Line scan fluorescence correlation spectroscopy for three-dimensional microfluidic flow velocity measurements

Xiaotao Pan
National University of Singapore
NUS Graduate Program in Bioengineering
28 Medical Drive
Singapore 117456, Singapore
    and
National University of Singapore
Department of Chemistry
3 Science Drive 3
Singapore 117543, Singapore

Xianke Shi
National University of Singapore
Department of Chemistry
3 Science Drive 3
Singapore 117543, Singapore

Vladimir Korzh
Institute of Molecular and Cell Biology
Proteos #08-13
61 Biopolis Drive
Singapore 138673, Singapore

Hanry Yu
Institute of Molecular and Cell Biology
Proteos #08-13
61 Biopolis Drive
Singapore 138673, Singapore
    and
Institute of Bioengineering and Nanotechnology
The Nanos #04-01
31 Biopolis Way
Singapore 138669, Singapore
    and
National University of Singapore
Department of Physiology and NUSTEP
2 Medical Drive
Singapore 117597, Singapore

Thorsten Wohland
National University of Singapore
NUS Graduate Program in Bioengineering
28 Medical Drive
Singapore 117456, Singapore
    and
National University of Singapore
Department of Chemistry
3 Science Drive 3
Singapore 117543, Singapore

Abstract. The flow direction of microfluidics in biological applications is not limited to two dimensions, but often extends to three dimensions. Currently there are optical methods available for the measurement of 3-D microfluidic flow vectors, but with low spatial resolution. Line scan fluorescence correlation spectroscopy (FCS) was proposed to determine flow directions in 2-D within microchannels and small blood vessels in our previous work. Importantly, its spatial resolution was demonstrated to be as good as 0.5 μm. In this work, we extend line scan FCS to the third dimension for the characterization of 3-D flow velocity vectors. The spatial resolution is close to the diffraction limit using a scan length of 0.5 μm in all three dimensions. The feasibility of line scan FCS for 3-D microfluidic flow is verified by measurements in microchannels and small blood vessels of zebrafish embryos. © *2009 Society of Photo-Optical Instrumentation Engineers.* [DOI: 10.1117/1.3094947]

The measurement of microfluidic flow velocity is of great significance in the field of microfluidic chips, perfusion culture tissue engineering, and developmental biology. The information obtained is either used to improve the performance of microfluidic chip systems or collect physiological parameters from the circulation system for further experimental analysis.

Currently there are several noninvasive optical methods developed for this purpose, including laser line scan velocimetry,[1,2] particle image velocimetry (PIV),[3] and fluorescence correlation spectroscopy (FCS).[4,5] From the principles, most of these methods can be applied only to situations when the flow axis is limited to a 2-D plane. However, with the development of miniature fabrication techniques and the

---

Address all correspondence to Thorsten Wohland, National University of Singapore, Department of Chemistry, 3 Science Drive 3, Singapore 117543, Singapore. E-mail: chmwt@nus.edu.sg
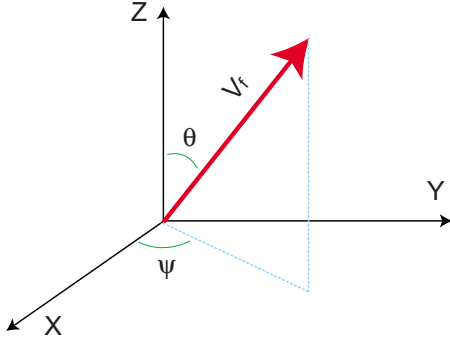
Fig. 1 A 3-D representation of flow velocity vector. Angle θ is the one formed between the flow vector and xy plane; angle ψ is the one between the x axis and the projection of flow vector in the xy plane.

growing interest in biological organism with complex 3-D structures, flows often have to be measured in a 3-D coordinate system. Therefore, we developed a noninvasive optical method that can measure 3-D microfluidic flow velocity vectors. Stereoscopic micro-PIV is one of the solutions for the measurement of three components of the velocity vector;[6] it uses two cameras to capture PIV images at two different angles by a stereomicroscope. However, the spatial resolution is limited by a low NA stereo objective $(44 \times 44 \times 15 \ \mu m^3)$ used in the stereomicroscope, and it requires a good and precise alignment of two focal planes through the objective in this case. Superresolution particle tracking velocimetry (PTV) is an alternative for 3-D velocity measurement,[7] but its low spatial resolution is still a disadvantage $(10 \times 10 \times 10 \ \mu m^3)$. In our previous work,[8] it was demonstrated that line scan FCS has a higher spatial resolution of 0.5 μm to resolve 2-D flow velocity. In this work, the line scan FCS was extended to the measurement of 3-D microfluidic flow velocity in both a microchannel and small blood vessel with a scan length of 0.5 μm in 3-D.

