Video Article

Micro-particle Image Velocimetry for Velocity Profile Measurements of Micro Blood Flows

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Abstract

Micro-particle image velocimetry (μPIV) is used to visualize paired images of micro particles seeded in blood flows. The images are cross-correlated to give an accurate velocity profile. A protocol is presented for μPIV measurements of blood flows in microchannels. At the scale of the microcirculation, blood cannot be considered a homogeneous fluid, as it is a suspension of flexible particles suspended in plasma, a Newtonian fluid. Shear rate, maximum velocity, velocity profile shape, and flow rate can be derived from these measurements. Several key parameters such as focal depth, particle concentration, and system compliance, are presented in order to ensure accurate, useful data along with examples and representative results for various hematocrits and flow conditions.

Video Link

The video component of this article can be found at http://www.jove.com/video/50314/

Introduction

The human body contains numerous vessels with diameters less than 50 μm, which are the main exchange site between blood and tissues. The study of blood flow in these vessels represents a considerable challenge due to both the scale of the measurements and the fluid properties of blood. These measurements, including the pressure gradient, the shear at the wall, and velocity profiles in arterioles and venules, are key factors linked with physiological responses. There are now unprecedented opportunities to resolve these measurement challenges, thanks to new experimental techniques at the micro scale to study the microcirculation and solve this multiscale problem.

Micro-particle image velocimetry (μPIV) is a particle-based flow visualization technique that is used to evaluate velocity profiles of blood flow in microchannels via cross correlation. μPIV, first developed by Santiago *et al*., has been used with hemorheology studies since Sugii *et al.* in 2001 used the technique to measure blood flow in 100 μm round glass tubes [1,2]. Different approaches to μPIV exist. High speed cameras can be used to correlate the movement of red blood cells (RBCs), and pulsed images can be used to correlate the movement of tracer particles. Either of these options can be coupled with an upright or inverted microscope, depending on the application. In both cases, the result is a 2D velocity profile. Another approach is to use a confocal microscope to achieve 2D and 3D profiles. This method has been applied to blood [3,4,5].

Micro scale PIV has several complications when compared with macro PIV. In macro PIV the data can be limited to a single plane through sheets of light, but at the micro scale volume illumination is necessary. Volume illumination is a greater problem for the imaging of micro blood flows, as the RBCs themselves are large in comparison to the channels, and using the RBCs as the tracer particles leads to a depth of correlation (DOC) which can significantly decrease the accuracy of the cross-correlation results [6,7,8]. Following Wereley *et al.* (1998) the DOC for a 40 μm tall channel with RBC as the tracers is 8.8 μm, while with a 1 μm tracer particle the DOC is 6.7 μm. This difference becomes more pronounced when changing channel height and magnification. Additionally, RBCs are opaque, and increasing the density of the RBCs in the flow causes imaging difficulties. Fluorescing tracer particles, first used by Santiago *et al.* (1998), have been advocated as a tool to decrease the influence of out-of-focus particles, when using the smallest particles possible. Using 1 μm diameter fluorescing microparticles coupled with a laser is one approach that may decrease the depth of focus problem in micro blood flow imaging [10]. There are several current reviews of the state of μPIV technology, each of which highlights the importance of μPIV to blood flow studies [11,12]. Several important considerations must be taken into account when using μPIV for blood. At the micro level, the scale of the microcirculation, blood cannot be considered a homogeneous fluid, as it is a suspension of flexible red blood cells (RBCs), large white blood cells, platelets and other proteins suspended in a Newtonian fluid (plasma).

The velocity profiles measured here can be used to measure certain characteristics of the micro blood flows. The important factors in microhemorheology are the flow rate of the blood, the shape of the velocity profile, and the shear stress at the wall of the vessel. This information

has clinical implications, as the microcirculation is the site for nutrient exchange in the body, and this exchange is shear-dependent. There are several current review studies on the state of research in the microcirculation as well [13,14,15].

