LETTER

Direct measurement of local oxygen concentration in the bone marrow of live animals

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Characterization of how the microenvironment, or niche, regulates stem cell activity is central to understanding stem cell biology and to developing strategies for the therapeutic manipulation of stem cells1. Low oxygen tension (hypoxia) is commonly thought to be a shared niche characteristic in maintaining quiescence in multiple stem cell types2–4. However, support for the existence of a hypoxic niche has largely come from indirect evidence such as proteomic analysis5, expression of hypoxia inducible factor-1α (Hif-1α) and related genes6, and staining with surrogate hypoxic markers (for example, pimonidazole)7. Here we perform direct in vivo measurements of local oxygen tension \( p_{O_2} \) in the bone marrow of live mice. Using two-photon phosphorescence lifetime microscopy, we determined the absolute \( p_{O_2} \) of the bone marrow to be quite low (<32 mm Hg) despite very high vascular density. We further uncovered heterogeneities in local \( p_{O_2} \), with the lowest \( p_{O_2} \) (~9.9 mm Hg, or 1.3%) found in deeper peri-sinusoidal regions. The endosteal region, by contrast, is less hypoxic as it is perfused with small arteries that are often positive for the marker nestin. These \( p_{O_2} \) values change markedly after radiation and chemotherapy, pointing to the role of stress in altering the stem cell metabolic microenvironment.

The precise location of a haematopoietic stem cell (HSC) niche within the bone marrow (BM) remains elusive, with evidence supporting the existence of both a vascular niche8–10 and an endosteal niche11–13. As the BM is densely perfused14–18, including in the endosteal region, we ask how such a highly vascularized tissue can harbour HSCs in a low oxygen microenvironment. The possibility of very steep \( p_{O_2} \) gradients forming hypoxic zones at short distances away from the blood vessels is suggested by an in silico model19,20. However, no direct measurement of local oxygen distribution within the BM has been reported.

Here we implemented two-photon phosphorescence lifetime microscopy (2PLM) on a two-photon microscope designed specifically for live animal imaging19. The all-optical design (Extended Data Fig. 1) enabled non-contact \( p_{O_2} \) measurements through the intact skull at precise locations within the BM with micrometer spatial resolution. Before imaging, a metalloporphyrin-based two-photon-enhanced phosphorescent nanoprobe, platinum porphyrin-coumarin-343 (PtP-C343)20,21, was injected systemically. The emissive triplet state of the PtP is highly sensitive to local oxygen concentration20,21. Bimolecular collisions with dissolved oxygen shorten the probe’s triplet lifetime and quench phosphorescence22. Thus, by measuring the phosphorescence decay time \( \tau \) after an excitation pulse, the absolute \( p_{O_2} \) value can be determined on the basis of a pre-established \( \tau \) versus \( p_{O_2} \) calibration curve (Extended Data Fig. 2). The metalloporphyrin in PtP-C343 is protected by a den- drimer with a polyethylene glycol (PEG) overcoat (Extended Data Fig. 2). The residual fluorescence of the C343 units in the probe enabled precise localization of the probe’s excitation cross-section, several C343 moieties are grafted onto the den- drimer shell as two-photon ‘antennas’ that funnel the captured energy to the metalloporphyrin by way of intramolecular energy transfer20,21.

The residual fluorescence of the C343 units in the probe enabled visualization of the BM vasculature by conventional two-photon excited fluorescence, while the bone was visualized simultaneously by the collagen second harmonic generation (SHG) signal4. We confirmed that the BM contains high vascular density (Fig. 1a–c), with >95% of vessels in the image stack located <25 μm from the nearest blood vessel (Fig. 1b, c). We then quantified BM \( p_{O_2} \) at several intravascular locations using 2PLM and obtained values that ranged from 11.7 to 31.7 mm Hg (1.5–4.2%) with a mean of 20.4 mm Hg (2.7%). These values are significantly lower than the \( p_{O_2} \) in the microvasculature of the brain24–26, the periosteum and the cortical bone (Fig. 1d). In many instances we were able to follow individual blood vessels as they penetrated from the bone into the BM cavity (Fig. 1e). When traced along individual vessels, we detected steep drops in \( p_{O_2} \) measured immediately before and after entrance into the BM cavity (Fig. 1e, f). The rapid depletion of oxygen along the direction of blood flow, where the blood transits from a region of low cellularity (cortical bone) to a region of high cellularity (BM), is reminiscent of the longitudinal \( p_{O_2} \) gradient observed when blood vessels enter solid tumours with high metabolic demand27.

