Red Blood Cell Velocity Measurement in Rodent Tumor Model: An *in vivo* Microscopic Study

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Abstract

Blood flow dynamics in microvessels in a small animal tumor model provide important physiological and pathological information. In this study, red blood cell velocity in microvessels distributed near the surface in a rodent tumor model is investigated using an approach based on optical flow estimation. A long-term experiment is performed to observe tumor growth on nude mice and to monitor the variation of blood flow velocity. The blood flow images are acquired using a capillaroscope with a spatial resolution of 1.42 μm and an image sampling rate of 30 frames per second. Frame-to-frame image registration with mutual information feature matching is used to stabilize images to eliminate the vessel shift that results from regular heartbeats. The blood flow results measured by the optical flow method are compared with visual inspection results to verify accuracy. The blood flow observations focus on the tumor surface of nude mice. Multiple weekly data are acquired from each nude mouse for five weeks after tumor implantation. The average red blood cell velocities of capillaries are measured and calculated at different weeks for each tumor. The distribution of blood flow velocity among all samples ranges from 40 to 350 μm/s. The differences in vessel diameter and blood flow velocity during tumor growth are studied. A significant difference is found between the velocity values at the initial week and the 2\(^{nd}\) week. The results suggest that an optical-based method can be used to develop experimental protocols for the numerical simulation of tumor growth. The proposed measurement system may also be useful in many other biotechnological evaluations.

**Keywords:** Microcirculation, Blood flow velocity, Tumor growth, Microscopy

1. Introduction

Microcirculation is crucial for the maintenance of healthy tissues and functional organs as it delivers oxygen and nutrients. In clinical studies, the relationship between blood flow in microcirculation and blood circulation has been extensively researched. Studies have shown that various risk factors of disease can be related to corresponding changes in microcirculation. For instance, Raynaud’s syndrome [1,2], hypertension [3,4], and diabetes [5,6] are usually accompanied by impaired microcirculation. Therefore, microcirculation information, namely vessel diameter, flow velocity, vessel density, permeability, leucocyte function, and tracer appearance times, plays an important role in health assessment and angiopathy prevention. In order to identify the characteristics of microcirculation during illness and its response to medicinal drugs, the hemodynamic characteristics of blood flow [7-9] must be obtained. The heterogeneous nature of blood flow in tumors results in a lack of nutrient supplement to tissues. Further investigations are needed to determine the mechanisms of this bizarre characteristic in tumor microcirculation [10]. The measurement of blood flow velocity at local microvessels is an effective method of observing and quantifying microvessel differences during tumor growth. Several studies have investigated tumor microcirculation using intravital video microscopy (IVVM), which allows dynamic observation of red blood cell (RBC) activity in the microcirculation of intact organs and tissues in live animals, such as laboratory mice [4,5].
Even though blood flow in large vessels can be measured using electro-magnetic blood flowmeters or ultrasonic Doppler flowmeters, measuring the flow of RBCs in microvessels is still a challenge. A major limitation of such measurements has been their inability to relate micro-vascular perfusion observed within individual microvessels to the topographical succession of arterioles, capillaries, and venules peculiar to a given tissue. Particle image velocimetry, which is based on the distribution of the apparent velocities of a brightness pattern in an image, has been applied to analyze the distribution of velocities in an arterial bifurcation under steady blood flow conditions. This non-contact procedure can be applied to analyze the cyclic variation of pulsatile blood flow through a microvessel if an electrocogastrogram (EGG) cannot be recorded [11].

Previously, the authors developed an optical instrument and a method for analyzing micro-vascular blood flow [12]. The present study observes the temporal heterogeneity in tumor blood flow using that system. The blood flow observations were focused on the tumor surface in nude mice. Data were obtained weekly from each nude mouse for 5 weeks after tumor implantation.