Presented here is a protocol for µPIV measurements of blood flows in polydimethylsiloxane (PDMS) microchannels. PDMS channels were fabricated in-house following the sources in section 1 of the protocol. Porcine blood samples were obtained from an accredited slaughterhouse and cleaned following section 2 of the protocol. All data was obtained using the LaVision MITAS µPIV system, as described in section 3 of the protocol. The set-up consists of a Nd:YAG laser (New Wave Research, USA) and CCD camera (Image Intense, Lavision) controlled by a programmable triggering unit, a fluorescent microscope coupled with a stage moving in 3 axes, and a computer, in addition to a high speed camera (Dalsa 1M150, Netherlands) was added for visualization of the RBCs themselves. Both cameras are connected to a 2 port optic box (Custom by Zeiss, Germany). In typical *in vivo* measurements of blood flow, an upright microscope is used to track the RBCs themselves, while in typical *in vitro* applications an inverted microscope is used to track the tracer particles. In this unique dual set-up, the optics box allows both tracers to be imaged using the inverted microscope. Blood was introduced into the microchips via a high precision syringe pump (Nexus3000, Chemyx Inc., USA). A diagram of the system is shown in **Figure 1**, where the top portion of the figure represents the 140 µm by 40 µm rectangular channels fabricated of PDMS, and the bottom portion represents the entire system including both cameras, the laser, the syringe pump and the microscope.

Current µPIV set-ups available, usually with proprietary software, include TSI, Dantec Dynamics, and LaVision. Standard cross-correlation algorithms can be achieved through numerous software options, including the MATLAB. The software is not the key, understanding what the dialogue boxes correspond to mathematically will serve the user much better. In this protocol DaVis, LaVision's proprietary software or MATLAB are utilized. The protocol is not software specific, but the menu options might be in different places in different software packages.

Protocol

1. Microchip Fabrication

The first step is to create or purchase your microchannel. There are many options for microchip material.

One of the most common materials chosen is poly(dimethylsiloxane) (PDMS). There are many publications on directions for PDMS fabrication through soft lithography [16,17,18].

Once the PDMS channel is fabricated, there are several surface treatments available to reverse its natural hydrophobicity. Oxygenated plasma treatment is a common option.

Zhou, Ellis, and Voelcker (2009) give a review of surface treatments and how they affect PDMS. These results are for water however, and blood has different properties. A study on what that surface treatment means for blood has been done by Pitts *et al.* (2012a).

For the results presented here, microchip surfaces and the glass slides they were bonded to were both exposed to oxygenated plasma for 45 seconds and then pressed together firmly resulting in a permanent bond.

2. Blood Preparation

1. Collect blood samples from an accredited facility. For example porcine blood can be used with ethylenediaminetetraacetic acid (EDTA) as an anticoagulant. Add 1 g EDTA with 4 ml of water, and then add the solution to 1 L of whole blood, mixing gently 10x.

When picking up blood samples, allow them to cool slowly to RT. Blood samples should be fresh, and ideally used the day they are collected to conserve the rheological properties of the blood.

Samples may be refrigerated once at RT; however whole blood without anticoagulant will be unusable after refrigeration. Depending on the anticoagulant, RBCs can be kept refrigerated for up to 42 days, and whole blood can be kept refrigerated for 35 days, but contamination would be possible in a non-medical facility [21].

Additionally, be careful of the collection method with bovine or porcine samples, and avoid contamination by bone marrow.

2. Centrifuge the blood samples 3x at 3,000 rpm for 10 min each time. After the first centrifugation, remove the plasma and buffy coat, discard. It is generally easiest to remove the plasma first, and then discard or keep for future use, then to remove the buffy coat alone.

Introduce the pipette slowly, so as to not mix the buffy coat back into the blood. After removing the buffy coat, add about 20 ml of phosphate buffered saline (PBS) and mix gently, recentrifuge. Repeat last step. After the third centrifugation, remove the PBS and remaining buffy coat, discard.

