**Controlling the sound of light: photoswitching optoacoustic imaging.**

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***Abstract***

Optoacoustic (photoacoustic) imaging has radically advanced the capabilities of optical investigations by allowing high-resolution imaging much deeper than optical microscopy. However, while label-free optoacoustic imaging has already entered into clinical application, optoacoustic biological imaging is in dire need of ubiquitous and easy-to-use labels that can impart cellular and molecular contrast, in ways similar to how fluorescent proteins propelled optical microscopy and imaging in biology. We review herein (photo-) switching advances that shine a new light or, in analogy, “bring a new sound” to biological optoacoustic imaging. The (photo-) switching concept uses light or other forms of energy to control a molecular property that affects optoacoustic signal generation. Light-controlled transitions have driven key applications in life-science research such as optogenetics or super-resolution fluorescence microscopy. Now, the combination of such transitions with novel devices that implement suitable molecular control and synchronous optoacoustic detection herald a new revolution in biological interrogations by enabling sensitive detection of cellular or molecular optoacoustic contrast at depths and resolutions that no other optical method is capable of. This review gives a brief background on optoacoustic agents, explains contrast enhancement in the spectral versus temporal domains, summarizes the key concepts of switching molecular properties and modulating optoacoustic signals, outlines the state of the art in systems and applications and discusses the outlook of optoacoustic imaging using photo-switching techniques over conventional contrast enhancement techniques.

***Introduction***

For more than three centuries, optical microscopy was limited by two major obstacles. The first, the diffraction limit, placed the upper boundary of optical investigations to resolutions of a few hundred nanometers. A series of techniques, however, have demonstrated how it is possible to overcome this obstacle, for example, by spatially confining the emission point-spread-function (deterministic methods: STED, RESOLFT, etc.)1 or by localization of temporally separated emissions from single events (stochastic methods: PALM, STORM, etc.)2 leading to the 2014 Nobel Prize in Chemistry. The second obstacle relates to the interaction of photons with matter, resulting in strong photon scattering and steep loss of resolution deeper than a few hundred microns in tissues, even with advanced techniques such as multiphoton intravital optical microscopy, one of the key tools for high-resolution biological interrogation of living tissues3,4. Optical imaging deeper in tissue is possible, for example, using fluorescence or bioluminescence imaging5, albeit with significant loss of resolution (several mm rather than µm) due to photon scattering6.

This second barrier, i.e., the effect of photon scattering, has been partially lifted by optoacoustic (OptA) methods. Through measuring ultrasound waves generated via the absorption of transient light by (tissue) absorbers, OptA imaging can form images based on ultrasound diffraction, allowing high-resolution optical imaging much deeper than optical methods. The resolution depends on the depth imaged, but it declines at a markedly slower rate as a function of depth than optical methods6-8, reaching resolutions in the tens of micrometers at millimeter depths and in the hundreds of micrometers at centimeter depths. This groundbreaking advance has been exploited in biomedical imaging to visualize endogenous tissue absorbers. Optoacoustic signals generated due to light absorption by hemoglobin in blood enable detailed visualization of the vasculature in-vivo9,10,11, whereby vascular and tissue blood oxygen saturation can be resolved by spectrally separating the relative contributions of oxy- and deoxy-hemoglobin12. Likewise, contrast from melanin or lipids has been demonstrated13,14. Besides technical demonstrations, this endogenous contrast has been employed in various human studies in dermatology15,16,17, oncology11,18, cardiology19, endocrinology20, gastroenterology21,22, muscle dystrophy 23 and other indications.

Conversely, while biological imaging can benefit from imaging endogenous absorbers, it also requires selective contrast from a broad range of cells and cellular components that typically do not emit OptA signals and thus do not inherently enable sensitive or specific detection. This is akin to the path taken towards fluorescence microscopy where the invention of fluorescent proteins24 (Nobel Prize 2008) overcame the challenge posed by the lack of fluorescence in most structures and functions of biological interest. Genetically encoded fluorescent labels can be targeted to most sub-cellular structures and cells, affording wide versatility in labeling cellular and sub-cellular moieties, and providing a key tool for the life sciences by fueling both in-vitro and in-vivo observations. On the other hand, such searches for a ubiquitous label for OptA imaging, including the use of fluorescence proteins as OptA labels, have been met with a range of challenges. As a result, even with the advantage of higher resolution over depth, OptA microscopy and imaging have not yet found broad acceptance in biology interrogations like optical methods.

This review outlines attempts to find contrast-generating technologies for OptA imaging that would allow efficient cellular and molecular imaging. First, it briefly summarizes progress with OptA contrast agents and early transgenic approaches and discusses the challenges that these early approaches met. Then, it focuses on photoswitching optoacoustic (psOptA) imaging25,26, an emerging technology that utilizes labels with optical absorption that is modulated by light over time to generate a temporally modulated OptA signal. In addition to light, other energy forms that impart modulation of the OptA signal emanating from a label are outlined. Then, the merits of detecting temporally modulated OptA signals are explained as the means to shift analysis from the spectral-domain to the time-domain for suppressing background signals and drastically improving detection sensitivity and specificity. Photoswitching labels could play a similar role in OptA imaging as fluorescence labels have for optical methods, i.e., provide a ubiquitous OptA platform for selectively increasing the contrast of the labeled moieties over the background. This review describes the progress with the development of time-domain modulation, detection schemes and labels for implementing such imaging. Furthermore, the review discusses how psOptA technology, and in general OptA with a temporally modulated signal, could enable cellular and sub-cellular contrast deeper in tissue than intravital optical microscopy.

***Optoacoustic methods for biological interrogations***

Optoacoustic imaging is based on the detection of pressure waves in tissues that are generated as the result of light absorption by (tissue) absorbers. Molecular movements due to the energy gained by transient light absorption translate to minute heating of the local molecular environment, leading to thermoelastic expansion, i.e. the rapid volumetric expansion and contraction of the heated volume. This process generates ultrasound waves that propagate within the surrounding medium and can be detected on the surface of the tissue by ultrasound detectors (USTs, **Figure 1a**). Using ultrasound detection to “listen” to optical absorption minimizes the sensitivity of OptA methods to photon scattering.

The imaging characteristics of OptA methods are markedly different than those of optical methods. Optical microscopy, including intravital implementations, operates by focusing light on or within tissue. However, due to photon scattering, it is not possible to focus light deep in tissues. Typical depths reached in the visible range are ~0.1‑0.5 mm, with the exact depth depending on the particular method employed and the scattering properties of the tissue under investigation. Optical imaging deeper in tissue operates with photons that experience multiple scattering events, without focusing ability, which results in a steep loss of resolution and consequently image fidelity. Therefore, macroscopic bioluminescence or fluorescence imaging, through several millimeters of tissue, rely on detection of the so-called diffuse photons, i.e. photons without high certainty on their spatial origin27 (Figure 1b). Such macroscopic images cannot accurately pinpoint the source of the activity imaged or differentiate the depth of the activity and for this reason also cannot quantify the activity recorded. Fluorescence Molecular Tomography28 restores the ability to resolve depth and improves quantification but is still fundamentally limited by photon diffusion and therefore by loss of resolution. Conversely, OptA image formation is not sensitive to photon scatter and therefore has a much gentler gradual loss of resolution as a function of depth. The OptA resolution depends on the geometrical characteristics of the ultrasound detector employed and on the frequency content of the ultrasound waves detected. Since waves of higher ultrasound frequency experience higher attenuation than waves of lower frequencies, deeper OptA sources in tissue with higher losses of high frequency components achieve consequently lower resolution. Nevertheless, depending on the frequency content of the OptA signal and the particular data collection scheme enabled by the OptA system, the resolutions in most implementations range from several micrometers to tens of micrometers (in the first mm) to a few hundred micrometers (at cm depths).

