Photoacoustic imaging of live chicken embryo at multiple developmental stages

Arunima Sharma[a], Noreen Ishak[a,b], Teoh Swee-Hin[a], Christine Cheung[b,c], and Manojit Pramanik[a,*]

[a] Nanyang Technological University, School of Chemical and Biomedical Engineering, Singapore.
[b] Agency for Science, Technology and Research, Institute of Molecular and Cell Biology, Singapore
[c] Nanyang Technological University, Lee Kong Chian School of Medicine, Singapore.

ABSTRACT

Chicken embryo remains a commonly used model for biomedical research due to its ease of availability, accessibility for surgical manipulations, and biological similarities with humans. Although, it is being extensively used for cancer, cardiovascular, and developmental studies, non-destructive imaging of live embryo vasculature with high optical contrast remains a challenge. In this work, photoacoustic tomography of chicken embryos developing in bioengineered eggshell was performed. Chicken embryos at different developmental stages were irradiated with laser pulses at wavelengths 532 nm and 740 nm to acquire cross sectional images at various depths. We have shown a label free method for imaging of live chicken embryo while maintaining its integrity. We demonstrated that our method has the potential to be a powerful non-invasive imaging method for studying vasculature growth and development in chicken embryos.

Keywords: Photoacoustic imaging, photoacoustic tomography, photoacoustic computed tomography, chicken embryo development, vasculature imaging, bioengineered eggshell.

1. INTRODUCTION

Chicken embryo remains an attractive model for biomedical research due to its ease of availability and manipulation [1-4]. Records of studies related to chicken embryo can be found since circa 330 BC when its growth was studied by Aristotle. Since then it has been extensively studied, especially in developmental biology and cardiovascular studies [5-7]. Since 60% of genes in chicken have corresponding human genes, chicken embryos are also being employed to study impacts of various genetic modifications [8]. Although multiple methods for visualizing chicken embryos have been reported, most of them are invasive methods which destroy the integrity of embryo while imaging, thus hindering normal growth of embryo. To study the same embryo for longer duration, several non-invasive imaging techniques like high frequency ultrasound [9], 7T magnetic resonance imaging [10], and bioluminescence imaging [11] have been employed, however, these methods suffer from limitations like poor resolution, low contrast, high cost etc. A low-cost non-invasive imaging modality which can provide both high resolution and good optical contrast may be a suitable option for studying growth of chicken embryo.

Photoacoustic imaging (PAI) is an emerging imaging technology which has gained importance in the last two decades due to its advantages of high optical contrast and good acoustic resolution [12-17]. PAT is a hybrid imaging modality wherein a nanosecond laser pulse is used to irradiate an object. Absorption of light by the chromophores present in object leads to rise and fall in temperature, resulting in production of photoacoustic (PA) waves. These waves can be detected using one of more ultrasound transducer (UST) present at different angles around the sample. Multiple configurations of PAI systems have been reported for different applications [18-21]. For acquiring deep tissue cross sectional images, circular scanning photoacoustic tomography (PAT) in orthogonal mode is commonly used [22-24].

Multiple studies have been previously performed for imaging of chicken embryos using PAI [25-27]. However, these studies have either helped in viewing only the top chorio-allantoic membrane (CAM) of the embryo, or have explanted the embryo prior to imaging, resulting in loss of structural integrity or even death of embryo post imaging. It was

* manojit@ntu.edu.sg;

hypothesized that high attenuation of light and PA waves due to the presence of hard calcium eggshells makes it difficult to image the embryo. Therefore, culturing the embryo outside the calcium eggshell may help in acquiring whole body embryonic images. In this work, we have shown PAI of chicken embryos cultured in 3D bioengineered transparent eggshells. The embryos were irradiated with laser pulses of 532 nm and 740 nm, and the generated PA signal was collected using an ultrasound transducer rotating around the sample. We have thus shown a low-cost method of acquiring high resolution images of live chicken embryo at multiple developmental stages.