It is also possible to use the native plasma instead of PBS if you wish to keep the aggregating properties of the blood. Make sure to not mix any buffy coat or white blood cells into the plasma.

3. Take cleaned red blood cells (RBCs) and suspend in PBS or plasma at desired concentrations (hematocrits). Check hematocrits with a microcentrifuge (CritSpin FisherSci, USA).

Blood at 10 to 20% hematocrit should be easier to visualize than 40 or 50% (physiological hematocrit). In the microcirculation, the blood is generally half the hematocrit of the macro circulation, so H=20 is adequate [15].

4. Add fluorescing tracer particles at the desired concentration to the blood samples. A general rule is to have 10 particles in a correlation window, which can be calculated ahead of time.

For example, 30 μl of particles are added to 1 ml of blood solution for the set-up depicted in the video. Alternatively, the RBCs themselves could be fluorescently tagged.

5. Additionally, make a calibration solution with water and fluorescing particles. It will be necessary to calibrate the system with a Newtonian solution.

For example, when using a 10X objective with a channel on the scale of 100 μm, a suitable concentration is 300 μl of particles mixed with 15 ml of distilled water. Another option is to use glycerol diluted with distilled water to a viscosity of 3cP, which is closer to blood's macro viscosity.

3. μPIV Measurements

1. Laser safety: Check temperature and humidity. Wear appropriate laser safety glasses. Close the laser curtain or turn on the laser sign on the lab door on to prevent people not protected from being exposed.
2. Procedure with Davis software is shown in the video. For more details refer to the user manual of a specific system.
3. Before taking data, the camera scale needs to be calibrated using a micrometer.
4. To reduce the compliance of the system, use the shortest amount of tubing and the smallest rigid syringe possible, such as a 15 or 50 μl Hamilton Gastight syringe. To reduce leakage of blood or air intake into the system, seal the tubing to the syringe and to the chip. This can be done with glue, PDMS or Vaseline (being careful not to contaminate the blood).

To fill the micro-syringe with water laced with tracer particles use backflow method because the volume of the micro-syringe is usually much smaller than the volume to fill.

Our backflow method consists to fill the micro-syringe and channel together via the output of the channel using a secondary plastic syringe. For that, first remove the micro-syringe plunger, then push the liquid using the classical plastic syringe attached to the outlet of the channel, let the liquid flow out the micro-syringe until all microbubbles are out of the micro -syringe, tubing or chip, finally put the plunger back, and unplug the plastic syringe. The presence or absence of microbubbles can be verified with a microscope.

5. Level the syringe pump to achieve horizontal tubing. Program the syringe pump to desired flow rate.

Be aware of the possible transitory effects, like the "bottleneck effect", for rigid system or the compliance of the system that modify the actual flow rate. Characteristic times of a system can be estimated as a function of the materials and dimensions of the system. (Chapter 2, pp. 77-81, Tabeling, 2005)

6. Place microchip on microscope stage and start the pump. Use the water with particles to calibrate the system to the middle velocity profile and calibrate the dT (the time between pulsed images).

The particles should move between 5 and 10 pixels between frames for a good correlation [11]. When calibrating, be careful to measure in the middle of the channel. The focus plane in the middle has the highest velocity [8].

If using a round channel, be careful of shadowing effects.

A sample image is shown in **Figure 2**, using a pulsed camera, whereas the top image is the first pulse and the bottom image is the second pulse. It can be seen in the figure that the brightest points (fluorescing particles) are the most in-focus.

