Cellular level near-wall unsteadiness of high-hematocrit erythrocyte flow using confocal μPIV

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Abstract:

In hemodynamics, the inherent intermittency of two-phase cellular-level flow has received little attention. Unsteadiness is reported and quantified for the first-time in the literature using a combination of fluorescent dye labelling, time-resolved scanning confocal microscopy, and micro-particle image velocimetry (μPIV). The near-wall red blood cell (RBC) motion of physiologic high-hematocrit blood in a rectangular microchannel was investigated under pressure driven flow. Intermittent flow was associated with (1) the stretching of RBCs as they passed through RBC clusters with twisting motions; (2) external flow through local obstacles; and (3) transitionary rouleaux formations. Velocity profiles are presented for these cases. Unsteady flow clustered in local regions. Extra-cellular fluid flow generated by individual RBCs was examined using submicron fluorescent microspheres. The capabilities of confocal μPIV post-processing were verified using synthetic raw PIV data for validation. Cellular interactions and oscillating velocity profiles are presented and 3D data are made available for computational model validation.

Keywords: Hemodynamics, microfluidics, confocal μPIV, Fluorescence, unsteady flow, rouleaux

1. Introduction

To achieve improved medical devices with very low blood damage and platelet activation, three-dimensional (3D) time-lapsed cellular deformation and fluid-induced mechanical red blood cell (RBC) loading must be quantified. In particular, near-wall regions of micron-sized, high-flow, blood-wetted components in cardiovascular devices (prosthetic valves, stents, bypass pumps and flow-assist devices) are critical. Typical jet flow velocities can reach 8 m/sec through the blood wetted narrow openings (150-500μm) of such devices, which can operate at turbulent jet flow regimes with Reynolds (Re) numbers around 800 (Fallon, Shah et al. 2006). The sluggish blood flow associated with very high abnormal hematocrit (>55%) is believed to increase the risks of thromboembolic events such as stroke or myocardial infarction (Sawka, Joyner et al. 1996). Unfortunately, the majority of past research has been limited to very low non-physiological hematocrit (Ht) levels, due to inadequate optical access at higher RBC concentrations, and to very low flow rates (Goldsmith and Marlow 1972). An early study using conventional microscopes to observe microchannel flow led to high levels of background noise which resulted from out-of focus emitted light (Meinhart, Wereley et al. 1999). Improvement was realized through high-speed μPIV experimentation to obtain velocities of both RBCs and plasma in a 100 μm square microchannel (Sugii, Okuda et al. 2005). RBCs have been used as natural particle seeds and *in vivo* measurements have been obtained (Bitsch, Olesen et al. 2005; Vennemann, Lindken et al. 2007; Poelma, Van der Heiden et al. 2009). To complement these efforts, methods that target higher spatial resolutions at the cellular scale should be further developed.

Densely crowded microscopic scenes of normal hematocrit (43%) present experimental challenges because cells pass in front of one another and occlude laser illumination. Recent advances in two-photon and confocal microscopy integrated with microscopic particle image velocimetry (μPIV) and advanced four-dimensional image processing can enable transformative measurement protocols. These methods can extend understanding of red blood cell flow and deformation to include physiologic, high-hematocrit,

high-flow regimes. Confocal μPIV offers higher 3D spatial resolution due to its unique optical slice capability (Park, Choi et al. 2004; Park and Kihm 2006); however, x-y resolutions are dependent upon the optical configurations of magnification, numerical aperture and wavelength. There is a growing interest in using this technique to observe RBC flow in microchannels. 3D velocity profiles were acquired to describe flow behaviour in 10% human blood suspension flow (Lima, Wada et al. 2006). Experiments with a higher 20% suspension flowing through a stenosed microchannel showed asymmetric trajectories due to the deformability of RBCs (Fujiwara, Ishikawa et al. 2009). Recently, confocal micro-PTV was used to analyze dynamic flow behaviour of RBC trajectories and their interactions (Lima, Ishikawa et al. 2009). In these previous studies, membrane labelling quality resulted in non-descript representations of RBCs and restricted its higher hematocrit (Ht) extensions. In our study, improved membrane labelling with an in-house long-wavelength fluorescent dye allowed time-resolved confocal microscopy scans, enabling investigations of RBC flow physics and clear depictions of membrane deformations.

The role of non-physiological turbulent and intermittent blood flow in the mechanism of RBC damage, specifically wall shear stress sensing and Reynolds stresses, has recently received increased attention (Poelma, Vennemann et al. 2008; Poelma, Van der Heiden et al. 2009). *In vitro* experiments indicate that turbulence induces significantly higher blood damage in comparison to laminar flow under the same mean wall shear stress (Kameneva, Burgreen et al. 2004). Turbulence-induced blood cell damage has been widely correlated with Reynolds stress components (Sallam and Hwang 1984; Ellis, Wick et al. 1998; Liu, Lu et al. 2000; Grigioni, Caprari et al. 2005), which is the averaged momentum flux that supplements the viscous stress terms of time-averaged Navier-Stokes equations. Several investigators (Sallam and Hwang 1984; Grigioni, Caprari et al. 2005; Quinlan 2006; Aziz, Werner et al. 2007; Quinlan and Dooley 2007; Ge, Dasi et al. 2008) suggested that the actual mechanical environment of the RBCs cannot be characterized by Reynolds stress since Reynolds stress does not represent any actual physical force exerted on the RBCs. Ge et al. showed that Reynolds stress in the wake of a mechanical heart valve predicts unrealistically high blood damage even under the laminar flow regime of the cardiac cycle (Ge,

Dasi et al. 2008). In contrast, true viscous stresses (~15 Pa) in turbulent flow of a homogeneous fluid (Ge, Dasi et al. 2008) or flow-induced stress (~60 Pa) on isolated RBC in turbulent flow (Quinlan and Dooley 2007) are considerably lower than the threshold bulk viscous shear stress (~150 to 560 Pa) required to lyse RBCs in laminar flow (Williams, Hughes et al. 1970; Leverett, Hellums et al. 1972; Hellums and Brown 1977). Recently, Antiga and Steinman performed gross order-of-magnitude assessments based on flow measurements near a mechanical heart valve (Liu, Lu et al. 2000) and elucidated the underestimation of the true viscous stresses by the overlooked cell–cell interactions in the previous studies (Antiga and Steinman 2009). The majority of these studies treat blood as a concentrated homogenous isotropic fluid, neglecting the multi-phase effects and cellular-level interactions on intermittent blood flow. In this article, it is demonstrated that these two-phase cellular-scale effects can indeed be studied through direct experiments with confocal μPIV.

