Microscopic imaging techniques for drug discovery

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Abstract | Microscopic imaging can enhance the drug discovery process by helping to describe how disease processes unfold and how potential therapies might intervene. Recently introduced technologies, and enhancements to existing techniques, are addressing technical issues that have limited the usefulness of microscopic imaging in the past. In particular, these innovations are improving spatial resolution, increasing tissue penetration, overcoming physical access issues and enhancing experimental throughput. Notable recent trends, which are discussed in this article, include the development of super-resolution microscopes, the incorporation of multiphoton techniques into intravital and fibre-optic microscopy and the automation of microscopy and image analysis for high-content screening. Together, these developments are augmenting the existing assays and disease models that are used in early drug discovery and, in some cases, enabling new ones.

An influx of new microscopic imaging methods and tools, including advanced imaging instruments, novel methods and new experimental reagents, are significantly enhancing assays and tissue models for early drug discovery, as well as more complicated disease models that are used later in drug discovery. Together, these innovations are facilitating more sensitive, specific and higher resolution measurements from cells and organisms, and so could have an impact on a wide range of discovery activities ranging from target biology, to compound screening and to animal models of disease.

Many of these innovations are important because they help ameliorate some of the technical limitations that are inherent in microscopic optical imaging (FIG. 1). Specifically, there are four main limitations that currently restrict this form of optical imaging: insufficient spatial resolution, modest depth or tissue penetration, poor physical access and low experimental throughput. In many experimental paradigms, one or more of these factors maybe limiting. For instance, the spatial resolution of light microscopy is often insufficient to adequately capture or characterize all of the underlying biology, especially at a molecular-level detail. However, new super-resolution microscopes are now available that resolve structures to well below the diffraction limit (that is, <200 nm) and thereby allow near molecular-level resolution images to be collected.

In other instances, interesting cells and tissues are inaccessible because they are buried deep within an organism or can only be studied when components of the tissue are artificially reconstituted in a dish. Innovations in intravital microscopy (IVM) and deep-tissue imaging are helping to overcome this problem by allowing image collection at greater tissue depths and in more physiologically relevant contexts. Poor depth penetration and other physical access issues are especially important in drug discovery, as *in vivo* disease models often require the examination of tissues deep within an organism. Parallel developments in optics and fibre-optic imaging technologies are driving the miniaturization of fluorescence microscopy. These developments are being used to facilitate better physical access during small-animal imaging studies.

Another limitation is the configuration of modern research-grade microscopes, which are often complicated and inflexible. This has made them difficult to automate and therefore incompatible with the high-throughput biology approaches that are often used in drug discovery, such as high-throughput screening (HTS). However, recent innovations in high-content screening (HCS) and 'imaging in flow' methodologies are now enabling improved image-based screening by increasing the information content and experimental throughput.

Together, these advanced and high-throughput microscopic imaging technologies are enabling a move away from simple *in vitro* assays with fixed end points. Instead, higher-order models (or model systems[1–3]) that emphasize both physiological relevance and throughput are gaining prominence. An advantage of models without fixed end points is that they allow repeated

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doi:10.1038/nrd2446
Published online 14 December 2007

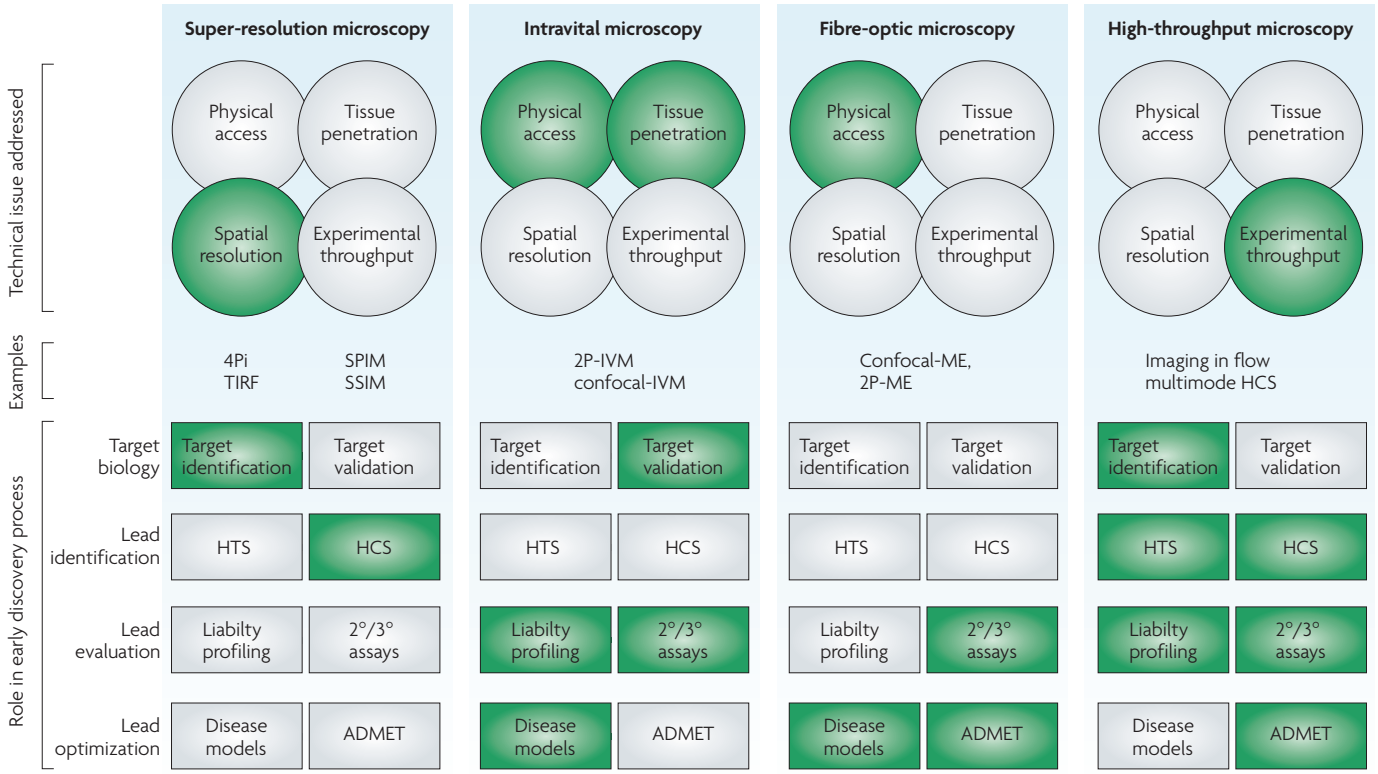


Figure 1 | **Microscopic imaging technologies for drug discovery.** This illustrates some of the important issues (shown in green) and developments in microscopic imaging relevant to early drug discovery. 2P-IVM, two photon intravital microscopy; 2P-ME, two photon microendoscopy; ADMET, absorption, distribution, metabolism, excretion and toxicology; confocal-IVM, confocal intravital microscopy; confocal-ME, confocal micro-endoscopy; HCS, high-content screening; HTS, high-throughput screening; SPIM, selective plan illumination microscopy; SSIM, saturated, structured illumination microscopy; TIRF, total internal reflection fluorescence microscopy.

measurements, chronic observations and/or kinetic determinations that are all important in characterizing the underlying systems biology. In fact, an extension of this theme is the capability to image the *in vivo* treatment response to test compounds, especially in animal models. Imaging the whole-body treatment response is a useful way of simultaneously quantifying the efficacy, time course and the specificity of therapeutic candidates. It is also potentially useful in identifying off-target effects and liabilities.

There is also a business rationale for introducing such technologies. For instance, a new technology may enable a completely novel assay platform, or allow an assay to be automated or reconfigured for greater throughput and/or saving of manpower. In other cases, new technologies can improve existing assays by enhancing information content, increasing data quality, reducing cycle times or by providing cost reductions in disposables and reagents.

This article surveys a range of new microscopic technologies and discusses how they are being used to support various research activities in early drug discovery. It is important to note that many related optical[4] and non-optical imaging[5–7] approaches also have a key role in drug discovery; however, the scope of this Review precludes their consideration here.

Super-resolution microscopes

New super-resolution microscopes are significantly improving the spatial resolution of light microscopy. These super-resolution microscopes are designed to operate at the macromolecular or submicroscopic resolution level and are commonly used to examine structural issues and/or physiological processes in single cells or subcellular organelles.

