24. Hüber, M. M. *et al.* Fluorescently detectable magnetic resonance imaging agents. *Bioconjug. Chem.* **9**, 242–249 (1998).
25. Dodd, S. J. *et al.* Detection of single mammalian cells by high-resolution magnetic resonance imaging. *Biophys. J.* **76**, 103–109 (1999).
26. Kayyem, J. F., Kumar, R. M., Fraser, S. E. & Meade, T. J. Receptor-targeted cotransport of DNA and magnetic-resonance contrast agents. *Chem. Biol.* **2**, 615–620 (1995).
27. Moore, A., Josephson, L., Bhorade, R. M., Basilion, J. P. & Weissleder, R. Human transferrin receptor gene as a marker gene for MR imaging. *Radiology* **221**, 244–250 (2001).
28. Basser, P. J., Mattiello, J. & LeBihan, D. Estimation of the effective self-diffusion tensor from the NMR spin echo. *J. Magn. Reson. B* **103**, 247–254 (1994).
29. Ahrens, E. T., Laidlaw, D. H., Readhead, C., Fraser, S. E. & Jacobs, R. E. Investigating white matter diffusion anisotropy using the demyelinating shiverer mutant mouse in *Proceedings of the Seventh International Society for Magnetic Resonance in Medicine* (Berkeley, California, 1999).
30. Song, S. K. *et al.* Dysmyelination revealed through MRI as increased radial (but unchanged axial) diffusion of water. *Neuroimage* **17**, 1429–1436 (2002).
31. Pierpaoli, C., Jezzard, P., Basser, P. J., Barnett, A. & Di Chiro, G. Diffusion tensor MR imaging of the human brain. *Radiology* **201**, 637–648 (1996).
32. Conturo, T. E. *et al.* Tracking neuronal fiber pathways in the living human brain. *Proc. Natl Acad. Sci. USA* **96**, 10422–10427 (1999).
33. Jones, D. K., Simmons, A., Williams, S. C. & Horsfield, M. A. Non-invasive assessment of axonal fiber connectivity in the human brain via diffusion tensor MRI. *Magn. Reson. Med.* **42**, 37–41 (1999).
34. Mori, S. & Barker, P. B. Diffusion magnetic resonance imaging: its principle and applications. *Anat. Rec.* **257**, 102–109 (1999).
35. Mori, S. *et al.* Diffusion tensor imaging of the developing mouse brain. *Magn. Reson. Med.* **46**, 18–23 (2001).
36. Frank, L. R. Characterization of anisotropy in high angular resolution diffusion-weighted MRI. *Magn. Reson. Med.* **47**, 1083–1099 (2002).
37. Govindaraju, V., Young, K. & Maudsley, A. A. Proton NMR chemical shifts and coupling constants for brain metabolites. *NMR Biomed.* **13**, 129–153 (2000).
38. Ross, B. & Bluml, S. Magnetic resonance spectroscopy of the human brain. *Anat. Rec.* **265**, 54–84 (2001).
39. Dydak, U., Weiger, M., Pruessmann, K. P., Meier, D. & Boesiger, P. Sensitivity-encoded spectroscopic imaging. *Magn. Reson. Med.* **46**, 713–722 (2001).
40. Tyszka, J. M. & Mamelak, A. N. Volumetric multishot echo-planar spectroscopic imaging. *Magn. Reson. Med.* **46**, 219–227 (2001).
41. Dowling, C. *et al.* Preoperative proton MR spectroscopic imaging of brain tumors: correlation with histopathologic analysis of resection specimens. *Am. J. Neuroradiol.* **22**, 604–612 (2001).
42. Graves, E. E. *et al.* Serial proton MR spectroscopic imaging of recurrent malignant gliomas after γ knife radiosurgery. *Am J. Neuroradiol.* **22**, 613–624 (2001).
43. Bluml, S., Moreno-Torres, A., Shic, F., Nguy, C. H. & Ross, B. D. Tricarboxylic acid cycle of glia in the *in vivo* human brain. *NMR Biomed.* **15**, 1–5 (2002).
44. Koretsky, A. P., Brosnan, M. J., Chen, L. H., Chen, J. D. & Van Dyke, T. NMR detection of creatine kinase expressed in liver of transgenic mice: determination of free ADP levels. *Proc. Natl Acad. Sci. USA* **87**, 3112–3116 (1990).
45. Weissleder, R. *et al.* MR imaging and scintigraphy of gene expression through melanin induction. *Radiology* **204**, 425–429 (1997).
46. Louie, A. Y. *et al. In vivo* visualization of gene expression using magnetic resonance imaging. *Nature Biotech.* **18**, 321–325 (2000).
47. Zhao, M., Kircher, M. F., Josephson, L. & Weissleder, R. Differential conjugation of tat peptide to superparamagnetic nanoparticles and its effect on cellular uptake. *Bioconjug. Chem.* **13**, 840–844 (2002).
48. Zook, A. L., Adhyaru, B. B. & Bowers, C. R. High capacity production of >65% spin polarized Xenon-129 for NMR spectroscopy and imaging. *J. Magn. Reson.* **159**, 175–182 (2002).

Online links

DATABASES

The following terms in this article are linked online to:

LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
creatine kinase | β-galactosidase

FURTHER INFORMATION

Edinburgh Mouse Atlas Project: http://genex.hgu.mrc.ac.uk/

Access to this interactive links box is free online.

