SCALING DOWN IMAGING: MOLECULAR MAPPING OF CANCER IN MICE

Ralph Weissleder

The development of miniaturized imaging equipment and reporter probes has improved our ability to study animal models of disease, such as transgenic and knockout mice. These technologies can now be used to continuously monitor *in vivo* tumour development, the effects of therapeutics on individual populations of cells, or even specific molecules. If these techniques prove effective in mice, they might be translated into the clinic in the future, where they could be used to non-invasively detect and monitor treatment of human cancers.

MAGNETIC RESONANCE IMAGING (MRI). A powerful diagnostic imaging method that uses radiowaves in the presence of a magnetic field to extract information from certain atomic nuclei (most commonly hydrogen). It is primarily used for producing anatomical images, but also gives information on: the physico-chemical state of tissues, flow, diffusion, motion and, more recently, molecular targets.

Harvard Medical School, Center for Molecular Imaging Research, Massachusetts General Hospital, Boston, Massachusetts 02129, USA.
e-mail: weissleder@helix.mgh.harvard.edu
DOI: 10.1038/nrc701

For many years, physicians and researchers could only detect tumours or observe their growth through surgery or biopsy. Despite recent advances in cancer research techniques, we are still limited in our ability to detect tumours in their earliest stage of formation, monitor tumour phenotype, quantify invasion or metastasis, or visualize the real-time *in vivo* activity of anticancer therapeutics. Techniques such as MAGNETIC RESONANCE IMAGING (MRI), X-RAY COMPUTED TOMOGRAPHY (CT) nuclear imaging, optical imaging and ULTRASOUND are likely to revolutionize the way we detect and monitor cancer over the next decade. These technologies, coupled with recently developed molecular probes designed to recognize tumour-specific markers, can already be used to visualize and quantify tumour growth or regression during therapy.

Many of these non-invasive technologies were originally developed for human use, but have recently been scaled down to allow high-resolution imaging of mice. This is important, because as the genomic era provides us with better animal models of cancer and more specific therapeutics, better methods are needed to monitor tumour development in animals. Mouse imaging technologies now range from a basic CHARGE-COUPLED DEVICE (CCD) camera for optical detection to highly sophisticated equipment capable of obtaining three-dimensional reconstructions of internal organs and tissues. The widespread availability and utility of miniaturized mouse-imaging systems are not as far off as many researchers might think — these systems are generally cheaper than their clinical counterparts and can be housed in basic science laboratories. In parallel, mouse models and imaging technologies will allow the continued development of new generations of injectable targeted and 'SMART' REPORTER PROBES that allow us to monitor tumours *in vivo*. Most importantly, these technologies will facilitate analysis of the numerous mouse models of cancer available, and will allow us to answer important questions about tumorigenesis, host–tumour interactions and the specific effects of pharmacological interventions.

Approaches to *in vivo* imaging

Imaging technologies allow for non-invasive visualization of the body based on different forms of energy interacting with tissues. They can be used to detect both primary and metastatic tumours, as well as to monitor cancer-associated physiological events, such as changes in blood volume or perfusion. Imaging can also be used to track tumour-specific molecular markers. Some imaging methods (MRI and CT) rely purely on energy–tissue interactions, whereas others (SINGLE PHOTON EMISSION TOMOGRAPHY (SPECT) and POSITRON EMISSION TOMOGRAPHY (PET)) require the administration of reporter probes or

Summary

- Traditional approaches of monitoring cancer growth in mouse models have largely used surface-implanted tumours and caliper measurements.
- A variety of non-invasive high-resolution imaging methods are now available for the detection and monitoring of deep-seated cancers, as well as their metastases, in transgenic models.
- Magnetic resonance imaging (MRI) and computed tomography (CT) are primarily used to display internal anatomy of mouse models and are useful tools for phenotyping.
- The availability of radioisotope, magnetic and/or fluorescent tags is continuously improving our ability to image specific molecular markers *in vivo*.
- The use of 'smart' sensors for MRI and optical imaging holds particular promise in sensing and reporting molecular signatures.
- Technologies are being developed that will ultimately allow cellular protein and signal-pathway profiling. This will further extend our understanding of the molecular pathology of cancer, speed up drug development and can lead to patient-tailored therapies.

contrast agents. Reporter probes are to *in vivo* imaging what stains are to histology — markers of a particular biological process. Imaging reporters can tell us what is happening at both the physiological and the molecular level. Typically, the probes consist of a label that is detectable by a given imaging technique (TABLE 1) and a targeting component. The targeting component might be a small molecule, peptide, enzyme substrate or antibody. More recently, 'smart probes' have been developed that can be activated and detected only when they interact with the target. These agents can significantly improve *in vivo* signal-to-noise ratios (BOX 1).

In the simplest image-acquisition mode, a 'picture' is acquired, essentially collapsing the volume of an animal or tumour into a single image (TABLE 2). This type of imaging, known as 'planar imaging', is generally fast, the data sets are small and imaging can be done in high-throughput fashion — at the expense of internal resolution. 'Tomographic imaging' allows slices of the subject to be obtained. Each slice has a certain thickness (z) that is usually thicker than the volume elements, or VOXELS, in the other two dimensions (x, y). Internal resolution refers to the size of these voxels, which can be as small as tens of µm. Tomographic methods are usually quantitative and capable of displaying internal anatomic structures, such as tumours. 'Volumetric image acquisition' methods show a volume of interest

X-RAY COMPUTED TOMOGRAPHY
(CT). As generated X-rays pass through different types of tissue, they are deflected or absorbed to different degrees. CT uses X-rays to obtain three-dimensional images by rotating an X-ray source around the subject and measuring the intensity of transmitted X-rays from different angles.

ULTRASOUND IMAGING
Acoustic waves with high frequencies (usually > 10 MHz) are used to generate images based on acoustic echoes.

CHARGE-COUPLED DEVICE
(CCD). Instrument containing semiconductors that are connected so that the output of one serves as the input of the next. Sensitive digital cameras used for image acquisition (for example, in CT, fluorescence and bioluminescence imaging) all use CCD arrays.

'SMART' REPORTER PROBE
Molecular probe that changes its physicochemical properties after target interaction. Occasionally referred to as a sensor, beacon or activatable probe.

SINGLE PHOTON EMISSION TOMOGRAPHY
(SPECT). The rotation of a photon detector array around the body to acquire data from many angles following the injection of a γ-emitting radionuclide (isotope decaying with γ-ray emission of 30–300 KeV energy).

