In vivo nanoparticle-mediated radiopharmaceutical-excited fluorescence molecular imaging

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Cerenkov luminescence imaging utilizes visible photons emitted from radiopharmaceuticals to achieve in vivo optical molecular-derived signals. Since Cerenkov radiation is weak, non-optimum for tissue penetration and continuous regardless of biological interactions, it is challenging to detect this signal with a diagnostic dose. Therefore, it is challenging to achieve useful activated optical imaging for the acquisition of direct molecular information. Here we introduce a novel imaging strategy, which converts γ and Cerenkov radiation from radioisotopes into fluorescence through europium oxide nanoparticles. After a series of imaging studies, we demonstrate that this approach provides strong optical signals with high signal-to-background ratios, an ideal tissue penetration spectrum and activatable imaging ability. In comparison with present imaging techniques, it detects tumour lesions with low radioactive tracer uptake or small tumour lesions more effectively. We believe it will facilitate the development of nuclear and optical molecular imaging for new, highly sensitive imaging applications.

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**Results**

**EO morphology and spectrophotometry.** Scanning electron microscopy (SEM) showed EO nanoparticles with peanut-like morphology (Fig. 1a). The average diameter of EO nanoparticles was $85 \pm 22$ nm. The $\zeta$ potential was 28.6 mV. The excitation profile is shown in Fig. 1b. There were multiple characteristic absorption peaks at 301, 323, 365, 384, 396, 467 and 536 nm. The emitted fluorescence spectra of EO excited by 400- and 535-nm lasers are shown in Fig. 1c,d. The peak emission was 620 nm in both cases. The absolute quantum yield of EO was 39%.

**The mechanism of radiopharmaceutical excitation.** The radiopharmaceutical imaging tracer $^{18}$F-FDG (fluorodeoxyglucose) emits $\gamma$-radiation (511 keV), $\beta^+$ particles and CL, whereas $^{99m}$Tc-MDP (methylene diphosphonate) only emits $\gamma$-radiation (140 keV). Therefore, we employed both radioactive tracers to reveal the mechanism of radioactive tracer-excited fluorescence from EO.

Our previous study illustrated that an excitation range from ultraviolet to blue can excite EO to generate red emission (620 nm). This indicated that CL (350–450 nm weighted) was also and underexplored. There is no comparison between this approach and other imaging techniques for highly sensitive *in vivo* tumour detection yet. Our technique combines the merits of CLI and FMI to boost the light intensity and signal-to-background ratio and achieve internal activatable imaging via the excitation from two different electromagnetic radiations. To investigate the excitation mechanisms, the signal enhancement, the emission spectrum, the tissue penetration ability and the difference in outcomes between REFI, CLI, FMI and PET, a series of *in vitro* phantom and *in vivo* xenograft studies are conducted. The benefits of REFI for highly sensitive tumour imaging are demonstrated in two scenarios. We believe that by combining the strengths of CLI and FMI, signal-enhanced optical imaging through internal radionuclide excitation with dual electromagnetic radiations will benefit preclinical research and clinical diagnosis in the future.
capable of exciting EO. However, it was not known whether it was the only radiation from radionuclides causing the excitation. Figure 2 demonstrates that neither blocking Cerenkov light (Row II) nor blocking β+ radiation (Row III) significantly influenced the induction of fluorescence from EO. Conversely, the fluorescent signal decreased dramatically when the γ radiation from 18F-FDG or 99mTc-MDP was blocked (Row IV). Therefore, γ radiation was the major cause of EO excitation. Both high (511 keV) and low energy (140 keV) γ-rays were able to achieve EO excitation.

The fluorescent emission of EO increased linearly with increasing activity of radioactive tracers (Fig. 3a,b). The excitation efficiency of 99mTc-MDP was better than that of 18F-FDG. Fluorescent emission strongly correlated with tracer activity with R² values of 0.97 (18F-FDG) and 0.99 (99mTc-MDP). For both 18F-FDG and 99mTc-MDP excitation, the emitted fluorescent intensity of EO increased exponentially with an increase in its mass (Fig. 3c,d). Fluorescent emission strongly correlated with the amount of EO with an R² value of 0.99. With increasing excitation distance, the EO emission intensity decreased exponentially (Fig. 3e,f). Fluorescent emission strongly and inversely correlated with distance with R² values of 0.99 for 18F-FDG excitation and 0.97 for 99mTc-MDP excitation. The fluorescent signal was almost undetectable when the excitation distance between 18F-FDG and EO was over 25 mm.

The fractions of γ- and Cerenkov excitation in RFI are dependent on radioactivity, γ- photon energy, EO mass, and excitation distance. For a single study (100 μCi 18F-FDG + 10 mg EO with 10 mm distance), we employed a lead partition and two sets of mirrors (Fig. 3g,h) to obtain dual-radiation excitation and CL radiation excitation separately. The induced mean optical signal of EO was (100.5 ± 5) × 10⁸ p s⁻¹ and (4.6 ± 0.1) × 10⁸ p s⁻¹, respectively (Fig. 3i). Therefore, the fractions of γ- and CL excitation were 95.4 and 4.6% in this case, which proved γ-radiation was the major source of excitation.

