Biochemistry

Perspective

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# Development of Photoacoustic Probes for *in Vivo* Molecular Imaging

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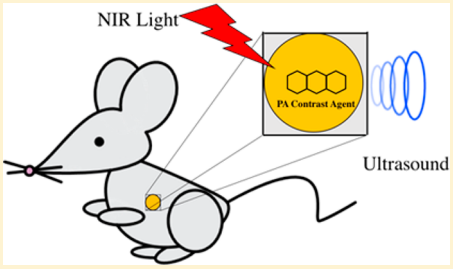
**ABSTRACT:** Photoacoustic (PA) imaging is an emerging, non-invasive imaging modality that encompasses attributes of both optical and ultrasound imaging. Because of the combination of optical excitation and acoustic detection, PA imaging enables high contrast and high resolution within deep tissue (centimeter depths). Recent advances in PA probe development have allowed for stimulus-responsive imaging in a variety of biological models with implications for basic, translational, and clinical sciences. This perspective highlights recent progress in the development of PA probes and their application to live-animal molecular imaging.






For most of the 20th and early 21st centuries, fluorescence microscopy functioned as the biologist's lens into the cellular world. Early fluorescence-based cellular imaging utilized fluorescent dyes or proteins to specifically label organelles and biomolecules. Later, this laid the groundwork for the development of stimulus-responsive probes, thereby expanding the capability of fluorescence imaging for the systematic detection of metals,[1] proteins,[2] voltage,[3] and reactive signaling molecules[4,5] with both improved selectivity and sensitivity.[6] However, cellular studies alone are rarely sufficient to recapitulate the complexity of biological processes in their native setting (e.g., within a whole animal). For instance, while hippocampal neurons can be cultured *ex vivo*, these conditions omit essential communication and architecture provided by the supportive neuronal cells, such as glial cells and oligodendrocytes. The application of purely optical methods to live-animal imaging is unfortunately limited by shallow imaging depths (~1 mm) due to extensive absorption and scattering of light within tissue. Thus, the development of new chemical tools and technologies for *in vivo* imaging is a particularly active area of research.

Photoacoustic (PA) imaging offers a promising alternative to optical methods by combining non-ionizing, deep-penetrating excitation light with acoustic detection. Through the detection of sound, rather than emitted light, PA imaging overcomes the limited imaging depths associated with fluorescence imaging. This enhancement stems from the decreased scattering of sound relative to light within biological tissue (~$10^3$-fold less), thereby enabling high-resolution imaging at centimeter depths.[7]

Since Alexander Graham Bell first discovered the PA effect more than a century ago,[8] this technology has significantly grown through key advances in instrumental design and data reconstruction methods, facilitating an impressive range of biological studies and preclinical trials.[9,10] Many of these studies have employed exogenous contrast agents (e.g., dyes and nanoparticles) to increase contrast and improve imaging resolution. However, these traditional contrast agents lack the capability to report on a given biological event (e.g., oxidative stress) or analyte. This perspective highlights recent advances in the development of new activatable PA probes with an emphasis on methods for expanding the utility of PA for molecular imaging. We also provide commentary on limitations that most urgently need attention.

## PHOTOACOUSTIC BASICS

In theory, any material that absorbs light can produce a PA signal; however, coupling signal generation with a specific biological stimulus remains a profound challenge. It is essential to consider the PA effect (Figure 1) to appreciate this challenge. In brief, irradiation of an optical absorber with light results in an excited state that can return to its ground state via radiative emission or nonradiative decay. In the case of nonradiative relaxation, the optical absorber releases energy in the form of heat, increasing the local temperature. This rapid increase in temperature results in thermoelastic expansion and produces a change in pressure. By pulsing the excitation source, one is able to generate fluctuating (megahertz) pressure waves, which propagate through the medium as acoustic waves. An array of ultrasound transducers converts the detected sound to electronic signals, which are reconstructed to afford three-dimensional images.[7,11]

## DEVELOPMENT OF ACTIVATABLE PA PROBES

Ideal PA probes must absorb significantly within the near-infrared (NIR)-I (620−950 nm)[11] or NIR-II (1000−1700 nm) biological windows.[15] Absorption in this range is necessary so the excitation light can penetrate several centimeters into tissue while minimizing the background signal from optically active

