Quantitative microfluidic fluorescence microscopy to study vaso-occlusion in sickle cell disease

Vaso-occlusive crisis is the primary reason for emergency medical care sought by Sickle Cell Disease (SCD) patients.[1] *In vivo* imaging in transgenic SCD mice has identified molecular events that may promote vaso-occlusion.[2-4] However, the relevance of these mechanisms is not completely understood in humans. As non-invasive *in vivo* imaging in humans is limited by low-resolution,[5-7] there is a need for *in vitro* approaches[8] that can allow visualization of single cell events in flowing human blood. Here, we introduce quantitative microfluidic fluorescence microscopy (qMFM) that enables visualization of cellular interactions in human blood flowing through silicone microfluidic channels. qMFM reproduces the leukocyte-endothelium adhesion cascade, starting from rolling, transition to arrest followed by crawling and platelet capture by crawling leukocytes in human blood. Remarkably, qMFM reveals that leukocyte rolling and arrest is several fold higher in SCD than in control human blood. qMFM also provides the first evidence to support the presence of slings in rolling and arresting human neutrophils. qMFM allows visualization of platelet-neutrophil interactions at single cell resolution and enables a numerical read-out of the vaso-occlusive events in the form of frequency and lifetime of interactions. This quantitative assessment renders qMFM a unique platform to study the molecular mechanism of vaso-occlusion and test the efficacy of anti-adhesion drugs in preventing vaso-occlusion.

SCD is an autosomal recessive genetic disorder that affects an estimated 100,000 Americans, and millions of people across the world.[1] Sickle Cell Anemia (SS), the most common form of SCD, leads to sickling of red blood cells (RBCs).[2] It is believed that sickle RBCs get trapped in blood vessels along with leukocytes and platelets to cause 'vaso-occlusion'.[2] Neutrophils are the most abundant leukocytes in human blood and their adhesion to the endothelium starts with rolling mediated by P-selectin on the endothelium binding to P-selectin glycoprotein ligand-1 (PSGL-1) on neutrophils.[9] Interleukin-8 (IL-8) on endothelium binds to CXCR2 on rolling neutrophils to activate β2-integrins CD11b-CD18 (Mac-1) and CD11a-CD18 (LFA-1) on neutrophils, which then bind to inter-cellular adhesion molecule-1 (ICAM-1) on endothelium to enable arrest.[9] Several studies have used polydimethylsiloxane (PDMS; Silicone) based microfluidic assays to extract invaluable insight into the mechanism of vaso-occlusion. However, these approaches were limited by the use of isolated SS-RBCs[10] or the inability to visualize cellular interactions at single cell resolution[11] and distinguish different cell types that constitute the vaso-occlusive plug.[8] We introduce qMFM (Figure 1), which enables visualization of molecular interactions between neutrophils and platelets at single cell resolution in SS blood. The methods used are described in detail in the *Online Supplementary Information*. A silicone chip with micro-channels engraved on its surface was gently placed on a glass coverslip (Figure 1A) either coated with a cocktail of P-selectin, ICAM-1, and IL-8 (*Online Supplementary Figure S1A*) or cultured with TNF-α treated human coronary artery endothelial cells (HCAECs) or human lung micro-vascular endothelial cells (HMVECs-L) and vacuum-sealed (*Online Supplementary Figure S1B*). The assembled device had an inlet, an outlet and four identical perfusion chambers (30 μm high and 500 μm wide). Alexa Fluor 647 conjugated anti-human CD16 and FITC-conjugated anti-human CD49b Abs to stain neutrophils and platelets, respectively, were added to the blood in the inlet reservoir. Finally, the microfluidic device was placed on the stage of the inverted microscope and the blood was perfused through the perfusion chambers at a wall shear stress of 6 dyn cm^{-2}. Rolling, arrest and crawling of fluorescent neutrophils in human blood was visualized in

