Measurement of Three-Dimensional Velocity upon Endothelial Cells Utilizing Confocal Micro-PIV

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ABSTRACT

To investigate an influence of a glycocalyx surface layer covering over endothelial cells (ECs) on the flow field or flow through the layer, a high spatial resolution measurement technique was improved for three-dimensional velocity distributions close to a glycocalyx layer by utilizing a confocal micro-PIV (Particle Image Velocimetry) technique. The developed technique achieved to measure three-dimensional velocity distributions in a $177 \times 177 \times 3 \ \mu\text{m}^3$ region with in-plane resolution of 7 μ m and with depth resolution of 0.5 μ m. By optimizing the PIV analysis, the measurement accuracy was improved even with small fluorescent particle of the diameter of 200 nm and large shear rate. Three-dimensional velocity distribution close to the layer of living ECs cultured in microchannel was measured. The results suggested that the developed technique was useful for investigation of velocity field close to the layer.

INTRODUCTION

Blood flow plays a critical role in maintaining homeostasis of tissue and organs such as delivering oxygen and nutrients. To investigate cause of vascular diseases such as cerebral infarction and cardiac infarction, a number of studies for biophysical behavior of blood flow in the vascular system have been carried out [1-4]. Fluid shear stress was related to physiological vascular functions such as maintenance of vessel tone, prevention of thrombosis and initiation of angiogenesis [5, 6]. Secretion of nitric oxide (NO) and intercellular calcium (Ca²⁺), and remodeling of blood vessel such as elongation and orientation in the flow direction were observed as response of Endothelial cells (ECs), which line inner surface of blood vessel, to fluid shear stress [7, 8]. One of the important factors in determining biophysical behavior of blood flow was considered to be glycocalyx surface layer [9, 10]. The thickness of the layer was measured using the transmission electron microscopy (TEM) and varied from 20 nm, 200 -500 nm to 2 - 4.5 μ m [11-14]. Blood flow resistance and vascular permeability, such as limiting access of certain molecules to the EC membrane, were notably affected by existence or non-existence of the layer [15- 16]. Morphological change and production of NO was also affected by the layer [17]. The layer was considered to play an important role in sensing of wall shear stress and in mechanotransduction [18]. Therefore, to clarify biophysical behavior of blood flow, it is important to investigate the flow field in vicinity of cell surface especially near the layer.

In order to investigate the influence of the layer on the flow field or flow through the layer, various theoretical studies have been carried out [19-25]. Since the glycocalyx layer shows bush like structure and elasticity, the layer has been modeled as a biphasic mixture, or porous layer, with a linearly elastic solid phase and an incompressible Newtonian fluid phase. These models indicated that the glycocalyx lead to lower fluid wall shear stresses at the endothelial surface than in the absence of the layer. Even a relatively thin glycocalyx with sufficiently low permeability impeded fluid flow near vessel wall and exerted a significant influence on microvascular resistance. The glycocalyx behaves as a molecular filter of water, ions and small hydrophilic solutes between the plasma and cells. Conversely, a few experimental studies have been reported due to difficulty of measurement in its small scale. Conventional micro particle image velocimetry (micro-PIV) technique was applied to flow field near the vessel wall in post-capillary venules in vivo [26]. The authors developed a high spatial resolution measurement technique for velocity distributions close to surface of cell utilizing confocal micro-PIV technique [27]. However, these measurement techniques were insufficient in a spatial resolution and accuracy to investigate the influence of the layer.

In the study, in order to investigate an influence of a glycocalyx surface layer on a flow field, a high spatial resolution measurement technique was improved for three-dimensional velocity distributions close to a glycocalyx layer by utilizing confocal micro-PIV technique. The optics was optimized and the PIV analysis was improved to obtain three-dimensional velocity distribution with high accuracy close to the layer by scanning very thin measurement plane. Three-dimensional velocity distribution near the layer of living ECs cultured in microchannel was investigated.