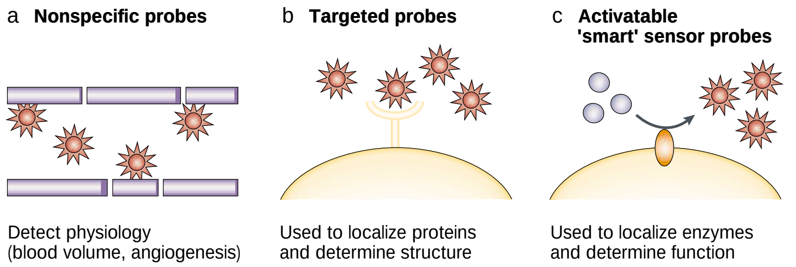
Table 1 | Overview of mouse imaging systems

Technique	Resolution	Depth	Time	Imaging agents	Cost	Primary use
Magnetic resonance imaging (MRI)	10–100 µm	No limit	Min/hours	Gadolinium, dysprosium, iron oxide particles	$$$	Best all-round imaging system with high contrast; used in phenotyping, physiological imaging and cell tracking
X-ray computed tomography (CT) imaging	50 µm	No limit	Min	Iodine	$$	Lung and bone-tumour imaging
Ultrasound imaging	50 µm	mm	Min	Microbubbles	$$	Vascular and interventional imaging
Positron emission tomography (PET) imaging	1–2 mm	No limit	Min	^{18}F, ^{11}C, ^{15}O	$$$	Imaging metabolism of molecules, such as glucose, thymidine and so on
Single photon emission tomography (SPECT) imaging	1–2 mm	No limit	Min	^{99m}Tc, ^{111}In	$$	Imaging of probes such as antibodies, peptides and so on
Fluorescence reflectance imaging (FRI)	1–2 mm	< 1 cm	Sec/min	Fluorescent proteins NIR fluorochromes	$	Rapid screening of molecular events in surface-based tumours
Fluorescence-mediated tomography (FMT)	1–2 mm	< 10 cm	Sec/min	NIR fluorochromes	$$	Quantitative imaging of targeted or 'smart' fluorochrome reporters in deep tumours
Bioluminescence imaging (BLI)	Several mm	cm	Min	Luciferin	$$	Gene expression, cell tracking
Intravital microscopy (confocal, multiphoton)	1 µm	< 400 µm	Sec/min	Fluorescent proteins, photoproteins, fluorochromes	$$$	All of the above at higher resolutions but at limited depths and coverage

Cost of system: $ < 100 K; $$ 100–300 K; $$$: > 300 K. C, carbon; F, fluorine; In, indium; NIR, near infrared; O, oxygen; ^{99m}Tc, technetium metastable.

Box 1 | Imaging probes

Probes have been developed to enhance all *in vivo* imaging techniques, and can be used to selectively highlight internal organs, tumours or molecular processes. **a** | The most commonly used imaging agents simply have compartmental distributions and can be used to image physiological processes such as changes in blood volume, perfusion and blood flow in angiogenesis. With many of these agents, the compartmental distribution changes over time so that fast imaging might be required. **b** | Imaging agents such as indocyanine green or fluorochromes can be coupled to molecules such as antibodies or proteins (for example, somatostatin), and targeted specifically to tumour cells. Depending on the nature of the imaging probe, background noise can be fairly high. **c** | 'Smart' sensors reduce signal-to-noise ratios because they can only be detected once they have interacted with their substrate. These agents, primarily developed for optical and magnetic resonance imaging, are relatively nondetectable in their native injected state. Examples of such agents include quenched near-infrared fluorochromes that can be activated by proteases such as matrix metalloprotease 2 (MMP-2), cathepsins, caspases or other enzymes[57,58]. Other examples include magnetic nanosensors that can interact with DNA or RNA sequences[16], activatable paramagnetic chelators[68,69] and paramagnetic substrates that are polymerized by peroxidases or tyrosinases[14].



(such as an organ or the tumour) in all three dimensions and result in the highest spatial information content. These images can be displayed in any plane or along any trajectory through the animal. Volumetric data sets can be enormous, resulting in up to hundreds of images per animal and often requiring special resources for data storage and image reconstruction. So what are the different imaging modalities that have been adapted for use in mice, and what types of study are each best-suited to?

Magnetic resonance imaging. The fundamental principle underlying MRI is that unpaired nuclear spins (such as hydrogen atoms in water and organic compounds) align themselves when placed into a magnetic field. A temporary radiofrequency pulse is then given to change the alignment of the spins, and their return to baseline is recorded as a change in electromagnetic flux. The timing parameters of pulse excitation and recording can be altered, resulting in images with different types of magnetic contrast. The two most frequently used timing parameters are known as T1 and T2 weighting (BOX 2). Paramagnetic metal cations such as chelated gadolinium or dysprosium, or SUPERPARAMAGNETIC NANOPARTICLES, can be used either as compartmental, targeted or smart probes (BOX 1) with this technique (TABLE 1).

High-resolution MRI is one of the most useful techniques for screening transgenic mice for tumours and other anatomical abnormalities. It can be used to visualize tumour size, location, metastatic burden and phenotype. These applications are particularly important as tumours frequently occur only in subsets of transgenic animals and at different stages of their lives[1–3]. More recently, techniques have been developed to measure angiogenic properties of tumours, and MRI is slowly replacing more invasive and usually terminal histological procedures that involve meticulous analysis of microvessel density. MRI analysis can be used to quantify vascular volume, capillary permeability, flow and several other vascular parameters[4–11], even in very deep tumours (BOX 2).

MRI also has considerable potential for imaging at the molecular level. A technique called MAGNETIC RESONANCE (MR) SPECTROSCOPY can be used to image metabolic activity in tumours[12]. For example, this technique has been used to detect lowered citrate and increased choline levels, which are markers of tumour aggressiveness in prostate cancers[12]. MR spectroscopy can detect individual molecules using magnetically labelled affinity molecules. Using an antibody-coupled paramagnetic liposome preparation that targets the endothelial integrin αVβ3, researchers have been able to obtain images of angiogenic vasculature in rabbit carcinomas[13]. MRI has also reportedly been used to detect expression of endothelial-cell-specific proteins such as E-selectin[14] and tumour-specific markers such as receptors and transgene products[15] (BOX 2).

Magnetic nanosensors have been developed that are capable of detecting certain DNA or mRNA sequences[16]. Superparamagnetic nanoparticles exert sensitive and reversible effects on spin–spin relaxation of adjacent water protons following hybridization of these probes to mRNA[16]. These magnetic nanosensors might be used with MRI to determine the phenotype of tumours *in vivo* or for rapid analysis of non-purified tumour samples.

A specific population of cells can also be magnetically labelled and followed using MRI. Magnetic nanoparticles have been used in this way to track and recover as few as 10–100 cells[17] *in vivo* (BOX 2). This technique might be useful in stem-cell-based therapies, which require long-term *in vivo* tracking of specific cell populations.

X-ray computed tomography. X-ray CT measures the absorption of X-rays as they pass through tissues. Intrinsic differences in absorption between bone, fat, air and water result in high-contrast images of anatomical structures. Unlike MRI, however, CT has relatively poor soft-tissue contrast and cannot detect differences between tumours and surrounding tissue. For this reason, iodinated contrast agents, which perfuse different tissue types at different rates, are commonly used to delineate organs and tumours.

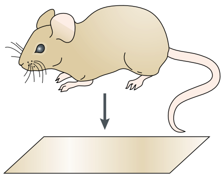
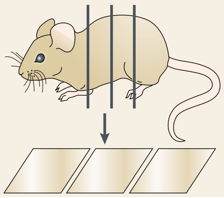
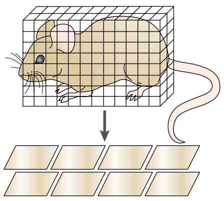
High-resolution CT imaging is possible in mice and has been primarily used to detect lung tumours[18] (FIG. 1) and to image bone metastases[19]. Similar to clinical systems, the source and detector rotate around the animal, acquiring volumetric data. But unlike clinical systems, most mouse CT images are collected with

POSITRON EMISSION TOMOGRAPHY
(PET). Tomographic imaging technique detects nuclides as they decay by positron emission. The emitted positrons collide with a free electron, resulting in the conversion of matter to two γ-rays, which emerge in opposite directions.

