Chapter 2

Hemodynamic Flow Visualization of Early Embryonic Great Vessels Using μPIV

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Abstract

Microparticle image velocimetry (μPIV) is an evolving quantitative methodology to closely and accurately monitor the cardiac flow dynamics and mechanotransduction during vascular morphogenesis. While PIV technique has a long history, contemporary developments in advanced microscopy have significantly expanded its power. This chapter includes three new methods for μPIV acquisition in selected embryonic structures achieved through advanced optical imaging: (1) high-speed confocal scanning of transgenic zebrafish embryos, where the transgenic erythrocytes act as the tracing particles; (2) microinjection of artificial seeding particles in chick embryos visualized with stereomicroscopy; and (3) real-time, time-resolved optical coherence tomography acquisition of vitelline vessel flow profiles in chick embryos, tracking the erythrocytes.

Key words Tissue morphogenesis, Particle image velocimetry, Hemodynamics, Cardiovascular development, Aortic arch, Congenital heart defects, Confocal microscopy, Chick embryo, Zebrafish, Optical coherence tomography

1 Introduction

Increasing number of patients suffering from cardiac diseases, particularly congenital heart defects [1] and emphasis on mechanotransduction during cardiac development [2], has urged the exploration of experimental tools for rapid and accurate quantification of cardiac flows. Syndromes and abnormalities influencing cardiac function start during early embryonic development, where the direct assessment of hemodynamics is even more challenging due to microfluidic scales [3]. Thus, advanced imaging and visualization methods need to be employed to precisely detect the malformations of the embryonic cardiac vessel systems and guide towards the necessary fetal treatments.

Various techniques have been utilized in order to noninvasively quantify the blood flow dynamics of embryonic cardiovascular structures, such as the Doppler ultrasound [4], Doppler optical

coherence tomography (Doppler-OCT) [5], and traditional microparticle image velocimetry (μPIV) [6, 7]. This chapter focuses on the use of μPIV technology for two model systems, namely the chick and zebrafish embryos. These model organisms have been widely examined for investigating vertebrate cardiovascular development and are well documented [8, 9]. μPIV is most applicable to chick embryos at stage 24 (approximately 4 days after the start of incubation) when the cardiac looping transforms the heart into a looped tube [10]. In the case of the zebrafish, embryos raised to 32 and 48 h post-fertilization periods can be utilized for predicting flow patterns [11]. μPIV technique requires the labeling of flowing fiducial fluid elements. However, injecting artificial particles to the microcirculation of the zebrafish embryos and subsequent long-duration time-lapsed imaging are practically difficult due to bleeding. Furthermore, particles will interfere with normal cardiac physiology. Major remedy is to utilize transgenic zebrafish embryos [12] or the OCT-μPIV.

2   Materials

2.1   Transgenic Embryo Sources and Staging

1. Fertile white Leghorn chicken eggs are from a local farm. Eggs should be placed in an incubator of 37 °C and 50–60 % relative humidity (*see* **Note 1**). To facilitate experimental work, eggs should be incubated blunt end up.

2. Zebrafish (*Danio rerio*) were maintained according to standard protocols [13]. *chrna1b*107/*b*107 [14], *Tg*(*kdrl:GFP*)*la*116 [15], *Tg*(*gata1:DsRed*)*sd*2 [16] zebrafish embryos were raised to 32 and 48 h post-fertilization (hpf) for hemodynamic quantification.

2.2   Solutions and Seeding Particles

1. Zebrafish anesthetic: 400 mg Tricaine powder (Sigma-Aldrich Corp., St. Louis, MO, USA), 97.9 mL deionized water, 1 M Tris-HCL (Sigma-Aldrich Corp., St. Louis, MO, USA), pH 7.

2. Chick Ringer's solution: 135 mM NaCl, 4 mM KCl, 10 mM Tris–HCl, 10 mM Tris base, 11 mM dextrose, pH 7.4 (*see* **Note 2**).

3. Fluorescent particles: 0.5 μm fluorescent polystyrene microspheres (Thermo Scientific Fluoro-Max Red Aqueous Fluorescent Particles, Waltham, MA, USA). Dilute the 1 % solid stock particle solution to 0.05 % solids with Ringer's solution. Add 150 μL of the 1 % solid solution to 2.85 mL Ringer's solution and vortex. The diameter and seeding concentration of artificial particles should be optimized for the specific PIV equipment (*see* **Note 3**).