FCS is traditionally implemented at a single focal point;[9] spatial FCS measurements utilize two-foci schemes for the characterization of diffusion[10] and microfluidic flow.[11] Scanning FCS is another representative technique of spatial FCS measurements, which collects fluctuation signals along the scan path.[12] The principles of FCS for microfluidic flow and line scan FCS for 2-D flow velocity have been discussed previously.[8] Briefly, the dwell time of molecules in the laser focus increases or decreases by scanning the laser in different directions. This time varies dramatically when the direction of the laser scan is the same as or opposite to the flow. In 2-D flow measurements, a line scan of the laser focus is performed in the xy plane in one direction as required for the measurement of a 2-D flow velocity vector. The scan can be extended to the z direction to resolve a 3-D flow velocity. In this case, two additional line scans have to be implemented, one perpendicular to the first scan line in the same xy plane, and the other along the optical axis (z direction). For simplicity we refer to the three line scans as x-scan, y-scan, and z-scan. Therefore, the three following velocity vector equations can be obtained, where V_n is the apparent flow, V_f is the microfluidic flow, and V_s is the line scan speed:

x-scan:

$$\vec{V_{nx}} = \vec{V_f} + \vec{V_{sx}} = \begin{pmatrix} V_{fx} + V_{sx} \\ V_{fy} \\ V_{fz} \end{pmatrix},$$

y-scan:

$$\vec{V_{ny}} = \vec{V_f} + \vec{V_{sy}} = \begin{pmatrix} V_{fx} \\ V_{fy} + V_{sy} \\ V_{fz} \end{pmatrix},$$

z-scan:

$$\vec{V_{nz}} = \vec{V_f} + \vec{V_{sz}} = \begin{pmatrix} V_{fx} \\ V_{fy} \\ V_{fz} + V_{sz} \end{pmatrix}.$$

The apparent flow velocity vector is actually the combination of three velocity components. The mathematical equations regarding this relationship can be written as,

$$\begin{cases} V_{nx}^2 = (V_{fx} + V_{sx})^2 + V_{fy}^2 + V_{fz}^2 \\ V_{ny}^2 = V_{fx}^2 + (V_{fy} + V_{sy})^2 + V_{fz}^2 \\ V_{nz}^2 = V_{fx}^2 + V_{fy}^2 + (V_{fz} + V_{sz})^2 \end{cases} . \qquad (1)$$

During calibration, the scan speeds V_{sx}, V_{sy}, and V_{sz} are determined. The apparent flow velocities V_{nx}, V_{ny}, and V_{nz} are measured during the experiment. Thus, the three unknown parameters (V_{fx}, V_{fy}, and V_{fz}) can be found from the equations using the known values of V_{nx}, V_{ny}, V_{nz}, V_{sx}, V_{sy}, and V_{sz}. From these, the angles of the flow velocity vector can be calculated. The schematic representation of a flow velocity vector is shown in Fig. 1. The two characteristic angles of the flow vector are defined in the figure:

$$\theta = \arcsin\left(\frac{V_{fz}}{V_f}\right); \quad \psi = \arctan\left(\frac{V_{fy}}{V_{fx}}\right).$$

All the calculated FCS data from the algorithm for scan length reduction[8] were fitted with the following flow model.

$$G(\tau) = \left[\frac{F_{\text{trip}}}{1 + F_{\text{trip}}} \exp(- \tau/\tau_{\text{trip}}) + 1 \right]$$
$$\times \frac{1}{N} g(\tau) \exp\left\{ - \left[(\tau/\tau_f)^2 \frac{1}{1 + \tau/\tau_d} \right] \right\}, \qquad (2)$$

with

$$g(\tau) = \frac{1}{1 + \tau/\tau_d} \frac{1}{[1 + \tau/(K^2\tau_d)]^{1/2}}. \qquad (3)$$