VOXEL
Also known as 'volume pixel element', it is the smallest distinguishable piece of a three-dimensional image. Clinical magnetic resonance images, for example, often consist of 256 × 256 or 512 × 512 voxel matrices.

SUPERPARAMAGNETIC NANOPARTICLES
Magnetic particles with dimensions in the order of tens of nanometres that are used as reporters in magnetic resonance imaging. Superparamagnetism refers to the fact that the particles do not have magnetic properties outside a magnetic field (unlike ferromagnets).

Table 2 | Planar, tomographic and volumetric image acquisition

Image analysis	Data size	Examples	Advantage	Disadvantage
Planar/projectional 	0.1–1 MB	X-ray, BLI, FRI, planar nuclear imaging	Simple, fast, high throughout	No internal anatomy
Tomographic slices 	1–10 MB	MRI, CT, SPECT, PET, FMT	Internal anatomy and function, quantitative	Limited resolution with some techniques
Volumetric 	100s of MB	MRI, CT, FMT	High resolution, reconstruction in any plane	Very large data sets, time consuming

BLI, bioluminescence imaging; CT, computed tomography; FMT, fluorescence-mediated tomography; FRI, fluorescence reflectance imaging; MB, megabytes; MRI, magnetic resonance imaging; PET, positron emission tomography; SPECT, single photon emission tomography.

MR SPECTROSCOPY
(MRS). Nuclear magnetic resonance spectroscopy method used to detect the concentration of specific metabolites in a particular region of a tumour or organ. Individual compounds or ratios might be predictive of response to radiation and chemotherapy.

CYCLOTRON
Device used to produce positron-emitting radionuclides for PET imaging by bombarding non-radioactive target atoms with nuclei (for example, protons or deuterons) that are accelerated to high energies.

LEAD COLLIMATORS
Perforated sheets of lead that are used to focus X-rays or γ-rays. Collimators are crucial elements that determine the sensitivity, resolution and contrast of obtained images.

FLUORESCENCE REFLECTANCE IMAGING
(FRI). Simple method of image acquisition similar to fluorescence microscopy, except that different optics allow image acquisition of whole animals. Mostly suited for surface tumours or surgically exposed tumours.

high-resolution phosphor screen/CCD detectors and a low-energy X-ray source (30–50 KVp[20,21]) to optimize image quality.

Although MRI is still the preferred technique for tumour screening, CT imaging might be preferable in certain situations — such as for lung and bone tumours — given its faster imaging times and higher spatial resolutions. For example, scanning an entire animal at 50 μm voxels takes only 10–15 minutes. High-throughput techniques such as CT are also a prerequisite for phenotyping large numbers of transgenic mice and detecting macroscopic abnormalities. The radiation dose, however, is not negligible (0.6 Gy per scan; 5% of LD_{50} for mice) and this can limit repeated imaging of the same animal.

Ultrasound. Ultrasound imaging involves scanning tissues with ultrasound waves (20–60 MHz; 2–10 MHz in humans) and detecting their reflections. Ultrasound is a quick, inexpensive and portable procedure, and images of up to 40 μm in resolution can be obtained within 5 mm of the detector[22]. In both mice and humans, the technique is most useful for rapid screening of pathologies such as bladder tumours (which are surrounded by sound-wave-transmitting urine[23]), measuring macroscopic blood flow[24–26], and imaging developing embryos[27]. In animals, it is also used to monitor interventional procedures such as *in utero* transgene or cell implantations[28]. Some of the limitations of ultrasound imaging include the presence of air and bone artefacts (which do not transmit sound waves) and limited depth penetration at the higher frequencies required to resolve the small organs of mice. Ultrasound imaging of mice is still not as widely used as MRI or CT.

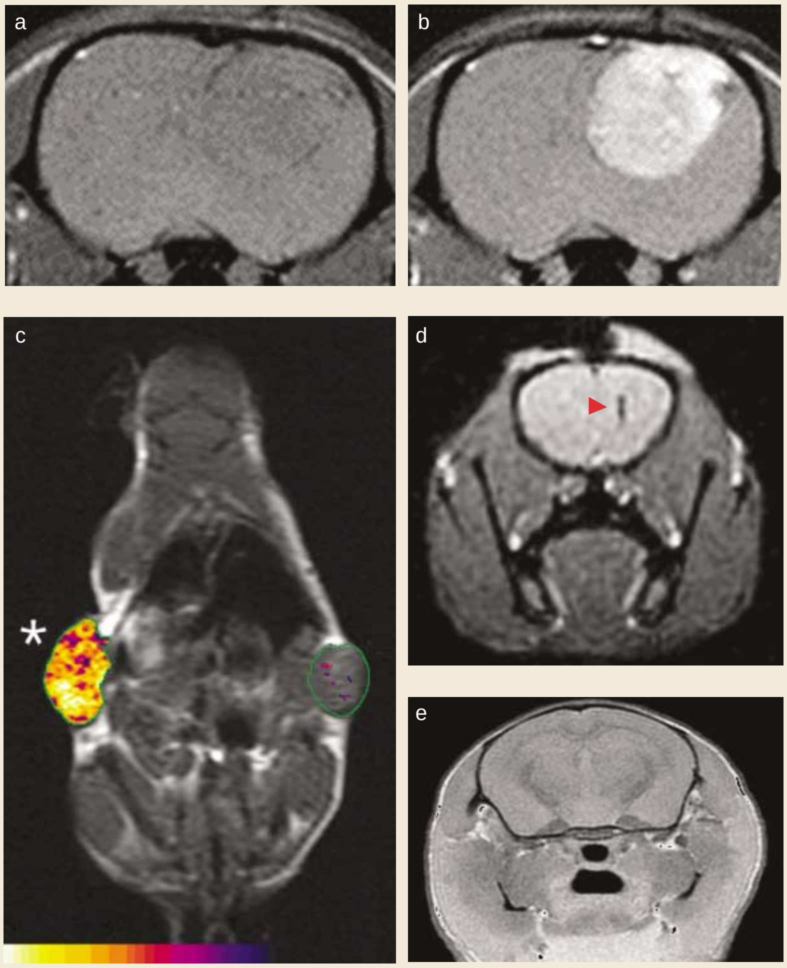
Positron emission tomography. Positron emission tomography (PET) is an imaging technique used to detect decaying nuclides such as ^{15}O, ^{13}N, ^{11}C, ^{18}F, ^{124}I and ^{94m}Tc (technetium). After these are administered to the subject, they begin to decay, emitting positrons that collide with a free electron. The interaction of the two subatomic particles results in the conversion of matter into energy in the form of two γ-rays emerging in opposite directions. These γ-rays are detected by an array of detectors that surround the subject. Positron-emitting radionuclides are primarily used to tag small molecules that are recognized by enzymes or bind to receptors and other targets (TABLE 1). For example, ^{18}F-labelled glycopeptides that contain the integrin-binding sequence Arg–Gly–Asp (RGD) have been used to image αVβ3 integrin expression by mouse tumours[29]. ^{18}F-labelled thymidine has been used to measure tumour proliferation in dogs and humans[30], and ^{18}F fluorodeoxyglucose is often used to detect hypermetabolic cancers in humans.