For a long time, the quality and underlying resolution of images captured with traditional microscopes was considered to be fixed by physical law (that is, image resolution was diffraction-limited)[8]. This finite spatial resolution arises from the fundamental physics involved in focusing light to a discrete spot and is described by Abbe's theory of image formation (see additional explanation in FIG. 2a). In the Abbe scheme, each image is constructed from an array of diffraction-limited spots. The best way to optimize spatial resolution and image contrast was to minimize the size and/or optimize the discreteness of these spots by decreasing the wavelength of excitation light, increasing numerical aperture or by increasing the refractive index of the specimen medium (for example, by using an oil immersion objective rather than an air objective). Even under ideal conditions, resolution is still limited to relatively modest levels (that is, ~0.2 μm laterally). Axial resolution, or a microscope's

optical sectioning capability, is even worse (~0.6 μm) and this makes three-dimensional determinations difficult. Moreover, when dealing with highly convoluted objects, such as cellular organelles, this poor axial sectioning capability can also degrade contrast in the imaging plane. Unfortunately, the overall sample contrast achieved in three-dimensional tissues is generally dominated by this poorer axial resolution.

However, it is important to note that these classical resolution limits are based on several underlying assumptions. In particular, they assume a traditional imaging geometry around a single objective lens; that excitation light is uniformly distributed; and, in the case of fluorescence imaging, that this fluorescence arises from the linear absorption and emission of a single photon. Recently, various groups[9,10] have shown that it is possible to construct instruments that bypass one or more of these limitations by using modified objective lens geometries that gather more light over a larger range of angles; for instance, by using various non-uniform excitation sources (for example, structured illumination) that can encode higher resolution information; and by utilizing nonlinear fluorescence processes. Alone, or in combination, these innovations have produced images that are no longer limited by diffractive phenomena. In some cases, the combined effect of these innovations has pushed the resolution of light microscopy to 30 nm and below[11]. Some examples of the ways used to circumvent these assumptions are shown schematically in FIG. 2b.

Many of the methods that use novel geometries (for example, 4Pi, I^{5}M)[8,11] use some combination of two objectives to optimize light gathering; tilted or rotated views to illuminate the sample in different ways and/or to maximize light capture from different parts of the sample[12]; image interference methods (at the detector and/or sample) to create additional contrast; and coherent light to further maximize optical contrast (often due to the constructive interference of in-phase but counter-propagating beams, also known as standing wave microscopy). In some cases, point scanning is also used to add confocality (that is, rejection of out-of-focus photons). Another geometry that uses a different approach is total internal reflection fluorescence (TIRF) microscopy[13]. TIRF illuminates the sample with a very thin evanescent field that is generated by the interaction of off-axis illumination with the surface. This kind of approach is particularly useful for imaging close to surfaces, because this evanescent field only excites fluorophores within 100 nm or less from the surface and thereby creates a very thin optical section. In general, the disadvantage of methods that use different geometries is that the geometries themselves are quite complicated and can only be applied to a subset of biology.

Methods that use non-homogeneous, or patterned, illumination improve resolution by exposing normally inaccessible high-resolution information in the observed image. These illumination patterns elicit Moiré fringes in the observed image. If the underlying illumination pattern in one image is known, then the Moiré fringes contain information about features in the other (and potentially unknown) image. For example, structured illumination systems capture multiple images ($n \geq 3$), each modulated by the same sinusoidal illumination pattern, but with a shifted phase relation. These images contain additional frequency components and when reconstructed as a weighted set of pair-wise difference images, the resulting image can possess higher spatial information with less out-of-focus light (that is, noise or blur) than a conventional wide field image. In fact, the resulting image quality and resolution is typically comparable with that of a confocal microscope. Advantages of this approach include the ease of integration into existing microscopes and the fact that no emission photons are wasted. Further advances in structured illumination schemes include the recent introduction of advanced axial and lateral patterns[14], three-dimensional structured illumination patterns and single-exposure optical sectioning with colour-structured patterns[15]. Transillumination[16] and reflectance[17] versions of structured illumination have also been introduced.

Methods that use nonlinear fluorescent processes such as multiphoton microscopy or reversible saturation microscopy are commonly used as part of scanning systems that generate improved spatial resolution by creating small fluorescing volumes. These fine volumes can be generated in several ways. For example, multiphoton excitation (BOX 1) is achieved when a fluorophore is excited by two photons, usually at twice their normal excitation wavelength. This process is only possible at the centre of a highly focused, femtosecond laser pulse, where the photon density is largest. This results in a focal excitation volume that is small and not compromised by light scattered from above and below the image plane. In a similar way, stimulated emission depletion (STED) microscopy[18–20] creates a small fluorescent volume by using two pulsed lasers, with structured beam geometries, that interact in a subtractive way to craft a small fluorescence volume.

Saturated structured illumination (SSIM) microscopy[9] is a further method that takes advantage of both patterned illumination and nonlinear fluorescent processes. In an extension to the regular structured illumination approach, SSIM introduces additional nonlinear frequencies into a structured illumination pattern by using peak excitation intensities above the saturation threshold of the fluorophore being used. The advantage of this approach is that it can be achieved with a simple wide-field microscope and no beam scanning is required. The disadvantage is that it is limited by photostability and signal-to-noise issues.

Two newly disclosed methods have pushed the limits of spatial resolution even lower. In one example, called photo-activated localization microscopy (PALM), Betzig and colleagues[21] were able to achieve even higher levels of spatial resolution (~2–25 nm). This method uses minimal stimulation of sparsely populated photoactivatable fluorescent protein (PAFP) molecules[22] (BOX 1), repeated many times, to incrementally sample all the fluorophores in a three-dimensional volume in isolation. By slowly mapping many thousands of sparsely populated

Numerical aperture
The numerical aperture is a measure of the light-gathering capability of an objective lens.

Moiré fringe
The Moiré effect is a well-known phenomenon that occurs when repetitive structures such as screens, grids or gratings are superposed or viewed against each other.

Multiphoton microscopy
This is also called two-photon excitation microscopy or nonlinear optical microscopy.

molecules over many hours, this group was able to effectively establish the absolute position of each individual PAFP, with little or no effect of scattering, and then reassemble this information into a single super-resolution image. Another example, stochastic optical reconstruction microscopy (STORM), also uses photoswitchable fluorophores and multiple imaging cycles to reconstruct a composite image with low nanometre resolution[23]. The disadvantage of both these methods is that images can take many hours to collect.

