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Multiplexed imaging in oncology

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In oncology, technologies for clinical molecular imaging are used to diagnose patients, establish the efficacy of treatments and monitor the recurrence of disease. Multiplexed methods increase the number of disease-specific biomarkers that can be detected simultaneously, such as the overexpression of oncogenic proteins, aberrant metabolite uptake and anomalous blood perfusion. The quantitative localization of each biomarker could considerably increase the specificity and the accuracy of technologies for clinical molecular imaging to facilitate granular diagnoses, patient stratification and earlier assessments of the responses to administered therapeutics. In this Review, we discuss established techniques for multiplexed imaging and the most promising emerging multiplexing technologies applied to the imaging of isolated tissues and cells and to non-invasive whole-body imaging. We also highlight advances in radiology that have been made possible by multiplexed imaging.

Diagnostic imaging methods widely employed in the clinic can determine whether cancer is present in the body, as well as its location and any metastatic dissemination. Results can be acquired within hours. However, the information provided by each scan is usually limited to the detection of a single molecular target or physiological characteristic. But cancers are typically heterogeneous and their molecular fingerprint changes over time, between loci and in response to therapy. The ability to perform molecular imaging of multiple biomarkers in a single session could transform patient care because it would extend the sensitivity of the test to diverse cell populations.

The term multiplexing, which first arose in the areas of telecommunications and computer networking, is defined as 'the simultaneous transmission of several messages along a single channel of communication'. In biomedicine, multiplexing is used to indicate the ability to detect and follow multiple processes or biomarkers. The accuracy and value of diagnostic information obtained by medical imaging can be augmented by performing, for example, liquid biopsies for the molecular and cellular analysis of circulating biomarkers. Medical imaging is also used for surgical guidance to obtain tumour tissue biopsies for more extensive molecular analyses, such as genetic testing, sequencing and protein-marker determination (for instance, via immunohistochemistry (IHC), flow cytometry or fluorescence in situ hybridization (FISH)). Advances in multimodal clinical imaging have enabled the integration of positron emission tomography (PET) with X-ray computed tomography (PET-CT) and with magnetic resonance imaging (PET-MRI). Such multimodal systems are powerful tools for the detection of multiple molecular targets and physiological biomarkers¹. The combination of molecular information with information from whole-body imaging has enabled the tracking of the onset and progression of disease, the monitoring of treatment efficacy and the anticipation of disease recurrence. These types of multiplexed data need to be sequentially acquired and generate large amounts of data that require advanced techniques for processing.

In this Review, we discuss the state-of-the-art in single imaging modalities that provide multiplexed information, including clinical, preclinical and ex vivo methods. We focus on the display of 'multichannel' information acquired during a single imaging session, either from images obtained in close succession or derived computationally from complex multivariate images, where each pixel contains measurements of different parameters (such as spectral intensity). Multiplexing can span the anatomic, physiological and molecular levels, and as more channels are imaged simultaneously, the information provided can increase considerably (Fig. 1). This information can then be displayed spatially over time, visualized individually in channels, or merged as combinations of multiple channels or in a quantitative graphical format.

Multiplexed imaging could enhance medical imaging in several ways. For example, it could facilitate the mapping of the heterogeneity of primary and metastatic tumours, where the different expression of key biomarkers is informative for patient stratification into different treatment arms². Current practice in clinical imaging does not account for this kind of heterogeneity; rather, tissue biopsies (obtained from fine needle aspirates (FNAs), core biopsies or intraoperative biopsies) represent the gold standard that directs the treatment regimen for most patients with cancer. However, biopsies only provide data on a small portion of a tumour; the remaining cancerous tissue remains unexamined. As a result, major treatment decisions are often made on the basis of incomplete or potentially misleading information. Equally challenging is the fact that the molecular profile of cancers can change over time, which cannot be easily followed by repeated biopsies³. This diagnostic challenge applies to both the primary tumour and to metastases, which are heterogeneous with respect to the primary tumour. Multiplexing can also improve the probing of spatial correlations between biomarkers to determine drug distribution and receptor occupancy, or to quantitate the activation of signalling pathways frequently overexpressed in cancer (Fig. 1). This Review focuses on cancer as one of the most pressing areas where multiplexed imaging could have a major impact on diagnosis, treatment and the monitoring of recurrence, yet similar needs are present in many other types of disease.

The value of multiplexing depends on the spatial resolution of the underlying imaging technology and on the clinical question at hand,

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Fig. 1 | Combinations of biomarkers for optimal multiplexed imaging. The simultaneous imaging of multiple and complementary target biomarkers can increase the information content in imaging data, provide a more comprehensive picture of a patient's condition and improve the analysis of a single medical scan. Each additional contrast agent detected (represented arbitrarily with a different colour) can enhance the understanding of the molecular underpinnings of physiology and disease.

and it is up to the physician or researcher to select the most suitable tool for their specific setting. The major modalities that lend themselves to molecular imaging of one or more targets are displayed schematically in Fig. 2, according to their multiplexing capabilities and spatial resolution. It is evident that fast modalities that currently form the mainstay of medical imaging are limited both in resolution and in degree of multiplexed detection. Optical methods fare better both in terms of resolution and multiplexing, but are primarily suited to the intraoperative setting, as optical imaging is limited by the penetration of light in tissue. High-resolution methods typically require excision and processing of the sample before imaging, and include the methods with the highest degree of multiplexing capacity, such as serial staining with fluorescent labels or imaging based on mass spectrometry. However, they are not suitable for quick diagnosis or for use in low-resource settings. Fast, cost-effective methods are primarily based on the analysis of cells, and can provide highly multiplexed molecular information^{4,5}. However, as these methods depend on collected cells ex situ, they do not provide spatial or anatomical information related to tumour spread.

Multiplexed imaging techniques that can detect multiple biomarkers simultaneously, mostly through the use of extrinsically applied contrast agents, can be classified into two categories: ex vivo modalities for the imaging of sampled tissues (fresh or fixed), and in vivo modalities for the imaging of patients (non-invasive or intraoperative). The different modalities are summarized in Table 1, along with synoptic information on resolution, multiplexing capabilities and primary (potential) clinical use. First, we describe single-cell ex vivo imaging technologies that lend themselves to multiplexing. Then we discuss the development of existing medical imaging modalities that have the potential for multiplexed imaging by leveraging new contrast agents and technical modifications. Lastly, we describe emerging experimental technologies that are being evaluated for their clinical potential.

