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High resolution, label-free photoacoustic imaging of live chicken embryo developing in bioengineered eggshell

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Abstract

Chicken embryos have been proven to be an attractive vertebrate model for biomedical research. They have helped in making significant contributions for advancements in various fields like developmental biology, cancer research, and cardiovascular studies. However, a non-invasive, label-free method of imaging live chicken embryo at high resolution still needs to be developed and optimized. In this work, we have shown the potential of photoacoustic tomography (PAT) for imaging live chicken embryos cultured in bioengineered eggshells. Laser pulses at wavelengths of 532 nm and 740 nm were used for attaining cross sectional images of chicken embryos at different developmental stages. Cross sections along different depths were imaged to gain knowledge of relative depth of different vessels and organs. Due to high optical absorption of vasculature and embryonic eye, images with good optical

contrast could be acquired using this method. We have thus reported a label-free method of performing cross sectional imaging of chicken embryos at high resolution demonstrating the capacity of PAT as a promising tool for avian embryo imaging.

1 INTRODUCTION

Chicken embryo has been a model organism for developmental biology for a very long time [1-3]. It is expansively studied in fields of developmental biology [4], cardiovascular studies [5], and angiogenesis [6, 7]. Research in chicken embryos has led to many fundamental breakthroughs in developmental and cancer biology including knowledge of formation and septation of heart, discovery of viral oncogenes, and identification of transcription factors [5, 8-10]. Experimental techniques for studying chicken embryo are well established and various methods have been reported for visualizing the embryo. Imaging the embryo through a window in the eggshell is a common method [11-13], but the small window size limits the view. This is overcome by transferring the embryo into a petri dish [14] or explanting [15] the embryo before imaging, which implies that the embryo needs to be sacrificed post imaging. *Ex ovo* cultures using weigh boats [16], polyurethane membrane hammocks [17], or surrogate shells [18] have been efficiently used for studying growth of embryo over time.

Recently, another *ex ovo* culture in the form of a transparent eggshell was developed using polydimethylsiloxane (PDMS) [19]. This culture system offers superiority over existing *ex ovo* culture systems as it provides transparency, sterility, reusability, and biocompatibility. In addition, chicken embryos cultured in this system remained alive for 19 days. Although the transparency of eggshell helps in visualizing the embryo and the vasculature, visibility of organs and vasculature within the embryo decreases over time as the embryo descends slightly into the yolk. This called for the need of an imaging technique which can help in

repeatedly observing an embryo cultured in bioengineered eggshell, throughout its incubation period, without making any incision and penetration in the embryo.

Several such non-invasive imaging modalities including magnetic resonance imaging (MRI), ultrasound, optical coherence tomography (OCT), micro computed tomography (CT) have been used for chicken embryo imaging. Studying vasculature growth is not possible using 7T MRI due to its poor resolution and low hemoglobin contrast [20]. Limited imaging depth of confocal microscopy [21] and OCT [22] makes it difficult to view the embryo at later stages of development. Other imaging techniques like bioluminescence imaging (BLI) [11], intravital imaging [16], laser speckle imaging (LSI) [23], and high frequency ultrasound imaging [13] are not widely used for vasculature imaging of chicken embryo as they either need external contrast agent to distinguish the embryonic tissues and organs, have poor imaging resolution, or need complicated image processing algorithms to enhance the image quality. Providing the advantages of high ultrasound resolution and good intrinsic optical contrast, photoacoustic imaging (PAI) seems like a promising choice for chicken embryo imaging.

PAI is a hybrid imaging modality based on photoacoustic (PA) effect, where absorption of optical energy by target objects (different chromophores in tissue) causes thermal expansion and generation of acoustic waves. In PAI, samples are irradiated with nanosecond laser pulses. The generated acoustic waves, in the form of ultrasound [also known as photoacoustic (PA) waves], are detected using an ultrasound transducer (UST) [24-27]. Based on system configuration, PAI can be broadly classified as photoacoustic tomography/photoacoustic computed tomography (PAT/PACT) and photoacoustic microscopy (PAM) [25, 28]. In a conventional PAM system, high resolution images are obtained using focused optical