Recent studies on hemodynamics have focused on cell-cell interactions and adhesion (Baskurt, Uyuklu et al. 2009; Burgmann, Grosse et al. 2009; Kaul, Finnegan et al. 2009; Ley 2009; Meiselman 2009; Ruggeri 2009), PIV and fluorescence measurement of *in vivo* flows (Poelma, Vennemann et al. 2008; Pacella, Kameneva et al. 2009; Wang, Dur et al. 2009), nuclear deformations (Dahl, Kalinowski et al. 2010), and simulations / analyses of artery and valve performance (Huang, Yang et al. 2009; Romano, Querzoli et al. 2009; Sun, Fan et al. 2009; Tanné, Bertrand et al. 2009). The cell-free layer has received much attention for its influence on blood rheological parameters (Kim, Ong et al. 2009). Computer simulation has been combined with microfluidic analyses to study interactions of all blood cell types (Munn and Dupin 2008). Interactions of blood cells with one another, including hemagglutination and rouleaux stacking formations, are characteristic of erythrocytes in high-hematocrit and readily form in pathogenic conditions. A rouleau, (*pl.* rouleaux) (Taber 2001) occurs when RBCs adhere to one another presenting a "stack-of-coins" appearance, usually in serum with increased amounts of globulin and fibrinogen (Platt 1979). Transitory rouleaux can form in the absence of pathogenic states during flow, but are not stable due to lack of hyperproteinemia or other factors. To our knowledge, dynamic velocity profiles generated

during selected rouleaux events are studied for the first time in this manuscript. Cell-cell interactions and adhesion are likely to have significant effects on the hemodynamics of medical device design.

Finally, methods that increase the capabilities of computational fluid dynamics (CFD) models that simulate 3D interactions among crowded blood cell clusters have recently been proposed by several research groups (Bagchi, Johnson et al. 2005; Jung, Lyczkowski et al. 2006; AlMomani, Udaykumar et al. 2008). However, in almost all cases, there is lack of direct experimental validation at the cellular scale which limits the application of these models to medical device design. Validation is particularly critical for emerging multi-scale CFD models since their solver structure is considerably different than the traditional continuum CFD solution techniques. It is intended that the methodology presented in this manuscript will provide high-quality 4D experimental data for CFD developers and computational researchers to validate test cases.

The organization of this manuscript is as follows: the Methods section is presented first including the experimental methodology and validation using synthetic confocal data. Next, the Results & Discussion section presents several test cases where unsteadiness and flow structure are quantified and individual RBC movements are analyzed. These test cases progress logically from representative cases of individual cellular flow to bulk near-wall flow physics, each immediately followed by pertinent discussion. The implications of these results in the context of physiologic and blood-wetted device flows are presented further along with their limitations. Finally, in the Conclusion section, the utility of this work is summarized.

2. Methods

2.1 Preparation of Blood Samples

Human whole blood was obtained through approved IRB protocol and was labelled with long-wavelength fluorescent dye diI-C_1-(7) using synthetic and labelling procedures published by Sims, et al. (Sims,

Waggoner et al. 1974). For motion studies using only erythrocytes, the final hematocrit was adjusted to 48% with 153 mM KCl-Tris buffer (pH 7.4). For particle tracking studies, 0.5 μm red fluorescent particles (Invitrogen, Carlsbad, CA) were combined with labelled RBCs and diluted with PBS buffer yielding 20% Ht containing ~0.5% microspheres. Human albumin, globulin free (Sigma Aldrich, St. Louis, MO - A8763-1G), when used, was prepared at 1% in Tris-KCl buffer for dilution of RBC to final hematocrit of 45%.

2.2 Quantification of membrane-dye uptake and high magnification confocal microscopy

In recent studies (Lima, Wada et al. 2006; Fujiwara, Ishikawa et al. 2009), the extent of RBC membrane labelling has not been known or quantified. As a result, arbitrary fluorescent signals caused by inconsistent dye labelling have resulted in excessive signals or poor distribution of the fluorescent label among the cells. The amount of dye that labels the RBC physically corresponds to the amount of fluorescence emission that occurs when the dye is excited by a laser. Too much fluorescence can result in saturation or quenching (thus loss of sensitivity to intensity differences that differentiate individual pixels, i.e. resolution), or not enough fluorescence can result in poor image quality. For one experiment of cell labelling with diI-C_1-(7), the amount of fluorescent dye taken up by the RBC (at 41% Ht) was quantified using analytical procedures described by Sims (Sims, Waggoner et al. 1974). To summarize, the amount of fluorescent dye that labelled the RBC membrane was quantified using a fluorescence spectroscopic method. This was done by measuring the difference in dye concentration (fluorescence) in labelling buffers before and after labelling the RBC using a PTI QuantaMaster fluorescence system configured with double grating excitation and emission monochromators and a Hamamatsu R5108 photomultiplier tube cooled with dry ice, run at 1100 V in photon-counting mode (PTI, Birmingham, NJ). After subtracting the amount dye that adheres to the cuvette used during measurement (solvated by butanol), the net concentration of dye that entered the cells was determined. Fluorescence spectra are presented in Figure 1 and a summary of the calculations is given in Table 1. The concentration of dye that labelled the membranes of 41% Ht RBC was 5.2 ± 0.4 nM. Based on these concentrations, and the assumption that an

erythrocyte has a volume of 90 fL, the amount of fluorescent dye per single RBC membrane in this labelling is estimated to be $4.7x10^{-22}$ moles or $1.9x10^{-19}$ grams of diI-C_1-(7).

2.3 Microchannel fabrication & experimental set-up

Rectangular PDMS microchannels, one 300 μm wide, 150 μm high, and 5 cm long, and another 330 μm wide, 100 μm high, and 5 cm long were fabricated (Duffy, McDonald et al. 1998; Zhao, Antaki et al. 2006) and bonded through oxygen plasma bombardment to a microscope coverslip (No.1, 0.15 ± 0.02 mm). For confocal μPIV, the coverslip thickness should be matched with the refractive index of the immersion oil and objective to ensure spatial accuracy and correct velocity computation. It should also be thin enough to minimize losses to depth of focus, yet thick enough to provide physical support of the PDMS. The rectangular geometry of the PDMS mold was confirmed by Scanning Electron Microscopy. In some experiments, human serum albumin (globulin-free, Sigma-Aldrich, St. Louis, MO - A8763-1G) was used to coat the glass surface of the microchannel by incubation at room temperature with 1% albumin (w/v) in Tris-KCl buffer for 2 hours (Xia, Goldsmith et al. 1993). P-100 tubing (Braintree Sci., Braintree, MA) was connected to the inlet and outlet of the microchannel. A calibrated micro-pump provided pressure driven flow at 0.7, 1.2 and 1.7 μl/min (corresponding to mean velocities of $2.6x10^{-4}$, $4.4x10^{-4}$, $6.3x10^{-4}$ m/s respectively) during confocal microscopy experiments as shown in Figure 2.

2.4 Confocal microscopy

Time-lapsed images were recorded at line scan speeds of 400 Hz, 1400 Hz, 8000 Hz (phase-matched resonant scanner) and 16000 Hz (bidirectional resonant scanning) using a Leica TCS SP5 Confocal Microscope fitted with Ar and HeNe lasers. Full size frame rates of the present experiments range from 1 to 100 frames per second (FPS) and are summarized in Table 2. Optical hardware consisted of a Leica S28 air condenser with numerical aperture (NA) of 0.55 and 28 mm working distance; and either a 20x oil immersion objective with (NA) of 0.70 and 260-170 μm working distance, a 40x oil immersion objective

with (NA) of 1.25 and 100 μm working distance or 100x oil immersion objective with (NA) of 1.4 and 90 μm working distance, each objective was model HCX PL APO CS manufactured by Leica Microsystems GmbH, Germany. Excitation wavelengths were 543 and 633 nm, with photo-multiplier tube (PMT) detection (Hamamatsu R6357) at 560-615 nm (red particles) and 750-800 nm (dye-labelled RBC membranes). Detailed images of membrane labelling in extremely high Ht (89%) are shown in Figure 1.