2.3 Zebrafish Confocal Microscopy and Microfluidic Platform

1. SU8 2075 photoresist and developer (MicroChem Corp., Newton, MA, USA).

2. Polydimethylsiloxane (PDMS) (Dow Corning Corp., Midland, MI, USA).

3. Acetone and Isopropanol (Sigma-Aldrich Corp., St. Louis, MO, USA): As an alternative, agarose gel (Sigma-Aldrich Corp., St. Louis, MO, USA) can be used to mount embryos during imaging (*see* ref. 12).

4. Hot plate.

5. Micro-poker.

6. Spin coater (Solitec Photoresist Spinner, WT Services, Inc., Fitchburg, MA, USA).

7. Silicon wafer.

8. Plasma cleaner.

9. Vacuum oven.

10. N_2 gun.

11. Petri dishes of 14 cm diameter (Fisher Scientific International Inc., Hampton, NH, USA).

12. Curing agent (Dow Corning Corp., Midland, MI, USA).

13. DaVis 7.2 PIV (LaVision, Inc., Goettingen, Germany) software is adopted for quantification of hemodynamics in blood vessels.

14. A Leica TCS SP5 confocal microscope with resonant scanner (Leica Microsystems GmbH, Wetzlar, Germany) is used for fluorescent imaging.

2.4 Chick Embryo Microscopy and Instrumentation

1. Leica MZ 16FC stereomicroscope with external Leica EL6000 mercury halide light source coupled through the collimator for episcopic fluorescence (Leica Microsystems GmbH, Germany) (*see* **Note 4**). The microscope binocular includes a beam splitter to direct light to both eyepieces or one eyepiece and a mounted camera.

2. Leitz 3-axis micromanipulator.

3. Dumont #55 and 5MS tweezers (WPI, Sarasota, FL, USA).

4. Vannas microscissors, 8 cm length with 5 mm blades and 0.1 mm tips (WPI, Sarasota, FL, USA).

5. 28 gauge MicroFil needle (WPI, Sarasota, FL, USA).

6. 10 μL Hamilton gastight syringe with Luer tip.

7. 20 gauge blunt tip needle.

8. PE-60 tubing (Braintree Scientific, Braintree, MA, USA).

9. PC-10 puller and EG-44 microgrinder (Narishige Inc., Japan) for generating 20–30 μm microneedles used for injection (*see* **Note 5**).

10. Photron SA4 high speed camera, Leica FX350 high speed camera.

2.5 Chick Embryo Optical Coherence Tomography

1. Thorlabs Ganymede Spectral Domain Optical Coherence Tomography (OCT) system: The OCT light source is a 930 nm superluminescent diode with a 100 nm spectral bandwidth. Axial resolution is 5.8 μm in air (4.3 μm in water) and lateral resolution is 8 μm. The maximum A-scan rate is 29 kHz (*see* **Note 6**). The spectrometer consists of a 12-bit high-sensitivity CCD camera with 2.0 μm pixel spacing. Data is transferred in real time over a GigE connection to a PC with a 3.3 GHz processor. A refraction index of 1.33 is used for the aqueous environment of the chick embryo. The OCT stage includes an XY-axis manipulator for fine adjustment of the embryo position. The position of the scanning plane can be further adjusted by shifting the Galvo mirrors enclosed in the probe.

2. A custom-built enclosed clear plastic chamber surrounding the OCT imaging probe and egg to maintain a 37 °C and 60 % humidity environment. Important features include a 250 W heat lamp connected to a temperature controller and a drug-store humidifier. Adjustable vents on opposing sides and a computer fan create adequate air circulation.

3 Methods

3.1 Embryo Staging

This protocol is designed to quantify microcirculation of embryos at selected developing stages. To ensure the healthy growth of the tested developing zebrafish, a zebrafish breeding protocol is followed (*see* ref. 13). The morphological changes of zebrafish embryos during this time window are quantified and described in Table 1. Chick embryos are staged according to Hamburger and Hamilton [17]. For early embryos (prior to stage 26), the number of somites and angle between the axial and cranial axes are primary indicators of the development stage. Blood flow begins between stages 11 and 12 (40–45 h).