Optoacoustic imaging, introduced in the late 1970’s and early 80’s29, is based on ultrasonic diffraction, i.e. it offers the so-called acoustic-based resolution (AR) using spatially broad illumination to excite signals within a volume that are captured by the ultrasound detector employed. A particular OptA case, also considered in the late 70’s, forms OptA images using focused light30, instead of broad illumination as in the case of AR-OptA methods. In this latter case, image resolution is dictated by optical diffraction, often referred to as optical resolution (OR) OptA and depth performance is similar to optical microscopy. Therefore, the major OptA advantage, i.e. the ability to visualize in high resolution at greater depths than optical microscopy is lost. While AR-OptA allows optical interrogation with performance parameters that extend beyond the ability of optical microscopy6,31, OR-OptA methods operate with imaging characteristics similar to optical microscopy and offer a limited set of applications compared to the wealth of contrast-generation methods developed already for optical microscopy. However, it is worth noting that, while only very few cellular components show fluorescence emission, many more show absorption. These specific absorption signatures can be exploited for label-free OptA biological imaging by tuning the wavelength to fall within bands of high intrinsic absorption for DNA32, cytochromes in mitochrondria33, or oxy- and deoxy-hemoglobin7. This premise has also been shown in the mid-infrared whereby detailed molecular contrast from proteins, carbohydrates or lipids can be detected based on their selective absorption due to distinct molecular vibrations34,35,36. While these developments are exciting and may enable key niche applications, biological imaging requires the ability to generate contrast in very diverse cellular and subcellular species, which generally cannot be achieved today by label-free OptA techniques. For example, it remains very challenging to visualize a particular type of cell or cell function *in-vivo* by harnessing intrinsic absorption contrast.

Therefore, despite the major potential of OptA methods, in particular AR-OptA, to achieve high resolution imaging beyond the depths reachable by optical microscopy, the use of OptA imaging in the life-sciences is so far limited. This is because, in analogy to optical methods, most biological targets of interest, i.e. cellular and sub-cellular moieties, are not major photon absorbers in the visible range and therefore do not present contrast on OptA images.

***In search of OptA contrast in the spectral domain***

Exogenous contrast, for example contrast agents or genetic reporters, could enhance OptA contrast but its application is not straightforward. Fluorescent (and bioluminescent) labels led to rapid adoption of optical microscopy and imaging in biomedical research not only because they reveal specific biological structure or function, but also because they operate with low background, since tissue offers virtually no bioluminescence and low to very low auto-fluorescence signals. Conversely, the absorption contrast offered by OptA contrast agents or genetic reporters competes with the strong background in tissue due to hemoglobin and other light-absorbing molecules such as melanin, water, or lipids, depending on the spectral region employed. Even in the near-infrared (NIR) where tissues have low absorption when optical methods are used, hemoglobin absorption remains significant and may confound OptA signals from labeled cells or cell components, especially for small numbers of cells.

Until recently, most strategies to develop OptA contrast, be they synthetic37 or transgene24, focused on three rules: i) maximize the absorption cross-section, i.e. create a signal that is as strong as possible, thus out-competing the endogenous contrast, ii) absorb in a spectral range with low tissue background absorption (650-900 nm and 1100-1350 nm for NIR optical window I and II, respectively) and iii) create a unique spectral signature to optimize discrimination of the label over background by spectral-unmixing strategies. Using these guiding rules, several synthetic/inorganic and semi-synthetic nanoparticle formulations38,39 as well as organic dyes37,40 were considered for OptA imaging. Inorganic nanoparticles include metal nanoparticles like gold41, palladium42, copper43 or molybdian44 carbon agents45,46, quantum dots (colloidal semiconductors) or polymer nanoparticles47. Organic dyes have also been considered, including the FDA approved agents methylene blue and indocyanine green48, the cyanine dye IRDye80049 or crocanines50. Numerous bio-inspired absorbers like porphyrins51, chlorophylls52 and synthetic melanin have been also used. Combinations of light absorbing materials have been reported, for example melanin-coated gold nanoparticles53, often in the context of additional functionalities like photothermal therapy or drug delivery54. An essential aspect of such agents is their functionalization to achieve preferential accumulation in target tissues, e.g. in tumors due to enhanced permeability and retention effects (EPR) or targeting55, e.g. via antibody conjugates. For some agents, especially inorganic ones, poor biocompatibility might challenge in-vivo imaging56. Hence, several attempts for more bio-derived or “bio-compatible” agents have been put forward, for example bacterial vesicles57 or micelle or liposome carriers58.

Moving away from contrast agents, the search for a genetic reporter that could move OptA methods closer to the requirements of a life-science imaging technique led to considering genetically encoded fluorescent proteins, already used in fluorescence microscopy59-64. Such proteins also emit OptA signals due to the part of their energy absorption that is not re-emitted as fluorescence photons. However, fluorescent proteins are not optimized for strong OptA signal generation but for strong fluorescence emission and the majority of them absorb below 600 nm, resulting in limited sensitivity that often requires more than a million cells for successful detection in tissue24. Dedicated developments focused on far-red shifted reporters with reduced fluorescence quantum yield to allow higher OptA signal generation, such as reduced-fluorescent forms of Crimson65 and cjBlue66, or explored protein classes with native far-red absorbances like phycobiliproteins67. Despite these efforts, the most suitable genetic reporter currently available is thought to be iRFP72068, a reporter used in fluorescence imaging63, due to its far-red shifted absorption (700 nm) and relatively high absorptivity (96,000 M-1cm-1). Other examples of successful application of genetically encoded fluorescent labels in OptA imaging include indicators for calcium (GCaMPs) 69,70 or apoptosis (caspase-3 cleavage side with NIR fluorescent protein Förster resonance energy transfer (FRET) pair)71, albeit only in less scattering brain tissue.

Besides single chromophore-bearing proteins, several pigment compounds that can be produced in the cell by introducing the necessary enzymatic pathways through genetic modification have been also explored. Violacein72 and β-galactosidase-accessed indigo73-75 show absorbance peaks below 600 nm and their contrast strongly competes with that of hemoglobin. Therefore, melanin has been examined as a biomolecule with absorption that extends in the NIR to improve the OptA contrast achieved over hemoglobin. Melanin is a natural polymeric pigment produced primarily due to the expression of the tyrosinase enzyme76-78 and attains very high absorption coefficients making it a strong OptA signal generator. However, the melanin spectrum lacks characteristic spectral peaks and, critically, its production is potentially associated with radical formation79. While sequestering strategies80 might alleviate toxicity to some extent, presently only bacteria81,82 or tumor77,83 cells have been labeled by tyrosinase expression. These challenges have limited the sensitivity to several tens of thousands of cells84 and hinder the investigation of detailed biological and (patho‑) physiological phenomena, in particular regarding delicate cell types like immune or neuronal cells. Overall, any labeling OptA strategy should be analytically studied in terms of toxicity and confounding effects, as commonly done for fluorescence reporters85. While whole cell labeling, prevalent in OptA imaging, does not face the challenge of ensuring functional fusions for tagging of specific subcellular structures, the required high overexpression can be potentially demanding for the cell. Furthermore, chemogenetic strategies like selective ICG uptake by a transport polypeptide (organic-anion-transporting polypeptide 1B3)86 have been discussed but not adopted yet.

***Detecting OptA contrast in the spectral domain***

The underlying premise for improving the sensitivity in detecting all the aforementioned agents is spectral unmixing, a computational technique that detects labels based on their distinct spectral signature and separates them from spectral contributions by background tissue chromophores. However, besides noise and computational approximations, a critical challenge for achieving high sensitivity and specificity in spectral OptA detection of synthetic labels or genetic reporters relates to spectral coloring12. Light at different wavelengths is attenuated at different rates as it propagates in tissue, depending on the tissue optical properties at each wavelength. Therefore, the effective spectrum that excites different volumes within tissue may change as a function of the location of the volume of interest, and this change becomes more prominent the deeper in tissue this volume is located. Consequently, the apparent spectrum of a known label may differ at different locations in tissue, complicating the accuracy and sensitivity of detection.

Several methods have been proposed to improve on the challenge of spectral coloring. A key issue in addressing the spectral un-mixing problem is the difficulty faced in determining a tissue’s optical properties in-vivo with sufficient precision and spatial resolution for accurate solvers87. A particular class of methods converts the unmixing problem from the spatial domain to the spectral domain, by identifying base (eigen-) spectral functions to model light fluence and quantify tissue oxygenation12. This method may be applied to the spectral unmixing of agents but is also limited by the strong tissue background absorption88. Therefore, to date, three-dimensional spectral unmixing in tissue is still a complex non-linear problem that does not have a straightforward solution, since even if a label with a distinct spectral signature is used, the appearance of the label’s spectrum will change as a function of location, in a manner that is difficult to predict, complicating its spectral detection. Deep learning, blind-unmixing methods and super-pixel techniques hold promise to improve performance89,90, but the need to overcome competing signals from the background and account for spectral coloring effects remains a factor that complicates the use of OptA labels. Consequently, efforts to develop efficient OptA labels have not yielded sufficient sensitivity or accuracy for broadly disseminated use in life-science applications so far.