illumination and ultrasound detection. This is in contrast to a PAT/PACT system in which the entire sample is illuminated homogeneously and single or multiple UST(s) are employed to detect the PA signal from different angles around the sample. Such alignment provides PAT the advantage of better imaging depth, but at the cost of a decrease in spatial resolution. Different configurations of PAI are used based on the desired application. The past two decades have seen a vast increase in pre-clinical and clinical applications of PAI like imaging of vasculature [29, 30], sentinel lymph node [31], brain [32, 33], breast [34, 35] etc. Photoacoustic imaging has been explored for chicken embryo imaging as well. PAM has been used for vasculature imaging of chicken embryo CAM, for flow speed measurement [36] and for studying the effects of angiogenesis inhibitors [37]. However, the imaging depth of PAM is limited hence PAT is normally used for whole body imaging [28]. Recently, Liu M. *et al.*, performed whole body imaging of explanted chicken embryos using dual modality OCT and PAT [15]. Although the images provided structural information of the embryo, image acquisition was done by explanting the embryos and placing them in paraformaldehyde solution on top of a polymer sensor. This made it impossible to image the same embryo over time.

In this work, we have performed dual wavelength photoacoustic tomography on chicken embryos. For the first time we have shown how PAT can be employed for imaging various cross-sections of chicken embryo while maintaining its shape and integrity. Initial experiments were performed on naturally growing embryos, wherein small window size and high attenuation of PA waves by the calcium shell prevented visualization of organs and blood vessels. Therefore, imaging was performed on chicken embryos cultured in bioengineered 3D transparent eggshell which highly mimics the natural eggshell environment. High hemoglobin absorption in visible spectrum of light (400-700 nm) helps achieve high-

contrast blood vessel imaging at these wavelengths. Further, in biological tissues, there is relatively decreased scattering and absorption of light of near-infrared (NIR) wavelengths (700-1200 nm). Therefore, greater imaging depth can be achieved with illumination in NIR region [38]. To achieve both high blood contrast and good imaging depth, PAT was performed at wavelengths of 532 nm and 740 nm. Intrinsic contrast from hemoglobin and eye of the embryo provided cross sectional images across different depths. Thus, we have shown a label-free method for imaging live chicken embryo cultured in bioengineered eggshells at different developmental stages.

2 MATERIALS AND METHODS

2.1 PAT System

A previously developed circular scan PAT system was used for acquiring cross sectional images of chicken embryo [38, 39]. Schematic of the system is shown in **Figure 1**. A Q-switched Nd:YAG laser (Continuum, Surelite Ex) delivering laser pulses at wavelengths of 532 nm and 1064 nm having a pulse width of 5 ns at a pulse repetition rate (PRR) of 10 Hz, was used for sample irradiation. For experiments at 532 nm, a mirror kept on a translational stage (TS-1) in front of Nd:YAG laser was used to divert the laser beam towards the sample. The 532 nm beam was instead pumped into a tunable optical parametric oscillator (OPO) (Continuum, Surelite OPO) laser to generate 740 nm radiation for sample illumination at 740 nm. The desired laser beam was then reflected using mirrors and right-angled prisms and then passed through an optical diffuser to homogeneously illuminate the embryo from the top.

Due to ease of availability, 532 nm is extensively used for photoacoustic imaging, therefore it was chosen for this work as well. For deeper penetration, wavelength in NIR-I region (700-900 nm) have been extensively used in the literature. In our work 740 nm was chosen for the

stability of the OPO laser and the higher laser energy output in this wavelength. Maximum fluence per pulse delivered at the surface of the sample was maintained at $\sim 6 \text{ mJ/cm}^2$ for 532 nm and $\sim 5.1 \text{ mJ/cm}^2$ for 740 nm wavelengths. For both the wavelengths, the fluence per pulse was well below the maximum permissible exposure limit, which is 20 mJ/cm^2 for 532 nm and 24 mJ/cm^2 at 740 nm for skin exposure [40].

PA signal generated from the sample was collected using an unfocused single element ultrasound transducer (UST) (Olympus NDT, V309-SU), having a central frequency of 5 MHz, a $\sim 70\%$ nominal bandwidth, and a 13 mm diameter active area. A stepper motor (Lin Engineering, Silverpak 23C) was used to rotate the UST continuously at a speed of $1.5^\circ/\text{sec}$ to collect time-resolved signals (A-lines) from different angles around the sample [41]. Both the sample and the UST were placed in a water tank to enable better coupling of PA signal by avoiding any air gap between the sample and the transducer. The water tank was kept on a laboratory scissor jack (lab jack) so that it could be easily moved in vertical direction. The acquired PA signal was amplified by $\sim 49 \text{ dB}$ and filtered using a pulse amplifier (Olympus-NDT, 5072PR). A data acquisition card (DAQ) (GaGe, compuscope 4227) inside a desktop computer was used to record the signal at a sampling rate of 25 MS/s . Data acquisition was synchronized with laser excitation using LabView program. PRR of 10 Hz and scan time of 240 seconds yielded a total of 2400 A-lines in one rotation. Once acquired, 10 consecutive A-lines were averaged into one A-line, thereby converting 2400 A-lines to 240 A-lines. These 240 A-lines were reconstructed using simple delay-and-sum reconstruction algorithm. This reconstruction provided the initial pressure rise distribution (which is related to absorption coefficient of the tissue) of the sample (tissue) in the imaging plane [42]. This system can provide a spatial resolution of $\sim 182 \mu\text{m}$ and an imaging depth of $\sim 3 \text{ cm}$ in biological tissues [39].

