Visualization of Near Surface Flow of Endothelial Cells based on Confocal Micro-PIV and Super-resolution Microscopy

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Abstract A glycocalyx layer located on the surface of vascular endothelial cells (ECs), whose thickness was to be 20 nm - 4.5 μm measured by transmission electron microscopy, is considered to play an important role in the sensing of fluid force. It is necessary to clarify an influence of the glycocalyx layer on the near surface flow field. However, it is difficult to measure the flow field near the layer because of lack of spatial resolution and accuracy. In this study, we developed a novel visualization technique which has high spatial resolution and high accuracy to investigate the influence of the glycocalyx layer on the near-surface flow field. A measurement technique for three dimensional velocity distributions with spatial resolution of 250 nm in the depth direction was developed by utilizing the confocal micro-PIV technique with optimization of the measurement parameter such as the objective lens, optical filters and by utilizing 100nm silica particles coated with polyethylene glycol (PEG). Super-resolution microscopy was used for the measurement of position and thickness of a glycocalyx layer with lateral resolution of 80 nm. The technique was applied to near surface flow field around EC cultured in a microchannel with a width of 400 μm and depth of 100 μm. The height of the EC and the thickness of the layer around the top of the EC were measured to be 4.67 and 1.33 μm. The velocities near the surface of the glycocalyx layer and the dimension of the layer were successfully measured. The results indicated that the measured velocity distributions show the influence of the layer on the near-surface flow field.
Keywords: Glycocalyx, Endothelial cell, Confocal micro-PIV, Super-resolution Microscopy

1 Introduction

Blood flow in micro vessels, such as velocity distributions and deformation of red blood cell, has been experimentally investigated by applying the micro PIV technique [1-4]. The measurement results showed multi-phase and non-Newtonian flow features. In order to clarify interaction between blood flow and endothelial cells (ECs) which line the inner surface of blood vessels, micro fluidic devices cultured with ECs was developed by utilizing micro fabrication technique and cell culture technique [5], and a measurement technique for wall shear stress distribution and cell topology in the device was developed [6].

It was reported that a glycocalyx layer coating on the ECs surface has the important function of keeping the endothelial responsive to blood flow [7, 8]. The thickness after the fixation was estimated to be 20 nm - 4.5 μm using transmission electron microscopy (TEM) [9-12]. It was experimentally investigated that the existence or non-existence of the glycocalyx layer affected blood flow resistance and vascular permeability [13-14], and morphological changes and production of NO [15]. To clarify the function the layer, the flow field was numerically calculated based on the model as a biphasic mixture or porous layer, consisting of linearly elastic solid phase and an incompressible Newtonian fluid phase [16-21]. These simulated results indicated that a glycocalyx layer leads to the reduction of the surface flow and lower fluid wall shear stresses at the endothelial surface compared to when absent from the layer. To investigate the influence of the layer on the near surface flow field, a high resolution measurement technique for near surface velocity distribution of ECs using confocal micro-PIV was developed [22]. However, the technique was insufficient in its spatial resolution and measurement accuracy to investigate the influence of the layer. The reason of low the measurement accuracy was mainly attachment of tracer particles on the cells and low intensity of the particles. Therefore, it is necessary to develop a stealth tracer particle with high intensity. Furthermore, a measurement technique for the dimension of the layer of living cell is also necessary.

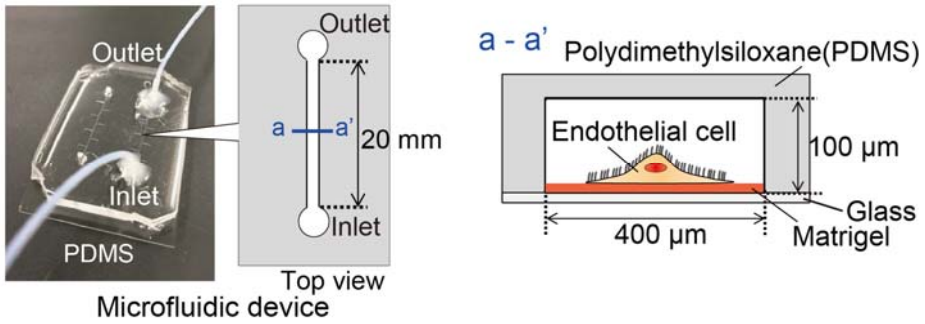
In the present study, in order to investigate an influence of the glycocalyx layer on the near surface flow field, a measurement technique with a high spatial resolution and high accuracy was developed for the flow field near the glycocalyx layer. A measurement technique for three dimensional velocity distributions with spatial resolution of 250 nm in the depth direction was developed by optimizing the measurement parameter such as the objective lens, optical filters and by utilizing 100nm silica particles coated with polyethylene

