

# *In vivo* high-speed imaging of individual cells in fast blood flow

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Imaging of individual cells in flow *in vivo* is potentially important for studying cell–cell interaction, blood transport (e.g., oxygen delivery), the response of cells to different interventions (e.g., drugs, radiation), and disease diagnosis and prevention (e.g., metastases, heart attack or stroke alert, diabetic shock, sickle-cell crisis, etc.). In particular, degree and dynamic of changes in cells' mechanical properties can be viewed as a new biological marker sensitive to early disease development because even small disturbances at the molecular level [e.g., modification of the protein-spectrin structure of red blood cells (RBCs)] may be accompanied by significant changes in cytoskeletal structures responsible for cell deformability and elasticity<sup>1,2</sup> (e.g., increased rigidity of cancer cells).

A number of well-developed, highly sensitive methods provide high-resolution imaging of RBCs *in vitro*, including monitoring of RBC deformability dynamic with advanced flexible microchannel.<sup>3</sup> Until now, these and others similar approaches were tested in only *in vitro* studies.<sup>4,5</sup> However, there is increasing recognition that *in vitro* data cannot correspond entirely to the physiological situation *in vivo* including complex environment within vessel, multilevel regulation of cell function, and cell-cell interactions.<sup>6,7</sup> Further, invasive isolation of cells from their native environment and their processing may not only introduce artifacts but also make it im-

**Abstract.** *In vivo*, label-free, high-speed (up to 10,000 with the potential for 40,000 frames per second), high-resolution (up to 300 nm) real-time continuous imaging with successive framing of circulating individual erythrocytes, leukocytes, and platelets in fast blood flow is developed. This technique, used in an animal model, reveals the extremely high dynamic deformability of erythrocytes in natural flow. Potential applications of this technique are discussed with focus on time-resolved monitoring of the cell deformation dynamics in the native biological environment, which may have diagnostic value for the early diagnosis of diseases. © 2006 Society of Photo-Optical Instrumentation Engineers. [DOI: 10.1117/1.2355666]

**Keywords:** *in vivo* imaging; high resolution; high-speed camera; blood cells; rat mesentery; transient cell deformability.

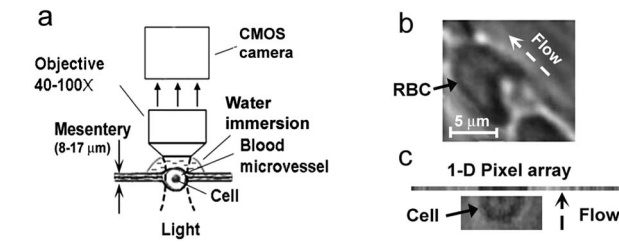
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possible to examine the same cell population over long time periods.

Until now, imaging of the flowing blood cells was realized at the moderate CCD camera speeds, in particular, at 750 to 2500 frame/s (fps) in blood flow,<sup>8,9</sup> and 500 fps in lymph flow<sup>10</sup> on an animal rat model. These speeds are not quite enough to image fast-moving RBCs in most of the blood microvessels. In addition, high optical resolution was not realized in these experiments (e.g., RBCs looked like spots with fuzzy edges). On other hand, the relatively high resolution vital optical microscopy has been used in different animal models including our previous studies<sup>11–16</sup> (e.g., rat and mouse mesentery, muscle, and ear) mainly in two modes: (1) a relatively slow, successive-framing mode (<30 fps) by which only slow-moving cells can be imaged (e.g., so-called “rolling” leukocytes with an average velocity of  $\sim 50 \mu\text{m/s}$ ), or (2) a short-time-exposure mode ( $\sim 0.1$  to 1 ms) by which just single images of fast-moving cells can be captured. To our knowledge, simultaneous high-optical-resolution and high-speed imaging of fast-moving cells (e.g., RBCs in arterioles with a velocity up to 5 to 10 mm/s) in natural conditions with successive high-speed framing that is crucial to time-resolved monitoring of cells' transient (i.e., dynamic) deformability has not yet been developed *in vivo*.

