Ultrasonic monitoring of early development of lower vertebrate embryos

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Abstract. In this paper, the scanning acoustic microscope is used for visualization of the lower vertebrate embryos in vivo. Compared to optical methods, high frequency ultrasonic imaging has a negligible effect on a developing organism, and chemical treatment of biological object is not required due to the high contrast of acoustic images and the deep penetration of ultrasound. A technique for continuous ultrasonic observation of a living embryo that grows in the immersion cell of the acoustic microscope is proposed and developed. Using this technique, it is possible to image the internal organs of the embryo, visualize the motion of the structural elements and estimate their velocity. The loach embryo (Misgurnus fossilis) at 11–12 stages of development was studied using the experimental setup based on the 50 MHz scanning acoustic microscope. The ultrasonic images of the yolk and blastula, animation of cell division and motion in the syncytial region and estimation of the velocity of the structural elements of the embryo were obtained by the developed method.

1. Introduction

Being sensitive to environmental conditions, lower vertebrate embryos can be recognized as model organisms for the research in the fields of developmental biology, ecology, and pharmacy [1]. Most developmental phenomena in lower vertebrates such as fish have been studied using optical microscopy. Cell growth and division, their motion and integration into organs and tissues are usually observed. However, visualization by means of electromagnetic radiation has some limitations in observing the interior of the embryo caused by low contrast of embryonic elements and light scattering on optical heterogeneities. Many optical microscopy methods have been developed to overcome these limitations [1–3]; however, all methods are somehow based on the use of high intensity radiation. The destructive effect of intense radiation on living organism (photo-toxicity) is a serious problem in the long lasting observation of embryos in vivo.

The ultrasonic waves have the minor effect on living organism [4]. Acoustic methods are effective for imaging the spatial three-dimensional distribution of variations in the elasticity, density and viscosity of an object. Ultrasonic diagnostic devices are widely used in medicine; however, the frequency range of medical scanners is low due to the deep location of the regions of interest inside the human body and high attenuation of ultrasound in the tissues. As a result, the spatial resolution of commercially available devices is not sufficient for visualization of fish embryos whose sizes do not exceed 1-2 mm. Scanning
acoustic microscopes provide better resolution [5–7]. The ultrasonic biomicroscope [5–6] allows observation of embryo general growth but it cannot be used for the real time visualization of tissue formation and movement. Several high frequency ultrasonic techniques were developed for the cardiac system visualization and for the blood flow velocity measurement in adult small animals and chick embryos [8–9]. The blood movement causes the Doppler frequency shift of the scattered ultrasonic waves. In these organisms, it is sufficiently large for the visualization and velocity measurement; however, in the fish embryos it is negligibly small. Therefore, the development of an ultrasonic imaging method for studying a continuously evolving biological system is an important and interesting problem.

In this paper, the time–lapse acoustic microscopy technique is proposed and developed for the visualization of the developmental processes in lower vertebrate embryos. The loach embryo (*Misgurnus fossilis*) was used in this study as a model biological object. The period of development of the early blastula was chosen since there is an intensive division and movement of cells at this stage. The developed ultrasonic technique was validated by imaging of the embryo structure and assessing the movement of cell clusters over time.

2. Experimental setup

The scheme of the experimental setup is presented in figure 1. The focused ultrasonic transducer 1 immersed in water generates the impulse ultrasonic waves. The waves reflected by the fish egg are received by the same transducer and are converted into digital form. The echoes from the focal area F are selected in the received waveforms using time windowing. To build an acoustic image the transducer is mechanically scanned over lateral coordinates \(x, y\), producing the output data set \(s(x, y, t)\), where \(s\) is the signal amplitude and \(t\) is the time of wave propagation. The central frequency of the ultrasonic transducer was 50 MHz, the angular aperture was 30 °. In accordance with the classical criteria [6], the lateral resolution of this imaging device was approximately 30 µm. The duration of the ultrasonic pulse (−6 dB) was about 40 ns, therefore the longitudinal resolution can be estimated within 20–30 µm.

