Ultrahigh-speed optical coherence tomography imaging and visualization of the embryonic avian heart using a buffered Fourier Domain Mode Locked laser

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Abstract

The embryonic avian heart is an important model for studying cardiac developmental biology. The mechanisms that govern the development of a four-chambered heart from a peristaltic heart tube are largely unknown due in part to a lack of adequate imaging technology. Due to the small size and rapid motion of the living embryonic avian heart, an imaging system with high spatial and temporal resolution is required to study these models. Here, an optical coherence tomography (OCT) system using a buffered Fourier Domain Mode Locked (FDML) laser is applied for ultrahigh-speed non-invasive imaging of embryonic quail hearts at 100,000 axial scans per second. The high scan rate enables the acquisition of high temporal resolution 2D datasets (195 frames per second or 5.12 ms between frames) and 3D datasets (10 volumes per second). Spatio-temporal details of cardiac motion not resolvable using previous OCT technology are analyzed. Visualization and measurement techniques are developed to non-invasively observe and quantify cardiac motion throughout the brief period of systole (less than 50 msec) and diastole. This marks the first time that the preseptated embryonic avian heart has been imaged in 4D without the aid of gating and the first time it has been viewed in cross section during looping with extremely high temporal resolution, enabling the observation of morphological dynamics of the beating heart during systole.

1. Introduction

Abnormalities that occur during heart formation in the embryo are responsible for congenital heart disease. Observations made in the avian embryo have contributed greatly to the...
understanding of human congenital heart disease, avian heart development being strikingly similar to heart development in humans. Aristotle, in fact, was the first to publish that the heart is the first functioning organ in the early vertebrate embryo from his observations of the avian heart [1]. The vertebrate heart begins as a straight midline tube fused from bilaterally symmetric structures that form on either side of the embryo [2]. The heart begins to beat after the process of looping ensues, which in the chicken and quail is approximately 33–45 hours after fertilization has taken place [2]. The heart initially bulges toward the right of the embryo in the shape of a “C”, then forms a complete loop leading to the process of septation that permits formation of a four chambered heart (septation takes approximately 6–23 hours [2]) This process has been well documented by researchers including Hamburger and Hamilton [3], after whom the current staging of avian embryos is named. Cardiac looping is an intricate process whose mechanisms have yet to be fully understood, although many intrinsic and extrinsic parameters are thought to affect this process [4–7]. Failure of looping results in embryonic demise and looping abnormalities lead to major congenital heart defects so an understanding of this process is critical. In addition, during looping the flow in the heart changes from peristaltic (a smooth contraction wave from the inflow tract to the outflow tract) to pulsatile (sequential and rapid ejection of blood from the atria to the ventricles and then the body). Due to a lack of appropriate imaging technology, there is much uncertainty and speculation regarding the cause and time frame of this transformation. To date researchers have had to rely upon bioassays, video microscopy [8] and mathematical models [4] to investigate the mechanisms underlying development during looping. Direct observation of looping has been problematic due to the diminutive size (< 2 mm) of the heart and the inability to measure the small and rapid events that influence heart development. An imaging tool with high spatial and temporal resolution and a field of view capable of imaging function and morphology simultaneously is critical to understanding the mechanisms of normal and abnormal cardiac development.

Optical coherence tomography (OCT) [9] measures coherently gated back-scattered light at resolutions of 2–15 μm and up to a depth of 1–2 mm in cardiac tissue. These specifications make OCT ideal for studying the developing heart. OCT researchers have imaged the embryonic heart in many animal models to demonstrate the potential of the imaging modality [10–16]. Regarding the avian model, Yelbuz et al imaged embryonic chick hearts in 2D (in vivo) and 3D (ex vivo) [17], Davis et al imaged blood flow in the developing chick heart using Doppler OCT [18] and Jenkins et al have demonstrated 4D imaging of paced, excised embryonic chick hearts by gating the acquisition of individual OCT frames (B-scans) [10] and in vivo gating from a laser Doppler velocimetry signal [19].

Gated OCT imaging [10,19] and gated Doppler OCT imaging [16] have provided a means for mitigating motion artifact when imaging the developing heart. Over multiple heart beats one can build up data sets of the beating heart, by timing data acquisition to the heart cycle. Advantages of gated imaging include the ability to observe larger areas, average frames without a loss in resolution and increased temporal resolution for observing periodic systolic dynamics. Some of the disadvantages of gating include increased imaging time, loss in resolution and increased noise due to abnormal beats or drifting, and an inability to capture non-periodic events during systole. Thus far, OCT systems have not been capable of

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imaging rapidly enough to capture the morphological dynamics of the beating heart in vivo during systole, which requires imaging speeds approaching 200 frames per second (fps).