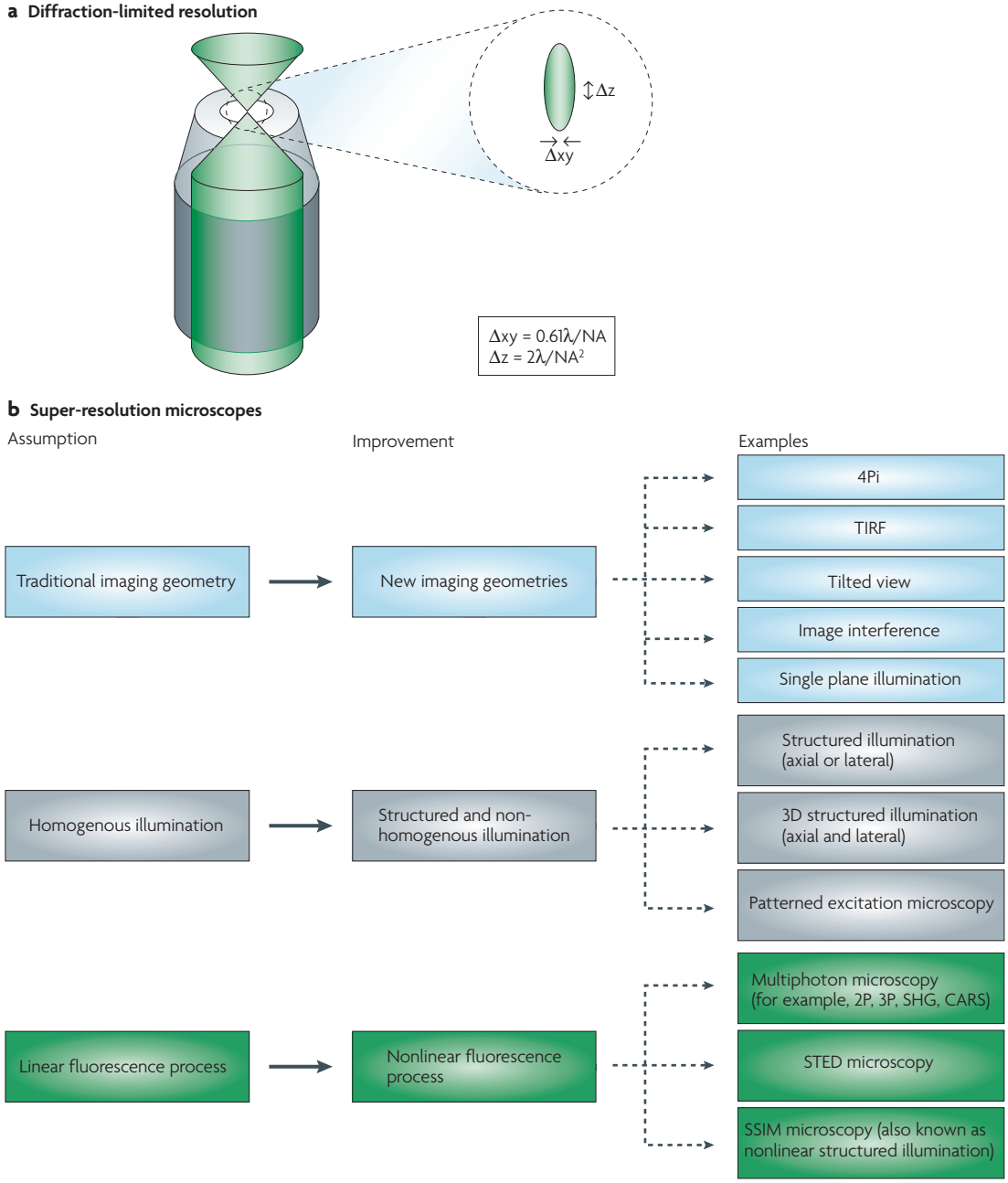


Figure 2 | **Super-resolution microscopes. a** | Diffraction-limited resolution. The smallest focal spot size generated with a traditional objective lens is indicative of the best possible resolution achievable with the larger microscope. This focal spot is laterally symmetrical (that is, in x and y) but elongated axially (that is, in z). Using the Rayleigh criterion to distinguish between two such adjacent spots gives a level of resolution described by the formulas shown, where NA represents numerical aperture. **b** | Super-resolution microscopes. These calculations of diffraction-limited resolution are based on three main assumptions about the imaging geometry, illumination homogeneity and the underlying photophysics. New techniques that extend spatial resolution beyond the diffraction limit have circumvented these assumptions in a number of clever ways. 2P, two photon microscopy; 3P, three photon microscopy; CARS, coherent anti-stokes Raman scattering microscopy; SHG, second harmonic generation microscopy; SSIM, saturated, structured illumination microscopy; STED, stimulated emission depletion microscopy; TIRF, total internal reflection fluorescence microscopy.

Box 1 | **Additional terms relating to microscopic imaging**

Multiphoton excitation. This is based on the idea that two or more photons of low energy can excite a fluorophore in a quantum event and thereby elicit fluorescent emission. The probability of the simultaneous absorption (that is, within 0.5 femtoseconds) of two or more photons is extremely low and therefore a high flux of excitation photons is typically required. In this scheme, excited fluorophores are confined to a small focal volume that translates into high spatial resolution imaging and an important optical sectioning capability. Infrared excitation light is commonly used in multiphoton microscopy and this has the added benefit of less scattering in complex three-dimensional tissues. The principles of two-photon excitation fluorescence and other nonlinear imaging approaches were recently reviewed by Oheim *et al*[96].

Photoactivatable fluorescent proteins (PAFPs). These are fluorescent proteins whose photo properties can be manipulated optically by irradiating them with light of a specific wavelength, intensity or duration[22]. The plasticity of PAFPs can be put to experimental use in several ways: photobleaching (that is, erasing existing fluorescence), photoactivation (that is, eliciting fluorescence from a nonfluorescent precursor) and photoconversion or photoswitching (that is, changing the wavelength of fluorescent emission).

High-brightness light-emitting diodes (HB-LEDs). These are a rapidly evolving[97] solid-state alternative to the traditional light sources (for example, mercury lamps and xenon arc lamps) that are commonly used in fluorescence microscopy. Although these HB-LEDs have yet to be incorporated into any high-content-screening (HCS) instrument, they do possess many favourable features that make them good candidates for inclusion. In particular, these HB-LEDs exhibit a consistent, stable[98] and instant source of light suitable for all types of fluorescence microscopy[99,100]. Moreover, they are available in a range of visible, ultraviolet and infrared wavelengths and when compared with xenon and mercury lamps they are easier to use, consume significantly less power, generate less heat and there are no limitations to the number of times they can be turned off and on. Recent versions of these HB-LEDs easily match the optical power output, and surpass the luminous efficiency, of mercury and xenon bulbs without any of the safety or convenience limitations. The unit cost of these HB-LEDs is small in comparison with traditional light sources and this may enable parallel illumination schemes for microtitre plates that use many miniaturized LED sources.

Supercontinuum lasers. These are a powerful, broadband excitation source that possesses all the favourable properties of regular lasers (that is, coherence, collimination and compatibility with scanning systems, especially confocal microscopes). The supercontinuum effect[101] occurs as a consequence of nonlinear optical processes that arise when very short laser pulses propagate through specially designed optical fibres (that is, photonic crystal fibres). Supercontinuum sources, although relatively new, have already been successfully applied to confocal and multiphoton microscopy[78,102–104]. The potential advantages of these sources for HCS include a simpler instrument configuration (that is, a single colliminated laser source), greater experimental flexibility (that is, a wider choice of excitation wavelengths) and potentially greater optical power at some wavelengths.

In summary, many of these super-resolution techniques have only recently evolved out of the physics laboratory into everyday use[11]. Additional work is still required to make them compatible with wet biology and high-throughput methods such as those used in drug discovery. Nonetheless, the reported improvements in spatial resolution are impressive and there are already examples of how these techniques can be incorporated into commercial microscopes (for example, the Leica 4PI microscope) and into screening instruments (for example, structured illumination capabilities into HCS readers).

IVM and deep-tissue imaging

In contrast to super-resolution microscopes, IVM and deep-tissue imaging seek to examine cellular behaviour in *in vivo* or *ex vivo* preparations with microscopic resolution. In both *in vivo* or *ex vivo* contexts, the key goal of these technologies is to collect functional information about dynamic processes occurring in tissues and organ systems that are maintained in their natural state or at least in a physiologically relevant state. IVM is different from whole animal or surface imaging, which is normally conducted with non-invasive methods and typically achieves only macroscopic resolution[4,7].

IVM and deep-tissue imaging present a different set of challenges to super-resolution microscopes: namely, physical access and light scattering. Specifically, obtaining *in vivo* access to interesting tissues is often incompatible with standard microscope optics and in many cases requires maintaining an animal or tissue in a tenuous imaging configuration for long periods of time. More importantly, biological tissues absorb and scatter light in a wavelength-dependent manner. This severely retards depth penetration, increases background haze and compromises image formation. Short or near-ultraviolet wavelengths (for example, ~400 nm) are normally scattered the most and cause the highest levels of tissue autofluorescence. For these reasons, IVM and deep-tissue imaging have traditionally been attempted with red or near infrared (NIR) excitation wavelengths. Although long wavelength illumination is not a particularly new idea, it is an area that has undergone considerable technological development recently and will probably have significant near-term impact in target biology and animal models of disease[24–33]. Some of these technological developments and novel applications for target biology and animal models are discussed below.

New optics and more flexible instruments are also helping to solve the physical access problem. For instance, new objective lens configurations such as those shown in FIG. 3a are allowing access to virtually any internal organ (accessible by keyhole surgery). In addition to their novel geometry, these objective lenses possess a relatively high numerical aperture and are optimized for use at red and NIR wavelengths. Furthermore, they can be fitted with drain tubes for aspiration of blood or conduits for the injection of dye, drugs or saline. Alencar *et al.*[34] were the first to demonstrate the utility of these 'stick objectives' for *in vivo* cancer imaging. The authors speculated that when combined with novel red and NIR imaging probes these stick objectives would be useful in a wide range of animal disease models.

