Chemical Imaging in Vivo: Photoacoustic-Based 4-Dimensional Chemical Analysis

We describe how 4-dimensional in vivo biochemical analysis can be performed using photoacoustic contrast nanoagents that have been designed to probe both structural and chemical information in vivo, enabling noninvasive, real time, spatially resolved chemical imaging. Early chemical imaging of a patient’s tumor can inform the decision of effective treatment, regarding choices of chemotherapy, radiation, or immunotherapy.

Chang H. Lee,‡§ Jeff Folz,¶⊥ Joel W. Y. Tan,§ Janggun Jo,§ Xueding Wang,§ and Raoul Kopelman†

†Department of Chemistry, University of Michigan, Ann Arbor, Michigan 48109, United States
‡Biophysics Program, University of Michigan, Ann Arbor, Michigan 48109, United States
§Department of Biomedical Engineering, University of Michigan, Ann Arbor, Michigan 48109, United States

In chemical imaging, each pixel essentially carries chemical information (comparable to a single point measurement using traditional chemical analysis) providing as an image a landscape of chemical measurements in real time. Despite its potentials, in vivo, real time chemical imaging is challenging and has not yet been accomplished in a straightforward way in clinical settings. Among several biomedical imaging modalities, photoacoustic (PA) imaging (PAI) is an attractive technique combining widely applicable optical imaging and ultrasound imaging. The combination of these approaches allows practitioners to overcome some of the limitations of either technique for chemical imaging, i.e., “thin” (superficial) tissue penetration of the optical methods and the “chemical blindness” of the ultrasound methods. Here, we show some of the most recent advances with photoacoustic chemical imaging, focusing on the spatially and temporally resolved chemical makeup of tumors in vivo.

While in vivo structural imaging has been a mainstay of medicine for a century or longer (e.g., X-ray/CT, MRI, ultrasound), chemical imaging has been largely limited to microscopy of ex vivo samples, such as cells or tissues. Present in vivo chemical analysis is limited in space and/or time, e.g., blood tests, biopsies, or even locally inserted electrodes. No spatially/temporally resolved chemical information is available, except postmortem. The presently available ex vivo chemical analysis and/or imaging methods have been largely established with optical-based chemical indicators, starting with so-called “molecular indicators” (commercially available) and reaching to nanoparticle-based “PEBBLEs” (photonic explorers by biologically localized embedding), which permit the active targeting of nanosensors to subcellular compartments and enable a larger spectrum of analytes, including hydrogen, calcium, sodium, magnesium, potassium, nitric oxide, iron, lithium, Glucose, zinc, chloride, oxygen, etc.

Real-time, spatially resolved quantitative chemical analysis can be crucial for biomedical applications considering that many pathological hallmarks are directly relevant to the chemical information. To re-emphasize, a typical chemical analysis measures the target analytes at only one point and time. Chemical imaging, on the other hand, allows multipoints and multitime measurements of the target analyte. In addition to the structural background information that any imaging technique provides, now each pixel also contains quantitative analyte information in situ. To the best of our knowledge, there is no chemical imaging modality available in clinical settings except for blood oxygen level dependent imaging through functional magnetic resonance imaging.

Despite successful attempts in preparing numerous optical sensors, it has been challenging to apply optical indicators directly to in vivo chemical imaging due to the well-known light penetration depth limit in biological tissues. Light gets scattered and absorbed by biological tissues. Since conventional optical imaging (i.e., fluorescence imaging) detects the

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light that is emanating out from an excited sample, it has been extremely challenging to perform in-depth, in vivo imaging (typically limited to around 1 mm even with two photon microscopy).[^59]

Overcoming the imaging depth limit of conventional optical imaging allows one to utilize already established optical sensors for in vivo chemical imaging. Having established the principle a decade ago using molecular probes, PA imaging has been utilized for in vivo chemical imaging.[^7,25,32,42] PA imaging is one of the fastest emerging biomedical imaging modalities, which combines conventional optical imaging and ultrasound imaging.[^48] At its core, a PA imaging setup requires an electromagnetic wave source (most commonly a pulsed laser) and an ultrasound detector. The energy from the pulsed laser of the laser gets absorbed by optical contrast agents (e.g., blood, nanosensors), and a portion or all of the absorbed light energy gets released as thermal energy. This heat induces local thermal expansion, which can be detected via an ultrasonic detector (Figure 1). Thus, PA signals correlate directly with the optical absorption of the contrast agents.

