The heart is the first organ of the embryo to reach a functional state, and the circulatory functions start in the early embryo [1, 2]. The pattern of blood flow in the developing embryo has long been proposed to play a significant role in the morphogenesis of the heart [3, 4]. Therefore the embryonic origin and early development of circulatory functions associated with morphogenesis in the embryonic heart and blood vessels have attracted sizable attention. However, ordinary physiological techniques for evaluating the circulation (e.g., use of a catheter) cannot be applied to such early embryonic preparations because of the small size and fragility of blood vessels. In this study, we optically monitored the blood flow dynamics in the early chick embryo, visualizing the circulation of juvenile blood cells in the dorsal aorta by using a video-imaging method without invasion to the circulatory system.

Some results presented herein have been communicated in a preliminary abstract [5].

Fertilized white Leghorn eggs were incubated for about 50 hours in a forced-draft incubator at a temperature of 37°C and 60% humidity and turned once each hour. In this study, chick embryos at stage 12+ of Hamburger and Hamilton [6] were used. Embryos isolated under a dissecting microscope were kept in Ringer’s solution of the following composition (in mM): NaCl, 138; KCl, 5.4; CaCl₂, 1.8; MgCl₂, 0.5; glucose, 10; and Tris-HCl buffer (pH 7.3), 10.0, equilibrated with oxygen. The embryo was then pinned onto the silicone bottom of the chamber with the ventral side up (Fig. 1A). A recording was carried out in a still chamber without continuous perfusion of the bathing solution at a room temperature of 28–30°C.

The preparation chamber was mounted on the stage of a microscope (Type FLUOPHOTO-VFD, Nikon Inc., Tokyo, Japan) with bright-field illumination provided by a 300 W tungsten-halogen lamp (Type JC-24V-300W, Kondo Sylvania Ltd., Tokyo, Japan) driven by a stable DC power supply (Model GPO35-20R, Takasago Ltd., Kawasaki, Japan). The microscope objective and eyepiece formed a magnified real image of the preparation on the target of a video camera.

The video-image processing system comprised a high signal/noise ratio video camera with a frame rate of 30 Hz (interlaced) (Model C1800, Hamamatsu Photonics Ltd., Hamamatsu, Japan); an image processor with an 8-bit video A/D converter and two 512(H)×485(V)×16-bit frame buffers (Model C1901 mark II, Hamamatsu Photonics Ltd.); a videocassette recorder; and a personal computer. The image processor and the computer were connected with an IEEE-488 bus line. The programs for controlling the image processor and for processing the image data were originally written in the Pascal language (Turbo Pascal, Borland International, Scotts Valley, CA, USA) by T.S. under the MS-DOS operating system.

In the chick embryo, the start of circulation of juvenile blood cells, which are suspended sparsely in the...
blood vessel, can be observed on the second day of incubation. In the embryo shown in Fig. 1, about 100 cells passed through the dorsal aorta in a minute. In this early stage of development, it is difficult to track individual blood cells because of the low contrast of the microscopic image of the preparation. We applied the video-imaging method to visualize the sparse juvenile blood cells and to quantitatively analyze their movement and the blood flow.

The image-processing procedure was based on the autosubtraction method [7]. The video image of the dorsal aorta, in which sparse juvenile blood cells circulated, was fed to the image processor. The maximum output of the video camera was set slightly below the saturation level of the input of the image processor by adjusting the intensity of the light source. First, the “control” image of the ventral view of the dorsal aorta was stored on the frame buffer of the image processor (Fig. 1B). Note that blood cells are hardly seen in this image. Then the control image was subtracted from the real-time image of the dorsal aorta. By means of digital contrast enhancement, each pixel within the subtracted image was classified into two categories: those for which the digitized value of the brightness was increased or remained unchanged, and those for which it decreased. These were painted in black and white, as shown in Fig. 1C. Using this procedure, we visualized the blood cells as white spots (indicated by arrowheads) on a black background. The processed image was recorded continuously on videotape. After the measurement, a series of frames with the time interval of 33.3 ms was obtained by means of “stop-motion” play. Each frame was stored on the frame buffer of the image processor, transferred to the computer, then stored as a series of images. A typical example of the processed image stored in the computer is shown in Fig. 1D. In this image the blood cells are displayed as black spots on a white background.

