

# Quantifying embolism: label free volumetric mapping of thrombus structure and kinesis in a microfluidic system with optical holography

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## Key points

1. Label- free imaging of a developing thrombus
2. Holographic QPM Imaging for real time volume quantification
3. Growth, contraction and stability of a thrombus formed on a microfluidic chip.
4. In vitro studies of embolization

1 **Abstract**

2  
3 Embolization of thrombotic material may lead to acute events such as ischaemia and myocardial  
4 infarction. The embolus is the physical detachment from a primary thrombus that has developed under  
5 fluid shear rates. The physical characteristics (surface area coverage, volume, mass and packing density)  
6 of a thrombus influence the overall flow dynamics of an occluding blood vessel. Here, we demonstrate  
7 the effectiveness of holographic quantitative phase microscopy (QPM) in identifying multiple  
8 morphological parameters of a thrombus (volume, surface area and height) formed over collagen-coated  
9 microfluidic channels by exerting a range of shear rates with anticoagulated platelet-rich plasma (PRP)  
10 and whole blood. QPM enables the recording of entire thrombus volumes in real-time using PRP and  
11 observed both growth and contraction trends of thrombi, without need for biochemical labelling. We  
12 quantified the process of emboli detachment in a microfluidic channel under pathophysiological shear  
13 rates ( $7500\text{ s}^{-1}$ ,  $12500\text{ s}^{-1}$ ). Rapid and direct quantification of an embolizing thrombus can enable the  
14 study of events during undesirable vessel occlusion and lead to targeting and early diagnosis of acute  
15 coronary and venous events.

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# 1 **Introduction**

2 Platelets are specialized anucleate blood cells that are essential for maintaining hemostasis. Hemostasis,  
3 the process of maintaining a normal blood volume, which often involves the forming of a blood clot  
4 (thrombus). Thrombus formation is initiated by adhesion of circulating blood platelets onto exposed  
5 extracellular matrix proteins, including collagen and von Willebrand Factor (VWF), within a damaged  
6 vascular wall. Adhered platelets then release secondary agonists/mediators and promote thrombin  
7 generation, which helps recruit additional platelets to the injured site, cumulatively building a thrombus.  
8 Under pathophysiological conditions, this normally protective cascade of events can result in arterial  
9 thrombosis or deep vein thrombosis, which can be responsible for major clinical events such as heart  
10 attack and stroke.

11 Real time monitoring of thrombus formation under fluidic settings is vital for studying the  
12 thrombotic process and provides significant insight to studying dynamic platelet function <sup>[1, 2]</sup>. Studying  
13 the initial molecular events as a thrombus develops are crucial to understanding the involvement of key  
14 platelet receptors and can also help in assessing platelet dysfunction in patients with genetic defects <sup>[2]</sup>.  
15 Measuring the growth of a thrombus would be useful in identifying targets to reduce pathophysiological  
16 events such as arterial thrombosis. Finally, the ability to study thrombus stability at pathological shear  
17 rates will inform on molecular processes involved in embolization, and facilitate the development of  
18 therapeutic avenues to treat venous thrombosis.

19 A developing thrombus can be classified into a series of morphological transitions along a high  
20 fluid shear. The transition starts off with the initial activation of platelets through engagement of platelet  
21 signalling receptors which trigger activation of platelet integrin receptors and drive the aggregation of  
22 platelets that leads to thrombus growth before undergoing contraction <sup>[3, 4]</sup>. The contractile forces <sup>[5]</sup> from  
23 the platelet are in part due to the interaction with the surrounding fibrin network <sup>[6]</sup>. On top of that, the  
24 degree of activation of each platelet in the thrombus influences how the platelets engage each other; fully

1 activated platelets are held together within the thrombus by fibrinogen binding and bridging adjacent  
2 integrin  $\alpha$ IIb $\beta$ 3 receptors. Over time, adherent and fully activated platelets form the stable core of a  
3 thrombus, and newly-arrived, less-activated platelets are more loosely packed and held together forming  
4 a shell-like zone. The molecular composition of the thrombus core and shell affect the overall structural  
5 stability of the thrombus [7]. To understand the basic biological processes underpinning thrombus growth  
6 and regulation of that growth, it is important to visualize the morphological development of a thrombus  
7 in real time using high speed imaging instruments that directly capture multiple views of a single  
8 thrombus and quantitates parameters including volume, height and area, simultaneously and temporally.

9 Fluorescence intensity emitted from platelet-targeted fluorescent probes and surface markers are  
10 used to map out the overall architecture of thrombus [8, 9]. The two main fluorescence imaging methods  
11 are wide field fluorescence microscopy (WFM) and confocal laser scanning microscopy (CLSM) [10].  
12 In WFM, the changes of fluorescence intensity over a given area are used to estimate the volume of the  
13 thrombus. The advantage of WFM imaging is the speed of acquisition, which is limited by the frame rate  
14 of the imaging camera and also the brightness of the fluorescence signals. The fluorescence signal  
15 collected on a digital imaging camera is integrated from a single imaging focal depth of around 8  $\mu$ m  
16 (based on objective of magnification 10x and NA 0.25) [11-13]. This means that the capacity to measure  
17 the thickness of thrombus is restricted by the focal depth of the imaging lenses used. Therefore, if a  
18 thrombus is larger than the focal depth, the quantification would encounter inaccuracies in volume  
19 quantification [14, 15]. To circumvent this, most researchers have turned to more precise CLSM imaging  
20 that captures the whole thrombus through multiple two dimensional thin imaging slices recorded by raster  
21 scanning at a focused laser spot. This technique offers more accurate depth and thickness quantification  
22 along with area and density of a thrombus [2, 16, 17]. The full 3D images acquired from CLSM imaging  
23 requires repeated scanning of a laser beam across the sample through mechanical devices such as  
24 galvanometer mirrors [2, 16, 17] of around 2 Hz at full frame or by spinning disc [18] that can reach 1000 Hz.

1 The scanning process also exposes sample to higher light irradiation that can result in photobleaching <sup>[19]</sup>  
2 or photodamage <sup>[20]</sup>. In fluorescence imaging, there is an additional complication introduced by binding  
3 fluorescently-conjugated antibodies to the platelet surface to provide specificity. Recently, quantitative  
4 phase microscopy (QPM) techniques have provided new opportunities to measure thrombus formation  
5 *in vitro* <sup>[21]</sup>. QPM maps out the difference in refractive index instead of using endogenous fluorescence  
6 signal and records thickness of the sample directly. However, since refractive index differences in  
7 biological samples are non-specific, this quantification tool requires a careful choice of biological  
8 samples <sup>[22, 23]</sup>.

9 QPM is usually in two forms, non-holographic and holographic type. The non-holographic QPM  
10 <sup>[23]</sup> combines existing imaging optics of a WFM with numerical phase calculation and a prior knowledge  
11 to estimate the phase (refractive index difference) before it can generate a reconstructed volume of the  
12 sample. Although it has the same optical limitation as a conventional WFM, it provides volumetric  
13 quantification that is not available in conventional WFM, albeit at the low imaging speed. Holographic  
14 QPM <sup>[24-26]</sup>, on the other hand, directly records the phase of the object and uses a non-focussed laser light.  
15 This method is not limited by the focal depth of the imaging optics in WFM or a focus laser beam in  
16 CLSM. The recorded phase also removes undesired optical distortion arising from any artefact or  
17 deformation within the sample <sup>[27, 28]</sup> which is important especially when dealing with samples of high  
18 scattering signals such as blood samples. Recently, Boudejltia and colleagues <sup>[29-31]</sup> showed that QPM  
19 can quantify individual platelet adhesion and other groups <sup>[32]</sup> have also began to using non-holographic  
20 QPM techniques to quantify the mass of the final thrombus. All in all, the application of QPM techniques  
21 in platelet and thrombus studies is in their infancy because of the lack of physiological relevant outcomes.  
22 We argue that QPM is uniquely positioned to present comprehensive and direct multi-dimensional  
23 morphological information of a developing thrombus, which would not be straightforward in other  
24 imaging modalities.