Because of the relatively high permeability of the BM vasculature, the injected probe (diameter ~3–4 nm) (ref. 20) diffused out of the blood vessels and accumulated in sufficient concentrations within minutes (Extended Data Fig. 4), allowing \( p_{O_2} \) measurements in the interstitial space. In accordance with mathematical models17,18, we found a steep lateral \( p_{O_2} \) gradient away from the blood vessels, with a mean extra- vascular \( p_{O_2} \) of 13.3 mm Hg (1.8%) and a range of 4.8 to 21.1 mm Hg (0.6–2.8%) (Fig. 1d).

Our measured \( p_{O_2} \) in the BM (~20.4 mm Hg intravascular, ~13.3 mm Hg extravascular) agreed well with a previously reported value of ~18 mm Hg obtained using an oxygen electrode2. However, that measurement lacked spatial resolution, and the insertion of the needle electrode probably damaged the microvasculature so that only an averaged reading of intravascular and extravascular \( p_{O_2} \) was recorded. Nevertheless, taken together these results support the notion that the BM as a whole is a hypoxic tissue despite its high vascularity.

To provide a finer-grained view of oxygen distribution within the BM, we measured \( p_{O_2} \) in different locations within the BM, as the vasculature is heterogeneous, with smaller vessels located closer to the endo- steel surface16 (Fig. 2a). 2PLM of blood vessels with diameters <15 μm shows higher \( p_{O_2} \) (22.7 mm Hg, 3.0%) than in blood vessels with diameters >15 μm (19.5 mm Hg, 2.6%, \( P < 0.03 \)). Accordingly, when we analysed the \( p_{O_2} \) values at different distances from the endosteum, we found the lowest \( p_{O_2} \) to be in the regions >40 μm from the bone, with...
values of 17.7 mm Hg (2.4%) in the vessels and 9.9 mm Hg (1.3%) outside the vessels (Fig. 2a). In the endosteal region (0–20 μm zone), where most of the smaller vessels are located, the \( p_{O_2} \) readings were slightly higher, with values of 21.9 mm Hg (2.9%; \( P < 0.03 \)) in the vessels and 13.5 mm Hg (1.8%; \( P < 0.01 \)) outside the vessels (Fig. 2a). These measurements uncovered a moderate \( p_{O_2} \) gradient with distance from the endostem, but the direction of the gradient was unexpected. Instead of the endosteal zone being the most hypoxic, we found that \( p_{O_2} \) decreased with increasing distance away from the endosteum towards the more hypoxic sinusoidal region.

As there are currently no HSC-specific markers for visualizing endogenous stem cells under homeostatic conditions, we used nestin-green fluorescent protein (nestin-GFP) mice to help identify the locations where HSCs reside. Nestin is expressed by a subset of BM mesenchymal stem cells that are involved in HSC maintenance and trafficking9, where HSCs reside. Nestin is expressed by a subset of BM mesenchymal stem cells that are involved in HSC maintenance and trafficking9, where HSCs reside. Nestin is expressed by a subset of BM mesenchymal stem cells that are involved in HSC maintenance and trafficking9, where HSCs reside. Nestin is expressed by a subset of BM mesenchymal stem cells that are involved in HSC maintenance and trafficking9, where HSCs reside.

Figure 1 | BM vascular density and oxygenation. a, Intravital maximum intensity image (~75 μm thick) of mouse calvarial BM showing blood vessels (red, Qtracker 655 vascular dye) and bone (blue, collagen SHG). b, Corresponding single plane of the three-dimensional intravital imaging stack showing blood vessels (red), bone SHG (blue), and the three-dimensional Euclidean distance measurement (EDM, green) to the nearest blood vessel wall for each extravascular pixel in the BM. c, Histogram of all EDMs from the full three-dimensional imaging stack. d, BM \( p_{O_2} \), is significantly lower compared with the \( p_{O_2} \) in periosteal and cortical bone vessels. Each point represents a \( p_{O_2} \) measurement in a separate blood vessel or interstitial position (\( n = 8, 21, 55 \) and 40 vessels/locations for periosteum, cortical bone, BM intravascular and BM extravascular, respectively, from 8 mice). The mean (black line) ± standard deviation (s.d.; shaded box) for each data set is shown. e, Maximum intensity projection image montage of a blood vessel entering the BM from the bone. Bone (blue) and blood vessels (yellow) are delineated with SHG and Rhodamine B–dextran/PtP-C343 fluorescence, respectively. The two arrows point to locations of \( p_{O_2} \) measurements just before and after the vessel enters the BM. f, Drop in \( p_{O_2} \), when tracking along individual vessels upon entry into the BM (\( n = 8 \) vessels from 4 mice). The mean (black line) is shown for each data set. Scale bars, ~100 μm.