glycol (PEG). Super-resolution microscopy was used for the measurement of position and thickness of a glycocalyx layer with lateral resolution of 80 nm. The technique was applied to near surface flow field around EC cultured in a microchannel with a width of 400 µm and depth of 100 µm.

2 Experiments

2.1 Cell culturing in microchannel

Human umbilical vein endothelial cells (HUVECs) were cultured in a straight-shape microchannel as shown in Fig.1 to investigate the near-surface flow and the glycocalyx dimension of the ECs. The microchannel was constructed in a microfluidic device composed of polydimethylsiloxane (PDMS) and glass bottom, of which the rectangular cross sectional shape is a width of 400 µm, a depth of 100 µm and a length of 20 mm. The microfluidic device fabricated by soft lithography technique [23], was coated with a solubilized basement membrane (BD Matrigel™) to enhance the attachment of the cells on the wall surface after sterilization of the channel by an ultraviolet lump and 70% ethanol. Then, HUVECs were injected in the channel and cultured in cell growth medium (EGM™-2) for few hours.



(a) Photo of the microfluidic device and outline of the channel structure.

(b) Schematic of ECs culturing in the microchannel.

Fig.1 Schematic of the microfluidic device for cell culturing.

2.2 Measurements

The red dash lines in Fig.2 show the measurement area for the near-surface flow field of the glycocalyx layer of EC cultured in the microchannel. The measurement volume of the flow field corresponded to 177 × 177 × 1.75 µm^3 by scanning with the objective lens of confocal micro-PIV system using a piezo actuator.
The blue dash lines in Fig.2 show the measurement area for the glycocalyx dimension using a super-resolution microscopy. The obtained image of the stained glycocalyx layer was 2048 × 2048 × 30 pixels, whose volume corresponded to 82 × 82 × 9 µm^3 with in-plane spatial resolution of 40 nm and depth resolution of 300 nm. By adjusting the velocity distribution and the glycocalyx dimension, an influence of the glycocalyx layer on the near surface flow field was investigated.

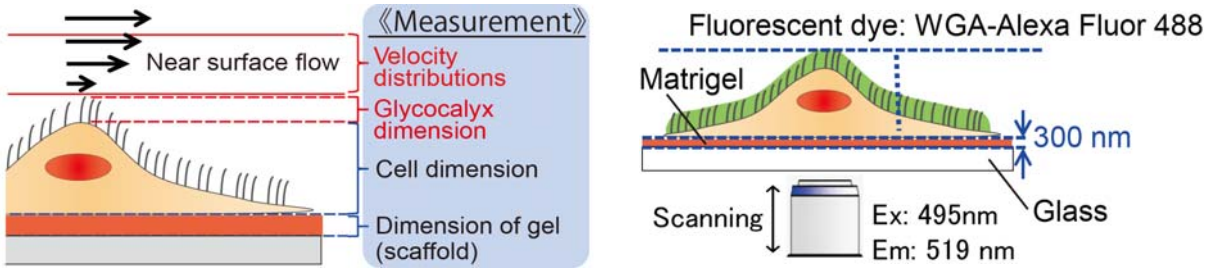


Fig.2 Measurement area for the near-surface flow field and the glycocalyx dimension.

(a) Measurement of near surface flow field of glycocalyx

In order to investigate the near surface flow field of the glycocalyx layer of ECs, a measurement technique with a high spatial resolution and high accuracy based on a confocal micro-PIV system was developed by optimizing the optical parameter and tracer particles as shown in Fig.3. 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 λ = 488 nm and a high speed camera (SV-200i; Photron, 10bit) sets 1000 fps with a resolution of 512 × 512 pixels. A 40× water immersion objective lens (CFI Apo 40XWI λS, Nikon, NA = 1.25), a piezo actuator (P-721K120; PI) with sub-nanometer resolution accuracy, and stealth fluorescent silica particles were used so that spatial resolution in depth direction was achieved to be 250 nm. The depth of focus in the system was estimated theoretically to be 0.49 μm [24].

