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Use of electron microscopy to study platelets and thrombi

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Abstract

Electron microscopy has been a valuable tool for the study of platelet biology and thrombosis for more than 70 years. Early studies using conventional transmission and scanning electron microscopy (EM) provided a foundation for our initial understanding of platelet structure and how it changes upon platelet activation. EM approaches have since been utilized to study platelets and thrombi in the context of basic, translational and clinical research, and they are instrumental in the diagnosis of multiple platelet function disorders. In this brief review, we provide a sampling of the many contributions EM based studies have made to the field, including both historical highlights and contemporary applications. We will also discuss exciting new imaging modalities based on EM and their utility for the study of platelets, hemostasis and thrombosis into the future.

Introduction

The study of platelet biology using electron microcopy methods has a long and rich history. It was not long after the introduction of the electron microscope that the first studies of platelet ultrastructure using transmission electron microscopy were published [1]. Conventional electron microscopy is divided into transmission electron microscopy (TEM) and scanning electron microscopy (SEM). In TEM, a beam of electrons is transmitted through a thin section in order to visualize the internal structures. By contrast in SEM, a focused beam of electrons is used to scan the surface of a specimen. A considerable amount of work went into developing the protocols to fix soft tissues so that they could be imaged by electron microscopy, work strongly motivated by the need to visualize tissues and their internal structures in ways that were not possible with any other microscopy technique before. Once the proper fixation protocols had been developed, electron microscopy became instrumental to study platelets, both for basic research [2] and as a diagnostic tool [3]. Today, conventional TEM and SEM approaches remain valuable tools in platelet and thrombosis research, even as EM approaches continue to evolve and new imaging modalities are developed.

A separate review in this series, by Scandola et al [4], focuses on the use of EM approaches for the study of megakaryocytes. The reader is referred to that review for an outstanding

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general description of the various EM methods that are in use today for the study of both megakaryocytes and platelets. In this brief review we summarize some of the contributions made using electron microscopy methods for the study of platelets and thrombus formation. Given the plethora of studies that have utilized EM approaches for the study of platelet biology, the goal of this review is not to be exhaustive, but only to provide examples that highlight the utility of EM approaches across a variety of experimental and clinical applications. We will also discuss recent advances in EM methods for visualization of platelets and thrombi, including the use of electron tomography, cryo-EM, serial block face-SEM, focused ion beam-SEM, and correlative light and electron microscopy (CLEM).

Transmission electron microscopy

Historical TEM studies of basic platelet biology

From the time that electron microscopes became available for the study of biological specimens, investigators have utilized EM approaches to study platelets. Using primitive fixation techniques, Bessis first showed crude platelet ultrastructure in the late 1940s and early 1950s [5]. These initial studies paved the way for a much deeper understanding and characterization of the subcellular components of platelets, and soon studies had described the presence of multiple granule subtypes, intracellular membrane systems and cytoskeletal features (Figure 1) [6–8]. The identification of the microtubule coil that is responsible for the discoid shape of resting platelets was an advance in the early understanding of platelet physiology made possible by EM [9–12]. EM studies also showed that the microtubule coil contracts following platelet activation, resulting in centralization of platelet organelles [13]. In the late 1980s and early 90s, fixation and permeabilization protocols were adapted to visualize multiple components of the platelet cytoskeleton using EM. These studies from Hartwig and others were foundational to our current understanding of the platelet cytoskeletal network. They identified the presence of a spectrin-based membrane skeleton in platelets, and characterized how the membrane, actin and microtubule skeletons interact with each other [14,15]. They also further characterized how changes in the platelet cytoskeleton drive platelet shape change [16]. For comprehensive reviews of the platelet cytoskeleton including outstanding EM images, the reader is referred to chapters devoted to the subject [17,18].

TEM studies were also critical to the early understanding of platelet intracellular granules. Reports from the 1960s determined that dense granules (dense bodies, δ-granules) were the storage compartment for serotonin, ADP and ATP and established the lack of dense granules as the basis for several platelet function disorders, including Hermansky-Pudlak syndrome (HPS). Indeed, White showed that electron opaque platelet dense granules were best visualized by TEM imaging of whole mount platelets [6]. Quantification of dense granules using such imaging remains the gold standard for identifying dense granule deficiencies, including HPS and Chediak-Higashi syndrome [19]. TEM studies demonstrated abnormal α-granules in multiple platelet disorders as well, such as gray platelet syndrome and Paris-Trousseau-Jacobsen syndrome [20,21]. Cytoskeletal defects that impact overall platelet morphology and granule number, such as Wiskott-Aldrich syndrome [22], may also be

readily identified by TEM. For additional information on the use of TEM to identify platelet disorders, the reader is referred to many excellent reviews on the subject [3,19,23–25].