**Different excitation efficiencies.** Figure 3b indicates that different radioisotopes with the same activity caused different excitation efficiencies, and 99mTc-MDP with lower γ photon energy (140 keV) was better than 18F-FDG (511 keV), when the radiotracer was separated with EO for excitation. However, when we co-mixed different radiotracers, 140 μCi 18F-FDG, 99mTc-MDP and 131I-NaI (131I-sodium iodide, major γ energy 364 keV), with 10 mg EO, respectively, and investigate their emission spectra, we found the opposite (Fig. 4a). 18F-FDG + EO showed the highest signal, and 99mTc-MDP + EO was the lowest. It was a surprise that 18F and 99mTc showed different relative excitation efficiency in conditions of separation and mixture. We hypothesized that the photoelectric interactions and Compton scattering of europium and γ-photons were related to the distance between radionuclide and europium. When mixing the 18F with EO, both β+ particles and γ-photons transferred energy to extranuclear electrons of EO and resulted in visible photons. However, 99mTc only emits γ photons, thus its excitation efficiency was lower than 18F in case of mixing with EO. When radiotracer was separated from EO for certain distance, the probability of β+ particles reaching EO was almost zero. It was γ photons that dominated the excitation for both isotopes. However, the probability of Compton scattering for 511 keV γ photon might be higher than that for 140 keV γ photon, which did not induce optical signal. The probability of photoelectric effect for 140 keV γ-photon might be higher than that for 511 keV γ-photon, which induced optical signal. That was the reason why separating and mixing radiotracers with EO showed different excitation efficiency.

To further explore the relationship between emission intensity and excitation photon energy below 140 keV, we built a hybrid X-ray and optical system (Supplementary Fig. 1a). X-ray with the energy range of 40 to 130 keV was used to excite the EO (Supplementary Fig. 1b), and the emission intensity increased linearly with an R² value of 0.95 (Supplementary Fig. 1c).

**Optical signal enhancement and spectral red shift.** With the presence of 10 mg EO, the optical signal enhancement showed a marked effect (Fig. 4a). The emission intensity was two orders of
Figure 3 | The investigation of different factors influencing the excitation. (a) The emission intensity of EO depends on the radioactivity and the radiotracers. (b) The linear relationship between emission intensity and radioactivity. $^{18}$F-FDG and $^{99m}$Tc-MDP shows different excitation efficiencies. (c) The emission intensity of EO depends on its mass. (d) The exponential relationship between emission intensity and EO mass. (e) The emission intensity of EO depends on the excitation distance. (f) The inverse relationship between emission intensity and excitation distance. (g,h) Using mirrors and a lead partition to achieve $\gamma$ and CL dual excitation (g) and pure CL excitation (h), respectively, to measure the fractions of $\gamma$- and Cerenkov excitation. (i) The quantification reveals that CL contributed a small portion of the dual-radiation excitation.

Figure 4b,c shows the difference in tissue penetration ability between CLI and REFI. The Cerenkov luminescent signal of the control well ($50 \mu$Ci $^{18}$F-FDG) was nearly ablated (from 6.4 to $0.4 \times 10^5$ $\text{p s}^{-1}$) after covering with a piece of porcine gastric mucosa tissue (1-mm thick). However, the REFI signal was greater in Wells 1 to 4, which contained a range of amounts of EO ($0.15$–$0.60 \text{ mg}$) mixed with $^{18}$F-FDG ($50 \mu$Ci each). The signal intensity of Well 4 ($50 \mu$Ci $^{18}$F-FDG + $0.6 \text{ mg}$ EO) was approximately fourfold greater ($1.8 \times 10^5 \text{p s}^{-1}$) than that of the control (Fig. 4d).

Combining the merits of CLI and FMI. CLI does not need external excitation as FMI does, which excludes background reflection and autofluorescence. Thus, an improved signal-to-background ratio is one of the strengths of CLI. REFI relies on radiation from a radiopharmaceutical to excite EO internally. Combining REFI with CLI confers the absence of external excitation and gives REFI the merit of a high signal-to-background. In both in vitro and in vivo phantom studies, the signal-to-background ratio of REFI was significantly larger than that of magnitude more than the normal Cerenkov luminescence, even with the only 2-min exposure (Note that it took 5-min exposure time for the rest of our studies of REFI and CLI). This together with our previous mechanism studies (Fig. 2) proved that EO was utilized to achieve fluorescent conversion of $\gamma$-radiation emitted from radiopharmaceuticals to boost the overall optical signal. Besides that, all three combinations ($^{18}$F-FDG, $^{99m}$Tc-MDP or $^{131}$I-NaI + EO) showed similar spectra between 570 and 740 nm. The peak of the emission profile was at 620 nm followed by a smaller peak at 700 nm. It is notable that for wavelength < 570 nm, the overall optical signal was weaker than Cerenkov signal emitted from $^{18}$F or $^{131}$I along. This indicated that the blue Cerenkov light also contributed the excitation of EO, therefore part of its energy was shifted from blue to red. The radio-pharmaceutical induced fluorescence showed greater signal intensity and shifted the optical spectrum from blue towards red. Both improvements suggested better ability to penetrate tissues. Since the emission profiles were similar for all three radiotracers, and the most clinically used $^{18}$F-FDG demonstrated the best excitation efficiency in the case of co-mixing EO, we focused on the $^{18}$F-FDG with EO for the rest of our studies.
FMI (in vitro $P < 0.001$, in vivo $P < 0.01$, Fig. 5). Regions of interest are shown with black circles, and quantifications are listed in Fig. 5 and Supplementary Table 1.