1 Table 1 and Figure 1 present the three imaging modalities; WFM, CLSM and QPM that are  
2 typically used to quantify thrombus. Table 1 lists out the performances of the three modalities. Figure  
3 1a) illustrates the dynamic growth of a thrombus under flow at different time points ( $t_1$  to  $t_3$ ). Figure 1b)  
4 shows how WFM imaging captures the integrated fluorescence over a given thrombus. Figure 1c)  
5 illustrates the multiple thin image slices ( $Z_0$  to  $Z_n$ ) that depict recorded fluorescence intensities at each  
6 slice,  $I(z_i)$ , from which CLSM retrieves the thrombus volume. The repeated scanning of a highly focused  
7 laser beam causes an ineluctable lag time of round trip scanning which increases the overall acquisition  
8 time. Figure 1d) shows how QPM imaging methods directly measure the refractive index difference of  
9 the thrombus which is directly translated to integrated volume. Another advantage in QPM is that the  
10 volume does not depend on the brightness emitted from the sample or the dynamic range of the detector  
11 as compared to WFM.

12 By demonstrating that QPM can measure dynamic events and parameters such as volume, height  
13 and area simultaneously and temporally at the different stages of thrombus formation and at multiple  
14 viewing points, this will enable a high throughput morphological mapping of thrombus development and  
15 greatly accelerate the understanding of early stages of thrombus development in a flow assay. A well  
16 developed and robust imaging framework using holographic QPM techniques also lends itself to on-chip  
17 holographic imaging techniques that be directly applied to diagnosing disorders of platelet function <sup>[33,</sup>  
18 <sup>34]</sup>.

## 19 **Results**

20 We used platelet-rich plasma (PRP) in a microfluidic environment to obtain optical holograms of  
21 thrombus formation (in this context, platelet-platelet aggregates formed through platelet deposition on to  
22 collagen), which were digitally recorded on a high speed digital camera <sup>[35]</sup>. We used automated image  
23 processing <sup>[36]</sup> for rapid quantification of individual thrombi over a given field of view. Within a single  
24 acquisition, we directly generate multi-parameter datasets including thrombus volume, height and surface

1 area. Since optical holograms record all volumetric information in a single snapshot, there is no  
2 requirement to actively change the focus position of the imaging lenses. As the holograms are essentially  
3 2D images, it enables directly volumetric imaging and the speed is limited only by the camera frame rate  
4 (150 fps). Figure 2 shows the diagram of holographic QPM imaging and microfluidic setup. In Figure  
5 2a) i) and ii) shows the actual microfluidic chips and the corresponding cross-sectional sketch  
6 respectively. Figure 2a) iii) depicts the position of the imaging microscopy objective (MO) that is placed  
7 under the microfluidic chip to collect the exiting laser light through the sample. The exiting laser light is  
8 recombined with a reference laser light at a CCD camera —using a beam splitter. The interference pattern  
9 of the two laser light fields creates the optical hologram. A syringe pump was used in suction mode to  
10 draw the sample; either whole blood (with heparin (10 U/mL) or 3.2% tri-sodium citrate used as an  
11 anticoagulant) or platelet-rich plasma (PRP), collected from heparinized or citrated blood, across a  
12 collagen-coated microfluidic chip surface, minimizing unnecessary sample shear stress prior to entry into  
13 the microfluidic slide. The direction of flow is represented by the arrows (Figure 2a). Due to difference  
14 in flow margination between platelet rich plasma (PRP) and whole blood, different regions within the  
15 flow channel are more prone to aggregation. For example, aggregates from PRP formed close to edges  
16 of the channel whereas aggregates in whole blood tended to form in the middle of the channel. To ensure  
17 fair comparison, we selected thrombi that were formed within defined regions 100  $\mu\text{m}$  from the channel  
18 side walls. Additional information of the sample and microfluidic chip preparations is discussed in the  
19 experimental section.

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## 21 • **From growth to reduction**

22 During thrombus formation, the different levels of activation of the platelets contribute to the overall  
23 packing density which affects physical stability of the adhering thrombus. During the initial stage of  
24 thrombus formation, there is a steady increase in volume which is followed by an overall thrombus

1 consolidation/contraction [37]. During physiological thrombus formation where fibrin is generated, the  
2 thrombus contraction is mediated in part by activated platelets pulling on fibrin networks to form a  
3 closely packed core [38]. The morphological change in the thrombus is orchestrated by mechanical and  
4 biochemical cues received by a formed thrombus. It has been showed that rate of diffusion of agonist  
5 within the thrombus is regulated by how platelets are packed [39]. The anticoagulated blood used in this  
6 work to generate thrombi refers to the quantification of platelet deposition and platelet-platelet  
7 aggregation and that the process of fibrin generation was not studied.

8 To investigate the morphological changes in thrombus formation, we perfused PRP over collagen-coated  
9 microfluidic channels at three different flow rates that produce fluid shear rates that are physiological  
10 relevant to thrombus formation at 72  $\mu\text{L}/\text{min}$  ( $1800\text{ s}^{-1}$  shear rate;  $18\text{ dyne}/\text{cm}^2$  shear stress), 144  $\mu\text{L}/\text{min}$   
11 ( $3600\text{ s}^{-1}$  shear rate;  $36\text{ dyne}/\text{cm}^2$  shear stress) and 288  $\mu\text{L}/\text{min}$  ( $7200\text{ s}^{-1}$  shear rate;  $72\text{ dyne}/\text{cm}^2$  shear  
12 stress) for 10 min. To investigate the parameters of thrombi formed under flow of whole blood, we  
13 perfused heparinized whole blood over collagen-coated microfluidic channels for 10 min at two different  
14 flow rates, with equivalent shear stress to PRP samples, 32  $\mu\text{L}/\text{min}$  ( $800\text{ s}^{-1}$  shear rate;  $36\text{ dyne}/\text{cm}^2$  shear  
15 stress) and 72  $\mu\text{L}/\text{min}$  ( $1600\text{ s}^{-1}$  shear rate;  $72\text{ dyne}/\text{cm}^2$  shear stress). The detailed conversion of shear  
16 stress, shear rate and flow rate can be found in Experimental section. The field of view for capturing  
17 dynamic thrombus formation was fixed on sections close to the inlet within  $\sim 800 \pm 200\text{ }\mu\text{m}$  after the  
18 inlet, for all the measurements to ensure consistency and minimal exposure to turbulent flow. Volumetric  
19 information was later reconstructed through classical holographic numerical approaches (see  
20 Experimental section). Figure 2 b) i) shows the result of the volumetric changes of thrombus formed at  
21 three different fluidic shear rates using PRP collected from heparinized blood from a single donor (d1).  
22 The x-coordinate indicates time in second and y-coordinate is for the volume in cubic micrometre. The  
23 blue, yellow and red lines indicate the thrombus formed under the  $1800\text{ s}^{-1}$ ,  $3600\text{ s}^{-1}$  and  $7200\text{ s}^{-1}$  shear  
24 rates respectively. Detailed statistic methods can be found in Experimental section. Figure 2 b) ii) shows