Figure 2 | Variation in BM \( p_{O_2} \) according to vessel diameter and distance from the endosteal surface. a, Average vessel diameter (green line; \( n = 38 \) vessels from 4 mice) and intravascular (red line; \( n = 38 \) vessels from 4 mice) and extravascular (blue line; \( n = 39 \) locations from 3 mice) \( p_{O_2} \) are plotted as a function of the distance from the bone surface to the measurement location. Error bars are ± s.d. b, The diameter of nestin+ (red circles; \( n = 9 \) vessels from 3 mice) and nestin− vessels (blue squares; \( n = 12 \) vessels from 3 mice) are plotted as a function of the distance from the endosteal surface. c, The \( p_{O_2} \), of the same nestin+ and nestin− vessels are plotted as a function of vessel diameter. d, Nestin+ (red circles; \( n = 17 \) vessels from 7 mice) and nestin− (blue squares; \( n = 16 \) vessels from 5 mice) vessel \( p_{O_2} \). The mean (black line) ± s.d. (shaded box) for each data set is shown.
irradiation, or after busulphan (35 mg kg\textsuperscript{-1}; n = 40 locations from 2 mice) conditioning. The untreated control condition. The untreated control

![Figure 3](image)

**Figure 3** | BM \(P_O_2\) after cytoreductive therapy and at sites of HSPC homing.  

- **a**. BM \(P_O_2\) 2 days after sub-lethal (4.5 Gy; n = 13 vessels and 29 extravascular locations from 5 mice) or lethal (9.5 Gy; n = 40 locations from 2 mice) gamma irradiation, or after busulphan (35 mg kg\textsuperscript{-1}; n = 40 locations from 6 mice) conditioning. The untreated control is plotted for comparison.  
- **b**. Extravascular \(P_O_2\) measurements are plotted as a function of distance to the bone surface from the measurement location 2 days after 4.5 Gy irradiation (n = 44 locations from 4 mice).  
- **c**. Comparison of \(P_O_2\) at sites of HSPC homing to the overall BM \(P_O_2\) in busulphan-treated recipients (n = 17 HSPCs from 6 mice). No statistically significant difference was observed. For **a** and **c**, the mean (black line) ± s.d. (shaded box) for each data set is shown.

Data Fig. 3). Nestin\textsuperscript{+} vessels are anatomically distinct from nestin\textsuperscript{-} vessels, being located closer to the bone surface (10.9 μm versus 25.5 μm; \(P < 0.02\)) and much smaller in diameter (9.5 μm versus 22.9 μm; \(P < 2 \times 10^{-6}\)) (Fig. 2b). Contrary to our expectation, we found \(P_O_2\) to be significantly higher in nestin\textsuperscript{+} compared with nestin\textsuperscript{-} vessels (22.8 mm Hg versus 17.6 mm Hg; \(P < 0.0007\)) (Fig. 2c, d).

Next we examined whether transplanted haematopoietic stem/progenitor cells (HSPCs) home to BM niches with distinct \(P_O_2\) values. Before transplantation, recipient animals were given cytoreductive conditioning with either radiation or chemotherapy (busulphan, 35 mg kg\textsuperscript{-1}). Remarkably, both treatments resulted in a substantial elevation in the overall BM \(P_O_2\) (Fig. 3a). The rise in \(P_O_2\) was notable considering that the vasculature was severely damaged after both radiation and chemotherapy (Extended Data Fig. 6a–c). Moreover, although the intravascular \(P_O_2\) values were essentially unchanged after sub-lethal irradiation (4.5 Gy), the gradient between intravascular and extravascular \(P_O_2\) disappeared, with the extravascular \(P_O_2\) rising to the same level as the intravascular \(P_O_2\) (Fig. 3a). The ‘negative’ gradient of decreasing \(P_O_2\) with increasing distance away from the endosteum also disappeared (Fig. 3b). In the case of lethal irradiation and busulphan treatment, the extensive vascular damage caused such widespread dye leakage that the boundary between intravascular and extravascular spaces could no longer be delineated.

