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Counting the platelets: a robust and sensitive quantification method for thrombus formation

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Summary

Flow chambers are common tools used for studying thrombus formation *in-vitro*. However, the use of such devices is not standardized and there is a large diversity among the flow chamber systems currently used, and also in the methods used for quantifying the thrombus development.

It was the study objective to evaluate a new method for analysis and quantification of platelet thrombus formation that can facilitate comparison of results between research groups.

Whole blood was drawn over a collagen patch in commercial Ibid or in-house constructed PDMS flow chambers. Five percent of the platelets were fluorescently labeled and z-stack time-lapse images were captured during thrombus formation. Images were processed in a Python script in which the number of platelets and their respective x-, y- and z-positions were obtained. For comparison with existing methods the platelets were also labeled and quantified using fluorescence intensity and thrombus volume estimations by confocal microscopy. The presented method was found less sensitive to microscope and image adjustments and provides more details on thrombus development dynamics than the methods for measuring fluorescence intensity and thrombus volume estimation. The platelet count method produced comparable results with commercial and PDMS flow chambers, and could also obtain information regarding the stability of each detected platelet in the thrombus. In conclusion, quantification of thrombus formation by platelet count is a sensitive and robust method that enables measurement of platelet accumulation and platelet stability in an absolute scale that could be used for comparisons between research groups.

Introduction

Platelets are essential to keep vascular integrity, but may at the same time cause thrombosis during pathological conditions (1). Due to this dual nature of platelets and their important function, platelets have been thoroughly studied in different models. One approach is to utilise in-vitro flow chambers for studies of platelet interactions, thereby attempting to replicate the process of thrombus formation under arterial or venous shear rates in a controlled setting (2). There are both commercially available and in-house constructed flow chambers, demonstrating a large diversity in both design and functionality (2–6). This diversity is further stimulated by the use of soft lithography with polydimethylsiloxane (PDMS), which facilitates prototyping and in-house manufacturing of flow chambers (7).

Flow chambers are commonly used with image capture in both wide-field and confocal fluorescence microscopes to detect platelet adhesion and thrombus formation. There are several methods for quantifying the thrombus in the acquired image data, but at present with little standardization, making comparison difficult between research groups. Commonly used quantification methods include; measurements of fluorescence intensity (8,9), surface coverage (10) and thrombus volume estimation based on confocal images (10,11). However, these measurements may not truly reflect the actual accumulation of platelets. Fluorescence intensity measured in a single focal plane close to the surface may lead to underestimation of aggregating platelets further out from the surface, since the out-of-focus platelets contribute with less intensity compared to platelets in the focal plane. Furthermore, thrombus volume and surface coverage measurements does not separate thrombus size increment by platelet accumulation from the parallel process of platelet-induced clot retraction, which simultaneously may reduce thrombus volume, as previously demonstrated by Ono et al. (11). This raises questions

regarding the individual contribution of these parallel processes to thrombus size when measuring thrombus volume or surface coverage.

Thrombus volume is generally estimated from z-stack image data acquired with confocal microscopy (5,10–12). Apart from requiring expensive instrumentation, the image capturing process is often slower on confocal microscopes (with the exception of some spinning disc configurations), making it less suitable for fast time-lapse acquisition. An increased temporal resolution have the benefit of capturing a more detailed view of the of thrombus formation dynamics. Considering these limitations, non-spinning disc confocal microscopy may be more suitable for measuring end-point results.

Another obstacle when trying to quantify thrombus volume/coverage in a standardized and reproducible manner is that manual adjustments are often included in the end result when setting the intensity threshold levels that separate the platelet from the background. To eliminate such user bias there are software with automated functions that makes these decisions. However, without exact knowledge of the underlying algorithms in such functions this will entail an uncertainty on how variations in fluorescence intensity, from e.g. aging light sources, photo bleaching or detector gain adjustments may translate into variations in the volume/coverage measurement.

The diversity of flow chamber design and quantification methods obstructs comparisons between thrombus formation experiments *in vitro*. We propose a robust and unbiased method for quantification of platelet thrombus formation suitable for both wide-field and confocal microscopy, based on an algorithm for time-resolved quantification and determination of individual platelet positions within the developing thrombus.

Material and methods

Ethic Statement

The research, blood sampling and consent procedure was approved by the local ethical review board in Linköping. Verbal informed consent was obtained from all blood donors, no documentation of the consent or any personal information about the blood donors was saved, thereby ensuring anonymization of the samples.

