Chapter 3

Only the Truth Would Enlighten Us—The Advantages and Disadvantages of Flow Cytometry as a Method of Choice in the Study of Mouse and Rat Platelets

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Abstract

Increasing number of transgenic and knockout strains of laboratory rodents has been developed to provide reliable models of human cardiovascular diseases. Due to apparent differences in platelet physiology, morphology, biochemistry, etc. between rodents and men, methods employed to study blood platelets in rodents should always consider these differences in a reasonably critical way. Flow cytometry is a convenient tool that enables to easily cope with the minute amounts of the available biological material and providing an extremely versatile information. This review focuses on the practical and methodological aspects of flow cytometry, pointing to the key elements of the commonly used protocols for determining of multiple parameters of blood platelet (patho)physiology in mice and rats. We summarized and critically reviewed the available procedures, as well as figured out how to overcome possible obstacles, shortcomings, drawbacks or artefacts that a researcher may encounter when monitoring various phenomena intimately associated with blood platelet biology. Flow cytometry assays have been also collated with some alternative techniques (intravital fluorescence microscopy, *in vitro* platelet adhesion under flow conditions). We hope that our paper may further facilitate other researchers to study mouse and rat platelets with the use of the most optimal and the least artefact-prone procedures.

Keywords: flow cytometry, mouse, platelets, rat, rodents

1. Introduction

Among various laboratory animals, mice and rats offer several benefits that make them convenient tools for preclinical *in vitro* and *in vivo* studies. Considering the variety of available methods used to study blood platelet activation and/or reactivity, flow cytometry seems an attractive technique that largely counterbalances the constraints of using rodents and as such it enables outcomes of laboratory research to be transferred successfully to clinical practice. However, the use of flow cytometry in the study of platelet function in laboratory rodents, such as mice and rats, is a quite distinguished field in the methodology of cytometry research. Differences in relation to the studying of human platelets do not concern so much the equipment requirements, but the protocols for blood sampling and preparation of collected biological material. Simple and straightforward implementations of the procedures commonly employed in human platelet research for the investigating of rodent platelets usually do not bode a researcher a success in obtaining reliable and repeatable results. This is an important issue because the use of laboratory animals in preclinical studies has become almost a universal practice, and the results of animal tests are often considered as an incentive to start further clinical trials. In this chapter, the particular attention was paid to methodological and technical aspects of research protocols, and especially concerning the used mice and rats strains, methods of animal anesthesia, blood withdrawal, preparation and anticoagulation, cell fixation, selection of platelet agonists, and the last but not the least important - the choice of antibodies. All the above could potentially lead to distortions in the collected results and/or drive to obtain false conclusions. In this chapter, the results obtainable with flow cytometry assays have been collated with those obtained with the use of alternative techniques applied in scientific research of particular objects, such as intravital fluorescence microscopy or *in vitro* platelet adhesion under flow conditions. Also, protocols for estimating blood platelet viability —the parameter that can potentially interfere with functional assays and may have an impact on final conclusion of an experiment—are critically reviewed. Further, we describe the methods invented for measuring intraplatelet calcium mobilization during platelet activation and emphasize the risk of artifacts in the course of these procedures. Another important topic is the selection of a flow cytometry approach for studying possible nitric oxide generation in blood platelets. We also summarize the available procedures, [as well] as we present our experience in the overcoming of the troubles with setting up the alternative approaches and the least artifact-prone protocols for the quantification of platelet-derived microvesicles. Moreover, one of the chapters describes possibilities of using flow cytometry in some challenging research applications, which, at the first side, appear quite unusual for this technique—the investigations of subcellular and paracellular objects, such as mitochondria. In particular, the characterization of parameters explored in functional mitochondria and applications of such methods to study platelet activation are presented. Finally, we summarize studies focused on monitoring apoptosis in blood platelets of rodents with the use of flow cytometric approach.

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2. From the beginning to the end—search for an optimal discriminative flow cytometric protocol for studying mouse and rat platelets

Because of the ethical limitations and restraints on human research, animal models, especially the ones involving rodents, constitute a convenient and recognized tool for studying platelet functioning. However, it should be pointed out that platelets from rodents differ in certain morphological characteristics from human platelets. For instance, murine platelets are much smaller than human (with a diameter in the range of 0.5 μm compared to 1–4 μm in human platelets), but they are more than two times abundant (0.7 to even 1.5 million cells per microliter of blood [1]). In the case of rat platelets, they are bigger (1.5–2.5 μm), but approximately equally abundant as in mice (ca. 1.3 million/μl of blood [2]). The commercially available beads, covered with a fluorescent label, of various diameters may be of a great convenience when determining the exact sizes of murine and rat platelets [3]. Such beads, added to the sample, may facilitate the determination of reference ranges and the estimation of platelet size at resting conditions and upon the *in vitro* stimulation of platelets with agonists (**Figure 1**).

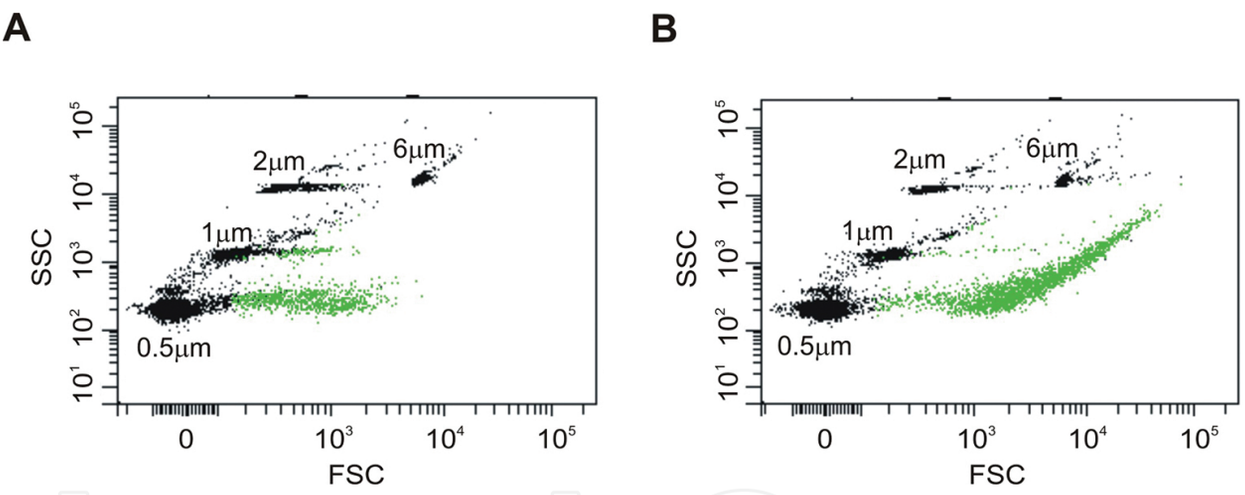

Figure 1. Scatter dot plots of polystyrene beads and resting and thrombin-stimulated isolated mouse platelets. The beads of the following sizes were applied: 0.5, 1, 2, and 6 μm. Mouse platelets were isolated by gel filtration and were labeled with FITC-conjugated anti-CD41/61 gating antibodies (green dots).

It should be noted that despite some similarities in terms of platelet surface antigens between men and rodents, it is crucial to use different antibodies for labeling human, murine, and rat blood platelets. Application of the same antibodies for these species is not indicated as any putative cross-reactivity of antibodies against human, murine, and rat platelet surface antigens is minimal or none [4]. The selection of proper antibodies can be a particular problem for the researcher, as the range of commercially available monoclonal antibodies against the rodent platelet antigens is quite limited, especially in the case of rat platelets. In such cases, it remains to use polyclonal antibodies, which are much less specific than monoclonal antibodies, because they recognize various nonspecific antigenic determinants. In the case of murine platelets, it should be noted that despite a significantly larger offer of commercially available antibodies,

they significantly differ from each other and hence are not equivalent in binding to target platelet surface antigens (**Figure 2**).

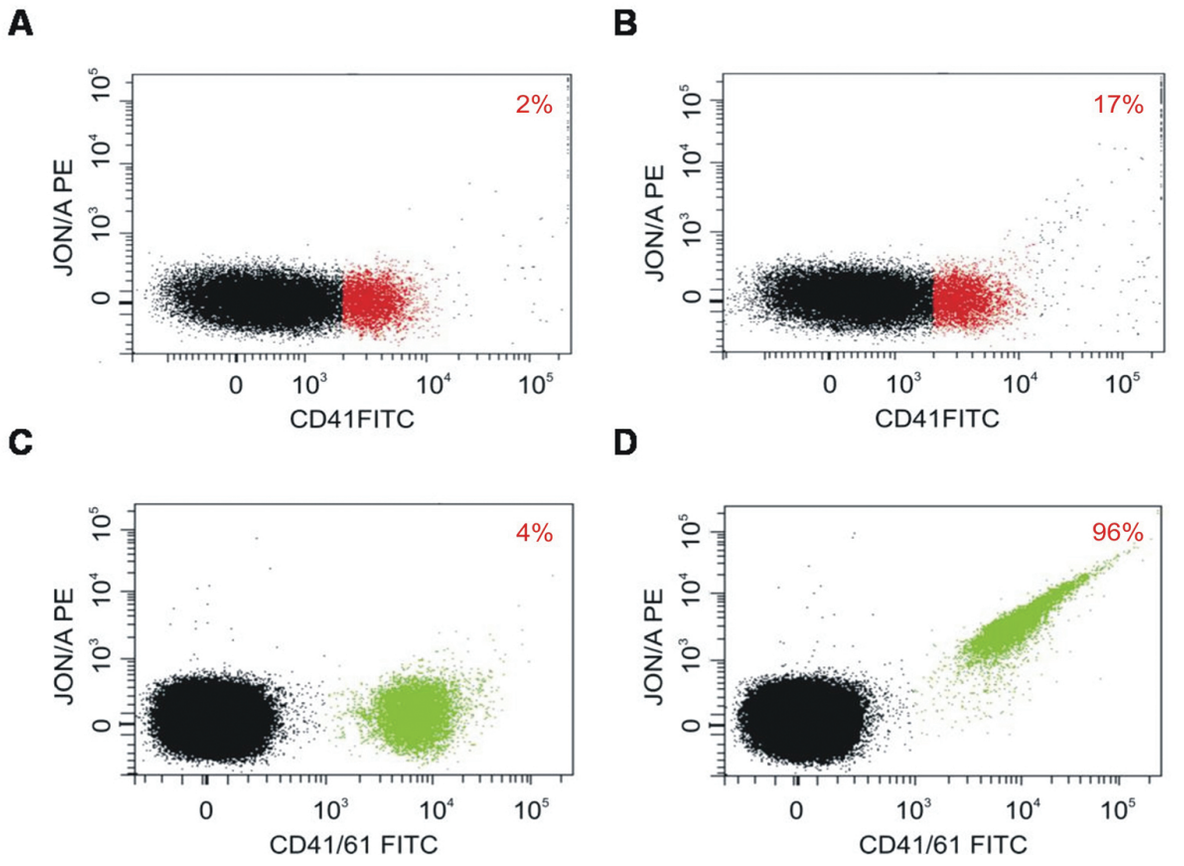

Figure 2. Fluorescence dot plots of resting and thrombin-activated mouse platelets labeled with FITC-conjugated anti-CD41 or anti-CD41/61 gating antibodies and with PE-conjugated JON/A (anti-theactivated form of integrin $\alpha_{IIb}\beta_3$) antibodies. Red dots represent the CD41-positive objects (A, B), whereas green dots represent the CD41/61-positive objects in samples of resting platelets (A, C) or platelets stimulated with 0.25 U/ml human thrombin (15 min, RT) (B, D). The percentage of JON/A-positive platelets was obtained after subtracting of nonspecific mouse IgG binding and was presented as a numerical value (in red).

As occasionally reported, merely the selected products are appropriate to identify the platelets without the interference from other cells [5]. Platelet reactivity is usually evaluated by the monitoring of the expressions of platelet surface membrane antigens after platelet stimulation with physiological or, rarely, nonphysiological agonists. Interestingly, while in the case of ADP and thrombin we receive the response of murine platelets similar to that observed in humans, platelet activation in mice after collagen stimulation is observed to be significantly lower than in human platelets (**Figure 3**).

When using flow cytometry, rodent platelets are usually identified in a whole blood according to the presence of a typical constitutive platelet surface membrane antigen (CD41, CD61, and CD41/61) on their surface [6, 7]. However, in some studies, the researchers used to rely only on the analysis of the FSC and SSC signals, which caused them to extract considerable pools

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of other cellular components (erroneously regarded as blood platelets) of whole blood or plasma-free blood [8]. It has been proven that there are some advantages of platelet gating on the basis of the presence of a given pan-platelet surface antigen over the identification of platelets on the virtue of FSC/SSC images [9]. First, such an approach enables to "pick up" all platelet-originated objects and to further distinguish normoplatelets from other objects (small aggregates, microparticles, dust, etc.). Moreover, platelets can be visualized in different cohorts as single platelets or aggregates with other blood cells. It clearly indicates that gating of platelets based on FSC/SSC instead of a demarcation with the use of a common platelet-specific marker can lead to the increased risk of collecting artifacts (i.e., objects resembling blood platelets in size and/or light scattering, missing the platelets in aggregates) (**Figure 4**).