2.5 PIV analysis

Velocity vectors were calculated with DaVis 7.2 PIV software, (LaVision, Inc., Ypsilanti, MI) using PIV time-series and multi-pass cross-correlation. A standard FFT cyclic-based algorithm was used to process images. Pre-processing was not used on confocal microscopy image data. For each data set, the size of the first and second iteration windows was varied from 256x256 to 8x8 on the first pass and 128x128 to 6x6 on the second pass. The resulting velocity vectors were overlaid on the original confocal microscopy data and accuracy was verified through qualitative comparison to particle or RBC displacements. Processing method test cases that did not accurately represent the displacements were discarded. These cases were typically characterized by vectors in the opposite the direction of flow and were attributed to poor matches between search dimensions and pixel displacements. Flow velocities that correlated well with the pixel displacements were further processed using a range of 1-2 cycles on the first pass and 2-4 cycles on the second pass. During PIV processing of RBC images, out-of-plane motions were common, and within a series of frames, some cells did move in or out of the focal plane. The effect this had on velocity vector calculation was largely determined by the accuracy of correlation and was evaluated with respect to the intended use of the PIV analysis. For analyses using vector averaging, the occasional departure of an RBC from the focal plane resulted in a net cancelation of vectors, which did not affect the overall directional component of averaging. For qualitative and velocity component analyses, excessive out-of-plane motions resulted in inaccurate data and masks were applied to exclude regions where correlation was poor. In other data sets, masks were applied to confine analyses to a single region of interest.

Post-processing protocols were optimized for each individual data set to address the different fields of view and flow speeds that were explored. In most sets, median filtering was used to remove outlier vectors (> 1.5 rms) and then reinserted (if < 2.3 rms. of neighbouring vectors). No masks were used when statistical methods were further applied to vector fields, as in section 2.6. Post-processed results were also compared to the original microscopy images. In some cases, differences produced by analyzing multiple cycles were very subtle. Individual frames were sometimes tracked more effectively with one method than another, but overall, processing was sufficient using either method. In order to choose the best method in these cases, all PIV result frames were time averaged (using threshold minimum 5) for each method and the method that produced the average vector field closest to the direction of net flow was used. After the optimal the processing method was determined, the velocity magnitude (|V|) and the x and y components of velocity (V_x and V_y) were extracted from the velocity vector maps.

2.6 Analysis of flow unsteadiness

As a measure of individual regional fluctuation or unsteadiness, u' was calculated $u' = u - \bar{u}$ where u is the instantaneous velocity component, and $\bar{u}$ is the time-average of all frames on each interrogation region. The time-averaged velocity vectors ($\bar{u}$ and $\bar{v}$ components) were typically calculated from a PIV time series comprising at least 20 converged image-pairs. PIV velocity component maps were further grouped spatially into 16 subzones of 10x20 μm (a region 3-4 times larger than the area of one erythrocyte) for raw confocal images 1024x512. The time averaged velocity magnitude at each subzone was compared against Reynolds stress components ($\overline{u'u'}$, $\overline{u'v'}$, and $\overline{v'v'}$), which were used here as fluctuation measures. The Turbulence Kinetic Energy (TKE) per unit mass was computed from the mean of turbulence normal Reynolds stress $\overline{u_1'^2} + \overline{u_2'^2}$, where $\overline{u_1'^2}$ and $\overline{u_2'^2}$ are the eigenvectors of the turbulence velocity fluctuation matrix (Tennekes and Lumley 1972).

2.7 Synthetic confocal data for PIV Validation (RBC motion)

To verify the applicability of various PIV processing parameters in the context of scanned confocal data, the rigid body motion of an RBC-sized ellipse was simulated with MATLAB (The MathWorks, Inc., MA, USA). The pixel-by-pixel scan mechanics of the confocal microscope were incorporated to generate stretched and scan-direction-biased synthetic ellipse images for low scanning speeds. This approach allowed us to test the effects that various scanning parameters (e.g. bi-directional vs. unidirectional scans) have on post-processing accuracy for standard experimental scan speeds. One ellipse was designed with simultaneous constant V_x (-6 μm/s) and V_y (7 μm/s) movements for 4 steps of 2D translational motion. RBC rotational movements had constant rotation steps (5° per 0.560 sec). Maximum and minimum velocities from PIV software vector maps were generated using the PIV processing procedures described in section 2.5. Specifically, synthetic image sets were pre-processed using 3x3 pixel smoothing. Velocity vector magnitudes were then calculated from V_x and V_y data and compared against theoretical MATLAB data. Comparisons of vector images generated using MATLAB and PIV software for translational and rotational cases are shown in Figure 3. For the translational case, the velocity magnitude calculated by PIV software was 9.90 μm/sec, which was 7% higher than the velocity magnitude specified in MATLAB (9.22 μm/sec). For the rotational case, the |V| magnitude calculated by PIV software was 3.50 μm/sec, which was 4% higher than specified in MATLAB (3.37 μm/sec). For both transitional and rotational 2D modes, membrane motions are captured with reasonable accuracy. While the reported cases are primarily two-dimensional, the out-of-plane motion would introduce additional uncertainty, which can be incorporated into synthetic test cases.

2.8. Near-wall velocity profiles across the microchannel

For our set-up the stream-wise velocities measured by conventional μPIV at h/2 (75 μm) agreed well with theoretical solutions and previously reported (Patrick, Chen et al. 2009). Near-wall velocity profiles of erythrocytes at high-hematocrit (45%) flowing at 0.73 μl/min (mean velocity = 350 μm/s) in a 330 μm x

100 μm albumin-coated microchannel at heights < h/5 were also determined. Confocal microscopy images (512x128, corresponding to a physical area of 0.40 x 0.10 mm) were scanned at 16000 Hz (bidirectional, 100.4 FPS). Use of 20x magnification oil objective and zoom level of 1.9 captured the entire width of the microchannel in one frame. Velocity vectors were obtained for each series of 250 images and time-averaged. Velocity profiles were extracted at Y positions 0.001, 0.025, 0.050, 0.075 and 0.099 mm and averaged. The average velocity profiles from each of the five Y positions were similar, but intermittent and oscillatory cellular scale flow regimes were observed due to cell-to-cell interactions. The averaged velocity profiles at different z-positions from the bottom wall of microchannel were plotted against the microchannel position, Figure 4 (left). When compared to theoretical velocity profiles calculated using the same z-level, channel dimensions and flow rate (Bruus 2004), the experimental profiles were shifted 3 μm lower than the z-level at which they were acquired in order to agree within two standard deviations (2σ) with theoretical profiles. This distance of 3 μm corresponds to the thickness of one layer of red blood cells. Peak velocities for experimental data taken at z-levels 3-19 μm are shown in Figure 4 (right). The experimental z-levels were also shifted 3 μm for comparison against theoretical maximum velocities and agreed well at h > 5 μm. In the effective range 0-4 μm (corresponding to the shifted experimental data), the deviation from theoretical values is greater than 2σ. This corresponds to an intermittent stationary layer of RBCs 1-2 cells thick. These effects can be attributed to gravitational erythrocyte sedimentation at high-hematocrit and low flow rates (< 400 μm/s). In clinical erythrocyte sedimentation rate measurements, healthy erythrocytes settle in a tube at an average rate of 15 mm/hr (4 μm/s). Sedimentation rates in these experiments are not as high, but sedimentation effects cannot be ignored. Despite the use of albumin to prevent RBC from adhering to the glass coverslip and flushing of the microchannel between measurement sets, sedimentation effects were observed in these experiments. Despite the uniform and steady macro-flow regime in a smooth channel, more realistic cellular-scale complex flow fields are observed through the confocal μPIV experiments. Stationary cells in crowded

near-wall regions and the collision of flexible cells with each other create patterns which deviate from the theoretical quadratic Newtonian flow profiles in this environment.