The recent development of spectral/Fourier domain [20–26] and swept source/Fourier domain [24,27–30] OCT technology (sometimes referred to as optical frequency domain imaging or OFDI) has increased acquisition speeds dramatically compared to time domain OCT systems. Conventional wavelength-swept lasers have been demonstrated for OCT imaging at up to 115,000 axial scans per second (115 kHz) [31] but are fundamentally limited in speed by the requirement to build up lasing in the cavity each time the tunable filter element is shifted [32]. The result is that swept source/Fourier domain OCT systems using conventional swept lasers are practical at speeds up to ~65 kHz [12,33]. Fourier Domain Mode Locked (FDML) lasers are a new class of wavelength-swept laser that virtually remove sweep speed limitations [29]. By tuning the intracavity wavelength filter synchronously to the cavity roundtrip time, quasi-continuous wave operation is achieved. All wavelengths are simultaneously active within the cavity and lasing builds up only once during initial power-up of the source. This enables rapid tuning, limited only by the maximum tuning rate of the filter. In addition extremely narrow instantaneous linewidths, broad total sweep ranges, and high phase stabilities are achieved [34]. Buffered FDML lasers use multiple output couplers in the fiber cavity to create unidirectional wavelength sweeps at even higher speeds [30]. Using buffered FDML lasers, OCT imaging has been demonstrated at up to 370 kHz [30].

In this paper a buffered FDML laser operating at 100 kHz is utilized to perform ultrahigh-speed OCT imaging of embryonic quail hearts during looping. The sweep speed was chosen to allow sufficiently high imaging depths given the 100 MHz bandwidth limitation of the A/D system. The excellent temporal resolution of the system allowed us to collect 3D (2D image data + time) datasets at 195 fps and 4D (3D volumetric data + time) datasets at 10 volumes per second. Several image processing techniques designed to demonstrate the capability of the buffered FDML OCT system were employed for enhanced visualization and quantitative measurements of the embryonic heart. Both autonomous (no manual segmentation) and supervised (manual segmentation) techniques were used to visualize the 3D + time data. Required imaging speeds and morphological dynamics during systole were examined by measuring the tube diameter versus time on a stage 13 2D + time dataset. This marks the first time that the preseptated embryonic avian heart has been imaged in vivo in 4D without the aid of cardiac gating and the first time it has been viewed in vivo in cross section during looping with extremely high temporal resolution, enabling the observation of morphological dynamics of the beating heart during systole. To summarize the observations, OCT imaging with a buffered FDML laser not only provides micron-scale spatial resolution, but supplies the extremely high temporal resolution needed to observe the complex morphological dynamics involved during cardiac contraction.

2. Experimental methods

2.1 Model preparation and imaging protocol

Avian embryos are an important biological model for cardiac development because their hearts develop structurally, functionally, and physiologically similarly to the human heart.
Furthermore, the absence of a uterus permits easier access to the embryo throughout development. Fertilized quail eggs were incubated in a humidified, forced draft incubator (GQF Manufacturing) at 39°C. At 36 to 50 hours of development (Hamburger-Hamilton stages 9–15 given the incubator temperatures and humidity levels) the eggs were taken out of the incubator, the eggshell was removed, and the contents were cultured in a sterilized 3.6 cm diameter Petri dish. Embryos are able to survive for weeks when cultured in a Petri dish. The embryos were positioned centrally on the yolk. Quail were chosen because they are similar to the chicken throughout development and are slightly smaller than the chicken, therefore more convenient to culture ex ovo. For comparison, several embryos were removed from the culture dish and fixed in 4% paraformaldehyde at 4 °C overnight for histologic evaluation. The fixed embryos were frozen, sectioned at 4 microns, and immunohistochemically labeled using MF20. MF20 is an antibody developed against myosin in striated muscle. Fixed embryos were incubated for 1 hour with 1:50 MF20 antibody, and then incubated for a second hour with 1:200 Alexa Fluor 594 goat anti-mouse LgG (H+L) antibody. Alexa Fluor attaches to the MF20 and provides the fluorescent signal. The sections were washed, cover-slipped, and photographed using a Nikon DIAPHOT 200 fluorescence microscope under bright-field and fluorescence microscopy.

After orienting the embryo in the culture dish, the beating hearts were imaged with the OCT system [30]. Operating at a sweep speed of 100 kHz, the buffered FDML laser provided a temporal resolution of 10 μs per axial image line (A-scan). The total tuning range of the laser was 117 nm at a center wavelength of 1287 nm, giving an axial resolution of ~ 7 μm in tissue. The laser was operated in a buffered configuration [30], giving unidirectional wavelength sweeps without sacrificing sweep rate or duty cycle. The output power was 7.3 mW and the system sensitivity was 102 dB for a reflector placed near zero path length mismatch with the reference arm. The sensitivity fell by 3 dB at a path length mismatch of 3.5 mm. Sensitivity rolloff in Fourier domain OCT is due to the inability to sample the entire contribution of every optical frequency independently.