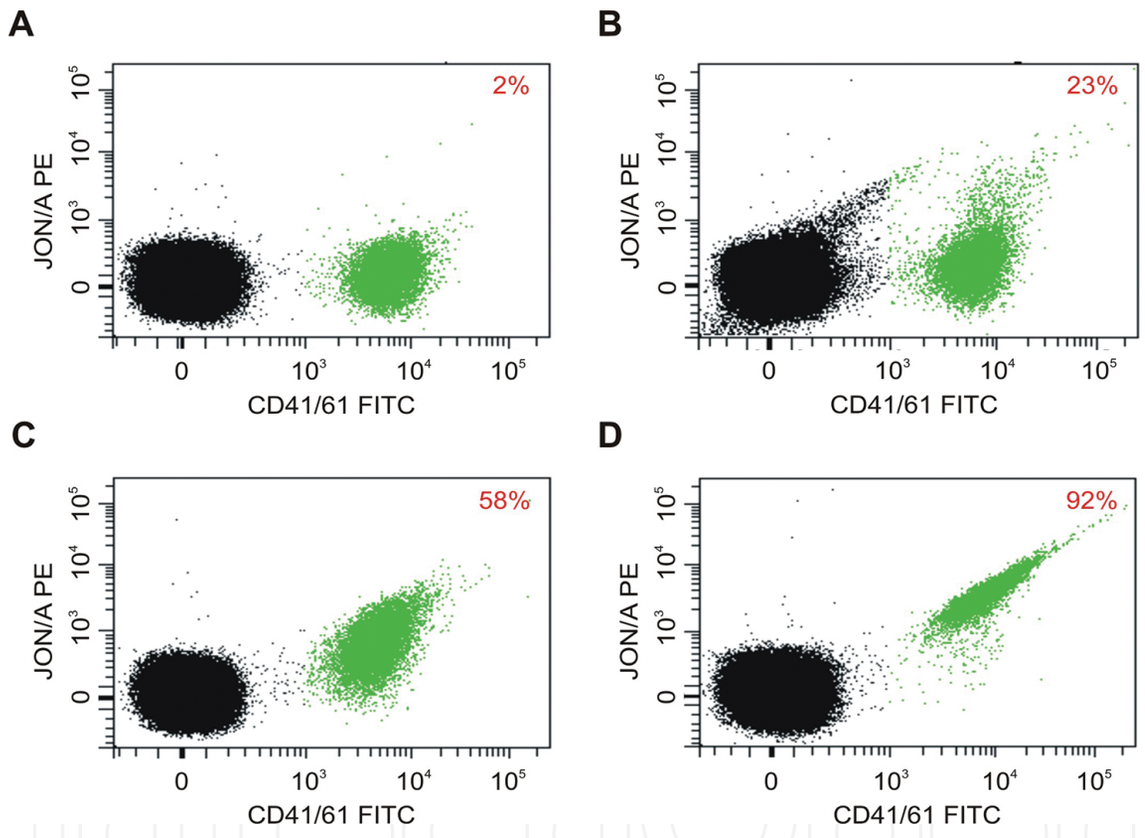

Figure 3. Fluorescence dot plots of resting mouse platelets and platelets stimulated with collagen, ADP, or thrombin. Platelets were labeled with FITC-conjugated anti-CD41/61 gating antibodies and with PE-conjugated JON/A (anti-the activated form of integrin $\alpha_{IIb}\beta_3$) antibodies. Green dots represent the CD41/61-positive objects in samples of resting platelets (A) and platelets stimulated with 20 μg/ml collagen (B), 20 μmol/l ADP (C), or 0.25 U/ml thrombin (D) (15 min, RT).). The percentage of JON/A-positive platelets was obtained after subtracting of nonspecific mouse IgG binding and was presented as a numerical value (in red).

The identification of platelets based on FSC/SSC images can be eventually made in the case of washed platelets. However, one has to keep in mind that in such an approach platelet aggregates still cannot be detected. Analyzing blood platelets from rodents, especially murine blood platelets, may be challenging due to some methodological restraints concerning the minute amount of blood available and the technical difficulties of collecting the blood, while

avoiding at the same time undesirable artifactual platelet activation. No uniform flow cytometry protocol has been recommended in the literature for studying mouse platelets. In the majority of the available reports, it has been demonstrated that none of the alternative methods of blood collection from mice (either from retrobulbar venous plexus, vena cava, by cardiac puncture or by tail bleeding) appear inferior with regard to artifactual platelet activation [10]. In contrast, some authors showed that blood withdrawal from retrobulbar venous plexus and inferior cava resulted in the least artifactual activation of platelets compared to blood collected directly from a heart, jugular vein, carotid artery, lateral saphenous vein, or tail vessels [11]. Moreover, the opinions on the effects of animal anesthesia on platelet artifactual activation vary a lot. In most studies, either inhalation or injected anesthesia has been utilized regardless of the likely effects of these methods on platelet functioning [12, 13]. However, some authors demonstrated that parenteral anesthesia reduced platelet reactivity in humans and various animal species and only a few studies on mice concerned this issue [14]. Also the effects of different blood anticoagulants on the outcomes of flow cytometric analysis have been compared. Heparin and citrate are the anticoagulants most widely used in the experiments with rodent blood samples [15, 16]. It has been proven, on the other hand, that the effects of these two compounds on the platelet functioning are quite dissimilar [10]. In this study, only the effects of citrate on platelet artifactual activation have been observed (**Figure 5**).

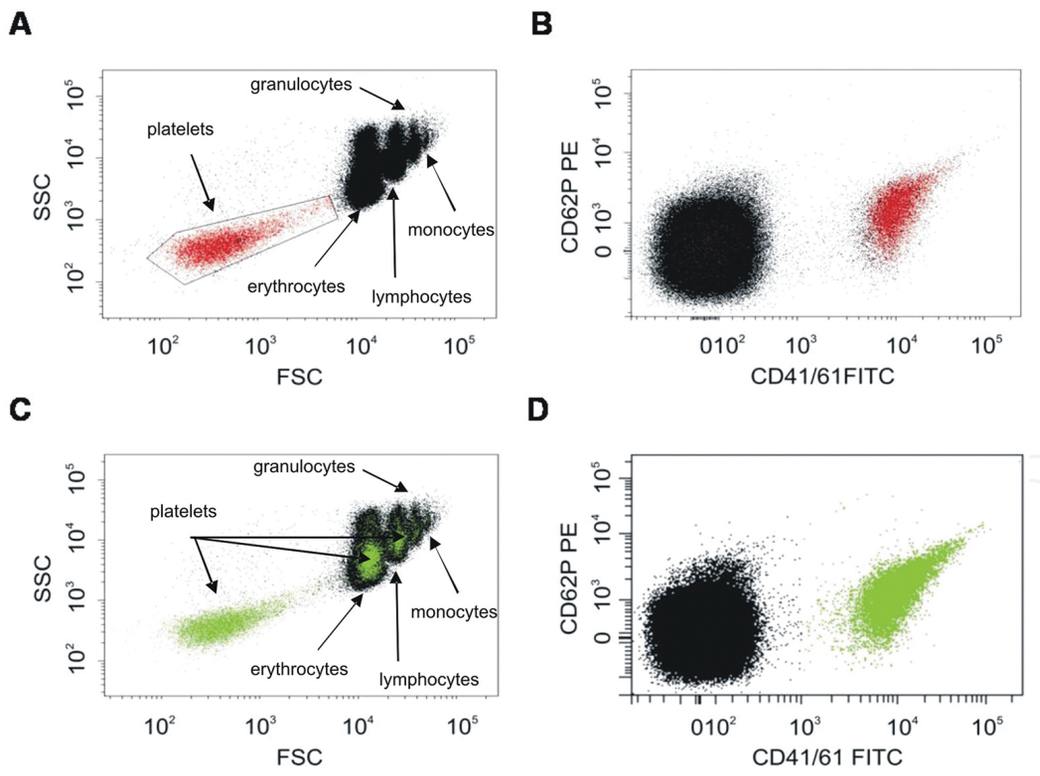

Figure 4. Fluorescence and scatter dot plots of thrombin-activated mouse platelets labeled with FITC-conjugated anti-CD41/61 and PE-conjugated anti-CD62P antibodies. Blood was incubated with 0.25 U/ml human thrombin (RT, 15 minutes). Green dots represent the CD41/61-positive objects (C, D), whereas red dots represent the objects referred to as "blood platelets" gated on the basis of the FSC/SSC scatter (A, B). Individual populations of blood cells and the aggregated platelets that are comprised in them were labeled.

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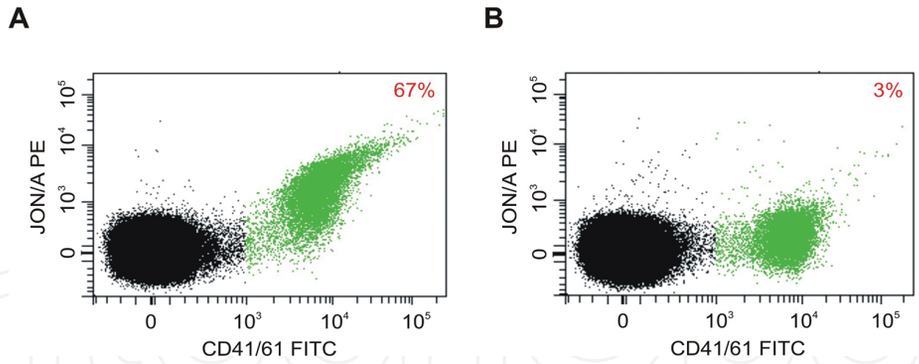

Figure 5. Fluorescence dot plots of resting mouse platelets in blood collected on citrate and low molecular weight heparin (LMWH). Platelets were labeled with FITC-conjugated anti-CD41/61 gating antibodies (green dots) and with PE-conjugated JON/A (anti-the activated form of integrin $\alpha_{IIb}\beta_3$) antibodies in blood collected on 3.2% sodium citrate (A) or 20 U/ml LMWH (B). The percentage of JON/A-positive platelets was obtained after subtracting of nonspecific mouse IgG binding and was presented as a numerical value (in red).

However, the contradictory reports have claimed that there is no firm evidence on the significance of different anticoagulants on platelet activation [11]. While working on optimizing the flow cytometry protocol, the impact of blood fixation on platelet functioning should certainly be evaluated. It has been proven that a fixation procedure may strongly affect the binding of antibodies to mouse platelets, thus supporting the conclusion that the flow cytometry protocols requiring shorter time of blood collection and preparation are preferential [10] (**Figure 6**).

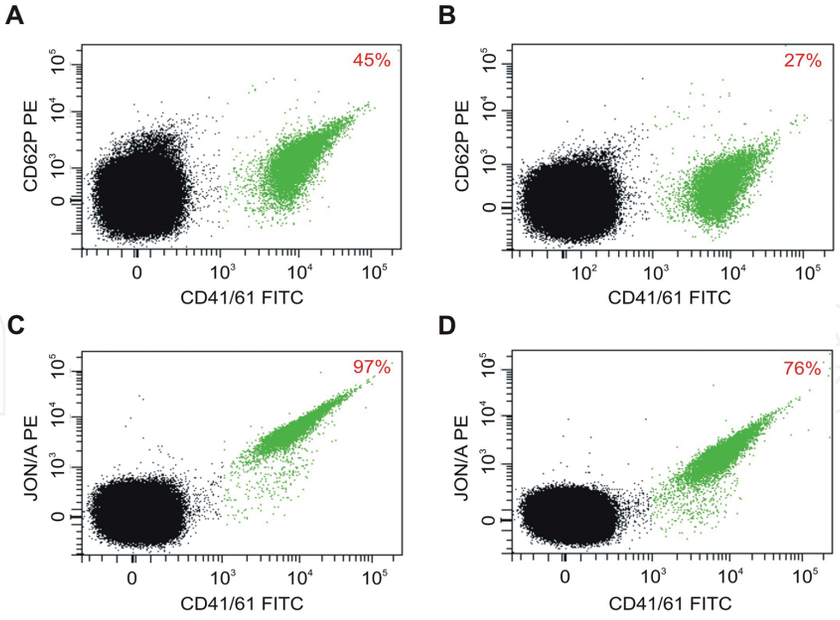

Figure 6. Fluorescence dot plots of thrombin-activated mouse platelets in unfixed and fixed blood. Platelets were labeled with FITC-conjugated anti-CD41/61 gating antibodies (green dots) and with PE-conjugated anti-CD62P (A, B) or JON/A (anti-the activated form of integrin $\alpha_{IIb}\beta_3$) (C, D) antibodies in unfixed blood(A, C) and in samples fixed with 1% paraformaldehyde (B, D). The percentages of CD62P-positive (A, B) and JON/A-positive (C, D) platelets were obtained after subtracting of nonspecific mouse IgG binding and presented as a numerical value (in red).

Furthermore, in the unfixed blood individual populations of blood cells seem to be better separated (**Figure 7**).

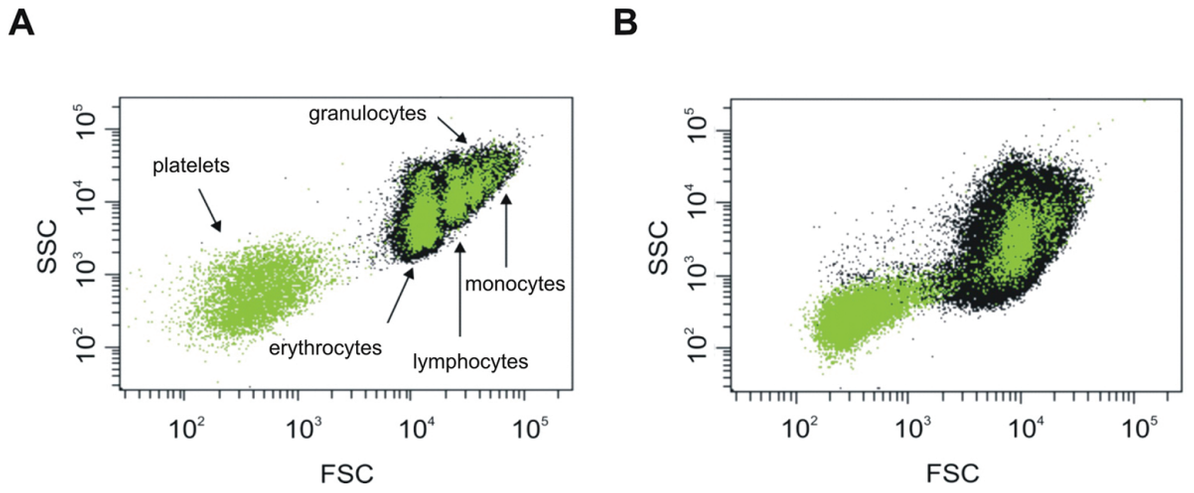

Figure 7. Scatter dot plots of resting mouse platelets in unfixed and fixed blood. Platelets were labeled with FITC-conjugated anti-CD41/61 gating antibodies (green dots) in unfixed blood (A) and in samples fixed with 1% paraformaldehyde (2 h, RT) (B).