We performed HSPC transplantation (~10\textsuperscript{5} lineage\textsuperscript{−} cKit\textsuperscript{+} Sca-1\textsuperscript{+} (LKS) cells per recipient) 24 h after busulphan treatment and measured the \(P_O_2\) around each LKS cell that homed to the calvarial BM 18–24 h later. We obtained \(P_O_2\) values that span nearly the entire range of the (elevated) BM \(P_O_2\) in the post-chemotherapy setting (Fig. 3c), indicating that HSPCs did not seek out specific niches defined by low \(P_O_2\) as sites for preferential homing.

**Figure 4** | BM vascular mapping and the effects of cellularity on local \(P_O_2\) after cytoreductive conditioning.  

- **a**. Vascular connectivity map of a nestin-GFP mouse reveals that nestin\textsuperscript{+} vessels are upstream of and drain into sinusoids. Arrows indicating blood flow direction, determined by tracking the movement of labelled RBCs with video-rate imaging, are superimposed onto a maximum intensity projection image of BM vasculature (red, vascular dye; green, nestin-GFP/RBCs; blue, SHG bone signal). A solid green line indicates a vessel with an adjacent nestin\textsuperscript{+} cell and a dashed white line indicates a nestin\textsuperscript{-} vessel.
- **b**. In vivo image of the bone marrow of a universal DsRed recipient 5 days after transplantation with 25 million total marrow cells from a universal GFP donor. Red, DsRed; green, GFP; blue, SHG from bone.  
- **c**. Ki-67 staining of green (donor) and red (host) BM cells by FACS on day 2 (n = 3 mice) and day 5 (n = 2 mice) after transplantation. Each symbol denotes a different mouse. The mean (black line) for days 2 and 5 ± s.d. (shaded box) for day 2 is shown.
- **d**. The in vivo \(P_O_2\) values in regions with large clusters of donor cells (n = 19 locations from 4 mice) compared with small donor clusters/single cells (n = 16 locations from 4 mice) and host clusters/single cells (n = 40 locations from 5 mice). The mean (black line) ± s.d. (shaded box) for each data set is shown. Scale bars, ~100 μm.
These observations compelled us to re-examine blood flow in the BM\textsuperscript{3}. Specifically we asked whether the differences in $p_{\text{O}_2}$ observed in nestin\textsuperscript{+} versus nestin\textsuperscript{−} vessels and in steady-state versus radiation/chemotherapy-treated animals could be accounted for, at least in part, by the blood flow profiles. We injected carboxyfluorescein succinimidyl ester (CFSE)-labelled red blood cells (RBCs) and increased our imaging speed to 120 frames per second (scanning one quarter of the frame size) to enable the tracking of individual RBC flow direction and speed. Interestingly, the highest RBC flow velocities are observed in nestin\textsuperscript{+} vessels ($>2 \text{ mm s}^{-1}$, compared with $0.2–1 \text{ mm s}^{-1}$ in sinusoidal vessels). The vascular network map shows that the nestin\textsuperscript{+} vessels are always upstream of and drain into sinusoidal vessels (Fig. 4a), suggesting that nestin\textsuperscript{+} vessels have arterial characteristics. In vivo immunostaining\textsuperscript{3} with fluorescent anti-Sca-1 antibody confirms that they are arteries (Extended Data Fig. 7a).

Flow imaging with labelled RBCs also helps to overcome the dye leakage problem after radiation or chemotherapy and enables us to delineate the functional blood vessels by analysing the tracks of the flowing RBCs in the video sequence. We found a surprisingly robust flow 2–6 days after lethal irradiation (Extended Data Fig. 7b and Supplementary Video 1), a time when BM cellularity is declining precipitously (Extended Data Fig. 8). A combination of ample blood supply and reduced oxygen consumption (reduced BM cellularity) may explain the elevation of $p_{\text{O}_2}$ after radiation and chemotherapy. Reduced BM cellularity may also explain why the gradient between intra- and extra-vascular $p_{\text{O}_2}$ disappears after myelosuppressive conditioning.