The OCT setup was similar to the system described in reference 29 [29]. 90% of the laser output power was directed into a fiber optic Michelson interferometer for OCT imaging, and 10% was directed into a fiber optic Mach Zehnder (MZ) interferometer for generation of a calibration clock signal. The OCT and clock signals were detected by dual-balanced detectors and recorded by a 2-channel, 14-bit analog-to-digital converter (A/D) operating at 200 Msamples/s with 2 GB of onboard memory. Because the PCI bus (32 bit, 66 MHz) is unable to transfer data as fast as it is collected, the maximum imaging time was determined by the onboard memory of the A/D board, which reached capacity in ~ 2 s. The fringe pattern from the calibration interferometer was used to recalibrate the image data from time to optical frequency by use of a fast next neighbor check algorithm [32]. The algorithm checks the two neighbors for every point in the fringe pattern from the calibration interferometer. The corresponding point in the image data is kept if the two neighbors are both below or above the point in question. The path mismatch in the MZ interferometer was 5 mm, which set the maximum imaging depth at 2.5 mm in air or ~1.9 mm in tissue. The spot size on the sample, which sets the lateral resolution, was 15 μm. The sample arm
consisted of 2 achromatic lens, which had a magnification of 1.5, followed by x-y scanning mirrors place after the lens.

The embryos were placed on a heating pad situated underneath the sample arm of the OCT system. The heating pad was set to 37 °C and a strip of Parafilm was placed over the Petri dish with a small hole cut out at the location of the heart. Figure 1 depicts an embryo in the sample arm of the OCT system. The Parafilm reduced the heat gradient across the surface of the yolk which reduced dehydration of the embryo over time. The imaging time was approximately 2 seconds. Immediately after imaging, the embryo was placed under a microscope, staged by an expert (F. Rothenberg) and returned to the incubator. The time the embryo spent outside of the incubator was not longer than 2 min. Two different imaging protocols were used in the experiments. In one protocol, 4D (3D volumetric data + time) datasets were collected at 10 volumes per second (70 frames of 150 A-lines). The 4D datasets provided 4–6 volumes per cardiac cycle depending on the heart rate. These datasets allow visualization of the systolic heart versus the diastolic heart. In order to resolve the fast dynamic events during systole, 3D (2D data + time) datasets were acquired at 195 fps (512 A-scans per frame). Each frame was acquired in 5.12 ms. The embryos were positioned so the frame was coronal to the body of the embryo, with the frame dissecting the tube centrally along the loop (Fig. 6).

By obtaining 2D + time data of the same embryo at 3 hour intervals, developmental progression of an individual embryo was visualized through approximately 3 stages. Four embryos were selected at varying stages of development (10, 13). Each embryo was imaged 3 times 3 hours apart. The embryo starting at stage 13 progressed to stage 15, while the embryo at stage 10 progressed to stage 12.

2.2 Image visualization and measurements

Several different visualizations of the embryonic heart were assembled from 2D + time data and 3D + time data. All visualizations were created using Amira (Mercury Computer Systems, San Diego, CA, USA) and Matlab R2006a (Mathworks, Inc.). For the 2D + time data, the aspect ratio was corrected and tile movies were constructed in Matlab showing multiple stages of the beating embryonic heart side by side. For the 3D + time data, 4–6 volumes were collected per heartbeat depending on the heart rate (average was ~120 bpm). Earlier stages (10–11) were only imaged in cross-section.

Because of the immense volume of data that can be generated with an FDML-based OCT system, visualization techniques which can be automated (requiring no manual segmentation) are useful because they allow the researcher to quickly assess the morphological structure of the heart. The first rapid visualization technique is called sum voxel projection [35] and was used on 3D + time datasets to highlight anatomic features while reducing speckle noise. This technique provides a 2D visualization of volumetric data by summing up voxel values along rays cast through the volume in a direction normal to the viewing plane. The 3D + time dataset was visualized by displaying a series of sum voxel projections corresponding to different viewing angles of the rotating and beating heart volume (see Fig. 2).
Another method used to quickly and automatically visualize the data was to apply intensity gradient filters to enhance boundaries and edges (see Fig. 3). A grayscale colormap (linear gray ramp in the data range) was used as a lookup table for visualizing the OCT data. First, a 3D median filter with a kernel size of 3 (i.e. \(3 \times 3 \times 3\)) was used for noise reduction. Then, a 3D Sobel mask was applied to the data volume corresponding to each phase of the cardiac cycle. The Sobel operator is a very popular image enhancement tool \([36,37]\) that detects the presence of horizontal, vertical and diagonal edges, and has been previously applied to OCT images \([38]\). The resulting gradient values were rescaled to lie in the original data range, so that the same colormap (gray ramp) could be used. The visualization tool in Amira was driven by a time series of these Sobel gradient volumes.