Ex vivo imaging of sampled tissues

Image-guided collection of tumour tissue (biopsies, such as FNAs) and image-guided surgical resections are increasingly common, prompted by the frequent need for molecular profiling before a patient's enrolment into a trial⁶, for tissue biobanking⁷ or for confirming the clearance of surgical resection margins8. Although all of these applications rely on rapid imaging technologies to be able to make real-time decisions, obtaining specific multiplexed molecular information from the acquired samples can take hours to days and is often limited to less than 5-10 markers because of the scarce amount of tissue resulting from biopsies. 'Virtual histology' technologies are emerging to address the need for a more robust and deeper molecular profiling of excised samples. Methods that profile multiple molecular and cellular biomarkers in collected cells and tissues are also being developed. When interrogating collected cells or tumour tissues (Table 1), collectively these methods are expected to enable broader analyses through an extremely high multiplexing potential and high resolution from little sample material, as well as fast point-of-care (POC) deployment capabilities.

Cell-based barcoding. An alternative to image-guided core biopsies has recently emerged in the form of FNAs, which yield cells rather than tissue samples. As a less-invasive method, FNAs are much better tolerated and are associated with lower patient complication rates⁹; however, the limited amount of sample that FNAs provide makes it more challenging to process them with traditional methods (such as staining and flow cytometry). Indeed, efficient pathway analysis of such cells in the clinic has proved difficult, time-consuming and costly (however, new promising methods have been reported¹⁰).

Recently, an antibody-DNA barcoding approach has allowed for the collected cells to be rapidly re-stained through the use of custom-designed oligonucleotide-fluorophore conjugates¹¹.

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Fig. 2 | Imaging modalities, arranged according to resolution and multiplexing capability. Clinically used imaging modalities and emerging imaging technologies are indicated by continuous boundaries and dashed boundaries, respectively. Techniques that are amenable to in vivo use (or to use with fresh tissue) are shown in green, and techniques that require considerable sample processing are shown in blue. CT, computed tomography; CyclF, cyclic immunofluorescence; H&E, haematoxylin and eosin; IF, immunofluorescence; IHC, immunohistochemistry; IOF, intraoperative fluorescence; LSM, light-sheet microscopy; MRI, magnetic resonance imaging; MSI, mass spectrometry imaging; MUSE, microscopy with ultraviolet surface excitation; OA, optoacoustic imaging; PET, positron emission tomography; SERS, surface-enhanced Raman spectroscopy imaging; SPECT, single-photon emission computed tomography; SRH, stimulated Raman histology; STED, stimulated emission depletion microscopy; STORM, stochastic optical reconstruction microscopy; VM, volumetric microscopy; US, ultrasound.

DNA-antibody barcoding is a powerful method: each probe is labelled with a customized readable sequence that can be matched uniquely to a database to identify the presence of the probe. In one exemplary application, antibodies for 90 specific protein targets were encoded with unique cleavable DNA sequences¹¹. The DNA tags were then sequentially hybridized with complementary DNA strands labelled with a fluorophore, or cleaved and sequenced downstream. A related method, single-cell analysis for tumour phenotyping (SCANT), showed that up to 12 DNA-conjugated antibodies could be multiplexed and used to sequentially image different sets of targets to interrogate drug-relevant pathways in scant clinical samples from FNAs⁴ (Fig. 3a). By probing the signalling pathway of phosphoinositide 3-kinase (PI3K), phosphatase and tensin homologue (PTEN) and cyclin-dependent kinases 4 and 6 (CDK4/6) in breast cancer, analyses could be performed in tandem with trial enrolment to evaluate downstream signalling following therapeutic inhibition. Approaches such as SCANT may allow for a more widespread use of limited single-cell material in clinical samples. Furthermore, the rich information obtained from a small sample and the ability to integrate it into portable or benchtop devices with integrated microfluidic, imaging and computational components for fast analysis could provide a meaningful snapshot of the state of a patient, for example by monitoring immune-cell populations.

Similar barcoding approaches based on FISH have been reported for highly multiplexed imaging of RNAs, and permit the spatially resolved gene-expression profiling of individual cells. Recently, such methods have been leveraged to map more than one hundred RNAs, by using binary-encoded fluorescent oligonucleotides¹²⁻¹⁴. These methods can in theory be extended to image thousands of RNA targets, which would provide spatially resolved information at the level of the transcriptome. Additionally, examples of functional imaging using CRISPR (for clustered regularly interspaced short palindromic repeats) have been reported in cells¹⁵, and although still preliminary, these can provide another paradigm for genome-level multiplexed functional imaging.

Cell-based microholography. Microholography is another POC methodology for the rapid analysis of collected tissue and cells¹⁶. Digital microholography is a lensless technology that enables microscopic bright-field imaging with a relatively large field-of-view (>1 mm²); instead of imaging a specific focal plane, it detects diffraction-based (holographic) patterns coming from out-of-focus microscopic objects. Molecular specificity is obtained by labelling cells with micrometre-sized beads that change the cells' holographic patterns. The presence and type of the beads can be established using available image-processing or computer-vision techniques. Because it does not necessitate sophisticated imaging equipment, digital microholography has been adopted in resource-limited settings as an advanced standalone diagnostic tool. In one example, antibody-conjugated microbeads of different sizes were used to impart a holographic signature on lymphoid cells, enabling the analysis of percutaneously sampled lymphoma¹⁷. More recently, digital microholography has been adapted for chromogenic staining using different colours (known as contrast-enhanced microholography)¹⁸, enabling the rapid multiplexing (four targets were detected in this study, yet more could be possible) of collected cells (Fig. 3b). The method has also been used for the rapid analysis of receptor status in breast cancer using deep learning¹⁹ and in cervical-cancer screening¹⁶.

Virtual histology. Although histology remains the gold standard for clinical and preclinical applications requiring the visualization of cells, it offers limited multiplexing potential. Emerging imaging technologies aim to replace or enhance traditional (haematoxylin and eosin, H&E) histological staining and provide 'slide-free histology' by inferring cellular and tissue structure computationally with minimal processing. Microscopy with UV surface excitation (MUSE) uses UV light to excite molecular contrast agents on a stained fresh sample, and can provide high-resolution images in minutes²⁰. Stimulated Raman histology (SRH) uses the vibrational (Raman) scattering energies characteristic of lipids, DNA and proteins to quickly provide a virtual H&E image of unprocessed surgical samples without the use of extrinsic contrast agents²¹. And a method based on light-sheet microscopy (LSM) seeks to preserve tissues intact while providing three-dimensional (3D) molecular images of large excised specimens²². Although these methods all supplement traditional histology, each has individual strengths and challenges, which affect their respective potential for clinical translation, regulatory approval and clinical use.

