Chapter

Platelet Imaging

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Abstract

The knowledge gained through imaging platelets has formed the backbone of our understanding of their biology in health and disease. Early investigators relied on conventional light microscopy with limited resolution and were primarily able to identify the presence and basic morphology of platelets. The advent of high resolution technologies, in particular, electron microscopy, accelerated our understanding of the dynamics of platelet ultrastructure dramatically. Further refinements and improvements in our ability to localize and reliably identify platelet structures have included the use of immune-labeling techniques, correlative-fluorescence light and electron microscopy, and super-resolution microscopies. More recently, the expanded development and application of intravital microscopy in animal models has enhanced our knowledge of platelet functions and thrombus formation in vivo, as these experimental systems most closely replicate native biological environments. Emerging improvements in our ability to characterize platelets at the ultrastructural and organelle levels include the use of platelet cryogenic electron tomography with quantitative, unbiased imaging analysis, and the ability to genetically label platelet features with electron dense markers for analysis by electron microscopy.

Keywords: platelets, platelet disorders, electron microscopy, confocal microscopy, fluorescence microscopy, intravital microscopy

1. Introduction

Platelets are small, anucleate cells with central roles in hemostasis, endothelial regulation, inflammation, and immune responses [1–5]. Critical to these biologic processes, platelets undergo several dynamic morphologic changes in overall shape and size. Furthermore, at the organelle level they alter and differentially release their cellular contents in signal dependent manners [6–10]. Detailed knowledge of these changes is essential for understanding platelet biology, including in the contexts of hemostasis and thrombosis, cellular and pathogen interactions, and intracellular signaling mechanisms [9, 11–14]. Within this chapter, we will review the methods and applications of multiple modalities of platelet imaging including light transmission, super-resolution, electron, and intravital microscopy. Advances in our ability to image platelets have enabled a more complete understanding of platelet biology in health and disease, and these techniques remain powerful clinical and research tools [15–19]. We will also detail the challenges of these imaging methods specific to platelets, and future barriers that should to be addressed.
2. Light and fluorescence microscopy

2.1 Overview

Owing to their small size, platelets were not discovered until long after red blood cells and leukocytes had been visualized with traditional microscopy. With the development of compound and achromatic microscopes, they were finally observed by several scientists in the nineteenth century, including the Italian scientist Giulio Bizzozero, who began to identify their roles in hemostasis and thrombosis [14, 20]. In their resting state, platelets are 2–3 μm in diameter and discoid in shape, appearing as small blue circular structures on traditional light microscopy with Giemsa staining (Figure 1). Despite the resolution limitations of light microscopy, many important advances have been made in our understanding of platelet structure and function with this technique. In fact, light microscopy remains an important diagnostic and research tool today [21, 22].

2.2 Diagnosis of platelet disorders with light and fluorescence microscopy

Many heritable platelet disorders can be diagnosed using light microscopy of blood smears when used in conjunction with immunofluorescent staining. Typically, light microscopy is initially used to identify abnormalities in platelet shape and size, narrowing the differential diagnosis, which is then followed by specific staining or flow cytometry to make a final diagnosis (Figure 2) [21]. This may be diagnostic in approximately 25–35% of cases of suspected inherited thrombocytopenia, and can obviate the need for further expensive genetic testing or complex imaging modalities such as electron microscopy [21, 22].

2.3 Platelet spreading assays

Differential interference contrast microscopy is a specialized form of light microscopy used to characterize platelet spreading and adhesion to proteins on transparent surfaces [24]. For example, the role of actin proteins in the platelet

Figure 1.
Standard light microscopy of platelets. Image from a light microscope (500×) from a Giemsa-stained peripheral blood smear showing platelets (blue dots) surrounded by red blood cells (pink circular structures). Image and caption by Dr. Graham Beards, reproduced under Creative Commons GNU Free Documentation 1.0 Generic license (https://creativecommons.org/licenses/by-sa/1.0/deed.en).
responses to collagen or fibrinogen can be studied (Figure 3) [25]. This form of microscopy enhances contrast in unstained platelet samples thereby allowing adequate visualization of shape change [24]. Platelet spreading experiments have led to several important advances in platelet biology such as the identification of sex differences in platelet adhesion [26], discovery of novel platelet structures such as actin nodules [27], and delineation of platelet-extracellular matrix interaction [25, 28].