The optical setup for fluorescence correlation microscopy (FCM) and xy line scan FCS, using the beam scanning mirrors, was described in a previous work.[13] To implement a line scan in the z direction, an extra device with a piezo scanner (LISA NanoAutomation Actuator, P753.2CD, Physik Instrumente, Germany) was designed and mounted on the microscope mechanical stage (Fig. 2). In the figure, the custom-made components of A, B, and C were fixed on the
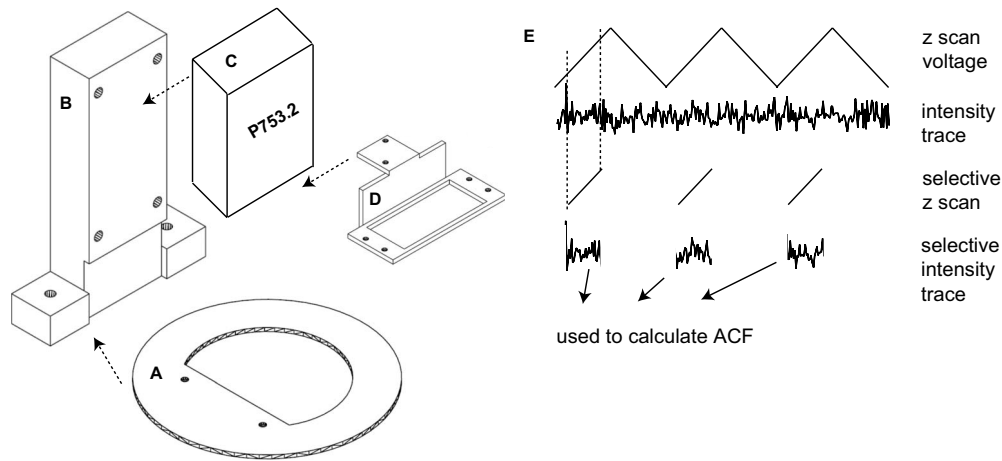
Fig. 2 Schematic diagram of z piezo scanner. A is the customized microscope stage insert; B is the support stand for the z scanner; C is the LISA NanoAutomation actuator (P753.2CD, Physik Instrumente); D is the adapter for #1 coverslip (50×22 mm) with samples (either microchannel or mounted zebrafish embryo); and E is the principle of scan length reduction.

microscope stage, while D, the sample mounting adapter, was connected to the piezo drive scanner. In this design, when the piezo scanner was moving vertically by applying a dc voltage, the sample attached to the adapter was being scanned in the z direction at the same frequency as the scanner. The mechanical stage and z-focus motor of the microscope were fixed, and an FCS point measurement was performed at the desired position. This is equivalent to line scan FCS in the z direction. The frequency of the sawtooth voltage wave applied to the piezo actuator/scanner was set to 300 Hz, and the maximum voltage amplitude was set to 1.2 V, which corresponded to a scan travel distance of 1.2 μm. The travel position of the scanner corresponded linearly to the applied dc voltage.

A polydimethylsiloxane (PDMS) microchannel was sealed on a glass coverslip, and its cross section was designed as a square of 100 μm width.[8] For this work, the fabricated microchannel included one inlet, one center channel, and one outlet (Fig. 3). During the fabrication process, the inlet channel (Fig. 3 part A) can be placed at different angles in the xz plane such as 30° and 60°. In this case, it was 30° or 60° for the angle of θ when measured in the inlet (Fig. 3 part A), 90° in the center channel (Fig. 3 part B), and 0° in the outlet (Fig. 3 part C). During the experiment, the center channel was manually placed at 45° for the angle ψ. Thus, 3-D microfluidic flow was generated when the dye solution (Atto 565) was perfused into the microchannel through the inlet.