Cyclic tissue-staining methods. Serial tissue sampling, usually obtained via core biopsy, has become essential to modern targeted and personalized cancer treatments. Traditionally, biopsy samples are obtained under image guidance (yielding tissue fragments of about 1×20 mm) or during a surgical procedure (where tumour or lymph nodes are resected as a whole), and are formalin-fixed and sliced, with each section stained to detect the presence of specific molecular targets. Recently, several multiplexed staining methods have been developed using cyclic immunofluorescence (CycIF) in formalin-fixed tissues, which can image a high number of targets on a single section or sample by sequentially applying a targeted fluorophore, imaging the sample, and then inactivating it before

Evvivo	number of viomarkers	resolution	Loverage	Time	Main application	Adoption in oncology	Examples	Limitations	Promising developments
Cyclic fluorescence > with DNA barcoding	≫10	1μm²	1-10 cm ²	Minutes to hours	FNAs, excised tissues	Experimental	CyclF (ref. ²³), MERFISH (ref. ¹⁴), SCANT (ref. ¹¹)	Slow and requires specialized processing	Varied applications with high multiplexing capacity
Microholography ²	4-6	NA	AN	Seconds	FNAs, point of care	Experimental	AIDA (ref. ¹⁹), CEM (ref. ¹⁸), D3 (ref. ¹⁶)		Portable automated systems
Flow cytometry	~10	NA	AA	Minutes	Lab analysis	Routine clinical/preclinical	Major vendors	Bulky and expensive	Miniaturization and microfluidics
Histology 3	3-4	1μm²	$1-10 \text{ cm}^2$	Hours	Fixed tissues	Routine clinical/preclinical	H&E, other chromogens	Limited stains	
Immunohistology	رج ح	1μm²	1-10 cm ²	Hours	Fixed tissues	Routine clinical/preclinical	Immunohistochemistry, immunofluorescence	Antibody dependent and requires extensive processing	Cyclic immunostaining
Histology alternatives	5-6	1μm²	1-10 cm ²	Minutes to hours	Excised tissues	Experimental/preclinical	MUSE (ref. ²⁰), LSM (ref. ²²), SRH (ref. ²¹)	Specialized optical components	Virtual histology with intrinsic and extrinsic contrast
Mass spectrometry > imaging	≫10	10-100 μm ²	1-10 cm ²	Days	Fixed tissues, drug development	Experimental/preclinical	SIMS, MALDI	Expensive and destructive of the sample	High multiplexing capacity
Tissue clearing	د ر ج	1,000 µm³	Tissues	Days	Excised tissues, drug development	Experimental/preclinical	CLARITY, CUBIC, 3DISCO	Slow and requires specialized processing	Fast clearing for specific applications (in particular, for the lungs)
In vivo									
PET	2-3	1-10 mm ³	Whole body	Ť	Medical imaging	Routine clinical; always needed	Major vendors	Radiation exposure and single-energy channel	Whole-body PET
MRI	3-5	1-10 mm ³	Whole body	Ť	Medical imaging	Routine clinical use for the characterization of tissue (typically, the musculoskeletal system and the brain)	Major vendors	Costly and requires complex infrastructure	Hyperpolarized MRI, MRS, MRI-CT
CT	2-3	0.1-1 mm ³	Whole body	Minutes	Medical imaging	Routine clinical	Major vendors	Low sensitivity and ionizing radiation	Dual-energy CT
Ultrasound	5-3	1-10 mm ³	Organ	Minutes	Medical imaging	Clinical use for specific indications (for example, for imaging the liver or the gallbladder, and for vascular assessment)	Major vendors	Low penetration depth, and dependence on the operator and on body habitus	Targeted microbubbles, ultrafast ultrasound
SPECT	3-5	100- 500 mm ³	Organ	Minutes	Medical imaging	Routine clinical	Major vendors	Radiation exposure and long acquisition times	Multi-energy γ -ray cameras
MSOT	3-5	0.1-1 mm ³	Organ	Minutes	Medical imaging	Multiple preclinical studies; clinical trials	Some vendors, experimental systems	Development and regulatory approval of the contrast agents	Assessment of lymph-node status in melanoma
Intraoperative 3	3-5	1-100 μm ³	Surgical bed	Seconds	Intraoperative imaging	Clinical trials	Some vendors	Wide overlapping bands and photobleaching	Hyperspectral imaging
Raman / SERS	~10	1-100 μm ³	1-10 cm ²	Minutes to hours	Intraoperative imaging, excised tissues	Preclinical only	Major vendors, experimental systems	No clinical scanners and need for regulatory approval of the nanoparticles	Highest multiplexing capacity (of all in vivo modalities)

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Fig. 3 | Cell-based multiplexed imaging techniques. a, Cell barcoding via cyclic imaging and quenching with different sets of fluorescent antibodies allows for the identification of multiple biological targets in individual cells. **b**, Microholography uses unfocused optics to image cells stained with targeted beads. Machine learning can easily distinguish different types of cells on the basis of their holographic patterns. **c**, Tissue-clearing techniques remove light-scattering materials from tissues and enable volumetric microscopy of multiple optical contrast agents in situ with cellular resolution at greater depths. **d**, Five-colour mass-spectrometric image of different fragment mass-to-charge ratio (*m/z*) peaks of the brain section of a rat (left) compared with its H&E stain (middle), and magnified decoupled signals from the boxed area (right). **e**, Histological sections labelled with metal-tagged antibodies and analysed by point-wise laser ablation. Thirty-two different antibody targets are detected simultaneously, providing a wealth of spatially correlated molecular data. PACT, passive clarity technique; CyTOF, mass cytometry time-of-flight. Panels adapted with permission from: **a**, ref. ⁴ under a Creative Commons licence CC BY 4.0; **b**, ref. ¹⁸, Springer Nature Ltd; **c**, ref. ²⁵, Elsevier; **d**, ref. ³⁰, Wiley; **e**, ref. ³², Springer Nature America, Inc.

applying the next fluorophore²³. In this way, small biopsy samples can provide information on the expression of a wide set of molecular biomarkers in the tumour. A similar strategy can be employed by using imaging methods other than fluorescence, for example by imaging and assigning pseudocolours to serially stained IHC images of the same tissue section. In this way, the distinct stains can be combined into a single multivariate image. In general, cyclic tissue-staining methods have proven to be most useful for fixed or embedded processed samples, rather than for bedside use²⁴. Similar methodologies combining cyclic staining and barcoding methods

with in situ hybridization have been reported for the imaging of nucleic acids¹²⁻¹⁴, and may pave the way for the functional imaging of gene expression based on RNA imaging.