Continuing use of TEM for basic science studies of platelet biology

The wealth of knowledge on platelet ultrastructure derived from early TEM studies continues to inform basic science research on platelets today. For example, investigators recently used TEM to investigate the effects of human aging on platelets, and reported that platelets from older patients have reduced α-granule content compared to young and middle age patients [26]. TEM-based measurements of mitochondrial content differences in platelets helped establish a link between aging-associated production of the proinflammatory cytokine TNF-α and the development of platelet hyper-reactivity [27]. The intracellular origin and structure of platelet-derived microparticles was also studied using TEM to provide a structural basis for the qualitative differences in microparticle formation induced by various platelet activators [28]. Multiple groups have utilized TEM to detail the molecular mechanisms and kinetics underlying platelet granule release [29–31]. All of these studies serve as examples of how TEM imaging of isolated platelets continues to inform our understanding of basic platelet biology in both health and disease.

TEM examination of hemostatic plugs and thrombi

Studies on ‘single’ platelets are key to define the building blocks of platelet biology. These studies are complemented by studies on platelet aggregates formed under various conditions *in vitro* and *in vivo* to understand aspects of hemostasis and thrombosis that emerge from multiple intercellular interactions. TEM observations were pivotal in describing the contents, structure, and temporal evolution of hemostatic plugs formed *in vivo* in both animal models and humans [32–36]. Similar studies also characterized defective hemostatic plug formation in various pathologic settings, such as hemophilia [35,37]. These studies were in large part the basis for our current models of hemostatic system function, including the initiation of platelet adhesion and activation by binding to collagen exposed at the site of injury. Similarly, thrombosis has been investigated using TEM in conjunction with other laboratory assays or imaging methods. Early studies from Mustard and others provided the initial characterization of platelet-rich thrombi, including the spatiotemporal evolution of platelet activation and fibrin formation [38]. Multiple recent studies highlight the continuing utility of conventional TEM imaging for examination of platelet ultrastructure to elucidate their role in hemostasis and thrombosis. For example, TEM was used to examine how clot contraction induces spatial re-distribution of procoagulant platelets in a murine model of thrombosis [39], and to study how platelet necrosis recruits neutrophils in the setting of ischemic stroke in mice [40].

Immunogold electron microscopy

Immunogold labeling is a staining technique that helps to localize cellular structures and macromolecules by loading antibodies or other specifically targeted proteins with colloidal gold particles of nanometer size. Gold is used for its high electron density that increases electron scatter to give dark spots in the TEM and SEM images. This technique has been applied successfully in many platelet studies. For example, immunogold labeling was used to demonstrate the spatial distribution of the GPIb-IX-V complex and $\alpha_{IIb}\beta_3$ on resting and

activated platelets (reviewed in [41]). Immunogold labeling in combination with TEM has been used to determine the subcellular localization of many platelet proteins, such as matrix metalloproteinase-9, which was found on the plasma membrane, α-granules, open canalicular system, and within the cytoplasm both in resting and activated platelets [42]. Immunogold labeling of the purine P2Y1 receptor and the thromboxane-prostanoid TP receptor revealed that, while present at the platelet surface, both receptors were also abundantly represented inside the platelet [43]. Immunogold labeling has also been critical for examining platelet α-granule constituents, providing evidence of heterogeneous localization of various protein constituents, such as von Willebrand factor and fibrinogen, within individual α-granules [44,45].

Scanning electron microscopy

SEM of platelets has been an instrumental technology for both basic science and clinical research. In contrast to TEM imaging of intracellular ultrastructure, in conventional SEM the electron beam scans the surface of specimens providing high resolution imaging of morphologic features. Conventional SEM requires different sample preparation than TEM since it involves fixation and dehydration of bulk samples instead of tissue embedding and sectioning. Rather than 2-dimensional information obtained by TEM of thin sections, SEM can give detailed 3-dimensional information of surface topology, making it particularly useful in a variety of situations where the presence and overall structure of blood clots is analyzed. Its applications include characterizing morphologic changes of single platelets following activation (Figure 2A–B) and examination of the composition and structure of *ex vivo* thrombi (Figure 3). Here, we provide a sampling of how SEM has been utilized to examine platelets in hemostasis and thrombosis, focusing primarily on basic science studies of platelets *in vitro* and clots or thrombi formed in animal models, as well as in clinical settings. It should be noted that there is an extensive literature utilizing SEM to study fibrin network structure in vitro and in vivo that will not be discussed.