Materials

Bovine serum albumin (BSA), HA1077, and Mix-n-Stain CF555A kit was from Sigma-Aldrich Co. (St. Louis, MO, USA). DiOC₆ were from Invitrogen Molecular Probes (Eugene, OR, USA). Monoclonal antibody against CD41, clone PM6/248 (preservative free) was from AbD Serotec. HORM Collagen was obtained from Takeda (Linz, Austria). Sylgard 184 base and curing agent were from Dow Corning Europe (Belgium). Photoresist SU-8 3035 was from Microchem (Newton, MA, USA). Cangrelor was a kind gift from the Medicines Company (Parsippany, NJ, USA).

Blood collection and fluorescent labelling

Whole blood was drawn from healthy volunteers into hirudin sampling tubes. The blood was used within 4 hours and was stored at room temperature as recommended by Roest et al. (6). Two labelling procedures were used: 1) Platelet count quantification: 5% of the blood was incubated for 15 minutes with 1 µg/mL of anti-CD41-CF555 antibody. Directly before the flow chamber experiment, the labelled blood fraction was returned to the unlabelled fraction and gently mixed. Flow cytometry was used to ensure that labelled platelet remained as a separate distinguishable population after mixture with unlabelled platelets for a minimum of 15 minutes (Suppl. Figure 1). 2) To determine the volume from confocal microscopy images and measure the fluorescence intensity all platelets (100%) were homogenously labelled using DiOC₆ (0.25 µM). Platelet-rich plasma (PRP) was prepared by

centrifugation at $60 \times g$ for 15 min and thereafter incubated with DiOC₆ in 15 minutes. The PRP was reconstituted with the remaining RBC fraction before the experiments.

Flow chamber construction and experiments

The PDMS flow chamber was moulded on a template of photo-patterned SU-8 resist on a silicon wafer. A collagen strip (250 μm wide) was coated on the glass slide with collagen solution (500 $\mu\text{g}/\text{mL}$), and a straight PDMS channel (height: 60 μm , width: 250 μm) was placed perpendicular over the collagen strip (Suppl. Figure 2). Flow chambers were blocked with BSA (1 mg/mL) for 15 minutes prior to use. The blood was drawn through the flow chamber with a Fusion 200 syringe pump from KR Chemyx (TX, USA) at 10 $\mu\text{L min}^{-1}$, resulting in a shear rate of approximately 1400 s^{-1} .

For comparative experiments, Ibidi Sticky-Slide I 0.1 Luer (Munich, Germany) flow chambers were mounted on collagen-coated glass slides as described above.

Image capture and analysis

Z-stack time-lapse images were captured with a wide-field 20x objective (NA 0.8) on a Zeiss Axio Observer Z1 with a Colibri LED-module and a Neo 5.5 sCMOS camera (Andor Technology Ltd., UK) controlled by $\mu\text{Manager}$ software (Vale lab, UCSF). Confocal images were acquired with a 20x objective (NA 0.8) on a Zeiss Axio Observer LSM 700 with Zen software. Image data were processed, analysed and visualized with the use of Python scripts (www.python.org), utilizing the Numpy (13), Scipy (14), Pandas (15), Matplotlib (16) and Seaborn (17) packages. Imaris (Bitplane AG, Switzerland) was used to render and calculate a 3D-volume from the Z-stack confocal images. Detailed descriptions of the image processing and analysis steps are provided in the supplemental materials and methods section, Suppl. Figure 3.

Results and Discussion

Using platelet count as quantification method

Counting the number of platelets in the forming thrombus may give a more reliable measure of thrombus size and also provide additional information regarding other thrombus properties, such as platelet distribution within the thrombus and thrombus stability. However, counting the platelets is not achievable when all platelets are fluorescently labelled, since their close packing in the thrombus mass makes it impossible to distinguish between individual platelets. Instead, by labelling a fraction of the platelets in blood, the labelled platelets will become separated and more easily distinguishable in the thrombus. This would enable counting of the labelled platelets with good precision also in wide-field microscopy, and since the ratio of labelled/non-labelled platelets is known, an accurate estimation of the total number of platelets can be determined. In this study we labelled 5% of the platelets with anti-CD41-CF555 and at this ratio the labelled platelets seemed dispersed in the volume and generally well separated from each other, as illustrated in Figure 1. Labelling a larger fraction of platelets could arguably result in an improved estimation of the total platelet population, at the expense of an increased risk for errors caused by two or more labelled platelets being close enough in the thrombus to falsely be counted as one.

