Small near-infrared photochromic protein for photoacoustic multi-contrast imaging and detection of protein interactions in vivo

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Photoacoustic (PA) computed tomography (PACT) benefits from genetically encoded probes with photochromic behavior, which dramatically increase detection sensitivity and specificity through photoswitching and differential imaging. Starting with a DrBphP bacterial phytochrome, we have engineered a near-infrared photochromic probe, DrBphP-PCM, which is superior to the full-length RpBphP1 phytochrome previously used in differential PACT. DrBphP-PCM has a smaller size, better folding, and higher photoswitching contrast. We have imaged both DrBphP-PCM and RpBphP1 simultaneously on the basis of their unique signal decay characteristics, using a reversibly switchable single-impulse panoramic PACT (RS-SIP-PACT) with a single wavelength excitation. The simple structural organization of DrBphP-PCM allows engineering a bimolecular PA complementation reporter, a split version of DrBphP-PCM, termed DrSplit. DrSplit enables PA detection of protein-protein interactions in deep-seated mouse tumors and livers, achieving 125-µm spatial resolution and 530-cell sensitivity in vivo. The combination of RS-SIP-PACT with DrBphP-PCM and DrSplit holds great potential for noninvasive multi-contrast deep-tissue functional imaging.

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To better understand the molecular mechanisms and dynamics involved in physiology and disease in a whole organism, biomedical studies increasingly employ non-invasive whole-body imaging with high-resolution in vivo1–3. Optical imaging offers valuable information and has been widely used in such studies4,5. However, photons are strongly scattered in biological tissue, limiting high-resolution pure optical imaging to a penetration depth within the optical diffusion limit (~1 mm)6. Photoacoustic (PA) computed tomography (PACT), by acoustically detecting photons absorbed by tissue, breaks the resolution and depth limitations of pure optical imaging and provides high-resolution imaging with optical contrast at depths up to centimeters7. PACT, highly sensitive to optical absorption by molecules, is inherently suited for molecular imaging using optically absorbing probes8–12.

Genetically encoded probes are advantageous due to their harmless non-invasiveness, precisely controllable targeting, and tissue-specific promoters. The combination of PACT and a reversibly photoswitchable near-infrared (NIR) absorbing full-length bacterial phytochrome (BphP) from Rhodopseudomonas palustris, RpBphP1, has resulted in an advanced differential imaging technique, termed reversibly switchable PACT (RS-PACT). RS-PACT provided substantially enhanced detection sensitivity in deep tissues13 in comparison with conventional PACT. PACT is now widely used with various proteins exhibiting reversible photochromic behavior14. Temporal unmixing has been applied to separate signals from two photoswitchable fluorescent proteins (FPs) in tissue phantoms15,16, however, their short absorption wavelengths make them less suited to deep-tissue PA imaging. Dual wavelength excitation has also been proposed to improve imaging sensitivity of BphPs, but is still limited to detecting only one phytochrome in vivo17,18.

Structurally, BphP proteins consist of a photosensory core module (PCM) and various so-called effector domains19–21 (Supplementary Fig. 1). The PCM is formed by the PAS (Per-ARNT-Sim), GAF (cGMP phosphodiesterase/adenylate cyclase/FhlA), and PHY (phytochrome-specific) protein domains connected with α-helix linkers, and typically has a molecular weight of 55–58 kDa. The spectral properties of BphPs are determined by a covalently attached chromophore, biliverdin IXα (BV). BV is an enzymatic product of heme and is widely present in mammalian cells and tissues. Incorporation of BV by an aoporphin of the BphP protein occurs in two steps. First, BV is secured to a chromophore-binding pocket in the GAF domain, and second, a covalent thioether bond is formed between the pyrrole ring of BphP (C32 atom) and the cysteine residues in the PAS domain22,23 (Supplementary Fig. 1). Canonical natural BphPs have two absorbing states, one of which absorbs at 670–700 nm (the Pr state) and the other at 740–780 nm (the Pfr state) (Fig. 1a). All BphPs exhibit natural photochromic behavior: they undergo reversible Pfr → Pr photoswitching upon 730–790 nm light irradiation and Pr → Pfr photoswitching upon 630–690 nm light irradiation. Here, we term the Pr state of the BphP-based probes the ON state and the Pr state the OFF state.

The RpBphP1, used in RS-PACT, consists of the PCM and two additional effector domains, named the PAS/PAC and HOS...
domains, and forms a dimer. Moreover, the HOS domain of one monomer interacts with the PCM of another monomer in the dimer. Because of its high molecular weight of ~82 kDa, RpBphP1 exhibits a limited folding efficiency and low expression level in mammalian cells. Attempts to delete the HOS domain resulted in the loss of photochromic behavior, suggesting that HOS binding to PCM is required for photoswitching. This finding was surprising, because in some BphPs, deletion of the effector domains does not affect reversible photoswitching, and only further truncation of the PHY domain starts to impair it. A PCM part of the DrBphP phytochrome from Deinococcus radiodurans (termed DrBphP-PCM below) does not interact with effector domains, preserves photochromism without effector domains, and is 1.5 times smaller than RpBphP1 (Supplementary Fig. 1). These features make it an attractive template for engineering advanced PA probes.