The maintenance and preparation of zebrafish embryos was described previously.[8] Briefly, treated embryos were mounted in 0.5% low melting temperature agarose (Invitrogen, Singapore) on a glass coverslip (25×75 mm) for the following measurements. The 3-D organization of blood vessels in zebrafish embryos made it a good model to study the 3-D microfluidic flow in small blood vessels. As found, some vessels in the embryo brain were naturally isolated from others, and were thus clearly visible. All the blood vessels in TG(fli1:EGFP)^{y1} zebrafish embryo[14] expressed green fluorescent protein (GFP) in endothelial cells clearly marking the blood vessels [Fig. 4(b), lateral view]. A region of interest was then identified in the embryo brain [Fig. 4(c), dorsal view] for

Fig. 4 (a) Picture of GFP expressed in blood vessel endothelial cells of zebrafish embryo. Zoom in from (a), (b), (c), to (d) until a single blood vessel in 3-D is identified.
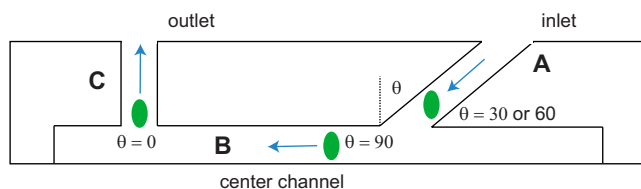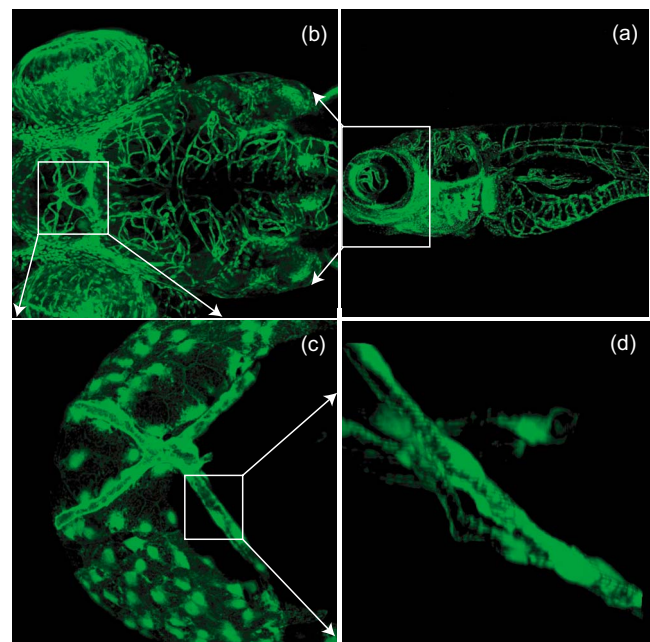

Fig. 3 Schematic diagram representation of a PDMS microchannel structure including inlet, center channel, and outlet. 3-D microfluidic flow can be measured in the inlet, center channel, or the outlet (green spot), namely at 30° or 60°, 90° and 0° for the angle of θ. The center channel is always placed at 45° for the angle of ψ. (Color online only.)
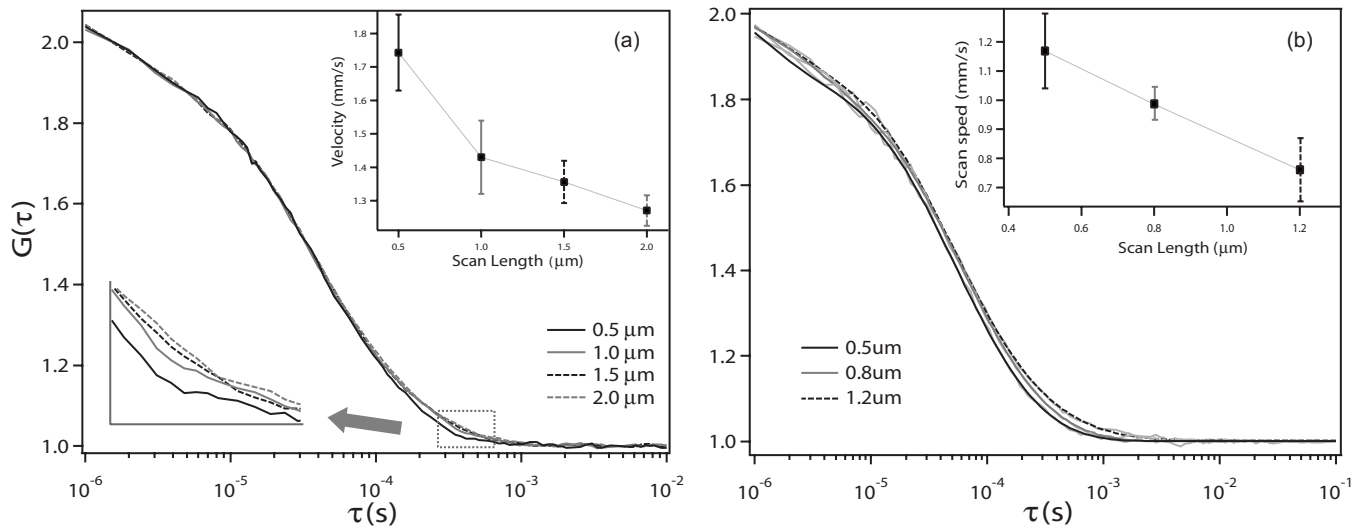
Fig. 5 ACF curves calculated from extracted photon counting data and scan speed graph (inset) of (a) xy scan and (b) z scan at different selective scan lengths.