![Photoacoustic imaging scheme](image)

**Figure 1.** Photoacoustic imaging scheme. Energy from a pulsed laser at specific wavelength illuminates and gets absorbed by optical contrast agents (either or both native, such as blood, or non-native, such as preinjected optical indicators). The absorbed energy causes thermal expansion, then ultrasonic emission, which can be detected by a conventional ultrasound transducer or ultrasound imaging system. We note that both ultrasound images (structural) and photoacoustic images (functional) can be acquired simultaneously if a conventional ultrasound imaging system is used. Reproduced with permission from ref 43. Copyright 2018 The Royal Society of Chemistry.

One of the unique features of PA imaging is its ability to scale its resolution at the expense of imaging depth. Since PA imaging utilizes both optical and ultrasound components, the resolution of PA imaging systems can be determined by either the optical or ultrasound system depending on the imaging depth or resolution required. Subwavelength lateral resolutions can be achieved by using optically focused PA imaging with an imaging depth up to a hundred micrometers.[^49] On the other end of the spectrum, submillimeter lateral resolutions can be achieved by using acoustically focused PA imaging but with a significantly improved imaging depth of up to several centimeters.[^560] The axial resolution of PA imaging systems is independent of the method of focusing and is mostly dependent on the speed of sound in the sample being imaged, the laser pulse width, imaging depth, and the ultrasound detector’s frequency response.[^68] Typically, this results in axial resolutions of tens to hundreds of micrometers.[^68] Temporal resolution is usually limited only by the laser’s pulse repetition rate and the time it takes for the ultrasound signal to reach the detector.[^51] Since the speed of sound in biological tissue is approximately 1.5 mm/μs, a 15 mm tissue sample would limit the frame time/rate to 1/10 μs or 100 kHz. However, since most PA systems have pulsed lasers with repetition rates much lower than this (between 10 and 5000 Hz), the frame rate of PA imaging is primarily limited by the pulsed laser repetition rate. This means that PA imaging can provide real-time (or near real-time) imaging. This, in combination with the enhanced imaging depth limit of up to several centimeters is significant for studying the heterogeneous chemical distribution of large in vivo tissues, such as tumors, in real time. Due to its versatility, PA imaging has been explored as a method for the study of many different cancers such as, but not limited to, breast cancer,[^52] melanoma,[^53] prostate cancer,[^54,55] cervical cancer,[^50] and gastrointestinal cancers.[^7] However, as the spatial resolution of PA imaging decreases with imaging depth, PA imaging tends to work best in near-surface tumors such as melanoma or in tumors that are accessible by endoscopes, such as gastrointestinal cancers. While there has been substantial effort of translation to clinical PA imaging,[^52,58,59] PA is still mostly limited to preclinical imaging.

Here, PA chemical imaging of analytes that are biomedically relevant are presented. Our central focus is cancer, which exhibits unique chemical properties such as low pH (acidosis), low O2 (hypoxia), and high [K+] (hyperkalemia). Acidosis and hypoxia of tumors are interrelated and explained through the Warburg effect.[^60] Cancer is essentially uncontrollable cell growth. As a tumor gets larger, tumor cells near the blood vessels take all the nutrients and oxygen that are needed for aerobic glycolysis. This effect causes local oxygen depletion, which grants the tumor resistance to radiation therapy. Due to the lack of oxygen at the core of the tumor (or far away from the blood vessels), the tumor cells change their major metabolic pathway from aerobic glycolysis, which consumes oxygen, into anaerobic glycolysis, which does not. A side product of anaerobic glycolysis is lactic acid, which causes acidosis in the tumor. The low pH of the tumor microenvironment can inhibit chemotherapeutics. Recently, hyperkalemia of cancer has been granted attention as a potential immune suppressing factor in the tumor microenvironment.[^61] Tumor cells burst and release potassium ions during necrosis, and this large excess of potassium ions is not easily removed from the tumor. The necrotic core of tumors is hypothesized to have significantly higher extracellular potassium concentrations (5–10-fold higher). This excess potassium inhibits immune cell function. Thus, the chemical makeup of the tumor microenvironment may have a large effect on the tumor therapy, including immunotherapy. If we are able to determine early on for a cancer patient what this microenvironment’s chemical content is, we would be able to apply the most promising therapy for that patient.