Edge enhanced imaging produces only a grayscale visualization of volumetric data. To better distinguish and quantify measurements between the heart, blood and the rest of embryo, manual segmentation of the structures of interest were performed and easily distinguishable colormaps were applied to each of these regions. An expert user segmented the outer contour of the heart in a set of 2D slices along the three orthogonal planes (including the plane of acquisition). These 2D slices were taken from heart volumes corresponding to different phases of the cardiac cycle. The input to the visualization program consisted of two separate volumes. One volume consisted of grayscale data from the whole embryo with a suitable colormap for visualization. The second volume consisted of grayscale data from the heart along with zero-valued voxels corresponding to regions not containing the heart. A different colormap was used for the heart as compared to that for the whole embryo. Amira was used to perform a direct volume rendering of these separate volumes to the same data window. A time series of these volumes was loaded corresponding to the different phases of the cardiac cycle to obtain a visualization of a rotating and beating heart with cut-away views (see Fig. 4).

The above visualization provided cut-away views along one orthogonal plane. This visualization scheme was then expanded to include all three orthogonal directions with the positions of the 2D slices marked on the beating heart (see Fig. 5). This visualization was included alongside the time-varying 2D image data from each of these slices. Multi-planar reformatting \([39]\) was used to enable visualization of 2D data from planes normal to the plane of acquisition. By selecting the position of the 2D slices along the heart, a user can investigate any region in more detail.

Tube diameter measurements were made on the 2D + time dataset, which enabled a quantitative determination of maximum wall velocity and contractile wave propagation velocity (i.e. how fast the contraction wave is propagating along the tube, CWPV). The maximum wall velocity was used to determine the theoretical imaging speeds that would be required to capture the dynamics of the beating heart with little displacement error. CWPV measurements showed two distinctly different propagation speeds in the first half and second half of the tube.

To quantify the diameter of the tubular heart, the outer contour of the heart was manually segmented in a time series of 2D images corresponding to one complete heart cycle. Here, the bulk motion of the heart volume was ignored, assuming that it causes only minor shifts.
in the anatomical position of these 2D slices. Next, a set of four marker lines was overlaid on the segmented heart region (refer to Fig. 8(A) and 8(D)) at two positions along the length of the tube. One set of lines was positioned at the proximal heart tube (atrio/ventricular end) while the other set was positioned at the distal heart tube (ventricular/outflow end). These line segments were placed approximately normal to the walls and were spatially static across all frames in the time sequence. The tube diameter was given by the distance between the two points on the perimeter where the lines intersected the manually segmented heart. In order to reduce sudden fluctuations in tube diameter arising from segmentation irregularities, a smoothing spline [40] was applied on these values across time. Next, each plot in this time series of diameter measurements was normalized by the maximum value observed during the cardiac cycle. The resulting tube diameter plots for the two sets of lines are shown in Fig. 8(B) and 8(E). Computing a gradient of tube diameter with respect to time and dividing by 2 gives the wall displacement velocity, as shown in Fig. 8(C) and 8(F). The wall displacement velocity is slightly underestimated due to out-of-plane motion arising from the torsion of the heartbeat and the inability to align the imaging plane exactly along the centerline of the entire tube. By comparing the approximate location of the coronal slice with other 4D datasets and video microscopy, the position of the 2 sets of four marker lines was chosen to minimize errors due to out-of-plane motion.

To measure CWPV, the cross-correlation was calculated between tube diameter waveforms corresponding to the different positions of the line, and calculating the delay that maximized this cross-correlation measure. The displacement estimates (in terms of physical distance traveled by the wave) were obtained by using known, calibrated pixel dimensions. These displacements and times gave us velocity estimates for the contractile wave. Errors due to misalignment of the 2 sets of four marker lines across the central part of the tube were considered relatively small and ignored.

For 2D + time and 3D + time data, lines in the frame or volume are recorded sequentially in time, but the image or volume is assumed to represent a single snapshot in time. In order to determine the maximum displacement error over an imaging region, the preceding tube diameter algorithm determined the maximum velocity, which was then used for computing the maximum possible displacement error (MDE) from the following formula.

\[
MDE = (L/R) \times V, \quad (1)
\]

where \(L\) is the number of lines per region that is assumed static, \(R\) is the A-scan rate, and \(V\) is the maximum wall velocity. The MDE is expected to be different for different heart stages, but can be determined experimentally in the same way.

### 3. Results

Figure 2 shows the summed voxel projection movie for a 3D + time dataset of a stage 14 quail heart, while Fig. 3 shows a movie depicting the top view of the same dataset with a gradient filter applied to it. Both methods produce clearly interpretable visualizations, without resorting to time-intensive manual segmentation. These approaches are suitable for improved throughput and rapid preliminary phenotyping of internal and external embryonic
structures. The summed voxel projection technique in Fig. 2 allows the viewer to assess the 3D morphology of the outer and inner lumen of the heart tube as it fills and ejects blood. The gradient filter technique in Fig. 3 clearly reveals the cardiac interaction with surrounding tissue.