Using this quantification approach, we aimed to make the image processing steps both reproducible and unbiased. Therefore all image processing and analysis were performed with the use of Python scripts that did not require user input or any adjustments during execution. Starting with a time-lapse image data set, acquired in the microscope as multiple z-stacks over time, we first reduced background noise and blurred signals from out of focus platelets with the use of a difference of Gaussians algorithm, which was performed on the individual images in the z-stack (Figure 1A). This background reduction step can likely be exchanged with a 3D-deconvolution step if it is available in the lab, or skipped if images are

captured with confocal microscopy. The optimal threshold level required to distinguish the labelled platelets from the background in the image data was set using a threshold probe. The probe uses a single z-stack volume from a time point when the thrombus has already formed, and in this volume counts the number of objects as a function of the threshold level as presented in Figure 1B (with corresponding thresholded images in Figure 1C). From this data the probe first finds a local minimum in the number of detected objects (Figure 1B II), which corresponds to a threshold level that is high enough to eliminate most background noise, but not sufficiently high to detect all individual platelets as single object, and several platelets are therefore detected as a conjoined object (Figure 1C II). The probe thereafter searches above this local minimum to locate a local maximum in the object count, which will represent the optimal threshold level (Figure 1B III). This is the point where the maximum number of single platelets is detected, and threshold levels above this point will tend to eliminate platelets with weaker fluorescence from detection (Figure 1C IV and V). It is generally sufficient to use a fixed threshold probe value for all time points in the time series, if the value is generated from a time point during the later stages of thrombus formation. By comparing the platelet count where the probe was run at a single time point, with a count from running the probe for all time points individually, it was evident that the difference was negligible, as shown in Suppl. Figure 4. When the optimal threshold level is found, all image data from that time series is passed through a script performing background reduction, thresholding, and finally, object counting. The object counting function also calculates and returns the centre-of-mass coordinates for each detected platelet. These x-, y- and z-coordinates can be used to visualize the thrombus formation process in e.g. open source scientific 3D visualization software as Paraview (Figure 2A) or by plotting the platelets in scatter plots as demonstrated in Figure 2B. The image processing and analysis is not particularly time consuming, an analysis of 80 time points with 3 megapixels images will take about 15 minutes. The image data analysed herein typically covers the

whole flow chamber width and about thrice the length of the collagen patch to ensure that all thrombus formation on the collagen is captured in the image.

To achieve a more dynamic view of the thrombus build-up, a high temporal resolution during image capture is desirable. A longer distance between images in the z-axis allows for a more rapid acquisition throughout the thrombus height and therefore a shorter interval between time-points. In order to optimize the z-axis distance we performed experiments to test which distances could be used without platelets being lost in between focal planes. We used z-stacks of thrombi ($n=3$) with 1 μm distance between images and created z-stacks with 2 to 8 μm between images by selecting a subset of images from the 1 μm stack. Increasing the z-axis distance from 1 to 4 μm causes less than 5% reduction in detected objects (Figure 3A), and increases the temporal resolution by a four-fold. However, the reduced number of detected objects may not only be a result of fewer detected platelets but could to some extent be a result of less pixel-sized noise, since the volume is constructed from fewer images, thereby decreasing the probability of such noise. We also investigated if the platelet count was affected by the starting position in z-axis when acquiring images with different z-axis resolution (Figure 3B). A shift in the starting position could theoretically affect the number of detected objects when the distance between images along the z-axis is increased. Studying the variation in counted objects in the z-stack image of a thrombus, where the starting image position was shifted up to 5 μm in 1 μm steps, we found that the variation with a z-axis resolution of 4 μm was still very small, demonstrated by a average CV of $\sim 1\%$ ($n=3$). It is important to note that the detection of platelets is always performed in the continuous 3D volume and not in individual images of the z-stack, and platelets that occur in two image planes is therefore only detected as single object and not as two or more platelets. The centre-of-mass calculation that is performed to return the platelet x-, y- and z-position will also be affected by an increasing distance between image planes in the z-stack. When the same platelet occur in several image planes the calculation of z-axis position can be interpolated between the image planes. However, this

interpolation is not possible for platelets only present in a single image plane. Longer distances between the image planes will increase the probability that a platelet only occurs in a single z-stack image, therefore leading to a larger number of platelets being constrained in z-position to one of the image planes, as demonstrated in Figure 3C. Therefore, depending on what may be of most importance for the study, it is possible to prioritize the temporal or z-axis resolution to obtain the most relevant information. We used the 4 μm z-axis resolution for the subsequent experiments in this study, where each z-stack covers 40 μm in height. With these settings and using an image exposure time of 150 milliseconds, the z-stack is captured in 4.5 seconds for each time point.