1 the height map for each thrombus in Figure 2 b) i) at different time points. The scale bar is 30  $\mu\text{m}$  and  
2 the colour bar indicates the height in micrometres. The growth trends shown in Figure 2 b) present a  
3 linear growth followed by plateau, which is similar with previously reported growth trends [38] and mostly  
4 similar shape to growth trends of citrated PRP (Supplementary Figure A2) . To quantify the growth rate,  
5 two linear fits (R-square above 0.9) were applied to the growth and plateau segments respectively,  
6 marked by black lines and  $S_1$  and  $S_2$ .  $S_1$  represents the speed of the volume accumulation before the  
7 maximum point.  $S_2$  represents the speed volume reduction after the maximum point. The statistics  
8 average of growth rate  $S_1$  (the positive Y axis) and reduction rate  $S_2$  (the negative Y axis) from all donors  
9 are presented in Figure 2 c) ii). The influence of the shear rate appears to be more dominant at  $7200\text{ s}^{-1}$   
10 compared with shear rates of  $1800\text{ s}^{-1}$  and  $3600\text{ s}^{-1}$ . While higher shear rates seem to suggest a rapid  
11 ascent, the speed ( $S_2$ ) of the decline appears to be small ( $< 1200\text{ }\mu\text{m}^3/\text{min}$ ) for all the thrombi formed at  
12 three different shear rates, which is likely to be related to platelet contraction [39, 40] under flow. The  
13 quantitative observation of the events is made possible because of the robustness of holographic QPM  
14 sensitivity.

### 15 • **Characteristics of individual thrombus**

16 After the 10 min formation process, we changed the flowing PRP or whole blood sample to the buffer  
17 (physiological saline; 0.9% NaCl) at the same shear rate previously used to remove excess PRP or whole  
18 blood and then translated the imaging field of view throughout the whole channel to capture images of  
19 individual thrombi. Using holographic QPM, we retrieved thrombus volume, surface area and maximum  
20 height after numerical reconstruction (see Experimental section) as shown in Figure 3. Figure 3 a) shows  
21 a perspective view of a reconstructed thrombus formed by PRP and whole blood. Figure 3 b) and c) show  
22 the measured volumes, surface areas and peak heights of thrombi formed by PRP and whole blood  
23 respectively. Each data point in the figure represents individual thrombi formed at different fluid shear  
24 ( $1800\text{ s}^{-1}$ ,  $3600\text{ s}^{-1}$ ,  $7200\text{ s}^{-1}$ ). Differences were observed between thrombi generated with heparinized

1 PRP and citrated PRP. The thrombi volumes appeared to be greater for citrated PRP thrombi that could  
2 be attributed to the increased in height without a significant increase in area of under which platelet is  
3 adhering to. The distribution of the thrombus peak height is presented in the Supplementary material Fig.  
4 A1. Within the microfluidic system the thrombus height (heparin PRP and citrated PRP) ranged from  
5  $1.43 \mu\text{m}$  to  $34.37 \mu\text{m}$  with median of  $8.114 \mu\text{m} \pm \text{SD} = 4.933$ . We observed that the volume and area of  
6 the individual thrombi from all the donors displayed significant degrees of variation. Hence, it would be  
7 of great interest to couple signatures between molecular and morphological information from each  
8 thrombus in future studies. However, this would require the combination of fluorescence imaging with  
9 holographic QPM <sup>[41]</sup> to give further information. By using the abundant characteristics information of  
10 individual thrombus morphology, it would be very informative to test functionality of platelets by  
11 controlled variables such as surface density of platelet receptors which control onset of platelet adhesion  
12 and thrombus formation. The phase images retrieved from digital holographic microscopy (DHM)  
13 provide the morphological information that is usually examined in platelet study using WFM or CLSM  
14 at the same temporal resolution. In addition to that, the morphological information would help developed  
15 a better thrombotic numerical model <sup>[42]</sup>.

16

### 17 • **Quantifying embolism**

18 The spontaneous embolism of a thrombus especially in acute coronary events may be a natural protective  
19 mechanism against infarction in organs <sup>[43]</sup>. Past studies have focused on individual platelet behaviour  
20 and more recently, there has been new interest in investigating factors related to thrombus stability that  
21 are able to be correlated with acute coronary events. Here, we investigate overall thrombus stability by  
22 perfusing a buffer at a high shear rate over a formed thrombus for a certain period of time and monitored  
23 thrombus parameters. The stability test could provide a new perspective to investigate the inner core and  
24 the outer shell regions of thrombus <sup>[44]</sup>. Figure 4 a) illustrates the experiment undertaken where a

1 thrombus that was previously formed with shear rates of either  $1800\text{ s}^{-1}$ ,  $3600\text{ s}^{-1}$  and  $7200\text{ s}^{-1}$  with PRP  
2 or at  $800\text{ s}^{-1}$ ,  $1600\text{ s}^{-1}$  with whole blood is then exposed to an additional saline buffer at a shear rate of  
3  $7500\text{ s}^{-1}$  for 5 minutes and  $12500\text{ s}^{-1}$  for 2 minutes. To ensure a fair comparison of the volume, we selected  
4 aggregates with equivalent surface areas ( $1000 - 1800\text{ }\mu\text{m}^2$ ) and similar distance ( $\sim 800 \pm 200\text{ }\mu\text{m}$ ) from  
5 the channel inlet. Structural change during the flush could reflect the stability of the thrombus formed.  
6 Figure 4 shows the stability test results. Figure 4 b) i) shows an example of the change in volume for  
7 three individual thrombi (pre-formed by  $1800\text{ s}^{-1}$ ,  $3600\text{ s}^{-1}$  and  $7200\text{ s}^{-1}$  shear rate, heparin PRP) from  
8 donor 4 (d4) during the stability test. Figure 4 c) i) shows the change in volume for thrombus pre-formed  
9 by  $800\text{ s}^{-1}$ ,  $1600\text{ s}^{-1}$  shear rate (equivalent shear stress as medium and high PRP shear rates) with  
10 heparinized whole blood from donor 4 during the stability test. The corresponding volumetric images  
11 are displayed in Figure 4 b) ii) and 4 c) ii). Figure 4 b) iii), iv) and v) as well as Figure c) iii), iv) and v)  
12 show the volume, surface area and height before and after the test. Each dot (circle for a thrombus formed  
13 under  $1800\text{ s}^{-1}$ , triangle for  $3600\text{ s}^{-1}$ , square for  $7200\text{ s}^{-1}$ ) in the bar represents the average data (obtained  
14 from  $n > 8$  data points) from each donor (D2, D3, D4 for citrated PRP, four donors for heparin PRP (d1-  
15 4) and four donors for heparinized whole blood). The statistical test performed was a paired t-test and  
16 significance was observed when  $p < 0.05$ . There was decreased volume post-perfusion with citrate PRP at  
17  $3600\text{ s}^{-1}$  ( $*p < 0.05$ ), due to a reduction in thrombus height ( $*p < 0.05$ ; Figure 4b) iii) and v). Reduction in  
18 height was also observed at the same shear rate with heparin PRP, although there was no significant  
19 reduction in volume. The rapid decrease in volume with citrated PRP suggests an unstable outer  
20 thrombus layer that breaks apart with a residual core thrombus remaining in the same site. This leaves a  
21 thrombus of smaller volume and height. However, the surface area of the thrombus is maintained  
22 relatively, except for thrombi formed by heparin PRP at  $3600\text{ s}^{-1}$ . In addition, the thrombus formed at  
23 lower shear rates ( $1800\text{ s}^{-1}$  and  $3600\text{ s}^{-1}$ ) retains a volume, area and height that are smaller than the

1 thrombus formed at  $7200\text{ s}^{-1}$ . We showed that holographic QPM can rapidly track, capture and quantify  
2 the rich dynamics of a developing thrombus.