To establish further the link between cellularity and oxygen consumption, we infused $25 \times 10^6$ total marrow cells from GFP donor mice into lethally irradiated DsRed recipients (Fig. 4b). We verified by fluorescence-activated cell sorting (FACS) on day 2 and 5 that most of the proliferating (Ki-67\textsuperscript{+}) cells are in the donor fraction (Fig. 4c). Then, by in vivo imaging, we showed that the BM contained heterogeneous patches of donor (GFP\textsuperscript{+}) and recipient (DsRed\textsuperscript{+}) cells, with corresponding differences in local $p_{\text{O}_2}$ (Fig. 4b). Lower $p_{\text{O}_2}$ was associated with large clusters of green cells, consistent with the notion that the proliferating cells consume oxygen more avidly (Fig. 4d). Direct measurements of the absolute $p_{\text{O}_2}$ in the BM have revealed a unique hypoxic landscape organized by its dense vascularity (oxygen supply) and high cellularity (consumption). The balance between supply and consumption can be altered by stress such as radiation and chemotherapy. The local topography is further defined by the positioning of different types of blood vessels within the BM (Extended Data Fig. 9). In particular, HSCs near nestin\textsuperscript{+} arteries and those near sinusoidal vessels will experience different metabolic milieus, highlighting the need to examine further the role of distinct vascular niches in HSC regulation.

**METHODS SUMMARY**

**Intravital imaging and $p_{\text{O}_2}$ measurement.** All $p_{\text{O}_2}$ measurements and intravital images were acquired with a home-built two-photon microscope (Extended Data Fig. 1). The arrival time of individual phosphorescence photons after pulse excitation was recorded using a custom photon counting circuit. The histogram of the photon arrival times was then analysed to obtain the triplet state lifetime.

For vascular mapping, RBCs were isolated from a donor C57BL/6 mouse, labelled with carboxyfluorescein diacetate succinimidyl ester (CFDA-SE; Life Technologies), and injected together with 200 kDa of 3 mg ml\textsuperscript{−1} 70 kDa Rhodamine B–dextran (Life Technologies) vascular label in PBS solution into the nestin-GFP mice immediately before imaging. Development of the HSC niche concept has turned 31. Kunisaki, Y. et al. Nature 466, 829–834 (2010).

Kiel, M. J. et al. SLAM family receptors distinguish hematopoietic stem and progenitor cells and reveal endothelial niches for stem cells. Cell 121, 1109–1121 (2005).

Calvi, L. M. et al. Osteoblastic cells regulate the haematopoietic stem cell niche. Nature 425, 841–846 (2003).

Wang, L. D. L. & Wagers, A. J. A. Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nature Rev. Mol. Cell Biol. 12, 643–655 (2011).

Lichtman, M. A. M. The ultrastructure of the hemopoietic environment of the marrow: a review. Exp. Hematol. 9, 391–410 (1981).

Lo Celso, C. et al. Live-animal tracking of individual haematopoietic stem/progenitor cells in their niche. Nature 457, 92–96 (2009).

Nombela-Arrieta, C. et al. Quantitative imaging of haematopoietic stem and progenitor cell localization and hypoxic status in the bone marrow microenvironment. Nature Cell Biol. 15, 533–543 (2013).

Kunisaki, Y. et al. Arteriolar niches maintain haematopoietic stem cell quiescence. Nature 502, 637–643 (2013).

Chow, D. C., Denning, L. A., Miller, W. M. & Papoutsakis, E. T. Modeling $p_{\text{O}_2}$ distributions in the bone marrow hematopoietic compartment. I. Krogh's model. Biophys. J. 81, 675–684 (2001).

Chow, D. C., Denning, L. A., Miller, W. M. & Papoutsakis, E. T. Modeling $p_{\text{O}_2}$ distributions in the bone marrow hematopoietic compartment. II. Modified Kroghian models. Biophys. J. 81, 685–696 (2001).

Veilleux, I., Spencer, J. A., Biss, D. P., Cote, D. & Lin, C. P. In vivo cell tracking with video rate multimodality laser scanning microscopy. IEEE J. Sel. Topics Quantum Electron. 14, 10–18 (2008).

Lebedev, A. Y., Trolle, T. & Vinogradov, S. A. Design of metalloporphyrin-based dendritic nanoprobes for two-photon microscopy of oxygen. J. Porphy. Phthalocyanines 12, 1261–1269 (2008).

Finikova, O. S. et al. Oxygen microscopy by two-photon-excited phosphorescence. PLoS One 8, e51483 (2013).