In conclusion, it has been shown that in order to establish an optimal protocol for measuring rodent platelets, some crucial factors, including blood preparation or platelet fixation, a type of the anticoagulant, and methods of the anesthesia, should be considered.

3. Not only flow cytometry—alternative methods for studying the phenomena of platelet activation in laboratory mouse and rat models

Recently, very much attention is paid to the methods based on measuring of platelet adhesion under flow conditions. The idea behind this approach is to test the response of platelets under conditions resembling those to which platelets are exposed in blood vessels. It is accepted that a shear force, to which blood platelets are subjected in a bloodstream, strongly modulates platelets' activation and reactivity. The first studies performed under flow conditions were conducted with the use of artificial chambers assembled by researchers. Nowadays numerous companies provide systems combining capillaries of standardized dimensions and equipped with the pumps able to generate a flow of defined parameters. The combination of a flow rate chosen by the researcher and dimensions of the capillary define the shear rate generated by a flowing sample (expressed in cm^{-1}). Using an approximated viscosity of the sample, the shear force (expressed in dyne/cm^2) can be calculated based on the shear rate. Depending on the researcher intentions, the capillary can be coated either with a protein or with the cell monolayer. The most often used proteins for this purpose are fibrinogen, von Willebrand factor (vWf), and collagen. The interaction with fibrinogen results in a firm adhesion of platelets, which form a layer coating the surface, but no aggregate formation is observed. The adhesion

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to vWf is unstable and platelets weakly interact with a capillary. In turn, in capillaries coated with a collagen the aggregate formation occurs, which can even lead to the occlusion of a capillary if the inhibitors of thrombin generation are not applied. The visualization and quantification of the adhesion can be performed using various approaches. One of the methods is to prestain the platelets prior to the experiment with a fluorescent dye and to record images at a very high frequency (more than 5 Hz) using a highly sensitive camera. The most often used dyes include $DiOC_6$, rhodamine 6G, and fluorescein derivatives. The biggest challenge of this approach is the reliable quantification of the results. Using a dedicated software, the researchers usually count the number of platelets that adhered to the surface in a defined period of time. The other parameter, often presented in publications, is the velocity of platelet "rolling" on the surface, which is calculated by measuring the trace left by a platelet within a given range of frames of the image. The other way is to image these blood platelets that formed stable contacts with a coating protein and remained adhered even after the perfusion of the capillary with a washing buffer. In this approach, we can evaluate either the number of firmly adhered platelets or the surface area covered by platelet aggregates. These adhering platelets can be additionally stained with antibodies against the active form of $\alpha_{IIb}\beta_3$ or P-selection in order to evaluate platelets' activation. In the case of a thrombus formation in capillaries coated with a collagen, the volume of thrombus is the mostly informative parameter. This can be measured with the use of a confocal microscopy.

3.1. *In vivo* methods

The *in vivo* methods can be categorized with respect to the way of inducing a specified platelet response. The most often used techniques are based on the experimental disruption of the endothelial layer, which inevitably leads to the exposure of the subendothelial matrix, thus triggering blood platelet activation and platelets' adherence to subendothelial matrix components, and in consequence the thrombus formation [17, 18]. The disruption of the endothelial layer is usually achieved by placing the vehicle saturated with $FeCl_3$ on the vessel. The diffusion of the highly reactive agents to the vessel wall results in a gradual endothelial denudation. The other approach is a disruption of the cells of a vessel wall by the applying of an electric impulse. A more sophisticated and a far more precise method utilizes the multiphoton excitation microscopy, which destroys the area covered by several endothelial cells with the use of a laser beam. The thrombus formation initialized by one of these methods can be subsequently quantified in one of the two manners. One is the measurement of the rate of cessation of blood flow resulting from the formation of the occlusive thrombus. Such a measurement utilizes Doppler flowmetry, based either on ultrasounds or a laser light. The probe of a dedicated device is usually located downstream from the site of an occlusion. The time required to achieve the flow cessation is accepted as the measure of the readiness of blood platelets to form the occlusive thrombus. The other way to quantify the extent of the *in vivo* thrombus formation is the microscopic visualization of the event. Usually, for this purpose the platelets are prestained with fluorescent dye or platelet-specific antibodies. Microscopic visualization with the use of a confocal microscopy allows to measure the volume of the thrombus. Moreover, the injecting of specific antibodies or fluorescent proteins to an animal provides the opportunity to monitor the structure of thrombus, its porosity, and functional architecture [19, 20].

The *in vivo* microscopy in animal models also allows the sight into the interactions between platelets and the intact endothelium (**Figure 8**).

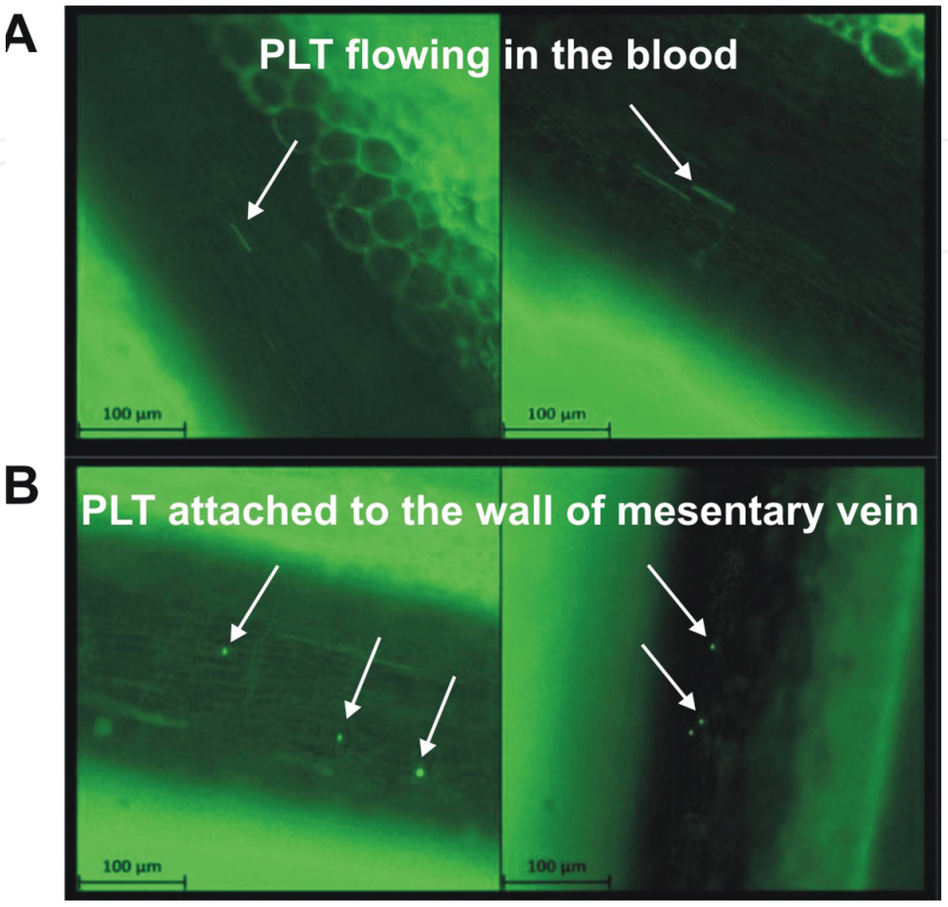

Figure 8. Platelet flow and adhesion in mesentery veins of healthy and diabetic mice. Anesthetized mice were injected with either DyLight488-conjugated anti-GPIbβ antibodies and placed on a stage of an upright microscope equipped with saline immersion objectives. Platelets flowing in the blood and those attached to the wall of a mesentery vein were evaluated in saline-injected (control) (A) and STZ-injected (diabetic) (B) mice.

Fluorescent-labeled platelets adhering transiently or firmly to the vessel wall are recorded and the interactions are quantified as described in the *in vitro* section of this paper. Another approach to test platelet reactivity *in vitro* is based on a systemic injection of a platelet agonist. In this case, the response of platelets can be quantified by two methods. In one of them, the measure of platelet reactivity is the death rate of animals, resulting from the agonist-induced thrombi formation in lungs [21]. The other method is based on the infusion of radiolabeled platelets to the animal's circulation and the monitoring of the rate at which radiolabeled platelets accumulate in lung vessels in response to nonlethal doses of an agonist [22]. We have recently proposed quite a novel approach to measure blood platelet aggregation in lungs induced by ADP injection. The protocol is based on the recording of the changes of blood flow in mesenteric vessels with the use of laser Doppler flowmetry [23].

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4. Investigation of platelet viability—critical appraisal of flow cytometric assays

Due to the lack of nucleus the commonly used methods for assessing the viability of nucleated cells, including the methods based on the measurement of a metabolic activity of cells (e.g. MTT, XTT, WST-1, and PrestoBlue™ assays), those based on the damage of a plasma membrane (e.g. lactate dehydrogenase assays, trypan blue staining) or the methods based on the analysis of DNA fragmentation cannot be adapted for a reliable measuring of the viability of blood platelets [24, 25]. Therefore, the most encouraged method for assessing the viability of platelets is the staining of blood platelets with the ester of calcein and the monitoring of the fraction of stained cells with the use of flow cytometry [26]. This assay may be applied for either a whole blood or isolated platelets. The application of this procedure is similar for platelets obtained from humans, mice or rats. The acetomethoxy ester of calcein (calcein AM) is a nonfluorescent lipophilic compound that trespasses plasma membranes of living cells and gets into the cells, where it is hydrolyzed by cytoplasmic esterases to the hydrophilic, highly green fluorescent compound—calcein [27, 28]. The free calcein is well retained within living cells with the intact plasma membrane, while the leakage of calcein to the extracellular environment is observed in the case of the loss of the integrity of cellular membranes. The application of the acetomethoxy ester of calcein, when using various protocols of platelet viability detection, has certain limitations. The presence of Ca^{2+} and Mg^{2+} in a strongly alkaline environment affects the growth of calcein fluorescence intensity, while the ions of Co^{2+}, Ni^{2+}, Cu^{2+}, Fe^{3+}, or Mn^{2+}, when present in a medium at physiological pH, quench the fluorescence of calcein [29–31]. The latter phenomenon is apparent when assessing the viability of platelets in a whole blood with the use of calcein. It was observed that platelets exhibit significantly stronger fluorescence derived from calcein than other blood cells. The probable reason for this is the fact that red blood cells, which are the most numerous cellular components of blood, contain hemoglobin, and thus the Fe^{3+} ions become responsible for the quenching of the calcein-derived fluorescence. Interestingly, it was also shown that the fluorescence of platelets stained with calcein and stimulated under *in vitro* conditions with physiological platelet agonists was increased compared to resting platelets [32]. One explanation could be that the higher concentration of Ca^{2+}, released from intracellular stores into the cytoplasm after platelet stimulation, may false-positively increase the fluorescence of calcein. However, this hypothesis was undermined, as the chelator of calcium ions, BAPTA AM, reduced platelet activation due to the binding of the intracellular Ca^{2+}; however, it did not affect the calcein fluorescence in stimulated platelets. This indicates that the increased population of calcein-positive platelets upon their *in vitro* stimulation does not result directly from the mobilization of intracellular calcium ions. Thus, it seems particularly important to provide the optimum reliable positive and negative controls for the protocols with calcein. As a potential agent used for the damaging of platelets (at present considered as a good positive control), formaldehyde (at a concentration above 1%) may be used. The reported protocol is also applicable in the case of mouse platelets (**Figure 9**).