**Special Issue:** Future of Biochemistry

**Received:** September 6, 2017
**Revised:** September 27, 2017
**Published:** October 12, 2017




DOI: 10.1021/acs.biochem.7b00888
*Biochemistry* 2018, 57, 194−199

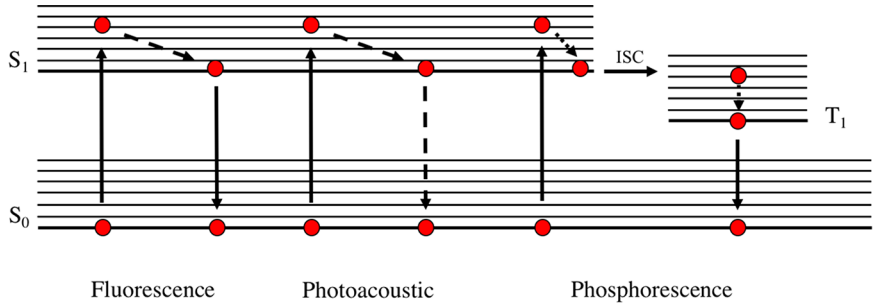



**Figure 1.** Stylized Jabłoński diagram depicting the origin of signals in fluorescence, photoacoustic, and phosphorescence imaging. Absorption, intersystem crossing (ISC), and emission events are indicated by solid lines, whereas nonradiative processes are depicted by dashed lines.

endogenous species (Figure 2). Additionally, chromophores with high extinction coefficients and low quantum yields are expected to be optimal for PA imaging because they absorb large amounts of light and preferentially relax through nonradiative processes.[11] One potential strategy for the design of PA probes is to utilize existing NIR fluorescence turn-off probes, strongly fluorescent molecules that undergo quenching upon interaction with a stimulus yielding a non-emissive, PA active product. Other photophysical properties, such as excited state absorption, relaxation kinetics, photobleaching, and triplet state contributions, are also key considerations in the context of PA probe development.[16−19] At the moment, these prove more difficult to predict, and a better connection between the property and chemical structure is required. Common PA probe platforms that satisfy these characteristics include cyanine, semicyanine, aza-BODIPY, squarine, and porphyrin/porphyrinoid platforms (Figure 3). Other excellent reviews discussing the development of activatable PA contrast agents can be found elsewhere.[11,20−22]

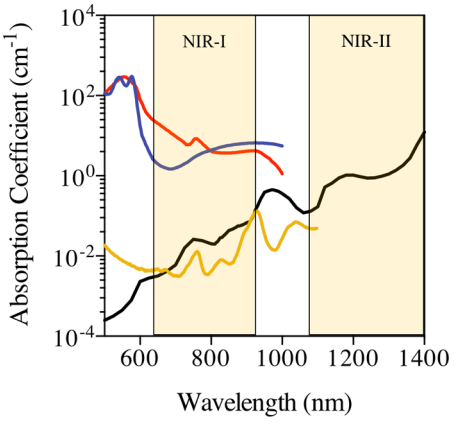



**Figure 2.** Absorption coefficient of biological chromophores: oxyhemoglobin (red), deoxyhemoglobin (blue), lipids (yellow),[12,13] and water (black).[14] Data from http://omlc.ogi.edu/spectra/.

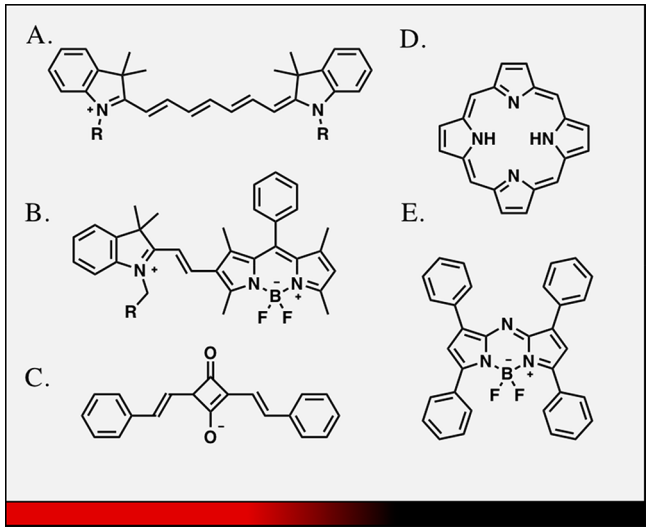



**Figure 3.** Representative structures for common NIR PA dye platforms: (A) cyanine, (B) semicyanine, (C) squaraine, (D) porphyrin/porphyrinoid, and (E) aza-BODIPY.