### pH (ACIDITY)

One of the earliest examples of PA pH imaging in vivo has been reported by Chen et al.[^47] A near-infrared absorbing pH indicating dye, benz[a]phenoxazine, and a reference dye, IR825, is encapsulated into glutaraldehyde cross-linked nanoparticles. The PA intensity ratio between the pH indicating dye and the reference dye grants ratiometric pH images in tumor models. Around the same time, Kimbrough et al. applied a pH sensitive insertion peptide as a PA pH indicator.[^46] The peptide recognizes acidic pH and inserts itself...
into the cellular membranes. This system has been demonstrated as a potent pH indicating agent both in vitro and in vivo.

We have recently published a method to quantitatively measure pH using PA imaging of xenograft tumors in vivo. A commercially available pH indicating dye, SNARF-5F, is used as an exogenous sensing component. SNARF-5F has a $pK_a$ of $\sim 7.2$, which is close to physiological neutral pH, and is, therefore, ideal for measurement of pH in biological samples. SNARF-5F changes its optical absorption maximum (or color) at different pH. SNARF-5F is encapsulated in a biocompatible polyacrylamide polymer-based nanoparticle (Figure 2). The surface of the nanoparticles is modified with tumor targeting F3-peptides. The nanoparticle encapsulation allows the sensor to be actively targeted to the tumor area and prevents premature dye degradation. SNARF-5F is a small molecule and interacts with proteins body, such as human serum albumin. Upon interaction with the human serum albumin, SNARF-5F changes its optical properties causing complications for in vivo chemical imaging. Nanoparticle encapsulated SNARF-5F does not alter its optical properties upon mixing with the same amount of the human serum albumins.

Since PA signal linearly correlates with the absorption characteristics of the dye, simple ratiometric PA images between 565 nm (pH independent isosbestic point) and 600 nm excitations (sensing point) are first applied. Although naive ratiometric images are correlated with different pH values, the ratiometric image no longer corresponds with different pH values with the addition of background blood (1% v/v). It would be crucial to consider blood PA signal for quantitative pH images, since blood presents a major background optical absorption in biological tissues.

In order to overcome the background issue, quad wavelength ratiometric PA pH imaging is developed. In this method, four wavelengths (565, 576, 584, and 600 nm) are used to compute quantitative pH. Using four wavelengths, three analogous calibration lines are generated by using 565 nm excitation and the PA signal in the range of 565 nm to 600 nm is used to determine the pH value. The calibration curves are determined by injecting the nanoparticles into the tumor region and measuring the PA signal at different pH values.

Figure 2. (a) SNARF-5F encapsulated polyacrylamide-based nanoparticle (NP) synthesis and pH-sensing scheme. Polyacrylamide matrix is formed in the presence of the pH indicator: SNARF-5F. The polymer matrix is further modified with PEG and the tumor homing F3 peptide. The absorption of this NP changes at different pH levels shown in the absorption spectra. (b) Since PA signals correlate with the optical absorption, PA signals from 600 nm excitation varies at different pH values while PA signals from 565 nm excitation stays relatively constant. (c) Representative in vivo pH image of the tumor (tumor boundary is shown by the red dotted line). The NPs have been injected through tail vein. (d) Representative in vivo pH image of the control muscle. The NPs have been directly injected subcutaneously. (e) Blood oxygenation image of tumor in vivo Reproduced with permission from ref 7. Copyright 2017 The Author(s). Published under the terms of the CC BY 4.0 license, https://creativecommons.org/licenses/by/4.0.
nm as an internal reference point. The calibration functions can be expressed as

\[
\frac{\epsilon_{NP}^\lambda}{\epsilon_{NP}^{565}} = (\alpha \times \text{pH}) + \beta
\]

(1)

where \(\epsilon_{NP}^\lambda\) is an optical absorption coefficient of the sensing peak wavelengths (576, 584, or 600 nm) and \(\epsilon_{NP}^{565}\) is the optical absorption coefficient of the reference absorption at 565 nm excitation. \(\alpha\) and \(\beta\) are constants from each calibration line (576 nm, 584 nm, 600 nm).