Many oncological studies make conclusions based solely on visual analysis of PET images, but the principal strength of PET is that quantitative kinetic data can be acquired repetitively — at least in human studies[31]. In mice, PET can be used to monitor expression of extracellular receptors[32,33] or efficacy of gene-therapy vectors[34,35]. The herpes simplex virus thymidine kinase gene, when expressed in mammalian cells, converts PET substrates into a detectable form and can be used as a marker of gene-transfer efficiency[34,36–38]. Recently, a number of sophisticated viral and non-viral gene-therapy vectors have been developed that can be imaged by PET[39]. Some of these constructs include promoters that allow for cell-specific gene expression or imaging of transcriptional regulation[40,41]. PET, however, is a costly approach to analysing mouse models. In addition to a dedicated mouse PET scanner, a CYCLOTRON and radiochemistry facility are required to use this technology efficiently and on a regular basis.

Single photon emission tomography imaging. Single photon emission tomography (SPECT) — the detection and quantification of gamma-emitting radionuclides — is commonly used in experimental oncology to track individual molecules or cells. In this approach, the molecule or cell of interest is labelled with a gamma-ray-emitting nuclide such as ^{99m}Tc, ^{111}In, ^{123}I or ^{125}I, injected into an animal and followed using sodium iodide gamma cameras or solid-state cadmium–zinc telluride (CZT) detectors[42]. SPECT has been used to detect radiolabelled annexin-V, to image programmed cell death[43], and to localize tumour-specific peptides *in vivo*[44]. Radiolabelling of DNA allows

Box 2 | Mouse magnetic resonance imaging

Magnetic resonance imaging (MRI) is a versatile imaging tool with high soft-tissue contrast and can be used to extract anatomical, physiological and molecular information from tumours. **a** | A glioma was implanted into the cerebral hemisphere of a mouse and imaged using native T1-weighted MRI. **b** | 24 hours after intravenous administration of superparamagnetic nanoparticles the tumour appears bright because of slow extravasation and accumulation of particles. **c** | *In vivo* detection of a transferrin-receptor-expressing tumour using MRI. A superparamagnetic reporter probe conjugated to transferrin detects tumour cells that transgenically express transferring receptor (left, marked by *). The control tumour on the right does not express transferrin[15]. Superparamagnetic imaging probes such as the one used in this example are being developed for tumour detection using a variety of other receptors, such as the bombesin and folate receptors. **d** | MR image of magnetically labelled progenitor cells implanted into the cerebral hemisphere as an antitumour-cell-based therapy. A total of 1,000 cells have been implanted (arrowhead) and imaging is carried out using T2-weighted spin-echo sequence using a clinical imaging system. **e** | Although dedicated mouse MRI systems operating at high field strengths are becoming more widely used, there are many more clinical imaging systems (operating at lower filed strengths) that could be adapted to high-resolution mouse imaging. This image shows a T2-weighted image of mouse brain obtained at 1.5 T using a clinical imaging system adapted with dedicated high-strength gradient coil inserts, resulting in spatial resolutions of 60 µm voxel sizes. (Image in **a** courtesy of B. Ross, University of Michigan, Michigan, USA; image in **d** courtesy of D. Hoegeman, MGH-CMIR, Boston, USA; image in **e** courtesy of B. Rutt, Imaging Research Laboratories, Robarts Research institute, London, Canada.)



therapeutic gene-delivery vectors[45] and antisense oligonucleotides to be monitored[46]. SPECT has also been used to follow the distribution of radioiodinated ERBB2-specific antibodies[45], receptor-binding molecules[47,48], hormones and metabolites[49], small molecules[50], lymphocytes[51] and herpesviruses[52].

Nuclides differ in their decay time (hours to days), energy and chemistry of attachment, so different labels are used for different applications. Spatial information is primarily obtained using LEAD COLLIMATORS, placed between the animal and the detectors, to absorb scattered gamma rays. By acquiring numerous projections using rotating detectors, researchers can tomographically reconstruct the distribution of the tracer within an animal. The spatial resolution with most mouse imaging systems is in the order of 1–2 mm. Gamma-emitting nuclides are inexpensive, readily available and their longer half-life obviates the need for a cyclotron.

Fluorescence imaging. One of the most inexpensive and rapid ways to image a particular molecule or cell in mice is through fluorescence imaging. In this technique, light of one wavelength (excitation) illuminates the specimen, resulting in a shifted (emission) wavelength that is collected by ultrasensitive CCD cameras[53]. Normal and cancer cells can be followed in this manner, labelled using a variety of techniques, including fluorescently labelled antibodies or inducible expression of green fluorescent protein (GFP). GFP-expressing metastatic peritoneal tumour nodules of 150 µm in diameter (about 1,000–10,000 cells) have been detected in mice[54]. One of the drawbacks of this technique, however, is that the tumour-bearing regions must often be exposed surgically (FIG. 2). Only occasionally can GFP-expressing tumours be seen through the abdominal wall or through organs — even in mice.

Tissue penetrance of photons is highly dependent on the wavelength of light used. Imaging in the near-infrared (NIR) spectrum (700–900 nm) maximizes tissue penetration as well as minimizing autofluorescence from non-target tissue. A number of NIR fluorochromes have recently become available that can be coupled to affinity molecules such as peptides[55] or antibodies[56]. Activatable NIR-imaging agents (BOX 1) have also been developed to measure enzymatic activities[57,58]. For example, injectable NIR-labelled probes have been used to measure enzyme activity of proteases such as cathepsin B[57] and cathepsin D[59]. In one study, tumour-bearing mice were treated with a model matrix metalloproteinase (MMP) inhibitor, which had been reported to slow tumour progression *in vivo*[60]. Using these reporters, the authors showed a clear reduction in tumour MMP activity within hours to days of treatment[58]. This study indicates that molecular targets can be used as biomarkers to measure early drug efficacy[58]. Although NIR FLUORESCENCE REFLECTANCE IMAGING is easy to use, it is still limited to situations in which the target is located relatively close to the illuminated surface, such as dysplastic adenomatous colonic polyps viewed endoscopically[61]. For deeper targets, FLUORESCENCE-MEDIATED TOMOGRAPHY (FMT) is the imaging method of choice.

