Measurements of the wall shear stress distribution in the outflow tract of an embryonic chicken heart

C. Poelma^{1,*}, K. Van der Heiden², B. P. Hierck³, R. E. Poelmann³
and J. Westerweel¹

¹*Laboratory for Aero and Hydrodynamics, Delft University of Technology, Leeghwaterstraat 21, 2628 CA Delft, The Netherlands*

²*British Heart Foundation Cardiovascular Sciences Unit, National Heart and Lung Institute, Imperial College London, London W12 0NN, UK*

³*Department of Anatomy and Embryology, Leiden University Medical Center, PO Box 9600, 2300 RC Leiden, The Netherlands*

In order to study the role of blood–tissue interaction in the developing chicken embryo heart, detailed information about the haemodynamic forces is needed. In this study, we present the first *in vivo* measurements of the three-dimensional distribution of wall shear stress (WSS) in the outflow tract (OFT) of an embryonic chicken heart. The data are obtained in a two-step process: first, the three-dimensional flow fields are measured during the cardiac cycle using scanning microscopic particle image velocimetry; second, the location of the wall and the WSS are determined by post-processing flow velocity data (finding velocity gradients at locations where the flow approaches zero). The results are a three-dimensional reconstruction of the geometry, with a spatial resolution of 15–20 µm, and provides detailed information about the WSS in the OFT. The most significant error is the location of the wall, which results in an estimate of the uncertainty in the WSS values of 20 per cent.

Keywords: *in vivo* measurement; physiology; wall shear stress; scanning micro-particle image velocimetry

1. INTRODUCTION

In the embryonic stage, the cardiovascular system starts functioning even before its development is completed. During this time, blood flow affects the development of the primitive heart and blood vessels, thereby strongly coupling form and function. This coupling stems from the interaction between haemodynamic forces and biological responses at the cell level, in particular in the endothelial cells, i.e. the cells that line the inner wall of blood vessels. These responses can be short term (e.g. the control of vascular tone) or long term (through gene expression). A more detailed discussion of the interaction between blood flow and the vessel wall during development is given in reviews by Reneman *et al.* (2006) and Hierck *et al.* (2008a). Note that the exact manner in which the blood flow is sensed by the cell and the subsequent biological response within the cell (the processes of mechanosensing and mechanotransduction, respectively) have not been completely clarified yet (see the reviews by Davies (1995), Hierck *et al.* (2008b) and Poelmann *et al.* (2008)). The response of endothelial cells to local flow phenomena is critical for the normal development of an embryo (Topper & Gimbrone 1999; Hove

et al. 2003). To illustrate this, we refer to recent studies using chicken embryos, a model system commonly used for human development. These studies have shown that an alteration of flow in the heart leads to malformations in the cardiovascular system later on (see work using the ‘venous clip model’ (Hogers *et al.* 1997; Groenendijk *et al.* 2007)). Flow analysis tools in this ongoing project have been extended significantly in the last decade, improving from qualitative visualization (Hogers *et al.* 1997) to detailed two-dimensional flow measurements (Vennemann *et al.* 2006) and numerical flow simulations (Groenendijk *et al.* 2005).

In this study, we focus on the flow pattern in the so-called outflow tract (OFT, figure 1) of the heart of a chicken embryo. This is the last segment of the embryonic heart, which resembles a looped, contracting tube at the stage under investigation (HH17, approximately 3 d after incubation; see Hamburger & Hamilton (1951) for a description of the developmental stages and Männer (2000) for a detailed discussion of the looping process). Changes in gene expression, which are linked to eventual birth defects, are most significant in this part of the heart after alteration of the cardiac inflow by an extra-embryonic venous clip (Groenendijk *et al.* 2005). The aim of this study is to document the haemodynamics in the OFT in detail. In particular, we aim to

*Author for correspondence (c.poelma@tudelft.nl).

Figure 1. A schematic of the embryonic chicken heart at HH17 stage (75 h post fertilization). The dashed rectangle indicates the location and orientation of the measurement region, including the coordinate system used. Scale bar, 100 μm .

determine the distribution of wall shear stress (WSS)—the friction force per unit area acting on the endothelium parallel to local blood flow. It has been hypothesized that this force is the major mechanism of interaction between flow and tissue (Topper & Gimbrone 1999).

Direct measurement of the WSS is nearly impossible, especially in a living system. The current practice is to estimate it using the mean velocity or flow rate (q) and the vessel diameter (D). Implicitly, one needs a model of the flow under investigation. Usually, it is assumed that the flow is parabolic (Poiseuille's law), so that the mean WSS $\bar{\tau}_w$ is found using

$$\bar{\tau}_w = \frac{32\eta q}{\pi D^3}, \quad (1.1)$$

where η is the dynamic viscosity, assuming a Newtonian fluid. In pulsating flows in a complex, non-stationary and curved geometry, the validity of equation (1.1) is questionable; often the flow profile deviates from the expected parabola (Vennemann *et al.* 2006; Poelma *et al.* 2008). Additionally, equation (1.1) gives only an integral value for, say, a blood vessel segment (hence the overbar indicating that we are dealing with an average of the WSS).

In this study, the WSS is measured without the need for assumptions about the flow. Furthermore, it can give insight into the spatial distribution of the flow quantity. The WSS is determined indirectly, in a two-step process: first, the three-dimensional flow field is measured using scanning microscopic particle image velocimetry (PIV). This technique determines local flow velocity by imaging the displacement of small tracer particles that are added to the flow (Adrian 1991). The microscopic implementation of this technique with a scanning measurement plane is discussed in §2.2. The resulting flow data are used to determine the location of the wall and the values of WSS by post-processing: the WSS is determined from the derivative of the velocity near the wall. These post-processing steps are discussed in §3.

The small scale and complexity of the moving geometry (see table 1 for an overview of some typical

Table 1. Typical flow characteristics in the OFT at HH stage 17.

maximum flow velocity, v_{max} (mm s^{-1})	40
OFT diameter, D (μm)	250
heart rate, f (b.p.m.)	147
maximum flow rate, q_{max} ($\text{mm}^3 \text{s}^{-1}$)	1
dynamic viscosity, η_{blood} (Pa s)	0.004
Reynolds number (max), $Re_{\text{max}} = \rho v_{\text{max}} D / \eta$ (—)	2.5
Womersley number, $\alpha = D/2 (2\pi f \rho / \eta)^{1/2}$ (—)	0.25

flow characteristics) require a sophisticated measurement system. As we will demonstrate in this paper, three-dimensional flow measurement—and derived WSS data—is possible with a resolution of typically 15–20 μm . The latter is mainly determined by the optical magnification that is used. The measurement technique can readily be applied to other model systems, provided that there is adequate optical access.