7. OPTIONAL: By taking data at different heights a 3D velocity profile can be reconstructed by adding the different vectors into a single image. The video presents a routine that was developed for the automation of the process.
8. When taking data with the blood, fill syringe with desired quantity of blood in the same method as the water. Program the syringe pump to the desired flow rate, and take desired data. An example of raw images obtained is presented **Figure 2**. Be careful of RBC sedimentation as well. Refilling the syringe every run or every other run will reduce the settling of the RBC.
9. After acquiring images, cross correlation is performed on the image pairs to obtain velocity vector fields between images. It is important to have good images to have a good correlation. Before correlating, pre-processing can be done to remove background noise.

Cross correlation is done between windows in the adjacent images, which can be varied in size and shape to affect the correlation. After cross correlation, image post processing can be done to remove erroneous vectors or outliers.

A detailed description of the different available options and their accuracies can be found in Pitts *et al.* (2012b), where the best processing for blood was found to be the method of "image overlapping" with windows extended in length and aligned with the flow. These operations can be done manually in a program like MATLAB, or within the software package with your system. The software is not important, the math behind the operations is more important and each operation can affect the accuracy of the final velocity profile.

The resulting vectors can be averaged in space across the channel to obtain instantaneous velocity profiles, and then averaged again in time to obtain an average, representative velocity profile. Kloosterman *et al.* (2011) give an explanation of the error in μPIV measurements where the depth of field is large. Discussions on the error of the data processing presented here can be found in Pitts *et al.* (2012b) for the pulsed measurements and Chayer *et al.* (2012) for the high speed measurements.

Examples of velocity profiles are presented in **Figures 3** and **4**. **Figure 3** presents a single velocity profile for the centerline of a 10 μl/hr flow of RBC at H=10 in a 140 μm wide channel.

This single profile is achieved by averaging the correlated vectors across the channel to get a velocity profile, and then averaged in time to get a representative measurement. In this case, 100 pairs were averaged in time across the field of view.

An example of the 3D reconstructed profile taken for a water calibration flow in a 100 µm square channel with a programmed flow rate of 30 µl/hr is found in **Figure 4**. In **Figure 4**, the profiles are measured at 1 µm intervals.

10. OPTIONAL: In the depicted set-up high speed images can be taken at the same conditions by switching cameras (and data acquisition systems). This can be useful for visualizing the cell-free layer in the channel, and for quantifying aggregation. The data analysis is slightly different (Chayer *et al.*, 2012).

Examples of white light images with and without RBC aggregation are presented in **Figure 5** at H=20. The top images shows H=20 in PBS, which removes the ability of the RBC to aggregate, at 1 µl/hr. The bottom image is H=20 in native plasma at 0.5 µl/hr. In the bottom image, aggregation is visible.

11. Shut down the system in a safe manner when measurements have been completed. Use proper laser safety procedures until laser power source is completely shut down.