If PA signals are only responsive to blood content (oxygenated hemoglobin and deoxygenated hemoglobin) and nanoparticle absorption, the PA signal can be expressed as

\[
PA = k(\epsilon_{\text{Hb}}^\lambda[Hb] + \epsilon_{\text{HbO2}}^\lambda[HbO2] + \epsilon_{\text{NP}}^\lambda[NP])
\]

(2)

where \(k\) is a PA signal constant, including the Grüneisen parameter of the tissue, light fluence, and sensitivity of the imaging system, \(\epsilon_{\text{Hb}}\) and \(\epsilon_{\text{HbO2}}\) are known optical absorption coefficients of deoxyhemoglobin and oxyhemoglobin, respectively, and \([[\text{Hb}], [[\text{HbO2}],\text{ and } [\text{NP}]\text{ refer to concentrations.}

Applying eq 1 into eq 2, the PA signal equations at each excitation can be expressed as

\[
PA_{565} = k(\epsilon_{\text{Hb}}^{565}[\text{Hb}] + \epsilon_{\text{HbO2}}^{565}[\text{HbO2}] + 0 \times \text{pH})
\]

(3)

\[
PA_{576} = k(\epsilon_{\text{Hb}}^{576}[\text{Hb}] + \epsilon_{\text{HbO2}}^{576}[\text{HbO2}] + \alpha_{576/565} \times \text{pH})
\]

(4)

\[
PA_{584} = k(\epsilon_{\text{Hb}}^{584}[\text{Hb}] + \epsilon_{\text{HbO2}}^{584}[\text{HbO2}] + \alpha_{584/565} \times \text{pH})
\]

(5)

\[
PA_{600} = k(\epsilon_{\text{Hb}}^{600}[\text{Hb}] + \epsilon_{\text{HbO2}}^{600}[\text{HbO2}] + \alpha_{600/565} \times \text{pH})
\]

(6)

The \(\epsilon_{NP}^\lambda[NP]\) term in eq 3 is essentially converted into the linear function based on using 565 nm excitation as both sensing and reference points (thus, slope, \(\alpha = 0\) and \(y\)-intercept, \(\beta = 1\)). The four unknown variables ([Hb], [HbO2], pH, and [NP]) in eqs 3–6 are computed during image processing.

Thus, the blood background PA signals are taken into consideration and direct quantitative pH information can be drawn onto the images (Figure 2c,e). Using this algorithm, quantitative PA pH images are taken of xenograft tumors in vivo. The average computed pH (6.71 ± 0.22) is always lower than the control, normal tissue pH (7.46 ± 0.095), which is similar to gold standard microelectrode measurements (Figure 2c,d). An example calculation is included in the Supporting Information.

Earlier this year, Baumann et al. introduced another PA pH probe based on a DNA triplex.66 This active DNA structure operates through a principal analogous to FRET, though the signal modulated is PA intensity rather than fluorescence intensity. An infrared fluorescent reporter and quenching agent are tagged onto the 3’ end of two bound DNA sequences of 21 and 26 units, respectively. At high pH (~8.0), the complex is disorganized, and the strands of the 3’ ends do not interact significantly. As the pH drops over the physiologically relevant range of 6.0–8.0, the DNA complex becomes increasingly ordered and the probe begins to fold onto itself, bringing the 3’ ends into tighter proximity. As such, the excited fluorescent probe begins to be actively quenched, and the photoacoustic signal increases. While this probe has not yet been demonstrated in vivo, it represents an exciting new approach to PA chemical imaging.

We note here that in vivo pH measurement has not been limited to PA imaging. Both positron emission tomography (PET) and magnetic resonance spectroscopy (MRS) techniques have been employed to this effort. One approach is to use hyperpolarized, C-13 enriched bicarbonate, whose transition, and associated relaxation times, between bicarbonate and carbon dioxide can be used to map pH in vivo.6 Recently, the resolution of this technique is extended to a voxel as small as 3.75 mm × 3.75 mm × 5 mm by using the same approach with zymonic acid.65 One drawback of this technique is that the hyperpolarization of these molecules quickly decays, and measurements must be made within 2 min of injection. P-31 MRS has also been used to measure pH in vivo via 3-aminopropyl phosphonate. The authors made use of a pH low insertion peptide, which targets the tumor microenvironment, bound to Cu-64. The subsequent accumulation of Cu-64 at the tumor sight permitted greatly enhanced PET imaging.64 Last, MRI-CEST (chemical exchange saturation transfer) has been employed to map pH in vivo using the contrast agent iopamidol, where a planar resolution of 117 μm is achieved along with probing metabolic information.66 In summary, however, the voxel resolution is significantly worse for MRI measurements, compared to PAI, as are the time limitations. Most importantly, MRI machines, not to mention PET, are orders of magnitude more expensive than PA setups, the latter’s price being on the order of 500,000 U.S. $. Furthermore, compared to PET, PAI only uses nonionizing radiation, with the same holding with respect to other potential methods employing X-rays, protons, or other high-energy photons or particles.