EXPERIMENTAL SETUP

Figure 1 shows the developed confocal micro-PIV system, which enables to remove out-of-focus light optically and obtain higher contrast image than conventional micro-PIV system [28], in order to improve a spatial resolution and measurement accuracy. The system consisted of an inverted microscope with a dual Nipkow disk-type confocal scanner, CSU-X1 (Yokogawa Elc. Cop., Japan), a CW laser (SAPPHIRE; Coherent) with a wavelength $\lambda = 488$ nm and a high speed camera (SV-200i; Photron, 10bit) set 1000 fps with resolution of 512 × 512 pixels. By using a high numerical aperture lens, a 40× oil immersion objective lens (Plan Fluor; Nikon, NA = 1.3), and a piezo actuator (P-721K120; PI) with sub-nm resolution accuracy, three dimensional velocity distributions with high spatial resolution was achieved. The depth of focus of the confocal optical system was estimated to be 0.58 µm. The measurement volume was corresponded to $177 \times 177 \times 3 \mu m^3$, in which depth resolution was 0.5 µm from Z = 13 to Z = 15.5 µm as shown in figure 2. Culture medium seeded with fluorescent particles with a diameter of 200 nm, which excites at 505 nm and emits at 515 nm, was used as a working fluid. The fluid was injected by using a microsyringe pump at the constant flow rate of 3.0 µL/min, Reynolds number Re = 0.21, which corresponded with flow rate of in vivo condition.



Figure 2 Measurement volume for velocity distributions around endothelial cell scanned by piezo actuator.

Since a dual Nipkow disk-type confocal scanner provides that an exposure time of a frame was almost equal to inverse of the frame rate, it is necessary to set enough higher frame rate compared with particle displacement to avoid a long streakline, which causes low measurement accuracy. Therefore high frame rate, 1000 fps, for all of focal planes compared with particle displacement was settled. To improve measurement accuracy by optimizing particle displacement, image pair from sequential images was settled varying the time interval Δt with focal position shown as figure 3. Appling the cross-correlation method and a Gaussian peak fitting method for sub-pixel analysis with an interrogation window of 40×40 pixels with 50% overlap, which was corresponded with in-plane spatial resolution of 7 \times 7 μ m², 200 velocity distributions over all image pair were obtained. Temporal averaging was applied over 200 velocity distributions to eliminate the effect of Brownian motion. The time interval at each focal plane at the minimum standard deviation was used for PIV analysis.



Figure 3 Optimization of PIV analysis by adjusting the time interval of image pairs.



Figure 4 Fluorescent particle image around the microchannel bottom with endothelial cells.

Flow field around living ECs cultured in a microchannel as shown in figure 4 (a) and (b) was used. A polydimethylsiloxane (PDMS) microchip consists of a single straight microchannel, which has a rectangular cross sectional shape with width of 400 µm, depth of 120 µm and long of 2 cm fabricated using soft-lithographic techniques as follows. A negative photoresist SU-8 was poured on a glass plate using spin-coater. After soft-bake on a hot plate, UV-light was exposed through a photolithography mask using a mask aligner to impress the channel pattern onto the SU-8. The exposed substrate was hard-baked and developed with a developer solution to remove photoresist in unexposed areas. A PDMS slurry on the master was baked in the oven. The glass slide with thickness of 170 µm was attached to the PDMS. Microtubes with the inner diameter of 500 µm were connected to microchip for fluid inlet and outlet. Human umbilical vein endothelial cells (HUVECs), which cultured in a 35 mm² dish with an EC culture medium (EGM-2, Lonza), were used in the experiments. After sterilization using ultraviolet lump and 70 % ethanol, the microchannel wall was coated with matrigel, which is one of an extracellular matrix gel and often used for surface coating of a dish. Solution of matrigel (0.1 mg/mL) was introduced into the microchannel. The microchip was incubated in a 5 % CO₂ incubator at 37 degree Celsius for 1 hour without flow to adsorb matrigel and then rinsed with the culture medium. ECs suspension at a cell density of 10⁶ cells/mL was introduced at the flow rate of 5.0 µl/min by withdrawing the micro syringe by the pump shown. The microchip was incubated in the 5 % CO₂ incubator at 37 degree Celsius for 2 hours to be attached ECs on the inner wall without flow. Figure 4 (c) shows a fluorescent particles image at the bottom of the microchannel, $Z = 0 \mu m$. White vertical line in the figure represents the side wall of the microchannel.