Representative Results

In all figures, flow is left to right in raw images, and upwards in calculated velocity profiles. An example of the raw data obtained with blood at hematocrit H=10 flowing at 10 µl/hr is shown in **Figure 2**. Raw data may be cross-correlated without any data processing to achieve velocity profiles. The impact of pre-processing and data processing methods is discussed by Pitts, *et al.,* (2012b). An example of a resultant velocity profiles from data similar to **Figure 2** at hematocrit H=10 and a flow rate of 10 µl/hr is shown in **Figure 3**, a 3D profile from water calibration is shown **Figure 4**. **Figure 3** and **4** included standard data processing. These velocity profiles can be used to make measurements such as the maximum velocity at the center of the channel, the flow rate in the channel, and the shear rate at the wall for various flow conditions, channel configurations, and physiologies. **Figure 5** gives an example of a high speed image with and without aggregation of the RBC. High speed images such as those in **Figure 5** can also be used to calculate velocity profiles using cross correlation.
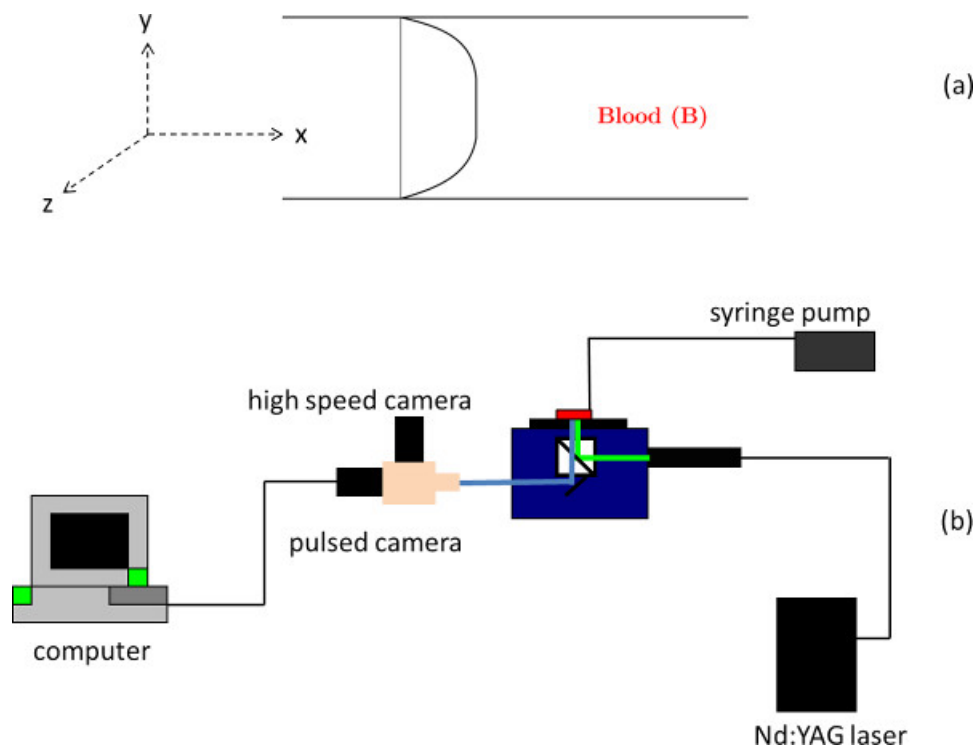

Figure 1. Diagram of the µPIV set-up where (a) represents the 140 µm by 40 µm rectangular channels fabricated of PDMS with flow moving left to right and (b) represents the entire system.
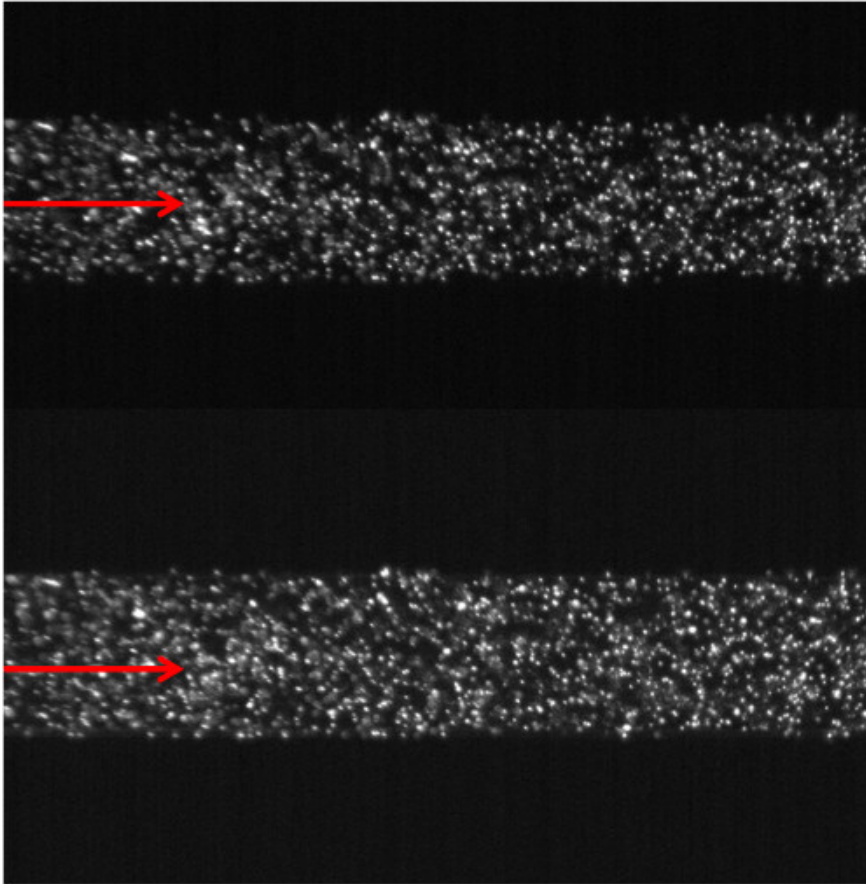
Figure 2. Resulting raw pulsed image data from the µPIV system with H=10 in PBS at a programmed flow rate of 10 µl/hr (flowing left to right). Top image is first pulse.