Volumetric microscopy with tissue clearing. The typical pathological workflow for surgical specimens is often a selective process because it is impractical to section, string and analyse hundreds of slides for each patient. There has thus been an intense interest in mesoscopic and macroscopic imaging that would allow surveying the entire resected tissue specimen, with the ability to zoom in onto

individual groups of cells. Light-based imaging methods at cellular resolution are often limited to a working depth of ~100 µm before resolution is degraded by light scattering. Tissue clearing facilitates the 3D optical imaging of large samples by rendering biological tissue transparent to light so that contrast agents can be detected without attenuation²⁵ (Fig. 3c). Thus far, the introduction of faster image-acquisition protocols and a variety of tissue-clearing methods have drastically improved deep-tissue imaging, primarily in neurobiological applications²⁵. Importantly, these methods are compatible with the imaging of multiple targets at different wavelengths at cellular resolution, and can be used for both preclinical and clinical investigations²⁶. However, some limitations remain for labelling and imaging tumours, such as slow antibody penetration (in the order of weeks for whole organs), uneven labelling and cost^{27,28}. A recently developed method for rapid tissue clearing and labelling²⁹ allowed for the imaging of cellular detail at desired locations throughout lung lobes. Whole-organ tumour burden, host-cell analysis and drug-delivery assessment could be obtained by imaging using a combination of fluorescent proteins, molecular probes administered in vivo, or topical stains. This type of volumetric multiplexed microscopy (4-8 channels) will likely have applications in cancer biology, but the long timelines required for the clearing process are not conducive to clinical applications.

Mass spectrometry imaging. Mass spectrometry imaging (MSI) uses different mass spectrometry approaches to obtain the spatial distribution of metabolites, drugs, peptides and proteins in tissue samples according to their molecular mass^{30,31}. It can also use antibodies with single metal-atom isotopes to provide molecularly specific contrast^{32,33} (Fig. 3d,e). The detected moieties, either fragments or whole molecules, are identified via comparison to specialized databases. The three most common MSI methods are secondary ion mass spectrometry imaging (SIMS, with resolutions <10 µm), matrix-assisted laser desorption ionization (MALDI) imaging (20 µm resolution) and desorption electrospray ionization (DESI) imaging (100 µm resolution). MSI is unique in its ability to profile biomolecules in tissues without a priori knowledge of them and to map the distribution of drugs and their metabolites in tissue. As such, the major applications of MSI are in research and drug development. The multiplexing ability of MSI is high (hundreds to thousands of peptides and proteins). The main disadvantages are the relatively high costs of the equipment, the destruction of the sample, and the complexity, time-consuming and semi-quantitative nature of the analyses.

In vivo whole-body imaging

CT, PET, MRI and, increasingly, fluorescence imaging, are used to inform key clinical decisions in patient care. Multiplexed information from these established modalities would substantially improve diagnostic accuracy, aid the process of differential diagnosis and facilitate decision making.

X-ray computed tomography. CT relies on endogenous contrast agents to map anatomical features. It is based on the differential absorption of X-ray photons in tissues of different chemical composition (for example, calcium-rich bones vs carbon-rich soft tissues). Therefore, CT provides exquisite anatomical images on the basis of the intrinsic contrast of body structures, and can be used for radiomic analysis to enable the identification and characterization of tumours according to their shape, texture and other features^{34,35}. Additionally, exogenous contrast agents can be used to enhance images of the vasculature (by using iodine) or the gastric system (by using barium). Dual-energy CT uses two X-ray emitters of different energies to provide differential element maps^{36,37} for, for example, calcium in bone and iodine in structures containing the contrast agent (Fig. 4a).

For a given element, the absorption of X-ray photons sharply increases when the photon energy is higher than the binding energy of the innermost electron shell. This leads to absorption spectra as seen in Fig. 4b (such a sharp increase is called the 'K edge'). Other less pronounced 'edges' are observed for the outer electronic orbitals. It may be possible to develop orthogonal contrast agents, directed to different targets, that can be distinguished by dual-energy CT via their differential absorption to provide a multiplexed 3D image by acquiring images just above the K edge of each agent (Fig. 4b)^{37,38}. For example, given two hypothetical contrast agents with different attenuation coefficients, an unknown mixture of the two can be determined by measuring at two energies just above the two K edges and calculating a linear fit. With additional contrast agents and measurements at additional appropriate energies, more complex mixtures can be determined via a simple linear fit.

However, CT suffers from limited sensitivity, requiring contrast agents at molar concentrations. This limitation still prevents CT from becoming a targeted molecular-imaging modality, and constrains its use to the imaging of anatomical features, such as vasculature or the gastrointestinal tract. Another disadvantage is that the inclusion of additional higher energies for imaging increases the risks of radiation exposure for the patient^{39–42}.

New CT technologies are currently being developed to markedly improve the capabilities of this imaging modality. One promising example is phase-contrast CT (PC-CT). The main advantage of PC-CT is that it results in far superior soft-tissue contrast compared with conventional absorption-based images. It has been shown that PC-CT can resolve ductal structures, the 3D visualization of ductal carcinoma and collagen architecture without requiring the administration of an exogenous contrast agent^{43,44}.

Another emerging CT technology is 'photon-counting detector' (PCD) CT^{45,46}. PCDs have the potential to increase the multiplexing power of dual-energy CT by resolving the incident X-ray spectrum into multiple energy bins^{45,47} and enabling the simultaneous differentiation of multiple contrast agents. The contrast agents gado-linium, iodine and bismuth have been simultaneously imaged and discriminated in vivo using PCD⁴⁵. Most studies reporting improvements in CT imaging have been performed in preclinical settings, yet they could be translated for use in the clinic relatively rapidly.