***Photoswitching optoacoustics – background suppression in the time domain***

A different direction to suppress background signals and overcome the challenges of spectral detection relates to moving the detection of OptA labels from the spectral to the time domain. Fundamentally, this shift can be achieved by controlling a property of the label that relates to the OptA signal generation (**Figure 1b**), while conducting OptA imaging. For example, control of the absorption coefficient, the OptA signal generation efficiency or the population of molecules **(Table 1)** can lead to corresponding control of the OptA signal generated from the label. Then, imparting a particular temporal pattern to that property of the label (i.e. a modulation pattern) generates a corresponding synchronous pattern of the OptA signal coming from the label, but not from the background tissue chromophores. Images acquired over time and processed to resolve the modulation pattern in each element of the image (i.e. picture element or pixel), allow the separation of the labeled cells or structures from the tissue background, i.e. enable temporal unmixing. The control pattern implemented can be a simple on-off pulse train, a sinusoidal modulation, or a more complex modulation pattern. Such control can be achieved with light, temperature, or possibly other forms of energy (see section “Other controls for state switching”). Commonly, a label is transferred in a controlled manner to different states that emit different OptA signals. The simplest case is a transfer from a state of high OptA signal to a state of low OptA signal, i.e. ON- and OFF-states, respectively. When the switch between the ON and OFF states can occur in a recursive manner, whereby an ON state can be driven to an OFF state and then to an ON state again and so on, then this process is referred to as a reversible process.

So far, the most broadly applied approach uses molecules that can be switched between two states by light of distinct wavelengths. The two states can simply be two different absorption levels at a wavelength where imaging is performed or, more elaborately, two different absorption spectra (**Figure 1c**). Illumination at wavelength A while the molecule is in one state, i.e. at one spectrum A, gradually switches the molecule to the second state, i.e. spectrum B. The result is that the OptA signal, generated at this first wavelength A, decreases. Then, illumination switches to wavelength B and similarly the OptA signal starts at a high value and gradually decreases, while the absorption spectrum of the molecules returns to the first state, i.e. spectrum A (**Figure 1c**). A common implementation of such psOptA system utilizes pulse trains at the two wavelengths (A, B) to switch the label between the two different spectral states **(Figure 1 e and f)**. The duration of the pulses in the pulse trains can be at the nanosecond to hundreds of nanosecond range, so that the same illumination switches the molecular state and modulates OptA signals to allow image acquisition during the switching process. Correspondingly, psOptA systems require machinery to generate and read a modulation pattern within the total OptA signal collected. Such systems also require methodology to separate the contribution of the label from the contribution of background absorbers. Therefore, many psOptA systems differ from their non photoswitching counterparts due to the need to implement temporal modulation and detection, imposing certain speed requirements in wavelength tuning and the synchronous sampling of OptA images. For example, operation with two different spectral states may require interleaving of pulsed trains at two wavelengths for control of the label. The speed of the transitions between the states, i.e. how many light pulses are required to drive the label to another state, depends on the illumination strength as well as the quantum yield of the transition and the absorption cross section of the label, that is, the intrinsic photophysical parameter of the label and the strength of the illumination reaching the label. The strength and dynamic range of the OptA signal generation (i.e. the OptA quantum yield) can differ between the two states, with one state giving stronger OptA responses and being primarily exploited for imaging. More generally however, using the decay kinetics of both states for analysis is possible. Exploiting photoswitching molecules with a fast recovery to the equilibrium state theoretically allows the implementation of the approach with only one pulsed wavelength. In this case, the signal decay at that wavelength is observed (e.g. only A) in the OptA images collected over time, followed by a time interval that allows the label to recover.

The ensuing repetitive switching kinetics imprinted in the time signal, e.g. time-series of images, can be subsequently analyzed in different ways, with the goal to identify the presence and amount of the label in different volume elements of the image resolved. The simplest approach is analyzing the difference in pixel intensities (Δ Signal) between the maximal (ON, e.g. label fully in state A) and minimal signal (OFF, e.g. label fully in state B, **Figure 1g**). This operation is achieved by subtracting images obtained at the first and at the last pulse of a transition. Since the background should show no pronounced change in signal, any statistically important difference in pixel intensities observed, possibly after averaging multiple images in each state, will be due to the presence of the photoswitching label. This scheme can be in principle implemented with a minimum number of three pulses per cycle (1st: WavelengthA (WLA), probing and concomitantly switching, 2nd: WLA, probing the switched state, 3rd: WLB, switching back). However, if sufficient points of the decay kinetics are sampled, the actual character of the transitions can be exploited by fitting a function that describes the expected modulation over time (**Figure 1h**). Many other methods can be employed to identify the modulation pattern of the OptA signal, including different implementations of lock-in detection, Fourier domain analytics91 (**Figure 1i**) or Principal Component Analysis (PCA), Vertex Component Analysis (VCA) and other similar techniques25 implemented in the time-domain. The accuracy by which the label is separated from the background can further be improved by taking into account the signal strength from neighboring pixels and the overall data quality to reduce false positives91, including consideration and separation of temporal patterns that may be introduced in the signal by electromagnetic interference or motion92, 93.

***Photoswitching Labels***

The photophysical photoswitching mechanism is a transition from the excited state of one form of a chromophore to the ground state of another form (**Figure 1d**). Chemically, this involves changes to the chromophore molecule – very often a *cis* / *trans* isomerization – which bestows distinctly different photophysical properties on the two switchable forms. Photoswitching is a well-known property of several dyes like azobenzene94 or cyanine95 but many chromophore-bearing proteins also show photoswitching with physiological functions e.g. photo-receptors96. Uses of such photoswitching compounds can range from proposed uses in data storage97 to vision restoration98. For life-science imaging, the interest in photoswitching largely began with the discovery of photoswitching in proteins of the Green Fluorescent Protein (GFP)-family99 and their use in fluorescence super-resolution imaging approaches. In these techniques, the switching is used to confine fluorescence emission to sub-diffraction sized spots (lens-based scanning, RESOLFT1) or, albeit more rarely, to more precisely control fluorescence events in single-molecule imaging super-resolution techniques2 (for example PALMIRA100). Beyond their use to enhance resolution, the reversibility of photoswitching was used to allow for repeated measurements within cells. For example, repeating multiple “fluorescence recovery after photoswitching” experiments in one cell allows more accurate measurement of diffusion properties than classical fluorescence recovery after photobleaching (FRAP) experiments 101. Repeated measurements also allow temporal marking of regions in dependence to cellular conditions. For example, photoswitchable (and photoconvertible) Ca2+ sensors have been used to allow for repeated marking of areas high in Ca2+ 102,103.

Beyond the benefits of using photoswitching in repeated measurements, the photophysical properties of photoswitching unique to each variant have been used in fluorescence imaging to distinguish labels beyond using their spectral properties. For example, to circumvent spectral crowding104 or to directly unmix labels based only on their temporal profiles. This allows the multiplexing of cell populations105 even in whole transparent organisms106. Knowledge about the transitions can also be used to allow absolute quantitative imaging in cells107,108. The possibility to single out only photoswitching molecules with the right choice of illumination schedule has been used to massively expand the size limits of time-resolved fluorescence anisotropy measurements of proteins in the cell from below ~10 nm to above 100 nm by using polarized photoswitching light to exclusively mark molecules of a specific sub-population109. The reversible transitions are further used to allow focusing of light in scattering media by tagging photons in tissue, based on their protein switching capability. This information is subsequently used for wavefront shaping110. Photoswitching also enhances the contrast-to-noise ratio (CNR) in fluorescence imaging by employing the same principle of signal modulation as in OptA to suppress tissue autofluorescence111-114, for example in light-sheet115 or 2-photon microscopy116. However, although photoswitching can contribute to CNR enhancement in fluorescence imaging, it is essential in OptA imaging due to the much stronger background OptA signals generated from tissue chromophores compared to tissue autofluorescence that is by comparison a much weaker signal. Presently, photoswitching proteins of the Bacteriophytochrome (BphP) class and first examples of photoswitching dyes are mainly employed in psOptA, as discussed in the following.