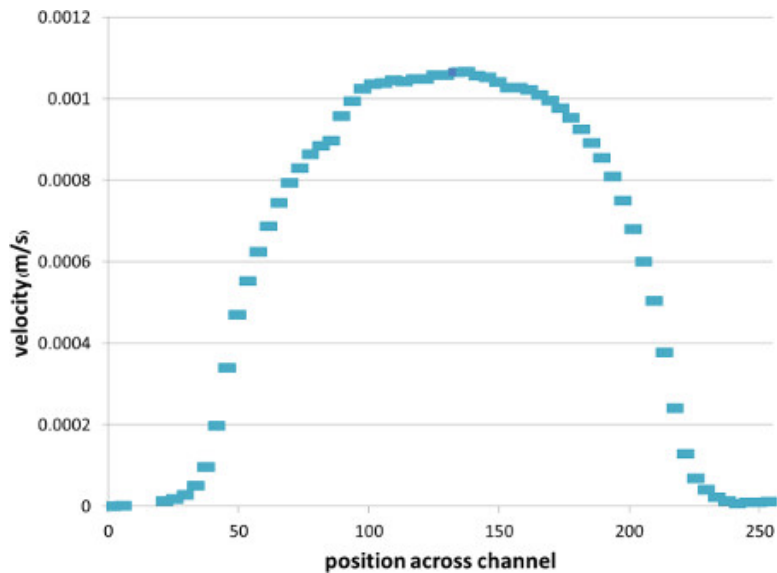**Figure 3. Resulting velocity profile from the µPIV system with H=10 in PBS at a programmed flow rate of 10 µl/hr.** Profile is rotated to show flow upward.
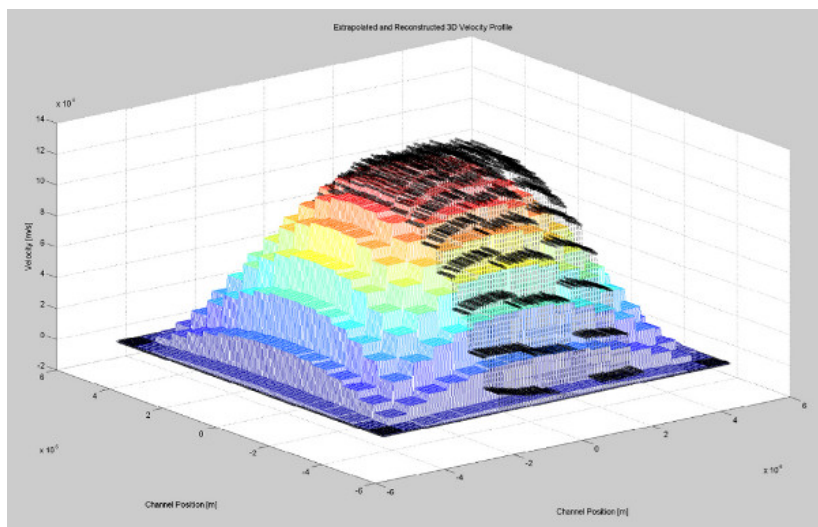