SEM for basic science studies of platelet biology

In the 1950s, early studies of platelet biology using SEM detailed the time-dependent changes in platelet shape following exposure to thrombin [46] and began classifying morphologically normal and abnormal platelets [47]. Not long after, early SEM studies examined the structure and composition of *in vivo* hemostatic plugs and thrombi, such as in artificially induced atherosclerotic lesions formed following endothelial denudation in primates [48]. In more recent years, SEM has been used to facilitate the understanding of thrombus formation *in vivo* in mouse models. For example, SEM was used to help understand the mechanism responsible for thrombus formation following ferric-chloride application in mouse carotid arteries, highlighting a previously unrecognized role for red blood cell adhesion [49]. In hemostasis models, SEM has been used to characterize the structure and composition of hemostatic platelet plugs following puncture injury (Figure 2D–F) [50]. *In vitro* models of platelet adhesion under flow using whole blood in microfluidic devices have become increasingly important to study hemostasis and thrombosis using human and mouse blood. Unsurprisingly, SEM has been a valuable imaging technique to examine the outcome of some of these experiments (Figure 2C). One

group, for instance, investigated the interaction between fibrinogen patch size and spacing with platelet deposition under flow [51,52], while our group used SEM to study physical attributes, such as porosity and interplatelet gap size distribution, of platelet aggregates under flow [53]. Other studies have utilized SEM to examine the spatiotemporal regulation of platelet procoagulant activity, membrane ballooning and microvesiculation [54,55]. Recent reports have uncovered important roles for platelets beyond hemostasis and thrombosis. The cross-talk between inflammation, platelets, and coagulation has important clinical implications, and multiple studies have detailed the effects of various cytokines on platelet activation and thrombin generation via SEM imaging [56–58].

SEM for translational research

Besides its use in primarily basic science, numerous studies have employed SEM imaging to address clinically related questions. For example, various animal models have been developed to investigate how a particular anticoagulant or antiplatelet therapy is able to inhibit excessive thrombin generation and/or platelet activation. In many of these models SEM was utilized, in combination with laboratory assays, to investigate the presence and structural characteristics of thrombi following experimental injury to study vascular remodeling [59], venous stasis [60], arterial crush injury [61], and thrombolysis [62], to provide just a few examples. One group used SEM to evaluate the effectiveness of a hemostatic powder against gastrointestinal bleeding, examining clots formed in trauma models [63]. Likewise, SEM was used in animal models to investigate the roles of thrombin and platelet integrins [64], and to assess the effectiveness of nitric oxide to reduce undesired platelet activation following balloon angioplasty [65].

SEM examination of human ex vivo thrombi

Many studies have employed SEM imaging to analyze the ultrastructural characteristics and cellular composition of thrombi formed in humans. In the past, these thrombi needed to be examined post mortem, potentially leading to artifacts. More recently, the advent of mechanical thrombectomy as a clinical intervention has provided freshly extracted intravital thrombi for analysis via a variety of imaging modalities that may then be related to clinical patient data. The structure and composition of thrombi from both myocardial infarction and ischemic stroke patients have been analyzed using SEM in multiple studies [66–71]. The results of those studies highlight the heterogeneity of clot composition in both settings. Arterial thrombi contained a surprisingly large amount of fibrin, in addition to platelets [72]. The composition of pulmonary emboli mirrored the most distal part of venous thrombi from which they originated, which differed from the structure of the body and head of the same thrombi. SEM studies of arterial and venous intravital thrombi as well as thrombotic emboli also revealed the abundance of polyhedral-shaped red blood cells named polyhedrocytes (Figure 3), a morphological sign of intravital platelet-driven contraction [72]. Polyhedrocytes and intermediate forms comprised the major constituents of venous thrombi and pulmonary emboli. The structures within all of the thrombi and emboli were very tightly packed, in contrast to clots formed *in vitro*. Clot contraction both in blood clots *in vitro* and in thrombi *in vivo* leads to a characteristic morphology in which platelets and fibrin are on the exterior (Figure 3A) and polyhedrocytes are on the interior (Figure 3B). SEM was also

used to examine the interaction of thrombi with retrieval devices [73], and the relationship of thrombus composition to resistance to different fibrinolytic regimes [74].

SEM in clinical device development

During their development, a multitude of clinical devices for cardiovascular intervention are evaluated and inspected for the presence of clots and their composition to help improve design. Stents for percutaneous coronary intervention are one category of such devices with the goal of preventing restenosis. Many designs with different materials and the ability of eluting a drug over time have been invented and are clinically available, but for each new iteration assessing the presence/absence of clots is validated via SEM imaging [75–80]. SEM imaging is also utilized to evaluate artificial heart valves to scan the valve leaflets for microscopic mechanical defects, such as tears, or to inspect platelet deposition and fibrin formation on different mechanical valve replacement materials [81–85]. Another example is provided by left ventricular assist devices, vital devices for the heart failure patients receiving them that also come in a variety of designs. Understanding which combination of factors including geometries, materials, shear rates, etc. is clinically better has direct life and death consequences. SEM imaging is used to visualize the presence or absence of platelets and fibrin clots in these devices, either during the development phase or post mortem [86–88]. Similarly, SEM imaging is used to assess the hemocompatibility and thrombogenicity of bioprosthetic total artificial hearts during the development stage [89]. Finally, SEM remains the obligate imaging choice to examine clot formation in hemodialysis catheters [90,91], as well as to examine the effectiveness of anticoagulant therapies in preventing such clots [92].

