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Fluorescence nanoscopy of platelets resolves platelet-state specific storage, release and uptake of proteins, opening for future diagnostic applications

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Author contributions:

JW and GA designed research

YY prepared samples

DR, HB and JW planned and built the STED instrument

DR and YY performed experiments

DR and JW analyzed data

JW, DR and YY wrote the paper

Abstract:

Dysregulation of how platelets store, sequester and release specific proteins seems to be implicated in many disease states, including cancer. Dual-color immunofluorescence stimulated emission depletion (STED) microscopy with 40 nm resolution was used to map pro-angiogenic VEGF, anti-angiogenic PF-4 and fibrinogen in more than 300 individual platelets. This imaging technique, offering distinct resolving advantages for protein localization studies in platelets, reveals that these proteins are stored in a segmented, zonal manner within regional clusters, significantly smaller than the size of an α -granule. No co-localization between the different proteins could be observed. Moreover, upon platelet activation by thrombin or ADP, the proteins were found to undergo significant spatial rearrangements, including alterations in the size and number of the protein clusters, which were specific for a certain protein and the type of activation induced. Following these observations, we devised a simple assignment procedure, showing that the three distinct states of platelets (non-, ADP- and thrombin-activated) can be identified based on the average size, number and peripheral localization profiles of the regional protein clusters within the platelets. This suggests that high-resolution spatial mapping in platelets of specific proteins is a useful procedure to detect and characterize deviations in the selective storage, release and uptake of these proteins in the platelets. Since these deviations seem to be specific for, and may even underlie certain patophysiological states including cancer, these findings may have interesting diagnostic and even therapeutic implications.

1. Introduction

It is becoming increasingly clear that there is a role for platelets beyond their participation in hemostasis. Platelet dysfunction can lead not only to bleeding disorders and thrombosis, but can also be associated with atherosclerosis, immunological disorders, as well as with cancer ^[1]. Cancer patients often present with changed coagulation parameters, increased platelet turnover, and various forms of thrombosis, whose severity correlate with the progress of the metastatic stage. The detailed mechanisms behind these observations have remained unclear, but recent findings have stimulated a renewed focus onto the involvement of platelets in tumor development and growth (for reviews, see ^[2] ^[3] ^[4]). Even the growth of very small tumors ($< 1\text{mm}^3$) is dependent on neovasculature development ^[5]. Studies on mice bearing human malignant tumour xenografts have shown that circulating platelets can specifically sequester angiogenesis regulatory proteins strongly correlated to micrometastasis, even in the presence of very small tumours ($< 1\text{mm}^3$) and when no significant change in the plasma content of the same angiogenesis regulatory proteins can be observed ^[6]. Similar alterations, representing a “metastatic/malignant platelet phenotype” has also been seen in patients with different, newly diagnosed metastatic diseases, displaying both increased and decreased platelet contents of specific, in particular angiogenesis regulating proteins ^[7]. This suggests that tumour-associated alterations in the protein content in platelets can be valuable biomarkers for early cancer progression.

Apart from mere up- and down-regulation of the protein content in platelets, further information with possible diagnostic and therapeutic implications may be obtained by studying the underlying regulation itself, i.e. changes in the selective harboring and subsequent release of platelet protein contents upon different activations. To date, more than 300 different molecules have been detected in platelet releasates ^[8]. Presumably, the regulation of secretion and uptake of proteins in the platelets is central for their multifaceted role and dys-regulation of this activity may to a large part underlie the pathophysiological states associated with aberrant platelet activation

and aggregation. Further understanding of this regulation is a key step to develop efficient therapies against cancer metastasis, and other unwanted effects associated to platelets, such as their pro-inflammatory role in atherosclerosis.

In platelets, α -granules act as the major protein storage and secretory organelle ^[1c;2]. α -granules may also contain proteins with apparently conflicting functions, including proangiogenic and antiangiogenic factors, and it remains an open question how release of such opposing factors can be physiologically balanced. Recent findings suggest that pro- and anti-angiogenic proteins are organized in separate α -granules in the platelets, which upon stimulation may be differentially released ^[5]. In contrast, other recent immunofluorescence co-localization data report little, if any, co-clustering of 15 angiogenesis-related proteins, instead indicating a stochastic distribution of the proteins into different α -granules, and that differential protein release is rather due to zonal segregation of proteins within individual granules ^[9]. This is supported by electron tomography data, providing evidence for morphological and molecular heterogeneities in the α -granule population, and high spatial segregation of cargo within individual α -granules ^[10].