2. MATERIALS AND METHODS

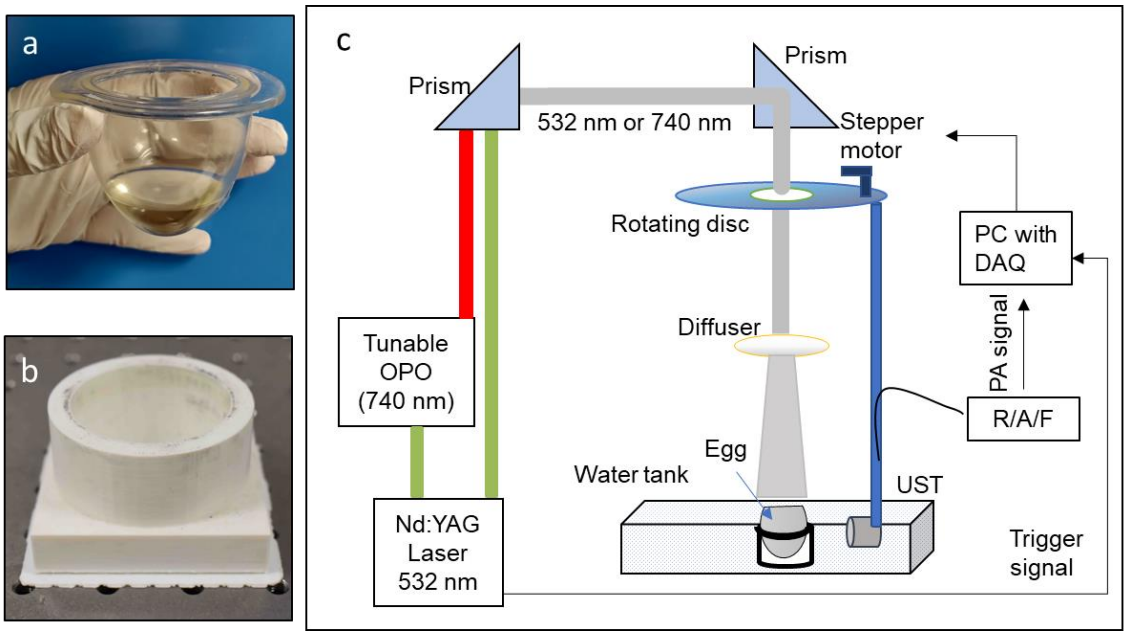

Figure 1. (a) Photograph of transparent bioengineered eggshell (b) Photograph of egg holder, and (c) Schematic of photoacoustic tomography system used in the experiment.

2.1 Bioengineered eggshell

A transparent eggshell (Fig. 1(a)) was prepared by mixing polydimethylsiloxane (PDMS) elastomer and a curing agent (Sylgard 184, Dow Corning, Midland MI) in the ratio 10:1 (wt/wt), using rotation molding [28]. The shape of this eggshell was similar to that of an oversized natural eggshell with some minor modifications. The modifications included removal of top curved portion and addition of excess PDMS at the bottom. These aided in making a wider opening to enable illumination of the entire embryo, and in increasing the weight of the embryo to prevent it from floating in water, respectively. The biocompatible constituents of the bioengineered eggshell along with its shape helped in providing a viable environment to the embryo while maintaining its structural integrity.

Figure 1(b) shows an egg holder made from acrylonitrile butadiene styrene filament using 3D printer (MakerBot® Replicator 2, USA) and designed using CAD software (SOLIDWORKS® 2016, USA). This holder helped in keeping the egg fixed and upright throughout the experiment.

2.2 Photoacoustic tomography system

Schematic for the circular photoacoustic tomography system used for acquiring cross sectional images of chicken embryo has been shown in Fig. 1(c) and has been described in detail previously [29]. Pulses of wavelength 532 nm having a 5 nm pulse width were generated using a Q-switched Nd:YAG laser (Continuum, Surelite Ex) at a pulse repetition rate of 10 Hz. Pumping these pulses to a tunable optical parametric oscillator (OPO) (Continuum, Surelite

OPO) laser generated 740 nm beam. The respective beams were deflected using prisms and mirrors and then passed through an optical diffuser to homogeneously illuminate the eggshell, placed inside the egg holder.

The egg holder was fixed to the surface of a water tank for better acoustic coupling of the PA signal. An unfocused single element ultrasound transducer (UST), having a central frequency of 5 MHz, a ~70% nominal bandwidth, and a 13 mm diameter active area was used to collect the generated PA signal. The UST was connected to a stepper motor (Lin Engineering, Silverpak 23C) which rotated continuously for 240 seconds and collected 2400 time resolved signals (A-lines) from different angles around the embryo [30]. Simple delay and sum algorithm was used to attain cross sectional images of the embryo by reconstructing these A-lines.

2.3 Phantom experiments

Prior to imaging the chicken embryos, experiments were conducted to validate that signal attenuation is due to presence of calcium eggshell and use of bioengineered eggshell can help in decreasing the attenuation. An absorbing target was irradiated with laser pulses of 532 nm wavelength and 10 A-lines were acquired. The optical or acoustic path was first blocked using natural eggshell and then using bioengineered eggshell and 10 A-lines were acquired for each set. The experiment was repeated by irradiating the sample with 740 nm beam instead of 532 nm. Peak to peak value of the PA signal acquired during each set were computed and compared.