2.2 Sample preparation for imaging

Phantom experiments

Experiments were first conducted to study the effects of presence of natural eggshell, and of PDMS on detected PA signal. In these experiments, an absorbing target was irradiated with laser pulses. 20 PA signals (A-lines) from the target were acquired and averaged. Experiments were repeated 10 times. Following this control experiment, a piece of natural calcium eggshell was placed, first in optical path and later in acoustic path. In both the cases, PA signal was acquired in the same manner as that of control experiment. Later, calcium eggshell was replaced with a piece of PDMS eggshell, and PA signal was acquired in the same manner. Finally, PDMS eggshell was placed in both acoustic and optical path to collect the PA signal from the target. This experiment was repeated for both 532 nm and 740 nm wavelength excitation. Peak to peak PA signal was calculated from the PA signal A-line by subtracting the minimum value of PA signal from the maximum value of PA signal.

Bioengineered eggshell design

A transparent bioengineered eggshell was designed to imitate the geometry of an oversized natural eggshell with a flat top surface. The bioengineered eggshell was prepared from a mixture of PDMS elastomer and a curing agent (Sylgard 184, Dow Corning, Midland MI) in the ratio of 10:1 (wt/wt), using rotation molding at speed ratio of 3:1 via a biaxial bioreactor. A removable transparent lid covered the top surface of the eggshell to maintain sterility. Minor changes were made to the design of a previously reported bioengineered eggshell [19] to accommodate photoacoustic imaging. **Figure 2A** shows a photograph of the bioengineered eggshell used for our experiments. Top narrow portion of bioengineered eggshell was removed as its wider circumference (having a top diameter of 50 mm) aided in increasing the

area of illumination during photoacoustic imaging. Further, the eggshell was weighted down by 10 ml of PDMS added to the base to prevent it from floating in the water tank during imaging. An egg holder was designed with CAD software (SOLIDWORKS® 2016, USA) and 3D printed (MakerBot® Replicator 2, USA) to hold the eggshell stably in the water tank during imaging. **Figure 2B** demonstrates the placement of the eggshell into the holder.

Chicken embryo preparation

Fertilized chicken eggs (Leghorn x Golden Comet) incubated at 37.9°C and 45% relative humidity (Brinsea Octagon 20 Advance Digital Egg Incubator) were used for the experiments. PAI with excitation at 532 nm and 740 nm was conducted on both chicken embryos incubated in natural eggshell and bioengineered eggshell respectively, at 6 days of embryonic life.

In the *in ovo* embryo, PA images of the embryo were acquired at three time points: before eggshell windowing, following windowing above the airsac, and after removal of the inner membrane. For windowing, a small portion of eggshell on the wider end of the egg was carefully removed to make an opening. Subsequently, the inner membrane was meticulously peeled off exposing the embryo and its vasculature. PA signals collected at different time points were reconstructed to obtain images.

For the *ex ovo* experiments, fertilized eggs were transferred into the bioengineered eggshell for imaging following 55 hours of incubation at 37.9°C and 45% relative humidity in the same incubator as mentioned above. The transfer of fertilized egg into the bioengineered eggshell involved perforating the narrow end of the egg to withdraw 5 ml of albumin, removal of shell above the air sac on the wider end of the egg, pouring the egg content along with the previously extracted albumin into the bioengineered eggshell, and sealing the bioengineered

eggshell with its transparent lid (**Figure 2C**). The sealed bioengineered eggshell was placed into the incubator until it was required for imaging. To acquire PA images, chicken embryos at different developmental stages were placed in the water tank and imaged using the PAT system.

For all the experiments, water temperature in the tank was $\sim 38^{\circ}\text{C}$ to provide a viable environment for the embryo. The embryos were immediately replaced in the incubator when they were not being imaged. Abrupt movements were minimized to maintain the viability of the embryos.

