Maps of in vivo oxygen pressure with submillimetre resolution and nanomolar sensitivity enabled by Cherenkov-excited luminescence scanned imaging

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Low signal-to-noise ratios and limited imaging depths restrict the ability of optical-imaging modalities to detect and accurately quantify molecular emissions from tissue. Here, by using a scanning external X-ray beam from a clinical linear accelerator to induce Cherenkov excitation of luminescence in tissue, we demonstrate in vivo mapping of the oxygenation of tumours at depths of several millimetres, with submillimetre resolution and nanomolar sensitivity. This was achieved by scanning thin sheets of the X-ray beam orthogonally to the emission-detection plane, and by detecting the signal via a time-gated CCD camera synchronized to the radiation pulse. We also show with experiments using phantoms and with simulations that the performance of Cherenkov-excited luminescence scanned imaging (CELSI) is limited by beam size, scan geometry, probe concentration, radiation dose and tissue depth. CELSI might provide the highest sensitivity and resolution in the optical imaging of molecular tracers in vivo.

Optical luminescence is the dominant preclinical imaging modality for the molecular probing of cells and tissue physiology, both in vitro and in vivo1–7. The sensitivity of optical detectors to small concentrations of molecular emitters and the ease of use of the detection technology make optical molecular imaging much more widely used than X-rays, ultrasonography, nuclear medicine or magnetic resonance imaging (MRI). However, because of elastic scattering of light in tissue, the tissue depths accessible by optical imaging in vivo are fundamentally limited8–10. Although fluorescence and bioluminescence have been used extensively to elucidate intracellular signalling in vitro, their use in vivo is largely limited to bulk temporal kinetics, surface tissue imaging8–9 or invasive/surgical measurements10. The inability to reconstruct the spatial origin of a detected photon generally limits non-invasive in vivo luminescent molecular imaging, where high spatial resolution has never been achieved beyond microscopy. Additionally, the decrease in signal intensity with depth into tissue is exponential, creating highly non-linear excitation during imaging, as illustrated in Fig. 1a.

Here, Cherenkov-excited luminescence scanned imaging (CELSI)11,12 was used to sense luminescent molecular probes deep within tissue, using megavoltage (MV) X-ray radiation, and adopting an orthogonal excitation geometry that allows scanning of the region of interest, similarly to the way fluorescent light sheet microscopy works12, as shown in Fig. 1b. As the illuminated area is consistently at a known distance from the detector, this geometry maximizes the signal-to-noise ratio, which tends to be the dominant factor in quantitative imaging of thick and optically turbid samples. Importantly, Cherenkov light of organic molecules is based on the electronic energy level of the excitation beam. Thus, with high MV X-ray sheet illumination, the spatial targeting of X-rays can be optimally combined with biological organic probes already widely used in animals for other imaging modalities, and is translatable to humans. CELSI is intended as an optical imaging tool that can preserve the linearity of the emitted optical signal with depth, and is not overly affected by scatter dominance in tissue. Further illustration of the value of CELSI relative to other imaging modalities can be seen in the Supplementary Fig. 1. The geometry used here is illustrated in Fig. 1c and temporal and spatial parameters in Fig. 1d.

Cherenkov light is generated in tissue from the scatter-induced secondary electrons produced from X-rays, here generated by a therapeutic MV linear accelerator (LINAC), that interact with the dielectric media. The spectrum of Cherenkov light is broadband, peaks in the ultraviolet (UV) and decays in intensity (I) with a wavelength dependence of \( I \propto \lambda^{-1/2} \) (Fig. 1c). Luminescence of the probe is therefore excited only within the volume that is directly in the path of the scanning radiation beam (Fig. 1b), so that all luminescent signals detected by the intensified charge-coupled device (ICCD) are considered as originating exclusively from within the pathway of the LINAC beam. This geometrical arrangement adopts some of the principles of directed excitation imaging tools, such as ultrasound, where depth-dependent attenuation correction can be applied to the data because of knowledge of the excitation or emission beam depth. The limiting factor remains the intensity attenuation with distance by diffusion scatter and absorption as the emitted light passes through the tissue. The luminescent probe used here, Platinum Oxyphor G4 (PtG4),

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Deep-tissue Cherenkov excitation from ionizing radiation of isotopes can also excite molecular luminescence\(^{11,15}\), with the signal increasing proportionally to the emitted \(\gamma\)-ray energy. Similarly, radiation delivered by a medical LINAC generates Cherenkov emission within the treated tissue\(^{17}\) and can be visualized for patient dosimetry studies\(^{14}\), as well as excitation of molecular probes\(^{19,20}\). This process can be used to excite fluorescent or phosphorescent probes in tissue to sense diagnostic molecular features, and has been demonstrated at low radiation doses\(^{18}\), where high-energy MV photons have the highest generation of Cherenkov emission, yet the lowest deposited dose level in tissue. The excitation light from Cherenkov emission is directed by the incident beam, and so the observation of emission need not be highly localized because the signal is backprojected along the line of the Cherenkov beam. This also enables attenuation correction to be readily applied to the signal, based on the depth of the beam. Additionally, altering the orthogonal angle of the excitation beam to the collection plane allows some advantages in contrast-to-background improvement, as used in thick tissue fluorescence microscopy\(^{12,21}\). This geometry makes CELSI’s approach to signal measurement much more useful for whole body imaging, as it eliminates the need to estimate the depth of the optical signal origin.

