intracellular metabolism in intact systems[19–21]. Some dyes are sensitive to electrical fields, and thus, when located in a membrane separating two compartments at different

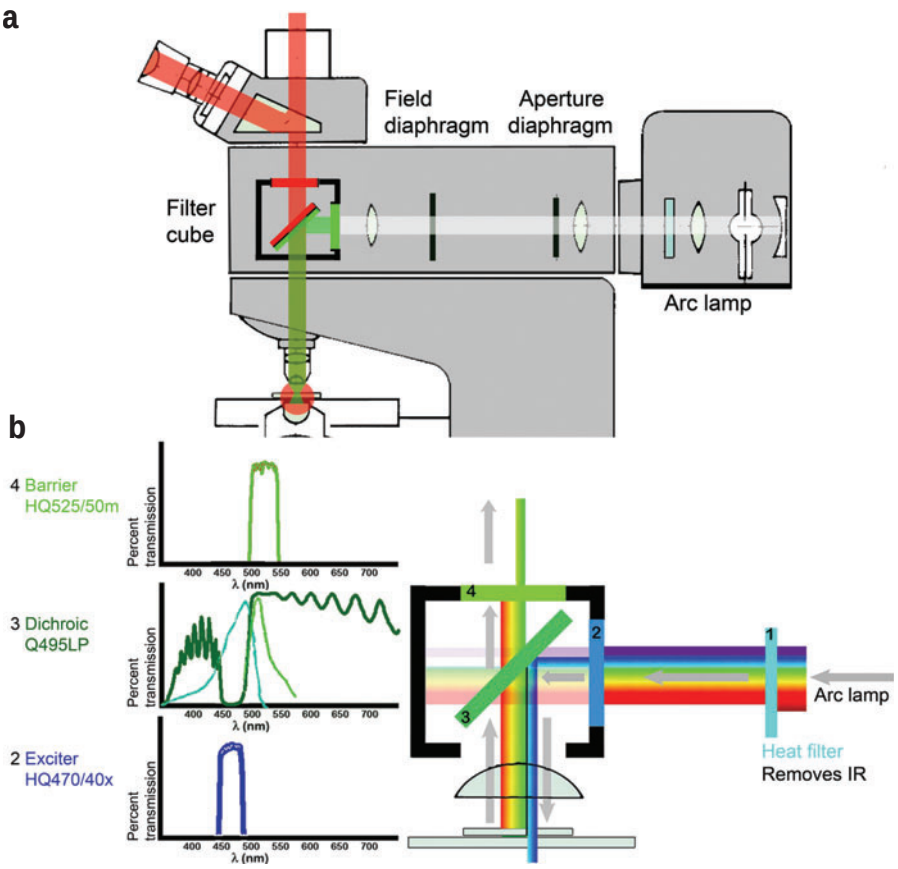

Figure 3 | The fluorescence microscope. (**a**) Epi-illumination fluorescence microscopes use the objective both to illuminate and image the specimen. Shown is an upright microscope with the slide at the bottom. The light source, in this case an arc lamp, sends full-spectrum light to the specimen by way of a fluorescence 'cube' that selectively illuminates the specimen with a wavelengths that excite a particular fluorophore (shown, green light to excite rhodamine). The red fluorescence that is excited sends photons in all directions and a fraction are collected by the objective and sent through the cube to the eye or camera port above. The cube has two filters, the dichroic mirror and barrier filter, to prevent the exciting wavelengths from reaching the detector. (**b**) The details of a cube designed by Chroma Technologies to excite and detect EGFP. The three main components (labeled 2, 3 and 4) have specific spectral features that are ideal for GFP. Note that the dichroic mirror[3] splits between reflection and transmission right between the absorption and emission peaks of the GFP, which are superimposed in blue and green, respectively.

potentials, they can provide an optical signal of transmembrane voltage[22,23]. Fluorescent proteins that sense membrane potential by coupling to voltage-sensitive ion channels offer the possibility of someday monitoring neural activity in many neurons simultaneously in living animals[24].