Vanderkooi, J. M. J., Maniara, G. G., Green, T. J. & Wilson, D. F. An optical microscope for detailed imaging of cerebral vessels. J. Neurosci. 23, 1292–1304 (2004).

Sakadzic, S. et al. Two-photon high-resolution measurement of partial pressure of oxygen in cerebral vasculature and tissue. Nature Methods 7, 755–759 (2010).

Lecocq, J. et al. Simultaneous two-photon imaging of oxygen and blood flow in deep cerebral vessels. Nature Med. 17, 893–898 (2011).

Kazmi, S. M. et al. Three-dimensional mapping of oxygen tension in cortical arterioles before and after occlusion. Biomed. Opt. Express 4, 1061–1073 (2013).

Dewhirst, M. W. et al. Quantification of longitudinal tissue $p_{\text{O}_2}$ gradients in window chamber tumours: impact on tumour hypoxia. Br. J. Cancer 79, 1717–1722 (1999).

Mazo, I. B. et al. Total body irradiation causes profound changes in endothelial trafficking of hematopoietic progenitor cell recruitment to bone marrow. Blood 99, 4182–4191 (2002).

Sipkins, D. A. et al. In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment. Nature 435, 969–973 (2005).

**Supplementary Information** is available in the online version of the paper.

**Online Content** Any additional Methods, Extended Data display items and Source Data are available in the online version of the paper; references unique to these sections appear only in the online paper.

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Author Contributions J.A.S. designed and built the microscope, designed experiments, conducted research, collected and analysed data and wrote the manuscript; F.F. designed experiments, conducted research, collected and analysed data, and wrote the manuscript; E.R. synthesized the PtP-C343 oxygen probe; A.K. helped conduct research and collected and analysed data; J.W., J.M.R., W.Z., L.J.M., R.T. and R.Y. helped conduct research; C.A. and D.C. helped build the microscope; S.A.V. synthesized the PtP-C343 oxygen probe and wrote the manuscript; D.T.S. designed experiments and wrote the manuscript; C.P.L. designed experiments, sponsored the project and wrote the manuscript.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Readers are welcome to comment on the online version of the paper. Correspondence and requests for materials should be addressed to C.P. L. (charles_lin@hms.harvard.edu).
Two-photon $\rho_{O_2}$ microscope. The microscope is composed of two excitation arms: a video-rate laser-scan scanning two-photon imaging arm and a point-detection two-photon phosphorescence lifetime sensing arm (Extended Data Fig. 3). Output from a 80 MHz femtosecond laser source (MaiTai) tuned to 840 or 913 nm is split into $s$ and $p$ polarizations by a polarizing beamsplitter (PBS1). The $s$-polarized beam passes through PBS1 and enters the imaging arm whereas the $p$-polarized beam is deflected 90° into the point detection arm. The power for each arm can be adjusted by rotating a half-wave plate in front of PBS1. These polarized beams are later recombined by a second polarizing beamsplitter (PBS2) immediately before entering the objective.

For the point-detection arm, the beam is swept across a slit aperture (NT38-560, Edmund Optics) using a galvanometric scanner (6220H, Cambridge Technology) and imaged onto the sample to form a $\sim 3.5 \mu m$ scan line. Such one-dimensional scanning simultaneously served two purposes. First, it generated an excitation gate with adjustable pulse duration (15–40 $\mu s$), determined by the scanning speed across the slit aperture. Second, it served to reduce the triplet state saturation effects that may accompany stationary excitation, thus helping to prevent degradation of the point spread function in the axial dimension. After application of the excitation gate, the arrival time of individual phosphorescence photons was recorded using a custom photon counting circuit. The histogram of the photon arrival times was then analysed to obtain the triplet state lifetime. We found that the standard deviation of the $\rho_{O_2}$ measurement was typically under 4 nm Hz and inversely proportional to the signal-to-noise ratio (SNR, data not shown). For the imaging arm, we use a similar scan engine as described previously and combine the beam proportional to the signal-to-noise ratio (SNR, data not shown). We then repeated the same protocol in a nestin-GFP mouse.