The activatable imaging strategy of external excitation in FMI is advantageous in terms of molecular specificity. Conversely, radioactive probes are constantly emitting CL during decay regardless of their interactions with biological molecules. This excludes CLI from the benefits of biologically specific, activatable imaging. However, REFI incorporates $\gamma$-radiation and CL to excite EO internally for fluorescent emission. This strategy conferred the merit of FMI for activatable imaging to REFI. For in vitro phantom imaging (Fig. 5a–h), the activated REFI signal was distinguished from conventional CLI by either optical signal enhancement (Fig. 5c,d, $P < 0.001$) or 620-nm filtering (Fig. 5e,f, $P < 0.001$). For in vivo phantom imaging (Fig. 5i–n), PET showed no significant differences ($P > 0.05$) between the two implanted capillary tubes (Fig. 5i), which indicated that the two tubes were filled with the same dose of $^{18}$F-FDG (50 $\mu$Ci). However, the signal intensity was significantly different between CLI and REFI with no filtering (Fig. 4j, $P < 0.05$) or filtering (Fig. 4k, $P < 0.001$).

**In vivo validation of the superiorities of REFI.** EO nanoparticles were directly injected into the tumours of Bcap-37 (human breast cancer cell) xenograft mice to simulate tumour-targeted nanoparticle delivery. Ten hours later, $^{18}$F-FDG was tail-vein injected. Figure 6a shows a comparison of REFI, CLI, and FMI in vivo. All three imaging techniques successfully detected optical signals from tumours. However, REFI exhibited both high intensity (REFI versus CLI: $8.43 \pm 1.35$ versus $3.69 \pm 0.84$, unit: $10^4$ ps$^{-1}$ cm$^{-2}$ sr$^{-1}$, $P < 0.01$) and high signal-to-background ratio (REFI versus FMI: $1.74 \pm 0.17$ versus $0.94 \pm 0.14$, $P < 0.01$). Therefore, REFI offered the best tumour to normal tissue contrast in all cases. The superiorities of REFI were consistent with the results of the previous phantom studies. We also performed a comparison between REFI and CLI, in which EO was tumour injected after the tail-vein injection of $^{18}$F-FDG. The signal enhancement effect was significant too ($P < 0.01$, Supplementary Fig. 2).

REFI and CLI were compared using U87MG (human glioblastoma cell) and Bcap-37 xenograft mice models to verify the passive accumulation of EO nanoparticles in tumours via enhanced permeability and retention (EPR) effect after intravenous (i.v.) injection.

**For in vivo imaging of U87MG xenografts, REFI with no filtering or 620-nm filtering showed significantly stronger optical signal** (both cases $P < 0.001$ compared with that of CLI (Fig. 6b). The mean intensity of REFI (no filtering: $135.7 \pm 12.1 \times 10^4$ ps$^{-1}$, $P < 0.01$) and high signal-to-background ratio (REFI versus FMI: $1.74 \pm 0.17$ versus $0.94 \pm 0.14$, $P < 0.01$). Therefore, REFI offered the best tumour to normal tissue contrast in all cases. The superiorities of REFI were consistent with the results of the previous phantom studies. We also performed a comparison between REFI and CLI, in which EO was tumour injected after the tail-vein injection of $^{18}$F-FDG. The signal enhancement effect was significant too ($P < 0.01$, Supplementary Fig. 2).

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Multimodality comparison for tumour detection. To investigate the advantages of REFI for sensitive imaging of tumour lesions, 4T1-luc2 (luciferase-expressing mouse adenocarcinoma cell) xenografts were used for multimodality imaging comparison. PET, CLI, FMI and REFI were applied for two scenarios, and REFI offered the best imaging performance in both cases. The advantages of REFI for sensitive imaging of tumour lesions, 4T1-luc2 (luciferase-expressing mouse adenocarcinoma cell) xenografts were used for multimodality imaging comparison. PET, CLI, FMI and REFI were applied for two scenarios, and REFI offered the best imaging performance in both cases. The same dose of $^{18}$F-FDG was i.v. injected into the xenografts for PET, CLI and REFI. All quantifications of optical intensity and signal-to-background ratio are listed in Figs 7 and 8, and Supplementary Table 2.

Six days after subcutaneous injection of 4T1-luc2 tumour cells, the tumour lesions were clearly visible on the upper and lower back of each mouse (Fig. 7a) with average diameter of

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**Figure 5** | The in vitro and in vivo phantom comparison between different imaging techniques. (a) A schematic illustration of the in vitro tissue-mimicking phantom. (b) FMI of the 100 μCi $^{18}$F-FDG + 1mg EO mixture. (c,d) With no filtering, CLI using $^{18}$F-FDG only (c) and REFI using the mixture of EO and $^{18}$F-FDG (d) show obvious different signal intensity. (e,f) With 620-nm filtering, CLI (e) and REFI (f) also demonstrate intensity differences. (g) The optical intensity comparison between CLI and REFI with no filtering and 620-nm filtering. (h) The signal-to-background ratio comparison between FMI and REFI. (i) The PET image of the in vivo phantom shows no significant difference between the two implanted glass tubes (left tube: 50 μCi of $^{18}$F-FDG + 0.15 mg of EO mixture, right tube: $^{18}$F-FDG, 50 μCi). (j,k) REFI and CLI show significant differences with no filtering (j) or 620-nm filtering (k). (l) FMI of the in vivo phantom. (m) The quantitative comparison of CLI and REFI in optical intensity with no filtering and 620 nm filtering. (n) The comparison of FMI and REFI in signal-to-background ratio.