Radioisotope-based imaging. PET, which is used widely in medical imaging, has many applications in cancer care. Today, most PET examinations are combined with CT to improve image reconstruction and interpretation. The two imaging techniques are thus highly complementary. On the one hand, although PET does not provide anatomical information and depends on injected radiotracers for the generation of imaging contrast from labelled cells and tissues, its sensitivity is in the picomolar range, well beyond that of other whole-body medical-imaging modalities. On the other hand, conventional CT uses the attenuation in X-ray projections to create a 3D image of a patient, revealing their internal anatomy.

PET relies on the annihilation of positrons emitted by radioisotopes, producing photons that are then detected and reconstructed to provide a 3D image. Different positron-emitting radionuclides, including ¹⁸F, ⁶⁸Ga, ⁶⁴Cu and ⁸⁹Zr, are used for diagnostic imaging in patients. These nuclides are either covalently attached or chelated into small-molecule compounds or antibodies to yield tracers such as ¹⁸F-fluorodeoxyglucose (FDG) for mapping glucose metabolism, or ⁸⁹Zr-MAb for antibody-based immuno-PET imaging⁴⁸. Many more tracers are currently in the translational pipeline^{49,50}. In PET, different tracers cannot be readily distinguished, as annihilated positrons all yield photons of the same energy (511 keV), regardless of their nuclide of origin. However, a series of technical approaches have been developed to identify multiple tracers. These approaches are based on kinetic modelling, where multiple images acquired in series are fitted mathematically to reveal the presence of the different

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Fig. 4 | Multiplexed clinical imaging modalities. a, Dual-energy CT provides contrast to different parts of the human anatomy on the basis of differences in X-ray attenuation by different tissues. After administration of a contrast agent, such as iodine, structures containing the contrast agent show high attenuation for 80 kilovolt peak (kVp) X-rays but lower attenuation at 140 kVp, which is further than the K edge of iodine (33.2 keV). **b**, It is possible to develop contrast agents with orthogonal scattering cross-sections derived from the K edge of different materials to enable multiplexed imaging with dual (or higher-order) energy CT. By imaging at the appropriate energies, indicated by the dotted lines, mixtures of the probes could be detected, revealing the presence and co-localization of the biological targets (for example, mixture 1 contains none of the agents whereas mixture 4 contains both). **c-e**, Dual-tracer serial PET imaging obtained after staggered administration of FLT (t = 0 min) and FDG (t = 30 min) reveals tumour margins and metabolic activity in the brain. The staggered injection in **c** allows for the monitoring of the total signal from the two tracers (**d**), which is then decoupled by fitting the kinetic model in **e**, which accounts for the signal lost to radioactive decay (λ) and the exchange rate (k_n) of the radiotracers between blood and tissues. K_1 is the primary uptake rate and k_n are the secondary transport events. An additional model with four parameters was used to account for dephosphorylation of FLT, indicated by the dotted arrow. **f**, MRF records the response of tissues to changes in the applied magnetic field, tracking multiple parameters simultaneously, and matches them to a library of known responses. TAC, time-activity curve; Δf , off-resonance frequency; M_0 , proton density; TR, time repetition. Panels adapted with permission from: **a**, **b**, ref. ³⁶, RSNA; **c-e**, ref. ⁵², under a Creative Commons licence CC BY 4.0; **f**, ref. ⁶³, Springer Nature Ltd

radiotracers. This can be done, for example, by administering isotopes with different half-lives sequentially and then demultiplexing them by considering the different rates of decay of each radiotracer to reveal their respective distribution⁵¹. Alternatively, the same radionuclide (isotope) can be used for different tracers that are administered via staggered injections and imaged during the same scan. For example, injecting ¹⁸F-fluorothymidine (FLT) followed by ¹⁸F-FDG 30 min later (Fig. 4c), and using dynamic acquisition over approximately 90 min allows for sufficient data to fit a kinetic model to time–activity curves (Fig. 4d) to determine the uptake of the two tracers⁵² (Fig. 4e). This method relies on estimated exchange rates of the radiotracer between the blood pool and tissue (k_n^{tracer}), and also takes into consideration the decrease in signal owing to nuclear decay (λ). By using a combination of different isotopes and the staggered injection of distinct PET tracers, PET imaging could be used to detect even more targets⁵³.

Single-photon emission computed tomography (SPECT) imaging relies on the detection of single photons (γ -rays) stemming from

nuclear decay (for example, via electron capture) rather than on the detection of positron annihilation (which is the basis of PET). As such, different radionuclides can be distinguished, as they emit γ -rays of different energies (35 keV for ¹²⁵I, 140 keV for ^{99m}Tc, and 200 keV for ¹¹¹In). By imaging in appropriate energy windows, it is possible to map multiple tracers at once. This has applications in oncology^{48,54} and cardiac imaging⁵⁵ in particular.

The combination of different isotopes or radiotracers during the same exam requires the careful weighing of risks. Concerns may arise about the increasing dose of radiation introduced with each additional administration of a radiotracer. However, these concerns can be addressed by recent progress in whole-body PET scanners that use detectors with improved sensitivity, which allow for up to 40 times faster acquisition times^{56–58}. As whole-body systems have many more detectors compared with the conventional ring-shaped scanner, they capture more of the annihilation photon pairs, resulting in reduction of the required radiation dose while improving image contrast. The virtually simultaneous detection of tracer uptake or distribution at all sites in the body⁵⁹ could perhaps also be leveraged towards true multiplexed capabilities for PET imaging.

Magnetic resonance imaging. MRI can provide rich anatomic and physiological information. It is based on the relaxivity of nuclear spins of protons (or other nuclei) in tissue. Typically, the spin relaxation times $(T_1 \text{ and } T_2)$ are used to generate images that are weighted to produce different types of contrast between tissues. Molecular-grade information can also be detected via exogenous contrast agents; for example, paramagnetic compounds or superparamagnetic particles can be synthesized with ligands that display high affinity for a specific target or receptor. Multiparametric MRI, often used for the imaging of prostate cancer⁶⁰⁻⁶², complements anatomical T_1 -weighted and T_2 -weighted information with functional information obtained by dynamic contrast-enhanced (DCE) imaging or diffusion-weighted imaging (DWI). In some instances, data obtained by magnetic resonance spectroscopy (MRS) can provide further chemical information, typically of metabolites in tissue. Magnetic resonance fingerprinting (MRF)^{63,64} is a newer technique that can assign each voxel in the scan to a library of known references on the basis of a 'fingerprint' response. As shown in Fig. 4f, a single acquisition provides an image on the basis of the timing of the excitation pulses and reveals an image from a single parameter (for instance, T_1). For MRF, the excitation radiofrequency pulses vary pseudorandomly, thus generating a unique sequence of signal evolutions that is recorded across multiple repetitions, as shown in Fig. 4f. This sequence of evolutions can subsequently be matched to a library of known evolutions from different tissue types, and in this way each voxel of the reconstructed image is identified, or used to extract the traditional clinical magnetic-resonance images (T_1 and T_2).