Image quantification. Using the axial stacks, the distance of each $\rho_{O_2}$ measurement point and vessel diameter was measured using the Pythagorean theorem. The vessel diameter was measured using the Rhodamine B–dextran signal. This measurement was only performed in the $x$–$y$ plane and is the diameter of the lumen of the vessel. Both the distance and vessel diameter measurements were performed in Imagej v1.47p. For display purposes, the brightness and contrast of Figs 1a, b, e, 4a, b, Extended Data Figs 4, 6, 7 and Supplementary Video 1 were adjusted. Image analysis was performed on raw images. In Fig 1a, the maximum intensity projection image displayed only $\sim 23 \mu m$ of depth in the blue channel. We did not include the full thickness for the blue channel because the blue SHG signal covers over the majority of the image at the most shallow depths.

For distance measurements to blood vessels, three-dimensional stacks were collected using Fiji (Image J v1.45b) into the vertical planes ($Y$–$Z$–$X$) and digitally rotated to remove the tilt of the skull relative to the imaging plane. Subsequently, for each R/G/B channel, the greyscale intensity profile along the $Z$ direction was measured and fitted into an empirical exponential decay curve, the inverse of which was then multiplied to each stack image to equalize the image intensity as a function of depth. Next, the three-dimensional stack was converted into binary images and manually verified for structural accuracy. Applying the exact Euclidean distance transform (EEDT) function on the binarized red (blood vessel) channel stack returned the three-dimensional distance of each non-vascular pixel to the nearest blood vessel wall as a 32-bit greyscale image stack. Pixels outside the bone marrow space were excluded using the binarized blue (bone) channel stack.

For whole BM transplantation, 4-month-old male C57BL/6j mice (Jackson Laboratory) or male actin-DsRed knock-in mice (Cg-Tg(CAG-DsRedMST)Nagy/J; Jackson Laboratory) were lethally irradiated with a single dose of 9.5 Gy gamma irradiation from a Cs137 source (Gammex Exactor, Nordion) 4h before rescue with $25 \times 10^6$ whole bone marrow cells from a donor actin-GFP knock-in mouse (C57BL/6j-Tg(CAG-EGFP)J11301Ob/LeySdp; Jackson Laboratory). On the fifth day after irradiation and transplantation, the actin-DsRed recipient mice were imaged and $\rho_{O_2}$ measurements acquired in the BM. C57BL/6j mice were killed, femurs and tibiae extracted, and the marrow prepared for analysis of cycling cells. RBCs were lysed on ice using Red Blood Cell lysing buffer (Sigma). The remaining BM cell fraction was counted, fixed, permeabilized and stained with PE-conjugated Ki-67 according to the manufacturer’s recommendations (BD Pharmingen/Biosciences). Analysis of cycling cells was performed on a SORP FACSAria II instrument (BD Biosciences).

For vascular mapping, we extracted blood from a donor C57BL/6j mouse, labeled the RBCs, labelled $7 \times 10^6$ RBCs with CFDA-SE (Life Technologies) at 15 $\mu M$ for 12 min at 37°C (labeling concentration $\sim 20 \times 10^6$ RBCs per ml in PBS without Ca plus 0.2% bovine serum albumin plus 0.2 g l$^{-1}$ glucose), and washed twice. We then suspended the RBCs in 200 ml of 400 nM Qtracker 655 (Life Technologies) vascular label PBS solution or 200 ml of 3 mg ml$^{-1}$ 70kDa Rhodamine B–dextran vascular label in PBS solution and intravenously injected the solution into the donor nestin-GFP mice immediately before imaging.

For in vivo anti-Sca-1 staining, we first demonstrated the in vivo specificity of the anti-Sca-1 antibody. We intravenously injected 20 mg of Trastuzumab (Genentech catalogue no. BI64564) into a C57BL/6j mouse to block non-specific Fc binding. Two hours later, we intravenously injected $\sim 15 \mu g$ of PE-Cy5.5 anti-Sca-1 antibody (BD Biosciences catalogue no. 108114) and $\sim 15 \mu g$ of PerCP-Cy5.5 Rat IgG2a isotype control antibody (eBioscience catalogue no. 45-4321-80). We then imaged the BM 24h later and found that anti-Sca-1 antibody had high specificity compared with the isotype control data (not shown). We then repeated the same protocol in a nestin-GFP mouse.