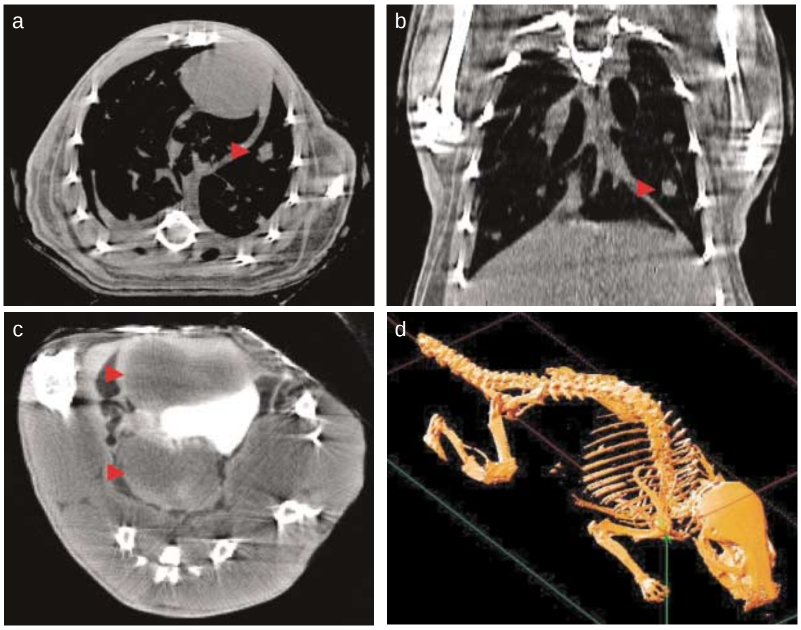


Figure 1 | **Mouse computed tomography imaging.** High-resolution computed tomography (CT) of a mouse showing **a, b** | metastatic lung tumours (arrowhead), and **c** | prostate tumours (arrowheads). **d** | The skeleton can be reconstructed in detail through segmenting and volume rendering. This technique is being developed to detect metastatatic bone tumours. (Parts **a** and **b** reproduced with permission from REF. 18 © (2000) Macmillan Magazines Ltd. Parts **c** and **d** are courtesy of M. Paulus, Oakridge National Labs, Tennessee, USA.)

FLUORESCENCE-MEDIATED TOMOGRAPHY (FMT). Tomographic reconstruction method developed for *in vivo* imaging of fluorescent probes. Images of deep structures are mathematically reconstructed by solving diffusion equations, under the assumption that photons have been scattered many times.

Fluorescence-mediated tomographic imaging. Tomographic imaging with fluorescent light is a more advanced technique for acquiring images of fluorescently labelled objects. In FMT, a fluorescently labelled probe is injected (or expressed transgenically), and the subject is exposed to continuous wave or pulsed light from different sources. The emitted light is captured by detectors arranged in a spatially defined order in an imaging chamber. The information is then mathematically processed, resulting in a reconstructed tomographic image[62]. In mice, this technique, at present, yields images with a resolution of 1–2 mm, and the fluorochrome detection threshold is in the nanomolar range[63] (FIG. 3). Not only does this technique provide higher resolution images than those obtained with a CCD camera, but it is also inherently quantitative (V. Ntziachristos, unpublished observations).

FMT imaging is useful in the laboratory because it can be used to quantify protein expression or localization *in vivo* without radioactive labelling. It has the potential for becoming more widespread in use, due to the number of fluorescently labelled molecules available. The technique has been used to image and quantify the expression of cathepsin B in gliomas (FIG. 3) and to associate protease expression levels with invasiveness in breast cancer xenografts using 'smart' (activatable) fluorescent enzyme substrates (V. Ntziachristos, personal communication). This technique might also be extended to clinical use[64], as patients could be injected with fluorescently labelled affinity molecules. Tumour reactivity to these probes could be used to identify the earliest forms of breast, prostate or colon cancers.

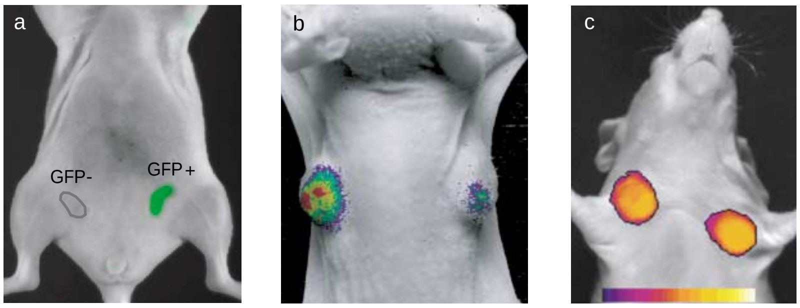


Figure 2 | **Optical imaging.** Fluorescent and bioluminescent signals emanating from superficial structures such as surface-implanted tumours can be imaged with sensitive charge-coupled device cameras. **a** | Fluorescence imaging in the visible light range (400–600 nm) can be used to detect green fluorescent protein (GFP) expressed by the right, but not by the left, tumour. **b** | Bilateral chest tumours expressing transgenic luciferase and imaged with a photon-counting camera after intraperitoneal injection of luciferin. The tumour on the left expresses higher levels of luciferase, indicated by areas of red, yellow and green colouration, than the tumour on the right. **c** | Near-infrared (NIR) fluorescence imaging (700–900 nm) can be used to image deeper tumours than can fluorescence imaging in the visible light range[53]. This example shows matrix metalloproteinase 2 (MMP-2) enzyme levels in bilaterally implanted breast tumours using an NIR fluorescence probe coupled to an MMP-2 substrate[58]. (Image in **a** courtesy of U. Mahmmod, MGH CMIR, Boston, USA; image in **b** courtesy of Y. Saeki and V. Ntziachristos, MGH CMIR, Boston, USA.)

Bioluminescence imaging. Bioluminescence imaging is used to detect the scant numbers of photons that emanate from cells that have been genetically engineered to express luciferases. Luciferases comprise a family of PHOTOPROTEINS, isolated from a variety of species, which modify their substrate (for example, luciferase from the firefly modifies luciferin, whereas luciferase from the sea pansy *Renilla reniformis* modifies coelenterazine) causing the release of a photon[65]. Luciferase can be transgenically expressed in mammalian cells, and when exposed to substrate, releases photons that can be detected and quantified using low-light photon-counting cameras. A number of genetically engineered luciferases from the firefly have been created, resulting in spectral shifts of released light photons. Using sensitive filters, these mutants can be used to track more than one protein, similar to what has been done with blue- and red-shifted GFP. Bioluminescence imaging has been used in mice to non-invasively monitor tumour-cell growth and regression[65], to visualize the kinetics of tumour-cell clearance by chemotherapeutics[64] and to track gene expression (FIG. 2).

This technique differs from fluorescence imaging in that the object to be imaged does not need to be exposed to light of an excitation wavelength. Like fluorescence imaging, however, the deeper the light source, the more photons are absorbed and scattered, making quantification difficult. One advantage of bioluminescent reporters is that there is the low level of background noise. It has been shown that as few as 1,000 luciferase-positive tumour cells can be detected within the peritoneal cavity[66] (FIG. 2b). Bioluminescence imaging in mice is fast, relatively inexpensive and represents an alternative to the expensive process of PET imaging in mice, at least for imaging reporter genes. Although bioluminescence imaging is a valuable technique for studying cancer in