The aim of this work was to evaluate the potential for Cherenkov excited luminescence scanned imaging to directly sample partial pressure of oxygen \((pO_2)\) in vivo for mammalian tissue, estimating the spatial resolution, depth and probe concentration sensitivity bounds, and estimate the contrast-to-background values available for realistic tumour imaging applications.

**Results**

**Beam size.** By measuring total luminescence signal and considering prior information about the position of the scanning beam, the distribution of optical signal along the direction of scanning can be recovered. In Fig. 2a–c, the possible beam geometries are illustrated, where the \(X\)-ray sheet can be shaped as a broad beam covering the entire tissue volume (as is commonly used in radiotherapy), or a 2D sheet of 5 mm thickness, or a pencil beam of 5×5 mm\(^2\), which can be raster scanned around the tissue. The benefit of a pencil beam would be the highest spatial resolution, in step sizes of 0.1 mm, whereas the benefit of a broad sheet is the fast imaging acquisition. However, the choices here make a large impact on the image quality and signal strength. In Fig. 2d, a broad homogenous phantom was imaged with a large square beam of 100×100 mm\(^2\), and then by a thin sheet beam of 5×100 mm\(^2\), and then a pencil beam of 5×5 mm\(^2\), as described in the Methods. The phantom had an embedded luminescent square of 10×10 mm\(^2\), at depth within it, to test for signal recovery, and the contrast-to-noise ratio (CNR) was plotted in the region of interest (ROI1) relative to the background (ROI2) (Fig. 2e) for each of the three cases at different depths of the object.

**Imaging geometry.** Radiation beam energies are carefully chosen to reach specific depths in the body, and a set of beams from different gantry angles can be delivered to the patient from these planned entrance positions. In general, arbitrary angles, beam energies and dose values are chosen based on an optimization algorithm to maximize dose to target regions and minimize dose to organs at risk of radiation damage. It is this flexibility of targeting that makes radiation-based excitation flexible for molecular imaging. The simplest angular geometries orthogonal or lateral to the subject are shown in
Fig. 2 | Radiation beam shape configuration and region of the tissue where Cherenkov light is generated affects contrast to noise measured. a, A single broad beam crossing the 3D volume of the animal generates broad Cherenkov emission. b, c, A 2D sheet beam generates an axial plane through the mouse (b), whereas a 1D pencil beam generates an axial excitation line (c). These geometric choices affect the possible contrast-to-noise ratio (CNR), and hence the sensitivity of other parameters such as minimum concentration and depth of imaging feasible, as well as acquisition time. d, Images from a phantom of the field light position, the Cherenkov emission and the luminescence of a single square target embedded in the phantom are shown for the geometries in a–c (duplicate images taken during separate imaging sessions show near-identical image quality). e, The CNR of the target relative to the background as measured by individual scans, where each data point represents an individual acquisition. Colour bars (d,e), detected photons cm⁻² s⁻¹. Scale bars, 1 cm.

Fig. 3a,b, where the beam could be placed roughly orthogonal to the camera direction for sheet-like imaging, or aligned along it for epi-illumination. While the range of possible angles is much greater than this, these were chosen as the extremes of contrast-to-background, as will be shown. A complete analysis of the effect of geometry on the singular value index, matrix condition number and matrix rank, for the Jacobian to be inverted, is shown in Supplementary Fig. 3, using coefficient values listed in Supplementary Table 1.

The experimental set-up was photographed and is shown in Fig. 3c,d, and a resulting luminescence image of a single 1 mm diameter capillary filled with 500 μM PtG4 at 5 mm depth into a tissue phantom solution is shown for each geometry in Fig. 3e,f, respectively. The phantom was a broad flat liquid media of 200 × 200 mm², with the single luminescent capillary within it. A scanned sheet beam was used for imaging with approximately 30 mGy per position of the beam. The colour bar for each result is the same and so the significantly higher background near 9,600 counts leads to a contrast ratio of 1.14 and CBR value of 14% for the epi-illumination, while the lower background near 2500 counts is seen for lateral illumination, leading to a contrast ratio of 3.5 and CBR of 220%. The higher background comes from the fact that the entire volume being imaged is being excited by Cherenkov, and so the signal comes from a mix of depths below the surface. Because of this observation, the lateral excitation geometry was used throughout most of the next experiments, providing a depth-selective excitation. A detailed analysis of the centroid position error and full width at half maximum (FWHM) values of recovered regions is shown in Supplementary Fig. 4.

Dose, concentration and depth of imaging. The dominant factors affecting image recovery were explored systematically by varying one parameter at a time (Fig. 4). The contrast-to-background ratio (CBR) was used as a metric of success, assessing how this varied with radiation dose delivered, concentration of PtG4 and depth of the object into the medium. This figure of merit was chosen because CELSI is a background-dominated imaging geometry, as are most luminescent and fluorescent imaging modalities22. CELSI of PtG4 in a 1 mm diameter capillary was assessed with different concentrations, depths (distance from capillary to phantom surface) and radiation doses. First, using a fixed depth of 5 mm, the concentrations range was varied logarithmically from 500 μM down to 0.78 μM inside a 1 mm capillary, measuring CBR for a 1 s acquisition time for a dose of 0.1 Gy, with the overlapping excitation volume being 3.9 × 10⁻⁴ l, making the number of moles used vary from 2.0 μmol down to 3 nmol. The results are shown in Fig. 4a, being monotonically in shape and reducing to a CBR of 1 at about 6–8 nmol. Next the CBR was tracked for a fixed concentration of 500 μM in the 1 mm capillary, for 200 nmol in the excitation volume, varying the depth down to 20 mm into the phantom, also using a 0.1 Gy dose (Fig. 4b). This was monotonically with depth and appears to decrease near CBR = 1 well beyond the 20 mm depth. Finally, the variation with radiation pulses added together was studied at a target depth of
5 mm and fixed concentration of 100 μM, or 2 nmol, showing monotonic dependence on dose in Fig. 4c, and decreasing to CBR of 1 at 2 radiation pulses, roughly equivalent to a 1.67 mGy dose.