**OXYGEN (O₂)**

Hypoxia is a typical pathological factor in cancer and other diseases.66 PA imaging is very well-suited to measure blood oxygenation levels. As mentioned in previous sections, the optical absorption of blood is from two forms of hemoglobin (oxygenated and deoxygenated), which exhibit different optical properties. Just as in the standard procedure of pulse oximetry, the blood oxygenation level can be computed by taking the ratio between PA signals at two or more wavelengths.67 We have also shown an example of tumor blood oxygenation PA images using the same algorithm discussed above (Figure 2e).7

Although simple algorithm based PA blood oxygenation images are highly useful for shallow samples (<5 mm), light fluence differences at different wavelengths (fewer photons of longer wavelength light get scattered and absorbed, thus, traveling farther) become more significant. In order to overcome this issue, Yao et al. reported a method to compute blood oxygenation levels using a single wavelength but with different pulse widths (3 ns and 3 ps).68 Oxygenated hemoglobin has different saturation intensities, resulting in the reduction of absorption coefficients. An initial 3 ps pulse excitation on oxygenated hemoglobin causes absorption coefficient reduction in comparison to a 3 ns pulse excitation as reference. On the other hand, deoxygenated hemoglobin is less influenced by the saturation. The functional imaging depth
is enhanced to \( \approx 0.7 \) mm. More recently, Tzoumas et al. reported a multispectral approach to minimize the light fluence difference.\textsuperscript{69} A total of 21 different wavelengths are used for the image processing, and this method allows for quantitative blood oxygen imaging with imaging depth up to 1 cm.

While the above techniques rely on the endogenous absorption of hemoglobin, there has been interest in developing \( \mathrm{O}_2 \) sensing probes that are able to measure \( \mathrm{O}_2 \) levels even in tissues that are devoid of the presence of blood. A technique known as PA lifetime imaging has emerged as a method that can determine tissue oxygenation levels using phosphorescent dyes.\textsuperscript{31–45,70} In short, two lasers are used to first excite the ground state phosphorescent dye into the primary triplet excited state (T1) and then to measure the PA signal generated by exciting the dye from the T1 state into the secondary triplet excited state (T2) (Figure 3a).\textsuperscript{70} The phosphorescent dye in the T1 state can return back to the ground state via phosphorescence or via quenching by other triplet state molecules such as oxygen. This means that the oxygen concentration will affect the T1 state lifetime and subsequently the PA signal generated by the probe laser, which is dependent on the amount of phosphorescent dye in the T1 state. By varying the time delay between the pump and the probe lasers (i.e., varying the amount of phosphorescent dye in the T1 state), a decay curve showing the decrease in the PA signal generated by the probe laser with increasing time delay will be indicative of the oxygen content of the tissue (Figure 3b).

Many alternative methods to measure both blood oxygenation (\( \mathrm{SO}_2 \)) and tissue oxygenation (\( \mathrm{pO}_2 \)) exist and are actively being pursued in research. Polarographic electrodes provide highly accurate measurements of \( \mathrm{pO}_2 \) and are considered the gold standard in oxygen measurement.\textsuperscript{8} However, the invasive nature of the electrode, and its inability to provide information on \( \mathrm{pO}_2 \) beyond a single location and time point has motivated researchers to seek other approaches. PET and single-photon emission computed tomography (SPECT) detect \( \gamma \) rays emitted from radionucleotides. While this approach can lend itself to whole body scans, for oxygen imaging it is often limited to hypoxic areas where radiotracers accumulate. It also measures \( \mathrm{pO}_2 \) indirectly and can be expensive to operate as many radiotracers need to be produced on-site due to their short half-lives.\textsuperscript{2} Magnetic resonance spectroscopy boasts a variety of oxygen sensing techniques. For example, F-19 oximetry makes use of perfluorocarbons, whose spin–lattice relaxation rates are linearly dependent on \( \mathrm{pO}_2 \) but requires specialized equipment capable of F-19 scanning.\textsuperscript{73} A full review of oxygen sensing techniques and approaches can be found here.\textsuperscript{4}