Red dashed lines represents ECs with size of $30 - 50 \mu m$, in which fluorescent particle was absent. The culture medium flowed in the microchannel from bottom to top in the figure.

RESULTS

Figure 7 shows three-dimensional temporal averaged velocity distributions at six measurement planes with depth resolution of 0.5 μ m in the microchannel with ECs obtained by the proposed technique after the optimization process as mention above. Height of the top of EC was roughly estimated to be 13 μ m around X = 120, Y = 80 μ m in the figure. Velocity vectors at all of the planes were almost parallel to the side wall, and velocity gradually increased from the side wall to the center of the channel. Maximum velocity was 0.76 mm/s around the center of the channel at Z = 15.5 μ m. Velocities were corresponded with theoretical value of laminar flow in the microchannel except them of upper parts of ECs. Velocities of upper parts of ECs were smaller than theoretical value. These velocities show the effect of the EC shape. The results indicated the developed technique was achieved high accuracy and high spatial resolution in spite of the large shear rate and close to wall.



Figure 5 Three dimensional temporal averaged velocity distributions at six planes.

CONCLUSIONS

We improved a high resolution measurement technique for three-dimensional velocity distributions close to a glycocalyx surface layer of living ECs by utilizing confocal micro-PIV to investigate the flow field in vicinity of vessel wall. The developed technique enabled to measure three-dimensional velocity distributions of micro-flows in a $177 \times 177 \times 3 \ \mu\text{m}^3$ region with in-plane resolution of 7 μm and with depth resolution of 0.5 μm . By optimizing the PIV analysis with adjusting the time interval, the measurement accuracy was improved even with small fluorescent particle of the diameter of 200 nm and large shear rate. Three-dimensional velocity distributions around living ECs culture in the PDMS microchannel with the rectangular cross sectional shape, width of 400 μm and depth of 120 μm were measured at six planes spaced 0.5 μm apart. The obtained velocity distributions indicated the influence of the EC on the flow field. The results showed the developed technique was achieved high accuracy and high spatial resolution in spite of the large shear rate and close to wall.

REFERENCES

- [1] Goldsmith HL "The microrheology of human blood" Microvascular Research 31 (1986) pp.121-142
- Hudetz AG "Blood Flow in the Cerebral Capillary Network: A Review Emphasizing Observations with Intravital Microscopy" Microcirculation 4 (1997) pp.233-252
- [3] Sugii Y, Nishio S, Okamoto K "in vivo PIV measurement of red blood cell velocity field in microvessels considering mesentery motion" Physiological measurement 23 (2002) pp.403