Clearly the evolution of fluorescence indicators of physiological functions is one of the triumphs of modern cell and molecular biology. The key point to their use, however, requires looking over time as well as space. Such multidimensional imaging requires highly efficient detectors and sometimes rapid switching of filters, and overcoming the many challenges of imaging living cells[14,18,25].

THE FLUORESCENCE MICROSCOPE

Given the Stokes shift, it is easy to imagine how one might build a fluorescence microscope: illuminate the specimen with one wavelength and filter the return light to only see longer wavelength–shifted fluorescence. Indeed this is similar to the way Sir George Gabriel Stokes first noted fluorescence: purportedly, a purple stained glass window filtered sunlight onto a flask of quinine water, which he then observed to emit blue light through a glass filled with white wine that blocked the purple light[3]. The preferred approach in modern fluorescence microscopes is epi-illumination (Fig. 3a). In this configuration the microscope objective not only has the familiar role of imaging and magnifying the specimen, but also serves as the condenser that illuminates it. The advantage of this approach over transmission, or diascopic, fluorescence microscopes (in which the exciting light comes through the condenser and the emission is collected by the objective) is that whereas the excitation of the fluorophore is equivalent in both epi- and transmitted microscopes, only the small percentage of the exciting light that is reflected off the sample needs to be blocked in the return light path in the epi-illumination mode. The main technical hurdle with this approach is that the exciting light and fluorescence emission overlap in the light path requiring a special kind of beam splitter, a dichroic mirror, to separate the excitation from the emission. The dichroic beamsplitter mirror is designed to be used in light paths at 45°. In ordinary fluorescence microscopes, the dichroic reflects shorter wavelength light originating from the light source and transmits the longer wavelengths of the emitted fluorescence. Each dichroic is designed to have a transition from reflection to transmission that resides between the excitation and emission peaks of the fluorophore it is designed to be used with (Fig. 3b). Dichroics are rarely used without two additional filters: the excitation filter, which preselects the exciting wavelengths, and a barrier filter that only allows passage of the longer wavelength light back to the detector. All three types of filter are typically interference filters which have very specific wavelength selectivity. These are marvels of engineering composed of many stacks of thin materials that alternate in index of refraction (Reichman, J. *Handbook of optical filters for fluorescence microscopy*. Chroma Technology Corp. (http://www.chroma.com/resources/PDF_files/handbook4.pdf; 2000). With these three elements, the exciter, beamsplitting dichroic and barrier, the separation of the exciting light from the emitted light can be very good. For example, a modern filter set might pass only one photon in 10,000 of the wrong excitation color to the specimen, and there is a similar ratio in the return light. Such high ratios are absolutely required for imaging small numbers or single fluorescent molecules.

Filter cubes

Many fluorescence microscopes provide a convenient way of selecting the precise wavelength bands for excitation and emission related to particular fluorophores by means of small block-shaped filter holders that are composed of the exciter, dichroic mirror and barrier filter

BOX 2 FACTORS THAT REDUCE THE QUALITY OF FLUORESCENCE IMAGES

There are many different reasons fluorescence imaging is sometimes not sufficiently good to provide useful data. Many of the issues have been touched upon above. In the following list, we itemize some of the most common factors that decrease the quality of fluorescence imaging and what can be done to improve the situation.

Choice of fluorophore. Maximize the absorption coefficient and quantum yield (http://probes.invitrogen.com/handbook/).

Concentration of the fluorophore. Especially when staining thick tissue blocks, it is sometimes difficult to get sufficient fluorophore inside the block. The potential solution of staining longer or with a higher concentration (for example, of a fluorescent secondary antibody) sometimes does not help as the background increases faster than the signal. Optical sectioning microscopes such as confocal can provide some help by removing background fluorescence from the image. In other cases the epitope or structure being labeled is just in low abundance, so the signal will be intrinsically dim. Brighter signals can be obtained by amplification methods[34].

Excitation intensity. With arc lamps, the brighter the light, the brighter the signal (and background). Unlike with a laser, it is not possible to saturate the fluorescence with an arc. The most intense arcs are the ones with the highest mean luminous density[26].