OPINION

Imagining imaging's future

Roger Y. Tsien

Imaging specific molecules and their interactions in space and time will be essential to understand how genomes create cells, how cells constitute organisms and how errant cells cause disease. Molecular imaging must be extended and applied from nanometre to metre scales and from milliseconds to days. This quest will require input from physics, chemistry, and the genetics and biochemistry of diverse organisms with useful talents.

Any attempt to forecast the future of a fast-moving subject is foolhardy, but at the Editors' request, I offer a few idiosyncratic extrapolations that indicate in which directions I feel imaging ought to progress.

Systematic cell and developmental biology began in the nineteenth century with morphological descriptions of visible structures, the chemical basis of which was largely unknown. Meanwhile, physiology began to describe the amazing, complex responses of living systems to real-time perturbations, but again molecular details were missing. The twentieth century witnessed explosive progress in macromolecular biochemistry and genetics, which started with the rediscovery of Mendelian genetics and the recognition of biopolymers, and culminated in the sequencing of complete genomes. However, genome sequences alone lack spatial and temporal information and are therefore as dynamic and informative as census lists or telephone directories. The challenge for the twenty-first century is to understand how these casts of molecular characters work together to make living cells and organisms, and how such understanding can be harnessed to improve health and well-being. I believe this quest will depend heavily on molecular imaging, which shows when and where genetically or biochemically defined molecules, signals or processes appear, interact and disappear, in time and space. Therefore, molecular imaging synergistically draws on physics, chemistry, anatomy, physiology, biochemistry and genetics.

Perhaps the first breakthroughs in molecular imaging were the development of fluorescent-antibody techniques in the 1940s and *in situ* hybridization techniques in the 1980s (for historical reviews, see REFS 1,2), which reveal the spatial distribution of protein and nucleotide sequences respectively, albeit only in dead fixed cells or tissue sections. Significant advances in cell physiology also came in the 1980s from the development of fluorescent organic dyes that could non-destructively image ionic signals such as pH, Ca^{2+} and membrane potential with high spatial and temporal resolution[3]. However, the range of signals for which satisfactory indicators could be devised was very limited, applications to intact organisms or non-mammalian cells were rare, and targeting to specific subcellular locations or cell types was nearly impossible. A paradigm-shifting advance that occurred in the past decade was the cloning[4] and expression[5] of the jellyfish green fluorescent protein (GFP), which enabled visible fluorescence to be genetically encoded by a single portable DNA sequence[6]. Much of the revolution wrought by GFP and its variants is reviewed in other articles in this supplement. The prevalence and success of GFP indicate that comparable revolutions might result from genetic sequences that robustly encode image contrast for other methods, such as electron microscopy (EM), NANOPROBE SCANNING, OPTICAL COHERENCE TOMOGRAPHY (OCT), magnetic resonance imaging (MRI), MAGNETOENCEPHALOGRAPHY (MEG) and near-infrared fluorescence. The spatial and temporal domains of these imaging techniques, and other techniques that are discussed in this article, are shown in FIG. 1.

One additional advantage of gene sequences is that they are easily transferred from one laboratory to another — for example, as a drop of complementary DNA on a filter or a file stating a useful mutation. Only software is as easily communicated and replicated, but high-performance imaging software often has to be highly optimized for a particular piece of hardware and its operating system, and therefore loses portability. We are lucky that the genetic code is so standardized. Advances in instrumentation hardware have the lowest rate of dissemination and all too often end up amusing only their creators unless a major manufacturer becomes a committed advocate.