Recent advances in electron microscopy for the study of platelets

Newer light microscopy techniques, collectively referred to as super resolution microscopy, have achieved resolutions breaking the limits of light diffraction. These approaches are particularly useful for determining subcellular localization and/or colocalization of proteins within cells and even intracellular organelles such as platelet α-granules [93]. However, with resolution limits in the 20–100 nm XY range [94], super resolution microscopy still does not achieve the single nanometer resolution possible using EM. Thus, rather than supplant EM, super resolution microscopy remains a complementary imaging modality. In fact, fluorescence microscopy (including super resolution techniques) may now be combined with EM, an approach referred to as correlative light and electron microscopy (CLEM). Further, EM approaches continue to advance as new sample preparation techniques and microscope technologies become available. For example, various chemical fixation methods are known to poorly preserve membrane structures and have the potential to introduce artifacts such as membrane blebbing [95]. Application of high-pressure freezing with freeze substitution protocols for fixation/dehydration, as well as vitrification of isolated platelets directly on EM grids, has opened new opportunities to examine platelet internal membranes in near native states (*e.g.* cryo-EM). Finally, multiple approaches for the examination of cells and tissues at EM resolution in 3-dimensions have been developed, including electron tomography (ET), serial block face SEM (SBF-SEM) and focused ion beam SEM (FIB-SEM). Each of these newer EM approaches overcomes specific limitations of conventional

EM approaches, while maintaining EM level resolution. Advantages and limitations of each are described in the sections below.

Correlative light and electron microscopy (CLEM)

Correlative light and electron microscopy (CLEM) combines the best features of both fluorescence imaging and EM techniques while overcoming some of their limitations. In CLEM, a fluorescently labeled sample is first analyzed with light microscopy followed by high-resolution EM of the same site. CLEM is usually applied for the following reasons: to locate a rare fluorescently labelled event followed by focusing on that signal for high-resolution EM imaging; to complement information from live imaging with ultrastructural information; and to identify the location of a fluorescently labelled molecule within the ultrastructure [96]. There are only a few examples of CLEM application to platelet studies [97,98]. While CLEM typically combines fluorescence microscopy with TEM or 3D-EM approaches (discussed below), a new frontier is combining fluorescence microscopy with SEM. We recently performed such a study examining hemostatic plug architecture utilizing fluorescence and SEM imaging (Figure 4). We were able to combine biochemical and morphologic information using these two imaging modalities to draw conclusions about the spatial regulation of platelet activation during hemostasis [52]. The fluorescence imaging also provided 3-dimensional information that was not obtainable with the conventional SEM alone, while the SEM yielded more detailed surface topology.

Electron tomography

A major limitation of conventional TEM is the lack of 3-dimensional information when imaging thin sections. Besides advances in tissue preservation protocols, advances in microscope technology coupled with computational tools and image processing techniques now provide for 3-dimensional reconstruction of images obtained from serial tissue sections. Moreover, electron tomography (ET) is an approach whereby a single relatively thick tissue section is imaged by TEM at multiple angles (see Engberts et al for a methodological review [97]). The resulting images may then be computationally reconstructed into a 3-dimensional volume. ET is characterized by outstanding axial resolution (as low as 2 nm), making it particularly useful for imaging of fine membranous structures, including membrane pores, and individual macromolecules can even be localized and identified. The thickness of the tissue section to be imaged is limited to 200–300 nm. ET may be coupled with cryopreservation of cells (cryo-ET) to achieve outstanding resolution of structures in near-native states, which is discussed in the next section.

Cryo-electron microscopy

Cryogenic electron microscopy (Cryo-EM) is a relatively new and rapidly developing type of TEM of hydrated biological samples that are instantaneously cooled to cryogenic temperatures (liquid ethane) without fixation and dehydration. The cooling procedure is so fast that water molecules do not form crystals that damage the structures but acquire a state of amorphous or vitreous ice that preserves the native biological organization of macromolecules, supramolecular structures, and cellular organelles. The cryogenically prepared samples can be analyzed using TEM, including tomography that involves acquisition of multiple images of the tilted specimen followed by 3D reconstruction [99].