2. METHODS

2.1. Chicken embryos

Fertilized White Leghorn eggs (*Gallus domesticus*) were incubated for 75 h at 37°C and 60 per cent humidity. The embryos were inspected and were used only if they had developed normally to HH stage 17. A small window (approx. $2 \times 2 \text{ cm}^2$) was removed from the shell and overlying membranes were carefully removed to allow access to the embryo (figure 2). The eggs were placed under an epifluorescent microscope (Leica MZ 16 FA), while partially submerged in a temperature-controlled water bath. Dehydration was reduced by adding a thin film of mineral oil. Furthermore, after manipulations of the embryo (in this case injection of tracer particles, see below), the window in the egg shell was covered using a microscope slide. The presence of the thin film of mineral oil and the glass slide did not noticeably decrease the image quality. All these experiments were performed according to national and institutional guidelines.

2.2. Flow measurement

The first step towards obtaining the WSS was the measurement of the flow velocities in the OFT. This was achieved using *in vivo* microscopic PIV. Detailed descriptions of this measurement technique—as applied to chicken embryos—are given by Vennemann *et al.* (2006) and Poelma *et al.* (2008); only a general description is given here.

To visualize the blood flow, bio-inert 1 μm polyethylene glycol (PEG)-coated polystyrene particles containing a fluorescent dye were introduced into the blood (Microparticles GmbH). The particles, suspended in phosphate-buffered saline, were injected into one of the extra-embryonic blood vessels. The total injection volume was $<1 \mu\text{l}$. After injection, the particles were rapidly transported throughout the embryo without any known adverse effects. Owing to their small size, tracer particles can be expected to follow the flow accurately; however, their size is sufficiently large so as not

Figure 2. Preparation of an egg for measurements. (a–d) Cutting the shell, removing the window, removing the membranes and protecting the embryo with a layer of phosphate-buffered saline and a cover glass. Images reproduced with permission from Vennemann (2008).

to penetrate the glycocalyx or endothelial cell layer (Vennemann 2008). As the particle Reynolds number is negligibly small, the particles do not exhibit Fåhræus–Lindqvist-like lateral migration towards the centre of the lumen (Segré & Silberberg 1962; Goldsmith *et al.* 1989). Even if there were a lower concentration of particles near the walls, this would not invalidate the measurements: with the PIV method, local velocity can be determined even if only a single tracer particle passes through the measurement location.

The tracer particles were illuminated by a pulsed dual-cavity Nd:YLF laser (New Wave Pegasus, 10 mJ/pulse at 527 nm). The particles emit fluorescent light that passes through the dichroic mirror of the epifluorescent microscope. The light with the original wavelength was blocked, thereby greatly enhancing the signal-to-noise ratio (Santiago *et al.* 1998). Tracer images were recorded using an intensified charge-coupled device camera (PCO Sensicam QE, 1376 × 1040 using 2 × 2 binning). The camera, laser and data acquisition were controlled by a PC running DaVIS 7.2 (LaVision GmbH). The camera recorded image pairs at 10 Hz, with a temporal separation of 500 μ s between the frames of each pair. The latter value was chosen in accordance with the optimization rules described by Keane & Adrian (1992) to optimize measurement reliability and precision. At this interval between the frames, the maximum tracer displacement at peak systole is 20–25 pixels, which is equivalent to 15–20 μ m.

Each measurement consisted of a series of 500 image pairs, with a duration of approximately 50 s. While the individual image pairs can be used to determine the instantaneous velocity field, accuracy can be greatly improved by phase averaging: the contracting motion of the heart is cyclic and, as the Reynolds number of the flow is of the order of unity, the flow can be expected to be periodic as well. For each image pair, the mean flow was first determined. Based on this mean flow, a phase ϕ was assigned (with $\phi = 0$ corresponding to systole). Subsequently, all image pairs with a similar phase were used to calculate the flow pattern at that particular phase. In practice, it suffices to divide the cardiac cycle into 10 phase steps, which results in an average of 50 image pairs per phase group. This procedure is described in detail by Poelma *et al.* (2008).

After recording the 500 image pairs, the measurement was repeated at a slightly lower z -location using the computer-controlled translation stage of the

microscope. The spacing between the planes was chosen to be 12 μ m, comparable to both the in-plane and out-of-plane resolution, determined by the correlation depth¹ (Olsen & Adrian 2000).

The displacement of the tracer particles was used to estimate the local flow velocity in each plane by means of a correlation-averaging PIV algorithm (Meinhart *et al.* 2000). In PIV, the mean displacement of the tracer particles is determined using local cross-correlation of image pairs. For each phase group, the intermediate correlation data are averaged to improve the signal-to-noise ratio (Meinhart *et al.* 2000). Processing was done with an in-house Matlab script using grid refinement (Willert 1997), with a final spatial resolution of 48 × 48 pixels; using a 50 per cent overlap between subsequent interrogation areas resulted in vector spacing of 17 μ m. Vectors were validated using the universal outlier detection algorithm (Westerweel & Scarano 2005), which finds usually <5 per cent outliers—this was within acceptable limits (Keane & Adrian 1992).

It was assumed that peak systole resulted in maximum velocity in all measured planes, so that the phases of the measured planes could be synchronized *a posteriori* (e.g. for peak systole, one selects the flow field with the highest velocity for all z -locations). This approach is valid only if pulsatile flow effects can be ignored. These effects can lead to phase differences in the velocity cycle for different radial positions (causing opposite signs of the flow velocity in the near-wall and core regions). As can be seen in table 1, the Womersley number of the flow was much smaller than unity, indicating that viscous effects dominated over transient inertial phenomena. The velocity profiles along the y -axis (figures 3 and 4) always remained unidirectional throughout the cardiac cycle, i.e. without flow reversal near the wall, as would occur for $\alpha > 1$. If it is assumed that the flow is more or less axisymmetric, any near-wall retrograde flow would also be visible in these measurement planes. As this was never observed, we concluded that the *a posteriori* sorting method was appropriate.

¹The correlation depth describes the maximum distance from the focal plane at which out-of-focus tracer particles still contribute to the velocity estimates, thereby effectively determining the depth of the measurement volume. Note that this depth is generally larger than the focal depth of the microscope and is a function of the particle diameter. This necessitates the use of small tracer particles: the use of, for example, erythrocytes as tracer particles (with a typical diameter of 8 μ m) would lead to considerable averaging effects in the z -direction (Vennemann *et al.* 2006).