3.2 Microfluidic Platform Fabrication

To immobilize zebrafish embryos during time-lapse confocal-μPIV imaging, a biocompatible polydimethylsiloxane (PDMS) platform is fabricated through a microfabrication procedure as described below:

1. Rinse a 4-in. silicon wafer and a 5-in. transparency (which defines the geometry of the platform) with acetone and

Table 1
Dimensions of zebrafish embryos during 24–48 hpf (hours post-fertilization)

(mm)				
28 hpf	0.56	0.60	1.07	1.78
36 hpf	0.47	0.55	1.38	2.14
48 hpf	0.34	0.39	1.30	1.94

isopropanol. Blow-dry the wafer and the transparency with N_2 gun after the rinse.

2. Place the wafer onto a spin-coater stage for SU8 2075 film spinning. The set spinning parameters are 10 s at 500 rpm and subsequent 30 s at 2,000 rpm.

3. Place the wafer with SU8 film (~150 μm thick) on top of a hot plate for 60 min at 65 °C (see **Note 7**).

4. Allow the wafer to cool to room temperature and harden after heating. Expose the wafer with a 365 nm ultraviolet (UV) light source for 145 s.

5. Heat the wafer for 22 min at 65 °C. Allow the wafer to cool to room temperature again.

6. Develop the exposed SU8 film using an SU-8 developer for approximately 20 min. Apply a 10-s rinse with isopropanol. Dry the wafer with N_2 gun and inspect it with a profilometer to examine the features of the SU8 mold.

7. To make a corresponding PDMS platform replica using the SU8 mold, firstly place the SU8 mold in a petri dish (*see* **Note 8**).

8. Mix PDMS base and curing agent in a 10:1 (base:agent) ratio of mixture by weight (example 30 g base, 3 g agent).

9. Place the mixture in a centrifuge tube and centrifuge for about 20 s to remove bubbles.

10. Bring a cup of PDMS mixture close to the surface of the SU8 mold. Pour it onto the center of the mold and spread around it (*see* **Note 9**).

11. Place the SU8 mold filled with PDMS in a vacuum oven. Wait for 2 min and then release the vacuum quickly. Repeat this procedure until the majority of small bubbles have been dispersed.
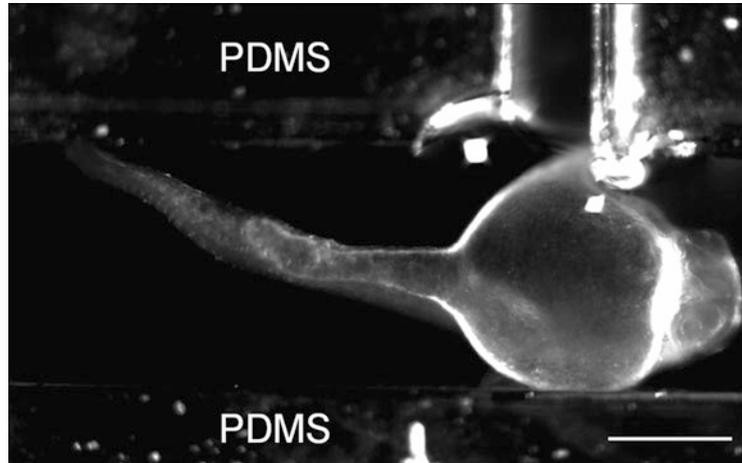
Fig. 1 A zebrafish embryo is immobilized in a PDMS microfluidic platform to facilitate the time-lapse imaging. Scale bar: 400 μm

12. Heat PDMS for 120 min at 60 °C. Remove cured PDMS from the SU8 mold by loosening edges with a scraping tool. Continue with plasma cleaning of the PDMS microfluidic platform (Fig. 1) [18].

***3.3 Volumetric Confocal Microscopy Imaging of Zebrafish***

1. Position embryos laterally on the stage of a confocal microscope to have a visual of erythrocyte flows.

2. Record time-lapse images (256 × 64 pixels) using a 20× oil objective on a confocal microscope fitted with Ar and HeNe lasers (488 and 543 nm) (*see* **Note 10**).

3. Acquire images using 8,000 Hz (phase-matched bidirectional resonant scanner). Set the full-frame acquisition rate at 175 frames per second (4,000 frames can be recorded per dataset). Record at least 20 cardiac cycles, with 60–90 data points sampled in each cardiac cycle.

