Photocontrollable Proteins for Optoacoustic Imaging

Photocontrollable proteins revolutionized life-science imaging due to their contribution to subdiffraction-resolution optical microscopy. They might have yet another lasting impact on photo-or optoacoustic imaging (OA). OA combines optical contrast with ultrasound detection enabling high-resolution real-time in vivo imaging well-beyond the typical penetration depth of optical methods. While OA already showed numerous applications relying on endogenous contrast from blood hemoglobin or lipids, its application in the life-science was limited by a lack of labels overcoming the strong signal from the aforementioned endogenous absorbers. Here, a number of recent studies showed that photocontrollable proteins provide the means to overcome this barrier eventually enabling OA to image small cell numbers in a complete organism in vivo. In this Feature article, we introduce the key photocontrollable proteins, explain the basic concepts, and highlight achievements that have been already made.

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In several organic chromophores such as azobenzenes or diarylethenes, photoexcitation leads to a reversible chemical rearrangement, primarily isomerization. Such rearrangements can be exploited in photosensing materials, nanorobotics, and other applications.1,2 Interestingly, nature utilizes similar photochemical transformations of chromophores in proteins to sense and react to light stimuli. In proteins like flavoproteins, phytochromes, or opsins, light-induced rearrangements result in subsequent signaling cascades leading to immediate or long-term physiological responses in the respective organisms. For example, in mammals, isomerization of the retinal chromophore in rhodopsin is the first step in visual perception.

Transgene technology allows us to use these photocontrollable proteins outside of their native hosts in organisms of interest and to harness their light-induced transformations as a way to control their function without direct (invasive) interference with the respective cells, tissue, or organism under observation. Together with a focused light source (laser or optical fiber), this allows the manipulation and study of molecular processes with high spatiotemporal precision in vivo (optogenetics3). For example “nerve impulses” can be triggered by light to study neuronal functioning,4 or cellular programs can be activated by light to study developmental processes.5

Next to the light-control of molecular function, the intrinsic photophysical characteristics of the chromophores of those proteins have heavily impacted optical imaging in the life sciences. Light control of photophysical characteristics has been a key to the success of super-resolution imaging (SR), a fluorescence microscopy method that allows the recording of images at subdiffraction resolution, providing insight into the structure and function inside a cell in unprecedented detail.5,7 Recently, photocontrollable proteins have begun to become equally important for photo- or optoacoustic imaging (OA), a method that allows tomographic imaging in vivo at centimeter depths and relatively large fields of view far beyond the reach of purely optical methods. OA achieves this unique performance by combining optical excitation and ultrasound detection.6

We will begin this Feature article with a brief introduction on the two classes of photocontrollable proteins most relevant to imaging: photocontrollable members of the fluorescent protein (FP) family and natively photocontrollable photoreceptors termed phytochromes. Broader overviews on photocontrollable proteins have recently been published.11,12 Here we focus on the applications of photocontrollable proteins in OA, since the application of these proteins to SR is already the focus of excellent reviews.13,14

PHOTOCONTROL IN FLUORESCENT PROTEINS AND PHYTOCHROMES

As a fluorescence-imaging technique, SR relies primarily on photocontrollable FPs, while imaging photocontrollable proteins in OA usually relies on phytochromes, which are advantageous due to their near-infrared (NIR) absorption but show little to no fluorescence. Similar to other photocontrollable proteins, both families predominantly show a light-
induced cis/trans isomerization of their chromophore as the key-element of photocontrol. This isomerization in context with the protein surroundings of the chromophore defines the photophysical characteristics of the proteins and thereby their applicability in different imaging contexts.

With very few exceptions,15,16 photocontrollable FPs are engineered derivatives of non-photo-controllable FPs known from conventional fluorescence imaging.17 They contain a hydroxybenzylidene-imidazolidone chromophore in the center of a barrell-like structure (Figure 1A). The chromophore is autocatalytically formed from three amino acids of the protein itself. The entirely self-contained nature of the chromophore means that expression of the single transgene encoding the chromophore, which stabilizes the protonated form, is decarboxylated by UV irradiation, which generates the fluorescent deprotonated form. In photoconvertible proteins, β-elimination extends the chromophore’s π-electron system, red-shifting the absorption of the deprotonated form. In reversible photoswitching proteins, which we refer to as reversibly switchable fluorescent proteins (RSFPs) and which are the most relevant photocontrollable FPs for OA imaging, light induces the cis/trans isomerization of the methine bridge linking the hydroxybenzyl and imidazole rings (Figure 1A). The isomerization changes the chromophore position with respect to its protein environment, altering the interactions with surrounding amino acids. This leads to different protonation, quenching, stabilization, and planarity of the chromophore. Thus, RSFPs undergo a reversible, light-induced change between forms with different photophysical characteristics (absorption spectra and de-excitation pathways).