Currently, because of the absence of PA probes with NIR absorbance, whole-body molecular imaging of protein–protein interactions (PPIs) employs bioluminescent luciferases and FPs. PPI studies utilize Förster resonance energy transfer (FRET), bioluminescence energy transfer (BRET), and bimolecular fluorescence complementation (BiFC) approaches. However, relatively small changes in the FRET and BRET signals make these techniques suboptimal for use in whole mammals. BiFC is based on the tagging of two proteins of interest, each with half of an FP. Upon interaction of the proteins, the two halves of the split FP associate with each other to form a fluorescent complex with the complemented FP, thus reporting the PPIs. Recently, we engineered several BiFC reporters from NIR FPs and demonstrated their ability to detect PPIs in mice. However, NIR BiFC did not provide high spatial resolution and sensitivity in imaging PPIs in deep tumors. PPIs were also imaged in vivo using split luciferase and thymidine kinase, resulting in bioluminescence and positron emission signals, respectively. However, these reporters require injection of substrates. Moreover, the emission of the most red-shifted split luciferase is limited to 615 nm, and thymidine kinase’s signal provides low contrast and a non-specific background in vivo.

Here, we report a PACT technique which combines three approaches, namely single-impulse panoramic PACT (SIP-PACT), RS-PACT, and real-time detection of the photoswitching rates of genetically encoded chromophoric probes. We term this combined technique RS-SIP-PACT. We also characterize DrBphP-PCM both in vitro and in vivo as an advanced NIR photochrome for PACT techniques and demonstrate that it outperforms RpBphP1. We introduce both BphPs into the same mammalian cells, resulting in a distinctive decay characteristic in comparison with the cells expressing DrBphP-PCM only. By discriminating the different decay characteristics, we successfully separate both cell types in deep tissue. Using a single illumination wavelength, we perform multi-contrast temporal frequency lock-in PA reconstruction (LIR) of two different tumors expressing the BphPs at depths in vivo. We next engineer a split version of DrBphP-PCM, resulting in the first bimolecular photoacoustic complementation (BiPC) reporter, termed DrSplit, and apply it to study intracellular PPIs in deep-seated mouse tumors and livers in vivo.

Results

Design and characterization of RS-SIP-PACT system. To characterize DrBphP-PCM as a PA probe and compare it with RpBphP1, we upgraded SIP-PACT for real-time reversible photoswitching, detection of photoswitching rates, and imaging, creating RS-SIP-PACT, which provides 125 µm in-plane resolution and ~1 mm elevational resolution (Supplementary Fig. 2). In order to image RpBphP1, DrBphP-PCM proteins, and the DrSplit reporter, we combined a Ti:Sapphire laser and an optical parametric oscillator (OPO) for illumination. These two lasers were synchronized and triggered by an FPGA-based controller (Methods). While the previous RS-PACT took 1.6 s to form a cross-sectional image with eight times multiplexing, RS-SIP-PACT requires only 50 µs to acquire data for one frame with a single laser pulse. Moreover, although its frame rate is currently limited by the imaging laser’s repetition rate (20 Hz), RS-SIP-PACT has achieved a 32-times greater frame rate than the previous RS-PACT. Due to the high-imaging speed of RS-SIP-PACT, we are able to capture the entire photoswitching process of the BphPs in real time, which enables temporal frequency analysis on each pixel. The result is a better contrast-to-noise ratio (CNR) in the images of BphPs, and a reduction in the impacts of motion (e.g., from respiration and heart beating) during in vivo imaging. In addition, the real-time detection of the photoswitching rates of BphPs allows a good separation of RpBphP1 and DrBphP-PCM, which have different photoswitching rates, using a single wavelength excitation.

Comparison of DrBphP-PCM and RpBphP1 as PA probes. We first measured the molar extinction coefficients for the ON states and the OFF states of DrBphP-PCM and RpBphP1. The ratios between the extinction coefficients of the ON state (Pfr form) at 780 nm and the OFF state (Pr form) at 630 nm of DrBphP-PCM and RpBphP1 were 9.9 and 4.1, respectively (Fig. 1a and Supplementary Fig. 3). We employed 780 nm light for PA imaging and photoswitching the BphPs to the OFF state, and used 630 nm light to switch the BphPs back to the ON state (Fig. 1b, c). The laser fluence on the sample surface at both wavelengths did not exceed 12 mJ cm⁻², which is below the American National Standards Institute safety limit. The imaging and photoswitching time sequences are shown in Fig. 1c. The change in RpBphP1 absorbance at 780 nm between the ON and OFF states was about four times, similar to earlier observations. The changes in DrBphP-PCM absorbance at 780 nm between the ON and OFF states were two times larger than that of RpBphP1 (Fig. 1d), which resulted in higher PA imaging contrast (Supplementary Table 1).

Tubes filled with DrBphP-PCM (~30 µM), hemoglobin (bovine blood with 90% oxygen saturation, sO₂), and RpBphP1 (~30 µM), respectively, were first embedded in clear gelatin (Fig. 1e). Although hemoglobin has the highest contrast in the ON state images (Fig. 1e, left column), in LIR, where a pixel-wise extraction of amplitudes of the harmonics of the illumination modulation frequency, both DrBphP-PCM and RpBphP1 signals stand out (Fig. 1e, middle column). The LIR method successfully separated the PA signals from two BphPs from the non-photoswitchable blood signals, even with 2.5 times higher CNR than previous differential methods (Methods and Supplementary Fig. 4). Typically, a threshold level of four times the noise level, estimated as the standard deviation of the background signal outside the imaged region, was globally applied to the PA LIR images.

Compared to RpBphP1, DrBphP-PCM took about three times longer time to photoswitch from the ON state to the OFF state (Fig. 1f, Supplementary Fig. 3c–f, and Supplementary Table 1). This photochemical feature enabled separating the PA signals of DrBphP-PCM and RpBphP1 by measuring the signal decay constants during imaging. Moreover, since hemoglobin is non-photoswitchable, its decay constant was close to zero, making it even more distinguishable from the BphP-based probes in RS-SIP-PACT (Fig. 1e, right column). The ON-to-OFF photoswitching rate (decay constant) here is defined as the reciprocal of the time it takes for the PA signal from the protein to drop to...
1/c of its maximum. The ON-to-OFF photoswitching rates of DrBphP-PCM and RpBphP1 were 0.54 s⁻¹ and 1.56 s⁻¹, respectively, as measured at a laser fluence of 4 mJ cm⁻² at 780 nm illumination (Supplementary Table 1).