### 3 **Discussion**

4 We have shown that the application of holographic QPM provides multi-parameter classification of  
5 morphological information in real time. Since depth of focus of the imaging system of QPM is not  
6 restricted, QPM retrieves the entire morphological information set of a developing thrombus without any  
7 form of labelling. Without QPM, it would require considerable sample preparation and imaging time to  
8 retrieve the morphological information. **Furthermore, whole blood presents a challenge in optical**  
9 **imaging as red blood cells (RBC) scatter light in a highly directional and ballistic manner, Mie scattering**  
10 **[45]. By combining advanced digital signal processing techniques with digital optical holography this**  
11 **enables the removal of optical scattering and increase imaging resolution. With deterministic**  
12 **microfluidic parameters, the ballistic scattering property of RBC could be removed. We have previously**  
13 **shown that incremental thresholding allows one to retrieve images of an object embedded in a scattering**  
14 **medium [36]. Further, Ferraro and colleagues have demonstrated the use of multi-frame holographic**  
15 **imaging to retrieve images of objects from flowing blood that are “hidden” [46, 47]. However, imaging**  
16 **through turbid or scattered medium remains a major imaging challenge, with holographic microscopy**  
17 **being a key technique [48, 49]. Many more recent techniques in optical holography [47-50] can be applied to**  
18 **the analysis of thrombus formation in whole blood.** However, one main limitation in the application of  
19 QPM is the non-specificity of the imaging technique that does not offer additional molecular information  
20 i.e. surface receptor levels. To achieve both morphological and molecular imaging, there is a need to  
21 combine QPM and high speed fluorescence imaging techniques such as lightsheet microscopy [1]. This  
22 provides a greater insight into the dynamic interaction of different receptors and adhesion molecules to  
23 thrombus formation. While high speed fluorescence microscopy requires specialized imaging sensors,  
24 holographic QPM can operate with more general imaging sensors [51]. Hence, it is plausible to incorporate

1 holographic QPM and microfluidic systems into a single portable unit. By reducing the footprint of  
2 holographic QPM and using unlabeled samples, it would be possible to use such a compact optofluidic  
3 system <sup>[52]</sup> to directly quantify dynamic thrombus morphology in a clinical setting. Here, we have used  
4 a limited number of healthy donors, which would need to be increased to permit comparative studies of  
5 thrombus growth rate with a wider distribution of shear rates. This will more accurately portray trends in  
6 volumetric growth and reduction over a wider distribution of shear rates. After which, from the clinical  
7 perspective, such a system may be effective in testing anticoagulation therapy, identifying platelet  
8 dysfunction and monitoring pro-thrombotic stages.

## 10 **Experimental Section**

### 11 **Blood collection**

12 Venous whole blood was collected from consenting, healthy drug-free volunteers into syringes  
13 containing 3.2% trisodium citrate or 10 U/mL heparin (Sigma) as an anticoagulant. Ethical approval was  
14 granted by the Australian National University Human Research Ethics Committee (2016/317). Whole  
15 blood was supplemented with 40  $\mu$ M PPACK (D-Phenylalanyl-prolyl-arginine Chloromethyl Ketone;  
16 Cayman Chemicals) 5 min before experimental procedure.

17 Platelet-rich plasma (PRP) was prepared from anticoagulated whole blood by centrifugation at 200 g for  
18 20 min and the upper PRP phase was collected. Prior to perfusion, blood samples were checked for  
19 absence of visible clots.

### 20 **Microfluidic channel preparation**

21 The microfluidic channel (400  $\mu$ m wide, 100  $\mu$ m height and 1.2 cm long) was made of transparent  
22 polydimethylsiloxane (PDMS) by soft lithography method <sup>[52]</sup>. Before the experiment, the entire  
23 microfluidic channel was coated with collagen Reagent HORM 100  $\mu$ g/mL (Takeda, Austria: a  
24 suspension of native equine tendon collagen type I) and incubated overnight at 4°C. The collagen was

1 added through the outlet to ensure that platelets initially contacted collagen from the entry of the channel  
2 beyond the inlet. Before the start of the experiment, the channel was washed using a wash buffer (25 mM  
3 TRIS HCl, 150 mM NaCl, pH 7.4) perfused at 300  $\mu$ L/min for 2 min in the opposite direction of  
4 experimental flow.

## 5 **Image and data processing**

6 The phase images were reconstructed from holograms recorded by CCD camera, using automatic  
7 numerical methods <sup>[53]</sup>. The volumetric images were calculated from the phase images according to  
8 equations below using Matlab (MATLAB R2017a). The standardization of the phase measurement were  
9 performed with the 3  $\mu$ m and 50  $\mu$ m polystyrene microspheres ( Polybead® Microspheres, polysciences,  
10 Inc.). The detail of the standardization can be found in Supplementary materials. In the equations, x and  
11 y are the coordinate of the image, representing each pixel. The Phase(x,y) is the phase image and the  
12 height (x,y) is the volumetric image. The  $\lambda$  is the wavelength of the laser, which is 632.8 nm in our  
13 system. The  $n_{pt} = 1.399$  and  $n_{pa} = 1.332$  are the refractive indexes of platelet and the buffer  
14 respectively <sup>[37]</sup>.

15 Individual thrombus image (where  $x, y \in T$ ) is selected from full field image by Otsu's threshold method  
16 <sup>[32, 54, 55]</sup>. The thrombus characteristics evaluated were thrombus peak height, surface area and volume.  
17 The thrombus peak height H is the highest point across an individual thrombus image. The thrombus  
18 surface area  $S_{thrombus}$  is calculated from the summation of pixels covering the thrombus. The  $S_p$  is the  
19 actual size of one pixel represents. The value of  $S_p$  is calibrated through a 1951 USAF Glass Slide  
20 Resolution Target. The volume of thrombus V is calculated by multiplying the number of pixels that the  
21 thrombus covered by the volume of each pixel.

$$22 \quad height(x, y) = -\frac{Phase(x, y) * \lambda}{2\pi * (n_{pt} - n_{pa})}$$

$$23 \quad H = -maximum[height(x, y)], \text{ where } x, y \in T$$

$$S_{thrombus} = \sum_{x,y} S_p, \text{ where } x, y \in T$$

$$V = \sum_{x,y} S_p * \text{height}(x, y), \text{ where } x, y \in T$$

### Shear stress conversion

Equation  $Q = \frac{Tbh^2}{6\mu}$  is used to calculate the experiment flow rates Q. In the equation, T is the shear stress, b and h are the width and height of the channel,  $\mu$  is the viscosity of the liquid sample. In our system, channel width is 400  $\mu\text{m}$ , channel height is 100  $\mu\text{m}$  and the viscosity of PRP sample is based on cell culture suspension which is 0.01  $\text{dyne}/\text{cm}^2 * \text{s}$ . The viscosity of whole blood sample is 0.045  $\text{dyne}/\text{cm}^2 * \text{s}$ .