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system. However, the intensity differences were not as great as in the U87MG xenograft experiment. This may have been because the injection of EO was performed 24 h earlier than the injection of $^{18}$F-FDG in the U87MG group, whereas the EO and $^{18}$F-FDG were injected together in the BCap-37 group. This may have resulted in further passive accumulation of EO inside U87MG tumour tissues.

We also performed the comparison between FMI using ICG (indocyanine green) and REFI using $^{11}$C-CHO (Choline) with EO through HepG2 (human hepatic cancer cell) orthotopic liver tumour mice models. The results further proved that REFI offered much better tumour to normal tissue contrast than FMI did ($P < 0.01$, Supplementary Fig. 3).
The signal-to-background ratio. Tissue contrast due to the non-specificity of the fluorescent probe. The black circle indicates the regions of interest (ROI) of the background for calculating the tumour tissue. This demonstrates the passive accumulation of the EO nanoparticle in the tumour tissue. The longitudinal observation comparing CLI and REFI in Bcap-37 xenografts. EO (0.1 mg) and tail-vein injection of 18F-FDG (500 μCi) in the U87MG-xenografted mice, REFI shows significantly greater signal than CLI did with both no filtering and 620-nm filtering. This demonstrates the passive accumulation of the EO nanoparticle in the tumour tissue. The comparison between different optical techniques for in vivo imaging xenografts. A) After direct intratumoural injection with EO (0.05 mg) and tail-vein injection of 18F-FDG (800 μCi) into the Bcap-37 xenografts, REFI shows the best tumour to normal tissue contrast among all three imaging modalities. B) After tail-vein injection with EO (0.1 mg, 24 h prior) and 18F-FDG (500 μCi) in the U87MG-xenografted mice, REFI shows significantly greater signal than CLI did with both no filtering and 620-nm filtering. This demonstrates the passive accumulation of the EO nanoparticle in the tumour tissue. C) The longitudinal observation comparing CLI and REFI in Bcap-37 xenografts. EO (0.1 mg) and 480 μCi of 18F-FDG are mixed and injected via the tail-vein. Both tumour and bladder show greater optical signal compared with that in the control mice at all time points.

Figure 7 | The multimodality comparison for in vivo imaging the dual-tumour xenografts. A) Six days after the subcutaneous injection of 4T1-luc2 tumour cells, two tumour lesions are visible on the back of a mouse model (red arrows). B) Three slices of PET (280 μCi 18F-FDG) in sagittal and axial directions show clear 18F-FDG uptake in the lower tumour (red arrows) but no significant uptake in the upper one (white arrows). The position of the three PET slices are indicated in (a) with black dotted lines. C) Without filtering, REFI (280 μCi 18F-FDG with 0.1 ml, 1 mg ml⁻¹ EO) shows optical signal of both tumours and brown adipose tissue (left mouse), but CLI does not visualize the upper back tumour (right mouse). D) With 620 nm filtering, two tumours are visualized in REFI (left mouse), but Cerenkov signal are nearly vanished. E) FMI of QD620 (0.1 ml, 10 mg ml⁻¹) does not show great tumour to normal tissue contrast due to the non-specificity of the fluorescent probe. The black circle indicates the regions of interest (ROI) of the background for calculating signal-to-background ratio.
5.6 ± 0.8 mm. PET can detect the lower back tumour (Fig. 7b red arrows), yet cannot detect the upper back one (Fig. 7b white arrows). This was because the brown adipose tissue (high 18F-FDG uptake) close to the upper tumour influenced its uptake of the radiotracer. CLI confirmed this phenomenon (Fig. 7c, right mouse). Optical signal were detected from brown adipose tissue and lower tumour region, but upper tumour was still not visualized. However, with the presence of EO and 18F-FDG in the same tumour lesion, the internal radiopharmaceutical excitation was activated. Significant optical signal enhancement (REFI versus CLI: upper tumour: \( P < 0.01 \), lower tumour: \( P < 0.01 \)) was detected in both tumours for REFI (Fig. 7c, left mouse). After 620-nm filtering, most of the Cerenkov luminescence was blocked (Fig. 7d, right mouse), including the optical signal from the brown adipose tissue (Fig. 7d, left and right mice). However, REFI offered significant optical signal for upper (REFI versus CLI: \( P < 0.001 \)) and lower (REFI versus CLI: \( P < 0.05 \)) tumour lesions (Fig. 7d, left mouse).

In previous phantom imaging and in vivo imaging studies, the FMI showed relatively worse performance partially due to the low quantum yield of EO. To make the comparison fair and thorough, the quantum dot 620 (QD620) was used in this study. It is ‘untargeted’, same as EO, but with absolute quantum yield of more than 80% (more than twice of EO). Besides that the dose of QD620 (0.1 ml, 10 mg ml\(^{-1}\)) was 10 times higher than the dose of EO (0.1 ml, 1 mg ml\(^{-1}\)) for in vivo imaging. However, REFI still demonstrated significant better signal-to-background ratio with the same excitation and emission setting up (REFI versus FMI: upper tumour: \( 2.74 ± 0.59 \) versus \( 0.55 ± 0.02 \), \( P < 0.05 \), lower tumour: \( 3.07 ± 0.63 \) versus \( 0.43 ± 0.05 \), \( P < 0.05 \), Fig. 7e).