3. Results & Discussion

The results are presented starting with the cellular-scale flow patterns followed by the larger-scale unsteady blood cell flow in the following order: (1) selected cases of individual near-wall RBC motion; (2) PIV analyses of rouleaux stacking and (3) analyses of unsteady flows in local regions. These case studies were selected from over 25 interesting time-lapsed flow phenomena. In these experiments, the microchannel is oriented such that the velocity vector component V_y is along the direction of flow and V_x is transverse to flow direction.

3.1 Qualitative RBC flow dynamics

For qualitative understanding, the confocal microscopy images of fluorescent dye-labelled RBC flowing through the microchannel (300x150 μm) are presented as Supplementary Movies and summarized in Table 3. These movies were selected from over 40 individual experiments with xyt (2D) and xyzt (3D) scans. The cell membranes were visualized clearly in physiologic high-hematocrit (48%) and allowed for the observation of shape changes, rotations, tumbling, collisions, and out-of-plane RBC motions in real time. MOV-1A shows fluorescently labelled RBC at 48% Ht with 40x objective and zoom level 3.8 (frame rate was 7.2 FPS). Presented in MOV-1B is a Bright Field image sequence of the same view, where light cannot penetrate due to high-hematocrit blood. This demonstrates the utility of the present technique. The remaining data sets were recorded with 40x objective and a zoom level of 6.0 to capture more detailed membrane deformations. Different scanning speeds were used to capture the data: 8000 Hz resonant-scan speed produced images (MOV-1A, MOV-1B, MOV-5) that were slightly nosier than those acquired at 1400 Hz (MOV-2, MOV-3 MOV-4), frame rate 4.9 FPS. These movies are provided

and are likely to be useful for experimental validation studies of computational two-phase blood flow simulations focusing on physiological flow conditions and cellular scales.

3.2. Selected instances of near-wall individual RBC motion

The technique was first applied to the analysis of subtle near-wall movements and adhesion characteristics of individual erythrocytes. The cellular deformations and bulk velocities were captured using PIV in these case studies from time lapse images (512x256 pixels, 1400 Hz, 4.9 FPS) in the crowded near-wall region of the microchannel 2.5 μm from the bottom at 48% Ht. The flow rate was 1.2 μl/min. and mean velocity was 4.4×10^{-4} m/s.

CASE-1: Rotational and counterflow of an RBC trapped inside a cell cluster. One RBC was trapped within a cell cluster perpendicular to the plane of the microscope slide and exhibited rigid-body rotation from 23 to 28 degrees (Figure 5A) and momentary counter-flow oscillation. The maximum velocity of the trapped RBC in the direction of flow was 1.0×10^{-6} m/sec.

CASE-2: Translational movement of RBC trapped inside an RBC cluster. One RBC was trapped within a cell cluster perpendicular to the plane of the microscope slide and exhibited translational oscillations perpendicular to the direction of flow due to interactions with other flowing cells and the draw of the plasma current. The maximum velocity of the sequence was 7.6×10^{-6} m/s. A series of images shows velocity vectors and V_x contour maps of the trapped RBC at points of interest. V_x is negligible at first ($+9.7 \times 10^{-10}$ m/s), then after contact with a cell in flow, V_x increases significantly to -1.69×10^{-6} m/s. The suction generated due to free-stream flow changes V_x to $+1.32 \times 10^{-6}$ m/s. A sudden collision event with another RBC reverses V_x to -1.89×10^{-6} m/s. (Figure 5B). This case illustrates the complex interplay between cellular interactions and plasma flow which affects individual cell motions and ultimately the translational component of Reynolds stresses on RBCs in flow.

CASE-3: Deformation of RBC through RBC cluster. One RBC perpendicular to the plane of the microscope slide curves and changes its shape to pass through three tethered RBC, creating a narrow passage (Figure 6A, MOV-2). PIV analysis shows the velocity vectors associated with the deformation of the erythrocyte membrane as it passes through the narrow openings. In the final frame, vectors along the edge of the membrane point towards the center plane of the RBC. This case illustrates the role of membrane elasticity as RBCs navigate through crowded near-wall environments.

CASE-4: Creeping motion of one RBC. Clustered RBCs can exhibit motions that are very different from the average fluid velocity. In Figure 6B, the maximum velocity of the RBC while passing through a cell cluster was found to be $9x10^{-6}$ m/s. This velocity was relatively low in comparison to the mean velocity in the microchannel, $4.4x10^{-4}$ m/s, generating an intermittent motion as the RBC passed through the cell cluster with axial rotation.

Cellular level sources of unsteadiness

The global intermittency in two-phase RBC flow that is highlighted in the present study is directly associated with the random cellular level phenomena. Several cases are isolated here that correspond to the fundamental modes. Flow-induced oscillation of a single RBC trapped in an RBC cluster (Figure 5A and 5B) illustrates one possible mode of micro-scale unsteadiness. In these cases, compared to other stationary RBCs which were parallel to the plane of the microscope slide, a single RBC oriented perpendicular to the plane of the microscope slide had oscillatory rotational motion spanning 10 degrees (Figure 5A). In CASE-2, the RBC had translational oscillatory motion due to cell-cell interactions (moving towards the wall) which was accompanied by suction from the external flow stream, which pulled the RBC away) (Figure 5B). Deformability of the erythrocyte membrane played an important role in this dynamic process as illustrated by the case of the perpendicularly oriented meandering RBC (Figure 6A). These crowded locations create a microenvironment that is very different from other cases of near-

wall flow where out-of-plane movements allow for alternate trajectories. Movement of the erythrocyte shown in Figure 6A is restricted to the plane perpendicular to the stationary cells. Axial rotation movements may also play a role in this RBC passage, however based on the PIV data, no conclusion can be made whether this erythrocyte was rotating as it passed through narrow openings. CASE-4 illustrates the unsteady flow generated as one RBC passes through an RBC cluster by creeping motions (Figure 6B). This particular movement pattern is likely to be advantageous through narrow gaps as it creates less resistance.