An alternative to these new stick objective configurations is gradient index (GRIN) lens technology. GRIN lenses exploit a radial variation in refractive index to focus light; by contrast, conventional optics refract light by variations in lens geometry (FIG. 3b). GRIN lenses can be fabricated with submillimetre diameters and typically possess planar optical surfaces that are easily assembled into a composite lens or mated with optical fibres. GRIN lenses in different forms can be used as an objective lens to focus light or as a relay lens to transfer an image some distance from the object plane. Together these properties make them ideally suited to be the terminal optical element in devices used to probe deep into tissues or restricted spaces. To date, most applications using GRIN lenses have positioned

these elements downstream of a regular objective lens and used the accompanying microscope for image capture. However, it should be noted that GRIN lenses can also play an important role in fibre-optic imaging devices (described later). The former approach has recently been used to image the vasculature and individual neurons at subsurface locations in the brain with subcellular resolution[35–37].

a

Magnification	Diameter (mm)	FOV (mm)	Length (mm)	Working distance	Numerical aperture
6×	1.3	0.67	20	0.20	0.14
20×	1.3	0.20	20	0.20	0.50
27×	3.5	0.2	25	0.20	0.70

b

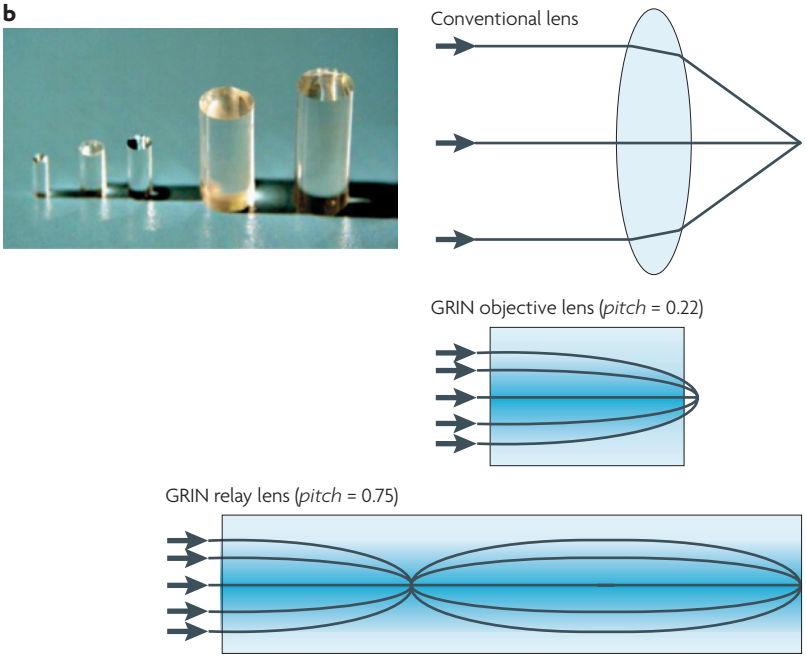


Figure 3 | **New optics for intravital microscopy. a** | The left panel shows a new objective lens configurations specialized for intravital microscopy (MicroProbe, Olympus). The right shows a representative image collected with MicroProbe objective lenses and the Olympus intravital laser scanning microscope (not shown) — an extended focus image of lucifer yellow labelled neuron in a fixed brain slice using 27× Microprobe lens. Image courtesy of Brendan Brinkman, Neuroscience Microscopy Shared Facility, University of California at San Diego, USA. **b** | Gradient index (GRIN) lenses. The left panel shows representative images of different size GRIN lenses. The right panel shows the light path through conventional and GRIN lenses of different path lengths. FOV, field of view.

In theory, it should also be possible to improve regular IVM by incorporating confocal detection techniques. However, various factors have meant that this method is not easily used for this purpose. The most important of these factors is light scattering and this arises because confocal excitation is achieved with an entire cone of excitation light. This cone interacts with the tissue it is penetrating and this elicits light scattering in direct proportion to the imaging depth. At any significant depth, this light scattering severely degrades the excitation spot intensity and discreteness, which weaken and blurs the resulting image. Increasing illumination intensity only confounds this problem and causes additional photodamage or phototoxicity. Instead, the real advance for IVM came with the inclusion of another technique: multiphoton microscopy. Several recent reviews have described this technique[38–41] and its application to various areas in *in vivo* or *ex vivo* biology[24,25,31,32,42–47]. To summarize, there are five main advantages of multiphoton approaches for IVM when compared with conventional microscopic methods and especially with confocal approaches. These advantages are less image degradation by light scattering (leading to higher resolution and contrast); greater depth penetration (approximately six-times that of confocal); better optical efficiency with no signal rejection by a pinhole; better z-registration, which leads to better signal-to-noise ratios and more successful three-dimensional image reconstructions; and less photodamage and phototoxicity, resulting in longer imaging times. Additionally, recent innovations in femtosecond laser pulse-shaping (both spatial and temporal) and pulse spacing will probably further improve the utility of multiphoton IVM by maximizing signal strength, reducing the amount of background excitation and significantly improving the depth penetration (BOXES 2,3). Similarly, developments in fast three-dimensional scanning methods[48] and multifocal–multipoint scanning techniques[49–51] will probably enable near-simultaneous measurements from many different points and imaging planes.

Although the advantages of multiphoton microscopy have been known for some time, the cost and complexity of setting up and maintaining a multiphoton laser system was initially a significant barrier to adoption[39]. However, recent advances in pulsed-laser technology have seen the development of turnkey laser systems that are suitable for incorporation into IVM setups and two-photon IVM is now more commonplace. In fact, this approach has now been applied to several disease biology disciplines, representative examples of which are described next.

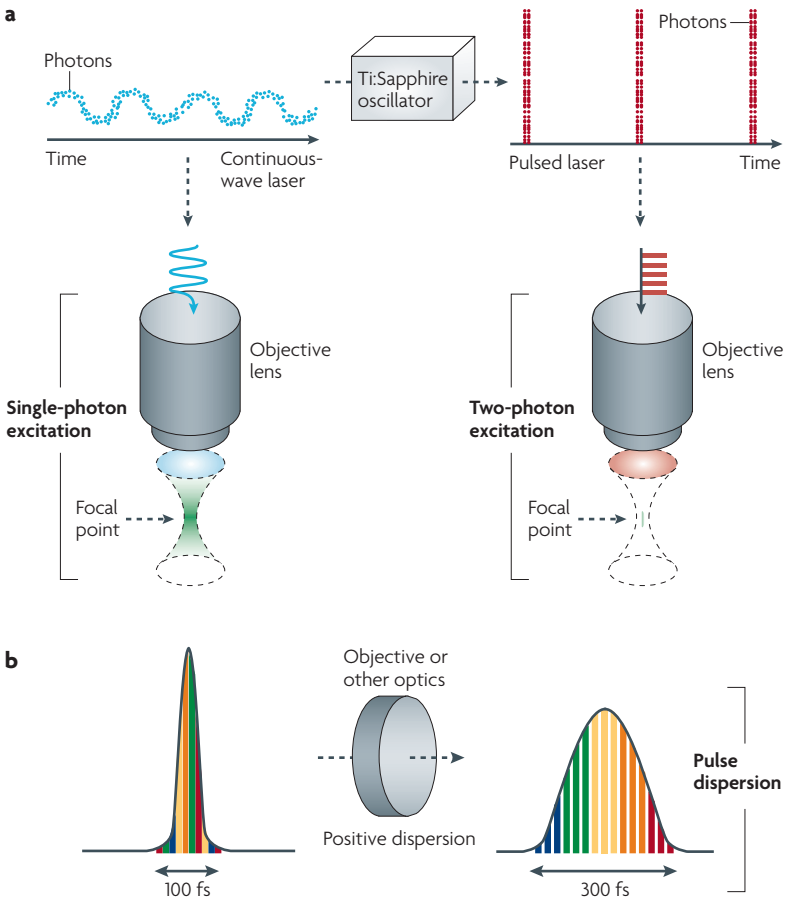
One area that has benefited from the incorporation of multiphoton microscopy into IVM is immunology[24,27,32]. Many important lymphocyte behaviours, such as antigen recognition, take place deep in densely populated lymphoid tissues. Until recently, examining these behaviours with microscopic resolution was only possible during *in vitro* experiments. However, several groups have now demonstrated that these behaviours can also be reliably assayed *in vivo* with multiphoton IVM. For instance,

Box 2 | **Multiphoton excitation**

Multiphoton excitation requires the concentration of photons in space and time. Spatial concentration of photons is normally achieved by focusing a laser beam to a small spot with a high numerical aperture objective lens as in traditional single-photon microscopy. By contrast, the temporal concentration of photons is accomplished by compressing photons from a continuous source into short (that is, femtosecond) pulses (right panel of figure **a**). Near-infrared pulses of this type are commonly produced by a Ti-sapphire oscillator driven by a continuous wave laser and typically exhibit high peak intensities but low average power. The combination of ultra-short pulses and spatial focusing elicits multiphoton excitation in a small focal volume that virtually eliminates the background fluorescence seen in single-photon microscopy.