Scan directions and tomographic reconstruction. The direction and range of scan parameters in CELSI is quite large, and three particular geometries were examined as analogous to other tomographic systems, and also used to compare CELSI to fluorescence tomography systems. First, a study of fluorescence tomography as acquired in the epi-illumination geometry was completed, as well as transmission geometry. For this, 1% noise was randomly added to the simulated forward data with either a single embedded object or the 3 objects, as shown in Fig. 5. The stopping criteria for the iterative reconstruction was when either a change in projection error of less than 0.01% was reached between iterations or the maximum iteration number of 40 was achieved. A Cartesian pixel basis of 30 × 30 squares was used in Fig. 5, and shown in Fig. 5a,b respectively with the test object shown; reconstructed images are shown in Fig. 5d,e. The CELSI tomography image with lateral excitation and vertical direction is shown in the schematic (Fig. 5c) and reconstructed object in Fig. 5f. In this case, CELSI signal is shown to improve signal detection compared to epi-illumination fluorescence tomography, and has higher contrast than transmission-based fluorescence tomography, with further numerical analysis of the Jacobian matrix improvement shown in Supplementary Fig. 3.

Next, the range of possible scans available with CELSI was explored to investigate how it would affect image recovery. As shown in Fig. 5g–i, the horizontal, vertical and diagonal aspects of a scan can more fully interrogate the image space, and reconstructed images for these three respective geometries are shown in Fig. 5j–l, with recovery from the combination of all of them shown in Fig. 5m. From these simulations, it is obvious that the excitation line directions distort the recovered objects in the axial direction of the source–detector lines. As with all tomography applications, the larger the range of angles sampled, the better the image recovery can become (Fig. 5m), better resolving the object in all directions. In this numerical study, three angles were used, as these were sufficient to resolve the three objects, yet in principle an unlimited number of angles could be used, limited only by the added dose given to the subject. So, as in CT imaging, ultimately there is a dose-resolution–contrast tradeoff that dictates the imaging system performance.

Spatial resolution. Both experiments and simulation studies were completed to assess the ultimate limits to spatial resolution with optimized conditions. The reconstruction set-up and convergence criteria were the same as in the previous section, but a larger pixel basis of 100 × 100 pixels was used to improve the quality of the reconstructed images in Fig. 6 (and Supplementary Fig. 4). Simulation studies were carried out with varying depths and using a standard resolution test, varying the distance between two small objects (Fig. 6a). The reconstructed images are shown with intensity in terms of the reconvened yield, as defined by the PtG4 absorption coefficient multiplied by the quantum yield of emission. The results reveal that when the depth of inclusions was smaller than 5 mm, the CELSI tomography has the ability to discriminate the two inclusions with edge-to-edge distance of 0.1 mm (Fig. 6b). When the depth of inclusions was increased to 2.5 mm, the two inclusions could still be discriminated with edge-to-edge distance but nearer 0.5 mm, and when it was increased to 3 cm, the two inclusions could be discriminated with edge-to-edge distance but nearer 1.8 mm.
The results show that CELSI tomography can yield high spatial resolution, but that this spatial resolution does degrade with depth into the medium, as expected, due to the loss of light penetration depth at these wavelengths. These CELSI results can be compared to epi-FT and full-FT results, which have significantly lower spatial resolution, ranging from a few millimetres to beyond 10 mm at depths of 15 and 25 mm into the medium. Fig. 6b shows the minimum spatial resolution as a function of depth of inclusions. Epi-FT could not reconstruct the inclusions accurately when the depth of inclusions was deeper than 15 mm, and so the spatial resolution was not calculated.

Experimental studies were carried out to attempt to match the simulations, but using a simpler geometry of a single capillary rod positioned at 5 mm into the tissue, simulating phantom medium. A range of capillaries with diameters varying from 1 mm down to 0.1 mm were used and filled with PtG4 at 50 μM. The FWHM recovered was extracted and the observed FWHM is plotted against the true value in Fig. 6d, showing nearly perfect linearity and matching the expected value of the smallest spatial resolution tested (100 μm).

Rodent and phantom imaging for sensitivity and resolution testing. To verify that imaging in a complex tissue shape would be possible, the XFM-2 phantom was imaged with 1 mM PtG4 within 7μl at the tip of the cylinder (for 7 nmol total PtG4) inserted into the central part of the body. For these mouse phantom experiments, 35 radiation sheets were used, and the vertical step of each sheet was approximately 0.52 mm. The 3D forward simulation mesh contained 18,496 nodes, 93,818 elements and 1,712 boundary nodes, and the absorption and reduced scattering coefficients for excitation and emission were both set at μs = 0.007 mm−1 and μa = 1.0 mm−1. The reconstruction pixel basis was 25×25×25 voxels. The initial guess of regularization parameter was 10, and the convergence criteria to stop the iterative update was when a change in projection error was less than 0.01% between successive iterations or when the maximum number of iterations was reached at n = 10. The recovered luminescence image from above is shown in Fig. 7a, with the Cherenkov beam in the plane of the tube, the luminescence from the tube showing a blurry image and FWHM of 10.4×11.9 mm in the two orthogonal directions. This signal is superimposed on the white light image of the mouse phantom. Reconstructed images are shown in the 3 orthogonal directions superimposed on CT scan images of the phantom (in grey scale). This recovery showed good localization and recovery of the shape as might be expected (Fig. 7b), with FWHM values of the reconstructed inclusion of 5.1×5.1×4.7 mm in total. Note that these dimensions are less than half of the FWHM values of planar projection imaging.