Velocity distributions were obtained using the cross-correlation method and a Gaussian peak fitting method for sub-pixel accuracy with an interrogation window of 40 × 40 pixels with 50% overlap, which corresponded with an in-plane spatial resolution of 6.8 × 6.8 μm^2. Since sequential images were captured at a constant frame rate of 1000 fps for all of the focal planes, the time interval of image pairs for PIV analysis was adjusted for maximum velocity at each plane to improve the measurement accuracy. In order to eliminate the effect of the Brownian motion, temporal averaging was applied to about 200 velocity distributions.

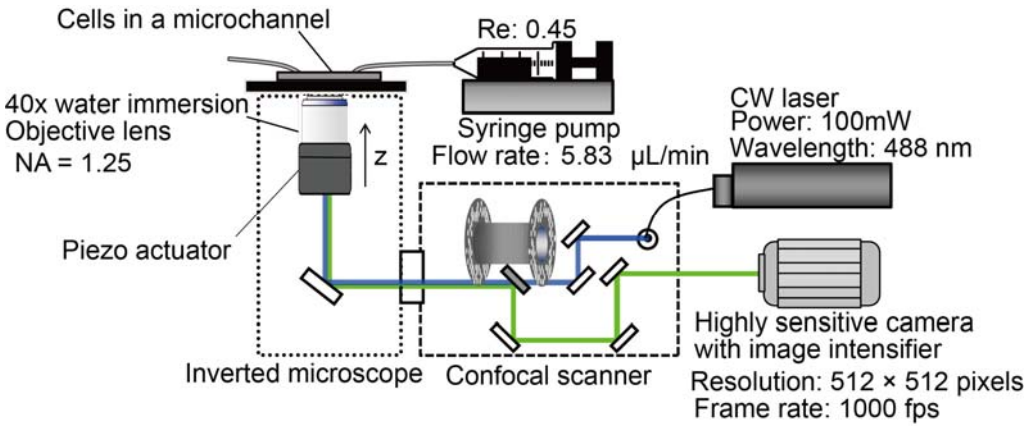
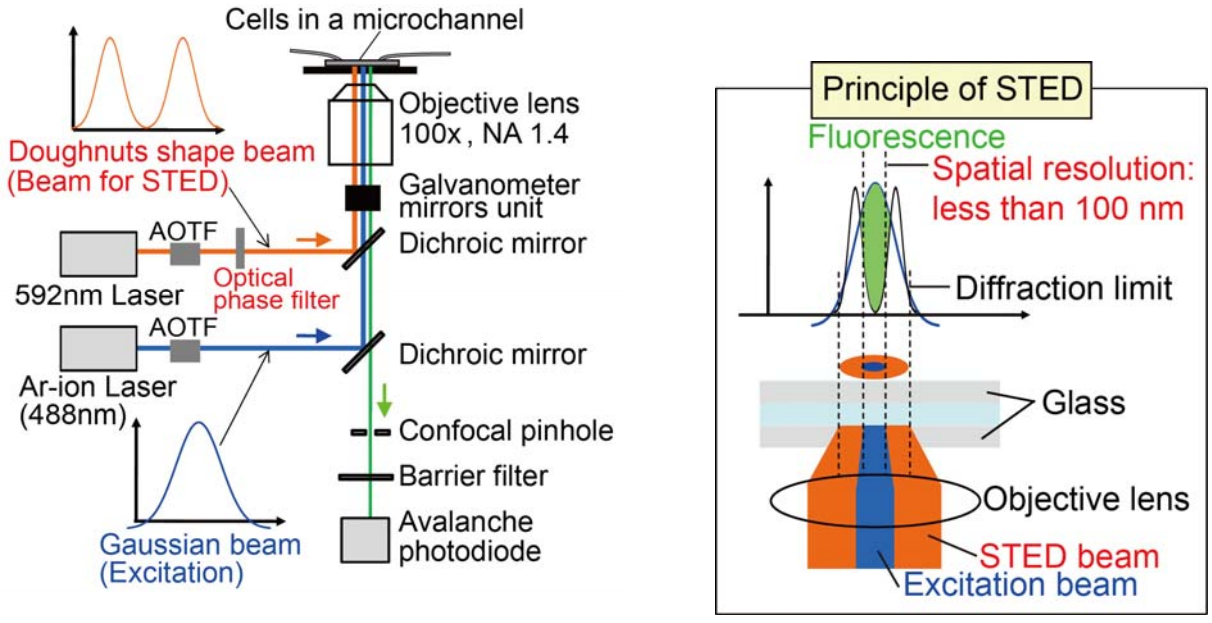