2.4 Imaging platelets under flow

In order to study platelet behavior in conditions that more closely replicate human vascular biology, techniques that incorporate the imaging of platelets under flow conditions have been developed [8, 29–31]. These include in vitro methods such as “flow chambers” and in vivo intravital microscopy (detailed in Section 5). Flow chambers include a wide range of home-made and commercially available

Figure 2.
Light microscopy of peripheral blood smear. Performed with Giemsa staining, showing a giant platelet characteristic of Bernard-Soulier syndrome. Diagnosis can then be confirmed by either immune-fluorescent microscopy or flow cytometry demonstrating defects in glycoprotein Ib. Image reproduced with permission from Sandrock et al. [23].

Figure 3.
Analysis of platelet spreading and actin organization. Washed platelets (2 × 10^7 platelets/ml) from wild type and HS1 (actin binding protein) −/− mice were added to cover slips coated with collagen (100 μg/ml), collagen related peptide (10 μg/ml) or fibrinogen (100 μg/ml) ± thrombin (1 U/ml) and allowed to settle for 45 or 90 min at 37°C. Spread platelets were fixed in formalin and imaged using differential interference contrast microscopy. Representative images of platelets at 45 min are shown. Image and caption reproduced with permission from Thomas et al. [25] under Creative Commons Attribution License (http://creativecommons.org/licenses/by/2.0).
devices such as parallel-plate flow chambers or biochips with micro channels through which fluid is passed at arterial or venous shear stresses [32, 33]. Confocal and fluorescence microscopy is then used to visualize platelet adhesion, aggregation and thrombus formation [30, 33]. Investigators can create a range of experimental conditions to study platelet behavior which has led to many advances in our understanding of platelet biology, thrombus formation, and responses to antiplatelet therapies [29, 32]. Most recently, for example, Tunstromer et al. used platelet staining and time-lapse microscopy to track the movements of individual platelets during thrombus formation using a flow chamber [29]. This model was applied to quantitatively study the effects of platelet inhibitors on platelet contraction, revealing differential impacts on platelet subpopulations [29].

3. Super-resolution microscopy

3.1 Overview

Standard light microscopy offers a maximum resolution of about 200–300 nm due to the limits on resolution to about half of the wavelength of visible light [34]. This allows platelets to be identified, but it is difficult to distinguish and examine intracellular structures. However, recent advances in fluorescence light microscopy with the development of super-resolution microscopy techniques have improved upon conventional light microscopy resolution by 2–10 fold, to the 10–200 nm range, and the pioneers of these advances were awarded Nobel prize in chemistry in 2014 [34, 35]. These novel techniques all utilize fluorescent labeling but broadly apply three different imaging techniques (wide-field microscopy, total internal reflection fluorescence, or confocal microscopy) to overcome the resolution limit of light microscopy, with resolutions at the 10–200 nm range [36, 37]. Further, they can be coupled to automated image analysis to systematically evaluate platelet granules and reveal three-dimensional structural details [34, 37].

3.2 Localization microscopy or pointillist imaging by single-molecule localization (SMLM)

Localization microscopy or pointillist imaging by single-molecule localization (SMLM) entails using many cycles of detecting and localizing single fluorescent labels within the cell achieving up to 10 nm resolution. By repeating the fluorescent imaging cycles thousands of times in conjunction with an on/off mechanism for fluorescence, the positions of molecules are precisely mapped out [37]. However, this process is quite slow given the need for several cycles of images to be taken. Sub-variants include photoactivation localization microscopy (PALM) and stochastic optical reconstruction microscopy (STORM) [34].