To further advance and test the performance of CELSI reconstruction, an in vivo experiment with breast cancer MDA-MB-231 xenograft tumours in a mouse was performed, with two separate injection sites on each of the mouse hind limbs. The mouse was injected with 50 nmol of PtG4 into the right-side tumour and 10 nmol into the left-side tumour, using a 50μM stock solution. These doses were specifically chosen to be just above and just below the detection threshold, respectively. Given the sizes of the tumours (9×9×6 mm3 ≈ 500 mm3), these corresponded to approximately 0.4 nM and 0.10 nM, when averaged over the tumour volume.

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Fig. 4 | Contrast-to-background ratio is affected by the concentration and depth of the object, and the radiation dose used in scanning. a-c. Data points are shown for increasing concentrations of PtG4 in the physiologically relevant range (a); increasing depth between the capillary and the surface of the phantom imaged (b); and increasing radiation dose between 1 and 500 radiation pulses, using 1.67 mGy per pulse (c). For each graph, the inset shows the line scan of the raw data. Each individual data point represents the extracted contrast-to-background ratio for a single scan of a test object. The red lines in each graph illustrate the linear trendline of the data on the log-log plots. d. The factors affecting CELSI signal strength are schematically illustrated as reciprocal in their effect on the contrast-to-background ratio, as illustrated by the red lines.
The voxel basis for reconstruction was 25 \(^{\mu}\)m. The forward simulation was carried out with a step between them of 0.8 mm. The optical properties of tissues in the mouse were estimated by average homogeneous values for a mouse, based on published values\(^{17}\). For these in vivo experiments, the mouse was scanned with a sheet illumination procedure. A total of 19 irradiation sheets were used with a step between them of 0.8 mm. The forward simulation mesh contained 13,185 nodes, 63,852 elements and 3,716 boundary nodes, and the absorption and scattering coefficients for excitation and emission were both set to \(\mu_a = 0.01 \text{ mm}^{-1}\) and \(\mu_s' = 1.0 \text{ mm}^{-1}\). The voxel basis for reconstruction was 25 \(^{\mu}\)m \times 25 \(^{\mu}\)m \times 25 pixels, and the optical properties of tissues in the mouse were estimated by average homogeneous values for a mouse, based on published values\(^{17}\).

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**Rodent imaging for \(P_{O_2}\) sensing.** A final murine experiment to map out \(P_{O_2}\) was carried out in mice with subcutaneous MDA-MB-231 tumours. A total of 4 animals and 8 tumours were imaged (2 tumours per animal), with local injections of 50 \(\mu\)l of 25 \(\mu\)M PtG4 (a total of 1.25 nmol per tumour). Each mouse was imaged while alive and then images were repeated 30 min after euthanasia, when the drop in blood circulation and respiration causes a marked decrease in \(P_{O_2}\) values. Temperature was controlled using a heating pad: 35.1 \(\pm\) 1.5 °C for live mice and 31.9 \(\pm\) 1.2 °C for dead mice. The CELSI scan was completed vertically (see Supplementary Video 2 for the experimental data capture). Images of the luminescence at different delay times between LINAC pulse and emission captured are shown in Fig. 8a. These were used for each mouse to create images of lifetime (Fig. 8b) and with the Stern-Volmer equation, the tissue \(P_{O_2}\) (Fig. 8c). The summary of total lifetimes and \(P_{O_2}\) values are shown in Fig. 8de, respectively, as box and whisker plots. The entire range of values in live or dead animals do not overlap, indicating a significant difference in both lifetime and \(P_{O_2}\) (\(P < 0.001\) for each). But, more importantly, the range of values is \(\pm 30\%\) in lifetime, and \(\pm 40\%\) in \(P_{O_2}\) value, as would be expected in heterogeneous tumours.

**Discussion**

Optical imaging of tissue has the potential for retrieving large amounts of molecular information, but image recovery has been plagued by the scattering of light in tissue, which makes the signal nonlinear and affected by the shape of tissue, its optical properties and its layers and regions. In practice, the signal-to-noise ratio is often unacceptably low, and doesn’t provide appreciable depth into the tissue. CELSI can use high-energy radiotherapy beams to launch light directly into the tissue, and by the knowledge of where the light was placed, it can allow high-resolution recovery of luminescent sources. Although this approach to imaging has been introduced in the past few years\(^{10,11}\), the factors that dictate the performance have not been exhaustively analysed, and nonlinear tomography has not been applied to fully yet the imaging-contrast recovery and spatial resolution. In this study, these issues have been fully examined, and the capabilities of this type of imaging can now be established for \(P_{O_2}\) imaging of tumours. A steered beam that provides localization of the excitation has inherent value, as it allows avoiding organs at risk and multiplexing the beam to multiple animals or multiple tissue volumes. The geometrical demonstrations of scanning explored in this study form the basis of what can be done with a LINAC that is designed for highly conformal radiotherapy delivery.