### Oxygen Imaging and Therapy

The combination of PA chemical imaging with therapy presents an easy step in the direction of personalized medicine. Combination approaches are already being presented for oxygen imaging. Treatments such as radiation and photodynamic therapy (PDT) are both heavily dependent on the availability of oxygen at the region of treatment. Indeed, changes in oxygen saturation are actively being used to inform therapy and predict treatment outcome. Mallidi and colleagues have used oxygenated and deoxygenated hemoglobin to monitor blood oxygen saturation in concert with photodynamic therapy.\textsuperscript{75} Murine tumors are treated with the FDA approved benzoporphyrin derivative, a photosensitizer. The authors have found that PDT is most effective approximately 1 h after initial treatment, which the authors attributed to the photosensitizer localizing to both the cellular and vascular components of the tumor. Blood oxygen saturation drops dramatically in mice treated 1 h after the photosensitizer injection compared to the control group and those who received PDT 3 h after injection of the photosensitizer. This drop in blood oxygen saturation is found to be predictive of treatment efficacy, and the authors are able to predict treatment regrowth within the first 24 h after treatment, a prediction that can previously only be made 10–30 days post-treatment, when regrowth had already begun. Similar techniques have been developed for real-time monitoring of blood oxygen saturation during PDT as well as prediction of treatment efficacy in a variety of cancers.\textsuperscript{68–60}

### POTASSIUM (K\(^{+}\))

Ion selective bulk optodes are a type of ion sensor that measures target analyte by color change. A cation selective bulk optode is composed of three lipophilic sensing components: an ionophore, a chromoionophore, and an anionic phase-transfer catalyst. All of the sensing components are embedded in highly lipophilic matrix, such as polydecyl methacrylate. Prior to target ion interaction, the chromoionophore in the sensor, which is essentially a lipophilic pH indicator, is kept protonated to neutralize the anionic phase-transfer catalyst’s negative charge. Once the target ions are introduced into the sensor, the ionophores chelate the analyte. During this process, excess protons are released from the sensor in order to keep the lipophilic sensor’s net neutrality. These cation (and anion) selective bulk optodes have been originally used in absorbance but later miniaturized into fluorescence based micro- or nanosized optode sensors for a variety of analytes.\textsuperscript{8,9,13–18,20–22,31–34,81–84}

A potassium ion selective nanosensor for PA imaging is prepared based on the K\(^{+}\) cation selective bulk optode design.\textsuperscript{23} The lipophilic sensing matrix is trapped inside poloxamer-based micelles. The calibration curve is generated by taking PA intensity ratios between two sensing (optical absorbance) peaks, at 540 and 660 nm (Figure 4). The nanosensor is capable of response to potassium concentrations ranging from 1 mM to 100 mM, even in the presence of the large physiological sodium concentration, 150 mM, as background. Simple ratiometric PA images between 540 and 660 nm show the differentiated PA images at 2 mM, 10 mM, and 100 mM (Figure 4c). However, as expected from the calibration curve, 100 mM and 200 mM ratiometric PA images do not differ by much. The chromoionophore used in this

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**Figure 3.** (a) Diagram to demonstrate the generation of the triplet state using a pump and probe laser and important processes linking the triplet (T) and singlet (S) states. Here, the PA signal generated by the probe laser is dependent on the molecules present in the T\(_1\) state. (b) Simulation showing the effects of oxygen on the decay curve of the photoacoustic amplitude with respect to the time delay between the pump and probe lasers. By measuring the decay rate and comparing to a calibration curve, the oxygen content can be determined.
sensor also has fluorescence characteristics. Interestingly, the sensor responds strongly from 20 mM to 1 M when measured by fluorescence spectroscopy.