We further compared the reversible photoswitching of both BphPs in scattering media at depths using 780 nm illumination. Tubes filled as before were embedded at a depth of 12 mm inside a scattering medium (10% gelatin and 1% intralipid in distilled water; reduced scattering coefficient of ~10 cm⁻¹) [36]. We defined the photoswitching ratio as the ratio of the measured PA signal amplitude of BphPs in the ON state to that in the OFF state. In both the clear medium (0 mm in depth) and scattering medium (12 mm in depth), DrBphP-PCM exhibited two times better photoswitching ratio than RpBphP1 (Fig. 1g).

**Multi-contrast RS-SIP-PACT imaging in cells and in vivo.** To compare expression levels of DrBphP-PCM and RpBphP1 in mammalian cells, we designed two similar plasmids where EGFP was co-expressed through a self-cleavable T2A peptide after the BphPs. The expression from these plasmids resulted in equimolar levels of a BphP and an EGFP control. Flow cytometry of HeLa cells transfected with these plasmids showed that the DrBphP-PCM expression level was 2.3 times higher than that of RpBphP1 (Supplementary Fig. 5a, b), likely because of the 1.5 times smaller size and simpler structural organization of the DrBphP-PCM, which enabled faster protein folding at 37 °C in mammalian cells.

We next used RS-SIP-PACT to image U87 glioblastoma cells stably expressing either RpBphP1 or DrBphP-PCM. With the RS-SIP-PACT system, we imaged pure bovine blood and either RpBphP1 or DrBphP-PCM expressing U87 cells embedded in gelatin (Fig. 2a, Supplementary Fig. 5c, and Supplementary Movie 1). The decay constants from the ON to the OFF state fitted with very different coefficients of \( b \approx c = 0 \), while the signals from U87 cells were fitted with very different coefficients of \( b > c = 0 \). Scale bar, 2 mm.

**Fig. 2** Photoacoustic characterization of the BphPs in cultured cells. a, LIR image of bovine blood, U87 cells expressing either RpBphP1 or DrBphP-PCM. Scale bar, 2 mm. b, PA signal decays and their fits from bovine blood. U87 cells expressing either RpBphP1 or DrBphP-PCM during 780 nm light illumination. c, The computed decay constants of the three types of cells; error bars are s.e.m. (\( n = 40 \)), calculated based on the pixel values from regions of interest. e, The contrast-to-noise ratio (CNR) of LIR signals from bovine blood and from U87 cells expressing either RpBphP1 or DrBphP-PCM in a clear medium (0 mm in depth) and a scattering medium (15 mm in depth). f, PA signal decays and their fits for the two types of cells—HEK-293 cells expressing both DrBphP-PCM and RpBphP1, and U87 cells expressing only DrBphP-PCM—under different illumination fluences. g, LIR images (top row) of U87 cells (left) and HEK-293 cells (right) and the images of computed coefficients of \( b \) (middle row) and \( c \) (bottom row) under different illumination fluences. The signal decays can be modeled in the form of \( g(t) = a + b \cdot e^{\frac{-t}{T_1}} + c \cdot e^{\frac{-t}{T_2}} \), where \( T_1 > T_2 \). The signals from HEK-293 cells were fitted with two similar coefficients \( b \approx c = 0.5 \), while the signals from U87 cells were fitted with very different coefficients of \( b > c = 0 \). Scale bar, 2 mm.

h, The computed coefficients \( k \), defined as \( k = \frac{\text{max}(b,c)}{\text{min}(b,c)} \), under different light fluences, showing a reliable separation of the two types of cells in f, g. Independent of the light fluence, the coefficient \( k \) for HEK-293 cells is ~1, and the coefficient \( k \) for U87 cells is much larger (>10); error bars are s.e.m. (\( n = 120 \)), calculated based on the pixel values from regions of interest.
The decay constants of the photoswitching processes depend on the local fluence. Under similar light fluence, DrBphP-PCM has a longer decay time than RpBphP1. However, due to optical absorption and scattering, the local optical energy delivery per unit area is unknown inside deep tissue, which poses a substantial challenge to unmixing multiple contrasts at depths using their decay constants. To address the unknown local optical fluence, we proposed a labeling strategy where we introduced both DrBphP-PCM and RpBphP1 into the same HEK-293 mammalian cells and introduced DrBphP-PCM only into U87 cells. Thus, the HEK-293 cells exhibited a distinctive decay characteristic in comparison with the U87 cells.

We used RS-SIP-PACT to image the HEK-293 cells expressing both BphPs in equimolar quantities from a single plasmid (Methods) and the U87 cells expressing only DrBphP-PCM. For each measurement voxel, we reasonably assumed that the local fluence was uniform within that voxel, because the 1/e optical penetration depth for NIR light is far greater than the voxel length. Experimental results showed that the photoswitching signals from HEK-293 cells expressing both BphPs contained two decay components, while the signals from U87 cells expressing DrBphP-PCM exhibited only one decay component, regardless of local fluence (Fig. 2f, Supplementary Fig. 7a–d, and Supplementary Table 2). With this labeling strategy, we took advantage of the number of decay components involved in the decay process to reliably separate two types of cells, instead of relying on the decay rates.