To our knowledge, the hematocrit levels presented in this study are higher than those used in earlier microchannel flow studies, but yet still enable comparison to studies based on lower RBC densities. It has reported that beyond a stenosis in 10% Ht flow, RBCs are initially located near-wall and then tend to migrate away from the wall. Flow at 20% Ht showed the opposite behaviour (Fujiwara, Ishikawa et al. 2009). Higher Ht flow (~48%), as described in the present paper lead to results that were similar to the 20% Ht case. Quantifying similar complex but physiologic flow conditions could aid in stent structure (He, Duraiswamy et al. 2005; Fujiwara, Ishikawa et al. 2009; Mejia, Mongrain et al. 2009) and device design (such as mechanical heart valves) for improved hemodynamics and reduced blood damage (Sallam and Hwang 1984; Ellis, Wick et al. 1998; Liu, Lu et al. 2000; Fallon, Shah et al. 2006; Quinlan and Dooley 2007). A more effective stent strut could be oriented parallel to the vessel axis to reduce flow recirculation, which can lead to restenosis (He, Duraiswamy et al. 2005). A computational simulation study (Mejia, Mongrain et al. 2009) investigated the hemodynamic performance of four different strut cross-sectional profiles (square, circular elliptical, and tear-drop) at cellular scale. In this study, the dynamics of a single-cell are illustrated experimentally as the cell passes through another near-wall stationary RBC that resembles a stent-like circular shape. This result enables numerical model validation. Similar comparisons between simulated and experimental data will increase working knowledge in the medical device field.

3.3 Analysis of transitionary rouleaux states

Rouleaux stacking was observed in flow as both stable and transitory formations, in free flowing and congested regions of the channel. In our experiments, such transitory formations were observed to occupy 0.5-3% of the area within a field of view at any given time instant, depending upon the dimensions of the acquisition images. The observed rouleaux stacking was infrequent, occurring in less than 10% of the data sets acquired for this study. PIV analysis of the rouleaux show characteristics unique to these formations at 48% Ht. The flow rate was 1.2 μl/min (mean velocity 440 μm/s).

Rolling and tumbling rouleau formation: A stable rouleau triplet was observed tumbling freely though steady flow regions in the center region of the microchannel, 7 μm from the bottom surface. 1024x1024 images were acquired at 8000 Hz scan speed (7.2 FPS). The maximum velocity at 7 μm from the bottom surface was 44.9 μm/sec. The cross-sectional velocity profile across the rouleau shows the velocity vectors of each of the cells in the triplet are similar and remain consistent throughout the flow, indicating that the formation has agglutinated as shown in Figure 7A and MOV-1A. This would suggest that stacking occurred prior to RBC isolation and dye-labelling, when the blood proteins needed for hemagglutination were available. Although the formation is stable, there were minute velocity differences between the cells in the formation, implying that the trio of cells comprising the rouleau were not rigidly fixed at all points and regularly observed membrane deformations still occur during rouleau formation.

Formation of rouleaux: Figure 7B shows a region of transitory rouleaux in a series of confocal microscopy images (512x256, 1400 Hz, 4.9 FPS) recorded at the near-wall 5 μm from the bottom of the channel. This region is characterized by stationary cells at one side and steadily moving cells at the opposite side having a maximum velocity of 12.7 μm/sec and an average velocity of 5.8 μm/sec. Cells that were positioned perpendicular to the coverslip were in the proper orientation to form rouleaux

(MOV-3). Cross-sectional velocity profiles across one rouleau, obtained by PIV analysis, show a momentary localized stoppage of flow when the ideal stacking presents, despite flow around the formation at much higher velocity. In physiologic micro flow, rouleaux formation will probably be more stable since the present experimental conditions reduce the concentration of proteins needed to cause hemagglutination.

Rouleau break-up: In this case, an initially intact rouleau triplet disintegrates upon impacting a stationary RBC cluster (MOV-4). 512x256 confocal microscope images were captured at 1400 Hz (4.9 FPS), 2.5 μm from the bottom surface and at the microchannel wall. PIV analysis initially shows uniform vector direction (Vy = 9.6 μm/sec), followed by divergence upon impact. This corresponds to disintegration of the rouleau (Figure 8). Subsequently, the rouleau components continue singularly in the original direction of flow and out-of-plane. Under such conditions, the attractive forces of hemagglutination that stabilize the rouleaux can be overcome by the kinetic forces from intercellular collisions.

The formation of transitionary rouleaux states is another phenomenon associated with near-wall intermittency which is unique to erythrocytes due to their flexible structures and deformability. The formation of transitory rouleau stacking was observed at the interface of moving and stagnant RBC regions. This suggests that in these regions, the orientation needed to form stacking can occur. In addition, the mere settling of erythrocytes during the stoppage of flow in the proper orientation can also result in formation. For stable rouleaux, high amounts of blood protein are required to initiate hemagglutination. In this study, successive buffer washes during isolation and dye-labelling dilute available blood proteins. To further study rouleaux formation using this method, additional high molecular weight blood proteins can be added after labelling to create the necessary hyperproteinemia. Here the velocity profiles of rouleaux formations in deep opaque blood were studied for the first time in literature.

3.4 Analysis of near-wall two-phase unsteadiness

To elucidate the differences between velocity fluctuations at different locations and velocities, Reynolds stress components, $\overline{u'u'}$, $\overline{u'v'}$, and $\overline{v'v'}$, were calculated along the scan plane for several data sets. An example data set is provided in Figure 9 where the distribution of time-averaged velocity fluctuations in the center of the microchannel with 48% Ht at different z positions (above the coverslip) is plotted. The flow rate was 1.2 μl/min (mean velocity = 4.4×10^{-4} m/s; Re= 3.0) and confocal images (1024x512) were captured 5 μm and 11 μm from the coverslip at the center of the microchannel scanned at a rate of 8000 Hz (14.1 FPS).

Reynolds stress components were further averaged on a subzone basis to analyze local variations. Spatial averages over each subzone were calculated to partially eliminate the effect of minimal out-of-plane motion and finite cell size. Subzones further away from channel walls generally had higher average velocity magnitudes, which were also influenced by temporary lower velocity cell clusters. For each subzone, the spatially averaged Reynolds stresses, $\overline{u'u'}$, $\overline{u'v'}$, and $\overline{v'v'}$, were compared to the corresponding averaged velocity magnitudes. A plot of these relationships is presented in Figure 10 for all data points. As the z-position increases (away from the bottom wall of the microchannel), Reynolds stress components increase, primarily contributing to the free stream Reynolds stress terms $\overline{u'u'}$ and $\overline{v'v'}$.

In contrast to the highly laminar and uniform single-phase micro-channel flow regimes, cellular scale complex flow fields are documented in the present study to examine the intermittent motion of RBCs and their interactions. Confocal μPIV is applied to further understand physiologic hemodynamics in near-wall and cell cluster environments, which is the main motivation. Based on the energy spectrum estimations of flow measurements near mechanical heart valves, Kolmogorov scale eddies range from 10

to 70μm (Ellis, Wick et al. 1998; Liu, Lu et al. 2000; Travis, Leo et al. 2002), which are on the same order of magnitude as RBC size (8 μm). Hence, several investigators have postulated that cardiovascular devices may contain flow features on the same order of magnitude as RBCs. However, as illustrated in the present study, direct observation of the microscopic environment of turbulent blood flow is essential and poses formidable experimental challenges. Present technology has allowed the study of flow dynamics as deep as 25 μm. In the future, faster scan rates and deeper imaging confocal objectives that can easily reach z- levels of ~1200μm could test the existence of turbulent eddies on the order of the RBC scale and resolve the applicability of Kolmogorov theory within blood flow (Sutera and Joist 1992; Antiga and Steinman 2009). Effects of turbulence at the cellular scale have recently been investigated computationally (Quinlan and Dooley 2007; Dooley and Quinlan 2009) based on flow measurements downstream of a mechanical heart valve (Liu, Lu et al. 2000; Ge, Dasi et al. 2008). Dooley and Quinlan (Dooley and Quinlan 2009) incorporated a two-dimensional immersed boundary fluid–structure interaction (FSI) model to simulate the flow-induced loading on a flexible RBC interacting with a spectrum of turbulent eddies of different length scales. These results suggested that RBC deformations are almost independent of the Kolmogorov length scale which is a function of turbulent kinetic energy of the flow field. In addition, the model predicts that within a given flow field, eddies of sub-cellular scale are less damaging than larger eddies. These findings highlight the need for deeper understanding of the micro-scale structure of turbulent blood flow.