Comparison with other techniques

In order to test the accuracy and robustness of the presented quantification method, we investigated how the platelet count method compares to two other frequently used methods for quantification of thrombus formation, i.e. fluorescence intensity measurement, and thrombus volume estimation from confocal microscopy images. Fluorescence intensity is commonly used to assess platelet accumulation, and the images are captured with the focal plane at the base of the thrombus, close to the glass surface. The accumulation of fluorescent platelets contributes to a higher total fluorescence intensity in the image, which is summed and used as a measure of thrombus size. However, we suspect that capturing intensity images in a single focal plane may underestimate aggregating platelets further away from that focal plane. To demonstrate such possible differences we chose a set of flow chamber experiments from normal donors (n=4) with pronounced variations in z-axis development. The fluorescence intensity method was then compared to the platelet count methods in an experiment where platelets in blood samples were labelled both for the intensity measurements (100% labelled) and the platelet count method (5% labelled). For fluorescence intensity measurements PRP was labelled with DiOC₆ and after incubation reconstituted with the remaining RBC-rich fraction. For the platelet count method 5% of the whole blood was labelled with anti-CD41-CF555. Z-stacks were acquired at alternating wavelengths

during thrombus formation on collagen in the PDMS flow chamber. The results are presented in Figure 4. When using fluorescence intensity, the baseline is greatly affected by the initial background fluorescence, as can be seen in the non-normalized data in the Figure 4B insert, and the results therefore need to be normalized. These large variations in baseline fluorescence may be a result of the less specific DiOC₆ labelling, and may be reduced using fluorescently labelled platelet specific antibodies. There is a visible difference in the quantitative relationship between experiments for the fluorescence intensity measurement (Figure 4A) and the platelet count results (Figure 4B). These differences become more pronounced during the later stages of thrombus build-up and we hypothesize that these differences may arise from an unequal contribution of fluorescence from the labelled platelets, which is dependent on the z-axis position of the accumulating platelets. Platelets further out from the surface will be out-of-focus and contribute with less fluorescence intensity to the image, making the thrombus size measure less and less sensitive to variations as the thrombus builds up further away from the surface and focal plane. To further illustrate this problem inherent to the method, we have also provided heatmaps where the time-resolved platelet distribution along the Z-axis is displayed for all four repetitions of the experiment (Figure 4C). For example, when comparing the relative flat thrombus development in repeat 1 with repeat 2, where the thrombus extends more in height, it appears that the platelets further out from the surface in repeat 2 are underestimated in the fluorescence intensity graph. To correct for this artefact it would be possible to use a complete z-stack image set for calculating the total fluorescence intensity, but this acquisition would be slower and generate much more image data, whereas the amount of information regarding the thrombus development would still be very limited, compared to the platelet count method.

With confocal microscopy it is possible to determine how the volume of the thrombus is distributed in height (z-axis). To investigate if the height distribution could likewise be used for the platelet count method we compared the quantitative z-axis distribution measured by confocal volume calculation and

wide-field platelet count in images of the same thrombus. We also included wide-field fluorescence intensity measurement for comparison. Thrombi were formed on collagen in a PDMS flow chamber using labels for both volume/intensity measurement (100% labelled platelets, DiOC₆) and platelet count (5% labelled platelets, anti-CD41-CF555). After formation of the thrombi (approx. 7 min) the channel was rinsed with Hepes-buffered saline and z-stacks were acquired for the platelet count and for measuring the fluorescence intensity. The flow chamber was thereafter directly moved to a confocal microscope where an additional z-stack of the DiOC₆ -labelled platelets was captured. The normalised distributions are displayed in Figure 5A, along with sample z-stack images used in the three methods (Figure 5B). The confocal images could be considered the golden standard for this type of measurements, and interestingly, the distribution of the platelet count was almost identical to the distribution in the confocal images, which verifies the platelet count methods capability in providing accurate information regarding the thrombus development along the z-axis. The fluorescence intensity distribution was however distinctly different and does not appear to provide reliable information regarding the platelet distribution in the z-axis. This is also evident in the z-stack images in Figure 5B, where a large part of the thrombus still appear in the wide-field intensity image at 24 μm above the surface, in contrast to confocal and the platelet count images, where only a small part of the top part remains visible.

Using specialized software packages it is possible to generate 3D reconstructions from confocal images and to produce thrombus volume estimations. However, it is not well known how robust such software is and to what extent user input and instrument variations can alter the outcome. We therefore investigated how sensitive such volume calculations are compared to the platelet count method presented here. Thrombi were formed on a collagen patch in the PDMS flow chamber with the two different labels for volume measurement (100% labelled platelets, DiOC₆) and platelet count (5% labelled platelets, anti-CD41-CF555). The software Imaris™ was used to exemplify the volume

measurements. Imaris™ uses a surface creation wizard to define the thrombus volume and gives two options of calculation methods for the threshold, absolute intensity and background subtraction. We used both methods and left all other parameters with default values. It would of course be possible to change the different available parameters in order to adjust the volume to what the user feels best resembles the thrombus, but such manual adjustments would inflict a considerable user bias. In Figure 6A the two resulting 3D reconstructions from the different threshold methods are compared to the original confocal image. From this choice of method the resulting volume calculation are considerably different, with a $40 \pm 2.4 \%$ ($n=3$) smaller volume when using the background subtraction method compared to the absolute intensity method.