The beam shape and angle of orientation have perhaps the largest effect on the signal, with larger beam shapes having substantially larger signals and resulting contrast-to-background ratio, as shown in Fig. 3d, presumably based on the fact that the light scatters and builds up within the tissue. Additionally, there is an output...
factor for the beam that reduces with smaller beam sizes, and so while smaller or thinner beams provide the best spatial resolution (as shown in Fig. 3), they inherently provide the lower signal intensities as well. The direction of the beam relative to the camera is perhaps the second most important factor, as a separation of the source from the surface being imaged inherently improves the observed contrast by suppressing background signals from surface tissues (as illustrated in the images of Fig. 4). For these reasons, much of the work following these two initial studies focused on using the lateral beam geometry predominantly, and with a wide sheet of radiation, to maximize build-up and light irradiance in the tissue. It is this localization of the excitation beam that provides the inherent value of CELSI relative to isotope-based Cherenkov excitation studies. In isotope-based molecular sensing, the sensitivity can be as high, or even higher, right down to the sub-nanomole range, but the ability to reconstruct and localize the emission is not dictated by anything other than the detected emission. In CELSI imaging, the high-precision knowledge of the beam allows both deconvolution and depth-dependent attenuation correction, leaving a reconstructed signal that is highly localized in space to better than 1 mm accuracy throughout the imaged volume of tissue.

Testing the key performance factors beyond these initial choices leads to a complex array of possible parameters. The radiotherapy dose, the probe concentration and the depth into the tissue each contribute to the detected signal in ways that would have reciprocity, as illustrated in Fig. 4d. The imaging of luminescent targets is possible within the range of concentrations, doses and depths that are suitable for small-animal imaging. The molecule number ranges near μM are typical for metabolites and higher concentration receptors, and the ability to resolve uptake within many millimetres of tissue is an important application in preclinical imaging. Within the volume of excitation, this corresponds to sensitivity needed near μmol to nmol levels. Radiation doses used here have been in the range of X-ray exams, so although the concept of using a linear accelerator for imaging appears dangerous from a radiation safety perspective, it is critical to appreciate that this is done with very a very low dose delivered. Additionally, the radiation dose is selectively deposited only where the scanned beam passes, so radiosensitive organs or dose-limiting volumes could be strategically avoided in an optimized scan.

A goal of this work has been to establish the feasibility of high spatial resolution several millimetres into animal tissue. The spatial resolution is largely dictated by the precision with which the X-ray beam can be controlled, because the signal origin can be localized to where the beam excited the luminescent agent. The imaging tests, both experimentally and computationally, support the belief that the spatial resolution is in the range of 100–300 μm for objects as deep as 5 mm into the tissue. Computationally, this spatial resolution appears to be resolvable down to 20 mm depth, and this fundamental limit is a fascinating goal for deep-tissue imaging, as it essentially implies achieving near-microscopic resolution deep into tissue with optical imaging. This realization is fundamental in molecular sensing in deep tissue.

Finally, the tests used with mouse phantoms and mouse tumours demonstrate both the localization concepts and how the accuracy of reconstruction is superior to diffuse tomography and to superficial MIP images of luminescence. This latter comparison to superficial imaging is important, because the vast majority of preclinical luminescence imaging is done with epi-illumination box systems, where a single superficial surface image of the animal is obtained.

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**Fig. 6 | The spatial resolution of CELSI is below 1 mm down to depths in tissue of 25 mm.** Simulated data for a pair of 5 mm diameter inclusions containing 50 μM PtG4 with a fixed edge-to-edge distance, placed at increasing depths in a phantom. a, For each depth, data was reconstructed for epi-illumination fluorescence tomography, diffuse fluorescence tomography and CELSI tomography. Data of representative simulations are shown in units of luminescent yield (μmol, colour bar). Scale bar, 30 mm. b, The minimum resolvable distance between the two inclusions was estimated for each geometry and illustrate CELSI’s near-linear resolution at depths of up to 30 mm. c, Capillaries, varying from 0.1 mm diameter up to 1.0 mm diameter, filled with 50 μM of PtG4 were embedded into a tissue mimicking phantom. The phantoms were scanned using CELSI. d, The reconstructed data was used to extract the full-width-at-half-maximum (FWHM) value for each tube size. Each data point reports a single measurement, and fitting was completed with $r^2 > 0.9$. 

| Capillary diameter (mm) | Minimum spatial resolution (mm) |
|------------------------|-------------------------------|
| 0.1                    | 2.5                           |
| 0.2                    | 2.0                           |
| 0.5                    | 1.5                           |
| 1.0                    | 1.0                           |