The live fish egg was placed in the special holder 2 immersed in the water cell of the microscope (figure 1). The holder was manufactured from bioinert plastic and had a cylindrical cavity with a diameter and depth of 2 mm. The egg placed in the cavity was protected from the disturbances caused by the movement of the transducer in the immersion liquid. The temperature of the water was kept at 23±2 ° throughout the entire experiment.

![Figure 1. Experimental setup: 1 – ultrasonic transducer; 2 – sample holder; 3 – yolk; 4 – blastula; 5 – perivitelline liquid.](image)

Eggs of loach *Misgurnus fossilis* were used in this work as biological objects. Although the embryo could develop in the immersion cell of the microscope for several days from the fertilization to hatching, and even longer, the 11-12 stages of development (early blastula) [1, 10] were considered in this work. At these stages, the diameter of the loach egg is 1.8–1.9 mm. There are the round yolk 3 and nonsymmetrical blastula 4 inside the egg (figure 1). The space between these organs and outer film is filled with the perivitelline liquid 5. The blastula consists of cells that constantly divide and move. The cell size is in a range of 10–20 µm which is less than the resolution of the acoustic microscope.
3. Ultrasonic imaging of embryo structure

To generate the ultrasonic image, the Hilbert transform $G$ of the recorded three-dimensional data set $s(x,y,t)$ is calculated over time $t$ and the envelop of the result is determined. Then, for all $x,y$, taking the maximum values of the signal envelop within the time gate $[t_1,t_2]$ the image $I(x,y)$ is constructed. Since the propagation distance of the ultrasonic waves in immersion liquid is proportional to the time $t$, the image $I(x,y)$ represents the lateral distribution of acoustic reflectivity in the layer $[z_1,z_2]$, where $z_1=C_w t_1/2$, $z_2=C_w t_2/2$, and $C_w=1485$ m/s is the ultrasound velocity in the liquid.

As an example, the ultrasonic images of the loach embryo are presented in figure 2 for several positions of the imaging planes $z = 100, 0, -100, -200$ µm. Only one experimental data set $s(x,y,t)$ was used for visualization. The length of the longitudinal window $\Delta z$ the was equal to 50 µm, the spatial sampling periods $\Delta x$, $\Delta y$ were equal to 10 µm. The focus of the transducer ($z=0$) was located slightly above the middle of the yolk (figure 2(b)). This image shows the best sharpness, however the resolution remains good enough to observe of the embryo structure within a defocusing range of 100–200 µm.

![Figure 2. Ultrasonic images $I(x,y)$ of the loach embryo, $z = 100$ µm (a), $z = 0$ µm (b), $z = -100$ µm (c), and $z = -200$ µm (d)](image)

The round dark yolk $Y$ and the bright blastula $R$ can be recognized in the images. Due to the cellular structure, the blastula has a high scattering coefficient of the ultrasonic waves compared to the yolk. The individual cells are not visible in the blastula area since the cell size is smaller than the diameter of the ultrasonic beam at the focus. Constructive and destructive interference of the waves reflected by several cells randomly located within the focus spot causes fluctuation of the received electrical signal. These fluctuations form a typical speckle pattern, which often appears in ultrasonic images of biological tissues [4]. The reflectivity of the yolk $Y$ is much smaller; however, the weak speckles can also be detected here. In general, the recorded ultrasonic images are consistent with knowledge of the fish embryo morphology obtained using optical techniques [1]. On the other hand, the ultrasonic images show the spatial distribution of the elastic properties of tissues that are not available in the optical range.

4. Ultrasonic visualization of organ motion

The measurement of a complete ultrasonic data set $s(x,y,t)$ takes several minutes due to the employment of a mechanically scanned single-element transducer in the device. This data acquisition speed is
insufficient to detect the movements of various organs in real time and to observe structural changes within the embryo. To overcome this restriction it was proposed to scan the transducer along one spatial coordinate and to record the data in the $s(x,t,T)$ format, where $T$ is the “slow” time associated with the development processes. The recording period of the $s(x,t)$ frames can be reduced to a fraction of second, which allows one to study fast changes for a long time.