Figure 3. (a) A typical example of a raw PIV image taken in the OFT (only one frame shown, inverted for clarity). (b) The corresponding flow field represented as a vector field; the false colours indicate the reconstructed out-of-plane component. The wall locations have been drawn schematically to illustrate the orientation of flow and the geometry.

Figure 4. (a) A volumetric dataset reconstructed by scanning the OFT (false colours indicate velocity magnitude). (b) An example of a slice of the absolute velocity, perpendicular to the mean flow direction (indicated by ‘cross-flow plane’ in (a)).

The scanning process yields a three-dimensional measurement of the in-plane velocities in the OFT for each step in the cardiac cycle. An example of such a scanning measurement result at systole is shown in figure 4.

2.3. Reconstruction of the out-of-plane velocity

As the fluid can be considered to be incompressible, the third velocity component could be derived from the volumetric measurement of the two measured components using the continuity equation (Robinson & Rockwell 1993)

$$\frac{\partial v_x}{\partial x} + \frac{\partial v_y}{\partial y} + \frac{\partial v_z}{\partial z} = 0. \quad (2.1)$$

Obviously, this approach is feasible only if the measurement is sufficiently resolved. As can be seen in figures 3 and 4, the velocity fields appear smooth and thus resolved. The continuity equation can be

rewritten in terms of central differences of the measured velocities v_x and v_y

$$\frac{\partial v_z}{\partial z} \approx - \frac{v_x(i+1, j) - v_x(i-1, j)}{2\delta x} - \frac{v_y(i, j+1) - v_y(i, j-1)}{2\delta y}. \quad (2.2)$$

In this equation, δx and δy represent the in-plane grid spacing of the vector field, and they are identical ($17 \mu\text{m}$) owing to uniform magnification of the microscope. The estimates for v_z can now easily be found by integrating from $z = 0$, using the trapezium rule. The integration step size in the z -direction is determined by a scanning step interval of $12 \mu\text{m}$. The boundary condition for the integration procedure is straightforward: the initial planes are usually recorded *outside* of the OFT, where the total flow is zero. An example of the reconstructed out-of-plane velocity component is shown as false colours in figure 3: red indicates flow towards the observer and blue indicates flow away from the observer. Note

that the out-of-plane velocities are an order of magnitude smaller than the in-plane velocities.

3. DETERMINATION OF WSS

Each measurement series of 500 image pairs resulted in one velocity field for each of the 10 steps in the cardiac cycle. As the measurements were performed in 14 planes in total, this resulted in a large four-dimensional matrix containing the velocity information for one single specimen. From this matrix, the geometries of the OFT and the local WSSs need to be determined.

In this section, two approaches are described to reconstruct the OFT geometry. In the first method, the wall was detected by an isosurface of the velocity magnitude. The WSS was subsequently determined by finding the gradient with respect to the local wall-normal vector. The second method tracked the flow in an iterative fitting/tracking process, using the inherent correlation between the geometry and the mean flow direction.

3.1. Isosurface method

In principle, the wall location can be found by finding the isosurface of the velocity, $v_{\text{abs}} = 0$. Here it was assumed that the flow velocity reached zero only at the wall because of the no-slip condition. We disregarded results outside the OFT; the sporadic velocity measurement there results from secondary scattering of the fluorescent signal by tissues, as there are no tracer particles outside the lumen. In practice, the measurement quality was affected by noise and the isosurfaces obtained would be somewhat irregular. This can be overcome by either smoothing the data or using an isosurface level with a small offset (e.g. 5% of the maximum velocity). However, both result in a bias in the estimated wall position (i.e. a geometry that is smaller than reality). This effect will be further evaluated in the next section. Once the isosurface is known, the local wall-normal vector $\mathbf{n}$ can be determined. Using the gradients in the flow velocity in the direction of the normal vectors, the value of WSS then follows from its definition,

$$\tau_w = \eta \frac{\partial u^*}{\partial \mathbf{n}}, \quad (3.1)$$

with $\mathbf{n}$ being the local wall-normal vector and u^* the local velocity component aligned with the wall direction. The gradient is obtained by interpolating along the wall-normal direction in the Cartesian dataset. As a first approximation, u^* can be replaced by the magnitude of the local velocity (Poelma *et al.* 2008). To derive the shear stress from the shear rate ($\gamma \equiv \partial u / \partial \mathbf{n}$), we need the value of the dynamic viscosity. Usually, a value of 0.004–0.005 Pa s is used for embryonic chicken blood (Ji *et al.* 2007). More details about haemorheology are given in §3.3.

3.2. Flow tracking

The second approach to reconstruct the OFT geometry was by an iterative fitting/tracking of the flow, referred

to here as flow tracking. The method starts by selecting a seed point in the flow. The local velocity vector was determined to find the direction of the flow. Perpendicular to this direction, a plane was cut from the measured data, as indicated by the slice S in figure 5a. A two-dimensional fit was used to describe the velocity data in this slice (figure 5b). This fit was used to find the location of the walls by extrapolation and then to find the zero-crossings of the function, as indicated by the green dots in the figure. The derivative of the fit function can be used to determine the value of the WSS. Subsequently, a new location was calculated by integrating the velocity at the seed point, and the entire fitting procedure was repeated at the next location. Note that this approach works only if the flow direction is reasonably uniform. For strong secondary flow patterns and recirculation zones—which are not observed in the current datasets—this method is not suitable.

Not all data in each slice were used for the fit: only the data above a certain threshold were taken into account. This improved the stability of the fitting process (velocity measurements inside or outside the wall, which are usually zero, should obviously be ignored) and avoided the influence of under-resolved gradients near the wall. In practice, using data above 20 per cent of the maximum in the slice gave good results. The choice for the fit function was a compromise between stability/robustness and flexibility. For example, a two-dimensional parabola would generally give good results and useful zero-crossings (i.e. a circle in the $v_{\text{fit}} = 0$ plane). In reality, the flow profile might be skewed, something that cannot be captured as only symmetric WSS patterns can be obtained with such a function. On the other hand, choosing a function with too many terms will lead to non-realistic zero-crossings. A compromise between an overly restrictive function and the stability of the fitting process leads us to use a second-order polynomial of the form²

$$v_{\text{fit}}(x = \text{const.}, y, z) = c_0 + c_1 y + c_2 y^2 + c_3 z + c_4 z^2 + c_5 yz. \quad (3.2)$$

Typical discrepancies between the fit result and the measured data (expressed as the standard deviation of the differences) are 3–5%. The points at which the function crosses zero are found numerically by evaluating the fitted function on a very fine grid and finding an isocontour for $v_{\text{fit}} = 0$; the derivatives at these points were found using a central difference scheme. For simple functions, an analytical solution would be tractable, but for increasing complexity of the fit polynomial, this soon becomes unfeasible.