Arc lamp alignment. If the arc is not focused sharply on the back aperture, the specimen plane will be unevenly illuminated. Ask a microscope company representative to show you how to align and focus the arc. Alternatively, you may consider a liquid light guide–based light source, which provides even illumination and no moving parts to get out of alignment once properly set up.

Wavelength of the exciting light. Another way to increase the intensity of fluorescence is to use a wide-band exciter—in this way more photons that are capable of exciting the fluorophore are impinging on it. Narrow-band exciters are only useful when there is potential cross-talk with another fluorophore (see http://www.chroma.com/ or http://www.omegafilters.com/ for further details).

Emission filters. Broad-band emission will give a larger signal and should always be used except when there is cross-talk with the emission of another fluorophore in the same sample.

NA of the objective. Fluorescence intensity dramatically increases with the objective's NA[26,27]. For low-NA objectives that are used both to illuminate and collect the light (the standard epi-illumination microscope), the amount of light reaching the detector is proportional to $(NA)^4$. For high-NA, the light excitation and collection efficiency is actually a bit better.

Magnification. The magnification of an image spreads the light out and decreases the intensity per pixel in proportion to 1/magnification squared . Sometimes in order to get a high resolution image that matches the optical characteristics of the objective (that is, a diffraction-limited image) with the pixel size of the detector, it is necessary to zoom beyond the magnification of the objective. The price for getting a diffraction-limited image may be a very dim image with insufficient signal to see the resolution. There is no simple solution other than being sure that there is no 'empty' magnification that magnifies more than is necessary to get to twice the diffraction limit (the Nyquist limit). It is worth noting that light loss caused by magnification is not an issue with laser scanning 'zoom', which concentrates more light over a smaller area—but bleaching can be almost instantaneous.

Ionic milieu (quenching). It is worth making sure that iodine and several other metal ions are not contaminating your samples.

Bleaching. Keep samples in the dark when not in use. Use only enough light to get the image you need. Use antifade agents. Keep oxygen tension low.

Background fluorescence. Autofluorescence background is progressively reduced at higher wavelengths. Several companies offer red and infrared dyes to mitigate background problems (Atto fluorophores from Sigma-Aldrich, and Alexa fluors from Molecular Probes). Long-wavelength fluorescence has its own challenges, however[35]. Fixatives often increase autofluorescence. Glutaraldehyde, which is used in electron microscopy fixation, is a great fixative but is totally unusable for fluorescence imaging as the background is huge. Use of 4% or even 2% paraformaldehyde has low autofluorescence and for many applications is often adequate. A simple way to reduce background is to use the field diaphragm on the epi-illumination port (**Fig. 3a**) to limit the illumination to the small region of interest. Sometimes fluorescence that is totally invisible can be revealed by stopping the field aperture to a very small spot. Of course this limits the field of view. The index of refraction mismatch between the immersion medium between the coverslip and the objective versus the sample medium can cause a blurry image with extra background glow owing to spherical aberration. Objectives such as the Leica AS MDW 60×, 1.3 NA that provide the opportunity to use the same proportions of water and glycerol on both sides of the objective can dramatically remove this background artifact.

Light passing properties of optical path. UV dyes are difficult to excite with ordinary glass objectives that are opaque to light <350 nm.

Degradation of fluorophore. If possible, store slide boxes in refrigerator or freezer.

Path length. Blue and near-UV emitting fluorophores are harder to see the deeper they are located within a thick tissue block owing to Rayleigh scattering. Since scattering is inversely proportional to λ^4 a red-shifted dye can help dramatically.

Detector sensitivity. Digital cameras are potentially very useful for detecting signals that seem too dim for the 'naked' eye. Two technical approaches for different applications are available. When exposure time can be long, a cooled charge-coupled device (CCD) is probably the best option. Recent improvements in CCDs make the quantum efficiency very high so that more exotic thinned, back illuminated CCDs are no longer typically required. Large pixel size or binning increases sensitivity further. When the signal needs to be obtained quickly, an intensifier in front of the CCD is the better alternative[26].

Membrane fluorescence optical artifact. The edges of membrane labeled cells that are orthogonal to the image plane are always brighter than membranes parallel to the plane. This artifact is due to the fact that within the optical thickness of an image plane there can be many more fluorescent molecules contributing to a pixel if the membrane is vertical than when the membrane is horizontal.