3.3 Target based inhibition of fluorescence emission by stimulated emission

Target based inhibition of fluorescence emission by stimulated emission uses an excitation and a depletion laser beam to manipulate fluorescent signals to further improve resolution, relying on diffraction properties of light from the microscope’s focal point [37, 38]. Some examples of this include stimulated emission depletion (STED), reversible saturable optical fluorescence transitions (RESOLFT), and conical diffraction [34]. This method requires a narrow field and imaging an entire sample is a lengthy process, though the resolution is excellent at up to 20 nm [34].
3.4 Structured illumination microscopy

Structured illumination microscopy only improves resolution by approximately 2 fold (up to 120 nm) compared to light microscopy, but this is a more efficient, faster method [35]. It employs mathematical processing of a series of illumination and laser interference patterns to improve resolution [37]. As a proof of concept, Westmoreland and colleagues used structured illumination microscopy to discriminate between three patients with Hermansky-Pudlak Syndrome (a platelet storage defect whereby dense granules are absent) and healthy controls [35]. This work highlights some of the inherent limitations of super-resolution microscopy. Because it relies upon fluorescent labeling of structures of interest, there are risks of mislabeling and therefore misidentification of structures. For example, in their paper, the authors noted that in order to stain dense granules fluorescently, a CD63 marker was used, however, off target effects such as identification of lysosomes were seen as these are also known to be CD63 positive. The description of platelet actin nodules required for platelet-platelet interactions and their potential relevance to Wiskot-Aldrich syndrome is another advance that was made using structured illumination microscopy [39].

In summary, super-resolution microscopy is a recent and notable advance over traditional light microscopy techniques in the identification and visualization of platelet ultrastructural features. Despite some limitations, it does offer the advantage, particularly for structured illumination microscopy, of being relatively less time consuming and intensive than other advanced imaging techniques (such as electron microscopy), and as such may ultimately become more widely available.

4. Electron microscopy of platelets: transmission, scanning, and cryogenic

4.1 Overview of electron microscopy

Electron microscopy (EM) utilizes a beam of accelerated electrons as the source of illumination, and because the wavelength of an electron is up to 100,000 times shorter than that of visible light, this provides resolution at the nanometer level with excellent visualization of platelet ultrastructural details (see Figure 4). EM was first developed in the 1930s, and much of our detailed knowledge of platelets has been gleaned through application of EM, including through transmission, scanning, and cryogenic EM [40, 41]. Substantial early contributions were made by James White who analyzed platelet ultrastructure in health and disease states using EM [13, 14, 16, 42–48], and by Marcel Bessis, who studied both megakaryocyte and platelet ultrastructure in addition to other blood cell morphologies [49, 50]. In this section, we will explore the applications of EM in clinical and research settings and discuss their respective advantages and drawbacks.

4.2 Scanning and transmission electron microscopy

Scanning (SEM) and transmission electron microscopy (TEM) can magnify up to a resolution of 0.2 nm [15]. In SEM, detailed topographical images are obtained of the platelet surface. However, to examine the contents of platelets, TEM is used which sends a beam of electrons through ultrathin sections, providing excellent internal details. EM relies on differences in electron density to differentiate individual structures; however, some structures may have similar electron densities. For example, the identification and study of platelet lysosomes with EM requires
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staining with acid phosphatase or by using arylsulfatase reactive granules to differentiate them from alpha granules [52]. EM has also been used to demonstrate how platelets may release organelles encapsulated in vesicles, such as mitochondria, to drive pathologic inflammation [53, 54].