Cryo-electron tomography (cryo-ET) of platelets combined with immunogold labeling was used to characterize the 3-dimensional organization of the platelet open canalicular system and its relationship to the dense tubular system, as well as to define α-granule subtypes [100]. Cryo-ET of intact platelets allowed visualization of the cytoskeletal architecture of spread platelets on extracellular matrix correlated with stiffness maps of the platelets determined with atomic force microscopy [101]. Using cryo-ET to examine frozen-hydrated platelets from patients with ovarian cancer revealed significant morphological differences between the cancer and control platelets, including disruption of the microtubule marginal band as well as differences in mitochondria and other subcellular structures [102]. These and other papers suggest the potential of cryo-EM as a powerful technology to study the ultrastructure of platelets in their native hydrated state at nanometer to subnanometer resolution [103].

SBF-SEM and FIB-SEM

To provide an even greater depth of 3-dimensional imaging at EM level resolution, newer techniques incorporate serial tissue sectioning within scanning electron microscopes. Serial block face-scanning electron microscopy (SBF-SEM) incorporates an ultramicrotome into an SEM. The surface of the tissue block is imaged with the electron beam after each section of tissue is removed to collect serial z-plane images that can subsequently be reconstructed in 3-D. Focused ion beam-SEM (FIB-SEM) is a similar application that utilizes a beam of gallium ions to mill the surface of a tissue block with high precision [104]. Both techniques can be used to obtain serial images with nanometer z plane spacing (~20–30 nm for SBF and ~5 nm for FIB), while overcoming the limitation of tissue thickness in tilt series electron tomography. The application of these approaches has been pioneered in the neuroscience community, where they have been used to map individual neurons and neural connections in brain tissue. In platelet biology, they have been used primarily to obtain 3D structures of whole, isolated platelets, in order to examine subcellular structures and changes that occur during the activation process. Pokrovskaya et al recently used SBF-SEM to provide a detailed characterization of platelet size, volume, granule number, OCS volume and other parameters obtained from 3D reconstructions of whole human platelets [105]. FIB-SEM was coupled with immunogold labeling to examine the distribution of clotting factors on procoagulant platelets under flow, highlighting the importance of platelet ‘cap’-like structures capable of increasing the local concentration of clotting factors [106]. Multiple studies have examined the membrane fusion events occurring during α-granule secretion [30,107]. SBF-SEM and FIB-SEM have also been used to examine additional platelet membrane components, such as Golgi [108], and to analyze granule repertoires in the presence of various molecular perturbations [109–111].

Given the large datasets generated when performing ET, SBF-SEM and FIB-SEM, newer computational tools, including machine learning approaches, are now being applied to EM imaging to permit analysis of complex 2- and 3-dimensional data sets. Such approaches allow semi-automated segmentation and analysis of cells within a multicellular tissue (such as a platelet aggregate), as well as intracellular constituents such as granules, other organelles and even microtubules. We anticipate that in time, it will be possible using these approaches to image and reconstruct in 3-dimensions entire hemostatic plugs and thrombi

formed *in vivo* at nanometer resolution. Such studies would allow observation and analysis of platelet subcellular events as they occur in (patho)physiologic settings at an unprecedented level of detail.

Conclusions

In conclusion, for over 70 years electron microscopy has proven to be a staple for clinical and basic research studies in hemostasis and thrombosis. Early studies using conventional EM techniques provided a foundation for our understanding of basic platelet cell biology, including identification of granule subtypes, morphologic changes upon platelet activation and the important role of platelet cytoskeletal components. Electron microscopy remains the gold standard for identifying various platelet abnormalities and storage pool deficiencies. Similarly, EM imaging continues to be a valuable tool for analyzing platelet and thrombus ultrastructure in both humans and animal models of disease, as well as in settings of clinical device development. Recent advances in EM technology coupled with computationally-based image analysis are now starting to open new possibilities for imaging of platelets and thrombi in 3-dimensions at nanometer level resolution. Such approaches hold promise to lead to new discoveries and address outstanding questions in hemostasis and thrombosis. These include a 3-dimensional analysis of α-granule cargoes to resolve once and for all questions regarding α-granule heterogeneity and regulated cargo release; analysis of the spatiotemporal regulation of secretion and other activation events during hemostasis and thrombosis *in vivo*; comparisons of the architecture of hemostatic plugs with thrombi of various etiologies; and alterations in platelets following non-traditional stimulation, e.g. in response to inflammatory stimuli, just to name a few. Further, the increasing inclusion of electron microscopes within dedicated core facilities that provide both hardware and technical expertise makes the use if EM more accessible to investigators than ever before. There is no doubt that electron microscopy will continue to be a valuable tool for the study of platelets and thrombosis into the foreseeable future.