Exogenous contrast agents for MRI can be engineered with substances (such as Gd, Mn or Fe) that alter the local magnetic properties. Such agents have been used for a variety of biomarker-imaging applications^{65–67}. Although examples of multiplexed MRI using a contrast agent date back 30 years⁶⁸, these concepts have recently been re-explored using the differences in T_1 and T_2 relaxation times for Mn and Gd to quantitatively decouple and identify them in phantoms⁶⁹. It should thus be possible to engineer other orthogonal contrast agents by taking advantage of the multiparametric capabilities of MRI to map multiple targets at once. However, as is the case with CT, the limited sensitivity of MRI makes it difficult to detect biomarkers of low abundance in vivo.

Conventional magnetic resonance spectroscopy imaging (MRSI) can in principle provide information on multiple different parameters or metabolites. However, this information is not often collected in practice, mostly because of technical challenges in acquiring the spectra in scenarios where there is patient motion. However, with

the advent of hyperpolarized MRI (HP-MRI), multiplexed imaging based on MRS may become a reality. HP-MRI is a fundamentally different technique for imaging the metabolization of systemically injected hyperpolarized substrates. Metabolites-such as pyruvate, bicarbonate or glutamine-can be chemically synthesized from a precursor using the stable isotope carbon-13. The hyperpolarized state is induced by aligning the nuclear and electron spins, usually via dynamic nuclear polarization, and results in a 10,000-100,000-fold increase in the signal-to-noise ratio, thus allowing for real-time measurements of metabolite transport, exchange, metabolism and perfusion via MRSI (ref. 70). Following a period of extensive preclinical studies, HP-MRI is being tested in early clinical trials. Although the technique is currently available only in a few centres worldwide, it has shown promise for the imaging of prostate cancer⁷¹ and brain tumours in patients⁷², and is currently being studied for the imaging of other cancer types. HP-MRI offers the potential to examine metabolism in vivo in real time throughout the entire human body. Importantly, it is possible to administer multiple carbon-13-enriched metabolites using a single intravenous bolus, and to record their respective kinetics simultaneously to enable multiplexed metabolic imaging^{73,74}. Once matured, this technology is expected to aid clinical decision-making, for example by allowing for patient stratification with regards to the optimal anticancer-drug regimen before any anatomical signs of efficacy (for example, tumour-size reduction) are discernible.

Intraoperative imaging. As surgical intervention is one of the primary modes of treatment in oncology, it is of paramount importance to aid surgeons in the visualization of tumour margins and residuals to improve the postoperative outlook. Fluorescence imaging, used in cellular and tissue-based ex vivo imaging methods, has been adapted for epifluorescence in situ imaging during surgery and microendoscopy (Fig. 5). There are several recent review articles on these topics^{75,76}, so here we limit our discussion to the use of molecular probes to enhance fluorescence-guided surgery. The underpinning principle and need is to augment native colour contrast (that is, what surgeons see) with molecular information to better define tumour margins and visualize difficult-to-see vital structures (nerves and ureters, for instance), or to probe for functional information (such as leaks, or the patency of vessels following anastomosis).

Approaches based on quantum dots for sentinel-lymph-node mapping have shown promise in preclinical studies. Quantum dots are attractive because their fluorescence emission wavelength can be tuned to different colours by varying their size (Fig. 5a). Newer compositions have yielded biocompatible quantum dots that could one day be used in the clinic^{77,78}.

For the intraoperative imaging of tumours, many examples using small-molecule near-infrared fluorophores in mice show that tumour margins can be detected in different cancer types and loci⁷⁹⁻⁸². By performing fluorescence imaging serially with different excitation and emission filters, it becomes possible to intraoperatively map multiple fluorophores. This approach has been used clinically to map sentinel lymph nodes with indocyanine green (ICG) and lymphatic ducts with fluorescein during laparoscopy⁸³ (Fig. 5d), and for the ratiometric detection of an enzymatically activatable dye that changes colour in the vicinity of tumours⁸⁴ (Fig. 5c).

Several molecularly targeted fluorescence-imaging probes have entered clinical testing. These include fluorochrome-tagged antibodies⁸⁵, nanoparticles⁵⁰, peptides⁸, small molecules (poly(ADP-ribose) polymerase inhibitors⁸⁶ and folate⁸⁷), as well as simple fluorochromes (ICG and others). Examples of molecular contrast agents, such as 5-aminolevulinic acid used successfully for the excision of brain tumours⁸⁸⁻⁹⁰, have propelled intraoperative imaging and have had direct impact on patients with cancer. The results from clinical trials have been most encouraging for some of the agents and

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Fig. 5 | Intraoperative multiplexed fluorescence imaging. a, Silicon-based quantum dots (left, colour image; right, emission spectra) have tunable fluorescence and low toxicity. **b**, Different 'colours' of quantum dots used to map five different lymphatic-flow pathways in living mice (left, schematic; right, pseudocolour image). **c**, A fluorescent dye that localizes in lymph nodes (LN) and changes its emission after enzymatic cleavage can provide a ratiometric signal in the presence of cancer. **d**, Lymph-node resection in humans using a laparoscopic system and two fluorescent dyes to identify sentinel lymph nodes (red star). **e**, Molecular detection at the microscopic scale can be achieved via intravital microscopy (IVM) to track a variety of fluorochromes, such as drugs and their metabolites. **f**, IVM can leverage genetically encoded (endogenous) fluorescent markers as well as exogenous contrast. Panels adapted with permission from: **a**, ref. ⁷⁸, American Chemical Society; **b**, ref. ⁷⁷, American Chemical Society; **c**, ref. ⁸⁴ under a Creative Commons licence CC BY 4.0; **d**, ref. ⁸³, Elsevier; **e**, ref. ¹⁴³ under a Creative Commons licence CC BY 4.0; **f**, ref. ⁹², AAAS.

devices tested; in particular, they have significantly decreased the re-operating rates of patients with breast cancer⁹¹. Although most clinical trials currently evaluate the diagnostic accuracy of a single imaging agent, several agents could eventually be combined.

