



In vivo imaging of the diseased nervous system

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Abstract | *In vivo* microscopy is an exciting tool for neurological research because it can reveal how single cells respond to damage of the nervous system. This helps us to understand how diseases unfold and how therapies work. Here, we review the optical imaging techniques used to visualize the different parts of the nervous system, and how they have provided fresh insights into the aetiology and therapeutics of neurological diseases. We focus our discussion on five areas of neuropathology (trauma, degeneration, ischaemia, inflammation and seizures) in which *in vivo* microscopy has had the greatest impact. We discuss the challenging issues in the field, and argue that the convergence of new optical and non-optical methods will be necessary to overcome these challenges.

Each neurological disease has a unique clinical course. For example, in stroke, a sudden onset is often followed by a slow recovery, whereas insidious progression is observed in neurodegenerative disease, and the waxing and waning of deficits is associated with multiple sclerosis. Underlying these clinical patterns are disturbances in neuronal and glial functions that are often highly localized, asynchronous and involve complex cell–cell interactions. Given how dynamic such cellular disturbances can be, they would be best studied at the single-cell level in real time¹. At present, there is no tool with sufficient spatial or temporal resolution to perform such studies in the human brain, but sophisticated evaluation has been achieved in animal models. Rodent models of neurological diseases have been extremely valuable in elucidating disease processes and in evaluating experimental therapies. The co-evolution of optical *in vivo* imaging (or *in vivo* microscopy) and mouse genetics now allows the investigation of neurological diseases in living animals at the level of single cells.

In this review, we provide a rationale for visualizing living cells in the diseased brain, and we outline the techniques that have made such studies possible. We give an overview of what has been learned so far by *in vivo* imaging, and discuss the challenge of integrating real-time observations of single cells with the molecular mechanisms and the clinical manifestations of neurological diseases.

Why image neuropathology *in vivo*?

In vivo imaging offers a unique insight into how neurons in the intact nervous system change in relation to

behavioural adaptations or experience². It also allows us to track how cells are altered by pathology. This provides several advantages over conventional post-mortem studies of neurological disease models (FIG. 1). First, only direct observation reveals the dynamism of cells in the brain. For example, most mature neurons are relatively stable, at least over a period of a day or two^{2,3}. By contrast, the so-called ‘resting’ microglial cells — the resident immune cells of the brain — are highly dynamic, continuously probing their vicinity and responding quickly to nearby damage^{4,5} (FIG. 1a). Knowledge of such behaviours is important for understanding disease processes: degenerative diseases might compromise the stability of synapses, whereas autoimmune processes might dysregulate microglial behaviour.

Second, observing the same cells over time is often more sensitive and fail-safe than trying to deduce how cells change from ‘snapshots’ of fixed tissue. Studies of nervous system injury, for example, require certainty that all axons in a tract have been severed before a putative growth-promoting agent is administered. Otherwise, axons that were never cut can be mistaken for axons that have regrown⁶ (FIG. 1b). Visually confirming the transection of a given axon in the living animal removes such doubts.

Third, observation of the sequence of pathological events can establish their causative relationship, or at least refute some hypotheses about how diseases evolve. For example, how axonal damage occurs in neuroinflammatory lesions could be studied in this way (FIG. 1c). During an immune attack, axons lose their myelin sheath and often degenerate. These three events — inflammation,

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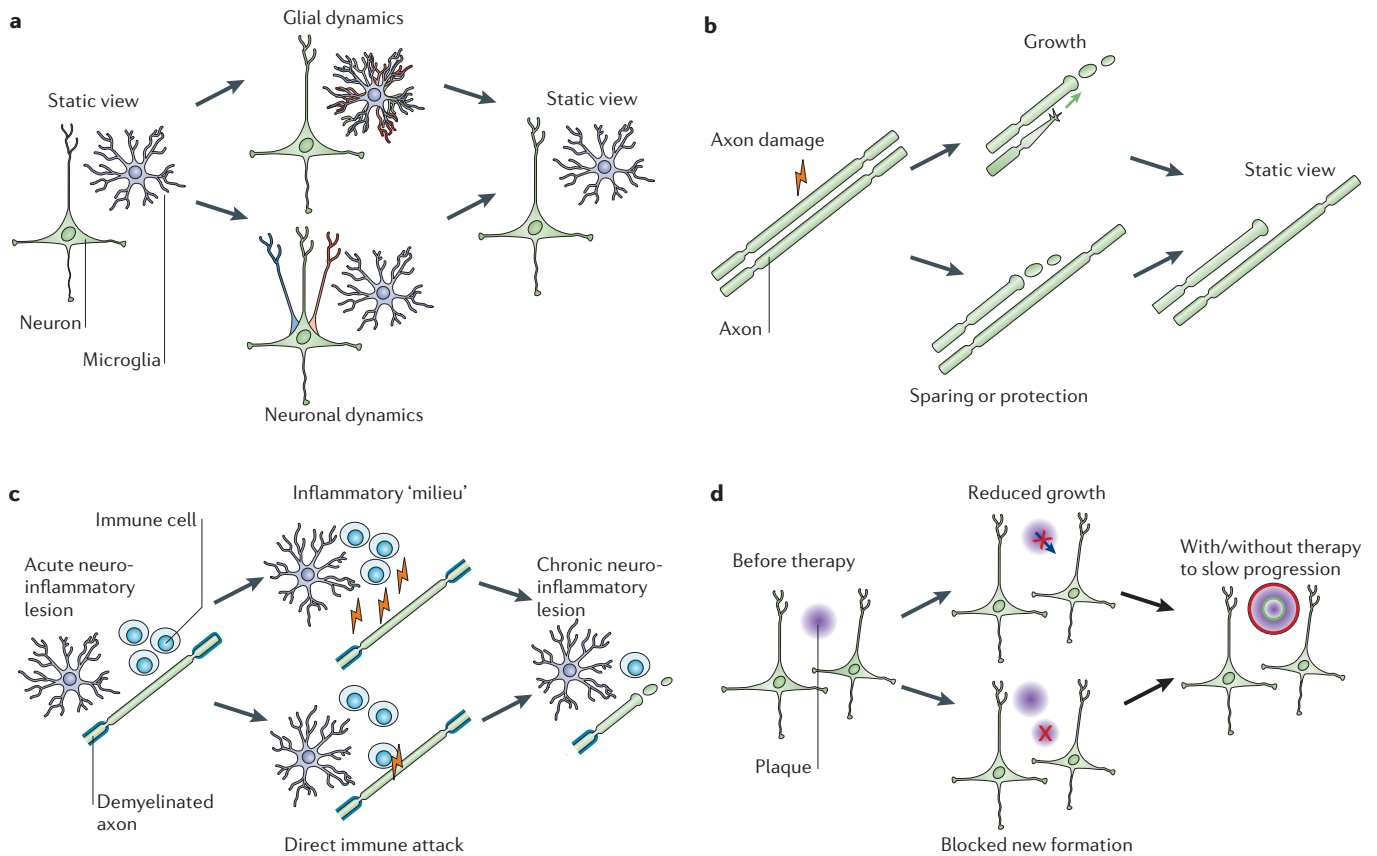


Figure 1 | Advantages of *in vivo* imaging in neurological disease models. **a** | *In vivo* imaging reveals the dynamics of cells in the nervous system. Static views of fixed tissue (left and right) cannot reveal that in the healthy brain dendritic branches are relatively stable, whereas microglial cells are highly dynamic. **b** | Intravital microscopy allows the identification of damage and assessment of repair in disease models. Trauma (flash) leads to axon transection and compensatory growth. When the nerve tissue is fixed and examined after trauma, a mixture of damaged and intact axons is often seen (right). *In vivo* imaging can clearly differentiate whether single fibres have regenerated (middle top, growth cone with arrow) or rather escaped damage (middle bottom). **c** | *In vivo* observations can give insight into disease pathogenesis. In neuroinflammatory lesions, demyelinated axons often degenerate, but the cause of this degeneration is unknown. Inflammatory and glial cells could create a neurotoxic environment (middle top); alternatively, immune cells could directly attack the denuded axons (middle bottom). **d** | Direct visualization of pathogenic structures before and after treatment can provide information about therapeutic mechanisms. Alzheimer's plaques can be visualized using vital dyes (violet). Whereas static views (left and right) can show that a therapy slows progression (on the right: plaque size without therapy (outlined in red) is bigger than with therapy (outlined in green)), imaging plaques over time can reveal how such an improvement is achieved, that is, whether growth of pre-existing plaques is slowed (middle top), or new seeding of plaques is prevented (middle bottom).

demyelination and axon damage — are highly correlated, but it is not clear which particular event is the cause of the others. *In vitro*, several possible scenarios have been demonstrated: immune cells can damage both oligodendrocytes and neurons^{7,8}; damage to oligodendrocytes can cause axon pathology that can, in turn, cause damage to myelin^{9,10}; and, finally, degenerating axons themselves can induce secondary immune activation¹¹. Direct *in vivo* observations could reveal which process occurs first and, therefore, which one is likely to cause other events.