Figure 4 shows a movie of the rotating and beating heart within the embryonic volume, where manual segmentation has been used to delineate cardiac borders within the thorax. Here, the same 3D + time data shown in Fig. 2 is presented with the segmented heart color-coded as compared to rest of the embryo. By segmenting the heart volume, one can better visualize the heart within the thorax in vivo. The movie permits visualization of the peristaltic motion and blood flow with the bolus of blood visible in the cut-away views. Segmentation is also necessary for making quantitative measurements on the data.

Figure 5 shows the visualization of a 4D dataset with multiplanar reformatting, along with slice positions on the beating heart volume. Imaging cardiac motion in three orthogonal planes permits investigations of the relationships between the myocardial wall, cardiac jelly, and endocardium as they relate to each other non-invasively and in vivo. For example, consider the changes that take place during cardiac contraction. As the contractile wave passes down the heart tube (Figure 5F, movie), the inner chamber closes, forcing blood forward. The cardiac jelly was not compressed symmetrically, but rather there were areas in which the cardiac wall (primarily the cardiac jelly) was thinner (green arrow) or thicker (green arrow). This suggests asymmetry of mechanical relationships in the early heart tube during systole, despite the fact that the heart appears symmetric during diastole. These relationships could only be inferred indirectly with prior techniques. OCT technology will enable the origins of these mechanical-anatomical relationships to be followed non-invasively within the same animal over time and measured quantitatively.

Figure 6 shows ultrahigh-speed OCT imaging of the same region in two embryonic hearts. The younger heart in panels A–C represents 6 hours of development from stage 10 to 12. The heart in panels D–F represents 6 hours of development from stage 13 to 15. The embryonic heart at stage 10 had just begun to beat. The myocardium and endocardium are uniform in thickness. Contraction of the myocardium was slow and peristaltic in nature with flow from right (inflow) to left (outflow). During diastole, the heart tube appeared asymmetric with the posterior wall cardiac jelly visibly thicker than the anterior wall. The cardiac jelly had rare cellular elements uniformly disbursed throughout the heart tube in all stages where cardiac jelly was present. Peristaltic contraction produced a symmetric thickening down the length of the heart tube. By stage 11, the posterior wall developed a gentle bend (again seen best during diastole) that was more evident by stage 12. The sinus venosus was observed in cross-section in panels A and B. This structure did not actively contract, although blood can be seen flowing through it during systole. Red blood cell content appeared to increase as the embryo aged, consistent with what is known of embryonic hematopoiesis [41]. The posterior bend in the heart tube was more evident at stage 12. After the contractile wave passed through the heart tube, regurgitant blood was seen reentering the cardiac chamber at the beginning of diastole. In all stages, the myocardial and endocardial thickness appears uniform throughout the length of the heart tube.
The second heart in panels D–F (stage 13–15) was U-shaped with a stricture that provided a dividing point between rapidly and slowly contracting myocardium. The endocardial surface was no longer uniform, with shallow wrinkles that were more evident during systole. These wrinkles suggest connections between the endocardium and myocardium that we call “tethers”. By stage 13 the myocardium and endocardium were in closer proximity (cardiac jelly was thinner) where the ventricle will form and remained thick where the endocardial cushions were developing (also Fig. 5). Endocardial cushions are thickenings of cardiac jelly that are precursors of the atrioventricular and semilunar valves. The myocardium was subtly thicker where the ventricle is forming (the base of the U), however trabeculations will not form until a stage or so after these data were recorded. The inflow region that will become the atrium advanced cranially behind the outflow tract (what will become the infundibular portion of the right ventricle and part of the base of the aorta and pulmonary artery) as the heart developed. The heart rate was higher than previous stages, and flow appeared continuous through the sinus venosus. The erythrocyte content appeared higher in the older heart tube, as well. Thinning cardiac jelly except in the endocardial cushions, folding of the endocardial lining, and myocardial thickening of the ventricle were more prominent as the heart developed. After contraction of the ventricle, the inflow portion of the heart relaxes and fills with blood in preparation for the next wave of contraction that is initiated in the inflow region. The heart will continue this rhythm until it dies or is interrupted by illness or toxicity.

As has been shown in other publications, fixation artifact is a common problem in histologically prepared specimens, as demonstrated by significant shrinkage of the tissue in Fig. 7, and the sharp angles and folds in the myocardial wall that are not normally seen in the living embryonic heart. Scallop ing of the myocardial edges was observed where the endocardium involutes toward the myocardium (regions where “tethers” are inferred – see arrows). These “tethers” were also observed with OCT (see the arrows in Fig. 6(D–F) and arrows in Fig. 6).

Figure 8(A–F) show measurements of heart tube diameter, CWPV and wall displacement velocity measurements using the 2D + time dataset. A lower time delay between successive lines in the atria/ventricular (inflow) part of the tube (Fig. 8(B)) as compared to the ventricular/outflow tract (outflow) end of the tube (Fig. 8(E)) suggests that the contractile wave propagates faster at the inflow than the outflow (approximately 9.2 mm/s compared to ~2.1 mm/s). Also, the systolic ejection causes a more pronounced and more rapid reduction in tube diameter at the inflow, which manifests as a higher wall displacement velocity at the inflow (approximately 1.5 mm/sec maximum, Fig. 8(C)) compared to the outflow (approximately 0.8 mm/sec maximum, Fig. 8(F)) of the heart loop. Methods for calculating wall velocity and CWPV are described in section 2.2.