To date, the majority of PA probes are activated via modification of the extinction coefficient, quantum yield, cell or tissue localization, or a combination of these factors. Overall, activatable PA probes fall into five main groups: (1) cleavage retention, (2) intermolecular assembly, (3) reversible binding, (4) reaction-based, and (5) electro-responsive (Figure 4).

**Cleavage-Retention PA Probes.** Gambhir and co-workers reported the first activatable PA probe in 2010. Their cleavage-retention-based probe consisted of two small-molecule chromophores linked by a matrix metalloprotease-2 (MMP-2) sequence. The approach utilized MMP-2-mediated cleavage of the linker to disconnect the two chromophores. Attachment of a cell-penetrating peptide sequence allowed targeted uptake of one fragment. Selective imaging of the cleavage product was achieved by subtracting the signal at the two absorbance maxima.[23] Although this approach was initially evaluated in tissue phantoms and fibrosarcoma cells, it has since been employed for *in vivo* imaging in preclinical studies (Figure 5).[24] In addition to MMP-2, other tumor-associated enzymes (MMP-9[24] and MMP-13[25]) have been studied using cleavage-retention-based probes. This general approach has potential to be applied to other enzyme-catalyzed cleavage reactions (e.g., RNA, DNA, carbohydrate, and lipid hydrolysis), although no other examples yet exist.

**Intermolecular Assembly PA Probes.** Intermolecular assembly-based PA probes utilize a host of intermolecular interactions (aggregation, polymerization, and assembly) to modulate the PA signal in the presence of a stimulus. This is an attractive alternative to the cleavage-retention mechanism because it omits the necessity for targeted retention and combines positive attributes of both small-molecule and nanomaterial probes (e.g., aggregation-based quenching). In 2013, Gambhir and co-workers introduced the first intermolecular assembly-based activatable PA probe. Much like their initial activatable probe, the furin-responsive PA probe for cancer detection featured a furin-specific peptide sequence (containing a thioethyl-protected cysteine), Atto740, and 2-