Fourth, by using *in vivo* imaging the same area of the diseased nervous system can be compared before and after therapeutic intervention¹². This allows the formulation of new ideas about how therapies act and predictions about synergistic combinations. For example, in Alzheimer's disease, the reduction in plaque

load in response to antibodies that bind amyloid could be due to several reasons (FIG. 1d): pre-existing lesions could just stop growing (or even shrink); alternatively, antibodies could prevent the 'seeding' of new lesions. *In vivo* microscopy allows the differentiation of these scenarios.

Development of *in vivo* neuroimaging

Two techniques have made *in vivo* microscopy in the nervous system possible (see BOX 1 for definitions of imaging techniques discussed in this article). The first is modern optical imaging, in particular multiphoton microscopy; the second is sophisticated transgenic technology that allows the labelling of single cells in the nervous system and permits their function to be monitored.

Box 1 | A summary of frequently used imaging techniques

Optical imaging. Techniques that use light to visualize the distribution of cells and molecules. Includes microscopy and biophotonic techniques.

Wide-field microscopy. Method in which the entire field of view is illuminated simultaneously to produce an image.

Scanning microscopy. Technique in which a focused beam of light (usually a laser) is moved (scanned) across the field of view to detect the signal of each spot sequentially (includes confocal microscopy and multiphoton microscopy).

Confocal laser scanning microscope (or confocal microscopy). Scanning microscopy technique that allows optical sectioning by placing a pinhole in front of the detector to exclude out-of-focus light and generate a sharp image of structures in the focal plane.

Multiphoton microscopy (includes two-photon microscopy). Scanning microscopy technique in which a pulsed near-infrared laser is used to achieve optical sectioning by limiting (multiphoton) excitation to the perifocal plane; widely used for *in vivo* imaging.

Second-harmonic generation microscopy. The use of near-infrared lasers to generate frequency-doubled signals from the scattering of light as it interacts with specific macromolecules; allows visualization of structures such as the cytoskeleton or collagen.

Intrinsic optical imaging. Imaging technique that detects neuronal activity by virtue of changes in the optical properties of brain tissue as neurons start to fire, such as changes in the degree of blood oxygenation or the osmotic status of cells.

Optical coherence tomography. Optical technique that uses interferometry of reflected or back-scattered light from biological tissues; for example, to visualize laminae in the retina.

Biophotonic whole-body imaging. A group of techniques that detect light emitted from intact animals by placing them in imaging chambers after introducing fluorescent or luminescent labels by intravenous injection or implantation.

Calcium imaging. Technique that uses calcium indicator dyes (that is, calcium-dependent fluorophores) and microscopy to measure changes in intracellular calcium concentration.

MRI. The standard clinical imaging technique used to visualize the brain in humans; detects the relaxation properties of hydrogen atoms in strong magnetic fields to generate three-dimensional views of the inside of the body.

Functional MRI (fMRI). Modification of MRI that detects changes in local blood flow and haemoglobin oxygenation in response to altered neuronal activity, and allows mapping of the functional centres of the human brain.

Diffusion tensor imaging. Variant of MRI that measures the restriction of local water diffusion; as diffusion is facilitated parallel to the axons in a tract, this technique allows 'tracing' of major axon tracts and detection of damage to such tracts in humans and animals.

'Micro-MRI'. Application of MRI to experimental animals; because of the smaller size of the animals, stronger magnetic fields can be used and higher (almost microscopic) resolution can be achieved.

Contrast-enhanced MRI. MRI using compounds with a high MRI contrast such as gadolinium or super-paramagnetic iron.

Manganese-enhanced MRI. MRI using manganese as a contrast agent. As manganese can be introduced to neurons and can replace calcium, it can be used to track axonal connections or map neuronal activity in animals.

Positron emission tomography (PET). Clinical imaging technique that measures the distribution of chemical compounds in which single atoms have been substituted with positron-emitting isotopes (like radio-substituted neurotransmitter ligands); used, for example, to visualize the distribution of neurotransmitter receptors or to map neuronal activity.

Nanoscopy. Term coined to describe a set of revolutionary optical techniques that potentially have nanometre resolution and can therefore overcome the diffraction limit of light microscopy.

Optical sectioning

The exclusion of signal from out-of-focus planes. In confocal microscopy this is achieved by placing a pinhole in front of the detector. In multiphoton microscopy, optical sectioning is an intrinsic property, as excitation is limited to the perifocal volume, where photon density peaks.

Diffraction limit

Fundamental limitation of the resolution achievable with conventional light microscopy. As a result of light diffraction, the focal 'spot' cannot be made smaller than 100–200 nm in the plane of focus and 0.5 μm in depth (depending on the objective, immersion media and wavelength).

Charge-coupled device cameras

(CCD cameras). Digital cameras used for wide-field microscopy that incorporate silicon chips as detectors; light that hits the photo-sensitive areas of the chip generates charge, which is measured to generate a digital image.

Photomultiplier tubes

Point detectors used in many scanning microscopes (for example, confocal laser scanning or multiphoton microscopes), which have a photocathode as a front surface that releases electrons on illumination, and a secondary electrode array that multiplies the signal before readout.

Optical microscopy had become an established technique by the mid-twentieth century. However, since then, the microscope has been converted from a tool used to visualize histological specimens to an imaging device that allows the observation of living cells. Pioneering time-lapse microscopy studies demonstrated the power of real-time observations; for example, by directly showing axonal transport or growth cone dynamics^{13,14}. Numerous technical developments have since converged to make such studies routine¹⁵, with the most important being the digitization of image acquisition by highly sensitive detectors, such as charge-coupled device (CCD) cameras or photomultiplier tubes, and the increasing power of computerized image processing. In parallel, microscopes

have been adapted for *in vivo* imaging: for example, long working distance objectives with increasing numerical aperture not only improve resolution, but also collect more light, which reduces phototoxicity. Improved multichromatic filter sets increase sensitivity and simplify fast imaging by avoiding filter cube switches. The automation of microscope controls allows long-term observations with little need for user intervention.

The decisive breakthrough for imaging in live animals, however, came with multiphoton microscopy¹⁶. The advantages of this technique have been the subject of a number of excellent reviews and are not discussed here in depth^{17,18}. In brief, multiphoton excitation depends on the near simultaneous absorption of two

Box 2 | *In vivo* imaging: pitfalls and potential solutions

In vivo microscopy is not without pitfalls¹. There are two fundamentally different modes of *in vivo* imaging, and each has its own problems. On the one hand, there are 'real-time' observations, in which a process is visualized as it happens. On the other hand, there is 'intermittent' imaging, in which multiple observations are collected, but the animal being studied is awake between imaging sessions. Although real-time imaging is often more direct, intermittent imaging alleviates many concerns about toxicity, as changes take place while the animal is not being imaged. However, finding and orienting cells for each session poses a new set of challenges. Excellent reference texts have been published that provide protocols for avoiding and dealing with artefacts (for an example, see REF. 57), so we highlight only a few problems here.

Artefacts induced by anaesthesia, labelling and surgery.

In vivo microscopy is mostly carried out in anaesthetized animals and requires at least minor surgical procedures. Such invasive procedures, or even transgenic labelling alone, can change the normal behaviour of cells, and the diseased brain might be particularly susceptible to such stressors. Anaesthesia can influence cellular behaviour, and limits the monitoring of neuronal activity.

Potential solutions.

- Minimize surgery and carefully monitor anaesthesia.
- Check *in vivo* observations against histological samples.
- Compare different anaesthetics and always include healthy animals as controls.
- All sudden changes in morphology that are not compatible with the known course of a disease should be treated with suspicion.
- In the future, improved forms of animal restraint¹⁶¹, novel miniature microscopes²³ and fibre-optics⁶² might permit experiments to be carried out in awake, behaving animals.

Phototoxicity.

Too much light is toxic to cells. Phototoxic damage is easily induced in the diseased brain, and can be misinterpreted as disease-related change.

Potential solutions.

- Use as little excitation light as possible and the most sensitive detector possible.
- Filter out irrelevant wavelengths from the excitation light.
- Consider multiphoton microscopy.
- Carefully control temperature, oxygenation and pH to reduce cellular stress.

Movement and orientation artefacts.

The living brain is in constant motion due to motor activity, respiration and heartbeat. Repeatedly identifying and orienting the same cells under the microscope can be challenging during 'intermittent' imaging protocols.

Potential solutions.

- Use well-engineered equipment (such as stereotactic head holders) to immobilize the animals and adjust your surgical access to reduce pressure gradients (for example, by closing a craniotomy with agarose and a cover slip).
- Consider triggering image acquisition or illumination from the electrocardiogram and interrupting ventilation for short periods of time (<1 min) while collecting images.
- Fast imaging allows the taking of images that are little affected by pulse-related blurring, and subsequent x–y alignment and averaging can improve the signal-to-noise ratio^{20,74}.

Cost.

In vivo imaging, especially multiphoton microscopy, can be expensive.

Potential solutions.