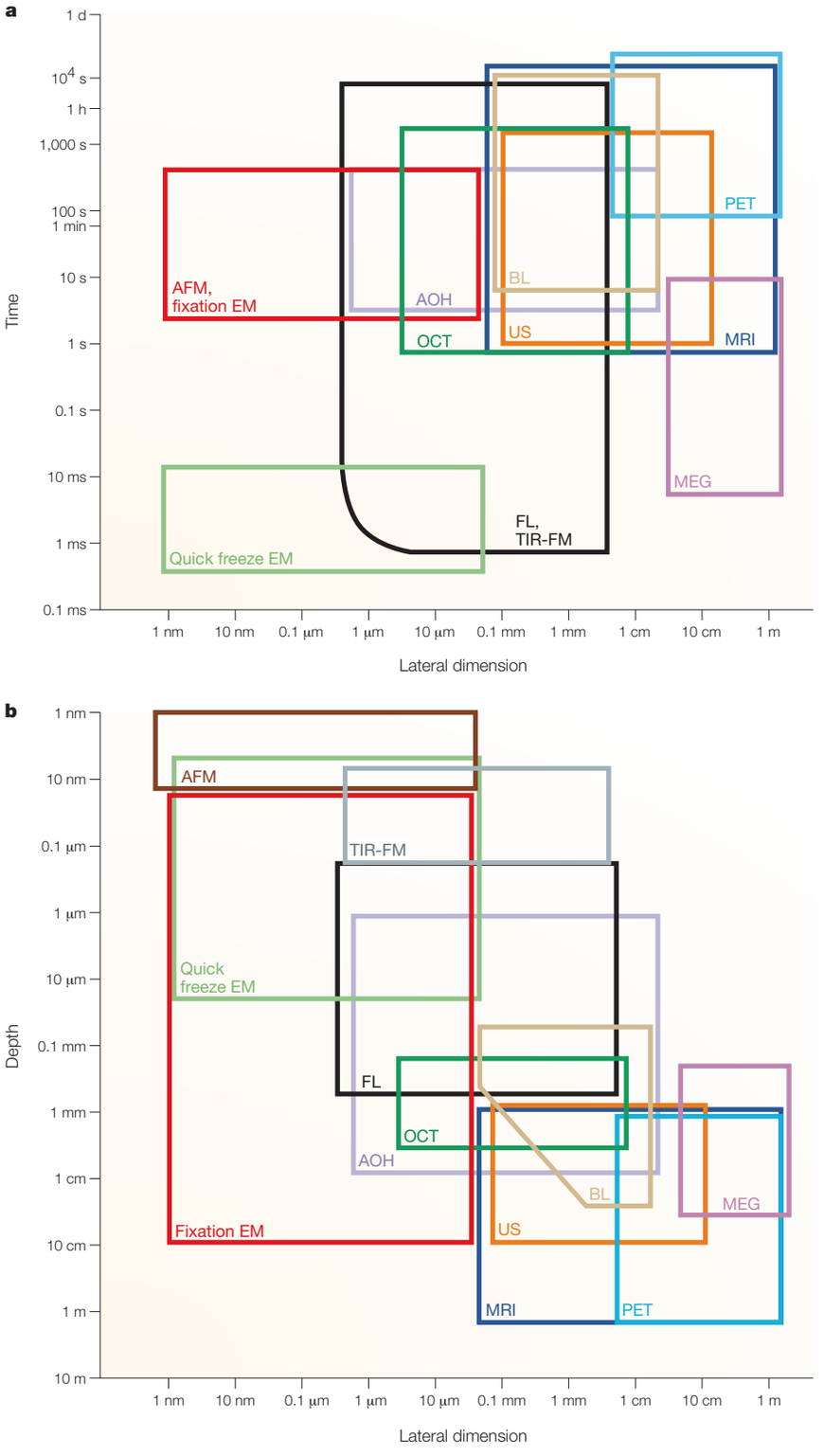

Figure 1 | **Imaging methods compared by their timescales, penetration depths and ranges of lateral dimensions.** All scales are logarithmic, and all box boundaries are estimates of typical present practices and are much fuzzier than the crisp lines shown. **a** | Each time span, except for electron microscopy (EM) techniques, indicates the range from the shortest time difference that can be comfortably resolved by a particular technique to the maximum duration of continuous observation. For EM techniques, the timescale indicates the estimated time required for freezing or fixing the tissue. Lateral dimensions range from the finest spacing over which separate objects can be discriminated up to the maximum size of a single field of view. **b** | Depth dimensions range from the minimum thickness for an adequate signal, to the maximum depth of imaging without a severe loss of sensitivity or lateral resolution. Note that far-red or near-infrared fluorescence can be comparable to bioluminescence in terms of depth resolution. Again, lateral dimensions range from the finest spacing over which separate objects can be discriminated up to the maximum size of a single field of view. AFM, atomic-force microscopy; AOH, all-optical histology; BL, bioluminescence; FL, fluorescence microscopy at visible wavelengths; MEG, magnetoencephalography; MRI, functional magnetic resonance imaging; OCT, optical coherence tomography; PET, positron emission tomography; TIR-FM, total internal reflection fluorescence microscopy; US, ultrasound.

Nanometre to micrometre imaging

Many important biological machines and signalling domains have dimensions of ~5–500 nm and therefore fall between the optimal scales for X-ray crystallography and optical microscopy. As pointed out by James Rothman, many of the most important mechanistic questions in cell biology might be rapidly answered if only we had molecular video EM — that is, the ability to non-destructively image specific molecules with the resolution of EM in real time. Fluorescence microscopy has all of these capabilities except that diffraction ordinarily limits spatial resolution to >200 nm. Finer spatial resolutions (30–100 nm) have been attained by using three innovative techniques: SCANNING NEAR-FIELD OPTICAL MICROSCOPY[7]; the coherent collection of fluorescences using a pair of diametrically opposed objectives[8]; and superlocalized depletion of the excited state by stimulated emission[9]. However, because these techniques are so new, the instrumentation

is complex, and the possible depth of imaging is limited, their widespread application has so far been prevented.

EM has the desired spatial resolution, but is inherently destructive and is normally dependent on antibody labelling for molecular selectivity. As pointed out on page SS6 of this supplement, efficient immunolabelling and fine structural preservation tend to be antithetical. Genetically encoded labels that create EM contrast in well-fixed sections without the need for antibody staining should avoid this problem, because the crucial reporter would be introduced into the live cell by transfection. The first such tag was a well-known reporter enzyme, horseradish peroxidase, that was genetically fused to targeting signals and various proteins in the secretory pathway, and was imaged by its ability to catalyse a reaction between H_2O_2 and diaminobenzidine (DAB), which results in a dense precipitate[10]. Unfortunately, however, this enzyme of 308 amino acids does not become functional when it is expressed in the cytosol, perhaps because it needs Ca^{2+} and four disulphide bonds. Re-engineering this peroxidase, or finding another that works naturally in the cytosol, would therefore be worthwhile.

