Review of Two Papers on Photoacoustic Dyes

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Introduction

Photoacoustic imaging (PI) uses a laser and ultrasound system to generate images of a target. Photoacoustic imagining is an attractive modality because it has favorable spatial and temporal resolution compared to existing imaging systems, while being non-invasive. The photoacoustic effect is highly dependent upon the optical properties of the imaging target, especially absorbance. This means that PI is well suited to observing not just physical structure, as with ultrasound, but specifically targeted molecules related to biological processes. Combined with PI's good space and time resolution, scanning for specific molecules can give real time feedback on processes inside the body. This attribute is extremely desirable for understanding the propagation of signals in the brain, in real time and at a size-scale close to a neuron, a feat that is challenging to this day.

Fluorescent compounds have long been used to observe cellular processes in the lab. However, in vivo use of fluorescent compounds requires the fluorescent signal to travel through tissue, and low signal magnitude and compound concentrations severally hampers the applications of pure fluorescent dye imaging. Photoacoustic imaging uses light from laser to induce an acoustic response from an illuminated target, turning the target into an acoustic point source. The acoustic signal can easily travel through tissue and be picked up by an ultrasound probe. The laser is capable of accurately penetrating and producing a response in the centimeter range, and careful choice of laser wavelength and output filtering can allow the response from specific compounds to dominate. Fluorescent dyes can be good compounds to use for photoacoustic imaging, since they have been used in biology for years and their absorption spectrums are known. Adapting fluorescent dyes to photoacoustic imaging, especially dyes that will produce an observable change in response to some biological quantity, is an important area of research. I will discuss two papers that investigate different fluorescent dyes in a photoacoustic imaging context.

Paper 1: Functional photoacoustic microscopy of pH (pH)

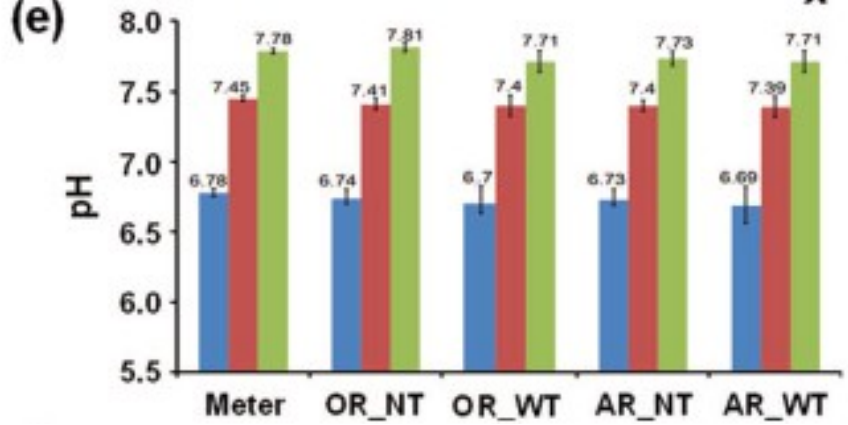
Muhammad Rameez Chatni, Junjie Yao, Amos Danielli, et. al in Journal of Biomedical Optics, 2011

Intro and Methods

This paper deals with the absolute detection of pH through tissue with two different photoacoustic imaging set-ups. A commercially available pH sensitive dye, SNARF-5F was chosen as the active photoacoustic indicator. The dye was mixed to the same dilution in 6.78, 7.45, and 7.80 pH buffer solutions (verified with pH meter).

Two different phantoms were constructed for the two different photoacoustic systems. For the optical resolution (OR-PAM) system, the dye samples were injected into silicone tubes. For the acoustic resolution (AR-PAM) system, the dye samples were placed in machined wells in an acrylic block. The laser systems both took rasterized readings of their respective phantoms, yielding a pixelated “image” of the acoustic response. The systems each had a single ultrasound transducer combined with lasers operating at two wavelengths: 581nm and 594 nm for OR-PAM, and 565 and 580 nm for the AR-PAM. The pixel readings for a dye sample region were averaged together, and a least-squares unmixing performed to retrieve the acoustic response for a dye sample, at the two wavelengths. The result for each imaging was a photoacoustic response at two wavelengths for each dye sample. For the OR-PAM system, results were collected with and without a ~200 micrometer thickness of mouse skin tissue separating the OR-PAM apparatus from the sample. For the AR-PAM system, results were collected with and without a ~2mm thick piece of chicken breast tissue separating the apparatus from

the sample.