The final microscopy image used for analysis is affected by several factors, which may or may not be within control of the user. Examples of such factors are microscope settings, photo-bleaching from repeated image capturing, aging light sources, variability in labelling and also image processing before analysis. We wanted to test how factors that cause variations in fluorescence intensity will affect volume estimation from confocal images, and compare this with corresponding tests for the platelet count method presented herein. Images, with different intensity, of the same thrombus were obtained by varying the master gain setting for the confocal detector. The results are presented in Figure 6B and C, and it is evident that the estimated thrombus volume was considerably affected by the intensity variations for both threshold methods. The volume estimation increases at both lower and higher gain (Figure 6C). An increase of 20 % in gain (510 to 600) resulted in an increase in the calculated volume of $5.8 \pm 3.7\%$ or $15.2 \pm 9.1\%$ ($n=3$) for absolute intensity and background subtraction respectively.

Increasing gain further, with 30% (510 to 650) resulted in considerably larger volume estimations, which in the case of absolute intensity thresholding amounted to a more than 25% increase in the estimated volume, and pronounced variation between the samples. These experiments emphasize the importance of keeping settings like gain fixed during several experiments if they are to be compared later on. The

variability exemplified above also presents an obstacle for comparing volume data between research groups since it is exceedingly difficult to ensure that images are captured with the same intensities on different microscopy systems, handled by different operators.

To investigate if the platelet count method was equally affected by variations in image intensity, images of thrombi (n=3) with 5% labelled platelets were captured with different exposure times, from 50 to 250 ms, using wide-field microscopy, and analysed with the platelet counting script with the threshold probe (Figure 6D). The threshold probe was able to compensate for the variations in intensity and the platelet count only demonstrated a minor decrease, with a maximum deviation of $2.6 \pm 2.3\%$ at 30% of the original exposure time. From these tests it appears that the platelet count method is more robust and can handle images with larger variations in intensity with better reproducibility.

To better understand these differences we also performed a more direct comparison of sensitivity between confocal volume measurement and wide-field platelet count. Z-stack fluorescence images of three thrombi formed on collagen in the PDMS flow chamber, labelled both for volume estimation (100% labelled platelets, DiOC₆) and platelet count (5% labelled platelets, anti-CD41-CF555), were subject for intensity adjustments by a multiplier from 0.92 to 1.08. All adjusted image stacks were thereafter thresholded with a single value set by the user for original image stacks (multiplier 1.00). Thrombus volume was simply calculated by summation of the white pixels in the thresholded image stack (Figure 7A). Platelet counting was likewise performed on the same three thrombi with the same intensity adjustment procedure, with and without the use of the threshold probe (Figure 7B). Notably, when comparing the volume measurement with the platelet count method it becomes evident that the volume calculation is affected by the intensity variations to a much greater extent than the platelet count method, even without the use of the threshold probe. Although most volume reconstructing software would utilize a more complex procedure to distinguish the fluorescent object from the

background it is likely that the greater variability demonstrated in this example also plays a role in the variations seen with the commercial software.

An additional layer of complexity inherent in the use of thrombus volume estimations to study thrombus development is that during thrombus formation there are in fact two parallel processes that will affect the measurements, both platelet accumulation and platelet-induced clot retraction. Platelet contractility may occur as a fibrin-independent process, which stabilizes the forming thrombus, causing an overall decrease in volume of 30-40% (11). When measuring thrombus volume or surface coverage it is not possible to differentiate between the counteracting processes of platelet accumulation and platelet clot-retraction. However, using platelet count in combination with volume measurements may be a possibility to better separate and study these processes.

Measuring thrombus characteristics

The platelets and their position in the thrombus can be used for further analysis that can be valuable in determining the characteristics of the growing thrombus, in particular in terms of thrombus stability. To investigate these possibilities we performed experiments with additions of the Rho kinase inhibitor HA1077 (120 μ M), which has previously been described to induce thrombus instability (11). As we have already shown, the information on platelet positions in x-, y- and z-position at specific time-points can be used to construct a 2D histogram of how thrombus height develops over time, as visualized in the Figure 8A heatmap. Platelet accumulation can also be presented as a summation of platelets in each time point, as displayed in Figure 8B. This type of measurement will of course give some information regarding the thrombus stability in terms of increasing or decreasing platelet number. However, information regarding the position of platelets can also be used to determine if individual platelets are stable, i.e. not moving more between two images than that the centre position of the platelet is still covered by the platelet volume in the subsequent image. We performed such analysis and the results