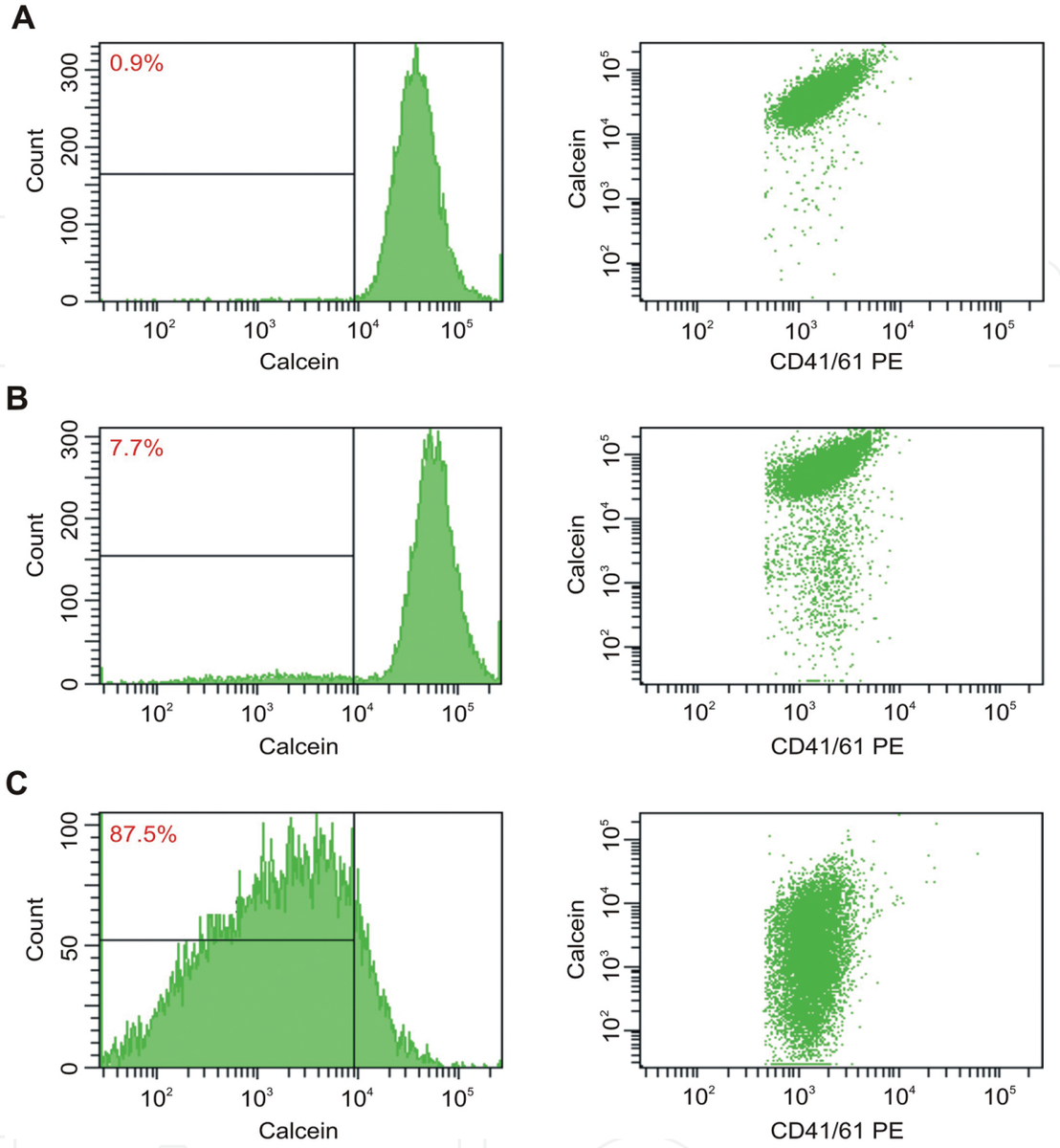

Figure 9. The viability of resting and collagen-activated mouse platelets. Platelets were labeled with calcein AM and PE-conjugated anti-CD41/61 gating antibodies. Platelets' viability was presented as percentage (numerical values in red) of calcein-negative objects in resting (A) and collagen-activated (20 μg/ml) platelets (B). Platelets incubated with 1% formaldehyde (2 h, RT) remain the positive control (C).

If we measure the viability of platelets in a whole blood, we have to use antibodies for gating the platelets. What is important is that they cannot be conjugated with the fluorochrome emitting a green fluorescence. The analysis of the changes in the intensity of a green fluorescence of gated platelets may become falsified due to the fact that in the case of platelet activation the objects showing the increased intensity of green fluorescence may be recorded, probably due to the formation of platelet aggregates. Therefore, the most convenient approach to distinguish between living platelet and those with damage cellular membrane is to set the appropriate cutoff on the histogram of green fluorescence and the subsequent recording of the appearance of calcein-negative objects [33].

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5. Monitoring of the ion mobilization and transport in platelets—flow cytometry protocols for the release of calcium ions into a cytoplasm

Changes in platelet cytosolic concentrations of calcium ions are fundamental conditions for the transmission of signals within the cell and between the cytoplasm and the external environment of platelets. These ions can be released from intracellular resources stored in granules, mainly the dense tubular system, or they can penetrate into a cytoplasm through the cell membrane from the outside [34–36]. This process takes place in a similar manner in both human platelets and platelets derived from laboratory rodents, such as mouse or rat [37, 38]. It is known that the increased cytosolic free calcium is a key for early events that follow platelet activation, and it underlies several activation responses, including shape change, aggregation, and secretion [39, 40]. Thus, the changes in the concentration of platelet intracellular calcium can be seen as a characteristic sign of platelets' stimulation by an external stimulus. For the measurements of Ca^{2+} release into the cytoplasm of platelets, the calcium-sensitive dyes have been employed. These can be divided into two groups: (i) those for which the intensity of the fluorescence emission grows when the concentration of Ca^{2+} in the cytoplasm increases (e.g., Fluo-3, Quin-2, and Rhod-2) [41, 42] and (ii) those characterized by a shift in the excitation and emission spectra (e.g., respectively, Fura-2, and Indo-1) with the increasing calcium concentrations [43, 44]. The second group of the calcium-sensitive fluorophores is much more appreciated by the researchers, as their use minimizes the risk of artifacts due to nonuniform intracellular Ca^{2+} distribution. A common problem encountered with the application of these compounds is their high polarity that hinders the penetration through the cell membrane and prevents the entering of the label into the cells [45]. To overcome this obstacle, the electroporation of cells is often performed; however, for the platelet membrane perforation (also referred to as membrane permeabilization) this procedure carries the risk of the change/loss of some cell functions [46]. Another way to increase the ability of calcium indicators to penetrate cell membranes is a chemical modification of these labels, e.g., their esterification. The esterified forms of fluorescent labels are re-released in a free form due to the action of cytoplasmic esterases. Due to their reduced polarity the esterified indicators easily penetrate the lipid bilayer membranes of platelets, and their ionization in the cytoplasm, following the hydrolysis of ester bonds, permits the binding of divalent ions, such as calcium ions. It is important that the fluorescence of the ionized forms of ion indicators is several orders of magnitude greater than their esterified derivatives, therefore the efficient hydrolysis of the labels in a cell cytoplasm is the intimate condition of the usefulness of these compounds in examining the changes in the ion concentration in platelet cytoplasm [47]. Moreover, the efficient transport of these labels into cells is also an experimental evidence of the proper functioning of cells and a preservation of the integrity of cytoplasmic membranes. Since the conversion of a nonfluorescent indicator into its fluorescent derivatives requires the presence of some active enzymes in the cell (esterases, oxidases, etc.), the calcium flux assay also provides the information on the activity of some intracellular enzymes. The excessively rapid and facilitated transport of the label and its redistribution in the cell (e.g., due to its excessively high concentrations) may result in its penetration into the intraplatelet granules (where the cell reservoirs of calcium ions are accumulated). Under such conditions the label may easily turn out useless for the moni-

toring of the release of calcium from the granules into the cell cytoplasm. The requirement of the proper adaptation of this method for the testing of a particular type of cells is, therefore, the selection of the optimal fluorescent label concentration and the optimal incubation time of the label with cells. Too short incubation time and/or low label concentration slow down its transport into the cytoplasm and result in the incomplete hydrolysis. In addition, in the case of an inefficient label concentration the assay is poorly sensitive to large changes in the concentrations of calcium in the cytoplasm because the pool of the available ionized label becomes quickly saturated with the calcium ions present in the overwhelming concentration. On the other hand, the excessively long incubation time and/or high concentration of fluorescent label may lead to its uniform distribution in the cell. In that case, the changes in the fluorescence of the labeled calcium ions released from the intracytoplasmic granules might have not been recorded because the entire pool of the label (present also in the granules) would be uniformly saturated with calcium ions. A typical flow cytometry protocol for measuring the concentration of calcium in platelets involves several crucial steps (it is similar for mouse, rat, and human platelets [48]). First, platelets are labeled with the appropriate calcium indicator for 10 minutes at 37°C. The fluorescence intensity is then recorded for about 1 minute to establish the baseline. At 60 seconds, a given platelet agonist is added to the sample and the time-dependent changes in the fluorescence of calcium-sensitive dye are measured over a period of 5 minutes. Then, the magnitude of the calcium concentration change is represented by the alterations in the calcium concentration at every second divided by basal calcium level. The key factor in the case of flow cytometric determination of intracellular calcium mobilization is the selection of appropriate ion indicator. The use of the fluorescent probes that are characterized by a shift of either the excitation or emission spectrum along with varying Ca^{2+} concentrations is limited since such shifts are typically not big enough to be recorded in the different flow cytometer channels [47]. Therefore, more available and useful are labels characterized by the changes in a fluorescence intensity along with the increasing calcium concentration. These changes can be recorded either as changes in the relative fluorescence or changes in the fraction of fluorescing cells. The commonly used calcium indicator is Fluo-3 because its excitation and emission spectra make it suitable for the use in flow cytometers equipped with a standard 488 nm argon laser. Preferably, two different calcium indicators may be used in which the emission varies in the opposite direction (increases for one and decreases for the other) with the increasing calcium ion concentration. One of the labels is then a reference indicator. When deciding to study the ion transport in platelets with the use of flow cytometry, a researcher has to face a number of methodological problems. First, since the calcium indicators are present in the ionized form in the platelet cytoplasm, there is a risk of their removal by the cellular membrane anion transporters. Hence, the concentration of the "active" ion indicator decreases inside a cell, as the time passes, which makes it impossible to reliably monitor the fluctuations in calcium concentration in such cells. Under such circumstances, the use of ion transporter blockers (like probenecid) may guarantee a collection of the reliable results [49]. However, as it turns out, some current reports undermine the use of some of these blockers due to their negative effect on platelet functioning. The above-mentioned probenecid is a good example of such a compound. It was applied in numerous studies to prevent a leakage of the used calcium indicators [50, 51]. Unfortunately, probenecid was also found to markedly

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hamper the increase in Ca^{2+} caused by physiological platelet agonists [52, 53]. Hence, paradoxically, a physiological event of calcium mobilization in activated platelets can be hardly monitored. Second, numerous ion indicators are characterized by a considerable toxicity and thus may cause a gradual leakage of fluorophores from the labeled cells due to cell membrane disruptions [54, 55]. A good way to confirm this kind of leakage may be the adding of Mn^{2+} ions to the buffer, in which the cells are suspended. These ions are capable of quenching the fluorescence of a variety of labels. If there is a leakage of ions through the damaged platelet membrane, Mn^{2+} ions may penetrate into the cytoplasm and—via competing with calcium ions—may bind to the molecules of indicator inside cells and quench its fluorescence [56]. Importantly, needless to emphasize, there are a few advantages of flow cytometry over the alternative classical spectrofluorometric plate- or cuvette-based methods for measuring Ca^{2+} release from the intracellular resources. First, sample preparation for flow cytometry measurement requires only a minute amount of blood. This fact is particularly important in the case of smaller laboratory rodent models, for which main methodological obstacle concerns the minimal portions of blood available (like in mice). Second, for plate- or cuvette-based methods, it is necessary to isolate platelets and separate them (wash out) from other cellular elements of blood [57]. In practice, it seems hardly possible to obtain a sufficient aliquot of isolated washed platelet suspension from the aforementioned small volume of blood withdrawn from small rodents. In the case of flow cytometry, we can monitor the process of calcium mobilization in platelets in whole blood samples. For this purpose, we have to use the antibodies against a common platelet-specific marker that discriminates between platelet-positive and platelet-negative blood cell subpopulations. This constitutes the guarantee that calcium concentration will be monitored only in these "gated" objects—platelets. Thus, the procedure of platelet "gating" truncates the calcium-specific signal originating from multiple cells types other than platelets, which possibly show differentiated responses to the same stimulus. As far as calcium mobilization constitutes one of the most initial stages of blood platelets, the protocols that allow to trace the phenomenon in a whole blood certainly minimizes the risk of artifacts due to sample preparation. Another advantage of flow cytometry may be that it enables the identification of varying extents of calcium-related responsiveness within a single population of platelets [58]. In turn, the possibility to record a high number of independent single cellular events provides a high statistical power of such analysis. In addition, in contrast to other methods, flow cytometry allows, with the use of the panel of appropriate antibodies, to distinguish between the fluorescent signals originating from either single platelets or platelet aggregates. It seems particularly important, and more even so, considering the fact that the total fluorescence of platelet aggregates appears artificially elevated compared to the fluorescence arising from single platelets, hence elevating the risk of collecting overestimated outcomes [58]. Finally, it should be remembered that for either plate- or cuvette-based methods the washing steps are required in order to cut off the background fluorescence [59, 60]. These washing steps can inevitably cause the artifactual platelet activation, and therefore, their further stimulation with an agonist may result in the apparently reduced response, also reflected in the extent of calcium mobilization. Immediate and continuous recording of a fluorescence wave upon platelet stimulation is very important since the oscillations of

cytoplasmic calcium levels occur rapidly within a few to several seconds from the occurrence of the stimulus (**Figure 10**).