4 Discussion

4.1 Temporal resolution

The high temporal resolution of the OCT system enabled us to view the contraction dynamics of the beating quail heart during looping. The tile movie in Fig. 9 shows the same stage 13 quail heart beating with different temporal sampling. The heart on the left is
composed from 90 phases in the cardiac cycle separated by approximately 5 ms (i.e. temporal sampling rate of 2D + time dataset). The heart on the right is assembled from 6 phases in the cardiac cycle separated by 75 ms (i.e. approximately equal to the temporal sampling rate of 4D data shown in Figures 2–5). One can observe the detail of the motion that is lost at low temporal resolution. At high temporal resolution, one can observe a “kickstart” of the heart beat occur on the right (inflow) side of the tube. This suggests an active contraction at the beginning of the tube initiating a wave which then propagates more passively through the rest of the tube and undergoes dampening. At low temporal resolution, this dynamic is not observable. In addition, blood flow dynamics are observed at high temporal resolution that are not observable at low temporal resolution. This provides a strong motivation for ultrahigh-speed OCT imaging.

In order to quantify the required imaging rate for capturing cardiac motion during systole over a given spatial range, the maximum displacement error was calculated by using equation 1 and the maximum wall velocity calculated with the tube diameter measurements in Fig. 8. Table 1 describes the displacement error (calculated from the wall velocity measurements obtained by measuring tube diameter) across a frame or volume under different imaging conditions. Row 1 represents the conditions identical to the 2D + time imaging presented in this paper. Since the lateral resolution of the OCT system was 15 μm, the 10 μm maximum displacement error is quite reasonable. Row 2, which represents the conditions of the 3D + time imaging presented in this paper, shows a much greater error. According to row 3, in order to accomplish 4D imaging with acceptable displacement error and similar spatial sampling as the 3D + time imaging presented in this paper, a sweep speed of 2,100,000 lines/s would be required. As A/D systems increase in speed, eventually a buffered FDML laser maybe able to accommodate a sweep rate of 2 MHz. Rows 4 and 5 represents 3D + time imaging with spatial sampling at the Nyquist rate (7.5 μm) for a 2 mm by 1 mm volume. As seen in row 4, the temporal requirements for capturing motion during systole for 3D + time imaging are much higher than present imaging capabilities (7,111,000 lines/sec.). 3D + time imaging without spatial understanding at 100,000 lines/s would result in significant displacement error. Table 1 does not account for frame averaging, which significantly reduces noise in the images but would drive imaging speed requirements even higher. Future work may circumvent the problem by gating the data acquisition to the cardiac cycle \[^{10}\] so that a 3D + time dataset would be built up over multiple heartbeats.

4.2 Cardiac structure and function

These data highlight the utility of swept source OCT using buffered FDML lasers for imaging the embryonic vertebrate heart non-invasively. Because of the superior depth penetration of OCT compared with confocal microscopy, these images are the first to demonstrate early looping in what will become a four-chambered heart. In addition, ultrahigh-speed OCT provide a unique opportunity to detect the relationships between myocardial contraction and the cardiac jelly and endocardium, such as the asymmetric contraction of the heart tube observed in Figures 5 and 6. Histology was able to detect this asymmetry (Fig 7(B) and 7(D)), but it was not possible to establish if this was due to fixation artifact. Ultrahigh-speed OCT reveals that there appear to be endocardial “tethers” on the myocardium of the heart tube. One possibility is that the endocardium is tethered to
the myocardium where the cardiac jelly is thinner, anchoring the tissues in place. This powerful imaging technology will make it possible to investigate the interplay between mechanical forces and molecular/cellular mechanisms that govern early cardiac modeling.

Ultrahigh-speed OCT permits high temporal and spatial resolution quantitation of motion through the heart tube that was previously not possible. The contractile wave propagation in the proximal heart tube is measured to be 4.5 times faster than wave propagation in the distal heart tube at very early stages of development. While differences in wave propagation down the length of the heart tube have been demonstrated previously using measurements of electrical conduction [42–44], OCT imaging demonstrates the ability to quantify the mechanical wave in vivo, non-invasively, and without the addition of contrast agents or surrogate markers. In the future by combining gated imaging with an ultrahigh-speed OCT system, the contractile wave can be measured on a 3D + time data set, which will provide a more accurate measurement. Investigators will be able to determine the effects of surrounding tissues on cardiac development and function as the heart remains in situ. This is critical because preload, the stretch on the myocardium prior to contraction, is known to exert a powerful effect on mature myocardial contraction. Preload is provided by the blood that enters the heart, exerting radial stretch on the cardiomyocytes in diastole. Afterload, the resistance to blood flow exerted on the embryonic heart by the distal cardiovascular system, is also maintained when ultrahigh-speed OCT is used to non-invasively investigate the specimen. The effects of preload and afterload would be altered by removal of the heart from the body, unless measures are taken to maintain the as yet unknown effect of preload and afterload on the heart tube. Ultrahigh-speed OCT will permit investigators to measure if and when preload and afterload become relevant to embryonic cardiac function and formation.