The strength of multiphoton excitation is inversely proportional to pulse duration. Although very short pulses (<100 femtosecond) give the strongest signal, they are no longer purely monochromatic and as they propagate through different optical elements (that is, optical fibres and objective lens) some dispersion, or pulse broadening, occurs (**b**). This chromatic dispersion arises because separate spectral components are retarded differentially depending on their wavelength. Pulse dispersion reduces the overall efficiency of multiphoton excitation in general but is most problematic in applications that use complicated optical configurations such as intravital microscopy (IVM).

Eliciting sufficient multiphoton excitation in IVM is also compromised by the loss of excitation intensity owing to scattering and linear absorption. This is especially true in cases in which the interesting biology is buried at depths beyond the reach of pulses with sufficient intensity. In theory, pulses of greater starting intensity could be used, but these can cause photodamage. Similarly, pulse power can be amplified at the expense of repetition rate[105] but again, only under conditions in which photodamage or background fluorescence are not limiting. Alternatively, new methods of pulse shaping are now available that counteract pulse dispersion and thereby improve the efficiency of multiphoton excitation in general and multiphoton IVM in particular. Such methods are described in BOX 3.



Witt *et al.*[52] have recently shown an interesting transition in the motility of developing T-cells (that is, thymocytes) undergoing positive selection in the thymus. Using multiphoton IVM to track green fluorescent protein (GFP)-labelled thymocytes in real-time and in three-dimensions, they showed that, before positive selection, these thymocytes had a distinct non-polar morphology and that their movement was effectively random. However, immediately following positive selection, these cells adopted a polar morphology with an obvious leading edge, and displayed rapid and directed migration towards the thymic medulla (FIG. 4a). In another study examining processes further downstream, Okada *et al.*[53] investigated interactions between B cells and T cells in intact lymph nodes. Interestingly, the authors documented a combination of random and directed behaviours. Antigen-engaged B cells migrate randomly along the follicular edge but then undergo directed migration near the follicle/T-cell border as they encounter their helper T-cell counterpart. The authors showed this directional movement occurred in a CCR7-dependent manner and thus provided evidence in support of lymphocyte chemotaxis *in vivo* (FIG. 4b).

Another area that has benefited from the incorporation of multiphoton microscopy into IVM is infectious diseases. For example, Frevert *et al.*[54] have used this approach to investigate the life cycle of malarial parasites once they infect a mouse liver. FIGURE 4c shows malarial parasites, tagged with red fluorescent protein, as they transverse the sinusoids of a mouse liver. Interestingly, this study showed that, contrary to previous thought, the main portal of entry into the liver for malaria parasites was through the Kupffer cells that line this sinus. Another group[55] used a similar approach to examine bacterial pathogenesis in a rat model of pyelonephritis. This group used real-time imaging to characterize the spatio-temporal pattern of bacterial proliferation and associated tissue inflammation. Novel mechanistic observations like this, and the potential to assay their modulation by therapeutic agents, are likely to have significant implications for disease biology and pharmaceutical discovery in the future.

Tumour biology has also benefited from the incorporation of multiphoton microscopy into IVM. In particular, multiphoton IVM has been used to examine cell migration in tumours and metastasis. Work from Condeelis and others[26,33] using this approach has provided new insights into the mechanisms of cell protrusion and chemotactic migration during invasion and metastasis, with important new roles being assigned to macrophages, elements of the extracellular matrix and stromal cells. These *in vivo* results show that the regulation of cancer-cell migration is more complicated than was first supposed from *in vitro* studies, and that there are important differences between *in vivo* and *in vitro* conditions[26]. Although multiphoton IVM is an essential technology for understanding the basic mechanisms of oncogenesis, it also has a potentially important role in characterizing the physiological barriers to the delivery of therapeutic agents[28,56]. For instance, IVM studies have been used to characterize

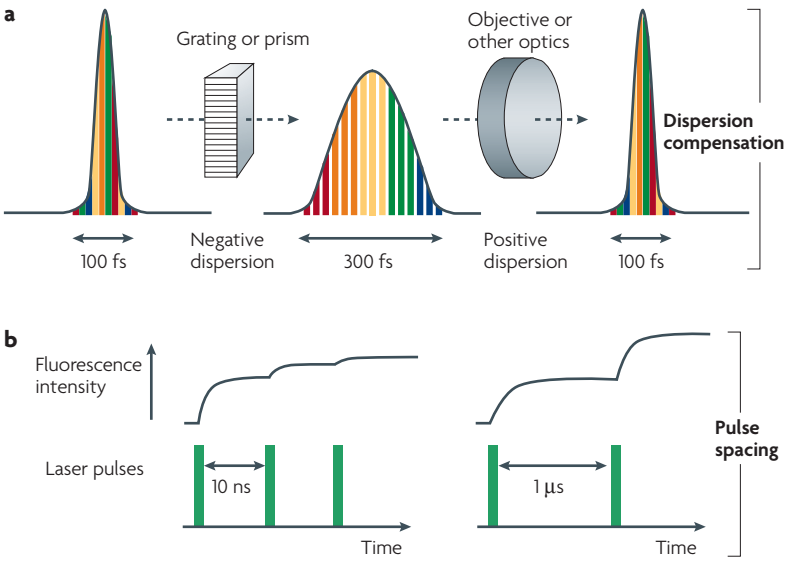
Box 3 | **Multiphoton innovations: making the most of your pulses**

Pulse shaping can counteract pulse dispersion and thereby improve the efficiency of multiphoton excitation in general and multiphoton intravital microscopy (IVM) in particular. Pulse-shaping methods (also called pre-chirping) impart a 'negative dispersion' (also known as negative group velocity dispersion or GVD) to longer wavelength components in each pulse and thereby allow pulses to travel further through a scattering tissue before they become too weak to be effective. This manipulation improves the effective depth penetration (**a**) by up to three times[106] relative to unconditioned pulses without causing any additional photodamage.

Higher order, or pulse phase, manipulations can also help improve the effectiveness and repeatability of these ultra-short pulses[107,108]. These coherence, or phase adjustments, minimize constructive/destructive interference normally occurring within each pulse and thereby promote more homogeneous excitation. Apparently, these phase corrections can increase signal intensity and improve contrast compared with uncompensated pulses. Conveniently, these coherence manipulations are maintained following passage through scattering tissues[109] and there is evidence to suggest that photodamage is also lessened[107]. Interestingly, this method can also be used for selective excitation[108,110] of closely spaced fluorophores or ratiometric imaging of different excitation bands.

Another particularly exciting development in ultra-short pulse-shaping technologies is that some variations may enable axial focusing without having to move the terminal focusing element (that is, without moving an objective lens)[111–113]. In fact, it has been shown that under specific optical conditions insertion of a variable GVD can be used to adjust z-focus. Such a remote axial focusing capability would be a major improvement to current methods in three-dimensional IVM. It may be possible to use this method in combination with lower numerical aperture lenses, which possess correspondingly longer working distance, to reach previously inaccessible tissue depths. Other configurations[41] that reduce the effective numerical aperture of an objective (that is, by incompletely illuminating its back focal plane) are also candidates to combine with this temporal focusing effect.

Another way to increase the efficiency of multiphoton excitation is to manipulate the spacing between pulses. Donnert and colleagues[114] have recently reported that a substantial improvement in multiphoton efficiency (that is, stronger fluorescence and less photobleaching) could be achieved by increasing the time between successive pulses enough to allow complete dark-state relaxation (**b**). Normally, the time between successive pulses in two-photon microscopy is 10–25 ns. By lengthening this time to >1 μs the authors were able to show a substantial augmentation (that is, 20–25×) in fluorescence intensity and significantly less photobleaching. Although this lengthened inter-pulse interval will probably translate into less pulses per pixel dwell time and/or slower frame rates, the magnitude of this signal augmentation is highly significant and will certainly improve image quality in many applications.



the *in vivo* therapeutic response during anti-angiogenic therapies aimed at destroying tumour vasculature. These studies have been useful in identifying novel mechanisms of action, suggesting that these therapies may actually work by normalizing tumour vasculature rather than by destroying it[57].