Fig.3 Schematic of the confocal micro-PIV system.

The part of the authors developed stealth liposomes coated with a polymer as a drug delivery system [25]. Synthesis technique of polyethylene glycol (PEG) coated nanoparticles was reported [26]. Fluorescent silica nanoparticle with thiol residues on the surface and high fluorescent intensity was developed [27]. By utilizing these techniques, stealth particle was synthesized to reduce the measurement error on the velocity caused by particles attached to the EC surface. Fluorescent silica particles with a diameter of 100 nm coated with long chains of PEG with molecular weight of 2000 were used. Thiol group of silica particles were combined with maleimide group of PEG. The fluorescent particles excited at 485 nm and emitted at 510 nm. The culture medium seeded with PEG coating fluorescent particles was used as a working fluid. The fluid was injected by using a microsyringe pump at the constant flow rate of 5.83 μL/min, which resulted in a Reynolds number of Re = 0.45, which corresponded to the flow rate of in vivo conditions.

(b) Measurement of glycocalyx dimension

The dimension of a glycocalyx layer such as its position and thickness of ECs was measured by using a stimulated emission depletion (STED) microscope system (Leica, TCS STED CW) as shown in Fig.4 (a), which is one of a super-resolution microscopy. Based on the STED microscope system, a measurement technique of ion distribution in the nanochannel with a spatial resolution of 87 nm was previously developed [28]. The system has two laser beams: one is an Ar-ion laser at 488 nm wavelength with a Gaussian intensity profile for fluorescent excitation, and the other is a fiber laser at 592 nm wavelength of a doughnut-shaped intensity profile for STED formed by an optical phase filter. The two laser beams were completely aligned and focused to fluorescent molecules through dichroic mirrors and an oil-immersion objective lens (×100, NA = 1.4, refractive index of immersion oil n = 1.52) as shown in Fig.4 (b). The fluorescent molecules in the center of the focal spot were excited to the singlet state and emitted fluorescence with de-excitation to the ground state. Those in the periphery of the focal spot, where the STED beam is illuminated, are also excited to the singlet state but forced to return to the ground state without fluorescence emission. Hence, only the fluorescence emitted from the molecules in the doughnut hole was detected. Therefore, it is possible to achieve a spatial resolution (10 - 100 nm), which is smaller than the width of the Gaussian focal spot under the optical diffraction limit [30]. Glycocalyx of ECs cultured in the microchannel was stained with WGA-Alexa Fluor 488 (Molecular Probes), which excited at 495 nm and emitted at 519 nm. Wheat germ agglutinin (WGA) is a lectin that binds to oligomers of N-acetylglucosamine and N-acetylmuramic acid, which are components of an endothelial glycocalyx. By optimizing the parameters of STED, a lateral spatial resolution of 80 nm was achieved.



