High Resolution Measurement Technique for Near Surface Velocity Distribution and Surface Topography of Endothelial Cells Using Confocal Micro-PIV

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Abstract To investigate cause of vascular diseases, it is necessary to investigate response of endothelial cells (ECs), which line inner surface of blood vessels, to wall shear stress caused by blood flow. Glycocalyx surface layer, which covers over EC surface with a thickness of 200-500 nm, played an important role of the response to wall shear stress such as maintaining the vascular permeability barrier and regulating the release of shear stress-induced nitric oxide (NO). The layer was considered to affect sensing of wall shear stress and vascular permeability, such as activation of ion channels and ion transportation. Therefore, we will develop the measurement technique of the position and thickness of glycocalyx surface layer and near surface velocity distributions to estimate the flow in glycocalyx surface layer. In the study, a novel high resolution measurement technique was developed for near surface velocity distribution by using confocal micro-PIV (Particle Image Velocimetry) system to estimate the flow field in glycocalyx surface layer. To improve the measurement accuracy, time interval of image pairs for PIV analysis was adjusted at different Z position. The technique was applied to living ECs cultured in a microchannel of a rectangular cross-sectional shape with a width of 400 µm, depth of 63 µm and long of 2 cm, made of polydimethylsiloxane (PDMS) and glass bottom. Velocity distributions at four planes (Z = 5, 10, 15, 20 µm) scanned by using piezo actuator at the flow rate of 3.0 µl/min (Re = 0.22) were obtained. The results suggest that the developed technique enabled to measure flow field around ECs.

1. Introduction

To investigate cause of vascular diseases such as cerebral infarction and cardiac infarction, it is necessary to clarify relationship of Endothelial cells (ECs), which line inner surface of blood vessels, with hemodynamic forces, especially fluid shear stress caused by blood flows. Fluid shear stress is a critical factor in determining physiological vascular functions such as maintenance of vessel tone, prevention of thrombosis, promotion of fibrinolysis and initiation of angiogenesis (Davies et al, 1984). Secretion of nitric oxide (NO) and intercellular calcium (Ca²⁺) were observed as response of ECs to shear stress. Remodeling of blood vessel such as elongation and orientation in the flow direction was also caused by the wall shear stress. It was considered that ECs changed their morphology measured by atomic force microscopy to reduce the maximum wall shear stress or stress gradient (Barbee et al, 1994). To determine the mechanisms of the response, the surface geometry of the cells was measured by confocal laser scanning microscopy, and the shear stress distribution on the measured cell surface was determined using the flow field simulated by computational fluid dynamics (Fukushima et al 2003). The results show the importance of not only the mean shear stress but also interaction with surrounding cells to the adaptive response. Sugii (2010) developed a non-intrusive measurement technique for wall shear stress distributions and morphology simultaneously around living ECs to investigate the relationship between them.

Glycocalyx surface layer, which covers over EC surface with a thickness of 200-500 nm, played an important role of the response to wall shear stress such as maintaining the vascular permeability barrier and regulating the release of shear stress-induced nitric oxide (NO) (Gouverneur et al 2006). It was reported that morphological change and production of NO was notably affected by existence...
or non-existence of the layer (Mochizuki et al 2003). The layer was considered to affect sensing of wall shear stress and vascular permeability, such as activation of ion channels and ion transportation (Gautam et al, 2006). Therefore, it is important to investigate the flow field in the layer. However, the layer is too thin to measure the flow field in the layer directly. The flow field in the layer shown in Figure 1 was estimated by the Brinkman equation by assuming the layer as a porous material, in which the thickness of the layer $\delta$ and velocity at $Z = \delta$ were estimated by using velocity distribution measured by conventional micro-PIV (Particle Image Velocimetry) (Damiano et al 2004). We will develop the measurement technique of the position and thickness of the layer and near surface velocity distributions to estimate the flow in the layer.

In the study, in order to estimate the flow field in the glycocalyx surface layer, a novel high resolution measurement technique was developed for near surface velocity distribution close to glycocalyx layer by using confocal micro-PIV system. The developed technique was applied to living ECs cultured in microchannel.