### Statistics

The volume growth data for thrombus formation is first smoothed by a moving average filter (span=50 sampling points, data contains more than 1800 sampling points in total). Then a fitting (polynomial fit with degree of 5 and R-square above 0.98) was applied to each smoothed volume growth line. Two linear fittings (R-square above 0.9) were applied to front and end zone of the polynomial fit, indicated by black lines and the gradient value of the lines are  $S_1$  and  $S_2$  in the unit of cubic micrometre per minute. One set of volume growth data is shown in the Figure 2 b) i) with the two linear fittings (n=1 for each line). All experimental volume growth data can be found in the Supplementary material.

The growth rate shown in the Figure 2 c) ii) are the averaged growth rate from all the experimental data (n=3 for heparin PRP at 1800  $\text{s}^{-1}$  and 3600  $\text{s}^{-1}$ , n=1 for heparin PRP at 7200  $\text{s}^{-1}$ , n=2 for citrated PRP at 1800  $\text{s}^{-1}$ , 3600  $\text{s}^{-1}$  and 7200  $\text{s}^{-1}$ ). Software used for data smoothing, fitting and line plots is Matlab (MATLAB R2017a).

The individual thrombus characteristic bar graphs in Figure 3 are shown as means  $\pm$  SD by One-way ANOVA with Tukey's post-hoc tests and paired t-tests were performed by Graph Pad Prism (version

1 7) software. Individual scatter dot in the bar graph is representative for each thrombus data. The number  
2 of thrombus is marked by n beside each bar. The number of donor is marked by N beside each bar.

3 The volume, surface area and peak height analysis of stability test in Figure 4 b) iii), iv) and v)  
4 and Figure 4 c) iii) iv) and v) are means  $\pm$  SD by One-way ANOVAs with Tukey's post-hoc tests by  
5 Graph Pad Prism software. Each dot represents average data from one donor. The number of donor N=  
6 3 for citrated PRP and N=4 for heparin PRP and whole blood.

7

### 8 **Contribution and acknowledgements**

9 WML and EEG conceived the project. XH designed and built the holographic optical microscopy system.  
10 TX and XH designed and fabricated microfluidic chip. SJM and EEG designed the platelet experiments.  
11 XH and SJM performed the experiments and analysis of the results. All authors interpreted the data and  
12 co-wrote the manuscript. We received funding from the National Health and Medical Research Council  
13 of Australia and the Australian Research Council. XH is the recipient of the CSC scholarship.

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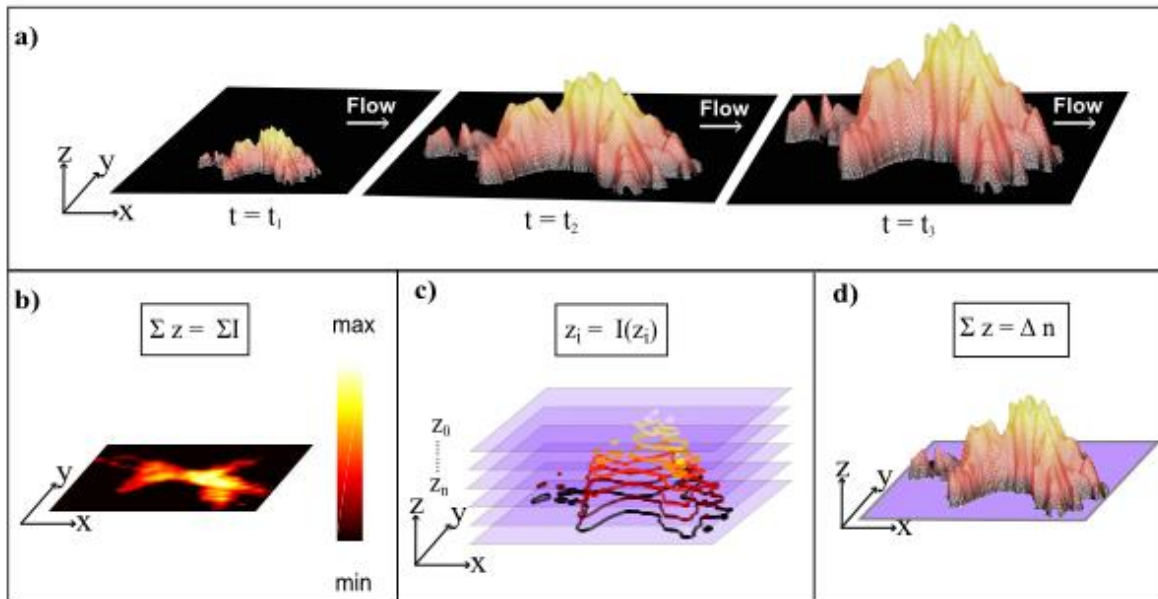
**Table 1 – Summary of three imaging modalities; Wide-Field Microscopy, (WFM), Confocal laser scanner microscopy (CLSM) and Digital holographic microscopy (DHM).**

<b>Technique</b>	<b>Wide-Field Fluorescence Microscopy (WFM)</b>	<b>Confocal laser scanning microscopy (CLSM)</b>	<b>Digital holographic microscopy (DHM)</b>
Molecular	Yes	Yes	No
Morphology	2D	2D and 3D	2D and 3D
Signal	Fluorescence brightness	Fluorescence brightness	Refractive index
Speed	0.0030 s camera frame rate	1 s	0.0030 s - 0.0006 s
Depth	0.4 to 5.8 $\mu\text{m}$ (60x, NA 0.85 to 20x, NA 0.4) <sup>[41]</sup>	1 to 100 $\mu\text{m}$	> 0.1 $\mu\text{m}$ *

\* Estimated based on 632 nm laser source with sample of refractive index difference at 0.067. (Please refer to Image and data processing in Experimental section)