- Many parts of the nervous system can be imaged with a relatively affordable wide-field microscope.
- A number of detailed descriptions have been published about how to build your own multiphoton microscope^{162,163}. These instruments can be tailored to specific needs, and in the past have often outperformed what was offered commercially¹⁷.

(or more) photons of near-infrared wavelength. Excitation is thereby limited to the perifocal volume, where the density of exciting photons is highest, which affords multiphoton microscopy with intrinsic optical sectioning. This reduces phototoxicity and bleaching outside the perifocal volume (which plague other forms of microscopy, including confocal laser scanning microscopy). At the same time, emitted photons can be detected regardless of whether they are scattered along the return light path, because they all originate in the perifocal volume. By careful instrument design, sensitivity and penetration depth can be improved (the penetration depth is normally below 500 μm , but can reach up to 1,000 μm with special instrumentation^{18,19}). Imaging depth can be further increased by surgery²⁰ and by the miniaturization of objective lenses that are inserted into the brain²¹. By creating devices that can be carried by awake animals, some of the confounding factors that still plague *in vivo* microscopy in anaesthetized animals might be overcome^{22,23} (BOX 2).

In addition to the development of multiphoton microscopy, long-term *in vivo* observations in the brain have depended on the advent of new molecular biological tools to label subsets of neurons²⁴. Of particular importance was the generation of transgenic mice that express high levels of fluorescent proteins in their neurons²⁵ (FIG. 2; TABLE 1). In these mice, neurons are labelled with different spectral variants of fluorescent proteins, which are expressed under the control of a modified **Thy1**-promoter element. These 'Thy1-XFP mice' had a huge impact on the imaging of the peripheral and central nervous systems, in particular because positional effects in some transgenic lines restrict transgene expression to a small percentage of neurons, which results in an intravital Golgi-like staining pattern (these transgenic lines are referred to as 'subset' Thy1-XFP lines). By choosing lines carefully, preferential labelling of dendrites, axons or even neuronal subtypes (such as interneurons) can be obtained in specific brain regions^{26,27}; axons can be reconstructed in their entirety²⁸; and synaptic partners can be labelled in different colours²⁵. Moreover, by using 'combinatorial' genetics in similar mouse lines with conditional expression of the Thy1-transgene, fluorescent neurons can be restricted to specific regions of the nervous system (for example, the cortex), and their projection tracts (for example, the corticospinal tract) can therefore be selectively marked²⁹.

There are other transgenic mouse lines in which glial cells are labelled with fluorescent probes. For Schwann cells of the PNS, transgenic lines using the **S100**-promoter have been described, including some in which only a fraction of the cells are labelled^{30,31}. Transgenic labels also exist for astrocytes and oligodendrocytes in the brain^{32–35}. However, labelling these 'macroglial' cells is not easy, in part because their gene expression pattern is often dependent on their state of activation. In addition, their density is often too high for single cells to be resolved clearly by light microscopy, and their morphology is complex, with some cell compartments dominated by membranous structures (for example, myelin) that make cytoplasmic labels difficult to detect.

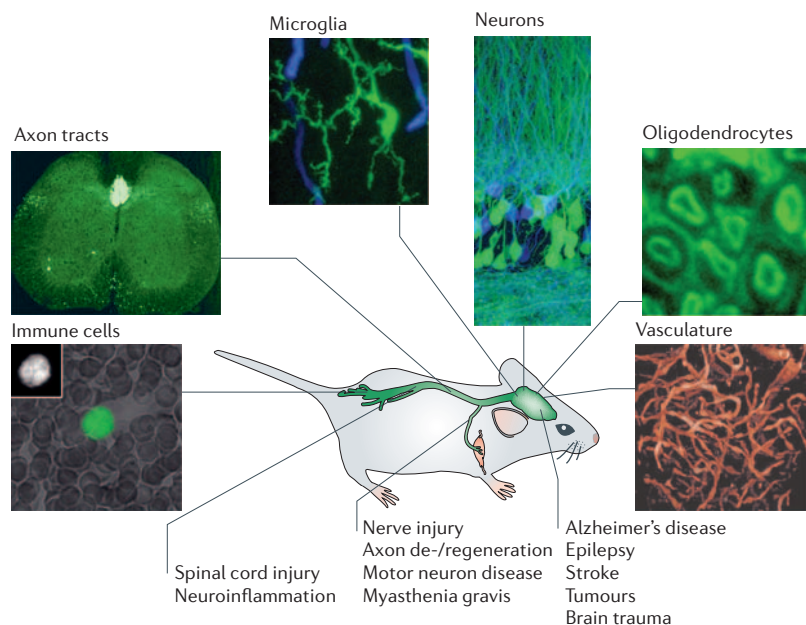


Figure 2 | The current scope of *in vivo* imaging. Currently, numerous parts of the nervous system can be visualized *in vivo*, including the cortex, cerebellum, olfactory bulb, retina, spinal cord, peripheral nerves and autonomic ganglia. There are transgenic labels for tracing many disease-relevant structures in the nervous system, including neurons, axon tracts, most glial populations, oligodendrocytes, brain vasculature and invading immune cells (TABLE 1). This has allowed a growing number of disease models, such as those listed in the figure, to be studied by *in vivo* imaging in mice. Picture of brain vasculature reproduced, with permission, from REF. 164 © (2003) Elsevier Science.

reveal subcellular alterations, such as changes in the morphology and function of organelles (for example, synaptic vesicles, endoplasmic reticulum, cytoskeletal components and mitochondria) or dysregulation of basic cell biological processes (for example, synaptic vesicle recycling or axonal transport). Originally, vital dyes were used for these purposes (for examples, see REF. 46 for an early neuronally enriched mitochondrial dye, and REFS 47,48 for studies on vesicle recycling), but many of these dyes are difficult to use in live animals. Now, cellular compartments can be labelled *in vivo* with fluorescent proteins by attaching them to membrane proteins or by using specific targeting sequences⁴⁹. Mice currently are available that allow the visualization of synaptic vesicles (and, therefore, the formation and loss of synapses)^{50,51}, synaptic vesicle recycling⁵² and neuronal mitochondria (T.M., M.K., F. M. Bareyre, R.W. Burgess and J. W. Lichtman, manuscript in preparation).

Optical methods can also be used to monitor neuronal activity in complex networks⁵³. As many neurological diseases first manifest as disturbances of neuronal activity, such tools have an obvious appeal to experimental neurologists. The two most widely used techniques are intrinsic optical imaging and calcium measurements. Intrinsic optical imaging depends on changes of light absorption in response to increased neuronal activity, and has been applied to the cortex⁵⁴ and the spinal cord⁵⁵, and even to humans undergoing surgery⁵⁶. However, this technique is limited by insufficient resolution to visualize single cells, as well as the caveat that intrinsic contrast can be influenced by pathological alterations that do not affect activity *per se*. An alternative way to measure activity in neurons is calcium imaging. Local calcium concentrations can be measured with calcium indicator dyes that chelate the ion and, in response, change their fluorescence intensity and/or spectrum⁵⁷. Loading single cells via recording pipettes⁵⁸ or slices by bath application has long been possible⁵⁹. A novel technique to stain large populations of cortical neurons with calcium dyes *in vivo*, called multicell bolus loading, was recently introduced⁶⁰. This technique has been used in mammals and other vertebrates to visualize calcium transients with cellular resolution. Recordings obtained from cells labelled in this way can be correlated with the large-scale maps obtained by intrinsic optical imaging⁶¹, and multicell bolus loading even allows calcium waves to be detected in awake, behaving animals⁶². The transgenic expression of fusion proteins that indicate calcium (or other ion) concentrations by fluorescence resonance energy transfer (FRET) will be another powerful tool with which to assign changes in activity to specific neural subpopulations^{63,64}. One limitation of intrinsic optical imaging and calcium measurements is poor temporal resolution. These methods rely on signals that are only indirectly linked to the neuronal membrane potential and action potential firing. Novel approaches based on additional intrinsic signals (such as second-harmonic generation microscopy^{65,66}; BOX 1), advanced voltage sensitive dyes⁶⁷ and genetic indicators of membrane potential⁶⁸ could allow more direct optical monitoring of neuronal activity in the future.

Microglial cells have been labelled alongside macrophages by knock-in strategies^{4,5,36}. Given that microglial cells are naturally sparse in the brain, the absence of subset lines has so far not been a limitation. For imaging studies of neuroinflammatory lesions, in which activated microglial cells intermix with macrophages, the differential labelling of blood-borne immune cells is desirable. Immune cells can be easily labelled *ex vivo*; in addition, transgenic lines with well-defined sets of labelled immune cells are available³⁷. Viral labelling strategies have also been described for most cell types and can be combined with transgenic mouse lines^{38,39}. Intrauterine electroporation allows the labelling of large populations of developing neurons⁴⁰, whereas single-cell electroporation is a versatile tool for producing transient overexpression in individual cells⁴¹. Numerous vital dyes specific for cells or extracellular components of the nervous system can be introduced as additional markers⁴², and these dyes allowed some of the early *in vivo* studies of the CNS to be carried out (for an example, see REF. 43). Finally, many structures in the brain generate intrinsic contrast, which allows them to be visualized — for example, by having a different refractive index from their surrounding tissues (such as myelin⁴⁴), or by containing autofluorescent molecules (such as serotonin-containing neurons⁴⁵).