Sixty-five hours after subcutaneous injection of 4T1-luc2 tumour cells, the tumour size was only 2.1 ± 0.3 mm (Fig. 8a, red arrow). BLI visualized the tumour lesion (Fig. 8b), but PET offered a negative scan (Fig. 8c). This insufficient sensitivity of PET is probably due to the partial volume effects that caused significant underestimation of radiotracer concentration in small lesions\(^{29–31}\). CLI confirmed this again (Fig. 8d,e, black arrows). It was really difficult to detect the weak Cerenkov signal from such a small lesion. However, REFI successfully achieved a positive imaging in both no filtering and 620-nm filtering modes (Fig. 8d,e, red arrows, REFI versus CLI: both \( P < 0.01 \)).

To make a comprehensive comparison between FMI and REFI, we employed two fluorescent probes this time, the ‘untargeted’ QD620 and targeted RediJect 2-DeoxyGlucosone 750 (RJ2-DG750). RJ2-DG750 is a probe for targeting of tumours that exhibit elevated glucose uptake rate in comparison with surrounding tissues. The FMI of QD620 showed multiple suspected lesions (Fig. 8f, white arrows), whereas the FMI of RJ2-DG750 showed an overestimated tumour lesion area (Fig. 8f, right mouse). Nevertheless, the signal-to-background ratios of FMI were still significantly smaller than that of REFI (REFI versus FMI: 4.42 ± 0.18 versus 0.64 ± 0.14 (QD620), \( P < 0.05 \), 4.42 ± 0.18 versus 1.48 ± 0.57 (RJ2-DG750), \( P < 0.01 \)).

To further validate above findings that REFI was more sensitive than PET for small tumour detection, another in vitro phantom and in vivo breast cancer mice model studies were conducted (Supplementary Figs 4 and 5). The results also proved that REFI was indeed more sensitive in small tumour detection.

**Biodistribution of EO and toxicity evaluation.** Cytotoxicity assay of EO was performed. Human umbilical cord mesenchymal stem cell morphology after 24h of incubation with 50 and 400 \( \mu \)g ml\(^{-1}\) EO was captured using fluorescence microscopy (Fig. 9a). Neither significant change of cell morphology nor cell aggregation was observed in the experimental sample compared with that of the control sample (Fig. 9a) without nanoparticles.

The biodistribution of EO was measured 40 min after the tail-vein injection of EO (0.1 ml, 1 mg ml\(^{-1}\)). Vital organs, such as heart, kidney, liver, lung and spleen, were ground using tissue-grinding pestles. Each sample (0.1 ml), including the sample of blood and urine, was mixed with \( 18^F \)-FDG (0.1 ml, 100 \( \mu \)Ci) respectively, so that the REFI signal was induced. By comparing the optical intensity with the control samples obtained
from mice without the injection of EO (pure CL signal), the signal difference reflected the biodistribution of EO per 0.1 ml (EO concentration). The results showed the spleen and heart had the highest EO concentration. Blood, liver, kidney and urine were very similar, and lung had the lowest EO concentration (Fig. 9b). This proved that EO was partially accumulated in liver, and partially excreted in urine through kidney.

Haematoxylin and eosin (H&E) microscopy (Fig. 9c) showed no obvious structural change in kidneys, lungs, spleen, heart or tumour of the experiment group with EO injection. Compared with QD620, the EO nanoparticles were much less toxic, as all mice injected with QD620 died within the next 4 h, but all mice injected with EO lived more than 4 weeks except killed mice.

**Discussion**

This study establishes REFI as a novel imaging strategy. Using EO nanoparticles and clinical radiopharmaceuticals, the γ- (major) and Cerenkov (minor) radiation can be converted to fluorescence to achieve internally excited optical imaging. Our series of *in vitro* studies identified the excitation source and illustrated the relationship between the emission intensity and various parameters, such as radioactivity, EO mass and excitation distance. The excitation efficiency of different radiotracers and X-ray photon energies was investigated. The optical signal enhancement, spectra red shift and signal tissue penetration were also demonstrated.

REFI utilized both γ- and Cerenkov radiation from radioactive tracers to achieve internal activatable imaging. This unique feature eliminates the adverse effects of autofluorescence and reflection from external excitation as occurs in conventional fluorescence molecular imaging and enhances emission signals through fluorescent conversion of γ-radiation to overcome the challenge of detecting a weak Cerenkov signal from radioisotopes. Comparison of REFI, CLI and FMI through phantom *in vitro* and Bcap-37, U87MG and 4T1-luc2 xenograft *in vivo* studies clearly demonstrated that REFI combined the merits of CLI and FMI with superb signal-to-background ratio and internal activatable imaging ability. Especially in the 4T1-luc2 xenograft study of small tumour detection, REFI showed significant better signal-to-background ratio than FMI did, no matter it was QD620 (higher quantum yield) or RJ2-DG750 (better targeting specificity) that was applied in FMI. Furthermore, in comparison of CLI, the optical signal of REFI was boosted remarkably and was spectrally shifted towards the deeper tissue-penetrating red range. All these features benefited the optical imaging performance in our *in vitro* and *in vivo* studies.

REFI employed both radiotracer and EO nanoparticles, and the internal signal activation highly relies on the distance of them. Therefore, if tumour lesion shows similar uptake of one tracer but...
higher uptake of the other, in comparison with surrounding normal tissues, the tumour to normal tissue contrast will stand out. Our dual tumour 4T1-luc2 xenograft study demonstrated this phenomenon through multimodality comparison of REFI, PET and CLI. The uptake of $^{18}$F-FDG in upper back tumour was influenced by brown adipose tissue nearby, but REFI was able to visualize the tumour unaffectedly, whereas PET and CLI failed.