Another EM-visible tag is the tetracysteine– ReAsH system, in which the protein of interest is fused to the amino-acid sequence Cys-Cys-Pro-Gly-Cys-Cys (where Cys is cysteine, Pro is proline and Gly is glycine)[11,12]. This sequence motif can be labelled in live cells or in fixed sections with ReAsH, which is a membrane-permeable biarsenical derivative of the red fluorophore resorufin. ReAsH is visible because of its red fluorescence in living cells, which survives harsh fixation. In addition, vigorous illumination of ReAsH catalyses the oxidation of DAB by O_2 to produce a precipitate that is even finer than that produced by horseradish peroxidase, DAB and H_2O_2. Dye-catalysed photodeposition of an EM-visible deposit (for the founding paper, see REF. 13) therefore becomes genetically encodable. Correlation between live-cell fluorescence movies and post-fixation EM snapshots at much higher spatial resolution provides a partial solution to the goal of molecular video EM. For an example of four-dimensional multi-photon time-lapse imaging correlated through photoconversion to three-dimensional electron microscopy, please see the Photoconversion example in the Online links. In addition, because ReAsH labelling can be performed in a pulse-chase manner, it can be used to stain young or old copies of the protein selectively and therefore to add some temporal resolution to EM. However, the technique still has scope for considerable improvements in sensitivity, selectivity and robustness.

Attempts to use GFP to catalyse the photoconversion of DAB by O_2 have generally been unsuccessful, presumably because the fluorophore inside GFP is sterically shielded from O_2 (REF. 6). A post-fixation protocol that allows GFP, or an engineered variant, to photo-oxidize DAB reliably would be valuable, because it would bypass the need for antibodies and would fit well with using GFP fusions to facilitate correlative EM.

A related challenge in EM is to image two or more distinct molecular species simultaneously — the EM equivalent of multicolour fluorescence. The nearest approximation has been double-immunoEM with different-sized gold particles. However, the size of such particles, the low percentage of antigens that get labelled and the technical difficulty of the procedures prevent routine detection of protein–protein associations or co-localization. Rather than using the size of the gold particle as the distinguishing characteristic, a spectroscopic parameter that is independent of morphology would be desirable. Perhaps the most attractive candidate is the energy loss of the electrons as they pass through the specimen and scatter inelastically off atoms of particular elements. Electron energy loss spectroscopy (EELS) is a well-established adjunct to biological EM; the challenge is therefore to devise contrast agents, preferably genetically encoded, that each cause the local deposition of elements with distinct EELS signatures[14]. An answer to this challenge could provide a powerful way to map the associations of specified pairs of proteins throughout cells and tissues.

Scanning microscopes, such as the ATOMIC-FORCE MICROSCOPE (AFM), are the only technologies that are presently capable of non-destructive continuous imaging with resolutions that are well below optical wavelengths. The AFM in TAPPING MODE can map surface topography, but it is often difficult to know which molecule corresponds to which bump on a complex biological surface. Perhaps molecular specificity might be derived from the distinctive sizes and shapes of fluorescent proteins or NANOPARTICLES, or from the detection of electron tunnelling between an AFM tip and electrochemical contrast agents, such as a redox protein in contact with a redox reagent in solution. Such tunnelling would be an extension of scanning electrochemical microscopy[15,16].

Micrometre to millimetre imaging

Cell biology from Hooke and Leeuwenhoek onward has relied on light microscopy for observations at the micrometre to millimetre length scale. GFP improvements, different colours (including the cloning of fluorescent proteins from numerous organisms)[17], and targetable dynamic indicators for physiological signals have been well covered in recent reviews[18], including several in this supplement. I therefore focus on a few unsolved problems.

How far can multi-photon excitation take us? Multi-photon excitation has been well established as the best way to recover fluorescence signals from endogenous or exogenous fluorophores that are relatively deep inside a scattering tissue such as the brain. This superiority is because the excitation is near-infrared, to which tissues are relatively transparent, and because the emitted light can be collected in a non-imaging mode (which includes photons that have undergone scattering)[19]. Nevertheless, multi-photon excitation still cannot reach much deeper than ~0.6 mm. Moreover, there are some hints that the ratio of quantum yields for fluorescence versus photobleaching — that is, the total number of photons that can be obtained before photodestruction — might be less favourable for multi-photon excitation than for conventional single-photon excitation[20].

How to get bigger spectral responses to biochemical signals? Despite the plethora of genetically encoded indicators for various biochemical signals, such as Ca^{2+}, cyclic nucleotides, phosphorylation or protein–protein associations, the maximal optical changes are often modest — that is, typically 10–50% changes in the ratio of intensities at two wavelengths. Larger responses are often obtainable from indicators in which fluorescent proteins have been split into two fragments and shuffled together with sensor domains[21–23]. However, these chimaeras are generally subject to pH interference and change their intensity in a wavelength-independent manner. Such wavelength-independent responses are subject to many more artefacts than responses in which signals at two wavelengths respond in opposite directions. The 'glass ceiling' on the dynamic range of the response might, in part, be because ratio changes of 10–20% are enough to enable biological experiments to be carried out in cultured cells. Once this threshold is attained, junior scientists find themselves under pressure to switch from improving the molecules to demonstrating biological applications. In a few cases, unusually patient investigators have made tens of constructs to optimize the response amplitude, and these seem to be the cases in which responses of twofold or greater were eventually obtained[24]. The logical extension

should be the high-throughput generation of thousands to millions of variants, coupled to automated screening for the best responders — a cycle that would probably need to be iterated many times.