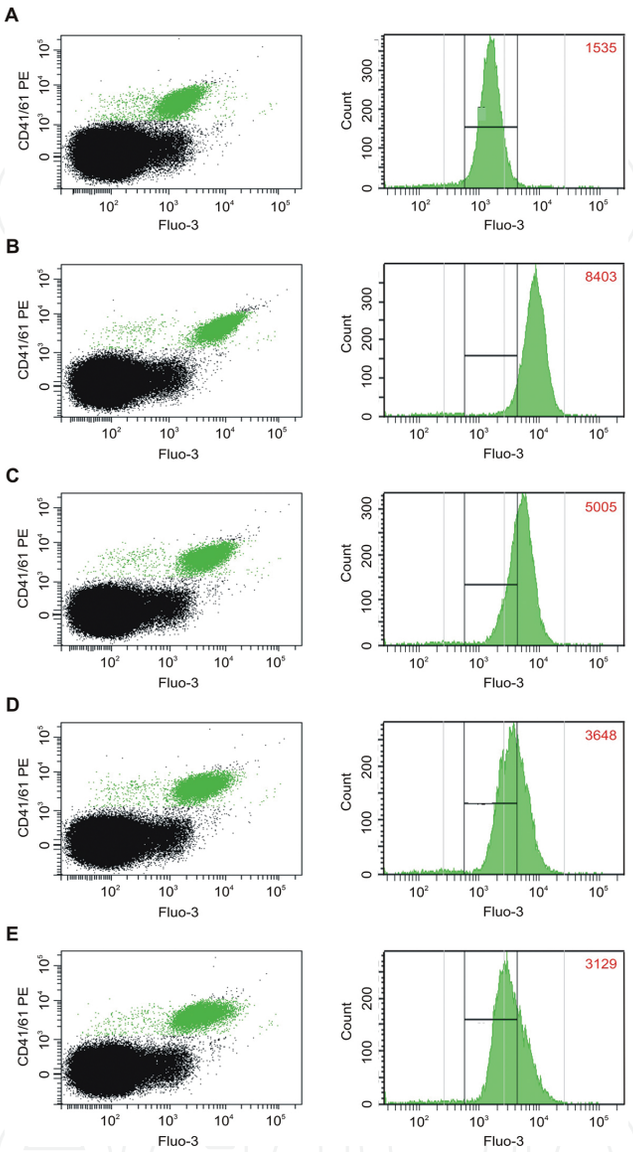

Figure 10. Calcium mobilization in resting and thrombin-activated mouse platelets. Platelets were labeled with calcium indicator, Fluo-3 AM, and PE-conjugated anti-CD41/61 gating antibodies. Changes in calcium concentration were monitored in resting (A) and thrombin-activated platelets at the following time points (starting from the addition of the agonist): 0 second (B), 30 seconds (C), 60 seconds (D), 90 seconds (E) and presented as mean fluorescence intensity (numerical values in red).

After this period there is usually a fast decrease in the calcium concentration to the level observed for the resting platelets [61]. This subsequent decrease in the calcium level results from the removal of Ca^{2+} ions from the cytosol into internal stores or their extrusion across the plasma membrane of cells. The main limitation of the flow cytometry method is that most flow cytometers have a pressurized system for aspirating the sample, and therefore, there is practically no opportunity for a continuous addition (within a certain period of time) of tested

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compounds, such as agonists, in order to stimulate the calcium mobilization in platelets [62]. Resulting gaps in the recordings, as the consequence of the addition of these compounds, may result in the loss of some details of the recorded signal in the course of a rapid calcium response. This approach requires the researcher to keep in mind that the aspiration of the sample is paused for a really very short time to supplement the tube with a needed component and of course that the pause time should be the same in each experiment. Only then the accuracy and reproducibility of the results is ensured. Fortunately, the new generations of flow cytometers resolve this problem by the use of peristaltic pump which allows to add tested compounds to the cell suspension placed in open tubes [62]. This provides the continuous measurement of the kinetics of calcium mobilization in platelets in a relatively repeatable manner. Summing up, we argue that flow cytometry provides simple and sensitive tools to assess the time course and intensity of the signal transduction of calcium mobilization in platelets in response to different platelet agonists under near-physiological conditions.

6. Nitric oxide synthase activity in blood platelets—a fact or a myth? Is there a place for the use of flow cytometry?

Platelets, the major components of primary hemostasis, are regulated by both procoagulant and anticoagulant factors. One of the factors inhibiting the activation of circulating platelets is nitric oxide. NO (as well as its precursor, L-citruline) is produced from L-arginine by the catalytic action of NO synthase (NOS) isoforms in various types of cells [63–65]. Upon diffusion across the plasma membrane, NO binds to its intracellular receptor, soluble guanylyl cyclase (sGC), triggering the production of increased cGMP levels [66]. The cyclic nucleotide directly activates protein kinase G (PKG) and indirectly activates PKA through the inhibition of phosphodiesterase 3. The activation of PKG inhibits almost all the agonist-induced events in a platelet, including intracellular calcium mobilization [67], integrin activation [68], cytoskeletal reorganization [69], and platelet granule secretion [70]. In addition, NO is able to exert cGMP-independent functions, however, at much higher concentrations than those needed for sGC activation [71]. In addition to endothelial cells, where the NOS activity is crucial for the controlling of platelet activation [72], human blood platelets are also considered to express functional NOS. Therefore, nowadays it is believed that platelets may be regulated not only by exogenous endothelium-derived NO, but also by nitric oxide produced endogenously in platelets themselves. Two isoforms of NO synthase, endothelial (eNOS) and inducible (iNOS), seem to participate in that [73]. According to the opinions raised by some researchers, the endogenous activity of NOS present in platelets might act in an autocrine manner to stabilize platelets or to reduce the activation of resting platelets during the recruitment phase [74]. Nowadays, a major question in a scientific debate is whether platelets really express endogenous, functionally active NOS proteins. The data concerning the expression, regulation, and function of eNOS and iNOS in platelets remain contradictory. Since platelets do not have nuclei, and thus do not have the ability to regulate protein expression transcriptionally, the observed elevated NO generation upon platelets' stimulation with agonists most likely results from the increased activities of these enzymes [75]. Some older reports confirm the presence

of eNOS (also referred to as NOS3) and iNOS (also referred to as NOS2) in human platelets, with NOS3 being predominant [76]. Platelet NOS3 has been characterized as the Ca^{2+}-sensitive enzyme, and therefore, each stimulus that triggers intracellular calcium mobilization, such as e.g. physiological platelet agonists, should demonstrate the ability to activate NOS and induce NO generation [77, 78]. Some recent evidence also suggests the possible calcium-independent mechanism of NOS3 activation [79]. It has been reported that the NOS activity in human platelets increased via the cAMP/PKA pathway. The mediator is involved in the nitric oxide synthesis induced by adenosine, forskolin, and potentially also by any other antiaggregating substance enhancing the intraplatelet cAMP concentration either via the receptor-dependent or receptor-independent mechanisms [80]. The agonist-mediated activation is physiologically probably the most natural pathway of NO generation in platelets, we should be aware, however, that also other compounds of either natural or exogenous origin may affect NOS activity in human platelets. It has been proven that both aspirin and indomethacin decrease the activity of NOS in human platelets [81]. On the other hand, however, some most recent reports put the putative presence of eNOS and iNOS in human or mouse platelets into question [82]. First of all, the authors showed that platelets of the mentioned species did not contain mRNA for these proteins. The suggested lack of an active NOS of platelet origin was further confirmed by the fact that neither human platelets treated with the NOS substrate (L-arginine) nor those subjected to the action of the NOS inhibitor (L-NAME), and nor even platelets from mice with NOS deficiency (eNOS/iNOS knockout mice), produced detectable functional effects in blood platelets. These findings may collectively indicate either the presence of nonfunctional NOS in nonstimulated or stimulated platelets, or even the complete absence of NOS isozymes in these cells. To possibly reconcile, at least partly, these contradictory findings reported in this chapter, note that some reports have shown that a possible contamination of platelets with NOS-expressing cells may stimulate NO production in human platelets [83]. Otherwise, some contrary reports not only suggest the presence of NOS in mouse platelets, but also propose a biphasic role of NO in platelet activation—promoting platelet aggregation and secretion at low NO concentrations (of the order of that produced by platelet NOS) or the inhibition of platelet activation at the higher levels of NO [84]. Such a view has only occasionally been raised and several studies have even questioned the possible pathophysiologic role of platelet-derived NO because of the evanescent nature of this oxide and very small amounts of NO produced by platelets (estimated at about 5×10^{-17} mole per platelet stimulated with 5 μM ADP) [85]. So far, definitely much less publications relate to the expression and activity of NOS in platelets from mice or rats in comparison to human studies. Some earlier studies have suggested that the expression of NOS in murine platelets is possible, and thus, the lack of platelet-derived NO would alter the *in vivo* hemostatic response by increasing the recruitment of platelets [86]. Furthermore, it has been suggested that NO generated by murine platelets upon their activation at the site of arterial damage is more effective than that generated by endothelium-derived NO, probably because it acts at short distances, exactly in the place where platelets get activated [87]. However, the most current research confirms the findings gathered for humans, i.e., that also in the case of mice blood platelets do not express NOS, and hence, the endogenous production of NO inside these cells seems unlikely. Animal studies concern in particular the use of genetic models

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of animals with the knockout NOS expression [88]. Under these conditions the potentiation of platelet response to agonists is observed to be similar to that recorded following the systemic NOS inhibition. The conclusion is that *in vivo* platelets are regulated primarily by NO originating from the outside of platelets, mainly from the vascular endothelium [89]. As for rat platelets, there is only a very few reports of possible expression and activity of NOS in these cells [90, 91]. The fact that there exist so many conflicting reports about the expression of NOS in platelets, also in rodent platelets, largely results from differences in experimental conditions and from nonspecific methods employed to detect NOS expression and activity. For example, the detection of platelet eNOS by Western blot and immunoprecipitation may provide false-positive results because of the variable specificity of commercially available eNOS antibodies [82]. A frequently used NOS activity assay is based on measuring of a radiolabeled L-citruline formed by the incubation of a radiolabeled L-arginine with intact or lysed platelets [73]. These methods seem not to be specific because platelets, as well as other cells, can utilize L-arginine in various pathways independent of NOS, including , for example, the urea cycle [73]. Undoubtedly, numerous reported findings apparently seem to be nothing more than artifacts, considering the fact that the reported platelet NOS activity, measured on the basis of the L-citruline production, ranged from 8 fmol [92] to 16 pmol/min/mg protein [93], which constitutes the difference of three orders of magnitude. In the collagen-stimulated platelets, the formation of 5 fmol/mg protein in 60 minutes was observed [94]. When we assume a protein content of 2.1 pg protein per platelet, this trace synthesis is equivalent to the formation of 6.3 molecules of NO per a single platelet per hour, and that minuscule amount simply seems negligible to cause any physiological effect [94]. Otherwise, this method does not provide the information on the concentration of NO produced from L-arginine. More specific GC-MS assay for NOS activity is based on the simultaneous measurement of nitrites and nitrates formed from the oxidized NO upon its NOS-catalyzed formation from L-arginine [95]. Remarkably, most studies did not verify NOS expression in platelets using stringent negative (NOS-deficient cells) or positive (endothelial cells, stimulated macrophages) controls [82]. There are also some doubts concerning the utilization of spectrophotometric, fluorescence-, and chemiluminescence-based assays to determine the level of NO production in platelets. The main objections concern the lack of a specificity of these methods. It has been suggested that such assays cannot discriminate between NO released by NOS-catalyzed oxidation of one of the guanidine nitrogen atoms of L-arginine and NO released by other sources [73]. The NO released by the oxidation of the α-NH_2 group of L-arginine or other amino acids and amines could falsely contribute to the measured NOS activity. As shown in the literature, despite the above-mentioned doubts, flow cytometry is still a widely used method for the monitoring of the intracellular NO production [96, 97]. The main advantage of this method is the fact that we can identify the selected population of cells, therefore, the presence of other cells does not interfere in the measurements of NO production. This aspect seems to be important since it has been suggested that the positive signs of the presence of NOS even in conventionally prepared, isolated platelets very likely result from the contaminants originating from other blood cells or their fragments [83]. The assessment of NOS activity by measuring NO concentration seems challenging because of its short half-life in a circulation and its high reactivity. It has been proven that the superoxide anion (O^{-2}) rapidly scavenges

NO to produce the cytotoxic peroxynitrite ($ONOO^-$) and this is one of the fastest chemical reaction occurring in the liquid phase ($k = 9.6 \times 10^9$ l/mol per s) [98]. Therefore, it is important to optimize the measurement conditions, especially the concentration and the incubation time with a fluorescent dye should be optimized and assessed using different concentrations and different incubation times with a probe. The most commonly used dye, DAF-FM (4-amino-5-methylamino-2'7'-difluorofluorescein) diacetate, is a pH-insensitive fluorescent dye that spontaneously penetrates the plasma membrane and is cleaved inside cells by esterases to generate the intracellular ionized form of DAF FM, which is subsequently oxidized by NO to the strongly fluorescing triazole product [99]. The fluorescence quantum efficiency increased more than 100 times after the transformation of DAF by NO. Typical flow cytometric protocol includes platelets labeling with DAF-FM diacetate for 30 minutes at 37°C. Following the addition of the NO donor (the NOS substrate, L-arginine) or the NOS inhibitor (L-NAME), the fluorescence intensity is measured at 515 nm upon the excitation at 494 nm [100]. Our own results are in line with other reports (**Figure 11**).