1

2 **Figure 1 – Imaging a developing thrombus.**

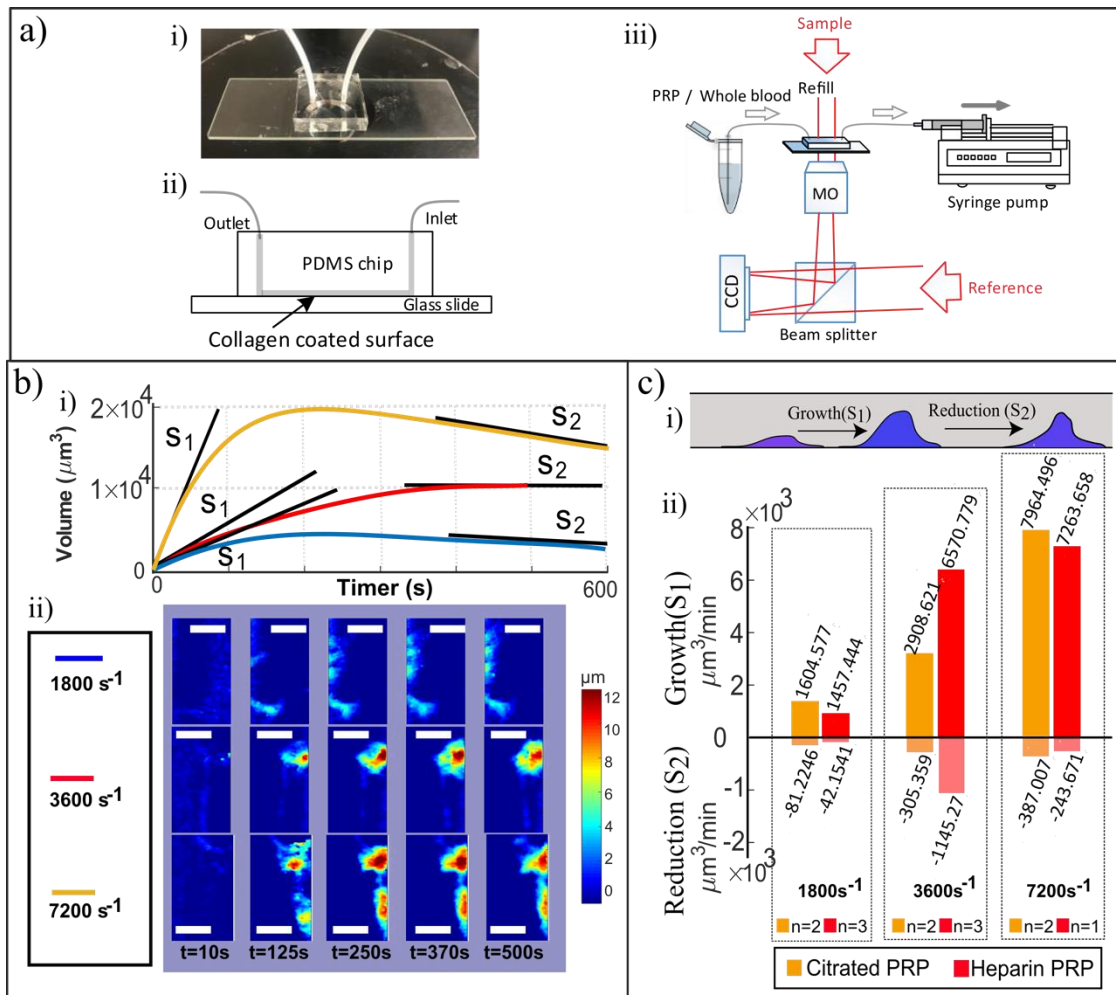


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4 Figure 1. (a) Schematic diagram of a thrombus formed under flow. (b), (c) and (d) Comparison of three  
5 imaging techniques, Wide-field fluorescence microscope, Confocal laser scanning microscope and  
6 Digital holographic microscopy (DHM) respectively.

7

1 **Figure 2 – Imaging growth and reduction of thrombus in microfluidic system.**

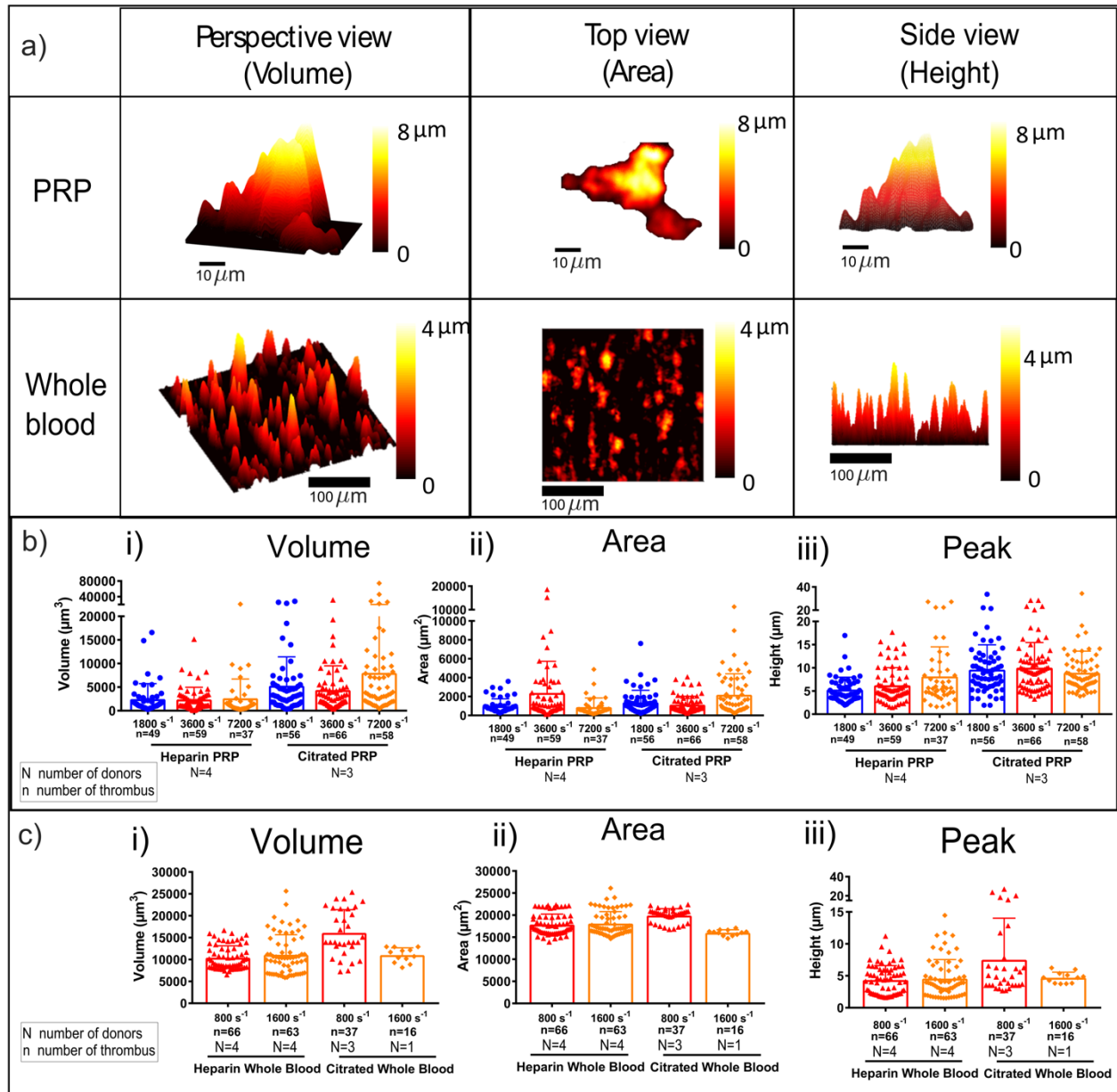


2  
3 Figure 2. (a), Schematic diagram of experiment setup. (i), picture of polydimethylsiloxane (PDMS) chip  
4 used for experiment. (ii), schematic diagram of PDMS microfluidic chip setup. (iii) Platelet Rich Plasma  
5 (PRP) and whole blood (heparinized) as sample are perfused over collagen coated PDMS chips. Arrows  
6 represent direction of flow. MO is the microscopy objective. The red lines represent the laser light. Figure  
7 2 (b-c). Thrombus formation result and analysis. (b) (i), Example volume growth of three thrombi formed  
8 by heparin PRP (from donor 1) under  $1800 \text{ s}^{-1}$ ,  $3600 \text{ s}^{-1}$  and  $7200 \text{ s}^{-1}$  shear rates during 10 min perfusion  
9 of PRP. The lines in three colours represent three different shear rates. The lines are polynomial fits (with  
10 degree of 5 and R-square above 0.98) of smoothed (moving average filter) raw data. The black lines are  
11 the linear fit (R-square above 0.9) of the curve lines, representing the speed of the volume accumulations  
12 (the front part S1) and volume reduction due to contraction (the end part S2). (b) (ii) The height images  
13 show the thrombus forming at different time points. The scale bar is  $30 \mu\text{m}$  and colour bar is in micron.  
14 Citrated PRP and whole blood thrombus volume growth curves are located in Supplementary materials.  
15 (c) (i) schematic diagram of thrombus formation trend observed in the experiments. (c) (ii) The bar chart  
16 of average speed of volume change during thrombus formation. The positive Y axis is the speed of  
17 volume accumulation (S1). The negative Y axis is the speed of volume reduction (S2). The heparin PRP  
18 sample is indicated by red bars and citrated PRP is indicated by orange bars the size of the sample is  
19 indicated below the bar chart.