Microscopy-based methods enhance the resolution of intraoperative fluorescence imaging in the mapping of the tumour microenvironment. One example is shown in Fig. 5e, where different components of the tumour microenvironment are visualized using a combination of a vascular probe, an antibody-conjugated fluorophore and an enzymatically activated fluorophore. A similar approach has been used to study the progression of autoimmune disease in mice⁹².

Emerging technologies

Optical and hybrid ultrasound-optical techniques can also be employed for multiplexed imaging in the clinic. Multiplexed optical imaging techniques use non-ionizing radiation, are relatively cost-effective, offer exquisite spatial resolution, and can be combined with fluorescent nanoparticles for tumour imaging as well as with Raman spectroscopy, as discussed elsewhere⁹³. Here we discuss optoacoustic and contrast-enhanced Raman imaging.

Optoacoustic imaging. Optoacoustic (or photoacoustic) imaging uses a combination of ultrasound and light to generate contrast in vivo. When molecules are excited by a pulse of light, varying amounts of the absorbed energy are transformed into heat. A pulsed laser therefore generates repeated thermoelastic expansion, each time emitting a sound wave that can be visualized via ultrasound imaging. By using a range of laser-excitation wavelengths and following a tomographic approach to illumination and detection, multispectral optoacoustic tomography (MSOT) exploits the optoacoustic effect to provide 3D images of light-absorbing molecules



Fig. 6 | Optoacoustic imaging techniques discriminate between multiplexed signals. a, Exogenous targeted contrast agents for MSOT can be decoupled from signals stemming from endogenous molecules. **b**, Endogenous contrast agents, such as oxyhaemoglobin and deoxyhaemoglobin, can be used to image tissue oxygenation. **c**, Dermal and subdermal blood vessels of different sizes can be detected using RSOM by post-acquisition filtering into two ultrasound frequency bands (red, larger vessels detected at 10–60 MHz; green, smaller vessels detected at 60–180 MHz). RSOM, raster-scan optoacoustic mesoscopy; EP, epidermis; CL, capillary loops; DR, dermis; VP, vascular plexus. Panels adapted with permission from: **a,b**, ref. ¹⁴⁴, RSNA; **c**, ref. ¹⁰⁶, Springer Nature Ltd.

in tissues⁹⁴. MSOT achieves markedly greater penetration depth (up to 5 cm) than purely optical imaging techniques (such as fluorescence), mainly because the generated ultrasound waves are not attenuated or scattered by tissue to the degree that photons are⁹⁵.

MSOT is ideally suited for imaging pigment-containing tissues such as melanoma because melanin is a strong light absorber and generates an endogenous optoacoustic signal. The outcomes of the first clinical trial of the use of MSOT to non-invasively discriminate metastasized lymph nodes from healthy lymph nodes were promising%. In a melanoma mouse model, MSOT was used to detect lymphatic metastases at an early stage ('in-transit metastases') when melanoma cells were still migrating from the primary tumour within lymphatic vessels towards the draining-lymph-node basin and before having reached the sentinel lymph node97. In addition to melanin, haemoglobin and other biological molecules in blood can provide endogenous contrast for MSOT. The spectral profile of haemoglobin absorption changes depending on whether the protein is bound to oxygen⁹⁸; therefore, measuring blood absorption with two or more wavelengths permits the mapping of blood oxygenation, allowing for the identification of hypoxic areas associated with tumours (Fig. 6) and the monitoring of the efficacy of vasculature-targeting therapies99,100.

Exogenous contrast agents for MSOT need to be strong light absorbers and have an absorption spectral profile that does not overlap with that of haemoglobin so that multi-wavelength excitation can be used to discriminate the signals from the exogenous contrast agent and from blood. Exogenous contrast agents for MSOT have been developed mostly for the imaging of tumours, pH sensing and targeting cell-death markers¹⁰¹⁻¹⁰³. Most often, infrared fluorescent dyes are used as optoacoustic contrast reporters; however, non-fluorescent light-absorbing agents generate a stronger photoacoustic signal because more incident laser energy is converted into thermoelastic expansion and therefore into sound waves^{104,105}.

By using the same principles of optoacoustic imaging and detecting a wide range of ultrasound frequencies, raster-scan optoacoustic mesoscopy allows for the detailed imaging of dermal and subdermal microvasculature, from which pathophysiological parameters can be derived on the basis of vessel diameters (Fig. 6c). Because microvasculature density is indicative of inflammation, the quantitative information obtained from broadband optoacoustic mesoscopy can be used to objectively extrapolate optoacoustic biomarkers for scoring systems that have been proposed for evaluating psoriasis plaques and diabetes¹⁰⁶.

Raman imaging. Raman imaging is an optical method where light scattered from a sample is collected and analysed. Raman scattering relies on energy exchange between the incident photons and the sample, and is therefore sensitive to the sample's molecular structure, so that different scattering spectra allow for the detection of specific chemical species¹⁰⁷. One limitation is that very few photons undergo Raman scattering (most photons experience elastic Rayleigh scattering); as a result, intrinsic Raman signals are much weaker than, for example, those emitted by fluorescence. To form an image, multiple spectra are needed for each pixel; however, because the signals of intrinsic Raman scattering are weak, the technique requires prohibitively long acquisition times for most in vivo applications. Related methods, such as stimulated Raman scattering (SRS)21,108-111 and coherent anti-Stokes Raman scattering (CARS)¹¹², can be used to increase the signal intensity. They allow for the fast imaging of intrinsic signals, but they can only image a single energy band at a time and do not provide the whole spectrum. SRS uses two lasers of different frequencies to excite bond vibrations of Raman energy shift corresponding to the laser-energy difference, whereas CARS makes use of coherent laser sources to excite vibrational energy levels corresponding to the energy of their laser interference patterns. Traditional Raman imaging, SRS and CARS can also be used in microscopy for the label-free high-resolution analysis of tissue sections and of other biological samples on the basis of intrinsic contrast¹⁰⁷.