*In vivo* flow image cytometry schematics<sup>11–14</sup> on the technical platform of an Olympus BX51 microscope was adopted to realize this approach with an advanced CMOS camera (model MV-D1024-160-CL-8, Photonfocus, Lachen, Switzerland).

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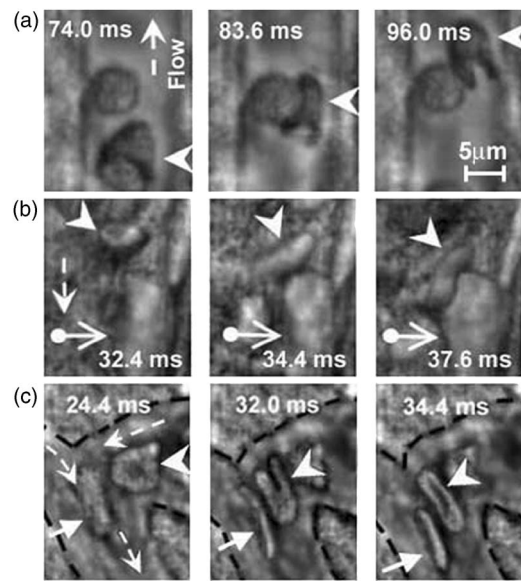


**Fig. 1** (a) Schematic of high-speed imaging of cells in blood vessels of rat mesentery with different areas of interest: (b)  $128 \times 128$  pixels and (c)  $512 \times 1$  pixels with magnifications  $100\times$ , and  $40\times$ , respectively.

land) [Fig. 1(a)]. Rat mesentery as an almost ideal animal model was chosen to test high-speed optical imaging of flowing cells because it consists of very thin (8 to  $17\text{-}\mu\text{m}$ ), relatively transparent connective tissue with a single layer of blood and lymph microvessels.<sup>11</sup> Male Sprague-Dawley rats weighting between 150 and 180 g were used for experiments according to animal protocol approved by the University of Arkansas for Medical Sciences (UAMS) Institutional Animal Care and Use Committee (IACUC). In accordance to well established procedure,<sup>11-14,17</sup> the anesthetized rats [ketamine/xylazine 50/10 mg/kg intramuscular (i.m.)] were laparotomized by midabdominal incision ( $\sim 1$  cm), and small-intestinal mesentery was placed on a thermostabilizing customized microscope stage and maintained at the same body temperature as the rat ( $37.7^\circ\text{C}$ ). The mesentery was bathed with a constant diffusion of warm Ringer's solution ( $37^\circ\text{C}$ , pH 7.4). As established, such procedures do not markedly influence the properties of flowing cells for at least 5 h.<sup>17</sup>

The minimum frame rate  $f_{\min}$  for avoiding optical resolution ( $\delta_{\text{opt}}$ ) distortion due to motion, was chosen on the basis of the condition  $f_{\min} \geq (V_F)_{\max} / \delta_{\text{opt}}$ , where  $(V_F)_{\max}$  is the maximum flow velocity. For the typical parameters  $(V_F)_{\max} \approx 5$  mm/s and  $\delta_{\text{opt}} \approx 0.5$   $\mu\text{m}$ ,  $f_{\min} \geq 10,000$  fps. High-speed imaging without loss of spatial resolution was achieved by adjusting the area of interest. For example, four typical areas of interests,  $512 \times 512$ ,  $128 \times 256$ ,  $128 \times 64$ , and  $512 \times 1$  pixels corresponded to frame rates of 500, 2500, 10,000, and 40,000 fps, respectively. At  $40\times$  magnification, the sizes of these areas of interest (at a  $10.6\text{-}\mu\text{m}$  pixel size) in the sample plane were  $136 \times 136$ ,  $34 \times 68$ ,  $34 \times 17$ , and  $136 \times 0.3$   $\mu\text{m}$ , respectively. The largest areas of interest were useful for providing an overview of the mesenteric vessel network, while small areas were better suited for high-resolution imaging of single cells [Fig. 1(b)]. The highest frame speed could be achieved with a linear pixel array, although further reconstruction of 2-D cell images was required [Fig. 1(c)]. Optical resolutions, estimated with nanoscale colored polystyrene beads, were approximately 700, 350, and 250 nm at  $10\times$ ,  $40\times$ , and  $100\times$  magnifications, respectively.

