Noninvasive vasculature detection using laser speckle imaging in avian embryos through intact egg in early incubation stage

Lin Yang,1,3 Sixian You,1,3 Liangkai Zhang,1 Tixiong Yang,2 Pengcheng Li,1 and Jinling Lu1,*

1Britton Chance Center for Biomedical Photonics, Wuhan National Laboratory for Optoelectronics—Huazhong University of Science and Technology, Wuhan 430074, China
2Zhong Nan Hospital, Wuhan 430000, China
These authors contributed equally
*lujinling@mail.hust.edu.cn

Abstract: Monitoring the vital signs of a developing embryo is very useful in avian breeding programs, especially during early days of incubation, so that dead or unfertilized eggs can be timely removed from incubator and new eggs can be placed in. A noninvasive system for detecting the vital signs of avian embryo through intact egg in early stage of incubation has been developed using laser speckle imaging (LSI). The system was based on the measurement of intensity fluctuations of speckle caused by the embryo’s blood flow in the intact egg under laser light illumination. This system was found to be feasible in imaging the vasculature in the egg as well as confirming its fertilization or survival from the second day to fifth day of incubation while other reported noninvasive methods cannot detect vital signs of the embryo until the sixth day of incubation.

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1. Introduction
Both researches and industry programs involving avian breeding often require artificial incubation of eggs. Confirmation of fertilization and survival of eggs at early stage are of practical importance in order to make full advantage of the resources used for incubation. Candling is often the method of choice for monitoring egg fertility and early embryonic development [1]. However, during the early days of incubation, the movement of the embryo is relatively weak, and candling does not allow precise evaluation of the embryo's status.

Invasive and noninvasive methods of detecting the vital signs of chicken embryos have been developed. Tazawa et al. reported that heart rate of chicken embryos can be recorded from the 12th to 18th day of incubation using invasive electrocardiogram [2]. The detection period was moved forward to the 3rd through 9th day of incubation by Akiyama et al. using semi-invasive impedance cardiography [3]. However, methods of monitoring the vital signs of embryos should be low risk and easy to perform. Attributable to the involvement of delicate and high-risk procedures, such as needle penetration of the eggshell, these invasive and semi-invasive methods are not suitable for routine embryo detection. Laser speckle phenomenon has been used to noninvasively measure the heart rate of chicken embryos from the 13th to 19th day of incubation by detecting movements of the eggshell caused by cardiac contractions of the embryo inside the egg [4]. Another noninvasive method has been proposed to monitor the vital signs of chicken embryos from the 6th day of incubation until hatching by measuring the amount of infrared light absorbed by embryonic blood [1]. Despite all these efforts, one often-underappreciated problem remains: effective detection of embryo development during the first quarter of incubation period is rarely achieved using noninvasive methods.

In this paper, a new method based on laser speckle imaging (LSI) has been developed to noninvasively detect early vital signs of avian embryos by recording intensity fluctuations of speckles attributable to the embryonic blood flow under laser light illumination. Over recent decades, LSI has become a powerful tool to investigate the spatio-temporal changes in blood flow under physiological and pathological conditions in biomedical applications [5,6]. LSI has advantages over other imaging methods such as Laser Doppler Perfusion Imaging because LSI perform full-field imaging without scan, which results in short measurement time. It is also attractive because non-cooled CCD camera is sufficient for this technique, which makes it relatively inexpensive while maintaining excellent spatial and temporal resolution.

Based on LSI, we are able to present the vascular structure of the embryo in the intact egg on the 2nd through 5th day of incubation. Information about whether the eggs survive can also be acquired based on the blood flow map of the eggs. Considering the simplicity, cost and the efficiency of this technique, LSI is a proper method for routine embryo’s assessment in both research and industry.

2. Materials and methods
2.1. Imaging setup
The schematic setup for the laser speckle imaging system is shown in Fig. 1. The egg is fixed by two rubber rings and illuminated by a laser beam from laser diode (780 nm, 50 mW) at horizontal incidence. 500 frames of laser speckle images were acquired by a 12-bit charge-couple device (CCD) camera (PixelFly QE, PCO Computer, Germany; pixel size = 6.45 × 6.45 μm) through an optical imaging system placed right above the egg. The CCD exposure duration is 20 ms and the frame interval time is approximately 55 ms. The raw data was
processed by the computer subsequently based on laser speckle contrast analysis. The whole setup was placed on a vibration-isolator table (VH3036W, Newport, USA).

![Diagram of laser speckle imaging system]

Fig. 1. Setup of the laser speckle imaging system.