Applying PIV analysis on confocal images of stationary RBC and particles at rest, the RBC membrane average velocity was 9.34 x 10^{-8} m/s and particle velocity was 2.59 x 10^{-7} m/s. These velocities attributed to Brownian motion are 10^{3}-10^{4} times smaller than the velocities presented in the experimental results used to analyze unsteadiness. The present unsteady flow analysis of erythrocytes indicated that as velocity magnitude increases, so do measures of unsteadiness ($\overline{u'u'}$ and $\overline{v'v'}$); however, $\overline{u'v'}$ remained nearly constant. To determine if this trend is consistent of at higher velocity magnitudes and at greater

heights from the bottom wall of the microchannel, additional experiments are required. Particularly at higher velocities, the higher RBC momentum in the free-stream direction results in an increase in cell-cell collision intensity and augmented out of plane movements, where the effective deviation from average velocity is increased. At the near-wall region, stationary RBCs create complex obstacles that disturb laminar flow and cause a reduction in the average velocity at the global scale.

Recent studies showed that Reynolds stress is not an adequate predictor of cell damage because it leads to overestimation of the force required to cause hemolysis (Quinlan and Dooley 2007; Ge, Dasi et al. 2008). However, these results were based on highly diluted suspension RBC flow. By considering the concentrated nature of cells in normal blood flow, the RBCs which constitute roughly half of the blood volume, become the main recipients of kinetic energy and give rise to viscous stresses on the order of Reynolds stresses (Antiga and Steinman 2009). In this study, averaged TKE was found to be proportional to corresponding velocity magnitude through direct measurements. These results support the Antiga and Steinman argument that RBCs and plasma can be equal contributors to turbulence in blood flow. Averaged TKE can be used to predict the blood cell trauma on a subzone basis and at high velocity magnitudes.

3.5 Differences in plasma vs. cellular flow fields at near-wall

Differences between plasma and cellular flows are presented from 2 data sets of a series of confocal images (512x512) captured at 400 Hz (0.8 FPS) at the near-wall region of the microchannel of RBC (20% Ht) seeded with 0.5 μm red fluorescent particles (0.5%). When fully developed, the flow rate was 0.7 μl/min (mean velocity = 260 μm/s). Particle and RBC fluorescence signals were acquired simultaneously through separate photo-multiplier tubes.

CASE-5: Sinusoidal pulsatile near-wall flow

A special case of pulsatile flow (the first cycle of sinusoidal forward and backward unidirectional mean flow) was analyzed at 3 μm from the bottom surface. Pulsatile flow was created by a microsyringe pump engaged from rest. The period of pulsation was 2.6 sec of back flow with a peak velocity of -1.0 μm/sec, followed by a maximum velocity of 4.4 μm/sec, 18.4 sec after reversal. Velocity vectors were calculated using PIV with post processing and V_x and V_y components were extracted (Figure 11A). RBC flow was slightly larger in V_y for several frames during the transition to forward direction. The maximum particle velocity was slightly larger in V_x opposite the wall (+1.4 μm/sec) as compared to RBC velocity (+0.9 μm/sec). The particles are 16 times smaller and move laterally when RBCs are blocking them. Due to the inertia of RBCs, cell-cell interactions around regions of stationary RBCs, and the much larger diameter of RBCs (as compared to particles), the particles have more space to move in the x direction. However the RBCs dominate during acceleration in the y direction when forward flow is being established.

In contrast, during steady flow 15 μm above the coverslip, the effects of stationary RBC attached to the wall or glass surface of the microchannel are minimized. The maximum velocities attained by the RBC and particles were 6.8 and 7.4 μm/sec, respectively (Figure 11B). The maximum V_y (7.3 μm/sec) was higher in the particles than the RBCs (6.7 μm/sec). The particle V_x was 1.4 μm/sec at maximum particle V_y, while the RBC V_x was 1.8 μm/sec at maximum RBC V_y. Movements of particles and RBCs in the x direction were both towards and away from the channel wall. However, particle motions tended toward the center of the channel while RBC motions were more equally split in both directions. One possible explanation for the equally bidirectional movements of the RBC observed can be explained by collisions caused by erythrocyte sedimentation during steady flow at this z-level. RBC from higher planes can collide with an RBC in plane and the collision result in movement in either direction. Since the particles are much smaller in size and weight, these effects are smaller and they are drawn towards the center region of higher flow. Lima and colleagues observed that erratic transversal displacements, induced by

the strong influence of velocity gradients at the near-wall contribute to the lateral movement of RBC at 20% hematocrit compared to the plasma flow. Thus, erythrocytes are subject to axial and lateral migrations, but in crowded regions wall collisions increase. Blood flow properties are affected by local Ht and velocity gradients induced by the wall. (Lima, Ishikawa et al. 2009)

Identifying differences in plasma and RBC velocity fields are critical in order to quantify the momentum interactions between the phases. Restricted to low near-wall velocities and confined flow regimes, these differences are measured both at pulsatile and steady conditions. During sinusoidal flow, the momentum of the RBCs is considerable in the y direction during flow, but the particles are free to shift with the plasma rather than collide with congested RBC (Figure 11A). During steady flow (Figure 11B), particles had higher V_y than RBC, which was opposite of that observed during pulsatile flow. V_x showed exactly the opposite trend than V_y for both flow scenarios illustrating that when movement of one phase is diminished in one direction, movement in the perpendicular direction is increased relative to the other phase. Labelling of both phases and acquiring the fluorescent signal simultaneously from both channels demonstrated that this unsteadiness is due to the irregularities at the cellular scale. In addition to medical device design, similar random two-phase flow regimes play an important role during very early cardiovascular development of great vessels. We also observed that RBCs can orient themselves from in-plane to out-of-plane movement in areas of high viscosity or crowded cell clusters and exhibit flow patterns that can be significantly different from the surrounding fluid (buffer). Such motions can be quantified through reduced image size, ultra fast multi-plane scans. These techniques are currently investigated and can be applied with the existing hardware (Chen 2010).

3.6 Limitations

Accuracy of the out-of-plane component of velocity is a well established limitation of the present study. To address this, the cases presented in this article were strictly selected from a larger set of experimental data to be two-dimensional and primarily confined to near-wall regions. 3D motion will generate

isovolumetric changes but will have limited effects on gross motions. To evaluate the extent of out-of-plane motions in large interrogation window sizes, an xyzt (time-volume) scan was collected. An xyz sum of intensities projection of each time point from the xyzt scan was created from z-slices spanning 4 μm and PIV analysis was applied to the projection images. This served to increase the pixel density and reduce z direction data losses since any part of the cell can be captured within the 4 μm z-direction. The projected image covers a 4 μm range whereas a single plane is limited to the voxel depth of the slice. However, in this case, the time between projection frames was large (8.5 sec) due to high resolution scanning parameters and thus impractical under these scanning conditions. Furthermore, averaging the velocity values over subzones that are sufficiently larger than the typical cell size partially cancels these 3D effects.