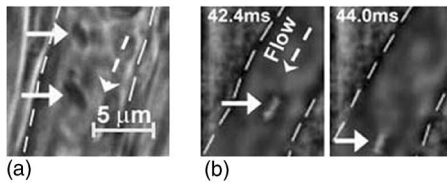
The combination of light-absorption and scattering effects on cells made it possible to visualize and identify most blood cells without conventional labeling and vital staining with the use just of bright-field microscopy. In particular, due to relatively strong light absorption by RBCs in the visible spectral range, these single cells in flow appeared mostly as dark objects in transillumination mode, while weakly absorbing white blood cells (WBCs) and platelets appeared either as light objects (e.g., in the presence of many more strongly absorbing



**Fig. 2** (a) Three frames of parachute-shaped RBC traveling through capillary at 0.4 mm/s (DI=1.4, 3.7, and 1.5, respectively, 1250 fps,  $40\times$ ); (b) interaction of fast-moving RBC (arrow head) with slow-rolling WBC (arrow with circle) (2500 fps,  $40\times$ ); (c) two RBCs in an area of merging flow streams with a velocity of 0.3 mm/s: left RBC DI=3.2, 6.7, and 7.3; right RBC DI=1.1, 2.2, and 3.4, respectively (2500 fps;  $40\times$ ).

RBCs in blood flow or with dominant scattering effects) or, in contrast, as slightly dark objects (e.g., in the transparent plasma without RBCs). In the "packed" flow, RBCs sometimes exhibited bright margins, or are seen even as light objects due to multiply scattering effects. This is because the light during propagation through many other RBCs before reaching the plane of focus was significantly scattered and attenuated through absorption resulted in dominant scattering light around imaged cells. The CMOS camera's sensitivity was sufficient to visualize most of these cells with conventional continuous mode illumination in the frame-rate range used.

This technique confirmed the well-known fact that normal RBCs (basically a 5 to  $7\text{-}\mu\text{m}$ -diam biconcave disk) flowing in single-file through capillaries or postcapillary venules with a velocity of 0.3 to 0.7 mm/s are usually deformed into parachute-like shapes<sup>16,18,19</sup> [Fig. 1(b)]. High-speed, high-resolution imaging, however, revealed for the first time that RBCs in fast blood flow undergo more significant (than previously believed) deformations in geometrically irregular regions (e.g., bifurcations, curves, narrowed areas, etc.), as well as in their interactions with other cells. In particular, Fig. 2(a) shows the high deformability of parachute-like RBCs as they squeezed through a narrow gap between the vessel wall and another cell (rigid RBC or small WBC), adherent to the opposite wall. High-speed imaging also demonstrated how quickly relatively fast flowing RBCs (velocity  $\geq 1$  mm/s) changed shape as they interacted with much more slowly moving ( $\sim 20$   $\mu\text{m/s}$ ) rolling WBCs [Fig. 2(b)]. Figure 2(c) shows a very considerable stretching up to at least 0.4 to 0.7  $\mu\text{m}$  (in 2-D projection) of two RBCs in the diverging flow streams in a bifurcation zone. The typical value of deformation index (DI), defined as the ratio of cell length to