The promise of imaging disease processes *in vivo* goes beyond documenting altered morphology. Sophisticated optical tools are becoming available to monitor neuronal cell biology and electrical activity. Such tools can

Perifocal volume

The volume surrounding the focus of an objective lens; real lenses do not focus light into a geometrical 'point' but into a small spheroid volume, the size of which depends on the wavelength of the light and the characteristics of the objective.

Phototoxicity

Toxic effects of light on cells that limits most *in vivo* microscopy experiments; caused at least in part by bleaching and associated photochemical generation of free radicals.

XFP

Green fluorescent proteins or coral fluorescent proteins and their spectral variants, such as yellow and cyan fluorescent proteins.

Table 1 | Selection of *in vivo* labels for imaging studies of animal models of neurological disease

Labelled structure	Compartment/subset	Transgenic approach	Fluorophore	References
Neurons				
Cytoplasm		Thy1-promoter	XFP, coFP	25,34,50
	Specific populations	Subtype-specific promoters	XFP	166–168
Subcellular	Membranes	Thy1-promoter, palmitoylation	XFP	50
	Synaptic vesicles	Thy1-promoter, synaptophysin-fusion	XFP	50,51
	Mitochondria	Thy1-promoter, coxVIII	CFP, YFP	Manuscript in preparation*
	Vesicular release	Thy1-promoter	SynaptopHluorin	52,169
	Calcium	CaMKII-promoter, tet-system	Camgaroo, pericam	63
	Chloride	Thy1-promoter	Clomeleon	170
Axon tracts	Corticospinal	Thy1-promoter, conditional	f1STOPfl-YFP	29
	Thalamocortical	Thy1-promoter	XFP	26
Glial cells				
Schwann cells		S100-promoter	YFP, GFP	31
Oligodendrocytes		PLP-promoter	GFP, coFP	34,171
Astrocytes		GFAP-promoter	GFP, coFP	32–34
		S100-promoter	YFP, GFP	31
Microglia		CX ₃ CR1-locus S100-promoter	YFP, GFP	4,5,31,36
Vascular component				
Serum		Vital dye	Fluor-dextran	93
Erythrocytes		Vital dye, negative stain	Fluor-dextran	93
Immune cells		Various immune cell promoters	XFP	37
Endothelium		TIE2-promoter	GFP	172

*T.M., M.K., F.M. Bareyre, R.W. Burgess & J.W. Lichtmen. CaMKII, calcium/calmodulin-dependent protein kinase II; CFP, cyan fluorescent protein; coFP, coral-derived fluorescent protein; CX₃CR1, chemokine (C-X₃-C motif) receptor 1; coxVIII: mitochondrial targeting sequence from human cytochrome oxidase subunit VIII; f1STOPfl-YFP, yellow fluorescent protein expression suppressed by floxed stop-sequence; fluor-, fluorophore conjugated; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; PLP, proteolipid protein; tet-system, tetracycline-derivative controlled conditional expression system; TIE2, tyrosine kinase with immunoglobulin and epidermal growth factor homology domains 2; XFP, green fluorescent or coral fluorescent proteins and their spectral variants YFP, yellow fluorescent protein.

Knock-in strategy

Technique by which an endogenous gene is replaced with a novel sequence; for example, to inactivate the gene and/or to characterize its expression pattern. Knock-in strategies differ from 'transgenic' approaches in which genetic material is randomly inserted into the genome.

Vital dye

Used in microscopy to denote any compound that can be applied to living organisms and cells to stain specific structures.

Intrinsic contrast

All features of normal tissue that allow the detection of structures without additional labelling. Techniques that use intrinsic contrast are phase contrast microscopy and skeletal X-rays.

Multicell bolus loading

Recent innovation in the field of calcium imaging in which large groups of neurons or glial cells are loaded with calcium indicator dyes through the injection of concentrated solutions of cell-permeant dye esters into brain tissue.

Fluorescence resonance energy transfer

(FRET). Non-radiative transfer of photons between molecules with overlapping excitation–emission spectra; depends strongly on the proximity of molecules and can therefore indicate molecular interactions.

Imaging neurological disease in animals

The number of *in vivo* imaging applications to neurological disease models is growing rapidly (FIG. 2). There are various parameters that determine how easy it is to image a given disease. First, superficial parts of the nervous system are the most accessible, as high-resolution optical studies are currently limited to within 500 μm of the pia mater. As a consequence, in the brain, high-resolution optical imaging techniques are more suitable for the examination of grey matter diseases than diseases that affect white matter, and favour studying pathology in the cortex (for example, Alzheimer's disease) over changes in deeper brain regions (such as those affected in Parkinson's disease). The geometry is reversed in the spinal cord, where the axon tracts lie superficially and white matter pathology (for example, axon trauma) is easier to study than grey matter pathology (for example, motor neuron degeneration). Second, it is crucial that robust labels exist for the cells that mediate a given disease. At present, this makes studies of neurons easier than studies of glial cells (thanks to the Thy1-XFP mice), and

poses a special challenge for diseases in which many cell types interact. Third, disease models with a well-defined trigger and time course are best suited to *in vivo* studies. In the following section, we highlight five areas of neurological research — trauma, degeneration, ischaemia, inflammation and seizures — that are favoured for *in vivo* imaging by at least some of these criteria.

Trauma. Axonal transection (axotomy) is a common form of neuronal damage. In the PNS, axotomy is followed by regeneration, reinnervation and recovery. This is not the case in the CNS, where regeneration fails with the dire consequences that are known from brain and spinal injuries. *In vivo* imaging is an attractive method with which to study axotomy — indeed, it was one of the first pathological alterations of the nervous system to be imaged *in vivo* using intrinsic contrast^{69,70}. Axons can be transected easily in optically accessible parts of the nervous system, where single fluorescently labelled axons can be followed in transgenic Thy1-XFP mice. Traumatic axotomy also has an obvious cause and