To date, practically all imaging studies on storage and release of proteins in platelets have been performed by either immunofluorescence, typically using confocal laser scanning microscopy (CLSM), or by electron microscopy (EM). Fluorescence readouts offer high sensitivity, specificity and throughput, and the labeling and readout can be done with minor perturbations on the cells. However, the dimensions of a platelet is 2-4 μm , a typical α -granule 200-500nm ^[5;10], and any structures within the α -granule are of even smaller dimensions. Consequently, the resolution of CLSM (about 250nm), and even of the more elaborate structured illumination microscopy (at best 100nm ^[11]), makes it difficult to evaluate the distribution of proteins within the platelets, and in particular any co-localization of proteins or zonal confinement of proteins within individual α -granules. This lack of adequate resolution most likely underlies the controversy in how specific proteins are stored and released from platelets ^[12].

In contrast, EM can provide the necessary resolution. However, prior to imaging by EM, the samples must undergo repetitive fixation, dehydration, embedding, ultrathin sectioning and several rounds of staining, an extensive preparation protocol with low throughput that may also distort the samples. Typically, the sections examined are not sufficiently thick to encompass entire organelles, and hence morphologic records are based on inferences from organelle fragments. Moreover, the degree of immunogold-labeling is lower than for immunofluorescence. Due to steric hindrance^[13], or reduced probe penetration into the samples^[14], immunogold-labeling using gold particles with diameters of 5-15nm (typical range used) display a size-dependent reduction in the labelling efficiency by as much as an order of magnitude. This further reduces the completeness of view of the protein content within the individual organelles.

To overcome the limitations of CLSM and EM, respectively, we introduce fluorescence-based nanoscopy, based on stimulated emission depletion (STED) microscopy^[15], and with dual-color readout capability^[16] to study protein storage, co-localisation and secretion in human platelets. By immunofluorescence STED microscopy we can encompass both a sufficient spatial resolution and degree of labeling, avoid extensive and perturbing sample preparations, make our observations on intact organelles and platelets, and on sufficient numbers of platelets to take platelet-to-platelet variations into account. In this work, we utilized a home-built STED instrument with a resolution of 40 nm to study how the pro-angiogenic protein VEGF (vascular endothelial growth factor), the anti-angiogenic protein PF-4 (platelet factor 4) and fibrinogen (Fg) are distributed within human platelets at rest, and upon activation by thrombin and by ADP. Our studies provide specific, high-resolution information at the subcellular level on the spatial distribution of the proteins, and on the size and number of the regional clusters in which the proteins accumulate. Analyzing this information, we find that we can resolve features in the distributions that are specific for the different proteins and the type of activation induced. This suggests that protein distribution patterns and redistribution of proteins within

platelets may have a diagnostic value for certain patophysiological states, including early malignancies.

2. Results and discussion

2.1. STED versus confocal images of platelets

VEGF, PF-4 and Fg were separately mapped in individual platelets together with actin using confocal and STED imaging. Deconvolution of the STED images (STED+) was performed to reduce noise and increase contrast allowing for easier analysis ^[17]. As illustrated by the VEGF proteins imaged in figure 1A, the spatial distribution of the labeled proteins can not be resolved in the confocal images, and the localization, size and numbers of the protein clusters are not quantifiable. In contrast, the STED images can reveal clear and separate intensity profiles, providing detailed information about cluster numbers and sizes, and where the proteins are stored in the platelets. Also the images of the actin cytoskeleton benefit from the higher resolution, and give more details to the structures and shapes of the platelets. For the STED images of the VEGF distribution in the platelets (figure 1A), we note that the sizes of the VEGF clusters are significantly smaller than the expected size of the α -granules (200-500nm). The same observation was also made in the STED images of platelets where PF-4 or Fg was labeled (figure 1B). This supports recent reports ^[9-10] indicating that proteins are stored in the α -granules in a segregated, zonal manner, rather than within the full reservoir volume of the α -granule.

Figure 1:

(A): Confocal, STED and STED+ images of VEGF (green) and actin (red) on an inactivated platelet.

Scale bars: 1 μ m.

(B): Confocal and corresponding STED+ images, co-stained with VEGF/PF-4 (left), Fg/VEGF (middle), and PF-4/Fg (right). Scale bars: 1 μ m.

2.2. Protein distribution patterns of VEGF, PF-4 and Fg +/- activation by thrombin or ADP

Based on the ability to resolve sub-granular protein distribution features, we investigated to what extent STED microscopy can also resolve protein distribution patterns and redistribution of proteins within platelets, specific for certain physiological or pathophysiological states. VEGF, PF-4 or Fg in platelets from three individual healthy donors were labeled together with actin, and for each donor and each target protein the platelets were activated by either Thrombin (1 U/ml) or ADP (10 mM) or kept inactivated as control. STED images were then recorded to study distribution patterns and possible rearrangements of the proteins due to the activation. For actin, ring-like actin structures at the cell periphery are present in the majority of the control platelets (Figure 2, A1,B1,C1). When activated by thrombin a major transformation occurs where the platelets form filopodia and spread out over a larger area (Figure 2, A2,B2,C2). Upon ADP activation actin depolymerizes into its monomer form^[18]. This disallows phalloidin staining, and results in dotted intensity structures in the images of the ADP treated platelets (Figure 2, A3,B3,C3).