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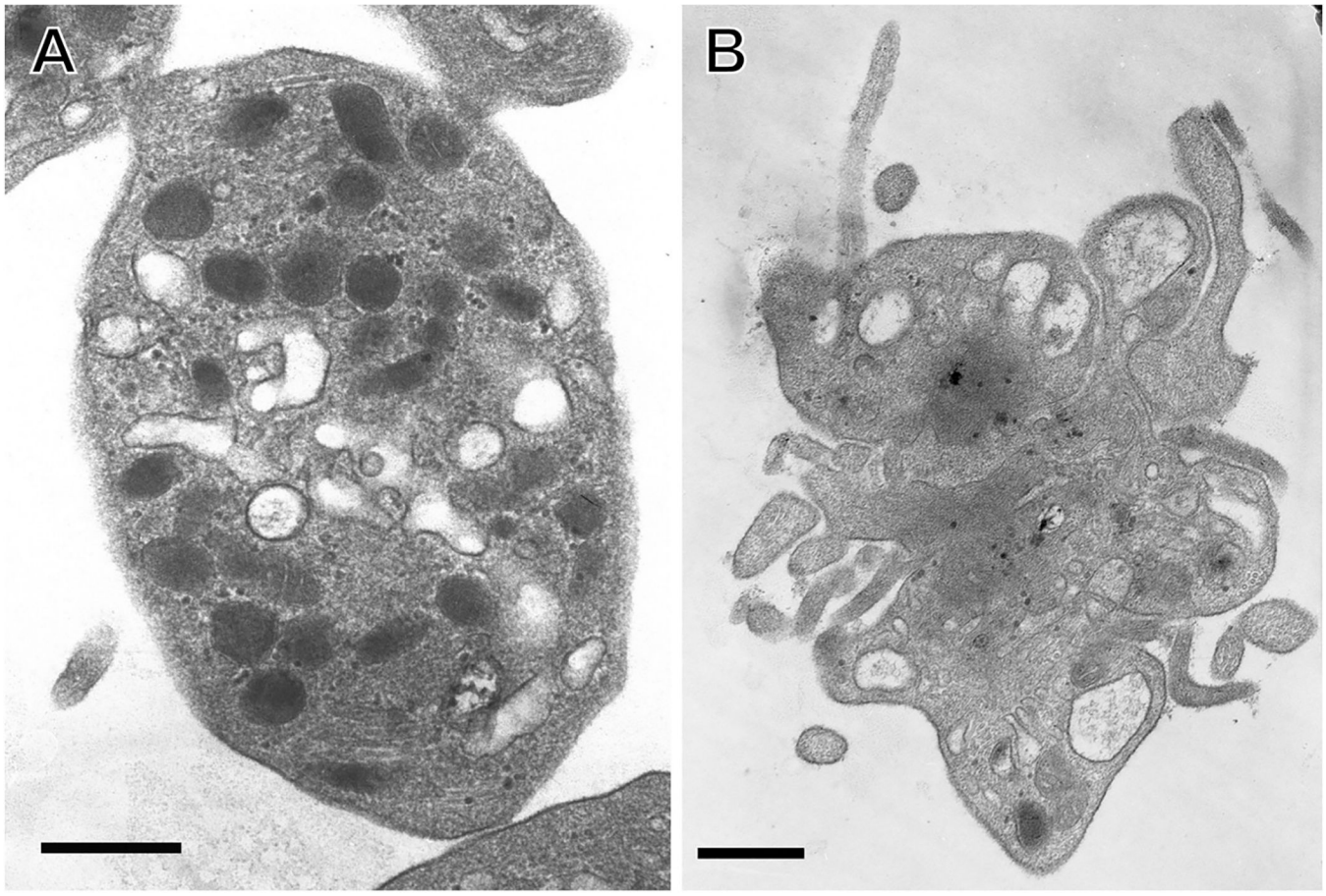

Figure 1: Use of TEM to study platelet structure.

TEM images show **A)** isolated resting human platelet; **B)** isolated thrombin-activated human platelet. Scale bars = 500 nm. Image credit: from the laboratory of John W. Weisel: Anastasia A. Ponomareva and Rustem I. Litvinov.