To improve the accuracy, an iteration loop was implemented at each point: the maximum of the fitted function was used as the new origin. Additionally, the mean direction of the data used in the fit was used to refine the direction of the flow (and thus the slice that

²We have assumed here that the coordinate system of the fit is identical to the Cartesian measurement coordinate system, with flow aligned in the x -direction.

Figure 5. (a) A slice S , perpendicular to the mean flow direction, is interpolated in the original data; the red surface approximates the location of the OFT walls for orientation purposes. (b) Fitting of a polynomial function; from the entire dataset (blue dots), only data above a certain threshold are used (red circles). The fit result is shown as black crosses, while the zero-crossing of the fitted function is indicated by green dots in the $z = 0$ plane.

needs to be cut out). This iteration usually converges after one or two steps.

3.3. Haemorheological consideration

It was implicitly assumed until now that the proportionality between the shear rate at the wall and the resulting shear stress is the viscosity of blood. However, there are complications to this. First, blood is effectively a non-Newtonian fluid. Fortunately, at the shear rates under consideration (approx. $100\text{--}1000\text{ s}^{-1}$), the shear dependence of viscosity becomes less significant (Chien 1970) and the viscosity approaches a constant value. To correct for the shear-thinning effect and to refine the results, a constitutive equation for η can be included in equation (3.1). A commonly accepted constitutive equation is currently not available, although, recently, rheological data from *in vitro* experiments in microchannels have become available (Ji *et al.* 2007). In our application of the current method, we focus on differences in WSS (both spatial variations in one OFT as well as comparisons between embryos). Therefore, the uncertainty in the exact value of the WSS is not critical.

Second, owing to the Fåhræus–Lindqvist effect, a cell-depleted layer close to the wall can occur (Goldsmith *et al.* 1989; Long *et al.* 2004). This means that the viscosity of blood *plasma* has to be used to calculate WSS, rather than the viscosity of whole blood (the former being approximately twice as low). Note that in the current experiments the small artificial tracer particles were distributed more or less homogeneously over the lumen, so that flow velocity measurements close to the wall could accurately reflect plasma velocity. Some recent studies have relied on the use of erythrocytes as flow tracers (Sugii *et al.* 2002; Hove *et al.* 2003; Lee *et al.* 2007). In these experiments, the presence of a cell-free layer can complicate meaningful estimation of the WSS, as there can be a ‘slip’ velocity. The thickness of the cell-free layer (if present) will be small compared with the diameter of the geometry. Note that the heart and OFT are contracting and the

Fåhræus–Lindqvist effect requires some time to create the cell-free layer (Segré & Silberberg 1962). In high-resolution measurements (discussed in appendix B), the velocity varied smoothly as the wall was approached—without any measurable change in slope. Therefore, the value of whole blood is used to calculate the WSS.

A final complication is the presence of the endothelial glycocalyx, a network of membrane-bound proteoglycans and glycoproteins, covering the endothelial wall (Pries *et al.* 2000; Reitsma *et al.* 2007). Depending on the mechanical properties of this endothelial surface layer (ESL), the plasma velocity may not approach zero close to the vessel wall, but an apparent slip velocity may occur due to the moving/deforming glycocalyx. If we consider the glycocalyx to be a very viscous layer (significantly more viscous than plasma), we can argue that this slip velocity will be irrelevant compared with the large gradients in the plasma close to the glycocalyx. Studies by Smith *et al.* (2003) and Secomb *et al.* (2001) suggest that the fluid shear rate effectively reaches zero within the ESL, so that the endothelial cells might not sense the fluid shear forces directly but only through the glycocalyx as an intermediate. A final result of the presence of the ESL (with a typical thickness of $0.5\text{--}1\text{ }\mu\text{m}$) is a decrease in the effective diameter: decreasing blood vessel diameter will lead to higher apparent flow resistance.

3.4. Effect of moving walls

The heart and OFT form a complex, dynamic geometry. It is difficult to determine the wall location and shear stress when the walls are moving together with the fluid: an offset in the flow magnitude profile is observed, complicating our method of extrapolating to zero velocity. Fortunately, during most of the cycle, the motion of the OFT is restricted to minor changes in the radial direction (i.e. no axial motion). As our method uses the gradient of the axial velocities, no

offset is observed and the wall can be determined accurately. During the actual contraction phase of the OFT, this is no longer the case. We have chosen not to report data during this contraction phase to avoid any ambiguity in the determination of the wall location. Furthermore, during this phase, the phase-averaging process becomes less reliable due to the rapid changes in geometry even at small temporal variations during the sorting process. Note that the maximum velocities are observed just *before* the OFT contraction, as the ventricle ejects fluid through the OFT (there is a phase delay between contractions of the different segments of the heart).

3.5. In vitro validation and estimation of the accuracy

To demonstrate the feasibility of measuring the WSS using the micro-PIV technique, we have validated the method using an *in vitro* experiment. The details can be found in appendix A. The experiment shows that an accurate reconstruction of the wall location and local WSS is possible. Obviously, this will not guarantee a similar accuracy for the *in vivo* measurements in the OFT. We have estimated the errors in the velocities (in-plane and out-of-plane), the location of the wall and the WSS in appendix B. Summarizing, the errors in the in-plane and out-of-plane velocities are 2–5% and 20 per cent, respectively (the latter is relatively high because the absolute value of the out-of-plane component is small). The largest source of error in the determination of the WSS is the inaccuracy in the determination of the wall location, which is accurate within 30 μm . This results in a conservative estimate of the error in the WSS at 20 per cent.

4. RESULTS

4.1. Isosurface method

An example of the reconstructed OFT is shown in figure 6. Owing to the blurring effect of erythrocytes, only the top half of the OFT could be acquired. The colour coding of the surface represents the shear rate, determined from the wall-normal gradient (the wall normals are shown as vectors, pointing to the inside of the OFT).