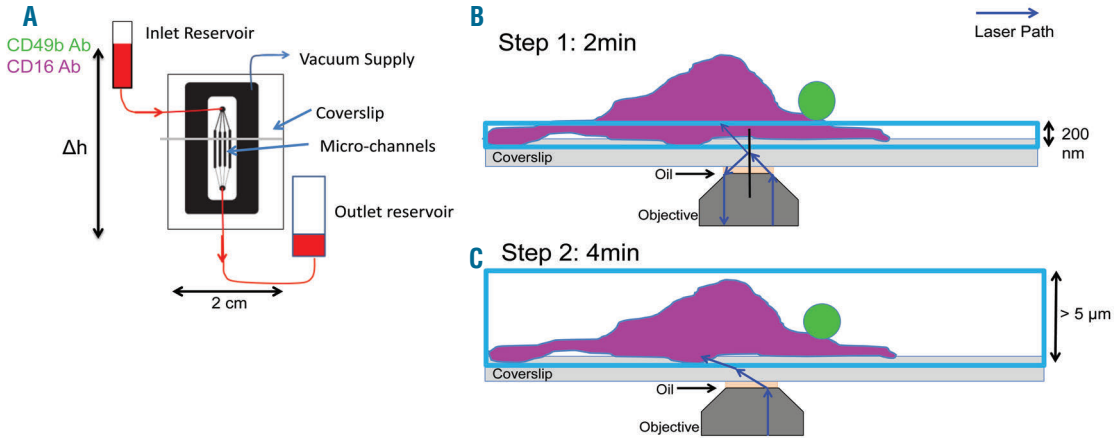

Figure 1. The working principle of qMFM. (A) Schematic of custom PDMS vacuum sealed microfluidic device. Δh is the difference in height between the inlet and the outlet reservoir. FITC-conjugated anti-human CD49b and Alexa Fluor 647 conjugated anti-human CD16 Abs were added to the blood in the inlet reservoir. The microfluidic device has four identical micro-channels. (B) Step 1 of the imaging technique uses qDF which requires that the laser is incident at the glass-cell interface at an angle greater than the critical angle (θ_c), resulting in total internal reflection and generation of a 200 nm evanescent wave (light blue box) which allows visualization of only the footprint of the neutrophil. (C) Step 2 of the imaging technique uses a laser incidence angle smaller than θ_c, which increases the illumination zone (light blue box) to greater than 5 μm allowing visualization of the platelets nucleating on top of neutrophils. Neutrophil (violet) and platelet (green) in B-C.

the perfusion chambers using quantitative dynamic footprinting (qDF).[12] In qDF, a laser is incident at the glass-cell interface at an angle greater than the critical angle. The laser is reflected back into the objective and an evanescent wave (Figure 1B - light blue box) is established on the cell side of the cover slip. The intensity of the evanescent wave becomes negligible within or greater than 200 nm above the cover slip (Figure 1B - light blue box). As a result, fluorescence is excited only in the cell membrane and cytosolic region that lies within 200 nm above the cover slip, while the remainder of the cell remains invisible. In order to observe platelets interacting with adhered neutrophils, the angle of the laser was reduced during imaging to increase the illumination zone from 200 nm to greater than 5 μm (Figure 1C - light blue box). Refer to *Online Supplementary Information* for details.

Neutrophil rolling has been shown to be facilitated by 'slings', which are long membrane cell-autonomous structures extended at the front of rolling neutrophils.[13] Although slings have been shown to exist on mouse neutrophils, the evidence to support their presence on human neutrophils does not exist. When SS or control blood was perfused through P-selectin coated microfluidic channels, the majority of neutrophils were rolling (*Online Supplementary Figure S2A,B; Online Supplementary Movie S1*) and formed slings (*Online Supplementary Figure S2C; Online Supplementary Movie S2*). This P-selectin dependent rolling was completely abolished by a function blocking antibody (Ab) against P-selectin or PSGL-1, thus confirming the specificity of the molecular interactions. When SS or control blood was perfused through micro-channels coated with a cocktail of P-selectin, ICAM-1 and IL-8, neutrophils were observed to roll and then quickly arrest (*Online Supplementary Figure S2D,E; Online Supplementary Movie S3*). As shown in the *Online Supplementary Movie S3*, arrested neutrophils were observed to spread over time and crawl, which is similar to observations made in mice vasculature *in vivo*.[14] Slings were also observed to exist on arrested neutrophils in SS (*Online Supplementary Figure S2E*) as well as control blood (*Online Supplementary Figure S2F; Online Supplementary Movie S4*). Neutrophil arrest in control blood was completely abolished by a function blocking Ab against Mac-1, and partially by a function blocking Ab against LFA-1 (*Online Supplementary Figure S3*), suggesting that Mac-1 is the predominant β2-integrin mediating human neutrophil arrest. We found that the number of neutrophils that rolled in P-selectin coated micro-channels was four-fold higher in SS than control blood (Figure 2A,B). Similarly, the number of neutrophils that arrested in P-