Phytochromes are multidomain photoreceptor proteins in plants, fungi, cyanobacteria, and other bacteria20 (Figure 1B). Natively, they relay light-induced structural changes to subsequent effector cascades regulating molecular, cellular, and developmental responses such as cell division. Their stronger absorption in far-red and NIR regions stems from the extended π-electron system of their tetrapyrrole chromophores. Similar to RSFPs, the light-induced change is a cis/trans isomerization of a methine bridge, in this case the one between the C and D rings of the tetrapyrrole (Figure 1B). The result is interconversion between two forms with different absorption characteristics: most phytochromes exhibit a red-absorbing state (Pfr; cis, 670–700 nm) and a far-red-absorbing state (Pfr; trans, 740–760 nm). The majority of phytochromes employ chromophores such as phytochromobilin (PFB) and phycocyanobilin (PCB), which are not found in mammalian cells, hampering their application to in vivo imaging. The exception are bacteriophytochromes (BphPs), which utilize the tetrapyrrole biliverdin (BV) as a chromophore. BV is a product of heme catabolism and is ubiquitous in mammalian cells. BphPs and most other phytochromes share a three-domain architecture, with the chromophore covalently anchored in the central GAF domain, which is flanked by the PAS and PHY domains. The PHY domain is a C-terminal domain that partially shields the chromophore from the solvent and is mainly responsible for relaying the chromophore isomerization to changes in the overall protein structure, which regulate downstream signaling cascades in the cell.

![Figure 1](image-url)  
**Figure 1.** Photocontrollable proteins: reversibly switchable fluorescent proteins (RSFPs) and bacteriophytochromes (BphPs). (A) RSFPs host a hydroxybenzylidene-imidazolidone chromophore inside a barrel-like protein structure. The chromophore undergoes reversible, light-driven cis/trans isomerization at the methine bridge linking the rings. (B) BphPs are multidomain proteins carrying a biliverdin chromophore in a central GAF domain. The chromophore undergoes light-driven cis/trans isomerization at the methine bridge linking the C and D rings.

| FUNDAMENTAL PHOTOPHYSICS OF SWITCHING IN RSFPs AND PHYTOCHROMES |
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| In RSFPs and phytochromes, the cis/trans isomerization induces a change in photophysical characteristics. In detail, these changes and the photophysical states involved are complex and differ from protein to protein. For reasons of simplification, we employ a model consisting of the protein and its chromophore being either in form A or B (Figure 2A). The two forms differ in the conformation of the chromophore and its position relative to residues in its vicinity. As a result, A and B differ in absorbance and emission spectra as well as in quantum yields (QY) and lifetimes of transitions. Effectively, this means a reversible light-controllable transition between a fluorescent (‘on’), which is usually cis in FPs, and a nonfluorescent (‘off’?) form; alternatively, or in addition, this transition results in a red- or blue-shift in absorption (photochromism) enabling OA. These different absorption characteristics between A and B mean that light of different...
wavelengths can be used to stimulate forward (A → B) and reverse (B → A) transitions. The interconversion kinetics depend on the QY of the respective photophysical transitions relative to competing transitions, e.g., fluorescence, non-radiative decay, or intersystem crossing (Figure 2B). A high QY for the cis/trans isomerization means a high likelihood that an absorbed photon will induce interconversion between A and B, rather than lead to some other transition. At the bulk level with millions of chromophoric proteins observed together in an imaging experiment, high QY for the transition between A and B translates to faster photocontrolled switching of the entire illuminated sample (Figure 2C). Consequently, the kinetics (k_{off} and k_{on}) also depend on the intensity of the light used, in other words, on the number of photons available for the chromophore to absorb and thereby induce the transition between A and B. A third conversion comes into play because A and B differ in absolute energy, resulting in a thermal relaxation of the chromophore in the dark (k_{dark}). The kinetics of this transition strongly depend on the relative stabilities of A and B and can be on the order of seconds to days.

■ PHOTOCONTROL IN SR

Presently, only the photoswitching of RSFPs has been employed for fluorescence SR because their on-state is strongly fluorescent. Fluorescent derivatives of the natively dark BphPs have been engineered by stabilizing the cis (P_{cis}) state, but they do not photoswitch efficiently, precluding their widespread use in SR, although they have recently been used in SR schemes similar to stimulated emission depletion (STED). BphPs that photoswitch between highly fluorescent and nonfluorescent states would be powerful tools for SR due to their favorable NIR absorption.