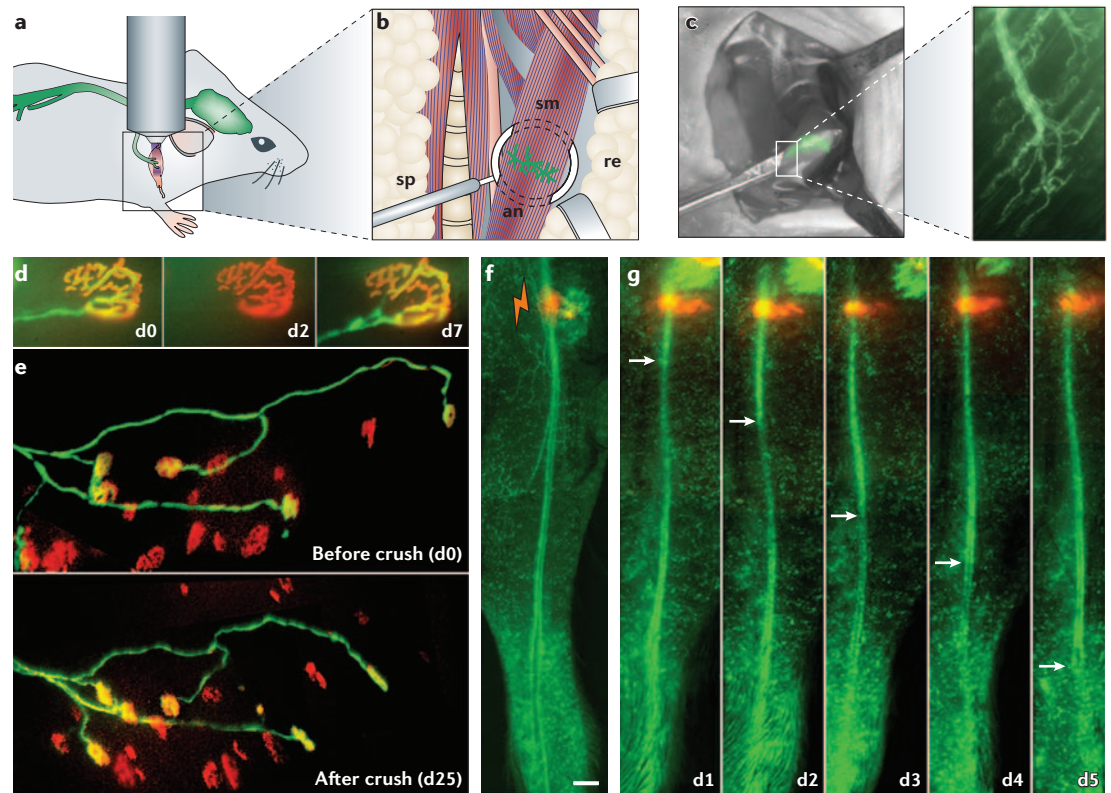


Figure 3 | *In vivo* imaging in peripheral nerves. *In vivo* imaging is a powerful tool with which to study how axons in peripheral nerves behave during degeneration and regeneration. **a** | Superficial muscles and nerves are easily accessible by small surgical procedures in anaesthetized mice, and water immersion objectives can be used to visualize transgenically labelled cells (in the schematic this is shown for a forelimb muscle). **b** | Drawing of the classical sternomastoid muscle (sm) preparation in the mouse. The muscle is surgically exposed and carefully lifted on a metal spatula (sp), while retractors (re) replace overlying tissue. In this way the innervation of the muscle (by the accessory nerve (an), green) can be studied. **c** | View of the sternomastoid preparation through a dissection microscope. The fluorescence picture of the transgenically labelled nerve is superimposed in green pseudo-colour at the centre of the greyscale image. The enlargement shows a low-resolution view of a small motor axon fascicle that innervates the sternomastoid muscle in a Thy1-XFP mouse. **d** | A single neuromuscular junction (axon, green; postsynaptic acetylcholine receptors, red) before (d0), two days (d2), and a week (d7) after crush, when reinnervation is complete. **e** | Part of a single labelled motor unit in a superficial muscle (the platysma) of a 'subset' Thy1-XFP mouse (axon, green; postsynaptic receptors, red). Note the striking similarity of the innervation pattern before (d0) and after (d25) reinnervation following crush. **f** | *In vivo* imaging can also be carried out transcutaneously in Thy1-XFP mice. The depilated leg of a Thy1-XFP mouse shows a superficial sensory nerve, the saphenous, which is crushed (flash; crush site is marked by red fluorescent beads). **g** | Nerve regeneration after prolonged denervation can be followed through the skin. The growth front of the nerve's axons is marked with an arrow. XFP, Green fluorescent proteins (GFP) or coral fluorescent proteins and their spectral variants, such as yellow and cyan fluorescent proteins. Panel **b** reproduced, with permission, from REF. 165 © (1987) Society for Neuroscience. Panels **d** and **e** reproduced, with permission, from REF. 72 © (2002) Macmillan Publishers Ltd. Panels **f** and **g** reproduced, with permission, from REF. 73 © (2003) Society for Neuroscience.

a largely cell-autonomous initial neuronal response — that is, Wallerian degeneration⁷¹.

With *in vivo* imaging, a number of unresolved issues surrounding axonal degeneration and regeneration have been addressed. One study used subset Thy1-XFP mice to document regeneration of single motor axons with unprecedented precision⁷² (FIG. 3a–e): axon growth was observed directly, and the innervation pattern of a single motor axon before and after regeneration was compared. When the nerve was only mildly damaged, so that the glial sheath was not destroyed (for example, by crushing rather than severing it), axons retraced the path to their original synaptic sites with remarkable fidelity. This

suggests that mechanical cues can have a central role in guiding axons. The brightness of axon labelling in Thy1-XFP mice has also allowed non-invasive monitoring of nerve regeneration through the skin of live mice, which could serve as a useful assay to measure pharmacological modulation of axonal growth⁷³ (FIG. 3f–g).

To conduct similar studies in the CNS, we have developed a technique that allows *in vivo* imaging of axons after spinal cord injury⁷⁴. Using wide-field microscopy in subset Thy1-XFP mice, individual fluorescently labelled sensory axons were visualized through a laminectomy opening before and after axotomy (FIG. 4a–d). Repeated imaging of the same

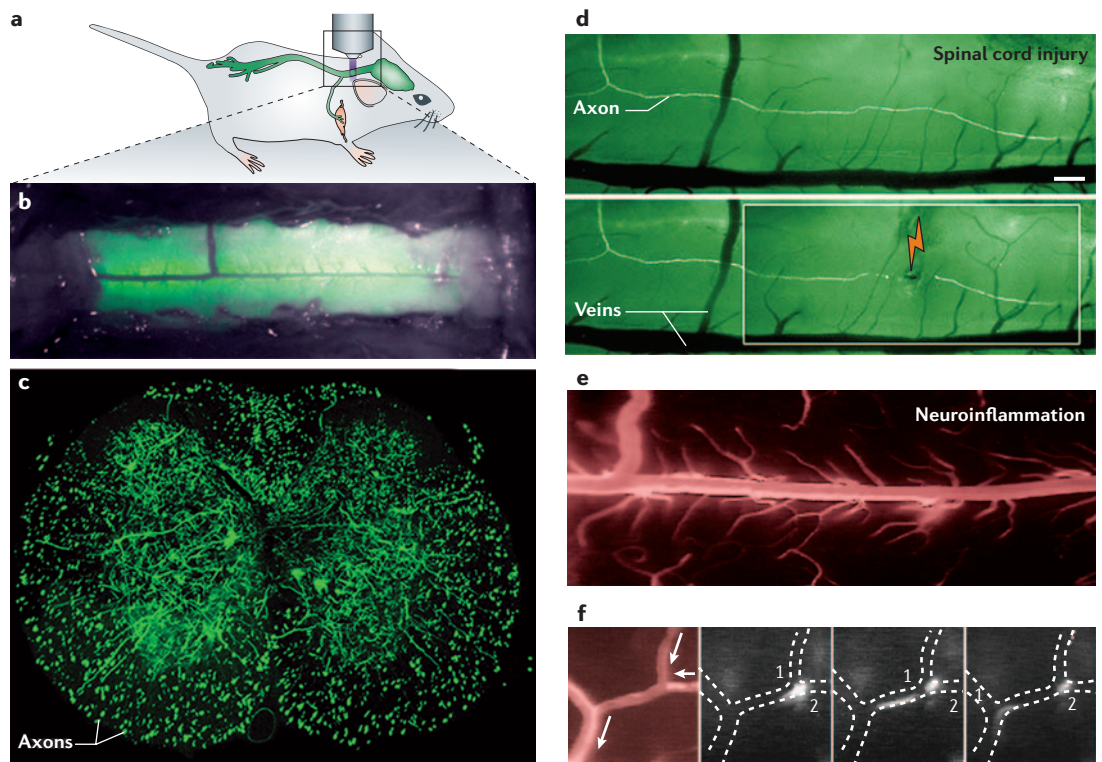


Figure 4 | *In vivo* imaging in the spinal cord. **a** | Visualization of axons and vasculature in spinal cord pathology is now possible in small rodents. **b** | A laminectomy opening allows viewing of the living cord. The fluorescence image of axons labelled with green fluorescent protein (green) is superimposed on a low-power greyscale view of the laminectomy (spinal vasculature appears dark; the animal's head is to the right). **c** | Thy1-XFP animals provide sparse axon labelling in the spinal cord, as shown on this cross-section (dorsal is up). **d** | This labelling pattern allows the visualization of single axons in dorsal views during spinal cord injury *in vivo*. The lesion (flash) can be targeted at specific axons (top, before lesion; bottom, after lesion). **e** | Neuroinflammatory diseases originate from the spinal vasculature (red), which can be highlighted by intravenous injection of fluorescent tracers. **f** | Cell tracer-labelled immune cells can be followed after re-injection as they move through the highlighted microvasculature. Here, three frames acquired at near video-rate show one immune cell passing by (1) and another attached to the endothelium (2). XFP, Green fluorescent proteins (GFP) or coral fluorescent proteins and their spectral variants, such as yellow and cyan fluorescent proteins. Panels **b**, **c** and **d** reproduced, with permission, from *Nature Medicine* REF. 74 © (2005) Macmillan Publishers Ltd. Panels **e** and **f** reproduced, with permission, from REF. 100 © (2001) The American Society for Clinical Investigation.

axons showed spontaneous but misguided growth that could be followed over several days. These observations also uncovered a form of acute post-traumatic degeneration that underlies the process of axonal 'die-back'. Related optical approaches have also been used to monitor cytotoxic mediators after blunt spinal trauma⁷⁵. Axon degeneration can be further visualized in the brain using multiphoton microscopy in Thy1-XFP mice with preferential transgene expression in thalamic neurons, which project to the cortex²⁶. Initial studies have focused on developmental axon remodeling, but, in principle, a similar approach could also be applied to the study of axonal changes following traumatic brain injury. In the immediate future, there are at least two major applications for *in vivo* imaging of axon trauma. One is the study of neuron–glia interactions underlying axonal growth inhibition using double-transgenic mice with differentially labelled axons and glial cells (FIG. 2). The second is the evaluation of therapies that aim to improve regeneration after trauma. With repeated imaging of the same

axon before and after lesioning, regeneration can be unequivocally differentiated from fibre-sparing or collateral sprouting⁶ (FIG. 1b).