2.2. Algorithms for data processing of LSI

Laser speckle imaging techniques are based on the measurement of light intensity fluctuations of speckle pattern generated by motion in the sample recorded with a CCD camera. Firstly, local speckle contrast was calculated based on the temporal speckle contrast analysis. The speckle temporal contrast image was constructed by calculating the speckle temporal contrast of each image pixel in the time sequence [7]. The value of the speckle temporal contrast $C$ at pixel $(x, y)$ was calculated as

$$C = \frac{\sigma}{T} = \frac{\sqrt{\frac{\sum_{i=1}^{N}(I_i - \overline{I})^2}{N-1}}}{T} = \frac{\sqrt{\frac{N\sum_{i=1}^{N}I_i^2 - (\sum_{i=1}^{N}I_i)^2}{N(N-1)}}}{\sum_{i=1}^{N}I_i/N},$$

where $I_i$ is the light intensity of the $i$th laser speckle image, $\sigma$ is the standard deviation of intensity, $i$ is the index of image in the sequence, and $N$ is equal to the total number of images recorded. Then, the speckle contrast has to be converted to relative velocity value. As the mean velocity of blood flow is proportional to the camera’s exposure time $T$ divided by correlation time $\tau_c$, the relationship between local temporal contrast and $T/\tau_c$ is defined as [8]

$$C = \sqrt{\exp(-2x) - 1 + 2x}/2x^2, \quad x = T/\tau_c.$$  

Cheng et al. proposed a simplified LSI analysis method [9]. When the correlation time is much smaller than the camera’s exposure time, $T/\tau_c$ is considered to be approximated to $1/C^2$.

2.3. Sample preparation

Thirty fertilized chicken eggs were incubated at 37.8 ± 0.1°C and 75% humidity for 21 days. The eggs were imaged using LSI technique every 24 hours to confirm its survival. For weakening the vital signs of embryo, ten chicken eggs on the second day of incubation were taken from the incubator (37.8 ± 0.1°C) and cooled for a period of 70 minutes at 5°C in the refrigerator, during which the embryonic structure in the intact eggs was imaged every 10 minutes.
3. Results

3.1. Imaging of chicken egg on the third day of incubation

To validate the application of laser speckle imaging on intact egg, we compared the blood flow map of chicken embryo obtained using LSI with other methods. Figure 2 presents the images of the same egg which were obtained by different methods on the 3rd day of incubation. Figure 2a shows the vasculature of the embryo obtained through intact egg using LSI. The vasculature of the embryo shown in the blood flow map is similar as that obtained through imaging the internal structure of the egg without shell under white light illumination(Fig. 2b), which validates the feasibility of laser speckle contrast analysis on noninvasively detecting the vital signs of chicken embryos. The raw laser speckle image of the egg (Fig. 2c) shows that the vasculature of the chicken embryo could not be acquired directly under laser illumination without the data processing of laser speckle contrast analysis. It could be observed in Fig. 2d that candling is not able to detect the vital signs of the embryo through the intact egg on the third day of incubation either. These comparisons demonstrate that LSI can be used to map the vasculature of the embryo through intact egg.

![Fig. 2. Images of the same egg on the third day of incubation using different methods. (a) LSI blood flow maps of the embryo obtained through intact egg. (b) Color image of the same egg after removing its shell. (c) Raw laser speckle image before removing the shell. (d) Color image obtained through intact egg under white light illumination.](image)

3.2. Imaging the development of chicken embryo in intact eggs at early stage

Long-term observation of chicken embryos was performed to monitor the embryonic development of chicken egg. The eggs were imaged every day at 8 o’clock pm. Figure 3 shows a typical spatio-temporal change of blood flow during the embryonic development of a chicken egg at early stage.

![Fig. 3. LSI blood flow maps of chicken embryo in an intact egg on different days during the early embryonic stage: (a) on the first day of incubation, (b) on the second day of incubation, (c) on the third day of incubation, (d) on the fourth day of incubation.](image)

The red spot in the image of chicken embryo on the second day (Fig. 3b) can be used to distinguish fertilized (living) eggs from unfertilized (or dead) ones as it indicates the movements of blood cells surrounding the red spot. It can be observed that feeding vessels are expanding from the location of the heart in the egg day by day (Figs. 3b–3d). Therefore, with LSI technique, confirmation of fertilization and survival of chicken embryos can be easily...
achieved on the second day of incubation, which is of practical importance for automatically egg screening during artificial incubation.

3.3. Imaging of cooled chicken egg on the second day

To demonstrate the feasibility of LSI on detecting the survival of eggs, LSI was performed to access the vital signs during the cooling of the egg in refrigerator for certain time. The longer time one egg is placed in low-temperature environment, the weaker its vital signs become. The eggs were imaged every 10 minutes after being placed in the refrigerator at 5 °C. As shown in Fig. 4, the contrast between the blood flow map of normal developing embryo and that of embryo cooled for different lengths of time reveals the relation between the blood flow and the temperature of egg. The red regions indicate higher blood flow velocity, which is the consequence of movements of blood cells in the red regions. The faster the blood in vessels flows, the redder it appears in the blood flow map. The weakening of vital signs of the embryo presented in the blood flow map demonstrates that it is the motion inside the living egg, rather than other factors such as the absorption of infrared light, that is responsible for red regions in the LSI map. This trial further demonstrates the reliability of LSI method of detecting the vital signs of avian embryo within the intact egg.