3-D blood flow velocity measurements using line scan FCS. Furthermore, a single blood vessel organized in 3-D (approximately $\theta=45°$, $\psi=45°$) was found using confocal laser scanning microscopy (LSM) imaging [Fig. 4(d), dorsal view]. A 3-D projection from z-stack of the blood vessel [Fig. 4(e)] and its orientation in 3-D was determined.

The length of the scan line in the x and y directions can be reduced to restrict the used range to the linear range of the scanner and simultaneously increase the spatial resolution.[8] In x and y directions, line scan FCS was implemented by the galvanometer scanning mirrors. The experiment of selective scan length was duplicated in this case. The resulting autocorrelation function (ACF) curves are shown in Fig. 5(a), where the scan length was reduced from 2 μm, 1.5 μm, and 1.0 μm to 0.5 μm. The respective ACF curve shifted to the left, indicating a shorter dwell time of molecules in the laser focus. The corresponding measured scan speeds are shown in the inset of Fig. 5(a). The shorter the scan length is, the faster the scan speed. The same principle and strategy applies to the line scan in the z direction. To reduce the scan length, one of the methods is to record the photon counting data only from a part of the scan line, while the galvanometer mirrors scan over a long distance. By selecting the middle part of the scan line, the linear range of the scanners is used, avoiding problems of nonlinearities of the scanner as well as reducing the scan line. The extracted photon trace data are then used to calculate the ACF. The original z line scan length (travel distance) was 1.2 μm. In this case, it can be selectively reduced down to 0.5 μm. As shown in Fig. 5(b), the scan speed is becoming faster when the selective length reduction algorithm is applied to shorten the scan length from 1.2 down to 0.5 μm, which is represented by the left-shift of the ACF curves and the derived scan speed graph [Fig. 5(b) inset].

The PDMS microchannel is a good model to study microfluidic flow, because it is an optically transparent material and easy to manipulate for a variety of 3-D microchannel structures. For this work, a 3-D microchannel was designed and perfused with a solution of Atto 565 (1 nM). Point FCS was

first implemented to measure the absolute flow speed at a position in the microchannel (Fig. 3, green spot), and line scan FCS was then performed at the same position in xy directions using the galvanometer scanning mirrors in the confocal LSM scan unit, and in the z direction using the piezo actuator scanner. The selective scan length reduction is additionally applied to line scan FCS in the xyz directions. All ACF curves were fitted with the one-flow model [see Eq. (2)]. The calculated microfluidic flow velocities were used in Eq. (1). Thus the three components of flow velocity, V_{fx}, V_{fy}, and V_{fz}, were measured. The angles θ and ψ were then calculated from the known V_f vector components. The measurements were performed at different positions in one microchannel with an inlet channel at 30° (Fig. 3, parts A, B, and C) and the other microchannel with inlet channel at 60°. The result of 3-D flow velocity angles is listed in Table 1. As shown, the angles of ψ were measured as 46.5°, 54.7°, 39.4°, and 30°, which were close to the aligned channel angle of 45°. Considering the measurement and alignment errors, the line scan FCS can accurately measure the angle ψ for the 3-D flow velocity vector with an error less than 10° or better. During the experiment, the x and y line scans are both at an angle of 45° with respect to the flow axis, so the measurement error by line scan FCS is optimal. This is further demonstrated by the results of flow angle θ, which shows a large error (21.6°) at 0°, but smaller errors at 30°, 60°, and 90° (3.6°, 5.7°, and 12.4°, respectively). The reason is that the z scan line is parallel to the flow axis when the angle θ is 0°, and the measurement error decreases when θ is close to 90° (Ref. 8). To make the measurements more accurate, flow angles of θ and ψ are chosen close to 45° as a compromise in the following work.