Meter is the pH meter readings, OR_NT is OR-PAM no tissue, OR_WT is OR-PAM with tissue, likewise for AR-PAM and AR_NT/AR_WT. The three bars per label are the readings for each of the 3 buffered solutions. So the green bars are the pH readings for the 7.78 pH dye solution, reported by each method.

Results

The SNARF-5F dye dissociates into two species in solution. The relative proportion of each species is directly related to the pH of the solution, and importantly, each species has a different absorption spectrum. This allows the proportions of each species to be determined and, together, the exact pH value you can be identified. With and without tissue barriers, both methods produced mean pH measurements that agreed well with the pH meter. These results validate the ability of photoacoustic imaging to determine absolute pH through at least 2 mm of tissue. The introduction of the tissue versus no tissue resulted in a worse signal-to-noise ratio and standard deviation, but the mean value remained good.

Analysis

The paper claims to have been, at the time of publication, the first absolute value pH determination using photoacoustic imaging. Further, the imaging depths are greater than that attainable with fluorescence microscopy (.1 mm limit), while the process has many advantages over MRI. It also demonstrated the strength of the dual wavelength design in obtaining good measurements from absorption spectrum changes of the target.

Despite the good results obtained, there are some limitations to the methods used. All the phantoms involved multiple material transitions along both the light and acoustic transmission paths. For example, acoustic signals traveled through the wall of the silicone tube, through tissue, and through a water tank in the OR-PAM phantom. Each of these transitions presents an opportunity for signal attenuation. The excitation laser energy was also rather low, in the nano-joule as opposed to milli-joule range, which would likely limit tissue depth capabilities. Nevertheless, it is heartening that good results could be obtained despite these limitations, leaving only room for improvement.

In regards to our project, the most useful lessons are the viability of the pH dye signal, and of the dual-wavelength measurement setup. This confirms that searching for effective pH dyes is a worthwhile pursuit. Further, we now know we should give special attention to finding dyes with a pair of wavelengths to sample at, as opposed to relying on a single wavelength spectrum shift.

Paper 2: Design, Synthesis, and Imaging of an Activatable Photoacoustic Probe (probe)
Jelena Levi, Sri Rajasekhar Kothapalli, Te-Jen Ma, et. al in Journal of American Chemical Society, 2010

Intro and Methods

This paper deals with designing a photoacoustic dye molecule, called the probe, that will indicate the progress of a molecular process occurring in a cell when imaged. The chosen process was the cleavage of proteins by the matrix metalloprotease 2 (MMP-2) protease, which is known to be overexpressed in cancer cells. Activatable cell-penetrating peptide (ACPP) is a protein that is cleaved by MMP-2, and to which fluorescent dye molecules can be attached. The design of the probe focused on choosing a pair of suitable dye compounds to attach to ACPP that would provide distinguishable

singles for pre and post cleavage. Specifically, the probe was designed with the intent that when the probe protein was cleaved, a fragment with the stronger photoacoustic chromophore would remain in the cell, and the weaker chromophore would remain out of the cell. So the strong emitter would accumulate in cells, while the weaker emitter would be flushed away and diluted outside the cell.

The first step involved determining the best dye pairs to attach to the ACPP substrate. The five dyes in the table to the right were tested for photoacoustic response at two target wavelengths, 675 and 750 nm. The dyes were contained in plastic capillaries in agar gel.