3.7 Experimental & Methodological Considerations

In conjunction with the experimental results presented in the previous section, the image acquisition parameters, implications of synthetic PIV data validation, differences in PIV processing methods, and practicalities of dye-labelled RBCs use with particles are discussed further.

3.7.1 Acquisition parameters and frame rates

The current state of the art favors high-sensitivity, meshless multialkali compact, electro-optic constructed PMT technology for the fastest imaging of long-wavelength fluorescence. Use of spinning disc confocal microscopy with CMOS cameras was investigated for this project; however, the long exposure times needed to obtain strong long-wavelength fluorescence signals resulted in frame rates significantly lower than those generated by PMTs and resonant scanners. The maximum recording speed in these experiments of 125 FPS was attained by using bidirectional resonant scanning and reducing image size to 512x128. The frame rates are dependent upon the line scan speeds and the frame dimensions in the Y direction. The frame rates attained experimentally are not as fast as the scanning acquisition parameters

are set. For instance, when set at 16000 line scan speed and 512x128, the calculated frame rate is 125 FPS; however the actual frame rates measured during the experiments (total number of frames / duration) were 100 FPS (20% lower). These differences are due to delay times inherent to the hardware, and are also are dependent upon the number of lines scanned and the length of each line. In recent our experiments to record blood flow of zebrafish embryos *in vivo* (Chen 2010) using the same Leica confocal microscope used here, recording rates of 250 FPS were possible by reducing the image size to 256x64. This produced actual image scan rates of 175 FPS (30% lower than setting). However, at faster frame rates, resolution is sacrificed. Therefore, very slow scan rates (0.8 FPS) were used to record RBCs seeded with particles at a line scan speed of 400 Hz for a 512x512 image. At this higher resolution, the shapes of RBC membranes and the position of 0.5 μm fluorescent particles were clearly defined. Using an image size of 512x512 permitted velocity mapping that corresponded to a physical area of 65 x 65 μm at the near-wall region with sufficient detail (Figure 11). This dimension is approximately 8 RBC wide and 8 RBC long, and thus allowed capture of a region large enough to study cell-cell and near-wall interactions.

3.7.2 Synthetic data validation

Synthetic PIV data generated in MATLAB enabled verification of the post-processing algorithms used in this study and applied to confocal PIV the first time in literature. The temporal and spatial scales selected here correspond to the near-wall motion of high-hematocrit cellular scale blood flow and agreed well (Figure 3). The errors in actual confocal microscopy data are smaller since the MATLAB data were generated as discrete pixels of uniform intensity. Fluorescent microscopy images obtained with the long-wavelength fluorescent membrane dye, DiI-C_1-(7), showed that the intensities of different pixels are non-uniform. DiI-C_1-(7) labels the erythrocyte membrane through a non-covalent mechanism that results in dye occupation of the outer layer of the membrane bi-layer leaflet (Sims, Waggoner et al. 1974). Because dye labelling occurs at the molecular level (and the bi-layer leaflet can be viewed as being comprised of

molecular sub-units), there will be non-uniform labelling throughout the surface area of the membrane, until dye-saturation of the membrane occurs. This non-uniform labelling creates spatial intensity variations that improve cross-correlation. For this reason, quantification of dye uptake is essential, so that non-uniform labelling conditions are achieved. In addition, the exporting of pure numerical synthetic data from MATLAB to a digitized image file is not exact and may contribute to errors in velocity prediction. The synthetic data can be made more realistic by introducing random and fixed intensity variations to incorporate these two effects. Furthermore, analysis of the whole RBC (or simulated ellipse) may transcend the capabilities of the PIV software, which is designed for particle clusters and not necessarily whole objects spanning many pixels of similar intensity. However, many other suitable motion estimation algorithms exist and can be explored further.

3.7.3 PIV processing differences

General PIV methods were applied to data as described in section 2.5. The application of PIV processing parameters and functions that gave good results with conventional PIV (Patrick, Chen et al. 2009) to RBC membrane processing was clearly incorrect when the RBC velocity vectors were compared against cell movement using the original confocal microscopy images. The main difference between PIV particle tracking and intact membranes tracking is the magnitude of displacement. The membrane is imaged as a collection of pixels and PIV processing can track pixel displacements. However, the desired outcome is movement of the membrane as a unit, not the individual pixels comprising it. For conventional PIV data (Patrick, Chen et al. 2009), 3x3 pixel smoothing was needed to achieve more accurate results. However, when pre-smoothing was applied to the RBC images, it led to poor image correlation and thus poor vector results. These can be attributed to differences in the hardware used to acquire the image sets, but validation of methods was needed for RBCs since their shape is much larger than traditional particles. The dimensions of the iteration windows and the use of masks can also affect results. Windows that are too large give too little vector data and windows that are too small give high density vector data with erroneous correlation. Post-processing techniques such as removing vectors that differ from neighboring

vectors often resulted in removal of significant portions of vector data. Recently, it has been shown that image overlapping is effective in improving the accuracy of time-averaged measurements by reducing measurement depth and is more reliable than correlation averaging (Nguyen 2010) and image overlapping was used in our methods.

3.7.4 Dye-labelled RBCs and optimum particle conditions

To maximize the utility of confocal μPIV hemodynamic data, the labelled RBCs must remain intact and provide strong fluorescent signals. RBCs are very sensitive to the ionic strength of the solutions to which they are exposed. If the ionic strength of the surrounding solution is lower than that of the intracellular fluid, then RBCs rupture, which increases the fluorescent contribution from the dye-labelled membrane fragments relative to the intact membranes. Hemoglobin interference is also a concern, and for this reason, a long wavelength fluorescent dye was used. It was found that RBCs could be labelled up to two weeks after isolation from fresh whole blood when stored at 4^{o}C in isotonic buffer, and were stable up to two weeks after labelling when stored at low hematocrit in isotonic buffer. The amount of fluorescent dye taken up by the erythrocyte membranes has been quantified for one batch of labelling although different values can be obtained for different blood samples, handling procedures, and labelling conditions. The determination of dye uptake for each labelling is impractical. However, the ability to quantify membrane dye uptake is extremely useful when experimental conditions are altered significantly, such as when proteins are added to cause hyperproteinemia. DiI-C_1-(7) labels the RBC membranes and not the interior of the cell, resulting in clear confocal images of membrane shape. These images can be used to study 3D morphology of erythrocytes.

Particles should be selected so that the emission wavelength is sufficiently removed from the excitation and emission wavelengths of the membrane dye fluorescence. The choice of surface coating for the particle tracers and the buffer solution are also significant. Particles must not interact with the cell membranes; they must move freely within the fluid. The buffer must be compatible with the RBCs to

prevent rupture, but not incompatible with the particles because this will lead to particle aggregation of and separation of the particles from the blood. This was observed when using Invitrogen carboxylate FluoSpheres, and buffer was changed to PBS when using these in mixed phase experiments. In addition the particles must not adhere to the glass or microchannel surfaces.