Figure 2: STED+ images of VEGF (A1-A3, green), PF4(B1-B3, green) and Fibrinogen (C1-C3, green), together with actin (red) in inactivated control (left), Thrombin-activated (middle) and ADP-activated (right) platelets. Scale bar 1 μ m.

For the targeted proteins VEGF, PF-4 and Fg, we observe systematic differences in sizes and numbers of the clusters, and in the spatial localization patterns between the different target proteins (figure 2, A-C,1-3). This indicates that the different proteins are not co-localized, but also not randomly distributed in granules and sub-granules within the platelets. To further verify this indication, STED imaging was performed with VEGF/PF-4, VEGF/Fg and PF-4/Fg co-stained in the same platelets. Overall, no co-localization of the protein clusters beyond a random level could be observed (figure 1B). Instead, an absence of co-localization, most prominent for the protein pairs VEGF/PF-4 and VEGF/Fg, indicates that there is even an anti-correlated localization of the proteins with respect to each other within the platelets. Further, the fact that the proteins also show distinctly different redistribution patterns upon activation, which are specific for a certain protein and the type of activation (figure 2,A-C,2-3), indicates that the proteins are subject to different storage, release and uptake mechanisms in the platelets.

2.3. Analysis of protein cluster sizes and numbers

To quantify the observed protein distribution patterns and the specific changes in protein storage coupled to platelet activation and to take platelet-to-platelet variations into account algorithm-based image analysis was performed on images of at least 30 individual platelets for each target protein and activation. For the recorded STED images, and for each of the imaged platelets, the number of fluorescent spots and their locations and sizes were analyzed (see material and methods). The size of the labeled protein spots indicates how the proteins are stored inside the platelets. Many small and separate intensity profiles, such as the ones for VEGF (Figure 2, A,1-3), suggest that the proteins are stored in small and separate sub-granules. While larger profiles, such as the ones observed for PF-4 or Fg in control platelets (Figure 2, B,1 and C,1, respectively), tell us that the proteins are either stored in larger sub-granules or even

over whole granules, or that the protein containing sub-granules are packed very tightly and cannot be separately resolved. The size of the imaged clusters are hereinafter denoted by D_c , the diameter of the fluorescence spots estimated as described in the material and methods section. Cumulative histograms of D_c for all clusters of VEGF, PF-4 and Fg identified in the control, ADP- and thrombin-activated platelets are shown in figure S1. The average D_c is significantly smaller for VEGF than for PF-4 and Fg. For all proteins, changes in both the average and the distribution of D_c can be observed upon activation. In figure 3(A1,B1,C1), the average protein cluster size within each imaged platelet, $\bar{D}_c$, is plotted versus the number of clusters per platelet, N_c . Each small dot in the graphs represents one individual platelet. The graphs display a large platelet-to-platelet variability of $\bar{D}_c$ and N_c , which could be observed also within the same samples. This indicates that several platelets need to be measured and analyzed to obtain protein cluster data of statistical meaning.

From the $\bar{D}_c$ versus N_c data in figure 3 (A1,B1,C1) it is possible to estimate if there is a release or an uptake of proteins following platelet activation. For VEGF a reduction in both $\bar{D}_c$ and N_c can be seen for the ADP treated platelets, as compared to the non-activated control. This indicates that VEGF is released from the platelets, and is in agreement with previous observations ^[19]. Activation by Thrombin leads to smaller changes, where $\bar{D}_c$ decreases but N_c increases, rather suggesting a reorganization of the VEGF protein. In contrast, for PF-4 both $\bar{D}_c$ and N_c decrease upon thrombin activation, and its spatial distribution is less centralized (figure 2-B2), indicating release of PF-4. On the other hand, upon ADP stimulation the PF-4 cluster distribution in the platelets look similar to that in the control platelets (figure 2-B3), $\bar{D}_c$ remains similar, but N_c increases. This indicates that PF-4 remains in the platelets, as recently reported ^[19b], and that there may even be an uptake of PF-4 by the platelets from the suspension. This uptake of anti-angiogenic PF-4, combined with release of pro-angiogenic VEGF may distinctly promote angiogenesis in local regions with high

ADP concentrations. Fg is very strongly released upon thrombin activation, with both $\bar{D}_c$ and N_c markedly reduced. Following ADP treatment $\bar{D}_c$ is decreased compared to in the control platelets, but N_c increases, and the Fg clusters are almost exclusively localized at the periphery of the platelets (Figure 2, C1, C3). This indicates that endogenous proteins have been transported to the edge of the cell, but the strongly peripheral distribution can also be due to external Fg binding to the activated platelets [20].