*Bacteriophytochromes*

Bacteriophytochromes are native photoswitching proteins, with the photoswitching leading to downstream effects on biochemical activity, resulting in a range of different cellular responses117-119. A BphP shows a photochromic behavior, with the Pr state absorbing at *red* wavelengths around 680 nm, while the Pfr state exhibits absorbance in the *far-red* around 760 nm. These far-red absorbances and high absorptivity (~100,000 M-1cm-1) make BphPs ideally suited for tissue imaging120. This far-red absorption is in contrast with photochromic photoswitching fluorescent proteins of the GFP-family, which may be used for psOptA imaging but are not optimally suitable due to their absorbance being generally below 600 nm25,121, and thus competing with that of hemoglobin. Bacteriophytochromes utilized so far in psOptA imaging have a biliverdin (BV) chromophore, which is readily available in most mammalian cell types as a catabolic product of heme122. However, there are existing limitations123. Biliverdin can be present in low quantities in certain tissues, e.g., brain124, and BphPs can have low affinity or specificity to BV in their binding pocket125. In those cases, the amount of holo-form available for imaging is limited by the lack of BV or binding in the pocket due to competition from other tetrapyrroles like protoporphyrin IXa126. Several strategies for rectification have been put forward, including the addition of BV127,128 or a precursor of its production (5-ALA)127, co-expression of BV-generating enzyme heme-oxygenase127,128 or knockout of BV-degrading enzyme BV Reductase-A129. Finally, down-regulation of BphP expression shifts the BphP to BV stoichiometry favoring chromophorylation and this can be a tractable way to increase the fraction of the holo-population130.

The BphPs used in psOptA imaging are close relatives to engineered fluorescent far-red BphPs like the iRFP720 mentioned previously. In fact, the fluorescence of those BphPs is achieved by preventing photoisomerization as a means of deexcitation and by generally strengthening the radiative decay pathway (increasing fluorescent quantum yield). Fluorescent BphPs like iRFPs131, wiPhys132 and iFPs133 have now been used for over a decade as genetically-encoded labels in fluorescence imaging showing an ease of use comparable to GFP-like fluorescent proteins with regards to toxicity, tagging and protein production. This amenability to life-science imaging workflows suggests similar advantages for the use of the BphP class of proteins for psOptA imaging. In particular, the so-called bathy BphPs are preferentially used in psOptA imaging. Their state at thermal equilibrium is the far-red absorbing Pfr state, known to be advantageous for tissue imaging, conventionally considered the ON state, which shows the maximal change of absorbance upon photoswitching. Thus, 760 nm illumination results in a decay of the OptA signal and 680 nm illumination results in a recovery. Alternating 760 and 680 nm illumination leads to the desired modulation. The two wavelengths also mean that probing and switching always happen concomitantly. So far, BphPs descending from five different native proteins have been used: i) RpBphP126, and RpBphP1-PCM91, ii) DrBphP, DrBphP-PCM92 and variant DrBphP-PCM-F469W134, iii) ReBphP-PCM91, iv) AGP1135,136, and v) sGPC2 and sGPC3137. Recent work, employing RpBphP1, showed the first transgene animal expressing a photoswitching agent imaged with psOptA138.

*Non-natural photoswitching compounds*

While several photochromic dyes exist, for the majority of them, at least one of the switching wavelengths is in the blue region of the spectrum, hence these dyes are of limited use for tissue imaging. Recently this challenge was circumvented by tethering a photoswitching diarylethene dye (dithienylethene-containing β-diketone, 3ThacacH) to an upconverting nanoparticle139. The pure dye photoswitched between an open and closed form with illumination at ~640 nm and ~365 nm while the nanoparticle shows emission bands between 300 and 400 nm after 980 nm excitation. Tethering both together allows switching with excitation at 980 and 640 nm, a wavelength range well suited for in-tissue applications, with the encapsulation of the dye in the nanoparticle ensuring high energy transfer (>80%)139. However, this photoswitching compound suffers from very long switching times in the minutes range despite the provision of light energies around 2 mJ/cm2. Beyond such strategies to circumvent the use of blue light, current efforts to develop photoswitching dyes entirely controllable in the NIR wavelength range are promising140,141. For example thermally reversibly (T-type) dyes142 could allow high speed psOptA imaging with only one wavelength for switching and readout.

***Implementation of photoswitching optoacoustics***

Photoswitching OptA imaging was first shown with photoswitching GFPs known from super-resolution fluorescence microscopy (Dronpa and Dronpa-M159T)25. Imaging was performed with a 256-element cup-shaped detector for real-time three-dimensional imaging and a 50 Hz tunable laser switching between 488 nm and 405 nm. Reading out OptA signals from a tissue-mimicking phantom, and analysis via VCA allowed suppression of signals from hemoglobin (**Figure 2a**), with a CNR over reference signals of ~3025. Optoacoustic photoswitching was also considered in the context of cytometry, i.e. single-point OptA measurements for detecting plated MTLn3 tumor cells labeled with the photoconvertible protein Dendra2143. Photoconversion in Dendra2 results in an irreversible change of absorption, from peaking at 490 nm to peaking at 553 nm. Optoacoustic signals were excited by 560 nm pulsed illumination before and after photoconversion using 405 nm illumination, resulting in OptA signal differences between the pre and post photo-conversion signals143. *In-vivo* imaging in mice was first demonstrated using a BphP label with absorption at the NIR. U87 cells labeled with RpBphP1were implanted in a mouse and imaged with alternating trains of 630 and 780 nm pulsed illumination and detected with a 512-element ring array26. Differential analysis allowed visualization of the location of the implanted cells up to 10 mm deep with a CNR of ~80 for ~3x103 cells/voxel as inferred from histology.

Since then, constant improvements in sensitivity have been made. Photoswitching OptA imaging using an off-the-shelf commercial OptA imaging device showcased that ~1000 cells/µl in-vivo and ~100 cell/µl in tissue mimicking phantoms could be detected91 at resolutions of ~ 200 µm. The study used Jurkat and 4T1 tumor lines labeled with BphP ReBphP-PCM and a decision tree-based classification incorporating differentials, exponential fitting and frequency-space for analysis. Similar cell numbers were also detected in-vivo in mouse imaging of MTLn3 cells labeled with a DrBphP-PCM-based complementation assay type label allowing the indication of interaction between two proteins, with the sensitivity reaching as low as ~500 labelled cells (**Figure 2b**)92. The temporal signatures captured and attributed to the presence and amount of a label can be coregistered with background OptA images collected at one or more wavelengths so that they can be referenced to anatomical or spectral (functional) features of the target visualized135. Acceleration of imaging can be achieved by using two independent continuous laser sources for switching (790 nm for OFF and 636 nm for ON), in addition to the 750 nm pulsed laser employed for imaging (and OFF switching) 138. Using this arrangement, the first transgene mouse was imaged with psOptA (loxP-BphP1-mCherry-TetR in ROSA26 locus)138. Such imaging can, for example, help to identify and study the relationships between immune or immunotherapeutic cells and the vascular network, tissue oxygenation, and tumor pathophysiology or treatment. In-vivo imaging was also shown using a photoswitching dye tethered to upconverting nanoparticles (see section on non-natural photoswitching compounds). Illumination with 680 nm and 980 nm light allowed effective photoswitching whereby the 680 nm illumination was used to probe for the OptA signal. With this strategy implanted HeLa cells labeled with the nanoparticles could be detected with a sensitivity down to 104 cells subcutaneously139. **Supplemental table 1** gives a comprehensive tabular overview of studies performed, along with their approaches to modulate the OptA signal.