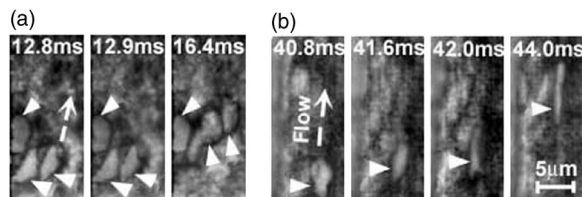
**Fig. 3** (a) Images of platelets in slow flow with velocity of  $20 \mu\text{m/s}$  (12 fps;  $100\times$ ) and (b) two (frames 106 and 110) images of platelets (arrows) in relatively fast flow with a velocity of  $2.5 \text{ mm/s}$  (2500 fps;  $40\times$ ); dashed lines show internal margin of microvessel.

width, was found to be approximately 7.3 in curved-vessel flow [Fig. 2(c), middle] compared to a DI of 1.4 to 1.5 in straight-microvessel flow [Fig. 2(a), left] and a DI of 1 for almost static or adherent cells [Fig. 2(a)].

Slow-moving ( $30$  to  $80 \mu\text{m/s}$ ), rolling WBCs were reliably visualized at a relatively slow frame rate of 20 to 30 fps, at which fast-flowing RBCs (velocity  $\sim 1$  to  $5 \text{ mm/s}$ ) looked like the nonstructural dark background due to motion distortion. Only a fast frame rate ( $>2000$  fps) provided time-resolved monitoring of the deformation of fast-moving RBCs as they interacted with rolling WBCs [Fig. 2(b)].

Labeling-free identification of single platelets is difficult to achieve because these weakly absorbing cells are also small ( $2$  to  $3 \mu\text{m}$ ). Nevertheless, our technique has provided relatively good contrast images of both slow- [Fig. 3(a)] and fast-flowing [ $2.5 \text{ mm/s}$ , Fig. 3(b)] single platelets in blood microvessels. The best-quality images of platelets were obtained in the RBC-free space of the microvessel lumen [Fig. 3(b)].

The most difficult task was to obtain images of RBCs in multifile cell flow in  $30$  to  $40\text{-}\mu\text{m}$  venules and especially in arterioles, which have the highest axial velocity (up to  $10 \text{ mm/s}$ ). Nevertheless, the monitoring of image sequences at a high frame rate of  $10,000$  fps by adjustment of the focus locations made it possible to record the size and shape dynamic of selected single RBCs in fast flow [Fig. 4(a)]. In addition, the continuous video framing has the potential to measure linear velocity of several cells traveling along a cross-section of a microvessel simultaneously [Fig. 4(a)]. High-speed imaging demonstrated for the first time the dynamic changes of RBC 2-D shape, and correspondingly, transient deformation, in relatively fast arteriolar straight flow ( $5 \text{ mm/s}$  and higher). In particular, extremely high cell dynamic deformation (DI up to 10) was observed in a very short time ( $\leq 0.4 \text{ ms}$ ) [Fig. 4(b)]. In comparison, for blood straight flow in a tube *in vitro* the maximum DI has been reported to



**Fig. 4** (a) Three subsequent images (frames 128, 129 and 164) of a few RBCs in different files of fast arteriolar flow with velocities  $1 \text{ mm/s}$  for left RBC (nearest to vessel wall),  $2 \text{ mm/s}$  for middle RBC, and  $2.5 \text{ mm/s}$  for right RBC (nearest to vessel axis) ( $10,000$  fps;  $40\times$ ); (b) fast transient deformation of single RBC in flow with a velocity of  $5 \text{ mm/s}$ ; DI=2.1, 3.0, 5.9, and 10.3, respectively ( $2500$  fps;  $40\times$ ).

be approximately 1.5 in healthy individuals, with a decrease to 1.05 in diabetes mellitus patients.<sup>21</sup>