3 RESULTS AND DISCUSSION

Figure 3 shows (**Figures 3 A-B**) PA signal A-line profiles, and (**Figures 3C-D**) the peak-to-peak PA signal amplitudes from the target. It was observed that no PA signal could be detected/observed when the natural eggshell was placed in either the optical or the acoustic path for both the wavelengths. The peak-to-peak PA signal amplitude in all these cases were less than 5% of the control peak-to-peak PA signal amplitudes (noise level). This is expected, as neither light nor sound can pass through the hard calcium natural eggshell. With PDMS, the PA signal attenuation was observed, but not significantly. Attenuation by $\sim 30\%$ and $\sim 35\%$ was observed on irradiation with 532 nm beam and 740 nm beam, respectively, due to the presence of PDMS eggshell in both the optical and acoustic paths together. Attenuation was quite low when PDMS eggshell blocked either the optical path or the acoustic path. It should also be noted that a slight delay in PA signal was observed when the PDMS shell was placed in path of the acoustic waves. This delay is due to difference in sound speed in the two mediums. Reconstruction algorithms are designed such that this delay does not alter the final reconstructed image.

Following phantom experiments, imaging was performed on chicken embryos to further validate the effects of presence of different eggshells. **Figure 4** shows (**Figures 4A-E**) photographs and (**Figures 4F-O**) photoacoustic images of chicken embryos on their 6th embryonic day of development (EDD). PA images were collected by irradiating the embryos at (**Figures 4F-J**) 532 nm and (**Figures 4K-O**) 740 nm. Initially, PA images of embryo growing naturally were acquired from (**Figures 4F,K**) intact eggshell, (**Figures 4G,L**) windowed eggshell, and (**Figures 4H,M**) eggshell with inner membrane also removed. No distinct features of the embryo were visible in the acquired PA images at both wavelengths. This is due to obstruction (and lack of generation) of PA waves because of the presence of calcium eggshell (as shown earlier).

Figures 4D,E show photographs of chicken embryos developing in bioengineered eggshells with (**Figure 4D**) lid intact and (**Figure 4E**) lid removed. **Figures 4I,N** and **Figures 4J,O** are corresponding PA images of **Figures 4D** and **4E**, respectively. Analogous to phantom results, attenuation of PA signal due to bioengineered eggshell was significantly less than the natural eggshell. No substantial difference was seen in PA images acquired with and without presence of the lid covering the bioengineered eggshell. However, when bioengineered eggshells covered with lids were placed outside the incubator for longer than 10 minutes, condensation on the internal side of the lid decreased the contrast of acquired PA images (not shown). To minimize condensation, the total scan time was kept within four minutes. In **Figure 4**, considerable differences in embryo structures were present between images collected at 532 nm and 740 nm. While acquiring PA images, it was necessary that the laser beam illuminated the embryo. However, there was a slight change in alignment of laser beam on changing the wavelength. Therefore, the position of eggshell was changed to ensure that

the beam illuminated the embryo. The change in position of eggshell led to change in embryonic position in PA images for different illumination wavelengths. Moreover, the dissimilarity in beam diameter, penetration depth, and absorbance of chromophores at different wavelengths resulted in variation of embryonic features visible at different illumination wavelengths. To study this variation further, embryos in different developmental stages were imaged at both the wavelengths separately.

Figures 5 and 6 show PA images of chicken embryo collected at 532 nm and 740 nm, respectively. Embryos growing in bioengineered eggshells were imaged from EDD-4 to EDD-7 after illumination at 532 nm. **Figures 5A-D** show the top view photos of chicken embryos developing in the bioengineered eggshell on different days of development. **Multiple embryos were imaged during the study. Figures 5A and 5C show images of the same embryo on different developmental days, while the rest are of different embryos.** Magnified view of the selected region in **Figures 5A-D** (black dotted square) is shown in **Figures 5E-H**, respectively. **Figures 5I-L** show the corresponding PA images acquired when the 532 nm beam was aligned to irradiate the area shown in **Figures 5E-H**. Numbers marked in **Figures 5E-H** show the corresponding regions detected in **Figures 5I-L**. Due to high absorbance of blood at 532 nm, superficial vasculature was clearly visible in the acquired PA images, however, it was difficult to clearly discern the embryonic structures owing to the limited penetration depth of 532 nm [38].