3.4 Microinjection of Seeding Particles into the Chick Embryo

1. Candle the egg by holding it in front of a bright white light source to view the position of the air sac. Place the egg in a holder made from an empty scotch tape ring, round bottom flask holder, or other stable platforms. Using the blunt end of the tweezers, gently crack the shell above the air sac and peel away to create a small window around 1 cm². Remove any underlying shell membrane blocking the embryo. With the tweezers, gently peel away the slightly opaque membrane covering the embryo and surrounding vitelline circulation. Only peel away enough to expose the embryo and area planned for injection and PIV acquisition (*see* **Note 11**). The embryo should appear right side up. Left side up or otherwise

dysmorphic embryos should be discarded unless the experiment calls for them. The window can be covered with Parafilm and the egg is placed back in the incubator if necessary.

2. Thread about 18 cm of PE-60 tubing onto the 20 gauge blunt-tip needle and connect to the Hamilton syringe. Attach the Hamilton syringe to the body of the micromanipulator with scotch tape. Prime the syringe and tubing with Ringer's solution using the MicroFil to perfuse from the syringe to the tubing. Replace the syringe plunger when done. Using the MicroFil, prime the microneedle with Ringer's solution. Attach the microneedle to the end of the tubing, being sure to avoid the introduction of air bubbles (*see* **Note 12**). Fix the microneedle to the end of the micromanipulator using a clamp or other devices, being sure to give enough length to reach the egg.

3. Put a small amount (3 μL) of the fluorescent dye solution in a shallow dish (the cap cut from a 2 mL centrifuge tube works well) and place on the microscope stage. Using the micromanipulator, carefully position the tip of the microneedle into the drop of solution. Draw the fluorescent particles into the tip of the microneedle by retracting the plunger of the Hamilton syringe. Verify that the microneedle has taken up some solution by examining with the microscope.

4. Place the windowed egg under the microscope. If the embryo is stage 18 or older, dissect away any additional membranes covering the desired injection site using the tweezers and microscissors. Do not remove more membrane than necessary.

5. Using the micromanipulator, position the microneedle at the desired injection site and gently advance to puncture the vascular tissue (*see* **Note 13**). Blood cells should be observed within the tip of the microneedle to ensure penetration into the lumen. Bleeding should not be observed, as it indicates a complete puncture through the vessel. If bleeding is observed, the microneedle is withdrawn and the embryo is discarded. Figure 2 shows a stage 21 (3.5-day) embryo with the needle in place.

6. Turn on the external fluorescent light source and rotate the filter carriage to the appropriate set. At this time, switch to a higher objective if possible (*see* **Note 4**).

7. Inject the desired volume of solution by depressing the plunger of the Hamilton syringe. Light pressure should be applied not to burst the delicate embryo. Injection volumes of up to 0.5 μL have been shown to minimally affect normal hemodynamics (<10 % change in stroke volume and mean arterial pressure) [19]. A section of the vitelline vasculature with seeded particles is shown in Fig. 3.
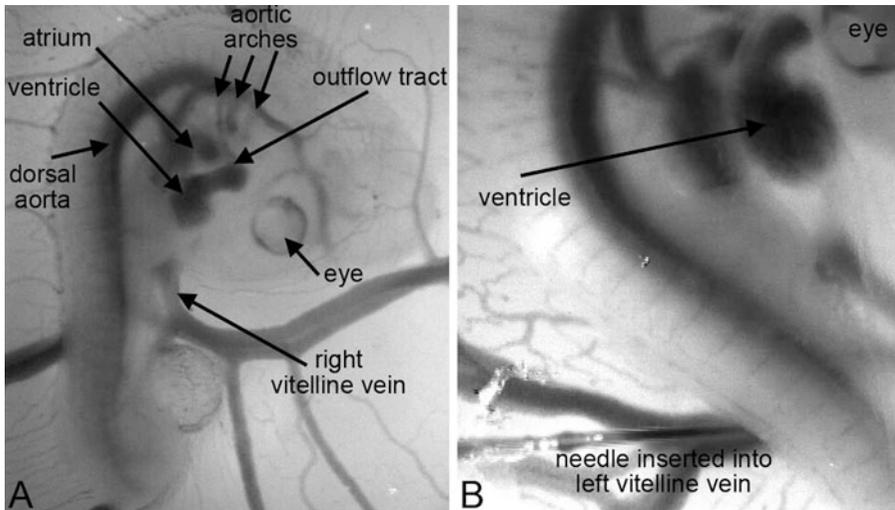
Fig. 2 A stage 21 (3.5-day) chick embryo. (**a**) The embryo vas viewed under a stereomicroscope with major cardiovascular structures labeled. (**b**) A microneedle is inserted into the left vitelline vein to facilitate injection of microparticles