“Tethers” – the ultra-fine connections observed between the endocardium and myocardium – may produce the involutions seen in the endocardium in the early heart tube. Investigation of the formation of these involutions or wrinkles in the endocardial lining in vivo with experimental intervention and information regarding changes in molecular composition of the cushions may permit a better understanding of cardiac morphogenesis and potentially cardiac valve formation. Studies of the endocardium at these stages of cardiac differentiation have been sparse.

Although Doppler OCT was beyond the scope of this report, the effect of the blood flow on developing morphology and vice versa may be critical to understanding the mechanisms of looping and abnormal development. Because of the excellent phase stability of the FDML laser, future Doppler studies should provide comparable phase noise as spectrometer based systems at much higher acquisition rates [34].

### 4.3 Longitudinal studies

The goal of this work was to image embryonic heart structure and function non-invasively over a 6 hour period. The time window in which cardiac contraction is in the process of changing from peristaltic to pulsatile was observed, and initial observations of interrelationships between tissues and mechanical function over time have been made. It is apparent, however that a more rigorous system for maintenance of temperature and humidity...
will be required to allow the embryos to develop naturally over many hours while imaging. During the observations presented here, the embryonic heart rate declined and an increase in regurgitant flow resulted despite our attempts at temperature control. The heart rate of the embryo that progressed from stage 10 to 12 started at 52 beats per minute (bpm) and declined to 31 bpm, while the heart rate of the embryo that progressed from stage 13–15 started at 130 bpm and declined to 76 bpm. An imaging environment with the goal of precisely measuring preload, afterload, and effects of blood flow on the morphological and mechanical changes that occur throughout the looping process is under development. This will serve as a baseline for future work that will define both genetic and mechanical abnormalities that impinge on the developing heart and can result in congenital defects.

5. Conclusions

Ultrahigh-speed OCT imaging provided unparalleled temporal and spatial resolution of living embryonic avian hearts. This marks the first time that the pre-septated embryonic avian heart has been imaged in vivo in 4D without the aid of gating. It is also the first time the embryonic heart has been viewed in cross section during looping with extremely high temporal resolution, enabling the observation of morphological dynamics of the beating heart during systole.

Ultrahigh-speed OCT with FDML lasers can effectively be used to quantify mechanical events in the embryonic heart with high temporal resolution (i.e. the fast systolic kick at the inflow of the heart tube shown in Fig. 9). These data suggest that the cardiac impulse is initiated at the inflow region of the embryonic heart tube and is more passively transmitted through the distal tube. Slower imaging systems cannot resolve these systolic dynamics, which provide another metric for looking at the mechanisms that drive the looping process and deciphering normal versus abnormal heart development.

Visualization methods were developed to rapidly reconstruct embryonic hearts in three and four dimensions for high spatial resolution non-invasive phenotyping of the embryonic hearts. This will permit rapid decision-making regarding experimental alterations that could potentially alter structure and/or function of the heart in the early phases of cardiac looping.

The appearance of invaginations in the endocardial lining of the early heart tube that correlate with the appearance of asymmetric contraction in the heart tube was also observed. This suggests the presence of tethers that may link the endocardium to the myocardium, which may influence cardiac physiology and morphology.