In general, the advantage of multiphoton IVM over competing technologies such as PET and MRI is the capability to chronically image structure, function and the therapeutic response at high resolution with superior contrast and maximal physiological relevance. The recent synergism demonstrated between multiphoton imaging and quantum dots[58] means that the prevalence and usefulness of IVM in disease models is only going to increase (BOX 4).

Fibre-optic imaging

Fibre-optic imaging is a rapidly evolving technique that permits *in vivo* visualization of cells and tissues under circumstances that preclude the use of a traditional light microscope[59–61]. It is particularly well suited for non-destructively investigating disease processes within hollow tracts, such as blood vessels, from deep within organ systems (for example, the brain) or from within solid tumours. Fibre-optic imaging is particularly valuable for drug discovery applications because it provides physical access to tissues and organisms that are of high clinical relevance. To date, this physical access has been achieved at the expense of some signal quality, spatial resolution and depth penetration. However, technological developments in this area are now appearing that help address many of these issues. Parallel innovations are also enabling chronic or long-term physical access, which is valuable for longitudinal animal studies. In many instances, this form of imaging is a portable alternative to IVM and the deep-tissue two-photon approaches described earlier.

Fibre-optic imaging technologies were initially based on optical fibres that were simple, efficient and flexible light conduits, but recently fibres have become available that act as filters (that is, spectral, spatial and polarization filters), as beam-splitters and as nonlinear devices that generate or manipulate particular kinds of light[62]. Moreover, the miniaturization of associated fibre-optic hardware, such as microelectromechanical system scanners[59] and microlenses, is driving the overall reduction in size or compactness of many *in vivo* imaging technologies[63–66]. Specifically, progress in this area has been driven by a significant reduction in probe size, an increase in bundle element numbers and the incorporation of new imaging modes. Portable fibre imaging devices (fibrescopes) have also been developed that allow chronic implantation for long-term imaging studies in small animals[67].

Fibre-based imaging devices are often categorized into three broad classes on the basis of their size and potential usage: microscopy, endoscopy and micro-endoscopy. These categories can be further subdivided into many variations (FIG. 5). Of these, portable microscopes and microendscopes are currently the most useful for disease biology and drug discovery applications.

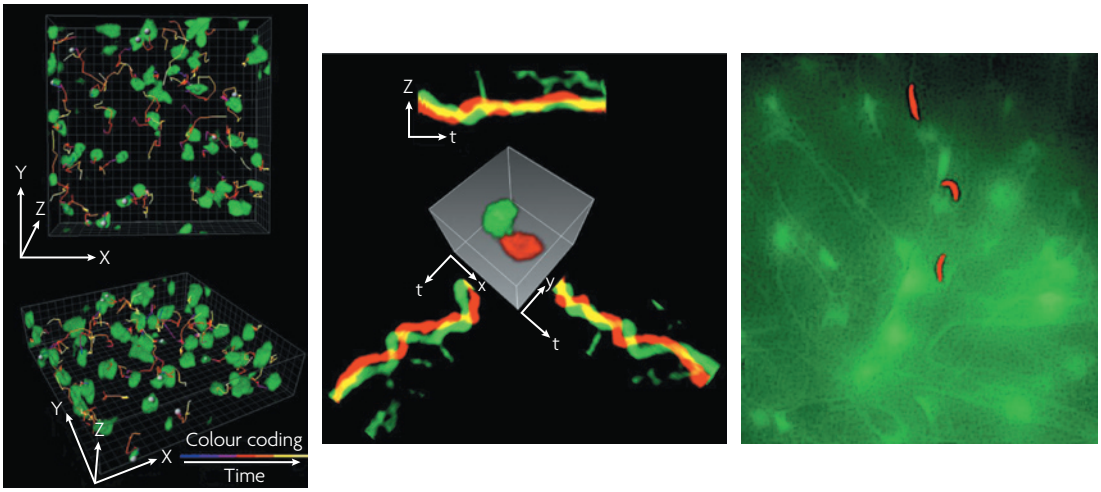


Figure 4 | **Disease biology applications of intravital microscopy. a** | Tracking thymocyte migration in three dimensions in the mammalian thymus using intravital microscopy. Trajectories of individual thymocytes at a single time point superimposed on cell tracks. Image reproduced from REF. 52. **b** | Time lapse, two-photon image reconstruction showing the three-dimensional dynamics of a B-cell (red) and T-cell (green) conjugate within live lymph tissue. Image reproduced from REF. 123. **c** | Malarial parasites (tagged with red fluorescent protein) in the sporozoite stage migrating along the sinusoids in a mouse liver imaged using two-photon intravital microscopy. Image reproduced from REF. 124.

In particular, fluorescence microendoscopy (FME), is the most frequently used form of minimally invasive deep-tissue imaging. FME is usually applied in cases in which cellular resolution is required deep within complex three-dimensional tissues. FME commonly uses probes of 1 mm or less. Initially, these endoscope categories were solely paired with traditional reflective light and epifluorescence optics. Recently, however, two new microendoscopy paradigms have emerged — confocal fluorescence and two-photon fluorescence — that possess several technical advantages[37,68] over simple FME. These advantages include improved spatial resolution, better optical sectioning and deeper tissue penetration.

The confocal versions of FME generate a spatially resolved image using fibre bundles in which each individual pixel is carried by an independent fibre or with a single fibre that is mechanically scanned in a raster pattern by a miniaturized imaging head[62]. In the former case, image capture can occur in direct contact (that is, no terminal focusing element) or focusing mode (that is, with an objective lens). In choosing between these approaches it is often necessary to make a trade-off between image quality and ease of implementation and/or miniaturization. Typically, fibre-bundle systems are simpler and easier to miniaturize but give poorer image quality. Systems that use a single fibre and distal scan heads are more complicated and harder to miniaturize but generally produce better images. Image quality is largely dictated by the numerical aperture of the fibre, or final focusing element, which determines the relative illumination spot size and light-gathering ability of these systems. This feature, together with the amount of scattering in the tissue and the geometry of the fibre bundle, determines the relative efficacy of these systems in terms of signal strength and image quality. Although fibre-bundle systems can generate images confocally, with some degree of optical sectioning, they generally collect less light and often suffer from image pixelation related to the spacing between bundle elements. Various signal-processing techniques (especially pixel dilations, background subtraction and spatial filtering) can be used to mitigate this problem, but the underlying deficiency in image resolution is not changed.

The utility of this fibred confocal approach for oncology applications was recently demonstrated by D'Hallewin *et al.*[69] who conducted *in situ* bladder tumour imaging following intravesical administration of rhodamine 123. Using this endoscopic approach, the authors were able to visualize different cell types present in the bladder epithelium. Tumours were distinguished as bright fluorescent blots surrounded by many small inflammatory cells. Commercialization of this hardware has also meant that it has moved from the purely research setting into the drug discovery realm. Vendors currently offer instruments (for example, Cellvizio; Mauna Kea Technologies, Paris) with submillimetre diameter endoscopic probes that exhibit micron-scale resolution and which have successfully enabled *in situ* molecular imaging at the cellular level. These instruments are based on a fibred, scanning confocal approach and have been used for various cardiovascular, oncology and absorption, distribution, excretion, metabolism and toxicity applications[60,61,68,70] (FIG. 6).

Applying multiphoton techniques to fibre-optic imaging has been more difficult because the transmission of ultra-short pulses through single-mode fibres is poor[71]. In particular, chromatic dispersion, self-phase modulation and peak power threshold limitation have limited the usefulness of these fibres for multiphoton fibre-optic imaging[59]. However, new microstructure fibres or photonic crystal fibres (PCFs)[72] that have less

CCR7
CCR7 is a chemokine receptor involved in the trafficking of B and T lymphocytes and dendritic cells.

Pyelonephritis
This is an infectious condition of the kidney whereby bacteria spread throughout the lumen of infected nephrons into surrounding tissues. It is accompanied by an acute inflammatory response and can result in significant tissue damage.

PET
Positron-emission tomography. A noninvasive, molecular imaging technique of high sensitivity that detects species labelled with positron-emitting radionuclides *in vivo*.

MRI
Magnetic resonance imaging. The use of radio waves in the presence of a magnetic field to extract information from certain atomic nuclei (most commonly hydrogen, for example, in water). Tissues can be differentiated by differences in their water densities.