Monitoring long-distance protein–protein interactions. One of the main tasks of proteomic analysis is to find and quantify protein–protein interactions, and imaging must have a crucial role whenever such interactions are to be monitored dynamically in space and time. The best established ways to image protein–protein interactions are resonance energy transfer (RET; of fluorescence (FRET) or bioluminescence (BRET)) and protein fragment complementation assays (PCA)[25,26]. Both approaches require the attachment of reporters to both putative interacting partners. In FRET and BRET, one reporter is a donor fluorophore or bioluminescent protein, the other reporter is a longer wavelength acceptor fluorophore, and the readout is energy transfer from the donor to the acceptor. The absolute efficiency of FRET can be measured *in situ* by several methods. The simplest method is to observe how much the donor brightens when the acceptor is selectively photobleached[24]. A non-destructive alternative is to measure the fluorescence at three sets of excitation and emission wavelengths and to apply correction factors for spectral overlaps and relative quantum yields[27–29]. Perhaps the most elegant, but expensive, method is to use fluorescence lifetime imaging to detect the effect of FRET on donor excited-state lifetime[30,31]. In PCA, the reporters are complementary halves of an enzyme, which can only function when the two fragments are brought together and fold around each other. The readout is the conversion of the enzyme's substrate to product. RET has a better-defined physical basis, better spatial and temporal resolution, better reversibility and less intrinsic perturbation of the interacting partners, whereas PCA has the benefits of enzymatic amplification and a much greater dynamic range over the background.

Both RET and PCA have distance ranges that are limited to single digits of nanometres, which is much less than the dimensions of known supramolecular machines, such as transcription complexes, ribosomes, chaperone assemblies, proteasomes and signal-transduction assemblies. Therefore, techniques that detect associations over tens of nanometres would be very desirable. Two-colour fluorescence correlation spectroscopy[32] monitors whether pairs of labelled macromolecules consistently diffuse, in unison, into and out of a femtolitre volume on which a laser is focused. If the partners are within the right concentration range and diffusion rate, their correlated migration strongly indicates their association. Another intriguing pair of reporters would be a generator and a detector of singlet oxygen, the diffusion range of which is ~10–50 nm through cells[33]. Clinical immunoassays that are based on singlet-oxygen proximity detection have been devised and commercialized[34], but extension to cell-biological imaging and proteomics seems to be unexplored. Placement of such reporters on antibodies might allow the proximities of unfused endogenous proteins to be assessed in fixed cells.

Imaging specific nucleic acids in living cells. So far, proteins are the only macromolecules that can be made fluorescent under genetic control. A few DNA and RNA sequences, lipids and carbohydrates can be visualized indirectly because of their ability to bind a protein that can be fused to GFP, but this strategy introduces considerable complexity and molecular bulk. For example, present ways to image RNA in living cells involve using six tandem repeats of a 19-nucleotide stem-loop sequence, each of which can bind a phage capsid protein (MS2) that is fused to GFP, which adds on a total bulk of ~270 kDa[35]. Is there any prospect of bypassing fluorescent proteins? RNA sequences of 38–90 nucleotides have been developed that can bind various fluorescent and non-fluorescent dyes with submicromolar dissociation constants[36,37], but they have not yet been optimized enough to be used for imaging in live cells. Ideally, these short RNA sequences could be genetically fused to RNAs of biological interest and would bind a non-fluorescent, non-toxic, membrane-permeable dye tightly and make it fluorescent. The many important roles and locations of various RNAs call for a range of techniques to image RNAs directly in living cells.

Imaging at millimetre scales and above

Imaging at this scale is required for non-invasive visualization inside intact organs and opaque organisms such as mammals.

The main readouts are transcriptional. At present, the most popular genetically encoded reporters for imaging at depths of more than about a millimetre in opaque organisms are firefly luciferase[38] and herpes simplex thymidine kinase[39]. The yellow–green bioluminescence from luciferase (which is fuelled by ATP, O_2 and exogenous luciferin) has a substantial component that is >600 nm, which can escape from several millimetres deep in an organism[38]. However, the deeper the source, the more scattering the photons undergo, which attenuates and blurs the image. Nevertheless, luciferase bioluminescence from just tens of cells is readily detectable in mice.

Viral thymidine kinase phosphorylates and traps ^{18}fluorine-labelled antiviral drugs inside cells and can therefore be imaged by POSITRON EMISSION TOMOGRAPHY (PET). PET requires much higher numbers of reporter-expressing cells and more expensive reagents and instrumentation compared with bioluminescence. However, PET gives true tomographic resolution at any depth of practical interest.

Genetically encoded contrast agents are largely missing (but might be developed) for several other *in vivo* imaging techniques on this scale, such as MRI, MEG, near-infrared fluorescence and OCT. MRI of β-galactosidase expression has been carried out with a gadolinium chelate that, after it is cleaved by β-galactosidase, becomes better able to relax the spins of water protons[40]. This contrast agent was only an early-stage proof-of-principle agent, because it required intracellular microinjection and was a very sluggish substrate for the enzyme. Substrates with better kinetics and larger changes in relaxivity would be highly desirable. An alternative is indicated by the well-established use of artificial SUPERPARAMAGNETIC MAGNETITE NANOPARTICLES as contrast agents in MRI. Many organisms, from microaerophilic bacteria to birds, create analogous nanoparticles ('MAGNETOSOMES') for navigation purposes[41]. Efforts are underway to clone the relevant proteins from bacterial magnetosomes. If magnetosomes can be heterologously expressed, they might be powerful focusing agents for all magnetic imaging techniques, including MRI and MEG.