2. Materials and methods

2.1 Animal preparation

7-12 week-old male nude mice with severe combined immune deficiency (SCID) and an average weight of 22 g were used for tumor blood flow measurements and vital microscopic observations under anesthesia. The mice were injected with subcutaneous tumors (2 × 10⁶ cells in 0.05 ml) under the skin of the rear dorsum, housed in groups of two in a standard cage at 21 °C in a 12-h light cycle, and supplied with laboratory chow and distilled water. The mice were randomly allocated into five groups for observation of micro-vascular blood velocity on the tumor surface for five weeks. The experimental setup is shown in Fig. 1(a). Microscopy without fluorescence labeling (M320, JMC Corporation, Kyoto, Japan) that uses a closed-circuit video system consisting of a CCD video camera, an LCD monitor, and a video recorder. The video recorder acquires real-time blood flow video of microcirculation at 380x magnification, with a spatial resolution of 1.42 µm and an image sampling rate of 30 frames per second. The static image is 720 × 480 pixels.

Before surgery, the mice were anesthetized with pentobarbital. The intraperitoneal induction dose used was 70 mg/kg of body weight. The animals were laid sideways on an operating table. The surface temperature of body was kept at 36.5-37.5 °C by a heat lamp. The skin area was carefully incised and separated (incision was 2-2.5 cm long) for microcirculation observation on the surface of the solid tumor (Fig. 1(b)). After measurements, the skin incision was closed by a suture. At the end of the experiment, the animals were killed by an overdose of pentobarbital.

Figure 1. (a) Experimental equipments. (b) Left: animal preparation. Tumor cell implantation was within the dotted rectangle in the nude mouse with SCID. (b) Right: microcirculation in the tumor region observed after a simple surgery on the skin. Microvessels with (c) low density, (d) medium density, and (e) high density.

2.2 Capillary microcirculation in nude mice

Microcirculation covers blood flows in arterioles, capillaries, and venules. The capillaries connect the arterioles with venules. Capillaries are the smallest and most numerous blood vessels in the human body. The extensive branching networks dramatically increase the surface area for the rapid exchange of molecules. Capillaries are small-diameter vessels that allow only one RBC to pass. Those that connect arteries are known as pre-capillaries. The diameter of pre-capillaries, which lack complete coats, is about 3-5 times that of capillaries. They are similar to capillaries in all other aspects. The density of capillary microcirculation on the tumor surfaces was observed using a microscope system without fluorescence labeling, as shown in Figs. 1(c)(e).

2.3 Microscopy and image acquisition

The developed microscope system provides precise and continuous quantitative data of the blood flow rate in individual small vessels. Wavelength and illumination are key parameters of the light source in a microcirculation imaging system. The microcirculation was imaged by the penetrating white light from the side. The hemoglobin of erythrocytes absorbed the green beam and thus appeared as dark cells. The image was projected by a magnifying lens onto a camera sensor. The imaging light, collected by the central part of the light guide, was optically isolated from the illuminating white light from light-emitting diodes (LEDs). The LEDs were arranged in a circle at the tip of the light guide, which directly illuminated the area of interest (Fig. 2). A window scanning technique was used. It could be helpful for sampling spatial information on a tumor surface. A comparison of two scanning orientations, namely horizontal and vertical, was performed. The scan in either orientation moved along the geometric construction by a distance of 1 mm per line. The image pixel matrix was up to 720 × 480 pixels.
2.4 Imaging processing and RBC velocity

Respiration and pulsatile heartbeats result in oscillatory movement of the body in IVVM observations. Therefore, image stabilization was the first step of image processing. With a cost (or similarity) function to quantify the quality of the alignment of two images, frame-to-frame image matching can effectively mitigate the motion [12,13]. Prior to the implementation of the optical flow algorithm, each pair of digitized images was processed by a homomorphic filter, a median filter, and a matching filter. The optical flow algorithm was implemented in Microsoft (MS) Visual C++, the pre-processing steps are described below.

**Homomorphic filtering.** Since the IVVM images of blood flow through microvessels were low contrast, a homomorphic filter was used to compress the dynamic range and enhance contrast [14].

**Median filtering.** Median filtering is a nonlinear operation that is implicitly edge-adaptive. A $5 \times 5$ window-sized median filter was used to denoise the image by reducing the blurring of edges.

**Matching filter.** Vascular images can be calculated using a simplistic matching filter after Gaussian smoothing to reduce the computation load and to segment microvessel trees [15]. Vessel density can be calculated as the number of vessels. Vessel size is then categorized by the diameter of vessels.