Box 4 | **Intravital multiphoton imaging of Alzheimer's disease**

Intravital multiphoton microscopy has been used to conduct longitudinal *in vivo* imaging studies of plaques in animal models of Alzheimer's disease (AD)[115–117]. Wider application of this approach should have a significant impact on discovery programmes in this and related areas. Clinical trials in this area are difficult to conduct because AD is a diagnosis of exclusion that is usually confirmed only after death.

AD is characterized by the appearance of senile plaques (that is, insoluble aggregates of amyloid-β peptide; Aβ) in the extracellular space and neurofibrillary tangles inside neurons. These plaques and tangles are clearly visible in the light microscope post-mortem, but so far it has been difficult to obtain an equivalent non-destructive readout from living animals. Moreover, it has been difficult to follow the evolution of these plaques and tangles in animal studies because of their size and the difficulty in accessing deep-brain structures. The application of intravital multiphoton microscopy in this area[117,118] was an important development because it has allowed repeated and kinetic observations from transgenic mouse models of AD at a scale and depth not previously possible[118]. Long-term multiphoton imaging from the same animal was made possible by exchanging a section of the skull with a permanently affixed cranial window[117]. Preliminary studies using this technique addressed the lifetime of individual plaques[116] and their clearance following first-generation immunotherapies[115,119]. It is likely that other potential therapeutic interventions (that is, small-molecule drugs) could also be evaluated in a similar manner.

Interestingly, this optical approach was also used to characterize some of the consequences of plaque formation. For instance, Spires *et al.*[120] documented a widespread pattern of change in neuronal architecture attributable to these plaques. In particular, there was a synaptotoxic effect that included a disruption to dendritic spine morphology. This effect extended well beyond the plaques themselves. The authors speculated that the wider effects of these plaques maybe responsible for some of the cognitive impairments associated with AD.

AD is also associated with vasculature pathologies such as amyloid angiopathy. In this condition, Aβ is deposited in the walls of arterial vessels and this can lead to haemorrhagic stroke. Intravital multiphoton microscopy has also proved useful in characterizing the kinetics of this disease process[121] and its potential reversibility with antibody-mediated therapies[122].

dispersion and increased power thresholds are now making the introduction of multiphoton excitation from remote sources, by optical fibres, possible[73–76]. These PCFs are elaborations of a wider class of photonic crystals[77] and have proved to be useful for multiphoton microscopy in general[78] and miniaturized fibre optic versions in particular[63–65].

One example of how multiphoton excitation can be usefully delivered through a highly miniaturized system utilizing PCFs is a novel hand-held optical biopsy probe recently developed by So and colleagues[79]. This high-speed, hand-held miniaturized multifocal, multiphoton microscope (H^2M^4) device was constructed to perform optical biopsies with subcellular resolution. In particular, it was optimized for the compactness and portability required in clinical use. To increase the speed of image acquisition, this device uses an array of microlenses to generate parallel excitation beamlets. This multifoci array is scanned over the tissue under study by a piezo-electric tip-tilt mirror that is capable of bidirectional scanning and subsequently descanned using a multi-anode photomultiplier tube. This parallel point-scanning configuration significantly reduced the time needed to generate high-quality images and enables the high frame rates that are necessary with hand-held clinical devices.

Flow cytometry
Flow cytometry is a well-established technique that is used to count, characterize and sometimes sort cells that are suspended in a stream of fluid.

Fibre-optic devices are also useful for other drug discovery and disease biology applications. For example, Verkman and colleagues[80] have recently used microfibre-optic epifluorescence photobleaching (MFEP) to address basic questions about diffusion in solid tumours. This group showed that, in addition to a known diffusion barrier at the tumour surface, there is also a previously unrecognized slowing of diffusion deep in tumours that may be an important barrier to drug delivery.

HT microscopy and image processing

HCS is an example of how the industrialization of microscopic imaging technologies can be successfully applied to support discovery sciences and large-scale cell biology[81,82]. HCS automates image acquisition and analysis of microtitre-plate assays by applying sophisticated robotic instrumentation together with powerful information processing and storage strategies. The development of HCS has paralleled the rise in popularity of cell-based assays. However, HCS assays are typically more powerful than equivalent HTS assays because they are inherently multi-parametric, and also because they consider individual cells rather than the population response of an entire well. Moreover, it is possible to consider subpopulations of cells based on their phenotypes and/or other secondary markers. Multiplexing of different assays is also possible using HCS techniques[83]. In general, HCS assays provide significantly more, and higher quality, information than equivalent non-imaging assays.

HCS is now considered a mainstream technology and has spread to all aspects of pharmaceutical discovery[84]. In recent years, the number and sophistication of HCS instruments has grown significantly[85,86]. New instruments are offering more excitation sources and emission channels together with multimode detection capabilities. Many innovations in the wider microscopy field are also being included in these instruments (for example, structured illumination and multipoint scanning). Perhaps the most exciting potential innovation in this area is the incorporation of novel illumination sources into HCS instruments. For instance, smaller, more compact and more energy efficient solid-state lasers are commonly being used with contemporary HCS instruments. Two other potential sources are more revolutionary: high-brightness light-emitting diodes and supercontinuum lasers (BOX 1).

A closely related development is image-based flow cytometry or 'imaging in flow' applications. Flow cytometry instruments typically make many measurements in parallel, which include multi-spectral determinations that are used to indicate protein expression, cell health and/or the presence of some label. In parallel, light scattering measurements are used to assay other cellular properties such as cell size using forward scatter and granularity by examining side scatter. These instruments are commonly accompanied by powerful software packages that categorize different cell populations based on a combination of these measurements.

The power of flow cytometry arises from the ability to quickly process individual cells in large numbers and then robustly characterize these cells into discrete subpopulations with subsequent statistical analysis. To date, flow

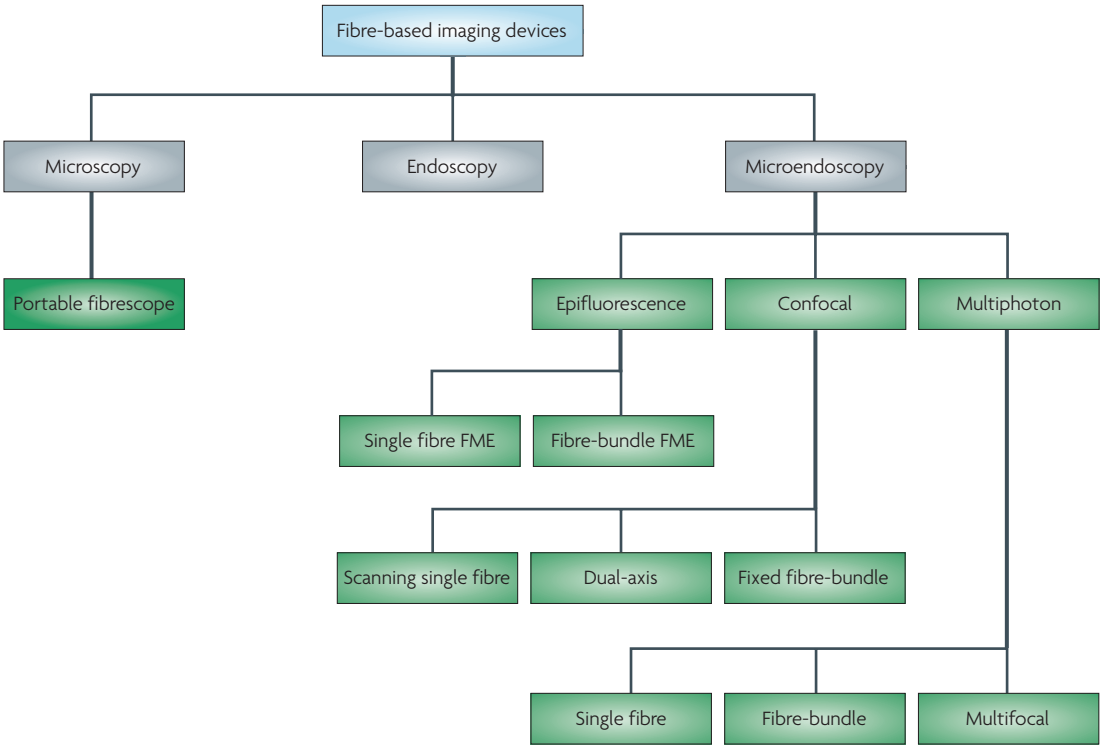


Figure 5 | **Fibre-optic imaging technique hierarchy.** There are a range of fibre-based imaging devices currently available; however only a subset of these (that is, microendoscopy) allow microscopic level resolution. These different microendoscopic modalities use different mechanisms of image formation (that is, wide field versus proximal scanning versus distal scanning), different types of fluorescence excitation (for example, single photon versus multiphoton) and different mechanisms of optical sectioning (for example, confocal versus multiphoton). The relative efficacy of each approach is discussed in more detail in the text.