After 1 hour after recording of the image presented in figure 2, the acquisition of the $s(x,t,T)$ data set was started. It was acquired 160 frames with a period of 7 s; therefore, the overall time of the recording was 1120 s. The coordinate $y=1$ mm was constant; the position of the scanning plane $(x,t)$ is shown in figure 2 by the dashed line. Two frames recorded at the moments $T = 7$ s and $T = 1100$ s are shown in figure 3 (a) and (b), respectively. The full data set $s(x,t,T)$ can be presented as an animation.

There are the blastula R and the yolk Y in the presented image $s(x,t)$ (figure 3). The reflectivity of the blastula is stronger than the reflectivity of the yolk as it is observed in the images shown in figure 2. The exterior boundary A of the blastula remains practically motionless. The interface S between the yolk and the blastula, known as the syncytial layer, slowly and smoothly moves upwards. This movement causes a thinning of the upper part of the blastula and the coordinated displacement of the yolk elements adjacent to the interface. Such morphological behaviour is typical for the development of fish embryos at stages 11–12. Inside the blastula, especially in the regions adjacent to the syncytial layer, more intense changes in the recorded ultrasonic signal are observed. Cell division, growth and movement can explain such activity in the blastula.

5. Data analysis and velocity estimation

To analyze the time transformation of the ultrasonic signals, the measured data are presented as functions of $t$ and $T$ for several values of $x$ (figure 4). At $x=0.25$ mm the ultrasonic waves propagate along the left vertical dashed line shown in figure 3. The signal $s(t,T)$ received at this position of the transducer is presented in figure 4(a). This measurement line is located on the slope of the blastula; therefore the reflection at the boundary A is weak. Inside the blastula in the region B, the slow changes in the reflectivity occur without significant vertical movement of the structural elements. The measurement line is tangent to the syncytial interface, and the reflection at this interface is not detected. In the neighbour area at $x=0.4$ mm (figure 4(b)) the movement of the scatters in the outer layer A in the vertical direction is practically not observed. However, in the layer C located deeper and closer to the yolk, intense chaotic motion and predominant upward drift are present. The average velocity of this predominant movement of the particles can be estimated from the slope of their spatio-temporal responses:
\[ v = \frac{\Delta t \cdot C_w}{2 \cdot \Delta T} , \]  

where \( \Delta t \) is the change in the echo delay acquired during the observation time \( \Delta T \). For this area the velocity can be estimated in the range of 0.08 – 0.1 \( \mu \text{m/s} \).

Figure 4. The \( s(t,T) \) data taken at \( x= 0.25; 0.4; 0.65; 1.05 \) mm, respectively.

At \( x=0.65 \) mm (figure 4(c)) the ultrasonic beam incidents at the outer boundary A at the normal angle producing a strong reflection. The vertical displacement of this boundary for the entire recording time is small whereas the local travel distance of the syncytial interface S is much larger. The movement of this interface is smooth; however the velocity \( v \) is not constant. The maximum value of \( v= 0.15 \mu \text{m/s} \) is achieved in the time interval of 300 – 400 s.

The measurement line \( x=1.05 \) mm (figure 4(d)) is located at the other slope of the blastula. Due to the oblique incidence of ultrasound on the object, the echoes from the external boundary A are not clear meanwhile the reflections from the internal cells are quite pronounced. The slope of the spatio-temporal responses is negative, therefore, the particles move down in this region of the blastula. The velocity of the movement can be estimated as \( v= 0.04 – 0.06 \mu \text{m/s} \).

6. Conclusion
The results of ultrasonic visualization of the live loach embryo obtained by the developed technique are consistent with modern knowledge. The ultrasonic images show a high contrast of organs without any chemical effect on the object under the study. Minor impact of the ultrasound waves allows long continuous monitoring of embryos that develop in the immersion cell of the scanning acoustic
microscope. Some ultrasonic data is difficult or impossible to obtain using optical methods. For instance, in this study, the cell motion in the inner layers of the blastula next to the syncytial interface was visualized, and the velocity of the structural components was estimated. Since there are many stages of development of the lower vertebrate embryos and many various phenomena associated with their development, the proposed technique can be considered as versatile and effective tool for future research.

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