Despite a large platelet-to-platelet variability in the $\bar{D}_c$ versus N_c plots in figure 3 (A1,B1,C1) and except for clusters of VEGF in the in-activated and thrombin-activated platelets, the $\bar{D}_c$ versus N_c distributions are distinctly different for all target proteins and categories of platelets. This indicates that size and number characteristics of clusters of specific proteins within platelets can provide signatures of underlying physiological conditions or states of activation of the platelets.

Figure 3: Regional protein cluster data, recorded from labeled VEGF (A), PF-4 (B), and Fg (C) in platelets. (A1, B1, C1): Scatter plots of the average protein cluster

diameters $\bar{D}_c$ versus the number of clusters, N_c , where each dot represents the determined values in one platelet, for inactivated (black squares), thrombin-activated (blue circles), and ADP-activated (red triangles) platelets. (A2, B2, C2): Average $\bar{D}_c$ and N_c values determined for each of the three blood donors, in their inactivated (black squares), thrombin-activated (blue circles), and ADP-activated (red triangles) platelets. Large symbols with error bars indicate average $\bar{D}_c$ and N_c values, and the average standard deviation in $\bar{D}_c$ and N_c for all the three donors. (A3, B3, C3): Peripheral localization profiles, $P(r)$, of regional protein clusters in inactivated, control (black), thrombin-activated (blue), and ADP-activated (red) platelets. (C4) Student t-test between the average $\bar{D}_c$, N_c and $P(r)$ values of the donors where “x” signifies $P \leq 0.05$, “xx” $P \leq 0.01$ and “xxx” $P \leq 0.001$, for Thrombin (blue) and ADP (red) activation as compared to control.

2.4. Analysis of localization patterns of the protein clusters within the platelets

To allow a more objective and quantifiable analysis of the protein localization patterns, the total area of each imaged platelet was obtained from the actin image and divided into five separate concentric zones of equal size (SI Figure S2). Summing up in which of these zones each of the identified protein cluster in the platelet resided yielded peripheral localization probability profiles, $P(r)$, with $r = \{1, \dots, 5\}$ and $\sum_{r=1}^5 P(r) = 1$, providing a measure of how centrally/peripherally the protein clusters are located in the platelet, and how activation affects their peripheral distribution. Due to the lower depth resolution, which is the same as in normal confocal microscopy (700-900nm), $P(r)$ is not an absolute value in terms of how peripherally located the proteins are but rather a two dimensional projection of the volume of the platelet (SI figure S3). On the other hand, since the thickness of the studied platelets is in the order of 1-1.5 μm we will, due to the lower resolution, detect almost all labeled proteins in the platelet. This effectively decreases the time to scan each individual platelet since there is no need to perform extensive z-stacking of images. The $P(r)$ for the different target protein clusters in control platelets, and in platelets upon ADP- and

thrombin-activation are shown in figure 3 (A3,B3,C3).

For VEGF a clear reallocation occurs upon ADP stimulation, towards the peripheral parts of the cell where VEGF can be released (figure 3,A3). For thrombin-activated and control platelets the distribution of VEGF is more homogenous. PF-4 (figure 3,B3) mainly resides in the central part of the control platelets but shows a strong reallocation towards the cell membrane upon thrombin activation. Upon ADP activation, PF-4 mainly remains in the central zones but not as clearly as for control cells. Fg (figure 3, C3) shows an even more central distribution than PF-4 for control cells. Upon thrombin treatment, the proteins move slightly towards the edge, while for ADP there is a significant change, with a majority of the proteins in the two outermost zones. Detailed information regarding the average location, amount and size of the labeled protein intensity profiles is given in table S1 (SI).

Corresponding $P(r)$ profiles were also generated for actin, based on the distribution of the fluorescence intensity between the five zones of the platelets (figure S4). Overall, compared to control platelets, labeled actin is to a larger extent found in the peripheral parts upon ADP-activation, and in the central parts upon thrombin-activation. However, the $P(r)$ differences are in general smaller than those for the target proteins in figure 3 (A3,B3,C3).