We modeled the decay process as a linear combination of two single exponential decay functions with different decay constants (Methods). By comparing the contributions of both decay functions to the overall decay, we established a criterion: If the contribution from the slower decay process is significantly larger (10×) than that from the faster decay process, the decay process is dominated by one component and the signals are from U87 cells; if the two contributions are similar (a ratio of ~1), the number of decay components is two and the signals are from HEK-293 cells. Therefore, by computing the number of decay components involved in the decay process, we can reliably separate the two types of cells in deep tissue, where knowledge of local fluence is limited (Fig. 2g, h, Supplementary Fig. 7e, and Supplementary Table 2). If the HEK-293 and U87 cells are mixed together and cannot be spatially resolved, we can quantify the concentration of each cell type by comparing the contributions from the two decay functions (Methods, Supplementary Fig. 7f, and Supplementary Table 2).

To study the performance of DrBphP-PCM in vivo, we first injected $1 \times 10^6$ U87 cells expressing DrBphP-PCM into a mouse brain and successfully detected the tumors using RS-SIP-PACT (Supplementary Fig. 8). To demonstrate the advantages of DrBphP-PCM in vivo, we imaged a mouse 2 weeks after injection of $1 \times 10^6$ U87 cells expressing DrBphP-PCM into the left front of the brain and $1 \times 10^6$ U87 cells expressing RpBphP1 into the right rear of the brain. A conventional PACT image reveals the brain’s cortical vasculature (Fig. 3a). In addition, LIR of ten...
Differential imaging

LIR

We injected HEK-293 cells expressing both BphPs (8 × 10^6) into the left lobe of the liver. At 2 h post injection, we then imaged the tumor-bearing mouse (n = 3) for 20 photoswitching cycles, each of which contained 160 frames. The LIR image clearly resolved the two tumors, with minimized motion artifacts (Fig. 5a). The HEK-293 tumors contain two different photochromic proteins, exhibiting two different decay constants in the decay process (Fig. 5b, c); while the U87 tumors contain only one photochromic protein, exhibiting only one decay constant in the decay process (Fig. 5b, c). Moreover, by analyzing the number of decay constants involved, we achieved reliable differentiation between the two tumors in deep tissue (~9.1 mm beneath skin, Fig. 5d–f).

Characterization of DrSplit for protein–protein interaction.

We next designed a BiPC reporter from DrBphP-PCM. For this, we genetically separated (split) DrBphP-PCM between the DrPAS domain and the DrGAF-PHY domains, and termed the set of these two constructs DrSplit (Fig. 6a). Notably, the PAS-GAF domains alone do not exhibit reversible photoswitching. Complementation of the PAS domain with the GAF-PHY domain reconstitutes the complete PIP (i.e., PAS-GAF-PHY domains), thus recovering its photoswitching property. To test DrSplit complementation, we used a rapamycin-induced PPIs between the FRB and FKBP proteins. We genetically fused the FRB protein to the DrPAS domain, and the FKBP protein to the DrGAF-PHY domains (Fig. 6b).
Because the main application of DrSplit was foreseen as in vivo imaging (see below), we made MTLn3 breast adenocarcinoma cells stably co-expressing DrPAS-FRB and FKBP-DrGAF-PHY fusions and studied their complementation using RS-SIP-PACT. Upon addition of rapamycin to the cells, a BiPC occurred, reconstituting the functional DrBphP-PCM (Fig. 7a–c). Complemented DrSplit retained 36% of the Pr state fluorescence of the non-split DrBphP-PCM (Supplementary Fig. 9a) and almost 100% of Pr ↔ Pfr photoswitching contrast measured by changes of intrinsic fluorescence (Fig. 7d, e). And the Pr ↔ Pfr photoswitching contrast measured by PA is retained ~50% (Supplementary Fig. 9b–d). DrSplit photoswitching at 780 nm was practically non-detectable without rapamycin, but was restored after FRB-FKBP binding induced by rapamycin, thus providing four times contrast (Supplementary Fig. 9d). The CNRs of the LIR images in the absence or presence of rapamycin were ~0.1 and 8.2, representing an ~82 times change in the CNRs of LIR images upon PPI induction (Fig. 7f). Complemented DrSplit in MTLn3 cells exhibited photoswitching ratios of 4.46 ± 0.49 in the clear medium and 2.60 ± 0.34 in the scattering medium (Fig. 7g, h). We then analyzed the expression of DrSplit in HeLa and U87 cells, and found that we could detect the fluorescence of EGFP co-expressed with DrPAS-FRB and mCherry co-expressed with FKBP-DrGAF-PHY, even 72 h after the transfection, indicating a low cytotoxicity of the DrSplit (Supplementary Fig. 10).

We have also demonstrated DrSplit’s application for microscopic imaging (Supplementary Fig. 11). MTLn3 cells, stably expressing DrPAS-FRB with EGFP and FKBP-DrGAF-PHY with mCherry, exhibited fluorescence of co-expressed fluorescent proteins in the absence of rapamycin. Upon induction of PPIs with rapamycin, we detected weak intrinsic NIR fluorescence of the DrBphP-PCM reconstituted from DrSplit (Supplementary Fig. 11a, b). The complementation of the DrSplit reporter provided approximately ten-time stronger fluorescence in the NIR channel (Supplementary Fig. 11c). Thus, DrSplit can be used as a multimodal reporter not only for BiPC, but also for BiFC.