**Velocity estimation.** A gradient-based deformable image registration algorithm, the optical flow method (OFM) [16,17], was applied to calculate the RBC velocity from two successive images. In our previous study, an automatic RBC velocity estimation system was developed to perform OFM-based measurements in a complete, clip-shape capillary [12]. A flow chart of velocity estimation is shown in Fig. 3. Based on Horn and Schunck’s method [18], an approach is developed here to constrain the optical flow field. Horn and Schunck’s method calculates the optical flow within the gradient of images by iterating Eq. (1).

$$
\begin{align*}
\hat{v}^{(k)}_x (x,t) &= \bar{v}^{(k)}_x (x,t) - \frac{\partial f}{\partial x} \left( \alpha^2 + \left( \frac{\partial f}{\partial x} \right)^2 + \left( \frac{\partial f}{\partial y} \right)^2 \right) \\
\hat{v}^{(k)}_y (x,t) &= \bar{v}^{(k)}_y (x,t) - \frac{\partial f}{\partial y} \left( \alpha^2 + \left( \frac{\partial f}{\partial x} \right)^2 + \left( \frac{\partial f}{\partial y} \right)^2 \right)
\end{align*}
$$

where $k$ is the iteration counter, the overbar denotes weighted local averaging (excluding the present pixel), and all partials are evaluated at the point $(x, y)$. The initial estimates of the velocities $\bar{v}^{(0)}_x (x,t)$ and $\bar{v}^{(0)}_y (x,t)$ are usually taken as zero. If object movement is large or no features overlap, the Horn and Schunck optical flow method does not give reliable velocity flow fields. To avoid this problem, the Horn and Schunck optical flow is preprocessed with a deformed image which depends on the velocity field estimated from the source image. The features are thus modified to the covered pattern according to the correct patterns in the next frame. This helps motion estimation, yielding a suitable velocity field. In another study, the authors discussed the use of three techniques for RBC velocity measurement using simulated blood flow images [17]. The OFM, a space-time diagram (Hough-transform-based estimation), and the cross-correlation method (CCM) are suitable for RBC velocity determination. Among these three approaches, the OFM has the highest accuracy in RBC velocity calculation with simulated blood flow patterns of microvessels. In the present study, a velocity estimation method based on analyzing the intensity variation of pixels on the skeleton of a vessel was used. A thinning method [12], which enables a continuous vessel skeleton including small and large microvessels, was applied to vascular tree segmentation. The obtained skeleton was independent of the position of interest (ROI) in an image pixel matrix of $256 \times 256$. Thus, the measurements of the RBC velocity are consistent and reproducible (Fig. 4).

3. Results

3.1 Tumors in mice

A total of 25 mice, which weighed 22.7 ± 0.88 g, were divided into two groups. Ten mice were in the control group and the remaining 15 were in the tumor experimental group. Figure 5 shows the weekly tumor growth on the back leg muscle of SCID mice. Table 1 lists the average sizes measured every week in the experiment group after tumor implantation.

Figure 6 shows the weekly average volumes. Tumors grew gradually in the first 2 weeks with mild rates of 49.58 mm³ in the 1st week and 63.37 mm³ in the 2nd week. The growth was rapid in the 3rd (228.22 mm³) and 4th weeks.
significant increase of growth average diameter reach 21 to 30 μm were defined as large vessels. Our study, showed the presence of a heartbeat in the vessels distribution in studies that used the IVVM technique to measure the velocity in tumor microvessels (mean ± SD). The size of small vessels, for the 4th week, the blood flow velocities were between 40 and 350 μm/s, varying with mouse and tumor area. The results also show that the difference between the initial and late phases of the large vessels was about twice that of the small vessels.

3.2 Blood flow velocity

The microcirculation blood flow velocity data are shown in Table 2 and Fig. 8. In general, the blood flow velocities were (470.45 mm²) but slowed down in the 5th week (503.50 mm²). This weekly measurement data agree with the typical tumor growth pattern, which is defined by 3 phases, namely initial, growth, and late phases.