Two major SR concepts relying on photocontrollable proteins exist to overcome the diffraction limit of optical resolution (~200 nm). One is a purely physical strategy (dubbed RESOLFT), which relies on RSFPs. Here a diffraction limited excitation spot is overlaid by a second, ring-shaped diffraction-limited beam, which effectively transfers a large portion of the originally fluorescent RSFPs to an off-state that no longer contributes to fluorescence emission. The resulting “leftover” spot is diffraction-unlimited. In the second approach, which is typically performed with photoactivatable or -convertible proteins but can also be done with RSFPs, the sample is illuminated with low-intensity light that stochastically transfers a sparsely distributed subpopulation of labels to the fluorescent state. The positions of those labels in the “on” state can be determined at subdiffraction accuracy using centroid analysis. Repeating this procedure and computationally combining the images allows the position of all labels to be determined, giving rise to a diffraction-unlimited image.

■ GENERAL CONCEPTS IN OA

One of the exciting recent developments in the field of photocontrollable proteins is their application to optoacoustic imaging, which offers several advantages over many other imaging modalities. In addition to the resolution limit, optical imaging is limited by two other frontiers: penetration depth and field of view. Optical imaging is usually restricted to a depth of ~400 μm due to strong light scattering and absorption in soft tissues. However, this is insufficient to provide a comprehensive understanding of many molecular processes in vivo, such as the functioning of the brain or immune system. Modalities that can provide such insights,
such as magnetic resonance imaging (MRI) and positron emission tomography (PET), have high infrastructure costs and often require very specialized contrast agents while ultrasound often shows relatively poor resolution. OA is much less expensive than MRI or PET, it can image tissues without exogenous contrast agents, and it provides better resolution than ultrasound. In OA, the sample is illuminated with short light pulses that are absorbed by chromophores, which show thermo-elastic expansion after nonradiative de-excitation, leading to the emission of transient acoustic waves (Figure 2A), which are detected as pressure signals and reconstructed into an image using various inverse algorithms. Since OA detects ultrasound instead of light, it can penetrate to depths of several millimeters in tissue (Figure 3B), because tissue scatters sound much less than light. As a result, a complete mouse brain with a diameter of ~1 cm can be imaged by OA but not by purely optical methods (Figure 3B). OA and its applications have been extensively reviewed (Figure 3C).

The lack of labels is frustrating given that essentially any light-absorbing molecule can function as an OA label. However, the signal strength is determined by the molar absorption as well as the QY and lifetime of nonradiative transitions that are on time scales detectable by transducers of common OA imaging systems (ns); this excludes contribution from very fast vibrational transitions (ps) as well as nonradiative transitions from triplet states (ms) (Figure 2). Molecules that emit sufficiently strong acoustic signals after light absorption are hard to come by, partially because most efforts to develop labels have focused on labels for fluorescence imaging and so have tried to minimize the QY of nonradiative transitions.

Genetically encoded labels known from fluorescence imaging, such as EGFP and mCherry, have been used in OA, but their absorption is too blue-shifted to allow in vivo imaging of anything but transparent organisms such as zebrafish, due to the overwhelming background signal from endogenous chromophores in the visible spectrum (e.g., hemoglobin) in mammals which hampers delineation of the label from other absorbers purely on the basis of spectral information. Another approach to generate OA labels is to express enzymes that catalyze reactions that generate light-absorbing products such as melanin, violacein, or galactopyranosides (e.g., X-Gal). This approach has proven less effective because most products absorb in the visible range, too. Moreover, the product melanin emits a strong acoustic signal, but its production stresses cells and its absorption spectrum lacks features that can easily distinguish it from the background. To overcome these limitations to some extent, NIR-fluorescing phytochromes have been employed, but their signal is often too weak and the concentration too low to give significant signal against the background of endogenous absorbers.

### PHOTOCONTROL AND SIGNAL UNMIXING IN OA

A more promising alternative for generating strong OA labels are proteins that, by virtue of their photoswitching, can generate a sufficiently strong signal over background even at lower, more physiological concentrations. Since the inter-converting forms A and B differ in their QY and absorption, the excitation light can be used to modulate the strength of the OA signal, allowing it to be differentiated from non-modulating background absorption, such as from the abundant blood hemoglobin. To achieve this, the sample is repeatedly illuminated: first at a wavelength that switches the label to either an OA-active or -inactive state and subsequently with a second wavelength that induces the reverse transition (Figure 2C). Since the frequency at which the sample is illuminated with the second wavelength is known, OA signal modulation with the same frequency can be assigned as label and other signals as background (locked-in detection). This process is referred to as signal unmixing.