2.4 Culturing of chicken embryos

Fertilized chicken eggs (Leghorn x Golden Comet) were incubated at 37.9ºC and 45% relative humidity to sustain their normal growth. For windowing of egg, a small portion on the wider end of the egg was removed above the airsac to make an opening called a window [31]. The embryo and its vasculature were then exposed by peeling off the inner membrane. For culturing chicken embryos in bioengineered eggshells, fertilized eggs were incubated for 55 hours before being perforated from the narrow end to extract albumin. After albumin extraction, window was made in the eggshell and the egg contents were slowly poured in a bioengineered eggshell. The extracted albumin was also added in the shell, which was sealed using a thin PDMS membrane and returned to the incubator.

3. RESULTS AND DISCUSSION

Figures 2(a-d) show the (Figs. 2(a, b)) averaged A-lines and (Figs. 2(c, d)) peak to peak values of absorbing target when irradiated with (Figs. 2(a, c)) 532 nm and (Figs. 2(b, d)) 740 nm beams. It can be seen in the figure that PA signal was hardly discernible when either the acoustic or the optical path was blocked using the calcium eggshell. This validated our hypothesis that the presence of natural eggshell results in high signal attenuation. A distinct PA signal could again be observed when the calcium eggshell was replaced with PDMS bioengineered eggshell.

To further analyze the effects of calcium eggshell, a fertilized chicken egg was imaged on its 6^{th} day of embryonic development (EDD-6). A windowed egg was imaged to permit irradiation of sample with laser pulses. The egg was placed in the egg holder and imaging was conducted in the manner described above. Images were acquired using both the illumination wavelengths. However, it was observed that no signal could be detected using either of the wavelengths. Figure 2(e) shows the photograph of windowed fertilized egg which was imaged during the experiment. The PA images acquired after illumination with 532 nm and 740 nm beams are shown in Fig. 2(f) and Fig. 2(g), respectively.

Following the phantom experiments, photoacoustic imaging was performed on chicken embryos cultured in bioengineered eggshells. Photographs and PA images of the chicken embryo acquired on 4^{th} day of embryonic development are shown in Fig. 3(a) and Figs. 3(b, c), respectively. After acquiring the PA images, the embryos were placed back in the incubator and allowed to grow further. Photographs and PA images of the chicken embryo acquired on 6^{th} day of embryonic development are shown in Fig. 3(d) and Figs. 3(e, f), respectively. On both the days, images were acquired after illuminating the embryos with both 532 nm and 740 nm laser beams. Figures 3(b, e) and Figs. 3(c, f) show images obtained after illumination at 532 nm and 740 nm, respectively.

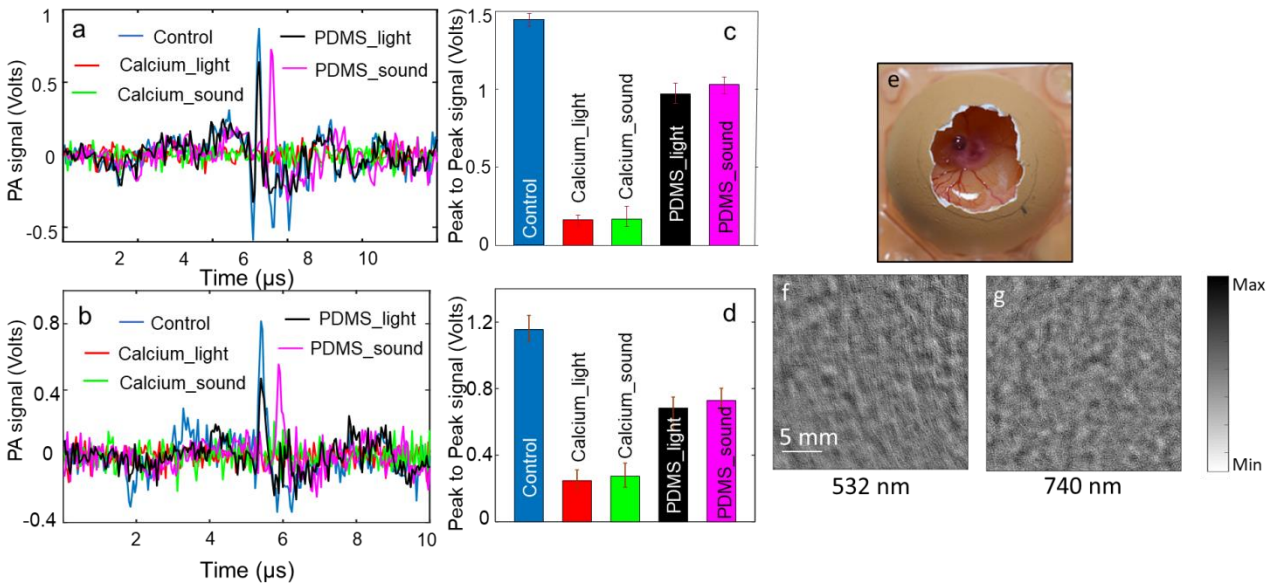

Figure 2. (a, b) Averaged A-line and (c, d) peak to peak PA signal from the absorbing target illuminated with (a, c) 532 nm beam and (b, d) 740 nm beam. Control denotes the signal acquired without any blockage. Blockage of optical and acoustic path with calcium eggshell generated Calcium_light and Calcium_sound, respectively, and with PDMS eggshell generated PDMS_light and PDMS_sound, respectively. (e) Photograph and (f, g) PA images of fertilized egg collected on its 6th day of embryonic development. Window shown in the eggshell was made before imaging. Scale bar for f and g is shown in f.