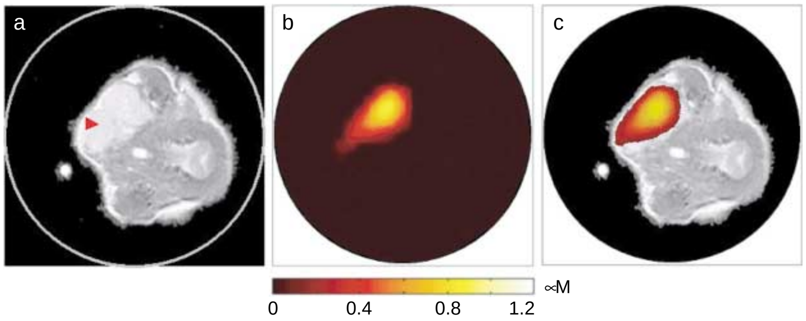


Figure 3 | **Fluorescence-mediated tomography.** Combined magnetic resonance (MR) and fluorescence-mediated tomography (FMT) imaging of a tumour implanted into the upper chest region of a mouse. **a** | A T2-weighted MR image shows the tumour as a bright structure (arrowhead). **b** | FMT imaging of the same tumour. The mouse was injected intravenously with a 'smart' probe that is activated by cathepsin B. Note that the tomographic fluorescence reconstruction is quantitative and so correlates with enzyme levels[62]. **c** | Superimposed fusion image, showing cathepsin B activity within the tumour. (Images courtesy of V. Ntziachristos, MGH-CMIR, Boston, USA.)

mice, it is not likely to be used in a clinical setting, because it can only monitor transgenically modified tumour cells and because it is difficult to detect photons released from deep tissues.

Challenges in mouse imaging research

Despite the phenomenal advances in adapting imaging technologies to mice, significant challenges remain to be overcome. These include a number of engineering and biological obstacles.

Chemistry. One of the main challenges for imaging is to select relevant, accessible and specific imaging targets. The completion of the mouse and human genome projects — along with technological advances such as microarray analysis, high-throughput and combinatorial chemistries — will speed the identification of tumour-specific markers. Therapeutic drug discovery has already been streamlined by these techniques, but imaging targets are still primarily discovered by trial-and-error or by chance. Imaging researchers must begin to make the most of these accepted and validated platform technologies to test larger numbers of small molecules and combinatorial libraries[67]. Improved approaches to deliver imaging reporters to target cells and to specific intracellular compartments are also needed[17].

Image acquisition. One of the reasons why clinical images can be of spectacular clarity is that images are made of large voxels, often up to several mm^3, to provide high signal-to-noise ratios. Decreasing the voxel size in mouse imaging reduces the signal obtained, per unit time. So, to image smaller and smaller voxels, new techniques must be developed to counteract this loss. This might be achieved in MRI by building higher efficiency coils or imaging at higher field strengths. Resolution might be improved in CT by using smaller focal spots with more X-ray photons. In nuclear imaging, resolution can be improved by increasing detector efficiency, the quantum yield or by using cooled CCDs to decrease background noise. Microfabrication techniques are also expected to result in improved minituarized imaging systems.

Data processing. Another challenge resulting from very high spatial resolution imaging is data analysis. The amount of memory it takes to reconstruct and store three-dimensional volumetric data sets can be staggering. For example, imaging a single mouse at 100 μm isotropic voxels results in 600 axial images and about 75 MB of data. Screening of 50 mice for a phenotypic abnormality (such as a brain tumour) during 1 day of high-throughput MRI typically results in 2,500 images for analysis. Scientists who use this type of analysis also spend a great deal of time performing THREE-DIMENSIONAL MAPPING, quantitative morphometrics and MULTIMODAL DATA FUSION, and sharing this data places a significant burden on intranet servers. Fortunately, most clinical imaging departments have learned to cope with similar demands, and software development is expeditious. However, considerable advances must be made before we will be able to analyse data from large-scale screening and high-throughput imaging experiments. The development of automated systems will allow the tasks to be performed off-line.

Outlook

Mouse imaging has become a reality, despite the enormous engineering challenges. In the near future, many high-resolution, fast imaging systems will become commercially available for mice, and miniaturized imaging technologies will begin to appear in basic science laboratories. But as we develop newer and more sophisticated imaging technologies, we must remember that the ultimate goal is to translate this technology back into clinical use. The clinical needs for more specific molecular imaging tools are obvious. The ability to non-invasively image cancer-associated molecular markers will ultimately allow earlier detection and phenotyping of tumours, and give us an opportunity to tailor therapies to individual patients[58]. Molecular imaging methods might also accelerate drug development by providing molecular biomarkers as objective end points of treatment efficacy.

Although several of these developments will occur in PET, SPECT, MR and CT imaging, the largest advances are likely to occur in optical imaging technologies that involve injectable fluorescent probes and photoproteins. NIR light can penetrate tissues of 10 cm; fluorescence emission can be easily quantified; and a growing number of near-infrared fluorochromes have recently become available. Furthermore, NIR microscopy does not require radiation; MULTI-WAVELENGTH IMAGING is feasible; and the method can be combined with traditional anatomical imaging modalities. As fluorescent technologies have changed biotechnology, they could also change *in vivo* imaging and provide more sophisticated methods for imaging molecular events — both in mice and in humans.

PHOTOPROTEIN
Class of proteins that give off light following oxidation or oxidative conversion of a substrate such as luciferin. Usually also requires ATP, oxygen and catalysts.

THREE-DIMENSIONAL MAPPING
Segmenting and volume reconstruction of specific targets, disease processes or organs after image acquisition. Mapping aids in visualizing complex information of tens to hundreds of images.

MULTIMODAL DATA FUSION
Fusion of anatomical (for example, derived from CT or MRI) and physiological or molecular (for example, derived from nuclear or optical imaging) images, into a new synthetic image.

MULTI-WAVELENGTH IMAGING
Fluorescence or absorption imaging at different, non-overlapping wavelengths (also referred to as multicolour imaging). Allows the imaging of multiple targets simultaneously.