Fig. 3 A section of the extraembryonic vitelline vascular network with seeded half micron microparticles after injection. Blood flow is right to left in the large vessel. The sparse background is due to particles within the smaller vessels and capillaries

8. Record the circulation of particles for PIV post-processing. The embryonic heart rate between stages 18 and 24 ranges from 1 to 3 Hz and peak dorsal aortic blood velocity is approximately 30 mm/s. Velocities in the primary vitelline veins range from 1 to 10 mm/s. Data acquisition at 100 frames per second is therefore required for vessels such as the dorsal aorta, while 30 frames per second is sufficient for the extraembryonic regions.
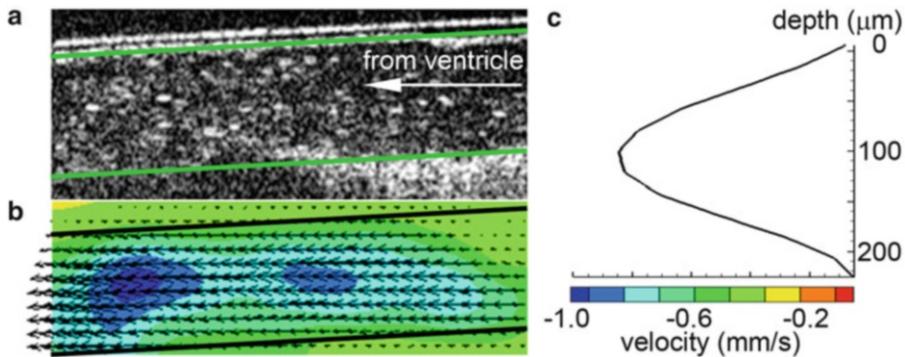
Fig. 4 Velocity vectors in the right vitelline vein of a stage 18 (3-day) embryo computed from OCT data. (**a**) Sample frame from a 2D OCT image sequence. *Green lines* mark the vessel boundaries. (**b**) Cycle-averaged velocity vectors computed from PIV of erythrocytes. *Black lines* mark vessel boundaries. (**c**) The velocity profile along the *dotted line* in **b** (Color figure online)

3.5 Optical Coherence Tomography Acquisition for PIV

1. Turn on the incubation chamber and allow it to stabilize temperature and humidity.

2. Window the egg to expose the embryo as described previously (*see* Subheading 3.4, **step 1**).

3. Place the egg on the OCT stage. Turn on the OCT system and apply appropriate settings, including scan width, number of A-lines, and index of refraction.

4. Begin acquiring 2D B-scans with live streaming. Position and rotate the embryo such that the scanning plane intersects the vessel longitudinally. Use the OCT Galvo controls to finely adjust the scanning plane angle and position. Ensure that the scanning direction and blood flow direction are the same.

5. Adjust the scanning width and number of A-lines to achieve the desired frame rate (*see* **Note 14**).

6. Stop live streaming and set the record options for the application. Record a set number of B-scans or for a set number of seconds.

7. Restart the scan and record the data (*see* **Note 15**). Figure 4 shows a sample processed dataset of blood flow in the stage 18 right vitelline vein.

3.6 Time-Lapse Flow Velocity Measurement

1. Calculate velocity vectors of blood flows using a fast Fourier transform cyclic algorithm with second-order correlation from the PIV software (*see* **Note 16**).