**RS-SIP-PACT imaging of PPIs in vivo with DrSplit.** Using DrSplit and RS-SIP-PACT, we next longitudinally imaged PPIs in the tumors and monitored tumor metastases in the liver of mice (n = 4) (Fig. 8a–d, Supplementary Fig. 12, and Supplementary Movie 4). DrSplit-expressing MTLn3 cells (1 × 10⁶) were first locally injected in the mouse liver. Then, rapamycin was injected through the tail vein ~40–44 h before the PA imaging. The LIR PA images highlighted the photoswitchable signals from the complemented DrSplit resulting from the PPIs. We detected exponential growth of the primary tumor in the right lobe of the liver over 1 month (Fig. 8a–e). From day 15, we detected a delayed exponential growth of secondary tumors on the left lobe of the liver, resulted from metastasizing MTLn3 cells spreading to the other liver lobe (Fig. 8b–e). The diameter of the secondary...
Another part of DrSplit makes possible the monitoring of proteinprotein interactions (PPIs) between protein A and protein B. Genetically fusing one protein of interest (protein A) to one part of DrSplit and another protein of interest (protein B) to another part of DrSplit makes possible the monitoring of protein-protein interactions (PPIs) between protein A and protein B.

We next compared non-split DrBphP-PCM and DrSplit in native mouse tissues, without MTLn3 cell transplantation. We first performed hydrodynamic transfection38 of the plasmid encoding DrBphP-PCM into the liver of mice (n = 4) (Supplementary Figs. 14 and 15). Using RS-SIP-PACT, we found significant changes in the PA signals in the liver-expressing DrBphP-PCM. We then hydrodynamically co-transfected mice with DrPAS-FRB and FKBP-DrGAF-PHY plasmids encoding the DrSplit reporter. As a baseline image, we imaged the mouse liver after 24 h (Fig. 9a). To induce the PPIs resulting in the DrSplit complementation, rapamycin was injected through the tail vein, and the PA signals were detected ~42 h later (~66 h after hydrodynamic injection) (Fig. 9b). Ex vivo PA imaging of the isolated liver from the rapamycin-injected mouse further confirmed the reconstitution of functional DrBphP-PCM from DrSplit (Fig. 9c–f), and the differential fluorescence images also validated the existence of reconstituted DrSplit (Supplementary Fig. 16). The CNRs of the photoswitchable signals in the mouse liver were 0.152 and 6.60 before and after the rapamycin injection, respectively, indicating the ~43 times CNR enhancement. Upon rapamycin injection, we observed a significant increase of the LIR PA signals, whereas vehicle injection alone did not cause any PA signal changes (Fig. 9g).

**Discussion**

PACT is well suited to take maximum advantage of the photochromic behavior of genetically encoded probes. Temporal unmixing for the detection of photoswitchable fluorescent proteins has been demonstrated previously15,16. Recently, a BphP from Agrobacterium tumefaciens, called AgP17,18, was applied for PA imaging in the same manner as RpBphP1 in earlier reported RS-PACT13. Here, we combined the advanced RS-SIP-PACT technique with two distinct BphP-based probes, DrBphP-PCM and RpBphP1, which enabled multi-contrast PA in vivo imaging with a single 780 nm excitation. We then designed the first BiFC reporter, DrSplit, and by combining it with RS-SIP-PACT, photoacoustically detected PPIs with high spatial resolution in deep tissues at the whole-body level in mice. These advances resulted from the photochemical and structural features of DrBphP-PCM, which are superior to those of the previously used RpBphP1, as well as from the high-imaging speed and the LIR approach of RS-SIP-PACT, which provided high-sensitivity, high-resolution imaging at depths beyond those achievable by pure optical imaging39.

DrBphP-PCM is two times smaller than RpBphP1 and free from interactions between the domains, which facilitates folding and results in its higher expression in mammalian cells. These beneficial differences do not affect cell properties and allow establishing cell lines that stably express DrBphP-PCM. Like RpBphP1, DrBphP-PCM has OFF and ON states in the far-red and NIR regions, where tissues have relatively low-light attenuation, and therefore can maintain its photoswitching efficiency at depths. Further, DrBphP-PCM exhibits a two times greater absorbance photoswitching ratio than RpBphP113. In our experiments, the combination of all these characteristics provided about four times enhancement of the image CNR at depths in vivo. This performance makes DrBphP-PCM the reporter of...
choice for switching-contrast PACT techniques, such as RS-PACT and the advanced RS-SIP-PACT reported here.

Both DrBphP-PCM and RpBphP1 are photochromic and share similar absorption spectra in the ON and OFF states, so it is not possible to discriminate between these BphPs using standard multi-wavelength unmixing or differential imaging methods. However, DrBphP-PCM has a three-time lower photoswitching rate from the ON to the OFF state than RpBphP1, which requires a longer imaging time to capture the photoswitching process. Considering the unknown fluence, we proposed a labeling strategy where the HEK-293 cells were labeled with both DrBphP-PCM and RpBphP1 in equimolar quantities and U87 cells were labeled with DrBphP-PCM only. By computing the fluorescence changes of the lysate of HeLa cells expressing DrBphP-PCM detected at 720 nm during recurrent illumination cycles with 780/20 nm and 636/20 nm, repeated fluorescence changes of the lysate of HeLa cells expressing DrSplit in the presence (black line) or absence (blue line) of rapamycin, detected at 720 nm during recurrent illumination cycles with 780/20 nm and 636/20 nm. Repeated fluorescence changes of the lysate of HeLa cells expressing DrSplit in the presence of rapamycin and blood (dilute 10×) show similar CNRs in the ON state PA image; while the LIR image shows an outstanding CNR for MTLn3 cells expressing DrSplit in the presence of rapamycin. LIR image of blood and MTLn3 cells expressing DrSplit in the presence of rapamycin (left) and MTLn3 cells expressing DrSplit in the presence of rapamycin (right). Scale bar, 2 mm. The induction with rapamycin reconstitutes the functional DrBphP-PCM, which responds to the periodical light modulation. Repeated fluorescence changes of the lysate of HeLa cells expressing DrSplit in the presence of rapamycin and blood (dilute 10×) show similar CNRs in the ON state PA image; while the LIR image shows an outstanding CNR for MTLn3 cells expressing DrSplit in the presence of rapamycin. LIR image of blood and MTLn3 cells expressing DrSplit in the presence of rapamycin (right). The induction with rapamycin reconstitutes the functional DrBphP-PCM, which responds to the periodical light modulation. Repeated fluorescence changes of the lysate of HeLa cells expressing DrSplit in the presence of rapamycin and blood (dilute 10×) show similar CNRs in the ON state PA image; while the LIR image shows an outstanding CNR for MTLn3 cells expressing DrSplit in the presence of rapamycin.
properties of the parental DrBphP-PCM. Therefore, the BiPC approach should allow simultaneous detection of several PPIs by using multiple BphP-derived split probes that exhibit different ON-to-OFF photoswitching rates. Moreover, unlike BiFC, detection of several PPIs using BiPC can be performed with a single excitation wavelength. Further application of the DrSplit reporter could be simplified by using a self-cleavable peptide, such as T2A, inserted between the DrBphP-PAS and DrBphP-GAF-PHY parts, allowing co-expression of the complete BiPC reporter from a single plasmid.