(Fig. 3b). The cube fits into a circular carousel or linear block that can hold from three to nine separate cubes. These cubes can be moved into position either manually or by a computer driven motor. Thus, to switch between imaging a red fluorescing fluorophore like TRITC, a green fluorescing dye such as fluorescein, and a blue fluorescing dye such as DAPI, is as easy and rapid as moving from one cube to another. One point of caution should be remembered. Because the cubes are not necessarily perfectly aligned (especially the 45° angle of the dichroic), the images with the various cubes may be slightly offset, requiring care when overlapping two images taken with different cubes. The cubes can be selected based on not only spectral matching to particular fluorophores, but also whether they are broad or narrow band. Broad-band cubes attempt to give the largest signal for a dye based on its excitation and emission spectra. Such cubes are preferred when one is looking for a signal from a single dye and you are not concerned with crosstalk. Alternatively, there are many situations in which the location of different fluorophores in the same sample is essential. In these cases, it is useful to use narrow-band cubes. In determining what is the right cube for a particular application the user must have access to the excitation and emission curves of the fluorophore as well as the spectral filtering properties of the three elements of the cube. An example, shown in Figure 3b, is of a cube that is designed to image EGFP.

It is also possible to use a dichroic mirror with filter wheels that rapidly switch between different exciting and barrier filters. This approach offers the user the possibility of rapid switching between fluorescence channels, and the alignment of the images is better because the same dichroic beamsplitter is used for each fluorophore. For this approach, the dichroic mirror must be more sophisticated. One may use a double, triple or even quadruple dichroic; these mirrors selectively reflect the exciting wavelengths for two, three or four fluorophores while at the same time transmitting the fluorescence for each of these dyes. Such dichroics are useful when scanning to find sites of overlap of two different fluorophores or for situations where filter wheels allow rapid switching in the excitation wavelengths. Given the large number of colors that are passed by such cubes, the background may appear more gray than black. Several companies (for example, Omega Optical and Chroma Technology) will even generate custom filter cubes and dichroics if you have a particular unconventional fluorophore.

Light sources

There are several different strategies for fluorescence illumination. Traditionally the intensities needed for comfortable viewing by eye or rapid camera exposure come from arc lamps. Mercury and xenon arc lamps are expensive, potentially dangerous, and require special lamp houses and power supplies. These two types of arc lamps differ in several important ways. Depending on your requirements xenon or mercury is the better choice. Xenon has the advantage of being relatively even in the coverage of wavelengths throughout the UV, visible and near infrared. Mercury, however, is a spectrally peaky light source with several extremely intense lines[26]. If these lines coincide with the excitation spectrum of the fluorophores you are using, it provides brighter light. Mercury is not a good light source for some ratiometric dyes, such as fura 2, for which comparisons between the signal at two nearby excitation wavelengths are confounded by the fact that one of the wavelengths overlaps with a mercury peak but the other does not. With such exceptions in mind, the most intense fluorescence usually comes from the 100-W mercury. The reason why this is the brightest source is that the mean luminous flux per surface area is highest.

Because the image of the arc is focused onto the back aperture of the objective in Kohler-style illumination, the key to bright illumination is the intensity of the image of the arc. The surprising fact is that whereas arcs with higher power (wattage) produce more light, they are larger, and their image needs to be reduced below its actual size to fit in the objective's back aperture, and this minification causes the image to be less intense. For this reason a small highly intense arc provides more intense excitation light.

With time, arcs will not ignite easily owing to blunting of the cathode and anode. The bulb will also begin to show variations in intensity and slight movements in arc position that cause flickering. Thus once a bulb has reached its useable lifetime (200 h for mercury and 400 h for many xenon), the bulb should be replaced. It is also possible for the alignment to drift so that the image of the arc is not centered on the objective's back aperture, requiring adjustment of the alignment every week or so. This requires setting a number of adjustments on the lamp house and requires a bit of practice, but ignoring this step will lead to uneven illumination and often unacceptably dim images.