As mentioned in the previous section, the choice of the threshold used for the isosurface has a significant effect on the result. In figure 7, a single slice of the OFT (at $x = 0.20$ mm) is shown for different values of this threshold. The labels in the figure refer to the value used: ‘1/3’ refers to a threshold value of 1/3 of the maximum velocity in the dataset. As can be seen, a decreasing threshold leads to a larger OFT. As the threshold increases, the result can be expected to approach the true location of the OFT walls. However, the relative contribution of measurement noise increases, which leads to an irregular, unrealistic shape of the OFT (see, in particular, the result labelled ‘1/40’).

Surprisingly, the value of the shear rate found decreases with an increase in size. One would expect

Figure 6. Local shear rate in the OFT at peak systole using the isosurface method. The colour coding represents the shear rate, while the vector indicates the wall-normal vector (pointing inwards). The mean flow direction is in the positive x -direction.

Figure 7. Effect of the threshold on the reconstructed wall location and resulting wall shear rate (a slice at $x = 0.20$ mm is shown). The labels ‘1/3’, ‘1/5’, ‘1/10’, ‘1/20’ and ‘1/40’ correspond to the isosurfaces using a threshold of $v = \frac{1}{3}v_{\text{max}}$, $\frac{1}{5}v_{\text{max}}$, etc. The data labelled ‘fit method’ use the flow-tracking method discussed in §4.2.

higher gradients of the local velocity as one approaches the wall. However, the velocities near the wall are affected by the well-documented spatial-filtering phenomenon in PIV (Olsen & Adrian 2000). This effect can be clearly observed by evaluating the flow profile in the y -direction, as shown in figure 8 (open circles). In the region $y = 300\text{--}350$ μm , a point of inflexion can be observed, resulting from spatial averaging: outside the OFT, there are no tracer particles and there is no contribution to the spatial average. The smeared-out velocity profile will lead to a decrease in the observed gradients at the wall. This in turn will lead to an underestimation of the WSS. Also, the width of the OFT will be overestimated if zero-crossings are used. Note also the skewness of the profile, with the maximum shifted slightly towards the inside wall (right-hand side of the figure), which was observed previously by Vennemann *et al.* (2006).

Figure 8. Flow velocity along a line in the midplane of the OFT. Open circles indicate results at typical measurement resolution (48×48 pixels), while the solid line is a polynomial fit. The dots indicate a measurement with high resolution (32×8 pixels; for details see appendix B), with the dashed line showing the corresponding polynomial fit. For both fits, only the data above the indicated threshold of $0.2 v/v_{\max}$ were used. The labels refer to lumen (L), near-wall (W) and tissue (T) regions of the measurement.

4.2. Flow tracking

Figure 9a shows the reconstructed OFT using the flow-tracking method. Again, the colour coding represents the shear rate at the wall. Each arc represents the result of a single fit result. The fit planes are nearly aligned with the y - z plane, as the main flow direction is in the x -direction. To compare these results with the previous method, the data at $x = 20$ mm have also been added to figure 7. As can be seen in this figure, the general shape agrees with the isosurface method using the ‘1/10’ or ‘1/20’ threshold. However, the value of the shear rate (and thus WSS) is significantly higher.

In figure 9b, a side view of the OFT is shown. The colour coding here represents the WSS (in dynes/cm², as is customary in physiology), using a viscosity value of $\eta = 0.004$ Pa s. The blue lines represent the number of streamlines, obtained by integrating the flow field, to indicate the general instantaneous flow pattern. Note the tilting of the fit planes as a result of the local flow direction.

An additional advantage of the flow-tracking method is that it allows automatic data reduction. After the procedure, the flow can be described in the form of a number of parameters (e.g. the diameter, maximum flow velocity, local shear, etc.) or solely as a function of a single parameter: the distance along the centreline of the OFT. This will facilitate considerably the systematic, quantitative comparison of different embryos in future work.

4.3. Temporal reconstruction

In figure 10, the reconstructed OFT geometry is shown (with shear rate as colour coding) for three time steps in the cardiac cycle ($\phi = 0.0, 0.2$ and 0.5). These time

steps were reconstructed using the *a posteriori* sorting method described briefly in §2.2 and in detail by Poelma *et al.* (2008). For this particular embryo, the heart rate was abnormally low: 71 b.p.m., compared with a typical range of 120–150 b.p.m. (Hu & Clark 1989), most probably because of hypothermia. As a result, there is some retrograde flow during the diastolic phase (there are no valves at this developmental stage). This is evident in the one-dimensional velocity profiles shown in figure 10d (taken along the z -axis, approximately in the middle of the OFT): the profile becomes negative at $\phi = 0.5$. As a result, the WSS changes sign. For the embryos with a normal heart rate, this was not the case. The wall shear rate varies from -100 to $+800$ s⁻¹, corresponding to WSSs of -0.4 to $+3.2$ Pa (the negative value indicating a flow reversal).

5. DISCUSSION AND CONCLUSION

The measurements presented here provide a first *in vivo* observation of the magnitude and spatial distribution of the WSS in the OFT of an embryonic chicken heart; the method does not rely on the assumption of Poiseuille flow. From detailed velocity measurements, the WSS was derived using two methods: the isosurface method uses velocity data close to the wall, while the flow-tracking method emphasizes the flow in the core of the geometry (as only data above a threshold are used). Nevertheless, the results of the two measurements are in reasonable agreement for both the obtained shape of the OFT and the range of values of the WSS. As the isosurface method is more sensitive to noise and resolution issues (leading to an underestimation of the WSS), the flow-tracking method is expected to be the more reliable. However, this method is applicable only if the flow geometry is relatively simple. For some applications, e.g. in bifurcating blood vessels, it is less suitable; in this case, the isosurface method can serve as an alternative.

In an *in vitro* experiment (see appendix A), we have shown that the technique can give an accurate estimate of the WSS. Typical errors in the *in vivo* measurement of the WSS are expected to be around 20 per cent, mainly owing to the uncertainty in the location of the wall.

The values of the WSS in the OFT obtained here (1–3 Pa) are lower than the value of the 5 Pa estimated in a previous experiment focusing on the flow in the ventricle of a chicken embryo at approximately the same stage (Vennemann *et al.* 2006). However, those results were obtained by a scaling analysis, based on results of a single measurement plane; this plane was assumed to be the midplane of the heart. The current three-dimensional results remove this uncertainty of the actual measurement location, as well as take into account all three velocity components, thereby improving the accuracy of the WSS estimate.