Can red (600–700 nm) or, even better, infrared (>700 nm) emission be genetically encoded? Deep-ocean dragonfish were reported to luminesce with a peak at 705 nm (REF. 42), but the isolated protein was very photobleachable and showed its longest wavelength emission maximum at only 626 nm. Furthermore, this luminescence probably depends on a tetrapyrrole chromophore, which is not genetically encoded and would have to be supplied exogenously if this protein were ever to be used as a genetic fusion. Meanwhile, coral-derived fluorescent proteins with self-generated chromophores now exist with excitation peaks above 600 nm and emission maxima beyond 630 nm (REF. 17). infrared wavelengths would have been better,

but not by a huge factor, so it would be worth trying to use these coral proteins for *in vivo* imaging. An interesting alternative reporter would be *cobA*, a bacterial methyltransferase, which converts ubiquitous, endogenous, non-fluorescent uroporphyrinogen III into red fluorescent porphyrins that have emission peaks at 605 nm (REF. 44). Yet another possibility relies on exogenous polymeric substrates that are labelled with numerous infrared fluorophores that quench each other. Proteases that attack the polymer split the fluorophores apart and allow them to fluoresce[45].

OCT is a radar-like echo-ranging technique that uses ultrashort infrared pulses of $\sim 10^{-13}$ seconds duration[46]. Reflections are detected from interfaces that are up to a few millimetres deep, with a resolution as fine as tens of micrometres. OCT detects reflecting or back-scattering interfaces but not fluorophores, so how could contrast be genetically encoded? Many organisms make reflectors, including fish scales, scallop eyes and the tapetum that is responsible for the reflectivity of cats'eyes[47]. However, the system that might be most convenient for this genetic dissection and reconstitution is the reflective stack of membranes behind the eyespot of green algae such as *Chlamydomonas*[48], because the genome of this single-cell organism has been sequenced.

Can other readouts be devised? The above *in vivo* reporters — present and projected — are essentially transcriptional. Although almost any biochemical signal can be coupled to some transcriptional response element, we would often like to bypass the complexity and slow kinetics of such networks. The fastest general mechanism to sense protein conformation or proximity would probably be RET from a bioluminescent protein to a red or near-infrared fluorescent protein. For somewhat slower events, the *in vivo* reporter proteins, such as luciferase or thymidine kinase, have already been split into fragments for *in vivo* PCA[49].

Pulse-chase techniques. When continuous high-resolution imaging at a significant depth is impossible, sometimes we might have to sacrifice continuous recording and be content with a snapshot that can be read post-mortem. EM and autoradiography already work in this way. Fluorescence microscopy with a submicron spatial resolution can now be performed in fixed tissue over several millimetres of both depth and lateral extent. This can be done by imaging the top ~0.1 mm with two-photon excitation and a scanning stage, and then using a much higher-power laser to ablate that layer and expose the next underlying layer, which can then be imaged in perfect registration. This iterative cycle of imaging and vapourization ('all-optical histology') could convert a mouse brain to two terabytes of volumetric image data in <1 day (REF. 50). There are many other untapped possibilities for reporters that can be triggered, by a pulse of photons or small molecules, to memorize transient biochemical events in a form that can be imaged with high spatial resolution after fixation and sectioning.

Circumventing the limitation to transgenic animals/xenografts. The obsessive focus above on genetically encoded contrast agents for molecular imaging leaves an important gap — will such agents ever be applicable to humans? Although the co-administration of GFP genes has been used to monitor the effectiveness of gene-therapy vectors[51], the practical and ethical challenges of gene delivery into humans argue for a longer-term focus on how to image life-threatening events such as tumour metastasis. Our growing understanding of fundamental oncogenic mechanisms and differential-expression profiles in tumour versus normal tissues[52] are providing lists of messenger RNAs and proteins that are causally related to, and highly indicative of, tumour formation, metastasis and angiogenesis. Important funding agencies have recognized that non-invasive imaging of endogenous mRNA is conceptually possible and very desirable, so many laboratories are pursuing complementary strategies towards this enormous, but worthwhile, goal.

Conclusion

The growing synergy between biochemistry, genetics, physiology, anatomy and medicine will be accelerated by new modes of molecular imaging and new genetically targetable contrast agents. The latter are particularly cost-effective and portable. However, to become optimal, they will require interdisciplinary cooperation, and the transplantion and optimization of many of the unusual talents of obscure organisms.