In the future, the 3D + time data can be acquired with the same temporal resolution as the 2D + time data by gating the FDML-based OCT image acquisition to the heart cycle, which will also improve the accuracy of wall velocity and CWPV measurements. This will enable more detailed investigations into systolic dynamics. Also, by controlling the environment (temperature and humidity) of the embryos more closely, longitudinal studies spanning more stages will be possible. Preload, afterload, and effects of blood flow on the morphological and mechanical changes that occur throughout the looping process can be examined more closely and serve as a baseline for future work that will define environmental, genetic and mechanical factors that impinge on the developing heart and can result in congenital defects.
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Fig. 1. Setup for imaging embryonic quail hearts. The embryos were placed on a heating pad situated underneath the sample arm of the OCT system. The heating pad was set to 37 °C and a strip of Parafilm was placed over the Petri dish with a small hole cut out at the location of the heart.
Fig. 2.
Sum voxel projection of a beating stage 14 quail heart. The heart tube is clearly visible and the inner lumen of the heart can be seen filling and ejecting blood. The arrows point in the direction of blood flow. Vent - ventricle.
Fig. 3.
Top view (ventral) of a beating stage 14 quail heart. A 3D Sobel mask was applied to the data volume corresponding to each phase of the cardiac cycle. Interaction between the heart tube and the surrounding tissue can be clearly visualized. Vent - ventricle.
Fig. 4.
Beating and rotating heart inside the embryonic volume of stage 14 quail. A cut-away operation is performed as the heart beats to enable visualization of internal structures.
Fig. 5.
Beating 2D slices from different orthogonal orientations of a stage 14 quail heart. Along the top row (A–C) are three different 2D slices sagittal to the body of the embryo. The 3D reconstruction to the right (D) shows the location of the three slices as planes cutting through the heart. The planes from top to bottom correspond to the images starting from the left and moving right. The middle row (E–G) represents three 2D slices cut coronal to the body of the embryo. The 3D reconstruction (H) of the heart to the right marks the left to right images as planes going from front to back in the image. The bottom row (I–K) shows three 2D slices of the heart cut transverse to the body of the embryo. The 3D reconstruction (L) to the right marks the left to right images as planes going from left to right in the image. White arrows point to the location of possible tethers connecting the endocardium to the myocardium. The green arrowheads point to asymmetry in the cardiac wall. The scale bar in F corresponds to all 2D slices, while the scale bar in L corresponds all 3D reconstructions.
Fig. 6.
Time series of coronal sections through the same location of two quail hearts using ultrahigh-speed OCT. A–C (2.29 MB) stage 10–12 from the same quail embryo, D–F (2.42 MB) stage 13–15 from a different quail embryo, same orientation and location. All images are oriented such that the inflow region is on the right, and the outflow tract is on the left. Blood flow was, therefore, right to left. (A–C) The playback rate of the movies has been slowed to ~1/7th the original speed. Stage 10–12: Heart was a single midline tube, looping not yet evident in panel A. Peristaltic contraction leading to forward flow of blood (to the left) was observed in all three panels. The sinus venosus does not contract, but blood flow is observed during systole. Individual red blood cells were observed in the earliest stages of development, with apparent increase in number as the embryo developed. The cardiac jelly had rare cellular content, and the endocardium appeared smooth throughout systole and diastole in these 2D + time movies. The heart showed symmetric contraction at stage 10. By stage 11, a shallow bend appeared on the dorsal aspect of the heart tube, more evident during systole, and more prominent at stage 12. The heart tube is distinctly asymmetric at stage 12 during systole and diastole. (D–F) Stage 13–15: A stricture formed separating the inflow and outflow regions into rapidly (inflow) and slowly (outflow) contracting myocardium. During systole, small wrinkles in the endocardial lining were observed extending outward to the myocardium. These bands became more prominent as the heart developed. Erythrocytes filled the cardiac chamber, and the heart rate appeared to increase. Contraction appears to be moving toward pulsatile flow. White arrows point to the location of possible tethers connecting the endocardium to the myocardium. Myo – myocardium, SV – sinus venosus, CJ – cardiac jelly, EC – endocardium, In – inflow and Out - outflow
Fig. 7.
Histologic sections through a stage 11 embryonic quail heart (A and B – same section) and a stage 13 heart, (panels C and D – same section) are shown. A and C were immunohistochemically labeled with anti-myosin antibody MF20 (green) that label striated muscle. B and D are images taken with Hoffman Optics of the same sections in A and C, performed to demonstrate the appearance of the myocardium relative to the endocardium. Tethers connect the endocardium (arrows) to the myocardium (arrowheads). The dotted outline shows tissue folding, an artifact of the histological process. Myo – myocardium, CJ – cardiac jelly and EC - endocardium
Fig. 8. Heart tube diameter, wall displacement velocity and CWPV measurements were computed from a 2D + time coronal dataset. (A) Line positions for the proximal end of the heart tube, (B) normalized tube diameter versus time (C) wall displacement velocity versus time, (D) Line positions for the distal end of the heart tube, (E) normalized tube diameter versus time, and (F) wall displacement velocity versus time.
Fig 9.
Movie showing the effects of high and low temporal sampling rate on the visualization of heart dynamics. A higher temporal sampling rate allows for the visualization of the fast systolic kick, which is missed in the case of lower temporal sampling. In – inflow and Out - outflow
Table 1

Maximum Displacement Error

| Max. Disp. Err. (um) | Lines Per Region | Required Imaging Speed | A-line Rate (lines/s) | Imaging conditions          |
|---------------------|------------------|------------------------|-----------------------|-----------------------------|
| 10                  | 512              | 195 fps                | 100,000               | Current study/2D + time     |
| 210                 | 10,500           | 10 volumes/s           | 100,000               | Current study/3D + time     |
| 10                  | 10,500           | 200 volumes/s          | 2,100,000             | Semi-ideal/3D + time        |
| 10                  | 35,555           | 200 volumes/s          | 7,111,000             | Ideal/3D + time             |
| 711                 | 35,555           | 2.8 volumes/s          | 100,000               | Ideal spatial sampling w/current line rate |