The current results can also be compared with the results obtained when one assumes a parabolic flow profile. In this case, the WSS is given by $\tau_{w,P} = 4\eta u_{\max}/D$. Using a typical centreline velocity of 40 mm s⁻¹ and an OFT diameter of 300 μm, we

Figure 9. (a) Reconstruction of the wall and associated shear rate at the wall using the flow-tracking approach. (b) Side view of the OFT; colour coding represents the wall shear rate and stress, respectively. The thin blue lines are streamlines to indicate the flow pattern.

Figure 10. (a–c) The OFT geometry and the wall shear rate at three time steps in the cardiac cycle ($\phi = 0.0, 0.2$ and 0.5). (d) A profile in the z -direction to indicate the global flow profile evolution. Note that the colour bar scaling is different for each time step because of the differences in range.

obtain a value for the WSS of 2.1 Pa. This is the same order of magnitude as is obtained using the current method. The assumption of Poiseuille flow gives an inherently uniform distribution of the WSS, while the current method shows that it varies over a factor of 3 within the present measurement domain. Additionally, there is a strong temporal variation, as is evident from figure 10. This means that the interaction at the endothelial layer cannot be characterized by a single value of the WSS.

Recently, numerical simulations of the same model system at a later developmental stage (HH21) have become available (Liu *et al.* 2007). While the typical centreline velocity and flow rate in the OFT reported are higher (owing to the later stage in development), the WSS is remarkably similar: 1–2 Pa on average, with a maximum of 3 Pa. Higher WSS values were found on the inner curvature of the OFT.

In this paper, data from a small sample group have been presented. The results in figures 6–9 all show

the same OFT, so as to facilitate a comparison of the methods. However, the results are representative: other embryos showed WSS values in the same range, despite small differences in heart rate and geometry. The exception was for the embryo with an abnormally low heart rate, which showed retrograde flow and thus a change in the sign of the WSS. Owing to the relatively small sample size ($n = 5$), it was decided to refrain from a systematic statistical analysis at present. Future work will include similar experiments with a larger sample size.

The results of the current experiment, together with the data from future work using a larger sample size, will be useful in a number of ways. First, they can shed light on the expression patterns of shear-responsive markers such as Krüppel-like factor-2, endothelin-1 and endothelial nitric oxide synthase (NOS-3). Based on earlier work, a qualitative correlation was found between WSSs and their expression patterns (Groenendijk *et al.* 2005). Using the methodology presented, the correlation can be studied in more quantitative detail. To facilitate this, the current results can be used to design *in vitro* experiments. In these experiments, endothelial cells are placed in flow chambers where they are subjected to controlled flow conditions (see recent work by Chien (2008) and Rossi *et al.* (in press)). While it is qualitatively known that certain markers respond to shear stress, the current results provide a range of parameters for quantitative *in vitro* studies. It will be interesting to see whether there is a gradation in the expression as a response to the range of shear stresses that is observed here (rather than, for example, ‘flow’ versus ‘no-flow’).

The authors would like to acknowledge the valuable contributions of Dr P. Vennemann in the initial stages of this project.

APPENDIX A: VALIDATION OF THE TECHNIQUE

The accuracy of the measured WSS distribution can be evaluated in a number of ways. One option is to integrate the WSS over the wall surface and compare the result with a measurement of the pressure drop. Unfortunately, such an *in vivo* check is not feasible with the expected small, fluctuating pressure drops in this system. As an alternative, the measurement methodology tested here has comparable geometry and flow conditions, yet with a known pressure drop: for this we used a stationary laminar flow in a straight circular tube. In this case, it can safely be assumed that Poiseuille’s law is valid, so that the mean value of the WSS can be calculated from the flow rate (equation 1.1) and compared with the results from the micro-PIV method.

For this validation experiment, most of the parameters chosen are similar to those of the *in vivo* experiments. Apart from the working fluid (water rather than blood), all other parameters were similar: nominal diameter (150 μm , note that the real OFT diameter varies during the cardiac cycle), flow rate/velocity, tracer particles, illumination source, laser pulse delay

Figure 11. Validation of the measurement methodology: reconstructed geometry and variations of the measured WSS of a capillary (as colour coding). The mean WSS using Poiseuille’s law is 0.50 Pa.

time, magnification, number of recorded image pairs, PIV settings, etc. To avoid optical aberrations, the capillary was immersed in a mineral oil droplet and covered with a glass cover plate (creating an approximately similar change in refractive index as the *in vivo* case). The flow was driven by a syringe pump at $10 \mu\text{l min}^{-1}$, leading to a Reynolds number of 1.5 and an expected WSS value of $\bar{\tau} = 0.50 \text{ Pa}$. The flow can be considered to be fully developed, as measurements were performed $O(10^5)$ diameters downstream of the pump.

In figure 11, a reconstruction, using the flow-tracking method, of the capillary wall, colour-coded with the measured variations in the WSS as obtained by the micro-PIV method, is shown. The mean flow rate, obtained by integrating the cross-flow profiles and averaged along the x -axis, is $10.1 \mu\text{l min}^{-1}$ with a standard deviation of 1.7 per cent. Similarly, the average diameter is $153 \mu\text{m}$ with a variation of 1.4 per cent. The mean value of the WSS (averaged over the entire visible surface of the capillary) is 0.510 Pa , close to the expected 0.50 Pa . The values vary over a range of $0.480\text{--}0.540 \text{ Pa}$ (standard deviation 0.010 Pa). There appears to be a small systematic error, visible as a pattern of lower WSS at the top of the capillary and a higher value at the sides. This can be attributed to the differences in resolution and accuracy of the in-plane and out-of-plane data.

This validation experiment confirms that, in principle, the method is capable of very accurate measurements. Obviously, by no means does it guarantee a similar accuracy for the *in vivo* measurements. An estimate of the latter is given in appendix B. Even if the real accuracy is an order of magnitude smaller than the *in vitro* case, the proposed method can still give valuable insight into the spatial distribution of the WSS in the OFT. As is shown in §4, the variations in the WSS in the OFT are considerable, yet gradual with respect to the measurement resolution. This may confirm an accuracy sufficient for meaningful studies.