1. DePinho, R. A. The age of cancer. *Nature* **408**, 248–254 (2000).
2. Attardi, L. D. & Jacks, T. The role of p53 in tumour suppression: lessons from mouse models. *Cell. Mol. Life Sci.* **55**, 48–63 (1999).
3. Artandi, S. *et al.* Telomere dysfunction promotes non-reciprocal translocations and epithelial cancers in mice. *Nature* **406**, 641–645 (2000).
4. Gillies, R. *et al*. Applications of magnetic resonance in model systems: tumor biology and physiology. *Neoplasia* **2**, 139–151 (2000).
5. Lewin, M. *et al. In vivo* assessment of vascular endothelial growth factor-induced angiogenesis. *Int. J. Cancer* **83**, 798–802 (1999).
6. Taylor, J. S. *et al.* MR imaging of tumor microcirculation: promise for the new millennium. *J. Magn. Reson. Imaging* **10**, 903–907 (1999).
7. Weissleder, R., Cheng, H., Marecos, E., Kwong, K. & Bogdanov, A. Mapping of tumor vascular and interstitial volume fractions non-invasively *in vivo*. *Eur. J. Cancer* **34**, 1448–1454 (1998).
8. Mandeville, J. B. *et al.* Dynamic functional imaging of relative cerebral blood volume during rat forepaw stimulation. *Magn. Reson. Med*. **39**, 615–624 (1998).
9. Neeman, M., Dafni, H., Bukhari, O., Braun, R. D. & Dewhirst, M. W. *In vivo* BOLD contrast MRI mapping of subcutaneous vascular function and maturation: validation by intravital microscopy. *Magn. Reson. Med.* **45**, 887–898 (2001).
10. Brasch, R. *et al.* Assessing tumor angiogenesis using macromolecular MR imaging contrast media. *J. Magn. Reson. Imaging* **7**, 68–74 (1997).
11. Dennie, J. *et al.* NMR imaging of changes in vascular morphology due to tumor angiogenesis. *Magn. Reson. Med.* **40**, 793–799 (1998).
12. Koutcher, J. A., Zakian, K. & Hricak, H. Magnetic resonance spectroscopic studies of the prostate. *Mol. Urol.* **4**, 143–152; discussion 153 (2000).
13. Sipkins, D. A. *et al.* Detection of tumor angiogenesis *in vivo* by αVβ3-targeted magnetic resonance imaging. *Nature Med.* **4**, 623–626 (1998).
14. Bogdanov, A., Matuszewski, L., Bremer, C., Petrovsky, A. & Weissleder, R. Oligomerization of paramagnetic substrates result in signal amplification and can be used for MR imaging of molecular targets. *Mol. Imaging* **1**, 1–9 (2001).
15. Weissleder, R. *et al. In vivo* magnetic resonance imaging of transgene expression. *Nature Med.* **6**, 351–355 (2000).
16. Josephson, L., Perez, M. & Weissleder, R. Magnetic nanosensors for the detection of oligonucleotide sequences. *Angew. Chem. (Int Ed.Engl.)* **40**, 3204–3206 (2001).
Describes the use of magnetic nanoparticles as sensors to read out DNA hybdridization and, potentially, interactions between large and small molecules. Similar nanoparticles are also used for imaging of other molecular targets by high-resolution MRI.
17. Lewin, M. *et al.* Tat peptide-derivatized magnetic nanoparticles allow *in vivo* tracking and recovery of progenitor cells. *Nature Biotechnol.* **18**, 410–414 (2000).
Describes a versatile magnetic label that can be used to image a variety of cell types *in vivo*. Labelled cells were isolated after intravenous injection using a magnetic separation column.
18. Kennel, S. J. *et al.* High resolution computed tomography and MRI for monitoring lung tumor growth in mice undergoing radioimmunotherapy: correlation with histology. *Med. Phys.* **27**, 1101–1107 (2000).
19. Borah, B. *et al.* Three-dimensional microimaging (MRmicroI and microCT), finite element modeling, and rapid prototyping provide unique insights into bone architecture in osteoporosis. *Anat. Rec.* **265**, 101–110 (2001).
20. Paulus, M. J., Gleason, S. S., Kennel, S. J., Hunsicker, P. R. & Johnson, D. K. High resolution X-ray computed tomography: an emerging tool for small animal cancer research. *Neoplasia* **2**, 62–70 (2000).
21. Paulus, M. J., Gleason, S. S., Easterly, M. E. & Foltz, C. J. A review of high-resolution X-ray computed tomography and other imaging modalities for small animal research. *Lab. Anim. (NY)* **30**, 36–45 (2001).
22. Turnbull, D. *et al*. Ultrasound backscatter microscope analysis of mouse melanoma progression. *Ultrasound Med. Biol.* **22**, 845–853 (1996).
23. Rooks, V., Beecken, W., Iordanescu, I. & Taylor, J. A. Sonographic evaluation of orthopic bladder tumors in mice treated with TNP-470, an angiogenesis inhibitor. *Acad. Radiol.* **8**, 121–127 (2001).
24. Drevs, J. *et al.* Effects of PTK787/ZK 222584, a specific inhibitor of vascular endothelial growth factor receptor tyrosine kinases, on primary tumor, metastasis, vessel density, and blood flow in a murine renal cell carcinoma model. *Cancer Res.* **60**, 4819–4824 (2000).
25. Zagar, B., Fornaris, R. & Ferrara, K. Ultrasonic mapping of the microvasculature: signal alignment. *Ultrasound Med. Biol.* **24**, 809–824 (1998).
26. Ferrara, K. W. *et al.* Evaluation of tumor angiogenesis with US: imaging, Doppler, and contrast agents. *Acad. Radiol.* **7**, 824–839 (2000).
27. Turnbull, D. H., Bloomfield, T. S., Baldwin, H. S., Foster, F. S. & Joyner, A. L. Ultrasound backscatter microscope analysis of early mouse embryonic brain development. *Proc. Natl Acad. Sci. USA* **92**, 2239–2243 (1995).
28. Liu, A., Joyner, A. L. & Turnbull, D. H. Alteration of limb and brain patterning in early mouse embryos by ultrasound-guided injection of Shh-expressing cells. *Mech. Dev.* **75**, 107–115 (1998).
29. Haubner, R. *et al.* Noninvasive imaging of α(v)β3 integrin expression using 18F-labeled RGD-containing glycopeptide and positron emission tomography. *Cancer Res.* **61**, 1781–1785 (2001).
30. Shields, A. F. *et al.* Imaging proliferation *in vivo* with [^{18}F]FLT and positron emission tomography. *Nature Med.* **4**, 1334–1336 (1998).
31. Price, P. Positron emission tomography (PET) in diagnostic oncology: is it a necessary tool today ? *Eur. J. Cancer* **36**, 691–693 (2000).
32. MacLaren, D. C. *et al.* Repetitive, non-invasive imaging of the dopamine D2 receptor as a reporter gene in living animals. *Gene Ther.* **6**, 785–791 (1999).
33. Yang, D. *et al.* Imaging, biodistribution and therapeutic potential of halogenated tamoxifen analogues. *Life Sci.* **55**, 53–67 (1994).
34. Tjuvajev, J. G. *et al.* Noninvasive imaging of herpes virus thymidine kinase gene transfer and expression: a pothential method for monitoring clinical gene therapy. *Cancer Res.* **56**, 4087–4095 (1996).
35. Gambhir, S. S. *et al.* Imaging adenoviral-directed reporter gene expression in living animals with positron emission tomography. *Proc. Natl Acad. Sci. USA* **96**, 2333–2338 (1999).
36. Luker, G. & Piwnica-Worms, D. Beyond the genome: molecular imaging *in vivo* with PET and SPECT. *Acad. Radiol.* **8**, 4–14 (2001).
37. Gambhir, S. *et al.* Imaging transgene expression with radionuclide imaging technologies. *Neoplasia* **2**, 118–138 (2000).
38. Gambhir, S., Barrio, J., Herschman, H. & Phelps, M. Assays for non-invasive imaging of reporter gene expression. *Nucl. Med. Biol.* **26**, 481–490 (1999).