2. Experiment set-up
2.1. Measurement System

Figure 2 shows a schematic diagram of the confocal micro-PIV system to measure velocity distribution close to glycocalyx surface layer. The system consisted of an inverted microscope with a $20\times$ objective lens (Plan Fluor; Nikon, NA = 0.75), the confocal scanner, a CW laser (SAPPHIRE; Coherent) with a wavelength $\lambda = 488$ nm and a high speed camera (SV-200i; Photron, 10bit) set 2000 fps with resolution of $512 \times 512$ pixels, which was corresponding to $228 \times 228 \mu m^2$. The confocal scanner and the high speed camera were synchronized. A dual Nipkow disk-type confocal scanner, CSU-X1 (Yokogawa Elc. Cop., Japan), was used in the confocal micro-PIV system since it offers the highest confocal scanning rate. It is possible to produce confocal images continuously at 2000 fps and the scanning time required for a single cross-sectional image is 0.5 ms. Focal position along the Z-axis was controlled by using piezo actuator.

Culture medium containing the fluorescent particles with diameter of 500 nm, which excites at 505 nm and emits at 515 nm, were injected at the constant flow rate of 3.0 $\mu l/min$ ($Re = 0.22$) by using a microsyringe pump. Velocity distributions were obtained using cross-correlation method with an interrogation window of $40 \times 40$ pixels with 50% overlap. A Gaussian peak fitting method for sub-pixel analysis was used. The depth of field (DOF) of the system was estimated to be 4.90 $\mu m$, theoretically (Park et al 2004).
2.2. Culture of ECs in PDMS microchip

The developed system was applied to flow field around living ECs cultured in a microchannel. A polydimethylsiloxane (PDMS) microchip consists of a single straight microchannel, which has a rectangular cross sectional shape with width of 400 µm, depth of 63 mm 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. Microtubes were connected to microchip for fluid inlet and outlet.

Human umbilical vein endothelial cells (HUVECs) were cultured in a 35 mm² dish with an EC culture medium (EGM-2, Lonza). It was observed that cell density increased from 2 hours to 96 hours after cell seeding in figure 3. ECs from 48 hours to 96 hours after seeding were used for culturing in the microchannel. 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 one 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 ml/min by withdrawing the micro syringe by the pump shown in figure 3. The microchip was incubated in the 5 % CO₂ incubator at 37 degree Celsius for two hours to be attached ECs on the inner wall without flow.
3. Result and Discussion

Red and blue squares in figure 4 (a) represent the observation area by phase contrast microscopy to confirm ECs attached on the bottom of the microchannel and the measurement region by the confocal micro-PIV system, respectively. The culture medium flowed in the microchannel from bottom to top in the figure. Figure 4 (b) shows a phase-contrast image of the microchip two hours after seeding of ECs. ECs attached to the bottom of the microchannel were observed as round or elliptical shape with size of 40 – 50 µm represented by red dashed lines. It was confirmed that ECs were cultured in microchannel successfully. Figure 4 (c) shows a fluorescent particles image at the bottom of the microchannel (Z = 0 mm) obtained by the confocal micro-PIV system. White vertical line in the figure represents the side wall of the microchannel. Red dashed lines represent ECs with sizes of 30 – 70 µm, in which fluorescent particle was absent because of EC shape.

![Microchannel used for culture of ECs](image1)

![Phase contrast image of ECs](image2)

![Fluorescent particle image at the bottom](image3)

Figure 4. ECs cultured in microchannel

In order to assess the developed technique, velocity distributions at four focal planes Z = 5, 10, 15, 20 µm scanned by piezo actuator were obtained. Figure 5 shows measurement planes for velocity distributions around ECs scanned by piezo actuator. Since pixel displacements varied at different Z position, time interval Δt of image pairs for PIV analysis was adjusted to improve the measurement accuracy shown in figure 6. In the case of Δt = 0.5 ms (Case 1), two successive images as image pair for analysis were settled, and in the case of Δt = 1.5 ms (Case 2), first and
fourth images as image pair were settled. By optimizing the time interval, it is possible to improve the PIV error and also to reduce the effect of the Brownian motion. The effect of Brownian motion $v_b$, which was given by equation (1) (Santiago et al, 1998), was estimated to be 0.063 mm/s at $\Delta t = 0.5$ ms, 0.033 mm/s at $\Delta t = 2$ mm/s, 0.022 mm/s at $\Delta t = 2.5$ mm/s, 0.018 mm/s at $\Delta t = 4$ ms and 0.016 mm/s at $\Delta t = 8$ ms, respectively.

$$v_b = \frac{2\kappa T}{3\pi \mu d_p \Delta t},$$

(1)

where $\kappa$ is Boltzman’s constant, $T$ is the absolute temperature of the fluid, $\mu$ is dynamic viscosity of the fluid.