Additionally, tracer particle distribution was checked in the data taken in the capillary: individual tracer particles in the flow were detected (using image processing in Matlab) and a histogram of their radial

Figure 12. (a) A histogram of the radial position of the tracer particles; as can be seen in the close-up of the wall region (b), the particles are not significantly excluded from the near-wall region due to lateral migration effects. For reference, interrogation areas of 48×48 pixels were used in the PIV algorithm. N_p , number of particles per radial region.

position was made. Results are shown in figure 12. A close-up of the near-wall region is shown in figure 12b. The locations of the wall, indicated in figure 12 by the dashed lines, were determined by visual inspection of the average image (using 500 images). As can be seen in the graph, the particles approach the wall sufficiently close to be able to perform accurate velocity measurements. The particles outside the capillary are a result of a small inclination ($<1^\circ$) of the capillary with respect to the image frame of reference. Note that, for this single planar PIV measurement, approximately half a million tracer particles were used (this number is probably an underestimation, as only bright particles were detected in the simple particle detection algorithm used for the figures).

APPENDIX B: ACCURACY OF THE IN VIVO RESULTS

In the flow-tracking method, it may seem counterintuitive to use the velocities in the core region of the flow, rather than the near-wall velocity data to determine the WSS. For a limited number of datasets, the data quality allowed an analysis at a higher spatial resolution; these images had a higher than average tracer density. In this case, interrogation areas of 32×8 pixels could be used (with the long axis of the interrogation areas aligned with the wall direction and no overlap in the radial direction), so that a spatial resolution of $5.7 \mu\text{m}$ could be achieved—a threefold increase in radial resolution. In figure 8, a typical result of this analysis is shown, together with a measurement at the original resolution. Also shown are the polynomial fits using the data above a threshold ($0.2 v/v_{\text{max}}$). As can be seen in the graph, both fits correspond closely. Moreover, the high-resolution data points in the near-wall region (indicated by ‘W’) do not deviate from the extrapolated fit, indicating that this fit is a reasonable description of the flow near the wall. For the normal resolution data (circles in the graph), the points in the near-wall region are more strongly affected by the

spatial averaging of the PIV method. However, these points are discarded as they are below the threshold. Under these flow conditions, the flow profiles never appear to be other than a smooth function with a single maximum. This validates the flow-tracking approach.

Typically, PIV measurement errors are of the order 0.1 pixel in the displacement (e.g. Westerweel 2000). For an average displacement of 5 pixels at systole (and lower during the remainder of the cardiac cycle), this corresponds to 2 per cent. This is in reasonable agreement with an alternative, *post hoc* indicator of the measurement error: the average residuals of the polynomial fits used in the flow-tracking method (3–5%, as mentioned in §3.3). The residuals did not show a trend (which would indicate a systematic error, for instance, because of an incorrect choice of the fitting function), but rather appeared as random scatter (as associated with random measurement errors).

Errors in the reconstructed out-of-plane component originate in the errors in the in-plane velocities. Assuming that all in-plane velocity estimates are uncorrelated, the mean divergence error is similar to the aforementioned displacement error of 2–5%. The actual value of the out-of-plane component at a given z -location is obtained by a cumulative integration of the divergence along the z -direction. During this integration, random errors average out and the final error is again similar to the in-plane error. As the absolute values of the out-of-plane velocities are an order of magnitude lower than the in-plane components, this leads to an estimate of 20 per cent error in the out-of-plane components. Note that the values shown in figure 3b (colour coding) are obtained individually; despite the significant error estimate, they show a relatively smooth result. Owing to their low absolute value, this error does not contribute significantly to the estimation of WSS.

To determine the WSS, a more significant error is introduced by the uncertainty in the location of the wall. If it is assumed, based on figure 8 and similar

plots, that the location of the wall can be determined within 30 μm , we can evaluate the uncertainty in the WSS by evaluating the derivative of the fit at the wall location $\pm 15 \mu\text{m}$. Such an evaluation using a number of datasets estimates the uncertainty at 20 per cent.