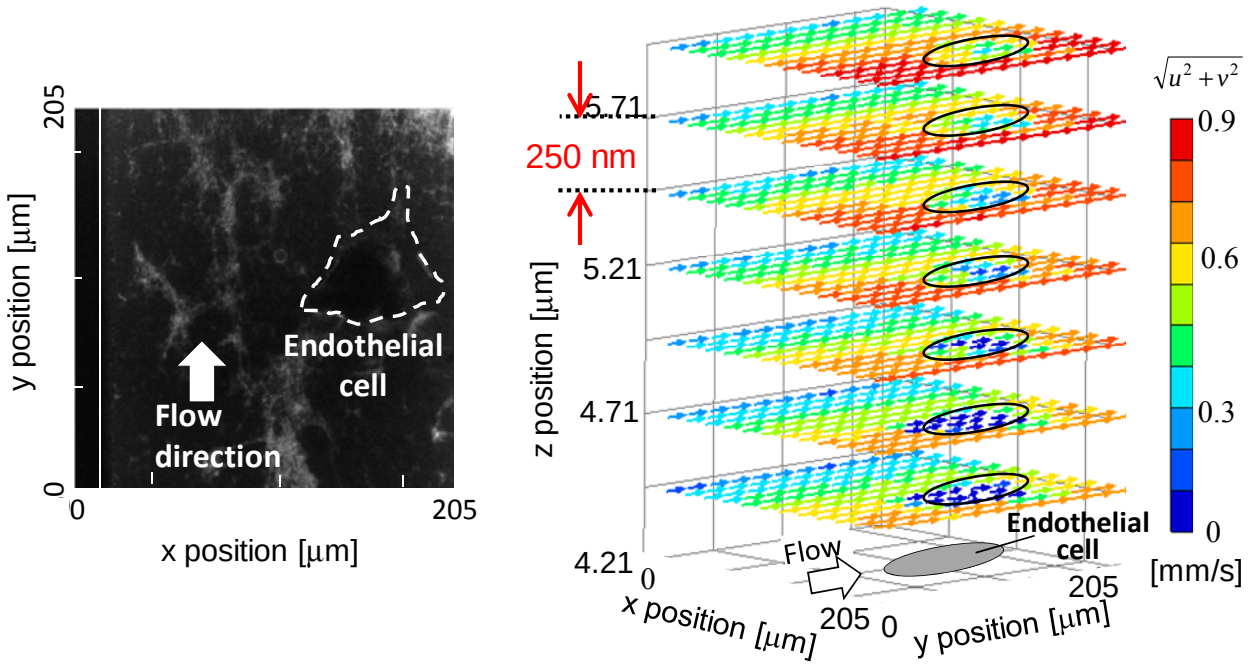
(a) Schematic of STED system. **(b)** Principle of STED.

Fig.4 Measurement system for glycocalyx dimension using stimulated emission depletion microscope system.

3 Results and Discussion

A target EC for measurement of velocity and dimension of the glycocalyx captured by the confocal micro-PIV system at 60 fps, in which the focal position set to be around bottom of the microchannel, is shown in Fig. 5 (a). The alignment of EC positions obtained by STED and by the confocal micro-PIV system was performed using the outlines of EC represented by the dashed lines. Although matrigel was observed as patchy pattern in the image due to very low frame rate, the intensities were small enough compared to that of the fluorescent particles. The white vertical line at x = 12 μm represents the side wall of the microchannel.

Figure 5 (b) shows three-dimensional temporal-averaged velocity distributions at seven planes with a spatial resolution of 6.8 × 6.8 × 0.25 μm. The black line represents the EC shape. The position of z = 0.0 μm was estimated using the velocity profile with an assumption of non-slip boundary condition in the area of absence of the cell around x = 85, y = 140 μm. The velocity vectors at all of the planes were almost parallel to the side wall and velocity magnitude gradually increased from the side wall to the center of the channel. The maximum velocity was 0.89 mm/s at the center of the channel, x = 165, z = 5.77 μm, in which velocity distribution agreed with theoretical one for laminar flow in the channel. Slower velocities around the upper area of the EC were observed in the broad area compared to the size of the EC and the velocities recovered away from the top of EC. The velocity at the upper area of the EC at z = 4.76 μm was 0.08 mm/s at x = 135, y = 115 μm, which was smaller than the velocity, 0.78 mm/s, in the area of absence of the cell. The tendency of the velocities shows the effect of the EC shape; velocities became minimum at a nucleus around the center of the EC and then gently recovered toward the edge of cell.



(a) Photo of the EC in microchannel. **(b)** Velocity distributions on the EC.

Fig.5 Three-dimensional temporal-averaged velocity distributions at seven planes.