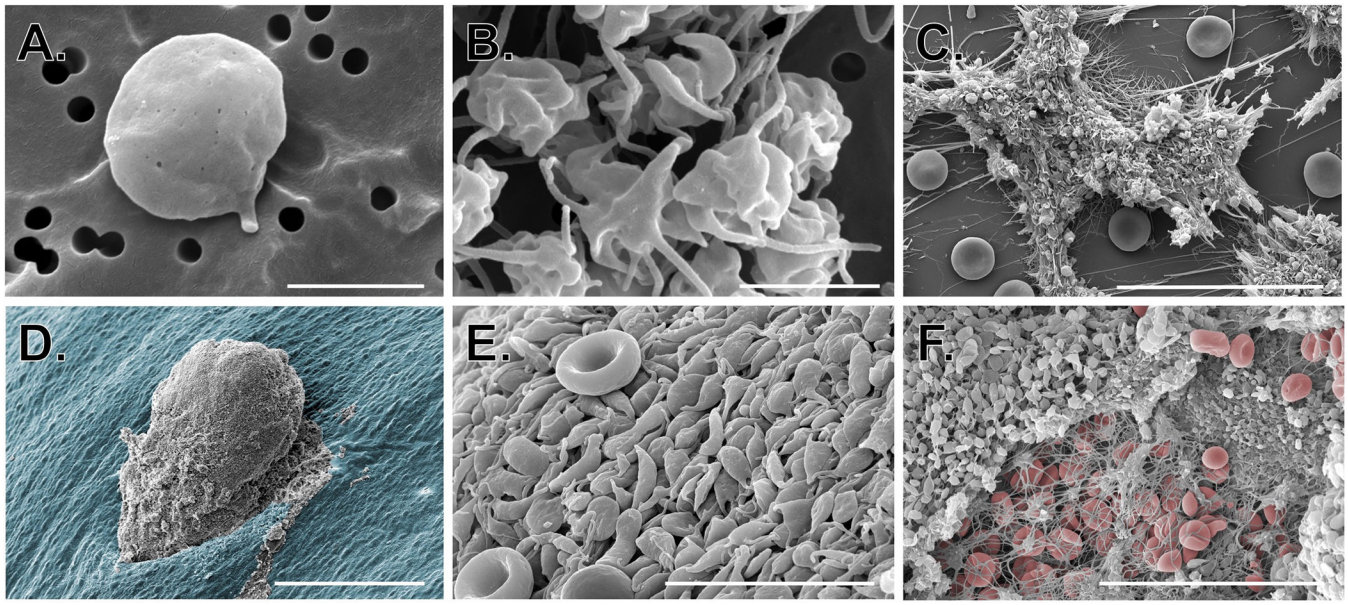

Figure 2: Use of SEM to study platelets in vitro and in vivo.

SEM images show **A-B)** isolated resting and activated human platelets (scale bars = 2 μm); **C)** human platelet-rich thrombi formed in a microfluidic flow chamber coated with collagen and tissue factor (scale bar = 30 μm); **D-F)** platelet-rich hemostatic plugs formed following puncture injury to a mouse jugular vein. **D)** hemostatic plug imaged from the intraluminal side (scale bar = 150 μm); the endothelium is pseudocolored blue. **E)** High magnification image of platelets on the luminal surface of a hemostatic plug (red blood cells can be seen for comparison; scale bar = 10 μm). **F)** Extraluminal component of a hemostatic plug showing platelets, fibrin and a few fibrin entrapped red blood cells (RBCs are pseudocolored red, scale bar = 30 μm). Image credit: M. Tomaiuolo and T.J. Stalker.