The most even illumination comes when a sharp image of the arc is placed on the back aperture of the objective lens. Although this sharp image means that there will be regions without light, that absence only has the effect of removing some potential angles of illumination of the specimen. Because fluorescence emission is typically not sensitive to the angle of illumination, the inhomogeneity in the angles of illumination is invisible. Conversely, when the arc is not well focused on the back aperture there can be inhomogeneities in the intensity at various positions on the sample. This artifact causes brighter and dimmer fluorescence within the image. Recently these issues have been tackled by the development of liquid light guides coupled to new ultrahigh pressure 120-W mercury halide bulbs similar to those used in LCD projectors. Some of these systems have been specifically developed for fluorescence microscopy illumination (for example, X-Cite 120 from Exfo Photonic Solutions). The emission spectra of these bulbs are similar to traditional mercury arc lamps but have some additional spectral broadening owing to the high-pressure collisions that alter the vibrational energy of the excited atoms in the arc. This broadening, especially in the fluorescein excitation region, makes these bulbs superior to traditional mercury arcs. Such systems, although expensive, provide much longer time between arc replacement (>1,000 h versus 200 h), the convenience of not having the lamp house attached directly to the microscope, no alignment problems and uniform illumination in the microscope field. The recent development of bright light–emitting diodes (LEDs) raises the possibility that arc lamps will someday be replaced by these lightweight, inexpensive light sources.

Objectives

Because the microscope objective is both the source of light that excites the fluorescence in the specimen and the optical element that collects the fluorescence, its properties have a large impact on the fluorescence image. The design of objectives is beyond our scope and is nicely described in other reviews[27]. It is important, however, to understand that because the design of microscope objectives has in the past centered on features that improve the quality of brightfield color images, much effort has been put into correcting chromatic aberrations. These corrections are somewhat less important to the fluorescent microscopist who is more typically concerned with excitation and collection efficiency, resolution, and contrast.

The ideal fluorescence microscope objective has a high numerical aperture (NA). The NA of an objective is a critical parameter and can be found on the barrel of the objective. It is more important than the magnification, as it sets both the resolving power and light efficiency of the objective. The value of the NA is derived by multiplying the sine of the 'half angle' (the angle between the vertical and most extremely angled ray that can be collected by the objective) with the index of refraction of the medium between the objective and the coverslip. The larger the half angle, the greater number of photons that can be collected and the greater the amount of light that can be used to excite the specimen. For this reason the amount of exciting light through an objective is roughly proportional to $(NA)^2$, and at the same time the amount of the fluorescence emission collected is also proportional to $(NA)^2$. The intensity observed is thus proportional to the $(NA)^4$. In addition, oil immersion lenses (in which the oil matches the index of refraction of the coverslip) prevent light losses owing to reflections and refraction off the coverslip and thus improve excitation and collection efficiency. Image intensity is also related inversely to magnification squared, so the most intense images will come from relatively low magnifications. High NA also increases the resolving power of the objective. The minimal resolvable distance is $0.61\,\lambda$ / NA. Additionally, the ideal fluorescence objective will have relatively few lens elements to reduce losses owing to reflection and glare from stray light. The lens elements and cements ideally have very low intrinsic fluorescence so the field appears absolutely black in the absence of fluorescence signal. Lastly, the ideal fluorescence objective will pass exciting wavelengths in the UV, visible and near infrared. All of this information is available (albeit, with some digging) from manufacturers.

It is fair to say that objectives designed particularly for the special needs of the fluorescence microscopist are becoming more common but that no objective is ideal in all circumstances. Users whose samples are thick may want to consider lenses that also have a correction collar for spherical aberrations, as these aberrations cause blurring and loss of contrast when samples have a different index of refraction than the material between the coverslip and the objective. If one is routinely dealing with thin sections, one may want a 'plan' lens that images a flat field in the specimen—otherwise the edges may be blurry. If the main aim is perfect alignment between blue, green and red fluorescent material in one sample plane, an apochromatic lens should be considered. It is appropriate to try out an expensive objective on your own sample before purchase to make sure it provides the kind of image you are expecting.