The blood vessel diameters on the surface of the tumors were measured weekly during the experiment (Fig. 7). Several studies that used the IVVM technique to measure the velocity distribution in blood vessels with a diameter of 20-30 μm showed the presence of a heartbeat in the vessels [19]. In this study, vessels with a diameter smaller than 30 μm were defined as small vessels and those with diameters larger than 30 μm were defined as large vessels. The sizes of small vessels did not change substantially in the first 4 weeks with the average diameter remaining to be 21 μm. In the 5th week, there was a noticeable growth of 6 μm for small vessels, with the average diameter reaching 27 μm. For the large vessels, a significant increase of growth (13 μm in the diameter) appeared in the 4th week. The average diameter was 38 μm in the first 3 weeks, and 51 μm in the 4th and 5th weeks (Table 2).

Table 1. Tumor dimensions and volume (mean ± SD) (n is the number of vessels)

<table>
<thead>
<tr>
<th></th>
<th>1st week (n = 17)</th>
<th>2nd week (n = 17)</th>
<th>3rd week (n = 17)</th>
<th>4th week (n = 17)</th>
<th>5th week (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Length (mm)</td>
<td>6.24 ± 0.43</td>
<td>7.35 ± 2.23</td>
<td>11.59 ± 3.66</td>
<td>12.39 ± 0.03</td>
<td>12.49 ± 0.67</td>
</tr>
<tr>
<td>Width (mm)</td>
<td>5.77 ± 0.25</td>
<td>6.12 ± 0.41</td>
<td>8.14 ± 0.48</td>
<td>11.27 ± 0.93</td>
<td>11.07 ± 0.56</td>
</tr>
<tr>
<td>Height (mm)</td>
<td>1.37 ± 0.15</td>
<td>1.45 ± 0.33</td>
<td>2.53 ± 0.67</td>
<td>3.40 ± 0.62</td>
<td>3.65 ± 0.21</td>
</tr>
<tr>
<td>Volume (mm³)</td>
<td>49.58 ± 10.26</td>
<td>63.37 ± 13.50</td>
<td>228.22 ± 45.66</td>
<td>470.45 ± 45.57</td>
<td>503.50 ± 27.59</td>
</tr>
</tbody>
</table>

Table 2. Vessel diameter and blood flow velocity in tumor microvessels (mean ± SD), (n is the number of vessels)

<table>
<thead>
<tr>
<th>Weight</th>
<th>1st week (n = 26)</th>
<th>2nd week (n = 17)</th>
<th>3rd week (n = 17)</th>
<th>4th week (n = 17)</th>
<th>5th week (n = 17)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diameter (μm)</td>
<td>≤ 30</td>
<td>17.34 ± 5.86</td>
<td>22.68 ± 4.24</td>
<td>20.51 ± 6.18</td>
<td>21.31 ± 4.78</td>
</tr>
<tr>
<td>Velocity (μm/s)</td>
<td>&gt; 30 (μm)</td>
<td>38.41 ± 2.41</td>
<td>38.35 ± 7.41</td>
<td>38.17 ± 4.61</td>
<td>38.03 ± 3.29</td>
</tr>
<tr>
<td>Diameter (μm)</td>
<td>≤ 30 (μm)</td>
<td>112.74 ± 57.62</td>
<td>131.20 ± 23.72</td>
<td>171.76 ± 82.22</td>
<td>130.92 ± 59.52</td>
</tr>
<tr>
<td>Velocity (μm/s)</td>
<td>&gt; 30 (μm)</td>
<td>150.54 ± 32.28</td>
<td>171.25 ± 98.07</td>
<td>225.35 ± 66.63</td>
<td>116.95 ± 46.71</td>
</tr>
</tbody>
</table>

Figure 4. (a) Image pixel matrix (1 mm²) with 720 × 480 pixels. The rectangular region is the ROI (256 × 256 pixels). (b) First image frame and (c) last image frame. Arrows indicate the direction of blood flow. (d) Estimated 2-D blood flow velocity map that corresponds to (a) and (b).

Figure 5. Weekly size of tumor on the back leg muscle of SCID mouse (indicated by circle).

Figure 6. Exponential tumor growth curve.