2.5. Identification of states based on protein cluster size, number of clusters, and the peripheral localization profiles

As a possible strategy to identify different pathophysiological states, we estimated the ability to distinguish the three different states of the platelets (non-, ADP- and thrombin-activated platelets) for an individual donor, by determining $\bar{D}_c$, N_c and $P(r)$ in individual platelets. In figure 3 (A2,B2,C2) the average $\bar{D}_c$ and N_c for the different individual donors are plotted for each of the targeted proteins VEGF, PF-4 and Fg, for control platelets as well as for thrombin- and ADP- activated platelets. We assumed for simplicity the corresponding distributions of $\bar{D}_c$ and N_c for the platelets

from all donors to be Gaussian distributed, and their average values and standard deviations to be given by the mean values of the corresponding average values and standard deviations of the individual donors.

From its $\bar{D}_c$ and N_c , each one of the platelets plotted in figure 3 (A1,B1,C1) was classified to be a control, ADP-activated, or thrombin-activated platelet, based on which of the platelet-state probability distributions for the particular target protein (VEGF, PF-4 or Fg) that showed the highest probability value for the $\bar{D}_c$ and the N_c values of that platelet, respectively. The outcome of these platelet state assignments are given in table S2, left and middle columns (SI). Overall, as also indicated in the graphs in figure 3(A1,B1,C1), $\bar{D}_c$ and N_c assignments of VEGF and PF-4 labeled platelets provides a good ability to discriminate ADP-activated from thrombin-activated and control platelets, and assignment of Fg labeled platelets show a good ability to discriminate thrombin- from ADP-activated and control platelets.

A similar platelet-identification procedure as for $\bar{D}_c$ and N_c was also applied based on the $P(r)$ profile of each platelet. For each imaged platelet, its $P(r)$ was compared to the $P(r)$ of the same target protein for the three different platelet states (figure 3, A3,B3,C3)). The platelets were then assigned to that platelet state, which $P(r)$ by least-squares analysis best resembled that of the platelet. For all targeted proteins and platelet states, this $P(r)$ -assignment always assigned the correct state of the individual platelets with the highest probability (SI, table S2, right column), and apart from thrombin-activated platelets labeled for VEGF (43%), and ADP-activated platelets labeled for PF-4 (42%), with better than 50% probability.

Combining the $\bar{D}_c$, N_c and $P(r)$ assignments above can further improve the identification strength. Here, all platelets assigned to the same state in at least two out of the three separate assignments above, were assigned to that state. The resulting combined assignment probabilities are listed in table 1. For all targeted proteins and platelet states the correct state was assigned with the highest probability. Labeled VEGF can distinguish ADP-activated platelets with 91% probability, and labeled PF-4

and Fg can distinguish thrombin-activated platelets with 73% and 85% probability, respectively. It should be noted that the diagonal elements in the assignment probability matrices in table 1, indicate the probabilities to correctly assign the state of one single platelet, and are based on the image of one target protein. By investigating a combination of proteins, and by imaging several platelets, the identification strength can be significantly increased. Moreover, in the calculation of the classification probabilities in table 1 no information about the spatial distribution patterns of actin has been included. This would further increase the identification strength, although the differences in $P(r)$ of actin between the different platelet states are smaller than those observed for the target proteins.

VEGF	Control	Thromb.	ADP	Unclass.
Control	0,49	0,30	0,05	0,16
Thrombin	0,24	0,28	0,21	0,26
ADP	0,02	0,03	0,91	0,04

PF-4	Control	Thromb.	ADP	Unclass.
Control	0,38	0,32	0,10	0,21
Thrombin	0,12	0,73	0,04	0,11
ADP	0,19	0,22	0,36	0,23

Fg	Control	Thromb.	ADP	Unclass.
Control	0,60	0,20	0,05	0,16
Thrombin	0,09	0,85	0,01	0,06
ADP	0,11	0,08	0,63	0,18

Table 1: Combined assignment probability matrices of individual platelets. Assignment probabilities for VEGF (top), PF-4 (middle), and Fg-labeled (bottom) platelets, based on the combined $\bar{D}_c$, N_c , and $P(r)$ assignments of their detected protein clusters. The matrix elements indicate the probability to assign a platelet with a state given by the vertical position in the matrix, to a state indicated by the horizontal position. The three diagonal elements in the matrices (marked gray) thus indicate the probability to correctly assign the different platelet states. Off-diagonal elements indicate miss-classified platelets. Right columns: Fraction of un-classified platelets, for which the three assignment steps resulted in three different assignments.