2. Employ Gaussian peak fit as a three-point estimator to obtain sub-pixel accuracy measurement. Apply a multi-pass post-processing median filter to remove bad vectors >2 root mean square (rms) velocity, and reinserted if <3 rms of neighboring vectors (Fig. 5).
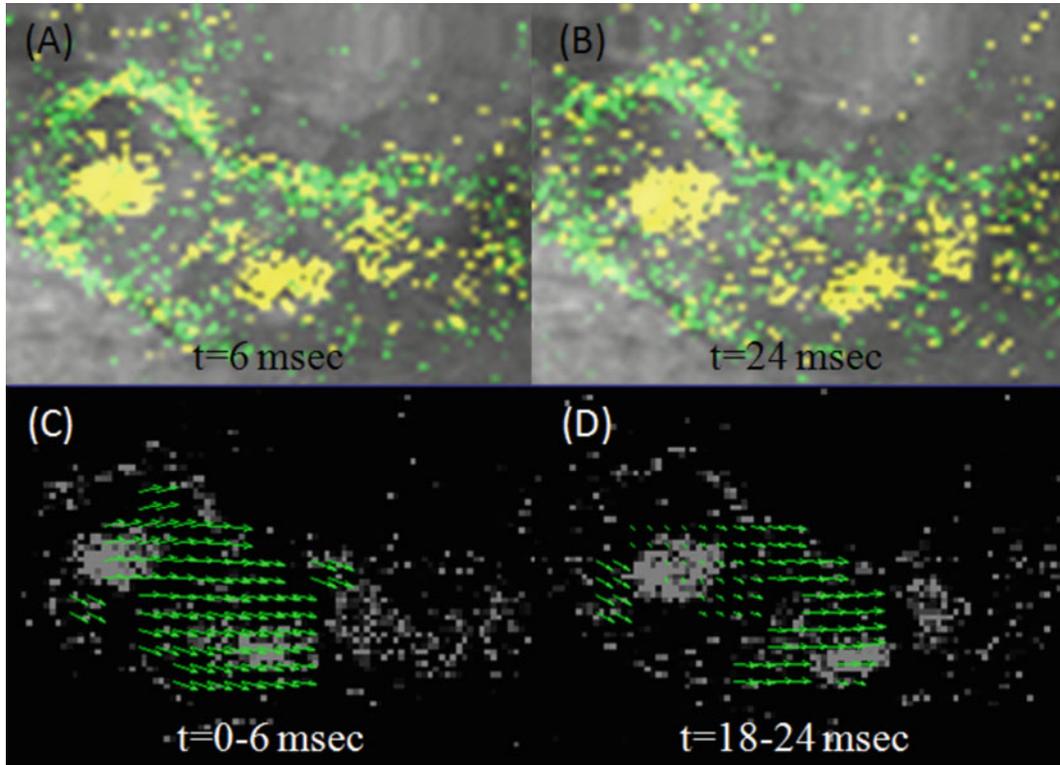
Fig. 5 An example of calculated velocity vectors of a blood flow in a zebrafish embryo at two instants. (**a**) and (**b**) show raw single-scan confocal images; (**c**) and (**d**) show velocity vectors (adapted from [11]) (reproduced with permission from IOS Press, The Netherlands)

3.7 Wall Shear Stress (WSS) Calculation

1. Extract instantaneous streamwise velocity from a 50×30 pixel window within a two-dimensional time-lapse PIV velocity map.

2. Integrate the streamwise velocity component along the vessel cross section to provide the instantaneous average blood flow rate (*see* **Note 17**).

3. Calculate the averaged diameters of the vessel using the average of systolic and diastolic diameters throughout three cardiac cycles.

4. Orient embryos to access a region of unidirectional flow.

5. Estimate the instantaneous WSS values from Eq. 1, as follows (*see* **Note 18**):

$$\tau = \mu \frac{\partial u}{\partial r} \qquad (1)$$

$\frac{\partial u}{\partial r}$ represents the streamwise normal velocity gradient along the diameter of a blood vessel of interest (*see* ref. 11).

4   Notes

1. Embryos will not begin development until the egg is placed in the incubator. To have the correct stage embryo for experiment, it is important to coordinate the start time of the incubation. Eggs that are not incubated soon after laying generally show delayed developmental stages, and this delay increases with the amount of time waited before incubation begins. Starting incubation more than 3 days after laying results in a low yield (<50 %).

2. Weigh 7.89 g NaCl, 0.30 g KCl, 1.58 g Tris–HCl, 1.21 g Tris base, and 1.98 g dextrose and transfer to the vessel. Dilute to a total volume of 900 mL with distilled water and mix until clear. Adjust the pH with 1 N HCl. Dilute to 1 L with distilled water. Store the solution in a closed container at 4 °C.