Howard Hughes Medical Institute, Departments of Pharmacology and of Chemistry and Biochemistry, University of California, San Diego, La Jolla, California 92093-0647, USA.
e-mail: rtsien@ucsd.edu

Please cite this article as a supplement to volume 4 of Nature Reviews Molecular Cell Biology, *pages SS16–SS21.*
doi:10.1038/nrm1196

1. Goldman, M. *Fluorescent Antibody Methods* (Academic Press, New York, 1968).
2. Trask, B. J. Human cytogenetics: 46 chromosomes, 46 years and counting. *Nature Rev. Genet.* **3**, 769–778 (2002).
3. Tsien, R. Y. Fluorescent probes of cell signaling. *Annu. Rev. Neurosci.* **12**, 227–253 (1989).
4. Prasher, D. C., Eckenrode, V. K., Ward, W. W., Prendergast, F. G. & Cormier, M. J. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* **111**, 229–233 (1992).
5. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. Green fluorescent protein as a marker for gene expression. *Science* **263**, 802–805 (1994).
6. Tsien, R. Y. The green fluorescent protein. *Ann. Rev. Biochem.* **67**, 509–544 (1998).
7. Edidin, M. Near-field scanning optical microscopy, a siren call to biology. *Traffic* **2**, 797–803 (2001).
8. Gustafsson, M. G., Agard, D. A. & Sedat, J. W. I5M: 3D widefield light microscopy with better than 100 nm axial resolution. *J. Microsc.* **195**, 10-16 (1999).
9. Dyba, M. & Hell, S. W. Focal spots of size λ/23 open up far-field fluorescence microscopy at 33 nm axial resolution. *Phys. Rev. Lett.* **88**, 163901–163904 (2002).
10. Hopkins, C., Gibson, A., Stinchcombe, J. & Futter, C. Chimeric molecules employing horseradish peroxidase as reporter enzyme for protein localization in the electron microscope. *Methods Enzymol.* **327**, 35–45 (2000).
11. Adams, S. R. *et al.* New biarsenical ligands and tetracysteine motifs for protein labeling *in vitro* and *in vivo*: synthesis and biological applications. *J. Am. Chem. Soc.* **124**, 6063–6076 (2002).
12. Gaietta, G. *et al.* Multicolor and electron microscopic imaging of connexin trafficking. *Science* **296**, 503–507 (2002).
13. Maranto, A. R. Neuronal mapping: a photooxidation reaction makes Lucifer yellow useful for electron microscopy. *Science* **217**, 953–955 (1982).
14. Radcke, C., Stroh, T., Dworkowski, F. & Veh, R. W. Specific visualization of precipitated cerium by energy-filtered transmission electron microscopy for detection of alkaline phosphatase in immunoenzymatic double labeling of tyrosine hydroxylase and serotonin in the rat olfactory bulb. *Histochem. Cell Biol.* **118**, 459–472 (2002).
15. Liebetrau, J. M., Miller, H. M. & Baur, J. E. Scanning electrochemical microscopy of model neurons: imaging and real-time detection of morphological changes. *Anal. Chem.* **75**, 563–571 (2003).
16. Macpherson, J. V., Jones, C. E., Barker, A. L. & Unwin, P. R. Electrochemical imaging of diffusion through single nanoscale pores. *Anal. Chem.* **74**, 1841–1848 (2002).
17. Matz, M. V., Lukyanov, K. A. & Lukyanov, S. A. Family of the green fluorescent protein: journey to the end of the rainbow. *Bioessays* **24**, 953–959 (2002).
18. Zhang, J., Campbell, R. E., Ting, A. Y. & Tsien, R. Y. Creating new fluorescent probes for cell biology. *Nature Rev. Mol. Cell Biol.* **3**, 906–918 (2002).
19. Denk, W. & Svoboda, K. Photon upmanship: why multiphoton imaging is more than a gimmick. *Neuron* **18**, 351–357 (1997).
20. Patterson, G. H. & Piston, D. W. Photobleaching in two-photon excitation microscopy. *Biophys. J.* **78**, 2159–2162 (2000).
21. Baird, G. S., Zacharias, D. A. & Tsien, R. Y. Circular permutation and receptor insertion within green fluorescent proteins. *Proc. Natl Acad. Sci. USA* **96**, 11241–11246 (1999).
22. Nakai, J., Ohkura, M. & Imoto, K. A high signal-to-noise Ca^{2+} probe composed of a single green fluorescent protein. *Nature Biotechnol.* **19**, 137–141 (2001).
23. Nagai, T., Sawano, A., Park, E. & Miyawaki, A. Circularly permuted green fluorescent proteins engineered to sense Ca^{2+}. *Proc. Natl Acad. Sci. USA* **98**, 3197–3202 (2001).
24. Miyawaki, A. & Tsien, R. Y. Monitoring protein conformations and interactions by fluorescence resonance energy transfer between mutants of green fluorescent protein. *Methods Enzymol.* **327**, 472–500 (2000).
25. Michnick, S. W., Remy, I., Campbell-Valois, F. X., Vallee-Belisle, A. & Pelletier, J. N. Detection of protein–protein interactions by protein fragment complementation strategies. *Methods Enzymol.* **328**, 208–230 (2000).
26. Wehrman, T., Kleaveland, B., Her, J. H., Balint, R. F. & Blau, H. M. Protein–protein interactions monitored in mammalian cells via complementation of β-lactamase enzyme fragments. *Proc. Natl Acad. Sci. USA* **99**, 3469–3474 (2002).
27. Gordon, G. W., Berry, G., Liang, X. H., Levine, B. & Herman, B. Quantitative fluorescence resonance energy transfer measurements using fluorescence microscopy. *Biophys. J.* **74**, 2702–2713 (1998).
28. Sorkin, A., McClure, M., Huang, F. & Carter, R. Interaction of EGF receptor and grb2 in living cells visualized by fluorescence resonance energy transfer (FRET) microscopy. *Curr. Biol.* **10**, 1395–1398 (2000).