| Capillary diameter (mm) | Observed deconvolved FWHM (mm) |
|------------------------|-------------------------------|
| 0.1                    | 2.5                           |
| 0.2                    | 2.0                           |
| 0.5                    | 1.5                           |
| 1.0                    | 1.0                           |
Being able to bring optical imaging to a significantly higher spatial resolution throughout the animal’s whole body would have a profound impact in molecular imaging. The mouse phantom tested the potential of CELSI imaging in conditions matching the geometry, size and average tissue optical properties of a mouse, where the reconstruction of the embedded 7 μl of PtG4 was found to be spatially accurate. CELSI imaging was then tested in the heterogeneous tissue conditions of a mouse in vivo, addressing the lack of realistic internal heterogeneities in the phantom. The more detailed CELSI images of tumour oxygenation shown in Fig. 8 illustrate how this method can be used to sense tumour $pO_2$, and potentially with high spatial resolution. The features mapped out in this figure have sub-millimetre lateral spatial resolution, showing the heterogeneity of the $pO_2$ ranging from 40–90 mmHg, and confirms existing reported values for these tumours. The heterogeneity seen within intratumoral regions and between the tumours on the left and right flanks presumably illustrates areas of high and low $pO_2$ variation. Areas that are high $pO_2$ are thought to be closer to blood vessels whereas those with lower $pO_2$ are consistent with areas distal from blood supply. The ability to map $pO_2$, pH and other features such as enzyme concentrations, protein expression, cell-receptor density or metabolites would be valuable to understand responses to therapy. This methodology will be most important for molecular features at higher spatial resolution, where tumour heterogeneity plays an important factor in therapeutic response.

In molecular imaging, there can be a separation between the physical capabilities of the imaging system and the pharmacokinetics and localization of the biochemical targeting moiety. Yet, the value of a system is defined by the intersection of these two properties, where the physical resolution and contrast of the biochemical agent gets localized in biological features of the tissue. In the current realization of CELSI, the agents were delivered directly into solid tumours, avoiding the issue of plasma pharmacokinetics and tumour retention. As such, the chosen focus here has been largely technological development, combined with localized delivery of the agent to tumours. While there are clear limitations to this, the approach does still match with radiotherapy delivery as well, where the location of delivery is well planned as targeted to the treatment volume. As such, the compound has its limitation at the site of injection, the knowledge of the oxygenation of these radiotherapy target sites is still of considerable value to radiation sensitization. Thus, we present the application

Fig. 7 | Animal phantom tomography and in vivo validation of the luminescence yield. a, Image for the luminescent yield of a 7 μl inclusion placed within an animal phantom and overlaid on the surface extracted from the microCT image. Colour bar, photons cm$^{-2}$s$^{-1}$. b–d, CELSI reconstructed images are shown with three orthogonal views: axial (b), sagittal (c) and transverse (d). Colour bar, μμaf. In vivo CELSI is shown from a single representative animal carrying two subcutaneous MDA-MB-231 tumours on the hindlimb flanks (arrows); 50 nmol PtG4 was injected into the tumour indicated with a blue arrow, and 10 nmol PtG4 was injected into the tumour with the orange arrow. The entire body of the animal was scanned and reconstructed with CELSI. e, An X-ray CT scan is overlaid to the summed intensity projection image of the luminescence. Colour bar, photons cm$^{-2}$s$^{-1}$. f–h, The reconstructions from the CELSI tomographic data are overlaid on the high resolution microCT images in axial (f), sagittal (g) and 3D perspective views (h). The reconstructed image shows recovery of the 50 nmol injection while the 10 nmol injection is not recovered; the data illustrate the detectable limit is in the range of tens of nanomoles. A full rotating 3D video of this mouse is available in Supplementary Video 7. Scale bars, 1 cm.
of localized oxygen sensing with high-resolution CELSI in this format with clear potential to be further advanced by improvements in the biotargeting of luminescent reporters. Recent studies have examined multiple agents for Cherenkov-excited sensitivity with fluorescence\(^{14,20,25}\) and for the capability of detecting multiple emitters at the same time\(^{26}\)—yet in all of these studies, PtG4 has been shown to be the most sensitive probe in terms of lower limit on concentration. Also, with its design for oxygen sensitivity from quenching, which is accessed by measurement of emission lifetime, it also provides one of the most practical tools for radiation therapy, where tissue oxygenation is known to be an important factor in therapy efficacy. However, there is also potential for other lifetime phosphor sensing agents such as pH\(^{27,28}\).

**Outlook.** Cherenkov-excited luminescence scanned imaging (CELSI) allows \(p_O^2\) imaging at a spatial resolution close to 0.1 mm, detected at tissue depths of several millimetres through an intact living mouse, by using nanomolar levels of an oxygen-sensitive biological probe. X-ray beams from a linear accelerator are shaped into thin sheets to scan a field of view in arbitrary directions, and collect the Cherenkov emissions via time-gating of the captured luminescence, enabling molecular sampling of a number of agents relevant to the tissue microenvironment. The depths and signal recovery possible are suitable for applications using small-animal biomedical imaging models. Results from computer simulations and experimental studies suggest that, when fully automated, CELSI may have the highest sensitivity and spatial resolution of any preclinical whole-body molecular-imaging system.

**Methods**

**LINAC and Cherenkov imaging.** Cherenkov was induced by a linear accelerator (Varian Linac 2100CD, Varian Medical System) based at the Norris Cotton Cancer Center, at Dartmouth-Hitchcock Medical Center. The LINAC multi-leaf collimator...
outside the ROI. In some studies, the contrast-to-noise ratio was used as a metric, which was defined as: \( CNR = (I_{ROI} - I_{BG})/\sigma_B \), where \( \sigma_B \) was defined as the standard deviation of intensities in the background region.

Spatial resolution assessment. The spatial resolution of imaging was assessed with a range of glass capillary tubes of varying inner diameter, from 0.1 mm up to 1.0 mm (Hampton Research Corp, glass number 50 capillaries). These tubes were placed into tissue simulating phantom at 5 mm depth one by one, and all were filled with 50 \( \mu \)l PtG4 for a high contrast target for resolution assessment. The sheet radiation beam was scanned laterally across the tube, to vary the signal with position, and the linescan full width at half maximum (FWHM) was recovered for each capillary experiment.