The sensitivity of PET is superior to many other imaging modalities, but it is still limited by the low resolution partially due to the partial volume effects$^{29-31}$. For small tumour lesion with the size smaller or close to its spatial resolution, the underestimation of radiotracer uptake becomes significant. The high superficial resolution of optical imaging, the signal enhancement effect and the better signal-to-background ratio empowered REFI to detect small subcutaneous tumour lesions more effectively. Our 4T1-luc2 xenograft study indicated that REFI was able to detect tumour lesions with the size $< 2 \text{mm}$, which were $< 3$ days after tumour transplanting in nude mice. This suggested a great potential of applying REFI for highly sensitive early tumour detection and tumour metastasis imaging, even with passive delivery of the nanoparticle. Different from bioluminescence imaging, REFI does not require incorporation of immunogenic proteins$^{32,33}$, such as luciferase and GFP (green fluorescent protein). It thus can be applied to a wider range of animal tumour models and holds better clinical translation potential.

Other studies of Cerenkov-induced fluorescence imaging (SCIIFI) have already demonstrated the mechanism of utilizing Cerenkov light solely for excitation and applications of SCIIFI in tumour marker detection using targeted fluorescence probes$^{19,22}$. The fundamental difference between REFI and SCIIFI (or other similar approaches) is the utilization of $\gamma$-radiation for optical excitation and the resulted signal enhancement, as the overall optical signal of SCIIFI was even weaker than Cerenkov light because of the inevitable energy loss during excitation. There were also studies of using radioluminescent nanoparticles and radiotracers, such as $^{18}$F-FDG, for biomedical imaging applications$^{23,25-27}$, but these works remained in phantom studies and did not reveal the dual-radiation excited imaging mechanism. To the best of our knowledge, this is the first in vivo small animal tumour model study using REFI or other similar approaches.

The EO nanoparticle is not the only choice for applying the strategy of REFI. The lanthanide-doped nanoparticle is likely to provide similar fluorescence conversion of both $\gamma$- and Cerenkov radiation from radioisotopes$^{26,27}$. These can be excited by collimated X-rays (approximately the same energy magnitude as $\gamma$-radiation from $^{99m}$Tc-MDP) and have already been applied in X-ray/optical imaging modalities, such as X-ray luminescence computed tomography$^{27,34,35}$. Therefore, our imaging technique converts external high-energy (X-ray) and low-energy (optical light) electromagnetic excitation to internal $\gamma$- and CL excitation via radiopharmaceuticals for in vivo optical molecular imaging.

We believe that REFI and its principle hold great promise for in vivo activatable imaging that can detect molecular targets or events and provide quantitative information of pathological processes on molecular levels with proper modification of the EO or other lanthanide-doped nanoparticle to overcome the issue of target specificity. REFI, with its intrinsic superiorsities, will benefit the imaging of tumour-to-tumour molecular heterogeneity, which can further facilitate the development of precision medicine and personalized patient care. Therefore, this imaging technique will expand the applicability of activatable nuclear and optical molecular imaging.

In conclusion, through utilizing EO nanoparticles mediators, we have achieved internal conversion of continuous radio-pharmaceutical radiation to activatable fluorescence for molecular imaging. REFI can merge the advantages of nuclear and optical molecular imaging techniques in vivo. This creates a strong motivation for further modification of the EO nanoparticle to obtain biomarker specificity and application of this highly sensitive imaging technique to early and small tumour detection in the near future.

### Methods

#### Nanoparticles and radionucleide tracers.

The EO nanoparticle (Eu2O3, 99.9% metal basis, molecular weight $= 351.91$) was purchased from the Aladdin Chemistry Co. Ltd. $^{18}$F-FDG, $^{99m}$Tc-MDP and $^{131}$I-NaI was provided by the department of nuclear medicine, Chinese PLA General Hospital, Beijing, China. The QD620 was purchased from China Beijing Beida Jubiang Science & Technology Co. Ltd, and the R12-DG2750 probe was purchased from PerkinElmer. The absolute quanta yield of EO and QD620 was measured using FLSP900 fluorescence spectrometer (Edinburgh Instruments). Both kinds of nanoparticle were dissolved in PBS for measurements.

#### SEM and spectrophotometry.

The size and morphology of EO nanoparticles were determined by SEM (Hitachi S-4700). As-prepared EO powder samples were dispersed and dropped on a copper grid for scanning. The particle size was quantified using Image J. The EO nanoparticles were characterized for fluorescent properties using an EnSpire Multimode Plate Readers (PerkinElmer). The samples were read using a transparent 96-well plate. The excitation profile was obtained using a 620-nm emission filter. The fluorescence profiles were obtained with excitation at 400 and 535 nm.

#### Mechanistic study of radiopharmaceutical excitation.

The imaging system used for these studies was the IVIS Spectrum system (Caliper Life Sciences). The imaging parameters were binning: 4, exposure: 5 min and aperture: f1, unless otherwise indicated.

In the identification of the excitation source experiment, two 1.5-ml tubes filled with 0.2 g EO and 600 $\mu$Ci of $^{18}$F-FDG were placed 15 mm apart inside the imaging chamber and a sequence of images were acquired. The first image was taken in the normal view. The second was taken with a black cardboard box covering the $^{18}$F-FDG tube. The third and fourth images were taken with an aluminium foil and a lead torus placed between the two tubes, respectively. Finally, a normal view of the two tubes was acquired again. Images of tubes with 990 $\mu$Ci of $^{99m}$Tc-MDP and 0.20 g of EO were acquired following the same protocol.