3. Conclusions

STED imaging combines the major advantages of immunofluorescence CLSM and immunogold EM for studies of protein distribution patterns in platelets: a sufficient spatial resolution and degree of labelling can be achieved, extensive and perturbative sample preparations can be avoided, and studies comprising a larger number of intact platelets are feasible. Owing to these features, our data provides solid support to the view that proteins are stored in a segmented, not co-localized manner in the α -granules. For the proteins studied (VEGF, PF-4 and Fg) the sizes, numbers and spatial distributions of their regional clusters display clear differences from one protein to another, and show significant rearrangements and alterations upon activation, specific for a certain protein and type of activation induced. This indicates that the proteins are not only stored differently, but also subject to different release and uptake mechanisms.

Inspired by recent evidence that platelet dysfunction and alterations in the protein contents in platelets correlate with a range of disease states, including cancer, we investigated if fluorescence nanoscopy of specific proteins in the platelets can be used as a diagnostic procedure, and if spatial mapping of certain proteins, subject to selective storage, release and uptake in the platelets, can reveal features of protein storage and turnover, specific for different physiological or pathophysiological states. We show that the average size, number, and peripheral localization profiles of regional protein clusters within the platelets can be used to identify three distinct platelet states (non-, ADP- and thrombin-activated), with high specificity at the single platelet level. Moreover, in a diagnostic scenario, given the huge concentration of platelets in peripheral blood, and since STED in contrast to EM can image a sufficient number of platelets in a reasonable time, several platelets from the same individual can be monitored, and the spatial distribution patterns of not only one protein, but of several specific proteins can be analyzed. Thereby, we expect that higher identification strengths can be reached, inherent platelet-to-platelet variations can be

taken into consideration, and that a broader range of also less distinct platelet states can be resolved. In summary, the use of high-resolution distribution patterns of specific proteins within platelets as a diagnostic marker opens interesting prospects. The association of platelet dysfunction with malignancy, and the possibility to detect and characterize aberrant protein storage, release and uptake in platelets with STED microscopy merit further investigations.

4. Materials and Methods

4.1. Platelet isolation

Buffy coat from healthy donors (Blood Central, Karolinska Hospital, Stockholm) was centrifuged (180g, 10 minutes) at room temperature to separate the platelet-containing plasma layer. Dulbecco's modified phosphate buffer saline (DPBS, without Ca^{2+} and Mg^{2+} , Hyclone) was added to the plasma and the suspension was centrifuged (900g, 10 minutes), resulting in clearly separated layers of red blood cells, platelets and supernatant. The supernatant and platelets were gently aspirated out and resuspended. In this suspension the cell count of platelets was determined using a hemacytometer and adjusted to $100 \times 10^6/\text{ml}$ with DPBS.

4.2. Platelet activation

Platelet suspension (300 μl) was added into a well of a 24-well microplate, where a piece of a polylysine coated coverslip (12 mm) was placed, incubated at 37°C for 1 hour under static condition for cell attachment, and then treated with thrombin (1 NIH unit/ml, Sigma, Sweden) or ADP (10 mM, Sigma, Sweden) for 5 minutes. The cells were then immediately fixed with 3.7% formaldehyde in DPBS for 15 minutes. Control samples were prepared in parallel without agonist treatment.

4.3. Immunostaining

Sheep anti-mouse and goat anti-rabbit antibodies (both from Dianova, Hamburg, Germany) was conjugated with ATTO 594 and ATTO 647N, respectively (Atto-Tec GmbH, Siegen, Germany). Mouse monoclonal antibodies were used for labeling against human PF-4 (R&D Systems, Europe), VEGF (BD Science, Europe), and Fg

(Santa Cruz Biotechnology, Heidelberg, Germany). Phalloidin linked with ATTO 647N (Atto-Tec GmbH, Siegen, Germany) was used to stain actin. For colocalization studies rabbit polyclonal antibodies were used for labeling against human PF-4, VEGF, and fibrinogen (all from Santa Cruz Biotechnology, Heidelberg, Germany).

Unless otherwise stated, DPBS was used throughout the study as buffer. The fixed platelets were permeabilized with Triton X-100 (0.2%) for 15 minutes and then blocked for nonspecific binding with bovine serum albumin (1%, Sigma, Sweden) over night at 4°C. The cells were then washed once with DPBS on an orbital shaker (300 rpm), followed by incubation with primary antibodies against PF-4 (2 µg/ml), VEGF (2.5 µg/ml) or Fg (2.5 µg/ml) over night under room temperature. The samples were then washed twice with DPBS on an orbital shaker (300 rpm), incubated with the secondary antibody (3 µg/ml) together with Phalloidin ATTO 647N for 3 hours under room temperature, and then washed three times on an orbital shaker (300 rpm) before being mounted onto microslides with Mowiol-Dabco mounting medium (Sigma, Sweden).