Table 1 shows standard deviation of velocities and standard deviation to magnitude of the velocity ratio for various $\Delta t$ at $X = 205$ $\mu$m, $Y = 175$ $\mu$m. Both of standard deviation and standard deviation to magnitude of the velocity ratio at all of plans became small by adjusting the time interval of image pair. After optimization, $\Delta t$ was adopted to be 8 ms at $Z = 5$ $\mu$m, 4 ms at $Z = 10$ $\mu$m, 2.5 ms at $Z = 15$ $\mu$m and 2 ms at $Z = 20$ $\mu$m, respectively. The results show that the adjustment of the time interval was effective to improve measurement accuracy.
Table 1. Standard deviation of velocities for various $\Delta t$ at $X = 205 \, \mu m$, $Y = 175 \, \mu m$

<table>
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<tr>
<th>$Z$ [Å]</th>
<th>$\Delta t$ [ns]</th>
<th>$\sqrt{U^2 + V^2}$ [mm/s]</th>
<th>$SD$ [mm/s]</th>
<th>$SD / \sqrt{U^2 + V^2}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
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<td>0.539</td>
<td>0.058</td>
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</tr>
<tr>
<td>8</td>
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<td>0.568</td>
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<td>0.04</td>
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<tr>
<td>10</td>
<td>0.5</td>
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<td>0.103</td>
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<td>0.033</td>
<td>0.03</td>
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<tr>
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<td>1.925</td>
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<tr>
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</tr>
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</table>

Figure 7 shows in-plane temporal averaged velocity distributions in four measurement planes. To eliminate effect of the Brownian motion, the temporal averaging was applied over 100 velocity distributions. Gray ellipses at the bottom represent the ECs. At $Z = 20 \, \mu m$, velocity vectors were almost parallel to the side wall and velocity gradually increased from the side wall to the center of the channel. Maximum velocity was to be 2.4 mm/s at the center of the channel. The velocity distribution agreed with theoretical one of the laminar flow in the channel. At $Z = 5, 10 \, \mu m$, close to the bottom of the channel, velocity gradually became smaller. Maximum velocities at $Z = 5, 10 \, \mu m$ were 0.6 and 1.3 mm/s, respectively. The variations of the velocity vectors were observed around $X = 112 \, \mu m$, $Y = 142 \, \mu m$, especially, whose position corresponded with upper area of ECs. To clarify the variations in detail, two-dimensional temporal averaged velocity distributions and contour maps of the velocity distributions at four planes were shown in figure 8 (a)-(d). Black dashed lines represent ECs observed at $Z = 0 \, \mu m$. The side wall was at $X = 5 \, \mu m$. At $Z = 5 \, \mu m$, significant reduction of velocity was observed around the upper areas of ECs. Velocities at the upper areas of three ECs, were 0.05 mm/s at $X = 36 \, \mu m$, $Y = 202 \, \mu m$, 0.17 mm/s at $X = 52 \, \mu m$, $Y = 60 \, \mu m$, 0.09 mm/s at $X = 112 \, \mu m$, $Y = 142 \, \mu m$. Around the upper area of ECs at $Z = 15, 20 \, \mu m$ reduction of velocity became small. To clarify the influence of the EC on flow, figure 9 shows cross-sectional velocity profiles at $Y = 112 \, \mu m$, in which the largest EC was attached at the bottom. Although velocities in the range of around $X > 100 \, \mu m$ should be constant theoretically, velocity around EC was smaller than them at the center of the microchannel due to EC shape, whose ratio were 99, 95, 82 % and 16 at $Z = 5, 10, 15$ and $20 \, \mu m$, respectively. Thus, the obtained results revealed that the technique is useful for measurement close to ECs surface.
Figure 7. In-plane temporal averaged velocity distributions at four planes

(a) $Z = 5 \, \mu m$
Figure 8. Two-dimensional temporal averaged velocity distributions and contour maps at four planes.
4. Conclusions

In the study, we developed a novel high resolution measurement technique for near surface velocity distributions close to endothelial cells surface by using confocal micro-PIV to investigate flow field in a glycocalyx surface layer. As a measurement of the flow field around ECs cultured in microchannel, we obtained following conclusions.

(1) The developed technique enabled to measure cross-sectional velocity distributions of micro-flows in a $228 \times 228 \mu m$ region with optical slice thickness of $4.9 \mu m$. The measurement accuracy by optimizing the time interval of image pairs for PIV analysis was improved.

(2) ECs attached to the bottom of the microchannel by coating the wall with matrigel, which is one of an extracellular matrix gel. Velocity distributions around ECs were measured at four planes by the developed technique. The variation of flow field influenced by three-dimensional shape of ECs was observed at the upper area on them.

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6. References