As expected, some limitations of the optical imaging used were revealed in fast multifile flow. In particular, even at a low hematocrit<sup>19,20</sup> ( $\sim 20\%$ ), several cells simultaneously appeared in a small focal area of detection, and up to six to eight cells appeared in a whole microvessel cross section, which led to the overlapping of cell images in 2-D projection making their identification quite difficult. It seems that this problem could be resolved partly by hemodilution and reduction of hematocrit in analogy to study of RBC aggregation.<sup>8,9</sup> However, hematocrit may effect significantly on RBC deformability.<sup>18</sup> The overcoming of this limitation may require developing of 3-D imaging *in vivo*. In some cases, fast radial displacement of cells in multifile flow prevented continuing high-resolution imaging of the same cell with a strongly focused micro-objective at high magnification. Small arterioles (diameter,  $20$  to  $30 \mu\text{m}$ ) were found to be the most suitable for such measurements with a fixed focal plane because cells in these vessels mainly moved along the flow axis. Furthermore, a high enough flow velocity in such vessels still decreased the probability of transient RBC aggregation<sup>19</sup> (in “rouleaux” or clumps), thus facilitating the study of individual cells.

In a future study, several modes (or digital cameras) operating at different frame rates and hence sensitivity levels (which usually decrease with increases in speed) might be useful for simultaneous imaging of cells moving at different velocities. This approach might include (1) imaging of static (i.e., adhered) or slow-moving cells (e.g., rolling WBCs) at  $10$  to  $30$  fps, (2) monitoring of the deformability of RBCs traveling in single-file flow in capillaries and postcapillary venules (velocity,  $500$  to  $1000 \mu\text{m/s}$ ) at  $500$  to  $1000$  fps, and (3) high-speed imaging of individual cells moving in multifile fast flow (velocity,  $5$  to  $10 \text{ mm/s}$ ) at  $\geq 10,000$  fps. Despite the distinctive optical contrast of RBCs, WBCs, and platelets, the low absorption sensitivity of transmission microscopy makes it difficult to differentiate cells with slightly different absorption properties (e.g., subtypes of WBCs). For this purpose, recently developed labeling-free, photothermal flow cytometry *in vivo* might be useful because its high absorption sensitivity makes it possible to image weakly absorbing cellular structures with specific “fingerprints.”<sup>11,12,14</sup>

The approach that we have developed also has the potential to be used *in vivo* in humans. Noninvasive mode can be realized with the use of thin, translucent structures, such as ear, eyelid, or interdigital membrane,<sup>11</sup> with optical clearing.<sup>5</sup> An invasive approach might involve incorporation of a fiber-chip-based catheter in vessels.

In summary, a high-speed (up to  $10,000$  with the potential for  $40,000$  fps), high-resolution (up to  $300 \text{ nm}$ ) continuous *in vivo* optical imaging technique was developed to monitor and identify RBCs, WBCs, and platelets in the blood flow of rat mesenteric microvessels without conventional labeling. It was demonstrated that the frame rate up to  $10,000$  fps at the high optical resolution could be sufficient for full estimation of individual cell behavior and cellular biomechanical properties *in vivo* in microvascular net.

Potential applications of this technique may include (1) fundamental study of cell-cell interactions in native flow, with



a focus on transient RBC deformability during RBC-RBC or RBC-WBC interactions; (2) estimation of the proportion of fast-moving WBCs traveling with RBCs in axial (core) flow to slow-moving (rolling) WBCs, which may have diagnostic value for the study of some pathologic conditions (e.g., inflammation, when the number of rolling WBCs dramatically increases);<sup>20</sup> (3) identification of rare abnormal cells (e.g., cancerous or sickled cells) on the basis of their different deformability in flow; (4) imaging of platelets during thrombus formation and interaction with metastatic cancer cells; (5) study of the influence of environmental factors (e.g., drugs or radiation) on individual blood cells; (6) estimation of blood viscosity in high-velocity flow through the principal determinants of blood rheology (hematocrit, cell deformability, and aggregation); (7) study of the dynamics of intravascular cell aggregation and adhesion to endothelial cells; (8) estimation of velocity profiles in fast flow; and (9) imaging and detection of individual cell dynamics in afferent lymph flow with cell velocity up to 7 mm/s.<sup>10</sup>

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