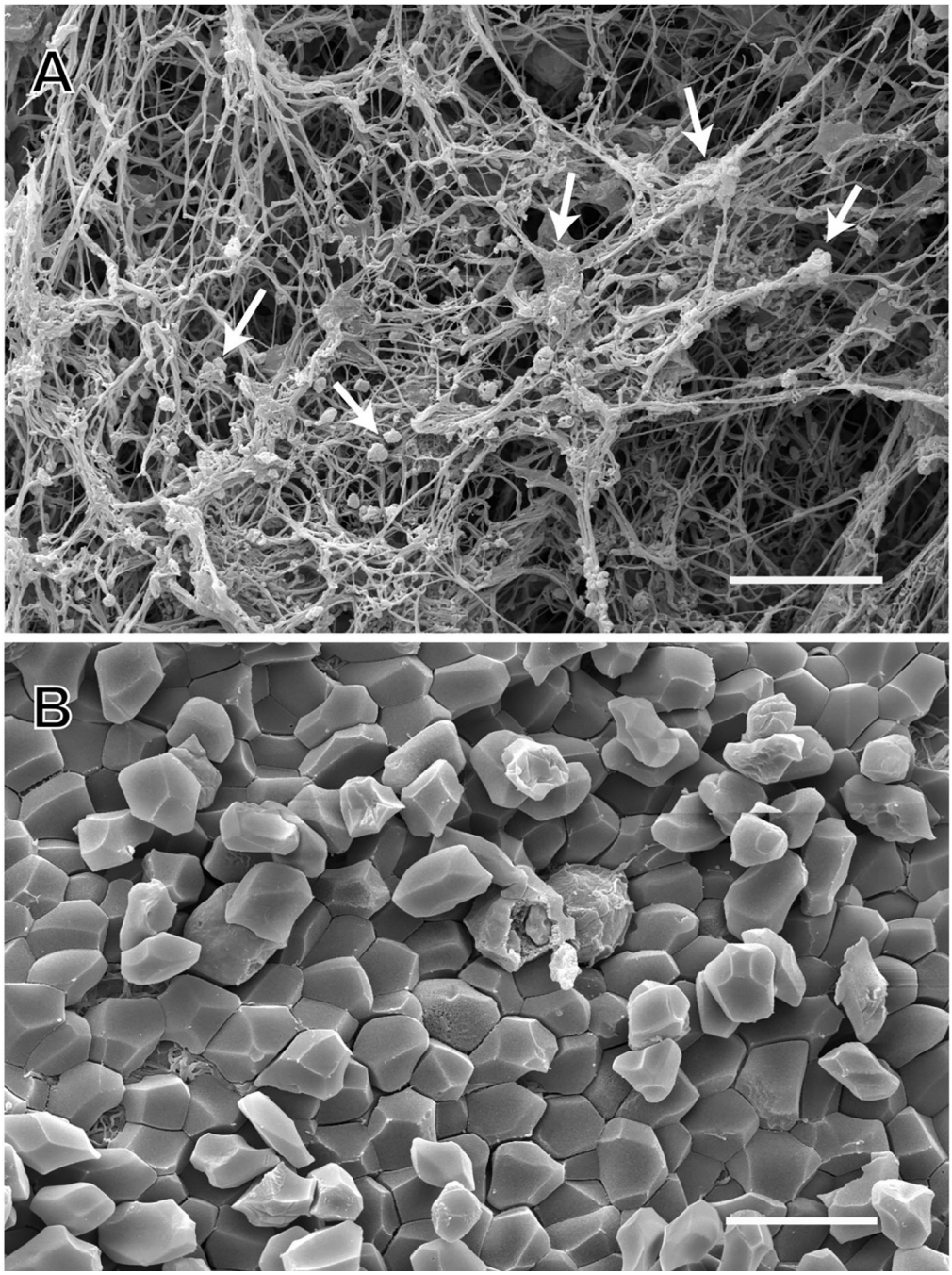

Figure 3: SEM of arterial thrombi taken from patients by thrombectomy.

SEM images show **A)** single and aggregated platelets (white arrows) and fibrin in an arterial thrombus; an example of the appearance of the outside of a contracted blood clot or thrombus; **B)** polyhedrocytes (compressed polyhedral erythrocytes) in an arterial thrombus; an example of the interior of a contracted blood clot. Scale bars = 10 μm. Image credit: from the laboratory of John W. Weisel: Rafael R. Khismatullin and Rustem I. Litvinov.

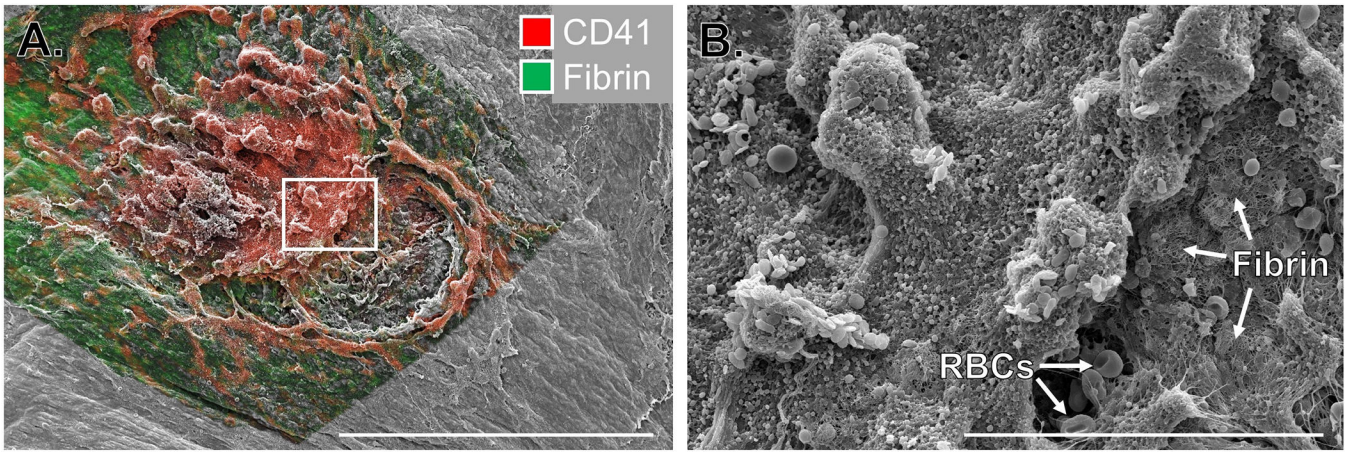

Figure 4: Correlative light and electron microscopy to study hemostatic plug architecture.
Images show the extravascular surface of a mouse jugular vein following puncture injury. **A)** Multiphoton fluorescence image of platelets (CD41, red) and fibrin (green) overlaid on an SEM image of the same region. Scale bar is 300 μm. **B)** SEM image of the region indicated by the white rectangle in A. Scale bar is 50 μm. Images adapted from Tomaiuolo et al [52].