BLEACHING

The phenomenon of bleaching

Although in principle a fluorophore can cycle between ground and excited states an unlimited number of times, the conditions in which organic fluorophores are used usually limit the number of cycles. Estimates of 10,000–40,000 cycles are often cited as the limit before permanent bleaching occurs for good fluorophores. Bleaching is a generic term for all of the processes that cause the fluorescent signal to fade permanently. Quenching on the other hand, is a reversible loss of fluorescence owing to noncovalent interactions between a fluorophore and its molecular milieu. FRET, mentioned above, is an example of collisional or dynamic quenching of the excited state fluorophore. Static quenching occurs when the ground-state fluorophore associates with another molecule (sometimes other identical fluorophores: self-quenching).

At the molecular level there are several different ways bleaching can occur, and it is fair to say that the photochemistry is not well worked out for most fluorophores. What is clear is that the long-lived triplet state provides more opportunities for a molecule with an excited electron to interact with other molecules than the briefer singlet states. Hence, most bleaching is thought to be associated with triplet states. It also appears that one of the important ingredients in bleaching is an interaction between a triplet state fluorophore and molecular oxygen. The triplet state can transfer its energy to oxygen (which is itself a triplet in the ground state), exciting oxygen to its singlet excited state. Singlet oxygen is a reactive molecule that can participate in may kinds of chemical reactions with organic molecules. These chemical reactions can covalently alter the fluorophore to inactivate its ability to fluoresce (that is, bleaching it). In addition, the singlet oxygen can interact with other organic molecules causing phototoxicity for living cells.

Dealing with bleaching

There are many reasons why fluorescence signals are not as bright as one might hope (see Box 2 for a list of problems and potential solutions), but bleaching is perhaps the most serious. One way to reduce bleaching is to use no more light than absolutely necessary to image a sample. If the microscope has an aperture diaphragm on the epi-illumination port (Fig. 3a), use it to titrate the light level to a low value. The field diaphragm can also be stopped down to illuminate only a small region to minimize general bleaching and also improve contrast. In fixed samples, the amount of bleaching appears to be directly related to the number of excitation emission cycles a fluorophore goes through, so dim light does not automatically eliminate bleaching. Slides that contain fluorescence samples should therefore always be stored in the dark when not in use because ambient light of long enough duration can bleach them.

It is important to realize that fluorophores with similar excitation and emission spectra can have dramatically different bleaching rates. Companies, like Invitrogen's Molecular Probes or Sigma-Aldrich, sell new-generation fluorophores that have greater photostability than previous well-known dyes such as fluorescein, rhodamine and Texas red. The Alexa dyes are one such example (Fig. 2), with bleaching rates that are several-fold slower than older dyes[28]. Not surprisingly, the relative lack of bleaching of newer fluorophores comes with another benefit; their intensity is high owing to their high quantum yield because efficient fluorescence emission antagonizes the tendency of molecules to end up in the triplet state.

Unfortunately even with the best dyes, it is sometimes necessary to use intensities that will cause bleaching, as for example when taking an image with a camera or prolonged time lapse imaging. In fixed samples, immersing a sample on a slide with a mounting medium that is designed to reduce bleaching is an effective strategy. A number of antifade agents can be made up in the lab rather inexpensively or purchased ready to use. All of these are designed only for fixed specimens for which there are a variety of options[29]. *p*-Phenylenediamine is very effective for FITC and rhodamine, but discolors with exposure to light, so slides must be stored in a dark container. Also, it is a reactive chemical that needs to be used with care to minimize contact[30]. DABCO ('Slow Fade' is the commercial version from Molecular Probes) is a bit less effective but has greater light resistance and less toxicity[31]. *N*-propylgallate is better than the previous two for rhodamine[32]. In addition, there are several proprietary antifade mounting media (Vectashield from Vector Labs, ProLong Gold from Molecular Probes).

It is worth emphasizing that not all dyes are equally susceptible to all anti-fade agents. An agent that works quite well for one dye may be almost useless for another, whereas the reverse may be true for another antifade agent. All of these antifade mountants typically contain a water-glycerine mix plus a chemical designed to reduce fading by reducing the generation of singlet oxygen or its lifetime. The water glycerin mix has a refractive index that is between water and oil. Thus several companies are now making objectives for immersion media between the objective and the coverslip to match the refractive index of the mountant on the other side of the coverslip. Index matching mitigates spherical aberration in the sample images. For living specimens, antifade agents are still not perfected although ascorbic acid, vitamins C and E, beta-carotene and low oxygen tension might provide some relief.