Figure 4. Example of a reconstructed 3D velocity profile of water calibration in a 100 μm square channel with multiple profiles taken 1 μm apart. Profile is rotated to show flow upward. Click here to view larger figure.
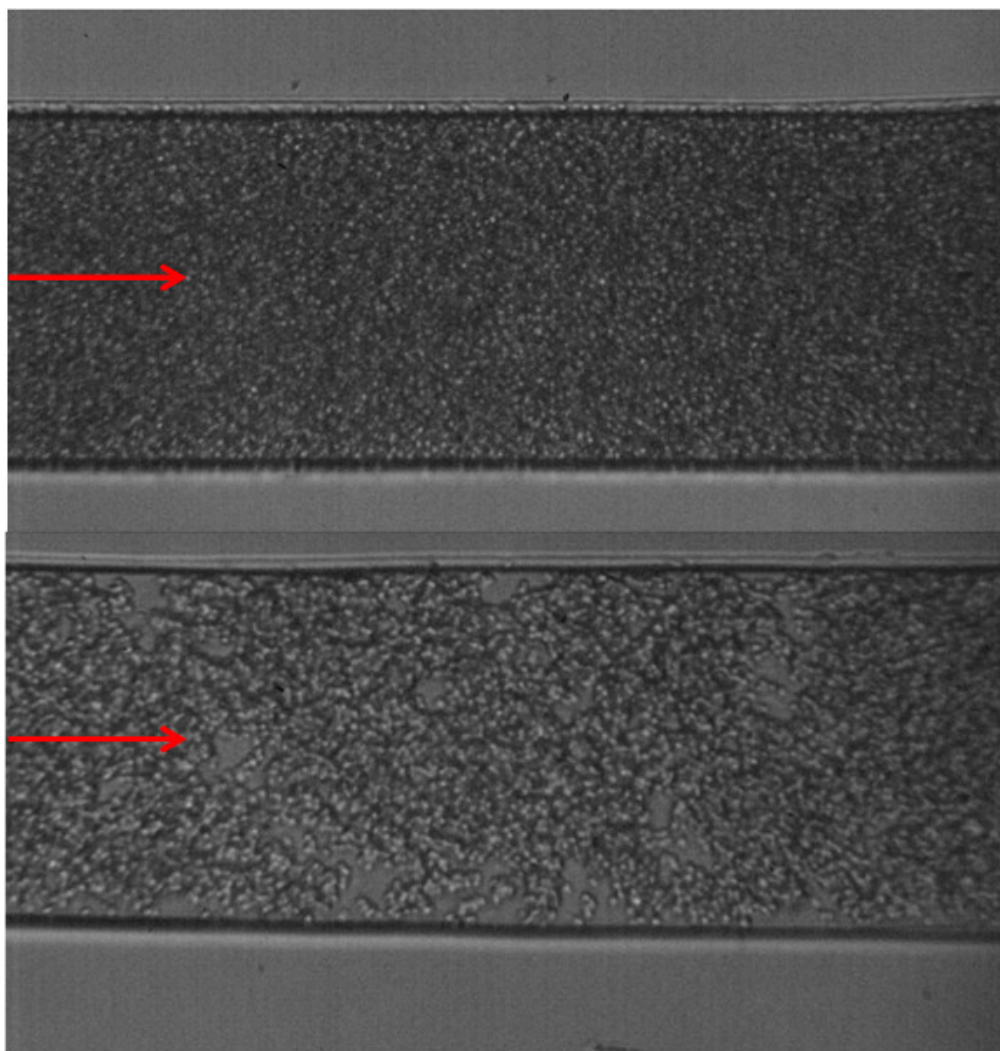