The photographs of the vasculature in **Figure 5** vary from the corresponding PA images due to difference in nature of the acquired image. Photographs show the top view of the egg, while the PA images reveal the chromophores present at a particular cross section. Cross sectional imaging can help in attaining three-dimensional information of the embryo

development, which is difficult to obtain in normal photographs. Dark regions visible in **Figure 5H** show the embryonic structure which is not visible in corresponding PA image (**Figure 5L**). This is due to the difference in depth at which the embryo was present and at which the PA image was collected. Similarly, some vessels (marked by arrows 3 and 4 in **Figures 5H and 5L**) were more clearly visible in the PA images than in the photographs. This is because they were present along the cross section which was imaged. Moreover, some vessels which were slightly covered by the yolk and not directly visible to the eye, could be seen in the PA images due to the higher absorption coefficient of blood at 532 nm as compared to that of yolk. Although this wavelength greatly aided in studying the development of superficial blood vessels of the embryo, a higher penetration depth was required to study the complete embryonic development.

For imaging at higher penetration depth, embryos developing in bioengineered eggshells were imaged at 740 nm. **Figure 6** shows top view photos (**Figures 6A-C**), magnified photos (**Figures 6D-F**), and photoacoustic images (**Figures 6G-I**) of chicken embryos at day 3 (**Figures 6A,D,G**), day 4 (**Figures 6B,E,H**), and day 6 (**Figures 6C,F,I**) of development, respectively. In the images it can be observed that fewer blood vessels were visible at this wavelength, as compared to 532 nm. This may be due to both decreased hemoglobin absorbance as well as a reduced spot size of beam at this wavelength. However, a higher penetration depth of 740 nm compared to 532 nm increased the visibility of embryonic structures, particularly of the eyes, in the acquired PA images. Arrows marked 1, 2, and 3 in **Figures 6G-J** show the top eye, bottom eye, and the heart of the embryo, respectively. The PA images clearly showed gradual development of the eyes over time. Our observations showed that the eyes were barely visible on day 3 however their contrast greatly increased on days 4 and 6. This was expected as development of eyes generally takes place after day 3 [10].

Images of different cross sections were collected by moving the water tank in vertical directions with the help of the lab jack. **Figures 6J(i-vi)** show images of six different cross sections acquired on EDD-6. Precise distance between different cross sections could not be calculated with the set-up used in this work. **Imaging of multiple cross-sections on EDD-3 and EDD-4 (data not shown) for an extended period of time probably affected the survivability of the embryo due to its removal from the ideal incubator conditions. Consequently, a different embryo was chosen for imaging on EDD-6. Decreasing the scan time by using circular array transducers can help in acquiring full body 3-D images of embryo in shorter time duration.**

Similar to **Figure 5**, many features of the embryo which were not visible in the photographs could easily be seen in the PA images. Only one eye was distinguishable in the photograph taken from a digital camera, while both eyes could be easily discerned in the PA images. Precisely measuring the displacement of water tank in vertical direction can help in accurately finding the relative depth of different vessels and organs. Further, decreasing the field of view of UST by using a cylindrically focused UST having a narrow profile of detection in the depth direction (vertical direction), can aid in mapping the embryonic structures in 3D. Taking these steps can aid in comparing the embryonic development on different days to quantitatively measure the dimension changes in blood vessels and organs.

Acquired images in **Figures 5 and 6** demonstrated the capability of photoacoustic imaging to systematically study the growth of chicken embryos. Use of non-ionizing radiations in PAT minimalizes the exposure of chicken embryo to harmful ionizing radiations (X-rays used in CT or gamma rays used in PET/SPECT) during imaging. Moreover, the fast acquisition time prevents the need of keeping the embryo outside the incubator for long. These factors aid in maintaining an ideal environment for normal growth of chicken embryo. **The laser radiation**

did not seem to have harmful effects on embryonic development. However, further investigation on a larger sample size may be required to confirm this finding, and to optimize the conditions for PA imaging. Since chicken embryos are vastly studied in developmental and cancer biology, PAT imaging of chicken embryos in bioengineered eggshells can be used for various applications i.e. to study angiogenesis, effects of genetic manipulations, tumor models, vascular perfusions etc. Different structural information can be attained by performing PAT at different wavelengths. Additionally, functional imaging can be performed by using multiple wavelengths. Wavelengths beyond 900 nm (e.g., 1064 nm is easily available) can also be employed to increase the penetration (imaging) depth. These wavelengths were not chosen for this work as absorption coefficient of blood is low in these wavelengths. Use of contrast agent with 1064 nm excitation is another possibility for embryo imaging, which can be explored in the future [43]. Different organs or structures of the developing embryo can be targeted using such external contrast agents [44]. Employing UST with higher central frequency can improve the spatial resolution of the system, thus enabling visualization of smaller vasculature [45]. Furthermore, decreasing the scanning time using multiple ultrasound transducers [33], or lasers with high repetition rate [39, 46] can help in performing real time imaging.