From the perspective of US detection, several implementations have been shown. Ultrasound transducer arrays, composed of multiple elements arranged on a line or a curve, have been employed26,91,92,137,138 affording concurrent acquisition of OptA signals at multiple positions on the surface of the imaging target and enabling real-time acquisition of cross-sectional images. Fast acquisition of volumetric images is also possible using a cup-like arrangement of transducer elements covering entire volumes within the sample for every light pulse excitation, an improvement on single cross-sections achieved using single dimension ultrasound transducer arrays 25. Conversely, setups that scan a single ultrasound detector to collect OptA signals from one location at a time can offer simpler instrumentation alternatives and can improve imaging resolution. On the other hand, single element detection leads to longer acquisition times than psOptA systems using multiple parallel channels for ultrasound detection. Using Fabry Perot interferometric detection scanning allowed the imaging of an implanted HT29 tumor with the cells expressing photoswitching protein AGB1, together with its vascular anatomy, at resolutions of ~50 µm135. The authors used a stepwise acquisition method in which the readout from the label states was obtained at each scanning position before moving to the next, leading to hours-long scan times. Other scanning acquisition schemes are possible, for example, by using continuous illumination with light of one switching wavelength together with probing and switching with light of a second pulsed wavelength. The simultaneous illumination keeps the population of photoswitching molecules in a steady state. Changing the intensity of the continuous illumination for each scan of the field-of-view results in a distinct variation of this steady state indicative of the presence of the photoswitching molecules. In this proof-of-concept study, a green photoswitching protein in a tissue-mimicking phantom was excited with pulsed 488 nm while simultaneously illuminated with continuous 405 nm light. The 405 nm light was used at different intensities per scanning round, resulting in the population of photoswitching labels being fully on, ¼ off, ½ off and so on. The confounding blood does not show a dependence on the 405 nm light and can thus be separated from the label signal. This scheme can speed up acquisition by enabling continuous scanning over the field of view (FOV) and was demonstrated using raster scanning optoacoustic mesoscopy (RSOM)144.

The impact of reconstruction methods on the ability to extract the photoswitching characteristics has only been explored to a limited extent. Although a majority of photoswitching studies have so far employed back-projection approaches (Supplemental Table 1), advanced model-based reconstruction approaches offer the ability to better model the spatial and frequency aspects of OptA signal generation and propagation138,145,146 and thus positively impact the quantification accuracy, image fidelity and sensitivity. Improvements in photo-switching detection when filtering high-frequency components have been demonstrated145, whereby a weighted frequency-band specific model-based (fbMB) reconstruction has been proposed as an optimal and ubiquitous approach in OptA inversion for employing broad-bandwidth OptA signals146, a methodology suitable for handling the frequency components of the OptA signal in photoswitching imaging as well.

*Multiplexing based on photoswitching time constants*

The photoswitching behavior of different labels may unequivocally characterize the label and allow multiplexing of multiple labels (**Figure 2c**). At the same energy, photoswitching proteins show different time constants for the photoswitching transitions, characteristic of the protein employed. The reason is that the individual amino acids surrounding the chromophore of a given protein result in unique photophysical properties, including different photoswitching quantum yields, i.e., the likelihood that an absorbed photon leads to photoswitching. This characteristic nature can be harnessed for multiplexing labels in time. Temporal unmixing can be based on identifying the characteristic decays of the ensemble transitions91,92. Multiplexing was first shown in a phantom experiment with purified protein variants of the green photoswitching protein Dronpa25. Subsequently, experiments in-vivo with different cell types expressing different BphPs91,92 showed that differentiation of three labels91. Additionally, differences in the absorption spectra of different labels can be exploited to achieve a combination between photoswitching based and spectral unmixing137. Such label identification and separation can be further augmented by using automated classification91,93. It is critical to note that the apparent time constant visible in the analysis depends not only on the photoswitching quantum yield but also on the light energy deposited at the given point in tissue. Hence, for imaging tissue with unknown light propagation, labels with sufficiently different transition kinetics are essential for clear discrimination. Furthermore, the decay characteristics for different labels can be also exploited for multiplexing by co-expressing two labels with distinct kinetics in one cell type of interest while expressing only one label in the second cell-type. Now the two cell types can be distinguished based on the necessity to model the observed data as a process with two or one exponential decays92. This approach is largely independent of light fluence but requires data quality suitable for meaningful models.

*Biosensing with photoswitching readouts in optoacoustics*

Biosensors, defined here as molecules that emit signals dependent on the *presence* of an analyte of interest, have also gained significant attention in life science imaging with numerous examples, especially in fluorescence imaging147. Using psOptA, biosensors can interrogate deeper in tissue than fluorescence methods, allowing the read out of cell states, e.g. monitoring of key metabolites deep within tissues, while retaining spatial certainty. To date, two concepts that combine photoswitching and sensing have been demonstrated. The first approach relies on the principle of complementation assays. DrBphP-PCM was split in two parts (PAS and GAF-PHY) which associate only in proximity and establish the integrity of the BV-binding pocket. This reassociation would then allow BV attachment, the generation of a signal, and, importantly, photoswitching92. The proof of concept was demonstrated with a complementation assay that allowed visualization of rapamycin-induced FRB and FKBP interaction when the proteins were in molecular proximity92. However, this concept is irreversible due to the covalent attachment of BV. A second approach which enables reversible sensing was demonstrated using calcium indicators, such as those well-known from fluorescence imaging. A green Ca2+ sensor was primarily developed for super-resolution Ca2+ imaging using RESOLFT fluorescence microscopy, but it could be used to show the general feasibility for psOptA imaging144. Despite being a sensor for Ca2+ imaging, its usage for neuronal OptA calcium imaging is unlikely due to dwell time constraints imposed by the required repetitive switching70. Photoswitching sensors add a layer of complexity to the protein-engineering endeavor since the capability to photoswitch can only be implemented with a deep understanding of the general photophysics of the chromophore defining the absorption and deexcitation pathways, as well as the chromophore maturation or attachment chemistry via the same set of residues interacting with the chromophore (pocket). However, with the recent emergence of fluorescent BphP-based Ca2+ sensors148-150, a transition to photoswitching BphP-based sensors is likely. FRET-based concepts, common to fluorescence imaging, are also being explored for use in OptA151, but no sensors have yet been put forward. The protein-engineering of FRET-based sensors would be easier since it will deal with the interaction of individual folds rather than more complicated chimeric proteins. Generally, it can be expected that additional sensors which are well-known for fluorescence imaging will be adapted to psOptA imaging. Beyond these existing sensors, OptA is already compatible with many types of activatable labels, also termed activatable probes, which can potentially be coupled with switching components. Since most of these activatable probes rely on synthetic chromophores, advances in the development of NIR photoswitching dyes could lead to promising developments in this area.

***Other controls for state switching***

Any controllable transition between states of distinct OptA signal generation can be used as a concept to control signal switching and temporal unmixing (**Figure 1b**). Such control can either be exerted by light with phenomena other than photoswitching or with different forms of energy (presently: heat, magnetic fields, radiofrequency electric fields).

*Controls exploiting photophysical phenomena other than photoswitching.*

As early as 2009, a pump-probe based scheme involving the luminescent dye Pt(II) Octaethylporphine showcased the difference between the absorption coefficient at the probe wavelength (760 nm pulsed) and the distinctly higher absorption coefficient after pumping with a second wavelength at 532 nm, which transiently drives the molecule into a triplet state that decays phosphorescently. The differential image recorded with a 40-element linear transducer allowed for discrimination between a tube containing Pt(II) Octaethylporphine dye and a tube containing a confounder (IR-783)152. A comparable approach using rose bengal-doped silica nanoparticles ex vivo in tissue achieved an approximately 20x increase in contrast over conventional imaging techniques153.

Another interesting approach is to control the population of molecules that can contribute to the OptA signal by stimulated emission. If a fluorescent molecule is illuminated with light within the wavelength range of the emission photons, the likelihood of this emission is greatly increased, i.e. leading to induced emission and the return of the molecule to the ground state. For an OptA experiment, if a molecule has a sufficiently high OptA quantum yield, under stimulated emission conditions, the faster return to ground state of the excited population effectively increases the contribution of the faster radiationless deexcitation. This is because more ground state molecules are available than in the case without stimulated emission where part of the population decays via the slower spontaneous fluorescence emission. This was demonstrated using the fluorescent dye Atto-680, where toggling between OptA measurements with and without stimulated emission light and analyzing the differential effectively suppressed a blood confounder in a phantom experiment154. The concept was also shown in vivo with Atto‑680 deposited as subcutaneous Matrigel implants on the back of a mouse for measurements using a Fabry-Perot interferometric detection approach135,155 (**Figure 2d**). Here the authors also implemented interleaved acquisition allowing probing with and without time-shifted stimulated emission light on a per-pixel basis to reduce the impact of motion artefacts135,155.

In contrast to approaches using BphPs, genetic encoding of labels for most of the techniques mentioned above may be challenging since they rely on transitions currently only observed for non-biological chromophores. It remains to be seen if, for example, stimulated emission could also be performed with STED-optimized NIR proteins like the tailored BphP SNIFP156 or if the fluorescent QY (i.e., the population that makes the differential) is too small compared to the rhodamine6G mentioned above. Alternatively, dye approaches could be used with chemogenetic systems like Halo tags157.