Table 1. Manufacturer-Reported Values for Extinction Coefficients (ε), Fluorescence Quantum Yields (Φ), and Maximum Extinction/ Emission Wavelengths for the Chromophores Tested in This Study[28]

chromophore	$\lambda_{ex/em}$ (nm)	ε (mol/cm·g)	Φ (%)
BHQ3	672	42 700	
QXL680	679	110 000	
Cy5.5	675/695	250 000	0.23
Alexa750	749/775	290 000	0.12
Hilyte750	754/778	275 000	0.12

Second, BHQ3 and Alexa750 were paired with ACPP to produce one candidate probe molecule (B-APP-A), while QXL680 and Hilyte750 were paired with ACPP to produce a second candidate probe (Q-APP-H). BHQ3 and QXL680 had the strongest photoacoustic responses in the first step, and thus were chosen as the cell-accumulating component, while Alexa750 and Hilyte750 both had lower responses and were chosen as the flushed component. Absorbance spectra were taken using fluoroscopy, followed by photoacoustic response measurements as in the first step.

For the photoacoustic response measurements, the whole probe molecule and the cell-accumulating fragment were separately characterized. Plastic tubes in agar gels were used for the phantom, and rasterized measurements at 675 and 750 nm were taken. The goal was to find which cell-accumulating fragment could be best distinguished from the whole probe molecule.

Finally, in cell trials were performed with B-APP-A. HT1080 fibrous carcinoma cells were used as a test platform. Three sets of cells were incubated with a different dye each. One set was incubated with B-APP-A, one set with a variant B-PP-A molecule (B-APP-A missing a component from the ACPP backbone), and the third set with the cell-accumulating B-CP. The authors chose not to rely on what would likely be very low-rate cleavage of the probe by the cells, so cells directly exposed to the cleaved component were used to see the imaging response that should appear after cleavage. The cells were grown and then rinsed to remove dye no absorbed by the cells. The cells were cultured in agar and samples used to fill wells in an agar phantom. Photoacoustic response of this phantom at 675 and 750nm was taken.