Computed tomography recovery. CELSI data can be scanned for different beam angles and the performance expected varies considerably based on these choices. There are many options is so large that experimental study of these is prohibitive, and so a series of computer simulations were carried out, in particular to compare CELSI images to blurred light images where traditional light excitation is used as compared to Cherenkov excitation. Additionally, different combinations of viewing angles and different depths and sizes of objects could be simulated much more quickly than carrying out all the experiments. The computer simulations were carried out using the NIFAST software package (available at www.nirfast.org), used to model light transport in tissue and carry out regularized iterative reconstruction of the luminescent reporter in the medium. The recovery of the reporter was in terms of units of luminescent yield, \( \eta \mu \text{M} \), where \( \eta \) is the reporter quantum yield for phosphorescence emission and \( \mu \text{M} \) is the absorptivity of the reporter (see the Supplementary Information for further explanation of the theory). This quantity is a direct report of the amount of light produced per unit volume. To assess spatial resolution of CELSI tomography, a classic resolution test was performed with two small inclusions and varying the spatial distance between them and assessing when they could not be resolved as separate objects. The edge-to-edge distance was varied from 0.1 mm to 5 mm, in the plausible range of limiting spatial resolutions given the physical constraints of the LINAC MLCS used. The yield contrast of inclusion was set as 1:1. A finer mesh with 6161 nodes and 12,000 linear triangular elements was used in reconstruction. Inspired by the results of combining scan modes, the following reconstructions were performed by summing up scan modes.

Animal phantom imaging. In order to assess performance in a more complex tissue geometry, a commercially available mouse phantom was used (Xfm-2, PerkinElmer Health Sciences), using rod of material inserted which was filled with 1 mM PtG4, in 7 \( \mu \)l PBS. The phantom has tissue optical properties within the NIR wavelengths 650–800 nm, approximated by \( \mu = 1.5–0.9 \text{mm}^{-1} \) and \( \mu' = 1.5–0.9 \text{mm}^{-1} \) (manufacturer-supplied data sheet). This phantom was created for phantom studies which would allow imaging in a realistic tissue volume and geometry that directly mimics the shape and absorption and reduced scattering coefficient values representative of murine tissue. The CELSI imaging was carried out with lateral beam excitation and vertical emission capture, using a long lifetime gate on the camera, to sync out the Cherenkov light and noise, and maximize luminescence emission detection. X-ray tomography was completed on the IVIS Spectrum CT (PerkinElmer Inc) with the full field of view 120 x 120 x 30 mm, with a 150 \( \mu \)m voxel scan acquired in 90 s.

Tumour cell lines. MDA-MB-231 cells were purchased directly from ATCC as a confirmed cell type and mycoplasma free, no further testing was performed, and the cells lines are not listed in the ICLAC database of cross-contaminated or misidentified cell lines. Cells were grown in culture media in an incubator at 37 °C in DMEM with 10% (v/v) fetal bovine serum (FBS) and 1% penicillin-streptomycin. When ready for use, cells were trypsinized, counted, spun down into a slurry, and used for inoculation into animals.

Animal studies. All animal procedures were approved by the Dartmouth Institutional Animal Care and Use Committee, and the studies here were carried out in compliance with these approved procedures. Nude female mice were purchased at 6 weeks of age from Charles River Labs. A week after a week of acclimatization, animals were used for injections of 10^5 cells under the skin on each flank of the mouse in individual 0.05 ml injections. After approximately 3 weeks of growth, when a tumour with an average of 6 mm diameter was observed on both flanks, the animals were then used for imaging studies. All mice were under general anaesthesia of inhaled isofluorane at 1.5% in flowing air through a nose cone. For the tomography study, the volume of injection and vertical emission capture, using a long lifetime gate on the camera, to sync out the Cherenkov light and noise, and maximize luminescence emission detection. X-ray tomography was completed on the IVIS Spectrum CT (PerkinElmer Inc) with the full field of view 120 x 120 x 30 mm, with a 150 \( \mu \)m voxel scan acquired in 90 s.

Dose, concentration and depth. These are factors that affect contrast. The signal contrast in all imaging systems is a function of key parameters and so for CELSI it was hypothesized that these key factors would be linearly related to the radiation dose used, the concentration of probe present in the tissue, and the logarithm of the depth in which the target was located. The depth dependence on signal is likely to be more complex. Two levels of the light diffusion characteristics, but much more complex, with a more complex tissue to model, and with the signal falling off over macroscopic distances with the effective attenuation coefficient of the medium, estimated by diffusion theory to be \( \mu = (3\pi \rho \mu_a)^{-1} \), where \( \rho \) is the absorption coefficient and \( \mu_a \) is the transport scattering coefficient of the tissue. The dose delivered was varied by simple linear summation of the signal over more LINAC pulse durations, to effectively build up signal with delivered dose. The concentration of PtG4 was varied in solution within the embedded object, from 500 \( \mu \)M down to 0.78 \( \mu \)M using a 1 mm capillary present at 5 mm depth into the medium. The excitation volume of this was given by the overlap of the 5 mm beam with the 1 mm capillary, which was \( V = \pi r^2 h = 3.9 \times 10^4 \). The contrast-to-background ratio (CBR) was used as a metric of recovery success, and was calculated using the simple calculation defined by the ratio of the average intensity values: \( CBR = (I_{ROI} - I_{BG})/I_{BG} \), where \( I_{ROI} \) is the mean pixel intensity in the region of interest (ROI) and \( I_{BG} \) is the mean pixel intensity in the background.
Statistical analysis. The differences between the live and dead conditions, as displayed in Fig. 8, with n = 8 paired samples each, was established by two-tailed Students t-test, with α = 0.05 and resulting P < 0.001. These indicate that both in live and as well as P<0.05, there are significant differences between the values of the animals in the two conditions. However, more pertinent to the value of the study is the observation of the total range of values, presented as a percentage of the median value. Figure 8d,e was generated using Python 3.4.3 with the library matplotlib 2.0.0. The function used to make the box plot is called boxplot, available online (https://matplotlib.org/api/_as_gen/matplotlib.pyplot.boxplot.html). The box extends from the lower to the upper quartile values of the data, with a line at the median. The whiskers extend from the box to show the range of the data. Filler points are those past the end of the whiskers.