39. Tjuvajev, J. G. *et al.* Imaging adenoviral-mediated herpes virus thymidine kinase gene transfer and expression *in vivo*. *Cancer Res.* **59**, 5186–5193 (1999).
40. Doubrovin, M. *et al.* Imaging transcriptional regulation of p53-dependent genes with positron emission tomography *in vivo*. *Proc. Natl Acad. Sci. USA* **98**, 9300–9305 (2001).
41. Boland, A. *et al.* Adenovirus-mediated transfer of the thyroid sodium/iodide symporter gene into tumors for a targeted radiotherapy. *Cancer Res.* **60**, 3484–3492 (2000).
42. Groch, M. W. & Erwin, W. D. Single-photon emission computed tomography in the year 2001: instrumentation and quality control. *J. Nucl. Med. Technol.* **29**, 12–18 (2001).
43. Blankenberg, F. G. *et al. In vivo* detection and imaging of phosphatidylserine expression during programmed cell death. *Proc. Natl Acad. Sci. USA* **95**, 6349–6354 (1998).
44. Rogers, B. *et al.* Localization of iodine-125-mIP-Des-Met14-bombesin (7-13)NH2 in ovarian carcinoma induced to express the gastrin releasing peptide receptor by adenoviral vector-mediated gene transfer. *J. Nucl. Med.* **38**, 1221–1229 (1997).
45. Bogdanov, A., Tung, C., Kayne, L., Hnatowich, D.J. & Weissleder, R. in *NASA–NCI Workshop on Sensors for Bio-Molecular Signatures* 170 (Pasadena,1999).
46. Hnatowich, D. J. *et al.* Technetium-99m labeling of DNA oligonucleotides. *J. Nucl. Med.* **36**, 2306–2314 (1995).
47. Zalutsky, M. R. *et al.* Radioiodinated antibody targeting of the HER-2/neu oncoprotein: effects of labeling method on cellular processing and tissue distribution. *Nucl. Med. Biol.* **26**, 781–790 (1999).
48. Zinn, K. R., Chaudhuri, T. R., Buchsbaum, D. J., Mountz, J. M. & Rogers, B. E. Simultaneous evaluation of dual gene transfer to adherent cells by gamma-ray imaging. *Nucl. Med. Biol.* **28**, 135–144 (2001).
49. Katzenellenbogen, J. A. *et al.* Tumor receptor imaging: proceedings of the National Cancer Institute workshop, review of current work, and prospective for further investigations. *Clin. Cancer Res.* **1**, 921–932 (1995).
50. Hom, R. K. & Katzenellenbogen, J. A. Technetium-99m-labeled receptor-specific small-molecule radiopharmaceuticals: recent developments and encouraging results. *Nucl. Med. Biol.* **24**, 485–498 (1997).
51. Melder, R. *et al.* Systemic distribution and tumor localization of adoptively transferred lymphocytes in mice: comparison with physiologically based pharmacokinetic model. *Neoplasia* (in the press).
52. Schellingerhout, D., Rainov, N., Breakefield, X. & Weissleder, R. Quantitation of HSV mass distribution in a rodent brain tumor model. *Gene Ther.* **7**, 1648–1655 (2000).
53. Mahmood, U., Tung, C. H., Bogdanov, A., Jr & Weissleder, R. Near-infrared optical imaging of protease activity for tumor detection. *Radiology* **213**, 866–870 (1999).
54. Kaneko, K. *et al.* Detection of peritoneal micrometastases of gastric carcinoma with green fluorescent protein and carcinoembryonic antigen promoter. *Cancer Res.* **61**, 5570–5574 (2001).
55. Becker, A. *et al.* Receptor targeted optical imaging of tumors with near infrared flurorescent ligands. *Nature Biotechnol.* **19**, 327–331 (2001).
56. Folli, S. *et al.* Antibody-indocyanin conjugates for immunophotodetection of human squamous cell carcinoma in nude mice. *Cancer Res.* **54**, 2643–2649 (1994).
57. Weissleder, R., Tung, C. H., Mahmood, U. & Bogdanov, A. Jr. *In vivo* imaging of tumors with protease-activated near-infrared fluorescent probes. *Nature Biotechnol.* **17**, 375–378 (1999).
58. Bremer, C., Tung, C.H. & Weissleder, R. *In vivo* molecular target assessment of matrix metalloproteinase inhibition. *Nature Med.* **7**, 743–748 (2001).
Describes novel fluorescent sensors that are specific for proteinases, and explains how they can be used to image therapeutic proteinase inhibition *in vivo.*
59. Tung, C. H., Mahmood, U., Bredow, S. & Weissleder, R. *In vivo* imaging of proteolytic enzyme activity using a novel molecular reporter. *Cancer Res.* **60**, 4953–4958 (2000).
60. Shalinsky, D. R. *et al.* Broad antitumor and antiangiogenic activities of AG3340, a potent and selective MMP inhibitor undergoing advanced oncology clinical trials. *Ann. NY Acad. Sci.* **878**, 236–270 (1999).
61. Marten, K. *et al.* Detection of dysplastic intestinal adenomas using enzyme sensing molecular beacons. *Gastroenterology* (in the press).
62. Ntziachristos, V. & Weissleder, R. Experimental three-dimensional fluorescence reconstruction of diffuse media by use of a normalized Born approximation. *Optics Lett.* **26**, 893–895 (2001).
63. Ntziachristos, V., Ripoll, J. & Weissleder, R. Can near-infrared fluorescence propagate through human organs for non–invasive clinical examinations? *Optics Lett.* (in the press).
64. Ntziachristos, V., Yodh, A. G., Schnall, M. & Chance, B. Concurrent MRI and diffuse optical tomography of breast after indocyanine green enhancement. *Proc. Natl Acad. Sci. USA* **97**, 2767–2772 (2000).
65. Contag, C. H. *et al.* Photonic detection of bacterial pathogens in living hosts. *Mol. Microbiol.* **18**, 593–603 (1995).
66. Contag, C. H., Jenkins, D., Contag, P. R. & Negrin, R. S. Use of reporter genes for optical measurements of neoplastic disease *in vivo*. *Neoplasia* **2**, 41–52 (2000).
67. Hoegeman, D., Ntziachristos, V., Josephson, L. & Weissleder, R. High throughput MR imaging for evaluating targeted magnetic nanoparticle probes. *Bioconj. Chem.* (in the press).
68. Louie, A. Y. *et al. In vivo* visualization of gene expression using magnetic resonance imaging. *Nature Biotechnol.* **18**, 321–325 (2000).
69. Moats, R. A., Fraser, S. E. & Meade, T. J. A 'smart' magnetic resonance imaging agent that reports on specific enzymatic activity. *Angew. Chem. Int. Ed. Engl.***36**, 726–731 (1997).

Online links

DATABASES

The following terms in this article are linked online to:

CancerNet: http://cancernet.nci.nih.gov/
bladder tumours | bone tumours | brain tumour | breast cancer | colon cancer | prostate cancer

LocusLink: http://www.ncbi.nlm.nih.gov/LocusLink/
bombesin receptor | caspases | cathepsins | cathepsin B | cathepsin D | ERBB2 | E-selectin | folate receptor | MMP | MMP-2 | somatostatin | thymidine kinase | transferrin receptor

FURTHER INFORMATION

Berkeley Labs, Center for Functional Imaging (associated with UCSF):
http://www.radiology.ucsf.edu/academics/labs/index.shtml
Biomedical imaging programme at NCI:
http://www.nci.nih.gov/bip/default.htm
Duke Center for *in vivo* microscopy:
http://wwwcivm.mc.duke.edu
Journal of Molecular Imaging:
http://www.molecularimaging.org/journal/index.php3
MGH Center for Molecular Imaging Research: www.mgh-cmir.org
Ralph Weissleder's lab: www.mgh-cmir.org
The Society for Molecular Imaging: www.molecularimaging.org
UCLA Crump Institute for Molecular Imaging:
http://www.crump.ucla.edu

Access to this interactive links box is free online.