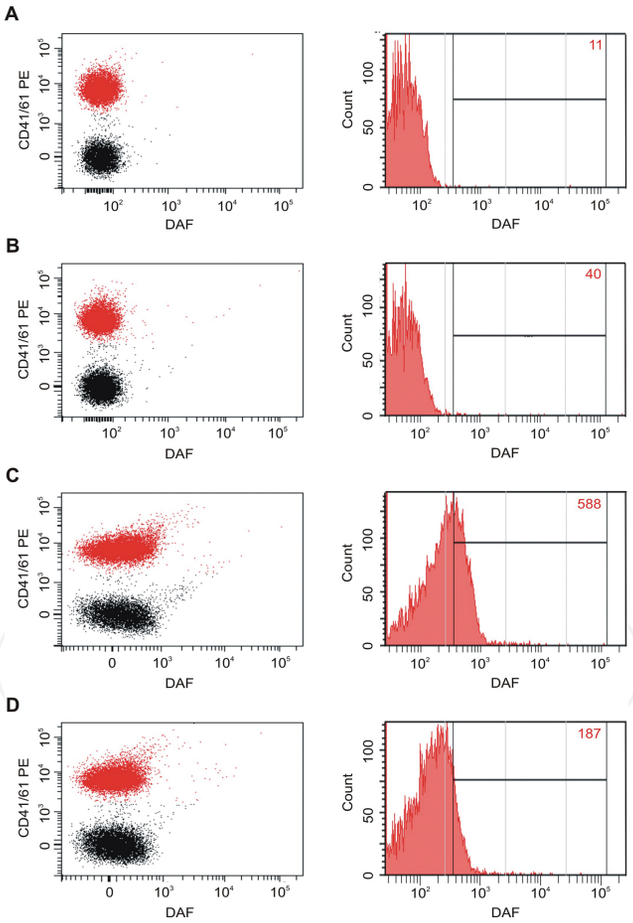

Figure 11. Monitoring of the presence of nitric oxide in mouse resting platelets. Platelets, labeled with PE-conjugated anti-CD41/61 gating antibodies, were incubated with NO indicator (DAF FM) alone (A) or with the addition of L-arginine (the substrate for NO synthase) (B), DEA/NO (the NO donor) (C), or both DEA/NO and L-NAME (the inhibitor of NO synthase) (D). Changes in DAF FM fluorescence were presented as mean fluorescence intensity (numerical values in red).

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We confirm that there is no NO production in murine platelets (**Figure 11A**). Furthermore, even providing a substrate for nitric oxide synthase (L-arginine) did not increase the fluorescence derived from DAF FM (**Figure 11B**). Nitric oxide was detected in platelets only when the NO donor (DEA/NO) was applied to these cells (**Figure 11C**). Interestingly, the detection of nitric oxide derived from DEA/NO significantly decreased when we used L-NAME, the inhibitor of NO synthase (**Figure 11D**). This may confirm the hypothesis claimed by other authors for human platelets that the presence of exogenous NO in platelets may stimulate NO production in these cells [83]. What is worth emphasizing is that DAF-FM is rather an endpoint dosimeter and it is not a reversible equilibrium sensor. This may further constitute a certain limitation in the monitoring of rapid fluctuations in the intracellular NO concentrations in a real time. The reaction, in which DAF FM is converted into the fluorescent benzotriazole derivative, is a two-step process. In fact, first DAF-FM must be nonspecifically oxidized to an anilinyl radical, which then reacts with NO to form the fluorescent triazole product [101]. When interpreting experimental data, this mechanistic complication must be kept in mind. Specifically, the question of whether a nonspecific preoxidation or rather a subsequent reaction with NO is the dominant factor controlling the recorded DAF-FM fluorescence signals requires a critical scrutiny [102].

7. Platelet-derived microparticles—what we are really supposed to measure?

Extracellular vesicles (EV)[1] are small membrane vesicles derived from cells upon their activation or apoptosis. The classification of EV is mostly based on their size, composition, and the process of their release from cells. Exosomes (50–100 nm in diameter) are stored in cells and are extracted by exocytosis of multivesicular bodies. Apoptotic bodies (1000–5000 nm in diameter) are formed during apoptosis, while microparticles (100–1000 nm in diameter) are produced - for example, upon platelet activation [103]. In the circulating blood, in a physiological state platelet-derived microparticles are widely distributed and constitute up to 70–90% of the total pool of various microparticles present in plasma [104, 105]. They are a convenient marker of platelet exhaustion and/or damage, and the increased number of platelet-derived microparticles may be associated with certain diseases [106]. Therefore, they are often considered as the specific diagnostic markers [107]. Microparticles originating as the consequence of platelet membrane shedding can exhibit substantial biological (procoagulant) activity due to phosphatidylserine exposed on the surface (although not all microparticles have it), and thus, they provide a convenient indicator of the reactivity and/or sensitivity of platelets for various states of disease [108, 109]. Indeed, diabetes mellitus, collagen-associated vascular disorders, hypertension, and acute coronary syndromes are associated with increased

[1] For years they have been erroneously termed—and in the literature they are still most often referred to as— "platelet microparticles" (PMPs). However, according to the latest terminological trends, they should be rather called "platelet microvesicles" (PMVs).

levels of platelet-derived microparticles [110]. Microparticles are also considered to have an important effect on atherosclerosis, thrombosis, and cancer metastasis [110]. In comparison to studies on humans, there is noticeably less reports describing the formation of microparticles in laboratory rodents. Nevertheless, even these few studies confirm the significant role of these small vesicles in the thrombogenic process. Ramaciotii and colleagues showed that platelet-derived microparticles correlate with thrombus weight and tissue factor activity in an experimental mouse model of venous thrombosis in [111]. In another study by Chen and coauthors, the authors proved the elevated formation of platelet-derived microparticles in a murine AIDS model, which exhibited procoagulant activity to accelerate the coagulation cascade and caused platelet activation in autocrine manner [112]. Interestingly, it was shown that the generation of platelet-derived microparticles did not differ between commonly used laboratory mouse strains (C57BL/6, BALB/c, and 129Sv) and that it was not possible to efficiently inhibit microparticle formation by the potent antiplatelet inhibitor, aspirin [113]. Increased production of platelet-derived microparticles was also observed for various rat laboratory models, including diabetes [114] and hypertension [115]. Although the formation of microparticles can be monitored with the use of many methods, such as atomic force microscopy, electrochemical impedance spectroscopy, ELISA, or functional assays, the use of a standard flow cytometry has several advantages in the study of these objects. Obviously, the possibility of enumeration and the possibility of using multiple fluorochromes for a simultaneous detection of multiple markers of these cells seem the most important. Remarkably, the isolation of microparticles, as well as their counting, and finally staining pose a number of methodological/technical limitations. Since the detection of microparticles in a whole blood is not recommended, the initial and the most crucial steps involve the selection of the optimized centrifugal forces to obtain the microparticles from platelet-rich plasma. This is particularly important in the case of platelet-derived microparticles because the platelets are the smallest cells of all morphological blood elements. The situation becomes even difficult when we want to isolate murine microparticles since platelets (and microparticles derived from these platelets) are much smaller in mouse in comparison to human or rat blood [1]. Unfortunately, as it clearly emerges from the profound review of the available literature, there is no suitable uniform protocol to obtain microparticles from human or rodent blood [116]. Optimally, in order to obtain cell-free plasma, blood should be subjected to the two-step purification procedure, in which the platelet-poor plasma (PPP) is first obtained. Microparticles can be directly quantified in PPP; however, the better results may be obtained for isolated microparticles. Therefore, PPP should be centrifuged again at a much higher centrifugal forces (very often the ultracentrifugation may be a reasonable choice) in order to obtain platelet-free plasma (PFP) and to get rid of the remaining small platelets and to further purify the pool of microparticles [117]. However, it was shown that microparticles counts may be lower in PFP than PPP [118, 119]. The possible explanation is that probably in the course of this procedure large microparticles may also become depleted. Indeed, we have also confirmed these observations in our studies (**Figure 12**).

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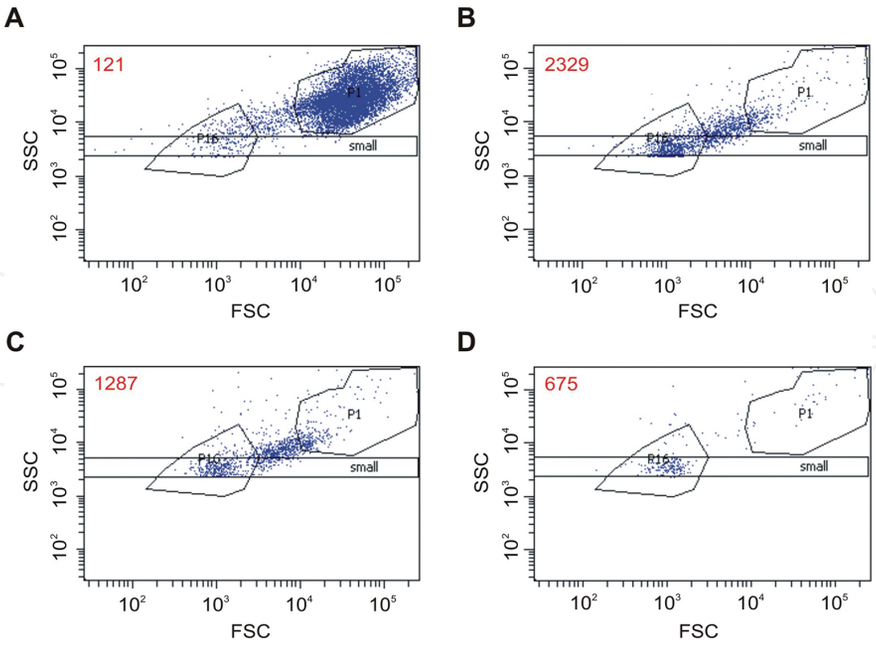

Figure 12. Detection of microparticles derived from thrombin-activated mouse platelets. Platelets and platelet-derived microparticles were labeled with FITC-conjugated anti-CD41/61 gating antibodies (blue dots). The gates P1 and P16 correspond to the areas where respectively the 1 μm and 0.2 μm beads (Megamix-Plus SSC beads) were found. The formation of microparticles was estimated in a whole blood (A), pellet obtained after centrifugation of PPP to PFP (B), PPP (C), and PFP (D). The number of CD41/61-positive objects in the size range of 0.2 μm was presented as a numerical value (in red).

We have recorded much smaller populations of platelet-derived microparticles in PFP (**Figure 12D**) than in PPP (**Figure 12C**). In that case, some part of the population of microparticles was found in the pellet resulting from the centrifugation of PPP to PFP (**Figure 12B**). When considering the measurement of platelet-derived microparticles using flow cytometry, first it is worthy to perform the analysis of FSC (with linear scale) vs. SSC (with logarithmic scale). Microparticles appear close to the electronic noise and may interfere with cellular debris and precipitates. Regrettably, the standard protocol of how efficiently discriminate between the objects of interest particularly with small dimensions and dust particles or cellular debris has not been established hitherto. One of the possibilities is to use the ultrapure saline solution (filtered twice through a filter with a pore size of 0.2 μm) and to set up the reference threshold value for objects registered in the background noise (per time unit) at a given flow rate [120]. It should be emphasized herein that the value of 0.5 μm has been commonly accepted for the minimum size of the microparticles that can be reliably measured by modern classical cytometry. Microparticles with sizes lower than 0.5 μm are practically impossible to be distinguished from cellular debris. This is because the lasers used in most cytometers excite at 488 nm and this wavelength is not suitable as a discriminator for the detection of microparticles of sizes less than 488 nm (i.e., 0.488 μm) [121, 122]. Multiple smaller microparticles can often be detected simultaneously and are erroneously considered as single objects. High-sensitivity flow cytometers have been recently developed and they provide sufficient size resolution for the identification of microparticle subtypes [123, 124]. However, as with the conventional flow cytometers, protein aggregates and potentially other factors present in

biological samples may also significantly interfere in a measurement performed using novel-generation flow cytometers. Neither conventional nor even the standard novel-generation cytometers are optimal for the evaluation of murine microparticles due to their extremely small sizes of less than 0.2 μm. Therefore, it is recommended to use a high-sensitivity flow cytometer with the small particle module equipped with a more powerful blue laser (100 mW rather than 20 mW) and a Fourier bar that provides a lower background and noise and increases the angle of light diffusion[125]. The best strategy to identify microparticles would be the simultaneous monitoring of the forward light scattering and fluorescence upon labeling of the microparticles with the antibody recognizing the antigen reflecting their cell of origin. An example of such a marker may be annexin V, which more strongly binds to the platelet-derived microparticles than to normal platelets (due to the strong procoagulant activity of the former) [126]. The identification of platelet microparticles can also be made on the virtue of the gating for one of the pan-platelet surface antigens (CD41, CD41/61, and CD42) or platelet surface activation markers (P-selectin, active form of GP IIb/IIIa complex). Alternatively, the CELLTracker (5-chloromethylfluorescein, CMFDA) can be used to determine the platelet-derived microparticles [125]. This compound freely passes through cellular membrane and is subsequently converted into a fluorescent cell-impermeant product product by cytosolic esterases. When activating the platelets preloaded with this reagent, the fluorescence gets encapsulated within microparticles, permitting their closer identification [127]. However, the uncritical application of such an approach may be associated with the collection of artifacts. First of all, we should use the simultaneous labeling of several platelet membrane antigens since various subpopulations of microparticles may carry on their different surface platelet antigens [128]. Otherwise, the number of microparticles largely depends on biological and individual variation depending on numerous factors, such as a strain, sex, age, and physiological or pathophysiological state of the organism. Therefore, it is impossible to establish any kind of standardization depending on the volume of body fluid or the volume of platelet suspension. In such circumstances, a good solution may be the enumeration of microparticles using fluorescently labeled beads [129] and the subsequent use of the relevant amount of antibodies or the relevant concentration of a fluorophore for the labeling of a known number of microparticles in a cell suspension [117]. On the other hand, it should be remembered that to work with the calibration beads of a size range similar to the size of microparticles is more troublesome due to the nonlinearity of FSC parameter vs. the diameter of the small-sized beads. Alternatively, the measurements of the appropriately calibrated flow rate with the use of Trucount™ beads can be applied [120, 130]. In general, the photomultiplier (PMTs) values should be validated for the sufficient resolution of the objects. Too low resolution makes the recording of the poor-light scattering objects difficult, whereas for too high PMT values the recording of noncellular objects is also risky. It should be kept in mind that the use of artificial beads for any size-related calibrations (FSCs) remains imprecise as far as beads and microparticles display different refractive indices. The development of "calibration vesicles," having a refractive index similar to that of cellular microparticles, seems challenging, but the correct determination of microparticle sizes and further, maybe, the comprehension of their physio(patho)logical functions may be possible only with this "tool." In summary, the proper determination of microparticles, especially those derived from murine platelets, requires not

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only the advanced and suitable flow cytometric instrumentation, but also, mostly, the properly validated protocols and the experience in this field of cytometric analysis.