Figure 5. Example of high speed images where Top) is H=20 in PBS at 1 μl/hr (no aggregation) and Bottom) is H=20 in native plasma at 0.5 μl/hr (aggregation). In both images flow is left to right.

Discussion

Using µPIV for blood flow measurements at the scale of the microcirculation can give insight into a great number of relevant biomedical, mechanical and chemical engineering processes. Some of the key factors to account for are the density of the RBC themselves, the aggregation and deformability of the RBC, aggregation or movement of the fluorescing micro particles, and the settling of the RBC in the channels. All of these can be accounted for if the general guidelines laid out above are followed. There is a basic checklist to keep in mind for good measurements using this system. First of all, determine how much compliance is in the system or proposed system. To minimize the compliance use the shortest amount of tubing possible and the smallest syringe possible. Rigid systems have less compliance, but can lead to bottlenecking. Despite the small scale of the measurements, gravity will affect the RBC. Keeping the syringe, tubing and channel at the same height is ideal. Secondly, determine the concentrations of the suspensions. Ensure there are enough fluorescing tracer particles in the solution and not too high of a concentration of RBC. Increasing the density of tracer particles of a sample will not overcome the inherent opacity of the RBCs. The amount of tracer particles will affect the correlation, but having too many RBCs will make the fluid opaque and the tracer particles difficult to image. The overall hematocrit of the sample must be kept low enough that the middle plane of the channel can be imaged clearly. This is a trade off between hematocrit and channel size. At an increased depth, the amount of RBC between the lens and the plane of measurement grows, even at low hematocrit. In order to image higher hematorcrit samples or larger channels, the "ghost cells" method can be utilized whereas the opaque interior of RBC is replaced with clear fluid for a portion of the RBC (Goldsmith and Skalak, 1975). Thirdly, ensure that the microscope is focusing on the middle plane of the channel. Knowing the exact height of the channel is vital to finding the middle plane. Keep in mind the size of the particles and the size of the channel, and calculate the depth of correlation (DOC) in your system (Wereley *et al.*, 1998). The DOC can greatly affect the accuracy necessary to achieve in finding the middle plane. A difference of one micron can adversely affect the accuracy. Once the amount of particles, the size of the channel, and the DOC are found, the difference in time between pulsed images (dT) needs to be determined. Ensure the 5 to 10 pixels of movement of the particles between windows. The movement of the particles must fit into the correlation windows outlined in section 3.9. Finally, decide on the method of pre-processing, processing or post-processing of vector data. Many methods are available in literature, but some are more successful than others when applied to blood. The method of "image overlapping" is advised for blood flows (Pitts *et al.*, 2012b).

Another factor to keep in mind is the "cell-free" layer, which is well documented in microhemodynamics. This is the lack of RBCs at the wall of the channel or vessel. In following the fluorescing tracer particles, the user is actually following the movement of the plasma. At the center of the channel or vessel, the velocity is at a maximum and both will be moving at the same speed. At the wall, the plasma will still have movement while there will be few or no RBCs present. At the scale presented here with 10X magnification, the cell-free layer is not measureable but at higher magnifications it must be accounted for. High speed video would allow for a measurement of the thickness of the cell-free layer.

Conclusions

µPIV has been used for hemorheology and biomedical studies since shortly after its development. When applied to blood flows at the scale of the microcirculation, the complex behavior of the blood must be accounted for. A protocol for µPIV measurements of blood flows in microchannels was presented here along with tips for dealing with the complex nature of blood. Several imaging options were outlined, including pulsed images, high speed images and a 3D option as well as data processing information. In using µPIV for blood microflow research, the effect of medications, diseases and therapeutic treatments on the shape of the velocity profile can be analyzed. These measurements useful to modern medicine and engineering since the uptake of nutrients and medications is shear dependent.

Disclosures

The authors have nothing to disclose.

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