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1 **Figure 3 – Reconstructed multi-view of individual thrombus.**

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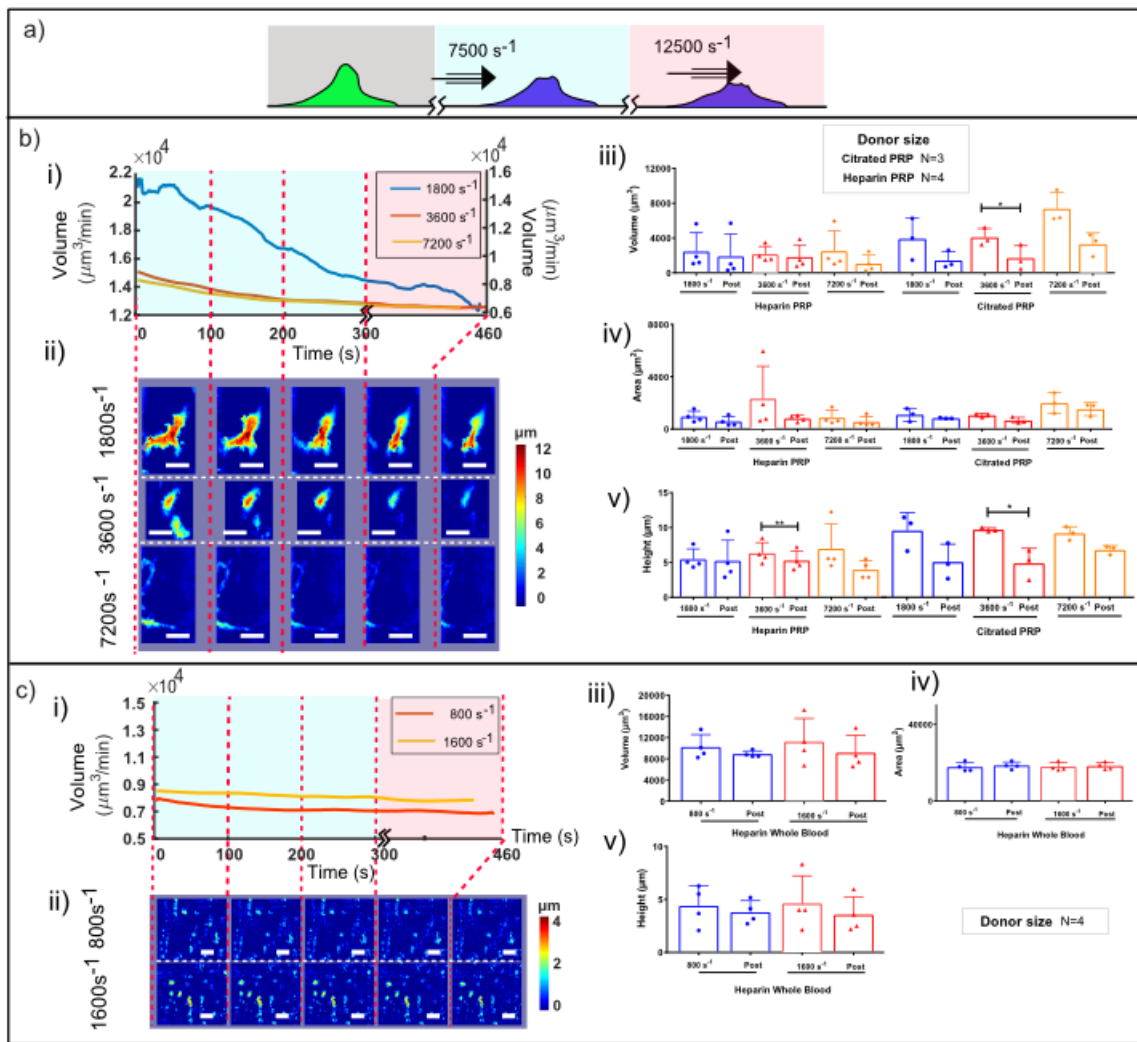


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4 Figure 3. Individual thrombus characteristics. (a) Three columns show the perspective, top and side view  
 5 of a thrombus formed by PRP and whole blood conditions. The side view is the cross sections of the  
 6 thrombus through the peak point of it by the direction along with (Y axis) and the direction vertical to  
 7 (X axis) the PRP and whole blood flow. (b-c) The volume, the surface area and the height of thrombus  
 8 formed under different shear rates (PRP flow condition: 1800  $\text{s}^{-1}$ , 3600  $\text{s}^{-1}$ , 7200  $\text{s}^{-1}$ ; whole blood flow  
 9 condition: 800  $\text{s}^{-1}$ , 1600  $\text{s}^{-1}$ ; *equivalent shear stress to mid and high PRP shear rates*). The blue circle,  
 10 red triangle and the yellow diamond are the individual thrombus formed under 1800  $\text{s}^{-1}$  PRP flow, 3600  
 11  $\text{s}^{-1}$  PRP flow (800  $\text{s}^{-1}$  whole blood flow), 7200  $\text{s}^{-1}$  PRP flow (800  $\text{s}^{-1}$  whole blood flow). The number of  
 12 thrombus is indicated by n beside each bar and the number of donor is indicated by N. Mean shown as  
 13 +SD.

14

1 **Figure 4 – Stability of thrombus.**



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3 Figure 4. (a) Stability test by exerting high shear rate; buffer flowing at 7500 s<sup>-1</sup> for 5 min and then 12500  
 4 s<sup>-1</sup> for 2 min) (b) Reduction of thrombus pre-formed by heparin PRP. i) Each line profile traces the  
 5 thrombus volume that was previously formed under shear rate of 1800 s<sup>-1</sup> (y-axis left), 3600 s<sup>-1</sup> (y-axis  
 6 right) and 7200 s<sup>-1</sup> (y-axis right) from same donor (d4) respectively. ii) The images below the line plots  
 7 are the corresponding thrombus at different time points. The scale bar is 40 μm and color bar is in micron.  
 8 (c iii-v) The bar graphs for average volume, surface area and the peak height data of the thrombus from  
 9 all donors. Number of thrombus quantified, n > 8 from each donor. The statistical test is a paired t-test,  
 10 \*p < 0.05. Mean shown +SD. (c) Reduction of thrombus pre-formed by heparin whole blood flow. i) Each  
 11 line profile traces the thrombus volume that was previously formed under shear rate of 800 s<sup>-1</sup> and 1600  
 12 s<sup>-1</sup> from same donor (d4) respectively. ii) The images below the line plots are the corresponding thrombus  
 13 at different time points. The scale bar is 40 μm and color bar is in micron. (iii) The statistics bar graph  
 14 for averaged volume, surface area and the peak data of the thrombus from all donors. Number of  
 15 thrombus quantified, n > 8 from each donor. The statistical test is a paired t-test. \*p < 0.05.