are shown as a percentage of unstable platelets in a histogram heatmap (Figure 8C), and as a summation with time (Figure 8D). Interestingly, from Figure 8C it is clear that an embolization event starts with platelet instability at the top of the thrombus, and within seconds this instability propagates down through the entire volume until the embolization occurs. The embolization event can also be visualized as a time-lapse reconstruction where unstable platelets are plotted in red and stable platelets in yellow (Figure 8E and Suppl. Video 1), which may also give more details about the spatial dynamics of the embolization process. An advantage with these stability measurements is that using a more general measurement such as percentage unstable platelets would facilitate comparisons between experiments not only within the lab but also between different research groups. Another possible extension of the method, which is not explored herein, is to acquire additional information regarding platelet activation state for each detected platelet. This could be facilitated by adding a fluorescent probe for intracellular calcium, P-selectin or Annexin V in another fluorescent channel, and since the x-, y- and z-position for each platelet is already known, it would be achievable to measure the intensity in the activation marker channel in these positions during thrombus development.

Comparing different flow chambers using platelet count quantification

To exemplify how the presented quantification method can be used for comparing results obtained in different flow chamber systems we performed the same experiment in a commercial flow chamber from Ibidi and an in-house constructed PDMS flow chamber (both at shear rate 1400 s^{-1}) ($n=5$). Thrombus formation was induced by a collagen patch in the flow chamber and experiments were performed with and without ADP receptor blockade by the P2Y₁₂ inhibitor Cangrelor ($5\text{ }\mu\text{M}$). To enable a straightforward comparison of the two systems the thrombus development was quantified as platelet count per area (mm^2) of collagen coating. As expected the P2Y₁₂ inhibition had a substantial impact on thrombus development (Figure 9A), resulting in less aggregation and development of the thrombus in z-axis, which is visible in the heat maps from a single donor (Figure 9B), presenting platelet count with

time and z-axis distribution. With P2Y12 inhibition the results are almost identical between chambers, whereas small differences can be seen between the two systems without P2Y12 inhibition when the thrombus can propagate via aggregation out from the collagen. These differences could be a result of dissimilar flow/shear profiles during thrombus development in the two systems since the flow chamber heights are different, and the flow rates in the chambers were set to match the shear rate at the flow chamber wall.

Conclusion

Herein we introduced a method for quantifying the platelet thrombus, compatible with both wide-field and confocal microscopy. A fraction of the platelets in blood are fluorescently labelled and thereafter detected and counted in time-lapse images of the forming thrombus. We have demonstrated that quantification performed with the platelet count method is very robust and not as easily influenced by image intensity variations as for example confocal volume measurements. The method not only counts the platelets but also obtains information regarding each detected platelets respective x-, y- and z-position in the thrombus at each captured time-point. This information can be used to visualize thrombus dynamics by plotting the platelets in 2D or 3D, and can also be used for describing other properties of thrombus development as for example how the thrombus expands in height (z-axis) with time, or determine the stability of each detected platelet in the thrombus. The latter analysis will thereby generate information on the local stability in the different parts of the thrombus.

The development of flow chamber techniques has indeed carried the research field of thrombosis and haemostasis forward. However, the great diversity in designs, quantification methods and other experimental parameters makes comparison of data between research groups problematic. The main advantage with the described quantification method is that the unit of measurement, i.e. platelet count, is absolute and not defined by the specific microscope configuration. Although this type of

quantification will not eliminate differences originating in experimental parameters such as anti-coagulation used, or flow chamber design, it could bring these experiments, however different, into a common scale and thereby enable them to be compared with one another. The described quantification approach could be an important step towards standardising the use of flow chambers.

Conflict of interest

None declared

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Addendum

K. Claesson, L. Faxälv contributed to the concept and design of the study, analysis and interpretation of the data, critical writing and final approval of the manuscript. T. Lindahl contributed to the interpretation of the data; and revising the intellectual content and final approval of the manuscript.

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Figure 1. Image processing steps for background reduction and determining a suitable threshold with a threshold probe. A PDMS flow chamber was used to form a thrombus on collagen with 5% labelled platelets (anti-CD41–CF555) at a shear rate of 1400 s^{-1} . A) Original fluorescence wide-field micrographs (20x objective, NA 0.8) of the formed thrombus were processed with a difference of Gaussians algorithm for reduction of the image background. The scale bar represents $50 \mu\text{m}$. B) The threshold level for each flow chamber experiment was determined by an automated threshold probe. The threshold probe finds the local maximum at III, which corresponds to the optimal threshold level where the number of detected platelets is maximized (insert with linear scale). C) Threshold images from different threshold levels corresponding to the numbers in the B. The scale bar represents $50 \mu\text{m}$.