To date this approach has been reported in five implementations: single temporal imaging, multicontrast temporal frequency lock-in photoacoustics reconstruction (LIR), and difference-spectra demixing (Figure 4). In single-wavelength differential imaging, the unmixing is done simply by subtracting the image acquired when the label is in the OA-active form (IA) from the image acquired after switching the label to the OA-inactive (IB) form with the appropriate wavelength (Figure 4A). (The OA-active and -inactive forms are named as depicted in Figure 2A.) Since only the label shows a change, it is readily distinguished from the background. The reversibility of switching allows for multiple repetitions, leading to robust enhancement of the contrast-to-noise ratio (CNR). In the dual-wavelength differential approach, the concept of single-wavelength differential imaging is extended to include an intermediate state in which both OA-active and -inactive molecules (IA+IB) are generated by simultaneously illuminating with both wavelengths. Difference images are obtained by subtracting the two single population images from the mixed...
one (Figure 4B). For example Märk and colleagues converted the label BphP Agp1 sequentially from Pfp to Pfr via an intermediate step (Pfr) and all three states could be differentiated based on time-resolved OA signals: Pfp absorbed primarily at 755 nm, the mixed population of Pfp and Pfr showed peaks at 755 and 700 nm, and Pfr showed a peak only at 700 nm. Subsequent differential imaging generates the unmix signal (S_{Pfp}-S_{Pfr}+S_{Pfr}).

One shortcoming of differential imaging is that it neglects the kinetics of the transition between OA-active and -inactive forms. Temporal unmixing and LIR take this kinetics into account, which can substantially improve the results. Temporal unmixing has been used with principal component analysis to extract modulation patterns from images, based on the known timing of illumination at different wavelengths or by extracting the amplitudes of the harmonics of the preset photocontrol frequency in LIR (Figure 4C).

Another advantage of considering the kinetics of the transition between OA-active and -inactive forms is that the signals from multiple labels can be distinguished if their transition kinetics differ enough (Figure 4D). This allows the simultaneous imaging of several labels (multiplexing) while using only two wavelengths, thus reducing “spectral crowding” that normally limits how many labels can be used for a given set of wavelengths. An intermediate approach is difference-spectra demixing. Here two proteins that both absorb strongly at the photocontrol wavelength but not at the second wavelength are imaged sequentially. The entire spectra of the proteins are recorded, and the final unmixred image is formed by taking advantage of the spectral differences between the two proteins. In this way, the crosstalk between the spectral overlap from the two reporter proteins can be minimized, facilitating multiplexing (Figure 4E).

It is interesting to note that similar concepts have already been applied in fluorescence imaging to separate modulated fluorescence signals from invariant background signal. These approaches include out-of-phase imaging after optical modulation, optical-locked-in-detection, and SAFIRE. However, invariant background noise in fluorescence imaging (autofluorescence) is usually relatively weak in biological samples, limiting the use of these methods.

**PHOTOCONTROLLABLE PROTEINS USED IN OA AND THEIR APPLICATIONS**

Conventional OA imaging relies on the signal of strong and abundant endogenous absorbers since most transgene labels tested for OA show too low CNR. In the future, employing photocontrollable labels and the unmixing strategies discussed above may provide the required robustness for efficient routine use of OA for the detection of small numbers of labeled cells, significantly expanding the possible uses of OA for life sciences. So far, a number of applications have showcased this potential, employing a number of photocontrollable proteins, most of them native BphPs. The conceptual basis and first experiments demonstrating the use of photocontrol for OA were reported by Galanzha in 2013, albeit only using phototransformable proteins. Since their transitions are irreversible (see above), the authors were able to observe only one differential signal. In vitro work using the RSFPs Dronpa and Dronpa-M159T demonstrated the use of reversible photocontrol and temporal unmixing of signals from blood. A subsequent study with a BphP from *Rhodopseudomonas palustris* reported the first in vivo work on OA photocontrol. The authors visualized U87 cancer xenografts expressing BphP1 in the liver despite strong intrinsic background from blood hemoglobin (Figure 5A). Two later studies based on the BphP Agp1 from *Agrobacterium tumefaciens* visualized subcutaneous HT29 tumors expressing AGP1 (Figure 5B). A drawback of native BphPs is that they are fairly large (~80 kDa) and must dimerize to be functional. Consequently, the first attempts to engineer a BphP for OA have focused on creating a smaller, monomeric protein. Promising examples reported so far are a ~55-kDa monomeric protein from *Deinococcus radiodurans* (DrBphP-PCM) and engineered monomeric cyanobacterial BphPs (~16 kDa). Expressing smaller labels is likely to place a smaller metabolic burden on host cells.