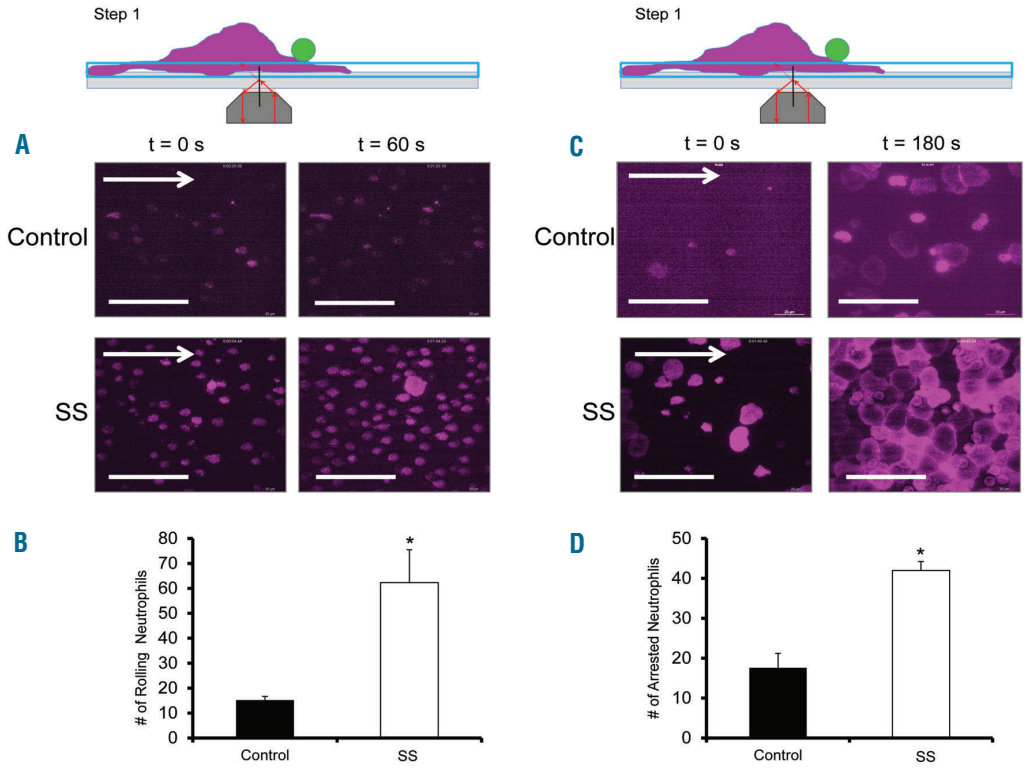

Figure 2. More neutrophils roll and arrest in SS than in control blood. SS or control blood was perfused through microfluidic channels coated with P-selectin (A-B) or a cocktail of P-selectin, ICAM-1 and IL-8 (C-D). Footprints were recorded using step 1 imaging. (A) Snapshots of the same FOV at t = 0 and 60 s showing the accumulation of rolling neutrophils in control (top row) or SS blood (bottom row) over 60 s. (B) Number of neutrophils that were observed to roll in a FOV~14,520 μm² during a 60 s observation period in control or SS blood. (C) Snapshots of the same FOV at t = 0 and 180 s showing the accumulation of arrested neutrophils in control (top row) or SS blood (bottom row) over 180 s. (D) Number of neutrophils that were observed to arrest in a FOV~14,520 μm² during a 180 s observation period in control or SS blood. *P<0.05 compared to control blood. Means based on 7 different FOVs from 3 SS or 3 control subjects and compared using Student's t-test. Error bars are SD. The schematic on top of each panel denotes the imaging strategy (step 1) used for visualization. Horizontal arrows denote direction of blood flow. Wall shear stress 6 dyn cm⁻². Scale bars 50 μm. Neutrophils (violet; AF647-anti-CD16 Ab). Excitation laser 640 nm.