29. Erickson, M. G., Alseikhan, B. A., Peterson, B. Z. & Yue, D. T. Preassociation of calmodulin with voltage-gated Ca^{2+} channels revealed by FRET in single living cells. *Neuron* **31**, 973–985 (2001).
30. Wouters, F. S., Verveer, P. J. & Bastiaens, P. I. Imaging biochemistry inside cells. *Trends Cell Biol.* **11**, 203–211 (2001).
31. Elangovan, M., Day, R. N. & Periasamy, A. Nanosecond fluorescence resonance energy transfer-fluorescence lifetime imaging microscopy to localize the protein interactions in a single living cell. *J. Microsc.* **205**, 3–14 (2002).
32. Medina, M. A. & Schwille, P. Fluorescence correlation spectroscopy for the detection and study of single molecules in biology. *Bioessays* **24**, 758–764 (2002).
33. Moan, J. On the diffusion length of singlet oxygen in cells and tissues. *J. Photochem. Photobiol. B* **6**, 343–347 (1990).
34. Ullman, E. F. *et al.* Luminescent oxygen channeling assay (LOCI™): sensitive, broadly applicable homogeneous immunoassay method. *Clin. Chem.* **42**, 1518–1526 (1996).
35. Bertrand, E. *et al.* Localization of ASH1 mRNA particles in living yeast. *Mol. Cell* **2**, 437–445 (1998).
36. Baugh, C., Grate, D. & Wilson, C. 2.8 Å crystal structure of the malachite green aptamer. *J. Mol. Biol.* **301**, 117–128 (2000).
37. Holeman, L. A., Robinson, S. L., Szostak, J. W. & Wilson, C. Isolation and characterization of fluorophore-binding RNA aptamers. *Fold. Des.* **3**, 423–431 (1998).
38. Contag, C. H. & Bachmann, M. H. Advances in *in vivo* bioluminescence imaging of gene expression. *Annu. Rev. Biomed. Eng.* **4**, 235–260 (2002).
39. Gambhir, S. S. Molecular imaging of cancer with positron emission tomography. *Nature Rev. Cancer* **2**, 683–693 (2002).
40. Louie, A. Y. *et al. In vivo* visualization of gene expression using magnetic resonance imaging. *Nature Biotechnol.* **18**, 321–325 (2000).
41. Schuller, D. The biomineralization of magnetosomes in *Magnetospirillum gryphiswaldense*. *Int. Microbiol.* **5**, 209–214 (2002).
42. Douglas, R. H., Mullineaux, C. W. & Partridge, J. C. Long-wave sensitivity in deep-sea stomiid dragonfish with far-red bioluminescence: evidence for a dietary origin of the chlorophyll-derived retinal photosensitizer of *Malacosteus niger*. *Phil. Trans. R. Soc. Lond. B* **355**, 1269–1272 (2000).
43. Campbell, A. K. & Herring, P. J. A novel red fluorescent protein from the deep-sea luminous fish *Malacosteus niger*. *Comp. Biochem. Physiol.* **86B**, 411–417 (1987).
44. Wildt, S. & Deuschle, U. *cobA*, a red fluorescent transcriptional reporter for *Escherichia coli*, yeast, and mammalian cells. *Nature Biotechnol.* **17**, 1175–1178 (1999).
45. Ntziachristos, V., Bremer, C. & Weissleder, R. Fluorescence imaging with near-infrared light: new technological advances that enable *in vivo* molecular imaging. *Eur. Radiol.* **13**, 195–208 (2003).
46. Tadrous, P. J. Methods for imaging the structure and function of living tissues and cells: 1. Optical coherence tomography. *J. Pathol.* **191**, 115–119 (2000).
47. Land, M. F. The physics and biology of animal reflectors. *Prog. Biophys. Mol. Biol.* **24**, 75–106 (1972).
48. Roberts, D. G. W., Lamb, M. R. & Dieckmann, C. L. Characterization of the EYE2 gene required for eyespot assembly in *Chlamydomonas reinhardtii*. *Genetics* **158**, 1037–1049 (2001).
49. Paulmurugan, R., Umezawa, Y. & Gambhir, S. S. Noninvasive imaging of protein–protein interactions in living subjects by using reporter protein complementation and reconstitution strategies. *Proc. Natl Acad. Sci. USA* **99**, 15608–15613 (2002).
50. Tsai, P. S. *et al.* All-optical histology using ultrashort laser pulses. *Neuron* **39**, 27–41 (2003).
51. Wahlfors, J., Loimas, S., Pasanen, T. & Hakkarainen, T. Green fluorescent protein (GFP) fusion constructs in gene therapy research. *Histochem. Cell Biol.* **115**, 59–65 (2001).
52. Boon, K. *et al.* An anatomy of normal and malignant gene expression. *Proc. Natl Acad. Sci. USA* **99**, 11287–11292 (2002).

Online links

DATABASES

The following terms in this article are linked online to:

Entrez: http://www.ncbi.nlm.nih.gov/Entrez/
horseradish peroxidase
Swiss-Prot: http://www.expasy.ch/
β-galactosidase | GFP | luciferase | MS2 | thymidine kinase

FURTHER INFORMATION

National Center for Imaging and Microscopy Research:
http://ncmir.ucsd.edu
Photoconversion example:
http://ncmir.ucsd.edu/biology/photoconversion
Roger Tsien's laboratory: http://tsienlab.ucsd.edu
Access to this interactive links box is free online.