Comparing axonal growth behaviour between species with differences in regeneration in the CNS might also provide hints as to what precludes regeneration in mammals. For example, imaging optically transparent zebrafish larvae has shown that the axons of some brain stem neurons, which do not normally regrow, can regenerate and functionally reinnervate their spinal targets following an increase in intracellular cyclic AMP⁷⁶. In mammals, similar pharmacological manipulations also enhance growth but lead to a less striking recovery⁷⁷. In non-mammalian organisms, it has been demonstrated that a multiphoton laser can serve as a 'micro-scalpel' to transect single axons^{78,79}. Similar miniature axon injury models can be established in rodents, and might help to dissect the pathogenesis of axon injury in mammals (see REFS 4,5 for examples of laser-induced photodamage in the mammalian brain).

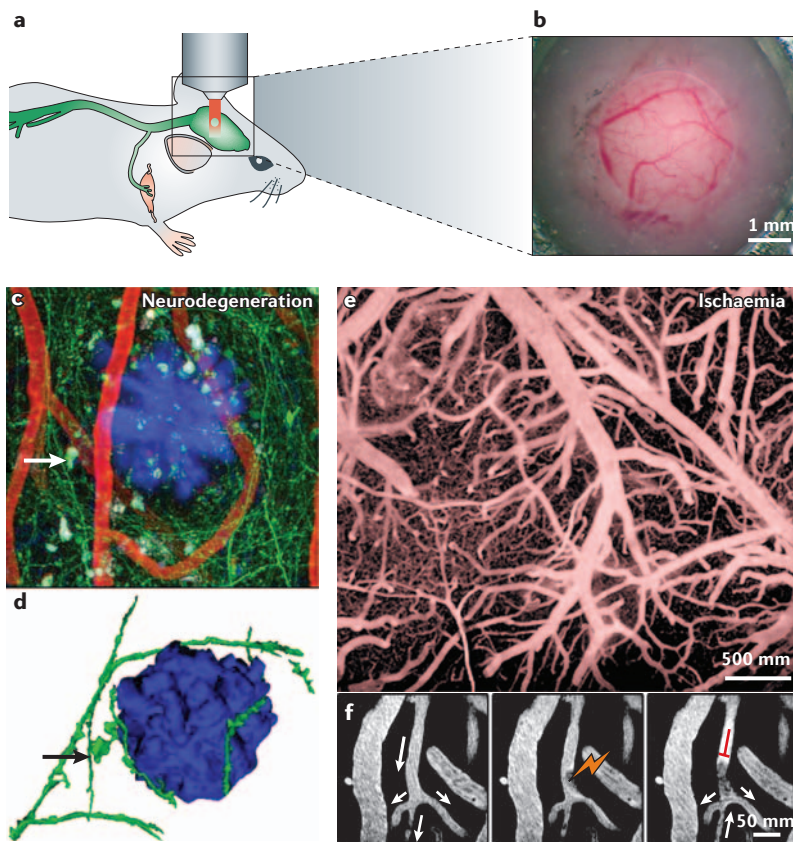


Figure 5 | Imaging the brain. **a** | *In vivo* multiphoton imaging of the brain allows numerous disease processes to be studied. **b** | A cranial window is created, either by a craniotomy covered with a cover slip as shown here (the brain vasculature can be seen in red) or by thinning the skull. **c** | Imaging in Alzheimer's disease models has significantly improved our understanding of plaque dynamics and the resulting cellular responses. Three-colour *in vivo* multiphoton image showing a plaque (blue, stained with a vital amyloid dye) surrounded by brain vasculature (red, filled with fluorescent dextran), and neurites labelled with fluorescent protein (green, expressed by viral infection). Neurites in close proximity to the plaque show signs of local dystrophy (arrow). **d** | Surface-rendered version of panel **c** to highlight the spatial relationship of plaques and neurites. **e** | Low-power overview of brain vasculature obtained *in vivo* by multiphoton microscopy. A highly complex and redundant vascular network that provides blood to CNS parenchyma is revealed, and its pathology can be studied in detail. **f** | Light can be used not only to visualize vessels and locally measure blood flow (note stripes in vessels, which are the negative images of passing erythrocytes) but also to induce localized ischaemia by vessel occlusion. On the left, a trifurcated vessel is shown with blood flow from top to bottom (arrows). Local illumination induces clot formation in the presence of a photosensitizer (middle, flash). After occlusion (right), flow reversal can be seen in the middle of the three secondary branches below the occlusion (arrows), whereas above it the stasis of blood flow is indicated by brighter labelling (red bar). Panels **c** and **d** reproduced, with permission, from REF. 83 © (2005) Society of Neuroscience. Panels **e** and **f** reproduced, with permission, from REF. 92. Panel **b** courtesy of M. Vaz Afonso and T. Bonhoeffer, Max-Planck Institute of Neurobiology, Martinsried, Germany.

Transcranial imaging

In the context of *in vivo* microscopy, this refers to imaging 'through' the skull; multiphoton imaging can image through thin slivers of bone, so that a craniotomy can be avoided by drilling away all but the inner table of the skull.

Degeneration. Most *in vivo* imaging studies of neurodegeneration so far have focused on Alzheimer's disease. The pathology of Alzheimer's disease and its animal models is mainly localized in the cerebral cortex, with easy access for multiphoton microscopy. Amyloid plaques, an important disease substrate, can be labelled *in vivo* by administering fluorescent dyes such as thioflavin-S or fluorescently labelled antibodies against amyloid-β¹². Repeated visualization of the same plaque allows

for the direct evaluation of plaque dynamics (FIG. 1d). *In vivo* analysis has, for example, shown that plaques remain at a remarkably stable size for many months, which suggests a dynamic equilibrium between amyloid deposition and clearance⁸⁰. Further insight can be gained by co-visualization of plaques and the presumed cytotoxic mediators that they induce (such as reactive oxygen species)⁸¹. How amyloid plaques contribute to neuronal damage has been explored by the concomitant imaging of neuronal processes; for example, by using transcranial imaging in Thy1-XFP mice^{82–84} (FIG. 5a–d). Although neurite damage can be readily correlated with plaque proximity in fixed tissue, the underlying dynamics only become apparent during intravital observations. For example, the results of a recent study suggest that spine loss — a feature of neuronal pathology in Alzheimer's disease — is the result of increased spine turnover with a shifted equilibrium of formation and loss⁸². This suggests that neurons can mount a compensatory reaction even in the hostile environment surrounding amyloid plaques. The relevance of such compensation has been emphasized by *in vivo* imaging of the neuronal response to therapy. When plaques are cleared by treatment with antibodies against amyloid-β, a significant proportion of dystrophic neurites recover within 3 days⁸⁴. Whether this can translate into the recovery of local function remains to be seen, perhaps by assessing local neuronal network activity with calcium imaging or electrophysiology⁸⁵. Other important contributors to Alzheimer's pathology, such as microglial cells, can now be incorporated into this dynamic view to explore their role in neuronal dystrophy, and amyloid deposition or clearance^{4,5}.

Amyotrophic lateral sclerosis (ALS) is another important neurodegenerative disease with an established animal model. The disease mainly affects motor neurons, the axons and synapses of which are accessible in living animals. *In vivo* microscopy of an ALS model revealed early synaptic pathology, which suggests that ALS might have a 'dying-back' component⁸⁶. Analysis in subset Thy1-XFP mice showed an asynchronous loss of branches in single axons, but also a dichotomy of extremely shrunken motor units and others that expanded massively by compensatory sprouting. Therefore, a population of motor neurons might exist that is relatively resistant to the early stages of this model. A number of other aspects of motor neuronal pathology, such as the role of axonal transport⁸⁷ and neuron–glia interactions⁸⁸ can be similarly tested *in vivo*.

Ischaemia. Diseases that affect the neurovascular unit of the CNS, like ischaemic stroke, are particularly suited to *in vivo* imaging studies. Ischaemia can be easily induced in animals, and vessels can be outlined by intravenous injection of fluorescent dextran-conjugates (FIG. 5e). The resultant negative contrast of erythrocytes (which exclude the dextran) can be used to assess blood flow. Originally, flow measurements were taken in the pia mater and in the upper cortical layers using wide-field or confocal microscopy^{89,90}. Later, measuring changes in cortical blood flow in response to sensory stimulation was one of the first applications of multiphoton microscopy,

which allowed the visualization of capillary flow down to cortical layer IV in rats⁹¹. This approach has more recently been transferred to animal models of ischaemic stroke. Imaging the redistribution of blood flow after vascular occlusion has demonstrated the importance of the cortical arteriole network for haemodynamic compensation^{92,93}. Photothrombotic occlusion of individual pial arterioles is counteracted by flow reversal in a single downstream arteriole, whereas occlusion of a major cerebral artery leads to the reversal of flow in many vessels. These recent studies have also shown that multiphoton microscopy not only allows the monitoring of the effects of vascular occlusion, but can also be used to develop ‘miniature’ models of a wide variety of vascular pathologies that complement more classic models of human stroke, such as occlusions of a major cerebral artery (FIG. 5f). Ultra-short laser pulses of different energy and duration can cause local extravasation of blood components, formation of an intravascular clot or vascular rupture with haemorrhage⁹³.