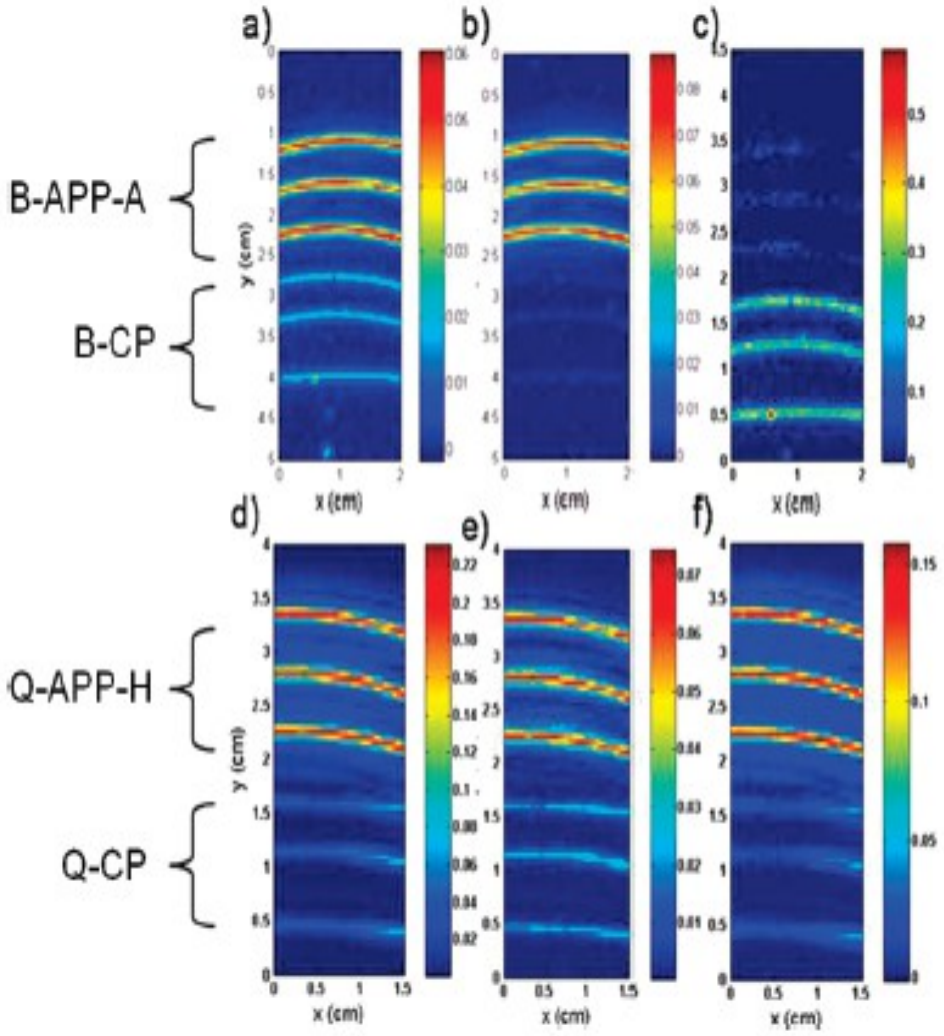
Results

Regarding the first step, the photoacoustic response of various commercially available dyes was found at two wavelengths. Importantly, the magnitude of the acoustic response was found to be not directly proportional to the absorbance of the dye. Absorbance is considered a good indicator of photoacoustic performance, as the absorption of the laser light is an integral part of acoustic response production. However, the dyes with the lowest absorbance values, in this case, produced the strongest response. The number of confounding variables involved in photoacoustic response, combined with these results, underscore the necessity of empirical determination of response.

BHQ3 and QXL680 were chosen as cell-accumulating probe components since of all the dyes they had the strongest responses. Their maximum absorption wavelengths are both near 675nm. Alexa750 and Hilyte750 both have max absorbances near 750nm and were chosen for companion components, having the additional benefit of creating a gap between the absorbance peaks.

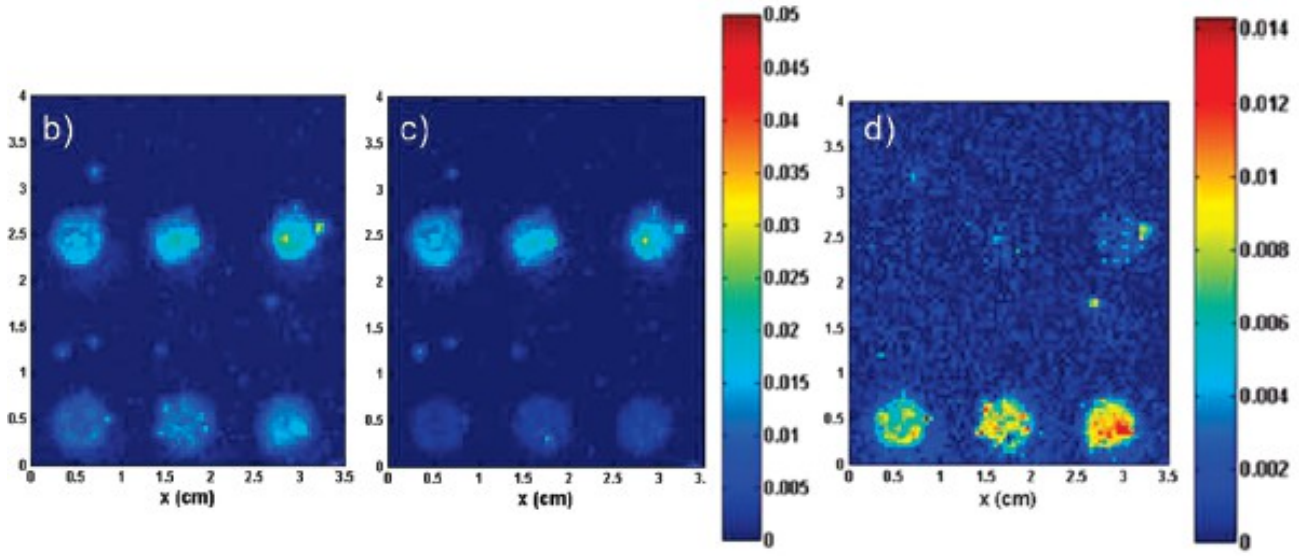
In the second step, evidence for dimerization affecting the acoustic response was found in the B-APP-A probe. Absorbance spectra were taken of the synthesized dye probe before and after cleavage of the molecule. At 675 nm, the absorbance decreased approximately .2 units, while at 750 nm the absorbance remained about the same. However, after cleavage, the photoacoustic response at 675 nm was larger than the cleaved response at 750 nm (though both responses were lower than the pre-cleavage response). This is in spite of the fact that the absorbance decreased at 675nm and remained the same at 750 nm.