3. Keane and Adrian demonstrated an optimal particle seeding concentration depending on the PIV optics [20]. For volumetric illumination, the depth of correlation (thickness that influences the correlation result) is related to particle diameter [21].

4. A stereomicroscope is useful for work with *in ovo* chick embryos, given the long working distance of the objective lens. It is helpful to have an external white light source. A gooseneck illuminator is preferable, as it allows for easy positioning and minimally obstructs access to the embryo. A 1.0× objective (working distance of 60 mm) can be used for embryo manipulation, including injection, while a 5.0× objective can be used for PIV data acquisition. The Leica MZ 16FC can be equipped with rotating objective turret (FluoCombi III) for this purpose. The Leica MZ 16FC uses a floating lens to adjust the zoom level, ranging between 0.63× and 11.5×. The focus can be controlled digitally and locked to preserve the focal plane. A variety of dichroic filter sets are manufactured for use with the fluorescence module.

5. The microneedle is pulled from a 1.0 mm inner diameter glass capillary tube. After pulling, the microneedle is beveled to a 30° tip angle using a microgrinder for about 30 min. The microneedle should be inspected for chips prior to use. The same microneedle can be used for up to four injections unless it is broken or blocked between the injections. Fluorescent particles tend to aggregate and block the microneedle. They can be flushed with distilled water using the MicroFil.

6. The maximum A-scan rate cannot be achieved due to data transfer from the OCT engine to the PC. Live streaming of the B-scan further degrades the actual scan rate. In the case of live streaming, frame rates of 25.1 fps and 17.1 fps are achieved for a 512 and 757 A-line image, respectively.

7. During the baking of the SU8 film, use a micro-poker (small sharp glass rod) to pop bubbles while heating.

8. A paperboard dam around the platform area can be prepared to reduce the amount of PDMS needed.

9. When pouring PDMS into SU8 mold, be sure to pour the mixture slowly and close to the mold in order to reduce the formation of bubbles.

10. Set high-sensitive photomultiplier (PMT) detection in the range of 495–536 nm and 555–640 nm for green fluorescent protein (GFP) and red fluorescent protein (DsRed) signals, respectively.

11. To peel away the extraembryonic membrane, it is easiest to find a place where the membrane has buckled, grab with the tweezers, and pull gently. Pulling in a circular pattern may help to bring as much membrane away as possible. It is important not to break any vessels, as the embryos cannot clot until day 8.

12. The outer diameter of the glass capillary is slightly larger than the inner diameter of the tubing to ensure a tight seal. It helps to expand the end of the tubing using the tweezers or a heated metallic probe.

13. Typical injection sites include the large vitelline veins. These vessels are external to the embryo and close to the surface, allowing easy access. If the embryo is stage 18 or older, remove the overlying vitelline membrane using the tweezers and microscissors. Vitelline vessels are around 300 μm at stage 18. The microneedle tip should be 1/10th the vessel diameter. Position the embryo such that the microneedle and vessel are parallel. If the microneedle pushes the vessel and does not puncture, it is likely dull and should be replaced.

14. The lateral scanning width and number of A-lines should be optimized to allow the fastest frame rate without sacrificing lateral pixel resolution. These parameters will set the maximum velocity that can be acquired. A faster frame rate and larger scanning width will increase the number of frames that contain the same particles, allowing higher velocities. However, increasing the frame rate, by reducing the number of A-lines, and expanding the scanning width quickly reduce the lateral pixel resolution (scan width divided by number of A-lines). In general, the maximum velocity that can be measured is slightly less than the scan width multiplied by the frame rate.

15. The Thorlabs OCT software writes data to a propriety file type. These files can be read easily with programs developed in MATLAB or another language. Each frame is exported as a TIFF image for use with the PIV processing software.

16. 16×16 pixel with 50 % overlap for two iterations is used as the first-pass interrogation window. The second pass is selected as 12×12-pixel interrogation size with 50 % overlap for two iterations.

17. The Womersley numbers of the embryonic microcirculation are generally low (<1). As such, an assumption of a quasi-steady parabolic plasma velocity profile is justified.

18. Equation 1 is applied with the assumptions of conservation of mass as well as the no-slip boundary conditions at the vessel walls. Embryos with significant out-of-plane velocity components at the imaging plane should be discarded.