Recent years saw a few FCS applications in blood vessels of small animals.[8,15] In this work, we extended our previous work of line scan FCS in zebrafish embryo to 3-D velocity measurements. The flow measurements without external label are made possible by any autofluorescent species within the blood. The major component would be erythrocytes, but au-

Table 1 Angles (θ and ψ) measured for 3-D flow velocity vector in a microchannel and a blood vessel of zebrafish embryo (unit: degree; μCh: microchannel; Fish: zebrafish embryo).

Sample	μCh	μCh	Fish	μCh	μCh
Angle θ	0	30	45	60	90
Measured θ	21.6	26.4	43.1	54.3	77.6
Angle ψ	45	45	45	45	45
Measured ψ	46.5	54.7	39.3	39.4	51.1

tofluorescent biomolecules within the blood will serve the same purpose, as flow can be observed even at embryonic development stages, at which erythrocytes are not present in the blood stream. It should be noted that due to the variety of fluorescent species, the amplitude of the FCS is difficult to interpret. However, the width of the FCS, which is dominated by the flow velocity, is reliable. This is confirmed by the injection of fluorescent labels into the blood stream of zebrafish embryos, which yield the same flow speeds (unpublished data). In the experiments, the zebrafish embryo was mounted at dorsal view in agarose gel on a coverslip glass. Then the coverslip was adapted to the z piezo scanner sitting on the microscopic mechanical stage. Using confocal LSM, a single blood vessel was identified in the brain of the embryo [Figs. 4(d) and 4(e)]. The orientation of the blood vessel was manually arranged at angles θ of 45° and ψ of 45°. Line scan FCS was performed in x and y directions using the galvanometer scan mirrors in slow scan mode with scan length of 3 μm, and in the z direction using the stage piezo scanner at a frequency of 300 Hz with scan length of 1.2 μm. Furthermore, the scan length of line scan FCS in the xyz directions was limited to 0.5 μm by the algorithm of selective scan length reduction described previously (Fig. 5). The line scan speeds in xyz directions were obtained during the system calibration ($V_{sx}=V_{sy}=1.60$ mm/s, and $V_{sz}=0.93$ mm/s), and all the ACF curves for blood flow coupled with line scan were fitted with the one-flow model [Eq. (2)]. From the fitting results, V_{nx}, V_{ny}, and V_{nz} are measured as 1.59, 1.53 and 1.30 mm/s, respectively. With the known parameters, the blood flow velocity in xyz directions is calculated as $V_{fx}=0.88$ mm/s, $V_{fy}=0.95$ mm/s, and $V_{fz}=1.06$ mm/s using Eq. (1), and the angles of θ and ψ were calculated as 43.1° and 39.3°, respectively. Therefore, the total flow velocity of blood at this position is about 1.67 mm/s. Corresponding to a report of blood flow velocity in the developing heart of zebrafish embryos,[2] the value ranged from 1 to 4 mm/s. Considering that FCS measures average flow velocity and organ difference, the prior measured velocity is physiologically plausible. The data are presented in Table 1, together with those in a microchannel. The resulting angles are quite close to the prealigned embryo orientation with small measurement errors. It demonstrates that line scan FCS can be extended to the z direction for 3-D flow velocity measurements in small animals. The scan length of line scan FCS in the z direction is kept to 0.5 μm, while the resolution of 2-D line scan FCS is maintained as 0.5 μm. Therefore, line scan FCS has potential applications in either tissue engineering or developmental bi-

ology, as the two fields may require an accurate measurement of 3-D microfluidic flow velocities to optimize or characterize the systems.