![Fig. 4. LSI blood flow maps of embryo within the egg on the 2nd day of incubation during cooling process at 5°C: (a) imaged when the egg was normally incubated, (b) imaged when the egg has been cooled for 50 min, (c) imaged when the egg has been cooled for 60 min, (d) imaged when the egg has been cooled for 70 min.](image)

4. Discussion and conclusion

In the current study, a noninvasive method was developed for detecting the vital signs of avian embryo through intact egg in early stage of incubation based on laser speckle imaging. It shows that the vasculature of a developing embryo can be noninvasively detected as early as on the second day of incubation, and the vital signs of embryos can be distinguished easily by the blood flow map.

Chicken embryo is a common model system of embryo developing, vitelline circulation and tumor perfusion. Recently, several imaging-based blood flow measurement techniques have been developed to measure intracardiac blood flow in the chick embryonic heart [10–12]. Particle image velocimetry (PIV) provides quantitative information about blood flow by tracing the exogenously added bio-inert, fluorescent particles [10]. Liu et al. presented a method that combined OCT imaging and finite element modeling to quantify the in vivo blood flow dynamics and wall shear stresses in the cardiac outflow tract of early chicken embryos [11]. These methods provide the temporal dynamic and spatial distribution of blood flow, benefiting applications ranging from developmental biology to tumor perfusion studies. However, because of the involvement of the invasive procedures such as injecting exogenous tracer or removing the egg shell, these imaging methods are not suitable for routine embryo detection of egg incubation.

The LSI-based imaging approach proposed in the present study offers a new angle for detection of vital signs of avian embryos. Through the intact egg and without any contact procedure, the blood flow map of developing embryo was obtained through LSI and temporal speckle contrast analysis, which not only presents the vasculature of avian embryos but also
indicates whether the embryos survive. When the fertile egg was cooled down at 5 °C, the blood flow velocity of chick embryo decreased significantly with the influence of low temperature, as shown in Fig. 4. Similar results can be found in the work of Lierz et al., who observed a decrease in the chick’s heart rate in the cooling process by measuring the amount of infrared light absorbed by embryonic blood [1]. These results further suggest that the blood flow map can distinguish the status of the embryos, confirming the reliability of LSI method of detecting the vital signs of avian embryo within the intact egg at early stage.

Another imaging technique, micro-magnetic resonance imaging (µMRI), is a good method for studying changes in the three-dimensional (3D) internal anatomy of the optically opaque avian eggs. It provided excellent anatomical details and MRI atlas of avian development in fixed avian embryos or live embryos in ovo [13,14]. Duce et al. presented 3D structure information of quail eggs obtained at 24-h intervals from Day 0 to Day 8, and followed the embryonic development and quantify volumetric changes in the embryo by µMRI in high static 7 T magnetic field [14]. Although µMRI can provide excellent structure information of intact eggs during early stage of incubation, it failed in judging the physiological state of embryo in one imaging trial. Furthermore, considering the expensive MRI instrument and time-consuming data acquirement and analysis, it is not suitable for routine embryo detection in industry either. LSI performs full-field imaging without scan results in short measurement time (within 30 sec for 500 frames) and it is relatively inexpensive as non-cooled CCD camera is sufficient for this technique while maintaining acceptable spatial and temporal resolution. The vital signs of embryos can be feasibly assessed by the blood flow map with enough spatial and temporal resolution. With the advantage of the simplicity and efficiency of this technique, LSI can be a proper method for routine embryo’s assessment.

For the safety of laser power applied for embryos, the laser power density is 10 mW/cm$^2$ on the shell of egg and the exposure time is 30 s for each detection so that the dose is 0.3 J/cm$^2$. No significant difference was observed on survivability and incubation duration between the laser irradiated and the control group eggs in our condition. For the detailed evaluation of biological effects of photon irradiation on chicken embryos, Yeager et al. investigated the effects of 670 nm phototherapy on the survival and hatching success of chickens (Gallus gallus), in which fertile chicken eggs were treated once per day from embryonic days 0–20 with 670 nm LED light at a dose of 4 J/cm$^2$ [15]. They reported that 670 nm phototherapy by itself does not adversely affect developing embryos and may improve the hatching survival rate [15].

On the penetrability of laser through the egg, our results showed that the vasculature can be clearly resolved during the first five days of the incubation. However, as chick embryos grow day by day, the blood flow images of the embryos obtained through egg became obscured since the fifth day of incubation as shown in Fig. 3 although the mean blood flow of living embryos remained significantly higher than that of dead embryos. This may be partly caused by the increase of scattering of embryo tissue as more muscles and organs began to be developed.

In summary, LSI can be a useful tool to detect the vital signs of avian embryos during the first quarter of incubation period. More efficient artificial incubation can be achieved using this method for routine embryo’s assessment in industry, which provides a potential tool for automatically egg screening during artificial incubation.

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