From the series of images, some blood cells were

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**Fig. 1. An example of the image-processing procedure.**

A: The ventral view of a stage 12+ chick embryo at a low magnification. The arrowhead indicates the heart, and the arrows indicate the left and right dorsal aorta and the somite. The square indicates the area of imaging shown in B, C, and D. B: The control image of the dorsal aorta. Note that sparse juvenile blood cells are hardly seen in this image. C: The subtracted image. The blood cells were visualized as white spots on a black background. D: The image stored in the computer. The blood cells are displayed as black spots (indicated by arrowheads) on a white background.
selected and their positions were plotted in chronological order with a time interval of 33.3 ms. In Fig. 2A, five examples of the plotted positions of a single blood cell are shown. These positions were obtained in a single continuous recording. The control image of the dorsal aorta, where the measurement was made, is shown in Fig. 2B. The right dorsal aorta runs obliquely in the center. The heart was located above the image so that in the plot the blood cells moved downward. The distances between the adjacent plots are not constant, indicating that the velocity of the blood flow continuously changed. From observation at lower magnification, the change in the velocity of the blood flow was rhythmical and synchronized with the cardiac cycle: The flow was fast in the systolic phase and slow in the diastolic phase. Note that at this stage of development, each juvenile blood cell was discriminated and the position of individual blood cells could be plotted.

To quantitatively analyze the movement of the blood cells (i.e., the blood flow), the position of each cell in the image was measured and its time course represented. An example is shown in Fig. 3. This figure was constructed from the plot shown as “#1” in Fig. 2A. In Fig. 3A, the cumulative distance of the movement of the blood cell in the dorsal aorta is shown. This graph shows the relative position of a blood cell in the dorsal aorta from the upper edge of the image. The distance increased slowly during the diastolic phase, then quickly in the systolic phase. This change in the cell’s velocity can be clearly observed in Fig. 3B, in which the instantaneous velocity of the blood cell is plotted against time. The velocity slowly decreased during the diastolic phase, then increased quickly in the systolic phase. As shown in this figure, the flow velocity of an individual blood cell during the cardiac cycle can be determined by using this method. It is plausible that this change of flow velocity of the blood cell reflects that of blood fluid.

In this study, we visualized juvenile blood cells in the blood vessel of the early chick embryo and were able to clearly show the dynamics of their flow. The video-imaging method reported here presents some important advantages over other techniques. First, it allows the visualization of the circulation of blood.

Fig. 2. Tracking of blood cells. A: Five examples of the plot of the position of the blood cell. The time interval between the spots is 33.3 ms. The heart was located above the image so that in the plot the blood cells moved downward. The plots were obtained in a single continuous recording. B: Image of the right dorsal aorta, where the measurement was made. Large arrowheads indicate the direction of the heart and the peripheral blood vessels. Small arrowheads with LW, MW, and NT indicate the lateral wall and the medial wall of the right dorsal aorta, and the neural tube at the midline. “S” indicates somite.

Fig. 3. Position and flow velocity of a blood cell. A: Cumulative distance of the movement of a blood cell in the dorsal aorta. B: Instantaneous velocity of the blood cell. This figure was constructed from the plot shown as “#1” in Fig. 2A.
cells in the small and weak blood vessels in the early embryo without any mechanical contact with the preparation. Second, blood vessels are kept intact during measurement and the loss of blood fluid is negligible. This means that the circulatory function of the early embryo is maintained in the physiological condition. Third, the spatiotemporal resolution of this method is sufficient to allow an analysis of the blood flow pattern in the early embryonic circulatory system. By using a similar video-imaging method, we have reported the contraction pattern of the early embryonic chick heart [8].

 Previously, Hu and Clark reported the flow velocity of blood cells in the dorsal aorta of the chick embryo at stage 12, using a Doppler velocity meter [9]. In their report, in the diastolic phase the velocity of the blood cells became zero, though a positive pressure was recorded in the vitelline artery. This finding does not coincide with our present observation that the blood cells were moving during the diastolic phase. This discrepancy seems to be based on the method they used: They did not observe the movement of individual blood cells, but measured the velocity of blood cells as a mass.

 In this video-imaging method we are able to track individual blood cells in the dorsal aorta. As the development proceeds, the density of the cells increases. The tracking of individual blood cells is impossible in the older embryo, in which the density of the blood cells is too high. Therefore the limitation of this method results from the increased density of blood cells, and the method is applicable only to embryos with sparse juvenile blood cells in the dorsal aorta at the beginning stages of circulation. However, at these stages of development it is quite difficult to apply other methods to evaluate the circulatory function.

 The study of the circulatory function during the early stage of ontogenetic development has been hampered by technical limitations because of the small size and fragility of embryonic blood vessels. The video-imaging method reported here would be a powerful tool for study in this field. Using this method, we can measure the change of flow velocity of a single blood cell, which reflects the pump function of the heart. We can also estimate the difference in the flow velocity between the center of the blood vessel and the vicinity of the vascular wall (i.e., velocity profile) in the early embryo. Further developmental study of the early embryonic circulation, including the blood vessel system and the heart, would be fruitful.

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REFERENCES