*Temperature-dependent control*

Temperature-dependent control of the OptA signal emitted by labels can be exerted in a variety of ways, most commonly by using so-called thermochromic dyes which change their absorbance profile with temperature, thus modulating the OptA signal at the probe wavelength. For example, heating and cooling cycles of organic and polymer-based thermochromic microcapsules between 20°C and 40°C were used to create absorption coefficient modulations. Those translate into modulations of the OptA signals generated after 532 nm excitation which can be exploited to delineate the subcutaneously implanted thermochromes *in vivo* in mice158 (e.g., see **Figure 2e**). A similar concept was shown with photothermal stimuli-responsive poly(n-isopropylacrylamide) nanogels together with gold or copper nanoparticles. Here, a continuous laser source was used to heat the nanogel and this resulted in an contraction of the material associated with a change in crowding and thus enhanced plasmonic coupling of the nanoparticles altering the absorption coefficient, which allowed the detection of implants in a live mouse159. Furthermore, thermochromic dyes can be coupled with magnetic nanoparticles (e.g. SPIONS), which can be heated when laser light or electric fields (hysteresis) are applied. As a result, the temperature change of a magnetic nanoparticle can facilitate an effective laser-induced spectral shift of the coupled thermochrome160. Such localized heating can also be exploited directly via changes of the Grüneisen parameter. This parameter relates the material properties to the thermoelastic expansion and is itself affected by temperature. Hence, altering the temperature of a nanoparticle’s surrounding environment with an electric field affects the local Grüneisen parameter, which leads to a modulated signal when the nanoparticle is illuminated by a laser161. The scale of temperature variation and the speed at which temperature is controlled in tissues are parameters that need careful examination to make temperature-dependent control methods suitable for biological imaging.

*Magneto-motive control*

Other control mechanisms are focused on the manipulation of the spatial distribution or density of the labels which emit OptA signals (e.g., nanoparticles). This manipulation can be achieved through the application of magnetic fields around the sample to reposition or temporally displace magnetic beads or nanoparticles. The signal at a given position would be modulated due to the presence or absence of the label in the interrogated voxel, depending on the direction and strength of the magnetic field applied. Initial work in phantom models demonstrated the proof of principle by differential analysis between data acquired at different magnetic states162-164. Applications of magneto-motive control were also demonstrated based on speckle tracking165 to analyze the magnetic displacement in the sample. In-vivo application of the technique in mice, under oscillating magnetic fields allowed cancer tissue to be distinguished from unlabeled healthy tissue163,166,167 (**Figure 2f**). In a related context, OptA magneto-motive differential imaging has also been used to analyze particle uptake in individual cells168.

*Activatable labels yielding one-time differential contrast*

Several labels only exhibit contrast when activated by exposure to a particular analyte or condition. Such activation can be achieved, for example, due to cleavage of a quencher169, the induced separation of two dyes allowing differential imaging at two wavelengths170, modification of a single dye so that it goes from a low to a strong signal state171 or shows a different absorbance172. Such approaches have previously been summarized extensively173,174. While the often-irreversible activation mechanism of these labels is fundamentally different from the (mostly) reversible mechanisms discussed in this review, the mechanisms share the common underlying principle of a controllable change in the OptA signal. Hence, several irreversible activatable labels have already been exploited for differential imaging: for example, gold-coated microbubbles have been applied for one-time differential imaging by bursting the bubbles, leading to denser gold aggregation and hence higher OptA signal175. Another example is the vaporization of nanoparticle-filled perfluoroalkoxy alkane-droplets, which generates bubbles and subsequently increased OptA signal due to the gas/water acoustic impedance mismatch176.

***Future perspectives***

The possibility for highly sensitive detection of cells, cellular moieties and functions, granted by psOptA technology, may become the critical missing link to enabling the disseminated use of optoacoustics in biological interrogations. Efficient generation of genetically encoded OptA contrast would allow researchers to capitalize on the particular strengths of OptA imaging over optical methods, in particular, the ability to offer high-resolution visualization deeper than optical microscopy. Such performance renders psOptA as an imaging modality that can complement optical microscopy, as evident from progress in hybrid optical and OptA microscopes, which enable both the capturing of broader contrast and deeper imaging.177-180 In particular, the development of psOptA is critical for increasing the sensitivity of the OptA method and bridging the gap that exists between OptA and the superior sensitivity of fluorescence methods in cellular and molecular detection24. In the same vein, psOptA systems could also be employed in optical imaging applications now served by macroscopic bio-luminescence and fluorescence techniques, allowing the ability to visualize cells and other biological contrast in high resolution deep in tissue for the first time, in addition to visualizing intrinsic tissue contrast based on hemoglobin, water and lipids.

From a technological perspective, psOptA imaging would benefit from efficient illumination sources that move away from bulky, expensive and slow laser sources. Here, efforts in laser-diode technologies offer a promising direction, especially for psOptA microscopy applications181, and can result in economic and significantly faster implementations182. Efficient systems could be based on the use of a single wavelength for OptA excitation and label switching or employ inexpensive light or other energy sources for modulating the state of the label. Single wavelength psOptA may also be implemented using photoswitching agents that are metastable in the photoswitched state and naturally relax to their equilibrium state in the dark, preferably with short recovering periods. For some reversibly switchable GFP-like proteins, recovery times in the dark are well below seconds, suggesting the possibility to engineer such fast dark state recoveries for BphPs as well. Nevertheless, for practical implementations, those relaxation times should become much shorter.

Furthermore, new ways of introducing specific signal modulation can be explored, like other transient states associated with nonlinear mechanisms183. For example, fluence-dependent nonlinearities could be exploited by alternating between low (agent and background linear regime) and high intensities (agent non-linear behavior) during imaging and differentially comparing the results of both intensities. Such non-linearities are commonly found among multi-layer nanoparticles184. However, this requires agents with effective non-linearities in fluence regimes that remain within the allowed ANSI limits for imaging organisms (< ~20 mJ cm−2, 10 Hz). Furthermore, magnetism can be used to alter the arrangement of labeled (e.g. melanin) chromophores contributing to the OptA signal, resulting in frequency shifts due to changes in effective emitter size (~30 MHz to > 100 MHz). Such frequency shifts could lead to the collection of temporal signatures usable for unmixing. While this has only been shown for switch-on probes to date, e.g., in the context of sensors185, such changes could also potentially be implemented in a reversible manner in the future.

For *in vivo* imaging, all aforementioned transitions are dependent on energy fluxes within the sample, which act as local controls. While some energy forms, such as magnetism-related fluxes are not as affected, light fluence is strongly dependent on depth and tissue composition. This dependence remains a major factor affecting the psOptA performance and the depth that a certain label can be detected by temporal unmixing. In addition, varying fluence at different depths may induce different kinetics, affecting the performance of photoswitchable labels (**Figure 2g**). Such kinetic differences may be exploited to map fluence distribution in tissues186 and provide the ground truth in research developing fluence models, addressing this remaining major challenge in bio-optics. Conversely, the temporal sensitivity of the photoswitching behavior of a label with depth complicates temporal unmixing when multiple labels are employed, but this sensitivity also creates an interesting and unique computational problem whereby the time constant of labels and the fluence could be concurrently resolved by time-space measurements. The accuracy of solutions to the time-space maps generated by psOptA is expected to be better when using labels with distinctively different fluence-dependent responses92.

Besides biological imaging, photoswitching agents could be also employed to augment clinical OptA imaging through the specific detection of otherwise hard to visualize biomarkers. One of the most promising potential applications would be the use of synthetic photoswitching compounds as they are often related to synthetic dyes approved for human use. However, an interesting further direction would be to explore whether the highly bio-compatible proteins or bio-nanoparticles built on photoswitching proteins could gain acceptance for human use to become an exciting and potentially more versatile OptA contrast enhancing possibility.

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V.N. is a founder and equity owner of sThesis GmbH, iThera Medical GmbH, Spear UG and I3 Inc.

***Author contributions:***

A.C.S. and V.N. wrote the manuscript together.