REFERENCES

- Adrian, R. J. 1991 Particle-imaging techniques for experimental fluid mechanics. *Annu. Rev. Fluid Mech.* **23**, 261–304. (doi:10.1146/annurev.fl.23.010191.001401)
- Chien, S. 1970 Shear dependence of effective cell volume as a determinant of blood viscosity. *Science* **168**, 977–979. (doi:10.1126/science.168.3934.977)
- Chien, S. 2008 Effects of disturbed flow on endothelial cells. *Ann. Biomed. Eng.* **36**, 554–562. (doi:10.1007/s10439-007-9426-3)
- Davies, P. F. 1995 Flow-mediated endothelial mechanotransduction. *Physiol. Rev.* **75**, 519–560.
- Goldsmith, H. L., Cokelet, G. R. & Gaehtgens, P. 1989 Robin Fahraeus: evolution of his concepts in cardiovascular physiology. *Am. J. Physiol. Heart Circ. Physiol.* **257**, 1005–1015.
- Groenendijk, B. C. W., Hierck, B. P., Vrolijk, J., Baiker, M., Pourquie, M. J. B. M., Gittenberger-de Groot, A. C. & Poelmann, R. E. 2005 Changes in shear stress-related gene expression after experimentally altered venous return in the chicken embryo. *Circ. Res.* **96**, 1291–1298. (doi:10.1161/01.RES.0000171901.40952.0d)
- Groenendijk, B. C. W., van der Heiden, K., Hierck, B. P. & Poelmann, R. E. 2007 The role of shear stress on ET-1, KLF2, and NOS-3 expression in the developing cardiovascular system of chicken embryos in a venous ligation model. *Physiology* **22**, 380. (doi:10.1152/physiol.00023.2007)
- Hamburger, V. & Hamilton, H. L. 1951 A series of normal stages in the development of the chick embryo. *J. Morphol.* **88**, 49–92. (doi:10.1002/aja.1001950404)
- Hierck, B. P., van der Heiden, K., Poelma, C., Westerweel, J. & Poelmann, R. E. 2008a Fluid shear stress and inner curvature remodeling of the embryonic heart. Choosing the right lane! *Sci. World J.* **8**, 212–222. (doi:10.1100/tsw.2008.42)
- Hierck, B. P. et al. 2008b Primary cilia sensitize endothelial cells for fluid shear stress. *Dev. Dyn.* **237**, 725. (doi:10.1002/dvdy.21472)
- Hogers, B., de Ruiter, M. C., Grittenberger-de Groot, A. C. & Poelmann, R. E. 1997 Unilateral vitelline vein ligation alters intracardiac blood flow patterns and morphogenesis in the chick embryo. *Circ. Res.* **80**, 473–481.
- Hove, J. R., Koster, R. W., Forouhar, A. S., Acevedo-Bolton, G., Fraser, S. E. & Gharib, M. 2003 Intracardiac fluid forces are an essential epigenetic factor for embryonic cardiogenesis. *Nature* **421**, 172–177. (doi:10.1038/nature01282)
- Hu, N. & Clark, E. B. 1989 Hemodynamics of the stage 12 to stage 29 chick embryo. *Circ. Res.* **65**, 1665–1670.
- Ji, H. S., Lee, J. Y. & Lee, S. J. 2007 *In-vitro* study on the hemorheological characteristics of chicken blood in microcirculation. *Korea-Australia Rheol. J.* **19**, 89–95.
- Keane, R. D. & Adrian, R. J. 1992 Theory of cross-correlation analysis of PIV analysis. *Appl. Sci. Res.* **49**, 191–215. (doi:10.1007/BF00384623)
- Lee, J. Y., Ji, H. S. & Lee, S. J. 2007 Micro-PIV measurements of blood flow in extraembryonic blood vessels of chicken embryos. *Physiol. Meas.* **28**, 1149–1162. (doi:10.1088/0967-3334/28/10/002)
- Liu, A., Rugonyi, S., Pentecost, J. O. & Thornburg, K. L. 2007 Finite element modeling of blood flow-induced mechanical forces in the outflow tract of chick embryonic hearts. *Comput. Struct.* **85**, 727–738. (doi:10.1016/j.compstruc.2007.01.014)
- Long, D. S., Smith, M. L., Pries, A. R., Ley, K. & Damiano, E. R. 2004 Microviscometry reveals reduced blood viscosity and altered shear rate and shear stress profiles in microvessels after hemodilution. *Proc. Natl. Acad. Sci. USA* **101**, 10 060. (doi:10.1073/pnas.0402937101)
- Männer, 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. (doi:10.1002/1097-0185(20000701)259:3<248::AID-AR30>3.0.CO;2-K)
- Meinhart, C. D., Wereley, S. T. & Santiago, J. G. 2000 A PIV algorithm for estimating time-averaged velocity fields. *J. Fluids Eng.* **122**, 285–289.
- Olsen, M. G. & Adrian, R. J. 2000 Out-of-focus effects on particle image visibility and correlation in microscopic particle image velocimetry. *Exp. Fluids* **29**, 166–174. (doi:10.1007/s003480070018)
- Poelma, C., Vennemann, P., Lindken, R. & Westerweel, J. 2008 *In vivo* blood flow and wall shear stress measurements in the vitelline network. *Exp. Fluids* **45**, 703–713. (doi:10.1007/s00348-008-0476-6)
- Poelmann, R. E., van der Heiden, K., Gittenberger-de Groot, A. & Hierck, B. P. 2008 Deciphering the endothelial shear stress sensor. *Circulation* **117**, 1124. (doi:10.1161/CIRCULATIONAHA.107.753889)
- Pries, A. R., Secomb, T. W. & Gaehtgens, P. 2000 The endothelial surface layer. *Pflügers Arch.* **440**, 653–666. (doi:10.1007/s004240000307)
- Reitsma, S., Slaaf, D. W., Vink, H., van Zandvoort, M. A. M. J. & Oude Egbrink, M. G. A. 2007 The endothelial glycocalyx: composition, functions, and visualization. *Pflügers Arch.* **454**, 345–359. (doi:10.1007/s00424-007-0212-8)
- Reneman, R. S., Arts, T. & Hoeks, A. P. G. 2006 Wall shear stress: an important determinant of endothelial cell function and structure in the arterial system *in vivo*; discrepancies with theory. *J. Vasc. Res.* **43**, 251–269. (doi:10.1159/000091648)
- Robinson, O. & Rockwell, D. 1993 Construction of three-dimensional images of flow structure via particle tracking techniques. *Exp. Fluids* **14**, 257–270. (doi:10.1007/BF00194017)
- Rossi, M., Lindken, R., Hierck, B. P. & Westerweel, J. In press. Tapered microfluidic chip for the study of biochemical and mechanical response at subcellular level of endothelial cells to shear flow. *Lab Chip*. (doi:10.1039/B822270N)
- Santiago, J. G., Wereley, S. T., Meinhart, C. D., Beebe, D. J. & Adrian, R. J. 1998 A particle image velocimetry system for microfluidics. *Exp. Fluids* **25**, 316–319. (doi:10.1007/s003480050235)
- Secomb, T. W., Hsu, R. & Pries, A. R. 2001 Effect of the endothelial surface layer on transmission of fluid shear stress to endothelial cells. *Biorheology* **38**, 143–150.
- Segré, G. & Silberberg, A. 1962 Behaviour of macroscopic rigid spheres in Poiseuille flow. Part 2. Experimental results and interpretation. *J. Fluid Mech.* **14**, 136–157. (doi:10.1017/S0022112062001111)
- Smith, M. L., Long, D. S., Damiano, E. R. & Ley, K. 2003 Near-wall μ -PIV reveals a hydrodynamically relevant endothelial surface layer in venules *in vivo*. *Biophys. J.* **85**, 637–645. (doi:10.1016/S0006-3495(03)74507-X)
- Sugii, Y., Nishio, S. & Okamoto, K. 2002 *In vivo* PIV measurement of red blood cell velocity field in microvessels

- considering mesentery motion. *Physiol. Meas.* **23**, 403–416. (doi:10.1088/0967-3334/23/2/315)
- Topper, J. N. & Gimbrone Jr, M. A. 1999 Blood flow and vascular gene expression: fluid shear stress as a modulator of endothelial phenotype. *Mol. Med. Today* **5**, 40–46. (doi:10.1016/S1357-4310(98)01372-0)
- Vennemann, P. 2008 Particle image velocimetry for micro-scale blood flow measurements. Thesis, Delft University of Technology, The Netherlands; available via repository.tudelft.nl.
- Vennemann, P. *et al.* 2006 *In vivo* micro particle image velocimetry measurements of blood-plasma in the embryonic avian heart. *J. Biomech.* **39**, 1191–1200. (doi:10.1016/j.jbiomech.2005.03.015)
- Westerweel, J. 2000 Theoretical analysis of the measurement precision in particle image velocimetry. *Exp. Fluids* **29**, 3–12. (doi:10.1007/s003480070002)
- Westerweel, J. & Scarano, F. 2005 Universal outlier detection for PIV data. *Exp. Fluids* **39**, 1096–1100. (doi:10.1007/s00348-005-0016-6)
- Willert, C. E. 1997 Stereoscopic digital particle image velocimetry for application in wind tunnel flows. *Meas. Sci. Technol.* **8**, 1465–1479. (doi:10.1088/0957-0233/8/12/010)