Despite the significant advances SEM and TEM contributed to the understanding of platelet structure and biology, they have important limitations. Both SEM and TEM require extensive processing of the samples. This includes fixation, dehydration, hardening or embedding, as well as sectioning. Given that platelets are prone to activation, degranulation, and microparticle release with even modest environmental stimuli, the concern is that these fixation processes risk significantly altering and activating platelets. The most common fixative used for SEM and TEM of platelets is glutaraldehyde, which preserves morphology best of the agents used. Despite these limitations, SEM and TEM have significantly contributed to our understanding of the intracellular organization of platelet contents with nanometer resolutions. Critically, SEM and TEM can be combined with immune-gold labeling to further localize structures [7]. For example, this labeling approach has been used to delineate the intracellular distribution of vWF [10], and to demonstrate that thrombin but not adenosine diphosphate treated platelets have upregulated GLUT-3 receptors [55].

4.3 Cryogenic-electron microscopy

Cryogenic electron microscopy (Cryo-EM), developed in the 1980s, is a technique in which EM is applied to samples which are nearly instantaneously cooled to cryogenic temperatures in aqueous solution [56]. This process (typically done by plunging into liquid ethane) occurs so rapidly that water molecules do not rearrange into crystalline form (also known as amorphous or vitreous ice). This preserves cellular structures such as lipid membranes in their near native states [41]. This is a particularly important advantage over TEM and SEM which require extensive fixation as described above because platelets are known to activate with minimal stimuli. Furthermore, samples can then be stored for long periods of time in liquid
nitrogen. Once cryogenically preserved, standard TEM techniques are applied to the samples. Additionally, multiple “tilt-series” or tomograms are obtained, in which images are captured at multiple angles, which can then be built into 3D reconstructions (also called cryogenic electron tomography or cryo-ET), which can detail internal structures such as the open canalicular system, organelles, and granules [57, 58] (Figure 5).

4.4 Diagnostic and research applications of electron microscopy

There are several important diagnostic applications of EM for assisting in the diagnosis of a variety of platelet disorders, particularly for granule defects [15]. Further, there have been recent increases in interest in utilizing EM for platelet research, in particular with the advent of cryo-ET paired with quantitative image analysis of platelet ultrastructural features.

The diagnosis of several granule defects is assisted or can even be made using EM. For example, in Gray Platelet Syndrome, which consists of a rare, heterogenous group of inherited platelet disorders, “gray” and enlarged platelets are observed by EM after Wright staining [59, 60]. More specifically, EM is able to reliably demonstrate the absence of alpha granules and presence of empty vacuoles (which are the immature alpha granule precursors), a key diagnostic criterion [15]. Furthermore, cryogenic electron microscopy studies have revealed different alpha granule subtypes, some of which are in fact tubular in nature and lack vWF [7]. Paris Trousseau syndrome is a rare platelet bleeding disorder also present in nearly all patients with Jacobsen syndrome (chromosomal disorder with multiple anomalies), and the diagnosis can be confirmed by detecting the presence of giant alpha granules by EM with immune-gold labeling [16]. Similarly, for Hermansky-Pudlak syndrome,

Figure 5.
Electron tomographic 3D model of the open canalicular system (OCS). The OCS is shown with a pore (P) connecting it with the surrounding milieu. The DTS is indicated by arrowheads. One virtual slice is shown in background. Abbreviations: MTC, microtubular coil DTS dense tubular system; α, α-granules; Mit, mitochondria; OCS, open canalicular system; P, pores of the OCS. Figure and caption reproduced from Neumüller et al. [51], under open access Creative Commons 3.0 Licensing (https://creativecommons.org/licenses/by-sa/3.0/deed.en).
the absence of dense granules on EM is criteria for diagnosis [17], while Chediak-Higashi syndrome can be confirmed by observing decreased dense bodies, normal alpha granules, and giant cytoplasmic inclusions [46]. While the clinical use of EM is not widely available, it remains an important tool for the diagnosis of rare, but important platelet disorders described above, as well as for several others [15, 46].

In addition to the current clinical applications of platelet EM for the diagnosis of platelet disorders, emerging research using cryo-ET shows promise for expanding our understanding of the role of platelets in a range of disease states including cancer [61], thrombo-inflammatory conditions, and for monitoring the quality of blood banking strategies for platelets [51, 58]. In a recent investigation using cryo-ET of platelets from patients with ovarian cancer, the authors identified important morphological changes in nine parameters between those with and without malignancy, including shortened microtubules, and increases in both size and number of platelet mitochondria [61]. These differences were then incorporated into a prediction model which accurately identified 20 out of 23 patients with ovarian cancer, demonstrating exciting potential for future diagnostic purposes.