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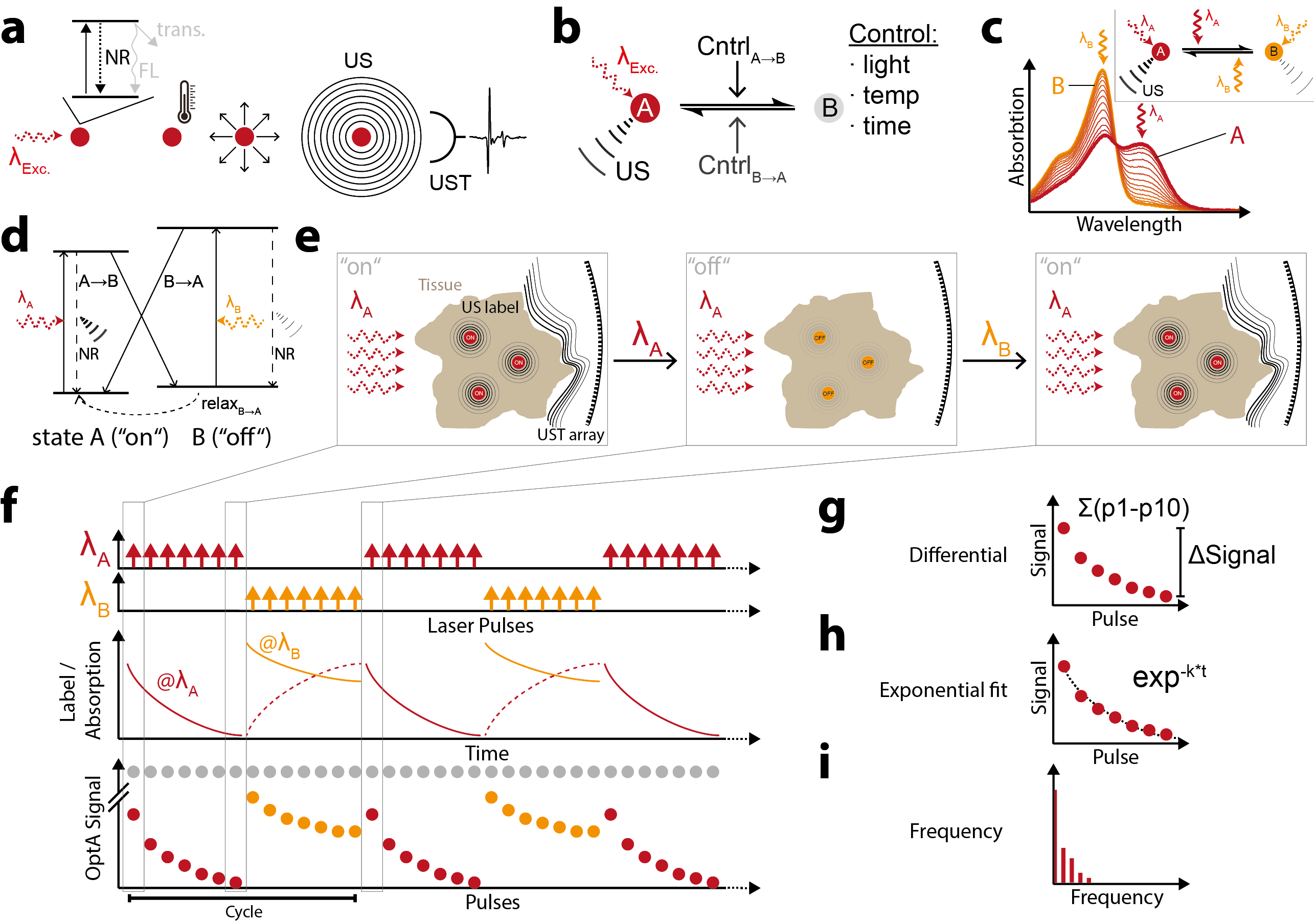
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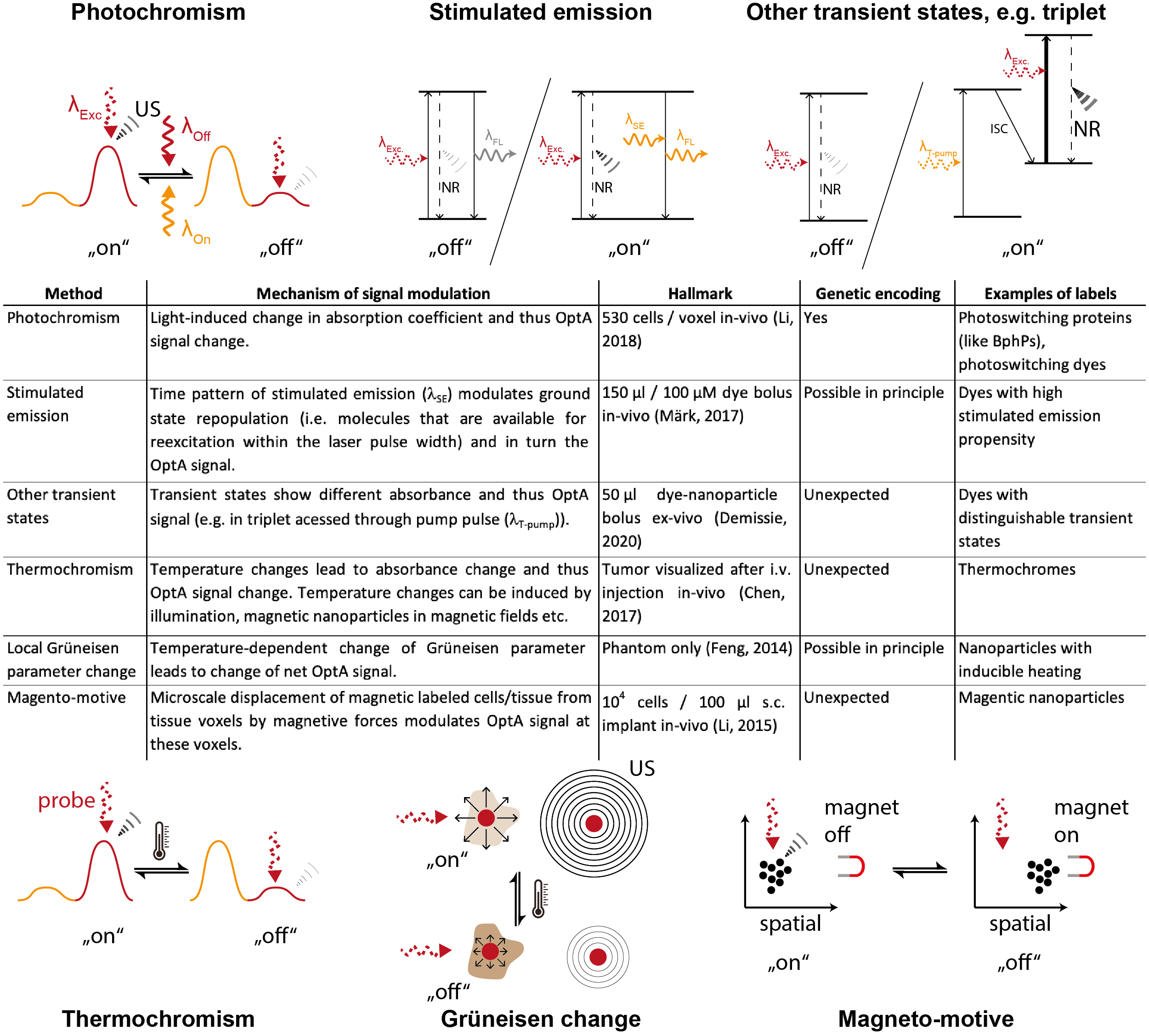
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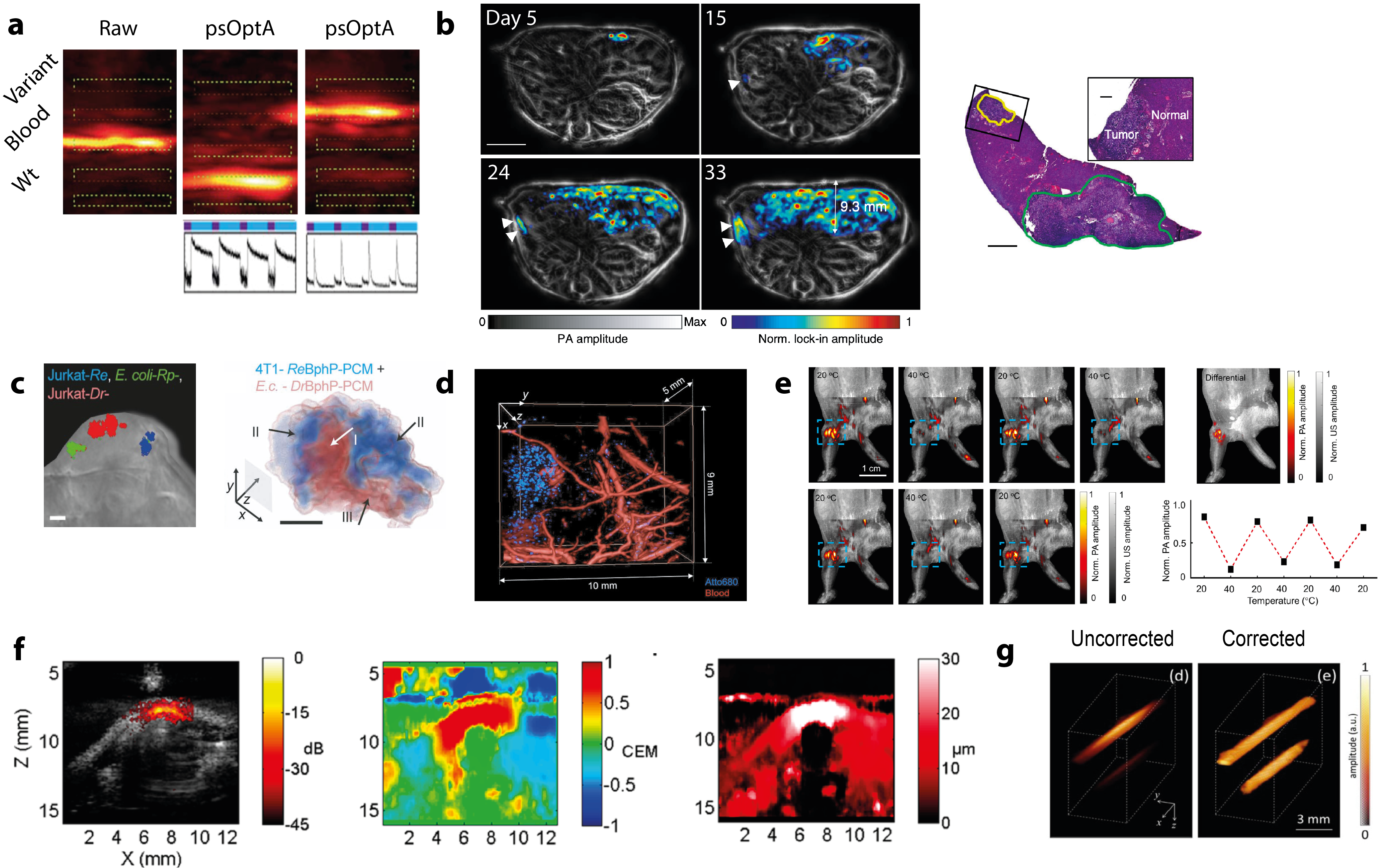
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**Figure 1 | Fundamental principles of photoswitching optoacoustics (psOptA). a**Fundamental principle of optoacoustic (OptA) signal generation. A chromophore (red dot) is excited by pulsed laser light (λExc.). The subsequent non-radiative deexcitation (NR) results in a local temperature rise and thermoelastic expansion which yields ultrasound pulses (US) that can be detected by a transducer (UST). Note: other deexcitation pathways (light grey arrows) like fluorescence (FL) or transition to other states (trans.) can also be present alongside NR. **b**Fundamentally, an agent for OptA with temporal analysis requires a state (A) that can be excited (by 𝛌Exc.) to generate OptA signals (US) and the agent needs to show a controllable and reversible transition to a second state (B). In principle, this control can also be exerted by other forms of energy other than light. Cntrl: control, temp: temperature. **c** Photoswitching scheme and arbitrary absorbance spectra (fully state A and fully state B indicated) of photochromic photoswitching agents which are primary used in psOptA. The agent can be switched between two states with light of two different wavelengths (λA and λB). **d** Simplified Jablonski diagram explaining the principle of photoswitching proteins commonly used in psOptA. **e**Graphical representation of the three essential stages of a psOptA experiment. Left panel: In the agent’s ON-state, illumination with laser light (𝛌A) excites the label, generates measurable OptA signals and concomitantly drives the label to the OFF-state. Center panel: In the OFF-state, illumination with 𝛌A results only in minimal OptA signal due to lack of absorption at this wavelength in the OFF-state (see c). Subsequent illumination with the second wavelength (𝛌B) drives the label back to the ON-state (right panel). The tissue chromophores always emit similar levels of OptA signal independent of previous light illumination. **f** Scheme of illumination, change of signal and resulting OptA signal. The three stages from d are indicated. Note, the OFF-to-ON-transition with limited dynamic range is also shown here and the increasing absorption of the A form is indicated for clarity (red dashed line). The OptA signal from tissue background (gray spheres) is always constant and not affected by the illumination schemes. The details of all transitions and wavelengths are strongly dependent on the agent used. **g**Fundamental analysis approaches: In the differential analysis, the first and last pulse(s) are compared over the whole modulation either after calculating a mean switching cycle or individually for each cycle (cycle = see f bottom). **h** The exponential characteristic of the transition is exploited again either on a mean cycle or individually per cycle. **i** For the frequency analysis, the harmonics of the known modulation are analyzed.