In the emission intensity versus radioactivity experiment, EO (10 mg) was excited using 0.1 ml of $^{18}$F-FDG and 0.1 ml of $^{99m}$Tc-MDP with 11 different activities (2, 5, 10, 19, 30, 63, 140, 240, 521, 1,010 and 1,990 $\mu$Ci). The images were analysed quantitatively to obtain the relationship between the emission intensity and radioactivity. The excitation distance was 10 mm.

In the emission intensity versus excitation distance experiment, the excitation distance was set to 1, 3, 5, 7, 10, 15, 20, 25, 43 and 60 mm, respectively. The tips of two EP tubes were 12 mm apart. After taking optical images, a lead partition (12-mm thick) was put in between the EP tubes to block $\gamma$-radiations from $^{18}$F. However, the CL was partially reflected to the EO because of the two mirrors reflection. Then optical imaging were taken again for pure CL excitation.

#### Emission spectra assessment.

$^{18}$F-FDG, $^{99m}$Tc-MDP and $^{131}$I-NaI (each 140 $\mu$Ci) were mixed with EO nanoparticles (10 mg), respectively. The emission spectra together with the Cerenkov luminescent spectra of $^{18}$F and $^{131}$I (both 140 $\mu$Ci) were measured using the IVIS system (exposure: 2 min). PBS was used as the Control.

#### X-ray excitation of EO nanoparticles.

During the X-ray excitation process, the voltage of the X-ray tube (Hamamatsu L9181-02 Microfocus X-ray Source, Japan) was increased gradually, while the tube current was kept constant. Therefore, X-ray spectra with different emission energies (40, 60, 80, 100, 110, 120 and 130 keV) were obtained to excite EO nanoparticles (2 mg) inside an EP tube (Supplementary Fig. 1b), but the total photon number of the X-ray beam was kept constant for each excitation. To protect the EMCCD (DU888 +, Andor, UK), the optical imaging system was perpendicular to the X-ray tube (Supplementary Fig. 1a). The white light image was acquired with a 0.1-s exposure inside room light, and the X-ray-excited fluorescence image was acquired with 5-s exposure inside a light sealed environment. The PBS was used as control.
Excitation efficiency of different radiopharmaceuticals. $^{18}$F-FDG, $^{99m}$Tc-MDP and $^{111}$In-NaI (each 100 μCi) were used to excite 10 mg EO, respectively (mixing excitation efficiencies). The optical images were acquired using a confocal laser scanning microscope (Caliper Life Sciences) with binning: 4, exposure: 2 min and aperture: f1. The excitation efficiencies of using different radiotracers were compared (Supplementary Fig. 1d).

**Biological tissue penetration assessment.** The biological tissue penetration ability of the fluorescence emission was evaluated using a piece of 1-mm thick porcine muscle tissue dissected from a freshly butchered pig. $^{18}$F-FDG at 50 μCi was injected into one well as the control. The other four wells were injected with the mixture of $^{18}$F-FDG (50 μCi) and EO (0.15, 0.30, 0.45 or 0.60 mg). Images without and with tissue blocking were taken (binning: 8, exposure: 2 min).

**Comparison of optical imaging techniques via phantoms.** Two identical tissue-mimicking phantoms were made from high-density polyethylene to simulate the optical properties of mouse muscle. Each was a cube that was 40 × 40 × 40 mm$^3$ in dimension. A small hole (diameter 2.5 mm, length 20 mm) was drilled 2 mm beneath the surface (Fig. 4a). A 0.1-ml mixture of $^{18}$F-FDG (100 μCi) and EO (1 mg) or 0.1 ml of $^{18}$F-FDG (100 μCi) alone was injected into each hole. CLI, REFI and FMI were performed for comparison. For CLI and REFI, both 620-nm filtered and unfiltered images were acquired (exposure 5 min). For FMI, the excitation and emission wavelengths were 465 and 620 nm (exposure 1 s). The experiment was performed in triplicate.

Two glass tubes were filled with 0.1 ml of $^{18}$F-FDG (50 μCi) or a 0.1-ml mixture of $^{18}$F-FDG (50 μCi) and EO (0.15 mg). They were then subcutaneously implanted into a euthanized nude mouse. PET (Genios PET, SoFi Biosciences), CLI, REFI and FMI were performed. The experiment was performed in triplicate.

**Animal experiments.** All animal experiments were conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee of General Hospital of Chinese People’s Armed Police Forces. All animal procedures were performed isoluorane gas anesthesia (3% isoluorane-air mixture), and all efforts were made to minimize suffering. The Balb/c nude mice were obtained from the Laboratory Animal Center of the Chinese Academy of Medical Sciences. Seven- to eight-week-old mice were used for experiments. Female mice were applied for Bcap-37 and 4T1-luc2 studies, and male mice were applied for U87MG and HepG2 eight-week-old mice. Female mice were applied for U87MG and HepG2 experiments. The Balb/c nude mice were obtained from the General Hospital of Chinese People’s Armed Police Forces. All animal procedures were conducted in compliance with the guidelines of the Institutional Animal Care and Use Committee of General Hospital of Chinese People’s Armed Police Forces.

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 Hu, Z. et al. Cerenkov luminescence tomography of aminopeptidase N (APN/CD13) expression in mice bearing HT1080 tumors. Mol. Imaging 12, 173–181 (2013).