Figure 7. Weekly average microvessel size measured after tumor implantation.
velocity in the large vessels was always faster than that in the small vessels. The average velocities were 150.54 ± 32.28 μm/s for the large vessels and 112.74 ± 57.62 μm/s for the small vessels. In the tumor group, for both large and small vessels, the highest and second highest flow velocities were found in the 2nd and 1st weeks, respectively. The averages of the highest velocities were 171.76 ± 82.22 μm/s for the small vessels and 225.35 ± 66.36 μm/s for the large vessels. The averages of the second highest velocities were 131.20 ± 23.72 μm/s and 171.25 ± 98.07 μm/s, respectively. The small and large vessels exhibited opposite tendencies from the 3rd to the 5th weeks. In small vessels, the blood flow gradually declined during the last 3 weeks. The average values for the small vessels from the 3rd to the 5th weeks were 130.92 ± 59.52, 126.96 ± 49.87, and 110.67 ± 63.20 μm/s, respectively. In the large vessels, the velocity had a sudden decrease in the 3rd week, and then increased gradually in the last 3 weeks. However, the velocities did not exceed those of the first two weeks. The values for the large vessels in the 3rd to the 5th weeks were 116.95 ± 46.71, 130.20 ± 56.30, and 148.37 ± 55.46 μm/s, respectively. Statistical analysis of blood flow velocity was done using the independent T test. According to the p values (p < 0.05) obtained for each week compared to week 0, a significant difference was found between the velocity values in the 2nd week. In the experimental group, a significant difference (p < 0.01) was found between the vessel diameter values in each week. The blood flow velocity in the large vessels was larger than that in the small vessels, except in the 3rd week. Since the definitions of arterioles and venules are not suitable for describing vessels in tumors, these terms are not used here [20]. The results obtained using the proposed imaging system and analysis method are consistent with those reported in a previous study [21]. In that study, the blood flow velocity measured at the tumor surface of SCID mice was on average 200 μm/s with a range of 0–600 μm/s.

4. Discussion

The differences in vessel diameter and blood flow velocity during tumor growth were found to be statistically significant. The blood vessel density increases with tumor growth, reaching its maximum at the late phase [22, 23]. According to the obtained tumor growth curves from experiments, tumors grew to a certain volume in the initial phase (1st and 2nd weeks). The regional nutrition and oxygen requirements in the initial phase are high. To satisfy this high demand, the incomplete microcirculation system in the initial phase is compensated for with high blood flow velocity. In the growth phase (3rd and 4th weeks), the slow blood flow in tumor microcirculation resulted from two factors. First, the microvessels in tumor tissues are different from those in normal tissues. In tumor tissues, the microvessels are lumens formed by a layer of endothelial cells as barrier walls. These lumens have incomplete differentiation, and are not controlled by nerves, which paralyzes the microvessels. The blood flow in such microvessels hence cannot be varied by varying the diameter, and thus tends to be constant. Second, a high-density microcirculation system is well established in this phase, which can provide sufficient nutrition and oxygen to the tumor cells even with a relatively slow blood flow. In the 4th and 5th weeks, tumor growth is still fast. The cells in the edge regions usually require more nutrition and oxygen than those in the tumor center. As microvessels are lower in density or even not present in the center volume, the blood flow velocity stops increasing. As a result, apoptosis is often found in the tumor cells in the center volume due to ischemia.

Other studies have used intravital microscopy techniques with fluorescence labeling to measure temporal instabilities in red blood cell flow in individual vessels in small sub-regions of tumors growing in window chambers [24]. However, fluorescence microscopy may have some detrimental effects on living tissues. For long-term observation, this study used a microscopy system without fluorescence labeling to evaluate the velocity range of blood flow through a solid tumor area.

5. Conclusion

A microcirculation imaging system that uses white LEDs as the illumination source and no fluorescence labeling was used for in vivo tumor surface microcirculation blood flow velocity measurements. Weekly blood flow velocity data were collected from the tumor surface microvessels in nude mice. The techniques used in this microcirculation study have potential applications in tumor development evaluation and prognosis. The tumor microcirculation data of small animals obtained in this study could be a reference for tumor and blood pathophysiology research in the future.

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