4. Conclusion

A methodology combining fluorescent dye labelling, scanning confocal microscopy, and particle image velocimetry has been developed to observe near-wall red blood cell motion of physiologic high-hematocrit blood (48%) in a rectangular microchannel. For the first time in RBC μPIV studies, the extent of fluorescent dye labelling has been quantified and thus can serve as baseline when altering this experimental variable to improve the quality of fluorescent confocal images. Verification tests used to characterize the 2D capability of this technique are summarized. The approach can be extended to realistic medical device geometries and higher speeds with technology advances. Measures of RBC membrane-phase unsteadiness were analyzed, indicating that cell-cell interactions, both stationary and moving, greatly affect local velocity oscillations during flow. Plasma flow seeded with particles showed that for near-wall high-hematocrit flow, the velocity components (V_x and V_y) of both phases are globally identical at steady flow conditions (although small regional fluctuations were observed). Compared to free-stream blood flow, near-wall phenomena may be more critical as most physiologically vital processes such as platelet activation, cell adhesion and wall shear stress sensing occur near-wall and at low speeds. The common paradigm of homogenous, single-phase, isotropic turbulent blood flow is challenged by direct observations from the present study of opaque blood. As velocity magnitude increased, fluctuations increased in the direction of flow, and the other components showed minimal to no increase. Selected snapshots of cellular scale fluid-RBC interactions demonstrated complex spatially variable unsteady regimes. In general, individual RBCs tended towards out-of-plane motions as they passed through stent-like stationary RBC clusters. In a high Ht flow, an RBC will initially approach and then move away from the stationary cell instead of following its contour. Velocity profiles of rouleaux

stacking formations were observed at the interface of moving and stagnant RBC regions. The utility of this analytical method is that the conditions required for the formation of stable rouleaux during flow can be created by controlled addition of proteins needed to induce hemagglutination in microchannels designed to contain planar regions that induce the RBC orientation needed to form rouleaux. The specific knowledge gained from this application can aid the design of blood devices that minimize conditions favorable to rouleaux formation. Out-of-plane motions are a limitation of the technique. Further analysis of 3D motion estimation errors can be studied using our synthetic confocal PIV framework. Employing ultrafast scans that generate reduced resolution coarse 3D datasets combined with advanced 4D image reconstruction and interpolation (Frakes, Pekkan et al. 2008) can be considered for highly three-dimensional flow regimes. Characterization of the physiologic RBC flow where there is minimal blood damage can inspire innovative alternatives in biomedical device design. Understanding these basic fluid dynamics will impact both the pediatric and adult patient populations with heart diseases and vascular disorders.

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Figure Captions

Figure 1: *Left Panel:* Fluorescence Spectra of diI-C_1-(7) and RBC suspensions for quantification of dye uptake in the RBC membrane (Excitation wavelength 710 nm). Curves and values are summarized in Table 1. *Right Panel, Top Row:* Confocal microscopy images of stationary diI-C_1-(7) labelled RBC at 100x objective and 89% HCT at zoom 2.5 (left) and 5.0 (right). *Right Panel, Bottom Row*: Bright field shows density of blood cells respectively. Images are 2.0 μm (left) and 4.2 μm (right) from the coverslip. Lasers Ar, HeNe (633nm), PMT 750 - 800nm; Dimensions 512x512 pixels; Voxel-Depth 199.8 nm, Scan Speed 400 Hz.

Figure 2: Cartoon of thin glass cover bonded PDMS micro-channel to minimize reflections and picture of experimental set-up on the microscope stage.

Figure 3: Comparison of synthetic PIV velocity vector data (*right*) of simulated cell movement (idealized ellipse) with simultaneous constant V_x and V_y movements (*top*) and rotation (*bottom*) against actual vector field (*left*).

Figure 4 *Left:* Theoretical Newtonian velocity profiles (dashed lines) and experimental time-averaged velocity profiles (solid lines) of RBC at 0.7 μl/min. Error bars indicate two standard deviation values of 5 velocity profiles for each z level. *Right:* Peak velocities of theoretical flow profiles (black squares) and experimental time-averaged RBC flow profiles (red deltas) at different z positions in a microchannel at 0.7 μl/min. Experimental values were shifted 3 μm (approximately the thickness of one RBC) due to a stationary sedimentation layer of cells that formed at the bottom of the microchannel at high Ht (45%).

Figure 5: Rotational Movements and Lateral Oscillations **(A)** *CASE-1* One RBC trapped within a RBC cluster perpendicular to the plane of the microscope slide exhibits 2D rotation and counter-flow between two consecutive frames. **(B)** *CASE-2* One RBC trapped within a RBC cluster perpendicular to the plane of the microscope slide exhibits translational oscillations in a series of non-sequential frames to illustrate the effects of cellular interactions and suction current streams.

Figure 6: (A) *CASE-3* One RBC perpendicular to the plane of the microscope slide curves and deforms to pass through three tethered RBC creating a narrow passage between them. The last frame shows narrowing of the RBC. Frames are shown 0.200 sec apart. **(B)** *CASE-4* Individual RBC motion (outlined) tracked as it squeezes through a near-wall cell cluster by axial rotation and edging motions.

Figure 7 (A) Stable Rouleau Formation In Channel Flow. Rouleau cluster tumbles and flows as a stable unit characterized by uniform velocity profile (indicated along the yellow section) in consecutive images 0.140 sec apart. Top row is Vy (direction of flow); Vx bottom row. Flow rate 1.2 μl/min at z= 7 μm. **(B)** Transitory Rouleaux (1.2 μl/min, z = 5 μm) in a sequence of images 0.818 sec apart. Velocity profiles (extracted through the horizontal yellow line) show momentary stoppage of flow (3rd frame) that characterizes rouleau formation during flow. All velocities are given in μm/sec.

Figure 8: Rouleau Break Up Event. Velocity profiles across a rouleau (indicated by yellow line), shown for consecutive frames 0.204 sec apart. Flow rate 1.2 μl/min, 2.5 μm from bottom glass surface. Top row is Vy (direction of flow); bottom row, Vx. Velocity values are given in μm/s

Figure 9 Distribution of 2D Reynolds stress components and averaged velocity magnitude at different z positions (left column, z=5 μm; right column, z=11 μm) obtained from confocal PIV experiment and interpolated by Tecplot at 48% Ht. Reynolds stress components are plotted in m^2/s^2; velocity magnitude is in m/s.

Figure 10 Comparison of the relationship of averaged velocity fluctuations terms as a function of velocity magnitude. Experimental data shown at 48% Ht, was obtained from subzones, each represented by delta symbols. Bottom right plot shows the linear trend line of averaged TKE as a function of the velocity magnitude.

Figure 11 *CASE-5* Selected frames showing sinusoidal pulsation of flow and steady flow of RBC and red fluorescent particles at 20% Ht. (A) Sinusoidal oscillation 3 μm from bottom of microchannel at near-wall, first showing deceleration, then acceleration phases (12 intermediate frames not shown; Δt between frames shown is 17.1 sec). (B) The steady flow 15 μm from bottom of microchannel at near-wall, showing maximum velocity. Images shown in rows (top to bottom): velocity vectors overlaid confocal microscopy images (RBC, Particles), velocity component contour maps of RBC V_y, Particle V_y, RBC V_x and Particle V_y. The yellow arrows indicate the main flow direction. Velocities are in μm/sec.

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