Figure 2: Visualization of thrombus formation.

Platelet thrombus formation on collagen was performed in a PDMS flow chamber at a shear rate of 1400 s^{-1} with 5% labelled platelets (anti-CD41–CF555). Time-lapse z-stack images were captured with wide-field fluorescence microscopy (20x objective, NA 0.8) and analysed with the platelet count method. A) The x-, y- and z- positions of all detected platelets were used to visualize the thrombus in 3D using the software Paraview™. The red dots represent the detected platelets and the surrounding blue spheres represent hypothetical volumes of the remaining non-labelled platelet fraction in the thrombus. B) Platelet positions were used to plot time-lapse graphs of the thrombus development viewed from

different angles. It should be noted that the z-axis is scaled differently from x- and y-axis to emphasise the thrombus-build up in height.

Figure 3. Characterizing the influence of Z-axis resolution on platelet detection

Platelet thrombi were formed on collagen in a PDMS flow chamber at a shear rate of 1400 s^{-1} with 5% labelled platelets (anti-CD41–CF555). Z-stacks with 1 µm distance between images were captured with wide-field microscopy (20x objective, NA 0.8). A set of z-stacks with the distance between 2 to 8 µm between focal planes were created by selecting a subset of images from the 1 µm stack. The z-stacks were analysed with the platelet count method. A) Platelet count as a function of z-axis resolution, presented as percent of platelet count at 1 µm resolution ($n=3$). B) Relation between z-axis resolution and variation in platelet count as a result of variations in z-axis starting position. Presented as CV % of six platelet counts of the same thrombus but with an incremental shift in starting position of 1 µm after each count ($n=3$). C) Visualization of the increased constrained position in z-axis of platelets to the image planes with decreasing z-axis resolution. Z-axis resolutions (distance between images) in the range of 1

to 8 μm is shown as D in the images. It should be noted that the z-axis and x-axis distance scales are different.

Figure 4. Comparison of fluorescence intensity and platelet count quantification methods.

The accumulation of platelets on collagen in four individual PDMS flow chamber experiments (shear rate: 1400 s^{-1}) was quantified using fluorescence intensity and platelet count. The blood samples were labelled both for the fluorescence intensity measurements (100% labelled platelets, DiOC₆) and the platelet count method (5% labelled platelets, anti-CD41–CF555). Time-lapse z-stack images were acquired with a 20x objective (NA 0.8) fluorescence wide-field microscope. A) Total fluorescence intensity in the images was measured over time in the visual field. The baselines are affected by the initial background fluorescence (as seen in the insert figure) and normalization is therefore required. B) Platelet count of labelled platelets in the visual field performed on the same experiments as in A. C)

Thrombus development in height for the four experiments in A and B, presented as 2D histogram heatmaps of platelet accumulation along the z-axis and over time.

Figure 5. Quantitative distribution in the z-axis for different quantification methods. Platelet thrombi

were formed on collagen in a PDMS flow chamber at a shear rate of 1400 s^{-1} with 5% labelled platelets

(anti-CD41–CF555) and 100% labelled platelets (DiOC₆). Z-stack images were acquired with a

fluorescence wide-field microscope (20x objective, NA 0.8) and a confocal microscope (20x objective, NA

0.8). The confocal volume (DiOC₆ platelets), wide-field platelet count (anti-CD41–CF555 platelets) and

wide-field fluorescence intensity (DiOC₆ platelets) were quantified on the same thrombus. A) The z-axis

distribution for the three methods are presented as values normalized to the range 0 to 100%. Note that

the captured z-stack started below the collagen coating and the thrombus starts at z-position 4 μm. B)

Sample fluorescence images used for quantification by the methods in A, showing three different

positions along the z-axis. The scale bar in the images represents 50 μm.

Figure 6. Method robustness of confocal volume estimation and platelet count quantification. Platelet thrombi were formed on collagen in a PDMS flow chamber (shear rate: 1400 s^{-1}) with fluorescent labels for volume measurement (100% labelled platelets, DiOC₆) and platelet count (5% labelled platelets, anti-CD41-CF555). Z-stack images were acquired with a fluorescence wide-field microscope (20x objective, NA 0.8) and a confocal microscope (20x objective, NA 0.8). A) Impact of image processing steps on confocal volume estimation. Comparing two available threshold methods in the software Imaris; absolute intensity and background subtraction. The original confocal image, surface reconstruction and volume calculations are presented from a representative flow chamber experiment. B) Volume estimations based on confocal images were compared from images captured with different master gain setting on the microscope. The confocal image for gain 510 and surface reconstruction for gain settings 510 and 600 are presented. The grid in the imaris images represents $25 \times 25 \mu\text{m}$. C) Volume calculation performed in Imaris using confocal images captured with different gain settings are presented as mean $\pm$

SD (n=3). D) Platelet count sensitivity was investigated by performing the platelet count method on images captured with different exposure times. The threshold probe optimized the threshold (black line) and adjusted for the intensity shift thereby allowing for a correct quantification. Values presented as mean $\pm$ SD (n=3).