Contrast-enhanced Raman imaging. Surface-enhanced Raman spectroscopy (SERS) holds great promise for ultrasensitive biomarker detection and cancer imaging¹¹³. Although it requires

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nanoscale contrast agents, it is based on a principle that is fundamentally different from those of many other nanoparticle imaging approaches¹¹⁴⁻¹¹⁸. In SERS, surface plasmons excited on the surfaces of noble metals massively amplify the Raman effect of nearby molecules without compromising signal specificity¹¹⁹. Nanoparticles can be synthesized with bright and distinct spectra (Fig. 7a), allowing for rapid SERS imaging. Such nanoparticles can be engineered as molecular probes and have been combined with a variety of imaging strategies¹²⁰⁻¹²³ in preclinical cancer imaging. Nanoparticles that do not contain toxic materials (such as quantum dots¹²⁴) have been used for intraoperative SERS imaging in animals to visualize tumour margins and residual tumours^{125,126}.

One main strength of SERS-based nanoparticles is their potential for multiplexed detection. Unlike fluorescence emission spectra, whose single and wide peaks make them hard to decouple, Raman spectra have multiple and fairly narrow peaks and serve as distinct spectral 'fingerprints'. In theory, by engineering molecularly targeted nanoprobes with bright and distinct SERS spectra, it should be possible to create fingerprints that would allow for high numbers of targets to be detected simultaneously. The feasibility of nanoparticle-based multiplexing in SERS has been shown in healthy mice with subcutaneously injected nanoparticles¹²⁷ (Fig. 7a,b).

Two notable applications of multiplexed imaging with SERS nanoparticles with potential for clinical translation have emerged. In particular, two high-performance SERS nanoprobes were used for the specific ratiometric imaging of ovarian cancer, after topical application in living mice (Fig. 7c). One probe was targeted to the folate receptor, which is overexpressed in some ovarian cancers, and a second non-targeted probe was used to account for non-specific background nanoparticle uptake. The spectral signals of the two nanoprobes were decoupled, and the ratio of the two probes was used to identify microscopic metastases¹²⁸. A similar ratiometric strategy employed up to four distinct targeted nanoparticles and one non-targeted control to identify breast cancer in excised samples from patients (Fig. 7d)^{129,130}. Ongoing research aims to increase the depth of detection via 'surface-enhanced spatially offset Raman spectroscopy'¹³¹, and to engineer faster Raman scanners^{132,133} and endoscopes¹³⁴ for use in humans.

Fig. 7 | Raman imaging with SERS nanoparticles. a, A selection of four SERS nanoparticles created with distinct spectral signatures can be combined for multiplexed imaging. **b**, In vivo multiplexed imaging of the four SERS nanoparticles, subcutaneously injected in a mouse at different picomolar concentrations, can be detected and discriminated via Raman imaging. The nanoparticles were detected via spectral unmixing and displayed in pseudocolour (the mixture of the four colours is displayed in brown and indicated by the white rectangle in the single-channel images). c, Ratiometric analysis of SERS nanoparticles targeted to the folate receptor (FR) reveals microscopic ovarian metastases (visible in red) against a background (blue) of non-targeted SERS nanoparticles in a mouse model of ovarian cancer. The base-10 logarithmic ratio allows for the representation of signals over four orders of magnitude. d, Multiplexed imaging of a human lumpectomy breast cancer specimen topically stained with five distinct nanoparticles (four of them targeted to the proteins EGFR, HER2, CD44 and CD24, and one being a non-targeted control). Ratiometric Raman imaging (of each targeted nanoparticle versus the control) can be used to determine the relative quantification of each biomarker, which can be validated via staining by immunohistochemistry (the dashed outlines indicate the area of a detailed view shown in the original paper). This information can be combined to detect the presence of residual tumour tissue around surgical resection margins. EGFR, epidermal growth-factor receptor; HER, human epidermal growth-factor receptor; CD44 and CD24, cluster-of-differentiation molecules. Panels adapted with permission from: **a,b**, ref. ¹²⁷, AAAS; **c**, ref. ¹²⁸, American Chemical Society; **d**, ref. ¹³⁰, Wiley-VCH.

Emerging optoacoustic and optical techniques should see further clinical development as their use in patients is approved. For optoacoustic imaging, the hurdles are lower as certain studies can be conducted without the need for an exogenous contrast agent. For SERS imaging, the requirement of the administration of a nanoparticle contrast agent demands separate regulatory approval. However, unlike many other nanoparticle agents, these can be designed using inert materials such as gold and silica, and several gold or gold–silica nanoparticles for therapeutic uses are being tested in advanced clinical trials. Studies examining SERS nanoparticles have shown favourable toxicity profiles¹³⁵; however, clinical trials will have to be designed to show non-inferiority, or even superior efficacy, over existing probes. Also, nanomaterial probes for multiplexed imaging can often be synthesized as theranostic agents; many are being developed and tested preclinically^{136–142}.



Outlook

The simultaneous imaging of more than one molecular target in a single scan would provide valuable clinical information (Fig. 1a), and may also reduce the time and costs associated with separate measurements. Multiplexed imaging could be further developed for the detection of whole signalling pathways or for multi-omics applications. Work in preclinical models suggests that, in principle, multiplexed imaging of signalling pathways is feasible. Nevertheless, the process of obtaining regulatory approval for use in humans presents substantial hurdles. Firstly, multiplexed imaging techniques may require the administration of multiple contrast agents, each requiring its own trials and approval. Also, serial imaging approaches may increase scan times beyond what a patient is expected to withstand. Additionally, the interpretation of multiple channels of data will require complex data processing, which although useful may not be immediately apparent from a clinical perspective. Therefore, to effect multiplexed clinical imaging, the patient benefits will need to be strong and not only limited to reducing the number of tests. The design of well-controlled prospective observational clinical trials that test the efficacy of diagnostic techniques for multiplexed imaging (such as computational methods to interpret the multichannel imaging data and to provide a meaningful diagnosis), will be of upmost importance, and should be coupled to interventional clinical trials where multiplexed imaging is used to guide a treatment or a surgical procedure. The roadmap for the development and implementation of multiplexed imaging follows the successful roadmap for the testing of combinatorial therapeutics and should drive innovation in radiology.

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Author contributions

All authors contributed to literature research, and to the writing and editing of the manuscript.

Competing interests

R.W. has received consultancy payments from ModeRNA, Tarveda Pharmaceuticals, Alivio Therapeutics and Accure Health, and is a shareholder of T2Biosystems, Lumicell and Accure Health. All patents associated with R.W. have been assigned to and are handled by the Massachusetts General Hospital. M.F.K is a co-founder of RIO Imaging, which did not contribute to this manuscript. All patents associated with M.F.K. have been assigned to and are handled by Stanford University or Memorial Sloan Kettering Cancer Center, respectively. C.A. declares no competing interests.

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