cytometry has largely been a non-imaging technique. However, recent innovations in this area have seen the incorporation of imaging detectors into flow-cytometry instruments. These imaging-in-flow instruments combine the power of quantitative microscopy with the statistical power of flow cytometry[87]. Conveniently, these imaging detectors also possess enough spatial resolution to undertake subcellular localization of fluorescence markers. Furthermore, some of these instruments can also collect brightfield and darkfield images from each cell under examination. The integration of these different image sources significantly increases the overall information content and allows powerful comparisons to be drawn between, and even within, subpopulations of cells that have previously been identified as the same by traditional flow cytometry. This type of system possesses many of the advantages of modern HCS systems but does not require that cells be adherent and/or fixed. Additionally, the relative throughput of these imaging-in-flow instruments (that is, 15,000 cells per min) is also greater than many HCS instruments.

Commercial devices for imaging flow cytometry are now available, such as ImageStream (Amnis, Seattle, USA). This instrument uses a novel implementation of a charge-coupled device-based image sensor. Specifically, this sensor uses time-delay integration to follow a cell across the imager at a rate that is carefully matched to the underlying fluid stream. This allows multiple image acquisitions from the same cell and thereby achieves better sensitivity, registration and image quality[88].An example of a study using an imaging flow cytometer that has potential pharmaceutical applications was recently documented by George and colleagues[89]. This group examined nuclear translocation events associated with the nuclear factor-κB (NFκB) system. Using the ImageStream instrument, the authors were able to robustly assay NFκB translocation in immunologically interestingly but technically challenging cell types (naturally non-adherent cells with small cytoplasm-to-nuclear ratios) such as murine lymph node cells and human blood plasmacytoid dendritic cells.

HCS techniques are being extended beyond cell-based assays[82]. In particular, automated analysis techniques are being applied to harness new biosensor technologies, to examine whole tissues and, in some cases, even consider whole organisms or model systems[2,90,91]. Other studies are using image-based screening approaches to search for novel mechanisms of action and/or to profile large compound sets for phenotypic changes and off-target effects[92,93].

An example of how automated image processing strategies can be combined with novel biosensors was recently demonstrated by Westwick and colleagues[94,95]. This group used reconstitution of split fluorescent proteins in a novel complementation assay to assess the activity of the tumour necrosis factor-α (TNFα)/NFκB

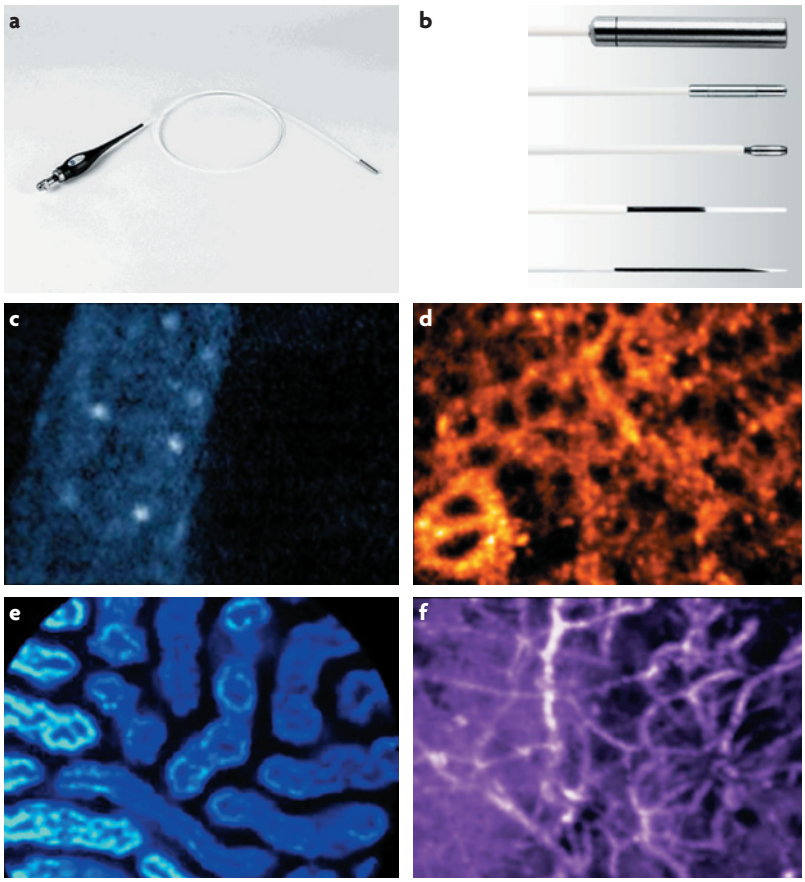


Figure 6 | **Fibre-optic fluorescence imaging technology and applications.** **a** | A typical flexible imaging microprobe (that is, ProFlex from Mauna Kea Technologies) used in fibre-optic microscopy. Shown with the high precision connector, the fibre bundle and the distal tip. **b** | A range of ProFlex distal tips for various uses and applications that range from 1.8 mm to 300 µm in diameter. **c** | Microcirculation images: rolling and adherent leukocytes imaged in the venule of a mouse cremaster muscle stained with Rhodamine 6G. **d** | Oncology images: endoscopic visualization and early detection of preneoplastic lesions of mice after instillation of acriflavine (that is, for nuclear staining). **e** | Renal physiology: mouse kidney imaged intravitally after intravenous injection of a nucleotide tagged with AlexaFluor680. **f** | Tumoral angiogenesis images: capillaries of a mouse subcutaneous prostate tumour stained with fluorescein isothiocyanate–albumin. All images courtesy of Mauna Kea Technologies (Paris, France).

pathway. By using automated microscopy and image analysis techniques, the authors were able to screen large numbers of potential modulators of this pathway. In fact, they applied this approach to a proof-of-concept HTS campaign that yielded potent inhibitors at many different points in this pathway. More recently, the authors[95] have extended this approach to include a large panel of protein-fragment complementation assays (PCAs), which they used to characterize a cross-section of marketed drugs (107 in total) from many therapeutic areas. This panel included 49 separate PCAs addressing a range of biochemical and cellular pathways. Each of these assays was conducted at several time points to yield 127 separate measurements. Analysis of this massive data set revealed both known and hidden phenotypes. These studies are a clear indication of the value that new probe technologies and sophisticated image-processing methods can bring to compound screening and profiling.

Outlook

When considering how the recent innovations described in this article might affect the range of drug discovery activities, several common themes become apparent. One theme is that the renaissance in IVM or *in vivo* microscopy is helping to better characterize disease states and assess the efficacy of therapeutic interventions. This renaissance is helping to emphasize both pharmacological and clinical relevance in animal and tissue models. In particular, imaging the treatment response is a useful way of quantifying the efficacy, time course and the specificity of therapeutic candidates. In parallel, this capability can also provide a simultaneous readout of off-target effects and liabilities.

Another theme is the significant increase in information content being derived from imaging studies in general. For instance, many of the technologies highlighted here enabled better image quality, improved image resolution and/or greater imaging depth. For example, super-resolution microscopy has radically changed expectations regarding the capability of light microscopy from a technique that is capable of subcellular resolution to one that is able to generate molecular-level detail. Likewise, *in vivo* microscopies are achieving better resolution at greater depths or from within less accessible tissues.

A final theme is how the miniaturization of fluorescence microscopy by fibre-optic technologies is helping to implement less invasive optical readouts and thereby enabling improved chronic or long-term animal models. In general, these technologies are facilitating a move away from acute experiments with fixed end points to models that allow repeated measurements, chronic observations and/or kinetic determinations, all of which are important in characterizing the underlying systems biology and long-term treatment effects.

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Acknowledgements
The author would like to thank B. Brission and A. Goodacre for their assistance in providing material for this Review. The editorial assistance of P. Prack and V. Shen is also greatly appreciated.

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