Reporting summary. Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

Code availability. Image-reconstruction code was custom developed for this study, and is available for download in bulk at www.nirfast.org. Individual MATLAB routines are available from the corresponding author upon request.

Data availability. The data that support the findings of this study are available within the paper and its Supplementary Information. Source datasets generated and analysed during the study are available from the corresponding author upon request.

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Author contributions

B.W. conceived the study, supervised all aspects of the work and drafted the manuscript; J.F., H.L., P.B., E.P.L., R.Z. and J.R.S. each completed measurements and data analysis as well as designed the experiments, wrote initial parts of the manuscript, and edited the entire manuscript. H.D. and S.C.D. helped design and analyse the tomography work with J.F., and each edited the manuscript. S.A.V. provided the molecular probe, provided advice on experimental design and data analysis and edited the manuscript. D.J.G. and L.A.I. each contributed advice on radiotherapy design and data interpretation, as well as edited the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

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- An indication of whether measurements were taken from distinct samples or whether the same sample was measured repeatedly
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- A description of all covariates tested
- A full description of the statistics including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
- For null hypothesis testing, the test statistic (e.g. F, t, r) with confidence intervals, effect sizes, degrees of freedom and P value noted. Give P values as exact values whenever possible.
- For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
- For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
- Estimates of effect sizes (e.g. Cohen’s d, Pearson’s r), indicating how they were calculated
- Clearly defined error bars

Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

| Data collection | n/a |
|-----------------|-----|
| Data analysis   | Image reconstruction code was custom developed for this study, and is available for download in bulk through our website www.nirfast.org, and individual MATLAB routines are available from the corresponding author. Public shareware Python code was used for plotting of the box plots, as well as for the students t-test, as described in the methods section. |

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Life sciences

Study design

All studies must disclose on these points even when the disclosure is negative.

| Sample size | The sample sizes were determined based upon the preliminary variance in the measurements of pO2 and secondary lifetime, and the goal of detecting a difference in the tumors when the animals were awake oxygenated versus after death. The change in pO2 was expected to be about 40 mmHg and the variance expected to be about 20 mmHg, which for student’s t-test predicts a sample size near 6 for an alpha of 0.05 and a power of 0.9. In this study, a sample of 4 mice were used with 2 tumors each, for a total sample of 8. This was thought to be sufficient to test the hypothesis that the sample of pO2 in living vs dead mice was significantly measured. |
| Data exclusions | No data were excluded in this study. |
| Replication | Replication number of all studies is explicitly described in each figure caption. In basic studies where the variance was very low compared to the signal, repeated measures were not used (such as in phantom data with high concentrations). However in selected experiments where variation was significant compared to the mean value, up to 3 repeated measures were taken. Each experiment has a description of the number of measurements in the figure legend. |
| Randomization | Randomization was not used, since there was not more than one arm of animals used. |
| Blinding | Blinding was not used, since this was a pilot study of a new technology, and there was not more than one arm of animals used. |

Materials & experimental systems

Policy information about availability of materials

n/a Involved in the study
☒ ☐ Unique materials
☒ ☐ Antibodies
☒ ☒ Eukaryotic cell lines
☐ ☐ Research animals
☐ ☐ Human research participants

Eukaryotic cell lines

Policy information about cell lines

Cell line source(s) ATCC
Authentication the cells came authenticated from ATCC and grown from sterile culture directly for these experiments.
Mycoplasma contamination the cells lines were confirmed mycoplasma free from ATCC, and no further testing was done from the cell culture samples.
Commonly misidentified lines (See ICLAC register) the cells lines are not listed in the ICLAC database of cross-contaminated or misidentified cell lines, and so we did no further testing or analysis of this.
Research animals
Policy information about studies involving animals; ARRIVE guidelines recommended for reporting animal research

Animals/animal-derived materials
Female, nude mice were used, purchased from Charles River Labs, at 6 weeks of age. Mice were acclimatized for 1 week prior to use, and the inoculated with MDA-MB-231 tumor cells at 10^5 cells in 50 ul injection in both flanks. When the tumors were an average diameter of 6mm, they were used for imaging experiments. Animals were sacrificed immediately after imaging, and no further tissue analysis was done.

Human research participants
Policy information about studies involving human research participants

Population characteristics n/a

Method-specific reporting
n/a Involved in the study

- [x] ChIP-seq
- [ ] Flow cytometry
- [x] Magnetic resonance imaging