Electron microscopy has also been used to characterize the properties of platelet microparticles, patterns of platelet activation, as well as the structure of platelet plug formation in *in vivo* models [18, 58, 62, 63]. For example, Ponomareva et al used both SEM and TEM to study the generation of microparticles from platelets in response to stimulation from adenosine diphosphate, arachidonic acid, and thrombin, and found that many microparticles were in fact smaller (less than 300 nm) than those typically able to be detected by flow cytometry [18]. Furthermore, they found that microparticles isolated from patients with conditions such as disseminated intravascular coagulation, heparin induced thrombocytopenia, or lupus were larger and displayed pathologic properties [18]. Lastly, in an *in vivo* mouse model of vascular injury, Tomaiuolo et al used a combination of fluorescence microscopy and SEM to detail different levels of platelet activation within a forming thrombus, and the effects of antiplatelet agents on the hemostatic plug’s architecture [63].

### 4.5 Current limitations and future directions in electron microscopy

Despite its clear potential as both a research and clinical tool, EM has important drawbacks. In addition to high costs, it requires specialized equipment and highly trained personnel, and therefore is neither widely available nor easy to automate. Furthermore, from a technical perspective, sample thickness is limited, as resolution dramatically decreases with increasing diameters, though this presents more of a challenge for imaging larger cells than it does for platelets [41]. Further, if an intracellular structure takes on an unexpected shape or localization, it can be very difficult to identify without the aid of immuno-labeling, which is not always feasible. Currently, techniques to label intracellular structures require fluorescent tagging and light microscopy, which can be correlated to electron microscopy images (correlative light and electron microscopy or CLEM). These have been improved upon with the use of super-resolution light microscopies as described above, but still does not reach the resolution of EM (super-resolution correlated light and electron microscopy) [35, 36, 41, 64]. However, emerging techniques using cloneable, genetic, electron dense markers, for direct localization with EM could have important applications if used with EM of platelets [65, 66]. Lastly, expanding the use of EM for diagnostic purposes, not just of rare platelet disorders, but in other conditions which affect platelets such as malignancy may provide important insights into disease processes as well as novel diagnostic approaches [61].
5. Intravital microscopy: imaging thrombus formation and platelet function \textit{in vivo}

5.1 Overview

Intravital microscopy is a well-established research tool that has been increasingly applied to study platelets, real-time, \textit{in vivo}. In this technique, the role of platelets in thrombus formation and other cellular activities such as interactions with leukocytes can be directly observed by the surgical creation of “imaging window” in anesthetized rat or mouse models. The major advantage of this approach is that it allows for observation of platelets in an environment that most closely matches what occurs \textit{in vivo} in humans compared to other assays of platelet function. Imaging cells in living organisms has been practiced since the early twentieth century [67]. However, it was not until recently with the development of multi-photon or non-linear optical microscopy that has allowed for imaging in tissues at deeper depths (up to 1 mm) whereas conventional confocal microscopy can only be used up to depths of 50–60 μm [68, 69]. Lastly, with the advent of advanced computational power and digital image acquisition technology integrating with microscopes, quantitative analysis of fluorescent markers and other image properties can now be performed to more comprehensively evaluate properties of clot formation, and distinctive platelet activation and signaling patterns [9, 11, 70, 71].