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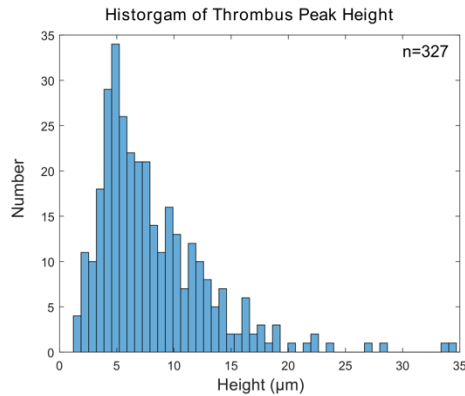


1 **Supplementary materials**

2

3 **Supplementary Figures**

4 **Figure A1 – distribution of thrombus peak height.**



5  
6 Figure A1. Histogram of thrombus peak height. Thrombus (n=327) were formed by the PRP from all  
7 donors (D1, D2, D3, D4 for citrated PRP and d1, d2, d3, d4 for heparin PRP) under all three  
8 experimental shear rates.

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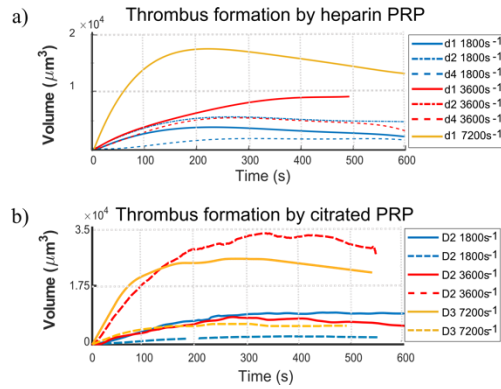
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1 **Figure A2 – Volume profiles for thrombus formation using PRP**



2  
3 Figure A.2. a) Volume growth of thrombus formed by heparin PRP under three different shear rates. b) Volume  
4 growth of thrombus formed by citrated PRP under three different shear rates. Each line stands for each  
5 thrombus. The colour of the lines indicates the shear rates of flowing PRP, blue for 1800  $\text{s}^{-1}$ , red for 3600  $\text{s}^{-1}$  and  
6 yellow for 7200  $\text{s}^{-1}$ . The type of lines distinguish the thrombus from different donors.  
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**Figure A3 – Stability test of thrombus formed by citrated PRP**

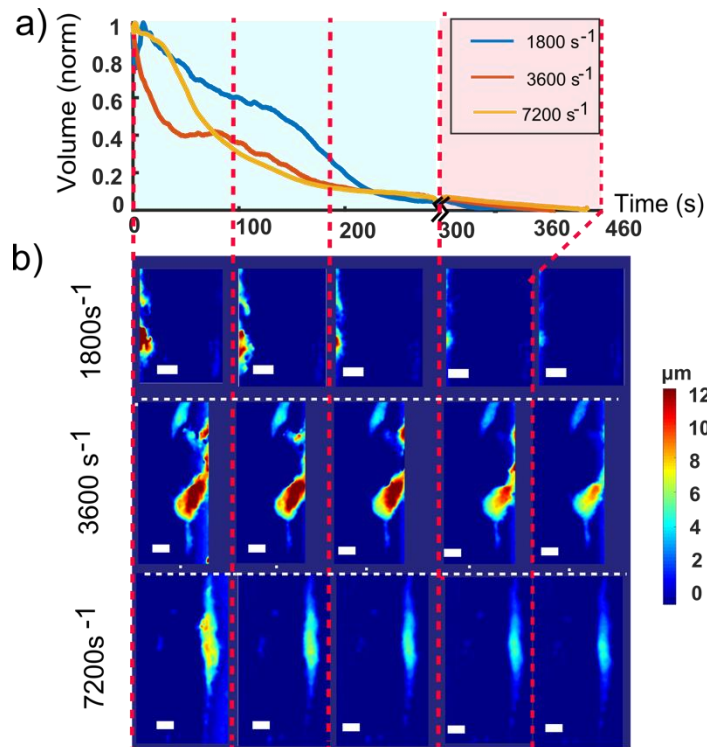
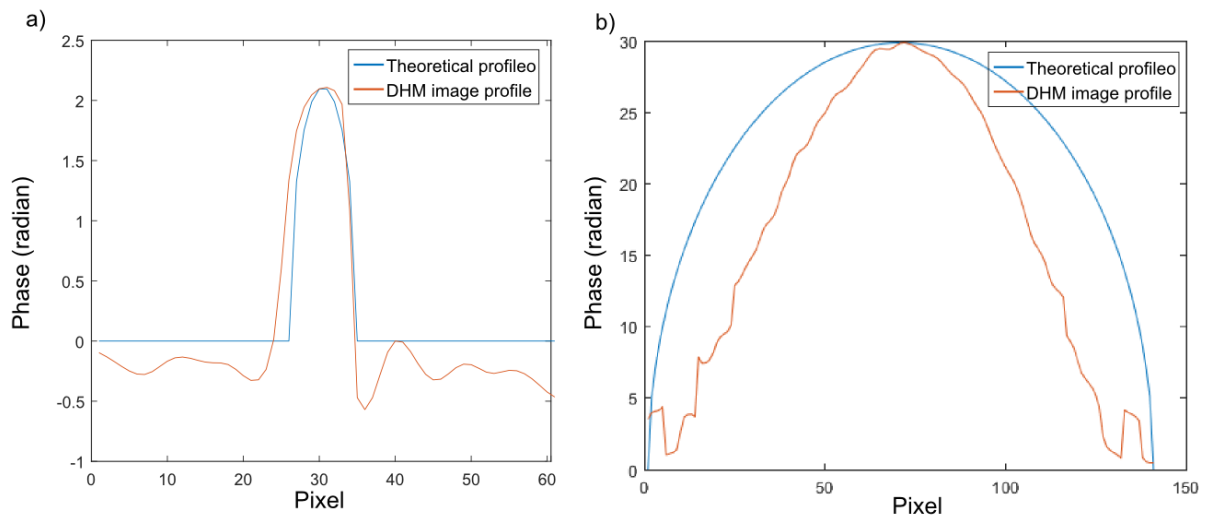


Figure A.3. a) Volume reduction of thrombus formed by citrated PRP during stability test (buffer flowing at 7500 s<sup>-1</sup> for 5 min and then 12500 s<sup>-1</sup> for 2 min). Each line profile traces the thrombus volume that was previously formed under shear rate of 1800 s<sup>-1</sup>, 3600 s<sup>-1</sup> and 7200 s<sup>-1</sup> from donor D2 respectively. b) The height map images below the line plots are the corresponding thrombus at different time points. The scale bar is 30 μm and colour bar is in micron.

1 **Figure A4 – Imaging standardization using microspheres**



2  
3 Figure A.4. a) Phase profiles of a 3 μm microsphere. b) Phase profiles of a 3 μm microsphere. The red  
4 lines are the reconstructed phase profiles from DHM system. The unit is in radian. The blue lines are  
5 the theoretical profiles of the spheres based on the projection of a sphere. The material of the  
6 microsphere is polystyrene with refractive index of 1.587.  
7