Although in vivo quantitative photoacoustic imaging of potassium has not yet been demonstrated, it presents a potential direction in combination with the algorithm used for the quantitative pH imaging. Current efforts to push potassium measurements in vivo have been made but challenges remain. Bischof and colleagues took a novel approach in developing their genetically encoded potassium ion indicator (GEPII); this FRET-based sensor binds potassium with excellent selectivity can be easily expressed in cell lines and even mice, and its dynamic range can be adjusted. However, this system does suffer from the same drawbacks of any fluorescent probe used in vivo: limited resolution and imaging depth. K-39 magnetic resonance spectroscopy has also been used to measure potassium in vivo. The ability to map potassium distributions in 3D and superimpose these images onto traditional MRI maps is of great diagnostic interest. However, the technology is still limited by relatively poor resolution (voxel sizes of ~1 mL) as well as the significant financial expense associated with MRI.

Other Analytes (Li	extsuperscript{+}). The Clark group has introduced an ionophore-based optical sensor for the PA monitoring of lithium. Lithium, which is used to treat bipolar disorders, has a narrow therapeutic window: 0.6–1.2 mM. Its toxic dose, however, is only 2 mM, making it a worthy candidate for continuous monitoring. Clark and colleagues used their particle to demonstrate dose dependent changes of blood lithium concentrations and found that the lithium ion reached its peak blood concentration 18 min after injection in mice.

**REMAINING PROBLEMS FOR PA BASED IN VIVO CHEMICAL IMAGING**

One of the most persistent challenges with PA imaging is the fact that any optical biosensors have to compete with endogeneous absorbers such as hemoglobin, melanin, and myoglobin. As a result, optical biosensors that have absorption in the near-infrared or in the so-called “optical window” are highly desired as the absorptions of the endogeneous absorbers are at a minimum within these wavelengths. Recently, photoswitchable contrast agents that can be switched “on” and “off” have been developed in an attempt to reduce the background signals and have largely been successful in improving the signal-to-noise ratios from these contrast agents. While such efforts have mostly been used to improve image contrast, this technology can prove to be extremely useful for PA chemical imaging if it can be successfully incorporated into biosensors. Besides changing the design of the optical biosensors directly, multiple wavelength unmixing techniques such as the quad wavelength imaging technique described in this paper are continually being developed in order to reduce the effects of these absorbers on the desired signal. These techniques can help to isolate each absorber contributing to the PA signal, provided that sufficient measurements at enough wavelengths are made. However, it is worth noting that multiple wavelength unmixing techniques usually suffer from inaccuracies due to the fluence differences between each of the wavelengths used. Since each wavelength is absorbed and scattered differently in the biological tissue, a nonhomogenous, wavelength-dependent fluence distribution is present in the tissue and will affect the accuracy of the measurements unless accounted for in these measurements.

**FUTURE PERSPECTIVES FOR PA CHEMICAL IMAGING (PACI)**

Most of the work presented here discuss about multi-wavelength photoacoustic imaging. Considering that light penetrates differently in tissues depending on its wavelength, it is hard to approximate the maximum chemical imaging depth. We note here that most tumors are entirely within reach from a distance of 1 cm (clinical potentials for superficial tumors, such as melanoma, head and neck cancer, breast cancer, etc.), the depth reachable by present methods, and even deeper than that with probes that work in the infrared spectrum. Furthermore, PACI’s application is not necessarily limited to monitoring the tumor microenvironment. Notably, blood tests are just “1-dimensional” (counting the analyte concentration as a dimension), compared to the “4-dimensional” recent advances in PACI summarized above, which provide X, Y, Z and time resolution, in addition to the concentration of the specific analyte. It is noteworthy to mention that PA imaging can essentially detect any colorimetric changes. Most of the applications of PACI were performed for small analytes, such as pH, K	extsuperscript{+}, and O	extsubscript{2}. However, the extension to any ion (cation or anion) selective PA optode would be trivial, as the only necessary change would be a different ionophore, which is optically silent, but with the same chromoionophore as used for the K	extsuperscript{+} nanosonophore. We also point out that there are numerous optical biosensors designed for numerous pathological biomarkers. Challenges still remain for direct PA biomarker imaging in vivo as most of the current biosensors are designed for ex vivo measurements of such biomarkers. We are optimized such that remaining challenges can be overcome, as the field continues to grow, noting that so far PACI has significantly improved on the in vivo imaging depth limit of conventional optical imaging and has made the possibility of universal in vivo chemical imaging a reality.

**ASSOCIATED CONTENT**

*Supporting Information*

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.analchem.8b04797.