selectin, ICAM-1 and IL-8 coated micro-channels was two-fold to three-fold higher in SS than control blood (Figure 2C,D).

The capture of activated platelets by adherent neutrophils is believed to play a role in the onset of vaso-occlusion[3,4] in the venules of SCD mice. Using the two-step imaging strategy shown in Figure 1B,C, neutrophils were observed to arrest and then crawl in P-selectin, ICAM-1 and IL-8 coated microfluidic channels (Figure 3A) which enabled nucleation of platelets on top of crawling neutrophils (Figure 3B). The crawling of neutrophils followed by nucleation of platelets, shown sequentially in the *Online Supplementary Movie S5*, is similar to observations reported in mice *in vivo*.[3,4,14] We observed that platelet nucleation on arrested neutrophils in SS blood led to the formation of aggregates which partially occluded the microfluidic channels (*Online Supplementary Figure S4*). As shown previously in SCD mice *in vivo*,[3] RBCs were found to be trapped in these aggregates (*Online Supplementary Figure S4D-F*). qMFM allowed visualization of platelet-neutrophil interaction at single cell resolution (Figure 3C). The time-series of qMFM images were analyzed using the spot detection algorithm (NIS-Elements; NIKON) to quantify the total number and lifetime of platelet-neutrophil interactions (Figure 3D). This methodology was used to evaluate the effect of the choice of anticoagulant on the platelet-neutrophil interaction in control blood. We observed that the number (Figure 3E) and the lifetime (Figure 3F) of platelet-neutrophil interactions were comparable in heparin or hirudin anticoagulated control blood. Thus platelet-neutrophil interactions were independent of the choice of anticoagulant. The *in vitro* microfluidic approach also allows fixation of interacting cells under flow followed by scanning electron microscopy. Figure 3G shows a scanning electron micrograph of a platelet interacting with an arrested neutrophil in control blood.

In order to establish that qMFM serves to visualize cellular interactions on cultured endothelium, blood from SS or control subjects was perfused through microfluidic micro-channels cultured with TNF-α activated HMVECs-L or HCAECs and cellular interactions were recorded using step 2 of the imaging technique (Figure 1C). In some experiments (*Online Supplementary Figure S5A-C*), cultured HMVECs-L were stained with a PE-conjugated Ab against endothelial PECAM-1 to visualize the endothelial cell borders. As shown in the *Online Supplementary Figure S5A-C* and the *Online Supplementary Movie S6*, neutrophils (violet) in control blood were observed to roll and arrest on activated HMVECs-L (green). Neutrophils in SS or control blood were also observed to roll (*Online Supplementary Figure S5D*), arrest (*Online Supplementary Figure S5E*) and then capture freely flowing platelets on activated HCAECs (*Online Supplementary Figure S5F*). The majority of neutrophils rolling on activated HCAECs were observed to form slings (*Online Supplementary Figure S5D*). As shown in the *Online Supplementary Figure S5G* and the *Online Supplementary Movie S7*, neutrophils following arrest were also observed to crawl on activated HCAECs.