Figure 7. Direct comparison of method robustness between the confocal volume calculation (A) and the platelet count method (B). Thrombi were formed on collagen in a PDMS flow chamber (shear rate: 1400 s^{-1}) with fluorescent labels for volume measurement (100% labelled platelets, DiOC₆) and platelet count (5% labelled platelets, anti-CD41-CF555). Z-stack images were acquired with a fluorescence wide-field microscope (20x objective, NA 0.8) and a confocal microscope (20x objective, NA 0.8). The z-stack images were subjected to intensity adjustments by multiplication with a factor in the range 0.92 to 1.08. Image stacks were then thresholded with a single threshold value optimized for the original image stacks (multiplier 1.00). The platelet count values are presented with and without the use of the threshold probe. All values are presented as mean $\pm$ SD of three experiments.

Figure 8. Quantification of thrombus formation and stability. Thrombus formation on collagen was performed in a PDMS flow chamber at a shear rate of 1400 s^{-1} , with 5% labelled platelets (anti-CD41–CF555). Time-lapse z-stack images were captured using wide-field fluorescence microscopy (20x objective, NA 0.8) and analysed with the platelet count method. Thrombus instability was induced by the addition of the Rho kinase inhibitor HA1077 ($120 \text{ } \mu\text{M}$). Platelet accumulation is presented as (A) heatmap 2D histogram of platelet count in time and z-axis, and (B) plotted as total platelet count in each time-point. The number of unstable platelets was calculated and are presented as a (C) percentage of platelets in a 2D heatmap corresponding to (A), and also plotted as the overall percentage unstable platelets in each time point (D). Stable/unstable platelets were also plotted in yellow/red as time-lapse images (E) to visualize the localization of these platelets during an embolization event. The images correspond to the red markers in B and D. The arrow represents $50 \text{ } \mu\text{m}$ and indicates the direction of flow.

Figure 9. Platelet count used for comparing two different flow chamber systems.

Flow chamber experiment was compared in a commercial flow chamber from Ibidi and an in-house constructed PDMS flow chamber at a shear rate of 1400 s^{-1} using a collagen patch to initiate thrombus formation. Experiments were performed with and without the ADP receptor inhibitor Cangrelor (5 μM). 5% labelled platelets (anti-CD41–CF555) was captured with a wide-field fluorescence microscope (20x objective, NA 0.8) as time-lapse z-stacks and analysed with the platelet count method. The thrombus development was quantified as platelet count per area (mm^2) of collagen coating. A) Flow chamber experiments from five donors are presented as mean with 95% confidence intervals (shaded area). B) Platelet count heatmaps from a single representative donor, presenting platelet count with time and z-axis for both flow chamber systems, with and without Cangrelor treatment.

Supplemental Figure S1. Stability of 5% platelet labeling

5% of the blood was fluorescently labeled using an Anti-CD41 antibody (For these experiments an AF 647 conjugated antibody was used, this since the CF-555 could not be detected in the flow cytometer). The portion of labeled platelets was measured using flow cytometry over 30 minutes. A and B) the unlabeled and labeled peaks (gate A) were well defined and separated throughout 30 minutes. C) The percentage of labeled platelets was unchanged during 30 minutes (n=3).

Supplemental Fig. S2 Experimental setup with the PDMS flow chamber.

The PDMS flow chamber is mounted on a collagen coated glass slide. The image capture zone covers the width of the flow chamber and about thrice the length of the collagen patch to ensure that all thrombus formation on collagen is covered in the image.

Supplemental Figure S3. Flow chart of image processing and analysis. Description of the components used in the python script for image processing and analysis. Each step is presented with the used python packages and modules.

Supplemental Figure S4. Platelet count is not affected by the time point used for the threshold probe.

The thresholding probe counts the number of objects as a function of the threshold level and finds a local maximum. The threshold for each experiment is set from one time point, it is therefore important that the object count does not differ depending on the used time point. A) The local maximum depending on the time point used. B) The platelet count was not affected of the time point used to set the threshold. A threshold set by the last time point was compared to a threshold that was set for each time point of the experiment.