4.4. STED microscopy

The home-built STED microscope has been described in detail before ^[21], but with a few minor differences ^[22]. In brief, two excitation beams (570 ± 5 and 647 ± 5 nm) and two STED beams (710 ± 10 and 750 ± 10 nm) are separated from the same pulsed supercontinuum laser (SC-450-PP-HE, Fianium Ltd., Southampton, UK). The STED beams are passed through a vortex phase plate (VPP-1, RPC Photonics, Rochester, NY, USA) imprinting a helical phase mask of 2π onto the wave-front. This, together with circular polarization of the STED beam creates effective destructive interference at the center of the focal point and thus the deep central minima in the doughnut shape of the STED-point spread function (PSF). The excitation and corresponding STED beams for the two different dyes are separated in time by 40 ns before they reach the sample. This time delay, accompanied by the same time separation in the detectors (SPCM AQR-12, Perkin-Elmer optoelectronics) efficiently reduces the cross-talk between the two dyes to only a few percent. The acquired images were taken with average STED powers of 2.2 mW at 710 ± 10 nm and 1.2 mW at 750 ± 10 nm,

achieving a resolution of about 40 nm in both channels as compared to the confocal resolution of 250-300 nm. The pixel size was kept constant at 20 nm for the STED images and 50 nm for the confocal images.

4.5. Image processing and analysis

Prior to image analysis the STED images were rescaled to 10 nm per pixel and then deconvoluted by 20 iterations of the Richardson-Lucy deconvolution algorithm using a 40 nm Lorentzian PSF ^[23]. Thereafter, the images were analyzed using a custom written code in Matlab (MathWorks Inc, Massachusetts, USA). In brief, the strategy was to use the protein images (green) to determine the sizes and numbers of their clusters, and the actin images (red) to determine the size and area of platelets. Thereby, combining the green and red images, the total number, density and distribution of the protein clusters within the platelets can be quantified.

The locations of the proteins are determined from the centers of the fluorescent peaks in the images, identified by having an intensity at least four times above the background level within the cell. In order to avoid background effects which may broaden low intensity profiles, the size of the profiles was only calculated for centers where the profile peak intensity was at least ten times higher than the average background level in the cell. The size was calculated for each profile separately as the average FWHM measured over 20 angles. The actin images were used to determine the contact area of the platelets and to create five zones of equal areas within each platelet, surrounding the center of the platelet at different peripheral distances. The distribution of protein clusters between these zones provides a measure of how peripherally localized the proteins are within each platelet.

4.6. Statistics

Samples from three different donors were investigated. From each individual blood donor three groups of samples were prepared for studying each of the proteins PF-4, VEGF and Fg, and for each protein, its distribution was monitored for resting platelets, as well as for thrombin- and ADP-treated platelets. From each individual microscopic sample at least eight images were captured on randomly chosen platelets. The number, density, size and distribution of the sub-granule proteins are represented as the

average plus and minus the standard deviation of the investigated sets, and taken as the average from the three donors, giving each donor the same impact. Two-tailed student t-tests with unequal variance were applied on the comparisons of interest, and with the results displayed in figure 3.

4.7. Ethical approval

Ethical approval for this study has been obtained from the local ethical committee of Karolinska Institutet, Stockholm, Dnr: 2010/504-31.

Supporting Information

[Supporting information is available online from the Wiley Online Library or from the author.](#)

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Supplementary information (SI)

Figure S1: Cumulative histograms showing the size (diameter) distribution of the detected regional VEGF (top row), PF-4 (middle row), and Fg (lowest row) protein clusters in inactivated, control platelets (left column), thrombin-activated platelets (middle column), and ADP-activated platelets (right column). Number of platelets investigated for each category is indicated in the upper right corner of each histogram, together with the average cluster size (diameter) $\pm$ standard deviation.

Figure S2: Determined total area of a platelet, based on the extension of actin (red) fluorescence in the image (left). The middle image shows located peaks (white pixels) for the protein size, number and location analysis. The total area of the platelet was divided into five peripheral zones (right) with equal areas. The five zones were created by finding the center of the platelets actin image and then extending each zone to cover 20% of the total area spanned by the actin (see also methods and materials section).

Figure S3: Each platelet image is a 2-dimensional projection of a three-dimensional volume with a thickness that is the same as the depth (Z) resolution. Due to the lower Z-resolution (700-900nm) as compared to the X-Y resolution (40nm) for the taken STED images, almost the entire volume of the platelet will be projected to the captured two-dimensional image. A protein laying close to the central part of the platelet will also be projected to the central part in the image (B) but a protein located near the top or bottom membrane of the platelet could be depicted to be more centrally located than it actually is (A). Thus $p(r)$ might underestimate the actual distribution of proteins in the platelet but still gives a good estimate as to how peripherally located the proteins are. (C) X-Y images of labeled VEGF and actin show higher resolution for STED as compared to confocal in both dimensions. Corresponding X-Z images of the same platelet (section of the X-Y plane recorded is indicated with a dashed white line) show only higher resolution in the X-dimension.