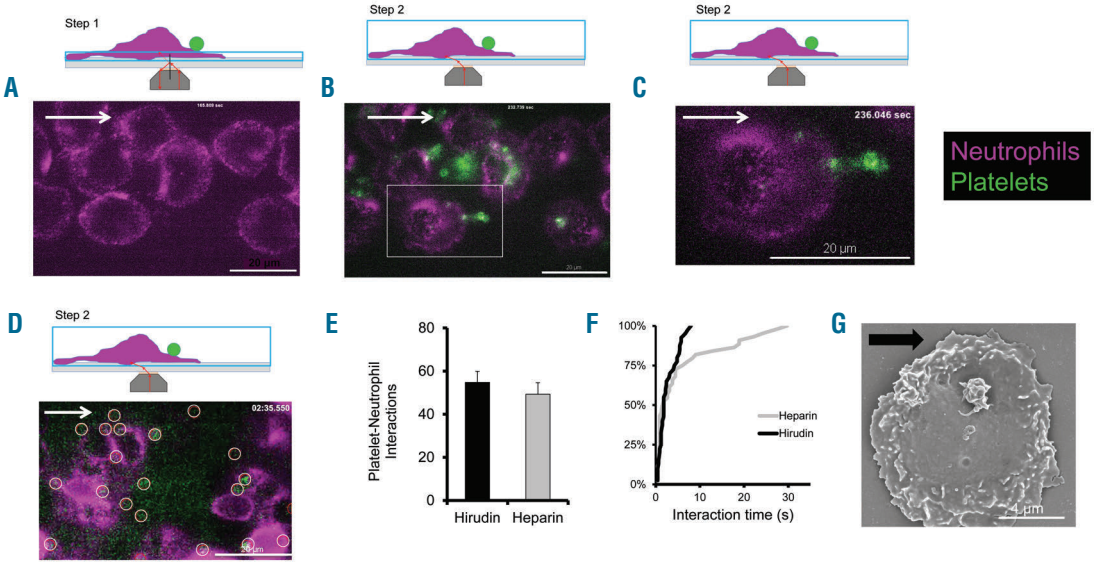

Figure 3. qMFM provides the choice to visualize neutrophil footprints or platelet-neutrophil interactions. (A) Imaging step 1 (schematic shown on top) visualizes footprints of neutrophils arrested on P-selectin, ICAM-1, and IL-8 coated substrate in control human blood. (B) The laser incidence angle was decreased to allow transition from imaging step 1 (shown in A) to step 2 (schematic shown on top) which enabled visualization of platelet-neutrophil interactions on P-selectin, ICAM-1 and IL-8 coated substrate in control human blood. The transition from A to B is shown in the *Online Supplementary Movie S5*. (C) The region marked by a dashed box in B is magnified to reveal platelet-neutrophil interaction at single cell resolution. (D) White circles mark the platelets interacting with arrested neutrophils in SS blood. Spot detection algorithm in NIS-Elements suite was used to detect platelets. (E) Comparison of total platelet-neutrophil interactions in hirudin *vs.* heparin anticoagulated control human blood. Error bars are SE. Student's *t*-test ($P>0.05$). (F) Cumulative probability distribution of the lifetime of platelet-neutrophil interactions in hirudin (n = 41 cells) *vs.* heparin (n = 44 cells) anticoagulated control human blood; n = 4 FOV, number of control human subjects = 2; distributions not significant based on non-parametric Kruskal-Wallis H-test. (G) Scanning electron micrograph showing a platelet attached to an arrested neutrophil. Control blood was fixed under flow. Wall shear stress = 6 dyn cm^2. Scale bars 20 μm. Horizontal arrows denote blood flow direction. The schematic shown on top of panels A-D denotes the imaging strategy used for visualization. Neutrophil (violet; AF647-anti-CD16 Ab), platelet (green; FITC-conjugated anti-human CD49bAb). Excitation lasers 488 nm and 640 nm.

Neutrophil rolling and arrest on activated HCAECs in SS blood was not affected by blocking E-selectin, but was completely abolished by simultaneous blocking of P-selectin on HCAECs and Mac-1 on neutrophils (*Online Supplementary Figure S5H*). Thus, neutrophil rolling on activated HCAECs is primarily mediated by P-selectin.

In conclusion, qMFM serves as an *in vitro* imaging platform that can be used to elucidate the cellular, molecular and biophysical mechanisms of single cell adhesive events that potentiate vaso-occlusion in SS blood. In addition, blood and endothelial cells[15] isolated from the same SS patient can be used in qMFM to evaluate the efficacy of a drug or treatment for individual patients.

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