In this work, line scan FCS was extended to the third dimension to measure 3-D microfluidic flow velocities. For this purpose we introduced three line scans. Two along the xy axes performed by using the scan mirrors of the confocal microscope, and a third one along the z axis using a piezo scanner to scan the sample. The method and its accuracy were proved in both the microchannel and zebrafish embryo brain blood vessels with 3-D flow microstructures. With the development of selective scan length reduction, the scan length of line scan FCS was reduced to 0.5 μm in all three directions. This is an advantage to other methods available for 3-D microfluidic flow vector measurements. Due to its noninvasive property and successful demonstration of application in zebrafish embryo brain blood vessels, line scan FCS for 3-D microfluidic flow velocity could assist in the system optimization of microelectromechanical systems (MEMS) research, microfluidic-flow-related tissue engineering, or blood flow physiological measurements in small animals.

References

1. E. A. V. Jones, M. H. Baron, S. E. Fraser, and M. E. Dickinson, "Measuring hemodynamic changes during mammalian development," *Am. J. Physiol. Heart Circ. Physiol.* **287**, H1561–H1569 (2004).
2. M. H. Malone, N. Sciaky, L. Stalheim, K. M. Hahn, E. Linney, and G. L. Johnson, "Laser-scanning velocimetry: a confocal microscopy method for quantitative measurement of cardiovascular performance in zebrafish embryos and larvae," *BMC Biotechnol.* **7**, 40 (2007).
3. J. G. Santiago, S. T. Wereley, C. D. Meinhart, D. J. Beebe, and R. J. Adrian, "A particle image velocimetry system for microfluidics," *Exp. Fluids* **25**, 316–319 (1998).
4. D. Magde, W. W. Webb, and E. L. Elson, "Fluorescence correlation spectroscopy. iii. uniform translation and laminar flow," *Biopolymers* **17**, 361–376 (1978).
5. M. Gösch, H. Blom, J. Holm, T. Heino, and R. Rigler, "Hydrodynamic flow profiling in microchannel structures by single molecule fluorescence correlation spectroscopy," *Anal. Chem.* **72**, 3260–3265 (2000).

6. R. Lindken, J. Westerweel, and B. Wieneke, "Stereoscopic micro particle image velocimetry," *Exp. Fluids* **41**, 161–171 (2006).
7. M. R. Bown, J. M. MacInnes, R. W. K. Allen, and W. B. J. Zimmerman, "Three-dimensional, three-component velocity measurements using stereoscopic micro-piv and ptv," *Meas. Sci. Technol.* **17**, 2175–2185 (2006).
8. X. Pan, H. Yu, X. Shi, V. Korzh, and T. Wohland, "Characterization of flow direction in microchannels and zebrafish blood vessels by scanning fluorescence correlation spectroscopy," *J. Biomed. Opt.* **12**, 014034 (2007).
9. E. L. Elson and D. Magde, "Fluorescence correlation spectroscopy. I. Conceptual basis and theory," *Biopolymers* **13**, 1–27 (1974).
10. R. Jaffiol, Y. Blancquaert, A. Delon, and J. Derouard, "Spatial fluorescence cross-correlation spectroscopy," *Appl. Opt.* **45**, 1225–1235 (2006).
11. M. Brinkmeier, K. Doerre, J. Stephan, and M. Eigen, "Two-beam cross-correlation: a method to characterize transport phenomena in micrometer-sized structures," *Anal. Chem.* **71**, 609–616 (1999).
12. N. O. Petersen, "Scanning fluorescence correlation spectroscopy. I. Theory and simulation of aggregation measurements," *Biophys. J.* **49**, 809–815 (1986).
13. X. Pan, M. Fok, W. Foo, W. Lim, P. Liu, H. Yu, I. Maruyama, and T. Wohland, "Multifunctional fluorescence correlation microscope for intracellular and microfluidic measurements," *Rev. Sci. Instrum.* **78**, 053711 (2007).
14. N. D. Lawson and B. M. Weinstein, "*In vivo* imaging of embryonic vascular development using transgenic zebrafish," *Dev. Biol.* **248**, 307–318 (2002).
15. A. A. Clancy and D. T. Cramb, "Dynamics of quantum dots in angiogenic blood vessels: a fluorescence correlation spectroscopy study," *Proc. SPIE* **6866**, P8660-P8660 (2008).