DOI: 10.1021/acs.biochem.7b00888
*Biochemistry* 2018, 57, 194−199

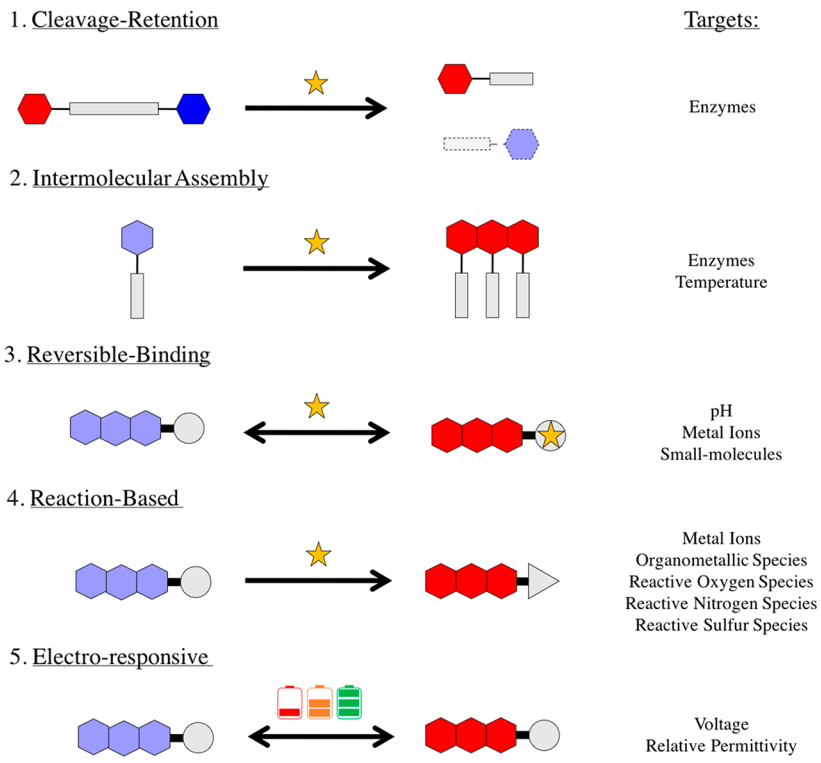



**Figure 4.** Schematic representations of methods for the development of activatable PA probes.

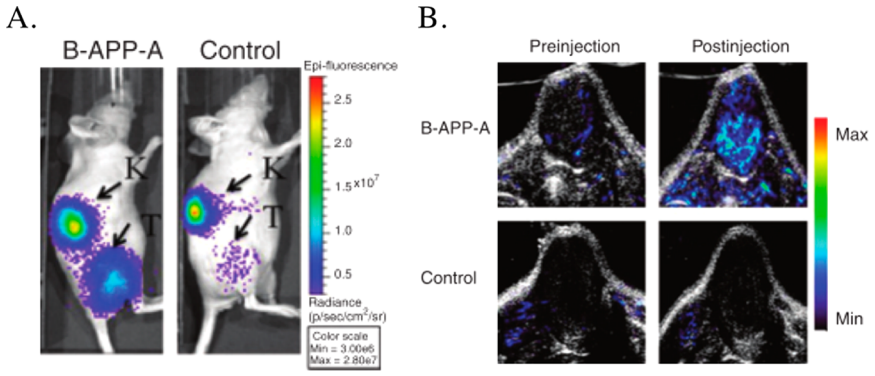



**Figure 5.** Comparison between (A) *in vivo* fluorescence and (B) PA imaging of follicular thyroid carcinoma in mice with a cleavage-retention-based PA probe, B-APP-A.[24] Reprinted with permission from ref 16. Copyright 2013 American Association for Cancer Research.

cyano-6-aminobenzothiazole (CABT). Upon cellular uptake, the protected disulfide is reduced, releasing an unprotected cysteine. Exposure of the free cysteine allows for recognition and enzyme-catalyzed peptide hydrolysis by furin. The unmasked N-terminal cysteine of the product is then available to undergo condensation with CABT, oligomerization, and finally nanostructure formation. In addition to accumulation, the aggregation of Atto740 within the nanostructures facilitates aggregation-based fluorescence quenching and PA intensity enhancement, allowing for successful identification of furin-positive cancer cells over furin-negative cancer cells.[26]

A notable expansion of the intermolecular assembly approach is the use of aggregation as a temperature-responsive trigger. Through temperature-based changes in J-aggregate formation, it was possible to measure temperature thresholds, or the temperature required for the loss of J-aggregates, within a xenograft model.[27] While advantageous in many ways, the generalizability of self-assembly- and aggregation-based methods depends on the development of new, stimulus-specific assembly and aggregation triggers.

**Reversible Binding PA Probes.** The direct detection of analytes through reversible binding greatly expands the number of accessible targets for molecular imaging. Wang and co-workers demonstrated the first example of this approach in 2013, with a commercially available pH probe within a hydrogel matrix. Importantly, the authors demonstrated the utility of encapsulation to minimize medium- or protein-based turn-on responses. Because the maximal absorbance occurs at 580 nm, the experiments were limited to imaging the tail vein of mice.[28] It was not until 2015 that turn-on pH measurements in deep tissue were made possible by Han and co-workers with a NIR fluorophore-containing carbohydrate-based nanoprobe. The initial report demonstrates the application of the targeted nanoprobe for imaging aberrant lysosomal acidity within subcutaneous and liver tumor models.[29] The utility of the small-molecule pH-responsive fluorophore, without the nano-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particle shell, was later expanded for the detection of inflammation.[30] While initial nanoprobe designs were largely applied to limit environmental interference and for targeting, Pu and co-workers have developed a generalizable approach to enhancing PA output through nanoparticle−dye photoinduced electron transfer quenching. Because of the electronic overlap between PA-active semiconducting oligomer nanoparticles and the selected pH-sensitive dye, the authors were able to enhance the PA output. While the seminal report detected tumor acidity within a HeLa xenograft model, we predict an expansion of this method to other stimuli of interest because of its ease of design and modularity.[31]