Figure S4: Peripheral localization profiles, $P(r)$, of actin in inactivated, control (black, dashed), thrombin-activated (blue dashed), and ADP-activated (red dashed) platelets. The $P(r)$ values for actin were calculated based on how the fluorescence from labeled actin was distributed between the five peripheral zones of each platelet.

Agonist		Diameter	Number	Density	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5	Periph.
[# Platelets]		[nm]	[#]	[#/μm ²]	[%]	[%]	[%]	[%]	[%]	[0-1]
Control [34]	Average	87	71	14	23%	23%	22%	18%	14%	0,44
	StdDev	13	21	3	7%	6%	5%	6%	6%	0,07
Thrombin [30]	Average	78	76	14	17%	20%	22%	20%	22%	0,52
	StdDev	15	17	3	6%	6%	6%	5%	8%	0,07
ADP [35]	Average	67	35	7	18%	18%	16%	16%	32%	0,56
	StdDev	7	14	2	9%	8%	6%	6%	10%	0,09

Agonist		Diameter	Number	Density	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5	Periph.
[# Platelets]		[nm]	[#]	[#/μm ²]	[%]	[%]	[%]	[%]	[%]	[0-1]
Control [48]	Average	122	65	12	33%	24%	18%	14%	11%	0,37
	StdDev	38	27	5	10%	8%	6%	7%	7%	0,09
Thrombin [56]	Average	93	54	11	19%	17%	17%	16%	31%	0,56
	StdDev	28	18	4	10%	7%	5%	6%	10%	0,10
ADP [40]	Average	123	96	20	26%	24%	20%	18%	12%	0,42
	StdDev	30	30	7	8%	6%	5%	6%	6%	0,07

Agonist		Diameter	Number	Density	Zone 1	Zone 2	Zone 3	Zone 4	Zone 5	Periph.
[# Platelets]		[nm]	[#]	[#/μm ²]	[%]	[%]	[%]	[%]	[%]	[0-1]
Control [38]	Average	158	72	14	48%	26%	14%	7%	6%	0,24
	StdDev	60	26	4	9%	8%	5%	5%	6%	0,07
Thrombin [36]	Average	108	28	6	27%	23%	18%	13%	18%	0,43
	StdDev	29	12	2	15%	15%	11%	10%	15%	0,16
ADP [41]	Average	128	89	18	11%	11%	14%	24%	40%	0,68
	StdDev	31	21	5	7%	6%	7%	8%	12%	0,10

Table S1: Average values and standard deviations of size, number, density and zone distribution for the three studied proteins and for control (green), thrombin (blue) and ADP (red) treatment. Periph is a value ranging from zero to one where zero means all proteins are in the innermost zone and one means all proteins are in the outermost zone.

	Diameter $\bar{D}_c$			Number, N_c			Distribution, $P(r)$		
	Control	Thromb	ADP	Control	Thromb	ADP	Control	Thromb	ADP
VEGF									
Control	20	15	5	12	19	9	27	10	3
Thrombin	8	4	18	12	15	3	9	13	8
ADP	3	5	28	2	1	33	5	5	26
PF-4									
Control	25	28	6	12	30	17	38	9	12
Thrombin	11	41	7	17	37	5	10	38	11
ADP	16	22	10	9	10	29	15	13	20
Fg									
Control	17	13	6	17	9	10	27	9	0
Thrombin	7	24	2	2	31	0	10	18	5
ADP	14	20	13	16	1	30	1	8	38

Table S2:

(A): 3x3 assignment matrices of individual platelets, for VEGF (top), PF-4 (middle), and Fg-labeled (bottom) platelets, based on the $\bar{D}_c$ (left), N_c (middle), and $P(r)$ (left) parameters of their detected protein clusters. Each element in the matrices gives the number of platelets in a state given by the vertical position in the matrix, which are assigned to a platelet state indicated by the horizontal position in the matrix. The three diagonal elements in the matrices thus indicate the number of correctly assigned platelets, i.e. a control assigned as a control, a thrombin-activated as a thrombin-activated, and an ADP-activated platelet assigned as an ADP-activated platelet, respectively. Off-diagonal elements indicate the number of mis-classified platelets.

(B): Schematic figure illustrating the combined, sequential assignment of platelets to states. It is based on three assignment steps, using the platelet parameters $\bar{D}_c$ (first step), N_c (second step), and $P(r)$ (third step). The platelet is assigned to a state if two out of the three assignments give the same result. If each assignment step leads to different results, the platelet is "unclassified".