- [4] Sugii Y "Simultaneous measurement of wall shear stress distribution and three-dimensional shape of living endothelial cells cultured in microchannel" Journal of Biomechanical Science and Engineering 5 (2010) pp.625-634
- [5] Davies PF, Dewey CF, Bussolari SR, Gordon EJ "Influence of hemodyanamic forces on vascular endothelial function. In vitro studies of shear stress and pinocytosis in bovine aortic cells" Journal of Clinical Investigation 73 (1984) pp.1121-1129
- [6] Pries AR, Secomb TW "Microvascular blood viscosity in vivo and the endothelial surface layer" American Journal of physiology 289 (2005) pp.2657-2664
- [7] Adams DJ, Barakeh J, Laskey R, van Breemen C "Ion channels and regulation of intracellular calcium in vascular endothelial cells" The FASEB Journal 3 (1989) pp.2389-2400
- [8] Barbee KA, Davies PF, Lal R "Shear stress-induced reorganization of the surface topography of living endothelial cells imaged by atomic force microscopy" Circulation Research 74 (1994) pp.163-171
- [9] Secomb TW, Hsu R, Pries AR "Effect of the endothelial surface layer on transmission of fluid shear stress to endothelial cells" Biorheology 38 (2001) pp.143-150
- [10] Weimnbaum S, Zhang X, Han Y, Vink H, Cowin SC "Mechanotransduction and flow across the endothelial glycocalyx" Proceedings of the National Academy of Sciences of the United States of America 100 (2003) pp.7988-7995
- [11] Luft JF "Fine structures of capillary and endocapillary layer as revealed by ruthenium red" Federation proceedings 25 (1966) pp.1773-1783
- [12] van den Berg BM, Vink H, Spaan JA "The endothelial glycocalyx protects against myocardial edema" Circulation Research 92 (2003) pp.592-594
- [13] van Haaren PMA, VanBavel E, Vink H, Spaan JA "Localization of the permeability barrier to solutes in isolated arteries by confocal microscopy" American Journal of Physiology - Heart and Circulatory Physiology 285 (2003) pp.2848-2856
- [14] Megens RTA, Reitsma S, Schiffers PHM, Hilgers RHP, De Mey JGR, Slaaf DW, oude Egbrink MGA, van Zandvoort MAMJ "Twophoton microscopy of vital murine elastic and muscular arteries" Journal of Vascular Research 44 (2007) pp.87-98
- [15] van Teeffelen JWGE, Dekker S, Fokkema DS, Siebes M, Vink H, Spaan JA "Hyaluronidase treatment of coronary glycocalyx increases reactive hyperemia but not adenosine hyperemia in dog hearts" American Journal of Physiology - Heart and Circulatory Physiology 289 (2005) pp.2508-2513
- [16] Henry CBS, Duling BR "Permeation of the luminal capillary glycocalyx is determined by hyaluronan" American Journal of Physiology - Heart and Circulatory Physiology 277 (1999) pp.508-514
- [17] Mochizuki S, Vink H, Hiramatsu O, Kajita T, Shigeto F, Spaan JAE, Kajiya F "Role of hyaluronic acid glycosaminoglycans in shearinduced endothelium-derived nitric oxide release" American Journal of physiology 285 (2003) pp.722-726.
- [18] Gouverneur M, van den Berg B, Nieuwdorp M, Stroes E, Vink H "Vasculoprotective properties of the endothelial glycocalyx: effects of fluid shear stress" Journal of Internal Medicine 259 (2006) pp.393-400
- [19] Barry SI, Parker KH, Aldis GK "Fluid flow over a thin deformable porous layer" Journal of Applied Mathematics and Physics 42 (1991) pp.633-648
- [20] Wang W, Parker KH "The effect of deformable porous surface layers on the motion of a sphere in a narrow cylindrical tube" Journal of Fluid Mechanics 283 (1995) pp.287–305
- [21] Damiano ER, Duling BR, Ley K, Skalak TC "Axisymmetric pressure-driven flow of rigid pellets through a cylindrical tube lined with a deformable porous wall layer" Journal of Fluid Mechanics 314 (1996) pp.163-189
- [22] Damiano ER, Stace TM "A mechano-electrochemical model of radial deformation of the capillary glycocalyx" Biophysical Journal 82 (2002) pp.1153-1175
- [23] Damiano ER, Long DS, Smith ML "Estimation of viscosity profiles using velocimetry data from parallel flows of linearly viscous fluid: application to microvascular haemodyanamics" Journal of Fluid Mechanics 512 (2004) pp.1-19
- [24] Damiano ER, Stace TM "Flow and deformation of the capillary glycocalyx in the wake of a leukocyte" Physics of Fluids 17 (2005) pp.031509-17
- [25] Han Y, Weinbum S, Spaan JA, Vink H "Large-deformation analysis of the elastic recoil of fiber layers in a Brinkman medium with application to the endothelial glycocalyx" Journal of Fluid Mechanics 554 (2006) pp.217-235
- [26] Smith ML, Long DS, Damiano ER, Ley K "Near-wall m-PIV reveals a hydrodynamically relevant endothelial surface layer in venules in vivo" Biophysical Journal 85 (2003) pp.637-645

- [27] Mukoyama T, Sugii Y, Hishida K "High resolution measurement technique for near surface velocity distribution and surface topography of endothelial cells using confocal micro-PIV" Proceedings of 16th International Symposium on Applications of Laser Techniques to Fluid Mechanics Lisbon, Portugal (2012)
- [28] Park JS, Choi CK, Kihm KD "Optically sliced micro-PIV using confocal laser scanning microscopy (CLSM)" Experiment in Fluids 37 (2004) pp.105-119