More recently, researchers have expanded the utility of pH contrast agents to assist in the detection of other analytes. Clark and co-workers developed a lithium-responsive nanoprobe composed of a lithium ionophore-based nanoparticle and a pH-responsive dye. Accumulation of lithium within the nanoprobe causes a pH change, deprotonation of the dye, and a corresponding PA turn-on. Ratiometric imaging allowed for the detection of systemic lithium concentrations following lithium dosing in a mouse model. The authors were able to clearly demonstrate the higher resolution obtained in deep tissue with PA imaging relative to that obtained in fluorescence imaging. Moreover, this report introduced an interesting concept of indirectly coupling an analyte to a PA readout.[32] Later work has expanded this approach for the detection of potassium.[33]

To generalize this approach for the detection of individual analytes, recent reports have employed both novel and well-known binding moieties for metal sensing. Specifically, analyte-specific PA probes have been developed for the detection of uranium[34] and calcium.[35] The former uses a novel porphyrinoid-containing nanoparticle that aromatizes following metal binding. The latter employs a well-known calcium binding moiety that disrupts the push−pull system following calcium binding. Non-metal targets are also beginning to emerge in the literature, such as the detection of heparin, an anionic polysaccharide therapeutic, using methylene blue.[36] Overall, reversible binding-based detection is favorable because it allows for the imaging of dynamic processes, for example, the changes in calcium concentrations before, during, and after neuron firing.

**Reaction-Based PA Probes.** In contrast to reversible binding methods, reaction-based probes undergo stimulus-selective modification to afford a PA response. Both irreversible and reversible methods are possible; however, current examples are limited to irreversible triggers. The lack of reversible reaction-based probes persists for both activatable fluorescence and PA probes, as only a handful exist to date.[37−39] It is important to note that reversible and irreversible approaches are complementary because reversibility is important for monitoring dynamics, while irreversibility allows for accumulation of the product and enhances sensitivity.

The first stimulus-specific PA probes utilized reaction-based oxidation of a NIR dye to monitor reactive oxygen species (ROS) and reactive nitrogen species (RNS) within a nanoparticle. As the dye reacted with hypochlorite and peroxynitrite, the intensity of the corresponding PA signal decreased. By monitoring the ratio of unreacted to oxidized nanoprobe, one is able to elicit a turn-on response, even while turn-off relative to the contrast agent.[40] More recently, analyte-specific degradation of PA-active semiconducting polymer nanoparticles has been reported for the ratiometric detection of hypochlorite in a murine allograph model of breast cancer.[41]

Methods for developing turn-on contrast agents and enhancing the selectivity of promiscuous dyes are beginning to emerge within the literature. For example, Pu and co-workers introduced the concept of analyte-specific dopants to render a known fluorescent contrast agent selective for the detection of peroxynitrite. The boronic acid trigger was initially intended for the detection of hydrogen peroxide, but it can display cross-reactivity with peroxynitrite. To increase selectivity for peroxynitrite, the authors doped their nanoprobe with bulky borane compounds (triphenylborane or perfluorotriphenylborane). Because these dopants react with only the analyte of interest, it is possible to permit peroxynitrite activation while occluding hydrogen peroxide from the dye. This nanoprobe was applied for the detection of endogenous peroxynitrite within small-animal cancer models.[42] Later examples of analyte-specific nanoprobes utilized custom, selective activatable NIR dyes within nanoparticle frameworks for the visualization of hydrogen sulfide[43] and methylmercury *in vivo*.[44]

Recently, Liu and co-workers have employed a liposomal nanoprobe containing horseradish peroxidase (HRP) and ABTS within the core to repurpose a conventional chromogenic assay for PA imaging. In the presence of hydrogen peroxide, HRP catalyzes the oxidation of ABTS into a radical cation chromophore. Because of the broad absoption from 600 to 900 nm, the authors were able to detect hydrogen peroxide in an impressive array of small-animal models (lipopolysaccharide-mediated inflammation, orthotropic cancer, and metastatic cancer models).[45]