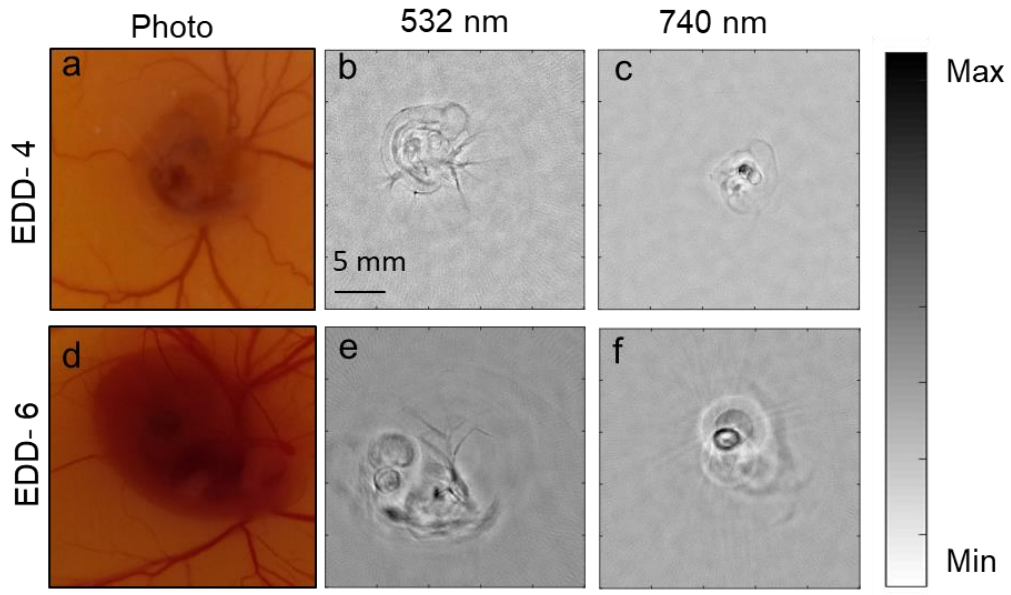

Figure 3. (a, d) Photos and (b, c, e, f) PA images of chicken embryo cultured in bioengineered eggshells on (a-c) 4th and (d-f) 6th day of embryonic development. PA images were collected after irradiation with (b, e) 532 nm and (c, f) 740 nm pulses. Scale bar is same as that shown in b.

The acquired PA images prove the capability of photoacoustic imaging for live imaging of chicken embryo. It was observed that high resolution imaging of embryonic vasculature is possible by irradiating the embryo with 532 nm laser pulses. Irradiation with 740 nm pulses can help in visualization of eyes, heart, and some big vessels. Due to low scattering of wavelengths in near infrared region, 740 nm has higher imaging depth than 532 nm [29]. Therefore, organs present deeper in yolk like eyes were more clearly visible in images acquired at 740 nm. However, in the experiments conducted, the diameter of 740 nm beam was smaller than that of 532 nm beam. This may be the reason why more vessels are visible in Figs 3(b, e) as compared to Figs. 3(c, f).

PA images acquired using this method gives images along a particular cross section. Cross sectional images can help in mapping the exact depth of the vessels, which may not be possible using camera photos. Further, this method can help in visualizing the embryonic structures and the vasculature which may not be visible to naked eyes due to presence of yolk above it. Moreover, it was observed that the shape and integrity of the chicken embryo was maintained during the process.

4. CONCLUSION

In this work, we have shown the potential of photoacoustic tomography for imaging live chicken embryos on multiple developmental days. Photoacoustic tomography of chicken embryo cultured in bioengineered eggshells can help in studying the growth of chicken embryo over time. Photoacoustic imaging of naturally growing chicken embryo was not possible due to high signal attenuation caused by the presence of calcium eggshell. Therefore, a bioengineered eggshell made of PDMS was used for culturing the embryo. This eggshell provided a viable environment for the growth of embryo. Signal attenuation due to bioengineered eggshell was much less than that due to the natural one. Photoacoustic imaging at 532 nm helped in visualizing the embryonic vasculature and at 740 nm helped in detecting growth in embryonic structures like eyes and heart. We have thus demonstrated a low cost, label free method for high resolution imaging of chicken embryo over time.

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