The effects of ischaemia on the brain parenchyma have been addressed by the combined imaging of cerebral blood flow and neuronal morphology after photothrombotic or micro-embolic stroke^{94,95}. Repeated imaging of dendrites for up to 5 hours after ischaemia showed that severe, but not moderate, ischaemia leads to rapid spine loss and dendritic alterations⁹⁵. Reperfusion within one hour after photothrombotic ischaemia allowed substantial recovery of the original dendrite structures, suggesting that even severe neuronal insults can be reversed by timely intervention. Using the new ‘optical’ micro-occlusion models⁹³, it should be possible to further define the neuronal consequences of finely graded ischaemia. It will be important to combine this with functional and molecular readouts of neuronal integrity by, for example, probing membrane potential, intracellular calcium load or activation of cell death cascades.

In vivo imaging also allows for the mechanistic dissection of neurovascular regulation, which tightly couples local blood flow and neuronal activity⁹⁶. Understanding this coupling is crucial, not only because it ensures proper substrate supply to the neuronal parenchyma (which makes it relevant to numerous neurological conditions), but also because it underlies many *in vivo* imaging methods aimed at monitoring neuronal activity, such as functional MRI. Microcirculatory regulation was recently explored with *in vivo* calcium imaging, and astrocytic calcium transients were found to herald vasodilation⁹⁷. Before this *in vivo* study, *in vitro* experiments had resulted in conflicting ideas about the role of glial calcium signalling in vasoregulation^{98,99}. Neurovascular regulation therefore provides a good example of how imaging in the intact brain can help to resolve controversies sparked by more reductionist approaches.

Inflammation. Another important aspect of the neurovascular unit is that it forms the blood–brain barrier, and therefore controls the access of immune cells to the brain. In neuroinflammatory conditions such as multiple sclerosis and its animal model, experimental autoimmune

encephalomyelitis, transmigration of immune cells across the blood–brain barrier is a crucial step in the formation of autoimmune lesions. As the study of transmigration through superficial vessels requires high temporal resolution but little depth penetration, most *in vivo* studies so far have relied on wide-field microscopy^{100–102} (FIG. 4e,f). Studies of peripheral immune responses have provided a number of protocols to label immune cells with fluorescent cell-tracker dyes or stable genetic markers, and to observe immune cells in vessels that are labelled by injecting fluorescent dextrans (for an example, see REF. 37). In studies of neuroinflammation, these techniques have been used to probe the molecular interactions that mediate immune cell migration into the inflamed CNS by, for example, using functional blocking antibodies to cell adhesion molecules. Such studies have helped to define the molecular interactions that mediate rolling, capture and adhesion of immune cells in CNS vasculature^{100,101}.

Considering the importance of these processes in multiple sclerosis, *in vivo* imaging is an attractive tool for developing therapeutic interventions. On the one hand, the efficacy of compounds known to target transmigration can be assessed in more detail. For example, the effects of targeting the interaction of the adhesion molecules $\alpha 4$ -integrin and vascular cell adhesion molecule 1 (VCAM1) with antibodies have been studied in this way. This treatment strategy has shown promising results, but also has grave side effects in patients with multiple sclerosis¹⁰³. On the other hand, *in vivo* imaging can help to unravel previously unknown mechanisms of action of established therapeutic compounds. An example is a recent study of the effects of intravenous immunoglobulins, which have been used successfully to treat many autoimmune disorders¹⁰⁴. *In vivo* imaging suggested that at least part of the beneficial effects of intravenously injected immunoglobulins was mediated by interference with $\alpha 4$ -integrin-dependent leukocyte recruitment. These studies underscore the power of combining *in vivo* imaging, pharmacological manipulation and quantitative evaluation to gain insight into molecular mechanisms of complicated cellular interactions and their therapeutic manipulation. It will be important to extend these *in vivo* studies to the actual neuroinflammatory lesions in the parenchyma, as has been accomplished in recent studies using tissue explants^{105,106}. By doing so, studies of the immunological events that initiate lesion formation become possible by, for example, co-visualizing antigen-presenting cells in their interaction with blood-borne lymphocytes¹⁰⁶. In addition, the co-labelling of cells that are damaged in neuroinflammatory lesions, such as oligodendrocytes and neurons, will improve our understanding of cell damage¹⁰⁵. Even following the molecular target of an autoimmune disease *in vivo* is possible, as originally shown by visualizing the nicotinic acetylcholine receptor in a model of myasthenia gravis¹⁰⁷.

Seizures. Many neurological deficits are primarily a reflection of altered neuronal activity. Epileptic seizures are a good example of this. Although it is well established that seizures are caused by bursts of synchronous,

Negative contrast

Form of contrast in which the object of interest is not labelled but surrounded by stained ‘background’ — for example, an unlabelled erythrocyte in fluorescently labelled serum.

Photothrombotic stroke

Form of small vessel occlusion that depends on ‘photosensitizers’ — that is, molecules like rose bengal that cause vessel damage in response to being illuminated. By injecting such molecules intravenously, local clotting (‘thrombosis’) can be targeted by light (‘optical’ micro-occlusion models).

Micro-embolic stroke

Miniature stroke model that is induced by injecting small particles into the blood stream. These particles lodge in microvessels and occlude them (‘emboli’). This model can be achieved by injecting microspheres or fragmented clots into a major artery.

rhythmic neuronal activity, important questions regarding the initiation of seizure activity and its consequences for cellular morphology and function remain unresolved. This is at least in part due to the low spatial resolution provided by surface electrophysiological recordings — the standard method for monitoring epileptic activity. Optical *in vivo* imaging studies now provide the opportunity to study epileptic seizures and their consequences at high resolution. Initial studies have used intrinsic optical imaging to follow altered neuronal activity *in vivo*^{56,108}. This technique can generate high-resolution maps of epileptic foci in minutes, thereby providing a dynamic view of the populations of neurons that participate in seizures¹⁰⁸. Notably, epileptic foci can be visualized by intrinsic imaging not only in animals, but also in humans during epilepsy surgery⁵⁶. Despite these advantages, the resolution of intrinsic optical imaging is still insufficient to study epileptic activity patterns with single-cell resolution. In contrast, multiphoton calcium imaging can resolve neuronal calcium activity in detail^{58,109}, to the point that an ‘optical encephalogram’ with cellular resolution can be obtained *in vivo*¹¹⁰. A related approach to calcium monitoring in astrocytes has recently revealed that calcium transients in glia precede neuronal bursting activity in a mouse model of epilepsy¹¹¹. Interestingly, several established anticonvulsant drugs, such as valproate, gabapentin and phenytoin, can directly suppress astrocytic calcium signals. This study provides a convincing example of how *in vivo* imaging can identify unexpected disease mechanisms and point to new therapeutic targets.

In vivo multiphoton imaging also allows analysis of the morphological consequences of seizures^{20,112}. Dendritic spines appear to remain remarkably stable, at least during the first hours after epileptic activity. This indicates that most of the seizure-related brain injury evolves only after prolonged or repetitive seizures. The stability of neuronal morphology, despite dramatically increased energy consumption, is testimony to the extent to which blood flow can match local demand. Indeed, multiphoton-based monitoring of cortical blood flow during epileptiform activity reveals greatly increased local perfusion¹¹³.

The road ahead

Many more neurological diseases are likely to be studied by *in vivo* microscopy in the near future, as improved techniques make more parts of the rodent brain accessible (for example, the cerebellum¹¹⁴) and transgenic labels for more cell types are established^{31,34,36}. Such studies will presumably include neurodevelopmental conditions (such as fragile X syndrome, Rett syndrome and storage disorders^{115,116}), as well as psychiatric disorders. However, rather than listing such future applications, we would like to conclude by discussing two major challenges for *in vivo* imaging of neurological diseases. The first is to elucidate the molecular pathology that underlies cellular changes. The second is to bridge the gap between the microscopic picture provided by *in vivo* microscopy and the macroscopic phenotype of experimental animals, and, eventually, of neurological patients.

Towards the molecular domain. Molecules ultimately mediate the pathogenesis of neurological diseases and offer the most promising targets for therapeutic interventions. Therefore, it is important for future imaging studies to monitor the molecular mechanisms underlying disease. Some techniques to image individual molecular species are already in place. A good example is calcium imaging, as it not only reveals disturbances in neuronal activity, but also provides a sensitive tool with which to visualize a central molecular mediator of cellular damage. Excessive calcium influx triggers well-characterized molecular pathways, which lead to axonal degeneration, demyelination or excitotoxic cell death^{71,117,118}. Inspired by the development of optical indicators for calcium influx, other molecular mediators of neurological disease have been monitored by imaging. For example, it is now possible to optically measure caspase activation during cell death¹¹⁹, gene expression during gliosis¹²⁰, transforming growth factor-induced signalling¹²¹, activity of matrix metalloproteinases¹²² and ubiquitin-dependent proteolysis¹²³.