While the vast majority of activatable PA contrast agents have been nanoprobes, we highlight the utility of activatable small-molecule contrast agents, or acoustogenic probes. We reported the first acoustogenic probe, acoustogenic probe for copper-1 (APC-1), and its water-soluble congener (APC-2) in 2015. The 2-picolinc acid-functionalized aza-BODIPY utilized selective Cu(II)-catalyzed hydrolysis for the ratiometric detection of copper(II) within tissue phantoms.[46] In contrast to nanoprobe-based structures, small molecules are more readily taken up via passive diffusion, show improved ability to permeate the blood−brain barrier, and exhibit faster clearance (minimizing toxicity because of accumulation). Moreover, small molecules tend to be more easily amenable to the measurement of enzymatic activities (due to less significant steric effects) and are historically more likely to pass clinical trials. For these reasons, we anticipate a rapid increase in the number of acoustogenic reports.

**Electro-Responsive PA Probes.** The electronic environment of the cell is closely linked to a variety of biological phenomena (e.g., neuronal signaling and cardiomyocyte function). While the majority of work has focused on neuronal and muscular cells, it is acknowledged that electronic properties such as membrane potential play key roles in both excitatory and non-excitatory cells. To enable PA imaging of these processes, researchers are employing existing and novel chromophores to image electronic features, such as voltage and polarity. Wang and co-workers published the first report of an electro-responsive PA probe in 2017. The authors employed dipicrylamine, a well-known voltage-responsive small molecule, to monitor voltage changes within a small-animal model. While unable to perform deep tissue voltage imaging, the authors demonstrate the ability to image changes in voltage potentials on the surface of a live brain following electronic stimulation


DOI: 10.1021/acs.biochem.7b00888
*Biochemistry* 2018, 57, 194−199

and 4-aminopyridine-induced epilepsy. Moreover, the authors report the detection of voltage changes in HEK-293 cells below 4.5 mm of rat brain tissue, exceeding the optical diffusion limit.[47]

In addition to voltage imaging, changes in polarity are thought to be a potential biomarker for several pathological states (e.g., cancer, neuronal impairment, and diabetes). To enable deep tissue imaging of diabetes-induced liver damage, Tang and co-workers developed a cyanine-based photoacoustic probe for the detection of endoplasmic reticulum polarity changes. The authors demonstrate that there is a significant change in polarity for diabetic mice relative to the control, indicating that the liver damage results in decreased polarity. Treatment with metformin, an oral hypoglycemic drug, prevented liver damage and the associated decrease in polarity.[48] To expand the utility of PA imaging for the detection of electronic changes, it will be essential to develop new NIR platforms capable of responding to electronic changes *in vivo*.

## ■ CONCLUSIONS AND OUTLOOK

PA imaging has emerged as a promising alternative for *in vivo* imaging because of its high resolution at extended imaging depths. Over the past decade, a series of PA nanoprobes and acoustogenic probes have been developed for a breadth of stimuli (enzyme activities, pH, ROS, RNS, reactive sulfur species, metals, temperature, voltage, and polarity). Along with the expanding number of targets, a variety of strategies for stimulus-specific detection have been developed. To expand the utility of PA imaging, it is important to develop new NIR platforms that exhibit the requisite characteristics for optimal *in vivo* performance and new methods for stimulus-driven modulation of PA signals (e.g., PA lifetime imaging). Moreover, it is essential to make a coordinated effort to optimize not only activatable contrast agents (to increase the imaging depth, resolution, and target specificity) but also advanced instrumentation (to enable diagnostic accuracy, aid in treatment planning, and guide surgical procedures). With advances in each of these areas, we anticipate that PA imaging will be at the forefront of the next generation of molecular and biomedical imaging.

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### Funding

C.J.R. was supported by Chemistry-Biology Interface Training Grant 2T32 GM070421. Additional financial support came from the Alfred P. Sloan fellowship (FG-2017-8964 to J.C.).

### Notes

The authors declare no competing financial interest.


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DOI: 10.1021/acs.biochem.7b00888
*Biochemistry* 2018, 57, 194−199