This expansion of photocontrollable proteins usable for in vivo OA imaging has already allowed the first examples of multiplexing using photocontrol: Li et al. injected U87 cells expressing RpBphP1 or DrBphP-PCM into the right rear or left front lobe of the brain. LIR was used to unmix the signals from the two probes based on the different transition kinetics. The same approach was used to resolve signals after injecting the two populations of cells into the kidney and liver (Figure 5C). Multiplexing has been used not only to image mammalian cells but also to image bacteria invading a mammalian host. Chee et al. injected *E. coli* expressing cyanobacterial BphP, BphP1, and non-photocontrollable mIFP into the hind flank of a mouse and used difference-spectra demixing to separate the labels from background hemoglobin and from one another (Figure 5D). Detection of small numbers of bacteria *in vivo* in a whole organism is highly relevant to studying infection...
pathways and efficacy of treatment strategies as well as following the effects of bacterial cancer therapies.\textsuperscript{49} Recently, the switching and photophysical characteristics of a range of RSFPs and BphPs have been compared spectroscopically.\textsuperscript{50} This work may facilitate the rational pairing of labels with appropriate imaging, switching, and unmixing approaches. It may also help guide future engineering to optimize OA performance.

\section*{Perspective}

Despite the number of promising studies using existing photocontrollable labels and unmixing algorithms, further work is needed to bring photocontrol in OA to maturity and achieve the long-term goal of single-cell sensitivity in whole-animal studies. This will require advancement on several fronts: (i) more efficient transitions between the OA-active and -inactive forms to achieve more transitions in a given dwell-time (the time required to record an image), allowing robust unmixing; (ii) advanced illumination schemes and unmixing algorithms that allow extraction of as much differentiating information per illumination time as possible, and that compensate for movement and bleaching artifacts; and (iii) more labels with clearly distinguishable kinetics for each set of photocontrol wavelengths in order to allow routine multiplexing in the time domain. Beyond GFP-like and BphPs, it would be relevant to engineer other classes of chromophoric proteins, preferably with absorption in the NIR, to be photocontrollable, e.g., phycobiliproteins.

Analogous to the historical development of labels for fluorescence imaging, the next crucial step for developing photocontrollable OA labels is to create functional labels (sensors), that is, labels that provide information beyond the spatial position of the labeled structure or cell. The first such OA sensor can track protein−protein interactions in live cells using OA whole-animal tomography. Borrowing the complementation concept from fluorescence imaging,\textsuperscript{51} Li et al. expressed the DrPAS and DrGAF-PHY domains of DrBphP-PCM and fused those individual domains to proteins that are known to interact. When the resulting fusion proteins are expressed and the binding partner come together, they reconstitute functional DrBphP-PCM \textsuperscript{39} (Figure 5E). OA detection and unmixing of this label allowed visualization of a population as small as $\sim 530$ cells in tomographic images of an entire mouse \textit{in vivo}. Along this road, further sensors or indicator concepts known from fluorescence imaging can be translated to OA enabling further functional studies. For example, voltage-sensors with enhanced contrast through photocontrol and temporal unmixing may enable the maturation of OA neuroimaging.\textsuperscript{52}

Another interesting approach is to translate the way photocontrollable proteins are used in SR to OA. Yao and colleagues showed that a RESOLFT-like approach could...
achieve superior lateral resolution in comparison to conventional OA microscopy\textsuperscript{55} (Figure 5F). Such “hybrid” approaches, which can combine the penetration depth of OA with the resolution enhancement of RESOLFT-like concepts, require adaptive optics to keep the quality of the photocontrol light pattern through scattering tissue. Even if it proves impossible to achieve subdiffraction resolution at centimeter depths, OA may still be able to mature from imaging only at those depths if the resolution of whole-animal imaging can be increased to the few-micrometer level.

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V.N. is a shareholder of iThera Medical GmbH manufacturing OA imaging devices. All other authors declare no competing interests.

\section*{Notes}

The authors declare the following competing financial interest(s): V.N. is a shareholder of iThera (Munich, Germany), a company manufacturing photoacoustic devices. However, none of the devices were used in the discussed works or discussed in the manuscript.

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