Figure 4. General schematic of photoacoustic potassium nanosensor (K\textsuperscript{+} NS). The prepared colorimetric potassium sensor can serve both as a ratiometric PA imaging agent and a fluorescence imaging agent. Reproduced with permission from ref 23. Copyright 2017 American Chemical Society.
An example calculation of multiwavelength PA imaging for quantifying pH and $\Delta \phi \gamma$.

**AUTHOR INFORMATION**

**Corresponding Author**

*E-mail: kopelman@umich.edu.*

**ORCID**

Chang H. Lee: 0000-0002-4649-833X

Raoul Kopelman: 0000-0002-7770-8272

**Present Address**

1C.H.L.: Applied Science Research Institute, Korea Advanced Institute of Science and Technology (KAIST), 291 Daehak-ro, Yuseong-gu, Daejeon, 34141, Republic of Korea.

**Author Contributions**

$^\dagger$C.H.L. and J.F. contributed equally to this work.

**Notes**

The authors declare no competing financial interest.

**Biographies**

Chang H. Lee received a B.S. in Chemistry (2013) from Stony Brook University and a Ph.D. in Chemistry (2018) from University of Michigan, Ann Arbor, under the joint supervision of Prof. Raoul Kopelman and Prof. Xueding Wang, where his research focused on nanosensor design and synthesis for photoacoustic chemical imaging. He is currently appointed as a postdoctoral researcher at KAIST, South Korea, under the supervision of Prof. Chan Beum Park.

Jeff Folz is a Ph.D. student under the supervision of Prof. Raoul Kopelman at the University of Michigan. He received his undergraduate degrees at Miami University in Oxford, Ohio. His current work focuses on applying nanotechnology to cancer diagnostics.

Joel W. Y. Tan received his BASc in Engineering Science (2015) from the University of Toronto, and a M.S. in Biomedical Engineering (2017) from the University of Michigan, Ann Arbor. His research focus is on nanoprobe-enabled photoacoustic imaging for cancer biosensing. He is currently pursuing his Ph.D. under the supervision of Prof. Xueding Wang in the Department of Biomedical Engineering at the University of Michigan, Ann Arbor.

Janggun Jo received his B.S. and M.S. degrees in Electronic Engineering from KoreaTech (Korea). He received his Ph.D. degree in Bioengineering at the University of Kansas. Dr. Jo works as a postdoc at the University of Michigan currently. His research interests include photoacoustic imaging modality including optical and ultrasound imaging as well as high intensity focused ultrasound treatment.

Xueding Wang is currently an Associate Professor at the Department of Biomedical Engineering, University of Michigan, holding an Adjunct Associate Professor position in the Department of Radiology. Dr. Wang has extensive experience in imaging system development and adaptation of diagnostic technology to research and clinical managements. Sponsored by NIH, NSF, DoD, and other funding agencies, his research has led to over 100+ peer-reviewed publications. Dr. Wang is the recipient of the Sontag Foundation Fellow of the Arthritis National Research Foundation in 2005, Joint Research Fund for Overseas Chinese Scholars and Scholars in Hong Kong and Macao from National Science Foundation of China in 2011, and the Distinguished Investigator Award of the Academy of Radiology Research in 2013.

Raoul Kopelman obtained B.S. and Dipl. Eng. Degrees in Chemical Engineering from the Technion, Israel Institute of Technology, as well as an M.S. in Physical Chemistry. He received a Ph.D. in Chemistry from Columbia University in 1960. He then moved to Harvard for a postdoctoral position. After 2 years as an instructor at the Technion, Israel Institute of Technology, he moved in 1964 to the California Institute of Technology as a senior research fellow. In 1966 he joined the faculty of The University of Michigan Department of Chemistry. He is now The Richard Smalley Distinguished University of Michigan Professor of Chemistry, Physics, and Applied Physics and Professor of Biomedical Engineering, Biophysics, Chemical Biology, and Member of MNIMBS (Michigan Nanotechnology Institute for Medicine and the Biological Sciences) as well as of the Rogel Cancer Center of The Medical School of The University of Michigan. His research interests include nanoconstruct-based targeted imaging and therapy in vitro and in vivo, including optical, MRI, X-ray (CT), and photoacoustic imaging methods, with emphasis on functional/chemical imaging and photodynamic therapy of cancer and heart disease.

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