**Table 1 | Different mechanisms of temporal control of the optoacoustic (OptA) signal.** The column "Hallmark" notes the most mature demonstration of the technology so far. "Genetic encoding" is the authors perspective on the likelihood that a genetically encoded agent for the given modality might be found. The imagery above and below the table depicts the different effects with visual codes as explained in figure 1. Supplemental figure 1 shows a potential imaging scheme for agents other than photoswitching agents in a visual style as in figure 1f. A complete overview of all current OptA studies using modulation of a label’s signal can be found in Supplemental Table 1.





**Figure 2 | Imaging examples of photoswitching optoacoustics (psOptA), optoacoustics (OptA) with thermo-control and magneto-control of label states and other applications of photocontrollable agents in OptA. a**Three tubes filled with two photoswitching proteins (Dronpa Wt and Dronpa Variant) and blood have been embedded in agarose and imaged with blue and UV light. Based on the photoswitching of the Dronpa proteins, they can be distinguished from the blood confounder tube as well as from each other. Adapted with permission from Stiel et al. (2015)25 © Optical Society of America. **b**MTLn3 cells expressing DrSplit, a DrBphP-based complementation reporter, develop tumor metastasis after implantation. Imaging tumor progression with psOptA and end-point histology verification suggests the cell number of the smallest metastases detected to be ~600 cells. Adapted from Li et al. (2018)92 under a CC-BY4.0 license. **c**Multiplexing in temporally unmixed OptA by exploiting the different kinetics of different photoswitching labels. Left: 2 Jurkat cell lines and bacteria expressing different BphPs unmixed. Right: A tumor with cells labeled with one BphP separated from implanted bacteria labeled with a second BphP, psOptA volume representation. Adapted from Mishra et al. (2020)91 under a CC-BY4.0 license. **d**Pump-probe based unmixing exploiting the stimulated-emission of Atto680. Shown is the dye injected *s.c.* in a live mouse­ together with the vasculature. Adapted from Mark et al. (2018)155 under a CC-BY4.0 license. **e**Use of thermochromics for temporal unmixing in OptA. The thermochromic agent is driven between states with high and low absorbance at 540 nm by changes between 20°C (high absorbance and thus OptA signal) and 40°C (low absorbance). Adapted from Ma et al. (2023)158 under a CC-BY4.0 license. **f** In-vivo magneto-motion imaging of *s.c.* implanted HeLa cells pre-tagged with magnetic nanoparticles. Leftmost image: unmixed image using 8-cycle 20 Hz magnetic "excitation". Middle: Correlation with excitation map utilized to produce photoacoustic image background suppression. Right: Peak to peak displacement image. Adapted with permission from Li et al. (2015)166. Copyright 2015 American Chemical Society. **g**The kinetics of the photoswitching transitions encodes the used fluence, hence the knowledge about the transitions can be used to normalize data for fluence differences. The left image shows 2 tubes with photoswitching protein embedded in absorbing material. The lower tube is clearly darker due to lower fluence. The right image is normalized to the switching kinetics obtained from the respective tubes. Adapted with permission from Dean-Ben et al (2020)187 © Optical Society of America.