Figure 6 shows a fluorescent image of a glycocalyx layer on the EC visualized by super-resolution microscopy, STED, with the spatial resolution of 40 × 40 × 300 nm; (a) cross-sectional image in the x-y plane focused around center of EC in depth direction and (b) cross-sectional image in the y-z plane at section b-b'. The glycocalyx layer was observed as a white round ring band shape. Since matrigel coated on the glass surface was also stained with the fluorescent dye, white cingulate pattern was observed around the left side in the image, outside the cell. Right and bottom images in the Fig.6 show the cross-sectional fluorescent images on the y-z plane at a-a' and on the z-x plane at b-b'. The position and thickness of the glycocalyx

layer were obtained from the image using image processing. The alignment of the z-axis between the velocity distributions and the glycocalyx dimension was performed by adjusting the cell shape. The height of the EC became maximum around the center of the cell shown in Fig.6, at which the thickness of the layer and the position of the surface were estimated to be 1.33 μm and z = 4.67 μm, respectively.

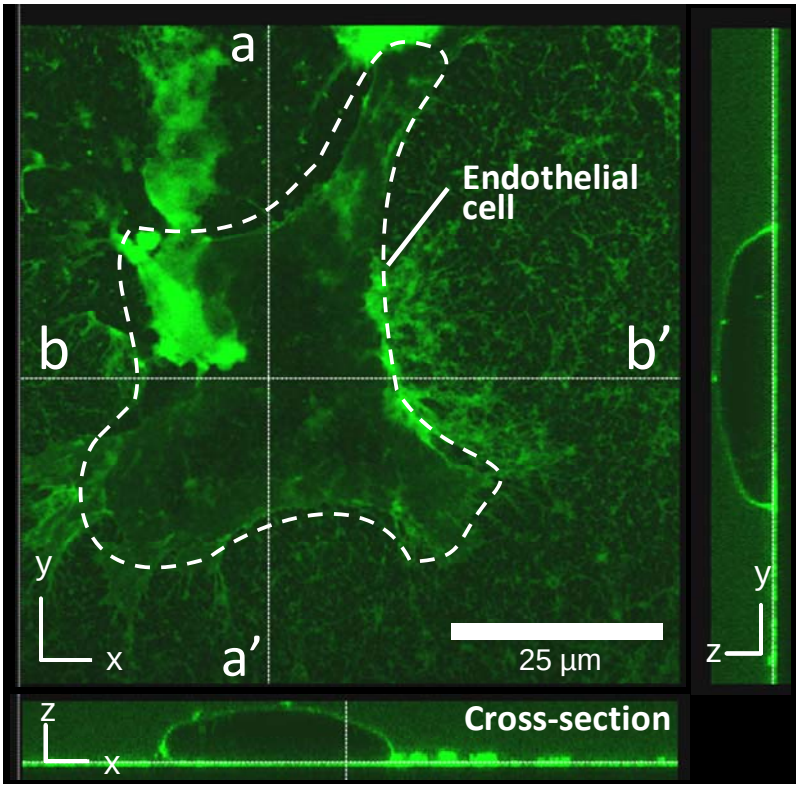


Fig.6 Fluorescent image of the glycocalyx layer visualized by super-resolution microscopy

4 Conclusion

In order to investigate the influence of a glycocalyx layer on the near surface flow field, a measurement technique with a high spatial resolution and high accuracy with confocal micro PIV and super-resolution microscopy was developed for the flow field near the glycocalyx layer. Spatial resolution was achieved to be 250 nm in the depth direction by utilizing the confocal micro-PIV technique with optimization of the measurement parameter such as the objective lens, optical filters and by utilizing 100 nm silica particles coated with polyethylene glycol (PEG). Super-resolution microscopy, a stimulated emission depletion (STED) microscope system, was used for the measurement of position and thickness of a glycocalyx layer stained with fluorescent dye, WGA-Alexa Fluor 488, with lateral resolution of 80 nm. The technique was applied to near surface flow field around EC cultured in a microchannel with a width of 400 μm and depth of 100 μm. The height of the EC and the thickness of the layer around the top of the EC were measured to be 4.67 and 1.33 μm, respectively. The velocities near the surface of the glycocalyx layer and the dimension of the layer were successfully measured. The results indicated that the measured velocity distributions show the influence of the layer on the near-surface flow field.

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