8. Mitochondria as a source of energy for platelets' activation—the applications of flow cytometry

Mitochondria are essential for the proper functioning of the cell. Their main role is to generate ATP and to regulate the metabolism of the cell [131], but they are also involved in a number of other biochemical processes, including the regulation of calcium homeostasis [132], the production of reactive oxygen species [133], and the control of apoptotic and necrotic cell death [134]. An increasing number of reports indicate the potential impact of changes in a mitochondrial bioenergetic on the activation and aggregation of blood platelets [135–138], and more even so, considering that both these physiological events definitely depend on the energy produced in platelet mitochondria [136–138]. Changes in blood platelet mitochondria mass, mitochondrial membrane potential (MMP), reactive oxygen species production (ROS), ATP level, or mitochondrial respiration were observed in pathological states and diseases, such as sepsis [139], diabetes [140–142], or major depressive disorder (MDD) [143]. Some studies revealed positive associations between the expressions of activation markers on the surface of blood platelets (like P-selectin) and the extent of mitochondrial respiration [144] or the value of mitochondrial membrane potential [143]. Moreover, the associations between the potentiated collagen-stimulated platelet aggregation and mitochondrial membrane hyperpolarization and/or the overproduction of mitochondrial ROS in blood platelets were observed [135]. Interestingly, it has also been revealed that the mechanism of the action of a strong inhibitor of platelet adhesion and aggregation, nitric oxide, can be associated with the inhibition of the complex IV of mitochondrial respiratory chain, thus implying a reduced mitochondrial energy production [138]. Therefore, more and more attention is paid to the mitochondrial functioning in blood platelets, and flow cytometry is certainly a well-suited technique often chosen for this purpose. The following examples help to understand the essence of the appropriate study design of the experiments in which the flow cytometry technique is used to study mitochondria and to show how our knowledge on the applied staining methods helps in assessing the reliability of the results.

8.1. Mitochondrial membrane potential

One of the most commonly evaluated parameters when studying these organelles is the mitochondrial membrane potential. This parameter is a sensitive indicator of the mitochondrial energetic state. The conventional techniques for MMP measurements are based on the potential-dependent movement of positively charged lipophilic dyes across biological membranes. This method can be applied in both isolated mitochondria and intact cells. Since the potential inside the cell is negative (about −60 mV for the majority of cells), the positively charged dye molecules freely pass through the cell membrane to get into the cytoplasm and negatively charged organelles. Due to the strong negative potential of the mitochondrial

matrix (approximately −160 mV), much more dye (by two or even three orders of magnitude) can accumulate in the mitochondria matrix than in the cytoplasm. Significant amounts of dyes also locate in the space between the two mitochondrial membranes. Therefore, the total amount of cationic dyes inside the cell highly depends on the mitochondrial potential [145]. Changes in mitochondrial membrane potential lead to the concentration-dependent alterations in a dye fluorescence. However, the use of cationic, lipophilic fluorescent probes is not devoid of traps regardless of whether we study cells derived from humans, animals, or cell cultures. Therefore, a careful selection of a probe and the knowledge about its limitations are extremely important.

8.2. Problems with the evaluation of MMP with the use of cationic, lipophilic fluorescence probes

The problems concerning the use of cationic lipophilic dyes to evaluate the MMP include (a) nonspecific binding of the dye, e.g., rhodamine 123 (Rh-123), which locates in a number of places regardless of the level of energetic level of mitochondria or (b) the need to use large amounts of biological material to record signals of sufficiently high intensity [146]. Moreover, numerous potential-sensitive fluorescent probes may nonspecifically inhibit mitochondrial respiration, and thus, indirectly contribute to the changes in mitochondrial potential. Such features are characteristic for carbocyanine dyes such as $DiOC_2$ (3), $DiOC_5$ (3), $DiOC_6$ (3), $DiOC_2$ (4) [147], or rhodamine 123 [148]. Due to the cytotoxicity of these compounds, it is recommended to avoid the use of these dyes at loading concentrations higher than 1 μM. In general, to restrain problems with fluorescent signal that is nonproportional to MMP, probe concentrations should not exceed 500 nM [149]. Therefore, the evaluation of a threshold concentration for the selected probe, above which a fluorescence quenching occurs, is always highly recommended. Otherwise, the accumulation of a probe used at the excessive concentrations may likely complicate the data interpretation [150, 151]. Furthermore, some literature reports indicate that rhodamine 123 has a high nonspecific fluorescence in the presence of the elevated level of reactive oxygen species in the sample (regardless of whether isolated mitochondria or whole cells are studied) and this type of fluorescence is not related to the mitochondrial membrane potential [152, 153]. Therefore, it may happen that inexperienced researchers may consider such a rhodamine 123-derived fluorescence as an indicator of high (or low) MMP, whereas in fact (usually unconsciously) they evaluate the level of ROS. Therefore, the best option, as far as we want to obtain the reliable results, is not to employ this dye in the measurements of MMP [154]. Unfortunately, relatively low cost of rhodamine vs. other more specific markers used to evaluate the MMP, the apparent ease of a "direct" interpretation of the obtained results and the widespread use of this dye, makes rhodamine still commonly used by many researchers to assess the potential of mitochondrial membrane in both the studies that use spectrofluorometry and those that employ flow cytometry.

8.3. The use of JC-1 to evaluate MMP with the addition of MP uncouplers: CCCP and FCCP

One of the most popular dyes to evaluate MMP is JC-1 (5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide). It is also one the most commonly used indicators of MMP in human blood platelets [155–160]. Although the studies of MMP in rodent platelets are very

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scarce, the occasionally collected data point out that JC-1 also remains the most commonly used probe for the evaluation of MMP in both rat [161, 162] and mouse [155, 163–167] blood platelets. At high concentrations, this dye forms aggregates. This change is accompanied by the shift in the emission wavelength from green fluorescence (FL1 channel), characteristic for the JC-1 monomers (occurring at low mitochondrial potential), to red fluorescence (channel FL2), characteristic for JC-1 aggregates (occurring at elevated mitochondrial potential) (**Figure 13**).

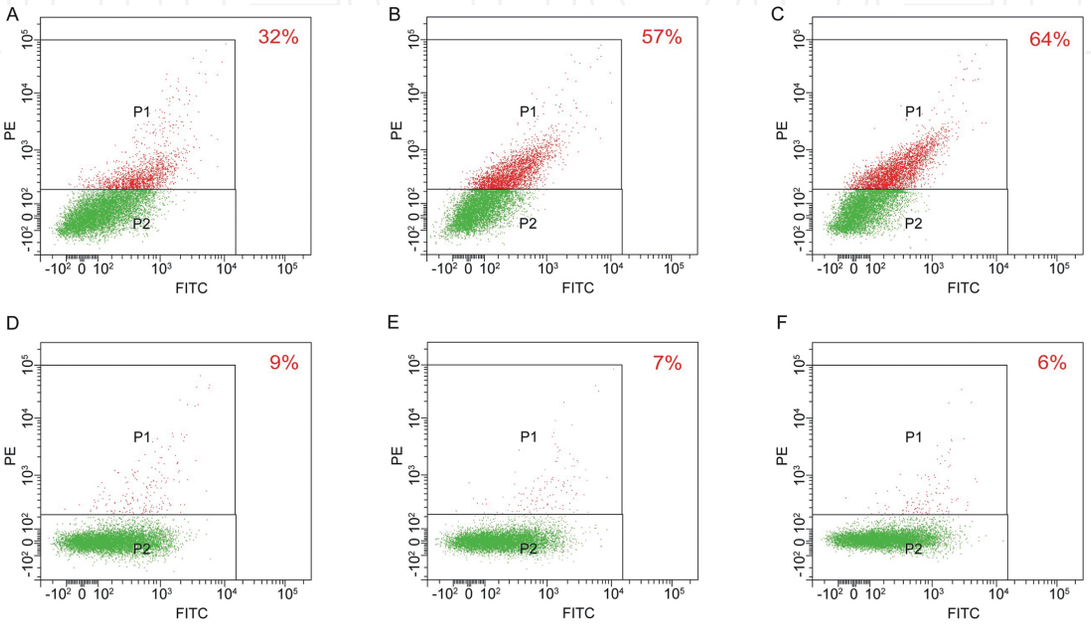

Figure 13. The flow cytometric analysis of MMP measured with the use of JC-1 in mitochondria isolated from rat hepatocytes. The fraction of JC-1 aggregates (region P1) in mitochondria (A, B, C) incubated with EtOH (solvent for FCCP) disappears after the incubation with 5 μM FCCP lasting for 1 minute (D), 6 minutes (E), and after 11 minutes (F). Green dots in the region P2 represent the fluorescence emitted by monomers, while red dots in the region P1 show the fluorescence emitted by JC-1 aggregates. The number of JC-1 aggregates was presented as a numerical value (in red).

In mitochondria with a high MMP, JC-1 spontaneously forms aggregates, which results in the intense red fluorescence dominating over a weak green fluorescence (**Figure 13A**, **B**, and **C**). With a decline in mitochondrial membrane potential, caused for example by the addition of a strong MMP uncoupler (such as CCCP or FCCP) to the sample, the dye concentration inside the mitochondrial matrix reduced, which is accompanied by the disintegration of JC-1 aggregates to monomeric forms. These changes lead to a distinct change in the color of the light emitted by JC-1, from red to green (**Figure 13C**, **D**, and **E**). A simple mechanism of action of this dye is based on changes in the fluorescence emitted by either monomers or aggregates or both. The use of JC-1 allows for a fast and direct assessment of the MMP without the considerable risk of committing interpretation errors compared to the protocols using rhodamine 123. In **Figure 13**, we can see that before the addition of the uncoupler FCCP, the mitochondrial potential is very high, which is reflected by a strong increase in the concentration of the tracer inside mitochondria and the efficient formation of JC-1 aggregates. FCCP leads to the uncoupling of mitochondria and causes a quick and drastic decrease in MMP so

that any later changes in MMP of the uncoupled mitochondria (from 1 to 11 minutes) are almost imperceptible. The ratio of red light to green fluorescence emitted by JC-1 is then a suitable indicator for the evaluation of the MMP because the resultant fluorescence ratio is not influenced by size, shape, or density of mitochondria inside the cells. Moreover, JC-1-staining can be used for both isolated mitochondria and whole cells [146, 168]. Advantages and disadvantages of the flow cytometry technique in the analysis of MMP with the use JC-1 probe are mainly derived by the characteristics of the probe. Like in the case of other cationic, lipophilic dyes, the use of JC-1 may also lead to collecting artifacts associated with too short time of incubation of cells or mitochondria with JC-1. Therefore, it is important to carry out preliminary tests to determine the optimal time of staining (**Figure 13**). To illustrate this problem, the mitochondria were incubated for 1 minute with JC-1 before measurement and then the samples were measured again 5 and 10 minutes after the first measurement. After 11-minute incubation of mitochondria with probe, the ratio of red to green fluorescence increased from 0.3 to 1.1 (**Figure 13A**, **B**, and **C**). Such an increase was not observed for FCCP-uncoupled mitochondria (**Figure 13D**, **E**, and **F**). This clearly shows that too short time designed for the dye accumulation results in the underestimation of the JC-1 aggregate/monomer ratio (and the misevaluation of MMP), and may lead to the blurring of the differences between mitochondria with different MMP. Hence, the rigorously controlled time of mitochondria incubation with the probe is essential. The recommended incubation time may vary depending on the studied sample and usually it is within the range of 1 and 15 minutes. Therefore, it seems highly desirable to evaluate the incubation time that would be appropriate to a given experimental protocol (which may differ in details since it closely depends on the type of examined mitochondria) and to stick to it faithfully. Due to the broad spectrum of JC-1 emissions, the additional staining of a sample with antibodies conjugated with FITC or PE may cause certain problems. This apparent obstacle, however, can be bypassed by choosing antibodies conjugated with fluorochromes emitting in the deep red (channel FL3). This creates additional opportunities for researchers, but it is not entirely devoid of drawbacks because such a choice may sometimes be quite problematic when setting up the compensation, e.g., in the case of simultaneous staining with propidium iodide and JC-1. Other commonly used indicators of MMP are TMRM (tetramethyl rhodamine methyl ester) and TMRE (tetramethyl rhodamine ethyl ester). However, TMRE was found to inhibit the mitochondrial respiration chain even stronger than Rh123 when used at approximately the same concentrations. Otherwise, TMRM has no effect on mitochondrial respiration, at least when used at low concentrations [151], and therefore, it became one of the most popular dyes to evaluate MMP in different cells [169–173]. TMRM is also very often used in the research of both human and rodents' blood platelets [174, 175]; according to the number of publications listed in PubMed, TMRM is actually the second most frequently used indicator of MMP in mouse blood platelet mitochondria [141, 176]. Both TMRE and TMRM exhibit a considerable fluorescence quenching upon their accumulation in mitochondria, and the characteristic red shifts in both the maximum excitation and emission wavelengths [151]. These dyes can be used in both nonquenching (below 5 nM) [177] and quenching modes, keeping in mind, however, that the appropriate approach and suitable calculations should always be carefully elaborated [178]. Moreover, it is important to remember that very often mitochondrial preparations are not

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homogeneous. They may significantly differ in both the number of mitochondria in cells (important for measurements in whole cells) and the level of mitochondrial membrane potential (important for measurements in whole cells or isolated organelles). Mitochondria are cellular organelles that are in a constant motion, undergoing fission and fusion, which are usually accompanied by the MMP changes passed on adjacent mitochondria [179]. The consequence of this is the difference in the accumulation of a tracer in the studied mitochondria preparation, which in turn affects the recorded differences in the fluorescence intensity of the probe [180]. The advantage of flow cytometry over spectrofluorometry lies in the fact that the fluorescence is collected from each object individually, thus allowing to calculate median or mean and to determine a variability for all objects in the sample. This gives an idea about the biological variation of mitochondria presented in the sample. Moreover, the possibility of multicolor staining, using different markers to study various parameters of mitochondrial function, seems the advantage hard to overestimate. However, since for the evaluation of mitochondrial parameters an ideal method has not yet been discovered, it is important to remember the limits of the presented methods.