Animal preparation. All procedures were approved by the Institutional Animal Care and Use Committee at Massachusetts General Hospital. Male wild-type C57BL/6 (Jackson Laboratory) and C57BL/6 nestin-GFP mice less than 6 months of age were used for all in vivo Pip-C343 experiments. In a typical experiment, anaesthesia was slowly induced in mice via inhalation of a mixture of 1.35–2% isoflurane and oxygen (O2). To maintain anaesthesia the mixture was reduced to 1.25% isoflurane with nitrous oxide. To maintain anaesthesia the mixture was reduced to 1.25% isoflurane with nitrous oxide. To maintain anaesthesia the mixture was reduced to 1.25% isoflurane with nitrous oxide. To maintain anaesthesia the mixture was reduced to 1.25% isoflurane with nitrous oxide.
31. Mignone, J. L., Kukekov, V., Chiang, A. S., Steindler, D. & Enikolopov, G. Neural stem and progenitor cells in nestin-GFP transgenic mice. J. Comp. Neurol. 469, 311–324 (2004).

32. Esipova, T. V. et al. Two new 'protected' oxyporphs for biological oximetry: properties and application in tumor imaging. Anal. Chem. 83, 8756–8765 (2011).
Extended Data Figure 1 | Schematic of the two-photon intravital imaging and 2PLM system. BP, bandpass filter; DAQ, data acquisition device; f, focal length; Galvo, galvanometer mirror; L, lens; LP, longpass filter; PBS, polarizing beam splitter; PMT, photomultiplier tube; z/2, half-wave plate. L13 and L14 have a focal length of 5 cm.
**Extended Data Figure 2 | In vitro calibration measurements.** The $p_O_2$ values of a solution of PtP-C343 are plotted as a function of the phosphorescent lifetime measurements recorded by our microscope. $p_O_2$ was determined by an oxygen microsensor inserted into the solution. The solid line is the published calibration curve for the same batch of PtP-C343 recorded by a different laboratory.  

![Graph showing $p_O_2$ values as a function of Tau (μs). The graph includes a solid line labeled 'Calibration from reference 24' and open circles labeled 'Calibration at 38°C and pH 7.4.']
Extended Data Figure 3 | Schematic of PtP-C343 platinum porphyrin oxygen sensor. The core porphyrin, PEG chains, C343 moieties and dendrimer are denoted in red, green and blue, respectively.
Extended Data Figure 4 | PtP-C343 leakage into extravascular space within the BM. Two-photon intravital image of PtP-C343 fluorescence ~60 min after intravenous administration. Scale bar, ~100 μm.
Extended Data Figure 5 | HSC homing to bone marrow. Scatter plot of HSC (lineage^− c-kit^+ Sca-1^+ CD150^+ CD48^-) homing to nestin^- vessels and bone (endosteum) in lethally irradiated recipients (9.5 Gy) 2 days after adoptive transfer (n = 19 cells from 3 mice).
Extended Data Figure 6 | Vascular leakage after cytoreductive conditioning. a–c, Intravital images of BM in untreated controls (a), lethally irradiated (b) and busulphan-treated (c) mice 2 days after conditioning reveals increased permeability and decreased contrast of BM vessels. Rhodamine B–dextran (red) and bone SHG (blue) signal is shown. Scale bar, ~50 μm.
Extended Data Figure 7 | *In vivo* immunostaining and blood flow analysis after cytoreductive conditioning.  

**a**, Intravital BM image of anti-Sca-1 immunostaining (red), nestin-GFP (green), blood vessels (blue) and bone (SHG, grey) demonstrating Sca-1+ nestin-GFP vessels. Scale bar, ~50 μm.  

**b**, Standard deviation image from 600 frames of a confocal video sequence showing the path of RBC flow (green) and bone signal (SHG, blue) on day 2 after lethal irradiation (9.5 Gy). Each pixel displays the standard deviation of the corresponding pixel location across all 600 frames of the video. Scale bar, ~100 μm.
Extended Data Figure 8 | Cytoreduction of the BM after myeloablative or myelosuppressive conditioning. BM cellularity as a function of treatment day is plotted for untreated (n = 3 mice), sub-lethally irradiated (4.5 Gy, n = 3 mice per time point), lethally irradiated (9.5 Gy, n = 3 mice per time point) and busulphan-treated (n = 3 mice per time point) mice.
Extended Data Figure 9 | Revised model of local oxygen tension in the BM. Instead of a poorly perfused hypoxic zone near the endosteum, we detected an opposite gradient, with the peri-sinusoidal region being more hypoxic than the endosteal region, which is perfused with small arteries. After irradiation, the sinusoids are greatly dilated and the blood flow is maintained, whereas the reduced oxygen consumption due to declining BM cellularity causes the pO₂ to become elevated in the entire marrow space, with no defined spatial gradients.