A powerful tool to detect signals that emerge from such probes is a technique known as ‘biophotonic whole-body imaging’, in which the entire anaesthetized animal is placed in an imaging chamber. The emitted photons are detected across large fields of view, preferentially using near-infrared fluorescence or red-shifted bioluminescence reporters, which take advantage of the low absorption of biological tissues in this spectral window¹²⁴. The attraction of such biophotonic approaches is twofold. First, individual animals can be followed longitudinally throughout the disease course. Second, relatively large cohorts of animals can be examined to screen for therapeutic effects in translational research. However, one limitation is the low spatial and temporal resolution of this technique. Although photons from deep within the body can reach the surface, they are scattered along the way so that their site of origin is obscured. One possible solution is to use tomographic approaches, which allow a three-dimensional reconstruction of the location of the cells that emit the signal¹²⁴. Furthermore, histological examination of tissues post mortem can provide more detailed spatial information in retrospect. Another limitation is that biophotonic signals are often weakened by absorption, which restricts temporal resolution by requiring long acquisition times. Some help in this area might come from photochemistry. New fluorophores that boost sensitivity are becoming available (such as ‘quantum dots’ — that is, semiconductor nanocrystals that have unique fluorescence features; reviewed in REF. 125). Such probes can be combined with innovative methods of providing excitation of biophotonic probes (for example, by conjugating light-emitting molecules to nanocrystals, thereby creating ‘self-illuminating’ beacons based on bioluminescence resonant energy transfer¹²⁶).

Multiphoton microscopy is likely to remain the method of choice for the monitoring of molecules with subcellular resolution. Modifications of this method allow important disease-related molecules to be examined using intrinsic autofluorescence signals, such as nicotinamide intermediates or

Tomographic approaches
Describes imaging approaches in which the object is imaged from multiple angles and mathematical algorithms are used to reconstruct the three-dimensional structure of the signal source.

Bioluminescence resonant energy transfer
(BRET). Energy transfer in which the donor is a bioluminescent molecule that emits light as part of a biochemical reaction and transfers it to a fluorescent acceptor. Green fluorescent protein is the acceptor in a BRET cascade that makes some jellyfish glow.

neurotransmitters^{45,127,128}. Signals that do not depend on fluorescence molecules but instead rely on light scattering (for example, second-harmonic signals) allow the visualization of macromolecular arrays with suitable geometries, such as cytoskeletal components or the extracellular matrix¹²⁹. Alternatively, molecular deposits that form under pathological conditions can be stained with vital dyes, for example, in Alzheimer's disease models or in other amyloidopathies¹². There is also potential for autofluorescence or vital dye-based approaches in the longitudinal monitoring of pathogenic deposits of macromolecules in developmental storage disorders¹¹⁶. Finally, fluorescent proteins can be fused to molecules of interest, and FRET can be used to quantitatively assess molecular interactions¹³⁰. This approach has been used recently to visualize the activity of calpain proteases in live mice¹³¹. Protease activity can be imaged by attaching a donor fluorophore to a quencher, using a protease-specific linker peptide. Such probes fluoresce strongly only after proteolytic cleavage, and can be expressed genetically. However, in many instances, such methods are challenging to implement in live animals in which movement artefacts compromise imaging stability (BOX 2). Therefore, it is often easier to combine *in vivo* studies with *in vitro* systems (such as cell culture or organotypic explants). For example, it was recently demonstrated in a technical *tour-de-force* that genetically encoded FRET-sensors can reveal local Ras-activation with single-spine resolution in brain slices¹³².

Towards the clinical domain. *In vivo* imaging has already contributed much to our understanding of the dynamic cellular changes that underlie neurological disease in animal models. However, how the behaviour of single cells cause the clinical manifestations of neurological diseases in humans still needs to be explained. For this, it will be important to monitor pathological changes in the entire nervous system, rather than just in small fields of view. Ultimately, work with human patients will be indispensable. What can *in vivo* imaging contribute to this?

In the superficial parts of the human nervous system, such as the retina, high-resolution laser-scanning imaging is possible¹³³. In rodents, this approach has been used to image single cells; for example, retinal ganglion cells¹³⁴ and microglia¹³⁵. Multiphoton imaging of intrinsic fluorophores has been applied to the explanted eye¹³⁶, and it is likely that the construction of multiphoton ophthalmoscopes is well underway. In parallel, 'ultra-high-resolution' optical coherence tomography, a technique that uses interferometry of back-scattered light (similar to light-based 'ultrasound'), has already been used to reveal retinal structure in humans with micrometre resolution¹³⁷. However, such optical techniques are largely limited to the eye, where CNS tissues are optically accessible, or perhaps to peripheral nerves that can be easily exposed. For other parts of the human nervous system, direct optical imaging is less likely to be possible. Therefore, a more promising avenue to converge microscopy in animals and clinical imaging in humans is to miniaturize clinical imaging techniques and apply them to animal models in parallel to *in vivo* microscopy.

The most important clinical technique for longitudinal imaging of the human brain is MRI. New MRI protocols allow visualization of the orientation and integrity of axon tracts (using diffusion tensor imaging^{138,139}), to trace axonal projections (for example, using manganese¹⁴⁰) and to quantify the extent of axon damage (using spectroscopic analysis of neuron-specific markers¹⁴¹). High-resolution MRI is widely used in small animals (micro-MRI)¹⁴², and reveals fine morphological details that can be correlated with the transgenic labelling approaches used for *in vivo* microscopy. For example, diffusion tensor imaging of axon tracts in mice with axon guidance defects has recently been combined with fluorescence microscopy in Thy1-XFP animals¹⁴³. In addition, the corticospinal tract can be highlighted in living mice using manganese-enhanced MRI¹⁴⁴, and also traced using genetic fluorescent markers²⁹. Peripheral nerve degeneration can be followed by contrast-enhanced MRI¹⁴⁵ and by transcutaneous *in vivo* imaging⁷³. Other pathological alterations, such as Alzheimer's plaques, can be detected by micro-MRI and the parallel use of fluorescent dyes¹⁴⁶. To develop more versatile 'surrogate' markers for the cellular and molecular mediators of neurological disease, novel contrast agents that can be detected by MRI^{147,148} or by positron emission tomography (PET) are rapidly being generated. Individual cell populations (such as macrophages¹⁴⁹, T cells¹⁵⁰ or stem cells¹⁵¹) can be followed, and biochemical alterations can be visualized (for example, during apoptosis¹⁵², or during plaque formation in Alzheimer's disease¹⁵³). Increasingly, bimodal contrast agents are becoming available. These generate MRI or PET signals and, at the same time, are detectable by microscopy¹⁵¹. In the future, such agents might even be genetically encoded¹⁴⁸; for example, by taking advantage of biology's own magnetic senses¹⁵⁴ or iron-handling metabolism¹⁵⁵. One example of bimodal agents are amyloid probes that, in principle, allow the visualization of Alzheimer's plaques in animals by MRI, and also by *in vivo* microscopy¹⁵⁶. Another example is that of iron-based tracers, which can be used to follow immune cells in traumatic¹⁴⁹ or inflammatory lesions¹⁵⁷, and that are detectable by histological methods. In the future, studies that correlate clinical imaging modalities with *in vivo* microscopy in small animals (rodents or even non-human primates¹⁵⁸) might provide surrogate markers of cellular and molecular changes. This would provide a dynamic framework of interpretation for clinical imaging in humans, just as correlated serial electron microscopy can allow ultrastructural interpretation of cellular dynamics as observed by *in vivo* microscopy².

Conclusions and future perspectives

In summary, an optimistic picture is emerging of what *in vivo* microscopy could offer basic and clinical neuroscientists. Further progress will depend on technological innovations, some of which might not be related to today's most powerful optical microscopy approaches. In the past, progress has often come from unexpected directions. For example, before the introduction of multiphoton microscopy, few experimental neurologists would have considered nonlinear optics to be within their realm. Indeed,

Interferometry

Optical tool used to measure differences in path length or travel time of light by interference — that is, the addition or cancellation of light waves as a consequence of phase shifts.

Contrast agent

Used in clinical context for agents that generate a strong signal in an imaging technique and can be injected into the blood stream, swallowed by a patient or introduced into a body cavity.

Bimodal contrast agent

A compound that enhances contrast in more than one imaging modality, for example, a substance that generates an MRI signal and is fluorescent at the same time.

there are hints that the ultimate frontier of light microscopy — that is, the diffraction limit of resolution — might be overcome. A new field, dubbed ‘nanoscopy’¹⁵⁹, aims to expand light microscopy into the subcellular or even the molecular realm by creating excitation volumes that are much smaller than those that can be generated in conventional microscopy. Current ‘nanoscopic’ approaches use highly complicated optical tools to accomplish this, which are unlikely to be easily applicable to living multicellular

organisms. However, it has been argued that newly identified proteins that have light inducible fluorescent properties⁶⁴ could allow ‘nanoscopic’ imaging to be accomplished with much simpler optics. Therefore, rather than depending on physicists alone, the future development of *in vivo* microscopy might equally depend on the contributions made by chemists and molecular biologists^{159,160}. The charm and challenge of *in vivo* imaging lies in the uncertainty of what will move into focus next.

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Competing interests statement

DATABASES

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