Acknowledgement

References

1. Pekkan K, Keller BB (2013) Developmental fetal cardiovascular biomechanics in the 21st century: another tipping point. Cardiovasc Eng Technol 4:231–233

2. Buskohl PR, Jenkins JT, Butcher JT (2012) Computational simulation of hemodynamic-driven growth and remodeling of embryonic atrioventricular valves. Biomech Model Mechanobiol 11:1205–1217

3. Kowalski WJ, Dur O, Wang Y, Patrick MJ, Tinney JP, Keller BB, Pekkan K (2013) Critical transitions in early embryonic aortic arch patterning and hemodynamics. PLoS One 8: e60271

4. Oosterbaan AM, Ursem NT, Struijk PC, Bosch JG, van der Steen AF, Steegers EA (2009) Doppler flow velocity waveforms in the embryonic chicken heart at developmental stages corresponding to 5–8 weeks of human gestation. Ultrasound Obstet Gynecol 33:638–644

5. Gu S, Jenkins MW, Peterson LM, Doughman YQ, Rollins AM, Watanabe M (2012) Optical coherence tomography captures rapid hemodynamic responses to acute hypoxia in the cardiovascular system of early embryos. Dev Dyn 241:534–544

6. Chen C, Menon PG, Kowalski W, Pekkan K (2013) Time-resolved OCT-μPIV: a new microscopic PIV technique for noninvasive depth-resolved pulsatile flow profile acquisition. Exp Fluids 54:1426

7. Vennemann P, Kiger KT, Lindken R, Groenendijk BC, Stekelenburg-de Vos S, ten Hagen TL, Ursem NT, Poelmann RE, Westerweel J, Hierck BP (2006) In vivo micro particle image velocimetry measurements of blood-plasma in the embryonic avian heart. J Biomech 39:1191–1200

8. Martinsen BJ (2005) Reference guide to the stages of chick heart embryology. Dev Dyn 233:1217–1237

9. Bakkers J (2011) Zebrafish as a model to study cardiac development and human cardiac disease. Cardiovasc Res 91:279–288

10. Manner J (2000) Cardiac looping in the chick embryo: a morphological review with special reference to terminological and biomechanical aspects of the looping process. Anat Rec 259:248–262

11. Chen CY, Patrick MJ, Corti P, Kowalski W, Roman BL, Pekkan K (2011) Analysis of early embryonic great-vessel microcirculation in zebrafish using high-speed confocal muPIV. Biorheology 48:305–321

12. Corti P, Young S, Chen CY, Patrick MJ, Rochon ER, Pekkan K, Roman BL (2011) Interaction between alk1 and blood flow in the development of arteriovenous malformations. Development 138:1573–1582

13. Westerfield M (2000) The Zebrafish book. University of Oregon Press, Eugene

14. Sepich DS, Wegner J, O'Shea S, Westerfield M (1998) An altered intron inhibits synthesis of the acetylcholine receptor alpha-subunit in the paralyzed zebrafish mutant nic1. Genetics 148:361–372

15. Choi J, Dong L, Ahn J, Dao D, Hammerschmidt M, Chen JN (2007) FoxH1 negatively modulates flk1 gene expression and vascular formation in zebrafish. Dev Biol 304:735–744

16. Traver D, Paw BH, Poss KD, Penberthy WT, Lin S, Zon LI (2003) Transplantation and in vivo imaging of multilineage engraftment in zebrafish bloodless mutants. Nat Immunol 4:1238–1246

17. Hamburger V, Hamilton HL (1951) A series of normal stages in the development of the chick embryo. J Morphol 88:49–92

18. Patrick MJ, Chen C, Frakes DH, Dur O, Pekkan K (2011) Cellular-level near-wall unsteadiness of high-hematocrit erythrocyte flow using confocal μpIV. Exp Fluids 50:887–904

19. Wagman AJ, Hu N, Clark EB (1990) Effect of changes in circulating blood volume on cardiac output and arterial and ventricular blood pressure in the stage 18, 24, and 29 chick embryo. Circ Res 67:187–192

20. Keane RD, Adrian RJ (1990) Optimization of particle image velocimeters. 1. Double pulsed systems. Meas Sci Technol 11:1202–1215

21. Olsen MG, Adrian RJ (2000) Out-of-focus effects on particle image visibility and correlation in microscopic particle image velocimetry. Exp Fluids 29:S166–S174