In turn, the developed RS-SIP-PACT technique, no longer requiring data multiplexing, substantially accelerates the PA imaging process and further improves the detection sensitivity. In addition, the LIR method is simple and reliable; the background signals and respiratory motion influence are removed without loss of spatial resolution and sensitivity.

The NIR photochrome DrBphP-PCM probe and DrSplit PPI reporter engineered here, combined with RS-SIP-PACT, open various possibilities in basic biology and biomedical research. Both probes can noninvasively monitor individual pathways in subsets of cells in deep tissue and provide analysis of multiple pathways in a whole organ. DrSplit will allow detection of various biological processes that involve PPIs, such as wound healing, host–pathogen interactions, and organ development, and also serve as a whole-cell sensor for metabolic changes. Although BiPC of split reporters, such as DrSplit, can be irreversible, as with BiFC, it will visualize the accumulation of transient PPIs and low-affinity complexes. The higher-detection sensitivity of BiPC can advance the monitoring of activities of drug targets, to identify potential off-target effects by detecting PPIs associated with downstream pathways. Furthermore, it will enable in vivo genome-wide studies of PPIs, which previously were tested with BiFC, outperforming it in depth and spatial resolution.

**Methods**

**Photoacoustic tomography.** In PAT, as photons propagate in tissue, some are absorbed by biomolecules and their energy is partially or completely converted into heat. The heat-induced pressure propagates in the tissue and is detected outside the tissue by an ultrasonic transducer or transducer array to form an image that maps the original optical energy deposition in the tissue. The scattering of acoustic waves, within the ultrasonic frequency range of interest, is about three orders of magnitude weaker than that of light in soft tissue, on a per unit path length basis, which means that PAT can provide high spatial resolution at depths reachable by diffuse photons. PACT is a major implementation of PAT: A multi-element ultrasonic transducer array, or a mechanical/electronic scanning equivalent, is used to detect photoacoustic waves. Then, an inverse algorithm—essentially a method for accurately locating photoacoustic sources and mapping the absorbed optical energy density from the time-resolved acoustic signals—is employed to reconstruct high-resolution images.

**Plasmid construction.** The DrBphP gene was kindly provided by J. Ihala (University of Jyväskylä, Finland). The RpBphP1 gene was kindly provided by E. Giraud (Institute for Research and Development, France). For mammalian expression, the RpBphP1 gene was cloned as described earlier. PCR part encodings of the first 502 amino acids of DrBphP gene were PCR amplified as a Nhel-KpnI fragment and cloned into the pACGFP1-Hyg-N1 plasmid (Takara/Clontech). The AsCFP1 gene was cut out using BamHI and NotI enzymes and swapped with IRES2-mCherry, which was a fragment of the pIRE2-mCherry plasmid (Takara/Clontech). The final pIRE2-mCherry-DrBphP-PCM plasmid bears the Hygromycin resistance. The resulting plasmid allows co-expression of DrBphP-PCM and mCherry proteins from the same bicistronic mRNA. For equimolar mammalian expression from individual plasmids, the RpBphP1 gene or the PCM part of the DrBphP gene were PCR amplified as BglII-AscI fragments and cloned into multi-cloning sites of the pMCS-T2A-EGFP vector developed in our laboratory. The resulting plasmids allowed equimolar co-expression of RpBphP1 or DrBphP-PCM and EGFP proteins. To obtain plasmid for equimolar expression of both BphPs in one mammalian cell, the PCM part of the DrBphP gene was amplified via PCR with SpeI-NotI fragment and cloned into the RpBphP1-T2A-EGFP plasmid instead of EGFP gene.

For mammalian expression of DrSplit, the DrPAS encoding DNA fragment was PCR amplified as an Xbal-Xbal fragment and cloned into the iSPLIT plasmid to generate the pCA-RHE-DrPAS. Then, the DrPAS-FRb encoding fragment was cut out with EcoRI and BamHI and inserted into the multiple cloning site of the pIRE2-EGFP plasmid (Takara/Clontech) to generate the final pIRE2-EGFP-DrPAS-FRb, which bears the neomycin resistance. The resulting plasmid allows...
co-expression of DrPAS-FRB and EGFP proteins from the same bicistronic mRNA. DrGAF-PHY encoding fragment was amplified without an NLS signal and inserted as a NheI-KpnI fragment into the pAcGFP1-Hyg-N1 plasmid (Takara/Clontech). Aequorin was cut out using BamHI and NotI enzymes and swapped with IRES2-mCherry, which was a fragment of the pIRES2-mCherry plasmid (Takara/Clontech). The final pIRES2-mCherry-FKB-PdrGAF-PHY plasmid bears the hygroycin resistance. The resulting plasmid allows co-expression of FKB-PdrGAF-PHY and mCherry proteins from the same bicistronic mRNA.