5.2 Choice of site and imaging technique

The cremaster muscle and mesenteric veins of mice are the most commonly used imaging windows for intravital microscopy, but other possible sites include carotid artery, brain, skin, bones, or liver [11, 72, 73]. In order for visualization and subsequent quantitative analyses, platelets must be labeled with fluorescent antibodies or genetically modified mouse models which express fluorescent platelet proteins. Both of these approaches provide adequate capacity to visualize platelets \textit{in vivo}, however, at least for some of the genetic approaches in mice, there may be untoward effects on platelet aggregation compared to antibody labeling approaches [74]. While any vascular bed theoretically may be used to study platelet function, the primary limitations include the anatomic challenges of isolating the vessels while minimizing trauma and inflammation, as well as vessel caliber, since it must be sufficiently transparent for light penetration [75]. Pros and cons of each anatomic site are summarized below (Table 1).

The essential imaging equipment includes a microscope, an illumination light source for fluorescence excitation, an ablation laser (to generate endothelial injury), an image intensifier, and a camera [75]. Endothelial injury and thrombosis can also be induced by mechanical or chemical means, but laser injury offers the advantage of greater temporal and spatial control than the other methods [75]. There are a number of different microscopes that may be used, depending on the type of imaging required, which is in large part dictated by the depth of penetration required by type of tissue being examined, see Masedunskas et al. [69]. Recommendations for camera characteristics include that they be sensitive enough to detect the lowest expected fluorescent signals and to be able to reset fast enough to allow frame rates of 10 per second [75]. For further details on methods of performing intravital microscopy to study platelet function, see Stalker et al. [9], Herr et al. [11], and Falati et al. [70].

5.3 Applications and examples

Intravital microscopy has several applications to identify underlying mechanisms of disease and new aspects of platelet biology. A few illustrative examples of the value
and versatility of the technique include studies demonstrating mechanisms of microvascular thrombosis, platelet production, and for examining platelet leukocyte interactions [19, 72, 73, 76]. For example, in a murine model of arteriolar thrombosis, Lu et al employed cranial intravital microscopy to study anti-platelet properties of caffeic acid (Figure 6) [72]. It can also be used to better characterize platelet interactions with other cell types. Using liver intravital microscopy to examine pathways involved in steatohepatitis, Malehmir et al. showed that platelet colonization depended on Kupffer cells and CD44 binding, and that treatment with anti-platelet therapy reduced infiltration of platelets into liver cells as well as platelet immune interactions and trafficking, ultimately leading to attenuation of liver damage [73]. Lastly, the identification of the lung microvasculature as a site of platelet production is another remarkable discovery made possible by the use of intravital microscopy [19].

5.4 Limitations and future advances

The main limitations of intravital microscopy include the depth of penetration depending on the type of microscope used, technical challenges, heterogeneous features and low transparency of certain tissues, and limitations on the number of fluorescent tags that can be used simultaneously [68, 69, 75]. Future advances may include incorporation of correlative light and electron microscopy (CLEM) to intravital approaches, technical advances in miniaturization of lenses and imaging equipment, and improvements in the array of genetic and molecular markers used to track platelet-cellular and platelet-ultrastructural processes [68].
6. Conclusions and future directions

An increasingly versatile armamentarium of imaging modalities is available for research and diagnostic purposes to study platelets [11, 61, 68, 71]. We anticipate these will continue to play instrumental roles in further discoveries in the field of platelet biology, revealing new insights into the dynamics of platelet signaling, ultrastructure, and function. Understanding how to utilize these evolving imaging technologies to best suit the visualization of platelets is essential. Given the inherently highly reactive nature of platelets, newer approaches that cause minimal manipulation and processing offer significant advantages, including intravital microscopy for studying the roles of platelets roles in thrombus formation and cellular interactions [70], and cryogenic electron tomography which preserves platelets in a near native state for ultrastructural analysis [61]. Important advances continue to be made in labeling approaches that can be paired with imaging modalities, including correlative light and electron microscopy, and genetically inducible fluorescent or electron dense markers [34, 65, 66, 77]. In addition to the technical advances in our imaging capabilities, their integration with advanced statistical and computational power will continue to reveal new opportunities to analyze and better understand platelet structure and function.

Conflict of interest

Dr. Kornblith and Dr. Matthay have nothing to disclose.
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