B-APP-A was also found to have superior characteristics to the Q-APP-H probe. BHQ3 and QXL680 were both chosen as the cell-accumulating components, and the B-CP fragment was compared with the B-APP-A probe while the Q-CP fragment was compared with the Q-APP-H probe. B-CP showed a good signal response at 675nm and very little wavelength response at 750nm, whereas the Q-CP fragment had a lesser distinction between the two wavelengths. When the results at one wavelength were normalized and subtracted from each other wavelength's results, B-CP was an obvious indicator, while Q-CP was not.



Images of the phantom with the photoacoustic response superimposed as colorization,Observe how in the first column (675 nm) B-CP has a strong (more colored) output, whereas Q-CP has a weak output. Compare to the second column (750 nm) where B-CP has nearly no output, and Q-CP has moderately strong output. The third column shows the difference between the first two columns. A strong B-CP signal remains, while a weak Q-CP signal remains.

For the in cell test, HT1080 cells were allowed to uptake B-APP-A, B-PP-A, and B-CP. Since only the B-CP portion of the probe should enter the cell, it was predicted that B-APP-A would not uptake appreciably, whereas B-CP should uptake. B-PP-A was a modified probe designed to uptake regardless of cleavage. As predicted, the cells exposed to B-APP-A did not display any photoacoustic response, while the cells exposed to B-CP did produce a response. The B-PP-A cells also produced a response. However, when the results from the two wavelengths were subtracted from each other, the B-PP-A response disappeared, while the B-CP was clear. This represents the ability of B-CP signal to be completely isolated from uncleaved background signal.



Images of cell containing phantoms, photoacoustic signal superimposed as color, with the scale corresponding to magnitude. From left to right, the first block shows 675 nm response, with the upper row of colored circles the B-PP-A wells, and the lowest row B-CP wells. The second block is 750 nm results, and the final block is the signal from the first block minus the signal from the second block.

Analysis

This paper shows the strength of using custom designed and synthesized photoacoustic probe molecules to image a process occurring in the body. In fact, it serves as a sort of walkthrough of the process, through dye selection to techniques of differentiation. Again, the merit of a dual-wavelength based system is shown. Drawbacks of this study include the realism of the phantom, and the lack of output directly derived from cellular processes, as opposed to the simulation performed by the authors. In addition, a consideration of the time spectrum of the process would have a useful contribution.

The most direct contributions to our project is the technique of designing a custom probe, support for the dual-wavelength system, and finding that "the photoacoustic signal did not correlate with the absorbance and fluorescence of the molecules, as the highest photoacoustic signal arose from the least absorbing quenchers." In our tests so far, we have encountered some dyes that produced unexpectedly low outputs at certain wavelengths. The ability of other factors to appreciably alter the output from that expected via absorbance provides some possible explanation for what we have seen. Additionally, we have more reason to prioritize finding dyes that can work well with the dual-wavelength imaging. The technique of neutralizing the baseline, background signal with simple subtraction between wavelength readings is very interesting as an easy signal processing step providing further motivation to prioritize such dyes for dynamic imaging.