 Thorek, D. L. et al. Cerenkov imaging - a new modality for molecular imaging. Am. J. Nucl. Med. Mol. Imaging 2, 163–173 (2012).

 Mitchell, G. S., Gill, R. K., Boucher, D. L., Li, C. & Cherry, S. R. In vivo Cerenkov luminescence imaging: a new tool for molecular imaging. Philos. Trans. A Math. Phys. Eng. Sci. 369, 4605–4619 (2011).

 Liu, H. et al. Intraoperative imaging of tumors using Cerenkov luminescence endoscopy: a feasibility experimental study. J. Nucl. Med. 53, 1579–1584 (2012).

 Liu, H. et al. Molecular optical imaging with radioactive probes. PLoS ONE 5, e9470 (2010).

 Spinelli, A. E. et al. First human Cerenkography. J. Biomed. Opt. 18, 20502 (2013).

 Thorek, D. L., Ried, C. C. & Grimm, J. Clinical Cerenkov luminescence imaging of (18)F-FDG. J. Nucl. Med. 55, 95–98 (2014).

 chin, P. et al. Optical imaging as an expansion of nuclear medicine: Cerenkov luminescence imaging in vivo. J. Nucl. Med. Mol. Imaging 40, 1283–1291 (2013).

 Thorek, D. L., Ogirala, A., Beattie, B. J. & Grimm, J. Quantitative imaging of disease signatures through radioactive decay signal conversion. Nat. Med. 19, 1345–1350 (2013).

 Thorek, D. L., Das, S. & Grimm, J. Molecular imaging using nanoparticle quenchers of Cerenkov luminescence. Small 10, 3729–3734 (2014).

 Dohtage, R. S., Goffin, R. J., Jackson, E., Harpstrite, S. & Pivonia-Worms, D. Cerenkov radiation energy transfer (CRET) imaging: a novel method for optical imaging of PET isotopes in biological systems. PLoS ONE 5, e13300 (2010).

 Axelsson, J., Davis, S. C., Gladstone, D. J. & Pogue, B. W. Cerenkov emission induced by external beam radiation stimulates molecular fluorescence. Med. Phys. 38, 4127–4132 (2011).

 Ma, X. et al. Enhancement of Cerenkov luminescence imaging by dual excitation of Eu¹⁶⁺, Yb³⁺-doped rare-earth microparticles. PLoS ONE 8, e77926 (2013).

 Zharavela, M., Friedrich, S. & Melcher, C. L. The europium oxidation state in CsSrI₃:Eu scintillators measured by X-ray absorption spectroscopy. Opt. Mater. 36, 670–674 (2014).

 Shen, J., Sun, L. D. & Yan, C. H. Luminescent rare earth nanomaterials for bioprobe applications. Dalton Trans. 24, 5887–5897 (2008).

 Carpenter, C. M. et al. Radioluminescent nanophosphors enable multiplexed small-animal imaging. Opt. Express 20, 11598–11604 (2012).

 Sun, C. et al. Synthesis and radioluminescence of PEgylated Eu(3+) doped nanophosphors as bioimaging probes. Adv. Mater. 23, H195–H199 (2011).

 Chen, H. et al. Monitoring pH-triggered drug release from radioluminescent nanocapsules with X-ray excited optical luminescence. ACS Nano 7, 1178–1187 (2013).

 Bellevire, D. et al. Staging the axilla in breast cancer patients with ¹⁸⁸F-FDG PET: how small are the metastases that we can detect with new generation clinical PET systems? Eur. J. Nucl. Med. Mol. Imaging 41, 1103–1112 (2014).

 Soret, M., Bachrach, S. L. & Buvat, I. Partial-volume effect in PET tumor imaging. J. Nucl. Med. 48, 932–945 (2007).

 Hoetjes, N. J. et al. Partial volume correction strategies for quantitative FDG PET in oncology. Eur. J. Nucl. Med. Mol. Imaging 37, 1679–1687 (2010).

 Bhaumik, S. & Gambhir, S. S. Optical imaging of Renilla luciferase reporter gene expression in living mice. Proc. Natl. Acad. Sci. USA 99, 377–382 (2002).

 So, M.-K., Xu, C., Loening, A.M., Gambhir, S.S. & Rao, J. Self-illuminating quantum dot conjugates for in vivo imaging. Nat. Biotechnol. 24, 339–343 (2006).

 Pratx, G., Carpenter, C. M., Sun, C., Rao, R. P. & Xing, L. Tomographic molecular imaging of x-ray-excitable nanoparticles. Opt. Lett. 35, 3345–3347 (2010).

 Pratx, G., Carpenter, C. M., Sun, C. & Xing, L. X-ray luminescence computed tomography via selective excitation: a feasibility study. IEEE Trans. Med. Imaging 29, 1992–1999 (2010).

 Cong, W. et al. Practical reconstruction method for bioluminescence tomography. Opt. Express 13, 6756–6771 (2005).

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 Author contributions

 J.T. and H.F.L. initiated the project and contributed to the experimental design as the principal investigators. Z.H., Y.Q., K.W. and X.Z. conceived the experiments, characterized the EO nanoparticles and performed in vitro and in vivo experiments. X.Z., J.Z., T.S., C.B., H.L., Z.W. and J.W. assisted in toxicity evaluation. K.W. and Z.H. analysed the data and wrote the manuscript. All authors commented and contributed to the manuscript.

 Additional information

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