In all imaging applications, high-quality optical filters that provide efficient passage of all or nearly all of the emitted wavelengths can also decrease the bleaching. Use of fast film or cameras with high quantum efficiency or low noise allow for shorter exposures and less bleaching[14,26]. Of course, fluorescent probes that don't bleach would be the best solution. This seemingly unlikely proposition has now been realized with the development of Quantum dots[33]. These complex three-layered nanocrystals use cadmium salt semiconductors as the equivalent of the fluorophore. The outer shell of the dot provides a convenient chemical substrate to attach the dots to biological ligands like avidin. They have many remarkable properties both in terms of an enormous range of absorption and wavelengths based in part on the particular cadmium salt and partly on their size. Because they can be small (nanometers) they are useful for a variety of labeling techniques. Their resistance to bleaching also makes them ideal for single molecule detection.

CONCLUSIONS

The use of fluorescence to probe biological phenomena is rapidly expanding into all fields of cell and molecular biology. The underlying principles in photophysics and chemistry that underlie fluorescence and fluorophores may seem far removed from biology but understanding them are central to good fluorescence microscopy. Moreover, many new modalities such as confocal, multiphoton, stimulated emission depletion (STED), structured illumination, total internal reflection fluorescence (TIRF), FRET, FLIM, fluorescence recovery after photobleaching (FRAP) and fluorescence correlation spectroscopy (FCS). Lastly, the genetic engineering of fluorescent proteins to monitor living cellular activities is among the most powerful new windows into biology. Thus for the foreseeable future, biology and fluorescence will be deeply intertwined.

COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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1. Tsien, R.Y. The green fluorescent protein. *Annu. Rev. Biochem.* **67**, 509–544 (1998).
2. Valeur, B. *Molecular Fluorescence: Principles and Applications.* (Wiley-VCH, Weinheim, 2002).
3. Lakowicz, J.R., ed. *Principles of Fluorescence Spectroscopy*. 2nd ed. (Plenum Press, New York, 1999).
4. Turro, N.J. *Modern Molecular Photochemistry*. (University Science Books, Sausalito, California, 1991).
5. Irvine, D.J., Purbhoo, M.A., Krogsgaard, M. & Davis, M.M. Direct observation of ligand recognition by T cells. *Nature* **419**, 845–849 (2002).
6. Wang, L., Jackson, W.C., Steinbach, P.A. & Tsien, R.Y. Evolution of new nonantibody proteins via iterative somatic hypermutation. *Proc. Natl. Acad. Sci. USA* **101**, 16745–16749 (2004).
7. Deerinck, T.J. *et al.* Fluorescence photooxidation with eosin: a method for high resolution immunolocalization and in situ hybridization detection for light and electron microscopy. *J. Cell Biol.* **126**, 901–910 (1994).
8. Suhling, K., French, P.M. & Phillips, D. Time-resolved fluorescence microscopy. *Photochem. Photobiol. Sci.* **4**, 13–22 (2005).
9. Elson, D. *et al.* Time-domain fluorescence lifetime imaging applied to biological tissue. *Photochem. Photobiol. Sci.* **3**, 795–801 (2004).
10. Jares-Erijman, E.A. & Jovin, T.M. FRET imaging. *Nat. Biotechnol.* **21**, 1387–1395 (2003).
11. Mizuno, H. *et al.* Photo-induced peptide cleavage in the green-to-red conversion of a fluorescent protein. *Mol. Cell* **12**, 1051–1058 (2003).
12. Ando, R., Hama, H., Yamamoto-Hino, M., Mizuno, H. & Miyawaki, A. An optical marker based on the UV-induced green-to-red photoconversion of a fluorescent protein. *Proc. Natl. Acad. Sci. USA* **99**, 12651–12656 (2002).
13. Mason, W.T., ed. *Fluorescent and Luminescent Probes for Biological Activity: A Practical Guide for Quantitative Real-Time Analysis.* (Academic Press Harcourt Brace and Co., Boston, 1993).
14. Periasamy, A., ed. *Methods in Cellular Imaging* (Oxford University Press, Oxford, 2001).
15. Rudolf, R., Mongillo, M., Rizzuto, R. & Pozzan, T. Looking forward to seeing calcium. *Nat. Rev. Mol. Cell Biol.* **4**, 579–586 (2003).
16. Tsien, R.Y. Fluorescent indicators of ion concentrations. *Methods Cell Biol.* **30**, 127–156 (1989).
17. Bassnett, S., Reinisch, L. & Beebe, D.C. Intracellular pH measurement using single excitation-dual emission fluorescence ratios. *Am. J. Physiol.* **258**, 171–178 (1990).
18. Yuste, R. & Konnerth, A., eds. *Imaging in Neuroscience and Development: A Laboratory Manual*. (Cold Spring Harbor Laboratory Press, New York, 2005).
19. Zhang, J., Campbell, R.E., Ting, A.Y. & Tsien, R.Y. Creating new fluorescent probes for cell biology. *Nat. Rev. Mol. Cell Biol.* **3**, 906–918 (2002).
20. Griesbeck, O. Fluorescent proteins as sensors for cellular functions. *Curr. Opin. Neurobiol.* **14**, 636–641 (2004).
21. Ohmichi, T. *et al.* DNA-based biosensor for monitoring pH *in vitro* and in living cells. *Biochemistry* **44**, 7125–7130 (2005).
22. Grinvald, A. & Hildesheim, R. VSDI: a new era in functional imaging of cortical dynamics. *Nat. Rev. Neurosci.* **5**, 874–885 (2004).
23. Kuhn, B., Fromherz, P. & Denk, W. High sensitivity of Stark-shift voltage-sensing dyes by one- or two-photon excitation near the red spectral edge. *Biophys. J.* **87**, 631–639 (2004).
24. Reiff, D.F. *et al.* In vivo performance of genetically encoded indicators of neural activity in flies. *J. Neurosci.* **25**, 4766–4778 (2005).
25. Goldman, R.D. & Spector, D.L., eds. *Live Cell Imaging: A Laboratory Manual.* (Cold Spring Harbor Laboratory Press, New York, 2004).
26. Inoué, S. & Spring, K.R. *Video Microscopy*. 2nd edn. (Plenum Publishing, New York, 1997).
27. Abramowitz, M., Spring, K.R., Keller, H.E. & Davidson, M.W. Basic principles of microscope objectives. *Biotechniques* **33**, 772–781 (2002).
28. Panchuk-Voloshina, N. *et al.* Alexa dyes, a series of new fluorescent dyes that yield exceptionally bright photostable conjugates. *J. Histochem. Cytochem.* **47**, 1179–1188 (1999).
29. Ono, M. *et al.* Quantitative comparison of anti-fading mounting media for confocal laser scanning microscopy. *J. Histochem. Cytochem.* **49**, 305–331 (2001).
30. Johnson, G.D. & Nogueira Araujo, G.M. A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods* **43**, 349–350 (1981).
31. Johnson, G.D. *et al.* Fading of immunofluorescence during microscopy: a study of the phenomenon and its remdedy. *J. Immunol. Methods* **55**, 231–242 (1982).
32. Giloh, H. & Sedat, J.W. Fluorescence microscopy: reduced photobleaching of rhodamine and fluorescein protein conjugates by n-propyl gallate. *Science* **217**, 1252–1255 (1982).
33. Alivisatos, A.P., Gu, W. & Larabell, C. Quantum dots as cellular probes. *Annu. Rev. Biomed. Eng.* **7**, 55–76 (2005).
34. van Gijlswijk, R.P. *et al.* Fluorochrome-labeled tyramides: use in immunocytochemistry and fluorescence *in situ* hybridization. *J. Histochem. Cytochem.* **45**, 375–382 (1997).
35. Cullander, C. Imaging in the far-red with electronic light microscopy: requirements and limitations. *J. Microsc.* **176**, 281–286 (1994).