9. Blood platelet apoptosis—do we need to bother with this?

Blood platelets are produced by megakaryocytes, then released into the bloodstream, where they circulate for around 10 days (anucleate human platelets) or much shorter—from a few hours to a few days (anucleate rodents' platelets). Afterward, platelets are destroyed by the reticuloendothelial system, mainly in spleen and liver [181]. While some mechanisms regulating the platelet biogenesis have been known very well [182], the factors that control their lifespan and death are still a subject of many speculations. Physiologically, the cells that are aged, redundant, damaged, or infected are eliminated by the organism in the course of a cell suicide mechanism termed apoptosis [183]. Nevertheless, apoptosis is thought to be unique to selected cells, and although the mechanisms controlling the lifespan of platelets is still not clear, a number of studies suggested that apoptosis may also concern blood platelets [184, 185]. Hence, some nontrivial questions may be raised. How do platelets undergo apoptosis living so shortly? Is it possible at all? Is it reasonable from the evolutionary point of view? What are the mechanisms of their apoptosis, if any? Willing to answer these questions, in this chapter we provide a brief insight into some peculiar and specific regularities of apoptosis in murine platelets. It has been evidenced quite well that platelets display all the typical events of apoptotic pathway. It suggests that we can consider the occurrence of apoptosis in platelets. Numerous studies have documented that apoptosis in platelets can be induced by various factors, such as (a) natural or artificial chemical agonists, i.e., ADP, collagen, thrombin, hydrogen peroxide, arachidonic acid, calcium ionophore-A23187, ionomycin, valinomycin, dibucaine, epinephrine, and cyclosporine A [186–188]; (b) oxidative-stress-associated factors, i.e., hyperlipidemia, altered cardiac functions, diabetes, and chronic uremia [189, 190]; (c) physical factors, i.e., hyperthermia, hypothermia, platelet storage, and shear stress [190–192]; and (d) others, i.e., resveratrol, doxorubicin, etc. [193, 194]. The majority of these proapoptotic factors have been identified when studying human platelets; however, it is likely that a

number of them may *per analogiam* also act in rodents' platelets (i.e., originating from rats or mice). Until now, to the best of our knowledge, merely very occasional papers related to this subject have been published. The first reports date back to the last decade, suggesting the possible mechanisms of apoptosis in blood platelets of these species [191]. Interestingly, at the very beginning of the researchers' interest focused on platelet apoptosis, different terms were used to define the phenomenon occurring specifically in these cells, e.g. "process that resembles apoptosis," "apoptosis-like events," "apoptosis-like process," or "constitutive death program" [191]. Most likely it happened because two intimately connected physiological phenomena, apoptosis and platelet activation, share common morphological and biochemical features. Nowadays, there is no longer doubt that anucleate platelets of higher vertebrates also have the ability to self-destruction in the course of the commonly known process of apoptosis. The symptoms of apoptosis concern the "bunch" of morphological changes, including the platelet shrinkage, plasma membrane scrambling, cytoplasm condensation, or phosphatidylserine (PS) redistribution to the outside leaflet of the surface membrane lipid bilayer. All of the above-mentioned alterations were found in murine platelets after the treatment with either thrombin or collagen [195]. Moreover, the other apoptosis markers, such as the enhanced depolarization of the mitochondrial membrane (reduced membrane potential), cytochrome c release from mitochondria, caspase pathway activation, and microparticle formation, have been well annotated to blood platelets from mice [196]. In the paper by Gyulkhandanyan et al., the authors indicated some murine models, in which the occurrence of apoptosis in platelets was studied. It was revealed that on exposure to various factors (antiplatelet antibodies, diabetes induction, aging processes, and genetic mutations), blood platelets of mice, rats, rabbits, or dogs exhibited all the hallmarks of classical apoptosis [196]. In another study, Zhao et al. demonstrated that lovastatin (a statin drug used for the lowering of plasma cholesterol) induced a dose-dependent apoptosis of murine platelets, and the revealed significant depolarization of mitochondrial membrane strongly suggested that apoptosis proceeded through the mitochondria-associated pathways. Furthermore, the authors demonstrated that lovastatin therapy lead to the upregulation of proapoptotic Bak protein, downregulation of antiapoptotic Bcl-X_L protein, and the activation of caspases-3 and 9, thus clearly indicating that these platelets were able to undergo the apoptosis using mitochondrial destruction [197]. The integrity of outer mitochondrial membrane (OMM) is regulated by proapoptotic and antiapoptotic members of Bcl-2 family proteins. Following proapoptotic treatment, the balance between Bcl-2 regulatory proteins shifts in a proapoptotic direction. Proapoptotic Bcl-2 proteins interact with the OMM, resulting in membrane permeabilization and release of apoptogenic factors [198, 199]. The investigations conducted by Leytin et al. have shown that *in vivo* administration of antiplatelet antibody, antiglycoprotein IIb (anti-GPIIb), causes apoptotic changes in murine platelets, involving the dissipation of mitochondrial transmembrane potential, the activation of caspase-3, and the augmented exposure of PS [200]. Other research demonstrated apoptosis of rabbit platelets exposed to the action of ßy-CAT (nonlens crystalline). The activation of caspase-3, augmented PS exposure, depolarization of mitochondrial membrane, cytochrome c release, as well as a strong expression of Bax and Bak proteins were recorded. Two of these phenomena, PS delocalization and mitochondrial membrane depolarization, appeared Ca^{2+} dependent [201]. Interestingly, it was

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also revealed that aspirin (a widely used antiplatelet and inflammatory drug) administered in mice caused significant reduction in the half-life of circulating platelets. Moreover, following the treatment with aspirin platelets exhibited features of typical apoptosis, including the decrease in mitochondrial membrane potential, the increase in PS exposure, the rise in the cytosolic ROS concentrations, and the activation of caspase-3 [202]. The above findings, briefly reviewed herein, indicate that murine platelets are intrinsically programed to undergo cell death and that their lifespan in the circulation is circumscribed by the initiation of the pathway of apoptosis. Moreover, it also seems that mitochondria play a central role in mediating the phenomena associated with platelet life and death cycle. It appeared that diverse stimuli (e.g., thrombin, calcium ionophore A23187, or antiglycoprotein (GP) IIb antibody) are able to induce depolarization of transmembrane mitochondrial potential in blood platelets. Mitochondria-associated apoptotic markers (caspase-9 and proapoptotic members of Bcl-2 family, caspase-3) have also been shown to get induced in animal platelets on the appropriate stimulation, both under *in vitro* and *in vivo* conditions [188, 203]. Remarkably, the contribution of the extrinsic apoptosis pathway to the regulation of platelet lifespan and death has not been hitherto extensively explored. Probably because until now platelets had been reported to lack the death FAS receptors, suggesting that classical extrinsic activation of apoptosis by FAS ligand is unlikely in these cells [204]. However, recently Schleicher et al. have evidenced that both human- and mouse-activated platelets present the death receptor Fas ligand (FasL) on their surface, which is able to trigger platelet apoptosis [205]. These findings suggest that platelets are probably not "slavishly" dependent on mitochondrial signaling to undergo apoptosis, but can also use other pathways to induce process of the programed death. In the face of these reports, probably the role of the extrinsic pathway of apoptosis in regulating platelet survival, particularly in pathological states, will still be explored and new facts may soon appear on this intriguing topic. The above-mentioned pieces of evidence seem supportive for the belief that apoptosis may also concern platelets in mice or, more generally, in rodents. This in turn implies another question: how apoptotic platelets could be identified and further characterized using flow cytometric tools? In 2009 The Nomenclature Committee on Cell Death (NCCD) has formulated the recommendations for the determination of apoptosis in nucleated cells. Later, based on these guidelines, Gyulkhandanyn et al. have proposed the bunch of methods useful for the characterization of apoptosis in anucleate cells, such as platelets. Among them, the flow cytometric analysis of the increased caspase-3 activity with the use of a cell-penetrating carboxyfluorescein probe, FAM-DEVED-FMK (the executioner of caspase-3 activation), has been strongly encouraged. Staining of fixed permeabilized platelets with anti-Bax and anti-Bak antibodies demonstrated the increased binding of these antibodies to proapoptotic Bax and Bak proteins following thrombin treatment [196]. Rapid development of cytometric tools and protocols suitable for the monitoring of apoptosis in anucleate platelets has soon revived the interest in the use of fluorescently labeled annexin V for the determination of the symmetrization of platelet membrane lipid bilayer. The loss of a natural asymmetry of phospholipid distribution, with PS being preferentially located in the inner leaflet of membrane lipid bilayer, appeared so intimately associated with the apoptotic pathway that nowadays the use of annexin V became an integral part of the protocol for determining apoptosis [195, 196]. One particular methodological issue has to be addressed

herein. The protocol with annexin V works appropriately, provided the concentration of Ca^{2+} ions is sufficient [206, 207]. To ensure the efficient annexin V binding, samples are supplemented with exogenous calcium (intraplatelet [Ca^{2+}] increased up to 2.5 mM), which is of particular significance in blood samples anticoagulated with calcium chelators (e.g., sodium citrate) [116, 208]. However, what is good for annexin binding appears devastating for blood platelets. In the milieu rich with Ca^{2+} ions platelets undergo facilitated spontaneous activation, and thus, they become more procoagulant [209–211]. In turn, the enhanced platelet priming and accelerated activation drive to the augmented consumption of platelets and a massive formation of microparticles [212–214]. Thus, the protocol may appear to drive artifactual outcomes, as far as it perpetuates platelet activation, although, paradoxically, it has been designed to determine the extent of platelets' readiness to get activated. Remarkably, all the above hallmarks, presented in the course of platelets' staining with annexin V, are strictly convergent with those typical for apoptosis [215–218]. Then, it is of no wonder that merely the determination of annexin V binding might discriminate reliably apoptotic from nonapoptotic platelets. Occasionally, another protocol for the determination of PS exposure in blood platelets, devoid of the above-mentioned drawbacks, has been elaborated and validated, which uses merocyanine 540 instead of annexin V. This alternate or compounding protocol is based on the membrane charge redistribution and may be used in a whole blood flow cytometry for the monitoring of platelet membrane symmetrization. Platelet staining with MC540 does not require Ca^{2+}; however, it is sensitive to the fluctuations in the intraplatelet [Ca^{2+}] during platelet activation, required for the increased PS exposure and membrane bilayer symmetrization [219, 220]. Furthermore, it has been revealed that the third upstream marker of apoptosis, the expression of proapototic members of Bcl-2 protein family, and their translocation to mitochondria can be successfully quantified by flow cytometry, allowing the detection of these proteins in both the cytosol and in the outer mitochondrial membrane. Another apoptotic hallmark, the changes in platelet mitochondrial membrane potential, may also be detected with the use of flow cytometry. Several cell-penetrating fluorescence dyes, such as $DiOC_6(3)$ or JC-1, have been recommended for that, as we have already much more profoundly described in the previous chapter [154, 155]. Platelet shrinkage, the consequence of shear-induced shedding of MPs, can be investigated using forward light scatter (FSC) and side scatter (SSC) flow cytometric histograms since FSC and SSC light signal intensities have been shown to correlate with cell size and internal cellular structure [221]. In order to decide whether platelets are apoptotic in the tested platelet population (e.g., platelet-isolated animal models) and to evaluate the sensitivity of the tested population to stimulation with appropriate apoptosis trigger, it has been recommended to use not one but several apoptosis markers simultaneously to determine a whole spectrum and the magnitude of apoptotic responses. All assays should be performed in parallel with positive and negative controls. Platelets may be considered apoptotic if the level of the specific apoptotic response is statistically higher than that in respective negative control population, keeping in mind that the extent of apoptotic changes can be determined in comparison with positive controls. In accordance with the policy raised by the Nomenclature Committee on Cell Death, it has been recommended to employ the following methodology for the determination of apoptotic platelets: simultaneous quantitative determination of different apoptosis markers (e.g., mitochondrial membrane depolariza-

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tion, MPTP formation, Bax and Bak expression, caspase-9 and caspase-3 activation, gelsolin and moesin cleavage, PS exposure, platelet shrinkage, and microplatelet formation) [196, 213, 222–225]. Healthy donor platelets treated with appropriate diluent buffer as a negative control and platelets treated with strong platelet agonists, such as calcium ionophore A23187 or thrombin or collagen, as a positive control, are strongly recommended [196]. In general, reviewing the literature related to the methodology employed for studying apoptotic events in blood platelets, flow cytometry seems one of the most often utilized and also one of the most universal for the confirmation of apoptosis. In summary, our literature review supports the argument that the phenomenon of apoptosis is not only theoretically possible, but that it really exists in murine platelets. Although it might seem unbelievable that platelets, the relatively short-lived cells, undergo apoptosis, there is quite a lot of data showing that apoptosis-like processes have been linked to these cells. Nevertheless, certainly the further studies on other apoptotic mechanisms could help in the understanding of the association between apoptotic pathway and platelets kinetics, platelet lifespan and their survival, and, as such, could add new insights into platelet biology.

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