For bacterial expression, the PCM encoding part of the DrBphP gene was cloned into a pBAD/HisB vector (Life Technologies). Mammalian cell culture. HeLa cells (ATCC, Cat#CCRL-1321) were grown in DMEM supplemented with 10% FBS, a penicillin-streptomycin mixture and 2 mM of glutamine (all from Invitrogen/Life Technologies) at 37 °C in 5% CO₂ air atmosphere. To compare expression level of RpBphP1 and DrBphP-PCM HeLa cells were transiently transfected with RpBphP1-T2A-EGFP or DrBphP-PCM-T2A-EGFP using an Effectene (Qiagen) according to the manufacturer’s protocol. Expression level of EGFP co-expressed downstream of T2A peptide with RpBphP1 or DrBphP-PCM cells was measured using a LSR II flow cytometer (BD Biosciences) equipped with a 488-nm laser and a 530/30 nm emission filter. To study the expression of RpBphP1 and DrBphP-PCM, HEK-293 cells (ATCC, Cat#CRL-11228) were transiently transfected with RpBphP1-T2A-EGFP using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol.

U87 cells (ATCC, Cat#HTB-14) were grown in DMEM supplemented with 10% FBS, a penicillin-streptomycin mixture and 2 mM of glutamine (all from Invitrogen/Life Technologies) at 37 °C in 5% CO₂ air atmosphere. We obtained a U87 stable precional mixture by transfecting cells with pIRES2-mCherry-DrBphP-PCM plasmid. Plasmid transfection was performed using an Effectene (Qiagen). Cells were further selected with 75 µg ml⁻¹ of Hygromycin B (Gold Biotechnology) and enriched using a FACS Aria sorter (BD Biosciences) equipped with a 561-nm laser and a 610/20 nm emission filter. For further culturing of U87 cells stably expressing DrBphP-PCM, the medium was supplemented with 75 µg ml⁻¹ of Hygromycin B.

MTLn3 cells (kindly provided by Dr. J. E. Segall, Albert Einstein College of Medicine) were grown in MEM Alpha supplemented with 5% FBS, a penicillin-streptomycin mixture, and 2 mM of glutamine (all from Life Technologies) at 37 °C in a 5% CO₂ air atmosphere. The MTLn3 precional mixture was obtained by transfecting with DrSplit plasmids, pRES2-EGFP-DrFAS-FRB, and pRES2-mCherry-FRB-DrGAF-PHY. Plasmid transfection was performed using an Effectene (Qiagen). Cells were further selected with 700 µg ml⁻¹ of G418 (Corning) and 300 µg ml⁻¹ of Hygromycin B (Gold Biotechnology) and enriched using a FACS Aria sorter (BD Biosciences) equipped with a 488-nm laser with a 530/30 nm emission filter and a 610/20 nm emission filter. After a week of growth, MTLn3 cells expressing DrSplit were then enriched again using a FACS Aria sorter (BD Biosciences) equipped with a 635-nm laser with a 720/20 nm emission filter. Before the second enrichment, the MTLn3 cells with DrSplit were treated with rapamycin for 24 h. For further culturing of MTLn3 cells stably expressing DrSplit, the medium was supplemented with 700 µg ml⁻¹ of G418 and 300 µg ml⁻¹ of Hygromycin B.

For induction of the FRB-FKBP protein–protein interaction in cultured cells, rapamycin (LC Laboratories) was dissolved in ethanol and 100 nM was added to...
cells 22–25 h before analysis. The cells are analyzed by fluorescence spectroscopy or flow cytometry in 48 h after transfection.

To test the cytotoxicity of DrSplit, either HeLa or U87 cells were transiently transfected with pRcRES-EGFP-DrPAS-FRB and pRcRES-mCherry-FKBP-DrGAP- PHY using LipofectaMin 2000 (Invitrogen) according to the manufacturer’s protocol. After 72 h of expression, the fluorescence intensity of EGFP and mCherry was analyzed by flow cytometry using an LSRII analyzer (BD Biosciences) equipped with a 488-nm laser with a 350/30-nm emission filter and a 561-nm laser with a 610/20-nm emission filter.

Preparation of animals. Adult 2- to 3-month-old female nude mice (Hsd: Athymic Nude-Foxn1nu, Harlan; body weight: ~20–30 g) were used for all in vivo experiments. All experimental procedures were carried out in conformity with laboratory animal protocols approved by the Animal Studies Committee at Washington University in St. Louis and the Office of Laboratory Animal Resources at California Institute of Technology. Throughout the experiment, the mouse was maintained under anesthesia with 1.5% vaporized isoflurane. The anesthetized mouse was taped to a lab-made motorized animal holder, which held the animal upright during imaging. The top of the holder was a small aluminum tube, providing anesthetix gas to the mouse, protecting the eye, nose, and mouth, and the bottom was an aluminum cylinder attached to a permanent magnet. The magnet securely held the animal holder to the scanning stage for elevational scanning. The taped to a lab-made motorized animal holder, which held the animal upright by an optical diffuser (RPC Photonics), and then passed through a 130° conical lens by an optical power meter. The laser beam was