In summary, we have shown the potential of photoacoustic tomography for imaging chicken embryos growing in bioengineered eggshells. High intrinsic contrast was achieved by using wavelengths in visible and near infrared-I range. Imaging at 532 nm aided in detecting vascular structure of the developing embryo while the higher penetration depth of 740 nm helped in imaging the embryonic structures (e.g., eyes and heart) which had sunk inside the yolk. We have thus reported an application of PAT for imaging the same embryo for consecutive days. This can help in studying embryonic development at different stages.

4 CONCLUSION

In this work, we have shown a label-free and high-resolution method of imaging live chicken embryo. Chicken embryos developing in bioengineered eggshells were imaged using photoacoustic tomography at 532 nm and 740 nm at different developmental stages. Due to high hemoglobin contrast, vasculature development was clearly discernible at 532 nm. Along with the vasculature, the eyes and the heart were visible at 740 nm. Furthermore, cross sectional imaging helped in identifying the depths at which different organs and vasculatures were present. By performing PAT on the same chicken embryo developing in bioengineered eggshell, its development over time can be easily monitored. Moreover, using this method, effects of various drugs, genetic modifications, or chemicals on embryonic development can also be examined. Therefore, we believe photoacoustic tomography can be a promising option for imaging the development of chicken embryos over time.

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Author Biographies

Please see Supporting Information online

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Figure 1. Schematic of photoacoustic tomography system used for imaging of chicken embryo. M1-M4: Mirror; P1-P3: Right-angled prism; OD: optical diffuser; R/A/F: Receiver/Amplifier/Filter; PA: photoacoustic; TS1, TS2: Translation stage; WT: Water tank; UST: Ultrasound transducer; SM: Stepper Motor; RD: rotating disc; DAQ: Data acquisition card; LJ: Lab jack.

Figure 2. Photographs of (A) bioengineered eggshell and (B) eggshell fixed inside the holder. (C) Schematic of process used to transfer the fertilized chicken embryo inside the bioengineered eggshell.

Figure 3. (A, B) Time resolved photoacoustic signal and (C, D) peak to peak photoacoustic signal from the absorbing target with (A, C) 532 nm illumination and (B, D) 740 nm illumination. Ca_light and Ca_sound denote obstruction of optical and acoustic path respectively using natural calcium shell. Similarly, PDMS_light, PDMS_sound, PDMS_both denote obstruction of optical path, acoustic path, and both paths, respectively, using PDMS eggshell.

Figure 4. Photos of chicken embryo in (A) natural eggshell, (B) natural eggshell with shell removed from top, (C) natural eggshell with shell and outer membrane removed from top, (D) bioengineered eggshell, and (E) bioengineered eggshell with lid removed. Photoacoustic image of A-E at 532 nm and 740 nm are given in F-J and K-O, respectively. Scale bar for F-O is shown in O.

Figure 5. (A-D) Photos of chicken embryo developing in bioengineered eggshell on embryo development (A) day 4, (B) day 5, (C) day 6, and (D) day 7. (E-H) Magnified images (area shown by dashed lines) and (I-L) photoacoustic images of A-D, respectively. PA images were collected after illumination at 532 nm wavelength. Arrows and numbers in E-H show corresponding structures in I-L, respectively. Scale bar for A-D and E-H are shown in A and E, respectively.

Figure 6. (A-C) Photos of chicken embryo growing in bioengineered eggshell on (A) day-3, (B) day-4, and (C) day-6 after incubation. Dotted area in A-C is shown in D-F, respectively. G, H, and I are photoacoustic images of A, B, and C, respectively, collected after illumination with laser pulses at 740 nm wavelength. (J) Photoacoustic images of C at different cross sections. Scale bar for G-I is shown in G.

Graphical Abstract

Photoacoustic (PA) tomography was performed on live chicken embryos cultured in bioengineered eggshells. Embryos at different developmental stages were imaged at 532 nm and 740 nm wavelengths. Cross sectional images along different depths were obtained to gain knowledge of relative depth of different vessels and organs. Intrinsic contrast of vasculature and embryonic eye helped in acquiring images with high optical contrast. Thus, a high-resolution, label-free, non-invasive PA imaging of live chicken embryos can become a promising tool for avian embryo imaging.