The injected volume was 150 µl and resulted in 4.5 mg kg⁻¹ rapamycin was diluted in an aqueous solution of 5.2% Tween 80 and 5.2% PEG400. In order to image DrBphP-PCM 2;3 0 m J cm⁻² were measured. Fluorescence imaging using an LSRII analyzer (BD Biosciences) with the same number of switching cycles, because it averaged over the entire decay process within a cycle as well as across different cycles. The direct differential method, moreover, completely ignores the repeatability of the decay process across cycles. Typically, a threshold level of four times the noise level, estimated as the standard deviation of the background signal outside the imaged region and corrected to a globally applied to the temporal frequency domain of reconstructed PA images. To minimize the influence of respiratory motion, a non-rigid image matching algorithm was applied to the whole-body image for registration. During registration, we first selected a series of frames where respiratory motions were not obvious, and then averaged them to a single frame as a reference image. The other frames were registered to the reference image through a non-rigid image matching method.

Calculation of decay constant. To quantify the decay constant of the switching process, raw data from multiple trials were acquired and averaged, and a sequence of PA images representing one complete decay cycle was reconstructed from the averaged data. Each PA image was smoothed by a 5-pixel-by-5-pixel square kernel to further increase the signal-to-noise ratio. The time sequence at each pixel was then fitted to an exponential decay function of the form \( f(t) = a \cdot b^{-c t} \), where \( a \) is the measured value at time \( t = 0 \), \( b \) is the fitted peak pixel value, and \( c \) is the fitted non-negative time constant.

When two PhPs are fully mixed in a fixed ratio inside the cells, the decay function can be expressed in the form \( g(t) = a \cdot b^{-c t} - c \cdot e^{-d t} \), where \( R = T_1/T_2 \) should be relatively stable. This hypothesis was supported by our experimental results, where \( R = 2.2 \pm 0.3 \). The ratio \( k = \frac{\text{max}(g)}{\text{min}(g)} \) should also be stable, because two phychromes were mixed in a fixed ratio. Inside HEK-293 cells, the two phychroms were expressed in equimolar concentrations. Indeed, the experimental results for HEK-293 cells showed that \( b/c < 0.5 \), and thus \( k = 1 \). Notice that \( g(t) \) has a more general form than \( f(t) \), thus \( g(t) \) could also be used to fit the decay curve of a single phychrome, such as DrBphP-PCM only.

Experimental results showed that \( k \) was much greater than 1 when only DrBphP-PCM was measured. Thus, \( k = \frac{\max(g)}{\min(g)} \) was used as a criterion to distinguish the two types of cells—HEK-293 cells expressing both DrBphP-PCM and RpbPhP1, and U87 cells expressing only DrBphP-PCM. Empirically, we determined that when \( 1 < k < 1.2 \), the signals were from HEK-293 cells, and when \( k > 10 \), the signals were from U87 cells. This criterion is independent of local optical fluence. To avoid infinite in computation, the upper limit of \( k \) was set to 50. During computation, a pixel-wise curve fitting operation was performed first. Then, we spatially averaged pixel values of \( k \) across the regions of interest, which were defined using the LIR.

If the two types of cells—HEK-293 cells and U87 cells—are mixed together and cannot be spatially separated, we can use \( g(t) \) to quantify the concentration of U87 cells, and thus \( s' = 1 - s \) denotes the concentration of HEK-293 cells.

Hametoxalin and eosin histology and fluorescence imaging. The tumor-bearing livers and kidneys and hydrodynamic-transfected livers were harvested and fixed in 4% paraformaldehyde for 24 h. After paraffin embedding, coronal sections (3 µm thick) of the livers were cut. Standard hematoxylin and eosin (H&E) staining was performed on the sections, which were examined using bright-field microscopy (NanosZoomer, Hamamatsu) with a 20 × 0.67 NA objective lens.

A lab-made fluorescence imager, including a CCD camera (D4Y121-VB, Andor) and camera lens (SP 272E, Tamron, 90 mm, F/2.8), was used for fluorescence imaging. A laser diode (HL6738MG, Thorlabs Inc, 690 nm, 30 mW) was used for excitation, and a bandpass filter (FP50-40, Thorlabs Inc. 750 nm, FW8HM = 40 ± 20 nm) was used as an emission filter. A near-infrared LED (M780LP1, Thorlabs Inc., 780 nm, 800 mW) was used to switch the DrBphP-PCM/DrSplit proteins to...
Repeatability. The experiments were not randomized. The investigators were not blinded to allocation during the experiments and outcome assessment. No sample-size estimation was performed to ensure adequate power to detect a pre-specified effect size.

Data availability. The data that support the findings of this study are available from the corresponding authors on reasonable request. The reconstruction algorithm and data processing methods are described in detail in the Methods. We have opted not to make the data acquisition, image reconstruction, and processing code available because the code is proprietary and used for other projects.

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Author contributions

V.V.V. conceived the study. L.L., A.A.S., M.B., L.V.W. and V.V.V. designed the experiments. A.A.S., M.B. and D.M.S. constructed the plasmids, characterized the purified proteins, and established the stable cell lines. L.L. and J.S. constructed the RS-SIP-PACT system. L.L. performed the photoacoustic experiments. L.L., P.H. and L.Z. analyzed the photoacoustic data. L.L. and R.Z. cultured the mammalian cells. L.V.W.
and V.V.V. supervised the study. L.L., A.A.S., L.V.W. and V.V.V. wrote the manuscript. All authors reviewed the manuscript.

Additional information
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Competing interests: L.V.W. has financial interests in Microphotoacoustics, Inc., CalPACT, LLC, and Union Photoacoustic Technologies, Ltd., which, however, did not support this work. The remaining authors declare no competing interests.

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